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# ⟨1⟩ INJECTIONS AND IMPLANTED DRUG PRODUCTS (PARENTERALS)—PRODUCT QUALITY TESTS

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## INTRODUCTION

Parenteral drug products include both injections and implanted drug products that are injected through the skin or other external boundary tissue, or implanted within the body to allow the direct administration of the active drug substance(s) into blood vessels, organs, tissues, or lesions. Injections may exist as either immediate- or extended-release dosage forms. Implanted parenteral drug products are long-acting dosage forms that provide continuous release of the active drug substance(s), often for periods of months to years. For systemic delivery, they may be placed subcutaneously; for local delivery, they may be placed in a specific region of the body. Routes of administration for parenteral drug products include intravenous, intraventricular, intra-arterial, intra-articular, subcutaneous, intramuscular, intrathecal, intracisternal, and intraocular.

Parenteral dosage forms include solutions, suspensions, emulsions, sterile powders for solutions and suspensions (including liposomes), implants (including microparticles), and products that consist of both a drug and a device such as drug-eluting stents. The definitions and descriptions of these dosage forms, and brief information about their composition and manufacturing processes, are found in *Pharmaceutical Dosage Forms* ⟨1151⟩. [NOTE—All references to chapters above 1000 are for informational purposes only, for use as a helpful resource. These chapters are not mandatory unless explicitly called out for application.]

This chapter is divided into three main sections: (1) universal product quality tests that are applicable to parenteral dosage forms; (2) specific product quality tests, which are tests that should be considered in addition to *Universal Tests*; and (3) product quality tests for specific dosage forms, which list applicable tests (universal and specific) for the specific dosage form.

This chapter applies, in whole or in part, when referenced in a drug product monograph (see *General Notices, 3.10 Applicability of Standards*).

The pharmacopeial definitions for sterile preparations for parenteral use may not apply to some biologics because of their special nature and licensing requirements (see *Biologics* ⟨1041⟩). However, some biological finished drug products containing "Injection" in the monograph title must meet the requirements of ⟨1⟩ or indicated chapter subparts, where it is specified in the monograph.

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## PRODUCT QUALITY TESTS COMMON TO PARENTERAL DOSAGE FORMS

### Universal Tests

Universal tests are listed below and are applicable to parenteral dosage forms.

#### IDENTIFICATION

Identification tests are discussed in *General Notices, 5.40 Identification* should establish the identity of the drug or drugs present in the article and should discriminate between compounds of closely related structure that are likely to be present..

#### ASSAY

A specific and stability-indicating test should be used to determine the strength (content) of the drug product. In cases where the use of a nonspecific assay is justified, other supporting analytical procedures should be used to achieve overall specificity. A specific procedure should be used when there is evidence of excipient interference with the nonspecific assay.

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### IMPURITIES

Tests for *Impurities* are discussed in *General Notices, 5.60 Impurities and Foreign Substances*. All articles should be tested to ensure that they meet the requirements.

### FOREIGN AND PARTICULATE MATTER

Articles intended for parenteral administration should be prepared in a manner designed to exclude particulate matter as defined in *Subvisible Particulate Matter in Therapeutic Protein Injections* ⟨787⟩, *Particulate Matter in Injections* ⟨788⟩, or *Particulate Matter in Ophthalmic Solutions* ⟨789⟩, as well as excluding other foreign matter as appropriate for the dosage form. Each final container of all parenteral preparations should be inspected to the extent possible for the presence of observable foreign and particulate matter (hereafter termed visible particulates) in its contents. The inspection process should be designed and qualified to ensure that every lot of all parenteral preparations is essentially free from visible particulates, as defined in *Visible Particulates in Injections* ⟨790⟩. Qualification of the inspection process should be performed with reference to particulates in the visible range and those particulates that might emanate from the manufacturing or filling process. Every container in which the contents show evidence of visible particulates must be rejected. The inspection for visible particulates may take place during examination for other defects such as cracked or defective containers or seals, or when characterizing the appearance of a lyophilized product.

When the nature of the contents or the container–closure system permits only limited inspection of the total contents, the 100% inspection of a lot should be supplemented with the inspection of constituted (e.g., dried) or withdrawn (e.g., from a dark amber container) contents of a sample of containers from the lot.

Large-volume injections for single-dose infusion, small-volume injections, and pharmacy bulk packages (PBPs) are subject to the light obscuration or microscopic procedures and limits for subvisible particulate matter set forth in ⟨788⟩, unless otherwise specified in the chapter or in the individual monograph. An article packaged as both a large-volume and a small-volume injection meets the requirements set forth for small-volume injections where the container is labeled as containing 100 mL or less. It meets the requirements set forth for large-volume injections for single-dose infusion where the container is labeled as containing more than 100 mL.

### STERILITY

The sterility of all drug products intended for parenteral administration should be confirmed by the use of methods described in *Sterility Tests* ⟨71⟩ or by an approved alternative method.

### BACTERIAL ENDOTOXINS

All articles intended for parenteral administration should be prepared in a manner designed to limit bacterial endotoxins as defined in *Bacterial Endotoxins Test* ⟨85⟩ or *Pyrogen Test* ⟨151⟩.

### CONTAINER CONTENT

Container contents should be determined when appropriate (see *Container Content for Injections* ⟨697⟩).

### PACKAGING SYSTEMS

The packaging system should not interact physically or chemically with the preparation to alter its strength, quality, or purity beyond the official or established requirements. The packaging system should meet the requirements in ▲*Elastomeric Components Used in Injectable Pharmaceutical Packaging/Delivery Systems* ⟨381⟩▲ (CN 1-Dec-2020), *Packaging and Storage Requirements* ⟨659⟩, *Containers—Glass* ⟨660⟩, *Plastic Packaging Systems and their Materials of Construction* ⟨661⟩, *Plastic Materials of Construction* ⟨661.1⟩, and *Plastic Packaging Systems for Pharmaceutical Use* ⟨661.2⟩. Further information regarding packaging systems testing may be found in *Assessment of Extractables Associated with Pharmaceutical Packaging/Delivery Systems* ⟨1663⟩ and *Assessment of Drug Product Leachables Associated with Pharmaceutical Packaging/Delivery Systems* ⟨1664⟩.

### CONTAINER–CLOSURE INTEGRITY

The packaging system should be closed or sealed in such a manner as to prevent contamination or loss of contents. Validation of container integrity must demonstrate no penetration of microbial contamination or gain or loss of any chemical or physical parameter deemed necessary to protect the product (see *Package Integrity Evaluation—Sterile Products* ⟨1207⟩, *Package Integrity Testing in the Product Life Cycle—Test Method Selection and Validation* ⟨1207.1⟩, *Package Integrity Leak Test Technologies* ⟨1207.2⟩, and *Package Seal Quality Test Technologies* ⟨1207.3⟩).

### LABELING

All articles intended for parenteral administration should meet the labeling requirements defined in *Labeling* ⟨7⟩.

## Specific Tests

In addition to the *Universal Tests* listed above, the following specific tests may be necessary depending on the dosage form.

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### UNIFORMITY OF DOSAGE UNITS

This test is applicable for parenteral drug products and dosage forms packaged in single-unit containers. It includes both the mass of the dosage form and the content of the active substance in the dosage form (see *Uniformity of Dosage Units* ⟨905⟩).

### VEHICLES AND ADDED SUBSTANCES

There are other vehicles, both aqueous and nonaqueous, beyond those that are discussed below. All vehicles should be suitable for their intended use and not impact drug product quality.

**Aqueous vehicles:** Aqueous vehicles must meet the requirements of ⟨151⟩ or ⟨85⟩, whichever is specified in the monograph. Water for injection is generally used as the vehicle. Sodium chloride or dextrose may be added to render the resulting solution isotonic, and sodium chloride injection or Ringer's injection may be used in whole or in part instead of water for injection.

**Nonaqueous vehicles:** Fixed oils are classified under *Nonaqueous vehicles*. Fixed oils used as vehicles are of vegetable origin and are odorless. They meet the requirements in the test for *Solid Paraffin* in the *Mineral Oil* monograph with the cooling bath maintained at 10°.

Nonaqueous vehicles should also meet the requirements of the following tests:

- *Fats and Fixed Oils* ⟨401⟩, *Saponification Value*: Between 185 and 200
- *Fats and Fixed Oils* ⟨401⟩, *Iodine Value*: Between 79 and 141
- *Fats and Fixed Oils* ⟨401⟩, *Unsaponifiable Matter*: NMT 1.5%
- *Fats and Fixed Oils* ⟨401⟩, *Acid Value*: NMT 0.2
- *Fats and Fixed Oils* ⟨401⟩, *Peroxide Value*: NMT 5.0
- *Water Determination* ⟨921⟩, *Method Ic*: NMT 0.1%
- *Limit of Copper, Iron, Lead, and Nickel*: [NOTE—The test for nickel is not required if the oil has not been subjected to hydrogenation, or a nickel catalyst has not been used in processing.] Proceed as directed in *Fats and Fixed Oils* ⟨401⟩, *Trace Metals* or *Elemental Impurities—Procedures* ⟨233⟩. Meet the requirements in *Elemental Impurities—Limits* ⟨232⟩.

Synthetic mono- or diglycerides of fatty acids may be used provided they are liquid and remain clear when cooled to 10° and have a *Iodine Value* of NMT 140.

**Added substances:** Suitable substances may be added to preparations in order to increase stability or usefulness unless they are proscribed in the monograph. No coloring agent may be added to a preparation solely for the purpose of coloring the finished preparation (see *General Notices, 5.20 Added Substances* and *Antimicrobial Effectiveness Testing* ⟨51⟩).

Observe special care in the choice and use of added substances in preparations with volumes that exceed 5 mL. The following limits prevail unless otherwise directed:

- Mercury and cationic surface-active agents: NMT 0.01%
- Chlorobutanol, cresol, phenol, and similar substances: NMT 0.5%
- Sulfur dioxide or an equivalent amount of sulfite, bisulfite, or metabisulfite of potassium or sodium: NMT 0.2%

### ANTIMICROBIAL PRESERVATIVES

Antimicrobial agents must be added to preparations intended for injection that are packaged in multiple-dose containers unless one of the following conditions prevails: (1) there are different directions in the individual monograph; (2) the substance contains a radionuclide with a physical half-life of less than 24 h; or (3) the active ingredients are themselves antimicrobial. Substances must meet the requirements of ⟨51⟩ and *Antimicrobial Agents—Content* ⟨341⟩.

### WATER CONTENT

The water content of freeze-dried (lyophilized) products should be determined when appropriate (see ⟨921⟩).

### ALUMINUM CONTENT

See *Labeling* ⟨7⟩, *Aluminum in Large-Volume Injections (LVIs), Small-Volume Injections (SVIs), and Pharmacy Bulk Packages (PBPs) Used in Parenteral Nutrition (PN) Therapy* for information related to specific labeling requirements associated with aluminum content.

### COMPLETENESS AND CLARITY OF SOLUTIONS

The following tests are performed to demonstrate suitability of constituted solutions prepared before administration. Constitute the solution as directed in the labeling supplied by the manufacturer:

- The solid dissolves completely, leaving no undissolved matter.
- The constituted solution is not significantly less clear than an equal volume of the diluent or of purified water contained in a similar vessel and examined similarly. Protein solutions may exhibit an inherent opalescence.

The constituted solution is free from particulate matter that can be observed on visual inspection (see ⟨790⟩).

## PRODUCT QUALITY TESTS FOR SPECIFIC PARENTERAL DOSAGE FORMS

Product quality tests for the specific dosage forms are listed below. Specific chapter(s) referenced for the test can be found in the *Universal Tests* and *Specific Tests* sections.

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## Solutions

A solution is a clear, homogeneous liquid dosage form that contains one or more chemical substances (e.g., drug substances or excipients) dissolved in a solvent (aqueous or nonaqueous) or a mixture of mutually miscible solvents. Solutions intended for parenteral administration (e.g., by injection or for irrigation) must be sterile and biocompatible with the intended administration site. This includes consideration of factors such as tonicity, pH, pyrogenicity, extraneous particulate matter, and physicochemical compatibility, among others.

Unless otherwise justified, the following tests are required for solutions for injection:

- *Universal Tests*
- *Specific Tests*
  - *Antimicrobial Preservatives*

## Sterile Powders for Solutions

Sterile powders for solutions (also referred to as sterile powders for injection) consist of drug substances and other components as dry-formulation ingredients to ensure the chemical and physical stability of the presentation within a final-use container. Companion sterile diluent or diluent compartments may be provided to facilitate constitution to the desired final volume.

The sterile article for injection may be presented in several forms: lyophilized powder intended for final solution, powdered solids intended for final solution, or dry solids that form viscous liquids upon constitution.

The description should include a section that deals with ease of dispersion and reconstitution. The dosage form is a homogeneous solid that is readily constituted to the final form with the specified diluent, and dispersion is completed with gentle agitation.

Unless otherwise justified, the following tests apply to sterile powders for injection:

- *Universal Tests*

The following applies to constituted solutions:

- Chapter ⟨905⟩: To ensure the consistency of dosage units, each unit in a batch should have a drug substance content within a narrow range around the label claim. Dosage units are defined as dosage forms that contain a single dose or a part of a dose of drug substance in each unit. For liquid dosage forms analysts should conduct the assay on an amount of well-mixed constituted material that is removed from an individual container under conditions of normal use, should express the results as delivered dose, and should calculate the acceptance value.

The following applies to dry cake:

- *Loss on Drying* ⟨731⟩: The procedure set forth in this chapter determines the amount of volatile matter of any kind that is driven off under the conditions specified.
- Chapter ⟨921⟩: Water or solvent content may have important effects on reconstitution and stability. For articles that require water or solvent content control, analysts should perform one of the methods described in ⟨921⟩ or a suitable replacement.
- *Appearance*: Analysts should assess the level of and the unit variation for the following parameters:
  - Color of Dry Cake: Varies within target parameters
  - Texture and Homogeneity of Dry Cake: Varies within target parameters
  - Presence of Foreign Material: All units with visible foreign material must be rejected

## Suspensions

Parenteral suspensions are liquid dosage forms that contain solid particles in a state of uniform dispersion. Suspensions for parenteral administration must be sterile and compatible with the administration site. Consideration should be given to pH and pyrogenicity, and appropriate limits should be identified. Physical stability evaluations of parenteral suspension preparations should include evaluations to confirm that the particle size range of suspended matter does not change with time and to confirm that the solids in the preparation can be readily resuspended to yield a uniform preparation.

The following tests are required for suspensions for injection unless otherwise justified:

- *Universal Tests*
- *Specific Tests*
  - *Uniformity of Dosage Units*
  - *Antimicrobial Preservatives*

## Liposomes

Liposomes are unique drug products with unique properties that can be either solutions or suspensions. Liposomes are aqueous dispersions of amphiphilic lipids and have low water solubility. They are organized as a bilayer sheet that encloses an internal aqueous compartment and are known as lipid bilayer vesicles. Liposomes can have a single lipid bilayer (unilamellar vesicle) or can have an onion-like multilayered structure (multilamellar vesicle). The amphiphilic lipids comprise a hydrated head group at the water interface of the bilayer attached to a hydrophobic group that forms the interior of the bilayer by association with the hydrophobic group of lipids from the opposite leaflet of the bilayer. The physical properties of the liposome and its bilayer can vary widely and depend on lipid composition, aqueous composition, and temperature relative to the acyl components' phase transition points. Because of the central aqueous compartment, a simple test for the presence of liposomes in a lipid dispersion is to determine the presence of an entrapped aqueous phase.

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A liposome drug product consists of the drug substance, liposome components, and other inactive but critical ingredients such as an aqueous dispersion unless the contents are a lyophilized product.

Unless otherwise justified, the following tests are required for liposomes:

- *Universal Tests*
- *Specific Tests*
  - *Globule Size Distribution in Lipid Injectable Emulsions* ⟨729⟩

## Sterile Powders for Suspensions

Sterile powders for suspensions consist of drug substances and other components as dry-formulation ingredients to ensure the chemical and physical stability of the presentation within a final-use container. Companion sterile diluent or diluent compartments may be provided to facilitate constitution to the desired final volume.

The sterile article for injection may be presented in several forms: lyophilized powder intended for final suspension, powdered solids intended for final suspension, and microparticles that retain their integrity and are delivered as a sterile suspension. The description should include a section that deals with ease of dispersion and reconstitution. The dosage form is a homogeneous solid that is readily constituted to the final form with the specified diluent, and dispersion is completed with gentle agitation.

Unless otherwise justified, the following tests apply to sterile powders for injection:

- *Universal Tests*

**Microparticles:** Some microparticles are provided as a sterile powder to be reconstituted as a suspension before injection. The majority of microparticle preparations are for reconstitution as a suspension for injection. For quality test requirements, please refer to *Implants*.

## Emulsions

Emulsions for parenteral dosage forms are liquid preparations of drug substances dissolved or dispersed in a suitable emulsion medium. Oil-in-water or water-in-oil emulsions typically entrap the drug substance.

Emulsions typically are white, turbid, homogeneous liquid dosage forms that contain one or more chemical substances (e.g., drug substances and excipients) dissolved in a solvent (aqueous or nonaqueous) or mixture of mutually miscible solvents. Emulsions intended for intravenous administration must be sterile and must be compatible with the intended administration site.

Unless otherwise justified, the following tests are required for emulsions for injection:

- *Universal Tests*
- *Specific Tests*
  - Chapter ⟨729⟩

## Implants

Implants for extended release consist of a matrix of drug substance and polymeric excipient that may or may not have an outer rate-controlling membrane. The polymeric excipient must be biocompatible but may or may not be bioresorbable. Some implants are made from medical-grade metal with an osmotic pump inside that effects the extended release of the drug substance. Implants must be sterile and usually are formed in the shape of a cylinder, although other shapes are used. Solvents used to dissolve the formulation can lead to sterilization, and thus the internal sterility test method should demonstrate that the sample preparation does not lead to sterilization of the test sample.

Cylindrically shaped implants for systemic delivery usually are provided in an inserter for subcutaneous or local administration such as local ocular delivery. Implants also can be surgically implanted for local delivery, e.g., ocular delivery.

Unless otherwise justified, the following tests are required for implants:

- *Universal Tests*
- *Specific Tests*
  - *Uniformity of Dosage Units*

## In situ Gels

Sterile in situ gels are liquid preparations that are intended for injection into specific therapeutic targets. Typically they consist of polymers in organic solvents, and upon injection the solvents migrate away from the site, leaving a gelled mass. The preparations may be injected as-is, upon reconstitution, from in situ formation, or from chemically initiated catalysis that results in the final form.

Unless otherwise justified, the following tests are required for in situ gels:

- *Universal Tests*
- *Specific Tests*
  - *Antimicrobial Preservatives*

## Microparticles

Injectable, resorbable microparticles for extended release generally range from 20 to 100 µm in diameter. They consist of drug substances embedded within a biocompatible, bioresorbable polymeric excipient, e.g., polyester excipients. Microparticles are provided as a sterile powder in a vial or syringe.

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Just before intramuscular or subcutaneous administration, the microparticle powder should be suspended in an aqueous injection vehicle (diluent). The injection vehicle usually consists of water for injection, surfactant, and a viscosity enhancer, and the vehicle may contain a compound that adjusts osmolality, e.g., a sugar with or without a compound that controls pH, e.g., an acid. The injection vehicle must be sterile and must be tested according to requirements for solutions that are intended for parenteral administration.

Unless otherwise justified, the following tests are required for microparticles for injection:

- *Universal Tests*
- *Specific Tests*
  - *Uniformity of Dosage Units*
  - *Water Content*

## Drug-Eluting Stents

Drug-eluting stents are tiny metal or polymer scaffolds used to keep arteries open following a medical intervention; the drug substance is incorporated into or onto the stent platform. Drug-eluting stents typically have two components of testing: (1) functional tests that generally are American Society for Testing and Materials (ASTM) International methods that fall outside the scope of this chapter and (2) analytical tests.

Unless otherwise justified, the following tests are required for drug-eluting stents:

- *Universal Tests*
- *Specific Tests*
  - *Uniformity of Dosage Units.* The content of the active substance in the dosage form is applicable for drug-eluting stents packaged in single-unit containers. The test can be performed by either content uniformity or weight variation (see ⟨905⟩). With appropriate justification, the number of stents needed for this test may be fewer than the recommended number of stents in ⟨905⟩.
  - ⟨88⟩ *Biological ReactivityTests, In Vivo*

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# ⟨2⟩ ORAL DRUG PRODUCTS—PRODUCT QUALITY TESTS

## INTRODUCTION

Oral delivery is the most common route of administration for drug products. All oral drug products lead to systemic and/or local action in the oral cavity and/or gastrointestinal tract. Oral drug products fall primarily into two main categories: solids and liquids. Solid oral drug products include but are not restricted to capsules, tablets, granules, and powders. Similarly, liquid oral drug products include but are not restricted to solutions, suspensions, and emulsions. The definitions and descriptions of these dosage forms and brief information about their composition and manufacturing process are found in *Pharmaceutical Dosage Forms* ⟨1151⟩. [NOTE—All references to chapters above ⟨1000⟩ are for informational purposes only, for use as a helpful resource. These chapters are not mandatory unless explicitly called out for application.]

This chapter focuses on the product quality tests that are generally necessary for oral drug products for a single or combination of small molecules of active ingredients. Biologics in solid dosage forms are not considered. In this chapter, the terms "drug substance" and "active ingredient" are used interchangeably. The contents of this chapter do not necessarily apply to drug products that are intended for use other than by oral administration. For example, the chapter does not address oromucosal dosage forms. Some of the tests indicated in this chapter may be performed on an in-process basis or omitted as routine tests based on process validation. However, the product must meet USP compendial requirements when sampled and tested, once the product is on the market.

This chapter provides lists of consolidated common product quality test requirements in a concise and coherent fashion. This chapter applies, in part or whole, when referenced in a drug product monograph (see *General Notices, 3.10 Applicability of Standards*). This chapter includes the quality tests for the specific route of administration. The quality tests listed can be used as appropriate by manufacturers toward the development of new drug product monographs for submission to USP. If a validated performance test procedure is available for the specific drug product, it is identified in a general chapter below ⟨1000⟩. In cases where a validated procedure cannot be recommended, but if the information is available for a product quality and/or product performance test, it is described in an informational chapter above ⟨1000⟩.

### Drug Product Quality Tests and Performance Tests

Monograph tests, analytical procedures, and acceptance criteria for testing oral drug products are divided into two categories: 1) those that assess general product quality attributes, and 2) those that assess product performance, which is a specific quality attribute typically linked to bioavailability and bioequivalence studies (see *Assessment of Solid Oral Drug Product Performance and Interchangeability, Bioavailability, Bioequivalence, and Dissolution* ⟨1090⟩). Drug product quality tests are intended to assess attributes such as identification, strength (assay), impurities , dose content uniformity, pH, minimum fill, alcohol content, volatile content, and microbial content. Drug product performance tests are designed to assess in vitro drug release from dosage forms (e.g., *Dissolution* ⟨711⟩ and *Drug Release* ⟨724⟩). For liquid oral drug products in solution, performance is considered optimal, and a monograph performance test is not included.

Each of these attributes is important for a primary understanding of the quality and performance of a drug product. Thus, they form the basis for the monograph. A compendial product should meet all drug product quality tests and drug product performance tests contained in its monograph.

[NOTE—Dissolution tests, specifically dissolution profile similarity between higher strengths and lower strengths of a given manufacturer's product and dissolution profile similarity between the generic product and the reference product, are used for granting biowaivers. See ⟨1090⟩.]

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## PRODUCT QUALITY TESTS FOR ORAL DRUG PRODUCTS

Drug product quality tests for oral drug products fall into two categories: 1) universal tests that are applicable to all oral drug products and should be included in the monograph, and 2) specific tests that should be considered for inclusion for specific types of oral products.

### Universal Tests for Oral Drug Products

Product quality attributes for oral dosage forms are important to ensure that commercialized products meet minimum quality requirements. Universal tests should be applied to all oral dosage forms and include *Description*, *Identification*, *Strength* (*Assay*), and *Impurities* (organic, inorganic, and residual solvents).

#### DESCRIPTION

Description is general in nature and is not a standard in itself. It communicates the appearance of an article that complies with monograph standards.

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### IDENTIFICATION

The identification test is defined in *General Notices, 5.40 Identification*. It is included in a monograph as an aid to confirm that the article contains the labeled drug substance by providing a positive identification of the drug substance or substances in a drug product.

One method of confirming the identity is to compare the retention time of the sample with that obtained for the standard injections in a chromatographic assay procedure. Other methods often used to orthogonally confirm the identity of the active ingredient are: *Thin-Layer Chromatographic Identification Test* ⟨201⟩, *Spectroscopic Identification Tests* ⟨197⟩, *Nuclear Magnetic Resonance Spectroscopy* ⟨761⟩, *Near-Infrared Spectroscopy—Theory and Practice* ⟨1856⟩, and ▲*Raman Spectroscopy—Theory and Practice* ⟨1858⟩▲ (CN 1-Aug-2020), among others. The analytical procedure must be able to distinguish the active ingredient from all excipients that are present or from potential degradation products likely to be present. Care must also be taken to ensure that the chromatographic system separates the article from other closely related drug substances, impurities, and additives. Infrared and ultraviolet absorption also can be used for identification (see ⟨197⟩), if the procedure has been demonstrated to be selective for the drug substance via an appropriate validation or verification study. The results of the identification test must be compared to the results obtained from a similarly prepared, suitable Reference Standard.

### ASSAY

The assay is a specific and stability-indicating test to determine the potency (content) of the drug product. When a nonspecific assay (e.g., titration) is justified, other supporting analytical procedures should ensure that any interfering species can be detected. In general the *a priori* acceptance of ±10% variation in limits of a quality attribute (e.g., assay) from the target label claim (100%) in most cases is intended to account for manufacturing variability and shelf-life stability and is primarily based on the notion that such variation in a quality attribute is less likely to have any noticeable adverse impact on the desired clinical outcome. Acceptance criteria of 95.0%–105.0% are used with justification (e.g., for drug products with narrow therapeutic index). Activity assays and absolute content assays also are acceptable when justified.

### IMPURITIES

Process impurities, synthetic by-products, and other inorganic and organic impurities may be present in the drug substance and in the excipients used in the manufacture of the drug product. These impurities are limited by drug substance and excipient monographs. During product manufacture and over the shelf life of the product, degradation products can form. These can be a result of degradation of the drug substance or from interactions between the drug substance and excipient(s), among other factors. The procedures and acceptance criteria should specifically limit toxic materials. See specific requirements in the *General Notices 5.60, Impurities and Foreign Substances*. [NOTE—For additional information, see *Impurities in Drug Substances and Drug Products* ⟨1086⟩.]

## Specific Tests for Tablets

In addition to the *Universal Tests for Oral Drug Products* described above, the following specific tests for tablets should be considered, depending upon the nature of the drug substance and formulation.

### VOLATILE CONTENT

The test and the specific method depend on the nature of the article. Special consideration should be given to dosage forms for which water content has been shown to be a potential quality attribute and to products where solvent is used in the manufacture of the drug product.

When the presence of moisture or other volatile material may become critical, analysts must determine the amount of unbound volatile solvents or volatile matter of any kind that is driven off by *Loss on Drying* ⟨731⟩ or another suitable technique (e.g., water activity). For substances that appear to contain water as the only volatile constituent, the procedure given in *Water Determination* ⟨921⟩ may be appropriate. For drug products, analysts also should consult *Residual Solvents* ⟨467⟩.

### DISINTEGRATION

Disintegration is an essential attribute of oral solids, except for those intended to be chewed before being swallowed and for delayed- or extended-release products. This test measures the time it takes for the dosage unit to disintegrate in an aqueous medium and is described in detail in *Disintegration* ⟨701⟩. Certain dosage forms (e.g., effervescent tablets and others) may require special procedures and identity tests for bicarbonate when dissolved in water. The disintegration test for some of the dosage forms in this chapter is included for completeness. For detailed procedures, refer to ⟨701⟩. The disintegration test, if included, is used only as a quality control test and not as a product performance test and should conform with the specifications in the monograph. Only when disintegration has been correlated with dissolution of a dosage form can a disintegration test be used as a product performance test [International Council for Harmonisation (ICH) Guidance Q6A, available at www.ich.org]. In all other instances, a dissolution test should be considered as a product performance test.

### TABLET FRIABILITY

The test procedure is applicable to most compressed, uncoated tablets. Friability determines the ability of tablets to withstand mechanical stresses and their resistance to chipping and surface abrasion. [NOTE—For additional information, see *Tablet Friability* ⟨1216⟩.]

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### TABLET BREAKING FORCE

Tablet breaking force measures the mechanical integrity of tablets, which is the force required to cause them to fail (i.e., break) in a specific plane. [NOTE—For additional information, see *Tablet Breaking Force* ⟨1217⟩.]

### UNIFORMITY OF DOSAGE UNITS

Uniformity of dosage units must be demonstrated by either content uniformity or weight variation. Content uniformity is based on the assay of the individual content of drug substance(s) in a number of dosage units to determine whether the individual contents are sufficiently close to label claim. Weight variation can be used as an alternative to estimate content uniformity under certain conditions (see *Uniformity of Dosage Units* ⟨905⟩).

## Specific Tests for Uncoated Tablets

Uncoated tablets include single-layer tablets that result from a single compression of particles and multilayer tablets that consist of concentric or parallel layers obtained by successive compression of particles of different composition. The excipients used generally are not specifically intended to modify the release of the active substance in the digestive fluids. Uncoated tablets include but are not limited to: effervescent tablets, buccal tablets, sublingual tablets, chewable tablets, orally disintegrating tablets, tablets for oral solution, and tablets for oral suspension. For uncoated tablets, disintegration should be tested as directed in ⟨701⟩.

### BUCCAL, SUBLINGUAL, AND ORALLY DISINTEGRATING TABLETS

These dosage forms are discussed in *Mucosal Drug Products—Product Quality Tests* ⟨4⟩. They are listed here for informational purposes and completeness.

### CHEWABLE TABLETS

Chewable tablets (intact) should undergo dissolution and disintegration testing, as a product performance test (if cited in the monograph), because they might be swallowed without proper chewing by a patient. In general, the dissolution and disintegration test conditions for chewable tablets should be the same as for nonchewable tablets of the same active ingredient or moiety.

### TABLETS FOR ORAL SOLUTION AND TABLETS FOR ORAL SUSPENSION

These are tablets intended to be dissolved or dispersed in water before administration, giving a homogeneous solution or dispersion. They are listed here for informational purposes and completeness.

## Specific Tests for Coated Tablets

Coated tablets are tablets covered with one or more layers of mixtures of various substances such as natural or synthetic resins, gums, gelatin, inactive and insoluble excipients, sugars, plasticizers, polyols, waxes, coloring matter authorized by the competent authority, and sometimes flavoring substances and active substances. Tablets coated by sugar or film include but are not limited to: plain coated tablets, extended-release tablets, and delayed-release tablets. A disintegration test, when applicable, should be performed as directed in ⟨701⟩.

There are no additional specific quality tests for extended-release tablets and delayed-release tablets. Universal quality tests should be applied to these products.

## Specific Tests for Capsules

In addition to the *Universal Tests for Oral Drug Products* described above, the specific tests included below should be considered, depending on the nature of the drug substance and formulation. Modified release capsules include delayed-release capsules and extended-release capsules.

Product quality tests that are considered specific to the type of capsule include those for volatile content (see ⟨731⟩ and ⟨921⟩). One-piece capsules typically are used to deliver a drug substance as a solution or suspension. Two-piece capsules consist of two telescoping cap-and-body pieces that are used to deliver solid material as powder, granules, or small tablets.

### UNIFORMITY OF DOSAGE UNITS

Uniformity of dosage units must be demonstrated by either content uniformity or weight variation. Content uniformity is based on the assay of the individual content of drug substance(s) in a number of dosage units to determine whether the individual contents are sufficiently close to label claim. Weight variation can be used as an alternative to estimate content uniformity under certain conditions (see ⟨905⟩).

### DISINTEGRATION

For capsules, proceed as directed in ⟨701⟩, if a disintegration test is required. Disintegration for modified release capsules may require special procedures.

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There are no additional specific quality tests for modified release capsules. Universal quality tests should be applied to these products.

## Specific Tests for Granules

In addition to the *Universal Tests for Oral Drug Products* described above, the specific tests included below should be considered, depending on the nature of the drug substance and formulation.

Granules are solid dosage forms that are composed of agglomerations of smaller particles. Granules include but are not limited to: effervescent granules, coated granules, extended-release granules, and delayed-release granules.

Tests that are considered specific to the type of granules include volatile content (see ⟨731⟩ and ⟨921⟩). For disintegration for effervescent granules, refer to ⟨701⟩. On the basis of the nature of the article and scientific criteria, additional tests may apply, including powder fineness and content uniformity (see ⟨905⟩) if granules are packaged in single-unit containers.

## Specific Tests for Powders

Oral powders should indicate: "For Oral Use Only". Tests that are considered specific to the type of powders include: *Minimum Fill* ⟨755⟩ for products packaged in multiple-dose containers, content uniformity (see ⟨905⟩) for products packaged in single-unit containers, and volatile content (see ⟨731⟩ and ⟨921⟩).

On the basis of the nature of the article and scientific criteria, additional tests may apply, including pH in an aqueous solution, powder fineness, microbial limits, and others.

## Specific Tests for Liquids

The recommended product quality tests for a liquid drug product include the *Universal Tests for Oral Drug Products* described above and the specific tests included below. Most of the quality tests for liquids require the evaluation of single-dose products to estimate the quality attribute. Specific directions to perform the quality tests (including preservative content) for either single-dose or multiple-dose products are provided in the monograph or the general chapter. For example, weight variation may be used when adequacy of mix for the active substance(s) and excipients in the blend is well controlled to ensure their uniform distribution, as in solutions.

### DELIVERABLE VOLUME

When the liquid formulation is packaged in a multiple-dose container, compliance with *Deliverable Volume* ⟨698⟩ is required.

### ALCOHOL DETERMINATION

If the liquid formulation contains a quantity of alcohol, *Alcohol Determination* ⟨611⟩ should be included. The limits may be an absolute concentration, in percentage, or relative to a labeled content.

### pH

Liquid oral products typically are aqueous formulations that are susceptible to pH changes from exposure to atmospheric carbon dioxide ($CO_2$). The uptake of atmospheric carbon dioxide ($CO_2$) and consequent pH change of oral liquid products is only relevant to aqueous-based products. The pH of an oral liquid formulation can affect flavor and stability. The pH range as outlined in *pH* ⟨791⟩ is indicated in the monograph.

### MICROBIAL CONTENT

The presence of certain microorganisms in nonsterile preparations may have the potential to reduce or even inactivate the therapeutic activity of the product and has a potential to adversely affect the health of the patient. Some liquid oral products can be subject to extreme microbiological control, and others require none. The needed microbial specification for a given liquid oral product depends on its formulation and use and is indicated in the monograph.

[NOTE—For additional information, see *Microbiological Examination of Nonsterile Products: Acceptance Criteria for Pharmaceutical Preparations and Substances for Pharmaceutical Use* ⟨1111⟩.]

### ANTIOXIDANT

Release testing should be performed. Shelf-life testing may be unnecessary where justified by development and stability data (ICH Guidance Q6A).

### EXTRACTABLES

Where development and stability data show no significant evidence of extractables, elimination of this test may be proposed. Where data demonstrate the need and acceptance criteria for oral solutions—rubber stopper, cap liner, plastic bottle—data should be collected as early in the development process as possible (ICH Guidance Q6A).

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TYPES OF LIQUID DOSAGE FORMS

Specific quality tests for these dosage forms are provided in their respective monographs.
**Solutions, powders, and granules for solution:** Tests of "for Solution" formulations are conducted on a well-mixed solution of the drug product constituted as described in the labeling.
**Emulsions, suspensions, and powders and granules for suspension:** Tests of "for Suspension" formulations are conducted on a well-mixed suspension of the drug product constituted as described in the labeling. Product quality tests for suspensions should include a test of suspendability.
**Powders and granules for solutions:** After dissolution or suspension, they comply with monograph requirements for the final dosage form. Volatile content (see ⟨731⟩ and ⟨921⟩) may be an additional quality test for powders and granules for reconstitution.

## Specific Tests for Miscellaneous Oral Dosage Forms

LYOPHILIZED ORAL PRODUCTS

***Water Determination*** ⟨921⟩**,** *Method I, Method Ia***:** Lyophilized oral products comply with the test. The limits are approved as indicated in the specific monograph.

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# ⟨3⟩ TOPICAL AND TRANSDERMAL DRUG PRODUCTS—PRODUCT QUALITY TESTS

## INTRODUCTION

Topically applied drug products fall into two general categories: those applied to achieve local action and those applied to achieve systemic effects after absorption through the skin into the blood circulation. Local action can occur at or on the surface of the application site (e.g., stratum corneum); in the underlying tissues (e.g., epidermis and/or dermis); and in subcutaneous tissues (e.g., muscle or joint). Topically applied drug products include, but are not limited to, creams, gels, ointments, pastes, suspensions, lotions, foams, sprays, aerosols, solutions, and topical and transdermal delivery systems (TDS). The definitions and descriptions of these dosage forms, as well as brief information on their composition and/or manufacturing processes, can be found in *Pharmaceutical Dosage Forms* ⟨1151⟩.

Procedures and acceptable criteria for testing topically applied drug products can be divided into those that assess general product quality attributes and those that assess product performance. The product quality attributes include the following: description, identification, assay (strength), impurities, physicochemical and structural properties, uniformity of dosage units, water content, pH, apparent viscosity, microbial limits, antimicrobial preservative content, antioxidant content, sterility (if applicable), penetration enhancer content, and other tests that may be product specific. Product performance testing assesses drug release and other attributes that affect drug release from the finished dosage form.

This chapter provides lists of consolidated common product quality test requirements in a concise and coherent fashion. This chapter applies, in whole or in part, when referenced in a drug product monograph (see *General Notices, 3.10 Applicability of Standards*) and includes the quality tests for the specific route of administration. The quality tests listed can be used as appropriate by manufacturers toward the development of new drug product monographs for submission to *USP–NF*.

TDS release their active ingredients by different mechanisms. They can be passive or active. This chapter covers only the tests related to passive TDS.

## PRODUCT QUALITY TESTS FOR TOPICAL AND TRANSDERMAL DRUG PRODUCTS

### Additional Procedure for Products Packaged in Containers with a Non-Metered Pump

For some tests (e.g., viscosity, assay, etc.), with the exception of the uniformity in containers test, samples need to be collected from the pumped-out product. In these instances, the samples should be collected as follows:

1. Remove cap from container.
2. Fully depress and release the actuator, disposing of any material dispensed, and allowing the pump actuator to return to the initial position after each actuation. Repeat the sequence, as indicated in the patient instructions, until a full amount of material is dispensed.
3. Collect the next material dispensed as the sample. Generally, collect NMT the amount needed to perform a single analysis (for the assay test, typically NMT 2 actuations).

### Universal Tests

Universal tests [see International Council for Harmonisation (ICH) guidance *Q6A—Specifications: Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Chemical Substances*, available at www.ich.org] are listed as follows and are applicable to all topically applied drug products.

#### DESCRIPTION

A qualitative description of the drug product should be provided. The acceptance criteria should include the final acceptable appearance of the finished dosage form and packaging. A visual examination should identify changes in color, adhesive migration (i.e., cold flow; see *Cold Flow Test*) for TDS, separations, crystallization, and others that are specific to the drug product. The description should specify the content or the label claim of the article. For TDS, a visual examination should also be done to assess potential use issues with the product. The examination should include an assessment of the difficulty of removing the TDS from the pouch (e.g., due to adhesive migration adhering the system to the pouch); inability to remove the TDS from the pouch without damage to the system; and adhesive residue remaining on the pouch after removal of the TDS. This is not a compendial test but is part of the manufacturer's specification for the drug product.

#### IDENTIFICATION

Identification tests are discussed in *General Notices, 5.40 Identification*. Identification tests should establish the identity of the drug or drugs present in the article and should discriminate between compounds of closely related structures that are likely to be present. Identification tests should be specific for the drug substance(s) (e.g., infrared spectroscopy). Near-infrared (NIR) or Raman spectrophotometric methods also could be acceptable for the identification of the drug product (for additional information, see *Near-Infrared Spectroscopy—Theory and Practice* ⟨1856⟩ and *Raman Spectroscopy—Theory and Practice* ⟨1858⟩). Identification solely by a single chromatographic retention time is not specific.

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### ASSAY

A specific and stability-indicating test should be used to determine the strength (active pharmaceutical ingredient content) of the drug product. This assay requirement can be satisfied for topical products containing antibiotics by a standard microbiological method (see *Antibiotics—Microbial Assays* ⟨81⟩). In cases when the use of a nonspecific assay (e.g., *Titrimetry* ⟨541⟩) is justified, other supporting analytical procedures should be used to achieve overall specificity.

### IMPURITIES

Process impurities, synthetic byproducts, impurities associated with the adhesive (e.g., residual monomers), residual solvents (see *Residual Solvents* ⟨467⟩), and other inorganic and organic impurities may be present in the drug substance and in the excipients used in the manufacture of the drug product and should be assessed and controlled. Impurities arising from the degradation of the drug substance and those arising during the manufacturing process of the drug product also should be assessed and controlled.

## Specific Tests

In addition to the *Universal Tests* listed previously, the following *Specific Tests* should be considered on a case-by-case basis.

### UNIFORMITY OF DOSAGE UNITS

This test is applicable for TDS and for topical dosage forms intended for systemic delivery, or where tight control of the dose is necessary to limit local irritation or undesired systemic exposure, packaged in single-unit containers, such as packets (see *Uniformity of Dosage Units* ⟨905⟩). The uniformity of dosage units specification is not intended to apply to solutions, suspensions, emulsions, ointments, or gels in single-unit containers intended for local action following external, cutaneous administration.

### WATER CONTENT

A test for water content should be included when appropriate (see *Water Determination* ⟨921⟩). This test is generally formulation dependent. Therefore, it is not included in the compendial drug product monograph but is part of the manufacturer's specification for the drug product.

### MICROBIOLOGICAL QUALITY

Microbiological examination of nonsterile drug products is performed according to the methods given in *Microbiological Examination of Nonsterile Products—Tests for Burkholderia cepacia Complex* ⟨60⟩ (as appropriate for aqueous formulations), *Microbial Enumeration Tests* ⟨61⟩, and *Tests for Specified Microorganisms* ⟨62⟩, as appropriate, unless the formulation itself is demonstrated to have antimicrobial properties. Recommended acceptance criteria for nonsterile pharmaceutical products based on total aerobic microbial count and total combined yeasts and molds count are given in *Microbiological Examination of Nonsterile Products: Acceptance Criteria for Pharmaceutical Preparations and Substances for Pharmaceutical Use* ⟨1111⟩.

### ANTIMICROBIAL PRESERVATIVE CONTENT

Acceptance criteria for antimicrobial preservative content in multiple-unit products should be established. They should be based on the levels of antimicrobial preservative necessary to maintain the product's microbiological quality at all stages throughout its proposed usage and shelf life (see *Antimicrobial Effectiveness Testing* ⟨51⟩).

### PENETRATION ENHANCER CONTENT

If an excipient is claimed to be a penetration enhancer, a test and acceptance criteria for its content should be established.

### ANTIOXIDANT CONTENT

If antioxidants are present in the drug product, tests of their content should be established unless oxidative degradation can be detected by another test method such as impurity testing. Acceptance criteria for antioxidant content should be established. They should be based on the levels of antioxidant necessary to ensure the product's stability at all stages throughout the product's proposed usage and shelf life.

### STERILITY

Depending on the use of the dosage form (e.g., products that will be applied to open wounds or burned areas), sterility of the product should be demonstrated as appropriate (see *Sterility Tests* ⟨71⟩).

### pH

When applicable, topically applied drug products should be tested for pH. Because some topically applied drug products contain very limited quantities of water or aqueous phase, pH measurements may not always be warranted. This test is generally formulation dependent. Therefore, it is not included in the compendial drug product monograph but is part of the manufacturer's specification for the drug product.

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### PARTICLE SIZE

When the finished product contains a suspended solid drug substance, the product should be examined for particle size. The particle size of the active drug substance(s) in topically applied drug products is usually established and controlled at the formulation development stage. However, topically applied drug products should be examined for evidence of particle alteration (i.e., change in drug polymorphic form, appearance of particles, size, shape, morphology, agglomeration, or aggregation) of the drug substance that may occur during the course of product processing and storage. These types of tests are generally formulation dependent. Therefore, such tests are not included in compendial drug product monographs but are part of the manufacturer's specification for the drug product.

### CRYSTAL FORMATION

When the drug substance is dissolved in the finished product, the product should be microscopically examined for evidence of crystal formation of the active drug substance. This test is generally formulation dependent. Therefore, it is not included in the compendial drug product monograph but is part of the manufacturer's specification for the drug product. It is recommended that the potential for the drug product to form crystals of drug substance be examined during product development.

### EMULSION GLOBULE SIZE

Because many semisolid dosage forms such as creams and lotions are multiphasic systems (e.g., emulsions) with co-existing oil and water/aqueous phases in the formulation, emulsion droplet size or globule size can be a critical quality attribute that characterizes the microstructure of these dosage forms. Therefore, control of the emulsion droplet and/or globule size should be considered a specific test for such products to ensure consistency, homogeneity, and physical stability in the drug product throughout the shelf life.

## IN VITRO DRUG RELEASE TEST

For TDS, the in vitro drug release test is required as a batch-to-batch quality control. See *Drug Release* ⟨724⟩ for apparatus and test conditions. The development and validation report of the in vitro drug release test needs to include sufficient detail and data to facilitate the assessment of whether the method is adequate as a quality control test for batch release and stability testing.

For semisolid dosage forms, an in vitro drug release test (IVRT) is currently not mandatory for batch release. See *Semisolid Drug Products—Performance Tests* ⟨1724⟩ for appropriate contexts of use for an IVRT, as well as discussions on method development, experimental design, data analysis, suitable equipment and their qualification, as well as other practical information.

## SPECIFIC TESTS FOR OPHTHALMIC DRUG PRODUCTS

See *Ophthalmic Products—Quality Tests* ⟨771⟩.

## SPECIFIC TESTS FOR TOPICAL AEROSOLS

See *Topical Aerosols* ⟨603⟩.

## SPECIFIC TESTS FOR TOPICALLY APPLIED SEMISOLID DRUG PRODUCTS

### Minimum Fill

Single- and multiple-unit containers must meet minimum fill requirements as established by testing described in *Minimum Fill* ⟨755⟩. For single-unit containers where the test for ⟨905⟩ is applied, the test for ⟨755⟩ is not required.

***Change to read:***

### Apparent Viscosity

Viscosity is a measure of a formulation's resistance to flow and is an assessment of a rheological property of a semisolid dosage form. The term "apparent viscosity" applies to non-Newtonian fluids, which comprise the majority of semisolid pharmaceutical dosage forms. For additional information, see *Rheometry* ⟨1911⟩ and *Measurement of* ▲*Yield Stress*▲ (CN 1-Dec-2023) *of Semisolids* ⟨1912⟩.

Measurement procedures should be developed as outlined in *Viscosity—Capillary Methods* ⟨911⟩, *Viscosity—Rotational Methods* ⟨912⟩, and *Viscosity—Rolling Ball Method* ⟨913⟩. For semisolids that show thixotropy and/or irreversible changes in viscosity after shearing, specific attention should be given to sample preparation procedures to minimize variability in the measurement of apparent viscosity caused by variable shear histories (e.g., mixing speed and temperature, filling operation, and sample handling). Furthermore, for some products it may be warranted to have apparent viscosity specifications at more

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than one stage of the manufacturing process (e.g., bulk in-process stage, final packaged product, etc.) or with more than one set of test conditions (e.g., high and low shear rates, different temperatures, etc.).

Apart from single-point viscosity measurements, more advanced rheological techniques (flow, oscillatory, creep, and stress relaxation testing) can be applied to develop a mechanistic understanding of a formulation and its structure. See ⟨1912⟩. These techniques may be useful for product development using the principles of quality by design or for comparative physicochemical and structural characterization of the test and reference products to support a demonstration of bioequivalence in an abbreviated new drug application. However, these techniques are not generally suitable for routine quality testing. Common parameters derived from rheological testing of semisolid pharmaceutical dosage forms that may be useful for characterization and comparison are the storage modulus, loss modulus, relaxation modulus, compliance, thixotropic index, and yield stress.

Acceptance criteria are product specific and defined to ensure that the apparent viscosity of each batch of semisolid drug product is within the the acceptable range determined during product development and is consistent between batches.

## Uniformity in Containers

Topically applied semisolid drug products may show physical separation during manufacturing processes and during their shelf life. To ensure the integrity of the drug product, it is essential to evaluate the uniformity of the finished product. This test applies only to multiple-unit containers, such as tubes and jars. This test does not apply to more fluid topical drug products in multiple-unit containers, such as emulsions, lotions, two-phase gels, or topical suspensions, in which the labeling directs the user to mix the product (e.g., shake well) before use.

### PRODUCTS PACKAGED IN TUBES

**Visual uniformity:** Carefully remove or cut off the bottom tube seal and make a vertical cut from the bottom to the top of the tube. Carefully cut the tube around the upper rim, open the two flaps, and lay the flaps open to expose the product.

Inspect the product visually for the presence of phase separation, change in physical appearance and texture (e.g., color change, crystallization, lumping), and other properties described in the product specification for *Description*. If there is no significant phase separation or change in physical appearance and texture, and if the product meets the *Description* criteria, the product passes the test. If the product exhibits significant phase separation or change in physical appearance or texture, the product fails the test.

**Uniformity of active ingredient(s):** The following procedures can be modified depending on the sensitivity of the quantitative procedure used to determine the amount of the drug substance(s) present in the formulation.

*For multiple-unit tubes that contain 5 g or more of product*

STAGE 1:

1. Using a single tube, after performing the test for *Visual uniformity*, remove an appropriate amount of the product from the top (i.e., cap end), middle, and bottom (i.e., seal end) portions of the tube. The sample size should be sufficient for at least one quantitative determination of the active ingredient(s) and should not exceed the maximum dose recommended by the product labeling for a single application.
2. Determine the amount of the active ingredient(s) in each portion of the product using any appropriate validated quantitative procedure, and evaluate the test results from the single tube using the *Stage 1* acceptance criteria outlined in number *3*.
3. *Stage 1* acceptance criteria are met if:
   - None of the 3 results are outside of the product assay range, and
   - The maximum difference in the amount of active ingredient(s) determined within the tube is NMT 10.0%. For example, if the 3 measurements within the tube are 97.0%, 95.2%, and 99.7%, the maximum difference would be 4.5% (i.e., 99.7% – 95.2% = 4.5%).
4. Proceed to *Stage 2* testing if *Stage 1* acceptance criteria are not met and none of the test results are outside the product assay range by more than 5.0% (e.g., if the product assay range is 90.0%–120.0%, the range will be 85.0%–125.0%), and the maximum difference in the amount of active ingredient(s) measured within the tube is NMT 10.0%. An example of a product that fails to meet *Stage 1* criteria: if the highest and lowest assay values were 106.0% and 94.7% of label claim, then the difference would be 106.0% – 94.7% = 11.3%.
5. Proceed to *Stage 3* testing if *Stage 1* acceptance criteria are not met, *Stage 2* acceptance criteria cannot be met, NMT 1 of the 3 test results is outside of the product assay range by more than ±5.0%, and the maximum difference of the amount of active ingredient(s) measured within the tube is NMT 15.0%.

STAGE 2:

1. Test an additional 2 tubes for *Visual uniformity* and *Uniformity of active ingredient(s)* for a total of 3 samples each from 3 tubes.
2. Determine the amount of the active ingredient(s) in each portion of the product using any appropriate validated quantitative procedure, and evaluate the test results from the 3 tubes using the *Stage 2* acceptance criteria.
3. *Stage 2* acceptance criteria are met if:
   - The *Visual uniformity* test is met for all tubes;
   - None of the 9 results (i.e., 3 each from 3 tubes) are outside of the product assay range by NMT 5.0%; and
   - The maximum difference of the amount of active ingredient(s) measured within each tube, for each of the samples tested, is NMT 10.0%.
4. Proceed to *Stage 3* testing if NMT 1 of the 9 test results is outside of the product assay range by ±5.0% and/or the maximum difference between the amount(s) of active ingredient(s) measured within each tube is more than 10% but NMT 15.0%.

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STAGE 3:

1. If *Stage 2* has been completed, test an additional 7 tubes for *Visual uniformity* and *Uniformity of active ingredient(s)* for a total of 3 samples each from 10 tubes. If *Stage 2* was skipped, test an additional 9 tubes for *Visual uniformity* and *Uniformity of active ingredient(s)* for a total of 3 samples each from 10 tubes.
2. Determine the amount of active ingredient(s) in each portion of the product using any appropriate validated quantitative procedure, and evaluate the test results from the 10 tubes using the *Stage 3* acceptance criteria as outlined in number *3*.
3. *Stage 3* acceptance criteria are met if:
    - The *Visual uniformity* test is met for all tubes;
    - NMT 1 of the 30 test results is outside of the product assay range by ±5.0%; and
    - The maximum difference of the amount of active ingredient(s) measured within each tube, for each of the 10 tubes tested, is NMT 15.0%.

*For multiple-unit tubes that contain less than 5 g of product*

STAGE 1:

1. Using a single tube, after performing the test for *Visual uniformity*, remove an appropriate amount of product from the top (i.e., cap end) and bottom (i.e., seal end) portions of the tube. The sample size should be sufficient for at least one quantitative determination of the active ingredient(s) and should not exceed the maximum dose recommended by the product labeling for a single application.
2. Determine the amount of the active ingredient(s) in each portion of the product using any appropriate validated quantitative procedure, and evaluate the test results from the tube using the *Stage 1* acceptance criteria outlined in number *3*.
3. *Stage 1* acceptance criteria are met if:
    - Neither result is outside of the product assay range; and
    - The difference between the amount of active ingredient(s) determined for the 2 samples within the tube tested is NMT 10.0%. For example, if the 2 measurements within a tube were 95.2% and 89.7%, the difference would be 5.5%.
4. Proceed to *Stage 2* testing if *Stage 1* acceptance criteria are not met and neither of the test results are outside the product assay range by more than ±5.0% (e.g., if the product assay range is 90.0%–120.0%, the range will be 85.0%–125.0%), and the difference between the amounts of active ingredient(s) measured within the tube is NMT 10.0%.
5. Proceed to *Stage 3* testing if *Stage 1* acceptance criteria are not met, *Stage 2* acceptance criteria cannot be met, NMT 1 of the test results is outside of the product assay range by more than ±5.0%, and the difference between the amounts of active ingredient(s) measured within the tube is NMT 15.0%.

STAGE 2:

1. Test an additional 2 tubes for *Visual uniformity* and *Uniformity of active ingredient(s)* for a total of 2 samples each from 3 tubes.
2. Determine the amount of the active ingredient(s) in each portion of the product using any appropriate validated quantitative procedure, and evaluate the test results from the 3 tubes using the *Stage 2* acceptance criteria as outlined in number *3*.
3. *Stage 2* acceptance criteria are met if:
    - The *Visual uniformity* test is met for all tubes;
    - None of the 6 test results (i.e., 2 each from 3 tubes) are outside of the product assay range by ±5.0%; and
    - The difference between the amount of active ingredient(s) determined for the 2 samples within each tube, for each of the 3 tubes tested is NMT 10.0%.
4. Proceed to *Stage 3* testing if NMT 1 of the 6 test results is outside of the product assay range by ±5.0%, and/or the maximum difference between the amount(s) of active ingredient(s) measured within each tube is more than 10% but NMT 15.0%.

STAGE 3:

1. If *Stage 2* has been completed, test an additional 7 tubes for *Visual uniformity* and *Uniformity of active ingredient(s)* for a total of 2 samples each from 10 tubes. If *Stage 2* was skipped, test an additional 9 tubes for *Visual uniformity* and *Uniformity of active ingredient(s)* for a total of 2 samples each from 10 tubes.
2. Determine the amount of active ingredient(s) in each portion of the product using any appropriate validated quantitative procedure, and evaluate the test results from the 10 tubes using the *Stage 3* acceptance criteria as outlined in number *3*.
3. *Stage 3* acceptance criteria are met if:
    - The *Visual uniformity* test is met for all tubes;
    - 19 of 20 test results are within ±5.0% of the product assay range; and
    - The difference between the amount of active ingredient(s) determined for the 2 samples within each tube, for each of the 10 tubes tested, is NMT 15.0%.

## PRODUCTS PACKAGED IN CONTAINERS OTHER THAN TUBES

For semisolid products packaged in a container other than a tube when the sampling method presented previously cannot be used, other sampling methods are acceptable, such as this method described for a jar:

1. Select a suitable syringe of sufficient length to extend to the bottom of the container.

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2. Remove and set aside the syringe plunger, and cut off the bottom of the syringe barrel. Sampling should take place from a location to the left or right of the mid-line of the jar surface to preserve an undisturbed region on the other side for any additional investigation (see *Figure 1*).

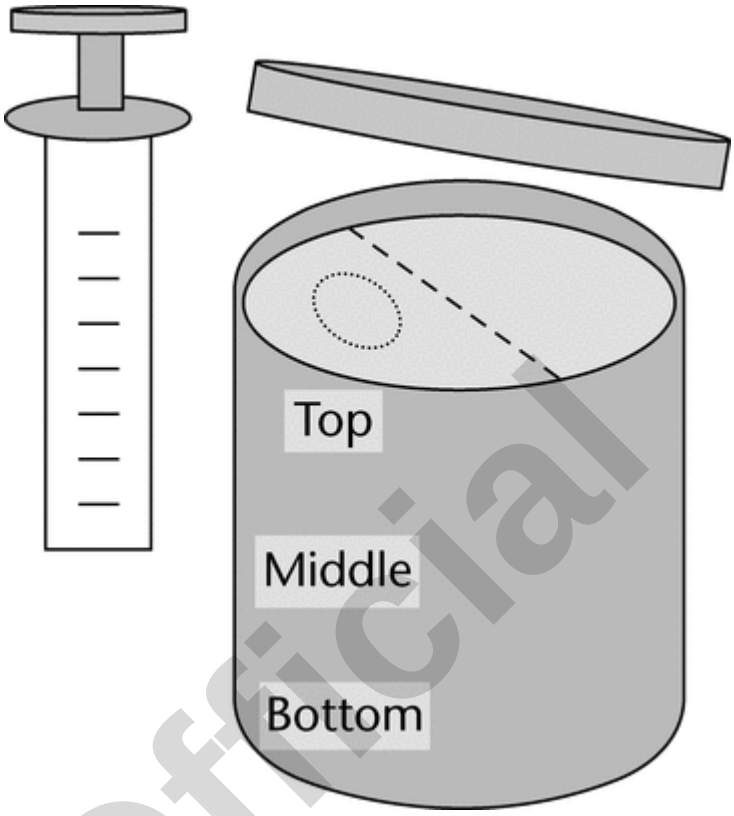


Figure 1. Sampling from a jar container.

3. Slowly push the syringe barrel into the container until it reaches the bottom. Then twist the syringe barrel containing the sample core, and remove the syringe from the container.
4. Insert the syringe plunger into the barrel, and carefully extrude the sample core onto a clean surface in three equal portions to represent the top, middle, and bottom portions of the container.
5. Remove an appropriate sample representative of the top, middle, and bottom portions of the container samples, and test according to the instructions outlined in *Products Packaged in Tubes*.

## Delivered-Dose Uniformity in Metered-Dose Containers

The test for delivered-dose uniformity is required for drug products contained either in metered-dose containers or in premetered-unit presentations. The test for delivered-dose uniformity includes dose uniformity over the entire unit shelf life. Select 1 container. Using separate collection vessels, quantitatively collect delivered doses representing the initial, middle, and the final dose from the container. Appropriately discard doses not collected for testing. Determine the drug substance content of each of the 3 collected samples using an appropriate and validated quantitative procedure or the procedure indicated in the individual monograph. A dose in this test is defined as the minimum recommended number of actuations specified in the product labeling or in the instructions for use but NMT 2 actuations. The target-delivered dose is specified by the product label claim, unless otherwise specified in the individual monograph.

## In-Process Testing

### PUMP FUNCTIONALITY TEST FOR METERED- AND NON-METERED-DOSE CONTAINER CLOSURES

This test is conducted during product development to establish that the airless pumps are performing as intended. This test is product dependent. Therefore, it is part of the manufacturer's controls for the drug product and it is not included in compendial drug product monographs.

During the filling process for semisolid or liquid products packaged in a non-metered-dose container closure requiring an actuator to dispense the product (for example, an airless pump container), in-process testing to verify pump functionality should be examined. Example tests include pumps to prime and total amount dispensed.

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***Change to read:***

# SPECIFIC TESTS FOR TDS

TDS are formulated with an adhesive layer to ensure intimate contact with the skin and allow the delivery of the desired dose of the drug. Adhesives in TDS must permit easy removal of the release liner before use, adhere properly to human skin upon application, maintain adhesion to the skin during the prescribed period of use, and permit easy removal of the TDS at the end of use without leaving a residue or causing damage to the skin or other undesirable effect(s). Additionally, adhesives must be able to maintain the performance of the TDS throughout the shelf life of the drug product.

Testing of the physical properties of the TDS generally include peel adhesion, release liner peel, tack, cold flow, shear, and crystal formation (see *Crystal Formation*). The peel adhesion, release liner peel, and tack tests measure the adhesion properties of the TDS. Each of these tests measures the force required to separate the TDS from another surface. The cold flow and shear tests measure the cohesive properties of the TDS. These latter tests measure the resistance to flow of the adhesive matrix.

Acceptance criteria are product specific and defined to ensure that adhesion of each batch of TDS is within the range defined by the product design and is consistent between batches based on the product development specifications. Acceptance criteria should be established using in vitro testing results of representative batches, including clinical batches for which satisfactory in vivo adhesion performance has been demonstrated. Release and shelf life limits should be the same, unless justified.

In addition to physical testing, this section also discusses the *Leak Test* applicable to form-fill-seal-type (reservoir or pouched) TDS.

## Peel Adhesion Test

This test measures the force required to remove (peel away) a TDS attached to a standard substrate surface (e.g., polished stainless steel). The TDS is applied to the substrate using specified techniques for application and is conditioned at a specified temperature and time. Then the TDS is peeled away from the substrate with an instrument that allows for control of the peel angle (e.g., 90° or 180°) and peel rate (e.g., 300 mm/min), and the force profile is recorded. This procedure is repeated using a minimum of 5 independent samples yielding results from a suitable method. The product fails the test if the overall mean adhesion force is outside of the acceptable range determined during product development. During method development, suitable methods (including test panel surface) need to be identified. The method should allow the TDS to be removed entirely and cleanly, leaving no visually noticeable matrix residue on the substrate surface (i.e., an indication of adhesive failure).

Other failure modes (e.g., delamination at an interface, cohesive failure, etc.) are not indicative of true peel adhesion. The method should be developed to ensure an adhesive failure mode. Suitable methods do not result in undesired adhesion failures, including delamination at an interface (e.g., between a membrane and an adhesive layer or between two different adhesive layers of a bilayer product) or transfer of adhesive to the test panel (i.e., cohesive failure).

Release and shelf life limits should be the same, unless justified.

The geometry of a TDS (e.g., round or rectangular shaped) and its design (e.g., TDS with different surfaces such as an inner part and with a peripheral adhesive ring) should be considered in the method.

The substrate surface should be cleaned regularly and checked for scratches, as this may influence test results.

## Release Liner Peel Test

This test measures the force required to separate the release liner from the adhesive layer of the TDS. The test is performed with a finished product sample. The test sample is conditioned using specific procedures (temperature and time). Then, the release liner is pulled away from the TDS with an instrument that allows for control of the peel angle (e.g., 90° or 180°) and peel rate (e.g., 300 mm/min), and the force profile is recorded. This procedure is repeated using a minimum of 5 independent samples. The product fails the test if the ▲overall▲ (ERR 1-Dec-2023) mean peel force is outside the acceptable range determined during product development.

## Tack Test

A few tack test methods have been developed and the current predominantly used method is the probe tack method. It is up to the TDS manufacturer to decide which tack test is most appropriate for each drug product. The product fails the test if the overall mean of the maximum (tack) force results, using a minimum of 5 independent samples, is outside the acceptable range determined during product development. For additional discussion on the probe tack method, see *Probe Tack Test* ⟨1212⟩.

## Cold Flow Test

Cold flow is the migration of the adhesive matrix beyond the edge of the TDS backing, and through the slit in the release liner, which may occur during the course of product processing and storage. Cold flow is an inherent property of TDS due to the use of pressure-sensitive adhesives that flow when force is applied (i.e., if the adhesive matrix did not flow, the TDS would not stick). The magnitude of the cold flow is generally dependent on the product formulation, packaging design, storage conditions, and storage time. Cold flow should be assessed using a combination of quantitative and qualitative methods. No single quantitative method has been identified to work universally for all TDS. The TDS manufacturer should determine the most suitable cold flow test, or tests, as cold flow may manifest differently for different products. Several different cold flow tests have been developed. Examples include the following:

- Linear measurement of the radial cold flow using microscopy
- Measuring the distance of migrated adhesive matrix at predefined and evenly spaced positions of a TDS

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- Measuring cold flow by applying a reference plate in the size of the TDS plus the acceptable cold flow
- Swabbing and stripping the migrated part of the matrix and determining it gravimetrically or by assay of the drug substance
- Die cutting and punching out the original size of the TDS and determining the amount of migrated matrix on the outside
- Overall area determining methods of cold flow using image analysis tools

Acceptance criteria are product specific and defined to ensure that the cold flow of each batch of TDS is within the range determined during product development and is consistent between batches.

## Shear Test

The shear test measures the cohesive strength of a TDS. It can be measured under static (see *Static Shear Test*) or dynamic conditions. Shear testing may not be feasible for all TDS because the presence of multiple layers of adhesive in the system, the presence of a membrane or scrim, or the use of an emulsion adhesive system may result in the inability to achieve cohesive failure. TDS that are constructed with a peripheral adhesive ring or form-fill-seal TDS may not be suitable for this test. The TDS manufacturer should decide if a shear test is appropriate, and if so, which shear test is most appropriate for each drug product. Acceptance criteria are product specific and defined to ensure that the shear of each batch of TDS is within the range determined during product development and is consistent between batches.

## Static Shear Test

For the static shear test, the time required to remove a standard area of the TDS from the substrate (i.e., stainless steel test panel) under a standard load (e.g., 250 g) is measured. The TDS is applied to a test panel, and the sample is subjected to a shearing force by means of a given weight suspended from the TDS. The test apparatus holds the test panels at 0°–2° from vertical to ensure that the TDS will not experience peeling action when the weight is attached. Dwell time, weight used, test panel type, mode of failure, and sample size should be noted; the time taken for the TDS sample to detach from the test panel is reported. Cohesive failure, i.e., adhesive matrix is left on the TDS and on the substrate plate, should occur. Suitable methods do not result in undesired adhesion failures, including delamination at an interface, e.g., between a membrane and an adhesive layer, or between two different layers of a bilayer product.This procedure is repeated using a minimum of 5 independent samples yielding results from a suitable method. The product fails the test if the overall mean result, i.e., arithmetic mean or geometric mean as determined by the manufacturer, of each TDS batch is outside of the range determined during product development.

## Leak Test

This test is applicable only for form-fill-seal-type (reservoir or pouched) TDS. Form-fill-seal TDS must be manufactured with zero tolerance for leaks because of their potential for dose dumping if leaking occurs.

In-process control methods to examine TDS for leaks or potential leaks are needed and require considerable development on the part of TDS manufacturers.

### IN-PROCESS TESTING

During the manufacturing process, the presence of leakage (or potential for leakage) due to TDS perforation, cuts, and faulty seals resulting from failures such as air bubbles, gel splash, or misalignment of a TDS backing and release liner layers must be examined. Unless automated process analytical technology is implemented, in-process testing to identify these defects should be performed using the following test procedures.

**Visual inspection:**

- A specified number of TDS, defined on the basis of batch size, should be examined randomly.
- Each sampled TDS should be thoroughly visually inspected for leakage.
- The product fails if any of the TDS examined are detected with a leak.

**Seal integrity:** TDS seals should be stress tested to ensure that the application of pressure does not force seals to open, thereby leading to leakage.

- A specified number of TDS, defined on the basis of batch size, should be randomly examined.
- Each sampled TDS should be thoroughly visually inspected for leakage.
- Each sampled TDS is placed on a hard, flat surface and overlaid with a weight so that it is subjected to 13.6 kg. The weight should be left in place for 2 min. Upon removal of the weight, the TDS should be visually inspected for leakage.
- The product fails if the number of TDS detected with a leak is greater than the acceptable limit established by the manufacturer.

**Packaged product testing:** TDS may leak after they have been individually placed in the primary packaging material as a result of the packaging operation itself or by a user opening the packaging. Therefore, TDS should be tested for leakage after they have been manufactured and packaged in their primary packaging material.

- A specified number of TDS, defined on the basis of batch size, should be randomly examined after they have been placed in their primary packaging material.
- The sampled TDS should be removed from their packaging and thoroughly visually inspected for leakage.
- Each sampled TDS should then be uniformly wiped with a solvent-moistened swab. Both the backing side and the release liner side of the TDS should be wiped. The inside surface of the pouch should also be wiped. The swab(s) is then extracted and assayed for the drug.

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- The product fails if the total amount of drug from the TDS, and the corresponding pouch, exceed the acceptable limit established by the manufacturer.

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# ⟨4⟩ MUCOSAL DRUG PRODUCTS—PRODUCT QUALITY TESTS

## INTRODUCTION

The mucosal route of drug administration is subdivided into seven membrane surfaces for the purposes of taxonomic distinction of dosage forms by route of administration. These membrane surfaces are characterized as otic, ophthalmic, nasal, oropharyngeal, urethral, vaginal, and rectal. This grouping does not include the pulmonary mucosal route addressed in *Inhalation and Nasal Drug Products—General Information and Product Quality Tests* ⟨5⟩. A drug product is administered to any of these seven mucosal surfaces to effect either local action or systemic absorption. Local action is to the area proximate to application. Where local action is intended, systemic absorption is not typically desired and is unnecessary for therapeutic effect. In some cases, however, the mucosal delivery of a drug for systemic absorption is used because it avoids first-pass metabolism, it provides more rapid systemic delivery, or it provides an alternative when oral delivery (to the gastrointestinal tract) is not possible due to a disease state. A large number of the dosage forms listed in *Pharmaceutical Dosage Forms* ⟨1151⟩ can be delivered by way of the various membrane surfaces in the mucosal category. [NOTE—All references to chapters above 1000 are for informational purposes only, for use as a helpful resource. These chapters are not mandatory unless explicitly called out for application.]

Analytical procedures and acceptance criteria for testing drug products are divided into two categories: those that assess general product quality attributes and those that assess product performance. Drug product quality tests assess attributes such as identification, assay (strength), dose content uniformity, and impurities and are usually part of the compendial monograph. Product performance tests include the dissolution test for a solid oral dosage form, *Dissolution* ⟨711⟩, and the drug release test, *Drug Release* ⟨724⟩. Taken together, quality and performance tests ensure the identity, strength, quality, and purity of a mucosal drug product.

This chapter provides lists of consolidated common product quality test requirements in a concise and coherent fashion. This chapter applies, in part or in its entirety, when referenced in a drug product monograph (see *General Notices, 3.10 Applicability of Standards*). The quality tests listed can be used, as appropriate, by manufacturers toward the development of new drug product monographs for submission to the *USP*.

## PRODUCT QUALITY TESTS FOR MUCOSAL DRUG PRODUCTS

This chapter provides product quality tests that are generally necessary, tests that apply to specific products, and tests that apply to one or more of the specific mucosal routes. Quality tests listed under a specific mucosal route in this chapter represent expectations for any dosage form administered by that specific route.

### Generally Necessary Tests

Product quality attributes for mucosal dosage forms should reflect acceptable requirements for marketed products. The following tests should be generally applied to all dosage forms intended for mucosal delivery. Tests that are generally necessary for any article include: *Definition*, *Identification*, *Assay*, and *Impurities* (organic, inorganic, and residual solvents). *Uniformity of Dosage Units* ⟨905⟩ is typically included in a *USP* product monograph.

#### DEFINITION

The definition section (see *General Notices, 4.10 Monographs*) in a *USP* monograph describes the drug product and specifies the range of acceptable assayed content of the drug substance(s) present in the dosage form. For certain products, the definition includes any relevant additional information, such as the presence or absence of other components, excipients, or adjuvants, and cautionary statements on toxicity and stability. Appearance information is used in a regulatory submission to aid in product identification. Because the size, shape, color, and other attributes are attributes of individual marketed products, a qualitative description is typically not required as part of a *USP* monograph (see ⟨1151⟩).

#### IDENTIFICATION

Identification is included in a monograph as an aid in verifying the identity of the article (see *General Notices, 5.40 Identification*.)

#### ASSAY

The assay is used to determine the strength (content) of the drug product. Typically, the assay is specific and stability-indicating. When a nonspecific assay is justified, other supporting analytical procedures should ensure that any interfering species will be detected and can be limited. Assay results are often reported as a percentage of the label claim, with acceptance criteria that are typically in the range from 90.0% to 110.0%. For some antibiotic products, the range may be wider. The width of these limits is intended to allow for manufacturing variability, including changes in stability, as well as analytical variation. The narrower acceptance range of 95.0%–105.0% is used less often and with justification.

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### IMPURITIES

Process impurities include those arising from starting materials, synthetic byproducts, and other inorganic and organic impurities that may be present in the drug substance and in the excipients used in the manufacture of the drug product. These impurities are limited, as specified within the drug substance and excipient monographs. Impurities in the drug product may also result from degradation of the drug substance or excipients, from interactions between the drug substance and an excipient, or from interactions between the drug substance and the packaging components. The procedures and acceptance criteria should specifically limit toxic degradation products as well as degradation products that compromise the quality of the article if they exceed certain levels. A more complete discussion of impurities is provided in *Impurities in Drug Substances and Drug Products* ⟨1086⟩ [1] and in ICH Q3B Impurities in New Drug Products.[1]

### UNIFORMITY OF DOSAGE UNITS

Chapter ⟨905⟩ is used to ensure the consistency of drug substance content in dosage units within a narrow range around the label claim. The test is applied only to dosage forms containing a single dose or a part of a dose of the drug substance in each unit. Uniformity of dosage units may be demonstrated by one of two methods: content uniformity or weight variation. Content uniformity is based on the assays of a number of individual dosage units. Weight variation can be used to estimate content uniformity under certain conditions.

## Dosage Forms by Specific Mucosal Route and Product-Specific Tests

In addition to the generally necessary product quality tests already discussed, the dosage form may require specific quality tests that are common across routes of administration. *Injections and Implanted Drug Products* ⟨1⟩ provides testing requirements common to injectable and implantable products. *Oral Drug Products—Product Quality Tests* ⟨2⟩ provides testing requirements for tablets and lozenges. *Topical and Transdermal Drug Products—Product Quality Tests* ⟨3⟩ provides testing requirements common to semisolids (creams, ointments, and gels). Chapter ⟨5⟩ presents testing requirements for sprays and aerosols. Where a dosage form has no specific test given in this chapter, no additional test is required unless included in the individual monograph specification.

### OTIC ROUTE

The otic route is characterized by administration of a preparation into, or by way of, the ear. Demonstration of sterility (see *Sterility Tests* ⟨71⟩) is not always required for products delivered to the ear. Typically, sterility is required where the product is administered to the inner ear or where the eardrum is damaged. Where sterility is not required, the quantitative enumeration of mesophilic bacteria and fungi that grow under anaerobic conditions, *Microbial Enumeration Tests* ⟨61⟩, or the determination of the absence or limited occurrence of specified organisms, *Tests for Specified Microorganisms* ⟨62⟩, may be required.

If an antimicrobial preservative is used, *Antimicrobial Effectiveness Testing* ⟨51⟩ and *Antimicrobial Agents—Content* ⟨341⟩ may be required.

Dosage forms given by the otic route include liquids, solutions, and suspensions.

### OPHTHALMIC ROUTE

The ophthalmic route is to the eye. In addition to the generally necessary tests, the following specific tests for ophthalmic drug products should be considered (see *Table 1*). For products that are injected or implanted into the eye, see ⟨1⟩. Some of the important product quality tests for products administered by the ophthalmic route are listed below. See *Ophthalmic Products—Quality Tests* ⟨771⟩ for details and other product quality information.

- Particulate and Foreign Matter
- Sterility
- Particle Size and Particle Size Distribution
- Antimicrobial Preservative

**Table 1. Drug Products Administered by the Ophthalmic Route, with Product-Specific Tests**

| Ophthalmic Route | |
|---|---|
| **Dosage Form** | **Product-Specific Tests** |
| Gels | *Minimum Fill* ⟨755⟩ |
| | ⟨3⟩ |

[1] ICH Q3B (R2) Impurities in New Drug Products, 2006, http://www.ich.org/products/guidelines/quality/article/quality-guidelines.html. Accessed 28 May 2015.

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**Table 1. Drug Products Administered by the Ophthalmic Route, with Product-Specific Tests** *(continued)*

| Ophthalmic Route | |
|---|---|
| **Dosage Form** | **Product-Specific Tests** |
| Emulsions | *Osmolality and Osmolarity* ⟨785⟩<br>*pH* ⟨791⟩<br>Surface tension<br>*Viscosity—Capillary Methods* ⟨911⟩<br>*Viscosity—Rotational Methods* ⟨912⟩<br>Zeta potential |
| Inserts | *Bacterial Endotoxins Test* ⟨85⟩ |
| Ointments | ⟨755⟩<br>⟨3⟩ |
| Solutions | *Particulate Matter in Ophthalmic Solutions* ⟨789⟩<br>⟨791⟩<br>⟨911⟩<br>⟨912⟩<br>*Viscosity—Rolling Ball Method* ⟨913⟩<br>⟨785⟩ |
| Strips | Currently no specific tests (additional specific monograph requirements may apply) |
| Suspensions | ⟨791⟩<br>⟨785⟩<br>Particle size and particle size distribution<br>⟨911⟩<br>⟨912⟩<br>⟨913⟩ |

## NASAL ROUTE

The nasal route is administration to the nose, or by way of the nose, for local or systemic effect (see *Table 2*).

**Table 2. Drug Products Administered by the Nasal Route, with Product-Specific Tests**

| Nasal Route | |
|---|---|
| **Dosage Form** | **Product-Specific Tests** |
| Aerosols | ⟨5⟩ |
| Gels (Jelly) | ⟨755⟩<br>⟨3⟩ |
| Ointments | ⟨755⟩<br>⟨3⟩ |
| Sprays | ⟨5⟩ |
| Solutions | ⟨5⟩ |

## OROPHARYNGEAL ROUTE

The oropharyngeal route is into the oral cavity and/or pharyngeal region. The oropharyngeal route is subclassified by the specific intra-oral surfaces, such as buccal or sublingual. Buccal and sublingual administrations are typically intended to promote systemic absorption by permeation through the respective mucosa. However, in this context, oral administration may mean topical application for local action (see *Table 3*). Product quality tests for products administered to oropharyngeal surfaces often conform to those for oral administration to the gastrointestinal tract (see ⟨2⟩).

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**Table 3. Drug Products Administered by the Oropharyngeal Route, with Product-Specific Tests**

| Oropharyngeal Route | |
|---|---|
| **Dosage Form** | **Product-Specific Tests** |
| Buccal patches | See ⟨3⟩ for testing requirements common to patches. |
| Films | Currently no specific tests (additional specific monograph requirements may apply) |
| Gels | ⟨755⟩ |
| | ⟨3⟩ |
| Gums | Currently no specific tests (additional specific monograph requirements may apply) |
| Lozenges | Currently no specific tests (additional specific monograph requirements may apply) |
| Ointments | ⟨3⟩ |
| | ⟨755⟩ |
| Solutions (Rinses) | Currently no specific tests (additional specific monograph requirements may apply) |
| Sprays | ⟨5⟩ |
| Tablets | ⟨2⟩ |

## URETHRAL ROUTE

The urethral route is into the urethra, typically for local action, but systemic distribution is also possible. Chapters ⟨61⟩ and ⟨62⟩ may apply. Drug products in this category include urethral inserts.

## VAGINAL ROUTE

The vaginal route is into the vagina, typically for local action, but systemic distribution is also possible. Chapters ⟨61⟩ and ⟨62⟩ may apply (see *Table 4*).

**Relative foam density:** Determine relative foam density by weighing a mass of foam (*m*) and a mass of the same volume of water (*e*) in a flat-bottom dish. Relative foam density = *m*/*e*.

**Volume of foam expansion:** Estimate the volume of foam expansion at 25° using a graduated buret and a foam-generating container equipped with a dose-actuating device and fitted to the buret.

**Table 4. Drug Products Administered by the Vaginal Route, with Product-Specific Tests**

| Vaginal Route | |
|---|---|
| **Dosage Form** | **Product-Specific Tests** |
| Creams | ⟨755⟩ |
| | ⟨3⟩ |
| Foams | ⟨755⟩ |
| | Physical appearance (of the foam and of the collapsed foam)<br>*Relative foam density*<br>*Volume of foam expansion* |
| Gels | ⟨755⟩ |
| | ⟨3⟩ |
| Inserts | Currently no specific tests (additional specific monograph requirements may apply) |

## RECTAL ROUTE

The rectal route is into the rectum. Rectally administered products may produce local effect(s) or delivery to the systemic circulation.

**Softening time of lipophilic suppositories:** The test is intended to determine, under defined conditions, the time that elapses until a suppository maintained in water at 37 ± 0.5° softens to the extent that it no longer offers resistance when a defined weight is applied (see *Table 5*).

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**Table 5. Drug Products Administered by the Rectal Route, with Product-Specific Tests**

| Rectal Route | |
|---|---|
| **Dosage Form** | **Product-Specific Tests** |
| Foams | ⟨755⟩ |
| | Physical appearance (of the foam and of the collapsed foam) |
| | *Relative foam density* |
| | *Volume of foam expansion* |
| Ointments | ⟨755⟩ |
| | ⟨3⟩ |
| Suppositories | Softening time of lipophilic suppositories |
| Solutions | Currently no specific tests (additional specific monograph requirements may apply) |
| Suspensions | Currently no specific tests (additional specific monograph requirements may apply) |

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# ⟨5⟩ INHALATION AND NASAL DRUG PRODUCTS—GENERAL INFORMATION AND PRODUCT QUALITY TESTS

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## 1. INTRODUCTION

Inhalation drug products deliver drugs into the lungs by oral inhalation and include inhalation aerosols, inhalation powders, inhalation sprays, inhalation solutions, inhalation suspensions, solutions for inhalation, and drugs for inhalation solutions dosage forms. Nasal drug products deliver drugs into the nasal cavity and include nasal sprays, nasal solutions, nasal aerosols, and nasal powder dosage forms. This chapter does not address nasal products in gel and ointment form. See *Table 1* for established names and definitions of these dosage forms. Definitions of these drug product dosage forms, brief information about their manufacture, and a glossary of dosage form names can be found in *Pharmaceutical Dosage Forms* ⟨1151⟩. [NOTE—All references to general information chapters are for informational purposes only, for use as a helpful resource. See *General Notices, 3.10 Applicability of Standards* for further information on the applicability of *USP* standards pertaining to a specific monograph requirement.]

**Table 1. Established Names and Definitions**

| Established Name | Definition |
|---|---|
| Inhalation Aerosol | A drug product for oral inhalation that is packaged under pressure, aerosolizes, and delivers a specified amount of active ingredient(s) upon activation of an accurate metering valve system in association with an actuator mechanism. Inhalation aerosol drug products are more commonly known as metered-dose inhalers or MDIs. |
| Inhalation Powder | Drug powder for oral inhalation with the use of a device that aerosolizes and delivers an accurately metered amount of active ingredient(s). Inhalation powder drug products are more commonly known as dry powder inhalers or DPIs. |
| Inhalation Spray | A nonpressurized liquid, sterile (if aqueous-based) drug dosage form for oral inhalation that is packaged in a container–closure system that upon activation aerosolizes and delivers in fine droplets of formulation an accurately metered amount of active ingredient(s). |
| Inhalation Solution | A sterile drug solution for oral inhalation with the use of a nebulization system. |
| Inhalation Suspension | A sterile drug suspension for oral inhalation with the use of a nebulization system. |
| Solution for Inhalation | A sterile drug solution for oral inhalation that must be diluted before it is administered with the use of a nebulization system. |
| [Drug] for Inhalation Solution | A sterile drug powder that, upon the addition of a suitable vehicle, yields a sterile solution conforming in all respects to an inhalation solution. |
| Nasal Spray | A nonpressurized, accurately metered, liquid drug dosage form for local application into the nasal passages that is packaged in a container–closure system that upon activation aerosolizes and delivers in droplets of formulation an accurately metered amount of active ingredient(s). |
| Nasal Aerosol | A drug product for local application into the nasal passages that is packaged under pressure, aerosolizes, and delivers a specified amount of active ingredient(s) upon activation of an accurate metering valve system in association with an actuator mechanism. |
| Nasal Solution | A nonpressurized, liquid drug dosage form for local application into the nasal passages. |
| Nasal Powder | Drug powder for local application into the nasal passages with the use of a device that aerosolizes and delivers an accurately metered amount of active ingredient(s). |

This chapter provides lists of consolidated common product quality test requirements in a concise and coherent fashion. This general chapter applies, in part or in its entirety, when referenced in a drug product monograph (see *General Notices, 3.10 Applicability of Standards*) and includes the quality tests for the specific route of administration. The quality tests listed can be used, as appropriate, by manufacturers toward the development of new drug product monographs for submission to the *USP–NF*. If a validated performance test procedure is available for the specific drug product, it is identified in a general chapter below 1000. Additional information, or information on promising technologies that have not yet been fully validated, may be presented in general information chapters.

### Drug Product General Quality Tests and Performance Quality Tests

A *USP* drug product monograph contains tests, analytical procedures, and acceptance criteria. Drug product tests are divided into two categories: 1) general quality tests; and 2) product performance tests, e.g., delivered dose uniformity and its physical characteristics such as aerodynamic particle size distribution and/or droplet ▲(or particle)▲ (USP 1-Aug-2023) size distribution. General

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quality tests assess the integrity of the dosage form, whereas product performance quality tests assess delivery of the drug and other attributes that may relate to in vivo drug performance.

Taken together, general quality and product performance tests ensure the identity, strength, quality, and purity of inhalation and nasal drug products.

The next two sections of this chapter list product quality attributes for inhalation drug products and for nasal drug products, respectively. The final section describes in greater detail the quality tests for inhalation and nasal drug products. *Inhalation and Nasal Drug Products: Aerosols, Sprays, and Powders—Performance Quality Tests* ⟨601⟩ contains product performance tests for inhalation and nasal drug products and ▲*Products for Nebulization—Characterization Tests* ⟨1601⟩ contains performance tests for nebulization products. These two chapters should be used in conjunction with this chapter, ⟨5⟩, as applicable.▲ (USP 1-Aug-2023)

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## 2. GENERAL QUALITY TESTS FOR INHALATION DRUG PRODUCTS

### Inhalation Aerosol

DESCRIPTION

Aerosol in this context is a dosage form consisting of a liquid preparation packaged under pressure and intended for administration as a fine mist. The descriptive term "aerosol" also refers to the fine mist of small droplets and/or solid particles that are emitted from the product. Inhalation aerosols, also known as metered-dose inhalers (MDIs), are preparations characterized by dispersion of the active pharmaceutical ingredient into the airways during oral inspiration for either local or systemic effect, and nasal aerosols, also known as nasal MDIs, are characterized by deposition in the nasal cavity for either local or systemic effect.

An aerosol formulation typically contains a drug substance(s) dissolved or suspended in a propellant or a mixture of propellant(s) and co-solvent(s) and possibly other suitable excipients. An inhalation aerosol drug product delivers a specified amount and quality of therapeutically active ingredient(s) upon activation of an accurate metering valve system. General quality tests for inhalation aerosol drug products include the following attributes (see *4. Description of Product Quality Tests* for more detailed discussions of each test):

- *Assay (strength and content uniformity)*
- *Co-Solvent Content [(e.g., ethanol) if present]*
- *Elemental Impurities*
- *Foreign Particulate Matter*
- *Identification*
- *Impurities and Degradation Products*
- *Leachables*
- *Leak Rate*
- *Microbial* ▲*Quality*▲ (USP 1-Aug-2023)
- *Net Fill Weight*
- *Residual Solvents*
- *Spray Pattern*
- *Valve Delivery*
- *Water Content*
- For performance quality tests, refer to ⟨601⟩

### Inhalation Solution

DESCRIPTION

Inhalation solution drug products typically are water-based sterile preparations. They are intended for delivery to the lungs by nebulization with a specified nebulizer. Such drug preparations typically are packaged in single-dose semipermeable containers within protective packaging to minimize ingress of volatile foreign contaminants, loss of solvent, and exposure to oxygen and light. Nebulization involves continuous generation and delivery to the patient of a fine mist of aqueous droplets containing a drug solution by means of ultrasonic energy, Venturi effect, or other appropriate mechanical/electrical means. General quality tests for inhalation solutions include the following attributes (see *4. Description of Product Quality Tests* for more detailed discussions of each test):

- *Assay (strength and content uniformity)*
- *Assay for Antimicrobial Preservative and Stabilizing Excipients (if present)*
- *Content Uniformity for Premetered Dosage Forms*
- *Elemental Impurities*
- *Foreign Particulate Matter*
- *Identification*
- *Impurities and Degradation Products*
- *Leachables*

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- *Net Fill Weight*
- *Osmolality* (if relevant)
- *pH*
- *Residual Solvents*
- *Sterility*
- *Viscosity*, if relevant
- *Weight Loss*
- For performance quality tests, refer to ▲⟨1601⟩▲ (USP 1-Aug-2023)

## Inhalation Suspension

DESCRIPTION

Inhalation suspension drug products typically are water-based sterile preparations. They are intended for delivery to the lungs by nebulization with a specified nebulizer. Such drug preparations typically are packaged in single-dose semipermeable containers within protective packaging to minimize ingress of volatile foreign contaminants, loss of solvent, and exposure to oxygen and light. Nebulization involves continuous generation and delivery to the patient of a fine mist of aqueous droplets containing the formulation components by means of ultrasonic energy, Venturi effect, or other appropriate mechanical means. General quality tests for inhalation suspensions include the following attributes (see *4. Description of Product Quality Tests* for more detailed discussions of each test):

- *Primary Particle Size Distribution*
- For all other general quality attributes, refer to the previous *Inhalation Solution* attributes

## Solution for Inhalation

DESCRIPTION

Solution for inhalation drug products typically are water-based sterile preparations. Upon dilution, in accordance with labeling, including identity and amount of dilution vehicle, they are intended for delivery to the lungs by nebulization with a specified nebulizer. Such drug preparations typically are packaged in single-dose semipermeable containers within protective packaging to minimize ingress of volatile foreign contaminants, loss of solvent, and exposure to oxygen and light. Nebulization involves continuous generation and delivery to the patient of a fine mist of aqueous droplets containing the formulation components by means of ultrasonic energy, Venturi effect, or other appropriate mechanical/electrical means. General quality tests for solutions for inhalation include the following attributes (see *4. Description of Product Quality Tests* for more detailed discussions of each test):

- *Clarity and Color of Solution upon Dilution* in accordance with the labeling
- For all other general quality attributes, refer to the previous *Inhalation Solution* attributes

## Drug for Inhalation Solution

DESCRIPTION (POWDER)

Drug for inhalation solution is a (specified color) sterile drug powder formulation that upon the addition of a suitable vehicle, in accordance with labeling, including identity and amount of dilution vehicle, yields a sterile solution conforming in all respects to the *Inhalation Solution* requirements. General quality tests for drug for inhalation solutions include the following attributes (see *4. Description of Product Quality Tests* for more detailed discussions of each test):

- *Reconstitution Time (powder)*
- *Water Content*
- Clarity, color, and completeness of solution within specified time, upon reconstitution
- For all other general quality attributes, refer to the previous *Inhalation Solution* attributes upon (re)constitution of the drug product

## Inhalation Spray

DESCRIPTION

Inhalation spray drug products typically are water-based sterile formulations packaged in a compact container–closure system containing an integral spray pump unit that, upon activation, delivers an accurately metered amount of fine droplets of the formulation. The droplets can be generated by various means such as mechanical action, power assistance, or energy from the patient's inspiration. The mechanisms by which they generate droplets distinguish the various types of inhalation sprays. These drug products may be unit-dose or multidose presentations. Inhalation spray drug products may be designed as premetered or device-metered presentations. A premetered unit contains a previously measured amount of liquid formulation in an individual container (e.g., a blister) that is inserted in the device by the patient before use. A device-metered product contains a sufficient amount of liquid formulation for a prescribed number of doses in a reservoir, and each dose is delivered as an accurately

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metered spray by the device throughout the unit's life. General quality tests for inhalation sprays include the following attributes (see *4. Description of Product Quality Tests* for more detailed discussions of each test):

- *Plume Geometry*
- For all other general quality attributes, refer to the previous *Inhalation Solution* attributes
- For performance quality tests, refer to ⟨601⟩

## Inhalation Powder

DESCRIPTION

Inhalation powder drug products, commonly known as dry powder inhalers (DPIs), dispense powders for inhalation with the use of a device that aerosolizes and delivers an accurately metered amount of active ingredient(s) with consistent physical characteristics alone or with a suitable excipient(s). Current designs include premetered and device-metered DPIs, all of which rely on various energy sources to create and disperse the aerosol during patient inspiration.

Premetered DPIs contain previously measured amounts of formulation in individual containers (e.g., capsules or blisters) that are inserted into the device before use. Premetered DPIs also may contain premetered dose units as ordered multidose assemblies in the delivery system. Device-metered DPIs have an internal reservoir that contains a sufficient quantity of formulation for multiple doses that are metered by the device itself during actuation by the patient. General quality tests for inhalation powders include the following attributes (see *4. Description of Product Quality Tests* for more detailed discussions of each test):

- *Assay (strength and content uniformity)*
- *Content Uniformity for Premetered Dosage Forms*
- *Elemental Impurities*
- *Foreign Particulate Matter*
- *Identification*
- *Impurities and Degradation Products*
- *Leachables*
- *Microbial* ▲*Quality*▲ (USP 1-Aug-2023)
- Net Content (device-metered)
- *Residual Solvents*
- *Water Content*
- For performance quality tests, refer to ⟨601⟩

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# 3. GENERAL QUALITY TESTS FOR NASAL DRUG PRODUCTS

## Nasal Aerosol

Refer to the above *Inhalation Aerosol* attributes.

## Nasal Spray

DESCRIPTION

Nasal spray drug products typically are water-based formulations applied to the nasal cavity for local and/or systemic effects. They contain therapeutically active ingredient(s) dissolved or suspended in solution or mixtures of excipients in a nonpressurized compact container–closure system. The container–closure system includes an integral spray pump unit that upon activation delivers an accurately metered amount of the formulation as a mist. Dispersion of the formulation as a spray typically is accomplished by forcing the formulation through the nasal actuator and its orifice. Often, such drug products are multidose device-metered (see *Inhalation Spray*) presentations in which the dose is metered by the spray pump. Nasal spray drug products also may be designed as premetered presentations. General quality tests for nasal sprays include the following attributes (see *4. Description of Product Quality Tests* for more detailed discussions of each test):

- *Assay (strength and content uniformity)*
- *Assay for Antimicrobial Preservative and Stabilizing Excipients (if present)*
- *Content Uniformity for Premetered Dosage Forms*
- *Elemental Impurities*
- *Foreign Particulate Matter*
- *Identification*
- *Impurities and Degradation Products*
- *Leachables*
- *Microbial* ▲*Quality*▲ (USP 1-Aug-2023)
- *Net Fill Weight*

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- *Osmolality*
- *pH*
- *Primary Particle Size Distribution* (for suspensions)
- *Pump Delivery*
- *Residual Solvents*
- *Spray Pattern*
- *Viscosity*
- For performance quality tests, refer to ⟨601⟩

### Nasal Powder

Refer to the previous *Inhalation Powder* quality attributes as applicable.

### Nasal Solution

DESCRIPTION

Nasal solutions are drug products that typically are water-based formulations applied to the nasal cavity for local effect. They may contain drug substance(s) dissolved in solution or mixtures of excipients in a nonpressurized compact container–closure system. The container–closure system includes a delivery system that administers nonmetered amounts of drops or a fine mist of droplets of the formulation. Typically, such drug products are multidose presentations. General quality tests for nasal solution drug products include the following attributes (see *4. Description of Product Quality Tests* for more detailed discussions of each test):

- *Assay (strength and content uniformity)*
- *Assay for Antimicrobial Preservative and Stabilizing Excipients (if present)*
- *Elemental Impurities*
- *Foreign Particulate Matter*
- *Identification*
- *Impurities and Degradation Products*
- *Leachables*
- *Microbial* ▲*Quality*▲ (USP 1-Aug-2023)
- *Net Fill Weight*
- *Osmolality*
- *pH*
- *Residual Solvents*
- *Viscosity*

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## 4. DESCRIPTION OF PRODUCT QUALITY TESTS

Product quality tests are listed as follows and should be applied to inhalation and nasal drug products and to products for nebulization. Product-specific quality tests are addressed in product monographs.

### Assay (strength and content uniformity)

The *USP* assay test of a drug substance in the drug product container is determined by means of a validated stability-indicating procedure following *Validation of Compendial Procedures* ⟨1225⟩. The *USP* assay test should measure the amount of drug substance(s) in the unit and its stability throughout the expiration dating period, including adherence of the drug substance to the container–closure components. Appropriate acceptance criteria can provide added assurance of manufacturing reproducibility and may ensure better conformance in performance attributes (e.g., delivered dose uniformity).

If a drug product is labeled to contain a single enantiomer of a chiral drug substance, analysts can use a chiral assay or a combination of an achiral assay and a validated procedure to control the presence of the undesired enantiomer as an impurity.

### Assay for Antimicrobial Preservative and Stabilizing Excipients (if present)

The assay of any preservative (e.g., an antimicrobial) in a multidose container or stabilizing excipient (e.g., an antioxidant, an agent specifically added to minimize or prevent degradation) in a formulation should be determined analytically, typically with a validated stability-indicating procedure following the current International Council for Harmonisation ICH Q2 guidance. The assay acceptance criteria for antimicrobial preservatives normally are based on appropriate preservative effectiveness demonstrated by a microbial challenge test.

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## Clarity and Color of Solution upon Dilution

Solution for inhalation and drug for inhalation solution dosage forms must be diluted and reconstituted in accordance with labeling before administration by nebulization. The type and amount of the vehicle used for dilution and reconstitution must be specified on the labeling. Appropriate studies must be undertaken to fully assess quantitatively the clarity and color of the solution upon dilution and reconstitution. The studies also should include appropriate physical and chemical stability studies as well as studies of performance characteristics.

## Content Uniformity for Premetered Dosage Forms

See *Uniformity of Dosage Units* ⟨905⟩.

## Co-Solvent Content [(e.g., ethanol) if present]

If a co-solvent is used in a drug product formulation, a specific assay with appropriate acceptance criteria should be included.

## Description

See the previous descriptions corresponding to applicable dosage forms, outlined in *2. General Quality Tests for Inhalation Drug Products* and *3. General Quality Tests for Nasal Drug Products* of this chapter, in association with their container–closure delivery systems and the respective labeling statement of the monograph of a drug product. This test should address the appearance of the contents of the container (i.e., dosage form) and the appearance and integrity of the components of the container–closure system for conformance to their respective descriptions.

## Elemental Impurities

Drug products should be evaluated for elemental impurities (see *Elemental Impurities—Limits* ⟨232⟩). Validated analytical procedures should be used as described in *Elemental Impurities—Procedures* ⟨233⟩.

## Foreign Particulate Matter

▲Foreign particulate matter in these drug products should be adequately controlled. Particulate matter in inhalation and nasal drug products may originate during the manufacturing process and from formulation and container–closure components. Method approaches presented in *Particulate Matter in Injections* ⟨788⟩ and *Light Diffraction Measurement of Particle Size* ⟨429⟩ may be utilized with proper modifications. Following a detailed toxicological assessment of the type, origin, amount, and size of any foreign particulates in the 10–25 µm range, greater than 25-µm range, and fine particulates (e.g., 1–10 µm), appropriate specifications should be established throughout the expiration dating period to confirm overall quality.▲ (USP 1-Aug-2023)

## Identification

A specific identification test or tests are used to verify the identity of the drug substance in the drug product. If a nonspecific method is used for identification, then it should be combined with a second independent and complementary method. A specific identification test for polymorphic forms should be carried out, if applicable. Moreover, if the drug substance is a salt, an appropriate identification test also should be included for the counterion.

## Impurities and Degradation Products

Validated stability-indicating analytical procedure(s) following ⟨1225⟩ should be used to determine the levels of impurities and degradation products in a drug product. Typically, the acceptance criteria are set for ▲specified▲ (USP 1-Aug-2023), unspecified, and total impurities and degradation products following current ICH Q3B guidance. For reporting, identification, and qualification thresholds and other relevant information, follow current ICH Q3B and ICH M7 guidelines.

## Leachables

Inhalation and nasal drug products should be evaluated for compounds that may leach from elastomeric and plastic components and from coatings of components of the container–closure system in direct contact with the formulation. Additionally, the drug product inadvertently may contain other residual contaminants from manufacturing and processing. Leachables may include polynuclear aromatics, nitrosamines, monomers, plasticizers, accelerators, antioxidants, and vulcanizing agents. Processing contaminants may include surface treatment or processing agents that may dissolve, chemically associate, or become suspended in the formulation.

Thus, throughout the expiration-dating period, the drug product should be evaluated for compounds that can migrate into the formulation from a variety of sources. The type of testing that should be performed depends on whether the formulation is a powder or a liquid and the composition of the container–closure system, e.g., a drug product packaged in a semipermeable container should be evaluated for ingress of volatile leachables. Appropriate specifications using validated analytical procedures should be applied to identify, monitor, and quantify the compounds in the drug product with appropriate minimum levels of quantification. Corresponding acceptance criteria should be established and justified from toxicological and safety perspectives.

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▲For additional information and guidance on these and other quality control related issues including extract and leachables identification, characterization, analytical techniques, validation of quantitative leachables, specifications, as well as safety assessment approaches, refer to *Assessment of Extractables Associated with Pharmaceutical Packaging/Delivery Systems* ⟨1663⟩, *Assessment of Drug Product Leachables Associated with Pharmaceutical Packaging/Delivery Systems* ⟨1664⟩, and *Orally Inhaled and Nasal Drug Products* ⟨1664.1⟩.▲ (USP 1-Aug-2023)

## Leak Rate

Leak rate studies of inhalation and nasal aerosol drug products can be used during drug product development and characterization to support the selection of appropriate container–closure components (e.g., valve and canister) and drug product manufacturing parameters, including the crimping process and 100% heat stress test prior to the equilibration/quarantine period. Release and stability specifications for the USP leak rate test can include multiple units from each batch based on weight difference determination with time at a specified temperature. See *Leak Rate* ⟨604⟩ for additional information.

## Microbial ▲Quality▲ (USP 1-Aug-2023)

The microbial quality of dosage forms, where indicated in *2. General Quality Tests for Inhalation Drug Products* and *3. General Quality Tests for Nasal Drug Products*, normally is controlled by appropriate validated test(s) and acceptance criteria for total aerobic count, total yeasts and molds count, and freedom from ▲specified microorganisms.▲ (USP 1-Aug-2023) Acceptance criteria can be expressed on a per-container, ▲per-gram, or per-milliliter▲ (USP 1-Aug-2023) basis. Refer to ▲*Microbiological Examination of Nonsterile Products—Tests for Burkholderia cepacia Complex* ⟨60⟩,▲ (USP 1-Aug-2023) *Microbial Enumeration Tests* ⟨61⟩, ▲*Tests for Specified Microorganisms* ⟨62⟩,▲ (USP 1-Aug-2023) *Alternative Microbiological Sampling Methods for Nonsterile Inhaled and Nasal Products* ⟨610⟩, *Microbiological Examination of Nonsterile Products: Acceptance Criteria for Pharmaceutical Preparations and Substances for Pharmaceutical Use* ⟨1111⟩, and ▲*Bioburden Control of Nonsterile Drug Substances and Products* ⟨1115⟩▲ (USP 1-Aug-2023) for additional information.

## Net Fill Weight

The total net weight of the formulation in the container should be assessed and controlled with a test and acceptance criteria. See *Minimum Fill* ⟨755⟩ for additional information.

## Osmolality

To control the tonicity of the formulation of dosage forms, where indicated in *2. General Quality Tests for Inhalation Drug Products* ▲and in *3. General Quality Tests for Nasal Drug Products*,▲ (USP 1-Aug-2023) the product should be tested for osmolality with appropriate specifications as described in *Osmolality and Osmolarity* ⟨785⟩.

## pH

Appropriate specification for the pH of the formulation of dosage forms, where indicated in *2. General Quality Tests for Inhalation Drug Products* and *3. General Quality Tests for Nasal Drug Products*, should be established as described in *pH* ⟨791⟩.

## Primary Particle Size Distribution

For suspension preparation drug products, appropriate method(s) and corresponding acceptance criteria should be used for the determination of the particle size distribution of the drug substance particles in the formulation within the container. Microscopic examination of the product formulation provides information and changes over product stability on the presence of large particles, morphology of the drug substance and carrier particles, extent of agglomerates, crystal growth, and foreign particulate matters. Additionally, where the crystalline form of the drug substance can affect the bioavailability, performance, stability, or other properties of the drug product, microscopic evaluation or other appropriate methods are recommended to control and monitor the crystalline and amorphous forms if changes are observed on stability. See *Optical Microscopy* ⟨776⟩ for additional information. For aerodynamic particle size distribution and droplet size distribution tests of inhalation and nasal drug products, as applicable, refer to ⟨601⟩.

## Plume Geometry

Because various factors can affect the plume characteristics of the spray of an inhalation aerosol, inhalation spray, nasal aerosol, nasal spray, or nasal powder (if device is pump-dependent) drug product, its full characterization is important for assessing the performance of the delivery system. Plume geometry can be determined by a variety of procedures using appropriately validated methods. Plume geometry also can be controlled by appropriate acceptance criteria that measure spray pattern characteristics, including shape and size of the evolving spray plume under defined experimental and instrumental test conditions.

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## Pump Delivery

A shot weight test (amount of formulation released per actuation) delivered from the individual pump of the drug product should be performed to better ensure reproducible performance of nasal spray drug products. Appropriate specifications for the pump delivery should be set.

## Reconstitution Time (powder)

Drug for inhalation solution dosage forms must be (re)constituted before administration with the use of a specified nebulization system. Hence, appropriate compatibility studies should be undertaken to fully assess the type and amount of the solvent(s), as well as (re)constitution time for preparation of the final solution for patient administration. The compatibility studies also should include appropriate physical and chemical stability studies on the reconstituted solution, including its performance characterization.

## Residual Solvents

Suitable and validated tests should be used to determine the levels of any solvent(s) in the drug product. Refer to *Residual Solvents* ⟨467⟩ for additional information.

## Spray Pattern

Because various factors can affect the spray pattern of an inhalation aerosol, nasal aerosol, or nasal spray drug product, full spray pattern characterization is important for assessing the performance of the specific valve and the actuator or the pump. The spray pattern can be determined using appropriately validated methods and corresponding acceptance criteria that measure the shape, density, and size of the pattern. The test procedure for spray patterns normally is specific to the drug product and may include, among others, the distance between the mouthpiece and the measurement plane or collection surface, minimum number of actuations per spray pattern to enable discrimination, orientation of the collection surface relative to the mouthpiece, and visualization procedure(s).

## Sterility

All aqueous-based inhalation dosage forms are sterile preparations and should meet the requirements of *Sterility Tests* ⟨71⟩.

## Valve Delivery

A shot weight test (amount of formulation released per actuation) delivered from the individual valve of the drug product should be performed to better ensure reproducible performance of inhalation and nasal aerosol drug products. Appropriate specifications for the valve delivery should be set.

## Viscosity

A test for viscosity with appropriate acceptance criteria should be included for dosage forms, where indicated in *2. General Quality Tests for Inhalation Drug Products* and *3. General Quality Tests for Nasal Drug Products*, as appropriate (see *Viscosity—Capillary Methods* ⟨911⟩, *Viscosity—Rotational Methods* ⟨912⟩, and *Viscosity—Rolling Ball Method* ⟨913⟩).

## Water Content

Appropriate specification for water content of dosage forms, where indicated in *2. General Quality Tests for Inhalation Drug Products* and *3. General Quality Tests for Nasal Drug Products*, should be established to ensure the drug product's continued stability and acceptable performance. Validated analytical procedures should be used as described in *Water Determination* ⟨921⟩. Proceed as directed in ⟨921⟩ with the following modification: provide the closed-system titrating vessel with an opening through which passes a coarse-porosity gas dispersion tube connected to a sampling cylinder.

## Weight Loss

Drug products should be evaluated for weight loss where applicable, e.g., drug products packaged in semipermeable containers, to assess the moisture-loss protective properties of the overall container–closure system.

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# ⟨7⟩ LABELING

## INTRODUCTION

This general chapter provides definitions and standards for labeling of official articles. Labeling standards for an article recognized in *USP–NF* are expressed in the article's monograph and applicable general chapters. It is intended that all articles in *USP* or *NF* will be subject to the labeling requirements specified in this chapter by means of a provision in *General Notices, 10 Preservation, Packaging, Storage, and Labeling*, unless different requirements are provided in a specific monograph. As with compendial standards for naming, identity, strength, quality, and purity, compendial requirements for labeling have a role in the adulteration and misbranding provisions of federal law [see the Federal Food, Drug, and Cosmetic Act (FDCA) sections 501(b), 502(e)(3)(b), 502(g), and 502(h)]. Exceptions or additional requirements specific to animal drug products and compounded preparations are provided in separate sections. Vaccine labeling is not included in this general chapter.

## DEFINITIONS

The term "labeling" includes all labels and other written, printed, or graphic matter on an article's immediate container or on, or in, any package or wrapper in which it is enclosed, except any outer shipping container. The term "label" is that part of the labeling on the immediate container.

A shipping container that contains a single article, unless the container also is essentially the immediate container or the outside of the consumer package, must be labeled with a minimum of product identification (except for controlled substances), lot number, expiration date, and conditions for storage and distribution.

Beyond-use dates (BUDs) and expiration dates are not the same. An expiration date identifies the time during which a conventionally manufactured product, active ingredient, or excipient can be expected to meet the requirements of a compendial monograph, if one exists, provided it is kept under the prescribed storage conditions. The expiration date limits the time during which the conventionally manufactured product, active pharmaceutical ingredient (API), or excipient may be dispensed or used. Expiration dates are assigned by manufacturers of conventionally manufactured products based on analytical and performance testing of the sterility, chemical and physical stability, and packaging integrity of the product. Expiration dates are specific for a particular formulation in its container and at stated exposure conditions of illumination and temperature.

The beyond-use date (BUD) is the date or time beyond which a compounded preparation must be discarded. The date or time is determined from the date the preparation was compounded.

## LABELS AND LABELING FOR DRUG PRODUCTS AND COMPOUNDED PREPARATIONS EXPRESSED AS ACTIVE MOIETY IN NAME AND STRENGTH

The names and strengths of drug products and compounded preparations formulated with a salt of an acid or base are to be expressed in terms of the active moiety on the label (see *Nomenclature* ⟨1121⟩*, Monograph Naming Policy for Salt Drug Substances in Drug Products and Compounded Preparations*).

### Labeling

The labeling clearly states the specific salt form of the active moiety that is present in the product or preparation because this information may be useful to practitioners and patients. The names and strengths of both the active moiety and specific salt form (when applicable) are provided in the labeling.

### Exceptions

In rare cases in which the use of the specific salt form of the active moiety in the title provides vital information from a clinical perspective, an exception to this policy may be considered. In such cases, when the monograph title contains the specific salt form of the active moiety, the strength of the product or preparation is also expressed in terms of the specific salt form.

## LABELS AND LABELING FOR INJECTABLE PRODUCTS

The labels[1] and the labeling state the following information:

- Name of the product
  - In the case of a liquid, the quantity or proportion of each active moiety or drug substance in a specified volume
  - In the case of any product to which a diluent must be added before use, the quantity or proportion of each active moiety or drug substance, name and volume of diluent to be added, the concentration after the diluent is added, directions for proper storage of the constituted solution, and a BUD (see *Expiration Date and Beyond-Use Date*)
- Route(s) of administration

[1] If there are space limitations, see 21 CFR§ 201.10(i), 21 CFR§ 201.105(b), 21 CFR§ 610.60.

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- Name and quantity or proportion of all inactive ingredients except ingredients added to adjust the pH or to make the drug isotonic may be declared by name with a statement of their effect; if the vehicle is *Water for Injection* it need not be named
- Statement of storage conditions
- Name and place of business of the manufacturer, packer, or distributor
- Identifying lot number and expiration date
- "Rx only" for human drugs
- The recommended or usual dosage.

The container must be labeled so that a sufficient area of the container remains uncovered for its full length or circumference to permit inspection of the contents.

The lot number must be traceable to the complete manufacturing history of the specific package, including all manufacturing, filling, sterilizing, and labeling operations.

If the individual monograph permits varying concentrations of active moiety or drug substance in a large-volume injection (LVI), the concentration of each active moiety or drug substance named in the official title is stated as if it were part of the official title (e.g., *5% Dextrose Injection*, or *Dextrose Injection 5%*, or *5% Dextrose and 0.2% Sodium Chloride Injection* or *Dextrose (5%) and Sodium Chloride (0.2%) Injection*).

## Quantity and Total Volume for Injectable Drug Products Packaged in Single- and Multiple-Dose Containers

For injectable drug products greater than 1 mL, whether packaged in single- or multiple-dose containers, the quantity per total volume should be the primary and prominent expression on the principal display panel of the label, followed in close proximity by quantity per milliliter enclosed by parentheses (quantity/mL).

For containers that hold a volume of less than 1 mL, the quantity per fraction of a milliliter should be the only expression of strength. For containers that hold a volume equal to 1 mL, the strength should be expressed as quantity per milliliter (quantity/mL), not quantity/1 mL.

The following example formats are acceptable:

1. For containers less than 1 mL: 12.5 mg/0.625 mL
2. For containers equal to 1 mL: 5 mg/mL (not 5 mg/1 mL)
3. For containers greater than 1 mL:

Example 1: 500 mg/10 mL
(50 mg/mL)

Example 2: 25,000 Units/5 mL
(5,000 Units/mL)

In certain cases, the primary and prominent expression of the total drug content per container is not effective in preventing medication errors and therefore in those cases, the total drug content per container should not be the primary and prominent expression of strength. Insulin products are an example of a product class that is an exception from the total drug content per container requirement. Another exception to expressing strength as quantity per total volume is lidocaine (or similar drugs for local anesthesia) where the product may be ordered and administered by percentage (e.g., 1% or 2%). In such cases, the percentage strength as well as the quantity per total volume followed in close proximity by quantity per milliliter enclosed by parentheses must be used.

Example 1: 1%
(100 mg/10 mL)
(10 mg/mL)

Example 2: 2%
(1000 mg/50 mL)
(20 mg/mL)

Dry solids that must be constituted should follow the same format with the exception that only the quantity of the drug in the container should be listed as the primary expression of strength, not the quantity per total volume or quantity per milliliter (quantity/mL).

Example: 500 mg/vial

## Ratio Expression of Strength

Single-entity injectable drug products must be labeled in terms of quantity per milliliter (quantity/mL) and not as a ratio expression.

Examples:

Epinephrine Injection, 1:1000 must be expressed as 1 mg/mL.
Epinephrine Injection, 1:10,000 must be expressed as 0.1 mg/mL.
Isoproterenol Hydrochloride Injection, 1:5000 must be expressed as 0.2 mg/mL.
Neostigmine Methylsulfate Injection, 1:1000 must be expressed as 1 mg/mL.

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Single-entity injectable drug products greater than 1 mL should be formatted as quantity per total volume on the principal display panel of the label followed in close proximity by quantity per milliliter (quantity/mL) enclosed by parentheses.

When combined with a local anesthetic, the concentration of epinephrine will be expressed as a ratio.

Examples:

Lidocaine Hydrochloride and Epinephrine Injection 1%/1:100,000
or
Lidocaine Hydrochloride 1%
and
Epinephrine Injection 1:100,000

## Pharmacy Bulk Package

Where a container is offered as a Pharmacy Bulk Package, the label must: 1) state prominently "Pharmacy Bulk Package—Not for Direct Infusion"; 2) contain or refer to information on proper techniques to help assure safe use of the product; and 3) bear a statement limiting the time frame in which the container may be used once it has been entered, provided it is held under labeled storage conditions (see *Packaging and Storage Requirements* ⟨659⟩).

## Imaging Bulk Package

Where a container is offered as an Imaging Bulk Package, the label must: 1) state prominently "Imaging Bulk Package" and, in juxtaposition with this statement, include the following use statement: "For use only with an automated contrast injection system, contrast management system, or contrast media transfer set approved or cleared for use with this contrast agent in this Imaging Bulk Package"; 2) bear a statement limiting the time frame in which the container may be used once it has been entered, provided it is held under the labeled storage conditions; 3) bear the statement "See drug and device labeling for information on devices indicated for use with this Imaging Bulk Package and techniques to help assure safe use." (See ⟨659⟩.)

## Ferrules and Cap Overseals

Healthcare practitioners using injectable products must be able to easily see and act on labeling statements that convey important safety messages critical for the prevention of imminent life-threatening situations. These cautionary labeling statements must be simple, concise, and devoid of nonessential information. Products that do not require cautionary statements should be free of information, so that those with cautionary statements are immediately apparent. Accomplishing this requires a systematic approach to the labeling of injectable products, and one that ensures that the ferrule and cap overseal—an area of these products that is highly visible to practitioners as they use these medicines—is reserved for critical safety messages. Accordingly:

1. Only cautionary statements may appear on the top (circle) surface of the ferrule and cap overseal of a vial containing an injectable product. The cautionary statement should appear on both the ferrule and cap, but may appear solely on the ferrule if the cap overseal is transparent and the cautionary statement beneath the cap is readily legible. A cautionary statement is one intended to prevent an imminent life-threatening situation and may include instructional statements that provide potency or other safety-related instructions if warranted. Examples of such statements include, but are not limited to: "Warning—Paralyzing Agent" and "Dilute before Using". The cautionary statement should be printed in a contrasting color and should be clearly visible under ordinary conditions of use.
2. If no cautionary statement is necessary, the top surface of the vial, including the ferrule and cap overseal, must remain blank.
3. Other statements or features including, but not limited to, identifying numbers or letters, such as code numbers, lot numbers, company names, logos, or product names, etc., may appear on the side (skirt) surface of the ferrule on vials containing injectable products, but not on the top (circle) surface of the ferrule or cap overseal. The appearance of such statements or features on the skirt surface of the ferrule should not detract from, or interfere with, the cautionary statement on the top surface.

## Potassium Chloride for Injection Concentrate

The use of a black closure system on a vial (e.g., a black cap overseal and a black ferrule to hold the elastomeric closure) or the use of a black band or series of bands above the constriction on an ampule is prohibited, except for Potassium Chloride for Injection Concentrate (see ⟨659⟩).

## Neuromuscular Blocking and Paralyzing Agents

All injectable neuromuscular blocking agents and paralyzing agents must be packaged in vials with a cautionary statement printed on the ferrules and cap overseals. Both the container cap ferrule and the cap overseal must bear in black or white print (whichever provides the greatest color contrast with the ferrule or cap color) the words: "Warning: Paralyzing Agent" or "Paralyzing Agent" (depending on the size of the closure system). Alternatively, the overseal may be transparent and without words, allowing for visualization of the warning labeling on the closure ferrule.

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## Aluminum in Large-Volume Injections (LVIs), Small-Volume Injections (SVIs), and Pharmacy Bulk Packages (PBPs) Used in Parenteral Nutrition (PN) Therapy[2]

1. The aluminum content of LVIs used in PN therapy must not exceed 25 mcg/L.
2. The package insert of LVIs used in PN therapy must state that the drug product contains no more than 25 mcg of aluminum per liter. This information must be contained in the *Precautions* section of the labeling of all LVIs used in PN therapy.
3. If the maximum amount of aluminum in SVIs and PBPs is 25 mcg/L or less, instead of stating the exact amount of aluminum that each contains, as in paragraph (4), the immediate container label for SVIs and PBPs used in the preparation of PN admixtures or formulations (with exceptions as noted below) may state: "Contains no more than 25 mcg/L of aluminum." If the SVI or PBP is a lyophilized powder, the immediate container label may state the following: "When reconstituted in accordance with the package insert instructions, the concentration of aluminum will be no more than 25 mcg/L."
4. The maximum level of aluminum at expiry must be stated on the immediate container label of all SVIs and PBPs used in the preparation of PN admixtures or formulations. The aluminum content must be stated as follows: "Contains no more than ___ mcg/L of aluminum." The immediate container label of all SVIs and PBPs that are lyophilized powders used in the preparation of PN solutions must contain the following statement: "When reconstituted in accordance with the package insert instructions, the concentration of aluminum will be no more than ___ mcg/L." This maximum amount of aluminum must be stated as the highest one of the following three levels:
   - The highest level for the batches produced during the past 3 years
   - The highest level for the latest 5 batches
   - The maximum level in terms of historical levels, but only until completion of production of the first 5 batches.

The package insert for all LVIs, SVIs, and PBPs used in the preparation of PN admixtures or formulations must contain the following statement in the *Warnings* section of the labeling:

> WARNING: This product contains aluminum that may be toxic. Aluminum may reach toxic levels with prolonged parenteral administration if kidney function is impaired. Premature neonates are particularly at risk because their kidneys are immature, and they require large amounts of calcium and phosphate solutions, which contain aluminum.
>
> Research indicates that patients with impaired kidney function, including premature neonates, who receive parenteral levels of aluminum at greater than 4 to 5 mcg/kg/day, accumulate aluminum at levels associated with central nervous system and bone toxicity. Tissue loading may occur at even lower rates of administration.

***Change to read:***

# LABELS AND LABELING FOR PRODUCTS IN OTHER CATEGORIES

## Labels and Labeling of Liquids and Constituted Products

The labels[1] and labeling state the following information:

1. In the case of a liquid product, the percentage content of each active moiety or drug substance or the amount of each active moiety or drug substance in a specified volume.
2. In the case of a product to which a diluent must be added before use, the amount of each active moiety or drug substance, the name and volume of diluent to be added, the final volume of solution, the concentration after the diluent is added (e.g., quantity/mL or quantity/5 mL), directions for proper storage of the constituted solution, and an expiration or BUD (see *Expiration Date and Beyond-Use Date*).

## Amount of Active Moiety or Drug Substance per Dosage Unit

The strength of a drug product is expressed on the container label in terms of micrograms, milligrams, grams, or percentage of the therapeutically active moiety or drug substance, whichever form is used in the title, unless otherwise indicated in an individual monograph. Both the active moiety and drug substance names and their equivalent amounts are then provided on the container label and in the labeling (see *Nomenclature* ⟨1121⟩, *Monograph Naming Policy for Salt Drug Substances in Drug Products and Compounded Preparations*).

Official articles in capsule, tablet, or other dosage forms must be labeled to express the quantity of each active moiety or drug substance contained in each unit. Unit-dose oral solutions or suspensions (whether supplied as liquid products or as liquid products that are constituted from solids upon addition of a designated volume of a specific diluent) must be labeled to express the quantity of each active moiety or drug substance delivered under the conditions prescribed in *Deliverable Volume* ⟨698⟩. Official drug products not in unit-dose packaging must be labeled to show the quantity of each active moiety or drug substance in each milliliter or in each gram, or to express the percentage of each such ingredient (see *General Notices, 8.140 Percentage Concentrations*). Exceptions are oral liquids or solids intended to be constituted to yield oral liquids that, alternatively, can be labeled in terms of each 5-mL portion of the liquid or resulting liquid. Unless otherwise required by regulation [e.g., over-the-counter (OTC) regulation 21 CFR §201.62(b)] or indicated in a monograph or chapter, declarations of strength or quantity must be stated only in metric units [see also *General Notices, 5.50.10 Units of Potency (Biological)*].

[2] See 21 CFR §201.323. USP uses the following terms: large-volume injections (LVIs), small-volume injections (SVIs), and parenteral nutrition (PN), rather than terminology used in 21 CFR §201.323: large-volume parenterals, small-volume parenterals, and total parenteral nutrition.

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# Expiration Date and Beyond-Use Date

## ▲EXPIRATION DATE▲ (IRA 1-Sep-2023)

The label of an official drug product ▲must▲ (IRA 1-Sep-2023) bear an expiration date. All products ▲must▲ (IRA 1-Sep-2023) display the expiration date so that it can be read by an ordinary individual under customary conditions of purchase and use. The expiration date ▲must▲ (IRA 1-Sep-2023) be prominently displayed in high contrast to the background ▲and it must▲ (IRA 1-Sep-2023) be ▲printed or▲ (IRA 1-Sep-2023) sharply embossed ▲or debossed,▲ (IRA 1-Sep-2023) and ▲be clear to the user. The term "expiration date" may be abbreviated on the drug package label in a manner that allows the reader to understand the abbreviation to mean "expiration date" (e.g., EXP, Exp Date). The year must always be in a 4-digit format.

When all-numeric dates are used, they must be formatted using the year, the month, and, if applicable, the day, separated by hyphens or forward slashes in one of the following formats:

YYYY-MM-DD (e.g., 2019-06-30, 2019/06/30)

YYYY-MM (e.g., 2019-06, 2019/06)

When alphanumeric dates are used, months must be displayed using at least three letters in one of the following formats:

YYYY-MMM-DD (e.g., 2019-JUN-30, 2019/JUN/30)

YYYY-MMM (e.g., 2019-JUN, 2019/JUN)

If an expiration date is stated only in terms of the year and the month, then the intended expiration date is the last day of the stated month.

For containers that have insufficient space on the primary container to accommodate the full expiration date format described previously (e.g., topical ophthalmic ointment container crimps, blow-fill-seal ampules), use the all-numeric format YYYY-MM or the alphanumeric format YYYYMMM (without a hyphen or forward slash to accommodate space constraints). If there is a lack of space in close proximity to the expiration date, the term designated to represent "expiration date" (e.g., EXP) may be omitted if the specific alphanumeric format, YYYYMMM, is used. However, all other packaging, including but not limited to a carton, tray, or overwrap, must have the full expiration date format as described previously.▲ (IRA 1-Sep-2023)

The monographs for some ▲drug products▲ (IRA 1-Sep-2023) state how the labeled expiration date ▲must▲ (IRA 1-Sep-2023) be determined. In the absence of a specific requirement in the individual monograph for a drug product, ▲▲ (IRA 1-Sep-2023) the label ▲must▲ (IRA 1-Sep-2023) bear an expiration date assigned for the particular formulation and package of the product. ▲The▲ (IRA 1-Sep-2023) label need not show an expiration date ▲for the following exception: if the product is a human OTC drug product for which the labeling states no dosage limitations, it is packaged in a container that is intended for sale without prescription, and if the product is stable for not less than (NLT) 3 years when stored under the prescribed conditions. Refer to 21 CFR 211.137(h).▲ (IRA 1-Sep-2023)

If an official ▲drug▲ (IRA 1-Sep-2023) product is required to bear an expiration date, the product ▲must▲ (IRA 1-Sep-2023) be dispensed solely in or from a container labeled with an expiration date, and the date on which the article is dispensed ▲and intended to be used must▲ (IRA 1-Sep-2023) be within the labeled expiry period. The expiration date identifies the time during which the article can be expected to meet the requirements of the compendial monograph, provided it is kept under the ▲labeled▲ (IRA 1-Sep-2023) storage conditions. The expiration date limits the time during which the article may be dispensed ▲and▲ (IRA 1-Sep-2023) used.

▲The label of a dietary supplement product recognized in *USP* and that claims conformance with *USP* must bear an expiration date for the specific dosage form formulation and package of the product, unless otherwise specified in the individual monograph. See *General Notices, 3.10.20 Applicability of Standards to Medical Devices, Dietary Supplements, and Their Components and Ingredients* for applicability of *USP* dietary supplement standards.

## BEYOND-USE DATE▲ (IRA 1-Sep-2023)

The beyond-use date (BUD) is the date after which a product ▲or preparation must▲ (IRA 1-Sep-2023) not be used. The dispenser ▲must▲ (IRA 1-Sep-2023) place on the label of the prescription container a suitable BUD to limit the patient's use of the article based on any information supplied by the manufacturer or this subsection. The BUD ▲must▲ (IRA 1-Sep-2023) not be later than the expiration date on the manufacturer's container. Also see the *Labels and Labeling for Compounded Preparations* section.

For articles that require constitution before use, a suitable BUD for the constituted product ▲must▲ (IRA 1-Sep-2023) be identified in the labeling.

For all other dosage forms, in determining a BUD the dispenser ▲must▲ (IRA 1-Sep-2023) take into account, in addition to any other relevant factors:

- Nature of the drug
- Container in which it was packaged by the manufacturer and the expiration date thereon
- Characteristics of the patient's container, if the article is repackaged for dispensing
- Expected storage conditions to which the article may be exposed
- Unusual storage conditions to which the article may be exposed
- Expected length of the course of therapy.

After considering these factors, the dispenser ▲must▲ (IRA 1-Sep-2023) label a container with a suitable BUD to limit the patient's use of the article. Unless otherwise specified in the individual monograph or in the absence of stability data to the contrary, the BUD ▲must▲ (IRA 1-Sep-2023) be ▲no▲ (IRA 1-Sep-2023) later than (a) the expiration date on the manufacturer's container; or (b) 1 year from the date the drug is ▲packaged and/or labeled by the dispenser,▲ (IRA 1-Sep-2023) whichever is earlier, unless stability data or the manufacturer's labeling indicates otherwise.

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## Labels and Labeling for Compounded Preparations

The labels and labeling state the following information:

In the case of a compounded preparation, list the name(s) and amount(s) or concentrations of active moiety(ies) or drug substance(s) on the immediate container (see *Pharmaceutical Compounding—Sterile Preparations* ⟨797⟩ and *Pharmaceutical Compounding—Nonsterile Preparations* ⟨795⟩.)

In the case of a compounded preparation prepared in 503A facilities as defined by FDCA §503A, the labeling should indicate that "this is a compounded preparation."

The label on the container or package of a compounded preparation must bear a BUD. The BUD is determined from the date the preparation is compounded. Because compounded preparations are intended for administration immediately or following short-term storage, their BUDs may be assigned based on criteria different from those applied to assigning expiration dates to manufactured drug products.

The monograph for an official compounded preparation typically includes a BUD that states the time period following the date of compounding during which the preparation, properly stored, may be used. For guidance regarding the BUD for compounded sterile and nonsterile preparations, see ⟨797⟩ and ⟨795⟩, respectively.

The label on the official compounded preparation must include the word "compounded" after the drug portion of a non-proprietary name (e.g., "*Baclofen Compounded Oral Suspension*").

## Dialysis, Hemofiltration, and Irrigation

Solutions that are intended for use as dialysis, hemofiltration, or irrigation, and are packaged in a container with a volume of more than 1 L, must be labeled to indicate that the contents are not to be administered either intravenously or intra-arterially.

## Use of Leading and Terminal Zeros

To help minimize the possibility of errors in drug dispensing and administration, when the quantity of active moiety or drug substance is expressed in whole numbers it must be shown without a decimal point followed by a terminal zero (e.g., express as 4 mg, not 4.0 mg). When the quantity of active moiety or drug substance is expressed as a decimal number smaller than 1, it must be shown with a zero preceding the decimal point (e.g., express as 0.2 mg, not .2 mg).

## Units

Abbreviations for the terms "Units" or "International Units" must not be used for labeling or prescribing purposes. Examples include "U", "u", and "IU". Medication errors have occurred when these abbreviations have been used. See *General Notices, 9.10 Use of Metric Units*.

## Alcohol

The alcohol content in a liquid formulation must be stated on the label as a percentage (v/v) of alcohol ($C_2H_5OH$).

## Botanicals

The label of an herb or other botanical intended for use as a dietary supplement shall bear the statement, "If you are pregnant or nursing a baby, seek the advice of a health professional before using this product."

## Electrolytes

The concentration of each electrolyte for replacement therapy (e.g., sodium, potassium, chloride) must be stated on the label in milliequivalents per volume (mEq/volume). Phosphorus containing injections must be expressed in milliMoles per volume (e.g., mM/volume). The label of the product must also indicate the quantity of ingredient(s) in terms of weight or percentage concentration.

## Non-Oral Products

Non-oral product labels and labeling must state the names of added substances (as defined in *General Notices, 5.20 Added Substances*) in compliance with 21 CFR §201.100(b)(5).

## Salts of Drugs

It is an established principle that official articles must have only one official title (see *General Notices, 2.20 Official Articles* and compendial nomenclature requirements in ⟨1121⟩). For purposes of saving space on labels and because chemical symbols for the most common inorganic salts of drugs are well known to practitioners, the following alternatives are permitted in labeling official articles that are salts: HCl for hydrochloride; HBr for hydrobromide; Na for sodium; and K for potassium. The symbols Na and K are intended for use in abbreviating names of the salts of organic acids, but these symbols are not used when the word Sodium or Potassium appears at the beginning of an official title (e.g., Phenobarbital Na is acceptable, but Na Salicylate is not).

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## Special Capsules and Tablets

The label of any form of capsule or tablet intended for administration other than by swallowing intact must bear a prominent indication of the manner in which it should be used (see *Compendial Nomenclature, Nomenclature Guidelines* on the USP website at www.usp.org).

## Products That Contain Vitamins

The vitamin content of a drug product must be stated on the label in metric units per dosage unit. The amounts of vitamins A, D, and E may also be stated in USP Units. Quantities of vitamin A declared in metric units refer to the equivalent amounts of retinol (vitamin A alcohol).

## Controlled Room Temperature

Articles may be labeled for storage at "controlled room temperature" or at "20° to 25°", or other equivalent wording based on the same mean kinetic temperature. All three labeling options must ensure not to exceed the mean kinetic temperature of 25° with excursions between 15° and 30° (see ⟨659⟩).

## Light-Resistant Container

When an opaque covering is used to provide protection from light for a light-sensitive product packaged in a clear or colorless or translucent container, the label of the container bears a statement that the opaque covering is needed until the contents are to be used or administered (see *Containers—Performance Testing* ⟨671⟩*, Spectral Transmission* and ⟨659⟩).

## Single-Unit Container

Each single-unit container must be labeled to indicate the identity; quantity and/or strength; name of the manufacturer, packer, or distributor; lot number; and expiration date of the article (see ⟨659⟩).

## Single-Dose Container

When space permits a single-dose container must be labeled as such, and should include on the label appropriate discard instructions (see ⟨659⟩).

## Multiple-Dose Container

When space permits, a multiple-dose container must be labeled as such (see ⟨659⟩).

## Unit-of-Use Container

A unit-of-use container must be labeled as such (see ⟨659⟩).

## Protection from Freezing

The container label must bear an appropriate instruction to protect the article from freezing if subject to loss of strength or potency, or to destructive alteration of its characteristics (see ⟨659⟩).

## Prescription Container Labeling

At a minimum, a prescription container must be labeled in a patient-centered manner. The label must contain essential information that is important for the patient's safe and effective use of the medicine. Labels should be designed and formatted to optimize readability and understanding (see *Prescription Container Labeling* ⟨17⟩).

# GENERAL LABELING

Users are reminded to always refer to the *General Notices* in assessing or applying any compendial standards. *General Notices* addresses a number of labeling-related aspects, including *3.20 Indicating Conformance* (when an article may be labeled *USP*, *NF*, or *USP–NF*, and requirements related to differences in identity, naming, strength, quality, or purity); *General Notices, 5.20.20 Added Substances (Excipients and Ingredients) in Official Products*; *6.70 Reagents*; and *8.240 Weights and Measures* (e.g., microgram may be represented as either µg or mcg. For labeling or prescribing purposes, only "mcg" is to be used).

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# LABELS AND LABELING FOR ANIMAL DRUG PRODUCTS

This section provides exceptions or additions to the previous requirements in this chapter. The following requirements are specific to the labeling for animal drug products.

## Definitions

The written printed or graphic matter on the outer shipping container for animal drugs is considered to be part of “labeling”. Shipping labeling for animal drugs should contain, at minimum, product identification, lot number, expiration date, and conditions for storage and handling.

## Labeling for Animal Drug Products

The labeling for animal drugs should identify the animal species and, if applicable, specific subset(s) of the animal species for which the drug is approved, conditionally approved, or indexed. In the case of a compounded preparation for animals, the labeling should indicate that “This is a compounded preparation.”

The labeling for prescription animal drug products must include the following statement:

- "Caution: Federal law restricts this drug to use by or on the order of a licensed veterinarian."

## Labeling for Injectable Animal Drug Products

Labeling for injectable animal drug products includes the following information:

- A statement limiting the time frame in which the container may be used once it has been entered (e.g., needle-punctured), provided it is held under the labeled storage conditions.

## Quantity and Total Volume for Single- and Multiple-Dose Injectable Animal Drug Products

Because of considerable variability in body weight within and among animal species, most injectable animal drugs are approved to be dosed on a mg/kg body weight basis. Thus, strength on labeling for single- and multiple-dose injectable animal drugs should be expressed on the basis of quantity per milliliter (quantity/mL), usually mg/mL. An exception would be for single-dose injectable animal drugs that are dosed regardless of the weight of the animal, in which case strength on labeling should be expressed as quantity per total volume (e.g., 50 mg/5 mL). For single-dose containers holding less than 1 mL, strength on labeling should be expressed as quantity per fraction of a mL (e.g., 12.5 mg/0.625 mL).

Dry solids that must be constituted should provide on labeling the total quantity followed by the quantity per milliliter (quantity/mL) after constitution.

## Compounded Veterinary Preparations

The label on the official compounded veterinary preparation must include the word “compounded” after the drug portion of a non-proprietary name and the word “veterinary” at the end of the full official name (e.g., Atenolol Compounded Oral Suspension, Veterinary).

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# ⟨11⟩ USP REFERENCE STANDARDS

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## ▲INTRODUCTION

Reference Standards provided by the United States Pharmacopeial Convention (USP Reference Standards or USP RS) are highly characterized materials demonstrated to have the appropriate qualities to support their intended use. USP RS are not for use in humans or animals.

USP RS are generally linked to relevant tests and assays in the *United States Pharmacopeia* (*USP*) or *National Formulary* (*NF*) documentary standards. They have been approved and established as suitable for use in the context of these applications. When approved as suitable for use in *USP* or *NF* tests and assays, USP RS also assume official status and legal recognition in the United States and other jurisdictions that recognize the *USP* or *NF* (see *General Notices, 2.30 Legal Recognition*). Where *USP* or *NF* tests or assays call for the use of a USP RS, only those results obtained using the specified USP RS are conclusive (see *General Notices, 5.80 USP Reference Standards*).

USP RS may also be used to support other measurements not necessarily prescribed in *USP–NF*. Assessment of the suitability for use in other applications is the responsibility of the user.

## ESTABLISHMENT APPROACHES AND VALUE ASSIGNMENT

USP RS, when they are physical materials, are Reference Materials as defined in the *International Vocabulary of Metrology—Basic and General Concepts and Associated Terms (VIM)*. The value assigned to quantitative standards relies on a mass determination by a primary reference measurement procedure such as a mass balance determination. If the value has been assigned by using a primary reference measurement procedure, the USP RS in metrology terms can be considered a primary measurement standard. If the value has been assigned by comparison or calibration to another material, the USP RS should be considered a secondary measurement standard; for example, when appropriate, USP RS are calibrated relative to international reference materials such as those provided by the World Health Organization (WHO).

USP may choose to develop USP RS that follow the requirements for the development of Certified Reference Materials (CRMs) in accordance with the relevant International Organization for Standardization (ISO) Guides. Correct use of these CRMs support traceability of results to SI units and comparability of procedures. USP may also provide RS that are traceable to a CRM established by a National Metrology Institute or other official provider as required or referenced by the documentary standard.

USP may issue USP RS that are not required in a documentary standard. The material and performance attributes for these USP RS are described in the supporting documentation supplied with the USP RS.

USP RS go through a rigorous characterization as part of their establishment process. The types and extent of testing (including the number of laboratories) are primarily driven by the official uses of the standard, but material characterization goes beyond the establishment of suitability for use. Typically, a comparison to the previous lot is performed during the study when establishing a replacement lot of a USP RS as an additional verification of the suitability of the new standard lot.

## USP REFERENCE STANDARDS FOR *USP* OR *NF*

Official applications of USP RS are specified in *USP* or *NF* monographs and general chapters. These applications are as follows. Some USP RS could be used for several types of applications listed below.

1. **Quantitative determinations**
   - A. The majority of USP RS for quantitative determinations support measurements for total amounts of material on a mass basis. This category includes USP RS for USP or NF articles and impurity standards labeled for quantitative use. The assigned value of the USP RS is stated on the labeling and should be included in calculations used in the monograph and applicable general chapters.
   - B. USP RS for relative determinations of potency or activity are often required for the measurement of complex materials (e.g., biologics, antibiotics, herbals, some dietary supplements) and quantitative amounts may be expressed in units or relative potency terms other than mass.
     These USP RS are established by calibration to a primary standard where the property of the material determines the unit. For these standards where an International Standard (IS) established by the WHO exist, USP RS are National Measurement Standards. The USP RS documentation will indicate when the USP RS has been established by comparison to an International Standard (IS) established by the WHO. Results may be expressed in USP Units, Units, or International Units. Additional statements about unit/mass relationship, specific activity, or other relevant information related to the measurement may be provided in the USP RS documentation.
     For antibiotics that use microbial assays to determine activity, the potency is determined in units or micrograms per milligram (µg/mg) of activity. The units or µg/mg of activity is established against a WHO IS when one exists for that antibiotic. Where no WHO IS is presently available, USP establishes and maintains the standard to which USP RS lots are calibrated. This approach may also be chosen for other complex materials for which no WHO IS exists. In these instances, the USP standard is established in such a way as to ensure long-term stability and fitness for purpose, which permits the calibration of successive lots of USP RS with increased confidence that drift in the assigned unit can be avoided.
2. **Qualitative determinations**
   - A. Identification USP RS: USP RS for identification tests are typically presented as single components of high chemical purity, but may also be complex materials of natural, synthetic, or recombinant origin (e.g., biologics, natural

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products, botanicals, complex nonbiologicals, others). For complex materials, the identification attributes are presented in a matrix of other materials and require highly specific measurement systems (e.g., nucleic acid-based identity determination for naturally derived materials).

B. Impurity USP RS: Impurity USP RS are typically used for system suitability or as impurity markers. They may be presented as single-component materials, as mixtures containing more than one impurity, or as drug substance(s) containing one or more impurities.

C. Digital and Visual USP RS: Unlike chemical reference materials, these USP RS are not physical materials used in chemical analyses. Instead, these visual images are used by analysts to compare test articles to ensure that they meet compendial requirements

3. **Performance verification.** These USP RS are typically called for in general tests and assays and are provided to analyze and, where appropriate, to facilitate adjustment of the operation of an instrument to ensure the results obtained are accurate and/or precise or otherwise give acceptable results. The use of these USP RS is generally described in associated general chapters and in the supporting documentation supplied with the USP RS.

## USP REFERENCE STANDARDS FOR OTHER MEASUREMENTS AND DETERMINATIONS

USP also develops Reference Standards that may not be required in official *USP–NF* tests or assays. USP provides RS specified in the current edition of the *Food Chemicals Codex*, the *Herbal Medicines Compendium*, and standards referenced in regulatory requirements.

USP RS without an official use in the *USP–NF* are developed following the same quality systems used for the characterization and release of USP RS used in official tests and assays. These USP RS are generally intended to address common quality issues and challenges inherent to technologies that cut across different types of products (e.g., system suitability samples, calibrators used to demonstrate performance of an analytical procedure, process, or equipment). Extensive characterization of the USP RS candidate is required and the testing plan takes into account the use of different methods to measure the same attribute, demonstrating broader applicability of these standards. In the absence of a companion monograph or chapter, the information generated from these studies may be disseminated to the user via other types of supporting documents including but not limited to the USP Certificate.

## LABELING

The labeling material consists of the label affixed to the USP RS and the associated USP Certificate. Both must be reviewed prior to handling or using the USP RS because in some cases not all of the necessary information can fit on the affixed label. USP Certificates are lot specific and are publicly available on the USP website (www.usp.org). Additional documentation may be provided with the USP RS as needed.

The affixed USP RS label typically contains the RS name, catalog number, lot number, package size, assigned value, storage conditions, handling instructions, and country of origin information. For multi-component items, there is also an outer package and label.

The affixed label also includes hazard and precautionary statements required by the Occupational Safety and Health Administration (OSHA) under the current revision of the Hazard Communication Standard (29 CFR 1910.1200). Terms used in these statements do not necessarily reflect specific definitions in the *USP–NF*. Safety Data Sheets for all USP RS are publicly available on the USP website (www.usp.org).

In addition to the information provided on the affixed USP RS label, the USP Certificate will generally contain the RS chemical name and structure, sequence (if applicable), CAS number, molecular formula, and molecular weight. A typical chromatogram may also be included if necessary for the intended use. Additional information may be included such as special handling instructions or information needed for the use of the USP RS. The USP Certificate also includes a copy of the label text and a series of general instructions.

## PACKAGING

The amount of material per individual USP RS container depends on the application of the standard. Some standards (mainly materials with significant handling requirements or materials that are available only in small amounts) are provided in single-use containers. Some single-use products may be lyophilized with content labeled in mass or activity units per container. If so labeled, the content of the container must be reconstituted in its entirety without any additional weighing. Instructions for use are given either on the label or USP Certificate, or in the monographs where the standard is used.

## STORAGE

USP RS should be stored in the packaging configuration provided by USP, according to the label and USP Certificate instructions. When storage in refrigerator or freezer is stated on the label, follow the definitions given in *Packaging and Storage Requirements* ⟨659⟩. If no specific directions or limitations are provided on the label, the conditions of storage shall be room temperature and protection from moisture, light, freezing, and excessive heat.

Any unused portions remaining after the container has been opened should be carefully stored in accordance with the user's Standard Operating Procedures and good laboratory practices. Decisions concerning the proper use of previously opened USP RS are the responsibility of the user, unless otherwise specified on the labeling. The user is responsible for ensuring that the contents of opened vials continue to be suitable for their intended use.

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## CONTINUED SUITABILITY FOR USE

All USP RS are periodically reevaluated by USP throughout their lifecycles. The USP Continued Suitability for Use (CSU) program is designed to monitor real-time suitability for use of all current lots of USP RS. Suitability testing intervals are established based on collaborative study data, manufacturer or supplier data, testing results, and CSU data trending and projections. When and where applicable, an accelerated degradation study may be performed to provide additional information on the stability of the USP RS and to support suitability testing intervals. The goal of the CSU program is to confirm the continued suitability of the material for use of a USP RS in its compendial applications during its valid use period.

## VALID USE DATE

USP RS lots are assigned a valid use date upon depletion. The valid use date is the last day upon which a particular lot of USP RS can be used. Typically, the valid use date assigned is one year from the date the last vial of a lot is sold.

It is the responsibility of the user to ascertain that a particular lot of a USP RS has official status either as a "Current Lot" or as a "Previous Lot" prior to the valid use date. Current and previous lot information, as well as the most current version of the catalog, can be found on the USP website (www.usp.org).

## PROPER USE

Many compendial tests and assays are based on comparison of a sample to a USP RS. In such cases, measurements are made on preparations of both the sample and the USP RS. Where it is directed that a standard solution or a standard preparation be prepared for a quantitative determination, it is intended that the USP RS substance be accurately weighed (see *Balances* ⟨41⟩) and subsequent dilutions be performed using volumetric apparatuses with, at least, the prescribed tolerances (see *Volumetric Apparatus* ⟨31⟩). Potential errors associated with the use of volumetric apparatus of small volume should be taken into account (see also *General Notices, 6.50.20.1 Adjustments to Solutions*).

Whenever the labeled directions for use require either drying or a correction for water and/or volatiles, this should be performed at the time of use. Further experimental details should be controlled by the user's Standard Operating Procedures and good laboratory practices.

The following list of label terms and definitions is provided as guidance for the handling and use of USP RS:

- **Assigned Value (Calculation Value):** The quantity value assigned to a USP RS for its use in the quantitative compendial applications.
- **As Is:** Use the USP RS as received, without drying or additional testing and apply the assigned value to correct the concentration of the standard solution and/or preparation. This is the preferred option, and is selected whenever data indicate the moisture content is constant over time. For the USP RS to be used on the as is basis, the assigned value has already been corrected for volatiles, including moisture.
- **Anhydrous Basis, Determine Water Content Titrimetrically at Time of Use:** Use the USP RS as received, and apply the water content determined to correct the weight of the standard. After the correction for water is applied, use the assigned value to correct the concentration of the standard solution and/or preparation. At the time a USP RS is to be weighed, proceed as directed under *Water Determination* ⟨921⟩, *Method I*, and determine the water content on a separate portion of material. Instrumental or microanalytical methods are acceptable for this purpose. When using typical amounts (about 50 mg of the USP RS), titrate with a 2- to 5-fold dilution of the reagent.
- **Dried Basis, Determine Loss on Drying at Time of Use:** Use the USP RS as received, and apply the loss on drying value obtained to correct the weight of the standard. After the correction for loss on drying is applied, use the assigned value to correct the concentration of the standard solution and/or preparation. Determine the loss on drying value on a separate portion of material, following the monograph procedure under *Loss on Drying*. Sample sizes smaller than those required in the general test chapter may be used for a USP RS provided that the user can obtain a sufficiently accurate result.
- **Dried Material, Dry Before Use, or Use Previously Dried Material:** Dry the USP RS before use. Use immediately after drying under stated conditions. Drying should not be performed in the original container. A portion of the material should be transferred to a separate drying vessel. Apply the assigned value to correct the concentration of the standard solution and/or preparation prepared with the dried USP RS.▲ (USP 1-Aug-2020)

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# ⟨ 17⟩ PRESCRIPTION CONTAINER LABELING

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## INTRODUCTION

This chapter applies to labeling instructions and information on prescription containers that are directly dispensed to the patient to promote better patient understanding. These standards do not apply when a prescription drug will be administered to a patient by licensed personnel who are acting within their scope of practice. Medication misuse has resulted in more than 1 million adverse drug events per year in the United States.[1] One of patients' primary sources▲ (USP 1-Dec-2021) of information regarding the medications they have been prescribed is on the prescription container label. Although other written information and oral counseling may be available, the prescription container label must fulfill the professional obligations of the prescriber and pharmacist. These obligations include giving patients the essential information they will need to understand how to safely and appropriately use the medication and how to adhere to the prescribed medication regimen.

Inadequate understanding of prescription directions for use and auxiliary ▲labels▲ (USP 1-Dec-2021) provided on dispensed containers is widespread. ▲An auxiliary label contains any instruction, warning, or informational statements in addition to primary instructions for use that are affixed to the prescription container.▲ (USP 1-Dec-2021) Studies have found that 46% of patients misunderstood one or more dosage instructions, and 56% misunderstood one or more auxiliary ▲labels.▲ (USP 1-Dec-2021) The problem of misunderstanding is particularly common and troublesome in patients with low or marginal literacy and in patients receiving multiple medications that are scheduled for administration using complex, nonstandardized time periods. In one study, patients with low literacy were 34 times more likely to misinterpret prescription ▲auxiliary▲ (USP 1-Dec-2021) labels than patients with adequate literacy. However, even patients with adequate literacy often misunderstand common prescription directions and ▲auxiliary labels.▲ (USP 1-Dec-2021) In addition, there ▲may be discrepancies between auxiliary label information and other written information provided by the pharmacy (e.g., medication guides).▲ (USP 1-Dec-2021) The specific evidence to support ▲the inclusion of▲ (USP 1-Dec-2021) a given auxiliary statement often is unclear, and patients often ignore such information. ▲▲ (USP 1-Dec-2021)Further study in comparison to explicit, simplified language alone ▲is needed to determine whether auxiliary information should be placed on auxiliary labels or just included as part of the language on the prescription container alone.▲ (USP 1-Dec-2021)

Lack of universal standards for labeling on dispensed prescription containers is a root cause of patient misunderstanding, nonadherence, and medication errors.

USP developed patient-centered label standards for the format, appearance, content, and language of prescription ▲container label▲ (USP 1-Dec-2021) medication instructions to promote patient understanding. These recommendations form the basis of this general chapter.

***Change to read:***

## PRESCRIPTION CONTAINER LABEL STANDARDS TO PROMOTE PATIENT UNDERSTANDING

### Organize the Prescription Label in a Patient-Centered Manner

Information shall be organized in a way that best reflects how most patients seek out and understand medication instructions. Prescription container labeling should feature only the most important patient information needed for safe and effective understanding and use.

### Emphasize Instructions and Other Information Important to Patients

Prominently display information that is critical for the patient's safe and effective use of the medicine. At the top of the label, specify the patient's name, the drug name (spell out full generic and brand name) and drug strength, and explicit clear directions for use in ▲plain▲ (USP 1-Dec-2021) language.

The prescription directions should follow a standard format so that the patient can expect that each element will be presented in a specific, predictable order each time a prescription is received. Use of a methodology that simplifies the administration instructions for the patient's medication should improve the individual's ability to understand prescription instructions, to take the correct dose, and to organize multidrug regimens. Employing best practices of patient-centered instructions—which utilize the principles of health literacy, medication therapy management, and education, to explicitly describe how to take daily-use, solid dose-form medications—has resulted in improved patient understanding. One such patient-centered method is the universal medication schedule (UMS). The UMS shifts medication-taking into four standardized time periods (morning, noon, evening, bedtime) and uses simplified language and formatting to promote understanding (e.g., "take 1 pill in the morning and 1 pill at bedtime" rather than "take one tablet twice daily"). Such methods may be particularly useful for simplifying daily medication regimens that include multiple oral therapies. [NOTE—The word "pill" is used in the UMS to enhance health literacy and may not reflect a USP definition for an oral dosage form (see ▲*Pharmaceutical Dosage Forms* ⟨ 1151⟩ ).▲ (USP 1-Dec-2021)]
▲▲ (USP 1-Dec-2021)

Other, less-critical but important content (e.g., pharmacy name and phone number, prescriber name, fill date, refill information, expiration date, prescription number, drug quantity, physical description, and evidence-based auxiliary information) should not supersede critical patient information. Such less-critical information should be placed away from dosing

[1] Available at https://www.nap.edu/catalog/12077/standardizing-medication-labels-confusing-patients-less-workshop-summary.

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instructions (e.g., at the bottom of the label or in another less prominent location) because it can distract patients, which can impair their recognition and understanding.

## ▲Oral Liquid Medication Dosing Tools

When oral liquid dosage forms are prescribed, the appropriate "dosing tool" (also known as a dosing component; examples include oral syringe or dosing cup) shall be provided to the patient or caregiver to accurately measure and administer the oral medication. Oral syringes are recommended when dosing accuracy is important, particularly for doses of 5 mL or less. The size of the dosing tool should be considered in the context of the recommended dose volume. The dosing tool volume provided should be the smallest one capable of holding the entire dose volume (e.g., for a 2-mL dose, a 5-mL capacity syringe is preferable to a 10-mL capacity syringe; for a 7.5-mL dose, a 10-mL capacity syringe is preferable to a 5-mL capacity syringe). The volume markings on the dosing tool shall be legible, indelible, and also limited to a single measurement scale that corresponds with the dose instructions on the prescription container label, typically volumetric metric units (see *Packaging and Storage Requirements* 〈 659〉 ).▲ (USP 1-Dec-2021)

## Simplify Language

The language on the label should be clear, simplified, concise, and familiar, and should be used in a standardized manner. Only common terms and sentences should be used. Do not use unfamiliar words (including Latin terms) or medical jargon.

Use of readability formulas and software is not recommended for simplifying short excerpts of text such as those on prescription labels. Instead, use simplified, standardized sentences that were developed by seeking feedback from samples of diverse consumers. Such language will promote correct understanding of the instructions.

## Give Explicit Instructions

Instructions for use (i.e., the SIG or signatura) should clearly separate the dose itself from the timing of each dose in order to explicitly convey the number of dosage units to be taken and when (e.g., specific time periods each day such as morning, noon, evening, and bedtime). Instructions shall include specifics on time periods. Do not use alphabetic characters for numbers. For example, write “Take 2 tablets in the morning and 2 tablets in the evening” rather than “Take two tablets twice daily”).

Whenever available, use standardized directions (e.g., write “Take 1 tablet in the morning and 1 tablet in the evening” if the prescription reads b.i.d.). Vague instructions based on dosing intervals such as twice daily or 3 times daily, or hourly intervals such as every 12 h, generally should be avoided because such instructions are implicit rather than explicit, they may involve numeracy skills, and patient interpretation may differ from prescriber intent. Although instructions that use specific hourly times (e.g., 8 a.m. and 10 p.m.) may seem to be more easily understood than implicit vague instructions, recommending dosing at precise hours of the day is less readily understood and may present greater adherence issues (due to individual lifestyle patterns such as shift work) than more general time frames such as in the morning, in the evening, after breakfast, with lunch, or at bedtime. Consistent use of the same terms should help avoid patient confusion. A set of standardized, explicit instructions [the universal medication schedule (UMS)] were developed and tested in English and other languages to improve patient understanding.[2]

Ambiguous directions such as “take as directed” should be avoided unless clear and unambiguous supplemental instructions and counseling are provided (e.g., directions for use that will not fit on the prescription container label). A clear statement referring the patient to such supplemental materials should be included on the container label.

## Include Purpose for Use

If the purpose of the medication is included on the prescription, it should be included on the prescription container label unless the patient prefers that it not appear. Practitioners should always ask patients their preference when writing the prescription. Confidentiality and FDA approval for intended use (e.g., labeled versus off-label use) may limit inclusion of the purpose on labels. Current evidence supports inclusion of purpose-for-use language in clear, ▲plain▲ (USP 1-Dec-2021) terms (e.g., “for high blood pressure” rather than “for hypertension”).

## Limit Auxiliary Information

Auxiliary information on the prescription container label should be evidence-based and presented in ▲plain▲ (USP 1-Dec-2021) explicit language that is minimized to avoid distracting patients with nonessential information. Most patients, particularly those with limited literacy, pay little attention to auxiliary information. The information should be presented in a standardized manner and should be critical for patient understanding and safe medication use (e.g., warnings and critical administration alerts). Icons are frequently misunderstood by patients. In addition, icons that provide abstract imagery for messages that are difficult to depict visually may be ineffective at improving understanding compared with simplified text alone. Use only icons for which there is adequate evidence, through consumer testing, that they improve patient understanding about correct use. Evidence-based auxiliary information, both text and icons, should be standardized so that it is applied consistently and does not depend on individual practitioner choice.

[2] Explicit and Standardized Prescription Medicine Instructions. December 2014. Agency for Healthcare Research and Quality, Rockville, MD. https://www.ahrq.gov/health-literacy/improve/pharmacy/instructions.html.

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## ▲Opioid Warning Label Development

Opioids are a class of drugs with a high risk of misuse, abuse, dependence, and addiction. It has been reported that many patients prescribed opioids for pain do not even know that they are taking an opioid. Providing this factual information to patients could be an important first step in helping them understand and minimize their own risks for misuse, abuse, dependence, and addiction.

For these reasons, opioids may warrant a warning label. An example of a warning label would be a message telling the patient the prescription contains an opioid. Another type of warning label would alert patients to the risk of overdose and addiction. Any brief warning could be printed on a sticker and placed on the cap of the prescription vial where it would be seen easily.

Warning labels for opioids may be useful because the prescription container label may be the only source of information patients review, according to some studies. And yet, 46% of patients in one study misunderstood one or more pieces of information on the label. Patients with low health literacy are more likely to ignore the labels.

Although the physical space for labeling on prescription containers is very limited, some of this space could be used in an impactful way to educate patients on the risk of opioid abuse and addiction. This could be an opportunity to provide clearer, more useful information to patients about the risks of prescription opioids. There is also concern, however, that a label identifying the drug as an opioid could lead to diversion.

When developing or choosing opioid warning labels that would be affixed to prescription container labels, the same health literacy principles used for auxiliary labels apply. The following key principles have been shown to convey messaging effectively in other health campaigns:

- All icons should be accompanied by text
- The label should address and emphasize the potential health consequences
- The warning should be frank and direct, clearly stating the serious—even fatal—consequences, in order to capture the reader's attention and have a strong impact
- Additional information should be accessible to the patient (e.g., pamphlet, guide, website)
- The labeling should be supported by established theory, such as behavior change theory, to have a public health impact
- The labeling should be linked to education and a media campaign with high visibility for the target populations▲ (USP 1-Dec-2021)

## Address Limited English Proficiency

Whenever possible, the directions for use on a prescription container label should be provided in the patient's preferred language. Otherwise there is a risk of misinterpretation of instructions by patients with limited English proficiency, which could lead to medication errors and adverse health outcomes. Additionally, whenever possible, directions for use should appear in English as well, to facilitate counseling; the drug name(s) shall be in English so that emergency personnel and other intermediaries can have quick access to the information. Standardized translations of universal medication schedule instructions are available.[3]

Translations of prescription medication labels should be produced using a high-quality translation process. An example of a high-quality translation process is:

- Translation by a trained translator who is a native speaker of the target language
- Review of the translation by a second trained translator and reconciliation of any differences
- Review of the translation by a pharmacist who is a native speaker of the target language and reconciliation of any differences
- Testing of comprehension with target audience

If a high-quality translation process cannot be provided, labels should be printed in English, with the use of trained interpreter services whenever possible to ensure patient comprehension. The use of computer-generated translations should be limited to programs with demonstrated quality because dosage instructions can be translated inconsistently or incorrectly, which is potentially hazardous. Standardized translated instructions and technology advances are needed to ensure the accuracy and safety of prescription container labeling for patients with limited English proficiency.

## Improve Readability

Labels should be designed and formatted so they are easy to read. Currently, no strong evidence supports the superiority, in terms of legibility, of serif versus sans serif typefaces, ▲however there are guidances that support sans serif typeface for labeling.[4, 5, 6]▲ (USP 1-Dec-2021)

Optimize typography by using the following techniques:

- High-contrast print (e.g., black print on white background).
- ▲Plain,▲ (USP 1-Dec-2021) uncondensed familiar fonts with sufficient space within letters and between letters (e.g., Times New Roman or Arial).

[3] Available at https://www.ahrq.gov/health-literacy/improve/pharmacy/instructions.html.
[4] Available at https://www.fda.gov/regulatory-information/search-fda-guidance-documents/instructions-use-patient-labeling-human-prescription-drug-and-biological-products-and-drug-device.
[5] Available at https://www.afb.org/blindness-and-low-vision/your-rights/rx-label-enable-campaign/summary-recommendations-pharmacists.
[6] Available at https://www.canada.ca/en/health-canada/services/drugs-health-products/drug-products/applications-submissions/guidance-documents/labelling-pharmaceutical-drugs-human-use-2014-guidance-document.html.

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- Sentence case (i.e., punctuated like a sentence in English: Initial capital letter followed by lower-case words except proper nouns).
- Large font size (e.g., minimum 12-point Times New Roman or 11-point Arial) for critical information. Note that point size is not the actual size of the letter, so two fonts with the same nominal point size can have different actual letter sizes. The height of the typeface, x-height, has been used as a more accurate indicator of apparent size than point size. For example, for a given point size, the x-height and apparent size of Arial are actually bigger than those for Times New Roman. Do not use type smaller than 10-point Times New Roman or the equivalent size in another font. Older adults, in particular, have difficulty reading small print.
- Adequate white space between lines of text (25%–30% of the point size).
- White space to separate sections on the label such as directions for use versus pharmacy information.
- Horizontal text only.

Other measures that can also improve readability:

- Minimize the need to turn the container in order to read lines of text
- Never truncate or abbreviate critical information
- Use highlighting, bolding, and other cues to preserve readability (e.g., high-contrast print and light color for highlighting) and emphasize patient-centered information or information that facilitates adherence (e.g., refill ordering)
- Limit the number of colors used for highlighting (i.e., no more than two)
- Use of separate lines to distinguish when each dose should be taken

## Alternative-Access Methods to Address Visual Impairment

Patients with visual impairment who are unable to read printed prescription container labels often report inadvertently taking the wrong medication or amount, or taking it at the wrong time or under the wrong instructions, compromising their own safety. Similarly, if a caregiver is visually impaired, the patient they care for is at risk for medication errors. The magnitude of this problem increases with aging, as the risk of visual impairment and the number of prescribed medications both increase with age.

- Follow standards for patient-centered prescription labels
- Provide alternative access for visually impaired patients (alternative-access methods include tactile, auditory, or enhanced visual systems that may employ advanced mechanics of assistive technology)[7]
- Enhance communication between the pharmacist and visually impaired patients (and their designated representatives) such that the pharmacist can explain alternative-access options and together they can identify those best suited to the patient's needs
- Once an alternative-access method is identified for the individual patient, the pharmacist shall provide the service or direct the patient to a pharmacy that offers that type of alternative access
- Ensure that duplicate accessible labels preserve the integrity of the print prescription drug container label and provide the same sequence of information as the printed label
- Follow specific best practices for each respective alternative-access format employed

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[7] ▲Available at▲ (USP 1-Dec-2021) https://www.acb.org/content/access-board-issues-report-best-practices-access-prescription-drug-label-information-melanie.

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# ⟨31⟩ VOLUMETRIC APPARATUS

Most of the volumetric apparatus available in the United States are calibrated at 20°, and the National Institute of Standards and Technology (NIST) has adopted 20° as the reference temperature for the calibration of laboratory glassware, although the temperatures generally prevailing in laboratories are usually between 20° and 25°. To minimize volumetric error, the temperature should be the same for the volumetric apparatus, the material being prepared, the solvents being used to prepare the volumetric solutions, the area in which they are prepared, and the final volume adjustment. To attain the degree of precision required in many Pharmacopeial assays involving volumetric measurements and directing that a quantity be "accurately measured", the apparatus must be chosen and used with care.

***Change to read:***

## STANDARDS OF ACCURACY

The capacity tolerances for volumetric flasks, transfer pipets, and burets are those accepted by NIST (Class A),[1] as indicated in *Table 1*, *Table 2*, and *Table 3*, respectively. [NOTE—The tables in this chapter list the tolerances for the most commonly used sizes. See the referenced ASTM standards for a complete list of tolerances and other applicable criteria.] Use Class A volumetric apparatus unless otherwise specified in the individual monograph. When a plastic volumetric apparatus is specified, the accepted capacity tolerances are equal to Class B glass.[2]

The capacity tolerances for measuring (i.e., "graduated") pipets of up to and including 10-mL capacity are somewhat larger than those for the corresponding sizes of transfer pipets, namely, 10, 20, and 30 µL for the 2-, 5-, and 10-mL sizes, respectively.[3]

Transfer and measuring pipets calibrated "to deliver" should be completely drained in a vertical position and then touched against the wall of the receiving vessel to drain the ▲pipet tip▲ (ERR 1-Nov-2020). Volume readings on burets should be estimated at least to the nearest one-half of a subdivision. Pipets calibrated "to contain" are called for in special cases, generally for measuring viscous fluids such as syrups; however, a volumetric flask may be substituted for a "to contain" pipet. In such cases, the pipet or flask should be washed clean after draining, and the washings should be added to the measured portion.

**Table 1. Volumetric Flasks**

| Designated volume, mL | 10 | 25 | 50 | 100 | 250 | 500 | 1000 |
|---|---|---|---|---|---|---|---|
| Limit of error, mL | 0.02 | 0.03 | 0.05 | 0.08 | 0.12 | 0.20 | 0.30 |
| Limit of error, % | 0.20 | 0.12 | 0.10 | 0.08 | 0.05 | 0.04 | 0.03 |

**Table 2. Transfer Pipets**

| Designated volume, mL | 1 | 2 | 5 | 10 | 25 | 50 | 100 |
|---|---|---|---|---|---|---|---|
| Limit of error, mL | 0.006 | 0.006 | 0.01 | 0.02 | 0.03 | 0.05 | 0.08 |
| Limit of error, % | 0.60 | 0.30 | 0.20 | 0.20 | 0.12 | 0.10 | 0.08 |

**Table 3. Burets**

| Designated volume, mL | 10 ("micro" type) | 25 | 50 |
|---|---|---|---|
| Subdivisions, mL | 0.02 | 0.1 | 0.1 |
| Limit of error, mL | 0.02 | 0.03 | 0.05 |

[1] See ASTM E288-10, ASTM E287-02, ASTM E1189-00, and ASTM E969-02.
[2] See ASTM E288-10 and ISO 384:2015.
[3] See ASTM E1293-02.

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# ⟨41⟩ BALANCES

This chapter states the requirements for balances used for materials that must be accurately weighed (see *General Notices, 8.20 About*). Unless otherwise specified, when substances must be "accurately weighed", the weighing shall be performed using a balance that is calibrated over the operating range and meets the requirements defined for repeatability and accuracy. For balances used for other applications, the balance repeatability and accuracy should be commensurate with the requirements for its use.

For a discussion of the theoretical basis of these requirements, see *Weighing on an Analytical Balance* ⟨1251⟩, which may be a helpful—but not mandatory—resource.

***Change to read:***

## REPEATABILITY

Repeatability is assessed by weighing one test weight NLT 10 times. [NOTE—The test weight must be within the balance's operating range, but the weight need not be calibrated. Because the standard deviation is virtually independent of sample mass within the balance's capacity, use of a small test weight, which may be difficult to handle, is not required.]

Repeatability is satisfactory if ▲twice▲ (USP 1-Aug-2019) the standard deviation of the weighed value, ▲▲$s$▲ (ERR 1-Aug-2019) ,▲ (USP 1-Aug-2019)divided by the desired smallest net weight (i.e., smallest net weight that the users plan to use on that balance), does not exceed 0.10%. ▲The repeatability measurement establishes the smallest net amount of material that may be weighed on the balance in conformance with the 0.10% limit. The minimum weight, $M_{min}$, is described by the inequality $M_{min} \geq 2000$▲$s$▲ (ERR 1-Aug-2019). For example, if ▲$s$ is found to be 0.00015, then $M_{min}$ must be ≥ 0.30000 g or 300.00 mg.▲ (ERR 1-Aug-2019)▲ (USP 1-Aug-2019) If the standard deviation, $s$, obtained is less than 0.41$d$, where $d$ is the scale ▲interval of the balance, then the inequality becomes $M_{min} \geq 2000\ (0.41d)$. For example, for a 4-place analytical balance, $d$ is 0.0001 so that $M_{min}$ must be ≥ 0.0820 g or 82 mg.▲ (USP 1-Aug-2019)

## ACCURACY

The accuracy of a balance is satisfactory if its weighing value, when tested with a suitable weight(s), is within 0.10% of the test weight value.

A test weight is suitable if it has a mass between 5% and 100% of the balance's capacity. The test weight's maximum permissible error (mpe), or alternatively its calibration uncertainty, shall be NMT one-third of the applied test limit of the accuracy test.

[NOTE—Applicable standards are the following: American Society for Testing and Materials (ASTM) E617 (available from http://www.astm.org) and International Organization of Legal Metrology (OIML) R 111 (available from http://www.oiml.org).]

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# ⟨51⟩ ANTIMICROBIAL EFFECTIVENESS TESTING

## INTRODUCTION

Antimicrobial preservatives are substances added to aqueous pharmaceutical products. Nonsterile dosage forms may have preservatives added to protect them from growth of microorganisms inadvertently introduced during or subsequent to the manufacturing process. In the case of sterile articles packaged in multiple-dose containers, antimicrobial preservatives are added to inhibit the growth of microorganisms that may be introduced from repeatedly withdrawing individual doses. One or more antimicrobial preservative(s) are expected in all sterile multidose units.

Antimicrobial preservatives should not be used as a substitute for good manufacturing practices, solely to reduce the viable microbial population of a nonsterile product, or control the presterilization bioburden of a multidose formulation during manufacturing. Antimicrobial preservatives in compendial dosage forms meet the requirements for *General Notices, 5.20 Added Substances*.

All useful antimicrobial agents are toxic substances. For maximum protection of patients, the concentration of the preservative shown to be effective in the final packaged product should be below a level that may be toxic to human beings based on the recommended dosage of the medicinal product.

The concentration of an added antimicrobial preservative can be kept to a minimum if the active ingredients of the formulation possess an intrinsic antimicrobial activity. Antimicrobial effectiveness, whether inherent in the product or produced because of the addition of an antimicrobial preservative, must be demonstrated for all injections packaged in multiple-dose containers or for other products containing antimicrobial preservatives. Antimicrobial effectiveness must be demonstrated for aqueous-based, multiple-dose topical and oral dosage forms and for other dosage forms such as ophthalmic, otic, nasal, irrigation, and dialysis fluids (see *Pharmaceutical Dosage Forms* ⟨1151⟩). For the purpose of the test, aqueous is defined as a water activity of more than 0.6 (see *Application of Water Activity Determination to Nonsterile Pharmaceutical Products* ⟨1112⟩).

Challenge organisms are generally based on likely contaminants to a drug product while considering its physical attributes, formulation, and intended use. The standard battery of challenge organisms described in this test need not prevent the inclusion of other species of microorganisms if deemed useful to measure the biological activity of the preservative system for a specific product. These supplemental challenge organisms are not within the scope of this chapter, but may be added in addition to the described test organisms.

## GENERAL PROCEDURES

This chapter provides procedures to demonstrate the effectiveness of added antimicrobial preservatives. Such antimicrobial preservatives must be declared on the label. The procedures and acceptance criteria for effectiveness apply to a product in the original, sealed container in which it was distributed by the manufacturer (see *Table 1* for categories of products). The test need not be conducted in these containers, but care should be taken to avoid using materials that can interact with the preservative in the containers that are used for antimicrobial effectiveness testing.

### Growth Promotion Procedure and Suitability of the Recovery Method

#### GENERAL CONSIDERATIONS

The ability of the procedure to detect challenge microorganisms in the presence of a suitably neutralized product to be tested must be established. The suitability of the procedure must be reconfirmed if a change is made in materials or methods or if a change is made in the product or direct product contact materials that may affect the outcome of the test.

The growth-promoting capabilities of media used in this procedure must be established.

#### PREPARATION OF TEST STRAINS

Use standardized suspensions of test strains or prepare as stated below. Seed-lot culture maintenance techniques (seed-lot systems) are used so that the viable microorganisms used for inoculation are NMT five passages removed from the original master seed lot. Grow each of the bacterial and fungal test strains separately (see *Table 2*).

Use cultures of the following microorganisms:[1] *Candida albicans* (ATCC No. 10231), *Aspergillus brasiliensis* (ATCC No. 16404), *Escherichia coli* (ATCC No. 8739), *Pseudomonas aeruginosa* (ATCC No. 9027), and *Staphylococcus aureus* (ATCC No. 6538). The viable microorganisms used in the procedure should be part of a freshly growing culture (e.g., in logarithmic growth phase) with the exception of *A. brasiliensis* spores. The culture conditions for the inoculum culture are described (see *Table 2*) in which the suitable media are Soybean–Casein Digest or Sabouraud Dextrose Agar Medium.

To harvest the bacterial and *C. albicans* cultures, use sterile saline TS to wash the surface growth, and collect it in a suitable vessel. To harvest the spores of *A. brasiliensis,* use sterile saline TS containing 0.05% of polysorbate 80. The spore suspension should be aseptically treated (e.g., filtration through sterile glass wool) to remove hyphae. All microbial suspensions should be prepared to ensure that there is no carry over of residual growth medium from the inoculum (e.g., centrifugation followed by resuspension in appropriate sterile suspending fluid.)

Alternatively, the stock culture organisms may be grown in a suitable liquid medium (i.e., Soybean–Casein Digest Broth or Sabouraud Dextrose Broth) and the cells harvested by centrifugation, then washed and resuspended in appropriate sterile suspending fluid. The microbial suspensions used for inoculations should be adjusted to obtain a microbial count of about 1 ×

[1] Available from American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110-2209 (http://www.atcc.org).

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$10^8$ cfu/mL. Use the bacterial and yeast suspensions within 2 h, or within 24 h if stored between 2° and 8°. A stable spore suspension can be prepared and then may be maintained at 2°–8° for up to 7 days. [NOTE—The estimate of inoculum concentration may be obtained by turbidimetric procedures for the challenge microorganisms and later confirmed by plate count.]

### GROWTH PROMOTION OF THE MEDIA

Media used in this procedure must be capable of supporting microbial growth. Test each batch of ready-prepared medium and each batch of medium prepared either from dehydrated medium or from the ingredients described.

For solid media, counts obtained must be at least 50% of the calculated value for a standardized inoculum. For a freshly prepared inoculum, growth of the microorganisms occurs comparable to that previously obtained with a previously tested and approved batch of medium.

## Suitability of the Counting Method in the Presence of Product

Prepare a $10^{-1}$ dilution by adding 1 mL of product (by volume) to 9 mL of saline or other neutralizing diluent. Continue this dilution scheme to $10^{-2}$ and $10^{-3}$ dilution levels. Add an appropriate number of challenge organisms to each tube of diluted product, mix, and then plate a suitable volume from each dilution to yield less than 250 cfu/plate for bacteria and yeast (ideally between 25 and 250 cfu) or less than 80 cfu/plate for *A. brasiliensis* (ideally between 8 and 80 cfu). This plating should be performed minimally in duplicate (although a greater number of replicates can be useful to minimize variability in the plate count estimate). A positive control for this procedure is to introduce the same inocula into saline, and transfer similar volumes of saline to agar plates. A suitable recovery scheme is the one that provides at least 50% of this saline control count (averaged).

If the diluted product exhibits antimicrobial properties, specific neutralizers may need to be incorporated into the diluents or the recovery media. See *Validation of Microbial Recovery from Pharmacopeial Articles* ⟨1227⟩ for more information.

The ability of the procedure to measure preservative efficacy may be compromised if the method suitability requires significant dilution ($10^{-2}$ or $10^{-3}$) as this will affect the measured recovery (e.g., it may be difficult to measure a 3 log unit reduction for a $10^5$–$10^6$ inoculum). If no suitable neutralizing agent or method is found and method suitability requires significant dilution, a higher level of inoculum (e.g., $10^7$–$10^8$) may be used so that a 3 log unit reduction can be measured. Reported recovery cannot be less than 1 cfu/plate on average (or 100 cfu/mL if 1 mL is plated in duplicate at the $10^{-2}$ dilution).

Membrane filtration may be used to filter larger volumes of dilutions to overcome this difficulty or to assist in the neutralization of antimicrobial properties.

## Testing of Products

### PRODUCT CATEGORIES

For the purpose of testing, compendial articles have been divided into four categories (see *Table 1*). The criteria of antimicrobial effectiveness for these products are a function of the route of administration. It is expected that formulations containing preservatives will meet minimal efficacy standards, whether packaged as multidoses or unit doses.

**Table 1. Compendial Product Categories**

| Category | Product Description |
|---|---|
| 1 | Injections; other parenterals including emulsions, otic products, sterile nasal products, and ophthalmic products made with aqueous bases or vehicles |
| 2 | Topically used products made with aqueous bases or vehicles; nonsterile nasal products and emulsions, including those applied to mucous membranes |
| 3 | Oral products other than antacids, made with aqueous bases or vehicles |
| 4 | Antacids made with an aqueous base |

### PROCEDURE

The procedure can be conducted either in five original containers if a sufficient volume of product is available in each container and if the product container can be entered aseptically (i.e., needle and syringe through an elastomeric rubber stopper), or in five sterile, capped bacteriological containers [inert relative to the antimicrobial agent(s)] of suitable size into which a sufficient volume of product has been transferred. Inoculate each container with one of the prepared and standardized inocula, and mix. The volume of the suspension inoculum used is between 0.5% and 1.0% of the volume of the product to minimize potential effects on the product. The concentration of test microorganisms that is added to the product (*Category 1, 2,* or *3*) is such that the final concentration of the test preparation after inoculation is between $1 \times 10^5$ and $1 \times 10^6$ cfu/mL of the product. For *Category 4* products (antacids), the final concentration of the test preparation after inoculation is between $1 \times 10^3$ and $1 \times 10^4$ cfu/mL of the product.

The initial concentration of viable microorganisms in each test preparation is estimated based on the concentration of microorganisms in each of the standardized inocula as determined by the plate-count method. Incubate the inoculated containers at 22.5 ± 2.5°. Sample each container at the appropriate intervals (specified in *Table 3*). Record any changes observed in appearance at these intervals. Determine, by the plate-count procedure, the number of cfu present in each test preparation for the applicable intervals (see *General Procedures* in *Microbial Enumeration Tests* ⟨61⟩). Plate counts will be conducted using a minimum of duplicate plates, with the cfu averaged before determination of deduced cfu/mL. If membrane filtration is used,

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duplicate membrane filters will be used for each estimate. Using the calculated concentrations of cfu/mL present at the start of the test, calculate the change in $\log_{10}$ values of the concentration of cfu/mL for each microorganism at the applicable test intervals, and express the changes in concentration in terms of log reductions. The log reduction is defined as the difference between the $\log_{10}$ unit value of the starting concentration of cfu/mL in the suspension and the $\log_{10}$ unit value of cfu/mL of the survivors at that time point.

**Table 2. Culture Conditions for Inoculum Preparation**

| Organism | Suitable Medium | Incubation Temperature | Inoculum Incubation Time | Microbial Recovery Incubation Time |
|---|---|---|---|---|
| *Escherichia coli* (ATCC No. 8739) | Soybean–Casein Digest Broth; Soybean–Casein Digest Agar | 32.5 ± 2.5° | 18–24 h | 3–5 days |
| *Pseudomonas aeruginosa* (ATCC No. 9027) | Soybean–Casein Digest Broth; Soybean–Casein Digest Agar | 32.5 ± 2.5° | 18–24 h | 3–5 days |
| *Staphylococcus aureus* (ATCC No. 6538) | Soybean–Casein Digest Broth; Soybean–Casein Digest Agar | 32.5 ± 2.5° | 18–24 h | 3–5 days |
| *Candida albicans* (ATCC No. 10231) | Sabouraud Dextrose Agar; Sabouraud Dextrose Broth | 22.5 ± 2.5° | 44–52 h | 3–5 days |
| *Aspergillus brasiliensis* (ATCC No. 16404) | Sabouraud Dextrose Agar; Sabouraud Dextrose Broth | 22.5 ± 2.5° | 6–10 days | 3–7 days |

## Criteria for Antimicrobial Effectiveness

The requirements for antimicrobial effectiveness are met if the criteria specified in *Table 3* are met (see *General Notices, 7. Test Results*). “No increase” in counts is defined as NMT 0.5 $\log_{10}$ unit more than the value to which it is compared.

**Table 3. Criteria for Tested Microorganisms**

| | |
|---|---|
| **For Category 1 Products** | |
| Bacteria | NLT 1.0 log reduction from the initial calculated count at 7 days, NLT 3.0 log reduction from the initial count at 14 days, and no increase from the 14 days’ count at 28 days |
| Yeast and molds | No increase from the initial calculated count at 7, 14, and 28 days |
| **For Category 2 Products** | |
| Bacteria | NLT 2.0 log reduction from the initial count at 14 days, and no increase from the 14 days’ count at 28 days |
| Yeast and molds | No increase from the initial calculated count at 14 and 28 days |
| **For Category 3 Products** | |
| Bacteria | NLT 1.0 log reduction from the initial count at 14 days, and no increase from the 14 days’ count at 28 days |
| Yeast and molds | No increase from the initial calculated count at 14 and 28 days |
| **For Category 4 Products** | |
| Bacteria, yeast, and molds | No increase from the initial calculated count at 14 and 28 days |

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# ⟨55⟩ BIOLOGICAL INDICATORS—RESISTANCE PERFORMANCE TESTS

## INTRODUCTION

A biological indicator (BI) is a well-characterized preparation of a specific microorganism with a known resistance to a specific sterilization process. The correct use of BIs in the development, validation, and control of sterilization processes requires that their population and resistance be accurately known. The population and resistance can be selected to confirm the adequacy of individual sterilization process conditions for an article. The recommendations of *Sterilization of Compendial Articles* ⟨1229⟩ should be followed for effective BI usage. The methods described below can be used to establish population and resistance, such that the response of the BI to the subject sterilization process is appropriate. Although the BI manufacturers are required to maintain rigorous control of population and resistance using the number of replicates as specified below, the end users are not required to use the same number of replicates for verification of those determinations. Conduct all of the tests described in this chapter under appropriate microbiological laboratory conditions (see *Microbiological Best Laboratory Practices* ⟨1117⟩).

## TOTAL VIABLE SPORE COUNT

### Sample Collection/Recovery

PAPER/FIBER INDICATORS

For paper/fiber carrier BIs, remove at least four test samples from their individual containers. Disperse the indicator into component fibers by placing the test samples in a sterile vessel containing 100 mL of sterilized *Purified Water* chilled to 2°–8°, and mechanically disrupt to achieve a homogeneous suspension. For self-contained BIs, aseptically remove four BI carriers from their containers and proceed as directed above.

INDICATORS ON OTHER SUBSTRATES

For all other biological indicators, remove at least four samples from their individual containers. Place the test samples in a sterile vessel containing 100 mL of sterilized *Purified Water* chilled to 2°–8°, and mechanically disrupt to achieve a homogeneous suspension of the spores in the water.

SPORE SUSPENSIONS

For spore suspensions of BIs, prepare an appropriate serial dilution of the original spore suspension in sterilized *Purified Water* chilled to 2°–8°, in a sterile container, and follow the viable spore count procedures as specified below. The requirements of the tests are met if the average number of viable spores is within 50%–300% of the labeled count of the spore suspension.

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### Viable Spore Count

Transfer a 10-mL aliquot of the suspension to a sterile tube. For BIs using spores of *Geobacillus stearothermophilus*, *Bacillus coagulans*, and other thermophilic spore formers, heat the tube containing the suspension in a water bath at 95°–100° for 15 min (heat shock), starting the timing when the temperature reaches 95°. For BIs containing nonthermophilic spore formers, heat the tube containing the suspension in a water bath at 80°–85° for 10 min, starting the timing when the temperature of the spore suspension reaches 80°. Cool suspensions rapidly in an ice-water bath at 0°–4°. Transfer two 1-mL aliquots to suitable tubes, and make appropriate serial dilutions in sterilized *Purified Water*. Calculate the dilutions to yield 30–300 colonies on each plate in a pair, when treated as described below. Where the BI has a low spore concentration, it may be necessary to modify the dilution series and to use more plates at each dilution.

Prepare a separate series of plates for each aliquot. Place 1.0 mL of each selected dilution in each of two 15 × 100-mm Petri dishes. Within 20 min, add to each plate 20 mL of *Soybean–Casein Digest Agar Medium* that has been melted and cooled to approximately 45°. Swirl to attain a homogeneous suspension, and allow it to solidify. Incubate the plates in an inverted position at 55°–60° for thermophilic spore formers and at 30°–35° for nonthermophilic spore formers or at the optimal recovery temperature specified by the manufacturer. Examine the plates after at least 48 h, recording for each plate the number of colonies. Calculate the average number of spores per test sample from the results, using the appropriate dilution factor. When evaluating vendor-supplied BIs, the viable spore count shall be between 50% and 300% of the manufacturer's stated value.

***Change to read:***

## D-VALUE DETERMINATION

### Apparatus

The test equipment used for the determination of microbial resistance ("D-value") is described in substantial detail in ISO 18472, Sterilization of Health Care Products—Biological and Chemical Indicators—Test Equipment (*1*). The details of individual Biological Indicator Evaluation Resistometers (BIERs) vary with the specifics of their design and the particular sterilization process for which they are used. Provided that the performance of the BIER vessel meets the requirements of the ISO standard for

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exposure of the BI, design differences are acceptable. For single-phase sterilization processes where an acceptable BIER has not been defined, the D-value determination can be accomplished by adapting a BIER design intended for a sterilization process operating in the same phase. There are no current methods available for D-value determination for multiple-phase sterilization processes.

## Procedure

Carry out the tests for D-value at sterilization conditions consistent with those intended for use. Use 20 replicate test sample BIs in their original individual containers, subjected to at least five exposure conditions for a total of 100 tests. The number of exposure conditions is chosen to provide a range of observations from NLT one labeled D-value below the expected survival time through NLT one labeled D-value above the expected kill time. Place each group on a separate suitable sample holder that permits each sample to be exposed to the prescribed sterilizing condition at a specific location in the sterilizing chamber of the BIER. Check the BIER apparatus for operating parameters using sample holders without test samples. Select a series of sterilizing times in increments from the shortest time for the samples to be tested. The differences in sterilizing times over the series are as constant as feasible, and the difference between adjacent times is NMT 75% of the expected D-value. Test procedures for the use of BIER vessels for the evaluation of microbial resistance are defined in a series of ISO standards under the 11138 series (*2*–*5*). The appropriate standard should be followed for the BI. The test methods and carriers used with the BIER may be adapted to the specifics of the BI. The method and apparatus used for paper carriers may differ from those for other carriers and will be substantially different from those used for suspensions of BIs.

The D-value exposure conditions for alternative material carriers are the same as the conditions used to determine the D-value for paper carriers. If a manufacturer's label permits usage of the BI carrier with multiple sterilization methods, then data on D-value, survival time, and kill time will need to be provided by the manufacturer for each sterilization method.

For BIs that are spore suspensions, conduct D-value determinations for each of the microorganisms that are provided as a liquid spore crop suspension. The test is conducted using appropriate serial dilutions predicated upon the stated spore ▲titer▲ (ERR 1-Nov-2019) of the suspension in *Purified Water* in a sterile tube. Where the suspension is placed on or in a substrate such as an elastomeric closure or formulated product, its resistance may differ from that determined in *Purified Water*. That difference may be significant to the usage of the BIs and appropriate measurements made before use in sterilization validation activities.

## Recovery

After completion of the sterilizing procedure for BIs and within a noted time (NMT 4 h), aseptically remove and add each BI to a suitable medium (see media in *Sterility Tests* ⟨71⟩) to submerge the BI completely in a suitable tube. For self-contained BIs, the paper strip is immersed in the self-contained medium according to manufacturers' instructions, within a noted time NMT 4 h. For insoluble items inoculated with a spore suspension, aseptically transfer these items individually to a suitable medium (see media in ⟨71⟩) to submerge the item completely in the medium. When a sealed aqueous filled container has been inoculated, test the units individually, as described within ⟨71⟩.

Incubate each tube at the optimal recovery temperature appropriate for the BI. Observe each inoculated medium-containing tube at appropriate intervals for a total of 7 days after inoculation. Where growth is observed at any particular observation time, further incubation of the test sample(s) concerned may be omitted. Note the number of samples showing no evidence of growth at any time.

Where *Clostridium sporogenes* or another anaerobic microorganism is used as a BI, methods for preparation, inoculation, and recovery methods and media must be adapted to accommodate the use of these anaerobic spore formers.

## Calculation

The determination of D-values of BIs can be performed using the Limited Spearman-Karber, Survival Curve Method, or Stumbo-Murphy-Cochran procedures (*6*–*8*). When the BI has been purchased, use the same method as that defined by the BI manufacturer to subsequently determine D-values. The use of an alternate method can result in differences that are more an artifact of the method than a variation in the performance of the BI.

## Survival Time and Kill Time

Take two groups of BIs, each consisting of 10 test samples, in their original, individual containers. Place the samples of each group in suitable sample holders that permit each sample to be exposed to the sterilizing conditions at a specific location in the BIER chamber.

Expose the samples for the required survival time, enter the chamber, and remove the holder(s) containing the 10 test samples. Repeat the above procedure immediately, or preheat if a substantial interval has elapsed, so as to subject the second holder(s) containing 10 test samples similarly to the first conditions, but for the required kill time. Recover BIs as described above. The *Survival Time and Kill Time* should be provided by the BI manufacturer and verified by the end user.

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# ▲⟨56⟩ METHODS FOR DETERMINATION OF RESISTANCE OF MICROORGANISMS TO STERILIZATION PROCESSES

## INTRODUCTION

Sterilization relies on the reliable destruction of microorganisms present in/on the materials being sterilized. Information regarding the resistance of the bioburden to the sterilization process (with the possible exception of overkill processes) is essential to confirm the efficacy of the sterilization process (see *Sterilization of Compendial Articles* ⟨1229⟩) and is also a required component of a parametric release program (*1*,*2*). Spores exhibit inherent and varying levels of resistance to all modalities of sterilization. There are limited resistance data available for some spore-forming and pathogenic microorganisms; however, for the vast majority of microorganisms (including most spores), there is no data available regarding their susceptibility to sterilization processes when present in or on pharmaceutical articles (*3*). Furthermore, wild type microorganisms and spores derived from manufacturing processes are likely distinct from their reference-standard culture-collection counterparts and may exhibit differing levels of resistance to sterilization processes (*3*). This chapter outlines methods that can be used to determine the presence of spores and the resistance of microorganisms, spores, and biologic indicators (BIs) to moist heat sterilization, including variations of the boil test mentioned in *Moist Heat Sterilization of Aqueous Liquids* ⟨1229.2⟩ and *Monitoring of Bioburden* ⟨1229.3⟩. The methods included in this chapter are not intended for use in radiation sterilization where the procedures described in ISO 11137 are to be followed (*4*).

## GENERAL PROCEDURES

Carry out the tests under conditions designed to avoid extrinsic microbial contamination of the materials to be examined. The precautions taken to avoid contamination must be such that they do not affect the microorganisms that are recovered in the test. If the product to be examined has antimicrobial activity, insofar as possible, this should be removed or neutralized during the presence or absence test for spores. If inactivators are used for this purpose, their efficacy and their nontoxicity to the microorganisms must be demonstrated. If surface-active substances are used for sample preparation, their nontoxicity to the microorganisms and compatibility with any inactivators used must be demonstrated.

### Preparation of the Sample

The method for sample preparation depends on the physical characteristics of the pre-sterilization material to be tested. For aqueous products, 10-mL samples should be taken from closed primary containers. Composite sampling may be used. For solid, water-insoluble, or immiscible products, follow the sample preparation details provided in *Microbial Enumeration Tests* ⟨61⟩. Samples should be aseptically transferred (either from 1 unit or composite) to sterile, screw-capped tubes. A minimum of 10 samples should be tested to minimize variability.

## PRESENCE/ABSENCE TEST FOR SPORES (ALL STERILIZATION PROCESSES EXCEPT FILTRATION AND RADIATION)

A universal assumption in overkill sterilization processes is that the resistance of the bioburden microorganisms as compared to the BI is negligible because the BI is specifically chosen as a "worst case" challenge to the sterilization process (*1*). This is predicated upon an assumption of the absence of spores in the pre-sterilization bioburden. A test in which bioburden samples are exposed to 70°–75° for 30 min can be used to confirm the absence of spores. Vegetative microorganisms are seldom resistant to temperatures >70° and are killed under these conditions. However, under these temperature conditions with the presence of non-nutrient germinants (agents not necessary for growth but which promote germination of spores, such as Ca-DPA and dodecylamine), spore germination is promoted by heat activation. This test is suitable where the presence of spores in the pre-sterilization bioburden is associated with a greater chance for survival (*5*). When spores are detected, resistance testing as outlined below for moist heat sterilization or in ISO 11138 series of standards (*6*,*7*) should be conducted to confirm that process lethality is sufficient.

### Heat Treatment

Transfer the screw-capped tubes to a water bath, heat the contents to 70°–75°, and hold at this temperature for 30 min.

### Recovery and Enumeration of Spores

Immediately cool the product to 30°–35°, and aseptically add sterile calcium dipicolinic acid (Ca-DPA) and dodecylamine (non-nutrient germinants) in Buffered Sodium Chloride–Peptone Solution pH 7.0 or in Phosphate Buffer Solution pH 7.2 to generate concentrations of 60 and 1.2 mM, respectively. Maintain the solutions at 30°–35° for 30 min. Use either membrane filtration or plate-count methods per ⟨61⟩ for the recovery and growth of spores with *Soybean–Casein Digest Agar* containing 60 mM Ca-DPA and 1.2 mM dodecylamine, incubated at 30°–35° for 5–7 days.

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## Positive and Negative Controls

To verify efficacy of heat treatment, a positive control is included by inoculating at least one additional test sample with a countable number of spores such as *Bacillus subtilis*. There must be growth of microorganisms. To verify testing conditions, a negative control is included using the chosen diluent in a screw-capped tube in place of the test sample. There must be no growth of microorganisms.

## Growth Promotion of Media

Test each batch of *Soybean–Casein Digest Agar* containing 60 mM Ca-DPA and 1.2 mM dodecylamine per *Microbial Enumeration Tests* ⟨61⟩, *Table 1*.

## Suitability of Recovery and Enumeration

Perform method suitability per ⟨61⟩ by adding a sufficient volume of microbial suspension of *Bacillus subtilis* ATCC 6633, NCIMB 8054, CIP 52.62, or NBRC 3134 to the cooled product and demonstrating acceptable recovery on *Soybean–Casein Digest Agar* containing 60 mM Ca-DPA and 1.2 mM dodecylamine.

## Interpretation of Results

The presence of colony forming units suggests that spores are on/in the product and more definitive estimation of their resistance should be considered (see below).

# RESISTANCE ESTIMATION (MOIST HEAT PROCESSES ONLY)

This test method, also known as Boil Test, can be applied to estimate moist heat resistance of spores present in pre-sterilization product. Initially, a range of times can be used to estimate moist heat resistance (*Preliminary Resistance Estimation*). The use of a single time period tailored to the pre-established limits for resistance (*Maximum Resistance Estimation*) can be used to support parametric release to confirm sterilization process suitability. Either of these tests can be performed with parallel testing of a biological indicator microorganism for comparison purposes.

## Preliminary Resistance Estimation

Transfer samples in screw-capped tubes to a water bath, heat the contents to 95°–100°, and incubate at this temperature using a range of time periods (see *Table 1*). *Recovery and Enumeration of Spores*, *Positive and Negative Controls*, *Growth Promotion of Media*, and *Suitability of Recovery and Enumeration* are performed as described above.

### INTERPRETATION OF RESULTS

The estimated moist heat resistance of spores is established by the maximum time where colony growth is determined as shown in *Table 1*. Adjustments in dwell time (shorter or longer) at 95°–100° can be used to accommodate the microorganisms detected.

## Maximum Resistance Estimation

Transfer samples in screw-capped tubes to a water bath, heat the contents to 95°–100°, and incubate at this temperature for the desired time. *Recovery and Enumeration of Spores*, *Positive and Negative Controls*, *Growth Promotion of Media*, and *Suitability of Recovery and Enumeration* are performed as described above.

### INTERPRETATION OF RESULTS

The presence of colony forming units suggests that spores are present with a level of moist heat resistance to sterilization and requires further investigation.

**Table 1. Moist Heat Resistance Estimation from Boil Test**

| Exposure Time at 95°–100° with Positive Growth (min) | Approximate $D_{121°}$-Value (min) |
|---|---|
| 10 | ≥0.1 |
| 20 | ≥0.2 |
| 30 | ≥0.3 |
| 40 | ≥0.4 |
| 50 | ≥0.5 |

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# *D*-VALUE DETERMINATION (MOIST HEAT PROCESSES ONLY)

The determination of *D*-value for microorganisms and BIs is a required aspect of sterilization cycle development and validation (*1,8*). The established procedures for this determination are focused on moist heat processes performed near 121° and rely upon equipment specifically designed to operate with saturated steam. Accurate resistance determination for biological indicators and bioburden isolates from moist heat processes operating at lower temperatures can benefit from the alternative practices described below.

## *D*-value Determination for >110° Processes

The methods described in *Biological Indicators—Resistance Performance Tests* ⟨55⟩ should be followed. Additional details regarding the procedures and equipment can be found in ISO 18472, *Sterilization of health care products—Biological and chemical indicators—Test equipment* (*9*).

## *D*-value Determination for ≤110° Processes

The apparatuses for *D*-value determination at 121° utilize saturated steam and resemble steam sterilizers in their design and operation. With the use of sterilization processes at temperatures ≤110°, adaptation of the test equipment and methods to better suit the use of lower temperatures is appropriate. Adjustments in dwell time (shorter or longer) and operating temperature may be necessary to accommodate the microorganism being tested (*10*). These methods apply directly to BI's used for low temperature moist heat processes and must be adapted for use with bioburden isolates.

### PROCEDURE

Carry out the tests for *D*-value at sterilization conditions consistent with those intended for use. Use 20 replicate (BI or bioburden isolate) test sample items subjected to NLT 5 exposure conditions for a total of 100 tests. The number of exposure conditions is chosen to provide a range of observations from NLT 1 *D*-value below the expected survival time through NLT 1 *D*-value above the expected kill time. Place each group in a separate suitable sample holder that permits each sample to be exposed to the prescribed temperature condition in a water bath at 95°–100° (other temperatures can be utilized as necessary). Check the apparatus for operating parameters using sample holders with dummy test samples. Select a series of sterilizing times in increments starting with the shortest time for the samples to be tested. The differences in sterilizing times over the series are as constant as feasible, and the difference between adjacent times is NMT 75% of the expected *D*-value. Test procedures for the evaluation of microbial resistance are defined in the ISO 11138-series standards (*6,7*). The test methods and carriers used may be adapted to the specifics of the microorganisms being evaluated.

For microorganisms that are spores in suspension, the test is conducted using appropriate serial dilutions predicated upon the stated spore titer of the suspension in *Sterilized Purified Water* in a sterile tube.

### RECOVERY

After completion of the sterilizing procedure and within a noted time (NMT 4 h), aseptically remove and add each sample to a suitable medium (see *Sterility Tests* ⟨71⟩, *Culture Media and Incubation Temperatures*), submerging the unit completely in a suitable tube. For self-contained BIs, the paper strip is immersed in the self-contained medium according to manufacturers' instructions, within a noted time of NMT 4 h. For insoluble items inoculated with a spore suspension, aseptically transfer these units individually to a suitable medium (see media in ⟨71⟩) supplemented to contain 60 mM Ca-DPA and 1.2 mM dodecylamine and submerge the items completely in the medium. When a sealed aqueous filled container has been inoculated, test the units individually, as described in ⟨71⟩.

Incubate each tube at the optimal recovery temperature. For bioburden isolates, recovery methods and media may be modified as needed. Observe each inoculated medium-containing tube at appropriate intervals for a total of 7 days after inoculation. Where growth is observed at any observation time, further incubation of the test sample(s) concerned may be omitted. Note the number of samples showing no evidence of growth at any time.

Where *Clostridium sporogenes* or another anaerobic microorganism is used as a BI, methods for preparation, inoculation, recovery, and media must be adapted to accommodate the use of these anaerobic spore-formers.

### CALCULATION

The determination of *D*-values of microorganisms can be performed using the limited Spearman-Karber procedure, survival curve method, or Stumbo-Murphy-Cochran procedure (*3,11,12*). When a BI has been purchased, use the same method as that defined by the BI manufacturer to subsequently determine *D*-values. The use of an alternate method can result in differences that may be an artifact of the method rather than a variation in the performance of the BI.

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# ▲⟨60⟩ MICROBIOLOGICAL EXAMINATION OF NONSTERILE PRODUCTS —TESTS FOR *BURKHOLDERIA CEPACIA* COMPLEX

## INTRODUCTION

The tests described in this chapter will allow determination of the absence of *Burkholderia cepacia* complex (Bcc), which can be detected under the conditions described.

The tests are designed to determine whether a substance or preparation complies with an established specification for microbiological quality and/or to evaluate whether products—especially those for inhalation use or aqueous preparations for oral, oromucosal, cutaneous, or nasal use—contain members of the Bcc.

## GROWTH-PROMOTING AND INHIBITORY PROPERTIES OF THE MEDIA AND SUITABILITY OF TESTS FOR ABSENCE OF Bcc

Test each batch of ready-prepared medium and each batch of medium prepared from either dehydrated medium or ingredients.

### Preparation of Test Strains

Use standardized stable suspensions of test strains (see *Table 1*) NMT 5 passages removed from the original strain culture.

**Table 1. Test Strains of Microorganisms for Growth Promotion and Suitability Testing**

| Microorganism | Standard Strain |
|---|---|
| *Burkholderia cepacia* | ATCC 25416, NCTC 10743, or CIP 80.24 |
| *Burkholderia cenocepacia* | ATCC BAA-245 or LMG 16656 |
| *Burkholderia multivorans* | ATCC BAA-247, LMG 13010, CCUG 34080, CIP 105495, DSM 13243, or NCTC 13007 |
| *Pseudomonas aeruginosa* | ATCC 9027, NCIMB 8626, CIP 82.118, or NBRC 13275 |
| *Staphylococcus aureus* | ATCC 6538, NCIMB 9518, CIP 4.83, or NBRC 13276 |

### Microorganisms

Grow each of the test strains separately in *Soybean–Casein Digest Broth* or on Soybean–Casein Digest Agar at 30°–35° for 18–24 h.

Use *Buffered Sodium Chloride–Peptone Solution pH 7.0* or *Phosphate Buffer Solution pH 7.2* to make the test suspensions. Use the suspensions within 2 h, or within 24 h if stored at 2°–8°. If purchased, follow the supplier's instructions. If self-prepared cultures are used, follow a validated procedure (such as in *Microbial Enumeration Tests* ⟨61⟩) for preparation. Use a challenge inoculum of NMT 100 colony-forming units (cfu) for growth promotion and suitability testing.

#### NEGATIVE CONTROLS

Include a negative control to verify the testing conditions. There must be no growth of microorganisms. A negative control is also performed when testing the products as described in *Testing of Products*.

#### TEST FOR GROWTH-PROMOTING PROPERTIES, SOLID MEDIA

Perform the *Surface-Spread Method* (see *Microbial Enumeration Tests* ⟨61⟩, *Growth Promotion Test, Suitability of the Counting Method and Negative Controls, Suitability of the Counting Method in the Presence of Product, Recovery of Microorganisms in the Presence of Product, Plate-Count Methods*), inoculating each plate with a small number (NMT 100 cfu) of the appropriate microorganism (see *Table 2*). Incubate at the specified temperature for NMT the shortest period of time specified in the test. Growth of the microorganism comparable to that previously obtained with a previously tested and approved batch of medium occurs.

**Table 2. Microorganisms for the Growth-Promoting, Inhibitory, and Indicative Properties of the Media**

| Medium | Property | Microorganism |
|---|---|---|
| *Burkholderia cepacia* selective agar | Growth-Promoting and Indicative | *Burkholderia cepacia*, *Burkholderia cenocepacia*, or *Burkholderia multivorans* |
| | Inhibitory | *Pseudomonas aeruginosa*, *Staphylococcus aureus* |

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TEST FOR INHIBITORY PROPERTIES, SOLID MEDIA

Inoculate the appropriate medium with at least 100 cfu of the appropriate microorganism. Incubate at the specified temperature for NLT the longest period of time specified in the test. Inhibition of growth of the indicated microorganisms occurs (see *Table 2*).

TEST FOR INDICATIVE PROPERTIES

Perform the *Surface-Spread Method* (see *Microbial Enumeration Tests* ⟨61⟩*, Growth Promotion Test, Suitability of the Counting Method and Negative Controls, Suitability of the Counting Method in the Presence of Product, Recovery of Microorganisms in the Presence of Product, Plate-Count Methods*), inoculating each plate with a small number (NMT 100 cfu) of the indicated microorganism. Incubate at the specified temperature for a period of time within the range specified in the test. Colonies are comparable in appearance and indication reactions to those previously obtained with a previously tested and approved batch of medium (see *Table 2*).

### Suitability of the Test Method

The ability of the test to detect Bcc in the presence of the product to be tested must be established. The incubation time for the method suitability should not exceed the shortest incubation period specified. Suitability must be confirmed if there is a change in testing performance or a change in the product that may affect the outcome of the test.

For each new product to be tested, perform the sample preparation as described in *Testing of Products*. At the time of mixing, add each test strain in the prescribed growth medium. Inoculate the test strains individually. Use a number of microorganisms equivalent to NMT 100 cfu in the inoculated test preparation.

Perform the test as described in *Testing of Products*, using the shortest incubation period prescribed. Bcc microorganisms must be detected with the indication reactions described in *Interpretation*.

Any antimicrobial activity of the product necessitates a modification of the test procedure (see *Microbial Enumeration Tests* ⟨61⟩*, Growth Promotion Test, Suitability of the Counting Method and Negative Controls, Suitability of the Counting Method in the Presence of Product, Neutralization/Removal of Antimicrobial Activity*).

## TESTING OF PRODUCTS

### Sample Preparation and Pre-Incubation

Prepare a sample using a 1-in-10 dilution of NLT 1 g of the product to be examined. Use 10 mL or the quantity corresponding to 1 g or 1 mL to inoculate a suitable amount (determined as described in *Suitability of the Test Method*) of *Soybean–Casein Digest Broth* or an appropriate dilution of *Soybean–Casein Digest Broth* as determined during method suitability (for example, a 1:10 dilution may be required when conducting optional testing of pharmaceutical waters). Then mix and incubate at 30°–35° for 48–72 h.

### Selection and Subculture

Subculture by streaking on a plate of *Burkholderia cepacia* selective agar (BCSA), and incubate at 30°–35° for 48–72 h.

### Interpretation

The possible presence of Bcc is indicated by the growth of greenish–brown colonies with yellow halos, or white colonies surrounded by a pink–red zone on BCSA. Any growth on BCSA is confirmed by identification tests. See *Microbial Characterization, Identification, and Strain Typing* ⟨1113⟩ for additional information.

The product complies with the test if colonies of the types described are not present or if the confirmatory identification tests are negative.

## RECOMMENDED CULTURE MEDIA

[NOTE—This section is given for information.]

The following solutions and culture media have been found satisfactory for the purposes for which they are prescribed in the tests in this Pharmacopeia. Other media may be used provided that their suitability can be demonstrated.

### Stock Buffer Solution

Transfer 34 g of potassium dihydrogen phosphate to a 1000-mL volumetric flask, dissolve in 500 mL of *Purified Water*, adjust with sodium hydroxide to a pH of 7.2 ± 0.2, add *Purified Water* to volume, and mix. Dispense in containers, and sterilize. Store at a temperature of 2°–8°.

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## Phosphate Buffer Solution pH 7.2

Prepare a mixture of *Purified Water* and *Stock Buffer Solution* (800:1 v/v), and sterilize.

## Buffered Sodium Chloride–Peptone Solution pH 7.0

Prepare *Buffered Sodium Chloride–Peptone Solution pH 7.0* as directed in *Table 3*. Sterilize in an autoclave using a validated cycle.

**Table 3**

| | |
|---|---|
| Potassium dihydrogen phosphate | 3.6 g |
| Disodium hydrogen phosphate dihydrate | 7.2 g (equivalent to 0.067 M of phosphate) |
| Sodium chloride | 4.3 g |
| Peptone (meat or casein) | 1.0 g |
| *Purified Water* | 1000 mL |

## Soybean–Casein Digest Broth

Prepare *Soybean–Casein Digest Broth* as directed in *Table 4*. Adjust the pH so that after sterilization it is 7.3 ± 0.2 at 25°. Sterilize in an autoclave using a validated cycle.

**Table 4**

| | |
|---|---|
| Pancreatic digest of casein | 17.0 g |
| Papaic digest of soybean | 3.0 g |
| Sodium chloride | 5.0 g |
| Dibasic hydrogen phosphate | 2.5 g |
| Glucose monohydrate | 2.5 g |
| *Purified Water* | 1000 mL |

## *Burkholderia cepacia* Selective Agar

Prepare BCSA as directed in *Table 5*. When preparing media in-house, first prepare the base ingredients without the antibiotics. Adjust the pH so that after sterilization it is 6.8 ± 0.3 at 25°. Sterilize in an autoclave using a validated cycle. Cool the base medium to 45°–50° and add a 1% solution of the sterile filtered antibiotics, mix, and pour into the plates.

**Table 5**

| | |
|---|---|
| Casein peptone | 10.0 g |
| Lactose | 10.0 g |
| Sucrose | 10.0 g |
| Sodium chloride | 5.0 g |
| Yeast extract | 1.5 g |
| Phenol red | 0.08 g |
| Gentamicin | 10.0 mg |
| Vancomycin | 2.5 mg |
| Crystal violet | 2.0 mg |
| Polymyxin B | 600,000 U |
| Agar | 14.0 g |
| Demineralized water | 1000 mL▲ (USP 1-Dec-2019) |

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# ⟨61⟩ MICROBIOLOGICAL EXAMINATION OF NONSTERILE PRODUCTS: MICROBIAL ENUMERATION TESTS

## INTRODUCTION

The tests described hereafter will allow quantitative enumeration of mesophilic bacteria and fungi that may grow under aerobic conditions.

The tests are designed primarily to determine whether a substance or preparation complies with an established specification for microbiological quality. When used for such purposes, follow the instructions given below, including the number of samples to be taken, and interpret the results as stated below.

The methods are not applicable to products containing viable microorganisms as active ingredients.

Alternative microbiological procedures, including automated methods, may be used, provided that their equivalence to the Pharmacopeial method has been demonstrated.

## GENERAL PROCEDURES

Carry out the determination under conditions designed to avoid extrinsic microbial contamination of the product to be examined. The precautions taken to avoid contamination must be such that they do not affect any microorganisms that are to be revealed in the test.

If the product to be examined has antimicrobial activity, this is, insofar as possible, removed or neutralized. If inactivators are used for this purpose, their efficacy and their absence of toxicity for microorganisms must be demonstrated.

If surface-active substances are used for sample preparation, their absence of toxicity for microorganisms and their compatibility with any inactivators used must be demonstrated.

## ENUMERATION METHODS

Use the *Membrane Filtration* method or one of the *Plate-Count Methods*, as directed. The *Most-Probable-Number (MPN) Method* is generally the least accurate method for microbial counts; however, for certain product groups with very low bioburden, it may be the most appropriate method.

The choice of a method is based on factors such as the nature of the product and the required limit of microorganisms. The method chosen must allow testing of a sufficient sample size to judge compliance with the specification. The suitability of the chosen method must be established.

## GROWTH PROMOTION TEST, SUITABILITY OF THE COUNTING METHOD AND NEGATIVE CONTROLS

### General Considerations

The ability of the test to detect microorganisms in the presence of product to be tested must be established.

Suitability must be confirmed if a change in testing performance or a change in the product that may affect the outcome of the test, is introduced.

### Preparation of Test Strains

Use standardized stable suspensions of test strains or prepare as stated below. Seed-lot culture maintenance techniques (seed-lot systems) are used so that the viable microorganisms used for inoculation are not more than five passages removed from the original master seed-lot. Grow each of the bacterial and fungal test strains separately as described in *Table 1*.

**Table 1. Preparation and Use of Test Microorganisms**

| | | Growth Promotion | | Suitability of Counting Method in the Presence of Product | |
|---|---|---|---|---|---|
| **Microorganism** | **Preparation of Test Strain** | **Total Aerobic Microbial Count** | **Total Yeasts and Molds Count** | **Total Aerobic Microbial Count** | **Total Yeasts and Molds Count** |
| *Staphylococcus aureus* such as ATCC 6538, NCIMB 9518, CIP 4.83, or NBRC 13276 | Soybean–Casein Digest Agar or Soybean–Casein Digest Broth<br>30°–35°<br>18–24 hours | Soybean–Casein Digest Agar and Soybean–Casein Digest Broth<br>≤100 cfu<br>30°–35°<br>≤3 days | | Soybean–Casein Digest Agar/MPN Soybean–Casein Digest Broth<br>≤100 cfu<br>30°–35°<br>≤3 days | |

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**Table 1. Preparation and Use of Test Microorganisms** *(continued)*

| | | Growth Promotion | | Suitability of Counting Method in the Presence of Product | |
|---|---|---|---|---|---|
| **Microorganism** | **Preparation of Test Strain** | **Total Aerobic Microbial Count** | **Total Yeasts and Molds Count** | **Total Aerobic Microbial Count** | **Total Yeasts and Molds Count** |
| *Pseudomonas aeruginosa* such as ATCC 9027, NCIMB 8626, CIP 82.118, or NBRC 13275 | Soybean–Casein Digest Agar or Soybean–Casein Digest Broth<br>30°–35°<br>18–24 hours | Soybean–Casein Digest Agar and Soybean–Casein Digest Broth<br>≤100 cfu<br>30°–35°<br>≤3 days | | Soybean–Casein Digest Agar/MPN Soybean–Casein Digest Broth<br>≤100 cfu<br>30°–35°<br>≤3 days | |
| *Bacillus subtilis* such as ATCC 6633, NCIMB 8054, CIP 52.62, or NBRC 3134 | Soybean–Casein Digest Agar or Soybean–Casein Digest Broth<br>30°–35°<br>18–24 hours | Soybean–Casein Digest Agar and Soybean–Casein Digest Broth<br>≤100 cfu<br>30°–35°<br>≤3 days | | Soybean–Casein Digest Agar/MPN Soybean–Casein Digest Broth<br>≤100 cfu<br>30°–35°<br>≤3 days | |
| *Candida albicans* such as ATCC 10231, NCPF 3179, IP 48.72, or NBRC 1594 | Sabouraud Dextrose Agar or Sabouraud Dextrose Broth<br>20°–25°<br>2–3 days | Soybean–Casein Digest Agar<br>≤100 cfu<br>30°–35°<br>≤5 days | Sabouraud Dextrose Agar<br>≤100 cfu<br>20°–25°<br>≤5 days | Soybean–Casein Digest Agar<br>≤100 cfu<br>30°–35°<br>≤5 days<br>MPN: not applicable | Sabouraud Dextrose Agar<br>≤100 cfu<br>20°–25°<br>≤5 days |
| *Aspergillus brasiliensis* such as ATCC 16404, IMI 149007, IP 1431.83, or NBRC 9455 | Sabouraud Dextrose Agar or Potato–Dextrose Agar<br>20°–25° 5–7 days, or until good sporulation is achieved | Soybean–Casein Digest Agar<br>≤100 cfu<br>30°–35°<br>≤5 days | Sabouraud Dextrose Agar<br>≤100 cfu<br>20°–25°<br>≤5 days | Soybean–Casein Digest Agar<br>≤100 cfu<br>30°–35°<br>≤5 days<br>MPN: not applicable | Sabouraud Dextrose Agar<br>≤100 cfu<br>20°–25°<br>≤5 days |

Use *Buffered Sodium Chloride–Peptone Solution pH 7.0* or *Phosphate Buffer Solution pH 7.2* to make test suspensions; to suspend *A. brasiliensis* spores, 0.05% of polysorbate 80 may be added to the buffer. Use the suspensions within 2 hours, or within 24 hours if stored between 2° and 8°. As an alternative to preparing and then diluting a fresh suspension of vegetative cells of *A. brasiliensis* or *B. subtilis,* a stable spore suspension is prepared and then an appropriate volume of the spore suspension is used for test inoculation. The stable spore suspension may be maintained at 2° to 8° for a validated period of time.

## Negative Control

To verify testing conditions, a negative control is performed using the chosen diluent in place of the test preparation. There must be no growth of microorganisms. A negative control is also performed when testing the products as described under *Testing of Products*. A failed negative control requires an investigation.

## Growth Promotion of the Media

Test each batch of ready-prepared medium and each batch of medium prepared either from dehydrated medium or from the ingredients described.

Inoculate portions/plates of *Soybean–Casein Digest Broth* and *Soybean–Casein Digest Agar* with a small number (not more than 100 cfu) of the microorganisms indicated in *Table 1*, using a separate portion/plate of medium for each. Inoculate plates of *Sabouraud Dextrose Agar* with a small number (not more than 100 cfu) of the microorganisms indicated in *Table 1*, using a separate plate of medium for each. Incubate according to the conditions described in *Table 1*.

For solid media, growth obtained must not differ by a factor greater than 2 from the calculated value for a standardized inoculum. For a freshly prepared inoculum, growth of the microorganisms comparable to that previously obtained with a previously tested and approved batch of medium occurs. Liquid media are suitable if clearly visible growth of the microorganisms comparable to that previously obtained with a previously tested and approved batch of medium occurs.

## Suitability of the Counting Method in the Presence of Product

### PREPARATION OF THE SAMPLE

The method for sample preparation depends on the physical characteristics of the product to be tested. If none of the procedures described below can be demonstrated to be satisfactory, a suitable alternative procedure must be developed.

**Water-Soluble Products—**Dissolve or dilute (usually a 1 in 10 dilution is prepared) the product to be examined in *Buffered Sodium Chloride–Peptone Solution pH 7.0, Phosphate Buffer Solution pH 7.2,* or *Soybean–Casein Digest Broth*. If necessary, adjust to a pH of 6 to 8. Further dilutions, where necessary, are prepared with the same diluent.

**Nonfatty Products Insoluble in Water—**Suspend the product to be examined (usually a 1 in 10 dilution is prepared) in *Buffered Sodium Chloride–Peptone Solution pH 7.0, Phosphate Buffer Solution pH 7.2,* or *Soybean–Casein Digest Broth*. A surface-active agent such as 1 g per L of polysorbate 80 may be added to assist the suspension of poorly wettable substances. If necessary, adjust to a pH of 6 to 8. Further dilutions, where necessary, are prepared with the same diluent.

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**Fatty Products**—Dissolve in isopropyl myristate sterilized by filtration, or mix the product to be examined with the minimum necessary quantity of sterile polysorbate 80 or another noninhibitory sterile surface-active reagent heated, if necessary, to not more than 40° or, in exceptional cases, to not more than 45°. Mix carefully and if necessary maintain the temperature in a water bath. Add a sufficient quantity of the prewarmed chosen diluent to make a 1 in 10 dilution of the original product. Mix carefully, while maintaining the temperature for the shortest time necessary for the formation of an emulsion. Further serial 10-fold dilutions may be prepared using the chosen diluent containing a suitable concentration of sterile polysorbate 80 or another noninhibitory sterile surface-active reagent.
**Fluids or Solids in Aerosol Form**—Aseptically transfer the product into a membrane filter apparatus or a sterile container for further sampling. Use either the total contents or a defined number of metered doses from each of the containers tested.
**Transdermal Patches**—Remove the protective cover sheets ("release liners") of the transdermal patches and place them, adhesive side upwards, on sterile glass or plastic trays. Cover the adhesive surface with a suitable sterile porous material (e.g., sterile gauze) to prevent the patches from sticking together, and transfer the patches to a suitable volume of the chosen diluent containing inactivators such as polysorbate 80 and/or lecithin. Shake the preparation vigorously for at least 30 minutes.

## INOCULATION AND DILUTION

Add to the sample prepared as directed above and to a control (with no test material included) a sufficient volume of the microbial suspension to obtain an inoculum of not more than than 100 cfu. The volume of the suspension of the inoculum should not exceed 1% of the volume of diluted product.

To demonstrate acceptable microbial recovery from the product, the lowest possible dilution factor of the prepared sample must be used for the test. Where this is not possible due to antimicrobial activity or poor solubility, further appropriate protocols must be developed. If inhibition of growth by the sample cannot otherwise be avoided, the aliquot of the microbial suspension may be added after neutralization, dilution, or filtration.

## NEUTRALIZATION/REMOVAL OF ANTIMICROBIAL ACTIVITY

The number of microorganisms recovered from the prepared sample diluted as described in *Inoculation and Dilution* and incubated following the procedure described in *Recovery of Microorganisms in the Presence of Product,* is compared to the number of microorganisms recovered from the control preparation.

If growth is inhibited (reduction by a factor greater than 2), then modify the procedure for the particular enumeration test to ensure the validity of the results. Modification of the procedure may include, for example,

1. An increase in the volume of the diluent or culture medium;
2. Incorporation of a specific or general neutralizing agents into the diluent;
3. Membrane filtration; or
4. A combination of the above measures.

**Neutralizing Agents**—Neutralizing agents may be used to neutralize the activity of antimicrobial agents (see *Table 2*). They may be added to the chosen diluent or the medium preferably before sterilization. If used, their efficacy and their absence of toxicity for microorganisms must be demonstrated by carrying out a blank with neutralizer and without product.

**Table 2. Common Neutralizing Agents/Methods for Interfering Substances**

| Interfering Substance | Potential Neutralizing Agents/Method |
|---|---|
| Glutaraldehyde, mercurials | Sodium hydrogen sulfite (Sodium bisulfite) |
| Phenolics, alcohol, aldehydes, sorbate | Dilution |
| Aldehydes | Glycine |
| Quaternary ammonium compounds (QACs), parahydroxybenzoates (parabens), bis-biguanides | Lecithin |
| QACs, iodine, parabens | Polysorbate |
| Mercurials | Thioglycollate |
| Mercurials, halogens, aldehydes | Thiosulfate |
| EDTA (edetate) | Mg or Ca ions |

If no suitable neutralizing method can be found, it can be assumed that the failure to isolate the inoculated organism is attributable to the microbicidal activity of the product. This information serves to indicate that the article is not likely to be contaminated with the given species of the microorganism. However, it is possible that the product inhibits only some of the microorganisms specified herein, but does not inhibit others not included among the test strains or those for which the latter are not representative. Then, perform the test with the highest dilution factor compatible with microbial growth and the specific acceptance criterion.

## RECOVERY OF MICROORGANISMS IN THE PRESENCE OF PRODUCT

For each of the microorganisms listed, separate tests are performed. Only microorganisms of the added test strain are counted.

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**Membrane Filtration**—Use membrane filters having a nominal pore size not greater than 0.45 µm. The type of filter material is chosen in such a way that the bacteria-retaining efficiency is not affected by the components of the sample to be investigated. For each of the microorganisms listed, one membrane filter is used.

Transfer a suitable quantity of the sample prepared as described under *Preparation of the Sample, Inoculation and Dilution,* and *Neutralization/Removal of Antimicrobial Activity* (preferably representing 1 g of the product, or less if large numbers of cfu are expected) to the membrane filter, filter immediately, and rinse the membrane filter with an appropriate volume of diluent.

For the determination of total aerobic microbial count (TAMC), transfer the membrane filter to the surface of the *Soybean–Casein Digest Agar*. For the determination of total combined yeasts and molds count (TYMC), transfer the membrane to the surface of the *Sabouraud Dextrose Agar.* Incubate the plates as indicated in *Table 1*. Perform the counting.

**Plate-Count Methods**—Perform plate-count methods at least in duplicate for each medium, and use the mean count of the result.

*Pour-Plate Method*—For Petri dishes 9 cm in diameter, add to the dish 1 mL of the sample prepared as described under *Preparation of the Sample, Inoculation and Dilution,* and *Neutralization/Removal of Antimicrobial Activity* and 15 to 20 mL of *Soybean–Casein Digest Agar* or *Sabouraud Dextrose Agar,* both media maintained at not more than 45°. If larger Petri dishes are used, the amount of agar medium is increased accordingly. For each of the microorganisms listed in *Table 1*, at least two Petri dishes are used.

Incubate the plates as indicated in *Table 1*. Take the arithmetic mean of the counts per medium, and calculate the number of cfu in the original inoculum.

*Surface-Spread Method*—For Petri dishes 9 cm in diameter, add 15 to 20 mL of *Soybean–Casein Digest Agar* or *Sabouraud Dextrose Agar* at about 45° to each Petri dish, and allow to solidify. If larger Petri dishes are used, the volume of the agar is increased accordingly. Dry the plates, for example, in a laminar-airflow cabinet or in an incubator. For each of the microorganisms listed in *Table 1*, at least two Petri dishes are used. Spread a measured volume of not less than 0.1 mL of the sample, prepared as directed under *Preparation of the Sample, Inoculation and Dilution,* and *Neutralization/Removal of Antimicrobial Activity* over the surface of the medium. Incubate and count as directed for *Pour-Plate Method*.

**Most-Probable-Number (MPN) Method**—The precision and accuracy of the *MPN Method* is less than that of the *Membrane Filtration* method or the *Plate-Count Method*. Unreliable results are obtained particularly for the enumeration of molds. For these reasons, the *MPN Method* is reserved for the enumeration of TAMC in situations where no other method is available. If the use of the method is justified, proceed as follows.

Prepare a series of at least three serial 10-fold dilutions of the product as described for *Preparation of the Sample, Inoculation and Dilution,* and *Neutralization/Removal of Antimicrobial Activity*. From each level of dilution, three aliquots of 1 g or 1 mL are used to inoculate three tubes with 9 to 10 mL of *Soybean–Casein Digest Broth*. If necessary a surface-active agent such as polysorbate 80, or an inactivator of antimicrobial agents may be added to the medium. Thus, if three levels of dilution are prepared, nine tubes are inoculated.

Incubate all tubes at 30° to 35° for not more than 3 days. If reading of the results is difficult or uncertain owing to the nature of the product to be examined, subculture in the same broth or in *Soybean–Casein Digest Agar* for 1 to 2 days at the same temperature, and use these results. From *Table 3,* determine the most probable number of microorganisms per g or mL of the product to be examined.

**Table 3. Most-Probable-Number Values of Microorganisms**

| Observed Combinations of Numbers of Tubes Showing Growth in Each Set | | | MPN per g or per mL of Product | 95% Confidence Limits |
|---|---|---|---|---|
| Number of g or mL of Product per Tube | | | | |
| 0.1 | 0.01 | 0.001 | | |
| 0 | 0 | 0 | <3 | 0–9.4 |
| 0 | 0 | 1 | 3 | 0.1–9.5 |
| 0 | 1 | 0 | 3 | 0.1–10 |
| 0 | 1 | 1 | 6.1 | 1.2–17 |
| 0 | 2 | 0 | 6.2 | 1.2–17 |
| 0 | 3 | 0 | 9.4 | 3.5–35 |
| 1 | 0 | 0 | 3.6 | 0.2–17 |
| 1 | 0 | 1 | 7.2 | 1.2–17 |
| 1 | 0 | 2 | 11 | 4–35 |
| 1 | 1 | 0 | 7.4 | 1.3–20 |
| 1 | 1 | 1 | 11 | 4–35 |
| 1 | 2 | 0 | 11 | 4–35 |
| 1 | 2 | 1 | 15 | 5–38 |
| 1 | 3 | 0 | 16 | 5–38 |
| 2 | 0 | 0 | 9.2 | 1.5–35 |
| 2 | 0 | 1 | 14 | 4–35 |

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**Table 3. Most-Probable-Number Values of Microorganisms** *(continued)*

| Observed Combinations of Numbers of Tubes Showing Growth in Each Set | | | MPN per g or per mL of Product | 95% Confidence Limits |
|---|---|---|---|---|
| Number of g or mL of Product per Tube | | | | |
| 2 | 0 | 2 | 20 | 5–38 |
| 2 | 1 | 0 | 15 | 4–38 |
| 2 | 1 | 1 | 20 | 5–38 |
| 2 | 1 | 2 | 27 | 9–94 |
| 2 | 2 | 0 | 21 | 5–40 |
| 2 | 2 | 1 | 28 | 9–94 |
| 2 | 2 | 2 | 35 | 9–94 |
| 2 | 3 | 0 | 29 | 9–94 |
| 2 | 3 | 1 | 36 | 9–94 |
| 3 | 0 | 0 | 23 | 5–94 |
| 3 | 0 | 1 | 38 | 9–104 |
| 3 | 0 | 2 | 64 | 16–181 |
| 3 | 1 | 0 | 43 | 9–181 |
| 3 | 1 | 1 | 75 | 17–199 |
| 3 | 1 | 2 | 120 | 30–360 |
| 3 | 1 | 3 | 160 | 30–380 |
| 3 | 2 | 0 | 93 | 18–360 |
| 3 | 2 | 1 | 150 | 30–380 |
| 3 | 2 | 2 | 210 | 30–400 |
| 3 | 2 | 3 | 290 | 90–990 |
| 3 | 3 | 0 | 240 | 40–990 |
| 3 | 3 | 1 | 460 | 90–1980 |
| 3 | 3 | 2 | 1100 | 200–4000 |
| 3 | 3 | 3 | >1100 | |

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RESULTS AND INTERPRETATION

When verifying the suitability of the *Membrane Filtration* method or the *Plate-Count Method,* a mean count of any of the test organisms not differing by a factor greater than 2 from the value of the control defined in *Inoculation and Dilution* in the absence of product must be obtained. When verifying the suitability of the *MPN Method,* the calculated value from the inoculum must be within 95% confidence limits of the results obtained with the control.

If the above criteria cannot be met for one of more of the organisms tested with any of the described methods, the method and test conditions that come closest to the criteria are used to test the product.

## TESTING OF PRODUCTS

### Amount Used for the Test

Unless otherwise directed, use 10 g or 10 mL of the product to be examined taken with the precautions referred to above. For fluids or solids in aerosol form, sample 10 containers. For transdermal patches, sample 10 patches.

The amount to be tested may be reduced for active substances that will be formulated in the following conditions: the amount per dosage unit (e.g., tablet, capsule, injection) is less than or equal to 1 mg, or the amount per g or mL (for preparations not presented in dose units) is less than 1 mg. In these cases, the amount of sample to be tested is not less than the amount present in 10 dosage units or 10 g or 10 mL of the product.

For materials used as active substances where the sample quantity is limited or batch size is extremely small (i.e., less than 1000 mL or 1000 g), the amount tested shall be 1% of the batch unless a lesser amount is prescribed or justified and authorized.

For products where the total number of entities in a batch is less than 200 (e.g., samples used in clinical trials), the sample size may be reduced to two units, or one unit if the size is less than 100.

Select the sample(s) at random from the bulk material or from the available containers of the preparation. To obtain the required quantity, mix the contents of a sufficient number of containers to provide the sample.

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## Examination of the Product

### MEMBRANE FILTRATION

Use a filtration apparatus designed to allow the transfer of the filter to the medium. Prepare the sample using a method that has been shown to be suitable as described in *Growth Promotion Test and Suitability of the Counting Method,* transfer the appropriate amount to each of two membrane filters, and filter immediately. Wash each filter following the procedure shown to be suitable.

For the determination of TAMC, transfer one of the membrane filters to the surface of *Soybean–Casein Digest Agar*. For the determination of TYMC, transfer the other membrane to the surface of *Sabouraud Dextrose Agar*. Incubate the plate of *Soybean–Casein Digest Agar* at 30° to 35° for 3 to 5 days and the plate of *Sabouraud Dextrose Agar* at 20° to 25° for 5 to 7 days. Calculate the number of cfu per g or per mL of product.

When examining transdermal patches, separately filter 10% of the volume of the preparation described for *Preparation of the Sample* through each of two sterile filter membranes. Transfer one membrane to *Soybean–Casein Digest Agar* for TAMC and the other membrane to *Sabouraud Dextrose Agar* for TYMC.

### PLATE-COUNT METHODS

**Pour-Plate Method**—Prepare the sample using a method that has been shown to be suitable as described in *Growth Promotion Test and Suitability of the Counting Method*. Prepare for each medium at least two Petri dishes for each level of dilution. Incubate the plates of *Soybean–Casein Digest Agar* at 30° to 35° for 3 to 5 days and the plates of *Sabouraud Dextrose Agar* at 20° to 25° for 5 to 7 days. Select the plates corresponding to a given dilution and showing the highest number of colonies less than 250 for TAMC and 50 for TYMC. Take the arithmetic mean per culture medium of the counts, and calculate the number of cfu per g or per mL of product.

**Surface-Spread Method**—Prepare the sample using a method that has been shown to be suitable as described in *Growth Promotion Test and Suitability of the Counting Method*. Prepare at least two Petri dishes for each medium and each level of dilution. For incubation and calculation of the number of cfu, proceed as directed for the *Pour-Plate Method.*

### MOST-PROBABLE-NUMBER METHOD

Prepare and dilute the sample using a method that has been shown to be suitable as decribed in *Growth Promotion Test and Suitability of the Counting Method*. Incubate all tubes for 3 to 5 days at 30° to 35°. Subculture if necessary, using the procedure shown to be suitable. Record for each level of dilution the number of tubes showing microbial growth. Determine the most probable number of microorganisms per g or mL of the product to be examined from *Table 3*.

## Interpretation of the Results

The total aerobic microbial count (TAMC) is considered to be equal to the number of cfu found using *Soybean–Casein Digest Agar;* if colonies of fungi are detected on this medium, they are counted as part of TAMC. The total combined yeasts and molds count (TYMC) is considered to be equal to the number of cfu found using *Sabouraud Dextrose Agar;* if colonies of bacteria are detected on this medium, they are counted as part of TYMC. When the TYMC is expected to exceed the acceptance criterion due to the bacterial growth, *Sabouraud Dextrose Agar* containing antibiotics may be used. If the count is carried out by the *MPN Method,* the calculated value is TAMC.

When an acceptance criterion for microbiological quality is prescribed, it is interpreted as follows:

- $10^1$ cfu: maximum acceptable count = 20;
- $10^2$ cfu: maximum acceptable count = 200;
- $10^3$ cfu: maximum acceptable count = 2000;

and so forth.

The recommended solutions and media are described in *Tests for Specified Microorganisms* ⟨62⟩.

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# ⟨62⟩ MICROBIOLOGICAL EXAMINATION OF NONSTERILE PRODUCTS: TESTS FOR SPECIFIED MICROORGANISMS

## INTRODUCTION

The tests described hereafter will allow determination of the absence of, or limited occurrence of, specified microorganisms that may be detected under the conditions described.

The tests are designed primarily to determine whether a substance or preparation complies with an established specification for microbiological quality. When used for such purposes, follow the instructions given below, including the number of samples to be taken, and interpret the results as stated below.

Alternative microbiological procedures, including automated methods, may be used, provided that their equivalence to the Pharmacopeial method has been demonstrated.

## GENERAL PROCEDURES

The preparation of samples is carried out as described in *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* ⟨61⟩.

If the product to be examined has antimicrobial activity, this is insofar as possible removed or neutralized as described in *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* ⟨61⟩.

If surface-active substances are used for sample preparation, their absence of toxicity for microorganisms and their compatibility with any inactivators used must be demonstrated as described in *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* ⟨61⟩.

## GROWTH-PROMOTING AND INHIBITORY PROPERTIES OF THE MEDIA, SUITABILITY OF THE TEST AND NEGATIVE CONTROLS

The ability of the test to detect microorganisms in the presence of the product to be tested must be established. Suitability must be confirmed if a change in testing performance or a change in the product that may affect the outcome of the test is introduced.

### Preparation of Test Strains

Use standardized stable suspensions of test strains as stated below. Seed-lot culture maintenance techniques (seed-lot systems) are used so that the viable microorganisms used for inoculation are not more than five passages removed from the original master seed-lot.

#### AEROBIC MICROORGANISMS

Grow each of the bacterial test strains separately in containers containing *Soybean–Casein Digest Broth* or on *Soybean–Casein Digest Agar* at 30° to 35° for 18 to 24 hours. Grow the test strain for *Candida albicans* separately on *Sabouraud Dextrose Agar* or in *Sabouraud Dextrose Broth* at 20° to 25° for 2 to 3 days.

| | |
|---|---|
| *Staphylococcus aureus* | such as ATCC 6538, NCIMB 9518, CIP 4.83, or NBRC 13276 |
| *Pseudomonas aeruginosa* | such as ATCC 9027, NCIMB 8626, CIP 82.118, or NBRC 13275 |
| *Escherichia coli* | such as ATCC 8739, NCIMB 8545, CIP 53.126, or NBRC 3972 |
| *Salmonella enterica* subsp. *enterica* serovar Typhimurium or, as an alternative, | such as ATCC 14028 |
| *Salmonella enterica* subsp. *enterica* serovar Abony | such as NBRC 100797, NCTC 6017, or CIP 80.39 |
| *Candida albicans* | such as ATCC 10231, NCPF 3179, IP 48.72, or NBRC 1594 |

Use *Buffered Sodium Chloride–Peptone Solution pH 7.0* or *Phosphate Buffer Solution pH 7.2* to make test suspensions. Use the suspensions within 2 hours or within 24 hours if stored at 2° to 8°.

#### CLOSTRIDIA

Use *Clostridium sporogenes* such as ATCC 11437 (NBRC 14293, NCIMB 12343, CIP 100651) or ATCC 19404 (NCTC 532 or CIP 79.3). Grow the clostridial test strain under anaerobic conditions in *Reinforced Medium for Clostridia* at 30° to 35° for 24 to 48 hours. As an alternative to preparing and then diluting down a fresh suspension of vegetative cells of *Cl. sporogenes,* a stable spore suspension is used for test inoculation. The stable spore suspension may be maintained at 2° to 8° for a validated period.

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## Negative Control

To verify testing conditions, a negative control is performed using the chosen diluent in place of the test preparation. There must be no growth of microorganisms. A negative control is also performed when testing the products as described under *Testing of Products*. A failed negative control requires an investigation.

## Growth Promotion and Inhibitory Properties of the Media

Test each batch of ready-prepared medium and each batch of medium prepared either from dehydrated medium or from ingredients. Verify suitable properties of relevant media as described in *Table 1*.

**Table 1. Growth Promoting, Inhibitory, and Indicative Properties of Media**

| Test/Medium | Property | Test Strains |
|---|---|---|
| *Test for bile-tolerant Gram-negative bacteria* | | |
| Enterobacteria Enrichment Broth Mossel | Growth promoting | *E. coli* |
| | | *P. aeruginosa* |
| | Inhibitory | *S. aureus* |
| Violet Red Bile Glucose Agar | Growth promoting + Indicative | *E. coli* |
| | | *P. aeruginosa* |
| *Test for Escherichia coli* | | |
| MacConkey Broth | Growth promoting | *E. coli* |
| | Inhibitory | *S. aureus* |
| MacConkey Agar | Growth promoting + Indicative | *E. coli* |
| *Test for Salmonella* | | |
| Rappaport Vassiliadis Salmonella Enrichment Broth | Growth promoting | *Salmonella enterica* subsp. *enterica* serovar *Typhimurium* or |
| | | *Salmonella enterica* subsp. *enterica* serovar *Abony* |
| | Inhibitory | *S. aureus* |
| Xylose Lysine Deoxycholate Agar | Growth promoting + Indicative | *Salmonella enterica* subsp. *enterica* serovar *Typhimurium* or |
| | | *Salmonella enterica* subsp. *enterica* serovar *Abony* |
| *Test for Pseudomonas aeruginosa* | | |
| Cetrimide Agar | Growth promoting | *P. aeruginosa* |
| | Inhibitory | *E. coli* |
| *Test for Staphylococcus aureus* | | |
| Mannitol Salt Agar | Growth promoting + Indicative | *S. aureus* |
| | Inhibitory | *E. coli* |
| *Test for Clostridia* | | |
| Reinforced Medium for Clostridia | Growth promoting | *Cl. sporogenes* |
| Columbia Agar | Growth promoting | *Cl. sporogenes* |
| *Test for Candida albicans* | | |
| Sabouraud Dextrose Broth | Growth promoting | *C. albicans* |
| Sabouraud Dextrose Agar | Growth promoting + Indicative | *C. albicans* |

### Test for Growth-Promoting Properties, Liquid Media

Inoculate a portion of the appropriate medium with a small number (not more than 100 cfu) of the appropriate microorganism. Incubate at the specified temperature for not more than the shortest period of time specified in the test. Clearly visible growth of the microorganism comparable to that previously obtained with a previously tested and approved batch of medium occurs.

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### Test for Growth-Promoting Properties, Solid Media

Perform *Surface-Spread Method* (see *Plate-Count Methods* under *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* ⟨61⟩), inoculating each plate with a small number (not more than 100 cfu) of the appropriate microorganism. Incubate at the specified temperature for not more than the shortest period of time specified in the test. Growth of the microorganism comparable to that previously obtained with a previously tested and approved batch of medium occurs.

### Test for Inhibitory Properties, Liquid or Solid Media

Inoculate the appropriate medium with at least 100 cfu of the appropriate microorganism. Incubate at the specified temperature for not less than the longest period of time specified in the test. No growth of the test microorganism occurs.

### Test for Indicative Properties

Perform *Surface-Spread Method* (see *Plate-Count Methods* under *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* ⟨61⟩), inoculating each plate with a small number (not more than 100 cfu) of the appropriate microorganism. Incubate at the specified temperature for a period of time within the range specified in the test. Colonies are comparable in appearance and indication reactions to those previously obtained with a previously tested and approved batch of medium.

## Suitability of the Test Method

For each new product to be tested perform sample preparation as described in the relevant paragraph under *Testing of Products*. At the time of mixing, add each test strain in the prescribed growth medium. Inoculate the test strains individually. Use a number of microorganisms equivalent to not more than 100 cfu in the inoculated test preparation.

Perform the test as described in the relevant paragraph under *Testing of Products* using the shortest incubation period prescribed.

The specified microorganisms must be detected with the indication reactions as described under *Testing of Products*.

Any antimicrobial activity of the product necessitates a modification of the test procedure (see *Neutralization/Removal of Antimicrobial Activity* under *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* ⟨61⟩).

For a given product, if the antimicrobial activity with respect to a microorganism for which testing is prescribed cannot be neutralized, then it is to be assumed that the inhibited microorganism will not be present in the product.

# TESTING OF PRODUCTS

## Bile-Tolerant Gram-Negative Bacteria

### Sample Preparation and Pre-Incubation

Prepare a sample using a 1 in 10 dilution of not less than 1 g of the product to be examined as described in *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* ⟨61⟩, but using *Soybean–Casein Digest Broth* as the chosen diluent, mix, and incubate at 20° to 25° for a time sufficient to resuscitate the bacteria but not sufficient to encourage multiplication of the organisms (usually 2 hours but not more than 5 hours).

### Test for Absence

Unless otherwise prescribed, use the volume corresponding to 1 g of the product, as prepared in *Sample Preparation and Pre-Incubation,* to inoculate *Enterobacteria Enrichment Broth Mossel*. Incubate at 30° to 35° for 24 to 48 hours. Subculture on plates of *Violet Red Bile Glucose Agar*. Incubate at 30° to 35° for 18 to 24 hours.

The product complies with the test if there is no growth of colonies.

### Quantitative Test

**Selection and Subculture**—Inoculate suitable quantities of *Enterobacteria Enrichment Broth Mossel* with the preparation as directed under *Sample Preparation and Pre-Incubation* and/or dilutions of it containing respectively 0.1 g, 0.01 g, and 0.001 g (or 0.1 mL, 0.01 mL, and 0.001 mL) of the product to be examined. Incubate at 30° to 35° for 24 to 48 hours. Subculture each of the cultures on a plate of *Violet Red Bile Glucose Agar*. Incubate at 30° to 35° for 18 to 24 hours.

**Interpretation**—Growth of colonies constitutes a positive result. Note the smallest quantity of the product that gives a positive result and the largest quantity that gives a negative result. Determine from *Table 2* the probable number of bacteria.

**Table 2. Interpretation of Results**

| Results for Each Quantity of Product | | | |
|---|---|---|---|
| **0.1 g or 0.1 mL** | **0.01 g or 0.01 mL** | **0.001 g or 0.001 mL** | **Probable Number of Bacteria per g or mL of Product** |
| + | + | + | more than $10^3$ |
| + | + | – | less than $10^3$ and more than $10^2$ |

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**Table 2. Interpretation of Results** *(continued)*

| Results for Each Quantity of Product | | | |
|---|---|---|---|
| **0.1 g or 0.1 mL** | **0.01 g or 0.01 mL** | **0.001 g or 0.001 mL** | **Probable Number of Bacteria per g or mL of Product** |
| + | – | – | less than $10^2$ and more than 10 |
| – | – | – | less than 10 |

## *Escherichia coli*

### Sample Preparation and Pre-Incubation

Prepare a sample using a 1 in 10 dilution of not less than 1 g of the product to be examined as described in *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* ⟨61⟩,and use 10 mL or the quantity corresponding to 1 g or 1 mL, to inoculate a suitable amount (determined as described under *Suitability of the Test Method*) of *Soybean–Casein Digest Broth,* mix, and incubate at 30° to 35° for 18 to 24 hours.

### Selection and Subculture

Shake the container, transfer 1 mL of *Soybean–Casein Digest Broth* to 100 mL of *MacConkey Broth,* and incubate at 42° to 44° for 24 to 48 hours. Subculture on a plate of *MacConkey Agar* at 30° to 35° for 18 to 72 hours.

### Interpretation

Growth of colonies indicates the possible presence of *E. coli*. This is confirmed by identification tests.
The product complies with the test if no colonies are present or if the identification tests are negative.

## *Salmonella*

### Sample Preparation and Pre-Incubation

Prepare the product to be examined as described in *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* ⟨61⟩, and use the quantity corresponding to not less than 10 g or 10 mL to inoculate a suitable amount (determined as described under *Suitability of the Test Method*) of *Soybean–Casein Digest Broth,* mix, and incubate at 30° to 35° for 18 to 24 hours.

### Selection and Subculture

Transfer 0.1 mL of *Soybean–Casein Digest Broth* to 10 mL of *Rappaport Vassiliadis Salmonella Enrichment Broth,* and incubate at 30° to 35° for 18 to 24 hours. Subculture on plates of *Xylose Lysine Deoxycholate Agar*. Incubate at 30° to 35° for 18 to 48 hours.

### Interpretation

The possible presence of *Salmonella* is indicated by the growth of well-developed, red colonies, with or without black centers. This is confirmed by identification tests.
The product complies with the test if colonies of the types described are not present or if the confirmatory identification tests are negative.

## *Pseudomonas aeruginosa*

### Sample Preparation and Pre-Incubation

Prepare a sample using a 1 in 10 dilution of not less than 1 g of the product to be examined as described in *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* ⟨61⟩, and use 10 mL or the quantity corresponding to 1 g or 1 mL to inoculate a suitable amount (determined as described under *Suitability of the Test Method*) of *Soybean–Casein Digest Broth,* and mix. When testing transdermal patches, filter the volume of sample corresponding to one patch of the preparation (see *Transdermal Patches* under *Preparation of the Sample* in *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* ⟨61⟩) through a sterile filter membrane, and place in 100 mL of *Soybean–Casein Digest Broth*. Incubate at 30° to 35° for 18 to 24 hours.

### Selection and Subculture

Subculture on a plate of *Cetrimide Agar,* and incubate at 30° to 35° for 18 to 72 hours.

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#### Interpretation

Growth of colonies indicates the possible presence of *P. aeruginosa*. This is confirmed by identification tests.
The product complies with the test if colonies are not present or if the confirmatory identification tests are negative.

### *Staphylococcus aureus*

#### Sample Preparation and Pre-Incubation

Prepare a sample using a 1 in 10 dilution of not less than 1 g of the product to be examined as described in *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* ⟨61⟩, and use 10 mL or the quantity corresponding to 1 g or 1 mL to inoculate a suitable amount (determined as described under *Suitability of the Test Method*) of *Soybean–Casein Digest Broth*, and homogenize. When testing transdermal patches, filter the volume of sample corresponding to one patch of the preparation (see *Transdermal Patches* under *Preparation of the Sample* in *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* ⟨61⟩) through a sterile filter membrane, and place in 100 mL of *Soybean–Casein Digest Broth*. Incubate at 30° to 35° for 18 to 24 hours.

#### Selection and Subculture

Subculture on a plate of *Mannitol Salt Agar*, and incubate at 30° to 35° for 18 to 72 hours.

#### Interpretation

The possible presence of *S. aureus* is indicated by the growth of yellow or white colonies surrounded by a yellow zone. This is confirmed by identification tests.
The product complies with the test if colonies of the types described are not present or if the confirmatory identification tests are negative.

## Clostridia

#### Sample Preparation and Heat Treatment

Prepare a sample using a 1 in 10 dilution (with a minimum total volume of 20 mL) of not less than 2 g or 2 mL of the product to be examined as described in *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* ⟨61⟩. Divide the sample into two portions of at least 10 mL. Heat one portion at 80° for 10 minutes, and cool rapidly. Do not heat the other portion.

#### Selection and Subculture

Use 10 mL or the quantity corresponding to 1 g or 1 mL of the product to be examined of both portions to inoculate suitable amounts (determined as described under *Suitability of the Test Method*) of *Reinforced Medium for Clostridia*. Incubate under anaerobic conditions at 30° to 35° for 48 hours. After incubation, make subcultures from each container on *Columbia Agar*, and incubate under anaerobic conditions at 30° to 35° for 48 to 72 hours.

#### Interpretation

The occurrence of anaerobic growth of rods (with or without endospores) giving a negative catalase reaction indicates the presence of Clostridia.
This is confirmed by identification tests. The product complies with the test if colonies of the types described are not present or if the confirmatory identification tests are negative.

### *Candida albicans*

#### Sample Preparation and Pre-Incubation

Prepare the product to be examined as described in *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* ⟨61⟩, and use 10 mL or the quantity corresponding to not less than 1 g or 1 mL, to inoculate 100 mL of *Sabouraud Dextrose Broth*, and mix. Incubate at 30° to 35° for 3 to 5 days.

#### Selection and Subculture

Subculture on a plate of *Sabouraud Dextrose Agar*, and incubate at 30° to 35° for 24 to 48 hours.

#### Interpretation

Growth of white colonies may indicate the presence of *C. albicans*. This is confirmed by identification tests.
The product complies with the test if such colonies are not present or if the confirmatory identification tests are negative.

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# RECOMMENDED SOLUTIONS AND CULTURE MEDIA

[NOTE—This section is given for information.]

The following solutions and culture media have been found satisfactory for the purposes for which they are prescribed in the test for microbial contamination in the Pharmacopeia. Other media may be used provided that their suitability can be demonstrated.

**Stock Buffer Solution—**Transfer 34 g of potassium dihydrogen phosphate to a 1000-mL volumetric flask, dissolve in 500 mL of Purified Water, adjust with sodium hydroxide to a pH of 7.2 ± 0.2, add Purified Water to volume, and mix. Dispense in containers, and sterilize. Store at a temperature of 2° to 8°.

**Phosphate Buffer Solution pH 7.2—**Prepare a mixture of Purified Water and *Stock Buffer Solution* (800:1 v/v), and sterilize.

| **Buffered Sodium Chloride–Peptone Solution pH 7.0** | |
|---|---|
| Potassium Dihydrogen Phosphate | 3.6 g |
| Disodium Hydrogen Phosphate Dihydrate | 7.2 g (equivalent to 0.067 M phosphate) |
| Sodium Chloride | 4.3 g |
| Peptone (meat or casein) | 1.0 g |
| Purified Water | 1000 mL |

Sterilize in an autoclave using a validated cycle.

| **Soybean–Casein Digest Broth** | |
|---|---|
| Pancreatic Digest of Casein | 17.0 g |
| Papaic Digest of Soybean | 3.0 g |
| Sodium Chloride | 5.0 g |
| Dibasic Hydrogen Phosphate | 2.5 g |
| Glucose Monohydrate | 2.5 g |
| Purified Water | 1000 mL |

Adjust the pH so that after sterilization it is 7.3 ± 0.2 at 25°. Sterilize in an autoclave using a validated cycle.

| **Soybean–Casein Digest Agar** | |
|---|---|
| Pancreatic Digest of Casein | 15.0 g |
| Papaic Digest of Soybean | 5.0 g |
| Sodium Chloride | 5.0 g |
| Agar | 15.0 g |
| Purified Water | 1000 mL |

Adjust the pH so that after sterilization it is 7.3 ± 0.2 at 25°. Sterilize in an autoclave using a validated cycle.

| **Sabouraud Dextrose Agar** | |
|---|---|
| Dextrose | 40.0 g |
| Mixture of Peptic Digest of Animal Tissue and Pancreatic Digest of Casein (1:1) | 10.0 g |
| Agar | 15.0 g |
| Purified Water | 1000 mL |

Adjust the pH so that after sterilization it is 5.6 ± 0.2 at 25°. Sterilize in an autoclave using a validated cycle.

| **Potato Dextrose Agar** | |
|---|---|
| Infusion from potatoes | 200 g |
| Dextrose | 20.0 g |
| Agar | 15.0 g |
| Purified Water | 1000 mL |

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Adjust the pH so that after sterilization it is 5.6 ± 0.2 at 25°. Sterilize in an autoclave using a validated cycle.

| Sabouraud Dextrose Broth | |
|---|---|
| Dextrose | 20.0 g |
| Mixture of Peptic Digest of Animal Tissue and Pancreatic Digest of Casein (1:1) | 10.0 g |
| Purified Water | 1000 mL |

Adjust the pH so that after sterilization it is 5.6 ± 0.2 at 25°. Sterilize in an autoclave using a validated cycle.

| Enterobacteria Enrichment Broth Mossel | |
|---|---|
| Pancreatic Digest of Gelatin | 10.0 g |
| Glucose Monohydrate | 5.0 g |
| Dehydrated Ox Bile | 20.0 g |
| Potassium Dihydrogen Phosphate | 2.0 g |
| Disodium Hydrogen Phosphate Dihydrate | 8.0 g |
| Brilliant Green | 15 mg |
| Purified Water | 1000 mL |

Adjust the pH so that after heating it is 7.2 ± 0.2 at 25°. Heat at 100° for 30 minutes, and cool immediately.

| Violet Red Bile Glucose Agar | |
|---|---|
| Yeast Extract | 3.0 g |
| Pancreatic Digest of Gelatin | 7.0 g |
| Bile Salts | 1.5 g |
| Sodium Chloride | 5.0 g |
| Glucose Monohydrate | 10.0 g |
| Agar | 15.0 g |
| Neutral Red | 30 mg |
| Crystal Violet | 2 mg |
| Purified Water | 1000 mL |

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Adjust the pH so that after heating it is 7.4 ± 0.2 at 25°. Heat to boiling; do not heat in an autoclave.

| MacConkey Broth | |
|---|---|
| Pancreatic Digest of Gelatin | 20.0 g |
| Lactose Monohydrate | 10.0 g |
| Dehydrated Ox Bile | 5.0 g |
| Bromocresol Purple | 10 mg |
| Purified Water | 1000 mL |

Adjust the pH so that after sterilization it is 7.3 ± 0.2 at 25°. Sterilize in an autoclave using a validated cycle.

| MacConkey Agar | |
|---|---|
| Pancreatic Digest of Gelatin | 17.0 g |
| Peptones (meat and casein) | 3.0 g |
| Lactose Monohydrate | 10.0 g |
| Sodium Chloride | 5.0 g |
| Bile Salts | 1.5 g |
| Agar | 13.5 g |
| Neutral Red | 30.0 mg |
| Crystal Violet | 1 mg |

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| MacConkey Agar | |
|---|---|
| Purified Water | 1000 mL |

Adjust the pH so that after sterilization it is 7.1 ± 0.2 at 25°. Boil for 1 minute with constant shaking, then sterilize in an autoclave using a validated cycle.

| Rappaport Vassiliadis Salmonella Enrichment Broth | |
|---|---|
| Soya Peptone | 4.5 g |
| Magnesium Chloride Hexahydrate | 29.0 g |
| Sodium Chloride | 8.0 g |
| Dipotassium Phosphate | 0.4 g |
| Potassium Dihydrogen Phosphate | 0.6 g |
| Malachite Green | 0.036 g |
| Purified Water | 1000 mL |

Dissolve, warming slightly. Sterilize in an autoclave using a validated cycle, at a temperature not exceeding 115°. The pH is to be 5.2 ± 0.2 at 25° after heating and autoclaving.

| Xylose Lysine Deoxycholate Agar | |
|---|---|
| Xylose | 3.5 g |
| L-Lysine | 5.0 g |
| Lactose Monohydrate | 7.5 g |
| Sucrose | 7.5 g |
| Sodium Chloride | 5.0 g |
| Yeast Extract | 3.0 g |
| Phenol Red | 80 mg |
| Agar | 13.5 g |
| Sodium Deoxycholate | 2.5 g |
| Sodium Thiosulfate | 6.8 g |
| Ferric Ammonium Citrate | 0.8 g |
| Purified Water | 1000 mL |

Adjust the pH so that after heating it is 7.4 ± 0.2 at 25°. Heat to boiling, cool to 50°, and pour into Petri dishes. Do not heat in an autoclave.

| Cetrimide Agar | |
|---|---|
| Pancreatic Digest of Gelatin | 20.0 g |
| Magnesium Chloride | 1.4 g |
| Dipotassium Sulfate | 10.0 g |
| Cetrimide | 0.3 g |
| Agar | 13.6 g |
| Purified Water | 1000 mL |
| Glycerol | 10.0 mL |

Heat to boiling for 1 minute with shaking. Adjust the pH so that after sterilization it is 7.2 ± 0.2 at 25°. Sterilize in an autoclave using a validated cycle.

| Mannitol Salt Agar | |
|---|---|
| Pancreatic Digest of Casein | 5.0 g |
| Peptic Digest of Animal Tissue | 5.0 g |
| Beef Extract | 1.0 g |

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| Mannitol Salt Agar | |
|---|---|
| D-Mannitol | 10.0 g |
| Sodium Chloride | 75.0 g |
| Agar | 15.0 g |
| Phenol Red | 0.025 g |
| Purified Water | 1000 mL |

Heat to boiling for 1 minute with shaking. Adjust the pH so that after sterilization it is 7.4 ± 0.2 at 25°. Sterilize in an autoclave using a validated cycle.

| Reinforced Medium for Clostridia | |
|---|---|
| Beef Extract | 10.0 g |
| Peptone | 10.0 g |
| Yeast Extract | 3.0 g |
| Soluble Starch | 1.0 g |
| Glucose Monohydrate | 5.0 g |
| Cysteine Hydrochloride | 0.5 g |
| Sodium Chloride | 5.0 g |
| Sodium Acetate | 3.0 g |
| Agar | 0.5 g |
| Purified Water | 1000 mL |

Hydrate the agar, and dissolve by heating to boiling with continuous stirring. If necessary, adjust the pH so that after sterilization it is about 6.8 ± 0.2 at 25°. Sterilize in an autoclave using a validated cycle.

| Columbia Agar | |
|---|---|
| Pancreatic Digest of Casein | 10.0 g |
| Meat Peptic Digest | 5.0 g |
| Heart Pancreatic Digest | 3.0 g |
| Yeast Extract | 5.0 g |
| Maize Starch | 1.0 g |
| Sodium Chloride | 5.0 g |
| Agar, according to gelling power | 10.0–15.0 g |
| Purified Water | 1000 mL |

Hydrate the agar, and dissolve by heating to boiling with continuous stirring. If necessary, adjust the pH so that after sterilization it is 7.3 ± 0.2 at 25°. Sterilize in an autoclave using a validated cycle. Allow to cool to 45° to 50°; add, where necessary, gentamicin sulfate corresponding to 20 mg of gentamicin base, and pour into Petri dishes.

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# ⟨63⟩ MYCOPLASMA TESTS

## INTRODUCTION

The genus Mycoplasma represents a group of minute bacteria which have no cell walls. The genus comprises more than 120 species. They are the smallest self-replicating prokaryotic organisms. The cells vary in size and morphology and cannot be Gram stained, but impressions of colonies on solid agar can be stained with methylene blue or equivalent stain. Mycoplasma are parasites and commensals, and some may be pathogenic to a variety of animal and plant hosts. In humans, Mycoplasma are usually surface parasites that colonize the epithelial lining of the respiratory and urogenital tracts. Mycoplasma are common and may cause serious contamination in cell and/or tissue cultures used to generate compendial articles. They may also cause contamination of filtered sterilized soybean casein digest broth. A cell culture infection may persist for an extended period of time without causing apparent cell damage. Infection of cells in a culture can affect nearly every pathway of cell metabolism, including alteration of the cells' phenotypical characteristics and normal growth. The presence of Mycoplasma species does not always result in turbid growth in cultures or visible alteration of the cells.

Testing for Mycoplasma is a necessary quality control requirement to assure reliably pure biotechnological products and allied materials used to generate these products. This general test chapter describes two methods required to detect Mycoplasma contamination of test articles, tissues and/or cell cultures used to produce test articles, digest broth, or any other material in which Mycoplasma contamination is suspected. These are: (A) the agar and broth media procedure and (B) the indicator cell culture procedure. These tests require careful aseptic technique and suitable laboratory conditions. In order to ensure appropriate testing and interpretation of results, personnel should be properly trained and qualified. A validated nucleic acid amplification technique (NAT) or an enzymatic activity based method may be used to detect Mycoplasma, provided such a method is shown to be comparable to both methods (A) and (B). Alternative methods must be suitably validated. Validation requirements for alternate methods will not be addressed in this chapter.

## CULTURE METHOD

### Choice of Media

The test is carried out using a sufficient number of both solid and liquid media to ensure growth in the chosen incubation conditions of small numbers (approximately 100 colony-forming units, cfu; or 100 color-changing units, ccu) of Mycoplasmas that may be present in the test article/material. Liquid media must contain phenol red. The range of media chosen is shown to have satisfactory nutritive properties for at least the microorganisms shown in *Quality Control Test Strain Organisms* (below). The nutritive properties of each new batch of medium are verified for the appropriate microorganisms in the list. When testing for Mycoplasmas include in each test at least two known Mycoplasma species or strains (listed in *Quality Control Test Strain Organisms*) as positive controls, one of which should be a dextrose fermenter (i.e., *M. pneumoniae* or equivalent species and strain) and one of which should be an arginine hydrolyzer (i.e., *M. orale* or equivalent species and strain). Only when testing insect cell lines should one include a *Spiroplasma* control strain (e.g., *S. citri* ATCC 29747, *S. melliferum* ATCC 29416, or equivalent species and strains). Additionally, these strains may be a little more fastidious in their nutritional requirements. They require lower incubation temperatures (as do insect cell lines).

### Quality Control Test Strain Organisms

Positive control cultures should be not more than 15 passages from isolation. Mycoplasma species or strains suitable for use are listed below:

- *Acholeplasma laidlawii* (vaccines and/or cell-derived materials/cultures for human and veterinary use when an antibiotic has been used during production)
- *M. gallisepticum* (when avian material has been used during production or when the vaccine or cell culture is intended for use in poultry)
- *M. hyorhinis* (nonavian veterinary vaccines or cell cultures)
- *M. orale* (vaccines for human and veterinary use)
- *M. pneumoniae* (vaccines or cell banks for human use) or another suitable species of D-glucose fermenter such as *M. fermentans*
- *M. synoviae* (when avian material has been used during production or when the vaccine or cell bank is intended for use in poultry)

The test strains may be field isolates that have undergone a limited number of subcultures (not more than 15), are stored frozen (−20° or lower) or freeze-dried, and are identified as being of the required species by comparison with type cultures, for example, those shown in *Table 1*.

**Table 1. Type Cultures for Identifying Field Isolates Used as Test Strains**

| Test Organism | NCTC Number | CIP Number | ATCC Number |
|---|---|---|---|
| *A. laidlawii* | NCTC 10116 | CIP 75.27 | ATCC 23206 |
| *M. gallisepticum* | NCTC 10115 | CIP 104967 | ATCC 19610 |

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**Table 1. Type Cultures for Identifying Field Isolates Used as Test Strains** *(continued)*

| Test Organism | NCTC Number | CIP Number | ATCC Number |
|---|---|---|---|
| *M. fermentans* | NCTC 10117 | CIP 105680 | ATCC 19989 |
| *M. hyorhinis* | NCTC 10130 | CIP 104968 | ATCC 17981 |
| *M. orale* | NCTC 10112 | CIP 104969 | ATCC 23714 |
| *M. pneumoniae* | NCTC 10119 | CIP 103766 | ATCC 15531 |
| *M. synoviae* | NCTC 10124 | CIP 104970 | ATCC 25204 |

## Incubation Conditions

Incubate liquid media in tightly stoppered containers at 36 ± 1°. Incubate solid media in microaerophilic conditions (hydrogen atmosphere containing < 0.5% oxygen and/or nitrogen containing 5%–10% carbon dioxide in nitrogen). Sufficient humidity should be available to prevent desiccation of the agar surface at 36 ± 1°.

## Nutritive Properties

Carry out the test for nutritive properties for each new batch of medium. Inoculate the chosen media with the appropriate test microorganisms; use not more than 100 cfu per plate containing at least 9 mL of solid media and per 100-mL container of liquid medium; use a separate plate and container for each species of microorganism. Incubate the media and make subcultures from 0.2 mL of liquid medium to solid medium at the specified intervals (see below under *Test for Mycoplasma in the Test Article/Material*). The solid medium complies with the test if a count within a 0.5-log unit range of the inoculate amount is found for each test microorganism. The liquid medium complies with the test if growth is found on agar plates subcultured from the broth, for at least 1 subculture for each test microorganism. The use of a microscope at 100× or greater may be helpful.

## Inhibitory Substances

The test for inhibitory substances is carried out once for a given product and is repeated whenever there is a change in production method that may affect the detection of Mycoplasma. To demonstrate absence of inhibitory substances, carry out the test for nutritive properties in the presence and absence of the test article/material. If growth of a test microorganism occurs more than 1 subculture sooner in the absence of the test article/material than in its presence, inhibitory substances are present. The same is true if plates directly inoculated with the test article/material are not within a 0.5-log unit range of the number of colonies of those inoculated without the test article/material. In both cases, inhibitory substances must be neutralized or their effect otherwise countered, by an appropriate method, for example, by passage in substrates not containing inhibitors or dilution in a larger volume of medium, before the test. If dilution is used, larger medium volumes may be used or the inoculums' volume may be divided among several 100-mL flasks. The effectiveness of the neutralization or other process is checked by repeating the test for inhibitory substances after neutralization.

## Test for Mycoplasma in the Test Article/Material

Inoculate no less than 10 mL of the test article/material per 100 mL of each liquid medium. If a significant pH change occurs upon the addition of the test article/material, the liquid medium is restored to its original pH value by the addition of a sterile solution of either sodium hydroxide or hydrochloric acid. Inoculate 0.2 mL of the test article/material on each plate of each solid medium. Incubate liquid media for 20–21 days. Incubate solid media for not less than 14 days, except those plates corresponding to the 20–21 day subculture, which are incubated for 7 days. Concurrently, incubate an uninoculated 100-mL portion of each liquid medium and agar plate, as a negative control. On days 2–4 after inoculation, subculture each liquid medium by inoculating 0.2 mL on at least 1 plate of each solid medium. Repeat the procedure between days 6 and 8, again between days 13 and 15, and again between days 19 and 21 of the test. Observe the liquid media every 2 or 3 days and if a color change occurs, subculture. If a liquid medium shows bacterial or fungal contamination, the test is invalid. The test is valid if at least 1 plate per medium and per inoculation day can be read. Include in the test positive controls prepared by inoculation of not more than 100 cfu of at least 1 test microorganism on agar medium or into broth medium. Where the test for Mycoplasmas is carried out regularly, it is recommended to use the test microorganisms in regular rotation. The test microorganisms used are those listed under *Choice of Media*. Incubate broths and plates in a humidified atmosphere with microaerophilic conditions (5%–10% $CO_2$).

## Interpretation of Results

At the end of the prescribed incubation period, examine all inoculated solid media for the presence of Mycoplasma colonies. The product complies with the test if growth of typical Mycoplasma colonies has not occurred. The product does not comply with the test if growth of typical Mycoplasma colonies has occurred on any of the solid media. The test is invalid if 1 or more of the positive controls do not show growth of Mycoplasmas on at least 1 subculture plate. The test is invalid if 1 or more of the negative controls show growth of Mycoplasmas. If suspect colonies are observed, use a suitable validated method to determine whether they are due to Mycoplasmas.

## Recommended Solutions and Media for the Culture Method

[NOTE—This section is provided for information.]

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## SOLUTIONS

### Beef Heart Infusion Broth

| | |
|---|---|
| Beef heart (for preparation of the infusion) | 500 g |
| Peptone | 10 g |
| Sodium chloride | 5 g |
| Distilled water | to 1000 mL |

### Essential Vitamins

| | |
|---|---|
| Biotin | 100 mg |
| Calcium pantothenate | 100 mg |
| Choline chloride | 100 mg |
| Folic acid | 100 mg |
| *i*-Inositol | 200 mg |
| Nicotinamide | 100 mg |
| Pyridoxal hydrochloride | 100 mg |
| Riboflavine | 10 mg |
| Thiamine hydrochloride | 100 mg |
| Distilled water | to 1000 mL |

### Agar, Purified

| A highly refined agar for use in microbiology and immunology, prepared by an ion-exchange procedure that results in a product having superior purity, clarity, and gel strength. It contains the following ingredients: | |
|---|---|
| Water | 12.2% |
| Ash | 1.5% |
| Acid-insoluble ash | 0.2% |
| Chlorine | 0 |
| Phosphate (calculated as $P_2O_5$) | 0.3% |
| Total nitrogen | 0.3% |
| Copper | 8 ppm |
| Iron | 170 ppm |
| Calcium | 0.28% |
| Magnesium | 0.32% |

### Hanks' Balanced Salt Solution (modified)

| | |
|---|---|
| Sodium chloride | 6.4 g |
| Potassium chloride | 0.32 g |
| Magnesium sulphate heptahydrate | 0.08 g |
| Magnesium chloride hexahydrate | 0.08 g |
| Calcium chloride, anhydrous | 0.112 g |
| Disodium hydrogen phosphate dihydrate | 0.0596 g |
| Potassium dihydrogen phosphate, anhydrous | 0.048 g |
| Distilled water | to 800 mL |

### Brain Heart Infusion

| | |
|---|---|
| Calf-brain infusion | 200 g |
| Beef-heart infusion | 250 g |
| Proteose peptone | 10 g |
| Glucose monohydrate | 2 g |
| Sodium chloride | 5 g |

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**Brain Heart Infusion** *(continued)*

| | |
|---|---|
| Disodium hydrogen phosphate, anhydrous | 2.5 g |
| Distilled water | to 1000 mL |

**PPLO Broth**

| | |
|---|---|
| Beef-heart infusion | 50 g |
| Peptone | 10 g |
| Sodium chloride | 5 g |
| Distilled water | to 1000 mL |

## MEDIA

The following media are recommended. Other media may be used, provided they meet the criteria given in the sections *Choice of Culture Media, Incubation Conditions, Nutritive Properties,* and *Inhibitory Substances.*

**Hayflick Media (Recommended for the general detection of Mycoplasmas)**

| | |
|---|---|
| **Liquid Medium** | |
| Beef heart infusion broth | 90.0 mL |
| Horse serum (unheated) | 20.0 mL |
| Yeast extract (250 g/L) (fresh yeast extract is recommended) | 10.0 mL |
| Phenol red (0.6 g/L solution) | 5.0 mL |
| Penicillin (20,000 IU/mL) | 0.25 mL |
| Deoxyribonucleic acid (2 g/L solution) | 1.2 mL |
| Adjust to a pH of 7.8 | |
| **Solid Medium** | |
| Prepare as described above replacing beef heart infusion broth by beef heart infusion agar containing 15 g/L of agar. | |

**Frey Media (Recommended for the detection of *M. synoviae*)**

| | |
|---|---|
| **Liquid Medium** | |
| Beef heart infusion broth | 90.0 mL |
| Essential vitamins | 0.025 mL |
| Glucose monohydrate (500 g/L solution) | 2.0 mL |
| Swine serum (inactivated at 56° for 30 min) | 12.0 mL |
| β-Nicotinamide adenine dinucleotide (10 g/L solution) | 1.0 mL |
| Cysteine hydrochloride (10 g/L solution) | 1.0 mL |
| Phenol red (0.6 g/L solution) | 5.0 mL |
| Penicillin (20,000 IU/mL) | 0.25 mL |
| Mix the solutions of β-nicotinamide adenine dinucleotide and cysteine hydrochloride and after 10 min add to the other ingredients. Adjust to a pH of 7.8. | |
| **Solid Medium** | |
| Beef heart infusion broth | 90.0 mL |
| Agar, purified | 1.4 g |
| Adjust to pH 7.8, sterilize by autoclaving then add: | |
| Essential vitamins | 0.025 mL |
| Glucose monohydrate (500 g/L solution) | 2.0 mL |
| Swine serum (unheated) | 12.0 mL |
| β-Nicotinamide adenine dinucleotide (10 g/L solution) | 1.0 mL |
| Cysteine hydrochloride (10 g/L solution) | 1.0 mL |
| Phenol red (0.6 g/L solution) | 5.0 mL |
| Penicillin (20,000 IU/mL) | 0.25 mL |

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**Friis Media (Recommended for the detection of nonavian Mycoplasmas)**

| | |
|---|---|
| **Liquid Medium** | |
| Hanks' balanced salt solution (modified) | 800 mL |
| Distilled water | 67 mL |
| Brain heart infusion | 135 mL |
| PPLO Broth | 248 mL |
| Yeast extract (170 g/L) | 60 mL |
| Bacitracin | 250 mg |
| Meticillin | 250 mg |
| Phenol red (5 g/L) | 4.5 mL |
| Horse serum | 165 mL |
| Swine serum | 165 mL |
| Adjust to a pH of 7.40–7.45 | |
| **Solid Medium** | |
| Hanks' balanced salt solution (modified) | 200 mL |
| DEAE-dextran | 200 mg |
| Agar, purified | 15.65 g |
| Mix well and sterilize by autoclaving. Cool to 100°. Add to 1740 mL of *Liquid Medium* as described above. | |

## INDICATOR CELL CULTURE METHOD

Cell cultures are stained with a fluorescent dye that binds to DNA. Mycoplasmas are detected by their characteristic particulate or filamentous pattern of fluorescence on the cell surface and, if contamination is heavy, in surrounding areas. Mitochondria in the cytoplasm may be stained but are readily distinguished from Mycoplasmas. For viral suspensions, if the interpretation of results is affected by marked cytopathic effects, neutralize the virus using a specific antiserum that has no inhibitory effects on Mycoplasmas, or use a cell culture substrate that does not allow growth of the virus. To demonstrate the absence of inhibitory effects of serum, carry out the positive control tests in the presence and absence of the antiserum.

### Verification of the Substrate

Use Vero cells or equivalent cell culture (for example, the production cell line) that is equivalent in effectiveness for detecting Mycoplasmas. Test the effectiveness of the cells to be used by applying the procedure shown below and inoculating not more than 100 cfu or ccu microorganisms of suitable reference strains of *M. hyorhinis* and *M. orale.* The cells are suitable if both reference strains are detected. The indicator cells must be subcultured without an antibiotic before use in the test.

### Test Method

[NOTE—The following is provided for information.]

SOLUTIONS

**Phosphate Buffered Saline**

*2.0 M Monobasic Potassium Phosphate*—Dissolve 13.61 g of anhydrous monobasic potassium phosphate in 50 mL of water.

*2.0 M Dibasic Potassium Phosphate*—Dissolve 17.42 g of anhydrous dibasic potassium phosphate in 50 mL of water.

*Phosphate Buffered Saline Solution (pH 7.4)*—Combine 3.6 mL of *2.0 M Monobasic Potassium Phosphate,* 16.4 mL of *2.0 M Dibasic Potassium Phosphate,* 8 g of sodium chloride, and 1 L of water. Mix thoroughly. Adjust the pH if necessary.

**Bisbenzimide Stock Solution—**Dissolve 5 mg of bisbenzimide in water, and dilute with the same solvent to 100 mL. Store in the dark.

**Bisbenzimide Working Solution**—Immediately before use, dilute 100 µL of *Bisbenzimide Stock Solution* with *Phosphate Buffered Saline Solution (pH 7.4)* to 100 mL.

**Phosphate-Citrate Buffer Solution pH 5.5—**Mix 56.85 mL of a 28.4-g/L solution of anhydrous disodium hydrogen phosphate and 43.15 mL of a 21-g/L solution of citric acid.

METHOD

1. Seed the indicator cell culture at a suitable density (for example, $2 \times 10^4$ to $2 \times 10^5$ cells/mL, $4 \times 10^3$ to $2.5 \times 10^4$ cells/$cm^2$) that will yield confluence after 3 days of growth. Inoculate 1 mL of the product to be examined into the cell culture vessel, and incubate at 36 ± 1°.

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2. After at least 3 days of incubation, when the cells have grown to confluence, make a subculture on cover slips in suitable containers or on some other surface (for example, chambered slides) suitable for the test procedure. Seed the cells at low density so that they reach 50% confluence after 3–5 days of incubation. Complete confluence impairs visualization of Mycoplasmas after staining and must be avoided.
3. Remove the medium and rinse the indicator cells with phosphate buffered saline, pH 7.4, then add a suitable fixing solution (a freshly prepared mixture of 1 volume of acetic acid, glacial, TS and 3 volumes of methanol, is suitable when bisbenzimide is used for staining).
4. Remove the fixing solution and wash the cells with sterile Purified Water. Dry the slides completely if they are to be stained more than 1 hour later (particular care is needed for staining of slides after drying owing to artifacts that may be produced).
5. Add a suitable DNA stain and allow standing for a suitable time (bisbenzimide working solution and a standing time of 10 minutes are suitable).
6. Remove the stain and rinse the monolayer with Purified Water.
7. Mount each coverslip, where applicable (a mixture of equal volumes of glycerol and *Phosphate-Citrate Buffer Solution pH 5.5* is suitable for mounting). Examine by fluorescence (for bisbenzimide stain a 330 nm/380 nm excitation filter and an LP 440 nm barrier filter are suitable) at 400× magnification or greater.
8. Compare the microscopic appearance of the test cultures with that of the negative and positive controls, examining for extranuclear fluorescence. Mycoplasmas produce pinpoints or filaments over the indicator cell cytoplasm. They may also produce pinpoints and filaments in the intercellular spaces. Multiple microscopic fields are examined according to the protocol established during validation.

## Interpretation of Results

The product to be examined complies with the test if fluorescence typical of Mycoplasmas is not present. The test is invalid if the positive controls do not show fluorescence typical of Mycoplasmas. The test is invalid if the negative controls show fluorescence typical of Mycoplasmas.

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***Add the following:***

# ▲⟨64⟩ PROBIOTIC TESTS

## INTRODUCTION

This chapter provides testing procedures for identification, enumeration, contamination, and other requirements for probiotics. Probiotics are live microorganisms that, when administered in adequate amounts, confer health benefits to the host. Probiotics are typically identified at the strain level as their characteristics are considered strain-specific.

This chapter applies to probiotics produced in specialized fermenters under strict hygiene conditions for dietary supplements or pharmaceutical applications. Fermentation media are tailored to the specific requirements of the microbial species or strain and typically contain nutrients such as proteins, carbohydrates, vitamins, and minerals. The culture is allowed to multiply and grow under carefully defined conditions. After the microbial cells are grown, the cultured cells are harvested, often by centrifugation. Suitable protectants may be added to the concentrated probiotic microorganisms (biomass), and the biomass is freeze-dried or spray-dried to a powdered form. The dried biomass then undergoes formulation, which may involve blending one or more strains with suitable excipients. Formulated probiotic ingredients can be further processed into dosage forms, e.g., tablets, capsules, softgels, powders, or gels.

## IDENTIFICATION

Probiotics as defined in this chapter can be identified at the genus and species level or at the strain level according to the monograph requirements. Since regulatory filings for commercial probiotics are commonly made at the strain level supported by information from safety assessment and human studies, identification at the strain level may be required to verify the strain declared in labeling of commercial products. In this chapter, strain-specific identification using polymerase chain reaction (PCR) with strain-specific primers is recommended for the identification of probiotics. Currently, a testing procedure for identification of *Lactobacillus* and *Bifidobacterium* strains by PCR with specific primers is listed in this chapter. Additional testing procedures for identification of probiotic strains including non-spore-forming bacteria, spore-forming bacteria, and yeasts or molds will be listed in this chapter once they become available.

• **IDENTIFICATION FOR NON-SPORE-FORMING BACTERIAL STRAINS**

**Identification for *Lactobacillus* and *Bifidobacterium* strains by PCR with specific primers**

Unless otherwise specified in the individual monograph, use the following general procedure. All reagents and solutions used in this test should be molecular biology grade.

**Buffer:** 10 mM tris-hydrochloride, 1 mM sodium ethylenediaminetetraacetic acid (EDTA) buffer[1]

**PCR master mix:** *Taq* DNA polymerase (62.5 U/mL), 2.5X *Taq* reaction buffer [125 mM potassium chloride (KCl), 75 mM tris-hydrochloride pH 8.3, and 4 mM magnesium ($Mg^{+2}$)], and 500 µM of each deoxynucleotide (dNTP). Prepared *PCR master mix* solutions are commercially available.[2]

**Sample:** 100 mg/mL suspension of the probiotic powder in *Buffer*

**Primer set:** Use the primers for the strain specified in the individual monograph. Dilute the forward and reverse primers in *Buffer* to a stock concentration of 100 µM, and dilute further with *Buffer* to 25 µM. The diluted forward primer and diluted reverse primer 25-µM dilutions can be stored at –20°.[3]

**PCR sample preparations:** For each *Primer set*, prepare a solution containing 1 µL of the *Sample*, 10 µL of *PCR master mix*, 1 µL of the diluted forward primer (25 µM), 1 µL of the diluted reverse primer (25 µM), and 12 µL of *Sterile Purified Water*.

**PCR negative control:** Prepare as directed for the *PCR sample preparations*, replacing the *Sample* with 1 µL of *Sterile Purified Water*.

**PCR positive control** (if available)**:** Prepare as directed for the *PCR sample preparations*, replacing the *Sample* with 1 µL of the corresponding Reference Standard.

**Analysis:** Perform PCR amplification on each of the *PCR sample preparations*, *PCR positive control*, and the *PCR negative control* using an appropriate thermal cycler.[4] Unless otherwise specified in the individual monograph, incubate at 95° for 7 min (step 1) for direct heat-induced DNA extraction.

**Thermal cycle:** 95° for 30 s (step 2); annealing temperature specified in the individual monograph for 30 s (step 3); and at 72° for 30 s (step 4). Repeat steps 2–4 for between 30 and 34 cycles, incubate at 72° for 5 min, and hold at 4°. Perform electrophoretic separation of the PCR amplification products for each of the *PCR sample preparations*, *PCR positive control*, and for the *PCR negative control*. An automated on-chip electrophoresis system with a DNA kit can be used.

Prepare or use a commercially available 1% (w/v) agarose gel in a 1X tris-acetic acid–EDTA buffer (40 mM tris-hydrochloride, 1% glacial acetic acid, and 1 mM EDTA). Stain the gel with 0.5 mg/mL of ethidium bromide in water and de-stain with water. [**CAUTION**—Ethidium bromide is considered a toxic substance and a potential mutagen. Use appropriate personal protective equipment (including nitrile gloves) when handling this reagent.]

Use a DNA ladder standard (1 KB plus)[5] suitable for determining the size of linear double-stranded DNA fragments between 100 and 12,000 base pairs. The ladder standard should be used in the first and last lanes on the gel to allow for proper comparison of amplicons. Analysis of the *PCR negative control* must result in the absence of any amplification products. If amplification occurs, repeat the *PCR sample preparations*, and the *PCR negative control*, followed by PCR amplification and analysis.

---

[1] Suitable buffers (e.g., TE Buffer 1X, molecular biology grade) are available from Promega (www.promega.com).

[2] 5 PRIME MasterMix Polymerase from VWR Scientific (www.vwr.com), AmpliTaq Gold DNA Polymerases from ThermoFisher Scientific (www.thermofisher.com), or other chemical/microbiology suppliers.

[3] DNA primers are commercially available (custom manufacture) from Integrated DNA Technologies (www.idtdna.com) and other commercial sources.

[4] Suitable thermal cyclers are available from Eppendorf (www.eppendorf.com/OC-en/).

[5] Suitable 1 KB plus DNA ladders are available from ThermoFisher Scientific (www.thermofisher.com).

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**Acceptance criteria:** Refer to the acceptance criteria in the individual monograph.

## ENUMERATION

Currently, a testing procedure for enumeration of *Lactobacillus* and *Bifidobacterium* species is listed in this chapter. Additional testing procedures for enumeration of probiotic strains, including non-spore-forming bacteria, spore-forming bacteria, and yeasts and molds, will be listed in this chapter once they become available. Alternative microbiological procedures, including automated methods, may be used provided that their equivalence has been demonstrated.

- **ENUMERATION FOR NON-SPORE-FORMING BACTERIA STRAINS**

***Lactobacillus* and *Bifidobacterium* species**

Unless otherwise specified in the individual monograph, use the following general procedure.

***Lactobacilli* MRS agar** (medium for *Lactobacillus* and *Bifidobacterium* species)**:** Prepare according to *Table 1* or use a commercially available mixture.[6]

**Table 1. *Lactobacilli* MRS Agar**

| Reagent | Quantity (g) |
|---|---|
| Proteose peptone #3[a] | 10.0 |
| Beef extract | 10.0 |
| Yeast extract | 5.0 |
| Dextrose | 20.0 |
| Polysorbate 80 | 1.0 |
| Ammonium citrate | 2.0 |
| Sodium acetate | 5.0 |
| Magnesium sulfate | 0.1 |
| Manganese sulfate[b] | 0.05 |
| Dipotassium phosphate | 2.0 |
| Agar | 15.0 |

[a] A suitable peptone for microbiological analysis is available from BD Bacto™ (www.bd.com).
[b] A suitable manganese sulfate is available from Sigma-Aldrich (www.sigmaaldrich.com).

Suspend the *Lactobacilli MRS agar* in 1 L of *Purified Water* in an appropriately sized conical flask or beaker (sufficiently large to not boil over). Cover the flask or beaker with aluminum foil and heat to boiling with stirring on a hot plate. Allow to boil for 1 min to completely dissolve the medium, and then autoclave the solution at 121° for 15 min. Cool to 45° and use immediately. Boiled agar medium may also be aseptically transferred into individual media bottles in 100- or 200-mL aliquots before sterilizing, and then autoclaved and stored for later use. The agar can be stored at a temperature between 2° and 8° in a sterile well-closed container unless it is intended for immediate use. Do not use the medium for a longer storage period than has been validated. Allow the agar to come to room temperature before use.

***Lactobacilli* MRS broth:** Prepare according to *Table 2* or use a suitable commercially available broth.[7]

**Table 2. *Lactobacilli* MRS Broth**

| Reagent | Quantity (g) |
|---|---|
| Proteose peptone #3[a] | 10.0 |
| Beef extract | 10.0 |
| Yeast extract | 5.0 |
| Dextrose | 20.0 |
| Polysorbate 80 | 1.0 |
| Ammonium citrate | 2.0 |
| Sodium acetate | 5.0 |
| Magnesium sulfate | 0.1 |
| Manganese sulfate[b] | 0.05 |
| Dipotassium phosphate | 2.0 |

[a] A suitable peptone for microbiological analysis is available from BD Bacto™ (www.bd.com).

[6] Difco™ *Lactobacilli* MRS Agar, or equivalent. Suitable *Lactobacilli* MRS agars are available from VWR Scientific (www.vwr.com) or other chemical/microbiological suppliers.
[7] Difco™ *Lactobacilli* MRS Broth or equivalent. Suitable *Lactobacilli* MRS broths are available from VWR Scientific (www.vwr.com) or other chemical/microbiological suppliers.

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[b] A suitable manganese sulfate is available from Sigma-Aldrich (www.sigmaaldrich.com).

Suspend *Lactobacilli MRS broth* in 1 L of *Purified Water* in an appropriately sized conical flask or beaker (sufficiently large to not boil over). Cover the flask or beaker with aluminum foil and heat to boiling with stirring on a hot plate. Allow to boil for 1 min to completely dissolve the broth ingredients, and then autoclave the solution at 121° for 15 min. Broth may also be aseptically transferred into individual media bottles in 100- or 200-mL aliquots before sterilizing, and then autoclaved and stored for later use. The broth can be stored at a temperature between 2° and 8° in a sterile well-closed container unless it is intended for immediate use. Do not use the medium for a longer storage period than has been validated. Allow broth to come to room temperature before use.

**Peptone diluent:** Prepare a solution of 0.1% peptone[8] in water (w/v) and adjust with a lactic acid solution to a pH of 7.0. Using an autoclave, steam sterilize the solution at 121° for NLT 15 min, and then allow to cool in the unopened autoclave. Dispense into sterile containers as needed for preparing samples.

**Sample preparation:** Unless otherwise specified in the individual monograph, transfer an amount equivalent to 11.0 g of probiotic powder into a sterile polyethylene bag, commercially known as a stomacher bag. Add 99 mL of previously sterilized (room temperature) *Lactobacilli MRS broth* to the bag. Blend in a mechanical blender until a uniform mixture is achieved or in a stomacher[9] at 230 rpm for 30 s. Hold the mixture at room temperature for 30 min to allow rehydration of the sample, and then blend for an additional 30 s at 230 rpm. This is the primary $10^{-1}$ dilution. Using sterilized, filtered pipet tips, make serial dilutions by aseptically transferring 1.0 mL of the primary $10^{-1}$ dilution to sterile media bottles, each containing 99.0 mL of *Peptone diluent* ($10^{-3}$ dilution). Repeat this operation until the desired dilution series is obtained. Dilutions are expected to contain 25–250 cfu/mL. Shake the media bottles to mix completely before proceeding with the *Analysis*.

**Analysis:** Using three sterile, filtered 1-mL pipet tips, aseptically transfer 1.0 mL of the *Sample preparation* separately into three appropriately labeled, sterile 15-mm × 100-mm Petri plates, and then pour about 10–15 mL of the *Lactobacilli MRS agar* at about 45° onto each plate, flaming the lip of the bottle between pours. Place the lid on each plate after adding the agar medium, and then gently swirl the plates to mix the *Sample preparation* and the agar medium. Avoid spillage onto the lid of the dish when swirling the plates. Repeat this procedure for additional dilutions of the *Sample preparation*. Prepare a blank plate containing only *Lactobacilli MRS agar* and a second blank plate containing 1.0 mL of *Peptone diluent* mixed with *Lactobacilli MRS agar*. Allow the plates to sit at room temperature on a level surface until the agar solidifies, and then incubate the plates at 36°–40° for 3–5 days under anaerobic conditions.[10] After the incubation period, count the colonies and record the results as viable colony-forming units (cfu) per gram, taking into account the appropriate dilution factor of the *Sample preparation*. Only count plates containing 25–250 colonies. Determine the average plate count, in colony-forming units per gram.

**Acceptance criteria:** Refer to the acceptance criteria in the individual monograph.

## PERFORMANCE TESTS

Performance tests are recommended for any dosage forms of probiotics, including tablets, capsules, softgels, gelcaps, powders, or chewable gels, etc.

- **Disintegration and Dissolution** ⟨2040⟩, *Disintegration*: Meet the requirements
- **Weight Variation** ⟨2091⟩: Meet the requirements

## CONTAMINANTS

The level of the standard contaminant-indicator organisms as well as the absence of pathogenic microorganisms specified in the individual monographs must be determined to ensure the quality of probiotics. Recommended standard indicator organisms and specified microorganisms are listed in *Table 3* and *Table 4*. Alternative microbiological procedures, including automated methods, may be used, provided that their equivalence has been demonstrated. Principles of validation of alternative microbiological methods are also described in *Validation of Alternative Microbiological Methods* ⟨1223⟩. Methods (e.g., dilution, buffer, or membrane filtration) may be suitably modified to overcome potential interferences such as acid production resulting from growth of probiotic organisms during the enrichment step, leading to possible false-negative results. Method suitability to detect the presence of microorganisms in the product to be tested must be established. Method suitability must be confirmed if changes in the testing performance occur, or changes in the product composition that may affect the outcome of the test are introduced.

- **Contaminant Microorganisms**

  Unless otherwise specified in the individual monograph, the recommended tests and acceptance criteria for contaminant microorganisms in probiotic ingredients or finished products for oral use are listed in *Table 3*.

**Table 3. Recommended Tests for Contaminant Microorganisms in Probiotic Ingredients or Finished Products for Oral Use**

| Probiotic Classification | Test | Test Methods | Acceptance Criteria (cfu/g) |
|---|---|---|---|
| Non-spore-forming bacteria | Non-lactic acid bacteria | ISO 13559 (IDF 153)[a] | NMT $5 \times 10^3$ |
| | Total yeasts and molds | *Microbial Enumeration Tests* ⟨2021⟩ | NMT 100 |
| Spore-forming bacteria | Total yeasts and molds | *Microbial Enumeration Tests* ⟨2021⟩ | NMT 100 |
| Yeasts and molds | Total aerobic microbial count | *Microbial Enumeration Tests* ⟨2021⟩ | NMT $1 \times 10^3$ |

[a] Available from the International Organization for Standardization (www.iso.org).

[8] Suitable peptone for microbiological analysis is available from BD Bacto (www.bd.com).
[9] Suitable stomacher (e.g., Stomacher 400 Circulator) for mechanical sample blending is available from Seward (www.seward.co.uk).
[10] Suitable anaerobic systems are available from BD GasPak EZ Container System (www.bd.com).

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- **Specified Microorganisms**

Unless otherwise specified in the individual monograph, the recommended tests and acceptance criteria for specified microorganisms in probiotics or finished products for oral use are listed in *Table 4*.

**Table 4. Recommended Tests for Specified Microorganisms in Probiotic Ingredients or Finished Products for Oral Use**

| Probiotic Strain | Test | Test Methods | Acceptance Criteria |
|---|---|---|---|
| Non-spore-forming bacteria, spore-forming bacteria, or yeasts and molds | *Escherichia coli* | *Absence of Specified Microorganisms* ⟨2022⟩ | None detected in 10 g |
| | *Salmonella* species | *Absence of Specified Microorganisms* ⟨2022⟩ | None detected in 10 g |

The absence of *Listeria monocytogenes*, *Staphylococcus aureus*, or *Pseudomonas aeruginosa* in addition to the absence of the specified microorganisms listed in *Table 4* should be tested and confirmed if a probiotic ingredient or a finished product containing a probiotic ingredient(s) poses a risk associated with the contamination of the above microorganisms based on formal risk assessment programs such as Hazard Analysis and Critical Control Points (HACCP). The absence of *Clostridium perfringens* and *Cronobacter sakazakii* in addition to the absence of the listed specified microorganisms should be tested and confirmed if a probiotic ingredient or finished product is intended for infant use. The methods for *Listeria monocytogenes*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Clostridium perfringens*, and *Cronobacter sakazakii* must be officially accepted methods or appropriately validated methods.

## ADDITIONAL REQUIREMENTS

- **Packaging and Storage**

Shipping and storage conditions will necessarily be adapted to the particular strains being sold. Unless otherwise specified in the individual monograph, probiotic ingredients should be stored in high-barrier foil-laminate bags and kept at or below 4° for long-term storage. Dosage forms of probiotics should be stored in tight containers in a cool, dry place.

- **Labeling**

For probiotic ingredients, the strain designation should be listed on the label for each strain. An ingredient or a dosage form of probiotics should be labeled with the genus, species, and strain names. In cases where there is suitable scientific rationale, such as scientific substantiation of health benefits that are not strain specific, a dosage form of probiotics may be labeled with the genus and species names. A total formulated enumeration of all probiotic ingredients throughout the product shelf life should be included at minimum in cfu/g or cfu/serving.▲ (USP 1-Aug-2019)

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# ⟨71⟩ STERILITY TESTS

•Portions of this general chapter have been harmonized with the corresponding texts of the European Pharmacopeia and/or the Japanese Pharmacopeia. Those portions that are not harmonized are marked with symbols (•,•) to specify this fact.•

These Pharmacopeial procedures are not by themselves designed to ensure that a batch of product is sterile or has been sterilized. This is accomplished primarily by validation of the sterilization process or of the aseptic processing procedures.

*The test is applied to substances, preparations, or articles which, according to the Pharmacopeia, are required to be sterile. However, a satisfactory result only indicates that no contaminating microorganism has been found in the sample examined under the conditions of the test.*

## PRECAUTIONS AGAINST MICROBIAL CONTAMINATION

The test for sterility is carried out under aseptic conditions. In order to achieve such conditions, the test environment has to be adapted to the way in which the sterility test is performed. The precautions taken to avoid contamination are such that they do not affect any microorganisms that are to be revealed in the test. The working conditions in which the tests are performed are monitored regularly by appropriate sampling of the working area and by carrying out appropriate controls.

## CULTURE MEDIA AND INCUBATION TEMPERATURES

Media for the test may be prepared as described below or equivalent commercial media may be used provided that they comply with the requirements of the *Growth Promotion Test of Aerobes, Anaerobes, and Fungi*.

The following culture media have been found to be suitable for the test for sterility. *Fluid Thioglycollate Medium* is primarily intended for the culture of anaerobic bacteria. However, it will also detect aerobic bacteria. *Soybean–Casein Digest Medium* is suitable for the culture of both fungi and aerobic bacteria.

**Fluid Thioglycollate Medium**

| | |
|---|---|
| L-Cystine | 0.5 g |
| Sodium Chloride | 2.5 g |
| Dextrose Monohydrate/Anhydrous | 5.5/5.0 g |
| Agar | 0.75 g |
| Yeast Extract (water-soluble) | 5.0 g |
| Pancreatic Digest of Casein | 15.0 g |
| Sodium Thioglycollate | 0.5 g |
| or Thioglycolic Acid | 0.3 mL |
| Resazurin Sodium Solution (1 in 1000), freshly prepared | 1.0 mL |
| Purified Water | 1000 mL |

pH after sterilization: 7.1±0.2.

Mix the L-cystine, agar, sodium chloride, dextrose, yeast extract, and pancreatic digest of casein with the purified water, and heat until solution is effected. Dissolve the sodium thioglycollate or thioglycolic acid in the solution and, if necessary, add 1 N sodium hydroxide so that, after sterilization, the solution will have a pH of 7.1 ± 0.2. If filtration is necessary, heat the solution again without boiling, and filter while hot through moistened filter paper. Add the resazurin sodium solution, mix, and place the medium in suitable vessels that provide a ratio of surface to depth of medium such that not more than the upper half of the medium has undergone a color change indicative of oxygen uptake at the end of the incubation period. Sterilize using a validated process. If the medium is stored, store at a temperature between 2° and 25° in a sterile, airtight container. If more than the upper one-third of the medium has acquired a pink color, the medium may be restored once by heating the containers in a water-bath or in free-flowing steam until the pink color disappears and by cooling quickly, taking care to prevent the introduction of nonsterile air into the container. Do not use the medium for a longer storage period than has been validated.

*Fluid Thioglycollate Medium* is to be incubated at 30°–35°. For products containing a mercurial preservative that cannot be tested by the membrane filtration method, *Fluid Thioglycollate Medium* incubated at 20°–25° may be used instead of *Soybean–Casein Digest Medium* provided that it has been validated as described in *Growth Promotion Test of Aerobes, Anaerobes, and Fungi*. Where prescribed or justified and authorized, the following alternative thioglycollate medium might be used. Prepare a mixture having the same composition as that of the *Fluid Thioglycollate Medium*, but omitting the agar and the resazurin sodium solution. Sterilize as directed above. The pH after sterilization is 7.1 ± 0.2. Heat in a water bath prior to use and incubate at 30°–35° under anaerobic conditions.

**Soybean–Casein Digest Medium**

| | |
|---|---|
| Pancreatic Digest of Casein | 17.0 g |
| Papaic Digest of Soybean Meal | 3.0 g |

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**Soybean–Casein Digest Medium** *(continued)*

| | |
|---|---|
| Sodium Chloride | 5.0 g |
| Dibasic Potassium Phosphate | 2.5 g |
| Dextrose Monohydrate/Anhydrous | 2.5/2.3 g |
| Purified Water | 1000 mL |

pH after sterilization: 7.3±0.2.

Dissolve the solids in the Purified Water, heating slightly to effect a solution. Cool the solution to room temperature, and adjust the pH with 1 N sodium hydroxide so that, after sterilization, it will have a pH of 7.3 ± 0.2. Filter, if necessary to clarify, dispense into suitable containers, and sterilize using a validated procedure. Store at a temperature between 2° and 25° in a sterile well-closed container, unless it is intended for immediate use. Do not use the medium for a longer storage period than has been validated.

*Soybean–Casein Digest Medium* is to be incubated at 22.5 ± 2.5°.

## ♦Media for Penicillins or Cephalosporins

Where sterility test media are to be used in the *Direct Inoculation of the Culture Medium* method under *Test for Sterility of the Product to be Examined*, modify the preparation of *Fluid Thioglycollate Medium* and the *Soybean–Casein Digest Medium* as follows. To the containers of each medium, transfer aseptically a quantity of β-lactamase sufficient to inactivate the amount of antibiotic in the specimen under test. Determine the quantity of β-lactamase required to inactivate the antibiotic by using a β-lactamase preparation that has been assayed previously for its penicillin- or cephalosporin-inactivating power. [NOTE—Supplemented β-lactamase media can also be used in the membrane filtration test.]

Alternatively (in an area completely separate from that used for sterility testing), confirm that an appropriate amount of β-lactamase is incorporated into the medium, following either method under *Method Suitability Test*, using less than 100 colony-forming units (cfu) of *Staphylococcus aureus* (see *Table 1*) as the challenge. Typical microbial growth of the inoculated culture must be observed as a confirmation that the β-lactamase concentration is appropriate.♦

**Table 1. Strains of the Test Microorganisms Suitable for Use in the Growth Promotion Test and the Method Suitability Test**

| | |
|---|---|
| **Aerobic bacteria** | |
| *Staphylococcus aureus* | ATCC 6538, CIP 4.83, NCTC 10788, NCIMB 9518, NBRC 13276 |
| *Bacillus subtilis* | ATCC 6633, CIP 52.62, NCIMB 8054, NBRC 3134 |
| *Pseudomonas aeruginosa*♦[1]♦ | ATCC 9027, NCIMB 8626, CIP 82.118, NBRC 13275 |
| **Anaerobic bacterium** | |
| *Clostridium sporogenes*♦[2]♦ | ATCC 19404, CIP 79.3, NCTC 532 or ATCC 11437, NBRC 14293 |
| **Fungi** | |
| *Candida albicans* | ATCC 10231, IP 48.72, NCPF 3179, NBRC 1594 |
| *Aspergillus brasiliensis* (*Aspergillus Niger*) | ATCC 16404, IP 1431.83, IMI 149007, NBRC 9455 |

♦[1] An alternative microorganism is *Kocuria rhizophila (Micrococcus luteus)* ATCC 9341.♦

♦[2] An alternative to *Clostridium sporogenes,* when a nonspore-forming microorganism is desired, is *Bacteroides vulgatus* (ATCC 8482).♦

The media used comply with the following tests, carried out before, or in parallel, with the test on the product to be examined.

## Sterility

Incubate portions of the media for 14 days. No growth of microorganisms occurs.

## Growth Promotion Test of Aerobes, Anaerobes, and Fungi

Test each lot of ready-prepared medium and each batch of medium prepared either from dehydrated medium or from ingredients. Suitable strains of microorganisms are indicated in *Table 1*.

Inoculate portions of *Fluid Thioglycollate Medium* with a small number (not more than 100 cfu) of the following microorganisms, using a separate portion of medium for each of the following species of microorganism: *Clostridium sporogenes*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. ♦Inoculate portions of alternative thioglycollate medium with a small number (not more than 100 cfu) of *Clostridium sporogenes*.♦ Inoculate portions of *Soybean–Casein Digest Medium* with a small number (not more than 100 cfu) of the following microorganisms, using a separate portion of medium for each of the following species of microorganism: *Aspergillus brasiliensis*, *Bacillus subtilis,* and *Candida albicans*. Incubate for not more than 3 days in the case of bacteria and not more than 5 days in the case of fungi. Seed lot culture maintenance techniques (seed-lot systems) are used so that the viable microorganisms used for inoculation are not more than five passages removed from the original master seed-lot.

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The media are suitable if a clearly visible growth of the microorganisms occurs.

## ♦DILUTING AND RINSING FLUIDS FOR MEMBRANE FILTRATION

### Fluid A

PREPARATION

Dissolve 1 g of peptic digest of animal tissue in water to make 1 L, filter or centrifuge to clarify, if necessary, and adjust to a pH of 7.1 ± 0.2. Dispense into containers, and sterilize using a validated process.

PREPARATION FOR PENICILLINS OR CEPHALOSPORINS

Aseptically add to the above *Preparation*, if necessary, a quantity of sterile β-lactamase sufficient to inactivate any residual antibiotic activity on the membranes after the solution of the test specimen has been filtered (see *Media for Penicillins or Cephalosporins*).

### Fluid D

To each L of *Fluid A* add 1 mL of polysorbate 80, adjust to a pH of 7.1 ± 0.2, dispense into containers, and sterilize using a validated process. Use this fluid for articles containing lecithin or oil, or for devices labeled as "sterile pathway."

### Fluid K

Dissolve 5.0 g of peptic digest of animal tissue, 3.0 g of beef extract, and 10.0 g of polysorbate 80 in water to make 1 L. Adjust the pH to obtain, after sterilization, a pH of 6.9 ± 0.2. Dispense into containers, and sterilize using a validated process.♦

## METHOD SUITABILITY TEST

Carry out a test as described below under *Test for Sterility of the Product to be Examined* using exactly the same methods, except for the following modifications.

### Membrane Filtration

After transferring the content of the container or containers to be tested to the membrane, add an inoculum of a small number of viable microorganisms (not more than 100 cfu) to the final portion of sterile diluent used to rinse the filter.

### Direct Inoculation

After transferring the contents of the container or containers to be tested (for catgut and other surgical sutures for veterinary use: strands) to the culture medium, add an inoculum of a small number of viable microorganisms (not more than 100 cfu) to the medium.

In both cases use the same microorganisms as those described above under *Growth Promotion Test of Aerobes, Anaerobes, and Fungi*. Perform a growth promotion test as a positive control. Incubate all the containers containing medium for not more than 5 days.

If clearly visible growth of microorganisms is obtained after the incubation, visually comparable to that in the control vessel without product, either the product possesses no antimicrobial activity under the conditions of the test or such activity has been satisfactorily eliminated. The test for sterility may then be carried out without further modification.

If clearly visible growth is not obtained in the presence of the product to be tested, visually comparable to that in the control vessels without product, the product possesses antimicrobial activity that has not been satisfactorily eliminated under the conditions of the test. Modify the conditions in order to eliminate the antimicrobial activity, and repeat the *Method Suitability Test*.

This method suitability is performed (a) when the test for sterility has to be carried out on a new product; and (b) whenever there is a change in the experimental conditions of the test. The method suitability may be performed simultaneously with the *Test for Sterility of the Product to be Examined*.

## TEST FOR STERILITY OF THE PRODUCT TO BE EXAMINED

### ♦Number of Articles to Be Tested

Unless otherwise specified elsewhere in this chapter or in the individual monograph, test the number of articles specified in *Table 3*. If the contents of each article are of sufficient quantity (see *Table 2*), they may be divided so that equal appropriate portions are added to each of the specified media. [NOTE—Perform sterility testing employing two or more of the specified

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media.] If each article does not contain sufficient quantities for each medium, use twice the number of articles indicated in *Table 3*.

**Table 2. Minimum Quantity to be Used for Each Medium**

| Quantity per Container | Minimum Quantity to be Used (unless otherwise justified and authorized) |
|---|---|
| *Liquids* | |
| Less than 1 mL | The whole contents of each container |
| 1–40 mL | Half the contents of each container, but not less than 1 mL |
| Greater than 40 mL, and not greater than 100 mL | 20 mL |
| Greater than 100 mL | 10% of the contents of the container, but not less than 20 mL |
| *Antibiotic liquids* | 1 mL |
| *Insoluble preparations, creams, and ointments to be suspended or emulsified* | Use the contents of each container to provide not less than 200 mg |
| *Solids* | |
| Less than 50 mg | The whole contents of each container |
| 50 mg or more, but less than 300 mg | Half the contents of each container, but not less than 50 mg |
| 300 mg–5 g | 150 mg |
| Greater than 5 g | 500 mg |
| Catgut and other surgical sutures for veterinary use | 3 sections of a strand (each 30-cm long) |
| •Surgical dressing/cotton/gauze (in packages) | 100 mg per package |
| Sutures and other individually packaged single-use material | The whole device |
| Other medical devices | The whole device, cut into pieces or disassembled• |

**Table 3. Minimum Number of Articles to be Tested in Relation to the Number of Articles in the Batch**

| Number of Items in the Batch* | Minimum Number of Items to be Tested for Each Medium (unless otherwise justified and authorized)** |
|---|---|
| *Parenteral preparations* | |
| Not more than 100 containers | 10% or 4 containers, whichever is the greater |
| More than 100 but not more than 500 containers | 10 containers |
| More than 500 containers | 2% or 20 containers, whichever is less |
| • *For large-volume parenterals* | 2% or 10 containers, whichever is less |
| *Antibiotic solids* | |
| Pharmacy bulk packages (<5 g) | 20 containers |
| Pharmacy bulk packages (≥5 g) | 6 containers |
| Bulks and blends | See *Bulk solid products*• |
| | |
| *Ophthalmic and other noninjectable preparations* | |
| Not more than 200 containers | 5% or 2 containers, whichever is the greater |
| More than 200 containers | 10 containers |
| If the product is presented in the form of single-dose containers, apply the scheme shown above for preparations for parenteral use. | |
| Catgut and other surgical sutures for veterinary use | 2% or 5 packages, whichever is the greater, up to a maximum total of 20 packages |
| •Not more than 100 articles | 10% or 4 articles, whichever is greater |
| More than 100, but not more than 500 articles | 10 articles |
| More than 500 articles | 2% or 20 articles, whichever is less• |
| *Bulk solid products* | |
| Up to 4 containers | Each container |
| More than 4 containers, but not more than 50 containers | 20% or 4 containers, whichever is greater |
| More than 50 containers | 2% or 10 containers, whichever is greater |

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* If the batch size is unknown, use the maximum number of items prescribed.
** If the contents of one container are enough to inoculate the two media, this column gives the number of containers needed for both the media together.

The test may be carried out using the technique of *Membrane Filtration* or by *Direct Inoculation of the Culture Medium* with the product to be examined. Appropriate negative controls are included. The technique of membrane filtration is used whenever the nature of the product permits; that is, for filterable aqueous preparations, for alcoholic or oily preparations, and for preparations miscible with, or soluble in, aqueous or oily solvents, provided these solvents do not have an antimicrobial effect in the conditions of the test.

## Membrane Filtration

Use membrane filters having a nominal pore size not greater than 0.45 µm, in which the effectiveness to retain microorganisms has been established. Cellulose nitrate filters, for example, are used for aqueous, oily, and weakly alcoholic solutions; and cellulose acetate filters, for example, are used for strongly alcoholic solutions. Specially adapted filters may be needed for certain products (e.g., for antibiotics).

The technique described below assumes that membranes about 50 mm in diameter will be used. If filters of a different diameter are used, the volumes of the dilutions and the washings should be adjusted accordingly. The filtration apparatus and membrane are sterilized by appropriate means. The apparatus is designed so that the solution to be examined can be introduced and filtered under aseptic conditions: it permits the aseptic removal of the membrane for transfer to the medium, or it is suitable for carrying out the incubation after adding the medium to the apparatus itself.

### AQUEOUS SOLUTIONS

If appropriate, transfer a small quantity of a suitable, sterile diluent such as ♦*Fluid A* (see *Diluting and Rinsing Fluids for Membrane Filtration*)♦ onto the membrane in the apparatus and filter. The diluent may contain suitable neutralizing substances and/or appropriate inactivating substances, for example, in the case of antibiotics.

Transfer the contents of the container or containers to be tested to the membrane or membranes, if necessary, after diluting to the volume used in the *Method Suitability Test* with the chosen sterile diluent, but using not less than the quantities of the product to be examined prescribed in *Tables 2* and *3*. Filter immediately. If the product has antimicrobial properties, wash the membrane not less than three times by filtering through it each time the volume of the chosen sterile diluent used in the *Method Suitability Test.* Do not exceed a washing cycle of five times 100 mL per filter, even if during method suitability it has been demonstrated that such a cycle does not fully eliminate the antimicrobial activity. Transfer the whole membrane to the culture medium or cut it aseptically into two equal parts, and transfer one half to each of two suitable media. Use the same volume of each medium as in the *Method Suitability Test.* Alternatively, transfer the medium onto the membrane in the apparatus. Incubate the media for not less than 14 days.

### SOLUBLE SOLIDS

Use for each medium not less than the quantity prescribed in *Tables 2* and *3* of the product dissolved in a suitable solvent, such as the solvent provided with the preparation, Sterile Water for Injection, sterile saline, or a suitable sterile solution such as ♦*Fluid A* (*Diluting and Rinsing Fluids for Membrane Filtration*),♦ and proceed with the test as described above for *Aqueous Solutions* using a membrane appropriate to the chosen solvent.

### OILS and OILY SOLUTIONS

Use for each medium not less than the quantity of the product prescribed in *Tables 2* and *3*. Oils and oily solutions of sufficiently low viscosity may be filtered without dilution through a dry membrane. Viscous oils may be diluted as necessary with a suitable sterile diluent such as isopropyl myristate shown not to have antimicrobial activity in the conditions of the test. Allow the oil to penetrate the membrane by its own weight, and then filter, applying the pressure or suction gradually. Wash the membrane at least three times by filtering through it each time about 100 mL of a suitable sterile solution such as ♦*Fluid A* (see *Diluting and Rinsing Fluids for Membrane Filtration*)♦ containing a suitable emulsifying agent at a concentration shown to be appropriate in the *Method Suitability Test,* for example polysorbate 80 at a concentration of 10 g per L ♦(*Fluid K*)♦. Transfer the membrane or membranes to the culture medium or media, or vice versa, as described above for *Aqueous Solutions*, and incubate at the same temperatures and for the same times.

### OINTMENTS and CREAMS

Use for each medium not less than the quantities of the product prescribed in *Tables 2* and *3*. Ointments in a fatty base and emulsions of the water-in-oil type may be diluted to 1% in isopropyl myristate as described above, by heating, if necessary, to not more than 40°. In exceptional cases it may be necessary to heat to not more than 44°. Filter as rapidly as possible, and proceed as described above for *Oils and Oily Solutions*.

### ♦PREFILLED SYRINGES

For prefilled syringes without attached sterile needles, expel the contents of each syringe into one or two separate membrane filter funnels or into separate pooling vessels prior to transfer. If a separate sterile needle is attached, directly expel the syringe contents as indicated above, and proceed as directed for *Aqueous Solutions*. Test the sterility of the needle, using *Direct Inoculation* under *Method Suitability Test*.

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### SOLIDS FOR INJECTION OTHER THAN ANTIBIOTICS

Constitute the test articles as directed on the label, and proceed as directed for *Aqueous Solutions* or *Oils and Oily Solutions*, whichever applies. [NOTE—If necessary, excess diluent can be added to aid in the constitution and filtration of the constituted test article.]

### ANTIBIOTIC SOLIDS FOR INJECTION

**Pharmacy Bulk Packages, <5 g**—From each of 20 containers, aseptically transfer about 300 mg of solids, into a sterile 500-mL conical flask, dissolve in about 200 mL of *Fluid A* (see *Diluting and Rinsing Fluids for Membrane Filtration*), and mix; or constitute, as directed in the labeling, each of 20 containers and transfer a quantity of liquid or suspension, equivalent to about 300 mg of solids, into a sterile 500-mL conical flask, dissolve in about 200 mL of *Fluid A*, and mix. Proceed as directed for *Aqueous Solutions* or *Oils and Oily Solutions*, whichever applies.
**Pharmacy Bulk Packages, ≥5 g**—From each of 6 containers, aseptically transfer about 1 g of solids into a sterile 500-mL conical flask, dissolve in about 200 mL of *Fluid A*, and mix; or constitute, as directed in the labeling, each of 6 containers and transfer a quantity of liquid, equivalent to about 1 g of solids, into a sterile 500-mL conical flask, dissolve in about 200 mL of *Fluid A*, and mix. Proceed as directed for *Aqueous Solutions*.

### ANTIBIOTIC SOLIDS, BULKS, and BLENDS

Aseptically remove a sufficient quantity of solids from the appropriate amount of containers (see *Table 2*), mix to obtain a composite, equivalent to about 6 g of solids, and transfer to a sterile 500-mL conical flask. Dissolve in about 200 mL of *Fluid A*, and mix. Proceed as directed for *Aqueous Solutions*.

### STERILE AEROSOL PRODUCTS

For fluid products in pressurized aerosol form, freeze the containers in an alcohol-dry ice mixture at least at –20° for about 1 hour. If feasible, allow the propellant to escape before aseptically opening the container, and transfer the contents to a sterile pooling vessel. Add 100 mL of *Fluid D* to the pooling vessel, and mix gently. Proceed as directed for *Aqueous Solutions* or *Oils and Oily Solutions*, whichever applies.

### DEVICES WITH PATHWAYS LABELED STERILE

Aseptically pass not less than 10 pathway volumes of *Fluid D* through each device tested. Collect the fluids in an appropriate sterile vessel, and proceed as directed for *Aqueous Solutions* or *Oils and Oily Solutions*, whichever applies.
In the case of sterile, empty syringes, draw sterile diluent into the barrel through the sterile needle, if attached, or through a sterile needle attached for the purpose of the test, and express the contents into a sterile pooling vessel. Proceed as directed above.

## Direct Inoculation of the Culture Medium

Transfer the quantity of the preparation to be examined prescribed in *Tables 2* and *3* directly into the culture medium so that the volume of the product is not more than 10% of the volume of the medium, unless otherwise prescribed.
If the product to be examined has antimicrobial activity, carry out the test after neutralizing this with a suitable neutralizing substance or by dilution in a sufficient quantity of culture medium. When it is necessary to use a large volume of the product, it may be preferable to use a concentrated culture medium prepared in such a way that it takes into account the subsequent dilution. Where appropriate, the concentrated medium may be added directly to the product in its container.

### OILY LIQUIDS

Use media to which have been added a suitable emulsifying agent at a concentration shown to be appropriate in the *Method Suitability Test,* for example polysorbate 80 at a concentration of 10 g per L.

### OINTMENTS and CREAMS

Prepare by diluting to about 1 in 10 by emulsifying with the chosen emulsifying agent in a suitable sterile diluent such as *Fluid A* (see *Diluting and Rinsing Fluids for Membrane Filtration*). Transfer the diluted product to a medium not containing an emulsifying agent.
Incubate the inoculated media for not less than 14 days. Observe the cultures several times during the incubation period. Shake cultures containing oily products gently each day. However, when *Fluid Thioglycollate Medium* is used for the detection of anaerobic microorganisms, keep shaking or mixing to a minimum in order to maintain anaerobic conditions.

### CATGUT and OTHER SURGICAL SUTURES FOR VETERINARIAN USE

Use for each medium not less than the quantities of the product prescribed in *Tables 2* and *3*. Open the sealed package using aseptic precautions, and remove three sections of the strand for each culture medium. Carry out the test on three sections, each 30-cm long, which have been cut off from the beginning, the center, and the end of the strand. Use whole strands from freshly opened cassette packs. Transfer each section of the strand to the selected medium. Use sufficient medium to cover adequately the material to be tested (20 mL to 150 mL).

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### ♦SOLIDS

Transfer a quantity of the product in the form of a dry solid (or prepare a suspension of the product by adding sterile diluent to the immediate container), corresponding to not less than the quantity indicated in *Tables 2* and *3*. Transfer the material so obtained to 200 mL of *Fluid Thioglycollate Medium,* and mix. Similarly, transfer the same quantity to 200 mL of *Soybean–Casein Digest Medium,* and mix. Proceed as directed above.

### PURIFIED COTTON, GAUZE, SURGICAL DRESSINGS, and RELATED ARTICLES

From each package of cotton, rolled gauze bandage, or large surgical dressings being tested, aseptically remove two or more portions of 100- to 500-mg each from the innermost part of the sample. From individually packaged, single-use materials, aseptically remove the entire article. Immerse the portions or article in each medium, and proceed as directed above.

### STERILE DEVICES

Articles can be immersed intact or disassembled. To ensure that device pathways are also in contact with the media, immerse the appropriate number of units per medium in a volume of medium sufficient to immerse the device completely, and proceed as directed above. For extremely large devices, immerse those portions of the device that are to come into contact with the patient in a volume of medium sufficient to achieve complete immersion of those portions.

For catheters where the inside lumen and outside are required to be sterile, either cut them into pieces such that the medium is in contact with the entire lumen or fill the lumen with medium, and then immerse the intact unit.♦

## OBSERVATION AND INTERPRETATION OF RESULTS

At intervals during the incubation period and at its conclusion, examine the media for macroscopic evidence of microbial growth. If the material being tested renders the medium turbid so that the presence or absence of microbial growth cannot be readily determined by visual examination, 14 days after the beginning of incubation transfer portions (each not less than 1 mL) of the medium to fresh vessels of the same medium, and then incubate the original and transfer vessels for not less than 4 days.

If no evidence of microbial growth is found, the product to be examined complies with the test for sterility. If evidence of microbial growth is found, the product to be examined does not comply with the test for sterility, unless it can be clearly demonstrated that the test was invalid for causes unrelated to the product to be examined. The test may be considered invalid only if one or more of the following conditions are fulfilled:

1. The data of the microbiological monitoring of the sterility testing facility show a fault.
2. A review of the testing procedure used during the test in question reveals a fault.
3. Microbial growth is found in the negative controls.
4. After determination of the identity of the microorganisms isolated from the test, the growth of this species (or these species) may be ascribed unequivocally to faults with respect to the material and or the technique used in conducting the sterility test procedure.

If the test is declared to be invalid, it is repeated with the same number of units as in the original test. If no evidence of microbial growth is found in the repeat test, the product examined complies with the test for sterility. If microbial growth is found in the repeat test, the product examined does not comply with the test for sterility.

## APPLICATION OF THE TEST TO PARENTERAL PREPARATIONS, OPHTHALMIC, AND OTHER NONINJECTABLE PREPARATIONS REQUIRED TO COMPLY WITH THE TEST FOR STERILITY

When using the technique of membrane filtration, use, whenever possible, the whole contents of the container, but not less than the quantities indicated in *Table 2,* diluting where necessary to about 100 mL with a suitable sterile solution, such as ♦*Fluid A* (see *Diluting and Rinsing Fluids for Membrane Filtration*).♦

When using the technique of direct inoculation of media, use the quantities shown in *Table 2,* unless otherwise justified and authorized. The tests for bacterial and fungal sterility are carried out on the same sample of the product to be examined. When the volume or the quantity in a single container is insufficient to carry out the tests, the contents of two or more containers are used to inoculate the different media.

## MINIMUM NUMBER OF ITEMS TO BE TESTED

The minimum number of items to be tested in relation to the size of the batch is given in *Table 3*.

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# ⟨81⟩ ANTIBIOTICS—MICROBIAL ASSAYS

## INTRODUCTION AND GENERAL INFORMATION

The activity (potency) of antibiotics can be demonstrated by their inhibitory effect on microorganisms under suitable conditions. A reduction in antimicrobial activity may not be adequately demonstrated by chemical methods. This chapter summarizes procedures for the antibiotics recognized in the *United States Pharmacopeia* (*USP*) for which the microbiological assay is the standard analytical method.

Two general techniques are employed: the cylinder-plate (or plate) assay and the turbidimetric (or tube) assay. *Table 1* lists all the antibiotics that contain microbial assays and specifies the type of assay (cylinder-plate or turbidimetric).

**Table 1**

| Antibiotic | Type of Assay |
|---|---|
| Amoxicillin | Cylinder-plate |
| Amphotericin B | Cylinder-plate |
| Bacitracin | Cylinder-plate |
| Bleomycin | Cylinder-plate |
| Capreomycin | Turbidimetric |
| Chloramphenicol | Turbidimetric |
| Chlortetracycline | Turbidimetric |
| Cloxacillin | Cylinder-plate |
| Colistemethate | Cylinder-plate |
| Colistin | Cylinder-plate |
| Dihydrostreptomycin | Cylinder-plate |
| | Turbidimetric |
| Erythromycin | Cylinder-plate |
| Gentamicin | Cylinder-plate |
| Gramicidin | Turbidimetric |
| Nafcillin | Cylinder-plate |
| Neomycin | Cylinder-plate |
| | Turbidimetric |
| Novobiocin | Cylinder-plate |
| Nystatin | Cylinder-plate |
| Oxytetracycline | Turbidimetric |
| Paromomycin | Cylinder-plate |
| Penicillin G | Cylinder-plate |
| Polymyxin B | Cylinder-plate |
| Tetracycline | Turbidimetric |
| Thiostrepton | Turbidimetric |
| Tylosin | Turbidimetric |
| Vancomycin | Cylinder-plate |

[NOTE—Perform all procedures under conditions designed to avoid extrinsic microbial contamination. Take adequate safety precautions while performing these assays because of possible allergies to drugs and because live cultures of organisms are used in the procedures.]

### Cylinder-Plate Assay

The cylinder-plate assay depends on diffusion of the antibiotic from a vertical cylinder through a solidified agar layer in a Petri dish or plate. The growth of the specific microorganisms inoculated into the agar is prevented in a circular area or "zone" around the cylinder containing the solution of the antibiotic.

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## Turbidimetric Assay

The turbidimetric assay depends on the inhibition of growth of a microorganism in a uniform solution of the antibiotic in a fluid medium that is favorable to the growth of the microorganism in the absence of the antibiotic.

## Units and Reference Standards

For substances like the antibiotics quantified using the method in this chapter which are not easily characterized by chemical or physical means, it is still necessary to express quantities of biological activity in units of biological potency, each defined by an authoritative reference standard. The potency of antibiotics is designated in either units (U) or µg of activity. In each case, the unit or µg of antibiotic activity was originally established against a US Federal Master Standard for that antibiotic. This practice started because originally the antibiotic selected as a reference standard was thought to consist entirely of a single chemical entity and was therefore assigned a potency of 1000 µg/mg. In several such instances, as the manufacturing and purification methods for particular antibiotics became more advanced, antibiotics containing more than 1000 µg of activity/mg became possible. Since relative potency was used to assign this value and not the mass of the antibiotics themselves, such antibiotics had an "activity" equivalent to a given number of µg of the original reference standard (rather than a µg of mass as would be expected). Over time, less complex antibiotics or those with a single primary active substance were demonstrated to have µg of activity equivalent numerically to the µg (weight) of the pure substance. In these cases, the antibiotics have moved away from the microbial assay methods and are assigned by a mass balance approach; until that exercise is completed for a given antibiotic, manufacturers cannot assume that the µg of activity corresponds to the µg (weight) of the antibiotic substance. Since the US Federal Master antibiotic standards are no longer available, USP Reference Standards for antibiotics that are assigned relative potency by the methods in this chapter are calibrated either against the World Health Organization (WHO) International Standard for Antibiotics (ISA), if it exists, or the previous USP lot when a WHO ISA or another authoritative reference standard calibrated against an ISA does not exist. Once assigned, the International Units (IUs), when used, are converted to USP units by the previously agreed upon bridging value between the two when the respective Reference Standards were originally assigned. Over time, it may be possible to convert the assay methods of more antibiotics from the microbial bioassay to a physicochemical method much like that suggested in *Validation of Alternative Methods to Antibiotic Microbial Assays* ⟨1223.1⟩ or other suitable strategies that include validated methods with associated bridging data.

## Apparatus

Labware used for the storage and transfer of test dilutions and microorganisms must be sterile and free of interfering residues (see *Cleaning Glass Apparatus* ⟨1051⟩). Use a validated sterilization method, such as dry heat, steam, or radiation; or use sterile, disposable labware.

## Temperature Control

Thermostatic control is required in several stages of a microbial assay: when culturing a microorganism and preparing its inoculum, and during incubation in plate and tube assays. Refer to specific temperature requirements below for each type of assay.

## Test Organisms

The test organism for each antibiotic is listed in *Table 3* for the cylinder-plate assay and *Table 8* for the turbidimetric assay. The test organisms are specified by the American Type Culture Collection (ATCC) number.

In order to ensure acceptable performance of test organisms, store and maintain them properly. Establish the specific storage conditions during method validation or verification. Discard cultures if a change in the organism's characteristics is observed.

## Prolonged Storage

For prolonged storage, maintain test organisms in a suitable storage solution such as 50% fetal calf serum in broth, 10%–15% glycerol in tryptic soy broth, defibrinated sheep blood, or skim milk. Prolonged-storage cultures are best stored in the freeze-dried state; temperatures of –60° or below are preferred; temperatures below –20° are acceptable.

## Primary Cultures

Prepare primary cultures by transferring test organisms from prolonged-storage vials onto appropriate media, and incubate under appropriate growth conditions. Store primary cultures at the appropriate temperature, usually 2°–8°, and discard after 3 weeks. A single primary culture can be used to prepare working cultures only for as many as 7 days.

## Working Cultures

Prepare working cultures by transferring the primary culture onto appropriate solid media to obtain isolated colonies. Incubate working cultures under appropriate conditions to obtain satisfactory growth for preparation of test inocula. Prepare fresh working cultures for each test day.

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## Uncharacteristic Growth or Performance of a Test Organism

Use new stock cultures, primary cultures, or working cultures when a test organism shows uncharacteristic growth or performance.

## Assay Designs

Suitable experimental designs are key to increasing precision and minimizing bias. Control of the incubation parameters, temperature distribution and time, is critical for minimizing bias; it can be accomplished by staging the plates and racks as described for each assay.

### CYLINDER-PLATE ASSAY

The comparisons are restricted to relationships between zone diameter measurements within plates, excluding the variation between plates. Individual plate responses are normalized on the basis of the relative zone size of the standard compared to the mean zone size of the standard across all plates.

### TURBIDIMETRIC ASSAY

To avoid systematic bias, place replicate tubes randomly in separate racks so that each rack contains one complete set of treatments. The purpose of this configuration is to minimize the influence of temperature distribution on the replicate samples. The turbidimetric assay, because of the configuration of the samples in test tube racks, is sensitive to slight variations in temperature. The influence of temperature variation can also be decreased by ensuring proper airflow or heat convection during incubation. At least 3 tubes for each sample and Standard concentration (one complete set of samples) should be placed in a single rack. The comparisons are restricted to relationships between the observed turbidities within racks.

## Potency Considerations

Within the restrictions listed above, the recommended assay design employs a five-concentration standard curve and a single concentration of each sample preparation.

For the cylinder-plate assay, each plate includes only 2 treatments, the reference treatment (median level standard, i.e., $S_3$) and one of the other 4 concentrations of the standard ($S_1$, $S_2$, $S_4$, and $S_5$) or the sample ($U_3$). The concentration of the sample is an estimate based on the target concentration. The sample should be diluted to give a nominal concentration that is estimated to be equivalent to the median reference concentration ($S_3$) of the standard. The purpose of diluting to the median reference concentration is to ensure that the sample result will fall within the linear portion of the standard curve. The test determines the relative potency of $U_3$ against the standard curve. The sample ($U_3$) should have a relative potency of about 100%. The final potency of the sample is obtained by multiplying the $U_3$ result by the dilution factor.

An assay should be considered preliminary if the computed potency value of the sample is less than 80% or more than 125%. In this case, the results suggest that the sample concentration assumed during preparation of the sample stock solution was not correct. In such a case, one can adjust the assumed potency of the sample on the basis of the preliminary potency value and repeat the assay. Otherwise, the potency will be derived from a portion of the curve where the Standard and sample responses will likely not be parallel.

Microbial determinations of potency are subject to inter-assay as well as intra-assay variables; therefore three or more independent assays are required for a reliable estimate of the potency of a given sample. Starting with separately prepared stock solutions and test dilutions of both the Standard and the sample, perform additional assays of a given sample. The mean potency should include the results from all the valid independent assays. The latter is assessed by the width of the confidence interval (refer to *Confidence Limits and Combination of Assays Calculations*). The combined result of a series of smaller, independent assays is a more reliable estimate of potency than one from a single large assay with the same total number of plates or tubes. Note that additional assays or lower variability allows the product to meet tighter specification ranges. Reducing assay variability achieves the required confidence limit with fewer assays.

# CYLINDER-PLATE METHOD

## Temperature Control

Use appropriately qualified and calibrated equipment to obtain the temperature ranges specified in *Table 3*.

## Apparatus

### PLATES

Use glass or disposable plastic Petri dishes (approximately 20 mm × 100 mm or other appropriate dimensions) with lids.

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CYLINDERS

Use stainless steel or porcelain cylinders; 8 ± 0.1-mm o.d.; 6 ± 0.1-mm i.d.; 10 ± 0.1-mm high. [NOTE—Carefully clean cylinders to remove all residues; occasional cleaning in an acid bath, e.g., with about 2 N nitric acid or with chromic acid (see ⟨1051⟩) is required.]

## Standard Solutions

To prepare a stock solution, dissolve a suitable quantity of the USP Reference Standard of a given antibiotic, or the entire contents of a vial of USP Reference Standard, where appropriate, in the solvent specified in *Table 2*; and dilute to the specified concentration. Store at 2°–8°, and use within the period indicated. On the day of the assay, prepare from the stock solution five or more test dilutions, in which the successive solutions increase stepwise in concentration, usually in the ratio of 1:1.25. Use the final diluent specified such that the median has the concentration suggested in *Table 2*.

## Sample Solutions

Assign an assumed potency per unit weight or volume to the sample. On the day of the assay, prepare a stock solution in the same manner specified for the USP Reference Standard (see *Table 2*). Dilute the sample stock solution in the specified final diluent to obtain a nominal concentration equal to the median concentration of the standard ($S_3$).

**Table 2**

| Antibiotic | Stock Solution | | | | | Test Dilution | |
|---|---|---|---|---|---|---|---|
| | Initial Solvent | Initial Concentration | Further Diluent | Final Concentration | Use Within (days) | Final Diluent | Median Concentration ($S_3$)[a, b] |
| Amoxicillin[c] | Water | — | — | 100 µg/mL | 7 | B.3[d] | 0.1 µg/mL |
| Amphotericin B[c, e] | Dimethyl sulfoxide | — | — | 1 mg/mL | Same day | B.10[d] | 1 µg/mL |
| Bacitracin[f] | 0.01 N hydrochloric acid | — | — | 100 U/mL | Same day | B.1[d] | 1 U/mL |
| Bleomycin | B.16[d] | — | — | 2 U/mL | 14 | B.16[d] | 0.04 U/mL |
| Cloxacillin | B.1[d] | — | — | 1 mg/mL | 7 | B.1[d] | 5 µg/mL |
| Colistimethate[c] | Water | 10 mg/mL | B.6[d] | 1 mg/mL | Same day | B.6[d] | 1 µg/mL |
| Colistin | Water | 10 mg/mL | B.6[d] | 1 mg/mL | 14 | B.6[d] | 1 µg/mL |
| Dihydrostreptomycin[g] | B.3[d] | — | — | 1 mg/mL | 30 | B.3[d] | 1 µg/mL |
| Erythromycin | Methanol | 10 mg/mL | B.3[d] | 1 mg/mL | 14 | B.3[d] | 1 µg/mL |
| Gentamicin | B.3[d] | — | — | 1 mg/mL | 30 | B.3[d] | 0.1 µg/mL |
| Nafcillin | B.1[d] | — | — | 1 mg/mL | 2 | B.1[d] | 2 µg/mL |
| Neomycin[g] | B.3[d] | — | — | 1 mg/mL | 14 | B.3[d] | 1 µg/mL |
| Novobiocin | alcohol | 10 mg/mL | B.3[d] | 1 mg/mL | 5 | B.6[d] | 0.5 µg/mL |
| Nystatin[c, h] | Dimethylformamide | — | — | 1000 U/mL | Same day | B.6[d] | 20 U/mL |
| Paromomycin | B.3[d] | — | — | 1 mg/mL | 21 | B.3[d] | 1 µg/mL |
| Penicillin G | B.1[d] | — | — | 1000 U/mL | 4 | B.1[d] | 1 U/mL |
| Polymyxin B[i] | Water | — | B.6[d] | 10,000 U/mL | 14 | B.6[d] | 10 U/mL |
| Vancomycin | Water | — | — | 1 mg/mL | 7 | B.4[d] | 10 µg/mL |

[a] It is acceptable to adjust the median concentration to optimize zone sizes if the data remain in the linear range.
[b] µg in this column refers to µg of activity.
[c] Prepare the USP Reference Standard and sample test dilutions simultaneously.
[d] The letter B refers to buffer. See *Buffers* for a description of each buffer listed in this table.
[e] Further dilute the stock solution with dimethyl sulfoxide to give concentrations of 12.8, 16, 20, 25, and 31.2 µg/mL before making the test dilutions. The test dilution of the sample should contain the same amount of dimethyl sulfoxide as the test dilutions of the USP Reference Standard.
[f] Each of the standard test dilutions should contain the same amount of hydrochloric acid as the test dilution of the sample.
[g] The turbidimetric assay can be used as an alternative procedure.
[h] Further dilute the stock solution with dimethylformamide to give concentrations of 256, 320, 400, 500, and 624 U/mL before making the test dilutions. Prepare the standard test dilutions simultaneously with test dilutions of the sample to be tested. The test dilution of the sample should contain the same amount of dimethylformamide as the test dilutions of the standard. Use low-actinic glassware.
[i] Prepare the stock solution by adding 2 mL of water for each 5 mg of the USP Reference Standard.

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## Inocula

Suspend the test organism from a freshly grown slant or culture in 3 mL of sterile saline TS. Glass beads can be used to facilitate the suspension. Spread the saline suspension onto the surface of two or more agar plates (covering the entire surface) or onto the surface of a Roux bottle containing 250 mL of the specified medium (see *Table 3*).

Incubate for the specified time and at the temperature as specified in *Table 3*, or until growth is apparent.

After incubation, harvest the organism from the plates or Roux bottle with approximately 50 mL of sterile saline TS (except use *Medium 34* for bleomycin; see *Media and Solutions*), using a sterile bent glass rod or sterile glass beads. Pipet the suspension into a sterile glass container. This is the harvest suspension.

To make the stock suspension, dilute an appropriate amount of the harvest suspension with sterile saline TS. Using the UV-Vis spectrophotometer, measure the percentage transmittance at 580 nm. The target value is approximately 25% transmittance at 580 nm. This value is used to standardize the harvest suspension volume added to the seed layer agar.

Starting with the suggested volumes indicated in *Table 3*, determine during method verification the proportions of stock suspension to be added to the inoculum medium that result in satisfactory zones of inhibition of approximately 14–16 mm in diameter for the median concentration of the Standard ($S_3$).

[NOTE—Zone sizes that are outside the 11–19-mm range are not desirable, because these contribute to assay variability.]

If the dilution percentage transmittance is above 25%, a ratio may be used to normalize the addition of organism to the seed layer. The normalization factor can be determined by dividing the percentage transmittance obtained from the dilution by 25. This ratio can then be multiplied by the suggested inoculum amount to obtain the volume (mL) of harvest suspension that needs to be added to the seed layer. Adjust the quantity of inoculum on a daily basis, if necessary, to obtain an optimum concentration–response relationship.

Alternatively, determine during method verification the proportion of harvest suspension to be incorporated into the inoculum, starting with the volumes indicated in *Table 3*, that result in satisfactory demarcation of the zones of inhibition of about 14–16 mm in diameter for the median concentration of the Standard ($S_3$) and giving a reproducible concentration–response relationship. Prepare the inoculum by adding a portion of stock suspension to a sufficient amount of agar medium that has been melted and cooled to 45°–50°. Swirl the mixture without creating bubbles in order to obtain a homogeneous suspension.

**Table 3**

| Antibiotic | Test Organism | ATCC[a] Number | Incubation Conditions | | | Suggested Inoculum Composition | |
|---|---|---|---|---|---|---|---|
| | | | Medium[b] | Temperature (°) | Time | Medium[b] | Amount (mL/100 mL) |
| Amoxicillin | *Kocuria rhizophila* | 9341 | 1 | 32–35 | 24 h | 11 | 0.5 |
| Amphotericin B | *Saccharomyces cerevisiae* | 9763 | 19 | 29–31 | 48 h | 19 | 1.0 |
| Bacitracin | *Micrococcus luteus* | 10240 | 1 | 32–35 | 24 h | 1 | 0.3 |
| Bleomycin | *Mycobacterium smegmatis* | 607 | 36 | 36–37.5 | 48 h | 35 | 1.0 |
| Cloxacillin | *Staphylococcus aureus* | 29737 | 1 | 32–35 | 24 h | 1 | 0.1 |
| Colistimethate | *Bordetella bronchiseptica* | 4617 | 1 | 32–35 | 24 h | 10 | 0.1 |
| Colistin | *Bordetella bronchiseptica* | 4617 | 1 | 32–35 | 24 h | 10 | 0.1 |
| Dihydrostreptomycin | *Bacillus subtilis* | 6633 | 32 | 32–35 | 5 days | 5 | As required |
| Erythromycin | *Kocuria rhizophila* | 9341 | 1 | 32–35 | 24 h | 11 | 1.5 |
| Gentamicin | *Staphylococcus epidermidis* | 12228 | 1 | 32–35 | 24 h | 11 | 0.03 |
| Nafcillin | *Staphylococcus aureus* | 29737 | 1 | 32–35 | 24 h | 1 | 0.3 |
| Neomycin | *Staphylococcus epidermidis* | 12228 | 1 | 32–35 | 24 h | 11 | 0.4 |
| Novobiocin | *Staphylococcus epidermidis* | 12228 | 1 | 32–35 | 24 h | 1 | 4.0 |
| Nystatin | *Saccharomyces Kudriavzevii* | 2601 | 19 | 29–31 | 48 h | 19 | 1.0 |
| Paromomycin | *Staphylococcus epidermidis* | 12228 | 1 | 32–35 | 24 h | 11 | 2.0 |
| Penicillin G | *Staphylococcus aureus* | 29737 | 1 | 32–35 | 24 h | 1 | 1.0 |

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**Table 3** *(continued)*

| Antibiotic | Test Organism | ATCC[a] Number | Incubation Conditions | | | Suggested Inoculum Composition | |
|---|---|---|---|---|---|---|---|
| | | | Medium[b] | Temperature (°) | Time | Medium[b] | Amount (mL/100 mL) |
| Polymyxin B | *Bordetella bronchiseptica* | 4617 | 1 | 32–35 | 24 h | 10 | 0.1 |
| Vancomycin | *Bacillus subtilis* | 6633 | 32 | 32–35 | 5 days | 8 | As required |

[a] American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110-2209 (www.atcc.org).
[b] See *Media*.

## Analysis

Prepare the base layer for the required number of assay Petri plates, using the medium and volume shown in *Table 4*. Allow it to harden into a smooth base layer of uniform depth. Prepare the appropriate amount of seed layer inoculum (see *Table 5*) as directed for the given antibiotic (see *Table 3*) with any adjustments made based on the preparatory trial analysis. Tilt the plate back and forth to spread the inoculum evenly over the base layer surface, and allow it to harden.

**Table 4. Base Layer**

| Antibiotic | Medium[a] | Target Volume (mL) |
|---|---|---|
| Amoxicillin | 11 | 21 |
| Amphotericin B[b] | — | — |
| Bleomycin | 35 | 10 |
| Colistimethate | 9 | 21 |
| Colistin | 9 | 21 |
| Dihydrostreptomycin | 5 | 21 |
| Erythromycin | 11 | 21 |
| Gentamicin | 11 | 21 |
| Neomycin | 11 | 21 |
| Nystatin[b] | — | — |
| Paromomycin | 11 | 21 |
| Polymyxin B | 9 | 21 |
| Vancomycin | 8 | 10 |
| All others | 2 | 21 |

[a] See *Media*.
[b] No base layer is used. [NOTE—The base layer may be warmed to facilitate a uniform seed layer.]

**Table 5. Seed Layer**

| Antibiotic | Medium[a] | Target Volume (mL) |
|---|---|---|
| Amphotericin B | Refer to *Table 3* | 8 |
| Bleomycin | | 6 |
| Nystatin | | 8 |
| All others | | 4 |

[a] See *Media*.

Drop 6 assay cylinders on the inoculated surface from a height of 12 mm, using a mechanical guide or other device to ensure even spacing on a radius of 2.8 cm, and cover the plates to avoid contamination. Fill the 6 cylinders on each plate with the same volume of dilutions of antibiotic containing the test levels ($S_1$–$S_5$ and $U_3$) specified in the following paragraph. Incubate the plates as specified in *Table 6* for 16–18 h, and remove the cylinders. Measure and record the diameter of each zone of growth inhibition to the nearest 0.1 mm.

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**Table 6**

| Antibiotic | Incubation Temperature (°) |
|---|---|
| Amphotericin B | 29–31 |
| Colistimethate | 36–37.5 |
| Colistin | 36–37.5 |
| Dihydrostreptomycin | 36–37.5 |
| Gentamicin | 36–37.5 |
| Neomycin | 36–37.5 |
| Novobiocin | 34–36 |
| Nystatin | 29–31 |
| Paromomycin | 36–37.5 |
| Polymyxin B | 36–37.5 |
| Vancomycin | 36–37.5 |
| All others | 32–35 |

The Standards ($S_1$–$S_5$) and a single test level of the sample ($U_3$) corresponding to $S_3$ of the standard curve, as defined in *Standard Solutions* and *Sample Solutions* will be used in the assay. For deriving the standard curve, fill alternate cylinders on each of 3 plates with the median test dilution ($S_3$) of the Standard and each of the remaining 9 cylinders with one of the other four test dilutions of the standard. Repeat the process for the three test dilutions of the Standard. For the sample, fill alternate cylinders on each of 3 plates with the median test dilution of the Standard ($S_3$), and fill the remaining 9 cylinders with the corresponding test dilution ($U_3$) of the sample.

## TURBIDIMETRIC METHOD

### Temperature Control

Use appropriately qualified and calibrated equipment to obtain the temperature ranges specified in *Table 8*. [NOTE—Temperature control can be achieved using either circulating air or water. The greater heat capacity of water lends it some advantage over circulating air.]

### Spectrophotometer

Measuring absorbance or transmittance within a fairly narrow frequency band requires a suitable spectrophotometer in which the wavelength can be varied or restricted by the use of 580- or 530-nm filters. Alternatively, a variable-wavelength spectrophotometer can be used and set to a wavelength of 580 or 530 nm.

The instrument may be modified as follows:

1. To accept the tube in which incubation takes place (see *Apparatus* below)
2. To accept a modified cell fitted with a drain that facilitates rapid change of contents
3. To contain a flow cell for a continuous flowthrough analysis

Autozero the instrument with clear, uninoculated broth prepared as specified for the particular antibiotic, including the same amount of test dilution (including formaldehyde if specified) as found in each sample.

Either absorbance or transmittance can be measured while preparing inocula.

### Apparatus

Use glass or plastic test tubes, e.g., 16 mm × 125 mm or 18 mm × 150 mm. [NOTE—Use tubes that are relatively uniform in length, diameter, and thickness and substantially free from surface blemishes and scratches. In the spectrophotometer, use matched tubes that are free from scratches or blemishes. Clean tubes thoroughly to remove all antibiotic residues and traces of cleaning solution. Sterilize tubes before use.]

### Standard Solutions

To prepare a stock solution, dissolve a quantity of the USP Reference Standard of a given antibiotic or the entire contents of a vial of USP Reference Standard, where appropriate, in the solvent specified in *Table 7*, and dilute to the required concentration. Store at 2°–8°, and use within the period indicated. On the day of the assay, prepare from the stock solution five or more test dilutions, the successive solutions increasing stepwise in concentration, usually in the ratio of 1:1.25. [NOTE—It may be necessary to use smaller ratios for the successive dilutions from the stock solution for the turbidimetric assay.] Use the final diluent specified such that the median level of the Standard ($S_3$) has the concentration suggested in *Table 7*.

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## Sample Solutions

Assign an assumed potency per unit weight or volume to the unknown, and on the day of the assay prepare a stock solution in the same manner specified for the USP Reference Standard (see *Table 7*). Dilute the sample stock solution in the specified final diluent at a nominal concentration equal to the median concentration of the Standard ($S_3$) as specified in *Table 7*.

**Table 7**

| Antibiotic | Stock Solution | | | | | Test Dilution | |
|---|---|---|---|---|---|---|---|
| | Initial Solvent | Initial Concentration | Further Diluent | Final Stock Concentration | Use Within (days) | Final Diluent | Median Concentration ($S_3$)[a] |
| Capreomycin | Water | — | — | 1 mg/mL | 7 | Water | 100 µg/mL |
| Chloramphenicol | Alcohol | 10 mg/mL | Water | 1 mg/mL | 30 | Water | 2.5 µg/mL |
| Chlortetracycline | 0.1 N hydrochloric acid | — | — | 1 mg/mL | 4 | Water | 0.06 µg/mL |
| Dihydrostreptomycin[b] | Water | — | — | 1 mg/mL | 30 | Water | 30 µg/mL |
| Gramicidin | Alcohol | — | — | 1 mg/mL | 30 | Alcohol | 0.04 µg/mL |
| Neomycin[b, c] | B.3[d] | — | — | 100 µg/mL | 14 | B.3[d] | 1.0 µg/mL |
| Oxytetracycline | 0.1 N hydrochloric acid | — | — | 1 mg/mL | 4 | Water | 0.24 µg/mL |
| Tetracycline | 0.1 N hydrochloric acid | — | — | 1 mg/mL | 1 | Water | 0.24 µg/mL |
| Thiostrepton | Dimethyl sulfoxide | — | — | 100 U/mL | Same day | Dimethyl sulfoxide | 0.80 U/mL |
| Tylosin | Methanol | 10 mg/mL | B.16[d] | 1 mg/mL | 30 | Methanol and B.3[d] (1:1) | 4 µg/mL |

[a] µg in this column refers to µg of activity.
[b] The cylinder-plate assay can be used as an alternative procedure.
[c] Dilute the 100-µg/mL stock solution with *Buffer B.3* to obtain a solution having a concentration equivalent to 25 µg/mL of neomycin. To separate 50-mL volumetric flasks add 1.39, 1.67, 2.00, 2.40, and 2.88 mL of this solution. Add 5.0 mL of 0.1 N hydrochloric acid to each flask, dilute with *Buffer B.3* to volume, and mix to obtain solutions having concentrations of 0.69, 0.83, 1.0, 1.2, and 1.44 µg/mL of neomycin. Use these solutions to prepare the standard response line.
[d] The letter B refers to buffer. See *Buffers* for a description of each buffer listed in this table.

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## Inocula

Suspend the test organism from a freshly grown slant or culture in 3 mL of sterile saline TS. Glass beads can be used to facilitate the suspension. *Enterococcus hirae* (ATCC 10541) (when used for the gramicidin assay), and *Staphylococcus aureus* (ATCC 9144) are grown in a liquid medium, not on agar. Spread the saline suspension onto the surface of two or more agar plates (covering the entire surface) or onto the surface of a Roux bottle containing 250 mL of the specified medium (see *Table 8*). Incubate at the time and temperature specified in *Table 8*, or until growth is apparent.

After incubation, harvest the organism from the plates or Roux bottle with approximately 50 mL of sterile saline TS, using a sterile bent glass rod or sterile glass beads. Pipet the suspension into a sterile glass bottle. This is the harvest suspension.

Determine during method verification the quantity of harvest suspension that will be used as the inoculum, starting with the volume suggested in *Table 8*. Prepare also an extra Standard ($S_3$) as a test of growth. Incubate the trial tests for the times indicated in *Table 11*. Adjust the quantity of inoculum daily, if necessary, to obtain the optimum concentration–response relationship from the amount of growth of the test organism in the assay tubes. At the completion of the specified incubation periods, tubes containing the median concentration of the Standard should have absorbance values as specified in *Table 9*. Determine the exact duration of incubation by observing the growth in the reference concentration (median concentration) of the standard ($S_3$).

**Table 8**

| Antibiotic | Test Organism | ATCC[a] Number | Incubation Conditions | | | Suggested Inoculum Composition | |
|---|---|---|---|---|---|---|---|
| | | | Medium[b] | Temperature (°) | Time (h) | Medium[b] | Amount (mL/100 mL) |
| Capreomycin | *Klebsiella pneumoniae* | 10031 | 1 | 36–37.5 | 16–24 | 3 | 0.05 |
| Chloramphenicol | *Escherichia coli* | 10536 | 1 | 32–35 | 24 | 3 | 0.7 |
| Chlortetracycline | *Staphylococcus aureus* | 29737 | 1 | 32–35 | 24 | 3 | 0.1 |

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**Table 8** *(continued)*

| Antibiotic | Test Organism | ATCC[a] Number | Incubation Conditions | | | Suggested Inoculum Composition | |
|---|---|---|---|---|---|---|---|
| | | | Medium[b] | Temperature (°) | Time (h) | Medium[b] | Amount (mL/100 mL) |
| Dihydrostrepto-mycin | *Klebsiella pneumo-niae* | 10031 | 1 | 36–37.5 | 16–24 | 3 | 0.1 |
| Gramicidin | *Enterococcus hirae* | 10541 | 3 | 36–37.5 | 16–18 | 3 | 1.0 |
| Neomycin | *Klebsiella pneumo-niae* | 10031 | 1 | 36–37.5 | 16–24 | 39 | 2 |
| Oxytetracycline | *Staphylococcus aureus* | 29737 | 1 | 32–35 | 24 | 3 | 0.1 |
| Tetracycline | *Staphylococcus aureus* | 29737 | 1 | 32–35 | 24 | 3 | 0.1 |
| Thiostrepton | *Enterococcus hirae* | 10541 | 40 | 36–37.5 | 18–24 | 41 | 0.2 |
| Tylosin | *Staphylococcus aureus* | 9144 | 3 | 35–39 | 16–18 | 39 | 2–3 |

[a] American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110-2209 (www.atcc.org).
[b] See *Media*.

**Table 9**

| Antibiotic | Absorbance, NLT (a.u.) |
|---|---|
| Capreomycin | 0.4 |
| Chlortetracycline | 0.35 |
| Gramicidin | 0.35 |
| Tetracycline | 0.35 |
| All others | 0.3 |

## Analysis

On the day of the assay, prepare the necessary concentration of antibiotic by dilution of stock solutions of the Standard and of each sample as specified under *Standard Solutions* and *Sample Solutions*. Prepare 5 test levels, each in triplicate, of the Standard ($S_1$–$S_5$) and a single test level ($U_3$), also in triplicate, of up to 20 samples corresponding to $S_3$ (median concentration) of the Standard.

Place the tubes in test tube racks or other carriers. Include in each rack 1–2 control tubes containing 0.1 mL of the test diluent for gramicidin, thiostrepton, and tylosin, or 1 mL of the test diluent for all others (see *Table 7*), but no antibiotic. Add the volumes of the Standard and sample test dilutions as indicated in *Table 10*. Randomly distribute one complete set, including the controls, in a tube rack. Add the volume of inoculum specified in *Table 10* to each tube in the rack in turn, and place the completed rack immediately in an incubator or a water bath maintained at 36.0°–37.5° for the time specified in *Table 11*.

**Table 10**

| Antibiotic | Volume of Test Dilution (mL) | Volume of Inoculum (mL) |
|---|---|---|
| Gramicidin | 0.10 | 9.0 |
| Thiostrepton | 0.10 | 10.0 |
| Tylosin | 0.10 | 9.0 |
| All others | 1.0 | 9.0 |

**Table 11**

| Antibiotic | Incubation Time (h) |
|---|---|
| Capreomycin | 3–4 |
| Chloramphenicol | 3–4 |
| Cycloserine | 3–4 |
| Dihydrostreptomycin | 3–4 |
| Tylosin | 3–5 |

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**Table 11** *(continued)*

| Antibiotic | Incubation Time (h) |
|---|---|
| All others | 4–5 |

After incubation, immediately inhibit the growth of the organism by adding 0.5 mL of dilute formaldehyde to each tube, except for tylosin. For tylosin, heat the rack in a water bath at 80°–90° for 2–6 min or in a steam bath for 5–10 min, and bring to room temperature. Read absorbance or transmittance at 530 or 580 nm, analyzing one rack at a time.

## MEDIA AND SOLUTIONS

The media required for the preparation of test organism inocula are made from the ingredients listed herein. Minor modifications of the individual ingredients are acceptable; and reconstituted dehydrated media can be substituted, provided that the resulting media possess equal or better growth-promoting properties and give a similar standard curve response.

### Media

Dissolve the ingredients in water to make 1 L, and adjust the solutions with either 1 N sodium hydroxide or 1 N hydrochloric acid as required, so that after steam sterilization the pH is as specified.

**Medium 1**

| | |
|---|---|
| Peptone | 6.0 g |
| Pancreatic digest of casein | 4.0 g |
| Yeast extract | 3.0 g |
| Beef extract | 1.5 g |
| Dextrose | 1.0 g |
| Agar | 15.0 g |
| Water | 1000 mL |
| pH after sterilization | 6.6 ± 0.1 |

**Medium 2**

| | |
|---|---|
| Peptone | 6.0 g |
| Yeast extract | 3.0 g |
| Beef extract | 1.5 g |
| Agar | 15.0 g |
| Water | 1000 mL |
| pH after sterilization | 6.6 ± 0.1 |

**Medium 3**

| | |
|---|---|
| Peptone | 5.0 g |
| Yeast extract | 1.5 g |
| Beef extract | 1.5 g |
| Sodium chloride | 3.5 g |
| Dextrose | 1.0 g |
| Dibasic potassium phosphate | 3.68 g |
| Monobasic potassium phosphate | 1.32 g |
| Water | 1000 mL |
| pH after sterilization | 7.0 ± 0.05 |

**Medium 4**

| | |
|---|---|
| Peptone | 6.0 g |
| Yeast extract | 3.0 g |
| Beef extract | 1.5 g |
| Dextrose | 1.0 g |

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**Medium 4** *(continued)*

| | |
|---|---|
| Agar | 15.0 g |
| Water | 1000 mL |
| pH after sterilization | 6.6 ± 0.1 |

**Medium 5**

| | |
|---|---|
| Peptone | 6.0 g |
| Yeast extract | 3.0 g |
| Beef extract | 1.5 g |
| Agar | 15.0 g |
| Water | 1000 mL |
| pH after sterilization | 7.9 ± 0.1 |

**Medium 8**

| | |
|---|---|
| Peptone | 6.0 g |
| Yeast extract | 3.0 g |
| Beef extract | 1.5 g |
| Agar | 15.0 g |
| Water | 1000 mL |
| pH after sterilization | 5.9 ± 0.1 |

**Medium 9**

| | |
|---|---|
| Pancreatic digest of casein | 17.0 g |
| Papaic digest of soybean | 3.0 g |
| Sodium chloride | 5.0 g |
| Dibasic potassium phosphate | 2.5 g |
| Dextrose | 2.5 g |
| Agar | 20.0 g |
| Water | 1000 mL |
| pH after sterilization | 7.2 ± 0.1 |

**Medium 10**

| | |
|---|---|
| Pancreatic digest of casein | 17.0 g |
| Papaic digest of soybean | 3.0 g |
| Sodium chloride | 5.0 g |
| Dibasic potassium phosphate | 2.5 g |
| Dextrose | 2.5 g |
| Agar | 12.0 g |
| Water | 1000 mL |
| Polysorbate 80 (added after boiling the medium to dissolve the agar) | 10 mL |
| pH after sterilization | 7.2 ± 0.1 |

**Medium 11**

| | |
|---|---|
| Peptone | 6.0 g |
| Pancreatic digest of casein | 4.0 g |
| Yeast extract | 3.0 g |
| Beef extract | 1.5 g |
| Dextrose | 1.0 g |
| Agar | 15.0 g |
| Water | 1000 mL |

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**Medium 11** *(continued)*

| | |
|---|---|
| pH after sterilization | 8.3 ± 0.1 |

**Medium 13**

| | |
|---|---|
| Peptone | 10.0 g |
| Dextrose | 20.0 g |
| Water | 1000 mL |
| pH after sterilization | 5.6 ± 0.1 |

**Medium 19**

| | |
|---|---|
| Peptone | 9.4 g |
| Yeast extract | 4.7 g |
| Beef extract | 2.4 g |
| Sodium chloride | 10.0 g |
| Dextrose | 10.0 g |
| Agar | 23.5 g |
| Water | 1000 mL |
| pH after sterilization | 6.1 ± 0.1 |

**Medium 32**

| | |
|---|---|
| Peptone | 6.0 g |
| Pancreatic digest of casein | 4.0 g |
| Yeast extract | 3.0 g |
| Beef extract | 1.5 g |
| Manganese sulfate | 0.3 g |
| Dextrose | 1.0 g |
| Agar | 15.0 g |
| Water | 1000 mL |
| pH after sterilization | 6.6 ± 0.1 |

**Medium 34**

| | |
|---|---|
| Glycerol | 10.0 g |
| Peptone | 10.0 g |
| Beef extract | 10.0 g |
| Sodium chloride | 3.0 g |
| Water | 1000 mL |
| pH after sterilization | 7.0 ± 0.1 |

**Medium 35**

| | |
|---|---|
| Glycerol | 10.0 g |
| Peptone | 10.0 g |
| Beef extract | 10.0 g |
| Sodium chloride | 3.0 g |
| Agar | 17.0 g |
| Water | 1000 mL |
| pH after sterilization | 7.0 ± 0.1 |

**Medium 36**

| | |
|---|---|
| Pancreatic digest of casein | 15.0 g |
| Papaic digest of soybean | 5.0 g |
| Sodium chloride | 5.0 g |

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**Medium 36** *(continued)*

| | |
|---|---|
| Agar | 15.0 g |
| Water | 1000 mL |
| pH after sterilization | 7.3 ± 0.1 |

**Medium 39**

| | |
|---|---|
| Peptone | 5.0 g |
| Yeast extract | 1.5 g |
| Beef extract | 1.5 g |
| Sodium chloride | 3.5 g |
| Dextrose | 1.0 g |
| Dibasic potassium phosphate | 3.68 g |
| Monobasic potassium phosphate | 1.32 g |
| Water | 1000 mL |
| pH after sterilization | 7.9 ± 0.1 |

**Medium 40**

| | |
|---|---|
| Yeast extract | 20.0 g |
| Polypeptone | 5.0 g |
| Dextrose | 10.0 g |
| Monobasic potassium phosphate | 2.0 g |
| Polysorbate 80 | 0.1 g |
| Agar | 10.0 g |
| Water | 1000 mL |
| pH after sterilization | 6.7 ± 0.2 |

**Medium 41**

| | |
|---|---|
| Pancreatic digest of casein | 9.0 g |
| Dextrose | 20.0 g |
| Yeast extract | 5.0 g |
| Sodium citrate | 10.0 g |
| Monobasic potassium phosphate | 1.0 g |
| Dibasic potassium phosphate | 1.0 g |
| Water | 1000 mL |
| pH after sterilization | 6.8 ± 0.1 |

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## Solutions

### BUFFERS

Prepare as directed in *Table 12*, or by other suitable means. The buffers are sterilized after preparation; the pH specified in each case is the pH after sterilization.

**Table 12. Buffers**

| Buffer | **Concentration of** Dibasic Potassium Phosphate **(g/L)** | **Concentration of** Monobasic Potassium Phosphate **(g/L)** | **Volume of** 10 N Potassium Hydroxide TS **(mL)** | **pH after Sterilization**[a] |
|---|---|---|---|---|
| Buffer B.1 (1%, pH 6.0) | 2 | 8 | — | 6.0 ± 0.05 |
| Buffer B.3 (0.1 M, pH 8.0) | 16.73 | 0.523 | — | 8.0 ± 0.1 |
| Buffer B.4 (0.1 M, pH 4.5) | — | 13.61 | — | 4.5 ± 0.05 |
| Buffer B.6 (10%, pH 6.0) | 20 | 80 | — | 6.0 ± 0.05 |
| Buffer B.10 (0.2 M, pH 10.5) | 35 | — | 2 | 10.5 ± 0.1 |

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**Table 12. Buffers** *(continued)*

| Buffer | **Concentration of** Dibasic Potassium Phosphate **(g/L)** | **Concentration of** Monobasic Potassium Phosphate **(g/L)** | **Volume of** 10 N Potassium Hydroxide TS **(mL)** | **pH after Sterilization**[a] |
|---|---|---|---|---|
| Buffer B.16 (0.1 M, pH 7.0) | 13.6 | 4 | — | 7.0 ± 0.2 |

[a] Adjust the pH with 18 N phosphoric acid or 10 N Potassium Hydroxide TS.

**Other solutions:** See *Reagents, Indicators, and Solutions*.
**Water:** Use *Purified Water*.
**Saline:** Use saline TS.
**Dilute formaldehyde:** Formaldehyde solution and water (1:3)

# CALCULATIONS

## Introduction

Antibiotic potency is calculated by interpolation from a standard curve using a log-transformed straight-line method with a least-squares fitting procedure (see below for calculation details). The analyst must consider three essential concepts in interpreting antibiotic potency results:

1. Biological concentration–response relationships generally are not linear. The antibiotic potency method allows fitting the data to a straight line by evaluating a narrow concentration range where the results approach linearity. The assay results can be considered valid only if the computed potency is 80%–125% of that assumed in preparing the sample stock solution. When the calculated potency value falls outside 80%–125%, the result for the sample may fall outside the narrow concentration range where linearity has been established. In such a case, adjust the assumed potency of the sample accordingly, and repeat the assay to obtain a valid result.
2. The most effective means of reducing the variability of the reportable value (the geometric mean potency across runs and replicates) is through independent runs of the assay procedure. The combined result of a series of smaller, independent assays is a more reliable estimate of potency than that from a single large assay with the same total number of plates or tubes. Three or more independent assays are required for antibiotic potency determinations.
3. The number of assays needed in order to obtain a reliable estimate of antibiotic potency depends on the required specification range and the assay variability. The confidence limit calculation described below is determined from several estimated log potencies that are approximately equal in precision. If the value calculated for the half-width of the confidence interval, *W*, is too wide, no useful decision can be made about whether the potency meets its specification.

The laboratory should predetermine in its standard operating procedures a maximum acceptable value for the confidence interval half-width. This maximum value should be determined during development and confirmed during validation or verification. If the calculated confidence interval half-width exceeds this limit, the analyst must perform additional independent potency determinations to meet the limit requirement. Note that the decision to perform additional determinations does not depend on the estimated potency but only on the uncertainty in that estimate as determined by the confidence interval half-width. *Assay* variability has a greater impact on the calculated confidence limit than does the number of independent potency determinations. As a result, the analyst should first consider decreasing variability to the extent possible before conducting potency determinations.

The following sections describe the calculations for determining antibiotic potency as well as for performing the confidence limit calculation. Methods for calculating standard error are also shown in order to allow estimates of assay variance. Where logarithms are used, any base log is acceptable. *Appendix 1: Formulas for Manual Calculations of Regression and Sample Concentration* provides formulas for hand calculations applicable when the concentrations are equally spaced in the log scale. Alternative statistical methods may be used if appropriately validated.

## Cylinder-Plate Assay

This section details analysis of the sample data and determination of the potency of an unknown, using the cylinder-plate assay.

### SAMPLE DATA

*Table 13* shows the data from one assay that will be used as an example throughout this section. For each of the 15 plates, zones 1, 3, and 5 are for the reference concentration, ($S_3$), and the other three zones are for one of the other four standard concentrations, ($S_1$, $S_2$, $S_4$, and $S_5$) or the sample ($U_3$), as shown. Other columns are needed for calculations and are explained below.

**Step 1:** Perform initial calculations and variability suitability check.

For each set of 3 plates, average the 9 reference values and average the 9 Standard values.

*Example:* See *Table 13*.

$$15.867 = \overline{X}(16.1, 15.6, \ldots, 15.8)$$
$$14.167 = \overline{X}(14.6, 14.1, \ldots, 14.8)$$

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For each set of 3 plates, determine the standard deviation of the 9 reference values and the standard deviation of the 9 Standard values. For each standard deviation, determine the corresponding relative standard deviation.

*Example:* See *Table 13*.

$$0.200 = \sigma(16.1, \ldots, 15.8)$$
$$1.3\% = (0.200/15.867) \times 100$$
$$0.324 = \sigma(14.6, \ldots, 14.1)$$
$$2.3\% = (0.324/14.167) \times 100$$

For a variability suitability criterion, each laboratory should determine a maximum acceptable value for the relative standard deviation. If any of the eight relative standard deviations (four for the reference and four for the Standard) exceed this predetermined maximum, the assay data are not suitable and should be discarded. [NOTE—The suggested limit for relative standard deviation is NMT 10%.]

**Step 2:** Perform a plate-to-plate variation correction.

This correction is applied to convert the average zone measurement obtained for each concentration to the value it would be if the average reference concentration measurement for that set of 3 replicate plates were the same as the value of the correction point:

$$\overline{X}_C = \overline{X}_S - (\overline{X}_R - P)$$

$\overline{X}_C$ = corrected standard mean
$\overline{X}_S$ = original standard mean
$\overline{X}_R$ = reference mean
$P$ = correction point

*Example:* For the first set of 3 plates in *Table 13* ($S_1$), the correction is:

$$14.022 = 14.167 - (15.867 - 15.722) = 14.167 - 0.145$$

**Step 3:** Determine the standard curve line.

Generate the standard curve line by plotting the corrected zone measurements versus the log of the standard concentration values. Calculate the equation of the standard curve line by performing a standard unweighted linear regression on these values, using appropriate software or the manual calculations of *Appendix 1: Formulas for Manual Calculations of Regression and Sample Concentration*. [NOTE—Use either the natural log or the base 10 log to plot the standard curve and determine the regression equation; both provide the same final test result.] Each laboratory should determine a minimum value of the coefficient of determination (%$R^2$) for an acceptable regression. The regression is acceptable only if the obtained %$R^2$ exceeds this predetermined value. [NOTE—The suggested limit for the percentage coefficient of determination is NLT 95%.]

**Table 13. Sample Data (Cylinder-Plate Assay)**

| Standard | Concentration (U/mL) | Plate Replicate | Reference ($S_3$) Zone 1 (mm) | Zone 3 (mm) | Zone 5 (mm) | Mean (mm) | SD | %RSD | Standard Zone 2 (mm) | Zone 4 (mm) | Zone 6 (mm) | Mean (mm) | SD | %RSD | Corrected Mean (mm) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 16.1 | 15.6 | 15.8 | | | | 14.6 | 14.1 | 13.5 | | | | |
| | | 2 | 16.0 | 15.9 | 16.2 | | | | 14.5 | 14.1 | 14.4 | | | | |
| $S_1$ | 3.20 | 3 | 15.7 | 15.7 | 15.8 | 15.867 | 0.200 | 1.3 | 14.0 | 14.2 | 14.1 | 14.167 | 0.324 | 2.3 | 14.022 |
| | | 1 | 15.8 | 15.6 | 15.5 | | | | 14.7 | 15.1 | 14.8 | | | | |
| | | 2 | 15.7 | 15.5 | 15.6 | | | | 14.7 | 14.9 | 15.2 | | | | |
| $S_2$ | 4.00 | 3 | 15.7 | 15.4 | 15.3 | 15.567 | 0.158 | 1.0 | 14.8 | 15.0 | 14.3 | 14.833 | 0.265 | 1.8 | 14.989 |
| | | 1 | 15.6 | 15.8 | 16.0 | | | | 16.6 | 16.8 | 16.3 | | | | |
| | | 2 | 15.8 | 15.6 | 15.7 | | | | 16.6 | 16.5 | 16.2 | | | | |
| $S_4$ | 6.25 | 3 | 16.1 | 15.7 | 15.8 | 15.789 | 0.169 | 1.1 | 16.9 | 16.5 | 16.8 | 16.578 | 0.233 | 1.4 | 16.511 |
| | | 1 | 15.6 | 15.6 | 15.5 | | | | 17.3 | 17.0 | 17.0 | | | | |
| | | 2 | 15.6 | 15.7 | 15.5 | | | | 17.3 | 17.4 | 17.2 | | | | |
| $S_5$ | 7.8125 | 3 | 15.9 | 15.8 | 15.8 | 15.667 | 0.141 | 0.9 | 17.3 | 17.3 | 16.7 | 17.167 | 0.224 | 1.3 | 17.222 |
| | | | | | | 15.722[a] | | | | | | | | | |
| Sample | | | Reference ($S_3$) | | | | | | Sample | | | | | | |
| | | 1 | 15.7 | 15.8 | 15.7 | | | | 15.3 | 15.8 | 15.7 | | | | |
| | | 2 | 15.9 | 15.7 | 15.7 | | | | 15.8 | 15.8 | 15.5 | | | | |
| $U_3$ | Unknown | 3 | 15.5 | 15.8 | 15.3 | 15.678 | 0.179 | 1.1 | 15.2 | 15.1 | 15.1 | 15.478 | 0.307 | 2.0 | 15.522 |

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[a] This is the value of the overall reference mean, referred to as the "correction point" below.

*Example:* *Table 14* summarizes the portion of *Table 13* needed for this part of the calculation.

**Table 14**

| Standard Set | Corrected Zone Measurements (mm) | Concentration (U/mL) |
|---|---|---|
| $S_1$ | 14.022 | 3.2 |
| $S_2$ | 14.989 | 4.0 |
| Reference ($S_3$) | 15.722 | 5 |
| $S_4$ | 16.511 | 6.25 |
| $S_5$ | 17.222 | 7.8125 |

LINEAR REGRESSION RESULTS

**Standard curve line:**

$$Z = [3.551 \times \ln(C)] + 9.978$$

$Z$ = corrected zone measurement
$C$ = concentration
$\%R^2$ = 99.7

SAMPLE POTENCY DETERMINATION

To estimate the potency of the unknown sample, average the zone measurements of the Standard and the zone measurements of the sample on the 3 plates used. Correct for plate-to-plate variation using the correction point determined above to obtain a corrected average for the unknown, $\overline{U}$. [NOTE—An acceptable alternative to using the correction point is to correct using the value on the estimated regression line corresponding to the log concentration of $S_3$.] Use the corrected average zone measurement in the equation of the standard curve line to determine the log concentration of the sample, $L_U$, by:

$$L_U = (\overline{U} - a)/b$$

$a$ = intercept of the regression line
$b$ = slope of the regression line

To obtain the potency of the unknown, take the antilog of $L_U$ and multiply the result by any applicable dilution factor. This value can also be expressed as a percentage of the reference concentration value.

**Example:** Corrected sample zone measurement (see *Table 13*) = 15.522

Natural log of the sample concentration:

$$L_U = (15.522 - 9.978)/3.551 = 1.561$$

Sample concentration:

$$C_U = e^{1.561} = 4.765$$

Percentage of reference concentration:

$$\text{Result} = (4.765/5.000) \times 100 = 95.3\%$$

## Turbidimetric Assay

This section details analysis of the sample data and determination of the potency of an unknown using the turbidimetric assay. The method assumes that the tubes are randomly distributed within the heat block or other temperature control device. If the device has a temperature profile that is not uniform, a randomized blocks design is preferred. In such a design, the rack is divided into areas ("blocks") of relatively uniform temperature and at least one tube of each Standard concentration and of each unknown is placed in each area. The data analysis of a randomized block design is different from the following.

SAMPLE DATA

*Table 15* shows the data from one assay that will be used for an example throughout this section. Other columns are needed for calculations and are explained below.

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**Table 15. Sample Data (Turbidimetric Assay)**

| Standard | Concentration (µg/mL) | Replicate | Absorbance (a.u.) | Average (a.u.) | Standard Deviation |
|---|---|---|---|---|---|
| | | 1 | 0.8545 | | |
| | | 2 | 0.8422 | | |
| $S_1$ | 64 | 3 | 0.8495 | 0.8487 | 0.0062 |
| | | 1 | 0.8142 | | |
| | | 2 | 0.8273 | | |
| $S_2$ | 80 | 3 | 0.8392 | 0.8269 | 0.0125 |
| | | 1 | 0.6284 | | |
| | | 2 | 0.6947 | | |
| $S_3$ | 100 | 3 | 0.7563 | 0.6931 | 0.0640 |
| | | 1 | 0.6933 | | |
| | | 2 | 0.6850 | | |
| $S_4$ | 125 | 3 | 0.6699 | 0.6827 | 0.0119 |
| | | 1 | 0.5299 | | |
| | | 2 | 0.5779 | | |
| $S_5$ | 156 | 3 | 0.5316 | 0.5465 | 0.0272 |
| | | 1 | 0.7130 | | |
| | | 2 | 0.7960 | | |
| $U_3$ | Unknown | 3 | 0.7201 | 0.7430 | 0.0460 |

**Step 1:** Perform initial calculations and variability suitability check.

For each concentration (including the sample), average the three absorbance values.

*Example:* See $S_1$ in *Table 15*.

$$0.8487 = \bar{X}(0.8545, 0.8422, 0.8495)$$

For each concentration, determine the standard deviation of the three readings and a combined standard deviation for all the concentrations.

*Example:* See $S_1$ in *Table 15*.

$$0.0062 = SD(0.8545, 0.8422, 0.8495)$$

The combined value is calculated by taking the square root of the average of the five variances:

$$0.0322 = \{[(0.0062)^2 + (0.0125)^2 + (0.0640)^2 + (0.0119)^2 + (0.0272)^2]/5\}^{1/2}$$

For a variability suitability criterion, each laboratory should determine a maximum acceptable combined standard deviation. If the combined standard deviation exceeds this predetermined maximum, the assay data are not suitable and should be discarded. [NOTE—The suggested limit for the combined standard deviation is NMT 10% of the average absorbance value across the five concentrations.] If the number of replicates per concentration is at least 5, then a relative standard deviation can be computed for each concentration after checking for outliers and compared to a maximum acceptable relative standard deviation. [NOTE—The suggested limit for the relative standard deviation is NMT 10%.]

**Step 2:** Determine the standard curve line.

Generate the standard curve line by plotting the average absorbance values versus the log of the standard concentration values. Calculate the equation of the standard curve line by performing an unweighted linear regression on these values using appropriate software or the manual calculations of *Appendix 1: Formulas for Manual Calculations of Regression and Sample Concentration*. [NOTE—Use either the natural log or the base 10 log to plot the standard curve and determine the regression equation; both provide the same final test result.] Each laboratory should determine a minimum value of the percentage coefficient of determination (%$R^2$) for an acceptable regression. The regression is acceptable only if the %$R^2$ value obtained exceeds this predetermined value. [NOTE—The suggested limit for the percentage coefficient of determination is NLT 90%.]

*Example: Table 16* summarizes the portion of *Table 15* needed for this part of the calculation.

**Table 16**

| Set of Standards | Average Absorbance Values (a.u.) | Concentration (µg/mL) |
|---|---|---|
| $S_1$ | 0.8487 | 64 |

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**Table 16** *(continued)*

| Set of Standards | Average Absorbance Values (a.u.) | Concentration (µg/mL) |
|---|---|---|
| $S_2$ | 0.8269 | 80 |
| $S_3$ | 0.6931 | 100 |
| $S_4$ | 0.6827 | 125 |
| $S_5$ | 0.5465 | 156 |

LINEAR REGRESSION RESULTS

**Standard curve line:**

$$\text{Absorbance} = 2.2665 - [0.7735 \times \log_{10}(\text{concentration})]$$

$\%R^2$ = 93.0%

SAMPLE POTENCY DETERMINATION

To estimate the potency of the unknown sample, average the three absorbance measurements to obtain an average for the unknown, $\overline{U}$. Use this average measurement in the equation of the standard curve line to determine the log concentration of the unknown sample, $L_U$, by:

$$L_U = (\overline{U} - a)/b$$

$a$ = intercept of the regression line
$b$ = slope of the regression line

To obtain the potency of the unknown, take the antilog of $L_U$ and multiply the result by any applicable dilution factor. This value can also be expressed as a percentage of the reference concentration value.
**Example:** Average sample absorbance (see *Table 15*) = 0.7430.

$$\log_{10}(C_U) = (0.7430 - 2.2665)/(-0.7735) = 1.9696$$
$$C_U = 10^{1.9696} = 93.2$$
$$\text{Percentage of reference concentration} = (93.2/100.0) \times 100 = 93.2\%$$

$C_U$ = concentration of the sample

## Confidence Limits and Combination of Assays Calculations

Because of interassay variability, three or more independent determinations are required for a reliable estimate of the sample potency. For each independent determination, start with separately prepared stock solutions and test dilutions of both the Standard and the sample, and repeat the assay of a given sample.

Given a set of at least three determinations of the unknown potency, use the method of *Appendix 2: Procedure for Checking for Outliers; Rejection of Outlying or Aberrant Measurements* to check for any outlier values. This determination should be done in the log scale.

To obtain a combined estimate of the unknown potency, calculate the average, $M$, and the standard deviation of the accepted log potencies. [NOTE—Use either the natural log or the base 10 log.] Determine the confidence interval for the potency as follows:

$$\text{antilog}[M - t(0.05, N - 1) \times SD/\sqrt{N}],\ \text{antilog}[M + t(0.05, N - 1) \times SD/\sqrt{N}]$$

$M$ = average
$t(0.05, N–1)$ = the two-sided 5% point of a Student's $t$-distribution with $N$–1 degrees of freedom
[NOTE—The $t$ value is available in spreadsheets, statistics texts, and statistics software.]
$SD$ = standard deviation
$N$ = number of assays

$$W = \text{antilog}\{[t(0.05, N - 1) \times SD/\sqrt{N}]\}$$

$W$ = half-width of the confidence interval

Compare the half-width of the confidence interval to a predetermined maximum acceptable value. If the half-width is larger than the acceptance limit, continue with additional assays.

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EXAMPLE

Suppose the sample is assayed four times, with potency results in the natural log scale of 1.561, 1.444, 1.517, and 1.535. Then:

$$N = 4$$
$$M = \overline{X}(1.561, 1.444, 1.517, 1.535) = 1.514$$
$$SD = \sigma(1.561, 1.444, 1.517, 1.535) = 0.050$$
$$t = 3.182$$

The confidence interval in the log scale is

$$1.514 \pm (3.182 \times 0.050/\sqrt{4}) = (1.434, 1.594)$$

Taking antilogs, the estimated potency is

$$e^{1.514} = 4.546$$

with a 95% confidence interval for the potency of $e^{1.434}$, $e^{1.594}$ = (4.197, 4.924).

The half-width of the confidence interval to compare to an acceptance value is the ratio 4.924/4.546 = 1.083.

***Change to read:***

# APPENDICES

## Appendix 1: Formulas for Manual Calculations of Regression and Sample Concentration

If the concentrations are equally spaced in the logarithmic scale, the calculations can be performed using the following formula. Let:

$\overline{S}_k$ = mean corrected zone measurement (cylinder-plate assay) or average absorbance value (turbidimetric assay) for standard set $k$

$k$ = 1, 2, 3, 4, 5

$\overline{S}$ = mean of the five $\overline{S}_k$ values

$L_k$ = logarithm of the $k$th concentration. [NOTE—Use either the natural log or the base 10 log. Slope of the regression line is calculated by:]

$$b = (Y_{high} - Y_{low})/(X_{high} - X_{low})$$
$$Y_{high} = \tfrac{1}{5}(3\overline{S}_5 + 2\overline{S}_4 + \overline{S}_3 - \overline{S}_1)$$
$$Y_{low} = \tfrac{1}{5}(3\overline{S}_1 + 2\overline{S}_2 + \overline{S}_3 - \overline{S}_5)$$

$X_{high}$ = $L_5$

$X_{low}$ = $L_1$

Combine and simplify to:

$$b = (4\overline{S}_5 + 2\overline{S}_4 - 2\overline{S}_2 - 4\overline{S}_1)/[5(L_5 - L_1)]$$

The log of the concentration of the sample is found using:

$$L_U = L_{reference} + [(\overline{U} - \overline{S})/b]$$

For example, using the data for the cylinder-plate assay in *Table 13* and natural logarithms:

$$b = [(4 \times 17.222) + (2 \times 16.511) - (2 \times 14.989) - (4 \times \text{▲}14.022\text{▲}_{(ERR\ 1\text{-}Jan\text{-}2024)})]/\{5[\ln(7.81) - \ln(3.2)]\} = 3.551$$
$$\overline{S} = (\text{▲}14.022\text{▲}_{(ERR\ 1\text{-}Jan\text{-}2024)} + 14.989 + 15.722 + 16.511 + 17.222)/5 = 15.693$$
$$\text{Natural log of sample concentration} = \ln(5) + [(15.522 - 15.693)/3.551] = 1.561$$
$$\text{Sample concentration} = e^{1.561} = 4.765$$

## Appendix 2: Procedure for Checking for Outliers—Rejection of Outlying or Aberrant Measurements

A measurement that is clearly questionable because of a failure in the assay procedure should be rejected, whether it is discovered during the measuring or tabulation procedure. The arbitrary rejection or retention of an apparently aberrant measurement can be a serious source of bias. In general, the rejection of measurements solely on the basis of their relative magnitudes is a procedure that should be used sparingly.

Each suspected potency measurement, or outlier, may be tested against the following criterion. This criterion is based on the variation within a single group of supposedly equivalent measurements from a normal distribution. At a confidence level

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of 99%, a valid observation will be rejected once in 100 trials (when the suspected outlier can occur at only one end) or once in 50 trials (when the suspected outlier can occur at either end), provided that relatively few, if any, responses within the group are identical. Arrange the responses in order of magnitude from $y_1$ to $y_N$, where $N$ is the number of observations in the group. Compute the relative gap by using *Table A2-1*, and the formulas below:

When $N$ = 3–7:

$$G_1 = (y_2 - y_1)/(y_N - y_1) \text{ Candidate Outlier is Smallest } (y_1)$$
$$G_1 = (y_N - y_{N-1})/(y_N - y_1) \text{ Candidate Outlier is Largest } (y_N)$$

When $N$ = 8–10:

$$G_2 = (y_2 - y_1)/(y_{N-1} - y_1) \text{ Candidate Outlier is Smallest } (y_1)$$
$$G_2 = (y_N - y_{N-1})/(y_N - y_2) \text{ Candidate Outlier is Largest } (y_N)$$

When $N$ = 11–13:

$$G_3 = (y_3 - y_1)/(y_{N-1} - y_1) \text{ Candidate Outlier is Smallest } (y_1)$$
$$G_3 = (y_N - y_{N-2})/(y_N - y_2) \text{ Candidate Outlier is Largest } (y_N)$$

If $G_1$, $G_2$, or $G_3$, as appropriate, exceeds the critical value in *Table A2-1*, for the observed $N$, there is a statistical basis for identifying the discordant measurement as an outlier and considering its removal.

**Table A2-1. Test for Outlier Measurements**

| In samples from a normal population, gaps equal to or larger than the following values of $G_1$, $G_2$, and $G_3$ occur with a probability $P$ = 0.01, when outlier measurements can occur only at one end; or with $P$ = 0.02, when they may occur at either end. | | | | | |
|---|---|---|---|---|---|
| $N$ | 3 | 4 | 5 | 6 | 7 |
| $G_1$ | 0.988 | 0.889 | 0.780 | 0.698 | 0.637 |
| $N$ | 8 | 9 | 10 | — | — |
| $G_2$ | 0.683 | 0.635 | 0.597 | — | — |
| $N$ | 11 | 12 | 13 | — | — |
| $G_3$ | 0.679 | 0.642 | 0.615 | — | — |

EXAMPLE

Estimated potencies of sample in log scale = 1.561, 1.444, 1.517, 1.535.

Check the lowest potency for outlier:

$$G_1 = (1.517 - 1.444)/(1.561 - 1.444) = 0.624 < 0.889$$

Therefore, 1.444 is not an outlier.

Check the highest potency for outlier:

$$G_1 = (1.561 - 1.535)/(1.561 - 1.444) = 0.222 < 0.889$$

Therefore, 1.561 is not an outlier.

Outlier potencies should be marked as outlier values and excluded from the assay calculations. NMT 1 potency can be excluded as an outlier.

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# ⟨85⟩ BACTERIAL ENDOTOXINS TEST

♦Portions of this general chapter have been harmonized with the corresponding texts of the *European Pharmacopoeia* and/or the *Japanese Pharmacopoeia*. Those portions that are not harmonized are marked with symbols (♦ ♦) to specify this fact.♦

The Bacterial Endotoxins Test (BET) is a test to detect or quantify endotoxins from Gram-negative bacteria using amoebocyte lysate from the horseshoe crab (*Limulus polyphemus* or *Tachypleus tridentatus*).

There are three techniques for this test: the gel-clot technique, which is based on gel formation; the turbidimetric technique, based on the development of turbidity after cleavage of an endogenous substrate; and the chromogenic technique, based on the development of color after cleavage of a synthetic peptide-chromogen complex. Proceed by any of the three techniques for the test. In the event of doubt or dispute, the final decision is made based upon the gel-clot limit test unless otherwise indicated in the monograph for the product being tested. The test is carried out in a manner that avoids endotoxin contamination.

## APPARATUS

Depyrogenate all glassware and other heat-stable materials in a hot air oven using a validated process.♦[1] ♦ A commonly used minimum time and temperature is 30 min at 250°. If employing plastic apparatus, such as microplates and pipet tips for automatic pipetters, use apparatus that is shown to be free of detectable endotoxin and does not interfere in the test. [NOTE—In this chapter, the term "tube" includes any other receptacle such as a microtiter well.]

## REAGENTS AND TEST SOLUTIONS

### Amoebocyte Lysate

A lyophilized product obtained from the lysate of amoebocytes (white blood cells) from the horseshoe crab (*Limulus polyphemus* or *Tachypleus tridentatus*). This reagent refers only to a product manufactured in accordance with the regulations of the competent authority. [NOTE—*Amoebocyte Lysate* reacts to some β-glucans in addition to endotoxins. *Amoebocyte Lysate* preparations that do not react to glucans are available: they are prepared by removing the G factor reacting to glucans from *Amoebocyte Lysate* or by inhibiting the G factor reacting system of *Amoebocyte Lysate* and may be used for endotoxin testing in the presence of glucans.]

### Water for Bacterial Endotoxins Test (BET)

Use Water for Injection or water produced by other procedures that shows no reaction with the lysate employed, at the detection limit of the reagent.

### Lysate TS

Dissolve *Amoebocyte Lysate* in *Water for BET*, or in a buffer recommended by the lysate manufacturer, by gentle stirring. Store the reconstituted lysate, refrigerated or frozen, according to the specifications of the manufacturer.

## PREPARATION OF SOLUTIONS

### Standard Endotoxin Stock Solution

A *Standard Endotoxin Stock Solution* is prepared from a USP Endotoxin Reference Standard that has been calibrated to the current WHO International Standard for Endotoxin. Follow the specifications in the package leaflet and on the label for preparation and storage of the *Standard Endotoxin Stock Solution*. Endotoxin is expressed in Endotoxin Units (EU). [NOTE—One USP Endotoxin Unit (EU) is equal to one International Unit (IU) of endotoxin.]

### Standard Endotoxin Solutions

After mixing the *Standard Endotoxin Stock Solution* vigorously, prepare appropriate serial dilutions of *Standard Endotoxin Solution*, using *Water for BET*. Use dilutions as soon as possible to avoid loss of activity by adsorption.

### Sample Solutions

Prepare the *Sample Solutions* by dissolving or diluting drugs using *Water for BET*. Some substances or preparations may be more appropriately dissolved, or diluted in other aqueous solutions. If necessary, adjust the pH of the solution to be examined (or dilution thereof) so that the pH of the mixture of the lysate and *Sample Solution* falls within the pH range specified by the

♦[1] For a validity test of the procedure for inactivating endotoxins, see *Dry-Heat Sterilization* under *Sterility Assurance* ⟨1211⟩. Use *Lysate TS* having a sensitivity of not less than 0.15 Endotoxin Unit per mL.♦

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lysate manufacturer, usually 6.0–8.0. The pH may be adjusted by use of an acid, base, or suitable buffer as recommended by the lysate manufacturer. Acids and bases may be prepared from concentrates or solids with *Water for BET* in containers free of detectable endotoxin. Buffers must be validated to be free of detectable endotoxin and interfering factors.

## DETERMINATION OF MAXIMUM VALID DILUTION (MVD)

The maximum valid dilution is the maximum allowable dilution of a specimen at which the endotoxin limit can be determined. Determine the MVD from the following equation:

$$MVD = (\text{endotoxin limit} \times \text{concentration of } \textit{Sample Solution})/(\lambda)$$

### Endotoxin Limit

The endotoxin limit for parenteral drugs, defined on the basis of dose, equals $K/M$ ♦[2] ♦, where $K$ is a threshold pyrogenic dose of endotoxin per kg of body weight, and $M$ is equal to the maximum recommended bolus dose of product per kg of body weight. When the product is to be injected at frequent intervals or infused continuously, $M$ is the maximum total dose administered in a single hour period. The endotoxin limit for parenteral drugs is specified in the individual monograph in units such as EU/mL, EU/mg, EU/Unit of biological activity, etc.

### Concentration of Sample Solution

mg/mL: in the case of endotoxin limit specified by weight (EU/mg);
Units/mL: in the case of endotoxin limit specified by unit of biological activity (EU/Unit);
mL/mL: when the endotoxin limit is specified by volume (EU/mL).
λ: the labeled sensitivity in the *Gel-Clot Technique* (EU/mL) or the lowest concentration used in the standard curve for the *Turbidimetric Technique* or *Chromogenic Technique*.

## GEL-CLOT TECHNIQUE

The gel-clot technique is used for detecting or quantifying endotoxins based on clotting of the lysate reagent in the presence of endotoxin. The minimum concentration of endotoxin required to cause the lysate to clot under standard conditions is the labeled sensitivity of the lysate reagent. To ensure both the precision and validity of the test, perform the tests for confirming the labeled lysate sensitivity and for interfering factors as described in *Preparatory Testing*, immediately below.

### Preparatory Testing

TEST FOR CONFIRMATION OF LABELED LYSATE SENSITIVITY

Confirm in four replicates the labeled sensitivity, λ, expressed in EU/mL of the lysate prior to use in the test. The test for confirmation of lysate sensitivity is to be carried out when a new batch of lysate is used or when there is any change in the test conditions that may affect the outcome of the test. Prepare standard solutions having at least four concentrations equivalent to 2λ, λ, 0.5λ, and 0.25λ by diluting the USP Endotoxin RS with *Water for BET*.

Mix a volume of the *Lysate TS* with an equal volume (such as 0.1-mL aliquots) of one of the *Standard Endotoxin Solutions* in each test tube. When single test vials or ampuls containing lyophilized lysate are used, add solutions directly to the vial or ampul. Incubate the reaction mixture for a constant period according to the directions of the lysate manufacturer (usually at 37 ± 1° for 60 ± 2 min), avoiding vibration. To test the integrity of the gel, take each tube in turn directly from the incubator, and invert it through about 180° in one smooth motion. If a firm gel has formed that remains in place upon inversion, record the result as positive. A result is negative if an intact gel is not formed. The test is considered valid when the lowest concentration of the standard solutions shows a negative result in all replicate tests.

The endpoint is the smallest concentration in the series of decreasing concentrations of standard endotoxin that clots the lysate. Determine the geometric mean endpoint by calculating the mean of the logarithms of the endpoint concentrations of the four replicate series and then taking the antilogarithm of the mean value, as indicated in the following formula:

$$\text{geometric mean endpoint concentration} = \text{antilog}\,(\Sigma e/f)$$

where $\Sigma e$ is the sum of the log endpoint concentrations of the dilution series used, and $f$ is the number of replicate test tubes. The geometric mean endpoint concentration is the measured sensitivity of the lysate (in EU/mL). If this is not less than 0.5λ and not more than 2λ, the labeled sensitivity is confirmed and is used in tests performed with this lysate.

♦[2] $K$ is 5 USP-EU/kg of body weight for any route of administration other than intrathecal (for which $K$ is 0.2 USP-EU/kg of body weight). For radiopharmaceutical products not administered intrathecally, the endotoxin limit is calculated as 175 EU/$V$, where $V$ is the maximum recommended dose in mL. For intrathecally administered radiopharmaceuticals, the endotoxin limit is obtained by the formula 14 EU/$V$. For formulations (usually anticancer products) administered on a per square meter of body surface, the formula is $K/M$, where $K$ = 100 EU/m$^2$ and $M$ is the maximum dose/m$^2$. ♦

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### TEST FOR INTERFERING FACTORS

Usually prepare solutions *(A–D)* as shown in *Table 1*, and perform the inhibition/enhancement test on the *Sample Solutions* at a dilution less than the MVD, not containing any detectable endotoxins, operating as described for *Test for Confirmation of Labeled Lysate Sensitivity*. The geometric mean endpoint concentrations of *Solutions B* and *C* are determined using the formula described in the *Test for Confirmation of Labeled Lysate Sensitivity*. The test for interfering factors must be repeated when any condition changes that is likely to influence the result of the test.

**Table 1. Preparation of Solutions for the Inhibition/Enhancement Test for Gel-Clot Techniques**

| Solution | Endotoxin Concentration/ Solution to Which Endotoxin Is Added | Diluent | Dilution Factor | Endotoxin Concentration | Number of Replicates |
|---|---|---|---|---|---|
| A[a] | None/*Sample Solution* | — | — | — | 4 |
| B[b] | 2λ/*Sample Solution* | *Sample Solution* | 1 | 2λ | 4 |
| | | | 2 | 1λ | 4 |
| | | | 4 | 0.5λ | 4 |
| | | | 8 | 0.25λ | 4 |
| C[c] | 2λ/*Water for BET* | *Water for BET* | 1 | 2λ | 2 |
| | | | 2 | 1λ | 2 |
| | | | 4 | 0.5λ | 2 |
| | | | 8 | 0.25λ | 2 |
| D[d] | None/*Water for BET* | — | — | — | 2 |

[a] *Solution A*: A *Sample Solution* of the preparation under test that is free of detectable endotoxins.
[b] *Solution B*: Test for interference.
[c] *Solution C*: Control for labeled lysate sensitivity.
[d] *Solution D*: Negative control of *Water for BET*.

The test is considered valid when all replicates of *Solutions A* and *D* show no reaction and the result of *Solution C* confirms the labeled sensitivity.

If the sensitivity of the lysate determined in the presence of *Solution B* is not less than 0.5λ and not greater than 2λ, the *Sample Solution* does not contain factors that interfere under the experimental conditions used. Otherwise, the *Sample Solution* to be examined interferes with the test.

If the sample under test does not comply with the test at a dilution less than the MVD, repeat the test using a greater dilution, not exceeding the MVD. The use of a more sensitive lysate permits a greater dilution of the sample to be examined, and this may contribute to the elimination of interference.

Interference may be overcome by suitable treatment such as filtration, neutralization, dialysis, or heating. To establish that the chosen treatment effectively eliminates interference without loss of endotoxins, perform the assay described above using the preparation to be examined to which Standard Endotoxin has been added and which has then been submitted to the chosen treatment.

## Limit Test

### PROCEDURE

Prepare *Solutions A, B, C,* and *D* as shown in *Table 2*, and perform the test on these solutions following the procedure above for *Preparatory Testing*, *Test for Confirmation of Labeled Lysate Sensitivity*.

**Table 2. Preparation of Solutions for the Gel-Clot Limit Test**

| Solution* | Endotoxin Concentration/ Solution to Which Endotoxin Is Added | Number of Replicates |
|---|---|---|
| A | None/Diluted *Sample Solution* | 2 |
| B | 2λ/Diluted *Sample Solution* | 2 |
| C | 2λ/*Water for BET* | 2 |
| D | None/*Water for BET* | 2 |

* Prepare *Solution A* and the positive product control *Solution B* using a dilution not greater than the MVD and treatments as described for the *Test for Interfering Factors* in *Preparatory Testing*. The positive control *Solutions B* and *C* contain the *Standard Endotoxin Solution* at a concentration corresponding to twice the labeled lysate sensitivity. The negative control *Solution D* consists of *Water for BET*.

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### INTERPRETATION

The test is considered valid when both replicates of *Solutions B* and *C* are positive and those of *Solution D* are negative. When a negative result is found for both replicates of *Solution A*, the preparation under test complies with the test. When a positive result is found for both replicates of *Solution A*, the preparation under test does not comply with the test.

When a positive result is found for one replicate of *Solution A* and a negative result is found for the other, repeat the test. In the repeat test, the preparation under test complies with the test if a negative result is found for both replicates of *Solution A*. The preparation does not comply with the test if a positive result is found for one or both replicates of *Solution A.* However, if the preparation does not comply with the test at a dilution less than the MVD, the test may be repeated using a greater dilution, not exceeding the MVD.

## Quantitative Test

### PROCEDURE

The test quantifies bacterial endotoxins in *Sample Solutions* by titration to an endpoint. Prepare *Solutions A, B, C,* and *D* as shown in *Table 3*, and test these solutions by following the procedure in *Preparatory Testing*, *Test for Confirmation of Labeled Lysate Sensitivity.*

**Table 3. Preparation of Solutions for the Gel-Clot Assay**

| Solution | Endotoxin Concentration/ Solution to Which Endotoxin Is Added | Diluent | Dilution Factor | Endotoxin Concentration | Number of Replicates |
|---|---|---|---|---|---|
| A[a] | None/*Sample Solution* | *Water for BET* | 1 | — | 2 |
| | | | 2 | — | 2 |
| | | | 4 | — | 2 |
| | | | 8 | — | 2 |
| B[b] | 2λ/*Sample Solution* | — | 1 | 2λ | 2 |
| C[c] | 2λ/*Water for BET* | *Water for BET* | 1 | 2λ | 2 |
| | | | 2 | 1λ | 2 |
| | | | 4 | 0.5λ | 2 |
| | | | 8 | 0.25λ | 2 |
| D[d] | None/*Water for BET* | — | — | — | 2 |

[a] *Solution A*: *Sample Solution* under test at the dilution, not to exceed the MVD, with which the *Test for Interfering Factors* was completed. Subsequent dilution of the *Sample Solution* must not exceed the MVD. Use *Water for BET* to make a dilution series of four tubes containing the *Sample Solution* under test at concentrations of 1, ½, ¼, and ⅛ relative to the concentration used in the *Test for Interfering Factors*. Other dilutions up to the MVD may be used as appropriate.
[b] *Solution B*: *Solution A* containing standard endotoxin at a concentration of 2λ (positive product control).
[c] *Solution C*: Two replicates of four tubes of *Water for BET* containing the standard endotoxin at concentrations of 2λ, λ, 0.5λ, and 0.25λ, respectively.
[d] *Solution D*: *Water for BET* (negative control).

### CALCULATION AND INTERPRETATION

The test is considered valid when the following three conditions are met: (1) Both replicates of negative control *Solution D* are negative; (2) Both replicates of positive product control *Solution B* are positive; and (3) The geometric mean endpoint concentration of *Solution C* is in the range of 0.5λ to 2λ.

To determine the endotoxin concentration of *Solution A*, calculate the endpoint concentration for each replicate by multiplying each endpoint dilution factor by λ. The endotoxin concentration in the *Sample Solution* is the endpoint concentration of the replicates. If the test is conducted with a diluted *Sample Solution*, calculate the concentration of endotoxin in the original *Sample Solution* by multiplying by the dilution factor. If none of the dilutions of the *Sample Solution* is positive in a valid assay, report the endotoxin concentration as less than λ (if the diluted sample was tested, report as less than λ times the lowest dilution factor of the sample). If all dilutions are positive, the endotoxin concentration is reported as equal to or greater than the greatest dilution factor multiplied by λ (e.g., initial dilution factor times eight times λ in *Table 3*).

The preparation under test meets the requirements of the test if the concentration of endotoxin in both replicates is less than that specified in the individual monograph.

# PHOTOMETRIC QUANTITATIVE TECHNIQUES

## Turbidimetric Technique

This technique is a photometric assay measuring increases in reactant turbidity. On the basis of the particular assay principle employed, this technique may be classified as either an endpoint-turbidimetric assay or a kinetic-turbidimetric assay. The endpoint-turbidimetric assay is based on the quantitative relationship between the concentration of endotoxins and the

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turbidity (absorbance or transmission) of the reaction mixture at the end of an incubation period. The kinetic-turbidimetric assay is a method to measure either the time (onset time) needed to reach a predetermined absorbance or transmission of the reaction mixture, or the rate of turbidity development. The test is carried out at the incubation temperature recommended by the lysate manufacturer (which is usually 37 ± 1°).

## Chromogenic Technique

This technique is an assay to measure the chromophore released from a suitable chromogenic peptide by the reaction of endotoxins with lysate. On the basis of the particular assay principle employed, this technique may be classified as either an endpoint-chromogenic assay or a kinetic-chromogenic assay. The endpoint-chromogenic assay is based on the quantitative relationship between the concentration of endotoxins and the release of chromophore at the end of an incubation period. The kinetic-chromogenic assay is a method to measure either the time (onset time) needed to reach a predetermined absorbance of the reaction mixture, or the rate of color development. The test is carried out at the incubation temperature recommended by the lysate manufacturer (which is usually 37 ± 1°).

## Preparatory Testing

To assure the precision or validity of the turbidimetric and chromogenic techniques, preparatory tests are conducted to verify that the criteria for the standard curve are valid and that the sample solution does not interfere with the test. Validation for the test method is required when conditions that are likely to influence the test result change.

### ASSURANCE OF CRITERIA FOR THE STANDARD CURVE

The test must be carried out for each lot of lysate reagent. Using the *Standard Endotoxin Solution,* prepare at least three endotoxin concentrations within the range indicated by the lysate manufacturer to generate the standard curve. Perform the assay using at least three replicates of each standard endotoxin concentration according to the manufacturer's instructions for the lysate (volume ratios, incubation time, temperature, pH, etc.). If the desired range is greater than two logs in the kinetic methods, additional standards should be included to bracket each log increase in the range of the standard curve. The absolute value of the correlation coefficient, *r*, must be greater than or equal to 0.980 for the range of endotoxin concentrations set up.

### TEST FOR INTERFERING FACTORS

Select an endotoxin concentration at or near the middle of the endotoxin standard curve. Prepare *Solutions A, B, C,* and *D* as shown in *Table 4*. Perform the test on *Solutions A, B, C,* and *D* at least in duplicate, according to the instructions for the lysate employed, for example, concerning volume of *Sample Solution* and *Lysate TS,* volume ratio of *Sample Solution* to *Lysate TS,* incubation time, etc.

**Table 4. Preparation of Solutions for the Inhibition/Enhancement Test for Photometric Techniques**

| Solution | Endotoxin Concentration | Solution to Which Endotoxin Is Added | Number of Replicates |
|---|---|---|---|
| A[a] | None | *Sample Solution* | Not less than 2 |
| B[b] | Middle concentration of the standard curve | *Sample Solution* | Not less than 2 |
| C[c] | At least three concentrations (lowest concentration is designated λ) | *Water for BET* | Each not less than 2 |
| D[d] | None | *Water for BET* | Not less than 2 |

[a] *Solution A*: The *Sample Solution* may be diluted not to exceed MVD.
[b] *Solution B*: The preparation under test at the same dilution as *Solution A,* containing added endotoxin at a concentration equal to or near the middle of the standard curve.
[c] *Solution C*: The standard endotoxin at the concentrations used in the validation of the method described for *Assurance of Criteria for the Standard Curve* under *Preparatory Testing* (positive controls).
[d] *Solution D*: *Water for BET* (negative control).

The test is considered valid when the following conditions are met.

1. The absolute value of the correlation coefficient of the standard curve generated using *Solution C* is greater than or equal to 0.980.
2. The result with *Solution D* does not exceed the limit of the blank value required in the description of the lysate reagent employed, or it is less than the endotoxin detection limit of the lysate reagent employed.

Calculate the mean recovery of the added endotoxin by subtracting the mean endotoxin concentration in the solution, if any (*Solution A, Table 4*), from that containing the added endotoxin (*Solution B, Table 4*). In order to be considered free of factors that interfere with the assay under the conditions of the test, the measured concentration of the endotoxin added to the *Sample Solution* must be within 50%–200% of the known added endotoxin concentration after subtraction of any endotoxin detected in the solution without added endotoxin.

When the endotoxin recovery is out of the specified range, the *Sample Solution* under test is considered to contain interfering factors. Then, repeat the test using a greater dilution, not exceeding the MVD. Furthermore, interference of the *Sample Solution* or diluted *Sample Solution* not to exceed the MVD may be eliminated by suitable validated treatment such as filtration, neutralization, dialysis, or heat treatment. To establish that the chosen treatment effectively eliminates interference without loss

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of endotoxins, perform the assay described above, using the preparation to be examined to which Standard Endotoxin has been added and which has then been submitted to the chosen treatment.

## Test Procedure

Follow the procedure described for *Test for Interfering Factors* under *Preparatory Testing*, immediately above.

## Calculation

Calculate the endotoxin concentration of each of the replicates of *Solution A*, using the standard curve generated by the positive control *Solution C*. The test is considered valid when the following three requirements are met.

1. The results of the control *Solution C* comply with the requirements for validation defined for *Assurance of Criteria for the Standard Curve* under *Preparatory Testing*.
2. The endotoxin recovery, calculated from the concentration found in *Solution B* after subtracting the concentration of endotoxin found in *Solution A*, is within the range of 50%–200%.
3. The result of the negative control *Solution D* does not exceed the limit of the blank value required in the description of the lysate employed, or it is less than the endotoxin detection limit of the lysate reagent employed.

## Interpretation

In photometric assays, the preparation under test complies with the test if the mean endotoxin concentration of the replicates of *Solution A*, after correction for dilution and concentration, is less than the endotoxin limit for the product.

**USP Reference Standards** ⟨11⟩

USP Endotoxin RS

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# ⟨87⟩ BIOLOGICAL REACTIVITY TESTS, IN VITRO

The following tests are designed to determine the biological reactivity of mammalian cell cultures following contact with the elastomeric plastics and other polymeric materials with direct or indirect patient contact or of specific extracts prepared from the materials under test. It is essential that the tests be performed on the specified surface area. When the surface area of the specimen cannot be determined, use 0.1 g of elastomer or 0.2 g of plastic or other material for every mL of extraction fluid. Exercise care in the preparation of the materials to prevent contamination with microorganisms and other foreign matter.

Three tests are described (i.e., the *Agar Diffusion Test*, the *Direct Contact Test*, and the *Elution Test*).[1] The decision as to which type of test or the number of tests to be performed to assess the potential biological response of a specific sample or extract depends upon the material, the final product, and its intended use. Other factors that may also affect the suitability of a sample for a specific use are the polymeric composition; processing and cleaning procedures; contacting media; inks; adhesives; absorption, adsorption, and permeability of preservatives; and conditions of storage. Evaluation of such factors should be made by appropriate additional specific tests before determining that a product made from a specific material is suitable for its intended use. Materials that fail the *in vitro* tests are candidates for the *in vivo* tests described in *Biological Reactivity Tests, In Vivo* ⟨88⟩.

## PROCEDURES

- **Test Control**

**Positive control:** Polyurethane film containing zinc diethyldithiocarbamate (ZDEC)[2] or zinc dibutyldithiocarbamate (ZDBC)

**Cell culture preparation:** Prepare multiple cultures of L-929 (ATCC cell line CCL 1, NCTC clone 929; alternative cell lines obtained from a standard repository may be used with suitable validation) mammalian fibroblast cells in serum-supplemented minimum essential medium having a seeding density of about $10^5$ cells per mL. Incubate the cultures at 37 ± 1° in a humidified incubator for NLT 24 h in a 5 ± 1% carbon dioxide atmosphere until a monolayer, with greater than 80% confluence, is obtained. Examine the prepared cultures under a microscope to ensure uniform, near-confluent monolayers. [Note—The reproducibility of the in vitro biological reactivity tests depends upon obtaining uniform cell culture density.]

**Extraction solvents:** *Sodium Chloride Injection* [see monograph—use *Sodium Chloride Injection* containing 0.9% of sodium chloride (NaCl)]. Alternatively, serum-free mammalian cell culture media or serum-supplemented mammalian cell culture media may be used. Serum supplementation is used when extraction is done at 37° for 24 h.

- **Apparatus**

**Autoclave:** Employ an autoclave capable of maintaining a temperature of 121 ± 2°, equipped with a thermometer, a pressure gauge, a vent cock, a rack adequate to accommodate the test containers above the water level, and a water cooling system that will allow for cooling of the test containers to about 20°, but not below 20°, immediately following the heating cycle.

**Oven:** Use an oven, preferably a mechanical convection model, that will maintain operating temperatures in the range of 50°–70° within ±2°.

**Incubator:** Use an incubator capable of maintaining a temperature of 37 ± 1° and a humidified atmosphere of 5 ± 1% carbon dioxide in air.

**Extraction containers:** Use only containers, such as ampuls or screw-cap culture test tubes, or their equivalent, of Type I glass. If used, culture test tubes, or their equivalent, are closed with a screw cap having a suitable elastomeric liner. The exposed surface of the elastomeric liner is completely protected with an inert solid disk 50–75 µm in thickness. A suitable disk can be fabricated from polytef.

**Preparation of apparatus:** Cleanse all glassware thoroughly with chromic acid cleansing mixture and, if necessary, with hot nitric acid followed by prolonged rinsing with *Sterile Water for Injection*. Sterilize and dry by a suitable process for containers and devices used for extraction, transfer, or administration of test material. If ethylene oxide is used as the sterilizing agent, allow NLT 48 h for complete degassing.

- **Procedure**

**Preparation of sample for extracts:** Prepare as directed in the *Procedure* in ⟨88⟩.

**Preparation of extracts:** Prepare as directed for *Preparation of extracts* in ⟨88⟩ using either *Sodium Chloride Injection* [0.9% sodium chloride (NaCl)] or serum-free mammalian cell culture media as *Extraction solvents*. [Note—If extraction is done at 37° for 24 h in an incubator, use cell culture media supplemented by serum. The extraction conditions should not in any instance cause physical changes, such as fusion or melting of the material pieces, other than a slight adherence.]

- **Agar Diffusion Test**

This test is designed for elastomeric closures in a variety of shapes. The agar layer acts as a cushion to protect the cells from mechanical damage while allowing the diffusion of leachable chemicals from the polymeric specimens. Extracts of materials that are to be tested are applied to a piece of filter paper.

**Sample preparation:** Use extracts prepared as directed, or use portions of the test specimens having flat surfaces NLT 100 $mm^2$ in surface area.

**Positive control preparation:** Proceed as directed for *Sample preparation*.

**Negative control preparation:** Proceed as directed for *Sample preparation*.

**Procedure:** Using 7 mL of cell suspension prepared as directed in *Cell culture preparation*, prepare the monolayers in plates having a 60-mm diameter. Following incubation, aspirate the culture medium from the monolayers, and replace it with serum-supplemented culture medium containing NMT 2% of agar. [Note—The quality of the agar must be adequate to

[1] Further details are given in the following publications of the American Society for Testing and Materials, 1916 Race St., Philadelphia, PA 19103: Standard test method for agar diffusion cell culture screening for cytotoxicity, ASTM Designation F 895-84; Standard practice for direct contact cell culture evaluation of materials for medical devices, ASTM Designation F 813-83.

[2] ZDEC and ZDBC polyurethanes are available from the Food and Drug Safety Center, Hatano Research Institute, Ochiai 729–5, Hadanoshi, Kanagawa 257, Japan.

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support cell growth. The agar layer must be thin enough to permit diffusion of leached chemicals.] Place the flat surfaces of *Sample preparation*, *Positive control preparation*, and *Negative control preparation* or their extracts in an appropriate extracting medium, in duplicate cultures in contact with the solidified agar surface. Use no more than three specimens per prepared plate. Incubate all cultures for NLT 24 h at 37 ± 1°, preferably in a humidified incubator containing 5 ± 1% of carbon dioxide. Examine each culture around each sample, negative control, and positive control under a microscope, using a suitable stain, if desired.

**Interpretation of results:** The biological reactivity (cellular degeneration and malformation) is described and rated on a scale of 0–4 (see *Table 1*). Measure the responses of the cell cultures to the *Sample preparation*, the *Positive control preparation*, and the *Negative control preparation*. The cell culture test system is suitable if the observed responses to the *Negative control preparation* is grade 0 (no reactivity) and to the *Positive control preparation* is at least grade 3 (moderate). The sample meets the requirements of the test if the response to the *Sample preparation* is not greater than grade 2 (mildly reactive). Repeat the procedure if the suitability of the system is not confirmed.

**Table 1. Reactivity Grades for Agar Diffusion Test and Direct Contact Test**

| Grade | Reactivity | Description of Reactivity Zone |
|---|---|---|
| 0 | None | No detectable zone around or under specimen |
| 1 | Slight | Some malformed or degenerated cells under specimen |
| 2 | Mild | Zone limited to area under specimen and less than 0.45 cm beyond specimen |
| 3 | Moderate | Zone extends 0.45–1.0 cm beyond specimen |
| 4 | Severe | Zone extends greater than 1.0 cm beyond specimen |

- **Direct Contact Test**

This test is designed for materials in a variety of shapes. The procedure allows for simultaneous extraction and testing of leachable chemicals from the specimen with a serum-supplemented medium. The procedure is not appropriate for very low- or high-density materials that could cause mechanical damage to the cells.

**Sample preparation:** Use portions of the test specimen having flat surfaces NLT 100 $mm^2$ in surface area.

**Positive control preparation:** Proceed as directed for *Sample preparation*.

**Negative control preparation:** Proceed as directed for *Sample preparation*.

**Procedure:** Using 2 mL of cell suspension prepared as directed in *Cell culture preparation*, prepare the monolayers in plates having a 35-mm diameter. Following incubation, aspirate the culture medium from the cultures, and replace it with 0.8 mL of fresh culture medium. Place a single *Sample preparation*, a *Positive control preparation*, and a *Negative control preparation* in each of the duplicate cultures. Incubate all cultures for NLT 24 h at 37 ± 1° in a humidified incubator containing 5 ± 1% of carbon dioxide. Examine each culture around each *Sample preparation*, a *Positive control preparation*, and a *Negative control preparation*, under a microscope, using a suitable stain, if desired.

**Interpretation of results:** Proceed as directed for *Interpretation of results* in *Agar Diffusion Test*. The sample meets the requirements of the test if the response to the *Sample preparation* is not greater than grade 2 (mildly reactive). Repeat the procedure if the suitability of the system is not confirmed.

- **Elution Test**

This test is designed for the evaluation of extracts of polymeric materials. The procedure allows for extraction of the specimens at physiological or nonphysiological temperatures for varying time intervals. It is appropriate for high-density materials and for dose-response evaluations.

**Sample preparation:** Prepare as directed in *Preparation of extracts*, using either *Sodium Chloride Injection* [0.9% sodium chloride (NaCl)] or serum-free mammalian cell culture media as *Extraction solvents*. If the size of the sample cannot be readily measured, a mass of NLT 0.1 g of elastomeric material or 0.2 g of plastic or polymeric material per mL of extraction medium may be used. Alternatively, use serum-supplemented mammalian cell culture media as the extracting medium to simulate more closely physiological conditions. Prepare the extracts by heating for 24 h in an incubator containing 5 ± 1% of carbon dioxide. Maintain the extraction temperature at 37 ± 1°, because higher temperatures may cause denaturation of serum proteins.

**Positive control preparation:** Proceed as directed for *Sample preparation*.

**Negative control preparation:** Proceed as directed for *Sample preparation*.

**Procedure:** Using 2 mL of cell suspension prepared as directed in *Cell culture preparation*, prepare the monolayers in plates having a 35-mm diameter. Following incubation, aspirate the culture medium from the monolayers, and replace it with extracts of the *Sample preparation*, *Positive control preparation*, or *Negative control preparation*. The serum-supplemented and serum-free cell culture media extracts are tested in duplicate without dilution (100%). The *Sodium Chloride Injection* extract is diluted with serum-supplemented cell culture medium and tested in duplicate at 25% extract concentration. Incubate all cultures for 48 h at 37 ± 1° in a humidified incubator preferably containing 5 ± 1% of carbon dioxide. Examine each culture at 48 h, under a microscope, using a suitable stain, if desired.

**Interpretation of results:** Proceed as directed for *Interpretation of results* in *Agar Diffusion Test* but use *Table 2*. The sample meets the requirements of the test if the response to the *Sample preparation* is not greater than grade 2 (mildly reactive). Repeat the procedure if the suitability of the system is not confirmed. For dose-response evaluations, repeat the procedure, using quantitative dilutions of the sample extract.

**Table 2. Reactivity Grades for Elution Test**

| Grade | Reactivity | Conditions of All Cultures |
|---|---|---|
| 0 | None | Discrete intracytoplasmic granules; no cell lysis |

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**Table 2. Reactivity Grades for Elution Test** *(continued)*

| Grade | Reactivity | Conditions of All Cultures |
|---|---|---|
| 1 | Slight | Less than or equal to 20% of the cells are round, loosely attached, and without intracytoplasmic granules; occasional lysed cells are present |
| 2 | Mild | Greater than 20% to less than or equal to 50% of the cells are round and devoid of intracytoplasmic granules; no extensive cell lysis and empty areas between cells |
| 3 | Moderate | Greater than 50% to less than 70% of the cell layers contain rounded cells or are lysed |
| 4 | Severe | Nearly complete destruction of the cell layers |

## ADDITIONAL REQUIREMENTS

- **USP Reference Standards** ⟨11⟩
  USP High-Density Polyethylene RS
  (Negative Control)

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# ⟨88⟩ BIOLOGICAL REACTIVITY TESTS, IN VIVO

The following tests are designed to determine the biological response of animals to elastomerics, plastics, and other polymeric material with direct or indirect patient contact, or by the injection of specific extracts prepared from the material under test. It is essential to make available the specific surface area for extraction. When the surface area of the specimen cannot be determined, use 0.1 g of elastomer or 0.2 g of plastic or other material for every mL of extraction fluid. Also, it is essential to exercise care in the preparation of the materials to be injected or instilled to prevent contamination with microorganisms and other foreign matter. Three tests are described. The *Systemic Injection Test* and the *Intracutaneous Test* are used for elastomeric materials, especially to elastomeric closures for which the appropriate *Biological Reactivity Tests, In Vitro* ⟨87⟩ have indicated significant biological reactivity. These two tests are used for plastics and other polymers, in addition to a third test, the *Implantation Test*, to test the suitability of these materials intended for use in fabricating containers and accessories thereto, for use in parenteral preparations, and for use in medical devices, implants, and other systems.

These three tests are applied to materials or medical devices, if there is a need for classification of plastics and other polymers based on in vivo biological reactivity testing.

For the purpose of this chapter, these definitions apply: the *Sample* is the specimen under test or an extract prepared from such a specimen. A *Blank* consists of the same quantity of the same extracting medium that is used for the extraction of the specimen under test, treated in the same manner as the extracting medium containing the specimen under test. A *Negative Control*[1] is a specimen that gives no reaction under the conditions of the test.

## CLASSIFICATION OF PLASTICS

Six Plastic Classes are defined (see *Table 1*). This classification is based on responses to a series of in vivo tests for which extracts, materials, and routes of administration are specified. These tests are directly related to the intended end-use of the plastic articles. The choice of extractants is representative of the vehicles in preparations with which the plastics are likely to be in contact. The *Table 1* classification facilitates communication among suppliers, users, and manufacturers of plastics by summarizing the tests to be performed for containers for injections and medical devices if a need for classification exists.

**Table 1. Classification of Plastics**

| Plastic Classes[a] | | | | | | Tests to be Conducted | | | |
|---|---|---|---|---|---|---|---|---|---|
| I | II | III | IV | V | VI | Test Material | Animal | Dose | Procedure[b] |
| x | x | x | x | x | x | Extract of *Sample* in *Sodium Chloride Injection* | Mouse | 50 mL/kg | A (IV) |
| x | x | x | x | x | x | | Rabbit or Guinea Pig | 0.2 mL/animal at each of 10 or 6 sites | B (IC) |
| | x | x | x | x | x | Extract of *Sample* in *1 in 20 Solution of Alcohol in Sodium Chloride Injection* | Mouse | 50 mL/kg | A (IP) |
| | x | x | x | x | x | | Rabbit or Guinea Pig | 0.2 mL/animal at each of 10 or 6 sites | B (IC) |
| | | x | | x | x | Extract of *Sample* in *Polyethylene Glycol 400* | Mouse | 10 g/kg | A (IP) |
| | | | | x | x | | Rabbit or Guinea Pig | 0.2 mL/animal at each of 10 or 6 sites | B (IC) |
| | | x | x | x | x | Extract of *Sample* in *Vegetable Oil* | Mouse | 50 mL/kg | A (IP) |
| | | | x | x | x | | Rabbit or Guinea Pig | 0.2 mL/animal at each of 10 or 6 sites | B (IC) |
| | | | x | | x | Implant strips of *Sample* | Rabbit | 4 strips/animal | C |
| | | | x | | x | Implant *Sample* | Rat | 2 *Samples*/animal | C |

[a] Tests required for each class are indicated by "x" in appropriate columns.
[b] Legend: A (IP)—Systemic Injection Test (intraperitoneal); B (IC)—Intracutaneous Test (intracutaneous); C—Implantation Test (intramuscular or subcutaneous implantation).

With the exception of the *Implantation Test*, the procedures are based on the use of extracts that, depending on the heat resistance of the material, are prepared at one of three standard temperatures: 50°, 70°, and 121°. Therefore, the class designation of a plastic must be accompanied by an indication of the temperature of extraction (e.g., IV-121°, which represents a class IV plastic extracted at 121°, or I-50°, which represents a class I plastic extracted at 50°).

Plastics may be classified as USP Plastic Classes I–VI only on the basis of the response criteria prescribed in *Table 1*.

This classification does not apply to plastics that are intended for use as containers for oral or topical products, or that may be used as an integral part of a drug formulation. *Table 1* does not apply to natural elastomers, which are to be tested in Sodium Chloride Injection and vegetable oils only.

The *Systemic Injection Test* and the *Intracutaneous Test* are designed to determine the systemic and local, respectively, biological responses of animals to plastics and other polymers by the single-dose injection of specific extracts prepared from a

[1] USP High-Density Polyethylene RS.

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*Sample*. The *Implantation Test* is designed to evaluate the reaction of living tissue to the plastic and other polymers by the implantation of the *Sample* itself into animal tissue. The proper preparation and placement of the specimens under aseptic conditions are important in the conduct of the *Implantation Test*.

These tests are designed for application to plastics and other polymers in the condition in which they are used. If the material is to be exposed to any cleansing or sterilization process prior to its end-use, then the tests are to be conducted on a *Sample* prepared from a specimen preconditioned by the same processing.

Factors such as material composition, processing and cleaning procedures, contacting media, inks, adhesives, absorption, adsorption and permeability of preservatives, and conditions of storage may also affect the suitability of a material for a specific use. Evaluation of such factors should be made by appropriate additional specific tests to determine the suitability of a material for its intended use.

*USP Reference Standards* ⟨11⟩—*USP High-Density Polyethylene RS*.

**Extracting Media—**

**SODIUM CHLORIDE INJECTION** (see monograph). Use Sodium Chloride Injection containing 0.9% of NaCl.

**1 IN 20 SOLUTION OF ALCOHOL IN SODIUM CHLORIDE INJECTION.**

**POLYETHYLENE GLYCOL 400** (see monograph).

**VEGETABLE OIL**—Use freshly refined Sesame Oil (see monograph) or Cottonseed Oil (see monograph) or other suitable vegetable oils.

**DRUG PRODUCT VEHICLE** (where applicable).

**WATER FOR INJECTION** (see monograph).

[NOTE—The Sesame Oil or Cottonseed Oil or other suitable vegetable oil meets the following additional requirements. Obtain, if possible, freshly refined oil. Use three properly prepared animals, and inject the oil intracutaneously in a dose of 0.2 mL into each of 10 sites per animal, and observe the animals at 24, 48, and 72 h following injection. Rate the observations at each site on the numerical scale indicated in *Table 2*. For the 3 rabbits or guinea pigs (30 or 18 injection sites), at any observation time, the average response for erythema is not greater than 0.5 and for edema is not greater than 1.0, and no site shows a tissue reaction larger than 10 mm in overall diameter. The residue of oil at the injection site should not be misinterpreted as edema. Edematous tissue blanches when gentle pressure is applied.]

**Table 2. Evaluation of Skin Reactions[a]**

| Erythema and Eschar Formation | Score |
|---|---|
| No erythema | 0 |
| Very slight erythema (barely perceptible) | 1 |
| Well-defined erythema | 2 |
| Moderate to severe erythema | 3 |
| Severe erythema (beet-redness) to slight eschar formation (injuries in depth) | 4 |
| **Edema Formation[b]** | **Score** |
| No edema | 0 |
| Very slight edema (barely perceptible) | 1 |
| Slight edema (edges of area well defined by definite raising) | 2 |
| Moderate edema (raised approximately 1 mm) | 3 |
| Severe edema (raised more than 1 mm and extending beyond the area of exposure) | 4 |

[a] Draize JH, Woodward G, Calvery HO. Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes. *J Pharmacol Exp Ther* 1944;82:377–390.

[b] Excludes noninflammatory (mechanical) edema from the blank or extraction fluid.

**Apparatus**—The apparatus for the tests includes the following.

**AUTOCLAVE**—Use an autoclave capable of maintaining a temperature of 121 ± 2.0°, equipped with a thermometer, a pressure gauge, a vent cock, a rack adequate to accommodate the test containers above the water level, and a water cooling system that will allow for cooling of the test containers to about, but not below, 20° immediately following the heating cycle.

**OVEN**—Use an oven, preferably a forced-circulation model, that will maintain operating temperatures of 50° or 70° within ±2°.

**EXTRACTION CONTAINERS**—Use only containers, such as ampuls or screw-cap culture test tubes, of Type I glass. If used, culture test tubes are closed with screw caps having suitable elastomeric liners. The exposed surface of the elastomeric liner is completely protected with an inert solid disk 0.05–0.075 mm in thickness. A suitable disk may be fabricated from a polytef resin.

**Preparation of Apparatus**—Cleanse all glassware thoroughly with chromic acid cleansing mixture, or if necessary, with hot nitric acid, followed by prolonged rinsing with water. Clean cutting utensils by an appropriate method (e.g., successive cleaning with acetone and methylene chloride) prior to use in subdividing a specimen. Clean all other equipment by thorough scrubbing with a suitable detergent and prolonged rinsing with water.

Render containers and equipment used for extraction, and in transfer and administration of test material, sterile and dry by a suitable process. [NOTE—If ethylene oxide is used as the sterilizing agent, allow adequate time for complete degassing.]

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**Procedure—**

**PREPARATION OF SAMPLE—**Both the *Systemic Injection Test* and the *Intracutaneous Test* may be performed using the same extract, if desired, or separate extracts may be made for each test. Select and subdivide into portions a *Sample* of the size indicated in *Table 3*. Remove particulate matter, such as lint and free particles, by treating each subdivided *Sample* or *Negative Control* as follows. Place the *Sample* into a clean, glass-stoppered, 100-mL graduated cylinder of Type I glass, and add about 70 mL of *Water for Injection*. Agitate for about 30 s, and drain off the water. Repeat this step, and dry those pieces prepared for the extraction with *Vegetable Oil* in an oven at a temperature not exceeding 50°. [NOTE—Do not clean the *Sample* with a dry or wet cloth or by rinsing or washing with an organic solvent, surfactant, etc.]

**Table 3. Surface Area of Specimen To Be Used[a]**

| Form of Material | Thickness | Amount of Sample for each 20 mL of Extracting Medium | Subdivided into |
|---|---|---|---|
| Film or sheet | <0.5 mm | Equivalent of 120 $cm^2$ total surface area (both sides combined) | Strips of about 5 × 0.3 cm |
| | 0.5–1 mm | Equivalent of 60 $cm^2$ total surface area (both sides combined) | |
| Tubing | <0.5 mm (wall) | Length (in cm) = 120 $cm^2$/(sum of ID and OD circumferences) | Sections of about 5 × 0.3 cm |
| | 0.5–1 mm (wall) | Length (in cm) = 60 $cm^2$/(sum of ID and OD circumferences) | |
| Slabs, tubing, and molded items | >1 mm | Equivalent of 60 $cm^2$ total surface area (all exposed surfaces combined) | Pieces up to about 5 × 0.3 cm |
| Elastomers | >1 mm | Equivalent of 25 $cm^2$ total surface area (all exposed surfaces combined) | Do not subdivide[b] |

[a] When surface area cannot be determined due to the configuration of the specimen, use 0.1 g of elastomer or 0.2 g of plastic or other polymers for every 1 mL of extracting fluid.
[b] Molded elastomeric closures are tested intact.

**PREPARATION OF EXTRACTS—**Place a properly prepared *Sample* to be tested in an extraction container, and add 20 mL of the appropriate extracting medium. Repeat these directions for each extracting medium required for testing. Also, prepare one 20-mL blank of each medium for parallel injections and comparisons. Extract by heating in an autoclave at 121° for 60 min, in an oven at 70° for 24 h, or at 50° for 72 h. Allow adequate time for the liquid within the container to reach the extraction temperature.

[NOTE—The extraction conditions should not in any instance cause physical changes such as fusion or melting of the *Sample* pieces, which result in a decrease in the available surface area. A slight adherence of the pieces can be tolerated. Always add the cleaned pieces individually to the extracting medium. If culture tubes are used for autoclave extractions with *Vegetable Oil*, seal screw caps adequately with pressure-sensitive tape.]

Cool to about room temperature but not below 20°, shake vigorously for several minutes, and decant each extract immediately, using aseptic precautions, into a dry, sterile vessel. Store the extracts at a temperature of 20°–30°, and do not use for tests after 24 h. Of importance are the contact of the extracting medium with the available surface area of the plastic and the time and temperature during extraction, the proper cooling, agitation, and decanting process, and the aseptic handling and storage of the extracts following extraction.

## SYSTEMIC INJECTION TEST

This test is designed to evaluate systemic responses to the extracts of materials under test following injection into mice. Alternate routes of injection may be used with justification.

**Test Animals—**Use healthy, not previously used albino mice weighing 17–23 g. For each test group use only mice of the same source. Allow water and food, commonly used for laboratory animals and of known composition, ad libitum.

**Procedure—** [NOTE—Agitate each extract vigorously prior to withdrawal of injection doses to ensure even distribution of the extracted matter.] Inject each of the five mice in a test group with the *Sample* or the *Blank* as outlined in *Table 4*, except to dilute each g of the extract of the *Sample* prepared with *Polyethylene Glycol 400*, and the corresponding *Blank*, with 4.1 volumes of *Sodium Chloride Injection* to obtain a solution having a concentration of about 200 mg of polyethylene glycol per mL.

**Table 4. Injection Procedure—Systemic Injection Test**

| Extract or Blank | Dose per kg | Route[a] |
|---|---|---|
| Sodium Chloride Injection | 50 mL | IV |
| 1 in 20 solution of Alcohol in Sodium Chloride Injection | 50 mL | IV |
| Polyethylene Glycol 400 | 10 g | IP |
| Drug product vehicle (where applicable) | 50 mL | IV |
| | 50 mL | IP |

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**Table 4. Injection Procedure—Systemic Injection Test** *(continued)*

| Extract or Blank | Dose per kg | Route[a] |
|---|---|---|
| Vegetable Oil | 50 mL | IP |

[a] IV = intravenous (aqueous sample and blank); IP = intraperitoneal (oleaginous sample and blank).

Observe the animals immediately after injection, again 4 h after injection, and then at least at 24, 48, and 72 h. If during the observation period none of the animals treated with the extract of the *Sample* shows a significantly greater biological reactivity than the animals treated with the *Blank*, the *Sample* meets the requirements of this test. If two or more mice die, or if abnormal behavior such as convulsions or prostration occurs in two or more mice, or if a body weight loss greater than 2 g occurs in three or more mice, the *Sample* does not meet the requirements of the test. If any animals treated with the *Sample* show only slight signs of biological reactivity, and not more than one animal shows gross symptoms of biological reactivity or dies, repeat the test using groups of 10 mice. On the repeat test, all 10 animals treated with the *Sample* show no significant biological reactivity above the *Blank* animals during the observation period.

## INTRACUTANEOUS TEST

This test is designed to evaluate local responses to the extracts of materials under test following intracutaneous injection into rabbits or guinea pigs.

**Test Animals—**Select healthy, rabbits or guinea pigs with fur that can be clipped closely and skin that is free from mechanical irritation or trauma. In handling the animals, avoid touching the injection sites during observation periods, except to discriminate between edema and an oil residue.

**Procedure—** [NOTE—Agitate each extract vigorously prior to withdrawal of injection doses to ensure even distribution of the extracted matter.] On the day of the test, closely clip the fur on the animal's back on both sides of the spinal column over a sufficiently large test area. Avoid mechanical irritation and trauma. Remove loose hair by means of vacuum. If necessary, swab the skin lightly with diluted alcohol, and dry the skin prior to injection. More than one extract from a given material can be used per rabbit or guinea pig, if it is determined that the test results will not be affected. For each *Sample* use two animals and inject each intracutaneously, using one side of the animal for the *Sample* and the other side for the *Blank*, as outlined in *Table 5*. [NOTE—Dilute each g of the extract of the *Sample* prepared with *Polyethylene Glycol 400*, and the corresponding *Blank*, with 7.4 volumes of *Sodium Chloride Injection* to obtain a solution having a concentration of about 120 mg of polyethylene glycol per mL.]

**Table 5. Intracutaneous Test**

| Extract or Blank | Number of Sites (per animal) | Dose (µL per site) |
|---|---|---|
| Sample | 5 | 200 |
| Blank | 5 | 200 |

Examine injection sites for evidence of any tissue reaction such as erythema, edema, and necrosis. Swab the skin lightly, if necessary, with diluted alcohol to facilitate reading of injection sites. Observe all animals at 24, 48, and 72 h after injection. Rate the observations on a numerical scale for the extract of the *Sample* and for the *Blank*, using *Table 2*. Reclip the fur as necessary during the observation period. The average erythema and edema scores for *Sample* and *Blank* sites are determined at every scoring interval (24, 48, and 72 h) for each rabbit or guinea pig. After the 72-hour scoring, all erythema scores plus edema scores are totalled separately for each *Sample* and *Blank*. Divide each of the totals by 12 (2 animals × 3 scoring periods × 2 scoring categories) to determine the overall mean score for each *Sample* versus each corresponding *Blank*. The requirements of the test are met if the difference between the *Sample* and the *Blank* mean score is 1.0 or less. If at any observation period the average reaction to the *Sample* is questionably greater than the average reaction to the *Blank*, repeat the test using three additional rabbits or guinea pigs. The requirements of the test are met if the difference between the *Sample* and the *Blank* mean score is 1.0 or less.

## IMPLANTATION TEST

The implantation test is designed for the evaluation of plastic materials and other polymeric materials in direct contact with living tissue. Of importance are the proper preparation of the implant strips and their proper implantation under aseptic conditions. The intramuscular implantation test requires healthy adult New Zealand rabbits. The test specimens are placed into needles as the delivery system for implantation. Although most materials lend themselves readily to this method, there are a number of materials that are unsuitable for intramuscular implantation. For materials with physical characteristics unsuitable for routine intramuscular implantation, the subcutaneous rat implantation model is a viable alternative.

### Intramuscular Implantation in Rabbits

Prepare for implantation 8 strips of the *Sample* and 4 strips of USP High-Density Polyethylene RS. Each strip should measure not less than 10 × 1 mm. The edges of the strips should be as smooth as possible to avoid additional mechanical trauma upon

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implantation. Strips of the specified minimum size are implanted by means of a hypodermic needle (15–19 gauge) with intravenous point and a sterile trocar. Use either presterilized needles into which the sterile plastic strips are aseptically inserted, or insert each clean strip into a needle, the cannula and hub of which are protected with an appropriate cover, and then subjected to the appropriate sterilization procedure. [NOTE—Allow for proper degassing if agents such as ethylene oxide are used.]

**Test Animals—**Select healthy, adult rabbits weighing not less than 2.5 kg, and with paravertebral muscles that are sufficiently large in size to allow for implantation of the test strips. Do not use any muscular tissue other than the paravertebral site. The animals must be anesthetized with a commonly used anesthetic agent to a degree deep enough to prevent muscular movements, such as twitching. See the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) guidelines.

**Procedure—**Perform the test in a clean area. On the day of the test or up to 20 h before testing, clip the fur of the animals on both sides of the spinal column. Remove loose hair by means of vacuum. Swab the skin lightly with diluted alcohol, and dry the skin prior to injection.

Implant four strips of the *Sample* into the paravertebral muscle on one side of the spine of each of two rabbits, 2.5–5 cm from the midline and parallel to the spinal column, and about 2.5 cm apart from each other. In a similar fashion implant two strips of USP High-Density Polyethylene RS in the opposite muscle of each animal. Insert a sterile stylet into the needle to hold the implant strip in the tissue while withdrawing the needle. If excessive bleeding is observed after implantation of a strip, place a duplicate strip at another site.

Keep the animals for a period of not less than 120 h, and sacrifice them at the end of the observation period by administering an overdose of an anesthetic agent or other suitable agents. Allow sufficient time to elapse for the tissue to be cut without bleeding. Examine macroscopically the area of the tissue surrounding the center portion of each implant strip. Use a magnifying lens and auxiliary light source. Observe the *Sample* and *Control* implant sites for hemorrhage, necrosis, discolorations, and infections, and record the observations. Measure encapsulation, if present, by recording the width of the capsule (from the periphery of the space occupied by the implant *Control* or *Sample* to the periphery of the capsule) rounded to the nearest 0.1 mm. Score encapsulation according to *Table 6*.

**Table 6. Evaluation of Encapsulation in the Implantation Test**

| Capsule Width | Score |
|---|---|
| None | 0 |
| Up to 0.5 mm | 1 |
| 0.6–1.0 mm | 2 |
| 1.1–2.0 mm | 3 |
| Greater than 2.0 mm | 4 |

Calculate the differences between average scores for the *Sample* and *Control* sites. The requirements of the test are met if the difference does not exceed 1.0, or if the difference between the *Sample* and *Control* mean scores for more than one of the four implant sites does not exceed 1 for any implanted animal.

## Subcutaneous Implantation in Rats

Prepare for implantation 10 sample specimens and 10 control specimens. The size and shape of the control specimens shall be as similar to that of the test specimens as practically possible. For example, specimens made of sheeting material shall be 10–12 mm in diameter and from 0.3–1 mm in thickness. The edges of the specimens should be as smooth as possible to avoid additional mechanical trauma upon implantation.

**Test Animals—**Select healthy albino rats weighing 225–350 g at the time of implantation.

**Procedure—**Perform the test in a clean area. Anesthetize (see AAALAC guidelines) the animal until a surgical plane is achieved. Clip the fur of the animals on both sides of the spinal column. Remove loose hair by means of vacuum. Clean the clipped area with povidone–iodine solution. Using aseptic technique, make two midline incisions (approximately 1.0 cm long) through the skin at the cranial and caudal regions on the dorsal surface. Using blunt dissection, separate the fascia connecting skin to muscle to form a pocket underneath the skin lateral to each side of the incision (base of pocket approximately 20 mm from the line of implant). Insert a sterile sample into each pocket, and close the incision with wound clips or sutures. Implant two test samples and two control samples in each of five rats. Keep the animals for a period of at least seven days, and sacrifice them at the end of the observation period by $CO_2$ induced hypoxia or administering an overdose of an anesthetic agent. Allow sufficient time to elapse for the tissue to be cut without bleeding. Cut the skin (dorsal surface) longitudinally and lay back. Carefully examine macroscopically the area of the tissue surrounding the implant. Cut the sample in half and remove for close examination of the tissue in direct contact with the sample. Use a magnifying lens and auxiliary light source, if appropriate. Observe the *Sample* and *Control* implant sites for hemorrhage, necrosis, discolorations, and infections, and record the observations. Measure encapsulation, if present, by recording the width of the capsule (from the periphery of the space occupied by the implant *Control* or *Sample* to the periphery of the capsule) rounded to the nearest 0.1 mm. Score encapsulation according to *Table 6*. Calculate the differences between average scores for the *Sample* and *Control* sites. The requirements of the test are met if the difference does not exceed 1.0.

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# SAFETY TESTS—BIOLOGICALS

The safety test set forth here is intended to detect in an article any unexpected, unacceptable biological reactivity. This in vivo test is provided for the safety assessment of biotechnology-derived products.

## Safety Test

Select five healthy mice not previously used for testing, weighing 17–23 g, unless otherwise directed in the individual monograph or elsewhere in this chapter, and maintained on an adequate balanced diet. Prepare a test solution as directed in the individual monograph. Unless otherwise directed in the individual monograph or elsewhere in this chapter, inject a dose of 0.5 mL of the test solution into each of the mice, using a 26-gauge needle of suitable length, or of the length specified below as applicable. Observe the animals over the 48 h following the injection. If, at the end of 48 h, all of the animals survive and not more than one of the animals shows outward symptoms of a reaction not normally expected of the level of toxicity related to the article, the requirements of this test are met. If one or more animals die or if more than one of the animals shows signs of abnormal or untoward toxicity of the article under test, repeat the test using at least another 10 mice similar to those used in the initial test, but weighing 20 ± 1 g. In either case, if all of the animals survive for 48 h and show no symptoms of a reaction indicative of an abnormal or undue level of toxicity of the article, the requirements of the test are met. Body weights of mice before and at the end of the test should be obtained to detect any untoward effects. Animals that show signs of toxicity should be grossly necropsied and subjected to histopathology if necessary.

For biologics, perform the test according to the procedures prescribed in the *Code of Federal Regulations,* Section 610.11.

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# ⟨89.1⟩ COLLAGENASE I

```
IANTNSEKYD FEYLNGLSYT ELTNLIKNIK WNQINGLFNY STGSQKFFGD
KNRVQAIINA LQESGRTYTA NDMKGIETFT EVLRAGFYLG YYNDGLSYLN
DRNFQDKCIP AMIAIQKNPN FKLGTAVQDE VITSLGKLIG NASANAEVVN
NCVPVLKQFR ENLNQYAPDY VKGTAVNELI KGIEFDFSGA AYEKDVKTMP
WYGKIDPFIN ELKALGLYGN ITSATEWASD VGIYYLSKFG LYSTNRNDIV
QSLEKAVDMY KYGKIAFVAM ERITWDYDGI GSNGKKVDHD KFLDDAEKHY
LPKTYTFDNG TFIIRAGDKV SEEKIKRLYW ASREVKSQFH RVVGNDKALE
VGNADDVLTM KIFNSPEEYK FNTNINGVST DNGGLYIEPR GTFYTYERTP
QQSIFSLEEL FRHEYTHYLQ ARYLVDGLWG QGPFYEKNRL TWFDEGTAEF
FAGSTRTSGV LPRKSILGYL AKDKVDHRYS LKKTLNSGYD DSDWMFYNYG
FAVAHYLYEK DMPTFIKMNK AILNTDVKSY DEIIKKLSDD ANKNTEYQNH
IQELADKYQG AGIPLVSDDY LKDHGYKKAS EVYSEISKAA SLTNTSVTAE
KSQYFNTFTL RGTYTGETSK GEFKDWDEMS KKLDGTLESL AKNSWSGYKT
LTAYFTNYRV TSDNKVQYDV VFHGVLTDNA DISNNKAPIA KVTGPSTGAV
GRNIEFSGKD SKDEDGKIVS YDWDFGDGAT SRGKNSVHAY KKAGTYNVTL
KVTDDKGATA TESFTIEIKN EDTTTPITKE MEPNDDIKEA NGPIVEGVTV
KGDLNGSDDA DTFYFDVKED GDVTIELPYS GSSNFTWLVY KEGDDQNHIA
SGIDKNNSKV GTFKSTKGRH YVFIYKHDSA SNISYSLNIK GLGNEKLKEK
ENNDSSDKAT VIPNFNTTMQ GSLLGDDSRD YYSFEVKEEG EVNIELDKKD
EFGVTWTLHP ESNINDRITY GQVDGNKVSN KVKLRPGKYY LLVYKYSGSG
NYELRVNK
```

$C_{5099}H_{7771}N_{1329}O_{1610}S_{14}$
113,897 daltons (for $\beta$ subtype)
CAS RN®: 9001-12-1

## DEFINITION

Collagenase I (EC 3.4.24.3), isolated from *Clostridium histolyticum* and encoded by *colG* gene (GenBank accession number BAA77453.1), is a key raw material used in the dissociation or destruction of a broad range of tissue types. Collagenase I is a metalloprotease that acts as an endoprotease and also exhibits a tripeptidylcarboxypeptidase activity. It shows endopeptidic activity with the main cleavage site found in front of the human collagen duplex amino acids glycine–proline. Hydrolysis takes place near the ends of the triple helical domains of collagen.

Collagenase I is also known as class I collagenase and consists of three subtypes: α, β, and γ. Collagenase I β is the full-length enzyme while collagenase I α (68,000 Da) and collagenase I γ (79,000 Da) are thought to be proteolytic degradation products of collagenase I β caused by other proteases present in *C. histolyticum* (mainly a trypsin-like enzyme and clostripain/endoproteinase Arg C).

Collagenase I can be provided in a liquid formulation consisting of 5 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) and 1 mM calcium chloride pH 7.5, and stored as a frozen liquid. The specific activity of collagenase I is 0.10–0.60 units/mg of protein using 4-phenylazobenzyloxycarbonyl (PZ)-Pro-Leu-Gly-Pro-D-Arg as the substrate described in the *Assay*. The peak area for collagenase I is NLT 90% as determined by HPLC described in the test for *Purity*. The test for *Clostripain Activity* is used to assess the activity of the clostripain impurity and the acceptance criterion is NMT 0.5 units/mg of protein. The test for *Trypsin Activity* is used to assess the activity of the trypsin-like enzyme impurity and the acceptance criterion is NMT 0.5 units/mg of protein.

## IDENTIFICATION

- **A.** It meets the requirements in the *Assay*.
- **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the test for *Purity*.

## ASSAY

- **PROCEDURE**

**Tris buffer:** 0.1 M Tris pH 7.1, prepared as follows. Dissolve 6.05 g of tris(hydroxymethyl)aminomethane (Tris) in 400 mL of water, and adjust with 2 N hydrochloric acid to a pH of 7.1 (at 25 ± 1°). Dilute with water to a final volume of 500 mL.

**Substrate solution:** Dissolve 10 mg of PZ-Pro-Leu-Gly-Pro-D-Arg in 0.2 mL of methanol, and dilute this solution with *Tris buffer* to a final volume of 10 mL. [NOTE—Use a freshly prepared solution only.]

**Calcium chloride solution:** 0.1 M, prepared as follows. Weigh 1.47 g of calcium chloride dihydrate in a volumetric flask, and dilute with water to a final volume of 100 mL.

**Citric acid solution:** 0.025 M, prepared as follows. Weigh 525 mg of citric acid monohydrate in a volumetric flask, and dilute with water to a final volume of 100 mL.

**Extraction mixture:** To one test tube per sample to be assayed, pipette 5.0 mL of ethyl acetate and 1.0 mL of *Citric acid solution*. [NOTE—Use a freshly prepared mixture only.]

**Drying tube:** Into one test tube per sample to be assayed, add 0.35–0.40 g of sodium sulfate anhydrous. Seal the test tube with parafilm.

**Standard solution:** Dilute USP Collagenase I RS with *Tris buffer* in the range of 1:50 to 1:100 (v/v). [NOTE—Avoid freezing and thawing USP Collagenase I RS. After withdrawing USP Collagenase I RS, wipe off the outside of the plastic pipette tips to remove any residual solution.]

**Sample solutions:** Dilute collagenase I with *Tris buffer* to an appropriate dilution to achieve the absorbance range of 0.3–0.9 from the *Analysis*. Prepare in triplicate. [NOTE—Avoid freezing and thawing the collagenase I sample. After withdrawing the collagenase I sample, wipe off the outside of the tip to remove any residual solution.]

**Instrumental conditions**

(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)

**Mode:** UV

**Analytical wavelength:** 320 nm

**Path length:** 1 cm

**Temperature:** 25°

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**Analysis**

**Samples:** *Standard solution* and *Sample solutions*

Transfer 1.0 mL of *Substrate solution* and 0.2 mL of *Calcium chloride solution* into a test tube, and equilibrate the test tube to 25°. Start the reaction by adding 0.05 mL of *Standard solution* or each *Sample solution*. Prepare a blank by replacing the *Standard solution* or *Sample solution* with 0.05 mL of *Tris buffer*. Mix and incubate for exactly 15 min at 25°. Transfer 0.5 mL of the reaction to the test tube containing 6.0 mL of *Extraction mixture*. Vortex immediately for 20 s. Transfer 3 mL of the ethyl acetate phase (upper layer) into a *Drying tube* using a glass pipette, and vortex thoroughly. Transfer the supernatant to a disposable, semi-micro cuvette suitable for UV absorbance with a Pasteur pipette. Record the absorbance.

Calculate the activity of collagenase I in units/mL:

$$\text{Result} = (A - A_B) \times [V_T \times V_E/(\varepsilon \times V \times V_R \times B \times T)] \times D$$

$A$ = absorbance of the *Standard solution* or *Sample solution*
$A_B$ = absorbance of the blank
$V_T$ = volume of the reaction mixture, 1.25 mL
$V_E$ = volume of ethyl acetate in the *Extraction mixture*, 5.0 mL
$\varepsilon$ = extinction coefficient for 320 nm, 21 ($1\ cm^2 \cdot \mu mol^{-1}$)
$V$ = volume of the *Standard solution* or *Sample solution*, 0.05 mL
$V_R$ = volume of the reaction transferred to *Extraction mixture*, 0.5 mL
$B$ = absorption path length, 1 cm
$T$ = incubation time, 15 min
$D$ = dilution factor

[NOTE—One unit will release the equivalent of 1 µmol of PZ-Pro-Leu from PZ-Pro-Leu-Gly-Pro-D-Arg per minute under the conditions of the *Assay*.]

Calculate the specific activity of collagenase I in units/mg of protein:

$$\text{Result} = \textit{Activity}/C$$

*Activity* = activity of collagenase I (units/mL)
$C$ = protein concentration (mg/mL)

**System suitability**

**Samples:** *Standard solution* and *Sample solutions*

**Suitability requirements**

**Average calculated activity:** 85%–115% of the value on the label, *Standard solution*

**Absorbance:** 0.3–0.9, *Standard solution* and *Sample solutions*

**Acceptance criteria:** 0.10–0.60 units/mg of protein

## PURITY

• **PROCEDURE**

**Solution A:** 20 mM Tris and 1 mM calcium chloride pH 7.5, prepared as follows. Dissolve 2.42 g of Tris and 147 mg of calcium chloride dihydrate in 900 mL of water. Adjust with 2 N hydrochloric acid to a pH of 7.5. Dilute with water to a final volume of 1000 mL.

**Solution B:** 20 mM Tris, 1 mM calcium chloride, and 1 M sodium chloride pH 7.5, prepared as follows. Dissolve 2.42 g of Tris, 147 mg of calcium chloride dihydrate, and 58.44 g of sodium chloride in 900 mL of water. Adjust with 2 N hydrochloric acid to a pH of 7.5. Dilute with water to a final volume of 1000 mL.

**Mobile phase:** See *Table 1*.

**Table 1**

| Time (min) | Solution A (%) | Solution B (%) |
|---|---|---|
| 0 | 100 | 0 |
| 2 | 100 | 0 |
| 22 | 85 | 15 |
| 32 | 0 | 100 |
| 34 | 100 | 0 |
| 40 | 100 | 0 |

**Storage buffer:** 5 mM HEPES and 1 mM calcium chloride pH 7.5, prepared as follows. Dissolve 1.19 g of HEPES and 147 mg of calcium chloride dihydrate in 900 mL of water. Adjust with 4 N sodium hydroxide solution to a pH of 7.5. Dilute with water to a final volume of 1000 mL.

**Standard solution:** Thaw USP Collagenase I RS at room temperature shortly before use and mix. Store on ice or at 5°. Dilute with *Storage buffer* to achieve a protein concentration of 5.5 mg/mL. Transfer to an HPLC vial and keep at 5°. Prepare in duplicate, and inject each duplicate once.

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**Collagenase II solution:** Thaw USP Collagenase II RS at room temperature shortly before use and mix. Store on ice or at 5°. Dilute with *Storage buffer* to achieve a protein concentration of 5.5 mg/mL. Transfer to an HPLC vial and keep at 5°. Prepare in duplicate, and inject each duplicate once.
**Sample solution:** Dilute collagenase I with *Storage buffer* to achieve a protein concentration of 5.5 mg/mL and keep at 5°.
**Blank:** *Storage buffer*
**Chromatographic system**
(See *Chromatography* ⟨621⟩, *System Suitability*.)
**Mode:** LC
**Detector:** UV 280 nm
**Column:** 5-mm × 5-cm; 10-µm packing L91
**Temperatures**
**Column:** 25°
**Autosampler:** 5°
**Flow rate:** 1.5 mL/min
**Injection volume:** 20 µL
**System suitability**
**Sample:** *Standard solution*
**Suitability requirement:** The chromatogram from the *Standard solution* corresponds to the typical chromatogram provided with USP Collagenase I RS.
**Analysis**
**Sample:** *Sample solution*
The *Blank* should be considered for integration. Identify the peak corresponding to collagenase II by comparing its retention time to the main peak from the *Collagenase II solution*. Evaluate the purity of collagenase I using the area–% method but excluding peaks associated with the *Blank*. All shoulders in the fronting and tailing of the main peak are integrated by dropping a perpendicular line at the inflection points and considered as separate impurities. Disregard any peaks having retention times greater than 25 min.
**Acceptance criteria:** NLT 90% for the main peak of collagenase I and NMT 3% of collagenase II

## IMPURITIES

• **CLOSTRIPAIN ACTIVITY**

**Potassium phosphate buffer:** 0.1 M pH 7.6, prepared as follows. Dissolve 1.36 g of monobasic potassium phosphate in water, and dilute to 100 mL. Dissolve 2.28 g of dibasic potassium phosphate trihydrate in water, and dilute to 100 mL. Adjust the pH of the second solution to 7.6 with the first solution.
**Dithiothreitol solution:** 0.194 M, prepared as follows. Dissolve 60 mg of dithiothreitol (DTT) in 2 mL of *Potassium phosphate buffer*.
**Calcium chloride solution:** 0.01 M, prepared as follows. Dissolve 147 mg of calcium chloride dihydrate in 100 mL of water.
**Substrate stock solution:** 38 mM, prepared as follows. Dissolve 13 mg of *N*-benzoyl-L-arginine ethyl ester hydrochloride (BAEE · HCl) in 1 mL of *Potassium phosphate buffer*.
**Substrate solution:** 0.73 mM BAEE · HCl, 7.8 mM DTT, and 0.4 mM calcium chloride, prepared as follows. Transfer 0.5 mL of *Substrate stock solution*, 1.0 mL of *Dithiothreitol solution*, and 1.0 mL of *Calcium chloride solution* to a 25-mL volumetric flask, and dilute with *Potassium phosphate buffer* to volume.
**Sample solution:** Prepare in such a way that $\Delta A$/min lies in the 0.02–0.06 range. Dilute with ice-cold *Potassium phosphate buffer* if necessary.
**Instrumental conditions**
(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)
**Mode:** UV
**Analytical wavelength:** 255 nm
**Path length:** 1 cm
**Temperature:** 25°
**Analysis**
**Sample:** *Sample solution*
Transfer 3.0 mL of *Substrate solution* into a cuvette, and equilibrate the cuvette to 25°. Start the reaction by adding 0.05 mL of *Sample solution*. Prepare a blank by replacing the *Sample solution* with 0.05 mL of *Potassium phosphate buffer*. Mix well. Determine the change in absorbance ($\Delta A$/min) from the linear range of the reaction. Assay the *Sample solution* in triplicate.
**System suitability**
**Sample:** *Sample solution*
**Suitability requirement:** 0.02–0.06 for $\Delta A$/min
Calculate the activity of clostripain in units/mL in the portion of collagenase I taken:

$$\text{Result} = [V_T/(\varepsilon \times V_U \times B)] \times \Delta A/\text{min} \times D$$

$V_T$ = volume of the reaction mixture, 3.05 mL
$\varepsilon$ = extinction coefficient for 255 nm, 0.81 ($1\ \text{cm}^2 \cdot \text{mmol}^{-1}$)
$V_U$ = volume of the *Sample solution*, 0.05 mL
$B$ = absorption path length, 1 cm
$\Delta A$/min = change in absorbance from the linear range of the reaction
$D$ = dilution factor

Calculate the specific activity in units/mg of protein:

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$$\text{Result} = Activity/C$$

*Activity* = activity of clostripain (units/mL)
*C* = protein concentration (mg/mL)

**Acceptance criteria:** NMT 0.5 units of clostripain activity per mg of protein

- **TRYPSIN ACTIVITY**

**Buffer:** 0.1 M Tris and 0.02 M calcium chloride pH 8.0, prepared as follows. Dissolve 6.05 g of Tris and 1.45 g of calcium chloride dihydrate in 400 mL of water. Adjust with 2 N hydrochloric acid to a pH of 8.0 (at 25 ± 1°). Dilute with water to a final volume of 500 mL.

**Substrate stock solution:** Dissolve 10 mg of carbobenzoxy-valyl-glycyl-arginine-4-nitril-anilide acetate,[1] accurately weighed, in 1.5 mL of water. Store on ice. [NOTE—Use freshly prepared solution only.]

**Substrate solution:** Prepare a solution by mixing 9.2 mL of *Buffer* and 1.0 mL of *Substrate stock solution*. Store on ice. [NOTE—Use freshly prepared solution only.]

**Sample solution:** Undiluted collagenase I solution

**Instrumental conditions**

(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)

**Mode:** Vis

**Analytical wavelength:** 405 nm

**Path length:** 1 cm

**Temperature:** 25°

**Analysis**

**Sample:** *Sample solution*

Transfer 1.02 mL of *Substrate solution* into a polystyrene, semi-micro cuvette, allow the temperature to stabilize, equilibrate the cuvette to 25°, and wait for 10 min. Start the reaction by adding 0.10 mL of *Sample solution*. Start recording the absorbance and continue for at least 5 min after the addition of the *Sample solution*. Determine the change in absorbance ($\Delta A$/min) from the linear range of the reaction. Assay the *Sample solution* in triplicate. [NOTE—Use polyethylene pipette tips to transfer the *Sample solution*. The pipette tip should not be wet before transfer, and each pipette tip should be used only for transferring one sample. After withdrawing the *Sample solution*, wipe off the outside of the tip to remove any residual solution. After adding the *Sample solution* to the *Substrate solution*, rinse the tip by pipetting the solution up and down 2–3 times, discard the tip, and mix.]

**System suitability**

**Sample:** *Sample solution*

**Suitability requirement:** >0.01 for $\Delta A$/min

Calculate the activity of trypsin in units/mL in the portion of collagenase I taken:

$$\text{Result} = [V_T/(\varepsilon \times V_U \times B)] \times \Delta A/\text{min} \times D$$

$V_T$ = volume of the reaction mixture, 1.12 mL
$\varepsilon$ = extinction coefficient for 405 nm, 10.4 ($1\ cm^2 \cdot mmol^{-1}$)
$V_U$ = volume of the *Sample solution*, 0.10 mL
$B$ = absorption path length, 1 cm
$\Delta A$/min = change in absorbance from the linear range of the reaction
$D$ = dilution factor

Calculate the specific activity in units/mg of protein:

$$\text{Result} = Activity/C$$

*Activity* = activity of trypsin (units/mL)
*C* = protein concentration (mg/mL)

**Acceptance criteria:** NMT 0.5 units of trypsin activity per mg of protein

## SPECIFIC TESTS

- **PROTEIN CONTENT**

**Sample solutions:** Dilute collagenase I in water. Prepare at least in triplicate. [NOTE—Prepare the dilution using plastic pipette tips and not glass pipettes. Carefully wipe off the outside of the tip to remove any residual solution.]

**Blank solution:** Water

**Instrumental conditions**

(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)

**Mode:** UV

**Analytical wavelength:** 280 nm

**Path length:** 1 cm

**System suitability**

**Samples:** *Sample solutions*

**Suitability requirement:** Absorbance is in the range of 0.10–1.00.

[1] A suitable carbobenzoxy-valyl-glycyl-arginine-4-nitril-anilide acetate is Chromozym TRY from Roche Applied Science (catalog number 10378496103) or equivalent.

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**Analysis**

**Samples:** *Sample solutions* and *Blank solution*

Determine the net absorbance of *Sample solutions* by subtracting the absorbance of the *Blank solution* from the absorbance of each *Sample solution*. Determine the average net absorbance of *Sample solutions*.

Calculate the protein concentration in mg/mL:

$$\text{Result} = A_U \times D/\varepsilon$$

$A_U$ = average net absorbance of the *Sample solutions*
$D$ = dilution factor
$\varepsilon$ = extinction coefficient ($A_{280}$0.1%/cm) for collagenase, 1.4

- **Bacterial Endotoxins Test** ⟨85⟩**:** NMT 50 USP Endotoxin Units/mg of protein
- **Microbial Enumeration Tests** ⟨61⟩**:** The total bacterial count is NMT 100 cfu/mL.

## ADDITIONAL REQUIREMENTS

- **Packaging and Storage:** Store in closed containers at –15° to –25°.
- **Labeling:** The labeling states that the material is derived from *Clostridium histolyticum* along with the lot number, product or catalog number, and storage conditions.
- **USP Reference Standards** ⟨11⟩
  USP Collagenase I RS
  USP Collagenase II RS
  USP Endotoxin RS

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# ⟨89.2⟩ COLLAGENASE II

VQNESKRYTV SYLKTLNYYD LVDLLVKTEI ENLPDLFQYS SDAKEFYGNK
TRMSFIMDEI GRRAPQYTEI DHKGIPTLVE VVRAGFYLGF HNKELNEINK
RSFKERVIPS ILAIQKNPNF KLGTEVQDKI VSATGLLAGN ETAPPEVVNN
FTPILQDCIK NIDRYALDDL KSKALFNVLA APTYDITEYL RATKEKPENT
PWYGKIDGFI NELKKLALYG KINDNNSWII DNGIYHIAPL GKLHSNNKIG
IETLTEVMKV YPYLSMQHLQ SADQIKRHYD SKDAEGNKIP LDKFKKEGKE
KYCPKTYTFD DGKVIIKAGA RVEEEKVKRL YWASKEVNSQ FFRVYGIDKP
LEEGNPDDIL TMVIYNSPEE YKLNSVLYGY DTNNGGMYIE PEGTFFTYER
EAQESTYTLE ELFRHEYTHY LQGRYAVPGQ WGRTKLYDND RLTWYEEGGA
ELFAGSTRTS GILPRKSIVS NIHNTTRNNR YKLSDTVHSK YGASFEFYNY
ACMFMDYMYN KDMGILNKLN DLAKNNDVDG YDNYIRDLSS NYALNDKYQD
HMQERIDNYE NLTVPFVADD YLVRHAYKNP NEIYSEISEV AKLKDAKSEV
KKSQYFSTFT LRGSYTGGAS KGKLEDQKAM NKFIDDSLKK LDTYSWSGYK
TLTAYFTNYK VDSSNRVTYD VVFHGYLPNE GDSKNSLPYG KINGTYKGTE
KEKIKFSSEG SFDPDGKIVS YEWDFGDGNK SNEENPEHSY DKVGTYTVKL
KVTDDKGESS VSTTTAEIKD LSENKLPVIY MHVPKSGALN QKVVFYGKGT
YDPDGSIAGY QWDFGDGSDF SSEQNPSHVY TKKGEYTVTL RVMDSSGQMS
EKTMKIKITD PVYPIGTEKE PNNSKETASG PIVPGIPVSG TIENTSDQDY
FYFDVITPGE VKIDINKLGY GGATWVVYDE NNNAVSYATD DGQNLSGKFK
ADKPGRYYIH LYMFNGSYMP YRINIEGSVG R

$C_{5028}H_{7666}N_{1300}O_{1564}S_{21}$
112,023 (for ζ subtype)
CAS RN®: 9001-12-1

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**DEFINITION**

Collagenase II (EC 3.4.24.3), isolated from *Clostridium histolyticum* and encoded by the *colH* gene (GenBank accession number BAA06251.1), is a key raw material used in the dissociation or destruction of a broad range of tissue types. Collagenase II is a metalloprotease that acts as an endoprotease and also exhibits a tripeptidylcarboxypeptidase activity. It shows endopeptidic activity with the main cleavage site found in front of the human collagen duplex amino acids glycine–proline. Hydrolysis takes place in the interior of the triple helical domains of collagen.

Collagenase II is also known as class II collagenase and consists of three subtypes: δ, ε, and ζ. Collagenase II ζ is the full-length enzyme, whereas collagenase II δ (100,000 Da) and collagenase II ε (110,000 Da) are thought to be proteolytic degradation products of collagenase II ζ caused by other proteases present in *C. histolyticum* (mainly a trypsin-like enzyme and clostripain/endoproteinase Arg C).

Collagenase II can be provided in a liquid formulation consisting of 5 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) and 1 mM calcium chloride pH 7.5 and stored as a frozen liquid. The specific activity of collagenase II is 10–16 units/mg of protein using 4-phenylazobenzyloxycarbonyl (PZ)-Pro-Leu-Gly-Pro-D-Arg as the substrate described in the *Assay*. The peak area for collagenase II is NLT 90% as determined by HPLC described in the test for *Purity*. The test for *Clostripain Activity* is used to assess the activity of the clostripain impurity and the acceptance criterion is NMT 0.5 units/mg of protein. The test for *Trypsin Activity* is used to assess the activity of the trypsin-like enzyme impurity and the acceptance criterion is NMT 0.5 units/mg of protein.

**IDENTIFICATION**

- **A.** It meets the requirements in the *Assay*.
- **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the test for *Purity*.

**ASSAY**

***Change to read:***

- **PROCEDURE**

**Tris buffer:** 0.1 M Tris pH 7.1, prepared as follows. Dissolve 6.05 g of tris(hydroxymethyl)aminomethane (Tris) in 400 mL of water, and adjust with 2 N hydrochloric acid to a pH of 7.1 (at 25 ± 1°). Dilute with water to a final volume of 500 mL.

**Substrate solution:** Dissolve 10 mg of PZ-Pro-Leu-Gly-Pro-D-Arg in 0.2 mL of methanol, and dilute this solution with *Tris buffer* to a final volume of 10 mL. [NOTE—Use a freshly prepared solution only.]

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**Calcium chloride solution:** 0.1 M, prepared as follows. Weigh 1.47 g of calcium chloride dihydrate in a volumetric flask, and dilute with water to a final volume of 100 mL.

**Citric acid solution:** 0.025 M, prepared as follows. Weigh 525 mg of citric acid monohydrate in a volumetric flask, and dilute with water to a final volume of 100 mL.

**Extraction mixture:** To one test tube per sample to be assayed, pipette 5.0 mL of ethyl acetate and 1.0 mL of *Citric acid solution*. [NOTE—Use a freshly prepared mixture only.]

**Drying tube:** Into one test tube per sample to be assayed, add 0.35–0.40 g of sodium sulfate anhydrous. Seal the test tube with parafilm.

**Standard solution:** Dilute USP Collagenase II RS with *Tris buffer* ▲to an appropriate dilution to achieve the absorbance range of 0.3–0.9 from the *Analysis*.▲ (IRA 1-Sep-2022) [NOTE—Avoid freezing and thawing USP Collagenase II RS. After withdrawing USP Collagenase II RS, wipe off the outside of the plastic pipette tip to remove any residual solution.]

**Sample solutions:** Dilute Collagenase II with *Tris buffer* to an appropriate dilution to achieve the absorbance range of 0.3–0.9 from the *Analysis*. Prepare in triplicate. [NOTE—Avoid freezing and thawing the Collagenase II sample. After withdrawing the Collagenase II sample, wipe off the outside of the pipette tip to remove any residual solution.]

**Instrumental conditions**

(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)

**Mode:** UV

**Analytical wavelength:** 320 nm

**Path length:** 1 cm

**Temperature:** 25°

**Analysis**

**Samples:** *Standard solution* and *Sample solutions*

Transfer 1.0 mL of *Substrate solution* and 0.2 mL of *Calcium chloride solution* into a test tube, and equilibrate the test tube to 25°. Start the reaction by adding 0.05 mL of *Standard solution* or each *Sample solution*. Prepare a blank by replacing the *Standard solution* or *Sample solution* with 0.05 mL of *Tris buffer*. Mix and incubate for exactly 15 min at 25°. Transfer 0.5 mL of the reaction to the test tube containing 6.0 mL of *Extraction mixture*. Vortex immediately for 20 s. Transfer 3 mL of the ethyl acetate phase (upper layer) into a *Drying tube* using a glass pipette, and vortex thoroughly. Transfer the supernatant to a disposable, semi-micro cuvette suitable for UV absorbance with a Pasteur pipette. Record the absorbance.

Calculate the activity of collagenase II in units per milliliter:

$$\text{Result} = (A - A_B) \times [V_T \times V_E/(\varepsilon \times V \times V_R \times B \times T)] \times D$$

$A$ = absorbance of the *Standard solution* or *Sample solution*
$A_B$ = absorbance of the blank
$V_T$ = volume of the reaction mixture, 1.25 mL
$V_E$ = volume of ethyl acetate in the *Extraction mixture*, 5.0 mL
$\varepsilon$ = extinction coefficient for 320 nm, 21 ($1\ cm^2 \cdot \mu mol^{-1}$)
$V$ = volume of the *Standard solution* or *Sample solution*, 0.05 mL
$V_R$ = volume of the reaction transferred to *Extraction mixture*, 0.5 mL
$B$ = absorption path length, 1 cm
$T$ = incubation time, 15 min
$D$ = dilution factor

[NOTE—One unit will release the equivalent of 1 µmol of PZ-Pro-Leu from PZ-Pro-Leu-Gly-Pro-D-Arg per minute under the conditions of the *Assay*.]

Calculate the specific activity of collagenase II in units per milligram of protein:

$$\text{Result} = \text{Activity}/C$$

Activity = activity of collagenase II (units/mL)
$C$ = protein concentration (mg/mL)

**System suitability**

**Samples:** *Standard solution* and *Sample solutions*

**Suitability requirements**

**Average calculated activity:** 85%–115% of the value on the label, *Standard solution*

**Absorbance:** 0.3–0.9, *Standard solution* and *Sample solutions*

**Acceptance criteria:** 10–16 units/mg of protein

## PURITY

• **PROCEDURE**

**Solution A:** 20 mM Tris and 1 mM calcium chloride pH 7.5, prepared as follows. Dissolve 2.42 g of Tris and 147 mg of calcium chloride dihydrate in 900 mL of water. Adjust with 2 N hydrochloric acid to a pH of 7.5. Dilute with water to a final volume of 1000 mL.

**Solution B:** 20 mM Tris, 1 mM calcium chloride, and 1 M sodium chloride pH 7.5, prepared as follows. Dissolve 2.42 g of Tris, 147 mg of calcium chloride dihydrate, and 58.44 g of sodium chloride in 900 mL of water. Adjust with 2 N hydrochloric acid to a pH of 7.5. Dilute with water to a final volume of 1000 mL.

**Mobile phase:** See *Table 1*.

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**Table 1**

| Time (min) | Solution A (%) | Solution B (%) |
|---|---|---|
| 0 | 100 | 0 |
| 2 | 100 | 0 |
| 22 | 85 | 15 |
| 32 | 0 | 100 |
| 34 | 100 | 0 |
| 40 | 100 | 0 |

**Storage buffer:** 5 mM HEPES and 1 mM calcium chloride pH 7.5, prepared as follows. Dissolve 1.19 g of HEPES and 147 mg of calcium chloride dihydrate in 900 mL of water. Adjust with 4 N sodium hydroxide solution to a pH of 7.5. Dilute with water to a final volume of 1000 mL.

**Standard solution:** Thaw USP Collagenase II RS at room temperature shortly before use and mix. Store on ice or at 5°. Dilute with *Storage buffer* to achieve a protein concentration of 5.5 mg/mL. Transfer to an HPLC vial and keep at 5°. Prepare in duplicate, and inject each duplicate once.

**Sample solution:** Dilute Collagenase II with *Storage buffer* to achieve a protein concentration of 5.5 mg/mL and keep at 5°.

**Blank:** *Storage buffer*

**Chromatographic system**

(See *Chromatography* ⟨621⟩, *System Suitability*.)

**Mode:** LC

**Detector:** UV 280 nm

**Column:** 5-mm × 5-cm; 10-µm packing L91

**Temperatures**

**Autosampler:** 5°

**Column:** 25°

**Flow rate:** 1.5 mL/min

**Injection volume:** 20 µL

**System suitability**

**Sample:** *Standard solution*

**Suitability requirement:** The chromatogram from the *Standard solution* corresponds to the typical chromatogram provided with USP Collagenase II RS.

**Analysis**

**Sample:** *Sample solution*

The *Blank* should be considered for integration. Evaluate the purity of Collagenase II using the percentage peak area method but excluding peaks associated with the *Blank*. All shoulders in the fronting and tailing of the main peak are integrated by dropping a perpendicular line at the inflection points and considered as separate impurities. Disregard any peaks having retention times greater than 25 min.

**Acceptance criteria:** NLT 90% for the main peak of collagenase II

## IMPURITIES

• **Clostripain Activity**

**Potassium phosphate buffer:** 0.1 M pH 7.6, prepared as follows. Dissolve 1.36 g of monobasic potassium phosphate in water, and dilute to 100 mL. Dissolve 2.28 g of dibasic potassium phosphate trihydrate in water, and dilute to 100 mL. Adjust the pH of the second solution to 7.6 with the first solution.

**Dithiothreitol solution:** 0.194 M, prepared as follows. Dissolve 60 mg of dithiothreitol (DTT) in 2 mL of *Potassium phosphate buffer*.

**Calcium chloride solution:** 0.01 M, prepared as follows. Dissolve 147 mg of calcium chloride dihydrate in 100 mL of water.

**Substrate stock solution:** 38 mM, prepared as follows. Dissolve 13 mg of *N*-benzoyl-L-arginine ethyl ester hydrochloride (BAEE · HCl) in 1 mL of *Potassium phosphate buffer*.

**Substrate solution:** 0.73 mM BAEE · HCl, 7.8 mM DTT, and 0.4 mM calcium chloride, prepared as follows. Transfer 0.5 mL of *Substrate stock solution*, 1.0 mL of *Dithiothreitol solution*, and 1.0 mL of *Calcium chloride solution* to a 25-mL volumetric flask, and dilute with *Potassium phosphate buffer* to volume.

**Sample solution:** Prepare in such a way that $\Delta A$/min lies in the 0.02–0.06 range. Dilute with ice-cold *Potassium phosphate buffer* if necessary.

**Instrumental conditions**

(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)

**Mode:** UV

**Analytical wavelength:** 255 nm

**Path length:** 1 cm

**Temperature:** 25°

**Analysis**

**Sample:** *Sample solution*

Transfer 3.0 mL of *Substrate solution* into a cuvette, and equilibrate the cuvette to 25°. Start the reaction by adding 0.05 mL of *Sample solution*. Prepare a blank by replacing the *Sample solution* with 0.05 mL of *Potassium phosphate buffer*. Mix

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well. Determine the change in absorbance ($\Delta A$/min) from the linear range of the reaction. Assay the *Sample solution* in triplicate.

**System suitability**

**Sample:** *Sample solution*

**Suitability requirement:** 0.02–0.06 for $\Delta A$/min

Calculate the activity of clostripain in units per milliliter in the portion of Collagenase II taken:

$$\text{Result} = [V_T/(\varepsilon \times V_U \times B)] \times \Delta A/\text{ min} \times D$$

$V_T$ = volume of the reaction mixture, 3.05 mL
$\varepsilon$ = extinction coefficient for 255 nm, 0.81 (1 $cm^2 \cdot mmol^{-1}$)
$V_U$ = volume of the *Sample solution*, 0.05 mL
$B$ = absorption path length, 1 cm
$\Delta A$/min = change in absorbance from the linear range of the reaction
$D$ = dilution factor

Calculate the specific activity in units per milligram of protein:

$$\text{Result} = \text{Activity}/C$$

Activity = activity of clostripain (units/mL)
$C$ = protein concentration (mg/mL)

**Acceptance criteria:** NMT 0.5 units of clostripain activity per milligram of protein

- **TRYPSIN ACTIVITY**

**Buffer:** 0.1 M Tris and 0.02 M calcium chloride pH 8.0, prepared as follows. Dissolve 6.05 g of Tris and 1.45 g of calcium chloride dihydrate in 400 mL of water. Adjust with 2 N hydrochloric acid to a pH of 8.0 (at 25 ± 1°). Dilute with water to a final volume of 500 mL.

**Substrate stock solution:** Dissolve 10 mg of carbobenzoxy-valyl-glycyl-arginine-4-nitril-anilide acetate,[1] accurately weighed, in 1.5 mL of water. Store on ice. [NOTE—Use freshly prepared solution only.]

**Substrate solution:** Prepare a solution by mixing 9.2 mL of *Buffer* and 1.0 mL of *Substrate stock solution*. Store on ice. [NOTE—Use freshly prepared solution only.]

**Sample solution:** Undiluted Collagenase II solution

**Instrumental conditions**

(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)

**Mode:** Vis

**Analytical wavelength:** 405 nm

**Path length:** 1 cm

**Temperature:** 25°

**Analysis**

**Sample:** *Sample solution*

Transfer 1.02 mL of *Substrate solution* into a polystyrene semi-micro cuvette, allow the temperature to stabilize, equilibrate the cuvette to 25°, and wait for 10 min. Start the reaction by adding 0.10 mL of *Sample solution*. Start recording the absorbance and continue for at least 5 min after the addition of the *Sample solution*. Determine the change in absorbance ($\Delta A$/min) from the linear range of the reaction. *Assay* the *Sample solution* in triplicate. [NOTE—Use polyethylene pipette tips to transfer the *Sample solution*. The pipette tip should not be wet before transfer, and each pipette tip should be used only for transferring one sample. After withdrawing the *Sample solution*, wipe off the outside of the tip to remove any residual solution. After adding the *Sample solution* to the *Substrate solution*, rinse the tip by pipetting the solution up and down 2–3 times, discard the tip, and mix.]

**System suitability**

**Sample:** *Sample solution*

**Suitability requirement:** >0.01 for $\Delta A$/min

Calculate the activity of trypsin in units per milliliter in the portion of Collagenase II taken:

$$\text{Result} = [V_T/(\varepsilon \times V_U \times B)] \times \Delta A/\text{min} \times D$$

$V_T$ = volume of the reaction mixture, 1.12 mL
$\varepsilon$ = extinction coefficient for 405 nm, 10.4 (1 $cm^2 \cdot mmol^{-1}$)
$V_U$ = volume of the *Sample solution*, 0.10 mL
$B$ = absorption path length, 1 cm
$\Delta A$/min = change in absorbance from the linear range of the reaction
$D$ = dilution factor

Calculate the specific activity in units per milligram of protein:

$$\text{Result} = \text{Activity}/C$$

[1] A suitable carbobenzoxy-valyl-glycyl-arginine-4-nitril-anilide acetate is Chromozym TRY from Roche Applied Science (catalog number 10378496103) or equivalent.

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Activity = activity of trypsin (units/mL)
*C* = protein concentration (mg/mL)

**Acceptance criteria:** NMT 0.5 units of trypsin activity per milligram of protein

**SPECIFIC TESTS**

- **PROTEIN CONTENT**

**Sample solutions:** Dilute Collagenase II in water. Prepare at least in triplicate. [NOTE—Prepare the dilution using plastic pipette tips and not glass pipettes. Carefully wipe off the outside of the tip to remove any residual solution.]
**Blank solution:** Water
**Instrumental conditions**
(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)
**Mode:** UV
**Analytical wavelength:** 280 nm
**Path length:** 1 cm
**System suitability**
**Samples:** *Sample solutions*
**Suitability requirement:** Absorbance is in the range of 0.10–1.00.
**Analysis**
**Samples:** *Sample solutions* and *Blank solution*
Determine the net absorbance of *Sample solutions* by subtracting the absorbance of the *Blank solution* from the absorbance of each *Sample solution*. Determine the average net absorbance of *Sample solutions*.
Calculate the protein concentration in mg/mL:

$$\text{Result} = A_U \times D/\varepsilon$$

$A_U$ = average net absorbance of the *Sample solutions*
$D$ = dilution factor
$\varepsilon$ = extinction coefficient for 280 nm, 1.4 (mL · $mg^{-1}$ · $cm^{-1}$)

- **BACTERIAL ENDOTOXINS TEST** ⟨85⟩**:** NMT 50 USP Endotoxin Units/mg of protein

***Change to read:***

- **MICROBIAL ENUMERATION TESTS** ⟨61⟩**:** The total ▲▲ (IRA 1-Sep-2022) count is NMT 100 cfu/mL.

**ADDITIONAL REQUIREMENTS**

- **PACKAGING AND STORAGE:** Store in closed containers at –60° to –90°.
- **LABELING:** The labeling states that the material is derived from *Clostridium histolyticum* along with the lot number, product or catalog number, and storage conditions.

***Change to read:***

- **USP REFERENCE STANDARDS** ⟨11⟩

USP Collagenase II RS
▲▲ (IRA 1-Sep-2022)

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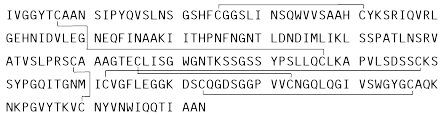
# ⟨89⟩ ENZYMES USED AS ANCILLARY MATERIALS IN PHARMACEUTICAL MANUFACTURING

## INTRODUCTION

The purpose of this chapter is to describe the quality attributes and associated tests with acceptance criteria for enzymatic preparations used in biopharmaceutical manufacturing. The quality of ancillary materials, including enzymes, used in biopharmaceutical manufacturing can have an impact on the therapeutic products. Several enzymes are used in this type of cell processing. Examples include trypsin, collagenase, pepsin, and papain. This chapter does not discuss applications of these enzymes but rather focuses on tests to assess their quality as process materials.

**Recombinant Trypsin**




$C_{1020}H_{1597}N_{287}O_{321}S_{14}$
23,463 (for β-Trypsin)
CAS RN®: 9002-07-7

## DEFINITION

Recombinant trypsin, a key raw material in biopharmaceutical manufacturing, is a serine protease that cleaves peptide chains mainly at the carboxyl end of the amino acids arginine and lysine. The amino acid sequence of recombinant trypsin is identical to that of trypsin from porcine pancreas, and the recombinant trypsin is produced by methods based on recombinant DNA technology in the yeast *Pichia pastoris*. Therefore, the specifications described in this chapter apply only to recombinant porcine trypsin produced in yeast. Because of the recombinant production process, recombinant trypsin is free of chymotrypsin. Two active forms of trypsin are known: β-trypsin (23,463 daltons) and α-trypsin (23,481 daltons). Autolysis of β-trypsin at the peptide bond between $Arg^{99}$ and $Val^{100}$, $Lys^{125}$ and $Ser^{126}$, or $Lys^{139}$ and $Ala^{140}$ results in three possible isoforms of α-trypsin. All isoforms are held together by disulfide bridges and remain correctly folded. As a consequence of hydrolysis of a peptide bond, the molecular weight of α-trypsin is more than that of β-trypsin by 18 daltons. The peak area for β-trypsin is NLT 70%, and the peak area for α-trypsin is NMT 20% as determined by the HPLC procedure described in the test for *Purity*. The specific activity is NLT 180 Units/mg of protein using carbobenzoxy-valyl-glycyl-arginine-4-nitril-anilide acetate as the substrate described in the *Assay*.

[NOTE—One Unit of trypsin activity using carbobenzoxy-valyl-glycyl-arginine-4-nitril-anilide acetate as the substrate corresponds to 21 USP Trypsin Units. One USP Trypsin Unit is the activity causing a change in absorbance of 0.003/min under the conditions specified in the *Assay* in the *Trypsin* monograph using *N*-benzoyl-L-arginine ethyl ester hydrochloride as the substrate. Therefore, the specific activity of 180 Units/mg of protein using carbobenzoxy-valyl-glycyl-arginine-4-nitril-anilide acetate as the substrate for recombinant trypsin corresponds to 3800 USP Trypsin Units/mg of protein.]

## IDENTIFICATION

- **A.** It meets the requirements in the *Assay*.
- **B.** The retention time of the major peak of β-trypsin from the *Sample solution* corresponds to that from the *Standard solution*, as obtained in the test for *Purity*.

## ASSAY

***Change to read:***

- **PROCEDURE**

**Buffer:** Dissolve 1.21 g of tris(hydroxymethyl)aminomethane and 0.29 g of calcium chloride dihydrate in 100 mL of water, and adjust with 2 N hydrochloric acid to a pH of 8.0 (at 25 ± 1°).

**Substrate stock solution:** Dissolve 20 mg of carbobenzoxy-valyl-glycyl-arginine-4-nitril-anilide acetate,[1] accurately weighed, in 3.0 mL of water. [NOTE—Use a freshly prepared solution only.]

**Substrate solution:** Prepare a solution by mixing 28 mL of *Buffer* and 2.8 mL of *Substrate stock solution*. [NOTE—Use a freshly prepared solution only.]

**Standard solutions:** Precool USP Trypsin Recombinant Porcine RS and water to approximately 4°. Start preparing *Standard solutions* immediately when the temperature has reached 4°. Prepare each *Standard solution* by diluting USP Trypsin Recombinant Porcine RS with water, at 1:68,921 dilution. Prepare at least five *Standard solutions* in parallel. Assay each *Standard solution* in duplicate. [NOTE—Use an adjustable pipettor for each measurement and dilution operation. Use ▲low protein binding▲ (USP 1-Dec-2021) test tubes for preparing *Standard solutions* and *Sample solutions*, and use ▲low protein binding▲ (USP 1-Dec-2021) pipet tips containing ▲low protein binding▲ (USP 1-Dec-2021) filters to transfer samples. The pipet tip should not be wet before transfer, and each pipet tip should be used only for transferring one sample.] [NOTE—A dilution of 1:68,921 can be achieved by three dilution steps in which each step has a 1:41 dilution (1:41/1:41/1:41). For example, for the initial dilution withdraw 0.1 mL of USP Trypsin Recombinant Porcine RS using a pipettor with a ▲low protein binding▲ (USP 1-Dec-2021) tip containing ▲low protein binding▲ (USP 1-Dec-2021) filters, wipe off the outside of the tip to remove any residual solution, add the Reference Standard to a ▲low protein binding▲ (USP 1-Dec-2021) test tube containing 4.0 mL of precooled water, rinse the tip by pipetting the solution up and down for 2–3 times, discard the tip, and mix the solution

[1] A suitable carbobenzoxy-valyl-glycyl-arginine-4-nitril-anilide acetate is Chromozym TRY from Roche ▲CustomBiotech▲ (USP 1-Dec-2021) (catalog number 10378496103) or equivalent.

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on a vortex mixer for approximately 2 s with maximum speed. For the second and third dilution steps, proceed as described for the initial dilution except for transferring 0.1 mL of solution from the previous dilution step.]

**Sample solutions:** Prepare at least five *Sample solutions* of recombinant trypsin in parallel as directed for the *Standard solutions* to obtain a final concentration of at least 0.16 Units/mL using precooled water, as the diluent. Each *Sample solution* is assayed in duplicate.

**Instrumental conditions**

(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)

**Mode:** UV

**Analytical wavelength:** 405 nm

**Path length:** 1 cm

**Temperature:** 25°

**System suitability**

**Samples:** *Standard solutions* and *Sample solutions*

**Suitability requirement:** $\Delta A$/min (calculated below) should be 0.03–0.07 for *Standard solutions* and *Sample solutions*. The average calculated activity for the *Standard solutions* is 90%–110% of the value on the label.

**Analysis**

**Samples:** *Standard solutions* and *Sample solutions*

Transfer 1.10 mL of *Substrate solution* into a ▲low protein binding▲ (USP 1-Dec-2021) semimicro cuvette, allow the temperature to stabilize, check the cuvette for the specified temperature, and wait for 10 min. Start the reaction by adding 0.020 mL of *Standard solution* or *Sample solution*. Record the absorbance for at least 5 min, and determine the change in absorbance per minute, $\Delta A$/min, from the linear range of the reaction.

Calculate the activity of recombinant trypsin in Units/mL:

$$\text{Activity} = [V_T/(\varepsilon \times V \times B)] \times (\Delta A/\text{min}) \times D$$

$V_T$ = volume of the reaction mixture, 1.12 mL
$\varepsilon$ = extinction coefficient for 405 nm, 10.4 ($\text{mmol}^{-1} \cdot 1\ \text{cm}^{-1}$)
$V$ = volume of *Standard solution* or *Sample solution*, 0.020 mL
$B$ = absorption path length, 1 cm
$\Delta A$/min = change in absorbance per minute
$D$ = dilution factor

[NOTE—One Unit will release the equivalent of 1 mmol of 4-nitril aniline from carbobenzoxy-valyl-glycyl-arginine-4-nitril-anilide acetate per min under the conditions of the *Assay*.]

Calculate the specific activity of recombinant trypsin in Units/mg of protein:

$$\text{Result} = \text{Activity}/C$$

Activity = activity of recombinant trypsin as previously calculated (Units/mL)
$C$ = protein concentration of recombinant trypsin (mg/mL)

**Acceptance criteria**

**Specific activity:** NLT 180 Units/mg of protein

**Relative standard deviation:** NMT 5% for the activities determined from 5 replicates

## PURITY

• **PROCEDURE**

**Solution A:** Dilute 1 mL of phosphoric acid (85%) with water, to 1000 mL.

**Solution B:** Dilute 1 mL of phosphoric acid (85%) with acetonitrile to 1000 mL.

**Mobile phase:** See *Table 1*.

**Table 1**

| Time (min) | Solution A (%) | Solution B (%) |
|---|---|---|
| 0 | 75 | 25 |
| 25 | 55 | 45 |
| 30 | 10 | 90 |
| 34 | 10 | 90 |
| 35 | 75 | 25 |
| 45 | 75 | 25 |

**Standard solution:** Thaw 100 µL of USP Trypsin Recombinant Porcine RS at room temperature for about 1 h, mix, and transfer to an HPLC vial. The target protein concentration should be 70 ± 10 mg/mL.

**Sample solution:** Thaw 100 µL of recombinant trypsin at room temperature for about 1 h, mix, and transfer to an HPLC vial. The target protein concentration should be 70 ± 10 mg/mL.

[NOTE—Keep *Standard solution* and *Sample solution* at 2°–8° if they are not ready to be injected immediately after preparation.]

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**Chromatographic system**
(See *Chromatography* ⟨621⟩, *System Suitability*.)
**Mode:** LC
**Detector:** UV 280 nm
**Column:** 4.6-mm × 25-cm; 3-µm packing L1 with a 200 Å pore size
**Column temperature:** 40°
**Flow rate:** 1.0 mL/min
**Injection volume:** 1 µL
**System suitability**
**Sample:** *Standard solution*
[NOTE—The retention time for the main peak of recombinant trypsin is 12–17 min.]
**Suitability requirements**
**Resolution:** NLT 1 between the peaks of α-trypsin and β-trypsin
**Analysis**
**Sample:** *Sample solution*
Record the chromatograms, and measure the peak areas. Evaluate the purity of trypsin using the area percentage method. Time of integration is 25 min. The blank should be considered for integration. Peaks that elute as a fronting shoulder of α-trypsin are integrated by perpendicular dropping if and only if a minimum is formed. Peaks that elute as a tailing shoulder of β-trypsin are evaluated by tangential integration if and only if a minimum is formed.
**Acceptance criteria:** NLT 70% for the peak area of β-trypsin and NMT 20% for the peak area of α-trypsin

## SPECIFIC TESTS

***Change to read:***

- **PROTEIN CONTENT**

**4 N hydrochloric acid solution:** Mix 10.4 mL of 25% hydrochloric acid with 9.6 mL of water.
**Storage buffer:** Dissolve 2.9 g of calcium chloride dihydrate in water, add 2.5 mL of *4 N hydrochloric acid solution*, and dilute with water, to a final volume of 1000 mL. Adjust with *4 N hydrochloric acid solution* to a pH of 2.0 ± 0.2, if necessary.
**Sample solutions:** Add 0.025 mL of recombinant trypsin to 3 mL of *Storage buffer*. Prepare at least in triplicate.
**Blank solution:** *Storage buffer*, 3 mL
**Instrumental conditions**
▲(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)▲ (USP 1-Dec-2021)
**Mode:** UV
**Analytical wavelength:** 280 nm
**Path length:** 1 cm
**System suitability**
**Sample:** *Sample solutions*
**Suitability requirement:** $\Delta A$ (as calculated below) is in the range of 0.13–1.8.
**Analysis**
**Samples:** *Sample solutions* and *Blank solution*
▲Calculate the change in absorbance ($\Delta A$):▲ (USP 1-Dec-2021)

$$\Delta A = A_U - A_B$$

$A_U$ = absorbance of the *Sample solution*
$A_B$ = absorbance of the *Blank solution*

Calculate the protein concentration in mg/mL:

$$\text{Result} = \left[\frac{(\Delta A \times F)}{A_{280}^{\frac{1\,\%}{1\text{cm}}}}\right] \times D$$

▲$\Delta A$ = change in absorbance, as calculated previously▲ (USP 1-Dec-2021)
$F$ = conversion factor from % to mg/mL, 10
$A_{280}^{\frac{1\,\%}{1\text{cm}}}$ = extinction coefficient for trypsin, 13.6
$D$ = dilution factor

***Change to read:***

- **MICROBIAL ENUMERATION TESTS** ⟨61⟩**:** The total ▲aerobic microbial count is NMT $10^2$ cfu/mL,▲ (USP 1-Dec-2021) the test being performed on 1 mL of recombinant trypsin in duplicate.

## ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Store in closed containers at –15° to –25°.
- **LABELING:** The labeling states that the material is of recombinant DNA origin, along with the product number and lot number, storage conditions, and expiration date.
- **USP REFERENCE STANDARDS** ⟨11⟩
USP Trypsin Recombinant Porcine RS

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# ⟨90⟩ FETAL BOVINE SERUM—QUALITY ATTRIBUTES AND FUNCTIONALITY TESTS

## INTRODUCTION

Fetal bovine serum (FBS) is the light-brown liquid fraction of clotted fetal bovine blood. It is depleted of cells, fibrin, and clotting factors. Although the complete composition of FBS is undefined, FBS contains high levels of growth factors and low levels of immunoglobulins. In addition, it contains other key ingredients that are essential in supporting proliferation of cells in culture. This product is used both in life science basic research and industrial manufacturing. FBS is a by-product of the meat industry and is collected from bovine fetuses removed from cattle found to be pregnant at slaughter. FBS is harvested from abattoirs that are inspected by the competent authority in the country of origin. Trained personnel following written and approved procedures should perform collection and processing. Blood is collected in a closed system in a dedicated area within the facility, and processed quickly to prevent hemolysis. The blood is allowed to clot and then typically is centrifuged in a refrigerated centrifuge to separate the serum from the other components. Serum typically is removed from the clot, transferred to labeled containers, and frozen. All manufacturers employ sterile filtration before final packaging. Additionally, gamma irradiation provides the highest assurance of the absence of viral activity. Gamma irradiation doses of 25–40 kGy provide significant log reduction of viral and other adventitious agents while preserving cellular growth performance.

The screening of FBS for viral contamination is accomplished by using all applicable testing described in the Code of Federal Regulations 9 CFR 113.53 (known as full 9 CFR testing). Mycoplasma assays are performed as described in *Mycoplasma Tests* ⟨63⟩.

## IDENTIFICATION

- **IDENTIFICATION—RADIAL IMMUNODIFFUSION**

**Reagents**

- FBS test samples
- Horse serum, negative control samples
- Bovine IgG calibrator (500 mg/L)
- Sheep albumin diluent (1% Sheep albumin, 0.18% EDTA, 1.75% NaCl, and 1.21% Tris/HCl pH 7.4).

**Materials/Apparatus:** Ring measuring device is calibrated in 0.1-mm increments. Radial immunodiffusion (RID) plates are commercially available and contain anti-bovine IgG antiserum in a 1.5% agarose gel, 0.1 M phosphate buffer, pH 7.0, 0.1% sodium azide as bacteriostatic agent, and 1 µg/mL amphotericin B as an antifungal agent. Store at 2°–8°. Use RID plates that can measure bovine IgG in the range of 50–500 mg/L.

**Standard curve:** Use the bovine IgG calibrators for system suitability and for generation of a calibration curve. Prepare two dilutions from a 500 mg/L bovine IgG stock solution. Dilute 120 µL of the 500 mg/L stock with 80 µL of diluent (medium dilution) and 25 µL of the 500 mg/L stock with 225 µL diluent (low dilution). Label each dilution respectively as 300 mg/L and 50 mg/L calibrators. Use the 500 mg/L, 300 mg/L, and 50 mg/L solutions to generate the standard curve. [NOTE—Prepare and analyse the calibrator bovine IgG solutions in duplicate.] Load 5 µL of each sample into the 2.5-mm wells of the plate. At 72 h of incubation, measure ring diameters to the nearest 0.1 mm using an appropriate ring measuring device. Record the results and proceed to the generation of a standard curve.

The ring diameter should develop to completion at room temperature for 72 h. Using the result from each data point of the standard curve, generate a single linearity plot where y is the squared diameter ($mm^2$) of precipitin ring around the well and x is the Bovine IgG concentration (mg/L). Calculate the linear least-squares-fit regression line of the form $y = m(x) + b$ with the help of suitable software and determine the values for slope (m), y-intercept (b), and coefficient of determination ($R^2$). The standard curve for the method is linear if $R^2$ is $\geq 0.98$.

**Analysis:** Frozen undiluted samples of FBS are thawed and tested within 24 h if stored at 4°. Testing of FBS test and USP Fetal Bovine Serum RS samples is performed in triplicate. Prepare RID plates containing anti-bovine IgG to be tested for the various types of sera. Allow plates and reagents to equilibrate to room temperature before use by leaving the plates open for 10–15 min at room temperature to allow any condensation in the wells or on the gel surface to evaporate. Samples should not be applied to wells where moisture is visible. Prepare serial dilutions, if necessary, of FBS test and USP Fetal Bovine Serum RS samples in diluent. Dilute the negative control horse serum in diluent. Load 5 µL of each sample into the 2.5-mm wells of the plate, and incubate at room temperature for 72 h. [NOTE—The test samples and the negative control are loaded on the same plate.]

**Calculation:** After 72 h, measure the diameters of the rings using the ring measuring device, and record the results. Using the regression equation developed under standard curve deviation, calculate the concentration of bovine IgG in FBS samples. Concentration is expressed as mg/L.

**Acceptance criteria:** Horse serum is negative (should not give a precipitation ring). FBS test and USP Fetal Bovine Serum RS samples are positive and contain NMT 500 mg/L of IgG.

- **HEMOGLOBIN CONTENT**

(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)

**Sample preparation:** FBS samples are thawed, are stored at 4°, and are tested within the same day.

**Analysis:** Determine the absorbance of the serum sample using a spectrophotometric cell of 1-cm path length at the wavelengths of absorbance at 576, 623, and 700 nm and using water as a blank. Calculate the concentration of hemoglobin in mg/dL:

$$(Abs_{576} \times 115) - (Abs_{623} \times 102) - (Abs_{700} \times 39.1)$$

**Acceptance criteria:** NMT 30 mg/dL

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## SPECIFIC TESTS

- **FBS Functionality Tests**

In the absence of a user-defined functionality assay, the following tests are suitable to determine the functionality of specific lots of FBS and to aid in the optimization of the growth conditions of mammalian cell cultures in the presence of FBS. For valid functionality confirmation independent of user-specific applications, tests are performed on the specified cell lines. For in-house validation of specialized cell culture applications, cell line(s) specific to those applications should be used and characterized. Use appropriate tissue culture vessels. Two tests described in this chapter are the *Growth-Promotion Curve* and the *Clonal Assay*. The decision about which type of test or the number of tests to be performed to assess suitability of a specific lot of FBS depends on the type of cell line used. For adherent cell lines, the number of colonies at the end of the culturing period represents a good assessment of the capacity of these cells, at low concentration, to grow in the presence of a specific lot of FBS. For cell lines growing in suspension cultures, the optimum growth kinetics is measured by counting viable cells after 7 days of culture.

**Cell lines:** Five cell lines are recommended for use:

1. HFL1 (ATCC CCL-153) normal lung, fibroblast
2. Mv1 Lu (ATCC CCL-64) mink lung, epithelial
3. HL-60 (ATCC CCL-240) peripheral blood promyeloblast, suspension
4. VERO (ATCC CCL-81) monkey kidney fibroblast
5. CHO (CCL-61) Chinese hamster ovary

The functionality tests described are to be performed on three cell lines, two of which are drawn from the five recommended cell lines and the third of which is the cell line relevant to the user's application. Cell lines are cultured with specific media as recommended by ATCC.

**Materials**

- Suitable growth vessel/container
- Biological Safety Cabinet Class II, Type A
- Cell counter/hemacytometer
- Inverted microscope with digital camera accessory
- Tissue culture vessels: T25 $cm^2$

**Preparation of cells for assays:** Quick-thaw a vial in a 37° water bath, and determine cell count and viability. Prepare multiple cultures from each cell line in serum-supplemented growth medium. Incubate the cultures at 37° following instructions provided by ATCC for each of the cell lines used for the test. Examine the prepared cultures under a microscope to ensure uniform, near-confluent monolayers or suspensions. Expand cells until there are enough for assay (about $1 \times 10^7$ total cells; >90% viability).

**Harvesting of cultures**

1. Remove and discard the growth medium, and then rinse each culture with media lacking FBS.
2. For adherent cells, add 1 mL of Trypsin/EDTA for a few minutes for cells to disperse. Incubate at 37°, if necessary. Neutralize with 1 mL culture medium containing at least 10% FBS.
3. Spin down the cells in a centrifuge. Aspirate off wash media, and resuspend cells in an appropriate volume for seeding.

**Seeding of cells**

1. On day 0: For the three cell lines to be tested prepare multiple cultures using seeding densities that range between $2 \times 10^3$ and $2 \times 10^4$ viable cells/mL. (Different inocula are chosen initially to determine optimum growth conditions. Once the appropriate inoculum is chosen, that condition is used to propagate the cells.) Following are the recommended seeding densities:
   Low seeding density: $2 \times 10^3$ viable cells/mL
   Mid seeding density: $6 \times 10^3$ viable cells/mL
   High seeding density: $2 \times 10^4$ viable cells/mL
2. Prepare cultures in triplicate for at least five time points (in days or hours according to the cell line), to determine the seeding density that will yield the optimal growth conditions for each cell line used.
3. Incubate the cultures at 37° in a humidified incubator saturated with 5% $CO_2$.
4. For each time point of measurement (days 0, 1, 2, 3, 4, and 7), take a photograph of each culture, in triplicate, for both the FBS test material and the USP Fetal Bovine Serum RS at each of the three concentrations for each cell line, and record the percentage of confluency for each of the conditions. [Note—Perform this step before trypsinization and cell counting.]
5. Harvest the cells from the three different seeding density cultures for each specific time point. For adherent cultures, harvest cells as described above.
6. Perform and record total cell count and viability for each of the nine cultures for the FBS test and the USP Fetal Bovine Serum RS for each cell line using an appropriate cell counter or hemacytometer. [Note—The schedule for counting may have to be changed for fast-growing cell lines or large cells that would become confluent before day 7 and/or for slow-growing lines that need to be in culture 8–10 days before reaching a plateau. Some adherent cell lines will never reach confluency.]

- **Growth-Promotion Curve**

Measurements of cell proliferation rates often are used to determine the response of cells to exogenous stimuli. Quantitative assessment of cell growth conditions is an important factor in monitoring consistency of culture conditions. The optimal cell concentration range for subculturing, optimum inoculum, and doubling time are parameters that can be quantified and trended. Information about the growth kinetics of a culture is critical in the design of cell-based experiments. Cultures

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vary significantly in their growth properties from lag phase, log phase, and stationary phase. Document the growth characteristics of the culture during the three growth stages to determine population doubling time and cell cycle time. Cells that have entered the stationary phase may demonstrate reduced growth potential and change in morphology. Cells may become polarized and may secrete more extracellular matrix, making them difficult to remove from the substratum. Cells at the end of the log phase give the highest yield and greatest reproducibility.

**Reagents**

- Growth media without FBS
- FBS test samples
- Growth medium + 10% FBS
- Trypsin/EDTA solution (0.25%/0.53 mM) in Hank's Balanced Salt Solution (HBSS)

**Analysis:** Once the cells have reached the end of the log phase, subculture the cells for the test. Follow the procedure described in *Specific Tests, FBS Functionality Tests, Seeding of cells* and prepare multiple cultures for the USP Fetal Bovine Serum RS, and test FBS for different cell lines at three seeding densities for which at least one growth curve displays a lag phase, log phase, and stationary phase and for which the log phase is linear at three or more time points. Viable cell counts are determined on days 0, 1, 2, 3, 4, and 7.

**Calculation** and **Data analysis:** Calculate the mean viable count [cells/$cm^2$ (adherent) or cells/mL (suspension)] and the mean percent viability for each data point. Plot the data on a semi-log scale graph with the viable count on the log scale on the *y*-axis and days (or hours) in culture on an arithmetic scale on the *x*-axis. Estimate the doubling time using a growth curve that is linear over three or more time points.

**Acceptance criteria:** The $R^2$ value of the line should be equal to or greater than 0.98 in order to support calculation of a valid doubling time. The doubling time of the test sample should be no less than 90% of the doubling time of USP Fetal Bovine Serum RS.

- **Clonal Assay**

This assay is designed to assess the optimal growth for adherent cell lines. Plating efficiency or colony formation at low cell density is a preferred method for analyzing the proliferative capacity and survival of single cells under optimal growth conditions. This is a very sensitive test and is often used for assessing the quality of serum lots. This technique reveals differences in the growth rate within the cell population and is capable of distinguishing between changes in growth rate (colony size) and cell survival (colony number). Because of the heterogeneous cell population of some cell cultures, remember that cells grow differently as isolated colonies at low densities. Consequently, few cells survive even under ideal conditions because all cell interaction is lost. Cloning is a survival assay that is also used for optimizing growth conditions (selection of medium and serum). If it can be confirmed that a single colony arose from a single cell, then cloning efficiency can be determined.

**Reagents**

- Growth medium + 10% FBS (test serum)—Eagle minimum essential medium (EMEM) with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium carbonate, 0.1 mM nonessential amino acids, and sodium pyruvate containing 100 U/mL penicillin and 100 g/mL streptomycin plus 10% FBS.
- Trypsin/EDTA solution (0.25%/0.53 mM) in HBSS.
- Dulbecco's Phosphate Buffer Saline without calcium or magnesium.
- Carbol Fuchsin–Methylene Blue Solution—Mix 20 g carbol fuchsin stock in 2 L methanol and stir for 10 min (1% carbol fuchsin). Mix 50 g methylene blue in 5 L methanol, and stir for 10 min (1% methylene blue). Prepare Carbol Fuschin–Methylene Blue working solution by mixing methylene blue, methanol, and carbol fuchsin in a ratio of 3:2:1. Mix for 20 min and filter through four folds of cheesecloth in a funnel. Aliquot and store in brown glass bottles at 15° to 25°.

**Sample:** Multiple lots of FBS are used for this assay. For each lot of serum to be tested, add 20 mL of FBS to 180 mL of EMEM, and use the same sample for the entire test. Sterilize using 0.22-m low protein binding filter units. Store growth medium at 4° until ready to use.

**Cell preparation:** This test is only for adherent cultures and is performed with the adherent cell lines described under *Cell Lines* (HFL1 and Mv 1 Lu). One week before testing serum, expand the cell lines as described under *Seeding of Cells*, change the medium every 2–3 days, and subculture the cells when they are about 90% confluent. Determine the cell count and viability (viability should be >90%) before performing the assay. Harvest cells as described under *Harvesting of Cultures*, wash twice, and resuspend cells in basal EMEM.

**Analysis:** The procedure involves plating single-cell suspension at low densities (2–50 cells/$cm^2$) from which discrete colonies will form. At the end of the assay, fix, stain, and count the number of colonies as directed below.

1. For each cell line label ten 60-mm × 15-mm tissue culture dishes for each serum lot that will be tested. Label the side of the lower half of each dish, including controls.
2. Transfer 5 mL of medium containing 10% of the appropriate test serum (10 replicates). Add 400 cells per culture dish (aim for a cell concentration of about 800 cells/mL).
3. Incubate for 10–14 days at 37° in a humidified incubator saturated with 5% $CO^2$.
4. Remove the supernatant and add enough Carbol Fuchsin–Methylene Blue Solution to cover each of the culture dishes for 10 min.
5. Remove the stain; rinse the culture dishes with several changes of distilled water; invert the dishes on paper towels; and allow to dry.
6. Count and record (1) the number of colonies and (2) the total surface of stained colonies ($mm^2$). Calculate means and standard deviations.

**Acceptance criteria:** Percent plating efficiency is expressed by counting the number of colonies in a defined area divided by the number of cells seeded multiplied by 100. Compare results between lots of FBS, and select a serum lot that is good for various types of cells and optimal for a specific cell culture application.

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- **pH** ⟨791⟩**:** 7.00–8.00, in undiluted serum samples

***Change to read:***

- **▲Osmolality and Osmolarity** ⟨785⟩
  **Osmolality:**▲ (Official 1-Aug-2022) 280–360 mOsmol/kg
- **Bacterial Endotoxins Test** ⟨85⟩**:** It contains not more than 10 USP Endotoxin Units/mL of serum.
- **Total Protein Content** ⟨1057⟩**:** 30–45 mg/mL
- **Sterility Tests** ⟨71⟩**:** Meets the requirements

**ADDITIONAL REQUIREMENTS**

- **Packaging and Storage:** Store in sealed containers at a temperature of –10° or below.
- **Labeling:** Label it to indicate that contents are Fetal Bovine Serum, and indicate lot number, expiration date, and storage conditions. Also, indicate country of origin on product labeling.
- **USP Reference Standards** ⟨11⟩
  USP Endotoxin RS
  USP Fetal Bovine Serum RS

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# ⟨91⟩ CALCIUM PANTOTHENATE ASSAY

## INTRODUCTION

The following liquid chromatographic procedures are provided for the determination of calcium pantothenate as an active pharmaceutical ingredient, a dietary supplement ingredient, or a component in dietary supplements or pharmaceutical dosage forms.

While conducting these procedures, protect solutions containing and derived from the test specimen and the Reference Standards from atmosphere and light, preferably with the use of low-actinic glassware. Use the appropriate USP Reference Standards.

## ASSAY

• **Chromatographic Methods, Procedure 1**

This procedure can be used to determine calcium pantothenate in:

- *Oil- and Water-Soluble Vitamins Capsules*
- *Oil- and Water-Soluble Vitamins Tablets*
- *Oil- and Water-Soluble Vitamins with Minerals Capsules*
- *Oil- and Water-Soluble Vitamins with Minerals Tablets*
- *Water-Soluble Vitamins Capsules*
- *Water-Soluble Vitamins Tablets*
- *Water-Soluble Vitamins with Minerals Capsules*
- *Water-Soluble Vitamins with Minerals Tablets*

This procedure involves the extraction of calcium pantothenate from the formulation by the *Internal standard solution*.

Unless specified in the individual monographs, the *Internal standard solution*, *Standard solution*, *Sample solution*, and reagent solutions are prepared as follows.

**Mobile phase:** Phosphoric acid and water (1:1000)

**Internal standard solution:** 80 mg of *p*-hydroxybenzoic acid in 3 mL of alcohol. Add 50 mL of water and 7.1 g of dibasic sodium phosphate, and dilute with water to 1000 mL. Adjust with phosphoric acid to a pH of 6.7.

**Standard solution:** 0.6 mg/mL of USP Calcium Pantothenate RS in *Internal standard solution*

**Sample solution** (for capsules)**:** Weigh NLT 20 capsules in a tared weighing bottle. Open the capsules, without losing shell material, and transfer the contents to a 100-mL beaker. Remove any contents adhering to the empty shells by washing, if necessary, with several portions of ether. Discard the washings, and dry the capsule shells with the aid of a current of dry air until the odor of ether is no longer perceptible. Weigh the empty capsule shells in the tared weighing bottle, and calculate the average net weight per capsule. Transfer a quantity of mixed capsule contents and a volume of *Internal standard solution* to a centrifuge tube to obtain a concentration of 0.6 mg/mL in the *Sample solution*.

**Sample solution** (for tablets)**:** Finely powder NLT 30 tablets. Transfer a portion of the powder, equivalent to a nominal amount of 15 mg of calcium pantothenate, to a centrifuge tube. Add 25.0 mL of the *Internal standard solution*, and shake vigorously for 10 min. Centrifuge, filter, and use the clear filtrate.

**Chromatographic system**

(See *Chromatography* ⟨621⟩*, System Suitability*.)

**Mode:** LC

**Detector:** UV 210 nm

**Column:** 3.9-mm × 15-cm; packing L1

**Flow rate:** 1.5 mL/min

**Injection volume:** 10 µL

**System suitability**

**Sample:** *Standard solution*

[Note—The relative retention times for calcium pantothenate and *p*-hydroxybenzoic acid are about 0.5 and 1.0, respectively.]

**Suitability requirements**

**Relative standard deviation:** NMT 3.0%

**Analysis**

**Samples:** *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of the sample taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

$R_U$ = peak response ratio of calcium pantothenate to *p*-hydroxybenzoic acid from the *Sample solution*
$R_S$ = peak response ratio of calcium pantothenate to *p*-hydroxybenzoic acid from the *Standard solution*
$C_S$ = concentration of USP Calcium Pantothenate RS in the *Standard solution* (mg/mL)
$C_U$ = nominal concentration of calcium pantothenate in the *Sample solution* (mg/mL)

• **Chromatographic Methods, Procedure 2**

This procedure can be used to determine calcium pantothenate in:

- *Oil- and Water-Soluble Vitamins Capsules*
- *Oil- and Water-Soluble Vitamins Tablets*
- *Oil- and Water-Soluble Vitamins with Minerals Capsules*

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- *Oil- and Water-Soluble Vitamins with Minerals Tablets*
- *Water-Soluble Vitamins Capsules*
- *Water-Soluble Vitamins Tablets*
- *Water-Soluble Vitamins with Minerals Capsules*
- *Water-Soluble Vitamins with Minerals Tablets*

This procedure involves dissolving an appropriate quantity of the sample equivalent to 10 mg of calcium pantothenate in 10 mL of methanol and diluting with water to 250 mL.

Unless specified in the individual monographs, the *Standard solution*, *Sample solution*, and reagent solutions are prepared as follows.

**Buffer solution:** Dissolve 10.0 g of monobasic potassium phosphate in 2000 mL of water, and adjust with phosphoric acid to a pH of 3.5.

**Mobile phase:** Methanol and *Buffer solution* (1:9)

**Standard stock solution:** 0.25 mg/mL of USP Calcium Pantothenate RS in water. Prepare fresh every 4 weeks. Store in a refrigerator.

**Standard solution:** 40 µg/mL of USP Calcium Pantothenate RS from the *Standard stock solution* diluted with water

**Sample solution** (for capsules)**:** Weigh NLT 20 capsules in a tared weighing bottle. Open the capsules, without losing shell material, and transfer the contents to a 100-mL beaker. Remove any contents adhering to the empty shells by washing, if necessary, with several portions of ether. Discard the washings, and dry the capsule shells with the aid of a current of dry air until the odor of ether is no longer perceptible. Weigh the empty capsule shells in the tared weighing bottle, and calculate the average net weight per capsule. Transfer a portion of the capsule contents, equivalent to 10 mg of calcium pantothenate, to a 250-mL volumetric flask. Add 10 mL of methanol, and swirl the flask to disperse the capsule contents. Dilute with water to volume, mix, and filter.

**Sample solution** (for tablets)**:** Finely powder NLT 20 tablets. Transfer a portion of the powder, equivalent to 10 mg of calcium pantothenate, to a 250-mL volumetric flask. Add 10 mL of methanol, and swirl the flask to disperse. Dilute with water to volume, mix, and filter.

**Chromatographic system**

(See *Chromatography* ⟨621⟩*, System Suitability*.)

**Mode:** LC

**Detector:** UV 205 nm

**Column:** 3.9-mm × 30-cm; 5-µm packing L1

**Column temperature:** 50°

**Flow rate:** 2 mL/min

**Injection volume:** 25 µL

**System suitability**

**Sample:** *Standard solution*

**Suitability requirements**

**Relative standard deviation:** NMT 3.0%

**Analysis**

**Samples:** *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of the sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

$r_U$ = peak response of calcium pantothenate from the *Sample solution*
$r_S$ = peak response of calcium pantothenate from the *Standard solution*
$C_S$ = concentration of USP Calcium Pantothenate RS in the *Standard solution* (mg/mL)
$C_U$ = nominal concentration of calcium pantothenate in the *Sample solution* (mg/mL)

- **Chromatographic Methods, Procedure 3**

This procedure can be used to determine calcium pantothenate in:

- *Oil- and Water-Soluble Vitamins Oral Solution*
- *Oil- and Water-Soluble Vitamins with Minerals Oral Solution*
- *Water-Soluble Vitamins with Minerals Oral Solution*

This procedure involves diluting an appropriate quantity of the liquid sample with water to a final concentration of 80 µg/mL of calcium pantothenate.

Unless specified in the individual monographs, the *Standard solution*, *System suitability solution*, *Sample solution*, and reagent solutions are prepared as follows.

**Mobile phase:** 0.2 M monobasic sodium phosphate and methanol (97:3). Adjust with 1.7 M phosphoric acid to a pH of 3.2 ± 0.1.

**Standard solution:** 80 µg/mL of USP Calcium Pantothenate RS in *Mobile phase*

**System suitability solution:** 80 µg/mL of USP Racemic Panthenol RS in *Mobile phase*. Mix the resulting solution and *Standard solution* (1:1).

**Sample solution:** Equivalent to 80 µg/mL of calcium pantothenate from the oral solution in *Mobile phase*

**Chromatographic system**

(See *Chromatography* ⟨621⟩*, System Suitability*.)

**Mode:** LC

**Detector:** UV 210 nm

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**Column:** 4.0-mm × 10-cm; packing L1
**Flow rate:** 1 mL/min
**Injection volume:** 20 µL

**System suitability**
**Samples:** *Standard solution* and *System suitability solution*
**Suitability requirements**
**Resolution:** NLT 1.5 between panthenol and calcium pantothenate, *System suitability solution*
**Tailing factor:** NMT 2.0 for the calcium pantothenate peak, *Standard solution*
**Relative standard deviation:** NMT 2.0%, *Standard solution*

**Analysis**
**Samples:** *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of the sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

$r_U$ = peak response of calcium pantothenate from the *Sample solution*
$r_S$ = peak response of calcium pantothenate from the *Standard solution*
$C_S$ = concentration of USP Calcium Pantothenate RS in the *Standard solution* (mg/mL)
$C_U$ = nominal concentration of calcium pantothenate in the *Sample solution* (mg/mL)

• **Chromatographic Methods, Procedure 4**
This procedure can be used to determine calcium pantothenate in:
- Active pharmaceutical ingredients
- Dietary ingredients

This procedure involves the dissolution of the sample directly into water.
Unless specified in the individual monographs, the *Standard solution*, *System suitability solution*, *Sample solution*, and reagent solutions are prepared as follows.
**Buffer solution:** Dissolve 3.2 g of monobasic sodium phosphate in 1 L of water and adjust with 1 N sodium hydroxide to a pH of 5.5.
**Mobile phase:** Acetonitrile and *Buffer solution* (2:98)
**System suitability solution:** 0.1 mg/mL of USP Calcium Pantothenate RS and 0.5 mg/mL each of USP Beta Alanine RS, USP Sodium D-Pantoate RS, and USP Pantolactone RS in water
**Standard solution:** 0.5 mg/mL of USP Calcium Pantothenate RS in water
**Sample solution:** 0.5 mg/mL of Calcium Pantothenate in water
**Chromatographic system**
(See *Chromatography* ⟨621⟩, *System Suitability*.)
**Mode:** LC
**Detector:** UV 200 nm
**Column:** 4.6-mm × 25-cm; 5-µm packing L1
**Column temperature:** 35°
**Flow rate:** 2 mL/min
**Injection volume:** 10 µL

**System suitability**
**Samples:** *System suitability solution* and *Standard solution*
[Note—The relative retention times for beta alanine, pantoic acid, pantothenic acid, and pantolactone are 0.3, 0.6, 1.0, and 1.9 min, respectively.]
**Suitability requirements**
**Resolution:** NLT 5.0 between the pantothenic acid and pantoic acid peaks, *System suitability solution*
**Tailing factor:** NMT 2.0, *Standard solution*
**Relative standard deviation:** NMT 2.0%, *Standard solution*

**Analysis**
**Samples:** *Standard solution* and *Sample solution*
Calculate the percentage of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of the sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

$r_U$ = peak response of calcium pantothenate from the *Sample solution*
$r_S$ = peak response of calcium pantothenate from the *Standard solution*
$C_S$ = concentration of USP Calcium Pantothenate RS in the *Standard solution* (mg/mL)
$C_U$ = concentration of Calcium Pantothenate in the *Sample solution* (mg/mL)

## ADDITIONAL REQUIREMENTS

• **USP Reference Standards** ⟨11⟩
USP Beta Alanine RS
USP Calcium Pantothenate RS
USP Pantolactone RS

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USP Racemic Panthenol RS
USP Sodium D-Pantoate RS

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# ⟨92⟩ GROWTH FACTORS AND CYTOKINES USED IN CELL THERAPY MANUFACTURING

## INTRODUCTION

Qualification of reagents, source materials, and control of the manufacturing process are key elements that ensure the quality and safety of cell therapies. Growth factors and cytokines are important for the maintenance, growth, selection, and purification of cultures of cell therapy products. This chapter describes the accepted tests, procedures, and acceptance criteria for growth factors and cytokines that may be involved in the manufacturing of cell therapy products.

**RECOMBINANT HUMAN INTERLEUKIN 4 (rhIL-4)**

| | | | |
|---|---|---|---|
| MHKCDITLQE | IIKTLNSLTE | QKTLCTELTV | TDIFAASKNT |
| TEKETFCRAA | TVLRQFYSHH | EKDTRCLGAT | AQQFHRHKQL |
| IRFLKRLDRN | LWGLAGLNSC | PVKEANQSTL | ENFLERLKTI |
| MREKYSKCSS | | | |

$C_{658}H_{1071}N_{193}O_{197}S_8$ 15,096 Da

rhIL-4 is a single-chain polypeptide of 130 amino acid residues expressed in *Escherichia coli*. It is produced as a lyophilized powder and contains NLT $0.5 \times 10^7$ USP Units of IL-4/mg of total protein. Process specific host-cell DNA impurities in IL-4 with limits of less than 1 ng/mg are determined as described in *Nucleic Acid-Based Techniques—Approaches for Detecting Trace Nucleic Acids (Residual DNA Testing)* ⟨1130⟩. Neither manufacturing license nor market approval is required for IL-4 intended for use as an ancillary material during manufacturing. Following are typical IL-4 quality attributes.

## IDENTIFICATION

- **A.** Amino-terminal sequence analysis of at least eight amino acids is performed with an automated sequencer. Stepwise-released phenylthiohydantoin amino acids are identified with on-line reversed-phase high-performance liquid chromatography, on the basis of their elution times.
- **B.** Use the electrophoresis method followed by western blotting analysis to visualize the IL-4 protein. The method is sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), described in the test for *Purity*.

  **Phosphate buffered saline; Laemmli sample buffer, reducing;** and **Laemmli sample buffer, nonreducing:** Proceed as directed in the test for *Purity* in the *Assay*.

  **Standard stock solution:** 50 µg/mL of reconstituted USP rHuman Interleukin 4 RS in *Phosphate buffered saline*. [NOTE—Do not agitate while mixing; swirl gently.]

  **Standard solution:** 20 µg/mL of IL-4, from *Standard stock solution*, in *Phosphate buffered saline*

  **Standard solution, reducing:** Combine 20 µL of *Standard solution* and 5 µL of *Laemmli sample buffer, reducing*.

  **Standard solution, nonreducing:** Combine 20 µL of *Standard solution* and 5 µL of *Laemmli sample buffer, nonreducing*.

  **Sample stock solution:** 50 µg/mL of reconstituted IL-4 in *Phosphate buffered saline*. [NOTE—Do not agitate while mixing; swirl gently.]

  **Sample solution:** 20 µg/mL of IL-4, from *Sample stock solution*, in *Phosphate buffered saline*

  **Sample solution, reducing:** Combine 20 µL of *Sample solution* and 5 µL of *Laemmli sample buffer, reducing*.

  **Sample solution, nonreducing:** Combine 20 µL of *Sample solution* and 5 µL of *Laemmli sample buffer, nonreducing*.

  **Analysis**

  **Samples:** *Standard solution, reducing; Standard solution, nonreducing; Sample solution, reducing;* and *Sample solution, nonreducing*

  **Western blotting:** After electrophoresis, the proteins are transferred onto a polyvinylidene fluoride (PVDF) membrane using standard procedures. Incubate the membrane for 1 h at room temperature with *Phosphate buffered saline* containing 0.1% Tween 20 and 5% skim milk powder. The membrane is then incubated with an anti-IL-4 antibody[1] (diluted appropriately in *Phosphate buffered saline*), followed by incubation with a secondary antibody at room temperature under gentle agitation for 1 h for each of the antibodies. The IL-4 protein band is identified by developing the membrane using a suitable detection system.[2]

  **Acceptance criteria:** The developed Western blot should give a positive signal equivalent to the USP rHuman Interleukin 4 RS.

## ASSAY

- **PURITY:** [NOTE—Purity is determined on the bulk material.] SDS-PAGE is performed as described under *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* ⟨1056⟩ under reducing and nonreducing conditions.

  **Molecular weight marker:** Use a suitable molecular weight marker containing protein bands between 10 and 200 kDa.

  **Phosphate buffered saline:** 2.67 mM of potassium chloride, 1.47 mM of potassium phosphate ($KH_2PO_4$), 137.93 mM of sodium chloride, and 8.06 mM of dibasic sodium phosphate in water. Adjust to a pH of 7.0–7.3.

  **Laemmli sample buffer, nonreducing:** 100 mM TRIS-HCl, pH 6.8, 50% glycerol, 0.25% bromophenol blue indicator, and 10% sodium lauryl sulfate in water

  **Laemmli sample buffer, reducing:** Add 2.5 µL mercaptoethanol to 50 µL of *Laemmli sample buffer, nonreducing*.

  **Sample stock solution:** 400 µg/mL of bulk IL-4 in *Phosphate buffered saline*

[1] A suitable anti-IL-4 antibody can be obtained from commercial sources (e.g., Dianova Inc.).
[2] A suitable detection system can be obtained from commercial sources (e.g., Pierce/Perbio Science).

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**Sample solution 1:** Combine 20 µL of *Sample stock solution* and 5 µL of *Laemmli sample buffer, nonreducing*.
**Sample solution 2:** Combine 20 µL of *Sample stock solution* and 5 µL of *Laemmli sample buffer, reducing*.
**Control A stock solution:** 4 µg/mL of IL-4, from *Sample stock solution*, in *Phosphate buffered saline*. [NOTE—*Control A solutions* are run in triplicates in both reducing and nonreducing conditions.]
**Control A solution 1:** Combine 20 µL of *Control A stock solution* and 5 µL of *Laemmli sample buffer, nonreducing*.
**Control A solution 2:** Combine 20 µL of *Control A stock solution* and 5 µL of *Laemmli sample buffer, reducing*.
**Control B stock solution:** 12 µg/mL of IL-4, from *Sample stock solution*, in *Phosphate buffered saline*. [NOTE—*Control B solutions* are run in duplicates in both reducing and nonreducing conditions.]
**Control B solution 1:** Combine 20 µL of *Control B stock solution* and 5 µL of *Laemmli sample buffer, nonreducing*.
**Control B solution 2:** Combine 20 µL of *Control B stock solution* and 5 µL of *Laemmli sample buffer, reducing*.
**Electrophoretic conditions**
(See *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* ⟨1056⟩.)
**Mode:** Discontinuous PAGE gel
**Stacking gel:** 4% acrylamide
**Resolving gel:** 12% acrylamide
**Run conditions:** 10 min at 100 V; then 30 min at 200 V
**Protein detection:** Silver stain
**Analysis**
**Samples:** *Sample solution 1, Sample solution 2, Control A solution 1, Control A solution 2, Control B solution 1*, and *Control B solution 2*
Incubate 25 µL of each *Sample solution* and *Control solution* under nonreducing conditions for 5 min at 60°, and load onto the gel. Incubate 20 µL of each *Sample solution* and *Control solution* under reducing conditions for 5 min at 60°, and load onto the gel. After silver staining and scanning the whole gel, determine the intensity of all detectable protein bands by densitometry, and calculate the percentage of each detectable protein band, in the *Sample solution*, twice by comparing the pixel intensity of each contaminating band with the mean value of *Control solutions A* and *B*, respectively, by the formulas:

$$\text{Result} = (A_{100}) \times 1/(A_1) \text{ and}$$

$$\text{Result} = (A_{100}) \times 3/(A_3)$$

$A_{100}$ = intensity of one contaminating band of the *Sample solution*
$A_1$ = mean intensity of all detectable bands of *Control A solution*
$A_3$ = mean intensity of all detectable bands of *Control B solution*

IL-4 control solutions analysis should yield one detectable band with an apparent molecular weight of approximately 15 kDa. If values calculated by means of *Control A solution* are different from those revealed by comparison with *Control B solution*, the value corresponding to the highest amount of impurity should be taken. If the intensity of one of the contaminating bands is lower than the value of *Control A solution* (corresponding to 1%), the value of this contamination is set to 1%. The purity of the sample solution is then calculated:

$$\text{Result} = 100 - \Sigma\, C_n$$

C = percentage of each contamination given in rounded whole numbers
n = number of contaminants of the IL-4 *Sample solution*

**Acceptance criteria:** The purity of IL-4 is NLT 97%, as determined by SDS-PAGE.

• **PROTEIN CONTENT:** [NOTE—Protein content is determined on the basis of the packaged product.]
**Phosphate buffered saline:** Proceed as directed in the test for *Purity*.
**Sample solution:** 50 µg/mL of IL-4 in *Phosphate buffered saline*. [NOTE—Do not agitate while mixing; swirl gently.]
**Blank:** *Phosphate buffered saline*
**Spectrometric conditions**
(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)
**Mode:** UV
**Pathlength:** 1 cm
**Analytical wavelength:** 280 nm
**Analysis**
**Samples:** *Sample solution* and *Blank*
Calculate the protein concentration:

$$C = A_{280}/0.63$$

C = IL-4 concentration of the *Sample solution* (mg/mL)
$A_{280}$ = absorbance at 280 nm

## SPECIFIC TESTS

• **BIOIDENTITY:** [NOTE—The biological activity measurement is determined on the basis of the packaged product.]
**RPMI 1640 medium with L-glutamine:** Prepare a mixture of the ingredients in the quantities shown in sufficient water to obtain 1 L of medium, and sterilize by filtration:

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| Material | Quantity |
|---|---|
| Calcium nitrate ($Ca(NO_3) \cdot 4H_2O$) | 100 mg |
| Magnesium sulfate ($MgSO_4 \cdot 7H_2O$) | 100 mg |
| Potassium chloride | 400 mg |
| Sodium chloride | 6000 mg |
| Sodium phosphate, dibasic anhydrous | 800 mg |
| Sodium bicarbonate | 2000 mg |
| Glycine | 10 mg |
| L-Arginine | 200 mg |
| L-Asparagine | 50 mg |
| L-Aspartic acid | 20 mg |
| L-Polyvinylidene fluoride L-cystine dihydrochloride | 20 mg |
| L-Glutamic acid | 20 mg |
| L-Glutamine | 300 mg |
| L-Histidine | 15 mg |
| L-Hydroxyproline | 20 mg |
| L-Isoleucine | 50 mg |
| L-Leucine | 50 mg |
| L-Lysine hydrochloride | 40 mg |
| L-Methionine | 15 mg |
| L-Phenylalanine | 15 mg |
| L-Proline | 20 mg |
| L-Serine | 30 mg |
| L-Threonine | 20 mg |
| L-Tryptophan | 5 mg |
| L-Tyrosine disodium salt dihydrate | 20 mg |
| L-Valine | 20 mg |
| Biotin | 0.2 mg |
| Choline chloride | 3 mg |
| D-Calcium pantothenate | 0.25 mg |
| Folic acid | 1 mg |
| *i*-Inositol | 35 mg |
| Niacinamide | 1 mg |
| *para*-Aminobenzoic acid | 1 mg |
| Pyridoxine hydrochloride | 1 mg |
| Riboflavin | 0.2 mg |
| Thiamine hydrochloride | 1 mg |
| Vitamin $B_{12}$ | 0.005 mg |
| D-Glucose (dextrose) | 2000 mg |
| Glutathione (reduced) | 1 mg |
| Phenol red | 5 mg |

**Growth medium:** Using aseptic procedures, prepare the following tissue culture medium:

| | |
|---|---|
| RPMI-1640 with L-glutamine | 500 mL |
| Sodium pyruvate 100 mM | 5 mL |
| Fetal bovine serum | 50 mL |

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| | |
|---|---|
| Human rGM-CSF[a] | $3 \times 10^4$ International Units |

[a] Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) is added extemporaneously.

Sterilize by filtration, and store at between 2° to 8°. Use within 1 month. Add GM-CSF immediately before use.

**Assay medium:** Use *Growth medium* containing no GM-CSF.

**Phosphate buffered saline:** Proceed as directed in the test for *Purity* in the *Assay*.

**Resazurin solution:** 11 mg of resazurin in 100 mL of *Phosphate buffered saline*. [NOTE—Sterile filter and store solution protected from light at 4°. *Resazurin solution* is stable for at least 6 months if treated under sterile conditions.]

[NOTE—For all *Standard* and *Sample solutions*, IL-4 concentration is determined by photometry at 280 nm using an extinction coefficient (ε) of 0.63 $mg^{-1}cm^{-1}$.]

**Standard stock solution:** 50 µg/mL of USP rHuman Interleukin 4 RS in *Phosphate buffered saline*. [NOTE—Do not agitate while mixing; swirl gently.]

**Standard solutions:** 36, 12, 4, 1.33, 0.44, 0.15, 0.05, 0.016, 0.006 ng/mL of IL-4, from *Standard stock solution* in *Assay medium*

**Sample stock solution:** 50 µg/mL of IL-4 in *Phosphate buffered saline*. [NOTE—Do not agitate while mixing; swirl gently.]

**Sample solutions:** 36, 12, 4, 1.33, 0.44, 0.15, 0.05, 0.016, 0.006 ng/mL of IL-4, from *Sample stock solution* in *Assay medium*

**Control solution:** Use the *Assay medium*.

**Cell culture preparation:** Prepare cell cultures of the human factor-dependent TF-1 cell line (ATCC No. CRL-2003), following the protocol described in the ATCC information sheet. Passage the cultures every 2–3 days, using 1:3 subcultures of the cells for up to 1 month. Seed density should be $0.5 \times 10^6$ cells/mL, and maximal density should be $3 \times 10^6$ cells/mL. Viability of the cells should be >90%. Maximal passage number is 24, and maximal cultivation time from thawing is 28 days. After 28 days, initiate a new culture. Cells are propagated using *Growth medium* at 37°, supplemented with air and 5% carbon dioxide.

**Analysis**

**Samples:** *Standard solutions, Sample solutions,* and *Control solution*

The activity of the *Sample solution* is determined in duplicate. Wash the cells three times in *Phosphate buffered saline*. Plate $2 \times 10^4$ TF-1 cells resuspended in 100 µL of *Assay medium* per well in 96-well, flat-bottom microplates. Incubate for 72 h at 37° and 5% $CO_2$ atmosphere in a humidified incubator in the presence or absence of various concentrations of *Standard solution, Sample solution*, or *Control solution* by adding 100 µL of the corresponding solution to each well. Add 30 µL of *Resazurin solution* to each well and incubate for another 24 h. Determine the fluorescence intensity per well by reading the plate with a microplate reader using 544 nm (excitation) and 590 nm (emission). Convert the fluorescence intensity in each well to a percentage of the maximum fluorescence intensity. For the *Sample solution* and *Standard solution*, plot the percentage of fluorescence intensity versus the concentration of the respective solution. By using the least squares method of regression analysis, compute the ED50 in ng/mL of the *Sample solution* and the *Standard solution*. The coefficient of determination for curve regression should be $\geq 0.98$. Calculate the potency in USP Interleukin 4 Units/mg:

$$\text{Result} = A \times E_S/E_U$$

A = activity of USP rHuman Interleukin 4 RS (USP units/mg)
$E_S$ = determined $ED_{50}$ of *Standard solution* (ng/mL)
$E_U$ = determined $ED_{50}$ of *Sample solution* (ng/mL)

**Acceptance criteria:** NLT $0.5 \times 10^7$ USP IL-4 Units/mg

- **STERILITY TESTS** ⟨71⟩: Meets the requirements
- **BACTERIAL ENDOTOXINS TEST** ⟨85⟩: It contains NMT 50 USP Endotoxin Units/mg.

## ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, and store at –80°.
- **LABELING:** Material is of recombinant DNA origin.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Endotoxin RS
USP rHuman Interleukin 4 RS

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# ⟨111⟩ DESIGN AND ANALYSIS OF BIOLOGICAL ASSAYS

## INTRODUCTION

The potency of several Pharmacopeial articles must be determined by bioassays. The aim of this chapter is to present a concise account of certain essential biometrical procedures for bioassays in chapters or monographs of *USP–NF*, namely outlier identification, confidence intervals for relative potency measurements, and combination of independent assays. For bioassays not in *USP–NF*, other methods may be appropriate. See general information chapter *Analysis of Biological Assays* ⟨1034⟩ which may be a helpful, but not mandatory, guidance.

## REJECTION OF OUTLYING OR ABERRANT OBSERVATIONS

A response that is questionable because of failure to comply with the procedure during the course of an assay is rejected. Other aberrant values may be discovered only after the responses have been tabulated, but can then be traced to assay irregularities that justify their omission. The arbitrary rejection or retention of an apparently aberrant response can be a serious source of bias. In general, the rejection of observations solely on the basis of their relative magnitudes, without investigation as to cause, is a procedure to be used sparingly. Should it be understood, either following an investigation into cause or based on practical assay experience, that an observation's discordance is unlikely to arise from a reasonable expectation of response to assay treatments, then a suspected aberrant response or outlier may be tested against one of two criteria, both of which assume that the data have an approximately normal distribution (which may be satisfied only after a suitable transformation of the original responses). Alternative statistically sound approaches to outlier detection may be used. The conditions under which outlier testing will be conducted and the criterion to be used should be specified a priori in the lab's procedures if not specified in the monograph or chapter.

### Criterion 1 (Dixon's Test)

The first criterion is based on the variation within a single group of supposedly equivalent responses, such as a group of animals given a common concentration of a sample. At a confidence level of 99%, a valid observation will be rejected once in 100 trials (when the suspected outlier can occur at only one end) or once in 50 trials (when the suspected outlier can occur at either end), provided that relatively few, if any, responses within the group are identical. Arrange the responses in order of magnitude from $y_1$ to $y_N$, where $N$ is the number of observations in the group. Compute the relative gap by using the formulas in *Table 1* below.

**Table 1**

| Sample Size ($N$) | Candidate Outlier is Smallest ($y_1$) | Candidate Outlier is Largest ($y_N$) |
|---|---|---|
| 3–7 | $G_1 = (y_2 - y_1)/(y_N - y_1)$ | $G_1 = (y_N - y_{N-1})/(y_N - y_1)$ |
| 8–10 | $G_2 = (y_2 - y_1)/(y_{N-1} - y_1)$ | $G_2 = (y_N - y_{N-1})/(y_N - y_2)$ |
| 11–13 | $G_3 = (y_3 - y_1)/(y_{N-1} - y_1)$ | $G_3 = (y_N - y_{N-2})/(y_N - y_2)$ |

If $G_1$, $G_2$, or $G_3$, as appropriate, exceeds the critical value in *Table 2*, for the observed $N$, there is a statistical basis for identifying the discordant measurement as an outlier and considering its removal. For $N$ larger than 13, use *Criterion 2*.

In samples from a normal population, at a confidence level of 99%, gaps equal to or larger than the following values of $G_1$, $G_2$, and $G_3$ occur with a probability $P = 0.01$, when outlier measurements can occur only at one end; or with $P = 0.02$, when they may occur at either end.

**Table 2. Test for Outlier Measurements**

| | | | | | |
|---|---|---|---|---|---|
| $N$ | 3 | 4 | 5 | 6 | 7 |
| $G_1$ | 0.988 | 0.889 | 0.780 | 0.698 | 0.637 |
| | | | | | |
| $N$ | 8 | 9 | 10 | — | — |
| $G_2$ | 0.683 | 0.635 | 0.597 | — | — |
| | | | | | |
| $N$ | 11 | 12 | 13 | — | — |
| $G_3$ | 0.679 | 0.642 | 0.615 | — | — |

### Criterion 2 (Grubbs, Extreme Studentized Deviate Test)

The second criterion may be used to examine for outlying values in groups of supposedly equivalent responses and may also be used in examining the set of residuals from a fitted model (linear or nonlinear) where there is constant variance. The final

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model (which yields the residuals for outlier detection) should include all important design variables. (For further discussion of design variables, see general information chapter *Design and Development of Biological Assays* ⟨1032⟩, which may be a helpful, but not mandatory, resource.) (Note that for application to residuals, the following is an approximation. If the statistical software provides studentized residuals, those values should be used instead of those from the following equation.) For the value, *R*, that is furthest from the sample mean, compute the standardized deviation *Z*:

$$Z = (R - \overline{R})/S$$

where $\overline{R}$ and *S* are the mean and standard deviation, respectively, of the set of values. For residuals from a least squares fit, such as for a parallel line assay, $\overline{R} = 0$, and *S* is the square root of the residual mean square from the analysis. If $|Z|$ is greater than *C* as determined below, then the value *R* is identified as a statistical outlier at the 1% level.

$$C = \frac{(N - 1)t_{df - 1,p}}{\sqrt{N(N - 2 + t^2_{df - 1,p})}}$$

where *N* is the sample size, *t* is the one-sided 100*p* percentage point from the *t* distribution with *df* the degrees of freedom associated with *S*:

$$p = 1 - \frac{0.01}{2N}$$

Alternative outlier methods are available that are intended for use on data sets that may contain multiple outliers and for detection of outliers associated with the bioassay design or model. For further discussion of outliers, see general information chapter *Analytical Data—Interpretation and Treatment* ⟨1010⟩, which may be a helpful, but not mandatory, resource.

## THE CONFIDENCE INTERVAL AND LIMITS OF POTENCY

The following method (Fieller's) is used to determine the confidence interval for an estimate of log relative potency from a parallel line assay or a slope ratio assay. Let $M = a/b$ be the ratio for which we need a confidence interval. For the estimates, *a* and *b*, we have their respective standard errors, $SE_a$ and $SE_b$, and a covariance between them, denoted *Cov*. The confidence interval, ($M_{Low}$, $M_{Up}$), for the estimated log relative potency then is as follows:

$$(M_{Low}, M_{Up}) = \left\{ \frac{M - \frac{gCov}{SE_b^2} \pm \frac{t}{b}\sqrt{\left(1 - g\right) SE_a^2 + M^2 SE_b^2 - 2MCov + \frac{gCov^2}{SE_b^2}}}{1 - g} \right\}$$

where:

$$g = \frac{t^2 SE_b^2}{b^2}$$

and $t = t_{df,\alpha/2}$ is the upper α/2 percentage point (or the two-sided α percentage point) with the residual degrees of freedom, *df*, from the statistical analysis and chosen confidence level, 100*(1–α), (usually 95%). If $g \geq 1$, it means that the denominator, *b*, is not statistically significantly different from 0 and the use of the ratio is not sensible for those data. The length, *L*, of this confidence interval is $M_{Up} - M_{Low}$.

For those cases in which the estimates of *a* and *b* are statistically uncorrelated (*Cov* = 0), the confidence interval formula simplifies to the following:

$$(M_{Low}, M_{Up}) = \left\{ \frac{M \pm \frac{t}{b}\sqrt{\left(1 - g\right) SE_a^2 + M^2 SE_b^2}}{1 - g} \right\}$$

For further discussion of confidence intervals for potency, see chapter ⟨1034⟩ which may be a helpful, but not mandatory, resource.

***Change to read:***

## COMBINATION OF INDEPENDENT ASSAYS

When the monograph or chapter permits, multiple independent assays may be performed until the combined results reduce the confidence interval width to within the limits specified in the pertinent monograph or chapter. Where two or more

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independent assays are required, each leading to a log-potency *M*, the *M*'s are combined using one of the following two methods.

## Method 1

Let $M_i$ denote the logarithm of the relative potency of the *i*th assay of *h* assay results to be combined. To combine the *h* results, the mean, standard deviation, and standard error of the $M_i$ are calculated in the usual way:

$$\text{Mean } \overline{M} = \sum_{i=1}^{h} M_i/h$$

$$\text{Standard Deviation } S = \sqrt{\frac{1}{h-1}\sum_{i=1}^{h}\left(M_i-\overline{M}\right)^2}$$

$$\text{Standard Error } SE = S/\sqrt{h}$$

A 100(1 – α)% confidence interval is then found as:

$$\overline{M} \pm t_{h-1,\alpha/2}SE$$

where $t_{h-1,\alpha/2}$ is the upper α/2 percentage point (or the two-sided α percentage point) of a *t*-distribution with *h* – 1 degrees of freedom. The width, *L*, of this interval is $2t_{h-1,\alpha/2}SE$.

## Method 2

It is assumed that the results of each of the *h* assays have been analyzed to give *h* values of log potency with associated confidence limits. For each assay, *i*, obtain the confidence interval for the log potency or log relative potency. Then compute value $L_i$ by subtracting the *i*th lower confidence limit from the *i*th upper confidence limit. A weight $w_i$ for each value of the log relative potency, $M_i$, is calculated as follows, where $t_i$ has the same *t*-distribution value as that used in the calculation of confidence limits in the *i*th assay and is based on $n_i$ degrees of freedom:

$$W_i = \frac{4t_i^2}{L_i^2}$$

The products $w_iM_i$ are formed for each assay, and their sum is divided by the total weight (*w*) for all assays to give the weighted mean log relative potency and its standard error as follows:

$$\left.\begin{aligned} \text{Mean, } \overline{M} &= \sum_{i=1}^{h} w_iM_i/w \\ \text{Approximate Standard Error, } SE &= 1/\sqrt{w} \\ \text{where } w &= \sum_{i=1}^{h} w_i \end{aligned}\right\} \quad (1)$$

Next compute an approximate chi-square:

$$\chi^2_M = \sum_{i=1}^{h} w_i\left(M_i-\overline{M}\right)^2 = \sum_{i=1}^{h} w_iM_i^2 - w\overline{M}^2$$

If the value of the approximate $\chi^2_M$ is *well under* the 5% value shown in *Table 3*, compute the confidence interval using the mean and approximate standard error equations in (1) above; otherwise use *Alternate weights* as described below. Labs need to specify in their procedures how to quantify "well under". Absent such a specification, the 20% values of *Table 3* are suggested.

A 100(1 – α)% confidence interval in the log scale is then found as:

$$\overline{M} \pm L/2$$

$$\text{where } L = \frac{2t_{df,\alpha/2}}{\sqrt{w}}\sqrt{1+\frac{4}{w^2}\times\sum_{i=1}^{h}\frac{w_i\left(w-w_i\right)}{n_i'}}$$

$$n_i' = n_i - 4\times\frac{h-2}{h-1}$$

$$\text{and } df = \left.w^2\middle/\sum_{i=1}^{h} w_i^2/n_i\right.$$

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where $t_{N,\alpha/2}$ is the upper $\alpha/2$ percentage point (or the two-sided $\alpha$ percentage point) of a *t*-distribution with degrees of freedom, *df*. The width of this interval is *L*.

**Table 3. Critical Values for Approximate Chi-Square Test**

| | Critical Values | |
|---|---|---|
| ***h*** | **5%** | **20%** |
| 2 | 3.841 | 1.642 |
| 3 | 5.991 | 3.219 |
| 4 | 7.815 | 4.642 |
| 5 | 9.488 | 5.989 |
| 6 | 11.070 | 7.289 |
| 7 | 12.592 | 8.558 |
| 8 | 14.067 | 9.803 |
| 9 | 15.507 | 11.030 |
| 10 | 16.919 | 12.242 |

**Alternate weights:** The observed variation among the estimated log potencies or relative potencies can be divided into two components:

- intra-assay variation for assay *i*:
  $V_i = 1/w_i$
- inter-assay component of variation:

$$S_B^2 = \max\left\{0, \frac{1}{h-1}\sum_{i=1}^{h}\left(M_i - \overline{M}\right)^2 - \frac{1}{h}\sum_{i=1}^{h} V_i\right\}$$

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For each assay, a weighting coefficient is then calculated as:

$$W_i' = \frac{1}{V_i + S_B^2}$$

The confidence interval is then found as:

$$\overline{M} \pm t \times SE',$$
$$\text{where } SE' = 1\sqrt{w'} \text{ and } w' = \sum_{i=1}^{h} w_i'$$

and *t*, the *t-distribution value*, is often approximated by the value 2.

For further discussion of combination of assays, see ⟨1034⟩, which may be a helpful, but not mandatory, resource.

## APPENDIX—KEY LITERATURE

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# ⟨115⟩ DEXPANTHENOL ASSAY

## INTRODUCTION

The following liquid chromatographic procedure is provided for the determination of dexpanthenol as a component in dietary supplements or pharmaceutical dosage forms.

While conducting this procedure, protect solutions containing and derived from the test specimen and the Reference Standards from the atmosphere and light, preferably with the use of low-actinic glassware. Use the appropriate USP Reference Standards.

## ASSAY

• **Chromatographic Methods, Procedure 1**

This procedure can be used to determine dexpanthenol in:

- *Oil- and Water-Soluble Vitamins Oral Solution*
- *Oil- and Water-Soluble Vitamins with Minerals Oral Solution*
- *Water-Soluble Vitamins with Minerals Oral Solution*

Unless specified in the monograph, the *Standard solution*, *System suitability solution*, and *Sample solution* are prepared as follows.

**Mobile phase:** 0.2 M monobasic sodium phosphate and methanol (97:3). Adjust with 1.7 M phosphoric acid to a pH of 3.2 ± 0.1.

**Standard solution:** 80 µg/mL of USP Dexpanthenol RS in *Mobile phase*

**System suitability solution:** 80 µg/mL of USP Calcium Pantothenate RS in *Mobile phase*. Mix the resulting solution and *Standard solution* (1:1).

**Sample solution:** Equivalent to 80 µg/mL of dexpanthenol from Oral Solution in *Mobile phase*

**Chromatographic system**

(See *Chromatography* ⟨621⟩, *System Suitability*.)

**Mode:** LC

**Detector:** UV 210 nm

**Column:** 4.0-mm × 10-cm; packing L1

**Flow rate:** 1 mL/min

**Injection volume:** 20 µL

**System suitability**

**Samples:** *Standard solution* and *System suitability solution*

**Suitability requirements**

**Resolution:** NLT 1.5 between dexpanthenol and calcium pantothenate, *System suitability solution*

**Tailing factor:** NMT 2.0 for the dexpanthenol peak, *Standard solution*

**Relative standard deviation:** NMT 2.0%, *Standard solution*

**Analysis**

**Samples:** *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of dexpanthenol ($C_9H_{19}NO_4$) in the portion of the sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

$r_U$ = peak response of dexpanthenol from the *Sample solution*

$r_S$ = peak response of dexpanthenol from the *Standard solution*

$C_S$ = concentration of USP Dexpanthenol RS in the *Standard solution* (mg/mL)

$C_U$ = nominal concentration of dexpanthenol in the *Sample solution* (mg/mL)

## ADDITIONAL REQUIREMENTS

• **USP Reference Standards** ⟨11⟩

USP Calcium Pantothenate RS

USP Dexpanthenol RS

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# ⟨121.1⟩ PHYSICOCHEMICAL ANALYTICAL PROCEDURES FOR INSULINS

## INTRODUCTION

Several physicochemical procedures are used for the assessment of the quality attributes of Insulin Human, various insulin analogues, and animal derived insulins, hereafter called insulins. Of these procedures, peptide mapping and determination of high molecular weight proteins are similar for the various insulin drug substances and drug products. This chapter describes the tests for peptide mapping and for quantitative analysis of high molecular weight proteins that can be used for the various insulins.

Specific instructions that deviate from the general procedures outlined here are given in the respective drug substance or drug product monographs for the different insulins.

### PEPTIDE MAPPING

Insulin digestion is performed using *Staphylococcus aureus* strain V8 (*S. aureus* V8) protease (also referred to as Endoproteinase Glu-C), a serine endoproteinase that cleaves at the C-terminal side of glutamyl and aspartyl residues in phosphate buffer at pH 7.8. This protease is specific for Glu-C digestion in ammonium bicarbonate or other non-phosphate-containing buffers at pH 7.8. The presence of proline on the carboxy side of the peptide bond inhibits cleavage. The buffer system used should cleave all glutamyl bonds in insulin without cleavage of the aspartyl bonds. In general, insulin is digested into four peptides in the buffer system described in the *Sample solution* below.

The following show the amino acid differences of the insulin analogues compared to Insulin Human and the *in silico* differences in the fragments obtained upon digestion with *S. aureus* V8 protease.

*Figure 1* shows the four fragments of Insulin Human following digestion with *S. aureus* V8 protease.

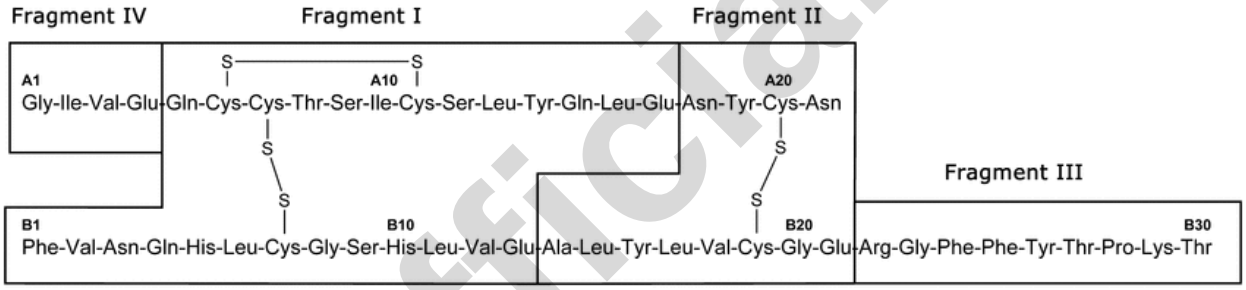


Figure 1. Insulin Human: *S. aureus* V8 protease digest fragments.

*Table 1* shows the amino acid differences between insulin analogues, animal derived insulins, and Insulin Human.

**Table 1. Amino Acid Differences between Insulin Analogues, Animal Derived Insulins, and Insulin Human**

| Insulin | Amino Acid Differences from Insulin Human |
|---|---|
| Insulin aspart | B28 Asp |
| Insulin glargine | A21 Gly, two Arg added to C-terminus of B chain |
| Insulin glulisine | B3 Lys, B29 Glu |
| Insulin lispro | B28 Lys, B29 Pro |
| Insulin porcine | B30 Ala |
| Insulin bovine | A8 Ala, A10 Val, B30 Ala |

*Table 2* shows specific *S. aureus* V8 protease digest fragments of insulin analogues and animal derived insulins. Amino acid differences compared to Insulin Human are highlighted.

**Table 2. Amino Acid Differences: Insulin Human, Insulin Analogues, and Animal Derived Insulins (Differences with Insulin Human Are Shown in Boldface)**

| Insulin | Amino Acid Differences Compared to Insulin Human[a] | |
|---|---|---|
| Insulin aspart | Fragment III | (B22) Arg-Gly-Phe-Phe-Tyr-Thr-**Asp**-Lys-Thr (B30) |
| Insulin glargine | Fragment II | (A18) Asn-Tyr-Cys-**Gly** (A21) |
| | Fragment III | (B22) Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Thr-**Arg**-**Arg** (B32) |
| Insulin glulisine | Fragment I | (B1) Phe-Val-**Lys**-Gln-His-Leu-Cys-Gly-Ser-His-Leu (B11) |
| | Fragment III | (B22) Arg-Gly-Phe-Phe-Tyr-Thr-Pro-**Glu**-Thr (B30) |
| Insulin lispro | Fragment III | (B22) Arg-Gly-Phe-Phe-Tyr-Thr-**Lys**-**Pro**-Thr (B30) |
| Insulin porcine | Fragment III | (B22) Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-**Ala** (B30) |

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**Table 2. Amino Acid Differences: Insulin Human, Insulin Analogues, and Animal Derived Insulins (Differences with Insulin Human Are Shown in Boldface)** *(continued)*

| Insulin | Amino Acid Differences Compared to Insulin Human[a] | |
|---|---|---|
| | Fragment I | (A5) Gln-Cys-Cys-**Ala**-Ser-**Val**-Cys-Ser-Leu-Tyr-Gln-Leu-Glu (A17) |
| Insulin bovine | Fragment III | (B22) Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-**Ala** (B30) |

[a] A and B denote the A and B chains of insulin, respectively; numbers denote amino acid position in the chain.

- **Peptide Mapping Procedure**

The following procedure is applicable for preparing peptide maps of Insulin Human, insulin analogues, and animal derived insulins.

**HEPES buffer:** Dissolve 2.38 g of HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) in about 90 mL of water in a 100-mL volumetric flask. Adjust with 5 M sodium hydroxide to a pH of 7.5, dilute with water to volume, and mix.

**Sulfate buffer:** 2.0 M ammonium sulfate and 0.5 M sulfuric acid (1:1). Mix, and filter.

- **Insulin Digestion**

The following procedure provides efficient cleavage of the glutamyl bonds of insulin. [Note—Volumes up to 20-fold higher can be used as long as the ratio of the solutions remains the same. If interfering autolysis byproducts are observed in the chromatogram when a digest of the enzyme alone is run, the enzyme-to-insulin ratio must be decreased and digestion time must be increased.]

**Enzyme solution:** Prepare a 1-mg/mL solution of *S. aureus* V8 protease in water (approximately 500 units/mg using casein as the substrate).

**Sample solution:** Prepare a 2.0-mg/mL solution of the insulin to be examined in 0.01 N hydrochloric acid. To a clean vial add 25 µL of the 2.0-mg/mL insulin solution, 100 µL of *HEPES buffer*, and 20 µL of *Enzyme solution* (final ratio is 25:100:20). Cap the vial, and incubate at 25° for 6 h. Stop the reaction by adding an equal volume of *Sulfate buffer*. Longer incubation times may be needed for analogues with poor solubility at pH 7.5.

**Standard solution:** Prepare at the same time and in the same manner a solution of the appropriate USP Insulin Reference Standard as directed in the *Sample solution*.

- **Peptide Fragment Determination**

Determine the peptide fragments using the following peptide mapping procedure (see *Biotechnology-Derived Articles—Peptide Mapping* ⟨1055⟩).

**Solution A:** Acetonitrile, water, and *Sulfate buffer* (100:700:200). Filter, and degas.

**Solution B:** Acetonitrile, water, and *Sulfate buffer* (400:400:200). Filter, and degas.

**Mobile phase:** See *Table 3*.

**Table 3**

| Time (min) | Solution A (%) | Solution B (%) |
|---|---|---|
| 0 | 95 | 5 |
| 3 | 95 | 5 |
| 30 | 41 | 59 |
| 35 | 20 | 80 |
| 40 | 95 | 5 |
| 50 | 95 | 5 |

**Chromatographic system**

(See *Chromatography* ⟨621⟩, *System Suitability*.)

**Mode:** LC

**Detector:** UV 214 nm

**Column:** 4.6-mm × 10-cm; 5-µm packing L1

**Column temperature:** 40°

**Flow rate:** 1 mL/min

**Injection volume:** 50–100 µL

**System suitability**

**Sample:** *Standard solution*

**Suitability requirements**

**Chromatogram comparability:** In the chromatogram obtained from the *Standard solution*, identify the peaks due to digest fragments I, II, III, and IV. The chromatogram of the *Standard solution* corresponds to that of the typical chromatogram provided with the appropriate USP Insulin Reference Standard.

**Resolution:** There should be complete separation of the peaks due to fragments II and III. The resolution is defined in the applicable insulin monograph.

**Tailing factor:** NMT 1.5 for digest fragments II and III

**Analysis**

**Samples:** *Sample solution* and *Standard solution*

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Condition the *Chromatographic system* by running at initial conditions, $t$ = 0 min, for at least 15 min. Carry out a blank gradient program before injecting the digests. Separately inject equal volumes of the *Standard solution* and the *Sample solution*, and record the responses of each peak.

**Acceptance criteria:** The chromatographic profile of the *Sample solution* corresponds to that of the *Standard solution*.

## LIMIT OF HIGH MOLECULAR WEIGHT PROTEINS

• **PROCEDURE**

**Solution A:** 1 mg/mL of L-arginine in water

**Mobile phase:** *Solution A*, acetonitrile, and glacial acetic acid (65:20:15). Filter, and degas.

**Resolution solution:** Store a suitable amount of insulin drug substance at room temperature for a sufficient period of time (5–10 days, or as needed) to obtain insulin with more than 0.4% high molecular weight proteins. Prepare a 4-mg/mL solution in 0.01 N hydrochloric acid. Store the solution in a refrigerator, and use within 7 days. Alternatively, dissolve about 4 mg of USP High Molecular Weight Insulin Human RS in 1 mL of 0.01 N hydrochloric acid.

**Sample solution:** In a small vial, prepare a 4-mg/mL solution of insulin in 0.01 N hydrochloric acid, and mix to dissolve. Store in a refrigerator, and use within 7 days.

**Chromatographic system**

(See *Chromatography* ⟨621⟩*, System Suitability*.)

**Mode:** LC

**Detector:** UV 276 nm

**Column:** 7.8-mm × 30-cm; 5- to 10-µm packing L20

**Temperatures**

**Autosampler:** It is advisable to use a refrigerated autosampler.

**Column:** Ambient

**Flow rate:** 0.5 mL/min

**Injection volume:** 100 µL

**System suitability**

**Sample:** *Resolution solution*

**Suitability requirements**

**Retention times:** Between 13 and 17 min for the polymeric insulin complexes, about 17.5 min for the covalent insulin dimer, and between 18 and 22 min for the insulin monomer, with salts eluting after the insulin monomer

**Peak-to-valley ratio:** The ratio of the height of the covalent insulin dimer peak to the height of the valley between the covalent insulin dimer peak and the insulin monomer peak is NLT 2.0.

**Analysis**

**Sample:** *Sample solution*

Disregard any peaks with retention times greater than that of the insulin monomer.

Calculate the percentage of high molecular weight proteins in the portion of Insulin taken:

$$\text{Result} = \Sigma r_H/(\Sigma r_H + r_M) \times 100$$

$\Sigma r_H$ = sum of the responses of all peaks with retention times less than that of the insulin monomer

$r_M$ = peak response of the insulin monomer

**Acceptance criteria:** As stated in the applicable insulin monograph

## ADDITIONAL REQUIREMENTS

• **USP REFERENCE STANDARDS** ⟨11⟩

USP High Molecular Weight Insulin Human RS

(alternative, optional)

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# ⟨121⟩ INSULIN ASSAYS

***Change to read:***

## INTRODUCTION

The most prominent manifestation of insulin activity, an abrupt decrease in blood glucose, was the basis for biologic assay from the time of its first clinical use. The ▲bio▲ (USP 1-Dec-2020) assay, although relatively cumbersome, has the great merit of accurately reflecting the effect on the diabetic patient. The advent of practical yet sophisticated physicochemical methods (e.g., liquid chromatography) to measure insulin potency quantitatively has resulted in a more accurate and precise compendial test for insulin and insulin products. However, the bioidentity of insulin and insulin products cannot be assessed by these methods. Thus, a bioidentity test ▲▲ (USP 1-Dec-2020)is called for in the appropriate monographs.

▲Two options are provided: The *Rabbit Blood Sugar Method* can be used for any compendial insulin or insulin analog. The *In Vitro Cell-Based Bioidentity Test* can be used for Insulin glargine and Insulin lispro.▲ (USP 1-Dec-2020) The *Rabbit Blood Sugar Method—Quantitative* is used to determine the potency of Insulin Reference Standards, for the validation of the stability of new insulin preparations, and to determine the specific activities of insulin analogs.

## ASSAY

***Change to read:***

- **Rabbit Blood Sugar Method—Quantitative**

**Diluent:** Prepare an aqueous solution containing 0.1%–0.25% (w/v) of either cresol or phenol, 1.4%–1.8% (w/v) of glycerin, and sufficient hydrochloric acid to produce a pH between 2.5 and 3.5, unless otherwise directed in the individual monograph.

**Standard stock solution:** Prepare a solution containing 40 USP Insulin Units/mL of the USP Insulin Reference Standard of the appropriate species in *Diluent* and having a pH between 2.5 and 3.5, unless otherwise directed in the individual monograph. Store in a ▲refrigerator,▲ (USP 1-Dec-2020) protected from freezing, and use within 6 months.

**Standard solution 1:** Dilute a portion of the *Standard stock solution* with *Diluent* to contain 1.0 USP Insulin Unit/mL.

**Standard solution 2:** Dilute a portion of the *Standard stock solution* with *Diluent* to contain 2.0 USP Insulin Units/mL.

**Sample stock solution:** Prepare as directed in the *Standard stock solution*, except to use a suitable quantity of the preparation under test in place of the USP Insulin Reference Standard of the appropriate species. The *Sample stock solution* contains about 40 USP Insulin Units/mL.

**Sample solution 1:** Dilute a portion of the *Sample stock solution* with *Diluent* to make a dilution of the preparation under test. On the basis of the assumed potency, it is expected to contain 1.0 USP Insulin Unit/mL. In the case of neutral insulin injection, adjust to a pH of 2.5–3.5 before making the dilution.

**Sample solution 2:** Dilute a portion of the *Sample stock solution* with *Diluent* to make a dilution of the preparation under test. On the basis of the assumed potency, it is expected to contain 2.0 USP Insulin Units/mL. In the case of neutral insulin injection, adjust to a pH of 2.5–3.5 before making the dilution.

**Doses of the solutions to be injected:** Select, on the basis of trial or experience, the dose of the dilutions to be injected, the volume of which usually will be between 0.30 and 0.50 mL. For each animal, the volume of the *Standard solution* is the same as that of the *Sample solution*.

**Preparation of animal:** Select suitable, healthy rabbits, each weighing NLT 1.8 kg. Keep the rabbits in the laboratory for NLT 1 week before use in the assay, maintaining them on an adequate uniform diet, with water available at all times.

**Analysis:** Divide the rabbits into four equal groups of preferably NLT 6 rabbits each. On the preceding day, approximately 20 h before performing the assay, provide each rabbit with an amount of food that will be consumed within 6 h. Follow the same feeding schedule before each test day. During the assay, withhold all food until after the final blood specimen is taken. Handle the rabbits with care to avoid undue excitement, and inject subcutaneously the doses indicated in the following design (see *Table 1*), the second injection being made on the day after the first injection, or NMT 1 week later. The time between the first and second injections is the same for all rabbits.

**Table 1**

| Group | First Injection | Second Injection |
|---|---|---|
| 1 | *Standard solution 2* | *Sample solution 1* |
| 2 | *Standard solution 1* | *Sample solution 2* |
| 3 | *Sample solution 2* | *Standard solution 1* |
| 4 | *Sample solution 1* | *Standard solution 2* |

**Blood samples:** At 1 h ± 5 min and 2.5 h ± 5 min after the time of ▲each▲ (USP 1-Dec-2020) injection, obtain from each rabbit a suitable blood specimen from a marginal ear vein. Blood can also be collected effectively from the central auricular artery.

**Dextrose determination:** Determine the dextrose content of the blood specimens by a suitable procedure that is adapted to automated analysis. The following procedure may be used.

**Anticoagulant solution:** Dissolve 1 g of edetate sodium and 200 mg of sodium fluoride in 1 L of water, and mix.

**Dextrose standard preparations:** Transfer known concentrations of USP Dextrose RS to suitable vessels, and dilute quantitatively and stepwise with *Anticoagulant solution* (1:9) to obtain a range of *Dextrose standard preparations* containing 20–100 mg/100 mL, having known concentrations similar to the concentrations in the rabbit blood samples.

**Sample preparations:** Pipet into separate, suitable vessels 0.1 mL of each *Blood sample* and 0.9 mL of *Anticoagulant solution*.

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**Analysis:** Subject the *Sample preparations* to dialysis across a semipermeable membrane for a sufficient time so that the dextrose passes through the membrane into a saline TS solution containing glucose oxidase, horseradish peroxidase, 3-methyl-2-benzothiazolinone hydrazone hydrochloride TS, and *N,N*-dimethylaniline. The absorbances of the *Sample preparations* are determined at 600 nm in a recording colorimeter. The absorbances of the *Dextrose standard preparations* are similarly determined at the start and the end of each run.

**Calculation:** Calculate the response of each rabbit to each injection from the sum of the two blood sugar values, and subtract its response, disregarding the chronological order in which the responses were observed, to obtain the individual differences, *y*, as shown in *Table 2*.

When the data for one or more rabbits are missing in an assay, do not use the confidence interval formulas given here, but seek statistical help. The data can still be analyzed with proper analysis of variance.

When the number of rabbits, *f*, carried through the assay is the same in each group, total the *y*'s in each group and compute:

$$T_a = -T_1 + T_2 + T_3 - T_4$$

and

$$T_b = T_1 + T_2 + T_3 + T_4$$

The logarithm of the relative potency of the test dilutions is $M' = 0.301 T_a/T_b$. The potency of the injection in USP Units/mg equals the antilog (log $R + M'$), where:

$$R = v_S/v_U$$

$v_S$ = number of USP Units/mL of insulin in the *Standard solution*
$v_U$ = number of mg/mL of insulin in the corresponding *Sample solution*

**Table 2**

| Group | Differences | Individual Response (*y*) | Total Response (*T*) | Standard Deviation of Differences (*S*) |
|---|---|---|---|---|
| 1 | *Standard solution 2 – Sample solution 1* | $y_1$ | $T_1$ | $S_1$ |
| 2 | *Sample solution 2 – Standard solution 1* | $y_2$ | $T_2$ | $S_2$ |
| 3 | *Sample solution 2 – Standard solution 1* | $y_3$ | $T_3$ | $S_3$ |
| 4 | *Standard solution 2 – Sample solution 1* | $y_4$ | $T_4$ | $S_4$ |

Determine the 95% confidence interval for the log-relative potency using *Fieller's Theorem for Determining the Confidence Interval for a Ratio* as shown below. ▲▲ (USP 1-Dec-2020) If the confidence interval ▲width $(U - L)$▲ (USP 1-Dec-2020) is more than 0.082, which corresponds at P = 0.95 to confidence limits of about ±10% of the computed potency, repeat the assay until the combined data of the two or more assays, redetermined as described in *Design and Analysis of Biological Assays* ⟨111⟩, *Combination of Independent Assays*, meet this acceptable limit.

***Change to read:***

▲▲ (USP 1-Dec-2020)**Fieller's Theorem for Determining the Confidence Interval for a Ratio**

This version of Fieller's Theorem is for the case where the numerator and denominator are uncorrelated. The equation assumes that the numerator and denominator are normally distributed and that the groups of rabbits are of equal sizes.

Then, the 95% confidence interval ▲($L$: lower, $U$: upper)▲ (USP 1-Dec-2020) for the ratio is:

$$(L,U) = \frac{M' \pm \frac{t}{T_b}\sqrt{\left(1-g\right)S_N^2 + (M')^2 S_D^2}}{1-g}$$

where $f$ (degrees of freedom in the standard errors) = $4(k - 1)$, where $k$ is the number of rabbits in a group, $t$ is the upper 97.5 percentile of the $t$-distribution with $f$ degrees of freedom, and

$$g = \frac{t^2 S_D^2}{T_b^2}$$

If $g \geq 1$, the denominator is not significantly different from 0 and the formula does not work.

$$S_N = 0.301\sqrt{k}\sqrt{S_1^2 + S_2^2 + S_3^2 + S_4^2}$$
$$S_D = \sqrt{k}\sqrt{S_1^2 + S_2^2 + S_3^2 + S_4^2}$$

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***Change to read:***

• ▲**IN VIVO**▲ (USP 1-Dec-2020) **BIOIDENTITY TEST**

Proceed as directed in *Rabbit Blood Sugar Method—Quantitative* with the following modifications.

**Procedure:** Divide the rabbits into four equal groups of 2 rabbits each.

**Calculation:** Proceed as directed for *Calculation* in *Rabbit Blood Sugar Method—Quantitative*, but do not determine the confidence interval of the log-relative potency, *M*′.

**Interpretation:** If the potency value obtained is NLT 15 USP Units/mg, then the ▲*In Vivo*▲ (USP 1-Dec-2020) *Bioidentity Test* requirement is met. If the potency value is less than 15 USP Units/mg, then repeat the test using 8 more rabbits. If the average potency of the two sets of tests is NLT 15 USP Units/mg, then the requirement of the test is met.

***Add the following:***

▲• **IN VITRO CELL-BASED BIOIDENTITY TEST FOR INSULIN GLARGINE AND INSULIN LISPRO**

**Minimal medium:** Ham's F-12 Nutrient Mixture containing 1 mM L-alanyl-L-glutamine[1]

**Growth medium:** 90% (v/v) Ham's F-12 Nutrient Mixture with 1 mM L-alanyl-L-glutamine, 10% (v/v) fetal bovine serum (FBS),[2] and 0.03% (w/v) hygromycin B

**D-PBS solution:** Dulbecco's phosphate-buffered saline without calcium and magnesium[3] containing 0.2 g/L of potassium chloride, 0.2 g/L of monobasic potassium phosphate, 8 g/L of sodium chloride, and 2.16 g/L of dibasic sodium phosphate.

**Fixing solution:** Prepare 3.7% (v/v) formaldehyde solution by diluting a commercially available 37% (w/v) formaldehyde solution with *D-PBS solution*. Prepare freshly before use.

**Permeabilization solution:** 0.1% (v/v) Triton® X-100 in *D-PBS solution*

**Washing solution:** 0.1% (v/v) polysorbate 20 in *D-PBS solution*

**Blocking buffer:** 2% (w/v) bovine serum albumin (BSA)[4] in *D-PBS solution*. [NOTE—Filtration of solution can be helpful to remove particles.]

**Blocking buffer for primary antibody:** 0.1% (v/v) polysorbate 20 in *Blocking buffer*

**Primary antibody solution:** Dilute an anti-phosphotyrosine antibody recognizing phosphotyrosines of insulin receptors in *Blocking buffer for primary antibody*. [NOTE—For example, dilute the anti-phosphotyrosine mouse monoclonal antibody[5] in *Blocking buffer* for primary antibody at 1:2000 when the antibody is at a concentration of 1 mg/mL. Use within 15 min after preparation.]

**Blocking buffer for secondary antibody:** 0.2% (v/v) polysorbate 20 in *Blocking buffer*

**Secondary antibody solution:** Dilute a suitable secondary antibody conjugated with a suitable fluorescent dye,[6] a fluorescent dye for cell staining,[7] and/or a suitable fluorescent dye for DNA staining[8] in *Blocking buffer for secondary antibody*. Protect the solution from light. [NOTE—See example on preparing a *Secondary antibody solution*.[9]]

**Cell culture preparation:** Culture CHO INSR 1284 cells[10] in *Growth medium* at 37° and 5% carbon dioxide ($CO_2$) atmosphere in a humidified incubator. Cells should be transferred when the cultures are approximately 70%–95% confluent. For cell transfer, remove the flasks from the incubator and discard the media. Rinse the cells with *D-PBS solution*. Add an appropriate amount of Accutase[11] to the cells and place in an incubator for about 5 min at 37°. Tap repeatedly on the flask in order to remove all cells from the flask bottom. Add an appropriate amount of *Growth medium* to stop the Accutase activity. Determine cell concentration and viability. Calculate the desired amount of cells for seeding depending on planned cultivation days. Cells are transferred into a T75 $cm^2$ flask with approximately 18 mL of *Growth medium* at $0.25 \times 10^5$ to $9 \times 10^5$ cells per flask or a T175 $cm^2$ flask with approximately 45 mL of *Growth medium* at $0.1 \times 10^6$ to $2 \times 10^6$ cells per flask.

**Preparation of cells for analysis**

**Plates:** Sterile 96-well, clear bottom, black[12]

**System suitability requirements for the cells**

**Viability:** NLT 85%

**Average % of cell aggregates:** NMT 15% of cell clumps. [NOTE—The clump is defined as a cluster containing more than 3 cells.]

For bioidentity test, a total of two plates of cells are needed to assay two independent dilutions of the *Standard solution* and the *Sample solution*. The maximal number of cell passages to be used is determined individually. Following the procedure described in *Cell culture preparation*, prepare an appropriate volume of cell solution at $0.5 \times 10^5$ to $1 \times 10^5$ viable cells/mL in *Growth medium* (see *Table 3* for examples of seeding densities).

---

[1] Ham's F-12 Nutrient Mixture, Glutamax™ Supplement, ThermoFisher #31765035 or #31765092, or equivalent.

[2] ThermoFisher #10082147 or equivalent.

[3] ThermoFisher #14190144 or equivalent.

[4] Sigma-Aldrich #A9647 or equivalent.

[5] A suitable primary antibody is anti-phosphotyrosine 4G10 mouse monoclonal antibody (1 mg/mL) from Millipore #05-321 or equivalent.

[6] A suitable secondary antibody is IRDye 800CW Goat-anti-mouse IgG from LI-COR #926-32210 or equivalent.

[7] A suitable fluorescent dye for cell staining is Sapphire700™ Stain #928-40022, or CellTag 700 Stain #926-41090 from LI-COR, or equivalent.

[8] A suitable fluorescent DNA dye is DRAQ5™ DNA Stain from Biostatus #DR50050 or #DR50200, or equivalent.

[9] An example for preparing a solution containing diluted IRDye® 800CW Goat-anti-mouse IgG,[6] Sapphire700 Stain,[7] and DRAQ5™ DNA Stain[8] is as follows. Prepare the solution containing 1 µg/mL of IRDye® 800CW Goat-anti-mouse-IgG5, Sapphire700™ (diluted 1:1000), and 0.5 µM DRAQ5™ in *Blocking buffer for secondary antibody*. Use this solution within 15 min after preparation.

[10] ATCC #CRL-3307.

[11] Sigma-Aldrich #A6964 or equivalent.

[12] Corning #3904 96-Well Flat Clear Bottom Black Polystyrene Microplates or equivalent.

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**Table 3**

| Initial Cell Concentration (cells/mL) | Number of Cells Seeded per Well | Time Needed for Growth Prior to Analysis (days) |
|---|---|---|
| 100,000 | 20,000 ± 20% | 2 |
| 70,000 | 14,000 ± 20% | 3 |
| 50,000 | 10,000 ± 20% | 4 |

To the sterile plates add 0.2 mL of cell solution per well under aseptic conditions. Mix the cell solution frequently during dispensing to prevent cells from settling and to ensure consistent density throughout the plate. Cover the plates. Let the plates seeded with cells stand at room temperature for 30 min before transferring them into an incubator. Arrange the plates side by side, and do not stack up the plates. Incubate plates at 37° with 5% carbon dioxide ($CO_2$) for the indicated time as shown in *Table 3*. Verify even growth and 90% or higher confluency via microscope before *Analysis*. Following the incubation, remove media from the cells and wash each well with 200 µL of sterile *D-PBS solution*. Add 160 µL of sterile *Minimal medium* to each well. Incubate plates for 3–5 h at 37° with 5% carbon dioxide ($CO_2$). This last step is for cell media starvation prior to *Analysis*.

**Preparation of solutions for insulin glargine and insulin lispro**

Up to three different insulin samples can be tested in one assay. For bioidentity test, independently prepare *Standard solutions* and *Sample solutions* in duplicate that are assayed in two separate plates to obtain two intermediate potency results per sample.

[NOTE—All described dilutions except for stock solutions are to be carried out just prior to their application to the cells. For each black plate seeded with cells as described in *Preparation of cells for analysis*, one separate 96-well polypropylene plate can be used for preparation of the serial dilutions from one of the *Standard solution* or *Sample solution* duplicates.]

Preparation of the *Standard solution* and *Sample solution* and delivery to the cells must occur within 15 min when using plasticware.

**Insulin glargine**

**Standard stock solution:** 17 USP Insulin Glargine Units/mL of USP Insulin Glargine RS in 0.01 N hydrochloric acid

**Standard solutions:** 0.34 USP Insulin Glargine Units/mL prepared by diluting the *Standard stock solution* with *D-PBS solution*. Prepare in duplicate.

**Sample solutions:** 0.34 USP Insulin Glargine Units/mL of insulin glargine in *D-PBS solution*. Prepare in duplicate.

**Diluted standard solutions and sample solutions:** For each assay plate, prepare three separate dilution sets from one of the *Standard solution* and *Sample solution* preparations. The range of the concentrations of insulin glargine solutions to be added to the cells needs to be determined during method verification. Successful testing was demonstrated for a range of $340 \times 10^{-3}$ to $0.16 \times 10^{-3}$ USP Insulin Glargine Units/mL. The dilution can be prepared as follows. Add 200 µL of *D-PBS solution* into each well of a 96-well polypropylene plate, except those wells in row A. Next, add 300 µL of *Standard solution* into wells A1, A5, and A9 of the dilution plate. Add 300 µL of the first *Sample solution* into wells A2, A6, and A10; the second *Sample solution* into wells A3, A7, and A11; the third *Sample solution* into wells A4, A8, and A12. Perform simultaneously serial three-fold dilutions on the plate by transferring 100 µL of solution from the first row (A1–A12) to the second row, and mix three times. Then transfer 100 µL of solution from the second row to the third row, and mix three times. Repeat this procedure across the whole plate up to row H. Repeat the above for the replicate preparation.

**Insulin lispro**

**Dilution buffer:** 0.1% (w/v) BSA[4] in *D-PBS solution*. Prepare freshly before use.

**Standard stock solution:** 10 USP Insulin Lispro Units/mL of USP Insulin Lispro RS in 0.01 N hydrochloric acid

**Standard solutions:** 0.3 USP Insulin Lispro Units/mL in duplicate prepared by diluting the *Standard stock solution* with *Dilution buffer*

**Sample solutions:** 0.3 USP Insulin Lispro Units/mL of insulin lispro in *Dilution buffer*. Prepare in duplicate.

**Diluted standard solutions and sample solutions:** For each assay plate, prepare three separate dilution sets from one of the *Standard solution* and *Sample solution* preparations. The range of the concentrations of insulin lispro solutions to be added to the cells needs to be determined during method verification. Successful testing was demonstrated for a range of $300 \times 10^{-3}$ to $0.004 \times 10^{-3}$ USP Insulin Lispro Units/mL. The dilution can be prepared as follows. Dispense 300 µL of *Dilution buffer* into each well of a 96-well polypropylene plate, except those wells in rows A, B, and C. Add 180 µL of *Dilution buffer* into the wells of row B and C. Next, add 300 µL of *Standard solution* into wells A1, A5, and A9. Add 300 µL of the first *Sample solution* into wells A2, A6, and A10; the second *Sample solution* into wells A3, A7, and A11; the third *Sample solution* into wells A4, A8, and A12. Perform simultaneously serial dilutions on the plate by transferring 120 µL of solution from the first row (A1–A12) to the second row, and mix three times. Then transfer 60 µL of solution from the second row to the third row, and mix three times. Next transfer 60 µL of solution from the third row to the fourth row, mix three times, and repeat this procedure across the whole plate up to row H. Repeat the above for the replicate preparation.

**Analysis:** All solutions are added by pipetting them into the plate wells carefully without touching the bottom. Following cell starvation, add 40 µL per well of each *Diluted standard solution* or *Diluted sample solution* into appropriate wells of the plate. Since each well has contained 160 µL of *Minimal medium*, the *Diluted standard solution* and *Diluted sample* solution are further diluted by a factor of 5.

[NOTE—See *Table 4* for an example of the plate layout, which can be changed column by column for randomization purposes. See *Design and Development of Biological Assays 1032, 5. Stages in the Bioassay Development Process, 5.1 Design: Assay Layout, Blocking, and Randomization* for helpful information on randomization of samples and plate layout.]

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**Table 4**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | St,#1 | S1,#1 | S2,#1 | S3,#1 | St,#1 | S1,#1 | S2,#1 | S3,#1 | St,#1 | S1,#1 | S2,#1 | S3,#1 |
| B | St,#2 | S1,#2 | S2,#2 | S3,#2 | St,#2 | S1,#2 | S2,#2 | S3,#2 | St,#2 | S1,#2 | S2,#2 | S3,#2 |
| C | St,#3 | S1,#3 | S2,#3 | S3,#3 | St,#3 | S1,#3 | S2,#3 | S3,#3 | St,#3 | S1,#3 | S2,#3 | S3,#3 |
| D | St,#4 | S1,#4 | S2,#4 | S3,#4 | St,#4 | S1,#4 | S2,#4 | S3,#4 | St,#4 | S1,#4 | S2,#4 | S3,#4 |
| E | St,#5 | S1,#5 | S2,#5 | S3,#5 | St,#5 | S1,#5 | S2,#5 | S3,#5 | St,#5 | S1,#5 | S2,#5 | S3,#5 |
| F | St,#6 | S1,#6 | S2,#6 | S3,#6 | St,#6 | S1,#6 | S2,#6 | S3,#6 | St,#6 | S1,#6 | S2,#6 | S3,#6 |
| G | St,#7 | S1,#7 | S2,#7 | S3,#7 | St,#7 | S1,#7 | S2,#7 | S3,#7 | St,#7 | S1,#7 | S2,#7 | S3,#7 |
| H | St,#8 (BG) | S1,#8 | S2,#8 | S3,#8 | St,#8 (BG) | S1,#8 | S2,#8 | S3,#8 | St,#8 (BG) | S1,#8 | S2,#8 | S3,#8 |

**Legend:**
St,#1–#8 = *Diluted standard solutions* prepared from the dilution series of *Standard solutions*.
S1, S2, S3, #1–#8 = *Diluted sample solutions* prepared from the dilution series of *Sample solutions* of insulin samples 1, 2, and 3.
BG = Background; *Diluted standard solutions* at the lowest concentration serve as background.

[NOTE—All the steps below are performed at room temperature, unless otherwise specified.]

Incubate the plates at 37° with 5% carbon dioxide ($CO_2$) for 20 min. Discard the media and replace with 150 µL of freshly prepared *Fixing solution* per well. Incubate the plates for 20 min with constant gentle shaking. Discard the *Fixing solution*, and add 200 µL of *Permeabilization solution* to each well. Incubate the plates for 10 min with constant gentle shaking. Repeat the permeabilization step once. Discard the *Permeabilization solution* and add 300 µL of *Blocking buffer* to each well. Cover the plates. Incubate the plates at 2°–8° overnight without shaking.

Remove *Blocking buffer* and add 50 µL of freshly prepared *Primary antibody solution* to each well. Cover the plates. Incubate the plates for about 2 h with constant gentle shaking. Alternatively, before removing the *Blocking buffer*, incubate the plates at room temperature for 1 h with constant gentle shaking. Then remove the *Blocking buffer*, add freshly prepared *Primary antibody solution*, and cover the plates. Incubate at 2°–8° overnight without shaking.

Discard the *Primary antibody solution*, wash each well 5 times using 200 µL of *Washing solution* per well each time. Perform each wash step under constant gentle shaking for about 5 min. Apply 50 µL of freshly prepared *Secondary antibody solution* to each well. Incubate the plates protected from light (use black lids) for about 1 h with constant gentle shaking. Discard the *Secondary antibody solution*. Wash each well 5 times using 200 µL of *Washing solution* per well each time. Perform each wash step under constant gentle shaking for about 5 min. Add 200 µL of *Washing solution* per well and leave the solution in until measurement. Protect the plates from light. [NOTE—The plates containing the *Washing solution* can be stored at 2°–8° for a maximum of 24 h and protected from light]

Read the plate in a suitable microplate reader or scanner with appropriate excitation and emission wavelengths.

**Instrumental conditions**

**Mode:** Fluorescence[13]

**Analytical wavelengths:** Appropriate excitation and emission wavelengths based on the fluorescent dyes used. Excitation wavelength for the secondary antibody signal needs to differ from the wavelength of the cell and/or DNA stain in order to enable parallel readout, for example, using about 800 nm to detect the secondary antibody and about 700 nm to detect DRAQ5™ DNA Dye and Sapphire700™.

**Calculations:** For each well of each assay plate, normalize (for number of cells per well) the assay signals detected from the secondary antibody that is bound to the primary antibody with the signals from the fluorescent dye for the cell staining and/or DNA fluorescent dye, which represent the total number of cells present for that well. The processed signal is derived from the normalized signal that is subtracted by the background signal. The background signal is the average of the normalized signals detected by the *Diluted standard solution* at the lowest concentration. If necessary, it can be multiplied with a factor smaller than 1.0 in order to avoid negative values at the lowest concentration after background subtraction.

Generate a dose-response curve by plotting the processed signal responses from each set of *Diluted standard solutions* or *Diluted sample solutions* from one assay plate against their concentrations on a logarithmic scale using a four parameter logistic (4-PL) constrained model. Therefore, three individual curves representing triplicate sets of *Diluted standard solutions* or *Diluted sample solutions* from each assay plate are generated. Determine the individual $EC_{50}$ value for the *Standard solution* or *Sample solution* from each of the curves of each assay plate.

[NOTE—The necessity of the responses to be weighted is to be determined during method verification. See *Design and Development of Biological Assays* ⟨1032⟩*, 4. Statistical Aspects of Bioassay Fundamentals, 4.8 Outliers* for helpful information on performing outlier detection and rejection, e.g., through a goodness-of-fit tool.]

Calculate the relative potency ($RP_{U,1\ or\ 2}$) percentage for each insulin sample from each assay plate:

$$RP_{U,x} = (EC_{50,S}/EC_{50,U}) \times 100$$

$EC_{50,S}$ = $EC_{50}$ value for the *Standard solution* from one assay plate
$EC_{50,U}$ = $EC_{50}$ value for the *Sample solution* from one assay plate

Calculate the geometric mean of the relative potency ($M_U$) of each insulin sample from both assay plates:

[13] Using a microplate reader with fluorescence detection, such as LI-COR Odyssey Infrared Imaging System or equivalent.

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$$M_U = \sqrt{RP_{U,1} \times RP_{U,2}}$$

$RP_{U,1}$ = relative potency of an insulin sample from assay plate 1
$RP_{U,2}$ = relative potency of an insulin sample from assay plate 2

Calculate the potency of the insulin sample in USP Insulin Glargine Units/mg or USP Insulin Lispro Units/mg:

$$\text{Potency} = (C_S \times M_U)/(C_U \times 100)$$

$C_S$ = concentration of USP Insulin Glargine RS or USP Insulin Lispro RS in the *Standard stock solution* (USP Insulin Glargine Units/mL or USP Insulin Lispro Units/mL)
$M_U$ = geometric mean of the relative potency of each insulin sample from both assay plates
$C_U$ = initial concentration of insulin sample (mg/mL)

**Parallelism between standard and sample solution curves:** See *Design and Development of Biological Assays* ⟨1032⟩*, 4. Statistical Aspects of Bioassay Fundamentals, 4.7 Suitability Testing* for helpful information on determining the parallelism by assessing the slope ratio and the upper and lower asymptotes.
**Relative standard deviation for $EC_{50}$:** NMT 25% for three $EC_{50}$ values derived from the triplicate sets of *Diluted sample solutions* from the same assay plate using the 4-PL free model
**Relative bias for relative potency of each individual sample:** NMT 35%
Determine the relative bias (RB) for relative potency of each individual sample:

$$\text{RB} = [(RP_{U,1\text{ or }2}/M_U) - 1] \times 100$$

$RP_{U,1\text{ or }2}$ = relative potency of an insulin sample from assay plate 1 or 2
$M_U$ = average of the relative potency of each insulin sample from both assay plates

**System suitability**
Generate a dose-response curve by plotting the processed signal responses of all three sets of *Diluted standard solutions* or *Diluted sample solutions* from one assay plate against their concentrations on a logarithmic scale using a 4-PL free model. Therefore, one curve representing all three sets of *Diluted standard solutions* and one curve for all three sets of *Diluted sample solutions* from one assay plate are generated. Determine the $EC_{50}$ value for the *Standard solution* ($EC_{50,S}$) and *Sample solution* ($EC_{50,U}$) of each assay plate from the corresponding curve.
**Samples:** *Diluted standard solution* and *Diluted sample solution*
**Suitability requirements**
**Relative standard deviation for $EC_{50}$:** NMT 25% for three $EC_{50}$ values derived from the triplicate sets of *Diluted standard solutions* from the same assay plate using the 4-PL free model
**Signal-to-noise ratio:** The mean signal for the *Diluted standard solutions* at the highest concentration is NLT 1.5-fold of the mean signal for all *Diluted standard solutions* at the lowest concentration from the same assay plate.
**Acceptance criteria:** NLT 15 USP Insulin Units/mg on the as-is basis▲ (USP 1-Dec-2020)

## ADDITIONAL REQUIREMENTS

• **USP Reference Standards** ⟨11⟩
USP Dextrose RS
USP Insulin Aspart RS
USP Insulin Glargine RS
USP Insulin Human RS
USP Insulin Lispro RS
USP Insulin Pork RS

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# ⟨123⟩ GLUCAGON BIOIDENTITY TESTS

## INTRODUCTION

Glucagon is a peptide hormone that increases blood glucose levels via release of liver glycogen stores. A robust and precise physicochemical chromatographic procedure is used in the glucagon assay to assign potency on a mass basis. Bioidentity is still required in *Glucagon for Injection*, and two procedure options are presented here: an in vivo procedure based on release of glucose from freshly prepared rat liver cells (hepatocytes) stimulated with glucagon ex vivo or production of cyclic adenosine monophosphate (cAMP) in vitro in response to glucagon stimulation of the glucagon receptor cell line. To meet the acceptance criteria of the bioidentity test, only one of these bioidentity tests is required.

## PROCEDURE

***Change to read:***

• **A. Primary Liver Cell Bioidentity Test**

[Note—All buffers are oxygenated, prepared with either *Sterile Water for Injection* or *Sterile Water for Irrigation*, warmed to 37°, and adjusted to a final pH of 7.4 unless otherwise indicated. At least two independent assays (replicates) must be performed utilizing two rat livers for each lot of glucagon. *Figure 1* demonstrates the process used to generate one replicate value. A minimum of two replicates are combined according to the *Calculations* section. The concentration range of the *Standard preparations* and *Assay preparations* may be modified to fall within the linear range of the *Assay*, and the calculations can be adjusted accordingly. Alternatively, full curve analysis using validated nonlinear statistical methods can be used, provided that similarity is demonstrated when analysts compare the responses of the *Standard preparations* and *Assay preparations*.]

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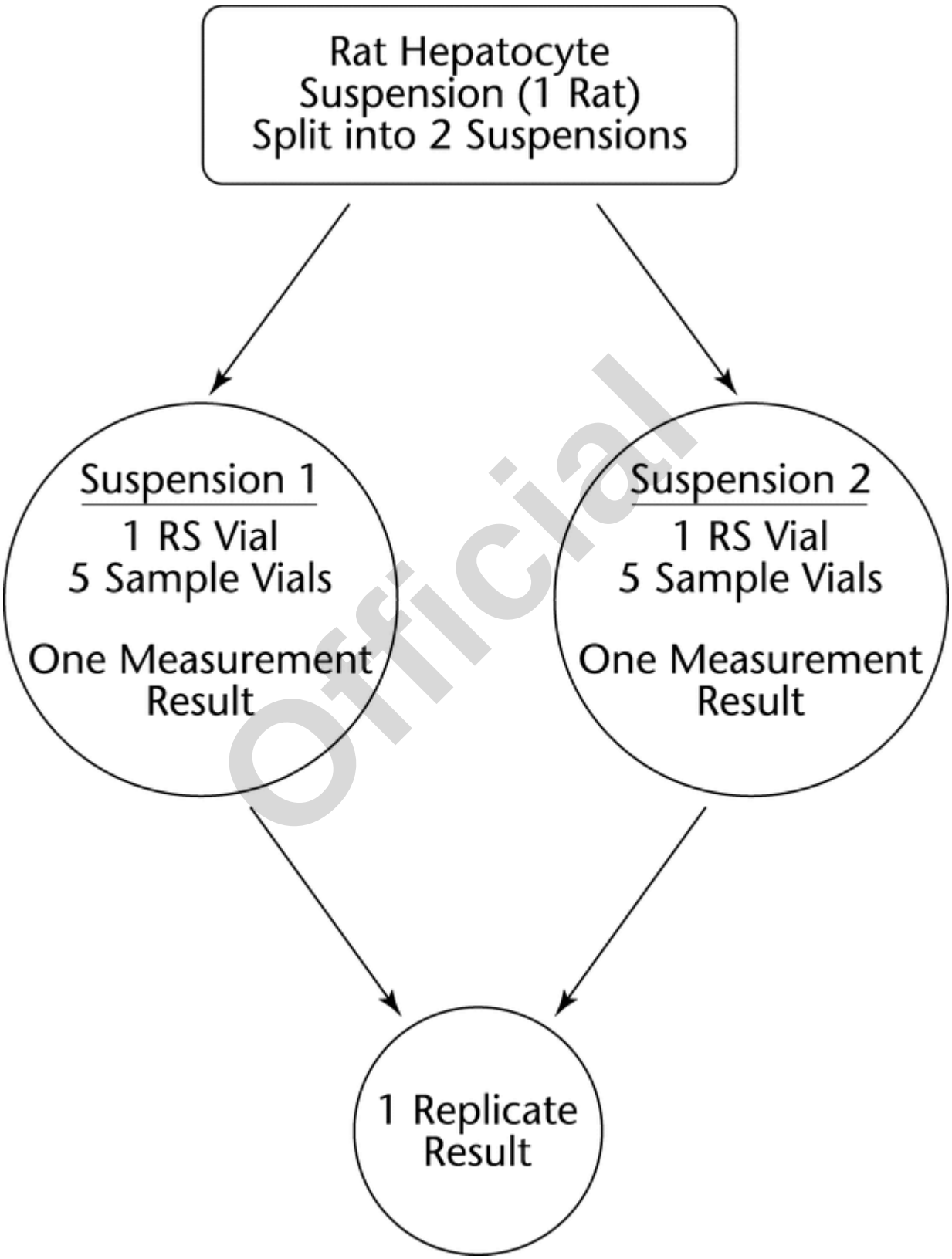


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Figure 1. Rat hepatocyte assay method flow diagram (RS = Reference Standard).

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**Hepatocyte preparation**

**Calcium-free perfusion buffer with dextrose:** Prepare a solution containing 7.92 g/L of sodium chloride, 0.35 g/L of potassium chloride, 1.80 g/L of dextrose, 0.19 g/L of edetic acid (EDTA), and 2.38 g/L of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). Oxygenate before use.

**Collagenase buffer:** Prepare a solution containing 3.62 g/L of sodium chloride, 23.83 g/L of HEPES, 0.35 g/L of potassium chloride, 0.52 g/L of calcium chloride, and 1.8 g/L of dextrose. Adjust to a pH of 7.6. Immediately before perfusion, dissolve a quantity of collagenase in this solution to obtain a concentration of 0.02%–0.05%. The exact concentration of collagenase is determined empirically for each new lot of enzyme and is the amount that can consistently dissociate the tissue within 10 min of buffer entry and produce a viable cell concentration of not less than $3 \times 10^6$ cells/mL.

**Wash buffer:** Prepare a solution containing 7.92 g/L of sodium chloride, 0.35 g/L of potassium chloride, 0.19 g/L of EDTA, 2.38 g/L of HEPES, 0.11 g/L of calcium chloride, and 0.06 g/L of magnesium sulfate.

**Incubation buffer:** Prepare a solution containing 6.19 g/L of sodium chloride, 0.35 g/L of potassium chloride, 0.22 g/L of calcium chloride, 0.12 g/L of magnesium sulfate, 0.16 g/L of monobasic potassium phosphate, 11.915 g/L of HEPES, and 10 g/L of bovine serum albumin (1% BSA). Adjust to a pH of 7.5.

**Test animals:** Male Sprague-Dawley rats are maintained on a standard rat chow diet, given water ad libitum, and allowed to adjust to their new housing before testing. On the morning of the test, select a healthy rat weighing approximately 300–400 g, and administer 100 units of Heparin Sodium subcutaneously.

**Procedure:** [NOTE—Conduct this procedure in the morning to ensure that the rat has optimal glycogen in its liver and so that the procedure can be completed in 1 day.] Anesthetize the rat with an appropriate anesthetic. Open the abdominal cavity and isolate the portal vein. Insert an angiocatheter and tie into the portal vein at the general location of the lienal branch and then connect to a perfusion pump. Start the perfusion (25 mL/min) in situ with the previously warmed, oxygenated, *Calcium-free perfusion buffer with dextrose*. As the liver enlarges, cut the inferior vena cava to allow pressure equilibrium. [NOTE—About 300 mL of the perfusate is needed to clear the liver of red blood cells at a flow rate of 25–60 mL/min.] Then circulate *Collagenase buffer* at an appropriate flow rate so that the liver leaks perfusate out of the lobes in approximately 10 min (typically 25–60 mL/min). When the liver significantly increases in size, changes color and consistency, and starts to leak perfusate out of the lobes, change the system to the oxygenated prewarmed *Wash buffer*. About 100 mL of *Wash buffer* is needed to wash the liver of collagenase at a flow rate of 25 mL/min. Surgically remove the liver from the animal, and place in a prewarmed Petri dish containing a small amount of oxygenated *Wash buffer* (37°). Gently comb the liver with a stainless steel, fine-toothed comb to free the hepatocytes. Filter and wash the hepatocytes with *Wash buffer*, through prewetted cheesecloth (three layers thick, or through a 150-µm mesh polyethylene net) into a beaker. Transfer the cells to 2 centrifuge tubes and spin for about 1 min at 600 rpm. Discard the supernatant fractions and resuspend the two pellets in *Incubation buffer*. Combine the two pellets in a suitable container and add sufficient *Incubation buffer* to make 150 mL.

**System suitability of cell preparation:** The cell yield may vary because of the collagenase activity and the viability of the hepatocytes. To check cell viability and to determine viable cell concentration, dilute a 100-µL aliquot of the cell suspension with 400 µL of *Wash buffer* and 500 µL of isotonic 0.4% trypan blue solution. Load aliquots of the cell suspension into both chambers of a hemocytometer and count the cells in all eight quadrants. To meet system suitability of the cell preparation method, a viable cell concentration of $3 \times 10^6$ cells/mL (acceptable range of $2.5 \times 10^6$ to $3.4 \times 10^6$ cells/mL) must be obtained to proceed with the bioassay. If the viable cell concentration exceeds the upper limit, additional *Incubation buffer* may be added to the cells to adjust the concentration to $3 \times 10^6$ cells/mL. In this case, the cells are counted again in a hemocytometer, as described above, to verify the concentration. [NOTE—Viable cells are those cells that exclude the trypan blue.]

**Glucose determination**

**Negative control solution:** Prepare a solution containing 0.5% BSA using *Sterile Water for Injection* or *Sterile Water for Irrigation*.

**Incubation flasks:** Use specially prepared 25-mL conical flasks, the bottoms of which have been heated and pushed inward to form a conically raised center, or similar flasks that allow sufficient mixing when swirling. Place the *Incubation flasks* in an orbital shaker water bath at 35°.

**Standard preparations:** On the day of the assay, dissolve 2 vials of ▲USP Glucagon (Human) RS▲ (USP 1-Aug-2022) in 0.01 N hydrochloric acid or other suitable diluent (volume based on the potency of the Reference Standard lot) to obtain two solutions each containing ▲1 USP Glucagon (Human) Unit/mL.▲ (USP 1-Aug-2022) All dilutions thereafter are made using *Negative control solution*. Accurately dilute measured volumes of each solution with *Negative control solution* to obtain an intermediate concentration of 400 µU/mL, and then dilute the intermediate to produce 5 concentrations: 200, 100, 50, 25, and 12.5 µU/mL (*Standard preparations*). Pipet 0.1 mL of each *Standard preparation* into separate *Incubation flasks*. Pipet 0.1 mL of *Negative control solution* into each of 2 flasks (*Negative control solutions 1* and *2*).

**Assay preparations:** Using accurately weighed quantities of glucagon samples, proceed as directed for *Standard preparations* or, if testing *Glucagon for Injection*, reconstitute 10 vials by slowly adding the contents of the accompanying prefilled syringes containing an appropriate glucagon diluent. Gently mix each vial until the glucagon is dissolved. Using the same syringes, withdraw the contents of 5 vials and place the solutions in a 25-mL volumetric flask. Repeat for the second set of 5 vials, transferring the contents to a second 25-mL volumetric flask. Dilute each flask with 0.01 N hydrochloric acid to volume. Dilute an accurate amount of each solution with 0.5% BSA to yield a concentration of 400 µU/mL, and dilute the intermediate to produce 5 *Assay preparation* concentrations: 200, 100, 50, 25, and 12.5 µU/mL. Then proceed as directed for the *Standard preparations*.

**Reference stock solution:** Dry USP Dextrose RS, and then transfer 1.0 g, accurately weighed, to a 100-mL volumetric flask. Dissolve in and dilute with saturated benzoic acid solution to volume.

**Reference solutions:** Transfer suitable quantities of *Reference stock solution* to 4 flasks, and dilute with saturated benzoic acid solution to obtain *Reference solutions* having concentrations of 100, 500, 1000, and 1500 mg/L.

**Potassium ferrocyanide solution:** Dissolve 1.25 g of trihydrate potassium ferrocyanide in 125 mL of *Sterile Water for Injection*, or use an appropriate commercial source.

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**System suitability:** Analyze the *Potassium ferrocyanide solution*, the *Reference solutions*, and an additional 5 replicates of either the 500- or 1000-mg/L *Reference solution* in an appropriate glucose analyzer. [NOTE—*Potassium ferrocyanide solutions* are only appropriate standards for glucose analyzers that measure glucose oxidase activity. The procedure can also be performed using alternative platforms.] Prepare a standard curve using the *Reference solutions* as directed for the *Standard preparations*. The square root of the residual error mean square from the regression divided by the average of the response multiplied by 100% (line %RSD) must be not more than 2.0%. In addition, the response of the *Potassium ferrocyanide solution* must be not more than 30 mg/L, and the relative standard deviation must be not more than 2.0% for the replicate analyses of the middle *Reference solution*.

**Procedure:** Dispense 5 mL of *Hepatocyte preparation* into the *Incubation flasks* in sequence from high glucagon concentration to low glucagon concentration, alternating the *Standard preparations* with the *Assay preparations*. Swirl the flasks in an orbiting water bath at 125 rpm at 30°–35° for approximately 30 min. Following incubation, remove 1.0-mL aliquots from each *Incubation flask*, transfer to labeled microcentrifuge tubes, and centrifuge at 13,000 rpm for 15 s. Place each supernatant fraction in a labeled sampling tube for a glucose analyzer, and determine the glucose concentration (mg/L) of each *Standard preparation* and *Assay preparation*. Measure the background reading of *Negative control solution 1* and *Negative control solution 2*, and calculate the average of the two responses.

To conform to the linear range of the instrument being used, analysts may find it necessary to adjust by dilution each of the *Standard preparations* and *Assay preparations*. Use a glucose analyzer that has demonstrated appropriate specificity, accuracy, precision, and linear response over the range of concentrations being determined. Determine the increase in glucose concentration for each *Standard preparation* and *Assay preparation* compared to the average value of the *Negative control solution*.

**Calculations**

Calculate the relative potency of the glucagon samples using statistical methods for parallel-line assays, comparing the Reference Standard curve (from the *Standard preparations*) to the glucagon sample curve (from the *Assay preparations*). No dose–response reversals may occur within a run for the 25, 50, or 100 µU/mL *Standard preparations* and *Assay preparations*. [NOTE—Either the low- or high-dose level, but not both, may be excluded from the calculation in order to meet linearity requirements.] Because a minimum of two valid assays (rats) are required, the estimated potencies are combined using the procedures in *Design and Analysis of Biological Assays* ⟨111⟩*, Combination of Independent Assays*, and the width, *L*, of a 95% confidence interval for the estimated logarithm of the relative potency is calculated. If *L* is not more than 0.1938, the results are valid. If *L* is >0.1938, additional assays may be performed and combined until a valid *L* term results, and the relative potency is then calculated from all valid independent runs. Calculate the potency of the glucagon samples in ▲USP Glucagon (Human) Units/mg▲ (USP 1-Aug-2022) by multiplying the relative potency result by the potency of ▲USP Glucagon (Human) RS.▲ (USP 1-Aug-2022)

**Acceptance criteria:** Not less than ▲0.80 USP Glucagon (Human) Units/mg▲ (USP 1-Aug-2022)

***Change to read:***

- **B. IN VITRO CELL-BASED BIOIDENTITY TEST**

**Medium A:** Dulbecco's Modified Eagle Medium (DMEM) containing 4.5 mg/mL of D-glucose and sodium pyruvate, 4 mM L-alanine-L-glutamine[1] or L-glutamine, 10% (v/v) fetal bovine serum,[2] and 0.5 mg/mL of G418[3]

**Buffer A:** Hank's Balanced Salt Solution[4] containing 5.3 mM potassium chloride, 0.4 mM potassium phosphate monobasic, 4.2 mM sodium bicarbonate, 137.9 mM sodium chloride, 0.3 mM sodium phosphate dibasic anhydrous, and 5.6 mM dextrose

**Cell culture preparation:** Remove glucagon receptor cell line[5] from cryostorage and immediately thaw at 37° until the cell suspension has just thawed. Aseptically transfer the cell suspension from the cryotube into a sterile test tube containing 10 mL of warmed *Medium A*. Mix and then pellet the cells by centrifugation for 5 min at 125 × g. Remove the supernatant and resuspend the cells in fresh, warmed *Medium A*. Measure the quantity of cells contained in an aliquot of the suspension by suitable methods, and adjust the cell concentration with *Medium A* such that the cell suspension is 0.5–5 × $10^3$ cells/$cm^2$ of tissue culture flask surface. Inoculate tissue culture flasks[6] and store in a humidified incubator at 37° containing 5% carbon dioxide. Cells should be passaged two to three times per week when they are not more than 90% confluent but never trypsinized 2 days in a row. If cells are not ready for passaging after 3 days of culture, the medium should be replaced with fresh *Medium A*. Cells are passaged by first removing the medium from the cell flasks, followed by adding sufficient *Buffer A* prewarmed to room temperature to cover the surface of the flasks. The flasks are gently rocked to wash the cells. This wash fluid is discarded and then sufficient trypsin[7] is added to the cell flasks to cover the surface, followed by gentle rocking and placement back in the incubator. After 3–5 min, the trypsinized cells are aseptically collected from the flasks and transferred to a sterile centrifuge tube. Then, a volume of *Medium A* is added that is a minimum of 2 times the volume of trypsinized cells, and an aliquot is counted. The cell suspension is further diluted with fresh *Medium A* to a final cell concentration of 0.5–5 × $10^3$ cells/$cm^2$ of tissue culture flask surface. After a minimum of 8 passages post-thaw but not more than 25 passages, the cells can be used in the assay. The day before an assay, follow the cell passaging instructions above, but resuspend the cells in fresh, warmed *Medium A* to a final cell concentration of 4–5 × $10^4$ cells/mL. [NOTE—Three identical, independent 96-well white plates[8] are needed for measurement of two test samples ("A" and "B") analyzed on each. All wells must be loaded with cells within 40 min of resuspension following trypsinization and counting

[1] Invitrogen catalog #31966-021 or suitable equivalent.
[2] Gibco catalog #10082-147 or suitable equivalent.
[3] Calbiochem catalog #345812 or suitable equivalent.
[4] Invitrogen catalog #14175 or suitable equivalent.
[5] Available from the American Type Culture Collection (ATCC).
[6] Corning catalog #3151 or suitable equivalent.
[7] Gibco catalog #12563-011 or suitable equivalent.
[8] PerkinElmer catalog #6005680 or suitable equivalent.

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in order to keep a homogeneous cell suspension.] Using constant, gentle mixing without foaming, each well is loaded with 0.1 mL of cell suspension and incubated overnight at 37° and 5% carbon dioxide (approximately 24 h ± 4 h) prior to starting the *Procedure*. [NOTE—All remaining solutions should be prepared on the day of the *Procedure*.]

**Medium B:** *Krebs salt solution* containing 0.3% (v/v) human serum albumin, 25 mM HEPES, 1.7 mM 3-isobutyl-1-methyl-xanthine, 0.2 mg/mL of glucose, 650 KIU/mL of aprotinin, and 0.0003% (v/v) polysorbate 80, pH 7.4

**Medium C:** *Krebs salt solution* containing 0.3% (v/v) human serum albumin and 25 mM HEPES, pH 7.4

**Standard stock solution:** Reconstitute ▲USP Glucagon (Human) RS▲ (USP 1-Aug-2022) in *Water for Injection* or another suitable diluent to a concentration of 4 mg/mL by gently mixing on a rotator for 10 min or until completely clear. Dilute this material 1:1000 with *Medium B* to a concentration of 4 µg/mL and then 1:200 with *Medium B* to 20 ng/mL.

**Standard solutions:** Within an hour of use, dilute the 20-ng/mL *Standard stock solution* with *Medium B* to make a 1:5 concentration series of 4 ng/mL (R8), 0.8 ng/mL (R7), 160 pg/mL (R6), 32 pg/mL (R5), 6.4 pg/mL (R4), and 1.3 pg/mL (R3). A final dilution is made from R3 in *Medium B* to make 65 fg/mL (R2). [NOTE—Alternative suitable concentrations can be similarly prepared if necessary and validated.] R1 is a blank solution of *Medium B*. [NOTE—For these *Standard solutions*, as well as the *Sample solutions* and *cAMP standard solutions* described below, it may be helpful to prepare these dilutions in a dilution microplate that mimics the assay plate layout to easily and quickly transfer the materials to the wells of the assay plate.]

**Sample solutions:** Reconstitute two independent preparations of glucagon from the same lot of material in *Water for Injection* or the same suitable diluent used to prepare the *Standard stock solution* to a concentration of 4 mg/mL. Prepare by diluting this material further with *Medium B* to prepare a concentration series similar to that suggested for the *Standard solutions*. One preparation series is *Sample solution* A8–A2 and the other preparation series is *Sample solution* B8–B2.

**cAMP standard solutions:** Prepare a 5 mM cAMP▲▲ (USP 1-Aug-2022) solution in water, and then prepare further solutions by dilution with *Medium B* as shown in *Table 1*. [NOTE—To conform to the linear range of the instrument being used, analysts may find it necessary to adjust by dilution each of the *Standard solutions*, *cAMP standard solutions*, and *Assay solutions*. Alternative suitable concentrations can be similarly prepared if necessary and validated.]

**Table 1. Preparation of cAMP Standard Solutions**

| Starting cAMP Solution | Fold Dilution with Medium B | Final cAMP Solution | Final cAMP Standard Solution Name |
|---|---|---|---|
| 5 mM | 1:10 | 0.5 mM | — |
| 0.5 mM | 1:20 | 25 µM | C1 |
| 25 µM | 1:3.3 | 7.5 µM | C2 |
| 25 µM | 1:10 | 2.5 µM | C3 |
| 7.5 µM | 1:10 | 0.75 µM | C4 |
| 2.5 µM | 1:10 | 250 nM | C5 |
| 0.75 µM | 1:10 | 75 nM | C6 |
| 250 nM | 1:10 | 25 nM | C7 |
| 75 nM | 1:10 | 7.5 nM | C8 |
| *Medium B* only | — | No cAMP | C9 |

**Donor biotin-cAMP beads:** Dissolve 10 nmol biotinylated cAMP in 0.5 mL of phosphate-buffered saline, pH 7.4. Add 80 µL of this biotinylated cAMP solution to 33.8 mL of a 5 mM HEPES lysis buffer containing 0.1% (w/v) BSA and 0.3% (v/v) polysorbate 20, pH 7.4. Add 270 µL of donor beads[9] and 2.4 mL of 120 mM magnesium chloride solution.

**Procedure:** Remove the plates seeded with cells the day before, and discard the *Medium A* in each well. Wash the cells with 350 µL of *Medium C* per well, then discard the wash solution. Rapidly add 20 µL of diluted 1 × anti-cAMP-acceptor beads per well. Add 30 µL each of *Standard solution*, *Sample solution*, *Medium B*, or *cAMP standard solution* per well as indicated in the plate layouts shown in *Table 2* and *Table 3*. [NOTE—The white plate for the *cAMP standard solutions* does not contain cells.] Cover the plate,[10] protecting it from light and evaporation, and incubate for 35–60 min in an incubator at 37° with gentle shaking. Next, dispense 60 µL of *Donor biotin-cAMP beads*[9] per well, mix thoroughly, then cover the plate again and wrap it with foil. [NOTE—The beads are light sensitive so plates should be kept in the dark or under green light conditions.] Gently rotate the plate on a shaker for at least 30 min at room temperature. Keep the plate in the dark at room temperature without shaking for 16–30 h before measuring the top luminescence from the plate wells on a suitable plate reader[11] and detection mode (excitation wavelength of about 680 nm; emission of 520–620 nm).

**Table 2. Schematic Representation of the Standard and Sample Assay Plates**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | R1 | R2 | A2 | B2 | R3 | A3 | B3 | R4 | A4 | B4 | R8 | — |
| B | R1 | R2 | A2 | B2 | R3 | A3 | B3 | R4 | A4 | B4 | R8 | — |
| C | R1 | R2 | A2 | B2 | R3 | A3 | B3 | R4 | A4 | B4 | R8 | — |

[9] PerkinElmer catalog #6760635D or suitable equivalent.
[10] PerkinElmer catalog #6050195 or suitable equivalent.
[11] PerkinElmer catalog #2300-0000 or suitable equivalent.

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**Table 2. Schematic Representation of the Standard and Sample Assay Plates** *(continued)*

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| D | R1 | R2 | A2 | B2 | R3 | A3 | B3 | R4 | A4 | B4 | R8 | — |
| E | R5 | A5 | B5 | R6 | A6 | B6 | R7 | A7 | B7 | A8 | B8 | C9 |
| F | R5 | A5 | B5 | R6 | A6 | B6 | R7 | A7 | B7 | A8 | B8 | C6 |
| G | R5 | A5 | B5 | R6 | A6 | B6 | R7 | A7 | B7 | A8 | B8 | C4 |
| H | R5 | A5 | B5 | R6 | A6 | B6 | R7 | A7 | B7 | A8 | B8 | C1 |

**LEGEND:**
A2–A8 = Dilution series of *Sample solution* A. (The same solution is used on 3 plates, with 4 ng/mL as the starting concentration.)
R1–R8 = Dilution series of *Standard solution* for each test plate, with 4 ng/mL as starting concentration; as a result, each *Sample solution* is only compared to the *Standard solution* on that plate.
B2–B8 = Dilution series of *Sample solution* B. (The same solution is used on 3 plates, with 4 ng/mL as the starting concentration.)
— = No glucagon (contains 30 µL *Medium B* instead), but cells are present.
C = cAMP standard controls, no glucagon. C1 contains 25 µM cAMP, C4 contains 0.75 µM cAMP, C6 contains 75 nM cAMP, and C9 contains no cAMP.

**Table 3. Schematic Representation of the cAMP Standards Assay Plate**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | — | — | — | — | — | — | — | — | — | — | — | — |
| B | — | — | — | — | — | — | — | — | — | — | — | — |
| C | C1 | C2 | C3 | C4 | C5 | C6 | C7 | C8 | C9 | — | — | — |
| D | C1 | C2 | C3 | C4 | C5 | C6 | C7 | C8 | C9 | — | — | — |
| E | C1 | C2 | C3 | C4 | C5 | C6 | C7 | C8 | C9 | — | — | — |
| F | — | — | — | — | — | — | — | — | — | — | — | — |
| G | — | — | — | — | — | — | — | — | — | — | — | — |
| H | — | — | — | — | — | — | — | — | — | — | — | — |

C1–C9 = Dilution series of cAMP standard controls starting with C1, which contains 25 µM cAMP, through C9, which does not contain cAMP.

**System suitability criteria:** The lower asymptote of the four-parameter logistic curves generated from *Sample solutions* A2–A8 and B2–B8 and *Standard solutions* R1–R8 must be above the lower limit (defined as C3) of the *cAMP standard solutions*. Not less than a three-fold difference between the signals obtained in the wells of cells treated without glucagon (R1) versus those with the maximal amount of glucagon (R8). Not more than 4 technical outliers may be omitted per standard curve. Any plate that fails one or more of these criteria is rejected and must be repeated. If either *Sample solution* A or B fails one of these criteria, then only the passing series can be used for the calculations. [NOTE—The upper asymptotes of the four-parameter logistic standard curves generated from *Sample solutions* A2–A8 and B2–B8 and *Standard solutions* R1–R8 should be approximately equal to or less than the C8 response of the *cAMP standard solutions* and within the linear range of the instrument used.]

**Calculations:** A series of two independent *Sample solution* preparations must be used for each test sample across 3 plates (assays). Outliers identified by Grubbs test (see ⟨111⟩; but not more than 4 per curve and not more than 1 data point from a replicate set) are omitted, and then the same number of *Standard solution* and *Sample solution* dose responses, including the 50% response ($EC_{50}$) of the standard/test sample within this range, are used to calculate the relative potency of the glucagon sample using statistical methods for parallel-curve analysis with a four-parameter logistic fit using all replicate values. For each individual *Sample solution* compared to the *Standard solution*, the statistical tests for slope and parallelism must pass at the 95% confidence level. Calculate the relative potency of the glucagon samples by comparing the Reference Standard curve (from the *Standard solutions*) to the glucagon sample curve (from the *Sample solutions*). Because a minimum of three valid assays are required, the estimated potencies are combined using the procedures in ⟨111⟩, *Combination of Independent Assays*, and the confidence interval is calculated using suitable statistical methods. If the confidence limits are between 64% and 156%, the results are valid. If not, up to 2 more assay plates may be performed and combined until a valid confidence interval results, and the relative potency is then calculated from all valid independent runs. Calculate the potency of the glucagon samples in ▲USP Glucagon (Human) Units/mg▲ (USP 1-Aug-2022) by multiplying the relative potency result by the potency of ▲USP Glucagon (Human) RS.▲ (USP 1-Aug-2022)

**Acceptance criteria:** Not less than ▲0.80 USP Glucagon (Human) Units/mg▲ (USP 1-Aug-2022)

## ADDITIONAL REQUIREMENTS

***Change to read:***

- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Dextrose RS
  ▲USP Glucagon (Human) RS▲ (USP 1-Aug-2022)

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# ⟨124⟩ ERYTHROPOIETIN BIOASSAYS

## INTRODUCTION

This chapter provides guidance about the use of bioassays to measure the biological activity of erythropoietin (EPO). Based on a relative potency approach described in general chapter *Design and Development of Biological Assays* ⟨1032⟩, the biological activity of an unknown EPO preparation typically is determined by comparison to the biological activity of a Reference Standard (RS). For this purpose, a USP Erythropoietin for Bioassays RS has been developed. The assays described in the chapter are not applicable to forms of EPO that have been engineered to prolong their half-life.

The USP Erythropoietin for Bioassays RS has been calibrated against the World Health Organization (WHO) International Standard for EPO using both the normocythemic mouse bioassay and the exhypoxic polycythemic mouse bioassay. Accordingly, the USP Erythropoietin for Bioassays RS has an assigned unitage in terms of International Units (IU) of EPO as defined by WHO. Of these two in vivo bioassays, the normocythemic mouse bioassay is less cumbersome and is more widely used for the determination of EPO potency. The assay is based on the measurement of EPO-stimulated reticulocyte maturation. Because the USP Erythropoietin for Bioassays RS has been calibrated against the WHO International Standard for EPO by in vivo bioassay, it can be used directly in the normocythemic mouse bioassay for the calibration of any process-specific EPO preparation.

However, if the intent is to use a validated in vitro bioassay to transfer the unitage from the USP Erythropoietin for Bioassays RS to a process-specific EPO preparation, then it is necessary to demonstrate that the USP Erythropoietin for Bioassays RS and the process-specific EPO preparation exhibit an equivalent ratio of in vitro to in vivo potency. This is because the biological activity of EPO shows a complex relationship between structure and function. In particular, the ratio of in vitro to in vivo activity is inversely correlated with type and degree of terminal sialylation. Highly sialylated products have a higher level of in vivo activity; thus, they have a relatively lower ratio of in vitro to in vivo activity. Because of differences in the manufacturing processes used in the preparation of EPO, the type and degree of terminal sialylation can be variable from one EPO preparation to another, including EPO preparations used as Reference Standards. As a result, the use of an in vitro assay to measure the biological activity of an EPO preparation requires a full understanding of the relationship between the EPO's in vivo and in vitro activity. This understanding is best achieved by comparing the test EPO preparation with the USP Erythropoietin for Bioassays RS in both the in vivo assay and in a specific in vitro assay.

The USP Erythropoietin for Bioassays RS is assigned a unitage that represents its activity in both in vivo assays and in vitro assays. If the ratios of in vitro to in vivo potency for the material being tested and the USP Erythropoietin for Bioassays RS are equivalent, then the USP Erythropoietin for Bioassays RS can be used directly in the in vitro assay to calibrate the material being tested. However, if the ratios are not equivalent, then the material being tested and the USP Erythropoietin for Bioassays RS have a different ratio of in vitro to in vivo potency, and the standard cannot be used with its assigned potency in the in vitro assay. Instead, this ratio determined for the material being tested should be used to assign a process-specific in vitro assay unitage to the USP Erythropoietin for Bioassays RS. The USP Erythropoietin for Bioassays RS, with its adjusted in vitro assay unitage, then can be used in the in vitro assay to transfer the unitage from the USP Erythropoietin for Bioassays RS to the material being tested.

## ASSAY

- **Normocythemic Mice Assay**

**Solution A:** Dissolve 10.75 g of disodium hydrogen phosphate dodecahydrate, 7.6 g of sodium chloride, and 10 g of bovine albumin in 1000 mL of water. Immediately before use adjust with sodium hydroxide solution or phosphoric acid to a pH of 7.2.

**Standard solution 1:** Dissolve USP Erythropoietin for Bioassays RS in *Solution A* to a concentration of 80 IU/mL.

**Standard solution 2:** Mix equal volumes of *Solution A* and *Standard solution 1*.

**Standard solution 3:** Mix equal volumes of *Solution A* and *Standard solution 2*.

**Sample solution 1:** Prepare the sample to be tested in *Solution A* to a concentration of 80 IU/mL.

**Sample solution 2:** Mix equal volumes of *Solution A* and *Sample solution 1*.

**Sample solution 3:** Mix equal volumes of *Solution A* and *Sample solution 2*.

The exact concentrations of each *Standard solution* and *Sample solution* may require adjustment based on the response range in the animals used.

**Preparation of animals:** At the beginning of the assay procedure, randomly distribute mice of a suitable age and strain (8-week-old B6D2F1 mice are suitable) into six cages. A minimum of eight mice per cage is recommended. Inject each animal subcutaneously with 0.5 mL of the appropriate treatment (one solution per cage), and put the animal in a new cage. Combine the mice in such a way that each cage housing the treated mice contains one mouse out of the six different treatments (each *Standard solution* and each *Sample solution*; six mice per cage).

**Analysis:** Four days after the injections, collect blood samples from the animals, and determine the number of reticulocytes by using a suitable procedure. The following method can be employed.

The volume of blood, dilution procedure, and fluorescent reagent may require modification to ensure maximum development and stability of fluorescence.

For concentrated colorant solution, use a solution of thiazole orange suitable for the determination of reticulocytes. Prepare at a concentration twice that necessary for the analysis.

Proceed with the following dilution steps. Dilute whole blood 500-fold in the buffer used to prepare the colorant solution. Dilute this solution two-fold in the concentrated colorant solution. After staining for 3–10 min, determine the reticulocyte count microfluorometrically in a flow cytometer. The percentage of reticulocytes is determined using a biparametric histogram: number of cells/red fluorescence (620 nm).

Calculate the potency by the usual statistical methods for a parallel-line assay. For additional information, see general chapter *Analysis of Biological Assays* ⟨1034⟩.

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**Acceptance criteria:** The activity of each *Sample solution* is compared with that of the USP Erythropoietin for Bioassays RS and is expressed in IU. The confidence limits of the estimated potency ($P = 0.95$) are NLT 64% and NMT 156% of potency as determined by the method given above. The estimated potency is NLT 80% and NMT 125% of the stated potency.

## ADDITIONAL REQUIREMENTS

- **USP REFERENCE STANDARDS** ⟨11⟩

  USP Erythropoietin for Bioassays RS

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***Change to read:***

# ⟨126⟩ SOMATROPIN BIOIDENTITY TEST▲ ▲ (USP 1-May-2023)

***Change to read:***

Somatropin is a protein hormone that contains the same amino acid sequence as the human growth hormone produced by the pituitary gland. A robust and precise physicochemical chromatographic procedure is used in the assay to assign potency on a mass basis. Bioidentity is still required,▲ so a▲ (USP 1-May-2023) rat-cell-line-based approach that measures production of ATP as a direct indicator of cell growth ▲is presented here.▲ (USP 1-May-2023) [NOTE—The bioidentity test may be performed either on the somatropin bulk drug substance or on the drug product.]

**PROCEDURE**

***Delete the following:***

▲• **IN VIVO BIOIDENTITY TEST**▲ (USP 1-May-2023)

• **IN VITRO CELL-BASED BIOIDENTITY TEST**

**Medium A:** Fischer's medium[1] containing 10% heat-inactivated fetal bovine serum,[2] 10% heat-inactivated horse serum,[3] 0.075% sodium bicarbonate, and 0.05 mM 2-mercaptoethanol. Filter sterilize. [NOTE—Store for up to 2 weeks at 2°–8°.]

**Medium B:** Fischer's medium containing 1% horse serum, 0.075% sodium bicarbonate, and 0.05 mM 2-mercaptoethanol. Filter sterilize. [NOTE—Store for up to 2 weeks at 2°–8°.]

**Phosphate buffered saline[4]:** Calcium- and magnesium-free phosphate buffered saline containing 1.5 mM monobasic potassium phosphate, 155 mM sodium chloride, and 3 mM dibasic sodium phosphate, pH 7.2–7.4

**Cell culture preparation:** Prepare cell suspension cultures of Nb2-11[5] cells in *Medium A* in a humidified incubator at 37° and containing 5% carbon dioxide. Cells should be passaged twice per week and reseeded at a density of $1 \times 10^5$ cells/mL for 2 days, $2 \times 10^4$ cells/mL for 3 days, and $1 \times 10^4$ cells/mL for 4 days in *Medium A*. [NOTE—Seeding densities may need adaptation when analysts qualify new lots of fetal bovine serum and horse serum.] On the day of an assay, cells are harvested from flasks and are pelleted by centrifugation for 7 min at about 218 × g. The supernatant is discarded, and the cells are washed twice with phosphate buffered saline followed by centrifugation. The cells are resuspended in *Medium B* and are counted, and the cell concentration is adjusted to $1 \times 10^5$ cells/mL with *Medium B*. Except for the wells of the first column, transfer 50 µL of cell suspension into each well of a 96-well black tissue culture plate with a clear bottom.[6] Pipet 50 µL of *Medium B* into each well of the first column of the plate. [NOTE—Cover the plate, and incubate at 37° for up to 1 h while preparing the *Standard solutions* and *Test solutions*.]

**Standard solutions:** Reconstitute USP Somatropin RS in 1 mL of phosphate buffered saline. From the reconstituted USP Somatropin RS, the *Standard solutions* and the *Positive control solution* are prepared for use in the *Procedure*. For the *Standard solutions*, the reconstituted USP Somatropin RS is further diluted with *Medium B* to a concentration of 2.0 ng/mL. [NOTE—Use polypropylene test tubes to make dilutions. For each plate approximately 1 mL of this solution is needed. Do not use single step dilutions of more than 1:100 or smaller transfer volumes than 40 µL.]

**Test solutions:** For each test solution, two results must be obtained by two independent preparations of somatropin. Reconstitute two independent preparations of somatropin in a suitable diluent (e.g., phosphate buffered saline or *Water for Injection*) to a concentration of not more than 5 mg/mL. Prepare *Test solutions* by diluting this material further with *Medium B* to a concentration of 2.0 ng/mL. [NOTE—Use polypropylene test tubes to make dilutions. For each plate approximately 1 mL of this solution is needed. Do not use single step dilutions of more than 1:100 or smaller transfer volumes than 40 µL.] One preparation is *Test solution A*, and the other preparation is *Test solution B*.

**Positive control solution:** Starting with the reconstituted stock preparation of USP Somatropin RS described in *Standard solutions*, dilute with *Medium B* to a concentration of 20 ng/mL. [NOTE—Use polypropylene test tubes to make dilutions. Do not use single step dilutions of more than 1:100 or smaller transfer volumes than 40 µL.]

**Procedure:** Dispense 100 µL of *Medium B* into each well of a U-bottom, clear 96-well plate,[7] except those wells in column 12 (wells A12–H12 = positive control) and wells A2–A10. [NOTE—For each black plate seeded with cells as described in *Cell culture preparation*, one separate U-bottom plate is used for preparation of the *Standard solutions* and *Test solutions* dilutions.] Next, dispense 200 µL of 2.0-ng/mL *Standard solutions* into wells A3, A6, and A9 of the dilution plate. Dispense 200 µL of 2.0-ng/mL *Test solution A* into wells A2, A5, and A8 of the dilution plate. Dispense 200 µL of 2.0-ng/mL *Test solution B* into wells A4, A7, and A10 of the dilution plate. Dispense 100 µL of 20-ng/mL *Positive control solution* into the last plate column (wells A12–H12) of the dilution plate. Using a 12-channel pipet, perform serial two-fold dilutions on the plate by aspirating 100 µL from the first row (A2–A10), transfer to the second row, and mix three times. Then aspirate 100 µL from the second row, transfer to the third row, and mix three times, etc. Repeat this procedure across the whole plate up to row H. Discard the 100 µL aspirated from the last row. [NOTE—The wells of column 11 (wells A11–H11) are negative controls containing 100 µL of *Medium B*; the wells of column 1 (wells A1–H1) are blanks.]

Starting with the lowest concentration and using a 12-channel pipet, transfer 50 µL of each solution in the dilution plate to the respective well of the black plate containing the cells. [NOTE—During the transfer, immerse the pipet tips into

[1] Quality Biological, catalog #112-032-101, or equivalent.
[2] Gibco, catalog #10082-147, or equivalent.
[3] Gibco, catalog #26050-088, or equivalent.
[4] Amimed, catalog #8-05F29I; Gibco, catalog #20012-019, or equivalent.
[5] HPA Culture Collections or Sigma-Aldrich, catalog #97041101.
[6] Costar, catalog #3904, or equivalent.
[7] Greiner, catalog #650160 plate, or equivalent.

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the cell solution. This transfer yields an additional 1:2 dilution of somatropin, or a final concentration of 1.0 ng/mL for the highest dose added to the cells and 10 ng/mL for the *Positive control solution*. A plate layout is shown in *Table 1*.] Following all transfers, gently agitate the black plate for 30 s, and then incubate it in a humidified 37° incubator containing 5% carbon dioxide for 30 ± 2 h. Next, add 100 µL of reconstituted luminescent substrate solution[8] to all wells. Incubate the plates for 15 min at room temperature while gently agitating the plates on an appropriate plate shaker, protected from light. Incubate the plates for an additional 15 min at room temperature without shaking, protected from light. Read the luminescence from the plate wells in a suitable plate reader with a luminescence detection mode.

**Table 1. Schematic Representation of the Final Assay Plate**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | BL | A,#1 | St,#1 | B,#1 | A,#1 | St,#1 | B,#1 | A,#1 | St,#1 | B,#1 | NEG | POS |
| B | BL | A,#2 | St,#2 | B,#2 | A,#2 | St,#2 | B,#2 | A,#2 | St,#2 | B,#2 | NEG | POS |
| C | BL | A,#3 | St,#3 | B,#3 | A,#3 | St,#3 | B,#3 | A,#3 | St,#3 | B,#3 | NEG | POS |
| D | BL | A,#4 | St,#4 | B,#4 | A,#4 | St,#4 | B,#4 | A,#4 | St,#4 | B,#4 | NEG | POS |
| E | BL | A,#5 | St,#5 | B,#5 | A,#5 | St,#5 | B,#5 | A,#5 | St,#5 | B,#5 | NEG | POS |
| F | BL | A,#6 | St,#6 | B,#6 | A,#6 | St,#6 | B,#6 | A,#6 | St,#6 | B,#6 | NEG | POS |
| G | BL | A,#7 | St,#7 | B,#7 | A,#7 | St,#7 | B,#7 | A,#7 | St,#7 | B,#7 | NEG | POS |
| H | BL | A,#8 | St,#8 | B,#8 | A,#8 | St,#8 | B,#8 | A,#8 | St,#8 | B,#8 | NEG | POS |

**LEGEND:**
BL = *Blank*: no somatropin, no cells.
A = Dilution series of *Test solution A* (with 1.0 ng/mL as starting concentration).
St = Dilution series of *Standard solutions* (with 1.0 ng/mL as starting concentration).
B = Dilution series of *Test solution B* (with 1.0 ng/mL as starting concentration).
NEG = Negative control: no somatropin, but cells are present.
POS = *Positive control solution*: maximum somatropin concentration (10 ng/mL).

**Calculations:** A minimum of two independent *Test solutions* preparations must be used for each test sample. The *Test solutions* and *Standard solutions* are normalized by protein content before calculation of the relative potency. [NOTE—1 mg of anhydrous somatropin is equivalent to 3.0 USP Somatropin Units.] Technical outliers (but not more than 4 per curve) are omitted, and then the same number of *Standard solutions* and *Test solutions* dose responses, including the 50% response ($EC_{50}$) of the standard per test sample within this range, are used to calculate the relative potency of each somatropin sample using statistical methods for parallel-line analysis. For each of the individual *Test solutions* compared to the *Standard solutions*, the statistical tests for linearity, slope, and parallelism must pass at the 95% level. The confidence limit for each calculated relative potency must be within 75%–133%. The relative potency of each of the *Test solutions* must be within the validated assay range (50%–200% relative potency) of the *Standard solutions*. The unweighted mean log relative potency is calculated from valid individual samples, and the potency is then calculated in USP Somatropin Units/mg relative to the USP Somatropin RS.

**System suitability criteria:** The signal-to-noise ratio of the mean chemiluminescence signal of the *Positive control solution* to the mean chemiluminescence signal of the negative control wells must be not less than 3. Not more than 4 technical outliers may be omitted per standard curve. Any plate that fails one or more of these criteria is rejected and must be repeated.

**Acceptance criteria:** Not less than 2 USP Somatropin Units/mg

• **USP REFERENCE STANDARDS** ⟨11⟩
USP Somatropin RS

[8] CellTiter-Glo Luminescent Kit, e.g., Promega, catalog #G7573, or equivalent.

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# ⟨127⟩ FLOW CYTOMETRIC ENUMERATION OF CD34+ CELLS

## INTRODUCTION

The CD34 antigen is expressed on the surface of almost all human hematopoietic stem and progenitor cells. The absolute number of CD34+ hematopoietic stem cells (HSCs) has been shown to correlate with in vitro colony-forming unit (CFU) assay activity and with clinical engraftment in hematopoietic grafts prepared from peripheral blood, bone marrow, and cord blood sources. The single-platform, flow cytometric CD34+ cell enumeration method described here is based on a clinical laboratory protocol,[1] established by the International Society of Hematotherapy and Graft Engineering (ISHAGE), now known as the International Society for Cellular Therapy (ISCT). This protocol can be used to enumerate CD34+ cells in samples of peripheral blood, leukapheresis products, bone marrow, and cord blood. CD34+ cell enumeration by flow cytometry is a rare event analysis, which requires specific gating instructions that are provided in this chapter. Furthermore, the USP CD34+ Cell Enumeration System Suitability RS has been developed to assess the reagents and ensure the correct gating during data acquisition and analysis.

## IDENTIFICATION OF CD34+ HEMATOPOIETIC STEM CELLS

CD34+ cell enumeration by flow cytometry is a rare event analysis. For the analysis of CD34+ HSCs, cell samples are stained with fluorescently labeled antibodies against both the HSC antigen CD34 and the pan-leukocyte antigen CD45. Five parameters —forward light scatter (FSC), side light scatter (SSC), CD34 staining, CD45 staining, and viability dye staining are combined in a sequential, or Boolean, gating strategy to identify viable CD34+ cells.

CD34+ HSCs have FSC and SSC characteristics similar to lymphocytes; expressing both CD45 and CD34, and exhibiting dim CD45 expression and low SSC characteristics. Viability dye does not stain live cells, allowing the exclusion of dead cells from the analysis of viable cell preparations. For analysis of nonviable, fixed (preserved) cell preparations such as the USP CD34+ Cell Enumeration System Suitability Reference Standard (RS), either the flow cytometer viable cell analysis gate is fully opened to include all cells, or the *Viability dye* is omitted from the analysis.

## ENUMERATION CONSIDERATIONS

The CD34+ cell enumeration method described here relies on the use of synthetic fluorescent microspheres (counting beads) as internal enumeration controls. Homogeneous counting beads are added to the cell sample at a known concentration and volume, or the counting beads may be procured, pre-aliquoted, and lyophilized in special sample tubes, in which all subsequent cell staining and processing steps are conducted. To avoid the loss of counting beads, the wash steps are omitted, and protein-containing sample buffers are used. An ammonium chloride-based, red blood cell (RBC)-lysing protocol is used for fresh cell preparations; no lysing is necessary for frozen, thawed, or fixed cell preparations.

After the cells are stained and processed, the counting beads and cells are simultaneously analyzed on a flow cytometer. The number of CD34+ cells/µL in the cell sample can be directly calculated by comparing the absolute number of target CD34+ cells and the number of counting beads detected in the same data file. The USP CD34+ Cell Enumeration System Suitability RS is used to verify that the correct reagents and flow cytometer gating parameters were used.

## EQUIPMENT SPECIFICATIONS

The following equipment is needed:

- A pipettor capable of accurately reverse-pipetting microliter volumes
- A flow cytometer with the following minimum specifications (see *Flow Cytometry* ⟨1027⟩):
  - Detection capabilities for FSC; SSC; "green" fluorescence emission (range, 510–550 nm); "yellow" fluorescence emission (range, 560–590 nm); and "red" fluorescence emission (>600 nm)
  - Light-scatter and fluorescence measurements that are correlated to time
  - Light-scatter resolution allowing for the identification of lymphocytes, monocytes, granulocytes, and fluorescent counting beads
  - Detection and data acquisition rates of at least 5000 cells/s
  - A 488-nm laser excitation source
  - Fluorescence intensity sensitivity allowing for the detection of cellular autofluorescence
  - Analysis software, including a data file structure in the Flow Cytometry Standard (FCS) or equivalent, with spectral overlap correction capability

## FLOW CYTOMETER INSTRUMENT SETUP AND CONSIDERATIONS

[NOTE—For general information, see ⟨1027⟩ and the flow cytometer manufacture's recommendations.]

[1] Sutherland DR, Keeney M, Gratama JW. Enumeration of CD34+ hematopoietic stem and progenitor cells. *Curr Protoc Cytom.* 2003;*6*:6.4.1–6.4.24.

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Ensure that the sheath fluid receptacle is filled, the waste fluid receptacle is empty, and all caps are tightly closed. Verify that the laser power, system vacuum, and pressure are properly set.

Using fluorochrome-conjugated calibration microspheres, verify proper system alignment by measuring the average detection rate, mean fluorescence, mean FSC, mean SSC, and the respective calculated coefficient of variance (CV) values as measured by each detector. All values should fall within the ranges recommended by the manufacturer of the flow cytometer instrument. Adjust the detector threshold to ensure that all counting bead events are included.

Use either antibody-binding microspheres or preserved cells, such as the USP CD34+ Cell Enumeration System Suitability RS, to establish a matrix of detector values while adjusting for spectral overlap (compensation). Note that although either microspheres or cells can be stained with anti-CD34 phycoerythrin (PE) and anti-CD45 fluorescein isothiocyanate (FITC) antibodies, only fresh cells can be stained with *Viability dye*.

## REAGENTS

**Phosphate-buffered saline (PBS):** 138 mM sodium chloride, 2.7 mM potassium chloride, 8 mM dibasic sodium phosphate, and 1.47 mM monobasic potassium phosphate. [NOTE—pH 7.0–7.4. If needed, adjust pH with hydrochloric acid.]

**Dilution buffer:** 1% (w/v) bovine serum albumin or human serum albumin in *PBS*

**10X Stock ammonium chloride RBC lysis buffer:** 1.5 M ammonium chloride, 0.01 M sodium bicarbonate, and 0.01 M ethylenediaminetetraacetic acid (EDTA)[2]

**Diluted (1X) RBC lysis buffer:** *10X Stock ammonium chloride RBC lysis buffer* diluted 1:10 with water

**Anti-CD34 PE:** PE-conjugated mouse IgG1 anti-human CD34 antibody (clones QBEnd10, 8G12, 581, Birma K3, or equivalent class II or class III PE-conjugated anti-CD34 antibodies), appropriately titrated

**Anti-CD45 FITC:** FITC-conjugated mouse IgG1 anti-human CD45 antibody (clones J33, T29/33, or HLE-1 or equivalent FITC-conjugated anti-CD45 antibodies), appropriately titrated

**Viability dye:** 1 µg/mL of 7-aminoactinomycin D (7-AAD) or equivalent, freshly diluted from a stock solution of 100 µg/mL[3]

**Instrument calibration beads:** Fluorochrome-conjugated instrument calibration microspheres[4]

**Suspended counting beads:** Fluorochrome-conjugated counting beads, formulated in liquid suspension[5]

**Lyophilized counting bead tube:** Fluorochrome-conjugated counting beads, formulated and lyophilized in a counting tube[6]

**System suitability standard USP CD34+ Cell Enumeration System Suitability RS:** Reconstitute the entire vial of USP CD34+ Cell Enumeration System Suitability RS with 0.5 mL of distilled water.

**Cell samples:** Fresh, fixed, or cryopreserved and thawed cell suspensions containing CD34+ cells, with a minimum of 100,000 nucleated cells/sample. Cell samples may include peripheral blood, leukapheresis product, cord blood, or bone marrow.

## SAMPLE PREPARATION

Use reverse-pipetting techniques for all sample dilutions and transfers. Dilute *Cell samples* with *Dilution buffer* to obtain a total nucleated cell concentration of 10–30 × $10^6$ cells/mL. Add *Anti-CD45 FITC* and *Anti-CD34 PE* to a 12-mm × 75-mm polystyrene tube (or a *Lyophilized counting bead tube*), followed by *Viability dye* to a final concentration of 1 µg/mL. Add 100 µL of well-mixed *Cell samples* to the bottom of the tube, and mix. Incubate for 20 min protected from light. Add 2 mL of *Diluted (1X) RBC lysis buffer* to fresh samples or add 2 mL of *Dilution buffer* to a fixed or frozen and thawed sample; vortex to mix. For fresh *Cell samples*, incubate for 10 min protected from light; for fixed or frozen then thawed *Cell samples*, no incubation time is needed. Place samples on ice. Samples should be analyzed within 1 h of lysis.

Immediately before acquiring data on the flow cytometer, pipet 100 µL of well-mixed *Suspended counting beads* (if not using *Lyophilized counting bead tubes*) to the prepared *Cell samples* described above. Cap the tube, and gently mix by inversion. Do not add counting beads to *Cell samples* intended to be used for adjustment of the instrumentation compensation matrix.

Immediately proceed to flow acquisition to collect a minimum of 75,000 CD45+ events and a minimum of 100 CD34+ cells. Acquire and analyze data by creating regions and logical gates (manually, or by using software appropriate for the flow cytometer), as described in *Table 1*.

### System Suitability Requirements

Use the USP CD34+ Cell Enumeration System Suitability RS to verify that the gating strategy described below allows the detection and quantitation of CD34+ HSCs. Note that for fixed *Cell samples*, the viability gate (*R1* in *Table 1*) must be opened to include all events; alternatively, the *Viability dye* can be omitted. Calculate the number of CD34+ HSCs/µL in USP CD34+ Cell Enumeration System Suitability RS.

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[2] A suitable fixative-free RBC lysis reagent can be obtained from BD Biosciences: BD Pharm Lyse™ Lysing Buffer Catalog No. 555899, BD Ammonium Chloride Lysing Solution (10X) Catalog No 344563, or equivalent.

[3] Suitable reagents can be obtained from BD Biosciences as part of a kit, Catalog No. 344563, or BD Biosciences Catalog No. 555899, or equivalent.

[4] A suitable reagent can be obtained from BD Biosciences: Catalog No. 641319, or equivalent.

[5] Suitable reagents can be obtained from Dako: Catalog No. K2370 (in a kit) or Dako Catalog No. S2366, or equivalent.

[6] A suitable reagent can be obtained from BD Biosciences: Catalog No. 340334.

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## Acceptance Criteria

The results should fall within the range provided in the USP CD34+ Cell Enumeration System Suitability RS certificate.

# DATA ACQUISITION AND ANALYSIS

For flow acquisition, collect a minimum of 75,000 CD45+ events and a minimum of 100 CD34+ cells. Acquire and analyze data by creating regions and logical gates (manually, or using software appropriate for the flow cytometer) as described in *Table 1*. The best results are obtained when cell and bead data (events) are displayed as bivariate dot plots. *Table 1* contains representative dot plots and gating strategies for fresh cell samples. Dot plot displays may vary depending on the cell sample, flow cytometer, and software used. Troubleshooting guidelines can be found in *Table 2*.

**Table 1. Dot Plot Descriptions and Gating Instructions**

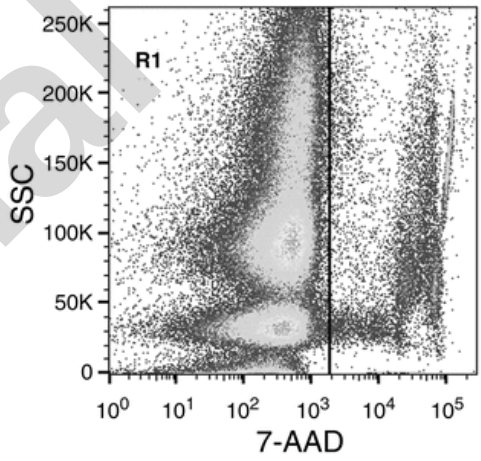
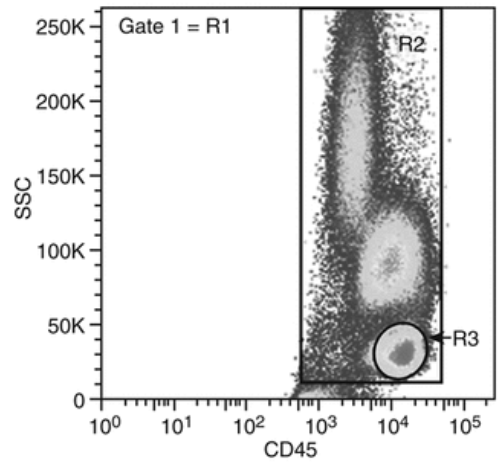
| Step | Format | Purpose | Gating Instructions | Representative Dot Plots and Gating Strategies |
|---|---|---|---|---|
| 1 | SSC (linear) vs. *Viability dye* fluorescence (log) | Define viable cells | • Display all unselected events. Create region *R1* around events with little-to-no fluorescence.<br>• Included in the region *R1*: viable cells, debris.<br>• Excluded from the region *R1*: dead and dying cells, counting beads. |  |
| 2 | SSC (linear) vs. CD45 fluorescence (log) | Define viable CD45+ leukocytes and lymphocytes | • Display *R1*-selected events. Create region *R2* around events with CD45+ fluorescence, excluding debris (i.e., events with very low SSC). Within *R2*, create region *R3* around events with high CD45+ fluorescence and low SSC.<br>• Included in the region *R2*: viable CD45+ leukocytes.<br>• Included in the region *R3*: viable lymphocytes.<br>• Excluded from the region *R2*: cell debris, platelets, and unlysed RBCs.<br>• Excluded from the region *R3*: granulocytes, monocytes, and other non-lymphocytes. |  |

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**Table 1. Dot Plot Descriptions and Gating Instructions** *(continued)*

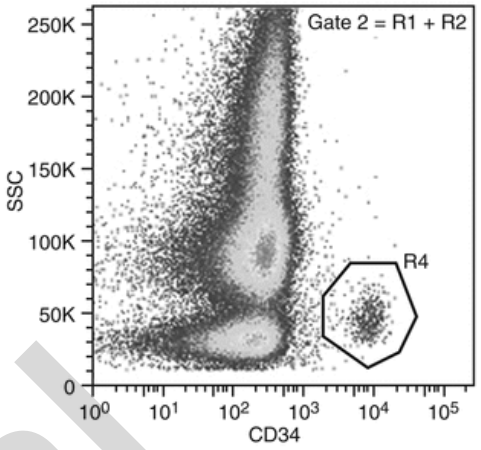
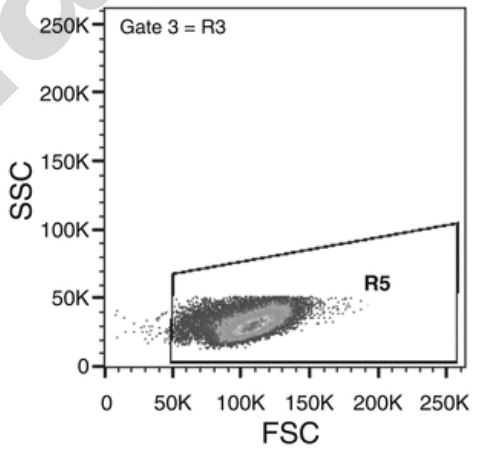
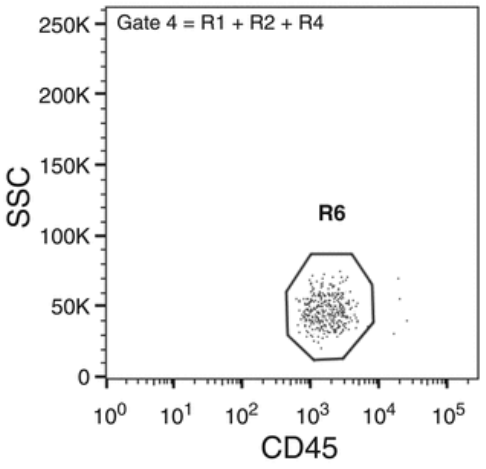
| Step | Format | Purpose | Gating Instructions | Representative Dot Plots and Gating Strategies |
|---|---|---|---|---|
| 3 | SSC (linear) vs. CD34 fluorescence (log) | Define viable CD34+ cells | • Display *R2*-selected events. Create region *R4* around events with CD34+ fluorescence and low SSC.<br>• Included in the region *R4*: viable CD34+ cells.<br>• Excluded from the region *R4*: CD34- cells. |  |
| 4 | SSC (linear) vs. FSC (linear) | Define lymphocytes and blasts | • Display *R3*-selected events. Create region *R5* to exclude debris (low SSC and low FSC).<br>• Included in the region *R5*: viable lymphocytes and blasts.<br>• Excluded from the region *R5*: small debris and events with high SSC values. |  |
| 5 | SSC (linear) vs. CD45 fluorescence (log) | Define CD45 dim CD34+ cells | • Display *R4*-selected events. Create region *R6* around events with low-to-intermediate SSC and intermediate CD45 expression.<br>• Included in the region *R6*: viable CD34+ HSCs.<br>• Excluded from the region *R6*: cells that express high levels of CD45. |  |

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**Table 1. Dot Plot Descriptions and Gating Instructions** *(continued)*

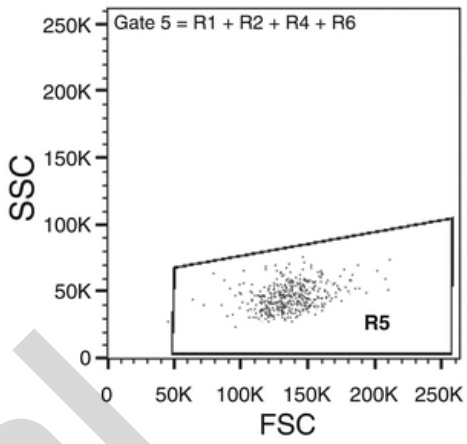
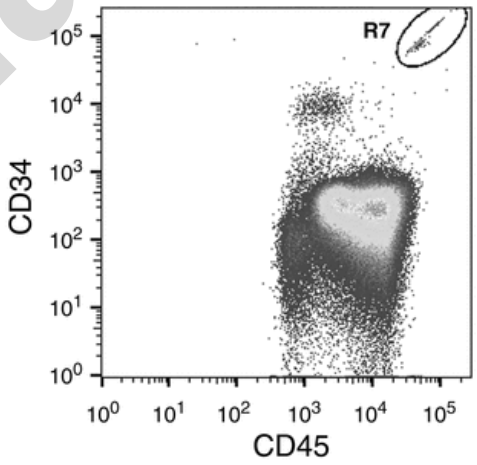
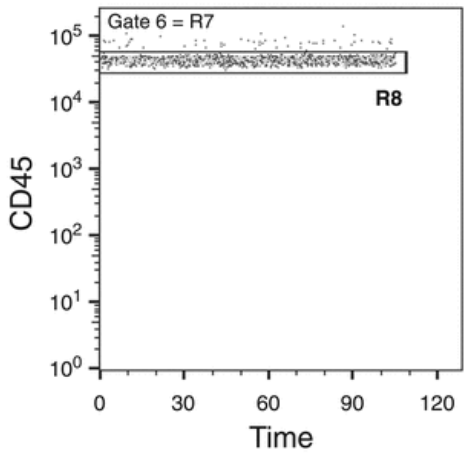
| Step | Format | Purpose | Gating Instructions | Representative Dot Plots and Gating Strategies |
|---|---|---|---|---|
| 6 | SSC (linear) vs. FSC (linear) | Confirm CD34+ HSCs | • Display *R6*-selected events, and paste a copy of the *R5* gate parameters into the plot. Events in *R5* have light-scatter characteristics similar to lymphocytes and blasts, confirming the identity of the events as CD34+ HSCs.<br>• Included in the region *R5*: confirmed viable CD34+ HSCs.<br>• Excluded from the region *R5*: debris and other irrelevant events.<br>• For counting bead enumeration, proceed to either Step 7 or Step 8 according to the recommendations of the bead manufacturer. |  |
| 7 | CD34 fluorescence (log) vs. CD45 fluorescence (log) | Quantify total counting beads | • Display all ungated events. Create region *R7* around events with both high CD34+ and high CD45+ fluorescence.<br>• Included in the region *R7*: total counting beads.<br>• Excluded from the region *R7*: cells and debris. |  |
| 8 | CD45 fluorescence (log) vs. time (linear) | Quantify "singlet" (e.g., unaggregated) counting beads | • When recommended by the counting bead manufacturer, display *R7*-selected events. Create *R8* around events with lower fluorescence intensity.<br>• Included in the region *R8*: singlet counting beads.<br>• Excluded from the region *R8*: counting bead aggregates. |  |

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**Table 2. Troubleshooting Guidelines (see also ⟨1027⟩)**

| Problems | Possible Reasons | Solutions | Comments |
|---|---|---|---|
| Unable to clearly define the bead gate (*R7*) in *Table 1*, graph 7 due to fluorescent debris | Degraded fresh cell sample; fixed sample; too many platelets; incomplete RBC lysis; nucleated RBCs (e.g., in cord blood) | Use a far-red fluorescence channel (>600 nm), instead of FITC, to gate counting beads. | Counting beads are highly fluorescent in all channels. Autofluorescence interference decreases dramatically at longer wavelengths (>600 nm). |
| Unable to find CD34+ population in *Table 1*, graph 3 | Incorrect compensation settings for PE and *Viability dye* | Repeat the spectral compensation setup procedure, and adjust settings accordingly. | Make sure that counting beads are not in the compensation matrix sample, because they can interfere with compensation settings. |
| Unsure of gating for singlet or total beads | High levels of counting bead aggregates | Follow the counting bead manufacturer's recommendation for including or excluding aggregates. | Counting bead concentration calculations will vary by manufacturer. |
| Want to use a different CD34 antibody clone | Multi-parametric flow cytometry considerations | Alternative antibody clones must be carefully validated against the specified CD34 antibodies. Choose a class II or class III antibody that detects all CD34 isoforms and glycoforms. | Class I antibodies do not detect all CD34 glycoforms. |
| Want to use a different CD45 antibody clone | Multi-parametric flow cytometry considerations | Alternative antibody clones must be carefully validated against the specified CD45 antibodies. Choose an antibody that detects all isoforms and all glycoforms of CD45. | — |

## CALCULATION OF ABSOLUTE CD34 NUMBER

The absolute number of viable CD34+ cells/µL is calculated as follows:

$$\text{Viable CD34+ cells/µL} = (\text{number of CD34+ cells})/(\text{number of counting beads}) \times (\text{bead concentration} \times DF)$$

*DF* = sample dilution factor

The number of CD34+ cells is determined from the viable CD34+ cells (region *R5* from *Table 1*, Step 6). Depending on the recommendations of the counting bead manufacturer, the number of counting beads is determined from either the total counting beads (region *R7* from *Table 1*, graph 7) or the singlet counting beads (region *R8* from *Table 1*, graph 8). The bead concentration is specified by the counting bead manufacturer.

## ADDITIONAL REQUIREMENTS

### USP Reference Standards ⟨11⟩

USP CD34+ Cell Enumeration System Suitability RS

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# ⟨129⟩ ANALYTICAL PROCEDURES FOR RECOMBINANT THERAPEUTIC MONOCLONAL ANTIBODIES

## INTRODUCTION

This chapter provides analytical procedures for murine, chimeric, and humanized IgG isotype monoclonal antibodies and subtypes (e.g., IgG1 and IgG2). Subclasses show differences in amino acid sequence and in the number of disulfide bonds, and in some cases they require subclass-specific analysis. This chapter applies to monoclonal antibodies for therapeutic and prophylactic use and for use as in vivo diagnostics. It does not apply to monoclonal antibodies used as reagents in the manufacture of medicinal products, for which applicable requirements are determined by the appropriate regulatory agency.

Naturally occurring polyclonal antibodies in serum or plasma can bind to many different targets as part of the immune system. In contrast, therapeutic monoclonal antibodies for human use are preparations of an immunoglobulin (or a fragment of an immunoglobulin) that have specificity for a target and are derived from a single clone of cells. Therapeutic monoclonal antibodies can bind to and neutralize soluble antigens, and their mechanism of action (MOA) often involves blocking of the ligand from binding to its cognate receptor. Alternatively, therapeutic monoclonal antibodies can recognize and bind to cell-bound antigens and in these cases may also engage the immune system through Fc (crystallizable fragment) mediated effector functions as part of their MOA. Monoclonal antibodies that have a single defined binding specificity are monospecific, whereas recombinantly engineered bispecific monoclonal antibodies can bind to two different targets (e.g., antigens). IgG-type monoclonal antibodies have a molecular weight of approximately 150 kD. Each molecule consists of two heavy and two light polypeptide chains that have a molecular weight of approximately 50 and 25 kD, respectively. Antibodies can be schematically represented as a Y joined by disulfide bridges, where each arm of the Y is called the Fab domain, and the stem of the Y is called the Fc domain. Monoclonal antibodies are glycoproteins that have a glycosylation site in the Fc portion located on each of the heavy chains and have possible additional glycosylation sites in the Fab domain, depending on the molecule.

The specificity of a monoclonal antibody is based on its antigen binding site, which is located in the Fab part of the molecule. The Fc domain contains receptor binding sites that are associated with antibody effector functions that have subclass-specific applications such as antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity. Pharmacokinetic effects via binding to the neonatal Fc receptor also are present in this domain.

Monoclonal antibodies are produced in cell culture using a seed lot system with master and working cell banks. After harvest, purification steps ensure that product- and process-related impurities are reduced to an acceptable level.

Currently licensed monoclonal antibody therapeutics include those involved in the activation of effector cells, cell killing, cross-linked induced apoptosis, antagonism against several targets, and agonist antibodies.

This chapter includes purity assessments by size-exclusion chromatography (SE-HPLC), capillary electrophoresis, and analysis of oligosaccharides, and it provides validated procedures for each of these.

Although this chapter is focused on IgG-type monoclonal antibodies, the principles of the tests included can apply to other related molecules, such as IgM or other isotype antibodies, antibody fragments, and Fc-fusion proteins. When the active substance is a conjugated antibody, such tests can be performed on the purified antibody before modification or conjugation.

Alternative methods and/or procedures may be used if they provide advantages in terms of accuracy, sensitivity, precision, selectivity, or adaptability to automation or computerized data reduction, or in other special circumstances. Such alternative procedures and methods shall be validated as described in *Validation of Compendial Procedures* ⟨1225⟩ and must be shown to give equivalent or better results.

### • Size-Exclusion Chromatography

Although SE-HPLC is robust for measuring monomer and high-molecular-weight species (HMWS, aggregates), the quantitation of low-molecular-weight species (LMWS, fragments) can be highly variable depending on the mAb studied. The measurement of LMWS is better quantitated by capillary electrophoresis sodium dodecyl sulfate (CE-SDS).

**Mobile phase:** Prepare by mixing 10.5 g of dibasic potassium phosphate, 19.1 g of monobasic potassium phosphate, and 18.6 g of potassium chloride per L of water. Verify that the pH is 6.2 ± 0.1. Pass through a membrane filter of ≤ 0.45-µm or smaller pore size.

**System suitability solution:** Prepare a 10-mg/mL USP Monoclonal IgG System Suitability RS solution in *Mobile phase* by reconstituting the contents of one vial with 200 µL of *Mobile phase*. Reconstituted *System suitability solution* should be used within 24 h after reconstitution, and should be stored at 2°–8° if not used immediately.

**System suitability blank:** Use *Mobile phase*.

**Sample solution:** Dilute the sample to 10 mg/mL in *Mobile phase* if dilution is required. Similarly, a blank should be prepared using an equivalent dilution of formulation buffer in *Mobile phase*.

**Chromatographic system**

(See *Chromatography* ⟨621⟩, *System Suitability*.)

**Mode:** LC

**Detector:** UV 280 nm

**Column:** 7.8-mm × 30-cm; 5-µm packing L59

**Temperatures**

**Column:** Ambient

**Autosampler:** Maintain at 2°–8°

**Flow rate:** 0.5 mL/min

**Injection volume:** 20 µL

**Run time:** 30 min

**System suitability**

**Sample:** *System suitability solution*

[Note—Approximate retention times for polymer, dimer, monomer, and LMWS are 12, 15, 18, and 22 min, respectively.]

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**Suitability requirements**

**Chromatogram:** The chromatogram of the *System suitability solution* is consistent with the typical chromatogram as illustrated in the USP Certificate for USP Monoclonal IgG System Suitability RS.

**Chromatogram similarity:** The chromatographic profiles of *System suitability solution* injections that bracket injections of *Sample solutions* in a sample set are consistent with each other and with the typical chromatographic profile as illustrated in the USP Certificate for USP Monoclonal IgG System Suitability RS. All named peaks in the typical chromatogram must be present, be well-resolved, and in the same elution order as that in the *System suitability solution* chromatograms.

**Quantitative criteria:** Bracketing *System suitability solution* injections must meet the following quantitative criteria:
The percent peak area of the HMWS in the *System suitability solution* must be 0.4%–0.67%.
The percent peak area of the main peak in the *System suitability solution* must be 99.1%–99.6%.
The percent peak area of the LMWS in the *System suitability solution* must be NMT 0.2%.

**Analysis**

**Samples:** *Blank, System suitability solution*, and *Sample solution*

Injections of the *Sample solution* should be bracketed minimally with single injections of the *System suitability solution*. An injection of a *Blank* should be included as the final injection before the final *System suitability solution* injection.

**Calculations:** Using a suitable electronic integrator or computer system, analyze the bracketing *System suitability solution* injections. Exclude from integration any peaks that are present in the *Blank*. Protein-related peaks that elute before the main peak are classified as HMWS; those that elute after the main peak are classified as LMWS. Report the percentage of peak areas for the HMWS, main peak, and LMWS.

- **Capillary SDS Electrophoresis (Reduced and Nonreduced)**

Capillary sodium dodecyl sulfate (CE-SDS) electrophoresis is a sensitive, analytical method for quantitative estimation of product-related impurities such as nonglycosylated molecules, half antibodies, and fragments, and thus is also useful as a stability-indicating assay. After denaturation with SDS, the method allows the analysis of the complete antibody under nonreducing conditions, and the analysis of light and heavy chains (LC and HC, respectively) under reducing conditions.

[Note—Slight variations in sample preparation may be necessary depending on the stability of the individual antibody. If 15 min incubation at 70° leads to fragmentation or cleavage of disulfide bonds for a particular antibody sample, adjust the incubation time accordingly.]

**SDS sample buffer:** Prepare a solution containing 100 mM tris-hydrochloride (tris-HCl), a pH of 8.95 ± 0.05, and 1% (w/w) SDS.

**SDS gel buffer:** Buffer at a pH of 8.0 containing 0.2% (w/w) SDS and an appropriate hydrophilic polymer acting as a molecular sieve[1]

**Alkylation solution:** 250 mM iodoacetamide in water prepared immediately before use

**System suitability solution:** Reconstitute 1 vial of USP Monoclonal IgG System Suitability RS with 0.5 mL of *Water for Injection* (WFI) to a final concentration of 4 mg/mL.

**Internal standard solution:** 5 mg/mL of 10 kDa internal standard[2] in water containing 0.5% (w/w) SDS and 0.2% (w/v) sodium azide

**Nonreduced system suitability solution:** Mix 25 µL of the *System suitability solution* with 4 µL of *Internal standard solution*. Mix 66 µL of *SDS sample buffer* with 5 µL of the *Alkylation solution*. Add the *SDS sample buffer* and *Alkylation solution* mixture to the *System suitability solution* and *Internal standard solution* mixture. Mix thoroughly, and heat the mixture at 70° for 15 min. Allow to cool for 3 min at room temperature, centrifuge at 300 × g for 1 min, and transfer 100 µL into autosampler vials.

**Reduced system suitability solution:** Mix 25 µL of the *System suitability solution* with 66 µL of *SDS sample buffer*. Add 4 µL of *Internal standard solution* and 5 µL of neat β-mercaptoethanol. Mix thoroughly, and heat the mixture at 70° for 15 min. Allow to cool for 3 min at room temperature, centrifuge at 300 × g for 1 min, and transfer 100 µL into autosampler vials.

**Blank solution:** Mix 45 µL of water with 50 µL of *SDS sample buffer*. Add 4 µL of *Internal standard solution* and 5 µL of neat β-mercaptoethanol for the reduced conditions or 5 µL of *Alkylation solution* for the nonreducing conditions. Mix thoroughly, and heat the mixture at 70° for 15 min. Allow to cool for 3 min at room temperature, centrifuge at 300 × g for 1 min, and transfer 100 µL into autosampler vials.

**Nonreduced sample solution:** Dilute 100 µg of the sample to be tested with 50–95 µL of *SDS sample buffer* to give a final volume of 95 µL. Add 4 µL of *Internal standard solution* and 5 µL of the *Alkylation solution*. Mix thoroughly, and heat the mixture at 70° for 15 min. Allow to cool for 3 min at room temperature, centrifuge at 300 × g for 1 min, and transfer 100 µL into autosampler vials.

**Reduced sample solution:** Dilute 100 µg of the sample to be tested with 50–95 µL of *SDS sample buffer* to give a final volume of 95 µL. Add 4 µL of *Internal standard solution* and 5 µL of neat β-mercaptoethanol. Mix thoroughly, and heat the mixture at 70° for 15 min. Allow to cool for 3 min at room temperature, centrifuge at 300 × g for 1 min, and transfer 100 µL into autosampler vials.

**Instrumental conditions**

**Mode:** CE-SDS

**Detector:** UV or diode array 220 nm

**Capillary:** Fused-silica capillary (i.d. 50 µm) cut to a total length of 30 cm with an effective length of 20 cm.[3] The following preconditioning and prefilling steps are required between each run.

**Preconditioning of the capillary:** Rinse for 3 min at 70 psi with 0.1 N sodium hydroxide followed by 0.1 N hydrochloric acid for 1 min at 70 psi, and water for 1 min at 70 psi.

**Prefilling of capillary:** Rinse for 10 min at 70 psi with *SDS gel buffer*.

[1] Available from Beckman Coulter as a component of test kit, catalog number A10663, or a suitable equivalent.
[2] Available from Beckman Coulter as a component of test kit, catalog number A10663, or a suitable equivalent.
[3] Beckman Coulter catalog number 338472 or equivalent.

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**Sample injection:** Electrokinetic injection, 5.0 kV reversed polarity for 20 s
**Voltage separation**
**Nonreduced conditions:** Run time 40 min at 15 kV, reversed polarity
**Reduced conditions:** Run time 40 min at 15 kV, reversed polarity
**Temperatures**
**Sample storage:** Approximately 25°
**Capillary:** Approximately 25°

**System suitability**

**Suitability requirements for reducing conditions**

**Sample:** *Reduced system suitability solution*

**Electropherogram:** The electropherogram of the *Reduced system suitability solution* is consistent with the typical electropherogram illustrated in the USP Certificate for USP Monoclonal IgG System Suitability RS.

**Resolution:** The main peak of the heavy chain and the peak of the nonglycosylated heavy chain can be clearly identified. The resolution between the nonglycosylated heavy chain and the intact heavy chain is NLT 1.2.

**Ratio of nonglycosylated to total heavy chain:** Use time-corrected peak areas (see *Capillary Electrophoresis* ⟨1053⟩, *Quantification*, which may be a helpful, but not mandatory resource) for calculation. Calculate the ratio of nonglycosylated to total heavy chain by dividing the relative amount of nonglycosylated heavy chain by the sum of all heavy chains, and multiply by 100. The ratio of nonglycosylated to total heavy chain in the *System suitability solution* should be within the limits of 0.75%–1.34%.

**Suitability requirements for nonreducing conditions**

**Sample:** *Nonreduced system suitability solution*

**Electropherogram:** The electropherogram of the *Nonreduced system suitability solution* is consistent with the typical electropherogram illustrated in the USP Certificate for USP Monoclonal IgG System Suitability RS.

**Resolution:** The IgG main peak can be clearly identified. The resolution between the IgG main peak and Fragment 1 (F1) is NLT 1.3.

**Amount of main peak:** Calculate the relative amount of the main peak by dividing the sum of the time-corrected peak area of the main IgG peaks by the sum of all time-corrected peak areas excluding internal standard peaks and all system peaks, and multiply by 100. The relative amount of the main IgG peak of the *System suitability solution* should be within the limits of 61.4%–86.4%.

**Relative standard deviation:** NMT 2% for the migration of the internal standard in the bracketing *Blank solution*. No other peaks migrating after the internal standard are detectable.

**Analysis**

**Samples:** *Blank solution, System suitability solution*, and *Sample solution*

[NOTE—Based on instrument requirements, the field applied across the capillary and the conditions for the sample injection may be adjusted to achieve *System suitability*.] Apply a field strength of 500 V/cm (reversed polarity) for 40 min, using the *SDS gel buffer* as the electrolyte in both buffer reservoirs. Electrophorese the samples, using a 20-s reversed polarity and electrokinetic injection at 5.0 kV, into the anodic end of the capillary, and record the electropherograms at 220 nm. Rinse for 3 min at 70 psi with 0.1 N sodium hydroxide followed by 0.1 N hydrochloric acid for 1 min at 70 psi, and water for 1 min at 70 psi. Inject at least in duplicate.

Calculate the time-corrected peak areas of all peaks migrating after the internal standard peak.

**Reduced conditions:** Calculate the relative amount of product peak by dividing the sum of the time-corrected peak areas of heavy chain, nonglycosylated heavy chain, and light chain by the sum of all time-corrected peak areas for peaks appearing after the internal standard, and multiply by 100.

**Nonreduced conditions:** Calculate the relative amount of product peak by dividing the sum of the time-corrected peak area of the main IgG peak by the sum of all time-corrected peak areas for peaks appearing after the internal standard, and multiply by 100.

- **OLIGOSACCHARIDE ANALYSIS—ANALYSIS OF *N*-LINKED OLIGOSACCHARIDES OF MONOCLONAL ANTIBODIES**

The inclusion of oligosaccharide analysis in a specification for a monoclonal antibody drug substance should be assessed using science- and risk-based approaches and should be defined on a product-specific basis. If applicable, one or more of the following analytical approaches can be employed for oligosaccharide profiling or quantitation of individual structures. Other validated methods that are suitable for determination of *N*-linked glycosylation utilizing different modes of separation, labeling, or detection can be used. [NOTE—For monoclonal antibodies with both Fc and Fab *N*-linked oligosaccharides, additional sample preparation procedures (e.g., treatment with the enzyme papain) can be employed to separate the domains before analyses so that information at individual glycosylation sites can be obtained. All additional sample preparation steps for the method require validation.]

[NOTE—Numerical acceptance criteria may be defined for individual glycan structures. If applicable, acceptance criteria must be derived from product-specific information taking into consideration a suitable range of historical batch data.]

**Method A: Capillary electrophoresis with laser-induced fluorescence detection**

**Run buffer:** Prepare 100 mM triethanolamine (TEA) and 10% glycerol at pH 7.5 by dissolving 1.492 g of TEA and 10 g of glycerol in 80 mL of water. Adjust with 1 N hydrochloric acid to a pH of 7.5, and dilute with water to a final volume of 100 mL.

**Sample buffer:** Dilute 1 mL of *Run buffer* with 9 mL of water.

**Enzyme reaction buffer:** Prepare 50 mM sodium phosphate, pH 7.5.

**APTS labeling reagent:** Dissolve 5 mg of 8-aminopyrene-1,3,6-trisulfonate (APTS) in 48 µL of 15% acetic acid, and mix.

**Sample solution:** Add 2 µL of peptide *N*-glycosidase F (PNGase F) (5 units/mL) to 50 µg of sample, and adjust with *Enzyme reaction buffer* to a volume of 50 µL. [NOTE—The unit definition for PNGase F may differ among commercial sources. It may be necessary to experimentally determine the appropriate quantity of PNGase F required for the complete release of *N*-linked oligosaccharides in the sample.] Incubate at 37° for 18 h. Separate released oligosaccharides by centrifugation using a 10,000 molecular-weight-cut-off (MWCO) centrifugal filter. Dry the released

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oligosaccharides in a centrifugal vacuum evaporator to dryness. Add 2 µL of *APTS labeling reagent* and 2 µL of 1 M sodium cyanoborohydride to the dried oligosaccharides. Incubate at 55° for 90 min. Add 46 µL of water to quench the reaction and mixture. Add 5 µL of APTS-labeled sample to 95 µL of *Sample buffer*.

**System suitability solution:** Resuspend each of the suitable glycan standards (10 µg) in 50 µL of water, and mix on a vortex mixer. Add 5 µL of a suitable glycan standard (1 µg) to a microcentrifuge tube. Dry the glycan standard using centrifugal vacuum evaporation at ambient temperature. Add 2 µL of sodium cyanoborohydride to each standard. Add 2 µL of the *APTS labeling reagent* to each standard. Mix the standards on a vortex mixer, and centrifuge briefly to settle the liquid before incubation at 55° for 90 min. Quench the reaction by the addition of 46 µL of water to each standard. Standards can be stored at –20° up to 5 weeks.

**Instrumental conditions**

**Mode:** CE

**Detector:** Laser-induced fluorescence detector (488-nm excitation wavelength; 520-nm emission wavelength)

**Capillary:** 50-µm inner diameter bare fused-silica capillary with 50-cm total length and 40-cm separation length

**Sample injection:** 10-s hydrodynamic injection

**Separation voltage:** 22kV for 50 min

**Samples:** *Sample solution* and *System suitability solution*

**Analysis:** Integrate peaks in the resultant electropherogram, and report relative percentage time-corrected peak areas of major glycan structures relevant to the product. Comparison to suitable product-specific reference standard can be performed.

**Method B: Liquid chromatography with fluorescence detection**

**Enzyme reaction buffer:** Prepare 100 mM sodium phosphate, a pH of 7.5.

**Labeling buffer:** Prepare 8% (w/v) sodium acetate trihydrate and 4% (w/v) boric acid solutions in methanol by dissolving 800 mg of sodium acetate trihydrate and 400 mg of boric acid in 10 mL of methanol.

**Labeling reagent solution:** Prepare a 100-mg/mL stock solution of anthranilic acid (2-AA) in *Labeling buffer.* Mix with an equal volume of 1 M sodium cyanoborohydride in methanol for a final *Labeling reagent solution* containing 50 mg/mL of 2-AA and 0.5 M sodium cyanoborohydride. [NOTE—Keep the labeling reagent protected from light.]

**Solution A:** Prepare 50 mM ammonium formate, pH 4.4.

**Solution B:** Acetonitrile

**Mobile phase:** See *Table 1*.

**Table 1**

| Time (min) | Solution A (%) | Solution B (%) |
|---|---|---|
| 0.0 | 32.5 | 67.5 |
| 48.0 | 42.5 | 57.5 |
| 49.0 | 100 | 0 |
| 53.0 | 100 | 0 |
| 54.0 | 32.5 | 67.5 |
| 60.0 | 32.5 | 67.5 |

**Sample preparation:** Transfer 80 µg of test sample to a microcentrifuge tube. Add 3 µL of PNGase F (2.5 units/mL). [NOTE—The unit definition for PNGase F may differ among commercial sources. It may be necessary to experimentally determine the appropriate quantity of PNGase F required for complete release of *N*-linked oligosaccharides in the sample.] Add 14 µL of *Enzyme reaction buffer.* Add water to 70 µL of total volume, and incubate at 37° for 18 h. Add 70 µL of freshly prepared *Labeling reagent solution*, and incubate at 70° for 2 h in a fume hood. Allow to cool, and add 60 µL of 50% methanol to each sample. Mix, then centrifuge. Remove 140 µL of the supernatant, and transfer to a microcentrifuge tube. Add 500 µL of acetonitrile to the supernatant-containing tube. Load the sample onto a hydrophilic-interaction solid-phase extraction cartridge to remove excess labeling reagent. Wash the cartridge with 95% acetonitrile (approximately 10 mL). Elute the labeled glycans from the cartridge with 0.5 mL of 20% acetonitrile. Dilute the eluate 1:1 with acetonitrile.

**Chromatographic system**

(See *Chromatography* ⟨621⟩*, System Suitability*.)

**Mode:** LC

**Column:** 4.6-mm × 15-cm; 3-µm packing L68

**Detector:** Fluorescence (360-nm excitation wavelength; 420-nm emission wavelength)

**Column temperature:** Maintain column at 30°.

**Flow rate:** 0.8 mL/min

**Injection volume:** 50 µL

**Analysis**

**Sample:** *Sample preparation*

Integrate peaks in the resulting chromatogram, and report relative percentage peak areas of major glycan structures relevant to the product. Comparison to a suitable product-specific reference standard can be performed.

- **OLIGOSACCHARIDE ANALYSIS—SIALIC ACID ANALYSIS**

[NOTE—The range of the standard curve or the mass of test sample may require modification depending on the sialic acid content of the monoclonal antibody.]

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**Solution A:** Prepare 100 mM sodium hydroxide by diluting 10.4 mL of a 50% (w/w) sodium hydroxide solution in 2 L of water. [NOTE—Use high-quality water of high resistivity (18 MΩ-cm or better) that contains as little dissolved oxygen as possible.]
**Solution B:** Prepare a solution containing 100 mM sodium hydroxide and 1 M sodium acetate by adding 82.0 g of sodium acetate to 800 mL of water. Add 5.2 mL of 50% (w/w) sodium hydroxide, and dilute with water to a final volume of 1000 mL.
**Mobile phase:** See *Table 2*.

**Table 2**

| Time (min) | Solution A (%) | Solution B (%) |
|---|---|---|
| 0.0 | 93 | 7 |
| 10.0 | 70 | 30 |
| 11.0 | 70 | 30 |
| 12.0 | 93 | 7 |
| 15.0 | 93 | 7 |

**Standard stock solution:** Prepare 0.2 mM solutions of USP *N*-Acetylneuraminic Acid RS (NeuAc) and USP *N*-Glycolylneuraminic Acid RS (NeuGc) in 20 mM sodium acetate buffer, pH 5.2. Dilute an appropriate volume of this solution with 20 mM sodium acetate buffer, pH 5.2, to obtain a 0.02 mM *Standard stock solution*.
**Internal standard stock solution:** Prepare a 0.1 mM solution of 3-deoxy-D-glycero-D-galacto-2-nonulosonic acid (KDN) in 20 mM sodium acetate buffer, adjusted to a pH of 5.2.
**Standard solutions:** Prepare as indicated in *Table 3*.

**Table 3**

| Standard | Concentration (µM) | Volume of Standard Stock Solution (µL) | Volume of 20 mM Sodium Acetate Buffer, pH 5.2 (µL) | Volume of Internal Standard Stock Solution (µL) |
|---|---|---|---|---|
| 1 | 10 | 250 | 235 | 15 |
| 2 | 5 | 125 | 360 | 15 |
| 3 | 4 | 100 | 385 | 15 |
| 4 | 2 | 50 | 435 | 15 |
| 5 | 1 | 25 | 460 | 15 |
| 6 | 0.4 | 10 | 475 | 15 |

**System suitability solution:** Prepare 3 µM solutions of NeuAc, NeuGc, and KDN in 20 mM sodium acetate buffer, adjusted to a pH of 5.2. Store the *System suitability solution* at –70°.
**Sample solution:** Pipet a volume of test sample equivalent to 0.5 mg into a microcentrifuge tube. Dilute with 20 mM sodium acetate buffer, pH 5.2, to a total volume of 475 µL. Confirm the pH with a test strip, and add 10 µL of 10 milliunit/µL of neuraminidase. Incubate for 5 h at 37°. Add 15 µL of *Internal standard stock solution*. Mix on a vortex mixer, and transfer the sample to an autosampler vial. [NOTE—A slight adjustment in sample preparation may be necessary depending on the test sample and the quality of the enzyme. Adjust the incubation time accordingly.]
**Chromatographic system**
(See *Chromatography* ⟨621⟩, *System Suitability*.)
**Mode:** LC
**Detector:** Integrated amperometric detector with gold electrode
**Column:** 4-mm × 25-cm; 10-µm packing L46
**Flow rate:** 1 mL/min
**Injection volume:** 50 µL
Use the following waveform for the electrochemical detector (see *Table 4*).

**Table 4**

| Time (s) | Potential (V) | Integration |
|---|---|---|
| 0.00 | +0.10 | — |
| 0.20 | +0.10 | Begin |
| 0.40 | +0.10 | End |
| 0.41 | –2.00 | — |
| 0.42 | –2.00 | — |
| 0.43 | +0.60 | — |

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**Table 4** *(continued)*

| Time (s) | Potential (V) | Integration |
|---|---|---|
| 0.44 | –0.10 | — |
| 0.50 | –0.10 | — |

**Analysis**

**Samples:** *Standard solutions*, *System suitability solution*, and *Sample solution*

Integrate the NeuAc, NeuGc, and *Internal standard stock solution* peaks in each chromatogram. For the *Standard solutions*, evaluate the NeuAc and NeuGc peak areas relative to the peak area of the *Internal standard stock solution* as follows.

Area of NeuAc peak/area of *Internal standard stock solution* peak × 100

Area of NeuGc peak/area of *Internal standard stock solution* peak × 100

Generate NeuAc and NeuGc standard curves. Quantify NeuAc and NeuGc in the *Sample solution* by comparison of the relative percent of NeuAc of the *Internal standard stock solution* and the relative percent of NeuGc of the *Internal standard stock solution*, using the standard curves.

Divide the determined NeuAc and NeuGc concentrations by the test sample concentration, and report as molar ratio (i.e., numbers of NeuAc and NeuGc per molecule of test sample).

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# ⟨151⟩ PYROGEN TEST

## INTRODUCTION

The pyrogen test is designed to limit to an acceptable level the risks of febrile reaction in the patient to the administration, by injection, of the product concerned. The test involves measuring the rise in temperature of rabbits following the intravenous injection of a test solution and is designed for products that can be tolerated by the test rabbit in a dose not to exceed 10 mL/kg injected intravenously within a period of NMT 10 min. For products that require preliminary preparation or are subject to special conditions of administration, follow the additional directions given in the individual monograph or, in the case of antibiotics or biologics, the additional directions given in the federal regulations (see *Biologics* ⟨1041⟩). A validated, equivalent in vitro pyrogen or bacterial endotoxin test may be used in place of the in vivo rabbit pyrogen test,[1] where appropriate.

## APPARATUS AND DILUENTS

Render the syringes, needles, and glassware free from pyrogens by heating at 250° for NLT 30 min or by any other suitable method. Treat all diluents and solutions for washing and rinsing of devices or parenteral injection assemblies in a manner that will assure that they are sterile and pyrogen-free. Periodically perform control pyrogen tests on representative portions of the diluents and solutions for washing or rinsing of the apparatus. Where *Sodium Chloride Injection* is specified as a diluent, use Injection containing 0.9% of sodium chloride (NaCl).

## TEMPERATURE RECORDING

Use an accurate temperature-sensing device such as a clinical thermometer, or thermistor probes or similar probes that have been calibrated to assure an accuracy of ±0.1° and have been tested to determine that a maximum reading is reached in less than 5 min. Insert the temperature-sensing probe into the rectum of the test rabbit to a depth of NLT 7.5 cm, and, after a period of time NLT that previously determined as sufficient, record the rabbit's body temperature.

## TEST ANIMALS

Use healthy, mature rabbits. House the rabbits individually in an area of uniform temperature between 20° and 23° and free from disturbances likely to excite them. The temperature varies NMT ±3° from the selected temperature. Before using a rabbit for the first time in a pyrogen test, condition it NMT 7 days before use by a sham test that includes all of the steps as directed in *Procedure* except injection. Do not use a rabbit for pyrogen testing more frequently than once every 48 h, nor prior to 2 weeks following a maximum rise of its temperature of 0.6° or more while being subjected to the pyrogen test, or following its having been given a test specimen that was adjudged pyrogenic.

## PROCEDURE

Perform the test in a separate area designated solely for pyrogen testing and under environmental conditions similar to those under which the animals are housed and free from disturbances likely to excite them. Withhold all food from the rabbits used during the period of the test. Access to water is allowed at all times, but may be restricted during the test. If rectal temperature-measuring probes remain inserted throughout the testing period, restrain the rabbits with light-fitting neck stocks that allow the rabbits to assume a natural resting posture. NMT 30 min prior to the injection of the test dose, determine the "control temperature" of each rabbit: this is the base for the determination of any temperature increase resulting from the injection of a test solution. In any one group of test rabbits, use only those rabbits whose control temperatures do not vary by more than 1° from each other, and do not use any rabbit with a temperature exceeding 39.8°.

Unless otherwise specified in the individual monograph, inject into an ear vein of each of three rabbits 10 mL of the test solution per kg of body weight, completing each injection within 10 min after start of administration. The test solution is *either* the product, constituted if necessary as directed in the labeling, *or* the material under test treated as directed in the individual monograph and injected in the dose specified therein. For pyrogen testing of devices or injection assemblies, use washings or rinsings of the surfaces that come in contact with the parenterally administered material or with the injection site or internal tissues of the patient. Assure that all test solutions are protected from contamination. Perform the injection after warming the test solution to a temperature of 37 ± 2°. Record the temperature at 30-min intervals between 1 and 3 h subsequent to the injection.

## TEST INTERPRETATION AND CONTINUATION

Consider any temperature decreases as zero rise. If no rabbit shows an individual rise in temperature of 0.5° or more above its respective control temperature, the product meets the requirements for the absence of pyrogens. If any rabbit shows an individual temperature rise of 0.5° or more, continue the test using five other rabbits. If NMT three of the eight rabbits show

[1] United States Food and Drug Administration. Guidance for industry. Pyrogen and endotoxins testing: questions and answers. Rockville, MD: Food and Drug Administration; June 2012. http://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm310098.pdf.

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individual rises in temperature of 0.5° or more and if the sum of the eight individual maximum temperature rises does not exceed 3.3°, the material under examination meets the requirements for the absence of pyrogens.

# RADIOACTIVE PHARMACEUTICALS

## Test Dose for Preformulated, Ready-to-Use Products Labeled with Radioactivity

### AGGREGATED ALBUMIN AND OTHER PARTICLE-CONTAINING PRODUCTS

For the rabbit pyrogen test, dilute the product with *Sodium Chloride Injection* to NLT 100 µCi/mL, and inject a dose of 3 mL/kg of body weight into each rabbit.

### OTHER PRODUCTS

**Where physical half-life of radionuclide is greater than 1 day:** Calculate the maximum volume of the product that might be injected into a human subject. This calculation takes into account the maximum recommended radioactive dose of the product, in µCi, and the radioactive assay, in µCi/mL, of the product at its expiration date or time. Using this information, calculate the maximum volume dose per kg to a 70-kg human subject.

For the rabbit pyrogen test, inject a minimum of 10 times this dose per kg of body weight into each rabbit. If necessary, dilute with *Sodium Chloride Injection*. The total injected volume per rabbit is NLT 1 mL and NMT 10 mL of solution.

**Where physical half-life of radionuclide is less than 1 day:** For products labeled with radionuclides with a half-life of less than 1 day, the dosage calculations are identical to those described in the first paragraph in *Other Products*. These products may be released for distribution prior to completion of the rabbit pyrogen test, but such test must be initiated at NMT 36 h after release.

## Test Dose for Pharmaceutical Constituents or Reagents to Be Labeled

The following test dose requirements pertain to reagents that are to be labeled or constituted prior to use by the direct addition of radioactive solutions such as *Sodium Pertechnetate Tc 99m Injection*, i.e., "cold kits".

Assume that the entire contents of the vial of nonradioactive reagent will be injected into a 70-kg human subject, or that $^{1}/_{70}$ of the total contents per kg will be injected. If the contents are dry, constitute with a measured volume of *Sodium Chloride Injection*.

For the rabbit pyrogen test, inject ($^{1}/_{7}$) of the vial contents per kg of body weight into each rabbit. The maximum dose per rabbit is the entire contents of a single vial. The total injected volume per rabbit is NLT 1 mL and NMT 10 mL of solution.

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# ⟨161⟩ MEDICAL DEVICES—BACTERIAL ENDOTOXIN AND PYROGEN TESTS

## INTRODUCTION

The methods and requirements in this chapter apply to assemblies or devices labeled sterile and nonpyrogenic that are in contact directly or indirectly with the cardiovascular system, lymphatic system, or cerebrospinal fluid. This includes, but may not be limited to, the following:

- Fluid pathways of catheters and administration sets such as solution administration sets, extension sets, transfer sets, blood administration sets, intravenous catheters, implants, extracorporeal oxygenator tubing, dialysis tubing, intramuscular drug delivery catheters, and transfusion and infusion assemblies
- Liquid medical devices such as dialysate
- Implantable medical devices such as heart valves and vascular grafts, and other medical devices with a nonpyrogenic claim that may come into contact with blood or cerebrospinal fluid
- Gels with a nonpyrogenic claim including demineralized bone matrices and drug delivery systems

## BACTERIAL ENDOTOXINS

For the bacterial endotoxins test (BET) assay parameters, procedures, standards, and controls for the gel-clot and kinetic and endpoint assays, proceed as directed in *Bacterial Endotoxins Test* ⟨85⟩, substituting the medical device eluate for the product sample.

## DEFINITIONS

### Endotoxin limit

- The endotoxin limit for the finished device is NMT 20 USP Endotoxin Units per device and NMT 2.15 USP Endotoxin Units for devices in contact with cerebrospinal fluid.
- For devices that directly or indirectly contact the intraocular environment, a lower endotoxin limit may apply (*1*).
- The endotoxin limit for the device extract in Endotoxin Units/mL is calculated as:

$$\text{Result} = (K \times N)/V$$

$K$ = limit for each device
$N$ = number of devices tested
$V$ = total volume of the extract

**Kit:** A kit is defined as a collection of individual devices in its primary package, or a variety of related devices. Each individual type of device may have its own product endotoxin limit and should be tested and evaluated on an individual basis; see *Table 1. Selection of Product Units for Testing* [refer to ANSI/AAMI ST72 (*2*)].

**Table 1. Selection of Product Units for Testing**

| Product Unit | Item for Testing |
|---|---|
| Individual medical device in a primary package where each medical device is used individually in clinical practice | Individual medical device |
| Set of components in a primary package where components are assembled as a product and are used together in clinical practice | Combination of components |
| A number of identical medical devices in one primary package where each medical device is used independently in clinical practice | Single medical device taken from the primary package |
| Kit of procedure-related medical devices where each medical device is used independently in clinical practice and where each medical device may have a different endotoxin limit | Each type of medical device that has a nonpyrogenic claim, or all items together |

**Lambda (λ):** λ is the test method sensitivity for gel-clot methods. For turbidometric or chromogenic technique, λ is equal to the lowest endotoxin concentration on the referenced standard curve.

**Product families:** Product families for the purposes of BET may be defined on the basis of common materials of construction and/or common components. The choice of family members should be justified and documented. If a product family contains devices of many sizes, the device with the largest surface area claimed to be nonpyrogenic is chosen as the representative member of the family to be used in suitability testing.

**Extracting or rinsing solution:** *Water for BET* [see *Bacterial Endotoxins Test* ⟨85⟩*, Reagents and Test Solutions, Water for Bacterial Endotoxins Test (BET)*] or other suitable solvent demonstrated to be noninterfering with the BET assay is used to extract or rinse the medical device.

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### Sampling

1. *Sample*: A sample for end-product testing must be in its final configuration and packaging, including all component parts that make up the final medical device.
2. *Representative sampling*: A representative sample is a sample plan that is created and justified based on the assumption that the manufacturing process is validated and in a state of control.
3. *Sample size*: The number of devices chosen for routine testing is dependent on the size of the lot, level of control, statistical considerations, and historical performance. In most cases, each lot of product must be tested using an appropriate number of samples, NMT 10, taken at random to represent the quality of the lot. Alternate sampling plans that utilize small sample sizes or do not test each lot of product must be clearly defined and must be supported/justified by a robust risk assessment.
4. *Pre-post sterilization samples*: When qualifying a BET assay for pre-sterilization samples, equivalency in test results must be shown with post-sterilization samples to ensure that sterilization and post-sterilization handling have no adverse impact on the accuracy of the test result. Pre-sterilization testing is inappropriate for products that support microbial growth.

**Suitability testing (also known as test for interfering factors):** Suitability studies of device extracts are designed to ensure that the device extract under test does not adversely impact the accuracy of the test result by either a) causing the assay to underestimate endotoxin (inhibition) or b) contributing to overestimation of endotoxin (enhancement).

## REAGENTS AND TEST SOLUTIONS

See ⟨85⟩.

## PREREQUISITES TO TESTING

### Apparatus

All equipment used in the extraction or testing process must be properly qualified and/or calibrated as appropriate. This equipment includes but is not limited to water baths, heat blocks, mechanical pipetters (fixed, adjustable, and repeating pipetters), thermometers, and plate/tube/cartridge readers.

Depyrogenate all glassware and other heat-stable materials in a hot air oven using a validated process. If employing plastic apparatus such as microplates and pipet tips for automatic pipetters, use apparatus that is shown to be free of detectable endotoxin and does not interfere with the test.

Analysts must be properly trained to perform the assay.

The assay sensitivity must be confirmed as described in *Bacterial Endotoxins Test* ⟨85⟩, *Gel-Clot Technique, Preparatory Testing, Test for Confirmation of Labeled Lysate Sensitivity* for gel-clot reagents and *Bacterial Endotoxins Test* ⟨85⟩, *Photometric Quantitative Techniques, Preparatory Testing, Assurance of Criteria for the Standard Curve* for quantitative methods. Assay sensitivity confirmation must be carried out when a new batch of reagent (lysate or endotoxin) is used or when there is any change in the test conditions that could affect the outcome of the test.

## PREPARATION OF DEVICES: STANDARD METHODS

### Endotoxin Limit

The endotoxin limit for the extracting or rinsing solution is:

$$\text{Result} = (K \times N)/V$$

$K$ = amount of endotoxin allowed per device (20 Endotoxin Units per device; 2.15 for intrathecal devices)
$N$ = number of devices tested
$V$ = total volume of the extract or rinse

[NOTE—The volume of the rinse or extract may be adjusted for the size and configuration of the device.]

The standard extracting fluid for extracting, rinsing, or soaking medical devices is *Water for BET*. If necessary, other solvents may be used after it has been demonstrated that they do not interfere with the performance of the assay. Extraction is not required for liquid medical devices. Either the entire device if claimed to be nonpyrogenic or all components of the device that are claimed to be nonpyrogenic, including fluid pathways, must come in contact with the extraction fluid for the entire course of the extraction.

The standard extraction method is to soak or immerse the device or flush the fluid pathway with extracting fluid that has been heated to 37 ± 1.0°, keeping the extracting fluid in contact with the relevant surface(s) for NLT 1 h at controlled room temperature. Alternate extraction or rinsing methods may be used, but must be demonstrated to be equivalent to or better than the standard method. Extracts from individual medical devices are typically pooled for testing.

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### Suitability

Suitability of the BET assay must be demonstrated for each product or product family. The number of batches of product chosen for the suitability study is reflective of the level of control of the process and must be justified.

For the suitability test, medical device extracts or liquid medical devices are considered to be the sample solutions under test and are tested as outlined for the chosen technique in *Bacterial Endotoxins Test* ⟨85⟩, *Gel-Clot Technique, Preparatory Testing, Test for Interfering Factors*.

If necessary, adjust the pH of the solution to be examined (or dilution thereof) so that the pH of the mixture of the lysate and sample solution falls within the pH range specified by the lysate manufacturer, usually pH 6.0–8.0. The pH may be adjusted by use of an acid, base, or suitable buffer as recommended by the lysate manufacturer. Acids and bases may be prepared from concentrates or solids with *Water for BET* in containers free of detectable endotoxin. Buffers must be shown to be free of detectable endotoxin and interfering factors.

If the undiluted rinsing or extracting solution or liquid medical device is determined to be unsuitable for ⟨85⟩, the test for interfering factors may be repeated utilizing dilution or a method that will neutralize or eliminate the interfering substance.

If glucan interference is suspected, the use of a glucan-blocking agent or endotoxin-specific lysate is permissible.

Dilution in *Water for BET* or other validated solvent by a factor not to exceed the maximum valid dilution (MVD) is permissible. The MVD for medical devices is the following:

$$\text{MVD} = \text{Endotoxin limit (of the rinsing, extracting, or diluting solution in Endotoxin Units/mL)}/\lambda$$

$\lambda$ = sensitivity of the test method in Endotoxin Units/mL

Interference may be overcome by suitable treatment of the device extract. Methods for overcoming interference and the qualification of such treatments are described in ⟨85⟩. To establish that the chosen treatment effectively eliminates interference without loss of endotoxins, perform the assay described in ⟨85⟩ using the preparation to be examined to which standard endotoxin has been added and which has then been submitted to the chosen treatment.

## DEMONSTRATION OF CONTINUED SUITABILITY

The continued suitability of the assay should be re-assessed when significant changes are made to the product, process, raw material supply, manufacturing and/or testing site, BET reagent source, or any other change that could adversely affect the accuracy of the BET result. The results of this re-assessment should be documented, and suitability studies re-executed as necessary.

Changes to extraction techniques require a new suitability study. Because of possible changes in interference patterns between standard BET methods, a change in BET methodology (e.g., from gel-clot limits test to kinetic chromogenic) requires a re-execution of the suitability study.

### Routine Testing

Routine testing of the finished medical devices must utilize the same extraction techniques that were documented in the successful suitability study.

Routine testing is to be performed as outlined in ⟨85⟩, following instructions for incubation and controls listed under the chosen assay technique.

### Interpretation of Test Results

See ⟨85⟩. The preparation under test complies with the test if the mean endotoxin concentration of the replicates of *Solution A*, after correction for dilution and concentration, is less than 20 Endotoxin Units per medical device (2.15 Endotoxin Units per medical device for those devices that come in contact with the cerebrospinal fluid.) For liquid medical devices, the limit meets the requirements set forth in *Bacterial Endotoxins Test* ⟨85⟩, *Determination of Maximum Valid Dilution (MVD), Endotoxin Limit, footnote 2*.

If a device fails to meet this requirement, an investigation may be initiated per documented procedures. A failed, valid BET assay may not be repeated using *Pyrogen Test* ⟨151⟩ in its place.

## PYROGENS

For samples that cannot be tested by BET because of nonremovable inhibition or enhancement of the test, ⟨151⟩ is applied. Select an appropriate number of devices, NMT 10, and obtain a pooled extract utilizing preparation methods appropriate to the device as directed for bacterial endotoxins, but with volumes of rinse or extraction fluid not to exceed 40 mL of sterile saline test solution per device. The requirements of ⟨151⟩ must be met.

## REFERENCES

1. U.S. Food and Drug Administration. Guidance for industry. Pyrogen and endotoxins testing: Questions and answers. Rockville, MD: Food and Drug Administration; June 2012. www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm314718.htm. Accessed 12 July 2016.

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2. ANSI/AAMI ST72. Bacterial endotoxins—Test methods, routine monitoring, and alternatives to batch testing. Arlington, VA: Association for the Advancement of Medical Instrumentation; 2011.

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# ⟨162⟩ DIPHTHERIA ANTITOXIN POTENCY TESTING FOR HUMAN IMMUNE GLOBULINS

An in vitro method is provided that is suitable for determining the potency of diphtheria antitoxin (antibodies against the diphtheria toxin) in preparations of plasma-derived human immune globulins. Diphtheria toxin is produced by *Corynebacterium diphtheriae* and has the ability to produce a cytopathogenic effect on susceptible epithelial cell lines. The test is based on the ability of diphtheria antitoxin to neutralize the diphtheria toxin, decreasing its cytotoxic effect. Specifically, the test determines the potency of the diphtheria antitoxin based on its ability to inhibit the cytotoxic effect of diphtheria toxin on cultured Vero cells (African green monkey kidney epithelial cells) relative to a reference standard. The mitochondrial dehydrogenases of live Vero cells can reduce the dye 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) to a blue–black product that is then measured by absorbance at 540 nm. If little or no diphtheria antitoxin is present, then diphtheria toxin induces cell death and the inability of cells to reduce MTT, resulting in the presence of white or colorless wells. Acceptance criteria are defined by the appropriate regulatory agencies.

## ASSAY

***Change to read:***

- **PROCEDURE**

**DMEM-5 solution:** Dulbecco's Modified Eagle's Medium,[1] supplemented to contain 5% fetal bovine serum (FBS) ▲or a suitable chemically defined alternative,▲ (USP 1-May-2022) 2 mM L-glutamine, 50 µg/mL of gentamicin, and 2.5 µg/mL of fungizone. [NOTE—Alternatively, a combination of 0.1 mg/mL of kanamycin sulfate, 0.1 units/mL of penicillin, and 0.1 mg/mL of streptomycin can be used in place of gentamicin.]

**DMEM-2 solution:** Dulbecco's Modified Eagle's Medium,[1] supplemented to contain 2% FBS ▲or a suitable chemically defined alternative,▲ (USP 1-May-2022) 2 mM L-glutamine, 50 µg/mL of gentamicin, and 2.5 µg/mL of fungizone. [NOTE—Alternatively, a combination of 0.1 mg/mL of kanamycin sulfate, 0.1 units/mL of penicillin, and 0.1 mg/mL of streptomycin can be used in place of gentamicin.]

**MTT solution:** 5 mg/mL of MTT in phosphate-buffered saline.[2] Prepare just before use and minimize exposure to room light.

**Extraction solution:** 0.4 N hydrochloric acid in isopropanol

**Toxin test solution:** Obtain a liquid preparation of toxin from a culture of *C. diphtheriae*.[3] It may have been rendered sterile by filtration, and sterility may be maintained by the inclusion of a suitable antimicrobial preservative. ▲If using fresh, noncommercially available toxin,▲ (USP 1-May-2022) store the filtrate in the dark at 2°–8° for several weeks until the activity is considered to be constant, as determined by testing. Dilute the toxin in *DMEM-5 solution* to obtain a suitable *Toxin test solution* that provides a working equivalence with the *Standard solution*. [NOTE—An appropriate *Toxin test solution* may be empirically determined as follows. ▲The example described here is for an assay performed using a toxin dose level of LCD/40 (where "LCD" stands for limit of cytotoxic dose, and "/40" refers to 1/40 units/mL [or 0.025 units/mL]).▲ (USP 1-May-2022) Titrate the toxin against a fixed concentration of antitoxin (e.g., ▲0.025▲ (USP 1-May-2022) units/mL) using volumes and media as defined below. Determine the lowest concentration of toxin that causes Vero cell toxicity in the presence of the ▲fixed▲ (USP 1-May-2022) antitoxin concentration. ▲▲ (USP 1-May-2022) This is ▲the toxin▲ (USP 1-May-2022) concentration that should be used in the *Assay*.]

**Trypsin–EDTA solution:** Dissolve 0.4 g of trypsin, 0.2 g of ethylenediaminetetraacetic acid (EDTA), and 0.85 g of sodium chloride in 100 mL of sterile water. Use cell culture grade and sterile reagents to prepare. Alternatively, a premade sterile preparation may be used.

**Cell culture preparation:** Prepare Vero cells[4] by culturing in 75-cm$^2$ tissue culture flasks in *DMEM-5 solution* at 36 ± 1°, 5% carbon dioxide in a humidified environment. [NOTE—Alternative flask sizes may be used by adjusting the volumes used below.] After 2–3 days of growth, the medium may be replaced by *DMEM-2 solution* and growth allowed to continue. When the cells have reached a confluent monolayer, discard the culture media. Wash the cell layer by pipetting 5 mL of *Trypsin–EDTA solution* into the flask and gently rocking the flask back and forth for approximately 30 s. Remove and discard the *Trypsin–EDTA solution*. Add an additional 5 mL of *Trypsin–EDTA solution*, and gently rock back and forth for ▲approximately▲ (USP 1-May-2022) 1 min. Remove and discard all but 1 mL. Incubate at 36 ± 1°, 5% carbon dioxide, in a humidified environment for approximately 10 min or until the cell sheet begins to slough from the flask, and tap the flask to release the cells. Add 10 mL of *DMEM-5 solution* to the trypsinized cells, count the cells, and adjust the cell suspension to 1 × 10$^5$ cells/mL in the same medium.

**Standard solutions:** In sterile tubes, prepare 4 concentrations of the standard by diluting ▲an appropriate diphtheria antitoxin standard suitable for the Vero cell assay▲ (USP 1-May-2022)[5] with *DMEM-2 solution* to 1 unit/mL, followed by serial dilutions in the same media that result in the additional concentrations of 0.5, 0.25, and 0.1 units/mL.

**Sample solutions:** ▲In sterile tubes, use *DMEM-2 solution* to dilute each human immune globulin test sample to 1.25% protein.▲ (USP 1-May-2022)Further dilute the samples with *DMEM-2 solution* to achieve an expected diphtheria antitoxin concentration that is within the range of the *Standard solutions* (i.e., 1–0.1 units/mL).

**Analysis**

**Samples:** *Toxin test solution*, *Standard solutions*, and *Sample solutions*

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[1] 11885092 ▲Thermo Fisher Scientific,▲ (USP 1-May-2022) or equivalent.

[2] 14190250 ▲Thermo Fisher Scientific,▲ (USP 1-May-2022) or equivalent.

[3] Diphtheria toxin code 12/282 from National Institute for Biological Standards and Control (NIBSC) or suitable alternative.

[4] American Type Culture Collection CCL-81.

[5] ▲Diphtheria Antitoxin Human IgG (1st International Standard) has been demonstrated to be suitable for the Vero cell assay.▲ (USP 1-May-2022)

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Test each *Standard solution* or *Sample solution* in duplicate. Label 96 well culture plates by marking off the 8-well columns in groups of two; all 4 *Standard solutions* and 1 *Sample solution* can be tested per plate. As described below, columns 11 and 12 can be used for the untreated cell control and the toxin control, respectively. [NOTE—Alternative plate layouts maintaining the dilutions can be used.] Add 112.5 µL of *DMEM-2 solution* to each well in row A, columns 1–10 only (these are standard- or sample-nonspecific toxicity controls). Add 75 µL of *DMEM-2 solution* to each well in rows B–H, columns 1–10 only.

Add 37.5 µL of a *Standard solution* dilution or a *Sample solution* to wells A1–A2, and add 75 µL of the same solution to wells B1–B2. Add 37.5 µL of a second *Standard solution* dilution or *Sample solution* to wells A3–A4, and add 75 µL of the same solution to wells B3–B4. Continue to make similar additions of *Standard solution* dilutions or *Sample solutions* to each group of two columns in columns 1–10. Make two-fold serial dilutions by transferring 75 µL from each well in row B to each well in row C of the same column. Mix and then continue transferring 75 µL in the same manner down each column. Discard the last 75 µL after mixing row H. Add 75 µL of the *Toxin test solution* to rows B–H in columns 1–10. Add 150 µL of *DMEM-2 solution* to all wells in column 11 (cell control), and add 75 µL of *DMEM-2 solution* plus 75 µL of *Toxin test solution* to all wells in column 12 (toxin control).

Cover the plates, and incubate them for 1 h at 36 ± 1°, at 5% carbon dioxide in a humidified environment. Add 150 µL of cell suspension to all wells, cover the plates, and incubate them at 36 ± 1°, at 5% carbon dioxide in a humidified environment. Incubate for 4–5 days, checking periodically for microbial contamination by microscopic examination. Once toxicity control wells in row A containing the lowest concentration of a *Standard solution* are nearly confluent, add 15 µL of *MTT solution* to each well. Cover the plates, and incubate them in the carbon dioxide incubator at 37° for 3 h. Discard the medium, and add 150 µL of *Extraction solution* to each well. Cover the plates, and place aluminum foil over them to minimize light exposure. Shake gently to solubilize the blue formazan formed by viable cells. Using a suitable plate reader, read the absorbance of each well at a wavelength of 540 nm.

**Calculation:** The cutoff dilution is the highest dilution in which there are still viable cells in the well but beyond which there are no viable cells. Cutoff dilution is defined by a corresponding absorbance (Abs). [NOTE—The average absorbance value can be calculated for the cell control wells in column 11 and divided by 2 to obtain a 50% control Abs value. This value can be used to determine cutoff dilutions. The cutoff dilution for each *Standard solution* and *Sample solution* can therefore be defined as the highest dilution where the Abs value is greater than the 50% control Abs value.] Determine the cutoff dilutions for each *Standard solution* and *Sample solution*. Plot the cutoff dilutions obtained for all *Standard solutions* (expressed as the reciprocal of the cutoff dilution) versus ▲the appropriate diphtheria antitoxin standard▲ (USP 1-May-2022) units. Calculate a linear regression line from the data. Calculate the geometric mean for the four cutoff dilutions associated with each *Sample solution*. Compare the mean cutoff dilution to the linear regression of the *Standard solution* data to determine a titer, in units per milliliter, for each *Sample solution*. To obtain the final potency value for the *Sample solution*, multiply the titer by any dilution that was made to the *Sample solution* prior to the *Assay*.

**System suitability:** The correlation coefficient of the standard curve must be >0.995. The slope of the standard curve must be between 26 and 38. The test is valid if the cells in wells of row A, columns 1–10, appear normal and similar to the cell control wells in column 11. In addition, the cutoff dilution values for *Standard solution* and *Sample solution* replicates should be within one serial dilution of each other, and the cutoff dilution must be within the range of the *Sample solution* serial dilutions.

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# ⟨165⟩ PREKALLIKREIN ACTIVATOR

## DEFINITION

Prekallikrein activator (PKA), also known as Factor XIIa, activates prekallikrein to kallikrein. Activated kallikrein cleaves kininogens into kinins (e.g., bradykinin) that can result in a loss of blood pressure. PKA is assayed by its ability to activate prekallikrein, which once activated cleaves a synthetic peptide substrate and releases a chromophore that can be measured spectrophotometrically. The amount of PKA is determined by comparison with reference standard material. The procedure presented in this chapter traditionally has been used to determine the amount of PKA in human plasma derivatives such as albumin and immunoglobulin preparations.

Some immunoglobulin preparations may contain kallikrein or kallikrein-like activity in the absence of the added prekallikrein substrate, resulting in turnover of the synthetic peptide substrate. If present, this kallikrein or kallikrein-like activity requires consideration when analysts verify the assay for suitability under the conditions of use. Some questions to consider when verifying the assay for suitability under the conditions of use include:

- Are sample matrix effects observed?
- Is the appropriate statistical model used for data analysis?
- Is an endpoint analysis sufficient, or is a kinetic analysis needed?

(For informational purposes only, refer to sections 2, 4, and 5 of *Design and Development of Biological Assays* ⟨1032⟩ when considering assay fitness for use.)

## ASSAY

• **PREKALLIKREIN ASSAY**

**Buffer A:** Prepare a solution of 0.05 M tris(hydroxymethyl)aminomethane and 0.15 M sodium chloride. Adjust with 2 N hydrochloric acid to a pH of 7.95–8.05.

**Buffer B:** Prepare a solution of 1.0 M tris(hydroxymethyl)aminomethane. Adjust with 2 N hydrochloric acid to a pH of 7.95–8.05.

**Solution A:** Prepare a solution of 0.33 N hydrochloric acid.

**Solution B:** Prepare a solution of 0.33 N sodium hydroxide.

**Solution C:** Prepare a solution of 50% acetic acid.

**Chromogenic substrate solution:** Prepare a solution of a suitable chromogenic substrate for amidolytic test specific for kallikrein in *Buffer A* to obtain a solution having a concentration of about 1.2 mM.

**Prekallikrein substrate:** Use a suitable *Prekallikrein substrate*. Test the *Prekallikrein substrate* for the absence of kallikrein activity by mixing 1 part with 20 parts of the *Chromogenic substrate solution* prewarmed at 37°, and incubate at 37° for 2 min. The substrate is suitable if the increase in absorbance at 405 nm is less than 0.001/min. [NOTE—To remove proteinase inhibitor contamination from the *Prekallikrein substrate*, acid treatment of the prekallikrein should be performed individually on each sample before use.] If frozen, thaw *Prekallikrein substrate* at 35°–39°. Add 2.0 mL of *Solution A* to 4 mL of *Prekallikrein substrate*, and incubate for 15 min at 23°–25°. Bring pH back to neutrality with 2.0 mL of *Solution B*. Add 200 µL of *Buffer B*, and store at room temperature for NMT 4 h.

**Standard solutions:** Dissolve the contents of a vial of USP Prekallikrein Activator RS in 0.5 mL of water. Dilute USP Prekallikrein Activator RS with *Buffer A* (1:5). Make five evenly spaced *Standard solutions* between 0 and 8.5 IU/mL.

**Sample solution:** Dilute sample appropriately to fall within the range of the standard dilutions with *Buffer A* performing at minimum (1:1).

**System suitability**

**Samples:** *Buffer A* and *Standard solutions*

**Suitability requirements**

**Assay range:** If the absorbance value of a sample is higher than the highest value of the standard curve, dilute the sample with *Buffer A* and repeat the *Assay*. If the absorbance of a sample is lower than the *Assay* limit of detection, the activity should be reported as less than the limit of detection.

**Correlation coefficient:** NLT 0.99

**Analysis**

**Samples:** *Buffer A*, *Standard solutions*, and *Sample solution*

[NOTE—This procedure also can be performed using alternative platforms. Care must be taken to minimize the time between the addition of *Prekallikrein substrate* to the *Standard solutions* and the *Sample solution* because some degradation of the *Prekallikrein substrate* can occur with time when thawed.]

To four separate individual wells of a suitable microtiter plate transfer 25 µL of either the *Standard solutions* or the *Sample solution*. Incubate the microtiter plate for 2–3 min at 35°–39°. Start to prewarm *Buffer A*, the *Prekallikrein substrate*, and the *Chromogenic substrate solution* at 35°–39°. For each *Standard solution* and *Sample solution*, in two of the four wells add 100-µL *Buffer A* and to the other two wells add 100-µL prewarmed *Prekallikrein substrate*; incubate at 35°–39° for 10 min. Add 125 µL of prewarmed *Chromogenic substrate solution* to all wells in the same order as the *Buffer A* and *Prekallikrein substrate* additions, and incubate at 35°–39° for 20 min. Stop the reactions with 50 µL of *Solution C*, and mix well. Read absorbance of each well of the plate at 405 nm within 2 h. Subtract the average $A_{405}$ of the two wells containing *Buffer A* for each *Standard solution* and *Sample solution* to give the corrected $A_{405}$ values. Using the least-squares method of linear regression, generate a standard curve from the activities of the *Standard solutions* (IU/mL) and their corresponding corrected absorbance values. Calculate prekallikrein activities of samples from the calibration curve of the standards. To obtain the corrected activity in IU/mL, multiply each activity by the sample's dilution factor.

## ADDITIONAL REQUIREMENTS

• **USP REFERENCE STANDARDS** ⟨11⟩

USP Prekallikrein Activator RS

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# ⟨171⟩ VITAMIN $B_{12}$ ACTIVITY ASSAY

## ASSAY

• **PROCEDURE**

**Assay preparation:** Place a suitable quantity of the material to be assayed, previously reduced to a fine powder if necessary and accurately measured or weighed, in an appropriate vessel containing, for each g or mL of material taken, 25 mL of an aqueous extracting solution prepared just prior to use to contain, in each 100 mL, 1.29 g of disodium phosphate, 1.1 g of anhydrous citric acid, and 1.0 g of sodium metabisulfite. Autoclave the mixture at 121° for 10 minutes. Allow any undissolved particles of the extract to settle, and filter or centrifuge, if necessary. Dilute an aliquot of the clear solution with water so that the final test solution contains vitamin $B_{12}$ activity approximately equivalent to that of the *Standard cyanocobalamin solution* which is added to the assay tubes.

**Standard cyanocobalamin stock solution:** To a suitable quantity of USP Cyanocobalamin (Crystalline) RS, accurately weighed, add sufficient 25 percent alcohol to make a solution having a known concentration of 1.0 µg of cyanocobalamin per mL. Store in a refrigerator.

**Standard cyanocobalamin solution:** Dilute a suitable volume of *Standard cyanocobalamin stock solution* with water to a measured volume such that after the incubation period as described for *Procedure*, the difference in transmittance between the inoculated blank and the 5.0-mL level of the *Standard cyanocobalamin solution* is not less than that which corresponds to a difference of 1.25 mg in dried cell weight. This concentration usually falls between 0.01 ng and 0.04 ng per mL of *Standard cyanocobalamin solution.* Prepare a fresh standard solution for each assay.

**Basal medium stock solution:** Prepare the medium according to the following formula and directions. A dehydrated mixture containing the same ingredients may be used provided that, when constituted as directed in the labeling, it yields a medium comparable to that obtained from the formula given herein.

Add the ingredients in the order listed, carefully dissolving the cystine and tryptophane in the hydrochloric acid before adding the next eight solutions in the resulting solution. Add 100 mL of water, mix, and dissolve the dextrose, sodium acetate, and ascorbic acid. Filter, if necessary, add the polysorbate 80 solution, adjust the solution to a pH between 5.5 and 6.0 with 1 N sodium hydroxide, and add purified water to make 250 mL.

| | |
|---|---|
| L-Cystine | 0.1 g |
| L-Tryptophane | 0.05 g |
| 1 *N* Hydrochloric Acid | 10 mL |
| Adenine–Guanine–Uracil Solution | 5 mL |
| Xanthine Solution | 5 mL |
| Vitamin Solution I | 10 mL |
| Vitamin Solution II | 10 mL |
| Salt Solution A | 5 mL |
| Salt Solution B | 5 mL |
| Asparagine Solution | 5 mL |
| Acid-hydrolyzed Casein Solution | 25 mL |
| Dextrose, Anhydrous | 10 g |
| Sodium Acetate, Anhydrous | 5 g |
| Ascorbic Acid | 1 g |
| Polysorbate 80 Solution | 5 mL |

**Acid-hydrolyzed casein solution:** Prepare as directed under *Calcium Pantothenate Assay* ⟨91⟩.

**Asparagine solution:** Dissolve 2.0 g of L-asparagine in water to make 200 mL. Store under toluene in a refrigerator.

**Adenine–guanine–uracil solution:** Prepare as directed under *Calcium Pantothenate Assay* ⟨91⟩.

**Xanthine solution:** Suspend 0.20 g of xanthine in 30 mL to 40 mL of water, heat to about 70°, add 6.0 mL of 6 N ammonium hydroxide, and stir until the solid is dissolved. Cool, and add water to make 200 mL. Store under toluene in a refrigerator.

**Salt solution A:** Dissolve 10 g of monobasic potassium phosphate and 10 g of dibasic potassium phosphate in water to make 200 mL. Add 2 drops of hydrochloric acid, and store under toluene.

**Salt solution B:** Dissolve 4.0 g of magnesium sulfate, 0.20 g of sodium chloride, 0.20 g of ferrous sulfate, and 0.20 g of manganese sulfate in water to make 200 mL. Add 2 drops of hydrochloric acid, and store under toluene.

**Polysorbate 80 solution:** Dissolve 20 g of polysorbate 80 in alcohol to make 200 mL. Store in a refrigerator.

**Vitamin solution I:** Dissolve 10 mg of riboflavin, 10 mg of thiamine hydrochloride, 100 µg of biotin, and 20 mg of niacin in 0.02 N glacial acetic acid to make 400 mL. Store, protected from light, under toluene in a refrigerator.

**Vitamin solution II:** Dissolve 20 mg of para-aminobenzoic acid, 10 mg of calcium pantothenate, 40 mg of pyridoxine hydrochloride, 40 mg of pyridoxal hydrochloride, 8 mg of pyridoxamine dihydrochloride, and 2 mg of folic acid in dilute neutralized alcohol (1 in 4) to make 400 mL. Store, protected from light, in a refrigerator.

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**Tomato juice preparation:** Centrifuge commercially canned tomato juice so that most of the pulp is removed. Suspend about 5 g per L of analytical filter-aid in the supernatant, and filter, with the aid of reduced pressure, through a layer of the filter-aid. Repeat, if necessary, until a clear, straw-colored filtrate is obtained. Store under toluene in a refrigerator.

**Culture medium:** [NOTE—A dehydrated mixture containing the same ingredients may be used provided that, when constituted as directed in the labeling, it yields a medium equivalent to that obtained from the formula given herein.] Dissolve 0.75 g of water-soluble yeast extract, 0.75 g of dried peptone, 1.0 g of anhydrous dextrose, and 0.20 g of potassium biphosphate in 60 mL to 70 mL of water. Add 10 mL of *Tomato juice preparation* and 1 mL of *Polysorbate 80 solution.* Adjust the solution with 1 N sodium hydroxide to a pH of 6.8, and add water to make 100 mL. Place 10-mL portions of the solution in test tubes, and plug with cotton. Sterilize the tubes and contents in an autoclave at 121° for 15 minutes. Cool as rapidly as possible to avoid color formation resulting from overheating the medium.

**Suspension medium:** Dilute a measured volume of *Basal medium stock solution* with an equal volume of water. Place 10-mL portions of the diluted medium in test tubes. Sterilize, and cool as directed above for the *Culture medium*.

**Stock culture of *Lactobacillus leichmannii*:** To 100 mL of *Culture medium* add 1.0 g to 1.5 g of agar, and heat the mixture, with stirring, on a steam bath, until the agar dissolves. Place approximately 10-mL portions of the hot solution in test tubes, cover the tubes suitably, sterilize at 121° for 15 minutes in an autoclave (exhaust line temperature), and allow the tubes to cool in an upright position. Inoculate three or more of the tubes, by stab transfer of a pure culture of *Lactobacillus leichmannii*.[*] (Before first using a fresh culture in this assay, make not fewer than 10 successive transfers of the culture in a 2-week period.) Incubate 16 to 24 hours at any selected temperature between 30° and 40° but held constant to within ±0.5°, and finally store in a refrigerator.

Prepare fresh stab cultures at least three times each week, and do not use them for preparing the inoculum if more than 4 days old. The activity of the microorganism can be increased by daily or twice-daily transfer of the stab culture, to the point where definite turbidity in the liquid inoculum can be observed 2 to 4 hours after inoculation. A slow-growing culture seldom gives a suitable response curve, and may lead to erratic results.

**Inoculum:** [NOTE—A frozen suspension of *Lactobacillus leichmannii* may be used as the stock culture, provided it yields an inoculum comparable to a fresh culture.] Make a transfer of cells from the *Stock culture of Lactobacillus leichmannii* to 2 sterile tubes containing 10 mL of the *Culture medium* each. Incubate these cultures for 16 to 24 hours at any selected temperature between 30° and 40° but held constant to within ±0.5°. Under aseptic conditions, centrifuge the cultures, and decant the supernatant. Suspend the cells from the culture in 5 mL of sterile *Suspension medium*, and combine. Using sterile *Suspension medium*, adjust the volume so that a 1 in 20 dilution in saline TS produces 70% transmittance when read on a suitable spectrophotometer that has been set at a wavelength of 530 nm, equipped with a 10-mm cell, and read against saline TS set at 100% transmittance. Prepare a 1 in 400 dilution of the adjusted suspension using *Basal medium stock solution*, and use it for the test inoculum. (This dilution may be altered, when necessary, to obtain the desired test response.)

**Calibration of spectrophotometer:** Check the wavelength of the spectrophotometer periodically, using a standard wavelength cell or other suitable device. Before reading any tests, calibrate the spectrophotometer for 0% and 100% transmittance, using water and with the wavelength set at 530 nm.

**Procedure:** Cleanse meticulously by suitable means, followed preferably by heating at 250° for 2 hours, hard-glass test tubes, about 20 mm × 150 mm in size, and other necessary glassware because of the high sensitivity of the test organism to minute amounts of vitamin $B_{12}$ activity and to traces of many cleansing agents.

To test tubes add, in duplicate, 1.0 mL, 1.5 mL, 2.0 mL, 3.0 mL, 4.0 mL, and 5.0 mL, respectively, of the *Standard cyanocobalamin solution.* To each of these tubes and to four similar empty tubes add 5.0 mL of *Basal medium stock solution* and water to make 10 mL.

To similar test tubes add, in duplicate, respectively, 1.0 mL, 1.5 mL, 2.0 mL, 3.0 mL, and 4.0 mL of the *Assay preparation.* To each tube add 5.0 mL of *Basal medium stock solution* and water to make 10 mL. Place one complete set of standard and assay tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes suitably to prevent bacterial contamination, and sterilize the tubes and contents in an autoclave at 121° for 5 minutes, arranging to reach this temperature in not more than 10 minutes by preheating the autoclave, if necessary. Cool as rapidly as practicable to avoid color formation resulting from overheating the medium. Take precautions to maintain uniformity of sterilizing and cooling conditions throughout the assay, since packing tubes too closely in the autoclave, or overloading it, may cause variation in the heating rate.

Aseptically add 0.5 mL of *Inoculum* to each tube so prepared, except two of the four containing no *Standard cyanocobalamin solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 40° held constant to within ±0.5°, for 16 to 24 hours.

Terminate growth by heating to a temperature not lower than 80° for 5 minutes. Cool to room temperature. After agitating its contents, place the container in a spectrophotometer that has been set at a wavelength of 530 nm, and read the transmittance when a steady state is reached. This steady state is observed a few seconds after agitation when the reading remains constant for 30 seconds or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 100% for the uninoculated blank, read the transmittance of the inoculated blank. If the difference is greater than 5% or if there is evidence of contamination with a foreign microorganism, disregard the results of the assay.

With the transmittance set at 100% for the uninoculated blank, read the transmittance of each of the remaining tubes. Disregard the results of the assay if the slope of the standard curve indicates a problem with sensitivity.

**Calculation:** Prepare a standard concentration-response curve by the following procedure. Test for and replace any aberrant individual transmittances. For each level of the standard, calculate the response from the sum of the duplicate values of

[*] Pure cultures of *Lactobacillus leichmannii* may be obtained as No. 7830 from the American Type Culture Collection, 10801 University Blvd., Manassas, VA 20110.

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the transmittances (Σ) as the difference, y = 2.00 – Σ. Plot this response on the ordinate of cross-section paper against the logarithm of the mL of *Standard cyanocobalamin solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, y, adding together the two transmittances for each level of the *Assay preparation.* Read from the standard curve the logarithm of the volume of the *Standard preparation* corresponding to each of those values of y that falls within the range of the lowest and highest points plotted for the standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Assay preparation* to obtain the difference, x, for each dosage level. Average the values of x for each of three or more dosage levels to obtain x = M′, the log-relative potency of the *Assay preparation.* Determine the quantity, in µg, of USP Cyanocobalamin (Crystalline) RS corresponding to the cyanocobalamin in the portion of material taken for assay by the equation:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = number of µg of cyanocobalamin that was assumed to be present in each mg (or capsule or tablet) of the material taken for assay

**Replication:** Repeat the entire determination at least once, using separately prepared *Assay preparations.* If the difference between the two log potencies M is not greater than 0.08, their mean, M, is the assayed log-potency of the test material (see *Vitamin $B_{12}$ Activity Assay* under *Design and Analysis of Biological Assays* ⟨111⟩). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of *M* that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

## ADDITIONAL REQUIREMENTS

- **USP Reference Standards** ⟨11⟩

  USP Cyanocobalamin (Crystalline) RS

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# ⟨181⟩ IDENTIFICATION—ORGANIC NITROGENOUS BASES

## INTRODUCTION

The purpose of this test is the identification of tertiary amine compounds. This spectroscopic test has a limited degree of specificity and, therefore, the conformance with all additional identification tests listed in a particular monograph is necessary to ensure the identity of the specimen under examination.

## ASSAY

• **PROCEDURE**

**Standard solution:** In a separator dissolve 50 mg of the corresponding USP Reference Standard in 25 mL of 0.01 N hydrochloric acid.

**Sample solution:** Depending upon the nature of the sample, dissolve 50 mg of the bulk substance under test in 25 mL of 0.01 N hydrochloric acid, or shake a quantity of powdered tablets or the contents of capsules, equivalent to 50 mg of the substance, with 25 mL of 0.01 N hydrochloric acid for 10 min. Transfer the liquid to a separator, filtering if necessary, and washing the filter and the residue with several small portions of water.

**Instrumental conditions**

(See *Mid-Infrared Spectroscopy* ⟨854⟩.)

**Mode:** IR

**Wavelength range:** 7–15 µm (1430 $cm^{-1}$ to 650 $cm^{-1}$)

**Cell:** 1-mm

**Blank:** Carbon disulfide

**Analysis**

**Samples:** *Standard solution* and *Sample solution*

Treat each solution as follows: Add 2 mL of 1 N sodium hydroxide and 4 mL of carbon disulfide, and shake for 2 min. Centrifuge if necessary to clarify the lower phase, and pass it through a dry filter, collecting the filtrate in a small flask provided with a glass stopper. Determine the absorption spectra of the filtered *Standard solution* and *Sample solution* without delay.

**Acceptance criteria:** The spectrum of the *Sample solution* must show all of the significant absorption bands present in the spectrum of the *Standard solution*.

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# ⟨191⟩ IDENTIFICATION TESTS—GENERAL

## INTRODUCTION

Procedures in this chapter are referenced in monographs for the identification of official articles and their components. Any acids, bases, or other reagents used in these procedures should not interfere with the results. Volumes may be scaled proportionally unless otherwise indicated. All of the tests include approximate amounts, except where specified.

Instrumental techniques described in this chapter may be used in lieu of chemical identification tests. Those instrumental techniques are not exhaustive and other techniques, such as nuclear magnetic resonance, ion-selective electrodes, and near-infrared, may be used in lieu of a chemical identification test provided that they are suitable and validated.

Unless otherwise specified in the monograph, if a chemical identification test is selected for an ion, then all chemical test procedures listed for the ion shall be met. If an instrumental identification test is selected, then only one instrumental technique is required for the ion(s).

## CHEMICAL IDENTIFICATION TESTS

**Acetate**

- **A.** Dissolve about 30 mg of the substance to be examined in 3 mL of water, or use 3 mL of the prescribed solution. Adjust the pH of the solution with sodium hydroxide to be slightly alkaline. Add 0.25 mL of lanthanum nitrate test solution (TS). If a white precipitate is formed, filter the solution. Add successively 0.1 mL of iodine and potassium iodide TS 3, and 0.1 mL of ammonia TS 2 to the solution. If no blue color is observed, heat carefully to boiling. In the presence of acetates, a dark color develops or a blue precipitate is formed.
- **B.** With neutral solutions of acetates, ferric chloride TS produces a red color that is destroyed by the addition of mineral acids.

**Aluminum**

- **A.** By using 6 N ammonium hydroxide, solutions of aluminum salts yield a gelatinous, white precipitate that is insoluble in an excess of 6 N ammonium hydroxide.
- **B.** 1 N sodium hydroxide or sodium sulfide TS with solutions of aluminum salts produces a similar gelatinous, white precipitate, which dissolves in an excess of either of the same reagents.

**Ammonium**

- **A.** Add 0.2 g of magnesium oxide to the solution under test. Pass a current of air through the mixture, and direct the gas that escapes to just beneath the surface of the indicator solution prepared previously by mixing 1 mL of 0.1 M hydrochloric acid and 0.05 mL of methyl red TS 2. In the presence of ammonium, the color of the indicator solution is changed to yellow. After directing the gas into the indicator solution for a sufficient period of time, add 1 mL of freshly prepared sodium cobaltinitrite TS to the indicator solution. Upon the addition of the sodium cobaltinitrite TS, a yellow precipitate is formed when ammonium is present.

**Antimony**

- **A.** With hydrogen sulfide, solutions of antimony (III) compounds, strongly acidified with hydrochloric acid, yield an orange precipitate of antimony sulfide that is insoluble in 6 N ammonium hydroxide but is soluble in ammonium sulfide TS.

**Barium**

- **A.** Solutions of barium salts yield a white precipitate with the addition of 2 N sulfuric acid. This precipitate is insoluble in hydrochloric acid and in nitric acid.

**Benzoate**

- **A.** In neutral solutions, benzoates yield a salmon-colored precipitate with ferric chloride TS.
- **B.** In moderately concentrated solutions, benzoates yield a precipitate of benzoic acid upon acidification when 2 N sulfuric acid is added. This precipitate is readily soluble in ethyl ether.

**Bicarbonate**

- **A.** Bicarbonates effervesce with acids, evolving a colorless gas that, when passed into calcium hydroxide TS, produces a white precipitate immediately.
- **B.** A cold solution (1 in 20) of a soluble bicarbonate or the solution prescribed in the specific monograph remains unchanged or is only slightly colored by phenolphthalein TS.

**Bismuth**

- **A.** When dissolved in a slight excess of nitric acid or hydrochloric acid, bismuth salts yield a white precipitate upon dilution with water. This precipitate is colored brown by hydrogen sulfide, and the resulting compound dissolves in a warm mixture of equal parts of nitric acid and water.

**Bisulfite** See *Sulfite*.

**Borate**

- **A.** To 1 mL of a borate solution acidified with hydrochloric acid, add 3 or 4 drops of iodine TS and 3 or 4 drops of polyvinyl alcohol solution (1 in 50): an intense blue color is produced.

**Bromide**

- **A.** Solutions of bromides, upon the addition of chlorine TS dropwise, liberate bromine, which is dissolved by shaking with chloroform, coloring the chloroform red-to-reddish brown.
- **B.** Silver nitrate TS produces, in solutions of bromides, a yellowish-white precipitate that is insoluble in nitric acid and is slightly soluble when 6 N ammonium hydroxide is added.

**Calcium**

- **A.** Solutions of calcium salts form insoluble oxalates when treated as follows. To a solution of the calcium salt (1 in 20) or the solution prescribed in the specific monograph, add 2 drops of methyl red TS and neutralize with 6 N ammonium hydroxide. Add 3 N hydrochloric acid dropwise, until the solution is acid to the indicator. Upon the addition of ammonium oxalate TS, a white precipitate is formed. This precipitate is insoluble when 6 N acetic acid is added but dissolves in hydrochloric acid.

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**Carbonate**
- **A.** Carbonates effervesce with acids, evolving a colorless gas that, when passed into calcium hydroxide TS, produces a white precipitate immediately.
- **B.** A cold solution (1 in 20) of a soluble carbonate or the solution prescribed in the specific monograph is colored red by phenolphthalein TS, whereas a similar solution of a bicarbonate remains unchanged or is only slightly colored.

**Chlorate**
- **A.** Solutions of chlorates yield no precipitate with silver nitrate TS. The addition of sulfurous acid to this mixture produces a white precipitate that is insoluble in nitric acid but is soluble in 6 N ammonium hydroxide.
- **B.** Upon ignition, chlorates yield chlorides, recognizable by appropriate tests.
- **C.** When sulfuric acid is added to a dry chlorate, decrepitation occurs and a greenish-yellow gas is evolved. [**CAUTION**—Use only a small amount of chlorate for this test, and exercise extreme caution in performing it.]

***Change to read:***

**Chloride**
- **A.** With silver nitrate TS, solutions of chlorides yield a white, curdy precipitate that is insoluble in nitric acid but is soluble in a slight excess of 6 N ammonium hydroxide. ▲When testing amine (including alkaloidal) hydrochlorides that do not respond to the previous test, add 1 drop of diluted nitric acid and 0.5 mL of silver nitrate TS to a solution of the substance being examined containing, unless otherwise directed in the monograph, about 2 mg of chloride ion in 2 mL: a white, curdy precipitate is formed. Centrifuge the mixture without delay, and decant the supernatant layer. Wash the precipitate with three 1-mL portions of nitric acid solution (1 in 100), and discard the washings. Add ammonia TS dropwise to this precipitate. It dissolves readily.▲ (USP 1-May-2021)

**Citrate**
- **A.** To 15 mL of pyridine add a solution or suspension of a few milligrams of a citrate salt in 1 mL of water or the solution prescribed in the specific monograph, and shake. To this mixture add 5 mL of acetic anhydride, and shake: a light red color is produced.

**Cobalt**
- **A.** Solutions of cobalt salts (1 in 20) in 3 N hydrochloric acid or the solution prescribed in the specific monograph yield a red precipitate when heated on a steam bath with an equal volume of a hot, freshly prepared solution of 1-nitroso-2-naphthol (1 in 10) in 9 N acetic acid.
- **B.** Solutions of cobalt salts, when saturated with potassium chloride and treated with potassium nitrite and acetic acid, yield a yellow precipitate.

**Copper**
- **A.** Cupric compounds, acidified with hydrochloric acid, deposit a red film of metallic copper upon a bright, untarnished surface of metallic iron.
- **B.** An excess of 6 N ammonium hydroxide, when added to a solution of a cupric salt, produces first a bluish precipitate and then a deep blue-colored solution.
- **C.** With potassium ferrocyanide TS, solutions of cupric salts yield a reddish-brown precipitate, insoluble in diluted acids.

**Hypophosphite**
- **A.** Hypophosphites in solution yield a white precipitate with mercuric chloride TS. This precipitate becomes gray when an excess of hypophosphite is present.
- **B.** Solutions of hypophosphites, acidified with sulfuric acid and warmed with cupric sulfate TS, yield a red precipitate.

**Iodide**
- **A.** Solutions of iodides, upon the addition of chlorine TS dropwise, liberate iodine, which colors the solution from yellow to red. When the solution is shaken with chloroform, the solution is colored violet. The iodine thus liberated gives a blue color with starch TS.
- **B.** Silver nitrate TS produces, in solutions of iodides, a yellow, curdy precipitate that is insoluble in nitric acid and insoluble in 6 N ammonium hydroxide.

**Iron**
- **A.** Ferrous and ferric compounds in solution yield a black precipitate with ammonium sulfide TS. This precipitate dissolves in cold 3 N hydrochloric acid and evolves hydrogen sulfide.

  **Ferric salts**
  - **A.** Acid solutions of ferric salts yield a dark blue precipitate with potassium ferrocyanide TS.
  - **B.** With an excess of 1 N sodium hydroxide added to the ferric salts solutions, a reddish-brown precipitate is formed.
  - **C.** With ammonium thiocyanate TS, solutions of ferric salts produce a deep red color that is not destroyed by dilute mineral acids.

  **Ferrous salts**
  - **A.** Solutions of ferrous salts yield a dark blue precipitate with potassium ferricyanide TS. This precipitate is insoluble in 3 N hydrochloric acid but is decomposed by 1 N sodium hydroxide.
  - **B.** With 1 N sodium hydroxide, solutions of ferrous salts yield a greenish-white precipitate; when shaken, the color changes rapidly to green and then to brown.

**Lactate**
- **A.** When solutions of lactates are acidified with sulfuric acid, potassium permanganate TS is added; the mixture is heated, and acetaldehyde evolves. Acetaldehyde can be detected by allowing the vapor to come into contact with a filter paper that has been moistened with a freshly prepared mixture of equal volumes of 20% aqueous morpholine and sodium nitroferricyanide TS: a blue color is produced.

**Lead**
- **A.** With 2 N sulfuric acid, solutions of lead salts yield a white precipitate that is insoluble in 3 N hydrochloric or 2 N nitric acid but is soluble in warm 1 N sodium hydroxide and in ammonium acetate TS.

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- **B.** With potassium chromate TS, solutions of lead salts, free or nearly free from mineral acids, yield a yellow precipitate that is insoluble in 6 N acetic acid but is soluble in 1 N sodium hydroxide.

**Lithium**

- **A.** With sodium carbonate TS, moderately concentrated solutions of lithium salts, made alkaline with sodium hydroxide, yield a white precipitate on boiling. The precipitate is soluble in ammonium chloride TS.
- **B.** Solutions of lithium salts are not precipitated by 2 N sulfuric acid or soluble sulfates (distinction from strontium).

**Magnesium**

- **A.** Solutions of magnesium salts in the presence of ammonium chloride yield NMT a slightly hazy precipitate when neutralized with ammonium carbonate TS, but on the subsequent addition of dibasic sodium phosphate TS, a white, crystalline precipitate, which is insoluble in 6 N ammonium hydroxide, is formed.

**Manganese**

- **A.** With ammonium sulfide TS, solutions of manganous salts yield a salmon-colored precipitate that dissolves in acetic acid.

**Mercury**

- **A.** When applied to bright copper foil, solutions of mercury salts, free from an excess of nitric acid, yield a deposit that, upon rubbing, becomes bright and silvery in appearance.
- **B.** With hydrogen sulfide, solutions of mercury compounds yield a black precipitate that is insoluble in ammonium sulfide TS and in boiling 2 N nitric acid.

**Mercuric salts**

- **A.** Solutions of mercuric salts yield a yellow precipitate with 1 N sodium hydroxide.
- **B.** Solutions of mercuric salts yield, in neutral solutions with potassium iodide TS, a scarlet precipitate that is very soluble in an excess of the reagent.

**Mercurous salts**

- **A.** Mercurous compounds are decomposed by 1 N sodium hydroxide, producing a black color.
- **B.** With hydrochloric acid, solutions of mercurous salts yield a white precipitate that is blackened by the addition of 6 N ammonium hydroxide.
- **C.** With potassium iodide TS, a yellow precipitate, which may become green upon standing, is formed.

**Nitrate**

- **A.** When a solution of a nitrate is mixed with an equal volume of sulfuric acid, the mixture is cooled, and a solution of ferrous sulfate is superimposed; a brown color is produced at the junction of the two liquids.
- **B.** When a nitrate is heated with sulfuric acid and metallic copper, brownish-red fumes evolve.
- **C.** Nitrates do not decolorize acidified potassium permanganate TS (distinction from nitrites).

**Nitrite**

- **A.** When treated with dilute mineral acids or with 6 N acetic acid, nitrites evolve brownish-red fumes. The solution colors starch–iodide paper blue.

**Oxalate**

- **A.** Neutral and alkaline solutions of oxalates yield a white precipitate with calcium chloride TS. This precipitate is insoluble in 6 N acetic acid but is dissolved by hydrochloric acid.
- **B.** Hot acidified solutions of oxalates decolorize potassium permanganate TS.

**Permanganate**

- **A.** Solutions of permanganates acidified with sulfuric acid are decolorized by hydrogen peroxide TS and by sodium bisulfite TS, in the cold, and by oxalic acid TS in a hot solution.

**Peroxide**

- **A.** Solutions of peroxides slightly acidified with sulfuric acid yield a deep blue color upon the addition of potassium dichromate TS. On shaking the mixture with an equal volume of ethyl ether and allowing the liquids to separate, the blue color is found in the ethyl ether layer.

**Phosphate**

[NOTE—Where the monograph specifies the identification test for *Phosphate*, use the tests for orthophosphates, unless the instructions specify the use of the pyrophosphate tests or indicate that the product is to be ignited before performing the test.]

**Orthophosphates**

- **A.** With silver nitrate TS, neutral solutions of orthophosphates yield a yellow precipitate that is soluble in 2 N nitric acid and in 6 N ammonium hydroxide.
- **B.** With ammonium molybdate TS, acidified solutions of orthophosphates yield a yellow precipitate that is soluble in 6 N ammonium hydroxide. This precipitate may be slow to form.

**Pyrophosphates**

- **A.** With silver nitrate TS, pyrophosphates obtained by ignition yield a white precipitate that is soluble in 2 N nitric acid and soluble in 6 N ammonium hydroxide.
- **B.** With ammonium molybdate TS, pyrophosphates obtained by ignition yield a yellow precipitate that is soluble in 6 N ammonium hydroxide.

**Potassium**

- **A.** In neutral, concentrated, or moderately concentrated solutions of potassium salts (depending upon the solubility and the potassium content), sodium bitartrate TS produces a white crystalline precipitate that is soluble in 6 N ammonium hydroxide and in solutions of alkali hydroxides and carbonates. The formation of the precipitate, which is usually slow, is accelerated by stirring or rubbing the inside of the test tube with a glass rod. The addition of a small amount of glacial acetic acid or alcohol also promotes the precipitation.

**Salicylate**

- **A.** In moderately concentrated solutions of salicylates, ferric chloride TS produces a violet color.
- **B.** Addition of acids to moderately concentrated solutions of salicylates produces a white, crystalline precipitate of salicylic acid that melts between 158° and 161°.

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**Silver**
- **A.** With hydrochloric acid, solutions of silver salts yield a white, curdy precipitate that is insoluble in nitric acid but is readily soluble in 6 N ammonium hydroxide.
- **B.** A solution of a silver salt, to which 6 N ammonium hydroxide and a small quantity of formaldehyde TS are added, deposits, upon warming, a mirror of metallic silver upon the sides of the container.

**Sodium**
- **A.** Unless otherwise specified in an individual monograph, prepare a solution to contain 0.1 g of the sodium compound in 2 mL of water. Add 2 mL of 15% potassium carbonate, and heat to boiling. No precipitate is formed. Add 4 mL of potassium pyroantimonate TS, and heat to boiling. Allow to cool in ice water and, if necessary, rub the inside of the test tube with a glass rod. A dense precipitate is formed.

**Sulfate**
- **A.** With barium chloride TS, solutions of sulfates yield a white precipitate that is insoluble in hydrochloric acid and in nitric acid.
- **B.** With lead acetate TS, neutral solutions of sulfates yield a white precipitate that is soluble in ammonium acetate TS.
- **C.** Hydrochloric acid produces no precipitate when added to solutions of sulfates (distinction from thiosulfates).

**Sulfite**
- **A.** When treated with 3 N hydrochloric acid, sulfites and bisulfites yield sulfur dioxide, which blackens filter paper moistened with mercurous nitrate TS.

**Tartrate**
- **A.** Dissolve a few mg of a tartrate salt in 2 drops of sodium metaperiodate solution (1 in 20). Add 1 drop of 1 N sulfuric acid and after 5 min add a few drops of sulfurous acid, followed by a few drops of fuchsin-sulfurous acid TS: a reddish-pink color is produced within 15 min.

**Thiocyanate**
- **A.** With ferric chloride TS, solutions of thiocyanates yield a red color that is not destroyed by moderately concentrated mineral acids.

**Thiosulfate**
- **A.** With hydrochloric acid, solutions of thiosulfates yield a white precipitate that soon turns yellow, and evolve sulfur dioxide, which blackens filter paper moistened with mercurous nitrate TS.
- **B.** The addition of ferric chloride TS to solutions of thiosulfates produces a dark violet color that disappears quickly.

**Zinc**
- **A.** In the presence of sodium acetate, solutions of zinc salts yield a white precipitate with hydrogen sulfide. This precipitate is insoluble in acetic acid but is dissolved by 3 N hydrochloric acid.
- **B.** Ammonium sulfide TS produces a similar precipitate in neutral and in alkaline solutions.
- **C.** With potassium ferrocyanide TS, zinc salts in solution yield a white precipitate that is insoluble in 3 N hydrochloric acid.

## INSTRUMENTAL IDENTIFICATION TESTS

Instrumental techniques described in this section may be used in lieu of procedures described in *Chemical Identification Tests*. Instrumental techniques provide flexibility in the choice of identification tests. All instrumental techniques shall follow method validation procedures for identification tests (see *Validation of Compendial Procedures* ⟨1225⟩, *Validation, Data Elements Required for Validation, Category IV*). *Instrumental Identification Tests* must demonstrate specificity. In addition, other suitable, validated instrumental techniques may be used.

The selection of the appropriate sample preparation depends on the material under test and must be appropriate for the technique being used. The analyst may use any of the following preparation procedures, with the appropriate verification. An electronic library spectrum of the Reference Standard may be used in comparison to the test sample provided adequate specificity is maintained. When using solvents, the solvent must be free of interfering species. Use *USP Reference Standards* where available (see *General Notices, 5.80 USP Reference Standards*).

### • Identification Using X-Ray Fluorescence Spectrometry

X-ray fluorescence spectrometry (XRF) can typically be used for identification of elements with atomic numbers from magnesium through uranium. The actual range of elements for which the instrument is capable is dependent on the instrument design. Guidance regarding the use of XRF may be found in *X-Ray Fluorescence Spectrometry* ⟨735⟩.

**Sample preparation**

**Powders/solids:** Powders and solids can be used without further manipulation, or material can be made into pellets as described in ⟨735⟩.

**Neat liquids:** Liquids can be used without further manipulation, provided the liquid is a single phase, is compatible with the sample holder, and has sufficiently low volatility.

**Samples in solution:** Samples may be dissolved in an appropriate solvent.

**Procedure**

**Blank:** For *Powders/solids* and *Neat liquids*, prepare a blank sample holder. For *Samples in solution*, use the solvent as a blank.

**Reference standard** and **Sample:** Prepare each *Reference standard* and *Sample* by using sufficient material as required for the specific manufacturer's instrumentation.

**Analysis:** Analyze the *Blank*, *Reference standard*, and *Sample* according to the manufacturer's suggestions for the specific instrument. If there is any interference, use the *Blank* to make the correction. The spectrum of the *Reference standard* exhibits radiation characteristic of the element(s) under investigation. The energy bands of the *Sample* exhibit radiation characteristic of the element(s) under investigation and compare qualitatively with that of the *Reference standard*.

### • Identification Using Atomic Spectroscopy Techniques: Atomic Absorption Spectroscopy, Inductively Coupled Plasma–Optical Emission Spectroscopy, Inductively Coupled Plasma–Mass Spectrometry

Atomic absorption (AA) spectroscopy techniques can be used for the identification of many elements. Guidance regarding the use of AA spectroscopy may be found in *Atomic Absorption Spectroscopy* ⟨852⟩ . Guidance regarding the use of

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inductively coupled plasma–optical emission spectroscopy (ICP–OES) or inductively coupled plasma–mass spectrometry (ICP–MS) may be found in *Plasma Spectrochemistry* ⟨730⟩.

**Sample preparation:** Samples should be dissolved in an appropriate solvent. If dissolving the sample is not possible, digestion may be necessary.

**Procedure**

**Blank:** Prepare an appropriate solution that does not contain the analyte(s) of interest and is compatible with the technique being used.

**Standard solution:** Standards must contain the analyte of interest. All solutions should be matrix-matched where possible.

**Sample solution:** Prepare the sample in the same solvent as the standard.

**Analysis:** Analyze the *Blank*, *Standard solution*, and *Sample solution* according to the manufacturer's suggestions for the specific instrument. When a wavelength-based technique is used, wherever possible select at least two wavelengths characteristic of the analyte(s) of interest. When a mass-based technique is used, wherever possible select at least two isotopes characteristic of the analyte(s) of interest. If two isotopes are unavailable, or if an element is monoisotopic, it may be possible to monitor the oxide of the analyte(s) ($m + 16$), if one is formed. If no oxide is formed, or if multiple isotopes cannot be evaluated because of interferences or because of a limited number of isotopes, then a different technique may be required. If interfering elements are present in the sample solution, wavelengths or masses examined must be selected to unequivocally identify the analyte(s) of interest.

- **Identification Using Ion Chromatography**

Ion chromatography (IC) can be used to identify a number of anions and cations found in drug substances (for additional information, see *Ion Chromatography* ⟨1065⟩).

**Apparatus:** Analyte detection will typically utilize suppressed conductivity, although other detection methods are possible, depending on the analyte (see ⟨1065⟩). The ion-exchange column must be capable of separating the analyte from any other ions of the same charge known to be in the sample at a concentration ≥5% of the analyte.

**Procedure**

**Blank:** Use the sample solvent as a blank.

**Standard** and **Sample solutions:** Dissolve or dilute the sample in water. Other solvents can be used if compatible with the IC column.

**Analysis:** Analyze an equal volume of the *Blank*, Reference Standard, and sample (according to the manufacturer's suggestions for the specific instrument and column dimensions). The counterion is identified if a peak in the sample has the same retention time as the peak in the Reference Standard, and there is not a peak of ≥5% of its size at the same retention time in the *Blank*.

- **Identification by Other Liquid Chromatography Techniques in Chromatography** ⟨621⟩

Some of the ions suitable for IC identification can also be identified by other forms of liquid chromatography. Sample and Reference Standard concentrations, as well as injection volumes, need to be adjusted, depending on the detection technique used. All high-concentration components in the test substance should be analyzed to judge whether there is interference with the analyte of interest.

- **Identification Using Raman Spectroscopy**

Raman spectroscopy can be used for identification of counterions. Guidance regarding the use of Raman spectroscopy may be found in *Raman Spectroscopy—Theory and Practice* ⟨1858⟩.

**Apparatus:** Prepare the spectrometer for operation according to the instrument instruction manual and the instrument manufacturer's recommendations. An instrument performance check and the quality of spectra collected should be evaluated at time of use or following manufacturer's instructions.

**Procedure**

**Reference standard** and **Sample:** All reference material and sample spectra should be collected using identical instrumental parameters. These instrumental parameters may be determined by the analyst based on the nature of the sample and the type of analysis and should be selected based on the quality of spectra needed. Use the appropriate sample container and/or sample holding apparatus, depending on the type of reference material and sample (powder, liquid, paste, film, or other) being analyzed. Transfer the sample or reference material into the appropriate sample container and/or holder, as needed, and acquire the spectrum for each.

**Analysis:** Qualitatively compare the Raman spectra obtained from the reference material and the *Sample*. The *Sample* complies with the identity test if the spectrum exhibits maxima only at the same wavelengths as that of a similar preparation of the corresponding USP Reference Standard, where available.

- **Identification Using Mid-Infrared Spectroscopy**

Mid-infrared spectroscopy techniques can be used for identification of counterions. Guidance regarding the use of mid-infrared spectroscopy may be found in *Mid-Infrared Spectroscopy* ⟨854⟩ and *Spectroscopic Identification Tests* ⟨197⟩. If the monograph contains an identification test by IR (e.g., ⟨197⟩) and a reference to ⟨191⟩, then mid-infrared cannot be used as an instrumental replacement for the chemical identification tests prescribed in this chapter.

**Procedure**

**Standard** and **Sample solutions:** Sample preparation may be conducted using any of the procedures described in ⟨854⟩ that are appropriate for the sample of concern. The standard preparation should be conducted in the same way but using the USP Reference Standard of the substance under test, where available.

**Analysis:** Record the spectra of the test specimen and the corresponding Reference Standard over the range from about 3800 to 650 $cm^{-1}$ (2.6–15 µm). The IR absorption spectrum of the preparation of the test specimen, previously dried under conditions specified for the corresponding Reference Standard, unless the Reference Standard is to be used without drying, exhibits maxima only at the same wavelengths as that of a similar preparation of the corresponding USP Reference Standard, where available.

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# ⟨193⟩ IDENTIFICATION—TETRACYCLINES

The following chromatographic procedures are provided to confirm the identity of Pharmacopeial drug substances that are of the tetracycline type, such as doxycycline, oxytetracycline, and tetracycline, and to confirm the identity of such compounds in their respective Pharmacopeial dosage forms. Two procedures are provided, one based on paper chromatography (*Method I*) and the other on thin-layer chromatography (*Method II*). *Method I* is to be used unless otherwise directed in the individual monograph.

## PROCEDURES

**Standard solution:** Unless otherwise directed in the individual monograph, dissolve the USP Reference Standard for the drug substance being identified in the same solvent and at the same concentration as for the *Test solution*.

**Test solution:** Prepare as directed in the individual monograph.

• **METHOD I**

**pH 3.5 Buffer:** Dissolve 13.4 g of anhydrous citric acid and 16.3 g of dibasic sodium phosphate in 1000 mL of water, and mix.

**Developing solvent:** On the day of use, mix 10 volumes of chloroform, 20 volumes of nitromethane, and 3 volumes of pyridine.

**Mixed test solution:** Mix equal volumes of the *Standard solution* and the *Test solution*.

**Chromatographic sheet:** Draw a spotting line 2.5 cm from one edge of a 20-cm × 20-cm sheet of filter paper (Whatman No. 1, or equivalent). Impregnate the sheet with *pH 3.5 Buffer* by passing it through a trough filled with *pH 3.5 Buffer*, and remove the excess solvent by firmly pressing the sheet between nonfluorescent blotting papers.

**Procedure:** To a suitable chromatographic chamber, prepared for ascending chromatography (see *Chromatography* ⟨621⟩) add *Developing solvent* to a depth of 0.6 cm. Apply at 1.5-cm intervals 2 µL each of the *Standard solution*, the *Test solution*, and the *Mixed test solution* to the spotting line of the *Chromatographic sheet*. Allow the sheet to dry partially, and while still damp place it in the chromatographic chamber with the bottom edge touching the *Developing solvent*. When the solvent front has risen about 10 cm, remove the sheet from the chamber, and expose the sheet to ammonia vapor. Examine the chromatogram under long-wavelength UV light. Record the positions of the major yellow fluorescent spots: the $R_F$ value of the principal spot obtained from the *Test solution* and from the *Mixed test solution* corresponds to that obtained from the *Standard solution*.

• **METHOD II**

**Resolution solution:** Unless otherwise directed in the individual monograph, prepare a solution in methanol containing 0.5 mg each of USP Chlortetracycline Hydrochloride RS, USP Doxycycline Hyclate RS, USP Oxytetracycline RS, and USP Tetracycline Hydrochloride RS per mL.

**Developing solvent:** Prepare a mixture of 0.5 M oxalic acid, previously adjusted with ammonium hydroxide to a pH of 2.0, acetonitrile, and methanol (80:20:20).

**Chromatographic plate:** Use a suitable thin-layer chromatographic plate (see *Thin-Layer Chromatography* under *Chromatography* ⟨621⟩) coated with a 0.25-mm layer of octylsilanized chromatographic silica gel mixture. Activate the plate by heating it at 130° for 20 minutes, allow to cool, and use while still warm.

**Procedure:** Separately apply 1 µL each of the *Standard solution*, the *Test solution*, and the *Resolution solution* to the *Chromatographic plate*. Allow the spots to dry, and develop the chromatogram in the *Developing solvent* until the solvent front has moved about three-fourths of the length of the plate. Remove the plate from the developing chamber, mark the solvent front, and allow to air-dry. Expose the plate to ammonia vapors for 5 minutes, and promptly locate the spots on the plate by viewing under long-wavelength UV light: the chromatogram of the *Resolution solution* shows clearly separated spots, and the principal spot obtained from the *Test solution* corresponds in $R_F$ value, intensity, and appearance to that obtained from the *Standard solution*.

## ADDITIONAL REQUIREMENTS

**USP Reference Standards** ⟨11⟩

USP Doxycycline Hyclate RS
USP Oxytetracycline RS
USP Tetracycline Hydrochloride RS

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# ⟨197⟩ SPECTROSCOPIC IDENTIFICATION TESTS

***Change to read:***

## INTRODUCTION AND SCOPE

This chapter provides several tests and procedures that are used to confirm the chemical identity of a specific material in its respective monograph. When one of the tests listed in this chapter is referenced in a monograph, it must be used to confirm the identity of the material. Alternative identification procedures may be used, provided they are demonstrated to be equivalent to or better than the specified procedure and if they meet the requirements specified in the *Equivalent/Alternative Tests* section.

Spectroscopic tests contribute meaningfully toward the identification of many official articles. The test procedures that follow are applicable to substances that absorb, transmit, reflect, or scatter electromagnetic radiation in the near-infrared (NIR), infrared (IR), visible or near-infrared (Raman), ultraviolet (UV), visible (Vis), or X-ray ranges (see *Mid-Infrared Spectroscopy* ⟨854⟩, ▲*Near-Infrared Spectroscopy* ⟨856⟩,▲ (ERR 1-Sep-2021)*Ultraviolet-Visible Spectroscopy* ⟨857⟩, ▲▲ (ERR 1-Sep-2021)*Raman Spectroscopy* ⟨858⟩, and *X-Ray Powder Diffraction* ⟨941⟩). The NIR, IR, and Raman spectra, or X-ray diffraction pattern of a substance, compared with the spectrum or diffraction pattern obtained with the corresponding USP Reference Standard, provides perhaps the most conclusive evidence of the identity of the substance that can be realized from any single test. The UV or Vis absorption spectrum of a substance, on the other hand, does not exhibit a high degree of specificity in most cases. To provide unambiguous confirmation of the identity of a substance, it may be necessary to execute two (or more) identity tests, as specified in a large proportion of compendial monographs.

## IDENTIFICATION METHODOLOGY

Suitable identification methodology must be used for the chemical identification of materials through comparison with the appropriate compendial standards. Where alternative techniques are used, it must be demonstrated that the alternative identification methodology is suitable for the intended application (see *Validation of Compendial Procedures* ⟨1225⟩). Identification procedures should be able to discriminate between materials similar in molecular structure. The lack of specificity of a single technique may be compensated by other supporting analytical procedure(s) or an application of an additional identification technique.

The IR spectrum of a substance, compared with that obtained using equivalent instruments and conditions for the corresponding USP Reference Standard, is the most widely used methodology for chemical identification in compendial monographs. In general practice, the analysis of the sample and the USP Reference Standard are completed at the same time; however, if the USP Reference Standard spectrum was obtained previously using equivalent instruments and conditions, it is appropriate to compare the sample spectrum with the stored USP Reference Standard spectrum.

Under conditions where the IR spectrum lacks specificity for definitive chemical identification, additional spectroscopic information can be used to supplement chemical identification. For example, conformance with both IR and UV test specifications, as specified in a large proportion of compendial monographs, provides complementary information for the definitive identity of the sample under examination. In these instances, the combined spectroscopic information enables discrimination between compounds similar in structure that would not be possible from either IR spectrum or UV spectrum alone.

For preparing the standards used in the applications of this chapter, unless the directions for preparing the USP Reference Standard are explicitly specified in the monograph procedure, the USP Reference Standard must be used in accordance with instructions on its label. For preparation of the test sample, the sample must be prepared in accordance with the directions in the individual monograph procedure. Where no specific instructions are provided for sample preparation, handle the sample in the same manner as described by the USP Reference Standard label. For example, dry the sample as per drying conditions on the label of the corresponding USP Reference Standard.

## INFRARED SPECTROSCOPY

Several methods are indicated for the preparation of test samples and USP Reference Standards for analysis by infrared spectroscopy (see *Table 1* and ⟨854⟩). The approaches for the techniques used in IR identity testing are summarized in *Table 1*.

**Table 1. Infrared Spectroscopy Sample Preparation Techniques[a]**

| Method Reference | Sample Preparation |
|---|---|
| ⟨197A⟩ | The substance under examination is intimately in contact with an internal reflection element for attenuated total reflection (ATR) analysis. |
| ⟨197D⟩ | The substance under examination is mixed intimately with potassium bromide and transferred to a sample container for diffuse reflection (DR) analysis. |
| ⟨197E⟩ | The substance under examination is pressed as a thin sample against a suitable plate for IR microscopic analysis. |
| ⟨197F⟩ | The substance under examination is a thin film of a neat liquid or semisolid between suitable (e.g., sodium chloride or potassium bromide) plates, or a thin microcrystalline or glassy film deposited from a solution or cooled from a melt. |
| ⟨197K⟩ | The substance under examination is mixed intimately with potassium bromide and compressed into a transparent pellet. |

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**Table 1. Infrared Spectroscopy Sample Preparation Techniques[a]** *(continued)*

| Method Reference | Sample Preparation |
|---|---|
| ⟨197M⟩ | The substance under examination (10–20 mg) is finely ground and dispersed in a drop of mulling agent (unless otherwise directed in the monograph, mineral oil is to be used). |
| ⟨197S⟩ | A solution of designated concentration is prepared in the solvent specified in the individual monograph. The solution is examined in 0.1-mm cells, unless a different cell path length is specified. |

[a] Each of the techniques in the table can be used as alternative methods when any other technique from the table is required in the monograph (see *General Notices, 6.30 Alternative and Harmonized Methods and Procedures*).

In each instance, infrared spectra of both the sample and corresponding USP Reference Standard are obtained using the same sample preparation technique and measurement parameters. Record and compare the spectra of the sample and the corresponding USP Reference Standard over the range from 3800 to 650 $cm^{-1}$, unless otherwise specified in the individual monograph. The comparison must establish that the IR spectrum of the preparation of the sample exhibits maxima only at the same wavenumbers as that of the appropriately prepared corresponding USP Reference Standard. If there are differences between the spectra, and the sample spectrum was compared with a previously obtained and electronically stored spectrum of the USP Reference Standard, the comparison must be repeated concomitantly with a freshly prepared USP Reference Standard.

Differences between the USP Reference Standard spectrum and sample spectrum that may be observed are sometimes attributable to differences in the solid-state form of the materials, if a solid-state technique is used (e.g., ⟨197A⟩, ⟨197K⟩, or ⟨197M⟩). If a specific crystal form is not specified in the monograph, where spectral differences between the sample and USP Reference Standard are observed, recrystallize both the sample and USP Reference Standard under identical conditions to produce the same solid-state form, unless specific procedures are provided in the individual monographs. Dissolve equal portions of the sample and the USP Reference Standard in equal volumes of a suitable solvent, evaporate the solutions to dryness in similar containers under identical conditions, and repeat the identification test on the residues. Other techniques for recrystallizing the sample and USP Reference Standard based on known scientific principles may be used with appropriate scientific justification.

***Change to read:***

## NEAR-INFRARED AND RAMAN SPECTROSCOPY

The reference ⟨197NIR⟩ in a monograph signifies that a test sample and a standard sample are examined by the NIR spectroscopic technique (see ▲⟨856⟩▲ (ERR 1-Sep-2021)), and the reference ⟨197R⟩ in a monograph signifies that a test sample and a standard sample are examined by the Raman spectroscopic technique (see ⟨858⟩). NIR and Raman spectroscopic procedures may be used to confirm the identity of materials. The approach is similar to that of IR identity testing but is often augmented through the use of spectral libraries and chemometrics. A sample can thus be analyzed by NIR or Raman techniques, with the resulting spectrum compared with stored spectra in the spectral library through the use of multivariate analysis. In general, a visual comparison or simple overlay of the spectra alone may not be sufficient and additional evaluation may be needed.

In both techniques, samples can be directly interrogated with minimal or no sample preparation. Measurement is nondestructive and noninvasive, and data collection can often be made through glass or plastic containers.

A description of these techniques, and the strategy for procedure and chemometrics model development and validation can be found in the appropriate associated chapters when available (see *Chemometrics* ⟨1039⟩, *Near-Infrared Spectroscopy—Theory and Practice* ⟨1856⟩, and *Raman Spectroscopy—Theory and Practice* ⟨1858⟩▲▲ (ERR 1-Sep-2021)).

## ULTRAVIOLET-VISIBLE SPECTROSCOPY

The reference ⟨197U⟩ in a monograph signifies that a sample solution and a Standard solution are examined spectroscopically, in 1-cm cells, over the spectral range from 200 to 400 nm, unless otherwise specified in the individual monograph (see ⟨857⟩). Dissolve a portion of the substance under examination in the designated medium to obtain a sample solution having the concentration specified in the monograph. Record and compare the spectra obtained for the sample solution and the Standard solution. Review or calculate the absorptivities and/or absorbance ratios and compare the results, and where appropriate, compare to criteria specified in an individual monograph.

The comparison must establish that the UV spectrum of the preparation of the sample exhibits absorption maxima and minima only at the same wavelengths as those of the appropriately prepared corresponding USP Reference Standard, and that the absorptivities and/or absorbance ratios are within the specified limits. If there are differences in the spectra, and the sample spectrum was compared with a previously obtained and electronically stored spectrum of the USP Reference Standard, the comparison must be concomitantly repeated with a freshly prepared USP Reference Standard.

Unless otherwise specified in the monograph, absorbances indicated for the calculations of the absorptivities and/or absorbance ratios are those measured at the maximum absorbance wavelength (within ±2 nm) specified in the individual monograph. Where the absorbance is to be measured at about the specified wavelength other than that of maximum absorbance, the abbreviations for minimum (min) and shoulder (sh) are used, respectively, in an absorption spectrum.

The reference ⟨197U-LC⟩ in a monograph signifies that, when a diode array detector is used in tandem with an LC procedure test in the monograph, the spectra of the major chromatographic peak(s) of the sample solution and Standard solution are examined spectroscopically.

The requirements are met if the UV spectra of the sample solution and of the Standard solution exhibit maxima and minima at the same wavelengths, and, if applicable, the absorptivities and/or absorbance ratios are within specified limits.

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## X-RAY POWDER DIFFRACTION

The reference ⟨197XR⟩ in a monograph signifies that a test sample and a standard sample are examined according to ⟨941⟩.

Prepare and mount the specimen as directed in ⟨941⟩. Unless otherwise indicated in the monograph, record the diffraction pattern in a 2θ-range from as near to 0° as possible to at least 32°. Unless otherwise specified in the monograph, the requirements are met if the X-ray diffraction pattern of the test specimen conforms to that of the corresponding USP Reference Standard obtained using equivalent instruments and conditions. Differences in the diffraction line intensities (but not line positions) between the sample and the Standard are acceptable.

The comparison must establish that the diffraction pattern of the preparation of the test specimen conforms to the diffraction pattern of the corresponding USP Reference Standard. If there are differences in the diffraction pattern and the sample diffraction pattern was compared with a previously obtained and electronically stored diffraction pattern of the USP Reference Standard, the comparison must be repeated concomitantly with a freshly prepared USP Reference Standard.

***Change to read:***

## EQUIVALENT/ALTERNATIVE TESTS

In addition to IR, NIR, Raman, X-ray, and UV absorption, several other spectroscopic methodologies can be utilized for the identification of the specimen under examination. The methods cited in this chapter may be used for identification of materials as an alternative method to the method referenced in the monograph, provided that the alternative technique has been determined to be suitable for identification. Suitable identification tests must be able to discriminate between compounds similar in molecular structure that are likely to be present. The choice of such potentially interfering materials must be based on sound scientific judgment, with consideration of interferences that could occur. It is not always possible to demonstrate that an analytical procedure is specific for a particular analyte (complete discrimination).

For information regarding sample preparation and measurement parameters associated with an alternative identification method, refer to the appropriate general chapter (see *Mass Spectrometry* ⟨736⟩, *Nuclear Magnetic Resonance Spectroscopy* ⟨761⟩, ⟨854⟩, ▲ ⟨856⟩,▲ (ERR 1-Sep-2021) ⟨857⟩, ▲▲ (ERR 1-Sep-2021) ⟨858⟩, and ⟨941⟩).

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***Add the following:***

# ▲⟨198⟩ NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY IDENTITY TESTING OF BACTERIAL POLYSACCHARIDES USED IN VACCINE MANUFACTURE

## 1. INTRODUCTION AND SCOPE

This chapter describes the application of nuclear magnetic resonance (NMR) spectroscopy to the identity testing of bacterial polysaccharides used in vaccine manufacture. The identity of the saccharide component in polysaccharide and glycoconjugate vaccines should be confirmed at various stages of the manufacturing process, including bulk monovalent polysaccharide, blended polysaccharide bulk, activated polysaccharide (if isolated), bulk monovalent conjugate, blended conjugate bulks, and final fills. NMR is an appropriate method to confirm the identity of polysaccharides, although it is most useful for bulk monovalent polysaccharides and activated polysaccharides (if isolated). This chapter describes the use of NMR spectroscopy for this purpose, but alternative validated approaches may also be suitable.

For additional information on NMR, including its general principles and applications, see *Applications of Nuclear Magnetic Resonance Spectroscopy* ⟨1761⟩. More specific information on instrument qualification, procedure validation, and reference standards are included in *Nuclear Magnetic Resonance Spectroscopy* ⟨761⟩.

### 1.1 NMR Spectra

NMR spectra for identity testing are typically collected for samples dissolved in deuterated water ($D_2O$) or a combination of deuterated water and small amounts of one or more internal standards for chemical shift calibration, quantification, or other purposes. The chemical shift of the resonance from residual water in deuterated solvent (HOD) is more temperature sensitive than other solvent resonances. The spectral separation and resolution of the HOD resonance may be optimized by the choice of temperature at which the spectrum is obtained.

### 1.2 *O*-Acetylated Polysaccharides

For those polysaccharides that are *O*-acetylated, the consistency of the degree of *O*-acetylation is considered part of the identity test. This could be evaluated through the use of the spectrum of a reference polysaccharide with an acceptable degree of *O*-acetylation, quantification of resonances characteristic of the *O*-acetylation in the spectrum of the intact polysaccharide, or chemical de-*O*-acetylation and relative quantification of the resulting acetate anion compared to the polysaccharide (see *2.4 Experimental Procedures*). The implementation of any given approach is likely to be product specific.

For some polysaccharides, a quantitative specification for the minimum degree of *O*-acetylation may exist; the approaches described in this chapter, appropriately validated, can be used to perform that assay. Requirements for the *O*-acetyl content of polysaccharides are typically quoted as a minimum quantity of "*O*-acetyl residues" (measured by a Hestrin assay) in millimoles per gram of dry weight of polysaccharide. This NMR assay method described in this chapter reports a value based on the average number of *O*-acetyl groups per repeat unit. A method to calibrate between the two approaches should be developed during method validation.

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## 2. PROCEDURE

### 2.1 Equipment Requirements

A description of a typical NMR spectrometer is given in ⟨1761⟩, and approaches to installation, performance, and operational qualification are given in ⟨761⟩. This section, and the rest of the chapter, refers to specific requirements for the identity testing of bacterial polysaccharides.

NMR SPECTROMETER

An NMR spectrometer with a minimal nominal field strength corresponding to a proton resonance frequency of NLT 400 MHz and with control of the sample temperature should be used. Temperature calibration within ±3° of the desired temperature is sufficient, provided a consistent sample temperature between the test and reference samples is achieved (or demonstrated in method validation), with temperature stability within a run of NMT 0.5°. NMR probes optimized for proton detection and suitable for 5-mm (outside diameter) NMR tubes should be used. Other probe options are possible but should be proven suitable before use.

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PROCESSING PARAMETERS

A weighting function may be applied to the free induction decay (FID) before Fourier transform: a suitable line broadening function may be applied, and in some cases 0.3 Hz has been found suitable. After transform of the FID, the phase is adjusted to pure ▲absorption▲ (ERR 1-Jul-2018) phase. Baseline correction should be applied consistently.

NMR TUBES

These should be of a quality suitable for use in high-field spectrometers and qualified for use in the assay.

## 2.2 Reagents for Vaccine Polysaccharide Sample Solutions

SOLVENTS

Deuterated solvents subject to proton exchange with the polysaccharide [e.g., deuterated water ($D_2O$)] must be selected with the highest deuterium proportion (e.g., >99.9 atom % D).

CHEMICAL SHIFT REFERENCE COMPOUNDS

Sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS; preferred), sodium trimethylsilylpropionate (TSP), or a deuterated analogue (TSP–$d_4$) are classically used and set to 0 ppm for the methyl signals. The reference material is usually added at low amounts [0.1%–0.01% (w/v) has been found to be appropriate] to the deuterated water used to dissolve the final sample.
[NOTE—Deuterated water >99.96% $^2H$ containing 0.01% DSS (w/v) is commercially available.]

SODIUM DEUTEROXIDE

Forty percent w/v (10 M) sodium deuteroxide (NaOD) in deuterated water (>99 atom % D) is commonly used to induce the de-*O*-acetylation of polysaccharides.

## 2.3 Sample Requirements

An aliquot of bulk purified polysaccharide or appropriate process intermediates in solid or liquid state containing 0.5–20 mg of saccharide content is suitable for preparation of NMR analytical samples. To obtain a solid aliquot, the relevant amount of material in liquid solution is dried under vacuum (using a freeze-drier, lyophilizer, or other solvent evaporator).

REFERENCE SPECTRA FOR ANTICIPATED IMPURITIES

Reference spectra for anticipated process-related impurities (e.g., ethanol, antifoaming agents, phenol, cetyl trimethylammonium bromide) should be obtained under the same experimental conditions as those used to analyze the test sample and at concentrations found similar to those in routine test samples. These spectra provide evidence for those resonances that may be excluded from further consideration when assigning the identity of the samples.

REFERENCE SPECTRA FOR POLYSACCHARIDES

Reference spectra for the polysaccharides samples being tested should be obtained by collecting spectra of authentic samples under identical sample preparation and spectrometer acquisition and data processing conditions to those used for test samples. Comparison of the spectra of test samples with these reference spectra allows the identity of the test samples to be confirmed.

## 2.4 Experimental Procedures

Two experimental protocols are described below. The first protocol is suitable for samples in which a single one-dimensional $^1H$ NMR spectrum will produce all of the required information. The second protocol includes in situ base-catalyzed de-*O*-acetylation of the polysaccharide to release *O*-acetyl groups as acetate anions, both simplifying the spectrum of the polysaccharide and allowing quantification of the degree of *O*-acetylation. A second one-dimensional $^1H$ NMR spectrum is collected after the de-*O*-acetylation step. The choice of which protocol to adopt will depend upon the product being tested and the information required; many stages in these two protocols are similar (e.g., sample preparation).

SAMPLE PREPARATION

NMR analytical samples of polysaccharide and its derivatives can be prepared as either solid- or liquid-state aliquots. NMR analytical samples are usually prepared by dissolving the solid aliquot in about 0.7 mL of deuterated solvent or, more rarely, by adding at least 10% (v/v) of deuterated solvent to the liquid aliquot. The solution is mixed to obtain a uniform concentration and is subsequently transferred to a 5-mm NMR tube.
[NOTE—NMR instrument design may allow or require the use of smaller volumes; users should confirm this for their instrument configuration.]

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DEUTERIUM EXCHANGE

With suitable method validation, lyophilization from deuterium oxide ("deuterium exchange") may be used to reduce the intensity of the resonance from residual water ($H_2O$)/HOD.

pH

Under some circumstances, control of the pH of the sample may be appropriate.

## 2.5 Procedure 1a

This procedure uses visual comparison with a reference spectra and/or comparison of test chemical shifts with reference chemical shift values.

SCOPE

This approach is compatible with polysaccharides that lack *O*-acetylation, such as *Haemophilus influenzae* type b or many pneumococcal polysaccharides, or with *O*-acetylated polysaccharides, such as *Neisseria meningitidis* or many pneumococcal polysaccharides where the product and spectral consistency allow identity to be established through direct comparison of test and reference spectra.

NMR SPECTRUM OF THE TEST SAMPLE

The NMR spectrum of the test sample is acquired under the same instrument operational and data processing parameters to those used to obtain the reference spectrum. Confirmation of identity can be based on visual comparison of the test and product-specific reference spectra, comparing resonance position, line width, relative intensity, and multiplicity. The complete spectrum, distinctive regions of the spectrum, or a series of at least five resonances (or three distinctive resonances in polysaccharides with monosaccharide or disaccharide repeat units) can be used in this comparison (as long as these are shown to be diagnostic).

RESONANCES

In a well-understood assay, resonances assigned as deriving from process-related impurities that are known to be removed at a later stage in the manufacturing process or otherwise proven not to impact product quality can be excluded from determination of identity.

## 2.6 Procedure 1b

This procedure uses analytical samples in deuterated water ($D_2O$) and a comparison with a reference spectrum by calculation of a correlation coefficient.

SCOPE

This approach is compatible with *Streptococcus pneumoniae* polysaccharides where the consistency of the product and the spectrum allow identity to be established through direct mathematical comparison of test and reference spectra.

With mathematical comparisons, a distinctive region of each spectrum (e.g., 4.64–5.89 ppm) is compared to spectra generated for designated reference samples for each polysaccharide of interest. The similarity of two spectral profiles for a test and reference sample acquired under identical conditions and in the same matrix is evaluated using a correlation coefficient ($\rho$). A positive identification of the sample polysaccharide is achieved when $\rho$ is NLT 0.95 (or another validated value) between the sample and reference spectra.

## 2.7 Procedure 2

The procedure is in-tube base-catalyzed de-*O*-acetylation and quantification of the acetate content.

SCOPE

In some cases, the degree of *O*-acetylation and their position, accompanied by spontaneous *O*-acetyl migration in solution, can complicate the NMR profile. Base-catalyzed de-*O*-acetylation of the polysaccharide in the NMR tube has proven useful for the *Salmonella typhi* Vi and the meningococcal Groups A, C, W135, and Y polysaccharides. Adding a base may also reduce line widths.

SAMPLE HANDLING

The sample is prepared as in *Sample Preparation* in *2.4 Experimental Procedures* and a one-dimensional $^1$H NMR spectrum is acquired as in *2.5 Procedure 1a*. This spectrum provides information on the presence/amount of acetate anion in the native sample. Sodium deuteroxide (NaOD) in deuterated water ($D_2O$) is added to the sample in the NMR tube to a final concentration of approximately 200 mM [equivalent to adding 14 µL of 10 M sodium deuteroxide (NaOD) in deuterated water ($D_2O$) to a

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0.7-mL sample]. De-*O*-acetylation is very rapid for almost all polysaccharides, but appropriate conditions should be evaluated in method development and validation. The NMR spectrum of the sample is reacquired after de-*O*-acetylation using acquisition conditions appropriate for quantitative spectroscopy. The spectrum of the de-*O*-acetylated material is characteristic of the polysaccharide backbone, and the degree of *O*-acetylation in the original sample is calculated from the integrals of the acetate anion and an appropriate resonance arising from the saccharide backbone. If the amount of acetate anion in the native sample is low, for example <5%, correction of the intensity of the acetate anion resonance in the de-*O*-acetylated sample is not necessary.

## 2.8 Assay Criteria

### TEMPERATURE

The temperature of the sample should be the same, within limits established during method validation, as that of the reference standard, and the sample temperature can be determined by the chemical shift difference between the HOD resonance and the internal chemical shift reference standard. For example, when using TSP-$d_4$ as the reference standard, calculate the actual temperature using the following equation based on the chemical shift of the HOD signal:

$$\delta = 5.051 - 0.0111(T)$$

where $\delta$ is the chemical shift of the HOD peak, and $T$ is the temperature in Celsius (°).

Other equations apply when different chemical shift reference standards are used. Consistency in the chemical shift difference between the HOD and chemical shift reference standard is a surrogate for sample temperature.

### CHEMICAL SHIFT REFERENCE STANDARD

The full width half-height (*fwhh*) line width of a defined resonance from a small molecule (e.g., the chemical shift reference standard) in the test sample should be within limits set during method validation to indicate both acceptable shimming of the magnet and sample temperature stability during the analysis. The signal-to-noise ratio of a defined signal within the spectrum of the polysaccharide material should exceed the specification established during method validation.

## 2.9 System Suitability

### SYSTEM SUITABILITY SOLUTION

Dissolve 1 vial of USP PS NMR System Suitability RS in deuterated water (>99.9% D, 1 mL) containing 0.01% DSS with brief mixing.

[NOTE—For example, for 10 s using a rotary mixer.]

Transfer 0.7 mL of this solution into an NMR tube (5-mm outside diameter).

[NOTE—NMR instrument design may allow or require the use of smaller volumes; users should confirm this for their instrument configuration.]

### SYSTEM SUITABILITY: PROCEDURE

Using an NMR spectrometer operating at NLT 400 MHz nominal frequency for $^1$H, acquire an FID using NLT 64 scans with a 90° pulse, a spectral width of 12 ppm, a recycle delay of NLT 30 s, a number of data points NLT 64,000, and with the spectral window centered at 4 ppm. Record the $^1$H NMR spectra of the *System suitability solution* at a stable temperature of 25 ± 3°. Obtain the spectrum using the above parameters. Transform the data after exponential multiplication (0.3 Hz line broadening). The DSS methyl signal should be set to 0.00 ppm.

### ACCEPTANCE CRITERIA

- The chemical shifts for the *N*-acetyl, GlcA H-2, and GlcNAc H-1 anomeric resonances in the *System suitability solution* should be observed at 2.01 ± 0.05, 3.33 ± 0.05, and 5.15 ± 0.05 ppm, respectively.
- The line width (*fwhh*) of the *N*-acetyl resonance at 2.01 ppm should be NMT 3.5 Hz.

## 2.10 USP Reference Standards ⟨11⟩

USP PS NMR System Suitability RS

▲ 1S (USP41)

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# ⟨201⟩ THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST

## PROCEDURES

- **General Procedure**

The following procedure is applicable as an aid in verifying the identities of many compendial drug substances as such and in their respective dosage forms.

Prepare a test solution as directed in the individual monograph. On a line parallel to and about 2 cm from the edge of a suitable thin-layer chromatographic plate, coated with a 0.25-mm layer of chromatographic silica gel mixture (see *Chromatography* ⟨621⟩) apply 10 µL of this solution and 10 µL of a *Standard solution* prepared from the USP Reference Standard for the drug substance being identified, in the same solvent and at the same concentration as the test solution, unless otherwise directed in the individual monograph. Allow the spots to dry, and develop the chromatogram in a solvent system consisting of a mixture of chloroform, methanol, and water (180:15:1), unless otherwise directed in the individual monograph, until the solvent front has moved about three-fourths of the length of the plate. Remove the plate from the developing chamber, mark the solvent front, and allow the solvent to evaporate. Unless otherwise directed in the individual monograph, locate the spots on the plate by examination under short-wavelength UV light. The $R_F$ value of the principal spot obtained from the test solution corresponds to that obtained from the Standard solution.

- **Procedure for Bacitracin, Neomycin, and Polymyxin B**

The following thin-layer chromatographic procedure is applicable as an aid in verifying the identities of bacitracin, neomycin, and polymyxin B active ingredients and in dosage forms when present singly and in two- and three-component mixtures. The reference ⟨201BNP⟩ in a monograph signifies that this procedure is intended.

Prepare a *Test solution* as follows, unless otherwise directed in the individual monograph.

**Test solution**

**For drug substances:** Dissolve a portion of Bacitracin, Bacitracin Zinc, Neomycin Sulfate, or Polymyxin B Sulfate in 0.1 N hydrochloric acid to obtain a solution containing about 500 USP Bacitracin Units per mL, 3.5 mg of neomycin (base) per mL, or 10,000 USP Polymyxin B Units per mL.

**For solutions:** Where the solution contains neomycin and polymyxin B, dilute a portion of it with 0.1 N hydrochloric acid to obtain a solution containing the equivalent of about 3.5 mg of neomycin (base) per mL. Where the Solution contains polymyxin B but not neomycin, dilute a portion of it with 0.1 N hydrochloric acid to obtain a solution containing about 10,000 USP Polymyxin B Units per mL.

**For creams, lotions, and ointments:** Where the Cream, Lotion, or Ointment contains Bacitracin or Bacitracin Zinc, transfer a portion of it equivalent to about 500 USP Bacitracin Units, to a 15-mL centrifuge tube. Where the Cream, Lotion, or Ointment contains neomycin, but not Bacitracin or Bacitracin Zinc, transfer a portion of it equivalent to about 3.5 mg of neomycin (base) per mL to a 15-mL centrifuge tube. Add 4 mL of chloroform to the centrifuge tube, and shake well to disperse the Cream, Lotion, or Ointment. Add 1 mL of 0.1 N hydrochloric acid, vortex for 4 minutes, centrifuge, and use the clear supernatant.

[Note—The *Modified test solution* as described below in the *Modified procedure* may be used in lieu of the *Test solution*.]

**Standard bacitracin solution:** Dissolve a portion of USP Bacitracin Zinc RS in 0.1 N hydrochloric acid to obtain a solution containing 500 USP Bacitracin Units per mL.

**Standard neomycin solution:** Dissolve a portion of USP Neomycin Sulfate RS in 0.1 N hydrochloric acid to obtain a solution containing the equivalent of 3.5 mg of neomycin (base) per mL.

**Standard polymyxin B solution:** Dissolve a portion of USP Polymyxin B Sulfate RS in 0.1 N hydrochloric acid to obtain a solution containing 10,000 USP Polymyxin B Units per mL. Where the article under test also contains Bacitracin or Bacitracin Zinc, dissolve a portion of USP Polymyxin B Sulfate RS in 0.1 N hydrochloric acid to obtain a solution containing 500*J* USP Polymyxin B Units per mL, *J* being the ratio of the labeled amount of USP Polymyxin B Units to the labeled amount of USP Bacitracin Units in each g of Cream, Lotion, or Ointment.

**Developing solvent solution:** Prepare a mixture of methanol, isopropyl alcohol, methylene chloride, ammonium hydroxide, and water (4: 2: 2: 2: 1.5).

**Procedure:** Apply 10 µL of the *Test solution* and each of the relevant *Standard solutions* to a suitable thin-layer chromatographic plate (see *Chromatography* ⟨621⟩) coated with a 0.25-mm layer of chromatographic silica gel. Place the plate in a presaturated chromatographic chamber, and develop the chromatogram with the *Developing solvent system* until the solvent front has moved about three-fourths of the length of the plate. Remove the plate from the chamber, and dry at 105° for 10 minutes. Spray the plate with a 0.2% solution of ninhydrin in butyl alcohol, and heat at 105° for 5 minutes. The $R_F$ value of each principal spot in the chromatogram of the *Test solution* corresponds to that of the principal spot in the chromatogram obtained from each relevant *Standard solution* as appropriate for the labeled active ingredient or ingredients specified on the label. If the chromatogram of the *Test solution* yields excessive streaking, proceed as directed for *Modified procedure*.

**Modified procedure:** Transfer the *Test solution* to a 15-mL centrifuge tube, add 10 mL of saturated aqueous picric acid solution (1.2%, w/v), vortex for 1 minute, centrifuge for 10 minutes, and discard the supernatant. Wash the residue with 1-mL portions of water until no yellow color is observed in the washing. Discard the washings, and dry the residue under a stream of nitrogen at 50°. Dissolve the residue in 1 mL of acetone, add 1 mL of a freshly prepared solution of sulfuric acid in acetone (1 in 100), shake, centrifuge for 5 minutes, and discard the supernatant. Rinse the residue with 1 mL of acetone, centrifuge briefly, and discard the washing. Repeat the washing until no yellow color is observed. Dry the residue under a stream of nitrogen at 50°. Dissolve the residue in 0.5 mL of 0.1 N hydrochloric acid (*Modified test solution*). Repeat the *Procedure* using this *Modified test solution* instead of the *Test solution*. The $R_F$ value of each principal spot in the chromatogram of the *Modified test solution* corresponds to that of the principal spot in the chromatogram obtained from each relevant *Standard solution* as appropriate for the active ingredient or ingredients specified on the label.

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**USP Reference Standards** ⟨11⟩

USP Bacitracin Zinc RS
USP Neomycin Sulfate RS
USP Polymyxin B Sulfate RS

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# ⟨202⟩ IDENTIFICATION OF FIXED OILS BY THIN-LAYER CHROMATOGRAPHY

## INTRODUCTION

The following procedure for the USP *Identification* test is used to identify fixed oils using high-performance thin-layer chromatography (HPTLC), with a suitable octadecylsilyl silica gel as the coating substance.

## IDENTIFICATION

• **METHOD I**

**Mobile phase 1:** Ethyl ether

**Mobile phase 2:** Methylene chloride, glacial acetic acid, and acetone (20:40:50)

**System suitability solution 1:** Dissolve about 20 mg (1 drop) of USP Corn Oil RS in 3 mL of methylene chloride.

**System suitability solution 2:** Dissolve about 20 mg (1 drop) of USP Olive Oil RS in 3 mL of methylene chloride.

**Standard solution:** Dissolve about 20 mg (1 drop) of the appropriate USP Reference Standard in 3 mL of methylene chloride.

**Sample solution:** Dissolve about 20 mg (1 drop) of a fixed oil sample in 3 mL of methylene chloride.

**Chromatographic system**

(See *Chromatography* ⟨621⟩*, Thin-Layer Chromatography*.)

**Mode:** HPTLC

**Plate:** 20 cm × 10 cm, silica gel 60 RP-18, 0.15–0.2 mm layer, 4–8 µm particle size[1]

**Application volume:** 1 µL, manually spot

**Spray reagent:** 100 mg/mL of phosphomolybdic acid in 96% alcohol

**System suitability**

**Samples:** *System suitability solution 1* and *System suitability solution 2*

**Suitability requirements**

**Resolution:** The four principal spots from corn oil are clearly identified and separated, and the two principal spots from olive oil are clearly identified and separated.

[NOTE—Retardation factors ($R_F$) are provided for informational purposes only to aid in spot identification. The $R_F$ values for the four principal spots for USP Corn Oil RS are 0.39, 0.45, 0.51, and 0.56, and the $R_F$ values for the two principal spots for USP Olive Oil RS are 0.39 and 0.45.]

**Analysis**

**Samples:** *Standard solution* and *Sample solution*

Apply the *Samples* in separate bands to the previously marked starting point on an HPTLC plate, and develop the plate in the following order:

1. Ensure that the spots are at least 3 mm above the surface of the mobile phase. Develop two times over a path of 0.5 cm using *Mobile phase 1*. Remove the plate from the chamber after each run, and allow the plate to dry in air.
2. Develop two times over a path of 8 cm using *Mobile phase 2*. Allow the plate to dry for about 5 min after each development and before spraying with a spray reagent.

Spray the plate with the *Spray reagent*.

Heat the plate at 120° for about 1 min, and examine in daylight.

**Acceptance criteria:** The $R_F$ values of the principal spots of the *Sample solution* correspond to those of the *Standard solution*.

• **METHOD II**

**Mobile phase:** Methylene chloride, glacial acetic acid, and acetone (20:40:50)

**System suitability solution 1:** Dissolve 25 µL of USP Corn Oil RS in 3 mL of methylene chloride.

**System suitability solution 2:** Dissolve 25 µL of USP Olive Oil RS in 3 mL of methylene chloride.

**Standard solution:** Dissolve 25 µL of the appropriate USP Reference Standard in 3 mL of methylene chloride.

**Sample solution:** Dissolve 25 µL of a fixed oil sample in 3 mL of methylene chloride.

**Chromatographic system**

(See *Chromatography* ⟨621⟩*, Thin-Layer Chromatography*.)

**Mode:** HPTLC

**Plate:** 20 cm × 10 cm, silica gel 60 RP-18 (or RP-18 $F_{254}$), 0.15–0.2 mm layer, 4–8 µm particle size

**Conditioning of plate:** Condition the plate to a relative humidity of about 33%.

**Application volume:** 2 µL as bands of 8 mm. A suitable automated apparatus is used.

**Spray reagent:** 25 mg/mL of phosphomolybdic acid in 96% alcohol

**System suitability**

**Samples:** *System suitability solution 1* and *System suitability solution 2*

**Suitability requirements**

**Resolution:** The four principal bands from corn oil are clearly identified and separated, and the two principal bands from olive oil are clearly identified and separated.

[NOTE—Retardation factors ($R_F$) are provided for informational purposes only to aid in band identification. The $R_F$ values for the four principal bands for USP Corn Oil RS are 0.20, 0.26, 0.30, and 0.34, and the $R_F$ values for the two principal bands for USP Olive Oil RS are 0.20 and 0.26.]

**Analysis**

**Samples:** *Standard solution* and *Sample solution*

Predevelop the plate with methylene chloride to the upper edge. Dry the plate at 120° for 10 min.

[1] HPTLC Silica Gel 60 RP-18 plate from Merck EMD, or equivalent.

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Apply the *Samples* in separate bands to the previously marked starting point on an HPTLC plate.
Ensure that the bands are at least 3 mm above the surface of the mobile phase.
Develop over a path of 7 cm using *Mobile phase*. Allow the plate to dry in air.
Treat the plate with the *Spray reagent*.
Heat the plate at 120° for 3 min, and examine in daylight.

**Acceptance criteria:** The $R_F$ values of the principal bands of the *Sample solution* correspond to those of the *Standard solution*.

## ADDITIONAL REQUIREMENTS

- **USP REFERENCE STANDARDS** ⟨11⟩

USP Almond Oil RS
USP Borage Seed Oil RS
USP Canola Oil RS
USP Corn Oil RS
USP Cottonseed Oil RS
USP Evening Primrose Oil RS
USP Flax Seed Oil RS
USP Olive Oil RS
USP Palm Oil RS
USP Peanut Oil RS
USP Safflower Oil RS
USP Sesame Oil RS
USP Soybean Oil RS
USP Sunflower Oil RS

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# ⟨203⟩ HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY PROCEDURE FOR IDENTIFICATION OF ARTICLES OF BOTANICAL ORIGIN

***Change to read:***

## INTRODUCTION

This chapter describes a procedure for use in a USP Identification test that ▲employs▲ (USP 1-May-2020) the technique of high-performance thin-layer chromatography (HPTLC). ▲Precise▲ (USP 1-May-2020) control of the ▲experimental▲ (USP 1-May-2020) variables ▲is essential for reproducible analysis. [NOTE—For additional information,▲ (USP 1-May-2020) see *Identification of Articles of Botanical Origin by High-Performance Thin-Layer Chromatography Procedure* ⟨1064⟩.]

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## ▲▲ (USP 1-MAY-2020)EQUIPMENT

The equipment used for HPTLC ▲analysis typically includes:▲ (USP 1-May-2020)

- Plates: Unless otherwise specified in the individual monograph, use 20 × 10 cm plates coated with a uniform 200-µm layer of porous (60-Å pore size) silica gel having irregular particles of 2–10 µm and an average particle size of 5 µm, a polymeric binder, and a fluorescence indicator ($F_{254}$). [NOTE—▲▲ (USP 1-May-2020)Glass plates are preferred over aluminum-backed sheets.▲▲ (USP 1-May-2020)]
- A device suitable for ▲application of indicated volumes as bands of▲ (USP 1-May-2020) specified length at the ▲designated▲ (USP 1-May-2020) positions.
- A device suitable for controlling the activity of the stationary phase ▲by preconditioning the plate to a prescribed▲ (USP 1-May-2020) relative humidity.
- A suitable chromatographic chamber ▲to maintain reproducible vapor phase composition and to allow for control of developing distance▲ (USP 1-May-2020) (for example, a twin-trough chamber).
- A device suitable for reproducible drying of the developed plate.
- A device suitable for treatment of the plate with derivatization reagent, if required.
- A device suitable for ▲controlled plate▲ (USP 1-May-2020) heating as part of the derivatization procedure, if required.
- A ▲device▲ (USP 1-May-2020) suitable for ▲illumination under prescribed observation conditions, typically, short-wave UV (nominally 254 nm), long-wave UV (nominally 365 or 366 nm),▲ (USP 1-May-2020) and white light. ▲ [NOTE—Light sources used for chromatogram visualization typically produce broad-band emission; therefore, the maxima quoted above carry only nominal significance.]
- A photographic system for documentation of chromatograms under the prescribed visualization conditions.▲ (USP 1-May-2020)

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## PROCEDURE

### Preparation of the Test Solution

Unless otherwise stated in an individual monograph, 100 mg of a powdered botanical ingredient, 10 mg of a dry extract, ▲▲ (USP 1-May-2020) or the amount of a dosage form containing the equivalent of the aforementioned quantities of the botanical ingredient is sonicated for 15 min with 1 mL of methanol. After ▲filtration or▲ (USP 1-May-2020) centrifugation, the filtrate or supernatant is used as the *Sample solution*. ▲For essential oils, 50 µL/mL solution in toluene is▲ (USP 1-May-2020) used as the *Sample solution*.

### Preparation of the Standard Solutions

Unless otherwise stated in an individual monograph, Reference Standards of individual marker compounds are dissolved ▲in methanol▲ (USP 1-May-2020) at a concentration of 1 mg/mL. The Reference Standard extracts are shaken and sonicated in methanol at a concentration of 10 mg/mL or, for essential oils, ▲in toluene at 50 µL/mL.▲ (USP 1-May-2020)

### Sample Application and Plate Layout

Samples are applied as narrow bands of ▲8-mm▲ (USP 1-May-2020) length at a distance of ▲8 mm▲ (USP 1-May-2020) from the lower edge of the plate ▲ [NOTE—Refer to ⟨1064⟩ for additional information.]▲ (USP 1-May-2020) The system suitability standards are applied to the ▲track▲ (USP 1-May-2020) nearest to the ▲lateral▲ (USP 1-May-2020) edge at NLT 20 mm from the ▲lateral▲ (USP 1-May-2020) edge of the plate. The distance between tracks, center-to-center, is NLT ▲11 mm.▲ (USP 1-May-2020) All application volumes are specified

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in the individual monographs, ▲and▲ (USP 1-May-2020) usually range from 2–10 µL. The developing distance is marked with a pencil close to one of the ▲lateral▲ (USP 1-May-2020) edges of the plate before the development; ▲alternatively,▲ (USP 1-May-2020) an electronic solvent front detection device ▲is used.▲ (USP 1-May-2020)

## Preconditioning of the Plate

Following sample application and unless otherwise stated in an individual monograph, the plate is conditioned at a relative humidity of 33% ▲by exposure to the atmosphere generated by vapors in equilibrium with saturated magnesium chloride solution in a sealed chamber▲ (USP 1-May-2020) for a minimum of 10 min. ▲Other frequently used saturated salt solutions of potassium thiocyanate and sodium chloride establish equilibrium relative humidity of 47% and 75%, respectively, at 25°.▲ (USP 1-May-2020)

## Preparation of the Developing Chamber and Development of the Plate

Where a twin-trough chamber is used, the rear trough is fitted with filter paper. The chamber is charged with a sufficient volume of developing solvent to wet the filter paper completely and achieve a level of developing solvent of exactly 5 mm in both troughs. With the lid closed, the chamber is ▲allowed to achieve saturation for▲ (USP 1-May-2020) 20 min. ▲▲ (USP 1-May-2020) The plate is introduced ▲vertically▲ (USP 1-May-2020) into the front trough of the chamber ▲with the chromatographic layer facing▲ (USP 1-May-2020) the filter paper. ▲Once the solvent front▲ (USP 1-May-2020) has ▲ascended 6 cm from the application line,▲ (USP 1-May-2020) the plate is removed from the chamber and dried in a vertical position in a current of cold air that does not affect the integrity of the separated zones. ▲Alternative▲ (USP 1-May-2020) chamber configurations and developing distances may be specified in an individual monograph. [NOTE—Other development chambers may be employed if the results obtained fulfill all of the system suitability criteria.]

## Derivatization Procedure

Where derivatization reagents are used, ▲appropriate▲ (USP 1-May-2020) volumes of reagents ▲▲ (USP 1-May-2020) (typically 1–2 mL) are ▲uniformly▲ (USP 1-May-2020) sprayed onto the plate or the plate is immersed into the reagent solution at a defined speed and for a defined dwell time. [NOTE—Immersion speed of 50 mm/s and the dwell time of 1 s ▲are recommended▲ (USP 1-May-2020) for most nonaqueous reagents.]

## Visualization

Chromatograms on the plate are visualized as directed in an individual monograph, ▲typically under short-wave UV, long-wave UV,▲ (USP 1-May-2020) or white light prior to and after derivatization.

## System Suitability

▲A general system suitability requirement is that the solvent front and the chromatographic bands are strictly parallel to the horizontal sides of the chromatographic plate. Specific suitability parameters are detailed in the monographs. Typically, to assess▲ (USP 1-May-2020) suitability of the chromatographic system for resolution, position, and color of the bands, ▲▲ (USP 1-May-2020) two or more reference substances are selected that have similar but just separable $R_F$ values under the chromatographic conditions to be used; for example, chlorogenic acid (blue) and hyperoside (yellow-orange) ▲are commonly▲ (USP 1-May-2020) used for flavonoids ▲methods. System suitability is required every time the analysis is performed; it is conducted on the same plate alongside the test articles. Failure to meet the system suitability requirements invalidates the analysis. In the event of failure, its cause needs to be identified and corrected (see ⟨1064⟩ for potential sources of failure), and the analysis repeated until the suitability requirements have been met. Individual▲ (USP 1-May-2020) Reference Standards, ▲or their▲ (USP 1-May-2020) mixtures, ▲are typically employed▲ (USP 1-May-2020) for system suitability ▲monitoring, and the use of▲ (USP 1-May-2020) the USP Reference Standard extracts ▲is common. The▲ (USP 1-May-2020) resolution, position, and colors of the key bands of the ▲Reference Standard chromatogram▲ (USP 1-May-2020) should match the description in the monograph within a specified tolerance. ▲▲ (USP 1-May-2020)

## Evaluation and Acceptance Criteria

Chromatograms of the *Sample solution* and *Standard solution* are compared against the descriptions in the *Acceptance criteria* section of the monograph with respect to ▲band▲ (USP 1-May-2020) position, ▲▲ (USP 1-May-2020)separation, color, and relative intensity.

## Documentation

▲Test results are required to be documented▲ (USP 1-May-2020) in an auditable manner to comply with the current good manufacturing practices. Proper documentation tools should be employed; for example, a ▲digital▲ (USP 1-May-2020) camera ▲capable of capturing images▲ (USP 1-May-2020) under ▲prescribed illumination, or a scanning densitometer along with the▲ (USP 1-May-2020) imaging software suitable for archival, retrieval, and analysis of the results.

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## ▲Standard Parameters

Unless otherwise indicated in an individual monograph, the following standard parameters, as specified in *Table 1*, are to be employed in every procedure referencing this chapter.

**Table 1**

| Parameter | Standard |
|---|---|
| Chromatographic plate, glass, 20 × 10 cm | 200-µm silica layer, 5-µm average particle size, with fluorescence indicator $F_{254}$ |
| Application position, from plate lower edge | 8 mm |
| Band application length | 8 mm |
| Band spacing, center-to-center | NLT 11 mm |
| Relative humidity | 33% |
| Plate conditioning time | 10 min |
| Chamber saturation time | 20 min |
| Developing distance | 6 cm |
| Temperature | 22 ± 5°▲ (USP 1-May-2020) |

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# ⟨206⟩ ALUMINUM

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## ▲INTRODUCTION

These procedures are▲ (Official 1-Jun-2023) provided to demonstrate that the content of aluminum (Al) does not exceed the limit given in the individual monograph of a substance labeled as intended for use in hemodialysis. ▲Use *Procedure 1*, *Procedure 2*, or *Procedure 3* as indicated in the individual monograph. *Procedure 2* or *Procedure 3* can be used in all circumstances, provided that suitability is demonstrated by meeting the *Requirements for Procedure Validation*.▲ (Official 1-Jun-2023)

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## ▲PROCEDURES

- **Procedure 1: Atomic Absorption Spectroscopy**▲ (Official 1-Jun-2023)

[Note—The *Standard preparations* and the *Test preparation* may be modified, if necessary, to obtain solutions of suitable concentrations adaptable to the linear or working range of the instrument.]

**Nitric acid diluent:** Transfer 40 mL of nitric acid to a 1000-mL volumetric flask, and dilute with water to volume.

**Standard preparations:** Treat some aluminum wire with 6 N hydrochloric acid at 80° for a few minutes. Dissolve about 100 mg of the treated wire, accurately weighed, in a mixture of 10 mL of hydrochloric acid and 2 mL of nitric acid by heating at about 80° for approximately 30 min. Continue heating until the volume is reduced to about 4 mL. Cool to room temperature, and add 4 mL of water. Evaporate to about 2 mL by heating. Cool, and transfer this solution, with the aid of water, to a 100-mL volumetric flask, dilute with water to volume, and mix. Transfer 10.0 mL of this solution to a second 100-mL volumetric flask, dilute with water to volume, and mix. Transfer 1.0 mL of this solution to a third 100-mL volumetric flask, dilute with water to volume, and mix. The concentration of aluminum in this *Standard preparation* is about 1.0 µg/mL. If a more diluted *Standard preparation* is required, transfer 1.0-, 2.0-, and 4.0-mL portions of this solution to separate 100-mL volumetric flasks, dilute with *Nitric acid diluent* to volume, and mix. These solutions contain 0.01, 0.02, and 0.04 µg of aluminum per milliliter, respectively.

**Test preparation:** Unless otherwise directed in the individual monograph, prepare as follows. Transfer an accurately weighed amount, in grams, of the test substance, as specified in the monograph, to a 100-mL plastic volumetric flask, add 50 mL of water, and sonicate for 30 min. Add 4 mL of nitric acid, dilute with water to volume, and mix.

**▲Analysis:▲** (Official 1-Jun-2023) Determine the absorbances of the *Standard preparations* and the *Test preparation* at the aluminum emission line at 309.3 nm with a suitable atomic absorption spectrophotometer (see *Atomic Absorption Spectroscopy* ⟨852⟩) equipped with an aluminum hollow-cathode lamp and a flameless electrically heated furnace, using the *Nitric acid diluent* as the blank. Plot the absorbances of the *Standard preparations* versus the content of aluminum, in micrograms per milliliter, drawing a straight line best fitting the three points. From the graph, determine the quantity, in micrograms, of aluminum in each milliliter of the *Test preparation*. Calculate the amount of aluminum in the specimen taken, in micrograms per gram, by multiplying this value by 100/*W*, where *W* is the weight, in grams, of the substance taken to prepare the *Test preparation*.

- **▲Procedure 2 and Procedure 3**

Both *Procedure 2* and *Procedure 3* are ICP-based procedures and can be used for the determination of aluminum. *Procedure 2* can be used for the determination of aluminum by inductively coupled plasma atomic (or optical) emission spectroscopy (ICP–AES or ICP–OES). *Procedure 3* can be used for the determination of aluminum by ICP–MS.

Before initial use, the analyst should verify that the procedure is appropriate for the instrument and sample used (procedural verification) by meeting the *Requirements for Procedure Validation*.

Where a monograph specifies a limit for aluminum concentration, the value listed in the monograph should be used as the *J* value for the purposes of this test.

System standardization and suitability evaluation using applicable reference materials should be performed on the day of analysis.

**Sample preparation:** Forms of sample preparation include neat, direct aqueous solution, direct organic solution, and indirect solution. The selection of the appropriate sample preparation depends on the material under test and is the responsibility of the analyst. When a sample preparation is not indicated in the monograph, an analyst may use any appropriately validated preparation procedure. In cases where spiking of a material under test is necessary to provide an acceptable signal intensity, the blank should be spiked with aluminum using, where possible, the same spiking solution. [Note—All liquid samples should be weighed.]

**Closed vessel digestion:** This sample preparation procedure is designed for samples that must be digested in a concentrated acid using a closed vessel digestion apparatus. Closed vessel digestion minimizes the loss of volatile impurities. The choice of a concentrated acid depends on the sample matrix. The use of any of the concentrated acids may be appropriate, but each introduces inherent safety risks. Therefore, appropriate safety precautions should be used at all times. [Note—Weights and volumes provided may be adjusted to meet the requirements of the digestion apparatus used.]

An example procedure that has been shown to have broad applicability is as follows. Dehydrate and predigest 0.5 g of the primary sample in 5 mL of freshly prepared concentrated acid. Allow to sit loosely covered for 30 min in a fume hood. Add an additional 10 mL of concentrated acid, and digest using a closed vessel technique until the digestion or extraction is complete. Repeat, if necessary, by adding an additional 5 mL of concentrated acid. [Note—Follow the manufacturer's recommended procedures to ensure safe use.]

**Reagents:** All reagents used for the preparation of sample and standard solutions should be free of elemental impurities, in accordance with *Plasma Spectrochemistry* ⟨730⟩.

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**Procedure 2: ICP–OES**
**Standardization solution 1:** 1.5*J* of aluminum in a matched matrix
**Standardization solution 2:** 0.5*J* of aluminum in a matched matrix
**Sample stock solution:** Prepare as directed in *Sample preparation*. Allow the sample to cool, if necessary.
**Sample solution:** Dilute the *Sample stock solution* with an appropriate solvent to obtain a final aluminum concentration of not more than 1.5*J*.
**Blank:** Matched matrix
**Elemental spectrometric system**
(See ⟨730⟩.)
**Rinse:** Use diluent.
**Standardization:** *Standardization solution 1*, *Standardization solution 2*, and *Blank*
**System suitability**
**Sample:** *Standardization solution 1*
**Suitability requirements**
**Drift:** Compare results obtained from *Standardization solution 1* before and after the analysis of the *Sample solution*.
**Suitability criteria:** Not more than 20% for aluminum. [NOTE—If samples are high in mineral content, rinse the system well before introducing the *Sample* in order to minimize carryover.]
**Analysis:** Analyze according to the manufacturer's suggestions for program and wavelength. Calculate and report results on the basis of the original sample size. [NOTE—Appropriate measures must be taken to correct for matrix-induced interferences (e.g., wavelength overlaps).]
**Procedure 3: ICP–MS**
Follow *Procedure 2* except for *Detector* and *Analysis*.
[NOTE—An instrument with a cooled spray chamber is recommended. (A collision cell or reaction cell may also be beneficial.)]
**Detector:** Mass spectrometer
**Analysis:** Analyze according to the manufacturer's suggestions for program and mass-to-charge ratio. Calculate and report results based on the original sample size. [NOTE—Appropriate measures must be taken to correct for matrix-induced interferences.]▲ (Official 1-Jun-2023)

***Add the following:***

## ▲REQUIREMENTS FOR PROCEDURE VALIDATION

The following section defines the validation parameters and the acceptance criteria for performance-based procedures. Meeting these requirements must be demonstrated experimentally using an appropriate system suitability procedure and reference materials. Any alternative procedure (e.g., an atomic-absorption-based procedure) that has been validated and meets the acceptance criteria that follow is considered to be suitable for use.

Meeting these validation acceptance criteria is sufficient to demonstrate that the procedure will produce comparable results to those obtained using the procedure prescribed in the monograph.

- **ACCURACY**
  **Standard solutions:** Prepare solutions containing aluminum at concentrations ranging from 50% to 150% of *J* using appropriate reference materials.
  **Test samples:** Spike the material under test with the appropriate reference materials before any sample preparation steps (digestion or solubilization). Prepare three replicate samples at concentrations ranging from 50% to 150% of *J* for aluminum.
  **Acceptance criteria**
  **Spike recovery:** 70%–150% for the mean of three replicate preparations at each concentration
- **PRECISION**
  **Repeatability**
  **Test samples:** Six independent samples of material under test (taken from the same lot), spiked with appropriate reference materials for aluminum, at the indicated concentration
  **Acceptance criteria**
  **Relative standard deviation:** Not more than 20% ($N = 6$) for aluminum
  **Intermediate precision (ruggedness)**
  **Analysis:** Perform the *Repeatability* analysis again on a different day, with different instrumentation, with a different analyst, or a combination thereof. Combine the results of this analysis with the *Repeatability* analysis so the total number of analyses is 12.
  **Acceptance criteria**
  **Relative standard deviation:** Not more than 25% ($N = 12$) for aluminum
- **SPECIFICITY:** The procedure must be able to unequivocally assess (see *Validation of Compendial Procedures* ⟨1225⟩) aluminum in the presence of components that may be expected to be present, including matrix components.
- **LIMIT OF QUANTITATION, RANGE, AND LINEARITY:** Demonstrated by meeting the *Accuracy* requirement.▲ (Official 1-Jun-2023)

***Add the following:***

## ▲GLOSSARY

**Concentrated acid:** Concentrated ultra-pure nitric, sulfuric, hydrochloric, or hydrofluoric acid or aqua regia.
**Aqua regia:** Aqua regia is a mixture of concentrated hydrochloric and nitric acids, typically at ratios of 3:1 or 4:1.
**Matched matrix:** Solutions having the same solvent composition as the *Sample solution*. In the case of an aqueous solution, a matched matrix would indicate that the same acids and acid concentrations are used in both preparations.

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**Target limit or target concentration:** The acceptance value for the elemental impurity being evaluated, in this case aluminum. Where a monograph specifies a threshold limit, this shall become the target limit or target concentration of aluminum for the material. Exceeding the target limit indicates that a material under test exceeds the acceptable value. The determination of compliance is addressed in other chapters.

***J*:** The concentration (w/w) of the element of interest, in this case aluminum, at the target limit, appropriately diluted to the working range of the instrument.

**Appropriate reference materials:** Where "appropriate reference materials" are specified in the chapter, certified reference materials (CRMs) from a national metrology institute (NMI), or reference materials that are traceable to the CRM of an NMI should be used. An example of an NMI in the United States is the National Institute of Standards and Technology (NIST).▲ (Official 1-Jun-2023)

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# ⟨207⟩ TEST FOR 1,6-ANHYDRO DERIVATIVE FOR ENOXAPARIN SODIUM

The following procedure is used to determine the levels of 1, 6-anhydro forms in enoxaparin sodium. [NOTE—The test for the 1,6-anhydro derivative is conducted only where specified in the individual monograph.]

## INTRODUCTION

The disaccharides specified in this general chapter are listed by name and structure in *Appendix 1*; the oligosaccharides are listed in *Appendix 2*.

Depolymerization of heparin into enoxaparin sodium produces a partial but characteristic conversion of glucosamines at the reducing termini of oligosaccharide chains with terminal glucosamine 6-*O* sulfate, yielding 1,6-anhydro derivatives (see *Figure 1*).

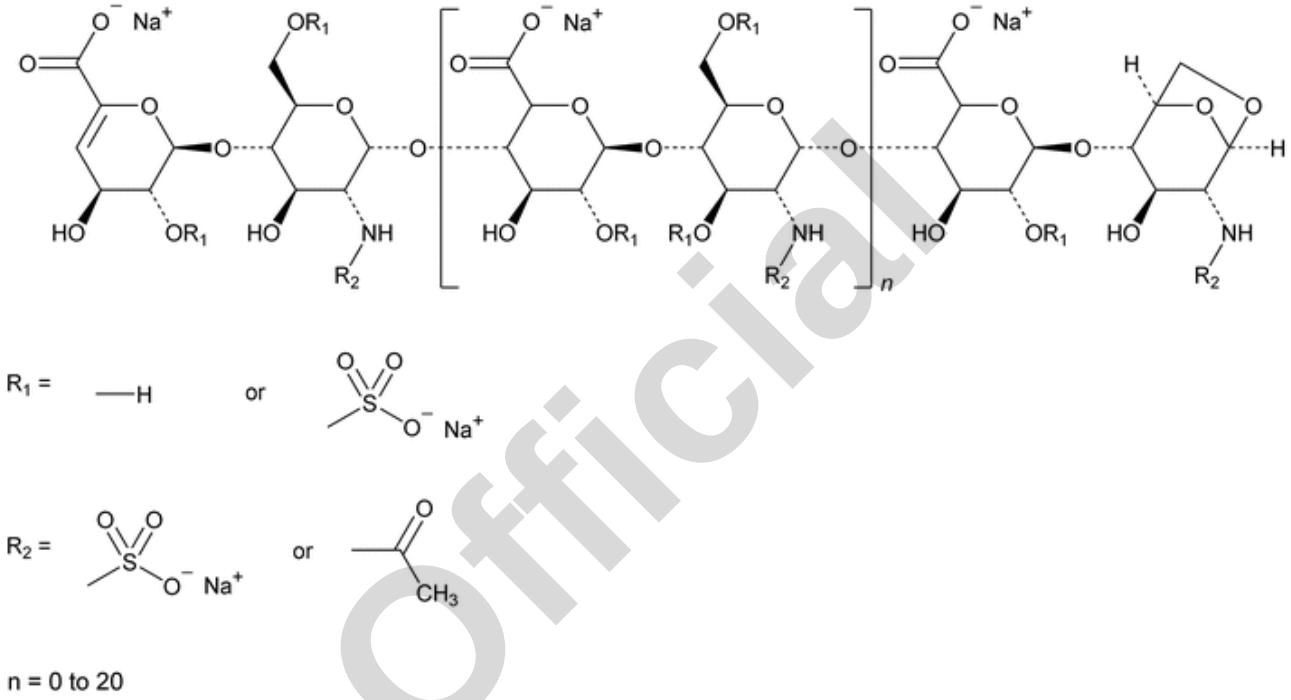


Figure 1. Structure of enoxaparin sodium containing a 1,6-anhydro derivative on the reducing end of the chain.

The percentage of oligosaccharide chains that are cyclized in a 1,6-anhydro ring is a characteristic of enoxaparin sodium.

## PROCEDURES

- **DEPOLYMERIZATION OF ENOXAPARIN SODIUM BY HEPARINASES AND RESULTING OLIGOSACCHARIDES**

The assay involves HPLC analysis of a depolymerized enoxaparin sodium solution by a mixture of heparinases. After enzymatic depolymerization, the main 1,6-anhydro residues of enoxaparin sodium observed are 1,6-anhydro ΔIIS and 1,6-anhydro $\Delta IIS^{epi}$, and 1,6-anhydro ΔIS and 1,6-anhydro $\Delta IS–IS^{epi}$ (see *Appendix 2*.).

The 1,6-anhydro $\Delta IS–IS^{epi}$ tetrasaccharide (2-*O*-sulfated mannosamine form) is not completely cleaved by the heparinases. The two disaccharides (1,6-anhydro ΔIIS and 1,6-anhydro $\Delta IIS^{epi}$), which generally co-elute, are poorly resolved with respect to ΔIIA (see *Appendix 1*), especially because the latter occurs as two anomers: α and β. To allow quantitation of 1,6-anhydro ΔIIS and 1,6-anhydro $\Delta IIS^{epi}$, the enoxaparin sodium sample already depolymerized by heparinases is then reduced by sodium borohydride (see *Figure 2*).

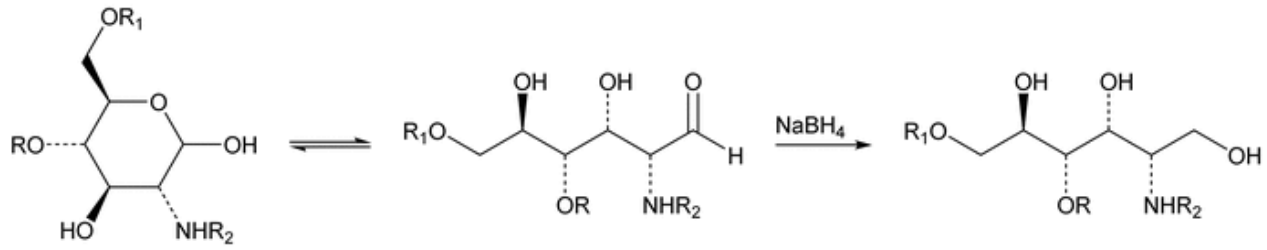


Figure 2. Reduction of oligosaccharides by sodium borohydride

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The sodium borohydride reduction eliminates the $\alpha \leftrightarrow \beta$ anomeric effect by opening the terminal oligosaccharide ring. The four 1,6-anhydro derivatives (see *Appendix 2*.) are not reduced by sodium borohydride because the ring opening is blocked by the 1,6-anhydro bridge. The reduction of the oligosaccharides decreases their retention time, whereas the retention time of the 1,6-anhydro derivatives remains unchanged. Thus, it is possible to separate the two compounds—1,6-anhydro ΔIIS and 1,6-anhydro $\Delta IIS^{epi}$—from the reduced ΔIIA disaccharide peak.

[NOTE—1,6-Anhydro ΔIIS and 1,6-anhydro $\Delta IIS^{epi}$ are eluted as two nonresolved peaks and are quantitated together as a single compound, 1,6-anhydro ΔIIS. Therefore, for the purpose of simplification, the epimeric form is not referred to in the remaining text.]

***Change to read:***

- **PROCEDURE**

**Solutions**

**Solution A:** Dissolve 0.280 g of monobasic sodium phosphate in 950 mL of water, adjust with phosphoric acid to a pH of 3.0, and dilute with water to 1000 mL.

**Solution B:** Dissolve 140 g of sodium perchlorate in 950 mL of *Solution A,* adjust with phosphoric acid to a pH of 3.0, and dilute with *Solution A* to 1000 mL.

**Mobile phase:** Use variable mixtures of filtered and degassed *Solution A* and *Solution B* as directed in *Chromatographic system*.

**Sodium/Calcium acetate pH 7.0 solution:** Dissolve 10 mg of bovine serum albumin and 32 mg of calcium acetate in 60 mL of water. Add 580 µL of glacial acetic acid, and adjust with 2 M sodium hydroxide to a pH of 7.0. Transfer to a 100-mL volumetric flask, and dilute with water to volume. Pass the solution through a filter having a porosity of 0.45 or 0.22 µm.

**Potassium phosphate pH 7.0 buffer:** Dissolve 68 mg of monobasic potassium phosphate and 10 mg of bovine serum albumin in 30 mL of water in a 50-mL volumetric flask. Adjust with potassium hydroxide, if necessary, to a pH of 7.0, and dilute with water to volume. Pass the solution through a filter having a porosity of 0.45 or 0.22 µm.

**Sodium borohydride solution:** Dissolve 12 mg of sodium borohydride in 400 µL of water, and mix on a vortex mixer. [NOTE—Prepare fresh *immediately* before use.]

**Heparinase 1 solution:** Dissolve heparinase 1 (see *Reagent Specifications* under *Reagents, Indicators,* and *Solutions*) [reference: heparin lyase I, EC 4.2.2.7] in *Potassium phosphate pH 7.0 buffer* to obtain a solution having an activity of 0.4 IU per mL. Store the solution at –20° until ready to use. [NOTE—Heparinase solutions can be stored for 3 months at –20°.]

**Heparinase 2 solution:** Dissolve heparinase 2 (see *Reagent Specifications* under *Reagents, Indicators,* and *Solutions* [no EC number] in *Potassium phosphate pH 7.0 buffer* to obtain a solution having an activity of 0.4 IU per mL. Store the solution at –20° until ready to use.

**Heparinase 3 solution:** Dissolve heparinase 3 (see *Reagent Specifications* under *Reagents, Indicators,* and *Solutions* [reference: heparitinase I, EC 4.2.2.8] in *Potassium phosphate pH 7.0 buffer* to obtain a solution having an activity of 0.4 IU per mL. Store the solution at –20° until ready to use.

**Heparinases 1, 2, 3, solution:** Prepare a 1:1:1 (v:v:v) mixture of *Heparinase 1 solution, Heparinase 2 solution,* and *Heparinase 3 solution.*

**Peak identification solutions**

[NOTE—The depolymerized test solutions and Standard solutions must be prepared at the same time. Depolymerized test solutions are stable for 1 month at –20°. Also, the reduced test solutions and Standard solutions must be prepared at the same time. Reduced solutions are also stable for 1 month at –20°.]

**Disaccharide solutions:** Separately prepare a 0.25 mg per mL solution of each disaccharide[1] ΔIA, ΔIIA, ΔIIIA, ΔIVA, ΔIS, ΔIIS, ΔIIIS, ΔIVS (see *Appendix 1*). Chromatograph each disaccharide solution, and record the peak responses.

**Reduced disaccharide solutions:** To 60 µL of each *Disaccharide solution,* add 10 µL of freshly prepared *Sodium borohydride solution.* Mix on a vortex mixer, and allow to stand at room temperature for at least 4 hours. Chromatograph each solution, and record the peak response.

**Blank solution:** Prepare a mixture of 20 µL of water, 70 µL of *Sodium/Calcium acetate pH 7.0 solution,* and 100 µL of the *Heparinases 1, 2, 3 solution*. Mix gently by inversion, and allow to stand for at least 48 hours in a 25° water bath. Prepare a mixture of 60 µL of this depolymerized solution with 10 µL of freshly prepared *Sodium borohydride solution*. Homogenize, and allow to stand at room temperature for at least 4 hours. Chromatograph the resulting solution, and record the peak responses.

**Test solution 1:** Prepare two solutions, each containing 20 mg of enoxaparin sodium in 1 mL of water.

**Standard solution 1:** Prepare one solution containing 20 mg of USP Enoxaparin Sodium RS in 1 mL of water.

**Test solution 2:** For each solution, prepare a mixture of 20 µL of *Test solution 1,* 70 µL of *Sodium/Calcium acetate pH 7 solution,* and 100 µL of *Heparinases 1, 2, 3 solution.* Mix gently by inversion, and allow to stand for at least 48 hours in a 25° water bath. After 48 hours of depolymerization, chromatograph the solution, and record the peak responses.

**Standard solution 2:** Prepare a mixture of 20 µL of *Standard solution 1,* 70 µL of *Sodium/Calcium acetate pH 7 solution,* and 100 µL of *Heparinases 1, 2, 3 solution.* Mix gently, and allow to stand for at least 48 hours in a 25° water bath. After 48 hours of depolymerization, chromatograph the solution, and record the peak responses.

**Test solution 3:** For each depolymerized test solution, prepare a mixture of 60 µL of *Test solution 2* and 10 µL of freshly prepared *Sodium borohydride solution*. Homogenize, and allow to stand loosely capped at room temperature for at least 4 hours before injecting into the chromatograph. *Test Solution 3* is stable for 48 hours at room temperature.

[1] Suitable disaccharides are available from Grampian Enzymes (GE-H1001, GE-G1002, GE-H1003, GE-H1004, GE-H1005, GE-H1006, GE-H1007, GE-H1008), Nisthouse, Harray, Orkney, KW17 2LQ, United Kingdom, Tel: 01856 771771, Scottish Local Authority: Orkney Islands.

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**Standard solution 3:** Prepare a mixture of 60 µL of *Standard solution 2* and 10 µL of freshly prepared *Sodium borohydride solution*. Homogenize and mix on a vortex mixer, and allow to stand loosely capped at room temperature for at least 4 hours before injecting into the chromatograph. *Standard solution 3* is stable for 48 hours at room temperature.

**Chromatographic system**

(See *Chromatography* ⟨621⟩.)

The liquid chromatograph is equipped with a 234-nm detector and a 3-mm × 25-cm column that contains 5-µm packing L14. A guard column packed with the same material should also be used. The flow rate is 0.45 mL per minute, the column temperature is maintained at 50°, and the injection volume is 10 µL. The chromatograph is programmed as follows.

| Time (min) | Solution A (%) | Solution B (%) | Elution |
|---|---|---|---|
| 0–20 | 97→65 | 3→35 | Linear gradient |
| 20–50 | 65→0 | 35→100 | Linear gradient |
| 50–60 | 0 | 100 | Isocratic |
| 60–61 | 0→97 | 100→3 | Linear gradient for re-equilibration |
| 61–79 | 97 | 3 | Isocratic for re-equilibration |

Chromatograph the reduced *Test solution 3* and the reduced *Standard solution 3,* and record the peak responses as directed for *Procedure.*

**Depolymerization suitability test:** The ratio of the peak area of 1,6-anhydro-ΔIS-IS to that of 1,6-anhydro ΔIS is not more than 1.15 for the depolymerized *Standard solution 2*.

**Column performance suitability test:** Identify the peaks corresponding to reduced ΔIA and 1,6-anhydro-ΔIS for the *Standard solution 3:* the retention time of reduced ΔIS is between 27 and 33 minutes for the depolymerized and reduced *Standard solution 3;* and the resolution, *R,* between reduced ΔIA and 1,6-anhydro-ΔIS is not less than 1.5.

**Reduction suitability test:** The ratio of the peak area of ΔIS disaccharide to that of reduced ΔIS in the depolymerized and reduced *Standard solution 3* and *Test solution 3* is not more than ▲0.02.▲ (ERR 1-Oct-2018)

**Procedure/Calculation:** Separately inject equal volumes of the reduced *Test solutions 3* and the reduced *Standard solution 3* into the chromatograph. Use the normalized area percentage method for calculation. Each peak is integrated from the dwell volume peak to the last detected peak. Measure the area of each analyte peak after excluding solvent peaks at the beginning of the chromatogram and in the *Blank solution*. Using the previously obtained chromatograms of the *Reduced disaccharide solutions,* identify peaks belonging to the eight reduced disaccharides in the chromatograms for *Test solution 3* and *Standard solution 3*. The peaks belonging to 1,6-Anhydro ΔIS, 1,6-Anhydro ΔIIS, and 1,6-Anhydro ΔIS-IS$^{epi}$ are identified from the relative retention times provided in *Table 1* and the Reference chromatogram provided with USP Enoxaparin Sodium RS. Once the peaks have been identified, use the values in *Table 1* to calculate the (w/w) percentage of the three main 1,6-anhydro derivatives obtained after depolymerization of enoxaparin sodium using the following formula:

$$\%\ 1,6\text{-anhydro i (w/w)} = (100 \times MW_i \times A_i)/\ \Sigma(MW_x \times A_x)$$

$MW_i$ = molecular weight of the 1,6-anhydro peak i
$A_i$ = area of the 1,6-anhydro peak i
$MW_x$ = molecular weight of either the peak X or the zone X specified by its retention time
$A_x$ = area of either the peak X or the zone X specified by its retention time

[NOTE—Once the method is established, the peaks belonging to the different di- and tetrasaccharides can be easily identified using the USP Enoxaparin Sodium RS chromatogram. Thus, the use of the disaccharide Standards is only needed during the method-implementation stage.]

Calculate the molar percentage of components containing a 1,6-anhydro structure at the reducing end of their chain in the enoxaparin sodium test sample according to the following formula:

$$\%\ 1.6anhydro = 100 \times \frac{\text{MW}}{\Sigma \text{MW}_\text{X} \times \text{Area}_\text{x}} \times \left(\text{Area}\Delta\text{I}s1.6anydro + \text{Area}\Delta\text{II}s1.6anhydro + \text{Area}\Delta\text{I}s - \text{I}s1.6anhydro\right)$$

in which MW is the mass-average molecular mass (see *Identification* test *D* under *Enoxaparin Sodium*); $MW_x$ and $Area_x$ are the molecular weight and the area, respectively, of either the peak X or the range X specified by its retention time. The molar percentage of components having a 1,6-anhydro structure at the reducing end of their chain is between 15% and 25%. Typical retention times and molecular masses attributed to different oligosaccharide structures are provided in *Table 1*.

**Table 1. Typical Relative Retention Times ($t_{RR}$) and Molecular Masses Attributed to Different Compounds***

| Compound | $t_{RR}$ | Molecular Mass (Daltons) |
|---|---|---|
| — | < 0.25 | 741 |
| Reduced ΔIVA | 0.25 | 401 |

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DOI: https://doi.org/10.31003/USPNF_M2740_04_01

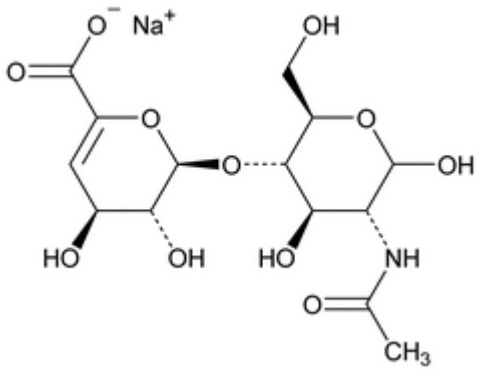
**Table 1. Typical Relative Retention Times ($t_{RR}$) and Molecular Masses Attributed to Different Compounds*** *(continued)*

| Compound | $t_{RR}$ | Molecular Mass (Daltons) |
|---|---|---|
| — | $0.25 < t_{RR} < 0.51$ | 741 |
| Reduced ΔIVS | 0.51 | 461 |
| — | $0.51 < t_{RR} < 0.55$ | 483 |
| Reduced ΔIIA | 0.55 | 503 |
| — | $0.55 < t_{RR} < 0.59$ | 503 |
| 1,6-Anhydro ΔIIS | 0.59 | 443 |
| — | $0.59 < t_{RR} < 0.64$ | 503 |
| Reduced ΔIIIA | 0.64 | 503 |
| — | $0.64 < t_{RR} < 0.72$ | 533 |
| Reduced ΔIIS | 0.72 | 563 |
| — | $0.72 < t_{RR} < 0.80$ | 563 |
| Reduced ΔIIIS | 0.80 | 563 |
| — | $0.80 < t_{RR} < 0.88$ | 583 |
| Reduced ΔIA | 0.88 | 605 |
| — | $0.88 < t_{RR} < 0.90$ | 635 |
| 1,6-Anhydro ΔIS | 0.90 | 545 |
| — | $0.90 < t_{RR} < 0.98$ | 635 |
| Reduced ΔIIA-IVSglu | 0.98 | 1066 |
| — | $0.98 < t_{RR} < 1.00$ | 635 |
| Reduced ΔIS | 1.00 | 665 |
| — | $1.00 < t_{RR} < 1.04$ | 665 |
| ΔIS | 1.04 | 665 |
| — | $1.04 < t_{RR} < 1.10$ | 1228 |
| Reduced ΔIIA-IISglu | 1.10 | 1168 |
| — | $1.10 < t_{RR} < 1.27$ | 1228 |
| 1,6-Anhydro ΔIS-IS | 1.27 | 1210 |
| — | $t_{RR} > 1.27$ | 1228 |

* Relative retention times were obtained with a depolymerized and reduced batch of enoxaparin sodium. They are expressed relative to the retention time of the main peak corresponding to reduced ΔIS. Note that according to the quality of the column, relative retention times can change slightly.

## ADDITIONAL REQUIREMENTS

- **USP Reference Standards** ⟨11⟩
  USP Enoxaparin Sodium RS
- **Appendix 1: Standard Disaccharide Structures**

| | | |
|---|---|---|
| ΔIVA |  | ΔUA-(1→4)α-GlcNAc |

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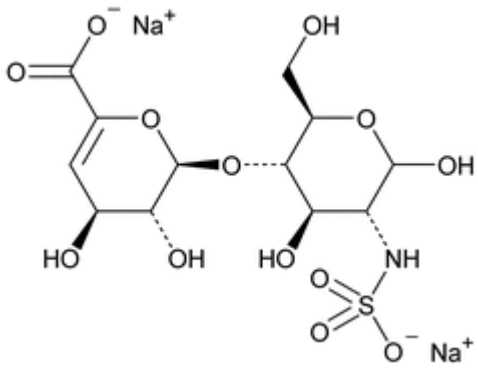
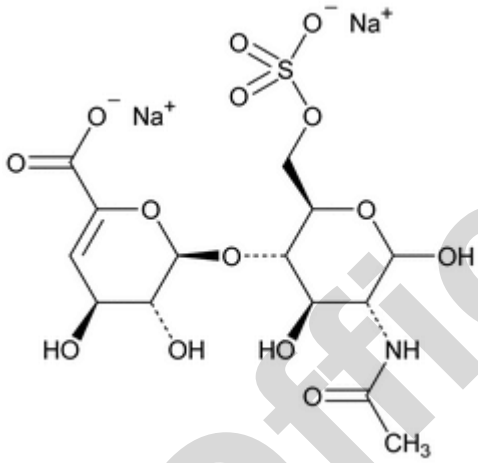
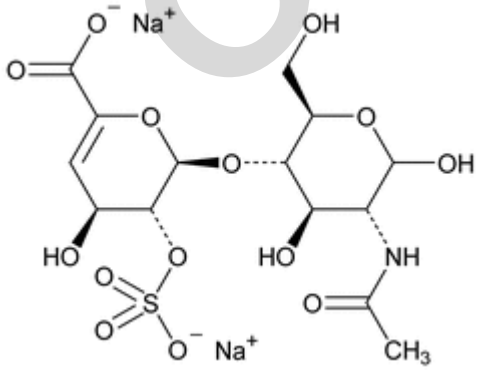
| | | |
|---|---|---|
| ΔIVS |  | ΔUA-(1→4)α-GlcN(NS) |
| ΔIIA |  | ΔUA-(1→4)α-GlcNAc(6S) |
| ΔIIIA |  | ΔUA-2S-(1→4)α-GlcNAc |

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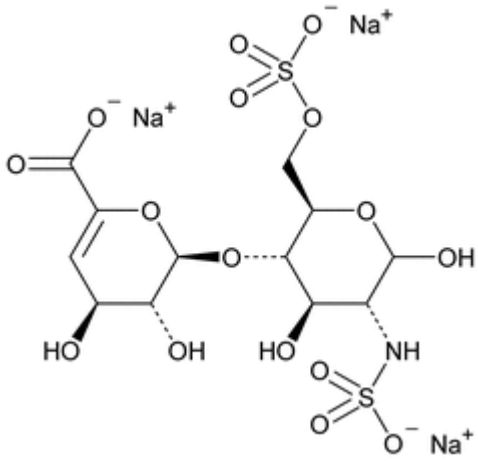
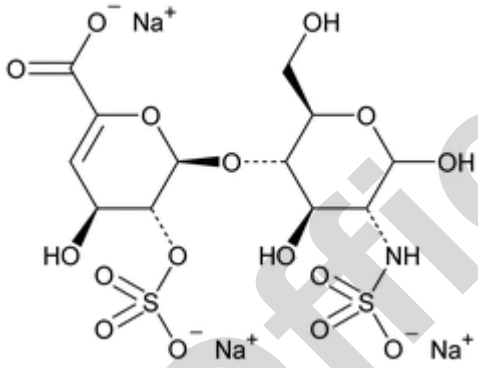
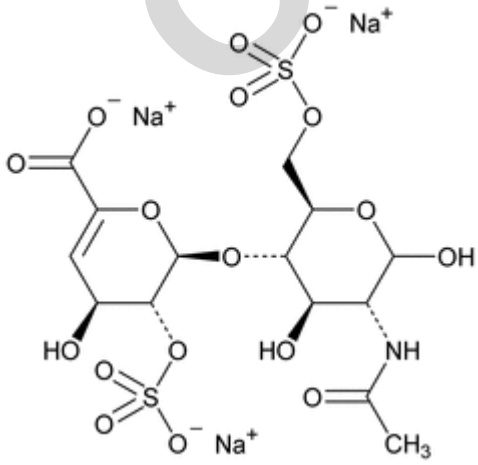
DOI: https://doi.org/10.31003/USPNF_M2740_04_01

| | | |
|---|---|---|
| ΔIIS |  | ΔUA-(1→4)α-GlcN (NS,6S) |
| ΔIIIS |  | ΔUA-2S-(1→4)α-GlcN (NS) |
| ΔIA |  | ΔUA-2S-(1→4)α-GlcNAc(6S) |

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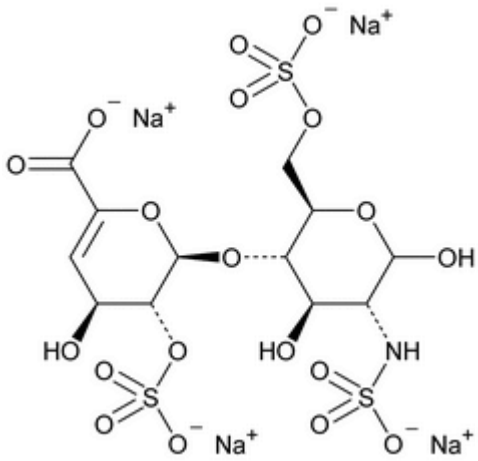
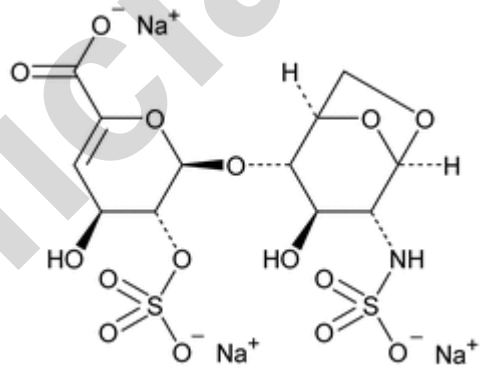
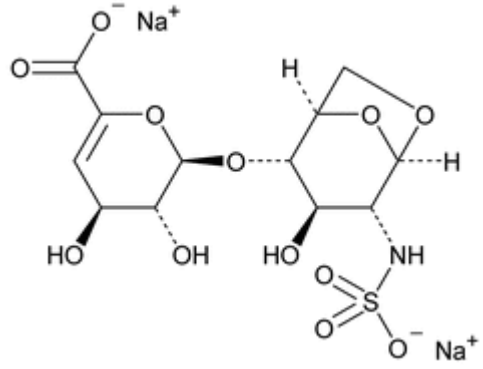
|---|---|---|
| ΔIS |  | UA-2S-(1→4)α-GlcN (NS,6S) |

## • Appendix 2: Oligosaccharide Structures

| | |
|---|---|
| 1,6-Anhydro ΔIS or<br>1,6-Anhydro ΔIS glucose |  |
| 1,6-Anhydro ΔIIS or<br>1,6-Anhydro ΔIIS glucose |  |

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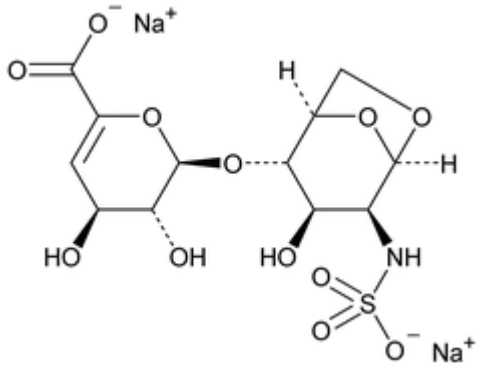
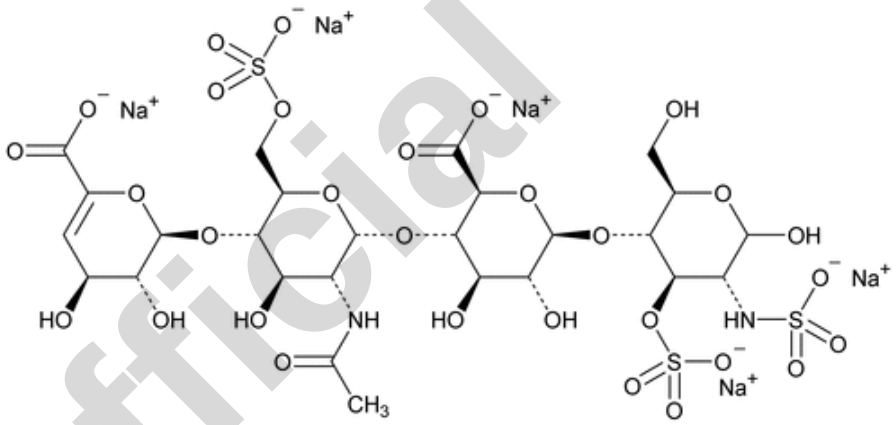
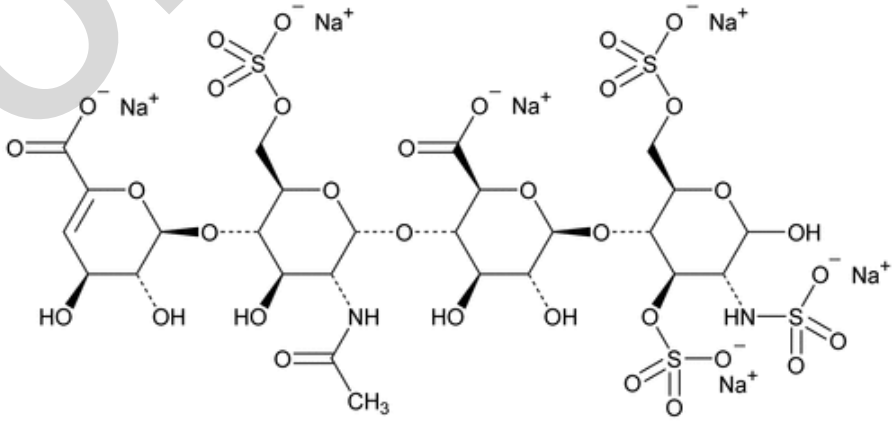
| | |
|---|---|
| 1,6-Anhydro ΔIIS epi or<br>1,6-Anhydro ΔIIS mannose |  |
| ΔIIA-IVSglu |  |
| ΔIIA-IISglu |  |

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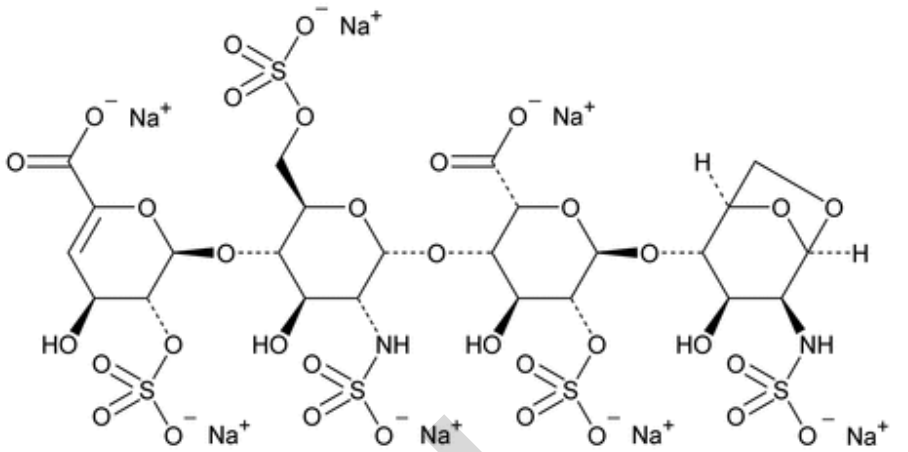
| | |
|---|---|
| 1,6-Anhydro ΔIS-IS epi or<br>1,6-Anhydro ΔIS-IS mannose |  |

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# ⟨208⟩ ANTI-FACTOR Xa AND ANTI-FACTOR IIa ASSAYS FOR UNFRACTIONATED AND LOW MOLECULAR WEIGHT HEPARINS

This chapter provides information and procedures to determine factor Xa inhibitory activity and factor IIa (thrombin) inhibitory activity for unfractionated heparin (UFH) and low molecular weight heparins (LMWH).

## INTRODUCTION

Unfractionated heparin and LMWH exert their anticoagulant effect by potentiating the activity of plasma coagulation inhibitors. Of all the commonly known glycosaminoglycans, only UFH, LMWH, and heparan sulfate (hereafter referred to generally as heparin) contain a specific pentasaccharide sequence that can bind to the plasma coagulation inhibitor, antithrombin (AT). AT-dependent assays, therefore, are developed to ensure the specificity of the methods for measuring the anticoagulant activity of heparin. Binding of heparin to AT induces a conformational change, thereby increasing AT's binding to and subsequent inactivation of activated blood coagulation factors. The major coagulation targets for the AT–heparin complex are factor IIa (thrombin) and coagulation factor Xa. Once AT is activated by the pentasaccharide sequence of heparin, it interacts with factors Xa and IIa via its reactive center loop. For efficient inhibition of thrombin, the heparin molecule also must bind to both AT and IIa. This interaction requires an extra length of approximately 13 monosaccharides attached at the nonreducing end of the AT-binding heparin pentasaccharide sequence. This minimum heparin motif for AT inhibition of thrombin, known as the C-domain, has an approximate molecular weight of 5400 Da. Although the potentiation of AT inactivation of factor Xa also depends on molecular weight, the additional saccharide units of the C-domain are not essential, and heparin with a molecular weight less than 5400 Da can potentiate AT to inactivate factor Xa. By convention, the potency ratio of anti-factor Xa to anti-factor IIa for UFH is 1. Unfractionated heparin is heterogenous and polydisperse but contains little or no material of molecular weight less than 5400 Da. The mean molecular weights of LMWH products are lower than those of UFH, and they contain a higher proportion of material that weighs less than 5400 Da. The potency ratio of anti-factor Xa to anti-factor IIa for LMWH products is greater than 1.5. This chapter describes assay procedures for the measurement of anti-factor Xa and anti-factor IIa activity of LMWH heparin in the presence of AT.

In the test system, heparin is bound to AT, and factor IIa or factor Xa added to the mixture binds to the heparin–AT complex. The residual factor IIa or factor Xa not inhibited by the heparin–AT complex is quantified by a chromogenic substrate that is specific for either factor IIa or factor Xa and is added in the final step. Analysts note an inverse relationship wherein more color is produced by more residual enzyme, which equates to less heparin activity.

As for any enzymatic assay, temperature and timing of the reaction, proper handling of the reagents, and the order in which the reagents are added are critical to the optimal performance of the assay.

### Anti-Factor Xa and Anti-Factor IIa Assays for Unfractionated Heparin

- **ANTI-FACTOR Xa ACTIVITY FOR UFH**

The following procedure is used where specified in the individual monographs. This assay can be performed manually in plastic tubes utilizing heated block stations or water bath. Microtiter plate equipment with a reader and automated coagulometer can improve reproducibility and throughput.

**pH 8.4 buffer:** Dissolve amounts of tris(hydroxymethyl)aminomethane, edetic acid or edetate sodium, and sodium chloride in water containing 0.1% of polyethylene glycol 6000 to obtain solutions having concentrations of 0.050 M, 0.0075 M, and 0.175 M, respectively. Adjust, if necessary, with hydrochloric acid or sodium hydroxide solution to a pH of 8.4.

**Antithrombin solution:** Reconstitute a vial of antithrombin (see *Reagents, Indicators, and Solutions—Reagent Specifications*) as directed by the manufacturer, and further dilute with *pH 8.4 buffer* to obtain a solution having a concentration of 1.0 Antithrombin IU/mL.

**Factor Xa solution:** Reconstitute bovine factor Xa as directed by the manufacturer (see *Factor Xa* in *Reagents, Indicators, and Solutions—Reagent Specifications*), and further dilute in *pH 8.4 buffer* to obtain a solution that gives an absorbance value of 0.65–1.25 at 405 nm when assayed as described below but using 30 µL of *pH 8.4 buffer* instead of 30 µL of the *Standard solutions* or the *Sample solutions*. [NOTE—*Factor Xa solution* contains about 3 nanokatalytic units/mL but can vary depending on the manufacturer of factor Xa or the substrate used.]

**Chromogenic substrate solution:** Prepare a solution of a suitable chromogenic substrate for amidolytic test (see *Reagents, Indicators, and Solutions—Reagent Specifications*) specific for factor Xa in water to obtain a concentration of 1 mM.

**Stopping solution:** 20% (v/v) solution of acetic acid

**Standard solutions:** Reconstitute the entire contents of an ampule of USP Heparin Sodium for Assays RS with water, and dilute with *pH 8.4 buffer* to obtain at least 5 dilutions in the concentration range of 0.03–0.375 USP Heparin Units/mL.

**Sample solutions:** Dissolve or dilute an accurately measured quantity of Heparin Sodium in *pH 8.4 buffer*, and dilute with the same buffer to obtain solutions having activities approximately equal to those of the *Standard solutions*.

**Analysis:** [NOTE—The procedure also can be performed using alternative platforms. Perform the test with each *Standard solution* and *Sample solution* in duplicate.]

To each of a series of suitable plastic tubes placed in a water bath set at 37°, transfer 120 µL of *pH 8.4 buffer*. Then separately transfer 30 µL of the different dilutions of the *Standard solutions* or the *Sample solutions* to the tubes. Add 150 µL of *Antithrombin solution*, prewarmed at 37° for 15 min, to each tube, mix, and incubate for 2 min. Add 300 µL of *Factor Xa solution*, prewarmed at 37° for 15 min, to each tube, mix, and incubate for 2 min. Add 300 µL of *Chromogenic substrate solution*, prewarmed at 37° for 15 min, to each tube, mix, and incubate for exactly 2 min. Add 150 µL of *Stopping solution* to each tube, and mix. Prepare a blank for zeroing the spectrophotometer by adding the reagents in reverse order, starting with the *Stopping solution* and ending with the addition of 150 µL of *pH 8.4 buffer*, and excluding the *Standard solutions* or the *Sample solutions*. Record the absorbance at 405 nm against the blank. The volume of the reactants can be increased or decreased to suit the assay format provided that the proportions of the reference sample or the test sample and the reagents are kept the same.

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**Calculations:** Plot the log of the absorbance values of the *Standard solutions* and the *Sample solutions* vs. heparin concentrations in USP Units. Calculate the activity of heparin sodium in USP Units/mg using statistical methods for slope ratio assays. Calculate the anti-factor Xa activity of heparin sodium:

$$\text{Result} = A \times (S_T/S_S)$$

$A$ = the potency of USP Heparin Sodium for Assays RS
$S_T$ = slope of the line for the *Sample solutions*
$S_S$ = slope of the line for the *Standard solutions*

Express the anti-factor Xa activity of the *Sample solution* as USP Heparin Units/mg, calculated on the dried basis. Calculate the ratio of anti-factor Xa activity against anti-factor IIa potency (see *Assay* below):

anti-factor Xa activity/anti-factor IIa potency

**Acceptance criteria:** 0.9–1.1

• **ANTI-FACTOR IIa ACTIVITY FOR UNFRACTIONATED HEPARIN**

**pH 8.4 buffer:** Dissolve 6.10 g of tris(hydroxymethyl)aminomethane, 10.20 g of sodium chloride, 2.80 g of edetate sodium, and, if suitable, 0–10.00 g of polyethylene glycol 6000 and/or 2.00 g of bovine serum albumin in 800 mL of water. [NOTE—2.00 g of human albumin may be substituted for 2.00 g of bovine serum albumin.] Adjust with hydrochloric acid to a pH of 8.4, and dilute with water to 1000 mL.

**Antithrombin solution:** Reconstitute a vial of antithrombin (see *Reagents, Indicators, and Solutions—Reagent Specifications*) in water to obtain a solution of 5 Antithrombin IU/mL. Dilute this solution with *pH 8.4 buffer* to obtain a solution having a concentration of 0.125 Antithrombin IU/mL.

**Thrombin human solution:** Reconstitute thrombin human (factor IIa) (see *Reagents, Indicators, and Solutions—Reagent Specifications*) in water to obtain a solution of 20 Thrombin IU/mL, and dilute with *pH 8.4 buffer* to obtain a solution having a concentration of 5 Thrombin IU/mL. [NOTE—The thrombin should have a specific activity of NLT 750 IU/mg.]

**Chromogenic substrate solution:** Prepare a solution of a suitable chromogenic thrombin substrate for amidolytic test (see *Reagents, Indicators, and Solutions—Reagent Specifications*) in water to obtain a concentration of 1.25 mM.

**Stopping solution:** 20% (v/v) solution of acetic acid

**Standard solutions:** Reconstitute the entire contents of an ampule of USP Heparin Sodium for Assays RS with water, and dilute with *pH 8.4 buffer* to obtain at least four dilutions in the concentration range of 0.005–0.03 USP Heparin Unit/mL.

**Sample solutions:** Proceed as directed for *Standard solutions* to obtain concentrations of Heparin Sodium similar to those obtained for the *Standard solutions*.

**Analysis:** [NOTE—The procedure also can be performed using alternative platforms.] For each dilution of the *Standard solutions* and the *Sample solutions*, at least duplicate samples should be tested. Label a suitable number of tubes depending on the number of replicates that will be tested. For example, if five blanks will be used: B1, B2, B3, B4, and B5 for the blanks; T1, T2, T3, and T4 each at least in duplicate for the dilutions of the *Sample solutions*; and S1, S2, S3, and S4 each at least in duplicate for the dilutions of the *Standard solutions*. Distribute the blanks over the series in such a way that they accurately represent the behavior of the reagents during the experiments. [NOTE—Treat the tubes in the order B1, S1, S2, S3, S4, B2, T1, T2, T3, T4, B3, T1, T2, T3, T4, B4, S1, S2, S3, S4, B5.] Note that after each addition of a reagent, the incubation mixture should be mixed without allowing bubbles to form. Add twice the volume (100–200 µL) of *Antithrombin solution* to each tube containing one volume (50–100 µL) of either the *pH 8.4 buffer* or an appropriate dilution of the *Sample solutions* or the *Standard solutions*. Mix, but do not allow bubbles to form. Incubate at 37° for at least 1 min. Add to each tube 25–50 µL of *Thrombin human solution*, and incubate for at least 1 min. Add 50–100 µL of *Chromogenic substrate solution*. Note that all reagents, *Standard solutions*, and *Sample solutions* should be prewarmed to 37° just before use. The volume of the reactants can be increased or decreased to suit the assay format provided that the proportions of the reference sample or the test sample and the reagents are kept the same.

Two different types of measurements can be recorded:

1. Endpoint Measurement: Stop the reaction after at least 1 min with 50–100 µL of *Stopping solution*. Measure the absorbance of each solution at 405 nm using a suitable spectrophotometer (see *Ultraviolet-Visible Spectroscopy* ⟨857⟩). The RSD over the blank readings is less than 10%.
2. Kinetic Measurement: Follow the change in absorbance for each solution over 1 min at 405 nm using a suitable spectrophotometer (see ⟨857⟩). Calculate the change in absorbance/min (ΔOD/min). The blanks for kinetic measurement are also expressed as ΔOD/min and should give the highest values because they are carried out in the absence of heparin. The RSD over the blank readings is less than 10%.

**Calculations:** The statistical models for *Slope ratio assay* or *Parallel-line assay* can be used depending on which model best describes the correlation between concentration and response.

**Slope ratio assay:** For each series, calculate the regression of the log absorbance or the log change in absorbance/min against concentrations of the *Sample solutions* and of the *Standard solutions*, and calculate the potency of heparin sodium in USP Units/mL using statistical methods for slope ratio assays. Express the potency of heparin sodium/mg, calculated on the dried basis.

**Parallel-line assay:** For each series, calculate the regression of the absorbance or change in absorbance/min against log concentrations of the *Sample solutions* and the *Standard solutions*, and calculate the potency of heparin sodium in USP Units/mL using statistical methods for parallel-line assays. Express the potency of heparin sodium/mg, calculated on the dried basis.

**Acceptance criteria:** The potency of heparin sodium, calculated on the dried basis, is NLT 180 USP Heparin Units in each mg.

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***Change to read:***

## Anti-Factor Xa and Anti-Factor IIa Assays for Low Molecular Weight Heparins

▲The following procedure is used where specified in the individual monographs. This assay can be performed manually in plastic tubes utilizing heated block stations or water bath. Microtiter plate equipment with a reader and automated coagulometer can improve reproducibility and throughput. Acetic acid solution (stopping solution) is used for manual and microtiter plate assay. Automated coagulometers measure initial kinetic rate, and because of that, stopping of the reaction is not needed.▲ (ERR 1-Aug-2020)

• **Anti-Factor Xa Activity for Low Molecular Weight Heparin**

**Acetic acid solution:** Glacial acetic acid and water (42:58)

**pH 7.4 polyethylene glycol 6000 buffer:** Dissolve 6.08 g of tris(hydroxymethyl)aminomethane and 8.77 g of sodium chloride in 500 mL of water. Add 1.0 g of polyethylene glycol 6000 or 10.0 g of bovine serum albumin, adjust with hydrochloric acid to pH 7.4, and dilute with water to 1000 mL.

**pH 7.4 buffer:** Dissolve 6.08 g of tris(hydroxymethyl)aminomethane and 8.77 g of sodium chloride in 500 mL of water. Adjust with hydrochloric acid to pH 7.4, and dilute with water to 1000 mL.

**pH 8.4 buffer:** Dissolve 3.03 g of tris(hydroxymethyl)aminomethane, 5.12 g of sodium chloride, and 1.40 g of edetate sodium in 250 mL of water. Adjust with hydrochloric acid to a pH of 8.4, and dilute with water to 500 mL.

**Human antithrombin solution:** Reconstitute a vial of human antithrombin (see *Reagents, Indicators, and Solutions—Reagent Specifications*) in water to obtain a solution containing 5 Antithrombin Units per mL. Dilute this solution with *pH 7.4 polyethylene glycol 6000 buffer* to obtain a solution having a concentration of 1.0 Antithrombin Unit/mL.

**Factor Xa solution:** Reconstitute an accurately weighed quantity of bovine factor Xa (see *Reagents, Indicators, and Solutions—Reagent Specifications*) as directed by the manufacturer's instructions. Further dilute the stock solution with *pH 7.4 polyethylene glycol 6000 buffer* to obtain a solution that gives an increase in absorbance value at 405 nm of NMT 0.20 absorbance units/min or 0.8 absorbance units after 4 min of incubation with the chromogenic substrate when assayed as described below but using as an appropriate volume, *V*, the volume in µL of *pH 7.4 buffer* instead of *V* µL of the standard or the sample solution.

**Chromogenic substrate solution:** Prepare a solution of a suitable chromogenic substrate for amidolytic test (see *Reagents, Indicators, and Solutions—Reagent Specifications*) for factor Xa in water to obtain a concentration of about 3 mM. Dilute with substrate *pH 8.4 buffer* to obtain a solution having a concentration of 0.5 mM. [Note—Preheat reagents to 37 ± 1° 15 min before use.]

**Standard solutions:** Reconstitute and dilute the entire contents of an ampule of USP Low Molecular Weight Heparin for Bioassays RS with distilled water and then further dilute with *pH 7.4 buffer* to obtain at least four dilutions in the concentration range of 0.025–0.2 USP anti-factor Xa Units/mL.

**Sample solutions:** Proceed as directed for *Standard solutions* to obtain concentrations of LMWH similar to those obtained for the *Standard solutions.*

**Analysis**

**Samples:** *Standard solutions, Sample solutions, Human antithrombin solution, pH 7.4 buffer, Factor Xa solution, Chromogenic substrate solution,* and *Acetic acid solution*

Label 18 suitable tubes: B1 and B2 for blanks; T1, T2, T3, and T4 each in duplicate for the dilutions of the *Sample solutions;* and S1, S2, S3, and S4 each in duplicate for the dilutions of the *Standard solutions.* [Note—Treat the tubes in the order B1, S1, S2, S3, S4, T1, T2, T3, T4, T1, T2, T3, T4, S1, S2, S3, S4, B2.] To each tube add 50 µL of *Human antithrombin solution* and an equal volume, *V,* of either the blank, *pH 7.4 buffer*, or an appropriate dilution of the *Sample solutions* or the *Standard solutions.* Mix, but do not allow bubbles to form. Incubate at 37° for 1.0 min. Add to each tube 100 µL of *Factor Xa solution,* and incubate for 1.0 min. Add 250 µL of *Chromogenic substrate solution.* Stop the reaction after about 4.0 min with 250 µL of *Acetic acid solution.* Measure the absorbance of each solution at 405 nm using a suitable spectrophotometer (see ⟨857⟩). Use quartz or disposable polystyrene cuvettes. The volume of the reactants can be increased or decreased to suit the assay format provided that the proportions of the reference sample or the test sample and the reagents are kept the same.

**System suitability:** The assay is valid if the following requirements are met:

1. A blank solution gives an increase in absorbance value at 405 nm of NMT 0.20 absorbance units/min (or 0.8 absorbance units in total) when assayed using an appropriate volume (50 µL) of *pH 7.4 buffer* instead of 50 µL of the *Standard solution* or the *Sample solution*.
2. The reading of the blank B2 is not more than ±0.05 absorbance units against blank B1.

**Calculations:** For this bioassay, parallel-line or slope ratio analysis can be applied.

Calculate the potency of the test sample in USP anti-factor Xa Unit/mL, using a statistical model for parallel assays, plotting absorbance against log concentrations of the *Sample solutions* and of the *Standard solutions.* In some cases, log transformation of absorbance may be needed to obtain linearity for the dose–response curves. The assay is valid when the data fulfill the acceptance criteria for regression, linearity, and parallelism as required for parallel line assay. For slope ratio analysis, plot absorbance against concentrations of *Sample solutions* and of the *Standard solutions.* In some cases, log transformation of absorbance may be needed to obtain linearity for the dose–response curves. The assay is valid when the data fulfill the acceptance criteria for regression, linearity, and common intercept as required for slope ratio assay (see *Design and Analysis of Biological Assays* ⟨111⟩ and *Analysis of Biological Assays* ⟨1034⟩).

Express the anti-factor Xa activity of sample/mg, calculated on the dried basis:

$$\text{Anti-factor Xa USP Unit/mg} = [\text{standard potency (USP Unit/mL)} \times \text{potency ratio}]/[\text{sample concentration (mg/mL)}]$$

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• **ANTI-FACTOR IIa ACTIVITY FOR LOW MOLECULAR WEIGHT HEPARIN**

**Acetic acid solution, pH 7.4 polyethylene glycol 6000 buffer, pH 7.4 buffer, pH 8.4 buffer,** and **Human antithrombin solution:** Proceed as directed in the *Anti-Factor Xa Activity*, except that the concentration of the *Human antithrombin solution* is 0.5 Antithrombin Unit/mL.

**Thrombin human solution:** Reconstitute thrombin (see *Reagents, Indicators, and Solutions—Reagent Specifications*) in water, and dilute in *pH 7.4 polyethylene glycol 6000 buffer* to obtain a solution having a concentration of 5 Thrombin Units/mL. Use the *Thrombin human solution* immediately after preparation.

**Chromogenic substrate solution:** Prepare a solution of a suitable chromogenic substrate for the amidolytic test (see *Reagents, Indicators, and Solutions—Reagent Specifications*) for thrombin in water to obtain a concentration of about 3 mM. Dilute immediately before use with *pH 8.4 buffer* to 0.5 mM.

**Standard solutions:** Reconstitute and dilute the entire contents of an ampule of USP Low Molecular Weight Heparin for Bioassays RS with distilled water, and further dilute with *pH 7.4 buffer* to obtain at least four dilutions having concentrations in the range of 0.015–0.075 USP Unit of anti-factor IIa activity/mL.

**Sample solutions:** Proceed as directed for *Standard solutions* to obtain concentrations of LMWH similar to those obtained for the *Standard solutions*.

**Procedure:** Proceed as directed under *Anti-Factor Xa activity*, but use *Thrombin human solution* instead of *Factor Xa solution* and use the *Human antithrombin solution* as described above.

**System suitability:** The assay is valid if the following requirement is met:

1. The reading of blank B2 is NMT ±0.05 absorbance units against blank B1.

**Calculations:** Proceed as directed for calculation of *Anti-Factor Xa Activity*.

## ADDITIONAL REQUIREMENTS

• **USP REFERENCE STANDARDS** ⟨11⟩

USP Heparin Sodium for Assays RS
USP Low Molecular Weight Heparin for Bioassays RS

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# ⟨209⟩ LOW MOLECULAR WEIGHT HEPARIN MOLECULAR WEIGHT DETERMINATIONS

This chapter provides procedures used to determine molecular weight (MW) distribution and weight-average molecular weight for low molecular weight heparins (LMWH).

## INTRODUCTION

Low molecular weight heparins are prepared from Heparin Sodium, USP, by partial depolymerization. LMWHs are polydisperse, i.e., they are made up of polysaccharide chains with a range of molecular weights. The MW distribution is a defining characteristic for each LMWH product. The *USP* contains specific monographs for LMWH products, including limits on molecular weight distribution parameters.

Most techniques for determination of the MW distribution of LMWHs make use of gel permeation chromatography (GPC), sometimes referred to as size exclusion chromatography (SEC). In order to derive molecular weight distribution from a chromatogram, it is necessary to know the relationship between retention time and MW. This chapter describes a GPC method that is calibrated using the USP Low Molecular Weight Heparin Molecular Weight Calibrant RS. This preparation is a single polydisperse standard for which the distribution of molecular weights is described in the form of a Broad Standard Table (see the USP Certificate for USP Low Molecular Weight Heparin Molecular Weight Calibrant RS) that lists the log of the molecular weight (log MW) of a number of reference points and the matching percent fractions by weight of the calibrant above or below these reference points. The Broad Standard Table is used together with a chromatogram of the calibrant to fit a suitable function, in this case a third degree polynomial, to the relationship between log MW and retention time, thus generating a calibration curve for the chromatographic system. With this calibration curve, the analyst calculates the weight-average molecular weight and distribution parameters from the chromatogram of a sample of LMWH.

All the calculations described in this chapter may be performed using a spreadsheet program, but this method is not recommended as it is laborious and open to human error. Proprietary software capable of automating the calibration and molecular weight calculations can be obtained from a number of chromatography systems manufacturers.

## PROCEDURE

***Change to read:***

- **Molecular Weight Measurements of Low Molecular Weight Heparins by Gel Permeation Chromatography**

The following procedure, with any necessary variations, is used where specified in the individual monographs.

**Ammonium acetate stock solution:** 1 M ammonium acetate in water

**Sodium azide solution:** 1% (w/v) sodium azide in water

**Mobile phase:** Transfer 100 mL of *Ammonium acetate stock solution* to a 1-L volumetric flask, add 20 mL of *Sodium azide solution*, and dilute with water to volume. Pass through a nylon membrane of 0.45-µm pore size prior to use.

**Calibration solution:** Add 2 mL of *Mobile phase* to a vial containing USP Low Molecular Weight Heparin Molecular Weight Calibrant RS. Filter using a nylon membrane of 0.45-µm pore size prior to use.

**Sample solution:** 5 mg/mL of low molecular weight heparin sample in *Mobile phase*. Pass through a nylon membrane of 0.45-µm pore size.

**System suitability solution:** As indicated in the product monograph. [Note—In the cases where no product monograph is available, 5 mg/mL of USP Dalteparin Sodium RS, in *Mobile phase* and filtered using a nylon membrane of 0.45-µm pore size, can be applied as the system suitability solution.]

**Chromatographic system**

(See *Chromatography* ⟨621⟩, *System Suitability*.)

[Note—The temperature of the refractive index detector must be set at the same temperature as that of the *Column temperature*.]

**Mode:** LC

**Detector:** Refractive index

**Columns**

**Analytical:** 7.8-mm × 30-cm; 5-µm packing L59 in series with a 7.8-mm × 30-cm; 5-µm packing L59[1]

**Guard:** 6-mm × 4-cm; 7-µm packing L59

**Column temperature:** 30°

**Flow rate:** 0.5 mL/min

**Column equilibration:** 0.5 mL/min for at least 2 h

**Injection volume:** 20 µL

**System suitability**

**Sample:** *System suitability solution*

**Suitability requirements**

**Resolution:** There is baseline resolution between the last peak of the USP Low Molecular Weight Heparin Molecular Weight Calibrant RS and the salt peak, or negative exchange peaks.

**Calibration curve:** The coefficient of determination of the calibration curve fitted to the Broad Standard Table values must be NLT 0.990, using a third-order polynomial equation.

**Weight-average molecular weight ($M_w$):** As indicated in the product monograph. [Note—In the cases where no product monograph is available and 5 mg/mL of USP Dalteparin Sodium RS is applied as the *System suitability solution*, take the mean of the calculated $M_w$ from the duplicate injections of the *System suitability solution*, and round

[1] The method was validated using a guard column TSK SWXL 6-mm × ▲4-cm▲ (ERR 1-Apr-2023); 7-µm in series with two analytical columns: TSK G3000 SWXL 7.8-mm × 30-cm; 5-µm in series with a TSK G2000 SWXL 7.8-mm × 30-cm; 5-µm.

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to the nearest 50 Da. The chromatographic system is suitable if the $M_w$ of the USP Dalteparin Sodium RS is within 150 Da of the labeled value as stated in the USP Certificate for USP Dalteparin Sodium RS.]

**Analysis**

**Samples:** Inject 20 µL of the *Calibration solution* (single injection), *System suitability solution* (duplicate injections), and *Sample solution* (duplicate injections), and record the chromatograms for a length of time to ensure complete elution, including salt and solvent peaks (about 50 min).

[NOTE—The *Calibration solution*, *System suitability solution*, or *Sample solution* will give a broad peak between about 25 and 45 min, followed by a later eluting narrow salt peak, as illustrated in the USP Certificate for USP Low Molecular Weight Heparin Molecular Weight Calibrant RS.]

**Calculations:** Calculate the total area under the low molecular weight heparin peak from the *Calibration solution* and the cumulative area at each point under the peak as a percent of the total. Do not include the salt peak. Using the Broad Standard Table provided in the USP Certificate for USP Low Molecular Weight Heparin Molecular Weight Calibrant RS, identify those points in the chromatogram for which the percent cumulative area is closest to the percent fractions listed in the Broad Standard Table, and assign the MW in this Table to the corresponding retention time (RT) in the chromatogram. For the set of retention times and molecular weights identified, fit log(MW) vs. RT to a third-order polynomial function using suitable GPC software, or find values of $a$, $b$, $c$, and $d$ such that $\log MW = a + (b \times RT) + [c \times (RT)^2] + [d \times (RT)^3]$.

Using the same GPC software, for each of the duplicate chromatograms of the *System suitability solution* and the *Sample solution*, with the calibration function derived as described above, calculate $M_w$:

$$M_w = \Sigma(RI_i \times M_i)/\Sigma RI_i$$

$RI_i$ = detector response at each point
$M_i$ = MW at each point

Round the mean value of $M_w$ to the nearest 50 Da. Using the same GPC software, determine for each of the duplicate *Sample solution* chromatograms the percentage of product with molecular weight as indicated in the product monograph.

**Acceptance criteria:** As indicated in the product monograph

## ADDITIONAL REQUIREMENTS

- **USP REFERENCE STANDARDS** ⟨11⟩

USP Dalteparin Sodium RS
USP Low Molecular Weight Heparin Molecular Weight Calibrant RS

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# ⟨210⟩ MONOSACCHARIDE ANALYSIS

## INTRODUCTION

The glycan composition of therapeutic glycoproteins may impact the biological activity, stability, pharmacokinetics, pharmacodynamics, efficacy, and immunogenicity of the molecule. The glycosylation pattern, or specific aspects of it such as the degree of sialylation, may form part of the product characterization and release specifications. One approach to ensuring the consistency of glycosylation and compliance of the product with specifications is to quantify specific monosaccharides present in the product. Monosaccharides are released by degradation of the product and quantified by chromatographic approaches. The most commonly determined sugars are *N*-acetylneuraminic acid (Neu5Ac) and its variant *N*-glycolylneuraminic acid (Neu5Gc). These monosaccharides can be selectively released from the protein either by dilute acid hydrolysis or by enzymatic methods, whereas increasingly aggressive hydrolysis conditions release neutral monosaccharides including mannose, galactose, and fucose, and then glucosamine and galactosamine. Separation and quantification of released monosaccharides by high-performance liquid chromatography (HPLC) can be achieved with or without fluorophore labeling.

## SAMPLE HANDLING PRIOR TO MONOSACCHARIDE RELEASE

The glycoprotein sample should be as free of salts, excipients, and other carrier materials as possible. This is typically achieved by: 1) dialysis against water or a volatile buffer using an appropriate membrane, or ultrafiltration through an appropriate membrane; 2) HPLC on an appropriate gel-permeation column eluted with water or a volatile buffer, monitored by UV absorbance or refractive index; 3) sample trapping on a solid-phase extraction (SPE) cartridge, followed by washing away salts and excipients and elution of the required sample. Where residual components are known to be present, their lack of interference in saccharide analysis should be confirmed during validation to verify either no bias or an acceptable level of bias due to matrix components. Sample concentration may be required.

***Change to read:***

## SIALIC ACID QUANTIFICATION

Sialic acids can be analyzed and quantified through the use of high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) without modification, or by the use of 1,2-diamino-4,5-methylenoxybenzene (DMB) labeling and reversed-phase HPLC (RP-HPLC) analysis equipped with a fluorescence detector. The combination of DMB labeling and RP-HPLC with fluorescence detection is significantly more sensitive than HPAEC-PAD analysis.

[NOTE—Under some circumstances, the presence of large amounts of protein may diminish the PAD response. For analysis by HPAEC-PAD, the use of 3-deoxy-D-glycero-D-galacto-2-nonulosonic acid (KDN), as an internal standard, can be an option to compensate for this effect. An example is to prepare an internal standard solution: 0.1 mM of ▲KDN▲ (USP 1-May-2021) in water. In the final dilution step, add 15 µL of the internal standard solution into each standard or sample solution, and reduce the amount of water added by 15 µL to keep the final volume of solution unchanged. Integrate the Neu5Ac, Neu5Gc, and KDN peaks in each chromatogram, and use the ratio of the Neu5Ac or Neu5Gc peak areas relative to the peak area of KDN for system suitability and sample analysis calculation.]

Sialic acids may be *O*-acetylated, and depending on the protein sample and the analytical procedure selected, it may be necessary to de-*O*-acetylate before sialic acid is released. Normally, dilute acid hydrolysis and anion exchange chromatography analysis lead to de-*O*-acetylation. Enzymatic methods of release can preserve *O*-acetyl groups, and the combined use of DMB labeling and RP-HPLC analysis (as in *Procedure 3*) retains the *O*-acetyl groups during the analytical steps.

A typical de-*O*-acetylation can be achieved by adding 10% of the sample's volume of 1 M sodium hydroxide to the sample, incubating at room temperature for 30 min, and then adding 10% of the sample's volume of 1 M hydrochloric acid to neutralize the reaction (e.g., to 20 µL of sample add 2 µL of 1 M sodium hydroxide, incubate at room temperature for 30 min, and then add 2 µL of 1 M hydrochloric acid to neutralize).

## PROCEDURES

***Change to read:***

- **PROCEDURE 1: ENZYMATIC RELEASE AND ANALYSIS BY HPAEC-PAD OF UNLABELED SIALIC ACIDS**

  **Sample buffer:** 20 mM sodium acetate prepared as follows. Dissolve 164 mg of sodium acetate anhydrous in 80 mL of water. Adjust with glacial acetic acid to a pH of 5.2, and dilute with water to 100 mL.

  **Standard stock solution:** 50 µmol/L (µM) of USP *N*-Acetylneuraminic Acid RS and 2 µM of USP *N*-Glycolylneuraminic Acid RS in water

  **Standard solutions:** See *Table 1* for typical five-level calibration *Standard solutions*.

**Table 1**

| Standard Solutions | Standard Stock Solution (µL) | Sample Buffer (µL) | Water (µL) | Concentration of Neu5Ac (µM) | Concentration of Neu5Gc (µM) |
|---|---|---|---|---|---|
| Blank | 0 | 200 | 300 | 0 | 0 |
| 1 | 10 | 200 | 290 | 1 | 0.04 |
| 2 | 20 | 200 | 280 | 2 | 0.08 |
| 3 | 30 | 200 | 270 | 3 | 0.12 |
| 4 | 40 | 200 | 260 | 4 | 0.16 |

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**Table 1** *(continued)*

| Standard Solutions | Standard Stock Solution (µL) | Sample Buffer (µL) | Water (µL) | Concentration of Neu5Ac (µM) | Concentration of Neu5Gc (µM) |
|---|---|---|---|---|---|
| 5 | 50 | 200 | 250 | 5 | 0.2 |

[NOTE—1 µM = 1 nmol/mL = 1 × $10^{-3}$ nmol/µL = 1 pmol/µL.]

**Sample solution:** To 50 µL of 6–60 µg/mL of protein sample (equivalent to about 1.5 nmol of sialic acid) in *Sample buffer* add 4 µL of 0.5 milliunit/µL (2 milliunits) of neuraminidase[1] in water and make up to a total volume of 200 µL with *Sample buffer*. Incubate for 5 h at 37°. [NOTE—Or use validated sample preparation and time/temperature ranges.] Add 300 µL of water, and mix.

**HPAEC analysis**

[NOTE—Use either *Chromatographic system 1* or *Chromatographic system 2*.]

**Solution A:** 0.1 M sodium hydroxide prepared as follows. Dilute 5.28 mL of 50%–52% sodium hydroxide solution with water to 1000 mL. [NOTE—The solution must be sealed well to avoid absorbing carbon dioxide.]

**Solution B:** 1 M sodium acetate in 0.1 M sodium hydroxide prepared as follows. Dissolve 82.0 g of anhydrous sodium acetate in 800 mL of water. Pass through a nylon filter of 0.2-µm pore size. Add 5.28 mL of 50%–52% sodium hydroxide solution, and dilute with water to 1000 mL.

**Chromatographic system 1**

(See *Chromatography* ⟨621⟩, *System Suitability*.)

**Mobile phase:** See *Table 2*.

**Table 2**

| Time (min) | Solution A (%) | Solution B (%) |
|---|---|---|
| 0 | 98 | 2 |
| 2 | 98 | 2 |
| 9.5 | 82 | 18 |
| 11 | 82 | 18 |
| 11.5 | 98 | 2 |
| 17 | 98 | 2 |

**Mode:** LC

**Detector:** Integrated amperometric detector with gold electrode (for an example of a waveform, see *Table 4*)

**Columns**

**Guard:** 3-mm × 3-cm; packing L69

**Analytical:** 3-mm × 15-cm; packing L69

**Flow rate:** 0.5 mL/min

**Injection volume:** 50 µL

[NOTE—For the system described, typical retention times for Neu5Ac and Neu5Gc are about 8 and 12 min, respectively.]

**Chromatographic system 2**

(See *Chromatography* ⟨621⟩, *System Suitability*.)

**Mobile phase:** See *Table 3*.

**Table 3**

| Time (min) | Solution A (%) | Solution B (%) |
|---|---|---|
| 0 | 93 | 7 |
| 10 | 70 | 30 |
| 11 | 70 | 30 |
| 12 | 93 | 7 |
| 15 | 93 | 7 |

**Mode:** LC

**Detector:** Integrated amperometric detector with gold electrode (for an example of a waveform, see *Table 4*)

**Columns**

**Guard:** 4-mm × 5-cm; 10-µm packing L46[2]

[1] Neuraminidase: recombinant *Arthrobacter ureafaciens* neuraminidase produced in *Escherichia coli* (*E. coli*), or similar enzyme, that releases both α2-3 and α2-6-linked sialic acid. One unit is defined as the amount of enzyme that will hydrolyze 1 µmol of 2′-*O*-substituted α-D-*N*-acetylneuraminic acid/min at the specified pH at 37°.

[2] Ethylvinylbenzene/divinylbenzene substrate available from Thermo as Carbopac PA10, or a suitable equivalent.

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**Analytical:** 4-mm × 25-cm; 10-µm packing L46[2]
**Flow rate:** 0.8 mL/min
**Injection volume:** 50 µL
[NOTE—For the system described, typical retention times for Neu5Ac and Neu5Gc are about 6 and 10 min, respectively.]

**Table 4**

| Time (s) | Potential (V) | Gain Region | Ramp | Integration |
|---|---|---|---|---|
| 0 | 0.1 | Off | Ramp | Off |
| 0.2 | 0.1 | On | Ramp | On |
| 0.4 | 0.1 | Off | Ramp | Off |
| 0.41 | −2 | Off | Ramp | Off |
| 0.42 | −2 | Off | Ramp | Off |
| 0.43 | 0.6 | Off | Ramp | Off |
| 0.44 | −0.1 | Off | Ramp | Off |
| 0.5 | −0.1 | Off | Ramp | Off |

**System suitability**
**Samples:** *Blank* and *Standard solutions*
**Suitability requirements**
**Chromatogram of the blank:** No peaks at the same retention time as Neu5Ac or Neu5Gc, *Blank*
**▲Coefficient of determination ($R^2$)▲ (USP 1-May-2021) calculated for the standard curve:** NLT ▲0.98▲ (USP 1-May-2021), *Standard solutions*
**Relative standard deviation:** NMT 5%, *Standard solution 3*

**Analysis**
**Samples:** *Standard solutions* and *Sample solution*
For the *Standard solutions*, generate a Neu5Ac and Neu5Gc standard curve of peak response versus concentrations using least square regression. From the standard curve, determine the concentration of Neu5Ac and Neu5Gc in the *Sample solution*. Divide the determined Neu5Ac and Neu5Gc concentrations by the protein concentration in the *Sample solution*, and report as molar ratio (nmol:nmol). [NOTE—Convert the protein concentration from mg/mL to µM (1 µM = 1 nmol/mL).]

• **PROCEDURE 2: ACID HYDROLYSIS AND ANALYSIS BY HPAEC-PAD OF UNLABELED SIALIC ACIDS**
**Sample buffer, HPAEC analysis, System suitability,** and **Analysis:** Proceed as directed in *Procedure 1*.
[NOTE—*Standard solutions* and *Sample solution* are to be prepared at the same time and subjected to the same hydrolysis and drying procedures. Using vacuum centrifuge for drying is described in the procedures; other suitable drying methods may also be used.]
[NOTE—Sialic standards subjected to the hydrolysis procedure may degrade to a different extent than sialic acids released from a glycoprotein during the procedure. A validated method in which the standard is not subjected to the hydrolysis conditions may be used.]
**Standard stock solution:** 50 µM of USP *N*-Acetylneuraminic Acid RS and 2 µM of USP *N*-Glycolylneuraminic Acid RS in water
**Standard solutions:** Add the amount of *Standard stock solution* to each microcentrifuge tube as indicated in *Table 5*.

**Table 5**

| Standard Solutions | Standard Stock Solution (µL) | Neu5Ac (nmol) | Neu5Gc (nmol) |
|---|---|---|---|
| Blank | 0 | 0 | 0 |
| 1 | 10 | 0.5 | 0.02 |
| 2 | 20 | 1 | 0.04 |
| 3 | 30 | 1.5 | 0.06 |
| 4 | 40 | 2 | 0.08 |
| 5 | 50 | 2.5 | 0.1 |

Dry each standard in a vacuum centrifuge without heat. Add 100 µL of 2 M acetic acid to each microcentrifuge tube, and hydrolyze all the standards series at approximately 80° for 2 h ± 15 min in a heating block. [NOTE—Or use validated time/temperature ranges.] Allow the tubes to cool to room temperature for approximately 10 min, and briefly centrifuge to ensure the entire sample is in the well of the tube. Dry the solutions in a vacuum centrifuge without heat, and resuspend in 300 µL of water. Before analysis, add 200 µL of *Sample buffer*. The final concentrations are summarized in *Table 6*.

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**Table 6**

| Standard Solutions | Concentration of Neu5Ac (μM) | Concentration of Neu5Gc (μM) |
|---|---|---|
| Blank | 0 | 0 |
| 1 | 1 | 0.04 |
| 2 | 2 | 0.08 |
| 3 | 3 | 0.12 |
| 4 | 4 | 0.16 |
| 5 | 5 | 0.2 |

**Sample solution:** Add desalted protein, approximately 6–60 μg (equivalent to about 1.5 nmol of sialic acid) into a microcentrifuge tube. Dry in a vacuum centrifuge without heat. Add 100 μL of 2 M acetic acid, and hydrolyze at approximately 80° for 2 h ± 15 min in a heating block. [NOTE—Or use validated sample preparation and time/temperature ranges.] Allow the tube to cool to room temperature for approximately 10 min and briefly centrifuge to ensure the entire sample is in the well of the tube. Dry the sample in a vacuum centrifuge without heat, and resuspend the sample in 300 μL of water. Before analysis add 200 μL of *Sample buffer*.

***Change to read:***

- **PROCEDURE 3: ENZYMATIC HYDROLYSIS AND ANALYSIS BY RP-HPLC OF DMB-LABELED SIALIC ACIDS**

**Labeling reagent:** Aqueous solution of DMB, acetic acid, 2-mercaptoethanol, and sodium hydrosulfite[3]

**Standard stock solution:** 200 μM of USP *N*-Acetylneuraminic Acid RS and 10 μM of USP *N*-Glycolylneuraminic Acid RS in water

**Standard solutions:** See *Table 7* for the typical dilutions. Then label each solution as directed by *Labeling*.

**Table 7**

| Standard Solutions | Standard Stock Solution (μL) | Water (μL) | Concentration of Neu5Ac before Labeling (μM) | Concentration of Neu5Gc before Labeling (μM) |
|---|---|---|---|---|
| Blank | 0 | 400 | 0 | 0 |
| 1 | 4 | 396 | 2 | 0.1 |
| 2 | 8 | 392 | 4 | 0.2 |
| 3 | 20 | 380 | 10 | 0.5 |
| 4 | 40 | 360 | 20 | 1 |
| 5 | 80 | 320 | 40 | 2 |

The concentrations of the resulting labeled *Standard solutions* are listed in *Table 8*.

**Table 8**

| Standard Solutions | Concentration of Neu5Ac after Labeling (μM) | Concentration of Neu5Gc after Labeling (μM) |
|---|---|---|
| Blank | 0 | 0 |
| 1 | 0.02 | 0.001 |
| 2 | 0.04 | 0.002 |
| 3 | 0.1 | 0.005 |
| 4 | 0.2 | 0.01 |
| 5 | 0.4 | 0.02 |

**Labeling:** Transfer 5 μL of the to-be-labeled *Standard solution* or *Sample solution* into a microcentrifuge tube, add 20 μL of *Labeling reagent*, cap the tube, mix by vortexing, and incubate for 3 h at 50° in the dark. Terminate the reaction by adding 475 μL of water, cap, and vortex thoroughly. This solution should be kept at 2°–8° and analyzed within 24 h. [NOTE—The *Standard solution* and *Sample solution* are to be labeled simultaneously.]

[3] Available from Ludger as LudgerTag Sialic Acid DMB Labeling Kit, catalog #LT-KDMB-A1, or a suitable equivalent. [Note—For example, prepare the DMB reagent in the order that follows. Add 1.5 mL of water to a glass vial. To this solution add 172 μL of glacial acetic acid, and mix well. Add 112 μL of 2-mercaptoethanol, and mix well. Add 4.9 mg of sodium hydrosulfite to the solution, and mix well. The solution may then become cloudy in appearance. Lastly, add 3.5 mg of DMB hydrochloride and 200 μL of water, and mix the solution well. This solution is aliquoted into 200-μL aliquots and can be stored in the dark at –20° for up to 12 months.]

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**Sample solution:** Transfer approximately 0.5–50 µg of protein (equivalent to about 5 pmol of sialic acid) into a 1.5-mL microcentrifuge tube. Perform de-*O*-acetylation if needed. Adjust to a pH of 6.0 ± 0.3. [NOTE—The pH needs to be optimized based on the enzyme used.] Add 2 µL of 5 units/mL (10 milliunits) of neuraminidase,[1] adjust to a total volume of 50 µL, and incubate for 16–24 h at 37°. [NOTE—Or use validated sample preparation and time/temperature ranges.] Label the solution as directed in *Labeling* before analysis.

**RP-HPLC analysis**

[NOTE—Use either *Chromatographic system 3* or *Chromatographic system 4*.]

**Mobile phase:** Acetonitrile, methanol, and water (9:7:84)

**Chromatographic system 3**

(See *Chromatography* ⟨621⟩, *System Suitability*.)

**Mode:** LC

**Detector:** Fluorescence detector with an excitation wavelength of 373 nm and an emission wavelength of 448 nm

**Column:** 2.1-mm × 10-cm; 1.9- to 2.2-µm packing L1

**Flow rate:** 0.25 mL/min

**Injection volume:** 5 µL

**Temperatures**

**Column:** 30°

**Autosampler:** 2°–8°

**Run time:** 10 min (isocratic)

[NOTE—For the system described, typical retention times for Neu5Ac and Neu5Gc are about 2.5 and 2 min, respectively.]

**Chromatographic system 4**

(See *Chromatography* ⟨621⟩, *System Suitability*.)

**Mode:** LC

**Detector:** Fluorescence detector with an excitation wavelength of 373 nm and an emission wavelength of 448 nm

**Column:** 4.6-mm × 15-cm; 3-µm packing L1

**Flow rate:** 0.5 mL/min

**Injection volume:** 5 µL

**Temperatures**

**Column:** 30°

**Autosampler:** 2°–8°

**Run time:** 30 min (isocratic)

[NOTE—For the system described, typical retention times for Neu5Ac and Neu5Gc are about 10 and 8 min, respectively.]

**System suitability**

**Samples:** *Blank* and *Standard solutions*

**Suitability requirements**

**Chromatogram of the blank:** No peaks at the same retention time as Neu5Ac or Neu5Gc, *Blank*

**▲Coefficient of determination ($R^2$)▲ (USP 1-May-2021) calculated for the standard curve:** NLT ▲0.98▲ (USP 1-May-2021), *Standard solutions*

**Relative standard deviation:** NMT 5%, *Standard solution 3*

**Analysis**

**Samples:** *Standard solutions* and *Sample solution*

For the *Standard solutions*, generate a Neu5Ac and Neu5Gc standard curve of peak response versus concentrations using least square regression. From the standard curve, determine the concentration of Neu5Ac and Neu5Gc in the *Sample solution*. Divide the determined Neu5Ac and Neu5Gc concentrations by the protein concentration in the *Sample solution*, and report as molar ratio (nmol:nmol). [NOTE—Convert the protein concentration from mg/mL to µM (1 µM = 1 nmol/mL).]

• **PROCEDURE 4: ACID HYDROLYSIS AND ANALYSIS BY RP-HPLC OF DMB-LABELED SIALIC ACIDS**

**Labeling reagent, Blank, Labeling, RP-HPLC analysis, System suitability,** and **Analysis:** Proceed as directed in *Procedure 3*.

[NOTE—*Standard solutions* and *Sample solution* are to be prepared at the same time and subjected to the same hydrolysis and drying procedures. Using vacuum centrifuge for drying is described in the procedures; other suitable drying methods may be used.]

[NOTE—Sialic standards subjected to the hydrolysis procedure may degrade to a different extent than sialic acids released from a glycoprotein during the procedure. A validated method in which the standard is not subjected to the hydrolysis conditions may be used.]

**Standard stock solution:** 50 µM of USP *N*-Acetylneuraminic Acid RS and 2.5 µM of USP *N*-Glycolylneuraminic Acid RS in water

**Standard solutions:** Add the amount of *Standard stock solution* as indicated in *Table 9* to each microcentrifuge tube. Dry in a vacuum centrifuge without heating.

**Table 9**

| Standard Solution | Standard Stock Solution (µL) | Neu5Ac (pmol) | Neu5Gc (pmol) |
|---|---|---|---|
| Blank | 0 | 0 | 0 |
| 1 | 1 | 50 | 2.5 |
| 2 | 2 | 100 | 5 |

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**Table 9** *(continued)*

| Standard Solution | Standard Stock Solution (µL) | Neu5Ac (pmol) | Neu5Gc (pmol) |
|---|---|---|---|
| 3 | 5 | 250 | 12.5 |
| 4 | 10 | 500 | 25 |
| 5 | 20 | 1000 | 50 |

Add 25 µL of 2 M acetic acid into the tube and briefly centrifuge to ensure the sample is entirely in the well of the tube. Incubate at 80° for 2 h ± 15 min. [NOTE—Or use validated time/temperature ranges.] Allow the tube to cool to room temperature for approximately 10 min, and then vortex and centrifuge. Label the solution as directed in *Labeling* before analysis. The concentrations of the resulting labeled *Standard solutions* are listed in *Table 10*.

**Table 10**

| Standard Solution | Concentration of Neu5Ac after Labeling (µM) | Concentration of Neu5Gc after Labeling (µM) |
|---|---|---|
| Blank | 0 | 0 |
| 1 | 0.02 | 0.001 |
| 2 | 0.04 | 0.002 |
| 3 | 0.1 | 0.005 |
| 4 | 0.2 | 0.01 |
| 5 | 0.4 | 0.02 |

**Sample solution:** Transfer a desalted sample containing approximately 0.5–50 µg of protein (equivalent to about 5 pmol of sialic acid) into a 0.5-mL microcentrifuge tube. Dry in a vacuum centrifuge without heating. Add 25 µL of 2 M acetic acid into the tube and briefly centrifuge to ensure all of the sample is in the well of the tube. Incubate at 80° for 2 h ± 15 min. [NOTE—Or use validated sample preparation and time/temperature ranges.] Allow the tube to cool to room temperature for approximately 10 min. Then vortex and centrifuge. Label the solution as directed in *Labeling* before analysis.

***Change to read:***

- **USP REFERENCE STANDARDS** ⟨11⟩
  USP *N*-Acetylneuraminic Acid RS
  USP *N*-Glycolylneuraminic Acid RS

▲▲ (USP 1-May-2021)

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# ⟨211⟩ ARSENIC

***Add the following:***

## ▲INTRODUCTION

This chapter describes four analytical procedures for the evaluation of the levels of arsenic (As). Use *Procedure 1*, *Procedure 2*, *Procedure 3*, or *Procedure 4* as indicated in the individual monograph. *Procedure 3* or *Procedure 4* can be used in all circumstances, provided that suitability is demonstrated by meeting the *Requirements for Procedure Validation*.▲ (Official 1-Jun-2023)

***Change to read:***

## PROCEDURES

- **▲PROCEDURE 1 AND PROCEDURE 2**

  Both *Procedure 1* and *Procedure 2* are▲ (Official 1-Jun-2023) designed to determine the presence of trace amounts of arsenic by converting the arsenic in a substance under test to arsine, which is then passed through a solution of silver diethyldithiocarbamate to form a red complex. The red color so produced is compared, either visually or spectrophotometrically, to the color produced similarly in a control containing an amount of arsenic equivalent to the limit given in the individual monograph. Limits are stated in terms of arsenic. The content of arsenic does not exceed the limit given in the individual monograph.

  ▲These procedures differ▲ (Official 1-Jun-2023) only in the preliminary treatment of the test substance and the standard. Generally, ▲*Procedure 1*▲ (Official 1-Jun-2023) is used for inorganic materials, ▲and *Procedure 2*▲ (Official 1-Jun-2023) is used for organic materials.

  **Apparatus**

  The apparatus (see *Figure 1*) consists of an arsine generator (*a*) fitted with a scrubber unit (*c*) and an absorber tube (*e*) with standard-taper or ground glass ball-and-socket joints (*b* and *d*) between the units. However, any other suitable apparatus embodying the principle of the assembly described and illustrated may be used.

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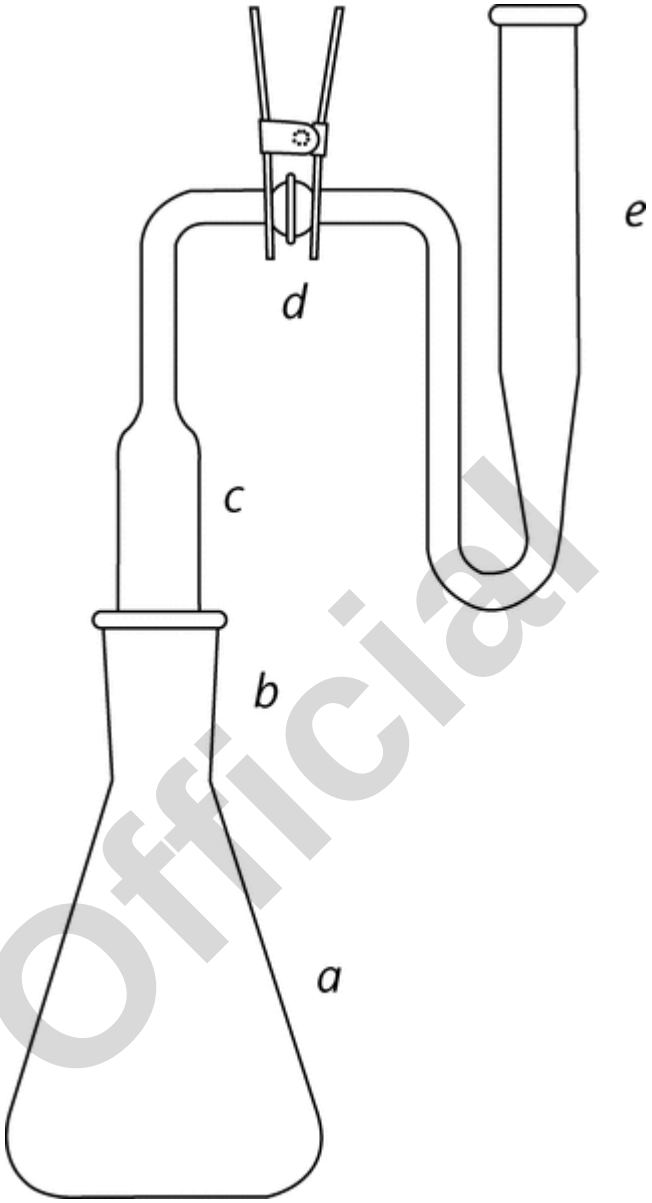


Figure 1. Arsenic test apparatus.

**Arsenic trioxide stock solution:** Dissolve 132.0 mg of arsenic trioxide, previously dried at 105° for 1 h and accurately weighed, in 5 mL of sodium hydroxide solution (1 in 5) in a 1000-mL volumetric flask. Neutralize the solution with 2 N sulfuric acid, add 10 mL more of 2 N sulfuric acid, then add recently boiled and cooled water to volume, and mix.

**Standard arsenic solution:** Transfer 10.0 mL of *Arsenic trioxide stock solution* to a 1000-mL volumetric flask, add 10 mL of 2 N sulfuric acid, then add recently boiled and cooled water to volume, and mix. Each milliliter of *Standard arsenic solution* contains the equivalent of 1 µg of arsenic. Keep this solution in an all-glass container, and use within 3 days.

▲**Procedure 1: Colorimetry**▲ (Official 1-Jun-2023)

**Standard preparation:** Pipet 3.0 mL of *Standard arsenic solution* into a generator flask, and dilute with water to 35 mL.

**Test preparation:** Unless otherwise directed in the individual monograph, prepare as follows. Transfer to the generator flask the quantity, in grams, of the test substance calculated as follows:

$$\text{Result} = 3.0/L$$

$L$ = limit of arsenic (ppm)

Dissolve in water and dilute with water to 35 mL.

▲**Analysis:**▲ (Official 1-Jun-2023) Treat the *Standard preparation* and the *Test preparation* similarly as follows. Add 20 mL of 7 N sulfuric acid, 2 mL of potassium iodide TS, 0.5 mL of stronger acid stannous chloride TS, and 1 mL of isopropyl alcohol, and mix. Allow to stand at room temperature for 30 min. Pack the scrubber tube (*c*) with two pledgets of cotton that have been soaked in saturated lead acetate solution, freed from excess solution by expression, and dried in vacuum at

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room temperature, leaving a 2-mm space between the two pledgets. Lubricate the joints (*b* and *d*) with a suitable stopcock grease designed for use with organic solvents, and connect the scrubber unit to the absorber tube (*e*). Transfer 3.0 mL of silver diethyldithiocarbamate TS to the absorber tube. Add 3.0 g of granular zinc (#20 mesh) to the mixture in the flask, immediately connect the assembled scrubber unit, and allow the evolution of hydrogen and the color development to proceed at room temperature for 45 min, swirling the flask gently at 10-min intervals. Disconnect the absorber tube from the generator and scrubber units, and transfer the absorbing solution to a 1-cm absorption cell. Any red color produced by the *Test preparation* does not exceed that produced by the *Standard preparation*. If necessary or desirable, determine the absorbance at the wavelength of maximum absorbance between 535 and 540 nm, with a suitable spectrophotometer or colorimeter, using silver diethyldithiocarbamate TS as the blank.

**Interfering chemicals:** Metals or salts of metals, such as chromium, cobalt, copper, mercury, molybdenum, nickel, palladium, and silver, may interfere with the evolution of arsine. Antimony, which forms stibine, produces a positive interference in the color development with silver diethyldithiocarbamate TS; when the presence of antimony is suspected, the red colors produced in the two silver diethyldithiocarbamate solutions may be compared at the wavelength of maximum absorbance between 535 and 540 nm, with a suitable colorimeter, since at this wavelength the interference due to stibine is negligible.

**▲Procedure 2: Colorimetry▲** (Official 1-Jun-2023)

[**CAUTION**—Some substances may react with explosive violence when digested with hydrogen peroxide. Exercise safety precautions at all times.]

[NOTE—If halogen-containing compounds are present, use a lower temperature while heating the test specimen with sulfuric acid, avoid boiling the mixture, and add the hydrogen peroxide with caution, before charring begins, to prevent loss of trivalent arsenic.]

[NOTE—If the test substance reacts too rapidly and begins charring with 5 mL of sulfuric acid before heating, use instead 10 mL of cooled dilute sulfuric acid (1 in 2), and add a few drops of the hydrogen peroxide before heating.]

**Standard preparation:** Pipet 3.0 mL of *Standard arsenic solution* into a generator flask, add 2 mL of sulfuric acid, mix, and add the total amount of 30 percent hydrogen peroxide used in preparing the *Test preparation*. Heat the mixture to strong fuming, cool, cautiously add 10 mL of water, and again heat to strong fumes. Repeat this procedure with another 10 mL of water to remove any traces of hydrogen peroxide. Cool, and dilute with water to 35 mL.

**Test preparation:** Unless otherwise directed in the individual monograph, transfer to a generator flask the quantity, in grams, of the test substance calculated as follows:

$$\text{Result} = 3.0/L$$

$L$ = limit of arsenic (ppm)

Add 5 mL of sulfuric acid and a few glass beads, and digest in a fume hood, preferably on a hot plate and at a temperature not exceeding 120°, until charring begins. (Additional sulfuric acid may be necessary to wet some specimens completely, but the total volume added should not exceed 10 mL.) Cautiously add, dropwise, 30 percent hydrogen peroxide, allowing the reaction to subside and again heating between drops. Add the first few drops very slowly with sufficient mixing, in order to prevent a rapid reaction. Discontinue heating if foaming becomes excessive. When the reaction has abated, heat cautiously, rotating the flask occasionally to prevent the specimen from caking on glass exposed to the heating unit. *Maintain oxidizing conditions at all times during the digestion by adding small quantities of the hydrogen peroxide solution whenever the mixture turns brown or darkens.* Continue the digestion until the organic matter is destroyed, gradually raising the temperature of the hot plate until fumes of sulfur trioxide are copiously evolved, and the solution becomes colorless or retains only a light straw color. Cool, add cautiously 10 mL of water, mix, and again evaporate to strong fuming, repeating this procedure to remove any trace of hydrogen peroxide. Cool, cautiously add 10 mL of water, wash the sides of the flask with a few milliliters of water, and dilute with water to 35 mL.

**▲Analysis:▲** (Official 1-Jun-2023) Proceed as directed for ▲*Analysis* in *Procedure 1*.▲ (Official 1-Jun-2023)

**Interfering chemicals:** See *Interfering chemicals* in ▲*Procedure 1*.

- **PROCEDURE 3 AND PROCEDURE 4**

Both *Procedure 3* and *Procedure 4* are ICP-based procedures and can be used for the determination of arsenic. *Procedure 3* can be used for the determination of arsenic by inductively coupled plasma atomic (or optical) emission spectroscopy (ICP–AES or ICP–OES). *Procedure 4* can be used for the determination of arsenic by ICP–MS.

Before initial use, the analyst should verify that the procedure is appropriate for the instrument and sample used (procedural verification) by meeting the *Requirements for Procedure Validation*.

Where a monograph specifies a limit for arsenic concentration, the value listed in the monograph should be used as the *J* value for the purposes of this test.

System standardization and suitability evaluation using applicable reference materials should be performed on the day of analysis.

**Sample preparation:** Forms of sample preparation include neat, direct aqueous solution, direct organic solution, and indirect solution. The selection of the appropriate sample preparation depends on the material under test and is the responsibility of the analyst. When a sample preparation is not indicated in the monograph, an analyst may use any appropriately validated preparation procedure. In cases where spiking of a material under test is necessary to provide an acceptable signal intensity, the blank should be spiked with arsenic using, where possible, the same spiking solution. [NOTE—All liquid samples should be weighed.]

**Closed vessel digestion:** This sample preparation procedure is designed for samples that must be digested in a concentrated acid using a closed vessel digestion apparatus. Closed vessel digestion minimizes the loss of volatile impurities. The choice of a concentrated acid depends on the sample matrix. The use of any of the concentrated acids may be appropriate, but each introduces inherent safety risks. Therefore, appropriate safety precautions should be used

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at all times. [NOTE—Weights and volumes provided may be adjusted to meet the requirements of the digestion apparatus used.]

An example procedure that has been shown to have broad applicability is as follows. Dehydrate and predigest 0.5 g of the primary sample in 5 mL of freshly prepared concentrated acid. Allow to sit loosely covered for 30 min in a fume hood. Add an additional 10 mL of concentrated acid, and digest using a closed vessel technique until digestion or extraction is complete. Repeat, if necessary, by adding an additional 5 mL of concentrated acid. [NOTE—Follow the manufacturer's recommended procedures to ensure safe use.]

**Reagents:** All reagents used for the preparation of sample and standard solutions should be free of elemental impurities, in accordance with *Plasma Spectrochemistry* ⟨730⟩.

**Procedure 3: ICP–OES**

**Standardization solution 1:** 1.5*J* of arsenic in a matched matrix

**Standardization solution 2:** 0.5*J* of arsenic in a matched matrix

**Sample stock solution:** Prepare as directed in *Sample preparation*. Allow the sample to cool, if necessary.

**Sample solution:** Dilute the *Sample stock solution* with an appropriate solvent to obtain a final arsenic concentration of not more than 1.5*J*.

**Blank:** Matched matrix

**Elemental spectrometric system**

(See ⟨730⟩.)

**Rinse:** Use diluent.

**Standardization:** *Standardization solution 1*, *Standardization solution 2*, and *Blank*

**System suitability**

**Sample:** *Standardization solution 1*

**Suitability requirements**

**Drift:** Compare results obtained from *Standardization solution 1* before and after the analysis of the *Sample solution*.

**Suitability criteria:** Not more than 20% for arsenic. [NOTE—If samples are high in mineral content, rinse the system well before introducing the *Sample* in order to minimize carryover.]

**Analysis:** Analyze according to the manufacturer's suggestions for program and wavelength. Calculate and report results on the basis of the original sample size. [NOTE—Appropriate measures must be taken to correct for matrix-induced interferences (e.g., wavelength overlaps).]

**Procedure 4: ICP–MS**

Follow *Procedure 3* except for *Detector* and *Analysis*.

[NOTE—An instrument with a cooled spray chamber is recommended. (A collision cell or reaction cell may also be beneficial.)]

**Detector:** Mass spectrometer

**Analysis:** Analyze according to the manufacturer's suggestions for program and mass-to-charge ratio. Calculate and report results based on the original sample size. [NOTE—Appropriate measures must be taken to correct for matrix-induced interferences (e.g., argon chloride interference with arsenic determinations).]▲ (Official 1-Jun-2023)

***Add the following:***

**▲REQUIREMENTS FOR PROCEDURE VALIDATION**

The following section defines the validation parameters and the acceptance criteria for performance-based procedures. Meeting these requirements must be demonstrated experimentally using an appropriate system suitability procedure and reference materials. Any alternative procedure (e.g., an atomic-absorption-based procedure) that has been validated and meets the acceptance criteria that follow is considered to be suitable for use.

Meeting these validation acceptance criteria is sufficient to demonstrate that the procedure will produce comparable results to those obtained using the procedure prescribed in the monograph.

- **ACCURACY**

**Standard solutions:** Prepare solutions containing arsenic at concentrations ranging from 50% to 150% of *J* using appropriate reference materials.

**Test samples:** Spike the material under test with the appropriate reference materials before any sample preparation steps (digestion or solubilization). Prepare three replicate samples at concentrations ranging from 50% to 150% of *J* for arsenic.

**Acceptance criteria**

**Spike recovery:** 70%–150% for the mean of three replicate preparations at each concentration

- **PRECISION**

**Repeatability**

**Test samples:** Six independent samples of material under test (taken from the same lot) spiked with appropriate reference materials for arsenic at the indicated concentration

**Acceptance criteria**

**Relative standard deviation:** Not more than 20% ($N = 6$) for arsenic

**Intermediate Precision (Ruggedness)**

**Analysis:** Perform the *Repeatability* analysis again on a different day, with different instrumentation, with a different analyst, or a combination thereof. Combine the results of this analysis with the *Repeatability* analysis so the total number of analyses is 12.

**Acceptance criteria**

**Relative standard deviation:** Not more than 25% ($N = 12$) for arsenic

- **SPECIFICITY:** The procedure must be able to unequivocally assess (see *Validation of Compendial Procedures* ⟨1225⟩) arsenic in the presence of components that may be expected to be present, including matrix components.
- **LIMIT OF QUANTITATION, RANGE, AND LINEARITY:** Demonstrated by meeting the *Accuracy* requirement.▲ (Official 1-Jun-2023)

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***Add the following:***

## ▲GLOSSARY

**Concentrated acid:** Concentrated ultra-pure nitric, sulfuric, hydrochloric, or hydrofluoric acid or aqua regia.

**Aqua regia:** Aqua regia is a mixture of concentrated hydrochloric and nitric acids, typically at ratios of 3:1 or 4:1.

**Matched matrix:** Solutions having the same solvent composition as the *Sample solution*. In the case of an aqueous solution, a matched matrix would indicate that the same acids and acid concentrations are used in both preparations.

**Target limit or target concentration:** The acceptance value for the elemental impurity being evaluated, in this case arsenic. Where a monograph specifies a threshold limit, this shall become the target limit or target concentration of arsenic for the material. Exceeding the target limit indicates that a material under test exceeds the acceptable value. The determination of compliance is addressed in other chapters.

***J*:** The concentration (w/w) of the element of interest, in this case arsenic, at the target limit, appropriately diluted to the working range of the instrument.

**Appropriate reference materials:** Where "appropriate reference materials" are specified in the chapter, certified reference materials (CRMs) from a national metrology institute (NMI), or reference materials that are traceable to the CRM of an NMI should be used. An example of an NMI in the United States is the National Institute of Standards and Technology (NIST).▲ (Official 1-Jun-2023)

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# ⟨212⟩ OLIGOSACCHARIDE ANALYSIS

## INTRODUCTION

Analysis of the asparagine (Asn-) linked oligosaccharide (also known as the *N*-linked oligosaccharide or *N*-glycan) composition of selected recombinant therapeutic proteins has become necessary for product characterization or release specifications of these proteins. For information on the characterization and assessment of protein glycosylation, see *Glycoprotein and Glycan Analysis—General Considerations* ⟨1084⟩. Chapter ⟨1084⟩ covers general analytical strategies and also highlights criteria for selecting appropriate methods for specific analytical challenges, thereby providing the context for this chapter. This chapter focuses on the analysis of *N*-linked oligosaccharides that are released from glycosylated recombinant therapeutic proteins. Therefore, the chapter provides 1) a generic procedure for enzymatic release of *N*-glycans using peptide *N*-glycosidase F (PNGase F), 2) two different approaches for fluorophore labeling of the released *N*-linked oligosaccharides, and 3) five analytical procedures based on chromatographic and electrophoretic separation, as well as performance criteria for the separation. Alternative analytical strategies are discussed in ⟨1084⟩. Although the procedures in this chapter are validated, the procedures must be verified with the individual specific product (see *Verification of Compendial Procedures* ⟨1226⟩). Furthermore, validation is required when the procedure is optimized for a specific product [e.g., outside the allowed parameter limits defined in *Chromatography* ⟨621⟩*, System Suitability* (see *Validation of Compendial Procedures* ⟨1225⟩)]. Data analysis, quantitation, and lot release specifications, which are expected to be product specific, will be found in individual product monographs; therefore, these aspects are not covered in this chapter.

Labeling oligosaccharides with a fluorophore before analysis provides an important advantage of enhanced detection of oligosaccharides during separation, because oligosaccharides have no or low absorptivity of either ultraviolet (UV) or visible light and no significant fluorophore groups. This chapter describes two different methods for fluorophore labeling of oligosaccharides: derivatization of oligosaccharides using 2-aminobenzamide (2-AB) for liquid chromatographic separation, and labeling of oligosaccharides with 8-aminopyrene-1,3,6-trisulfonic acid (APTS) for capillary electrophoresis (CE) separation. Although electrochemical detection (pulsed amperometric detection or pulsed electrochemical detection) is also used in oligosaccharide analysis by using a strong base elution, the electrochemical detection method is less sensitive and has a higher noise-to-signal ratio. Therefore, this detection method is not addressed in this chapter.

Many different chromatography and electrophoresis methods have been developed to analyze oligosaccharides, and the procedures described in this chapter have been used successfully for this purpose. The choice of procedures discussed is meant to reflect the most commonly and broadly used approaches for supporting current recombinant glycosylated biological medicines in commerce. These analytical procedures include normal phase or hydrophilic interaction liquid chromatography (HILIC), high-performance anion exchange liquid chromatography (HPAEC), and CE. Several other chromatographic modes that separate oligosaccharides by size, shape, and polarity are also commonly used for oligosaccharide separation, but those other chromatographic modes are not discussed in this chapter. The general principles of chromatography are covered in ⟨621⟩, and therefore ⟨621⟩ should be considered within that context.

USP Reference Standards (RS) have been developed to assess system suitability for these analytical procedures:

- USP Oligosaccharide System Suitability Mixture A RS consists of a mixture of partially sialylated, partially galactosylated, and partially fucosylated biantennary *N*-linked oligosaccharides that were released from human polyclonal immunoglobulin G (IgG) by PNGase F.
- USP Oligosaccharide System Suitability Mixture B RS consists of a mixture of high-mannose *N*-linked oligosaccharides with traces of hybrid chains that were released from bovine ribonuclease B (RNase B) by PNGase F.
- USP Oligosaccharide System Suitability Mixture C RS is a mixture of bi-, tri-, and tetra-antennary *N*-linked oligosaccharides with variable sialylation with or without fucose (Fuc; also containing $Le^x$-type structures); the mixture was released from human α1 acid-glycoprotein by PNGase F.
- USP Oligosaccharide System Suitability Mixture D RS is a mixture of sialylated bi- and tri-antennary complex type structures with an additional sialic acid on one of the tri-antennary structures; the mixture was released from bovine fetuin by PNGase F.

### ANALYTICAL PROCEDURES OVERVIEW

*Table 1* outlines the applications and separation principles of the analytical procedures included in this chapter. The choice of the appropriate RS will depend on the expected glycan content of the product being analyzed.

**Table 1**

| Analytical Procedures | Type of *N*-linked Oligosaccharides to Be Separated | Applicable USP Reference Standards |
|---|---|---|
| *Normal phase liquid chromatography/HILIC procedure 1*:<br>• Column: 2.0-mm × 15-cm; 3-µm packing L68<br>• More oligosaccharide peaks compared to *HILIC procedure 2*<br>• Longer mobile phase with shallow gradient compared to *HILIC procedure 2*<br>• Enhanced sensitivity for smaller sample size | Relatively simple biantennary chains with no or low levels of sialylated structures, after 2-AB labeling | USP Oligosaccharide System Suitability Mixture A RS |
| | High-mannose chains, after 2-AB labeling | USP Oligosaccharide System Suitability Mixture B RS |

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**Table 1** *(continued)*

| Analytical Procedures | Type of *N*-linked Oligosaccharides to Be Separated | Applicable USP Reference Standards |
|---|---|---|
| *Normal phase liquid chromatography/HILIC procedure 2*:<br>• Column: 4.6-mm × 25-cm; 5-µm packing L68<br>• Fewer oligosaccharide peaks observed compared to *HILIC procedure 1*<br>• Shorter mobile phase with less shallow gradient compared to *HILIC procedure 1* | Relatively simple biantennary chains with no or low levels of sialylated structures, after 2-AB labeling | USP Oligosaccharide System Suitability Mixture A RS |
| | High-mannose chains, after 2-AB labeling | USP Oligosaccharide System Suitability Mixture B RS |
| *HPAEC procedure 1*<br>• Guard column: 4.0-mm × 5-cm; 10-µm packing L46<br>• Column: 4.0-mm × 25-cm; 10-µm packing L46<br>• Mobile phase: 0–75 mM sodium acetate in isocratic 0.15 N sodium hydroxide<br>• Selectivity is different from *HILIC procedures 1* and *2* with respect to the size and composition of oligosaccharide | Relatively simple biantennary chains with no or low levels of sialylated structures, after 2-AB labeling | USP Oligosaccharide System Suitability Mixture A RS |
| | High-mannose chains, after 2-AB labeling | USP Oligosaccharide System Suitability Mixture B RS |
| *HPAEC procedure 2*<br>• Guard column: 4.0-mm × 5-cm; 10-µm packing L46<br>• Column: 4.0-mm × 25-cm; 10-µm packing L46<br>• Mobile phase: 150–450 mM sodium acetate in isocratic 0.15 N sodium hydroxide<br>• Suitable for separation of charged *N*-linked oligosaccharides | Bi-, tri-, and tetra-antennary *N*-linked oligosaccharides with variable sialylation, after 2-AB labeling | USP Oligosaccharide System Suitability Mixture C RS |
| | Sialylated bi- and tri-antennary complex type structures with an additional sialic acid on one of the tri-antennary structure, after 2-AB labeling | USP Oligosaccharide System Suitability Mixture D RS |
| Capillary electrophoresis<br>• Appropriate for APTS- oligosaccharides<br>• Capillary: 50-µm inner diameter with 50 cm of total length and 40 cm of separation length | Relatively simple biantennary chains with no or low levels of sialylated structures, after APTS labeling | USP Oligosaccharide System Suitability Mixture A RS |
| | High-mannose chains, after APTS labeling | USP Oligosaccharide System Suitability Mixture B RS |

## SAMPLE PREPARATION

See *Appendix* (*Table 17*) for abbreviations and structures of glycans. The sample preparation is for all of the procedures that follow.

Ideally, the samples should be free of salts, excipients, and other carrier materials that may interfere with the analysis. This can be achieved by either 1) dialysis against water, a suitable buffer, or a volatile buffer using an appropriate membrane, 2) sample trapping on a solid-phase extraction (SPE) cartridge followed by washing away of salts and excipients and elution of the required sample, or 3) ultrafiltration using an appropriate membrane.

[NOTE—In addition to RS, a control sample with a known glycan profile should be included in the overall procedure to confirm correct performance of the analysis. A reaction blank control only containing the buffer matrix of the glycoprotein sample can also be included in the overall procedure.]

### • ENZYMATIC RELEASE OF *N*-GLYCANS

[NOTE—The following is a generic method that should be optimized for individual products depending on the amount of glycoprotein to be digested and the glycan structures, especially on the basis of the protein-to-glycan ratio in the molecule and the accessibility of the sugars.]

**Procedure 1 for chromatographic separation**

**Enzyme reaction buffer:** 50 mM sodium phosphate pH 7.5 or 15 mM tris(hydroxymethyl)-aminomethane, and adjust with hydrochloric acid to a pH of 7.0.

**Digestion with PNGase F:** Transfer 0.1–2.25 mg of glycoprotein to a vial, and adjust with *Enzyme reaction buffer* to a final volume of 30–100 µL. Add PNGase F to the glycoprotein sample in a ratio of 0.5–15 units of PNGase F to 0.1 mg of glycoprotein. Incubate at 37° for 12–24 h. Cool at room temperature for about 5 min. Vortex gently and centrifuge briefly. [NOTE—One unit of PNGase F is defined as the amount of enzyme required to catalyze the release of *N*-linked oligosaccharides from 1.0 nmol of denatured ribonuclease B per min at a pH of 7.5 at 37°, and is equal to 1 IUB milliunit. Complete digestion by PNGase F can be assessed by observing a mobility shift of the deglycosylated proteins on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by Coomassie Blue staining or on SDS-capillary gel electrophoresis (SDS-CGE), as shown by reduction in mass of approximately 2 kDa per glycan chain cleaved.]

Two methods are described below for performing the separation of released glycans from glycoprotein.

**Method 1, using an ultrafiltration membrane with a molecular weight cutoff of 30 kDa:**[1,2] [NOTE—Ultrafiltration membranes with a lower molecular weight cutoff may be used for proteins < 150 kDa.]

Remove trace amounts of glycerin by centrifuging at 11,000 × *g* and rinsing the membrane with 0.5 mL of water. Discard the permeate. Add 0.1 mL of water into the sample reservoir and then add the digested glycoprotein

[1] A suitable ultrafiltration membrane is YM-30 microcon centrifugal filter device from Millipore (catalog numbers 42422, 42409, and 42410) or an equivalent.
[2] A suitable ultrafiltration membrane is EMD Millipore Ultra-0.5 Centrifugal Filter Unit (catalog number UFC501096) or an equivalent.

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sample to the reservoir. Rinse the reaction vial with 0.1 mL of water and add to the sample reservoir. Centrifuge at 11,000 × *g* and collect the permeate. Rinse the sample reservoir twice using 0.1 mL of water for each wash and collect the permeate. Combine the permeates (approximately 0.5 mL) and bring the samples to complete dryness using a centrifugal evaporator without heat.

**Method 2, using a reversed-phase SPE cartridge:**[3] Add 2.0 mL of methanol into a syringe, attach the syringe to the cartridge, and use the syringe plunger to pass methanol through to waste. Add into a syringe 6.0 mL of 5% (v/v) acetic acid prepared in water. Attach the syringe to the cartridge and use the syringe plunger to pass the acetic acid solution through to waste. Gently apply the digested sample onto an individual cartridge. Into a 1-mL syringe add 0.5 mL of 5% (v/v) acetic acid, attach the syringe to the cartridge, and use the syringe plunger to pass the acetic acid through to waste. To a 2- to 3-mL syringe add 1.5 mL of 5% (v/v) acetic acid. Attach the syringe to the cartridge, and use the syringe plunger to pass the acetic acid solution through, collecting the eluting solution into a 1.5-mL tube. Bring the samples to complete dryness using a centrifugal evaporator without heat.

**Procedure 2 for capillary electrophoresis separation**

**Enzyme reaction buffer:** 50 mM sodium phosphate, pH 7.5

**Digestion with PNGase F:** Add 2 µL of PNGase F (5 units/µL) to 50 µg of glycoprotein sample, and adjust with *Enzyme reaction buffer* to a final volume of 50 µL. Incubate at 37° for 18 h. Separate released oligosaccharides by centrifugation using a centrifugal filter with a 10,000 molecular weight cut-off.[2] In a centrifugal vacuum evaporator, dry the released oligosaccharides to dryness.

[NOTE—Follow the guidelines listed in *Table 1* to select the appropriate USP RS and label.]

- **2-AB LABELING[4] FOR LIQUID CHROMATOGRAPHIC SEPARATION**

**Solution A:** Mix glacial acetic acid and dimethyl sulfoxide (3:7, v/v).

**Solution B:** Add 1.5 mL of *Solution A* to 75 mg of 2-AB. Mix well by gentle vortexing to completely dissolve 2-AB.

**Labeling solution:** Add 1 mL of *Solution B* to 63 mg of sodium cyanoborohydride. Mix well by gentle vortexing. Cap the mixture tightly and incubate at 70° for 1–2 min for complete dissolution. Cool at room temperature for 10 min. Use this *Labeling solution* within 1 h of its preparation, and protect the solution from light.

**2-AB labeled standard solution:** Add 5–15 µL of *Labeling solution* to 1 vial of USP Oligosaccharide System Suitability Mixture A RS, USP Oligosaccharide System Suitability Mixture B RS, USP Oligosaccharide System Suitability Mixture C RS, or USP Oligosaccharide System Suitability Mixture D RS, and mix well.

**2-AB labeled sample solution:** Add 5–15 µL of *Labeling solution* to the dried glycan sample after enzymatic release and separation steps, and mix well.

**Procedure:** Immediately incubate the *2-AB labeled standard solution* and the *2-AB labeled sample solution* at 37° for 16–18 h or at 65° for 2 h. Allow to cool at room temperature for 10 min. Centrifuge briefly.

**Dried 2-AB labeled standard or Dried 2-AB labeled sample:** Two methods for the removal of free 2-AB are described below.

**Method 1, using a gel filtration spin column:** Prepare suitable G-10 microcentrifuge spin columns[5] by tapping the column gently to ensure that the dry resin is settled at the bottom. Remove the caps, and place the column in a 2-mL collection tube. Add 0.5 mL of water to the resin, and let it swell for at least 15 min. A longer swelling time (up to 24 h) is acceptable if maintained at 2°–8°. Centrifuge the column at maximum speed for 5–10 s. Remove the water from the collection tube. Wash the resin by adding 0.5 mL of water, and centrifuge the column. Repeat the washing step one more time. After removing the water from the final wash step, spin at maximum speed for 10 s to remove residual water from the resin. Residual water is sufficiently removed if the resin appears white. Place the column in a new, labeled collection tube. Add 100 µL of water to the *2-AB labeled standard solution* and the *2-AB labeled sample solution,* and then apply the entire solution to the center of each washed G-10 column, respectively. Place the G-10 columns in the microcentrifuge and spin at approximately 200 × *g* for 1 min. Apply the flow-through (approximately 90–120 µL) to the center of the second unused but previously washed G-10 column and spin at approximately 200 × *g* for 1 min. Transfer the repeat flow-through (approximately 60–100 µL) to a 0.5-mL microcentrifuge tube. Dry the eluates by centrifugal evaporation with no heat.

**Method 2, using an SPE cartridge:** Prepare suitable SPE cartridges[6] by washing with 1.0 mL of water, followed by 5 × 1.0 mL of 30% (v/v) acetic acid, and then 1.0 mL of acetonitrile. Apply the *2-AB labeled standard solution* and the *2-AB labeled sample solution* to the center surface of the cartridge discs, and allow the solution to incubate on the disc for 15–20 min. Wash each disc with 1 mL of acetonitrile, followed by 6 × 1.0 mL of 96% (v/v) acetonitrile, allowing each aliquot to drain before the next is applied. Elute the *2-AB labeled standard solution* and the *2-AB labeled sample solution* with 3 × 0.5 mL of water, allowing each aliquot to drain before the next is applied. Dry the eluates by centrifugal evaporation with no heat. [NOTE—Very small hydrophobic glycans may be lost during this procedure. During method verification, it should be confirmed that no oligosaccharide species are lost during the 2-AB removal step by comparing the chromatographic profiles of an unextracted reaction blank, an unextracted sample, and an extracted sample.]

- **APTS LABELING FOR CAPILLARY ELECTROPHORESIS SEPARATION**

**APTS labeling reagent:** Dissolve 5 mg of trisodium APTS in 48 µL of 15% (v/v) acetic acid.

**1 M sodium cyanoborohydride:** 1 M sodium cyanoborohydride in tetrahydrofuran

**Run buffer:** Dissolve 1.492 g of triethanolamine (TEA) and 10 g of glycerol, accurately weighed, in 80 mL of water. Adjust with 1 N hydrochloric acid to a pH of 7.5, and dilute with water to a final volume of 100 mL.

**Sample buffer:** Dilute 1.0 mL of *Run buffer* with 9.0 mL of water.

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[3] A suitable SPE cartridge is Glyko GlycoClean R Cartridge from Prozyme (catalog number GKI-4756) or an equivalent.

[4] A suitable 2-AB labeling kit is Glyko Signal 2-AB Labeling Kit from Prozyme (catalog number GKK-404), LudgerTage 2-AB labeling kit from QA Bio (catalog number LT-KAB-A2), or an equivalent.

[5] A suitable prepacked Sephadex G-10 column is Macro Spin G-10 mini SEC column from Harvard Apparatus (catalog number 743900) or The Nest Group (catalog number SMM S010), or an equivalent.

[6] A suitable cartridge to remove free 2-AB is Glyko GlycoClean S Cartridge from Prozyme (catalog number GKI-4726) or an equivalent.

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**APTS-labeled standard solution:** Add 2 µL of the *APTS labeling reagent* and 2 µL of *1 M sodium cyanoborohydride* to a vial of USP Oligosaccharide System Suitability Mixture A RS or USP Oligosaccharide System Suitability Mixture B RS. Incubate at 55° for 90 min. Add 46 µL of water to quench the reaction and mix. Transfer 5 µL of the APTS-labeled USP RS to 1.995 mL of *Sample buffer* before separation. [NOTE—The volume of *Sample buffer* may need to be adjusted so that the fluorescence signals of the oligosaccharides are similar to those from the *APTS-labeled sample solution*.]

**APTS-labeled sample solution:** Add 2 µL of *APTS labeling reagent* and 2 µL of *1 M sodium cyanoborohydride* to the dried glycan sample after enzymatic release and separation steps. Incubate at 55° for 90 min. Add 46 µL of water to quench the reaction and mix. Transfer 5 µL of the APTS-labeled glycan solution to 95 µL of *Sample buffer* before separation.

[NOTE—Follow the guidelines listed in *Table 1* to select the appropriate USP RS and label.]

## SEPARATION AND IDENTIFICATION OF OLIGOSACCHARIDES

- **NORMAL PHASE CHROMATOGRAPHY/HILIC**

**Procedure 1**

**1.4 M formic acid solution:** Mix 273 mL of water with 15 mL of 98%–100% formic acid.

**1.4 M ammonia solution:** Mix 155 mL of water with 40 mL of 26% ammonia solution.

**Ammonium formate buffer:** Add *1.4 M ammonia solution* to *1.4 M formic acid solution* until a pH of 4.4 is obtained.

**Solution A:** Acetonitrile, *Ammonium formate buffer*, and water (75: 4.3: 20.7)

**Solution B:** Acetonitrile, *Ammonium formate buffer*, and water (54: 8.3: 37.7)

**Mobile phase:** See *Table 2*.

**Table 2**

| Time (min) | Solution A (%) | Solution B (%) |
|---|---|---|
| 0.0 | 79 | 21 |
| 80.0 | 47 | 53 |
| 81.0 | 0 | 100 |
| 92.0 | 0 | 100 |
| 93.0 | 79 | 21 |
| 113.0 | 79 | 21 |

**Standard solution:** Reconstitute the *Dried 2-AB labeled standard* with NMT 500 µL of water. [NOTE—The volume of water may need to be adjusted so that the fluorescence signals from the *Standard solution* are similar to those from the *Sample solution*.]

**Sample solution:** Reconstitute the *Dried 2-AB labeled sample* with an appropriate volume of water. [NOTE—Use a ratio of 30 µL of water per 100 µg of glycoprotein used in the *Enzymatic Release of N-Glycans* test as a starting point.]

**Blank solution:** Buffer matrix of glycoprotein sample carried through the *Sample Preparation* procedure

**Chromatographic system**

(See *Chromatography* ⟨621⟩, *System Suitability*.)

**Mode:** LC

**Detector:** Fluorescence (250-nm excitation wavelength and 428-nm emission wavelength)

**Column:** 2.0-mm × 15-cm; 3-µm packing L68

**Temperatures**

**Column:** 45°

**Autosampler:** 2°–8°

**Flow rate:** 0.2 mL/min

**Injection volume:** 2 µL

**System suitability**

**Samples:** *Standard solution* and *Blank solution*

**Suitability requirements**

**Blank:** No peak in the chromatogram of the *Blank solution* within the retention time window at 5–113 min

**Chromatogram similarity:** The chromatogram obtained from the *Standard solution* corresponds to that of the reference chromatogram provided with the Certificate for either USP Oligosaccharide System Suitability Mixture A RS or USP Oligosaccharide System Suitability Mixture B RS.

**Presence of *N*-glycan species:** If USP Oligosaccharide System Suitability Mixture A RS is used, identify peaks corresponding to *G0, G0F, G1Fa, G1Fb, G2F, A1F,* and *A2F* (see *Table 3*; see also *Appendix*) in the chromatogram obtained from the *Standard solution* by comparing the reference chromatogram provided with the USP Certificate for USP Oligosaccharide System Suitability Mixture A RS. If USP Oligosaccharide System Suitability Mixture B RS is used, identify peaks corresponding to oligomannose (MAN) *MAN-5, MAN-6, MAN-7, MAN-8,* and *MAN-9* (see *Table 4*; see also *Appendix*) in the chromatogram obtained from the *Standard solution* by comparing the reference chromatogram provided with the USP Certificate for USP Oligosaccharide System Suitability Mixture B RS.

**Relative retention times:** Corresponding to the relative retention times of *N*-glycans listed in *Table 3* or *Table 4*

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**Table 3. Standard Solution Using USP Oligosaccharide System Suitability Mixture A RS**

| *N*-Glycan | Approximate Relative Retention Time |
|---|---|
| G0 (NGA2) | 0.47 |
| G0F (NGA2F) | 0.57 |
| G1Fa (NA1F, FA2G1, or NA2G1F) | 0.75 |
| G1Fb (NA1F, FA2G1, or NA2G1F) | 0.78 |
| G2F (NA2F) | 1.00 |
| A1F (G2fS1) | 1.39 |
| A2F (G2fS2) | 1.76 |

**Table 4. Standard Solution Using USP Oligosaccharide System Suitability Mixture B RS**

| *N*-Glycan | Approximate Relative Retention Time |
|---|---|
| MAN-5 | 0.74 |
| MAN-6 | 1.00 |
| MAN-7 | 1.29 |
| MAN-8 | 1.60 |
| MAN-9 | 1.85 |

**Analysis:** Equilibrate the *Chromatographic system* for at least 1 h with starting conditions described in *Table 2*. Inject the *Blank solution* until the baseline is stable (1–3 injections).

**Sample:** *Sample solution*

Integrate peaks in the resulting chromatogram, and report the relative peak areas of glycan structures relevant to the product. A baseline is drawn from the first to the last peak. For general information on chromatographic peak integration, see ⟨621⟩.

**Procedure 2**

**Solution A:** 250 mM ammonium formate, pH 4.0, prepared as follows. Mix 500 mL of water with 9.8 mL of 96% formic acid. Adjust with 30% ammonium hydroxide to a pH of 4.0 ± 0.1. Dilute with water to a final volume of 1000 mL. Pass the solution through a 0.22-µm pore size filter composed of cellulose acetate membrane.

**Solution B:** Acetonitrile

**Mobile phase:** See *Table 5*.

**Table 5**

| Time (min) | Solution A (%) | Solution B (%) |
|---|---|---|
| 0.0 | 20 | 80 |
| 2.0 | 30 | 70 |
| 67.0 | 52 | 48 |
| 67.1 | 80 | 20 |
| 73.0 | 80 | 20 |
| 73.1 | 20 | 80 |
| 85.0 | 20 | 80 |

**Standard solution:** Reconstitute the *Dried 2-AB labeled standard* with NLT 500 µL of water. [NOTE—The volume of water may need to be adjusted so that the fluorescence signals from the *Standard solution* are similar to those from the *Sample solution*.]

**Sample solution:** Reconstitute the *Dried 2-AB labeled sample* with an appropriate volume of water. [NOTE—Use a ratio of 50 µL of water per 100 µg of glycoprotein used in the *Enzymatic Release of N-Glycans* test as a starting point.]

**Blank solution:** Buffer matrix of glycoprotein sample carried through the *Sample Preparation* procedure

**Chromatographic system**

(See *Chromatography* ⟨621⟩, *System Suitability*.)

**Mode:** LC

**Detector:** Fluorescence; set the excitation and emission wavelengths as listed in *Table 6*.

**Table 6**

| Time (min) | Excitation (nm) | Emission (nm) |
|---|---|---|
| 0.00 | 330 | 420 |

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**Table 6** *(continued)*

| Time (min) | Excitation (nm) | Emission (nm) |
|---|---|---|
| 1.00 | 330 | 420 |
| 1.01 | 400 | 500 |
| 10.00 | 400 | 500 |
| 10.01 | 330 | 420 |
| 85.00 | 330 | 420 |

**Column:** 4.6-mm × 25-cm; 5-µm packing L68
**Temperatures**
**Column:** 35°
**Autosampler:** 2°–8°
**Flow rate:** 0.4 mL/min
**Injection volume:** 10 µL
**System suitability**
**Sample:** *Standard solution*
**Suitability requirements**
**Chromatogram similarity:** The chromatogram obtained from the *Standard solution* corresponds to that of the reference chromatogram provided with the Certificate for either USP Oligosaccharide System Suitability Mixture A RS or USP Oligosaccharide System Suitability Mixture B RS.
**Presence of *N*-glycan species:** If USP Oligosaccharide System Suitability Mixture A RS is used, identify peaks corresponding to *G0*, *G0F*, *G1Fa*, *G1Fb*, *G2*, *G2F*, *A1F*, and *A2F* (see *Table 7*; see also *Appendix*) in the chromatogram obtained from the *Standard solution* by comparing the reference chromatogram provided with the USP Certificate for USP Oligosaccharide System Suitability Mixture A RS. If USP Oligosaccharide System Suitability Mixture B RS is used, identify peaks corresponding to *MAN-5*, *MAN-6*, *MAN-7*, *MAN-8*, and *MAN-9* (see *Table 8*; see also *Appendix*) in the chromatogram obtained from the *Standard solution* by comparing the reference chromatogram provided with the USP Certificate for USP Oligosaccharide System Suitability Mixture B RS.
**Relative retention times:** Corresponding to the relative retention times of *N*-glycans listed in *Table 7* or *Table 8*

**Table 7. Standard Solution Using USP Oligosaccharide System Suitability Mixture A RS**

| *N*-Glycan | Approximate Relative Retention Time |
|---|---|
| G0 (NGA2) | 0.78 |
| G0F (NGA2F) | 0.83 |
| G1Fa (NA1F, FA2G1, or NA2G1F) | 0.91 |
| G1Fb (NA1F, FA2G1, or NA2G1F) | 0.92 |
| G2 (NA2) | 0.96 |
| G2F (NA2F) | 1.00 |
| A1F (G2fS1) | 1.11 |
| A2F (G2fS2) | 1.20 |

**Table 8. Standard Solution Using USP Oligosaccharide System Suitability Mixture B RS**

| *N*-Glycan | Approximate Relative Retention Time |
|---|---|
| MAN-5 | 0.77 |
| MAN-6 | 0.86 |
| MAN-7 | 0.93 |
| MAN-8 | 1.00 |
| MAN-9 | 1.05 |

**Analysis**
**Sample:** *Sample solution*
Integrate peaks in the resulting chromatogram and report the relative peak areas of glycan structures relevant to the product. For general information on chromatographic peak integration, see ⟨621⟩.

- **HIGH-PERFORMANCE ANION EXCHANGE LIQUID CHROMATOGRAPHY WITH FLUOROMETRIC DETECTION**

**Procedure 1**
**Solution A:** Water; degas before use.

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**Solution B:** 0.5 M sodium acetate, prepared as follows. Dissolve 20.5 g of anhydrous sodium acetate in 450 mL of water and mix well. Dilute with water to a final volume of 500 mL. Filter the solution through a membrane of NMT 0.45-µm pore size, and degas before use.
**Solution C:** 0.5 M sodium acetate, prepared as follows. To 972 mL of water, add 26 mL of 50% (w/w) sodium hydroxide solution. Pass the solution through an alkaline-resistant filter membrane of NMT 0.45-µm pore size, and degas before use.
**Mobile phase:** See *Table 9*.

**Table 9**

| Time (min) | Solution A (%) | Solution B (%) | Solution C (%) | Elution |
|---|---|---|---|---|
| 0.0 | 70 | 0 | 30 | Initial condition |
| 15.0 | 70 | 0 | 30 | 0–75 mM sodium acetate, isocratic 0.15 N sodium hydroxide |
| 65.0 | 55 | 15 | 30 | |
| 66.0 | 0 | 50 | 50 | 250 mM sodium acetate, 0.25 N sodium hydroxide wash |
| 74.0 | 0 | 50 | 50 | |
| 75.0 | 70 | 0 | 30 | |
| 90.0 | 70 | 0 | 30 | Re-equilibrium |

**Standard solution:** Reconstitute the *Dried 2-AB labeled standard* with NMT 500 µL of water. [NOTE—The volume of water may need to be adjusted so that the fluorescence signals from the *Standard solution* are similar to those from the *Sample solution*.]
**Sample solution:** Reconstitute the *Dried 2-AB labeled sample* with an appropriate volume of water. [NOTE—Use a ratio of 20 µL of water per 100 µg of glycoprotein used in the *Enzymatic Release of N-Glycans* test as a starting point.]
**Blank solution:** Buffer matrix of glycoprotein sample carried through the *Sample Preparation* procedure
**Chromatographic system**
(See *Chromatography* ⟨621⟩, *System Suitability*.)
**Mode:** LC
**Detector:** Fluorescence (330-nm excitation wavelength and 420-nm emission wavelength)
**Columns**
**Guard:** 4.0-mm × 5-cm; 10-µm packing L46
**Analytical:** 4.0-mm × 25-cm; 10-µm packing L46
**Temperatures**
**Column:** 25°
**Autosampler:** 4°
**Flow rate:** 0.8 mL/min
**Injection volume:** 50 µL
**System suitability**
**Sample:** *Standard solution*
**Suitability requirements**
**Chromatogram similarity:** The chromatogram obtained from the *Standard solution* corresponds to that of the reference chromatogram provided with the Certificate for either USP Oligosaccharide System Suitability Mixture A RS or USP Oligosaccharide System Suitability Mixture B RS.
**Presence of *N*-glycan species:** If USP Oligosaccharide System Suitability Mixture A RS is used, identify peaks corresponding to *G0F, G1F, G0, G2F, A1F,* and *A2F* (see *Table 10*; see also *Appendix*) in the chromatogram obtained from the *Standard solution* by comparing the reference chromatogram provided with the USP Certificate for USP Oligosaccharide System Suitability Mixture A RS. If USP Oligosaccharide System Suitability Mixture B RS is used, identify peaks corresponding to *MAN-5, MAN-6, MAN-7, MAN-8,* and *MAN-9* (see *Table 11*; see also *Appendix*) in the chromatogram obtained from the *Standard solution* by comparing the reference chromatogram provided with the USP Certificate for USP Oligosaccharide System Suitability Mixture B RS.
**Relative retention times:** Corresponding to the relative retention times of *N*-glycans listed in *Table 10* or *Table 11*

**Table 10. Standard Solution Using USP Oligosaccharide System Suitability Mixture A RS**

| *N*-Glycan | Approximate Relative Retention Time |
|---|---|
| G0F (NGA2F) | 0.67 |
| G1F (NA1F, FA2G1, or NA2G1F) | 0.86 |
| G0 (NGA2) | 0.92 |
| G2F (NA2F) | 1.00 |
| A1F (G2fS1) | 1.75 |
| A2F (G2fS2) | 2.22 |

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**Table 11. Standard Solution Using USP Oligosaccharide System Suitability Mixture B RS**

| *N*-Glycan | Approximate Relative Retention Time |
|---|---|
| MAN-5 | 0.86 |
| MAN-6 | 1.00 |
| MAN-7 | 1.09 |
| MAN-8 | 1.15 |
| MAN-9 | 1.24 |

**Analysis:** Equilibrate the *Chromatographic system* with the initial *Mobile phase* conditions described in *Table 9* for a minimum of 15 min. Inject 50 µL of water for the first injection.

**Sample:** *Sample solution*

Integrate peaks in the resulting chromatogram and report the relative peak areas of glycan structures relevant to the product. For general information on chromatographic peak integration, see ⟨621⟩.

**Procedure 2**

**Solution A:** Water; degas before use

**Solution B:** 0.5 M sodium acetate, prepared as follows. Dissolve 41.0 g of sodium acetate anhydrous in 900 mL of water. Transfer the solution to a 1-L volumetric flask, and bring with water to volume. Filter the solution through a nylon membrane with NMT 0.45-µm pore size, and degas before use.

**Solution C:** 0.5 M sodium hydroxide, prepared as follows. To 900 mL of water, add 26 mL of 50% (w/w) sodium hydroxide solution, and mix well. Bring the solution with water to the final volume of 1000 mL. Filter the solution through an alkaline-resistant nylon membrane with NMT 0.45-µm pore size, and degas before use.

**Mobile phase:** See *Table 12*.

**Table 12**

| Time (min) | Solution A (%) | Solution B (%) | Solution C (%) | Elution |
|---|---|---|---|---|
| 0.0 | 80 | 10 | 10 | Initial condition |
| 15.0 | 80 | 10 | 10 | 50–150 mM sodium acetate, isocratic 0.05 N sodium hydroxide |
| 70.0 | 60 | 30 | 10 | 150–450 mM sodium acetate, isocratic 0.05 N sodium hydroxide |
| 94.0 | 0 | 90 | 10 | Sodium acetate wash, no gradient |
| 99.0 | 0 | 90 | 10 | |
| 105 | 0 | 10 | 90 | Sodium hydroxide wash, no gradient |
| 110 | 00 | 10 | 90 | |
| 111 | 80 | 10 | 10 | Re-equilibrium |
| 130 | 80 | 10 | 10 | |

**Standard solution:** Reconstitute the *Dried 2-AB labeled standard* with NMT 500 µL of water. [NOTE—The volume of water may need to be adjusted so that the fluorescence signals from the *Standard solution* are similar to those from the *Sample solution*.]

**Sample solution:** Reconstitute the *Dried 2-AB labeled sample* with an appropriate volume of water. [NOTE—Use a ratio of 20 µL of water per 100 µg of glycoprotein used in the *Enzymatic Release of N-Glycans* test as a starting point.]

**Blank solution:** Buffer matrix of sample carried through the *Sample Preparation* procedure.

**Chromatographic system**

(See *Chromatography* ⟨621⟩*, System Suitability*.)

**Mode:** LC

**Detector:** Fluorescence (330-nm excitation wavelength and 420-nm emission wavelength)

**Columns**

**Guard:** 4.0-mm × 5-cm; 10-µm packing L46

**Analytical:** 4.0-mm × 25-cm; 10-µm packing L46

**Temperatures**

**Column:** 25°

**Autosampler:** 4°

**Flow rate:** 0.5 mL/min

**Injection volume:** 25 µL

**System suitability**

**Samples:** *Standard solution* and *Blank solution*

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**Suitability requirements**

**Blank:** The chromatogram with *Blank solution* must not have any interfering components in the regions of *N*-glycan elution.

**Chromatogram similarity:** The chromatogram obtained from the *Standard solution* corresponds to that of the reference chromatogram provided with the Certificate for either USP Oligosaccharide System Suitability Mixture C RS or USP Oligosaccharide System Suitability Mixture D RS.

**Presence of *N*-glycan species:** If USP Oligosaccharide System Suitability Mixture C RS is used, identify peaks corresponding to *A1* (peak 1 and peak 2), *A2*, and *A3* (see *Table 13*; see also *Appendix*) in the chromatogram obtained from the *Standard solution* by comparing the reference chromatogram provided with the Certificate. If USP Oligosaccharide System Suitability Mixture D RS is used, identify peaks corresponding to *A1* (peak 1), *A2*, *A3*, and *A3G3S4* (see *Table 14*; see also *Appendix*) in the chromatogram obtained from the *Standard solution* by comparing the reference chromatogram provided with the Certificate.

**Relative retention times:** Corresponding to the relative retention times of *N*-glycans listed in *Table 13* or *Table 14*

**Table 13. Standard Solution Using USP Oligosaccharide System Suitability Mixture C RS**

| *N*-Glycan | Approximate Relative Retention Time |
|---|---|
| A1 (G1S1), peak 1 | 0.31 |
| A1 (G1S1), peak 2 | 0.32 |
| A2 (G2S2) | 0.73 |
| A3 (G3S3) | 1.00 |

**Table 14. Standard Solution Using USP Oligosaccharide System Suitability Mixture D RS**

| *N*-Glycan | Approximate Relative Retention Time |
|---|---|
| A1 (G1S1), peak 1 | 0.23 |
| A2 (G2S2) | 0.57 |
| A3 (G3S3) | 0.78 |
| A3G3S4 (S4NA3, or A3 + Sa) | 1.00 |

**Analysis:** [NOTE—Equilibrate the column with initial mobile phase conditions for a minimum of 15 min.]

**Sample:** *Sample solution*

Inject 25 µL of water, and run the gradient program at least once to equilibrate the column and system. Integrate peaks in the resulting chromatogram, and report the relative peak areas of glycan structures relevant to the product. For general information on chromatographic peak integration, see ⟨621⟩.

- **CAPILLARY ELECTROPHORESIS**

**Run buffer:** Dissolve 1.492 g of TEA and 10 g of glycerol in 80 mL of water. Adjust with 1 N hydrochloric acid to a pH of 7.5 and dilute with water to a final volume of 100 mL.

**Standard solution:** *APTS-labeled standard solution* prepared as directed in the test for *APTS Labeling for CE Separation*

**Sample solution:** *APTS-labeled sample solution* prepared as directed in the test for *APTS Labeling for CE Separation*

**Blank solution:** Buffer matrix of glycoprotein sample carried through the *Sample Preparation* procedure

**Instrumental conditions**

**Mode:** CE

**Detector:** Laser-induced fluorescence (488-nm excitation wavelength and 520-nm emission wavelength)

**Capillary:** 50-µm inner diameter bare fused-silica with 50 cm total length and 40 cm separation length

**Preconditioning of the capillary:** Between each run, rinse for 5 min at 40 psi with 0.5 N sodium hydroxide, followed by water for 1 min at 40 psi. [NOTE—Precondition a new capillary or as needed: Rinse for 5 min at 20 psi with methanol, followed by water for 1 min at 50 psi, 1 N hydrochloric acid for 5 min at 20 psi, water for 1 min at 50 psi, 0.5 N sodium hydroxide for 25 min at 20 psi, and water for 5 min at 50 psi.]

**Prefilling of the capillary:** Rinse for 5 min at 40 psi with *Run buffer.*

**Sample injection:** 10 s hydrodynamic (pressure) injection

**Voltage:** 22 kV for 60 min

**Temperatures**

**Capillary cartridge:** 18°

**Sample storage:** 20°

**System suitability**

**Sample:** *Standard solution*

**Suitability requirements**

**Electropherogram similarity:** The electropherogram obtained from the *Standard solution* corresponds to that of the reference electropherogram provided with the Certificate for either USP Oligosaccharide System Suitability Mixture A RS or USP Oligosaccharide System Suitability Mixture B RS.

**Presence of *N*-glycan species:** If USP Oligosaccharide System Suitability Mixture A RS is used, identify peaks corresponding to *G2F*, *G2*, *G1Fa*, *G1Fb*, *G0F*, *MAN-5*, *G0*, and *A1F* (see *Table 15*; see also *Appendix*) in the electropherogram obtained from the *Standard solution* by comparing the reference electropherogram provided with the USP Certificate for USP Oligosaccharide System Suitability Mixture A RS. If USP Oligosaccharide System Suitability Mixture B RS is used, identify peaks corresponding to *MAN-5*, *MAN-6*, *MAN-7*, *MAN-8*, and *MAN-9* (see

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*Table 16*; see also *Appendix*) in the electropherogram obtained from the *Standard solution* by comparing the reference electropherogram provided with the USP Certificate for USP Oligosaccharide System Suitability Mixture B RS.

**Relative migration times:** Corresponding to relative migration times listed in *Table 15* or *Table 16*

**Table 15. Standard Solution Using USP Oligosaccharide System Suitability Mixture A RS**

| *N*-Glycan | Approximate Relative Migration Time |
|---|---|
| G2F (NA2F) | 0.79 |
| G2 (NA2) | 0.84 |
| G1Fb (NA1F, FA2G1, or NA2G1F) | 0.87 |
| G1Fa (NA1F, FA2G1, or NA2G1F) | 0.90 |
| G0F (NGA2F) | 1.00 |
| MAN-5 | 1.03 |
| G0 (NGA2) | 1.12 |
| A1F (G2fS1) | 1.18 |

**Table 16. Standard Solution Using USP Oligosaccharide System Suitability Mixture B RS**

| *N*-Glycan | Approximate Relative Migration Time |
|---|---|
| MAN-9 | 0.82 |
| MAN-8 | 0.85 |
| MAN-7 | 0.91, 0.92, 0.94 |
| MAN-6 | 1.00 |
| MAN-5 | 1.11 |

**Analysis**

**Sample:** *Sample solution*

Integrate peaks in the resultant electropherogram and report the relative peak areas of glycan structures relevant to the product.

## ADDITIONAL REQUIREMENTS

- **USP REFERENCE STANDARDS** ⟨11⟩

USP Oligosaccharide System Suitability Mixture A RS
USP Oligosaccharide System Suitability Mixture B RS
USP Oligosaccharide System Suitability Mixture C RS
USP Oligosaccharide System Suitability Mixture D RS

## APPENDIX

**Table 17. Glycan Descriptions**

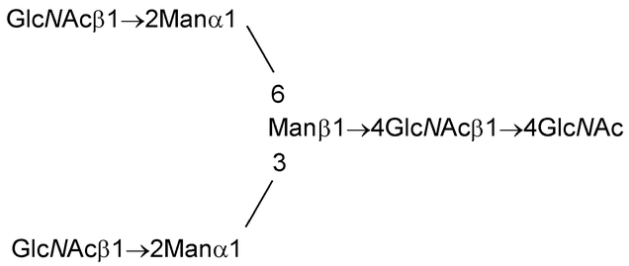
| Glycan | Description |
|---|---|
| G0 (NGA2) | Asialo-, agalacto- biantennary oligosaccharide  |



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**Table 17. Glycan Descriptions** *(continued)*

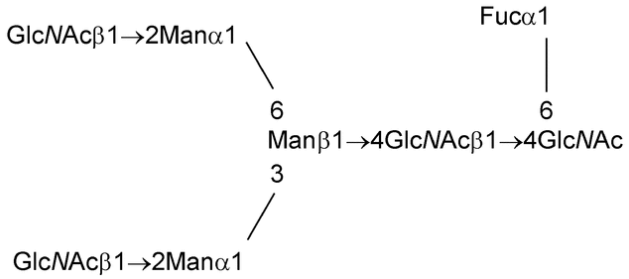
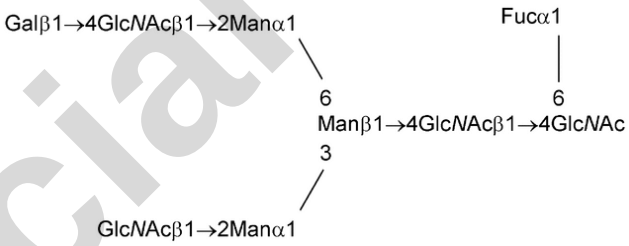
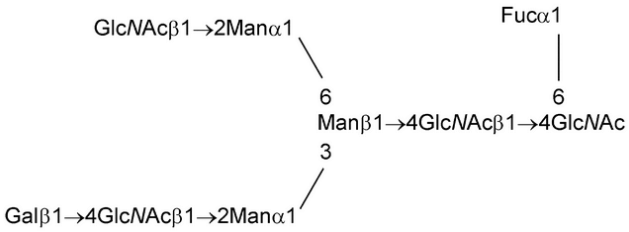
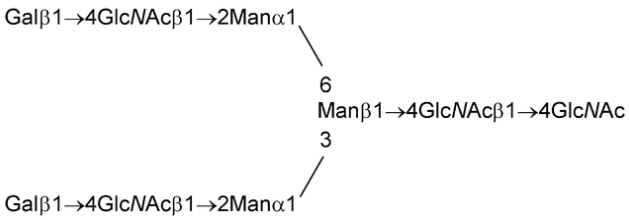
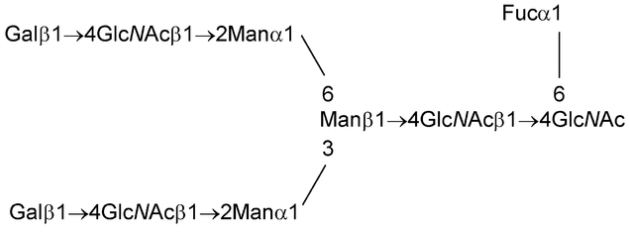
| Glycan | Description |
|---|---|
| G0F (NGA2F) | Asialo-agalacto-fucosylated biantennary oligosaccharide   |
| G1Fa (NA1F, FA2G1, or NA2G1F) with the α(1,6) linkage | Asialo-, monogalactosylated, fucosylated biantennary oligosaccharide with galactosylation at the α(1,6) linkage   |
| G1Fb (NA1F, FA2G1, or NA2G1F) with the α(1,3) linkage | Asialo-, monogalactosylated, fucosylated biantennary oligosaccharide with galactosylation at the α(1,3) linkage   |
| G2 (NA2) | Asialo, galactosylated, biantennary oligosaccharide   |
| G2F (NA2F) | Asialo-fucosylated biantennary oligosaccharide   |

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**Table 17. Glycan Descriptions** *(continued)*

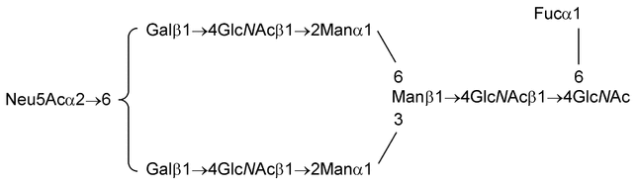
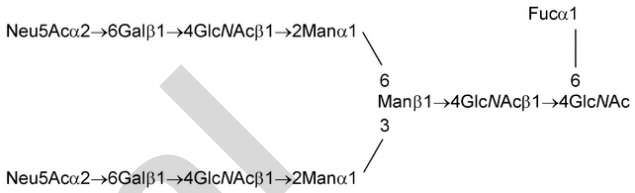
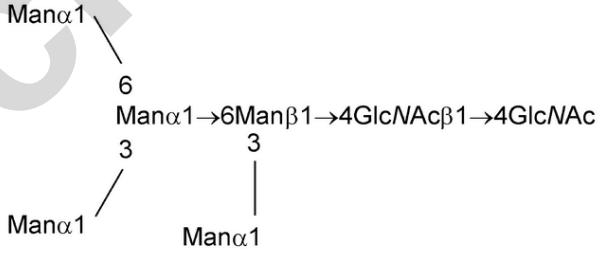
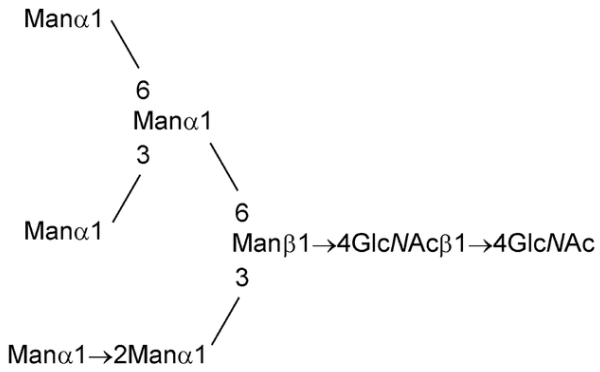
| Glycan | Description |
|---|---|
| A1F (G2fS1) | Monosialo-fucosylated biantennary oligosaccharide<br>  |
| A2F (G2fS2) | Disialo-fucosylated biantennary oligosaccharide<br>  |
| MAN-5 | Oligomannose 5<br>Oligomannose *N*-linked oligosaccharide with 5 mannosyl residues<br>  |
| MAN-6 | Oligomannose 6<br>Oligomannose *N*-linked oligosaccharide with 6 mannosyl residues<br>  |

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**Table 17. Glycan Descriptions** *(continued)*

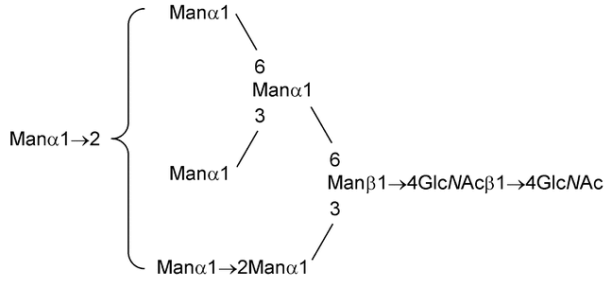
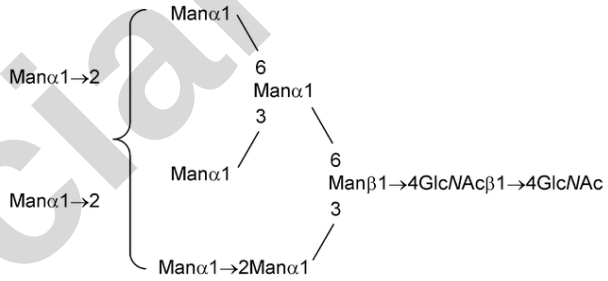
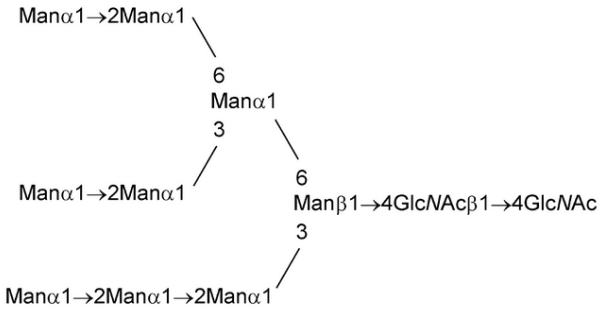
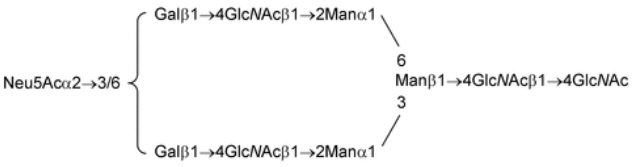
| Glycan | Description |
|---|---|
| MAN-7 | Oligomannose 7<br>Oligomannose *N*-linked oligosaccharide with 7 mannosyl residues<br>  |
| MAN-8 | Oligomannose 8<br>Oligomannose *N*-linked oligosaccharide with 8 mannosyl residues<br>  |
| MAN-9 | Oligomannose 9<br>Oligomannose *N*-linked oligosaccharide with 9 mannosyl residues<br>  |
| A1 (G1S1) | Mono-sialylated, galactosylated, biantennary oligosaccharide<br>  |
| A2 (G2S2) | Di-sialylated, galactosylated, biantennary oligosaccharide<br> |

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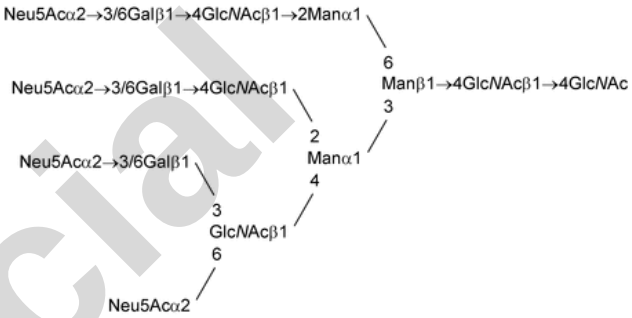
**Table 17. Glycan Descriptions** *(continued)*

| Glycan | Description |
|---|---|
| A3 (G3S3) | Tri-sialylated, galactosylated, triantennary oligosaccharide |
| A3G3S4 (S4NA3, or A3 + Sa) | Tetra-sialylated, galactosylated, triantennary oligosaccharide   |

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# ⟨221⟩ CHLORIDE AND SULFATE

***Change to read:***

The following limit tests are provided as general procedures for use where limits for chloride and sulfate are specified in the individual monographs.

Perform the tests and the controls in glass cylinders of the same diameter and matched as closely as practicable in other respects (see ▲*Visual Comparison* ⟨630⟩▲ (CN 1-May-2019)). Use the same quantities of the same reagents for both the solution under test and the control solution containing the specified volume of chloride or sulfate. If, after acidification, the solution is not perfectly clear, pass it through a filter paper that gives negative tests for chloride and sulfate. Add the precipitant, silver nitrate TS or barium chloride TS as required, to both the test solution and the control solution in immediate sequence.

Where the individual monograph calls for applying the test to a specific volume of a solution of the substance, and the limit for chloride or sulfate corresponds to 0.20 mL or less of 0.020 N hydrochloric acid or sulfuric acid, respectively, apply the test to the solution without further dilution. In such cases maintain the same volume relationships for the control solution as specified for the solution under test. In applying the test to the salts of heavy metals, which normally show an acid reaction, omit the acidification and do not neutralize the solution. Dissolve bismuth salts in a few mL of water and 2 mL of nitric acid before treating with the precipitant.

**CHLORIDE—**Dissolve the specified quantity of the substance under test in 30 to 40 mL of water, or, where the substance is already in solution, add water to make a total volume of 30 to 40 mL, and, if necessary, neutralize the solution with nitric acid to litmus. Add 1 mL each of nitric acid and of silver nitrate TS and sufficient water to make 50 mL. Mix, and allow to stand for 5 minutes protected from direct sunlight. Unless otherwise specified in the monograph, compare the turbidity, if any, with that produced in a solution containing the volume of 0.020 N hydrochloric acid specified in the monograph.

**SULFATE—**Dissolve the specified quantity of the substance under test in 30 to 40 mL of water, or, where the substance is already in solution, add water to make a total volume of 30 to 40 mL, and, if necessary, neutralize the solution with hydrochloric acid to litmus. Add 1 mL of 3 N hydrochloric acid, 3 mL of barium chloride TS, and sufficient water to make 50 mL. Mix, and allow to stand for 10 minutes. Unless otherwise specified in the monograph, compare the turbidity, if any, with that produced in a solution containing the volume of 0.020 N sulfuric acid specified in the monograph.

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# ⟨223⟩ DIMETHYLANILINE

The following limit test is provided as a general procedure, when specified in the individual monographs for the gas chromatographic determination in compendial articles of traces of dimethylaniline, a hydrochloric acid scavenger that may have been carried over during processing.

**PROCEDURE**

**Internal standard solution:** Unless otherwise specified in the individual monograph, prepare a solution of naphthalene in cyclohexane containing about 50 µg per mL.

**Standard preparation:** Unless otherwise specified in the individual monograph, transfer 50.0 mg of *N*,*N*-dimethylaniline to a 50-mL volumetric flask, add 25 mL of 1 N hydrochloric acid, swirl to dissolve, dilute with water to volume, and mix. Transfer 5.0 mL of the resulting solution to a 250-mL volumetric flask, dilute with water to volume, and mix. To a suitable centrifuge tube add 1.0 mL of this solution, 5.0 mL of 1 N sodium hydroxide, and 1.0 mL of *Internal standard solution*, shake vigorously for 1 minute, and centrifuge. Use the clear supernatant as the *Standard preparation.*

**Test preparation:** Unless otherwise specified in the individual monograph, transfer 1.0 g of the substance to be tested to a suitable centrifuge tube, add 5 mL of 1 N sodium hydroxide, swirl to dissolve the specimen, add 1.0 mL of *Internal standard solution*, shake vigorously for 1 minute, and centrifuge. Use the clear supernatant as the *Test preparation.*

**Chromatographic system**

(See *Chromatography* ⟨621⟩.)

The gas chromatograph is equipped with a flame-ionization detector, maintained at about 250°, and a 0.53-mm × 30-m fused silica capillary column bonded with a 1.0-µm film of phase G42. The carrier gas is helium, with a linear velocity of about 30 cm per second and a split ratio of 10:1. The column temperature is maintained at 110° for the first 4 minutes after an injection is made, then increased from 110° to 200° at 8° per minute, and then held at 200° for 5 minutes. The injection port temperature is maintained at 250°. Chromatograph the *Standard preparation*, and record the responses as directed for *Procedure* identify the dimethylaniline and naphthalene peaks by their relative retention times, which are 1.0 and 1.3, respectively. The signal-to-noise ratio for the dimethylaniline peak is not less than 10.

**Procedure:** Inject equal volumes (about 1 µL) of the *Standard preparation* and the *Test preparation* into the chromatograph, record the chromatograms, and measure the areas for the major peaks. The ratio of the response of any dimethylaniline peak to the response of the naphthalene peak obtained from the *Test preparation* is not greater than that obtained from the *Standard preparation* (0.002%).

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# ⟨226⟩ 4-EPIANHYDROTETRACYCLINE

This chromatographic procedure is provided to demonstrate that the content of 4-epianhydrotetracycline, a degradation product of tetracycline, does not exceed the limit given in the individual monograph.

**PROCEDURE**

**EDTA buffer:** Dissolve 37.2 g of edetate disodium in 800 mL of water, adjust with ammonium hydroxide to a pH of 7.8, dilute with water to 1000 mL, and mix.

**Support phase:** Add 5 mL of *EDTA buffer* to 10 g of acid-washed chromatographic siliceous earth for column chromatography, and mix until the siliceous earth is uniformly moistened.

**Test solution:** Prepare as directed in the individual monograph.

**Procedure:** Prepare a 15-mm × 170-mm chromatographic tube with a 4-mm × 50-mm outlet by packing it, in increments, with *Support phase*, firmly tamping down each increment, until the tube is filled to a height of about 10 cm. In a beaker, prepare a mixture of 1 g of acid-washed chromatographic siliceous earth for column chromatography and 1 mL of *Test solution.* Transfer the mixture to the top of the column. Dry-wash the beaker with *Support phase*, and transfer to the column to provide an additional 1-cm layer on top of the mixture containing the *Test solution.* Within 30 minutes, pass chloroform through the column, and collect successive fractions of 5.0 mL, 5.0 mL, 10.0 mL, 10.0 mL, and 5.0 mL. Observe the column during elution, and note the appearance of two separate yellow bands. The fraction or fractions containing the first yellow band contain the anhydrotetracyclines. Discard these fractions. The fractions after the first yellow band contain the 4-epianhydrotetracycline. Determine the absorbance of each 4-epianhydrotetracycline fraction at the wavelength of maximum absorbance at about 438 nm, with a suitable spectrophotometer, diluting each fraction, if necessary, with chloroform, and using chloroform as the blank. Calculate the quantity, in mg, of 4-epianhydrotetracycline in each fraction by the formula:

$$\text{Result} = A_U VD/A_S$$

$A_U$ = absorbance of the fraction taken
$V$ = volume of the fraction taken (mL)
$D$ = dilution factor, if the fraction was diluted
$A_S$ = absorptivity of 4-epianhydrotetracycline at 438 nm, 20.08

From the sum of the quantities of 4-epianhydrotetracycline found in the fractions, calculate the percentage of 4-epianhydrotetracycline in relation to the tetracycline hydrochloride equivalent contained in the *Test solution.*

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# ⟨227⟩ 4-AMINOPHENOL IN ACETAMINOPHEN-CONTAINING DRUG PRODUCTS

## INTRODUCTION

This chapter provides a procedure and acceptance criterion (limit) to control the principal degradation product of acetaminophen, 4-aminophenol, an impurity that can form by hydrolysis of acetaminophen.

### SOLUTION PREPARATIONS

All solution preparations that contain acetaminophen or 4-aminophenol should be protected from light and should be stored only for as long as can be supported by solution stability data acquired during verification under actual conditions of use.

**Buffer:** 4.0 g/L of sodium citrate dihydrate and 1.5 g/L of anhydrous citric acid, in water

**Diluent:** Acetonitrile and *Buffer* (10:90)

**Solution A:** 10 mM phosphate buffer prepared as follows. Add 0.60 g of monobasic potassium phosphate and 0.82 g of anhydrous dibasic sodium phosphate to a 1-L volumetric flask. Dissolve and dilute with water to volume to a pH of 7.0.

**Solution B:** Water

**Solution C:** Acetonitrile

**Mobile phase:** See *Table 1*.

**Table 1**

| Time (min) | Solution A (%) | Solution B (%) | Solution C (%) |
|---|---|---|---|
| 0 | 90 | 5 | 5 |
| 5 | 90 | 5 | 5 |
| 7 | 10 | 10 | 80 |
| 7.1 | 90 | 5 | 5 |
| 10 | 90 | 5 | 5 |

**Standard stock solution:** 25 µg/mL of USP 4-Aminophenol RS in *Diluent*. Prepare fresh in conjunction with the other solution preparations described below. Discard after 4 h or as supported by solution stability data. [NOTE—See *Chromatographic Adjustments*, solution stability, below.]

**System suitability solution:** 2.5 µg/mL of USP 4-Aminophenol RS in *Diluent*, from the *Standard stock solution*

**Sample stock solution:** Nominally 10 mg/mL of acetaminophen from a suitable quantity of drug product in *Diluent*. [NOTE—Either component of the *Diluent* may be introduced to the drug product first, followed by addition of the other component to maintain the proportions of acetonitrile and *Buffer* and to achieve the appropriate final volume defined for the *Diluent*.]

[NOTE—It is recommended that the *Sample solution* and *Standard solution* be prepared concurrently within a narrow window of time (e.g., 30 min) for each drug product sample.]

**Standard solution:** Add 25.0 mL of the *Sample stock solution* and 15.0 mL of the *Standard stock solution* to a 50-mL volumetric flask, and dilute with *Diluent* to volume. Pass through a suitable filter of 0.45-µm pore size, discarding the first 3 mL of filtrate.

**Sample solution:** Add 25.0 mL of the *Sample stock solution* to a 50-mL volumetric flask, and dilute with *Diluent* to volume. Pass through a suitable filter of 0.45-µm pore size, discarding the first 3 mL of filtrate.

### CHROMATOGRAPHIC METHOD

**Chromatographic system**

(See *Chromatography* ⟨621⟩, *System Suitability*.)

**Mode:** LC

**Detector:** UV 300 nm

**Column:** 4.6-mm × 15-cm; 5-µm packing L85

**Column temperature:** 30°

**Flow rate:** 1 mL/min

**Injection volume:** 10 µL

**System suitability**

**Samples:** *System suitability solution* and *Standard solution*

[NOTE—The typical retention time for 4-aminophenol is about 4.2–5.3 min.]

**Suitability requirements**

**Resolution:** NLT 1.0 between 4-aminophenol and the nearest peak, *Standard solution*

**Tailing factor:** NMT 1.5 for the 4-aminophenol peak, *Standard solution*

**Relative standard deviation:** NMT 5.0%, *Standard solution*

**Signal-to-noise ratio:** NLT 20 for the 4-aminophenol peak, *System suitability solution*

### ANALYSIS

**Samples:** *Standard solution* and *Sample solution*

Inject the *Sample solution* and *Standard solution* for each drug product sample sequentially, i.e., back-to-back.

Calculate the percentage of 4-aminophenol ($C_6H_7NO$) relative to acetaminophen in the portion of drug product taken:

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$$\text{Result} = [r_U/(r_S - r_U)] \times (W_S/W_U) \times 100$$

$r_U$ = peak response of 4-aminophenol from the *Sample solution*
$r_S$ = peak response of 4-aminophenol from the *Standard solution*
$W_S$ = amount of USP 4-Aminophenol RS added to the *Standard solution* (mg)
$W_U$ = amount of acetaminophen in the *Sample solution* (mg)

**Acceptance criteria** (unless otherwise stated in the monograph): NMT 0.15% of 4-aminophenol relative to acetaminophen

**CHROMATOGRAPHIC ADJUSTMENTS**

The retention time of 4-aminophenol can be tuned to achieve specificity for a given product matrix. This allowance supersedes provisions in ⟨621⟩ for adjusting chromatographic conditions and is intended to provide a measure of flexibility when needed. Suggestions for changing 4-aminophenol retention are given in *Table 2*. The use of a ternary mobile phase system affords ready changes to the ionic strength (water from *Solution B*) and organic strength (acetonitrile from *Solution C*), but this can be simplified to a binary mobile phase system.

**Table 2**

| Condition Change | Change in 4-Aminophenol Retention |
|---|---|
| Increase in organic strength (*Solution C*) | Decreases 4-aminophenol retention |
| Decrease in pH (*Solution A*) | Increases 4-aminophenol retention |
| Increase in ionic strength (*Solution B*) | Decreases 4-aminophenol retention |
| Increase in column temperature | Increases 4-aminophenol retention |

Adjustments to the chromatographic procedure may require verification or validation. See *Validation of Compendial Procedures* ⟨1225⟩ and *Verification of Compendial Procedures* ⟨1226⟩ for guidance. Adjusted chromatographic conditions must meet all system suitability requirements.

Solution stability must be verified under actual conditions of use to ensure 4-aminophenol is stable in the *Sample solution* and the *Standard solution* as evidenced by NMT ±10% change of the 4-aminophenol peak areas.

• **USP REFERENCE STANDARDS** ⟨11⟩

USP 4-Aminophenol RS

4-Amino-1-hydroxybenzene.
$C_6H_7NO$
109.13

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# ⟨228⟩ ETHYLENE OXIDE AND DIOXANE

The following procedure is used to determine the contents of residual ethylene oxide and dioxane in the products prepared from ethylene oxide. Unless otherwise directed in the individual monograph, use *Method I*.

**Method I**

[**CAUTION**—Ethylene oxide is toxic and flammable. Prepare these solutions in a well-ventilated fume hood, using great care. Protect both hands and face by wearing polyethylene protective gloves and an appropriate face mask. Store all solutions in hermetic containers, and refrigerate between 4° and 8°.]

[NOTE—Before using the polyethylene glycol 200 in this test, remove any volatile components from it by placing 500 mL of polyethylene glycol 200 in a 1000-mL round-bottom flask and attaching the flask to a rotary evaporator maintained at 60° and under a vacuum of 10–20 mm Hg for 6 h.]

**Acetaldehyde solution:** 10 µg/mL of acetaldehyde. [NOTE—Prepare immediately before use.]

**Ethylene oxide stock solution:** 2.5 mg/g of ethylene oxide.

Prepare as follows: Tare a glass-stoppered conical flask, add 50 mL of polyethylene glycol 200, and reweigh the flask. Transfer 5 mL of the liquid ethylene oxide to a 100-mL beaker chilled in a mixture of sodium chloride and ice (1:3). Transfer 300 µL (corresponding to 250 mg) of liquid ethylene oxide to the polyethylene glycol 200, and swirl gently to mix. Replace the stopper, reweigh the flask, and determine the amount of ethylene oxide absorbed by weight difference. Adjust the weight of the mixture with polyethylene glycol 200 to 100.0 g, replace the stopper, and swirl gently to mix. [NOTE—Fill a chilled pressure bottle with liquid ethylene oxide, and store in a freezer when not in use. Use a small piece of polyethylene film to protect the liquid from contact with the rubber gasket. Use an adequately chilled apparatus where appropriate. Prepare this stock solution immediately prior to use, and store in a refrigerator after preparation.]

**Ethylene oxide solution:** Tare a glass-stoppered conical flask, and chill it in a refrigerator. Add 35 mL of polyethylene glycol 200, and reweigh the flask. Transfer 1 g of chilled *Ethylene oxide stock solution* to the tared conical flask. Adjust the weight of the solution with polyethylene glycol 200 to 50.0 g, replace the stopper, and swirl gently to mix. Transfer 10 g of this solution to a 50-mL volumetric flask. Add 30 mL of water, and mix. Dilute with water to volume, and mix to obtain a solution containing 10 µg/mL of ethylene oxide. [NOTE—Use an adequately chilled apparatus where appropriate. Prepare immediately before use.]

**Dioxane solution:** 500 µg/mL of dioxane

**Standard solution A:** Transfer 0.1 mL of *Ethylene oxide solution* to a 10-mL pressure headspace vial. [NOTE—Other sizes such as a 22-mL pressure headspace vial may be used, depending on operating conditions; however, the same size must be used for *Standard solution A*, *Standard solution B*, and the *Sample solution*.] Add 0.1 mL of *Acetaldehyde solution* and 0.1 mL of *Dioxane solution*, seal the vial, and mix.

**Standard solution B:** Transfer 1.0 g of the test substance to a 10-mL pressure headspace vial, and add 0.1 mL of *Ethylene oxide solution*, 0.1 mL of *Dioxane solution*, and 1.0 mL of *N*,*N*-dimethylacetamide. Seal the vial, and mix.

**Sample solution:** Transfer 1.0 g of the test substance to a 10-mL pressure headspace vial, and add 1.0 mL of *N*,*N*-dimethylacetamide and 0.2 mL of water. Seal the vial, and mix.

**Chromatographic system**

(See *Chromatography* ⟨621⟩, *System Suitability*.)

**Mode:** Headspace GC

**Detector:** Flame ionization

**Column:** 0.32-mm × 30-m glass or quartz capillary; 1.0-µm layer of phase G1

**Temperature**

**Injector port:** 150°

**Detector:** 250°

**Column:** See the column temperature table below.

| Initial Temperature (°) | Temperature Ramp (°/min) | Final Temperature (°) | Hold Time at Final Temperature (min) |
|---|---|---|---|
| 50 | — | 50 | 5 |
| 50 | 5 | 180 | — |
| 180 | 30 | 230 | 5 |

**Carrier gas:** Helium

**Linear velocity:** 20 cm/s

**Injection volume:** 1 mL (gaseous headspace)

**Injection type:** Split ratio 20:1

**Headspace sampler**

**Temperature equilibration time:** 45 min

**Equilibration temperature**

70° for *Standard solution A*

90° for *Standard solution B*

90° for *Sample solution*

**Transfer line temperature:** 150°

**Pressurization time:** 1 min

**Injection time:** 12 s

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**System suitability**
**Sample:** *Standard solution A*
[NOTE—The relative retention times for acetaldehyde and ethylene oxide are 0.94 and 1.0, respectively.]
**Suitability requirements**
**Resolution:** NLT 2.0 between acetaldehyde and ethylene oxide
**Signal-to-noise ratio:** NLT 5, determined from the dioxane peak
**Relative standard deviation:** NMT 15%
**Analysis**
**Samples:** *Standard solution B* and *Sample solution*
[NOTE—The relative retention times for ethylene oxide and dioxane are 1.0 and 2.5, respectively.]
Calculate the content of ethylene oxide, in ppm, in the portion of the test substance taken:

$$\text{Result} = A_E \times r_U/[(r_S \times W_U) - (r_U \times W_S)]$$

$A_E$ = quantity of ethylene oxide added to *Standard solution B* (µg)
$r_U$ = ethylene oxide peak responses from the *Sample solution*
$r_S$ = ethylene oxide peak responses from *Standard solution B*
$W_U$ = weight of the test substance taken to prepare the *Sample solution* (g)
$W_S$ = weight of the test substance taken to prepare *Standard solution B* (g)

Calculate the content of dioxane, in ppm, in the portion of the test substance taken:

$$\text{Result} = A_D \times r_U/[(r_S \times W_U) - (r_U \times W_S)]$$

$A_D$ = quantity of dioxane added to *Standard solution B* (µg)
$r_U$ = dioxane peak responses from the *Sample solution*
$r_S$ = dioxane peak responses from *Standard solution B*
$W_U$ = weight of the test substance taken to prepare the *Sample solution* (g)
$W_S$ = weight of the test substance taken to prepare *Standard solution B* (g)

## Method II

**Ethylene oxide standard solution:** Dilute 0.5 mL of ethylene oxide in methylene chloride (50 mg/mL)[1] with water to 50.0 mL. [NOTE—The solution is stable for 3 months if stored in vials with polytetrafluoroethylene (polytef)-coated silicon membrane crimped caps at –20°.] Allow to reach room temperature. Dilute 1.0 mL with water to 250.0 mL to obtain a solution having a concentration of 2 µg/mL of ethylene oxide. [NOTE—Use this solution immediately after preparation.]
**Dioxane standard solution:** 0.05 µL/mL of dioxane
**Acetaldehyde standard solution:** 10 µg/mL of acetaldehyde. [NOTE—Prepare immediately before use.]
**Resolution solution:** Add 2.0 mL of *Acetaldehyde standard solution* and 2.0 mL of *Ethylene oxide standard solution* to a 10-mL headspace vial. Seal the vial immediately with a polytef-coated silicon membrane and an aluminum cap, and mix carefully.
**Standard solution A:** 0.48 µg/mL of ethylene oxide, from *Ethylene oxide standard solution*, and 0.005 µL/mL of dioxane, from *Dioxane standard solution*, in water
**Standard solution B:** Transfer 1.0 g of the test substance into a 10-mL headspace vial. Add 2.0 mL of *Standard solution A*, seal the vial immediately with a polytef-coated silicon membrane and an aluminum cap, and mix carefully.
**Sample solution:** Transfer 1.0 g of the test substance into a 10-mL headspace vial. Add 2.0 mL of water, seal the vial immediately with a polytef-coated silicon membrane and an aluminum cap, and mix carefully.
**Chromatographic system**
(See *Chromatography* ⟨621⟩*, System Suitability*.)
**Mode:** Headspace GC
**Detector:** Flame ionization
**Column:** 0.53-mm × 50-m fused-silica capillary column; 5.0-µm layer of phase G27
**Temperature**
**Injector port:** 85°
**Detector:** 250°
**Column:** See the column temperature table below.

| Initial Temperature (°) | Temperature Ramp (°/min) | Final Temperature (°) | Hold Time at Final Temperature (min) |
|---|---|---|---|
| 70 | 10 | 250 | 5 |

**Carrier gas:** Helium
**Flow rate:** 4 mL/min
**Injection volume:** 1 mL (gaseous headspace)
**Injection type:** Split ratio 3.5 : 1
**Headspace sampler**
**Temperature equilibration time:** 30 min
**Equilibration temperature:** 80°

[1] This is a commercially available solution.

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**System suitability**

**Sample:** *Resolution solution*

[NOTE—The relative retention times for acetaldehyde and ethylene oxide are 0.9 and 1.0, respectively.]

**Suitability requirements**

**Resolution:** NLT 2.0 between acetaldehyde and ethylene oxide

**Analysis**

**Samples:** *Standard solution B* and *Sample solution*

[NOTE—The relative retention times for ethylene oxide and dioxane are 1.0 and 1.9, respectively.]

Calculate the content of ethylene oxide, in ppm, in the portion of the test substance taken:

$$\text{Result} = C_E \times V \times r_U/[(r_S \times W_U) - (r_U \times W_S)]$$

$C_E$ = concentration of ethylene oxide in the *Standard solution A* (µg/mL)
$V$ = volume of *Standard solution A* added to *Standard solution B* (2.0 mL)
$r_U$ = ethylene oxide peak responses from the *Sample solution*
$r_S$ = ethylene oxide peak responses from *Standard solution B*
$W_U$ = weight of the test substance taken to prepare the *Sample solution* (g)
$W_S$ = weight of the test substance taken to prepare *Standard solution B* (g)

Calculate the content of dioxane, in ppm, in the portion of the test substance taken:

$$\text{Result} = C_D \times V \times \rho \times F \times r_U/[(r_S \times W_U) - (r_U \times W_S)]$$

$C_D$ = concentration of dioxane in *Standard solution A* (µL/mL)
$V$ = volume of *Standard solution A* added to *Standard solution B* (2.0 mL)
$\rho$ = density of dioxane (1.03 g/mL = 1.03 mg/µL)
$F$ = conversion factor (1000 µg/mg)
$r_U$ = dioxane peak responses from the *Sample solution*
$r_S$ = dioxane peak responses from *Standard solution B*
$W_U$ = weight of the test substance taken to prepare the *Sample solution* (g)
$W_S$ = weight of the test substance taken to prepare *Standard solution B* (g)

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# ⟨232⟩ ELEMENTAL IMPURITIES—LIMITS

## INTRODUCTION

This chapter specifies limits for the amounts of elemental impurities in drug products. Regardless of the approach used, compliance with the limits specified is required for all drug products unless otherwise specified in an individual monograph or specifically excluded in this *Introduction*.

Elemental impurities include catalysts and environmental contaminants that may be present in drug substances, excipients, or drug products. These impurities may occur naturally, be added intentionally, or be introduced inadvertently (e.g., by interactions with processing equipment and the container–closure system). When elemental impurities are known to be present, have been added, or have the potential for introduction, assurance of compliance to the specified levels is required. A risk-based control strategy may be appropriate when analysts determine how to assure compliance with this standard. Due to the ubiquitous nature of arsenic, cadmium, lead, and mercury, they (at the minimum) must be considered in the risk assessment.

This chapter does not apply to the following:

- Radiopharmaceuticals
- Articles intended only for veterinary use
- Vaccines
- Cell metabolites
- DNA products
- Allergenic extracts
- Cells, whole blood, cellular blood components, or blood derivatives, including plasma and plasma derivatives
- Products based on genes (gene therapy)
- Cells (cell therapy)
- Tissue (tissue engineering)
- Dialysate solutions not intended for systemic circulation
- Total parenteral nutrition (TPN)
- Elements that are intentionally included in the drug product for therapeutic benefit
- Dietary supplements and their ingredients, which are addressed in *Elemental Contaminants in Dietary Supplements* ⟨2232⟩

The limits presented in this chapter do not apply to excipients and drug substances, except where specified in an individual monograph. However, manufacturers of pharmaceutical products need certain information about the content of elemental impurities in drug substances or excipients in order to meet the criteria of this chapter. Drug product manufacturers can use elemental impurity test data on components from tests performed by drug substance or excipient manufacturers, who may provide test data, or if applicable, risk assessments. Elemental impurity data generated by a qualified supplier of drug product components are acceptable for use by a drug product manufacturer to demonstrate compliance with this chapter in the final drug product. Drug substance or excipient manufacturers who choose to perform a risk assessment must conduct that risk assessment using *Table 2* in this chapter. Elements that are inherent in the nature of the material, as in the case of some naturally sourced materials, must be considered in the risk assessment.

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## SPECIATION

The determination of the oxidation state, organic complex, or combination is termed “speciation”. Each of the elemental impurities has the potential to be present in differing oxidation or complexation states. However, arsenic and mercury are of particular concern because of the differing toxicities of their inorganic and complexed organic forms.

The arsenic limits are based on the inorganic (most toxic) form. Arsenic can be measured using a total-arsenic procedure under the assumption that all arsenic contained in the material under test is in the inorganic form. Where the limit is exceeded using a total-arsenic procedure, it may be possible to show, via a procedure that quantifies the different forms, that the inorganic form meets the specification.

The mercury limits are based upon the inorganic ($2^+$) oxidation state. The methyl mercury form (most toxic) is rarely an issue for pharmaceuticals. Thus, the limit was established assuming the most common (mercuric) inorganic form. Limits for articles that have the potential to contain methyl mercury (e.g., materials derived from fish) are to be provided in the monograph.

## ROUTES OF EXPOSURE

The elements included in the tables below have been placed into three classes, based on their toxicity and likelihood of occurrence in the drug product. The classification scheme is intended to focus the risk assessment on those elements that are the most toxic but also have a reasonable probability of inclusion in the drug product (see *Table 2*).

The toxicity of an elemental impurity is related to its extent of exposure (bioavailability). The extent of exposure has been determined for each of the elemental impurities of interest for three routes of administration: oral, parenteral, and inhalational. These limits are based on chronic exposure. Consider the oral permitted daily exposures (PDEs) in *Table 1* as a starting point in developing specific PDEs for other routes of administration, except where otherwise stated in the individual monograph.

[NOTE—The routes of administration of drug products are defined in *Pharmaceutical Dosage Forms* ⟨1151⟩.]

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# DRUG PRODUCTS

The limits described in the third through fifth columns of *Table 1* are the base daily dose PDEs of the elemental impurities of interest for a drug product taken by a patient according to the indicated routes of administration.

## Parenteral Products

Parenteral drug products with maximum daily volumes up to 2 L may use the maximum daily volume to calculate permitted concentrations from PDEs. For products whose daily volumes, as specified by labeling and/or established by clinical practice, may exceed 2 L (e.g., saline, dextrose, and solutions for irrigation), a 2-L volume may be used to calculate permitted concentrations from PDEs.

**Table 1. Permitted Daily Exposures for Elemental Impurities**

| Element | Class | Oral PDE (µg/day) | Parenteral PDE (µg/day) | Inhalation PDE (µg/day) |
|---|---|---|---|---|
| Cadmium | 1 | 5 | 2 | ▲3▲ (USP 1-Dec-2020) |
| Lead | 1 | 5 | 5 | 5 |
| Arsenic | 1 | 15 | 15 | 2 |
| Mercury | 1 | 30 | 3 | 1 |
| Cobalt | 2A | 50 | 5 | 3 |
| Vanadium | 2A | 100 | 10 | 1 |
| Nickel | 2A | 200 | 20 | 5 |
| Thallium | 2B | 8 | 8 | 8 |
| Gold | 2B | 100 | 100 | 1 |
| Palladium | 2B | 100 | 10 | 1 |
| Iridium | 2B | 100 | 10 | 1 |
| Osmium | 2B | 100 | 10 | 1 |
| Rhodium | 2B | 100 | 10 | 1 |
| Ruthenium | 2B | 100 | 10 | 1 |
| Selenium | 2B | 150 | 80 | 130 |
| Silver | 2B | 150 | 10 | 7 |
| Platinum | 2B | 100 | 10 | 1 |
| Lithium | 3 | 550 | 250 | 25 |
| Antimony | 3 | 1200 | 90 | 20 |
| Barium | 3 | 1400 | 700 | 300 |
| Molybdenum | 3 | 3000 | 1500 | 10 |
| Copper | 3 | 3000 | 300 | 30 |
| Tin | 3 | 6000 | 600 | 60 |
| Chromium | 3 | 11000 | 1100 | 3 |

## Recommendations for Elements to Be Considered in the Risk Assessment

*Table 2* identifies elemental impurities for inclusion in the risk assessment. This table can be applied to all sources of elemental impurities in the drug product.

**Table 2. Elements to Be Considered in the Risk Assessment**

| Element | Class | If Intentionally Added (All Routes) | If Not Intentionally Added | | |
|---|---|---|---|---|---|
| | | | Oral | Parenteral | Inhalation |
| Cadmium | 1 | Yes | Yes | Yes | Yes |
| Lead | 1 | Yes | Yes | Yes | Yes |
| Arsenic | 1 | Yes | Yes | Yes | Yes |
| Mercury | 1 | Yes | Yes | Yes | Yes |

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**Table 2. Elements to Be Considered in the Risk Assessment** *(continued)*

| Element | Class | If Intentionally Added (All Routes) | If Not Intentionally Added | | |
|---|---|---|---|---|---|
| | | | Oral | Parenteral | Inhalation |
| Cobalt | 2A | Yes | Yes | Yes | Yes |
| Vanadium | 2A | Yes | Yes | Yes | Yes |
| Nickel | 2A | Yes | Yes | Yes | Yes |
| Thallium | 2B | Yes | No | No | No |
| Gold | 2B | Yes | No | No | No |
| Palladium | 2B | Yes | No | No | No |
| Iridium | 2B | Yes | No | No | No |
| Osmium | 2B | Yes | No | No | No |
| Rhodium | 2B | Yes | No | No | No |
| Ruthenium | 2B | Yes | No | No | No |
| Selenium | 2B | Yes | No | No | No |
| Silver | 2B | Yes | No | No | No |
| Platinum | 2B | Yes | No | No | No |
| Lithium | 3 | Yes | No | Yes | Yes |
| Antimony | 3 | Yes | No | Yes | Yes |
| Barium | 3 | Yes | No | No | Yes |
| Molybdenum | 3 | Yes | No | No | Yes |
| Copper | 3 | Yes | No | Yes | Yes |
| Tin | 3 | Yes | No | No | Yes |
| Chromium | 3 | Yes | No | No | Yes |

## Options for Demonstrating Compliance

### DRUG PRODUCT ANALYSIS OPTION

The results obtained from the analysis of a typical dosage unit, scaled to a maximum daily dose, are compared with the daily dose PDE.

$$\text{Daily dose PDE} \geq \text{measured value } (\mu g/g) \times \text{maximum daily dose } (g/\text{day})$$

The measured amount of each impurity is NMT the daily dose PDE, unless otherwise stated in the individual monograph.

### SUMMATION OPTION

Separately, add the amounts of each elemental impurity (in µg/g) present in each of the components of the drug product:

$$\text{Daily dose PDE} \geq [\Sigma^{M}{}_{1}(C_M \times W_M)] \times D_D$$

$M$ = each ingredient used to manufacture a dosage unit
$C_M$ = element concentration in component (drug substance or excipient) (µg/g)
$W_M$ = weight of component in a dosage unit (g/dosage unit)
$D_D$ = number of units in the maximum daily dose (unit/day)

The result of the summation of each impurity is NMT the daily dose PDE, unless otherwise stated in the individual monograph. Before products can be evaluated using this option, the manufacturer must ensure that additional elemental impurities cannot be inadvertently added through the manufacturing process or via the container–closure system over the shelf life of the product.

### INDIVIDUAL COMPONENT OPTION

For drug products with a daily dose of NMT 10 g, if all drug substances and excipients in a formulation meet the concentration limits shown in *Table 3*, then these components may be used in any proportion. No further calculation is necessary. Although elemental impurities derived from the manufacturing process or the container–closure system are not specifically provided for in the *Individual Component Option*, it is expected that the drug product manufacturer will ensure that these sources do not contribute significantly to the total content of elemental impurities.

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## DRUG SUBSTANCE AND EXCIPIENTS

The acceptable levels of elemental impurities depend on the material's ultimate use. Therefore, manufacturers of pharmaceutical products need certain information about the content of elemental impurities in drug substances or excipients in order to meet the criteria of this chapter. Drug product manufacturers can use elemental impurity test data on components from tests performed by drug substance manufacturers or excipient manufacturers, who may provide test data, or, if applicable, risk assessments. Elemental impurity data generated by a qualified supplier of drug product components are acceptable for use by a drug product manufacturer to demonstrate compliance with this chapter in the final drug product. Drug substance or excipient manufacturers who choose to perform a risk assessment must conduct that risk assessment using *Table 2* in this chapter. Elements that are inherent in the nature of the material, as in the case of some naturally sourced materials, must be considered in the risk assessment.

The values provided in *Table 3* are example concentration limits for components (drug substances and excipients) of drug products dosed at a maximum daily dose of 10 g/day. These values serve as default concentration limits to aid discussions between drug product manufacturers and the suppliers of the components of their drug products. [NOTE—Individual components may need to be limited at levels different from those in the table depending on monograph-specific mitigating factors.]

**Table 3. Permitted Concentrations of Elemental Impurities for Individual Component Option**

| Element | Class | Oral Concentration (µg/g) | Parenteral Concentration (µg/g) | Inhalation Concentration (µg/g) |
|---|---|---|---|---|
| Cadmium | 1 | 0.5 | 0.2 | ▲0.3▲ (USP 1-Dec-2020) |
| Lead | 1 | 0.5 | 0.5 | 0.5 |
| Arsenic | 1 | 1.5 | 1.5 | 0.2 |
| Mercury | 1 | 3 | 0.3 | 0.1 |
| Cobalt | 2A | 5 | 0.5 | 0.3 |
| Vanadium | 2A | 10 | 1 | 0.1 |
| Nickel | 2A | 20 | 2 | 0.5 |
| Thallium | 2B | 0.8 | 0.8 | 0.8 |
| Gold | 2B | 10 | 10 | 0.1 |
| Palladium | 2B | 10 | 1 | 0.1 |
| Iridium | 2B | 10 | 1 | 0.1 |
| Osmium | 2B | 10 | 1 | 0.1 |
| Rhodium | 2B | 10 | 1 | 0.1 |
| Ruthenium | 2B | 10 | 1 | 0.1 |
| Selenium | 2B | 15 | 8 | 13 |
| Silver | 2B | 15 | 1 | 0.7 |
| Platinum | 2B | 10 | 1 | 0.1 |
| Lithium | 3 | 55 | 25 | 2.5 |
| Antimony | 3 | 120 | 9 | 2 |
| Barium | 3 | 140 | 70 | 30 |
| Molybdenum | 3 | 300 | 150 | 1 |
| Copper | 3 | 300 | 30 | 3 |
| Tin | 3 | 600 | 60 | 6 |
| Chromium | 3 | 1100 | 110 | 0.3 |

## ANALYTICAL TESTING

If, by process monitoring and supply-chain control, manufacturers can demonstrate compliance, then further testing may not be needed. When testing is done to demonstrate compliance, proceed as directed in *Elemental Impurities—Procedures* ⟨233⟩.

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# ⟨233⟩ ELEMENTAL IMPURITIES—PROCEDURES

## INTRODUCTION

This chapter describes two analytical procedures (*Procedures 1* and *2*) for the evaluation of the levels of the elemental impurities. The chapter also describes criteria for acceptable alternative procedures. By means of validation studies, analysts will confirm that the analytical procedures described herein are suitable for use on specified material.

### Use of Alternative Procedures

The chapter also describes criteria for acceptable alternative procedures. Alternative procedures that meet the validation requirements herein may be used in accordance with *General Notices, 6.30 Alternative and Harmonized Methods and Procedures*. Information on the *Requirements for Alternate Procedure Validation* is provided later in this chapter.

### Speciation

The determination of the oxidation state, organic complex, or combination is termed *speciation*. Analytical procedures for speciation are not included in this chapter, but examples may be found elsewhere in *USP–NF* and in the literature.

## PROCEDURES

- **Compendial Procedures 1 and 2**

System standardization and suitability evaluation using applicable reference materials should be performed on the day of analysis.

**Procedure and detection technique:** *Procedure 1* can be used for elemental impurities generally amenable to detection by inductively coupled plasma–atomic (optical) emission spectroscopy (ICP–AES or ICP–OES). *Procedure 2* can be used for elemental impurities generally amenable to detection by ICP–MS. Before initial use, the analyst should verify that the procedure is appropriate for the instrument and sample used (procedural verification) by meeting the alternative procedure validation requirements below.

**Sample preparation:** Forms of sample preparation include *Neat*, *Direct aqueous solution*, *Direct organic solution*, and *Indirect solution*. The selection of the appropriate sample preparation depends on the material under test and is the responsibility of the analyst. When a sample preparation is not indicated in the monograph, an analyst may use any of the following appropriately validated preparation procedures. In cases where spiking of a material under test is necessary to provide an acceptable signal intensity, the blank should be spiked with the same *Target elements*, and where possible, using the same spiking solution. Standard solutions may contain multiple *Target elements*. [Note—All liquid samples should be weighed.]

**Neat:** Used for liquids or alternative procedures that allow the examination of unsolvated samples.

**Direct aqueous solution:** Used when the sample is soluble in an aqueous solvent.

**Direct organic solution:** Used where the sample is soluble in an organic solvent.

**Indirect solution:** Used when a material is not directly soluble in aqueous or organic solvents. Total metal extraction is the preferred sample preparation approach to obtain an *Indirect solution*. Digest the sample using the *Closed vessel digestion* procedure provided below or one similar to it. The sample preparation scheme should yield sufficient sample to allow quantification of each element at the limit specified in the corresponding monograph or chapter.

**Closed vessel digestion:** This sample preparation procedure is designed for samples that must be digested in a *Concentrated acid* using a closed vessel digestion apparatus. *Closed vessel digestion* minimizes the loss of volatile impurities. The choice of a *Concentrated acid* depends on the sample matrix. The use of any of the *Concentrated acids* may be appropriate, but each introduces inherent safety risks. Therefore, appropriate safety precautions should be used at all times. [Note—Weights and volumes provided may be adjusted to meet the requirements of the digestion apparatus used.]

An example procedure that has been shown to have broad applicability is the following. Dehydrate and predigest 0.5 g of primary sample in 5 mL of freshly prepared *Concentrated acid*. Allow to sit loosely covered for 30 min in a fume hood. Add an additional 10 mL of *Concentrated acid*, and digest, using a closed vessel technique, until digestion or extraction is complete. Repeat, if necessary, by adding an additional 5 mL of *Concentrated acid*. [Note—Where closed vessel digestion is necessary, follow the manufacturer's recommended procedures to ensure safe use.]

Alternatively, leachate extraction may be appropriate with justification following scientifically validated metal disposition studies, which may include animal studies, speciation, or other means of studying disposition of the specific metal in the drug product.

**Reagents:** All reagents used for the preparation of sample and standard solutions should be free of elemental impurities, in accordance with *Plasma Spectrochemistry* ⟨730⟩.

- **Procedure 1: ICP–OES**

**Standardization solution 1:** 1.5*J* of the *Target element(s)* in a *Matched matrix*

**Standardization solution 2:** 0.5*J* of the *Target element(s)* in a *Matched matrix*

**Sample stock solution:** Proceed as directed in *Sample preparation* above. Allow the sample to cool, if necessary. For mercury determination, add an appropriate stabilizer.

**Sample solution:** Dilute the *Sample stock solution* with an appropriate solvent to obtain a final concentration of the *Target elements* at NMT 1.5*J*.

**Blank:** *Matched matrix*

**Elemental spectrometric system**

(See *Plasma Spectrochemistry* ⟨730⟩.)

**Mode:** ICP

**Detector:** Optical detection system

**Rinse:** Diluent used

**Standardization:** *Standardization solution 1*, *Standardization solution 2*, and *Blank*

**System suitability**

**Sample:** *Standardization solution 1*

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**Suitability requirements**

**Drift:** Compare results obtained from *Standardization solution 1* before and after the analysis of the *Sample solution*.

**Suitability criteria:** NMT 20% for each *Target element*. [NOTE—If samples are high in mineral content, rinse system well before introducing the *Sample* in order to minimize carryover.]

**Analysis:** Analyze according to the manufacturer's suggestions for program and wavelength. Calculate and report results on the basis of the original sample size. [NOTE—Appropriate measures must be taken to correct for matrix-induced interferences (e.g., wavelength overlaps).]

- **PROCEDURE 2: ICP–MS**

**Standardization solution 1:** 1.5*J* of the *Target element(s)* in a *Matched matrix*

**Standardization solution 2:** 0.5*J* of the *Target element(s)* in a *Matched matrix*

**Sample stock solution:** Proceed as directed for *Sample preparation* above. Allow the sample to cool, if necessary. For mercury determination, add an appropriate stabilizer.

**Sample solution:** Dilute the *Sample stock solution* with an appropriate solvent to obtain a final concentration of the *Target elements* at NMT 1.5*J*.

**Blank:** *Matched matrix*

**Elemental spectrometric system**

(See *Plasma Spectrochemistry* ⟨730⟩.)

**Mode:** ICP. [NOTE—An instrument with a cooled spray chamber is recommended. (A collision cell or reaction cell may also be beneficial.)]

**Detector:** Mass spectrometer

**Rinse:** Diluent used

**Standardization:** *Standardization solution 1*, *Standardization solution 2*, and *Blank*

**System suitability**

**Sample:** *Standardization solution 1*

**Suitability requirements**

**Drift:** Compare results obtained from *Standardization solution 1* before and after the analysis of the *Sample solution*.

**Suitability criteria:** *Drift* NMT 20% for each *Target element*. [NOTE—If samples are high in mineral content, rinse system well before introducing the *Sample* in order to minimize carryover.]

**Analysis:** Analyze according to the manufacturer's suggestions for program and $m/z$. Calculate and report results based on the original sample size. [NOTE—Appropriate measures must be taken to correct for matrix-induced interferences (e.g., argon chloride interference with arsenic determinations).]

## REQUIREMENTS FOR ALTERNATE PROCEDURE VALIDATION

If the specified compendial procedures do not meet the needs of a specific application, an alternative procedure may be developed (see *General Notices, 6.30 Alternative and Harmonized Methods and Procedures*). Alternative procedures must be validated and shown to be acceptable, in accordance with the validation requirements for alternative procedures as described below. The level of validation necessary to ensure that an alternative procedure is acceptable depends on whether a limit test or a quantitative determination is specified in the monograph. The requirements for the validation of an elemental impurities procedure for each type of determination are described below. Any alternative procedure that has been validated and meets the acceptance criteria that follow is considered to be suitable for use.

## LIMIT PROCEDURES

The following section defines the validation parameters for the acceptability of alternative limit procedures. Meeting these requirements must be demonstrated experimentally using an appropriate system suitability procedure and reference material.

The suitability of the method must be determined by conducting studies with the material or mixture under test supplemented with known concentrations of each *Target element* of interest at the appropriate acceptance limit concentration. The material or mixture under test must be spiked before any sample preparation steps are performed.

- **DETECTABILITY**

**Standard solution:** A preparation of reference materials for the *Target element(s)* at the *Target concentration*

**Spiked sample solution 1:** Prepare a solution of sample under test, spiked with appropriate reference materials for the *Target elements* at the *Target concentration*, solubilized or digested as described in *Sample preparation*.

**Spiked sample solution 2:** Prepare a solution of the sample under test, spiked with appropriate reference materials at 80% of the *Target concentration* for the *Target elements*, solubilized or digested as described in *Sample preparation*.

**Unspiked sample solution:** A sample of material under test, solubilized or digested in the same manner as the *Sample solutions*

**Acceptance criteria**

**Non-instrumental procedures:** *Spiked sample solution 1* provides a signal or intensity equivalent to or greater than that of the *Standard solution*. *Spiked sample solution 2* must provide a signal or intensity less than that of *Spiked sample solution 1*. [NOTE—The signal from each *Spiked sample solution* is NLT the *Unspiked sample solution* determination.]

**Instrumental procedures:** The average value of the three replicate measurements of *Spiked sample solution 1* is within ±15% of the average value obtained for the replicate measurements of the *Standard solution*. The average value of the replicate measurements of *Spiked sample solution 2* must provide a signal intensity or value less than that of the *Standard solution*. [NOTE—Correct the values obtained for each of the spiked solutions using the *Unspiked sample solution*.]

- **PRECISION FOR INSTRUMENTAL METHODS (REPEATABILITY)**

[NOTE—Non-instrumental precision is demonstrated by meeting the *Detectability* requirement above.]

**Sample solutions:** Six independent samples of the material under test, spiked with appropriate reference materials for the *Target elements* at the *Target concentration*

**Acceptance criteria**

**Relative standard deviation:** NMT 20% for each *Target element*

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- **SPECIFICITY**

The procedure must be able to unequivocally assess (see *Validation of Compendial Procedures* ⟨1225⟩) each *Target element* in the presence of components that may be expected to be present, including other *Target elements*, and matrix components.

**QUANTITATIVE PROCEDURES**

The following section defines the validation parameters for the acceptability of alternative quantitative procedures. Meeting these requirements must be demonstrated experimentally, using an appropriate system suitability procedure and reference materials. Meeting these requirements demonstrates that the procedure is equivalent to the compendial procedure for the purpose of quantifying the *Target elements*.

- **ACCURACY**

**Standard solutions:** Prepare solutions containing the *Target elements* at concentrations ranging from 50% to 150% of *J*, using appropriate reference materials.

**Test samples:** Prepare samples of the material under test spiked with appropriate reference materials before any sample preparation steps (digestion or solubilization) at concentrations ranging from 50% to 150% of *J* for each *Target element*.

**Acceptance criteria**

**Spike recovery:** 70%–150% for the mean of three replicate preparations at each concentration

- **PRECISION**

**Repeatability**

**Test samples:** Six independent samples of material under test (taken from the same lot) spiked with appropriate reference materials for the *Target element(s)* at the indicated level

**Acceptance criteria**

**Relative standard deviation:** NMT 20% ($N = 6$) for each *Target element*

**Intermediate precision (ruggedness)**

Perform the *Repeatability* analysis again either on a different day, with a different instrumentation, with a different analyst, or a combination thereof. Combine the results of this analysis with the *Repeatability* analysis so the total number of analyses is 12.

**Acceptance criteria**

**Relative standard deviation:** NMT 25% ($N = 12$) for each *Target element*

- **SPECIFICITY**

The procedure must be able to unequivocally assess (see ⟨1225⟩) each *Target element* in the presence of components that may be expected to be present, including other *Target elements*, and matrix components.

- **LIMIT OF QUANTITATION, RANGE, AND LINEARITY**

Demonstrated by meeting the *Accuracy* requirement.

## GLOSSARY

**Concentrated acid:** Concentrated ultra-pure nitric, sulfuric, hydrochloric, or hydrofluoric acids or *Aqua regia*

**Aqua regia:** *Aqua regia* is a mixture of concentrated hydrochloric and nitric acids, typically at ratios of 3:1 or 4:1, respectively.

**Matched matrix:** Solutions having the same solvent composition as the *Sample solution*. In the case of an aqueous solution, *Matched matrix* would indicate that the same acids, acid concentrations, and mercury stabilizer are used in both preparations.

**Target elements:** Elements with the potential of being present in the material under test. Include arsenic (As), cadmium (Cd), lead (Pb), and mercury (Hg) in the target element evaluation when testing is done to demonstrate compliance. *Target elements* should also include any elements that may be added through material processing or storage.

**Target limit or Target concentration:** The acceptance value for the elemental impurity being evaluated. Exceeding the *Target limit* indicates that a material under test exceeds the acceptable value. The determination of compliance is addressed in other chapters. [NOTE—When applying this chapter to *Elemental Impurities—Limits* ⟨232⟩ and *Elemental Contaminants in Dietary Supplements* ⟨2232⟩, *Target limits* can be approximated by dividing the *Daily Dose PDEs* by the maximum daily dose for the *Drug Product Analysis Option* in ⟨232⟩ or the *Daily Serving PDE* divided by the maximum daily serving size in ⟨2232⟩.]

**J:** The concentration (w/w) of the element(s) of interest at the *Target limit*, appropriately diluted to the working range of the instrument. For example, if the target elements are lead and arsenic for an analysis of an oral solid drug product with a daily dose of 10 g/day using inductively coupled plasma–mass spectrometry (ICP–MS), the target limit for these elements would be 0.5 µg/g and 1.5 µg/g (see *Table 2* in ⟨232⟩). However, in this case, the linear dynamic range of the ICP–MS is known to extend from 0.01 ng/mL to 0.1 µg/mL for these elements. Therefore, a dilution factor of at least 1:100 is required to ensure that the analysis occurs in the linear dynamic range of the instrument. *J* would thus equal 5 ng and 15 ng/mL for lead and arsenic, respectively, when the dilution factor is added.

**Appropriate reference materials:** Where *Appropriate reference materials* are specified in the chapter, certified reference materials (CRM) from a national metrology institute (NMI), or reference materials that are traceable to the CRM of an NMI should be used. An example of an NMI in the United States is the National Institute of Standards and Technology.

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# ⟨241⟩ IRON

***Add the following:***

## ▲INTRODUCTION

This chapter describes three analytical procedures for the evaluation of the levels of iron (Fe). Use *Procedure 1*, *Procedure 2*, or *Procedure 3* as indicated in the individual monograph. *Procedure 2* or *Procedure 3* can be used in all circumstances, provided that suitability is demonstrated by meeting the *Requirements for Procedure Validation*.▲ (Official 1-Jun-2023)

***Change to read:***

## ▲PROCEDURES

**• PROCEDURE 1: CHEMICAL METHOD**▲ (Official 1-Jun-2023)

This limit test is provided to demonstrate that the content of iron, in either the ferric or the ferrous form, does not exceed the limit for iron specified in the individual monograph. The determination is made by concomitant visual comparison with a control prepared from a standard iron solution.

**Standard iron solution:** Dissolve 863.4 mg of ferric ammonium sulfate [$FeNH_4(SO_4)_2 \cdot 12H_2O$] in water, add 10 mL of 2 N sulfuric acid, and dilute with water to 100.0 mL. Pipet 10 mL of this solution into a 1000-mL volumetric flask, add 10 mL of 2 N sulfuric acid, dilute with water to volume, and mix. Each milliliter of *Standard iron solution* contains the equivalent of 0.01 mg (10 µg) of iron.

**Ammonium thiocyanate solution:** Dissolve 30 g of ammonium thiocyanate in water to make 100 mL.

**Standard preparation:** Into a 50-mL color-comparison tube pipet 1 mL of *Standard iron solution* (10 µg of iron), dilute with water to 45 mL, add 2 mL of hydrochloric acid, and mix.

**Test preparation:** Into a 50-mL color comparison tube place the solution prepared for the test as directed in the individual monograph, and, if necessary, dilute with water to 45 mL; or, dissolve in water and dilute with water to 45 mL the quantity, in grams, of the substance to be tested, calculated as follows:

$$\text{Result} = 1.0/(1000L)$$

$L$ = limit of iron (%)

Add 2 mL of hydrochloric acid, and mix.

**▲Analysis:**▲ (Official 1-Jun-2023) To each of the tubes containing the *Standard preparation* and the *Test preparation* add 50 mg of ammonium peroxydisulfate crystals and 3 mL of *Ammonium thiocyanate solution*, and mix. The color of the solution from the *Test preparation* is not darker than that of the solution from the *Standard preparation.*

**▲• PROCEDURE 2 AND PROCEDURE 3**

Both *Procedure 2* and *Procedure 3* are ICP-based procedures and can be used for the determination of iron. *Procedure 2* can be used for the determination of iron by inductively coupled plasma atomic (or optical) emission spectroscopy (ICP–AES or ICP–OES). *Procedure 3* can be used for the determination of iron by ICP–MS.

Before initial use, the analyst should verify that the procedure is appropriate for the instrument and sample used (procedural verification) by meeting the *Requirements for Procedure Validation*.

Where a monograph specifies a limit for iron concentration, the value listed in the monograph should be used as the *J* value for the purposes of this test.

System standardization and suitability evaluation using applicable reference materials should be performed on the day of analysis.

**Sample preparation:** Forms of sample preparation include neat, direct aqueous solution, direct organic solution, and indirect solution. The selection of the appropriate sample preparation depends on the material under test and is the responsibility of the analyst. When a sample preparation is not indicated in the monograph, an analyst may use any appropriately validated preparation procedure. In cases where spiking of a material under test is necessary to provide an acceptable signal intensity, the blank should be spiked with iron using, where possible, the same spiking solution. [NOTE—All liquid samples should be weighed.]

**Closed vessel digestion:** This sample preparation procedure is designed for samples that must be digested in a concentrated acid using a closed vessel digestion apparatus. Closed vessel digestion minimizes the loss of volatile impurities. The choice of a concentrated acid depends on the sample matrix. The use of any of the concentrated acids may be appropriate, but each introduces inherent safety risks. Therefore, appropriate safety precautions should be used at all times. [NOTE—Weights and volumes provided may be adjusted to meet the requirements of the digestion apparatus used.]

An example procedure that has been shown to have broad applicability is as follows. Dehydrate and predigest 0.5 g of the primary sample in 5 mL of freshly prepared concentrated acid. Allow to sit loosely covered for 30 min in a fume hood. Add an additional 10 mL of concentrated acid, and digest using a closed vessel technique until the digestion or extraction is complete. Repeat, if necessary, by adding an additional 5 mL of concentrated acid. [NOTE—Follow the manufacturer's recommended procedures to ensure safe use.]

**Reagents:** All reagents used for the preparation of sample and standard solutions should be free of elemental impurities, in accordance with *Plasma Spectrochemistry* ⟨730⟩.

**Procedure 2: ICP–OES**

**Standardization solution 1:** 1.5*J* of iron in a matched matrix

**Standardization solution 2:** 0.5*J* of iron in a matched matrix

**Sample stock solution:** Prepare as directed in *Sample preparation*. Allow the sample to cool, if necessary.

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**Sample solution:** Dilute the *Sample stock solution* with an appropriate solvent to obtain a final concentration of iron of not more than 1.5*J*.
**Blank:** Matched matrix
**Elemental spectrometric system**
(See ⟨730⟩.)
**Mode:** ICP
**Detector:** Optical detection system
**Rinse:** Use diluent.
**Standardization:** *Standardization solution 1*, *Standardization solution 2*, and *Blank*
**System suitability**
**Sample:** *Standardization solution 1*
**Suitability requirements**
**Drift:** Compare results obtained from *Standardization solution 1* before and after the analysis of the *Sample solution*.
**Suitability criteria:** Not more than 20% for iron. [NOTE—If samples are high in mineral content, rinse the system well before introducing the *Sample* in order to minimize carryover.]
**Analysis:** Analyze according to the manufacturer's suggestions for program and wavelength. Calculate and report results on the basis of the original sample size. [NOTE—Appropriate measures must be taken to correct for matrix-induced interferences (e.g., wavelength overlaps).]
**Procedure 3: ICP–MS**
Follow *Procedure 2* except for *Detector* and *Analysis*.
[NOTE—An instrument with a cooled spray chamber is recommended. (A collision cell or reaction cell may also be beneficial.)]
**Mode:** ICP
**Detector:** Mass spectrometer
**Analysis:** Analyze according to the manufacturer's suggestions for program and mass-to-charge ratio. Calculate and report results based on the original sample size. [NOTE—Appropriate measures must be taken to correct for matrix-induced interferences.]▲ (Official 1-Jun-2023)

***Add the following:***

## ▲REQUIREMENTS FOR PROCEDURE VALIDATION

The following section defines the validation parameters and the acceptance criteria for performance-based procedures. Meeting these requirements must be demonstrated experimentally using an appropriate system suitability procedure and reference materials. Any alternative procedure (e.g., an atomic-absorption-based procedure) that has been validated and meets the acceptance criteria that follow is considered to be suitable for use.

Meeting these validation acceptance criteria is sufficient to demonstrate that the procedure will produce comparable results to those obtained using the procedure prescribed in the monograph.

- **ACCURACY**
  **Standard solutions:** Prepare solutions containing iron at concentrations ranging from 50% to 150% of *J* using appropriate reference materials.
  **Test samples:** Spike the material under test with the appropriate reference materials before any sample preparation steps (digestion or solubilization). Prepare three replicate samples at concentrations ranging from 50% to 150% of *J* for iron.
  **Acceptance criteria**
  **Spike recovery:** 70%–150% for the mean of three replicate preparations at each concentration
- **PRECISION**
  **Repeatability**
  **Test samples:** Six independent samples of material under test (taken from the same lot) spiked with appropriate reference materials for iron at the indicated concentration
  **Acceptance criteria**
  **Relative standard deviation:** Not more than 20% ($N = 6$) for iron
  **Intermediate precision (ruggedness)**
  **Analysis:** Perform the *Repeatability* analysis again on a different day, with different instrumentation, with a different analyst, or a combination thereof. Combine the results of this analysis with the *Repeatability* analysis so the total number of analyses is 12.
  **Acceptance criteria**
  **Relative standard deviation:** Not more than 25% ($N = 12$) for iron
- **SPECIFICITY:** The procedure must be able to unequivocally assess (see *Validation of Compendial Procedures* ⟨1225⟩) iron in the presence of components that may be expected to be present, including matrix components.
- **LIMIT OF QUANTITATION, RANGE, AND LINEARITY:** Demonstrated by meeting the *Accuracy* requirement.▲ (Official 1-Jun-2023)

***Add the following:***

## ▲GLOSSARY

**Concentrated acid:** Concentrated ultra-pure nitric, sulfuric, hydrochloric, or hydrofluoric acid or aqua regia.
**Aqua regia:** Aqua regia is a mixture of concentrated hydrochloric and nitric acids, typically at ratios of 3:1 or 4:1.
**Matched matrix:** Solutions having the same solvent composition as the *Sample solution*. In the case of an aqueous solution, a matched matrix would indicate that the same acids and acid concentrations are used in both preparations.

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**Target limit or target concentration:** The acceptance value for the elemental impurity being evaluated, in this case iron. Where a monograph specifies a threshold limit, this shall become the target limit or target concentration of iron for the material. Exceeding the target limit indicates that a material under test exceeds the acceptable value. The determination of compliance is addressed in other chapters.

***J*:** The concentration (w/w) of the element of interest, in this case iron, at the target limit, appropriately diluted to the working range of the instrument.

**Appropriate reference materials:** Where "appropriate reference materials" are specified in the chapter, certified reference materials (CRMs) from a national metrology institute (NMI), or reference materials that are traceable to the CRM of an NMI should be used. An example of an NMI in the United States is the National Institute of Standards and Technology (NIST).▲ (Official 1-Jun-2023)

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# ⟨251⟩ LEAD

***Change to read:***

**▲INTRODUCTION▲** (Official 1-Jun-2023)

The imposition of stringent limits on the amounts of lead (Pb) that may be present in pharmaceutical products has resulted in the use of ▲three▲ (Official 1-Jun-2023) methods, of which ▲*Procedure 1*,▲ (Official 1-Jun-2023) set forth in this chapter, depends upon extraction of lead by solutions of dithizone.

▲Use *Procedure 1*, *Procedure 2*, or *Procedure 3* as indicated in the individual monograph. *Procedure 2* or *Procedure 3* can be used in all circumstances, provided that suitability is demonstrated by meeting the requirements in *Requirements for Procedure Validation*.▲ (Official 1-Jun-2023)

***Change to read:***

**▲PROCEDURES**

• **PROCEDURE 1: CHEMICAL METHOD▲** (Official 1-Jun-2023)

Select all reagents for this test to have as low a content of lead as practicable, and store all reagent solutions in containers of borosilicate glass. Thoroughly rinse all glassware with warm dilute nitric acid (1 in 2), followed by water.

**Ammonium cyanide solution:** Dissolve 2 g of potassium cyanide in 15 mL of ammonium hydroxide, and dilute with water to 100 mL.

**Dithizone extraction solution:** Dissolve 30 mg of dithizone in 1000 mL of chloroform, and add 5 mL of alcohol. Store the solution in a refrigerator. Before use, shake a suitable volume of the *Dithizone extraction solution* with about half its volume of dilute nitric acid (1 in 100), discarding the nitric acid.

**Ammonium citrate solution:** Dissolve 40 g of citric acid in 90 mL of water. Add 2 or 3 drops of phenol red TS, then cautiously add ammonium hydroxide until the solution acquires a reddish color. Remove any lead that may be present by extracting the solution with 20-mL portions of *Dithizone extraction solution*, until the dithizone solution retains its orange–green color.

**Diluted standard lead solution:** Dilute an accurately measured volume of standard lead solution TS (containing 10 µg of lead per milliliter), with 9 volumes of dilute nitric acid (1 in 100) to obtain a solution that contains 1 µg of lead per milliliter.

**Hydroxylamine hydrochloride solution:** Dissolve 20 g of hydroxylamine hydrochloride in sufficient water to make approximately 65 mL. Transfer to a separator, add 5 drops of thymol blue TS, then add ammonium hydroxide until the solution assumes a yellow color. Add 10 mL of sodium diethyldithiocarbamate solution (1 in 25), mix, and allow to stand for 5 min. Extract this solution with successive 10- to 15-mL portions of chloroform until a 5-mL portion of the chloroform extract does not assume a yellow color when shaken with cupric sulfate TS. Add 3 N hydrochloric acid until the solution is pink (if necessary, add 1 or 2 drops more of thymol blue TS), then dilute with water to 100 mL.

**Potassium cyanide solution:** Dissolve 50 g of potassium cyanide in sufficient water to make 100 mL. Remove the lead from this solution by extraction with successive portions of *Dithizone extraction solution*, as described in *Ammonium citrate solution*, then extract any dithizone remaining in the cyanide solution by shaking with chloroform. Finally, dilute the cyanide solution with sufficient water so that each 100 mL contains 10 g of potassium cyanide.

**Standard dithizone solution:** Dissolve 10 mg of dithizone in 1000 mL of chloroform. Keep the solution in a glass-stoppered, lead-free bottle, suitably wrapped to protect it from light, and store in a refrigerator.

**Test preparation:** [NOTE—If, in the following preparation, the substance under test reacts too rapidly and begins charring with 5 mL of sulfuric acid before heating, instead use 10 mL of cooled dilute sulfuric acid (1 in 2), and add a few drops of hydrogen peroxide before heating.] Where the monograph does not specify preparation of a solution, prepare a *Test preparation* as follows.

[**CAUTION**—Exercise safety precautions in this procedure, because some substances may react with explosive violence when digested with hydrogen peroxide.]

Transfer 1.0 g of the substance under test to a suitable flask, add 5 mL of sulfuric acid and a few glass beads, and digest on a hot plate in a hood until charring begins. Other suitable means of heating may be substituted. (Add additional sulfuric acid, if necessary, to wet the substance completely, but do not add more than a total of 10 mL.) Add, dropwise and with caution, 30 percent hydrogen peroxide, allowing the reaction to subside and heat between drops. Add the first few drops very slowly, mix carefully to prevent a rapid reaction, and discontinue heating if foaming becomes excessive. Swirl the solution in the flask to prevent unreacted substance from caking on the walls of the flask. [NOTE—Add peroxide whenever the mixture turns brown or darkens.]

Continue the digestion until the substance is completely destroyed, copious fumes of sulfur trioxide are evolved, and the solution is colorless. Cool, and cautiously add 10 mL of water. Evaporate until sulfur trioxide again is evolved, and cool. Repeat this procedure with another 10 mL of water to remove any traces of hydrogen peroxide. Cautiously dilute with 10 mL of water, and cool.

**Analysis:** Transfer the *Test preparation*, rinsing with 10 mL of water, or the volume of the prepared sample specified in the monograph to a separator, and, unless otherwise directed in the monograph, add 6 mL of *Ammonium citrate solution* and 2 mL of *Hydroxylamine hydrochloride solution*. (For the determination of lead in iron salts, use 10 mL of *Ammonium citrate solution*.) Add 2 drops of phenol red TS, and make the solution just alkaline (red in color) by the addition of ammonium hydroxide. Cool the solution if necessary, and add 2 mL of *Potassium cyanide solution*. Immediately extract the solution with 5-mL portions of *Dithizone extraction solution*, draining off each extract into another separator, until the dithizone solution retains its green color. Shake the combined dithizone solutions for 30 s with 20 mL of dilute nitric acid (1 in 100), and discard the chloroform layer. Add to the acid solution 5.0 mL of *Standard dithizone solution* and 4 mL of ▲*Ammonium*▲ (ERR 1-Jun-2023) *cyanide solution*, and shake for 30 s.

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**Acceptance criteria:** The color of the chloroform layer is of no deeper shade of violet than that of a control made with a volume of *Diluted standard lead solution* equivalent to the amount of lead permitted in the sample under examination and made with the same quantities of the same reagents and in the same manner as in the test with the sample.

▲• **PROCEDURE 2 AND PROCEDURE 3**

Both *Procedure 2* and *Procedure 3* are ICP-based procedures and can be used for the determination of lead. *Procedure 2* can be used for the determination of lead by inductively coupled plasma atomic (or optical) emission spectroscopy (ICP–AES or ICP–OES). *Procedure 3* can be used for the determination of lead by ICP–MS.

Before initial use, the analyst should verify that the procedure is appropriate for the instrument and sample used (procedural verification) by meeting the *Requirements for Procedure Validation*.

Where a monograph specifies a limit for lead concentration, the value listed in the monograph should be used as the *J* value for the purposes of this test.

System standardization and suitability evaluation using applicable reference materials should be performed on the day of analysis.

**Sample preparation:** Forms of sample preparation include neat, direct aqueous solution, direct organic solution, and indirect solution. The selection of the appropriate sample preparation depends on the material under test and is the responsibility of the analyst. When a sample preparation is not indicated in the monograph, an analyst may use any appropriately validated preparation procedure. In cases where spiking of a material under test is necessary to provide an acceptable signal intensity, the blank should be spiked with lead using, where possible, the same spiking solution. [NOTE—All liquid samples should be weighed.]

**Closed vessel digestion:** This sample preparation procedure is designed for samples that must be digested in a concentrated acid using a closed vessel digestion apparatus. Closed vessel digestion minimizes the loss of volatile impurities. The choice of a concentrated acid depends on the sample matrix. The use of any of the concentrated acids may be appropriate, but each introduces inherent safety risks. Therefore, appropriate safety precautions should be used at all times. [NOTE—Weights and volumes provided may be adjusted to meet the requirements of the digestion apparatus used.]

An example procedure that has been shown to have broad applicability is as follows. Dehydrate and predigest 0.5 g of the primary sample in 5 mL of freshly prepared concentrated acid. Allow to sit loosely covered for 30 min in a fume hood. Add an additional 10 mL of concentrated acid, and digest using a closed vessel technique until the digestion or extraction is complete. Repeat, if necessary, by adding an additional 5 mL of concentrated acid. [NOTE—Follow the manufacturer's recommended procedures to ensure safe use.]

**Reagents:** All reagents used for the preparation of sample and standard solutions should be free of elemental impurities, in accordance with *Plasma Spectrochemistry* ⟨730⟩.

**Procedure 2: ICP–OES**

**Standardization solution 1:** 1.5*J* of lead in a matched matrix

**Standardization solution 2:** 0.5*J* of lead in a matched matrix

**Sample stock solution:** Prepare as directed in *Sample preparation*.

**Sample solution:** Dilute the *Sample stock solution* with an appropriate solvent to obtain a final lead concentration of not more than 1.5*J*.

**Blank:** Matched matrix

**Elemental spectrometric system**

(See ⟨730⟩.)

**Rinse:** Use diluent.

**Standardization:** *Standardization solution 1*, *Standardization solution 2*, and *Blank*

**System suitability**

**Sample:** *Standardization solution 1*

**Suitability requirements**

**Drift:** Compare results obtained from *Standardization solution 1* before and after the analysis of the *Sample solution*.

**Suitability criteria:** Not more than 20% for lead. [NOTE—If samples are high in mineral content, rinse the system well before introducing the *Sample* in order to minimize carryover.]

**Analysis:** Analyze according to the manufacturer's suggestions for program and wavelength. Calculate and report results on the basis of the original sample size. [NOTE—Appropriate measures must be taken to correct for matrix-induced interferences (e.g., wavelength overlaps).]

**Procedure 3: ICP–MS**

Follow *Procedure 2* except for *Detector* and *Analysis*.

[NOTE—An instrument with a cooled spray chamber is recommended. (A collision cell or reaction cell may also be beneficial.)]

**Detector:** Mass spectrometer

**Analysis:** Analyze according to the manufacturer's suggestions for program and mass-to-charge ratio. Calculate and report results based on the original sample size. [NOTE—Appropriate measures must be taken to correct for matrix-induced interferences.]▲ (Official 1-Jun-2023)

***Add the following:***

## ▲REQUIREMENTS FOR PROCEDURE VALIDATION

The following section defines the validation parameters and the acceptance criteria for performance-based procedures. Meeting these requirements must be demonstrated experimentally using an appropriate system suitability procedure and reference materials. Any alternative procedure (e.g., an atomic-absorption-based procedure) that has been validated and meets the acceptance criteria that follow is considered to be suitable for use.

Meeting these validation acceptance criteria is sufficient to demonstrate that the procedure will produce comparable results to those obtained using the procedure prescribed in the monograph.

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- **ACCURACY**
  **Standard solutions:** Prepare solutions containing lead at concentrations ranging from 50% to 150% of *J* using appropriate reference materials.
  **Test samples:** Spike the material under test with the appropriate reference materials before any sample preparation steps (digestion or solubilization). Prepare three replicate samples at concentrations ranging from 50% to 150% of *J* for lead.
  **Acceptance criteria**
  **Spike recovery:** 70%–150% for the mean of three replicate preparations at each concentration
- **PRECISION** ▲ (ERR 1-Jun-2023)
  **Repeatability**
  **Test samples:** Six independent samples of material under test (taken from the same lot) spiked with appropriate reference materials for lead at the indicated concentration
  **Acceptance criteria**
  **Relative standard deviation:** Not more than 20% ($N = 6$) for lead
  **Intermediate precision (Ruggedness)**
  **Analysis:** Perform the *Repeatability* analysis again on a different day, with different instrumentation, with a different analyst, or a combination thereof. Combine the results of this analysis with the *Repeatability* analysis so the total number of analyses is 12.
  **Acceptance criteria**
  **Relative standard deviation:** Not more than 25% ($N = 12$) for lead
- **SPECIFICITY:** The procedure must be able to unequivocally assess (see *Validation of Compendial Procedures* ⟨1225⟩) lead in the presence of components that may be expected to be present, including matrix components.
- **LIMIT OF QUANTITATION, RANGE, AND LINEARITY:** Demonstrated by meeting the *Accuracy* requirement. ▲ (Official 1-Jun-2023)

***Add the following:***

## ▲GLOSSARY

**Concentrated acid:** Concentrated ultra-pure nitric, sulfuric, hydrochloric, or hydrofluoric acid or aqua regia.
**Aqua regia:** Aqua regia is a mixture of concentrated hydrochloric and nitric acids, typically at ratios of 3:1 or 4:1.
**Matched matrix:** Solutions having the same solvent composition as the *Sample solution*. In the case of an aqueous solution, a matched matrix would indicate that the same acids and acid concentrations are used in both preparations.
**Target limit or target concentration:** The acceptance value for the elemental impurity being evaluated, in this case lead. Where a monograph specifies a threshold limit, this shall become the target limit or target concentration of lead for the material. Exceeding the target limit indicates that a material under test exceeds the acceptable value. The determination of compliance is addressed in other chapters.
***J*:** The concentration (w/w) of the element of interest, in this case lead, at the target limit, appropriately diluted to the working range of the instrument.
**Appropriate reference materials:** Where "appropriate reference materials" are specified in the chapter, certified reference materials (CRMs) from a national metrology institute (NMI), or reference materials that are traceable to the CRM of an NMI should be used. An example of an NMI in the United States is the National Institute of Standards and Technology (NIST). ▲ (Official 1-Jun-2023)

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# ⟨261⟩ MERCURY

***Add the following:***

## ▲INTRODUCTION

This chapter describes five analytical procedures for the evaluation of the levels of mercury (Hg). Use *Procedure 1*, *Procedure 2*, *Procedure 3*, *Procedure 4*, or *Procedure 5* as indicated in the individual monograph. *Procedure 4* or *Procedure 5* can be used in all circumstances, provided that suitability is demonstrated by meeting the *Requirements for Procedure Validation*.▲ (Official 1-Jun-2023)

***Change to read:***

## ▲PROCEDURES

• **PROCEDURE 1: TITRATION**▲ (Official 1-Jun-2023)

[NOTE—Mercuric dithizonate is light sensitive. Perform this test in subdued light.]

**Dithizone stock solution:** Dissolve 40 mg of dithizone in 1000 mL of chloroform.

**Dithizone titrant:** Dilute 30.0 mL of *Dithizone stock solution* with chloroform to 100.0 mL. This solution contains approximately 12 mg of dithizone per liter.

**Mercury stock solution:** Transfer 135.4 mg of mercuric chloride to a 100-mL volumetric flask, and dilute with 1 N sulfuric acid to volume. This solution contains the equivalent of 100 mg of mercury in 100 mL.

**Mercury solution for standardizing dithizone titrant:** Transfer 2.0 mL of *Mercury stock solution* to a 100-mL volumetric flask, and dilute with 1 N sulfuric acid to volume. Each milliliter of this solution contains the equivalent of 20 µg of mercury.

The following solutions are called for in the limit test for mercury that is specified in the monographs for *Ferrous Fumarate*, *Ferrous Sulfate*, and *Dried Ferrous Sulfate*:

**Hydroxylamine hydrochloride solution:** ▲Dissolve 20 g of hydroxylamine hydrochloride in sufficient water to make approximately 65 mL. Transfer to a separator, add 5 drops of thymol blue TS, then add ammonium hydroxide until the solution assumes a yellow color. Add 10 mL of sodium diethyldithiocarbamate solution (1 in 25), mix, and allow to stand for 5 min. Extract this solution with successive 10- to 15-mL portions of chloroform until a 5-mL portion of the chloroform extract does not assume a yellow color when shaken with cupric sulfate TS. Add 3 N hydrochloric acid until the solution is pink (if necessary, add 1 or 2 drops more of thymol blue TS), then dilute with water to 100 mL.▲ (Official 1-Jun-2023)

**Standard mercury solution:** On the day of use, quantitatively dilute 1.0 mL of *Mercury stock solution* with 1 N sulfuric acid to 1000 mL. Each milliliter of the resulting solution contains the equivalent of 1 µg of mercury.

**Dithizone extraction solution:** ▲Dissolve 30 mg of dithizone in 1000 mL of chloroform, and add 5 mL of alcohol. Store the solution in a refrigerator. Before use, shake a suitable volume of the *Dithizone extraction solution* with about half its volume of dilute nitric acid (1 in 100), discarding the nitric acid.▲ (Official 1-Jun-2023)

**Diluted dithizone extraction solution:** Just prior to use, dilute 5 mL of *Dithizone extraction solution* with 25 mL of chloroform.

**Standardization of dithizone titrant:** Transfer 1.0 mL of *Mercury solution for standardizing dithizone titrant* to a 250-mL separator, and add 100 mL of 1 N sulfuric acid, 90 mL of water, 1 mL of glacial acetic acid, and 10 mL of hydroxylamine hydrochloride solution (1 in 5). Titrate the solution with *Dithizone titrant* from a 10-mL microburet, shaking the mixture 20 times after each addition and allowing the chloroform layer to separate, then discarding the chloroform layer. Continue until a final addition of *Dithizone titrant* is green in color after shaking. Calculate the quantity, in micrograms, of mercury equivalent to each milliliter of *Dithizone titrant*:

$$\text{Result} = 20/V$$

$V$ = volume of *Dithizone titrant* added (mL)

**Test preparation:** Transfer about 2 g of the substance under test, accurately weighed, to a glass-stoppered, 250-mL conical flask, add 20 mL of a mixture of equal volumes of nitric acid and sulfuric acid, attach a suitable condenser, reflux the mixture for 1 h, cool, cautiously dilute with water, and boil until fumes of nitrous acid are no longer noticeable. Cool the solution, cautiously dilute with water, transfer to a 200-mL volumetric flask, dilute with water to volume, mix, and filter.

**▲Analysis:**▲ (Official 1-Jun-2023) Transfer 50.0 mL of *Test preparation* to a 250-mL separator, and extract with successive small portions of chloroform until the last chloroform extract remains colorless. Discard the chloroform extract, and add to the extracted *Test preparation* 50 mL of 1 N sulfuric acid, 90 mL of water, 1 mL of glacial acetic acid, and 10 mL of hydroxylamine hydrochloride solution (1 in 5). Proceed as directed in *Standardization of dithizone titrant*, beginning with "Titrate the solution". Calculate the amount of mercury.

• **▲PROCEDURE 2 AND PROCEDURE 3**▲ (Official 1-Jun-2023)

[NOTE—Wash all glassware associated with the test with nitric acid, and rinse thoroughly with water before use.]

**Mercury detection instrument:** Use any suitable atomic absorption spectrophotometer equipped with a fast-response recorder and capable of measuring the radiation absorbed by mercury vapors at the mercury resonance line of 253.6 nm.

**Aeration apparatus:** The apparatus (see *Figure 1*) consists of a flow meter capable of measuring flow rates from 500–1000 mL/min, connected via a three-way stopcock fitted with a polytef plug to an aeration vessel (250-mL gas washing bottle), followed by a trap, a drying tube packed with magnesium perchlorate, a 10-cm × 25-mm flow-through cell with quartz windows, and terminating with a vent to a fume hood.

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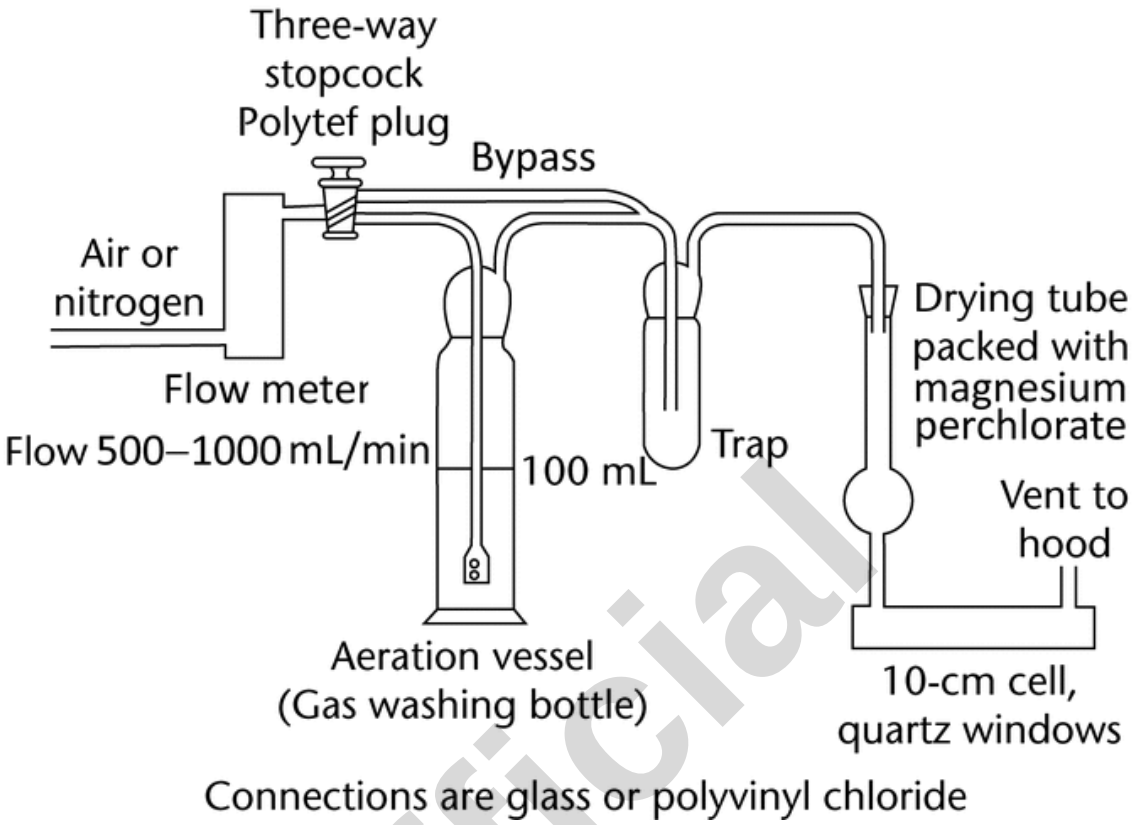


Figure 1. Mercury aeration apparatus.

**Potassium permanganate solution:** Dissolve 5 g of potassium permanganate in 100 mL of water.
**Hydroxylamine hydrochloride solution:** Dissolve 10 g of hydroxylamine hydrochloride in 100 mL of water.
**Stannous chloride solution:** Dissolve 10 g of stannous chloride dihydrate ($SnCl_2 \cdot 2H_2O$) in 20 mL of warm hydrochloric acid, and add 80 mL of water. Prepare fresh each week.
**Standard mercury solution:** Prepare from *Mercury stock solution* as directed under ▲Procedure 1.▲ (Official 1-Jun-2023) Each milliliter of the *Standard mercury solution* contains the equivalent of 1 µg of mercury.
**Test ▲sample▲** (Official 1-Jun-2023)**:** Unless otherwise directed in the individual monograph, use the quantity, in grams, of the test substance calculated as follows:

$$\text{Result} = 2.0/L$$

$L$ = limit of mercury (ppm)

**▲Procedure 2: Atomic Absorption Spectroscopy▲** (Official 1-Jun-2023)

**Standard preparation:** Pipet 2.0 mL of *Standard mercury solution* into a 100-mL beaker, and add 35 mL of water, 3 mL of sulfuric acid, and 1 mL of potassium permanganate solution. Cover the beaker with a watch glass, boil for a few seconds, and cool.

**Test preparation:** Transfer the calculated amount of the test substance to a 100-mL beaker, and add 35 mL of water. Stir, and warm to assist solution, if necessary. Add 2 drops of phenolphthalein TS, and, as necessary, slowly neutralize with constant stirring, using 1 N sodium hydroxide or 1 N sulfuric acid. Add 3 mL of sulfuric acid and 1 mL of *Potassium permanganate solution*. Cover the beaker with a watch glass, boil for a few seconds, and cool.

**▲Analysis:▲** (Official 1-Jun-2023) Assemble the aeration apparatus (see *Figure 1*), with the aeration vessel and the trap empty, and the stopcock in the bypass position. Connect the apparatus to the absorption cell, and adjust the air or nitrogen flow rate so that, in the following procedure, maximum absorption and reproducibility are obtained without excessive foaming in the test solution. Obtain a smooth baseline reading at 253.6 nm, following the manufacturer's instructions for operating the instrument.

Treat the *Standard preparation* and the *Test preparation* similarly, as follows. Destroy the excess permanganate by adding *Hydroxylamine hydrochloride solution*, dropwise, until the solution is colorless. Immediately wash the solution into the aeration vessel with water, and dilute with water to 100 mL. Add 2 mL of *Stannous chloride solution*, and immediately reconnect the aeration vessel to the aeration apparatus. Turn the stopcock from the bypass position to the aerating position, and continue the aeration until the absorption peak has been passed and the recorder pen returns to the baseline. Disconnect the aeration vessel from the apparatus, and wash with water after each use. After correcting for any reagent blank, any absorbance produced by the *Test preparation* does not exceed that produced by the *Standard preparation*.

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▲**Procedure 3: Atomic Absorption Spectroscopy**▲ (Official 1-Jun-2023)

[**CAUTION**—Some substances may react with explosive violence when digested with hydrogen peroxide. Exercise safety precautions at all times.]

**Standard preparation:** Pipet 2.0 mL of *Standard mercury solution* into a 125-mL conical flask, add 3 mL each of nitric acid and sulfuric acid, mix, and add an amount of 30 percent hydrogen peroxide equal to the total amount used in preparing the *Test preparation*. Attach a suitable water-cooled condenser with a standard-taper joint to fit the flask, and reflux the mixture in a fume hood for 1 h. Turn off the water circulating through the condenser, and heat until white fumes appear in the flask. Cool, and cautiously add 10 mL of water through the condenser, while swirling the flask. Again heat until white fumes appear, cool, and add an additional 15 mL of water. Remove the condenser, and rinse the sides of the flask to obtain a volume of 35 mL. Add 1 mL of *Potassium permanganate solution*, boil for a few seconds, and cool.

**Test preparation:** Transfer the calculated amount of the test substance to a 125-mL conical flask. Add 5 mL each of nitric acid and sulfuric acid and a few glass beads. Attach a suitable water-cooled condenser with a standard-taper joint to fit the flask, and digest in a fume hood, preferably on a hot plate, and at a temperature not exceeding 120°, until charring begins. (If additional sulfuric acid is necessary to wet the specimen completely, add it carefully through the condenser, but do not allow the total volume added to exceed 10 mL.) After the test substance has been decomposed by the acid, cautiously add, dropwise through the condenser, 30 percent hydrogen peroxide, allowing the reaction to subside and again heating between drops (add the first few drops very slowly with sufficient mixing, in order to prevent a rapid reaction; discontinue heating if foaming becomes excessive). When the reaction has abated, heat cautiously, rotating the flask occasionally to prevent the specimen from caking on glass exposed to the heating unit. Maintain oxidizing conditions at all times during the digestion by adding small quantities of the hydrogen peroxide solution whenever the mixture turns brown or darkens. Continue the digestion until the organic matter is destroyed, and then reflux the mixture for 1 h. Turn off the water circulating through the condenser, and heat until fumes of sulfur trioxide are copiously evolved and the solution becomes colorless or retains only a light straw color. Cool, and cautiously add 10 mL of water through the condenser, while swirling the flask. Again heat until white fumes appear. Cool, and cautiously add 15 mL of water. Remove the condenser, and rinse the sides of the flask with a few milliliters of water to obtain a volume of 35 mL. Add 1 mL of *Potassium permanganate solution*, boil for a few seconds, and cool.

▲**Analysis:**▲ (Official 1-Jun-2023) Proceed as directed for *Analysis* under ▲*Procedure 2*.

- **PROCEDURE 4 AND PROCEDURE 5**

Both *Procedures 4* and *Procedure 5* are ICP-based procedures and can be used for the determination of mercury. *Procedure 4* can be used for the determination of mercury by inductively coupled plasma atomic (or optical) emission spectroscopy (ICP–AES or ICP–OES). *Procedure 5* can be used for the determination of mercury by ICP–MS.

Before initial use, the analyst should verify that the procedure is appropriate for the instrument and sample used (procedural verification) by meeting the *Requirements for Procedure Validation*.

Where a monograph specifies a limit for mercury concentration, the value listed in the monograph should be used as the *J* value for the purposes of this test.

System standardization and suitability evaluation using applicable reference materials should be performed on the day of analysis.

**Sample preparation:** Forms of sample preparation include neat, direct aqueous solution, direct organic solution, and indirect solution. The selection of the appropriate sample preparation depends on the material under test and is the responsibility of the analyst. When a sample preparation is not indicated in the monograph, an analyst may use any appropriately validated preparation procedure. In cases where spiking of a material under test is necessary to provide an acceptable signal intensity, the blank should be spiked with mercury using, where possible, the same spiking solution. [NOTE—All liquid samples should be weighed.]

**Closed vessel digestion:** This sample preparation procedure is designed for samples that must be digested in a concentrated acid using a closed vessel digestion apparatus. Closed vessel digestion minimizes the loss of volatile impurities. The choice of a concentrated acid depends on the sample matrix. The use of any of the concentrated acids may be appropriate, but each introduces inherent safety risks. Therefore, appropriate safety precautions should be used at all times. [NOTE—Weights and volumes provided may be adjusted to meet the requirements of the digestion apparatus used.]

An example procedure that has been shown to have broad applicability is as follows. Dehydrate and predigest 0.5 g of primary sample in 5 mL of freshly prepared concentrated acid. Allow to sit loosely covered for 30 min in a fume hood. Add an additional 10 mL of concentrated acid, and digest, using a closed vessel technique, until digestion or extraction is complete. Repeat, if necessary, by adding an additional 5 mL of concentrated acid. [NOTE—Follow the manufacturer's recommended procedures to ensure safe use.]

**Reagents:** All reagents used for the preparation of sample and standard solutions should be free of elemental impurities, in accordance with *Plasma Spectrochemistry* ⟨730⟩.

**Procedure 4: ICP–OES**

**Standardization solution 1:** 1.5*J* of mercury in a matched matrix

**Standardization solution 2:** 0.5*J* of mercury in a matched matrix

**Sample stock solution:** Prepare as directed in *Sample preparation*. Allow the sample to cool, if necessary. For mercury determination, add an appropriate stabilizer.

**Sample solution:** Dilute the *Sample stock solution* with an appropriate solvent to obtain a final concentration of mercury at not more than 1.5*J*.

**Blank:** Matched matrix

**Elemental spectrometric system**

(See ⟨730⟩.)

**Rinse:** Use diluent.

**Standardization:** *Standardization solution 1*, *Standardization solution 2*, and *Blank*

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**System suitability**
**Sample:** *Standardization solution 1*
**Suitability requirements**
**Drift:** Compare results obtained from *Standardization solution 1* before and after the analysis of the *Sample solution*.
**Suitability criteria:** Not more than 20% for mercury. [NOTE—If samples are high in mineral content, rinse the system well before introducing the *Sample* in order to minimize carryover.]
**Analysis:** Analyze according to the manufacturer's suggestions for program and wavelength. Calculate and report results on the basis of the original sample size. [NOTE—Appropriate measures must be taken to correct for matrix-induced interferences (e.g., wavelength overlaps).]

**Procedure 5: ICP–MS**
Follow *Procedure 4* except for *Detector* and *Analysis*.
[NOTE—An instrument with a cooled spray chamber is recommended. (A collision cell or reaction cell may also be beneficial.)]
**Detector:** Mass spectrometer
**Analysis:** Analyze according to the manufacturer's suggestions for program and mass-to-charge ratio. Calculate and report results based on the original sample size. [NOTE—Appropriate measures must be taken to correct for matrix-induced interferences.]▲ (Official 1-Jun-2023)

***Add the following:***

**▲REQUIREMENTS FOR PROCEDURE VALIDATION**

The following section defines the validation parameters and the acceptance criteria for performance-based procedures. Meeting these requirements must be demonstrated experimentally using an appropriate system suitability procedure and reference materials. Any alternative procedure (e.g., an atomic-absorption-based procedure) that has been validated and meets the acceptance criteria that follow is considered to be suitable for use.

Meeting these validation acceptance criteria is sufficient to demonstrate that the procedure will produce comparable results to those obtained using the procedure prescribed in the monograph.

- **ACCURACY**
  **Standard solutions:** Prepare solutions containing mercury at concentrations ranging from 50% to 150% of *J* using appropriate reference materials.
  **Test samples:** Spike the material under test with the appropriate reference materials before any sample preparation steps (digestion or solubilization). Prepare three replicate samples at concentrations ranging from 50% to 150% of *J* for mercury.
  **Acceptance criteria**
  **Spike recovery:** 70%–150% for the mean of three replicate preparations at each concentration
- **PRECISION**
  **Repeatability**
  **Test samples:** Six independent samples of material under test (taken from the same lot) spiked with appropriate reference materials for mercury at the indicated concentration
  **Acceptance criteria**
  **Relative standard deviation:** Not more than 20% ($N = 6$) for mercury
  **Intermediate precision (ruggedness)**
  **Analysis:** Perform the *Repeatability* analysis again on a different day, with different instrumentation, with a different analyst, or a combination thereof. Combine the results of this analysis with the *Repeatability* analysis so the total number of analyses is 12.
  **Acceptance criteria**
  **Relative standard deviation:** Not more than 25% ($N = 12$) for mercury
- **SPECIFICITY:** The procedure must be able to unequivocally assess (see *Validation of Compendial Procedures* ⟨1225⟩) mercury in the presence of components that may be expected to be present, including matrix components.
- **LIMIT OF QUANTITATION, RANGE, AND LINEARITY:** Demonstrated by meeting the *Accuracy* requirement.▲ (Official 1-Jun-2023)

***Add the following:***

## ▲GLOSSARY

**Concentrated acid:** Concentrated ultra-pure nitric, sulfuric, hydrochloric, or hydrofluoric acid or aqua regia.
**Aqua regia:** Aqua regia is a mixture of concentrated hydrochloric and nitric acids, typically at ratios of 3:1 or 4:1.
**Matched matrix:** Solutions having the same solvent composition as the *Sample solution*. In the case of an aqueous solution, matched matrix would indicate that the same acids, acid concentrations are used in both preparations.
**Target limit or target concentration:** The acceptance value for the elemental impurity being evaluated, in this case mercury. Where a monograph specifies a threshold limit, this shall become the target limit or target concentration for mercury for the material. Exceeding the target limit indicates that a material under test exceeds the acceptable value. The determination of compliance is addressed in other chapters.
***J*:** The concentration (w/w) of the element of interest, in this case mercury, at the target limit, appropriately diluted to the working range of the instrument.
**Appropriate reference materials:** Where "appropriate reference materials" are specified in the chapter, certified reference materials (CRMs) from a national metrology institute (NMI), or reference materials that are traceable to the CRM of an NMI should be used. An example of an NMI in the United States is the National Institute of Standards and Technology (NIST).▲ (Official 1-Jun-2023)

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# ⟨267⟩ POROSIMETRY BY MERCURY INTRUSION

In general, different types of pores may be pictured as apertures, channels, or cavities within a solid body or as space (i.e., interstices or voids) between solid particles in a bed, compact, or aggregate. *Porosity* is a term that is often used to indicate the porous nature of solid material and is more precisely defined as the ratio of the volume of accessible pores and voids to the total volume occupied by a given amount of the solid. In addition to the accessible pores, a solid may contain closed pores, which are isolated from the external surface and into which fluids are not able to penetrate. The characterization of closed pores, i.e., cavities with no access to an external surface, is not covered in this general chapter.

Porous materials may take the form of fine or coarse powders, compacts, extrudates, sheets, or monoliths. Their characterization usually involves the determination of the total pore volume or porosity as well as the pore size distribution.

It is well established that the performance of a porous solid (e.g., its strength, reactivity, permeability, or adsorbent power) is dependent on its pore structure. Many different methods have been developed for the characterization of pore structure. In view of the complexity of most porous solids, it is not surprising to find that the results obtained are not always in agreement and that no single technique can be relied upon to provide a complete picture of the pore structure. The choice of the most appropriate method depends on the application of the porous solid, its chemical and physical nature, and the range of pore size.

This chapter provides guidance for measurement of porosity and pore size distribution by mercury porosimetry. It is a comparative test, usually destructive, in which the volume of mercury penetrating a pore or void is determined as a function of an applied hydrostatic pressure that can be related to a pore diameter. Other information such as pore shape and interconnectivity, the internal and external surface area, powder granulometry, and bulk and tapped density can also be inferred from volume–pressure curves; however, these aspects of the technique do not fall under the scope of this chapter.

Practical considerations presently limit the maximum applied absolute pressure reached by some equipment to about 400 MPa, corresponding to a minimum equivalent pore diameter of approximately 0.003 µm. The maximum diameter will be limited for samples having a significant depth because of the difference in the hydrostatic head of mercury from the top to the bottom of the sample. For most purposes this limit may be regarded as 400 µm.

Interparticle and intraparticle porosity can be determined, but the method does not distinguish between these porosities where they coexist.

The method is suitable for the study of most porous materials. Samples that amalgamate with mercury, such as certain metals, may be unsuitable for this technique or may require a preliminary passivation. Other materials may deform or compact under the applied pressure. In some cases, it may be possible to apply sample compressibility corrections, and useful comparative data may still be obtained.

The mercury porosimetry technique should be considered to be comparative, because for most porous media, a theory is not available to allow an absolute calculation of results of pore size distribution. Therefore, this technique is mainly recommended for development studies.

Mercury is toxic. Appropriate precautions must be observed to safeguard the health of the operator and others working in the area. Waste material must also be disposed of in a suitable manner, according to local regulations.

## PRINCIPLE

The technique is based on the measurement of the mercury volume intruded into a porous solid as a function of the applied pressure. The measurement includes only those pores into which mercury can penetrate at the pressure applied.

A nonwetting liquid penetrates into a porous system only under pressure. The pressure applied is in inverse proportion to the inner width of the pore aperture. In the case of cylindrical pores, the correlation between pore diameter and pressure is given by the Washburn equation:

$$d_p = -\frac{4 \cdot \sigma}{p}\cos(\theta) \quad [1]$$

$p$ = applied pressure, in pascals
$d_p$ = pore diameter, in meters
$\sigma$ = surface tension of mercury, in newtons per meter
$\theta$ = contact angle of mercury on the sample, in degrees

## APPARATUS

The sample holder, referred to as a penetrometer or dilatometer, has a calibrated capillary tube, through which the sample can be evacuated and through which mercury can enter. The capillary tube is attached to a wider tube in which the test sample is placed. The change in the volume of mercury intruded is usually measured by the change in capacitance between the mercury column in the capillary tube and a metal sleeve around the outside of the capillary tube. If precise measurements are required, the internal volume of the capillary tube should be between 20% and 90% of the expected pore and void volume of the sample. Because different materials exhibit a wide range of open porosities, a number of penetrometers with different diameter capillary tubes and sample volumes may be required. A typical setup for a mercury porosimeter instrument is given in *Figure 1*. The porosimeter may have separate ports for high- and low-pressure operation, or the low-pressure measurement may be carried out on a separate unit.

The pressure range is typically 4 kPa to 300 kPa for low-pressure operation and above 300 kPa for high-pressure operation, depending on the design of the particular apparatus and on the intended use.

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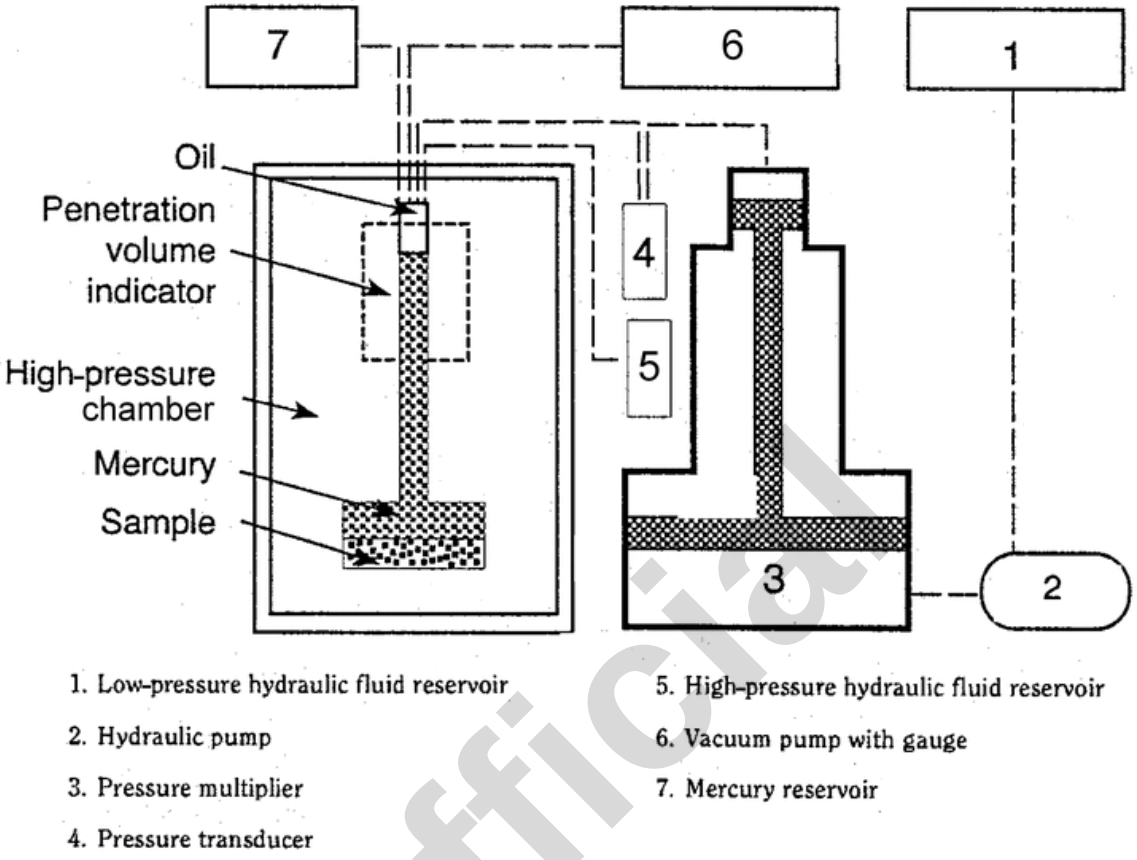


Figure 1. Example of the setup of a mercury porosimeter instrument.

## METHOD

### Sample Preparation

The sample is pretreated to remove adsorbed material that can obscure its accessible porosity by either heating and/or evacuation or by flowing inert gas. It may be possible to passivate the surface of wettable or amalgam-forming solids by producing a thin layer of oxide, or by coating with stearate. The sample of the pretreated solid is weighed and transferred to the penetrometer. The pore system of the sample is then degassed in a vacuum to a maximum residual pressure of 7 Pa.

### Filling the Penetrometer with Mercury

Use mercury of analytical quality. The sample is overlaid with mercury under vacuum. The vacuum is required to ensure the transfer of mercury from the reservoir to the penetrometer. In a filled penetrometer, the filling pressure is comprised of the applied pressure plus the pressure contribution created by the head of mercury contacting the sample. A typical filling pressure would be about 4 kPa. The hydrostatic pressure of the mercury over the sample can be minimized by filling the penetrometer in the horizontal positions.

### Low-Pressure Measurement

Admit air or nitrogen in a controlled manner to increase the pressure either in stages corresponding to the particular pore sizes of interest, or continuously at a slow rate. The concomitant change in the length of the mercury column in the capillary tube is recorded. When the maximum required pressure has been reached, reduce the pressure to ambient.

### High-Pressure Measurement

After measurement at low-pressure conditions, the penetrometer filled with mercury is transferred to the high-pressure port or unit of the instrument and overlaid with hydraulic fluid. Mercury is intruded into the pore system via the hydraulic fluid. Increase the pressure in the system to the maximum pressure reached in the low-pressure measurement, and record the intrusion volume at this pressure, because subsequent intrusion volumes are calculated from this initial volume. Increase the pressure

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either in stages corresponding to the particular pore sizes of interest, or continuously at a slow rate. The fall in the mercury column is measured up to the maximum required pressure. If required, the pressure may be decreased either in stages or continuously at a slow rate to determine the mercury extrusion curve. Make corrections to take account of changes in the volume of the mercury, the penetrometer, and other components of the volume detector system under elevated pressure. The extent of the corrections may be determined by means of blank measurements under the same conditions. An experimentally determined volume–pressure curve is shown in *Figure 2*.

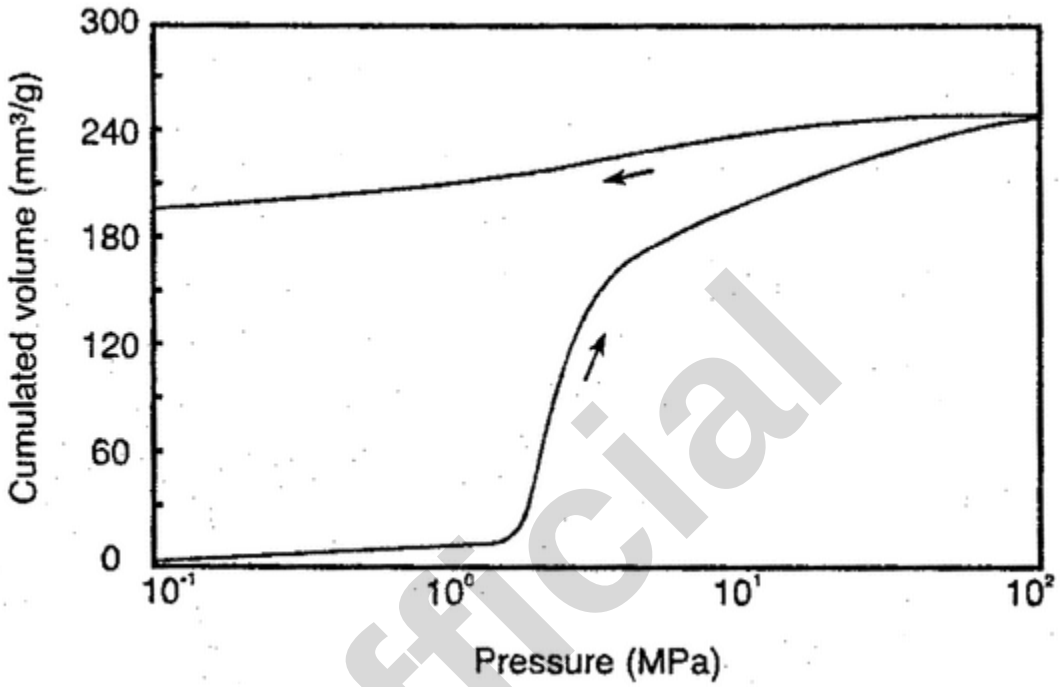


Figure 2. Volume–pressure curve as semilogarithmic plot.

## REPORTING OF RESULTS

The pressure readings are converted to pore diameter by means of the Washburn equation or by another model.

The surface tension of mercury, $\sigma$, depends not only on the temperature and the material, but also—in the case of markedly curved surface areas—on the radius of curvature. In general, values between 0.41 $N \cdot m^{-1}$ and 0.52 $N \cdot m^{-1}$ are measured at room temperature. If the value is not known, $\sigma = 0.48\ N \cdot m^{-1}$ can be used.

The contact angle of mercury $\theta$ in most cases is more than 90°. It may be determined using a contact angle instrument. If the $\theta$ value is not known, 130° can be used. Report the values of contact angle, surface tension, and the model used in the calculation. Visualization of the data can be done with several types of graphs. Frequently, in a graphical representation, the pore diameter is plotted on the abscissa and the dependent intruded specific volume on the ordinate to give the pore size distribution. It is appropriate here to choose a logarithmic scale for the abscissa (see *Figure 3*). The spaces between the particles of the solid sample are included as pores in the calculation. If the pores differ in size from the voids, the latter can be separated by choosing the relevant pore size range.

Extrusion curves may not be used for calculating the pore size distribution (for hysteresis, see *Figure 2*), because an intruded part of the mercury always remains in the pore system. The retention ratio may be useful for the qualitative characterization of pores that are only accessible via narrow openings (“ink-bottle pores”).

The most common characteristic values, such as the total intruded specific volume, the mean, and the median pore diameter are calculated from the pore size distribution. Moreover, sufficient information should be documented about the sample, the sample preparation, the evacuation conditions, and the instrument used.

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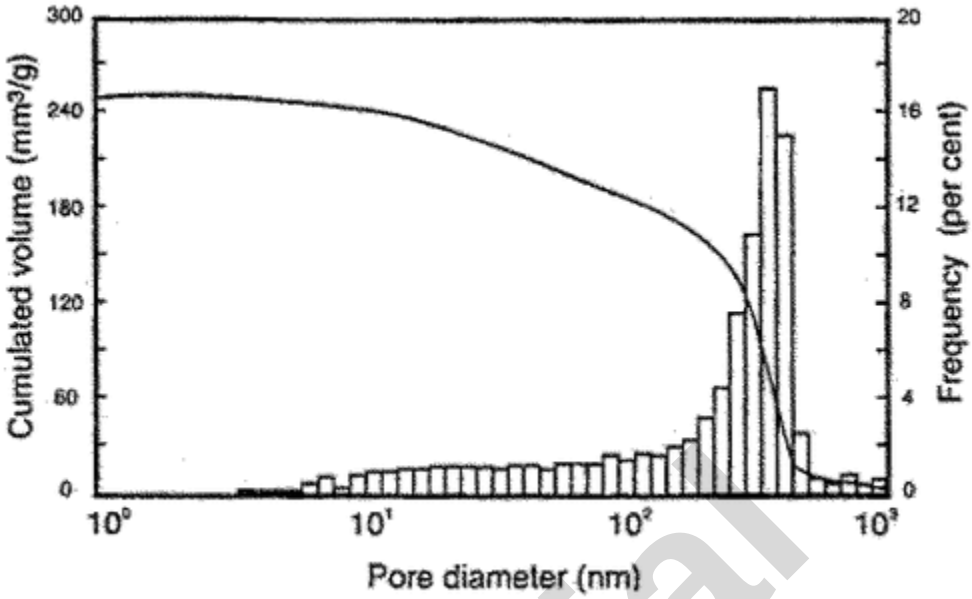


Figure 3. Pore volume distribution as semilogarithmic plot.

## CONTROL OF THE INSTRUMENT'S PERFORMANCE

As the mercury porosimetry technique is considered as a comparative test, no details are given in this chapter. However, it is recommended that a stable comparison material should be tested on a regular basis to monitor instrument calibration and performance.

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# ⟨268⟩ POROSITY BY NITROGEN ADSORPTION–DESORPTION

## INTRODUCTION

*Porosity* is a term typically used to indicate the porous nature of solid material and is more precisely defined as the ratio of the volume of accessible pores and voids to the total volume occupied by a given amount of the solid. Closed or inaccessible pores that are isolated from the external surface are excluded from this definition of pore volume. Pores (or voids) may consist of apertures, channels, or cavities within a solid body or spaces between solid particles in a compact or aggregate. Pores exist in a variety of solid materials beyond compacts and aggregates, such as powders and tablets, and their characterization usually involves the determination of the total pore volume or porosity, as well as the pore size distribution. Pores generally are classified by size into the following groups:

- Micropores—less than 2 nm
- Mesopores—2 to 50 nm
- Macropores—greater than 50 nm

The method in *Porosity by Nitrogen Adsorption–Desorption* ⟨268⟩ is complementary to that in general chapter *Porosimetry by Mercury Intrusion* ⟨267⟩. Mercury porosimetry may, in principle (in theory), be used with pore diameters from 3 nm to 400 µm but is most applicable in the range from 100 nm to 200 µm. Nitrogen adsorption–desorption may be used to characterize pores smaller than about 300 nm but is most appropriate for the analysis of mesopores and into the low macropore range from 2 to 100 nm.

## APPARATUS

Measurements are most commonly performed using the static volumetric procedure, although dynamic flow methods also may be employed. Users of commercially available equipment should refer to the manufacturer's literature and manual for a description of their particular apparatus. For example, a static volumetric apparatus must provide: evacuation of the system to a pressure of less than 10 Pa, delivery of known volumes of high-purity nitrogen and helium, accurate measurement of pressure and temperature, and a means of cooling the sample to liquid nitrogen temperature.

## MEASUREMENT PRINCIPLE

The adsorption of an inert gas onto solid surfaces at low temperatures is a well-known phenomenon and is the basis for measurement of the surface area of solids (see general chapter *Specific Surface Area* ⟨846⟩). As gas adsorbs to a surface, it can condense into accessible pores. The total pore volume and pore size distribution can be derived from the gas adsorption isotherm, which is the measure of amount adsorbed as a function of adsorbate partial pressure. Adsorption isotherms fall into six general categories, depending on the relative energetics of adsorption and the presence of pores. *Figure 1* shows six general categories of adsorption isotherms.

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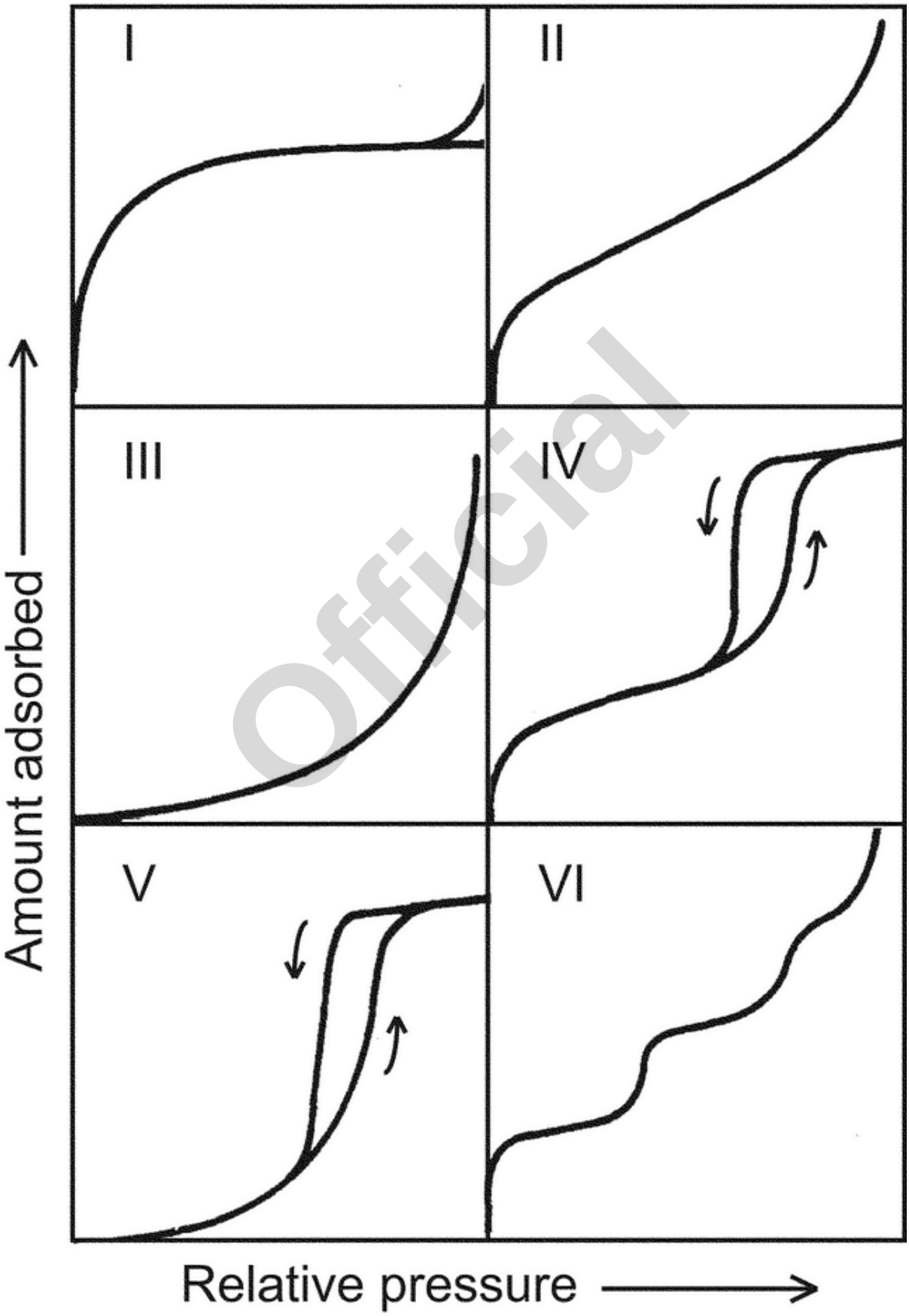


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Figure 1. Isotherm Types. [Reproduced with permission and modification from: Sing KSW, Everett DH, Haul RAW, et al.

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Reporting physisorption for gas/solid systems with special reference to the determination of surface area and porosity (recommendations 1984). *Pure Appl Chem.* 1985;57(4):603–619, *Figure 2*.]

Micropores (pore sizes less than 2 nm) frequently give rise to type I isotherms. Mesopores and macropores normally yield type IV isotherms, but for pores greater than about 100 nm hysteresis may be difficult to observe, thus yielding a type II isotherm. Although some information, such as the total porosity, can be derived for microporous materials, the determination of pore size distributions in that size range is outside the scope of this chapter.

The preferred adsorbate is nitrogen, and the isotherm is determined at liquid nitrogen temperature (77.4 K). Other adsorbates may be used for special purposes but are not discussed in this chapter.

# PROCEDURE

## Sample Preparation

Before analysis, analysts should outgas the sample to remove gases and vapors that may have become physically adsorbed onto the surface. The outgassing conditions must be demonstrated to yield reproducible adsorption–desorption, a constant sample weight, and no detectable physical or chemical changes in the sample. Outgassing of many substances often is achieved by applying a vacuum, by purging the sample in a flowing stream of a nonreactive, dry gas, or by applying an adsorption–desorption cycling procedure. If appropriate, analysts can apply elevated temperatures to increase the rate at which the contaminants leave the surface. Analysts should be cautious when outgassing samples using elevated temperatures to avoid affecting the nature of the surface and the integrity of the sample. If heating is employed, the recommended temperature and time of outgassing should be the minimum needed to achieve reproducible measurement of the adsorption–desorption isotherm.

Analysts should determine the sample mass after outgassing or, alternatively, should determine the mass after the adsorption–desorption measurement. The total surface area of the sample should be greater than 1 $m^2$ and preferably greater than 5 $m^2$.

## Isotherm Measurement

Specific details of the measurement process depend on the procedure used. Analysts should follow the manufacturer's instructions for the particular instrument used. The following description is applicable generally:

- Analysts should determine the saturated vapor pressure of the adsorbate, $p_0$. It is preferable to determine $p_0$ experimentally at the time of measurement, but analysts can use a calculated value.
- Analysts should determine the nitrogen sorption isotherm and should measure the volume adsorbed, $V_a$, at the lowest desired relative pressure ($p/p_0$, the ratio of the measured adsorbate pressure to its saturated vapor pressure).
- Analysts repeat the measurement of $V_a$ at successively higher relative pressure values to the maximum desired relative pressure (generally 0.99). Then they successively decrease the relative pressure to determine sorbed amounts on the desorption portion of the isotherm. Analysts should measure at least 20 points on both the adsorption and desorption segments, covering a relative pressure ($p/p_0$) range of approximately 0.05–0.99. The $p/p_0$ values can be distributed to achieve the best resolution of the pore size distribution. If only the desorption segment is being used to calculate the pore size distribution, fewer points can be used on the adsorption segment.

# DATA ANALYSIS

## Examination of the Isotherm

The isotherm is depicted as a plot of quantity of nitrogen adsorbed (as volume, $V_a$, or moles, $n_a$) vs. $p/p_0$. The isotherm data also can be presented in tabular form. From the graph, determine the isotherm and hysteresis types by comparison to the examples in *Figures 1* and *2*. A type I isotherm is common for microporous materials. A type IV isotherm usually is exhibited by materials that contain mesopores or small macropores.

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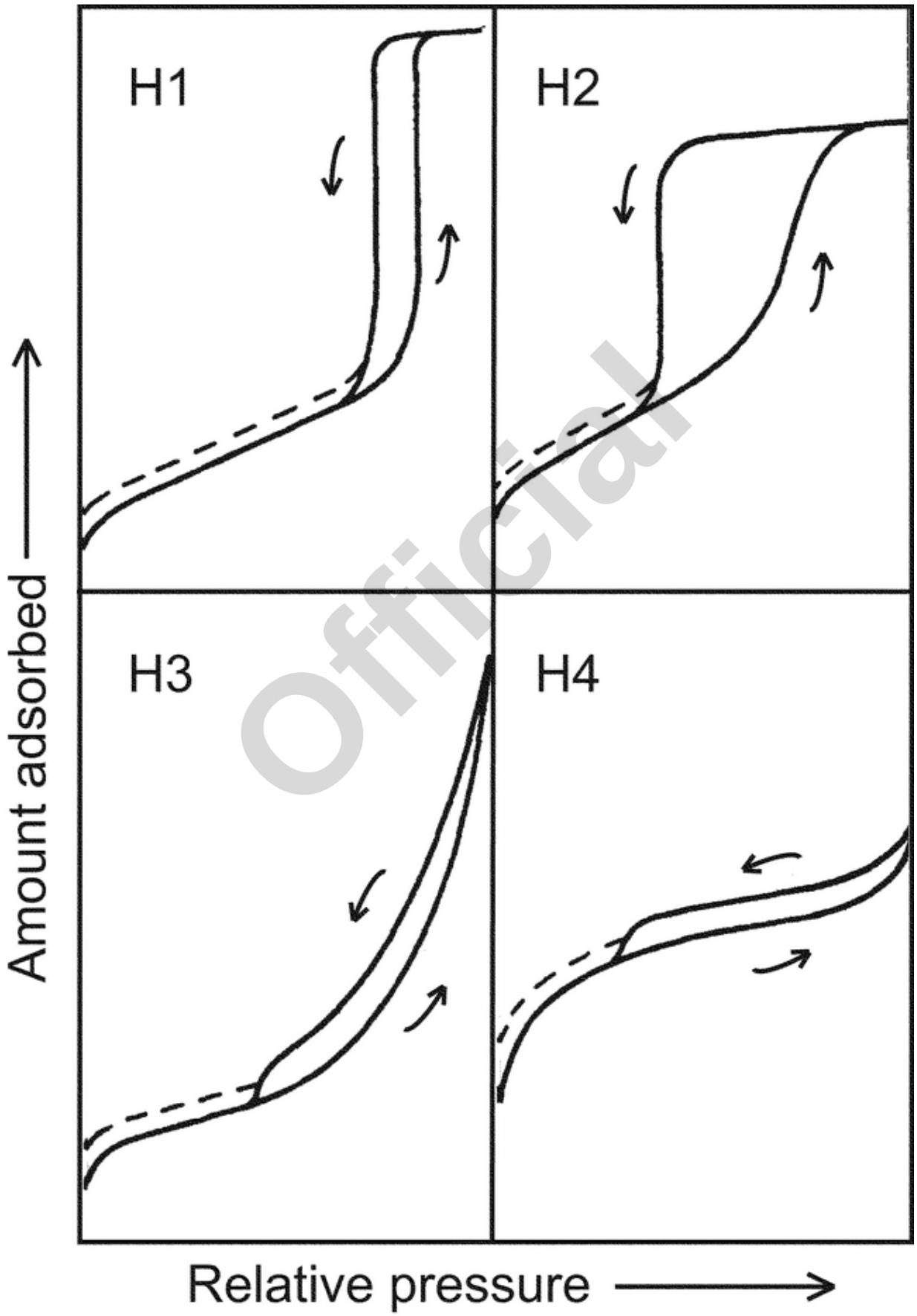


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Figure 2. Hysteresis Loop Types. [Reproduced with permission and modification from: Sing KSW, Everett DH, Haul RAW, et

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al. Reporting physisorption for gas/solid systems with special reference to the determination of surface area and porosity (recommendations 1984). *Pure Appl Chem.* 1985;57(4):603–619, *Figure 3*.]

Constructing a *t*-plot or $\alpha_S$-plot to compare the isotherm of the test sample to that of a reference isotherm also helps to illustrate the presence of micro- and mesoporosity. The reference isotherm may be calculated using a mathematical expression, but it is recommended that an experimentally determined reference isotherm be used when the adsorbent has chemical properties that are similar to those of the test sample.

The *t*-plot method is based on the *t*-curve, which is a plot of the amount of nitrogen adsorbed on the nonporous solid as a function of *t*, the statistical thickness of the adsorbed layer. The *t* value is calculated:

$$t = \frac{n_a \times \delta_a}{n_m}$$

$n_m$ = monolayer amount

$\delta_a$ = thickness of a single molecular layer, usually taken as 0.354 nm for nitrogen

In the $\alpha_S$-plot method, the amount of nitrogen adsorbed by the reference nonporous solid is normalized by using the amount adsorbed at some fixed relative pressure ($n'_{a,x}$), often taken as 0.4. The normalized adsorption $\alpha_S$ (equal to $n_a/n'_{a,x}$) then is plotted against $p/p_0$ to obtain an $\alpha_S$-curve.

The *t*-plot or $\alpha_S$-plot is constructed by plotting the amount of nitrogen adsorbed by the test sample against *t* or $\alpha_S$ for the reference material, rather than $p/p_0$. The conversion of $p/p_0$ to *t* or $\alpha_S$ is carried out by reference to the *t*-curve or $\alpha_S$-curve. The shape of the plot depends on the nature of the porosity present in the test sample, as follows:

1. if the *t*- or $\alpha_S$-plot is linear and passes through the origin, the test sample is nonporous or macroporous
2. if the test sample contains mesopores, the plot shows an upward deviation at the relative pressure corresponding to the onset of capillary condensation in the smallest mesopores
3. if the test sample contains micropores, the plot exhibits a downward deviation because multilayers cannot fully develop within the constricted space inside the micropores.

Some materials contain combinations of pores, which may result in a complex plot that is difficult to interpret. In such cases analysts should be cautious when they analyze the isotherm.

## Calculation of Pore Size Distribution

This analysis is valid only for calculations of the size distributions of mesopores.

Calculation of the pore size distribution is based on the Kelvin equation:

$$r_k = \frac{2 \times \sigma_l \times v_l \times \cos(\theta) \times 10^3}{R \times T \times \ln(p/p_0)}$$

$r_k$ = core radius of the pore (or Kelvin radius) (nm)

$\sigma_l$ = adsorbate (nitrogen) liquid surface tension (N/m)

$v_l$ = molar volume of the condensed adsorbate (nitrogen) ($cm^3/mol$)

$R$ = universal gas constant, 8.3144 ($J \cdot K^{-1} \cdot mol^{-1}$)

$T$ = temperature (K)

$\theta$ = adsorbate contact angle (0 for a wetted surface)

For nitrogen, equation 2 reduces to:

$$r_k = \frac{-0.953}{\ln(p/p_0)}$$

The actual pore radius, $r_p$, is calculated from the Kelvin radius by correcting for the thickness, *t*, of the adsorbate on the pore walls. For cylindrical pores, $r_p = r_k + t$, and the pore diameter, $d_p$, is given by $d_p = 2(r_k + t)$. Because of the different geometry of parallel-sided slit-shaped pores, the slit width is given by $r_k + 2t$.

Analysts can calculate the volume pore size distribution using the method of Barrett, Joyner, and Halenda. This model assumes that the pores are rigid and of regular shape (e.g., cylindrical or slit-shaped), micropores are absent, and the pore size distribution does not extend continuously above the largest pores measurable by this procedure, which implies that all of the pores evaluated are filled at the highest relative pressure.

Porosity and pore size distribution calculations that employ the Kelvin equation must be performed using the desorption isotherm. The Kelvin equation was derived for macroscopic systems and is not strictly valid at the molecular scale. Thus, the Kelvin equation relies on an intact meniscus in order to accurately describe experimental phenomena. For the systems discussed in this chapter, this is achieved only for the desorption isotherm. However, for desorption the application of the Kelvin equation at lower pore sizes is limited by the surface tension of the adsorbate. The limit is illustrated by the point of closure of the hysteresis loop in the isotherm. For nitrogen, this point occurs at a relative pressure of about 0.45, corresponding to a limiting cylindrical pore radius of about 2 nm. Thus the Kelvin equation is not applicable for micropores.

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### Calculation of Micropore Volume

If the $t$- or $\alpha_s$-plot indicates the presence of micropores, the micropore volume can be obtained from the intercept of the extrapolated linear portion of the curve.

### Reported Results

Typically reported results may include total pore volume or porosity, micropore volume, median or mean pore diameter, pore size distribution, and pore surface area.

## CALIBRATION AND VERIFICATION OF SYSTEM PERFORMANCE

Analysts should carry out calibration of individual components in accordance with the manufacturer's recommendations. Calibration of pressure transducers and temperature sensors is accomplished with reference to standard pressure- and temperature-measuring devices that have calibrations traceable to national standards. Manifold volume calibration is achieved by appropriate pressure and temperature measurements using constant-temperature volumetric spaces or solids of known, traceable volume.

A certified reference material or locally defined reference material that is traceable to a certified reference material should be tested on a regular basis in order to monitor instrument calibration and performance.

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# ⟨271⟩ READILY CARBONIZABLE SUBSTANCES TEST

In tests for readily carbonizable substances, unless otherwise directed, add the specified quantity of the substance, finely powdered if in solid form, in small portions to the comparison container, which is made of colorless glass resistant to the action of sulfuric acid and contains the specified volume of sulfuric acid (see under *Reagent Specifications* in the section *Reagents, Indicators, and Solutions*).

Stir the mixture with a glass rod until solution is complete, allow the solution to stand for 15 minutes, unless otherwise directed, and compare the color of the solution with that of the specified Matching Fluid (see *Color and Achromicity* ⟨631⟩) in a comparison container, which also is of colorless glass and has the same internal and cross-section dimensions, viewing the fluids transversely against a background of white porcelain or white glass.

When heat is directed in order to effect solution of the substance in the sulfuric acid, mix the sample and the acid in a test tube, heat as directed, and transfer the solution to the comparison container for matching with the designated Matching Fluid (see *Color and Achromicity* ⟨631⟩).

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# ⟨281⟩ RESIDUE ON IGNITION

Portions of this general chapter have been harmonized with the corresponding texts of the *European Pharmacopoeia* and the *Japanese Pharmacopoeia*. The portions that are not harmonized are marked with symbols (•,). The harmonized texts of these pharmacopeias are therefore interchangeable, and the methods of the *European Pharmacopoeia* and/or the *Japanese Pharmacopoeia* may be used for demonstration of compliance instead of the present *United States Pharmacopeia* general chapter. These pharmacopeias have undertaken not to make any unilateral change to this harmonized chapter.

The *Residue on Ignition/Sulfated Ash* test uses a procedure to measure the amount of residual substance not volatilized from a sample when the sample is ignited in the presence of sulfuric acid according to the procedure described below. This test is usually used for determining the content of inorganic impurities in an organic substance.

## PROCEDURE

Ignite a suitable crucible (for example, silica, platinum, quartz, or porcelain) at 600 ± 50° for 30 minutes, cool the crucible in a desiccator (silica gel or other suitable desiccant), and weigh it accurately. Weigh accurately •1 to 2 g of the substance, or,• the amount specified in the individual monograph, in the crucible.

Moisten the sample with a small amount (usually 1 mL) of sulfuric acid, then heat gently at a temperature as low as practicable until the sample is thoroughly charred. Cool; then,•unless otherwise directed in the individual monograph,• moisten the residue with a small amount (usually 1 mL) of sulfuric acid; heat gently until white fumes are no longer evolved; and ignite at 600 ± 50°, •unless another temperature is specified in the individual monograph,• until the residue is completely incinerated. Ensure that flames are not produced at any time during the procedure. Cool the crucible in a desiccator (silica gel or other suitable desiccant), weigh accurately, and calculate the percentage of residue.

Unless otherwise specified, if the amount of the residue so obtained exceeds the limit specified in the individual monograph, repeat the moistening with sulfuric acid, heating and igniting as before, using a 30-minute ignition period, until two consecutive weighings of the residue do not differ by more than 0.5 mg or until the percentage of residue complies with the limit in the individual monograph.

•Conduct the ignition in a well-ventilated hood, but protected from air currents, and at as low a temperature as is possible to effect the complete combustion of the carbon. A muffle furnace may be used, if desired, and its use is recommended for the final ignition at 600 ± 50°.

Calibration of the muffle furnace may be carried out using an appropriate digital temperature meter and a working thermocouple probe calibrated against a standard thermocouple traceable to the National Institute of Standards and Technology.

Verify the accuracy of the measuring and controlling circuitry of the muffle furnace by checking the positions in the furnace at the control set point temperature of intended use. Select positions that reflect the eventual method of use with respect to location of the specimen under test. The tolerance is ±25° at each position measured.•

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# ⟨291⟩ SELENIUM

***Add the following:***

## ▲INTRODUCTION

This chapter describes three analytical procedures for the evaluation of the levels of selenium (Se). Use *Procedure 1*, *Procedure 2*, or *Procedure 3* as indicated in the individual monograph. *Procedure 2* or *Procedure 3* can be used in all circumstances, provided that suitability is demonstrated by meeting the *Requirements For Procedure Validation*.▲ (Official 1-Jun-2023)

***Change to read:***

## ▲PROCEDURES

- **PROCEDURE 1: SPECTROSCOPY**▲ (Official 1-Jun-2023)

**Stock solution:** Dissolve 40.0 mg of metallic selenium in 100 mL of dilute nitric acid (1 in 2) in a 1000-mL volumetric flask, warming gently on a steam bath if necessary to effect solution, add water to volume, and mix. Pipet 5 mL of this solution into a 200-mL volumetric flask, add water to volume, and mix. Each milliliter of the resulting solution contains the equivalent of 1 µg of selenium.

**Diaminonaphthalene solution:** Dissolve 100 mg of 2,3-diaminonaphthalene and 500 mg of hydroxylamine hydrochloride in 0.1 N hydrochloric acid to make 100 mL. Prepare this solution fresh on the day of use.

**Standard solution:** Pipet 6 mL of *Stock solution* into a 150-mL beaker, and add 25 mL of dilute nitric acid (1 in 30) and 25 mL of water.

**Test solution:** Clean combustion of the test material is an important factor in conducting the test. For compounds that burn poorly and produce soot, the addition of magnesium oxide usually results in more thorough combustion and reduces soot formation. Where the need to add magnesium oxide has been identified, it is specified in the individual monograph. Using a 1000-mL combustion flask and using 25 mL of dilute nitric acid (1 in 30) as the absorbing liquid, proceed as directed in *Oxygen Flask Combustion* ⟨471⟩, using a test specimen weighing 100–200 mg, unless directed otherwise in the individual monograph. Upon completion of the combustion, place a few milliliters of water in the cup, loosen the stopper, and rinse the stopper, the specimen holder, and the sides of the flask with about 10 mL of water. Transfer the solution with the aid of about 20 mL of water to a 150-mL beaker, and heat gently to the boiling temperature. Boil for 10 min, and allow the solution to cool to room temperature.

**▲Analysis:**▲ (Official 1-Jun-2023) Treat the *Standard solution*, the *Test solution*, and the reagent blank consisting of 25 mL of dilute nitric acid (1 in 30) and 25 mL of water, concomitantly and in parallel, as follows. Add ammonium hydroxide solution (1 in 2) to adjust to a pH of 2.0 ± 0.2. Dilute with water to 60 mL, and transfer to a low-actinic separator with the aid of 10 mL of water, adding the 10 mL of rinsings to the separator. Add 200 mg of hydroxylamine hydrochloride, swirl to dissolve, immediately add 5.0 mL of *Diaminonaphthalene solution*, insert the stopper, and swirl to mix. Allow the solution to stand at room temperature for 100 min. Add 5.0 mL of cyclohexane, shake vigorously for 2 min, and allow the layers to separate. Discard the aqueous layer, and centrifuge the cyclohexane extract to remove any dispersed water. Determine the absorbances of the cyclohexane extracts of the *Test solution* and the *Standard solution* in a 1-cm cell at the wavelength of maximum absorbance at about 380 nm, with a suitable spectrophotometer, using the cyclohexane extract of the reagent blank as the blank, and compare the absorbances: the absorbance of the *Test solution* is not greater than that of the *Standard solution* where a 200-mg test specimen has been taken, or is not greater than one-half that of the *Standard solution* where a 100-mg test specimen has been taken.

▲- **PROCEDURE 2 AND PROCEDURE 3**

Both *Procedure 2* and *Procedure 3* are ICP-based procedures and can be used for the determination of selenium. *Procedure 2* can be used for the determination of selenium by inductively coupled plasma atomic (or optical) emission spectroscopy (ICP–AES or ICP–OES). *Procedure 3* can be used for the determination of selenium by ICP–MS.

Before initial use, the analyst should verify that the procedure is appropriate for the instrument and sample used (procedural verification) by meeting the *Requirements for Procedure Validation*.

Where a monograph specifies a limit for selenium concentration, the value listed in the monograph should be used as the *J* value for the purposes of this test.

System standardization and suitability evaluation using applicable reference materials should be performed on the day of analysis.

**Sample preparation:** Forms of sample preparation include neat, direct aqueous solution, direct organic solution, and indirect solution. The selection of the appropriate sample preparation depends on the material under test and is the responsibility of the analyst. When a sample preparation is not indicated in the monograph, an analyst may use any appropriately validated preparation procedure. In cases where spiking of a material under test is necessary to provide an acceptable signal intensity, the blank should be spiked with selenium using, where possible, the same spiking solution. [NOTE—All liquid samples should be weighed.]

**Closed vessel digestion:** This sample preparation procedure is designed for samples that must be digested in a concentrated acid using a closed vessel digestion apparatus. Closed vessel digestion minimizes the loss of volatile impurities. The choice of a concentrated acid depends on the sample matrix. The use of any of the concentrated acids may be appropriate, but each introduces inherent safety risks. Therefore, appropriate safety precautions should be used at all times. [NOTE—Weights and volumes provided may be adjusted to meet the requirements of the digestion apparatus used.]

An example procedure that has been shown to have broad applicability is as follows. Dehydrate and predigest 0.5 g of primary sample in 5 mL of freshly prepared concentrated acid. Allow to sit loosely covered for 30 min in a fume hood. Add an additional 10 mL of concentrated acid, and digest using a closed vessel technique until digestion or

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extraction is complete. Repeat, if necessary, by adding an additional 5 mL of concentrated acid. [NOTE—Follow the manufacturer's recommended procedures to ensure safe use.]

**Reagents:** All reagents used for the preparation of sample and standard solutions should be free of elemental impurities, in accordance with *Plasma Spectrochemistry* ⟨730⟩.

**Procedure 2: ICP–OES**

**Standardization solution 1:** 1.5*J* of selenium in a matched matrix

**Standardization solution 2:** 0.5*J* of selenium in a matched matrix

**Sample stock solution:** Prepare as directed in *Sample preparation*. Allow the sample to cool, if necessary.

**Sample solution:** Dilute the *Sample stock solution* with an appropriate solvent to obtain a final selenium concentration of not more than 1.5*J*.

**Blank:** Matched matrix

**Elemental spectrometric system**

(See ⟨730⟩.)

**Rinse:** Use diluent.

**Standardization:** *Standardization solution 1*, *Standardization solution 2*, and *Blank*

**System suitability**

**Sample:** *Standardization solution 1*

**Suitability requirements**

**Drift:** Compare results obtained from *Standardization solution 1* before and after the analysis of the *Sample solution.*

**Suitability criteria:** NMT 20% for selenium. [NOTE—If samples are high in mineral content, rinse the system well before introducing the *Sample* in order to minimize carryover.]

**Analysis:** Analyze according to the manufacturer's suggestions for program and wavelength. Calculate and report results on the basis of the original sample size. [NOTE—Appropriate measures must be taken to correct for matrix-induced interferences (e.g., wavelength overlaps).]

**Procedure 3: ICP–MS**

Follow *Procedure 2* except for *Detector* and *Analysis*.

[NOTE—An instrument with a cooled spray chamber is recommended. (A collision cell or reaction cell may also be beneficial.)]

**Detector:** Mass spectrometer

**Analysis:** Analyze according to the manufacturer's suggestions for program and mass-to-charge ratio. Calculate and report results based on the original sample size. [NOTE—Appropriate measures must be taken to correct for matrix-induced interferences.]▲ (Official 1-Jun-2023)

***Add the following:***

## ▲REQUIREMENTS FOR PROCEDURE VALIDATION

The following section defines the validation parameters and the acceptance criteria for performance-based procedures. Meeting these requirements must be demonstrated experimentally using an appropriate system suitability procedure and reference materials. Any alternative procedure (e.g., an atomic-absorption-based procedure) that has been validated and meets the acceptance criteria that follow is considered to be suitable for use.

Meeting these validation acceptance criteria is sufficient to demonstrate that the procedure will produce comparable results to those obtained using the procedure prescribed in the monograph.

- **ACCURACY**

**Standard solutions:** Prepare solutions containing selenium at concentrations ranging from 50% to 150% of *J* using appropriate reference materials.

**Test samples:** Spike the material under test with the appropriate reference materials before any sample preparation steps (digestion or solubilization). Prepare three replicate samples at concentrations ranging from 50% to 150% of *J* for selenium.

**Acceptance criteria**

**Spike recovery:** 70%–150% for the mean of three replicate preparations at each concentration

- **PRECISION**

**Repeatability**

**Test samples:** Six independent samples of material under test (taken from the same lot) spiked with appropriate reference materials for selenium at the indicated concentration

**Acceptance criteria**

**Relative standard deviation:** Not more than 20% ($N = 6$) for selenium

**Intermediate precision (ruggedness)**

**Analysis:** Perform the *Repeatability* analysis again on a different day, with different instrumentation, with a different analyst, or a combination thereof. Combine the results of this analysis with the first *Repeatability* analysis so the total number of analyses is 12.

**Acceptance criteria**

**Relative standard deviation:** Not more than 25% ($N = 12$) for selenium

- **SPECIFICITY:** The procedure must be able to unequivocally assess (see *Validation of Compendial Procedures* ⟨1225⟩) selenium in the presence of components that may be expected to be present, including matrix components.
- **LIMIT OF QUANTITATION, RANGE, AND LINEARITY:** Demonstrated by meeting the *Accuracy* requirement.▲ (Official 1-Jun-2023)

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## ▲GLOSSARY

**Concentrated acid:** Concentrated ultra-pure nitric, sulfuric, hydrochloric, or hydrofluoric acid or aqua regia.

**Aqua regia:** Aqua regia is a mixture of concentrated hydrochloric and nitric acids, typically at ratios of 3:1 or 4:1.

**Matched matrix:** Solutions having the same solvent composition as the *Sample solution*. In the case of an aqueous solution, a matched matrix would indicate that the same acids and acid concentrations are used in both preparations.

**Target limit or target concentration:** The acceptance value for the elemental impurity being evaluated, in this case selenium. Where a monograph specifies a threshold limit, this shall become the target limit or target concentration of selenium for the material. Exceeding the target limit indicates that a material under test exceeds the acceptable value. The determination of compliance is addressed in other chapters.

***J*:** The concentration (w/w) of the element of interest, in this case selenium, at the target limit, appropriately diluted to the working range of the instrument.

**Appropriate reference materials:** Where "appropriate reference materials" are specified in the chapter, certified reference materials (CRMs) from a national metrology institute (NMI), or reference materials that are traceable to the CRM of an NMI should be used. An example of an NMI in the United States is the National Institute of Standards and Technology (NIST).▲ (Official 1-Jun-2023)

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# ⟨301⟩ ACID-NEUTRALIZING CAPACITY

[NOTE—All tests shall be conducted at a temperature of 37 ± 3°.]

**Standardization of pH meter:** Standardize a pH meter using the 0.05 m potassium biphthalate and 0.05 m potassium tetraoxalate standardizing buffers as described under *pH* ⟨791⟩.

**Magnetic stirrer:** Transfer 100 mL of water to a 250-mL beaker containing a 40- × 10-mm (or other suitable size) magnetic stirring bar that is coated with solid perfluorocarbon and has a spin ring at its center. Adjust the power setting of the magnetic stirrer to produce a stirring rate of 300 ± 30 rpm when the stirring bar is centered in the beaker, as determined by a suitable optical tachometer.

**Test preparation**

**Powders:** Transfer the accurately weighed portion of the substance specified in the individual monograph to a 250-mL beaker, add 70 mL of water, and mix on the *Magnetic stirrer* for 1 minute.

**Effervescent solids:** Transfer an accurately weighed quantity, equivalent to the minimum labeled dosage, to a 250-mL beaker, add 10 mL of water, and swirl the beaker gently while allowing the reaction to subside. Add another 10 mL of water, and swirl gently. Wash the walls of the beaker with 50 mL of water, and mix on the *Magnetic stirrer* for 1 minute.

**Suspensions and other liquids:** Shake the container until the contents are uniform, and determine the density. Transfer an accurately weighed quantity of the uniform mixture, equivalent to the minimum labeled dosage, to a 250-mL beaker, add water to make a total volume of about 70 mL, and mix on the *Magnetic stirrer* for 1 minute.

**Lozenges:** Accurately weigh not fewer than 20 lozenges, and determine the average weight. Select and weigh 2 lozenges, and transfer them to a 250-mL beaker containing 70 mL of water.

**Nonchewable tablets:** Weigh not fewer than 20 tablets, and determine the average tablet weight. Grind the tablets to a fine powder, mix to obtain a uniform mixture, and transfer an accurately weighed quantity of it, equivalent to the minimum labeled dosage, to a 250-mL beaker. If wetting is desired, add not more than 5 mL of alcohol (neutralized to an apparent pH of 3.5), and mix to wet the specimen thoroughly. Add 70 mL of water, and mix on the *Magnetic stirrer* for 1 minute.

**Chewable tablets:** Prepare as directed for *Nonchewable tablets*.

**Tablets that are required to be chewed:** Transfer 1 Tablet to a 250-mL beaker, add 50 mL of water, and mix on the *Magnetic stirrer* for 1 minute.

**Capsules:** Weigh accurately not fewer than 20 capsules. Remove the capsule contents completely, with the aid of a cotton swab if necessary. Accurately weigh the empty capsules, and determine the average weight of the contents per capsule. Mix the combined capsule contents to obtain a uniform mixture, and proceed as directed for *Nonchewable tablets*, beginning with "transfer an accurately weighed quantity of it."

**PROCEDURES**

**• PROCEDURE FOR POWDERS, EFFERVESCENT SOLIDS, SUSPENSIONS AND OTHER LIQUIDS, LOZENGES, NONCHEWABLE TABLETS, CHEWABLE TABLETS, AND CAPSULES**

Pipet 30.0 mL of 1.0 N hydrochloric acid VS into the *Test preparation* while continuing to stir with the *Magnetic stirrer*. [NOTE—Where the acid-neutralizing capacity of the specimen under test is greater than 25 mEq, use 60.0 mL of 1.0 N hydrochloric acid VS, and make the appropriate modifications in the calculation.] Stir for 15 minutes, accurately timed, after the addition of the acid, begin to titrate immediately, and in a period not to exceed an additional 5 minutes, titrate the excess hydrochloric acid with 0.5 N sodium hydroxide VS to attain a stable (for 10 to 15 seconds) pH of 3.5. Calculate the number of mEq of acid consumed by the formula:

$$\text{Total mEq} = (30 \times N_{HCl}) - (V_{NaOH} \times N_{NaOH})$$

$N_{HCl}$ = normality of hydrochloric acid VS
$V_{NaOH}$ = volume of sodium hydroxide VS used for titration
$N_{NaOH}$ = normality of sodium hydroxide VS

Express the result in terms of mEq of acid consumed per g of the substance tested.

**• PROCEDURE FOR TABLETS THAT ARE REQUIRED TO BE CHEWED**

Pipet 30.0 mL of 1.0 N hydrochloric acid VS into the *Test preparation* while continuing to stir with the *Magnetic stirrer* for 10 minutes, accurately timed, after the addition of the acid. Discontinue stirring briefly, and without delay remove any gum base from the beaker using a long needle. Promptly rinse the needle with 20 mL of water, collecting the washing in the beaker, and resume stirring for 5 minutes, accurately timed, then begin to titrate immediately, and in a period not to exceed an additional 5 minutes, titrate the excess hydrochloric acid with 0.5 N sodium hydroxide VS to attain a stable (for 10 to 15 seconds) pH of 3.5. Calculate the number of mEq of acid consumed by the Tablet tested by the formula:

$$\text{Total mEq} = (30 \times N_{HCl}) - (V_{NaOH} \times N_{NaOH})$$

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# ⟨311⟩ ALGINATES ASSAY

## APPARATUS

The required apparatus (see *Figure 1*) contains a capillary metering valve, A, followed by a flowmeter, B, to control and monitor the flow of nitrogen through the system. Halogenated vinyl plastic tubing* and a rubber fitting, C, are used to connect the flowmeter to a sidearm of a reaction flask, D. Flask D is a 250-mL round-bottom, boiling flask, resting in a suitable heating mantle, E. Flask D is provided with a 225-mm Hopkins coil reflux condenser, F. The condenser terminates in a U-shaped trap, G, which contains two 25-g bands of 20-mesh zinc, the bands being bounded and separated by three 3-inch plugs of glass wool. The trap terminates in an adapter, H, that by means of a halogenated vinyl plastic tubing and a twistcock connector, I, connects with a 250-mL gas washing bottle, J. The inlet (bubbling) tube extends almost to the bottom of the gas washing bottle, and it terminates in a fritted disk having a coarse porosity. The size of all glass joints is $^{24}/_{40}$, except for the $^{45}/_{50}$ joint of the gas washing bottle.

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* This type of tubing is commonly referred to as Tygon tubing. This note is added for clarity and it does not constitute USP's endorsement of this product.

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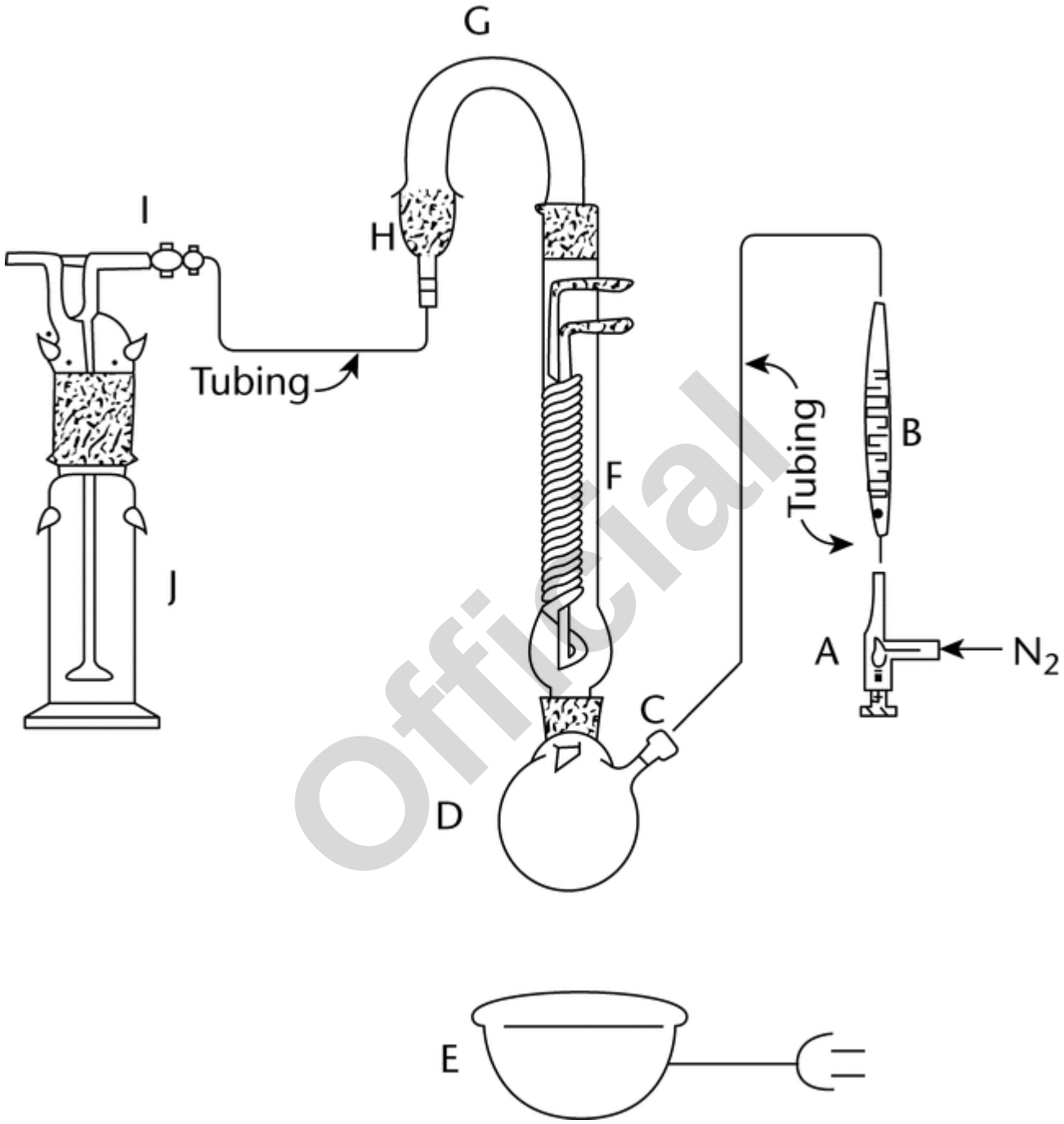


Figure 1. Apparatus for Alginates Assay

## SYSTEM SUITABILITY

Using D-glucuronolactone as the standard, proceed as directed for *Procedure,* but do not perform the preboiling steps. The system is suitable if the following criteria are met: (1) a blank determination results in a net titration value, C, between 0.02 and 0.06 mEq, calculated as follows:

$$A_b - B_b$$

in which $A_b$ is the number of mEq of 0.25 N sodium hydroxide in the 25 mL used, and $B_b$ is the number of mEq of 0.1 N hydrochloric acid used in the blank titration; and (2) the percentage of carbon dioxide, $CO_2$, obtained from the standard is between 24.2% and 25.7%.

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## PROCEDURE

Unless otherwise directed in the individual monograph, transfer a specimen of about 250 mg, accurately weighed, into the reaction flask, D, add 50 mL of 0.1 N hydrochloric acid, insert several boiling chips, and connect the flask to the reflux condenser, F, using phosphoric acid as a lubricant. [NOTE—Stopcock grease may be used for the other connections.] Connect the nitrogen line to the sidearm of the flask, and adjust the flow of cooling water to about 2 L per minute.

[NOTE—The following preboiling steps, outlined in this paragraph, are optional and need only be performed when the presence of inorganic carbonates is suspected.] Maintain the flow of nitrogen through the apparatus at 90 to 100 mL per minute. Raise the heating mantle, E, to the flask, heat the specimen to boiling, and boil gently for 2 minutes. Turn the heat off, lower the mantle, E, and allow to cool for about 10 minutes.

Connect the empty gas washing bottle assembly, J, and sweep the system with nitrogen at a rate of 90 to 100 mL per minute for 5 minutes. Reduce the nitrogen flow to 60 to 65 mL per minute, add 10 drops of butyl alcohol, 25.0 mL of 0.25 N sodium hydroxide VS, and 50 mL of distilled water into the bottle, rinsing down the inside of the gas washing bottle, and replace the cap. Detach the rubber fitting, C, from sidearm, and add 46 mL of hydrochloric acid through the sidearm of the boiling flask. Reattach the nitrogen line, raise the heating mantle, and heat the reaction mixture to boiling. After 2 hours of boiling, increase the nitrogen flow to 90 to 100 mL per minute, discontinue the heating, and lower the mantle. Allow to cool for 10 minutes. Disconnect, and disassemble the gas washing bottle. Using a directed stream of distilled water, thoroughly rinse all parts of the bubbling tube and cap, collecting the washings in the gas washing bottle. Use nitrogen to gently force all water out of the bubbling tube. To the bottle immediately add 10 mL of 10% barium chloride solution and a stirring bar. Insert a tight stopper, and stir gently for 1 minute. Allow to stand for at least 5 minutes. Add three drops of phenolphthalein TS, and titrate with 0.1 N hydrochloric acid VS. Perform a blank determination (see *Residual Titrations* under *Titrimetry* ⟨541⟩). Calculate the percentage of carbon dioxide, $CO_2$, by the formula:

$$2200[(A - B) - C]/(1000W)(1 - D)$$

in which A is the number of mEq of 0.25 N sodium hydroxide in the 25 mL used; B is the number of mEq of 0.1 N hydrochloric acid used for the titration of the sample or the standard; C is the net titration value calculated in the blank determination; W is the weight, in g, of the sample or the standard taken; and D is the percentage expressed as a decimal (1 decimal place), obtained in the test for *Loss on drying* for the sample or for the standard.

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***Add the following:***

# ▲⟨313⟩ MOLECULAR WEIGHT AND POLYMER CHAIN LENGTH DETERMINATION FOR POLYPROPYLENE GLYCOL FATTY ETHERS

Average molecular weight, polydispersity, and average polymer chain length are critical properties in understanding and control of excipient composition and variability during formulation development, manufacturing, and testing of polymeric excipients. The following gel permeation chromatography (GPC)/size exclusion chromatography (SEC) and nuclear magnetic resonance (NMR) spectroscopy procedures are used to determine average molecular weight, polydispersity, and average polymer chain length for polypropylene glycol fatty ethers. These procedures are suitable for the following substances:

- Polypropylene Glycol 11 Stearyl Ether
- Polypropylene Glycol 15 Stearyl Ether

## METHOD 1: APPARENT WEIGHT-AVERAGE MOLECULAR WEIGHT AND POLYDISPERSITY

**Mobile phase:** Tetrahydrofuran. [NOTE—Filter the solvent using a polytetrafluoroethylene (PTFE) filter of 0.45-µm pore size if necessary.]

**Standard solutions:** Prepare 1.0 mg/mL each of at least 5 polypropylene glycol (PPG) standards with peak molecular weights ranging from approximately 70–4500 Da (g/mol)[1] in *Mobile phase* separately.

**System suitability solution:** 1.0 mg/mL of PPG standard with a peak molecular weight of approximately 1000 Da (g/mol)[1] in *Mobile phase*

**Sample solution:** 1.0 mg/mL of the test substance in *Mobile phase*

**Chromatographic system**

(See *Chromatography* ⟨621⟩, *System Suitability*.)

**Mode:** LC

**Detector:** Refractive index

**Columns:** Two 7.5-mm × 30-cm; 3-µm packing L21 columns in series

**Temperatures**

**Detector:** 30°

**Column:** 30°

**Flow rate:** 1.0 mL/min

**Injection volume:** 20 µL

**Run time:** 25 min

**System suitability**

**Samples:** *Standard solutions* and *System suitability solution*

**Calibration curve:** Plot the retention times on the *x* axis against the log $M_P$ ($M_P$: peak molecular weight) on the *y* axis for the PPG peaks from the *Standard solutions* to generate a calibration curve using suitable GPC/SEC software. The correlation coefficient for the calibration curve is NLT 0.99.

Determine weight-average molecular weights, $M_w$, in g/mol (Da), from the chromatograms of the *System suitability solution*.

**Suitability requirements**

**Relative standard deviation:** NMT 5% for determined weight-average molecular weights of the PPG standard from 6 replicates, *System suitability solution*

**Analysis**

**Samples:** *Standard solutions* and *Sample solution*

Compute the data using the same GPC/SEC software from the chromatograms of the *Standard solutions* and *Sample solution* and determine the number- and weight-average molecular weights, $M_n$ and $M_w$, in g/mol (Da), respectively, for the tested substance.

Calculate the polydispersity for the tested substance:

$$\text{Result} = M_w/M_n$$

$M_w$ = weight-average molecular weight from the *Sample solution* (g/mol)
$M_n$ = number-average molecular weight from the *Sample solution* (g/mol)

## METHOD 2: AVERAGE POLYMER LENGTH

**Diluent:** Deuterated chloroform containing 0.03% (v/v) tetramethylsilane[2]

**Sample solution:** Weigh about 100 mg of the tested substance. Dissolve it in 0.6 mL of *Diluent*.

**Instrumental conditions**

**Mode:** An NMR spectrometer that is capable of performing quantitative analysis (see *Nuclear Magnetic Resonance Spectroscopy* ⟨761⟩, *Qualitative and Quantitative NMR Analysis, Quantitative Applications*)

**Flip angle:** 90°

[1] A PPG standard kit from PSS (Polymer Standards Service; part number PSS-ppgkit) was used. The kit contained a total of 7 PPG standards with the peak molecular weight ($M_P$) range of approximately 70–4500 Da (g/mol). The PPG standard with an $M_P$ of approximately 1000 Da (g/mol) was used for the *System suitability solution* preparation. An equivalent standard kit can also be used.

[2] The reagent from Sigma Aldrich (product #225789) was used. An equivalent reagent can also be used.

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**Analysis:** Place the tube in the NMR spectrometer, and record the NMR spectrum. Integrate the peak areas from 0.6–1.8 ppm ($A_1$) and from 2.8–4.2 ppm ($A_2$).

The following are the equations for the total number of protons in the two regions ($N_1$ and $N_2$):

$$N_1 = N_{N\,1} + N_{N\,2} \times n$$

$N_{\,1}$ = total number of protons in the region of 0.6–1.8 ppm
$N_{N1}$ = total number of protons in the fatty alcohol residual group (stearyl group for the two excipients currently listed in the chapter) not activated by oxygen, 35
$N_{N\,2}$ = number of protons not activated by oxygen in each oxypropylene unit, 3
$n$ = number of oxypropylene units per molecule

$$N_2 = N_{A1} + N_{A2} \times n$$

$N_2$ = total number of protons in the region of 2.8–4.2 ppm
$N_{A1}$ = total number of oxygen activated protons not included in the oxypropylene unit, 3
$N_{A2}$ = number of oxygen activated protons in each oxypropylene unit, 3
$n$ = number of oxypropylene units per molecule

The areas in the two regions ($A_1$ and $A_2$) are correlated to the total number of protons in the regions ($N_1$ and $N_2$) as in the following equation:

$$A_2/A_1 = N_2/N_1$$

Calculate the number of oxypropylene units per molecule ($n$):

$$n = (N_{N\,1} \times A_{\,2} - N_{A1} \times A_1)/(N_{A2} \times A_1 - N_{N2} \times A_2)$$

$N_{N1}$ = total number of protons in the fatty alcohol residual group (stearyl group for the two excipients currently listed in the chapter) not activated by oxygen, 35
$A_2$ = area from 2.8–4.2 ppm by integration
$N_{A1}$ = total number of oxygen activated protons not included in the oxypropylene unit, 3
$A_1$ = area from 0.6–1.8 ppm by integration
$N_{A2}$ = number of oxygen activated protons in each oxypropylene unit, 3
$N_{N2}$ = number of protons not activated by oxygen in each oxypropylene unit, 3▲ (USP 1-Aug-2023)

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# ▲⟨314⟩ MOLECULAR WEIGHT DETERMINATION FOR COPOLYMERS CONTAINING ALKYL METHACRYLATE OR ALKYL ACRYLATE

Average molecular weight and polydispersity are critical properties in understanding and control of excipient composition and variability during formulation development, manufacturing, and testing of polymeric excipients. The following gel permeation chromatography (GPC)/size exclusion chromatography (SEC) procedures are used to determine the average molecular weight and polydispersity for copolymers containing alkyl methacrylate or alkyl acrylate. The procedure is suitable for the following substances:

- Methacrylic Acid and Methyl Methacrylate Copolymer including Methacrylic Acid and Methyl Methacrylate Copolymer (1:1) and Methacrylic Acid and Methyl Methacrylate Copolymer (1:2)
- Methacrylic Acid and Ethyl Acrylate Copolymer
- Methacrylic Acid and Ethyl Acrylate Copolymer Dispersion

## APPARENT WEIGHT-AVERAGE MOLECULAR WEIGHT AND POLYDISPERSITY

**Mobile phase:** Tetrahydrofuran. [NOTE—Filter the solvent using a polytetrafluoroethylene (PTFE) filter of 0.45-µm pore size if necessary.]

**Standard solutions:** Prepare 1.0 mg/mL each of at least 7 poly(methyl methacrylate) (PMMA) standards with peak molecular weights ranging from approximately 600–2,000,000 Da (g/mol)[1] in *Mobile phase* separately.
Pass each solution using a suitable syringe compatible with tetrahydrofuran through a suitable filter of 1-µm pore size into a high-performance liquid chromatography (HPLC) vial with the first 1–2 mL discarded before analysis.[2]

**System suitability solution:** 1.0 mg/mL of a PMMA standard with a peak molecular weight of approximately 190,000 Da (g/mol)[1] in *Mobile phase*. Pass the solution using a suitable syringe compatible with tetrahydrofuran through a suitable filter of 1-µm pore size into an HPLC vial with the first 1–2 mL discarded before analysis.[2]

**Sample solution:** 1.0 mg/mL of the test substance in *Mobile phase* for a sample in solid form; 1.0 mg/mL equivalent to the corresponding solid form substance in *Mobile phase* for a sample in dispersion form. [NOTE—A sample in dispersion form can be dissolved in *Mobile phase* directly (without any pretreatment).] Pass the solution using a suitable syringe compatible with tetrahydrofuran through a suitable filter of 1-µm pore size into an HPLC vial with the first 1–2 mL discarded before analysis.[2]

**Chromatographic system**
(See *Chromatography* ⟨621⟩, *System Suitability*.)
**Mode:** LC
**Detector:** Refractive index
**Columns:** One suitable guard column[3] and two 7.5-mm × 30-cm; 5-µm packing L128 columns in series
**Temperatures**
**Detector:** 30°
**Column:** 30°
**Flow rate:** 1.0 mL/min
**Injection volume:** 20 µL
**Run time:** 30 min

**System suitability**
**Samples:** *Standard solutions* and *System suitability solution*
**Calibration curve:** Plot the retention times on the *x* axis against the log $M_P$ ($M_P$: peak molecular weight) on the *y* axis for the PMMA peaks from the *Standard solutions* to generate a calibration curve using suitable GPC/SEC software. The correlation coefficient for the calibration curve is NLT 0.99.
Determine weight-average molecular weights, $M_w$, in g/mol (Da), from the chromatograms of the *System suitability solution* using the GPC/SEC software.
**Suitability requirements**
**Relative standard deviation:** NMT 10% for determined weight-average molecular weights of the PMMA standard from 6 replicates, *System suitability solution*

**Analysis**
**Samples:** *Standard solutions* and *Sample solution*
Compute the data using the same GPC/SEC software from the chromatograms of the *Standard solutions* and *Sample solution* and determine the number- and weight-average molecular weights, $M_n$ and $M_w$, in g/mol (Da), respectively, for the tested substance.
Calculate the polydispersity for the tested substance:

$$\text{Result} = M_w/M_n$$

[1] A poly(methyl methacrylate) (PMMA) standard kit from PSS (Polymer Standards Service; part number PSS-mmkith) was used. The kit contained a total of 12 PMMA standards with the peak molecular weight ($M_P$) range that covers the region of approximately 600–2,000,000 Da (g/mol). The PMMA standard with an $M_P$ of approximately 190,000 Da (g/mol) was used for the *System suitability solution* preparation. An equivalent standard kit can also be used.
[2] A glass syringe and a PTFE filter were used. An equivalent syringe and an equivalent filter can also be used.
[3] A 5-µm, 7.5-mm × 5-cm Agilent PLgel Mixed guard column (part # PL1110-1520) was used. An equivalent guard column can also be used.

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$M_w$ = weight-average molecular weight from the *Sample solution* (g/mol)
$M_n$ = number-average molecular weight from the *Sample solution* (g/mol)▲ (USP 1-Dec-2023)

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# ⟨341⟩ ANTIMICROBIAL AGENTS—CONTENT

An essential component of Injections preserved in multiple-dose containers is the antimicrobial agent or antimicrobial agents present to reduce the hazard of having introduced, in the course of removing some of the contents, accidental microbial contamination of the contents remaining. It is a Pharmacopeial requirement that the presence and amount added of such antimicrobial agent(s) be declared on the label of the container. This general chapter provides methods for the most commonly used antimicrobial agents. These methods or other suitably validated methods are to be used to demonstrate that the declared antimicrobial agent is present but does not exceed the labeled amount by more than 20%.

The concentration of an antimicrobial preservative added to a multiple-dose or single-dose parenteral, otic, nasal, and ophthalmic preparation may diminish during the shelf life of the product. Therefore, the manufacturer shall determine the lowest level at which the preservative is effective, and the product should be so formulated as to assure that this level is exceeded throughout the product's shelf life. At the time of its manufacture, the product should contain the declared amount of antimicrobial preservative (within ±20% to allow for manufacturing and analytical variations). The quantitative label statement of the preservative content is not intended to mean that the labeled quantity is retained during the shelf life of the product; rather, it is a statement of the amount added, within process limits, and which is not exceeded by more than 20%. An example of such a label statement is "____(unit) added as preservative". [NOTE—"____(unit)" would be a number followed by the unit of measurement, e.g., 0.015 mg/mL or 0.1%.]

The most commonly used antimicrobial agents include benzyl alcohol; chlorobutanol; phenol; the four homologous esters of *p*-hydroxybenzoic acid (methyl, ethyl, propyl, and butyl parabens); and the two mercurials, phenylmercuric nitrate and thimerosal. The method used for phenylmercuric nitrate is polarographic, whereas quantitative liquid chromatography was used for thimerosal and the four homologous esters of *p*-hydroxybenzoic acid. Gas chromatography is used in the determination of phenol, benzyl alcohol, and chlorobutanol.

## GENERAL GAS AND LIQUID CHROMATOGRAPHIC METHODS

The general gas chromatography procedures set forth in the following paragraphs are applicable to the quantitative determination of benzyl alcohol, chlorobutanol, and phenol. Prepare the *Internal standard solution* and the *Standard solution* for each antimicrobial agent as directed below. Unless otherwise directed by the individual monograph, prepare the *Sample solution* from accurately measured portions of the sample under test and the *Internal standard solution* such that the concentration of the antimicrobial agent and the composition of the solvent correspond closely to the concentration and composition of the *Standard solution*. Suggested operating parameters of the gas chromatograph are provided in this section.

The general high-pressure liquid chromatography (HPLC) procedures set forth in the following paragraphs are applicable to the quantitative determination of parabens and *Thimerosal*. Prepare the *Internal standard solution* and the *Standard solution* for each antimicrobial agent as directed below. Unless otherwise directed, prepare the *Sample solution* from accurately measured portions of the sample under test and the *Internal standard solution*, if applicable, such that the concentration of the antimicrobial agent and the composition of the solvent is about the same as the concentration and composition of the *Standard solution*. Suggested operating parameters of the liquid chromatograph are provided in this section.

• **BENZYL ALCOHOL**

**Diluent:** Methanol and water (20:80)

**Internal standard solution:** 3.8 mg/mL of phenol prepared as follows. Dissolve a suitable amount of phenol in 10% of the flask volume of methanol, and dilute with water to volume.

**Standard solution:** 1.8 mg/mL of USP Benzyl Alcohol RS and 1.5 mg/mL of phenol prepared as follows. Dissolve 180 mg of USP Benzyl Alcohol RS in 20 mL of methanol contained in a 100-mL volumetric flask. Add 40.0 mL of *Internal standard solution*, and dilute with water to volume.

**Chromatographic system**

(See *Chromatography* ⟨621⟩*, System Suitability*.)

**Mode:** GC

**Detector:** Flame ionization

**Column:** 30-m × 0.32-mm fused-silica; bonded with a 0.5-µm film of phase G16

**Temperatures**

**Injection port:** 200°

**Detector:** 310°

**Column:** See *Table 1*.

**Table 1**

| Initial Temperature (°) | Temperature Ramp (°/min) | Final Temperature (°) | Hold Time at Final Temperature (min) |
|---|---|---|---|
| 150 | 0 | 150 | 5 |
| 150 | 10 | 230 | 7 |

**Carrier gas:** Helium

**Flow rate (constant):** 2 mL/min

**Injection volume:** 1 µL

**Split ratio:** 10:1

**Run time:** 20 min

**System suitability**

**Sample:** *Standard solution*

[NOTE—The relative retention times for benzyl alcohol and phenol are about 1.0 and 1.25, respectively.]

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**Suitability requirements**
**Resolution:** NLT 2.0 between the benzyl alcohol and phenol peaks
**Tailing factor:** NMT 2.0 for the benzyl alcohol peak
**Relative standard deviation:** NMT 2.0% for the peak response ratio of benzyl alcohol to phenol

**Analysis**
**Samples:** *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of benzyl alcohol ($C_7H_8O$) in the portion of the sample taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

$R_U$ = peak response ratio of benzyl alcohol to phenol from the *Sample solution*
$R_S$ = peak response ratio of benzyl alcohol to phenol from the *Standard solution*
$C_S$ = concentration of USP Benzyl Alcohol RS in the *Standard solution*
$C_U$ = nominal concentration of benzyl alcohol in the *Sample solution*

- **CHLOROBUTANOL**

**Diluent:** Methanol and water (50:50)
**Internal standard solution:** 10 mg/mL of 2,2,2-trichloroethanol in *Diluent*
**Standard stock solution:** 5 mg/mL of USP Chlorobutanol RS in methanol
**Standard solution:** 1.25 mg/mL of USP Chlorobutanol RS and 2 mg/mL of 2,2,2-trichloroethanol prepared as follows. Transfer 2.5 mL of *Standard stock solution*, 2.0 mL of *Internal standard solution*, and 0.5 mL of methanol to a 10-mL volumetric flask. Dilute with water to volume.
**Sample stock solution:** Quantitatively dilute, if necessary, an accurately measured volume corresponding to 2.5 mg/mL of chlorobutanol in water.
**Sample solution:** Combine 5.0 mL of *Sample stock solution* with 2.0 mL of *Internal standard solution* in a 10-mL volumetric flask, and dilute with *Diluent* to volume.
**Chromatographic system**
(See *Chromatography* ⟨621⟩, *System Suitability*.)
**Mode:** GC
**Detector:** Flame ionization
**Column:** 30-m × 0.32-mm fused-silica; bonded with a 0.25-µm film of phase G16
**Temperatures**
**Injection port:** 260°
**Detector:** 280°
**Column:** 135°
**Carrier gas:** Helium
**Flow rate:** 1 mL/min
**Injection volume:** 0.5 µL
**Split ratio:** 10:1
**Run time:** 12 min
**System suitability**
**Sample:** *Standard solution*
[NOTE—The relative retention times for chlorobutanol and 2,2,2-trichloroethanol are about 1.0 and 1.4, respectively.]
**Suitability requirements**
**Resolution:** NLT 2.0 between chlorobutanol and 2,2,2-trichloroethanol
**Relative standard deviation:** NMT 1.0% for the peak response ratio of chlorobutanol to 2,2,2-trichloroethanol
**Analysis**
**Samples:** *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of chlorobutanol ($C_4H_7Cl_3O$), on the anhydrous basis, in the portion of the sample taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

$R_U$ = peak response ratio of chlorobutanol to 2,2,2-trichloroethanol from the *Sample solution*
$R_S$ = peak response ratio of chlorobutanol to 2,2,2-trichloroethanol from the *Standard solution*
$C_S$ = concentration of USP Chlorobutanol RS in the *Standard solution*
$C_U$ = nominal concentration of chlorobutanol in the *Sample solution*

- **PHENOL**

**Internal standard solution:** 2 mg/mL of USP Benzyl Alcohol RS in methanol
**Standard stock solution:** 4 mg/mL of USP Phenol RS in water
**Standard solution:** 0.4 mg/mL each of USP Phenol RS and USP Benzyl Alcohol RS prepared as follows. Combine 5.0 mL of *Standard stock solution* with 10.0 mL of *Internal standard solution* in a 50-mL volumetric flask, and dilute with water to volume.
**Chromatographic system**
(See *Chromatography* ⟨621⟩, *System Suitability*.)
**Mode:** GC
**Detector:** Flame ionization
**Column:** 30-m × 0.32-mm fused-silica; bonded with a 0.5-µm film of phase G16

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**Temperatures**
**Injection port:** 200°
**Detector:** 310°
**Column:** See *Table 2*.

**Table 2**

| Initial Temperature (°) | Temperature Ramp (°/min) | Final Temperature (°) | Hold Time at Final Temperature (min) |
|---|---|---|---|
| 150 | 0 | 150 | 5 |
| 150 | 10 | 230 | 7 |

**Carrier gas:** Helium
**Flow rate (constant flow):** 2 mL/min
**Injection volume:** 1 µL
**Split ratio:** 10:1
**Run time:** 20 min

**System suitability**
**Sample:** *Standard solution*
[NOTE—The relative retention times for benzyl alcohol and phenol are about 0.85 and 1.0, respectively.]
**Suitability requirements**
**Resolution:** NLT 2.0 between benzyl alcohol and phenol
**Tailing factor:** NMT 2.0 for the phenol peak
**Relative standard deviation:** NMT 1.0% for the peak response ratio of phenol to benzyl alcohol

**Analysis**
**Samples:** *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of phenol ($C_6H_6O$) in the portion of the sample taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

$R_U$ = peak response ratio of phenol to benzyl alcohol from the *Sample solution*
$R_S$ = peak response ratio of phenol to benzyl alcohol from the *Standard solution*
$C_S$ = concentration of USP Phenol RS in the *Standard solution*
$C_U$ = nominal concentration of phenol in the *Sample solution*

• **METHYLPARABEN AND PROPYLPARABEN**
**Buffer:** 7 g/L of monobasic potassium phosphate in water
**Mobile phase:** Methanol and *Buffer* (65:35)
**Internal standard solution:** 0.013 mg/mL of USP Ethylparaben RS in *Mobile phase*
**System suitability solution:** 0.01 mg/mL each of USP Butylparaben RS, USP Propylparaben RS, USP Ethylparaben RS, USP Methylparaben RS, and *p*-hydroxybenzoic acid in *Mobile phase*
**Standard stock solution:** 0.2 mg/mL of USP Methylparaben RS and 0.03 mg/mL of USP Propylparaben RS in *Mobile phase*
**Standard solution:** Combine 5 mL of *Standard stock solution* with 5 mL of *Internal standard solution*, and extract three times with 10-mL aliquots of diethyl ether. Filter the combined ether layers through anhydrous sodium sulfate. Evaporate the ether extract to dryness, and dissolve the residue in 50 mL of *Mobile phase*.
**Sample solution:** Combine 5 mL of the specimen under test with 5 mL of *Internal standard solution*, and extract three times with 10-mL aliquots of diethyl ether. Filter the combined ether layers through anhydrous sodium sulfate. Evaporate the ether extract to dryness, and dissolve the residue in 50 mL of *Mobile phase*.
**Chromatographic system**
(See *Chromatography* ⟨621⟩, *System Suitability*.)
**Mode:** LC
**Detector:** UV 272 nm
**Columns**
**Guard:** 4.0-mm × 3-mm; packing L1
**Analytical:** 4.6-mm × 15-cm; 5-µm packing L1
**Flow rate:** 1.3 mL/min
**Injection volume:** 10 µL
**Run time:** 10 min
**System suitability**
**Samples:** *System suitability solution* and *Standard solution*
[NOTE—The relative retention times for *p*-hydroxybenzoic acid, methylparaben, ethylparaben, and propylparaben are about 0.58, 1.0, 1.4, and 2.1, respectively.]
**Suitability requirements**
**Resolution:** NLT 2.0 between *p*-hydroxybenzoic acid and methylparaben, NLT 2.0 between methylparaben and ethylparaben; *System suitability solution*
**Tailing factor:** NMT 2.0 for the methylparaben and propylparaben peaks, *Standard solution*
**Relative standard deviation:** NMT 2.0% for the peak response ratio of methylparaben to ethylparaben, NMT 2.0% for the peak response ratio of propylparaben to ethylparaben; *System suitability solution*

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**Analysis**
**Samples:** *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of methylparaben ($C_8H_8O_3$) in the portion of the sample taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

$R_U$ = peak response ratio of methylparaben to ethylparaben from the *Sample solution*
$R_S$ = peak response ratio of methylparaben to ethylparaben from the *Standard solution*
$C_S$ = concentration of USP Methylparaben RS in the *Standard solution*
$C_U$ = nominal concentration of methylparaben in the *Sample solution*

Calculate the percentage of the labeled amount of propylparaben ($C_{10}H_{12}O_3$) in the portion of the sample taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

$R_U$ = peak response ratio of propylparaben to ethylparaben from the *Sample solution*
$R_S$ = peak response ratio of propylparaben to ethylparaben from the *Standard solution*
$C_S$ = concentration of USP Propylparaben RS in the *Standard solution*
$C_U$ = nominal concentration of propylparaben in the *Sample solution*

Ethylparaben and butylparaben may be determined in a similar manner using appropriate internal standard solutions. However, because the extraction recovery is matrix dependent, the user should verify the suitability of the procedure for their drug product and for different product formulations.

- **THIMEROSAL**

**Solution A:** Trifluoroacetic acid and water (0.5: 1000)
**Mobile phase:** Methanol and *Solution A* (60:40)
**Standard solution:** 25 µg/mL of USP Thimerosal RS in water
**Chromatographic system**
(See *Chromatography* ⟨621⟩, *System Suitability*.)
**Mode:** LC
**Detector:** UV 222 nm
**Column:** 2.1-mm × 10-cm; 2-µm packing L1
**Autosampler temperature:** 4°
**Flow rate:** 0.35 mL/min
**Injection volume:** 2.5 µL
**System suitability**
**Sample:** *Standard solution*
**Tailing factor:** NMT 1.5
**Relative standard deviation:** NMT 1.0%
**Analysis**
**Samples:** *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of thimerosal ($C_9H_9HgNaO_2S$) in the portion of the sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

$r_U$ = peak response of thimerosal from the *Sample solution*
$r_S$ = peak response of thimerosal from the *Standard solution*
$C_S$ = concentration of USP Thimerosal RS in the *Standard solution*
$C_U$ = nominal concentration of thimerosal in the *Sample solution*

## POLAROGRAPHIC METHOD

- **PHENYLMERCURIC NITRATE**

**Standard stock solution:** 0.1 mg/mL of phenylmercuric nitrate in sodium hydroxide solution (1 in 250). Warm, if necessary, to dissolve.
**Standard solution:** Pipet 10 mL of *Standard stock solution* into a 25-mL volumetric flask, and proceed as directed under *Sample solution* beginning with "add 2 mL of potassium nitrate solution (1 in 100)".
**Sample solution:** Pipet 10 mL of the specimen under test into a 25-mL volumetric flask, add 2 mL of potassium nitrate solution (1 in 100) and 10 mL of pH 9.2 alkaline borate buffer (see in *Buffer Solutions* in the section *Reagents, Indicators, and Solutions*), and adjust to a pH of 9.2, if necessary, by the addition of 2 N nitric acid. Add 1.5 mL of freshly prepared gelatin solution (1 in 1000), then add the pH 9.2 alkaline borate buffer to volume.
**Analysis:** Pipet a portion of the *Sample solution* into the polarographic cell, and deaerate by bubbling nitrogen through the solution for 15 min. Insert the dropping mercury electrode of a suitable polarograph (see *Polarography* ⟨801⟩), and record the polarogram from –0.6 to –1.5 volts versus the saturated calomel electrode.
Calculate the quantity, in µg/mL, of phenylmercuric nitrate ($C_6H_5HgNO_3$) in the portion of the sample taken:

$$\text{Result} = 2.5C[(i_d)_U/(i_d)_S]$$

$C$ = concentration of phenylmercuric nitrate in the *Standard solution* (µg/mL)

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$(i_d)_U$ = diffusion current of the *Sample solution*, as the difference between the residual current and the limiting current
$(i_d)_S$ = diffusion current of the *Standard solution*, as the difference between the residual current and the limiting current

- **USP Reference Standards** ⟨11⟩
  USP Benzyl Alcohol RS
  USP Butylparaben RS
  USP Chlorobutanol RS
  USP Ethylparaben RS
  USP Methylparaben RS
  USP Phenol RS
  USP Propylparaben RS
  USP Thimerosal RS

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# ⟨345⟩ ASSAY FOR CITRIC ACID/CITRATE AND PHOSPHATE

## INTRODUCTION

The following ion chromatographic general procedure is provided for the determination of citric acid/citrate and phosphate in compendial articles, when specified in the individual monographs. See *Ion Chromatography* ⟨1065⟩ for discussion of the theory and principles of measurements using ion chromatography.

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**ASSAY** ▲[NOTE—Use procedure A if the monograph does not specify a procedure.]▲ (USP 1-May-2023)

***Change to read:***

• **PROCEDURE** ▲**A**▲ (USP 1-May-2023)

**Mobile phase:** 20 mM sodium hydroxide or potassium hydroxide from an appropriate volume of carbonate-free sodium hydroxide or potassium hydroxide solution of known concentration and water (resistivity NLT 18 megohm-cm). Alternatively, *Mobile phase* can be generated electrolytically using an automatic eluent generator. Protect the *Mobile phase* from atmospheric carbon dioxide.

**Standard solution 1 (for the assay of citric acid/citrate only):** 20 µg/mL of citrate ($C_6H_5O_7$) in freshly prepared 1 mM sodium hydroxide from USP Citric Acid RS

**Standard solution 2 (for the concomitant assay of citrate and phosphate):** 20 µg/mL of citrate ($C_6H_5O_7$) and 12 µg/mL of phosphate ($PO_4$) in freshly prepared 1 mM sodium hydroxide from USP Citric Acid RS and monobasic sodium phosphate

**Sample solution (for the assay of citric acid/citrate):** Nominally 20 µg/mL of citrate in freshly prepared 1 mM sodium hydroxide, unless otherwise stated in the monograph

**Sample solution (for the assay of phosphate):** Nominally 12 µg/mL of phosphate in freshly prepared 1 mM sodium hydroxide, unless otherwise stated in the monograph

**Chromatographic system**

(See *Chromatography* ⟨621⟩, *System Suitability*.)

**Mode:** LC

**Detector:** Conductivity with suppression

**Columns**

**Analytical:** 4-mm × 25-cm; 13-µm packing L61

**Guard:** 4-mm × 5-cm; 13-µm packing L61

**Temperatures**

**Column:** 30°

**Detector:** 35°

**Suppressor:** 4-mm membrane anionic autosuppressor or a suitable chemical suppression system

**Flow rate:** 2 mL/min

**Injection volume:** 10 µL

**System suitability**

**Samples:** *Standard solution 1* and/or *Standard solution 2*, as appropriate

[NOTE—The relative retention times for phosphate and citrate are 0.57 and 1.0, respectively.]

**Suitability requirements**

**Tailing factor:** NMT 2.0 for the citrate and/or phosphate peaks, as appropriate

**Relative standard deviation:** NMT 1.5% for six replicate injections for the citrate and/or phosphate peaks, as appropriate

**Analysis**

**Samples:** *Standard solution 1* and/or *Standard solution 2*, and *Sample solution*

Unless otherwise stated in the monograph, calculate the concentration of citrate or phosphate in the portion of the *Sample solution* taken:

$$\text{Result} = (r_U/r_S) \times C_S$$

$r_U$ = peak response of citrate or phosphate from the *Sample solution*

$r_S$ = peak response of citrate or phosphate from *Standard solution 1* or *Standard solution 2*

$C_S$ = concentration of citrate or phosphate from *Standard solution 1* or *Standard solution 2* (µg/mL)

***Add the following:***

▲• **PROCEDURE B**

**Mobile phase, Standard solution 1, Standard solution 2,** and **Sample solution(s):** Prepare as directed in *Procedure A*.

**Chromatographic system**

(See *Chromatography* ⟨621⟩, *System Suitability*.)

**Mode, Detector, Temperatures, Suppressor,** and **Injection volume:** Proceed as directed in *Procedure A*.

**Columns**

**Guard:** 4-mm × 5-cm; 10-µm packing L21

**Analytical:** 4.6-mm × 25-cm; 9-µm packing L46

**Flow rate:** 1 mL/min

**Run time:** 15 min

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**System suitability**

**Samples:** *Standard solution 1* and/or *Standard solution 2*, as appropriate

[NOTE—The relative retention times for phosphate and citrate are 0.65 and 1.0, respectively.]

**Suitability requirements**

**Resolution:** NLT 6.5 between phosphate and citrate, *Standard solution 2*

**Tailing factor:** NMT 1.5 for citrate and/or phosphate, as appropriate

**Relative standard deviation:** NMT 1.5% for 6 replicate injections for citrate and/or phosphate, as appropriate

**Analysis:** Proceed as directed in *Procedure A.*▲ (USP 1-May-2023)

## ADDITIONAL REQUIREMENTS

• **USP REFERENCE STANDARDS** ⟨11⟩

USP Citric Acid RS

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# ⟨351⟩ ASSAY FOR STEROIDS

The following procedure is applicable for determination of those Pharmacopeial steroids that possess reducing functional groups such as α-ketols.

## STANDARD PREPARATION

Dissolve in alcohol a suitable quantity of the USP Reference Standard specified in the individual monograph, previously dried under the conditions specified in the individual monograph and accurately weighed, and dilute quantitatively and stepwise with alcohol to obtain a solution having a concentration of about 10 µg per mL. Pipet 20 mL of this solution into a glass-stoppered, 50-mL conical flask.

## ASSAY PREPARATION

Prepare as directed in the individual monograph.

## PROCEDURE

To each of the two flasks containing the *Assay Preparation* and the *Standard Preparation,* respectively, and to a similar flask containing 20.0 mL of alcohol to serve as the blank, add 2.0 mL of a solution prepared by dissolving 50 mg of blue tetrazolium in 10 mL of methanol, and mix. Then to each flask add 2.0 mL of a mixture of alcohol and tetramethylammonium hydroxide TS (9:1), mix, and allow to stand in the dark for 90 minutes. Without delay, concomitantly determine the absorbances of the solutions from the *Assay Preparation* and the *Standard Preparation* at about 525 nm, with a suitable spectrophotometer, against the blank. Calculate the result by the formula given in the individual monograph, in which C is the concentration, in µg per mL, of the Reference Standard in the *Standard Preparation;* and $A_U$ and $A_S$ are the absorbances of the solutions from the *Assay Preparation* and the *Standard Preparation,* respectively.

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# ⟨381⟩ ELASTOMERIC ▲COMPONENTS IN INJECTABLE PHARMACEUTICAL PRODUCT PACKAGING/DELIVERY SYSTEMS

1. INTRODUCTION
2. SCOPE
3. TEST SAMPLES
4. PROCEDURES
    4.1 Biological Reactivity
    4.2 Physicochemical Tests
    4.3 Functionality Tests

## 1. INTRODUCTION

Packaging systems, also referred to as container–closure systems, are defined in *Packaging and Storage Requirements* ⟨659⟩; these systems are the sum of components that together contain and protect the drug product. Elastomeric components are formulated with elastomeric substances and can be either thermoset or thermoplastic in nature.

Every elastomeric component used in a pharmaceutical packaging/delivery system should be proven suitable for its intended use. The purpose of this chapter is to provide baseline chemical and biological reactivity requirements for the selection of elastomeric injectable packaging/delivery system components.

The establishment of the potential suitability of an elastomeric component does not rely on a single testing strategy. No single strategy can cover all elastomeric component attributes that have the potential to impact suitability. The chemical testing prescribed includes physicochemical tests. Extractable elements may also be relevant to the selection of a packaging system's materials of construction and therefore a relevant aspect of material characterization. Materials of construction can vary widely in terms of their intentionally and unintentionally added elements and their potential use. Because of this, it is challenging to provide universally effective and efficient test methodologies, lists of target elements, and reporting requirements. It is the material user's responsibility to evaluate the need for extractable elements testing and, if such testing is necessary, to establish and justify the means by which testing is accomplished, taking into account extraction conditions, target elements, and reporting requirement. Elastomeric components can vary widely in terms of their intentionally and unintentionally added elements as well as the components' potential use. Because of this, it is challenging to provide universally effective and efficient test methodologies, lists of target elements, and reporting requirements. It is the elastomeric component user's responsibility to evaluate the need for extractable elements testing and, if such testing is necessary, to establish and justify the means by which testing is accomplished, taking into account extraction conditions, target elements, and reporting requirements. The physicochemical tests are also augmented with biological reactivity tests.

If elastomeric components comply with the requirements outlined in this chapter, studies should follow to determine their suitability as recommended in *Assessment of Extractables Associated with Pharmaceutical Packaging/Delivery Systems* ⟨1663⟩ and *Assessment of Drug Product Leachables Associated with Pharmaceutical Packaging/Delivery Systems* ⟨1664⟩.

In summary, establishing chemical suitability of elastomeric components for injectable product packaging/delivery systems involves multiple tests and testing procedures including:

- Elastomeric component screening—baseline requirements for biological reactivity and physicochemical tests described in this chapter.
- Controlled extraction studies—studies as described in ⟨1663⟩ to create extractables profile(s) of particular pharmaceutical packaging/delivery systems and/or packaging components.
- Pharmaceutical product assessment—Actual-case measurement of confirmed leachables in the pharmaceutical product in the packaging/delivery system intended for the commercial market. (For additional information, see ⟨1664⟩.)

Additional information about elastomeric components, such as their composition, manufacturing processes, considerations for use, and testing procedures is found in ⟨1381⟩.

## 2. SCOPE

Elastomeric components within scope are those used in the packaging systems of products described in *Injections and Implanted Drug Products* ⟨1⟩.

Elastomeric components utilized for injectable products within chapter scope include, but are not limited to, those used for vials and bottles (stoppers and cap liners), prefilled syringes (plungers, needle shields, and tip caps), cartridges (plungers and seal liners), flexible bags (injection ports), and blow-fill-seal containers (cap liners). The specified procedures and requirements outlined in this chapter are not applicable to elastomeric components used in products regulated by the Center for Devices and Radiological Health. All elastomeric components in direct or indirect contact with the pharmaceutical product are within scope. An example of indirect contact is an elastomeric layer of a multilayer cap liner that does not directly contact the product but may leach into the product via migration through the product contact layer. Another example is an elastomeric cap liner that may contact the product after being punctured to attain product access.

Also within scope are elastomeric components of systems or packages that are intended for transient product storage and/or product delivery intended for specific pharmaceutical products.

Elastomeric components outside of scope include those components of containers and closures that do not have direct or indirect contact with the pharmaceutical product or hold intermediate compounds, active pharmaceutical ingredients (APIs), and excipients. Also outside chapter scope are elastomeric components of containment and/or transport systems used in product, intermediate compound, API, or excipient manufacturing. Although outside of the chapter scope, chapter tests and requirements may be applied.

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Chapter procedures and requirements are specified for physicochemical and biological reactivity tests. Elastomeric component identification tests fall beyond the chapter scope. Elastomeric components are made of a wide variety of elastomeric materials and optional polymeric coatings. For this reason, it is not possible to have identification tests that encompass all possible elastomeric component presentations.

Elastomeric component functional suitability tests described in this chapter are within scope until *Elastomeric Component Functional Suitability in Parenteral Product Packaging/Delivery Systems* ⟨382⟩ is fully implemented. As part of a finished product packaging system, elastomeric components must appropriately function to seal the container and, in some cases, aid in safe and effective product delivery. The essential principles and demonstrated best practices for such assessments for injectable product packaging/delivery systems can be found in ⟨382⟩ and *Assessment of Elastomeric Component Functional Suitability in Parenteral Product Packaging/Delivery Systems* ⟨1382⟩.

## 3. TEST SAMPLES

Test samples should mimic finished elastomeric components after the completion of all manufacturing and processing steps (e.g., molding conditions, sterilization, etc.), and surface modifications (such as siliconization, chlorinated surface treatments, fluoropolymer coatings and films).

## 4. PROCEDURES

**4.1 BIOLOGICAL REACTIVITY:** Elastomeric components (Type I and Type II) meet the requirements of *Biological Reactivity Tests, In Vitro* ⟨87⟩.[1] If components do not meet the requirements of ⟨87⟩, they can be subjected to in vivo testing set forth in *Biological Reactivity Tests, In Vivo* ⟨88⟩, *Systemic Injection Test* and ⟨88⟩, *Intracutaneous Test*. Components that meet the requirements of ⟨87⟩ are not required to undergo ⟨88⟩ testing.

Type I and Type II closures must both conform to the requirements of either the in vitro or the in vivo biological reactivity tests.

**Acceptance criteria:** Test selection and results are consistent with ⟨87⟩ and/or ⟨88⟩.

**4.2 PHYSICOCHEMICAL TESTS**

**Sample solution:** Place whole, uncut elastomeric components corresponding to a surface area of 100 ± 10 $cm^2$ into a borosilicate glass, wide-necked flask (see *Containers—Glass* ⟨660⟩). If it is not possible to achieve the prescribed closure surface area (100 ± 10 $cm^2$) using uncut elastomeric components, select the number of components that will most closely approximate 100 $cm^2$ and adjust the volume of water used to the equivalent of 2 mL/1 $cm^2$ of the actual elastomeric component's surface area. Add 200 mL of *Purified Water* or *Water for Injection* to the elastomeric components, and weigh. Cover the mouth of the flask with a borosilicate glass beaker, or similar non interacting container. Immerse the temperature probe for the autoclave program control in water in a container comparable to that used for the sample. Heat in an autoclave so that a temperature of 121 ± 2° is reached within 20–30 min, and maintain this temperature for 30 min. Cool to room temperature over a period of about 30 min. Add *Purified Water* or *Water for Injection* to bring it up to the original mass. Shake, and immediately decant and collect the solution. [NOTE—This solution must be shaken before being used in each of the tests.]

**Blank:** Prepare a blank solution similarly, using 200 mL of *Purified Water* or *Water for Injection*, omitting the elastomeric components.

**Elastomeric component categories, Type I and II:** Elastomeric components may be classified in two types: Type I elastomeric closures meet the strictest requirements and are preferred; Type II elastomeric closures have mechanical properties suitable for special uses (e.g., multiple piercing) but cannot meet the Type I acceptance criteria for *4.2.1 Appearance, 4.2.4 Absorbance*, and *4.2.5 Reducing Substances*. For these tests, Type II elastomeric closures have alternative acceptance criteria that must be met. The intended final product application will determine whether a Type I or Type II elastomeric component is more appropriate.

**4.2.1 Appearance (Turbidity/Opalescence):** The determination of turbidity may be performed using either a visual or instrumental comparison. For a discussion of turbidimetry, see *Nephelometry and Turbidimetry* ⟨855⟩. Instrumental assessment of clarity provides a more discriminatory test that does not depend on the visual acuity of the analyst.

**Hydrazine sulfate solution:** Dissolve 1.000 g of analytical grade hydrazine sulfate in particle-free water and dilute with particle-free water to 100.0 mL. Allow this solution to stand for 4–6 h.

**Hexamethylenetetramine solution:** Dissolve 2.5 g of analytical grade hexamethylenetetramine in 25.0 mL of particle-free water in a 100-mL glass-stoppered flask.

**Formazin stock suspension:** Add 25.0 mL of *Hydrazine sulfate solution* to the *Hexamethylenetetramine solution* in the 100-mL flask. Mix, and allow to stand for 48 h at 25 ± 1° before using. This suspension is stable for 2 months, provided it is stored in a glass container free from surface defects. The suspension must not adhere to the glass and must be well mixed before use.

**Formazin standard suspension:** Prepare a suspension by diluting 15.0 mL of the *Formazin stock suspension* with particle-free water to 1000.0 mL. It is stable for about 24 h after preparation.

**Reference suspensions:** Prepare according to *Table 1*. Mix and shake before use. [NOTE—Stabilized formazin suspensions that can be used to prepare stable, diluted turbidity standards are available commercially and may be used after comparison with the standards prepared as described.]

**Table 1. Reference Suspensions**

| | Reference Suspension A | Reference Suspension B | Reference Suspension C | Reference Suspension D |
|---|---|---|---|---|
| Standard of opalescence | 5.0 mL | 10.0 mL | 30.0 mL | 50.0 mL |
| Particle-free water | 95.0 mL | 90.0 mL | 70.0 mL | 50.0 mL |

[1] Biological reactivity testing in support of elastomeric components used for final pharmaceutical product packaging/delivery systems (drugs and drug/device combination products) provides baseline information and will often not be sufficient to assess the final suitability for use expectations of regulatory authorities. Thus, it is important to work with the appropriate regulatory authority for guidance regarding a product-specific application.

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**Table 1. Reference Suspensions** *(continued)*

| | Reference Suspension A | Reference Suspension B | Reference Suspension C | Reference Suspension D |
|---|---|---|---|---|
| Nephelometric turbidity units (NTU) | 3 NTU | 6 NTU | 18 NTU | 30 NTU |

**Procedure A (Visual Comparison ⟨630⟩):** Use identical test tubes made of colorless, transparent, neutral glass with a flat base and an internal diameter of 15–25 mm. Fill one tube to a depth of 40 mm with *Sample solution*, one tube to the same depth with water, and 4 other tubes to the same depth with *Reference suspension A*, *Reference suspension B*, *Reference suspension C*, and *Reference suspension D*. Compare the solutions in diffuse daylight 5 min after preparation of the *Reference suspensions*, viewing vertically against a black background. The light conditions must be such that *Reference suspension A* can be readily distinguished from water, and *Reference suspension B* can be readily distinguished from *Reference suspension A*.

**Acceptance criteria:** *Type I—Sample solution* is not more opalescent than *Reference suspension B*. *Type II—Sample solution* is not more opalescent than *Reference suspension C*.

**Procedure B (instrumental comparison):** Measure the turbidity of the *Reference suspensions* in a suitable calibrated turbidimeter (see *Nephelometry and Turbidimetry* ⟨855⟩). The *Blank* should be run and the results corrected for the *Blank*. *Reference suspension A, Reference suspension B, Reference suspension C*, and *Reference suspension D* represent 3, 6, 18, and 30 NTUs, respectively. Measure the turbidity of *Sample solution* using the calibrated turbidimeter.

**Acceptance criteria:** *Type I*—The turbidity of *Sample solution* [in nephelometric turbidity units (NTUs) or formazin turbidity units (FTUs) corrected for the blank] is NMT that for *Reference suspension B* (6 NTU/FTU). *Type II*—The turbidity of *Sample solution* (in nephelometric turbidity units or formazin turbidity units, corrected for the blank) is NMT that for *Reference suspension C* (18 NTU/FTU).

**4.2.2 Color**

**Color standard:** Prepare a solution by diluting 3.0 mL of *Matching Fluid O* (see *Color and Achromicity* ⟨631⟩) with 97.0 mL of diluted hydrochloric acid (10 ± 0.5%).

**Procedure:** Use identical tubes made of colorless, transparent, neutral glass with a flat base and an internal diameter of 15–25 mm. Fill one tube to a depth of 40 mm with *Sample solution*, and fill the second tube with the *Color standard*. Compare the liquids in diffuse daylight, viewing vertically against a white background.

**Acceptance criteria:** *Sample solution* is not more intensely colored than the *Color standard*.

**4.2.3 Acidity or Alkalinity**

**Bromothymol blue solution:** Dissolve 50 mg of bromothymol blue in a mixture of 4 mL of 0.02 M sodium hydroxide and 20 mL of alcohol. Dilute with water to 100 mL.

**Test solution:** To 20 mL of *Sample solution* add 0.1 mL of *Bromothymol blue solution*.

**Procedure:** If the solution is yellow, titrate with 0.01 N sodium hydroxide until a blue endpoint is reached. If the solution is blue, titrate with 0.01 N hydrochloric acid until a yellow endpoint is reached. If the solution is green, it is neutral and no titration is required.

**Blank correction:** Test 20 mL of *Blank* similarly. Correct the results obtained for *Sample solution* by subtracting or adding the volume of titrant required for the *Blank*, as appropriate. (See *Titrimetry* ⟨541⟩.)

**Acceptance criteria:** NMT 0.3 mL of 0.01 N sodium hydroxide produces a blue color or NMT 0.8 mL of 0.01 N hydrochloric acid produces a yellow color, or no titration is required.

**4.2.4 Absorbance**

[NOTE—Perform this test within 5 h of preparing *Sample solution*.]

**Procedure:** Pass *Sample solution* through an inert filter of 0.45-µm pore size, discarding the first few milliliters of filtrate. Measure the absorbance of the filtrate at wavelengths between 220 and 360 nm in a 1-cm cell using the *Blank* in a matched cell in the reference beam. If dilution of the filtrate is required before measurement of the absorbance, correct the test results for the dilution.

**Acceptance criteria:** *Type I*—NMT 0.2 *Type II*—NMT 4.0.

**4.2.5 Reducing Substances**

[NOTE—Perform this test within 4 h of preparing *Sample solution*.]

**Procedure:** To 20.0 mL of *Sample solution* add 1 mL of diluted sulfuric acid and 20.0 mL of 0.002 M potassium permanganate. Boil for 3 min. Cool, add 1 g of potassium iodide, and titrate immediately with 0.01 M sodium thiosulfate using 0.25 mL of starch solution TS as the indicator. Perform a titration using 20.0 mL of *Blank* and note the difference in volume of 0.01 M sodium thiosulfate required.

**Acceptance criteria:** *Type I*—The difference between titration volumes is NMT 3.0 mL of 0.01 M sodium thiosulfate. *Type II*—The difference between titration volumes is NMT 7.0 mL of 0.01 M sodium thiosulfate.

**4.2.6 Volatile Sulfides**

**Procedure:** Place elastomeric components, cut if necessary, with a total surface area of 20 ± 2 $cm^2$ in a 100-mL flask, and add 50 mL of a 20-g/L citric acid solution. In the same manner and at the same time, prepare a control solution in a separate flask by dissolving 0.154 mg of sodium sulfide in 50 mL of a 20-g/L citric acid solution. Place a piece of lead acetate paper over the mouth of each flask, and hold the paper in position by placing an inverted weighing bottle over it. Immerse the temperature probe for the autoclave program control in water in a container comparable to that used for the sample. Heat the flasks in an autoclave so that a temperature of 121 ± 2° is reached within 20–30 min, and then maintain this temperature for 30 min. Cool to room temperature over a period of about 30 min.

**Acceptance criteria:** Any black stain on the paper produced by the test solution is not more intense than that produced by the control solution.

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#### 4.2.7 Ammonium

**Alkaline potassium tetraiodomercurate solution:** Prepare a 100-mL solution containing 11 g of potassium iodide and 15 g of mercuric iodide in water. Immediately before use, mix one volume of this solution with an equal volume of a 250-g/L solution of sodium hydroxide.

**Test solution:** Dilute 5 mL of *Sample solution* with water to 14 mL. Make alkaline, if necessary, by adding 1 N sodium hydroxide, and dilute with water to 15 mL. Add 0.3 mL of *Alkaline potassium tetraiodomercurate solution* and close the container.

**Ammonium standard solution:** Prepare a solution of ammonium chloride in water [1 ppm of ammonium ($NH_4^+$)]. Mix 10 mL of the 1 ppm ammonium chloride solution with 5 mL of water and 0.3 mL of *Alkaline potassium tetraiodomercurate solution*. Close the container.

**Acceptance criteria:** After 5 min, any yellow color in the *Test solution* is no darker than the *Ammonium standard solution* [NMT 2 ppm of ammonium ($NH_4^+$) in *Sample solution*].

### 4.3 FUNCTIONALITY TESTS

[NOTE—Samples treated as described for preparation of *Sample solution* and air-dried should be used for the functionality tests of *4.3.1 Penetrability, 4.3.2 Fragmentation,* and *4.3.3 Self-Sealing Capacity.* Functionality tests are performed on closures intended to be pierced by a hypodermic needle. The *4.3.3 Self-Sealing Capacity* test is required only for closures intended for multiple-dose containers. The needle specified for each test is a lubricated, long-bevel (bevel angle 12 ± 2°) hypodermic needle with an external diameter of 0.8 mm (21 gauge).[2] ]

#### 4.3.1 Penetrability

**Procedure:** Fill 10 suitable vials to the nominal volume with water, fit the closures to be examined, and secure with a cap. Using a new hypodermic needle as described above for each closure, pierce the closure with the needle perpendicular to the surface.

**Acceptance criteria:** The force for piercing is no greater than 10 N (1 kilogram-force) for each closure, determined with an accuracy of ±0.25 N (25 gram-force).

#### 4.3.2 Fragmentation

**Closures for liquid preparations:** Fill 12 clean vials with water to 4 mL less than the nominal capacity. Fit the closures to be examined, secure with a cap, and allow to stand for 16 h.

**Closures for dry preparations:** Fit closures to be examined into 12 clean vials, and secure each with a cap.

**Procedure:** Using a hypodermic needle as described above fitted to a clean syringe, inject into each vial 1 mL of water while removing 1 mL of air. Repeat this procedure 4 times for each closure, piercing each time at a different site. Use a new needle for each closure, checking that it is not blunted during the test. Filter the total volume of liquid in all the vials through a single filter with a nominal pore size NMT 0.5 µm. Count the rubber fragments on the surface of the filter visible to the naked eye.

**Acceptance criteria:** There are no more than 5 fragments visible. This limit is based on the assumption that fragments with a diameter >50 µm are visible to the naked eye. In case of doubt or dispute, the particles are examined microscopically to verify their nature and size.

#### 4.3.3 Self-Sealing Capacity

**Procedure:** Fill 10 suitable vials with water to the nominal volume. Fit the closures that are to be examined, and cap. Using a new hypodermic needle as described above for each closure, pierce each closure 10 times, piercing each time at a different site. Immerse the 10 vials in a solution of 0.1% (1 g/L) methylene blue, and reduce the external pressure by 27 kPa for 10 min. Restore to atmospheric pressure, and leave the vials immersed for 30 min. Rinse the outside of the vials.

**Acceptance criteria:** None of the vials contain any trace of blue solution.▲ (USP 1-Dec-2020)

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[2] Refer to ISO 7864: *Sterile Hypodermic Needles for Single Use.*

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# ⟨391⟩ EPINEPHRINE ASSAY

## ASSAY

**Ferro-citrate solution:** On the day needed, dissolve 1.5 g of ferrous sulfate in 200 mL of water to which have been added 1.0 mL of dilute hydrochloric acid (1 in 12) and 1.0 g of sodium bisulfite. Dissolve 500 mg of sodium citrate in 10 mL of this solution, and mix.

**Buffer solution:** In a 50-mL volumetric flask mix 4.2 g of sodium bicarbonate, 5.0 g of potassium bicarbonate, and 18 mL of water (not all of the solids will dissolve at this stage). To another 18 mL of water add 3.75 g of aminoacetic acid and 1.7 mL of 6 N ammonium hydroxide, mix to dissolve, and transfer this solution to the 50-mL volumetric flask containing the other mixture. Dilute with water to volume, and mix until solution is complete.

**Standard preparation:** Transfer about 18 mg of USP Epinephrine Bitartrate RS, accurately weighed, to a 100-mL volumetric flask with the aid of 20 mL of sodium bisulfite solution (1 in 50), dilute with water to volume, and mix. Transfer 5.0 mL of this solution to a 50-mL volumetric flask, dilute with sodium bisulfite solution (1 in 500) to volume, and mix. [NOTE—Make the final dilution when the assay is carried out.] The concentration of USP Epinephrine Bitartrate RS in the *Standard preparation* is about 18 µg per mL.

**Assay preparation:** Transfer to a 50-mL volumetric flask an accurately measured volume of the Injection under assay, equivalent to about 500 µg of epinephrine, dilute with sodium bisulfite solution (1 in 500) to volume, if necessary, and mix. [NOTE—The final concentration of sodium bisulfite is in the range of 1 to 3 mg per mL, any bisulfite present in the Injection under assay being taken into consideration.]

**Procedure:** Into three 50-mL glass-stoppered conical flasks transfer, separately, 20.0-mL aliquots of the *Standard preparation*, the *Assay preparation*, and sodium bisulfite solution (1 in 500) to provide the blank. To each flask add 200 µL of *Ferro-citrate solution* and 2.0 mL of *Buffer solution*, mix, and allow the solutions to stand for 30 minutes. Determine the absorbances of the solutions in 5-cm cells at the wavelength of maximum absorbance at about 530 nm, with a suitable spectrophotometer, using the blank to set the instrument.

Calculate the quantity, in mg, of epinephrine ($C_9H_{13}NO_3$) in each mL of the Injection taken by the formula, in which 183.21 and 333.30 are the molecular weights of epinephrine and epinephrine bitartrate, respectively:

$$\text{Result} = (183.21/333.30)(0.05C/V)(A_U/A_S)$$

$C$ = concentration of USP Epinephrine Bitartrate RS in the *Standard preparation* (µg/mL)
$V$ = volume of Injection taken (mL)
$A_U$ = absorbance of the *Assay preparation*
$A_S$ = absorbance of the *Standard preparation*

## ADDITIONAL REQUIREMENTS

- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Epinephrine Bitartrate RS

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# ⟨401⟩ FATS AND FIXED OILS

The following definitions and general procedures apply to fats, fixed oils, waxes, resins, balsalms, and similar substances.

• **Preparation of Specimen**

If a specimen of oil shows turbidity owing to separated stearin, warm the container in a water bath at 50° until the oil is clear, or if the oil does not become clear on warming, pass it through dry filter paper in a funnel contained in a hot-water jacket. Mix thoroughly, and weigh at one time as many portions as are needed for the various determinations, preferably using a bottle that has a pipet dropper or a weighing buret. If the specimen is solid at room temperature, keep it melted until the desired portions of specimen are withdrawn.

## PROCEDURES

• **Specific Gravity:** Determine the specific gravity of a fat or oil as directed in *Specific Gravity* ⟨841⟩.

• **Melting Temperature:** Determine the melting temperature as directed for *Class II* substances (see *Melting Range or Temperature* ⟨741⟩).

• **Acid Value (Free Fatty Acids)**

The acidity of fats and fixed oils in this pharmacopeia may be expressed as the number of mL of 0.1 N alkali required to neutralize the free acids in 10.0 g of substance. Acidity frequently is expressed as the Acid Value, which is the number of mg of potassium hydroxide required to neutralize the free acids in 1.0 g of the substance. Unless directed otherwise in the individual monograph, use *Method I*.

### Method I

**Procedure:** Unless directed otherwise, dissolve about 10.0 g of the substance, accurately weighed, in 50 mL of a mixture of equal volumes of alcohol and ether (which has been neutralized to phenolphthalein with 0.1 N potassium hydroxide or 0.1 N sodium hydroxide, unless otherwise specified) contained in a flask. If the test specimen does not dissolve in the cold solvent, connect the flask with a suitable condenser and warm slowly, with frequent shaking, until the specimen dissolves. Add 1 mL of phenolphthalein TS, and titrate with 0.1 N potassium hydroxide VS or 0.1 N sodium hydroxide VS until the solution remains faintly pink after shaking for 30 s. Calculate either the Acid Value or the volume of 0.1 N alkali required to neutralize 10.0 g of specimen (free fatty acids), whichever is appropriate. Calculate the Acid Value:

$$\text{Result} = (M_r \times V) \times (N/W)$$

$M_r$ = molecular weight of potassium hydroxide, 56.11
$V$ = volume (mL)
$N$ = normality of the potassium hydroxide solution or the sodium hydroxide solution
$W$ = weight of the sample taken (g)

If the volume of 0.1 N potassium hydroxide VS or 0.1 N sodium hydroxide VS required for the titration is less than 2 mL, a more dilute titrant may be used, or the sample size may be adjusted accordingly. The results may be expressed in terms of the volume of titrant used or in terms of the equivalent volume of 0.1 N potassium hydroxide or 0.1 N sodium hydroxide.

If the oil has been saturated with carbon dioxide for the purpose of preservation, gently reflux the alcohol–ether solution for 10 min before titration. The oil may be freed from carbon dioxide also by exposing it in a shallow dish in a vacuum desiccator for 24 h before weighing the test specimens.

### Method II

**Procedure:** Prepare 125 mL of a solvent mixture consisting of equal volumes of isopropyl alcohol and toluene. Before use, add 2 mL of a 1% solution of phenolphthalein in isopropyl alcohol to the 125-mL mixture, and neutralize with alkali to a faint but permanent pink color. Accurately weigh the appropriate amount of well-mixed liquid sample indicated in *Table 1*, and dissolve it in the neutralized solvent mixture. If the test specimen does not dissolve in the cold solvent, connect the flask with a suitable condenser and warm slowly, with frequent shaking, until the specimen dissolves. Shake vigorously while titrating with 0.1 N potassium hydroxide VS or 0.1 N sodium hydroxide VS to the first permanent pink of the same intensity as that of the neutralized solvent before mixing with the sample. Calculate the Acid Value as indicated in *Method I*.

**Table 1**

| Acid Value | Sample Weight (g) |
|---|---|
| 0–1 | 20 |
| 1–4 | 10 |
| 4–15 | 2.5 |
| 15–74.9 | 0.5 |
| ≥75.0 | 0.1 |

• **Ester Value**

The Ester Value is the number of mg of potassium hydroxide required to saponify the esters in 1.0 g of the substance. If the Saponification Value and the Acid Value have been determined, the difference between these two represents the Ester Value (i.e., Ester Value = Saponification Value – Acid Value).

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**Procedure:** Place 1.5–2 g of the substance, accurately weighed, in a tared, 250-mL flask, add 20–30 mL of neutralized alcohol, and shake. Add 1 mL of phenolphthalein TS, and titrate with 0.5 N alcoholic potassium hydroxide VS until the free acid is neutralized. Add 25.0 mL of 0.5 N alcoholic potassium hydroxide VS, and proceed as directed in *Saponification Value*, beginning with "Heat the flask" and omitting the further addition of phenolphthalein TS. Calculate the Ester Value:

$$\text{Result} = [M_r \times (V_B - V_T) \times N]/W$$

$M_r$ = molecular weight of potassium hydroxide, 56.11
$V_B$ = volume of 0.5 N hydrochloric acid consumed in the blank test (mL)
$V_T$ = volume of 0.5 N hydrochloric acid consumed in the actual test (mL)
$N$ = exact normality of the hydrochloric acid
$W$ = weight of the substance taken for the test (g)

• **Hydroxyl Value**

The Hydroxyl Value is the number of mg of potassium hydroxide equivalent to the hydroxyl content of 1.0 g of the substance.

**Pyridine–acetic anhydride reagent:** Just before use, mix 3 volumes of freshly opened or freshly distilled pyridine with 1 volume of freshly opened or freshly distilled acetic anhydride.

**Procedure:** Transfer a quantity of the substance, determined by reference to *Table 2* and accurately weighed, to a glass-stoppered, 250-mL conical flask, and add 5.0 mL of *Pyridine–acetic anhydride reagent*. Transfer 5.0 mL of *Pyridine–acetic anhydride reagent* to a second glass-stoppered, 250-mL conical flask to provide the reagent blank. Fit both flasks with suitable glass-jointed reflux condensers, heat on a steam bath for 1 h, add 10 mL of water through each condenser, and heat on the steam bath for 10 min more. Cool, and to each add 25 mL of butyl alcohol, previously neutralized to phenolphthalein TS with 0.5 N alcoholic potassium hydroxide, by pouring 15 mL through each condenser and, after removing the condensers, washing the sides of both flasks with the remaining 10-mL portions. To each flask add 1 mL of phenolphthalein TS, and titrate with 0.5 N alcoholic potassium hydroxide VS, recording the volume, in mL, consumed by the residual acid in the test solution as *T* and that consumed by the blank as *B*. In a 125-mL conical flask, mix about 10 g of the substance, accurately weighed, with 10 mL of freshly distilled pyridine, previously neutralized to phenolphthalein TS, add 1 mL of phenolphthalein TS, and titrate with 0.5 N alcoholic potassium hydroxide VS, recording the volume, in mL, consumed by the free acid in the test specimen as *A*.
Calculate the Hydroxyl Value:

$$\text{Result} = [(M_r \times N)/W] \times \{B + [(W \times A)/C] - T\}$$

$M_r$ = molecular weight of potassium hydroxide, 56.11
$N$ = exact normality of the alcoholic potassium hydroxide
$W$ = weight of the substances taken for the acetylation (g)
$C$ = weight of the substances taken for the free acid determination (g)

If the Acid Value for the test substance is known, calculate the Hydroxyl Value:

$$\text{Result} = [(M_r \times N)/W] \times (B - T) + \text{Acid Value}$$

$M_r$ = molecular weight of potassium hydroxide, 56.11
$N$ = exact normality of the alcoholic potassium hydroxide
$W$ = weight of the substance taken for the acetylation (g)

**Table 2**

| Hydroxyl Value Range | Weight of Test Specimen (g) |
|---|---|
| 0–20 | 10 |
| 20–50 | 5 |
| 50–100 | 3 |
| 100–150 | 2 |
| 150–200 | 1.5 |
| 200–250 | 1.25 |
| 250–300 | 1.0 |
| 300–350 | 0.75 |

• **Iodine Value**

The Iodine Value represents the number of g of iodine absorbed, under the prescribed conditions, by 100 g of the substance. Unless otherwise specified in the individual monograph, determine the Iodine Value by *Method I*.

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**Method I (Hanus method)**

**Procedure:** Transfer an accurately weighed quantity of sample, as determined from *Table 3*, into a 250-mL iodine flask, dissolve it in 10 mL of chloroform, add 25.0 mL of iodobromide TS, insert the stopper in the vessel securely, and allow it to stand for 30 min protected from light, with occasional shaking. Then add, in the order named, 30 mL of potassium iodide TS and 100 mL of water, and titrate the liberated iodine with 0.1 N sodium thiosulfate VS, shaking thoroughly after each addition of thiosulfate. When the iodine color becomes quite pale, add 3 mL of starch TS, and continue the titration with 0.1 N sodium thiosulfate VS until the blue color is discharged. Perform a blank test at the same time with the same quantities of the same reagents and in the same manner (see *Titrimetry* ⟨541⟩, *Residual Titrations*).

Calculate the Iodine Value:

$$\text{Result} = [A_r \times (V_B - V_S) \times N]/(10 \times W)$$

$A_r$ = atomic weight of iodine, 126.90
$V_B$ = volume of 0.1 N sodium thiosulfate VS consumed by the blank test (mL)
$V_S$ = volume of 0.1 N sodium thiosulfate VS consumed by the actual test (mL)
$N$ = exact normality of the sodium thiosulfate VS
$W$ = weight of the substance taken for the test (g)

[NOTE—If more than half of the iodobromide TS is absorbed by the portion of the substance taken, repeat the determination, using a smaller portion of the substance under examination.]

**Table 3. Sample Weights**

| Iodine Value Expected | Weight (g) ±0.1 |
|---|---|
| <5 | 3.0 |
| 5–20 | 1.0 |
| 21–50 | 0.4 |
| 51–100 | 0.2 |
| 101–150 | 0.13 |
| 151–200 | 0.1 |

**Method II**

**Potassium iodide solution:** Dissolve 10.0 g of potassium iodide in water to make 100 mL. Store in light-resistant containers.

**Starch indicator solution:** Mix 1 g of soluble starch with sufficient cold water to make a thin paste. Add, while stirring, to 100 mL of boiling water. Mix, and cool. Use only the clear solution.

**Procedure:** Melt the sample, if it is not already liquid. [NOTE—The temperature during melting should exceed the melting point of the sample by not more than 10°.] Pass through two pieces of filter paper to remove any solid impurities and the last traces of moisture. The filtration may be performed in an air oven at 100° but should be completed within 5 min ± 30 s. The sample must be absolutely dry. All glassware must be absolutely clean and completely dry. After filtration, allow the filtered sample to achieve a temperature of 68°–71 ± 1° before weighing the sample. Once the sample has achieved a temperature of 68°–71 ± 1°, immediately weigh the sample into a 500-mL iodine flask, using the weights and weighing accuracy noted in the accompanying table. [NOTE—The weight of the substance must be such that there will be an excess of iodochloride TS of 50%–60% of the amount added, that is, 100%–150% of the amount absorbed.] Add 15 mL of a fresh mixture of cyclohexane and glacial acetic acid (1:1), and swirl to dissolve the sample. Add 25.0 mL of iodochloride TS, insert the stopper securely in the flask, and swirl to mix. Allow it to stand at 25 ± 5°, protected from light, with occasional shaking, for 1.0 or 2.0 h, depending on the Iodine Value (IV) of the sample: IV < 150, 1.0 h; IV ≥ 150, 2.0 h. Then, within 3 min after the indicated reaction time, add, in the order named, 20 mL of *Potassium iodide solution* and 150 mL of recently boiled and cooled water, and mix. Within 30 min, titrate the liberated iodine with 0.1 N sodium thiosulfate VS, while stirring by mechanical means after each addition of thiosulfate. When the yellow iodine color has almost disappeared, add 1–2 mL of *Starch indicator solution*, and continue the titration with 0.1 N sodium thiosulfate VS until the blue color is discharged. Perform a blank test at the same time with the same quantities of the same reagents and in the same manner (see ⟨541⟩, *Residual Titrations*). Calculate the Iodine Value as indicated in *Method I*.

• **PEROXIDE VALUE**

The Peroxide Value is the number that expresses, in milliequivalents of active oxygen, the quantity of peroxide contained in 1000 g of the substance. [NOTE—This test must be performed promptly after sampling to avoid oxidation of the test specimen.]

**Procedure:** Unless otherwise directed, place about 5 g of the substance, accurately weighed, in a 250-mL conical flask fitted with a ground-glass stopper. Add 30 mL of a mixture of glacial acetic acid and chloroform (3:2), shake to dissolve, and add 0.5 mL of saturated potassium iodide solution. Shake for exactly 1 min, and add 30 mL of water. Titrate with 0.01 N sodium thiosulfate VS, adding the titrant slowly with continuous shaking, until the yellow color is almost discharged. Add 5 mL of starch TS, and continue the titration, shaking vigorously, until the blue color is discharged. Perform a blank determination under the same conditions. [NOTE—The volume of titrant used in the blank determination must not exceed 0.1 mL.]

Calculate the Peroxide Value:

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$$\text{Result} = [1000\ (V_T - V_B) \times N]/W$$

$V_T$ = volume of 0.01 N sodium thiosulfate consumed in the actual test (mL)
$V_B$ = volume of 0.01 N sodium thiosulfate consumed in the blank test (mL)
$N$ = exact normality of the sodium thiosulfate solution
$W$ = weight of the substance taken for the test (g)

- **Saponification Value**

The Saponification Value is the number of mg of potassium hydroxide required to neutralize the free acids and saponify the esters contained in 1.0 g of the substance.

**Procedure:** Place 1.5–2 g of the substance in a tared, 250-mL flask, weigh accurately, and add to it 25.0 mL of 0.5 N alcoholic potassium hydroxide. Heat the flask on a steam bath, under a suitable condenser to maintain reflux for 30 min, frequently rotating the contents. [Note—Reflux time can be up to 90 min to ensure complete saponification, depending on the type of ester to be tested.] Then add 1 mL of phenolphthalein TS, and titrate the excess potassium hydroxide with 0.5 N hydrochloric acid VS. Perform a blank determination under the same conditions (see ⟨541⟩, *Residual Titrations*). The titration also can be carried out potentiometrically.

Calculate the Saponification Value:

$$\text{Result} = [M_r \times (V_B - V_T) \times N]/W$$

$M_r$ = molecular weight of potassium hydroxide, 56.11
$V_B$ = volume of 0.5 N hydrochloric acid consumed in the blank test (mL)
$V_T$ = volume of 0.5 N hydrochloric acid consumed in the actual test (mL)
$N$ = exact normality of the hydrochloric acid
$W$ = weight of the substance taken for the test (g)

If the oil has been saturated with carbon dioxide for the purpose of preservation, expose it in a shallow dish in a vacuum desiccator for 24 h before weighing the test specimens.

- **Unsaponifiable Matter**

The term "unsaponifiable matter" in oils or fats refers to those substances that are not saponifiable by alkali hydroxides but are soluble in the ordinary fat solvents, and to products of saponification that are soluble in such solvents.

**Procedure:** Transfer about 5.0 g of the oil or fat, accurately weighed, to a 250-mL conical flask, add 50 mL of an alcoholic potassium hydroxide solution prepared by dissolving 12 g of potassium hydroxide in 10 mL of water and diluting this solution with alcohol to 100 mL, and heat the flask on a steam bath under a suitable condenser to maintain reflux for 1 h, swirling frequently. Cool to a temperature below 25°, and transfer the contents of the flask to a separator with a polytetrafluoroethylene stopcock, rinsing the flask with two 50-mL portions of water that are added to the separator (do not use grease on stopcock). Extract with three 100-mL portions of ether, combining the ether extracts in another separator containing 40 mL of water. Gently rotate or shake the separator for a few minutes. [Note—Violent agitation may result in the formation of a difficult-to-separate emulsion.] Allow the mixture to separate, and discard the lower aqueous phase. Wash the ether extract with two additional 40-mL portions of water, and discard the lower aqueous phase. Wash the ether extract successively with a 40-mL portion of potassium hydroxide solution (3 in 100) and a 40-mL portion of water. Repeat this potassium hydroxide solution–water wash sequence three times. Wash the ether extract with 40-mL portions of water until the last washing is not reddened by the addition of 2 drops of phenolphthalein TS. Transfer the ether extract to a tared flask, and rinse the separator with 10 mL of ether, adding the rinsings to the flask. Evaporate the ether on a steam bath, and add 6 mL of acetone to the residue. Remove the acetone in a current of air, and dry the residue at 105° until successive weighings differ by not more than 1 mg.

Calculate the percentage of unsaponifiable matter in the portion of oil or fat taken:

$$\text{Result} = 100 \times (W_R/W_S)$$

$W_R$ = weight of the residue (g)
$W_S$ = weight of the oil or fat taken for the test (g)

Dissolve the residue in 20 mL of alcohol, previously neutralized to the phenolphthalein endpoint, add phenolphthalein TS, and titrate with 0.1 N alcoholic sodium hydroxide VS to the first appearance of a faint pink color that persists for not less than 30 s. If the volume of 0.1 N alcoholic sodium hydroxide required is greater than 0.2 mL, the separation of the layers was incomplete; the residue weighed cannot be considered "unsaponifiable matter," and the test must be repeated.

- **Solidification Temperature of Fatty Acids**

**Preparation of the fatty acids:** Heat 75 mL of glycerin–potassium hydroxide solution (made by dissolving 25 g of potassium hydroxide in 100 mL of glycerin) in an 800-mL beaker to 150°, and add 50 mL of the clarified fat, melted if necessary. Heat the mixture for 15 min with frequent stirring, but do not allow the temperature to rise above 150°. Saponification is complete when the mixture is homogeneous and no particles cling to the beaker at the meniscus. Pour the contents of the beaker into 500 mL of nearly boiling water in an 800-mL beaker or casserole, add slowly 50 mL of dilute sulfuric acid [made by adding water and sulfuric acid (3:1)], and heat the solution, with frequent stirring, until the fatty acids separate cleanly as a transparent layer. Wash the acids with boiling water until free from sulfuric acid, collect them in a small beaker, place on a steam bath until the water has settled and the fatty acids are clear, filter into a dry beaker while hot, and dry at 105° for 20 min. Place the warm fatty acids in a suitable container, and cool in an ice bath until they congeal.

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**Test for complete saponification:** Place 3 mL of the dry acids in a test tube, and add 15 mL of alcohol. Heat the solution to boiling, and add an equal volume of 6 N ammonium hydroxide. A clear solution results.

**Procedure:** Using an apparatus similar to the *Congealing Temperature Apparatus* specified therein, proceed as directed in *Congealing Temperature* ⟨651⟩*, Procedure*, reading "solidification temperature" for "congealing point" (the terms are synonymous). The average of not less than four consecutive readings of the highest point to which the temperature rises is the solidification temperature of the fatty acids.

• **FATTY ACID COMPOSITION**

**Standard solution:** Prepare an ester mixture of known composition containing the esters required in the individual monograph. This *Standard solution* may contain other components. [NOTE—Ester mixtures are available commercially from Nu-Chek-Prep, Inc., PO Box 295, Elysian, MN 56028. Typical Nu-Chek-Prep ester mixtures useful in this test include Nu-Chek 17A and Nu-Chek 19A.] Nu-Chek mixture 17A has the following composition:

**Table 4**

| Percentage | Fatty Acid Ester | Carbon-Chain Length | Number of Double Bonds |
|---|---|---|---|
| 1.0 | Methyl myristate | 14 | 0 |
| 4.0 | Methyl palmitate | 16 | 0 |
| 3.0 | Methyl stearate | 18 | 0 |
| 3.0 | Methyl arachidate | 20 | 0 |
| 3.0 | Methyl behenate | 22 | 0 |
| 3.0 | Methyl lignocerate | 24 | 0 |
| 45.0 | Methyl oleate | 18 | 1 |
| 15.0 | Methyl linoleate | 18 | 2 |
| 3.0 | Methyl linolenate | 18 | 3 |
| 20.0 | Methyl erucate | 22 | 1 |

Nu-Chek mixture 19A has the following composition:

**Table 5**

| Percentage | Fatty Acid Ester | Carbon-Chain Length | Number of Double Bonds |
|---|---|---|---|
| 7.0 | Methyl caprylate | 8 | 0 |
| 5.0 | Methyl caprate | 10 | 0 |
| 48.0 | Methyl laurate | 12 | 0 |
| 15.0 | Methyl myristate | 14 | 0 |
| 7.0 | Methyl palmitate | 16 | 0 |
| 3.0 | Methyl stearate | 18 | 0 |
| 12.0 | Methyl oleate | 18 | 1 |
| 3.0 | Methyl linoleate | 18 | 2 |

**0.5 N Methanolic sodium hydroxide solution:** Dissolve 2 g of sodium hydroxide in 100 mL of methanol.

**Test solution:** [NOTE—If fatty acids containing more than 2 double bonds are present in the test specimen, remove air from the flask by purging it with nitrogen for a few minutes.] Transfer about 100 mg of the test specimen to a 50-mL conical flask fitted with a suitable water-cooled reflux condenser and a magnetic stir bar. Add 4 mL of *0.5 N Methanolic sodium hydroxide solution,* and reflux until fat globules disappear (usually 5–10 min). Add 5 mL of a solution prepared by dissolving 14 g of boron trifluoride in methanol to make 100 mL, swirl to mix, and reflux for 2 min. Add 4 mL of chromatographic *n*-heptane through the condenser, and reflux for 1 min. Cool, remove the condenser, add about 15 mL of saturated sodium chloride solution, shake, and allow the layers to separate. Pass the *n*-heptane layer through 0.1 g of anhydrous sodium sulfate (previously washed with chromatographic *n*-heptane) into a suitable flask. Transfer 1.0 mL of this solution to a 10-mL volumetric flask, dilute with chromatographic *n*-heptane to volume, and mix.

**System suitability solution:** Transfer about 20 mg each of stearic acid, palmitic acid, and oleic acid to a 25-mL conical flask fitted with a suitable water-cooled reflux condenser and a magnetic stir bar, and prepare as directed for *Test solution* beginning with "Add 5.0 mL of a solution prepared by dissolving".

**Chromatographic system**

(See *Chromatography* ⟨621⟩.)

The gas chromatograph is equipped with a flame-ionization detector, maintained at a temperature of about 260°, a splitless injection system, and a 0.53-mm × 30-m fused-silica capillary column bonded with a 1.0-µm layer of phase G16. The chromatograph is programmed to maintain the column temperature at 70° for about 2 min after injection, then to increase the temperature at the rate of 5°/min to 240°, and finally to maintain this temperature for 5 min. The

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injection port temperature is maintained at about 220°. The carrier gas is helium with a linear velocity of about 50 cm/s.

Chromatograph the *System suitability solution*, and record the peak responses as directed for *Procedure:* the relative retention times are about 0.87 for methyl palmitate, 0.99 for methyl stearate, and 1.0 for methyl oleate; the resolution, *R,* between methyl stearate and methyl oleate is not less than 1.5; and the relative standard deviation of the peak area responses for the palmitate and stearate peaks for replicate injections is not more than 6.0%. The relative standard deviation of the peak area response ratio of the palmitate to stearate peaks from these replicate injections is not more than 1.0%.

**Procedure:** Separately inject equal volumes (about 1 µL) of the *Standard solution* and the *Test solution* into the chromatograph, record the chromatograms, identify the fatty acid ester peaks in the chromatogram of the *Test solution* by comparing the retention times of these peaks with those in the chromatogram of the *Standard solution*, and measure the peak areas for all of the fatty acid ester peaks in the chromatogram from the *Test solution*.

Calculate the percentage of each fatty acid component in the test specimen:

$$\text{Result} = 100 \times (A/B)$$

*A* = area of the peak response obtained for each individual fatty acid ester component
*B* = sum of the peak areas of all of the peaks, excluding the solvent peak, in the chromatogram from the *Test solution*

***Change to read:***

• **Omega-3 Fatty Acids Determination and Profile**

The following procedure may be used for the determination of eicosapentaenoic acid (EPA) (C20:5 n–3), docosahexaenoic acid (DHA) (C22:6 n–3), and total omega-3 acids obtained from fish, plant, or microbial sources in bulk oils and encapsulated oil, either as triglycerides or as ethyl esters. The term "triglyceride" is applicable to algal oils, fish oils, fish liver oils, and products containing omega-3-acids in triglycerides form. The results are expressed as free fatty acids or ethyl esters. Perform the tests as rapidly as possible. Protect the solutions from actinic light, oxidizing agents, oxidation catalysts, and air.

**Content of EPA and DHA**

**Antioxidant solution:** Dissolve an accurately weighed quantity of butylated hydroxytoluene in 2,2,4-trimethylpentane to obtain a solution with a concentration of 0.05 mg per mL.

**Internal standard solution:** Transfer an accurately weighed quantity of USP Methyl Tricosanoate RS to a volumetric flask. Dissolve in *Antioxidant solution*, and dilute with the same solvent to obtain a solution with a concentration of about 7.0 mg/mL. [Note—Guard the solution against evaporation during use.]

**Test solution 1** (for triglycerides): ▲ (USP 1-May-2022)Dissolve the mass of sample to be examined, according to *Table 6*, in ▲10.0 mL of▲ (USP 1-May-2022) *Antioxidant solution.*▲ (USP 1-May-2022)Transfer 2.0 mL of this solution to a glass tube, and evaporate the solvent with a gentle stream of nitrogen. Add 1.5 mL of a 2% (w/v) solution of sodium hydroxide in methanol, cap tightly with a polytetrafluoroethylene-lined cap, mix, and heat in a boiling water bath for 7 min. Cool, add 2 mL of boron trichloride–methanol solution (120 g in 1000 mL of methanol), cover with nitrogen, cap tightly, mix, and heat in a boiling water bath for 30 min. Cool to 40°–50°, add 1.0 mL of 2,2,4-trimethylpentane, cap, and mix on a vortex mixer or shake vigorously for at least 30 s. Immediately add 5 mL of saturated sodium chloride solution containing 1 volume of sodium chloride and 2 volumes of water. [Note—Shake from time to time. Before use, decant the solution from any undissolved substance, and filter if necessary.] Cover with nitrogen, cap, and mix on a vortex mixer or shake thoroughly for at least 15 s. Allow the upper layer to become clear, and transfer to a separate tube. Shake the methanol layer once more with 1.0 mL of 2,2,4-trimethylpentane, and combine the 2,2,4-trimethylpentane extracts. Wash the combined extracts with two quantities, 1 mL each, of water, and dry over anhydrous sodium sulfate.

**Table 6**

| Approx. Sum EPA + DHA | Amount of Sample to Be Weighed (g) |
|---|---|
| 30%–50% | 0.4–0.5 |
| 50%–70% | 0.3 |
| >70% | 0.25 |

**Test solution 2** (for triglycerides): ▲Dissolve▲ (USP 1-May-2022) the equivalent amount of sample used to prepare *Test solution 1* ▲in 10.0 mL of *Internal standard solution.*▲ (USP 1-May-2022)Gentle heating (up to 60°) may be applied to obtain a clear solution. Then prepare as directed in *Test solution 1* starting with "Transfer 2.0 mL".

**Test solution 3** (for ethyl esters): ▲ (USP 1-May-2022)Dissolve the mass of sample to be examined, according to *Table 6*, ▲in 10.0 mL of▲ (USP 1-May-2022) the *Internal standard solution.*▲ (USP 1-May-2022) Gentle heating (up to 60°) may be applied to obtain a clear solution.

**Test solution 4** (for ethyl esters): ▲Dissolve▲ (USP 1-May-2022) the equivalent amount of sample used to prepare *Test solution 3* ▲in 10.0 mL of▲ (USP 1-May-2022) *Antioxidant solution.*▲ (USP 1-May-2022)

**Standard solution 1a:** ▲Dissolve▲ (USP 1-May-2022) 60 mg of USP Docosahexaenoic Acid Ethyl Ester RS, accurately weighed, ▲in 10.0 mL of *Internal standard solution.*▲ (USP 1-May-2022) Gentle heating (up to 60°) may be applied to obtain a clear solution.

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**Standard solution 1b:** ▲Dissolve▲ (USP 1-May-2022) 90 mg of USP Eicosapentaenoic Acid Ethyl Ester RS, accurately weighed, ▲in 10.0 mL of *Internal standard solution*.▲ (USP 1-May-2022) Gentle heating (up to 60°) may be applied to obtain a clear solution.

*Standard solution 1a* and *Standard solution 1b* are ready for ethyl esters analysis. For triglycerides analysis, continue with *Standard solution 2a* and *Standard solution 2b*.

**Standard solution 2a:** Transfer 2.0 mL of *Standard solution 1 a* to a glass tube, and evaporate the solvent with a gentle stream of nitrogen. Then prepare as directed for *Test solution 1* starting with, "Add 1.5 mL".

**Standard solution 2b:** Transfer 2.0 mL of *Standard solution 1b* to a glass tube, and evaporate the solvent with a gentle stream of nitrogen. Then prepare as directed for *Test solution 1* starting with, "Add 1.5 mL".

**System suitability solution 1:** Transfer 0.300 g of methyl palmitate, 0.300 g of methyl stearate, 0.300 g of methyl arachidate, and 0.300 g of methyl behenate, accurately weighed, to a 10-mL volumetric flask, and dissolve in and dilute with *Antioxidant solution* to volume.

**System suitability solution 2:** [NOTE—This solution must be prepared only for triglycerides and only if tetracos-15-enoic acid methyl ester is not clearly observed in the chromatogram obtained with *Test solution 2*.] Transfer 55.0 mg of docosahexaenoic acid methyl ester and about 5.0 mg of tetracos-15-enoic acid (nervonic acid) methyl ester, accurately weighed, to a 10-mL volumetric flask, and dissolve in and dilute with *Antioxidant solution* to volume.

**Chromatographic system**

(See *Chromatography* ⟨621⟩, *System Suitability*.)

**Mode:** GC

**Detector:** Flame ionization

**Column:** 0.25-mm × 25-m fused–silica capillary column bonded with a 0.20-µm film of phase G16

**Temperatures**

**Injection port**

**Split injection:** 250°

**Splitless injection:** 90°–250°

**Detector:** 270°

**Column:** See *Table 7a* or *Table 7b*.

**Table 7a (Split Injection)**

| Initial Temperature (°) | Hold Time at 170° (min) | Temperature Ramp (°/min) | Final Temperature (°) | Hold Time at Final Temperature (min) |
|---|---|---|---|---|
| 170 | 2 | 3 | 240 | 2.5 |

**Table 7b (Splitless Injection)**

| Initial Temperature (°) | Hold Time at 90° (min) | Temperature Ramp Number 1 (°/min) | To Temperature (°) | Temperature Ramp Number 2 (°/min) | To Final Temperature (°) | Hold Time at Final Temperature (min) |
|---|---|---|---|---|---|---|
| 90 | 2 | 30 | 170 | 3 | 240 | 2 |

**Carrier gas:** Helium

**Flow rate:** 1 mL/min

**Split flow ratio:** 200:1. [NOTE—If necessary, adjust the split ratio and/or sample dilution to obtain a tailing factor of 0.8–1.5 for the fatty acid methyl ester peaks in *System suitability solution 1*, the methyl or ethyl ester peaks of eicosapentaenoic acid and docosahexaenoic acid in *Test solution 1* and *Test solution 4*, at the same time observing that for *Test solution 1* or *Test solution 4* any peaks due to the corresponding esters of C18:3 n-3, C18:4 n-3, C20:4 n-3, C21:5 n-3, and C22:5 n-3 are clearly detectable. If splitless injection mode is used, solutions should be further diluted 1 in 200 with *Antioxidant solution*.]

**Injection volume:** 1 µL

**System suitability** (for triglycerides)

**Samples:** *System suitability solution 1* and *Test solution 2* or *System suitability solution 2* (if applicable)

**System suitability** (for ethyl esters)

**Sample:** *System suitability solution 1*

**System suitability requirements**

**Theoretical area percentages** (System suitability solution 1)**:** The area percentages, after adjusting for the actual weights, of the peaks of methyl palmitate, methyl stearate, methyl arachidate, and methyl behenate, each should be within ±1.0% (absolute) of theoretical values provided in *Table 8*.

**Table 8**

| Fatty Acid Methyl Ester | Theoretical Area (%) in a Solution of Equal Weights of Methyl Palmitate, Methyl Stearate, Methyl Arachidate, and Methyl Behenate |
|---|---|
| Methyl palmitate | 24.37 |
| Methyl stearate | 24.84 |
| Methyl arachidate | 25.23 |

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**Table 8** *(continued)*

| Fatty Acid Methyl Ester | Theoretical Area (%) in a Solution of Equal Weights of Methyl Palmitate, Methyl Stearate, Methyl Arachidate, and Methyl Behenate |
|---|---|
| Methyl behenate | 25.56 |

[NOTE—The theoretical area (%) in a solution of equal weights (*Table 8*) is derived from the calculated theoretical response factors, which are 1.049, 1.029, 1.013, and 1.000 for methyl palmitate, methyl stearate, methyl arachidate, and methyl behenate, respectively.]

**Resolution:** For triglycerides (*Test solution 2* or *System suitability solution 2*): Not less than 1.2 between the docosahexaenoic acid methyl ester and tetracos-15-enoic acid methyl ester peaks

**Analysis** (for triglycerides)

**Samples:** *Standard solution 2a, Standard solution 2b, Test solution 1*, and *Test solution 2*

Inject each sample in duplicate and measure the peak ▲areas.▲ (USP 1-May-2022) Compare the chromatographic profile of *Test solution 2* with that of *Test solution 1* and identify the internal standard peak present in *Test solution 2*. Calculate the percentage of EPA or DHA in the triglycerides sample taken:

$$\text{Result} = (R_U/R_S) \times (W_S/W_U) \times F \times 100$$

$R_U$ = ratio of peak ▲area▲ (USP 1-May-2022) of either EPA or DHA to the corrected peak ▲area▲ (USP 1-May-2022) of the internal standard from *Test solution 2*, calculated:

[NOTE—If $r_{U1} = 0$ due to no peak observed at the locus of the internal standard from *Test solution 1*, then $R_U = r_{T2}/r_{U2}$.]

$$\text{Result} = 1/[(r_{U2}/r_{T2}) - (r_{U1}/r_{T1})]$$

$r_{U2}$ = peak ▲area▲ (USP 1-May-2022) of the internal standard from *Test solution 2*
$r_{T2}$ = peak ▲area▲ (USP 1-May-2022) of EPA or DHA from *Test solution 2*
$r_{U1}$ = peak ▲area▲ (USP 1-May-2022) of any peak at the locus of the internal standard from *Test solution 1*
$r_{T1}$ = peak ▲area▲ (USP 1-May-2022) of EPA or DHA from *Test solution 1*
$R_S$ = ratio of peak ▲area▲ (USP 1-May-2022) of either DHA or EPA to the peak ▲area▲ (USP 1-May-2022) of the internal standard from *Standard solution 2a* or *Standard solution 2b*
$W_S$ = weight of DHA taken for preparing *Standard solution 1a* or weight of EPA taken for preparing *Standard solution 1b* (mg)
$W_U$ = weight of sample taken for preparing *Test solution 2* (mg)
$F$ = factor to express content of DHA as free fatty acids, 0.921; or factor to express content of EPA as free fatty acids, 0.915

**Analysis** (for ethyl esters)

**Samples:** *Standard solution 1a, Standard solution 1b, Test solution 3*, and *Test solution 4*

Inject each sample in duplicate and measure the peak ▲areas.▲ (USP 1-May-2022) Compare the chromatographic profile of *Test solution 3* with that of *Test solution 4* and identify the internal standard peak present in *Test solution 3*. Calculate the percentage of EPA or DHA in the ethyl esters sample taken:

$$\text{Result} = (R_U/R_S) \times (W_S/W_U) \times 100$$

$R_U$ = ratio of peak ▲area▲ (USP 1-May-2022) of either EPA or DHA to the corrected peak ▲area▲ (USP 1-May-2022) of the internal standard from *Test solution 3*, calculated as follows:

$$\text{Result} = 1/[(r_{U3}/r_{T3}) - (r_{U4}/r_{T4})]$$

[NOTE—If $r_{U4} = 0$ due to no peak observed at the locus of the internal standard from *Test solution 4*, then $R_U = r_{T3}/r_{U3}$.]

$r_{U3}$ = peak ▲area▲ (USP 1-May-2022) of the internal standard from *Test solution 3*
$r_{T3}$ = peak ▲area▲ (USP 1-May-2022) of EPA or DHA from *Test solution 3*
$r_{U4}$ = peak ▲area▲ (USP 1-May-2022) of any peak at the locus of the internal standard from *Test solution 4*
$r_{T4}$ = peak ▲area▲ (USP 1-May-2022) of EPA or DHA from *Test solution 4*
$R_S$ = ratio of peak ▲area▲ (USP 1-May-2022) of either DHA or EPA to the peak ▲area▲ (USP 1-May-2022) of the internal standard from *Standard solution 1a* or *Standard solution 1b*
$W_S$ = weight of DHA taken for preparing *Standard solution 1a* or weight of EPA taken for preparing *Standard solution 1b* (mg)
$W_U$ = weight of sample taken for preparing *Test solution 3* (mg)

**Content of total omega-3 acids** (for triglycerides)

Calculate the percentage of the total omega-3 acids in the portion of triglycerides sample taken:

$$\text{Result} = \text{EPA} + \text{DHA} + [(A_{n\text{-}3} \times (\text{EPA} + \text{DHA})/(A_{EPA} + A_{DHA})]$$

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EPA = content of EPA, from the test for *Content of EPA and DHA* (%)
DHA = content of DHA, from the test for *Content of EPA and DHA* (%)
$A_{n\text{-}3}$ = sum of the areas of the peaks corresponding to C18:3 n-3, C18:4 n-3, C20:4 n-3, C21:5 n-3, and C22:5 n-3 methyl esters from *Test solution 1* ▲or *Test solution 2*▲ (USP 1-May-2022)
$A_{EPA}$ = area of the peak corresponding to the EPA methyl ester from *Test solution 1* ▲or *Test solution 2*▲ (USP 1-May-2022)
$A_{DHA}$ = area of the peak corresponding to the DHA methyl ester from *Test solution 1* ▲or *Test solution 2*▲ (USP 1-May-2022)

**Content of total omega-3 acids** (for ethyl esters)
Calculate the percentage of the total omega-3 acids in the portion of ethyl esters sample taken:

$$\text{Result} = \text{EPA} + \text{DHA} + [(A_{n\text{-}3} \times (\text{EPA} + \text{DHA})/(A_{EPA} + A_{DHA})]$$

EPA = content of EPA, from the test for *Content of EPA and DHA* (%)
DHA = content of DHA, from the test for *Content of EPA and DHA* (%)
$A_{n\text{-}3}$ = sum of the areas of the peaks corresponding to C18:3 n-3, C18:4 n-3, C20:4 n-3, C21:5 n-3, and C22:5 n-3 ethyl esters from ▲*Test solution 3* or▲ (USP 1-May-2022) *Test solution 4*
$A_{EPA}$ = area of the peak corresponding to the EPA ethyl ester from ▲*Test solution 3* or▲ (USP 1-May-2022) *Test solution 4*
$A_{DHA}$ = area of the peak corresponding to the DHA ethyl ester from ▲*Test solution 3* or▲ (USP 1-May-2022) *Test solution 4*

• **Water and Sediment in Fixed Oils**
**Apparatus:** The preferred centrifuge has a diameter of swing (*d* = distance from tip to tip of whirling tubes) of 38–43 cm and is operated at a speed of about 1500 rpm. If a centrifuge of different dimensions is used, calculate the desired rate of revolution:

$$\text{rpm} = 1500\sqrt{40.6ld}$$

The centrifuge tubes are pear-shaped and are shaped to accept closures. The total capacity of each tube is about 125 mL. The graduations are clear and distinct, reading upward from the bottom of the tube according to the scale shown in *Table 9*.

**Table 9**

| Volume (mL) | Scale Division (mL) |
|---|---|
| 0–3 | 0.1 |
| 3–5 | 0.5 |
| 5–10 | 1.0 |
| 10–25 | 5.0 |
| 25–50 | 25.0 |
| 50–100 | 50.0 |

**Procedure:** Place 50.0 mL of benzene in each of two centrifuge tubes, and to each tube add 50.0 mL of the oil, warmed if necessary to re-incorporate separated stearin, and mixed thoroughly at 25°. Insert the stopper tightly into the tubes, shake them vigorously until the contents are mixed thoroughly, and then immerse the tubes in a water bath at 50° for 10 min. Centrifuge for 10 min. Read the combined volume of water and sediment at the bottom of each tube. Centrifuge repeatedly for 10-min periods until the combined volume of water and sediment remains constant for three consecutive readings. The sum of the volumes of combined water and sediment in the two tubes represents the percentage, by volume, of water and sediment in the oil.

• **Anisidine Value**
The Anisidine Value is defined as 100 times the optical density measured in a 1-cm cell of a solution containing 1 g of the substance to be examined in 100 mL of a mixture of solvents and reagents according to the following method described. [Note—Carry out the operations as rapidly as possible, avoiding exposure to actinic light.]
**Test solution A:** Dissolve 0.500 g of the substance to be examined in isooctane, and dilute with the same solvent to 25.0 mL.
**Test solution B:** To 5.0 mL of *Test solution A* add 1.0 mL of a 2.5-g/L solution of *p*-anisidine in glacial acetic acid, shake, and store protected from light.
**Standard solution:** To 5.0 mL of isooctane add 1.0 mL of a 2.5-g/L solution of *p*-anisidine in glacial acetic acid, shake, and store protected from light.
**Procedure:** Measure the absorbance of *Test solution A* at 350 nm using isooctane as the blank. Measure the absorbance of *Test solution B* at 350 nm exactly 10 min after its preparation, using the *Standard solution* as the compensation liquid. Calculate the Anisidine Value:

$$\text{Result} = [25 \times (1.2A_S - A_B)]/m$$

$A_S$ = absorbance of *Test solution B* at 350 nm
$A_B$ = absorbance of *Test solution A* at 350 nm
$m$ = weight of the substance to be examined in *Test solution A* (g)

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• **TOTAL OXIDATION VALUE (TOTOX)**
Total Oxidation Value is defined:

$$\text{Result} = 2PV + AV$$

*PV* = Peroxide Value
*AV* = Anisidine Value

• **TRACE METALS**
**Apparatus**
The apparatus typically consists of the following:
**Digestion flasks:** Use a polytetrafluoroethylene flask with a volume of about 120 mL, fitted with an airtight closure, a valve to adjust the pressure inside the container, and a polytetrafluoroethylene tube to allow the release of gas.
**System:** Make the flask airtight, using the same torsional force for each of them.
**Microwave oven:** It has a magnetron frequency of 2450 MHz with a selectable output from 0 to 630 ± 70 W in 1% increments, a programmable digital computer, a polytetrafluoroethylene-coated microwave cavity with a variable speed exhaust fan, a rotating turntable drive system, and exhaust tubing to vent fumes.
**Atomic absorption spectrometer:** It is equipped with a hollow-cathode lamp as the source of radiation and a deuterium lamp as a background corrector. The system is fitted with the following:
1. A graphite furnace as the atomization device for cadmium, copper, iron, lead, nickel, and zinc.
2. An automated continuous-flow hydride vapor generation system for arsenic and mercury.

**General procedure**
[**CAUTION**—When using closed high-pressure digestion vessels and microwave laboratory equipment, the safety precautions and operating instructions given by the manufacturer must be followed.]
[NOTE—If an alternative apparatus is used, adjustment of the instrument parameters may be necessary.]
**Cleaning:** Clean all the glassware and laboratory equipment with a 10-mg/mL solution of nitric acid before use.
**Trace metal–free nitric acid:** Nitric acid meets the requirements with the maximum values for arsenic (As), cadmium (Cd), copper (Cu), iron (Fe), mercury (Hg), lead (Pb), nickel (Ni), and zinc (Zn) equal to 0.005, 0.005, 0.001, 0.02, 0.002, 0.001, 0.005, and 0.01 ppm, respectively.
**Trace metal–free hydrochloric acid:** Hydrochloric acid meets the requirements with the maximum values for arsenic (As), cadmium (Cd), copper (Cu), iron (Fe), mercury (Hg), lead (Pb), nickel (Ni), and zinc (Zn) equal to 0.005, 0.003, 0.003, 0.05, 0.005, 0.001, 0.004, and 0.005 ppm, respectively.
**Trace metal–free sulfuric acid:** Sulfuric acid meets the requirements with the maximum values for arsenic (As), cadmium (Cd), copper (Cu), iron (Fe), mercury (Hg), lead (Pb), nickel (Ni), and zinc (Zn) equal to 0.005, 0.002, 0.001, 0.05, 0.005, 0.001, 0.002, and 0.005 ppm, respectively.
**Test stock solution:** In a digestion flask place about 0.5 g of fatty oil, accurately weighed, as indicated in each individual monograph. Add 6 mL of *Trace metal–free nitric acid* and 4 mL of *Trace metal–free hydrochloric acid*. Close the flask.
**Blank stock solution:** Mix 6 mL of *Trace metal–free nitric acid* and 4 mL of *Trace metal–free hydrochloric acid* in a digestion flask.
**Test solution 1:** Place the digestion flask containing the *Test stock solution* in the microwave oven. Carry out the digestion in three steps according to the following program: 80% power for 15 min, 100% power for 5 min, and 80% power for 20 min.
At the end of the cycle allow the flask to cool. Add 4 mL of *Trace metal–free sulfuric acid* to the flask. Repeat the digestion program. After completing the digestion, allow the flask to cool to room temperature. Open the digestion flask, and transfer the clear, colorless solution obtained into a 50-mL volumetric flask. Rinse the digestion flask with 2 quantities, 15 mL each, of water, and collect the rinsings in the volumetric flask. Add 1.0 mL of a 10-mg/mL solution of magnesium nitrate and 1.0 mL of a 100-mg/mL solution of ammonium dihydrogen phosphate to the volumetric flask. Dilute with water to volume, and mix. This solution is *Test solution 1*.
**Blank solution 1:** Place the digestion flask containing *Blank stock solution* in the microwave oven. Prepare as directed in *Test solution 1* beginning with "Carry out the digestion in three steps according to the following program".
**Direct calibration:** [NOTE—Concentrations of the standard solutions will depend on the metal contents of the test substance.] For routine measurements, 3 standard solutions, *Blank solution 1*, and *Test solution 1* are prepared and examined. Use *Test solution 1* and *Blank solution 1* as prepared previously or as indicated in the monograph. Prepare not less than 3 standard solutions containing all the metal elements to be tested. The expected absorbance value in *Test solution 1* for each metal element should be within its corresponding calibrated absorbance range, preferably in the middle of the calibrated absorbance range. Any reagents used in the preparation of *Test solution 1* are added at the same concentration to the standard solutions.
Introduce each of the solutions into the instrument using the same number of replicates for each of the solutions to obtain a steady reading.
Prepare a calibration curve from the mean of the readings obtained with the standard solutions by plotting the means as a function of concentration. Determine the concentration of the element in *Test solution 1* from the curve obtained.
**Standard additions:** Add to at least four identical volumetric flasks equal volumes of *Test solution 1* as prepared previously or as indicated in the monograph. Add to all but one of the flasks progressively larger volumes of a standard solution containing a known concentration of the test element to produce a series of solutions containing steadily increasing concentrations of that element known to give responses in the linear part of the curve. Dilute the contents of each flask with the solvent specified in the monograph to volume, and mix. The flask without an addition of standard solution is labeled as the test solution.
Introduce each of the solutions into the instrument, using the same number of replicates for each of the solutions, to obtain a steady reading.

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Plot the absorbances of the standard solutions and the test solution versus the added quantity of test element. [NOTE—The test solution should be plotted as if it had a content of added test element equivalent to 0 mg or µg.] Extrapolate the line joining the points on the graph until it meets the concentration axis. The distance between this point and the intersection of the axes represents the concentration of test element in the test solution.

**Specific Tests**

**Cadmium (Cd), copper (Cu), iron (Fe), lead (Pb), nickel (Ni), and zinc (Zn)**

**Standard stock solution:** Prepare a solution containing known concentrations of 5 µg/mL for each test element.

**Standard solutions:** In three identical 10-mL volumetric flasks introduce 10, 20, and 40 µL of *Standard stock solution*, respectively. To each flask add 5.0 mL of *Test solution 1*, dilute with water to volume, and mix.

**Test solution 2:** In a 10-mL volumetric flask add 5.0 mL of *Test solution 1*, dilute with water to volume, and mix.

**Blank solution 2:** In a 10-mL volumetric flask add 5.0 mL of *Blank solution 1*, dilute with water to volume, and mix.

**Procedure:** Measure the content of Cd, Cu, Fe, Pb, Ni, and Zn using a suitable graphite furnace atomic absorption spectrophotometer. Concomitantly determine the absorbances of *Blank solution 2,* the *Standard solutions,* and *Test solution 2* at least three times each. The absorbance value of *Blank solution 2* is subtracted from the value obtained using the *Standard solutions* and *Test solution 2*. Proceed as directed in the *Standard additions* method in *General Procedure*. See *Table 10* for instrumental parameters that may be used.

**Table 10**

| | Cd | Cu | Fe | Pb | Ni | Zn |
|---|---|---|---|---|---|---|
| Wavelength (nm) | 228.8 | 324.8 | 248.3 | 283.5 | 232 | 213.9 |
| Slit (nm) | 0.5 | 0.5 | 0.2 | 0.5 | 0.2 | 0.5 |
| Lamp current (mA) | 6 | 7 | 5 | 5 | 10 | 7 |
| Ignition temperature (°) | 800 | 800 | 800 | 800 | 800 | 800 |
| Atomization temperature (°) | 1800 | 2300 | 2300 | 2200 | 2500 | 2000 |
| Background corrector | On | Off | Off | Off | Off | Off |
| Nitrogen flow (L/min) | 3 | 3 | 3 | 3 | 3 | 3 |

**Arsenic and mercury**

Measure the content of arsenic and mercury against their standard solutions at a known concentration using the *Direct calibration* method in *General Procedure*, with an automated continuous-flow hydride vapor generation system.

For the 1 ppm arsenic specification limit and 1 ppm mercury specification limit, prepare three working calibration solutions with known concentrations of 5, 10, and 20 ng/mL for each test element, respectively.

The absorbance value of the blank solution is automatically subtracted from the value obtained using the test solution.

**Arsenic**

**Blank solution 3:** Add 1.0 mL of a 200-mg/mL solution of potassium iodide to 19.0 mL of *Blank solution 1* prepared previously. Allow this solution to stand at room temperature for about 50 min or at 70° for about 4 min.

**Test solution 3:** Add 1.0 mL of a 200-mg/mL solution of potassium iodide to 19.0 mL of *Test solution 1* prepared previously. Allow this solution to stand at room temperature for about 50 min or at 70° for about 4 min.

**Acid reagent 1:** *Trace metal-free hydrochloric acid*

**Reducing reagent 1:** A 6-mg/mL solution of sodium tetrahydroborate in a 5-mg/mL solution of sodium hydroxide

The instrumental parameters in *Table 11* may be used.

**Mercury**

**Blank solution 4:** Prepare as directed in *Blank solution 3*.

**Test solution 4:** Prepare as directed in *Test solution 3*.

**Acid reagent 2:** A 515-mg/mL solution of *Trace metal–free hydrochloric acid*

**Reducing reagent 2:** A 10-mg/mL solution of stannous chloride in a 200-mg/mL solution of *Trace metal–free hydrochloric acid*

The instrumental parameters in *Table 11* may be used.

**Table 11**

| | As | Hg |
|---|---|---|
| Wavelength (nm) | 193.7 | 253.7 |
| Slit width (nm) | 0.2 | 0.5 |
| Lamp current (mA) | 10 | 4 |
| Acid reagent flow rate (mL/min) | 1.0 | 1.0 |
| Reducing reagent flow rate (mL/min) | 1.0 | 1.0 |
| Flow rate for the blank, standard, test solutions (mL/min) | 7.0 | 7.0 |
| Absorption cell | Quartz (heated) | Quartz (unheated) |
| Background corrector | Off | Off |

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**Table 11** *(continued)*

| | As | Hg |
|---|---|---|
| Nitrogen flow rate (L/min) | 0.1 | 0.1 |

- **STEROL COMPOSITION**

**Separation of the sterol fraction**

**Reference solution A:** Dissolve an accurately weighed quantity of cholesterol in chloroform to obtain a solution of 5% (w/v).

**Developing solvent system:** A mixture of toluene and acetone (95:5) or a mixture of hexane and ether (65:35)

**Test solution A:** Accurately weigh 5 g of the test substance into a 250-mL flask. Add 50 mL of alcoholic potassium hydroxide TS 2 (2 N alcoholic potassium hydroxide), and heat to gentle boiling with continuous vigorous stirring until saponification takes place (the solution becomes clear). Continue heating for a further 20 min, and add 50 mL of water from the top of the condenser. Cool the flask to approximately 30°. Transfer the contents of the flask to a 500-mL separating funnel with several rinses of water, amounting in all to about 50 mL. Add approximately 80 mL of ether, shake vigorously for approximately 30 s, and allow to settle. [NOTE—Any emulsion can be destroyed by adding small quantities of ethyl or methyl alcohol by means of a spray.] Separate the lower aqueous phase, and collect it into a second separating funnel. Perform two further extractions on the water–alcohol phase in the same way, using 60–70 mL of ether on each occasion. Pool the ether extracts into a single separating funnel, and wash with water, 50 mL at a time, until the wash water is no longer alkaline to phenolphthalein. Dry the ether phase with anhydrous sodium sulfate, and filter on anhydrous sodium sulfate into a previously weighed 250-mL flask, washing the funnel and filter with small quantities of ether. Distill the ether down to a few mL, and bring to dryness under a slight vacuum or in a stream of nitrogen. Complete the drying at 100° for approximately 15 min, and then weigh after cooling in a desiccator. Dissolve the unsaponifiables so obtained in chloroform to obtain a solution with a concentration of approximately 5%.

**Test solution B:** Treat 5 g of canola oil in the same way as prescribed for the test substance in *Test solution A*, beginning with "Add 50 mL of alcoholic potassium hydroxide TS 2 (2 N alcoholic potassium hydroxide)".

**Test solution C:** Treat 5 g of sunflower oil in the same way as prescribed for the test substance in *Test solution A*, beginning with "Add 50 mL of alcoholic potassium hydroxide TS 2 (2 N alcoholic potassium hydroxide)".

**Procedure:** Immerse the thin-layer chromatographic plate (see ⟨621⟩), 20-cm × 20-cm silica gel on polyester with a layer thickness of 200 µm and particle size of 5–17 µm,[1] completely in the 0.2 N alcoholic potassium hydroxide for 10 s, allow to dry in a fume cupboard for 2 h, and finally place at 100° for 1 h. [NOTE—Remove from the validated heating device, and keep the plate in a desiccator until required for use. The plates must be used within 15 days. Thin-layer chromatographic plates that do not require the preconditioning also are commercially available.] Use a separate plate for each test solution.

Place a mixture of toluene and acetone (95:5) or a mixture of hexane and ether (65:35) in the chamber to a depth of approximately 1 cm. Close the chamber with the appropriate cover, and leave for at least 30 min. Strips of filter paper dipping into the eluant may be placed on the internal surfaces of the chamber. [NOTE—The developing mixture should be replaced for every test to ensure reproducible elution conditions.] Apply 0.3 mL of *Test solution A* approximately 2 cm from the lower edge in a streak that is as thin and as uniform as possible. In line with the streak, place 2–3 µL of *Reference solution A* at one end of the plate. Develop the chromatograms in an equilibrated chamber with the *Developing solvent system* until the solvent front reaches approximately 1 cm from the upper edge of the plate. Remove the plate from the developing chamber, and evaporate the solvent under a current of hot air [NOTE—avoid excessive heat] or by leaving the plate for a short while under a hood. Spray the plate with a 0.2% alcoholic solution of 2,7-dichlorofluorescein, and examine in UV light at 254 nm. [NOTE—The plates pretreated with UV indicator also are commercially available and are used equivalently.] In each of the plates, mark the limits of the sterol band identified by being aligned with the stain obtained from *Reference solution A* along the edges of the fluorescence, and additionally include the area of the zones 2–3 mm above and below the visible zones corresponding to *Reference solution A*. Remove the silica gel in the marked area into a filter funnel with a G3 porous septum.[2] Add 10 mL of hot chloroform, mix carefully with the metal spatula, filter under vacuum, and collect the filtrate in the conical flask attached to the filter funnel. Wash the residue in the funnel three times with ether, about 10 mL each time, and collect the filtrate in the same flask attached to the funnel. Evaporate the filtrate to a volume of 4–5 mL, transfer the residual solution to a previously weighed 10-mL test tube with a tapering bottom and a sealing stopper, and evaporate to dryness by mild heating in a gentle stream of nitrogen. Dissolve the residue in a few drops of acetone, and evaporate again to dryness. Place at 105° for approximately 10 min, allow to cool in a desiccator, and weigh.

Treat *Test solution B* and *Test solution C* the same way as directed for *Test solution A*.

**Determination of the sterols**

**Test solution D:** To the test tube containing the sterol fraction separated from the test substance by thin-layer chromatography add a freshly prepared mixture of anhydrous pyridine, hexamethyldisilazane, and chlorotrimethylsilane (9:3:1) [NOTE—This reagent also is commercially available and is used equivalently.] in the ratio of 50 µL for every mg of sterols, avoiding any uptake of moisture. Insert the stopper into the test tube, and shake carefully until the sterols are completely dissolved. Allow it to stand for at least 15 min at ambient temperature, and centrifuge for a few minutes if necessary. Use the supernatant. [NOTE—The slight opalescence that may form is normal and does not cause an anomaly. However, the formation of a white floc or the appearance of a pink color is indicative of the presence of moisture or deterioration of the reagent. If these occur, the test must be repeated.]

[1] A commercial TLC plate may be obtained from Sigma-Aldrich, catalog #z122785.
[2] A commercial product may be obtained from Kimble/Kontes as a filter, Buchner with fritted disc, Kimax 28400-152.

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**Reference solution E:** To 9 parts of the sterols separated from canola oil by thin-layer chromatography, add 1 part of cholesterol. Treat the mixture in the same way as directed in *Test solution D*.

**Reference solution F:** Treat the sterols separated from sunflower oil by thin-layer chromatography in the same way as directed in *Test solution D*.

**Chromatographic system**

(See *Chromatography* ⟨621⟩.)

The gas chromatograph is equipped with a flame-ionization detector and a glass or fused-silica capillary column 20–30 m in length, internal diameter 0.25–0.32 mm, entirely coated with a 0.10- to 0.30-µm layer of stationary phase G27 or G36. The injection port temperature is maintained at 280°, the detector temperature is maintained at 290°, and the column temperature is maintained at 260 ± 5°. The carrier gas is either helium with a linear velocity of 20–35 cm/s or hydrogen with a linear velocity of 30–50 cm/s. A split ratio of 1:50 to 1:100 is used. Chromatograph *Reference solution E* and *Reference solution F,* and record the peak responses as directed in *Procedure:* the retention time should be 20 ± 5 min for β-sitosterol, and all the sterols present must be separated. The chromatogram obtained with *Reference solution E* shows four principal peaks corresponding to cholesterol, brassicasterol, campesterol, and β-sitosterol; and the chromatogram obtained with *Reference solution F* shows four principal peaks corresponding to campesterol, stigmasterol, β-sitosterol, and Δ7-stigmastenol. The retention times of the sterols with reference to β-sitosterol are given in *Table 12*.

**Table 12. Relative Retention Times of Sterols for Two Different Columns**

| Identification | G36 Column | G27 Column |
|---|---|---|
| Cholesterol | 0.67 | 0.63 |
| Brassicasterol | 0.73 | 0.71 |
| 24-Methylene-cholesterol | 0.82 | 0.80 |
| Campesterol | 0.83 | 0.81 |
| Campestanol | 0.85 | 0.82 |
| Stigmasterol | 0.88 | 0.87 |
| Δ7-Campesterol | 0.93 | 0.92 |
| Δ5,23-Stigmastadienol | 0.95 | 0.95 |
| Clerosterol | 0.96 | 0.96 |
| β-Sitosterol | 1.00 | 1.00 |
| Sitostanol | 1.02 | 1.02 |
| Δ5-Avenasterol | 1.03 | 1.03 |
| Δ5,24-Stigmastadienol | 1.08 | 1.08 |
| Δ7-Stigmastenol | 1.12 | 1.12 |
| Δ7-Avenasterol | 1.16 | 1.16 |

**Procedure:** Separately inject equal volumes (about 1 µL) of *Test solution D, Reference solution E,* and *Reference solution F* into the chromatograph, record the chromatograms, and measure the peak areas for the sterols.

Calculate the percentage of each individual sterol in the sterol fraction of the test substance taken:

$$\text{Result} = 100 \times (A/S)$$

$A$ = area of the peak due to the sterol component to be determined
$S$ = sum of the areas of the peaks due to the components indicated in *Table 12*

## ADDITIONAL REQUIREMENTS

- **USP Reference Standards** ⟨11⟩

USP Docosahexaenoic Acid Ethyl Ester RS
USP Eicosapentaenoic Acid Ethyl Ester RS
USP Methyl Tricosanoate RS

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# ⟨411⟩ FOLIC ACID ASSAY

## INTRODUCTION

The following liquid chromatographic procedures are provided for the determination of folic acid as an active pharmaceutical ingredient, a dietary supplement ingredient, or a component of dietary supplements or pharmaceutical dosage forms.

Throughout these procedures, protect solutions containing and derived from the test specimen and the Reference Standards from the atmosphere and light, preferably by using low-actinic glassware.

## ASSAY

• **PROCEDURE 1**

This procedure can be used to determine folic acid in the following:

- Oil- and Water-Soluble Vitamins with Minerals Tablets
- Oil- and Water-Soluble Vitamins with Minerals Capsules
- Oil- and Water-Soluble Vitamins Tablets
- Oil- and Water-Soluble Vitamins Capsules
- Water-Soluble Vitamins with Minerals Tablets
- Water-Soluble Vitamins with Minerals Capsules
- Water-Soluble Vitamins Tablets
- Water-Soluble Vitamins Capsules

This procedure involves the extraction of analytes from the formulation by using an *Internal standard solution* that contains methylparaben, tetrabutylammonium hydroxide, and pentetic acid in alcoholic phosphate buffer and by mechanically shaking to release the analytes from the matrices.

Unless specified in the individual monographs, the reagent solutions, *Internal standard solution*, *Standard solution*, and *Sample solutions* are prepared as follows.

**Reagent A:** 25% solution of tetrabutylammonium hydroxide in methanol

**Reagent B:** Transfer 5.0 g of pentetic acid to a 50-mL volumetric flask. Using sonication if necessary, dissolve in and dilute with 1 N sodium hydroxide to volume.

**Mobile phase:** 2 g of monobasic potassium phosphate in 650 mL of water. Add 12.0 mL of *Reagent A*, 7.0 mL of 3 N phosphoric acid, and 240 mL of methanol. Cool to room temperature, adjust with phosphoric acid or ammonia TS to a pH of 7.0, dilute with water to 1000 mL, and filter. Recheck the pH before use by adding water or methanol to the prepared *Mobile phase* to obtain baseline separation of folic acid and the internal standard. The pH may be increased up to 7.15 to obtain better separation. [NOTE—The methanol and water content may be varied (1%–3%).]

**Internal standard solution:** Transfer 40 mg of methylparaben to a 1000-mL volumetric flask, and add 220 mL of methanol to dissolve. Dissolve 2.0 g of monobasic potassium phosphate in 300 mL of water in a separate beaker, quantitatively transfer this solution to the flask containing the methylparaben solution, and add an additional 300 mL of water. Add 19 mL of *Reagent A*, 7 mL of 3 N phosphoric acid, and 30 mL of *Reagent B*. Adjust with ammonia TS to a pH of 9.8, bubble nitrogen through the solution for 30 min, dilute with water to volume, and mix.

**Standard solution:** 0.016 mg/mL of USP Folic Acid RS in the *Internal standard solution*

**Sample solution for Tablets:** Crush not less than 30 Tablets until finely powdered. Transfer a portion of powder, equivalent to 0.4 mg of folic acid, to a 50-mL amber-colored centrifuge tube. Add 25.0 mL of the *Internal standard solution*, shake by mechanical means for 10 min, and centrifuge. Filter a portion of the clear supernatant, and use the filtrate.

**Sample solution for Capsules:** Weigh not less than 20 Capsules in a tared weighing bottle. Open the Capsules, without the loss of shell material, and transfer the contents to a 100-mL beaker. Remove any contents adhering to the empty shells by washing, if necessary, with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air until the odor of ether is no longer perceptible. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the average net weight per Capsule. Transfer an amount of the Capsule contents to a suitable centrifuge tube, and add a volume of the *Internal standard solution* to obtain a concentration of 0.016 mg/mL of folic acid. Shake by mechanical means for 10 min, and centrifuge. Filter a portion of the clear supernatant, and use the filtrate.

**Chromatographic system**

(See *Chromatography* ⟨621⟩*, System Suitability*.)

**Mode:** LC

**Detector:** UV 280 nm

**Column:** 4.6-mm × 25-cm; packing L1

**Flow rate:** 1 mL/min

**Injection volume:** 15 µL

**System suitability**

**Sample:** *Standard solution*

[NOTE—The relative retention times for folic acid and methylparaben are about 0.8 and 1.0, respectively.]

**Suitability requirements**

**Relative standard deviation:** NMT 3.0%

**Analysis**

**Samples:** *Standard solution* and appropriate *Sample solution*

Calculate the percentage of the labeled amount of folic acid ($C_{19}H_{19}N_7O_6$) in the portion of the *Sample* taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

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$R_U$ = internal standard ratio (peak response of folic acid/peak response of the internal standard) from the appropriate *Sample solution*
$R_S$ = internal standard ratio (peak response of folic acid/peak response of the internal standard) from the *Standard solution*
$C_S$ = concentration of USP Folic Acid RS in the *Standard solution* (µg/mL)
$C_U$ = nominal concentration of folic acid in the appropriate *Sample solution* (µg/mL)

- **PROCEDURE 2**

This procedure can be used to determine folic acid in the following:

- Oil- and Water-Soluble Vitamins with Minerals Tablets
- Oil- and Water-Soluble Vitamins with Minerals Capsules
- Oil- and Water-Soluble Vitamins Tablets
- Oil- and Water-Soluble Vitamins Capsules
- Water-Soluble Vitamins with Minerals Tablets
- Water-Soluble Vitamins with Minerals Capsules
- Water-Soluble Vitamins Tablets
- Water-Soluble Vitamins Capsules

This procedure involves the extraction of analytes from the formulation by using an extracting solution that contains either a mixture of edetate disodium and ammonium hydroxide or a mixture of methanol, glacial acetic acid, and ethylene glycol, and by mechanically shaking to release the analytes from the matrices.

Unless specified in the individual monographs, the reagent solutions, *Diluent*, *Standard stock solution*, *Standard solutions*, and *Sample solutions* are prepared as follows.

**Reagent:** Dissolve 7.5 g of edetate disodium, with stirring, in 500 mL of water containing 10 mL of ammonium hydroxide.

**Diluent:** 60 µg/mL of ammonium hydroxide

**Mobile phase:** Transfer 0.4 mL of triethylamine, 15 mL of glacial acetic acid, and 350 mL of methanol to a 2000-mL volumetric flask, and dilute with 0.008 M sodium 1-hexanesulfonate to volume.

**Standard stock solution:** 60 µg/mL of USP Folic Acid RS in *Diluent*. Prepare this solution fresh daily.

**Standard solution for Tablets:** Mix 5.0 mL of the *Standard stock solution* with 10.0 mL of methanol and 35.0 mL of *Reagent*. Shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few milliliters of the filtrate.

**Standard solution for Capsules:** Mix 5.0 mL of the *Standard stock solution* with 10.0 mL of a mixture of methanol and glacial acetic acid (9:1) and 30.0 mL of a mixture of methanol and ethylene glycol (1:1). Shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few milliliters of the filtrate.

**Sample solution for Tablets:** Transfer a portion of finely powdered Tablets, equivalent to 0.3 mg of folic acid, to a 125-mL stoppered flask. Add 10.0 mL of methanol and 35.0 mL of *Reagent*. Shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few milliliters of the filtrate.

**Sample solution for Capsules:** Weigh not less than 20 Capsules in a tared weighing bottle. Open the Capsules, without the loss of shell material, and transfer the contents to a 100-mL beaker. Remove any contents adhering to the empty shells by washing, if necessary, with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air until the odor of ether is no longer perceptible. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the average net weight per Capsule. Transfer an amount of the Capsule contents, equivalent to 0.3 mg of folic acid, to a 125-mL stoppered flask. Add 10.0 mL of a mixture of methanol and glacial acetic acid (9:1) and 30.0 mL of a mixture of methanol and ethylene glycol (1:1). Shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few milliliters of the filtrate.

**Chromatographic system**

(See *Chromatography* ⟨621⟩*, System Suitability*.)

**Mode:** LC

**Detector:** UV 270 nm

**Column:** 4.6-mm × 25-cm; packing L7

**Column temperature:** 50°

**Flow rate:** 2 mL/min

**Injection volume:** 5 µL

**System suitability**

**Sample:** *Standard solution*

**Suitability requirements**

**Relative standard deviation:** NMT 2.0%

**Analysis**

**Samples:** Appropriate *Standard solution* and appropriate *Sample solution*

Calculate the percentage of the labeled amount of folic acid ($C_{19}H_{19}N_7O_6$) in the portion of the *Sample* taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

$r_U$ = peak response of folic acid from the appropriate *Sample solution*
$r_S$ = peak response of folic acid from the appropriate *Standard solution*
$C_S$ = concentration of USP Folic Acid RS in the appropriate *Standard solution* (µg/mL)
$C_U$ = nominal concentration of folic acid in the appropriate *Sample solution* (µg/mL)

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- **PROCEDURE 3**

This procedure can be used to determine folic acid in the following:

- Active pharmaceutical ingredient
- Dietary ingredient

This procedure involves the dissolution of analytes and internal standard in *Mobile phase*.

Unless specified in the individual monographs, the reagent solutions, *Internal standard solution*, *Standard stock solution*, *Standard solution*, *Sample stock solution*, and *Sample solution* are prepared as follows.

**3 N phosphoric acid:** 98 g/L of phosphoric acid in water

**6 N ammonium hydroxide:** Dilute 40 mL of ammonium hydroxide with water to 100 mL.

**Mobile phase:** Transfer 2.0 g of monobasic potassium phosphate to a 1000-mL volumetric flask, and dissolve in 650 mL of water. Add 15.0 mL of a solution of 0.5 M tetrabutylammonium hydroxide in methanol, 7.0 mL of *3 N phosphoric acid*, and 270 mL of methanol. Cool to room temperature, adjust with *3 N phosphoric acid* or *6 N ammonium hydroxide* to a pH of 5.0, and dilute with water to volume. Recheck the pH before use.

**Internal standard solution:** 2 mg/mL of methylparaben in *Mobile phase*. Dissolve the methylparaben first with methanol (about 4% of the final volume), and dilute with *Mobile phase* to volume.

**Standard stock solution:** 1 mg/mL of USP Folic Acid RS in *Mobile phase*. Dissolve the folic acid with the aid of 10% ammonium hydroxide (about 1% of the final volume), and dilute with *Mobile phase* to volume.

**Standard solution:** Transfer 4.0 mL of the *Standard stock solution* and 4.0 mL of the *Internal standard solution* to a 50-mL volumetric flask, and dilute with *Mobile phase* to volume.

**Sample stock solution:** Transfer 100 mg of folic acid to a 100-mL volumetric flask, and dissolve in 40 mL of *Mobile phase* and 1 mL of 10% ammonium hydroxide. Dilute with *Mobile phase* to volume.

**Sample solution:** Transfer 4.0 mL of the *Sample stock solution* and 4.0 mL of the *Internal standard solution* to a 50-mL volumetric flask, and dilute with *Mobile phase* to volume.

**Chromatographic system**

(See *Chromatography* ⟨621⟩, *System Suitability*.)

**Mode:** LC

**Detector:** UV 280 nm

**Column:** 4.6-mm × 25-cm; packing L1

**Flow rate:** 1.2 mL/min

**Injection volume:** 10 µL

**System suitability**

**Sample:** *Standard solution*

**Suitability requirements**

**Resolution:** NLT 3.6 between methylparaben and folic acid

**Relative standard deviation:** NMT 2.0% for the ratios of the folic acid peak response to the internal standard peak response

**Analysis**

**Samples:** *Standard solution* and *Sample solution*

Calculate the percentage of folic acid ($C_{19}H_{19}N_7O_6$) in the portion of the *Sample* taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

$R_U$ = internal standard ratio (peak response of folic acid/peak response of the internal standard) from the *Sample solution*
$R_S$ = internal standard ratio (peak response of folic acid/peak response of the internal standard) from the *Standard solution*
$C_S$ = concentration of USP Folic Acid RS in the *Standard stock solution* (mg/mL)
$C_U$ = concentration of folic acid in the *Sample stock solution* (mg/mL)

***Change to read:***

- **PROCEDURE 4**

This procedure can be used to determine folic acid in the following:

- Folic Acid Tablets
- Folic Acid Injection

This procedure involves the dissolution of analytes in a *Diluent* that contains 2 mL of ammonium hydroxide and 1 g of sodium perchlorate per 100 mL of water.

Unless specified in the individual monographs, *Diluent*, *System suitability solution*, *Standard solution*, and *Sample solutions* are prepared as follows.

**Mobile phase:** Transfer 35.1 g of sodium perchlorate and 1.40 g of monobasic potassium phosphate to a 1-L volumetric flask. Add 7.0 mL of 1 N potassium hydroxide and 40 mL of methanol, dilute with water to volume, and mix. Adjust with 1 N potassium hydroxide or phosphoric acid to a pH of 7.2.

**Diluent:** Aqueous solution containing 2 mL of ammonium hydroxide and 1 g of sodium perchlorate per 100 mL

**System suitability solution:** 0.2 mg/mL each of USP Folic Acid RS and ▲USP Leucovorin Calcium RS▲ (IRA 1-Nov-2022) in *Diluent*. [NOTE—Before use, pass through a filter of 1-µm or finer pore size.]

**Standard solution:** 0.20 mg/mL of USP Folic Acid RS in *Diluent*

**Sample solution for Tablets:** Equivalent to 0.2 mg/mL of folic acid from not less than 20 powdered Tablets in *Diluent*. Shake gently to aid dissolution, and filter, discarding the first few milliliters of the filtrate.

**Sample solution for Injection:** Dilute with *Diluent* an accurately measured volume of Injection, quantitatively and stepwise, to obtain a solution having a concentration close to that of the *Standard solution* and between 0.20 and 0.80 mg/mL.

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**Chromatographic system**
(See *Chromatography* ⟨621⟩, *System Suitability*.)
**Mode:** LC
**Detector:** UV 254 nm
**Column:** 4.6-mm × 25-cm; packing L1
**Flow rate:** 1 mL/min
**Injection volume:** 25 µL
**System suitability**
**Samples:** *System suitability solution* and *Standard solution*
**Suitability requirements**
**Resolution:** NLT 3.6 between ▲leucovorin▲ (IRA 1-Nov-2022) and folic acid, *System suitability solution*
**Relative standard deviation:** NMT 2.0%, *Standard solution*
**Analysis**
**Samples:** *Standard solution* and appropriate *Sample solution*
Calculate the percentage of folic acid ($C_{19}H_{19}N_7O_6$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

$r_U$ = peak response of folic acid from the *Sample solution for Tablets*
$r_S$ = peak response of folic acid from the *Standard solution*
$C_S$ = concentration of USP Folic Acid RS in the *Standard solution* (mg/mL)
$C_U$ = nominal concentration of folic acid in the *Sample solution for Tablets* (mg/mL)

Calculate the quantity, in milligrams, of folic acid ($C_{19}H_{19}N_7O_6$) in each milliliter of Injection taken:

$$\text{Result} = (r_U/r_S) \times (C_S/V_U) \times V$$

$r_U$ = peak response of folic acid from the *Sample solution for Injection*
$r_S$ = peak response of folic acid from the *Standard solution*
$C_S$ = concentration of USP Folic Acid RS in the *Standard solution* (mg/mL)
$V_U$ = volume of Injection taken for preparation of the *Sample solution for Injection* (mL)
$V$ = total volume used to dilute the Injection (mL)

## ADDITIONAL REQUIREMENTS

***Change to read:***

- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Folic Acid RS
  ▲USP Leucovorin Calcium RS▲ (IRA 1-Nov-2022)

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# ⟨413⟩ IMPURITIES TESTING IN MEDICAL GASES

## INTRODUCTION

This general test chapter defines the safe and proper means to sample high-pressure gas containers of different medical gas compositions using the detector tube manufacturer's suggested total gas volume for the purpose of conducting detector tube analysis to satisfy the *USP* monographs. See *Reagents* in the section *Reagents, Indicators, and Solutions* for information on each referenced detector tube.

There are two types of detector tubes currently manufactured: those to be used with a manual hand pump of fixed volume (e.g., 100 mL/pump stroke) and those used in a continuous flow system that can be set to pass a volume of gas through the detector tube at approximately 1 atmosphere. It is important to match the appropriate detector tube type to the mode of gas volume exchange.

To ensure that the required gas volume has passed through the detector tube, measure the gas volume at the time of the analysis either by using a hand pump or using a flowmeter that is calibrated to the subject gas or corrected via a calibration chart. Flowmeter manufacturers generally provide a chart of the gas volume flow of common gases for each of the flow tubes identified in this general chapter.

[NOTE—See general chapter *Medical Gases Assay* ⟨415⟩ for sampling.]

## CONTINUOUS FLOW SYSTEM

Identify the gas contained in the container, and select the appropriate gas regulator. Secure the regulator to the gas container. Do not apply lubricant or Teflon tape to the container-to-regulator connections. Purge the regulator with the gas under test. Select float setting to obtain the proper flow rate to achieve total gas volume as recommended by the manufacturer. Attach the detector tube, then adjust the flow rate to the required level as indicated by the charts that accompany the flowmeter and the detector tube. Time the gas flow to achieve the desired total gas volume ±10 s. At the allotted time close the regulator valve, then the main container valve. Observe the tube while it is still attached to determine the degree of color change, and record the result. Remove the tube, and disconnect the apparatus, vent the regulator gas pressure to atmosphere, and remove the regulator from the container. Dispose of the tube after use.

## HAND PUMP—FIXED VOLUME

The alternative approach is to apply the gas detector hand pump. The system draws a consistent volume with each pump stroke. To ensure the accuracy of the total gas volume, the user must follow the pump manufacturer's suggested recommendation about how to pump.

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# ⟨415⟩ MEDICAL GASES ASSAY

## INTRODUCTION

The evaluation of the purity of a gas used for medical treatment or as a component of a pharmaceutical process is the purpose of a *USP* medical gas monograph. The purity generally is evaluated by an assay for the content of the article and by analyses for trace impurities. The application of gas chromatography, paramagnetic analysis, and detector tubes to medical gases is somewhat different from traditional procedures used for analytes in the liquid phase and therefore warrants a separate description. This general test chapter focuses on the assay for content tests. Sampling for impurities is addressed in general chapter *Impurities Testing in Medical Gases* ⟨413⟩.

This chapter includes sampling and qualification aspects of gas chromatographic and paramagnetic analyses of medical gases. In addition, it includes a description of the initial set-up, validation, and calibration of these instruments. The specific assay procedures are defined in the specific monograph for that gas.

The basic definitions of instrumental qualification and validation are included in general information chapters *Analytical Instrument Qualification* ⟨1058⟩ and *Validation of Compendial Procedures* ⟨1225⟩, respectively, and will not be repeated. However, when variations of the materials presented in these chapters are necessary due to the character of the analyte, this chapter will define those variations.

## METHODS

### Gas Chromatography (GC)

See *Chromatography* ⟨621⟩.

### Detectors for Medical Gases Assay

The two most common detectors used in the analyses of medical gases are the thermal conductivity detector (TCD) and flame ionization detector (FID).

The TCD will detect any gas or vapor that has a thermal conductivity (TC) that differs significantly from the high TC of the reference gas, usually helium, therefore it is virtually universal. However, the generally accepted lower detection limit for the TCD is 50 ppm v/v. This represents a limitation for the evaluation of trace impurities in medical gases.

The FID is also used for the evaluation of trace impurities in medical gases, because it is more sensitive to organic compounds but does not produce a signal for most common medical gases.

## QUALIFICATION

### Installation Qualification (IQ)

The IQ requirements ensure the gas chromatograph hardware and software (or readout device) is installed safely and in accordance with the GC manufacturer's instructions.

Consideration should be given to the following as applicable:

- Suitability of the sample system (including connections);
- Leakage (should be leak free);
- Representative sampling;
- Sample flow rates;
- Response time;
- Correct output signals;
- Power supply (including voltage regulation); and
- Appropriate environmental conditions of the instrument and of the sample itself (e.g., temperature and pressure).

### Operational Qualification (OQ)

OQ verifies that the GC performs as intended within its anticipated operating range. For medical gas final product testing, the GC is tested to ensure repeatability (verification that relative standard deviation is consistent with claims) for each analyte of interest. Due to the specific nature of medical gas testing and the limited number of analytes, routine calibration and periodic calibration verification of a GC instrument and the testing procedure may be used in place of initial or periodic OQ. When an instrument is used for a broader range of analytes, the replacement of OQ with calibration and periodic calibration verification is inappropriate.

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## Performance Qualification (PQ)

For medical gas final product testing, the GC is periodically checked at appropriate intervals during analytical runs with a calibration gas (i.e., verifying that the results are consistent with a named concentration within acceptable accuracy and precision ranges after a specific number of sample injections).

## Paramagnetic Oxygen Measurement

### THEORY

The paramagnetic analyzer measures the displacement of a diamagnetic gas (nitrogen) by a paramagnetic gas (oxygen), in a strong magnetic field. A measuring cell typically employs a glass dumbbell with nitrogen-filled spheres that is suspended on a torsion strip between magnets that concentrate the flux around the dumbbell. When oxygen molecules enter the measuring cell, the dumbbell is deflected by the force exerted by the oxygen molecules that are attracted to the strongest part of the magnetic field. By using optical sensors, a feedback coil, and suitable electronics, analysts measure an output that is directly proportional to the partial pressure of oxygen.

Oxygen is the only paramagnetic gas present above trace levels in the atmosphere. However, paramagnetic analyzers can be affected by the magnetic susceptibility of the background gas. Therefore changes to background gases in *USP* monographs should be avoided.

## Design Considerations

The design considerations for the purchase of new instruments may include the following parameters.

### DRIFT

A change of the output of the instrument for a given concentration over a stated period of time under constant conditions and without any adjustments being made to the instrument by external means. Drift is the summation of two components, zero drift and span drift. Drift determines the frequency of instrument calibration.

### ZERO DRIFT

A change in the output when zero gas is being measured.

### SPAN DRIFT

A change in the output at the level of oxygen concentration that is being measured.

### OPERATING TEMPERATURE

The ambient temperature range for which the stated performance specification of the instrument will remain valid. A larger temperature coefficient will indicate that a smaller change in ambient temperature is permitted before re-calibration is required.

### OPERATING PRESSURE

The instrument should operate at the inlet pressures of the samples to be tested.

## Qualification Aspects

### INSTALLATION QUALIFICATION (IQ)

The IQ requirements ensure the oxygen analyzer hardware and software (or readout device) is installed safely and in accordance with the oxygen analyzer manufacturer's instructions.

Consideration should be given to the following as applicable:

- Suitability of the sample system (including connections);
- Leakage (should be leak free);
- Representative sampling;
- Sample flow rates;
- Response time;
- Correct output signals;
- Power supply (including voltage regulation); and
- Appropriate environmental conditions of the instrument and of the sample itself (e.g., temperature and pressure).

### OPERATIONAL QUALIFICATION (OQ)

The OQ requirements verify that the paramagnetic analyzer performs as intended within its anticipated operating range and is suitable for the actual conditions of use. Instruments and apparatus should be calibrated and used in accordance with the

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oxygen analyzer manufacturer's instructions. Because of the specific nature of the instrument, routine calibration may be used in place of initial or periodic OQ testing.

### PERFORMANCE QUALIFICATION (PQ)

The PQ requirements verify that the paramagnetic analyzer performs as intended in its normal operating environment. For medical gas final product testing, the paramagnetic analyzer is initially calibrated (zeroed and spanned using a certified standard) in accordance with the oxygen analyzer manufacturer's instructions and is periodically recalibrated to ensure continued acceptable performance.

### ZEROING THE INSTRUMENT (ESTABLISHING THE LOWER LIMIT)

Using the certified standard defined in the monograph, establish a zero setting on the analyzer by passing the zero gas into the analyzer at the oxygen analyzer manufacturer's suggested flow rate. Maintain the flow until a stable reading is observed on the instrument. As necessary, adjust the zero setting to a value of 0.0% according to the oxygen analyzer manufacturer's instructions. Confirm the reading is stable.

[NOTE—Depending on the intended use of the instrument, zeroing to a setting other than 0.0% is an acceptable alternative to this procedure if it provides greater measurement precision.]

### SPANNING THE RANGE OF USE

Establish the upper limit (span) with a span gas defined in the monograph and appropriate for the range of use. Pass the span gas through the instrument at the manufacturer's suggested flow rate. Confirm the reading is stable. Adjust the span setting to the certified value of the reference standard according to the oxygen analyzer manufacturer's instructions. Confirm the reading is stable.

## VALIDATION

Validation of this instrument is generally completed during the (IQ/OQ) process. Routine verification is performed as described in the OQ/PQ sections of this chapter; and, therefore, specific information on instrument validation is unnecessary.

## PROCEDURE

### For Off-line Instrument

Before analysis, the instrument is calibrated by zeroing and spanning as described in the PQ section.

[NOTE—The calibration need not be run concomitantly with the test samples.]

Connect the sample gas to the instrument, and establish a constant flow into the analyzer at the analyzer manufacturer's suggested flow rate. Maintain the flow until a constant reading is observed on the instrument. The definition of a constant reading is included in the analyzer manufacturer's instructions or in the user's instrument qualification documentation.

### For On-line Instrument

The calibration intervals are defined by the analyzer manufacturer, by past history, or by statistical means. Establish a constant flow into the analyzer at the manufacturer's suggested flow rate.

## SAMPLING

### Sampling from Liquid Phase

Cylinders containing a dip tube allow a liquid sample to be obtained from the valve outlet with the cylinder in the upright position. If a dip tube is not present, the cylinder should be placed in an inverted position with the cylinder and main valve safely supported (so the liquid phase is in contact with the valve).

Sampling of medical gases should always be conducted using the required regulator. Regulators should be purged with the gas that will be sampled. When necessary, the flow to the analyzer should be measured using a calibrated flow-measuring device.

### Sampling from Gaseous Phase

Cylinders that do not contain a dip tube allow a gaseous sample to be obtained from the valve outlet with the cylinder in the upright position. If a dip tube is present, the cylinder should be in an inverted position with the cylinder and main valve safely supported (so the gaseous phase is in contact with the end of the dip tube). Sampling of medical gases should always be conducted using the required regulator.

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# CERTIFIED STANDARDS FOR MEDICAL GAS ANALYSIS

*USP* monographs for medical gases require tests that use certified standards for instrument calibration and analytical determinations. Such compendial testing may be conducted using reference materials that are traceable to the U.S. National Institute of Standards and Technology or other National standards-setting organizations, e.g., Institute for National Measurement Standards (Canada). The individual monographs and the reagents, indicators, and solutions section refer to the nominal percent of various certified standards required to perform medical gas analysis. The requirements for the actual certified concentrations in terms of variance from the nominal value are indicated in the *Reagents, Indicators, and Solutions* section for each respective certified standard.

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# ⟨425⟩ IODOMETRIC ASSAY—ANTIBIOTICS

The following method is provided for the assay of most of the Pharmacopeial penicillin antibiotic drugs and their dosage forms, for which iodometric titration is particularly suitable.

## ASSAY

**Standard preparation:** Dissolve in the solvent specified in the table of *Solvents and Final Concentrations* a suitable quantity of the USP Reference Standard specified in the individual monograph, previously dried under the conditions specified in the individual monograph and accurately weighed, and dilute quantitatively and stepwise with the same solvent to obtain a solution having a known concentration of about that specified in the table. Pipet 2.0 mL of this solution into each of two 125-mL glass-stoppered conical flasks.

**Solvents and Final Concentrations**

| Antibiotic | Solvent* | Final Concentration |
|---|---|---|
| Amoxicillin | Water | 1.0 mg/mL |
| Ampicillin | Water | 1.25 mg/mL |
| Ampicillin Sodium | *Buffer B.1* | 1.25 mg/mL |
| Cloxacillin Sodium | Water | 1.25 mg/mL |
| Cyclacillin | Water | 1.0 mg/mL |
| Dicloxacillin Sodium | *Buffer B.1* | 1.25 mg/mL |
| Methicillin Sodium | *Buffer B.1* | 1.25 mg/mL |
| Nafcillin Sodium | *Buffer B.1* | 1.25 mg/mL |
| Oxacillin Sodium | *Buffer B.1* | 1.25 mg/mL |
| Penicillin G Potassium | *Buffer B.1* | 2,000 units/mL |
| Penicillin G Sodium | *Buffer B.1* | 2,000 units/mL |
| Penicillin V Potassium | *Buffer B.1* | 2,000 units/mL |
| Phenethicillin Potassium | *Buffer B.1* | 2,000 units/mL |

* Unless otherwise noted, the *Buffers* are the potassium phosphate buffers defined in the section *Media and Diluents* under *Antibiotics—Microbial Assays* ⟨81⟩, except that sterilization is not required before use.

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**Assay preparation:** Unless otherwise specified in the individual monograph, dissolve in the solvent specified in the table of *Solvents and Final Concentrations* a suitable quantity, accurately weighed, of the specimen under test, and dilute quantitatively with the same solvent to obtain a solution having a known final concentration of about that specified in the table. Pipet 2 mL of this solution into each of two 125-mL glass-stoppered conical flasks.

### Procedure

**Inactivation and titration:** To 2.0 mL of the *Standard preparation* and of the *Assay preparation*, in respective flasks, add 2.0 mL of 1.0 N sodium hydroxide, mix by swirling, and allow to stand for 15 minutes. To each flask add 2.0 mL of 1.2 N hydrochloric acid, add 10.0 mL of 0.01 N iodine VS, immediately insert the stopper, and allow to stand for 15 minutes. Titrate with 0.01 N sodium thiosulfate VS. As the endpoint is approached, add 1 drop of starch iodide paste TS, and continue the titration to the discharge of the blue color.

**Blank determination:** To a flask containing 2.0 mL of the *Standard preparation* add 10.0 mL of 0.01 N iodine VS. If the *Standard Preparation* contains amoxicillin or ampicillin, immediately add 0.1 mL of 1.2 N hydrochloric acid. Immediately titrate with 0.01 N sodium thiosulfate VS. As the endpoint is approached, add 1 drop of starch iodide paste TS, and continue the titration to the discharge of the blue color. Similarly treat a flask containing 2.0 mL of the *Assay preparation.*

### Calculations

Calculate the microgram (or unit) equivalent (F) of each mL of 0.01 N sodium thiosulfate consumed by the *Standard preparation* by the formula:

$$\text{Result} = (2CP)/(B - I)$$

$C$ = concentration of Reference Standard in the *Standard preparation* (mg/mL)
$P$ = potency of the Reference Standard (µg/mg or units/mg)
$B$ = volume of 0.01 N sodium thiosulfate consumed in the *Blank determination* (mL)
$I$ = volume of 0.01 N sodium thiosulfate consumed in the *Inactivation and titration* (mL)

Calculate the potency of the specimen under test by the formula given in the individual monograph.

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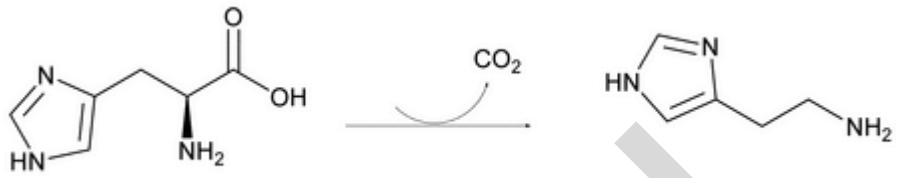
***Add the following:***

# ▲⟨426⟩ HISTAMINE TEST METHOD

## INTRODUCTION

High levels of histamine in drug products can lead to severe adverse drug reactions, including flushing, itching, hives, shortness of breath, decreased blood pressure, increased heart rate, and even fatality.

A potential source of histamine impurity is peptone of animal origin, particularly fish peptone, which is a critical raw material used as a protein source in the fermentation process. Free histidine is a naturally-occurring amino acid present in animals that can be converted to histamine by histidine decarboxylase.




**Histidine** **Histamine**

Fish peptone has a higher histamine contamination risk than other animal-derived peptones because some species of fish have larger quantities of histidine. Special attention must be paid to histamine levels when peptones of animal origin are used in the manufacturing process. This general chapter is intended to provide users with validated analytical methods to quantify the histamine levels in antibiotics.

Applicable requirements for histamine content are determined by the appropriate regulatory agency.

This chapter contains an LC-MS method to determine the histamine content. This method is validated for use with gentamicin sulfate, and must be validated for use with other drug substances.

## HISTAMINE CONTENT IN GENTAMICIN SULFATE

• **PROCEDURE**

**Solution A:** Acetonitrile

**Solution B:** 10 mM ammonium formate in 0.1% formic acid

**Sensitivity solution:** 2 ng/mL solution of USP Histamine Dihydrochloride RS

**Standard solutions:** Accurately weigh an appropriate amount of USP Histamine Dihydrochloride RS, and dissolve and dilute with *Solution B*. Dilute further with *Solution B* to obtain a series of standard solutions (NLT 3) appropriately spaced and at concentrations appropriate for the assay within the range from 2 to 20 ng/mL.

**Sample solution:** Accurately weigh a suitable quantity of gentamicin sulfate, and dissolve and dilute with *Solution B* to prepare a 1 mg/mL solution.

**Check standard:** Accurately weigh an appropriate amount of USP Histamine Dihydrochloride RS, and dissolve and dilute with *Solution B*. Dilute further with *Solution B* to obtain a check standard within the range of the concentrations prepared for the *Standard curve*.

**Mobile phase gradient:** See *Table 1*.

**Table 1**

| Time (min) | Solution A (% v/v) | Solution B (% v/v) |
|---|---|---|
| 0 | 90 | 10 |
| 4 | 40 | 60 |
| 5 | 40 | 60 |
| 5.01 | 90 | 10 |
| 7 | 90 | 10 |

**Chromatographic system**

(See *Chromatography* ⟨621⟩, *System Suitability*.)

**Mode:** LC

**Detector:** MS

**MS conditions**

**Ionization mode:** Electrospray (+) ionization

**Single-ion monitoring (SIM) mode:** m/z 112.1

**Probe temperature:** 600°

**Column:** 2.1-mm × 10-cm; 1.7-µm packing L68

**Column temperature:** 35°

**Flow rate:** 0.5 mL/min

**Injection volume:** 1 µL

[NOTE—The approximate retention time of the histamine peak is 2.8 min.]

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**Standard curve**

**Samples:** *Standard solutions*

Inject 1 µl of a series of *Standard solutions* and record the chromatograms and peak area of MS signals. Using the linear regression method, plot the histamine peak areas of the *Standard solutions* versus the histamine concentrations, and determine the standard curve best fitting the plotted points.

**System suitability**

**Samples:** *Sensitivity solution*, *Standard solutions*, and *Check standard*

**Suitability requirements**

**Linearity:** The correlation coefficient (*R*) of the *Standard curve* should be NLT 0.99.

**Percent recovery of the *Check standard*:** 80%–120%

**Relative standard deviation:** Inject 3 replicates of the *Standard solution* prepared at 10 ng/mL, and record the histamine peak areas. The percent relative standard deviation (%RSD) of the histamine peak areas is NMT 10%. If the sample MS signals are lower than the *Standard curve*, the %RSD is not required.

**Signal-to-noise ratio:** NLT 10 of the histamine peak, *Sensitivity solution*

**Analysis**

**Sample:** *Sample solution*

Inject 1 µl of the *Sample solution*, and record chromatograms and peak area of MS signals.

**Calculation:** From the *Standard curve* obtained and the histamine peak area of the *Sample solution*, determine the histamine concentration, *C*, in the *Sample solution*. Calculate the content of histamine, in ng/mg (ppm), in the gentamicin sulfate sample:

$$\text{Result} = C/S$$

$C$ = concentration of histamine in the *Sample solution* (ng/mL)
$S$ = concentration of gentamicin sulfate in the *Sample solution* (mg/mL)

## ADDITIONAL REQUIREMENTS

• **USP REFERENCE STANDARDS** ⟨11⟩

USP Histamine Dihydrochloride RS

$C_5H_9N_3 \cdot 2HCl$

184.07▲ (USP 1-May-2022)

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# ⟨429⟩ LIGHT DIFFRACTION MEASUREMENT OF PARTICLE SIZE

## INTRODUCTION

*The method is based on the ISO standards 13320-1(1999) and 9276-1(1998).*

This general chapter has been harmonized with the corresponding texts of the *European Pharmacopoeia* and/or the *Japanese Pharmacopoeia.*

The laser light diffraction technique used for the determination of particle-size distribution is based on the analysis of the diffraction pattern produced when particles are exposed to a beam of monochromatic light. Historically, the early laser diffraction instruments only used scattering at small angles. However, the technique has since been broadened to include laser light scattering in a wider angular range and application of the Mie theory, in addition to the Fraunhofer approximation and anomalous diffraction.

The technique cannot distinguish between scattering by single particles and scattering by clusters of primary particles, i.e., by agglomerates or aggregates. As most particulate samples contain agglomerates or aggregates and as the focus of interest is generally on the size distribution of primary particles, the clusters are usually dispersed into primary particles before measurement.

For nonspherical particles, an equivalent sphere-size distribution is obtained because the technique assumes spherical particles in its optical model. The resulting particle-size distribution may differ from those obtained by methods based on other physical principles (e.g., sedimentation, sieving).

This chapter provides guidance for the measurement of size distributions of particles in different dispersed systems (e.g., powders, sprays, aerosols, suspensions, emulsions, and gas bubbles in liquids), through analysis of their angular light-scattering patterns. It does not address specific requirements of particle-size measurement of specific products.

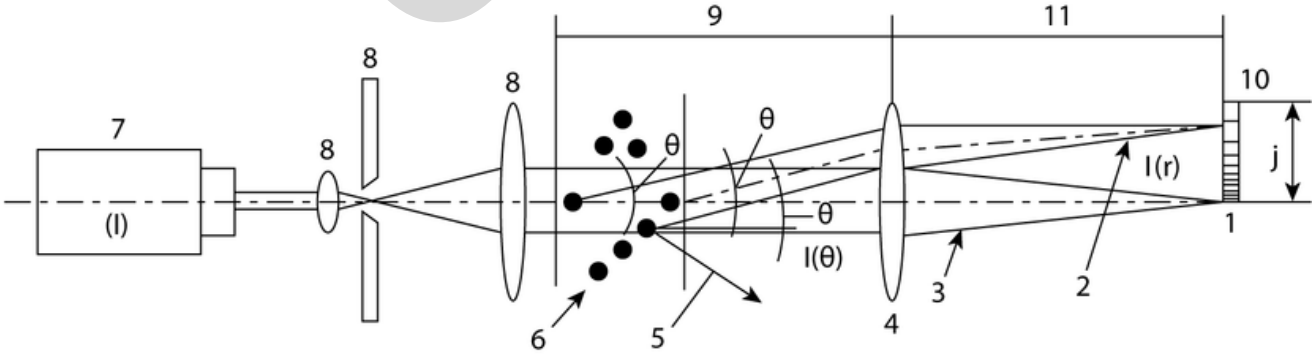
## PRINCIPLE

A representative sample, dispersed at an adequate concentration in a suitable liquid or gas, is passed through a beam of monochromatic light, usually a laser. The light scattered by the particles at various angles is measured by a multi-element detector. Numerical values representing the scattering pattern are then recorded for subsequent analysis. These scattering pattern values are then transformed, using an appropriate optical model and mathematical procedure, to yield the proportion of total volume to a discrete number of size classes, forming a volumetric particle-size distribution.

## INSTRUMENT

The instrument is located in an environment where it is not affected by electrical noise, mechanical vibrations, temperature fluctuations, humidity, or direct bright light.

An example of a setup of a laser light diffraction instrument is given in *Figure 1.* Other equipment may be used.




1. Obscuration detector
2. Scattered beam
3. Direct beam
4. Fourier lens
5. Scattered light not collected by lens (4)
6. Particle ensemble
7. Light source laser
8. Beam processing unit
9. Working distance lens (4)
10. Multi-element detector
11. Focal distance of lens (4)

Figure 1. Example of a set-up of a laser light diffraction instrument.

The instrument comprises a laser light source, beam processing optics, a sample measurement region (or cell), a Fourier lens, and a multi-element detector for measuring the scattered light pattern. A data system is also required for deconvolution of the scattering data into a volumetric size distribution and associated data analysis and reporting.

The particles can enter the laser beam in two positions. In the conventional case the particles enter the parallel beam before the collecting lens and within its working distance. In so-called reversed Fourier optics the particles enter behind the collecting

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lens and thus in a converging beam. The advantage of the conventional setup is that a reasonable path length for the sample is allowed within the working distance of the lens. The second setup allows only small path lengths but enables measurement of scattered light at larger angles, which is useful when submicron particles are present.

The interaction of the incident light beam and the ensemble of dispersed particles results in a scattering pattern with different light intensities at various angles. The total angular intensity distribution, consisting of both direct and scattered light, is then focused onto a multi-element detector by a lens or a series of lenses. These lenses create a scattering pattern that, within limits, does not depend on the location of the particles in the light beam. Hence, the continuous angular intensity distribution is converted into a discrete spatial intensity distribution on a set of detector elements.

It is assumed that the measured scattering pattern of the particle ensemble is identical to the sum of the patterns from all individual single scattering particles presented in random relative positions. Note that only a limited angular range of scattered light is collected by the lens(es) and, therefore, by the detector.

## DEVELOPMENT OF THE METHOD

The measurement of particle size by laser diffraction can give reproducible data, even in the submicron region, provided the instrument used and the sample tested are carefully controlled to limit variability of the test conditions (e.g., dispersion medium, method of preparation of the sample dispersion).

Traditionally, the measurement of particle size using laser diffraction has been limited to particles in the range of approximately 0.1 µm to 3 mm. Because of recent advances in lens and equipment design, newer instruments are routinely capable of exceeding this range. With the validation report, the user demonstrates the applicability of the method for its intended use.

### Sampling

The sampling technique must be adequate to obtain a representative sample of a suitable volume for the particle-size measurement. Sample splitting techniques such as rotating riffler or the cone and quartering method may be applied.

### Evaluation of the Dispersion Procedure

The sample to be analyzed is inspected, visually or with the aid of a microscope, to estimate its size range and particle shape. The dispersion procedure must be adjusted to the purpose of the measurement. The purpose may be such that it is preferable to deagglomerate clusters into primary particles as far as possible, or it may be desirable to retain clusters as intact as possible. In this sense, the particles of interest may be either primary particles or clusters.

For the development of a method, it is highly advisable to check that comminution of the particles does not occur and, conversely, that dispersion of particles or clusters is satisfactory. This can usually be done by changing the dispersing energy and monitoring the change of the particle-size distribution. The measured size distribution must not change significantly when the sample is well dispersed and the particles are neither fragile nor soluble. Moreover, if the manufacturing process (e.g., crystallization, milling) of the material has changed, the applicability of the method must be verified (e.g., by microscopic comparison).

Sprays, aerosols, and gas bubbles in a liquid should be measured directly, provided that their concentration is adequate, because sampling or dilution generally alters the particle-size distribution.

In other cases (such as emulsions, pastes, and powders), representative samples may be dispersed in suitable liquids. Dispersing aids (wetting agents, stabilizers) and/or mechanical forces (e.g., agitation, sonication) are often applied for deagglomeration or deaggregation of clusters and stabilization of the dispersion. For these liquid dispersions, a recirculating system consisting of an optical measuring cell, a dispersion bath usually equipped with stirrer and ultrasonic elements, a pump, and tubing is most commonly used. Nonrecirculating, stirred cells are useful when only small amounts of a sample are available or when special dispersion liquids are used.

Dry powders can also be converted into aerosols through the use of suitable dry powder dispersers that apply mechanical force for deagglomeration or deaggregation. Generally, the dispersers use the energy of compressed gas or the differential pressure of a vacuum to disperse the particles to an aerosol that is blown through the measuring zone, usually into the inlet of a vacuum unit that collects the particles. However, for free-flowing, coarser particles or granules, the effect of gravity may be sufficient to disperse the particles adequately.

If the maximum particle size of the sample exceeds the measuring range of the instrument, the material that is too coarse can be removed by sieving, and the mass and percentage of removed material are reported. However, after presieving, note that the sample is no longer representative, unless proven otherwise.

### Optimization of the Liquid Dispersion

Liquids, surfactants, and dispersing aids used to disperse powders must

- be transparent at the laser wavelength and practically free from air bubbles or particles;
- have a refractive index that differs from that of the test material;
- be a nonsolvent of the test material (pure liquid or prefiltered, saturated solution);
- not alter the size of the test materials (e.g., by solubility, solubility enhancement, or recrystallization effects);
- favor easy formation and stability of the dispersion;
- be compatible with the materials used in the instrument (such as O-rings, gaskets, tubing, etc.); and
- possess a suitable viscosity to facilitate recirculation, stirring, and filtration.

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Surfactants and/or dispersing aids are often used to wet the particles and to stabilize the dispersion. For weak acids and weak bases, buffering of the dispersing medium at low or high pH, respectively, can assist in identifying a suitable dispersant.

A preliminary check of the dispersion quality can be performed by visual or microscopic inspection. It is also possible to take fractional samples out of a well-mixed stock dispersion. Such stock dispersions are formed by adding a liquid to the sample while mixing it with, for example, a glass rod, a spatula or a vortex mixer. Care must be taken to ensure the transfer of a representative sample and that settling of larger particles does not occur. Therefore, a sample paste is prepared or sampling is carried out quickly from a suspension maintained under agitation.

## Optimization of the Gas Dispersion

For sprays and dry powder dispersions, a compressed gas free from oil, water, and particles may be used. To remove such materials from the compressed gas, a dryer with a filter can be used. Any vacuum unit should be located away from the measurement zone, so that its output does not disturb the measurement.

## Determination of the Concentration Range

In order to produce an acceptable signal-to-noise ratio in the detector, the particle concentration in the dispersion must exceed a minimum level. Likewise, it must be below a maximum level in order to avoid multiple scattering. The concentration range is influenced by the width of the laser beam, the path length of the measurement zone, the optical properties of the particles, and the sensitivity of the detector elements.

In view of the above, measurements must be performed at different particle concentrations to determine the appropriate concentration range for any typical sample of material. [NOTE—In different instruments, particle concentrations are usually represented by differently scaled and differently named numbers, e.g., obscuration, optical concentration, proportional number of total mass.]

## Determination of the Measuring Time

The time of measurement, the reading time of the detector, and the acquisition frequency is determined experimentally in accordance with the required precision. Generally, the time for measurement permits a large number of detector scans or sweeps at short time intervals.

## Selection of an Appropriate Optical Model

Most instruments use either the Fraunhofer or the Mie theory, though other approximation theories are sometimes applied for calculation of the scattering matrix. The choice of the theoretical model depends on the intended application and the different assumptions (size, absorbance, refractive index, roughness, crystal orientation, mixture, etc.) made for the test material. If the refractive index values (real and imaginary parts for the used wavelength) are not exactly known, then the Fraunhofer approximation or the Mie theory with a realistic estimate of the refractive index can be used. The former has the advantages that it is simple and it does not need refractive index values; the latter usually provides less-biased particle-size distributions for small particles. For instance, if the Fraunhofer model is used for samples containing an appreciable amount of small, transparent particles, a significantly larger amount of small particles may be calculated. In order to obtain traceable results, it is essential to document the refractive index values used, because small differences in the values assumed for the real and imaginary part of the complex refractive index may cause significant differences in the resulting particle-size distributions. Small values of the imaginary part of the refractive index (about 0.01–0.1 i) are often applied to allow the correction of the absorbance for the surface roughness of the particles. It should be noted, in general, that the optical properties of the substance to be tested, as well as the structure (e.g., shape, surface roughness, and porosity) bear upon the final result.

## Validation

Typically, the validity of a procedure may be assessed by the evaluation of its specificity, linearity, range, accuracy, precision, and robustness. In particle-size analysis by laser light diffraction, specificity as defined by ICH is not applicable as it is not possible to discriminate different components into a sample, nor is it possible to discriminate between agglomerates from dispersed particles unless properly complemented by microscopic techniques. Exploring a linear relationship between concentration and response, or a mathematical model for interpolation, is not applicable to this procedure. Rather than evaluating linearity, this method requires the definition of a concentration range within which the result of the measurements does not vary significantly. Concentrations below that range produce an error due to a poor signal-to-noise ratio, while concentrations above that range produce an error due to multiple scattering. The range depends mostly on the instrument hardware. Accuracy should be confirmed through an appropriate instrument qualification and comparison with microscopy, while precision may be assessed by means of a repeatability determination.

The attainable repeatability of the method mainly depends on the characteristics of the material (milled/not milled, robust/fragile, width of its size distribution, etc.), whereas the required repeatability depends on the purpose of the measurement. Mandatory limits cannot be specified in this chapter, as repeatabilities (different sample preparations) may vary appreciably from one substance to another. However, it is good practice to aim at acceptance criteria for repeatability such as % RSD $\leq$ 10% [n = 6] for any central value of the distribution (e.g., for $x_{50}$). Values at the sides of the distribution (e.g., $x_{10}$ and $x_{90}$) are oriented towards less stringent acceptance criteria such as % RSD $\leq$ 15% [n = 6]. Below 10 µm, these values must be doubled. Robustness may be tested during the selection and optimization of the dispersion media and forces. The change of the dispersing energy may be monitored by the change in the particle-size distribution.

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# MEASUREMENT

## Precautions

The instructions given in the instrument manual are followed:

- never look into the direct path of the laser beam or its reflections;
- earth all instrument components to prevent ignition of solvents or dust explosions;
- check the instrument set-up (e.g., warm-up, required measuring range and lens, appropriate working distance, position of the detector, no direct bright daylight); and
- in the case of wet dispersions, avoid air bubbles, evaporation of liquid, schlieren or other inhomogeneities in the dispersion; similarly, avoid improper mass-flow from the disperser or turbulent airflow in the case of dry dispersions; such effects can cause erroneous particle-size distributions.

## Measurement of the Light Scattering of Dispersed Sample(s)

After proper alignment of the optical part of the instrument, a blank measurement of the particle-free dispersion medium must be performed using the same method as that used for the measurement of the sample. The background signal must be below an appropriate threshold. The detector data are saved in order to subtract them later from the data obtained with the sample. The sample dispersion is measured according to the developed method.

For each detector element, an average signal is calculated, sometimes together with its standard deviation. The magnitude of the signal from each detector element depends upon the detection area, the light intensity, and the quantum efficiency. The coordinates (size and position) of the detector elements together with the focal distance of the lens determine the range of scattering angles for each element. Most instruments also measure the intensity of the central (unscattered) laser beam. The ratio of the intensity of a dispersed sample to that in its absence (the blank measurement) indicates the proportion of scattered light and hence the particle concentration.

## Conversion of Scattering Pattern Into Particle-Size Distribution

This deconvolution step is the inverse of the calculation of a scattering pattern for a given particle-size distribution. The assumption of spherical particle shape is particularly important as most algorithms use the mathematical solution for scattering from spherical particles. Furthermore, the measured data always contain some random and systematic errors, which may vitiate the size distributions. Several mathematical procedures have been developed for use in the available instruments. They contain some weighting of deviations between measured and calculated scattering patterns (e.g., least squares), some constraints (e.g., non-negativity for amounts of particles), and/or some smoothing of the size distribution curve.

The algorithms used are specific to each make and model of equipment, and are proprietary. The differences in the algorithms between different instruments may give rise to differences in the calculated particle-size distributions.

## Replicates

The number of replicate measurements (with individual sample preparations) to be performed, depends on the required measurement precision. It is recommended to set this number in a substance-specific method.

# REPORTING RESULTS

The particle-size distribution data are usually reported as cumulative undersize distribution and/or as density distribution by volume. The symbol x is used to denote the particle size, which in turn is defined as the diameter of a volume-equivalent sphere. Q3(x) denotes the volume fraction undersize at the particle size x. In a graphical representation, x is plotted on the abscissa and the dependent variable Q3 on the ordinate. Most common characteristic values are calculated from the particle-size distribution by interpolation. The particle sizes at the undersize values of 10%, 50%, and 90% (denoted as $x_{10}$, $x_{50}$, and $x_{90}$, respectively) are frequently used. $x_{50}$ is also known as the median particle size. It is recognized that the symbol d is also widely used to designate the particle size, thus the symbol x may be replaced by d.

Moreover, sufficient information must be documented about the sample, the sample preparation, the dispersion conditions, and the cell type. Because the results depend on the particular instrument, data analysis program, and optical model used, these details must also be documented.

# CONTROL OF THE INSTRUMENT PERFORMANCE

Use the instrument according to the manufacturer's instructions and carry out the prescribed qualifications at an appropriate frequency, according to the use of the instrument and substances to be tested.

## Calibration

Laser diffraction systems, although assuming idealized properties of the particles, are based on first principles of laser light scattering. Thus, calibration in the strict sense is not required. However, it is still necessary to confirm that the instrument is

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operating correctly. This can be undertaken using any certified reference material that is acceptable in industrial practice. The entire measurement procedure is examined, including sample collection, sample dispersion, sample transport through the measuring zone, measurement, and the deconvolution procedure. It is essential that the total operational procedure is fully described.

The preferred certified reference materials consist of spherical particles of a known distribution. They must be certified as to the mass-percentage size distribution by an absolute technique, if available, and used in conjunction with an agreed, detailed operation procedure. It is essential that the real and imaginary parts of the complex refractive index of the material are indicated if the Mie theory is applied in data analysis. The representation of the particle-size distribution by volume will equal that of the distribution by mass, provided that the density of the particles is the same for all size fractions.

The response of a laser diffraction instrument meets the requirements if the mean value of $x_{50}$ from at least three independent measurements does not deviate by more than 3% from the certified range of values of the certified reference material. The mean values for $x_{10}$ and $x_{90}$ must not deviate by more than 5% from the certified range of values. Below 10 µm, these values must be doubled.

Although the use of materials consisting of spherical particles is preferable, nonspherical particles may also be employed. Preferably, these particles have certified or typical values from laser diffraction analysis performed according to an agreed, detailed operating procedure. The use of reference values from methods other than laser diffraction may cause a significant bias. The reason for this bias is that the different principles inherent in the various methods may lead to different sphere-equivalent diameters for the same nonspherical particle.

Although the use of certified reference materials is preferred, other well-defined reference materials may also be employed. They consist of substances of typical composition and particle-size distribution for a specified class of substances. Their particle-size distribution has proven to be stable over time. The results must comply with previously determined data, with the same precision and bias as for the certified reference material.

## Qualification of the System

In addition to the calibration, the performance of the instrument must be qualified at regular time intervals or as frequently as appropriate. This can be undertaken using any suitable reference material as mentioned in the previous paragraph.

The qualification of the system is based on the concept that the equipment, electronics, software, and analytical operations constitute an integral system, which can be evaluated as an entity. Thus the entire measurement procedure is examined, including sample collection, sample dispersion, sample transport through the measuring zone, and the measurement and deconvolution procedure. It is essential that the total operational procedure is fully described.

In general, unless otherwise specified in the individual monograph, the response of a laser diffraction instrument is considered to meet the requirements if the $x_{50}$ value does not deviate by more than 10% from the range of values of the reference material. If optionally the values at the sides of the distribution are evaluated (e.g., $x_{10}$ and $x_{90}$), then these values must not deviate by more than 15% from the certified range of values. Below 10 µm, these values must be doubled.

[NOTE—For calibration of the instrument, stricter requirements are laid down in the paragraph on *Calibration*.]

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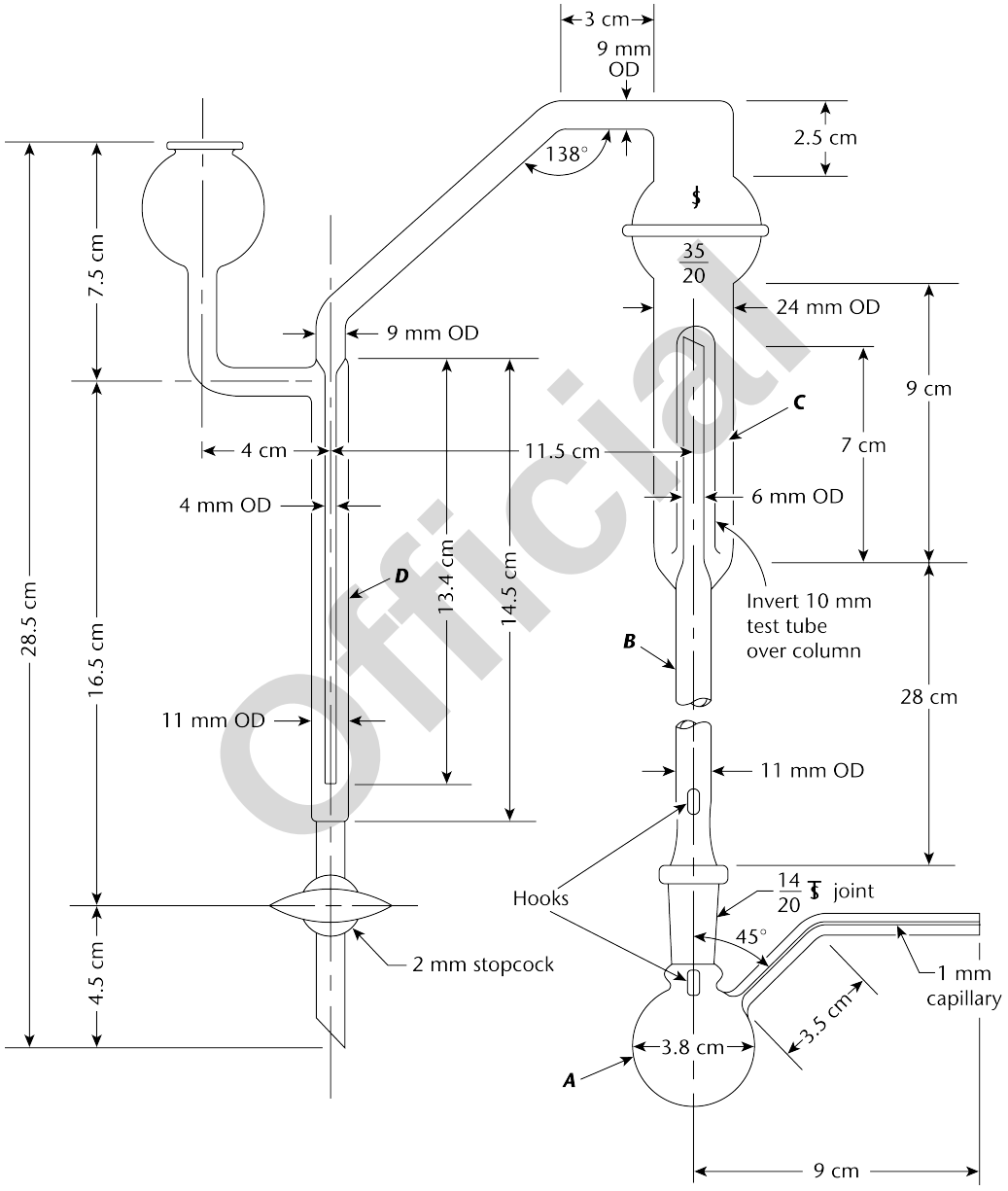
# ⟨431⟩ METHOXY DETERMINATION

## APPARATUS

The apparatus for methoxy determination is shown diagrammatically in the *accompanying figure.*




Apparatus for Methoxy Determination

The boiling flask, A, is fitted with a capillary side-arm for the introduction of carbon dioxide or nitrogen and is connected to a column, B, which serves to separate aqueous hydriodic acid from the more volatile methyl iodide. The methyl iodide passes through water in a scrubber trap, C, and is finally absorbed in the bromine–acetic acid solution in absorption tube D. The carbon dioxide or nitrogen is introduced through a pressure-regulating device and connected to the apparatus by a small capillary containing a small cotton pledget.

[NOTE—Avoid the use of organic solvents in cleaning this apparatus, since traces remaining may interfere with the determination. This test is used also for ethoxy determination with an 80-minute reaction time and a titrant equivalent of 0.751 mg of ($OC_2H_5$).]

For greater convenience in use and cleaning, a ground-glass ball joint connects the two upright columns of the apparatus. The top of the scrubber C consists of a 35/20 ball joint, the upper half of which is connected to the side-arm leading into tube

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D. This permits taking the apparatus apart and facilitates adding the water to the trap. Also, it allows access to the loose inverted (10-mm) test tube that serves as the trap over the inner tube of the scrubber C.

## REAGENTS

### Bromine-Acetic Acid Solution

Dissolve 100 g of potassium acetate in 1000 mL of a solution consisting of 900 mL of glacial acetic acid and 100 mL of acetic anhydride. On the day of use, to 145 mL of this solution add 5 mL of bromine.

### Hydriodic Acid

A colorless, or nearly colorless, constant-boiling reagent solution, prepared for this purpose, is available commercially. If not obtained commercially, it may be prepared by distilling hydriodic acid over red phosphorus, passing carbon dioxide or nitrogen through the apparatus during the distillation. Use the constant-boiling mixture (between 55% and 58% of HI) distilling between 126° and 127°, which is colorless or nearly colorless. [**Caution**—Exercise safety precautions when distilling Hydriodic Acid.] Place the acid in small, amber, glass-stoppered bottles previously flushed with carbon dioxide, or nitrogen, seal with paraffin, and store in a cool, dark place.

## PROCEDURE

Prepare the apparatus by disconnecting the ball joint and pouring water into trap C until it is half-full. Connect the two parts, using a minimal amount of a suitable silicone grease to seal the ball joint. Add 7 mL of *Bromine-Acetic Acid Solution* to absorption tube D. Weigh the sample in a tared gelatin capsule, and add it to the boiling flask along with a few boiling chips or pieces of porous plate. Finally add 6 mL of *Hydriodic Acid* and attach the flask to the column, using a minimal amount of a suitable silicone grease to seal the junction. Bubble the carbon dioxide or nitrogen through the apparatus at the rate of 2 bubbles per second, place the boiling flask in an oil bath or heating mantle heated to 150°, and continue the reaction for 40 minutes for methoxy determination, or 80 minutes for ethoxy determination. Drain the contents of the absorption tube into a 500-mL conical flask containing 10 mL of sodium acetate solution (1 in 4). Rinse the tube with water, adding the rinsings to the flask, and finally dilute with water to about 125 mL. Add formic acid, dropwise, with swirling, until the reddish brown color of the bromine is discharged, then add 3 additional drops. A total of 12 to 15 drops usually is required. Allow to stand for 3 minutes, and add 15 mL of diluted sulfuric acid and 3 g of potassium iodide, and titrate immediately with 0.1 N sodium thiosulfate VS, using 3 mL of starch TS as the indicator. Perform a blank determination, including also a gelatin capsule, and make any necessary correction. Each mL of 0.1 N sodium thiosulfate is equivalent to 0.517 mg of ($OCH_3$).

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# ⟨432⟩ DETERMINATION OF ZETA POTENTIAL BY ELECTROPHORETIC LIGHT SCATTERING

## INTRODUCTION

The zeta potential determination test method is based on the International Organization for Standardization (ISO) 13099-2:2012, *Colloidal systems—Methods for zeta-potential determination—Part 2: Optical methods*, as well as the scientific and technological developments that followed.

Zeta potential, denoted by the Greek letter ζ (hence the name), is a physicochemical characteristic of colloidal systems (suspensions and emulsions) that describes the electric potential difference between the mobile dispersion medium and the stationary layer of the dispersion medium attached to the dispersed particle. It is expressed in units of millivolts (mV). Zeta potential is often used to infer surface properties and to assess colloidal stability. Zeta potential cannot be directly measured but may be derived from experimentally measurable properties, including electroacoustic, electrokinetic, and electrophoretic phenomena.

This chapter provides procedures for the measurement of electrophoretic mobility based on light scattering phenomena, method development, and control of instrument performance. It does not address specific requirements of zeta potential measurement, or the acceptance value for particular products.

## PRINCIPLE

A representative sample dispersed at an adequate concentration in a suitable dispersion medium is placed in a cell equipped with a pair of electrodes to apply an electric potential. Although cell configuration can vary considerably, typically cells consist either of a cylindrical or rectangular capillary with electrodes at both ends, a cuvette, or another type of vessel into which a pair of electrodes are inserted at a known fixed distance. The cell is illuminated by a monochromatic coherent light beam, usually generated by a laser. Because of the applied electric potential, the particles that bear a net electric charge are attracted toward the oppositely charged electrode, a process known as electrophoresis. In the case of charged capillary walls, the dispersion medium stream flows along the capillary wall, a process known as electroosmosis. The overall movement of particles in the cell is the result of both processes. The electrophoretic light scattering technique uses a photoelectric detector to measure the Doppler shift (observed change in the frequency of a wave when the measuring apparatus and the wave source are in relative motion) of the scattered light. The Doppler shift is used to determine the velocity and direction of particle movement from which the electrophoretic mobility (μ) is calculated. Zeta potential is derived from the electrophoretic mobility using the Henry function, which can be approximated by the Smoluchowski equation or Hückel equation according to the relative thickness of the electrical double layer compared to the hydrodynamic radius of the particle.

For discussion of the theory and principles of measurements, see *Analytical Methodologies Based on Scattering Phenomena—Electrophoretic Light Scattering (Determination of Zeta Potential)* ⟨1430.4⟩, which is a helpful, but not mandatory resource.

***Change to read:***

## INSTRUMENT

The instrument should be located in a controlled environment with reduced dust, ▲▲ (USP 1-May-2023) electrical noise, vibrations, temperature fluctuations, humidity, and direct light. The instrument should contain a rigid internal optical system fixed on a bench with a vibration damp setup, and should be installed on a rigid table or bench.

An example of a setup of a zeta potential instrument is given in ⟨1430.4⟩, *Figure 1*. Other possibilities for optical arrangements are shown in ⟨1430.4⟩, *Figure 2*.

As shown in those figures, a typical zeta potential measurement instrument is comprised of the following components:

1. A sample cell to contain the test suspension between two electrodes so that an electric field can be applied
2. A suitable laser to illuminate the particles within the sample
3. A suitable detector to capture the scattered light and Doppler shifts in the scattered light when the electric field is applied
4. A software and computer system to process the collected scattering data and compute the mobility and zeta potential using appropriate models and algorithms

Typically, the Doppler shifts that occur during electrophoresis are quite small (ranging from a few to several hundred Hertz) and cannot be measured directly. To overcome this issue, interferometers are used to combine light that is frequency shifted by the sample with a reference beam. When the scattered light and the reference beam interfere, the result is a beat. The frequency of the beat is the frequency difference between the two underlying beams, which is the Doppler shift induced by the particles.

In practice, however, a simple interferometer cannot determine the sign of the Doppler shift and thus cannot determine the sign of the zeta potential. The measured beat frequency is always positive, whether the Doppler shift is positive or negative.

To overcome this problem, modern zeta potential instruments use a frequency modulator in the reference beam. This modulator causes a controlled Doppler shift at the frequency of the reference light. This means that the measured Doppler shift is the sum of the Doppler shift induced by the modulator plus the Doppler shift induced by the motion of the particles. Thus, the use of a frequency modulator enables the determination of the sign (positive or negative voltage) of the zeta potential.

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# DEVELOPMENT OF THE METHOD

## Sample Considerations—Effect of Physicochemical Parameters of the Colloidal System on the Zeta Potential

### pH

Depending on particle surface chemistry, a change of the dispersion's pH affects the zeta potential. When the pH changes, the ion concentration changes, and it affects the composition of the double layer on the surface of the particles. This, in turn, changes the electrophoretic mobility and the resulting zeta potential.

### CONDUCTIVITY

The conductivity of the dispersion should be ▲appropriate to avoid▲ (USP 1-May-2023) heating and electrolysis.▲▲ (USP 1-May-2023) It is recommended to use a 10 mM saline or similar media. High conductivity media may result in increased Joule–Thomson heating, and thus a reduced voltage should be used. Typically, the conductivity should be within 1–5 millisiemens per cm (mS/cm).

### CONCENTRATION RANGE OF THE COLLOIDAL SYSTEM

The effect of the dispersed material concentration on the zeta potential must be evaluated, and the concentration range of a constant zeta potential established (see *Determination of the Concentration Range*).

### CONCENTRATION OF OTHER ADDED COMPONENTS IN THE SYSTEM

The effects of other system components that need to be part of the colloidal system in a particular application must be evaluated.

### VISCOSITY

The electrophoretic mobility of the moving particles is related to the dispersion medium viscosity as shown in ⟨1430.4⟩, *Equation 3*. The dispersion medium's viscosity should preferably be lower than 10 mPa · s, so that the system does not deviate from Newtonian fluid. For high concentration dispersions, the viscosity of the dispersion medium is used, and consideration is given to other factors.

## Sample Preparation

### SAMPLE STABILITY CONSIDERATIONS

The following two aspects of sample stability are considered when performing zeta potential measurements:

1. Based on the nature of the dispersion being tested, the electrodes in the sample cell and the dispersion itself may change after a zeta potential measurement is performed. Therefore, when reusing the same sample volume or cell for replicate zeta potential testing, the data should be reviewed carefully to ensure the measurements are acceptable. With certain dispersions, a fresh dispersant aliquot may need to be tested for each replicate measurement.
2. The zeta potential determination of a concentrated dispersion may require further dilution of the dispersion using filtered dispersion medium prior to the measurement, in order to lower the particle concentration, and to avoid measurement artifacts due to multiple scattering or inter-particle interactions. It is important to ensure that the diluting dispersion medium has the same electrolyte composition and pH as the dispersion being tested, especially when the goal of the study is to understand the formulation properties of that particular medium. However, the resulting conductivity of the media needs to be within the workable range as shown under *Conductivity*. If the diluted dispersion is prepared well before measurement, the stability of this diluted dispersion should be evaluated by measuring the zeta potential over the expected measurement time, to ensure that there is no time-dependent behavior on the measured zeta potential. Testing of serial dilutions bracketing the expected target dilution factor may also be useful.

## Determination of the Concentration Range

A reliable measurement requires reaching an acceptable signal-to-noise ratio in the detector. To achieve this feat, the dispersed material concentration in the colloidal system being measured must exceed a certain level. Likewise, it must be below a maximum level in order to avoid multiple scattering and ▲colloidal particles▲ (USP 1-May-2023) interaction. The concentration range is influenced by the width of the laser beam, the path length between the electrodes, the optical properties of the materials, and the sensitivity of the detector. Therefore, measurements must be performed at different dispersion concentrations to determine the appropriate concentration range for any specific sample being tested.

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## Method Validation

Typically, the validity of an analytical procedure is assessed by the evaluation of its specificity, linearity, range, accuracy, precision, robustness, and quantitation limit (QL) or detection limit (DL) as needed (see *Validation of Compendial Procedures* ⟨1225⟩). In zeta potential analysis by electrophoretic light scattering, specificity as defined by the International Council for Harmonisation (ICH) is not applicable because it is not possible to discriminate different components of a sample. The range, linearity, DL, and QL as defined in ICH Q2 guidelines and ⟨1225⟩ are also not applicable. Exploring a linear relationship between concentration and response, or a mathematical model for interpolation is not applicable to this procedure since Zeta potential should be a constant value, and should be constant for the optimized concentration range. The DL and QL are also not applicable because the concentration range of the method for the zeta potential measurement represents a different concept from the minimum concentration above which the property could be detected or quantified.

Rather than evaluating linearity, DL, and QL, this method requires the definition of a concentration range and measurement conditions within which the result of the measurements does not vary significantly. Concentrations below that range produce an error due to poor signal-to-noise ratio, while concentrations above that range produce an error due to multiple scattering and ▲colloidal particles▲ (USP 1-May-2023) interaction (e.g., flocculation, aggregation). Evaluation of accuracy is not applicable to the actual sample, but it should be confirmed through an appropriate verification of instrument calibration using appropriate reference materials (see *Verification of Calibration*). Precision may be assessed by means of a repeatability determination and intermediate precision determination (on different days or with different analysts) using the same instrument.

The feasible repeatability of the method depends mainly on the characteristics of the material and dispersion medium, whereas the required repeatability depends on the purpose of the measurement. Mandatory limits cannot be specified in this chapter, as repeatability (different sample preparations) may vary appreciably from one substance to another. However, it is good practice to aim for a variance lower than 10% as acceptance criteria for repeatability, and lower than 15% for intermediate precision.

The robustness assessment should address the sensitivity of the method to other likely sources of variation, and should establish a set of acceptable measurement conditions (see *Measurement Procedure*) and sample considerations (see *Sample Considerations—Effect of Physicochemical Parameters of the Colloidal System on the Zeta Potential*).

# MEASUREMENT PROCEDURE

## Precautions

The instructions provided in the instrument manual should be followed:

- Never look into the direct path of the laser beam or its reflections
- Check the instrument set-up (e.g., warm-up time, required measuring range and lens position, angle of analysis, temperature equilibration)
- Avoid air bubbles, evaporation of liquid, or other inhomogeneities in the suspension (emulsion) being tested

## Dispersion Media

It is critical to ensure that the sample and the sample cell/cuvette are compatible with the dispersion medium. The electrophoretic mobility of the sample is dependent on the chemical characteristics of the dispersion medium. Specifically, the pH and the ion type and concentration need to be controlled if the sample is diluted prior to measurement.

## Measurement Cell

To avoid contamination, the measurement cell must be thoroughly rinsed prior to and after measurement. It is important to control and monitor the temperature of the measurement cell because the dispersion medium viscosity is a function of temperature, and has a significant impact on the measured results.

## Sample Inspection

It is important to visually inspect the sample prior to analysis to check for large particles or sedimentation, which can negatively impact the results and/or reproducibility.

## Preparation

It is important to avoid changing the electrophoretic mobility of the sample during the sample preparation process. Many materials such as pipettes, spatulas, or syringes may have ions on their surface that can contaminate the sample. Disposable plastic materials are recommended if they are chemically compatible with the sample.

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### Measurement of the Light Scattering of Dispersed Sample(s)

REPLICATES

The number of replicate measurements (with individual sample preparations) to be performed depends on the required measurement precision. It is recommended to set this number in a substance-specific method. Typically, three consecutive measurements are performed.

## DATA PROCESSING AND INTERPRETATION

Data processing is done by spectrum analysis, or autocorrelation function, or modulated Brownian motion power spectrum method.

For a more detailed information on data processing, see ⟨1430.4⟩, *5. Data Analysis*.

### Conversion of Doppler Shifts into Electrophoretic Mobility

The relationship between observed Doppler frequency shifts ($\Delta\omega$) of scattered light and corresponding electrophoretic mobility, μ, is shown in *Equation 1* and *Equation 2* for a reference beam optic alignment and a cross beam optic alignment, respectively:

$$\mu = \frac{\Delta\omega\lambda}{4\pi n E \sin(\theta/2)\sin[(\theta/2)+\xi]} \quad \text{Equation 1}$$

$$\mu = \frac{\Delta\omega\lambda}{4\pi n E \sin(\theta'/2)} \quad \text{Equation 2}$$

### Calculation of Zeta Potential

For large solute particles (the hydrodynamic radius is large compared to the thickness of the electrical double layer), the Smoluchowski equation is used to calculate ζ from μ:

$$\zeta = \frac{\eta_0 \mu}{\varepsilon_r \varepsilon_0} \quad \text{Equation 3}$$

For small solute particles (the hydrodynamic radius is small compared to the thickness of the electrical double layer), the Hückel equation is used to calculate ζ from μ:

$$\zeta = \frac{3\eta_0 \mu}{2\varepsilon_r \varepsilon_0} \quad \text{Equation 4}$$

For more detailed information on these equations, see ⟨1430.4⟩, *5.2 Calculation of Zeta Potential*.

***Change to read:***

## CONTROL OF THE INSTRUMENT PERFORMANCE

Use the instrument according to the manufacturer's instructions and carry out the prescribed qualifications at an appropriate frequency for the use of the instrument and substances to be tested.

### Verification of Calibration

The method is based on first principles and no calibration is needed. Verification is usually done with a standard sample.▲▲ (USP 1-May-2023) In general, it is not necessary to do a standard measurement each time a sample is being measured. Rather, it is recommended to run the standard sample "regularly" at predefined intervals.

### Qualification of the System

The performance of the instrument must be qualified at regular time intervals or as frequently as appropriate. This can be achieved using any suitable standard sample as mentioned in the previous paragraph.

The qualification of the system is based on the concept that the equipment, electronics, software, and analytical operations constitute an integral system, which can be evaluated as an entity. Thus, the entire measurement procedure is examined, including sample collection, sample dispersion, sample transport through the measuring zone, and the measurement and deconvolution procedure. It is essential that the total operational procedure is fully described.

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An instrument meets the requirement if the coefficient of variation for the mean electrophoretic mobility values from each measurement for a standard sample is less than 10%, provided that the absolute value of the standard sample mobility is higher than $2 \times 10^{-8}$ $m^2/V \cdot s$.

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# ⟨441⟩ NIACIN OR NIACINAMIDE ASSAY

## ASSAY

### Chemical Method

- **PROCEDURE**

The following photometric procedure involves the colorimetric reaction of niacin or niacinamide with cyanogen bromide in the presence of sulfanilic acid. It can be used for the determination of niacin or niacinamide in the monographs such as *Niacin Injection*, *Niacinamide Injection*, and *Niacinamide Tablets*.

[NOTE—Determine from the labeling if the vitamin in the assay specimen is niacin or niacinamide, and use the corresponding standard preparation (either *Standard niacin preparation* or *Standard niacinamide preparation*) as directed in the *Procedure*.]

**Cyanogen bromide solution:** Dissolve 5 g of cyanogen bromide in water to make 50 mL. [**CAUTION**—Prepare this solution under a hood, as cyanogen bromide volatilizes at room temperature, and the vapor is highly irritating and poisonous.]

**Sulfanilic acid solution:** To 2.5 g of sulfanilic acid add 15 mL of water and 3 mL of 6 N ammonium hydroxide. Mix, and add while stirring, more 6 N ammonium hydroxide, if necessary, until the acid dissolves. Adjust the solution with 3 N hydrochloric acid to a pH of about 4.5 using bromocresol green TS as an external indicator, and dilute with water to 25 mL.

**Standard niacin stock solution:** Transfer 25.0 mg of USP Niacin RS to a 500-mL volumetric flask, dissolve in alcohol solution (1 in 4), dilute with alcohol solution (1 in 4) to volume, and mix. Store in a refrigerator. Each mL of this solution contains 50 µg of USP Niacin RS.

**Standard niacin preparation:** Transfer 10.0 mL of *Standard niacin stock solution* to a 100-mL volumetric flask, dilute with water to volume, and mix. Each mL of this solution contains 5 µg of USP Niacin RS.

**Standard niacinamide stock solution:** Transfer 50.0 mg of USP Niacinamide RS to a 500-mL volumetric flask, dissolve in alcohol solution (1 in 4), dilute with alcohol solution (1 in 4) to volume, and mix. Store in a refrigerator. Each mL of this solution contains 100 µg of USP Niacinamide RS.

**Standard niacinamide preparation:** Transfer 10.0 mL of *Standard niacinamide stock solution* to a 100-mL volumetric flask, dilute with water to volume, and mix. Each mL of this solution contains 10 µg of USP Niacinamide RS.

**Assay preparation:** Prepare as directed in the individual monograph.

**Procedure:** Pipet into four marked tubes the quantities of the appropriate *Standard preparation*, *Assay preparation*, *Ammonia dilution*, and *Water* indicated in *Table 1*. Then add the other constituents, respectively, as listed in the table, according to the directions given herein.

**Table 1. Reaction Mixtures for Niacin or Niacinamide Assay—Chemical Method**

| Constituent | Tube 1 (mL) | Tube 2 (mL) | Tube 3 (mL) | Tube 4 (mL) |
|---|---|---|---|---|
| Standard preparation | 1.0 | 1.0 | — | — |
| Assay preparation | — | — | 1.0 | 1.0 |
| Ammonia dilution (ammonium hydroxide, diluted to 1 in 50) | 0.5 | 0.5 | 0.5 | 0.5 |
| Water | 6.5 | 1.5 | 6.5 | 1.5 |
| Cyanogen bromide solution | — | 5.0 | — | 5.0 |
| Sulfanilic acid solution | 2.0 | 2.0 | 2.0 | 2.0 |
| Hydrochloric acid | 1 drop | — | 1 drop | — |

To *Tube 1* add the *Sulfanilic acid solution*, shake well, and add the *Hydrochloric acid*. Mix, place in a suitable spectrophotometer, and adjust to zero absorbance at 450 nm. To *Tube 2* add the *Cyanogen bromide solution*, mix, and 30 s accurately timed after completion of the addition of the cyanogen bromide, add the *Sulfanilic acid solution*, with swirling. Close the tube, place it in the spectrophotometer, and after 2 min measure its absorbance at 450 nm against *Tube 1* as a blank, designating the absorbance as $A_S$. Repeat the procedure with *Tube 3* (as blank) and *Tube 4*, designating the absorbance of *Tube 4* as $A_U$. Calculate the quantity of niacin or niacinamide in the sample as directed in the individual monograph.

### Chromatographic Methods

The following liquid chromatographic procedures are provided for the determination of niacin or niacinamide as an active pharmaceutical ingredient, a dietary supplement ingredient, or a component in the dietary supplements or pharmaceutical dosage forms.

Use the appropriate USP Reference Standard for the niacin or niacinamide present in the formulation.

Throughout these procedures, protect solutions containing and derived from the test specimen and the Reference Standards from the atmosphere and light, preferably by the use of low-actinic glassware.

- **PROCEDURE 1**

  - This procedure can be used to determine niacin or niacinamide in:
    - Oil- and water-soluble vitamins with minerals tablets
  - This procedure involves the extraction of analytes from the formulation by hot water or mechanical shaking
  - Unless specified in the individual monographs, the *Standard solution*, *Sample solution*, and reagent solutions are prepared as follows.

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**Diluent:** Acetonitrile, glacial acetic acid, and water (5:1:94)
**Mobile phase:** A mixture of methanol, glacial acetic acid, and water (27:1:73) containing 140 mg of sodium 1-hexanesulfonate per 100 mL
**Standard solution:** Transfer 80 mg of either USP Niacin RS or USP Niacinamide RS to a 200-mL volumetric flask, and add 180 mL of *Diluent*. Immerse the flask in a hot water bath maintained at 65°–70° for 10 min with regular shaking or using a vortex mixer, until all the solid materials are dissolved. Chill rapidly in a cold water bath for 10 min to room temperature, and dilute with *Diluent* to volume.
**Sample solution:** For tablets, finely powder NLT 30 tablets. Transfer a portion of the powder, equivalent to 10 mg of niacinamide and 2.5 mg each of pyridoxine hydrochloride, riboflavin, and thiamine hydrochloride, to a 50-mL centrifuge tube. Add 25.0 mL of *Diluent*, and mix using a vortex mixer for 30 s to completely suspend the powder. Immerse the centrifuge tube in a hot water bath maintained at 65°–70°, heat for 5 min, and mix on a vortex mixer for 30 s. Return the tube to the hot water bath, heat for another 5 min, and mix on a vortex mixer for 30 s. Filter a portion of the solution, cool to room temperature, and use the clear filtrate. [NOTE—Use the filtrate within 3 h of filtration.]
**Chromatographic system**
(See *Chromatography* ⟨621⟩, *System Suitability*.)
**Mode:** LC
**Detector:** UV 280 nm
**Column:** 3.9-mm × 30-cm; packing L1
**Flow rate:** 1 mL/min
**Injection volume:** 10 µL
**System suitability**
**Sample:** *Standard solution*
**Suitability requirements**
**Relative standard deviation:** NMT 3.0%
**Analysis**
**Samples:** *Standard solution* and *Sample solution*
Measure the peak areas for niacin or niacinamide.
Calculate the percentage of the labeled amount of niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$) in the portion of the *Sample* taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

$r_U$ = peak response of niacin or niacinamide from the *Sample solution*
$r_S$ = peak response of niacin or niacinamide from the *Standard solution*
$C_S$ = concentration of USP Niacin RS or USP Niacinamide RS in the *Standard solution* (mg/mL)
$C_U$ = nominal concentration of niacin or niacinamide in the *Sample solution* (mg/mL)

- **PROCEDURE 2**
  - This procedure can be used to determine niacin or niacinamide in:
    - Oil- and water-soluble vitamins with minerals tablets
  - This procedure involves the extraction of analytes from the formulation by the *Extraction solvent*, heat, and mechanical shaking
  - Unless specified in the individual monographs, the *Standard solution*, *Sample solution*, and reagent solutions are prepared as follows.

**Solution A:** Transfer 1 mL of glacial acetic acid and 2.5 g of edetate disodium to a 100-mL volumetric flask. Dissolve in and dilute with water to volume.
**Extraction solvent:** *Solution A* and methanol (3:1)
**Mobile phase:** 0.1 M sodium acetate solution (13.6 mg/mL of sodium acetate in water). Adjust with acetic acid to a pH of 5.4. [NOTE—A small amount of methanol (up to 1%) may be added to the *Mobile phase* to improve resolution.]
**Standard stock solution:** 1 mg/mL of USP Niacin RS or USP Niacinamide RS in *Extraction solvent*
**Standard solution:** Transfer 5.0 mL of *Standard stock solution* to a 25-mL volumetric flask, and dilute with *Extraction solvent* to volume.
**Sample solution:** [NOTE—This preparation is suitable for the determination of niacin or niacinamide, pyridoxine, and riboflavin, when present in the formulation.] For tablets, finely powder NLT 20 tablets. Transfer a portion of the powder, equivalent to 2 mg of riboflavin, to a 200-mL volumetric flask. If riboflavin is not present in the formulation, transfer a portion of the powder, equivalent to 2 mg of pyridoxine. If pyridoxine is not present in the formulation, transfer a portion of the powder, equivalent to 20 mg of niacin or niacinamide. Add 100.0 mL of *Extraction solvent*, and mix for 20 min, using a wrist-action shaker. Immerse the flask in a water bath maintained at 70°–75°, and heat for 20 min. Mix on a vortex mixer for 30 s, cool to room temperature, and filter. Use the clear filtrate.
**Chromatographic system**
(See *Chromatography* ⟨621⟩, *System Suitability*.)
**Mode:** LC
**Detector:** UV 254 nm
**Column:** 4.6-mm × 25-cm; packing L1
**Flow rate:** 1 mL/min
**Injection volume:** 20 µL
**System suitability**
**Sample:** *Standard solution*

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**Suitability requirements**

**Relative standard deviation:** NMT 3.0% [NOTE—If necessary, flush the column with methanol between injections.]

**Analysis**

**Samples:** *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$) in the portion of the *Sample* taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

$r_U$ = peak response of niacin or niacinamide from the *Sample solution*
$r_S$ = peak response of niacin or niacinamide from the *Standard solution*
$C_S$ = concentration of USP Niacin RS or USP Niacinamide RS in the *Standard solution* (mg/mL)
$C_U$ = nominal concentration of niacin or niacinamide in the *Sample solution* (mg/mL)

- **PROCEDURE 3**
  - This procedure can be used to determine niacin or niacinamide in:
    - Oil- and water-soluble vitamins with minerals tablets
  - This procedure involves the extraction of analytes from the formulation by mixtures of organic solvents, heat, and mechanical shaking
  - Unless specified in the individual monographs, the *Standard solution*, *Sample solution*, and reagent solutions are prepared as follows.

**Reagent:** 25 mg/mL of edetate disodium in water

**Mobile phase:** Transfer 0.4 mL of triethylamine, 15.0 mL of glacial acetic acid, and 350 mL of methanol to a 2000-mL volumetric flask. Dilute with 0.008 M sodium 1-hexanesulfonate to volume.

**Standard stock solution:** 1.5 mg/mL of USP Niacin RS or USP Niacinamide RS in the *Reagent*, with heating if necessary

**Standard solution:** Transfer 5.0 mL of *Standard stock solution* to a stoppered 125-mL flask. Add 10.0 mL of a mixture of methanol and glacial acetic acid (9:1) and 30.0 mL of a mixture of methanol and ethylene glycol (1:1). Insert the stopper, shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

**Sample solution:** For tablets, weigh and finely powder NLT 20 tablets. Transfer a portion of the powder, equivalent to 7.5 mg of niacin or niacinamide, to a stoppered 125-mL flask. Add 10.0 mL of a mixture of methanol and glacial acetic acid (9:1) and 30.0 mL of a mixture of methanol and ethylene glycol (1:1). Insert the stopper, shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

**Chromatographic system**

(See *Chromatography* ⟨621⟩, *System Suitability*.)

**Mode:** LC

**Detector:** UV 270 nm

**Column:** 4.6-mm × 25-cm; packing L7

**Column temperature:** 50°

**Flow rate:** 2 mL/min

**Injection volume:** 5 µL

**System suitability**

**Sample:** *Standard solution*

**Suitability requirements**

**Relative standard deviation:** NMT 2.0%

**Analysis**

**Samples:** *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$) in the portion of the *Sample* taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

$r_U$ = peak response of niacin or niacinamide from the *Sample solution*
$r_S$ = peak response of niacin or niacinamide from the *Standard solution*
$C_S$ = concentration of USP Niacin RS or USP Niacinamide RS in the *Standard solution* (mg/mL)
$C_U$ = nominal concentration of niacin or niacinamide in the *Sample solution* (mg/mL)

- **PROCEDURE 4**
  - This procedure can be used to describe niacin as:
    - An active pharmaceutical ingredient
    - A dietary supplement ingredient
    - An ingredient in the niacin extended-release tablets
  - This procedure involves either dissolving the analyte in the *Diluent*, a mixture of methanol and water (82:18), or extracting the analyte from the formulation with *Diluent* and mechanical shaking
  - Unless specified in the individual monographs, the *System suitability solution*, *Standard solution A*, *Standard solution B*, *Sample solution A*, *Sample solution B*, and reagent solutions are prepared as follows.

**Diluent:** Methanol and water (82:18)

**Mobile phase:** Methanol and water (82:18), adjusted with glacial acetic acid to a pH of 3.15 ± 0.05

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**System suitability solution:** 0.25 mg/mL of USP Niacin RS, 0.050 mg/mL of USP 6-Hydroxynicotinic Acid RS, and 0.10 mg/mL of pyridine in *Diluent*

**Standard solution A:** For active pharmaceutical ingredient or dietary supplement ingredient, use 0.25 mg/mL of USP Niacin RS in *Diluent*.

**Standard solution B:** For extended-release tablets, use 0.25 mg/mL of USP Niacin RS, 0.050 mg/mL of USP 6-Hydroxynicotinic Acid RS, and 0.0978 mg/mL of pyridine in *Diluent*.

**Sample solution A:** For active pharmaceutical ingredient or dietary supplement ingredient, use 0.25 mg/mL of Niacin in *Diluent*.

**Sample solution B:** For extended-release tablets, transfer a quantity of powder, equivalent to 50 mg of niacin from NLT 20 finely powdered tablets, to a suitable flask. Add *Diluent*, and stir for 2 h. Dilute with *Diluent* to a final concentration of 0.25 mg/mL of niacin.

**Chromatographic system**

(See *Chromatography* ⟨621⟩, *System Suitability*.)

**Mode:** LC

**Detector:** UV 260 nm

**Column:** 4.6-mm × 15-cm; 5-µm packing L8

**Flow rate:** 1 mL/min

**Injection volume:** 25 µL

**System suitability**

**Samples:** *System suitability solution* and *Standard solution A* or *Standard solution B*

[NOTE—The relative retention times for pyridine, 6-hydroxynicotinic acid, and niacin are 0.14, 0.64, and 1.0, respectively, *System suitability solution*.]

**Suitability requirements**

**Resolution:** NLT 1.5 between pyridine and 6-hydroxynicotinic acid, and NLT 1.5 between 6-hydroxynicotinic acid and niacin, *System suitability solution*

**Relative standard deviation:** NMT 2.0% for the niacin peak from replicate injections, *Standard solution A* or *Standard solution B*

**Analysis**

**Samples:** *Standard solution A* and *Sample solution A* or *Standard solution B* and *Sample solution B*

Calculate the percentage of niacin ($C_6H_5NO_2$) in the portion of the *Sample* taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

$r_U$ = peak response of niacin from *Sample solution A* or *Sample solution B*
$r_S$ = peak response of niacin from *Standard solution A* or *Standard solution B*
$C_S$ = concentration of USP Niacin RS in *Standard solution A* or *Standard solution B* (mg/mL)
$C_U$ = concentration of Niacin in *Sample solution A* or nominal concentration of niacin in *Sample solution B* (mg/mL)

- **PROCEDURE 5**
  - This procedure can be used to determine niacinamide as:
    - An active pharmaceutical ingredient
    - A dietary supplement ingredient
  - This procedure involves dissolving the niacinamide as the active pharmaceutical ingredient or a dietary supplement ingredient in the *Mobile phase*
  - Unless specified in the individual monographs, the *System suitability solution*, *Standard solution*, *Niacin solution*, *Sample solution*, and reagent solutions are prepared as follows.

**Mobile phase:** Methanol and 0.005 M sodium 1-heptanesulfonate (30:70)

**Standard solution:** Transfer 50 mg of USP Niacinamide RS to a 100-mL volumetric flask. Add 3 mL of water to dissolve, and dilute with *Mobile phase* to volume. Dilute with *Mobile phase* to 0.04 mg/mL.

**Niacin solution:** Prepare as directed in the *Standard solution*, using USP Niacin RS instead of USP Niacinamide RS.

**System suitability solution:** Mix equal volumes of the *Standard solution* and *Niacin solution*.

**Sample solution:** Prepare as directed in the *Standard solution*, using Niacinamide instead of USP Niacinamide RS.

**Chromatographic system**

(See *Chromatography* ⟨621⟩, *System Suitability*.)

**Mode:** LC

**Detector:** UV 254 nm

**Column:** 3.9-mm × 30-cm; packing L1

**Flow rate:** 2 mL/min

**Injection volume:** 20 µL

**System suitability**

**Samples:** *System suitability solution* and *Standard solution*

**Suitability requirements**

**Resolution:** NLT 3.0 between niacin and niacinamide, *System suitability solution*

**Relative standard deviation:** NMT 2.0% for the niacinamide peak from replicate injections, *Standard solution*

**Analysis**

**Samples:** *Standard solution* and *Sample solution*

Calculate the percentage of niacinamide ($C_6H_6N_2O$) in the portion of the *Sample* taken:

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$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

$r_U$ = peak response of niacinamide from the *Sample solution*
$r_S$ = peak response of niacinamide from the *Standard solution*
$C_S$ = concentration of USP Niacinamide RS in the *Standard solution* (mg/mL)
$C_U$ = concentration of Niacinamide in the *Sample solution* (mg/mL)

- **PROCEDURE 6**
  - This procedure can be used to determine niacin as:
    - An active ingredient in niacin tablets
  - This procedure involves the extraction of the niacin from the formulation by water, heat, and mechanical shaking
  - Unless specified in the individual monographs, the *Standard solution*, *Sample solution*, and reagent solutions are prepared as follows.

**Solution A:** 5-mM solution of sodium 1-hexanesulfonate in water
**Mobile phase:** Methanol, acetonitrile, glacial acetic acid, and *Solution A* (14:7:1:78)
**Standard solution:** 0.050 mg/mL of USP Niacin RS in water. Dissolve with the aid of heat in a steam bath.
**Sample solution:** For tablets, transfer an equivalent to 500 mg of niacin from NLT 20 finely powdered tablets to a suitable flask. Add 50 mL of water, and heat on a steam bath for 30 min. Sonicate for 2 min, shake by mechanical means for 15 min, and cool to room temperature. Dilute with water to 0.050 mg/mL, and filter.
**Chromatographic system**
(See *Chromatography* ⟨621⟩*, System Suitability*.)
**Mode:** LC
**Detector:** UV 262 nm
**Column:** 3.9-mm × 30-cm; packing L1
**Flow rate:** 1.3 mL/min
**Injection volume:** 20 µL
**System suitability**
**Sample:** *Standard solution*
**Suitability requirements**
**Column efficiency:** NLT 1000 theoretical plates for the niacin peak
**Tailing factor:** NMT 2.0 for the niacin peak
**Relative standard deviation:** NMT 2.0% for the niacin peak from replicate injections, *Standard solution*
**Analysis**
**Samples:** *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of niacin ($C_6H_5NO_2$) in the portion of the *Sample* taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

$r_U$ = peak response of niacin from the *Sample solution*
$r_S$ = peak response of niacin from the *Standard solution*
$C_S$ = concentration of USP Niacin RS in the *Standard solution* (mg/mL)
$C_U$ = nominal concentration of niacin in the *Sample solution* (mg/mL)

## ADDITIONAL REQUIREMENTS

- **USP REFERENCE STANDARDS** ⟨11⟩
  USP 6-Hydroxynicotinic Acid RS
  (see *Procedure 4*)
  USP Niacin RS
  USP Niacinamide RS

[NOTE—The previously dried Reference Standards may be stored in a desiccator over silica gel, protected from light.]

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# ⟨451⟩ NITRITE TITRATION

The following general method is provided for the determination of most of the Pharmacopeial sulfonamide drugs and their dosage forms, as well as of other Pharmacopeial drugs for which nitrite titration is particularly suitable.

**PROCEDURE**

Accurately weigh about 500 mg in the case of a sulfonamide, or otherwise the quantity specified in the individual monograph, and transfer to a suitable open vessel. Add 20 mL of hydrochloric acid and 50 mL of water, stir until dissolved, cool to about 15°, and slowly titrate with 0.1 M sodium nitrite VS that previously has been standardized against USP Sulfanilamide RS.

Determine the endpoint electrometrically, using suitable electrodes (platinum-calomel or platinum-platinum). Place the buret tip below the surface of the solution to eliminate air oxidation of the sodium nitrite, and stir the solution gently, using a magnetic stirrer, without pulling a vortex of air under the surface, maintaining the temperature at about 15°. The titration may be carried out manually, or by means of an automatic titrator. In performing it manually, add the titrant until the titration is within 1 mL of the endpoint, and then add it in 0.1-mL portions, allowing not less than 1 minute between additions. (The instrument needle deflects and then returns to approximately its original position until the endpoint is reached.)

The weight, in mg, of the substance to which each mL of 0.1 M sodium nitrite VS is equivalent is as stated in the individual monograph.

For the assay of Tablets of the sulfonamides or other drugs, reduce not less than 20 tablets to a fine powder, weigh accurately a portion of the powder, equivalent to about 500 mg if a sulfonamide, or the quantity of drug specified in the individual monograph, and proceed as directed in the foregoing, beginning with “transfer to a suitable open vessel.”

For the assay of Injections and other liquid forms where the nitrite titration is specified, pipet a portion, equivalent to about 500 mg if a sulfonamide, or the quantity of drug specified in the individual monograph, into a suitable open vessel, and proceed as directed in the foregoing, beginning with “Add 20 mL of hydrochloric acid.”

**ADDITIONAL REQUIREMENTS**

- **USP Reference Standards** ⟨11⟩
  USP Sulfanilamide RS

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# ⟨461⟩ NITROGEN DETERMINATION

Some alkaloids and other nitrogen-containing organic compounds fail to yield all of their nitrogen upon digestion with sulfuric acid; therefore these methods cannot be used for the determination of nitrogen in all organic compounds.

## METHOD I

### Nitrates and Nitrites Absent

Place about 1 g of the substance, accurately weighed, in a 500-mL Kjeldahl flask of hard borosilicate glass. The material to be tested, if solid or semisolid, may be wrapped in a sheet of nitrogen-free filter paper for convenience in transferring it to the flask. Add 10 g of powdered potassium sulfate or anhydrous sodium sulfate, 500 mg of powdered cupric sulfate, and 20 mL of sulfuric acid. Incline the flask at an angle of about 45°, and gently heat the mixture, keeping the temperature below the boiling point until frothing has ceased. Increase the heat until the acid boils briskly, and continue the heating until the solution has been clear green in color or almost colorless for 30 minutes. Allow to cool, add 150 mL of water, mix the contents of the flask, and again cool. Add cautiously 100 mL of sodium hydroxide solution (2 in 5), in such manner as to cause the solution to flow down the inner side of the flask to form a layer under the acid solution. Immediately add a few pieces of granulated zinc, and without delay connect the flask to a Kjeldahl connecting bulb (trap), previously attached to a condenser, the delivery tube from which dips beneath the surface of 100 mL of boric acid solution (1 in 25) contained in a conical flask or a wide-mouth bottle of about 500-mL capacity. Mix the contents of the Kjeldahl flask by gentle rotation, and distill until about four-fifths of the contents of the flask has distilled over. Titrate with 0.5 N sulfuric acid VS, determining the endpoint potentiometrically. Perform a blank determination, and make any necessary correction. Each mL of 0.5 N sulfuric acid VS is equivalent to 7.003 mg of nitrogen.

When the nitrogen content of the substance is known to be low, the 0.5 N sulfuric acid VS may be replaced by 0.1 N sulfuric acid VS. Each mL of 0.1 N sulfuric acid VS is equivalent to 1.401 mg of nitrogen.

### Nitrates and Nitrites Present

Place a quantity of the substance, accurately weighed, corresponding to about 150 mg of nitrogen, in a 500-mL Kjeldahl flask of hard borosilicate glass, and add 25 mL of sulfuric acid in which 1 g of salicylic acid previously has been dissolved. Mix the contents of the flask, and allow the mixture to stand for 30 minutes with frequent shaking. To the mixture add 5 g of powdered sodium thiosulfate, again mix, then add 500 mg of powdered cupric sulfate, and proceed as directed under *Nitrates and Nitrites Absent*, beginning with "Incline the flask at an angle of about 45°."

When the nitrogen content of the substance is known to exceed 10%, 500 mg to 1 g of benzoic acid may be added, prior to digestion, to facilitate the decomposition of the substance.

## METHOD II

### Apparatus

Select an appropriate 300-mL Kjeldahl flask, from which the nitrogen is first liberated by acid digestion and then transferred quantitatively to the titration vessel by steam distillation.

### Procedure

Place an accurately weighed or measured quantity of the material, equivalent to 2 to 3 mg of nitrogen, in the digestion flask of the apparatus. Add 1 g of a powdered mixture of potassium sulfate and cupric sulfate (10:1), and wash down any adhering material from the neck of the flask with a fine jet of water. Add 7 mL of sulfuric acid, allowing it to rinse down the wall of the flask, then, while swirling the flask, add 1 mL of 30 percent hydrogen peroxide cautiously down the side of the flask. (Do not add hydrogen peroxide during the digestion.)

Heat the flask over a free flame or an electric heater until the solution has a clear blue color and the sides of the flask are free from carbonaceous material. Cautiously add to the digestion mixture 70 mL of water, cool the solution, and arrange for steam distillation. Add through a funnel 30 mL of sodium hydroxide solution (2 in 5) in such manner as to cause the solution to flow down the inner side of the flask to form a layer under the acid solution, rinse the funnel with 10 mL of water, tightly close the apparatus, and begin the distillation with steam immediately. Receive the distillate in 15 mL of boric acid solution (1 in 25), to which has been added 3 drops of methyl red-methylene blue TS and sufficient water to cover the end of the condensing tube. Continue the distillation until the distillate measures 80 to 100 mL. Remove the absorption flask, rinse the end of the condensing tube with a small quantity of water, and titrate the distillate with 0.01 N sulfuric acid VS. Perform a blank determination, and make any necessary correction. Each mL of 0.01 N sulfuric acid VS is equivalent to 140.1 µg of nitrogen.

When a quantity of material containing more than 2 to 3 mg of nitrogen is taken, 0.02 N or 0.1 N sulfuric acid may be employed, provided that at least 15 mL is required for the titration. If the total dry weight of material taken is greater than 100 mg, increase proportionately the quantities of sulfuric acid and sodium hydroxide.

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# ⟨466⟩ ORDINARY IMPURITIES

This test, where called for in the individual monograph, is provided to evaluate the presence of ordinary impurities in official articles. Ordinary impurities are defined as those species in drug substances and/or drug products that have no significant, undesirable biological activity in the amounts present. These impurities may arise out of the synthesis, preparation, or degradation of compendial articles. In certain instances, impurities that pose a potential health risk may be detected. Because these impurities would not be individually identified by the strict use of this General Chapter, a separate evaluation may be necessary to ensure that the detected impurities fit the requirements set forth in the definition of Ordinary Impurities. Selections of tests and assays allow for anticipated amounts of impurities that are unobjectionable for the customary use of the article.

## REPORTING AND SPECIFICATIONS

The value of 2.0%, unless otherwise specified in the individual monograph, was selected as the general limit for the total amount of ordinary impurities in monographs where documentation did not support adoption of other values.

Where a monograph sets limits on concomitant components and/or specified impurities/degradation products, these species are not to be included in the estimation of ordinary impurities unless so stated in the individual monograph. Concomitant components are defined as species characteristic of many drug substances that are not considered to be impurities in the Pharmacopeial sense. Examples of concomitant components are geometric and optical isomers (or racemates) and antibiotics that are mixtures. Any component that can be considered a toxic impurity because of significant undesirable biological effect is not considered to be a concomitant component.

## METHODOLOGY

Unless otherwise specified in an individual monograph, estimation of the amount and number of ordinary impurities is made by relative methods rather than by strict comparison to individual Reference Standards. Nonspecific detection of ordinary impurities is also consistent with this classification.

Typical evaluation methods used for ordinary impurities are thin-layer chromatographic (TLC) techniques. See *Chromatography* ⟨621⟩ for a general discussion of the thin-layer chromatographic technique. Tests for related substances or chromatographic purity may also be used to evaluate the presence of ordinary impurities. Other methods (e.g., HPLC, HPTLC, etc.) may also be used with adequate justification as an alternate method. Unless otherwise specified in the individual monograph, use the following method.

### Test Solution

Prepare, in the solvent specified in the monograph, a solution of the substance under test having an accurately known final concentration of about 10 mg per mL. [NOTE—Heat or sonication may be used to dissolve the drug substance where use of such does not adversely affect the compound.]

### Standard Solutions

Prepare, in the solvent specified in the monograph, solutions of the USP Reference Standard or designated substance having accurately known concentrations of 0.01 mg per mL, 0.05 mg per mL, 0.1 mg per mL, and 0.2 mg per mL.

[NOTE—Heat or sonication may be used to dissolve the drug substance where use of such does not adversely affect the compound.]

### Procedure

Use a thin-layer chromatographic plate coated with a 0.25-mm layer of chromatographic silica gel mixture, and the *Eluant* specified in the monograph. Apply equal volumes (20 µL) of the *Test Solution* and *Standard Solutions* to the plate, using a stream of nitrogen to dry the spots.

Allow the chromatogram to develop in a pre-equilibrated chamber until the solvent front has moved about three-fourths of the length of the plate. Remove the plate from the chamber, and air-dry. View the plate using the visualization technique(s) specified. Locate any spots other than the principal spot in the chromatogram of the *Test Solution,* and determine their relative intensities by comparison with the chromatograms of the appropriate *Standard Solutions.* See discussion above with regard to reporting and specifying total ordinary impurities.

***Change to read:***

## KEY FOR VISUALIZATION TECHNIQUES

1. Use UV light at 254 nm and at about 366 nm.
2. Use Iodoplatinate TS.
▲3. *Solution A*—Mix 850 mg of bismuth subnitrate with 40 mL of water and 10 mL of glacial acetic acid.

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*Solution B*—Dissolve 8 g of potassium iodide in 20 mL of water. Mix A and B together to obtain a Stock Solution which can be stored for several months in a dark bottle. Mix 10 mL of the Stock Solution with 20 mL of glacial acetic acid, and dilute with water to make 100 mL, to prepare the spray reagent.

4.▲ (ERR 1-Oct-2018) *Ninhydrin Spray*—Dissolve 200 mg of ninhydrin in 100 mL of alcohol. Heat the plate after spraying.

▲5.▲ (ERR 1-Oct-2018) *Acid Spray*—In an ice bath, add slowly and cautiously, with stirring, 10 mL of sulfuric acid to 90 mL of alcohol. Spray the plate, and heat until charred.

▲6.▲ (ERR 1-Oct-2018) *Acid–Dichromate Spray*—Add sufficient potassium dichromate to 100 mL of sulfuric acid to make a saturated solution. Spray the plate, and heat until charred.

▲7.▲ (ERR 1-Oct-2018) *Vanillin*—Dissolve 1 g of vanillin in 100 mL of sulfuric acid.

▲8.▲ (ERR 1-Oct-2018) *Chloramine T–Trichloroacetic Acid*—Mix 10 mL of a 3% aqueous solution of chloramine T with 40 mL of a 25% alcoholic solution of trichloroacetic acid. Prepare immediately before use.

▲9.▲ (ERR 1-Oct-2018) *Folin-C*—Add 10 g of sodium tungstate and 2.5 g of sodium molybdate to 70 mL of water, add 5 mL of 85% phosphoric acid and 10 mL of 36% hydrochloric acid, and reflux this solution for 10 hours.

▲10.▲ (ERR 1-Oct-2018) $KMnO_4$—Dissolve 100 mg of Potassium Permanganate in 100 mL of water.

▲11.▲ (ERR 1-Oct-2018) *DAB*—Mix 1 g of *p*-dimethylaminobenzaldehyde in 100 mL of 0.6 N hydrochloric acid.

▲12.▲ (ERR 1-Oct-2018) *DAC*—Mix 100 mg of *p*-dimethylaminocinnamaldehyde in 100 mL of 1 N hydrochloric acid.

▲13.▲ (ERR 1-Oct-2018) *Ferricyanide*—Mix equal volumes of a 1% ferric chloride solution and a 1% potassium ferricyanide solution. Use immediately.

▲14.▲ (ERR 1-Oct-2018) *Fast Blue B*—Reagent A—Dissolve 500 mg of Fast Blue B Salt in 100 mL of water. Reagent B—0.1 N sodium hydroxide.
Spray first with A, then with B.

▲15.▲ (ERR 1-Oct-2018) *Alkaline Ferric Cyanide*—Dilute 1.5 mL of a 1% potassium ferricyanide solution with water to 20 mL, and add 10 mL of 15% sodium hydroxide solution.

▲16.▲ (ERR 1-Oct-2018) *Iodine Spray*—Prepare a 0.5% solution of iodine in chloroform.

▲17.▲ (ERR 1-Oct-2018) Expose the plate for 10 minutes to iodine vapors in a pre-equilibrated closed chamber, on the bottom of which there are iodine crystals.

▲18.▲ (ERR 1-Oct-2018) *Solution A*—Dissolve 0.5 g of potassium iodide in 50 mL of water. *Solution B*—Prepare a solution of 0.5 g of soluble starch in 50 mL of hot water.
Just prior to use, mix equal volumes of *Solution A* and *Solution B*.

▲19.▲ (ERR 1-Oct-2018) *PTSS*—Dissolve 20 g of *p*-toluenesulfonic acid in 100 mL of alcohol, spray the plate, dry for 15 minutes at 110°, and view under UV light at 366 nm.

▲20.▲ (ERR 1-Oct-2018) *o-Tolidine Spray*—Dissolve 160 mg of *o*-tolidine in 30 mL of glacial acetic acid, dilute with water to make 500 mL, add 1 g of potassium iodide, and mix until the potassium iodide has dissolved.

▲21.▲ (ERR 1-Oct-2018) Mix 3 mL of chloroplatinic acid solution (1 in 10) with 97 mL of water, followed by the addition of 100 mL of potassium iodide solution (6 in 100) to prepare the spray reagent.

▲22.▲ (ERR 1-Oct-2018) *Iodine–Methanol Spray*—Prepare a mixture of iodine TS and methanol (1:1).

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# ⟨467⟩ RESIDUAL SOLVENTS

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## 1. INTRODUCTION

For pharmacopeial purposes, residual solvents in pharmaceuticals are defined as organic volatile chemicals that are used or produced in the manufacturing of drug substances, excipients, or dietary ingredients, or in the preparation of drug products or dietary supplement products. Appropriate selection of the solvent for the synthesis of a drug substance or an excipient may enhance the yield or determine characteristics such as crystal form, purity, and solubility. Therefore, the solvent may sometimes be a critical element in the synthetic process and may not be completely removed by the manufacturing process. Because residual solvents do not provide therapeutic benefit, they should be removed, to the extent possible, to meet safety-based limits, ingredient and product specifications, good manufacturing practices, or other quality-based requirements.

The objective of this general chapter is to define acceptable amounts of residual solvents in pharmaceutical drug products and dietary supplement products for the safety of the patient. Tests for residual solvents are not generally mentioned in specific monographs because the solvents used may vary from one manufacturer to another; however, the limits to be applied must comply with those specified in this general chapter.

This chapter provides procedures for the analysis of residual solvents, although alternative validated methodologies may also be used to demonstrate compliance with the defined limits. For guidance on verification of *USP* procedures or validation of alternative methods for residual solvents, see *Residual Solvents—Verification of Compendial Procedures and Validation of Alternative Procedures* ⟨1467⟩. This chapter does not address solvents deliberately used as excipients, nor does it address solvates. The limits specified in this chapter do not apply directly to excipients, drug substances, or dietary ingredients except where specified in the individual monographs. However, residual solvent levels present in drug substances, excipients, and dietary ingredients may be used to demonstrate compliance as an integral part of the control strategy, thereby reducing or eliminating the need for analysis in the product (see *3. Control Strategy*).

Information on residual solvents in coating materials, colorants, flavors, capsules, and imprinting inks is generally not needed unless Class 1 solvents are used in the manufacture of these components.

Throughout this chapter, the term “likely to be present” (LTBP) refers to 1) solvents used or produced in the final manufacturing step; 2) solvents used or produced in earlier manufacturing steps that are not consistently removed by a validated process; and 3) solvents properly declared by a validated supplier of a drug substance, excipient, or dietary ingredient.

## 2. CLASSIFICATION OF RESIDUAL SOLVENTS BY RISK ASSESSMENT

Residual solvents assessed in this general chapter are listed in *Appendix 1* by common names and structures. USP is aligned with the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) Harmonised Tripartite Guideline for Residual Solvents Q3C (R5) approach for the classification of residual solvents

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(see *Appendix 2* for additional details). These solvents were evaluated for their possible risk to human health and were placed into one of three classes based on their toxicity data and their environmental impact as shown in *Table 1*.

**Table 1. Classification of Residual Solvents and Their Assessments**

| Residual Solvent Classes | Assessment |
|---|---|
| Class 1 (solvents to be avoided) | Known human carcinogens<br>Strongly suspected human carcinogens<br>Solvents particularly known to have ozone-depleting properties |
| Class 2 (solvents to be limited) | Nongenotoxic animal carcinogens or possible causative agents of other irreversible toxicity, such as neurotoxicity or teratogenicity<br>Solvents suspected of other significant but reversible toxicities |
| Class 3 (solvents with low toxic potential) | Solvents with low toxic potential to humans; no health-based exposure limit is needed |

The limits for Class 2 solvents are based on the toxicological permitted daily exposure (PDE), calculated as defined in *Appendix 3*, whereas Class 3 solvents are considered less toxic, and control to 50 mg/day or less for each of these solvents is acceptable without justification. The lists in this chapter are not exhaustive and are subject to revision. For solvents not listed, *Appendix 3* may be used to define PDE limits if sufficient toxicological data are available. A flow diagram for potential sources of residual solvents in pharmaceutical drug products and dietary supplements is shown in *Figure 1*.

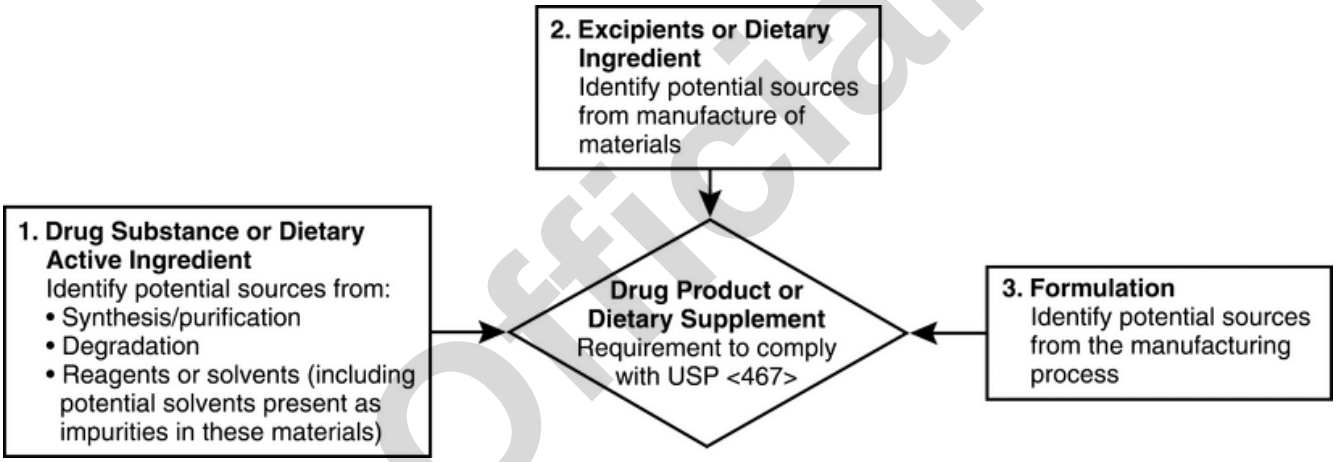


Figure 1. Potential sources of residual solvents in pharmaceutical drug products and dietary supplements.

There are three potential sources of residual solvents in the pharmaceutical drug products and dietary supplement products that should be considered (see *Figure 1*):

1. Drug substance or dietary active ingredient
   - Potential solvent sources include their use or formation during the synthesis or purification of the drug substance; their presence in raw materials or reagents used in their synthesis; or degradation of the drug substance.
2. Excipients and/or dietary ingredient
   - Potential solvent sources include their use or formation during the manufacture or purification of excipients.
3. Formulation
   - Potential solvent sources are associated with their use or formation during the official product manufacturing process.

The potential presence of other solvents as impurities in the solvents used must be taken into consideration in the assessment of solvents LTBP. Because of the high toxicity of benzene and other Class 1 solvents, all likely sources of these solvents must be considered. For example, potential sources of benzene may include its presence as an impurity in a solvent used in the manufacturing process; its use in the manufacture of starting material; or its production as a reaction by-product. In the event that the user has insufficient information to complete a thorough assessment of the potential sources of residual solvents (or as an alternative to performing this assessment), solvent screening may be used (see *8. Analytical Procedures for Class 1 and Class 2 Residual Solvents*).

Pharmaceutical drug products and dietary supplement products should contain no higher levels of residual solvents than can be supported by safety data. All solvents included in this general chapter are listed in *Appendix 1*. Those solvents that show toxicity of special concern or carcinogenicity, and/or atmospheric ozone-depletion effects (Class 1, see *Table 2*), should be avoided in the production of drug substances, dietary supplement ingredients, excipients, pharmaceutical drug products, and dietary supplement products unless their use can be strongly justified in a risk-benefit assessment. Those solvents associated with less severe but still significant toxicity (Class 2, see *Table 3*) should be limited to protect patients from potential adverse effects. Whenever it is practicable, less toxic solvents (Class 3, see *Table 4*) should be used. For the purposes of this pharmacopeia, when a manufacturer has received approval from a regulatory authority for the use of a solvent not currently listed in this

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chapter, or for a level of a listed solvent higher than the limit currently given in this chapter, it is the responsibility of that manufacturer to notify USP of the identity and level of the solvent, and the appropriate test procedure. USP will then address the information in the individual monograph. A new solvent or revised limit that has been approved through the ICH process will be added to the appropriate list in this general chapter. See *Appendix 2* for additional background information related to residual solvents.

## 3. CONTROL STRATEGY

The user may choose to demonstrate compliance with this chapter by analysis for residual solvents in the official product or by analysis of the official substances. These control strategy options are presented in *Figure 2*.

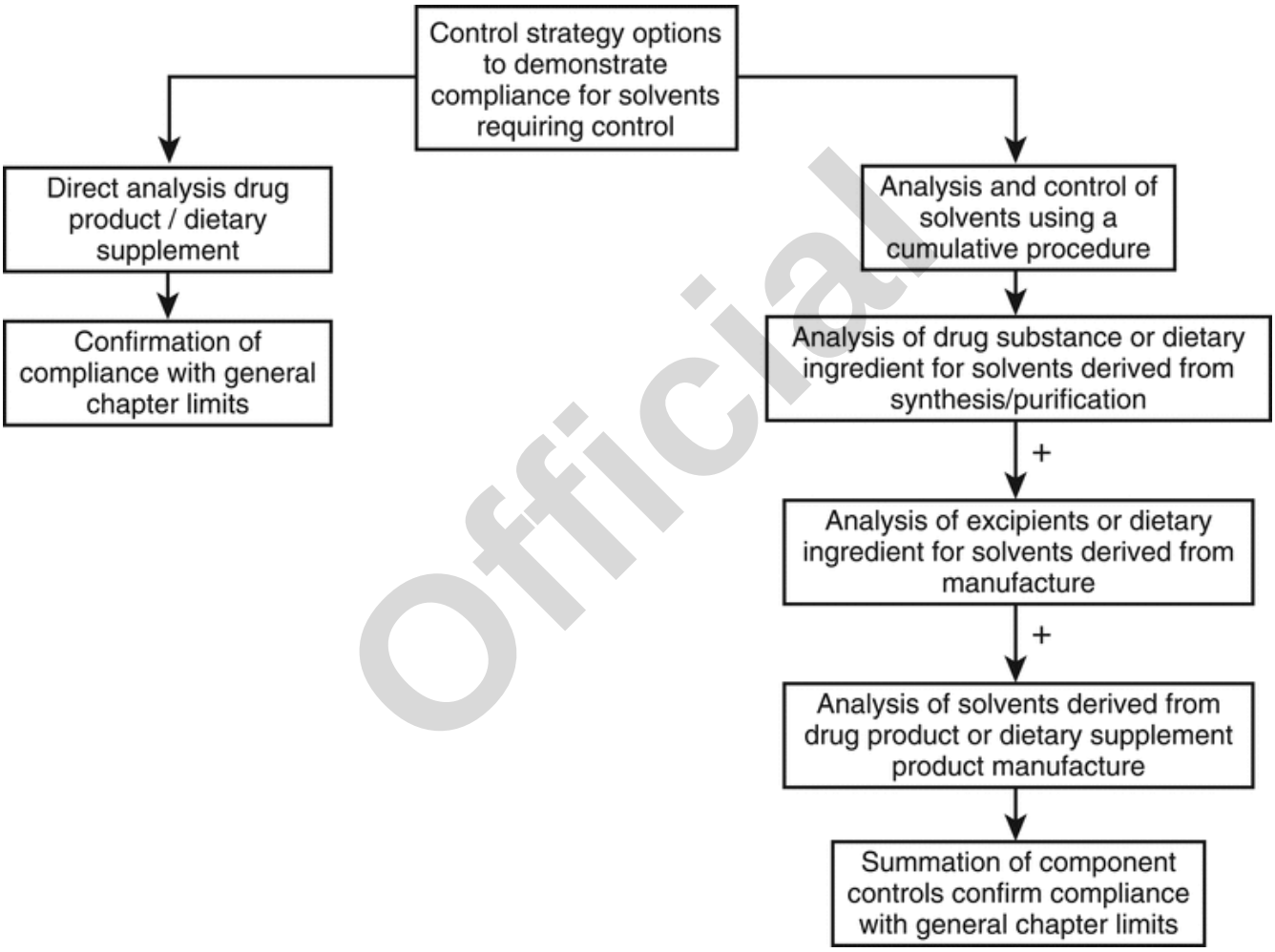


Figure 2. Control strategy options.

Compliance with the chapter requires that all solvents LTBP comply with the control limits.
The following options are to be considered when designing the control strategy.

1. **Testing the official product:** This approach is acceptable in all cases.
2. **Analysis of the official substances and using a cumulative approach to determine the solvent content in the official product:**
    A. **If no solvent(s) are used during the manufacture of the official product,** use the cumulative approach to determine the solvent content in the active pharmaceutical ingredient (API) and excipients or dietary ingredients to calculate solvent levels in the official product.
    B. **If solvent(s) are used during the manufacture of the official product,** use the cumulative approach as described in (a) above. Then, for each solvent used in the manufacture, determine the content in the finished product or after the manufacturing step where the solvent(s) were used.

In all cases, the solvent levels in the final official product must not exceed the limits defined in the chapter.

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# 4. LIMITS OF RESIDUAL SOLVENTS

## 4.1 Class 1: Solvents to Be Avoided

Class 1 residual solvents (*Table 2*) should not be used in the manufacture of drug substances, excipients, dietary ingredients, or official products because of their unacceptable toxicities or deleterious environmental effects. However, if their use in order to produce an official product with a significant therapeutic advance is unavoidable, their levels should be restricted as shown in *Table 2*, unless otherwise stated in the individual monograph. The solvent 1,1,1-trichloroethane is included in *Table 2* because it is a severe environmental hazard. The stated limit of 1500 ppm is based on a review of safety data.

The procedures described in *7. Identification, Control, and Quantification of Residual Solvents* in this general chapter are to be applied wherever possible. Otherwise an appropriate validated procedure is to be used.

**Table 2. Control Limits for Class 1 Residual Solvents in Official Products: Solvents to Be Avoided**

| Solvent | Concentration Limit (ppm) | Concern |
|---|---|---|
| Benzene | 2 | Carcinogen |
| Carbon tetrachloride | 4 | Toxic and environmental hazard |
| 1,2-Dichloroethane | 5 | Toxic |
| 1,1-Dichloroethene | 8 | Toxic |
| 1,1,1-Trichloroethane | 1500 | Environmental hazard |

## 4.2 Class 2: Solvents to Be Limited

Class 2 residual solvents (*Table 3*) should be limited in drug substances, excipients, dietary ingredients, and official products because of the inherent toxicities of these residual solvents. PDEs are given to the nearest 0.1 mg/day, and concentrations are given to the nearest 10 ppm. The method used to establish PDEs for residual solvents is presented in *Appendix 3*.

**Table 3. Class 2 Residual Solvents in Official Products**

| Solvent | PDE (mg/day) | Concentration Limit (ppm) |
|---|---|---|
| Acetonitrile | 4.1 | 410 |
| Chlorobenzene | 3.6 | 360 |
| Chloroform | 0.6 | 60 |
| Cumene | 0.7 | 70 |
| Cyclohexane | 38.8 | 3880 |
| 1,2-Dichloroethene | 18.7 | 1870 |
| 1,2-Dimethoxyethane | 1.0 | 100 |
| *N,N*-Dimethylacetamide | 10.9 | 1090 |
| *N,N*-Dimethylformamide | 8.8 | 880 |
| 1,4-Dioxane | 3.8 | 380 |
| 2-Ethoxyethanol | 1.6 | 160 |
| Ethylene glycol | 6.2 | 620 |
| Formamide | 2.2 | 220 |
| Hexane | 2.9 | 290 |
| Methanol | 30.0 | 3000 |
| 2-Methoxyethanol | 0.5 | 50 |
| Methylbutylketone | 0.5 | 50 |
| Methylcyclohexane | 11.8 | 1180 |
| Methylene chloride | 6.0 | 600 |
| Methylisobutylketone | 45 | 4500 |
| *N*-Methylpyrrolidone | 5.3 | 530 |
| Nitromethane | 0.5 | 50 |
| Pyridine | 2.0 | 200 |
| Sulfolane | 1.6 | 160 |

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**Table 3. Class 2 Residual Solvents in Official Products** *(continued)*

| Solvent | PDE (mg/day) | Concentration Limit (ppm) |
|---|---|---|
| Tetrahydrofuran | 7.2 | 720 |
| Tetralin | 1.0 | 100 |
| Toluene | 8.9 | 890 |
| Trichloroethylene | 0.8 | 80 |
| Xylene[a] | 21.7 | 2170 |

[a] Usually 60% *m*-xylene, 14% *p*-xylene, and 9% *o*-xylene with 17% ethyl benzene.

## 4.3 Class 3: Solvents with Low Toxic Potential

Class 3 solvents (*Table 4*) are regarded as less toxic and of lower risk to human health than Class 1 and Class 2 residual solvents. Class 3 includes no solvent known to be a human health hazard at levels normally accepted in pharmaceuticals. However, there are no long-term toxicity or carcinogenicity studies for many of the residual solvents in Class 3. Available data indicate that they are less toxic in acute or short-term studies and negative in genotoxicity studies.

It is considered that amounts of these residual solvents of 50 mg/day or less of each solvent (corresponding to 5000 ppm or 0.5% w/w in *Option 1*, which is described below) would be acceptable for each solvent without justification. Higher amounts may also be acceptable, provided that they are realistic in relation to manufacturing capability and good manufacturing practice. If a Class 3 solvent limit in an individual monograph is greater than 0.5%, that residual solvent should be identified and quantified. The procedures described in *7. Identification, Control, and Quantification of Residual Solvents*, with appropriate modifications to the standard solutions, are to be applied wherever possible. Otherwise, an appropriate validated procedure is to be used.

**Table 4. Class 3 Residual Solvents in Official Products**

| | |
|---|---|
| Acetic acid | Isobutyl acetate |
| Acetone | Isopropyl acetate |
| Anisole | Methyl acetate |
| 1-Butanol | 3-Methyl-1-butanol |
| 2-Butanol | Methylethylketone |
| Butyl acetate | |
| *tert*-Butylmethyl ether | 2-Methyl-1-propanol |
| Dimethyl sulfoxide | Pentane |
| Ethanol | 1-Pentanol |
| Ethyl acetate | 1-Propanol |
| Ethyl ether | 2-Propanol |
| Ethyl formate | Propyl acetate |
| Formic acid | Triethylamine |
| Heptane | |

# 5. OPTIONS FOR DESCRIBING LIMITS OF CLASS 2 AND CLASS 3 RESIDUAL SOLVENTS

This chapter provides two options for establishing compliance. Both *Option 1* and *Option 2* are based on the PDE.

## 5.1 Option 1—Concentration Limit

The concentration limits in ppm stated in *Table 3* for Class 2 solvents and the general requirement for Class 3 (5000 ppm, equivalent to 0.5% w/w) are used. The values for Class 2 solvents were calculated using the equation below by assuming a product weight of 10 g administered daily.

$$\text{Concentration (ppm)} = (1000\ \mu g/mg \times \text{PDE})/\text{dose}$$

Here, PDE is given in terms of milligrams per day (mg/day), and dose is given in grams per day (g/day). These limits are considered acceptable for all drug substances, excipients, dietary ingredients, and official products. Therefore, *Option 1* may be applied if the daily amount is not known or does not exceed 10 g. If all official substances (drug substances, excipients, and/or dietary ingredients) in a formulation or dietary supplement meet the limits given in *Option 1*, these components may be used in any proportions. No further calculation is necessary, provided that the daily amount does not exceed 10 g. Products

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that are administered in doses (daily intake for dietary supplements) greater than 10 g/day are to be considered under *Option 2*.

## 5.2 Option 2—Summation of Components Content

*Option 2* uses the PDE and the actual maximum daily amount of the product to calculate solvent exposure and assess compliance.

*Option 2* must be used to demonstrate compliance with this chapter where the maximum daily dose of the official product exceeds 10 g/day or where at least one component in the formulation exceeds the *Option 1* limits.

These limits are applied by summing the amounts of a residual solvent present in each of the components of the official product. The contribution of each solvent per day should result in a total amount that does not exceed the PDE.

Consider the example of the application of *Option 1* and *Option 2* limits to acetonitrile concentration in an official product. The PDE for acetonitrile is 4.1 mg/day, thus the *Option 1* limit is 410 ppm. The maximum administered daily mass of an official product is 5.0 g, and the official product contains two excipients. Assuming that there is no other source of acetonitrile in the manufacturing process, the calculated official product content of acetonitrile and the daily exposure are given in *Table 5*.

**Table 5. Example of Meeting the Requirement for Acetonitrile as per Option 2**

| Component | Amount in Formulation (g) | Acetonitrile Content (ppm) | Daily Exposure (mg) |
|---|---|---|---|
| Drug substance | 0.3 | 800 | 0.24 |
| Excipient 1 | 0.9 | 400 | 0.36 |
| Excipient 2 | 3.8 | 800 | 3.04 |
| Official product | 5.0 | 728 | 3.64 |

*Excipient 1* meets the *Option 1* limit, but the official substance, *Excipient 2*, and *Official product* do not meet the *Option 1* limit. Nevertheless, the product meets the *Option 2* limit of 4.1 mg/day and thus conforms to the recommendations in this chapter.

Consider another example using acetonitrile as a residual solvent. The maximum administered daily mass of an official product is 5.0 g, and the official product contains two excipients. Assuming no other source of acetonitrile in the manufacturing process, the *Official product's* content of acetonitrile and the maximum content of residual acetonitrile are given in *Table 6*.

**Table 6. Example of Failing the Requirement for Acetonitrile as per Option 2**

| Component | Amount in Formulation (g) | Acetonitrile Content (ppm) | Daily Exposure (mg) |
|---|---|---|---|
| Drug substance | 0.3 | 800 | 0.24 |
| Excipient 1 | 0.9 | 2000 | 1.8 |
| Excipient 2 | 3.8 | 800 | 3.04 |
| Official product | 5.0 | 1016 | 5.08 |

In this example, the product meets neither the *Option 1* nor the *Option 2* limit according to the summation. Compliance with the chapter may still be demonstrated via analysis of the *Official product* as it is possible that the formulation process may have reduced the level of acetonitrile below the calculated content. If the level of acetonitrile was not reduced during formulation to the allowed limit, then the manufacturer of the *Official product* should take other steps to reduce the amount of acetonitrile in the official product. If the manufacturer receives approval from the regulatory authority for such a higher level of residual solvent, it is the responsibility of that manufacturer to notify USP regarding the identity of this solvent and the approved residual solvent limit in the article. USP will then address this topic in the individual monograph.

# 6. REPORTING LEVELS OF RESIDUAL SOLVENTS

Manufacturers of pharmaceutical products need certain information about the content of residual solvents in drug substances and excipients to meet the criteria of this chapter. The following statements are given as acceptable examples of the information that could be provided from a supplier of drug substances or excipients to a pharmaceutical manufacturer. The supplier might choose one of the following as appropriate:

- If Class 1 solvents are present, they should be identified and quantified.
- Only Class 2 solvents X, Y,… are LTBP. All are below the *Option 1* limit. (Here the supplier would name the Class 2 solvents represented by X, Y,…)
- Only Class 2 solvents X, Y,…, and Class 3 solvents are LTBP. Residual Class 2 solvents are below the *Option 1* limit and residual Class 3 solvents are below 0.5% each.
- Only Class 3 solvents are LTBP. Loss on drying (LOD) is not greater than 0.5%.
- If only Class 3 solvents are LTBP and LOD is more than 0.5%, they should be identified and quantified.

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- If solvents of Class 2 or Class 3 are present at greater than their *Option 1* limits or 0.5%, respectively, they should be identified and quantified to allow compliance with *Option 2*.

The term LTBP as used in the above examples refers to the solvent used or produced in the final manufacturing step and to solvents used or produced in earlier manufacturing steps and not removed consistently by a validated process.

## 7. IDENTIFICATION, CONTROL, AND QUANTIFICATION OF RESIDUAL SOLVENTS

Whenever possible, the substance under test needs to be dissolved to release the residual solvent. The control strategy may involve analysis of official products, active ingredients, excipients, and dietary ingredients. In some cases the sample does not dissolve completely. In such cases it may be acceptable to pulverize the sample into a fine powder carefully, avoiding the generation of frictional heat and in a manner that minimizes the loss of volatile solvents. This technique ensures that any residual solvent that may be present can be released. It should be performed as fast as possible immediately prior to dispersion in the sample solvent, to minimize the loss of volatile solvents during the procedure.

[NOTE—The organic-free water specified in the following procedures produces no significantly interfering peaks when chromatographed.]

### 7.1 Class 1 and Class 2 Residual Solvents

This chapter describes analytical procedures (*Procedures A*, *B*, and *C*) for evaluating the levels of all Class 1 and the majority of Class 2 residual solvents. For each test matrix, verification is needed to demonstrate reliability of the compendial procedure, as described in ⟨1467⟩.

When the solvents that are present or potentially present in the sample are known, they can be determined using a limit test such as *Procedure A* or *Procedure B*, or by a quantitative test, such as *Procedure C*.

When the solvents LTBP are not known, use the screening tests of *Procedure A* and *Procedure B* as required. *Procedure A* and *Procedure B* can separate most of the solvents listed in this chapter. Additionally, *Figure 3* can be used as a decision tree to demonstrate compliance.

In a screening test, if the article does not meet the acceptance criteria of *Procedure A*, then *Procedure B* is to be used to demonstrate compliance. If the article does not meet the criteria using *Procedure A* and *Procedure B*, then *Procedure C* must be used to quantify the residual solvent(s) present in the article.

When the information about solvents LTBP in the material is known, the system suitability requirements only need to be demonstrated for the solvents expected to be present. Several residual solvents listed in this chapter are not detected at the limit concentration using the analytical procedure described below. Those solvents include formamide, 2-ethoxyethanol, 2-methoxyethanol, ethylene glycol, *N*-methylpyrrolidone, sulfolane, *N,N*-dimethylacetamide, and *N,N*-dimethylformamide. Those solvents should be determined using an alternative method that has been appropriately validated to demonstrate that the method is suitable for its intended purpose, i.e., quantitative determination of residual solvent or limit tests for them (see *Category II* in *Validation of Compendial Procedures* ⟨1225⟩, *Table 2*, which provides definitions and general guidance on analytical procedures validation without indicating specific validation criteria for each characteristic). Chapter ⟨1467⟩ provides additional guidance on the specific validation criteria for alternative methods used for identification and quantification of residual solvents.

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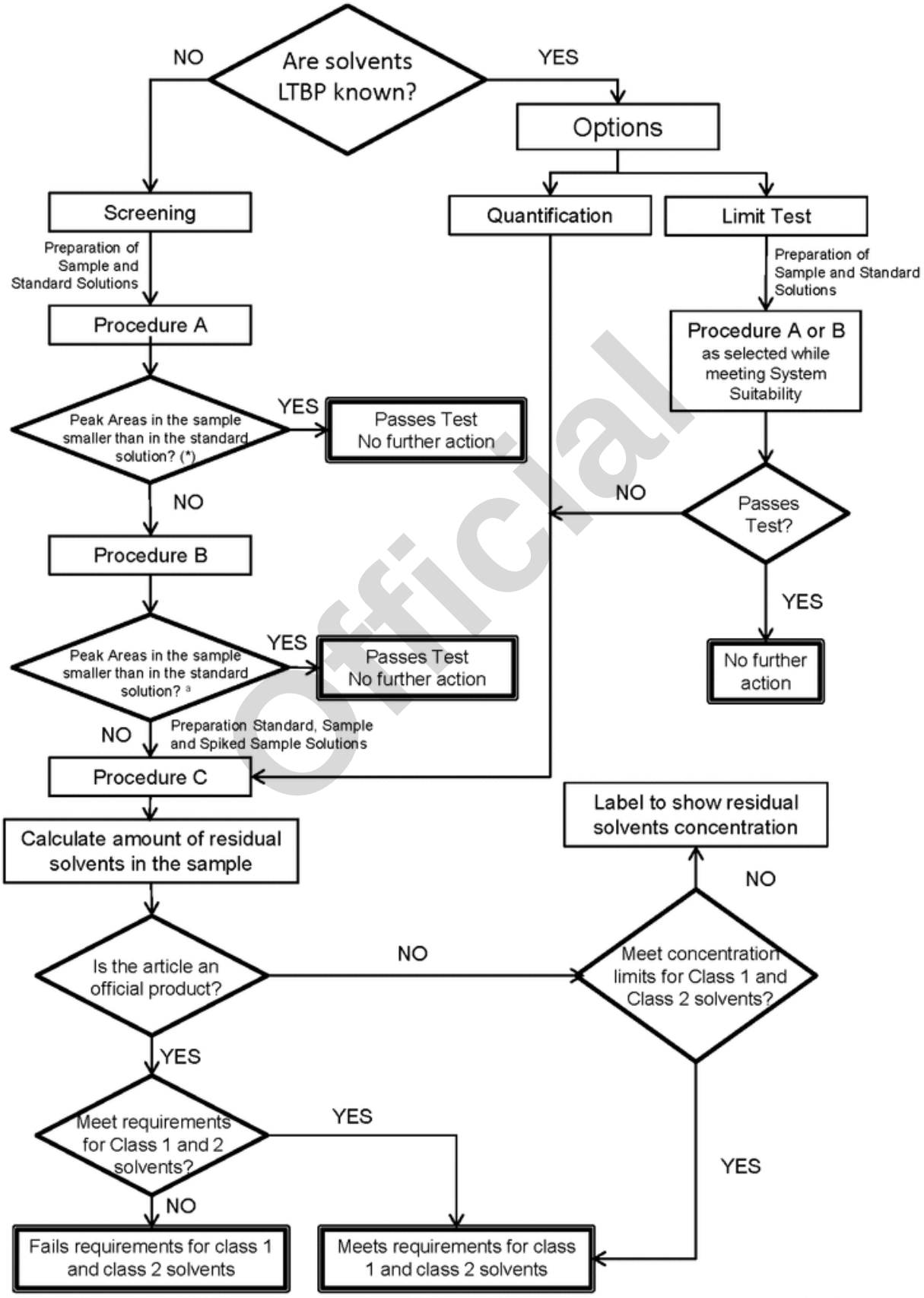


[a] Except 1,1,1-trichloroethane (TCE), which should be smaller than 150 times the size of the corresponding peak response from the Class 1 standard solution.

Figure 3. Determination of residual solvents and decision tree using *Procedures A, B,* and *C*.

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### 7.2 Class 3 Residual Solvents

*Procedures A* and *B* can separate most solvents in Classes 1, 2, and 3. However, the procedures have not been validated for Class 3 solvents, and appropriate validation will be required (see the entry for alternative methods under *7.1 Class 1 and Class 2 Residual Solvents*).

## 8. ANALYTICAL PROCEDURES FOR CLASS 1 AND CLASS 2 RESIDUAL SOLVENTS

### 8.1 Chromatographic Systems

(See *Chromatography* ⟨621⟩, *System Suitability* and *Figure 4*.)

#### PROCEDURE A

**Mode:** GC
**Injector:** Headspace (see *Table 9* for headspace settings)
**Injection type:** Split ratio, 1:5. [NOTE—Split ratio can be modified to optimize sensitivity.]
**Detector:** Flame ionization
**Column:** 0.32-mm × 30-m capillary fused silica coated with 1.8-µm layer of phase G43 or 0.53-mm × 30-m wide-bore coated with a 3.0-µm layer of phase G43
**Column temperature:** See *Table 7*.

**Table 7. Procedure A Column Temperature**

| Initial Temperature (°) | Temperature Ramp (°/min) | Final Temperature (°) | Hold Time at Final Temperature (min) |
|---|---|---|---|
| 40 | 0 | 40 | 20 |
| 40 | 10 | 240 | — |
| 240 | 0 | 240 | 20 |

**Temperatures**
*Injection port:* 140°
*Detector:* 250°
**Carrier gas:** Helium, nitrogen, or hydrogen
**Linear velocity:** About 35 cm/s for helium (adjustment and verification are necessary for other carrier gases)

#### PROCEDURE B

**Mode:** GC
**Injector:** Headspace (see *Table 9* for headspace settings)
**Injection type:** Split ratio, 1:5. [NOTE—Split ratio can be modified to optimize sensitivity.]
**Detector:** Flame ionization
**Column:** 0.32-mm × 30-m fused-silica coated with a 0.25-µm layer of phase G16 or a 0.53-mm × 30-m wide-bore coated with a 0.25-µm layer of phase G16
**Column temperature:** See *Table 8*.

**Table 8. Procedure B Column Temperature**

| Initial Temperature (°) | Temperature Ramp (°/min) | Final Temperature (°) | Hold Time at Final Temperature (min) |
|---|---|---|---|
| 50 | 0 | 50 | 20 |
| 50 | 6 | 165 | — |
| 165 | 0 | 165 | 20 |

**Temperatures**
*Injection port:* 140°
*Detector:* 250°
**Carrier gas:** Helium, nitrogen, or hydrogen
**Linear velocity:** About 35 cm/s for helium (adjustment and verification are necessary for other carrier gases)

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## PROCEDURE C

Use the chromatographic conditions, from either *Procedure A* or *Procedure B*, that provide the best performance for the solvent(s) being tested.

## HEADSPACE PARAMETER SETTINGS

There are three general sets of headspace operating parameters that are used for *Procedures A* and *B* (*Table 9*).

**Table 9. Headspace Operating Parameters**

| Procedure Parameters | Headspace Instrumental Setting | | |
|---|---|---|---|
| | Set 1 | Set 2 | Set 3 |
| Equilibration temperature (°) | 80 | 105 | 80 |
| Equilibration time (min) | 60 | 45 | 45 |
| Transfer-line temperature (°) [if appropriate][a] | 85 | 110 | 105 |
| Syringe temperature (°) [if appropriate] | 80–90 | 105–115 | 80–90 |
| Carrier gas: helium, nitrogen,[b] or hydrogen[b] at an appropriate pressure[b] | — | — | — |
| Pressurization time(s) (sec) [if appropriate] | ≥60 | ≥60 | ≥60 |
| Injection volume (mL)[c] | 1 | 1 | 1 |

[a] It is recommended to increase the temperature of the transfer line between runs to eliminate any potential condensation of solvents.
[b] The use of nitrogen or hydrogen as the carrier gas requires adjustment and verification.
[c] Injection volume may be expressed in terms of the injection time.

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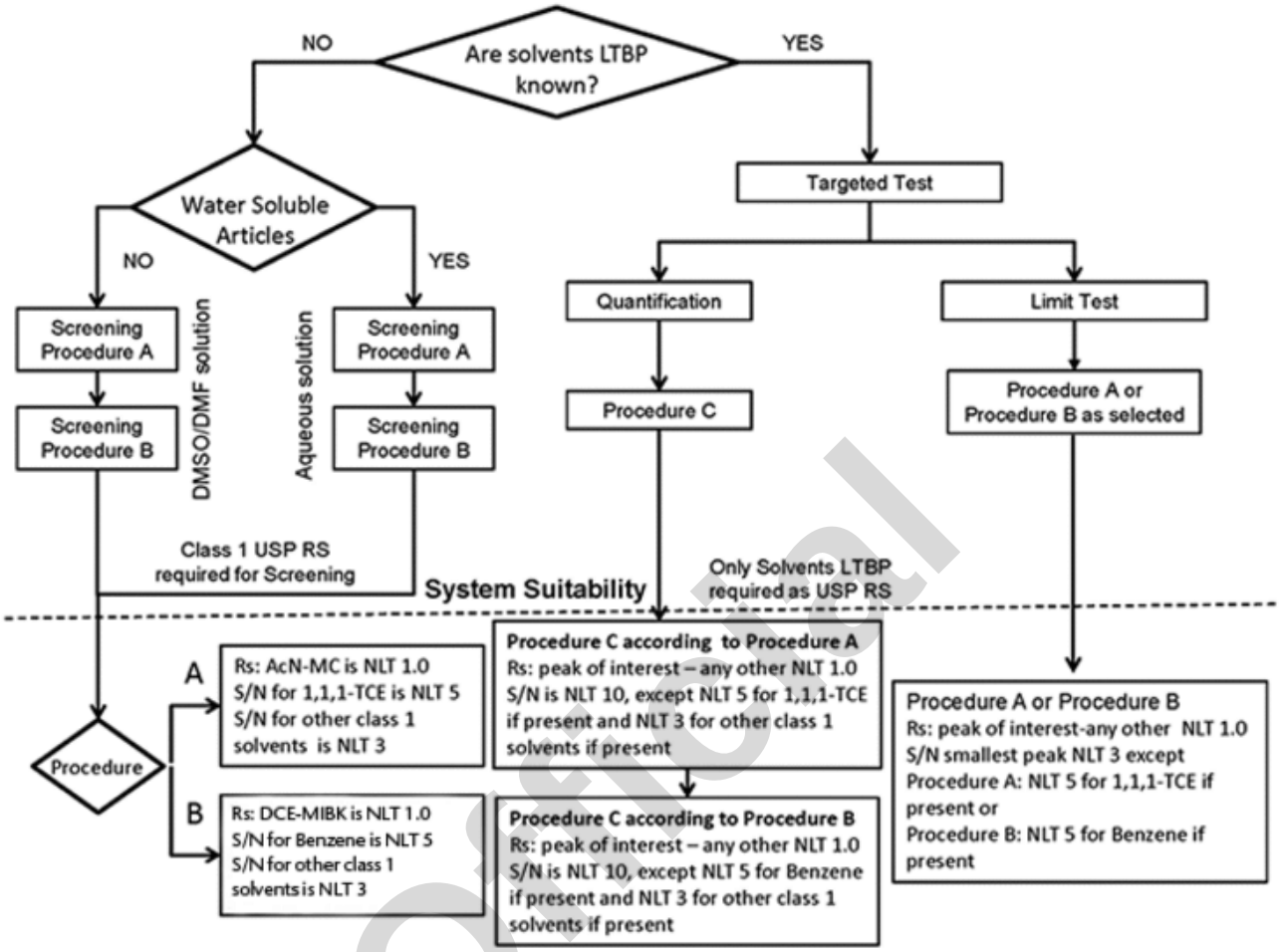


Figure 4. System suitability requirements based on the method of choice and previous knowledge about solvents LTBP. The acceptance criteria are based on the solvents LTBP (AcN: acetonitrile; 1,1,1-TCE: 1,1,1-trichloroethane; DCE: *cis*-dichloroethene; MC: methylene chloride; MIBK: Methylisobutylketone).

## 8.2 Screening of Water-Soluble Articles

STOCK SOLUTIONS

**Class 1 standard stock solution:** [NOTE—When transferring solutions, place the tip of the pipet just below the surface of the liquid and mix.] Transfer 1.0 mL of USP Residual Solvents Mixture—Class 1 RS to a 100-mL volumetric flask containing about 9 mL of dimethylsulfoxide, dilute with water to volume, and mix. Transfer 1.0 mL of this solution to a 100-mL volumetric flask containing about 50 mL of water, dilute with water to volume, and mix. Transfer 10 mL of this solution to a 100-mL volumetric flask containing about 50 mL of water, dilute with water to volume, and mix.
**Class 2 mixture A standard stock solution:** Transfer 1.0 mL of USP Residual Solvents Class 2—Mixture A RS to a 100-mL volumetric flask, dilute with water to volume, and mix.
**Class 2 mixture B standard stock solution:** Transfer 1.0 mL of USP Residual Solvents Class 2—Mixture B RS to a 100-mL volumetric flask, dilute with water to volume, and mix.
**Sample stock solution:** Transfer about 250 mg of the article under test, accurately weighed, to a 25-mL volumetric flask, dissolve in and dilute with water to volume, and mix.

SYSTEM SUITABILITY SOLUTION

**Class 1 system suitability solution:** Transfer 1.0 mL of *Class 1 standard stock solution* to an appropriate headspace vial, add 5.0 mL of *Sample stock solution*, apply the stopper, cap, and mix.

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STANDARD SOLUTIONS

**Class 1 standard solution:** [NOTE—When transferring solutions, place the tip of the pipet just below the surface of the liquid and mix.] Transfer 1.0 mL of *Class 1 standard stock solution* to an appropriate headspace vial containing 5.0 mL of water. Apply the stopper, cap, and mix.
**Class 2 mixture A standard solution:** Transfer 1.0 mL of *Class 2 mixture A standard stock solution* to an appropriate headspace vial containing 5.0 mL of water, apply the stopper, cap, and mix.
**Class 2 mixture B standard solution:** Transfer 5.0 mL of *Class 2 mixture B standard stock solution* to an appropriate headspace vial, add 1.0 mL of water, apply the stopper, cap, and mix.

SAMPLE SOLUTION

**Sample solution:** Transfer 5.0 mL of *Sample stock solution* to an appropriate headspace vial, add 1.0 mL of water, apply the stopper, cap, and mix.

PROCEDURE A

**Analysis:** Using *Procedure A Chromatographic System*, separately inject (using one of the headspace operating parameter sets described in *Table 9*) equal volumes of headspace of the *Class 1 standard solution*, *Class 1 system suitability solution*, *Class 2 mixture A standard solution*, *Class 2 mixture B standard solution*, and *Sample solution* into the chromatograph, record the chromatograms, and measure the responses for the peaks of interest.
**System suitability requirements (**see *Figure 4***):** The signal-to-noise (S/N) ratio for 1,1,1-trichloroethane in the *Class 1 standard solution* is NLT 5. The S/N ratio of each peak in the *Class 1 system suitability solution* is NLT 3, and the resolution between acetonitrile and methylene chloride in the *Class 2 mixture A standard solution* is NLT 1.0.
**Acceptance criteria:** If a response of any peak, other than the peak for 1,1,1-TCE, in the *Sample solution* is greater than or equal to a corresponding peak in either the *Class 1 standard solution*, or either *Class 2 mixture A standard solution* or *Class 2 mixture B standard solution*, or a peak response of 1,1,1-trichloroethane is greater than or equal to 150 times the peak response corresponding to 1,1,1-trichloroethane in the *Class 1 standard solution*, proceed to *Procedure B* to verify the identity of the peak; otherwise the article meets the requirements of this test.

PROCEDURE B

**Analysis:** Using *Procedure B Chromatographic System*, separately inject (using one of the headspace operating parameter sets described in *Table 9*) equal volumes of headspace of the *Class 1 standard solution*, *Class 1 system suitability solution*, *Class 2 mixture A standard solution*, *Class 2 mixture B standard solution*, and *Sample solution* into the chromatograph, record the chromatograms, and measure the responses for the peaks of interest.
**System suitability requirements (**see *Figure 4***):** The S/N ratio for benzene in the *Class 1 standard solution* is NLT 5. The S/N ratio of each peak in the *Class 1 system suitability solution* is NLT 3, and the resolution between methylisobutylketone and *cis*-dichloroethene in the *Class 2 mixture A standard solution* is NLT 1.0.
**Acceptance criteria:** If the response(s) in the *Sample solution* of the peak(s) identified in *Procedure A* is/are greater than or equal to corresponding peak(s) in either the *Class 1 standard solution*, or either *Class 2 mixture A standard solution* or *Class 2 mixture B standard solution*, proceed to *Procedure C* to quantify the peak(s); otherwise the article meets the requirements of this test.

## 8.3 Screening of Water-Insoluble Articles

STOCK SOLUTIONS

[NOTE—Dimethyl sulfoxide may be substituted as an alternative solvent to dimethylformamide. When transferring solutions, place the tip of the pipet just below the surface of the liquid and mix.]
**Class 1 standard stock solution:** Transfer 1.0 mL of USP Residual Solvents Mixture—Class 1 RS to a 100-mL volumetric flask previously filled with about 80 mL of dimethylformamide, dilute with dimethylformamide to volume, and mix. Transfer 1.0 mL of this solution to a 100-mL volumetric flask, previously filled with about 80 mL of dimethylformamide, dilute with dimethylformamide to volume, and mix (reserve a portion of this solution for the *Class 1 system suitability solution*). Transfer 1.0 mL of this solution to a 10-mL volumetric flask, dilute with dimethylformamide to volume, and mix.
**Class 2 mixture A standard stock solution:** Transfer 1.0 mL of USP Residual Solvents Class 2—Mixture A RS to a 100-mL volumetric flask containing about 80 mL of dimethylformamide, dilute with dimethylformamide to volume, and mix.
**Class 2 mixture B standard stock solution:** Transfer 0.5 mL of USP Residual Solvents Class 2—Mixture B RS to a 10-mL volumetric flask, dilute with dimethylformamide to volume, and mix.
**Sample stock solution:** Transfer about 500 mg of the article under test, accurately weighed, to a 10-mL volumetric flask, dissolve in and dilute with dimethylformamide to volume, and mix.

SYSTEM SUITABILITY SOLUTION

**Class 1 system suitability solution:** Mix 5 mL of *Sample stock solution* with 0.5 mL of the intermediate dilution reserved from *Class 1 standard stock solution*. Transfer 1 mL of this solution to an appropriate headspace vial containing 5 mL of water. Apply the stopper, cap, and mix.

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STANDARD SOLUTIONS

**Class 1 standard solution:** Transfer 1.0 mL of *Class 1 standard stock solution* to an appropriate headspace vial containing 5.0 mL of water, apply the stopper, cap, and mix.
**Class 2 mixture A standard solution:** Transfer 1.0 mL of *Class 2 mixture A standard stock solution* to an appropriate headspace vial containing 5.0 mL of water, apply the stopper, cap, and mix.
**Class 2 mixture B standard solution:** Transfer 1.0 mL of *Class 2 mixture B standard stock solution* to an appropriate headspace vial containing 5.0 mL of water, apply the stopper, cap, and mix.

SAMPLE SOLUTION

**Sample solution:** Transfer 1.0 mL of *Sample stock solution* to an appropriate headspace vial containing 5.0 mL of water, apply the stopper, cap, and mix.

PROCEDURE A

**Analysis:** Proceed as directed for *Procedure A* in *8.2 Screening of Water-Soluble Articles* with a split ratio of 1:3. [NOTE—The split ratio can be modified in order to optimize sensitivity.] Separately inject (using one of the headspace operating parameter sets described in *Table 9* with a recommended vial pressure of 10 psi) equal volumes of headspace of the *Class 1 standard solution*, *Class 2 mixture A standard solution*, *Class 2 mixture B standard solution*, and *Sample solution* into the chromatograph, record the chromatograms, and measure the responses for the major peaks.
**System suitability requirements (**see *Figure 4***):** The S/N ratio for 1,1,1-trichloroethane in the *Class 1 standard solution* is NLT 5. The S/N ratio of each peak in the *Class 1 system suitability solution* is NLT 3; and the resolution between acetonitrile and methylene chloride in the *Class 2 mixture A standard solution* is NLT 1.0.
**Acceptance criteria:** If a response of any peak, other than the peak for 1,1,1-TCE in the *Sample solution* is greater than or equal to a corresponding peak in either the *Class 1 standard solution*, or either *Class 2 mixture A standard solution* or *Class 2 mixture B standard solution*, or a peak response of 1,1,1-trichloroethane is greater than or equal to 150 times the peak response corresponding to 1,1,1-trichloroethane in the *Class 1 standard solution*, proceed to *Procedure B* to verify the identity of the peak; otherwise the article meets the requirements of this test.

PROCEDURE B

**Analysis:** Proceed as directed for *Procedure B* in *8.2 Screening of Water-Soluble Articles* with a split ratio of 1:3. Separately inject (using one of the headspace operating parameter sets described in *Table 9* with a recommended vial pressure of 10 psi) equal volumes of headspace of the *Class 1 standard solution*, *Class 2 mixture A standard solution*, *Class 2 mixture B standard solution*, *Sample solution*, and *Class 1 system suitability solution* into the chromatograph, record the chromatograms, and measure the responses for the peaks of interest.
**System suitability requirements (**see *Figure 4***):** The S/N ratio for benzene in the *Class 1 standard solution* is NLT 5. The S/N ratio of each peak in the *Class 1 system suitability solution* is NLT 3; and the resolution between methylisobutylketone and *cis*-dichloroethene in the *Class 2 mixture A standard solution* is NLT 1.0.
**Acceptance criteria:** If the peak response(s) in the *Sample solution* of the peak(s) identified in *Procedure A* is/are greater than or equal to corresponding peak(s) in either the *Class 1 standard solution*, or either *Class 2 mixture A standard solution* or *Class 2 mixture B standard solution*, proceed to *Procedure C* to quantify the peak(s); otherwise the article meets the requirements of this test.

## 8.4 Limit Tests When Solvents LTBP Are Known

SOLUTIONS

Prepare the standard solution and the *Sample solution* as directed in *8.2 Screening of Water-Soluble Articles* or *8.3 Screening of Water-Insoluble Articles* according to the procedure chosen based on the sample solubility. Use USP Reference Standards for those solvents LTBP to prepare the standard solution.

When solvents LTBP are known, only the standards of those solvents LTBP are needed, and determination may be done using a limit test or a quantitative test. Only quantitative tests can be used for *Option 2*.
**System suitability solution:** Prepare the *System suitability solution* according to the solubility of the sample for screening for water-soluble or water-insoluble articles at the concentration of the standard solutions there. Use only those solvents LTBP.

PROCEDURE A

**Analysis (limit test):** Separately inject (using one of the headspace operating parameter sets described in *Table 9*) equal volumes of headspace of the *System suitability solution*, *Standard solution*, and *Sample solution* into the chromatograph, record the chromatograms, and measure the responses for the peaks of interest.
**System suitability requirements (**see *Figure 4***):** The S/N ratio for 1,1,1-trichloroethane, if present, is NLT 5. The S/N ratio is NLT 3 for any other solvent. If there is more than one solvent LTBP, the resolution between a peak of interest and any adjacent peak is NLT 1.0.
**Acceptance criteria:** If the peak response of every peak of interest in the *Sample solution* is smaller than the corresponding peak in the *Standard solution*, the article meets the requirements of this test.

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PROCEDURE B

**Analysis (limit test):** Separately inject (following one of the headspace operating parameter sets described in *Table 9*) equal volumes of headspace of the *Standard solution* and *Sample solution* into the chromatograph, record the chromatograms, and measure the responses for the peaks of interest.
**System suitability requirements (**see *Figure 4*): The S/N ratio for benzene, if present, is NLT 5. The S/N ratio is NLT 3 for any other solvent. If there is more than one solvent LTBP, the resolution between a peak of interest and any other peak is NLT 1.0.
**Acceptance criteria:** If the peak response of every peak of interest in the *Sample solution* is smaller than the corresponding peak in the *Standard solution*, the article meets the requirements of this test.

## 8.5 Quantitative Tests—Procedure C

QUANTIFICATION FOR WATER-SOLUBLE ARTICLES

(Use the requirement for *General* or *Targeted test*, as applicable.)
**Sample stock solution:** Prepare a *Sample stock solution* as directed in *8.2 Screening of Water-Soluble Articles, Stock Solutions*.
**Standard stock solution:** [NOTE—Prepare a separate *Standard stock solution* for each solvent LTBP or potentially LTBP, or identified and verified by *Procedure A* and *Procedure B*. For the Class 1 solvents other than 1,1,1-trichloroethane, prepare the first dilution as directed for the first dilution under *Class 1 standard stock solution*.] Transfer an accurately measured volume of each individual USP Reference Standard corresponding to each residual solvent peak LTBP or identified and verified by *Procedure A* and *Procedure B* to a suitable container, and dilute quantitatively and stepwise, if necessary, with water to obtain a solution with a final concentration of 1/20 of the value stated in *Table 2* or *Table 3* under *Concentration Limit*.
**Standard solution:** Transfer 1.0 mL of *Standard stock solution* to an appropriate headspace vial, add 5.0 mL of water, apply the stopper, cap, and mix.
**Sample solution:** Prepare as directed in *8.2 Screening of Water-Soluble Articles, Sample Solution*.
**Spiked sample solution:** [NOTE—Prepare a separate *Spiked sample solution* for each peak LTBP or potentially LTBP or identified and verified by *Procedure A* and *Procedure B*.] Transfer 5.0 mL of each *Sample stock solution* to an appropriate headspace vial, add 1.0 mL of the *Standard stock solution*, apply the stopper, cap, and mix. The use of a vial containing the sample spiked with multiple standards is permitted, provided that the procedure is validated accordingly.
**Analysis:** Proceed as directed for *Procedure A*. [NOTE—If the performance of *Procedure B* is better than that shown by *Procedure A*, then *Procedure B* may be used for quantification.] Separately inject (using one of the headspace operating parameter sets described in *Table 9* with a recommended vial pressure of 10 psi) equal volumes of headspace of the *Standard solution*, *Sample solution*, and *Spiked sample solution* into the chromatograph, record the chromatograms, and measure the responses for the peaks of interest.
**System suitability requirements for Procedure A** (see *Figure 4*)**:**
**General:** Same as in *8.2 Screening of Water-Soluble Articles*, *Procedure A*.
**Targeted test:** The S/N ratio is NLT 10 for any solvent except NLT 5 for 1,1,1-trichloroethane and NLT 3 for any other Class 1 solvent, if present. If there is more than one solvent LTBP, the resolution between a peak of interest and any adjacent peak is NLT 1.0.
**System suitability requirements for Procedure B** (see *Figure 4*)**:**
**General:** Same as in *8.2 Screening of Water-Soluble Articles*, *Procedure B*.
**Targeted test:** The S/N ratio is NLT 10 for any solvent except NLT 5 for benzene and NLT 3 for any other Class 1 solvent, if present. If there is more than one solvent LTBP, the resolution between a peak of interest and any adjacent peak is NLT 1.0.
Calculate the amount, in ppm, of each residual solvent found in the article under test:

$$\text{Result} = 5(C/W)[r_U/(r_{ST} - r_U)]$$

$C$ = concentration of the appropriate USP Reference Standard in the *Standard stock solution* (µg/mL)
$W$ = weight of the article under test taken to prepare the *Sample stock solution* (g)
$r_U$ = peak responses of each residual solvent obtained from the *Sample solution*
$r_{ST}$ = peak responses of each residual solvent obtained from the *Spiked sample solution*

QUANTIFICATION FOR WATER-INSOLUBLE ARTICLES

(Use the requirement for *General* or *Targeted test* as applicable.)
**Sample stock solution:** Prepare a *Sample stock solution* as directed in *8.3 Screening of Water-Insoluble Articles, Stock Solutions*. Only the Reference Standard for those solvents identified by *Procedure A* or *Procedure B* or LTBP need to be used.
**Standard stock solution:** [NOTE—Prepare a separate *Standard stock solution* for each solvent LTBP or potentially LTBP, or identified and verified by *Procedure A* and *Procedure B*. For the Class 1 solvents other than 1,1,1-trichloroethane, prepare the first dilution as directed for the first dilution under *Class 1 standard stock solution*.] Transfer an accurately measured volume of each individual USP Reference Standard corresponding to each residual solvent peak LTBP or identified and verified by *Procedure A* and *Procedure B* to a suitable container, and dilute quantitatively and stepwise, if necessary, with water to obtain a solution with a final concentration of 1/20 of the value stated in *Table 2* or *Table 3* under *Concentration Limit*.
**Standard solution:** Transfer 1.0 mL of *Standard stock solution* to an appropriate headspace vial, containing 5.0 mL of water, apply the stopper, cap, and mix.
**Sample solution:** Transfer 1.0 mL of *Sample stock solution* to an appropriate headspace vial, containing 5.0 mL of water, apply the stopper, cap, and mix.
**Spiked sample solution:** [NOTE—Prepare a separate *Spiked sample solution* for each peak LTBP or potentially LTBP, or identified and verified by *Procedure A* and *Procedure B*.] Transfer 1.0 mL of each *Sample stock solution* to an appropriate headspace vial,

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add 1.0 mL of *Standard stock solution* and 4.0 mL of water, and apply the stopper, cap, and mix. The use of a vial containing the sample spiked with multiple standards is permitted, provided that the procedure is validated accordingly.
**Analysis:** Proceed as directed for *Procedure A*. [NOTE—If the performance of *Procedure B* is better than that shown by *Procedure A*, then *Procedure B* may be used for quantification.] Separately inject (using one of the headspace operating parameter sets described in *Table 9* with a recommended vial pressure of 10 psi) equal volumes of headspace of the *Standard solution*, *Sample solution*, and *Spiked sample solution* into the chromatograph, record the chromatograms, and measure the responses for the peaks of interest. If Class 1 solvents are LTBP, also inject the *Class 1 system suitability solution*.
**System suitability requirements for Procedure A** (see *Figure 4*)**:**
**General:** Same as in *8.3 Screening of Water-Insoluble Articles*, *Procedure A*.
**Targeted test:** The S/N ratio is NLT 10 for any solvent except NLT 5 for 1,1,1-trichloroethane and NLT 3 for any other Class 1 solvent, if present. If there is more than one solvent LTBP, the resolution between a peak of interest and any adjacent peak is NLT 1.0.
**System suitability requirements for Procedure B** (see *Figure 4*)**:**
**General:** Same as in *8.3 Screening of Water-Insoluble Articles, Procedure B*.
**Targeted test:** The S/N ratio is NLT 10 for any solvent except NLT 5 for benzene and NLT 3 for any other Class 1 solvent, if present. If there is more than one solvent LTBP, the resolution between a peak of interest and any adjacent peak is NLT 1.0.

Calculate the amount, in ppm, of each residual solvent found in the article under test:

$$\text{Result} = 10(C/W)[r_U/(r_{ST} - r_U)]$$

$C$ = concentration of the appropriate USP Reference Standard in the *Standard stock solution* (µg/mL)
$W$ = weight of the article under test taken to prepare the *Sample stock solution* (g)
$r_U$ = peak responses of each residual solvent obtained from the *Sample solution*
$r_{ST}$ = peak responses of each residual solvent obtained from the *Spiked sample solution*

## 9. ANALYTICAL PROCEDURES FOR CLASS 3 RESIDUAL SOLVENTS

If only Class 3 solvents are present, and the monograph for the article under test contains an LOD procedure specifying an upper limit of NMT 0.5% (per *Option 1* in this chapter), then the level of residual solvent can be determined as directed in *Loss on Drying* ⟨731⟩. If Class 3 solvents are present with other solvents, e.g., Class 1 or Class 2 solvents, or if there is no LOD procedure in the monograph for the article under test, or if the limit for Class 3 solvent(s) in an individual monograph is greater than 50 mg/day (corresponding to 5000 ppm or 0.5% under *Option 1*), then the Class 3 residual solvent or solvents present in the article under test should be identified and quantified. In that case, the procedures described in this chapter, with appropriate adjustments, are to be applied wherever possible and USP Reference Standards, where available, should be used in these procedures. Such procedures should be properly validated as described in ⟨1467⟩. If Class 1 or Class 2 solvents are present and LOD is less than 0.5%, then identification of Class 3 solvents is not required and only Class 1 and Class 2 solvents should be identified and quantified. See *Figure 5*.

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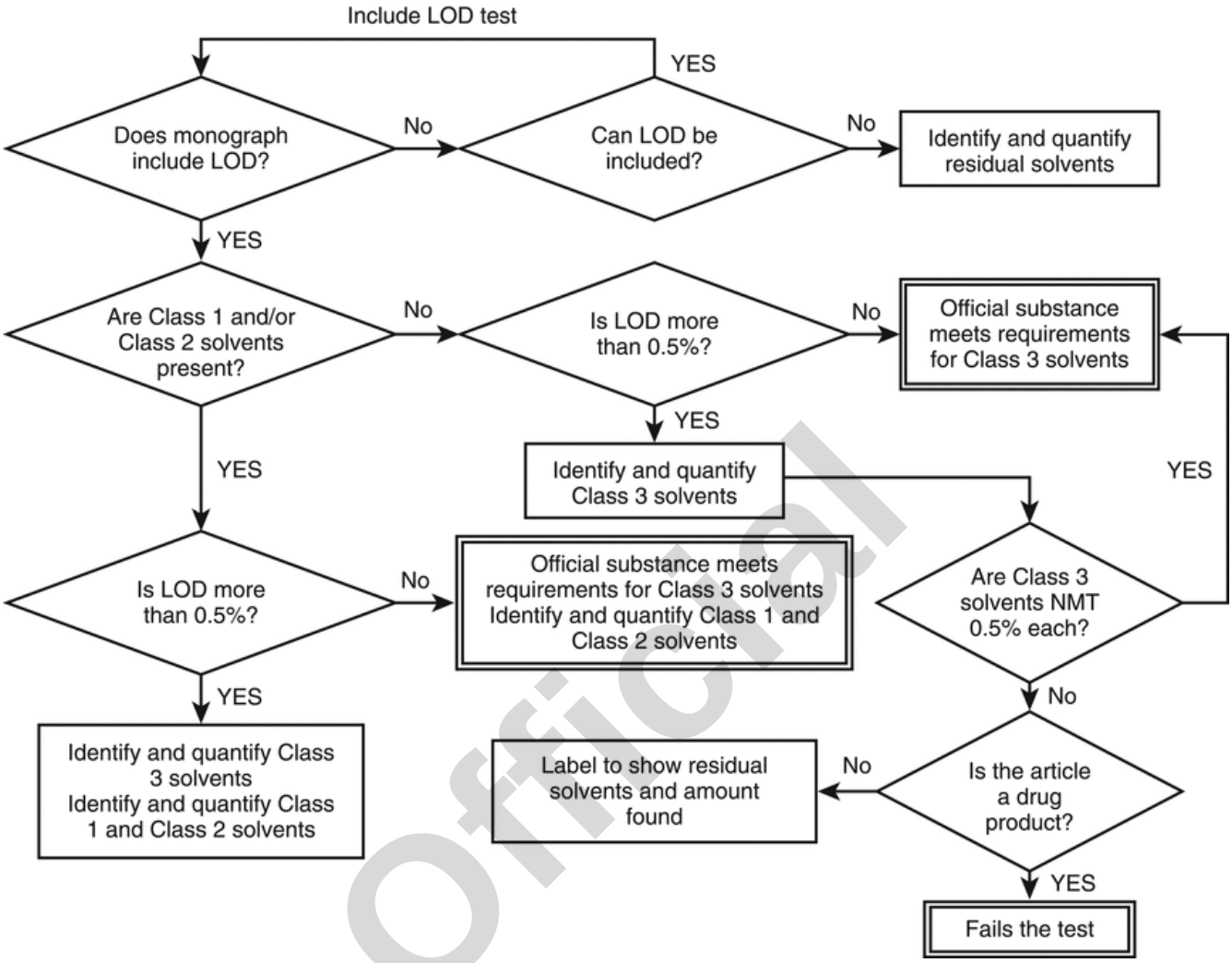


Figure 5. Decision tree for the control of Class 3 residual solvents. LOD: Loss on drying.

## GLOSSARY

**Genotoxic carcinogens:** Carcinogens that produce cancer by affecting genes or chromosomes.

**Likely to be present (LTBP):** Those solvents that are 1) used or produced in the final manufacturing step; 2) used or produced in earlier manufacturing steps but they are not consistently removed by a validated process; 3) the solvents properly declared by a validated supplier of a drug substance, excipient, or dietary ingredient.

**Lowest-observed-effect level (LOEL):** The lowest dose of a substance in a study or group of studies that produces biologically significant increases in frequency or severity of any effects in exposed humans or animals.

**Modifying factor:** A factor determined by professional judgment of a toxicologist and applied to bioassay data so that the data can be safely related to humans.

**Neurotoxicity:** The ability of a substance to cause adverse effects on the nervous system.

**No-observed-effect level (NOEL):** The highest dose of a substance at which there are no biologically significant increases in frequency or severity of any effects in exposed humans or animals.

**Permitted daily exposure (PDE):** The maximum acceptable intake per day of a residual solvent in pharmaceutical products or dietary supplements

**Reversible toxicity:** The occurrence of harmful effects that are caused by a substance and then disappear after the exposure to the substance ends.

**Strongly suspected human carcinogen:** A substance for which there is no epidemiological evidence of carcinogenesis but for which there are positive genotoxicity data and clear evidence of carcinogenesis in rodents.

**Teratogenicity:** The occurrence of structural malformations in a developing fetus when a substance is administered during pregnancy.

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# APPENDICES

## Appendix 1: Residual Solvents Included in this General Chapter

See *Table A-1* for a list of residual solvents included in this chapter.

**Table A-1. List of Residual Solvents Included in this General Chapter**

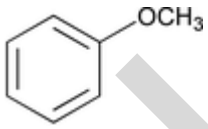
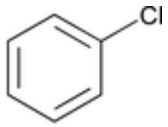
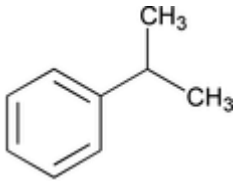
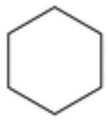
| Solvent | Other Names | Structure | Class |
|---|---|---|---|
| Acetic acid | Ethanoic acid | $CH_3COOH$ | Class 3 |
| Acetone | 2-Propanone; Propan-2-one | $CH_3COCH_3$ | Class 3 |
| Acetonitrile | AcN | $CH_3CN$ | Class 2 |
| Anisole | Methoxybenzene |  | Class 3 |
| Benzene | Benzol | | Class 1 |
| 1-Butanol | *n*-Butyl alcohol; Butan-1-ol | $CH_3(CH_2)_3OH$ | Class 3 |
| 2-Butanol | *sec*-Butyl alcohol; Butan-2-ol | $CH_3CH_2CH(OH)CH_3$ | Class 3 |
| Butyl acetate | Acetic acid butyl ester | $CH_3COO(CH_2)_3CH_3$ | Class 3 |
| *tert*-Butylmethyl ether | 2-Methoxy-2-methylpropane | $(CH_3)_3COCH_3$ | Class 3 |
| Carbon tetrachloride | Tetrachloromethane | $CCl_4$ | Class 1 |
| Chlorobenzene | |  | Class 2 |
| Chloroform | Trichloromethane | $CHCl_3$ | Class 2 |
| Cumene | Isopropylbenzene; (1-Methylethyl)benzene |  | Class 2 |
| Cyclohexane | Hexamethylene |  | Class 2 |
| 1,2-Dichloroethane | *sym*-Dichloroethane; Ethylene dichloride; Ethylene chloride | $CH_2ClCH_2Cl$ | Class 1 |
| 1,1-Dichloroethene | 1,1-Dichloroethylene; Vinylidene chloride | $H_2C=CCl_2$ | Class 1 |
| 1,2-Dichloroethene | 1,2-Dichloroethylene; Acetylene dichloride; DCE | ClHC=CHCl | Class 2 |

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**Table A-1. List of Residual Solvents Included in this General Chapter** *(continued)*

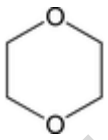
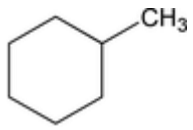
| Solvent | Other Names | Structure | Class |
|---|---|---|---|
| 1,2-Dimethoxyethane | Ethyleneglycol dimethyl ether; Monoglyme; Dimethyl cellosolve | $H_3COCH_2CH_2OCH_3$ | Class 2 |
| *N*,*N*-Dimethylacetamide | DMA | $CH_3CON(CH_3)_2$ | Class 2 |
| *N*,*N*-Dimethylformamide | Dimethylformamide | $HCON(CH_3)_2$ | Class 2 |
| Dimethyl sulfoxide | Methylsulfinylmethane; Methyl sulfoxide; Dimethyl sulfoxide | $(CH_3)_2SO$ | Class 3 |
| 1,4-Dioxane | *p*-Dioxane; [1,4]Dioxane |  | Class 2 |
| Ethanol | Ethyl alcohol | $CH_3CH_2OH$ | Class 3 |
| 2-Ethoxyethanol | Cellosolve | $CH_3CH_2OCH_2CH_2OH$ | Class 2 |
| Ethyl acetate | Acetic acid ethyl ester | $CH_3COOCH_2CH_3$ | Class 3 |
| Ethylene glycol | 1,2-Dihydroxyethane; 1,2-Ethanediol | $HOCH_2CH_2OH$ | Class 2 |
| Ethyl ether | Diethyl ether; Ethoxyethane; 1,1′-Oxybisethane | $CH_3CH_2OCH_2CH_3$ | Class 3 |
| Ethyl formate | Formic acid ethyl ester | $HCOOCH_2CH_3$ | Class 3 |
| Formamide | Methanamide | $HCONH_2$ | Class 2 |
| Formic acid | | HCOOH | Class 3 |
| Heptane | *n*-Heptane | $CH_3(CH_2)_5CH_3$ | Class 3 |
| Hexane | *n*-Hexane | $CH_3(CH_2)_4CH_3$ | Class 2 |
| Isobutyl acetate | Acetic acid isobutyl ester | $CH_3COOCH_2CH(CH_3)_2$ | Class 3 |
| Isopropyl acetate | Acetic acid isopropyl ester | $CH_3COOCH(CH_3)_2$ | Class 3 |
| Methanol | Methyl alcohol | $CH_3OH$ | Class 2 |
| 2-Methoxyethanol | Methyl cellosolve | $CH_3OCH_2CH_2OH$ | Class 2 |
| Methyl acetate | Acetic acid methyl ester | $CH_3COOCH_3$ | Class 3 |
| 3-Methyl-1-butanol | Isoamyl alcohol; Isopentyl alcohol; 3-Methylbutan-1-ol | $(CH_3)_2CHCH_2CH_2OH$ | Class 3 |
| Methylbutylketone | 2-Hexanone; Hexan-2-one | $CH_3(CH_2)_3COCH_3$ | Class 2 |
| Methylcyclohexane | Cyclohexylmethane |  | Class 2 |
| Methylene chloride | Dichloromethane; MC | $CH_2Cl_2$ | Class 2 |
| Methylethylketone | 2-Butanone; MEK; Butan-2-one | $CH_3CH_2COCH_3$ | Class 3 |
| Methylisobutylketone | 4-Methylpentan-2-one; 4-Methyl-2-pentanone; MIBK | $CH_3COCH_2CH(CH_3)_2$ | Class 2 |
| 2-Methyl-1-propanol | Isobutyl alcohol; 2-Methylpropan-1-ol | $(CH_3)_2CHCH_2OH$ | Class 3 |

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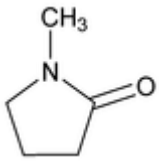
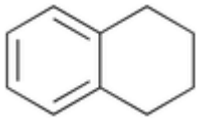
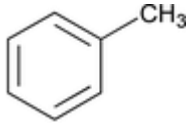
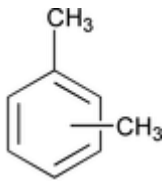
**Table A-1. List of Residual Solvents Included in this General Chapter** *(continued)*

| Solvent | Other Names | Structure | Class |
|---|---|---|---|
| *N*-Methylpyrrolidone | 1-Methylpyrrolidin-2-one; 1-Methyl-2-pyrrolidinone |  | Class 2 |
| Nitromethane | | $CH_3NO_2$ | Class 2 |
| Pentane | *n*-Pentane | $CH_3(CH_2)_3CH_3$ | Class 3 |
| 1-Pentanol | Amyl alcohol; Pentan-1-ol; Pentyl alcohol | $CH_3(CH_2)_3CH_2OH$ | Class 3 |
| 1-Propanol | Propan-1-ol; Propyl alcohol | $CH_3CH_2CH_2OH$ | Class 3 |
| 2-Propanol | Propan-2-ol; Isopropyl alcohol | $(CH_3)_2CHOH$ | Class 3 |
| Propyl acetate | Acetic acid propyl ester | $CH_3COOCH_2CH_2CH_3$ | Class 3 |
| Pyridine | | | Class 2 |
| Sulfolane | Tetrahydrothiophene 1,1-dioxide | | Class 2 |
| Tetrahydrofuran | Tetramethylene oxide; Oxacyclopentane | | Class 2 |
| Tetralin | 1,2,3,4-Tetrahydronaphthalene |  | Class 2 |
| Toluene | Methylbenzene |  | Class 2 |
| 1,1,1-Trichloroethane | Methylchloroform; 1,1,1-TCE | $CH_3CCl_3$ | Class 1 |
| Trichloroethylene | 1,1,2-Trichloroethene | $HClC{=}CCl_2$ | Class 2 |
| Triethylamine | *N*,*N*-Diethylethanamine | $(CH_3CH_2)_3N$ | Class 3 |
| Xylene[a] | Dimethylbenzene; Xylol |  | Class 2 |

[a] Usually 60% *m*-xylene, 14% *p*-xylene, and 9% *o*-xylene with 17% ethyl benzene.

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## Appendix 2: Additional Background

### ENVIRONMENTAL REGULATION OF ORGANIC VOLATILE SOLVENTS

Several of the residual solvents frequently used in the production of pharmaceuticals are listed as toxic chemicals in *Environmental Health Criteria* (EHC) monographs and in the *Integrated Risk Information System* (IRIS) and other similar reference material. The approach to risk assessment described therein relates primarily to long-term or lifetime exposure of the general population via the ambient environment (i.e., inhaled air, food, drinking water, and other sources). The objectives of such groups as the International Programme on Chemical Safety (IPCS), the U.S. Environmental Protection Agency (EPA), and the U.S. Food and Drug Administration (FDA) include the determination of acceptable exposure levels. The goal is maintenance of environmental integrity and protection of human health against the possible deleterious effects of chemicals resulting from long-term environmental exposure. The procedures involved in the estimation of maximum safe exposure limits are usually based on long-term studies. When long-term study data are unavailable, shorter-term study data can be used with modification of the approach, such as use of larger safety factors.

### RESIDUAL SOLVENTS IN PHARMACEUTICALS

Exposure limits in this chapter were established by referring to methodologies and toxicity data described in EHC and IRIS monographs. However, the following specific assumptions about residual solvents to be used in the synthesis and formulation of pharmaceutical products should be taken into account in establishing exposure limits.

1. Patients (not the general population) use pharmaceuticals to treat their diseases or for prophylaxis to prevent infection or disease.
2. The assumption of lifetime patient exposure is not necessary for most pharmaceutical products but may be appropriate as a working hypothesis to reduce risk to human health.
3. Residual solvents are unavoidable components in pharmaceutical production and will often be a part of pharmaceutical drug products.
4. Residual solvents should not exceed recommended levels except in exceptional circumstances.
5. Data from toxicological studies that are used to determine acceptable levels for residual solvents should have been generated using appropriate protocols such as those described by the Organization for Economic Cooperation and Development (OECD), the EPA, and the FDA *Redbook* or derived from alternative sources also considered to be adequate to support PDE limit setting.

## Appendix 3: Procedures for Establishing Exposure Limits

The Gaylor–Kodell method of risk assessment (Gaylor, DW, Kodell, RL. Linear interpolation algorithm for low dose risk assessment of toxic substances. *J Environ Pathol, Toxicol*, 1980;4:305–312) is appropriate for Class 1 carcinogenic solvents. Only in cases where reliable carcinogenicity data are available should extrapolation by the use of mathematical models be applied to setting exposure limits. Exposure limits for Class 1 residual solvents could be determined with the use of a large safety factor (i.e., 10,000–100,000) with respect to the no-observed-effect level (NOEL). Detection and quantification of these residual solvents should be performed using state-of-the-art analytical techniques.

Acceptable exposure levels in this chapter for Class 2 residual solvents were established by calculation of PDE values according to the procedures for setting exposure limits in pharmaceuticals (page 5748 of *Pharmacopeial Forum* [Nov.–Dec. 1989]), and the method adopted by IPCS for *Assessing Human Health Risks of Chemicals* (*Environmental Health Criteria 170*, WHO, 1994), detailed calculations are published in *Pharmeuropa*, Vol. 9, No. 1, Supplement, April 1997. These procedures are similar to those used by the U.S. EPA (IRIS) and the U.S. FDA (*Redbook*) and others. The method is outlined here to give a better understanding of the origin of the PDE values. It is not necessary to perform these calculations in order to use the PDE values presented in *Table 3* of this document.

PDE is derived from the NOEL, or the lowest-observed-effect level (LOEL), in the most relevant animal study as follows:

$$\text{PDE} = (\text{NOEL} \times \text{Weight Adjustment})/(F1 \times F2 \times F3 \times F4 \times F5)$$

The PDE is derived preferably from a NOEL. If no NOEL is obtained, the LOEL may be used. Modifying factors proposed here, for relating the data to humans, are the same kind of "uncertainty factors" used in *Environmental Health Criteria* (*Environmental Health Criteria 170*, WHO, 1994) and "modifying factors" or "safety factors" used in *Pharmacopeial Forum*. The assumption of 100% systemic exposure is used in all calculations regardless of the route of administration.

The modifying factors are as follows:

*F1* = A factor to account for extrapolation between species.
- *F1* = 5 for extrapolation from rats to humans
- *F1* = 12 for extrapolation from mice to humans
- *F1* = 2 for extrapolation from dogs to humans
- *F1* = 2.5 for extrapolation from rabbits to humans
- *F1* = 3 for extrapolation from monkeys to humans
- *F1* = 10 for extrapolation from other animals to humans

*F1* takes into account the comparative surface area to body weight ratios for the species concerned and for humans. Surface area (*S*) is calculated as:

$$S = kM^{0.67}$$

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*k* = a constant that has been taken to be 10
*M* = body weight

The body weights used in the equation are those shown below in *Table A-2*:

*F2* = A factor of 10 to account for variability between individuals.

A factor of 10 is generally given for all organic solvents, and 10 is used consistently in this chapter.

*F3* = A variable factor to account for toxicity studies of short-term exposure

*F3* = 1 for studies that last at least one half lifetime (1 year for rodents or rabbits; 7 years for cats, dogs, and monkeys)

*F3* = 1 for reproductive studies in which the whole period of organogenesis is covered

*F3* = 2 for a 6-month study in rodents, or a 3.5-year study in non-rodents

*F3* = 5 for a 3-month study in rodents, or a 2-year study in non-rodents

*F3* = 10 for studies of a shorter duration

In all cases, the higher factor has been used for study durations between the time points (e.g., a factor of 2 for a 9-month rodent study).

*F4* = A factor that may be applied in cases of severe toxicity, e.g., nongenotoxic carcinogenicity, neurotoxicity, or teratogenicity. In studies of reproductive toxicity, the following factors are used:

*F4* = 1 for fetal toxicity associated with maternal toxicity

*F4* = 5 for fetal toxicity without maternal toxicity

*F4* = 5 for a teratogenic effect with maternal toxicity

*F4* = 10 for a teratogenic effect without maternal toxicity

*F5* = A variable factor that may be applied if the no-effect level was not established.

When only a LOEL is available, a factor of up to 10 can be used, depending on the severity of the toxicity. The weight adjustment assumes an arbitrary adult human body weight for either sex of 50 kg. This relatively low weight provides an additional safety factor against the standard weights of 60 or 70 kg that are often used in this type of calculation. It is recognized that some adult patients weigh less than 50 kg; these patients are considered to be accommodated by the built-in safety factors used to determine a PDE. If the solvent was present in a formulation specifically intended for pediatric use, an adjustment for a lower body weight would be appropriate.

As an example of the application of this equation, consider a toxicity study of acetonitrile in mice that is summarized in *Pharmeuropa*, 1997;9(1): S24. The NOEL is calculated to be 50.7 mg $kg^{-1}$ $day^{-1}$. The PDE for acetonitrile in this study is calculated as follows:

$$PDE = (50.7 \text{ mg kg}^{-1} \text{ day}^{-1} \times 50 \text{ kg})/(12 \times 10 \times 5 \times 1 \times 1) = 4.22 \text{ mg day}^{-1}$$

In this example,

*F1* = 12 to account for the extrapolation from mice to humans

*F2* = 10 to account for differences between individual humans

*F3* = 5 because the duration of the study was only 13 weeks

*F4* = 1 because no severe toxicity was encountered

*F5* = 1 because the no-effect level was determined

**Table A-2. Values Used in the Calculations in This Chapter**

| | |
|---|---|
| Rat body weight | 425 g |
| Pregnant rat body weight | 330 g |
| Mouse body weight | 28 g |
| Pregnant mouse body weight | 30 g |
| Guinea-pig body weight | 500 g |
| Rhesus monkey body weight | 2.5 kg |
| Rabbit body weight (pregnant or not) | 4 kg |
| Beagle dog body weight | 11.5 kg |
| Rat respiratory volume | 290 L/day |
| Mouse respiratory volume | 43 L/day |
| Rabbit respiratory volume | 1440 L/day |
| Guinea-pig respiratory volume | 430 L/day |
| Human respiratory volume | 28,800 L/day |
| Dog respiratory volume | 9000 L/day |
| Monkey respiratory volume | 1150 L/day |
| Mouse water consumption | 5 mL/day |

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**Table A-2. Values Used in the Calculations in This Chapter** *(continued)*

| | |
|---|---|
| Rat water consumption | 30 mL/day |
| Rat food consumption | 30 g/day |

The equation for an ideal gas, $PV = nRT$, is used to convert concentrations of gases used in inhalation studies from units of ppm to units of mg/L or mg/m$^3$. Consider as an example the rat reproductive toxicity study by inhalation of carbon tetrachloride (molecular weight, 153.84) summarized in *Pharmeuropa*, 1997:*9*(1):S9.

$$\frac{n}{V} = \frac{P}{RT} = \frac{300 \times 10^{-6}\text{atm} \times 153{,}840 \ \text{mg mol}^{-1}}{0.082\text{L atm K}^{-1}\text{mol}^{-1} \times 298 \ \text{K}} = \frac{46.15 \ \text{mg}}{24.45 \ \text{L}} = 1.89 \ \text{mg/L}$$

The relationship 1000 L = 1 m$^3$ is used to convert to mg/m$^3$.

***Change to read:***

## USP Reference Standards ⟨11⟩

USP 1-Butanol RS
USP 2-Butanol RS
USP 2-Methyl-1-Propanol RS
USP 3-Methyl-1-butanol RS
USP 1-Pentanol RS
USP 1-Propanol RS
USP 2-Propanol RS
USP Acetone RS
USP Alcohol Determination—Alcohol RS
USP Anisole RS
USP Butyl Acetate RS
USP tert-Butylmethyl Ether RS
USP Dimethyl Sulfoxide RS
USP Ethyl Acetate RS
USP Ethyl Formate RS
USP Formic Acid RS
USP Glacial Acetic Acid RS
USP Heptane RS
USP Isobutyl Acetate RS
USP Isopropyl Acetate RS
USP Methyl Acetate RS
USP Methyl Ethyl Ketone RS
USP Pentane RS
USP Propyl Acetate RS
USP Residual Solvents Mixture—Class 1 RS
USP Residual Solvent Class 1—Benzene RS
USP Residual Solvent Class 1—Carbon Tetrachloride RS
USP Residual Solvent Class 1—1,2-Dichloroethane RS
USP Residual Solvent Class 1—1,1-Dichloroethene RS
USP Residual Solvent Class 1—1,1,1-Trichloroethane RS
USP Residual Solvents Class 2—Mixture A RS
USP Residual Solvents Class 2—Mixture B RS
▲USP Residual Solvents Class 2—Mixture C RS▲ (ERR 1-Sep-2022)
USP Residual Solvent Class 2—Acetonitrile RS
USP Residual Solvent Class 2—Chlorobenzene RS
USP Residual Solvent Class 2—Chloroform RS
USP Residual Solvent Class 2—Cumene RS
USP Residual Solvent Class 2—Cyclohexane RS
USP Residual Solvent Class 2—1,2-Dichloroethene RS
USP Residual Solvent Class 2—1,2-Dimethoxyethane RS
USP Residual Solvent Class 2—N,N-Dimethylacetamide RS
USP Residual Solvent Class 2—N,N-Dimethylformamide RS
USP Residual Solvent Class 2—1,4-Dioxane RS
USP Residual Solvent Class 2—2-Ethoxyethanol RS
USP Residual Solvent Class 2—Ethylene Glycol RS
USP Residual Solvent Class 2—Hexane RS
USP Residual Solvent Class 2—Methanol RS
USP Residual Solvent Class 2—2-Methoxyethanol RS
USP Residual Solvent Class 2—Methylbutylketone RS
USP Residual Solvent Class 2—Methylcyclohexane RS
USP Residual Solvent Class 2—Methylene Chloride RS
USP Residual Solvent Class 2—Methylisobutylketone RS

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USP Residual Solvent Class 2—N-Methylpyrrolidone RS
USP Residual Solvent Class 2—Nitromethane RS
USP Residual Solvent Class 2—Pyridine RS
USP Residual Solvent Class 2—Tetrahydrofuran RS
USP Residual Solvent Class 2—Tetralin RS
USP Residual Solvent Class 2—Toluene RS
USP Residual Solvent Class 2—Trichloroethylene RS
USP Residual Solvent Class 2—Xylenes RS
USP Triethylamine RS

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# ⟨469⟩ ETHYLENE GLYCOL, DIETHYLENE GLYCOL, AND TRIETHYLENE GLYCOL IN ETHOXYLATED SUBSTANCES

The following procedure is used to determine the concentration of residual ethylene glycol, diethylene glycol, and triethylene glycol in ethoxylated products. Ethoxylated products may contain residual ethylene glycol, diethylene glycol, and triethylene glycol as a result of the manufacturing process. The procedure is suitable for the following substances:

1. Polyethylene glycol 200
2. Polyethylene glycol 300
3. Polyethylene glycol 400
4. Polyethylene glycol 600
5. Polyethylene glycol 1000
6. Polysorbate 20
7. Polysorbate 40
8. Polysorbate 60
9. Polysorbate 80
10. Polyethylene glycol monomethyl ether 350
11. Polyethylene glycol monomethyl ether 550
12. Polyoxyl 35 castor oil
13. Polyoxyl 15 hydroxystearate
14. Polyoxyl 20 cetostearyl ether
15. Polyoxyl 8 stearate
16. Octoxynol 9
17. Nonoxynol 9

## IMPURITIES

• **PROCEDURE**

**Diluent:** Acetone

**Standard solution:** 25 µg/mL of USP Ethylene Glycol RS, 40 µg/mL of USP Diethylene Glycol RS, 40 µg/mL of USP Triethylene Glycol RS, and 40 µg/mL of USP Butane-1,3-diol RS (internal standard) in *Diluent*

**Sample solution:** 40 mg/mL of the test substance and 40 µg/mL of USP Butane-1,3-diol RS (internal standard) in *Diluent*

**Chromatographic system**

(See *Chromatography* ⟨621⟩, *System Suitability*.)

**Mode:** GC

**Detector:** Flame ionization

**Column:** 0.53-mm × 30-m capillary column bonded with a 1.0-µm layer of phase G3

**Temperatures**

**Detector:** 290°

**Injection port:** 270°

**Column:** See *Table 1*.

**Table 1**

| Initial Temperature (°) | Temperature Ramp (°/min) | Final Temperature (°) | Hold Time at Final Temperature (min) |
|---|---|---|---|
| 40 | 10 | 60 | 5 |
| 60 | 10 | 170 | 0 |
| 170 | 15 | 280 | 0, 60[a] |

[a] Hold time was 0 min for the *Standard solution* and 60 min for the *Sample solution* and *Diluent*.

**Carrier gas:** Helium

**Flow rate:** 5.0 mL/min

**Injection volume:** 1.0 µL

**Injection type:** Split injection, split ratio is 2:1

**Liner:** General-purpose split/splitless, tapered, glass wool, deactivated

**Run time:** 26 min for the *Standard solution*; 86 min for the *Sample solution* and *Diluent*

**System suitability**

**Sample:** *Standard solution*

[NOTE—See *Table 2* for the relative retention times.]

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**Table 2**

| Name | Relative Retention Time |
|---|---|
| Ethylene glycol | 0.45 |
| Butane-1,3-diol (internal standard) | 1.00 |
| Diethylene glycol | 1.25 |
| Triethylene glycol | 1.70 |

**System suitability requirements**

**Resolution:** NLT 20 between ethylene glycol and butane-1,3-diol; NLT 20 between butane-1,3-diol and diethylene glycol; NLT 20 between diethylene glycol and triethylene glycol

**Tailing factor:** 0.8–1.8 for each of the four peaks assigned to ethylene glycol, butane-1,3-diol, diethylene glycol, and triethylene glycol

**Relative standard deviation:** NMT 5.0% for the peak response ratio of the respective glycol (ethylene glycol, diethylene glycol, or triethylene glycol) to the internal standard

**Analysis**

**Samples:** *Standard solution* and *Sample solution*

Identify the ethylene glycol, diethylene glycol, and triethylene glycol peaks in the *Sample solution* by comparison with those in the *Standard solution*.

Calculate the content of ethylene glycol, diethylene glycol, or triethylene glycol, in µg/g, in the portion of test substance taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times F$$

$R_U$ = peak response ratio of the respective glycol to the internal standard (peak response of the respective glycol/peak response of the internal standard) from the *Sample solution*

$R_S$ = peak response ratio of the respective glycol to the internal standard (peak response of the respective glycol/peak response of the internal standard) from the *Standard solution*

$C_S$ = concentration of the respective glycol (ethylene glycol, diethylene glycol, or triethylene glycol) in the *Standard solution* (µg/mL)

$C_U$ = concentration of the test substance in the *Sample solution* (mg/mL)

$F$ = conversion factor (1000 mg/g)

## ADDITIONAL REQUIREMENTS

- **USP REFERENCE STANDARDS** ⟨11⟩

USP Butane-1,3-diol RS
USP Diethylene Glycol RS
USP Ethylene Glycol RS
USP Triethylene Glycol RS

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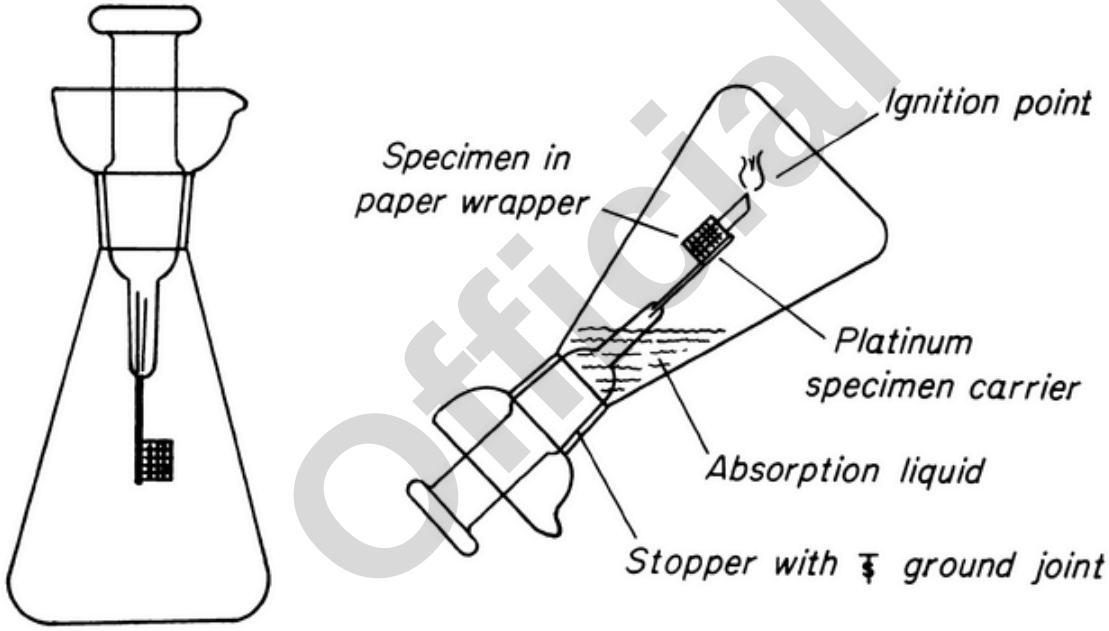
# ⟨471⟩ OXYGEN FLASK COMBUSTION

The oxygen flask combustion procedure is provided as the preparatory step in the determination of bromine, chlorine, iodine, selenium, and sulfur in some Pharmacopeial articles. Combustion of the material under test (usually organic) yields water-soluble inorganic products, which are analyzed for specific elements as directed in the individual monograph or general chapter.

The caution statement given for *Procedure* covers minimum safety precautions only, and serves to emphasize the need for exceptional care throughout.

## APPARATUS

The apparatus[1] consists of a heavy-walled conical, deeply lipped or cupped 500-mL flask (unless a larger flask is specified), fitted with a ground-glass stopper to which is fused a test specimen carrier consisting of heavy-gauge platinum wire and a piece of welded platinum gauze measuring about 1.5 × 2 cm.




Apparatus for Oxygen Flask Combustion

## PROCEDURE

[**CAUTION**—Wear safety glasses and use a suitable safety shield between yourself and the apparatus. Exercise care to ensure that the flask is scrupulously clean and free from even traces of organic solvents.]

Weigh the substance, if a solid, on a piece of halide-free filter paper measuring about 4 cm square, and fold the paper to enclose it. Liquid substances are weighed in tared capsules, polycarbonate capsules[1] being used for liquids in volumes not exceeding 200 µL, and gelatin capsules being satisfactory for use for larger volumes. [NOTE—Gelatin capsules may contain significant amounts of combined halide or sulfur. If such capsules are used, perform a blank determination, and make any necessary correction.] Place the specimen, together with a filter paper fuse-strip, in the platinum gauze specimen holder. Place the absorbing liquid specified in the individual monograph or general chapter in the flask, moisten the joint of the stopper with water, and flush the air from the flask with a stream of rapidly flowing oxygen, swirling the liquid to favor its taking up oxygen. [NOTE—Saturation of the liquid with oxygen is essential for the successful performance of the combustion procedure.] Ignite the fuse-strip by suitable means. If the strip is ignited outside the flask, immediately plunge the specimen holder into the flask, invert the flask so that the absorption solution makes a seal around the stopper, and hold the stopper firmly in place. If the

[1] A suitable apparatus [Catalog Nos. 6513-C20 (500-ml capacity) and 6513-C30 (1000-ml capacity)] and suitable capsules [Catalog No. 6513-84 (1000 capsules)] are obtainable from Thomas Scientific, 99 High Hill Road, Swedesboro, NJ 08085.

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ignition is carried out in a closed system, the inversion of the flask may be omitted. After combustion is complete, shake the flask vigorously, and allow to stand for not less than 10 minutes with intermittent shaking. Then proceed as directed in the individual monograph or general chapter.

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***Add the following:***

# ▲⟨476⟩ CONTROL OF ORGANIC IMPURITIES IN DRUG SUBSTANCES AND DRUG PRODUCTS

## INTRODUCTION

This chapter covers requirements for controlling drug-related organic impurities in drug substances and drug products described in *USP* monographs. A threshold-based approach described in the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) Q3A(R2) *Impurities in New Drug Substances* and Q3B(R2) *Impurities in New Drug Products* guidelines may be used for the control of organic impurities in drug substances or drug products generated during the manufacturing process and/or storage. (For additional information and definition of terms, see *Impurities in Drug Substances and Drug Products* ⟨1086⟩.)

This chapter applies to drug substances and drug products described in the *USP* when referenced in the individual monograph. This chapter addresses organic impurities arising during the manufacturing process and/or storage of the drug substance. Also the chapter covers those impurities in drug products classified as degradation products of the drug substance or reaction products of the drug substance with an excipient and/or immediate container–closure system (collectively referred to as "degradation products").

This chapter does not cover veterinary products, biological/biotechnological products, peptides, oligonucleotides, fermentation products and semisynthetic products derived from them, polymorphic forms, radiopharmaceuticals, herbal products, or crude products of animal or plant origin. In addition, inorganic/elemental impurities, residual solvents, impurities in excipients, and leachables from the container–closure system are out of the scope of this chapter.

This chapter does not cover mutagenic impurities as described in ICH Guideline M7(R1) *Assessment and Control of DNA Reactive (Mutagenic) Impurities in Pharmaceuticals to Limit Potential Carcinogenic Risk*.

## ANALYTICAL PROCEDURES FOR IMPURITIES

Manufacturers shall validate or verify, as appropriate, analytical procedures and must demonstrate their suitability for the detection and quantitation of impurities in drug substances and drug products. Manufacturers shall develop acceptance criteria for impurities that are justified by appropriate safety considerations and consistent with current applicable regulatory guidances.

For impurities known or suspected to be unusually toxic (e.g., mutagenic impurities) or to produce undesired pharmacological effects, the limit of detection and limit of quantitation of the analytical procedures shall be commensurate with the acceptance criteria and the current applicable regulatory guidances to ensure patient safety.

## REPORTING IMPURITIES

The reporting threshold can be established using current applicable regulatory guidances or other acceptable scientific means. The preferred terms applicable to organic impurities procedures are "impurity" and "total impurities" in the drug substance monographs and "degradation product" and "total degradation products" in the drug product monographs. Impurities presenting above the reporting threshold shall be reported according to the relevant analytical procedure.

Impurity results shall be reported as numerical values and rounded according to conventional rules (see *General Notices, 7.20. Rounding Rules*). All impurities at a level greater than (>) the reporting threshold shall be summed and reported as a value for total impurities, unless otherwise indicated in the monograph.

## IDENTIFICATION OF IMPURITIES

Impurities present above the identification threshold for drug substances and drug products at release and on storage shall be investigated and all reasonable attempts shall be made to identify these impurities. The identification threshold can be established using current applicable regulatory guidances or other acceptable scientific means. Lower thresholds may be required for impurities known or suspected to be unusually toxic (e.g., mutagenic impurities) or to produce undesired pharmacological effects. Higher thresholds may be applied if scientifically justified, according to ICH Q3A(R2) and ICH Q3B(R2).

## QUALIFICATION OF IMPURITIES

Qualification is the process of establishing the biological safety of impurities at the specified level(s). Qualification of impurities shall be based on a combination of factors including safety, intended use, applicable guidances, and scientific rationale.

## SETTING ACCEPTANCE CRITERIA FOR IMPURITIES

Acceptance criteria shall be set for all impurities present above the reporting thresholds for drug substances and drug products. Specified impurities/degradation products can be either identified or unidentified. Specified identified impurities/degradation products should be included along with specified unidentified impurities/degradation products estimated to be

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present at a level greater than (>) the identification threshold. Unspecified impurities/degradation products are limited by a general acceptance criterion, which is NMT (≤) the identification threshold and NLT (>) the reporting threshold. A rationale for the inclusion or exclusion of impurities in the specification should be documented. The acceptance criteria shall be based on applicable guidances or other acceptable scientific means, with safety as the primary consideration and not solely based on process capability.

A monograph for a drug substance shall include acceptance criteria for the following, where applicable:

- Each specified identified impurity
- Each specified unidentified impurity
- Any unspecified impurity with an acceptance criterion of NMT the identification threshold
- Total impurities

Unless otherwise indicated, total impurities in the drug substance monographs are the sum of all specified (identified and unidentified) and unspecified impurities above the reporting threshold.

A monograph for a drug product shall include acceptance criteria for the following, where applicable:

- Each specified identified degradation product
- Each specified unidentified degradation product
- Any unspecified degradation product with an acceptance criterion of NMT the identification threshold
- Total degradation products

Unless otherwise indicated, total degradation products in the drug product monographs are the sum of all specified and unspecified degradation products above the reporting threshold.

The preferred terminology applicable to organic impurities procedures is “impurity” and “total impurities” in the drug substance monographs, and “degradation product” and “total degradation products” in the drug product monographs, respectively. Drug substance process impurities need not be controlled in the drug product unless they are also degradation products. However, in some cases drug substance process-related impurities may be included in the drug product specifications, if appropriate, and limited by appropriate acceptance criteria. Drug substance process-related impurities also detected in the drug product and included in its specification may have a note that certain drug substance process-related impurities are listed only for information and should not be included in the total degradation products. When this note is included, the total degradation products should only include all specified and unspecified impurities/degradation products above the reporting threshold, with the exception of these designated process-related impurities.

Acceptance criteria for impurities (including unusually toxic, for example, mutagenic impurities) should be supported by appropriate toxicological evaluation, using current applicable guidances. Lower thresholds may be required for impurities known or suspected to be unusually toxic.

## ORGANIC IMPURITIES IN DRUG SUBSTANCES

Organic impurities in drug substances arising from the manufacturing process and/or storage should be controlled. A rationale for the inclusion or exclusion of impurities in the specification should be documented. The organic impurities to be controlled in the drug substance are the process-related impurities and degradation products. They can be identified or unidentified. Process-related impurities are generated during drug substance manufacturing, such as byproducts, residual starting materials, intermediates, reagents, and ligands. Impurities that increase over time on storage are considered degradation products.

When there are changes to the chemistry, manufacturing, and/or controls of the drug substance (e.g., a different manufacturer or manufacturing site, scale/equipment, starting materials, synthetic pathways, and/or purification steps) described in a monograph, they should be evaluated to determine if the differences affect the impurity profile listed in the existing monograph. If the individual monograph does not include a procedure for quantifying an impurity or acceptance criterion for an observed impurity, the manufacturer is responsible for developing and validating analytical procedures and then establishing appropriate acceptance criterion. USP requests submission of the alternative/new procedure to evaluate for potential inclusion in the appropriate monograph(s).

A threshold-based approach as described in ICH Q3A(R2) may be used for the reporting, identification, and/or qualification of impurities in drug substances. Because toxicity is a dose-related phenomenon, the thresholds are set based on the amount of drug substance administered per day (see *Table 1*). Lower thresholds can be appropriate if the impurity is unusually toxic or produces undesirable pharmacological effects.

**Table 1. ICH Recommended Thresholds for Impurities in Drug Substances**

| Maximum daily dose | ≤2 g | >2 g |
|---|---|---|
| Reporting threshold | 0.05% | 0.03% |
| Identification threshold | 0.10% (1.0 mg)[a] | 0.05% |
| Qualification threshold | 0.15% (1.0 mg)[a] | 0.05% |

[a] The total daily intake (TDI; in parentheses) applies if it is lower than the calculated value.

*Principles for Setting Acceptance Criteria for Impurities* in drug substances are discussed in this chapter and also in ICH guidelines and FDA guidances for New Drug Applications (NDAs) and Abbreviated New Drug Applications (ANDAs). The acceptance criteria shall be based on applicable guidances or other acceptable scientific means, with safety as the primary consideration and not solely based on process capability.

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# ORGANIC IMPURITIES IN DRUG PRODUCTS

Usually, the organic impurities to be controlled in the drug product are only the degradation products resulting from the degradation of the drug substance or the interaction of the drug substance with excipients and/or the primary packaging configuration. They can be identified or unidentified. A rationale for the inclusion or exclusion of impurities in the specification should be documented. Drug substance process-related impurities need not be monitored or specified in drug products unless they are also degradation products.

A threshold-based approach as described in ICH Q3B(R2) may be used for the reporting, identification, and/or qualification of impurities in drug products. Because the toxicity is dose-related, the thresholds are based on the amount of drug substance administered per day (see *Table 2*). The amount of drug substance administered per day is based upon the manufacturer's maximum recommended labeled dosage per day. Lower thresholds can be appropriate if the degradation product is unusually toxic or produces undesirable pharmacological effects.

**Table 2. ICH Recommended Thresholds for Degradation Products in Drug Products**

| Maximum daily dose | <1 mg | ≥1 to <10 mg | 10 mg | >10–100 mg | >100 mg–1 g | >1–2 g | >2 g |
|---|---|---|---|---|---|---|---|
| Reporting threshold | 0.1% | 0.1% | 0.1% | 0.1% | 0.1% | 0.05% | 0.05% |
| Identification threshold | 1.0% (5 µg)[a] | 0.5% (20 µg)[a] | 0.5% (20 µg) [a] | 0.2% (2 mg)[a] | 0.2% (2 mg)[a] | 0.2% (2 mg)[a] | 0.10% |
| Qualification threshold | 1.0% (50 µg)[a] | 1.0% (50 µg)[a] | 0.5% (200 µg)[a] | 0.5% (200 µg)[a] | 0.2% (3 mg)[a] | 0.2% (3 mg)[a] | 0.15% |

[a] Whichever is lower, calculated value or TDI (in parentheses).

*Principles for Setting Acceptance Criteria for Impurities* in drug products are discussed in this chapter and also in ICH guidelines and FDA guidances for NDAs and ANDAs. The acceptance criteria shall be based on applicable guidances or other acceptable scientific means, with safety as the primary consideration and not solely based on process capability. In cases of complex impurity profiles, limits may be established based on the grouping of impurities, if appropriate and scientifically justified [according to ICH Q3B(R2)].

For drug products that contain multiple drug substances, degradation products from each active ingredient should be controlled. Manufacturers should provide rationale and supporting data to justify the acceptance criteria for impurities associated with each drug substance, as applicable.▲ (USP 1-May-2021)

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# ⟨481⟩ RIBOFLAVIN ASSAY

## ASSAY

• **Chemical Methods, Procedure 1**

The following procedure is suitable for preparations in which riboflavin is a constituent of a mixture of several ingredients. In using the procedure, keep the pH of solutions below 7.0, and protect the solutions from direct sunlight at all stages.

**Standard riboflavin stock solution:** To 50.0 mg of USP Riboflavin RS, previously dried and stored protected from light in a desiccator over phosphorus pentoxide, add about 300 mL of 0.02 N acetic acid, and heat the mixture on a steam bath with frequent agitation until the riboflavin has dissolved, then cool. To this solution add 0.02 N acetic acid to make 500 mL; then mix. Store the solution under toluene in a refrigerator.

Dilute an accurately measured portion of this solution by using 0.02 N acetic acid to a concentration of 10.0 µg/mL of the dried USP Riboflavin RS to obtain the *Standard riboflavin stock solution*. Store the solution under toluene in a refrigerator.

**Standard solution:** Dilute with water 10.0 mL of *Standard riboflavin stock solution* in a 100-mL volumetric flask to volume, and mix. Each mL represents 1.0 µg of USP Riboflavin RS. Prepare a fresh *Standard solution* for each assay.

**Sample solution:** Place an amount of the material to be assayed in a flask of suitable size, and add a volume of 0.1 N hydrochloric acid equal in mL to NLT 10 times the dry weight of the material in grams, but the resulting solution will contain NMT 100 µg/mL of riboflavin. If the material is not readily soluble, comminute the material so that it may be evenly dispersed in the liquid. Agitate vigorously, and wash down the sides of the flask with 0.1 N hydrochloric acid.

Heat the mixture in an autoclave at 121°–123° for 30 min, and cool. If clumping occurs, agitate the mixture until the particles are evenly dispersed. Adjust the mixture, with vigorous agitation, with sodium hydroxide solution[1] to a pH of 6.0–6.5, then add hydrochloric acid solution[1] immediately until no further precipitation occurs (usually at a pH of approximately 4.5, which is the isoelectric point of many of the proteins present). Dilute the mixture with water to make a measured volume that contains about 0.11 µg of riboflavin in each mL, and filter through paper known not to adsorb riboflavin. To an aliquot of the filtrate add, with vigorous agitation, sodium hydroxide solution[1] to produce a pH of 6.6–6.8, dilute the solution with water to make a final measured volume that contains approximately 0.1 µg of riboflavin in each mL, and if cloudiness occurs, filter again.

**Instrumental conditions**

(See *Fluorescence Spectroscopy* ⟨853⟩.)

**Mode:** Fluorescence

**Excitation wavelength:** 444 nm

**Emission wavelength:** 530 nm

**Analysis**

**Samples:** *Standard solution*, *Sample solution*, and *Blank*

To each of four or more tubes (or reaction vessels) add 10.0 mL of the *Sample solution*. To each of two or more of these tubes add 1.0 mL of the *Standard solution*, and mix; to each of two or more of the remaining tubes add 1.0 mL of water, and mix. To each tube add 1.0 mL of glacial acetic acid, mix, then add, with mixing, 0.50 mL of potassium permanganate solution (1 in 25), and allow to stand for 2 min. To each tube add, with mixing, 0.50 mL of hydrogen peroxide solution, whereupon the permanganate color is destroyed within 10 s. Shake the tubes vigorously until excess oxygen is expelled. Remove any gas bubbles remaining on the sides of the tubes after foaming has ceased by tipping the tubes so that the solution flows slowly from end to end.

Measure the fluorescence of all tubes, designating the average reading from the tubes containing only the *Sample solution* as $I_U$, and designating the average from the tubes containing both the *Sample solution* and the *Standard solution* as $I_S$. Then to each of one or more tubes of each kind add, with mixing, 20 mg of sodium hydrosulfite, and within 5 s again measure the fluorescence, designating the average reading as $I_B$.

Calculate the quantity, in mg, of riboflavin ($C_{17}H_{20}N_4O_6$) in each mL of the *Sample solution* taken:

$$\text{Result} = [0.0001 \times (I_U - I_B)]/(I_S - I_U)$$

$I_U$ = average reading of the tubes containing only the *Sample solution*
$I_B$ = average reading of fluorescence of the tubes after adding 20 mg of sodium hydrosulfite
$I_S$ = average reading of the tubes containing both the *Sample solution* and *Standard solution*

Calculate the quantity, in mg, of riboflavin ($C_{17}H_{20}N_4O_6$) in each capsule or tablet.

• **Chemical Methods, Procedure 2**

This procedure is suitable for the determination of riboflavin as a dietary ingredient or active pharmaceutical ingredient. [Note—Conduct the entire *Analysis* without exposure to direct sunlight.]

**Standard solution:** Transfer 50 mg of USP Riboflavin RS to a 1000-mL volumetric flask containing 50 mL of water. Add 5 mL of acetic acid and sufficient water to make 800 mL. Heat on a steam bath, protected from light, with frequent agitation until dissolved. Cool to 25°, and dilute with water to volume. Dilute this solution with water to bring it within the operating sensitivity of the fluorometer used.

**Sample solution:** Transfer 50 mg of Riboflavin to a 1000-mL volumetric flask containing 50 mL of water. Add 5 mL of acetic acid and sufficient water to make 800 mL. Heat on a steam bath, protected from light, with frequent agitation until

[1] The concentrations of the hydrochloric acid and sodium hydroxide solutions used are not stated in each instance, because these concentrations may be varied depending upon the amount of material taken for assay, volume of test solution, and buffering effect of material.

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dissolved. Cool to 25°, and dilute with water to volume. Dilute this solution with water to bring it to the same concentration as that of the *Standard solution*.

**Blank:** Prepare as directed for the *Sample solution*, except omit the test specimen.

**Instrumental conditions**

(See *Fluorescence Spectroscopy* ⟨853⟩.)

**Mode:** Fluorescence

**Excitation wavelength:** 444 nm

**Emission wavelength:** 530 nm

**Analysis**

**Samples:** *Standard solution*, *Sample solution*, and *Blank*

Measure the fluorescence intensity of the *Standard solution*. Immediately after the reading, add to the solution 10 mg of sodium hydrosulfite, stirring with a glass rod until dissolved, and at once measure the fluorescence again. [NOTE—Depending on the final concentration of riboflavin in the solution, it may be necessary to increase the amount of sodium hydrosulfite to suppress the fluorescence activity completely.] The difference between the two readings represents the fluorescence intensity ($I_S$) due to the *Standard solution*. Similarly, measure the fluorescence intensity ($I_U$) due to the *Sample solution*. Perform the blank determination, and make any necessary correction.

Calculate the percentage of riboflavin ($C_{17}H_{20}N_4O_6$) in the portion of Riboflavin taken:

$$\text{Result} = (I_U/I_S) \times (C_S/C_U) \times 100$$

$I_U$ = fluorescence of the *Sample solution*
$I_S$ = fluorescence of the *Standard solution*
$C_S$ = concentration of USP Riboflavin RS in the *Standard solution* (µg/mL)
$C_U$ = concentration of riboflavin in the *Sample solution* (µg/mL)

The following liquid chromatographic procedures are provided for the determination of riboflavin as an active pharmaceutical ingredient, a dietary supplement ingredient, or a component in the dietary supplements or pharmaceutical dosage forms. Use the appropriate USP Reference Standards.

Throughout these procedures, protect solutions containing and derived from the test specimen and the Reference Standards from the atmosphere and light, preferably by the use of low-actinic glassware.

**Chromatographic Methods, Procedure 1**

This procedure can be used to determine riboflavin in:

- *Oil- and Water-Soluble Vitamins Capsules*
- *Oil- and Water-Soluble Vitamins Tablets*
- *Oil- and Water-Soluble Vitamins with Minerals Capsules*
- *Oil- and Water-Soluble Vitamins with Minerals Tablets*
- *Water-Soluble Vitamins Capsules*
- *Water-Soluble Vitamins Tablets*
- *Water-Soluble Vitamins with Minerals Capsules*
- *Water-Soluble Vitamins with Minerals Tablets*

This is the procedure that involves the extraction of riboflavin from the formulation by the *Diluent*, heat, and mechanical shaking.

Unless specified in the individual monographs, the *Standard solution*, *Sample solutions*, and reagent solutions are prepared as follows.

**Diluent:** Acetonitrile, glacial acetic acid, and water (5:1:94)

**Mobile phase:** A mixture of methanol, glacial acetic acid, and water (27:1:73) containing 140 mg of sodium 1-hexanesulfonate per 100 mL

**Standard solution:** Transfer 20 mg of USP Riboflavin RS to a 200-mL volumetric flask, and add 180 mL of *Diluent*. Immerse the flask in a hot water bath maintained at 65°–70° for 10 min with regular shaking, or using a vortex mixer, until all of the solid materials are dissolved. Chill rapidly in a cold water bath for 10 min to room temperature, and dilute with *Diluent* to volume.

**Sample solution for capsules:** Weigh NLT 20 capsules in a tared weighing bottle. Open the capsules, without loss of shell material, and transfer the contents to a 100-mL beaker. Remove any contents adhering to the shells by washing with several portions of ether. Discard the washings, and dry the capsule shells with the aid of a current of dry air until the odor of ether is no longer perceptible. Weigh the empty capsule shells in the tared weighing bottle, and calculate the average net weight per capsule. Transfer a portion of the capsule contents, equivalent to 2.5 mg of riboflavin, to a 50-mL centrifuge tube. Add 25.0 mL of *Diluent*, and mix using a vortex mixer for 30 s to completely suspend the powder. Immerse the centrifuge tube in a hot water bath maintained at 65°–70°, heat for 5 min, and mix on a vortex mixer for 30 s. Return the tube to the hot water bath, heat for another 5 min, and mix on a vortex mixer for 30 s. Filter a portion of the solution, cool to room temperature, and use the clear filtrate. [NOTE—Use the filtrate within 3 h of filtration.]

**Sample solution for tablets:** Finely powder NLT 30 tablets. Transfer a portion of the powder, equivalent to 2.5 mg of riboflavin, to a 50-mL centrifuge tube. Add 25.0 mL of *Diluent*, and mix using a vortex mixer for 30 s to completely suspend the powder. Immerse the centrifuge tube in a hot water bath maintained at 65°–70°, heat for 5 min, and mix on a vortex mixer for 30 s. Return the tube to the hot water bath, heat for another 5 min, and mix on a vortex mixer for 30 s. Filter a portion of the solution, cool to room temperature, and use the clear filtrate. [NOTE—Use the filtrate within 3 h of filtration.]

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**Chromatographic system**
(See *Chromatography* ⟨621⟩, *System Suitability*.)
**Mode:** LC
**Detector:** UV 280 nm
**Column:** 4.6-mm × 25-cm; packing L1
**Flow rate:** 1 mL/min
**Injection volume:** 10 µL
**System suitability**
**Sample:** *Standard solution*
**Suitability requirements**
**Relative standard deviation:** NMT 3.0%
**Analysis**
**Samples:** *Standard solution* and appropriate *Sample solution*
Calculate the percentage of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$) in the portion of sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

$r_U$ = peak response of riboflavin from the appropriate *Sample solution*
$r_S$ = peak response of riboflavin from the *Standard solution*
$C_S$ = concentration of USP Riboflavin RS in the *Standard solution* (mg/mL)
$C_U$ = nominal concentration of riboflavin in the appropriate *Sample solution* (mg/mL)

- **Chromatographic Methods, Procedure 2**

This procedure can be used to determine riboflavin in:
- *Oil- and Water-Soluble Vitamins Capsules*
- *Oil- and Water-Soluble Vitamins Tablets*
- *Oil- and Water-Soluble Vitamins with Minerals Capsules*
- *Oil- and Water-Soluble Vitamins with Minerals Tablets*
- *Water-Soluble Vitamins Capsules*
- *Water-Soluble Vitamins Tablets*
- *Water-Soluble Vitamins with Minerals Capsules*
- *Water-Soluble Vitamins with Minerals Tablets*

This is the procedure that involves the extraction of riboflavin from the formulation by the *Extraction solvent*, heat, and mechanical shaking.
Unless specified in the individual monographs, the *Standard solution*, *Sample solutions*, and reagent solutions are prepared as follows.
**Extraction solvent:** Transfer 1 mL of glacial acetic acid and 2.5 g of edetate disodium to a 100-mL volumetric flask. Dissolve in and dilute with water to volume. Mix the resulting solution with methanol (3:1).
**Solution A:** 6.8 g of sodium acetate per 1000 mL of water
**Mobile phase:** Prepare a mixture of *Solution A* and methanol (13:7). Add 2 mL of triethylamine per L of the mixture, and adjust with glacial acetic acid to a pH of 5.2.
**Standard stock solution:** Transfer 20 mg of USP Riboflavin RS to a 200-mL volumetric flask, and add 180 mL of *Extraction solvent*. Immerse the flask for 5 min in a water bath maintained at 65°–75°. Mix well, and repeat if necessary until dissolved. Chill rapidly in a cold water bath to room temperature, and dilute with *Extraction solvent* to volume.
**Standard solution:** Dilute 5.0 mL of the *Standard stock solution* with *Extraction solvent* to 25.0 mL.
**Sample solution for capsules:** Weigh NLT 20 capsules in a tared weighing bottle. Open the capsules, without loss of shell material, and transfer the contents to a beaker. Remove any contents adhering to the shells by washing with several portions of ether. Discard the washings, and dry the capsule shells with the aid of a current of dry air. Weigh the empty capsule shells in the tared weighing bottle, and calculate the net weight of the capsule contents. Transfer a portion of the capsule contents, equivalent to 2 mg of riboflavin, to a 200-mL volumetric flask. Add 100.0 mL of *Extraction solvent*, and mix for 20 min using a wrist-action shaker. Immerse the flask in a water bath maintained at 70°–75°, and heat for 20 min. Mix on a vortex mixer for 30 s, cool to room temperature, and filter. Use the clear filtrate.
**Sample solution for tablets:** Finely powder NLT 20 tablets. Transfer a portion of the powder, equivalent to 2 mg of riboflavin, to a 200-mL volumetric flask. Add 100.0 mL of *Extraction solvent*, and mix for 20 min using a wrist-action shaker. Immerse the flask in a water bath maintained at 70°–75°, and heat for 20 min. Mix on a vortex mixer for 30 s, cool to room temperature, and filter. Use the clear filtrate.
**Chromatographic system**
(See *Chromatography* ⟨621⟩, *System Suitability*.)
**Mode:** LC
**Detector:** UV 254 nm
**Column:** 4.6-mm × 25-cm; packing L1
**Flow rate:** 1 mL/min
**Injection volume:** 20 µL
**System suitability**
**Sample:** *Standard solution*
**Suitability requirements**
**Relative standard deviation:** NMT 3.0%

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**Analysis**

**Samples:** *Standard solution* and appropriate *Sample solution*

Calculate the percentage of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$) in the portion of sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

$r_U$ = peak response of riboflavin from the appropriate *Sample solution*
$r_S$ = peak response of riboflavin from the *Standard solution*
$C_S$ = concentration of USP Riboflavin RS in the *Standard solution* (mg/mL)
$C_U$ = nominal concentration of riboflavin in the appropriate *Sample solution* (mg/mL)

- **Chromatographic Methods, Procedure 3**

This procedure can be used to determine riboflavin in:

- *Oil- and Water-Soluble Vitamins Capsules*
- *Oil- and Water-Soluble Vitamins Tablets*
- *Oil- and Water-Soluble Vitamins with Minerals Capsules*
- *Oil- and Water-Soluble Vitamins with Minerals Tablets*
- *Water-Soluble Vitamins Capsules*
- *Water-Soluble Vitamins Tablets*
- *Water-Soluble Vitamins with Minerals Capsules*
- *Water-Soluble Vitamins with Minerals Tablets*

This is the procedure that involves the extraction of riboflavin from the formulation by mixtures of organic solvents, heat, and mechanical shaking.

Unless specified in the individual monographs, the *Standard solutions*, *Sample solutions*, and reagent solutions are prepared as follows.

**Diluent:** 25 mg/mL of edetate disodium in water

**Mobile phase:** Transfer 0.4 mL of triethylamine, 15.0 mL of glacial acetic acid, and 350 mL of methanol to a 2000-mL volumetric flask. Dilute with 0.008 M sodium 1-hexanesulfonate to volume.

**Standard stock solution:** 0.08 mg/mL of USP Riboflavin RS in *Diluent*, with heating if necessary

**Standard solution for capsules/tablets:** Transfer 5.0 mL of *Standard stock solution* to a stoppered 125-mL flask. Add 10.0 mL of a mixture of methanol and glacial acetic acid (9:1) and 30.0 mL of a mixture of methanol and ethylene glycol (1:1). Insert the stopper, shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

**Standard solution for oral solution:** 8 µg/mL of USP Riboflavin RS in *Diluent*, diluted from the *Standard stock solution*

**Sample solution for capsules:** Weigh NLT 20 capsules in a tared weighing bottle. Open the capsules, without the loss of shell material, and transfer the contents to a 100-mL beaker. Remove any contents adhering to the empty shells by washing, if necessary, with several portions of ether. Discard the washings, and dry the capsule shells with the aid of a current of dry air until the odor of ether is no longer perceptible. Weigh the empty capsule shells in the tared weighing bottle, and calculate the average net weight per capsule. Transfer a portion of the capsule contents, equivalent to 0.4 mg of riboflavin, to a stoppered 125-mL flask. Add 10.0 mL of a mixture of methanol and glacial acetic acid (9:1) and 30.0 mL of a mixture of methanol and ethylene glycol (1:1). Insert the stopper, shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

**Sample solution for oral solution:** Equivalent to 8 µg/mL of riboflavin from oral solution in the *Diluent*

**Sample solution for tablets:** Weigh and finely powder NLT 20 tablets. Transfer a portion of the powder, equivalent to 0.4 mg of riboflavin, to a stoppered 125-mL flask. Add 10.0 mL of a mixture of methanol and glacial acetic acid (9:1) and 30.0 mL of a mixture of methanol and ethylene glycol (1:1). Insert the stopper, shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

**Chromatographic system**

(See *Chromatography* ⟨621⟩*, System Suitability*.)

**Mode:** LC

**Detector:** UV 270 nm

**Column:** 4.6-mm × 25-cm; packing L7

**Column temperature:** 50°

**Flow rate:** 2 mL/min

**Injection volume:** 5 µL

**System suitability**

**Sample:** *Standard solution*

**Suitability requirements**

**Relative standard deviation:** NMT 2.0%

**Analysis**

**Samples:** Appropriate *Standard solution* and appropriate *Sample solution*

For capsules and tablets, calculate the percentage of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$) in the portion of sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

$r_U$ = peak response of riboflavin from the appropriate *Sample solution*

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$r_S$ = peak response of riboflavin from the *Standard solution for capsules/tablets*
$C_S$ = concentration of USP Riboflavin RS in the *Standard solution for capsules/tablets* (mg/mL)
$C_U$ = nominal concentration of riboflavin in the appropriate *Sample solution* (mg/mL)

For oral solution, calculate the percentage of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$) in the portion of sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

$r_U$ = peak response of riboflavin from the *Sample solution for oral solution*
$r_S$ = peak response of riboflavin from the *Standard solution for oral solution*
$C_S$ = concentration of USP Riboflavin RS in the *Standard solution for oral solution* (mg/mL)
$C_U$ = nominal concentration of riboflavin in the *Sample solution for oral solution* (mg/mL)

- **Chromatographic Methods, Procedure 4**

This procedure can be used to determine riboflavin in:

- *Oil- and Water-Soluble Vitamins Capsules*
- *Oil- and Water-Soluble Vitamins Tablets*
- *Oil- and Water-Soluble Vitamins with Minerals Capsules*
- *Oil- and Water-Soluble Vitamins with Minerals Tablets*
- *Water-Soluble Vitamins Capsules*
- *Water-Soluble Vitamins Tablets*
- *Water-Soluble Vitamins with Minerals Capsules*
- *Water-Soluble Vitamins with Minerals Tablets*

This is a newly added procedure as part of the USP monograph modernization efforts. The procedure uses hydrophilic interaction liquid chromatography (HILIC), and the sample preparation involves the extraction of riboflavin from the formulation by the *Diluent*, heat, and mechanical shaking.

Unless specified in the individual monographs, the *Standard solution*, *Sample solutions*, and reagent solutions are prepared as follows.

**Diluent:** Methanol, glacial acetic acid, and water (50:1:49)
**Solution A:** 50 mM ammonium formate; adjust with ammonium hydroxide to a pH of 9.0.
**Solution B:** Acetonitrile
**Mobile phase:** Gradient elution. See *Table 1*.

**Table 1**

| Time (min) | Solution A (%) | Solution B (%) |
|---|---|---|
| 0 | 11 | 89 |
| 8 | 17 | 83 |
| 15 | 23 | 77 |
| 20 | 30 | 70 |
| 21 | 50 | 50 |
| 24 | 50 | 50 |
| 25 | 11 | 89 |
| 30 | 11 | 89 |

**Standard solution:** Transfer 20 mg of USP Riboflavin RS to a 200-mL volumetric flask, and add 160 mL of *Diluent*. Immerse the flask in a hot water bath maintained at 65°–70° for 10 min with regular shaking or using a vortex mixer, until all of the solid materials are dissolved. Chill rapidly in a cold water bath for 10 min to room temperature, and dilute with *Diluent* to volume.

**Sample solution for capsules:** Weigh NLT 20 capsules in a tared weighing bottle. Open the capsules, without the loss of shell material, and transfer the contents to a 100-mL beaker. Remove any contents adhering to the empty shells by washing, if necessary, with several portions of ether. Discard the washings, and dry the capsule shells with the aid of a current of dry air until the odor of ether is no longer perceptible. Weigh the empty capsule shells in the tared weighing bottle, and calculate the average net weight per capsule. Transfer a portion of the capsule contents, equivalent to 2.5 mg of riboflavin, to a 50-mL centrifuge tube. Add 25.0 mL of *Diluent*, and mix using a vortex mixer for 30 s to completely suspend the powder. Immerse the centrifuge tube in a hot water bath maintained at 68°, heat for 10 min, and mix on a vortex mixer for 30 s. Return the tube to the hot water bath, heat for another 10 min, and mix on a vortex mixer for 30 s. Filter a portion of the solution, cool to room temperature, and use the clear filtrate.

**Sample solution for tablets:** Finely powder NLT 30 tablets. Transfer a portion of the powder, equivalent to 2.5 mg of riboflavin, to a 50-mL centrifuge tube. Add 25.0 mL of *Diluent*, and mix using a vortex mixer for 30 s to completely suspend the powder. Immerse the centrifuge tube in a hot water bath maintained at 65°–70°, heat for 10 min, and mix on a vortex mixer for 30 s. Return the tube to the hot water bath, heat for another 10 min, and mix on a vortex mixer for 30 s. Filter a portion of the solution, cool to room temperature, and use the clear filtrate. [Note—Use the filtrate within 3 h of filtration.]

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**Chromatographic system**
(See *Chromatography* ⟨621⟩, *System Suitability*.)
**Mode:** LC
**Detector:** UV 267 nm
**Column:** 4.6-mm × 15-cm; 3.5-µm packing L68
**Flow rate:** 1.2 mL/min
**Injection volume:** 10 µL
**System suitability**
**Sample:** *Standard solution*
**Suitability requirements**
**Relative standard deviation:** NMT 2.0%
**Analysis**
**Samples:** *Standard solution* and appropriate *Sample solution*
Calculate the percentage of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$) in the portion of sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

$r_U$ = peak response of riboflavin from the appropriate *Sample solution*
$r_S$ = peak response of riboflavin from the *Standard solution*
$C_S$ = concentration of USP Riboflavin RS in the *Standard solution* (mg/mL)
$C_U$ = nominal concentration of riboflavin in the appropriate *Sample solution* (mg/mL)

## ADDITIONAL REQUIREMENTS

• **USP Reference Standards** ⟨11⟩
USP Riboflavin RS

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# ⟨501⟩ SALTS OF ORGANIC NITROGENOUS BASES

## STANDARD PREPARATION

Unless otherwise directed, prepare a solution in dilute sulfuric acid (1 in 70) containing, in each mL, about 500 µg of the specified USP Reference Standard, calculated on the anhydrous basis, and accurately weighed.

## ASSAY PREPARATION

If the dosage form is a tablet, weigh and finely powder not less than 20 tablets, weigh accurately a portion of the powder, equivalent to about 25 mg of the active ingredient, and transfer to a 125-mL separator; or, if the dosage form is a liquid, transfer a volume of it, equivalent to about 25 mg of the active ingredient and accurately measured, to a 125-mL separator. Then to the separator add 20 mL of dilute sulfuric acid (1 in 350), and shake vigorously for 5 minutes. Add 20 mL of ether, shake carefully, and filter the acid phase into a second 125-mL separator. Shake the ether phase with two 10-mL portions of dilute sulfuric acid (1 in 350), filter each portion of acid into the second separator, and discard the ether. To the acid extract add 10 mL of sodium hydroxide TS and 50 mL of ether, shake carefully, and transfer the aqueous phase to a third 125-mL separator containing 50 mL of ether. Shake the third separator carefully, and discard the aqueous phase. Wash the two ether solutions, in succession, with a single 20-mL portion of water, and discard the water. Extract each of the two ether solutions with 20-, 20-, and 5-mL portions of dilute sulfuric acid (1 in 70), in the order listed, but each time extract first the ether solution in the third separator and then that in the second separator. Combine the acid extracts in a 50-mL volumetric flask, dilute with the acid to volume, and mix.

[NOTE—Hexane or heptane may be substituted for ether if the distribution ratio of the nitrogenous base between water and hexane, or between water and heptane, favors complete extraction by the organic phase.]

## PROCEDURE

Unless otherwise directed, dilute 5.0 mL each of the *Standard Preparation* and the *Assay Preparation* with dilute sulfuric acid (1 in 70) to 100.0 mL, and determine the absorbance of each solution at the specified wavelength, using dilute sulfuric acid (1 in 70) as the blank. Designate the absorbance of the solution from the *Standard Preparation* as $A_S$ and that from the *Assay Preparation* as $A_U$, and calculate the result of the assay as directed in the individual monograph.

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# ⟨503.1⟩ TRIFLUOROACETIC ACID (TFA) IN PEPTIDES

## INTRODUCTION

The following procedures are to be used to determine the amount of trifluoroacetic acid (TFA) in peptides. TFA/Trifluoroacetate is a common residual process impurity in the preparation of peptides or a counter ion in active pharmaceutical ingredients (API).

- **PROCEDURE**

**Solution A:** Add 7.0 mL of phosphoric acid and 5.0 mL of ammonium hydroxide to 900 mL of water. Mix and dilute with water to 1000 mL. [NOTE—The pH of the solution is approximately 2.5.] Pass through a filter of 0.45-µm pore size and degas. Add 20 mL of methanol, mix, and degas for an additional 2 min.

**Solution B:** Acetonitrile and water (50:50). Mix and degas.

**Mobile phase:** See *Table 1*.

**Table 1**

| Time (min) | Solution A (%) | Solution B (%) |
|---|---|---|
| 0 | 100 | 0 |
| 5 | 100 | 0 |
| 6 | 0 | 100 |
| 14 | 0 | 100 |
| 15 | 100 | 0 |
| 25 | 100 | 0 |

**Diluent:** 0.5% phosphoric acid in water (v/v)

**TFA stock solution** (option 1): 12 mg/mL of USP Sodium Trifluoroacetate RS in water

Calculate the concentration ($C_S$), in mg/mL, of TFA in the *TFA stock solution* taken:

$$C_S = 0.838 \times C$$

0.838 = molecular weight conversion factor (114.02/136.01)

$C$ = concentration of USP Sodium Trifluoroacetate RS in the *TFA stock solution* (mg/mL)

**TFA stock solution** (option 2): 10 mg/mL of TFA in water prepared as follows. Add about 50 mL of water to a 100-mL volumetric flask with a stopper. Tare the stoppered flask on an analytical balance until there is no further significant drift in the reading. Transfer 670 µL of TFA to the flask, stopper immediately, and weigh. Dilute with water to volume.

**System suitability solution:** 0.025 mg/mL of TFA in *Diluent* prepared from the *TFA stock solution*

**Standard solution** (when TFA content is tested as a process impurity): 0.01 mg/mL of TFA in *Diluent* prepared from the *TFA stock solution*

**Standard solution** (when TFA content is tested as a counter ion in the API): 0.25 mg/mL of TFA in *Diluent* prepared from the *TFA stock solution*. [NOTE—The concentration can be adjusted depending on the amount of TFA expected to be present in the test material.]

**Sample solution:** NLT 4 mg/mL of test sample in *Diluent*. [NOTE—The sample concentration can be adjusted so that the limit (when TFA content is tested as a process impurity) or the mid-range (when TFA content is tested as a counter ion) of the amount of TFA stated in the specification for the test material corresponds to that of the *Standard solution*.]

**Chromatographic system**

(See *Chromatography* ⟨621⟩, *System Suitability*.)

**Mode:** LC

**Detector:** UV 210 nm

**Column:** 4.6-mm × 25-cm; 5-µm packing L1. [NOTE—A guard column of 4-mm × 2-cm; 5-µm packing L1 can be used.]

**Flow rate:** 1.5 mL/min

**Injection volume:** 20 µL

**System suitability**

**Sample:** *System suitability solution*

[NOTE—The retention time is about 3 min for TFA.]

**Suitability requirements**

**Relative standard deviation:** NMT 5%

**Analysis**

**Samples:** *Standard solution* and *Sample solution*

Calculate the percentage of TFA in the portion of sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

$r_U$ = TFA peak responses from the *Sample solution*

$r_S$ = TFA peak responses from the *Standard solution*

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$C_S$ = concentration of TFA in the *Standard solution* (mg/mL)
$C_U$ = concentration of the *Sample solution* (mg/mL)

## ADDITIONAL REQUIREMENTS

- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Sodium Trifluoroacetate RS

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# ⟨503⟩ ACETIC ACID IN PEPTIDES

## INTRODUCTION

This chapter provides procedures to be used to determine the amount of acetic acid in peptides. Acetic acid/acetate is a common counter ion in peptide preparations.

• **METHOD 1**

**Strong sodium hydroxide solution:** Dissolve 42 g of sodium hydroxide in water, and dilute with water to 100 mL.

**Solution A:** Add 0.7 mL of phosphoric acid to 1000 mL of water, and adjust with *Strong sodium hydroxide solution* to a pH of 3.0.

**Solution B:** Methanol

**Mobile phase:** See *Table 1*.

**Table 1**

| Time (min) | Solution A (%) | Solution B (%) |
|---|---|---|
| 0 | 95 | 5 |
| 5 | 95 | 5 |
| 10 | 50 | 50 |
| 20 | 50 | 50 |
| 22 | 95 | 5 |

**Diluent:** Prepare a mixture of *Solution A* and *Solution B* (95:5).

**Standard solution:** Dissolve an accurately weighed quantity of USP Glacial Acetic Acid RS in *Diluent* to obtain a solution having a known concentration of about 0.1 mg/mL. [NOTE—The concentration can be adjusted, depending on the amount of acetate or acetic acid expected to be present in the test material.]

**Sample solution:** Prepare as directed in the individual monograph. If no direction is given in the individual monograph, the sample concentration can be adjusted so that the mid-range of the amount of acetic acid stated in the specification for the test material corresponds to that of the *Standard solution*.

**Chromatographic system**

(See *Chromatography* ⟨621⟩, *System Suitability*.)

**Mode:** LC

**Detector:** UV 210 nm

**Column:** 4.6-mm × 25-cm; 5-µm packing L1

**Flow rate:** 1.2 mL/min

**Injection volume:** 10 µL

**System suitability**

**Sample:** *Standard solution*

**Suitability requirements**

**Relative standard deviation:** NMT 5%

**Retention time of acetic acid:** 3–4 min

**Analysis**

**Samples:** *Standard solution* and *Sample solution*

Calculate the percentage of acetic acid in the portion of test material taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

$r_U$ = peak response from the *Sample solution*
$r_S$ = peak response from the *Standard solution*
$C_S$ = concentration of USP Glacial Acetic Acid RS in the *Standard solution* (mg/mL)
$C_U$ = concentration of the *Sample solution* (mg/mL)

• **METHOD 2**

**Solution A, Solution B, Mobile phase, Diluent,** and **Chromatographic system:** See *Trifluoroacetic Acid (TFA) in Peptides* ⟨503.1⟩.

**Standard solution:** 0.7 mg/mL of USP Sodium Acetate Trihydrate RS in *Diluent*

Calculate the concentration of acetic acid in the *Standard solution* ($C_S$), in mg/mL, taken:

$$C_S = 0.441 \times C$$

0.441 = molecular weight conversion factor (60.05/136.08)
$C$ = concentration of USP Sodium Acetate Trihydrate RS in the *Standard solution* (mg/mL)

**Sample solution:** About 4 mg/mL of the test sample in *Diluent*. [NOTE—The sample concentration can be adjusted so that the mid-range of the amount of acetic acid stated in the specification for the test material corresponds to that of the *Standard solution*.]

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[NOTE—Alternatively, the *Standard solution* and *Sample solution* can be prepared as described in *Method 1*.]

**System suitability**

**Sample:** *Standard solution*

**Suitability requirements**

**Relative standard deviation:** NMT 5%

**Analysis:** Proceed as described in *Method 1*.

## ADDITIONAL REQUIREMENTS

- **USP REFERENCE STANDARDS** ⟨11⟩

USP Glacial Acetic Acid RS

USP Sodium Acetate Trihydrate RS

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# ⟨507⟩ PROTEIN DETERMINATION PROCEDURES

## INTRODUCTION

Several procedures exist to determine the total protein in pharmaceutical drug substances and products. Procedures may differ based on the physical characteristic of the protein, which serves as the basis of the measurement principle (e.g., absorbance of ultraviolet light by aromatic amino acid residues or by dye binding). Understanding the protein structure and sample matrix is important when selecting a total protein quantitation procedure. Users must verify that the chosen validated method below is suitable for their specific purposes (see *Verification of Compendial Procedures* ⟨1226⟩).

## PROCEDURE

Two options for Method I are presented: *Method IA*, using denaturing conditions; and *Method IB*, using native conditions. Depending on the protein structure and the nature of the protein sample (e.g., denaturing conditions may be more suitable for a strong coiled structure with most aromatic amino acids in the interior of the protein), one method may prove superior to the other, and users should select which is the most suitable method based on their protein and verify, as appropriate. At a minimum, both methods are dependent on the following conditions being met: 1) the amino acid sequence and molecular weight of the protein must be known; and 2) the protein must contain tyrosines or tryptophans, and no other chromophores (other than cystines) that absorb near 280 nm (including nucleic acids and diluent components) should be present.

- **Method IA. Ultraviolet Light Absorbance Under Denaturing Conditions**

**Sample buffer A:** Prepare 20 mM sodium phosphate, pH 6.5 by dissolving 0.24 g of sodium dihydrogen phosphate in about 80 mL of water. Adjust the pH with sodium hydroxide solution (10% w/w), and fill with water to 100 mL. Filter.

**Sample buffer B:** Prepare 7 M guanidine hydrochloride and 20 mM sodium phosphate, pH 6.5 by dissolving 0.24 g of sodium dihydrogen phosphate and 66.87 g of guanidine hydrochloride in about 50 mL of water. Adjust with sodium hydroxide solution (10% w/w), and fill with water to 100 mL. Filter.

**Reference solution:** Prepare 6 M guanidine hydrochloride in *Sample buffer A* by mixing 330 µL of *Sample buffer A* and 2000 µL of *Sample buffer B*.

**Sample preparation A:** Dilute the test sample by adding *Sample buffer A* and *Sample buffer B* to achieve a final concentration of 6 M guanidine hydrochloride and an absorbance of about 0.4–0.6. [Note—Gravimetric dilutions can be used and sometimes improve accuracy.] Prepare at least three replicates per test sample.

**Analysis**

**Samples:** *Sample buffer A*, *Sample buffer B*, *Reference solution*, and *Sample preparation A*

**Determination of the protein concentration in denaturing buffer:** Using a suitable spectrophotometer (see *Ultraviolet-Visible Spectroscopy* ⟨857⟩), measure the absorbance of *Sample preparation A* in 1-cm cuvettes at an optical density of 280 nm against the *Reference solution*, and subtract the absorbance value at 330 nm to obtain $A_{corrected}$.

Calculate the protein concentration ($c$) in the test sample:

$$c = A_{corrected} \times DF \times MW/(\varepsilon_{280\ nm} \times 1\ cm)$$

$A_{corrected}$ = absorbance at 280 nm minus the absorbance at 330 nm
$DF$ = dilution factor
$MW$ = calculated molecular weight of the protein
$\varepsilon_{280\ nm}$ = extinction coefficient of the test sample at 280 nm

$$\varepsilon_{280\ nm} = (n_{Trp} \times 5690 + n_{Tyr} \times 1280 + n_{Cys} \times 120)\ M^{-1} \times cm^{-1}$$

$n_{Trp}$ = number of tryptophans
$n_{Tyr}$ = number of tyrosines
$n_{Cys}$ = number of cystines

**System suitability**

The test sample UV spectra must show a maximum between 270 and 285 nm and must show a minimum between 243 and 257 nm. The measured absorbance at 280 nm must be NLT 0.4 and NMT 0.6. The relative standard deviation (RSD) of the calculated concentration of all replicates must be NMT 3.0%.

- **Method IB. Ultraviolet Light Absorbance Under Native Conditions**

**Sample buffer A:** Prepare 20 mM sodium phosphate, pH 6.5 by dissolving 0.24 g of sodium dihydrogen phosphate in about 80 mL of water. Adjust the pH with sodium hydroxide solution (10% w/w), and fill with water to 100 mL. Filter.

**Sample preparation A:** Dilute the test sample by adding *Sample buffer A* to achieve an absorbance of about 0.4–0.6. [Note—Gravimetric dilutions can be used and sometimes improve accuracy.] Prepare at least three replicates per test sample.

**Analysis**

**Samples:** ***Sample buffer A*** and ***Sample preparation A***

**Determination of the protein concentration in native buffer:** Using a suitable spectrophotometer (see ⟨857⟩), measure the absorbance of *Sample preparation A* in 1-cm cuvettes at 280 nm against *Sample buffer A* and subtract the absorbance value at 330 nm to obtain $A_{corrected}$.

Calculate the protein concentration ($c$) of the test sample:

$$c = A_{corrected} \times DF \times MW/(\varepsilon_{280\ nm\text{-}native2} \times 1\ cm)$$

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$A_{corrected}$ = absorbance at 280 nm minus the absorbance at 330 nm
$DF$ = dilution factor
$MW$ = calculated molecular weight of the protein
$\varepsilon_{280\ nm\text{-}native2}$ = extinction coefficient of the test sample at 280 nm

where the extinction coefficient $\varepsilon_{280\ nm\text{-}native2}$ is calculated as:

$$\varepsilon_{280\ nm\text{-}native2} = (n_{Trp} \times 5500 + n_{Tyr} \times 1490 + n_{Cys} \times 125)\ M^{-1} \times cm^{-1}$$

$n_{Trp}$ = number of tryptophans
$n_{Tyr}$ = number of tyrosines
$n_{Cys}$ = number of cystines

**System suitability**

The test sample UV spectra must show a maximum between 270 and 285 nm and must show a minimum between 243 and 257 nm. The measured absorbance at 280 nm must be NLT 0.4 and NMT 0.6. The RSD of the calculated concentration of all replicates must be NMT 3.0%.

- **Method II. Bicinchoninic Acid Method**

**BCA reagent solution:**[1] Prepare a solution of 10 g/L of bicinchoninic acid (BCA), 20 g/L of sodium carbonate monohydrate, 1.6 g/L of sodium tartrate, 4 g/L of sodium hydroxide, and 9.5 g/L of sodium bicarbonate in water to a pH of 11.25.

**Copper sulfate reagent solution:** Prepare a solution of 40 g/L of cupric sulfate pentahydrate in water.

**BCA working reagent solution:** Mix 50 volumes of *BCA reagent solution* with 1 volume of *Copper sulfate reagent solution*. Mix until the green solution is clear.

**Sample dilution buffer:** Water or another suitable, non-interfering buffer

**Standard solutions:** Ideally, use the USP Reference Standard for the protein under test. If the protein of interest is unknown, is a mixture, or is a specific USP Reference Standard that is unavailable, use USP BSA for Protein Quantitation RS. Dilute portions of this solution with the *Sample dilution buffer* to obtain NLT five evenly spaced *Standard solutions* in triplicate. The selected *Standard solution* concentrations should result in a linear curve, typically in the range of 25–1000 µg/mL of protein.

**Suitability standard solution:** Prepare an independent dilution of the same protein used to make the *Standard solutions* above using *Sample dilution buffer* to obtain a *Suitability standard solution* with a concentration near the midpoint of the standard curve. Prepare the *Suitability standard solution* in triplicate.

**Sample solution:** Prepare a *Sample solution* with an expected concentration within the standard curve by diluting with the *Sample dilution buffer*. Prepare triplicate samples.

**Analysis**

**Samples:** *Sample dilution buffer*, *Standard solutions*, *Suitability standard solution*, and *Sample solution*

Accurately transfer 100 µL of *Sample dilution buffer*, *Standard solutions*, *Suitability standard solution*, and *Sample solution* to individual test tubes. [Note—96-well, plate-based, and automated methods can also be used with suitable volume adjustments to the procedure below.] Add 2.0 mL of *BCA working reagent solution* to each tube, and mix well.

Cover and incubate the solutions at 37 ± 2° for 30 ± 1 min, then allow the samples to cool to room temperature for NLT 5 min and NMT 60 min. Determine the absorbances of the solutions at 562 nm with a suitable spectrophotometer (see ⟨857⟩). Autozero the instrument with the *Sample dilution buffer* sample.

Measure the absorbance of all *Sample dilution buffer*, *Standard solutions*, *Suitability standard solution*, and *Sample solution* samples within 10 min.

**Calculations:** Plot the absorbances of the *Standard solutions* versus the protein concentrations of the *Standard solutions*, and prepare a standard curve using the linear regression method. Use this standard curve and the absorbance of the *Sample solution* to determine the concentration of protein in each *Suitability standard solution* and *Sample solution*.

**System suitability**

**Samples:** *Standard solutions* and *Suitability standard solution*

**Suitability requirements**

**Linearity:** The coefficient of determination ($r^2$) for a linear fit of all *Standard solutions* is NLT 0.99.

**Relative standard deviation:** NMT 5% for triplicate results of the *Suitability standard solution*

**Accuracy:** The calculated concentration of the *Suitability standard solution* must be within 90%–110% of the theoretical concentration.

**Acceptance criteria**

**Relative standard deviation:** NMT 5% for triplicate *Sample solution* results

- **Method III. Bradford Method**

**Sample dilution buffer:** Water or another suitable, non-interfering buffer

**Standard solutions:** Use the USP Reference Standard for the protein under test or USP BSA for Protein Quantitation RS if the protein of interest is unknown, is a mixture, or is a specific USP Reference Standard that is unavailable. Dilute portions of this solution with the *Sample dilution buffer* to obtain NLT five evenly spaced *Standard solutions* in triplicate. Two standard curve ranges are provided for use: either 0.1–1.0 mg/mL of protein (see *Analysis* procedure *A*, below) or 5–25 µg/mL of protein (see *Analysis* procedure *B*, below).

**Suitability standard solution:** Dilute the USP Reference Standard for the protein under test or USP BSA for Protein Quantitation RS with *Sample dilution buffer* to obtain a *Suitability standard solution* having a concentration near the midpoint of the standard curve. Prepare the *Suitability standard solution* in triplicate.

[1] Suitable BCA reagents are available from Thermo Scientific catalog #23225 or equivalent.

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**Sample solution:** Prepare a *Sample solution* with an expected concentration within one of the two concentration ranges in options *A* or *B* below by diluting with the *Sample dilution buffer*. Prepare triplicate samples.

**Coomassie reagent solution:** Dissolve 100 mg of brilliant blue G[2] in 50 mL of alcohol. Add 100 mL of phosphoric acid, dilute with water to 1 L, and mix. Pass the solution through filter paper (Whatman No. 1 or equivalent), and store the filtered reagent in an amber bottle at room temperature. [NOTE—Slow precipitation of the dye will occur during storage of the reagent. Filter the reagent before use.]

**Analysis:** Select one of the two concentration ranges listed below that is suitable for the expected concentrations of the *Sample solutions*.

**A. Protein concentration range:** 0.1–1.0 mg/mL

**Samples:** *Sample dilution buffer*, *Standard solutions*, and *Sample solution*

Add 1 mL of the *Coomassie reagent solution* to 20 µL of each *Sample dilution buffer*, *Standard solution*, and *Sample solution* sample. Mix by inversion, avoiding foaming. Incubate 10–30 min. By using a suitable spectrophotometer (see ⟨857⟩) [NOTE—Do not use quartz (silica) cuvettes.] , use the *Sample dilution buffer* to set the instrument to zero at 595 nm. Autozero the instrument with the *Sample dilution buffer* sample.

Measure the absorbance of all *Sample dilution buffer*, *Standard solutions*, *Suitability standard solution*, and *Sample solution* samples within 60 min.

**B. Protein concentration range:** 5–25 µg/mL

**Samples:** *Sample dilution buffer*, *Standard solutions*, and *Sample solution*

Add 0.5 mL of the *Coomassie reagent solution* to 0.5 mL each of *Sample dilution buffer*, *Standard solution*, and *Sample solution* sample. Mix by inversion, avoiding foaming. Incubate 10–30 min. By using a suitable spectrophotometer (see ⟨857⟩) [NOTE—Do not use quartz (silica) cuvettes.] , use the *Sample dilution buffer* to set the instrument to zero at 595 nm. Autozero the instrument with the *Sample dilution buffer* sample. Measure the absorbance of all *Sample dilution buffer*, *Standard solutions*, *Suitability standard solution*, and *Sample solution* samples within 60 min.

**Calculations:** [NOTE—The relationship of absorbance to protein concentration is nonlinear; however, if the standard curve concentration range is sufficiently small, it will approach linearity.] Plot the absorbances of the *Standard solutions* versus the protein concentrations, and determine the standard curve best fitting the plotted points. From this standard curve and the absorbance of the *Sample solution*, determine the concentration of protein in the *Sample solution*.

**System suitability**

**Samples:** *Standard solutions* and *Suitability standard solutions*

**Suitability requirements**

**Linearity:** $r^2$ for a fit of all *Standard solutions* is NLT 0.99.

**Relative standard deviation:** NMT 15% for triplicate results of the *Suitability standard solution*

**Recovery:** The calculated concentration of the *Suitability standard solution* must be within 90%–110% of the theoretical concentration.

**Acceptance criteria:** %CV of triplicates of the *Sample solution* is NMT 15%.

• **METHOD IV. LOWRY METHOD**

**SDS solution:** If required, prepare a solution of 50 g/L of sodium dodecyl sulfate (SDS).

**Lowry reagent A:** Prepare a solution of 10 g/L of sodium hydroxide in water, and prepare a solution of 50 g/L of sodium carbonate anhydrous in water. Mix equal volumes (2 volumes and 2 volumes) of each solution, and dilute with water or *SDS solution*, if required, to 5 volumes. [NOTE—SDS may be added to *Lowry reagent A* when the test protein contains detergents or lipids or is poorly soluble after treatment for interfering substances.]

**Lowry reagent B:** Prepare a fresh solution of 29.8 g/L of disodium tartrate dihydrate in water. Prepare a solution of 12.5 g/L of cupric sulfate pentahydrate in water. Mix equal volumes (2 volumes and 2 volumes) of each solution, and dilute with water to 5 volumes.

**Lowry reagent C:** Mix 50 volumes of *Lowry reagent A* with 1 volume of *Lowry reagent B*. [NOTE—Prepare fresh and protect from light.]

**Sample dilution buffer:** Purified water or other non-interfering buffer

**Standard solutions:** Use the USP Reference Standard for the protein under test or USP BSA for Protein Quantitation RS if the protein of interest is unknown, is a mixture, or is a specific USP Reference Standard that is unavailable. Dilute portions of this solution with the *Sample dilution buffer* to obtain NLT five evenly spaced *Standard solutions*. The standard concentrations should be selected to result in a linear curve, typically in the range of 10–200 µg/mL of protein.

**Suitability standard solution:** Dilute the USP Reference Standard for the protein under test or USP BSA for Protein Quantitation RS with *Sample dilution buffer* to obtain a *Suitability standard solution* having a concentration near the midpoint of the standard curve. Prepare and analyze the *Suitability standard solution* in triplicate.

**Sample solution:** Prepare a *Sample solution* with an expected concentration within the standard curve by diluting with the *Sample dilution buffer*. Prepare and analyze samples in triplicate.

**Diluted Folin–Ciocalteu's phenol reagent:** Dilute Folin–Ciocalteu's phenol reagent with water (1:1). Alternative dilutions may be used provided that the pH of the samples (i.e., *Standard solutions* and *Sample solutions* after addition of *Lowry reagent C* and the *Diluted Folin–Ciocalteu's phenol reagent*) is 10.0–10.6.

**Analysis**

**Samples:** *Sample dilution buffer*, *Standard solutions*, *Suitability standard solution*, and *Sample solution*

Accurately transfer 1.0 mL of *Sample dilution buffer*, *Standard solutions*, *Suitability standard solution*, and *Sample solution* samples to individual test tubes. To each test tube add 5.0 mL of *Lowry reagent C*. Allow to stand at room temperature for 10 min. Add 0.5 mL of *Diluted Folin–Ciocalteu's phenol reagent* to each test tube, mix immediately, and incubate at room temperature for 30 min. Determine the absorbance of each solution at 750 nm with a suitable spectrophotometer (see ⟨857⟩). Autozero the instrument with the *Sample dilution buffer*.

[2] Suitable brilliant blue G dye is available from Sigma–Aldrich, catalog #B0770.

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**Calculations:** Plot the absorbances of the *Standard solutions* versus the protein concentrations of the *Standard solutions*, and prepare a standard curve using the linear regression method. Use this standard curve and the absorbances of the *Sample solutions* to determine the concentration of protein in each of the *Sample solution* samples and *Suitability standard solution*.

**System suitability**

**Sample:** *Suitability standard solution*

**Suitability requirements**

**Linearity:** $r^2$ for a linear fit of all standards is NLT 0.995.

**Relative standard deviation:** NMT 5% for triplicate results of the *Suitability standard solution*

**Recovery:** The calculated concentration of the *Suitability standard solution* must be within 90%–110% of the theoretical concentration.

**Acceptance criteria**

**Relative standard deviation:** NMT 5% for triplicate sample results

• **Method V. Amino Acid Analysis**

Separation and determination of hydrolyzed amino acids by ion-exchange chromatography, followed by postcolumn ninhydrin derivatization, is described for protein samples containing a protein of known molecular weight and amino acid composition. [Note—For additional information, see *Biotechnology-Derived Articles—Amino Acid Analysis* ⟨1052⟩, which may be a helpful but not mandatory resource.]

**Hydrolysis solution:** 6 N hydrochloric acid

**Sample hydrolysate preparations:** Prepare a protein sample such that the content of amino acids is within the established linear working range of the procedure. [Note—Glassware used for hydrolysis must be clean.] Place suitable volumes of the sample and *Hydrolysis solution* in a hydrolysis tube. [Note—Common volumes range from 100 to 500 µL for the sample and from 1 to 2 mL for the *Hydrolysis solution* such that complete protein digestion is demonstrated within the hydrolysis incubation time.] Cool the mixture until solidification, and evacuate until the pressure is <0.2 bar. Flame-seal the hydrolysis tube in vacuum by melting the neck of the tube. [Note—Alternative commercially available hydrolysis vials also can be used.] Hydrolyze at 110° for 22 h. Allow the tube to cool, and remove the solvent by centrifugal vacuum concentrator.

**Chromatography**

**Solution A:** Sodium citrate buffer (0.2 N in sodium), containing 2.0% trisodium citrate dihydrate, 0.1% phenol, 0.2% thiodiglycol, and 2.0% propan-2-ol. Adjust with concentrated hydrochloric acid to a pH of 3.2.

**Solution B:** Sodium citrate buffer (0.2 N in sodium), containing 2.0% trisodium citrate dihydrate and 0.1% phenol. Adjust with concentrated hydrochloric acid to a pH of 4.25.

**Solution C:** Sodium citrate buffer (1.2 N in sodium), containing 2.0% trisodium citrate dihydrate, 0.1% phenol, and 5.8% sodium chloride. Adjust with concentrated hydrochloric acid to a pH of 6.45.

**Solution D:** 0.4 M sodium hydroxide containing 0.1% ethylenediaminetetraacetic acid

**Mobile phase:** See *Table 1*. Equilibrate the system before each run.

**Table 1**

| Time (min) | Solution (100%) | Column Temperature | Flow, Mobile Phase (mL/h) | Flow, Postcolumn Reagent (mL/h) |
|---|---|---|---|---|
| 0 | A | 49° | 35 | 25 |
| 8.5 | A | 49° | 35 | 25 |
| 8.51 | B | 51° | 35 | 25 |
| 23.0 | B | 51° | 35 | 25 |
| 23.01 | C | 51° | 35 | 25 |
| 24.0 | C | 51° | 35 | 25 |
| 24.01 | C | 95° | 35 | 25 |
| 41.5 | C | 95° | 35 | 25 |
| 41.51 | D | 95° | 35 | 25 |
| 45.5 | D | 95° | 35 | 25 |
| 45.51 | A | 49° | 35 | 25 |
| 49.0 | A | 49° | 35 | 25 |
| 49.01 | 50% Isopropanol | 49° | 0 | 0 |
| 51.0 | 50% Isopropanol | 49° | 0 | 0 |
| 51.01 | A | 49° | 44 | 0 |
| 62.0 | A | 49° | 44 | 0 |
| 62.01 | A | 49° | 35 | 25 |
| 64.0 | A | 49° | 35 | 25 |

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**Loading buffer:** Sodium citrate buffer (0.2 N in sodium), containing 2.0% trisodium citrate dihydrate, 0.1% phenol, and 2.0% thiodiglycol. Adjust with concentrated hydrochloric acid to a pH of 2.2.

**Postcolumn reagent**

**Solution 1:** Solution containing 7.6% acetic acid, 47.7% ethylene glycol, 28.7% water, and 16.0% potassium acetate

**Solution 2:** Solution containing 9% methanol, 8% ninhydrin, 46.4% diethylene glycolmethyl ether, 36% ethylene glycol, and 0.6% hydrindantin. Prepare *Postcolumn reagent* by mixing 1.75 L of *Solution 1* and 0.25 L of *Solution 2*. Use nitrogen sparging during preparation and mixing. Keep the solution under nitrogen pressure.

**Standard solution:** Prepare a solution having known equimolar amounts of L-alanine, L-arginine, L-aspartic acid, L-glutamic acid, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tyrosine, and L-valine with half the equimolar amount of L-cystine. [NOTE—Suitable concentrations are 250 and 125 nmol/mL, respectively.]

**Sample solution:** Reconstitute the *Sample hydrolysate preparations* in a suitable volume of *Loading buffer*.

**Blank:** *Hydrolysis solution* that has been taken through analytical steps including hydrolysis

**Chromatographic system**

(See *Chromatography* ⟨621⟩*, System Suitability*.)

**Mode:** LC

**Detector:** 440 and 570 nm

**Columns**

**Prewash:** 4.6-mm × 10-cm; 20-µm sodium form, packing L58

**Analytical:** 4.6-mm × 20-cm; 8-µm sodium form, packing L58

**Injection volume:** 40 µL, at 1 min with baseline reset

**Postcolumn reaction:** As the amino acids are eluted from the column, *Postcolumn reagent* is added at a rate of 25 mL/h. After mixing, the column effluent and the *Postcolumn reagent* are passed through a reaction coil of approximately 10-m × 0.3-mm PTFE tubing at a temperature of approximately 135°, where a purple color is developed.

**Reaction coil temperature:** 135°

**System suitability**

**Sample:** *Standard solution*

**Suitability requirements:** All 17 amino acid peaks must be visible in the *Standard solution*.

**Resolution:** NLT 1.2 between the pairs of L-threonine and L-serine, L-cystine and L-valine, and L-isoleucine and L-leucine

**Tailing factor:** 0.8–1.5 for the peak of L-aspartic acid

**Relative standard deviation:** NMT 2.0% determined from the L-aspartic acid peak

**Analysis**

**Samples:** *Blank*, *Standard solution*, and *Sample solution*

Record and measure the responses for each amino acid peak in the *Standard solution* and *Sample solution*. Use the responses obtained from the *Standard solution* to calculate the content of each amino acid in the sample. Analyze the *Blank* to confirm purity of the reagent and diluent.

**Calculations:** Use well-recovered amino acids to quantify the protein. [NOTE—For additional information, see *Biotechnology-Derived Articles—Amino Acid Analysis* ⟨1052⟩*, Protein Hydrolysis*, which may be a helpful but not mandatory resource.] Divide the quantity, in nmol, of each of the well-recovered amino acids by the expected number of residues for that amino acid to obtain the protein content based on each well-recovered amino acid. Average the protein content results calculated. Discard protein content values that deviate >5% from the mean. Recalculate the mean protein content from the remaining values to obtain the protein content of the sample.

• **USP REFERENCE STANDARDS** ⟨11⟩

USP BSA for Protein Quantitation RS

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# ▲⟨509⟩ RESIDUAL DNA TESTING

## INTRODUCTION

The following method is suitable for measurement of residual host cell DNA in recombinant therapeutic products produced in either *Escherichia coli* (*E. coli*) or Chinese hamster ovary (CHO) cell lines. Also provided is an extraction procedure that is suitable to be used prior to a quantitative polymerase chain reaction (qPCR)-based method for the measurement of residual host cell DNA. For discussion of the principles and best practices for this type of testing, see *Nucleic Acid-Based Techniques—Approaches for Detecting Trace Nucleic Acids (Residual DNA Testing)* ⟨1130⟩, which may be a helpful resource.

## PROCEDURE

- **Sample Preparation**

There are several procedures for isolating DNA that may be appropriate for biopharmaceutical sample testing. One such procedure is described in detail below and validated for starting DNA concentrations ranging from 0.01 to 50 pg/µL.

**Resuspension solution:** Dissolve tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) and ethylenediaminetetraacetic acid (EDTA) to obtain a solution of 10 mM and 1.0 mM, respectively. Add hydrochloric acid or sodium hydroxide to adjust to a pH of 8.0.

**DNA standard stock solution:** Dilute either the USP CHO Genomic DNA RS or the USP *E. coli* Genomic DNA RS, as appropriate, to a concentration of 1 µg/mL in *Resuspension solution*.

**Sample solutions:** Samples for testing may require dilution or reconstitution to: 1) overcome matrix interference affecting the DNA recovery, 2) yield an appropriate starting volume, or 3) bring the analyte concentration within the quantitative range of the qPCR method. *Sample solutions* may be diluted in water or in *Resuspension solution* if necessary. For drug substance samples, *Sample solutions* should contain sufficient starting material to allow determination of the residual DNA content, if present at the specification limit.

**Positive control solution:** Prepare by spiking *DNA standard stock solution* to *Sample solutions* at a concentration appropriate for the assay (specification, or otherwise justified).

**Negative control solution:** Water or *Resuspension solution* is used in place of *Sample solutions* in the extraction procedures and will be extracted with any samples (if extraction is necessary). The *Negative control solution* is tested using the qPCR-based method to determine the DNA content contributed by the background and to demonstrate that there is no potential cross-contamination during the assay.

**70% Ethanol:** Prepare by adding water to ethanol to give a final concentration of 70% (v/v).

**Proteinase K solution:** Dissolve Tris-HCl, EDTA, and sodium dodecyl sulfate (SDS) to obtain solutions of 100 mM (pH 8.0), 5 mM, and 100 mg/mL, respectively. Add proteinase K[1] to this solution to a final concentration of 10 mg/mL.

**Sodium iodide solution:** 6 M sodium iodide, 15 mM EDTA, 0.25% sodium sulfite, 0.5% sodium *N*-lauroyl sarcosinate, 25 mM Tris-HCl pH 8.0, and 35 µg/mL linear acrylamide. For 100 mL (scale as appropriate), while mixing with a stir bar on a magnetic plate, add in the following order: 50 mL of nuclease-free water, 0.25 g of sodium sulfite, 3.0 mL of 0.5 M EDTA solution, and 2.5 mL of 1 M Tris-HCl pH 8.0; then slowly add 89.93 g of sodium iodide. Add nuclease-free water to a final volume of 100 mL. Filter the solution using a 0.2-µm nylon membrane. [Note—This stock solution can be stored in the dark at 4° for up to 1 year.] On the day of use, add linear acrylamide and sodium *N*-lauroyl sarcosinate to final concentrations of 35 µg/mL and 0.5% (w/v), respectively. [Note—Addition of sodium *N*-lauroyl sarcosinate to sodium iodide solution may result in turbidity. If so, warming to 50–55° for 2–3 min is helpful before using for extraction.]

**Extraction:** Add 50 µL of *Proteinase K solution* to 450 µL of triplicate samples each of *Sample solutions* and *Positive control solution*, and at least one sample of *Negative control solution*, in a 2.0-mL centrifuge tube. Mix and incubate at 60 ± 2° for 1–24 h. Add 500 µL of *Sodium iodide solution* to each tube. Mix and incubate at 40° for 15 min. Add 900 µL of 100% ethanol, mix well, and incubate at room temperature for 15 min. Centrifuge at 10,000 × g for 15 min to pellet the DNA. [Note—Centrifugation at 2°–8° may be helpful to improve DNA recovery.] Remove and discard the supernatant. Wash the pellet by adding 1 mL of 70% ethanol containing 3 µg/mL linear acrylamide, centrifuge again, and discard the supernatant. Air dry the pellet for 5–10 min until no visible liquid is observed. Resuspend the pellet and record the volume of water or *Resuspension solution* used, which may be needed when reporting the final results in *Calculations.*

- **qPCR Analysis**

**2X Master mix:** A suitable buffer containing magnesium chloride, deoxyadenosine triphosphate, deoxyguanosine triphosphate, deoxycytidine triphosphate, deoxyuridine triphosphate, deoxythymidine triphosphate, and highly purified *Thermus aquaticus* DNA polymerase.[2] Mix well immediately before use.

**DNA stock primers and probes:** According to the DNA of the species tested, prepare individual 10-µM solutions of the following primer pairs and probe, using DNAse-free water (see *Table 1*).

**Table 1**

| | | |
|---|---|---|
| *E. coli* | Forward primer | 5′-CCTTACGACCAGGGCTACACA-3′ |
| | Reverse primer | 5′-CTCGCGAGGTCGCTTCTC-3′ |
| | Probe[a] | 5′-CGTGCTACAATGGCGCATACA-3′ |

[1] Sigma catalog #P2308 or a suitable alternative.
[2] ThermoFisher catalog #4440038 or a suitable alternative.

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**Table 1** *(continued)*

| | | |
|---|---|---|
| CHO | Forward primer | 5′-CCTGAGTTCAATTCCCAGCAA-3′ |
| | Reverse primer | 5′-ACATTCTGCTTCCATGTATATCTGCA-3′ |
| | Probe[a] | 5′-TGGCTCACAACCATCCGTTATGAGACCT-3′ |

[a] The probe can be 5′-labeled with 6-carboxyfluorescein and 3′-labeled with 6-carboxytetramethylrhodamine or a suitable alternative.

**DNA probe solution:** Dilute *DNA stock probe* to 2.5 µM with DNAse-free water.

**Standard solutions:** Dilute the *DNA standard stock solution* to obtain five or more suitable standards within the concentration range of 0.001–100 pg/µL.

**Analysis**

**Samples:** *Sample solutions*, *Positive control solution*, *Negative control solution*, and *Standard solutions*

[NOTE—If samples are extracted, then extracted *Sample solutions* and extracted *Control solutions* will be used.]

Transfer 25 µL of the *2X Master mix* to each well of a 96-well qPCR plate. Add 5 µL each of the *DNA stock forward primer*, the *DNA stock reverse primer*, and the *DNA probe solution* of the appropriate species to each well. Add 10 µL of either (extracted) *Sample solutions*, *Standard solutions*, (extracted) *Negative control solution*, or (extracted) *Positive control solution* to their respective wells. [NOTE—The qPCR reaction volume may be scaled as appropriate to accommodate different instruments.] Mix, seal the plate tightly, and centrifuge for 1 min at 1000 × g. Place the plate in a suitable qPCR thermal cycler. Incubate for 2 min at 50°, then for 10 min at 95°, followed by 40 cycles, with each cycle consisting of 95° for 15 s and 60° for 1 min. [NOTE—Some instruments and reagents require a preincubation step. Carefully follow specific instrument/reagent recommendations.] Monitor the signal of the labeled probe using a suitable fluorescent detector. Determine the threshold value using the instrument-specific recommendations. Record the cycle thresholds ($C_t$) for each sample.

**Calculations**

Plot the log quantity of DNA of the *Standard solutions* versus the $C_t$.

Calculate the slope and the intercept.

Using these values and the following equation, calculate the quantity of DNA in each well:

$$\text{Result} = 10^{\left(\frac{C_t - b}{m}\right)}$$

$C_t$ = cycle threshold of the *Sample solutions*
$b$ = intercept of the line for the *Standard solutions*
$m$ = slope of the line for the *Standard solutions*

Calculate the quantity of DNA in each of the *Sample solutions*. Correct for any dilution or concentration of the sample.

**System suitability**

**Samples:** *Negative control solution* and *Standard solutions*

**Suitability requirements**

**Negative control solution:** The $C_t$ corresponding to the *Negative control solution*, if any, is NLT the $C_t$ of all of the lowest concentrations of the *Standard solutions*.

**Sensitivity:** The $C_t$ corresponding to the lowest concentration of the *Standard solutions* is NMT 39.

**Linearity:** The regression coefficient associated with the *Standard solutions* is NLT 0.98. The slope is between –3.1 and –3.8.

**Acceptance criteria**

Any measurable sample must fall within the standard curve.

**Accuracy:** The mean spike recovery of three replicates of *Positive control solution* is between 50% and 150%. [NOTE—If needed due to the extraction procedure, correct for the sample dilution.]

**Relative standard deviation:** NMT 30% for three replicates of the *Sample solutions*; NMT 30% for three replicates of *Positive control solution*

The limit of residual DNA is defined in the product monograph.

## ADDITIONAL REQUIREMENTS

• **USP REFERENCE STANDARDS** ⟨11⟩

USP CHO Genomic DNA RS

USP *E. coli* Genomic DNA RS▲ (USP 1-Dec-2019)

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# ⟨511⟩ SINGLE-STEROID ASSAY

In the following procedure, the steroid to be assayed is separated from related foreign steroids and excipients by thin-layer chromatography and determined following recovery from the chromatogram.

## PREPARATION OF THE PLATE

Prepare a slurry from 30 g of chromatographic silica gel with a suitable fluorescing substance by the gradual addition, with mixing, of about 65 mL of a mixture of water and alcohol (5:2). Transfer the slurry to a clean, 20- × 20-cm plate, spread to make a uniform layer 250 µm thick, and allow to dry at room temperature for 15 minutes. Heat the plate at 105° for 1 hour, and store in a desiccator.

## SOLVENT A

Mix methylene chloride with methanol (180:16).

## SOLVENT B

Mix chloroform with acetone (4:1).

## STANDARD PREPARATION

Dissolve in a mixture of equal volumes of chloroform and alcohol a suitable quantity of the USP Reference Standard specified in the individual monograph, previously dried as directed (see *USP Reference Standards* ⟨11⟩) and accurately weighed, to obtain a solution having a known concentration of about 2 mg per mL.

## ASSAY PREPARATION

Prepare as directed in the individual monograph.

## PROCEDURE

Divide the area of the chromatographic plate into three equal sections, the left and right sections to be used for the *Assay Preparation* and the *Standard Preparation,* respectively, and the center section for the blank. Apply 200 µL each of the *Assay Preparation* and the *Standard Preparation* as streaks 2.5 cm from the bottom of the appropriate section of the plate. Dry the solution as it is being applied, with the aid of a stream of air. Using the *Solvent* specified in the individual monograph, develop the chromatogram in a suitable chamber, previously equilibrated and lined with absorbent paper, until the solvent front has moved 15 cm above the initial streaks.

Remove the plate, evaporate the solvent, and locate the principal band occupied by the *Standard Preparation* by viewing under UV light. Mark this band, as well as corresponding bands in the *Assay Preparation* and blank sections of the plate. Remove the silica gel from each band separately, either by scraping onto glazed weighing papers or by using a suitable vacuum collecting device, and transfer it to a glass-stoppered, 50-mL centrifuge tube. To each tube add 25.0 mL of alcohol, and shake for not less than 2 minutes. Centrifuge the tubes for 5 minutes, pipet 20 mL of the supernatant from each tube into a glass-stoppered, 50-mL conical flask, add 2.0 mL of a solution prepared by dissolving 50 mg of blue tetrazolium in 10 mL of methanol, and mix. Proceed as directed for *Procedure* under *Assay for Steroids* ⟨351⟩, beginning with "Then to each flask."

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# ⟨525⟩ SULFUR DIOXIDE

The following methods are provided for the determination of sulfur dioxide in pharmaceutical excipients.

## METHOD I

### Procedure

Mix 20 g of the test specimen, accurately weighed, with 200 mL of an appropriate solvent as indicated in each individual monograph, and stir until a smooth suspension is obtained. Allow the test specimen mixture to remain undisturbed until most of the test specimen has settled, and filter the aqueous portion through paper (Whatman No. 1 or equivalent). To 100 mL of the clear filtrate add an additional solvent as indicated in each individual monograph, add 3 mL of starch TS, and titrate with 0.01 N iodine solution VS to the first permanent blue or purple color. Each 1.0 mL of 0.01 N iodine solution VS consumed corresponds to 0.003% of sulfur dioxide found.

## METHOD II

### Procedure

Transfer about 50 to 100 g of the substance to be tested, accurately weighed, to a 250-mL conical flask, add 100 to 150 mL of water, and mix. Cool to between 5° and 10°. While stirring with a magnetic stirrer, add 10 mL of cold 1.5 N sodium hydroxide (at a temperature between 5° and 10°). Stir for an additional 20 seconds, and add 10 mL of starch indicator solution, prepared as follows: mix 10 g of soluble starch with 50 mL of cold water, transfer to 1000 mL of boiling water, stir until completely dissolved, cool, and add 1 g of salicylic acid preservative.

[NOTE—Discard the solution after 1 month.]

Add 10 mL of 2.0 N sulfuric acid (at a temperature between 5° and 10°), and titrate immediately with 0.005 N iodine VS until a light blue color persists for 1 minute (see *Titrimetry* ⟨541⟩). Perform a blank determination, using 200 mL of water treated similarly to the solution under test, and make any necessary correction. Each mL of 0.005 N iodine is equivalent to 0.16 mg of $SO_2$.

## METHOD III

### Procedure

Dissolve 20.0 g of the test specimen in 150 mL of hot water in a flask having a round bottom and a long neck, add 5 mL of phosphoric acid and 1 g of sodium bicarbonate, and at once connect the flask to a condenser.

[NOTE—Excessive foaming can be alleviated by the addition of a few drops of a suitable antifoaming agent.]

Distill 50 mL, receiving the distillate under the surface of 50 mL of 0.1 N iodine. Acidify the distillate with a few drops of hydrochloric acid, add 2 mL of barium chloride TS, and heat on a steam bath until the liquid is nearly colorless. The precipitate of barium sulfate, if any, when filtered, washed, and ignited, weighs not more than 3 mg, corresponding to not more than 0.004% of sulfur dioxide, correction being made for any sulfate that may be present in 50 mL of the 0.1 N iodine.

## METHOD IV

In this test, sulfur dioxide is released from the test specimen in a boiling acid medium and is removed by a stream of carbon dioxide. The separated gas is collected in a dilute hydrogen peroxide solution, in which the sulfur dioxide is oxidized to sulfuric acid and titrated with standard alkali, using a pH meter to control the pH value and titration. This test is performed under conditions such that the requirements specified in the system suitability test are met.

### Special Reagents

#### CARBON DIOXIDE

Use carbon dioxide with a flow regulator that will maintain a flow of 100 ± 10 mL per minute.

#### HYDROGEN PEROXIDE SOLUTION

Dilute 30% hydrogen peroxide with water to obtain a 3% solution. Neutralize the 3% hydrogen peroxide solution with 0.01 N sodium hydroxide to a pH of 4.1 determined potentiometrically.

#### POTASSIUM METABISULFITE SOLUTION

Transfer 0.87 g of potassium metabisulfite ($K_2S_2O_5$) and 0.2 g of edetate disodium to a 1000-mL volumetric flask. Dilute with water to volume before mixing. [NOTE—Edetate disodium is used to protect sulfite ion from oxidation.]

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## Apparatus

A suitable apparatus for sulfur dioxide determination is shown in the accompanying diagram (*Figure 1*).

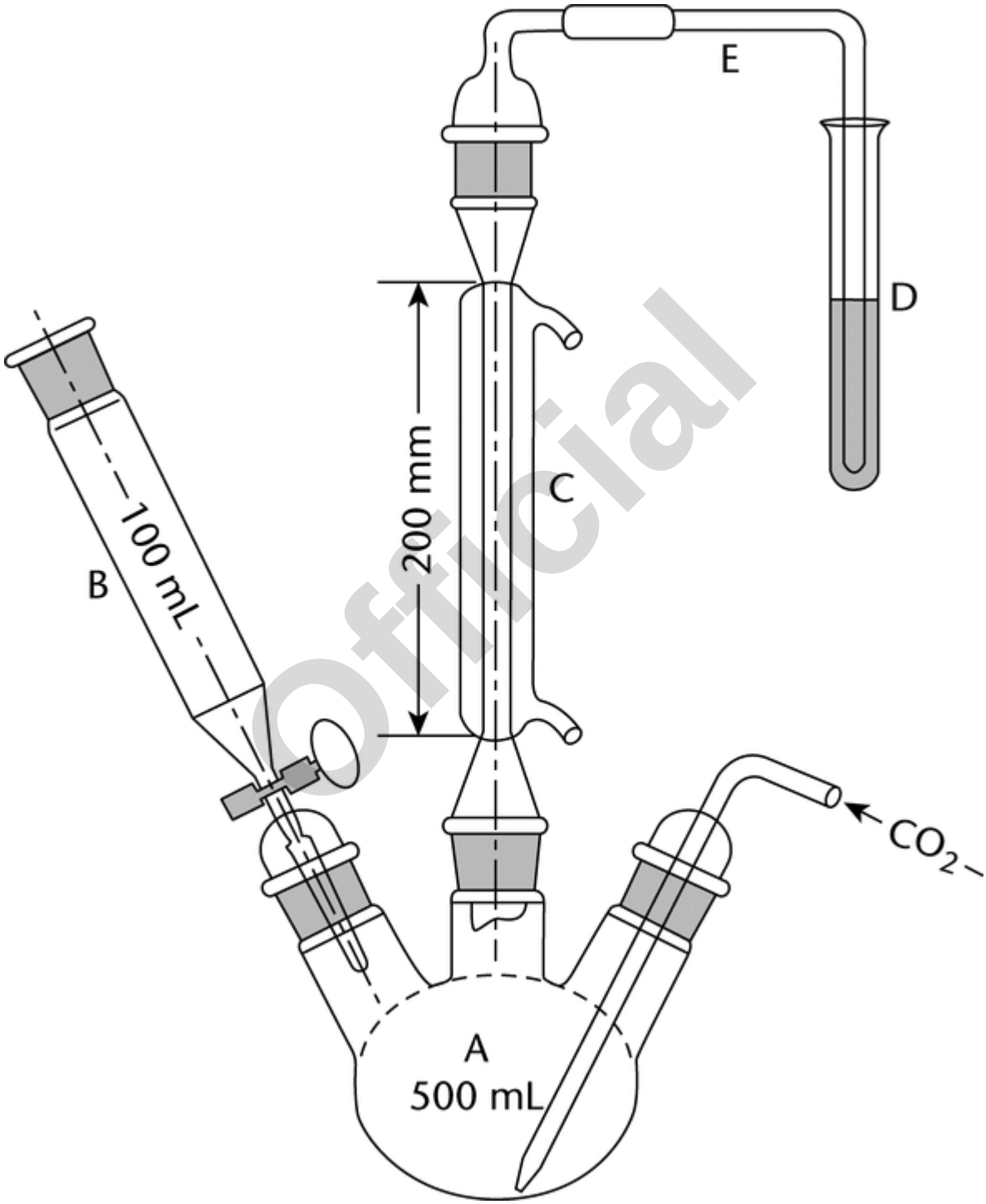


Figure 1. Apparatus for Method IV.

The apparatus consists of a 500-mL three-neck, round-bottom boiling flask, *A;* a separatory funnel, *B,* having a capacity of 100 mL or greater; a gas inlet tube of sufficient length to permit introduction of the carbon dioxide within 2.5 cm of the bottom of the boiling flask; a reflux condenser, *C,* having a jacket length of 200 mm; and a delivery tube, *E,* connecting the upper end of the reflux condenser to the bottom of a receiving test tube, *D.* Apply a thin film of stopcock grease to the sealing surfaces of all joints except the joint between the separatory funnel and the boiling flask, and clamp the joints to ensure tightness.

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## System Suitability Test

TEST A

Using the *Potassium Metabisulfite Solution* as the standard, proceed as directed for *Procedure*, except replace the 25.0 g of test substance with 20 mL of *Potassium Metabisulfite Solution*. Calculate the content, in µg per mL, of sulfur dioxide in the *Potassium Metabisulfite Solution* taken by the formula:

$$1000(32.03)VN/V_P$$

in which the factor 1000 converts mg to µg; 32.03 is the milliequivalent weight of sulfur dioxide; V is the volume, in mL, of titrant consumed; N is the normality of the titrant; and $V_P$ is the volume, in mL, of the *Potassium Metabisulfite Solution* taken for the test.

TEST B

In a 100-mL conical flask, add 20 mL of 0.02 N iodine solution and 5 mL of 2 N hydrochloric acid. Add 1 mL of starch TS, and titrate with the *Potassium Metabisulfite Solution* until the first discoloration is observed. Calculate the content, in µg per mL, of sulfur dioxide in the *Potassium Metabisulfite Solution* by the formula:

$$1000(32.03)V_IN_I/V_P$$

in which 1000 and 32.03 are defined above; $V_I$ is the volume, in mL, of the iodine solution used in the test; $N_I$ is the normality of the iodine solution; and $V_P$ is the volume, in mL, of the *Potassium Metabisulfite Solution* consumed.

The difference between the sulfur dioxide contents obtained from *Test A* and *Test B* is not more than 5% of their mean value. *Test B* shall be performed within 15 minutes after completion of *Test A*. [NOTE—This avoids a potential variation of the sulfur dioxide content in the *Potassium Metabisulfite Solution* when stored at room temperature.]

## Procedure

Add 150 mL of water to the boiling flask (*A*). Close the stopcock of the separatory funnel, and begin the flow of carbon dioxide at a rate of 100 ± 5 mL per minute through the apparatus. Start the condenser coolant flow. Place 10 mL of *Hydrogen Peroxide Solution* in the receiving test tube (*D*). After 15 minutes, without interrupting the flow of carbon dioxide, remove the separatory funnel (*B*) from the boiling flask, and transfer 25.0 g of the test specimen to the boiling flask with the aid of 100 mL of water. Apply stopcock grease to the outer joint of the separatory funnel, and replace the separatory funnel in the boiling flask. Close the stopcock of the separatory funnel, and add 80 mL of 2 N hydrochloric acid to the separatory funnel. Open the stopcock of the separatory funnel to permit the hydrochloric acid solution to flow into the boiling flask, guarding against escape of sulfur dioxide into the separatory funnel by closing the stopcock before the last few mL of hydrochloric acid drain out. Boil the mixture for 1 hour. Open the stopcock of the funnel, stop the flow of carbon dioxide, discontinue heating the flask, and turn off the cooling water in the condenser. Remove the receiving test tube, and transfer its contents to a 200-mL wide-necked, conical flask. Rinse the receiving test tube with a small portion of water, add the rinsing to the 200-mL conical flask, and mix. Heat on a water bath for 15 minutes, and allow to cool. Add 0.1 mL of bromophenol blue TS, and titrate the contents with 0.1 N sodium hydroxide VS until the color changes from yellow to violet-blue, with the color change lasting for at least 20 seconds. Perform a blank determination and make any necessary correction (see *Titrimetry* ⟨541⟩). Calculate the content, in µg per g, of sulfur dioxide in the test specimen taken by the formula:

$$1000(32.03)VN/W$$

in which the factor 1000 converts mg to µg; 32.03 is the milliequivalent weight of sulfur dioxide; V is the volume, in mL, of titrant consumed; N is the normality of the titrant; and W is the weight, in g, of the test specimen taken.

# METHOD V

In this method, similar to *Method IV,* sulfur dioxide is released from the test specimen in a boiling acid medium and is removed by a stream of nitrogen. The separated gas is collected in a dilute hydrogen peroxide solution, in which the sulfur dioxide is oxidized to sulfuric acid and titrated with standard alkali, using methyl red as an indicator. This test is performed under conditions such that the requirements specified in the system suitability test are met.

## Special Reagents

HYDROGEN PEROXIDE SOLUTION

Dilute a portion of 30 percent hydrogen peroxide with water to obtain a 3% solution. Just before use, add 3 drops of methyl red TS, and neutralize to a yellow endpoint with 0.01 N sodium hydroxide. Do not exceed the endpoint.

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NITROGEN

Use high-purity nitrogen with a flow regulator that will maintain a flow of 200 ± 10 mL per minute. Guard against the presence of oxygen by passing the nitrogen through a scrubber, such as alkaline pyrogallol, prepared as follows: add 4.5 g of pyrogallol to a gas-washing bottle, purge the bottle with nitrogen for 3 minutes, and add a solution containing 85 mL of water and 65 g of potassium hydroxide while maintaining an atmosphere of nitrogen in the bottle.

POTASSIUM METABISULFITE SOLUTION

Transfer 0.87 g of potassium metabisulfite ($K_2S_2O_5$) and 0.2 g of edetate disodium to a 1000-mL volumetric flask. Dilute with water to volume before mixing. [NOTE—Edetate disodium is used to protect sulfite ion from oxidation.]

## Apparatus

The apparatus (see *Figure 2*) is designed to effect the selective transfer of sulfur dioxide from the specimen in boiling aqueous hydrochloric acid to the *Hydrogen Peroxide Solution* in vessel *G*. The backpressure is limited to the unavoidable pressure due to the height of the *Hydrogen Peroxide Solution* above the tip of the bubbler, *F.* Keeping the backpressure as low as possible reduces the likelihood that sulfur dioxide will be lost through leaks. Preboil vinyl and silicone tubing. Apply a thin film of stopcock grease to the sealing surfaces of all joints, except the joint between the separatory funnel and the flask, and clamp the joints to ensure tightness. The separatory funnel, *B,* has a capacity of 100 mL or greater. The inlet adapter, *A,* with a hose connector, provides a means of applying headpressure over the solution.

[NOTE—A pressure-equalizing dropping funnel is not recommended because condensate, which may contain sulfur dioxide, is deposited in the funnel and the side arm.]

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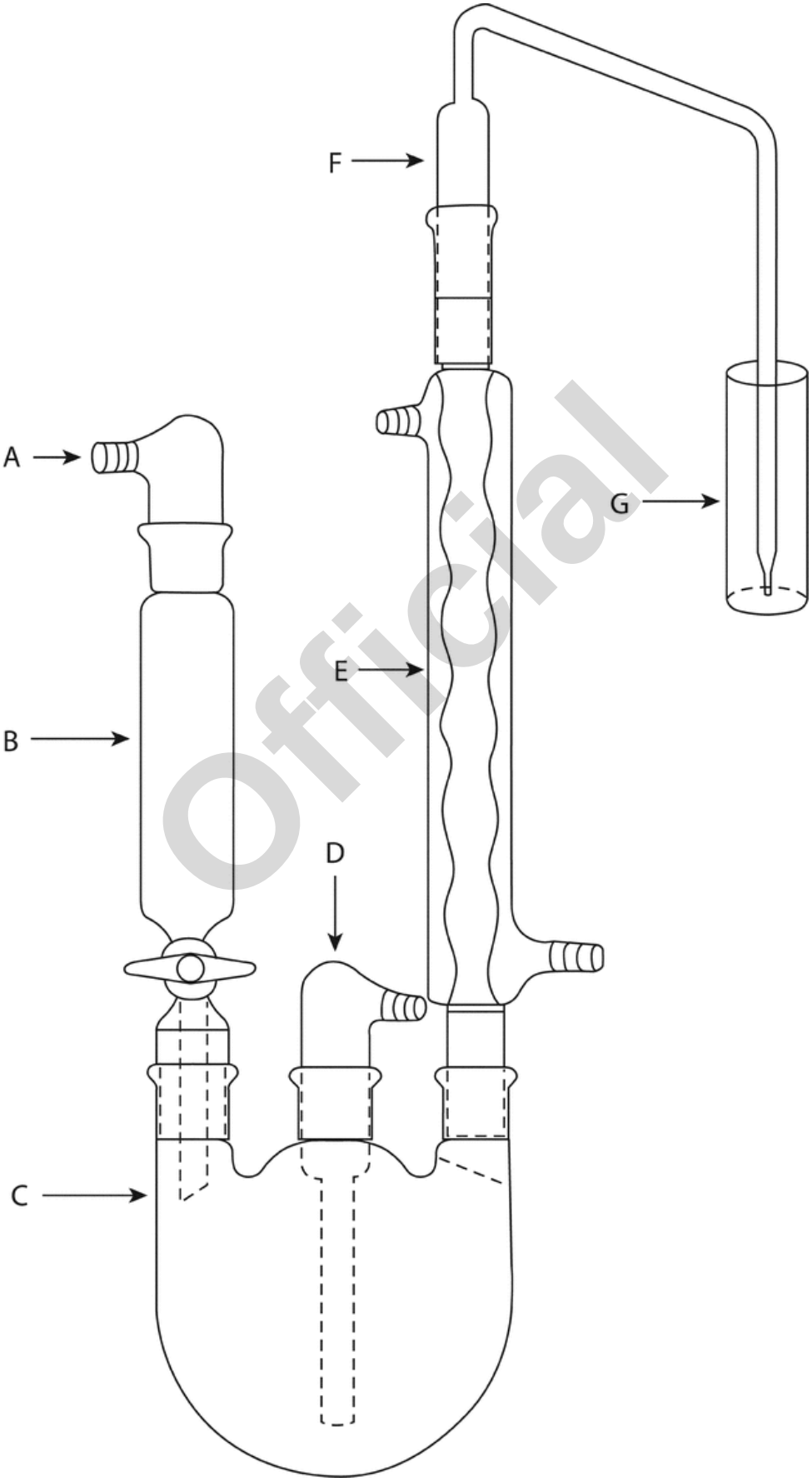


Figure 2. Apparatus for Method V.

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The round-bottom flask, *C,* is a 1000-mL flask with three 24/40 tapered joints. The gas inlet tube, *D,* is long enough to permit introduction of the nitrogen to within 2.5 cm of the bottom of the flask. The Allihn condenser, *E,* has a jacket length of 300 mm. The bubbler, *F* (see *Figure 3*), is fabricated from glass according to the dimensions given in *Figure 3*. The *Hydrogen Peroxide Solution* is contained in the vessel, *G*, having an inside diameter of about 2.5 cm and a depth of about 18 cm. Circulate coolant, such as a mixture of water and methanol (4:1) maintained at 5°, to chill the condenser.

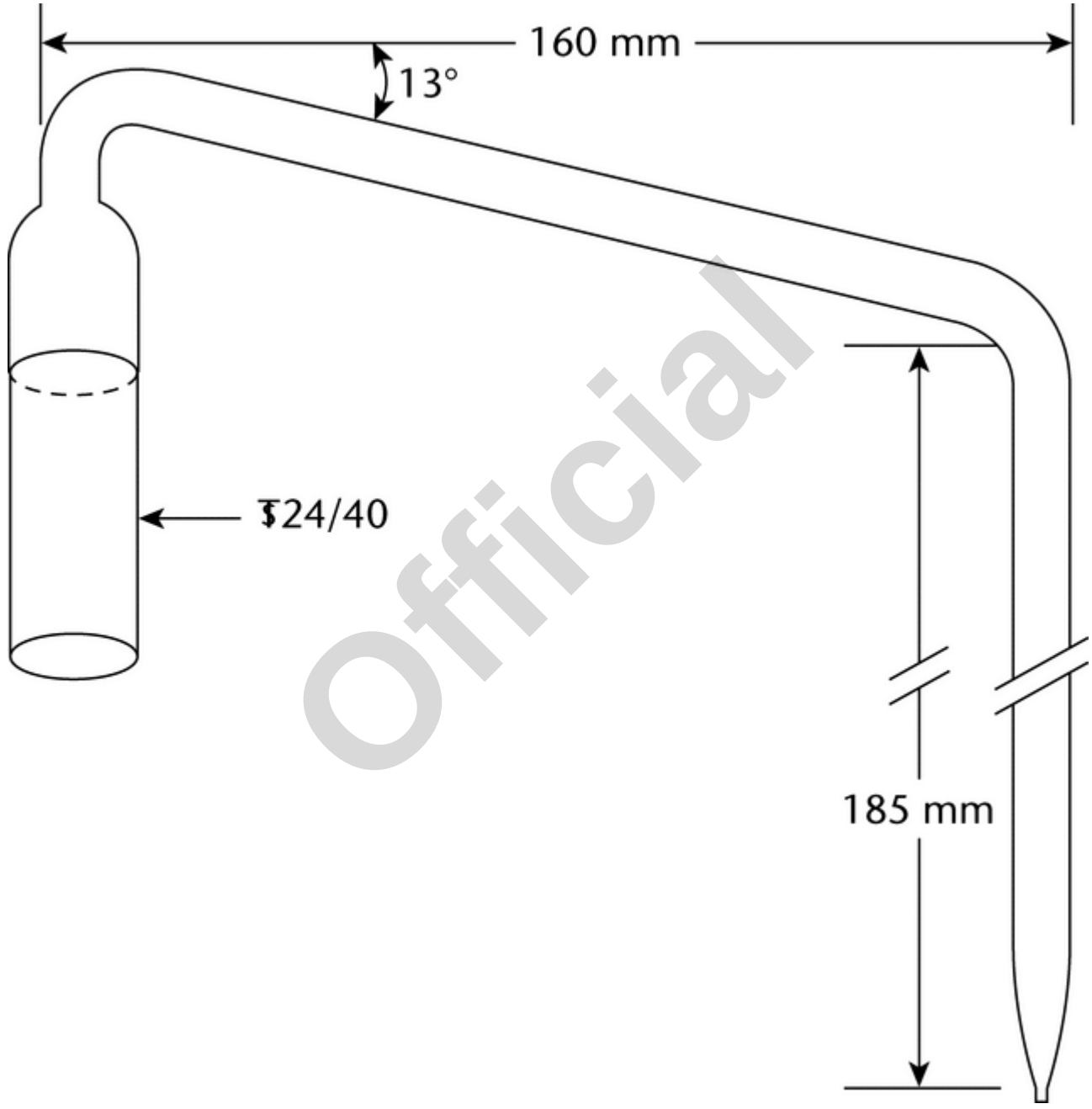


Figure 3. Bubbler (F) for apparatus in Method V.

## System Suitability Test

TEST A

Using the *Potassium Metabisulfite Solution* as the standard, proceed as directed for *Procedure*, except replace the 50.0 g of test substance with 20 mL of *Potassium Metabisulfite Solution*. Calculate the content, in µg per mL, of sulfur dioxide in the *Potassium Metabisulfite Solution* taken by the formula:

$$1000(32.03)VN/V_P$$

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in which the factor 1000 converts mg to µg; 32.03 is the milliequivalent weight of sulfur dioxide; V is the volume, in mL, of titrant consumed; N is the normality of the titrant; and $V_P$ is the volume, in mL, of *Potassium Metabisulfite Solution* taken for the test.

TEST B

In a 100-mL conical flask, add 20 mL of 0.02 N iodine solution and 5 mL of 2 N hydrochloric acid. Add 1 mL of starch TS, and titrate with the *Potassium Metabisulfite Solution* until the first discoloration is observed. Calculate the content, in µg per mL, of sulfur dioxide in the *Potassium Metabisulfite Solution* by the formula:

$$1000(32.03)V_IN_I/V_P$$

in which 1000 and 32.03 are defined above; $V_I$ is the volume, in mL, of iodine solution used in the test; $N_I$ is the normality of the iodine solution; and $V_P$ is the volume, in mL, of *Potassium Metabisulfite Solution* consumed.

The difference between the sulfur dioxide contents obtained from *Test A* and *Test B* is not more than 5% of their mean value. *Test B* shall be performed within 15 minutes after completion of *Test A*. [NOTE—This avoids a potential variation of the sulfur dioxide content in the *Potassium Metabisulfite Solution* when stored at room temperature.]

## Procedure

Position the apparatus in a heating mantle controlled by a power-regulating device. Add 400 mL of water to the flask. Close the stopcock of the separatory funnel, and add 90 mL of 4 N hydrochloric acid to the separatory funnel. Begin the flow of nitrogen at a rate of 200 ± 10 mL per minute. Start the condenser coolant flow. Add 30 mL of *Hydrogen Peroxide Solution* to the vessel *(G)*. After 15 minutes, remove the separatory funnel, and transfer a mixture of 50.0 g of the test specimen, accurately weighed, and 100 mL of alcohol solution (5 in 100) to the flask. Apply stopcock grease to the outer joint of the separatory funnel, return the separatory funnel to the tapered joint flask, and concomitantly resume the nitrogen flow. Apply headpressure above the hydrochloric acid solution in the separatory funnel with a rubber bulb equipped with a valve. Open the stopcock of the separatory funnel to permit the hydrochloric acid solution to flow into the flask. Continue to maintain sufficient pressure above the hydrochloric acid solution to force it into the flask.

[NOTE—The stopcock may be temporarily closed, if necessary, to increase the pressure.]

To guard against escape of sulfur dioxide into the separatory funnel, close the stopcock before the last few mL of hydrochloric acid drain out. Apply power to the heating mantle sufficient to cause about 85 drops of reflux per minute. After refluxing for 1.75 hours, remove the vessel *(G)*, add 3 drops of methyl red TS, and titrate the contents with 0.01 N sodium hydroxide VS, using a 10-mL buret with an overflow tube and a hose connection to a carbon dioxide-absorbing tube, to a yellow endpoint that persists for at least 20 seconds. Perform a blank determination, and make any necessary correction (see *Titrimetry* ⟨541⟩). Calculate the quantity, in µg, of $SO_2$ in each g of the test specimen taken by the formula:

$$1000(32.03)VN/W$$

in which the factor 1000 converts mg to µg; 32.03 is the milliequivalent weight of sulfur dioxide; V is the volume, in mL, of titrant consumed; N is the normality of the titrant; and W is the weight, in g, of the test specimen taken.

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# ⟨531⟩ THIAMINE ASSAY

## ASSAY

• **Chemical Methods, Procedure 1**

The following procedure is provided for the determination of thiamine as an ingredient of pharmacopeial preparations containing other active constituents. The procedure involves thiamine reacting with potassium ferricyanide and subsequently being determined by fluorescence detection. Throughout the procedure, protect solutions containing and derived from the test specimen and the Reference Standard from the atmosphere and light, preferably by the use of low-actinic glassware.

**Potassium ferricyanide solution:** Dissolve 1.0 g of potassium ferricyanide in water to make 100 mL. Prepare fresh on the day of use.

**Oxidizing reagent:** Mix 4.0 mL of *Potassium ferricyanide solution* with a sufficient amount of 3.5 N sodium hydroxide to make 100 mL. Use this solution within 4 h.

**Quinine sulfate stock solution:** Dissolve 10 mg of quinine sulfate in 0.1 N sulfuric acid to make 1000 mL. Preserve this solution, protected from light, in a refrigerator.

**Quinine sulfate standard solution:** Dilute 0.1 N sulfuric acid with *Quinine sulfate stock solution* (39:1). This solution fluoresces to approximately the same degree as the thiochrome obtained from 1 µg of thiamine hydrochloride and is used to correct the fluorometer at frequent intervals for variation in sensitivity from reading to reading within an assay. Prepare this solution fresh on the day of use.

**Standard thiamine hydrochloride stock solution:** Transfer about 25 mg of USP Thiamine Hydrochloride RS, accurately weighed, to a 1000-mL volumetric flask. Dissolve the weighed Standard in about 300 mL of dilute alcohol solution (1 in 5) adjusted with 3 N hydrochloric acid to a pH of 4.0, and add the acidified, dilute alcohol to volume. Store in a refrigerator in a light-resistant container. Prepare this stock solution fresh each month.

**Standard solution:** Dilute a portion of *Standard thiamine hydrochloride stock solution* quantitatively and stepwise with 0.2 N hydrochloric acid to obtain the *Standard solution*, each mL of which represents 0.2 µg of USP Thiamine Hydrochloride RS.

**Sample solution:** Place in a suitable volumetric flask a sufficient amount of the material to be assayed, accurately weighed or measured by volume as directed, such that when diluted with 0.2 N hydrochloric acid to volume, the resulting solution will contain about 100 µg of thiamine hydrochloride (or mononitrate) per mL. If the sample is difficult to dissolve, the solution may be heated on a steam bath, and then cooled and diluted with the acid to volume. Dilute 5 mL of this solution, quantitatively and stepwise, using 0.2 N hydrochloric acid, to an estimated concentration of 0.2 µg of thiamine hydrochloride (or mononitrate) per mL.

**Instrumental conditions**

(See *Fluorescence Spectroscopy* ⟨853⟩.)

**Mode:** Fluorescence

**Excitation wavelength:** 365 nm

**Emission wavelength:** 435 nm

**Analysis:** To each of three or more tubes (or other suitable vessels) of about 40-mL capacity, pipet 5 mL of *Standard solution*. To each of two of these tubes, add rapidly (within 1 to 2 s), with mixing, 3.0 mL of *Oxidizing reagent*, and within 30 s add 20.0 mL of isobutyl alcohol, then mix vigorously for 90 s by shaking the capped tubes manually, or by bubbling a stream of air through the mixture. Prepare a blank in the remaining tube of the standard by substituting for the *Oxidizing reagent* with an equal volume of 3.5 N sodium hydroxide and proceed in the same manner. Into each of three or more similar tubes, pipet 5 mL of the *Sample solution*. Treat these tubes in the same manner as directed for the tubes containing the *Standard solution*. Into each of the six tubes, pipet 2 mL of dehydrated alcohol, swirl for a few seconds, allow the phases to separate, and decant or draw off about 10 mL of the clear, supernatant isobutyl alcohol solution into the standardized cells; then measure the fluorescence in a suitable fluorometer, having an input filter of narrow transmittance range with a maximum at about 365 nm and an output filter of narrow transmittance range with a maximum at about 435 nm.

Calculate the quantity, in µg, of thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) in each 5 mL of the *Sample solution*:

$$\text{Result} = (A - b)/(S - d)$$

$A$ = average fluorometer readings of the portions of the *Sample solution* treated with the *Oxidizing reagent*
$b$ = reading for the blank of the *Sample solution*
$S$ = average fluorometer readings of the portions of the *Standard solution* treated with the *Oxidizing reagent*
$d$ = reading for the blank of the *Standard solution*

Calculate the quantity, in mg, of thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) in the assay material on the basis of the aliquots taken. Where indicated, the quantity, in mg, of thiamine mononitrate ($C_{12}H_{17}N_5O_4S$) may be calculated by multiplying the quantity of thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) found by 0.9706.

The following liquid chromatographic procedures are provided for the determination of thiamine as an active pharmaceutical ingredient, a dietary supplement ingredient, or a component in dietary supplements or pharmaceutical dosage forms.

Throughout these procedures, protect solutions containing and derived from the test specimen and the Reference Standards from the atmosphere and light, preferably by the use of low-actinic glassware.

• **Chromatographic Methods, Procedure 1**

This procedure can be used to determine thiamine in:

- *Oil- and Water-Soluble Vitamins Capsules*
- *Oil- and Water-Soluble Vitamins Tablets*

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- *Oil- and Water-Soluble Vitamins with Minerals Capsules*
- *Oil- and Water-Soluble Vitamins with Minerals Tablets*
- *Water-Soluble Vitamins Capsules*
- *Water-Soluble Vitamins Tablets*
- *Water-Soluble Vitamins with Minerals Capsules*
- *Water-Soluble Vitamins with Minerals Tablets*

This is the procedure that involves the extraction of thiamine from the formulation by the *Diluent*, heat, and mechanical shaking.

Unless specified in the individual monographs, the *Standard solution*, *Sample solutions*, and reagent solutions are prepared as follows.

**Diluent:** Acetonitrile, glacial acetic acid, and water (5:1:94)

**Mobile phase:** A mixture of methanol, glacial acetic acid, and water (27:1:73) containing 140 mg of sodium 1-hexanesulfonate per 100 mL

**Standard solution:** Transfer 20 mg of USP Thiamine Hydrochloride RS to a 200-mL volumetric flask, and add 180 mL of *Diluent*. Immerse the flask in a hot water bath maintained at 65°–70° for 10 min with regular shaking or using a vortex mixer until all of the solid materials are dissolved. Chill rapidly in a cold water bath for 10 min to room temperature, and dilute with *Diluent* to volume.

**Sample solution for capsules:** Weigh NLT 20 capsules in a tared weighing bottle. Open the capsules, without loss of shell material, and transfer the contents to a 100-mL beaker. Remove any contents adhering to the shells by washing with several portions of ether. Discard the washings, and dry the capsule shells with the aid of a current of dry air until the odor of ether is no longer perceptible. Weigh the empty capsule shells in the tared weighing bottle, and calculate the average net weight per capsule. Transfer a portion of the capsule contents, equivalent to 2.5 mg of thiamine hydrochloride, to a 50-mL centrifuge tube. Add 25.0 mL of *Diluent*, and mix using a vortex mixer for 30 s to completely suspend the powder. Immerse the centrifuge tube in a hot water bath maintained at 65°–70°, heat for 5 min, and mix on a vortex mixer for 30 s. Return the tube to the hot water bath, heat for another 5 min, and mix on a vortex mixer for 30 s. Filter a portion of the solution, cool to room temperature, and use the clear filtrate. [NOTE—Use the filtrate within 3 h of filtration.]

**Sample solution for tablets:** Finely powder NLT 30 tablets. Transfer a portion of the powder, equivalent to 2.5 mg of thiamine hydrochloride, to a 50-mL centrifuge tube. Add 25.0 mL of *Diluent*, and mix using a vortex mixer for 30 s to completely suspend the powder. Immerse the centrifuge tube in a hot water bath maintained at 65°–70°, heat for 5 min, and mix on a vortex mixer for 30 s. Return the tube to the hot water bath, heat for another 5 min, and mix on a vortex mixer for 30 s. Filter a portion of the solution, cool to room temperature, and use the clear filtrate. [NOTE—Use the filtrate within 3 h of filtration.]

**Chromatographic system**

(See *Chromatography* ⟨621⟩, *System Suitability*.)

**Mode:** LC

**Detector:** UV 280 nm

**Column:** 4.6-mm × 25-cm; packing L1

**Flow rate:** 1 mL/min

**Injection volume:** 10 µL

**System suitability**

**Sample:** *Standard solution*

**Suitability requirements**

**Relative standard deviation:** NMT 3.0%

**Analysis**

**Samples:** *Standard solution* and appropriate *Sample solution*

For products containing thiamine hydrochloride, calculate the percentage of the labeled amount of thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) in the portion of sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

$r_U$ = peak response of thiamine from the appropriate *Sample solution*
$r_S$ = peak response of thiamine from the *Standard solution*
$C_S$ = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)
$C_U$ = nominal concentration of thiamine hydrochloride in the appropriate *Sample solution* (mg/mL)

For products containing thiamine mononitrate, calculate the percentage of the labeled amount of thiamine mononitrate ($C_{12}H_{17}N_5O_4S$) in the portion of sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

$r_U$ = peak response of thiamine from the appropriate *Sample solution*
$r_S$ = peak response of thiamine from the *Standard solution*
$C_S$ = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)
$C_U$ = nominal concentration of thiamine mononitrate in the appropriate *Sample solution* (mg/mL)
$M_{r1}$ = molecular weight of thiamine mononitrate, 327.36
$M_{r2}$ = molecular weight of thiamine hydrochloride, 337.27

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- **Chromatographic Methods, Procedure 2**

This procedure can be used to determine thiamine in:

- *Oil- and Water-Soluble Vitamins Capsules*
- *Oil- and Water-Soluble Vitamins Tablets*
- *Oil- and Water-Soluble Vitamins with Minerals Capsules*
- *Oil- and Water-Soluble Vitamins with Minerals Tablets*
- *Water-Soluble Vitamins Capsules*
- *Water-Soluble Vitamins Tablets*
- *Water-Soluble Vitamins with Minerals Capsules*
- *Water-Soluble Vitamins with Minerals Tablets*

This is the procedure that involves the extraction of thiamine from the formulation by dilute hydrochloric acid, heat, and mechanical shaking.

Unless specified in the individual monographs, the *Standard solution*, *Sample solutions*, and reagent solutions are prepared as follows.

**Solution A:** 1.88 g/L of sodium 1-hexanesulfonate in 0.1% phosphoric acid

**Mobile phase:** *Solution A* and acetonitrile (46:9)

**Standard stock solution:** 0.1 mg/mL of USP Thiamine Hydrochloride RS in 0.2 N hydrochloric acid

**Standard solution:** 0.02 mg/mL of USP Thiamine Hydrochloride RS from *Standard stock solution* diluted with 0.2 N hydrochloric acid

**Sample solution for capsules:** Weigh NLT 20 capsules in a tared weighing bottle. Open the capsules, without the loss of shell material, and transfer the contents to a 100-mL beaker. Remove any contents adhering to the empty shells by washing, if necessary, with several portions of ether. Discard the washings, and dry the capsule shells with the aid of a current of dry air until the odor of ether is no longer perceptible. Weigh the empty capsule shells in the tared weighing bottle, and calculate the average net weight per capsule. Mix a portion of the capsule contents with a volume of 0.2 N hydrochloric acid to obtain a concentration of 0.02 mg/mL of thiamine. Shake the solution for 15 min with a wrist-action shaker, and heat to boiling for 30 min. Cool to room temperature, and filter. Use the clear filtrate.

**Sample solution for tablets:** Weigh and finely powder NLT 20 tablets. Mix a portion of the powder with a volume of 0.2 N hydrochloric acid to obtain a concentration of 0.02 mg/mL of thiamine. Shake for 15 min with a wrist-action shaker, and heat to boiling for 30 min. Cool to room temperature, and filter. Use the clear filtrate.

**Chromatographic system**

(See *Chromatography* ⟨621⟩, *System Suitability*.)

**Mode:** LC

**Detector:** UV 254 nm

**Column:** 4.6-mm × 25-cm; packing L1

**Flow rate:** 2 mL/min

**Injection volume:** 20 µL

**System suitability**

**Sample:** *Standard solution*

**Suitability requirements**

**Relative standard deviation:** NMT 3.0%

**Analysis**

**Samples:** *Standard solution* and appropriate *Sample solution*

For products containing thiamine hydrochloride, calculate the percentage of the labeled amount of thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) in the portion of sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

$r_U$ = peak response of thiamine from the appropriate *Sample solution*
$r_S$ = peak response of thiamine from the *Standard solution*
$C_S$ = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)
$C_U$ = nominal concentration of thiamine hydrochloride in the appropriate *Sample solution* (mg/mL)

For products containing thiamine mononitrate, calculate the percentage of the labeled amount of thiamine mononitrate ($C_{12}H_{17}N_5O_4S$) in the portion of sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

$r_U$ = peak response of thiamine from the appropriate *Sample solution*
$r_S$ = peak response of thiamine from the *Standard solution*
$C_S$ = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)
$C_U$ = nominal concentration of thiamine mononitrate in the appropriate *Sample solution* (mg/mL)
$M_{r1}$ = molecular weight of thiamine mononitrate, 327.36
$M_{r2}$ = molecular weight of thiamine hydrochloride, 337.27

- **Chromatographic Methods, Procedure 3**

This procedure can be used to determine thiamine in:

- *Oil- and Water-Soluble Vitamins Capsules*

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- *Oil- and Water-Soluble Vitamins Oral Solution*
- *Oil- and Water-Soluble Vitamins Tablets*
- *Oil- and Water-Soluble Vitamins with Minerals Capsules*
- *Oil- and Water-Soluble Vitamins with Minerals Oral Solution*
- *Oil- and Water-Soluble Vitamins with Minerals Tablets*
- *Water-Soluble Vitamins Capsules*
- *Water-Soluble Vitamins Tablets*
- *Water-Soluble Vitamins with Minerals Capsules*
- *Water-Soluble Vitamins with Minerals Oral Solution*
- *Water-Soluble Vitamins with Minerals Tablets*

This is the procedure that involves the extraction of thiamine from the formulation by mixtures of organic solvents, heat, and mechanical shaking.

Unless specified in the individual monographs, the *Standard solutions*, *Sample solutions*, and reagent solutions are prepared as follows.

**Diluent:** 25 mg/mL of edetate disodium in water

**Mobile phase:** Transfer 0.4 mL of triethylamine, 15.0 mL of glacial acetic acid, and 350 mL of methanol to a 2000-mL volumetric flask. Dilute with 0.008 M sodium 1-hexanesulfonate to volume.

**Standard stock solution:** 0.24 mg/mL of USP Thiamine Hydrochloride RS in *Diluent*, with heating if necessary

**Standard solution for capsules/tablets:** Transfer 5.0 mL of *Standard stock solution* to a stoppered 125-mL flask. Add 10.0 mL of a mixture of methanol and glacial acetic acid (9:1) and 30.0 mL of a mixture of methanol and ethylene glycol (1:1). Insert the stopper, shake for 15 min in a water bath maintained at 60°, and cool. Filter, and discard the first few mL of the filtrate.

**Standard solution for oral solution:** 24 µg/mL of USP Thiamine Hydrochloride RS, diluted from the *Standard stock solution* with *Diluent*

**Sample solution for capsules:** Weigh NLT 20 capsules in a tared weighing bottle. Open the capsules, without the loss of shell material, and transfer the contents to a 100-mL beaker. Remove any contents adhering to the empty shells by washing, if necessary, with several portions of ether. Discard the washings, and dry the capsule shells with the aid of a current of dry air until the odor of ether is no longer perceptible. Weigh the empty capsule shells in the tared weighing bottle, and calculate the average net weight per capsule. Transfer a portion of the capsule contents, equivalent to 1.2 mg of thiamine hydrochloride, to a stoppered 125-mL flask. Add 10.0 mL of a mixture of methanol and glacial acetic acid (9:1) and 30.0 mL of a mixture of methanol and ethylene glycol (1:1). Insert the stopper, shake for 15 min in a water bath maintained at 60°, and cool. Filter, and discard the first few mL of the filtrate.

**Sample solution for oral solution:** Equivalent to 24 µg/mL of thiamine hydrochloride or thiamine mononitrate from oral solution in *Diluent*

**Sample solution for tablets:** Weigh and finely powder NLT 20 tablets. Transfer a portion of the powder, equivalent to 1.2 mg of thiamine, to a stoppered 125-mL flask. Add 10.0 mL of a mixture of methanol and glacial acetic acid (9:1) and 30.0 mL of a mixture of methanol and ethylene glycol (1:1). Insert the stopper, shake for 15 min in a water bath maintained at 60°, and cool. Filter, and discard the first few mL of the filtrate.

**Chromatographic system**

(See *Chromatography* ⟨621⟩, *System Suitability*.)

**Mode:** LC

**Detector:** UV 270 nm

**Column:** 4.6-mm × 25-cm; packing L7

**Column temperature:** 50°

**Flow rate:** 2 mL/min

**Injection volume:** 5 µL

**System suitability**

**Sample:** *Standard solution*

**Suitability requirements**

**Relative standard deviation:** NMT 2.0%

**Analysis**

**Samples:** Appropriate *Standard solution* and appropriate *Sample solution*

For products containing thiamine hydrochloride, calculate the percentage of the labeled amount of thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) in the portion of sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

$r_U$ = peak response of thiamine from the appropriate *Sample solution*
$r_S$ = peak response of thiamine from the appropriate *Standard solution*
$C_S$ = concentration of USP Thiamine Hydrochloride RS in the appropriate *Standard solution* (mg/mL)
$C_U$ = nominal concentration of thiamine in the appropriate *Sample solution* (mg/mL)

For products containing thiamine mononitrate, calculate the percentage of the labeled amount of thiamine mononitrate ($C_{12}H_{17}N_5O_4S$) in the portion of sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

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$r_U$ = peak response of thiamine from the appropriate *Sample solution*
$r_S$ = peak response of thiamine from the appropriate *Standard solution*
$C_S$ = concentration of USP Thiamine Hydrochloride RS in the appropriate *Standard solution* (mg/mL)
$C_U$ = nominal concentration of thiamine mononitrate in the appropriate *Sample solution* (mg/mL)
$M_{r1}$ = molecular weight of thiamine mononitrate, 327.36
$M_{r2}$ = molecular weight of thiamine hydrochloride, 337.27

- **Chromatographic Methods, Procedure 4**

This procedure can be used to determine thiamine in:

- An active pharmaceutical ingredient
- A dietary ingredient

This is the procedure that involves the dissolution of the sample directly into the *Mobile phase*. The procedure is applicable for the determination of thiamine hydrochloride or thiamine mononitrate as active pharmaceutical or dietary supplement ingredients.

Unless specified in the individual monographs, the *Standard solution*, *Sample solution*, and reagent solutions are prepared as follows.

**Solution A:** 0.005 M sodium 1-octanesulfonate in dilute glacial acetic acid (1 in 100)
**Solution B:** Methanol and acetonitrile (3:2)
**Mobile phase:** *Solution A* and *Solution B* (60:40)
**Internal standard solution:** 2% (v/v) of methylbenzoate in methanol
**Standard solution:** Prepare a 1-mg/mL solution of USP Thiamine Hydrochloride RS in *Mobile phase*. Transfer 20.0 mL of this solution and 5.0 mL of *Internal standard solution* to a 50-mL volumetric flask, and dilute with *Mobile phase* to volume. The *Standard solution* contains 400 µg/mL of thiamine hydrochloride.
**Sample solution:** Prepare a 2-mg/mL solution of thiamine hydrochloride or thiamine mononitrate in *Mobile phase*. Transfer 10.0 mL of this solution and 5.0 mL of *Internal standard solution* to a 50-mL volumetric flask, and dilute with *Mobile phase* to volume.
**Chromatographic system**
(See *Chromatography* ⟨621⟩, *System Suitability*.)
**Mode:** LC
**Detector:** UV 254 nm
**Column:** 4.6-mm × 25-cm; packing L1
**Flow rate:** 1 mL/min. [NOTE—The flow rate may be adjusted to obtain a retention time of about 12 min for thiamine.]
**Injection volume:** 10 µL
**System suitability**
**Sample:** *Standard solution*
**Suitability requirements**
**Resolution:** NLT 4.0 between the thiamine and methylbenzoate peaks
**Tailing factor:** NMT 2.0 for the thiamine peak
**Column efficiency:** NLT 1500 theoretical plates for thiamine
**Relative standard deviation:** NMT 2.0% for the ratios of thiamine peak response to the internal standard peak response
**Analysis**
**Samples:** *Standard solution* and *Sample solution*
Calculate the percentage of thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) in the portion of sample taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

$R_U$ = peak response ratio of thiamine to methylbenzoate from the *Sample solution*
$R_S$ = peak response ratio of thiamine to methylbenzoate from the *Standard solution*
$C_S$ = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)
$C_U$ = concentration of thiamine hydrochloride in the *Sample solution* (mg/mL)

If the active pharmaceutical or dietary supplement ingredient is thiamine mononitrate, calculate the percentage of thiamine mononitrate ($C_{12}H_{17}N_5O_4S$) in the portion of sample taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

$R_U$ = peak response ratio of thiamine to methylbenzoate from the *Sample solution*
$R_S$ = peak response ratio of thiamine to methylbenzoate from the *Standard solution*
$C_S$ = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)
$C_U$ = concentration of thiamine mononitrate in the *Sample solution* (mg/mL)
$M_{r1}$ = molecular weight of thiamine mononitrate, 327.36
$M_{r2}$ = molecular weight of thiamine hydrochloride, 337.27

- **Chromatographic Methods, Procedure 5**

This procedure can be used to determine thiamine in:

- *Thiamine Hydrochloride Injection*

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- *Thiamine Hydrochloride Oral Solution*
- *Thiamine Mononitrate Oral Solution*

This is the procedure that involves the dissolution of the sample directly into the *Mobile phase*. The procedure is applicable for the determination of thiamine hydrochloride or thiamine mononitrate as an active ingredient in the formulations such as those used in *Thiamine Hydrochloride Injection*, *Thiamine Hydrochloride Oral Solution*, and *Thiamine Mononitrate Oral Solution*.

Unless specified in the individual monographs, the *Standard solution*, *Internal standard solution*, and *Sample solution* are prepared as follows.

**Mobile phase:** Methanol and 0.04 M aqueous monobasic potassium phosphate (45:55)
**Internal standard solution:** 100 µg/mL of methylparaben in *Mobile phase*
**Standard stock solution:** 500 µg/mL of USP Thiamine Hydrochloride RS in *Mobile phase*
**Standard solution:** Dilute a mixture of equal volumes of the *Standard stock solution* and *Internal standard solution* with *Mobile phase* to obtain a concentration of USP Thiamine Hydrochloride RS of about 50 µg/mL.
**Sample stock solution:** Equivalent to 500 µg/mL of thiamine hydrochloride or thiamine mononitrate in *Mobile phase* from an accurately measured volume of oral solution or injection
**Sample solution:** Dilute a mixture of equal volumes of the *Internal standard solution* and *Sample stock solution* with *Mobile phase* to obtain a concentration of thiamine hydrochloride or thiamine mononitrate of about 50 µg/mL.
**Chromatographic system**
(See *Chromatography* ⟨621⟩, *System Suitability*.)
**Mode:** LC
**Detector:** UV 254 nm
**Column:** 4.6-mm × 25-cm; packing L1
**Flow rate:** 1 mL/min
**Injection volume:** 25 µL
**System suitability**
**Sample:** *Standard solution*
[NOTE—The relative retention times for thiamine and methylparaben are about 0.35 and 1.0, respectively.]
**Suitability requirements**
**Resolution:** NLT 6.0 between thiamine and methylparaben
**Column efficiency:** NLT 1500 theoretical plates for thiamine
**Relative standard deviation:** NMT 2.0% for the ratios of thiamine peak response to the methylparaben peak response
**Analysis**
**Samples:** *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) in the portion of sample taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

$R_U$ = peak response ratio of thiamine to methylparaben from the *Sample solution*
$R_S$ = peak response ratio of thiamine to methylparaben from the *Standard solution*
$C_S$ = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)
$C_U$ = nominal concentration of thiamine hydrochloride in the *Sample solution* (mg/mL)

If the products contain thiamine mononitrate, calculate the percentage of the labeled amount of thiamine mononitrate ($C_{12}H_{17}N_5O_4S$) in the portion of sample taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

$R_U$ = peak response ratio of thiamine to methylparaben from the *Sample solution*
$R_S$ = peak response ratio of thiamine to methylparaben from the *Standard solution*
$C_S$ = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)
$C_U$ = concentration of thiamine mononitrate in the *Sample solution* (mg/mL)
$M_{r1}$ = molecular weight of thiamine mononitrate, 327.36
$M_{r2}$ = molecular weight of thiamine hydrochloride, 337.27

• **CHROMATOGRAPHIC METHODS, PROCEDURE 6**

This procedure can be used to determine thiamine in the following:

- *Thiamine Hydrochloride Tablets*

This is the procedure that involves the dissolution of the sample directly into water. The procedure is applicable to the performance (dissolution) test for *Thiamine Hydrochloride Tablets*.

Unless specified in the individual monographs, the *Standard solution* and *Sample solution* are prepared as follows.
**Medium:** Water, 900 mL
**Apparatus 2:** 50 rpm
**Time:** 45 min
**Mobile phase:** A mixture of methanol, glacial acetic acid, and water (27:1:73) containing 140 mg of sodium 1-hexanesulfonate per 100 mL
**Standard solution:** A known concentration of USP Thiamine Hydrochloride RS in *Medium* (water)
**Sample solution:** Filtered portion of the solution under test, suitably diluted with *Medium* (water) if necessary

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**Chromatographic system**
(See *Chromatography* ⟨621⟩, *System Suitability*.)
**Mode:** LC
**Detector:** UV 280 nm
**Column:** 4.6-mm × 25-cm; packing L1
**Flow rate:** 1 mL/min
**Injection volume:** 10 µL
**System suitability**
**Sample:** *Standard solution*
**Suitability requirements**
**Relative standard deviation:** NMT 3.0%
**Analysis**
**Samples:** *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) in the portion of sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S \times D \times V/L) \times 100$$

$r_U$ = peak response of thiamine from the *Sample solution*
$r_S$ = peak response of thiamine from the *Standard solution*
$C_S$ = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)
$D$ = dilution factor for the *Sample solution*
$V$ = volume of *Medium*, 900 mL
$L$ = labeled amount of thiamine hydrochloride (mg/Tablet)

• **Chromatographic Methods, Procedure 7**
This procedure can be used to determine thiamine in:
- *Oil- and Water-Soluble Vitamins Capsules*
- *Oil- and Water-Soluble Vitamins Oral Solution*
- *Oil- and Water-Soluble Vitamins Tablets*
- *Oil- and Water-Soluble Vitamins with Minerals Capsules*
- *Oil- and Water-Soluble Vitamins with Minerals Oral Solution*
- *Oil- and Water-Soluble Vitamins with Minerals Tablets*
- *Water-Soluble Vitamins Capsules*
- *Water-Soluble Vitamins Tablets*
- *Water-Soluble Vitamins with Minerals Capsules*
- *Water-Soluble Vitamins with Minerals Oral Solution*
- *Water-Soluble Vitamins with Minerals Tablets*

This is a newly added procedure as part of the USP monograph modernization efforts. The procedure uses hydrophilic interaction liquid chromatography (HILIC), and the sample preparation involves the extraction of thiamine from the formulation by the *Diluent*, heat, and mechanical shaking.
Unless specified in the individual monographs, the *Standard solution*, *Sample solutions*, and reagent solutions are prepared as follows.
**Diluent:** Methanol, glacial acetic acid, and water (50:1:49)
**Solution A:** 50 mM ammonium formate. Adjust with ammonium hydroxide to a pH of 9.0.
**Solution B:** Acetonitrile
**Mobile phase:** Gradient elution. See *Table 1*.

**Table 1**

| Time (min) | Solution A (%) | Solution B (%) |
|---|---|---|
| 0 | 11 | 89 |
| 8 | 17 | 83 |
| 15 | 23 | 77 |
| 20 | 30 | 70 |
| 21 | 50 | 50 |
| 24 | 50 | 50 |
| 25 | 11 | 89 |
| 30 | 11 | 89 |

**Standard solution:** Transfer 20 mg of USP Thiamine Hydrochloride RS to a 200-mL volumetric flask, and add 160 mL of *Diluent*. Immerse the flask in a hot water bath maintained at 65°–70° for 10 min with regular shaking or using a vortex

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mixer, until all of the solid materials are dissolved. Chill rapidly in a cold water bath for 10 min to room temperature, and dilute with *Diluent* to volume.

**Sample solution for capsules:** Weigh NLT 20 capsules in a tared weighing bottle. Open the capsules, without the loss of shell material, and transfer the contents to a 100-mL beaker. Remove any contents adhering to the empty shells by washing, if necessary, with several portions of ether. Discard the washings, and dry the capsule shells with the aid of a current of dry air until the odor of ether is no longer perceptible. Weigh the empty capsule shells in the tared weighing bottle, and calculate the average net weight per capsule. Transfer a portion of the capsule contents, equivalent to 2.5 mg of thiamine hydrochloride, to a 50-mL centrifuge tube. Add 25.0 mL of *Diluent*, and mix using a vortex mixer for 30 s to completely suspend the powder. Immerse the centrifuge tube in a hot water bath maintained at 68°, heat for 10 min, and mix on a vortex mixer for 30 s. Return the tube to the hot water bath, heat for another 10 min, and mix on a vortex mixer for 30 s. Filter a portion of the solution, cool to room temperature, and use the clear filtrate.

**Sample solution for oral solution:** Equivalent to 0.1 mg/mL of thiamine hydrochloride in *Diluent*, from an accurately measured volume of oral solution. In an appropriate volumetric flask, dissolve the sample with *Diluent* to about 80% of the total volume, immerse the flask in a water bath maintained at 68° for 10 min, chill rapidly in a cold water bath to room temperature, and dilute with *Diluent*. Mix well, and filter a portion of the solution; finally, use the clear filtrate.

**Sample solution for tablets:** Finely powder NLT 30 tablets. Transfer a portion of the powder, equivalent to 2.5 mg of thiamine hydrochloride, to a 50-mL centrifuge tube. Add 25.0 mL of *Diluent*, and mix using a vortex mixer for 30 s to completely suspend the powder. Immerse the centrifuge tube in a hot water bath maintained at 65°–70°, heat for 10 min, and mix on a vortex mixer for 30 s. Return the tube to the hot water bath, heat for another 10 min, and mix on a vortex mixer for 30 s. Filter a portion of the solution, cool to room temperature, and use the clear filtrate. [NOTE—Use the filtrate within 3 h of filtration.]

**Chromatographic system**

(See *Chromatography* ⟨621⟩, *System Suitability*.)

**Mode:** LC

**Detector:** UV 267 nm

**Column:** 4.6-mm × 15-cm; 3.5-µm packing L68

**Column temperature:** 40°

**Flow rate:** 1.2 mL/min

**Injection volume:** 10 µL

**System suitability**

**Sample:** *Standard solution*

**Suitability requirements**

**Relative standard deviation:** NMT 2.0%

**Analysis**

**Samples:** *Standard solution* and appropriate *Sample solution*

For products containing thiamine hydrochloride, calculate the percentage of the labeled amount of thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) in the portion of sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

$r_U$ = peak response of thiamine from the appropriate *Sample solution*
$r_S$ = peak response of thiamine from the *Standard solution*
$C_S$ = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)
$C_U$ = nominal concentration of thiamine in the appropriate *Sample solution* (mg/mL)

For products containing thiamine mononitrate, calculate the percentage of the labeled amount of thiamine mononitrate ($C_{12}H_{17}N_5O_4S$) in the portion of sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

$r_U$ = peak response of thiamine from the appropriate *Sample solution*
$r_S$ = peak response of thiamine from the *Standard solution*
$C_S$ = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)
$C_U$ = nominal concentration of thiamine mononitrate in the appropriate *Sample solution* (mg/mL)
$M_{r1}$ = molecular weight of thiamine mononitrate, 327.36
$M_{r2}$ = molecular weight of thiamine hydrochloride, 337.27

## ADDITIONAL REQUIREMENTS

- **USP REFERENCE STANDARDS** ⟨11⟩

USP Thiamine Hydrochloride RS

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# ⟨541⟩ TITRIMETRY

## DIRECT TITRATIONS

Direct titration is the treatment of a soluble substance, contained in solution in a suitable vessel (the titrate), with an appropriate standardized solution (the titrant), the endpoint being determined instrumentally or visually with the aid of a suitable indicator.

The titrant is added from a suitable buret and is so chosen, with respect to its strength (normality), that the volume added is between 30% and 100% of the rated capacity of the buret.

[NOTE—Where less than 10 mL of titrant is required, a suitable microburet is to be used.]

The endpoint is approached directly but cautiously, and finally the titrant is added dropwise from the buret in order that the final drop added will not overrun the endpoint. The quantity of the substance being titrated may be calculated from the volume and the normality or molarity factor of the titrant and the equivalence factor for the substance given in the individual monograph.

## RESIDUAL TITRATIONS

Some Pharmacopeial assays require the addition of a measured volume of a volumetric solution, in excess of the amount actually needed to react with the substance being assayed, the excess of this solution then being titrated with a second volumetric solution. This constitutes a residual titration and is known also as a "back titration." The quantity of the substance being titrated may be calculated from the difference between the volume of the volumetric solution originally added, corrected by means of a blank titration, and that consumed by the titrant in the back titration, due allowance being made for the respective normality or molarity factors of the two solutions, and the equivalence factor for the substance given in the individual monograph.

## COMPLEXOMETRIC TITRATIONS

Successful complexometric titrations depend on several factors. The equilibrium constant for formation of the titrant-analyte complex must be sufficiently large that, at the endpoint, very close to 100% of the analyte has been complexed. The final complex must be formed rapidly enough that the analysis time is practical. When the analytical reaction is not rapid, a residual titration may sometimes be successful.

In general, complexometric indicators are themselves complexing agents. The reaction between metal ion and indicator must be rapid and reversible. The equilibrium constant for formation of the metal-indicator complex should be large enough to produce a sharp color change but must be less than that for the metal-titrant complex. Indicator choice is also restricted by the pH range within which the complexation reaction must be carried out and by interference of other ions arising from the sample or the buffer. Interfering ions may often be masked or "screened" via addition of another complexing agent. (The masking technique is also applicable to redox titrations.)

## OXIDATION-REDUCTION (REDOX) TITRATIONS

Determinations may often be carried out conveniently by the use of a reagent that brings about oxidation or reduction of the analyte. Many redox titration curves are not symmetric about the equivalence point, and thus graphical determination of the endpoint is not possible; but indicators are available for many determinations, and a redox reagent can often serve as its own indicator. As in any type of titration, the ideal indicator changes color at an endpoint that is as close as possible to the equivalence point. Accordingly, when the titrant serves as its own indicator, the difference between the endpoint and the equivalence point is determined only by the analyst's ability to detect the color change. A common example is the use of permanganate ion as an oxidizing titrant since a slight excess can easily be detected by its pink color. Other titrants that may serve as their own indicators are iodine, cerium (IV) salts, and potassium dichromate. In most cases, however, the use of an appropriate redox indicator will yield a much sharper endpoint.

It may be necessary to adjust the oxidation state of the analyte prior to titration through use of an appropriate oxidizing or reducing agent; the excess reagent must then be removed, e.g., through precipitation. This is nearly always the practice in the determination of oxidizing agents since most volumetric solutions of reducing agents are slowly oxidized by atmospheric oxygen.

## TITRATIONS IN NONAQUEOUS SOLVENTS

Acids and bases have long been defined as substances that furnish, when dissolved in water, hydrogen and hydroxyl ions, respectively. This definition, introduced by Arrhenius, fails to recognize the fact that properties characteristic of acids or bases may be developed also in other solvents. A more generalized definition is that of Brönsted, who defined an acid as a substance that furnishes protons, and a base as a substance that combines with protons. Even broader is the definition of Lewis, who defined an acid as any material that will accept an electron pair, a base as any material that will donate an electron pair, and neutralization as the formation of a coordination bond between an acid and a base.

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The apparent strength of an acid or a base is determined by the extent of its reaction with a solvent. In water solution all strong acids appear equally strong because they react with the solvent to undergo almost complete conversion to oxonium ion and the acid anion (leveling effect). In a weakly protophilic solvent such as acetic acid the extent of formation of the acetate acidium ion shows that the order of decreasing strength for acids is perchloric, hydrobromic, sulfuric, hydrochloric, and nitric (differentiating effect).

Acetic acid reacts incompletely with water to form oxonium ion and is, therefore, a weak acid. In contrast, it dissolves in a base such as ethylenediamine, and reacts so completely with the solvent that it behaves as a strong acid. The same holds for perchloric acid.

This leveling effect is observed also for bases. In sulfuric acid almost all bases appear to be of the same strength. As the acid properties of the solvent decrease in the series sulfuric acid, acetic acid, phenol, water, pyridine, and butylamine, the bases become progressively weaker until all but the strongest have lost their basic properties. In order of decreasing strength, the strong bases are sodium 2-aminoethoxide, potassium methoxide, sodium methoxide, and lithium methoxide.

Many water-insoluble compounds acquire enhanced acidic or basic properties when dissolved in organic solvents. Thus the choice of the appropriate solvent permits the determination of a variety of such materials by nonaqueous titration. Furthermore, depending upon which part of a compound is the physiologically active moiety, it is often possible to titrate that part by proper selection of solvent and titrant. Pure compounds can be titrated directly, but it is often necessary to isolate the active ingredient in pharmaceutical preparations from interfering excipients and carriers.

The types of compounds that may be titrated as acids include acid halides, acid anhydrides, carboxylic acids, amino acids, enols such as barbiturates and xanthines, imides, phenols, pyrroles, and sulfonamides. The types of compounds that may be titrated as bases include amines, nitrogen-containing heterocyclic compounds, oxazolines, quaternary ammonium compounds, alkali salts of organic acids, alkali salts of weak inorganic acids, and some salts of amines. Many salts of halogen acids may be titrated in acetic acid or acetic anhydride after the addition of mercuric acetate, which removes halide ion as the unionized mercuric halide complex and introduces the acetate ion.

For the titration of a basic compound, a volumetric solution of perchloric acid in glacial acetic acid is preferred, although perchloric acid in dioxane is used in special cases. The calomel-glass electrode system is useful in this case. In acetic acid solvent, this electrode system functions as predicted by theory.

For the titration of an acidic compound, two classes of titrant are available: the alkali metal alkoxides and the tetraalkylammonium hydroxides. A volumetric solution of sodium methoxide in a mixture of methanol and toluene is used frequently, although lithium methoxide in methanol-benzene solvent is used for those compounds yielding a gelatinous precipitate on titration with sodium methoxide.

The alkali error limits the use of the glass electrode as an indicating electrode in conjunction with alkali metal alkoxide titrants, particularly in basic solvents. Thus, the antimony-indicating electrode, though somewhat erratic, is used in such titrations. The use of quaternary ammonium hydroxide compounds, e.g., tetra-*n*-butylammonium hydroxide and trimethylhexadecylammonium hydroxide (in benzene-methanol or isopropyl alcohol), has two advantages over the other titrants in that (a) the tetraalkylammonium salt of the titrated acid is soluble in the titration medium, and (b) the convenient and well-behaved calomel-glass electrode pair may be used to conduct potentiometric titrations.

Because of interference by carbon dioxide, solvents for acidic compounds need to be protected from excessive exposure to the atmosphere by a suitable cover or by an inert atmosphere during the titration. Absorption of carbon dioxide may be determined by performing a blank titration. The blank should not exceed 0.01 mL of 0.1 N sodium methoxide VS per mL of solvent.

The endpoint may be determined visually by color change, or potentiometrically, as indicated in the individual monograph. If the calomel reference electrode is used, it is advantageous to replace the aqueous potassium chloride salt bridge with 0.1 N lithium perchlorate in glacial acetic acid for titrations in acidic solvents or potassium chloride in methanol for titrations in basic solvents.

Where these or other mixtures are specified in individual monographs, the calomel reference electrode is modified by first removing the aqueous potassium chloride solution and residual potassium chloride, if any, by rinsing with water, then eliminating residual water by rinsing with the required nonaqueous solvent, and finally filling the electrode with the designated nonaqueous mixture.

In nearly all cases, except those where silver ion might interfere, a silver-silver chloride reference electrode may be substituted for the calomel electrode. The silver-silver chloride electrode is more rugged, and its use helps to eliminate toxic mercury salts from the laboratory. Generally, a salt bridge may be used to circumvent interference by silver ion.

The more useful systems for titration in nonaqueous solvents are listed in *Table 1*.

**Table 1. Systems for Nonaqueous Titrations**

| Type of Solvent | Acidic (for titration of bases and their salts) | Relatively Neutral (for differential titration of bases) | Basic (for titration of acids) | Relatively Neutral (for differential titration of acids) |
|---|---|---|---|---|
| Solvent[1] | Glacial Acetic Acid | Acetonitrile | Dimethylformamide | Acetone |
| | Acetic Anhydride | Alcohols | *n*-Butylamine | Acetonitrile |
| | Formic Acid | Chloroform | Pyridine | Methyl Ethyl Ketone |
| | Propionic Acid | Benzene | Ethylenediamine | Methyl Isobutyl Ketone |
| | Sulfuryl Chloride | Toluene | Morpholine | *tert*-Butyl Alcohol |
| | | Chlorobenzene | | |
| | | Ethyl Acetate | | |
| | | Dioxane | | |

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**Table 1. Systems for Nonaqueous Titrations** *(continued)*

| Type of Solvent | Acidic (for titration of bases and their salts) | Relatively Neutral (for differential titration of bases) | Basic (for titration of acids) | Relatively Neutral (for differential titration of acids) |
|---|---|---|---|---|
| Indicator | Crystal Violet | Methyl Red | Thymol Blue | Azo Violet |
| | Quinaldine Red | Methyl Orange | Thymolphthalein | Bromothylmol Blue |
| | *p*-Naphtholbenzein | *p*-Naphtholbenzein | Azo Violet | *p*-Hydroxyazobenzene |
| | Alphezurine 2-G | | *o*-Nitroaniline | Thymol Blue |
| | Malachite Green | | *p*-Hydroxyazobenzene | |
| Electrodes | Glass–calomel | Glass–calomel | Antimony–calomel | Antimony–calomel |
| | Glass–silver–silver chloride | Calomel–silver–silver chloride | Antimony–glass | Glass–calomel |
| | Mercury–mercuric acetate | | Antimony–antimony[2] | Glass–platinum[2] |
| | | | Platinum–calomel | |
| | | | Glass–calomel | |

[1] Relatively neutral solvents of low dielectric constant such as benzene, toluene, chloroform, or dioxane may be used in conjunction with any acidic or basic solvent in order to increase the sensitivity of the titration end-points.
[2] In titrant.

## INDICATOR AND POTENTIOMETRIC ENDPOINT DETECTION

The simplest and most convenient method by which the equivalence point, i.e., the point at which the stoichiometric analytical reaction is complete, may be determined is with the use of indicators. These chemical substances, usually colored, respond to changes in solution conditions before and after the equivalence point by exhibiting color changes that may be taken visually as the endpoint, a reliable estimate of the equivalence point.

A useful method of endpoint determination results from the use of electrochemical measurements. If an indicator electrode, sensitive to the concentration of the species undergoing titrimetric reaction, and a reference electrode, whose potential is insensitive to any dissolved species, are immersed in the titrate to form a galvanic cell, the potential difference between the electrodes may be sensed by a pH meter and used to follow the course of the reaction. Where such a series of measurements is plotted correctly (i.e., for an acid-base titration, pH versus mL of titrant added; for a precipitimetric, complexometric, or oxidation-reduction titration, mV versus mL of titrant added), a sigmoid curve results with a rapidly changing portion (the "break") in the vicinity of the equivalence point. The midpoint of this linear vertical portion or the inflection point may be taken as the endpoint. The equivalence point may also be determined mathematically without plotting a curve. However, it should be noted that in asymmetrical reactions, which are reactions in which the number of anions reacting is not the same as the number of cations reacting, the endpoint as defined by the inflection of the titration curve does not occur exactly at the stoichiometric equivalence point. Thus, potentiometric endpoint detection by this method is not suitable in the case of asymmetric reactions, examples of which are the precipitation reaction,

$$2Ag^{+} + CrO_4^{-2}$$

and the oxidation-reduction reaction,

$$5Fe^{+2} + MnO_4^{-}.$$

All acid-base reactions, however, are symmetrical. Thus, potentiometric endpoint detection may be employed in acid-base titrations and in other titrations involving symmetrical reversible reactions where an indicator is specified, unless otherwise directed in the individual monograph.

Two types of automatic electrometric titrators are available. The first is one that carries out titrant addition automatically and records the electrode potential differences during the course of titration as the expected sigmoid curve. In the second type, titrant addition is performed automatically until a preset potential or pH, representing the endpoint, is reached, at which point the titrant addition ceases.

Several acceptable electrode systems for potentiometric titrations are summarized in *Table 2*.

**Table 2. Potentiometric Titration Electrode Systems**

| Titration | Indicating Electrode | Equation[1] | Reference Electrode | Applicability[2] |
|---|---|---|---|---|
| Acid-base | Glass | $E = k + 0.0591\ pH$ | Calomel or silver–silver chloride | Titration of acids and bases |
| Precipitimetric (silver) | Silver | $E = E° + 0.0591 \log [Ag^{+}]$ | Calomel (with potassium nitrate salt bridge) | Titration with or of silver involving halides or thiocyanate |
| Complexometric | Mercury–mercury(II) | $E = E° + 0.0296(\log k' - pM)$ | Calomel | Titration of various metals (M), e.g., $Mg^{+2}$, $Ca^{+2}$ $Al^{+3}$, $Bi^{+3}$, with EDTA |

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**Table 2. Potentiometric Titration Electrode Systems** *(continued)*

| Titration | Indicating Electrode | Equation[1] | Reference Electrode | Applicability[2] |
|---|---|---|---|---|
| Oxidation–reduction | Platinum | $E = E° + (0.0591/n) \times \log [ox]/[red]$ | Calomel or silver–silver chloride | Titrations with arsenite, bromine, cerate, dichromate, exacyonoferrate(III), iodate, nitrite, permanganate, thiosulfate |

[1] Appropriate form of Nernst equation describing the indicating electrode system: k = glass electrode constant; k′ = constant derived from Hg–Hg(II)–EDTA equilibrium; M = any metal undergoing EDTA titration; [ox] and [red] from the equation,

$$ox + ne \rightleftarrows red.$$

[2] Listing is representative but not exhaustive.

## BLANK CORRECTIONS

As previously noted, the endpoint determined in a titrimetric assay is an estimate of the reaction equivalence point. The validity of this estimate depends upon, among other factors, the nature of the titrate constituents and the concentration of the titrant. An appropriate *blank correction* is employed in titrimetric assays to enhance the reliability of the endpoint determination. Such a blank correction is usually obtained by means of a *residual blank titration*, wherein the required procedure is repeated in every detail except that the substance being assayed is omitted. In such instances, the actual volume of titrant equivalent to the substance being assayed is the difference between the volume consumed in the residual blank titration and that consumed in the titration with the substance present. The corrected volume so obtained is used in calculating the quantity of the substance being titrated, in the same manner as prescribed under *Residual Titrations*. Where potentiometric endpoint detection is employed, the blank correction is usually negligible.

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# ⟨551⟩ VITAMIN E ASSAY

## INTRODUCTION

The following liquid chromatographic procedures are provided for the determination of vitamin E as an active pharmaceutical ingredient, as a dietary supplement ingredient, or as a component in compendial dosage forms in the forms of alpha tocopherol ($C_{29}H_{50}O_2$), alpha tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$).

Throughout this assay, protect solutions containing, and derived from, the test specimen and the Reference Standard from the atmosphere and light, preferably by the use of a blanket of inert gas and low-actinic glassware.

Where vitamin E (alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard.

## ASSAY

- **PROCEDURE 1**
  - This procedure can be used to determine vitamin E in:
    - Oil-Soluble Vitamins Tablets
    - Oil-Soluble Vitamins Capsules
    - Oil-Soluble Vitamins with Minerals Tablets
    - Oil-Soluble Vitamins with Minerals Capsules
    - Oil- and Water-Soluble Vitamins Tablets
    - Oil- and Water-Soluble Vitamins Capsules
    - Oil- and Water-Soluble Vitamins with Minerals Tablets
    - Oil- and Water-Soluble Vitamins with Minerals Capsules
  - This is a neutral procedure that involves the use of dimethyl sulfoxide to dissolve the excipients in the sample, followed by a liquid–liquid extraction of vitamin E with hexane. The hexane extract is then evaporated in vacuum to dryness, and the residue is reconstituted in methanol prior injection into the chromatograph.
  - Unless specified in the individual monographs, the *System suitability solution*, *Standard solution*, *Sample solutions*, and reagent solutions are prepared as follows.

**Solution A:** Phosphoric acid solution (1 in 100) in water

**Mobile phase:** Methanol and *Solution A* (19:1)

**System suitability solution:** Prepare a 0.65-mg/mL solution of USP Ergocalciferol RS in methanol. Transfer 1.0 mL of this solution to a 100-mL volumetric flask containing 100 mg of USP Alpha Tocopheryl Acetate RS. Dissolve in 30 mL of methanol, with the aid of sonication if necessary, and dilute with methanol to volume. Store this solution in a refrigerator.

**Standard solution:** 2 mg/mL of USP Alpha Tocopherol RS, USP Alpha Tocopheryl Acetate RS, or USP Alpha Tocopheryl Acid Succinate RS in methanol

**Sample solution for Tablets:** Finely powder NLT 20 Tablets. Transfer a portion of the powder typically equivalent to 20 mg of the vitamin E form under testing but not exceeding 7.5 g of the powder, to a centrifuge tube having a polytef-lined screw cap. Add about 2 mL of dimethyl sulfoxide per each g of powdered Tablets, and about 3 mL of *n*-hexane each per g of powdered Tablets, and shake for 45 min on a shaker in a water bath maintained at 60°. [NOTE—Set up the shaker to ensure that the contents of the container are mixed vigorously and thoroughly.] Centrifuge at 3000 rpm for 10 min, and transfer the hexane layer by means of a pipet to a volumetric flask. [NOTE—Volumetric flask size: NLT 20 mL.] Add 3 mL of *n*-hexane per each g of powdered Tablets to the dimethyl sulfoxide layer, shake thoroughly for 5 min, and transfer the hexane layer by means of a pipet to the same volumetric flask. Repeat this extraction with three additional portions of *n*-hexane. Dilute the extracts in the volumetric flask with *n*-hexane to volume. Transfer NLT 20 mL of this solution to a suitable container, and evaporate in vacuum at room temperature to dryness. Transfer the residue with the aid of methanol to a suitable volumetric flask, and dilute with methanol to volume to obtain a concentration of 2 mg/mL of alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate.

**Sample solution for Capsules:** Transfer the contents of NLT 20 Capsules to a suitable container, mix, and weigh. Transfer a portion of the mixture, typically equivalent to 20 mg of the vitamin E form under testing but not exceeding 7.5 g of the mixture, to a centrifuge tube having a polytef-lined screw cap. [NOTE—For hard gelatin Capsules, remove, as completely as possible, the contents of NLT 20 Capsules by cutting open the Capsule shells, transferring the shells and their contents to a suitable container, and triturating to a homogeneous mass. Transfer a portion of the mass, typically equivalent to 20 mg of the vitamin E form under testing but not exceeding 7.5 g of the mixture to a centrifuge tube having a polytef-lined screw cap.] Add about 2 mL of dimethyl sulfoxide per each g of Capsule contents, and about 3 mL of *n*-hexane per each g of Capsule contents, and shake for 45 min on a shaker in a water bath maintained at 60°. [NOTE—Set up the shaker to ensure that the contents of the container are mixed vigorously and thoroughly.] Centrifuge at 3000 rpm for 10 min, and transfer the hexane layer by means of a pipet to a volumetric flask. [NOTE—Volumetric flask size: NLT 20 mL.] Add 3 mL of *n*-hexane per each g of Capsule contents to the dimethyl sulfoxide layer, shake thoroughly for 5 min, and transfer the hexane layer by means of a pipet to the same volumetric flask. Repeat this extraction with three additional portions of *n*-hexane. Dilute the extracts in the volumetric flask with *n*-hexane to volume. Transfer NLT 20 mL of this solution to a suitable container, and evaporate in vacuum at room temperature to dryness. Transfer the residue with the aid of methanol to a suitable volumetric flask, and dilute with methanol to volume to obtain a concentration of 2 mg/mL of alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate.

**Chromatographic system**

(See *Chromatography* ⟨621⟩, *System Suitability*.)

**Mode:** LC

**Detector:** UV 254 nm

**Column:** 8-mm × 10-cm; 5-µm packing L1

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**Flow rate:** 2 mL/min
**Injection volume:** 100 µL

**System suitability**

**Samples:** *System suitability solution* and *Standard solution*

[NOTE—The relative retention times for ergocalciferol and alpha tocopheryl acetate are about 0.5 and 1.0, respectively.]

**Suitability requirements**

**Resolution:** NLT 12 between ergocalciferol and alpha tocopheryl acetate, *System suitability solution*
**Tailing factor:** 0.8–1.2, *System suitability solution*
**Relative standard deviation:** NMT 3.0%, *Standard solution*

**Analysis**

**Samples:** *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate in the portion of the sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

$r_U$ = peak response of the relevant vitamin E form from the *Sample solution*
$r_S$ = peak response of the relevant vitamin E form from the *Standard solution*
$C_S$ = concentration of the relevant vitamin E form of the corresponding USP Reference Standard in the *Standard solution* (mg/mL)
$C_U$ = nominal concentration of the corresponding form of vitamin E in the *Sample solution* (mg/mL)

- **PROCEDURE 2**
  - This procedure can be used for the formulations containing vitamins A, D, and E. Application includes:
    - Oil-Soluble Vitamins Tablets
    - Oil-Soluble Vitamins Capsules
    - Oil-Soluble Vitamins with Minerals Tablets
    - Oil-Soluble Vitamins with Minerals Capsules
    - Oil- and Water-Soluble Vitamins Tablets
    - Oil- and Water-Soluble Vitamins Capsules
    - Oil- and Water-Soluble Vitamins with Minerals Tablets
    - Oil- and Water-Soluble Vitamins with Minerals Capsules
  - It involves the treatment of sample with methanolic sulfuric acid, followed by extraction with 2,2,4-trimethylpentane.
  - Unless specified in the individual monographs, the *System suitability solution*, *Standard solution*, *Sample solutions*, and reagent solutions are prepared as follows.

**Mobile phase:** Mix 240 mL of methanol with 10 mL of water, followed by 0.5 mL of 50% phosphoric acid, and dilute with acetonitrile to 1000 mL.

**3 N methanolic sulfuric acid solution:** Cautiously add 9 mL of sulfuric acid to 80 mL of methanol in a 100-mL volumetric flask. Cool, and dilute with methanol to volume.

**Sodium ascorbate–pyrogallol solution:** Transfer 10 g of sodium ascorbate and 5 g of pyrogallol to a 100-mL volumetric flask, and add sufficient water to dissolve. Add 1.7 mL of sulfuric acid, and dilute with water to volume.

**Lecithin solution:** 5 mg/mL of lecithin in 2,2,4-trimethylpentane

**System suitability solution:** 2 mg/mL each of USP Alpha Tocopherol RS, USP Alpha Tocopheryl Acetate RS, and USP Alpha Tocopheryl Acid Succinate RS in methanol

**Standard solution:** 2 mg/mL of USP Alpha Tocopherol RS, USP Alpha Tocopheryl Acetate RS, or USP Alpha Tocopheryl Acid Succinate RS in methanol

**Sample solution for Tablets:** [NOTE—This preparation is suitable for the determination of vitamin A, vitamin D, and vitamin E when present in the formulation. The sample amount may be adjusted depending on the presence or absence of the appropriate vitamins.] Finely powder NLT 20 Tablets. Use a portion of the powder nominally equivalent to an amount 90 mg of alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate. Add 0.5 g of sodium bicarbonate, 1.5 mL of *Lecithin solution*, and 12.5 mL of 2,2,4-trimethylpentane, and disperse on a vortex mixer. Add 6 mL of *Sodium ascorbate–pyrogallol solution*, shake slowly, and allow the solution to degas. Continue shaking until the evolution of gas has ceased, and then shake for an additional 12 min. Add 6 mL of dimethyl sulfoxide, mix on a vortex mixer to form a suspension, and shake for 12 min. Add 6 mL of *3 N methanolic sulfuric acid solution*, mix on a vortex mixer to form a suspension, and shake for 12 min. Add 12.5 mL of 2,2,4-trimethylpentane, mix on a vortex mixer to form a suspension, and shake for 10 min. Centrifuge for 10 min to break up the emulsion and to clarify the supernatant. Transfer a volume of the supernatant 2,2,4-trimethylpentane layer to a suitable volumetric flask, the volume of the specimen withdrawn from the 2,2,4-trimethylpentane layer and the size of the volumetric flask being such that the final concentration of the *Sample solution* is equivalent to that of the *Standard solution*. Evaporate nearly to dryness, add several mL of methanol, and evaporate the remaining 2,2,4-trimethylpentane. Dilute with methanol to volume.

**Sample solution for Capsules:** [NOTE—This preparation is suitable for the determination of vitamin A, vitamin D, and vitamin E when present in the formulation. The sample amount may be adjusted depending on the presence or absence of the appropriate vitamins.] Weigh NLT 20 Capsules in a tared weighing bottle. Using a sharp blade if necessary, carefully open the Capsules, without loss of shell material, and transfer the contents to a 100-mL beaker. Remove any contents adhering to the empty shells by washing with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the net

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weight of the Capsule contents. Transfer a portion of the Capsule contents, equivalent to 55 mg of vitamin E, to a container having a polytef-lined screw cap. Add 0.5 g of sodium bicarbonate, 1.5 mL of *Lecithin solution*, and 12.5 mL of 2,2,4-trimethylpentane, and disperse on a vortex mixer. Add 6 mL of *Sodium ascorbate–pyrogallol solution*, shake slowly, and allow the solution to degas. Continue shaking until the evolution of gas has ceased, and then shake for an additional 12 min. Add 6 mL of dimethyl sulfoxide, mix on a vortex mixer to form a suspension, and shake for 12 min. Add 6 mL of *3 N methanolic sulfuric acid solution*, mix on a vortex mixer to form a suspension, and shake for 12 min. Add 12.5 mL of 2,2,4-trimethylpentane, mix on a vortex mixer to form a suspension, and shake for 10 min. Centrifuge for 10 min to break up the emulsion and to clarify the supernatant layer. Transfer a volume of the supernatant 2,2,4-trimethylpentane layer to a suitable volumetric flask, the volume of the specimen withdrawn from the 2,2,4-trimethylpentane layer and the size of the volumetric flask being such that the final concentration of the *Sample solution* is equivalent to that of the *Standard solution*. Evaporate nearly to dryness, add several mL of methanol, and evaporate the remaining 2,2,4-trimethylpentane. Dilute with methanol to volume.

**Chromatographic system**

(See *Chromatography* ⟨621⟩, *System Suitability*.)

**Mode:** LC

**Detector:** UV 280 nm

**Column:** 4.6-mm × 25-cm; 5-µm packing L1

**Flow rate:** 1.5 mL/min

**Injection volume:** 25 µL

**System suitability**

**Samples:** *System suitability solution* and *Standard solution*

[NOTE—The relative retention times for alpha tocopheryl acid succinate, alpha tocopherol, and alpha tocopheryl acetate are about 0.6, 0.8, and 1.0, respectively]

**Suitability requirements**

**Resolution:** NLT 4.0 between alpha tocopheryl acid succinate and alpha tocopherol; NLT 3.0 between alpha tocopherol and alpha tocopheryl acetate, *System suitability solution*

**Relative standard deviation:** NMT 3.0%, *Standard solution*

**Analysis**

**Samples:** *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of alpha tocopherol, alpha tocopheryl acetate, alpha tocopheryl acid succinate in the portion of the sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

$r_U$ = peak response of the relevant vitamin E form from the *Sample solution*

$r_S$ = peak response of the relevant vitamin E form from the *Standard solution*

$C_S$ = concentration of the relevant vitamin E form of the corresponding USP Reference Standard in the *Standard solution* (mg/mL)

$C_U$ = nominal concentration of the corresponding form of vitamin E in the *Sample solution* (mg/mL)

[NOTE—Account for the initial extraction volume of 26.5 mL of 2,2,4-trimethylpentane and the dilution factor to exchange the solvent from 2,2,4-trimethylpentane to methanol to calculate the nominal concentration.]

- **PROCEDURE 3**
  - This procedure can be used for the determination of vitamin E in:
    - Oil-Soluble Vitamins Tablets
    - Oil-Soluble Vitamins Capsules
    - Oil-Soluble Vitamins with Minerals Tablets
    - Oil-Soluble Vitamins with Minerals Capsules
    - Oil- and Water-Soluble Vitamins Tablets
    - Oil- and Water-Soluble Vitamins Capsules
    - Oil- and Water-Soluble Vitamins with Minerals Tablets
    - Oil- and Water-Soluble Vitamins with Minerals Capsules
    - Oil- and Water-Soluble Vitamins Oral Solution
    - Oil- and Water-Soluble Vitamins with Minerals Oral Solution
  - It involves the saponification of the sample, followed by a liquid–liquid extraction of vitamin E from the sample with *n*-hexane. Evaporate the hexane extract to dryness, and reconstitute the residue in a mixture of acetonitrile and ethyl acetate (1:1).
  - Unless specified in the individual monographs, the *Standard solution*, *Sample solutions*, and reagent solutions are prepared as follows.

**Mobile phase:** Methanol, acetonitrile, and *n*-hexane (46.5: 46.5: 7.0)

**Diluent:** Acetonitrile and ethyl acetate (1:1)

**Potassium hydroxide solution:** [NOTE—Used for the Oral Solution sample.] Transfer 90 g of potassium hydroxide pellets to a 100-mL volumetric flask containing 60 mL of water. Mix to dissolve, cool, and dilute with water to volume.

**Standard solution:** 0.3 mg/mL of USP Alpha Tocopherol RS in *Diluent*

**Sample solution for Tablets:** Finely powder NLT 20 Tablets. Transfer a portion of the powder, equivalent to 8 mg of alpha tocopherol, to a 125-mL flask fitted with a ground-glass joint. Add 25.0 mL of water, 25.0 mL of dehydrated alcohol, and 3.5 g of potassium hydroxide pellets. Shake for 1 h in a water bath maintained at 55°. Cool, and transfer with the aid of a

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minimum volume of water to a 125-mL separatory funnel. Rinse the flask with 50 mL of *n*-hexane, and add the rinsing to the separatory funnel. Insert the stopper, shake vigorously for 60 s, and allow the layers to separate. Drain the aqueous layer into a second 250-mL separatory funnel, and repeat the extraction with 50 mL of *n*-hexane. Discard the aqueous layer, and combine the hexane extracts. Wash the combined extracts with 25 mL of water, allow the layers to separate, and discard the aqueous layer. Add 3 drops of glacial acetic acid, and repeat the washing procedure two more times. Filter the washed hexane layer through anhydrous sodium sulfate into a 250-mL round-bottom flask. Rinse the funnel and sodium sulfate with a few mL of *n*-hexane, and add the rinsing to the hexane solution in the flask. Place the flask in a water bath maintained at 50°, and evaporate the hexane solution with the aid of a rotary evaporator to dryness. Immediately add 25.0 mL of *Diluent*, and swirl to dissolve the residue.

**Sample solution for Capsules:** Weigh NLT 20 Capsules in a tared weighing bottle. Open the Capsules, without loss of shell material, and transfer the contents to a 100-mL beaker. Remove any contents adhering to the empty shells by washing with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the net weight of the Capsule contents. Transfer a portion of the Capsule contents, equivalent to an amount of 8.0 mg of alpha tocopherol, to a glass-stoppered conical flask. Add 25.0 mL of water, 25.0 mL of dehydrated alcohol, and 3.5 g of potassium hydroxide pellets. Shake for 1 h in a water bath maintained at 55°. Cool, and transfer with the aid of a minimum volume of water to a 125-mL separatory funnel. Rinse the flask with 50 mL of *n*-hexane, and add the rinsing to the separatory funnel. Insert the stopper, shake vigorously for 60 s, and allow the layers to separate. Drain the aqueous layer into a second 250-mL separatory funnel, and repeat the extraction with 50 mL of *n*-hexane. Discard the aqueous layer, and combine the hexane extracts. Wash the combined extracts with 25 mL of water, allow the layers to separate, and discard the aqueous layer. Add 3 drops of glacial acetic acid, and repeat the washing procedure two more times. Filter the washed hexane layer through anhydrous sodium sulfate into a 250-mL round-bottom flask. Rinse the funnel and sodium sulfate with a few mL of *n*-hexane, and add the rinsing to the hexane solution in the flask. Place the flask in a water bath maintained at 50°, and evaporate the hexane solution with the aid of a rotary evaporator to dryness. Immediately add 25.0 mL of *Diluent*, and swirl to dissolve the residue.

**Sample solution for Oral Solution:** Transfer an amount of Oral Solution equivalent to 1.5 mg of alpha tocopherol to a 125-mL conical flask fitted with a ground-glass joint, and add 25.0 mL of dehydrated alcohol. Attach a reflux condenser, and reflux in a boiling water bath for 1 min. Cautiously add 3 mL of *Potassium hydroxide solution* through the condenser, and continue to reflux for 30 min. Remove the flask from the bath, and rinse the condenser with about 15 mL of water. Cool, and transfer with a minimum volume of water to a 250-mL separatory funnel. Rinse the flask with 50 mL of *n*-hexane, and add the rinsings to the separatory funnel. Insert the stopper, shake vigorously for 1 min, and allow the layers to separate. Drain the aqueous layer into a second 250-mL separatory funnel, and repeat the extraction with 50 mL of *n*-hexane. Discard the aqueous layer, and combine the hexane extracts. Wash the combined extracts with 25 mL of water, allow the layers to separate, and discard the aqueous layer. Add 3 drops of glacial acetic acid, and repeat the washing procedure two more times. Filter the washed hexane layer through anhydrous sodium sulfate into a 250-mL round-bottom flask. Rinse the funnel and sodium sulfate with *n*-hexane, and add the rinsing to the hexane solution in the flask. Evaporate the hexane solution to dryness with the aid of a rotary evaporator over a water bath maintained at about 50°. Immediately add 5.0 mL of *Diluent*, and swirl to dissolve the residue.

**Chromatographic system**

(See *Chromatography* ⟨621⟩, *System Suitability*.)

**Mode:** LC
**Detector:** UV 291 nm
**Column:** 4.6-mm × 25-cm; packing L1
**Column temperature:** 40°
**Flow rate:** 3 mL/min
**Injection volume:** 20 µL

**System suitability**

**Sample:** *Standard solution*
**Suitability requirements**
**Relative standard deviation:** NMT 0.5%

**Analysis**

**Samples:** *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of vitamin E, as alpha tocopherol, in the portion of the sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

$r_U$ = peak response of alpha tocopherol from the *Sample solution*
$r_S$ = peak response of alpha tocopherol from the *Standard solution*
$C_S$ = concentration of USP Alpha Tocopherol RS in the *Standard solution* (mg/mL)
$C_U$ = nominal concentration of vitamin E, as alpha tocopherol, in the *Sample solution* (mg/mL)

[NOTE—Calculate the alpha tocopherol equivalent of alpha tocopheryl acetate, or alpha tocopheryl acid succinate by multiplying their contents by the factors 0.91 or 0.81, respectively.]

- **PROCEDURE 4**
  - This gas chromatographic procedure is provided for the determination of vitamin E as an active pharmaceutical ingredient, as a dietary supplement ingredient, or as a component in compendial dosage forms. It can be used for:
    - Vitamin E
    - Vitamin E Capsules

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- Vitamin E Preparation
- Unless specified in the individual monographs, the *Standard solutions*, *Sample solutions*, and reagent solutions are prepared and used appropriately as follows.

**Internal standard solution:** 10 mg/mL of squalane in cyclohexane

**System suitability solution:** 0.1 mg/mL each of USP Alpha Tocopherol RS and USP Alpha Tocopheryl Acetate RS in cyclohexane

**Standard solution 1:** 10 mg/mL of USP Alpha Tocopherol RS in *Internal standard solution*

**Standard solution 2:** 10 mg/mL of USP Alpha Tocopheryl Acetate RS in *Internal standard solution*

**Standard solution 3:** Transfer 30.0 mg of USP Alpha Tocopheryl Acid Succinate RS into a 20-mL vial. Add 2.0 mL of methanol, 1.0 mL of 2,2-dimethoxypropane, and 0.1 mL of hydrochloric acid to the vial. Cap tightly, and sonicate. Allow to stand in the dark for 1 h ± 5 min. Remove from the dark, uncap, and evaporate just to dryness on a steam bath with the aid of a stream of nitrogen. Add 3.0 mL of *Internal standard solution*, and mix on a vortex mixer to dissolve.

**Sample solutions for Active Pharmaceutical Ingredient**

**Sample solution 1** (vitamin E as alpha tocopherol or alpha tocopheryl acetate)**:** 10 mg/mL of Vitamin E (*d*- or *dl*-alpha tocopherol or *d*- or *dl*-alpha tocopheryl acetate) in *Internal standard solution*

**Sample solution 2** (vitamin E as alpha tocopheryl acid succinate)**:** Transfer 30.0 mg of Vitamin E (*d*- or *dl*-alpha tocopheryl acid succinate) into a 20-mL vial. Add 2.0 mL of methanol, 1.0 mL of 2,2-dimethoxypropane, and 0.1 mL of hydrochloric acid to the vial. Cap tightly, and sonicate. Allow to stand in the dark for 1 h ± 5 min. Remove from the dark, uncap, and evaporate just to dryness on a steam bath with the aid of a stream of nitrogen. Add 3.0 mL of *Internal standard solution*, and mix on a vortex mixer to dissolve.

**Sample solutions for Vitamin E Preparation**

**Sample solution 1** (vitamin E as alpha tocopherol or alpha tocopheryl acetate in liquid form)**:** Dissolve a portion of Preparation in *Internal standard solution* to prepare a Vitamin E (*d*- or *dl*-alpha tocopherol or *d*- or *dl*-alpha tocopheryl acetate) solution with nominal concentration of 10 mg/mL.

**Sample solution 2** (vitamin E as alpha tocopheryl acid succinate in liquid form)**:** Transfer a portion of Preparation, equivalent to 30.0 mg of Vitamin E (*d*- or *dl*-alpha tocopheryl acid succinate), into a 20-mL vial. Add 2.0 mL of methanol, 1.0 mL of 2,2-dimethoxypropane, and 0.1 mL of hydrochloric acid to the vial. Cap tightly, and sonicate. Allow to stand in the dark for 1 h ± 5 min. Remove from the dark, uncap, and evaporate just to dryness on a steam bath with the aid of a stream of nitrogen. Add 3.0 mL of *Internal standard solution*, and mix on a vortex mixer to dissolve.

**Sample solution 3** (vitamin E as alpha tocopherol or alpha tocopheryl acetate in solid form)**:** Transfer a portion of Preparation, equivalent to 50 mg of alpha tocopherol or alpha tocopheryl acetate, into a flask suitable for refluxing. Add 5 mL of water, and heat on a water bath at 60° for 10 min. Add 25 mL of alcohol, and reflux for 30 min. Cool, and transfer to a separator with the aid of 50 mL of water and 50 mL of ether. Shake vigorously, allow the layers to separate, and collect each layer in individual separators. Extract the aqueous layer with two 25-mL portions of ether, combining the extracts with the original ether layer. Wash the combined extract with one 25-mL portion of water, filter the ether solutions through 1 g of anhydrous sodium sulfate, and with the aid of a stream of nitrogen evaporate the ether solution on a water bath, controlled at a temperature that will not cause the ether solution to boil over. Remove the container from the water bath when 5 mL remains, and complete the evaporation without the application of heat. Dissolve the residue in *Internal standard solution* to prepare a Vitamin E (*d*- or *dl*-alpha tocopherol or *d*- or *dl*-alpha tocopheryl acetate) solution with nominal concentration of 10 mg/mL.

**Sample solution 4** (vitamin E as alpha tocopheryl acid succinate in solid form)**:** Transfer a portion of Preparation, equivalent to 30 mg of Vitamin E (*d*- or *dl*-alpha tocopheryl acid succinate), into a flask suitable for refluxing. Add 5 mL of water, and heat on a water bath at 60° for 10 min. Add 25 mL of alcohol, and reflux for 30 min. Cool, and transfer to a separator with the aid of 50 mL of water and 50 mL of ether. Shake vigorously, allow the layers to separate, and collect each layer in individual separators. Extract the aqueous layer with two 25-mL portions of ether, combining the extracts with the original ether layer. Wash the combined extract with one 25-mL portion of water, filter the ether solutions through 1 g of anhydrous sodium sulfate, and with the aid of a stream of nitrogen evaporate the ether solution on a water bath, controlled at a temperature that will not cause the ether solution to boil over. Remove the container from the water bath when 5 mL remains. Quantitatively transfer the remains into a 20-mL vial, and complete the evaporation without the application of heat. Add 2.0 mL of methanol, 1.0 mL of 2,2-dimethoxypropane, and 0.1 mL of hydrochloric acid to the vial. Cap tightly, and sonicate. Allow to stand in the dark for 1 h ± 5 min. Remove from the dark, uncap, and evaporate just to dryness on a steam bath with the aid of a stream of nitrogen. Add 3.0 mL of *Internal standard solution*, and mix on a vortex mixer to dissolve.

**Sample solutions for Vitamin E Capsules**

**Sample solution 1** (vitamin E as alpha tocopherol or alpha tocopheryl acetate)**:** Weigh NLT 10 Capsules in a tared weighing bottle. With a sharp knife or by other appropriate means, carefully open the Capsules, without loss of the shell material, and transfer the combined Capsule content to a 100-mL beaker. Remove any adhering substance from the emptied Capsules by washing with several small portions of *n*-hexane. Discard the washings, and allow the empty Capsules to dry in a current of dry air until the odor of *n*-hexane is no longer perceptible. Weigh the empty Capsules in the original tared weighing bottle, and calculate the average net weight per Capsule. Dissolve a portion of the combined Capsule contents in *Internal standard solution* to prepare a Vitamin E (*d*- or *dl*-alpha tocopherol or *d*- or *dl*-alpha tocopheryl acetate) solution with a nominal concentration of 10 mg/mL.

**Sample solution 2** (vitamin E as alpha tocopheryl acid succinate)**:** Weigh NLT 10 Capsules in a tared weighing bottle. With a sharp knife or by other appropriate means, carefully open the Capsules, without loss of the shell material, and transfer the combined Capsule content to a 100-mL beaker. Remove any adhering substance from the emptied Capsules by washing with several small portions of *n*-hexane. Discard the washings, and allow the empty Capsules to dry in a current of dry air until the odor of *n*-hexane is no longer perceptible. Weigh the empty Capsules in the original tared weighing bottle, and calculate the average net weight per Capsule. Transfer a portion of the combined Capsule contents, equivalent to 30.0 mg of Vitamin E (*d*- or *dl*-alpha tocopheryl acid succinate), into a 20-mL vial. Add 2.0 mL

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of methanol, 1.0 mL of 2,2-dimethoxypropane, and 0.1 mL of hydrochloric acid to the vial. Cap tightly, and sonicate. Allow to stand in the dark for 1 h ± 5 min. Remove from the dark, uncap, and evaporate just to dryness on a steam bath with the aid of a stream of nitrogen. Add 3.0 mL of *Internal standard solution*, and mix on a vortex mixer to dissolve.

**Chromatographic system**
(See *Chromatography* ⟨621⟩, *System Suitability*.)
**Mode:** GC
**Detector:** Flame ionization
**Column:** 0.25-mm × 30-m fused-silica capillary, bonded with a 0.25-µm film of phase G2
**Temperatures**
**Column:** 280°
**Injection port:** 290°
**Detector:** 290°
**Carrier gas:** Helium
**Flow rate:** 1 mL/min
**Split ratio:** 100:1
**Injection volume:** 1 µL
**System suitability**
**Samples:** *System suitability solution* and appropriate *Standard solution*
**Suitability requirements**
**Resolution:** NLT 3.5 between alpha tocopherol and alpha tocopheryl acetate, *System suitability solution*
**Relative standard deviation:** NMT 2.0% for ratios of relevant vitamin E form to internal standard peak responses from replicate injections, appropriate *Standard solution*
**Analysis**
**Samples:** Appropriate *Standard solution* and appropriate *Sample solution*
Calculate the percentage of vitamin E in terms of alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate in the portion of the sample taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

$R_U$ = internal standard ratio (peak response of relevant vitamin E form/peak response of the internal standard) from the appropriate *Sample solution*
$R_S$ = internal standard ratio (peak response of relevant vitamin E form/peak response of the internal standard) from the appropriate *Standard solution*
$C_S$ = concentration of the corresponding USP Reference Standard in the appropriate *Standard solution* (mg/mL)
$C_U$ = nominal concentration of the corresponding form of vitamin E in the appropriate *Sample solution* (mg/mL)

- **PROCEDURE 5**
  - This gas chromatographic procedure is provided for the determination of Vitamin E (as *d*- or *dl*-alpha tocopherol) in:
    - Vitamin E Polyethylene Glycol Succinate (Vitamin E polyethylene glycol succinate is a mixture formed by the esterification of *d*-alpha tocopheryl acid succinate and polyethylene glycol).
  - Unless specified in the individual monographs, the *Standard solutions*, *Sample solutions*, and reagent solutions are prepared as follows.

**Solvent:** 0.25 mL of phenolphthalein TS in 1 L of alcohol
**Internal standard solution:** 12 mg/mL of ethyl arachidate in isooctane
**Standard solution:** Transfer 32.5 mg of USP Alpha Tocopherol RS to a suitable reaction flask. Add 2 mL of pyridine and 0.5 mL of *N,O*-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane, and heat the flask at 100° for 10 min. Cool the flask, add 5.0 mL of *Internal standard solution* followed by 20 mL of isooctane, and shake.
**Sample solution:** Transfer a quantity equivalent to 0.100–0.160 g of Vitamin E Polyethylene Glycol Succinate molten at 60° to a culture tube (about 20 cm long and 2.5 cm in diameter) equipped with a screw cap. Add 40–50 mg of ascorbic acid and a few boiling chips, followed by 20 mL of *Solvent*. [NOTE—Reflux the solution gently without emission of contents. Place the tube in a heating block set at 100°–150°.] When the sample is fully dissolved, add 0.25 g of potassium hydroxide, and continue to reflux for 30 min. Remove the tube from heat, and while contents are still hot, add 1–2 mL of hydrochloric acid dropwise until the pink coloration disappears. [**CAUTION**—Exothermic reaction. Allow the acid to trickle down the inside of the tube to prevent splashing.] Cool the tube, then wash the sides of the tube with 20 mL of water. Add 5.0 mL of *Internal standard solution*, cap, and shake to ensure thorough mixing. Allow the tube to stand until two distinct layers are formed. Transfer 2.5–3.5 mL of the upper layer into a suitable reaction flask, and add 2.0 mL of pyridine followed by 2.5 mL of *N,O*-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane. Heat the flask at 100° for 10 min. Cool, and then add 12 mL of isooctane.
**Chromatographic system**
(See *Chromatography* ⟨621⟩, *System Suitability*.)
**Mode:** GC
**Detector:** Flame ionization
**Column:** 0.25-mm × 15-m fused-silica capillary, bonded with a 0.25-µm film of phase G27
**Temperatures**
**Injection port:** 280°
**Detector:** 345°
**Column:** See *Table 1*.

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**Table 1**

| Initial Temperature (°) | Temperature Ramp (°/min) | Final Temperature (°) | Hold Time at Final Temperature (min) |
|---|---|---|---|
| 260 | 20 | 340 | 1 |

**Carrier gas:** Helium
**Flow rate:** 1.5 mL/min
**Split ratio:** 200:1
**Injection volume:** 1 µL

**System suitability**
**Sample:** *Standard solution*
**Suitability requirements**
**Tailing factor:** NMT 2.0 for the alpha tocopherol peak
**Relative standard deviation:** NMT 2.0% for the ratio of the alpha tocopherol peak response to the internal standard peak response from replicate injections

**Analysis**
**Samples:** *Standard solution* and *Sample solution*
Calculate the percentage of vitamin E as *d*-alpha tocopherol in the portion of the sample taken:

$$\text{Result} = (R_U/R_S) \times (W_S/W_U) \times 100$$

$R_U$ = internal standard ratio (peak response of alpha tocopherol/peak response of the internal standard) from the *Sample solution*
$R_S$ = internal standard ratio (peak response of alpha tocopherol/peak response of the internal standard) from the *Standard solution*
$W_S$ = weight of USP Alpha Tocopherol RS used to prepare the *Standard solution* (mg)
$W_U$ = weight of Vitamin E Polyethylene Glycol Succinate taken to prepare the *Sample solution* (mg)

## ADDITIONAL REQUIREMENTS

- **USP Reference Standards** ⟨11⟩
  USP Alpha Tocopherol RS
  USP Alpha Tocopheryl Acetate RS
  USP Alpha Tocopheryl Acid Succinate RS
  USP Ergocalciferol RS

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# ⟨561⟩ ARTICLES OF BOTANICAL ORIGIN

## SAMPLING

To reduce the effect of sampling bias in qualitative and quantitative results, it is necessary to ensure that the composition of the sample used be representative of the batch of drugs being examined. The following sampling procedures are the minimum considered applicable to vegetable drugs. Some articles, or some tests, may require more rigorous procedures involving more containers being sampled or more samples per container.

### Gross Sample

Where external examination of containers, markings, and labels indicates that the batch can be considered to be homogeneous, take individual samples from the number of randomly selected containers indicated below. Where the batch cannot be considered to be homogeneous, divide it into sub-batches that are as homogeneous as possible, and then sample each one as a homogeneous batch. It is recommended to include samples from the first, middle, and last containers where the *No. of Containers in Batch* (*N*) is 11 or more, and each container in the batch is numbered or lettered in order.

| No. of Containers in Batch (*N*) | No. of Containers to Be Sampled (*n*) |
|---|---|
| 1–10 | All |
| 11–19 | 11 |
| >19 | $n = 10 + (N/10)$ |

(Rounding was calculated as "*n*" to next highest whole number.)

Samples are taken from the upper, middle, and lower sections of each container. If the crude material consists of component parts that are 1 cm or less in any dimension, and in the case of all powdered or ground materials, withdraw the sample by means of a sampling device that removes a core from the top to the bottom of the container, NLT two cores being taken from different angles. For materials with component parts >1 cm in any dimension, withdraw samples by hand. In the case of large bales or packs, samples should be taken from a depth of 10 cm, because the moisture content of the surface layer may be different from that of the inner layers.

Prepare the gross sample by combining and mixing the individual samples taken from each opened container, taking care not to increase the degree of fragmentation or significantly affect the moisture content.

For articles in containers holding <1 kg, mix the contents, and withdraw a quantity sufficient for the tests. For articles in containers holding 1–5 kg, withdraw equal portions from the upper, middle, and lower parts of the container, each of the samples being sufficient to carry out the tests. Thoroughly mix the samples, and withdraw an amount sufficient to carry out the tests. For containers holding more than 5 kg, withdraw three samples, each weighing NLT 250 g, from the upper, middle, and lower parts of the container. Thoroughly mix the samples, and withdraw a portion sufficient to carry out the tests.

### Laboratory Sample

Prepare the *Laboratory Sample* by repeated quartering of the gross sample.

[NOTE—Quartering consists of placing the sample, adequately mixed, as an even and square-shaped heap and dividing it diagonally into four equal parts. The two opposite parts are then taken and carefully mixed. The process is repeated as necessary until the required quantity is obtained.]

The *Laboratory Sample* should be of a size sufficient for performing all the necessary tests.

### Test Sample

Unless otherwise directed in the individual monograph or test procedure below, prepare the *Test Sample* as follows.

Decrease the size of the *Laboratory Sample* by quartering, taking care that each withdrawn portion remains representative. In the case of unground or unpowdered drugs, grind the withdrawn sample so that it will pass through a No. 20 standard-mesh sieve, and mix the resulting powder well. If the material cannot be ground, reduce it to as fine a state as possible, mix by rolling it on paper or sampling cloth, spread it out in a thin layer, and withdraw the portion for analysis.

## METHODS OF ANALYSIS

### Foreign Organic Matter

TEST SAMPLE

Unless otherwise specified in the individual monograph, weigh the following quantities of the *Laboratory Sample*, taking care that the withdrawn portion is representative (quartering if necessary).

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| | |
|---|---|
| Roots, rhizomes, bark, and herbs | 500 g |
| Leaves, flowers, seeds, and fruit | 250 g |
| Cut vegetable drugs (average weight of the pieces is <0.5 g) | 50 g |

Spread the sample out in a thin layer, and separate the foreign organic matter by hand as completely as possible. Weigh it, and determine the percentage of foreign organic matter in the weight of drug taken.

## Total Ash

Accurately weigh a quantity of the *Test Sample*, representing 2–4 g of the material, in a tared crucible and incinerate, gently at first, and gradually increase the temperature to 675 ± 25° until free from carbon, and determine the weight of the ash. [NOTE—For botanicals, a material in conformance with the *Loss on Drying* test in individual monographs should be used.] If a carbon-free ash cannot be obtained in this way, extract the charred mass with hot water, collect the insoluble residue on an ashless filter paper, incinerate the residue and filter paper until the ash is white or nearly so, then add the filtrate, evaporate it to dryness, and heat the whole to a temperature of 675 ± 25°. If a carbon-free ash cannot be obtained in this way, cool the crucible, add 15 mL of alcohol, break up the ash with a glass rod, burn off the alcohol, and again heat the whole to a temperature of 675 ± 25°. Cool in a desiccator, weigh the ash, and calculate the percentage of total ash from the weight of the drug taken.

## Acid-Insoluble Ash

Boil the ash obtained as directed in *Total Ash* with 25 mL of 3 N hydrochloric acid for 5 min, collect the insoluble matter on a tared filtering crucible or ashless filter, wash with hot water, ignite, and weigh. Determine the percentage of acid-insoluble ash calculated from the weight of drug taken.

## Water-Soluble Ash

Boil the ash obtained as directed in *Total Ash* with 25 mL of water for 5 min. Collect the insoluble matter in a sintered-glass crucible or on an ashless filter paper. Wash with hot water, and ignite for 15 min at a temperature not exceeding 450°. Subtract the weight of this residue, in mg, obtained in *Total Ash*, and calculate the percentage of water-soluble ash with reference to the weight of sample as determined in *Total Ash*.

## Alcohol-Soluble Extractives

### METHOD 1 (HOT EXTRACTION METHOD)

Transfer about 4 g of air-dried, coarsely powdered material, accurately weighed, to a glass-stoppered conical flask. Add 100 mL of alcohol, and weigh the flask. Shake, and allow to stand for 1 h. Attach a reflux condenser to the flask, boil gently for 1 h, cool, and weigh. Readjust to the original weight with alcohol. Shake, and filter rapidly through a dry filter. Transfer 25 mL of the filtrate to a tared flat-bottomed dish, and evaporate on a water bath to dryness. Dry at 105° for 6 h, cool in a desiccator for 30 min, and weigh without delay. Calculate the content, in mg/g, of alcohol-extractable matter in the test specimen.

### METHOD 2 (COLD EXTRACTION METHOD)

Transfer about 4 g of air-dried, coarsely powdered material, accurately weighed, to a glass-stoppered conical flask. Add 100 mL of alcohol, insert a stopper into the flask, and macerate for 24 h, shaking frequently during the first 8 h, and then allowing to stand. Filter rapidly, taking precautions against loss of alcohol. Evaporate 25 mL of the filtrate to dryness in a tared, flat-bottomed, shallow dish, and dry at 105° to constant weight. Calculate the content, in mg/g, of alcohol-extractable matter in the test specimen.

## Water-Soluble Extractives

### METHOD 1 (HOT EXTRACTION METHOD)

Proceed as directed in *Method 1 (Hot Extraction Method)* in *Alcohol-Soluble Extractives*, except use water in place of alcohol.

### METHOD 2 (COLD EXTRACTION METHOD)

Proceed as directed in *Method 2 (Cold Extraction Method)* in *Alcohol-Soluble Extractives*, except use water in place of alcohol.

## Crude Fiber

Exhaust a weighed quantity of the *Test Sample*, representing about 2 g of the material, with ether. Add 200 mL of boiling dilute sulfuric acid (1 in 78) to the ether-exhausted marc, in a 500-mL flask, and connect the flask to a reflux condenser. Reflux the mixture for 30 min, accurately timed, then pass through a linen or hardened-paper filter, and wash the residue on the filter with boiling water until the effluent washing is no longer acid. Rinse the residue back into the flask with 200 mL of boiling sodium hydroxide solution, adjusted to 1.25% by titration and free from sodium carbonate. Again reflux the mixture for 30 min,

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accurately timed, then rapidly pass through a tared filter, wash the residue with boiling water until the last washing is neutral, and dry it at 110° to constant weight. Incinerate the dried residue, ignite to constant weight, cool in a desiccator, and weigh the ash: the difference between the weight obtained by drying at 110° and that of the ash represents the weight of the crude fiber.

[NOTE—The boiling with acid and alkali should continue for 30 min, accurately timed, from the time that the liquid (which is cooled below the boiling point by being added to the cold flask) again boils. After the solution has been brought to boiling, the heat should be turned low enough just to maintain boiling. During the boiling, the flask should be gently rotated from time to time to wash down any particles that may adhere to the walls of the flask. A slow current of air introduced into the flask during the boiling operation aids in preventing excessive frothing.]

## Starch Content

### METHOD 1

The following is a general procedure for all reducing sugars and may be used to determine the starch content in botanical articles.

**Malt extract:** Use clean new barley malt of known efficacy, and grind just before use. Prepare malt extract just before use. For every 80 mL of malt extract needed, digest 5 g of ground malt with 100 mL of water at room temperature for 2 h. [NOTE—If an electric mixer is used, stir the mixture for 20 min.] Filter to obtain a clear extract, filtering again, if necessary, and mix the infusion well.

**Test solution:** Extract about 5 g of the finely ground test specimen with five 10-mL portions of ether, using a filter that will completely retain the smallest starch granule. Allow the ether to evaporate from the residue, and wash with 250 mL of aqueous alcohol solution (10 in 100). Carefully wash the residue from the paper into a 500-mL beaker with about 100 mL of water. Heat to about 60° (avoiding, if possible, gelatinizing starch), and allow to stand for about 1 h, stirring frequently to effect complete solution of sugars. Transfer to a wide-mouth bottle, rinse the beaker with a little warm water, and cool. Add an equal volume of alcohol, mix, and allow to stand for NLT 1 h.

Centrifuge until the precipitate is closely packed on the bottom of the bottle, and decant the supernatant. Wash the precipitate with successive 50-mL portions of alcohol solution (50 in 100) by centrifuging and decanting through a suitable filter until the washings are sugar free. [NOTE—To test for the presence of sugar, transfer a few drops of the washings to a test tube, and add 3 or 4 drops of a 20% solution of 1-naphthol in alcohol prepared by dissolving 200 mg of 1-naphthol in 1 mL of alcohol and 2 mL of water. Shake the test tube well to allow uniform mixing, allow 2–4 mL of sulfuric acid to flow down the sides of the test tube, and hold the test tube upright. If sugar is present, the interface of the two liquids is colored faint to deep violet, and on shaking, the whole solution becomes blue-violet.]

Transfer the residue from the bottle and hardened filter to a beaker with about 50 mL of water. Immerse the beaker in boiling water, and stir constantly for 15 min or until all of the starch is gelatinized. Cool the beaker to 55°, add 20 mL of *Malt extract*, and hold at this temperature for 1 h. Heat again to boiling for a few min, cool to 55°, add 20 mL of *Malt extract*, and hold at this temperature for 1 h or until the residue when treated with iodine TS shows no blue tinge upon microscopic examination. Cool, dilute with water to 250 mL, and filter.

**General procedure:** Transfer 200 mL of the *Test solution* to a flask fitted with a reflux condenser, add 20 mL of hydrochloric acid, and heat in a boiling water bath for 2.5 h. Cool, nearly neutralize with sodium hydroxide TS, complete neutralization with sodium carbonate TS, dilute with water to 500 mL, mix, and filter. The volume of aliquot taken depends on the starch content of the specimen under test (see *Table 1*). The aliquot should contain between 100 and 200 mg of dextrose. Transfer 50 mL of the filtrate to a 400-mL alkali-resistant glass beaker, add 50 mL of alkaline cupric tartrate TS, cover the beaker with a water glass, and heat. Adjust the flame in the burner so that the contents of the flask begin to boil in 4 min, and continue boiling for exactly 2 min. Pass the hot solution at once through a sintered-glass filter. Wash the precipitate of cuprous oxide thoroughly with water at about 60°, then with 10 mL of alcohol, and finally with 10 mL of ether.

**Table 1. Determination of the Optimum Aliquot**

| Expected Starch Content (%) | Aliquot (mL) |
|---|---|
| 60 | 25 |
| 50 | 35 |
| 40 | 50 |
| 30 | 50 |
| 20 | 50 |

For solutions of reducing sugars of comparatively high purity, proceed as directed in *Method 1A* to determine the amount of reduced copper obtained by weighing the dried cuprous oxide. For solutions of reducing sugars containing large amounts of organic impurities, including sucrose, proceed as directed in *Method 1B* to determine the amount of reduced copper obtained by titration with sodium thiosulfate.

*Method 1A:* Dry the precipitate obtained in *General procedure* for 30 min in an oven at 110 ± 2°, cool to room temperature in a desiccator, and weigh. Refer to *Table 2* to find the quantity of dextrose, in mg, corresponding to the weight of cuprous oxide found. Determine the percentage of dextrose and then the content of starch by the following formula:

$$\text{Percentage of dextrose} = (\text{wt. of dextrose in mg} \times 0.1 \times 500)/(\text{wt. of sample in g} \times \text{aliquot in mL})$$

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Content of starch = % dextrose × 0.9

**Table 2. Calculating Dextrose (mg) [applicable when Cuprous Oxide ($Cu_2O$) is weighed directly]**

| Cuprous Oxide ($Cu_2O$) | Dextrose (D-Glucose) | Cuprous Oxide ($Cu_2O$) | Dextrose (D-Glucose) | Cuprous Oxide ($Cu_2O$) | Dextrose (D-Glucose) | Cuprous Oxide ($Cu_2O$) | Dextrose (D-Glucose) | Cuprous Oxide ($Cu_2O$) | Dextrose (D-Glucose) | Cuprous Oxide ($Cu_2O$) | Dextrose (D-Glucose) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 10 | 4.0 | 90 | 38.9 | 170 | 75.1 | 250 | 112.8 | 330 | 152.2 | 410 | 193.7 |
| 12 | 4.9 | 92 | 39.8 | 172 | 76.0 | 252 | 113.7 | 332 | 153.2 | 412 | 194.7 |
| 14 | 5.7 | 94 | 40.6 | 174 | 76.9 | 254 | 114.7 | 334 | 154.2 | 414 | 195.8 |
| 16 | 6.6 | 96 | 41.5 | 176 | 77.8 | 256 | 115.7 | 336 | 155.2 | 416 | 196.8 |
| 18 | 7.5 | 98 | 42.4 | 178 | 78.8 | 258 | 116.6 | 338 | 156.3 | 418 | 197.9 |
| | | | | | | | | | | | |
| 20 | 8.3 | 100 | 43.3 | 180 | 79.7 | 260 | 117.6 | 340 | 157.3 | 420 | 199.0 |
| 22 | 9.2 | 102 | 44.2 | 182 | 80.6 | 262 | 118.6 | 342 | 158.3 | 422 | 200.1 |
| 24 | 10.0 | 104 | 45.1 | 184 | 81.5 | 264 | 119.5 | 344 | 159.3 | 424 | 201.1 |
| 26 | 10.9 | 106 | 46.0 | 186 | 82.5 | 266 | 120.5 | 346 | 160.3 | 426 | 202.2 |
| 28 | 11.8 | 108 | 46.9 | 188 | 83.4 | 268 | 121.5 | 348 | 161.4 | 428 | 203.3 |
| | | | | | | | | | | | |
| 30 | 12.6 | 110 | 47.8 | 190 | 84.3 | 270 | 122.5 | 350 | 162.4 | 430 | 204.4 |
| 32 | 13.5 | 112 | 48.7 | 192 | 85.3 | 272 | 123.4 | 352 | 163.4 | 432 | 205.5 |
| 34 | 14.3 | 114 | 49.6 | 194 | 86.2 | 274 | 124.4 | 354 | 164.4 | 434 | 206.5 |
| 36 | 15.2 | 116 | 50.5 | 196 | 87.1 | 276 | 125.4 | 356 | 165.4 | 436 | 207.6 |
| 38 | 16.1 | 118 | 51.4 | 198 | 88.1 | 278 | 126.4 | 358 | 166.5 | 438 | 208.7 |
| | | | | | | | | | | | |
| 40 | 16.9 | 120 | 52.3 | 200 | 89.0 | 280 | 127.3 | 360 | 167.5 | 440 | 209.8 |
| 42 | 17.8 | 122 | 53.2 | 202 | 89.9 | 282 | 128.3 | 362 | 168.5 | 442 | 210.9 |
| 44 | 18.7 | 124 | 54.1 | 204 | 90.9 | 284 | 129.3 | 364 | 169.6 | 444 | 212.0 |
| 46 | 19.6 | 126 | 55.0 | 206 | 91.8 | 286 | 130.3 | 366 | 170.6 | 446 | 213.1 |
| 48 | 20.4 | 128 | 55.9 | 208 | 92.8 | 288 | 131.3 | 368 | 171.6 | 448 | 214.1 |
| | | | | | | | | | | | |
| 50 | 21.3 | 130 | 56.8 | 210 | 93.7 | 290 | 132.3 | 370 | 172.7 | 450 | 215.2 |
| 52 | 22.2 | 132 | 57.7 | 212 | 94.6 | 292 | 133.2 | 372 | 173.7 | 452 | 216.3 |
| 54 | 23.0 | 134 | 58.6 | 214 | 95.6 | 294 | 134.2 | 374 | 174.7 | 454 | 217.4 |
| 56 | 23.9 | 136 | 59.5 | 216 | 96.5 | 296 | 135.2 | 376 | 175.8 | 456 | 218.5 |
| 58 | 24.8 | 138 | 60.4 | 218 | 97.5 | 298 | 136.2 | 378 | 176.8 | 458 | 219.6 |
| | | | | | | | | | | | |
| 60 | 25.6 | 140 | 61.3 | 220 | 98.4 | 300 | 137.2 | 380 | 177.9 | 460 | 220.7 |
| 62 | 26.5 | 142 | 62.2 | 222 | 99.4 | 302 | 138.2 | 382 | 178.9 | 462 | 221.8 |
| 64 | 27.4 | 144 | 63.1 | 224 | 100.3 | 304 | 139.2 | 384 | 180.0 | 464 | 222.9 |
| 66 | 28.3 | 146 | 64.0 | 226 | 101.3 | 306 | 140.2 | 386 | 181.0 | 466 | 224.0 |
| 68 | 29.2 | 148 | 65.0 | 228 | 102.2 | 308 | 141.2 | 388 | 182.0 | 468 | 225.1 |
| | | | | | | | | | | | |
| 70 | 30.0 | 150 | 65.9 | 230 | 103.2 | 310 | 142.2 | 390 | 183.1 | 470 | 226.2 |
| 72 | 30.9 | 152 | 66.8 | 232 | 104.1 | 312 | 143.2 | 392 | 184.1 | 472 | 227.4 |
| 74 | 31.8 | 154 | 67.7 | 234 | 105.1 | 314 | 144.2 | 394 | 185.2 | 474 | 228.3 |
| 76 | 32.7 | 156 | 68.6 | 236 | 106.0 | 316 | 145.2 | 396 | 186.2 | 476 | 229.6 |
| 78 | 33.6 | 158 | 69.5 | 238 | 107.0 | 318 | 146.2 | 398 | 187.3 | 478 | 230.7 |
| | | | | | | | | | | | |
| 80 | 34.4 | 160 | 70.4 | 240 | 108.0 | 320 | 147.2 | 400 | 188.4 | 480 | 231.8 |

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**Table 2. Calculating Dextrose (mg) [applicable when Cuprous Oxide ($Cu_2O$) is weighed directly]** *(continued)*

| Cuprous Oxide ($Cu_2O$) | Dextrose (D-Glucose) | Cuprous Oxide ($Cu_2O$) | Dextrose (D-Glucose) | Cuprous Oxide ($Cu_2O$) | Dextrose (D-Glucose) | Cuprous Oxide ($Cu_2O$) | Dextrose (D-Glucose) | Cuprous Oxide ($Cu_2O$) | Dextrose (D-Glucose) | Cuprous Oxide ($Cu_2O$) | Dextrose (D-Glucose) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 82 | 35.3 | 162 | 71.4 | 242 | 108.9 | 322 | 148.2 | 402 | 189.4 | 482 | 232.9 |
| 84 | 36.2 | 164 | 72.3 | 244 | 109.9 | 324 | 149.2 | 404 | 190.5 | 484 | 234.1 |
| 86 | 37.1 | 166 | 73.2 | 246 | 110.8 | 326 | 150.2 | 406 | 191.5 | 486 | 235.2 |
| 88 | 38.0 | 168 | 74.1 | 248 | 111.8 | 328 | 151.2 | 408 | 192.6 | 488 | 236.3 |

*Method 1B*

SODIUM THIOSULFATE SOLUTION: Transfer 3.9 g of sodium thiosulfate, accurately weighed, to a 100-mL volumetric flask, dissolve in and dilute with water to volume, and mix.

POTASSIUM IODIDE SOLUTION: Dissolve 42 g of potassium iodide in 100 mL of water.

SODIUM ACETATE SOLUTION: Dissolve 5.74 g of sodium acetate in 10 mL of water.

COPPER SOLUTION: Transfer about 0.3 g of pure electrolytic copper, accurately weighed, to a 250-mL flask, add 5 mL of nitric acid to dissolve the copper, add about 25 mL of water, and boil to expel red fumes. Add about 5 mL of bromine TS, and boil until the bromine is completely removed. Cool, add 10 mL of *Sodium acetate solution* followed by 10 mL of *Potassium iodide solution*, and titrate with *Sodium thiosulfate solution* to a light yellow color. Add enough starch TS to produce a marked blue color, and continue the titration. As the endpoint nears, add 2 g of potassium thiocyanate, and stir until completely dissolved. Continue titration until the precipitate is completely white. One mL of *Sodium thiosulfate solution* is equivalent to about 10 mg of copper. [NOTE—It is essential that the concentration of *Potassium iodide solution* be carefully regulated. If the solution contains less than 320 mg of copper at the completion of titration, add 4.2–5 g of potassium iodide to make a total solution of 100 mL. If greater amounts of copper are present, add *Potassium iodide solution* slowly, with constant agitation, from the buret in amounts proportionately greater.]

PROCEDURE: Wash the precipitated cuprous oxide obtained in *General procedure* with water, cover this filter with a watch glass, and dissolve the cuprous oxide with 5 mL of nitric acid directed under the watch glass with a pipette. Collect the filtrate in a 250-mL flask, wash the watch glass, and the filter with water. Collect all the washings in the flask. Boil the contents of the flask to expel red fumes. Add about 5 mL of bromine TS, and boil until the bromine is completely removed. Cool, and proceed as directed in *Copper solution* beginning with "add 10 mL of *Sodium acetate solution*". From the volume of *Sodium thiosulfate solution* consumed, obtain the weight of copper, in mg, and multiply the weight of copper by 1.1259 to obtain the weight, in mg, of cuprous oxide. From *Table 2*, find the quantity of dextrose, in mg, corresponding to the weight of cuprous oxide. The content of starch is equivalent to the weight, in mg, of dextrose obtained times 0.9. Conduct a blank determination, using 50 mL of alkaline cupric tartrate TS and 50 mL of *Malt extract*. If the weight of the cuprous oxide so obtained exceeds 0.5 mg, correct the result of the determination accordingly. [NOTE—The alkaline cupric tartrate TS deteriorates on standing, and the quantity of cuprous oxide obtained in the blank determination increases.]

## METHOD 2

The following method is specific for dextrose (glucose), and because of its extreme sensitivity it may account for differences noted between values obtained from the same specimen. Duplicate determinations do not vary more than 2%.

**Glucoamylase solution:** Prepare a solution of glucoamylase in water containing 30 International Units (IU)/mL. Use glucoamylase obtained preferably from *Rhizopus delemar*. The total glucoamylase activity of the test specimen being used should be NLT 150 IU.

**Acetate buffer solution:** Dissolve 16.4 g of sodium acetate in 100 mL of water, add 12.0 mL of glacial acetic acid, and mix. The pH of this solution is 4.8.

**Phosphate buffer:** Dissolve 3.63 g of tris(hydroxymethyl) aminomethane and 5.0 g of monobasic sodium phosphate in 50.0 mL of water. At 37°, adjust with phosphoric acid to a pH of 7.0, dilute with water to 100.0 mL, and mix. [NOTE—The pH of the buffer medium is sensitive to temperature and should be adjusted to the desired pH at the temperature to be used during incubation.]

**Enzyme solution:** Dissolve 30 mg of glucose oxidase (Type II from *Aspergillus niger*), 3 mg of peroxidase (Type I from horseradish), and 10 mg of potassium ferrocyanide in 100 mL of *Phosphate buffer*. [NOTE—This mixture can be stored in a refrigerator for up to 10 days.]

**18 N sulfuric acid:** Add slowly, while stirring, 54 mL of sulfuric acid to 102 mL of water, allow to cool to 25°, and mix.

**Standard solutions:** Dissolve an accurately weighed quantity of USP Dextrose RS in water to obtain a solution containing 1.0 mg/mL of USP Dextrose RS. Quantitatively dilute a known volume of this solution with water to obtain *Standard solutions A*, *B*, *C*, *D*, and *E*, having known concentrations of 10, 20, 25, 40, and 50 µg/mL of USP Dextrose RS, respectively. [NOTE—Allow 4 h for complete mutarotation before use.]

**Test solutions:** Extract about 5 g of finely ground test specimen with five 25-mL portions of 80% alcohol, and filter. Remove all the alcohol from the residue by drying in an air oven at 105° for about 8 h. [NOTE—Any traces of alcohol remaining in the residue will inhibit glucoamylase.] Cool, and transfer the flask containing the dried test specimen to a desiccator. Transfer about 1 g, accurately weighed, of the test specimen to a previously tared flask, add 25 mL of water, and adjust with phosphoric acid to a pH of 5.0–7.0, if necessary. Boil the suspension for about 3 min, transfer the flask to an autoclave, and heat to 135° for 2 h. Remove the flask from the autoclave, maintain the temperature near 55°, and add 2.5 mL of *Acetate buffer solution* and sufficient water to adjust the total weight of the solution to 45 ± 1 g. Immerse the flask in a water bath maintained at 55 ± 1°, and add 5 mL of *Glucoamylase solution*. Continuously swirl the flask for 2 h to effect hydrolysis, pass through filter paper into a 250-mL volumetric flask, wash quantitatively with water, and collect all the washings in the flask. Dilute the contents of the flask with water to volume, and mix. Transfer 1 mL of an aliquot containing 20–60 µg of D-glucose to each of five test tubes.

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[NOTE—To obtain the range of concentration of glucose in the hydrolysate, quantitatively dilute, if necessary, with water to volume.] Add 2 mL of *Enzyme solution* to each of the five test tubes, and place the test tubes in the dark at 37 ± 1° for exactly 30 min to develop the color. At the end of 30 min, add 2 mL of *18 N sulfuric acid* to each of the test tubes to stop the reaction, and mix.

**Control solution:** Transfer an accurately weighed quantity of about 0.4 g of starch to a previously tared flask, and proceed as directed in *Test solutions* beginning with "add 25 mL of water, and adjust the pH with phosphoric acid".

**Procedure:** Concomitantly determine the absorbances of the *Standard solutions* and the *Test solutions* at the wavelength of maximum absorbance at about 540 nm, with a suitable spectrophotometer, using the *Control solution* as the blank to set the instrument. Plot the absorbance values of the *Standard solutions* versus concentration, in µg/mL, of dextrose, and draw the straight line best fitting the five plotted points. From the graph, determine the concentration, *C*, in µg/mL, of dextrose in each of the *Test solutions*, and calculate the average concentration, in µg/mL, of the solution under test. The percentage of starch content in the weight of the test specimen taken is calculated by the formula:

$$(0.9C/10^6) \times V_1 \times (250/V_0)(100/E)(100/W) = 2.25CV_1/V_0EW$$

$C$ = average concentration of dextrose in the *Test solutions* (µg/mL)
$V_1$ = volume of the *Test solution* if extra dilution is done (mL) (see *Note 2* in *Test solutions*)
$V_0$ = volume of the aliquot taken from the 250-mL volumetric flask (mL)
$E$ = weight of the test specimen (g)
$W$ = percentage of dry weight of the test specimen

[NOTE—$V_0$ is 1.0 when no extra dilution is done.]

## Volatile Oil Determination

Set up a round-bottom, shortneck, 1-L flask in a heating mantle set over a magnetic stirrer. Insert an egg-shaped stirring bar magnet in the flask, and attach a cold-finger condenser and an appropriate volatile oil trap of the type illustrated (see *Figure 1)*).

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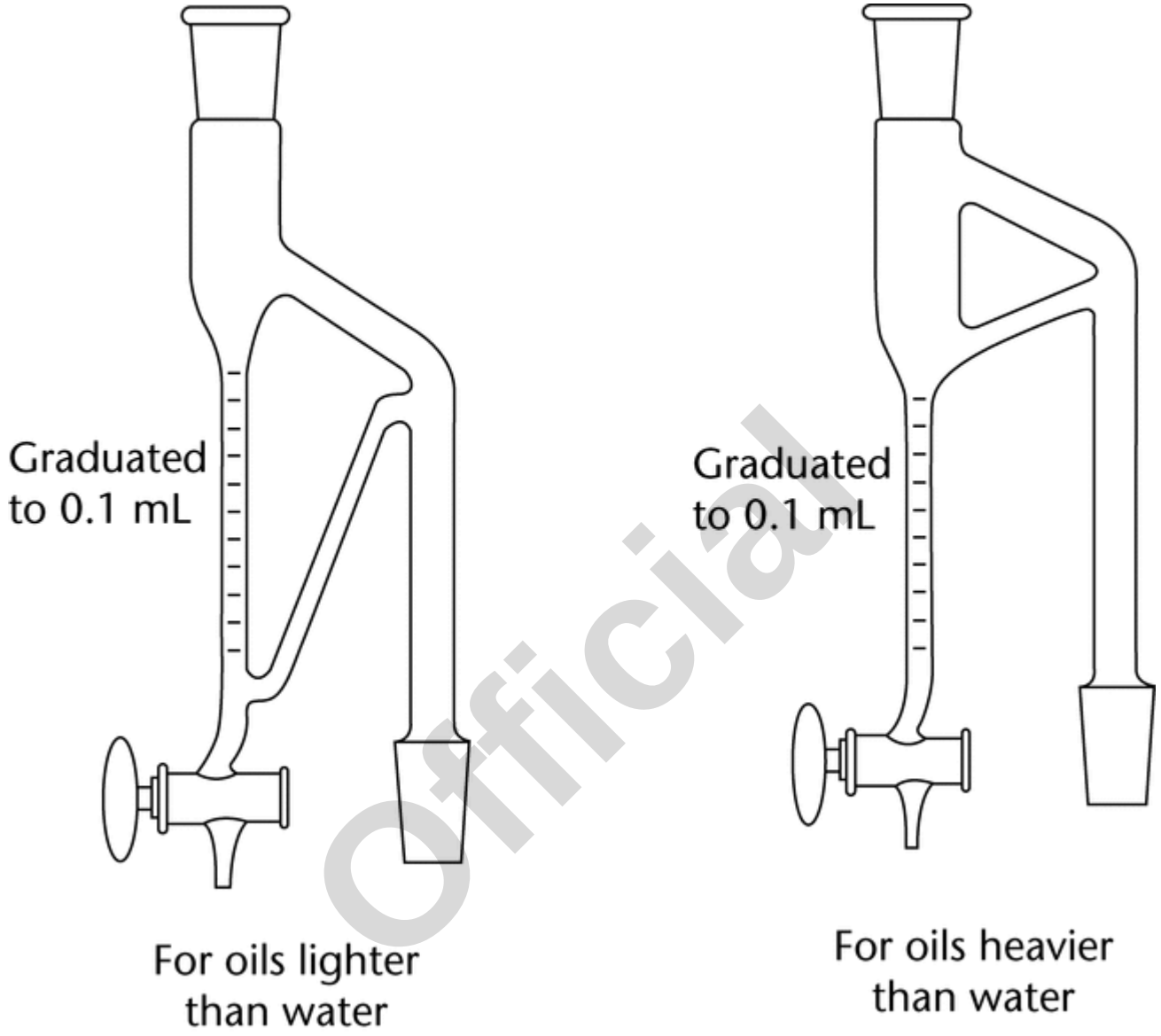


Figure 1. Traps for volatile oil apparatus.

Coarsely comminute a sufficient quantity of the material to yield from 1–3 mL of volatile oil. Small seeds, fruits, or broken leaves of herbs ordinarily do not need comminution. Very fine powders are to be avoided. If this is not possible, it may be necessary to mix them with purified sawdust or purified sand. Place a suitable quantity of the drug, accurately weighed, in the flask, and fill it one-half with water. Attach the condenser and the proper separator. Boil the contents of the flask, using a suitable amount of heat to maintain gentle boiling for 2 h, or until the volatile oil has been completely separated from the material and no longer collects in the graduated tube of the separator.

If a proper quantity of the volatile oil has been obtained in the graduated tube of the separator, it can be read to tenths of 1 mL, and the volume of volatile oil from each 100 g of material can be calculated from the weight of the drug taken. The graduations on the separator "for oils heavier than water" are so placed that oil remains below the aqueous condensate that automatically flows back into the flask.

## Water Content

For unground or unpowdered materials, prepare about 10 g of the *Laboratory Sample* by cutting, granulating, or shredding, so that the parts are about 3 mm in thickness. Seeds or fruits smaller than 3 mm should be cracked. Avoid the use of high-speed mills in preparing the sample, and exercise care that no appreciable amount of moisture is lost during the preparation and that the portion taken is representative of the *Laboratory Sample*. Determine the water content as directed in *Water Determination* ⟨921⟩, *Method III (Gravimetric), Procedure for Articles of Botanical Origin*.

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# TEST FOR AFLATOXINS

[**CAUTION**—Aflatoxins are highly dangerous, and extreme care should be exercised in handling aflatoxin materials.]

Where the individual monograph calls for compliance with the limits for aflatoxins, the limits are NMT 5 ppb for aflatoxin $B_1$ ($AFB_1$) and NMT 20 ppb for the sum of aflatoxins $B_1$ ($AFB_1$), $B_2$ ($AFB_2$), $G_1$ ($AFG_1$), and $G_2$ ($AFG_2$). The extent of testing may be determined using a risk-based approach that considers the likelihood of contamination. The presence of unexpected contamination with aflatoxins is to be considered in determining compliance. The following analytical procedures are provided for determining compliance. Unless otherwise specified in the individual monograph, use *Method I*. If system suitability fails, use either *Method II* or *Method III*.

## Method I

This TLC test is provided to detect the possible presence of $AFB_1$, $AFB_2$, $AFG_1$, and $AFG_2$ in any material of plant origin.

### ZINC ACETATE–ALUMINUM CHLORIDE REAGENT

Dissolve 20 g of zinc acetate and 5 g of aluminum chloride in sufficient water to make 100 mL.

### SODIUM CHLORIDE SOLUTION

Dissolve 5 g of sodium chloride in 50 mL of water.

### TEST SOLUTION 1

Grind about 200 g of plant material to a fine powder. Transfer about 50 g of the powdered material, accurately weighed, to a glass-stoppered flask. Add 200 mL of a mixture of methanol and water (17:3). Shake vigorously by mechanical means for NLT 30 min, and filter. [NOTE—If the solution has interfering plant pigments, proceed as directed for *Test Solution 2*.] Discard the first 50 mL of the filtrate, and collect the next 40-mL portion. Transfer the filtrate to a separatory funnel. Add 40 mL of *Sodium Chloride Solution* and 25 mL of solvent hexane, and shake for 1 min. Allow the layers to separate, and transfer the lower aqueous layer to a second separatory funnel. Extract the aqueous layer in the separatory funnel twice, each time with 25 mL of methylene chloride, by shaking for 1 min. Allow the layers to separate each time, separate the lower organic layer, and collect the combined organic layers in a 125-mL conical flask. Evaporate the organic solvent on a water bath. Transfer the remaining extract to an appropriate sample tube, and evaporate to dryness on a water bath. Cool the residue. If interferences exist in the residue, proceed as directed for *Cleanup procedure* in *Test Solution 2*. Otherwise, dissolve the residue obtained above in 0.2 mL of a mixture of chloroform and acetonitrile (9.8: 0.2), and shake by mechanical means if necessary.

### TEST SOLUTION 2

Collect 100 mL of the filtrate from the start of the flow, and transfer to a 250-mL beaker. Add 20 mL of *Zinc Acetate–Aluminum Chloride Reagent* and 80 mL of water. Stir, and allow to stand for 5 min. Add 5 g of a suitable filtering aid, such as diatomaceous earth, mix, and filter. Discard the first 50 mL of the filtrate, and collect the next 80-mL portion. Proceed as directed for *Test Solution 1*, beginning with "Transfer the filtrate to a separatory funnel".

**Cleanup procedure:** Place a medium-porosity sintered-glass disk or a glass wool plug at the bottom of a 10-mm × 300-mm chromatographic tube. Prepare a slurry of 2 g of silica gel with a mixture of ethyl ether and solvent hexane (3:1), pour the slurry into the column, and wash with 5 mL of the same solvent mixture. Allow the absorbent to settle, and add to the top of the column a layer of 1.5 g of anhydrous sodium sulfate. Dissolve the residue obtained above in 3 mL of methylene chloride, and transfer it to the column. Rinse the flask twice with 1-mL portions of methylene chloride, transfer the rinses to the column, and elute at a rate NMT 1 mL/min. Add successively to the column 3 mL of solvent hexane, 3 mL of ethyl ether, and 3 mL of methylene chloride; elute at a rate NMT 3 mL/min; and discard the eluates. Add to the column 6 mL of a mixture of methylene chloride and acetone (9:1), and elute at a rate NMT 1 mL/min, preferably without the aid of vacuum. Collect this eluate in a small vial, add a boiling chip if necessary, and evaporate to dryness on a water bath. Dissolve the residue in 0.2 mL of a mixture of chloroform and acetonitrile (9.8: 0.2), and shake by mechanical means if necessary.

### TEST SOLUTION 3

If interferences still exist in the residue, proceed as directed for *Cleanup procedure with IAC* in *Test Solution* in *Method II*.

### AFLATOXIN STANDARD SOLUTION

[**CAUTION**—Aflatoxins are highly toxic. Handle with care.]

Prepare an *Aflatoxin Standard Solution* containing $AFB_1$, $AFB_2$, $AFG_1$, and $AFG_2$ at 2.0, 0.50, 2.0, and 0.50 µg/mL, respectively, in acetonitrile as follows. First prepare individual stock solutions of aflatoxins $AFB_1$, $AFB_2$, $AFG_1$, and $AFG_2$ in acetonitrile, each having a nominal concentration of 10 µg/mL.[1] Determine the concentration of each aflatoxin stock solution by measuring the absorbance (*A*) at a wavelength of maximum absorption close to 360 nm and using the equation:

[1] Suitable aflatoxin individual standards are available from Romer Labs, catalog #001012, 001013, 001014, 001015; and Sigma-Aldrich, catalog #A6636, A9887, A0138, A0263.

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$$\text{Aflatoxin (µg/mL)} = (A \times M_r \times 1000)/\varepsilon$$

$A$ = absorbance
$M_r$ = molecular weight
$\varepsilon$ = molar absorptivity (see *Table 3*)

**Table 3. Molecular Weights and Molar Absorptivities for Aflatoxins**

| Aflatoxin | Molecular Weight ($M_r$) | Solvent | Molar Absorptivity ($\varepsilon$) |
|---|---|---|---|
| $AFB_1$ | 312 | Acetonitrile | 20700 |
| $AFB_2$ | 314 | Acetonitrile | 22500 |
| $AFG_1$ | 328 | Acetonitrile | 17600 |
| $AFG_2$ | 330 | Acetonitrile | 18900 |

Finally, to obtain the *Aflatoxin Standard Solution*, transfer an accurate volume of each aflatoxin stock solution to the same volumetric flask, and dilute with acetonitrile to volume. Calculate the concentration of each aflatoxin in the *Aflatoxin Standard Solution*:

$$C_{AF} = C'_{AF} \times V_{TS}/V_{AF}$$

$C_{AF}$ = concentration of the relevant aflatoxin in the *Aflatoxin Standard Solution*
$C'_{AF}$ = concentration of the relevant aflatoxin in the corresponding stock solution, as determined above
$V_{TS}$ = volume of the volumetric flask used to prepare the final *Aflatoxin Standard Solution*
$V_{AF}$ = volume of each individual aflatoxin stock solution transferred to the volumetric flask used to prepare the *Aflatoxin Standard Solution*

Store in a refrigerator. Equilibrate to room temperature before use.

### AFLATOXIN SOLUTION

Dilute the *Aflatoxin Standard Solution* with acetonitrile to obtain a solution having a concentration of 0.4 µg/mL each of $AFB_1$ and $AFG_1$, and 0.1 µg/mL each of $AFB_2$ and $AFG_2$.

### PROCEDURE

Separately apply 2.5, 5, 7.5, and 10 µL of the *Aflatoxin Solution* and three 10-µL applications of either *Test Solution 1*, *Test Solution 2*, or *Test Solution 3* to a suitable TLC plate (see *Chromatography* ⟨621⟩) coated with a 0.25-mm layer of chromatographic silica gel mixture. Superimpose 5 µL of the *Aflatoxin Solution* on one of the three 10-µL applications of the *Test Solution*. Allow the spots to dry, and develop the chromatogram in an unsaturated chamber containing a solvent system consisting of a mixture of chloroform, acetone, and isopropyl alcohol (85:10:5) until the solvent front has moved NLT 15 cm from the origin. Remove the plate from the developing chamber, mark the solvent front, and allow the plate to air-dry. Locate the spots on the plate by examination under UV light at 365 nm.

### SYSTEM SUITABILITY

The four applications of the *Aflatoxin Solution* appear as four clearly separated blue fluorescent spots. Observe any spot obtained from the *Test Solution* that coincides in hue and position with those of the *Aflatoxin Solution*. Any spot obtained from the *Test Solution* with the superimposed *Aflatoxin Solution* is not less intense than that of the corresponding *Aflatoxin Solution*.

### ACCEPTANCE CRITERIA

No spot from any of the other applications of the *Test Solution* corresponds to any of the spots obtained from the applications of the *Aflatoxin Solution*. If any spot of aflatoxins is obtained in the *Test Solution*, match the position of each fluorescent spot of the *Test Solution* with those of the *Aflatoxin Solution* to identify the type of aflatoxin present. The intensity of the aflatoxin spot, if present in the *Test Solution*, when compared with that of the corresponding aflatoxin in the *Aflatoxin Solution* will give an approximate concentration of aflatoxin in the *Test Solution*. Where the individual monograph calls for compliance with the limits for aflatoxins, the limits are NMT 5 ppb for $AFB_1$ and NMT 20 ppb for the sum of $AFB_1$, $AFB_2$, $AFG_1$, and $AFG_2$, except when otherwise indicated.

## Method II

### SODIUM CHLORIDE SOLUTION

See *Method I*.

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### PHOSPHATE BUFFERED SALINE SOLUTION

Prepare 10 mM phosphate buffer solution containing 0.138 M sodium chloride and 0.0027 M potassium chloride in water, and adjust with 2 M sodium hydroxide to a pH of 7.4.[2]

### IMMUNOAFFINITY COLUMN

Before conditioning, adjust the immunoaffinity column (IAC) to room temperature. For conditioning, apply 10 mL of *Phosphate Buffered Saline Solution* onto the column and let it flow through the column by gravity force at a rate of 2–3 mL/min. Leave 0.5 mL of the *Phosphate Buffered Saline Solution* on top of the column until the *Test Solution* is applied.

### TEST SOLUTION

**Sample extraction:** Transfer about 5 g of a representative powdered sample, accurately weighed, to a glass-stoppered flask. Add 20 mL of a mixture of methanol and water (17:3). Shake vigorously by mechanical means for NLT 30 min, and filter. Discard the first 5 mL of the filtrate, and collect the next 4-mL portion. Transfer the filtrate to a separatory funnel. Add 4 mL of *Sodium Chloride Solution* and 2.5 mL of hexane, and shake for 1 min. Allow the layers to separate, and transfer the lower aqueous layer to a second separatory funnel. Extract the aqueous layer in the separatory funnel twice, each time with 2.5 mL of methylene chloride, by shaking for 1 min. Allow the layers to separate each time, separate the lower organic layer, and collect the combined organic layers in a 50-mL conical flask. Evaporate the organic solvent on a water bath. Transfer the remaining extract to an appropriate sample tube, and evaporate to dryness on a water bath. Cool the residue. If interferences exist in the residue, proceed as directed for *Cleanup procedure with IAC*. Otherwise, dissolve the residue obtained above in 200 µL of acetonitrile, and shake by mechanical means if necessary.
**Cleanup procedure with IAC:** The residue is dissolved in 5 mL of a mixture of methanol and water (6:4) and then diluted with 5 mL of water. This extract is applied onto a conditioned IAC. The IAC is rinsed twice with 10 mL of *Phosphate Buffered Saline Solution*, and the elution is performed slowly with 2 mL of methanol. Evaporate the eluate with nitrogen, and dissolve the residue in 200 µL of acetonitrile.

### AFLATOXIN SOLUTION

[**CAUTION**—Aflatoxins are highly toxic. Handle with care.]
Dilute the *Aflatoxin Standard Solution* with acetonitrile to obtain a solution containing 0.04 µg/mL each of $AFB_1$ and $AFG_1$, and 0.01 µg/mL each of $AFB_2$ and $AFG_2$.

### ANALYSIS

Separately apply 5, 7.5, and 10 µL of *Aflatoxin Solution* and three 10-µL applications of the *Test Solution* to a suitable HPTLC plate (see *Chromatography* ⟨621⟩) coated with a 200-µm layer of chromatographic silica gel mixture. Superimpose 5 µL of *Aflatoxin Solution* on one of the three 10-µL applications of the *Test Solution*. Allow the spots to dry, and develop the chromatogram in a saturated chamber containing a solvent system consisting of a mixture of chloroform, acetone, and water (140: 20: 0.3) until the solvent front has moved NLT 72 mm from the origin (80 mm from the lower edge of the plate). Remove the plate from the developing chamber, mark the solvent front, and allow the plate to air-dry for 5 min. Locate the spots on the plate by scanning fluorescence density (>400 nm) under UV light at 366 nm. Match the position of each fluorescent spot of the *Test Solution* with those of *Aflatoxin Solution* to identify the type of aflatoxin present. The concentration of aflatoxins in the *Test Solution* can be calculated from the calibration curve obtained from the scan data with *Aflatoxin Solution*.

### SYSTEM SUITABILITY

The four applications of *Aflatoxin Solution* appear as four clearly separated blue fluorescent spots. Observe any spot obtained from the *Test Solution* that coincides in hue and position with those of *Aflatoxin Solution*. Any spot obtained from the *Test Solution* with the superimposed *Aflatoxin Solution* is not less intense than that of the corresponding *Aflatoxin Solution*. The mean recovery of spiked $AFB_1$ and $AFG_1$ is NLT 70%.

### ACCEPTANCE CRITERIA

Where the individual monograph calls for compliance with the limits for aflatoxins, the limits are NMT 5 ppb for $AFB_1$ and NMT 20 ppb for the sum of $AFB_1$, $AFB_2$, $AFG_1$, and $AFG_2$, except when otherwise indicated.

## Method III

This test method is provided as an example for the detection of the possible presence of $AFB_1$ and total aflatoxins (AF: sum of $AFB_1$, $AFB_2$, $AFG_1$, and $AFG_2$). It has been shown to be suitable for powdered ginseng and ginger. Its suitability to other articles of botanical origin must be demonstrated.

[2] A suitable powder mixture is available from Sigma as PBS P-3813.

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## 0.1 M PHOSPHATE BUFFER SOLUTION

Dissolve 8.69 g of anhydrous disodium phosphate and 4.66 g of anhydrous monosodium phosphate or 5.36 g of monosodium phosphate monohydrate in 800 mL of water, adjust with 2 M sodium hydroxide to a pH of 7.4, add 10 mL of polysorbate 20, and dilute with water to 1 L.

## PHOSPHATE BUFFERED SALINE SOLUTION

Prepare as directed in *Method II*.

## WORKING AFLATOXIN STANDARD SOLUTIONS

Prepare six solutions by diluting suitable volumes of *Aflatoxin Standard Solution* with methanol and water (1:1). The final aflatoxin concentrations in each *Working Aflatoxin Standard Solution* are shown in *Table 4*. Store in a refrigerator, and equilibrate to room temperature before use. Prepare the solutions daily.

**Table 4. Preparation of Working Aflatoxin Standard Solutions**

| Working Aflatoxin Standard Solutions | Aflatoxin Standard Solution (µL) | Final Aflatoxin Concentration of Working Aflatoxin Standard Solution (ng/mL) | | | | |
|---|---|---|---|---|---|---|
| | | $AFB_1$ | $AFB_2$ | $AFG_1$ | $AFG_2$ | ΣAF |
| 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| 2 | 12.5 | 0.25 | 0.0625 | 0.25 | 0.0625 | 0.625 |
| 3 | 25 | 0.5 | 0.125 | 0.5 | 0.125 | 1.25 |
| 4 | 50 | 1 | 0.25 | 1 | 0.25 | 2.5 |
| 5 | 100 | 2 | 0.5 | 2 | 0.5 | 5 |
| 6 | 200 | 4 | 1 | 4 | 1 | 10 |

## IMMUNOAFFINITY COLUMN[3]

Use an IAC that contains monoclonal antibodies cross reactive toward $AFB_1$, $AFB_2$, $AFG_1$, and $AFG_2$. The immunoaffinity columns have a minimum capacity of NLT 100 ng of total aflatoxin and give a recovery of NLT 80% for $AFB_1$, $AFB_2$, $AFG_1$, and $AFG_2$ when 5 ng of each $AFB_1$, $AFB_2$, $AFG_1$, and $AFG_2$ is applied in 10 mL of 10% methanol in *Phosphate Buffered Saline Solution* (v/v).

## TEST SOLUTION

**Extraction:** Weigh 5 g of a representative test sample in a 50-mL centrifuge tube. Add 1 g of sodium chloride and 25 mL of a mixture of methanol and 0.5% sodium bicarbonate (7:3). Mix on a vortex mixer until sample particles and extract solvent are well mixed. Shake at 400 rpm for 10 min. Centrifuge for 10 min at 7000 rpm (*g* value = 5323 $mm/s^2$) or at a speed that can result in a firm pellet of residues. Immediately pipette 7 mL into a 50-mL centrifuge tube, add 28 mL of *0.1 M Phosphate Buffer Solution*, mix, and filter through glass microfiber paper. Collect 25 mL of filtrate (equivalent to 1 g of test sample) into a 25-mL graduated cylinder, and proceed immediately with IAC chromatography.
**IAC cleanup:** [NOTE—For IAC cleanup, columns must be kept at room temperature for at least 15 min before use.] Remove the top cap from the column, and connect it with the reservoir. Remove the end cap from the column, and attach it to the column manifold (the fit must be tight). Let the liquid in the column pass through until the liquid is about 2–3 mm above the column bed. Pass 25 mL of filtrate into the reservoir. Let the filtrate flow through the column by gravity force. Let the column run dry. To start the flow again easily, remove the column from the manifold, add about 2 mL of *Phosphate Buffered Saline Solution* into the column, reattach the column to the reservoir, and wash the column with an additional 3 mL of *Phosphate Buffered Saline Solution* and then with 5 mL of water (the 5 mL of *Phosphate Buffered Saline Solution* can be added directly to the column reservoir if other techniques are used to dislodge the air bubble at the end of the column and to start flow again easily). Let the column run dry, then force 3 mL of air through the column with a syringe. Elute with 1 mL of methanol, and collect the analytes in a 3-mL volumetric flask, letting the eluate drip freely. Let the column run dry. Let stand for 1 min, then elute with an additional 1 mL of methanol, and collect in the same volumetric flask. Let the column run dry, and force 10 mL of air through the column. Dilute the eluate with water to volume. Use this as the *Test Solution*, and perform the analysis of aflatoxins immediately.

## SYSTEM SUITABILITY SOLUTION

Prepare a spiked sample by adding 5 mL of *Working Aflatoxin Standard Solution 5* to a 5-g sample and repeating the procedure for the *Test Solution*, using 20 mL instead of 25 mL of the mixture of methanol and 0.5% sodium bicarbonate (700:300).

[3] AflaOchraTest column (G1017; Vicam, Watertown, MA, USA) or equivalent. Aflatoxin/OTA immunoaffinity columns are suitable.

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## CHROMATOGRAPHIC SYSTEM

**Detector:** Fluorescence, excitation wavelength (Ex) 362 nm and emission wavelength (Em) 440 nm
**Column:** 4.6-mm × 15-cm; 3-µm packing L1
**Flow rate:** 0.8 mL/min
**Mobile phase:** Isocratic
*For post-column derivatization with PHRED cell:*[4] Water, methanol, and acetonitrile (60:25:15)
*For post-column derivatization with Kobra cell:*[5] A solution prepared by mixing 1 L of a mixture of water, methanol, and acetonitrile (60:25:15); 350 µL of 4 M nitric acid; and 120 mg of potassium bromide
**Post-column derivatization (PCD) systems**
*PHRED cell:* Post-column photochemical derivatization cell
*Kobra cell:* Electrochemical cell, post-column bromination derivatization cell

## ANALYSIS

**Post-column derivatization for aflatoxins:** Use a UV or Kobra cell. Inject 50 µL of reagent blank *(Working Aflatoxin Standard Solution 1)*, *Working Aflatoxin Standard Solutions 2–6*, or the *Test Solution* into the LC column. Identify the aflatoxin peaks in the *Test Solution* by comparing the retention times with those of the working standards. The aflatoxins elute in the order $AFG_2$, $AFG_1$, $AFB_2$, and $AFB_1$. After passing through the PHRED or Kobra cell, the $AFG_1$ and $AFB_1$ have been derivatized to form $AFG_{2a}$ (derivative of $AFG_1$) and $AFB_{2a}$ (derivative of $AFB_1$). [NOTE—The chemical structures of the derivatives resulting from electrochemical bromination and photolysis are not the same. The structures of $AFB_1$ and $AFG_1$ photolysis products have not been established.] The retention times of $AFG_2$, $AFG_{2a}$, $AFB_2$, and $AFB_{2a}$ are between about 14 and 27 min using the PHRED cell; retention times are shorter using the Kobra cell. The peaks should be baseline resolved. Construct standard curves for each aflatoxin. Determine the concentration of each aflatoxin in the *Test Solution* from the calibration curve.
**Aflatoxins calibration curves:** Calibration curves are prepared for each of the aflatoxins using the *Working Aflatoxin Standard Solutions* containing the four aflatoxins described. These solutions cover the range of 0.25–4 ng/mL for $AFB_1$ and $AFG_1$, and the range of 0.0625–1 ng/mL for $AFB_2$ and $AFG_2$. Make the calibration curves before analysis according to *Table 4*, and check the plot for linearity. If the test portion area response is outside (higher) the calibration range, then the *Test Solution* should be diluted with a mixture of methanol and water (1:1, v/v) and reinjected into the LC column.
**Quantitation of aflatoxins:** Quantitation of aflatoxins is performed by measuring peak areas at each aflatoxin retention time and comparing them with the corresponding calibration curve.

## SYSTEM SUITABILITY

The mean recovery of spiked $AFB_1$ (2 µg/kg) and the total of aflatoxins (5 µg/kg) is NLT 68% and 70%, respectively. The relative standard deviation (RSD) is NMT 10% for $AFB_1$ and for the total of aflatoxins.

## CALCULATIONS

Plot the peak area (response, *y*-axis) of each of the toxin standards against the concentration (ng/mL, *x*-axis) and determine the slope ($S$) and *y*-intercept ($a$). Calculate the level of toxin in the sample by the following formula:

$$\text{Toxin (µg/kg)} = \{[(R - a)/S] \times V/W\} \times F$$

$R$ = peak area of the *Test Solution*
$a$ = *y*-intercept of the calibration curve
$S$ = slope of the calibration curve
$V$ = final volume of the injected *Test Solution* (mL)
$W$ = 1 g of test sample passed through the immunoaffinity column
$F$ = dilution factor, 1 when $V = 3$ mL

The total of aflatoxins is the sum of $AFG_2$, $AFG_1$, $AFB_2$, and $AFB_1$.

## ACCEPTANCE CRITERIA

Where the individual monograph calls for compliance with the limits for aflatoxins, the limits are NMT 5 ppb for $AFB_1$ and NMT 20 ppb for the sum of $AFB_1$, $AFB_2$, $AFG_1$, and $AFG_2$, except when otherwise indicated.

[4] PHRED™ Photochemical Reactor (AURA Industries, New York, NY, USA) or equivalent. Avoid looking at the UV lamp.
[5] Kobra Cell™ (R-Biopharm Inc., Marshall, MI, USA) or equivalent. Set at 100 mA. Do not turn on the current until the LC pump is operating to avoid overheating the cell membrane.

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# PESTICIDE RESIDUE ANALYSIS

## Definition

Where used in this Pharmacopeia, the designation “pesticide” applies to any substance or mixture of substances intended to prevent, destroy, or control any pest, unwanted species of plants, fungus, or animals causing harm during or otherwise interfering with the production, processing, storage, transport, or marketing of pure articles. The designation includes substances intended for use as growth regulators, defoliants, or desiccants, and any substance applied to crops before or after harvest to protect the product from deterioration during storage and transport.

## Limits

Within the United States, many botanicals are treated as dietary supplements and are subject to the statutory provisions that govern foods but not drugs in the Federal Food, Drug, and Cosmetic Act. Limits for pesticides in foods are determined by the Environmental Protection Agency (EPA) as indicated in the Code of Federal Regulations (40 CFR §180) or the Federal Register (FR). In addition, the FDA establishes action levels for unavoidable pesticide residues (21 CFR §109 and 21 CFR §509). For pesticide chemicals without EPA-established tolerance levels or FDA action levels, the residues should be below the detection limit of the specified method. Results less than the EPA detection limits are considered zero values. The limits contained herein, therefore, are not applicable in the United States when articles of botanical origin are labeled for food purposes. The limits, however, may be applicable in other countries. Unless otherwise indicated in the monograph, the article to be examined complies with the limits given in *Table 5*. The limits for suspected pesticides that are not listed in *Table 5* must comply with the regulations of the EPA. For instances in which a pesticide is not listed in *Table 5* or in EPA regulations, calculate the limit by the formula:

$$\text{Limit (mg/kg)} = AM/100B$$

where $A$ is the acceptable daily intake (ADI), as published by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO), in mg/kg of body weight; $M$ is body weight, in kilograms (60 kg); and $B$ is the daily dose of the article, in kilograms.

If the article is intended for the preparation of extracts, tinctures, or other pharmaceutical forms of which the preparation method modifies the content of pesticides in the finished product, calculate the limits by the formula:

$$\text{Limit (mg/kg)} = AME/100B$$

where $E$ is the extraction factor of pesticide in the preparation method, determined experimentally as the ratio between the original pesticide content in the plant material and the final pesticide content in the preparation; $B$ is the daily dose of the preparation in kilograms; and $A$ and $M$ are as defined above.

A total or partial exemption from the test may be granted when the complete history (nature and quantity of the pesticides used, date of each treatment during cultivation and after harvest) of the treatment of the batch is known and can be checked precisely according to good agricultural and collection practices (GACP).

**Table 5**

| Substance | Limit (mg/kg) |
|---|---|
| Acephate | 0.1 |
| Alachlor | 0.05 |
| Aldrin and dieldrin (sum of) | 0.05 |
| Azinphos-ethyl | 0.1 |
| Azinphos-methyl | 1 |
| Bromide, inorganic (calculated as bromide ion) | 125 |
| Bromophos-ethyl | 0.05 |
| Bromophos-methyl | 0.05 |
| Bromopropylate | 3 |
| Chlordane (sum of *cis*-, *trans*-, and oxychlordane) | 0.05 |
| Chlorfenvinphos | 0.5 |
| Chlorpyriphos-ethyl | 0.2 |
| Chlorpyriphos-methyl | 0.1 |
| Chlorthal-dimethyl | 0.01 |
| Cyfluthrin (sum of) | 0.1 |

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**Table 5** *(continued)*

| Substance | Limit (mg/kg) |
|---|---|
| λ-Cyhalothrin | 1 |
| Cypermethrin and isomers (sum of) | 1 |
| DDT (sum of *o,p′*-DDE, *p,p′*-DDE, *o,p′*-DDT, *p,p′*-DDT, *o,p′*-TDE, and *p,p′*-TDE) | 1 |
| Deltamethrin | 0.5 |
| Diazinon | 0.5 |
| Dichlofluanid | 0.1 |
| Dichlorvos | 1 |
| Dicofol | 0.5 |
| Dimethoate and omethoate (sum of) | 0.1 |
| Dithiocarbamates (expressed as $CS_2$) | 2 |
| Endosulfan (sum of isomers and endosulfan sulphate) | 3 |
| Endrin | 0.05 |
| Ethion | 2 |
| Etrimphos | 0.05 |
| Fenchlorophos (sum of fenchlorophos and fenchlorophos-oxon) | 0.1 |
| Fenitrothion | 0.5 |
| Fenpropathrin | 0.03 |
| Fensulfothion (sum of fensulfothion, fensulfothion-oxon, fensulfothion-oxon sulfone, and fensulfothion sulfone) | 0.05 |
| Fenthion (sum of fenthion, fenthion-oxon, fenthion-oxon sulfone, fenthion-oxon sulfoxide, fenthion sulfone, and fenthion-sulfoxide) | 0.05 |
| Fenvalerate | 1.5 |
| Flucythrinate | 0.05 |
| τ-Fluvalinate | 0.05 |
| Fonophos | 0.05 |
| Heptachlor (sum of heptachlor, *cis*-heptachlorepoxide, and *trans*-heptachlorepoxide) | 0.05 |
| Hexachlorbenzene | 0.1 |
| Hexachlorocyclohexane (sum of isomers α-, β-, δ-, and ε-) | 0.3 |
| Lindan (γ-hexachlorocyclohexane) | 0.6 |
| Malathion and malaoxon (sum of) | 1 |
| Mecarbam | 0.05 |
| Methacriphos | 0.05 |
| Methamidophos | 0.05 |
| Methidathion | 0.2 |
| Methoxychlor | 0.05 |
| Mirex | 0.01 |
| Monocrotophos | 0.1 |
| Parathion-ethyl and paraoxon-ethyl (sum of) | 0.5 |
| Parathion-methyl and paraoxon-methyl (sum of) | 0.2 |
| Pendimethalin | 0.1 |
| Pentachloranisole | 0.01 |
| Permethrin and isomers (sum of) | 1 |
| Phosalone | 0.1 |
| Phosmet | 0.05 |
| Piperonyl butoxide | 3 |
| Pirimiphos-ethyl | 0.05 |

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**Table 5** *(continued)*

| Substance | Limit (mg/kg) |
|---|---|
| Pirimiphos-methyl (sum of pirimiphos-methyl and *N*-desethyl-pirimiphos-methyl) | 4 |
| Procymidone | 0.1 |
| Profenophos | 0.1 |
| Prothiophos | 0.05 |
| Pyrethrum (sum of cinerin I, cinerin II, jasmolin I, jasmolin II, pyrethrin I, and pyrethrin II) | 3 |
| Quinalphos | 0.05 |
| Quintozene (sum of quintozene, pentachloraniline, and methyl pentachlorphenyl sulfide) | 1 |
| S-421 | 0.02 |
| Tecnazene | 0.05 |
| Tetradifon | 0.3 |
| Vinclozolin | 0.4 |

**Qualitative and Quantitative Analysis of Pesticide Residues** Use analytical procedures validated e.g., in accordance with the latest version of the EU guideline on analytical quality control and validation procedures for pesticide residue analysis [NOTE—Current version Document No. ▲SANTE/12682/2019, https://ec.europa.eu/food/system/files/2020-01/pesticides_mrl_guidelines_wrkdoc_2019-12682.pdf▲ (ERR 1-Nov-2021)] or the EPA method validation principles (OPPTS 860.1340) that satisfy the following criteria. The method, especially with respect to its purification steps, is suitable for the combination of pesticide residue and substance under test, and is not susceptible to interference from co-extractives; the limit of quantification for each pesticide matrix combination to be analyzed is NMT the corresponding tolerance limit: the method is shown to recover between 70% and 120% of each pesticide with a repeatability NLT 20% RSD [NOTE—lower recoveries may be acceptable in certain cases as discussed in SANTE/11813/2017] ; and the concentrations of test and reference solutions and the setting of the apparatus are such that a linear response is obtained from the analytical detector.

## LIMITS OF ELEMENTAL IMPURITIES

The levels of elemental impurities should be restricted as shown in *Table 6* unless otherwise stated in the individual monograph. Specific monographs may provide different limits for articles that are typically used in large quantities.

**Table 6. Limits of Elemental Impurities**

| Element | Limit (µg/g) |
|---|---|
| Arsenic (inorganic)[a] | 2 |
| Cadmium | 0.5 |
| Lead | 5 |
| Mercury (total) | 1 |
| Methylmercury (as Hg)[b] | 0.2 |

[a] Arsenic may be measured using a nonspeciation procedure under the assumption that all arsenic contained in the supplement is in the inorganic form. Where the limit is exceeded using a nonspeciation procedure, compliance with the limit for inorganic arsenic shall be demonstrated on the basis of a speciation procedure.
[b] Methylmercury determination is not necessary when the content for total mercury is less than the limit for methylmercury.

Articles are tested according to the procedures set forth in *Elemental Impurities—Procedures* ⟨233⟩. Where speciation is required, the procedures given in *Elemental Contaminants in Dietary Supplements* ⟨2232⟩ are used for testing.

## USP REFERENCE STANDARDS ⟨11⟩

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# ⟨563⟩ IDENTIFICATION OF ARTICLES OF BOTANICAL ORIGIN

## INTRODUCTION

Identification of raw plant material intended for use in the manufacture of pharmaceuticals, excipients, or dietary supplements is carried out by examining the morphological and histological features of the article under test and by performing diagnostic chemical tests on the article. The botanical and chemical characteristics of the test article are then compared to the known botanical and chemical characteristics of the plant species. Reference articles may be specified to assist in the proper botanical and chemical identification of the plant and plant part. A reference article may be either a USP Authenticated Reference Material, which may be used for both botanical and chemical identification, or a USP Reference Standard, which is used for chemical identification only.

## USP AUTHENTICATED REFERENCE MATERIALS

USP Authenticated Reference Materials are plant organs or tissues certified to have come from a plant that has been properly identified as belonging to the species listed on the label. The authentication is performed by botanical taxonomists, plant anatomists, phytochemists, or other plant scientists contracted by USP. A USP Authenticated Reference Material is typically a dried, pulverized plant organ or tissue, and it may be obtained from USP. Herbarium samples that may include roots, stems, leaves, flowers, fruits, and seeds of the authenticated plants are archived and made available for examination upon request. Standard herbarium samples usually consist of the entire mature plant. USP Authenticated Reference Materials undergo the same botanical and chemical diagnostic tests as those applied to test raw materials. A test article must have all botanical and chemical characteristics specified and found in the USP Authenticated Reference Material. To serve its intended purpose, each USP Authenticated Reference Material is properly stored, handled, and used. Generally, USP Authenticated Reference Materials are stored in their original containers under cool and dry conditions and protected from light and insect infestation. Where special storage conditions are necessary, directions are given on the label. Active principles and marker compounds typically degrade with time; therefore, expiration dates are assigned to USP Authenticated Reference Materials for their use in chemical identification. USP Authenticated Reference Materials are not intended for use in the manufacture of pharmaceuticals, excipients, or dietary supplements.

## BOTANICAL IDENTIFICATION

The botanical identification of raw plant materials used in the manufacture of pharmaceuticals, excipients, or dietary supplements consists of ascertaining the macroscopic characteristics of the plant part, such as root, stem, leaf, flower, fruit, or seed, used in the manufacture of the article, as well as ascertaining its histological (microscopic) features. It may also include the inspection of organoleptic features of the botanical tissue such as the presence or absence of a characteristic odor. Individual compendial monographs may include botanical information on possible adulterant species to help ensure their absence in the raw material. For a proper identification of the plant, plant organ, or plant tissue, it is necessary to have a basic knowledge of plant anatomy.

### Diagnostic Plant Morphology and Anatomy

This section exclusively addresses the diagnostic morphological and anatomical features of vascular plants and the various plant parts, such as roots, stems, leaves, flowers, fruits, and seeds, from which pharmaceuticals, excipients, or dietary supplements are derived. Vascular plants include pteridophytes (ferns and fern allies; for example, genera *Aspidium*, *Equisetum*, and *Lycopodium*), gymnosperms (seed plants, in which the seed is not enclosed within a fruit; for example, genera *Ephedra*,*Gingko*, and *Pinus*), and angiosperms (seed plants, in which the seed is enclosed within a fruit; for example, genera *Allium*, *Digitalis*, *Panax*, *Matricaria*, and *Rauwolfia*). Anatomical diagnostic features that are specified in an individual monograph (see *Botanic characteristics* in individual monographs) may include, but are not limited to, the presence of a particular tissue within an organ; the arrangement and type of cells within a tissue; the presence and type of secretory canal, oil, or resin duct or laticifers within an organ; the number of epithelial cells surrounding a secretory canal; and the presence and type of ergastic substances such as starch, inulin, fat globules, essential oils, calcium oxalate crystals, cystoliths, polyphenols, fluids, or other materials occurring in the cytoplasm, organelles, vacuoles, cavities, or cell wall.

#### ROOTS

The tissues present in young roots, starting with the most external tissue, include an epidermis with root hairs, cortex, endodermis, pericycle, phloem, xylem, and, in some species, pith. In some species, the outermost layer or layers of cortex are distinct from the inner layers, in which case they are referred to as a hypodermis. In species that undergo secondary growth in the roots, it is typical for all tissues external to the pericycle to be sloughed off. Roots that exhibit secondary growth have a periderm or bark, composed of a phellum (cork), phellogen (cork cambium), and phelloderm as the outermost tissue. Underneath the periderm, remnants of primary phloem, secondary phloem, vascular cambium, primary xylem, and secondary xylem can be found. Secondary vascular tissues have medullary rays separating clusters of the principal conducting cells of phloem (sieve elements or sieve cells) and the principal conducting cells of xylem (vessels and tracheids). Most species of plants that undergo secondary growth lack pith in the root. The type and arrangement of the principal conducting cells of the vascular tissues may be diagnostic of the species. Roots of many species develop into food storage organs. Abundant parenchyma and

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large amounts of starch or other polysaccharides characterize these types of roots. The presence, type, and arrangement of fibers, sclereids, and other tissues, and the presence and location of ergastic material, may also be diagnostic features. Morphologically, roots may be distinguished from rhizomes (the underground stems) primarily by the absence of nodes and internodes, which are present in rhizomes.

## STEMS

Several external macroscopic features of stems that may be diagnostic of the species include the attributes of the nodes, internodes, leaf scars, vascular bundle scars, lenticles, and buds; the growth pattern of the buds; position and arrangement of the leaves along the stem; and the presence of tendrils, spines, thorns, or prickles. Starting with the outermost tissue, the internal arrangement of tissues in the young stems of most species is epidermis, cortex, a concentric ring of vascular bundles separated from each other by parenchymatous medullary rays, and pith. Depending on the species, stomata or trichomes or both structures may be present in the epidermis. The cortex of some species may include a hypodermis or an endodermis or both. In most monocotyledons, the vascular bundles are not arranged concentrically; instead, they are scattered throughout a mass of parenchyma tissue internal to the epidermis. Because of this arrangement, neither cortex, medullary rays, nor pith can be discerned. In woody plant stems that undergo secondary growth, it is typical for the epidermis to be sloughed off and replaced by a periderm composed of a phellum, phellogen, and phelloderm. Some species are characterized by having multiple periderms (rhytidome). Lenticles may be present in the periderm and their attributes may serve as diagnostic features. Underneath the periderm are the remnants of the cortex, primary phloem, secondary phloem, vascular cambium, secondary xylem, primary xylem, and pith. Medullary rays are also present. As in the root, the type and arrangement of the principal conducting cells of the vascular tissues; the presence, type, and arrangement of fibers, sclereids, and other tissues; and the presence and location of ergastic material may also be diagnostic features. Rhizomes may have some morphological characteristics similar to those of roots and therefore they may be mistaken for roots. However, rhizomes can be correctly identified as stems because they have distinct nodes and internodes.

## LEAVES

Several macroscopic features of leaves that may be diagnostic of the species include the attributes of the leaf blade, petiole, and stipules and the phyllotaxy. The outermost tissue of a leaf blade is the epidermis, followed by mesophyll and vascular tissues. Microscopic diagnostic features of epidermal cells include the cuticle thickness and markings, the shape and arrangement of stomata and guard cells, the arrangement and size of subsidiary cells, stomatal number (number of stomata per unit area), and stomatal index (number of stomata per unit number of epidermal cells). Additional features useful in the identification of leaf material include types and arrangement of trichomes (plant hairs) present; type and arrangement of mesophyll and vascular tissues; palisade mesophyll ratio; presence and appearance of accessory tissues such as parenchymatous or sclerenchymatous bundle sheaths, paraveinal mesophyll, endodermis, and transfusion tissue; type and arrangement of the principal conducting cells of the vascular tissues; presence, type, and arrangement of fibers, sclereids, and other tissues; and presence, location, and physical appearance of ergastic material.

## FLOWERS

Flowers are the best diagnostic morphological features of any flowering plant, and the floral structure is the principal criterion used in plant taxonomy. The diagnostic features of flowers include type of inflorescence; presence, number, and appearance of the primary floral parts (sepals, petals, stamens, and carpels); type of symmetry displayed by the floral parts; relative position of the ovaries in regard to the other parts of the flower; the number of ovules per ovary; type of placentation of the ovary; physical appearance of the pollen grains; presence of nectaries; presence of covering or glandular trichomes; and physical features of accessory structures such as the receptacle and bracts. The histological features and the presence of ergastic materials in the tissues of floral parts are also diagnostic of the species.

## FRUITS

The identification of the species of plant from which a fruit was derived may be determined by observing several macroscopic criteria. These criteria include the number of pistils found in the fruit; the number of carpels within each pistil; the number of seeds within each carpel; the placentation of the fruit; and the determination of whether the fruit is dehiscent, indehiscent, or fleshy. Additional diagnostic features include the number of sutures in a dehiscent fruit, the determination of whether the seeds are fused to or free from the pericarp wall, physical features of the three layers of the pericarp of fleshy fruits (epicarp, mesocarp, and endocarp), and presence and physical appearance of accessory tissues such as the receptacle and bracts. Histological features of fruit tissues may aid in identification. The characteristics of the seeds within the fruit are also diagnostic features of the species.

## SEEDS

The macroscopic features of seeds used in identification include the shape and size of the seed; appearance of the seed-coat surface; placement of the hilum and micropyle; and presence of accessory structures of the seed coat such as the arils, caruncle, or oil bodies. Physical features of the embryo, such as its size, shape, position, and the number and appearance of the cotyledons, as well as the presence and appearance of accessory nutritive tissues such as the remnants of a megagametophyte (in gymnosperms), perisperm (nucellus), or endosperm, are also diagnostic of the species. Histological features of the seed coat and other structures and tissues of the seed may also be used for species identification.

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## Microtechnique

Histological analysis of botanical specimens can be performed on whole plant material or plant powder. The use of cytological stain or other reagents may be necessary to visualize certain histological features. Crossed polarizers can be used to detect structures that rotate plane-polarized light. These structures include starch grains, calcium oxalate crystals, some fibers, and grains of sand (present as a contaminant) that can be observed as bright objects against a dark background. One polarizer is commonly placed in the condenser or the light source, and the second polarizer is placed in the ocular. Light entering the slide from below is plane polarized, permitting only some light waves in a specific plane to pass through. When the two polarizers are aligned, the field becomes bright; when the two polarizers are crossed, the field becomes dark.

### PROCEDURE FOR TEMPORARY MOUNTS AND POWDERED MATERIAL

**General procedure:** Plant samples are observed under the microscope by employing different mounting media, stains, or other solutions to assist in the correct identification of the test article. If a USP Authenticated Reference Material is available, prepare it with the same mounting media or reagent solutions used for the test article. Place 1 or 2 drops of water, *Glycerin–alcohol solution*, *Chloral hydrate solution*, or another reagent solution (see *Preparation and use of reagent solutions, optical devices, and mountants*) in the center of a clean slide. Transfer a small plant tissue section or a portion of plant powder into the mountant or reagent solution, and cover with a clean coverslip. (For specific preparation techniques, see *Preparation of temporary mounts and hand sections*, *Maceration*, or *Preparation of powdered materials*, as appropriate.) To prevent the formation of air bubbles, the coverslip may be carefully placed at an appropriate angle with its edge making the first contact with the slide and then pressed until it covers the specimen. Using a piece of filter paper, remove excess fluid from the margin of the coverslip. Air bubbles can be removed by placing the slide in a vacuum desiccator. When using chloral hydrate, air bubbles can be removed by gently boiling the sample over a small flame such as that from an alcohol lamp. To replace the mountant or reagent solution, place drops of the new mountant or reagent solution on one edge of the coverslip. Place a strip of filter paper at the opposite edge of the coverslip to remove the old mountant or reagent solution and to cause the new mountant or reagent solution to be drawn over the powdered material or tissue. Plant oils can be also washed away from the tissue in this manner when solvent hexane or acetone are washed through the slide followed by water and, if necessary, *Chloral hydrate solution*. Do not use *Chloral hydrate solution* immediately after treating the plant tissue with flammable solvents without thoroughly washing the tissue with water. This is to avoid setting fire to residual solvent when the microscope slide is later placed over a small flame to boil the tissue. Care must be taken when using reagent solutions that are volatile or corrosive to the microscope. To prevent drying of aqueous or chloral hydrate solutions during observation, add a small drop of glycerin to the slide. Observe the mounted sample under an optical microscope (see *Optical Microscopy* ⟨*776*⟩), and examine for histological features.

**Preparation and use of reagent solutions, optical devices, and mountants:** The following reagents, optical devices, and mounting media are used to assist in the identification of cells, tissues, structural features, and ergastic substances in the tissue or powdered material (see *Table 1* and *Table 2*).

**Table 1. The Use of Reagent Solutions and Optical Devices**

| Detection | Reagent Solutions and Optical Devices |
|---|---|
| Calcium carbonate concretion | *Diluted acetic acid* |
| Calcium oxalate crystals | *Crossed polarizers* |
| Cellulose | *Carmine alum–methyl green solution*<br>*Hydriodic acid*<br>*Zinc chloride–iodine solution* |
| Cytoplasm | *Alcoholic picric acid solution* |
| 1,8-Dihydroxyanthra-quinones | *1 M Potassium hydroxide solution* |
| Essential oils | *Osmium tetroxide solution*<br>*Sudan III solution* |
| Inulin | *Naphthol–sulfuric acid solution* |
| Lignin | *Carmine alum–methyl green solution*<br>*Phloroglucinol–hydrochloric acid solution*<br>*Universal reagent* |
| Lipids (cutin, waxes, and suberin included) | *Carmine alum–methyl green solution*<br>*Osmium tetroxide solution*<br>*Sudan III solution*<br>*Universal reagent* |
| Pectin and mucilage | *Ruthenium red solution*<br>*Thionine solution*<br>*Toluidine blue solution* |
| Phytoglycogen | *Ruthenium red solution* |
| Protein bodies | *Alcoholic picric acid solution*<br>*Osmium tetroxide solution* |

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**Table 1. The Use of Reagent Solutions and Optical Devices** *(continued)*

| Detection | Reagent Solutions and Optical Devices |
|---|---|
| Saponin | *Blood–gelatin mixture*<br>*Iodine–glycerin solution* (confirm by testing with *Blood–gelatin mixture*) |
| Starch | *Crossed polarizers*<br>*Iodine solution*<br>*Universal reagent* |
| Tannins and other polyphenols | *Ferric chloride solution*<br>*Osmium tetroxide solution* |

**Table 2. Bleaching and Clarifying Agents and Mountants**

| Use | Mountants and Agents |
|---|---|
| Bleaching agents | *Sodium hypochlorite solution* |
| Clarifying agents | *Chloral hydrate solution*<br>*Lactochloral solution*<br>*Lactophenol solution* |
| Mountants | *Glycerin*<br>*Glycerin–alcohol solution*<br>*Glycerin–gelatin mixture*<br>*Water* |

*Alcoholic picric acid solution*—Prepare a 1% solution of picric acid in alcohol. Picric acid is useful to stain cells having dense cytoplasm such as aleurone cells in seeds. Place a small amount of powdered plant material in a test tube, and shake with about 1 mL of solvent hexane to remove plant oils, which would interfere with the reaction. Centrifuge, and discard solvent hexane. Soak the plant powder in *Alcoholic picric acid solution* for about 30 min. Transfer a portion of the powder to a microscope slide, and observe under a microscope. Cytoplasm and protein bodies turn bright yellow. [**CAUTION**—Picric acid is explosive when dry. Handle appropriately.]

*Blood–gelatin mixture*—Add 4.5 g of gelatin powder to 100 mL of a 0.9% sodium chloride solution, and allow to swell for 30 min. Heat the gel, while stirring, to about 80° in a water bath. Cool to 40°, and add 6 mL of defibrinated bovine blood. Heat to 45°–50°, and pour onto a microscope slide in a thin layer of about 1 mm while the slide is in a horizontal position. To prevent loss of blood-gelatin mixture from the sides, seal the microscope slide edge with a 1-cm wide adhesive tape to form a tray. After cooling and solidification, it is ready for use. [NOTE—Store in a humid chamber for NMT 1–2 days at 3°–4°.] To test for saponins, place small clusters of the powdered plant material on the blood–gelatin layer, spacing them a few mm apart from each other, transfer to a humidifier for a few h, and observe. Saponin-containing particles will cause light-transparent zones to appear in the blood–gelatin.

*Carmine alum–methyl green solution*—Boil 1.5 g of carmine for 30 min in a 15% solution of aluminum potassium sulfate. Cool, filter, and add 10 mL of a 0.75% methyl green solution while stirring. Add 1–2 drops to the plant material. Lignin and suberin turn green and cellulose turns red-violet.

*Chloral hydrate solution*—Use chloral hydrate TS. When using the solution as a clarifying agent, add a few drops to the plant material, and boil briefly over a small flame. Chloral hydrate dissolves cellular contents and intercellular substances and allows cell walls and shapes to be easily observed. It can be used to assist in the identification of cork, fibers, vessels, calcium oxalate crystals (with the aid of crossed polarizers), trichomes, stomata, and pollen.

*Crossed polarizers*—This optical device is used to detect calcium oxalate crystals and starch grains (amyloplasts). In polarized light, calcium oxalate crystals and starch grains appear as bright, birefringent objects on a dark background. Starch grains observed under polarized light will also have a Maltese-cross effect with the arms of the cross intersecting at the hilum. Calcium oxalate crystals are usually best viewed after the sample has been clarified with *Chloral hydrate solution* or another clarifying agent.

*Diluted acetic acid*—Add 1–2 drops to the plant material, and immediately observe under a microscope. Calcium carbonate deposits dissolve with effervescence.

*Ferric chloride solution*—Dilute 1 mL of ferric chloride TS with 9 mL of water. For the detection of phenol hydroxyl groups, such as tannins and flavonoids, from the side of the coverslip add the solution to the aqueous sample. Tannins and other polyphenols become blue-black to green.

*Glycerin*—Use as a mountant to prevent the drying of aqueous and chloral hydrate solutions.

*Glycerin–alcohol solution*—Mix equal volumes of glycerin and alcohol. Use as a mounting medium.

*Glycerin–gelatin mixture*—Add 10.0 g of powdered gelatin to 60 mL of water. Allow to stand for 2 h, and add 70 mL of glycerin containing 1.5 g of dissolved phenol. Heat in a water bath, and filter through a preheated funnel containing glass wool. The filtered mixture is liquefied before use, and it serves as a mounting medium. Add a few drops to the cut or powdered plant material, and cover with a heated coverslip. This preparation is used for long-term storage of specimen mounts. The margins of the coverslip may be sealed with Canada balsam after a few months of drying.

*Hydriodic acid*—Add 1–2 drops to plant material. Cellulosic cell walls become blue to blue-violet.

*Iodine solution*—Add 1–2 drops of 0.1 N iodine VS to the plant material. Starch particles become dark-blue to blue-violet; this reaction is reversible on heating. [NOTE—Proteins, lipids, and cellulose turn yellow to brown; guaiac powder particles become green to blue, but this reagent is not used for diagnostic identification of these features.]

*Iodine–glycerin solution*—Dissolve 0.3 g of iodine and 1.0 g of potassium iodide in a small quantity of water, and add 10 mL of a mixture of glycerin and water (1:1). Add 1–2 drops to the powdered plant material. Samples containing saponins form

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yellow lumps or aggregates. If a sample tests positive for saponin, the result has to be confirmed by testing the sample with *Blood–gelatin mixture* as well.

*Lactochloral solution*—Dissolve 50.0 g of chloral hydrate in 50 mL of lactic acid with gentle heating. Add a few drops to the plant material. Place the microscope slide in a small vacuum desiccator if it is necessary to eliminate air bubbles. *Chloral hydrate solution* and *Lactochloral solution* are used for the same type of identification, except that *Lactochloral solution* is a stronger clarifying agent and it is used for plant material that is more difficult to clarify.

*Lactophenol solution*—Mix 20 g of lactic acid, 40 g of glycerin, and 20 mL of water. Add 20 g of phenol, and mix. This is a strong clarifying agent suitable for the examination of pollen grains.

*Naphthol–sulfuric acid solution*—Prepare a 20% solution of 1-naphthol in alcohol. To the plant material add 1 drop of 1-napthol solution and 1 drop of sulfuric acid. Inulin crystals turn brownish red and then dissolve.

*Osmium tetroxide solution*—Dissolve 0.1 g of osmium tetroxide in 5 mL of distilled water. Add 1–2 drops of the solution so obtained to the plant material. Essential oils, fatty oils and other lipids, tannins, and protein bodies become brown to black.

*Phloroglucinol–hydrochloric acid solution*—This solution is used for the identification of lignin and other hydroxyphenylpropane derivatives; lignified tissues, such as sclereids, vessels, fibers, and stone cells; and lignified parenchyma. Moisten the powder or the cut sample with phloroglucinol TS, and allow to dry for 2–3 min before placing the coverslip. Add a few drops of a 25% hydrochloric acid solution, and cover with the coverslip. Lignified cell walls turn carmine red. [NOTE—This stain is not stable.] Cells with hydroxyphenylpropane derivatives, such as vanillin and ferulic acid, also turn red. Alternatively, hydroxyphenylpropane derivatives can be extracted from the plant material and the plant material then examined. To extract hydroxyphenylpropane derivatives repeatedly immerse the untreated material in alcohol, mix on a vortex mixer, centrifuge, and discard the alcohol between washings. Then treat the plant material as specified previously, beginning with the addition of phloroglucinol TS.

*1 M Potassium hydroxide solution*—Add 1 drop to plant material. Cells containing 1,8-dihydroxyanthraquinones will stain red.

*Ruthenium red solution*—Add a few drops of ammonium hydroxide to ruthenium red TS. [NOTE—Store the solution protected from light.] Add 1–2 drops to the plant material. Pectin-containing cell membranes, acidic mucilage, and phytoglycogen turn red.

*Sodium hypochlorite solution*—This solution is used to bleach deeply colored sections. Immerse the plant material in the solution for a few min until sufficiently bleached. Wash the tissue with water, and mount with a suitable mounting agent. [NOTE—Sodium hypochlorite will extract lignin; plant tissue so treated will test negative for lignin.]

*Sudan III solution*—Dissolve 0.5 g of sudan III in 50 mL of alcohol or isopropyl alcohol with reflux boiling. Cool, filter, and add 50 mL of glycerin. Add 1–2 drops of this solution to plant powder. Essential oils, waxes, cutin, suberin, and fatty oils and other lipids combine with this lipophilic colorant and become orange-red to red after a short time.

*Thionine solution*—Prepare a 0.2% thionine acetate solution in 25% alcohol. Immerse the dry sample in this solution. After about 15 min, wash out the excess of stain with 25% alcohol. Mucilage will have swollen into spherical globules and turned red-violet, while cellulose, pectin, and lignified septa will turn blue or blue-violet.

*Toluidine blue solution*—Using toluidine blue, proceed as directed for *Thionine solution*.

*Universal reagent*

SOLUTION A: Dilute 20 mL of a lactic acid-saturated solution of sudan III with 30 mL of lactic acid.

SOLUTION B: Dissolve 0.55 g of aniline sulfate in 35 mL of water.

SOLUTION C: Dissolve 0.55 g of potassium iodide and 0.05 g of iodine in 5 mL of water, and add 5 mL of alcohol.

PROCEDURE: Combine *Solution A*, *Solution B*, and *Solution C*, and add 2.5 mL of hydrochloric acid while stirring. [NOTE—The solution is used without filtering.] For identification, add 2–3 drops to the sample, and gently boil over a small flame. If necessary, small amounts of *Universal reagent* may be added during boiling. Cover with the coverslip. Lignified elements turn yellow, suberin turns red-brown, lipids turn red, and starch turns blue-violet.

*Water*—Use as a mounting medium. [NOTE—All grades of water are acceptable for this purpose.]

*Zinc chloride–iodine solution*—Dissolve 20.0 g of zinc chloride and 6.5 g of potassium iodide in 10.5 mL of water. Add 0.5 g of 0.1 N iodine VS, and shake for 15 min. Filter if necessary. Store in low-actinic glassware. Add 1–2 drops to the plant material, and allow to stand for a few min. Cellulosic cell walls are stained blue to blue-violet.

**Preparation of temporary mounts and hand sections:** When using the dry plant tissue, soak or gently boil in water until soft. Do not soften too much. Material can then be treated like fresh plant material. When appropriate, use the mountants or reagent solutions listed for use with plant powder to help visualize features of the tissue (see *Preparation and use of reagent solutions, optical devices, and mountants*).

To make an epidermal peel of the leaf, petal, sepal, bract, and other leaf-like appendages, roll the tissue into a cylinder, and nick with a sharp, polytef-coated razor blade that has been wetted with water. Grasp the nicked piece of tissue with forceps, and strip back removing a clear section of the epidermis. Mount in water on a microscope slide, place a coverslip over the tissue, and examine under a microscope.

If it is difficult to obtain an epidermal peel using the previous procedure, proceed as follows. Soak the tissue in a 40%–60% nitric acid solution at 60° for 3–4 min or until the epidermis can be easily peeled. The peel is then washed 3–5 times in water to remove the excess of nitric acid. Neutralize the tissue in a 1% potassium hydroxide solution or a 1% sodium hydroxide solution. Wash the tissue again with water, mount in water on a microscope slide, place a coverslip over the tissue, and examine under a microscope.

An alternative method of preparing leaf tissue for the examination of the epidermis is to heat a leaf fragment (about 5 mm × 5 mm) for 15 min in *Chloral hydrate solution* on a water bath. Transfer the tissue to a microscope slide, add a drop of water, and cover with a coverslip. These procedures can be used to determine the stomatal type, distribution, number, and index.

Stomatal number is determined by counting the number of stomata per unit area of a microscopic field. Determine the stomatal number on at least 10 different sites of the specimen, and calculate a mean value. Keep track of which leaf surface is being observed, abaxial or adaxial, as the stomatal number for different surfaces is frequently significantly different.

To calculate the stomatal index, the specimen is observed under a microscope at a low magnification. The size of the surface is determined with a calibrated micrometer ocular, and the number of stomata and the number of epidermal cells for that area are determined. The stomatal index is calculated:

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$$\text{Result} = (100 \times S)/(E + S)$$

$S$ = number of stomata for a given area
$E$ = number of epidermal cells of the same area

Determine the stomatal index on at least 10 different sites of the specimen, and calculate a mean value. Again, keep track of which leaf surface is being observed, abaxial or adaxial, as the stomatal indices for different surfaces is frequently significantly different.

To make a cross section of a leaf or thin roots, stems, or other thin appendages, lay the appendage to be sectioned on a microscope slide. Place another microscope slide over the appendage with a portion of the tissue exposed. Using a sharp, polytef-coated razor blade that has been wetted, cut straight down along the edge of upper slide. Without moving the upper slide, cut down again with the razor blade at an angle. Some practice may be necessary to be able to get sections thin enough so that when they are mounted and covered with a coverslip, these sections can be used to determine tissue arrangements (for instance, the number of palisade layers in leaf, thickness of cuticle, types of trichomes, types of vascular bundles, and the like). Because razor blades dull quickly, they have to be replaced frequently.

Use the cross section of leaf tissue so obtained to determine the palisade mesophyll ratio. Alternatively, boil leaf fragments of about 2 $mm^2$ in *Chloral hydrate solution*, mount, cover with a coverslip, and observe under a microscope. Identify groups of four adaxial epidermal cells, and count the palisade mesophyll cells that are lying below and are at least 50% covered by the epidermal cells. This value divided by 4 is the palisade mesophyll ratio. Determine the palisade mesophyll ratio of at least 10 groups of epidermal cells, and calculate a mean value. Palisade mesophyll ratio can also be determined on powdered leaf material.

To make a cross section of thick stems, roots, or other plant parts, including woody tissues, hold the tissue in one hand and using a sharp, polytef-coated razor blade that has been wetted with water, shave a cross section from the appendage. Mount in water, another medium, or reagent solution, place a coverslip over the material, and examine under a microscope. Sections thin enough to determine vascular tissue arrangement, ray type, parenchyma distribution, presence of crystals, and the like can usually be made with a little practice.

**Maceration:** For the proper identification of a plant material, it is sometimes necessary to macerate the tissue into its individual cells before microscopic examination. This can be an especially useful technique for woody or other hard tissues. The material is cut into small pieces of about 2-mm thickness and 5-mm length or sliced into pieces of about 1-mm thickness. Depending on the nature of the cell wall, one of the following methods is used. For hard or highly lignified tissues, use *Method I*. For tissues that are not extensively lignified, use *Method II*.

*Method I*

SOLUTION A: Use 4 N nitric acid solution.

SOLUTION B: Prepare a mixture of 1.2 M chromium trioxide solution and sulfuric acid (7:4).

PROCEDURE: Place the plant material in a test tube containing about 5 mL of a mixture of *Solution A* and *Solution B* (1:1). Heat in a water bath for 20 min. Wash the tissue repeatedly with water, and transfer to a microscope slide. Tease the tissue apart with a dissecting needle, add 1–2 drops of mountant, cover with a coverslip, and examine under a microscope. If necessary, cells can be further separated from each other by pressing down on the coverslip with a gentle, sliding motion. The macerated tissue will test negative for lignin.

*Method II*

PROCEDURE: Place the plant material in a test tube containing about 5 mL of 2 M potassium hydroxide solution. Heat in a water bath for 30 min. Wash the tissue repeatedly with water, and transfer to a microscope slide. Add 1–2 drops of mountant. Place a coverslip over the tissue, press down, squashing the tissue, and examine under a microscope. The macerated tissue will test negative for lignin.

**Preparation of powdered materials:** Place 1 or 2 drops of water, another mountant, or a reagent solution in the center of a clean slide. Moisten the tip of a dissecting needle with water, and dip into the powder under test. Transfer a small amount of material that adheres to the needle into the fluid on the slide, and stir thoroughly and carefully. Cover with a clean coverslip. Because the arrangement of the tissue structures within the plant tissue has been destroyed, the important features for observation of the powdered plant material are the chemical and physical features of tissues and cell types, as well as the presence and chemical and physical features of ergastic substances. The specific tissues, cells, and ergastic substances to be examined are specified in the individual monograph.

## PROCEDURE FOR THIN, PERMANENT MOUNTS

When it is necessary to reveal detailed histological features of a plant specimen, thin tissue sections have to be obtained. The sections need to be thin enough to transmit light, and they have to be cut in such a plane that the desired features are exposed. The plant material is properly killed, fixed, dehydrated, and embedded in paraffin or other embedding media. The embedding medium is used as a solid-support matrix during tissue sectioning. After sectioning and mounting, staining of the specimen is frequently performed to aid in the differentiation of certain structures. [NOTE—The process of fixing, dehydration, embedding, and staining can be significantly expedited by utilizing a microwave oven specifically designed for histological work.]

**Killing and fixation:** As a first step in preparing plant material for sectioning, living cells are killed, and the tissue is preserved. This is most frequently done by employing a chemical fixative. A good general purpose fixative for plant material is a mixture of formaldehyde, acetic acid, and alcohol (FAA).

*FAA solution*—Mix 50 mL of alcohol, 5 mL of glacial acetic acid, 10 mL of formaldehyde solution, and 35 mL of water. [NOTE—Periodically prepare fresh solution, as it loses effectiveness with storage.]

*Procedure*—Completely immerse the plant material in the *FAA solution*. Allow the material to remain immersed for 18–24 h at room temperature. Plant material can be kept indefinitely in *FAA solution*, as long as it remains completely immersed and is not allowed to dry out. Certain plant tissues may require vacuum infiltration to facilitate the penetration of the fixative. Vacuum infiltration is required if the tissue has abundant air spaces or epidermal hairs or if it floats on top of the fixative solution. Place the tissue in a small vial containing the fixative. Place the uncapped vial into a bell jar or desiccator that is connected to a vacuum

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source, preferably an oil-sealed vacuum pump. The vacuum is vented into a fume hood to prevent fixative vapors from filling the room. Slowly turn on the vacuum. Do not use a strong vacuum because the fixative may start to boil and damage the tissue. As residual air is pulled from the tissue, it will rise to the surface. Turn the vacuum on and off through several cycles until the tissue stays at the bottom of the container during an "on" cycle.

**Tissue dehydration:** Paraffin and other embedding media are hydrophobic; thus, water must be removed from the plant tissue after fixation. This is accomplished by immersing the fixed tissue in dehydration solutions, which are a series of mixtures of alcohol and water with increasing alcohol concentration. The final solution in the series is dehydrated alcohol. Begin by washing the fixed tissue once or twice with fresh 50% alcohol to remove traces of FAA. Remove this solution, and subsequently remove any other dehydration solution, by decanting the solution or removing it with the aid of a glass pipet. Add the first dehydration solution (70% alcohol) to the vial, completely immersing the tissue. The graded alcohol–water series and the suggested times for tissue immersion are listed in *Table 3*.

**Table 3**

| Dehydration Solution | Time (h) |
|---|---|
| 50% alcohol | 1–2 |
| 70% alcohol | 1–2 |
| 90% alcohol | 1–2 |
| 95% alcohol | 1–2 |
| Dehydrated alcohol containing 0.1% of safranin O | 2–4 |
| Dehydrated alcohol | 1 |

Safranin O is added to the penultimate dehydration solution in the series to visualize the tissue when it has become embedded in paraffin. If the tissue to be sectioned is hard or woody, the time for each step in the series may need to be increased to up to 24 h. If necessary, the tissue can be stored for several days in 70% alcohol or in solutions of even higher alcohol concentrations.

**Embedding**

*Preparation for embedding*

ALCOHOL REMOVAL: Paraffin is the most common embedding medium, although other embedding media are available. After dehydration, alcohol is removed from the tissue by using a graded series of dehydrated alcohol–xylene solutions, because paraffin is not soluble in alcohol. The graded dehydrated alcohol–xylene series and the suggested times for tissue immersion are listed in *Table 4*.

**Table 4**

| Alcohol Removal Solution | Time (h) |
|---|---|
| A mixture of dehydrated alcohol and xylene (3:1) | 1 |
| A mixture of dehydrated alcohol and xylene (1:1) | 1 |
| A mixture of dehydrated alcohol and xylene (1:3) | 1 |
| Xylene | 1 |
| Xylene | 1 |

XYLENE REMOVAL: Once xylene has completely replaced alcohol, paraffin is added slowly to infiltrate the tissue and remove xylene. Proceed as follows:

1. For each mL of xylene add about 1 paraffin chip to the tissue vial, cap, and allow to stand at room temperature for 4 h. Add additional paraffin chips until no more chips dissolve.
2. Place the tissue in an oven maintained at 42°–45°. Add 2–3 paraffin chips every h until no more chips dissolve at that temperature.
3. Pour off one-third of the volume, and replace with an equal volume of melted paraffin. Do not cap, and transfer the vial to an oven maintained at 58°–60°.
4. After the paraffin remelts (about 4 h later) pour off one-half of the volume, and replace with an equal volume of melted paraffin. Transfer the vial to the oven maintained at 58°–60° if the paraffin begins to solidify.
5. Repeat the fourth step twice more, then pour off the entire volume of paraffin–xylene. Replace with pure melted paraffin. About 4 h later, pour off the paraffin, and replace with fresh pure melted paraffin. Repeat the pouring off and replacement 4 h later, and allow to stand overnight. [NOTE—Transfer the vial to the oven maintained at 58°–60° if the paraffin begins to solidify at any point.]

*Embedding procedure*—Pour the tissue with the paraffin to an embedding boat. Paraffin has to completely cover the tissue by about 3–5 mm. Place the embedding boat on top of a preheated warming platform that is designed for histology work. Adjust the tissue in the boat to its proper orientation for sectioning. Slowly cool the paraffin by sliding the boat down to the cool side of the platform until the paraffin has solidified. Immerse the paraffin block in ice water to rapidly cool the block and to prevent paraffin crystals from forming. Store the paraffin block at 4°.

**Sectioning and mounting:** Cut the paraffin block into pieces, each containing one tissue sample. Trim the paraffin block, as close to the tissue mass as possible, to form a rectangle or a slight trapezoid. Such trimming will prevent sectioning problems due to excess paraffin around the tissue. To make transverse sections, orient the tissue at a right angle to a wooden tissue block

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of which the face has been soaked in melted paraffin. Affix the paraffin block to the face of the tissue block. Add a small amount of melted paraffin to the base of the paraffin block to help form a tighter seal. Cool the block to 4°.

Properly mount and adjust the tissue and paraffin block in a microtome. Use a sharp stainless-steel microtome knife that has been properly honed. Set the microtome to cut sections 8–15 μm thick (10-μm thickness is optimal for most tissues). Cut individual or serial sections. Prepare a microscope slide as follows. An adhesive may be prepared as a solution containing 1% of gelatin and 0.5% of sodium benzoate that is heated to 30°–35° to dissolve the gelatin. Smear a thin film of the adhesive so obtained onto the slide, allow to dry, rinse with a 4% solution of formaldehyde TS, and add a small amount of water. Plate the cut sections upside down on the slide, so that they float on water, and flood with a 4% solution of formaldehyde TS. The sections will immediately spread out and wrinkles will disappear.

Place the slide on a warming platform, maintained at 42°, to relax the sections. Pipet, and blot the excess water and formaldehyde solution. Dry overnight in an oven at 42° to ensure adherence of the tissue section to the slide.

**Staining**

*Preparation for staining*—Immerse the microscope slide with the affixed tissue twice into xylene, each time for 10–15 min, to remove paraffin. Then immerse the slide into the following sequence of solutions, leaving it in each solution for 5 min and taking care not to dislodge the tissue: a mixture of dehydrated alcohol and xylene (1:1), dehydrated alcohol, alcohol, and a 70% alcohol solution. The tissue is bleached prior to staining if it is opaque because of the presence of tannins or other ergastic materials. To bleach, dip the slide into a 1% potassium permanganate solution for 1 min, rinse with water, dip into a 5% oxalic acid solution for 1 min, and rinse thoroughly with water. The material is ready for staining. One of the following two staining procedures is recommended for most botanical identification work. The first staining procedure uses safranin O counterstained with fast green. An alternative staining procedure uses safranin O counterstained with orange G.

*Safranin O–fast green staining*

SAFRANIN O STAINING SOLUTION: Prepare a mixture of methoxyethanol, dehydrated alcohol, water, and formaldehyde solution (50:25:25:2). Add a sufficient quantity of sodium acetate to obtain a solution containing 1% of sodium acetate, and mix. Add a sufficient quantity of safranin O to obtain a solution containing 1% of safranin O, and mix.

FAST GREEN STAINING SOLUTION: Prepare a mixture of methoxyethanol, dehydrated alcohol, and methyl salicylate (1:1:1) containing 0.05% of fast green FCF.

PROCEDURE: Once the tissue has been rehydrated to 70% alcohol as described in *Preparation for staining*, immerse for 2–24 h, depending on the tissue, in *Safranin O staining solution*. Remove excess stain by immersing the slide in water several times. Transfer the slide to an alcohol solution containing 0.5% of picric acid for 2–10 s to further remove excess stain from the section and to assist in differentiation of the tissue structures. To stop the action of the picric acid, transfer the slide for 10 s to 1 min to an alcohol solution containing 4 drops of ammonium hydroxide in each 100 mL of alcohol. Transfer the slide to dehydrated alcohol for 10 s. Visually inspect the stained tissue under a microscope to see if further destaining with picric acid is necessary. Counterstain for 10–15 s in *Fast green staining solution*. Transfer slide through two changes of a mixture of methyl salicylate, dehydrated alcohol, and xylene (2:1:1), each change lasting for 5–10 s. Then transfer the slide to a mixture of xylene and dehydrated alcohol (95:5) for 1 min. Transfer through two changes of xylene. Store in xylene until ready to mount the coverslip. Chromosomes, nuclei, and lignified, cutinized, or suberized cell walls will be stained red. Cytoplasm and cellulosic cell walls will be stained green to blue, depending on the pH of the tissue.

*Safranin O–orange G staining*

SAFRANIN O STAINING SOLUTION: Prepare a 0.004% solution of safranin O.

ORANGE G STAINING SOLUTION: Dissolve 2 g of orange G, 5 g of tannic acid, and 4 drops of hydrochloric acid in water, and dilute with water to 100 mL.

PROCEDURE: Once the tissue has been rehydrated to 70% alcohol as described in *Preparation for staining*, sequentially transfer the slide through the series of solutions in *Table 5*.

**Table 5**

| Solution | Time |
|---|---|
| 35% alcohol | 5 min |
| A filtered 2% zinc chloride solution | 1 min |
| Water | 5 s |
| *Safranin O staining solution* | 5 min |
| Water | 5 s |
| *Orange G staining solution* | 1 min |
| Water | 5 s |
| A filtered 5% tannic acid solution | 5 min |
| Water | 3 s |
| A 1% ferric ammonium sulfate solution | 2 min |
| Water | 15 s |
| 45% alcohol | 10 s |
| 90% alcohol | 10 s |
| Dehydrated alcohol | 10 s |
| A mixture of dehydrated alcohol and xylene (1:1) | 1–2 min |

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Finally, store in xylene until ready to mount the coverslip. Cellulosic cell walls will stain blue-black, nuclei will stain yellow, starch grains will stain black, and lignified cell walls will stain red.

**Mounting the coverslip:** The mounting of a coverslip over the tissue completes the preparation of the slide. Canada balsam, diluted with a small portion of xylene, can be used as an adhesive. Other mountants are also commercially available. Upon drying of the mountant, the slide can then be examined under a microscope. The entire process of making permanent microscope slides can be expected to take 5 or more days.

**Scanning electron microscopy:** Botanicals in commerce are often encountered in the form of powder or in pieces, thus making authentication by the routine method of cross-sectioning of the article difficult and often impossible. Structures such as xylem vessels and trachids may be broken into smaller bits, making detection of pitting and lignifications on the walls difficult if not impossible using an optical microscope. Structures that are resistant to these processes are most useful in identification. Scanning electron microscopy (SEM) is useful for characterizing the size and morphology of microscopic specimens. The more detailed differential characteristics in the structure of trichomes and peculiar elements in the epidermis, along with superficial granular material containing specific compounds, can be observed and identified with SEM, which assists in the identification of particular species. SEM has been used extensively to investigate surface topology of a wide variety of plant materials. It can play a vital role in authentication of an entire botanical, those in powder form, distinguishing between closely related species, and can be used to examine a mixture of powders.

Introduction and general information about SEM as applied to pharmacopeial articles can be found in general information chapter *Scanning Electron Microscopy* ⟨1181⟩.

SEM produces a higher resolution compared to that possible using an optical microscope, and the images obtained are three-dimensional. SEM has the advantage of providing images with a large depth of field, which allows a substantial thickness of the sample to be in focus at one time. It allows the analysis of specimens as large as 50 mm, making it possible to produce detailed topographical electron micrographs of an object clearly visible to the naked eye. The maximum resolution for SEM (minimum distance by which the two objects can be separated and observed as distinct objects) is 10–20 nm compared to 200–300 nm for optical microscopy. Typical SEM magnification ranges from ×10 to ×300,000. Commercial SEM instruments also are available with magnifications as low as ×5 and as high as ×2,000,000. In comparison, typical modern optical microscopes have a magnification range of ×10 to ×2000. At low magnification, images obtained with SEM provide more information than those of optical microscopy. SEM can produce images for which contrast is based on compositional variations of specimens.

***Change to read:***

## CHEMICAL IDENTIFICATION

To help ensure the authenticity of the article, chemical identification is performed in conjunction with the botanical identification outlined previously. Chemical identification typically employs chromatographic procedures to detect the presence of marker compounds specified in the individual monograph. Spectroscopic or chromatographic profiles can be used to achieve chemical identification by fingerprint comparison against that of a reference sample or standard. Examples of spectroscopic methods include ultraviolet (UV), infrared (IR), and Fourier transformed IR (see ▲*Spectroscopic Identification Tests* ⟨197⟩▲ (CN 1-May-2020)). Examples of chromatographic methods include high-pressure liquid chromatography (HPLC), thin-layer chromatography (TLC), two-dimensional-TLC, and gas chromatography (GC) (see *Chromatography* ⟨621⟩). Analytical methods used for fingerprinting should be capable of detecting as many chemical constituents as possible. Multiple fingerprints, using a combination of analytical methods with different separation principles and test conditions, may be useful. In addition to the spectroscopic chromatographic methods, qualitative wet-chemistry methods may also be specified in the individual monograph.

### Chemotaxonomy

Chemotaxonomy is the classification of the plants based on their chemical constituents and it may be useful in botanical articles identification. Metabolic compounds found within plant tissues can be divided into two broad categories based on their functions. The first category comprises primary metabolites—metabolites involved in the physiological plant processes that are absolutely necessary for life and ubiquitous throughout the plant kingdom. These processes include photosynthesis; respiration; and nucleic acid, protein, carbohydrate, and lipid metabolism. The second category comprises secondary metabolites—compounds that are thought not to be absolutely necessary for plant processes, although they may have important functions in the plant's interactions with other organisms, such as allelopathic interactions; in chemical defense against herbivores and plant pathogens; and in signaling to attract pollinating and seed-dispersing animals. Many secondary metabolites are known to have pharmacological activity. They are also the basis for the chemotaxonomy of plants. Secondary metabolites fall into several different chemical classes such as nonprotein amino acids, flavonoids, xanthones, coumarins, polyacetylenes, cyclic polyketides, monoterpenes, sesquiterpenes, iridoids, triterpenes, sterols, nitrogen-containing terpenes, and alkaloids. These chemical classes are not ubiquitous throughout the plant kingdom, but tend to be specific to certain botanical classes, orders, and families. Moreover, many chemical subclasses and individual secondary compounds are specific to certain subfamilies, genera, or species. It is these chemical subclasses and individual compounds that can be used as marker compounds to aid in the proper identification of plant material.

### Active Principles and Marker Compounds

For chemical identification of botanical articles, extracts are prepared. Such extracts are usually complex mixtures of several chemical constituents. For a large majority of botanical extracts it is not known with certainty which of the various components is responsible for the reported pharmacological effect. It is generally believed that several constituents act synergistically to provide the reported effect. For articles for which compendial monographs are provided, certain chemical constituents of the

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article are chosen and quantitative test procedures for determining their content are provided. The choice of such constituents, known generally as marker compounds, is based on certain considerations. Currently, the following types of marker compounds are specified in compendial monographs and may be identified in raw materials:

ACTIVE PRINCIPLES: These are constituents that have proven clinical activity. A minimum content or range for the active principles is usually specified in the individual monograph. A quantitative determination of active principles during stability studies of botanical dosage forms provides necessary information for arriving at suitable expiration dates.

ACTIVE MARKERS: These are constituents that have known pharmacological activity contributing in some extent to efficacy. However, the clinical efficacy for these constituents may not be proven. A minimum content or range for active markers is usually specified in individual monographs. A quantitative determination of active markers during stability studies of botanical dosage forms provides necessary information for arriving at suitable expiration dates.

ANALYTICAL MARKERS: Where neither defined active principles nor active markers are known, other constituents of the botanical extract amenable to quantitative determination are chosen. These markers aid in the positive identification of the article under test. In addition, maintaining a minimum content or a specified range of the analytical markers helps to achieve standardization of the plant extract and to arrive at a suitable expiration date during stability studies.

NEGATIVE MARKERS: These are constituents that may have allergenic or toxic properties, rendering their presence in the botanical extract undesirable. For example, ginkgolic acids from ginkgo belong to this category. A stringent limit for these negative markers may be specified in individual monographs.

### Use of USP Reference Articles

Reference articles are used to assist in the identification of marker compounds within the test article. Reference articles are either USP Authenticated Reference Materials or USP Reference Standards (see *USP Reference Standards* ⟨11⟩), whichever is specified in the individual monograph. USP Reference Standards used to identify marker compounds in the test articles may be a single purified chemical entity, a mixture of purified chemical entities, or a standardized extract prepared from the authenticated plant article. USP Reference Standards may also be used to quantitate marker compounds, as specified in the individual monograph.

A pulverized test article undergoes a specified extraction procedure (see *Methods of Extraction* in *Botanical Extracts* ⟨565⟩) and is prepared for chromatographic or wet-chemistry analysis. If a USP Authenticated Reference Material is available, then it undergoes the same extraction procedure as the test article. The test preparation and reference articles then undergo the same chromatographic or wet-chemistry procedure specified in the individual monograph. The response of the test preparation is compared to the response of reference articles to determine the presence of the marker compounds in the test article.

## DNA-BASED METHODS FOR AUTHENTICATION OF ARTICLES OF BOTANICAL ORIGIN

Because morphological identification often is not possible when the original plant material consists of dried, cut and shifted, or processed plant parts or when the material consists only of a whole, single plant part containing no taxonomic characters, additional identification methods, such as DNA-based identification, often are required for these sample types. DNA-based methods have been shown to be efficient in distinguishing genuine plant materials from adulterants in complex botanical matrixes and can complement traditional botanical identification methods that rely on morphological features or chemistry. In addition, DNA-based methods often are more reliable than traditional methods, especially when applied to single-organ specimens that lack diagnostic taxonomic characters, to powdered materials in which the distinguishing characteristics are no longer visible, or when it is difficult to distinguish among closely related or morphologically similar species.

### DNA Barcoding

DNA barcoding is a particular type of DNA sequence-based identification method that uses short sequences of specific nuclear or plastid DNA loci for identification of plant species. The assays rely on comparison of nucleotide sequences from a specific stretch of DNA (DNA sequences or DNA barcode) to perform DNA sequence-based identification. Further, DNA-based methods, such as next-generation sequencing (NGS) technologies, are able to identify multiple species in a mixture, including expected and unexpected species.

### Botanical Identification Using DNA (Sanger) Sequencing

The process for botanical identification using DNA (Sanger) sequencing includes marker selection, DNA extraction, polymerase chain reaction (PCR) primers and amplification, DNA sequencing, and comparison with reference materials, as described in the following sections. See *Nucleic Acid-Based Techniques—Extraction, Detection, and Sequencing* ⟨1126⟩, *Nucleic Acid-Based Techniques—Amplification* ⟨1127⟩, and *Nucleic Acid-Based Techniques—Genotyping* ⟨1129⟩ for additional information.

#### MARKER SELECTION

The chosen sequence must be sufficiently specific to capture any potential primary and adulterant species in the sample but also sufficiently universal to avoid false-negative reactions for closely related species. For example, a species-specific primer is not appropriate for most identification procedures because adulterants cannot be detected and amplification failure can result because of either the absence of the species or degraded DNA. In many cases, a single marker may be sufficient for identification, but multiple markers from different parts of the genome (e.g., plastid or nuclear material) ensure that hybrids can be detected.

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Typically, regions used for DNA sequence identification range from 100–1500 base pairs in length. Smaller DNA fragments may be less susceptible to DNA degradation.

## DNA EXTRACTION

Before amplification of the desired marker can be performed, the total genomic DNA must be extracted. The suitability of a procedure for genomic extraction depends on the starting material and the purity of the DNA required for downstream applications. The principal procedures are described as follows, and several commercial kits are available to accommodate different sample types and applications.

Total genomic DNA must be extracted from ground plant material. Plant materials can be homogenized manually using a mortar and pestle, mechanical grinder, or other apparatus depending on the nature of the material. Total genomic DNA extraction and purification can be challenging because of the abundance of secondary metabolites (polysaccharides, tannins, essential oils, phenolics, alkaloids, and waxes) in many medicinal plant species. Some of the secondary metabolites may coprecipitate with DNA during extraction and may inhibit further enzymatic reactions, including restriction digestion and PCR. In particular, large amounts of complex polysaccharides can make extraction of usable DNA impossible, rendering the aqueous portion of the extract too viscous to allow efficient separation of DNA from the contaminating polysaccharides. This type of contamination can lead to poor DNA yield and can prevent access by modifying enzymes.

Numerous commonly used DNA extraction methods are appropriate for a wide range of fresh and dried plant materials, including cetyl trimethylammonium bromide, silica-based methods, and a variety of commercially available kits that use silica columns or glass-coated magnetic beads. Although many of these methods work well on both fresh and dried materials and on any plant part, those that are degraded or contain significant levels of secondary compounds or other PCR inhibitors may require minor adjustments to standard extraction protocols.

## PCR PRIMERS AND AMPLIFICATION

Typically, PCR primers are between 18 and 30 bases in length and amplify a region between 100 and 1500 base pairs in length. As noted, PCR primers may be universal, meaning they are capable of amplifying all potential organisms that are present in a test sample (including fungi, plants, and animals or an important and predicable subset), or they are taxon specific, meaning they have been designed to amplify only organisms in a targeted set (i.e., family, genus, species, or subspecies). A number of nuclear, mitochondrial, and plastid gene regions are used for universal amplification, including nrITS, nrITS1, nrITS2, *matK*, *rbcL*, *psbA–trnH* intergenic spacer, *cox3*, *COI* (also known as *cox1*), external transcribed spacer, 18S, 5S, *trnL–trnF* intergenic spacer, and *trnL* intron. Taxon-specific primers may be designed on the basis of proteins found in specific plant groups (e.g., the soy lectin gene found in soy) or by obtaining sequences of any variable DNA region for the target taxon and designing primers that specifically bind only to sequences from the taxon of interest.

## DNA SEQUENCING

Most commonly, DNA sequencing is conducted using the Sanger protocol that has been modified to use fluorescent dye terminators on a capillary electrophoresis apparatus, although a number of emerging sequencing technologies [e.g., next-generation sequencing (NGS)] are now taking root. Once fluorescent dye has been incorporated into the amplified DNA, the bases are identified by their emission of light at different wavelengths. The resulting data are a chromatogram that can be visualized and analyzed by several sequence-analysis computer programs.

## COMPARISON WITH REFERENCE MATERIALS

DNA sequences of test articles are compared to sequences obtained from multiple reference materials in an aligned matrix (commonly referred to as an alignment), which allows the sequences to be visually inspected to identify diagnostic nucleotide positions. Although numerous computer programs are able to automate comparisons between sequences from test articles and reference sequences, performance varies widely. Investigators should always manually check the results suggested by computer programs to confirm identity. Positive identifications are not possible when the sequences from test articles fall outside the range of known variation represented in the reference sequences. If a sufficiently large number of reference materials have been used to develop the assay, most test materials should be identified without ambiguity based on DNA sequence data.

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# ⟨565⟩ BOTANICAL EXTRACTS

In the extraction practice for articles of botanical origin, the constituents of interest are completely or partially separated from other components with the aid of water, alcohol, alcohol-water mixtures, or other suitable solvents. This extraction process involves the removal of the desired constituents from the plant matter with suitable menstrua, the evaporation of all or nearly all of the solvent, and the adjustment of the residual fluids, masses, or powders to the prescribed standards. Suitable inert substances may be added as carriers or diluents to improve physical characteristics. Suitable antimicrobials and other preservatives may be added to preserve the integrity. Extracts may be subjected to processes that increase the content of characterized constituents, decrease the content of unwanted constituents, or both. Extracts with no added inert substances and no processing beyond the extraction are called native extracts. In some preparations, the plant matter may be pretreated by inactivation of enzymes and microbial contaminants, grinding, defatting, or a similar procedure.

Extracts may be defined as preparations with liquid, solid, or semisolid consistency. The products obtained by extraction are fluidextracts, powdered extracts, semisolid extracts, and tinctures.

## METHODS OF EXTRACTION

### Percolation

In the manufacture of extracts, percolation is a commonly used method. The crude material being extracted is reduced to pieces of suitable size, if necessary, then mixed thoroughly with a portion of the specified solvent, and allowed to stand for about 15 minutes. The mixture is transferred to a percolator, sufficient amount of the specified solvent is added to cover the entire solid mass, and the mixture is allowed to percolate slowly (at a rate of not more than 1 mL per minute for 1000 g of material), the matter to be extracted being always covered with a layer of solvent. The residue may be pressed, and the obtained fluid is combined with the percolate. The entire percolates are concentrated, generally by distillation under reduced pressure, so as to subject the constituents of interest in the article under extraction to as little heat as possible.

### Maceration

Unless otherwise specified, the crude material being extracted is reduced to pieces of suitable size, mixed thoroughly with the specified extracting solvent, and allowed to stand at room temperature in a closed container for an appropriate time, with frequent agitation until soluble matter is dissolved. The mixture is filtered, the insoluble material is washed with the same solvent used for maceration, and the filtrates are combined and concentrated, usually under reduced pressure, to the desired consistency.

***Change to read:***

## PREPARATIONS

### Fluidextracts

FLUIDEXTRACTS, also known as liquid extracts, are preparations of plant matter, containing alcohol as a solvent or as a preservative, or both, and are so made that each mL contains the extracted constituents of 1 g of the crude material that it represents, unless otherwise specified in the individual monograph. They may be prepared from suitable extracts and may contain suitable antimicrobial or other preservatives.

Pharmacopeial fluidextracts are made by percolation, often following a period of maceration. The required solvent is specified in the individual monograph. The common manufacturing procedure includes concentration of the more diluted portion of percolate by evaporation or distillation under vacuum at temperatures below 60°. The time of maceration and the rate of flow during percolation may be varied to adjust for the quantity and nature of the crude material under extraction, provided that the composition of the extracted constituents of interest is not adversely affected.

The rate of flow of the percolate can be slow, moderate, or rapid. With reference to the extraction of 1000 g of the starting material, at a slow rate, not more than 1 mL of percolate is produced per minute; at a moderate rate, between 1 and 3 mL per minute is produced; and at a rapid rate, between 3 and 5 mL per minute is produced. A fluidextract that tends to deposit sediment may be aged and filtered, or the clear portion may be decanted, provided that the resulting clarified liquid conforms to the Pharmacopeial standards.

### Powdered Extracts

POWDERED EXTRACTS are solid preparations having a powdery consistency obtained by evaporation of the solvent used for extraction. They may contain suitable added substances such as excipients, stabilizers, and preservatives. Standardized powdered extracts are adjusted to the defined content of constituents, using suitable inert materials or a powdered extract of the plant matter used for preparation. Where applicable, a limit for the solvent used for extraction is specified in the individual monograph.

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## Semisolid Extracts

SEMISOLID EXTRACTS, also known as soft extracts or pillular extracts, are preparations having consistencies between those of fluidextracts and those of powdered extracts, and are obtained by partial evaporation of the solvent, water, alcohol, or hydroalcoholic mixtures being used as extracting solvents. They may contain suitable antimicrobial or other preservatives. A semisolid extract and a powdered extract obtained from the same material are interchangeable as drugs or as supplements, but each has its own advantages.

## General Pharmacopeial Requirements

Unless otherwise specified in the individual monographs, Pharmacopeial requirements for the fluidextracts, powdered extracts, and semisolid extracts are as follows.

PACKAGING AND STORAGE—Store in tight, light-resistant containers. [NOTE—See *Packaging and Storage Requirements* ⟨659⟩*, General Definitions*.]

LABELING—Label it to indicate the name of the plant part used; the names of solvents, other than the hydroalcoholic solvents, used in preparation; the content, in percentage, of active principles or marker compounds identified in the individual monograph; and the name and concentration of any added antimicrobial or other preservative. Where active principles are unknown, the ratio of starting material to final product is stated. For semisolid extracts and powdered extracts, the identity and quantity of any added excipient is also indicated. In such cases the percentage of native extract may also be stated.

RESIDUE ON EVAPORATION—Transfer promptly about 2 mL, accurately measured, of Fluidextract, about 0.5 g of Powdered Extract, or about 2 g of Semisolid Extract to a suitable tared, round-bottom flask. Evaporate to dryness on a water bath, and dry the residue at 100° to 105° for 3 hours. Allow to cool in a desiccator over phosphorus pentoxide, and determine the weight of the residue obtained: not less than 95% of Powdered Extract specimen remains as residue; or not less than 70% of Semisolid Extract specimen remains as residue. [NOTE—Limits for Fluidextracts are specified in the individual monographs.]

RESIDUAL SOLVENTS—If prepared with solvents other than alcohol, water, or alcohol-water mixtures, it meets the requirements for *Residual Solvents* ⟨467⟩. [NOTE—See ICH document *Impurities: Residual Solvents* for related information.]

PESTICIDE RESIDUES—Botanical extracts, tinctures, or other pharmaceutical forms might contain pesticide residues at either enriched or reduced levels compared to their native form as plant materials. Unless otherwise indicated in the monograph, the limits for pesticides in extracts of botanical articles are calculated by the following formula:

$$\text{If } E \leq 10\text{: Limit (mg/kg)} = L \times E$$

$$\text{If } E > 10\text{: Limit (mg/kg)} = AM/100B$$

where $L$ is the limit in the original article as listed in ▲*Table 5*▲ (ERR 1-Jun-2022) (see *Pesticide Residue Analysis* under *Articles of Botanical Origin* ⟨561⟩), or EPA tolerance or the FDA action level; $E$ is the plant to extract ratio (i.e. the ratio between the quantity of botanical article used in the manufacture of the extract and the quantity of the extract obtained); $A$ is the acceptable daily intake (ADI), as published by FAO-WHO, in mg/kg of body weight; $M$ is body weight, in kg (60 kg); and $B$ is the daily dose of the extract, in kg.

[NOTE—The higher pesticide limits for extracts of botanical ingredients may be justified if the suggested intake or dose of the extract is reduced by a factor which is higher than $E$.]

A total or partial exemption from the test may be granted when the complete history (nature and quantity of the pesticides used, date of each treatment during cultivation and after harvest) of the treatment of the batch is known and can be checked precisely according to good agricultural and collection practice (GACP).

ALCOHOL DETERMINATION, *Method II* ⟨611⟩ (IF PRESENT): Between 90% and 110% of the labeled amount of $C_2H_5OH$ is found in Fluidextract and Semisolid Extract.

## Tinctures

TINCTURES are liquid preparations usually prepared by extracting plant materials with alcohol or hydroalcoholic mixtures. Traditionally, tinctures of potent articles of botanical origin represent the activity of 10 g of the drug in each 100 mL of tincture, the strength being adjusted following the test for content of active principles or marker compounds. Most other plant tinctures represent 20 g of the respective plant material in each 100 mL of tincture.

Different tinctures are not always diluted to obtain the same ratio of starting plant material to final tincture. This ratio will depend on the requirements prescribed in the specific tests for content of active principles or marker compounds included in the individual monographs. As tinctures are being prepared, they are assayed in accordance with these content tests. Using the values obtained from such assays, the final concentration of a tincture is adjusted by adding more solvent or by evaporating part of the solvent.

Unless otherwise specified, tinctures are usually prepared from coarse powder or fine cuttings of plant materials either by a percolation process or a maceration process.

### PERCOLATION PROCESS

Carefully mix the ground mixture of ingredients with a sufficient quantity of the prescribed extracting solvent to render it evenly and distinctly damp, allow it to stand for 15 minutes, transfer it to a suitable percolator, and pack the mass firmly. Pour on enough of the specified extracting solvent to saturate the drug, and cover the top of the percolator. When the liquid is about to drip from the percolator, close the lower orifice, and allow the drug to macerate for 24 hours or for the time specified in the

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monograph. If the test for content of active principles or marker compounds is not required in the individual monograph, allow the percolation to proceed slowly or at the specified rate (for definitions of flow rates, see under *Fluidextracts*), gradually adding sufficient quantity of extracting solvent to produce 1000 mL of tincture, and mix. If a test for content of active principles or marker compounds is required, collect only 950 mL of percolate, mix, and test a portion of it as directed in the individual monograph. Dilute the remainder of the percolate with as much of the prescribed extracting solvent as calculation from the content test indicates is necessary to produce a tincture that conforms to the requirements, and mix.

## MACERATION PROCESS

Macerate the drug with 750 mL of the prescribed extracting solvent in a closed container, and put in a warm place. Agitate it frequently during 3 days or until the soluble matter is dissolved. Transfer the mixture to a filter. When most of the liquid has drained, wash the residue on the filter with a sufficient quantity of the prescribed extracting solvent, combining the filtrates, to produce 1000 mL of tincture, and mix.

## GENERAL PHARMACOPEIAL REQUIREMENTS

Unless otherwise specified in the individual monographs, Pharmacopeial requirements for the tinctures are as follows.
**Packaging and Storage—**Store in tight, light-resistant containers, and avoid exposure to direct sunlight and excessive heat. [NOTE—See *Packaging and Storage Requirements* ⟨659⟩*, General Definitions*.]
**Labeling—**Label it to indicate the name of the plant part used for preparation; the name of the solvent or solvent mixture used for extraction; and the content of the constituents of interest and the ratio of starting material to final product.

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# ⟨571⟩ VITAMIN A ASSAY

## ASSAY

### Chemical Methods

- **PROCEDURE 1**

The following procedure is provided for the determination of vitamin A in dietary ingredients or pharmaceutical ingredients. It conforms to the procedure that was adopted in 1956 for international use by the International Union of Pure and Applied Chemistry.

Complete the assay promptly, and exercise care throughout the procedure to keep to a minimum the exposure to actinic light and to atmospheric oxygen and other oxidizing agents, preferably by the use of low-actinic glassware and an atmosphere of an inert gas.

For the test articles that contain tocopherol, an appropriate chromatographic method should be used.

**Sample solution:** Accurately weigh, count, or measure a portion of the test specimen expected to contain the equivalent of NLT 0.15 mg of retinol but containing NMT 1 g of fat. If in the form of capsules, tablets, or other solid, so that it cannot be saponified efficiently by the ensuing instructions, reflux the portion taken in 10 mL of water on a steam bath for about 10 min, crush the remaining solid with a blunt glass rod, and warm for about 5 min longer.

Transfer to a suitable borosilicate glass flask, and add 30 mL of alcohol, followed by 3 mL of potassium hydroxide solution (9 in 10). Reflux in an all-borosilicate glass apparatus for 30 min. Cool the solution, add 30 mL of water, and transfer to a conical separator. Add 4 g of finely powdered sodium sulfate decahydrate. Extract by shaking with one 150-mL portion of ether for 2 min, and then, if an emulsion forms, with three 25-mL portions of ether. Combine the ether extracts, if necessary, and wash by swirling gently with 50 mL of water. Repeat the washing more vigorously with three additional 50-mL portions of water. Transfer the washed ether extract to a 250-mL volumetric flask, add ether to volume, and mix.

Evaporate a 25.0-mL portion of the ether extract to about 5 mL. *Without applying heat and with the aid of a stream of inert gas or vacuum,* continue the evaporation to about 3 mL. Dissolve the residue in sufficient isopropyl alcohol to give an expected concentration of the equivalent of 3 µg–5 µg of vitamin A per mL or to give an absorbance in the range 0.5–0.8 at 325 nm.

**Instrumental conditions**

(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)

**Mode:** UV

**Analytical wavelengths:** 310, 325, and 334 nm

**Cell:** 1 cm

**Blank:** Isopropyl alcohol

**Analysis**

**Sample:** *Sample solution*

Determine the absorbances of the *Sample solution* at 310, 325, and 334 nm. Calculate the vitamin A, as retinol ($C_{20}H_{30}O$) content, in mg, in the portion of sample taken, using one of the following formulas:

$$\text{Result} = (0.549 \times A_{325})/(L \times C)$$

or

$$\text{Result} = (0.549 \times [A_{325}])/(L \times C)$$

$L$ = length of the absorption cell (cm)

$C$ = concentration in the final isopropyl alcohol solution of test specimen (g/100 mL) or capsules or tablets (units/100 mL)

$[A_{325}]$ = *corrected* absorbance at 325 nm, calculated:

$$\text{Result} = (6.815 \times A_{325}) - (2.555 \times A_{310}) - (4.260 \times A_{334})$$

Each mg of vitamin A, as retinol ($C_{20}H_{30}O$), represents 3333 USP Units of vitamin A.

Use the first formula when $A_{325}$, the observed absorbance at 325 nm, is between $[A_{325}]/1.030$ and $[A_{325}]/0.970$. Use the second formula when $[A_{325}]$ has a value less than $A_{325}/1.030$.

[NOTE—The range of the limits of error for this analytical procedure, indicating the extent of discrepancy to be expected in the results of different laboratories at $P = 0.05$, is approximately ±8%.]

- **PROCEDURE 2**

This procedure is used for dietary ingredients or pharmaceutical ingredients in the form of pure retinyl esters or prepared from pure retinyl esters into a vehicle excipient.

**Sample solution:** Dissolve 25–100 mg, accurately weighed, in 5 mL of pentane, and dilute with isopropyl alcohol to give an expected concentration of the equivalent of 3–4.5 µg/mL of retinol.

**Instrumental conditions**

(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)

**Mode:** UV

**Analytical wavelength:** 326 nm

**Cell:** 1 cm

**Blank:** Isopropyl alcohol

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**Analysis**

**Sample:** *Sample solution*

Calculate the vitamin A, as retinol ($C_{20}H_{30}O$) content, in mg, in the portion of sample taken. Each mg of vitamin A, as retinol ($C_{20}H_{30}O$), represents 3333 USP Units of vitamin A.:

$$\text{Result} = (0.570 \times A_{326})/(L \times C)$$

$A_{326}$ = absorbance at 326 nm
$L$ = length of the absorption cell (cm)
$C$ = concentration of the *Sample solution* (g/100 mL)

**Chromatographic Methods**

The following liquid chromatographic procedures are provided for the determination of vitamin A as an active pharmaceutical ingredient, a dietary supplement ingredient, or a component in the dietary supplements or pharmaceutical dosage forms.

Throughout these procedures, protect solutions containing and derived from the test specimen and the Reference Standards from the atmosphere and light, preferably by the use of a blanket of inert gas and low-actinic glassware.

Where an ester form of vitamin A (retinyl acetate or retinyl palmitate) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard.

• **PROCEDURE 1**

This is a neutral procedure that involves either simply dissolving the sample directly into hexane and injecting into the liquid chromatograph or sample extraction by first dissolving the sample in dimethyl sulfoxide, followed by a liquid–liquid extraction of the vitamin A with hexane. Although its chromatographic system can separate the 13-*cis* and all-*trans*-isomers of vitamin A, only the all-*trans*-isomer peak is used for the quantitation of vitamin A. The procedure can be used to determine vitamin A in raw material, Oil-Soluble Vitamins Tablets, Oil-Soluble Vitamins Capsules, Oil- and Water-Soluble Vitamins Tablets, Oil- and Water-Soluble Vitamins Capsules, Oil- and Water-Soluble Vitamins with Minerals Tablets, and Oil- and Water-Soluble Vitamins with Minerals Capsules.

Unless specified in the individual monographs, the *Standard solutions*, *Sample solutions*, and *System suitability solution* are prepared as follows.

**Mobile phase:** *n*-Hexane

**Standard solution 1:** 15 µg/mL of retinol[1] from USP Retinyl Acetate RS in *n*-hexane

**Standard solution 2:** 15 µg/mL of retinol[2] from USP Retinyl Palmitate RS in *n*-hexane

**System suitability solution:** Mix equal volumes of *Standard solution 1* and *Standard solution 2*.

**Sample solution for raw materials:** Transfer retinyl acetate or retinyl palmitate, accurately weighted, equivalent to 15 mg of retinol, to a 100-mL volumetric flask, dissolve in and dilute with *n*-hexane to volume, and mix. Pipet 5.0 mL of this solution into a 50-mL volumetric flask, dilute with *n*-hexane to volume, and mix.

**Sample solution for tablets:** Finely powder NLT 20 Tablets. Transfer a portion of the powder, not exceeding 7.5 g, equivalent to NLT 1 mg of vitamin A, as retinol ($C_{20}H_{30}O$), to a centrifuge tube having a polytef-lined screw cap. Add about 2 mL of dimethyl sulfoxide and about 3 mL of *n*-hexane per each g of powdered Tablets, and shake for 45 min on a shaker in a water bath maintained at 60°. [NOTE—Set up the shaker to ensure that the contents of the container are mixed vigorously and thoroughly.] Centrifuge at 3000 rpm for 10 min, and transfer the hexane layer by means of a pipet to a volumetric flask. Add 3 mL of *n*-hexane per each g of powdered Tablets to the dimethyl sulfoxide layer, shake thoroughly for 5 min, and transfer the hexane layer by means of a pipet to the same volumetric flask. Repeat this extraction with three additional portions of *n*-hexane. Dilute the extracts in the volumetric flask with *n*-hexane to volume. Dilute a volume of this solution with *n*-hexane to obtain a solution having a nominal concentration of 15 µg/mL of vitamin A, as retinol ($C_{20}H_{30}O$). [NOTE—Dilution may not be necessary.]

**Sample solution for capsules:** Transfer the contents of NLT 20 Capsules to a suitable container, mix, and weigh. Transfer a portion of the mixture, not exceeding 7.5 g equivalent to NLT 1 mg of vitamin A, as retinol ($C_{20}H_{30}O$), to a centrifuge tube having a polytef-lined screw cap. [NOTE—For hard gelatin Capsules, remove, as completely as possible, the contents of NLT 20 Capsules by cutting open the Capsule shells, transferring the shells and their contents to a suitable container, and triturating to a homogeneous mass. Transfer a portion of the mass, equivalent to NLT 1 mg of vitamin A, as retinol ($C_{20}H_{30}O$), to a centrifuge tube having a polytef-lined screw cap.] Add about 2 mL of dimethyl sulfoxide and about 3 mL of *n*-hexane per each g of Capsule contents, and shake for 45 min on a shaker in a water bath maintained at 60°. [NOTE—Set up the shaker to ensure that the contents of the container are mixed vigorously and thoroughly.] Centrifuge at 3000 rpm for 10 min, and transfer the hexane layer by means of a pipet to a volumetric flask. Add 3 mL of *n*-hexane per each g of Capsule contents to the dimethyl sulfoxide layer, shake thoroughly for 5 min, and transfer the hexane layer by means of a pipet to the same volumetric flask. Repeat this extraction with three additional portions of *n*-hexane. Dilute the extracts in the volumetric flask with *n*-hexane to volume. Dilute a volume of this solution with *n*-hexane to obtain a solution with a concentration of 15 µg/mL of vitamin A, as retinol ($C_{20}H_{30}O$). [NOTE—Dilution may not be necessary.]

**Chromatographic system**

(See *Chromatography* ⟨621⟩, *System Suitability*.)

**Mode:** LC

**Detector:** UV 325 nm

**Column:** 4.6-mm × 15-cm; 3-µm packing L8

**Flow rate:** 1 mL/min

[1] Use the value of 0.872 to convert retinyl acetate to its retinol equivalent.
[2] Use the value of 0.546 to convert retinyl palmitate to its retinol equivalent.

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**Injection volume:** 40 µL

**System suitability**

**Samples:** *System suitability solution* and *Standard solution 1* or *Standard solution 2*

**Suitability requirements**

**Resolution:** NLT 10 between all-*trans*-retinyl acetate and all-*trans*-retinyl palmitate, *System suitability solution*

**Relative standard deviation:** NMT 3.0%, *Standard solution 1* or *Standard solution 2*

**Analysis**

**Samples:** *Standard solution 1* or *Standard solution 2* and appropriate *Sample solution*

Calculate the percentage of the labeled amount of vitamin A, as retinol ($C_{20}H_{30}O$), in the portion of sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

$r_U$ = peak response of all-*trans* retinyl ester from the appropriate *Sample solution*
$r_S$ = peak response of all-*trans* retinyl ester from *Standard solution 1* or *Standard solution 2*
$C_S$ = concentration of retinol ($C_{20}H_{30}O$) in *Standard solution 1* or *Standard solution 2* (µg/mL)
$C_U$ = concentration of vitamin A, as retinol ($C_{20}H_{30}O$), in the *Sample solution* (µg/mL)

- **PROCEDURE 2**

This procedure involves the treatment of sample with methanolic sulfuric acid, followed by extraction with 2,2,4-trimethylpentane. Sample preparation can be used for the formulation containing vitamins A, D, and E. Application includes Oil-Soluble Vitamins Tablets, Oil-Soluble Vitamins Capsules, Oil- and Water-Soluble Vitamins Tablets, Oil- and Water-Soluble Vitamins Capsules, Oil- and Water-Soluble Vitamins with Minerals Tablets, and Oil- and Water-Soluble Vitamins with Minerals Capsules.

Unless specified in the individual monographs, the *Standard solutions*, *Sample solutions*, *System suitability solution,* and reagent solutions are prepared as follows.

**Mobile phase:** *n*-Hexane and ethyl acetate (99.7: 0.3)

**3 N methanolic sulfuric acid solution:** Cautiously add 9 mL of sulfuric acid to 80 mL of methanol in a 100-mL volumetric flask. Cool, and dilute with methanol to volume.

**Sodium ascorbate–pyrogallol solution:** Transfer 10 g of sodium ascorbate and 5 g of pyrogallol to a 100-mL volumetric flask, and add sufficient water to dissolve. Add 1.7 mL of sulfuric acid, and dilute with water to volume.

**Lecithin solution:** 5 mg/mL of lecithin in 2,2,4-trimethylpentane

**Standard solution 1:** 15 µg/mL of retinol[1] from USP Retinyl Acetate RS in 2,2,4-trimethylpentane

**Standard solution 2:** 15 µg/mL of retinol[2] from USP Retinyl Palmitate RS in 2,2,4-trimethylpentane

**System suitability solution:** Mix equal volumes of *Standard solution 1* and *Standard solution 2*.

**Sample solution for tablets:** [NOTE—This preparation is suitable for the determination of vitamin A, vitamin D, and vitamin E when present in the formulation. The sample amount may be adjusted depending on the presence or absence of the appropriate vitamins.] Finely powder NLT 20 Tablets. Use a portion of the powder nominally equivalent to an amount between 0.4 mg and 2.5 mg of retinol. Add 0.5 g of sodium bicarbonate, 1.5 mL of *Lecithin solution*, and 12.5 mL of 2,2,4-trimethylpentane, and disperse on a vortex mixer. Add 6 mL of *Sodium ascorbate–pyrogallol solution*, shake slowly, and allow the solution to degas. Continue shaking until the evolution of gas has ceased, and then shake for an additional 12 min. Add 6 mL of dimethyl sulfoxide, mix on a vortex mixer to form a suspension, and shake for 12 min. Add 6 mL of *3 N methanolic sulfuric acid solution*, mix on a vortex mixer to form a suspension, and shake for 12 min. Add 12.5 mL of 2,2,4-trimethylpentane, mix on a vortex mixer to form a suspension, and shake for 10 min. Centrifuge for 10 min to break up the emulsion and to clarify the supernatant. If necessary, quantitatively dilute a volume of the supernatant with 2,2,4-trimethylpentane to obtain a concentration close to that of the *Standard solution*.

**Sample solution for capsules:** [NOTE—This preparation is suitable for the determination of vitamin A, vitamin D, and vitamin E when present in the formulation. The sample amount may be adjusted depending on the presence or absence of the appropriate vitamins.] Weigh NLT 20 Capsules in a tared weighing bottle. Using a sharp blade if necessary, carefully open the Capsules, without loss of shell material, and transfer the contents to a 100-mL beaker. Remove any contents adhering to the empty shells by washing with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the net weight of the Capsule contents. Transfer a portion of the Capsule contents, equivalent to 2.5 mg of the labeled amount of vitamin A, as retinol. Add 0.5 g of sodium bicarbonate, 1.5 mL of *Lecithin solution*, and 12.5 mL of 2,2,4-trimethylpentane, and disperse on a vortex mixer. Add 6 mL of *Sodium ascorbate–pyrogallol solution*, shake slowly, and allow the solution to degas. Continue shaking until the evolution of gas has ceased, and then shake for an additional 12 min. Add 6 mL of dimethyl sulfoxide, mix on a vortex mixer to form a suspension, and shake for 12 min. Add 6 mL of *3 N methanolic sulfuric acid solution*, mix on a vortex mixer to form a suspension, and shake for 12 min. Add 12.5 mL of 2,2,4-trimethylpentane, mix on a vortex mixer to form a suspension, and shake for 10 min. Centrifuge for 10 min to break up the emulsion and to clarify the supernatant. If necessary, quantitatively dilute a volume of the supernatant with 2,2,4-trimethylpentane to obtain a concentration close to that of the *Standard solution*.

**Chromatographic system**

(See *Chromatography* ⟨621⟩*, System Suitability*.)

**Mode:** LC

**Detector:** UV 325 nm

**Column:** 4.6-mm × 25-cm; 5-µm packing L24

**Flow rate:** 1.5 mL/min

**Injection volume:** 40 µL

**System suitability**

**Sample:** *System suitability solution*

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**Suitability requirements**

**Resolution:** NLT 8.0 between all-*trans* retinyl acetate and all-*trans* retinyl palmitate

**Relative standard deviation:** NMT 3.0%

**Analysis**

**Samples:** *Standard solution 1* or *Standard solution 2* and *Sample solution*

Calculate the percentage of the labeled amount of vitamin A, as retinol ($C_{20}H_{30}O$), in the portion of sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

$r_U$ = peak response of all-*trans* retinyl ester from the *Sample solution*
$r_S$ = peak response of the all-*trans* retinyl ester from *Standard solution 1* or *Standard solution 2*
$C_S$ = concentration of retinol ($C_{20}H_{30}O$) in *Standard solution 1* or *Standard solution 2* (µg/mL)
$C_U$ = nominal concentration of vitamin A, as retinol ($C_{20}H_{30}O$), in the *Sample solution* (µg/mL).

[NOTE—Use 26.5 mL as the final volume of the *Sample solution* to calculate the nominal concentration.]

- **PROCEDURE 3**

This procedure involves the saponification of both the standard and sample, followed by a liquid–liquid extraction of vitamin A from the sample with a mixture of *n*-hexane and methylene chloride (3:1). The 13-*cis*- and all-*trans*- isomers of vitamin A can be characterized and quantitated. The procedure can be used for Oil-Soluble Vitamins Tablets, Oil-Soluble Vitamins Capsules, Oil- and Water-Soluble Vitamins Tablets, Oil- and Water-Soluble Vitamins Capsules, Oil- and Water-Soluble Vitamins with Minerals Tablets, and Oil- and Water-Soluble Vitamins with Minerals Capsules.

Unless specified in the individual monographs, the *Standard solution*, *Sample solutions*, and reagent solutions are prepared as follows.

**Mobile phase:** *n*-Hexane and isopropyl alcohol (92:8)

**Extraction solvent:** *n*-Hexane and methylene chloride (3:1)

**Potassium hydroxide solution:** 800 mg/mL of potassium hydroxide in water. [NOTE—Cautiously add potassium hydroxide in water. Mix, and cool.]

**Diluent:** 10 mg/mL of pyrogallol in alcohol

**Standard solution:** Dilute USP Retinyl Acetate RS or USP Retinyl Palmitate RS with *Diluent* to obtain a concentration of 8.5 µg/mL of retinol[1,2] ($C_{20}H_{30}O$). Transfer 10.0 mL of this solution to a stoppered 125-mL flask, and add 5 mL of water, 5 mL of *Diluent*, and 3 mL of *Potassium hydroxide solution*. Insert the stopper tightly, shake for 15 min over a water bath maintained at 60 ± 5°, and cool to room temperature. Add 7 mL of water and 25.0 mL of *Extraction solvent*. Insert the stopper tightly, and shake vigorously for 60 s. Rinse the sides of the flask with 60 mL of water, and allow to stand for 10 min until the layers separate. Withdraw a portion of the organic layer for injection into the chromatograph. This *Standard solution* contains 3.4 µg/mL of retinol.

**Sample solution for capsules:** Weigh NLT 20 Capsules in a tared weighing bottle. Open the Capsules, without loss of shell material, and transfer the contents to a 100-mL beaker. Remove any contents adhering to the empty shells by washing with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the net weight of the Capsule contents. Transfer a portion of the Capsule contents, equivalent to 1.3 mg of retinol, to a stoppered 125-mL flask. Add 5 mL of water, 15 mL of *Diluent*, and 3 mL of *Potassium hydroxide solution*. Insert the stopper tightly, shake for 15 min over a water bath maintained at 60 ± 5°, and cool to room temperature. Add 7 mL of water and 25.0 mL of *Extraction solvent*. Insert the stopper tightly, and shake vigorously for 60 s or longer, if necessary, for complete extraction. Rinse the sides of the flask with 60 mL of water, and allow to stand for 10 min until the layers separate. [NOTE—Do not shake, because an emulsion may form.] Withdraw a portion of the organic layer, and dilute quantitatively and stepwise if necessary, with *Extraction solvent*, to obtain a concentration of 3.4 µg/mL of retinol.

**Sample solution for tablets:** Finely powder a counted number of Tablets. Transfer a portion of the powder, equivalent to 1.3 mg of retinol, to a stoppered 125-mL flask. Add 5 mL of water, 15 mL of *Diluent*, and 3 mL of *Potassium hydroxide solution*. Insert the stopper tightly, shake for 15 min over a water bath maintained at 60 ± 5°, and cool to room temperature. Add 7 mL of water and 25.0 mL of *Extraction solvent*. Insert the stopper tightly, and shake vigorously for 60 s or longer, if necessary, for complete extraction. Rinse the sides of the flask with 60 mL of water, and allow to stand for 10 min until the layers separate. [NOTE—Do not shake, because an emulsion may form.] Withdraw a portion of the organic layer, and dilute with *Extraction solvent* to obtain a concentration of 3.4 µg/mL of retinol.

**Chromatographic system**

(See *Chromatography* ⟨621⟩, *System Suitability*.)

**Mode:** LC

**Detector:** UV 335 nm

**Column:** 6.2-mm × 8-cm; packing L3

**Column temperature:** 40°

**Flow rate:** 4 mL/min

**Injection volume:** 50 µL

**System suitability**

**Sample:** *Standard solution*

**Suitability requirements**

**Relative standard deviation:** NMT 3.0%

**Analysis**

**Samples:** *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of vitamin A, as retinol ($C_{20}H_{30}O$), in the portion of sample taken:

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$$\text{Result} = (r_{T1}/r_{T2}) \times (C_S/C_U) \times 100$$

$r_{T1}$ = sum of peak responses of all-*trans* retinyl ester and 13-*cis*-retinyl ester peaks from the *Sample solution*
$r_{T2}$ = sum of peak responses of all-*trans* retinyl ester and 13-*cis*-retinyl ester peaks from the *Standard solution*
$C_S$ = concentration of retinol ($C_{20}H_{30}O$) in the *Standard solution* (µg/mL)
$C_U$ = nominal concentration of vitamin A, as retinol ($C_{20}H_{30}O$), in the *Sample solution* (µg/mL)

• **PROCEDURE 4**

This procedure involves a liquid–liquid extraction of vitamin A from the sample with hexane, followed by the evaporation of hexane and reconstitution of the residue in tetrahydrofuran and acetonitrile mixture (1:1). It can be used for the determination of vitamin A in Oil- and Water-Soluble Vitamins Oral Solution and Oil- and Water-Soluble Vitamins with Minerals Oral Solution.

Unless specified in the individual monographs, the *Standard solution*, *Sample solution*, and *Diluent* are prepared as follows.

**Mobile phase:** Methanol, acetonitrile, and *n*-hexane (46.5: 46.5: 7.0)

**Diluent:** Tetrahydrofuran and acetonitrile (1:1)

**Standard solution:** 0.33 mg/mL of retinol[1,2] ($C_{20}H_{30}O$) from USP Retinyl Acetate RS or USP Retinyl Palmitate RS in *Diluent*

**Sample solution for liquid dosage forms:** Transfer an accurately measured volume of Oral Solution, equivalent to 3.3 mg of retinol, to a 500-mL separatory funnel containing 10 mL of water and 20 mL of dehydrated alcohol. Add 150 mL of solvent hexane, insert the stopper, and shake for 1 min. Add another 150 mL of solvent hexane, insert the stopper, shake, and allow the layers to separate. Discard the aqueous layer, and filter the solvent hexane extract through anhydrous sodium sulfate into a 500-mL, round-bottom flask. Evaporate the solution to dryness with the aid of a rotary evaporator over a water bath maintained at about 65°. Immediately add 10.0 mL of *Diluent*, swirl to dissolve the residue, and filter.

**Chromatographic system**

(See *Chromatography* ⟨621⟩, *System Suitability*.)

**Mode:** LC

**Detector:** UV 265 nm

**Column:** 4.6-mm × 50-cm (prepared from two concatenated 4.6-mm × 25-cm columns); packing L1

**Column temperature:** 40°

**Flow rate:** 1.5 mL/min

**Injection volume:** 20 µL

**System suitability**

**Sample:** *Standard solution*

**Suitability requirements**

**Relative standard deviation:** NMT 5.0%

**Analysis**

**Samples:** *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of vitamin A, as retinol ($C_{20}H_{30}O$), in the portion of sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

$r_U$ = peak response of all-*trans* retinyl ester from the *Sample solution*
$r_S$ = peak response of all-*trans* retinyl ester from the *Standard solution*
$C_S$ = concentration of retinol ($C_{20}H_{30}O$) in the *Standard solution* (µg/mL)
$C_U$ = nominal concentration of vitamin A, as retinol ($C_{20}H_{30}O$), in the *Sample solution* (µg/mL)

## ADDITIONAL REQUIREMENTS

• **USP REFERENCE STANDARDS** ⟨11⟩

USP Retinyl Acetate RS
USP Retinyl Palmitate RS

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# ⟨580⟩ VITAMIN C ASSAY

## INTRODUCTION

The following procedures are provided for analysis of different forms of vitamin C as ascorbic acid ($C_6H_8O_6$), sodium ascorbate ($C_6H_7NaO_6$), and calcium ascorbate dihydrate ($C_{12}H_{14}CaO_{12} \cdot 2H_2O$) or their mixtures in finished dosage forms, as Capsules, Tablets, or Oral Solutions.

### METHOD I—TITRIMETRIC METHOD

• **PROCEDURE**

Unless specified in the individual monographs, proceed as follows.

**Sample solution for Capsules:** Weigh NLT 20 Capsules in a tared weighing bottle. Open the Capsules, without the loss of shell material, and transfer the contents to a 100-mL beaker. Remove any contents adhering to the empty shells by washing, if necessary, with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air until the odor of ether is no longer perceptible. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the average net weight per Capsule. Transfer a portion of the Capsule contents, equivalent to 100 mg of ascorbic acid, to a 200-mL volumetric flask, and add 75 mL of metaphosphoric–acetic acids TS. Insert a stopper into the flask, and shake by mechanical means for 30 min. Dilute with water to volume.

**Sample solution for Oral Solutions:** Transfer a volume of Oral Solution equivalent to 50 mg of ascorbic acid, previously diluted with water if necessary, to a 100-mL volumetric flask. Add 20 mL of metaphosphoric–acetic acid TS, dilute with water to volume, and mix.

**Sample solution for Tablets:** Finely powder NLT 20 Tablets. Transfer a portion of the powder, equivalent of 100 mg of ascorbic acid, to a 200-mL volumetric flask, and add 75 mL of metaphosphoric–acetic acids TS. Insert a stopper into the flask, and shake by mechanical means for 30 min. Dilute with water to volume.

**Blank:** A mixture of 5.5 mL of metaphosphoric–acetic acids TS and 15 mL of water.

**Titrimetric system**

(See *Titrimetry* ⟨541⟩.)

**Mode:** Direct titration

**Titrant:** Standard dichlorophenol–indophenol solution VS

**Endpoint detection:** Visual

**Analysis:** Transfer a portion of the *Sample solution* to a centrifuge tube, and centrifuge until a clear supernatant is obtained. Transfer a volume of the *Sample solution*, equivalent to 2 mg of ascorbic acid into a 50-mL conical flask, and add 5 mL of metaphosphoric–acetic acids TS. Titrate with *Titrant* to a rose-pink color that persists for at least 5 s. Correct for the volume of *Titrant* consumed by the *Blank*.

Calculate the percentage of ascorbic acid ($C_6H_8O_6$) in the portion of sample taken:

$$\text{Result} = \{[(V_S - V_B) \times F]/W\} \times 100$$

$V_S$ = *Titrant* volume consumed by the *Sample solution* (mL)
$V_B$ = *Titrant* volume consumed by the *Blank*
$F$ = concentration of *Titrant* in terms of its equivalent of ascorbic acid (mg/mL)
$W$ = nominal amount of ascorbic acid taken for *Analysis* (mg)

### METHOD II—CHROMATOGRAPHIC METHOD

• **PROCEDURE 1**

Unless specified in the individual monographs, the *Diluent*, *Standard solution*, and *Sample solutions* are prepared as follows. [NOTE—Protect samples from air, light, and heat.]

**Buffer:** 2.04 g/L of monobasic potassium phosphate in water. Adjust with phosphoric acid to a pH of 3.0.

**Mobile phase:** *Buffer*

**Diluent:** 0.56 g of edetate disodium dihydrate and 2.04 g of monobasic potassium phosphate per 1000 mL of water. Adjust with phosphoric acid to a pH of 3.0.

**Standard solution:** 0.25 mg/mL of USP Ascorbic Acid RS in *Diluent*

**Sample solution for Capsules:** Weigh NLT 20 Capsules in a tared weighing bottle. Open the Capsules, without the loss of shell material, and transfer the contents to a 100-mL beaker. Remove any contents adhering to the empty shells by washing, if necessary, with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air until the odor of ether is no longer perceptible. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the average net weight per Capsule. Transfer a portion of the Capsule contents, equivalent to about 25 mg of ascorbic acid, into a 100-mL volumetric flask. Add 60 mL of *Diluent*, shake mechanically for 15 min, dilute with *Diluent* to volume, mix well, and pass through a membrane filter of 0.45-µm pore size, discarding the first 4 mL.

**Sample solution for Oral Solutions:** Dilute an accurately measured volume of Oral Solution with *Diluent* to obtain a solution with a concentration of 0.25 mg/mL of ascorbic acid. Mix carefully.

**Sample solution for Tablets:** Transfer a portion from NLT 20 finely powdered Tablets, nominally equivalent to about 25 mg of ascorbic acid, into a 100-mL volumetric flask. Add 60 mL of *Diluent*, shake mechanically for 15 min, dilute with *Diluent* to volume, mix well, and pass through a membrane filter of 0.45-µm pore size, discarding the first 4 mL.

**Chromatographic system**

(See *Chromatography* ⟨621⟩, *System Suitability*.)

**Mode:** LC

**Detector:** UV 245 nm

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**Column:** 4.6-mm × 25-cm; 5-µm packing L1
**Flow rate:** 1.0 mL/min
**Injection volume:** 5 µL
**System suitability**
**Sample:** *Standard solution*
**Suitability requirements**
**Relative standard deviation:** NMT 2.0%
**Analysis**
**Samples:** *Standard solution* and *Sample solution*
Calculate the percentage of vitamin C, as ascorbic acid ($C_6H_8O_6$), in the portion of sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

$r_U$ = peak area of ascorbic acid from the *Sample solution*
$r_S$ = peak area of ascorbic acid from the *Standard solution*
$C_S$ = concentration of USP Ascorbic Acid RS in the *Standard solution* (mg/mL)
$C_U$ = nominal concentration of ascorbic acid in the *Sample solution* (mg/mL)

- **PROCEDURE 2**

Unless specified in the individual monographs, the *Diluent*, *Standard solution*, and *Sample solutions* are prepared as follows. [NOTE—Protect samples from air, light, and heat. All prepared samples must be analyzed within 4 h.]
**Buffer:** 7.8 g/L of sodium dihydrogen phosphate dihydrate in water. Adjust with phosphoric acid to a pH of 2.5.
**Mobile phase:** *Buffer* and methanol. See *Table 1* for gradient.

**Table 1**

| Time (min) | Buffer (%) | Methanol (%) |
|---|---|---|
| 0 | 100 | 0 |
| 3 | 100 | 0 |
| 5 | 0 | 100 |
| 6 | 50 | 50 |
| 7 | 100 | 0 |
| 10 | 100 | 0 |

**Diluent:** Dissolve 73 g of metaphosphoric acid in 800 mL of water, add 84 mL of glacial acetic acid, and dilute with water to 1000 mL.
**Standard stock solution:** 1 mg/mL of USP Ascorbic Acid RS in *Diluent*. [NOTE—Sonicate with intermittent shaking to help dissolve, if necessary. Prepare fresh every time.]
**Standard solution:** Dilute *Standard stock solution* with *Diluent* to obtain a solution containing 0.05 mg/mL of USP Ascorbic Acid RS.
**Sample solution for Capsules:** Weigh NLT 20 Capsules in a tared weighing bottle. Open the Capsules, without the loss of shell material, and transfer the contents to a 100-mL beaker. Remove any contents adhering to the empty shells by washing, if necessary, with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air until the odor of ether is no longer perceptible. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the average net weight per Capsule. Transfer a portion of the Capsule contents, equivalent to about 25 mg of ascorbic acid, into a 50-mL centrifuge tube. Add 25.0 mL of *Diluent*, sonicate for 15 min, and centrifuge at about 2000 rpm for 5 min. Quantitatively dilute the clear supernatant with *Diluent* to obtain a solution containing 0.05 mg/mL of ascorbic acid. Mix and pass through a membrane filter of 0.45-µm pore size.
**Sample solution for Oral Solutions:** Dilute an accurately measured volume of Oral Solution with *Diluent* to obtain a solution with a concentration of 0.05 mg/mL of ascorbic acid. Mix carefully.
**Sample solution for Tablets:** Transfer a portion from NLT 20 ground Tablets, nominally equivalent to about 25 mg of ascorbic acid, into a 50-mL centrifuge tube. Add 25.0 mL of *Diluent*, sonicate for 15 min, and centrifuge at about 2000 rpm for 5 min. Quantitatively dilute the clear supernatant with *Diluent* to obtain a solution containing 0.05 mg/mL of ascorbic acid. Mix and pass through a membrane filter of 0.45-µm pore size.
**Chromatographic system**
(See *Chromatography* ⟨621⟩, *System Suitability*.)
**Mode:** LC
**Detector:** UV 245 nm
**Column:** 4.6-mm × 15-cm; 3.5-µm packing L7
**Flow rate:** 0.8 mL/min
**Injection volume:** 10 µL
**System suitability**
**Sample:** *Standard solution*
**Suitability requirements**
**Relative standard deviation:** NMT 2.0%

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**Analysis**

**Samples:** *Standard solution* and *Sample solution*

Calculate the percentage of vitamin C, as ascorbic acid ($C_6H_8O_6$), in the portion of sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

$r_U$ = peak area of ascorbic acid from the *Sample solution*
$r_S$ = peak area of ascorbic acid from the *Standard solution*
$C_S$ = concentration of USP Ascorbic Acid RS in the *Standard solution* (mg/mL)
$C_U$ = nominal concentration of ascorbic acid in the *Sample solution* (mg/mL)

## ADDITIONAL REQUIREMENTS

- **USP REFERENCE STANDARDS** ⟨11⟩

  USP Ascorbic Acid RS

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# ⟨581⟩ VITAMIN D ASSAY

***Change to read:***

## ASSAY

### Chromatographic Methods

The following liquid chromatographic procedures are provided for the determination of vitamin D as an active pharmaceutical ingredient, as a dietary supplement ingredient, or as a component in compendial dosage forms.

Throughout this assay, protect solutions containing and derived from the test specimen and the Reference Standard from the atmosphere and light, preferably by the use of a blanket of inert gas and low-actinic glassware.

Where vitamin D (cholecalciferol or ergocalciferol) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard.

- **PROCEDURE 1**

This procedure uses a sample preparation without pH adjustment and involves the use of dimethyl sulfoxide to dissolve the excipients in the sample, followed by a liquid–liquid extraction of the vitamin D with hexane. The chromatographic separation is achieved using normal phase on an L8 column. It can be used to determine vitamin D alone or in combination with other vitamins and minerals in compendial dosage forms.

Unless specified in the individual monographs, the *Standard solution*, *Sample solution*, and *System suitability solution* are prepared as follows.

**Mobile phase:** *n*-hexane and isopropyl alcohol (99:1)

**Standard solution:** 2 µg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in *n*-hexane

**System suitability solution:** Heat a volume of the *Standard solution* at 60° for 1 h to partially isomerize vitamin D (cholecalciferol or ergocalciferol) to its corresponding precursor.

**Sample solution for tablets:** Finely powder NLT 20 Tablets. Transfer a portion of the powder, not exceeding 7.5 g, equivalent to NLT 0.1 mg of vitamin D as cholecalciferol or ergocalciferol, to a centrifuge tube having a polytef-lined screw cap. Add about 2 mL of dimethyl sulfoxide and about 3 mL of *n*-hexane per each g of powdered Tablets, and shake for 45 min on a shaker in a water bath maintained at 60°. [NOTE—Set up the shaker to ensure that the contents of the container are mixed vigorously and thoroughly in order to achieve accurate recoveries.] Centrifuge at 3000 rpm for 10 min, and transfer the hexane layer by means of a pipet to a volumetric flask. Add 3 mL of *n*-hexane per each g of powdered Tablets to the dimethyl sulfoxide layer, shake thoroughly for 5 min, and transfer the hexane layer by means of a pipet to the same volumetric flask. Repeat this extraction with three additional portions of *n*-hexane. Dilute the extracts in the volumetric flask with *n*-hexane to volume. Dilute a volume of this solution with *n*-hexane to obtain a solution with a concentration of 2 µg/mL of cholecalciferol or ergocalciferol. [NOTE—Dilution may not be necessary.]

**Sample solution for capsules:** Transfer the contents of NLT 20 Capsules to a suitable container, mix, and weigh. Transfer a portion of the mixture, not exceeding 7.5 g equivalent to NLT 0.1 mg of vitamin D as cholecalciferol or ergocalciferol, to a centrifuge tube having a polytef-lined screw cap. [NOTE—For hard gelatin Capsules, remove, as completely as possible, the contents of NLT 20 Capsules by cutting open the Capsule shells, transferring the shells and their contents to a suitable container, and triturating to a homogeneous mass. Transfer a portion of the mass, equivalent to NLT 0.1 mg of vitamin D as cholecalciferol or ergocalciferol, to a centrifuge tube having a polytef-lined screw cap.] Add about 2 mL of dimethyl sulfoxide and about 3 mL of *n*-hexane per each g of Capsule contents, and shake for 45 min on a shaker in a water bath maintained at 60°. [NOTE—Set up the shaker to ensure that the contents of the container are mixed vigorously and thoroughly in order to achieve accurate recoveries.] Centrifuge at 3000 rpm for 10 min, and transfer the hexane layer by means of a pipet to a volumetric flask. Add 3 mL of *n*-hexane per each g of Capsule contents to the dimethyl sulfoxide layer, shake thoroughly for 5 min, and transfer the hexane layer by means of a pipet to the same volumetric flask. Repeat this extraction with three additional portions of *n*-hexane. Dilute the extracts in the volumetric flask with *n*-hexane to volume. Dilute a volume of this solution with *n*-hexane to obtain a solution with a concentration of 2 µg/mL of vitamin D as cholecalciferol or ergocalciferol. [NOTE—Dilution may not be necessary.]

**Chromatographic system**

(See *Chromatography* ⟨621⟩*, System Suitability*.)

**Mode:** LC

**Detector:** UV 265 nm

**Column:** 4.6-mm × 15-cm; 3-µm packing L8

**Flow rate:** 1 mL/min

**Injection volume:** 100 µL

**System suitability**

**Samples:** *Standard solution* and *System suitability solution*

**Suitability requirements**

**Resolution:** NLT 10 between the vitamin D form present and its corresponding precursor, *System suitability solution*

**Relative standard deviation:** NMT 3.0%, *Standard solution*

**Analysis**

**Samples:** *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$) in the portion of the sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

$r_U$ = peak response of cholecalciferol or ergocalciferol from the *Sample solution*

$r_S$ = peak response of cholecalciferol or ergocalciferol from the *Standard solution*

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$C_S$ = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* (µg/mL)
$C_U$ = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* (µg/mL)
$F$ = correction factor to account for the average amount of previtamin D present in the *Sample solution*, 1.09

- **PROCEDURE 2**

This procedure involves the treatment of sample with sodium bicarbonate, an antioxidant acid solution generating gas evolution; lecithin as a surfactant; and dimethyl sulfoxide, followed by methanolic sulfuric acid to create a dispersion and efficiently extract vitamin D from the matrix components into the 2,2,4-trimethylpentane phase. Separation is achieved in normal-phase mode on an L24 column. The sample preparation and *Chromatographic system* used in this procedure also are suitable to determine vitamins A and E when present in the formulation; analysts should make corresponding adjustments in sample size and detection wavelength.

Unless specified in the individual monographs, the *Standard solution*, *Sample solution*, *System suitability solution*, and reagent solutions are prepared as follows.

**Mobile phase:** *n*-hexane and tertiary butyl alcohol (98.75: 1.25)

**3 N methanolic sulfuric acid solution:** Cautiously add 9 mL of sulfuric acid to 80 mL of methanol in a 100-mL volumetric flask. Cool, and dilute with methanol to volume.

**Sodium ascorbate–pyrogallol solution:** Transfer 10 g of sodium ascorbate and 5 g of pyrogallol to a 100-mL volumetric flask, and add sufficient water to dissolve. Add 1.7 mL of sulfuric acid, and dilute with water to volume.

**Lecithin solution:** 5 mg/mL of lecithin in 2,2,4-trimethylpentane

**Standard solution:** 1 µg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in 2,2,4-trimethylpentane

**System suitability solution:** Heat a volume of the *Standard solution* at 60° for 1 h to partially isomerize vitamin D (cholecalciferol or ergocalciferol) to its corresponding precursor.

**Sample solution for tablets:** ▲▲ (USP 1-Dec-2023) Finely powder NLT 20 Tablets. Use a portion of the powder nominally equivalent to an amount NLT 0.1 mg of vitamin D. Add 0.5 g of sodium bicarbonate, 1.5 mL of *Lecithin solution*, and 12.5 mL of 2,2,4-trimethylpentane, and disperse on a vortex mixer. Add 6 mL of the *Sodium ascorbate–pyrogallol solution*, shake slowly, and allow the solution to degas. Continue shaking until the evolution of gas has ceased, and then shake for an additional 12 min. Add 6 mL of dimethyl sulfoxide, mix on a vortex mixer to form a suspension, and shake for 12 min. Add 6 mL of the *3 N methanolic sulfuric acid solution*, mix on a vortex mixer to form a suspension, and shake for 12 min. Add 12.5 mL of 2,2,4-trimethylpentane, mix on a vortex mixer to form a suspension, and shake for 10 min. Centrifuge for 10 min to break up the emulsion and to clarify the supernatant. If necessary, quantitatively dilute a volume of the supernatant with 2,2,4-trimethylpentane to obtain a concentration close to that of the *Standard solution*.

**Sample solution for capsules:** ▲▲ (USP 1-Dec-2023) Weigh NLT 20 Capsules in a tared weighing bottle. Using a sharp blade if necessary, carefully open the Capsules, without loss of shell material, and transfer the contents to a 100-mL beaker. Remove any contents adhering to the empty shells by washing with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the net weight of the Capsule contents. Transfer a portion of the Capsule contents, equivalent to NLT 0.1 mg of the labeled amount of vitamin D, as cholecalciferol or ergocalciferol. Add 0.5 g of sodium bicarbonate, 1.5 mL of *Lecithin solution*, and 12.5 mL of 2,2,4-trimethylpentane, and disperse on a vortex mixer. Add 6 mL of the *Sodium ascorbate–pyrogallol solution*, shake slowly, and allow the solution to degas. Continue shaking until the evolution of gas has ceased, and then shake for an additional 12 min. Add 6 mL of dimethyl sulfoxide, mix on a vortex mixer to form a suspension, and shake for 12 min. Add 6 mL of the *3 N methanolic sulfuric acid solution*, mix on a vortex mixer to form a suspension, and shake for 12 min. Add 12.5 mL of 2,2,4-trimethylpentane, mix on a vortex mixer to form a suspension, and shake for 10 min. Centrifuge for 10 min to break up the emulsion and to clarify the supernatant. If necessary, quantitatively dilute a volume of the supernatant with 2,2,4-trimethylpentane to obtain a concentration close to that of the *Standard solution*.

**Chromatographic system**

(See *Chromatography* ⟨621⟩, *System Suitability*.)

**Mode:** LC

**Detector:** UV 265 nm

**Column:** 4.6-mm × 25-cm; 5-µm packing L24

**Flow rate:** 1 mL/min

**Injection volume:** 40 µL

**System suitability**

**Samples:** *Standard solution* and *System suitability solution*

**Suitability requirements**

**Resolution:** NLT 4.0 between the vitamin D form present and its corresponding precursor, *System suitability solution*

**Relative standard deviation:** NMT 3.0%, *Standard solution*

**Analysis**

**Samples:** *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$) in the portion of the sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

$r_U$ = peak response of cholecalciferol or ergocalciferol from the *Sample solution*
$r_S$ = peak response of cholecalciferol or ergocalciferol from the *Standard solution*
$C_S$ = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* (µg/mL)
$C_U$ = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* (µg/mL)

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- **PROCEDURE 3**

This procedure is suitable for matrices with excipients that are soluble or degradable in alkaline conditions. It involves the saponification of the sample solution, followed by a liquid–liquid extraction with hexane and clean-up on a solid phase extraction (SPE) using a mixture of methylene chloride and isopropyl alcohol (99.8: 0.2) as eluent. The *Standard solution* undergoes similar treatment to compensate for losses in recovery due to isomerization. The eluate then is evaporated, and the residue is reconstituted in acetonitrile before injection into the chromatograph. Separation is achieved in reversed phase mode.

Unless specified in the individual monographs, the *Standard solution*, *Sample solution*, and reagent solutions are prepared as follows.

**Mobile phase:** Acetonitrile and methanol (91:9)

**Diluted acetic acid:** Glacial acetic acid solution (1 in 10) in water

**Phenolphthalein solution:** 10 mg/mL of phenolphthalein in alcohol

**Potassium hydroxide solution:** Slowly dissolve 14 g of potassium hydroxide in a mixture of 31 mL of dehydrated alcohol and 5 mL of water. Prepare fresh daily.

**Extraction solvent:** Methylene chloride and isopropyl alcohol (99.8: 0.2)

**Extraction column:** Silica packing with a sorbent mass-to-column volume ratio of 500 mg to 2.8 mL or equivalent. [NOTE—Condition the column by initially washing with 4.0 mL of a mixture of methylene chloride and isopropyl alcohol (4:1), followed by 5.0 mL of the *Extraction solvent*. Do not allow the column to dry.]

**Standard stock solution:** 0.2 mg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in dehydrated alcohol. [NOTE—Prepare fresh every 4 weeks. Store in a freezer.]

**Standard solution:** Dilute a volume of the *Standard stock solution* with dehydrated alcohol to obtain a concentration of 5 µg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS. Prepare this solution fresh daily. Transfer 2.0 mL of this solution to a stoppered 125-mL flask. Add 15.0 mL of water and 15.0 mL of the *Potassium hydroxide solution*, insert the stopper, and shake for 30 min in a water bath maintained at 60°. Allow to cool to room temperature, and transfer the contents of the flask to a 250-mL separatory funnel. Add 15.0 mL of water to the flask, insert the stopper, shake vigorously, and transfer this solution to the separatory funnel. Rinse the flask with 60 mL of *n*-hexane, and transfer the rinsing to the separatory funnel. Insert the stopper, shake vigorously for 90 s, and allow to stand for 15 min until the layers separate. Drain, and discard the aqueous layer. Add 15.0 mL of water to the hexane layer in the separatory funnel, insert the stopper, and shake vigorously. Allow to stand for 10 min until the layers separate, and discard the aqueous layer. Add 1 drop of *Phenolphthalein solution* and 15.0 mL of water to the separatory funnel. Add *Diluted acetic acid* dropwise, with shaking, until the washing is neutral. Allow to stand for 10 min until the layers separate. Drain, and discard the aqueous layer. Filter the hexane layer through anhydrous sodium sulfate supported by a small pledget of cotton into a 100-mL, round-bottom flask. Rinse the funnel and sodium sulfate with a few milliliters of *n*-hexane, and collect the rinsings in the same flask. Evaporate the hexane in the flask on a rotary evaporator at 50° to dryness. Immediately add 2.0 mL of the *Extraction solvent* to dissolve the residue. Transfer this solution to a freshly conditioned solid-phase *Extraction column*, rinse the round-bottom flask with 1.0 mL of the *Extraction solvent*, and transfer to the column. Elute the *Extraction column* with 2.0 mL of the *Extraction solvent*, and discard this fraction. Elute the column with 7.0 mL of the *Extraction solvent*, and collect the eluate in a suitable flask. Place the flask in a warm water bath maintained at 42°, and evaporate the solvent with the aid of a stream of nitrogen. Immediately add 2.0 mL of acetonitrile to the residue, and use the solution for injection into the chromatograph.

**Sample solution for tablets:** Finely powder NLT 20 Tablets. Transfer a portion of the powder, equivalent to 10 µg of cholecalciferol or ergocalciferol, to a stoppered 125-mL flask. Add 15.0 mL of water and 15.0 mL of the *Potassium hydroxide solution*, insert the stopper, and shake for 30 min in a water bath maintained at 60°. Allow to cool to room temperature, and transfer the contents of the flask to a 250-mL separatory funnel. Add 15.0 mL of water to the flask, insert the stopper, shake vigorously, and transfer this solution to the separatory funnel. Rinse the flask with 60 mL of *n*-hexane, and transfer the rinsing to the separatory funnel. Insert the stopper, shake vigorously for 90 s, and allow to stand for 15 min until the layers separate. Drain and discard the aqueous layer. Add 15.0 mL of water to the hexane layer in the separatory funnel, insert the stopper, and shake vigorously. Allow to stand for 10 min until the layers separate, and discard the aqueous layer. Add 1 drop of *Phenolphthalein solution* and 15.0 mL of water to the separatory funnel. Add *Diluted acetic acid* dropwise, with shaking, until the washing is neutral. Allow to stand for 10 min until the layers separate. Drain, and discard the aqueous layer. Filter the hexane layer through anhydrous sodium sulfate supported by a small pledget of cotton into a 100-mL, round-bottom flask. Rinse the funnel and sodium sulfate with a few milliliters of *n*-hexane, and collect the rinsings in the same flask. Evaporate the hexane in the flask on a rotary evaporator at 50° to dryness. Immediately add 2.0 mL of the *Extraction solvent* to dissolve the residue. Transfer this solution to a freshly conditioned solid-phase *Extraction column*, rinse the round-bottom flask with 1.0 mL of the *Extraction solvent*, and transfer to the column. Elute the *Extraction column* with 2.0 mL of the *Extraction solvent*, and discard this fraction. Elute the column with 7.0 mL of the *Extraction solvent*, and collect the eluate in a suitable flask. Place the flask in a warm water bath maintained at 42°, and evaporate the solvent with the aid of a stream of nitrogen. Immediately add 2.0 mL of acetonitrile to the residue, and use the solution for injection into the chromatograph.

**Sample solution for capsules:** Weigh NLT 20 Capsules in a tared weighing bottle. Open the Capsules, without loss of shell material, and transfer the contents to a 100-mL beaker. Remove any contents adhering to the empty shells by washing with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the net weight of the Capsule contents. Transfer a portion of the Capsule contents, equivalent to 10 µg of ergocalciferol or cholecalciferol, to a stoppered 125-mL flask. Add 15.0 mL of water and 15.0 mL of the *Potassium hydroxide solution*, insert the stopper, and shake for 30 min in a water bath maintained at 60°. Allow to cool to room temperature, and transfer the contents of the flask to a 250-mL separatory funnel. Add 15.0 mL of water to the flask, insert the stopper, shake vigorously, and transfer this solution to the separatory funnel. Rinse the flask with 60 mL of *n*-hexane, and transfer the rinsing to the separatory funnel. Insert the stopper, shake vigorously for 90 s, and allow to stand for 15 min until the layers separate.

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Drain, and discard the aqueous layer. Add 15.0 mL of water to the hexane layer in the separatory funnel, insert the stopper, and shake vigorously. Allow to stand for 10 min until the layers separate, and discard the aqueous layer. Add 1 drop of *Phenolphthalein solution* and 15.0 mL of water to the separatory funnel. Add *Diluted acetic acid* dropwise, with shaking, until the washing is neutral. Allow to stand for 10 min until the layers separate. Drain, and discard the aqueous layer. Filter the hexane layer through anhydrous sodium sulfate supported by a small pledget of cotton into a 100-mL, round-bottom flask. Rinse the funnel and sodium sulfate with a few milliliters of *n*-hexane, and collect the rinsings in the same flask. Evaporate the hexane in the flask on a rotary evaporator at 50° to dryness. Immediately add 2.0 mL of the *Extraction solvent* to dissolve the residue. Transfer this solution to a freshly conditioned solid-phase *Extraction column*, rinse the round-bottom flask with 1.0 mL of the *Extraction solvent*, and transfer to the column. Elute the *Extraction column* with 2.0 mL of the *Extraction solvent*, and discard this fraction. Elute the column with 7.0 mL of the *Extraction solvent*, and collect the eluate in a suitable flask. Place the flask in a warm water bath maintained at 42°, and evaporate the solvent with the aid of a stream of nitrogen. Immediately add 2.0 mL of acetonitrile to the residue, and use the solution for injection into the chromatograph.

**Chromatographic system**

(See *Chromatography* ⟨621⟩, *System Suitability*.)

**Mode:** LC

**Detector:** UV 265 nm

**Column:** 4.6-mm × 25-cm; 5-µm packing L1

**Column temperature:** 27°

**Flow rate:** 0.7 mL/min

**Injection volume:** 15 µL

**System suitability**

**Sample:** *Standard solution*

**Suitability requirements**

**Relative standard deviation:** NMT 4.0%

**Analysis**

**Samples:** *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$) in the portion of the sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \text{ ▲(USP 1-Dec-2023)} \times 100$$

$r_U$ = peak response of cholecalciferol or ergocalciferol from the *Sample solution*

$r_S$ = peak response of cholecalciferol or ergocalciferol from the *Standard solution*

$C_S$ = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* (µg/mL)

$C_U$ = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* (µg/mL)

▲ (USP 1-Dec-2023)

- **PROCEDURE 4**

This procedure is applicable to liquid dosage forms. It involves a liquid–liquid extraction of the sample with hexane, followed by the evaporation of hexane, and reconstitution of the residue in a tetrahydrofuran and acetonitrile mixture (1:1). Separation is achieved in reversed-phase mode.

Unless specified in the individual monographs, the *Standard solution*, *Sample solution*, and *Diluent* are prepared as follows.

**Mobile phase:** Methanol, acetonitrile, and *n*-hexane (46.5: 46.5: 7.0)

**Diluent:** Tetrahydrofuran and acetonitrile (1:1)

**Standard solution:** 5 µg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in *Diluent*

**Sample solution:** Transfer an accurately measured volume of the sample, equivalent to 50 µg of cholecalciferol or ergocalciferol, to a 500-mL separatory funnel containing 10 mL of water and 20 mL of dehydrated alcohol. Add 150 mL of solvent hexane, insert the stopper, and shake for 1 min. Add another 150 mL of solvent hexane, insert the stopper, shake, and allow the layers to separate. Discard the aqueous layer. Drain the solvent hexane extract through anhydrous sodium sulfate into a 500-mL, round-bottom flask. Evaporate the solution to dryness with the aid of a rotary evaporator over a water bath maintained at about 65°. Immediately add 10.0 mL of *Diluent*, swirl to dissolve the residue, and filter.

**Chromatographic system**

(See *Chromatography* ⟨621⟩, *System Suitability*.)

**Mode:** LC

**Detector:** UV 265 nm

**Column:** Two columns, 4.6-mm × 25-cm, connected in series; both with packing L1

**Column temperature:** 40°

**Flow rate:** 1.5 mL/min

**Injection volume:** 20 µL

**System suitability**

**Sample:** *Standard solution*

**Suitability requirements**

**Relative standard deviation:** NMT 5.0%

**Analysis**

**Samples:** *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$) in the portion of the sample taken:

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$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

$r_U$ = peak response of cholecalciferol or ergocalciferol from the *Sample solution*
$r_S$ = peak response of cholecalciferol or ergocalciferol from the *Standard solution*
$C_S$ = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* (µg/mL)
$C_U$ = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* (µg/mL)
$F$ = correction factor to account for the average amount of previtamin D present in the *Sample solution*, 1.09

- **PROCEDURE 5**

This procedure involves the dissolution of vitamin D in toluene, and the solution is injected into the liquid chromatograph. It is applicable to simple matrices such as pure vitamins and ingredients that do not require saponification and that are soluble in toluene. Separation is achieved in normal-phase mode.

Unless specified in the individual monographs, the *Standard solution*, *Sample solution*, and *System suitability solution* are prepared as follows.

**Dehydrated hexane:** Prepare a chromatographic column by packing a chromatographic tube, 8-cm × 60-cm, with 500 g of 50- to 250-µm chromatographic siliceous earth, activated by drying at 150° for 4 h. (See *Chromatography* ⟨621⟩, *Column Chromatography*.) Pass 500 mL of hexane through the column, and collect the eluate in a glass-stoppered flask.

**Mobile phase:** *n*-Amyl alcohol in *Dehydrated hexane* (3 in 1000)

**System suitability solution:** 250 mg of USP Vitamin D Assay System Suitability RS in 10 mL of a mixture of toluene and *Mobile phase* (1:1). Heat this solution, under reflux, at 90° for 45 min, and cool. [NOTE—This solution contains cholecalciferol, precholecalciferol, and *trans*-cholecalciferol. For the stock solutions, follow these procedures: use low-actinic glassware, dissolve the samples without heating, and prepare the solutions fresh daily.]

**Standard stock solution:** 0.6 mg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in toluene

**Standard solution:** 120 µg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in *Mobile phase*, prepared from the *Standard stock solution*

**Sample stock solution:** 0.6 mg/mL of cholecalciferol or ergocalciferol in toluene

**Sample solution:** 120 µg/mL of cholecalciferol or ergocalciferol in *Mobile phase*, prepared from the *Sample stock solution*

**Chromatographic system**

(See *Chromatography* ⟨621⟩, *System Suitability*.)

**Mode:** LC

**Detector:** UV 254 nm

**Column:** 4.6-mm × 25-cm; packing L3

**Column temperature:** 40°

**Flow rate:** Can be varied to meet the *System suitability requirements*

**Injection volume:** 5–10 µL

**System suitability**

**Sample:** *System suitability solution*

[NOTE—The relative retention times for precholecalciferol, *trans*-cholecalciferol, and cholecalciferol are 0.4, 0.5, and 1.0, respectively.]

**Suitability requirements**

**Resolution:** NLT 1.0 between *trans*-cholecalciferol and precholecalciferol

**Relative standard deviation:** NMT 2.0% for the peak response of cholecalciferol

**Analysis**

**Samples:** *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$) in the portion of the sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

$r_U$ = peak response of cholecalciferol or ergocalciferol from the *Sample solution*
$r_S$ = peak response of cholecalciferol or ergocalciferol from the *Standard solution*
$C_S$ = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* (µg/mL)
$C_U$ = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* (µg/mL)

- **PROCEDURE 6**

The procedure involves the dissolution of the sample in *Mobile phase*, and the solution is injected into the liquid chromatograph. The application includes cholecalciferol and ergocalciferol dissolved in edible vegetable oil, polysorbate 80, or propylene glycol, none of which will interfere with the corresponding precursor so that it can be quantitated. Separation is achieved in normal-phase mode on an L3 column, and *System suitability* requires separation of the *trans* forms from the vitamin D precursors.

Unless specified in the individual monographs, the *Standard solutions* and *Sample solution* are prepared as follows.

**Mobile phase:** Hexane and pentanol (997:3)

**Standard stock solution:** Dissolve USP Cholecalciferol RS or USP Ergocalciferol RS in toluene, and dilute with *Mobile phase* to 50 µg/mL. [NOTE—Prepare this solution fresh daily.]

**Standard solution A:** 5 µg/mL from the *Standard stock solution* in *Mobile phase*. [NOTE—Store at a temperature not above 0°.]

**Standard solution B:** Transfer 5.0 mL of the *Standard stock solution* to a round-bottom flask fitted with a reflux condenser. Displace the air with nitrogen, and reflux for 1 h in a water bath under a nitrogen atmosphere to obtain a solution

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containing cholecalciferol and precholecalciferol. Cool, transfer the solution with the aid of several portions of *Mobile phase* to a 50-mL volumetric flask, and dilute with *Mobile phase* to volume.

**Sample solution:** Equivalent to 5 µg/mL of cholecalciferol or ergocalciferol in *Mobile phase* from an accurately measured volume of the sample

**Chromatographic system**

(See *Chromatography* ⟨621⟩*, System Suitability*.)

**Mode:** LC

**Detector:** UV 254 nm

**Column:** 4.6-mm × 25-cm; packing L3

**Flow rate:** 1–2 mL/min

**Injection volume:** 10–20 µL

**System suitability**

**Sample:** *Standard solution B* (USP Cholecalciferol RS or USP Ergocalciferol RS)

[NOTE—The relative retention times for precholecalciferol and cholecalciferol are about 0.4 and 1.0, respectively. Those for pre-ergocalciferol and ergocalciferol are about 0.8 and 1.0, respectively.]

**Suitability requirements**

**Resolution:** NLT 1.0 between precholecalciferol and cholecalciferol; NLT 1.0 between pre-ergocalciferol and ergocalciferol

**Relative standard deviation:** NMT 2.0% for the peak response of cholecalciferol or ergocalciferol

**Analysis**

**Samples:** *Standard solution A*, *Standard solution B*, and *Sample solution*

**Cholecalciferol or ergocalciferol response factor**

Calculate the cholecalciferol or ergocalciferol response factor, $F_C$:

$$F_C = C_S / r_S$$

$C_S$ = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in *Standard solution A* (µg/mL)
$r_S$ = peak area of cholecalciferol or ergocalciferol from *Standard solution A*

**Precholecalciferol or pre-ergocalciferol response factor**

Calculate the concentration, $C'_S$, in µg/mL, of cholecalciferol or ergocalciferol in *Standard solution B*:

$$C'_S = F_C \times r'_S$$

$F_C$ = response factor for cholecalciferol or ergocalciferol
$r'_S$ = peak area of cholecalciferol or ergocalciferol from *Standard solution B*

Calculate the concentration, $C'_{pre}$, in µg/mL, of precholecalciferol or pre-ergocalciferol:

$$C'_{pre} = C_S - C'_S$$

$C_S$ = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in *Standard solution A* (µg/mL)
$C'_S$ = concentration of cholecalciferol or ergocalciferol in *Standard solution B* (µg/mL)

Calculate the response factor, $F_{pre}$, for precholecalciferol or pre-ergocalciferol:

$$F_{pre} = C'_{pre} / r_p$$

$C'_{pre}$ = concentration of precholecalciferol or pre-ergocalciferol (µg/mL)
$r_p$ = peak response of precholecalciferol or pre-ergocalciferol from *Standard solution B*

**Content of vitamin D**

Calculate the percentage of the labeled amount of vitamin D as cholecalciferol ($C_{27}H_{44}O$) or as ergocalciferol ($C_{28}H_{44}O$) in the portion of the sample taken:

$$\text{Result} = \{[(F_C \times r_C) + (F_{pre} \times r_{pre})]/C_U\} \times 100$$

$F_C$ = response factor for cholecalciferol or ergocalciferol
$r_C$ = peak area of cholecalciferol or ergocalciferol from the *Sample solution*
$F_{pre}$ = response factor for precholecalciferol or pre-ergocalciferol
$r_{pre}$ = peak area of precholecalciferol or pre-ergocalciferol from the *Sample solution*
$C_U$ = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* (µg/mL)

- **PROCEDURE 7**

This procedure involves the saponification of the sample, followed by a liquid–liquid extraction with ether–solvent hexane, and by the evaporation of ether–hexane and reconstitution of the residue in a mixture of toluene and *Mobile phase* (1:4). It is applicable to solutions in oil and capsules containing vitamin D solutions in oil, in which the oil does not interfere with the corresponding precursor so that it can be quantitated. Separation is achieved in normal-phase mode on an L3 column, and *System suitability* requires separation of the *trans* forms from the vitamin D precursors.

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Unless specified in the individual monographs, the *Standard solutions*, *Sample solution*, and reagent solutions are prepared as follows.

**Dehydrated hexane:** Prepare a chromatographic column by packing a chromatographic tube, 8-cm × 60-cm, with 500 g of 50- to 250-µm chromatographic siliceous earth, activated by drying at 150° for 4 h. (See *Chromatography* ⟨621⟩, *Column Chromatography*.) Pass 500 mL of hexane through the column, and collect the eluate in a glass-stoppered flask.

**Mobile phase:** *n*-Amyl alcohol in *Dehydrated hexane* (3 in 1000)

**Butylated hydroxytoluene solution:** 10 mg/mL of butylated hydroxytoluene in chromatographic hexane

**Aqueous potassium hydroxide solution:** 1 g/mL of potassium hydroxide in freshly boiled water. [NOTE—Prepare this solution fresh daily.]

**Alcoholic potassium hydroxide solution:** 3 g of potassium hydroxide in 50 mL of freshly boiled water. Add 10 mL of alcohol, and dilute with freshly boiled water to 100 mL. [NOTE—Prepare this solution fresh daily.]

**Sodium ascorbate solution:** 175 mg/mL of ascorbic acid in 1 N sodium hydroxide. [NOTE—Prepare fresh daily.]

**Sodium sulfide solution:** 12 g of sodium sulfide in 20 mL of water. Dilute with glycerin to 100 mL.

**Standard stock solution:** 0.5 mg/mL of USP Ergocalciferol RS or USP Cholecalciferol RS in toluene. [NOTE—Prepare this solution fresh daily.]

**Standard solution A:** 20 µg/mL from the *Standard stock solution* in *Mobile phase*. [NOTE—Store this solution at a temperature not above 0°.]

**Standard solution B:** Pipet 4 mL of the *Standard stock solution* into a round-bottomed flask fitted with a reflux condenser, and add 2 or 3 crystals of butylated hydroxytoluene. Displace the air with nitrogen, and heat in a water bath maintained at a temperature of 90° in subdued light under a nitrogen atmosphere for 45 min to obtain a solution containing vitamin D and previtamin D. Cool, transfer with the aid of several portions of *Mobile phase* to a 100-mL volumetric flask, and dilute with *Mobile phase* to volume.

**System suitability solution:** Add 100 mg of USP Vitamin D Assay System Suitability RS to a 10-mL volumetric flask. Add a mixture (1 in 5) of toluene and *Mobile phase* to volume, and mix. Heat a portion of this solution under reflux at 90° for 45 min, and cool.

**Sample solution:** Reflux NLT 10 Capsules with a mixture of 10 mL of the *Sodium ascorbate solution* and 2 drops of the *Sodium sulfide solution* on a steam bath for 10 min, crush any remaining solids with a blunt glass rod, and continue heating for 5 min. Cool, and add 25 mL of alcohol and 3 mL of the *Aqueous potassium hydroxide solution*. Reflux the mixture on a steam bath for 30 min. Cool rapidly under running water, and transfer the saponified mixture to a conical separator, rinsing the saponification flask with two 15-mL portions of water, 10 mL of alcohol, and two 50-mL portions of ether. [NOTE—Use ether within 24 h after opening the container.] Shake the combined saponified mixture and rinsings vigorously for 30 s, and allow to stand until both layers are clear. Transfer the aqueous phase to a second conical separator, add a mixture of 10 mL of alcohol and 50 mL of solvent hexane, and shake vigorously. Allow to separate, transfer the aqueous phase to a third conical separator, and transfer the solvent hexane phase to the first separator, rinsing the second separator with two 10-mL portions of solvent hexane and adding the rinsings to the first separator. Shake the aqueous phase in the third separator with 50 mL of solvent hexane, and add the solvent hexane phase to the first separator. Wash the combined ether–solvent hexane extracts by shaking vigorously with three 50-mL portions of the *Alcoholic potassium hydroxide solution*, and wash with 50-mL portions of water vigorously until the last washing is neutral to phenolphthalein. Drain any remaining drops of water from the combined ether–solvent hexane extracts, add 2 sheets of 9-cm filter paper, in strips, to the separator, and shake. Transfer the washed ether–solvent hexane extracts to a round-bottom flask, rinsing the separator and paper with solvent hexane. Combine the solvent hexane rinsings with the ether–solvent hexane extracts, add 100 µL of the *Butylated hydroxytoluene solution*, and mix. Evaporate under vacuum to dryness by swirling in a water bath maintained at a temperature not higher than 40°. Cool under running water, and introduce nitrogen sufficient to restore atmospheric pressure. Without delay, dissolve and dilute the residue in an accurately measured volume of a mixture (1 in 5) of toluene and *Mobile phase*, until the concentration of vitamin D is about 25 µg/mL.

**Chromatographic system**

(See *Chromatography* ⟨621⟩, *System Suitability*.)

**Mode:** LC

**Detector:** UV 254 nm

**Column:** 4.6-mm × 25-cm; packing L3

**Flow rate:** 1–2 mL/min

**Injection volume:** 10–20 µL

**System suitability**

**Sample:** *System suitability solution*

[NOTE—The relative retention times for precholecalciferol, *trans*-cholecalciferol, and cholecalciferol are 0.4, 0.5, and 1.0, respectively.]

**Suitability requirements**

**Resolution:** NLT 1.0 between *trans*-cholecalciferol and precholecalciferol

**Relative standard deviation:** NMT 2.0% for the peak response of cholecalciferol

**Analysis**

**Samples:** *Standard solution A*, *Standard solution B*, and *Sample solution*

**Cholecalciferol and ergocalciferol response factor**

Calculate the cholecalciferol or ergocalciferol response factor, $F_D$:

$$F_D = C_S / r_S$$

$C_S$ = concentration of USP Ergocalciferol RS in *Standard solution A* (µg/mL)

$r_S$ = peak area of cholecalciferol or ergocalciferol from *Standard solution A*

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**Precholecalciferol and pre-ergocalciferol response factor**

Calculate the concentration, $C'_S$, in µg/mL, of cholecalciferol or ergocalciferol in *Standard solution B*:

$$C'_S = F_D \times r'_S$$

$F_D$ = response factor for cholecalciferol or ergocalciferol
$r'_S$ = peak area of cholecalciferol or ergocalciferol from *Standard solution B*

Calculate the concentration, $C'_{pre}$, in µg/mL, of pre-ergocalciferol:

$$C'_{pre} = C_S - C'_S$$

$C_S$ = concentration of USP Ergocalciferol RS in *Standard solution A* (µg/mL)
$C'_S$ = concentration of cholecalciferol or ergocalciferol in *Standard solution B* (µg/mL)

Calculate the response factor, $F_{pre}$, for precholecalciferol or pre-ergocalciferol:

$$F_{pre} = C'_{pre}/r_p$$

$C'_{pre}$ = concentration of precholecalciferol or pre-ergocalciferol (µg/mL)
$r_p$ = peak response of precholecalciferol or pre-ergocalciferol from *Standard solution B*

**Content of vitamin D**

Calculate the percentage of the labeled amount of vitamin D as ergocalciferol ($C_{28}H_{44}O$) or as cholecalciferol ($C_{27}H_{44}O$) in the portion of the sample taken:

$$\text{Result} = \{[(F_D \times r_C) + (F_{pre} \times r'_{pre})]/C_U\} \times 100$$

$F_D$ = response factor for cholecalciferol or ergocalciferol
$r_C$ = peak area of cholecalciferol or ergocalciferol from the *Sample solution*
$F_{pre}$ = response factor for precholecalciferol or pre-ergocalciferol
$r'_{pre}$ = peak area of precholecalciferol or pre-ergocalciferol from the *Sample solution*
$C_U$ = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* (µg/mL)

- **PROCEDURE 8**

This procedure is suitable for the determination of cholecalciferol in oils or fatty matrices. It involves two chromatographic systems, one for sample clean-up and the other for vitamin D determination. The *Standard*, *Internal standard*, and *Sample solutions* are subjected to saponification, followed by a liquid–liquid extraction with a mixture of ether and hexane (1:1). The extract is evaporated and reconstituted in the *Butylated hydroxytoluene solution*.

Unless specified in the individual monographs, the *Standard solution*, *Sample solutions*, *Internal standard solution*, and reagent solutions are prepared as follows.

**Solution A:** *n*-Amyl alcohol and dehydrated hexane (3:997)

**Solution B:** Acetonitrile, water, and phosphoric acid (96: 3.8: 0.2)

**Butylated hydroxytoluene solution:** 10 mg/mL of butylated hydroxytoluene in chromatographic hexane

**Aqueous potassium hydroxide solution:** Dissolve 800 g of potassium hydroxide in 1000 mL of freshly boiled water, mix, and cool. [NOTE—Prepare this solution fresh daily.]

**Alcoholic potassium hydroxide solution:** Dissolve 3 g of potassium hydroxide in 50 mL of freshly boiled water, add 10 mL of alcohol, and dilute with freshly boiled water to 100 mL. [NOTE—Prepare this solution fresh daily.]

**Ascorbic acid solution:** 100 mg/mL of ascorbic acid in water. [NOTE—Prepare this solution fresh daily.]

**Internal standard solution:** 5 µg/mL of USP Ergocalciferol RS in alcohol

**Standard stock solution:** 5 µg/mL of USP Cholecalciferol RS in alcohol

**Standard solution:** Transfer 2.0 mL of the *Standard stock solution* and 2.0 mL of the *Internal standard solution* to a round-bottom flask. Proceed as directed for *Sample solution 1* beginning with "Add 5 mL of…".

**Sample solution 1:** Transfer 4.00 g of oil to a round-bottom flask. Add 5 mL of the *Ascorbic acid solution*, 100 mL of alcohol, and 10 mL of the *Aqueous potassium hydroxide solution*, and mix. Reflux the mixture on a steam bath for 30 min. Add 100 mL of a 10-mg/mL sodium chloride solution. Cool rapidly under running water, and transfer the saponified mixture to a 500-mL separator, rinsing the saponification flask with 75 mL of a 10-mg/mL sodium chloride solution, and then with 150 mL of a mixture of ether and hexane (1:1). Shake the combined saponified mixture and rinsings vigorously for 30 s, and allow to stand until both layers are clear. Discard the lower layer. Wash the ether–hexane extracts by shaking vigorously with 50 mL of the *Alcoholic potassium hydroxide solution*, and then washing with three 50-mL portions of a 10-mg/mL sodium chloride solution. Filter the upper layer through 5 g of anhydrous sodium sulfate on a fast filter paper into a 250-mL flask suitable for a rotary evaporator. Wash the filter with 10 mL of a mixture of ether and hexane (1:1), and combine with the extract. Evaporate the solvent at reduced pressure at a temperature not exceeding 30°, and fill with nitrogen when the evaporation is complete. Alternatively evaporate the solvent under a gentle stream of nitrogen at a temperature not exceeding 30°. Dissolve the residue in 1.5 mL of the *Butylated hydroxytoluene solution*. [NOTE—Gentle heating in an ultrasonic bath may be required. A large fraction of the white residue is cholesterol.]

**Sample solution 2:** To 4.00 g of oil add 2.0 mL of the *Internal standard solution*, and proceed as directed for *Sample solution 1* beginning with "Add 5 mL of…".

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**Clean-up chromatographic system**
(See *Chromatography* ⟨621⟩, *System Suitability*.)
**Mode:** LC
**Detector:** UV 265 nm
**Mobile phase:** *Solution A*
**Clean-up column:** 4.6-mm × 25-cm stainless steel; packing L10
**Flow rate:** 1.1 mL/min
**Injection volume:** 350 µL

**Analysis (clean-up)**
**Samples:** *Standard solution*, *Sample solution 1*, and *Sample solution 2*
Collect separately the eluates from 2 min before to 2 min after the retention time of cholecalciferol in a glass tube containing 1 mL of the *Butylated hydroxytoluene solution* and fitted with a hermetic closure. Evaporate each tube under a stream of nitrogen at a temperature not exceeding 30°. Dissolve each residue in 1.5 mL of acetonitrile, and inject into the analytical chromatographic system below.

**Analytical chromatographic system**
(See *Chromatography* ⟨621⟩, *System Suitability*.)
**Mode:** LC
**Detector:** UV 265 nm
**Mobile phase:** *Solution B*
**Analytical column:** 4.6-mm × 15-cm stainless steel; 5-µm packing L1
**Flow rate:** 1.0 mL/min
**Injection volume:** 200 µL

**System suitability**
**Sample:** *Standard solution* (after the clean-up)
**Suitability requirements**
**Resolution:** NLT 1.4 between cholecalciferol and ergocalciferol
**Relative standard deviation:** NMT 2.0% for the cholecalciferol peak from replicate injections

**Analysis**
**Samples:** *Standard solution*, *Sample solution 1*, and *Sample solution 2* (after the clean-up)
Calculate the content of vitamin D, in µg/g, in the portion of the sample taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U)$$

$R_S$ = peak response of cholecalciferol relative to the internal standard in the *Standard solution*
$C_S$ = concentration of USP Cholecalciferol RS in the *Standard solution* (µg/mL)
$C_U$ = concentration of oil in *Sample solution 2* (g/mL)
$R_U$ = peak response of cholecalciferol relative to the internal standard in *Standard solution 2*, calculated as follows:

[NOTE—If $r_{IS1} = 0$ due to no peak observed at the locus of the internal standard in the chromatogram of *Sample solution 1*, then $R_U = r_{U2}/r_{IS2}$.]

$$R_U = r_{U2}/[r_{IS2} - (r_{IS1} \times r_{U2}/r_{U1})]$$

$r_{U2}$ = peak response of cholecalciferol from *Sample solution 2*
$r_{IS2}$ = peak response of the internal standard from *Sample solution 2*
$r_{IS1}$ = peak response of the internal standard from *Sample solution 1*
$r_{U1}$ = peak response of cholecalciferol from *Sample solution 1*

## ADDITIONAL REQUIREMENTS

- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Cholecalciferol RS
  USP Ergocalciferol RS
  USP Vitamin D Assay System Suitability RS

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# ⟨591⟩ ZINC DETERMINATION

## INTRODUCTION

A quantitative determination of zinc in drug substance and drug product monographs containing zinc is achieved by the *Dithizone Method*, *Ion Chromatographic Method*, or *Atomic Absorption Method*. Unless a specific method is indicated in the individual monograph, any of these methods can be used.

## PROCEDURE

### • Dithizone Method

Select all reagents for this test to have as low a content of heavy metals as practicable. If necessary, distill water and other solvents into a hard or borosilicate glass apparatus. Rinse thoroughly all glassware with warm diluted nitric acid (1 in 2) followed by water. Avoid using on the separator any lubricants that dissolve in chloroform.

**Special solutions and solvents**

**Alkaline ammonium citrate solution:** Dissolve 50 g of dibasic ammonium citrate in water to make 100 mL. Add 100 mL of ammonium hydroxide. Remove any heavy metals that may be present by extracting the solution with 20-mL portions of the *Dithizone extraction solution* (see *Lead* ⟨251⟩) until the *Dithizone extraction solution* retains a clear green color, then extract any dithizone remaining in the citrate solution by shaking with chloroform.

**Chloroform:** Distill chloroform in a hard or borosilicate glass apparatus, receiving the distillate in sufficient dehydrated alcohol to make the final concentration 1 mL of alcohol for each 100 mL of distillate.

**Dithizone solution:** Use the *Standard dithizone solution* (see ⟨251⟩), prepared with the distilled *Chloroform*.

**Standard zinc solution:** Dissolve 625 mg of zinc oxide, accurately weighed and previously gently ignited to constant weight, in 10 mL of nitric acid, and add water to make 500.0 mL. This solution contains 1.0 mg/mL of zinc.

**Diluted standard zinc solution:** Dilute 1 mL of the *Standard zinc solution*, accurately measured, with 2 drops of nitric acid and sufficient water to make 100.0 mL. This solution contains 10 µg/mL of zinc. Use this solution within 2 weeks.

**Trichloroacetic acid solution:** Dissolve 100 g of trichloroacetic acid in water to make 1000 mL.

**Procedure:** Transfer 1–5 mL of the preparation to be tested, accurately measured, to a centrifuge tube graduated at 40 mL. If necessary, add 0.25 N hydrochloric acid, dropwise, to obtain a clear solution. Add 5 mL of the *Trichloroacetic acid solution* and sufficient water to make 40.0 mL. Mix and centrifuge.

Transfer to a hard-glass separator an accurately measured volume of the supernatant believed to contain 5–20 µg of zinc, and add water to make about 20 mL. Add 1.5 mL of the *Alkaline ammonium citrate solution* and 35 mL of the *Dithizone solution*. Shake vigorously 100 times. Allow the chloroform phase to separate. Insert a cotton plug in the stem of the separator to remove any water emulsified with the chloroform. Collect the chloroform extract (discarding the first portion that comes through) in a test tube, and determine the absorbance at 530 nm, with a suitable spectrophotometer.

Calculate the amount of zinc present by reference to a standard absorbance–concentration curve obtained by using 0.5, 1.0, 1.5 mL, and, if the zinc content of the sample extracted exceeds 15 µg, 2.0 mL of the *Diluted standard zinc solution*, corrected as indicated by a blank determination run concomitantly, using all of the reagents but no added zinc.

### • Ion Chromatographic Method

The following ion chromatographic general procedure is provided for the determination of zinc in compendial articles. See *Ion Chromatography* ⟨1065⟩ for discussion of the theory and principles of measurements using ion chromatography.

Use water with a resistivity of not less than 18 megohm-cm to prepare the solutions.

**Diluent:** 0.2% (w/v) hydrochloric acid

**Mobile phase:** 7.0 mM dipicolinic acid, 66.0 mM potassium hydroxide, 5.6 mM potassium sulfate, and 74.0 mM formic acid in water; adjust with 2 N potassium hydroxide TS to a pH of 4.2. Pass through a suitable filter of 0.2-µm pore size.

**Post-column derivatization reagent:** 0.5 mM 4-(2-pyridylazo)resorcinol monosodium salt, 1.0 M 2-dimethylaminoethanol, 0.50 M ammonium hydroxide, and 0.30 M sodium bicarbonate in water. Stir and sonicate until the solid is completely dissolved. Pass through a suitable filter of 0.2-µm pore size.

**Standard stock solution:** 1500 µg/mL of zinc from USP Zinc Oxide RS prepared as follows. Transfer an appropriate portion of USP Zinc Oxide RS to a suitable volumetric flask. Add 6 N hydrochloric acid to about 10% of the final flask volume to dissolve. Dilute with water to volume.

**Standard solution:** 15.0 µg/mL of zinc in *Diluent* from the *Standard stock solution*

**Sample stock solution:** Prepare as directed in the monograph.

**Sample solution:** Equivalent to 15.0 µg/mL of zinc in *Diluent* from the *Sample stock solution*, unless otherwise stated in the monograph

**Chromatographic system**

(See *Chromatography* ⟨621⟩, *System Suitability*.)

**Mode:** LC

**Detector:** Vis 530 nm

**Columns**

**Guard:** 4.0-mm × 5-cm; 9-µm packing L100

**Analytical:** 4.0-mm × 25-cm; 9-µm packing L100

**Column temperature:** 30°

**Flow rate:** 1.2 mL/min

**Flow rate of post-column reagent:** 0.6 mL/min. Introduce using a pulseless flow of reagent through a 375-µL polymeric mixing coil or other suitable volume coil.[1]

**Injection volume:** 10 µL

**Run time:** Not less than 2 times the retention time of zinc

[1] A knitted reaction coil, part number 043700, available from ThermoFisher Scientific (www.thermofisher.com), may be suitable.

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**System suitability**
**Sample:** *Standard solution*
**Suitability requirements**
**Tailing factor:** Not more than 2.0
**Relative standard deviation:** Not more than 0.73%, unless otherwise stated in the monograph
**Analysis**
**Samples:** *Standard solution* and *Sample solution*
Unless otherwise stated in the monograph, calculate the concentration of zinc in the portion of *Sample solution* taken:

$$\text{Result} = (r_U/r_S) \times C_S$$

$r_U$ = peak response from the *Sample solution*
$r_S$ = peak response from the *Sample solution*
$C_S$ = concentration of zinc in the *Standard solution* (µg/mL)

***Change to read:***

• **Atomic Absorption Method**
The following atomic absorption general procedure is provided for the determination of zinc in compendial articles.
**Diluent:** 0.01 N hydrochloric acid
**Zinc stock standard solution:** Use a certified 1000-µg/mL standard.
▲▲ (USP 1-May-2022)
**Zinc calibration standard solutions:** Prepare not less than 3 calibration standard solutions within the range of 0.2–1.6 µg/mL, or suitable concentrations to span anticipated zinc concentrations in the sample(s). Prepare these solutions by two-step dilutions of the *Zinc stock standard solution* with the *Diluent*.
**Drug substance sample solution:** Accurately weigh an amount of the sample and dissolve in *Diluent*. Dilute with *Diluent* to volume in a volumetric flask; dilute the solution such that the zinc concentration is within the concentration range of the prepared *Zinc calibration standard solutions*.
**Drug product sample solution:** If the sample is a suspension, resuspend and add 4–7 µL of 6 N hydrochloric acid,▲to each milliliter of the suspension▲ (USP 1-May-2022) as needed to dissolve prior to dilution. Dilute the sample with *Diluent* to a zinc concentration within the concentration range of the prepared *Zinc calibration standard solutions*.
**Instrumental conditions**
(See *Atomic Absorption Spectroscopy* ⟨852⟩.)
**Mode:** Atomic absorption spectrophotometry
**Analytical wavelength:** 213.9 nm
**Lamp:** Zinc hollow-cathode, 5–15 mA
**Flame:** Air–acetylene of suitable composition
**Blank:** 0.01 N hydrochloric acid
**Analysis:** Zero the instrument using the *Blank*. Determine the absorbance in triplicate, of the *Blank*, ▲▲ (USP 1-May-2022)the *Zinc calibration standard solutions*, and the *Sample solutions*.
**Calibration:** Prepare a calibration curve from the mean of the readings of the absorbance of the *Blank* and the *Zinc calibration standard solutions* (linear or quadratic fit).
**System suitability**
▲**Samples:** *Zinc calibration standard solutions*
**Suitability requirements**
**Correlation coefficient:** NLT 0.997, determined from the standard curve
**Relative standard deviation:** NMT 2.5%, for the triplicate measurements for each aspiration of the *Zinc calibration standard solutions* with the highest concentration▲ (USP 1-May-2022)
**Calculations:** Read the concentration of zinc in the sample solution using the calibration curve and calculate the concentration of zinc in the sample.

## ADDITIONAL REQUIREMENTS

• **USP Reference Standards** ⟨11⟩
USP Zinc Oxide RS

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# ⟨601⟩ INHALATION AND NASAL DRUG PRODUCTS: AEROSOLS, SPRAYS, AND POWDERS—PERFORMANCE QUALITY TESTS

***Change to read:***

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C.5 Next Generation Impactor with Pre-separator for Inhalation Powders and Nasal Powders

## INTRODUCTION

The major performance measures for inhalation and nasal aerosols, sprays, and powders relate to dose delivery to the patient, including *Delivered Dose Uniformity* (DDU) and relevant measures of particle size (optical and/or aerodynamic) depending on the dosage form. [NOTE—The word "dose" should not be confused with the clinical term but is used for quality purposes to refer to the minimum amount required to make the test functional.] Each of these is described in the following sections. A nomenclature table appears in *Inhalation and Nasal Drug Products—General Information and Product Quality Tests* ⟨5⟩ from which the descriptive terms for various dosage forms can be obtained. [NOTE—Typically, the word "spray" is used for spray drug products (e.g., inhalation and nasal spray drug products), and "actuation" is used for inhalation aerosol and powder drug products. However, actuation(s) and spray(s) are used interchangeably in the methods in this chapter.]

## A. DELIVERED DOSE UNIFORMITY

### A.1 Inhalation Aerosols and Inhalation Sprays

The following tests are applicable to inhalation aerosols [commonly known as (pressurized) metered-dose inhalers] and inhalation sprays. Inhalation aerosols are formulated as suspensions or solutions of one or more drug substances in propellant(s) and possibly other suitable excipients and are presented as multidose units. Inhalation sprays typically are aqueous-based liquid formulations packaged in a compact container–closure system containing an integral spray pump unit. Refer to ⟨5⟩ for additional information. The following performance quality test methods are specific to these products and may require modification when alternative inhalation products (for example, breath-actuated inhalation aerosols or inhalation sprays) are tested. However, pharmacopeial requirements for all metered inhalation dosage forms require determination of the DDU and *Aerodynamic Particle Size Distribution* (APSD). In all cases and for all tests, prepare and test the product as directed on the label and the instructions for use. When these directions are not provided by the product manufacturer, follow the precise discharge directions included in the following tests.

#### A.1.1 DELIVERED DOSE UNIFORMITY OF INHALATION AEROSOLS AND INHALATION SPRAYS

The test for *Delivered Dose Uniformity* is required for inhalation aerosols and inhalation sprays containing drug formulation either in device-metered or in premetered unit presentations. The test for *Delivered Dose Uniformity* includes dose uniformity over the entire unit life. (For products packaged in premetered dosage units, see also *Uniformity of Dosage Units* ⟨905⟩.) A dose in this test is defined as the minimum recommended number of actuations/sprays specified in the product labeling or instructions for use but NMT 2 actuations/sprays per determination. For inhalation aerosols and inhalation sprays, the drug delivered from the mouthpiece is specified by the label claim. Its value reflects the expected mean drug content for a large number of delivered actuations/sprays collected from many units of the product using the method specified in the monograph. For drug products equipped with an integral spacer, it should be emphasized that the quantity to be measured (label claim) is the drug delivered at the mouthpiece of the spacer, under specified in vitro test conditions.

Unless otherwise directed in the individual monograph, the drug content of the delivered dose collected at the beginning of unit life (after priming as described on the label or instructions for use) and end of unit life of the label claim, will be determined from each of 10 separate containers (i.e., a total of 20 determinations).

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**A.1.1.1 Sampling the delivered dose from inhalation aerosols and inhalation sprays**

***DDU sampling apparatus A procedure:*** To determine the content of active ingredient in the discharged plume from an inhalation aerosol and inhalation spray, use DDU sampling apparatus A (see *Figure 1a* and *Table 1*).

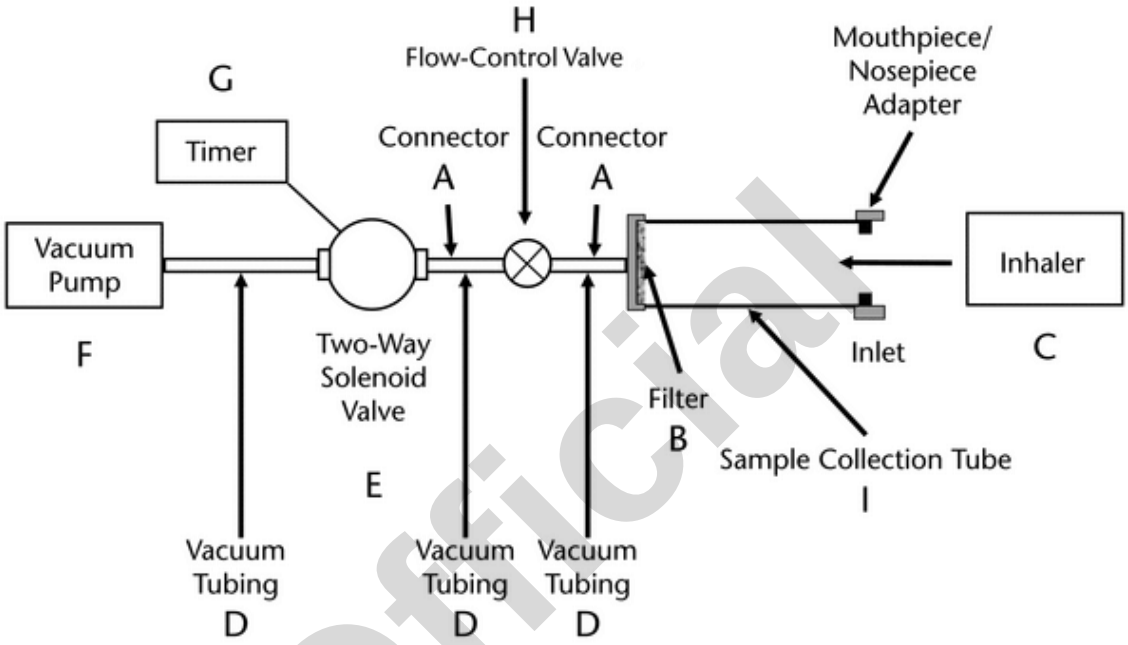


Figure 1a. DDU sampling apparatus for inhalation aerosol, inhalation spray, nasal aerosol, and nasal spray drug products. (See *Table 1* for component specifications for DDU sampling apparatus A.) [Note—The volumetric flow rate must be determined and fixed before the inhaler is inserted in *Figure 1a*.]

Unless otherwise specified in the individual monograph, with the vacuum pump running, ensure an airflow rate through the device of 28.3 L of air/min (±5%) or 30 L/min (±5%) as applicable for the apparatus selected for APSD determination. Prepare the product for use according to the label instructions for shaking, priming, and firing. Collect the minimum recommended number of actuations following priming. Discharge the first actuation of the minimum recommended actuations into the sampling apparatus through the mouthpiece adapter by actuating the metering system for a duration sufficient to ensure that the dose has been completely discharged. The volume of air sampled per actuation should not exceed 2.0 L (e.g., a timer solenoid valve can be used to achieve the required volume). If the minimum recommended dose consists of more than one actuation, wait for 5 s and collect the next actuation. The actuations between the two test samples (i.e., beginning and end of unit life) should be disposed of appropriately. Note that for inhalation aerosols the rate of discharges (number of discharges per unit time) to waste should not cause canister cooling that may affect subsequent test results. This should be evaluated for each drug product. Following each collection of the minimum number of actuations from each unit at each sequential test sample (NMT 2 actuations), detach the product from DDU sampling apparatus A (see *Figure 1a*), and disconnect the vacuum.

Separately assay the contents of the apparatus for drug for each delivered dose at the beginning and end of sequential test samples after rinsing the filter and the interior of the collection tube with a suitable solvent. A validated analytical method is employed to determine the amount of drug in each delivered dose, and data are reported as amount delivered and as a percentage of label claim.

***DDU sampling apparatus A:*** The DDU sampling apparatus is presented in *Figure 1a*. DDU Sampling Apparatus A consists of different accessories (see *Figure 1a* and *Table 1*) and a sample collection tube assembly (*Figure 2*). The latter includes a filter support base with an open mesh filter support such as a stainless steel screen. The sample collection tube is clamped or screwed to the filter support base, with a mouthpiece adapter to ensure an airtight seal between the sample collection tube and the mouthpiece. Use a mouthpiece adapter that ensures that the opening of the product's mouthpiece is flush with the front face

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or 2.5-mm indented shoulder in the sample collection tube to make an airtight seal. The sample collection tube is connected to a system comprising a vacuum pump and flow-control valve (see *Figure 1a*).

The vacuum pump should be capable of pulling air through the complete assembly, including the filter and the product to be tested, at the desired flow rate. Connect the flowmeter to set the flow rate for DDU analysis (e.g., 28.3 L/min (±5%) or 30 L/min (±5%) as applicable). Replace the flowmeter with the inhaler for DDU determination. The flow rate should be in accordance with the test requirements of the drug product being evaluated.

During tests of inhalation aerosols and sprays, air should be drawn continuously through the system to avoid loss of drug into the atmosphere. The filter support base is designed to accommodate 25-mm diameter filter disks (see *Figure 2*). At the flow rate used, the sample collection tube and the filter must be capable of quantitatively collecting the delivered dose. The filter and other materials used in the construction of the apparatus must be compatible with the drug and the solvents that are used to extract the drug from the filter. One end of the sample collection tube is designed to hold the filter tightly against the filter support base using an O-ring as needed. When assembled, the joints between the components of the apparatus are airtight so that when a vacuum is applied to the base of the filter, all of the air drawn through the DDU sampling apparatus passes through the device component. The description for *Figure 1b* appears in *A.3.1.1. Sampling the delivered dose from inhalation powders*.

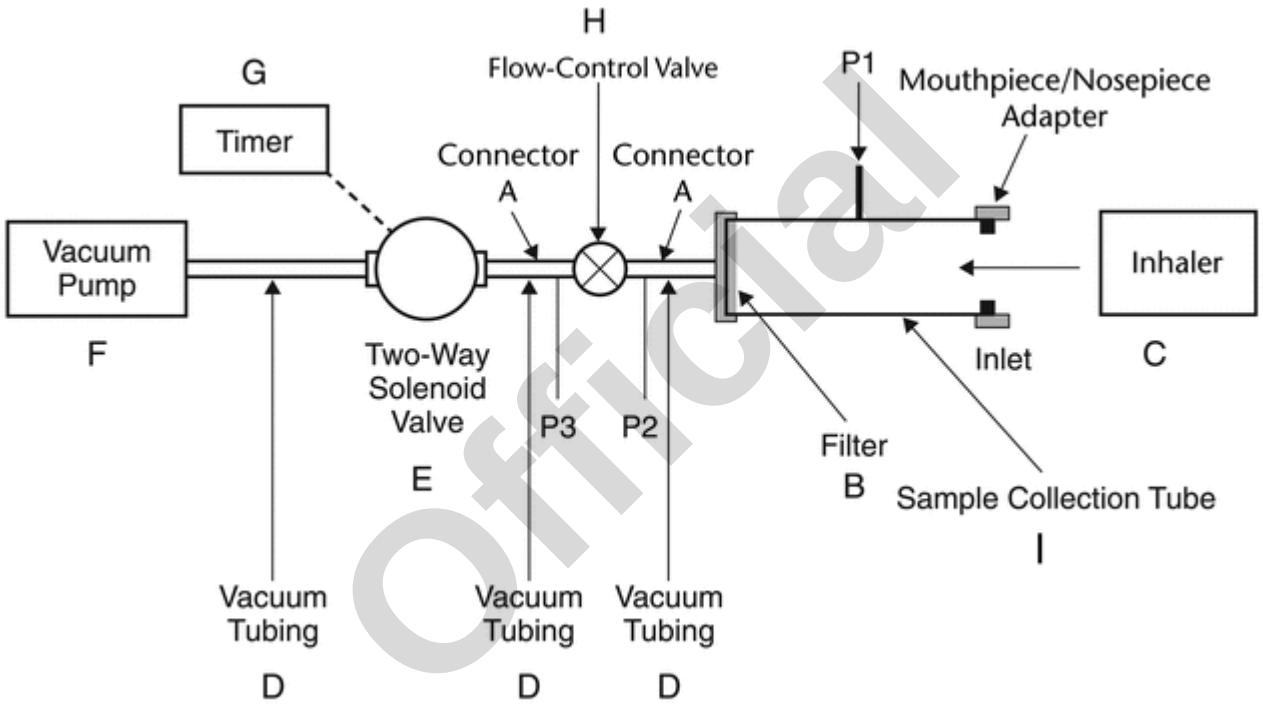


Figure 1b. DDU sampling apparatus for inhalation powder and nasal powder drug products. (See *Table 1* for component specifications for DDU sampling apparatuses A and B.) [Note—The volumetric flow rate must be determined and fixed before the inhaler is inserted.]

**Table 1. DDU Sampling Apparatuses A and B** (see *Figure 1a* and *Figure 1b*)

| Code | Item | Descriptions | |
|---|---|---|---|
| | | **DDU Sampling Apparatus A (inhalation aerosols, inhalation sprays, nasal aerosols, and nasal sprays)** | **DDU Sampling Apparatus B (inhalation powders and nasal powders)** |
| A | Connector | A short length of suitable vacuum tubing, e.g. silicone tubing with an inside diameter (ID) of 16 mm | A short length of suitable vacuum tubing, e.g. silicone tubing with an inside diameter of 16 mm with connectors to pressure taps at P2 and P3 |
| B | Filter | 25-mm glass fiber filter, stainless steel fiber filter, and microfiber polypropylene filter | 47-mm glass fiber filter, stainless steel fiber filter, and microfiber polypropylene filter |
| C | Flowmeter or inhaler | The flowmeter is for flow rate setting, and the inhaler is the product being tested. | |
| D | Vacuum tubing | See Code A. | |
| E | Two-way solenoid valve | Optional | 2-way, 2-port solenoid valve with an opening response time of ≤100 ms |

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**Table 1. DDU Sampling Apparatuses A and B** (see *Figure 1a* and *Figure 1b*) *(continued)*

| Code | Item | Descriptions | |
|---|---|---|---|
| | | **DDU Sampling Apparatus A (inhalation aerosols, inhalation sprays, nasal aerosols, and nasal sprays)** | **DDU Sampling Apparatus B (inhalation powders and nasal powders)** |
| F | Vacuum pump | The vacuum pump should be capable of pulling air through the complete assembly, including the filter and the product to be tested, at the desired flow rate. | Pump must be capable of drawing the required flow rate under critical (sonic) conditions (P3/P2 < 0.5) through the assembled apparatus with the test product seated in the mouthpiece/nosepiece adapter. |
| G | Timer | Optional | The timer switches current directly to the solenoid valve for the required duration. |
| H | Flow-control valve | Adjustable regulating valve with flow coefficient ($C_V$)[a] ≥ 1 | |
| I | Sample collection tube | 26.70-mm ID × 9.4-cm internal length | 34.85-mm ID × 12-cm internal length |
| P1 | Pressure tap | N/A | 2.2-mm ID, 3.1-mm outside diameter flush with the internal surface of the sample collection tube, centered and burr-free, 59 mm from its inlet. Pressure measurements determined under steady-state flow conditions with a differential pressure transducer. The pressure tap must not be open to the atmosphere during dose collection. |
| P2, P3 | Pressure tap | N/A | Pressure measurements determined under steady-state flow conditions with an absolute pressure transducer. The pressure taps must not be open to the atmosphere during dose collection. |

[a] Flow coefficient, as defined by ISA S75.02 Control valve capacity test procedures in *Standards and Recommended Practices for Instrumentation and Control*, 10th ed., Vol. 2. Research Triangle Park, NC: Instrument Society of America; 1989.

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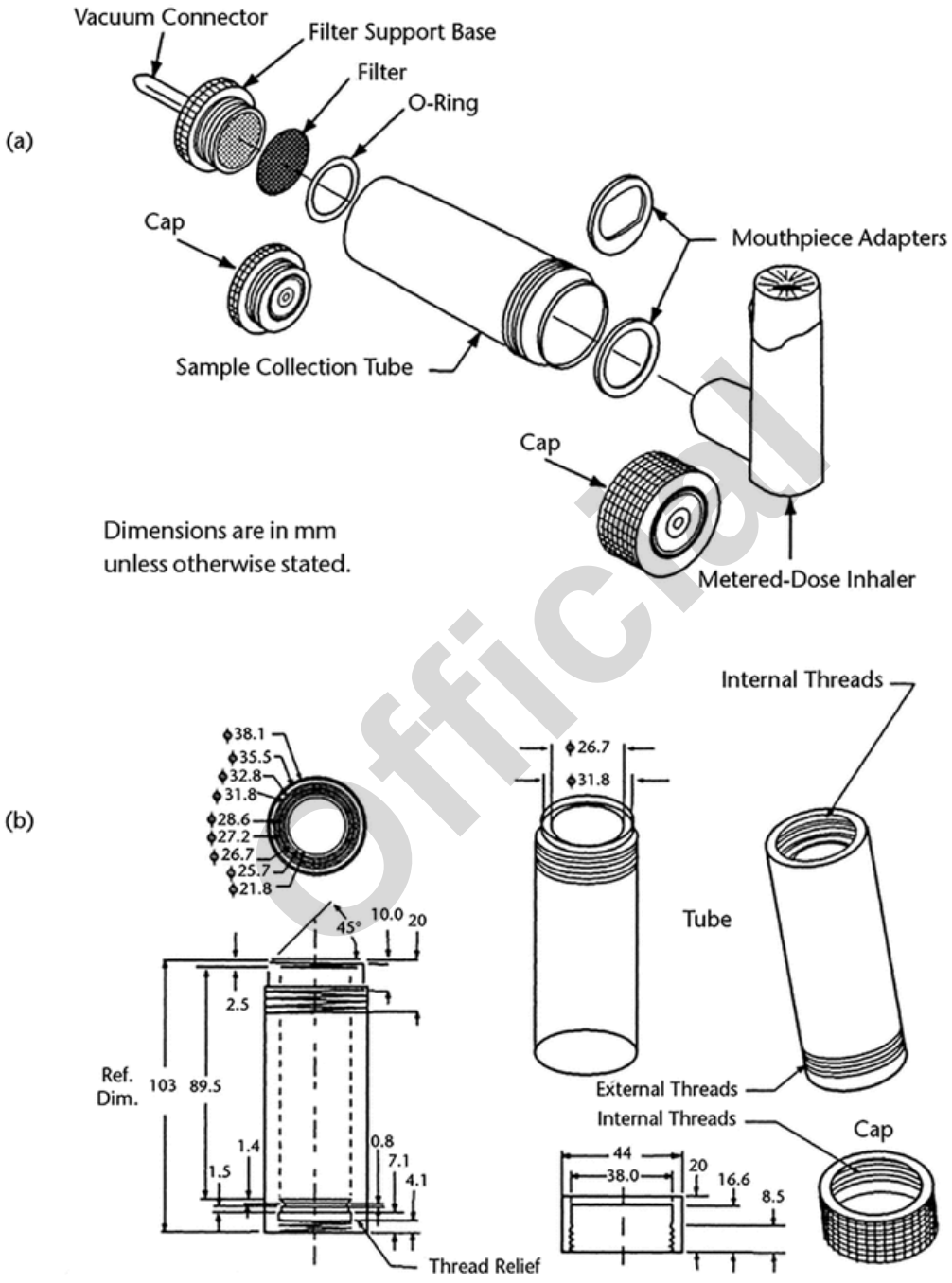


Figure 2. DDU sample collection tube (*Table 1*) assembly for inhalation and nasal aerosol, nasal spray, and nasal powder drug products. (a) tube assembly components and (b) dimensions of the components of Apparatus A, see *Table 1*. [Note—The caps as shown in *Figure 2* are used to seal the tube during the content evaluation. The figure is a visualization of the table, not an engineering drawing.]

[NOTE—The screw thread rinsing cap shown in *Figure 2* may be replaced by cap utilizing a bayonet fitting without changing any other aspect of the apparatus.]

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## A.2 Nasal Aerosols and Nasal Sprays

The following test is applicable to nasal aerosols and sprays formulated, respectively, as nonaqueous and aqueous suspensions or solutions of drug, presented typically in multidose containers, and fitted with dose-metering valves or pumps. In all cases and for all tests, prepare and test the spray as directed in the labeling and the instructions for use.

### A.2.1 DELIVERED DOSE UNIFORMITY OF NASAL AEROSOLS AND NASAL SPRAYS

Unless otherwise directed in the individual monograph, the drug content of the delivered dose collected at the beginning of unit life (after priming as described on the label or instructions for use) and end of unit life of the label claim, will be determined from each of 10 separate containers (i.e., a total of 20 determinations). This represents a total of 20 determinations. Sampling and measurements shall be made after priming as described in the labeling or instructions for use. A dose in this test is defined as the minimum recommended number of actuations/sprays specified in the product labeling or instructions for use but NMT 2 actuations per determination. For single-use products, *Delivered Dose Uniformity* of 10 dosage units should be determined separately.

**A.2.1.1 Sampling the delivered dose from nasal aerosols:** See *A.1.1.1 Sampling the delivered dose from inhalation aerosols and inhalation sprays*.

**A.2.1.2 Sampling the delivered dose from nasal sprays**

*Procedure:* To determine the content of active ingredient in the discharged plume from a nasal spray, use DDU sampling apparatus A (see *Figure 1a* and *Table 1*) described above. The use of a mechanical means of actuating the metering system or pump assembly to deliver doses for collection is recommended to ensure reproducible in vitro dose collection. The mechanical actuation procedure should have adequate controls for the critical mechanical actuation parameters (e.g., actuation force, actuation speed, stroke length, and rest periods). The test must be performed on units that have been thoroughly shaken and primed according to the instructions for patient use. The test unit should be actuated in a vertical or near-vertical, pump up position. The sprays at the beginning of each of the 10 test containers should be the sprays immediately following priming and the sprays at the end (label claim) of each from the same container. This represents a total of 20 determinations. The doses between the beginning and end sequential test samples must be disposed of appropriately. A validated analytical method is employed to determine the amount of drug in each delivered dose, and data are reported as amount delivered and as a percentage of label claim.

## A.3 Inhalation Powders

The following tests are applicable to inhalation powders (commonly known as dry powder inhalers (DPIs)) presented as premetered or device-metered units. Pharmacopeial requirements for all of these drug products require determination of each of the delivered doses and *Aerodynamic Particle Size Distribution*. In all cases and for all tests, prepare and test the product as directed in the labeling and the instructions for use. When these directions are not provided by the product manufacturer, follow the precise dose-discharge directions included in the following tests.

### A.3.1 DELIVERED DOSE UNIFORMITY OF INHALATION POWDERS

Where specified in the individual monograph, perform this test under conditions of controlled temperature and humidity.

The test for *Delivered Dose Uniformity* is required for inhalation powders in premetered (including ordered multiple dose) and device-metered presentations as labeled for use with the specified delivery system. The test for *Delivered Dose Uniformity* over the entire unit life is required for drug products packaged in device-metered or in ordered multiple-dose metering units of nominal premetered dosage units that have a predetermined dose sequence. For formulations packaged in premetered dosage units, an additional test, *Uniformity of Dosage Units* ⟨905⟩ is also required. Note that the target-delivered dose is determined by the mean drug content for a large number of delivered doses collected from many units under defined experimental conditions as specified in the product labeling. In many cases, the target value may depend on the manner in which the test for delivered dose uniformity is performed. For inhalation powders, where the label claim usually is the nominal premetered or device-metered dose of drug, the target-delivered dose and the method are specified in the individual monograph, and usually the target-delivered dose is less than the label claim.

Unless otherwise directed in the individual monograph, the drug content of the delivered dose from each of 10 separate units is determined separately.

The drug content of the delivered dose from each unit will be collected at the beginning and end of unit life per the label claim. Thus, two determinations will be obtained from each of 10 separate drug product units. This represents a total of 20 determinations.

For single-use products and for inhalation powders packaged in premetered single dosage form units, *Delivered Dose Uniformity* of 10 dosage units should be determined separately.

**A.3.1.1 Sampling the delivered dose from inhalation powders:** DDU sampling apparatus B should be used to determine the content of active ingredient emitted from the mouthpiece of an inhalation powder (see *Figure 1b* and *Table 1*). This apparatus is capable of sampling the emitted dose at a variety of flow rates.

***DDU sampling apparatus B procedure:*** Adjust the flow-control valve to provide the flow that has been determined to induce a 4-kPa pressure drop across the product to be tested and for a period of *T* seconds consistent with the withdrawal of 2.0 L of air from the mouthpiece of the product. [NOTE—If the flow rate and duration are defined otherwise in the monograph, adjust the system to within 5% of those values.] The volume of air sampled should not exceed 2.0 L. This flow rate should be fixed for all determinations for a specific drug product, established and validated during development studies as the suitable flow rate through the device to produce such pressure drop on the average. The flow rate should not be changed to compensate for device-to-device variability in flow resistance for the same product.

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Determine the test flow rate using DDU sampling apparatus B as follows. Insert the product into the mouthpiece adapter to ensure an airtight seal. For premetered products, use a device loaded with an empty capsule or blister. For device-metered products, use an unused device. In both cases, the flow path should be free of drug. Connect one port of a differential pressure transducer to the pressure tap, P1 (in *Figure 1b*), and leave the other open to the atmosphere. Switch on the pump, and open the two-way solenoid valve. Adjust the flow-control valve until the pressure drop across the product is 4.0 kPa. Ensure that critical (sonic) flow occurs in the flow-control valve by measuring the individual values for absolute pressure, P2 and P3, so that their ratio P3/P2 is ≤0.5. If this ratio of P3/P2 is not achieved, switch to a more powerful pump and remeasure the test flow rate. Critical (sonic) flow conditions in the flow-control valve are required in order to ensure that the volumetric flow rate drawn from the mouthpiece is unaffected by pump fluctuations and changes in flow resistance of the product. Remove the product and the mouthpiece adapter, and without disturbing the flow-control valve, measure the flow rate drawn from the mouthpiece, $Q_{out}$. A flowmeter is connected in an airtight fashion at the inlet to the sampling apparatus (see *Figure 1b*) using a suitable adapter. Use a flowmeter calibrated for the volumetric flow leaving the meter in an airtight fashion to directly determine $Q_{out}$. If the flow rate is >100 L of air/min, adjust the flow-control valve until $Q_{out}$ equals 100 L/min; otherwise, record the value of $Q_{out}$, and leave the flow-control valve undisturbed. Define the test flow duration in seconds, e.g., at 60 L/min, $T = 120/Q_{out}$, so that a volume of 2.0 L of air (±5%) is withdrawn from the product at the test flow rate $Q_{out}$, and adjust the timer controlling the operation of the two-way solenoid valve accordingly.

Prepare the device with powder for inhalation according to the labeled instructions. With the vacuum pump running and the solenoid valve closed, insert the product's mouthpiece horizontally into the mouthpiece adapter. Discharge the powder into DDU sampling apparatus B by activating the timer controlling the solenoid valve and withdrawing 2.0 L of air from the product at the previously defined flow rate. Repeat the whole operation $n - 1$ times beginning with the text, "Prepare the device with powder," where $n$ is the number of actuations defined in the labeling as the minimum recommended dose, but NMT 2 actuations per determination. Detach the inhalation powder device container from DDU sampling apparatus B, and disconnect the vacuum tubing, D (*Figure 1b*). *Assay*the contents of the apparatus for drug after rinsing the filter and the interior of the apparatus with a suitable solvent.

***DDU sampling apparatus B:*** The sample collection tube assembly is similar to that described in *Figure 2* for testing inhalation aerosols. In this case, however, the filter and sample collection tube have a larger internal diameter to accommodate a 47-mm diameter filter (*Table 1*). This feature enables dosage collection at higher flow rates—up to 100 L of air/min—when necessary. A mouthpiece adapter ensures an airtight seal between the sample collection tube and the mouthpiece of the inhalation powder being tested. The mouthpiece adapter must ensure that the tip of the product's mouthpiece is flush with the open end of the sample collection tube. Tubing connectors, if they are used, should have an internal diameter of ≥10 mm to preclude their own internal diameters from creating significant flow resistance. A vacuum pump with excess capacity must be selected in order to draw air at the designated volumetric flow rate through both the sampling apparatus and the product simultaneously. A timer-controlled, low-resistance, solenoid-operated, two-way valve is interposed between the vacuum pump and the flow-control valve to control the duration of flow. Flow control is achieved by ensuring that critical (sonic) flow occurs in the flow-control valve (absolute pressure ratio P3/P2 is ≤0.5 under conditions of steady-state flow).

A validated analytical method is employed to determine the amount of drug in each delivered dose, and data are reported as amount delivered and as a percentage of target-delivered label claim.

## A.4 Nasal Powders

The following test is applicable to nasal powders presented in premetered and device-metered units. In all cases and for all tests, prepare and test the powder as directed in the labeling and the instructions for use, but NMT two actuations per determination.

### A.4.1 DELIVERED DOSE UNIFORMITY OF NASAL POWDERS

Unless otherwise directed in the individual monograph, the drug content of the minimum number of delivered dose(s) will be determined at the beginning of unit life and also at the end of unit life from each of 10 separate containers. This represents a total of 20 determinations. For nasal powders packaged in single-dose units, *Delivered Dose Uniformity* can be applied on 10 dosage units.

**A.4.1.1 Sampling the delivered dose from nasal powders:** To ensure reproducible dose collection, it is recommended that appropriate means of operating the device assembly be used to deliver doses for collection. (See *A.3.1 Delivered Dose Uniformity of Inhalation Powders*, *A.3.1.1 Sampling the delivered dose from inhalation powders* for additional information regarding the sampling procedure.) The two separate determinations (NMT 2 actuations) include samples of the beginning and end of the unit life corresponding to the label claim from each of 10 units. The discharges between the two sequential beginning and end test samples for each unit must be disposed of appropriately. A validated analytical method is used to determine the amount of drug in each delivered dose. Data are reported as amount delivered and as a percentage of target-delivered label claim. DDU sampling apparatus B (*Figure 1b* and *Figure 2*) is recommended for nasal powders.

# B. DROPLET/PARTICLE SIZE DISTRIBUTION—NASAL AEROSOLS AND NASAL SPRAYS

For suspension or solution nasal aerosols and nasal sprays, the emitted droplet/particle size distribution should be determined for the delivered plume subsequent to delivery under specified experimental conditions. Assuming that the density of the liquid under investigation is constant irrespective of droplet size, the volume-based size distributions may be treated as mass-based size distributions for the purpose of data interpretation. If a laser diffraction method is used (for more detail, refer to the description of this method in *B.1 Droplet/Particle Size Determination by Laser Diffraction*), droplet size distribution can be estimated in terms of ranges for the 10th ($D_{10}$), 50th ($D_{50}$), and 90th ($D_{90}$) percentiles of the cumulative volume-based size

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distribution, as well as the span of the distribution, expressed as $[(D_{90} - D_{10})/D_{50}]$, and the percentage of droplets less than 10 µm. Note that $D_{50}$ is identical with the volume median diameter if the distribution is unimodal. Appropriate and validated or calibrated emitted droplet/particle size analytical procedures should be described in sufficient detail to allow accurate and reproducible assessment including the following:

- Complete information about the apparatus and accessories
- Theoretical model (Lorenz–Mie or Fraunhofer approximation)
- The application of a disabling option for one or more of the innermost detectors to mitigate beam steering effects (only applicable to nasal aerosols)
- Software version
- Sample placement with respect to the optical bench of the laser diffractometer
- Measurement range
- Beam width
- Laser trigger condition in connection with the initiation and termination of the measurement sequence
- Lower limit of detection (if laser triggering is not used)
- Obscuration limit (upper bound of detection range in terms of particle concentration)

## B.1 Droplet/Particle Size Determination by Laser Diffraction

Suspension and solution drug products intended for application to the nasal cavity produce liquid droplets that typically are much larger than the operating range for multistage inertial impactors. Thus, laser diffraction (sometimes referred to as low-angle laser light scattering) could be a complimentary and/or an alternative approach for determining the size distribution.

The theory and operating principles for laser diffraction are well described in the most recent version of ISO 13320. These systems use the light-scattering pattern produced by passage of a cloud of droplets through the measurement zone to develop the volume-based size distribution in an iterative process in which a theoretical model (either Fraunhofer or Lorenz–Mie) is used to interpret the data. The process is summarized schematically in *Figure 3*. [NOTE—If the Lorenz–Mie model option is chosen, it will be necessary to input values for the real (refraction) and imaginary (absorption) components of refractive index for the liquid being studied. Manufacturers of laser diffraction equipment provide this information for commonly encountered liquid media either on their website or upon inquiry.]

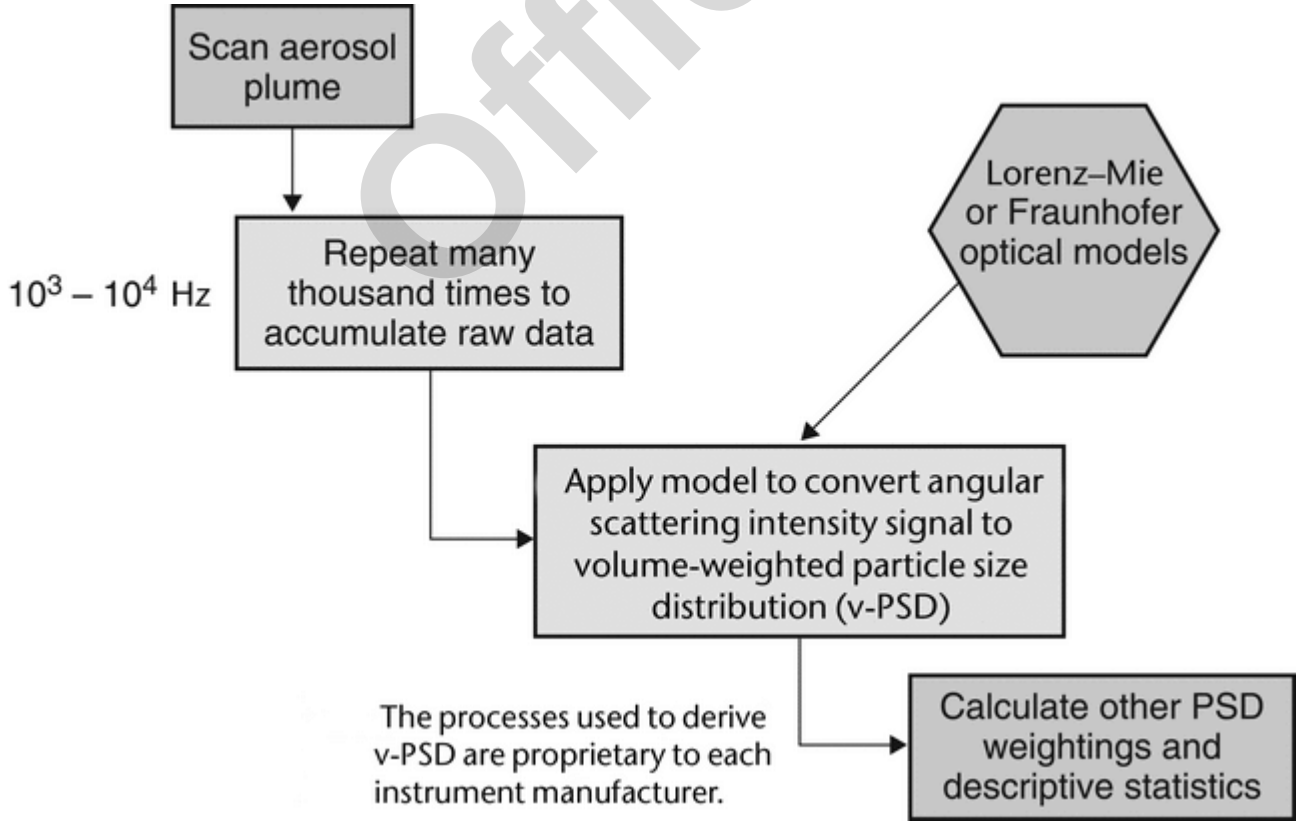


Figure 3. Conversion of light-scattering data into a droplet/particle size distribution by laser diffractometry. (PSD is particle size distribution.)

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An apparatus that has the capability to assess aerosols or sprays is required. [NOTE—Not all commercially available laser diffractions possess this capability.] Set up the measurement system in accordance with the procedure outlined schematically in *Figure 4*.

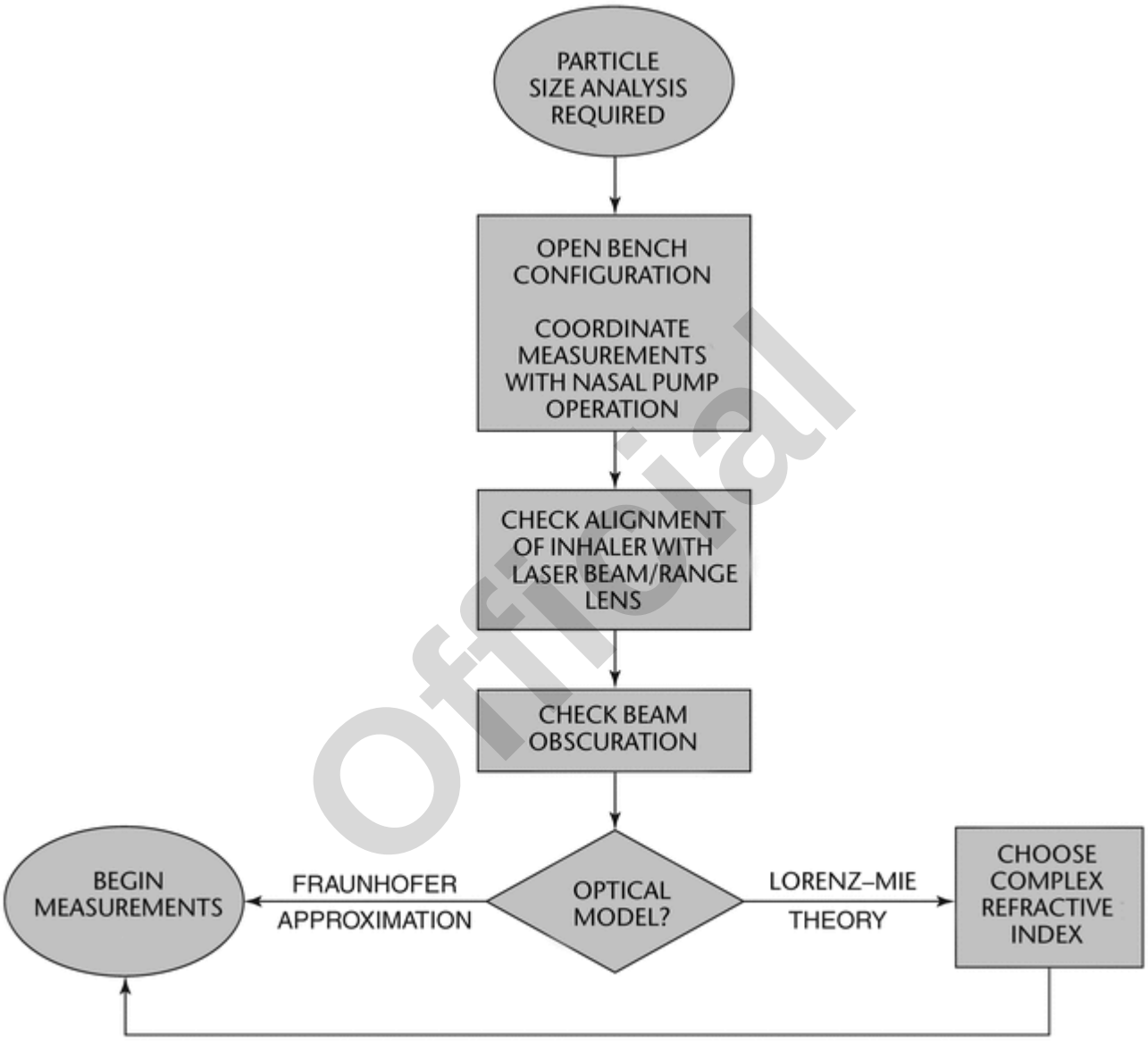


Figure 4. Setup for laser diffractometry with a nasal drug product.

Nasal spray drug products generally are determined with an open-bench arrangement with an automated actuation station setup so that reproducibility in nasal product operation from one actuation to the next can be optimized. Check the alignment of the inhaler plume intersecting with the optical configuration of the laser diffractometer using the tools provided by the manufacturer to optimize alignment of the optical bench. Check that the light beam obscuration is within the upper and lower limits specified by the manufacturer, consulting the operator's manual for the laser diffraction system being used. Vacuum extraction is required to efficiently remove droplets after they pass through the laser. An unrepresentative result may occur if this value is set too low (typically <5%). Unquantifiable error due to multiple scattering will happen if the obscuration is above the recommended upper limit. [NOTE—This limit varies from one manufacturer to another and may also vary from one laser diffractometer system to another from the same manufacturer.]

Conduct as many replicate measurements as needed to achieve a representative data set. Inspect each size distribution presented, ideally in differential volume-based format or equivalent to assess whether or not it is unimodal and symmetrical. A unimodal distribution is a requirement for data analysis.

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### B.2 Aerodynamic Particle Size Distribution—Determination By Cascade Impaction

The particle or droplet size distribution in the plume discharged from nasal aerosols may be determined similar to inhalation aerosols as described in *C. Aerodynamic Particle/Droplet Size Distribution—Inhalation Aerosols, Inhalation Sprays, Inhalation Powders, Nasal Aerosols, and Nasal Powders*.

## C. AERODYNAMIC PARTICLE/DROPLET SIZE DISTRIBUTION—INHALATION AEROSOLS, INHALATION SPRAYS, INHALATION POWDERS, NASAL AEROSOLS, AND NASAL POWDERS

### C.1 General Principles of Aerodynamic Particle/Droplet Size Distribution Measurement

The particle or droplet size distribution in the plume discharged from inhalation aerosols, inhalation sprays, nasal aerosols, and the particle size distribution in the cloud discharged from inhalation powders and nasal powders are important characteristics used in judging product quality and performance. The aerodynamic diameter of an aerosol particle is equal to the diameter of a sphere of unit density whose gravimetric settling velocity is the same. The aerodynamic size distribution defines the manner in which aerosol droplets/particles deposit during inhalation. In use, many inhalers discharge drug in the form of large droplets or particles (the “ballistic fraction”) that leave the inhaler at high velocity and impact on and are captured by the moist surfaces in the mouth, throat, or nose. The remainder of the discharge from the inhaler is the "nonballistic fraction" that is inhaled into the remainder of the respiratory tract.

Cascade impaction devices classify aerosol particles and droplets on the basis of aerodynamic diameters. The principle of their operation whereby they separate aerosol particles and droplets from a moving airstream on the basis of particle or droplet inertia is shown in *Figure 5*. The internal geometry, surface smoothness, and dimensions of the induction port (and pre-separator if used) may affect the mass of the drug that enters the cascade impactor. These dimensions are carefully defined and are held constant for all apparatuses. The surface roughness of the inlet should be around 0.40 μm.

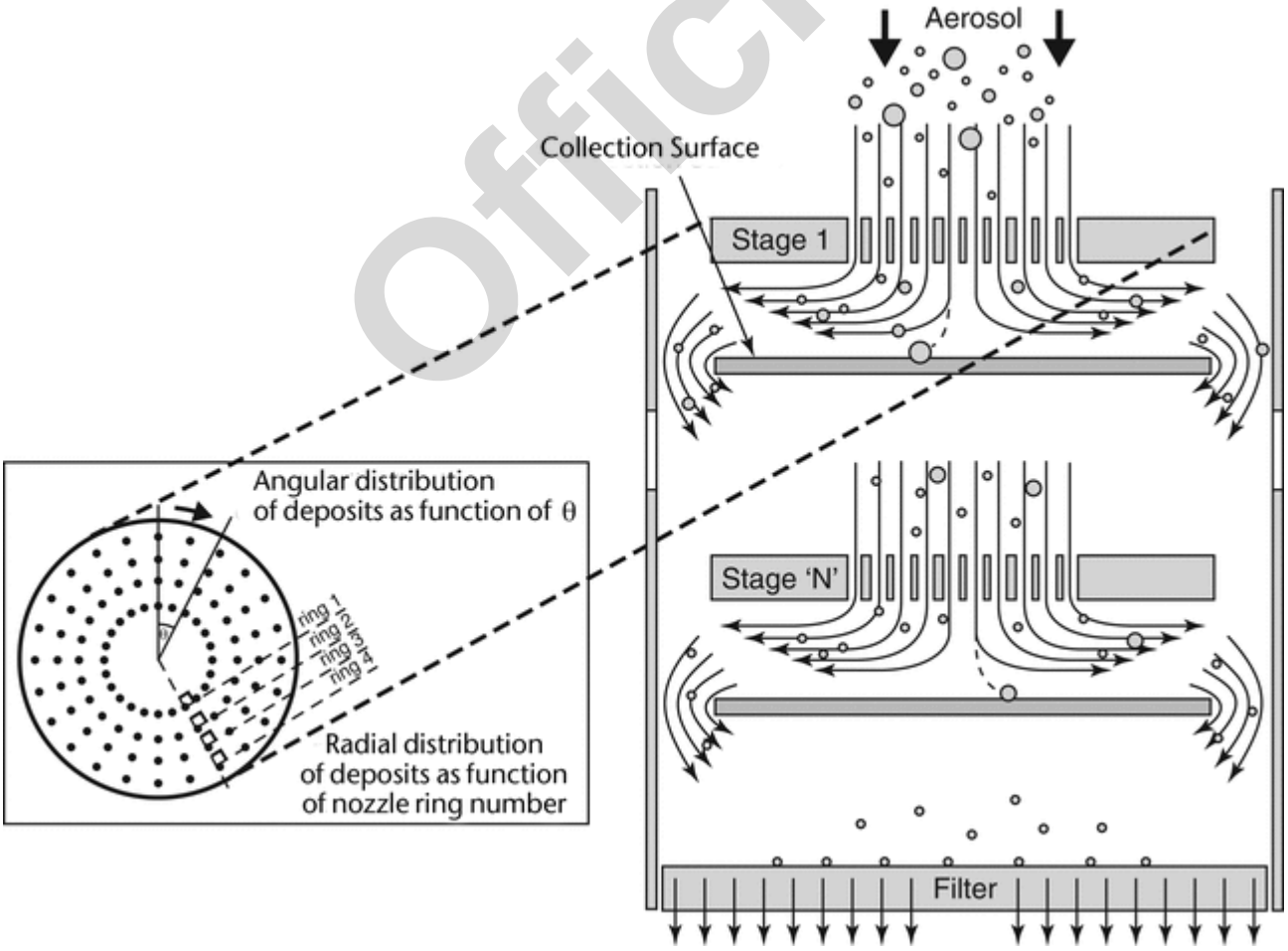


Figure 5. Schematic representation of the principle of operation of cascade impactors. [1]

[1] International Pharmaceutical Aerosol Consortium on Regulation & Science (IPAC-RS), used with permission.

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### C.1.1 STAGE MENSURATION

Calibration of a cascade impactor is a property of the nozzle dimensions, the spatial arrangement of the nozzles and its collection surface, and the flow rate passing through it. Because nozzles can corrode and wear over time, the effective diameter of the nozzles of each stage, which are related to the impaction stage's cut-off size, must be measured precisely on a regular basis. This process, known as stage mensuration, replaces the need for repetitive calibration using standard aerosols and ensures that only impactors that conform to the manufacturer's specifications for the stage nozzles are used for testing product output.

### C.1.2 INTERSTAGE DRUG LOSSES (WALL LOSSES)

The selected *Aerodynamic Particle Size Distribution* determination procedure should ensure that NMT 5% of the product's total delivered drug mass (into the impactor) is subject to wall loss between the impaction apparatus's collection surfaces. In the event that interstage drug losses are predetermined to be >5% for a given drug product, either the procedure should be modified (e.g. coating of impactor plates should be explored) in such a way that wall losses are decreased below this level or an alternative apparatus should be used. Wall losses on a particular stage should not be combined with the plate deposition to assay the mass deposited on a specific stage. It is appropriate to include wall deposition in the determination of the mass balance.

### C.1.3 RE-ENTRAINMENT

Where method variations are possible, the selected method should seek to minimize particle re-entrainment (from an upper to a lower impaction stage) on stages that contribute to size fractions defined in the individual monograph, especially where this may affect the amounts of drug collected. Minimizing the number of actuations, using coated particle-collection surfaces, and proving that multiple-actuation procedures produce results that are statistically similar to those from smaller numbers of actuations are all methods that can be used for this purpose. The number of actuations collected, the time interval between actuations, and the total duration of flow through the cascade impactor should be standardized for the measurement program by appropriately validated approaches.

By using validated analytical methods and a suitable mensurated impaction device, analysts can determine aerodynamic particle size distributions for drugs leaving the mouthpiece/nosepiece of inhalation aerosols, inhalation sprays, inhalation powders, nasal aerosols, or nasal powders. If temperature or humidity limits for use of the product are stated on the label, it may be necessary to control the temperature and humidity of the air surrounding and passing through the device to conform to those limits. Ambient conditions are presumed unless otherwise specified in individual monographs.

### C.1.4 MASS BALANCE

In addition to the size distribution, good analytical practice dictates that a mass balance be performed in order to confirm that the amount of the drug discharged from the product and recovered from the mouthpiece/nosepiece adapter, the induction port through to the after-filter of the cascade impactor apparatus, is within an acceptable range around the target-delivered label claim. The result for the recovered mass can be expressed on a per-actuation basis as a percent of the labeled delivered dose, which represents the dose delivered from the mouthpiece/nosepiece.

The total mass of drug collected in all of the components (mass balance) divided by the total number of actuations discharged per determination typically is NLT 85% and NMT 115% of the target-delivered label claim. This is not a test of the product but serves to ensure that the test results are valid.

### C.1.5 PROCEDURE

Use one of the following multistage impaction apparatuses to determine aerodynamic particle size distributions of drug(s) leaving the mouthpiece/nosepiece of inhalation aerosols, inhalation sprays, inhalation powders, nasal aerosols, or nasal powders. An appropriate minimum number of drug product units (e.g. 5) should be tested individually, and the determination for each unit should be performed with the minimum number of actuations justified by the sensitivity of the analytical procedure used to quantitate the deposited drug. The Andersen Cascade Impactor and Next Generation Impactor without pre-separator are intended for use with inhalation aerosols, inhalation sprays, and nasal aerosols at a single flow rate. The Andersen Cascade Impactor and Next Generation Impactor with pre-separator are intended for use with inhalation powders and nasal powders, unless it has been shown to be unnecessary, at the appropriate flow rate, $Q_{out}$, determined earlier during testing for *Delivered Dose Uniformity*, see *C.3.1 Design—Andersen Cascade Impactor with Pre-Separator* and *C.5.1 Design—Next Generation Impactor with Pre-Separator*. The volume of total air sampled per actuation should be 4.0 L.

*Table 2* indicates the apparatuses used to determine aerodynamic particle size and the products that they are used to evaluate. *Table 3a* and *Table 3b* provide the cut-off diameters of the impactors listed in *Table 2* at specified flow rates.

**Table 2. Aerodynamic Particle Size Apparatuses: Description and the Products That They Can Be Used to Evaluate**

| Description | Product |
|---|---|
| Andersen Cascade Impactor without pre-separator | Inhalation aerosols, inhalation sprays, and nasal aerosols |
| Andersen Cascade Impactor with pre-separator | Inhalation powders and nasal powders |
| Next Generation Impactor without pre-separator | Inhalation aerosols, inhalation sprays, and nasal aerosols |
| Next Generation Impactor with pre-separator | Inhalation powders and nasal powders |

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**Table 3a. Cut-Off Diameters (µm) for Andersen Cascade Impactor with and without Pre-Separator at 28.3 L/min Compared with Use at 60 and 90 L/min**

| Stage | 28.3 L/min | 60 L/min | 90 L/min |
|---|---|---|---|
| –2 | — | — | 8.0 |
| –1 | — | 8.6 | 6.5 |
| 0[a] | 9.0 | 6.5 | 5.2 |
| 1 | 5.8 | 4.4 | 3.5 |
| 2 | 4.7 | 3.2 | 2.6 |
| 3 | 3.3 | 1.9 | 1.7 |
| 4 | 2.1 | 1.2 | 1.0 |
| 5 | 1.1 | 0.55 | 0.22 |
| 6 | 0.7 | 0.26 | — |
| 7 | 0.4 | — | — |

[a] The version of Stage 0 used at 60 and 90 L/min (i.e. –0) has external modifications, permitting another stage rather than the inlet adapter cone to be fitted above it. Its internal characteristics and performance are unaltered.

**Table 3b. Cut-off diameters (µm) for Next Generation Impactor with and without Pre-separator at 30, 60, and 100 L/min**

| Stage | Flow Rate (30 L/min) | Flow Rate (60 L/min) | Flow Rate (100 L/min) |
|---|---|---|---|
| 1 | 11.72 | 8.06 | 6.12 |
| 2 | 6.40 | 4.46 | 3.42 |
| 3 | 3.99 | 2.82 | 2.18 |
| 4 | 2.30 | 1.66 | 1.31 |
| 5 | 1.36 | 0.94 | 0.72 |
| 6 | 0.83 | 0.55 | 0.40 |
| 7 | 0.54 | 0.34 | 0.24 |
| MOC[a] | 0.36 | 0.14 | 0.07 |

[a] MOC, micro-orifice collector. Sizes correspond to 80% collection efficiency for this back-up stage.

## C.2 Andersen Cascade Impactor without Pre-separator for Inhalation Aerosols, Inhalation Sprays, and Nasal Aerosols

Use the Andersen Cascade Impactor without pre-separator at a flow rate of 28.3 L/min (±5%) as specified by the manufacturer of the cascade impactor.

### C.2.1 DESIGN—ANDERSEN CASCADE IMPACTOR WITHOUT PRE-SEPARATOR

The design and assembly of this apparatus are shown in *Figure 6a*. The expanded view of the induction port and entrance cone to connect the impactor through the induction port to a drug product are shown, respectively, in *Figure 6b* and *Figure 6c*. The device is a cascade impactor with eight stages and an after filter. The material can be aluminum or stainless steel. Use this apparatus at a flow rate of 28.3 L/min (±5%) unless otherwise prescribed in the individual monograph.

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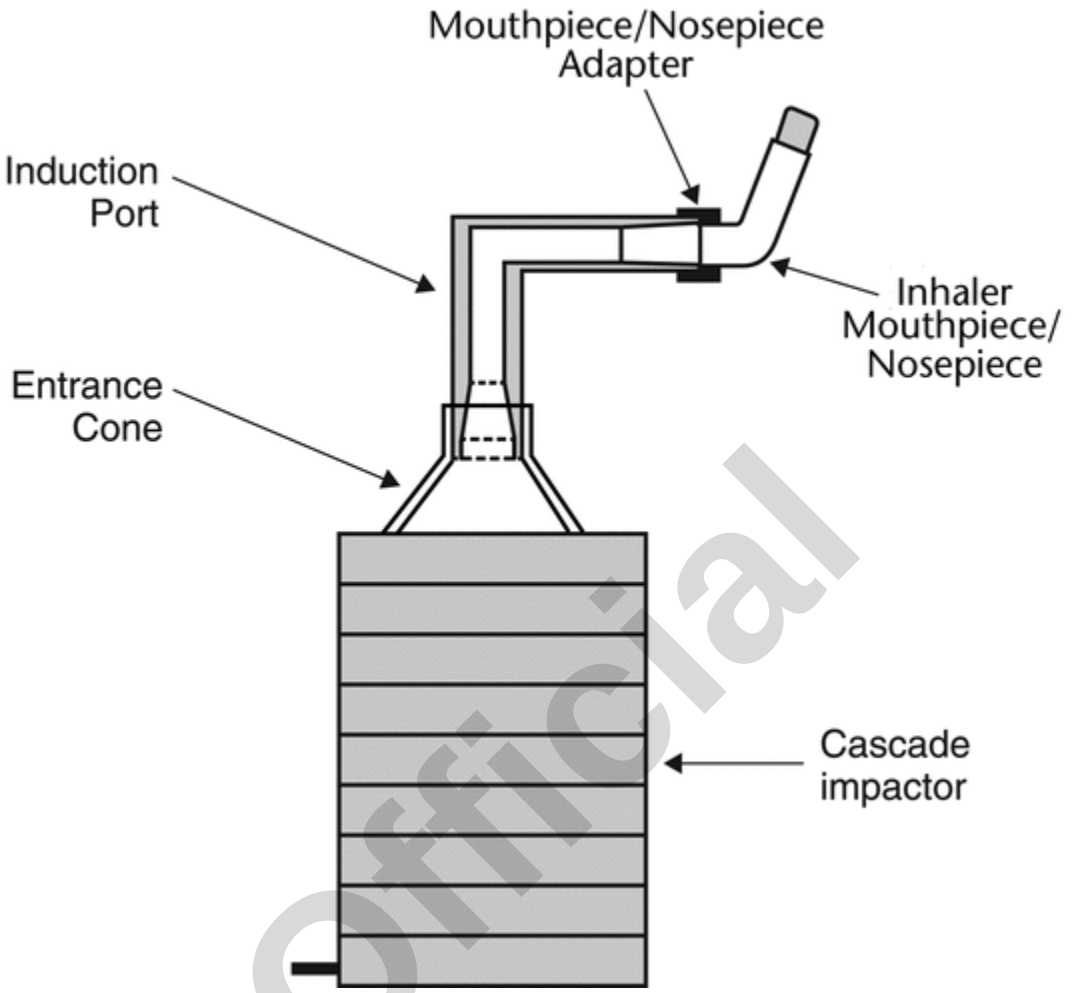


Figure 6a. Andersen Cascade Impactor without pre-separator: Assembly of induction port and entrance cone mounted onto cascade impactor.

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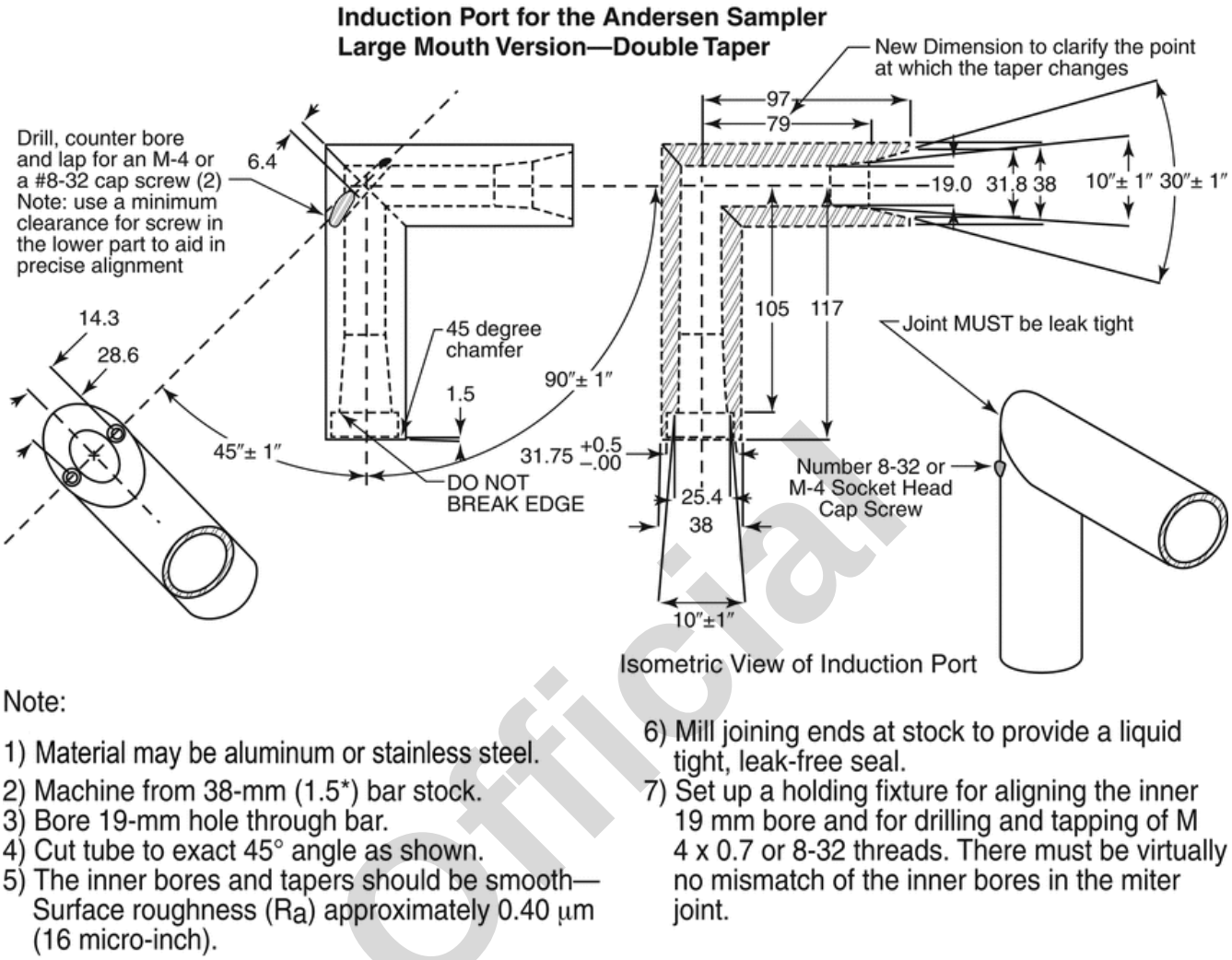


Note:

1) Material may be aluminum or stainless steel.
2) Machine from 38-mm (1.5*) bar stock.
3) Bore 19-mm hole through bar.
4) Cut tube to exact 45° angle as shown.
5) The inner bores and tapers should be smooth—Surface roughness ($R_a$) approximately 0.40 μm (16 micro-inch).
6) Mill joining ends at stock to provide a liquid tight, leak-free seal.
7) Set up a holding fixture for aligning the inner 19 mm bore and for drilling and tapping of M 4 x 0.7 or 8-32 threads. There must be virtually no mismatch of the inner bores in the miter joint.

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Figure 6b. Expanded view of induction port for use with cascade impactors.

[NOTE—All inhalation aerosol and powder drug products should employ the USP induction port (see *Figure 6b*). Specific monographs exist with a non-USP induction port because the product was developed before the USP induction port was available and subsequently approved by the FDA.] [NOTE—The external dimensions of the Next Generation Impactor version of the induction port are different than shown.]

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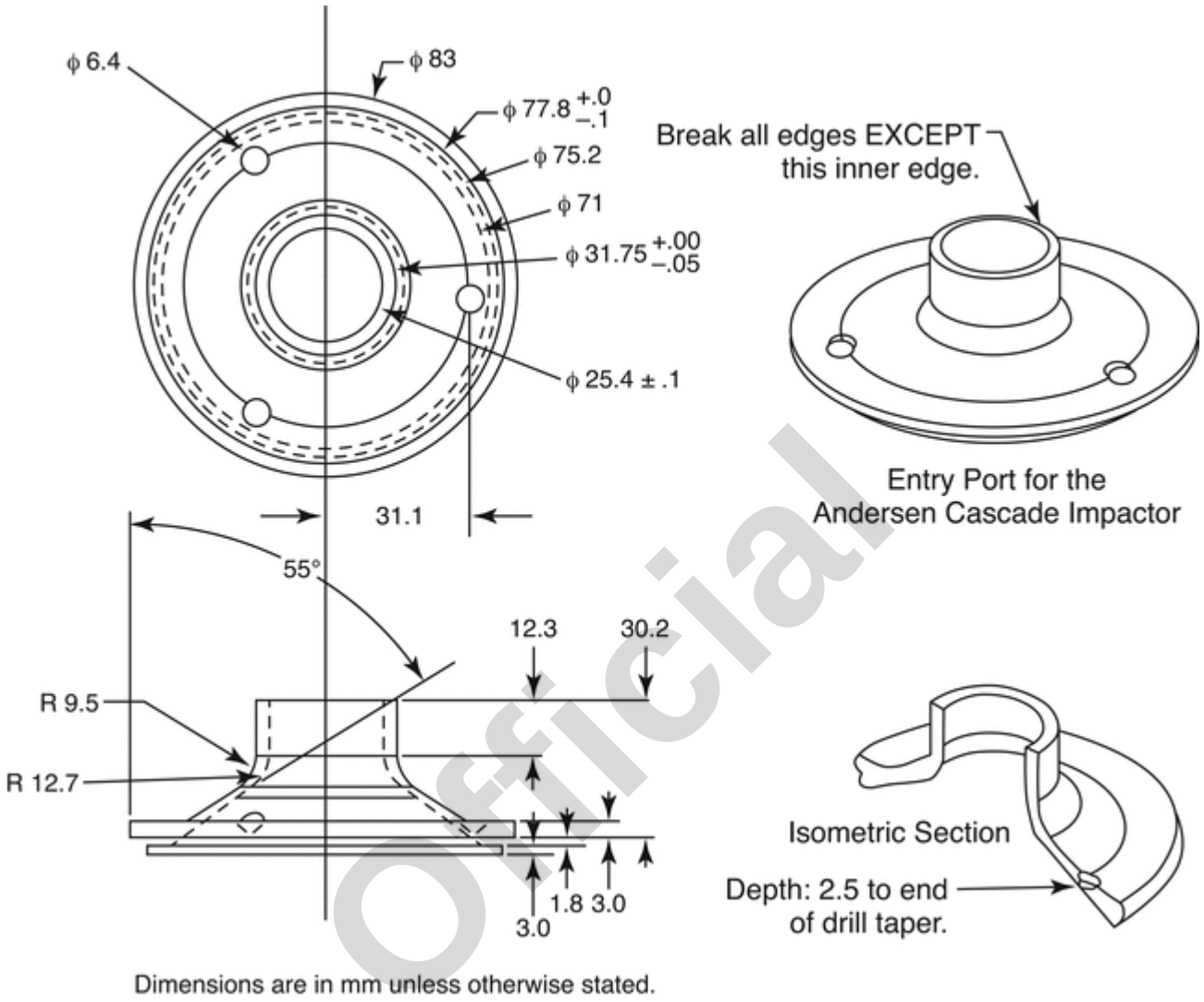


Figure 6c. Expanded view of the entrance cone for mounting induction port on the Andersen Cascade Impactor without pre-separator. Material can be aluminum or stainless steel. Surface roughness ($R_a$) should be approximately 0.40 µm.

Manufacturers' critical engineering dimensions for the stages of the Andersen Cascade Impactor are provided in *Table 4*. During use, some occlusion and blockage of nozzles may occur, and therefore, in-use mensuration tolerances must be justified.

**Table 4. Critical Dimensions for the Nozzles of the Andersen Cascade Impactor**

| Stage Number | Number of Nozzles | Nominal Nozzle Diameter (mm)[a] |
|---|---|---|
| 0 | 96 | 2.550 ± 0.025 |
| 1 | 96 | 1.890 ± 0.025 |
| 2 | 400 | 0.914 ± 0.013 |
| 3 | 400 | 0.711 ± 0.013 |
| 4 | 400 | 0.533 ± 0.013 |
| 5 | 400 | 0.343 ± 0.013 |
| 6 | 400 | 0.254 ± 0.013 |
| 7 | 201 | 0.254 ± 0.013 |

[a] Refer to manufacturer for specifications on tolerance/precision of the dimensions.

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### C.2.2 PROCEDURE—ANDERSEN CASCADE IMPACTOR WITHOUT PRE-SEPARATOR

Set up the multistage cascade impactor as described in the manufacturer's literature with an after-filter below the final stage to capture any fine particles that otherwise would escape from the impactor. To ensure efficient particle capture, coat the particle collection surface of each stage appropriately with glycerol, silicone oil, or other suitable liquid unless this has been demonstrated to be unnecessary. Attach the mouthpiece/nosepiece adapter to the end of the induction port to produce an airtight seal between the product mouthpiece/nosepiece and the induction port as shown in *Figure 6a*. Use a mouthpiece adapter that ensures that the tip of the product's mouthpiece/nosepiece is flush with the open end of the induction port. Ensure that the various stages of the cascade impactor are connected with airtight seals to prevent leaks (seals should be periodically inspected for cracks and replaced as necessary). Turn on the vacuum pump to draw air through the cascade impactor, and calibrate the flow rate through the system with an appropriate flowmeter attached to the open end of the induction port.

Adjust the flow-control valve on the vacuum pump to achieve steady flow through the system at 28.3 L/min (±5%). Prepare the product for use according to the label instructions for shaking, priming, and firing. With the vacuum pump running, insert the mouthpiece/nosepiece of the inhaler into the mouthpiece/nosepiece adapter and immediately fire into the cascade impactor the first of the minimum recommended number of actuations. When testing, keep the valve depressed for a duration sufficient to ensure that the actuation has been completely discharged. If additional sprays are required for the sample, wait for 5 s before firing the next actuation. Repeat until the required number of actuations has been discharged. The number of minimum recommended actuations discharged per determination must be sufficient to ensure an accurate and precise determination of the *Aerodynamic Particle Size Distribution*, but not excessive to mask the variability of individual doses. After the last actuation has been discharged, remove the product from the mouthpiece/nosepiece adapter. Rinse the mouthpiece/nosepiece adapter and the induction port with a suitable solvent, and dilute quantitatively to an appropriate volume. Disassemble the cascade impactor, place each collection plate or filter in a separate container, and rinse the drug from each of them. [NOTE—Analyze all components separately. Only data from analysis of deposition on the collection plates should be used for the purpose of determination of stage deposition. The data from analysis of deposition on the other components should only be used for the purpose of establishing the determination of mass balance and should not be combined with the plate deposition for determination of aerodynamic particle size distribution.] Dilute each sample quantitatively to an appropriate volume. Using an appropriately validated method of analysis, determine the mass of drug collected in each of the components.

## C.3 Andersen Cascade Impactor with Pre-separator for Inhalation Powders and Nasal Powders

### C.3.1 DESIGN—ANDERSEN CASCADE IMPACTOR WITH PRE-SEPARATOR

The Andersen Cascade Impactor with pre-separator (*Figure 7a*) is identical to the Andersen Cascade Impactor without pre-separator (*Figure 6a*) except that the manufacturer's pre-separator is added atop modified Stage 0 (28.3 L/min), Stage –1 (60 L/min), or Stage –2 (90 L/min) to collect large particles of noninhalable powder prior to their entry into the impactor. (Note that there are different external fittings for the pre-separator for the 28.3, 60, and 90 L/min configurations of the Andersen Cascade Impactor.) To connect the pre-separator of the impactor to the induction port, use the top shown in *Figure 7b*. The impactor, therefore, has eight stages, a pre-separator, and an after-filter. A pre-separator may not be required for certain engineered powders, notably those of low bulk density. Connect the cascade impactor into the control system specified in *Figure 7c*.

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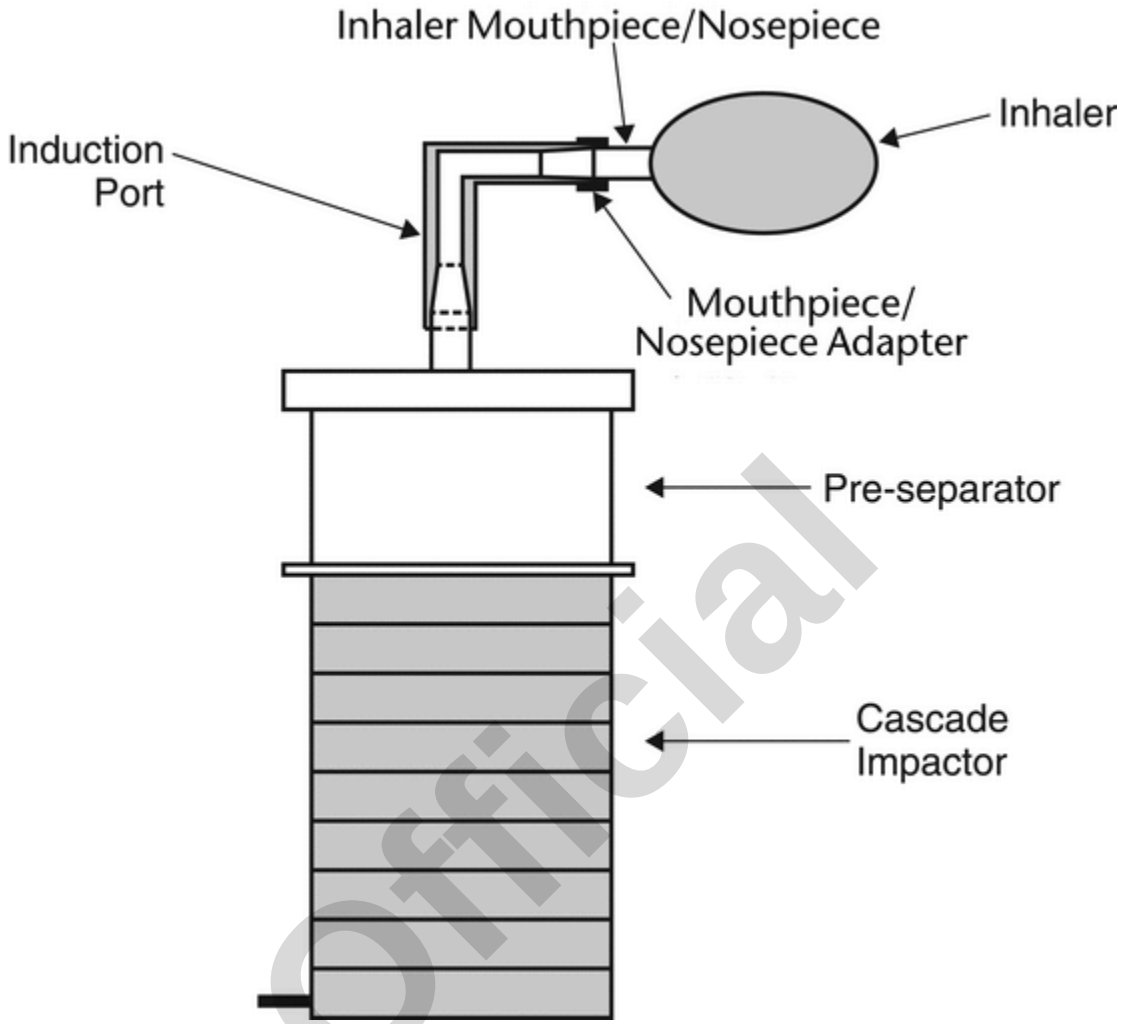


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Figure 7a. Schematic representation of Andersen Cascade Impactor with pre-separator: Assembly of induction port mounted on cascade impactor with pre-separator.

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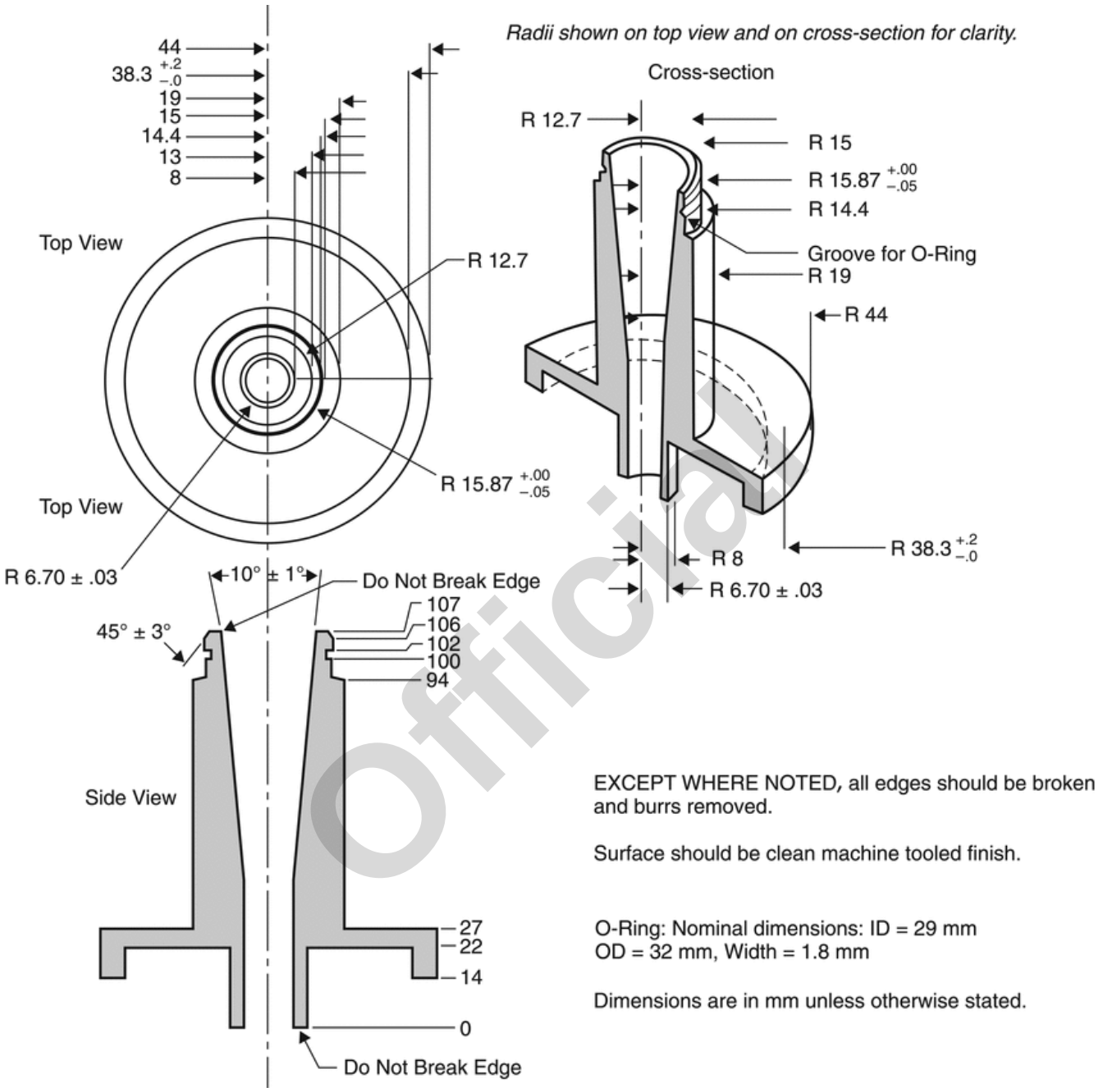


Figure 7b. Expanded views of top for the Andersen Cascade Impactor pre-separator for use at 28.3 L/min, adapted to the USP induction port. Material may be aluminum or stainless steel; interior bore should be polished to a surface roughness ($R_a$) of approximately 0.40 µm.

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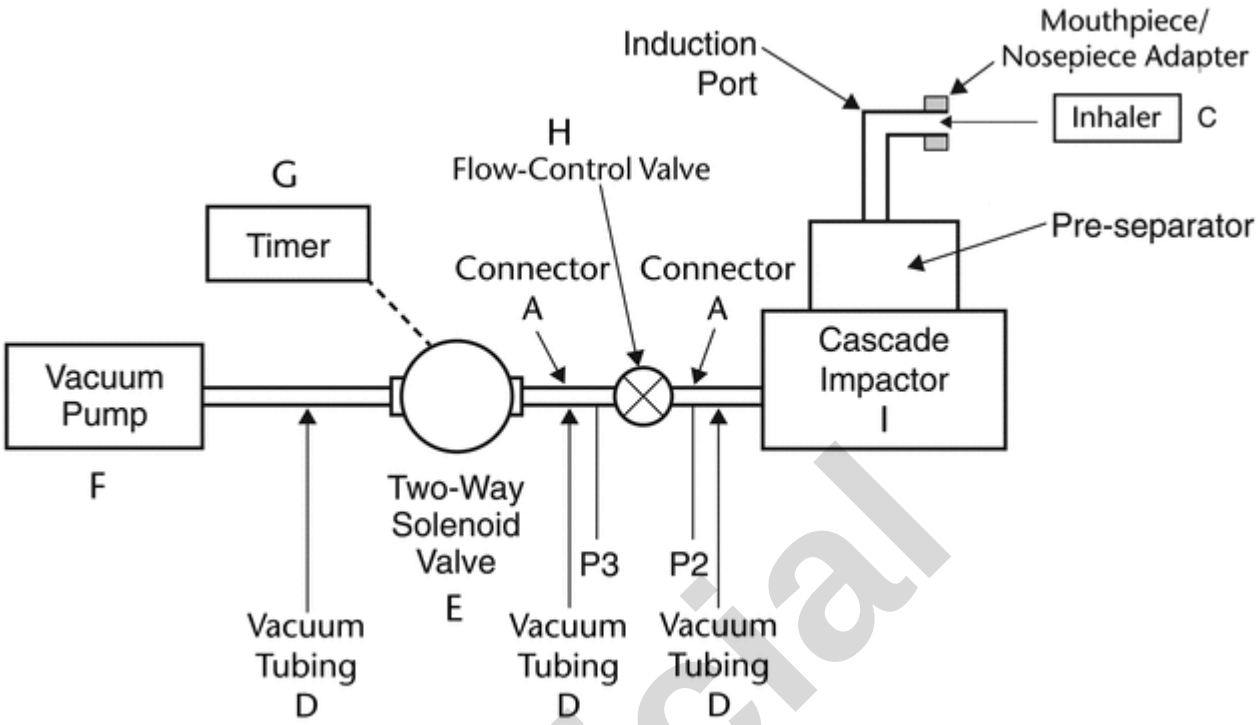


Figure 7c. Aerodynamic particle size distribution determination apparatus using Andersen Cascade Impactor or the Next Generation Impactor with pre-separator. The pre-separator is omitted for inhalation and nasal aerosol and inhalation spray drug products. (See *Table 5* for component specifications.) [Note—The volumetric flow rate must be determined and fixed before the inhaler is inserted in *Figure 7c*.]

**Table 5. Component Specifications for** *Figure 7c*

| Code | Item | Description | Other specifications |
|---|---|---|---|
| A | Connector | A short length of suitable vacuum tubing, e.g., silicone tubing with an inside diameter (ID) of 16 mm with connectors to pressure taps at P2 and P3 with an internal volume of 25 ± 5 mL | — |
| B | Not applicable | | |
| C | Inhaler | — | — |
| D | Vacuum tubing | A short length of suitable vacuum tubing, e.g. silicone tubing with an inside diameter of 16 mm. The internal volume of the tubing between the impactor and the flow control valve should be 25 ± 5 mL. | — |
| E | Two-way solenoid valve | See *Figure 7c* | 2-way, 2-port solenoid valve with an opening response time of ≤100 ms |
| F | Vacuum pump | See *Figure 7c* | Pump must be capable of drawing the required flow rate through the assembled apparatus with the test product seated in the mouthpiece/nosepiece adapter. Connect the pump to the solenoid valve using a short length of suitable vacuum tubing (as described in D) and connectors to minimize pump capacity requirements. |
| G | Timer | N/A | The timer switches current directly to the solenoid valve for the required duration. |
| H | Flow-control valve | See *Figure 7c* | Adjustable regulating valve with flow coefficient ($C_v$)[a] ≥ 1 |

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**Table 5. Component Specifications for** *Figure 7c (continued)*

| Code | Item | Description | Other specifications |
|---|---|---|---|
| I | Cascade impactor | See *Table 2* | Andersen Cascade Impactor with or without preseparator or Next Generation Impactor with or without preseparator |
| P2, P3 | Pressure tap | N/A | Pressure measurements determined under steady-state flow conditions with an absolute pressure transducer. The pressure taps must not be open to the atmosphere during sample collection. |

[a] Flow coefficient, as defined by ISA S75.02 Control valve capacity test procedures in *Standards and Recommended Practices for Instrumentation and Control*, 10th ed., Vol. 2. Research Triangle Park, NC: Instrument Society of America; 1989.

For operation at flow rates of 60 and 90 L/min, use manufacturers' alternate stage configurations. To ensure efficient particle capture, coat the particle collection surface of each stage appropriately with glycerol, silicone oil, or other suitable liquid unless this has been demonstrated to be unnecessary. Assemble the impactor as described in the manufacturer's literature with an after-filter below the final stage to capture any fine particles that otherwise would escape from the impactor. Place an appropriate predefined volume (up to 10 mL) of an appropriate solvent into the pre-separator, or coat the particle collection surfaces of the pre-separator to prevent re-entrainment of impacted particles. [**CAUTION**—Some solvents form flammable vapor–air mixtures that may be ignited during passage through a vacuum pump. Take appropriate precautions (alternative solvents, use of vapor traps, minimal pump operating times, etc.) to ensure operator safety during testing.] Attach a mouthpiece/nosepiece adapter to the end of the induction port to produce an airtight seal between the product's mouthpiece/nosepiece and the induction port. Use a mouthpiece/nosepiece adapter that ensures that the tip of the product's mouthpiece/nosepiece is flush with the open end of the induction port. Ensure that the various stages of the cascade impactor are connected with airtight seals to prevent leaks.

For flow rate calibration, turn on the vacuum pump, open the two-way solenoid valve, and determine the flow rate testing through the system as follows. Connect a flowmeter to the induction port. Use a flowmeter calibrated for the volumetric flow leaving the meter to directly determine $Q_{out}$. Adjust the flow-control valve to achieve a steady flow through the system at the required rate, $Q_{out}$, so that $Q_{out}$ is within ±5% of the value determined during testing for *Delivered Dose Uniformity*. Under steady-state flow conditions at the valve and at the flow rate to be used during testing ensure critical flow exists, by using the following procedure. With the product in place and the intended flow running, measure the absolute pressure on both sides of the flow-control valve (P2 and P3 in *Figure 7c*). A ratio of P3/P2 $\leq$0.5 indicates critical flow. If this ratio of P3/P2 is not achieved, switch to a more powerful pump, and remeasure the test flow rate. Adjust the timer controlling the operation of the two-way solenoid valve so that it opens this valve for a duration of 2$T$ seconds based on determination of duration of $T$ seconds for testing of *Delivered Dose Uniformity*.

### C.3.2 PROCEDURE—ANDERSEN CASCADE IMPACTOR WITH PRE-SEPARATOR

Perform the test using the Andersen Cascade Impactor with pre-separator at the flow rate, $Q_{out}$ determined earlier during testing for *Delivered Dose Uniformity*. Connect the apparatus to a flow-control system that is based on critical (sonic) flow as specified in *Figure 7c* (see also *Table 5*). Coat the particle collection surface of each of the stages of the cascade impactor appropriately to ensure that particles that have impacted on a given stage are not re-entrained in the flowing airstream or to reduce particle bouncing unless this has been shown to be unnecessary.

Prepare the inhalation powder or nasal powder according to the labeled instructions. With the vacuum pump running and the two-way solenoid valve closed, insert the product's mouthpiece/nosepiece, held horizontally, into the mouthpiece/nosepiece adapter. Once the product is positioned, discharge the powder into the apparatus by opening the two-way solenoid valve for the required duration of 2$T$ s, to sample 4.0 L of air, based on determined duration of $T$ seconds for testing of *Delivered Dose Uniformity*. After the total volume of 4.0 L of air per actuation has been sampled, the two-way solenoid valve is closed and the product is removed. If additional actuations are required for a determination, reload the inhalation powder according to the labeled instructions while inserted into the induction port mouthpiece/nosepiece adapter, and repeat the operation until the required number of actuations has been discharged. Certain types of oral or nasal DPIs, e.g., capsule-based premetered or other DPI designs, may need removal of the DPI from the mouthpiece/nosepiece adapter after each discharged dose for reloading of the device according to the labeled instructions. After each reload, reinsert the mouthpiece of the DPI into the mouthpiece/nosepiece adapter. Repeat the same operation until the required minimum number of actuations has been discharged. After discharge of the last actuation, remove the product from the mouthpiece/nosepiece adapter, and switch off the vacuum pump.

Carefully disassemble the apparatus. Using a suitable solvent, rinse the drug from the mouthpiece/nosepiece adapter, induction port, pre-separator, each stage, and the collection plate of each stage and the filter immediately into appropriately sized flasks. [NOTE—Analyze all components separately. Only data from analysis of deposition on the collection plates should be used for the purpose of determination of stage deposition. The data from analysis of deposition on the other components should only be used for the purpose of establishing the determination of mass balance and should not be combined with the plate deposition for determination of aerodynamic particle size distribution.] Quantitatively dilute each flask to an appropriate volume. Using an appropriately validated method of analysis, determine the mass of drug collected in each of the samples.

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## C.4 Next Generation Impactor without Pre-separator for Inhalation Aerosols, Inhalation Sprays, and Nasal Aerosols

### C.4.1 DESIGN—NEXT GENERATION IMPACTOR WITHOUT PRE-SEPARATOR

The design and assembly of the Next Generation Impactor without pre-separator are shown in *Figure 8a* and *Figure 8b*. The induction port used to connect the impactor to a product has the same internal dimensions as shown in *Figure 6b*. The device is a cascade impactor with seven stages and a micro-orifice collector (MOC). Use this apparatus at a flow rate of 30 L/min (±5%) unless otherwise prescribed in the individual monograph.

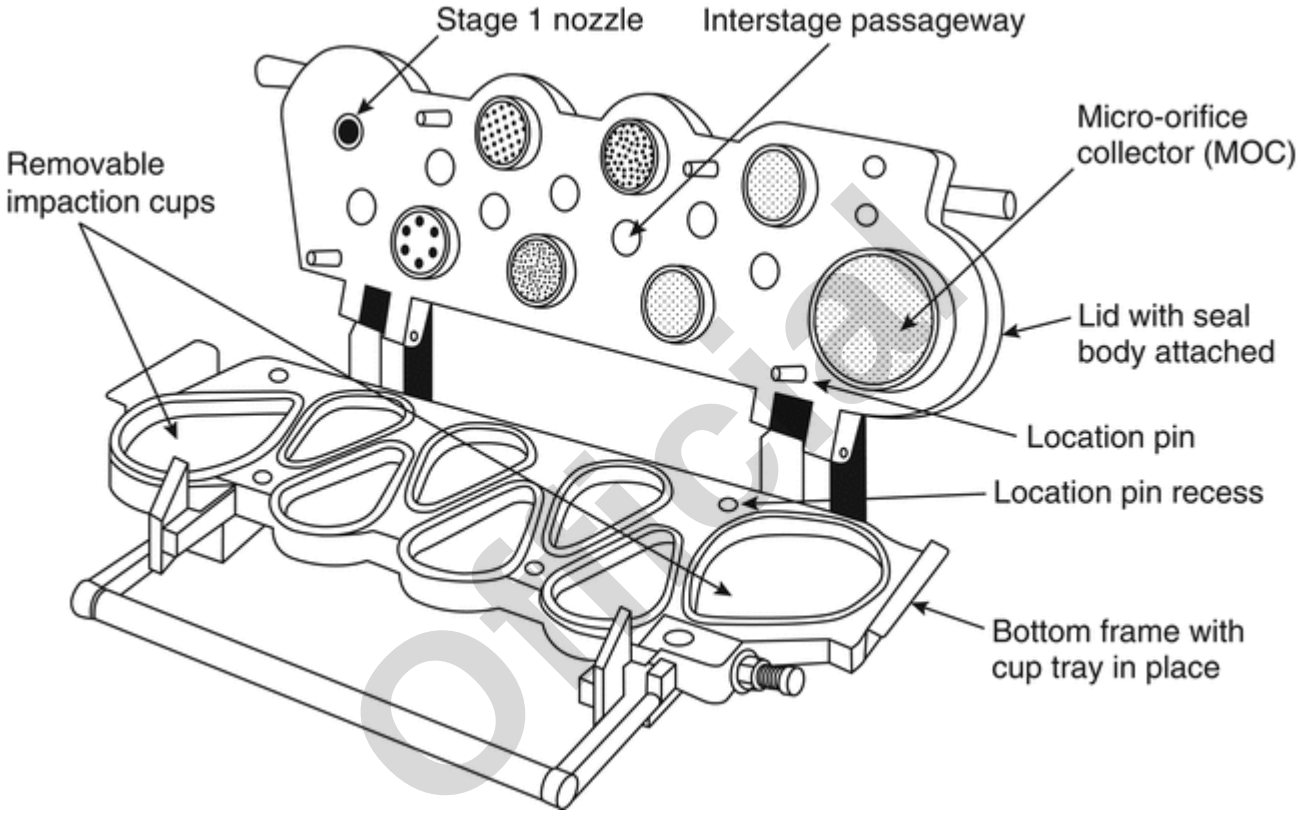


Figure 8a. Components and Nozzle Configuration of the Next Generation Impactor.

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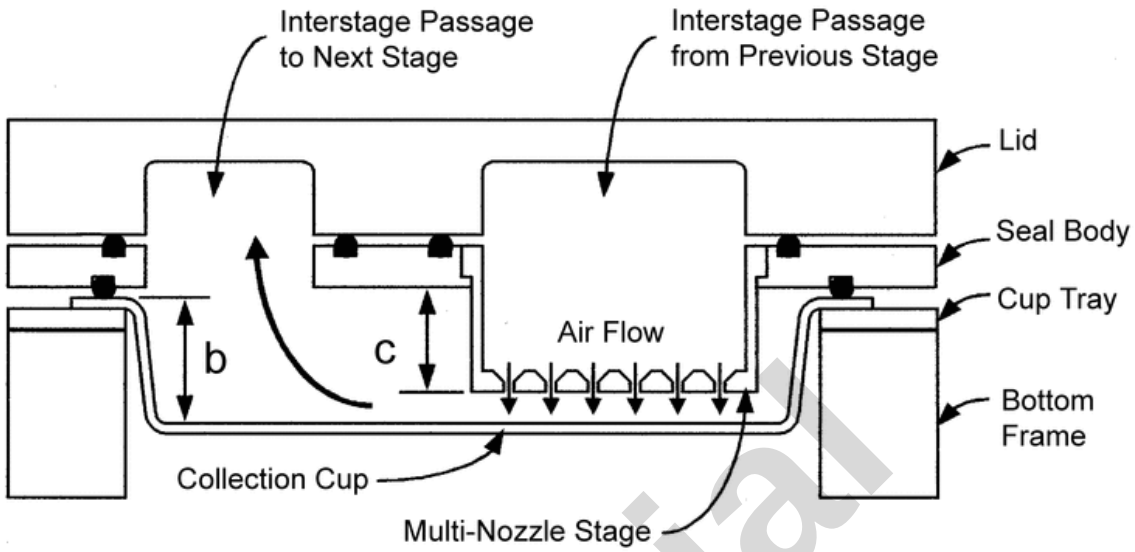


Figure 8b. A layout of interstage passageways of the Next Generation Impactor.

The Next Generation Impactor with pre-separator in place is presented in *Figure 9a*.

The impactor layout has removable impaction cups with all of the cups in one plane. There are three main sections to the impactor: the bottom frame that holds the impaction cups, the seal body that holds the nozzles, and the lid that contains the interstage passageways. Multiple nozzles are used at all but the first stage. The flow passes through the impactor in a saw-tooth pattern.

Stage mensuration is performed periodically (see *C.1.1 Stage Mensuration*) together with confirmation of other dimensions critical to the effective operation of the impactor. Critical dimensions are provided in *Table 6*.

**Table 6. Critical Dimensions for Next Generation Impactor**

| Description | Nominal Nozzle Diameter (mm) | Nominal Nozzle to Seal Body Distance (mm)[a, b] |
|---|---|---|
| Pre-separator[c] | 12.80 ± 0.05 | — |
| Stage 1[d] | 14.30 ± 0.05 | 1.18 |
| Stage 2[d] | 4.88 ± 0.04 | 5.236 ± 0.736 |
| Stage 3[d] | 2.185 ± 0.020 | 8.445 ± 0.410 |
| Stage 4[d] | 1.207 ± 0.010 | 11.379 ± 0.237 |
| Stage 5[d] | 0.608 ± 0.010 | 13.176 ± 0.341 |
| Stage 6[d] | 0.323 ± 0.010 | 13.999 ± 0.071 |
| Stage 7[d] | 0.206 ± 0.010 | 14.000 ± 0.071 |
| MOC[d] | ~0.070 | 14.429–14.571 |
| Optional cup depth (see *b* in *Figure 8b*) | 14.625 ± 0.100 | — |
| Collection cup surface roughness | 0.5–2 µm | — |

[a] See *Figure 8a*.
[b] Refer to manufacturer for specifications on tolerance/precision of the dimensions.
[c] See *Figure 9b*.
[d] See *Figure 8a*.

During routine operation, the seal body and lid are held together as a single assembly. The impaction cups are accessible when this assembly is opened at the end of a product test. The cups are held in a support tray so that all cups can be removed from the impactor simultaneously by lifting out the tray.

The USP induction port (*Figure 6b*) is connected to the impactor inlet. A suitable mouthpiece/nosepiece adapter is used to provide an airtight seal between the product's mouthpiece/nosepiece and the induction port.

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The apparatus contains a terminal MOC that for most formulations may eliminate the need for a final filter if this capability is demonstrated by method validation. The MOC is an impactor nozzle plate and collection cup. The nozzle plate contains, nominally, 4032 holes, each approximately 70 µm in diameter. Most particles not captured on Stage 7 of the impactor may be captured on the cup surface below the MOC. There is an optional filter holder that can replace the MOC for formulations with a significant fraction of particles not captured by the MOC. Alternatively, an after-filter (glass fiber is often suitable) can be placed in a filter holder external to the Next Generation Impactor with and without pre-separator, which is located downstream of the MOC.

### C.4.2 PROCEDURE—NEXT GENERATION IMPACTOR WITHOUT PRE-SEPARATOR

Assemble the apparatus without the pre-separator. Place cups into the apertures in the cup tray. To ensure efficient particle capture, coat the particle collection surface of each stage with glycerol, silicone oil, or other suitable liquid, unless this has been demonstrated to be unnecessary. Insert the cup tray into the bottom frame, and lower into place. Close the impactor lid with the seal body attached, and operate the handle to lock the impactor together so that the system is airtight. Connect the USP induction port (*Figure 6b*) to the impactor inlet. Attach a mouthpiece/nosepiece adapter to the end of the induction port to produce an airtight seal between the product's mouthpiece/nosepiece and the induction port as shown in *Figure 6a*. Use a mouthpiece/nosepiece adapter that ensures that the tip of the product's mouthpiece/nosepiece is flush with the open end of the induction port. Turn on the vacuum pump to draw air through the impactor, and calibrate the flow rate through the system with an appropriate flowmeter attached to the open end of the induction port.

Adjust the flow-control valve (see H in *Figure 7c*) on the vacuum pump to achieve steady flow through the system at 30 L/min (±5%). Prepare the product for use according to the label instructions for shaking, priming, and firing. With the vacuum pump running, insert the mouthpiece/nosepiece into the mouthpiece/nosepiece adapter and immediately fire into the cascade impactor the first of the minimum recommended number of actuations. Keep the valve depressed, as applicable, for a duration sufficient to ensure that the actuation has been completely discharged. If additional sprays are required for the sample, wait for 5 s before firing the next actuation. Repeat until the required number of actuations has been discharged. The number of actuations discharged must be sufficient to ensure an accurate and precise determination of aerodynamic size distribution. After the last actuation has been discharged, remove the product from the mouthpiece/nosepiece adapter. Rinse the mouthpiece/nosepiece adapter and induction port with a suitable solvent, and dilute quantitatively to an appropriate volume.

Dismantle the apparatus, and recover the drug for analysis as follows. Remove the induction port and mouthpiece/nosepiece adapter from the apparatus, and extract the drug from each component of the system into an aliquot of solvent and recover quantitatively the active ingredient from all inner surfaces. Open the impactor by releasing the handle and lifting the lid; remove the cup tray with the collection cups; and extract the active ingredient in each cup into an aliquot of solvent. Using a validated method of analysis, determine the mass of drug contained in each of the aliquots of solvent. Only data from analysis of deposition on the collection cups should be used for the determination of stage deposition.

## C.5 Next Generation Impactor with Pre-separator for Inhalation Powders and Nasal Powders

### C.5.1 DESIGN—NEXT GENERATION IMPACTOR WITH PRE-SEPARATOR

The Next Generation Impactor is identical to that described in *C.4.1 Design—Next Generation Impactor without Pre-separator* (*Figure 8a* and *Figure 8b*), except that the pre-separator (*Figure 9b*) is used. A pre-separator may not be required for certain engineered powders, notably those of low bulk density. Connect the cascade impactor into the control system specified in *Figure 7c*.

### C.5.2 PROCEDURE—NEXT GENERATION IMPACTOR WITH PRE-SEPARATOR

Assemble the apparatus with the pre-separator (*Figure 9a*).

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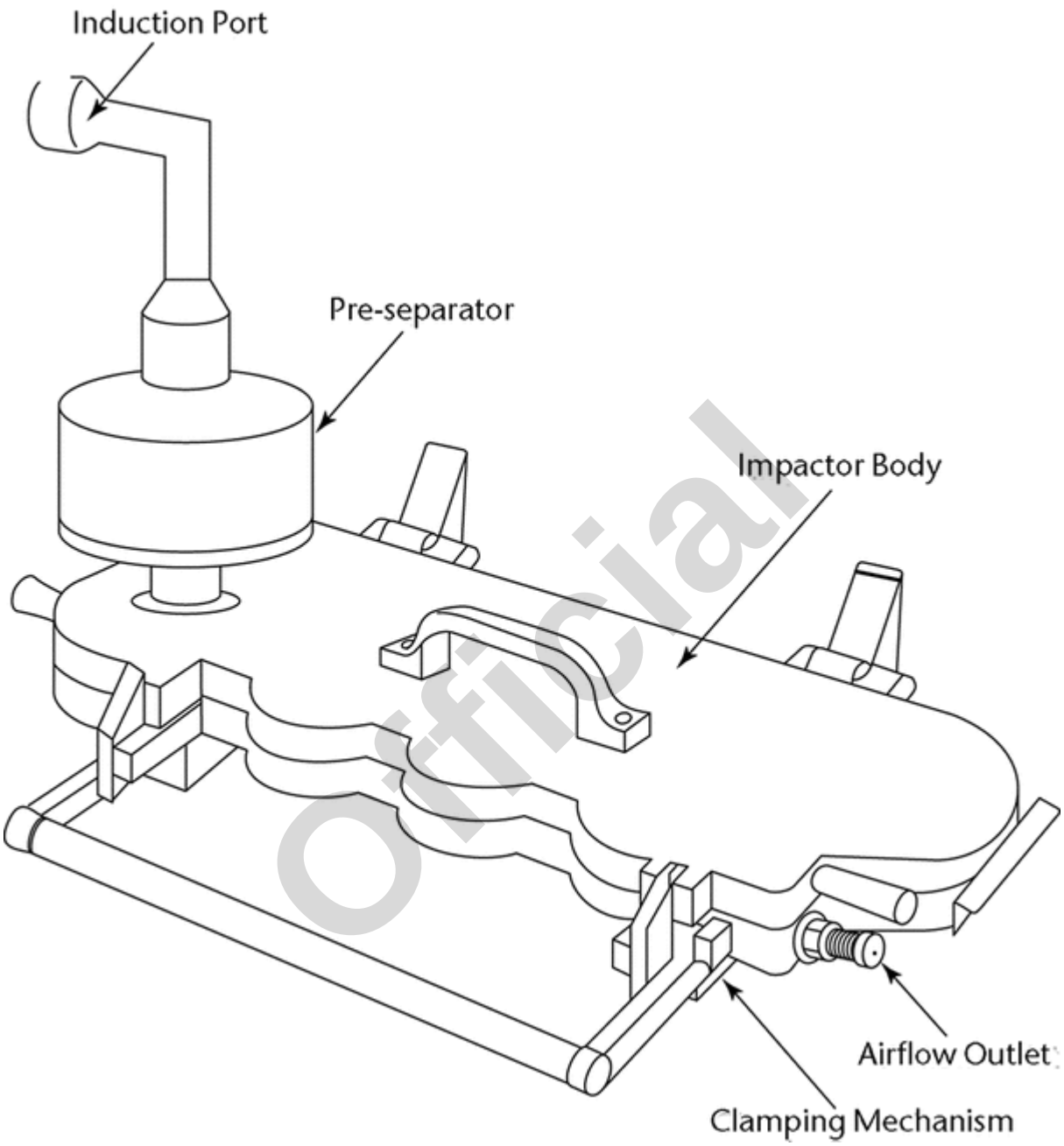


Figure 9a. Next Generation Impactor with pre-separator (shown with the pre-separator in place).

Place cups into the apertures in the cup tray (*Figure 8a*). To ensure efficient particle capture, coat the particle collection surface of each stage with glycerol, silicone oil, or other suitable liquid, unless it has been demonstrated to be unnecessary. Insert the cup tray into the bottom frame, and lower into place. Close the impactor lid with the seal body attached, and operate the handle to lock the impactor together so that the system is airtight.

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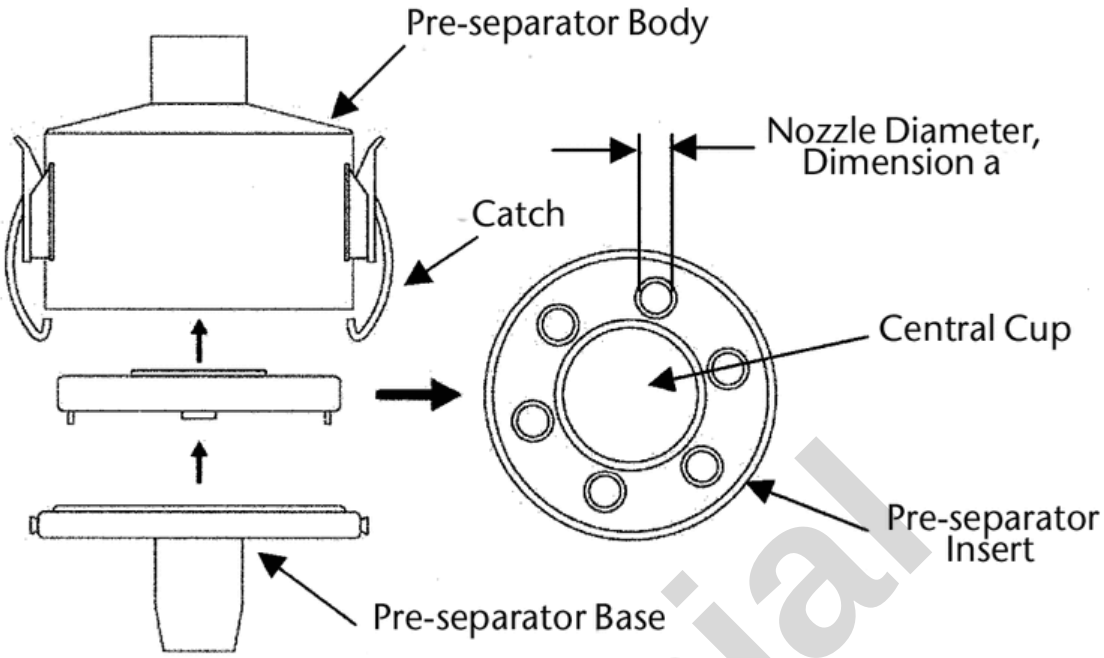


Figure 9b. Pre-separator layout for Next Generation Impactor.

The pre-separator can be assembled as follows. Assemble the pre-separator insert into the pre-separator base; fit the pre-separator base to the impactor inlet; add an appropriate predefined volume (up to 15 mL) of appropriate solvent used for sample recovery to the central cup of the pre-separator insert; place the pre-separator body on top of this assembly; and close the two catches. [**CAUTION**—Some solvents form flammable vapor–air mixtures that can be ignited during passage through a vacuum pump. Take appropriate precautions (e.g., alternative solvents, use of vapor traps, minimal pump operating times, etc.) to ensure operator safety during testing.]

Connect the USP induction port (*Figure 6b*) to the pre-separator inlet atop the cascade impactor (*Figure 9a*). Place a suitable mouthpiece/nosepiece adapter to the inlet of the induction port so that the mouthpiece/nosepiece end of the product, when inserted, lines up along the horizontal axis of the induction port. The front face of the product's mouthpiece/nosepiece is flush with the front face of the induction port, producing an airtight seal. When attached to the mouthpiece/nosepiece adapter, the product should be positioned in the same orientation as intended for use. Connect the apparatus to a flow system according to the scheme specified in *Figure 7c*.

Conduct the test at the flow rate used in the test for *Delivered Dose Uniformity*, drawing 4.0 L of air from the mouthpiece/nosepiece of the product and through the apparatus. Connect a flowmeter to the induction port. Use a flowmeter calibrated for the volumetric flow leaving the meter. Adjust the flow-control valve (see H in *Figure 7c*) to achieve steady flow through the system at the required rate, $Q_{out}$ (±5%). Ensure that critical flow occurs in the flow-control valve at the flow rate to be used during testing by using the following procedure. With the product in place, and the intended flow running, measure the absolute pressure on both sides of the flow-control valve (P2 and P3 in *Figure 7c*). A ratio of P3/P2 $\leq$0.5 indicates critical flow. If this ratio of P3/P2 is not achieved, switch to a more powerful pump, and remeasure the test flow rate.

Prepare the inhalation powder or nasal powder according to the labeled instructions. With the vacuum pump running and the two-way solenoid valve closed, insert the product's mouthpiece/nosepiece, held horizontally, into the mouthpiece/nosepiece adapter. Adjust the timer controlling the operation of the two-way solenoid valve so that it opens the valve for a duration of 2*T* s to sample 4.0 L of air, based on determined duration of *T* seconds for testing of *Delivered Dose Uniformity*. After total air volume of 4.0 L sampled per actuation, the two-way solenoid valve is closed and the product is removed. If additional actuations are required for a determination, reload the inhalation powder or nasal powder according to the labeled instructions while inserted into the mouthpiece/nosepiece adapter, and repeat the operation until the required number of actuations has been discharged. Certain types of oral or nasal DPIs, e.g., capsule-based premetered or other DPI designs, may need removal of the DPI from the mouthpiece/nosepiece adapter after each discharged dose for reloading of the device according to the labeled instructions. After each reload, reinsert the mouthpiece of the DPI into the mouthpiece/nosepiece adapter. Repeat the same operation until the required number of actuations has been discharged. After discharge of the last actuation, remove the product from the mouthpiece/nosepiece adapter, and switch off the vacuum pump.

Dismantle the apparatus, and recover drug for analysis as follows. Remove the induction port and mouthpiece/nosepiece adapter from the pre-separator, and extract the drug from each component of the system into an aliquot of appropriate solvent; remove the pre-separator from the impactor without spilling the solvent into the impactor; and recover quantitatively the active ingredient from all inner surfaces. Open the impactor by releasing the handle and lifting the lid. Remove the cup tray, with the collection cups, and recover the active ingredient from each cup into an aliquot of solvent. Using a validated method of analysis, determine the mass of drug contained in each of the aliquots of solvent.▲ (USP 1-May-2021)

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# ⟨602⟩ PROPELLANTS

[**Caution**—Hydrocarbon propellants are highly flammable and explosive. Observe precautions, and perform sampling and analytical operations in a well-ventilated fume hood.]

## GENERAL SAMPLING PROCEDURE

This procedure is used to obtain test specimens for those propellants that occur as gases at about 25° and that are stored in pressurized cylinders. Use a stainless steel sample cylinder equipped with a stainless steel valve with a capacity of NLT 200 mL and a pressure rating of 240 psi or more. Dry the cylinder with the valve open at 110° for 2 h, and evacuate the hot cylinder to less than 1 mm of mercury. Close the valve, cool, and weigh. Connect one end of a charging line tightly to the propellant container and the other end loosely to the sample cylinder. Carefully open the propellant container, and allow the propellant to flush out the charging line through the loose connection. Avoid excessive flushing, which causes moisture to freeze in the charging line and connections. Tighten the fitting on the sample cylinder, and open the sample cylinder valve, allowing the propellant to flow into the evacuated cylinder. Continue sampling until the desired amount of specimen is obtained, then close the propellant container valve, and finally close the sample cylinder valve.

[**Caution**—Do not overload the sample cylinder. Hydraulic expansion due to temperature change can cause overloaded cylinders to explode.]

Again weigh the charged sample cylinder, and calculate the weight of the specimen.

## APPROXIMATE BOILING TEMPERATURE

Transfer a 100-mL specimen to a tared, pear-shaped, 100-mL centrifuge tube containing a few boiling stones, and weigh. Suspend a thermometer in the liquid, and place the tube in a medium maintained at a temperature of 32° above the expected boiling temperature. When the thermometer reading becomes constant, record as the boiling temperature the thermometer reading after at least 5% of the specimen has distilled. Retain the remainder of the specimen for the determination of high-boiling residues.

## HIGH-BOILING RESIDUES, METHOD I

Allow 85 mL of the specimen to distill as directed in the test for *Approximate Boiling Temperature*, and transfer the centrifuge tube containing the remaining 15 mL of specimen to a medium maintained at a temperature 10° above the boiling temperature. After 30 min, remove the tube from the water bath, blot dry, and weigh. Calculate the weight of the residue.

## HIGH-BOILING RESIDUES, METHOD II

Prepare a cooling coil from copper tubing (about 6 mm outside diameter × about 6.1 m long) to fit into a vacuum-jacketed flask. Immerse the cooling coil in a mixture of dry ice and acetone in a vacuum-jacketed flask, and connect one end of the tubing to the propellant sample cylinder. Carefully open the sample cylinder valve, flush the cooling coil with about 50 mL of the propellant, and discard this portion of liquefied propellant. Continue delivering liquefied propellant from the cooling coil, and collect it in a previously chilled 1000-mL sedimentation cone until the cone is filled to the 1000-mL mark. Allow the propellant to evaporate, using a warm water bath maintained at about 40° to reduce evaporating time. When all of the liquid has evaporated, rinse the sedimentation cone with two 50-mL portions of pentane, and combine the rinsings in a tared 150-mL evaporating dish. Transfer 100 mL of the pentane solvent to a second tared 150-mL evaporating dish, place both evaporating dishes on a water bath, evaporate to dryness, and heat the dishes in an oven at 100° for 60 min. Cool the dishes in a desiccator, and weigh. Repeat the heating for 15-min periods until successive weighings are within 0.1 mg, and calculate the weight of the residue obtained from the propellant as the difference between the weights of the residues in the two evaporating dishes.

## WATER CONTENT

Proceed as directed in *Water Determination* ⟨921⟩, with the following modifications: (a) Provide the closed-system titrating vessel with an opening through which passes a coarse-porosity gas dispersion tube connected to a sampling cylinder. (b) Dilute the *Reagent* with anhydrous methanol to give a water equivalence factor of between 0.2 and 1.0 mg/mL, and age this diluted solution for NLT 16 h before standardization. (c) Obtain a 100-g specimen as directed under *General Sampling Procedure*, and introduce the specimen into the titration vessel through the gas dispersion tube at a rate of about 100 mL of gas per min. If necessary, heat the sample cylinder gently to maintain this flow rate.

## OTHER DETERMINATIONS

For those aerosols that use propellants, perform the tests specified in the individual *NF* propellant monographs.

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# ⟨603⟩ TOPICAL AEROSOLS

## INTRODUCTION

Topical aerosol products contain the drug in solution or in suspension, packaged under pressure, and released upon activation of an appropriate valve system. The topical aerosol products should follow the product quality test requirements described in general test chapters *Inhalation and Nasal Drug Products—General Information and Product Quality Tests* ⟨5⟩, *Microbial Enumeration Tests* ⟨61⟩, *Tests for Specified Organisms* ⟨62⟩, *Minimum Fill* ⟨755⟩, and *Leak Rate* ⟨604⟩. The topical aerosols include dermatological foams and sprays. Topical aerosol foams should include a physical appearance of both the foam and also of the collapsed foam.

## DELIVERY RATE AND DELIVERED AMOUNT

Perform these tests only on containers fitted with continuous valves.

**Delivery Rate:** Select NLT four aerosol containers; shake, if the label includes this directive; remove the caps and covers; and actuate each valve for 2–3 s. Weigh each container accurately, and immerse in a constant-temperature bath until the internal pressure is equilibrated at a temperature of 25° as determined by constancy of internal pressure, as directed under the *Pressure Test* below. Remove the containers from the bath; remove excess moisture by blotting with a paper towel; shake, if the label includes this directive; actuate each valve for 5.0 s (accurately timed by use of a stopwatch); and weigh each container again. Return the containers to the constant-temperature bath, and repeat the foregoing procedure three times for each container. Calculate the average *Delivery Rate*, in g/s, for each container.

**Delivered Amount:** Return the containers to the constant-temperature bath, continuing to deliver 5 s actuations to waste, until each container is exhausted. [NOTE—Ensure that sufficient time is allowed between each actuation to avoid significant canister cooling.] Calculate the total weight loss from each container. This is the *Delivered Amount*.

## PRESSURE TEST

Perform this test only on topical aerosols fitted with continuous valves.

Select NLT four aerosol containers, remove the caps and covers, and immerse in a constant-temperature bath until the internal pressure is constant at a temperature of 25°. Remove the containers from the bath, shake, and remove the actuator and water, if any, from the valve stem. Place each container in an upright position, and determine the pressure in each container by placing a calibrated pressure gauge on the valve stem, holding firmly, and actuating the valve so that it is fully open. The gauge is of a calibration approximating the expected pressure and is fitted with an adapter appropriate for the particular valve stem dimensions. Read the pressure directly from the gauge.

## MINIMUM FILL

Topical aerosols meet the requirements for aerosols in *Minimum Fill* ⟨755⟩.

## LEAKAGE TEST

Proceed as directed in *Leak Rate* ⟨604⟩.

## NUMBER OF DISCHARGES PER CONTAINER

Perform this test only on topical aerosols fitted with dose-metering valves, at the same time as, and on the same containers used for, the test for *Delivered-Dose Uniformity*. Determine the number of discharges or deliveries by counting the number of priming discharges plus those used in determining the spray contents, and continue to fire until the label claim number of discharges. The requirements are met if all the containers or inhalers tested contain NLT the number of discharges stated on the label.

***Change to read:***

## DELIVERED-DOSE UNIFORMITY

The test for *Delivered-Dose Uniformity* is required for topical aerosols fitted with dose-metering valves. For collection of the minimum dose, proceed as directed in ▲*Inhalation and Nasal Drug Products: Aerosols, Sprays, and Powders—Performance Quality Tests* ⟨601⟩*, A. Delivered-Dose Uniformity/A.1 Inhalation Aerosols and Inhalation Sprays*▲ (CN 1-May-2021), and ▲*A.3 Inhalation Powders*▲ (CN 1-May-2021), as described in ⟨601⟩, except modify the dose sampling apparatus so that it is capable of quantitatively capturing the delivered dose from the preparation being tested.

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# ⟨604⟩ LEAK RATE

Select 12 aerosol containers, and record the date and time to the nearest half hour. Weigh each container to the nearest mg, and record the weight, in mg, of each as $W_1$. Allow the containers to stand in an upright position at a temperature of 25.0 ± 2.0° for not less than 3 days, and again weigh each container, recording the weight, in mg, of each as $W_2$ and recording the date and time to the nearest half hour. Determine the time, $T$, in hours, during which the containers were under test. Calculate the leakage rate, in mg per year, of each container taken by the formula:

$$(365)(24/T)(W_1 - W_2).$$

Where plastic-coated glass aerosol containers are tested, dry the containers in a desiccator for 12–18 h, and allow them to stand in a constant-humidity environment for 24 h prior to determining the initial weight as indicated above. Conduct the test under the same constant-humidity conditions. Empty the contents of each container tested by employing any safe technique (e.g., chill to reduce the internal pressure, remove the valve, and pour). Remove any residual contents by rinsing with suitable solvents, then rinse with a few portions of methanol. Retain as a unit the container, the valve, and all associated parts, and heat them at 100° for 5 min. Cool, weigh, record the weight as $W_3$, and determine the net fill weight ($W_1 - W_3$) for each container tested. [NOTE—If the average net fill weight has been determined previously, that value may be used in place of the value ($W_1 - W_3$) above.] The requirements are met if the average leakage rate per year for the 12 containers is not more than 3.5% of the net fill weight, and none of the containers leaks more than 5.0% of the net fill weight per year. If 1 container leaks more than 5.0% per year, and if none of the containers leaks more than 7.0% per year, determine the leakage rate of an additional 24 containers as directed herein. Not more than 2 of the 36 containers leak more than 5.0% of the net fill weight per year, and none of the 36 containers leaks more than 7.0% of the net fill weight per year. Where the net fill weight is less than 15 g and the label bears an expiration date, the requirements are met if the average leakage rate of the 12 containers is not more than 525 mg per year and none of the containers leaks more than 750 mg per year. If 1 container leaks more than 750 mg per year but not more than 1.1 g per year, determine the leakage rate of an additional 24 containers as directed herein. Not more than 2 of the 36 containers leak more than 750 mg per year, and none of the 36 containers leaks more than 1.1 g per year. This test is in addition to the customary in-line leak testing of each container.

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***Add the following:***

# ▲⟨607⟩ PHARMACEUTICAL FOAMS—PRODUCT QUALITY TESTS

## INTRODUCTION

Foams are a dispersion of gas in a liquid or a semisolid continuous phase wherein the liquid or semisolid contains the drug substance and suitable excipients. Typical foam dosage forms include aqueous or nonaqueous carriers, foam structuring agents, surfactants, and propellants (for pressurized systems). Foams are produced by mechanical means or via interaction of propellant (vapor or gas) and the formulation under pressure. Foams dispensed from nonpressurized containers use mechanical forces allowing the mixing of the formulation and air, resulting in foam generation. Foams dispensed from pressurized containers use propellant present in gas phase to increase pressure inside the container. When the nozzle of the actuator is opened, the liquid phase containing a dispersed vapor or gas is emitted through an actuator. As a result, a foam is generated.

Foams are primarily used to apply drug substances to topically accessible sites. The most significant of these sites is the skin where direct application is frequently used to alleviate symptoms (e.g., inflammation, infection). An extension of this approach is to apply foams to accessible but not superficial sites such as the ear canal, or to mucosal sites such as the rectum, vagina, or oropharynx. Invasive approaches such as application at surgical sites or insertion in blood vessels extend the application of foams and their purpose.

This chapter provides a list of product quality tests that may be applied to pharmaceutical foam products. In part or in its entirety, this chapter applies when referenced in a drug product monograph (see *General Notices, 3.10 Applicability of Standards*). Manufacturers may use quality tests listed in this chapter, as appropriate, when considering the content of new drug product monographs for submission to USP.

## PRODUCT QUALITY TESTS FOR PHARMACEUTICAL FOAMS

Drug product quality tests fall into two categories: 1) universal tests that are applicable to all drug products and should be included in the monograph, and 2) specific tests that should be considered for inclusion for specific types of products. Universal tests include identification, assay, and impurities and are discussed in the following *USP* chapters, based on route of administration: *Injections and Implanted Drug Products* ⟨1⟩, *Oral Drug Products—Product Quality Tests* ⟨2⟩, *Topical and Transdermal Drug Products—Product Quality Tests* ⟨3⟩, *Mucosal Drug Products—Product Quality Tests* ⟨4⟩, and *Inhalation and Nasal Drug Products—General Information and Product Quality Tests* ⟨5⟩. Pharmaceutical foams are delivered by the injection, topical, and mucosal routes. For more information on specific tests by those routes of administration, see ⟨1⟩, ⟨3⟩, and ⟨4⟩, respectively.

## QUALITY TESTS FOR FOAM PRODUCTS BY ALL ROUTES OF ADMINISTRATION

- **Dispersed Content Uniformity Throughout Canister Life**

  **Non-metered products:** Select one container and determine its weight. Dispense quantities according to the labeled instructions separately collecting an appropriate amount, individually weighed. The sample size should be sufficient for at least one quantitative determination of the active ingredients(s) and should not exceed the maximum dose recommended by the product labeling for a single application. [Note—Refer to the labeled use instructions to determine whether or not the can is shaken prior to expelling foam and to determine the orientation (upright or inverted) when dispensing. Use the labeled content and the weights of samples collected and material discarded for the following sampling procedure.] Retain the portions of foam corresponding to: 1) an initial portion from the filled canister, 2) a portion from the middle of the canister (in the range of 40%–60% of labeled canister content), and 3) the portion corresponding to the canister contents with 85% of the labeled contents delivered. [Note—The canister should be dispensed at room temperature. If the canister cools as a result of dispensing, the canister should be warmed to room temperature before subsequent delivery. Properly discard the material removed from the canister by valve actuations required to collect specified samples. Using an appropriate sample preparation (such as outgassing) and analytical method, determine the drug substance concentration in each of the three portions.]

  **Acceptance criteria:** None of the three results are outside of the product assay range. The maximum difference in the amount of active ingredient(s) determined within the canister is NMT 10.0%. For example, if the three measurements within the canister are 97.0%, 95.2%, and 99.7%, the maximum difference would be 4.5% (i.e., 99.7%–95.2% = 4.5%).

  **Metered-dose products:** Delivered dose uniformity measures the drug substance content as dispensed over the entire unit life. Select one container. Refer to the labeled use instructions to determine whether or not the can is shaken prior to expelling foam. Using separate collection vessels, quantitatively collect delivered doses representing actuations for the initial dose, a middle dose, and the final dose from the container. [Note—Between actuations, warm the canister in a water bath at 25° for 3 min.] Discard the material removed from the canister by valve actuations required to collect specified samples. Determine the drug substance content of each of the three collected samples.

  **Acceptance criteria:** None of the three results are more than 10% outside of the labeled amount per dose, and the maximum difference between any of the results is NMT 10% of the claim for delivered dose.

- **Minimum Fill:** Proceed as directed for *Minimum Fill* ⟨755⟩, *Procedure for Aerosols and Sprays*. This procedure also can be applied to foams.

  **Acceptance criteria:** Meets the requirements

## OTHER TESTS

- **Leak Rate (Aerosol Products):** Proceed as directed in *Leak Rate* ⟨604⟩.

  **Acceptance criteria:** Meets the requirements

- **Propellant (Aerosol Products):** Proceed as directed in *Propellants* ⟨602⟩.

  **Acceptance criteria:** Meets the requirements as specified in the monograph

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- **Pressure in Canister (Aerosol Products):** See *Topical Aerosols* ⟨603⟩, *Pressure Test*. Pressure in the canister is only measured for products with continuous valves using a mechanism that attaches to a pressure gauge while sealing against the valve in a manner that contains the expelled gas and foam.
  **Acceptance criteria:** Meets the requirements for the pressure test as specified in the monograph
- **pH:** Apply *pH* ⟨791⟩ for the collapsed foam, as appropriate. In order to measure this attribute, the collapsed foam may require dilution.
- **Sterility:** If applicable, see *Sterility Tests* ⟨71⟩.
- **Microbial Limits:** Apply microbial limits tests (see *Microbial Enumeration Tests* ⟨61⟩ and *Tests for Specified Microorganisms* ⟨62⟩), as appropriate.
- **Antimicrobial Preservative Content:** If applicable, see *Antimicrobial Effectiveness Testing* ⟨51⟩ and *Antimicrobial Agents—Content* ⟨341⟩.
- **Antioxidant Content:** If applicable, see *Topical and Transdermal Drug Products—Product Quality Tests* ⟨3⟩, *Product Quality Tests for Topical and Transdermal Drug Products, Specific Tests, Antioxidant preservative content*.
- **Osmolarity and Osmolality** If applicable, see *Osmolality and Osmolarity* ⟨785⟩.

**ADDITIONAL QUALITY TESTS APPLICABLE FOR TOPICAL FOAM PRODUCTS**

- **Time to Break:** Rate of foam liquid drainage and collapse can be observed by dispensing a foam into a graduated cylinder maintained at an appropriate temperature. Record the foam volume as freshly dispensed and again after a period (e.g., complete collapse or as specified in the monograph). The ratio of the freshly dispensed volume to the volume of drained liquid on complete collapse of the foam or at the time specified in the monograph is as specified in the monograph.
- **Delivery Rate:** See *Topical Aerosols* ⟨603⟩, *Delivery Rate and Delivered Amount, Delivery Rate*.
  **Acceptance criteria:** Meets the requirements as specified in the monograph
- **Delivered Amount:** See *Topical Aerosols* ⟨603⟩, *Delivery Rate and Delivered Amount, Delivered Amount*.
  **Acceptance criteria:** Meets the requirements as specified in the monograph
- **Relative Density of Uncollapsed Foam:** If applicable, determine the relative foam density by weighing a mass of uncollapsed foam ($m$) and a mass of the same volume of water ($e$) in a suitable container:

$$\text{Relative density of uncollapsed foam} = m/e$$

$m$ = mass of uncollapsed foam (g/mL)
$e$ = volume of water (g/mL)

- **Water Content:** If applicable (where stability may be affected, primarily for nonaqueous formulations), see *Water Determination* ⟨921⟩.
- **Particle Size:** When the finished product contains a suspended drug substance or is an emulsion, the product should be examined for particle size (in case of suspended solid drug substance) or globule size (in case of an emulsion). These tests are generally formulation dependent. Therefore, such tests are not included in compendial monographs but are part of the manufacturer's approved regulatory specification for the product.▲ (USP 1-Dec-2020)

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# ⟨610⟩ ALTERNATIVE MICROBIOLOGICAL SAMPLING METHODS FOR NONSTERILE INHALED AND NASAL PRODUCTS

## INTRODUCTION

Proper microbiological sampling of microbiologically susceptible nonsterile products can be difficult because these products are often filled into unique primary containers that are designed to protect the product from inadvertent contamination during storage and use. These unique designs may increase the difficulty of taking an aseptic sample of sufficient size or volume for microbiological testing. Unless special approaches are used, products such as inhaled, nasal liquid, or powder dosage forms can be difficult to sample without potential exposure to extraneous microbial contamination. This general test chapter provides these special approaches for sampling either low- or high-content inhaled or nasal dosage forms. Alternative sampling approaches may provide better ways to sample containers in an aseptic manner. Any alternative methodology should employ aseptic techniques and should be conducted under environmental and other conditions that are appropriate for aseptic sampling.

## INHALED OR NASAL DOSAGE FORMS

Low-content inhaled and nasal drug products (low-content INDP) are products that have a target fill of less than 100 mg of powder or 1 mL of liquid formulation per unit (primary container). Examples are pre-metered inhalation powders, more commonly known as dry powder inhalers (DPIs), and single-dose nasal sprays.

High-content INDP are multidose drug products that have a target fill of more than 100 mg of powder or more than 1 mL of liquid formulation per unit. Examples are aerosols for inhalation and nasal delivery, known as metered-dose inhalers (MDIs); device-metered inhalation powders; and multidose nasal sprays.

The appropriate sample quantity or volume should be based on the test methodology, including any relevant general test chapters, such as ⟨61⟩ *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* and ⟨62⟩ *Microbiological Examination of Nonsterile Products: Tests for Specified Microorganisms*. Testing may be performed on the unpackaged bulk dry powder or liquid formulation or the finished product. If testing is performed on the bulk material alone, then the process leading from the bulk to the finished product should be validated for its ability to prevent microbial contamination. Testing should be performed on the finished product if this process is not validated.

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## SAMPLE SIZE DETERMINATION

For each microbiological test, sample 10 drug product containers or units or a number of units that can provide a minimum of 1 gram of product that are representative of the batch. For batch sizes smaller than 200 units (e.g., batches used in clinical trials), sample size may be reduced to 1% of the units or 1 unit, whichever is greater. The contents of individual containers may be pooled for testing.

### Bulk Testing for Low-Content INDP

Bulk lot testing may be preferable for low-content INDP in lieu of finished product testing to allow larger sample sizes that are representative of the batch, without unduly increasing the risk of inadvertent microbial contamination. Bulk testing can be performed on the bulk powder or liquid formulation just before filling. If bulk testing is performed in lieu of finished product testing, then manufacturing processes following bulk sampling (e.g., filling and packaging) must be validated in accordance with current good manufacturing practice (CGMP) for their ability to prevent microbial contamination. For microbial enumeration tests, at least 10 g or 10 mL of bulk material, or, for specified microorganisms tests, 1 g or 1 mL of bulk material may be sampled. For small batch sizes (i.e., less than 1000 g or 1000 mL), the recommended sample size is 1% of the batch for both microbial enumeration and specified microorganisms tests.

## SAMPLING METHODS FOR HIGH-CONTENT INDP

### Dry Powder Inhalers

DPIs have an internal reservoir that contains a sufficient quantity of formulation for multiple doses that are metered by the device itself during activation by the patient. For DPIs, appropriate validated procedures should be used to sample a nonsterile drug product container.

### Inhalation Aerosols

Consider safety issues related to both inhalation of the drug substance and the potential of a flammability hazard. Avoid contamination of samples by employing aseptic techniques whenever necessary.

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### AUTOMATIC ACTUATION METHOD

The contents of the inhalation aerosol containers may be collected by automatically actuating each aerosol container and collecting the delivered formulation on a suitable sterile filter.

### ROOM TEMPERATURE METHOD

Disinfect the outside of the test containers with an appropriate disinfectant, and allow the containers to dry in a controlled environment. Empty the contents of the aerosol container into a sterile vessel using a needle apparatus or similar device (e.g., icemaker water line tap). If it has been demonstrated that the propellant does not inhibit the growth of microorganisms, the contents of the sterile vessel may be added directly to the liquid media or buffer for the test. Otherwise, allow the propellant to evaporate from the vessel by leaving the vessel at room temperature for several minutes. Remove any residual gaseous propellant by tilting the vessel slightly or by allowing a slow stream of microbiologically inert sterile gas to pass over the surface. For some less volatile propellants such as chlorofluorocarbon (CFC) 11/12 combinations, the vessel may be heated slightly (to temperatures $\leq$ 45°) to assist with evaporation. After the propellant has evaporated, add the liquid media or buffer, and mix the contents to prepare for testing.

Direct expulsion into the broth media or buffer may be feasible if a needle apparatus that is thin and strong enough to puncture the container and to allow slow removal of the contents is available. In this case, the contents may be expelled into and mixed with the aqueous medium. Layering of the propellant and aqueous medium may occur, in which case a longer time period and slight heating (not to exceed 45°) may be required for propellant evaporation.

### CHILLING METHOD

Place the disinfected aerosol containers in dry ice, or a dry ice slurry (ensure the microbial quality of the dry ice and slurry-forming liquid), or a cryofreezer for the period required to liquefy the contents. Disinfect the outside of the test containers with an appropriate disinfectant, and allow the containers to dry in an aseptic environment. Aseptically open the aerosol containers using an appropriate tool. Be aware that freezing can affect the viability of microorganisms. For CFC-based products, pour the contents of the containers into sterile vessels. Allow the propellant to escape, and combine residues with a diluent appropriate for the drug product. Other drug-specific procedures may also be employed. For hydrofluoroalkane-based products, pour the contents of the containers into sterile vessels partially immersed in larger vessels containing dry ice. Drive off the propellant, for example by allowing a slow stream of sterile, filtered, oil-free compressed air to evaporate the material to dryness. Combine residues with a diluent appropriate for the drug product. An alternative procedure when testing the entire contents of a previously chilled container is to pour the contents of the opened container onto a sterile membrane filtration unit, allow the propellant to escape, and then rinse with an appropriate amount of sterile diluent.

## Multidose Nasal Sprays

Multidose nasal spray containers usually have a cap that is screwed on, crimped on, or forms a snap-fit. The container can be opened by unscrewing the cap, cutting the seal, or using a decrimping tool, taking care to avoid microbial contamination during the process. Following cap removal, traditional sampling methods as described in general test chapters such as ⟨61⟩ *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* and ⟨62⟩ *Microbiological Examination of Nonsterile Products: Tests for Specified Microorganisms* are typically appropriate for use.

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# ⟨611⟩ ALCOHOL DETERMINATION

## PROCEDURES

### Method I—Distillation Method

Method I is to be used for the determination of alcohol, unless otherwise specified in the individual monograph. It is suitable for examining most fluidextracts and tinctures, provided the capacity of the distilling flask is sufficient (commonly two to four times the volume of the liquid to be heated) and the rate of distillation is such that clear distillates are produced. Cloudy distillates may be clarified by agitation with talc, or with calcium carbonate, and filtered, after which the temperature of the filtrate is adjusted and the alcohol content determined from the specific gravity. During all manipulations, take precautions to minimize the loss of alcohol by evaporation.

Treat liquids that froth to a troublesome extent during distillation by rendering them strongly acidic with phosphoric, sulfuric, or tannic acid, or treat with a slight excess of calcium chloride solution or with a small amount of paraffin or silicone oil before starting the distillation.

Prevent bumping during distillation by adding porous chips of insoluble material such as silicon carbide, or beads.

**For liquids presumed to contain 30% of alcohol or less:** By means of a pipet, transfer to a suitable distilling apparatus not less than 25 mL of the liquid in which the alcohol is to be determined, and note the temperature at which the volume was measured. Add an equal volume of water, distill, and collect a volume of distillate about 2 mL less than the volume taken of the original test liquid, adjust to the temperature at which the original test liquid was measured, add sufficient water to measure exactly the original volume of the test liquid, and mix. The distillate is clear or not more than slightly cloudy, and does not contain more than traces of volatile substances other than alcohol and water. Determine the specific gravity of the liquid at 25°, as directed under *Specific Gravity* ⟨841⟩, using this result to ascertain the percentage, by volume, of $C_2H_5OH$ contained in the liquid examined by reference to the *Alcoholometric Table* in the section *Reference Tables.*

**For liquids presumed to contain more than 30% of alcohol:** Proceed as directed in the foregoing paragraph, except to do the following: dilute the specimen with about twice its volume of water, collect a volume of distillate about 2 mL less than twice the volume of the original test liquid, bring to the temperature at which the original liquid was measured, add sufficient water to measure exactly twice the original volume of the test liquid, mix, and determine its specific gravity. The proportion of $C_2H_5OH$, by volume, in this distillate, as ascertained from its specific gravity, equals one-half that in the liquid examined.

#### Special Treatment

**Volatile acids and bases:** Render preparations containing volatile bases slightly acidic with diluted sulfuric acid before distilling. If volatile acids are present, render the preparation slightly alkaline with sodium hydroxide TS.

**Glycerin:** To liquids that contain glycerin add sufficient water so that the residue, after distillation, contains not less than 50% of water.

**Iodine:** Treat all solutions containing free iodine with powdered zinc before the distillation, or decolorize with just sufficient sodium thiosulfate solution (1 in 10), followed by a few drops of sodium hydroxide TS.

**Other volatile substances:** Spirits, elixirs, tinctures, and similar preparations that contain appreciable proportions of volatile materials other than alcohol and water, such as volatile oils, chloroform, ether, camphor, etc., require special treatment, as follows:

**For liquids presumed to contain 50% of alcohol or less:** Mix 25 mL of the specimen under examination, accurately measured, with about an equal volume of water in a separator. Saturate this mixture with sodium chloride, then add 25 mL of solvent hexane, and shake the mixture to extract the interfering volatile ingredients. Draw off the separated, lower layer into a second separator, and repeat the extraction twice with two further 25-mL portions of solvent hexane. Extract the combined solvent hexane solutions with three 10-mL portions of a saturated solution of sodium chloride. Combine the saline solutions, and distill in the usual manner, collecting a volume of distillate having a simple ratio to the volume of the original specimen.

**For liquids presumed to contain more than 50% of alcohol:** Adjust the specimen under examination to a concentration of approximately 25% of alcohol by diluting it with water, then proceed as directed in *For liquids presumed to contain 50% of alcohol or less,* beginning with "Saturate this mixture with sodium chloride."

In preparing *Collodion* or *Flexible Collodion* for distillation, use water in place of the saturated solution of sodium chloride directed above.

If volatile oils are present in small proportions only, and a cloudy distillate is obtained, the solvent hexane treatment not having been employed, the distillate may be clarified and rendered suitable for the specific gravity determination by shaking it with about one-fifth its volume of solvent hexane, or by filtering it through a thin layer of talc.

### Method II—Gas Chromatographic Method

Use *Method IIa* when *Method II* is specified in the individual monograph. For a discussion of the principles upon which it is based, see *Gas Chromatography* under *Chromatography* ⟨621⟩.

• **METHOD IIA**

**Apparatus:** Under typical conditions, use a gas chromatograph equipped with a flame-ionization detector and a 4-mm × 1.8-m glass column packed with 100- to 120-mesh chromatographic column packing support S3, using nitrogen or helium as the carrier. Prior to use, condition the column overnight at 235° with a slow flow of carrier gas. The column temperature is maintained at 120°, and the injection port and detector temperatures are maintained at 210°. Adjust the carrier flow and temperature so that acetonitrile, the internal standard, elutes in 5 to 10 minutes.

#### Solutions

**Test stock preparation:** Dilute the specimen under examination stepwise with water to obtain a solution containing approximately 2% (v/v) of alcohol.

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**Test preparation:** Pipet 5 mL each of the *Test stock preparation* and the USP Alcohol Determination—Acetonitrile RS [NOTE—Alternatively, a 2% aqueous solution of acetonitrile of suitable quality may be used as the internal standard solution] into a 50-mL volumetric flask, dilute with water to volume, and mix.

**Standard preparation:** Pipet 5 mL each of the USP Alcohol Determination—Alcohol RS and the USP Alcohol Determination—Acetonitrile RS. [NOTE—Alternatively, a 2% aqueous solution of acetonitrile of suitable quality may be used as the internal standard solution] into a 50-mL volumetric flask, dilute with water to volume, and mix.

**Procedure:** Inject about 5 µL each of the *Test preparation* and the *Standard preparation,* in duplicate, into the gas chromatograph, record the chromatograms, and determine the peak response ratios. Calculate the percentage of alcohol (v/v) in the specimen under test according to the formula:

$$\text{Result} = CD(R_U/R_S)$$

$C$ = labeled concentration of USP Alcohol Determination—Alcohol RS
$D$ = dilution factor (the ratio of the volume of the *Test stock preparation* to the volume of the specimen taken)
$R_U$ = peak response ratio obtained from the *Test preparation*
$R_S$ = peak response ratio obtained from the *Standard preparation*

**System suitability test:** In a suitable chromatogram, the resolution factor, R, is not less than 2; the tailing factor of the alcohol peak is not greater than 2.0; and six replicate injections of the *Standard preparation* show a relative standard deviation of not more than 2.0% in the ratio of the peak of alcohol to the peak of the internal standard.

- **METHOD IIB**

**Apparatus:** The gas chromatograph is equipped with a split injection port with a split ratio of 5:1, a flame-ionization detector, and a 0.53-mm × 30-m capillary column coated with a 3.0-µm film of phase G43. Helium is used as the carrier gas at a linear velocity of 34.0 cm per second. The chromatograph is programmed to maintain the column temperature at 50° for 5 minutes, then to increase the temperature at a rate of 10° per minute to 200°, and maintain at this temperature for 4 minutes. The injection port temperature is maintained at 210° and the detector temperature at 280°.

**Solutions**

**Test stock preparation:** Dilute the specimen under examination stepwise with water to obtain a solution containing approximately 2% (v/v) of alcohol.

**Test preparation:** Pipet 5 mL each of the *Test stock preparation* and the USP Alcohol Determination—Acetonitrile RS [NOTE—Alternatively, a 2% aqueous solution of acetonitrile of suitable quality may be used as the internal standard solution] into a 25-mL volumetric flask, dilute with water to volume, and mix.

**Standard preparation:** Pipet 5 mL each of the USP Alcohol Determination—Alcohol RS and the USP Alcohol Determination—Acetonitrile RS. [NOTE—Alternatively, a 2% aqueous solution of acetonitrile of suitable quality may be used as the internal standard solution] into a 25-mL volumetric flask, dilute with water to volume, and mix.

**Procedure:** Inject about 0.2 to 0.5 µL each of the *Test preparation* and the *Standard preparation,* in duplicate, into the gas chromatograph, record the chromatograms, and determine the peak response ratios. Calculate the percentage of alcohol (v/v) in the specimen under test according to the formula:

$$\text{Result} = CD(R_U/R_S)$$

$C$ = labeled concentration of USP Alcohol Determination—Alcohol RS
$D$ = dilution factor (the ratio of the volume of the *Test stock preparation* to the volume of the specimen taken)
$R_U$ = peak response ratio obtained from the *Test preparation*
$R_S$ = peak response ratio obtained from the *Standard preparation*

**System suitability test:** In a suitable chromatogram, the resolution factor, R, between alcohol and the internal standard is not less than 4; the tailing factor of the alcohol peak is not greater than 2.0; and six replicate injections of the *Standard preparation* show a relative standard deviation of not more than 4.0% in the ratio of the peak of alcohol to the peak of the internal standard.

## ADDITIONAL REQUIREMENTS

- **USP REFERENCE STANDARDS** ⟨11⟩

USP Alcohol Determination—Acetonitrile RS
USP Alcohol Determination—Alcohol RS

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# ⟨616⟩ BULK DENSITY AND TAPPED DENSITY OF POWDERS

## BULK DENSITY

This general chapter has been harmonized with the corresponding texts of the *European Pharmacopoeia* and/or the *Japanese Pharmacopoeia*. ♦The portion that is not harmonized is marked with symbols (♦,♦) to specify this fact.♦

The bulk density of a powder is the ratio of the mass of an untapped powder sample and its volume including the contribution of the interparticulate void volume. Hence, the bulk density depends on both the density of powder particles and the spatial arrangement of particles in the powder bed. The bulk density is expressed in grams per mL (g/mL) although the international unit is kilograms per cubic meter (1 g/mL = 1000 kg/m$^3$) because the measurements are made using cylinders. It may also be expressed in grams per cubic centimeter (g/cm$^3$). The bulking properties of a powder are dependent upon the preparation, treatment, and storage of the sample, i.e., how it was handled. The particles can be packed to have a range of bulk densities; however, the slightest disturbance of the powder bed may result in a changed bulk density. Thus, the bulk density of a powder is often very difficult to measure with good reproducibility and, in reporting the results, it is essential to specify how the determination was made. The bulk density of a powder is determined by measuring the volume of a known weight of powder sample, that may have been passed through a sieve, into a graduated cylinder (*Method I*), or by measuring the mass of a known volume of powder that has been passed through a volumeter into a cup (*Method II*) or a measuring vessel (*Method III*).

*Method I* and *Method III* are favored.

### Method I—Measurement in a Graduated Cylinder

#### PROCEDURE

Pass a quantity of material sufficient to complete the test through a sieve with apertures greater than or equal to 1.0 mm, if necessary, to break up agglomerates that may have formed during storage; this must be done gently to avoid changing the nature of the material. Into a dry graduated 250-mL cylinder (readable to 2 mL) introduce, without compacting, approximately 100 g of test sample, *M*, weighed with 0.1% accuracy. Carefully level the powder without compacting, if necessary, and read the unsettled apparent volume ($V_0$) to the nearest graduated unit. Calculate the bulk density in g/mL by the formula $m/V_0$. Generally, replicate determinations are desirable for the determination of this property. If the powder density is too low or too high, such that the test sample has an untapped apparent volume of either more than 250 mL or less than 150 mL, it is not possible to use 100 g of powder sample. Therefore, a different amount of powder has to be selected as the test sample, such that its untapped apparent volume is 150–250 mL (apparent volume greater than or equal to 60% of the total volume of the cylinder); the weight of the test sample is specified in the expression of results. For test samples having an apparent volume between 50 mL and 100 mL, a 100-mL cylinder readable to 1 mL can be used; the volume of the cylinder is specified in the expression of results.

### Method II—Measurement in a Volumeter

#### APPARATUS

The apparatus (*Figure 1*) consists of a top funnel fitted with a 1.0-mm sieve.[1] The funnel is mounted over a baffle box containing four glass baffle plates over which the powder slides and bounces as it passes. At the bottom of the baffle box is a funnel that collects the powder and allows it to pour into a cup of specified capacity mounted directly below it. The cup may be cylindrical (25.00 ± 0.05 mL volume with an inside diameter of 30.00 ± 2.00 mm) or cubical (16.39 ± 0.20 mL volume with inside dimensions of 25.400 ± 0.076 mm).

[1] The apparatus (the Scott Volumeter) conforms to the dimensions in ASTMB329–06(2012).

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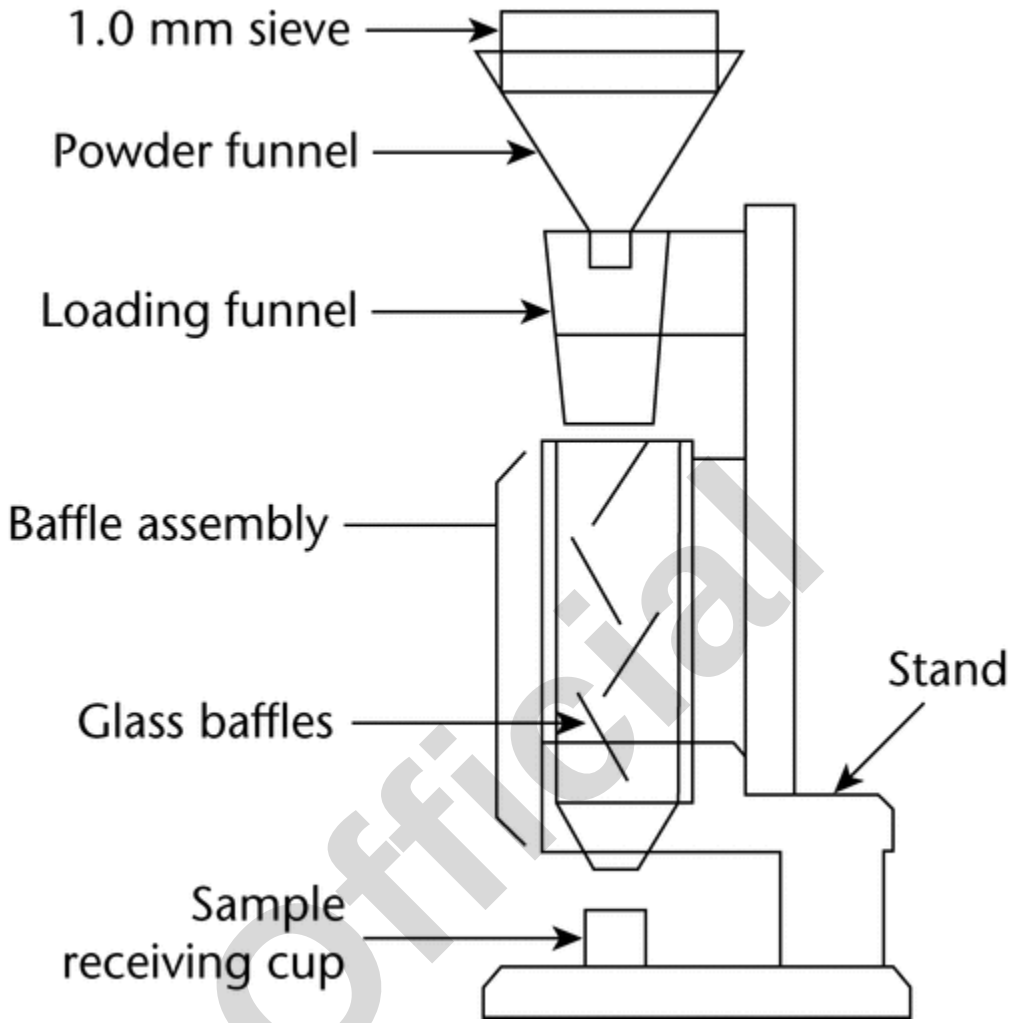


Figure 1.

PROCEDURE—Allow an excess of powder to flow through the apparatus into the sample receiving cup until it overflows, using a minimum of 25 $cm^3$ of powder with the square cup and 35 $cm^3$ of powder with the cylindrical cup. Carefully scrape excess powder from the top of the cup by smoothly moving the edge of the blade of a spatula perpendicular to and in contact with the top surface of the cup, taking care to keep the spatula perpendicular to prevent packing or removal of powder from the cup. Remove any material from the sides of the cup, and determine the weight, *M*, of the powder to the nearest 0.1%. Calculate the bulk density, in g/mL, by the formula:

$$(M)/(V_0)$$

in which $V_0$ is the volume, in mL, of the cup. Record the average of three determinations using three different powder samples.

## Method III—Measurement in a Vessel

APPARATUS

The apparatus consists of a 100-mL cylindrical vessel of stainless steel with dimensions as specified in *Figure 2*.

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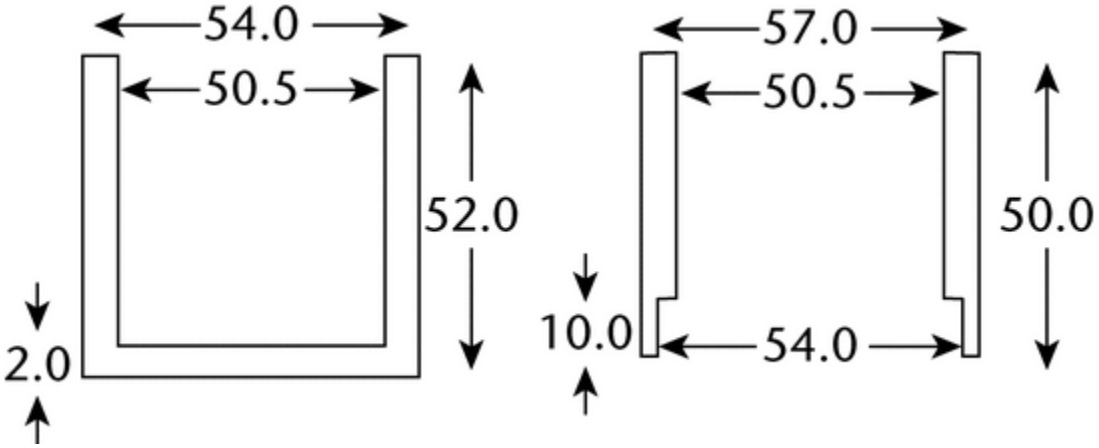


Figure 2.

PROCEDURE

Pass a quantity of powder sufficient to complete the test through a 1.0-mm sieve, if necessary, to break up agglomerates that may have formed during storage, and allow the obtained sample to flow freely into the measuring vessel until it overflows. Carefully scrape the excess powder from the top of the vessel as described for *Method II*. Determine the weight ($M_0$) of the powder to the nearest 0.1% by subtraction of the previously determined mass of the empty measuring vessel. Calculate the bulk density (g/mL) by the formula $M_0/100$, and record the average of three determinations using three different powder samples.

## TAPPED DENSITY

The tapped density is an increased bulk density attained after mechanically tapping a container containing the powder sample. Tapped density is obtained by mechanically tapping a graduated measuring cylinder or vessel containing a powder sample. After observing the initial powder volume or weight, the measuring cylinder or vessel is mechanically tapped, and volume or weight readings are taken until little further volume or weight change is observed. The mechanical tapping is achieved by raising the cylinder or vessel and allowing it to drop under its own weight a specified distance by either of three methods as described below. Devices that rotate the cylinder or vessel during tapping may be preferred to minimize any possible separation of the mass during tapping down.

### Method I

APPARATUS

The apparatus (*Figure 3*) consists of the following:

- A 250-mL graduated cylinder (readable to 2 mL with a mass of 220 ± 44 g)
- A settling apparatus capable of producing, in 1 min, either nominally 250 ± 15 taps from a height of 3 ± 0.2 mm, or nominally 300 ± 15 taps from a height of 14 ± 2 mm. The support for the graduated cylinder, with its holder, has a mass of 450 ± 10 g.

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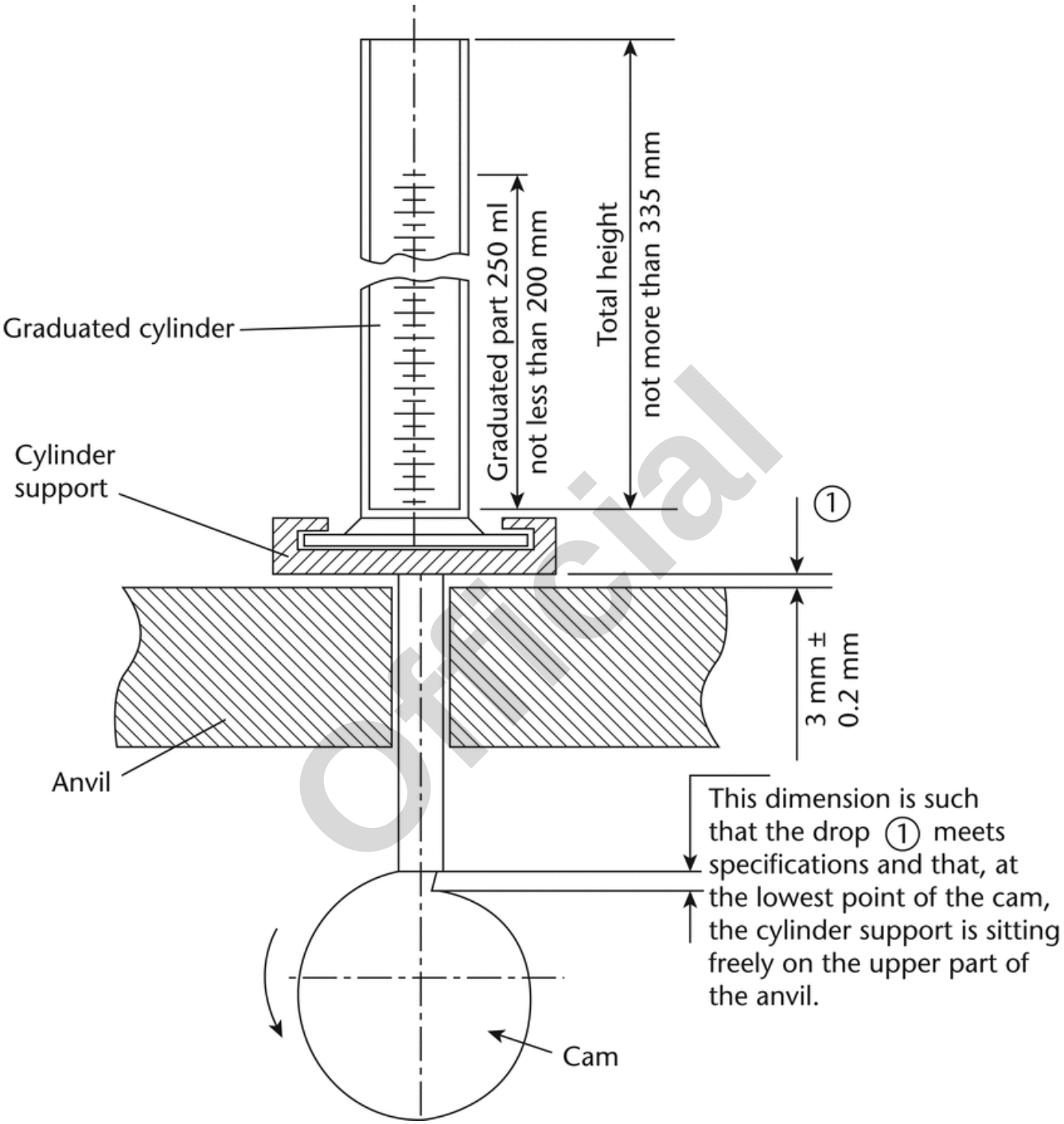


Figure 3.

PROCEDURE

Proceed as described above for the determination of the bulk volume ($V_0$). Secure the cylinder in the holder. Carry out 10, 500, and 1250 taps on the same powder sample and read the corresponding volumes $V_{10}$, $V_{500}$, and $V_{1250}$ to the nearest graduated unit. If the difference between $V_{500}$ and $V_{1250}$ is less than or equal to 2 mL, $V_{1250}$ is the tapped volume. If the difference between $V_{500}$ and $V_{1250}$ exceeds 2 mL, repeat in increments such as 1250 taps, until the difference between succeeding measurements is less than or equal to 2 mL. Fewer taps may be appropriate for some powders, when validated. Calculate the tapped density (g/mL) using the formula $m/V_F$, in which $V_F$ is the final tapped volume. Generally, replicate determinations are desirable for the determination of this property. Specify the drop height with the results. If it is not possible to use a 100-g test sample, use a reduced amount and a suitable 100-mL graduated cylinder (readable to 1 mL) weighing 130 ± 16 g and mounted on a holder weighing 240 ± 12 g. If the difference between $V_{500}$ and $V_{1250}$ is less than or equal to 1 mL, $V_{1250}$ is the tapped

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volume. If the difference between $V_{500}$ and $V_{1250}$ exceeds 1 mL, repeat in increments such as 1250 taps, until the difference between succeeding measurements is less than or equal to 1 mL. The modified test conditions are specified in the expression of the results.

### Method II

APPARATUS and PROCEDURE

Proceed as directed under *Method I* except that the mechanical tester provides a fixed drop of 3 ± 0.2 mm at a nominal rate of 250 taps per min.

### Method III

APPARATUS and PROCEDURE

Proceed as directed in *Method III—Measurement in a Vessel* for measuring bulk density using the measuring vessel equipped with the cap shown in *Figure 2*. The measuring vessel with the cap is lifted 50–60 times per min by the use of a suitable tapped density tester. Carry out 200 taps, remove the cap, and carefully scrape excess powder from the top of the measuring vessel as described in *Method III—Measurement in a Vessel* for measuring the bulk density. Repeat the procedure using 400 taps. If the difference between the two masses obtained after 200 and 400 taps exceeds 2%, carry out a test using 200 additional taps until the difference between succeeding measurements is less than 2%. Calculate the tapped density (g/mL) using the formula $M_F/100$, where $M_F$ is the mass of powder in the measuring vessel. Record the average of three determinations using three different powder samples. The test conditions including tapping height are specified in the expression of the results.

## MEASURES OF POWDER COMPRESSIBILITY

Because the interparticulate interactions influencing the bulking properties of a powder are also the interactions that interfere with powder flow, a comparison of the bulk and tapped densities can give a measure of the relative importance of these interactions in a given powder. Such a comparison is often used as an index of the ability of the powder to flow, for example the *Compressibility Index* or the *Hausner Ratio* as described below.

The *Compressibility Index* and *Hausner Ratio* are measures of the propensity of a powder to be compressed as described above. As such, they are measures of the powder's ability to settle, and they permit an assessment of the relative importance of interparticulate interactions. In a free-flowing powder, such interactions are less significant, and the bulk and tapped densities will be closer in value. For poorer flowing materials, there are frequently greater interparticle interactions, and a greater difference between the bulk and tapped densities will be observed. These differences are reflected in the *Compressibility Index* and the *Hausner Ratio*.

### Compressibility Index

Calculate by the formula:

$$100(V_0 - V_F)/V_0$$

$V_0$ = unsettled apparent volume
$V_F$ = final tapped volume

### Hausner Ratio

$$V_0/V_F$$

Depending on the material, the compressibility index can be determined using $V_{10}$ instead of $V_0$. [NOTE—If $V_{10}$ is used, it will be clearly stated in the results.]

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# ⟨621⟩ CHROMATOGRAPHY

***Change to read:***

**(The sections *System Sensitivity* and *Peak Symmetry* will become official on ▲May 1, 2025▲** (RB 1-Oct-2023) **as indicated.)**

To view the Notice from the Expert Committee that posted in conjunction with this accelerated revision, please click https://www.uspnf.com/rb-gc-621-20230929.

## INTRODUCTION

Chromatographic separation techniques are multistage separation procedures in which the components of a sample are distributed between two phases, one of which is stationary while the other is mobile. The stationary phase may be a solid or a liquid supported on a solid or a gel. The stationary phase may be packed in a column, spread as a layer, or distributed as a film, etc. The mobile phase may be gaseous or liquid or supercritical fluid. The separation may be based on adsorption, mass distribution (partition), ion exchange, etc., or may be based on differences in the physicochemical properties of the molecules such as size, mass, volume, etc.

Portions of the present general chapter text that are national *USP–NF* text, and therefore not part of the harmonized text, are marked with symbols (♦♦) to specify this fact.

♦This chapter describes general procedures, definitions, and calculations of common parameters and generally applicable requirements for system suitability.

The types of chromatography useful in qualitative and quantitative analysis employed in USP procedures are column, gas, paper, thin-layer (including high-performance thin-layer chromatography), and pressurized liquid chromatography (commonly called high-pressure or high-performance liquid chromatography).

## GENERAL PROCEDURES

This section describes the basic procedures used when a chromatographic method is described in a monograph. The following procedures are followed unless otherwise indicated in the individual monograph.

### Paper Chromatography

#### STATIONARY PHASE

The stationary phase is a sheet of paper of suitable texture and thickness. Development may be ascending, in which the solvent is carried up the paper by capillary forces, or descending, in which the solvent flow is also assisted by gravitational force. The orientation of paper grain with respect to solvent flow is to be kept constant in a series of chromatograms. (The machine direction is usually designated by the manufacturer.)

#### APPARATUS

The essential equipment for paper chromatography consists of a vapor-tight chamber with inlets for addition of solvent and a rack of corrosion-resistant material about 5 cm shorter than the inside height of the chamber. The rack serves as a support for solvent troughs and for antisiphon rods that, in turn, hold up the chromatographic sheets. The bottom of the chamber is covered with the prescribed solvent system or mobile phase. Saturation of the chamber with solvent vapor is facilitated by lining the inside walls with paper wetted with the prescribed solvent system.

#### SPOTTING

The substance or substances analyzed are dissolved in a suitable solvent. Convenient volumes delivered from suitable micropipets of the resulting solution, normally containing 1–20 µg of the compound, are placed in 6- to 10-mm spots not less than 3 cm apart.

#### DESCENDING PAPER CHROMATOGRAPHY PROCEDURE

1. A spotted chromatographic sheet is suspended in the apparatus, using the antisiphon rod to hold the upper end of the sheet in the solvent trough. [NOTE—Ensure that the portion of the sheet hanging below the rods is freely suspended in the chamber without touching the rack, the chamber walls, or the fluid in the chamber.]
2. The chamber is sealed to allow equilibration (saturation) of the chamber and the paper with the solvent vapor. Any excess pressure is released as necessary.
3. After equilibration of the chamber, the prepared mobile phase is introduced into the trough through the inlet.
4. The inlet is closed, and the mobile solvent phase is allowed to travel the desired distance down the paper.
5. The sheet is removed from the chamber.
6. The location of the solvent front is quickly marked, and the sheet is dried.
7. The chromatogram is observed and measured directly or after suitable development to reveal the location of the spots of the isolated drug or drugs.

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### ASCENDING PAPER CHROMATOGRAPHY PROCEDURE

1. The mobile phase is added to the bottom of the chamber.
2. The chamber is sealed to allow equilibration (saturation) of the chamber and the paper with the solvent vapor. Any excess pressure is released as necessary.
3. The lower edge of the stationary phase is dipped into the mobile phase to permit the mobile phase to rise on the chromatographic sheet by capillary action.
4. When the solvent front has reached the desired height, the chamber is opened, the sheet is removed, the location of the solvent front is quickly marked, and the sheet is dried.
5. The chromatogram is observed and measured directly or after suitable development to reveal the location of the spots of the isolated drug or drugs.

## Thin-Layer Chromatography

### STATIONARY PHASE

The stationary phase is a relatively thin, uniform layer of dry, finely powdered material applied to a glass, plastic, or metal sheet or plate (typically called the plate). The stationary phase of thin-layer chromatography (TLC) plates has an average particle size of 10–15 µm, and that of high-performance TLC (HPTLC) plates has an average particle size of 5 µm. Commercial plates with a preadsorbent zone can be used if they are specified in a monograph. Sample applied to the preadsorbent region develops into sharp, narrow bands at the preadsorbent–sorbent interface. The separations achieved may be based on adsorption, partition, or a combination of both effects, depending on the particular type of stationary phase.

### APPARATUS

A chromatographic chamber made of inert, transparent material and having the following specifications is used: a flat-bottom or twin trough, a tightly fitted lid, and a size suitable for the plates. The chamber is lined on at least one wall with filter paper. Sufficient mobile phase or developing solvent is added to the chamber so that, after impregnation of the filter paper, a depth appropriate to the dimensions of the plate used is available. The chromatographic chamber is closed and allowed to equilibrate. [NOTE—Unless otherwise indicated, the chromatographic separations are performed in a saturated chamber.]

### DETECTION/VISUALIZATION

An ultraviolet (UV) light source suitable for observations under short- (254 nm) and long- (365 nm) wavelength UV light and a variety of spray reagents to make spots visible are often used.

### SPOTTING

Solutions are spotted on the surface of the stationary phase (plate) at the prescribed volume in sufficiently small portions to obtain circular spots of 2–5 mm in diameter (1–2 mm on HPTLC plates) or bands of 10–20 mm × 1–2 mm (5–10 mm × 0.5–1 mm on HPTLC plates) at an appropriate distance from the lower edge and sides of the plate. [NOTE—During development, the application position must be at least 5 mm (TLC) or 3 mm (HPTLC) above the level of the mobile phase.] The solutions are applied on a line parallel to the lower edge of the plate with an interval of at least 10 mm (5 mm on HPTLC plates) between the centers of spots, or 4 mm (2 mm on HPTLC plates) between the edges of bands, then allowed to dry.

### PROCEDURE

1. Place the plate in the chamber, ensuring that the spots or bands are above the surface of the mobile phase.
2. Close the chamber.
3. Allow the mobile phase to ascend the plate until the solvent front has traveled three-quarters of the length of the plate, or the distance prescribed in the monograph.
4. Remove the plate, mark the solvent front with a pencil, and allow to dry.
5. Visualize the chromatograms as prescribed.
6. Determine the chromatographic *retardation factor* ($R_F$) values for the principal spots or zones.
7. Presumptive identification can be made by observation of spots or zones of identical $R_F$ value and about equal magnitude obtained, respectively, with an unknown and a standard chromatographed on the same plate. A visual comparison of the size or intensity of the spots or zones may serve for semiquantitative estimation. Quantitative measurements are possible by means of densitometry (absorbance or fluorescence measurements).

## Column Chromatography

### SOLID SUPPORT

Purified siliceous earth is used for normal-phase separation. Silanized chromatographic siliceous earth is used for reverse-phase partition chromatography.

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### STATIONARY PHASE

The solid support is modified by the addition of a stationary phase specified in the individual monograph. If a mixture of liquids is used as the stationary phase, mix the liquids before the introduction of the solid support.

### MOBILE PHASE

The mobile phase is specified in the individual monograph. If the stationary phase is an aqueous solution, equilibrate with water. If the stationary phase is a polar organic fluid, equilibrate with that fluid.

### APPARATUS

Unless otherwise specified in the individual monograph, the chromatographic tube is about 22 mm in inside diameter and 200–300 mm in length. Attached to it is a delivery tube, without stopcock, about 4 mm in inside diameter and about 50 mm in length.

**Apparatus preparation:** Pack a pledget of fine glass wool in the base of the tube. Combine the specified volume of stationary phase and the specified amount of solid support to produce a homogeneous, fluffy mixture. Transfer this mixture to the chromatographic tube, and tamp using gentle pressure to obtain a uniform mass. If the specified amount of solid support is more than 3 g, transfer the mixture to the column in portions of approximately 2 g, and tamp each portion. If the assay or test requires a multisegment column with a different stationary phase specified for each segment, tamp after the addition of each segment, and add each succeeding segment directly to the previous one. Pack a pledget of fine glass wool above the completed column packing. [NOTE—The mobile phase should flow through a properly packed column as a moderate stream or, if reverse-phase chromatography is applied, as a slow trickle.]

If a solution of the analyte is incorporated into the stationary phase, complete the quantitative transfer to the chromatographic tube by scrubbing the beaker used for the preparation of the test mixture with a mixture of about 1 g of solid support and several drops of the solvent used to prepare the sample solution before adding the final portion of glass wool.

### PROCEDURE

1. Transfer the mobile phase to the column space above the column packing, and allow it to flow through the column under the influence of gravity.
2. Rinse the tip of the chromatographic column with about 1 mL of mobile phase before each change in composition of mobile phase and after completion of the elution.
3. If the analyte is introduced into the column as a solution in the mobile phase, allow it to pass completely into the column packing, then add mobile phase in several small portions, allowing each to drain completely, before adding the bulk of the mobile phase.
4. Where the procedure indicates the use of multiple chromatographic columns mounted in series and the addition of mobile phase in divided portions is specified, allow each portion to drain completely through each column, and rinse the tip of each with mobile phase before the addition of each succeeding portion.

## Gas Chromatography (GC)

### LIQUID STATIONARY PHASE

This type of phase is available in packed or capillary columns.

### PACKED COLUMN GC

The liquid stationary phase is deposited on a finely divided, inert solid support, such as diatomaceous earth, porous polymer, or graphitized carbon, which is packed into a column that is typically 2–4 mm in internal diameter and 1–3 m in length.

### CAPILLARY COLUMN GC

In capillary columns, which contain no packed solid support, the liquid stationary phase is deposited on the inner surface of the column and may be chemically bonded to it.

### SOLID STATIONARY PHASE

This type of phase is available only in packed columns. In these columns the solid phase is an active adsorbent, such as alumina, silica, or carbon, packed into a column. Polyaromatic porous resins, which are sometimes used in packed columns, are not coated with a liquid phase. [NOTE—Packed and capillary columns must be conditioned before use until the baseline and other characteristics are stable. The column or packing material supplier provides instructions for the recommended conditioning procedure.]

### APPARATUS

A gas chromatograph consists of a carrier gas source, injection port, column, detector, and recording device. The injection port, column, and detector are temperature controlled and may be varied as part of the analysis. The typical carrier gas is helium, nitrogen, or hydrogen, depending on the column and detector in use. The type of detector used depends on the nature of the

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compounds analyzed and is specified in the individual monograph. Detector output is recorded as a function of time, and the instrument response, measured as peak area or peak height, is a function of the amount present.

### TEMPERATURE PROGRAM

The length and quality of a GC separation can be controlled by altering the temperature of the chromatographic column. When a temperature program is necessary, the individual monograph indicates the conditions in table format. The table indicates the initial temperature, rate of temperature change (ramp), final temperature, and hold time at the final temperature.

### PROCEDURE

1. Equilibrate the column, injector, and detector with flowing carrier gas until a constant signal is received.
2. Inject a sample through the injector septum, or use an autosampler.
3. Begin the temperature program.
4. Record the chromatogram.
5. Analyze as indicated in the monograph.

## Liquid Chromatography

The term "liquid chromatography" (LC), as used in the compendia, is synonymous with high-pressure liquid chromatography and high-performance liquid chromatography. LC is a separation technique based on a solid stationary phase and a liquid mobile phase.

### STATIONARY PHASE

Separations are achieved by partition, adsorption, or ion-exchange processes, depending on the type of stationary phase used. The most commonly used stationary phases are modified silica or polymeric beads. The beads are modified by the addition of long-chain hydrocarbons. The specific type of packing needed to complete an analysis is indicated by the “L” designation in the individual monograph (see also the section *Chromatographic Columns*). The size of the beads is often described in the monograph as well. Changes in the packing type and size are covered in the *System Suitability* section of this chapter.

### CHROMATOGRAPHIC COLUMN

The term "column" includes stainless steel, lined stainless steel, and polymeric columns, packed with a stationary phase. The length and inner diameter of the column affects the separation, and therefore typical column dimensions are included in the individual monograph. Changes to column dimensions are discussed in the *System Suitability* section of this chapter. Compendial monographs do not include the name of appropriate columns; this omission avoids the appearance of endorsement of a vendor’s product and natural changes in the marketplace. See the section *Chromatographic Columns* for more information.

In LC procedures, a guard column may be used with the following requirements, unless otherwise is indicated in the individual monograph: (a) the length of the guard column must be NMT 15% of the length of the analytical column, (b) the inner diameter must be the same or smaller than that of the analytical column, and (c) the packing material should be the same as the analytical column (e.g., silica) and contain the same bonded phase (e.g., C18). In any case, all system suitability requirements specified in the official procedure must be met with the guard column installed.

### MOBILE PHASE

The mobile phase is a solvent or a mixture of solvents, as defined in the individual monograph.

### APPARATUS

A liquid chromatograph consists of a reservoir containing the mobile phase, a pump to force the mobile phase through the system at high pressure, an injector to introduce the sample into the mobile phase, a chromatographic column, a detector, and a data collection device.

### GRADIENT ELUTION

The technique of continuously changing the solvent composition during the chromatographic run is called gradient elution or solvent programming. The gradient elution profile is presented in the individual monograph as a gradient table, which lists the time and proportional composition of the mobile phase at the stated time.

### PROCEDURE

1. Equilibrate the column and detector with mobile phase at the specified flow rate until a constant signal is received.
2. Inject a sample through the injector, or use an autosampler.
3. Begin the gradient program.
4. Record the chromatogram.
5. Analyze as directed in the monograph.

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## CHROMATOGRAPHIC COLUMNS

A complete list of packings (L), phases (G), and supports (S) used in *USP–NF* tests and assays is located in *USP–NF*, Reagents, Indicators, and Solutions—Chromatographic Columns. This list is intended to be a convenient reference for the chromatographer in identifying the pertinent chromatographic column specified in the individual monograph.♦

## DEFINITIONS

The system suitability and acceptance criteria in monographs have been set using parameters as defined below. With some equipment, certain parameters, such as the signal-to-noise ratio and resolution, can be calculated using software provided by the manufacturer. It is the responsibility of the user to ensure that the calculation methods used in the software are equivalent to the requirements of the *US Pharmacopeia* and to make any necessary corrections if this is not the case.

**Chromatogram:** A graphical or other representation of detector response, effluent concentration, or other quantity used as a measure of effluent concentration versus time or volume. Idealized chromatograms are represented as a sequence of Gaussian peaks on a baseline (*Figure 1*).

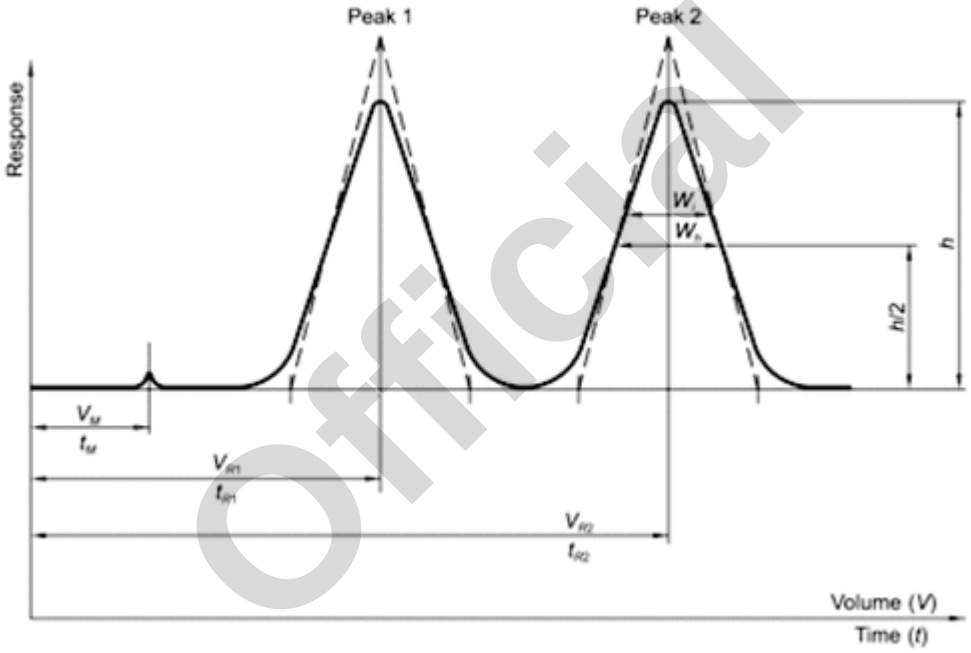


Figure 1.

$V_M$ = hold-up volume
$t_M$ = hold-up time
$V_{R1}$ = retention volume of peak 1
$t_{R1}$ = retention time of peak 1
$V_{R2}$ = retention volume of peak 2
$t_{R2}$ = retention time of peak 2
$W_h$ = peak width at half-height
$W_i$ = peak width at the inflection point
$h$ = height of the peak
$h/2$ = half-height of peak

**Distribution constant ($K_0$):** In size-exclusion chromatography, the elution characteristics of a component in a particular column may be given by the distribution constant (also referred to as distribution coefficient), which is calculated using the following equation:

$$K_0 = \frac{t_R - t_0}{t_t - t_0}$$

$t_R$ = retention time
$t_0$ = retention time of an unretained compound
$t_t$ = total mobile phase time

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**Dwell volume (*D*) (also referred to as $V_D$):** The dwell volume (also known as gradient delay volume) is the volume between the point at which the eluents meet and the inlet of the column. It can be determined using the following procedure.

COLUMN: Replace the chromatographic column by an appropriate capillary tubing (e.g., 1 m × 0.12 mm).

MOBILE PHASE: See *Table 1*.

MOBILE PHASE A: Water

MOBILE PHASE B: 0.1% v/v solution of acetone in water

**Table 1**

| Time (min) | Mobile phase A (% v/v) | Mobile phase B (% v/v) |
|---|---|---|
| 0–20 | 100→0 | 0→100 |
| 20–30 | 0 | 100 |

FLOW RATE: Set to obtain sufficient back-pressure (e.g., 2 mL/min).

DETECTION: Spectrophotometer at 265 nm

Determine the time ($t_{0.5}$), in minutes, when the absorbance has increased by 50% (*Figure 2*).

$$D = t_D \times F$$

$t_D$ = $t0.5 — 0.5tG$ (min)
$t_G$ = pre-defined gradient time, 20 min
$F$ = flow rate (mL/min)

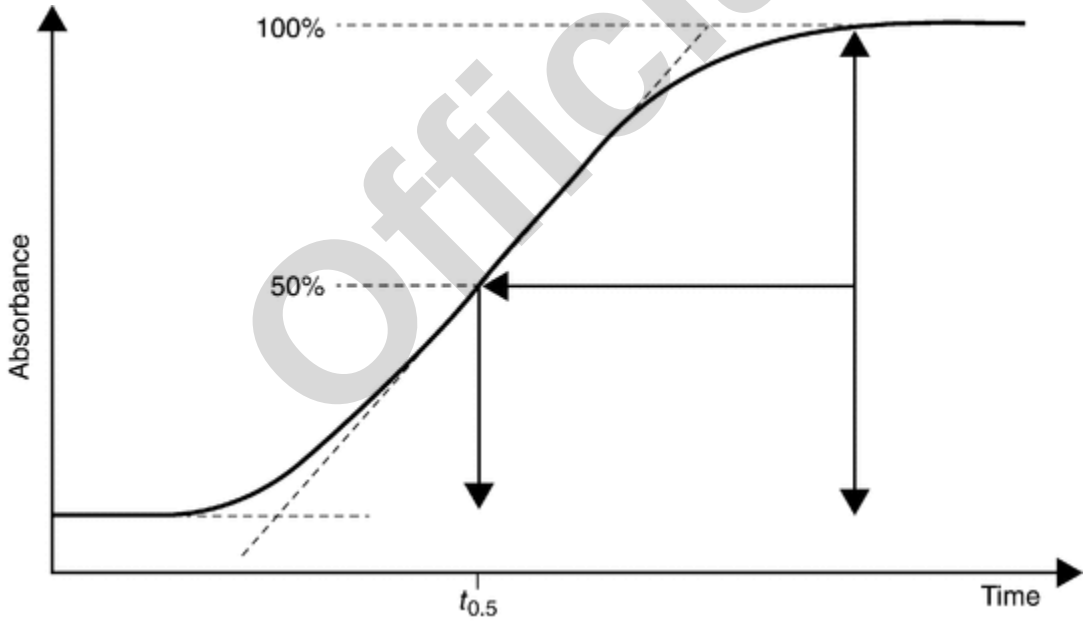


Figure 2.

[NOTE—where applicable, this measurement is performed with the autosampler in the "inject" position so as to include the injection loop volume in the dwell volume.]

**Hold-up time ($t_M$):** Time required for elution of an unretained component (see *Figure 1*, baseline scale being in minutes or seconds).

In size-exclusion chromatography, the term retention time of an unretained compound ($t_0$) is used.

**Hold-up volume ($V_M$):** Volume of the mobile phase required for elution of an unretained component. It may be calculated from the hold-up time and the flow rate ($F$) in milliliters per minute using the following equation:

$$V_M = t_M \times F$$

In size-exclusion chromatography, the term "retention volume" of an unretained compound $V_0$ is used.

**Peak:** Portion of a chromatogram recording the detector response when a single component (or two or more unresolved components) is eluted from the column.

The peak response may be represented by the peak area or the peak height ($h$).

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**Peak-to-Valley Ratio (*p*/*v*):** The peak-to-valley ratio may be employed as a system suitability criterion when baseline separation between two peaks is not achieved (see *Figure 3*).

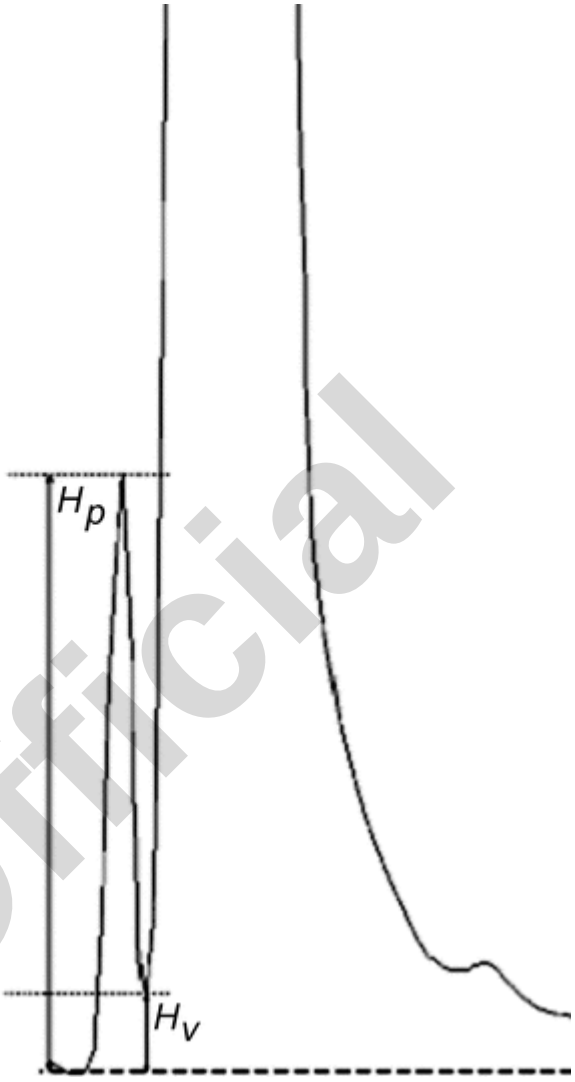


Figure 3.

$$p/v = \frac{H_p}{H_v}$$

$H_p$ = height above the extrapolated baseline of the minor peak
$H_v$ = height above the extrapolated baseline at the lowest point of the curve separating the minor and major peaks

**Plate height (*H*) (height equivalent to one theoretical plate [HETP]):** Ratio of the column length (*L*), in micrometers, to the plate number (*N*):

$$H = \frac{L}{N}$$

**Plate number (*N*) (number of theoretical plates):** A number indicative of column performance (column efficiency) can only be calculated from data obtained under either isothermal, isocratic, or isodense conditions, depending on the technique, as the plate number, using the following equation, the values of $t_R$ and $W_h$ being expressed in the same units:

$$N = 5.54 \left(\frac{t_R}{W_h}\right)^2$$

$t_R$ = retention time of the peak corresponding to the component
$W_h$ = peak width at half-height ($h$/2)

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The plate number varies with the component as well as with the column, the column temperature, the mobile phase, and the retention time.

**Reduced plate height (*h*):** Ratio of the plate height (*H*), in micrometers, to the particle diameter ($d_p$) in micrometers:

$$h = \frac{H}{d_p}$$

**Relative retardation ($R_{rel}$):** The relative retardation, used in thin-layer chromatography, is calculated as the ratio of the distances travelled by the spot of the compound of interest and a reference compound (*Figure 4*).

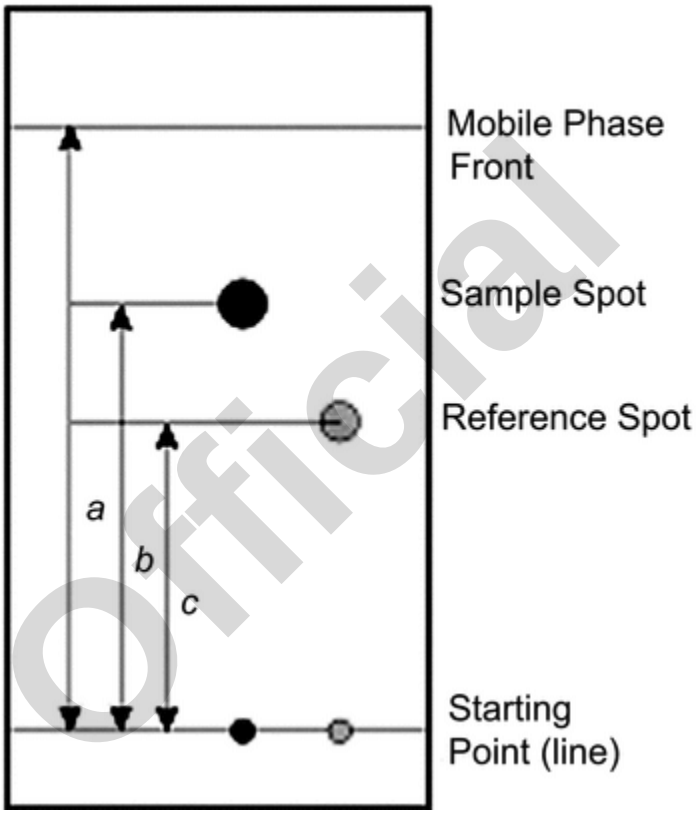


Figure 4.

$$R_{rel} = b/c$$

*a* = migration distance of the mobile phase
*b* = migration distance of the compound of interest
*c* = migration distance of the reference compound

**Relative retention (*r*):** Relative retention is calculated as an estimate using the following equation:

$$r = \frac{t_{Ri} - t_M}{t_{Rst} - t_M}$$

$t_{Ri}$ = retention time of the peak of interest
$t_{Rst}$ = retention time of the reference peak (usually the peak corresponding to the substance to be examined)
$t_M$ = hold-up time

### Relative retention, unadjusted ($r_G$)♦or (RRT)♦

The unadjusted relative retention is calculated using the following equation:

$$r_G = \frac{t_{Ri}}{t_{Rst}}$$

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Unless otherwise indicated, values for relative retention stated in monographs correspond to unadjusted relative retention.

♦**Relative retention time (RRT):** See *Relative retention, unadjusted*♦

**Resolution ($R_s$):** The resolution between peaks of two components (*Figure 1*) may be calculated using the following equation:

$$R_S = \frac{1.18(t_{R2} - t_{R1})}{W_{h1} + W_{h2}}$$
$$t_{R2} > t_{R1}$$

$t_{R1}$, $t_{R2}$ = retention times of the peaks
$W_{h1}$, $W_{h2}$ = peak widths at half-height

In quantitative thin-layer chromatography, using densitometry, the migration distances are used instead of retention times and the resolution between peaks of two components may be calculated using the following equation:

$$R_S = \frac{1.18a(R_{F2} - R_{F1})}{W_{h1} + W_{h2}}$$
$$R_{F2} > R_{F1}$$

$R_{F1}$, $R_{F2}$ = retardation factors of the peaks
$W_{h1}$, $W_{h2}$ = peak widths at half-height
$a$ = migration distance of the solvent front

**Retardation factor ($R_F$):** The retardation factor, used in thin-layer chromatography, is the ratio of the distance from the point of application to the center of the spot and the distance simultaneously travelled by the solvent front from the point of application (*Figure 4*).

$$R_F = \frac{b}{a}$$

$b$ = migration distance of the component
$a$ = migration distance of the solvent front

**Retention factor ($k$):** The retention factor (also known as mass distribution ratio ($D_m$) or capacity factor ($k'$)) is defined as:

$$k = \frac{amount\ of\ component\ in\ stationary\ phase}{amount\ of\ component\ in\ mobile\ phase} = K_C \frac{V_S}{V_M}$$

$K_C$ = distribution constant (also known as equilibrium distribution coefficient)
$V_S$ = volume of the stationary phase
$V_M$ = volume of the mobile phase

The retention factor of a component may be determined from the chromatogram using the following equation:

$$k = \frac{t_R - t_M}{t_M}$$

$t_R$ = retention time
$t_M$ = hold-up time

**Retention time ($t_R$):** Time elapsed between the injection of the sample and the appearance of the maximum peak response of the eluted sample zone (*Figure 1*, baseline scale being in minutes or seconds).

**Retention volume ($V_R$):**

Volume of the mobile phase required for elution of a component. It may be calculated from the retention time and the flow rate ($F$), in milliliters per minute, using the following equation:

$$V_R = t_R \times F$$

**Retention time of an unretained compound ($t_0$):** In size-exclusion chromatography, retention time of a component whose molecules are larger than the largest gel pores (*Figure 5*).

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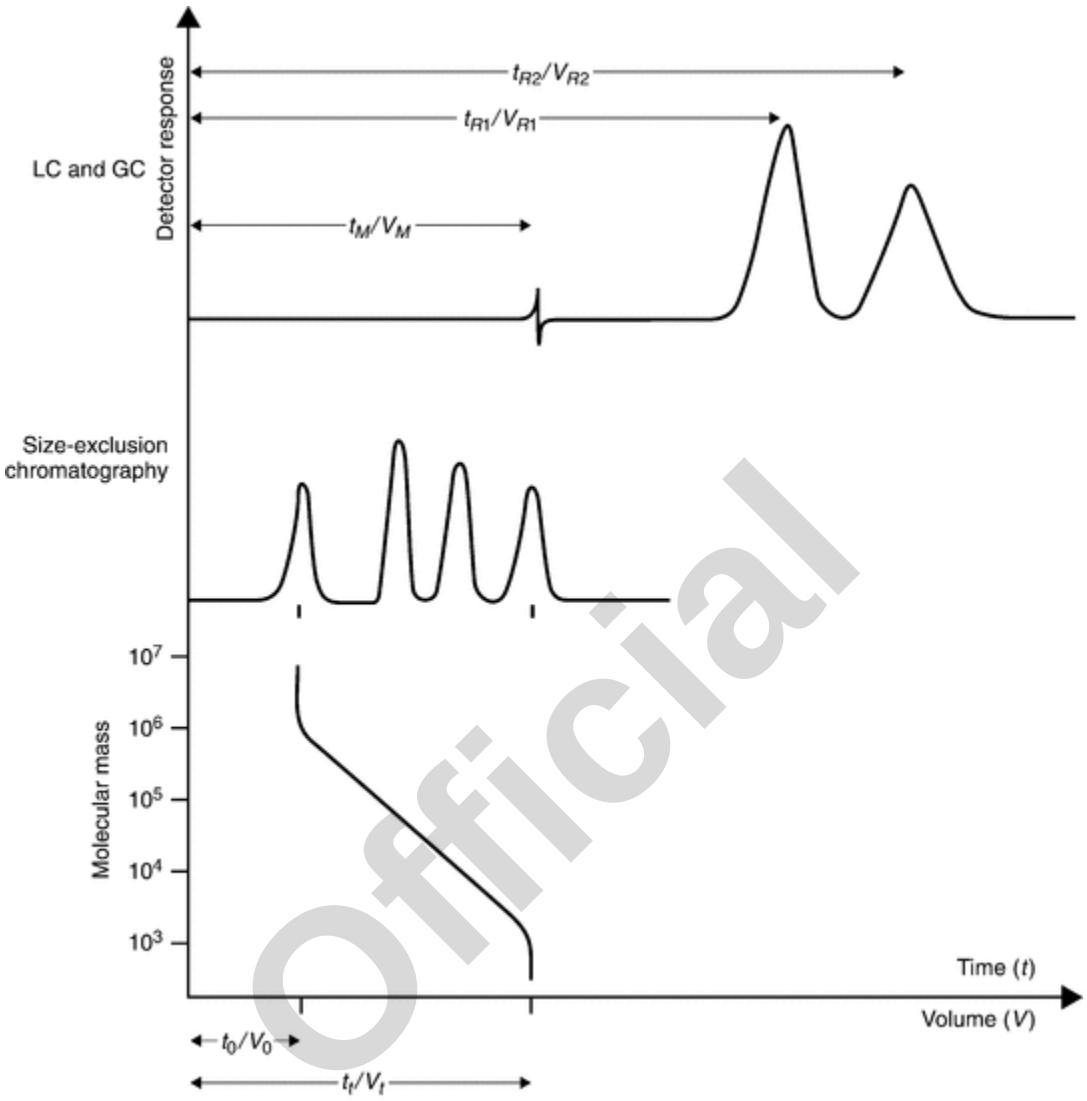


Figure 5.

**Retention volume of an unretained compound ($V_0$):** In size-exclusion chromatography, retention volume of a component whose molecules are larger than the largest gel pores. It may be calculated from the retention time of an unretained compound and the flow rate ($F$), in milliliters per minute, using the following equation:

$$V_0 = t_0 \times F$$

**Separation factor ($\alpha$):** Relative retention calculated for two adjacent peaks (by convention, the value of the separation factor is always >1):

$$\alpha = k_2/k_1$$

$k_1$ = retention factor of the first peak
$k_2$ = retention factor of the second peak

**Signal-to-noise ratio ($S/N$):** The short-term noise influences the precision and accuracy of quantitation. The signal-to-noise ratio is calculated using the following equation:

$$S/N = \frac{2H}{h}$$

$H$ = height of the peak (*Figure 6*) corresponding to the component concerned, in the chromatogram obtained with the prescribed reference solution, measured from the maximum of the peak to the extrapolated baseline of the signal observed over a distance of at least 5 times the width at half-height

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*h* = range of the noise in a chromatogram obtained after injection of a blank (*Figure 7*), observed over a distance of at least 5 times the width at half-height of the peak in the chromatogram obtained with the prescribed reference solution and, if possible, situated equally around the place where this peak would be found

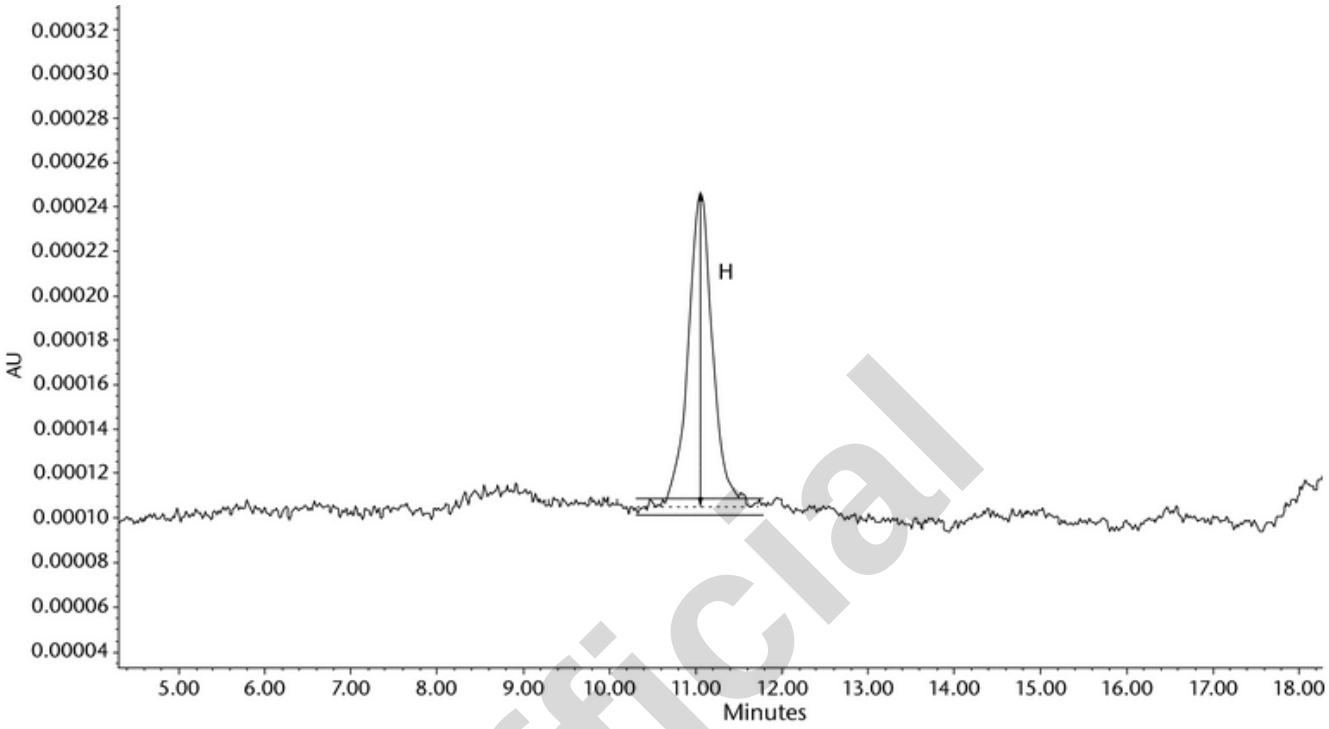


Figure 6. Chromatogram of the reference solution

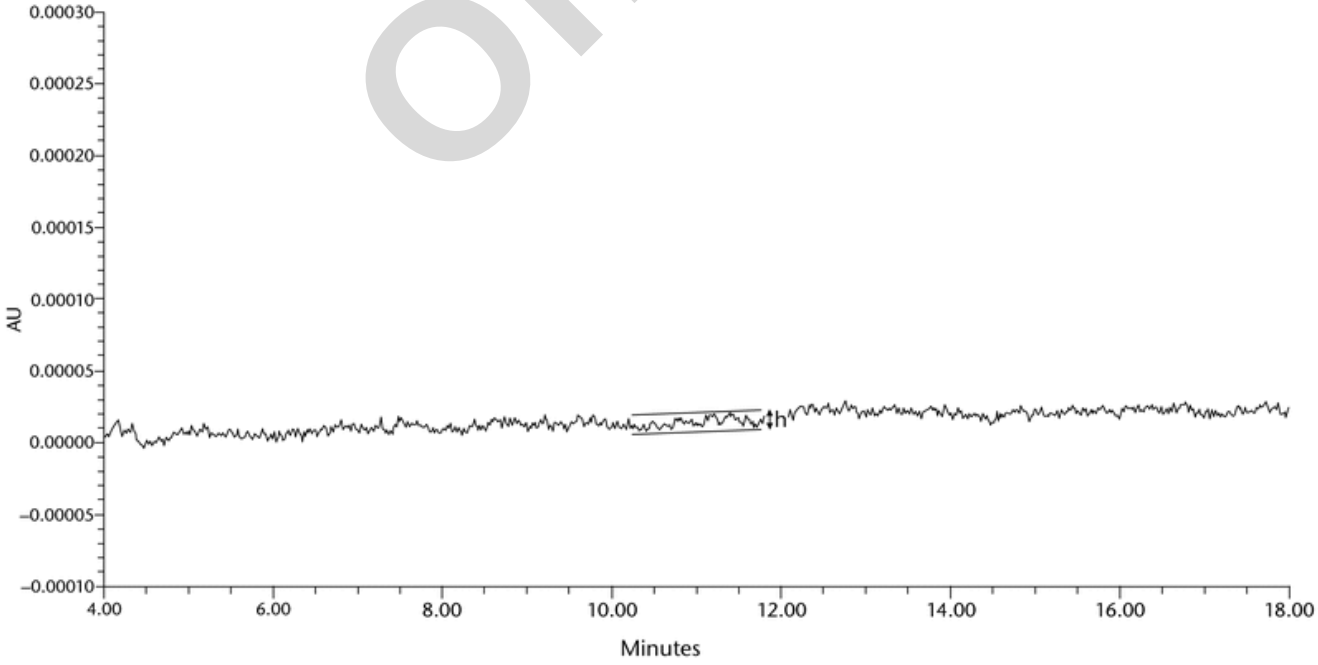


Figure 7.

**Symmetry factor ($A_S$):** The symmetry factor of a peak (also known as the asymmetry factor or tailing factor) (*Figure 8*) is calculated using the following equation:

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$$A_s = \frac{W_{0.05}}{2d}$$

$W_{0.05}$ = width of the peak at one-twentieth of the peak height
$d$ = distance between the perpendicular dropped from the peak maximum and the leading edge of the peak at one-twentieth of the peak height

An $A_s$ value of 1.0 signifies symmetry. When $A_s > 1.0$, the peak is tailing. When $A_s < 1.0$, the peak is fronting.

Figure 8.

**System repeatability:** The repeatability of response is expressed as an estimated percentage relative standard deviation (%RSD) of a consecutive series of measurements for not fewer than three injections or applications of a reference solution, and is calculated using the following equation.

$$\%\,RSD = \frac{100}{\overline{y}}\sqrt{\frac{\Sigma(y_i - \overline{y})^2}{n-1}}$$

$y_i$ = individual values expressed as peak area, peak height, or ratio of areas by the internal standardization method
$\overline{y}$ = mean of individual values
$n$ = number of individual values

**Total mobile phase time ($t_t$):** In size-exclusion chromatography, retention time of a component whose molecules are smaller than the smallest gel pores (*Figure 5*).

**Total mobile phase volume ($V_t$):** In size-exclusion chromatography, retention volume of a component whose molecules are smaller than the smallest gel pores. It may be calculated from the total mobile phase time and the flow rate ($F$), in milliliters/minute, using the following equation:

$$V_t = t_t \times F$$

***Change to read:***

## SYSTEM SUITABILITY

[NOTE—This section only covers liquid chromatography and gas chromatography]

The various components of the equipment employed must be qualified and be capable of achieving the performance required to conduct the test or assay. The system suitability tests represent an integral part of the analytical procedure and are used to ensure adequate performance of the chromatographic system. Column plate number, retention factor (mass distribution ratio), system repeatability, signal-to-noise, symmetry factor, and resolution/peak-to-valley ratio are the parameters that may be employed in assessing the performance of the chromatographic system. When it is indicated in the individual monograph, in case of complex chromatographic profiles (e.g., for biotechnological and biological products) visual comparison of the profiles can be used as a system suitability test. Factors that may affect the chromatographic behavior include:

- Composition and temperature of the mobile phase
- Ionic strength and pH of the aqueous component, of the mobile phase
- Flow rate, column dimensions, column temperature, and pressure
- Stationary phase characteristics including, type of chromatographic support (particle-based or monolithic), particle or pore size, and porosity, and specific surface area
- Reverse phase and other surface modification of the stationary phases, the extent of chemical modification (as expressed by end-capping, carbon loading, etc.)

Retention times and relative retentions may be provided in monographs for information purposes only, unless otherwise stated in the monograph. There are no acceptance criteria applied to relative retentions.

Compliance with the system suitability criteria is required throughout the chromatographic procedure. No sample analysis is acceptable unless the suitability of the system has been demonstrated.

The following requirements are to be fulfilled, in addition to any other system suitability criteria stated in the monograph. When specific requirements are stated in the monograph, they supersede the requirements mentioned in this chapter.

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## System Repeatability

♦When a relative standard deviation requirement is specified in an individual monograph, if the requirement is 2.0 or less the calculation is based on data from five replicate injections of the analyte, if the requirement is more than 2.0% data from six replicate injections are used.♦

In an assay of an active substance or an excipient, where the target value is 100% for a pure substance, and a system repeatability requirement is not specified, the maximum permitted relative standard deviation (%$RSD_{max}$) for the defined limits is calculated for a series ($n$ = 3 to 6) of injections of the reference solution. The maximum permitted relative standard deviation of the peak response does not exceed the appropriate value given in *Table 2*.

$$\%\,RSD_{\max} = \frac{KB\sqrt{n}}{t_{90\,\%,\,n-1}}$$

$K$ = constant (0.349), obtained from the expression $K = \frac{0.6}{\sqrt{2}} \times \frac{t_{90\,\%,\,n-1}}{\sqrt{6}}$ in which $\frac{0.6}{\sqrt{2}}$ represents the required percentage relative standard deviation determined on 6 injections for $B$ = 1.0
$B$ = upper limit given in the definition of the individual monograph minus 100%
$n$ = number of replicate injections of the reference solution ($3 \le n \le 6$)
$t_{90\%,n-1}$ = Student's $t$ at the 90% probability level (double sided) with $n$–1 degrees of freedom

**Table 2. Maximum Permitted Relative Standard Deviation of Peak Response (Assay)**

| | Number of Individual Injections ($n$) | | | |
|---|---|---|---|---|
| | 3 | 4 | 5 | 6 |
| $B$ (%) | Maximum Permitted Relative Standard Deviation (%) | | | |
| 2.0 | 0.41 | 0.59 | 0.73 | 0.85 |
| 2.5 | 0.52 | 0.74 | 0.92 | 1.06 |
| 3.0 | 0.62 | 0.89 | 1.10 | 1.27 |

$B$ = upper limit of content given in the individual monograph minus 100%

## ▲System Sensitivity

The signal-to-noise ratio is used to define the system sensitivity. The limit of quantitation (corresponding to a signal-to-noise ratio of 10) is equal to or less than the reporting threshold.

## Peak Symmetry

Unless otherwise stated, in a test or assay, the symmetry factor (tailing factor) of the peak used for quantification is 0.8–1.8.▲ (Official 1-May-2025)

# ADJUSTMENT OF CHROMATOGRAPHIC CONDITIONS

The chromatographic conditions described have been validated during the elaboration of the monograph.

The extent to which the various parameters of a chromatographic test may be adjusted without fundamentally modifying the pharmacopeial analytical procedures are listed below. Changes other than those indicated require revalidation of the procedure.

Multiple adjustments can have a cumulative effect on the performance of the system and are to be properly evaluated by the users. This is particularly important in cases where the separation pattern is described as a profile. In those cases, a risk assessment has to be carried out.

Any adjustments must be made on the basis of the pharmacopeial procedure.

If adjustments are made to a pharmacopeial procedure, additional verification tests may be required. To verify the suitability of the adjusted pharmacopeial procedure, assess the relevant analytical performance characteristics potentially affected by the change.

When a pharmacopeial procedure has been adjusted according to the requirements stated below, no further adjustments are allowed without appropriate revalidation.

Compliance with the system suitability criteria is required to verify that conditions for satisfactory performance of the test or assay are achieved.

Adjustment of conditions with gradient elution (HPLC) or temperature programming (GC) is more critical than with isocratic (HPLC) or isothermal (GC) elution, since it may shift some peaks to a different step of the gradient or to different elution temperatures, potentially causing partial or complete coelution of adjacent peaks or peak inversion, and, thus leading to the incorrect assignment of peaks and to the masking of peaks or a shift such that elution occurs beyond the prescribed elution time.

For some parameters the adjustments are explicitly defined in the monograph to ensure the system suitability.

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## Thin-Layer Chromatography

*Composition of the mobile phase*: the amount of the minor solvent components may be adjusted by ±30% relative or ±2% absolute, whichever is the larger; no other component is altered by more than 10% absolute. A minor component comprises less than or equal to (100/*n*)%, *n* being the total number of components of the mobile phase. For a minor component at 10% of the mobile phase, a 30% relative adjustment allows a range of 7%–13% whereas a 2% absolute adjustment allows a range of 8%–12%, the relative value therefore being the larger; for a minor component at 5% of the mobile phase, a 30% relative adjustment allows a range of 3.5%–6.5% whereas a 2% absolute adjustment allows a range of 3%–7%, the absolute value being the larger in this case.

*pH of the aqueous component of the mobile phase*: ±0.2 pH units, unless otherwise prescribed

*Concentration of salts in the buffer component of a mobile phase*: ± 10%

*Application volume*: 10%–20% of the prescribed volume if using fine particle size plates (2–10 µm)

## Liquid Chromatography: Isocratic Elution

### COLUMN PARAMETERS AND FLOW RATE

*Stationary phase*: No change of the identity of the substituent (e.g., no replacement of C18 by C8); the other physicochemical characteristics of the stationary phase, i.e., chromatographic support, surface modification and extent of chemical modification must be similar; a change from totally porous particle (TPP) columns to superficially porous particle (SPP) columns is allowed provided the above-mentioned requirements are met.

*Column dimensions (particle size, length)*: The particle size and/or length of the column may be modified, provided that the ratio of the column length (*L*) to the particle size (*dp*) remains constant or in the range between –25% to +50% of the prescribed *L*/*dp* ratio.

*Adjustments from totally porous to superficially porous particles*: For the application of particle-size adjustment from totally porous to superficially porous particles, other combinations of *L* and *dp* can be used, provided that the plate number (*N*) is within –25% to +50%, relative to the prescribed column. These changes are acceptable, provided that system suitability criteria are fulfilled, and selectivity and elution order of the specified impurities to be controlled are demonstrated to be equivalent.

*Internal diameter*: In absence of a change in particle size and/or length, the internal diameter of the column may be adjusted.

Caution is necessary when the adjustment results in smaller peak volumes due to a smaller particle size or smaller internal column diameter, a situation that may require adjustments to minimize extra-column band broadening by factors such as instrument connections, detector cell volume and sampling rate, and injection volume.

When the particle size is changed, the flow rate requires adjustment, because smaller-particle columns will require higher linear velocities for the same performance (as measured by reduced plate height). The flow rate is adjusted for both the change in column diameter and particle size using the following equation:

$$F_2 = F_1 \times \left[\left(dc_2^2 \times dp_1\right) / \left(dc_1^2 \times dp_2\right)\right]$$

$F_1$ = flow rate indicated in the monograph (mL/min)
$F_2$ = adjusted flow rate (mL/min)
$dc_1$ = internal diameter of the column indicated in the monograph
$dc_2$ = internal diameter of the column used (mm)
$dp_1$ = particle size indicated in the monograph (µm)
$dp_2$ = particle size of the column used (µm)

When a change is made from ≥3-µm to <3-µm particles in isocratic separations, an additional increase in linear velocity (by adjusting the flow rate) may be justified, provided that the column performance does not drop by more than 20%. Similarly, when a change is made from <3-µm to ≥3-µm particles, an additional reduction of linear velocity (flow rate) may be justified to avoid reduction in column performance by more than 20%.

After an adjustment due to a change in column dimensions, an additional change in flow rate of ±50% is permitted.

*Column temperature*: ± 10°C, where the operating temperature is specified, unless otherwise prescribed

Further adjustments in analytical procedure conditions (mobile phase, temperature, pH, etc.) may be required, within the permitted ranges described under *System Suitability* and *Adjustment of Chromatographic Conditions* in this chapter.

### MOBILE PHASE

*Composition*: The amount of the minor components of the mobile phase can be adjusted by ±30% relative. However, the change in any component cannot exceed ±10% absolute. A minor component comprises less or equal than (100/n) %, *n* being the total number of components of the mobile phase:

♦Examples of adjustments for binary and ternary mixtures are given below.

### BINARY MIXTURES

*Specified ratio of 50:50:* 30% of 50 is 15% absolute, but this exceeds the maximum permitted change of ±10% absolute in either component. Therefore, the mobile phase ratio may be adjusted only within the range of 40:60–60:40.

*Specified ratio of 2:98:* 30% of 2 is 0.6% absolute. Therefore, the maximum allowed adjustment is within the range of 1.4: 98.6–2.6: 97.4.

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TERNARY MIXTURES

*Specified ratio of 70:25:5*: For the second component, 30% of 25 is 7.5% absolute. Therefore, the second component may be adjusted within the range of 32.5%–17.5% absolute. For the third component, 30% of 5 is 1.5% absolute. In all cases, a sufficient quantity of the first component is used to give a total of 100%. Therefore, mixture ranges of 62.5: 32.5: 5 to 77.5: 17.5: 5 or 68.5: 25: 6.5 to 71.5: 25: 3.5 would meet the requirement.♦

- *pH of the aqueous component of the mobile phase*: ±0.2 pH units, unless otherwise prescribed
- *Concentration of salts in the buffer component of a mobile phase*: ±10%
- *Flow rate*: In absence of a change in column dimensions, an adjustment of the flow rate by ±50% is permitted

DETECTOR WAVELENGTH

No adjustment permitted.

INJECTION VOLUME

When the column dimensions are changed, the following equation may be used for adjusting the injection volume:

$$V_{inj2} = V_{inj1}\left(L_2\ {d_{c2}}^2\right)/\left(L_1\ {d_{c1}}^2\right)$$

$V_{inj1}$ = injection volume indicated in the monograph (µL)
$V_{inj2}$ = adjusted injection volume (µL)
$L_1$ = column length indicated in the monograph (mm)
$L_2$ = new column length (mm)
$dc_1$ = column internal diameter indicated in the monograph (mm)
$dc_2$ = new column internal diameter (mm)

This equation may not be applicable to changes from TPP columns to SPP columns.

Even in the absence of any column dimension change, the injection volume may be varied provided System Suitability criteria remain within their established acceptability limits. When the injection volume is decreased, special attention is given to (limit of) detection and repeatability of the peak response(s) to be determined. An increase is permitted provided, in particular, linearity and resolution of the peak(s) to be determined remain satisfactory.

## Liquid Chromatography: Gradient Elution

Adjustment of chromatographic conditions for gradient systems requires greater caution than for isocratic systems.

COLUMN PARAMETERS AND FLOW RATE

*Stationary phase*: No change of the identity of the substituent (e.g., no replacement of C18 by C8); the other physicochemical characteristics of the stationary phase i.e., chromatographic support, surface modification, and extent of chemical modification must be similar; a change from totally porous particle (TPP) columns to superficially porous particle (SPP) columns is allowed provided the above-mentioned requirements are met.

*Column dimensions (particle size, length)*: The particle size and/or length of the column may be modified provided that the ratio of the column length (*L*) to the particle size (*dp*) remains constant or in the range between –25% to +50% of the prescribed *L/dp* ratio.

*Adjustments from totally porous to superficially porous particles*: For the application of particle-size adjustment from totally porous to superficially porous particles, other combinations of *L* and *dp* can be used provided that the ratio $(t_R/W_h)^2$ is within –25% to +50%, relative to the prescribed column ♦for all the peaks used to determine the system suitability parameters♦. These changes are acceptable provided system suitability criteria are fulfilled, and selectivity and elution order of the specified impurities to be controlled are demonstrated to be equivalent.

*Internal diameter*: In absence of a change in particle size and/or length, the internal diameter of the column may be adjusted.

Caution is necessary when the adjustment results in smaller peak volume resulting from smaller particle size or smaller internal column diameter, a situation which may require adjustments to minimize extra-column band broadening by factors such as instrument connections, detector cell volume and sampling rate, and injection volume.

When the particle size is changed, the flow rate requires adjustment, because smaller-particle columns will require higher linear velocities for the same performance (as measured by reduced plate height). Flow rate is adjusted for both the change in column diameter and particle size using the following equation:

$$F_2 = F_1 \times \left[\left({dc_2}^2 \times dp_1\right)/\left({dc_1}^2 \times dp_2\right)\right]$$

$F_1$ = flow rate indicated in the monograph (mL/min)
$F_2$ = adjusted flow rate (mL/min)
$dc_1$ = internal diameter of the column indicated in the monograph (mm)
$dc_2$ = internal diameter of the column used (mm)
$dp_1$ = particle size indicated in the monograph (µm)
$dp_2$ = particle size of the column used (µm)

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A change in column dimensions, and thus in column volume, impacts the gradient volume which controls selectivity. Gradients are adjusted to the column volume by changing the gradient volume in proportion to the column volume. This applies to every gradient segment volume. Since the gradient volume is the gradient time, $t_G$, multiplied by the flow rate, $F$, the gradient time for each gradient segment needs to be adjusted to maintain a constant ratio of the gradient volume to the column volume (expressed as $L \times dc^2$). Thus, the new gradient time, $t_{G2}$ can be calculated from the original gradient time, $t_{G1}$, the flow rate(s), and the column dimensions as follows:

$$t_{G2} = t_{G1} \times (F_1/F_2) \left[\left(L_2 \times {dc_2}^2\right)/\left(L_1 \times {dc_1}^2\right)\right]$$

Thus, the change in conditions for gradient elution requires three steps:

1. Adjust the column length and particle size according to *L/dp*.
2. Adjust the flow rate for changes in particle size and column diameter.
3. Adjust the gradient time of each segment for changes in column length, diameter, and flow rate. The example below illustrates this process.

**Table 3**

| Variable | Original Conditions | Adjusted Conditions | Comment |
|---|---|---|---|
| Column length (*L*), in mm | 150 | 100 | User's choice |
| Column diameter (*dc*), in mm | 4.6 | 2.1 | User's choice |
| Particle size (*dp*), in µm | 5 | 3 | User's choice |
| *L/dp* | 30.0 | 33.3 | (1) |
| Flow rate, in mL/min | 2.0 | 0.7 | (2) |
| Gradient adjustment factor ($t_{G2}/t_{G1}$) | — | 0.4 | (3) |
| Gradient conditions | — | — | — |
| B (%) | Time (min) | Time (min) | |
| 30 | 0 | 0 | — |
| 30 | 3 | (3 × 0.4) = 1.2 | — |
| 70 | 13 | [1.2 + (10 × 0.4)] = 5.2 | — |
| 30 | 16 | [5.2 + (3 × 0.4)] = 6.4 | — |

1. An 11% increase within allowed *L/dp* change of –25% to +50%
2. Calculated using $F_2 = F_1\ [(dc_2^2 \times dp_1)/(dc_1^2 \times dp_2)]$
3. Calculated using $t_{G2} = t_{G1} \times (F_1/F_2)\ [(L_2 \times dc_2^2)/(L_1 \times dc_1^2)]$

*Column temperature*: ±5° C, where the operating temperature is specified, unless otherwise prescribed

Further adjustments in procedure conditions (mobile phase, temperature, pH, etc.) may be required, within the permitted ranges described under *System Suitability* and *Adjustment of Chromatographic Conditions* in this chapter.

## MOBILE PHASE

*Composition/gradient*: Adjustments of the composition of the mobile phase and the gradient are acceptable provided that:

- The system suitability criteria are fulfilled.
- The principal peak(s) elute(s) within ±15% of the retention time(s) obtained with the original conditions; this requirement does not apply when the column dimensions are changed.
- The composition of the mobile phase and the gradient are such that the first peaks are sufficiently retained and the last peaks are eluted.

*pH of the aqueous component of the mobile phase*: ±0.2 pH units, unless otherwise prescribed

*Concentration of salts in the buffer component of a mobile phase*: ±10%

Where compliance with the system suitability criteria cannot be achieved, it is often preferable to consider the dwell volume or to change the column.

## DWELL VOLUME

The configuration of the equipment employed may significantly alter the resolution, retention time, and relative retentions described. Should this occur, it may be due to a change in dwell volume. Monographs preferably include an isocratic step before the start of the gradient program so that an adaptation can be made to the gradient time points to take account of differences in dwell volume between the system used for analytical procedure development and that actually used. It is the user's responsibility to adapt the length of the isocratic step to the analytical equipment used. If the dwell volume used during the elaboration of the monograph is given in the monograph, the time points ($t$ min) stated in the gradient table may be replaced by adapted time points ($t_c$ min), calculated using the following equation:

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$$t_c = t - \frac{(D - D_0)}{F}$$

$D$ = dwell volume (mL)
$D_0$ = dwell volume used for development of the method (mL)
$F$ = flow rate (mL/min)

The isocratic step introduced for this purpose may be omitted if validation data for application of the analytical procedure without this step is available.

Detector wavelength: No adjustment permitted

Injection volume: When the column dimensions are changed, the following equation may be used for adjusting the injection volume:

$$V_{inj2} = V_{inj1}\left(L_2 dc_2^2\right)/\left(L_1 dc_1^2\right)$$

$V_{inj1}$ = injection volume indicated in the monograph (µL)
$V_{inj2}$ = adjusted injection volume (µL)
$L_1$ = column length indicated in the monograph (cm)
$L_2$ = new column length (cm)
$d_{c1}$ = column internal diameter indicated in the monograph (mm)
$d_{c2}$ = new column internal diameter (mm)

This equation may not be applicable to changes from TPP columns to SPP columns.

Even in the absence of any column dimension change, the injection volume may be varied provided system suitability criteria remain within their established acceptability limits. When the injection volume is decreased, special attention is given to (limit of) detection and repeatability of the peak response(s) to be determined. An increase is permitted provided, in particular, linearity and resolution of the peak(s) to be determined remain satisfactory.

## Gas Chromatography

COLUMN PARAMETERS

**Stationary phase**
*Particle size*: Maximum reduction of 50%; no increase permitted (packed columns)
*Film thickness*: –50% to +100% (capillary columns)
**Column dimensions**
*Length*: –70% to +100%
*Internal diameter*: ±50%
**Column temperature:** ±10%
**Temperature program:** Adjustment of temperatures is permitted as stated above; adjustment of ramp rates and hold times of up to ±20% is permitted.
**Flow rate:** ±50%

The above changes are acceptable provided system suitability criteria are fulfilled and selectivity and elution order of the specified impurities to be controlled are demonstrated to be equivalent.
**Injection volume and split ratio:** May be varied provided system suitability criteria remain within their established acceptability limits. When the injection volume is decreased, or the split ratio is increased special attention is given to (limit of) detection and repeatability of the peak response(s) to be determined. An increase in injection volume or decrease in split ratio is permitted provided, in particular, linearity and resolution of the peak(s) to be determined remain satisfactory.
**Injection port temperature and transfer-line temperature in static headspace conditions:** ±10° C, provided no decomposition or condensation occurs

# QUANTITATION

The following quantitation approaches may be used in general texts or monographs.

## External Standard Method

Using a calibration function: Standard solutions with several graded amounts of a reference standard of the compound to be analyzed are prepared in a range that has been demonstrated to give a linear response, and a fixed volume of these standard solutions is injected. With the chromatograms obtained, a calibration function is prepared by plotting the peak areas or peak heights on the ordinate against the amount of reference standard on the abscissa. The calibration function is generally obtained by linear regression. Then, a sample solution is prepared according to the procedure specified in the individual monograph. The chromatography is performed under the same operating conditions as for the preparation of the calibration function, the peak area or peak height of the compound to be analyzed is measured, and the amount of the compound is read out or calculated from the calibration function.

Using one-point calibration: In an individual monograph, generally one of the standard solutions with a concentration within the linear range of the calibration function and a sample solution with a concentration close to that of the standard solution

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are prepared, and the chromatography is performed under fixed conditions to obtain the amount of the component by comparing the responses obtained. In this method, all procedures, such as the injection procedure, must be carried out under constant conditions.

### Internal Standard Method

Using a calibration function: In the internal standard method, a stable compound is chosen as an internal standard which shows a retention time close to that of the compound to be analyzed, and whose peak is well separated from all other peaks in the chromatogram. Several standard solutions containing a fixed amount of the internal standard and graded amounts of a reference standard of the compound to be analyzed are prepared. Based on the chromatograms obtained by injection of a fixed volume of individual standard solutions, the ratio of peak area or peak height of the reference standard to that of the internal standard is calculated. A calibration function by plotting these ratios on the ordinate against the amount of the reference standard or the ratio of the amount of reference standard to that of the internal standard on the abscissa is prepared. The calibration function is generally obtained by linear regression. Then, a sample solution containing the internal standard in the same amount as in the standard solutions used for the preparation of the calibration function is prepared according to the procedure specified in the individual monograph. The chromatography is performed under the same operating conditions as for the preparation of the calibration function. The ratio of the peak area or peak height of the compound to be analyzed to that of the internal standard is calculated, and the amount of the compound is read out or calculated from the calibration function.

Using one-point calibration: In an individual monograph, generally one of the standard solutions with a concentration within the linear range of the calibration function and a sample solution with a concentration close to that of the standard solution, both containing a fixed amount of the internal standard, are prepared, and the chromatography is performed under fixed conditions to determine the amount of the compound to be analyzed by comparing the ratios obtained.

*Normalization procedure*: Provided linearity of the peaks has been demonstrated, individual monographs may prescribe that the percentage content of a component of the substance to be examined is calculated by determining the area of the corresponding peak as a percentage of the total area of all the peaks, excluding those due to solvents or reagents or arising from the mobile phase or the sample matrix, and those at or below the disregard limit or reporting threshold.

## OTHER CONSIDERATIONS

Detector response: ♦The detector sensitivity is the signal output per unit concentration or unit mass of a substance (also known as response factor) in the mobile phase entering the detector. The relative detector response factor, commonly referred to as relative response factor, expresses the sensitivity of a detector for a given substance relative to a standard substance.

In tests for related substances, any correction factors indicated in the monograph are applied.♦

Interfering peaks: Peaks due to solvents and reagents or arising from the mobile phase or the sample matrix are disregarded.

Measurement of peaks: Integration of the peak area of any impurity that is not completely separated from the principal peak is preferably performed by tangential skim (*Figure 9*).

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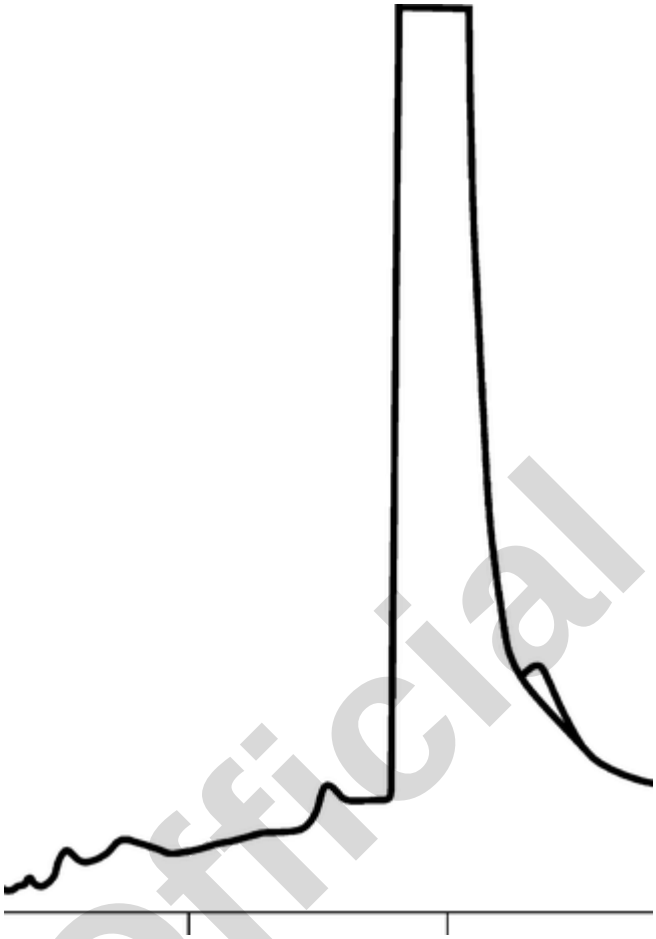

Figure 9.

**reporting threshold**

When the related substances test prescribes a limit for the total of impurities or a quantitative determination of an impurity, it is important to choose an appropriate reporting threshold and appropriate conditions for the integration of the peak areas.

In such tests the reporting threshold, i.e., the limit above which a peak is reported, is defined generally 0.05%.

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***Add the following:***

# ▲⟨630⟩ VISUAL COMPARISON

The purpose of this test is to provide the details for the visual comparison of the color and/or turbidance of sample solutions of certain concentration to a standard solution or a series of standard solutions of known concentration. Where a color or turbidity comparison is directed, follow the procedures and conditions outlined below for performing these tests.

**Comparison vessels:** Color-comparison tubes matched as closely as possible in internal diameter, in depth of sample solution, and in all other respects should be used.

**Viewing conditions for turbidity comparison:** Tubes should be viewed horizontally against a dark background with the aid of a light source directed from the sides of the tubes.

**Viewing conditions for color comparison:** Tubes should be viewed downward against a white background. Most of the time, common room lighting is sufficient to perform the assessment. A light source directed from beneath the bottoms of the tubes may be used if needed and if the practice is consistent between the materials under comparison.▲ (USP 1-May-2019)

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# ⟨631⟩ COLOR AND ACHROMICITY

## INTRODUCTION

The purpose of this chapter is to provide well-controlled methods for the assessment of color and achromicity of uniform liquid samples. The methods described in this chapter may be applied to the liquid phase of dispersed systems in cases where the dispersed phase can be removed prior to color measurements. Color is an organoleptic characteristic, which means that the value is based on the human response to stimuli. To reliably evaluate color, it is important to control both the illumination and observation parameters. By using a characteristic response for a standard observer, it is possible to perform precise instrumental evaluations of color. This chapter provides guidance for both an organoleptic assessment (*Method I*) and an instrumental assessment (*Method II*) of color and achromicity in liquid samples.

Color may be defined as an observer's perception of or subjective response to a stimulus of radiant energy in the visible spectrum extending over the wavelength range of 400–700 nm. Perceived color is a function of three variables: spectral properties of the object, spectral power distribution of the source of illumination, and visual perception of the observer.

Two objects are said to have a color match for a particular source of illumination when an observer cannot detect a color difference. Where a pair of objects exhibit a color match for one source of illumination and not another, they constitute a metameric pair. Color matches of two objects occur for all sources of illumination if the absorption and reflectance spectra of the two objects are identical.

Achromicity or colorlessness is one extreme of any color scale for transmission of light. It implies the complete absence of color; therefore, the visible spectrum of the sample lacks absorbances. For practical purposes, the observer in this case perceives little if any absorption taking place in the visible spectrum and is unable to discern the difference between an almost colorless sample and a colorless reference.

## COLOR ATTRIBUTES AND COLOR SPACE COORDINATES

Because the sensation of color has both a subjective and an objective part, color cannot be described solely in spectrophotometric terms. The common attributes of color therefore cannot be given a one-to-one correspondence with spectral terminology.

Three attributes are commonly used to identify a color: 1) hue (angle), or the color dimension dominant in its purest form, such as red, yellow, blue, green, and intermediate terms; 2) lightness, or the quality that distinguishes a light color from a dark one; and 3) chroma, or the quality that distinguishes an intense color from a weak one, such as the extent to which a color differs from a gray of the same lightness.

The three attributes of color may be used to define a three-dimensional color space in which any color is located by its coordinates. The color space chosen is visually uniform if the geometric distance between two colors in the color space is correlated with the perceived difference between the two colors. Cylindrical coordinates are often chosen for convenience. Points along the vertical axis represent the lightness from dark to light (or black to white) and have indeterminate hue (angle) and no chroma. Focusing on a cross-section perpendicular to the lightness axis, hue is determined by the angle about the long axis and chroma is determined by the geometric distance from the vertical axis. Red, yellow, green, blue, and intermediate hues are differentiated by various hue angles. Colors of the same hue angle become more intense (i.e., chromatic) as they move further from the vertical axis (larger chroma). For example, colorless or achromic water has an indeterminate hue angle, high lightness, and little to no chroma. If a colored solute is added, the water takes on a particular hue (angle). As more is added, the color becomes darker, more intense, or deeper; that is, the chroma increases and lightness decreases. However, if the solute is a neutral color, such as gray, the lightness decreases, no increase in chroma occurs, and the hue (angle) remains indeterminate.

Laboratory spectroscopic measurements can be converted to measurements of the color attributes by using the response factors for a standardized observer. Spectroscopic results are weighted by three distribution functions to yield the tristimulus values X, Y, and Z (for additional information, see *Color—Instrumental Measurement* ⟨1061⟩).

The tristimulus values are not coordinates in a visually uniform color space; however, they can be transformed into a more uniform chroma–hue color space known as the CIELAB color space, defined by the International Commission on Illumination (CIE) in 1976. Also known as CIE L*a*b* or sometimes abbreviated as simply "Lab" color space, CIELAB was designed so that the same amount of numerical change in these values corresponds to roughly the same amount of visually perceived change. The resulting $L^*$, $a^*$, $b^*$, $C^*_{ab}$, and $h^*_{ab}$ values indicate the following:

- $L^*$ is the lightness coordinate. $L$ is always positive and ranges from 0 to 100; 100 is the colorless (white) standard and 0 is the zero (black) standard.
- $a^*$ is the red/green coordinate. $+a^*$ is red, and $-a^*$ is green.
- $b^*$ is the yellow/blue coordinate. $+b^*$ is yellow, and $-b^*$ is blue.
- $C^*_{ab}$ is the chroma in the ($a^*$, $b^*$) plane. $C^*_{ab} = [(a^*)^2 + (b^*)^2\ ]^{1/2}$.
- $h^*_{ab}$ is the hue angle in the ($a^*$, $b^*$) plane, measured from the $a^*$ axis increasing counterclockwise, and is reported as a value from 0 to 360 degrees. The hue angles for the elementary colors are approximately 25 (red), 92 (yellow), 162 (green), 220 (cyan), 271 (blue), and 337 (magenta) degrees.

Additionally, the difference between two points in the CIELAB color space ($\Delta E^*$) can be calculated as follows:

$$\Delta E^* = \sqrt{\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2}}$$

Slight differences in colors are not perceptible in visual comparisons. Although it is possible for the human eye to differentiate between colors with a $\Delta E^*$ of about 1, it is frequently difficult for an observer with normal color vision to reliably differentiate

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between colors with a $\Delta E^*$ of less than 3. Therefore, it is preferable to rely on quantitative assessment of color and color differences when precise, reliable comparisons are required.

## COLOR DETERMINATION AND STANDARDS

The perception of color and color matches is dependent on conditions of viewing and illumination. Determinations should be made using diffuse, uniform illumination under conditions that reduce shadows and minimize non-spectral reflectance. Liquids should be compared in matched color-comparison containers that are transparent to the illumination light. Either organoleptic (qualitative) or instrumental (quantitative) assessment of color may be used.

### Containers for Liquid Samples and Matching Fluids

For liquid samples, the containers used to hold the samples during the color measurements should be non-absorbing in the 400–700 nm range and must be identical for the sample and matching fluids. The containers may be round or square, and the diameter or depth of the container may be adjusted to provide a shorter path length for darker samples or a longer path length for lighter samples. The use of cuvettes or glass tubes with a path length of about 10 mm is recommended.

### Turbid and Opaque Samples

When using transparent color-matching fluids, the color evaluation of the sample should be based on evaluation of the clarified sample. If the sample is turbid or opaque, the dispersed phase should be removed by centrifugation or filtration before evaluating the color. If the sample or matching fluid contains air bubbles or foam, these should be removed by centrifugation before evaluation of the color.

### Matching Fluids

The colors of standards should be as close as possible to those of test specimens for quantifying color differences. Any well-defined and well-characterized matching fluid is permitted (*1*). For example, matching fluids may be prepared according to *Table 1* or may be prepared as described in the *European Pharmacopoeia* (*2*) (*2.2.2. Degree of Coloration of Liquids*) or *Chinese Pharmacopoeia* (*3*) (*Appendix IX, Colour of Solution*). To prepare the matching fluids in *Table 1*, pipet the prescribed volumes of the colorimetric test solutions (see *Reagents, Indicators, and Solutions—Colorimetric Solutions*) and water into one of the matching containers and mix the solution in the container.

**Table 1. Matching Fluids**

| Matching Fluid | Parts[1] of Cobaltous Chloride CS | Parts of Ferric Chloride CS | Parts of Cupric Sulfate CS | Parts of Water |
|---|---|---|---|---|
| A | 0.1 | 0.4 | 0.1 | 4.4 |
| B | 0.3 | 0.9 | 0.3 | 3.5 |
| C | 0.1 | 0.6 | 0.1 | 4.2 |
| D | 0.3 | 0.6 | 0.4 | 3.7 |
| E | 0.4 | 1.2 | 0.3 | 3.1 |
| F | 0.3 | 1.2 | 0.0 | 3.5 |
| G | 0.5 | 1.2 | 0.2 | 3.1 |
| H | 0.2 | 1.5 | 0.0 | 3.3 |
| I | 0.4 | 2.2 | 0.1 | 2.3 |
| J | 0.4 | 3.5 | 0.1 | 1.0 |
| K | 0.5 | 4.5 | 0.0 | 0.0 |
| L | 0.8 | 3.8 | 0.1 | 0.3 |
| M | 0.1 | 2.0 | 0.1 | 2.8 |
| N | 0.0 | 4.9 | 0.1 | 0.0 |
| O | 0.1 | 4.8 | 0.1 | 0.0 |
| P | 0.2 | 0.4 | 0.1 | 4.3 |
| Q | 0.2 | 0.3 | 0.1 | 4.4 |
| R | 0.3 | 0.4 | 0.2 | 4.1 |
| S | 0.2 | 0.1 | 0.0 | 4.7 |
| T | 0.5 | 0.5 | 0.4 | 3.6 |

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[1] The composition in Table 1 is described on a v/v basis and indicates the relative volume of each ingredient required to make the reference solution.

If a matching fluid is prepared, it must be freshly prepared on the day of use unless suitable data demonstrating the stability of the color in the matching fluid are available to support the use for longer time periods. For purchased color standards or matching fluids, follow the appropriate use instructions and expiry dates.

## METHOD I: ORGANOLEPTIC (QUALITATIVE) ASSESSMENT OF COLOR AND COLOR MATCHES

### Illuminant

A standard illuminant is a theoretical source of visible light with a profile (its spectral power distribution) that is published. Standard illuminants are designated by a letter or by a letter-number combination. Illuminants A, B, and C were introduced in 1931, with the intention of representing average incandescent light, direct sunlight, and average daylight, respectively. The illumination of the sample(s) and matching fluid(s) should be solely due to the controlled illuminant source. Any additional illumination from room lighting or windows should be minimized (for example, by use of a light box or light booth). A daylight illuminant capable of providing a correlated color temperature (CCT) of 6000–7000 K should be used. Filtered tungsten halogen lamps are recommended, but daylight fluorescent lamps with comparable spectral and illuminance characteristics also can be used. The spectral power distribution should conform to ISO 23603/CIE S 012 (*4*) for the D65 illuminant with a grade of B/C or better. The illumination of the sample and matching fluid should be diffuse and not at an angle that will provide specular reflections to the observer. Recommended geometries include 0:45 and 45:0 illuminant viewer, in degrees.

### Observer

The observer angle may be normal (vertical) or at 90 degrees (horizontal) if the illumination angle is controlled to avoid specular reflection and provides diffuse illumination relative to the observer angle. The sample and one or more matching fluids should subtend a viewing angle of 2–10 degrees and be in the field of view simultaneously. Using identical tubes of colorless, transparent, and neutral glass of 12-mm external diameter, compare 2.0 mL of the liquid to be examined with 2.0 mL of water or matching fluid. A small, discernible gap should exist between the sample and matching fluid. The surrounding field should be white, black, or neutral gray.

### Procedures

Evaluate the sample and at least one other matching fluid simultaneously. Evaluate the brightness, saturation, and hue (angle). Hue (angle) differences between the sample and the reference should be interpreted as a failure of the comparative test unless there are explicit instructions in the monograph to ignore hue (angle) differences.

When analyzing samples that are expected to have color, there are three approaches:

1. Establish a minimum level of color. If the sample matches the reference sample or has more color, it passes the test.
2. Establish a maximum level of color. If the sample matches the reference sample or has less color, it passes.
3. Select a matching (or reference) solution to determine an indiscernible difference between the test sample and the reference. In this case, the sample must match the matching solution. It is preferred, in this case, to establish a standard of the same compound as the analyte of interest to avoid situations of metamerism.

*Table 2* illustrates how the results of these color evaluations should be interpreted and reported.

**Table 2. Interpretation of Color Comparisons for Qualitative Color Evaluations**

| Color Evaluation Test | Test Requirement | Sample Has More Color | Reference Has More Color | Sample Matches Reference |
|---|---|---|---|---|
| Almost Colorless | Compare sample to Purified Water. | Fails the test | Passes the test | Passes the test |
| Maximum Level of Color | Compare to a matching fluid that should exhibit discernibly more color than sample. | Fails the test | Passes the test | If used for achromicity, fails the test. Otherwise, passes the test |
| Minimum Level of Color | Identify a matching fluid that represents minimum acceptable color. | Passes the test | Fails the test | Passes the test |
| Indiscernible from Color Standard | Prepare color standard; compare test sample to color standard. | Fails the test | Fails the test | Passes the test |

Achromicity is a special case of an evaluation of the maximum level of color. For achromicity evaluations, one must either perform an *Indiscernible from Color Standard* test using Purified Water as the standard or perform a *Maximum Level of Color* test with a color standard with a low level of color that is discernibly different from Purified Water (i.e., a $\Delta E^*$ relative to Purified Water of 3–6). Matching fluids that are almost colorless are generally not useful for qualitative color evaluations and should be avoided. *Table 3* lists the matching fluids that should be avoided and those that are recommended for achromicity evaluations using the *Maximum Level of Color* test.

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**Table 3. Matching Fluids for Use in Achromicity Evaluations (*5*)**

| Source of Matching Fluid | Matching Fluids Almost Colorless (not recommended for use) | Matching Fluids Darker and Differentiated from Colorless (recommended for maximum level of color in an achromicity test) |
|---|---|---|
| This chapter | None | A, P, Q, S |
| *European Pharmacopoeia*,*2.2.2. Degree of Coloration of Liquids* | B7, B8, B9, BY7, Y7, GY7, R7 | B6, BY5, Y5, GY5, R6 |
| *Chinese Pharmacopoeia*, Color Standard | YG1, Y1, OY1, OR1, BR1 | YG2, YG3, Y2, Y3, OY3, OY4, OR3, OR4, BR3, BR4, BR5 |

***Change to read:***

# METHOD II: INSTRUMENTAL (QUANTITATIVE) ASSESSMENT OF COLOR AND COLOR MATCHES

## Instrumentation

For evaluating the color of solutions, it is acceptable to use any spectrometer or colorimeter (in transmission mode) that has been qualified for use, according to *Ultraviolet–Visible Spectroscopy* ⟨857⟩, in the operating range of 400–700 nm and has a bandwidth of 10 nm or less.

## Calibration

For verification of the calibration in transmission mode, a black tile (or block of the light path) is used to determine the zero end of the lightness scale; Purified Water (or relevant solvent) is used for 100% transmission (for more information, see *Analytical Instrument Qualification* ⟨1058⟩).

## Recording of Spectroscopic Data

The spectroscopic data should be recorded from 400–700 nm at 10-nm intervals. If results are acquired at more frequent intervals, only the results from the 10-nm intervals will be used to calculate the instrumental color values. If the samples are turbid, they should be clarified by centrifugation or filtration prior to loading into the cuvette. Samples also should be checked to ensure that the light transmission path is free from air bubbles or foam.

## Calculation of CIELAB Values

This instrumental method relies on calculation of the color values in the CIELAB color space using either illuminant C with the 2-degree observer or illuminant D65 with the 10-degree observer. *Table 4* and *Table 5* contain the weighting factors for illuminant C with the 2-degree observer (C/2) and for illuminant D65 with the 10-degree observer (D65/10), respectively. These weighting factors should be used to convert the spectroscopic data into CIELAB values (for additional information, see ⟨1061⟩). As an example, *Table 6* contains three sets of spectroscopic transmittance data for the ideal Lovibond 10R, 10Y, and 10B glasses (*6*). *Table 7* shows the CIELAB (C/2) and CIELAB (D65/10) values calculated from these data using the weighting factors in *Table 4* and *Table 5*.

**Table 4. Weighting Factors for Illuminant C with 1931 (2-degree) Observer (400–700 nm, 10-nm spacing)[a, b]**

| $\lambda$ (nm) | $Wx(\lambda) = x\lambda.P\lambda.$ | $Wy(\lambda) = y\lambda.P\lambda.$ | $Wz(\lambda) = z\lambda.P\lambda.$ |
|---|---|---|---|
| 400 | 0.099 | 0.003 | 0.463 |
| 410 | 0.325 | 0.009 | 1.547 |
| 420 | 1.292 | 0.038 | 6.207 |
| 430 | 2.968 | 0.123 | 14.496 |
| 440 | 3.959 | 0.261 | 19.860 |
| 450 | 3.931 | 0.443 | 20.728 |
| 460 | 3.360 | 0.692 | 19.286 |
| 470 | 2.283 | 1.061 | 15.022 |
| 480 | 1.116 | 1.612 | 9.479 |
| 490 | 0.363 | 2.358 | 5.286 |
| 500 | 0.048 | 3.414 | 2.868 |
| 510 | 0.092 | 4.842 | 1.512 |

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**Table 4. Weighting Factors for Illuminant C with 1931 (2-degree) Observer (400–700 nm, 10-nm spacing)[a, b]** *(continued)*

| *λ (nm)* | *Wx(λ)* = xλPλ | *Wy(λ)* = yλPλ | *Wz(λ)* = zλPλ |
|---|---|---|---|
| 520 | 0.578 | 6.449 | 0.720 |
| 530 | 1.519 | 7.936 | 0.381 |
| 540 | 2.786 | 9.145 | 0.195 |
| 550 | 4.285 | 9.831 | 0.086 |
| 560 | 5.877 | 9.834 | 0.038 |
| 570 | 7.323 | 9.148 | 0.020 |
| 580 | 8.414 | 7.990 | 0.015 |
| 590 | 8.985 | 6.629 | 0.010 |
| 600 | 8.958 | 5.321 | 0.007 |
| 610 | 8.324 | 4.177 | 0.003 |
| 620 | 7.055 | 3.146 | 0.001 |
| 630 | 5.327 | 2.196 | 0.000 |
| 640 | 3.692 | 1.442 | 0.000 |
| 650 | 2.352 | 0.887 | 0.000 |
| 660 | 1.360 | 0.503 | 0.000 |
| 670 | 0.713 | 0.261 | 0.000 |
| 680 | 0.364 | 0.132 | 0.000 |
| 690 | 0.172 | 0.062 | 0.000 |
| 700 | 0.154 | 0.055 | 0.000 |
| Sum | 98.074 | 100.000 | 118.230 |
| White point[c] | 98.074 | 100.000 | 118.232 |

[a] Adapted from ASTM E308 (*Standard Practice for Computing the Colors of Objects by Using the CIE System*).
[b] The weighting factors (*Wx*, *Wy*, *Wz*) are obtained by multiplying the illuminant power at the particular wavelength (*P*) by the response factor at that wavelength (*x*,*y*,*z*).
[c] The white point value is the exact value for the colorless/white standard. A slight difference may exist between the sum and white point values due to rounding in the last decimal place.

**Table 5. Weighting Factors for Illuminant D65 with 1964 (10-degree) Observer (400–700 nm, 10-nm spacing)[a, b]**

| *λ (nm)* | *Wx(λ)* = xλPλ | *Wy(λ)* = yλPλ | *Wz(λ)* = zλPλ |
|---|---|---|---|
| 400 | 0.145 | 0.015 | 0.643 |
| 410 | 0.676 | 0.069 | 3.110 |
| 420 | 1.603 | 0.168 | 7.627 |
| 430 | 2.451 | 0.300 | 12.095 |
| 440 | 3.418 | 0.554 | 17.537 |
| 450 | 3.699 | 0.890 | 19.888 |
| 460 | 3.064 | 1.290 | 17.695 |
| 470 | 1.933 | 1.838 | 13.000 |
| 480 | 0.802 | 2.520 | 7.699 |
| 490 | 0.156 | 3.226 | 3.938 |
| 500 | 0.039 | 4.320 | 2.046 |
| 510 | 0.347 | 5.621 | 1.049 |
| 520 | 1.070 | 6.907 | 0.544 |
| 530 | 2.170 | 8.059 | 0.278 |
| 540 | 3.397 | 8.668 | 0.122 |
| 550 | 4.732 | 8.855 | 0.035 |
| 560 | 6.070 | 8.581 | 0.001 |
| 570 | 7.311 | 7.951 | 0.000 |

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**Table 5. Weighting Factors for Illuminant D65 with 1964 (10-degree) Observer (400–700 nm, 10-nm spacing)[a, b]** *(continued)*

| *λ (nm)* | *Wx(λ)* = xλPλ | *Wy(λ)* = yλPλ | *Wz(λ)* = zλPλ |
|---|---|---|---|
| 580 | 8.291 | 7.106 | 0.000 |
| 590 | 8.634 | 6.004 | 0.000 |
| 600 | 8.672 | 5.079 | 0.000 |
| 610 | 7.930 | 4.065 | 0.000 |
| 620 | 6.446 | 2.999 | 0.000 |
| 630 | 4.669 | 2.042 | 0.000 |
| 640 | 3.095 | 1.290 | 0.000 |
| 650 | 1.859 | 0.746 | 0.000 |
| 660 | 1.056 | 0.417 | 0.000 |
| 670 | 0.570 | 0.223 | 0.000 |
| 680 | 0.274 | 0.107 | 0.000 |
| 690 | 0.121 | 0.047 | 0.000 |
| 700 | 0.109 | 0.043 | 0.000 |
| Sum | ▲94.809▲ (ERR 1-Dec-2023) | 100.000 | 107.307 |
| White point | ▲94.811▲ (ERR 1-Dec-2023) | 100.000 | 107.304 |

[a] Adapted from ASTM E308 (*Standard Practice for Computing the Colors of Objects by Using the CIE System*).
[b] The weighting factors (*Wx*, *Wy*, *Wz*) are obtained by multiplying the illuminant power at the particular wavelength (*P*) by the response factor at that wavelength (*x*,*y*,*z*).

**Table 6. Ideal Lovibond Transmission Spectra for 10R, 10Y, and 10B plates (*6*)**

| *λ (nm)* | 10R | 10Y | 10B |
|---|---|---|---|
| 400 | 0.3368 | 0.0000 | 0.9007 |
| 410 | 0.3429 | 0.0001 | 0.8938 |
| 420 | 0.3480 | 0.0013 | 0.8815 |
| 430 | 0.3514 | 0.0073 | 0.8630 |
| 440 | 0.3538 | 0.0271 | 0.8414 |
| 450 | 0.3527 | 0.0750 | 0.8154 |
| 460 | 0.3475 | 0.1704 | 0.7780 |
| 470 | 0.3370 | 0.3024 | 0.7112 |
| 480 | 0.3144 | 0.4441 | 0.6119 |
| 490 | 0.2753 | 0.5671 | 0.5013 |
| 500 | 0.2164 | 0.6615 | 0.4136 |
| 510 | 0.1472 | 0.7319 | 0.3172 |
| 520 | 0.0939 | 0.7801 | 0.2373 |
| 530 | 0.0709 | 0.8116 | 0.1722 |
| 540 | 0.0866 | 0.8354 | 0.1525 |
| 550 | 0.1483 | 0.8486 | 0.1822 |
| 560 | 0.2509 | 0.8521 | 0.2231 |
| 570 | 0.3703 | 0.8530 | 0.1967 |
| 580 | 0.4763 | 0.8472 | 0.1235 |
| 590 | 0.5666 | 0.8382 | 0.0674 |
| 600 | 0.6351 | 0.8264 | 0.0631 |
| 610 | 0.6861 | 0.8127 | 0.0712 |
| 620 | 0.7251 | 0.7989 | 0.0750 |
| 630 | 0.7558 | 0.7860 | 0.0709 |
| 640 | 0.7788 | 0.7776 | 0.0601 |

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**Table 6. Ideal Lovibond Transmission Spectra for 10R, 10Y, and 10B plates (*6*) *(continued)***

| *λ (nm)* | 10R | 10Y | 10B |
|---|---|---|---|
| 650 | 0.7968 | 0.7740 | 0.0645 |
| 660 | 0.8115 | 0.7734 | 0.0786 |
| 670 | 0.8230 | 0.7774 | 0.1457 |
| 680 | 0.8330 | 0.7834 | 0.2734 |
| 690 | 0.8419 | 0.7869 | 0.4701 |
| 700 | 0.8485 | 0.7918 | 0.6607 |

**Table 7. CIELAB Values Calculated from the Data in *Table 6* and Using the Weighting Factors from *Table 4* and *Table 5***

| CIELAB Values | Calculation with Illuminant C and 1931 (2-degree) Observer | | | Calculation with Illuminant D65 and 1964 (10-degree) Observer | | |
|---|---|---|---|---|---|---|
| | 10R | 10Y | 10B | 10R | 10Y | 10B |
| $L^*$ | 64.32 | 91.20 | 51.83 | 63.67 | 90.02 | 54.75 |
| $a^*$ | 49.79 | –21.97 | 23.36 | 47.75 | –15.48 | 9.65 |
| $b^*$ | –0.27 | 71.64 | –65.31 | –1.77 | 73.38 | –61.29 |
| $C^*_{ab}$ | 49.79 | 74.93 | 69.37 | 47.79 | 75.00 | 62.05 |
| $h^*_{ab}$ | 359.69 | 107.05 | 269.68 | 357.88 | 101.91 | 278.95 |

## METHOD IIA: COMPARATIVE TEST OF COLORS USING CIELAB VALUES

The CIELAB values for a sample and a matching solution may be evaluated to determine the acceptability of the sample. *Table 8* lists the different metrics that may be used to compare the color values of a sample to the color values of a matching solution. In evaluating stability, the color values may be tracked over time to assess trends. The variability in the color values may be used to assess process control and variability due to raw materials or processing.

**Table 8. Interpretation of Color Comparisons[a, b]**

| Color Evaluation Test | Test | Requirement |
|---|---|---|
| Almost Colorless | Compare the sample to Purified Water. | Measure the sample and Purified Water. Calculate $\Delta E^*$ relative to Purified Water. Requirement is that $\Delta E^* < 1$. |
| Maximum Level of Color | Identify a matching fluid of similar hue angle ($\Delta h^*_{ab} < 15$) that is discernibly more colored than the sample. | Measure the matching fluid and calculate the $\Delta E0^*$ relative to Purified Water. Measure the sample and calculate the $\Delta E^*$ relative to Purified Water. Requirement is $\Delta E^* < \Delta E0^*$. |
| Minimum Level of Color | Identify a matching fluid of similar hue angle ($\Delta h^*_{ab} < 15$) that is discernibly less colored than the sample. | Measure the matching fluid and calculate the $\Delta E0^*$ relative to Purified Water. Measure the sample and calculate the $\Delta E^*$ relative to Purified Water. Requirement is $\Delta E^* > \Delta E0^*$. |
| Indiscernible from Color Standard | Prepare color standard; compare the test sample to the color standard. | Calculate $\Delta E^*$ relative to selected color standard. Requirement is that $\Delta E^* < 3$. |

[a] Comparisons rely on the numerical values of $L^*$, $a^*$, and $b^*$, and the comparison of these values to those of the matching solution or standard.
[b] *CIELAB values must be calculated with the same illuminant and observer for evaluating differences between the sample and matching fluid.*

## METHOD IIB: INSTRUMENTAL COLOR ASSESSMENT

The CIELAB values (calculated as described in *Method IIa: Comparative Testing of Colors Using CIELAB Values*) may be used directly as a quantitative measure of the color attributes of a sample. Acceptable specifications for the color attributes can be established in the CIELAB color space and applied to color values obtained for a sample.

For new monographs under development, which do not already have a visual color determination method, the following approach harnesses the advantages of instrumental colorimetry to its fullest and removes drawbacks associated with limiting the control space to what is encompassed by existing visual standards. Also, for new monographs under development, acceptable numerical limits within color space should be determined based on process capability, stability, and analytical variability. A specification would then take the form of one of the following:

- A solution with $L^*$ NLT XX, NMT XX; $a^*$ NMT XX, NLT XX; and $b^*$ NMT XX, NLT XX (XX being a numerical value).
- $\Delta E^*$ versus a single point in 3-dimensional space. Ideally this point would represent the average based on multiple batches or based on representative material, such as a well-characterized reference standard. Other points may be justified.

Limits may be set with individual color parameters (e.g., $L^*$, $a^*$, and $b^*$ or another suitably validated color space) or as a $\Delta E^*$ versus a single point in color space when considering batch release or stability studies.

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## Reporting of Results

The calculated CIELAB color values are dependent on the illuminant and observer used and, therefore, should be reported. The CIELAB values for the coordinates *L**, *a**, and *b** should be reported. The values for chroma ($C^*_{ab}$) and hue angle ($h^*_{ab}$) are derived from *a** and *b** and do not need to be reported. A recommended method for reporting the CIELAB results is to report the color values as shown in the following examples:

CIELAB ([illuminant]/[observer]) = (*L**, *a**, *b**)
CIELAB (C/2) = (50.70, −11.30, −33.30)
CIELAB (D65/10) = (52.69, −19.43, 29.61)

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# ⟨641⟩ COMPLETENESS OF SOLUTION

***Add the following:***

▲The purpose of these methods is to verify that one or more materials are completely dissolved in a solution. These methods may be used to verify the descriptive solubility of a material according to the solubility table in *General Notices, 5.30 Description and Solubility*.

To verify the descriptive solubility, use the maximum amount of solvent specified for the descriptive solubility in the solubility table (see *General Notices, 5.30 Description and Solubility*). For example, for "soluble" use 30 parts of solvent. Into this amount of solvent, add 1 part of solute weighed accurately to 0.5%. Unless otherwise specified in the monograph, completeness of solution should be evaluated at 25°.

Evaluation of the completeness of solution, as described in this chapter, does not provide a precise measure of compound solubility. The descriptive solubility terms above should also not be confused with the terms "high solubility" and "low solubility" as applied to drug substances in the *Biopharmaceutical Classification System*.[1,2] For more information on precise solubility measurements and biorelevant solubility determinations, see *Solubility Measurements* ⟨1236⟩.

Unless otherwise directed in the individual monograph, use *Method I*.▲ (USP 1-Aug-2020)

***Change to read:***

## ▲METHOD I▲ (USP 1-AUG-2020)

Place the quantity of the substance specified in the individual monograph in a meticulously cleansed, glass-stoppered, 10-mL ▲color-comparison tube.▲ (USP 1-Aug-2020) Using the solvent that is specified in the monograph or on the label of the product, fill the ▲color-comparison tube to the 10-mL mark.▲ (USP 1-Aug-2020) Shake gently to dissolve: the solution is not less clear than an equal volume of the same solvent contained in a ▲matched color-comparison tube▲ (USP 1-Aug-2020) and examined similarly ▲(see *Visual Comparison* ⟨630⟩*, Viewing conditions for turbidity comparison*).▲ (USP 1-Aug-2020)

***Add the following:***

## ▲METHOD II

Place the quantity of the substance specified in the individual monograph into a meticulously cleansed 10-mL flask. Add 10 mL of the solvent that is specified in the monograph or on the label of the product. Shake gently to dissolve. Transfer the solution to a suitable measurement tube and measure the turbidity of the prepared solution per *Nephelometry and Turbidimetry* ⟨855⟩. The acceptance criterion is that the turbidity of the sample preparation is NMT that of *Reference suspension II* in ⟨855⟩, *Table 1* [NMT 6 nephelometric turbidity units (NTU)].▲ (USP 1-Aug-2020)

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[1] US Food and Drug Administration. Guidance for industry. Waiver of in vivo bioavailability and bioequivalence studies for immediate-release solid oral dosage forms based on a biopharmaceutics classification system. Rockville, MD: US Food and Drug Administration; December 2017.

[2] USP. Reagents and Reference Tables, Solutions, Buffer Solutions, 4. Standard Buffer Solutions, 4.1 Preparation. In: *USP–NF*. Rockville, MD: USP; 1 May 2018. https://online.uspnf.com/uspnf/document/GUID-0E4CE941-0762-456C-94B0-9209A58834FC.

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# ⟨643⟩ TOTAL ORGANIC CARBON

## INTRODUCTION

Total organic carbon (TOC) is an indirect measure of organic molecules present in pharmaceutical waters measured as carbon. Organic molecules are introduced into the water from the source water, from purification and distribution system materials, from biofilm growing in the system, and from the packaging of sterile and nonsterile waters. TOC also can be used as a process control attribute to monitor the performance of unit operations comprising the purification and distribution system. A TOC measurement is not a replacement test for endotoxin or microbiological control. Although there can be a qualitative relationship between a food source (TOC) and microbiological activity, there is no direct numerical correlation.

A number of acceptable methods exist for analyzing TOC. This chapter does not endorse, limit, or prevent any technologies from being used, but this chapter provides guidance on how to qualify these analytical technologies as well as how to interpret instrument results for use as a limit test.

Apparatuses commonly used to determine TOC in water for pharmaceutical use have in common the objective of oxidizing the organic molecules in the water to produce carbon dioxide followed by the measurement of the amount of carbon dioxide produced. Then the amount of carbon dioxide produced is determined and used to calculate the organic carbon concentration in the water.

All technologies must discriminate between the inorganic carbon, which may be present in the water from sources such as dissolved carbon dioxide and bicarbonate, and the carbon dioxide generated from the oxidation of organic molecules in the sample. The discrimination may be accomplished either by determining the inorganic carbon and subtracting it from the total carbon (the sum of organic carbon and inorganic carbon), or by purging inorganic carbon from the sample before oxidation. Although purging may entrain volatile organic molecules, such purgeable organic carbon is present in negligible quantities in water for pharmaceutical use.

***Change to read:***

## PROCEDURES

**1. Bulk Water**

The following sections apply to tests for bulk *Purified Water*, *Water for Injection*, *Water for Hemodialysis*, and the condensate of *Pure Steam*.

**1.1 Instrumentation requirements:** This test method is performed either as an on-line test or as an off-line laboratory test using a calibrated instrument. The suitability of the instrument must be periodically demonstrated as described below. In addition, it must have a manufacturer's specified limit of detection of 0.05 mg/L (0.05 ppm) or lower of carbon.

When testing water for quality control purposes, ensure that the instrument and its data are under appropriate control and that the sampling approaches and locations of both on-line and off-line measurements are representative of the quality of the water used. The water purification process, distribution, and use should be considered when selecting either on-line or off-line measurement.

**1.2 Reagent water:** Use water with a TOC level of NMT 0.10 mg/L. [NOTE—A conductivity requirement may be necessary to ensure method reliability.]

**1.3 Container preparation:** Organic contamination of labware and sample containers results in higher TOC values. Therefore, use labware and containers that have been scrupulously cleaned of organic residues. Any method that is effective in removing organic matter can be used (see *Cleaning Glass Apparatus* ⟨1051⟩). Use *Reagent water* (section 1.2) for the final rinse.

**1.4 System suitability solution:** Dissolve an accurately weighed quantity of USP 1,4-Benzoquinone RS in *Reagent water* (section 1.2) to obtain a solution with a concentration of 0.75 mg/L (0.50 mg/L of carbon).

**1.5 Standard solution:** Unless otherwise directed in the individual monograph, dissolve an accurately weighed quantity of USP Sucrose RS in the *Reagent water* (section 1.2) to obtain a solution with a concentration of 1.19 mg/L of sucrose (0.50 mg/L of carbon).

**1.6 Reagent water control:** Use a suitable quantity of *Reagent water* (section 1.2) obtained at the same time as those used in the preparations of the *Standard solution* (section 1.5) and the *System suitability solution* (section 1.4).

**1.7 Water sample:** Obtain an on-line or off-line sample that suitably reflects the quality of water used.

**1.8 Other control solutions:** Prepare appropriate reagent blank solutions or other specified solutions needed for establishing the apparatus baseline or for calibration adjustments following the manufacturer's instructions, and run the appropriate blanks to zero the instrument, if necessary.

**1.9 Limit response:** Measure the TOC of the *Reagent water control* (section 1.6) in the instrument, and record the response ($r_w$). Also measure the TOC of the *Standard solution* (section 1.5) in the instrument, and record the response ($r_S$). Calculate the corrected *Standard solution* response, which is also the *Limit response* ($r_L$), for the contribution from the *Reagent water* (section 1.2), by subtracting the *Reagent water control* response from the *Standard solution* response:

$$r_L = r_S - r_W$$

$r_S$ = instrument response to the *Standard solution* (section 1.5)
$r_W$ = instrument response to the *Reagent water control* (section 1.6)

The *Limit response* ($r_L$) of 0.50 mg/L of carbon will be equal to this corrected *Standard solution* response.

**1.10 System suitability:** Measure the TOC of the *System suitability solution* (section 1.4) in the instrument, and record the response ($r_{SS}$). Calculate the corrected *System suitability solution* response ($r_C$) by subtracting the *Reagent water control* response from the *System suitability solution* response:

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$$r_C = r_{SS} - r_W$$

$r_{SS}$ = instrument response to the *System suitability solution* (section 1.4)
$r_W$ = instrument response to the *Reagent water control* (section 1.6)

Calculate the percent response efficiency ($r_E$):

$$r_E = 100 \times \blacktriangle\blacktriangle_{\text{(ERR 1-Sep-2021)}}(r_C / r_L)$$

$r_C$ = corrected instrument response to the *System suitability solution* (section 1.4)
$r_L$ = corrected instrument response to the *Standard solution* (section 1.5) (also known as the *Limit response*)

The TOC measuring system is suitable if the percent response efficiency ($r_E$) is NLT 85% and NMT 115%. The suitability of the instrument must be periodically demonstrated.

**1.11 Procedure:** Measure the TOC of the *Water sample* (section 1.7) and record the response ($r_U$). The *Water sample* meets the requirements if $r_U$ is NMT $r_L$. This method can be performed using on-line or off-line instrumentation that meets the *Instrumentation requirements* (section 1.1).

**2. Sterile Water**

The following sections apply to tests for *Sterile Water for Injection*, *Sterile Purified Water*, *Sterile Water for Irrigation*, *Sterile Water for Inhalation*, and any other monographs that specify this section. The sterile waters are derived from *Purified Water* or *Water for Injection*, and therefore have been determined to be compliant with the *Bulk Water* (section 1) requirements before being stored and sterilized in the container.

Follow the requirements for *Container preparation* (section 1.3) and *Other control solutions* (section 1.8). Prepare the *Standard solution* (section 2.4) and the *System suitability solution* (section 2.3) that correspond to the *Limit response* (section 2.7) for the volume of the container being tested as specified in *Table 1* and as described in steps 2 and 4 in *Procedure* (section 2.9).

**Table 1. TOC Limit Based on Container Volume**

| Nominal Container Volume (mL) | Limit 1 (*L1*) (mg/L of carbon) | Limit 2 (*L2*)[a] (mg/L of carbon) |
|---|---|---|
| ≤5 | 32.00 | 48.00 |
| >5 and ≤100 | 24.00 | 36.00 |
| >100 | 8.00 | 12.00 |

[a] *Limit 2* concentrations are utilized to determine the system suitability requirements for the container volume being tested.

**2.1 Instrument requirements:** The suitability of the instrument must be periodically demonstrated as described below. In addition, it must have a manufacturer's specified limit of detection of 0.10 mg/L (0.10 ppm) or lower of carbon.

**2.2 Reagent water:** Use water with a TOC level of NMT 0.50 mg/L. [NOTE—See *General Notices, 8.230.30 Water in a Compendial Procedure*. A conductivity requirement may be necessary in order to ensure method reliability.]

**2.3 System suitability solution:** /Dissolve an accurately weighed quantity of USP 1,4-Benzoquinone RS in *Reagent water* (section 2.2) to obtain a solution with a concentration that corresponds to the container volume being tested, as specified in *Table 2*:

**Table 2. System Suitability Solution Based on Container Volume**

| Nominal Container Volume (mL) | 1,4-Benzoquinone (mg/L) | Equivalent Carbon Concentration (mg/L of carbon) |
|---|---|---|
| ≤5 | 72.00 | 48.00 |
| >5 and ≤100 | 54.00 | 36.00 |
| >100 | 18.00 | 12.00 |

**2.4 Standard solution:** Unless otherwise directed in the individual monograph, dissolve an accurately weighed quantity of USP Sucrose RS in the *Reagent water* (section 2.2) to obtain a solution with a concentration that corresponds to the *Standard solution* (section 2.4), measured for the container volume being tested, as specified in *Table 3*:

**Table 3. Standard Solution Based on Container Volume**

| Nominal Container Volume (mL) | Limit 1 (*L1*) | | Limit 2 (*L2*) | |
|---|---|---|---|---|
| | Sucrose Concentration (mg/L) | Equivalent Carbon Concentration (mg/L of carbon) | Sucrose Concentration (mg/L) | Equivalent Carbon Concentration (mg/L of carbon) |
| ≤5 | 76.00 | 32.00 | 114.00 | 48.00 |
| >5 and ≤100 | 57.00 | 24.00 | 85.50 | 36.00 |

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**Table 3. Standard Solution Based on Container Volume** *(continued)*

| Nominal Container Volume (mL) | Limit 1 (*L1*) | | Limit 2 (*L2*) | |
|---|---|---|---|---|
| | Sucrose Concentration (mg/L) | Equivalent Carbon Concentration (mg/L of carbon) | Sucrose Concentration (mg/L) | Equivalent Carbon Concentration (mg/L of carbon) |
| >100 | 19.00 | 8.00 | 28.50 | 12.00 |

**2.5 Reagent water control:** Use a suitable quantity of *Reagent water* (section 2.2) obtained at the same time as those used in the preparations of the *Standard solution* and the *System suitability solution* (section 2.3).

**2.6 Water sample:** Obtain water samples that suitably reflect the quality of the sterile water batch being tested. [NOTE—See *Container preparation* (section 1.3).] Before opening the water packages to remove water samples for analysis, vigorously agitate the packages to homogenize any TOC residues that may be present in packages. For small packages, several packages may be required in order to collect a sufficient water volume for analysis. Otherwise test water samples individually.

**2.7 Limit response:** Measure the TOC of the *Reagent water control* (section 2.5) and record the response ($r_W$). Also measure the TOC of the *Standard solution* (section 2.4) prepared at concentrations corresponding to the appropriate *Limit 1*, and, if required for step 4 in *Procedure* (section 2.9), *Limit 2* values from *Table 1* for the container volume being tested. The values in this table are the TOC limits based on container volume. Finally, record the instrument response to each of the *Standard solutions* (section 2.4) for *Limit 1* ($r_{S1}$) and, if required, *Limit 2* ($r_{S2}$) from *Table 1*.

Calculate the corrected instrument response to the *Standard solutions* prepared at the *Limit 1* ($r_{L1}$) or *Limit 2* ($r_{L2}$) concentrations, as appropriate, by subtracting the *Reagent water control* response ($r_W$) from the *Limit 1 Standard solution* response ($r_{S1}$) and, if required, the *Limit 2 Standard solution* response ($r_{S2}$):

$$r_{L1} = r_{S1} - r_W$$

and if required:

$$r_{L2} = r_{S2} - r_W$$

$r_{S1}$ = instrument response to the *Limit 1 Standard solution*
$r_{S2}$ = instrument response to the *Limit 2 Standard solution*
$r_W$ = instrument response to the *Reagent water control*

**2.8 System suitability:** Prepare the *Standard solution* (section 2.4) at the TOC concentrations corresponding to the *Limit 2* in *Table 3* for the container volume being tested. Prepare the *System suitability solution* (section 2.3) according to *Table 2* for the container volume being tested. Measure the TOC of the *Reagent water control* (section 2.5) in the instrument, and record the response ($r_W$). Measure the TOC of the *Standard solution* (section 2.4), and record the response ($r_S$). Calculate the corrected *Standard solution* response ($r_S - r_W$), by subtracting the *Reagent water control* response ($r_W$) from the *Standard solution* response ($r_S$). Measure the TOC of the *System suitability solution* (section 2.3) in the instrument, and record the response ($r_{SS}$). Calculate the corrected *System suitability solution* response ($r_{SS} - r_W$) by subtracting the *Reagent water control* response ($r_W$) from the *System suitability solution* response ($r_{SS}$) (section 2.3). Calculate the percent response efficiency ($r_E$):

$$r_E = 100 \times [(r_{SS} - r_W)/(r_S - r_W)]$$

$r_{SS}$ = instrument response to the *System suitability solution*
$r_W$ = instrument response to the *Reagent water control*
$r_S$ = instrument response to the *Standard solution*

The system is suitable if the percent response efficiency is NLT 85% and NMT 115%. The suitability of the instrument must be periodically demonstrated.

**2.9 Procedure**

1. Measure the TOC of the *Water sample* (section 2.6) and record the response ($r_U$).
2. Compare the $r_U$ to the corrected instrument response ($r_{L1}$) for *Limit 1* [see *Limit Response* (section 2.7)] for the appropriate nominal container volume.
3. If $r_U$ is NMT $r_{L1}$, then the *Water sample* (section 2.6) meets the requirements and the test is completed.
4. If $r_U$ is greater than $r_{L1}$, then compare $r_U$ from step 1 to $r_{L2}$ for the appropriate nominal container volume.
5. If $r_U$ is greater than $r_{L2}$, then the *Water sample* does not meet the requirements and the test is completed.
6. If $r_U$ is greater than $r_{L1}$ but NMT $r_{L2}$, then use suitable analytical procedures appropriate for the intended use to identify and quantify each individual organic impurity exceeding a concentration of 0.20 mg/L of carbon.
7. If there are no individual impurities that exceed 0.20 mg/L of carbon, then the *Water sample* (section 2.6) meets requirements and the test is completed.
8. If there are individual impurities that exceed 0.20 mg/L of carbon, then evaluate them for safety at the concentrations found in this testing.

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9. If the evaluation of the organic impurities is deemed safe, then the *Water sample* (section 2.6) meets the requirements and the test is completed.
10. If the evaluation of the organic impurities is deemed to impact patient safety, then the *Water sample* (section 2.6) does not meet the requirements and the test is completed.

## ADDITIONAL REQUIREMENTS

- **USP Reference Standards** ⟨11⟩
  USP 1,4-Benzoquinone RS
  USP Sucrose RS

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# ⟨644⟩ CONDUCTIVITY OF SOLUTIONS

Portions of the chapter text that are national *USP* text and are not part of the harmonized text, are marked with symbols (••) to specify this fact.

## INTRODUCTION

This chapter provides information on how to apply electrical conductivity measurements (hereafter referred to as "conductivity") of fluid solutions, including pure fluids. •This chapter does not replace the official *Water Conductivity* ⟨645⟩ procedure, which is used to ensure the ionic purity of compendial waters such as *Water for Injection*, *Purified Water*, *Pure Steam* condensate, and *Sterile Water for Injection*, among others.• This chapter is intended for •other• fluid applications when conductivity is used to measure, monitor, or control chemical dispensing, chemical purity, ionic concentration, and other applications where the ionic character of the fluid needs to be known or controlled.

Applications include, but are not limited to, solutions that may be used in clean-in-place, chromatography detection, ionic solution preparations, endpoint detection, dosing, fermentation, and buffer production. In some cases, conductivity measurements can be extended to pure organic fluids such as alcohols and glycols where a weak conductivity signal exists, and the signal can be significantly increased if the organics become contaminated with water or salts. • [NOTE—For additional background information, see *Theory and Practice of Electrical Conductivity Measurements of Solutions* ⟨1644⟩.] •

Conductivity is the measurement of the ability of a fluid to conduct electricity via its chemical ions. The ability of any ion to electrically conduct is directly related to its ion mobility. Conductivity is directly proportional to the concentrations of ions in the fluid, according to *Equation 1*:

$$\kappa = 1000 \sum_{i}^{all\ ions} C_i \lambda_i$$

$\kappa$ = conductivity (S/cm)
$C_i$ = concentration of chemical ion *i* (mol/L)
$\lambda_i$ = specific molar conductance of ion *i* ($S \cdot cm^2/mol$)

Although the SI unit S/m is the appropriate SI unit for conductivity, historically the unit S/cm has been selected by industry as the accepted unit.

On the basis of *Equation 1*, conductivity is not ion selective because it responds to all ions. Furthermore, the specific molar conductance of each ion is different. As a result, unless the percentage composition of ions of the solution is limited and known, the precise concentrations of ionic species cannot be determined from conductivity measurements. However, for examples such as a solution of a single salt or acid or base, such as a caustic solution used in cleaning, the precise concentration can be directly determined. Despite the lack of ionic specificity, conductivity is a valuable laboratory and process tool for measurement and control of total ionic content because it is proportional to the sum of the concentrations of all ionic species (anions and cations) for diluted solutions as described in *Equation 1*. At higher concentrations, conductivity measurements are not perfectly linear with concentration. Conductivity measurements cannot be applied to solids or gases, but they can be applied to the condensate of gases.

Another variable that influences conductivity measurements is the fluid temperature. As the fluid temperature increases, the ion conductance increases, making this physicochemical phenomenon the predominant reason for the temperature-compensation requirement when testing conductive fluids.

The conductivity, $\kappa$, is proportional to the conductance, *G* (S), of a fluid between two electrodes (*Equation 2*):

$$\kappa = G \times \left(\frac{d}{A}\right) = G \times K$$

$\kappa$ = conductivity (S/cm)
*G* = conductance (S)
*d* = distance between the electrodes (cm)
*A* = area of the conducting electrodes ($cm^2$)
*K* = cell constant ($cm^{-1}$), which also equals the ratio of *d*/*A*

The resistivity *p* (Ω-cm) of the fluid is, by definition, the reciprocal of the conductivity (*Equation 3*):

$$\rho = 1/\kappa = 1/(G \times K) = R/K$$

$\rho$ = resistivity (Ω-cm)
$\kappa$ = conductivity (S/cm)
*G* = conductance (S)
*K* = cell constant ($cm^{-1}$)
*R* = resistance (Ω), which is the reciprocal of the conductance, *G*

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## APPARATUS

An electrical conductivity measurement consists of the determination of resistance of the fluid between and around the electrodes of the conductivity sensor. To achieve this measurement, the primary instrumentation is the resistance-measuring circuit and the conductivity sensor, and they are usually connected by a cable when the sensor and the user interface are separated.

The resistance measurement is made by applying an alternating current (AC, meaning the flow of electric charge periodically reverses direction) voltage (or current) to the electrodes, measuring the current (or voltage), and calculating the resistance according to Ohm's law. The alternating source is used to prevent the polarization (collection of ions) at the electrodes. Depending on the instrument, the measuring frequency of the measuring system adjusts automatically according to the measuring conditions of the instrument, and there may be multiple resistance-measuring circuits embedded in the measuring system. The resistance-measurement circuit may be embedded in the transmitter or in the sensor.

The conductivity sensor consists of at least two electrical conductors of a fixed size and geometry, separated by an electrical insulator. The electrodes, insulator, and any other wetted materials should be constructed of materials that are unreactive to fluids with which they may come into contact. Also, the sensor construction should withstand the environmental conditions (process or ambient temperature, pressure, cleaning applications) that it would be subjected to.

Most conductivity sensors have temperature devices such as a platinum resistance temperature device (RTD) or negative temperature coefficient (NTC) thermistor embedded inside the sensor, although external temperature measurement is possible. The purpose of the temperature measurement is for temperature compensation of the conductivity measurement.

## CELL CONSTANT DETERMINATION

The purpose of the sensor's cell constant is to normalize the conductance (or resistance) measurement for the geometrical construction of the two electrodes.

The cell constant is determined by immersing the conductivity sensor in a solution of known conductivity. Solutions of known conductivity can be obtained by preparation of specific mixtures according to national authoritative sources or procurement of commercially available certified and traceable standard solutions. These recipes or certified solutions can range from 5 to 200,000 µS/cm, depending on the level of accuracy desired. Alternatively the cell constant is determined by comparison to other reference conductivity measuring systems (also available as an accredited calibration service). [NOTE—Conductivity measurements are not perfectly linear with concentration.]

The measured cell constant of the conductivity sensor must be within 5% of the nominal value indicated by the sensor certificate, unless otherwise prescribed.

Modern conductivity sensors normally do not change their cell constant over their lifetime. If a change of the cell constant is detected during calibration, a cleaning of the sensor is appropriate according to the manufacturer's recommendations. Following that, the calibration procedure should be repeated. Sometimes "memory effects" appear, particularly when changing from high to low concentrations if the sensor is not well flushed.

## CALIBRATION OF TEMPERATURE

In addition to verifying the sensor's cell constant, the embedded temperature device (or external temperature device) should be appropriately calibrated for the application to apply the temperature compensation algorithm accurately. The temperature accuracy that is required depends on the criticality of the temperature to the application. An accuracy of ±1° typically suffices.

## CALIBRATION OF MEASUREMENT ELECTRONICS

The measurement circuit of the system is fundamentally an AC resistance measuring device. Appropriate verification and/or calibration of the measuring circuit is required for measurement systems with signal transfer via analog cable. This is accomplished by disconnecting the measuring circuit from the sensor's electrodes, attaching traceable resistors of known value with the cable of the measurement system to the measuring circuit, and verifying that the measured resistance agrees with the resistor value to an acceptable level. A typical acceptance criterion for the resistance accuracy is <2% of the reading at resistances >100 Ω, and increasing to 5% at lower resistances. However, the application criticality should ultimately determine the desired accuracy.

For conductivity systems that cannot have the resistance-measuring circuit disconnected from the electrodes (e.g., measurement circuit and electrodes in one mutual housing), it may be difficult to directly adjust or verify the circuit accuracy, depending on the sensor design. An alternative method of verifying the measurement system integrity is a system calibration according to the procedures for the cell constant determination for each measuring circuit that is intended to be used.

If verification/calibration of the sensor's cell constant, temperature device, and measuring circuit are done at the same service interval, the measuring circuit should be verified first, the temperature device next, and the cell constant last. Because all of these parameters are typically very stable due to modern electronics and stable sensor construction, frequent calibration (such as daily) is not usually required. Comparison to qualified reference systems is also a proper means of calibration. Calibration is performed at appropriate intervals as defined in the quality management system.

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***Change to read:***

## TEMPERATURE COMPENSATION

Because the conductivity of a fluid is temperature dependent, temperature compensation of the conductivity measurement is necessary unless otherwise prescribed (e.g., purified water, water for injection). An appropriate temperature compensation algorithm will ensure that changes in the conductivity measurement can be ascribed to concentration changes and not temperature changes. Conductivity measurements are normally referenced to 25°. A common form of linear temperature compensation uses *Equation 4*:

$$\kappa_{25} = \frac{\kappa_T}{[1 + \alpha(T - 25)]}$$

$\kappa_{25}$ = conductivity compensated to 25°
$\kappa_T$ = conductivity at $T$
$\alpha$ = temperature coefficient of the conductivity
$T$ = measured temperature

A temperature coefficient of 2.1% per 1° is commonly used for many salt solutions. Most salt-based solutions have linear compensation factors ranging from 1.9% to 2.2% per 1°. Depending on the fluid samples, other forms of temperature compensation may be appropriate. Non-linear temperature compensation data for a variety of solutions is widely available (e.g., as described in ISO 7888 *Water quality—Determination of electrical conductivity*). In cases of very low conductivity (<10 µS/cm), such as purified ▲water for cleaning/rinsing purposes,▲ (USP 1-May-2021) two compensations need to be made. One is for the intrinsic conductivity of water, and the other is for the other ionic species in water. These compensations are normally combined and embedded in the microprocessor-controlled conductivity measurement systems. This is not supplied in all conductivity measurement technologies.

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## CONDUCTIVITY MEASUREMENT OF FLUIDS

For off-line ▲or at-line▲ (USP 1-May-2021) batch measurements, rinse the cleaned sensor with the fluid to be measured. Then immerse the sensor in the fluid to be measured, and record the temperature and the temperature-compensated conductivity as required. Be sure that the position of the sensor in the container does not affect the conductivity measurement, because the container walls can affect the measurement for some electrode designs.

For continuous on-line or ▲in▲ (USP 1-May-2021)-line measurements, install the cleaned sensor into the pipe, tank, or other containment vessel, and flush, if necessary. Make sure proper installation procedures are applied to prevent bubbles or particles from collecting between the electrodes. Be sure that the position of the sensor in the pipe or tank does not affect the conductivity measurement, because the nearby surfaces can affect the measurement for some electrode designs.

Record the temperature and the temperature-compensated conductivity as required.

For all batch or continuous measurements, ensure that the wetted components of the sensor are compatible with the fluid and the temperature to be measured.

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# ⟨645⟩ WATER CONDUCTIVITY

## INTRODUCTION

Electrical conductivity in water is a measure of the ion-facilitated electron flow through it. Water molecules dissociate into ions as a function of pH and temperature and result in a very predictable conductivity. Some gases, most notably carbon dioxide, readily dissolve in water and interact to form ions, which predictably affect conductivity also. For the purpose of this discussion, these ions and their resulting conductivity can be considered intrinsic to the water.

Water conductivity is also affected by the presence of extraneous ions. The extraneous ions used in modeling the conductivity specifications described below are the chloride and ammonia ions. The conductivity of the ubiquitous chloride ion (at the theoretical endpoint concentration of 0.47 ppm when chloride was a required attribute test in *USP 22* and earlier revisions) and the ammonium ion (at the limit of 0.3 ppm) represents a major portion of the allowed water ionic impurity level. A balancing quantity of anions (such as chloride, to counter the ammonium ion) and cations (such as sodium, to counter the chloride ion) is included in this allowed impurity level to maintain electroneutrality. Extraneous ions such as these may have a significant effect on the water's chemical purity and suitability for use in pharmaceutical applications.

The procedure in the section *Bulk Water* is specified for measuring the conductivity of waters such as *Purified Water*, *Water for Injection*, *Water for Hemodialysis*, and the condensate of *Pure Steam*. The procedure in the section *Sterile Water* is specified for measuring the conductivity of waters such as *Sterile Purified Water*, *Sterile Water for Injection*, *Sterile Water for Inhalation*, and *Sterile Water for Irrigation*.

The procedures below shall be performed using instrumentation that has been calibrated, has conductivity sensor cell constants that have been accurately determined, and has a temperature compensation function that has been disabled for *Bulk Water* Stage 1 testing. For both online and offline measurements, the suitability of instrumentation for quality control testing is also dependent on the sampling location(s) in the water system. The selected sampling instrument location(s) must reflect the quality of the water used.

## INSTRUMENT SPECIFICATIONS AND OPERATING PARAMETERS

Water conductivity must be measured accurately with calibrated instrumentation. An electrical conductivity measurement consists of the determination of the conductance, $G$ (or its inverse, resistance, $R$), of the fluid between and around the electrodes. The conductance ($1/R$) is directly affected by the geometrical properties of the electrodes; i.e., the conductance is inversely proportional to the distance ($d$) between the electrodes and proportional to the area ($A$) of the electrodes. This geometrical ratio ($d/A$) is known as the cell constant, $\Theta$. Thus the measured conductance is normalized for the cell constant to determine the conductivity, $\kappa$, according to the following equation:

$$\text{conductivity, } \kappa \text{ (S/cm)} = \Theta \text{ (cm}^{-1}\text{)}/R \text{ } (\Omega)$$

It is the cell constant and the resistance measurement that must be verified and adjusted, if necessary.

### Cell Constant

The cell constant must be known within ±2%. The cell constant can be verified directly by using a solution of known or traceable conductivity, or indirectly by comparing the instrument reading taken with the conductivity sensor in question to readings from a conductivity sensor of known or traceable cell constant. If necessary, adjust the cell constant following the manufacturer's instrument protocol. The frequency of verification/calibration is a function of the sensor design.

### Resistance Measurement

Calibration (or verification) of the resistance measurement is accomplished by replacing the conductivity sensor electrodes with precision resistors having standards traceable to NIST or equivalent national authorities in other countries (accurate to ±0.1% of the stated value) to give a predicted instrument conductivity response. The accuracy of the resistance measurement is acceptable if the measured conductivity with the traceable resistor is within ±0.1 µS/cm of the calculated value according to the equation above. For example, the traceable resistor is 50 kΩ, and the cell constant, Θ, is 0.10 $cm^{-1}$. The calculated value is $2.0 \times 10^{-6}$ S/cm or 2.0 µS/cm. The measured value should be 2.0 ± 0.1 µS/cm. The instrument must have a minimum resolution of 0.1 µS/cm on the lowest range.

The target conductivity value(s) should be based on the type of water to be analyzed, and it should be equal to or less than the water conductivity limit for that type of water. Multiple measuring circuits may be embedded in the meter or the sensor, and each circuit may require separate verification or calibration before use. The frequency of recalibration is a function of instrument system design.

### System Verification

The cell constant of the user's sensor can be determined with the user's resistance measurement system, or the cell constant can be determined with an independent resistance measurement system. If the cell constant is determined with an independent resistance measurement system, it is recommended that the user verify that the sensor has been properly connected to the resistance measurement system to ensure proper performance. Verification can be made by comparing the conductivity (or

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resistivity) values displayed by the measuring equipment with those of an external calibrated conductivity-measuring device. The two non–temperature-compensated conductivity (or resistivity) values should be equivalent to or within ±5% of each other, or should have a difference that is acceptable on the basis of product water criticality and/or the water conductivity ranges in which the measurements are taken. The two conductivity sensors should be positioned close enough together to measure the same water sample at the same temperature and water quality.

### Temperature Compensation and Temperature Measurements

Because temperature has a substantial effect on conductivity readings of specimens at high and low temperatures, many instruments automatically correct the actual reading to display the value that theoretically would be observed at the nominal temperature of 25°. This is typically done using a temperature sensor embedded in the conductivity sensor and a software algorithm embedded in the instrument. This temperature compensation algorithm may not be accurate for the various water types and impurities. For this reason, conductivity values used in the *Stage 1* test for *Bulk Water* are non–temperature-compensated measurements. Other conductivity tests that are specified for measurement at 25° can use either temperature-compensated or non–temperature-compensated measurements.

A temperature measurement is required for the *Stage 1* test or for the other tests at 25°. It may be made using the temperature sensor embedded in the conductivity cell sensor. An external temperature sensor positioned near the conductivity sensor is also acceptable. Accuracy of the temperature measurement must be ±2°.

## BULK WATER

The procedure and test limits in this section are intended for *Purified Water*, *Water for Injection*, *Water for Hemodialysis*, the condensate of *Pure Steam*, and any other monographs that specify this section.

This is a three-stage test method to accommodate online or offline testing. Online conductivity testing provides real-time measurements and opportunities for real-time process control, decision, and intervention. Precautions should be taken while collecting water samples for offline conductivity measurements. The sample may be affected by the sampling method, the sampling container, and environmental factors such as ambient carbon dioxide concentration and organic vapors. This procedure can be started at *Stage 2* if offline testing is preferred.

### Procedure

#### STAGE 1

*Stage 1* is intended for online measurement or may be performed offline in a suitable container.

1. Determine the temperature of the water and the conductivity of the water with a non–temperature-compensated conductivity reading.
2. Using *Table 1*, find the temperature value that is NMT the measured temperature, i.e., the next lower temperature. The corresponding conductivity value on this table is the limit.

[NOTE—Do not interpolate.]

3. If the measured conductivity is NMT the table value determined in step 2, the water meets the requirements of the test for conductivity. If the conductivity is higher than the table value, proceed with *Stage 2*.

**Table 1. Stage 1—Temperature and Conductivity Requirements** (for non–temperature-compensated conductivity measurements only)

| Temperature | Conductivity Requirement (µS/cm) |
|---|---|
| 0 | 0.6 |
| 5 | 0.8 |
| 10 | 0.9 |
| 15 | 1.0 |
| 20 | 1.1 |
| 25 | 1.3 |
| 30 | 1.4 |
| 35 | 1.5 |
| 40 | 1.7 |
| 45 | 1.8 |
| 50 | 1.9 |
| 55 | 2.1 |
| 60 | 2.2 |
| 65 | 2.4 |
| 70 | 2.5 |

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**Table 1. Stage 1—Temperature and Conductivity Requirements** (for non–temperature-compensated conductivity measurements only) *(continued)*

| Temperature | Conductivity Requirement (µS/cm) |
|---|---|
| 75 | 2.7 |
| 80 | 2.7 |
| 85 | 2.7 |
| 90 | 2.7 |
| 95 | 2.9 |
| 100 | 3.1 |

STAGE 2

4. Transfer a sufficient amount of water to a suitable container, and stir the test specimen. Adjust the temperature, if necessary, and, while maintaining it at 25 ± 1°, begin vigorously agitating the test specimen while periodically observing the conductivity. When the change in conductivity (due to uptake of atmospheric carbon dioxide) is less than a net of 0.1 µS/cm per 5 min, note the conductivity.

[NOTE—Conductivity measurements at this stage may be temperature-compensated to 25° or non–temperature-compensated.]

5. If the conductivity is not greater than 2.1 µS/cm, the water meets the requirements of the test for conductivity. If the conductivity is greater than 2.1 µS/cm, proceed with *Stage 3*.

STAGE 3

6. Perform this test within approximately 5 min of the conductivity determination in step 5, while maintaining the sample temperature at 25 ± 1°. Add a saturated potassium chloride solution to the same water sample (0.3 mL per 100 mL of the test specimen), and determine the pH to the nearest 0.1 pH unit, as directed in *pH* ⟨791⟩.

7. Referring to *Table 2*, determine the conductivity limit at the measured pH value. If the measured conductivity in step 4 is NMT the table value determined in step 6, the water meets the requirements of the test for conductivity. If either the measured conductivity is greater than this value or the pH is outside the range of 5.0–7.0, the water does not meet the requirements of the test for conductivity.

**Table 2. Stage 3—pH and Conductivity Requirements** (for atmosphere- and temperature-equilibrated samples only)

| pH | Conductivity Requirement (µS/cm) |
|---|---|
| 5.0 | 4.7 |
| 5.1 | 4.1 |
| 5.2 | 3.6 |
| 5.3 | 3.3 |
| 5.4 | 3.0 |
| 5.5 | 2.8 |
| 5.6 | 2.6 |
| 5.7 | 2.5 |
| 5.8 | 2.4 |
| 5.9 | 2.4 |
| 6.0 | 2.4 |
| 6.1 | 2.4 |
| 6.2 | 2.5 |
| 6.3 | 2.4 |
| 6.4 | 2.3 |
| 6.5 | 2.2 |
| 6.6 | 2.1 |
| 6.7 | 2.6 |
| 6.8 | 3.1 |
| 6.9 | 3.8 |
| 7.0 | 4.6 |

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# STERILE WATER

The procedure and test limits are intended for *Sterile Purified Water*, *Sterile Water for Injection*, *Sterile Water for Inhalation*, and *Sterile Water for Irrigation*, and any other monographs that specify this section. The sterile waters are derived from *Purified Water* or *Water for Injection*, and therefore have been determined to be compliant with the *Bulk Water* requirements before being stored in the container. The specification provided represents the maximum allowable conductivity value, taking into consideration the limitation of the measurement method and reasonable container leaching. Such specification and the sampling volume choices should be defined and validated on the basis of the intended purpose of the water.

## Procedure

Obtain a sample that suitably reflects the quality of water used. Before opening, vigorously agitate the package to homogenize the water sample. Several packages may be required to collect sufficient water for analysis.

Transfer a sufficient amount of water to a suitable container, and stir the test specimen. Adjust the temperature, if necessary, and, while maintaining it at 25 ± 1°, begin vigorously agitating the test specimen while periodically observing the conductivity. When the change in conductivity (due to uptake of ambient carbon dioxide) is less than a net of 0.1 µS/cm per 5 min, note the conductivity.

For containers with a nominal volume of 10 mL or less, if the conductivity is NMT 25 µS/cm, the water meets the requirements. For containers with a nominal volume greater than 10 mL, if the conductivity is NMT 5 µS/cm, the water meets the requirements.

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# ⟨651⟩ CONGEALING TEMPERATURE

The temperature at which a substance passes from the liquid to the solid state upon cooling is a useful index to purity if heat is liberated when the solidification takes place, provided that any impurities present dissolve in the liquid only, and not in the solid. Pure substances have a well-defined freezing point, but mixtures generally freeze over a range of temperatures. For many mixtures, the congealing temperature, as determined by strict adherence to the following empirical methods, is a useful index of purity. The method for determining congealing temperatures set forth here is applicable to substances that melt between –20° and 150°, the range of the thermometer used in the bath. The congealing temperature is the maximum point (or lacking a maximum, the point of inflection) in the temperature-time curve.

## APPARATUS

Assemble an apparatus similar to that *illustrated,*

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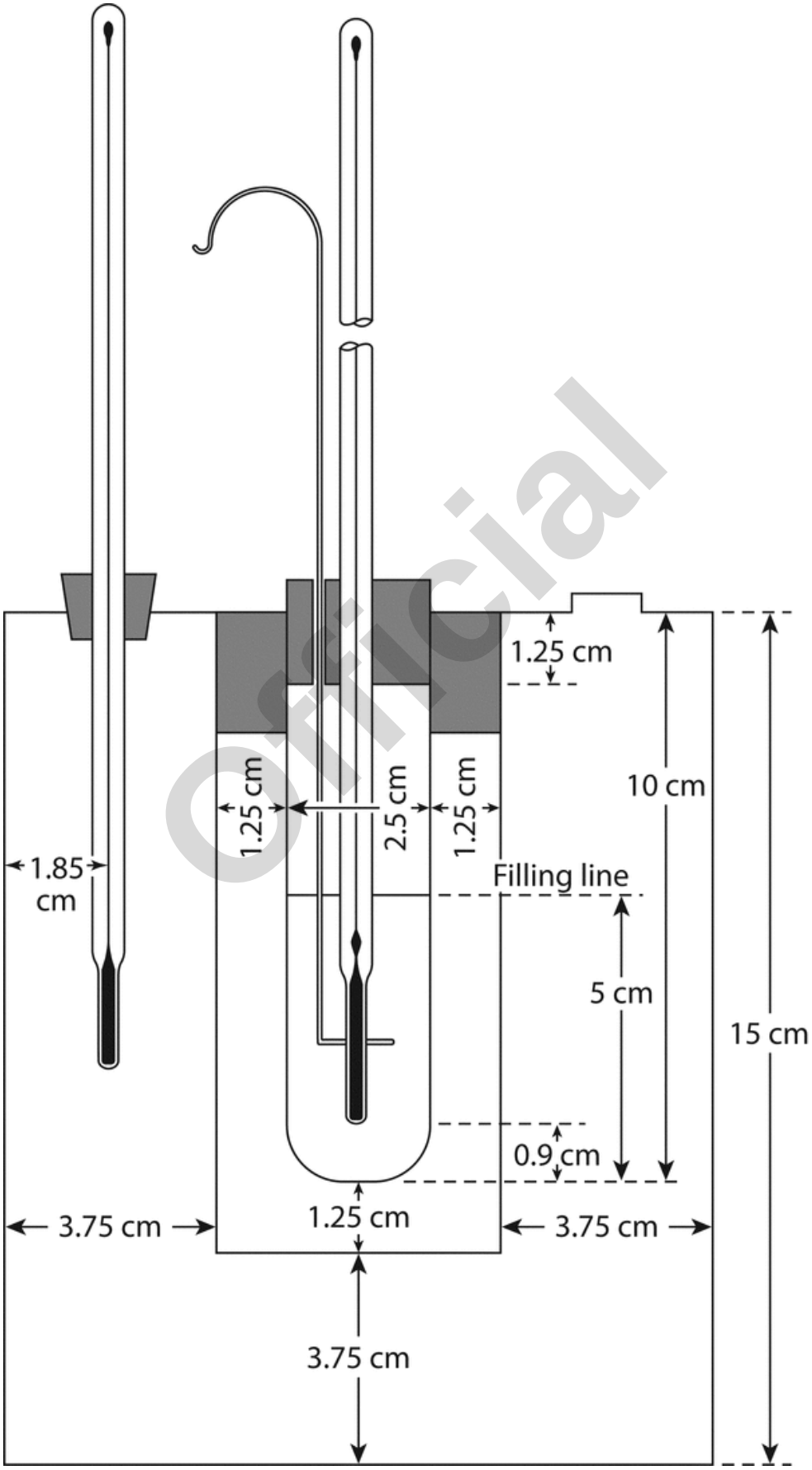


Congealing Temperature Apparatus

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in which the container for the substance is a 25- × 100-mm test tube. This is provided with a suitable, short-range thermometer suspended in the center, and a wire stirrer, about 30 cm long, bent at its lower end into a horizontal loop around the thermometer. Use a thermometer having a range not exceeding 30°, graduated in 0.1° divisions, and calibrated for, but not used at, 76-mm immersion. A suitable series of thermometers, covering a range from −20° to +150°, is available as the ASTM E1 series 89C through 96C. Other temperature-measuring devices may be used if they are validated for this procedure (see *General Notices, 6.80.30. Temperature Reading Devices*). Dimensions should be within ±20% of those given in the illustration.

The specimen container is supported, by means of a cork, in a suitable water-tight cylinder about 50 mm in internal diameter and 11 cm in length. The cylinder, in turn, is supported in a suitable bath sufficient to provide not less than a 37-mm layer surrounding the sides and bottom of the cylinder. The outside bath is provided with a suitable thermometer.

## PROCEDURE

Melt the substance, if a solid, at a temperature not exceeding 20° above its expected congealing point, and pour it into the test tube to a height of 50 to 57 mm. Assemble the apparatus with the bulb of the test tube thermometer immersed halfway between the top and bottom of the specimen in the test tube. Fill the bath to about 12 mm from the top of the tube with suitable fluid at a temperature 4° to 5° below the expected congealing point.

In case the substance is a liquid at room temperature, carry out the determination using a bath temperature about 15° below the expected congealing point.

When the test specimen has cooled to about 5° above its expected congealing point, adjust the bath to a temperature 7° to 8° below the expected congealing point. Stir the specimen continuously during the remainder of the test by moving the loop up and down between the top and bottom of the specimen, at a regular rate of 20 complete cycles per minute.

Congelation frequently may be induced by rubbing the inner walls of the test tube with the thermometer, or by introducing a small fragment of the previously congealed substance. Pronounced supercooling may cause deviation from the normal pattern of temperature changes. If the latter occurs, repeat the test, introducing small particles of the material under test in solid form at 1° intervals as the temperature approaches the expected congealing point.

Record the reading of the test tube thermometer every 30 seconds. Continue stirring only so long as the temperature is gradually falling, stopping when the temperature becomes constant or starts to rise slightly. Continue recording the temperature in the test tube every 30 seconds for at least 3 minutes after the temperature again begins to fall after remaining constant.

The average of not less than four consecutive readings that lie within a range of 0.2° constitutes the congealing temperature. These readings lie about a point of inflection or a maximum, in the temperature-time curve, that occurs after the temperature becomes constant or starts to rise and before it again begins to fall. The average to the nearest 0.1° is the congealing temperature.

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# ⟨659⟩ PACKAGING AND STORAGE REQUIREMENTS

**(A portion of the *Packaging* section of this chapter will become official on December 1, 2025 as indicated. Early adoption of the requirements in this chapter and *Plastic Materials of Construction* ⟨661.1⟩ and *Plastic Packaging Systems for Pharmaceutical Use* ⟨661.2⟩ is permitted by USP.)**

## INTRODUCTION

The purpose of this chapter is to provide packaging definitions, auxiliary packaging information, and storage condition definitions relevant to the storage and distribution of active ingredients, excipients, and medical products, such as pharmaceuticals, combination products, and,when labeled as being USP compliant, dietary supplements.

***Change to read:***

## PACKAGING

Packaging materials must not interact physically or chemically with a packaged article in a manner that causes its safety, identity, strength, quality, or purity to fail to conform to established requirements. ▲Any plastic material used to construct a *Packaging system* must meet the applicable requirements of *Plastic Materials of Construction* ⟨661.1⟩.▲ (Official 1-Dec-2025) All *Packaging systems* must meet the applicable requirements specified in *Containers—Glass* ⟨660⟩, *Plastic Packaging Systems and Their Materials of Construction* ⟨661⟩, ▲*Plastic Packaging Systems for Pharmaceutical Use* ⟨661.2⟩,▲ (Official 1-Dec-2025) and *Auxiliary Packaging Components* ⟨670⟩. All elastomeric closures must meet the applicable requirements in *Elastomeric Components in Injectable Pharmaceutical Product Packaging/Delivery Systems* ⟨381⟩.

Every monograph in *USP–NF* must have packaging and storage requirements. For the packaging portion of the statement, the choice of containers is provided in this chapter. For active pharmaceutical ingredients (APIs), the choice would be a tight or well-closed container (as specified in the monograph), and, where needed, a light-resistant container. For excipients, given their typical presentation as large-volume commodity items (*Packaging systems* ranging from drums to tank cars), a well-closed container is an appropriate default requirement. Articles must be protected from moisture, freezing, and excessive heat (see *General Definitions*) when no specific directions or limitations are provided.

The compendial requirements for the use of specified containers apply also to articles packaged by *Dispensers*, *Repackagers*, or other individuals, unless otherwise indicated in the individual drug product monograph.

## POISON PREVENTION PACKAGING ACT

This act, which is administered by the US Consumer Product Safety Commission (CPSC), requires special packaging for most human oral prescription drugs, oral controlled drugs, certain non-oral prescription drugs, certain dietary supplements, and many over-the-counter (OTC) drug preparations, to protect the public from personal injury or illness from misuse of these preparations [16 Code of Federal Regulations (CFR) §1700].

The primary packaging of substances regulated under the Poison Prevention Packaging Act (PPPA) must comply with the special packaging standards. These apply to all packaging types, including reclosable, non-reclosable, and unit-dose types.

Special packaging is not required for drugs dispensed within a hospital setting for inpatient administration. Also, special packaging does not need to be used by manufacturers and packagers of bulk-packaged prescription drugs that will be repackaged by the pharmacist. PPPA-regulated prescription drugs may be dispensed in non-*Child-resistant packaging* upon the request of the purchaser or when directed in a legitimate prescription.

Manufacturers or packagers of PPPA-regulated OTC preparations are allowed to package one size in non-*Child-resistant packaging* as long as popular-size, special packages are also supplied. The non-*Child-resistant packaging* requires special labeling.

## TEMPERATURE AND STORAGE

Specific directions are stated in some monographs with respect to storage conditions (e.g., the temperature or humidity) at which an article must be stored and shipped. Storage and shipping directions apply except where the label on the article or its prescribing information has different storage conditions that are based on stability studies. Where no specific directions or limitations are provided in the article's labeling or prescribing information, articles must be protected from moisture, freezing, and excessive heat, and, where necessary, from light during shipping and distribution; drug substances are exempt from this requirement.

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# GENERAL DEFINITIONS

## Packaging Definitions

**Packaging system** (also referred to as a Container–closure system)**:** The sum of *Packaging components* and materials that together contain and protect the article. This includes *Primary packaging components* as well as *Secondary packaging components* when such components are required to provide additional protection.

**Container:** A receptacle that holds an intermediate compound, API, excipient, or dosage form, and is in direct contact with the article (e.g., ampules, vials, bottles, syringes, and pen injectors).

**Closure:** A material that seals an otherwise open space of a *Container* and provides protection for the contents. It also provides access to the contents of the *Container* (e.g., screw caps and stoppers).

**Packaging component:** Any single part of the *Package* or *Container–closure system,* including: the *Container*; *Closures*; ferrules and overseals; *Closure* liners; *Container* liners (e.g., tube cartridge liners); inner seals; administration ports; overwraps; administration accessories; labels; cardboard boxes; and shrink wrap.

**Primary packaging component:** A *Packaging component* that is in direct contact with or may come into direct contact with the article.

**Secondary packaging component:** A *Packaging component* that is in direct contact with a *Primary packaging component* and may provide additional protection for the article.

**Tertiary packaging component:** A *Packaging component* that is in direct contact with a *Secondary packaging component* and may provide additional protection for the article during transportation and/or storage.

**Ancillary component:** A component or entity that may come into contact with a *Tertiary packaging component* during the distribution, storage, and/or transportation of the packaged article (e.g., pallets, skids, and shrink wrap).

**Associated component:** A *Packaging component* that is typically intended to deliver the drug article to the patient but is not stored in contact with the article for its entire shelf life (e.g., spoons, *Dosing cups*, and dosing syringes).

**Materials of construction:** The materials (e.g., glass, plastic, elastomers, and metal) of which a *Packaging component* consists.

**Small-volume injection** (also referred to as Small-volume parenteral)**:** An injectable dosage form that is packaged in *Containers* labeled as containing 100 mL or less.

**Large-volume injection** (also referred to as Large-volume parenteral)**:** An injectable dosage form that is packaged in *Containers* labeled as containing more than 100 mL.

**Child-resistant packaging:** A *Packaging system* designed or constructed to meet CPSC standards pertaining to opening by children (16 CFR §1700.20 et seq. and 16 CFR §1700.15).

**Senior-friendly packaging:** A *Packaging system* designed or constructed to meet CPSC standards pertaining to opening by senior adults (16 CFR §1700.15 and 16 CFR §1700.20).

**Restricted delivery system:** A *Packaging system* designed or constructed to restrict (control) the amount of the drug product that may be delivered in order to limit unintended access by children and other similarly vulnerable populations. *Restricted delivery systems* should meet and may exceed CPSC standards for special packaging [*Child-resistant* and *Senior-friendly packaging* (16 CFR §1700.15 et seq.)]. For oral medicinal liquids, surface and flow characteristics vary. It is the responsibility of the manufacturer to ensure that all components of the *Restricted delivery system* provide the intended safety protection. One component of the *Restricted delivery system* is the flow restrictor, which is a *Packaging component* that restricts the flow of liquid. The flow restrictor may be used as part of a *Restricted delivery system* or as an adapter to facilitate use of a measuring device for oral medicinal liquids. A flow restrictor should not compromise CPSC standards for special packaging [*Child-resistant* and *Senior-friendly packaging* (16 CFR §1700.15 et seq.)].

**Tamper-evident packaging:** A *Packaging system* that may not be accessed without obvious destruction of the seal or some portion of the *Packaging system*. *Tamper-evident packaging* must be used for sterile drug products intended for ophthalmic or otic use, except where extemporaneously compounded for immediate dispensing on prescription. Drug products intended for sale without prescription are also required to comply with the *Tamper-evident packaging* and labeling requirements of the FDA where applicable (21 CFR §211.132). Preferably, the immediate *Container* and/or the outer *Container* or protective packaging used by a manufacturer or distributor for all dosage forms that are not specifically exempt is designed to show evidence of any tampering with the contents.

**Reclosable packaging:** A package that after it has been initially opened is capable of being reclosed with a similar degree of security and is capable of being used a sufficient number of times to dispense the total contents without loss of security. *Reclosable packaging* may incorporate child-resistance capabilities.

**Non-reclosable packaging:** A package or part of a package that cannot be closed again after all or part of the contents have been removed. Examples of *Non-reclosable packaging* are blisters, sachets, strips, and other *Single-unit containers*. *Non-reclosable packaging* may include cold-formed foil blisters, foil strip packs, and polyvinyl chloride (PVC)/Aclar combining multilayer materials that are thermo-formed or cold-formed foil blisters. *Non-reclosable packaging* may be child resistant depending on the intended use and place of use. Household non-reclosables are subject to the PPPA as defined in 16 CFR §1700.14.

**Hermetic container:** A *Container–closure system* that is impervious to air or any other gas under the ordinary or customary conditions of handling, shipment, storage, and distribution.

**Tight container:** A *Container–closure system* that protects the contents from contamination by extraneous liquids, solids, or vapors; from loss of the article; and from efflorescence, deliquescence, or evaporation under the ordinary or customary conditions of handling, shipment, storage, and distribution, and is capable of tight reclosure. Where a tight container is specified, it may be replaced by a hermetic container for a single dose of an article. [NOTE—Where packaging and storage in a tight container or well-closed container is specified in the individual monograph, the container used for an article when dispensed on prescription meets the requirements in *Containers—Performance Testing* ⟨671⟩.]

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**Well-closed container:** A *Container–closure system* that protects the contents from contamination by extraneous solids and from loss of the article under the ordinary or customary conditions of handling, shipment, storage, and distribution. See ⟨671⟩.

**Light-resistant container:** A *Container–closure system* that protects the contents from the effects of light by virtue of the specific properties of the material of which it is composed, including any coating applied to it. A clear and colorless or a translucent container may be made light-resistant by means of an opaque covering or by use of secondary packaging, in which case the label of the container bears a statement that the opaque covering or secondary packaging is needed until the articles are to be used or administered. Where it is directed to "protect from light" in an individual monograph, preservation in a light-resistant container is intended. See ⟨671⟩, *Spectral Transmission* ▲*for Light-Resistant Packaging Components or Systems*▲ (ERR 1-Apr-2021) or ⟨661.2⟩, *Functionality* ▲*Test Method,*▲ (ERR 1-Dec-2025) *Spectral Transmission Requirements for Light-Resistant Components and Systems*.

**Equivalent container–closure system:** A *Container–closure system* that is as protective as or more protective than the original manufacturer's *Packaging system* in terms of moisture vapor transmission rate, oxygen transmission, light transmission, and compatibility. System equivalency extends to any special protective materials, such as those for seals or desiccants associated with the original *Packaging system*.

**Table 1. Packaging Systems Definitions: Injection versus Noninjection**

| Injection | Noninjection |
|---|---|
| Multiple-dose | Multiple-unit |
| Single-dose | Single-unit |
| — | Unit-dose |
| — | Unit-of-use |
| Pharmacy bulk package | — |
| Imaging bulk package | — |

## Injection Packaging Systems

**Multiple-dose container** (also referred to as Multi-dose)**:** A *Container–closure system* that holds a sterile medication for parenteral administration (injection or infusion) that has met antimicrobial effectiveness testing requirements, or is excluded from such testing requirements by FDA regulation. A *Multiple-dose container* is intended to contain more than one dose of a drug product. When space permits, a *Multiple-dose container* is labeled as such. *Multiple-dose containers* are generally expected to contain 30 mL or less of medications. The beyond-use date for an opened or entered (e.g., needle-punctured) *Multiple-dose container* is 28 days unless otherwise specified by the manufacturer on the label. An example of a *Multiple-dose container* is a vial.

**Single-dose container:** A *Container–closure system* that holds a sterile medication for parenteral administration (injection or infusion) that is not required to meet the antimicrobial effectiveness testing requirements. A *Single-dose container* is designed for use with a single patient as a single injection/infusion.[1] When space permits, a *Single-dose container* is labeled as such and should include on the label appropriate discard statements. Examples of *Single-dose containers* are vials, ampules, and prefilled syringes.

**Pharmacy bulk package:** A *Container–closure system* of a sterile preparation for parenteral use that contains many single doses. The contents are intended for use in a pharmacy admixture program and are restricted to the preparation of admixtures for infusion or, through a sterile transfer device, for the filling of empty sterile syringes. The *Closure* must be penetrated only one time after constitution, if necessary, with a suitable sterile transfer device or dispensing set that allows measured dispensing of the contents. The *Pharmacy bulk package* is to be used only in a suitable work area such as a laminar flow hood (or an equivalent clean-air compounding area). Designation as a *Pharmacy bulk package* is limited to injection, for injection, or injectable emulsion dosage forms as defined in *Nomenclature* ⟨1121⟩, *General Nomenclature Forms*.

*Pharmacy bulk packages*, although containing more than one single dose, are exempt from the *Multiple-dose container* volume limit of 30 mL and the requirement that they contain a substance or suitable mixture of substances to prevent the growth of microorganisms. See *Labeling* ⟨7⟩ for labeling requirements.

**Imaging bulk package:** A container of a sterile preparation for parenteral use that contains many single doses of a contrast agent (medical imaging drug product) for use with a medical imaging device. The contents are restricted to use in direct conjunction with a device with features to mitigate the risk of cross-contamination (i.e., an automated contrast injection system, contrast management system, or contrast media transfer set approved or cleared for use with an *Imaging bulk package*). The sterility assurance of the *Imaging bulk package* contents in part is dependent upon the automated contrast injection system, the contrast management system, or the contrast media transfer set.

The *Imaging bulk package* is to be used only in a room designated for radiological procedures that involve intravascular administration of a contrast agent. Using aseptic technique, the *Imaging bulk package* closure must be penetrated only one time with a suitable sterile component of the automated contrast injection system, the contrast management system,or the contrast media transfer set. If the integrity of the *Imaging bulk package* and the delivery system cannot be assured through direct continuous supervision, the *Imaging bulk package* and all associated disposables for the automated contrast injection system, the contrast management system, or the contrast media transfer set should be discarded.

Designation as an *Imaging bulk package* is limited to injection, for injection, or injectable emulsion dosage forms as defined in ⟨1121⟩, *General Nomenclature Forms*. *Imaging bulk packages*, although containing more than one single dose, are exempt from the multiple-dose container volume limit of 30 mL. The contents of the *Imaging bulk package* must have demonstrated the ability to limit the growth of microorganisms over the labeled period of use.

[1] Exceptions may be considered only under conditions described in *Pharmaceutical Compounding—Sterile Preparations* ⟨797⟩.

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Where a container is offered as an *Imaging bulk package*, the label must: 1) state prominently "Imaging Bulk Package" and, in juxtaposition with this statement, include the following use statement: "For use only with an automated contrast injection system, contrast management system, or contrast media transfer set approved or cleared for use with this contrast agent in this Imaging Bulk Package"; 2) bear a statement limiting the time frame in which the container may be used once it has been entered, provided it is held under the labeled storage conditions; and 3) bear the statement, "See drug and device labeling for information on devices indicated for use with this Imaging Bulk Package and techniques to help assure safe use".

## Noninjection Packaging Systems

**Multiple-unit container:** A *Container–closure system* that permits withdrawal of successive portions of a noninjection article without changing the safety, strength, quality, or purity of the remaining portion (e.g., bottle of capsules, tablets, oral or topical liquids, and semisolids).

**Single-unit container:** A *Container–closure system* that holds a quantity of a noninjection article intended for administration as a single dose or a single finished device intended for use promptly after the *Packaging system* is opened.

**Unit-dose container:** A single-unit *Container–closure system* for an article intended for administration by other than the parenteral route as a single dose.

**Unit-of-use container:** A *Container–closure system* that contains a specific quantity of an article that is intended to be dispensed as such without further modification except for the addition of appropriate labeling (see ⟨7⟩). It is not permitted to repackage *Unit-of-use containers* for sale.

## Miscellaneous

**Repackaging:** The act of removing a drug product from the original manufacturer's *Packaging system* and placing it into another *Packaging system*, usually one of smaller size.

**Repackager:** A firm that repackages drug products for distribution (e.g., for resale to distributors, hospitals, or pharmacies). For drug products, this applies to a function that is beyond the regular practice of a pharmacy. The distribution is not patient-specific, in that there are no prescriptions.

**Contract packager/contract repackager:** A firm that is contracted by another organization, such as a manufacturer, to package bulk into a marketed *Container* of a drug product. A *Contract packager* does not take ownership from the manufacturer and generally receives the assigned expiration date from the manufacturer.

**Dispenser:** A licensed or registered practitioner who is legally responsible for providing the patient with a preparation that is in compliance with a prescription or a medication order and contains a specific patient label. In addition, dispensers may prepare limited quantities in anticipation of a prescription or medication order from a physician. *Dispensers* are governed by the board of pharmacy of the individual state. The terms "dispenser" and "pharmacy" are used interchangeably.

**Beyond-use date:** See ⟨7⟩.

**Expiration date:** See ⟨7⟩.

**Black closure system or black bands:** The use of a *Black closure system* on a vial (e.g., a black cap overseal and a black ferrule to hold the elastomeric closure) or the use of a *Black band* or series of bands above the constriction on an ampule is prohibited, except for ⟨7⟩, *Labels and Labeling for Injectable Products, Potassium Chloride for Injection Concentrate*.

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# INJECTION PACKAGING

Packaging for sterile products intended for injection must be validated as meeting the containment, protection, and compatibility requirements that are essential for maintaining the article's strength, quality, and purity. Refer to *Package Integrity Evaluation—Sterile Products* ⟨1207⟩, *Package Integrity Testing in the Product Life Cycle—Test Method Selection and Validation* ⟨1207.1⟩, *Package Integrity Leak Test Technologies* ⟨1207.2⟩, and *Package Seal Quality Test Technologies* ⟨1207.3⟩ for further information regarding sterile product container–closure integrity testing and validation. *Closures* for *Multiple-dose containers* permit the withdrawal of the contents without removal or destruction of the *Closure*. The *Closure* permits penetration by a needle and, upon withdrawal of the needle, closes at once, protecting the contents against contamination. Refer to ⟨381⟩ for *Closure* reseal tests that are useful for screening multiple-dose *Closures* for their reseal properties. Additional testing may be needed to ensure that the specific *Closure* selected for a product package is able to prevent loss of product contents and microbial contamination under anticipated conditions of multiple entry and use. Piggyback *Packaging systems* are usually intravenous infusion *Container–closure systems* that are used to administer a second infusion through a connector of some type or an injection port on the administration set of the first fluid, thereby avoiding the need for another injection site on the patient's body. Piggyback *Packaging systems* also are known as secondary infusion containers.

The volume of injection in a *Single-dose container* provides the amount specified for one-time parenteral administration, and in no case is more than sufficient to permit the withdrawal and administration of 1 L. Preparations intended for intraspinal, intracisternal, or peridural administration are packaged in *Single-dose containers* only. Unless otherwise specified in the individual monograph, a *Multiple-dose container* contains a volume of injection sufficient to permit the withdrawal of NMT 30 mL.

The following injections are exempt from the 1-L restriction of the foregoing requirements relating to packaging:

- Injections packaged for extravascular use as irrigation solutions or peritoneal dialysis solutions
- Injections packaged for intravascular use as parenteral nutrition or as replacement or substitution fluid to be administered continuously during hemofiltration

Injections packaged for intravascular use that may be used for intermittent, continuous, or bolus replacement fluid administration during hemodialysis or other procedures, unless excepted above, must conform to the 1-L restriction. Injections labeled for veterinary use are exempt from the packaging and storage requirements concerning the limitation to single-dose *Packaging systems* and the limitation on the volume of *Multiple-dose containers*.

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## Packaging for Constitution

*Containers*, including the *Closures*, for dry solids intended for injection must not interact physically or chemically with the preparation in any manner that alters the strength, quality, or purity beyond the official requirements under the ordinary or customary conditions of handling, shipment, storage, sale, and use. A *Packaging system* for a sterile solid permits the addition of a suitable solvent and withdrawal of portions of the resulting solution or suspension in such manner that the sterility of the product is maintained. Where the assay in a monograph provides a procedure for the sample solution, in which the total withdrawable contents are to be withdrawn from a *Single-dose container* with a hypodermic needle and syringe, the contents are to be withdrawn as completely as possible into a dry hypodermic syringe of a rated capacity not exceeding 3 times the volume to be withdrawn and fitted with a 21-gauge needle NLT 2.5 cm (1 inch) in length. Care must be taken to expel any air bubbles, and the contents are then discharged into a *Container* for dilution and assay.

## MEDICAL GAS PACKAGING

**Gas cylinder:** A metallic *Packaging system* constructed of steel or aluminum and designed to hold medical gases under pressure; these gases may include: *Carbon Dioxide USP*, *Helium USP*, *Medical Air USP*, *Nitrogen NF*, and *Oxygen USP*. As a safety measure, for carbon dioxide, helium, medical air, nitrous oxide, and oxygen, the Pin Index Safety System of matched fittings is recommended for cylinders of Size E or smaller.

## ASSOCIATED COMPONENTS

Many *Associated Components* are graduated for measurement and dose administration. *Associated Components* can be packaged with the drug product or sold and purchased separately. It is the responsibility of the manufacturer to ensure that the appropriate measurement and dosing component is provided or that a general purpose component, such as those described in this section, is specified for delivering the appropriate amount/dose with the intended accuracy. Liquid preparations have unique surface and flow characteristics. Consequently, the volume delivered from a measurement/dosing component may vary for each preparation.

The graduated *Associated Components* described in this section are for general use and should be composed of safe materials. Graduated markings should be legible and indelible, and on an extraoral surface that does not contact the product.

The markings on associated components must be in metric units only and limited to a single measurement scale that corresponds with the dosing instructions on the OTC or prescription container label (see *Prescription Container Labeling* ⟨17⟩). Under expected conditions of use, the volume error incurred in measuring an individual dose by means of such graduated components should be NMT 10% of the indicated amount of the preparation with which the graduated component will be used.

**Dosing cup:** A measuring device consisting of a small cup that may be packaged with oral liquid articles.

**Dosing spoon:** A measuring device consisting of a bowl and handle that may be packaged with oral liquid articles. The handle may be a graduated tube.

**Medicine dropper:** A measuring device consisting of a transparent or translucent barrel or tube that is generally fitted with a collapsible bulb. It may be packaged with liquid articles.

**Oral syringe:** A measuring device consisting of a plunger and barrel made of transparent or translucent plastic material and a seal on the end. It may be packaged with oral liquid articles. The syringe should deliver a measured amount of a liquid drug product.

## TEMPERATURE AND STORAGE DEFINITIONS

**Freezer:** A place in which the temperature is controlled between –25° and –10° (–13° and 14° F). It is noted that, in some instances, articles may have a recommended storage condition below –20° (–4° F). In such cases, the temperature of the storage location should be controlled to ±10° of the recommended storage condition.

**Refrigerator:** A cold place in which the temperature is controlled between 2° and 8° (36° and 46° F).

**Cold:** Any temperature not exceeding 8° (46° F).

**Cool:** Any temperature between 8° and 15° (46° and 59° F). [NOTE—An article for which storage in a cool place is directed may, alternatively, be stored and shipped as refrigerated, unless otherwise specified by the individual monograph.]

**Room temperature** (also referred to as Ambient temperature)**:** The temperature prevailing in a working environment.

**Controlled cold temperature:** The temperature maintained thermostatically between 2° and 8° (36° and 46° F), which allows for temperature excursions between 2° and 15° (36° and 59° F) that may be experienced during storage, shipping, and distribution, but not to exceed 24 h, such that the allowable calculated mean kinetic temperature (MKT) is NMT 8° (46° F) with no excursions below 2° (36° F) or above 15° (59° F). These limits (time and temperature) and the calculated MKT must be documented (see *Mean Kinetic Temperature in the Evaluation of Temperature Excursions During Storage and Transportation of Drug Products* ⟨1079.2⟩). Additionally, controlled cold excursions may only occur one time during the possession of the product within the supply chain unless directed otherwise by the manufacturer. The length of time the product is held at 2° and 15° (36° and 59° F) should be supported by stability data. Other limits may be permitted if the manufacturer so instructs as supported by the manufacturer's stability data and/or thermal cycling studies.

**Controlled room temperature:** The temperature maintained thermostatically that encompasses the usual and customary working environment of 20°–25° (68°–77° F).

MKT may be used during an excursion provided: 1) MKT does not exceed 25° (77° F); 2) excursion between 15° and 30° (59° and 86° F); 3) transient excursions are NMT 40° (104° F); and 4) excursion time is NMT 24 h.[2] These limits (time and temperature) and the calculated MKT must be documented.

[2] Anderson C, Seevers R, Hunt D. The use of mean kinetic temperature to aid evaluation of temperature excursions: proper and improper application. *Pharm Forum.* 2018;44(4). www.usppf.com/pf/pub/index.html.

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Articles may be labeled for storage at "controlled room temperature" or at "20°–25°", or other wording based on the same MKT. (See ⟨1079.2⟩.)

An article for which storage at *Controlled room temperature* is directed may, alternatively, be stored and shipped in a cool place or refrigerated, unless otherwise specified in the individual monograph or on the label. Storage time in controlled cold or cool place cannot be used to calculate excursion temperature outside of controlled room temperature ranges.

**Warm:** Any temperature between 30° and 40° (86° and 104° F).

**Excessive heat:** Any temperature above 40° (104° F).

**Dry place:** A place that does not exceed 40% average relative humidity at 20° (68° F) or the equivalent water vapor pressure at other temperatures. The determination may be made by direct measurement at the place. Determination is based on NLT 12 equally spaced measurements that encompass either a season, a year, or, where recorded data demonstrate, the storage period of the article. There may be values of up to 45% relative humidity provided that the average value does not exceed 40% relative humidity. Storage in a *Container* validated to protect the article from moisture vapor, including storage in bulk, is considered a *Dry place*.

**Do not refrigerate:** The *Container* label will bear an appropriate instruction to protect the article from refrigeration in cases where refrigeration exposes the article to loss of strength or potency or destructive alteration of its characteristics (e.g., precipitation, cloudiness). These risks are increased when a drug product with the storage temperature of 20°–25° (68°–77° F) is formulated at near-maximum solubility.

**Protect from freezing:** The *Container* label will bear an appropriate instruction to protect the article from freezing in cases where freezing exposes an article to loss of strength or potency or to destructive alteration of its characteristics. These risks are present in addition to the risk that the *Container* may break if exposed to freezing temperatures.

**Protect from light:** Where light subjects an article to loss of strength or potency or to destructive alteration of its characteristics, the *Container* label bears an appropriate instruction to protect the article from light. The article must be packaged in a light-resistant *Container*.

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# ⟨660⟩ CONTAINERS—GLASS

***Change to read:***

## DESCRIPTION

▲Glass containers for pharmaceutical use are intended to come into direct contact with pharmaceutical products.▲ (IRA 1-Oct-2023) The following recommendations can be made as to the suitability of ▲a▲ (IRA 1-Oct-2023) glass ▲container▲ (IRA 1-Oct-2023) for pharmaceutical products, based on the tests for hydrolytic resistance. ▲Glass▲ (IRA 1-Oct-2023) containers ▲meeting Type I performance▲ (IRA 1-Oct-2023) are suitable for most products for parenteral and nonparenteral uses ▲(e.g., borosilicate). Glass▲ (IRA 1-Oct-2023) containers ▲meeting Type II performance▲ (IRA 1-Oct-2023) are suitable for most acidic and neutral aqueous products for parenteral and nonparenteral uses ▲(e.g., treated soda-lime silica). Glass▲ (IRA 1-Oct-2023) containers ▲meeting Type II performance▲ (IRA 1-Oct-2023) may be used for alkaline parenteral products where stability data demonstrate their suitability. ▲Glass▲ (IRA 1-Oct-2023) containers ▲meeting Type III performance (e.g., soda-lime silica)▲ (IRA 1-Oct-2023) usually are not used for parenteral products or for powders for parenteral use, except where suitable stability test data indicate that ▲containers meeting Type III performance are▲ (IRA 1-Oct-2023) satisfactory.

The inner surface of glass containers may be treated to improve hydrolytic resistance. The outer surface of glass containers may be treated to reduce friction or for protection against abrasion or breakage. The outer surface treatment is such that it does not contaminate the inner surface of the container.

Information on chemical composition of glass types, the formation of glass containers, and factors that influence inner surface durability of glass containers is provided in *Evaluation of the Inner Surface Durability of Glass Containers* ⟨1660⟩. This chapter also contains recommended approaches to evaluate the potential of a drug product to cause the formation of glass particles and delamination.

Glass may be colored to provide protection from light by the addition of small amounts of metal oxides and is tested as described in *Spectral Transmission for Colored Glass Containers*. A clear and colorless container that is made light resistant by means of an opaque enclosure (see *Packaging and Storage Requirements* ⟨659⟩*, General Definitions, Packaging Definitions, Light-resistant container*) is exempt from the requirements for spectral transmission.

***Change to read:***

## SPECIFIC TESTS

The *Glass Grains Test* combined with the *Surface Glass Test* for hydrolytic resistance determines glass ▲performance.▲ (IRA 1-Oct-2023) The hydrolytic resistance is determined by the quantity of alkali released from the glass under the conditions specified. This quantity of alkali is extremely small in the case of the more resistant glasses, thus calling for particular attention to all details of the tests and the use of apparatus of high quality and precision. Conducting these tests in conjunction with a glass standard reference material (SRM) on a routine basis will help to ensure the accuracy of the method. Reference materials are available for both borosilicate glass (SRM 623) and soda-lime-silica glass (SRM 622) from the National Institute of Standards and Technology. The tests should be conducted in an area relatively free from fumes and excessive dust. Test selection is shown in *Table 1* and *Table 2*.

**Table 1. Determination of Glass ▲Performance▲ (IRA 1-Oct-2023)**

| ▲Performance▲ (IRA 1-Oct-2023) Type | Test | Reason |
|---|---|---|
| I, II, III | *Glass Grains Test* | Distinguishes Type I ▲▲ (IRA 1-Oct-2023) from Type II and III ▲▲ (IRA 1-Oct-2023) |

The inner surface of glass containers is the contact surface for pharmaceutical preparations, and the quality of this surface is determined by the *Surface Glass Test* for hydrolytic resistance. The *Surface Etching Test* may be used to determine whether high hydrolytic resistance is due to chemical composition or to surface treatment. Alternatively, the comparison of data from the *Glass Grains Test* and the *Surface Glass Test* may be used in *Table 2*.

**Table 2. Determination of Inner Surface Hydrolytic Resistance**

| ▲Performance▲ (IRA 1-Oct-2023) Type | Test | Reason |
|---|---|---|
| I, II, III | *Surface Glass Test* | Determines hydrolytic resistance of inner surface; distinguishes between *Type I* and *Type II* containers with high hydrolytic resistance and *Type III* containers with moderate hydrolytic resistance |
| I, II | *Surface Etching Test* or comparison of *Glass Grains Test* and *Surface Glass Test* data | Where it is necessary, determines whether high hydrolytic resistance is due to inner surface treatment or to the chemical composition of the glass containers |

Glass containers must comply with their respective specifications for ▲▲ (IRA 1-Oct-2023) surface hydrolytic resistance ▲as noted in *Table 1* and *Table 2*.▲ (IRA 1-Oct-2023) Type I or Type II containers for aqueous parenteral products are tested for extractable arsenic.

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## Hydrolytic Resistance

APPARATUS

**Autoclave:** For these tests, use an autoclave capable of maintaining a temperature of 121 ± 1°, equipped with a thermometer, or a calibrated thermocouple device, allowing a temperature measurement independent of the autoclave system; a suitable recorder; a pressure gauge; a vent cock; and a tray of sufficient capacity to accommodate the number of containers needed to carry out the test above the water level. Clean the autoclave and other apparatus thoroughly with purified water before use.
**Mortar and pestle:** Use a hardened-steel mortar and pestle, made according to the specifications in *Figure 1*.

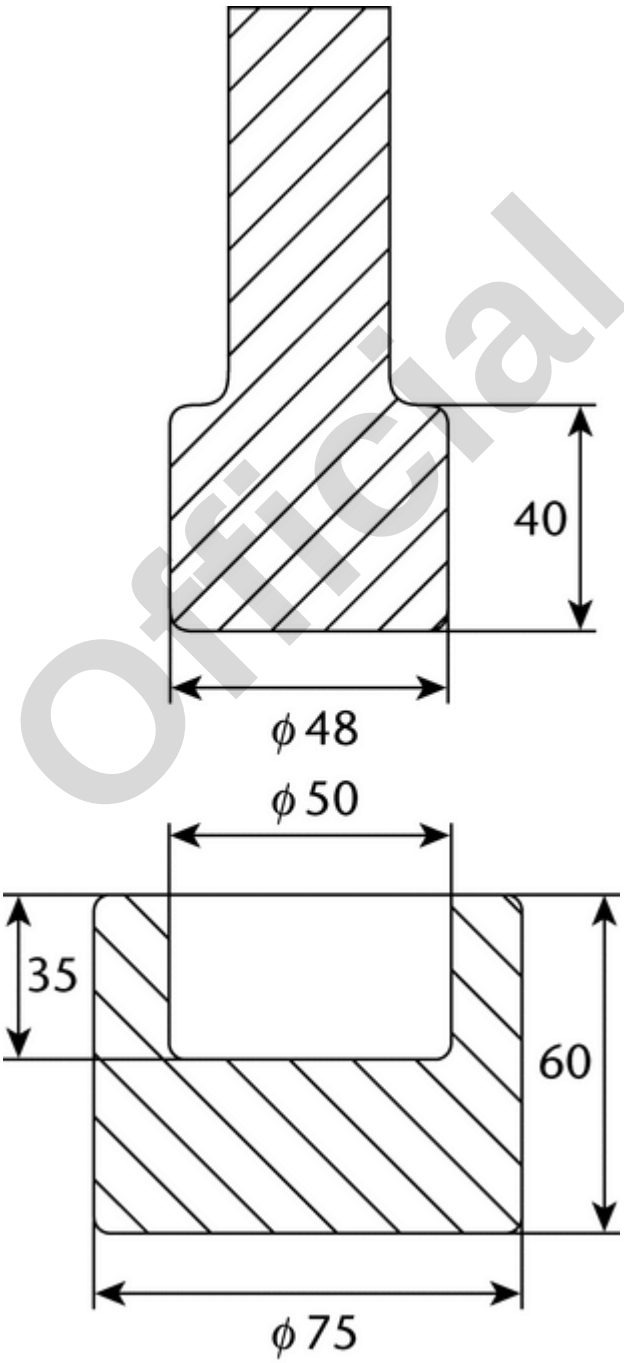


Figure 1. Mortar and pestle for pulverizing glass.

**Other apparatus:** Also required are a set of three square-mesh stainless steel sieves mounted on frames consisting of US Sieve Nos. 25, 40, and 50 (see *Particle Size Distribution Estimation by Analytical Sieving* ⟨786⟩, *Table 1. Sizes of Standard Sieve Series in Range of Interest*); a mechanical sieve-shaker or a sieving machine that may be used to sieve the grains; a tempered, magnetic

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steel hammer; a permanent magnet; weighing bottles; stoppers; metal foil (e.g., aluminum, stainless steel); a hot air oven, capable of maintaining 140 ± 5°; a balance, capable of weighing up to 500 g with an accuracy of 0.005 g; a desiccator; and an ultrasonic bath.

### REAGENTS

**Carbon dioxide-free water:** This is purified water that has been boiled vigorously for 5 min or more and allowed to cool while protected from absorption of carbon dioxide from the atmosphere, or purified water that has a resistivity of not less than 18 Mohm-cm.

**Methyl red solution:** Dissolve 50 mg of methyl red in 1.86 mL of 0.1 M sodium hydroxide and 50 mL of ethanol (96%), and dilute with purified water to 100 mL. To test for sensitivity, add 100 mL of carbon dioxide-free water and 0.05 mL of 0.02 M hydrochloric acid to 0.1 mL of the methyl red solution. The resulting solution should be red. NMT 0.1 mL of 0.02 M sodium hydroxide is required to change the color to yellow. A color change from red to yellow corresponds to a change in pH from pH 4.4 (red) to pH 6.0 (yellow).

## Glass Grains Test

The *Glass Grains Test* may be performed either on the canes used for the manufacture of tubing glass containers or on the containers.

### SAMPLE PREPARATION

Rinse the containers to be tested with purified water, and dry in the oven. Wrap at least three of the glass articles in clean paper, and crush to produce two samples of about 100 g each in pieces NMT 30 mm across. Place in the mortar 30–40 g of the pieces between 10 and 30 mm across taken from one of the samples, insert the pestle, and strike it heavily with the hammer once only. Alternatively, transfer samples into a ball mill-breaker, add the balls, and crush the glass. Transfer the contents of the mortar or ball mill to the coarsest sieve (No. 25) of the set. Repeat the operation until all fragments have been transferred to the sieve. Shake the set of sieves for a short time by hand, and remove the glass that remains on sieves No. 25 and No. 40. Submit these portions to further fracture, repeating the operation until about 10 g of glass remains on sieve No. 25. Reject this portion and the portion that passes through sieve No. 50. Reassemble the set of sieves, and shake for 5 min. Transfer to a weighing bottle the glass grains that passed through sieve No. 40 and are retained on sieve No. 50. Repeat the crushing and sieving procedure with the second glass sample until two samples of grains are obtained, each of which weighs more than 10 g.

Spread each sample on a piece of clean glazed paper, and remove any iron particles by passing the magnet over them. Transfer each sample into a beaker for cleaning. Add 30 mL of acetone to the grains in each beaker, and scour the grains, using suitable means such as a rubber-tipped or plastic-coated glass rod. After scouring the grains, allow to settle, and decant as much acetone as possible. Add another 30 mL of acetone, swirl, decant, and add a new portion of acetone. Fill the bath of the ultrasonic vessel with water at room temperature, then place the beaker in the rack, and immerse it until the level of the acetone is at the level of the water; apply the ultrasound for 1 min. Swirl the beaker, allow to settle, and decant the acetone as completely as possible; then repeat the ultrasonic cleaning operation. If a fine turbidity persists, repeat the ultrasonic cleaning and acetone washing until the solution remains clear. Swirl, and decant the acetone. Dry the grains, first by putting the beaker on a warm plate, then by heating at 140° for 20 min in a drying oven. Transfer the dried grains from each beaker into separate weighing bottles, insert the stoppers, and cool in a desiccator.

### METHOD

**Filling and heating:** Weigh 10.00 g of the cleaned and dried grains into two separate conical flasks. Pipet 50 mL of carbon dioxide-free purified water into each of the conical flasks (test solutions). Pipet 50 mL of carbon dioxide-free purified water into a third conical flask that will serve as a blank. Distribute the grains evenly over the flat bases of the flasks by shaking gently. Close the flasks with neutral glass dishes or aluminum foil rinsed with purified water or with inverted beakers so that the inner surfaces of the beakers fit snugly down onto the top rims of the flasks. Place all three flasks in the autoclave containing the water at ambient temperature, and ensure that they are held above the level of the water in the vessel. Carry out the following operations:

1. Insert the end of a calibrated thermometric device in a filled container through a hole of approximately the diameter of the thermocouple and connect it to an external measuring device. If the container is too small to insert a thermocouple, apply a thermocouple in a suitable, similar container. Alternatively, use the internal thermometer of the autoclave.
2. Close the autoclave door or lid securely but leave the vent-cock open.
3. Start automatic recording of the temperature versus time, and heat the autoclave at a regular rate such that steam issues vigorously from the vent-cock after 20–30 min, and maintain a vigorous evolution of steam for a further 10 min. For autoclaves using a steam generator, it is not necessary to maintain the temperature for 10 min at 100°.
4. Close the vent-cock, and raise the temperature from 100° to 121° at a rate of 1°/min within 20–22 min.
5. Maintain the temperature at 121 ± 1° for 30 ± 1 min from the time when the holding temperature is reached.
6. Cool down to 100° at a rate of 0.5°/min, venting to prevent formation of a vacuum, within 40–44 min.
7. Do not open the autoclave until it has cooled to 95°.
8. Remove the hot samples from the autoclave using appropriate safety precautions, and cool the samples cautiously down to room temperature within 30 min, avoiding thermal shock.

**Titration:** To each of the three flasks add 0.05 mL of *Methyl red solution*. Titrate the blank solution immediately with 0.02 M hydrochloric acid, then titrate the test solutions until the color matches that obtained with the blank solution. Subtract the titration volume for the blank solution from that for the test solutions. Calculate the mean value of the results in mL of 0.02 M

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hydrochloric acid per g of the sample. Repeat the test if the highest and lowest observed values differ by more than the permissible range given in *Table 3*.

**Table 3. Permissible Range for Values Obtained**

| Mean of the Values Obtained for the Consumption of Hydrochloric Acid Solution per g of Glass Grains (mL/g) | Permissible Range of the Values Obtained |
|---|---|
| NMT 0.10 | 25% of the mean |
| 0.10–0.20 | 20% of the mean |
| NLT 0.20 | 10% of the mean |

[NOTE—Where necessary to obtain a sharp endpoint, decant the clear solution into a separate 250-mL flask. Rinse the grains by swirling with three 15-mL portions of carbon dioxide-free water, and add the washings to the main solution. Add 0.05 mL of the *Methyl red solution*. Titrate, and calculate as before. In this case also add 45 mL of carbon dioxide-free purified water and 0.05 mL of *Methyl red solution* to the blank solution.]

LIMITS

The volume does not exceed the values indicated in *Table 4*.

**Table 4. Test Limits for Glass Grains Test**

| Filling Volume (mL) | Maximum Volume of 0.02 M HCl per g of Test Glass (mL) | |
|---|---|---|
| | Type I | Types II and III |
| All | 0.1 | 0.85 |

## Surface Glass Test

DETERMINATION OF THE FILLING VOLUME

The filling volume is the volume of purified water to be added to the container for the purpose of the test. For vials, bottles, cartridges, and syringes, the filling volume is 90% of the brimful capacity. For ampules, it is the volume up to the height of the shoulder.

**Vials and bottles:** Select six dry vials or bottles from the sample lot, or three if their capacity exceeds 100 mL, and remove any dirt or debris. Weigh the empty containers with an accuracy of 0.1 g. Place the containers on a horizontal surface, and fill them with purified water to about the rim edge, avoiding overflow and the introduction of air bubbles. Adjust the liquid levels to the brimful line. Weigh the filled containers to obtain the mass of the water expressed to two decimal places, for containers having a nominal volume less than or equal to 30 mL, and expressed to one decimal place, for containers having a nominal volume greater than 30 mL. Calculate the mean value of the brimful capacity in mL, and multiply it by 0.9. This volume, expressed to one decimal place, is the filling volume for the particular container lot.

**Cartridges and syringes:** Select six dry syringes or cartridges, and seal the small opening (mouth of cartridges, Luer cone, or staked needle of syringes), using an inert material. Determine the mean brimful capacity and filling volume according to *Vials and Bottles*.

**Ampules:** Place at least six dry ampules on a flat, horizontal surface, and fill them with purified water from a buret until the water reaches point A, where the body of the ampule starts to decrease to the shoulder of the ampule (see *Figure 2*). Read the capacities, expressed to two decimal places, and calculate the mean value. This volume, expressed to one decimal place, is the filling volume for the particular ampule lot. The filling volume may also be determined by weighing.

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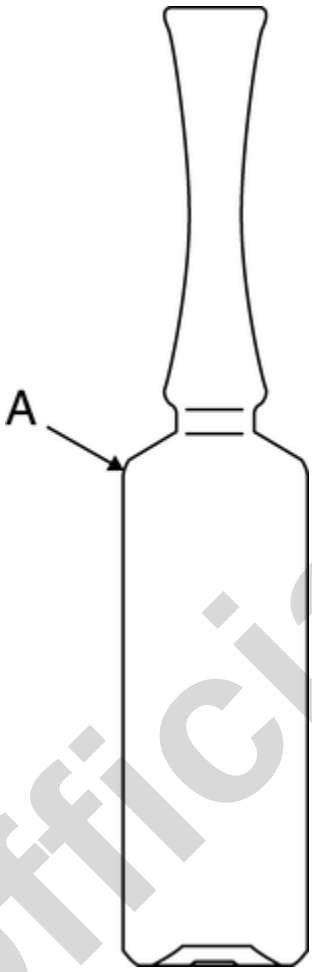


Figure 2. Filling volumes of ampules up to point A.

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TEST

The determination is carried out on unused containers. The volumes of the test solution necessary for the final determination are shown in *Table 5*.

**Table 5. Volume of Test Solution and Number of Titrations**

| Filling Volume (mL) | Volume of Test Liquid for One Titration (mL) | Number of Titrations |
|---|---|---|
| NMT 3 | 25.0 | 1 |
| 3–30 | 50.0 | 2 |
| 30–100 | 100.0 | 2 |
| NLT 100 | 100.0 | 3 |

METHOD

**Cleaning:** Remove any debris or dust. Shortly before the test, rinse each container carefully at least twice with purified water, refilled, and allow to stand. Immediately before testing, empty the containers; rinse once with purified water, then with carbon dioxide-free water; and allow to drain. Complete the cleaning procedure from the first rinsing within 20–30 min. Closed ampules may be warmed in a water bath or in an air oven at about 40° for approximately 2 min before opening to avoid container pressure when opening. Do not rinse before testing.

**Filling and heating:** The containers are filled with carbon dioxide-free water up to the filling volume. Containers in the form of cartridges or prefillable syringes are closed in a suitable manner with material that does not interfere with the test. Each container, including ampules, shall be loosely capped with an inert material such as a dish of neutral glass or aluminum foil previously rinsed with purified water. Place the containers on the tray of the autoclave. Place the tray in an autoclave containing a quantity of water such that the tray remains clear of the water. Close the autoclave, and carry out autoclaving procedure steps 1–8 as described in the *Glass Grains Test*, except that the temperature is maintained at 121 ± 1° for 60 ± 1 min. If a water bath

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is used for cooling samples, take care that the water does not make contact with the loose foil caps to avoid contamination of the extraction solution. The extraction solutions are analyzed by titration according to the method described below.
**Titration:** Carry out the titration within 1 h of the removal of the containers from the autoclave. Combine the liquids obtained from the containers, and mix. Introduce the prescribed volume (see *Table 5*) into a conical flask. Transfer the same volume of carbon dioxide-free water, to be used as a blank, into a second similar flask. Add to each flask 0.05 mL of *Methyl red solution* for each 25 mL of liquid. Titrate the blank with 0.01 M hydrochloric acid. Titrate the test solution with the same acid until the color of the resulting solution is the same as that obtained for the blank. Subtract the value found for the blank titration from that found for the test solution, and express the results in mL of 0.01 M hydrochloric acid per 100 mL of test solution. Express titration values of less than 1.0 mL to two decimal places; express titration values of greater than or equal to 1.0 mL to one decimal place.

### LIMITS

The results, or the average of the results if more than one titration is performed, are not greater than the values stated in *Table 6*.

**Table 6. Limit Values for the Surface Glass Test**

| Filling Volume (mL) | Maximum Volume of 0.01 M HCl per 100 mL of Test Solution (mL) | |
|---|---|---|
| | Types I and II | Type III |
| NMT 1 | 2.0 | 20.0 |
| 1–2 | 1.8 | 17.6 |
| 2–3 | 1.6 | 16.1 |
| 3–5 | 1.3 | 13.2 |
| 5–10 | 1.0 | 10.2 |
| 10–20 | 0.80 | 8.1 |
| 20–50 | 0.60 | 6.1 |
| 50–100 | 0.50 | 4.8 |
| 100–200 | 0.40 | 3.8 |
| 200–500 | 0.30 | 2.9 |
| NLT 500 | 0.20 | 2.2 |

## Surface Etching Test

The *Surface Etching Test* is used in addition to the *Surface Glass Test* when it is necessary to determine whether a container has been surface treated and/or to distinguish between Type I and Type II glass containers. Alternatively, the *Glass Grains Test* and *Surface Glass Test* may be used. The *Surface Etching Test* may be carried out either on unused samples or on samples used in the *Surface Glass Test*.

### METHOD

**Vials and bottles:** The volumes of test solution required are shown in *Table 5*. Rinse the containers twice with purified water, fill to the brimful point with a mixture of one volume of hydrofluoric acid and nine volumes of hydrochloric acid, and allow to stand for 10 min. Empty the containers, and rinse carefully five times with purified water. Immediately before the test, rinse once again with purified water. Submit these containers to the same autoclaving and determination procedure as described in the *Surface Glass Test*. If the results are considerably higher than those obtained from the original surfaces (by a factor of about 5–10), the samples have been surface treated.
[**CAUTION**—Hydrofluoric acid is extremely aggressive. Even small quantities can cause life threatening injuries.]
**Ampules, cartridges, and syringes:** Apply the test method as described in *Vials and Bottles*. If the ampules, cartridges, and syringes are not surface treated, the values obtained are slightly lower than those obtained in the previous tests.
[NOTE—Ampules, cartridges, and syringes made from Type I glass tubing are not normally subjected to internal surface treatment.]

### DISTINCTION BETWEEN TYPE I AND TYPE II GLASS CONTAINERS

The results obtained from the *Surface Etching Test* are compared to those obtained from the *Surface Glass Test*. For Type I glass containers, the values obtained are close to those found in the *Surface Glass Test*. For Type II glass containers, the values obtained greatly exceed those found in the *Surface Glass Test*; and they are similar to, but not greater than, those obtained for Type III glass containers of the same filling volume.

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# IMPURITIES

## Arsenic ⟨211⟩

Use as the *Test Preparation* 35 mL of the water from one Type I or Type II glass container, or, in the case of smaller containers, 35 mL of the combined contents of several Type I or Type II glass containers, prepared as directed in the *Surface Glass Test*. The limit does not exceed 0.1 µg/g.

# FUNCTIONALITY

## Spectral Transmission for Colored Glass Containers

### APPARATUS

A UV-Vis spectrophotometer, equipped with either a photodiode detector or a photomultiplier tube coupled with an integrating sphere

### SAMPLE PREPARATION

Break the glass container or cut it with a circular saw fitted with a wet abrasive wheel, such as a carborundum or a bonded diamond wheel. Select sections representative of the wall thickness, and trim them as suitable for mounting in a spectrophotometer. After cutting, wash and dry each specimen, taking care to avoid scratching the surfaces. If the specimen is too small to cover the opening in the specimen holder, mask the uncovered portion of the opening with opaque paper or tape, provided that the length of the specimen is greater than that of the slit. Before placing in the holder, wash, dry, and wipe the specimen with lens tissue. Mount the specimen with the aid of wax, or by other convenient means, taking care to avoid leaving fingerprints or other marks.

### METHOD

Place the specimen in the spectrophotometer with its cylindrical axis parallel to the slit and in such a way that the light beam is perpendicular to the surface of the section and the losses due to reflection are at a minimum. Measure the transmission of the specimen with reference to air in the spectral region of 290–450 nm, continuously or at intervals of 20 nm.

### LIMITS

The observed spectral transmission for colored glass containers for products for nonparenteral use does not exceed 10% at any wavelength in the range of 290–450 nm, irrespective of the type and capacity of the glass container. The observed spectral transmission in colored glass containers for parenteral products does not exceed the limits given in *Table 7*.

**Table 7. Limits of Spectral Transmission for Colored Glass Containers for Parenteral Products**

| Nominal Volume (mL) | Maximum Percentage of Spectral Transmission at Any Wavelength between 290 and 450 nm | |
|---|---|---|
| | Flame-Sealed Containers | Containers with Closures |
| NMT 1 | 50 | 25 |
| 1–2 | 45 | 20 |
| 2–5 | 40 | 15 |
| 5–10 | 35 | 13 |
| 10–20 | 30 | 12 |
| NLT 20 | 25 | 10 |

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# ⟨661⟩ PLASTIC PACKAGING SYSTEMS AND THEIR MATERIALS OF CONSTRUCTION

**(If the option of early adoption of** *Plastic Materials of Construction* ⟨661.1⟩ **and** *Plastic Packaging Systems for Pharmaceutical Use* ⟨661.2⟩ **is not used prior to December 1, 2025, the requirements under this chapter will apply.)**

## INTRODUCTION

The purpose of this chapter is to provide standards for plastic articles (materials, components, and systems) used to package medical articles (pharmaceuticals, biologics, and dietary supplements). Definitions that apply to this chapter are provided in *Packaging and Storage Requirements* ⟨659⟩. Standards and tests for the functional properties of containers and their components are provided in *Containers—Performance Testing* ⟨671⟩.

Plastics are composed of a mixture of homologous polymers, having a range of molecular weights. Plastics may contain other substances such as residues from the polymerization process and additives such as plasticizers, stabilizers, antioxidants, pigments, and lubricants.

Plastic articles are identified and characterized by infrared (IR) spectroscopy and differential scanning calorimetry (DSC). Standards are provided in this chapter for the identification and characterization of the different types of plastic, and the test procedures are provided at the end of the chapter. Furthermore, plastics are characterized to establish that they are suited for their intended use. Plastics and their additives, regardless of their risk and application, must meet the requirements for food contact as provided in Title 21 of the *Code of Federal Regulations* (CFR). Ingredients added to these items, must conform to the requirements in the applicable sections of 21 CFR, Indirect Food Additives, or have been evaluated by the FDA and determined to be acceptable substances for the intended use.

Physicochemical tests are performed on plastics used in high risk applications (inhalation, parenteral preparation, and ophthalmics) to characterize the extracted components and identify possible migrants. To this end, resin-specific extraction tests are provided in this chapter for polyethylene, polypropylene, polyethylene terephthalate, and polyethylene terephthalate G used in such high risk applications. Test all other plastics as directed for *Physicochemical Tests*. Conduct the *Buffering Capacity* test only when the containers are intended to hold a liquid product. *Physicochemical Tests* are not required for articles used in low risk applications (those intended for oral or topical administration). The degree or extent of biological testing is also dependent on the intended use of the plastic article and the degree of risk that extracted substances could adversely impact the efficacy of the compendial article (drug, biologic, dietary supplement, or device).

Plastic articles used for high risk products, such as those intended for inhalation, parenteral preparation, and ophthalmics, are tested using *Biological Tests*. Plastic articles used for low risk products, such as those intended for oral or topical administration, do not require *Biological Tests*. Standards are also provided for polyethylene containers used to package dry oral dosage forms that are not meant for constitution into solutions.

## POLYETHYLENE CONTAINERS

### Scope

The standards and tests provided in this section characterize containers and components, produced from either low-density polyethylene or high-density polyethylene of either homopolymer or copolymer resins that are interchangeably suitable for packaging dry oral dosage forms not meant for constitution into solution. All polyethylene components are subject to testing by IR spectroscopy and DSC. Where stability studies have been performed to establish the expiration date of a particular dosage form in the appropriate polyethylene container, then any other polyethylene container meeting these requirements may be similarly used to package such a dosage form, provided that the appropriate stability programs are expanded to include the alternative container, in order to ensure that the identity, strength, quality, and purity of the dosage form are maintained throughout the expiration period.

### Background

High-density and low-density polyethylene are long-chain polymers synthesized under controlled conditions of heat and pressure, with the aid of catalysts from NLT 85.0% ethylene and NLT 95.0% total olefins. Other olefin ingredients that are most frequently used are butene, hexene, and propylene. High-density polyethylene and low-density polyethylene both have an IR absorption spectrum that is distinctive for polyethylene, and each possesses characteristic thermal properties. High-density polyethylene has a density between 0.941 and 0.965 g/cm$^3$. Low-density polyethylene has a density between 0.850 and 0.940 g/cm$^3$. Other properties that may affect the suitability of polyethylene include modulus of elasticity, melt index, environmental stress crack resistance, and degree of crystallinity after molding.

### High-Density Polyethylene

#### INFRARED SPECTROSCOPY

Proceed as directed for *Multiple Internal Reflectance*. The corrected spectrum of the specimen exhibits major absorption bands only at the same wavelengths as the spectrum of USP High-Density Polyethylene RS.

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DIFFERENTIAL SCANNING CALORIMETRY

Proceed as directed for *Thermal Analysis*. The thermal analysis curve of the specimen is similar to the thermal analysis curve of USP High-Density Polyethylene RS, and the melting peak temperature obtained from the thermal analysis curve of the specimen does not differ from that of the Reference Standard by more than 6.0°.

HEAVY METALS AND NONVOLATILE RESIDUE

Prepare extracts of specimens for these tests as directed for *Physicochemical Tests*, except that for each 20.0 mL of *Extracting medium* under the *Physicochemical Tests*, the portion shall be 60 $cm^2$, regardless of thickness.
**Heavy metals:** Containers meet the requirements for *Physicochemical Tests, Heavy Metals*.
**Nonvolatile residue:** Proceed as directed for *Physicochemical Tests, Nonvolatile Residue*, except that the *Blank* shall be the same solvent used in each of the following test conditions: the difference between the amounts obtained from the *Sample preparation* and the *Blank* is NMT 12.0 mg when water maintained at a temperature of 70° is used as the *Extracting medium*; is NMT 75.0 mg when alcohol maintained at a temperature of 70° is used as the *Extracting medium*; and is NMT 100.0 mg when hexanes maintained at a temperature of 50° is used as the *Extracting medium*.

COMPONENTS USED IN CONTACT WITH ORAL LIQUIDS

Proceed as directed for *Physicochemical Tests, Buffering Capacity*.

### Low-Density Polyethylene

INFRARED SPECTROSCOPY

Proceed as directed for *Multiple Internal Reflectance*. The corrected spectrum of the specimen exhibits major absorption bands only at the same wavelengths as the spectrum of USP Low-Density Polyethylene RS.

DIFFERENTIAL SCANNING CALORIMETRY

Proceed as directed for *Thermal Analysis*. The thermal analysis curve of the specimen is similar to the thermal analysis curve of USP Low-Density Polyethylene RS, and the melting peak temperature obtained from the thermal analysis curve of the specimen does not differ from that of the Reference Standard by more than 8.0°.

HEAVY METALS AND NONVOLATILE RESIDUE

Prepare extracts of specimens for these tests as directed for *Physicochemical Tests, Testing Parameters, Sample preparation*, except that for each 20.0 mL of *Extracting medium* the portion shall be 60 $cm^2$, regardless of thickness.
**Heavy metals:** Containers meet the requirements for *Physicochemical Tests, Heavy Metals*.
**Nonvolatile residue:** Proceed as directed for *Physicochemical Tests, Nonvolatile Residue*, except that the *Blank* shall be the same solvent used in each of the following test conditions: the difference between the amounts obtained from the *Sample preparation* and the *Blank* is NMT 12.0 mg when water maintained at a temperature of 70° is used as the *Extracting medium*; is NMT 75.0 mg when alcohol maintained at a temperature of 70° is used as the *Extracting medium*; and is NMT 350.0 mg when hexanes maintained at a temperature of 50° is used as the *Extracting medium*.

COMPONENTS USED IN CONTACT WITH ORAL LIQUIDS

Proceed as directed for *Physicochemical Tests, Buffering Capacity*.

## POLYPROPYLENE CONTAINERS

### Scope

The standards and tests provided in this section characterize polypropylene containers, produced from either homopolymers or copolymers, that are interchangeably suitable for packaging dry solid and liquid oral dosage forms. Where suitable stability studies have been performed to establish the expiration date of a particular dosage form in the appropriate polypropylene container, then any other polypropylene container meeting these requirements may be similarly used to package such a dosage form, provided that the appropriate stability programs are expanded to include the alternative container, in order to ensure that the identity, strength, quality, and purity of the dosage form are maintained throughout the expiration period.

### Background

Propylene polymers are long-chain polymers synthesized from propylene or propylene and other olefins under controlled conditions of heat and pressure, with the aid of catalysts. Examples of other olefins most commonly used include ethylene and butene. The propylene polymers, the ingredients used to manufacture the propylene polymers, and the ingredients used in the fabrication of the containers conform to the applicable sections of 21 CFR.

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Factors such as plastic composition, processing and cleaning procedures, contacting media, inks, adhesives, absorption, adsorption and permeability of preservatives, and conditions of storage may also affect the suitability of a plastic for a specific use. The suitability of a specific polypropylene must be established by appropriate testing.

Polypropylene has a distinctive IR spectrum and possesses characteristic thermal properties. It has a density between 0.880 and 0.913 g/cm$^3$. The permeation properties of molded polypropylene containers may be altered when reground polymer is incorporated, depending on the proportion of reground material in the final product. Other properties that may affect the suitability of polypropylene used in containers for packaging drugs are the following: oxygen and moisture permeability, modulus of elasticity, melt flow index, environmental stress crack resistance, and degree of crystallinity after molding. The requirements in this section are to be met when dry solid and liquid oral dosage forms are to be packaged in a container defined by this section.

### Infrared Spectroscopy

Proceed as directed for *Multiple Internal Reflectance*. The corrected spectrum of the specimen exhibits major absorption bands only at the same wavelengths as the spectrum of the respective USP Homopolymer Polypropylene RS or copolymer polypropylene standard, similarly determined.

### Differential Scanning Calorimetry

Proceed as directed for *Thermal Analysis*. The melting peak temperature in the thermal analysis curve does not differ from that of USP Homopolymer Polypropylene RS by more than 12.0°.

### Heavy Metals and Nonvolatile Residue

Prepare extracts of specimens for these tests as directed for *Physicochemical Tests, Testing Parameters, Sample preparation*, except that for each 20 mL of *Extracting medium* the portion shall be 60 cm$^2$, regardless of thickness.

HEAVY METALS

Containers meet the requirements for *Physicochemical Tests, Heavy Metals*.

NONVOLATILE RESIDUE

Proceed as directed for *Physicochemical Tests, Nonvolatile Residue*, except that the *Blank* shall be the same solvent used in each of the following test conditions: the difference between the amounts obtained from the *Sample preparation* and the *Blank* is NMT 10.0 mg when water maintained at a temperature of 70° is used as the *Extracting medium*; is NMT 60.0 mg when alcohol maintained at a temperature of 70° is used as the *Extracting medium*; and is NMT 225.0 mg when hexanes maintained at a temperature of 50° is used as the *Extracting medium*. Containers meet these requirements for *Nonvolatile Residue* for all of the above extracting media.

[NOTE—Hexanes and alcohol are flammable. When evaporating these solvents, use a current of air with the water bath; when drying the residue, use an explosion-proof oven.]

### Components Used in Contact with Oral Liquids

Proceed as directed for *Physicochemical Tests, Buffering Capacity*.

***Change to read:***

## POLYETHYLENE TEREPHTHALATE BOTTLES AND POLYETHYLENE TEREPHTHALATE G CONTAINERS

### Scope

The standards and tests provided in this section characterize polyethylene terephthalate (PET) and polyethylene terephthalate G (PETG) bottles that are interchangeably suitable for packaging liquid oral dosage forms. Where stability studies have been performed to establish the expiration date of a particular liquid oral dosage form in a bottle meeting the requirements set forth herein for either PET or PETG bottles, any other PET or PETG bottle meeting these requirements may be similarly used to package such a dosage form, provided that the appropriate stability programs are expanded to include the alternative bottle in order to ensure that the identity, strength, quality, and purity of the dosage form are maintained throughout the expiration period. The suitability of a specific PET or PETG bottle for use in the dispensing of a particular pharmaceutical liquid oral dosage form must be established by appropriate testing.

### Background

PET resins are long-chain crystalline polymers prepared by the condensation of ethylene glycol with dimethyl terephthalate or terephthalic acid. PET copolymer resins are prepared in a similar way, except that they may also contain a small amount of

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either isophthalic acid (NMT 3 mole percent) or 1,4-cyclohexanedimethanol (NMT 5 mole percent). Polymerization is conducted under controlled conditions of heat and vacuum, with the aid of catalysts and stabilizers.

PET copolymer resins have physical and spectral properties similar to PET and for practical purposes are treated as PET. The tests and specifications provided in this section to characterize PET resins and bottles apply also to PET copolymer resins and to bottles fabricated from them.

PET and PET copolymer resins generally exhibit a large degree of order in their molecular structure. As a result, they exhibit characteristic composition-dependent thermal behavior, including a glass transition temperature of about 76° and a melting temperature of about 250°. These resins have a distinctive IR absorption spectrum that allows them to be distinguished from other plastic materials (e.g., polycarbonate, polystyrene, polyethylene, and PETG resins). PET and PET copolymer resins have a density between 1.3 and 1.4 g/cm$^3$ and a minimum intrinsic viscosity of 0.7 dL/g, which corresponds to a number average molecular weight of about 23,000 Da.

PETG resins are high molecular weight polymers prepared by the condensation of ethylene glycol with dimethyl terephthalate or terephthalic acid and 15–34 mole percent of 1,4-cyclohexanedimethanol. PETG resins are clear, amorphous polymers, having a glass transition temperature of about 81° and no crystalline melting point, as determined by DSC. PETG resins have a distinctive IR absorption spectrum that allows them to be distinguished from other plastic materials, including PET. PETG resins have a density of approximately 1.27 g/cm$^3$ and a minimum intrinsic viscosity of 0.65 dL/g, which corresponds to a number average molecular weight of about 16,000 Da.

PET and PETG resins, and other ingredients used in the fabrication of these bottles, conform to the requirements in the applicable sections of 21 CFR regarding use in contact with food and alcoholic beverages. PET and PETG resins do not contain any plasticizers, processing aids, or antioxidants. Colorants, if used in the manufacture of PET and PETG bottles, do not migrate into the contained liquid.

## Infrared Spectroscopy

Proceed as directed for *Multiple Internal Reflectance*. The corrected spectrum of the specimen exhibits major absorption bands only at the same wavelengths as the spectrum of USP Polyethylene Terephthalate RS, or USP Polyethylene Terephthalate G RS, similarly determined.

## Differential Scanning Calorimetry

Proceed as directed for *Thermal Analysis*. For polyethylene terephthalate, the thermal analysis curve of the specimen is similar to the thermal analysis curve of USP Polyethylene Terephthalate RS and the melting peak temperature obtained from the thermal analysis curve of the specimen does not differ by more than 4.0°. For polyethylene terephthalate G, the thermal analysis curve of the specimen is similar to the thermal analysis curve of USP Polyethylene Terephthalate G RS. The melting peak temperature obtained from the thermal analysis curve of the specimen does not differ by more than 6.0°.

## Colorant Extraction

Select 3 test bottles. Cut a relatively flat portion from the side wall of 1 bottle, and trim it as necessary to fit the sample holder of the spectrophotometer. Obtain the visible spectrum of the side wall by scanning the portion of the visible spectrum from 350 to 700 nm. Determine, to the nearest 2 nm, the wavelength of maximum absorbance. Fill the remaining 2 test bottles, using 50% alcohol for PET bottles and 25% alcohol for PETG bottles. Fit the bottles with impervious seals, such as aluminum foil, and apply closures. Fill a glass bottle having the same capacity as that of the test bottles with the corresponding solvent, fit the bottle with an impervious seal, such as aluminum foil, and apply a closure. Incubate the test bottles and the glass bottle at 49° for 10 days. Remove the bottles, and allow them to equilibrate to room temperature. Concomitantly determine the absorbances of the test solutions in 5-cm cells at the wavelength of maximum absorbance (see *Ultraviolet-Visible Spectroscopy* ⟨857⟩), using the corresponding solvent from the glass bottle as the blank. The absorbance values so obtained are less than 0.01 for both test solutions.

## Heavy Metals, Total Terephthaloyl Moieties, and Ethylene Glycol

EXTRACTING MEDIA

▲*Purified Water*: See monograph.▲ (ERR 1-Nov-2020)
**50 percent alcohol:** Dilute 125 mL of alcohol with water to 238 mL, and mix.
**25 percent alcohol:** Dilute 125 mL of *50 percent alcohol* with water to 250 mL, and mix.
▲***n*-Heptane**▲ (ERR 1-Nov-2020)
**General procedure:** [NOTE—Use an *Extracting medium* of *50 percent alcohol* for PET bottles and *25 percent alcohol* for PETG bottles.] For each *Extracting medium*, fill a sufficient number of test bottles to 90% of their nominal capacity to obtain NLT 30 mL. Fill a corresponding number of glass bottles with *Purified Water*, a corresponding number of glass bottles with *50 percent alcohol* or *25 percent alcohol*, and a corresponding number of glass bottles with *n*-heptane for use as *Extracting media* blanks. Fit the bottles with impervious seals, such as aluminum foil, and apply closures. Incubate the test bottles and the glass bottles at 49° for 10 days. Remove the test bottles with the *Extracting media* samples and the glass bottles with the *Extracting media* blanks, and store them at room temperature. Do not transfer the *Extracting media* samples to alternative storage vessels.

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HEAVY METALS

Pipet 20 mL of the ▲*Purified Water Extracting medium*▲ (ERR 1-Nov-2020) extract of the test bottles, filtered if necessary, into one of two matched 50-mL color-comparison tubes, and retain the remaining ▲*Purified Water Extracting medium*▲ (ERR 1-Nov-2020) extract in the test bottles for use in the test for *Ethylene Glycol*. Adjust the extract with 1 N acetic acid or 6 N ammonium hydroxide to a pH between 3.0 and 4.0, using short-range pH paper as an external indicator. Dilute with water to about 35 mL, and mix.

Into the second color-comparison tube, pipet 2 mL of freshly prepared (on day of use) *Standard lead solution* (see *Physicochemical Tests, Heavy Metals*), and add 20 mL of ▲*Purified Water Extracting medium*▲ (ERR 1-Nov-2020). Adjust with 1 N acetic acid or 6 N ammonium hydroxide to a pH between 3.0 and 4.0, using short-range pH paper as an external indicator. Dilute with water to about 35 mL, and mix.

To each tube add 1.2 mL of thioacetamide–glycerin base TS and 2 mL of *pH 3.5 acetate buffer* (see *Physicochemical Tests, Heavy Metals*), dilute with water to 50 mL, and mix: any color produced within 10 min in the tube containing the ▲*Purified Water Extracting medium*▲ (ERR 1-Nov-2020) extract of the test bottles is NMT that in the tube containing the *Standard lead solution*, both tubes being viewed downward over a white surface (1 ppm in extract).

TOTAL TEREPHTHALOYL MOIETIES

Determine the absorbance of the *50 percent alcohol* or *25 percent alcohol* extract in a 1-cm cell at the wavelength of maximum absorbance at about 244 nm (see ⟨857⟩), using the corresponding *Extracting medium* as the blank: the absorbance of the extract NMT 0.150, corresponding to NMT 1 ppm of total terephthaloyl moieties.

Determine the absorbance of the *n-heptane* extract in a 1-cm cell at the wavelength of maximum absorbance at about 240 nm (see ⟨857⟩), using *n-heptane* as the blank: the absorbance of the extract is NMT 0.150, corresponding to NMT 1 ppm of total terephthaloyl moieties.

ETHYLENE GLYCOL

**Periodic acid solution:** Dissolve 125 mg of periodic acid in 10 mL of water.
**Dilute sulfuric acid:** To 50 mL of water add slowly and with constant stirring 50 mL of sulfuric acid, and allow to cool to room temperature.
**Sodium bisulfite solution:** Dissolve 0.1 g of sodium bisulfite in 10 mL of water. Use this solution within 7 days.
**Disodium chromotropate solution:** Dissolve 100 mg of disodium chromotropate in 100 mL of sulfuric acid. Protect this solution from light, and use within 7 days.
**Standard solution:** Dissolve an accurately weighed quantity of ethylene glycol in water, and dilute quantitatively, and stepwise if necessary, to obtain a solution having a known concentration of about 1 µg/mL.
**Test solution:** Use the ▲*Purified Water Extracting medium*▲ (ERR 1-Nov-2020) extract.
**Procedure:** Transfer 1.0 mL of the *Standard solution* to a 10-mL volumetric flask. Transfer 1.0 mL of the *Test solution* to a second 10-mL volumetric flask. Transfer 1.0 mL of the ▲*Purified Water Extracting medium*▲ (ERR 1-Nov-2020) to a third 10-mL volumetric flask. To each of the 3 flasks, add 100 µL of *Periodic acid solution*, swirl to mix, and allow to stand for 60 min. Add 1.0 mL of *Sodium bisulfite solution* to each flask, and mix. Add 100 µL of *Disodium chromotropate solution* to each flask, and mix.

[NOTE—All solutions should be analyzed within 1 h after addition of the *Disodium chromotropate solution*.]

Cautiously add 6 mL of sulfuric acid to each flask, mix, and allow the solutions to cool to room temperature. [**CAUTION**—Dilution of sulfuric acid produces substantial heat and can cause the solution to boil. Perform this addition carefully. Sulfur dioxide gas will be evolved. Use of a fume hood is recommended.] Dilute each solution with *Dilute sulfuric acid* to volume, and mix. Concomitantly determine the absorbances of the solutions from the *Standard solution* and the *Test solution* in 1-cm cells at the wavelength of maximum absorbance at about 575 nm (see ⟨857⟩), using ▲*Purified Water Extracting medium* as the blank▲ (ERR 1-Nov-2020): the absorbance of the solution from the *Test solution* is NMT that of the solution from the *Standard solution*, corresponding to NMT 1 ppm of ethylene glycol.

# TEST METHODS

## Multiple Internal Reflectance

APPARATUS

Use an IR spectrophotometer capable of correcting for the blank spectrum and equipped with a multiple internal reflectance accessory.

SPECIMEN PREPARATION

Cut 2 flat sections representative of the average wall thickness of the container, and trim them as necessary to obtain segments that are convenient for mounting in the multiple internal reflectance accessory. Taking care to avoid scratching the surfaces, wipe the specimens with dry paper or, if necessary, clean them with a soft cloth dampened with methanol, and permit them to dry. Securely mount the specimens ensuring adequate surface contact. Prior to mounting the specimens on the plate, they may be compressed to thin uniform films by exposing them to temperatures of about 177° under high pressures (15,000 psi or more).

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GENERAL PROCEDURE

Place the mounted specimen sections within the multiple internal reflectance accessory, and place the assembly in the specimen beam of the IR spectrophotometer. Adjust the specimen position and mirrors within the accessory to permit maximum light transmission of the unattenuated reference beam. (For a double-beam instrument, upon completing the adjustments in the accessory, attenuate the reference beam to permit full-scale deflection during the scanning of the specimen.) Determine the IR spectrum from 3500 to 600 $cm^{-1}$ for polyethylene and polypropylene and from 4000 to 400 $cm^{-1}$ for PET and PETG.

## Thermal Analysis

GENERAL PROCEDURE

Cut a section weighing about 12 mg, and place it in the test specimen pan. [NOTE—Intimate contact between the pan and the thermocouple is essential for reproducible results.] Determine thermal analysis curve under nitrogen, using the heating and cooling conditions as specified for the resin type and using equipment capable of performing the determinations as specified under *Thermal Analysis* ⟨891⟩.

FOR POLYETHYLENE

Determine the thermal analysis curve under nitrogen at temperatures between 40° and 200° at a heating rate between 2° and 10°/min followed by cooling at a rate between 2° and 10°/min to 40°.

FOR POLYPROPYLENE

Determine the thermal analysis curve under nitrogen at temperatures ranging from ambient to 30° above the melting point. Maintain the temperature for 10 min, then cool to 50° below the peak crystallization temperature at a rate of 10° to 20°/min.

FOR POLYETHYLENE TEREPHTHALATE

Heat the specimen from room temperature to 280° at a heating rate of about 20°/ min. Hold the specimen at 280° for 1 min. Quickly cool the specimen to room temperature, and reheat it to 280° at a heating rate of about 5°/min.

FOR POLYETHYLENE TEREPHTHALATE G

Heat the specimen from room temperature to 120° at a heating rate of about 20°/min. Hold the specimen at 120° for 1 min. Quickly cool the specimen to room temperature, and reheat it to 120° at a heating rate of about 10°/min.

## Biological Tests

The in vitro biological tests are performed according to the procedures set forth under *Biological Reactivity Test, In Vitro* ⟨87⟩[1] Materials that do not meet the requirements of the in vitro tests are not suitable for containers for drug products.

## Physicochemical Tests

The following tests, designed to determine physical and chemical properties of plastics and their extracts, are based on the extraction of the plastic material, and it is essential that the designated amount of the plastic be used. Also, the specified surface area must be available for extraction at the designated temperature.

TESTING PARAMETERS

**Extracting medium:** Unless otherwise directed in a specific test below, use *Purified Water* as the *Extracting medium*, maintained at a temperature of 70° during the extraction of the *Sample preparation*.
**Blank:** Use *Purified Water* where a blank is specified in the tests that follow.
**Apparatus:** Use a water bath and the *Extraction Containers* as described in *Biological Reactivity Tests, In Vivo* ⟨88⟩, *Classification of Plastics, Apparatus*. Proceed as directed in the first paragraph of *Classification of Plastics, Preparation of Apparatus*. [NOTE—The containers and equipment need not be sterile.]
**Sample preparation:** From a homogeneous plastic specimen, use a portion, for each 20.0 mL of *Extracting medium*, equivalent to 120 $cm^2$ total surface area (both sides combined), and subdivide into strips approximately 3 mm in width and as near to 5 cm in length as is practical. Transfer the subdivided sample to a glass-stoppered, 250-mL graduated cylinder of Type I glass, and add about 150 mL of *Purified Water*. Agitate for about 30 s, drain off and discard the liquid, and repeat with a second washing.
**Sample preparation extract:** Transfer the prepared *Sample preparation* to a suitable extraction flask, and add the required amount of *Extracting medium*. Extract by heating in a water bath at the temperature specified for the *Extracting medium* for

[1] Biological reactivity testing in support of plastic packaging materials, components, and systems used for final pharmaceutical product packaging/delivery systems (drugs and drug/device combination products) provides baseline information and will often not be sufficient to assess the final suitability for use expectations of regulatory authorities. Thus, it is important to work with the appropriate regulatory authority for guidance regarding a product specific application.

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24 h. Cool, but not below 20°. Pipet 20 mL of the prepared extract into a suitable container. [NOTE—Use this portion in the test for *Buffering Capacity*.] Immediately decant the remaining extract into a suitably cleansed container, and seal.

### NONVOLATILE RESIDUE

Transfer, in suitable portions, 50.0 mL of the *Sample preparation extract* to a suitable, tared crucible (preferably a fused-silica crucible that has been acid-cleaned), and evaporate the volatile matter on a steam bath. Similarly evaporate 50.0 mL of the *Blank* in a second crucible. [NOTE—If an oily residue is expected, inspect the crucible repeatedly during the evaporation and drying period, and reduce the amount of heat if the oil tends to creep along the walls of the crucible.] Dry at 105° for 1 h: the difference between the amounts obtained from the *Sample preparation extract* and the *Blank* is NMT 15 mg.

### RESIDUE ON IGNITION ⟨281⟩

[NOTE—It is not necessary to perform this test when the *Nonvolatile Residue* test result is NMT 5 mg.] Proceed with the residues obtained from the *Sample preparation extract* and from the *Blank* in the test for *Nonvolatile Residue* above, using, if necessary, additional sulfuric acid but adding the same amount of sulfuric acid to each crucible: the difference between the amounts of residue on ignition obtained from the *Sample preparation extract* and the *Blank* is NMT 5 mg.

### HEAVY METALS

**Lead nitrate stock solution:** Dissolve 159.8 mg of lead nitrate in 100 mL of water to which has been added 1 mL of nitric acid, then dilute with water to 1000 mL. Prepare and store this solution in glass containers free from soluble lead salts.
**Standard lead solution:** On the day of use, dilute 10.0 mL of *Lead nitrate stock solution* with water to 100.0 mL. Each milliliter of *Standard lead solution* contains the equivalent of 10 µg of lead. A comparison solution prepared on the basis of 100 µL of *Standard lead solution* per gram of substance being tested contains the equivalent of 1 part of lead per million parts of substance being tested.
**pH 3.5 acetate buffer:** Dissolve 25.0 g of ammonium acetate in 25 mL of water, and add 38.0 mL of 6 N hydrochloric acid. Adjust, if necessary, with 6 N ammonium hydroxide or 6 N hydrochloric acid to a pH of 3.5, dilute with water to 100 mL, and mix.

Pipet 20 mL of the *Sample preparation extract*, filtered if necessary, into one of two matched 50-mL color-comparison tubes. Adjust with 1 N acetic acid or 6 N ammonium hydroxide to a pH between 3.0 and 4.0, using short-range pH paper as an external indicator, dilute with water to about 35 mL, and mix.

Into the second color-comparison tube pipet 2 mL of *Standard lead solution*, and add 20 mL of the *Blank*. Adjust with 1 N acetic acid or 6 N ammonium hydroxide to a pH between 3.0 and 4.0, using short-range pH paper as an external indicator, dilute with water to about 35 mL, and mix. To each tube add 1.2 mL of thioacetamide–glycerin base TS and 2 mL of *pH 3.5 acetate buffer*, dilute with water to 50 mL, and mix: any brown color produced within 10 min in the tube containing the *Sample preparation extract* is NMT that in the tube containing the *Standard lead solution*, both tubes being viewed downward over a white surface (1 ppm in extract).

### BUFFERING CAPACITY

Titrate the previously collected 20-mL portion of the *Sample preparation extract* potentiometrically to a pH of 7.0, using either 0.010 N hydrochloric acid or 0.010 N sodium hydroxide, as required. Treat a 20.0-mL portion of the *Blank* similarly: if the same titrant was required for both the *Sample preparation extract* and the *Blank*, the difference between the two volumes is NMT 10.0 mL; and if acid was required for either the *Sample preparation extract* or the *Blank* and alkali for the other, the total of the two volumes required is 10.0 mL.

**(The text above is official until November 30, 2025. The text beginning below becomes official on December 1, 2025.)**

***Change to read:***

## ▲INTRODUCTION

Packaging systems used for drug products (pharmaceuticals, biologics) are defined in *Packaging and Storage Requirements* ⟨659⟩ and such systems may be constructed from plastic materials and components. Plastics are made of polymers, which can have a range of molecular weights and may contain other substances such as residues from the polymerization process, plasticizers, stabilizers, antioxidants, pigments, and lubricants. The nature and amount of additives in the plastics used for packaging systems are dictated by the type of polymer, the polymer's use, and the process used to convert the polymer into components, containers, or packaging systems.

Drug products will be in direct contact with the primary packaging system and may result in an interaction between the product and the packaging system. Such an interaction should not adversely affect the dosage form, and this will be facilitated by the use of well-characterized plastic materials of construction in components and packaging systems and by the appropriate testing of packaging systems.

## SCOPE

Establishing the suitability of plastic packaging systems for drug products involves multiple tests and testing procedures, as briefly outlined below:

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- Material screening: Characterization of a packaging system's materials of construction to evaluate ingredients as probable extractables and potential leachables. Such a characterization facilitates the identification of materials that are suitable for use in packaging systems.
- Controlled extraction (simulation) study: Worst-case controlled extraction (simulation) study to determine the extent to which extractables may become probable leachables (for additional information, see *Assessment of Extractables Associated with Pharmaceutical Packaging/Delivery Systems* ⟨1663⟩).
- Product assessment: Actual-case measurement of confirmed leachables in the therapeutic product in the pharmaceutical packaging/delivery system intended for the commercial market (for additional information, see *Assessment of Drug Product Leachables Associated with Pharmaceutical Packaging/Delivery Systems* ⟨1664⟩).

Additionally, information provided by the vendor(s) of plastic packaging systems and their associated materials or components of construction can facilitate suitability for use assessments, as such information may be appropriate additions to or surrogates for the results obtained by performing the tests noted previously.

Testing of these plastic materials of construction to establish that they are well characterized and suitable for use in packaging components or systems is addressed in *Plastic Materials of Construction* ⟨661.1⟩.

Testing of packaging systems and components to establish that they are suited for their intended uses is addressed in *Plastic Packaging Systems for Pharmaceutical Use* ⟨661.2⟩. The requirements of ⟨661⟩ are met by performing applicable tests in ⟨661.1⟩ or by meeting the requirements of ⟨661.2⟩.

For more information on the scope of, applicability of, and other topics related to the ⟨661⟩ suite of general chapters, see *Evaluation of Plastic Packaging Systems and Their Materials of Construction with Respect to Their User Safety Impact* ⟨1661⟩.▲ (Official 1-Dec-2025)

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# ⟨670⟩ AUXILIARY PACKAGING COMPONENTS

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Auxiliary packaging components are articles that are used to support or enhance container–closure systems. These articles include, but are not limited to, pharmaceutical coil, desiccants, and ▲odor adsorbents▲ (USP 1-Dec-2023) for containers. The components covered in this chapter must meet the applicable requirements provided and the additional applicable requirements provided in other specified chapters.

***Change to read:***

## PHARMACEUTICAL COIL

Pharmaceutical coil is used as a filling material in multiple-unit containers for solid oral dosage forms to prevent breakage of tablets or capsules during shipment. The filling material should be discarded once the bottle is opened.

- **Solutions**

**Iodinated zinc chloride solution:** Dissolve 20 g of zinc chloride and 6.5 g of potassium iodide in 10.5 mL of *Purified Water*. Add 0.5 g of iodine, and shake for 15 min. Filter if necessary. Protect from light.

**Zinc chloride–formic acid solution:** Dissolve 20 g of zinc chloride in 80 g of an 850-g/L solution of anhydrous formic acid.

**1% DuPont Fiber Identification Stain No. 4[1] solution:** Dissolve 3.8 g of powdered stain in 378.5 mL of deionized water.

- **Cotton Pharmaceutical Coil**

Purified cotton is the hair of the seed of cultivated varieties of *Gossypium hirsutum* Linné, or of other species of *Gossypium* (Fam. Malvaceae). It is deprived of fatty matter and bleached and does not contain more than traces of leaf residue, pericarp, seed coat, or other impurities. Cotton pharmaceutical coil is used in bottles of solid oral dosage forms to prevent breakage.

**Identification**

**A.** When examined under a microscope, each fiber is seen to consist of a single cell, up to about 4 cm long and 40 µm wide, in the form of a flattened tube with thick and rounded walls that are often twisted.

**B.** When treated with *Iodinated zinc chloride solution*, the fibers become violet.

**C.** To 0.1 g of fibers add 10 mL of *Zinc chloride–formic acid solution*, heat to 40°, and allow to stand for 2 h, shaking occasionally: the fibers do not dissolve.

**D.** Weigh about 5 g of fibers, wet with water, and squeeze out the excess. Add fibers to 100 mL of a boiling solution of a *1% DuPont Fiber Identification Stain No. 4 solution*, and gently boil for at least 1 min. Remove the fibers, rinse well in cold water, and squeeze out the excess moisture: the fibers become green.

**Acidity or alkalinity:** Immerse about 10 g of fibers in 100 mL of recently boiled and cooled *Purified Water*, and allow to macerate for 2 h. Decant 25-mL portions of the water, with the aid of a glass rod, into each of two dishes. To one portion add 3 drops of phenolphthalein TS, and to the other portion add 1 drop of methyl orange TS. Neither portion appears pink when viewed against a white background.

**Fluorescence:** Examine a layer of about 5 mm in thickness under UV light at 365 nm. It displays only a slight brownish-violet fluorescence and a few yellow particles. It shows no intense blue fluorescence, apart from that which may be shown by a few isolated fibers.

**Residual hydrogen peroxide concentration:** Place 1 g of fibers in a beaker containing 30 mL of *Purified Water*, and stir for 3 min with a stirring rod. Pour contents into another clean container (do not squeeze sample), or alternatively, remove the fibers from the solution with clean tweezers. Remove a peroxide analytical test strip[2] from its container, and immerse the test end into the sample liquid for 2 s. Shake to remove the excess liquid, immediately insert the test strip into a suitable reflectometry instrument, and record the reading in mg/kg (ppm), and calculate residual hydrogen peroxide concentration in ppm.

For an alternate method, place 20 g in a beaker, add 400 mL of *Purified Water*, stir, add 20 mL of 20% sulfuric acid, and stir the contents. Titrate with 0.100 N potassium permanganate solution to a faint pink color that remains for 30 s. Record the amount of titer, and calculate the concentration in ppm.

**▲Calculation**

$$\%\ \text{Hydrogen peroxide} = \frac{(\text{mL of Titrant})(0.0017)}{20} \times 100$$

To calculate ppm of hydrogen peroxide, multiply % hydrogen peroxide (w/w) by 10,000.▲ (USP 1-Dec-2023)
Not more than 50 ppm is found using either method.

**Loss on Drying** ⟨731⟩

**Analysis:** Dry 5 g of fibers in an oven at 105° to constant weight.

**Acceptance criteria:** NMT 8.0%

**Residue on Ignition** ⟨281⟩

**Analysis:** Place 5 g of fibers in a porcelain or platinum dish, and moisten with 2 N sulfuric acid. Gently heat the cotton until it is charred, then ignite more strongly until the carbon is completely consumed.

**Acceptance criteria:** NMT 0.20%

**Water-soluble substances:** Place 10 g of fibers in a beaker containing 1000 mL of *Purified Water*, and boil gently for 30 min, adding water as required to maintain the volume. Pour the water through a funnel into another vessel, and press out the excess water from the cotton with a glass rod. Wash the cotton in the funnel with two 250-mL portions of boiling water,

[1] DuPont Fiber Identification Stain No. 4 is available from Pylam Products Co., 2175 East Cedar Street, Tempe, AZ 85281; www.pylamdyes.com.
[2] A suitable analysis system consisting of Reflectoquant® peroxide test strips and a RQflex® reflectometry instrument may be obtained from Millipore Sigma; www.emdmillipore.com.

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pressing the cotton after each washing. Filter the combined extract and washings, and wash the filter thoroughly with hot water. Evaporate the combined extract and washings to a small volume, transfer to a tared porcelain or platinum dish, evaporate to dryness, and dry the residue at 105° to constant weight. The residue weighs NMT 0.35%.

**Fatty matter:** Pack 10 g of fibers in a Soxhlet extractor provided with a tared receiver, and extract with ethyl ether for 4 h at a rate such that the ether siphons over not less than 4 times per hour. The ethyl ether solution in the flask shows no trace of blue, green, or brownish color. Evaporate the extract to dryness, and dry at 105° for 1 h. The weight of the residue does not exceed 0.7%.

**Dyes:** Pack about 10 g of fibers in a narrow percolator ▲or a porcelain Büchner funnel,▲ (USP 1-Dec-2023) and extract slowly with alcohol until the percolate measures 50 mL. When observed downward through a column 20 cm in depth, the percolate may show a yellowish color, but not a blue or a green tint.

**Other foreign matter:** Pinches contain no oil stains or metallic particles by visual inspection.

• **RAYON PHARMACEUTICAL COIL**

Rayon pharmaceutical coil is a fibrous form of bleached, regenerated cellulose, to be used as a filler in bottles of solid oral dosage forms to prevent breakage. It consists exclusively of rayon fibers except for a few isolated foreign fibers that may be present. [NOTE—Rayon pharmaceutical coil has been found to be a potential source of dissolution problems for gelatin capsules or gelatin-coated tablets resulting from gelatin cross-linking.]

**Identification**

**A.** When treated with *Iodinated zinc chloride solution*, the fibers become violet.

**B.** Add 10 mL of *Zinc chloride–formic acid solution* to 0.1 g of fibers, heat to 40°, and allow to stand for 2 h, shaking occasionally: the fibers dissolve completely, except for mat rayon fibers where titanium particles remain.

**C.** Weigh about 5 g of fibers, wet with water, and squeeze out the excess. Add fibers to 100 mL of a boiling solution of a *1% DuPont Fiber Identification Stain No. 4 solution*, and gently boil for at least 1 min. Remove the fibers, rinse well in cold water, and squeeze out the excess moisture: the fibers become blue–green.

**Acidity or alkalinity, Fluorescence, Fatty matter, Dyes,** and **Other foreign matter:** Proceed as directed under *Cotton Pharmaceutical Coil*, except use rayon pharmaceutical coil. The sample weight for fatty matter is 5 g and weight of the residue does not exceed 0.5%.

**Loss on Drying** ⟨731⟩

**Analysis:** Dry 5 g of fibers in an oven at 105° to constant weight.

**Acceptance criteria:** NMT 11.0%

**Residue on Ignition** ⟨281⟩**:** NMT 1.50%, determined on a 5-g test specimen

**Acid-insoluble ash:** To the residue obtained in the test for *Residue on Ignition*, add 25 mL of 3 N hydrochloric acid, and boil for 5 min. Collect the insoluble matter on a tared filtering crucible, wash with hot water, ignite, and weigh: the residue weighs not more than 1.25%.

**Water-soluble substances:** Proceed as directed under *Cotton Pharmaceutical Coil*, except to use rayon pharmaceutical coil. The residue weighs not more than 1.0%.

• **POLYESTER PHARMACEUTICAL COIL**

Polyester pharmaceutical coil is a white odorless material, to be used as a filler in bottles of solid oral dosage forms to prevent breakage.

**Identification**

**A.** Proceed as directed under *Infrared spectroscopy* in the *Test Methods* section. Determine the IR spectrum from 4000 to 650 $cm^{-1}$ (2.5 to 15 µm). The spectrum obtained from the specimen exhibits major absorption bands only at the same wavelengths as the spectrum of USP Polyethylene Terephthalate RS.

**B.** Weigh about 5 g of fibers, wet with water, and squeeze out excess. Add fibers to 100 mL of a boiling solution of a *1% DuPont Fiber Identification Stain No. 4 solution*, and gently boil for at least 1 min. Remove the fibers, rinse well in cold water, and squeeze out the excess moisture: the fibers become pale orange.

**Acidity or alkalinity:** Proceed as directed under *Cotton Pharmaceutical Coil*, except to use polyester pharmaceutical coil.

**Loss on Drying** ⟨731⟩

**Analysis:** Dry 5 g of fibers in an oven at 105° to constant weight.

**Acceptance criteria:** NMT 1.0%

**Residue on Ignition** ⟨281⟩**:** NMT 0.5%, determined on a 5-g test specimen

**Finish on fibers:** The finish on fibers used for processing should comply with FDA food contact regulations.

• **TEST METHODS**

**Infrared spectroscopy**[3]

**Apparatus:** FTIR or a double-beam spectrophotometer capable of scanning from 4000 to 650 $cm^{-1}$ (2.5–15 µm).

**Specimen preparation:** The ATR *Spectroscopic Identification Tests* ⟨197⟩, *Infrared Spectroscopy* 197A technique can be used as alternative methods where the Reference Standard spectra is similarly obtained.

**Method 1** (potassium bromide disc)**:** Use scissors to cut polyester fibers (1–3 mg) into short lengths (less than 1 mm long), mix with 200 mg of powdered potassium bromide, and grind in a ball mill for 1–2 min. Transfer to potassium bromide-disc die, and form a disc.

**Method 2** (melt film)**:** Produce film by pressing polyester fibers between tetrafluoroethylene-fluorocarbon sheets and place between heated plates.

***Change to read:***

**DESICCANT▲** ▲ (USP 1-Dec-2023)

Desiccants are used to remove moisture from air in containers in order to protect drug products, particularly solid oral dosage forms. They are supplied in a number of different packaging materials including cotton, Kraft paper, rayon and polyester

[3] Additional information on fiber identification methods may be found in "Standard Test Methods for Identification of Fibers in Textiles". Current version of ASTM Method D276, published by ASTM International, 100 Barr Harbor Drive, P.O. Box C700, West Conshohocken, PA 19428-2959; www.astm.org.

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cloth bags, perforated plastic, polymer films, or polymer housings▲▲ (USP 1-Dec-2023). The most common types of commercial desiccants are bentonite; calcium chloride; calcium oxide; ▲carbon, activated;▲ (USP 1-Dec-2023) molecular sieves; and silica gel. Other desiccants are subject to appropriate testing to ensure suitability for the intended application.

Where desiccants are incorporated directly into the wall or cap of packaging containers or bound by a carrier material, use unincorporated desiccant in the test methods. For desiccants that are loaded with a predetermined moisture content, perform the testing before the moisture has been loaded or after the desiccant has been regenerated.

- **BENTONITE**

Bentonite clay (also referred to as montmorillonite clay) is a native, colloidal, hydrated aluminum silicate.

**Appearance:** Grayish-white powder or pellets with a yellowish or pinkish tint

**Identification—X-Ray Powder Diffraction** ⟨941⟩**:** For *Sample preparation A*, the largest peak corresponds to a *d* value between 15.0 and 17.2 Å. The major peak in the region between 1.48 and 1.54 Å from the pattern of *Sample preparation B* is between 1.492 and 1.504 Å.

**Identification—precipitation:** Formation of a gelatinous white precipitate

**Inorganic impurities**

**Arsenic:** NMT 10 mg/kg

**Lead:** NMT 15 mg/kg

**Specific tests**

**pH** ⟨791⟩**:** 4.5–10.5. Disperse 4.0 g in 200 mL of water, mix vigorously to facilitate wetting.

**Loss on Drying** ⟨731⟩

**Sample:** 5–10-g sample

**Analysis:** Dry the *Sample* at 110° to a constant weight.

**Acceptance criteria:** It loses NMT 3.0%.

[NOTE—Conduct the assay immediately after opening the original container.]

**Moisture adsorption capacity**

NLT 13% at 40% ± 5% relative humidity (RH) and 25 ± 2°

NLT 23% at 80% ± 5% RH and 25 ± 2°

**Test methods**

**Identification—X-ray diffraction**

**Sample preparation A:** Add 2 g in small portions to 100 mL of water with intense agitation. Allow to stand for 12 h to ensure complete hydration. Place 2 mL of the mixture so obtained on a suitable glass slide, and allow to air-dry at room temperature to produce an oriented film. Place the slide in a vacuum desiccator over a free surface of ethylene glycol. Evacuate the desiccator, and close the stopcock so that ethylene glycol saturates the desiccator chamber. Allow the slide to stand for 12 h.

**Sample preparation B:** Prepare a random powder specimen of the sample.

**Analysis:** *Sample preparation A* and *Sample preparation B*. Record the X-ray diffraction pattern of the samples, and determine the *d* values.

**Identification—precipitation**

**Sample:** 5 g

**Analysis:** Add 1 g of potassium nitrate and 3 g of anhydrous sodium carbonate to the *Sample* contained in a metal crucible, heat until the mixture has melted, and allow it to cool. Add 20 mL of boiling water to the residue, mix, filter, and wash the residue with 50 mL of water. Add 1 mL of hydrochloric acid and 5 mL of water to the residue, and filter. Add 1 mL of 10 N sodium hydroxide to the filtrate, filter, and add 3 mL of 2 M ammonium chloride.

**Arsenic**

**Sample solution:** Transfer 8.0 g of dried sample into a 250-mL beaker containing 100 mL of dilute hydrochloric acid (1 in 25), mix, and cover with a watch glass. Boil gently, with occasional stirring, for 15 min without allowing excessive foaming. Pass the hot supernatant liquid through a rapid-flow filter paper into a 200-mL volumetric flask, and wash the filter with four 25-mL portions of hot dilute hydrochloric acid (1 in 25), collecting the washings in the volumetric flask. Cool the combined filtrates to room temperature, add dilute hydrochloric acid (1 in 25) to volume and mix.

**Arsenic trioxide stock solution:** Accurately weigh 132.0 mg of arsenic trioxide, previously dried at 105° for 1 h, and dissolve in 5 mL of sodium hydroxide solution (1 in 5) in a 1000-mL volumetric flask. Neutralize the solution with 2 N sulfuric acid, add an additional 10 mL of 2 N sulfuric acid, and bring to volume with recently boiled and cooled water and mix.

**Standard arsenic solution:** Dilute the *Arsenic trioxide stock solution* to obtain solutions of suitable concentrations, adaptable to the linear or working range of the instrument. Keep in an all-glass container, and use within 3 days.

**Analysis:** Proceed according to *Elemental Impurities—Procedures* ⟨233⟩.

**Lead**

**Sample solution:** Transfer 3.75 g of sample into a 250-mL beaker containing 100 mL of dilute hydrochloric acid (1 in 25), stir, and cover with a watch glass. Boil for 15 min, then cool to room temperature, and pass through a rapid-flow filter paper into a 400-mL beaker. Wash the filter with four 25-mL portions of hot water, collecting the washings in the 400-mL beaker. Concentrate the combined extracts by gentle boiling to approximately 20 mL. If a precipitate forms, add 2–3 drops of nitric acid, heat to boiling, and cool to room temperature. Pass the concentrated extracts through a rapid-flow filter paper into a 50-mL volumetric flask. Transfer the remaining contents of the 400-mL beaker through the filter paper and into the flask with water. Dilute with water to volume.

**Lead nitrate stock solution:** Dissolve 159.8 mg of lead nitrate in 100 mL of water to which has been added 1 mL of nitric acid, then dilute with water to 1000 mL. Prepare and store this solution in glass containers free from soluble lead salts.

**Standard lead solution:** On the day of use, dilute the *Lead nitrate stock solution* to obtain solutions of suitable concentrations, adaptable to the linear or working range of the instrument.

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**Analysis:** Proceed according to ⟨233⟩.

**Moisture adsorption capacity**

**Equipment:** Temperature-humidity chambers capable of controlled humidity at 40% ± 5% RH and 80% ± 5% RH at 25 ± 2° or a desiccator containing water-saturated salts that provide %RH at the required level plus an oven capable of maintaining 25 ± 2°.

**Method:** 5–10 g. Remove the sample from the packaging material. Where absorbents are incorporated directly into the wall or cap of packaging containers, use unincorporated desiccant. Add the sample to the humidity chamber or desiccator and measure the weight gain over time until this reaches equilibrium when two successive consecutive weighings do not differ by more than 3 mg/g of substance taken, the second weighing following an additional 3 ± 1 h of storage at the required temperature and humidity conditions. Calculate the adsorption capacity as percentage weight gained over the initial sample weight. Where two absorbents are packaged in combination, the moisture adsorption capacity specification must be calculated in proportion of the mix. For example, for a mixture of 60% molecular sieve and 40% silica gel, the moisture adsorption capacity would be not less than 16.6% (9.0% + 7.6%) when the test condition is 40% ± 5% RH and 25 ± 2° and the moisture adsorption capacities taken at their minimum specification.

**Calculation**

**Molecular sieve:** 60% by weight × 15% moisture adsorption capacity = NLT 9.0% moisture adsorption capacity

**Silica gel:** 40% by weight × 19% moisture adsorption capacity = NLT 7.6% moisture adsorption capacity

- **CALCIUM CHLORIDE, ANHYDROUS**

**Identification—calcium:** Passes tests

**Identification—chloride:** Passes tests ▲(USP 1-Dec-2023)

**Assay:** NLT 93.0% and NMT 100.5% of calcium chloride ($CaCl_2$)

**Inorganic impurities**

**Arsenic:** NMT 3 ppm

**Fluoride:** NMT 0.004%

**Lead:** NMT 5 ppm

**Magnesium and alkali salts:** NMT 25 mg of residue (NMT 5.0%)

**Specific tests**

**pH** ⟨791⟩**:** 4.5–11.0 (1:20 aqueous solution)

**Moisture adsorption capacity:** NLT 28% at 80% ± 5% RH and 25 ± 2°

**Test methods**

**Identification—calcium**

**Sample solution—100 mg/mL:** Insoluble oxalate salts are formed when solutions of calcium salts are treated in the following manner. Using 2 drops of methyl red TS as the indicator, neutralize a 1:20 solution of a calcium salt with 6 N ammonia, then add 2.7 N hydrochloric acid, dropwise, until the solution is acid. A white precipitate of calcium oxalate forms upon the addition of ammonium oxalate TS. This precipitate is insoluble in acetic acid but dissolves in hydrochloric acid.

**Identification—chloride**

**Sample solution—100 mg/mL:** Solutions of chlorides yield with silver nitrate TS a white, curdy precipitate that is insoluble in nitric acid but soluble in a slight excess of 6 N ammonia.

**Assay**

**Sample:** 1.5 g

**Analysis:** Transfer the *Sample* into a 250-mL volumetric flask, dissolve it in a mixture of 100 mL of water and 5 mL of 2.7 N hydrochloric acid, dilute with water to volume, and mix. Transfer 50 mL of this solution into a suitable container and add 50 mL of water. While stirring, preferably with a magnetic stirrer, add about 30 mL of 0.05 M disodium ethylenediaminetetraacetic acid (EDTA) from a 50-mL buret. Then, add 15 mL of 1 N sodium hydroxide and 300 mg of hydroxy naphthol blue indicator. Continue the titration to a blue endpoint. Each milliliter of 0.05 M disodium EDTA is equivalent to 5.55 mg of calcium chloride ($CaCl_2$).

**Arsenic**

**Sample solution:** 1 g in 10 mL

**Arsenic trioxide stock solution** and **Standard arsenic solution:** Prepare as directed under *Bentonite*.

**Analysis:** Proceed as directed under *Bentonite*.

**Fluoride**

**Sodium fluoride solution—5 µg/mL:** Transfer 2.210 g of sodium fluoride, previously dried at 200° for 4 h and accurately weighed, into a 400-mL plastic beaker, add 200 mL of water, and stir until dissolved. Quantitatively transfer this solution into a 1000-mL volumetric flask with the aid of water, dilute with water to volume, and mix. Store this stock solution in a plastic bottle. On the day of use, transfer 5.0 mL of the stock solution into a 1000-mL volumetric flask, dilute with water to volume, and mix.

**Calibration curve:** Transfer 1.0, 2.0, 3.0, 5.0, 10.0, and 15.0 mL of the *Sodium fluoride solution* into separate 250-mL plastic beakers. Add 50 mL of water, 5 mL of 1 N hydrochloric acid, 10 mL of 1 M sodium citrate, and 10 mL of 0.2 M disodium EDTA to each beaker and mix. Transfer each solution into separate 100-mL volumetric flasks, dilute with water to volume, and mix. Transfer a 50-mL portion of each solution into separate 125-mL plastic beakers, and measure the potential of each solution with a suitable ion-selective electrode apparatus,[4] using a suitable reference electrode.[5] Plot the calibration curve on two-cycle semi-logarithmic paper[6] or with the use of a suitable graphing calculator or spreadsheet program. ▲▲(USP 1-Dec-2023)

[4] A suitable apparatus such as the Orion Model No. 94–09, with solid-state membrane may be used.
[5] A suitable electrode such as the Orion Model No. 90–01, with single junction may be used.
[6] A suitable semi-logarithmic paper such as K & E No. 465130 may be used.

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**Analysis:** Transfer 1.00 g of the sample into a 150-mL glass beaker, add 10 mL of water, and, while stirring continuously, slowly add 20 mL of 1 N hydrochloric acid to dissolve the sample. Boil rapidly for 1 min, then transfer into a 250-mL plastic beaker, and cool rapidly in ice water. Add 15 mL of 1 M sodium citrate and 10 mL of 0.2 M disodium EDTA, and mix. Adjust the pH to 5.5 ± 0.1 with 1 N hydrochloric acid or 1 N sodium hydroxide, if necessary. Transfer into a 100-mL volumetric flask, dilute with water to volume, and mix. Transfer a 50-mL portion of this solution into a 125-mL plastic beaker, and measure the potential of the solution with the apparatus described under *Calibration curve*. Determine the fluoride content, in µg, of the sample from the *Calibration curve*. Determine the percentage of fluoride in the sample taken:

$$\text{Result} = (C/W_S) \times F \times 100$$

$C$ = content of fluoride (µg)
$W_S$ = sample weight (g)
$F$ = factor converting µg to g, 0.000001

**Lead**
**Sample solution:** 1 g in 20 mL
**Lead nitrate stock solution** and **Standard lead solution:** Prepare as directed under *Bentonite*.
**Analysis:** Proceed as directed under *Bentonite*.
**Magnesium and alkali salts**
**Sample:** 1 g
**Analysis:** Dissolve the *Sample* in 50 mL of water, add 500 mg of ammonium chloride, mix, and boil for 1 min. Rapidly add 40 mL of oxalic acid TS and stir vigorously until precipitation is well established. Immediately add 2 drops of methyl red TS. Then add 6 N ammonium hydroxide, dropwise, until the mixture is just alkaline, and cool. Transfer the mixture to a 100-mL cylinder, dilute with water to 100 mL, and let it stand for 4 h or overnight. Decant the clear, supernatant liquid through a dry filter paper, and transfer 50 mL of the clear filtrate to a platinum dish. Add 0.5 mL of sulfuric acid to the dish and evaporate the mixture on a steam bath to a small volume. Carefully evaporate the remaining liquid to dryness over a free flame and continue heating until the ammonium salts have been completely decomposed and volatilized. Finally, ignite the residue to constant weight.
**Moisture adsorption capacity:** Proceed as directed under *Bentonite*.

• **CALCIUM OXIDE**
**Identification—calcium:** Passes tests
**Assay:** NLT 95.0% and NMT 100.5% of calcium oxide (CaO), on the ignited basis
**Inorganic impurities**
**Acid-insoluble substances:** NMT 1%
**Arsenic:** NMT 3 ppm
**Fluoride:** NMT 0.015%
**Lead:** NMT 2 mg/kg
**Magnesium and alkali salts:** NMT 3.6%
**Specific tests**
**Loss on ignition:** NMT 10.0%
**Moisture adsorption capacity:** NLT 28% at 80% ± 5% RH and 25 ± 2°
**Test methods**
**Identification—calcium**
**Sample solution:** Shake 1 g of sample with 20 mL of water and add glacial acetic acid until the sample is dissolved.
**Analysis:** Proceed as directed under *Calcium Chloride, Anhydrous*.
**Assay**
**Sample:** 1 g of sample ignited to a constant weight (see *Loss on ignition* below)
**Analysis:** Dissolve the *Sample* in 20 mL of 2.7 N hydrochloric acid. Cool the solution, dilute with water to 500.0 mL, and mix. Pipet 50.0 mL of this solution into a suitable container, and add 50 mL of water. While stirring, preferably with a magnetic stirrer, add about 30 mL of 0.05 M disodium EDTA from a 50-mL buret. Then, add 15 mL of 1 N sodium hydroxide and 300 mg of hydroxy naphthol blue indicator. Continue the titration with disodium EDTA to a blue endpoint. Each milliliter of 0.05 M disodium EDTA is equivalent to 2.804 mg of calcium oxide (CaO).
**Arsenic**
**Sample solution:** 1 g in 15 mL of 2.7 N hydrochloric acid
**Arsenic trioxide stock solution** and **Standard arsenic solution:** Prepare as directed under *Bentonite*.
**Analysis:** Proceed as directed under *Bentonite*.
**Fluoride**
**Sample:** 1.0 g
**Analysis:** Proceed as directed under *Calcium Chloride, Anhydrous*.
**Lead**
**Sample solution:** 1 g in 15 mL of 2.7 N hydrochloric acid
**Lead nitrate stock solution** and **Standard lead solution:** Prepare as directed under *Bentonite*.
**Analysis:** Proceed as directed under *Bentonite*.
**Acid-insoluble substances**
**Sample solution:** Shake 5 g of sample, and then mix it with 100 mL of water and sufficient hydrochloric acid, added dropwise, to dissolve it.

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**Analysis:** Boil the *Sample solution*, cool, add hydrochloric acid, if necessary, to make the solution distinctly acid, and pass through a tared glass filter crucible. Wash the residue with water until free of chlorides, dry at 105° for 1 h, cool, and weigh.

**Magnesium and alkali salts**

**Sample:** 500 mg

**Analysis:** Dissolve the *Sample* in 30 mL of water and 15 mL of 2.7 N hydrochloric acid. Heat the solution, boil for 1 min, and rapidly add 40 mL of oxalic acid TS, and stir vigorously. Add 2 drops of methyl red TS, and neutralize the solution with 6 N ammonium hydroxide to precipitate the calcium completely. Heat the mixture on a steam bath for 1 h and allow it to cool. Dilute the mixture with water to 100 mL, mix well, and filter. Add 0.5 mL of sulfuric acid to 50 mL of the filtrate. Then evaporate to dryness and ignite to constant weight in a tared platinum crucible at 800 ± 25°.

**Loss on ignition**

**Sample:** 1 g

**Analysis:** Ignite the *Sample* to constant weight in a tared platinum crucible at 1100 ± 50°.

**Moisture adsorption capacity:** Proceed as directed under *Bentonite*.

▲• **CARBON, ACTIVATED**

Carbon, activated (also known as "active carbon") is a solid, porous material obtained from carbonaceous source materials (vegetable) matter by suitable carbonization processes, treated to increase its adsorptive power.

**Appearance:** Black-colored powder or granule, or a mixture of powder and granule

**Solubility:** Insoluble in water and in organic solvents

**Identification:** When heated to redness in atmosphere, it burns slowly without a flame.

**Inorganic impurities**

**Arsenic:** NMT 10 mg/kg (ppm)

**Lead:** NMT 15 mg/kg (ppm)

**Specific tests**

**Loss on Drying** ⟨731⟩

**Sample:** 5–10 g

**Analysis:** Dry the *Sample* at 105°–120° to a constant weight.

**Acceptance criteria:** It loses NMT 5% of its weight. [NOTE—Conduct assay immediately after opening the original container.]

**Residue on Ignition** ⟨281⟩

**Sample:** 1.0 g

**Acceptance criteria:** NMT 5.0%

**Moisture adsorption capacity:** NLT 21% weight at 80% ± 5% relative humidity (RH) and 25 ± 2°

**Higher aromatic hydrocarbons:** The sample shows no more color or fluorescence than the *Control solution* when observed under ultraviolet light at 365 nm.

**Water extractables:** NMT 5.0%

**Test methods**

**Inorganic impurities**

**Sample:** 2.0 g

**Analysis:** Combine the *Sample* and 50 mL of dilute hydrochloric acid in a conical flask with a ground glass neck. Boil gently under a reflux condenser for 1 h and wash the filter with dilute hydrochloric acid. Evaporate the combined filtrate and washings to dryness on a water bath. Dissolve the residue in 0.1 M hydrochloric acid and dilute with 0.1 M hydrochloric acid to 50 mL.

**Arsenic trioxide stock solution** and **Standard arsenic solution:** Prepare as directed under *Bentonite*.

**Lead nitrate stock solution** and **Standard lead solution:** Prepare as directed under *Bentonite*.

**Analysis:** Proceed as directed under *Bentonite*.

**Moisture adsorption capacity:** Proceed as directed under *Bentonite*.

**Higher aromatic hydrocarbons**

**Sample:** 1.0 g

**Analysis:** In a continuous-extraction apparatus, treat the *Sample* with 12 mL of cyclohexane for 2 h. Collect the liquid and dilute with cyclohexane to 12 mL. Place the *Sample* in a Nessler tube.

**Control solution:** Prepare a solution of 83 ng/mL of quinine in 0.1 N sulfuric acid and transfer into a matching Nessler tube.

**Water extractables**

**Sample:** 5.0 g

**Analysis:** Transfer about 5 g of the *Sample*, accurately weighed, into a 250-mL flask provided with a reflux condenser and a Bunsen valve. Add 100 mL of water and several glass beads, and reflux for 1 h. Cool slightly, and filter through filter paper,[7] discarding the first 10 mL of filtrate. Cool the filtrate to room temperature, and pipet 25.0 mL into a tared dish. Evaporate the filtrate in the dish to incipient dryness on a hot plate, never allowing the solution to boil. Dry for 1 h at 100° in a vacuum oven, cool, and weigh. Calculate the percentage of water extractables in the filtrate, based on the sample weight and volume of the sample taken for gravimetric measurement.▲ (USP 1-Dec-2023)

• **MOLECULAR SIEVES**

Molecular sieves are synthetic porous crystalline alkali-metal aluminosilicates in a beaded form with a tightly controlled pore size. The most commonly used molecular sieves for desiccants are described as types 3A, 4A, 5A, and 13X with a pore size range of approximately 3–10 Å.

**Identification A:** The drop of water becomes turbid.

**Identification B:** Passes tests

[7] A suitable filter paper such as Whatman No. 2 or equivalent may be used.

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**Inorganic impurities**
**Lead:** NMT 5 ppm
**Specific tests**
**pH** ⟨791⟩: 6.5–12 (200 mg/mL in carbon dioxide-free water)
**Loss on Drying** ⟨731⟩
**Sample:** 5–10 g sample
**Analysis:** Dry the *Sample* at 575 ± 25° to a constant weight.
**Acceptance criteria:** It loses NMT 4.5%.
[NOTE—Conduct assay immediately after opening the original container.]
**Moisture adsorption capacity**
NLT 15.0% weight at 40% ± 5% RH and 25 ± 2°
NLT 16.5% weight at 80% ± 5% RH and 25 ± 2°
**Test methods**
**Identification A**
**Sample:** 500 mg
**Analysis:** Mix the *Sample* with 2.5 g of anhydrous potassium carbonate, and heat the mixture in a platinum or nickel crucible until it melts completely. Cool, add 5 mL of water, and allow to stand for 3 min. Heat the bottom of the crucible gently, detach the melt, and transfer it into a beaker with the aid of about 50 mL of water. Gradually add hydrochloric acid until no effervescence is observed, add 10 mL more of the acid, and evaporate to dryness on a steam bath. Cool, add 20 mL of water, boil, and pass through ash-free filter paper. An insoluble residue of silica remains. [NOTE—Retain the filtrate for *Identification B*.] Transfer the gelatinous residue to a platinum dish, and cautiously add 5 mL of hydrofluoric acid. [**CAUTION—**Handle hydrofluoric acid in a fume hood with appropriate precautions.] The precipitate dissolves. (If it does not dissolve, repeat the treatment with hydrofluoric acid.) Heat the solution and introduce a glass stirring rod with a drop of water on the tip into the resulting vapors.
**Identification B—aluminum**
**Sample:** Use 2 portions of the filtrate obtained in *Identification A*.
**Analysis:** The solution of a first portion of the filtrate obtained in *Identification A* yields a white, gelatinous precipitate with 6 N ammonia that is insoluble in an excess of this reagent. The solution of a second portion of the filtrate obtained in *Identification A* yields a white precipitate with a 1 N sodium hydroxide that is dissolved in an excess of this reagent.
**Lead**
**Sample solution:** Transfer 10.0 g of sample into a 250-mL beaker, add 50 mL of 0.5 N hydrochloric acid, cover with a watch glass, and heat slowly to boiling. Boil gently for 15 min, cool, and let the undissolved material settle. Decant the supernatant liquid through suitable filter paper[8] into a 100-mL volumetric flask, retaining as much as possible of the insoluble material in the beaker. Wash the slurry and beaker with three 10-mL portions of hot water, decanting each washing through the filter into the flask. Finally, wash the filter paper with 15 mL of hot water, cool the filtrate to room temperature, dilute with water to volume, and mix.
**Lead nitrate stock solution** and **Standard lead solution:** Prepare as directed under *Bentonite*.
**Analysis:** Proceed as directed under *Bentonite*.
**Moisture adsorption capacity:** Proceed as directed under *Bentonite*.

• **SILICA GEL**

Silica gel is silicon dioxide ($SiO_2 \cdot H_2O$) that has been manufactured by the addition of sodium silicate solution to a mineral acid to produce a gelatinous precipitate that is washed, then dehydrated to produce colorless silica gel in a bead, granular, or micronized form in a range of mesh sizes.
**Appearance:** A white or translucent bead or granule
**Identification**▲▲ (USP 1-Dec-2023)**:** A deep yellow color is produced.
▲▲ (USP 1-Dec-2023)
**Assay:** NLT 94.0% of silicon dioxide ($SiO_2$) on the ignited basis
**Inorganic impurities**
**Lead:** NMT 5 ppm
**Soluble ionizable salts** (as $Na_2SO_4$)**:** The conductance produced by the sample is not more than that produced by the control solution (equivalent to NMT 5.0%).
**Specific tests**
**pH** ⟨791⟩: 4–8 in a slurry (1 in 20)
**Loss on Drying** ⟨731⟩
**Sample:** 5–10 g
**Analysis:** Dry the *Sample* at 145° for 3 h.
**Acceptance criteria:** It loses NMT 3.0%.
[NOTE—Conduct assay immediately after opening the original container.]
**Moisture adsorption capacity**
NLT 19% weight at 40% ± 5% RH and 25 ± 2°
NLT 27% weight at 80% ± 5% RH and 25 ± 2°
**Test methods**
**Identification**▲▲ (USP 1-Dec-2023)
**Sample:** 5 mg

[8] A suitable filter paper such as Whatman No. 4 or equivalent may be used.

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**Analysis:** Place into a platinum crucible, mix with 200 mg of anhydrous potassium carbonate, and ignite over a burner at a red heat for about 10 min. Cool, dissolve the melt in 2 mL of freshly distilled water, warming if necessary, and slowly add 2 mL of ammonium molybdate TS.

▲▲ (USP 1-Dec-2023)

**Assay**

**Sample:** 1 g, previously dried

**Analysis:** Transfer the *Sample* into a tared platinum crucible, ignite at 1000 ± 25° to constant weight, cool in a desiccator, and weigh to obtain the ignited sample weight ($W_1$). Moisten the residue with a few drops of alcohol, add 3 drops of sulfuric acid, then add enough hydrofluoric acid to cover the wetted sample.

[**CAUTION**—Handle hydrofluoric acid in a well-ventilated fume hood with appropriate precautions.]

Evaporate to dryness on a hot plate, using medium heat (95°–105°), then add a few milliliters of hydrofluoric acid enough to cover the *Sample*, swirl the dish carefully to wash down the sides, and again evaporate to dryness taking care that the *Sample* does not spatter as dryness is approached. Heat the crucible to a red heat using a Meker burner, a propane torch, or in a muffle furnace. Ignite the residue at 1000 ± 25° for 30 min, cool in a desiccator, and weigh to obtain the final weight ($W_2$). If a residue remains, repeat the *Analysis* beginning with the addition of hydrofluoric acid until a constant weight is obtained. The difference between the ignited sample weight and the final weight ($W_1 - W_2$) represents the weight, in g, of silicon dioxide ($SiO_2$) in the initially ignited sample. Express the result as a percentage of the initially ignited basis.

**Lead**

**Sample solution:** Transfer 5.0 g of sample into a 250-mL beaker, add 50 mL of 0.5 N hydrochloric acid, cover with a watch glass, and slowly heat to boiling. Boil gently for 15 min, cool, and let the undissolved material settle. Decant the supernatant liquid through a suitable filter paper[9] into a 100-mL volumetric flask, retaining as much as possible of the insoluble material in the beaker. Wash the slurry and beaker with three 10-mL portions of hot water, decanting each washing through the filter into the flask. Finally, wash the filter paper with 15 mL of hot water, cool the filtrate to room temperature, dilute with water to volume, and mix.

**Lead nitrate stock solution** and **Standard lead solution:** Prepare as directed under *Bentonite*.

**Analysis:** Proceed as directed under *Bentonite*.

**Soluble ionizable salts** (as $Na_2SO_4$)

**Sample:** 5 g, previously dried

**Control solution:** 1 mg/mL of anhydrous sodium sulfate, made to 250 mL

**Analysis:** Stir the *Sample* with 150 mL of water for at least 5 min in a high-speed mixer ▲or homogenizer capable of >1500 rpm.▲ (USP 1-Dec-2023) Filter with the aid of suction, and wash the mixer and filter with 100 mL of water in divided portions, adding the washings to the filtrate. Dilute the filtrate with water to 250 mL. Determine the conductances of the diluted filtrate and of the *Control solution* with a suitable conductance bridge assembly.

**Moisture adsorption capacity:** Proceed as directed under *Bentonite*.

**Reagents—test solutions**

**Ammonium molybdate TS:** Dissolve 6.5 g of finely powdered molybdic acid in a mixture of 14 mL of water and 14.5 mL of ammonium hydroxide. Cool the solution, and add it slowly, with stirring, to a well-cooled mixture of 32 mL of nitric acid and 40 mL of water. Allow to stand for 48 h, and filter through a fine-porosity, sintered-glass crucible. This solution deteriorates upon standing and is unsuitable for use if, upon the addition of 2 mL of dibasic sodium phosphate TS to 5 mL of the solution, an abundant yellow precipitate does not form at once or after slight warming. Store it in the dark. If a precipitate forms during storage, use only the clear supernatant.

**Oxalic acid TS:** Dissolve 6.3 g of oxalic acid in water to make 100 mL.

***Add the following:***

## ▲ODOR ADSORBENT

• **CARBON, ACTIVATED**[10]

Proceed as directed under *Carbon, Activated* as a desiccant plus the addition of the following test.

**Volatiles Organic Compounds (VOC) Adsorption Power**

**Butane activity**

This method determines the ability of carbon, activated to adsorb butane from dry air under controlled conditions and provides an indication of the pore size of carbon, activated for air/vapor applications. Butane activity is defined as the percent ratio of butane mass adsorbed by the mass of carbon, activated utilized when carbon, activated is saturated by butane under specified conditions.

**Method:** Dry the sample of charcoal, activated as per ⟨731⟩ and determine the bulk density (*Bulk Density and Tapped Density of Powders* ⟨616⟩). Assemble a perforated porcelain disk or an extra coarse fritted disk to a glass Schwartz drying tube to hold the sample. Accurately weigh the glass assembly together with the two glass stoppers to within 0.01 g. Transfer a sample of dried charcoal, activated equivalent to 16.70 ± 0.05 mL into the drying tube, add the two glass stoppers and weigh the assembly to within 0.01 g. Assemble a flow meter to a source of n-butane and connect the flow meter to a 1.9-m-long gas heat exchange coil. Attach the exchange coil to the inlet of the glass drying tube above the sample and an outlet tube to vent the butane. Securely clamp the drying tube in the vertical position in a water bath maintained at 25 ± 0.2° in a fume hood. Remove the right-angle stop cock and regulate the flow of butane through the carbon, activated bed at 250 ± 5 mL/min for at least 15 min.

[9] A suitable filter paper such as Whatman No. 3 or equivalent may be used.

[10] Additional information on the odor adsorption method may be found in "Standard Test Method for Determination of Butane Activity of Activated Charcoal". Current version of ASTM Method D5745, published by ASTM International, 100 Barr Harbor Drive, P.O. Box C700, West Conshohocken, PA 19428-2959; www.astm.org.

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[**CAUTION**—n-Butane is highly flammable and can act as a narcotic and may cause asphyxiation and ventricular fibrillation.]

Turn off the butane flow, disconnect the tubing and immediately stopper the drying tube. Remove the drying tube from the water bath and thoroughly dry the outer surface. Inspect visually for any condensed water vapor within the tube. Repeat the test procedure if any condensed water is observed. Weigh the sample tube plus stoppers to the nearest 0.01 g.

Reconnect the sample tubing and repeat the procedure for consecutive 10-min intervals until the butane activity is constant within ±0.02 g.

**Calculation**

Calculate according to the following equation, where:

$$\%\ \text{Butane activity} = \frac{(D - C)}{(C - M)} \times 100$$

*D* = mass of butane-saturated carbon, sample tube, and stoppers
*C* = mass of carbon, sample tube, and stoppers
*M* = mass of sample tube and stoppers

**Acceptance criteria:** Butane activity is NLT 40%. ▲ (USP 1-Dec-2023)

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# ⟨671⟩ CONTAINERS—PERFORMANCE TESTING

***Change to read:***

INTRODUCTION
MOISTURE VAPOR TRANSMISSION FOR ▲PLASTIC▲ (USP 1-Dec-2020) PACKAGING SYSTEMS
Introduction
Desiccant Methods for Packaging Systems▲▲ (USP 1-Dec-2020)
▲Water Method for Packaging Systems▲ (USP 1-Dec-2020)
CLASSIFICATION SYSTEM FOR PLASTIC PACKAGING SYSTEMS
Introduction
Classification Based on Desiccant Method ▲for Solid Oral Dosage Forms▲ (USP 1-Dec-2020)
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SPECTRAL TRANSMISSION ▲FOR LIGHT-RESISTANT PACKAGING COMPONENTS OR SYSTEMS▲ (USP 1-Dec-2020)
Apparatus
Procedure
Limits
▲▲ (USP 1-Dec-2020)

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## INTRODUCTION

It is the purpose of this chapter to provide standards for the functional properties of packaging systems used for solid oral dosage forms (SODFs) and liquid oral dosage forms (LODFs). The tests in this chapter may be applied to ▲packaging systems for▲ (USP 1-Dec-2020) other dosage forms with the appropriate justification. ▲These▲ (USP 1-Dec-2020) tests ▲▲ (USP 1-Dec-2020) determine the moisture vapor transmission rate (MVTR) i.e., water vapor permeation rate, for plastic packaging systems for manufacturers, packagers, and repackagers (*Table 1*). This chapter also includes a classification system that allows pharmacists and institutional repackagers to select appropriate containers to repackage SODFs and LODFs. Definitions that apply to this classification system are provided in *Packaging and Storage Requirements* ⟨659⟩.

▲Guidance on the application of these methods to determine the MVTR and the use of MVTR data to support the demonstration of the equivalence of plastic packaging systems when repackaging SODFs is contained in *The Application of Moisture Vapor Transmission Rates for Solid Oral Dosage Forms in Plastic Packaging Systems* ⟨1671⟩. Definitions of a blister, blister card, barrier blister types, moisture vapor transmission rate, and test specimen are contained in ⟨1671⟩, *Glossary*.

The chapter also contains a method for the determination of spectral transmission of plastic containers.▲ (USP 1-Dec-2020)

**Table 1. Moisture Vapor Transmission Test Methods for ▲Plastic▲ (USP 1-Dec-2020) Packaging Systems**

| Section | Moisture Vapor Transmission for ▲Plastic▲ (USP 1-Dec-2020) Packaging Systems | Classification System for ▲Plastic▲ (USP 1-Dec-2020) Packaging Systems | | |
|---|---|---|---|---|
| Subsection | ▲Desiccant Method (*Methods 1*, *2*, and *3*)<br>Water Method (*Method 4*)▲ (USP 1-Dec-2020) | Classification Based on Desiccant Method ▲for SODF (*Methods 5*, *6*, and *7*)▲ (USP 1-Dec-2020) | | Classification Based on Water Method ▲for LODF (*Method 8*)▲ (USP 1-Dec-2020) |
| Application | Multiple-unit containers ▲▲ (USP 1-Dec-2020) and single-unit or unit-dose containers | Multiple-unit containers ▲▲ (USP 1-Dec-2020) | Single-unit and unit-dose containers ▲▲ (USP 1-Dec-2020) | Multiple- ▲▲ (USP 1-Dec-2020)unit containers |
| Users | Manufacturers<br>▲Packagers<br>Repackagers▲ (USP 1-Dec-2020) | Manufacturers<br>Packagers<br>Repackagers<br>Pharmacies | Manufacturers<br>Packagers<br>Repackagers<br>Pharmacies | Manufacturers<br>Packagers<br>Repackagers<br>Pharmacies |

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## MOISTURE VAPOR TRANSMISSION FOR ▲PLASTIC▲ (USP 1-DEC-2020) PACKAGING SYSTEMS

### Introduction

The purpose of the moisture vapor transmission test method, which is based upon American Society for Testing and Materials (ASTM) method D7709,[1] is to obtain reliable and specific MVTRs that can be used to distinguish barrier performance of

[1] ASTM D7709. *Standard Test Methods for Measuring Water Vapor Transmission Rate (WVTR) of Pharmaceutical Bottles and Blisters* published by ASTM International, 100 Barr Harbor Drive, P.O. Box C700, West Conshohocken, PA 19428-2959.

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packaging systems used for regulated articles by manufacturers, packagers, and repackagers. This test method will establish a value that represents the MVTR of the container–closure system being evaluated. There may be additional packaging systems where the test methods in this section could be applied; however, any deviation should be described. If other methods are used to measure MVTR, these methods should be described in sufficient detail to justify their use.

## Desiccant Methods for Packaging Systems ▲▲ (USP 1-Dec-2020)

This section describes moisture vapor transmission test methods for multiple-unit containers (see *Method 1*), high-barrier ▲and ultra-high barrier▲ (USP 1-Dec-2020) single-unit and unit-dose containers (see *Method 2*), and low-barrier single-unit and unit-dose containers (see *Method 3*). ▲▲ (USP 1-Dec-2020)

These methods include the following:

- A specific moisture vapor transmission value for a container rather than a classification
- Sufficient sensitivity and precision to allow differentiation among moisture barrier performance for containers
- Conditions used for testing packaging systems that are the same as those used for accelerated stability testing of the primary packaging of regulated articles [typically 40°/75% relative humidity (RH)]

### EQUIPMENT

The following equipment should be used to complete the test method:

- A balance for weighing the test specimens that has sufficient capacity to weigh the test specimens throughout the period of the test. ▲▲ (USP 1-Dec-2020) The balance must have sensitivity adequate to measure small differences in weight from one time point to the next. The weighing uncertainty must be less than 5% of the weight gain from one time point to the next. The weighing uncertainty is typically three times the balance resolution/sensitivity. As an example, a balance with a resolution of 0.1 mg is acceptable for packaging systems whose weight gain per time interval is more than or equal to 6 mg [(0.1 × 3)/5%], which is 60 times the balance sensitivity.
- A chamber capable of maintaining 40 ± 2° and 75 ± 5% RH. Other chamber conditions can be used, ▲but should be noted in the report,▲ (USP 1-Dec-2020) as long as the balance sensitivity is adequate to measure small weight differences from one time point to the next time point.

### DESICCANT

**Method 1:** The desiccant is anhydrous calcium chloride in granular form. Other desiccants, such as a molecular sieve or silica gel, may be suitable. If anhydrous calcium chloride is used, pre-dry at 215 ± 5° for 7¼±¼ h to ensure that any hexahydrate present is fully converted to the anhydrate. Cool the desiccant in a desiccator for at least 2 h before use. [NOTE—It has been shown[2] that anhydrous calcium chloride may contain calcium hexahydrate, which loses water only when the temperature reaches 200°.]

**Methods 2 and 3:** ▲USP Desiccant, Small RS; USP Desiccant, Medium RS; and USP Desiccant, Large RS. The Reference Standards for desiccants are molecular sieves molded in a form to fit the size and shape of the blister cavity used. These desiccants should be at or near their original values when stored in their original and unopened containers, and thus drying should not be required. Prior to use, if the average weight of 10 tablets exceeds the weight specified on the USP certificate by more than 3%, drying shall be performed. Dry at 250 ± 5° for 1–2 h and cool in a desiccator for at least 2 h before use. Store desiccant in a tightly closed container in the driest conditions possible after being removed from original container.▲ (USP 1-Dec-2020)

### PROCEDURE

**Method 1:** Use 15 multiple-unit containers and 15 closures representative of the system to be tested. Prepare the test specimens by filling each multiple-unit container to two-thirds ▲of its nominal volume▲ (USP 1-Dec-2020) with desiccant and then, for screw-type closures, applying the closure using the torque that is within the range of tightness specified in *Table 2*. For other closure types, apply the closure according to the intended method. ▲If the intended use of the container shall have an impervious seal, then▲ (USP 1-Dec-2020) ensure that a proper seal has been made with the intended membrane to the land area of the bottle ▲opening.▲ (USP 1-Dec-2020) Identify each multiple-unit container with indelible ink. Do not use a label. If there is a need to increase the precision of the method, the user can test the system without the closure as long as an impervious seal remains on the container. If desired, weigh each multiple-unit container at ambient temperature and RH. Record this weight for time 0, but do not use it in the calculation of permeation. Place all containers in the test chamber (40°/75% RH) within 1 h of weighing. Weigh all multiple-unit containers at time intervals of 7 days ± 1 h. Weigh the multiple-unit containers at 7, 14, 21, 28, and 35 days to get ▲five▲ (USP 1-Dec-2020) steady-state data points. (The time interval from time 0 to day 7 is the period of equilibration.) Prior to weighing at each time interval, equilibrate the containers for about 30 min at the weighing temperature and RH. Limit the time out of the chamber to less than 2 h. Record the weights in an appropriate manner for later computation of the regression line.

[2] Chen Y, Li Y. Determination of water vapor transmission rate (WVTR) of HDPE bottles for pharmaceutical products. *Int J Pharm.* 2008;358(1-2):137–143.

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**Table 2. Torque Applicable to Screw-Type Container**

| Closure Diameter[a] (millimeters) | Suggested Tightness Range with Manually Applied Torque[b] (inch-pounds) | Suggested Tightness Range with Manually Applied Torque[b] (Newton-meters) |
|---|---|---|
| 8 | 5 | 0.56 |
| 10 | 6 | 0.68 |
| 13 | 8 | 0.90 |
| 15 | 5–9 | 0.56–1.02 |
| 18 | 7–10 | 0.79–1.13 |
| 20 | 8–12 | 0.90–1.36 |
| 22 | 9–14 | 1.02–1.58 |
| 24 | 10–18 | 1.13–2.03 |
| 28 | 12–21 | 1.36–2.37 |
| 30 | 13–23 | 1.47–2.60 |
| 33 | 15–25 | 1.69–2.82 |
| 38 | 17–26 | 1.92–2.94 |
| 43 | 17–27 | 1.92–3.05 |
| 48 | 19–30 | 2.15–3.39 |
| 53 | 21–36 | 2.37–4.07 |
| 58 | 23–40 | 2.60–4.52 |
| 63 | 25–43 | 2.82–4.86 |
| 66 | 26–45 | 2.94–5.08 |
| 70 | 28–50 | 3.16–5.65 |
| 83 | 32–65 | 3.62–7.35 |
| 86 | 40–65 | 4.52–7.35 |
| 89 | 40–70 | 4.52–7.91 |
| 100 | 45–70 | 5.09–7.91 |
| 110 | 45–70 | 5.09–7.91 |
| 120 | 55–95 | 6.22–10.74 |
| 132 | 60–95 | 6.78–10.74 |

[a] The torque designated for the next larger closure diameter is to be applied in testing containers having a closure diameter intermediate to the diameters listed.
[b] A suitable apparatus is available from SecurePak, www.secure-pak.com. An MRA model of a spring torque tester with indicators on both the removal and application sides is available in the following ranges: 1) 0–25 inch-pounds, read in 1-inch-pound increments; 2) 0–50 inch-pounds, read in 2-inch-pound increments; and 3) 0–100 inch-pounds, read in 5-inch-pound increments.

**Method 2:** Use 10 test units for this method. Provide a minimum of 10 blister cavities for each test unit. If the ▲blister▲ (USP 1-Dec-2020) card contains fewer than 10 cavities, bundle the cards to form a ▲▲ (USP 1-Dec-2020) test unit of at least 10 cavities. This is required to provide sufficient weight gain at each time interval. Fill with predried desiccant, and seal the blisters on equipment that is capable of correctly filling and sealing the blister. The desiccant should fill the cavity. The total weight of the desiccant must be sufficient to meet the quantity required to avoid partial saturation of the *Desiccant* before completion of the test. Fill the blisters in a low-humidity atmosphere (as low as possible, but not greater than 50% RH). Do not expose the desiccants to room humidity for more than 30 min before sealing. Identify each test specimen with indelible ink; do not use a label. ▲▲ (USP 1-Dec-2020) It should be noted that bundling of the blisters during storage can impact air flow and correspondingly increase data variability.

Weigh each test unit at ambient temperature and RH. Record this weight for time 0. Place all test units in the test chamber (40°/75% RH) within 1 h of weighing. Weigh all test units at time intervals of 7 days ± 1 h. Weigh the test units at 7, 14, 21, 28, and 35 days to get five steady-state data points. (The time interval from time 0 to day 7 is the period of equilibration.) Prior to weighing at each time interval, equilibrate the containers for 30 ± 5 min at the controlled weighing temperature and RH. Limit the time out of the chamber to less than 2 h. Record the weights in an appropriate manner for later computation of the regression line.

Ultra-high barrier blisters may not show the full measure of precision and sensitivity this method can provide. For ultra-high barrier blisters, test units should have more than 10 cavities, but NMT 30 cavities. Examples are foil–foil blisters or very small blisters formed from other materials. An alternative approach is to double or triple the length of weighing intervals to achieve at least a 6-mg weight gain per time interval by the test specimen.

**Method 3:** Prepare the test units as directed in *Method 2*. Place all test units in the test chamber (40°/75% RH) within 1 h of weighing. Weigh the test units at the end of 2 days (48 ± 1 h). At this time, the difference in weight (the weight gain) is divided

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by the number of blister ▲cavities▲ (USP 1-Dec-2020) and days (2) in each test unit and this is taken as the MVTR in mg/day/▲cavity.▲ (USP 1-Dec-2020) The number of blisters tested depends on the barrier characteristics of the material, the size of the blister, and the sensitivity of the balance used in the test. For this method, the requirement of five consecutive weighings is waived because the desiccant quickly becomes saturated when packed in a low-barrier package and stored at 40°/75% RH. [NOTE—For low-barrier single-unit and unit-dose containers, the weight gain after the second day displays a curvilinear profile typical of approaching saturation of the desiccant. To obtain five weighings within 2 days is not viable and is likely to increase variability. *Methods 2* and *3* may require that the blister cards be bundled in multiples to achieve periodic weight gains of sufficient magnitude to use the balance sensitivity; when bundled, these cards or test specimens are called test units. The weight gain in each weighing period must be 20 times the sensitivity of the balance, and the balance sensitivity is three times the balance precision. In other words, the minimal weight gain per time interval should be at least 60 times the balance precision.]

## CALCULATIONS

**Methods 1 and 2:** Perform the regression analysis for each test unit (see *Regression equation*). Typically, the initial data point (at day 0) is not included in fitting the regression line. The slope of the regression line is the MVTR of each test unit. For *Method 1*, the slope is the MVTR for the corresponding multiple-unit container. For *Method 2*, the MVTR of each blister cavity is calculated by dividing the slope by the number of cavities in each test unit.
**Method 3:** Calculate the weight gain in mg/day/▲cavity▲ (USP 1-Dec-2020) from day 0 to day 2 using the 10 test units (see *Calculations*). The MVTR of each blister ▲cavity▲ (USP 1-Dec-2020) is calculated by dividing the weight gain by 2 (for 2 days) and the number of ▲blister cavities▲ (USP 1-Dec-2020) in each test unit.
**Regression equation:**

$$W = I + MT$$

**Calculations:**

$$Slope\left(M\right) = \sum_{i=1}^{N}\left[\left(W_i - \overline{W}\right)\left(T_i - \overline{T}\right)\right] / \sum_{i=1}^{N}\left(T_i - \overline{T}\right)^2$$

$$\text{Intercept } (I) = \overline{W} - M\overline{T}$$

$W$ = measured weight (mg)
$I$ = regression line intercept (point where regression line intersects the vertical axis)
$M$ = regression line slope
$T$ = time point
$N$ = number of data points (each point consists of a weight and a time)
$\overline{W}$ = overall weight mean
$\overline{T}$ = overall time point mean

$$\sum_{i=1}^{N}\left[\left(W_i - \overline{W}\right)\left(T_i - \overline{T}\right)\right]$$

equals the sum of cross-products (for example, for each of the $N$ data points, subtract the overall weight mean from the measured weight and the overall time point mean from the time point and multiply the two differences to get a cross-product, then sum all $N$ cross-products).

$$\sum_{i-1}^{N}\left(T - \overline{T}\right)^2$$

equals the sum of squared deviations (for example, for each of the $N$ data points, subtract the overall time point mean from the time point and square the difference, then use the sum of all $N$ squared differences).

## RESULTS

**Method 1:** Report the MVTR as the average value, in mg/day/container, and the standard deviation of the 15 slopes. Properly describe the container–closure system tested ▲(e.g., material, size, shape, and color).▲ (USP 1-Dec-2020)
**Method 2:** Report the MVTR as the average value, in mg/day/cavity, and the standard deviation of the 10 test unit slopes. Properly describe the container–closure system tested ▲(e.g., material, size, shape, and color).▲ (USP 1-Dec-2020)
**Method 3:** Report the MVTR as the average value from day 0 to day 2, in mg/day/▲cavity,▲ (USP 1-Dec-2020) and the standard deviation of the 10 test unit slopes. Properly describe the container–closure system tested ▲(e.g., material, size, shape, and color).

# Water Method for Packaging Systems

This section describes moisture vapor transmission test methods (see *Method 4*) for low-barrier, high-barrier, and ultra-high barrier single-unit and unit-dose containers.

*Method 4* includes the following:

- A specific moisture vapor transmission value for a packaging system rather than a classification.

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- Sufficient sensitivity and precision to allow differentiation among moisture barrier performance for plastic blister packaging systems.
- Inverse environmental conditions (40°/25% RH) from those that are used for the desiccant method and stability testing of the primary packaging of regulated articles (40°/75% RH). These inverse conditions achieve the same pressure difference when water is used in place of desiccant.
- MVTR determination is based upon weight loss instead of weight gain.

## EQUIPMENT

The following equipment should be used to complete the test method:

- A humidity-controlled glove box capable of maintaining relative humidity at 25% ± 5%.
- A balance for weighing the test specimens that has sufficient capacity to weigh the test specimens throughout the period of the test. The balance must have sensitivity adequate to measure small differences in weight from one time point to the next. The weighing uncertainty must be less than 5% of the weight gain from one time point to the next. The weighing uncertainty is typically three times the balance resolution/sensitivity. As an example, a balance with a resolution of 0.1 mg is acceptable for packaging systems whose weight gain per time interval is more than or equal to 6 mg [(0.1 × 3)/5%], which is 60 times the balance sensitivity.
- A chamber capable of maintaining 40 ± 2°/25% ± 5% RH.

## AMOUNT OF WATER FOR TEST CONTAINERS

For blisters, approximately 10–100 mg of water per blister cavity (about 1–10 drops), depending upon expected MVTR, is adequate for all blister types at 40°/25% RH. As an example, consider the preparation of a low-barrier MVTR package, e.g., a polyvinyl chloride (PVC) water-filled blister. The expected MVTR is about 1.5 mg water/(day × cavity) at 40° and a 75% RH vapor–pressure difference. The initial amount of water required to create 100% RH at 40° in this blister (approximately 0.5 cc volume) is 0.025 mg, on the basis of absolute humidity. For a typical MVTR study of a low-barrier blister, 7 days are allowed for equilibration, followed by a study period of 28 days for obtaining 3–5 weight determinations. This is a total exposure time, or permeation time, of 35 days. According to the *Water-based method equation*, a minimum of 52.5 mg of water is needed.
**Water-based method equation:** 1.5 mg water/(day × cavity) × 35 days + 0.025 mg = 52.5 mg water/cavity

The recommended amount of water for this blister system is about 100 mg per cavity. The water-based method equation can be used to determine the minimum amount of water for all blister types, and this amount should then be multiplied by a factor between 2 and 3 to achieve an excess.

## PROCEDURE

**Method 4:** Use 10 test units for this method. Provide a minimum of 10 blister cavities for each test unit. If the blister card contains fewer than 10 cavities, bundle the cards to form a test unit of at least 10 cavities. This is required to provide sufficient weight loss at each time interval.

Fill blister cavities with a sufficient quantity of water. Care should be taken to insure the cavities are not overfilled and that water does not end up on the seal area.

Identify each test specimen with indelible ink; do not use a label. High-barrier plastic blister cards may be bundled to increase weight loss between weight determinations. Low-barrier plastic blister cards do not need to be bundled, regardless of the number of cavities per card. For bundled test units, it should be noted that bundling of the blisters during testing can impact air flow and correspondingly increase data variability.

Place test units in the humidity-controlled glove box at 25% RH for 60 min. Remove test units from the glove box and record initial weight to nearest 0.1 mg and time. Place all test units in the test chamber (40°/25% RH) and weigh at time intervals of 7 days ± 1 h. Weigh the test units at 7, 14, 21, 28, and 35 days to get five steady-state data points. Additional time points can be added to provide for laboratory operations and higher barrier blisters. At each time point, place the test units in the humidity-controlled glove box and allow to equilibrate for 60 min, then weigh, and record the time. After weighing, samples are placed back into the test chamber. [NOTE—It is imperative that the weighing order of test units be consistent among all time point weight determinations.]

Ultra-high barrier blisters may not show the full measure of precision and sensitivity this method can provide. For ultra-high barrier blisters, test units should have more than 10 cavities, but NMT 30 cavities. Examples are foil–foil blisters or very small blisters formed from other materials. An alternative approach is to double or triple the length of weighing intervals to achieve at least a 6-mg weight gain per time interval by the test specimen.

All test units should be inspected for the presence of water at each time point. Opaque and cold-form foil blisters should be opened at the conclusion of the test to insure the presence of water in each cavity since visual inspection is not possible.

## CALCULATIONS

**Method 4:** Perform the linear regression analysis for each test unit by following the *Calculations* in *Methods 1 and 2* in *Desiccant Methods for Packaging Systems*, except the slope in *Water Method for Packaging Systems* is negative because of the moisture egresses and weight loss with time in the water method. The MVTR of each blister cavity is calculated by dividing the absolute value of the slope by the number of cavities in each test unit.

## RESULTS

**Method 4:** Report the MVTR as the average value, in mg/day/cavity, and the standard deviation of the 10 test unit slopes. Properly describe the container–closure system tested.▲ (USP 1-Dec-2020)

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# CLASSIFICATION SYSTEM FOR PLASTIC PACKAGING SYSTEMS

## Introduction

A classification system is included to provide methods that allow pharmacists and institutional repackagers to select appropriate containers for repackaging. Repackaging is the act of taking a finished drug product from the container in which it was distributed by the original manufacturer and placing it into a different container without further manipulation of the drug. The classification system provided can also be used by the drug product manufacturer for testing packaging systems that have had the primary seal broached.

## Classification Based on Desiccant Method ▲for Solid Oral Dosage Forms▲ (USP 1-Dec-2020)

This section describes the methods for assigning the classification for multiple-unit containers (*Method ▲5*),▲ (USP 1-Dec-2020) multiple-unit containers with induction seals (*Method ▲6*),▲ (USP 1-Dec-2020) and single-unit and unit-dose containers (*Method ▲7*)▲ (USP 1-Dec-2020) used by pharmacists and institutional repackagers.

### DESICCANT

**Methods ▲5 and 6:**▲ (USP 1-Dec-2020) Place a quantity of 4- to 8-mesh, anhydrous calcium chloride[3] in a shallow container, taking care to exclude any fine powder, dry at 215 ± 5° for 7¼±¼ h, and cool in a desiccator ▲for at least 2 h before use.▲ (USP 1-Dec-2020) [NOTE—It has been shown[2] that anhydrous calcium chloride may contain calcium hexahydrate, which loses water only when the temperature reaches 200°.]

**Method ▲7:** Use USP Desiccant, Small RS; USP Desiccant, Medium RS; or USP Desiccant, Large RS. The USP Reference Standards for desiccants are molecular sieves molded in a form to fit the size and shape of the blister cavity used. These desiccants should be at or near their original values when stored in their original and unopened containers, and thus drying should not be required. Prior to use, if the average weight of 10 tablets exceeds the weight specified on the USP certificate by more than 3%, drying shall be performed. Dry at 250 ± 5° for 1–2 h, and cool in a desiccator for at least 2 h before use. Store desiccant in a tightly closed container in the driest conditions possible after being removed from original container.▲ (USP 1-Dec-2020)

### PROCEDURE

**Method ▲5:**▲ (USP 1-Dec-2020) Select 12 containers of a uniform size and type. Clean the sealing surfaces with a lint-free cloth, and close and open each container 30 times within the range of tightness specified in *Table 2*. Add desiccant to 10 of the containers, designated "test containers", filling each to within 13 mm of the closure if the container volume is 20 mL or more, or filling each to two-thirds of ▲its nominal volume▲ (USP 1-Dec-2020) if the container volume is less than 20 mL. If the interior of the container is more than 63 mm in depth, an inert filler or spacer ▲(e.g., glass beads)▲ (USP 1-Dec-2020) may be placed in the bottom to minimize the total weight of the container and desiccant; the layer of desiccant in such a container must be NLT 5 cm in depth. Close each container immediately after adding desiccant, and apply the torque designated in *Table 2* when closing screw-capped containers. To each of the remaining two containers, designated "controls", add a sufficient number of glass beads to attain a weight approximately equal to that of each of the test containers, and close, applying the torque designated in *Table 2* when closing screw-capped containers. Record the weight of the individual containers to the nearest 0.1 mg if the container volume is less than 20 mL, to the nearest milligram if the container volume is 20 mL or more but less than 200 mL, or to the nearest centigram (10 mg) if the container volume is 200 mL or more, and store at 75 ± 3% RH and a temperature of 23 ± 2°. [NOTE—A saturated system of 35 g of sodium chloride with each 100 mL of water placed in the bottom of a desiccator maintains the specified humidity. Other methods may be employed to maintain these conditions.] After 336 ± 1 h (14 days), record the weight of the individual containers in the same manner. Completely fill 5 empty containers of the same size and type as that of the containers under test with water or a noncompressible, free-flowing solid, such as well-tamped fine glass beads, to the level indicated by the closure surface when in place. Transfer the contents of each to a graduated cylinder, and determine the average container volume, in milliliters.

**Method ▲6:**▲ (USP 1-Dec-2020) Proceed as directed under *Method ▲5*,▲ (USP 1-Dec-2020) except fit containers with impervious seals obtained by heat-sealing the bottles with an aluminum foil–polyethylene laminate or other suitable seal. If there is a need to increase the precision of the method, the user can test the system without the closure as long as an impervious seal remains on the container. Keeping the cap on causes variation and the absorption of water by the cap liner is not part of the moisture permeation.

**Method ▲7:**▲ (USP 1-Dec-2020) Use this procedure for packs (e.g., punch-out cards) and blister cards that incorporate one or more separately sealed unit-dose containers or blisters. Seal a sufficient number of packs, such that NLT 4 packs and a total of NLT 10 unit-dose containers or blisters filled with 1 pellet in each unit are tested. Seal a corresponding number of empty packs, each pack containing the same number of unit-dose containers or blisters as used in the test packs, to provide the controls. Store all of the containers at 75 ± 3% RH and at a temperature of 23 ± 2°. [NOTE—A saturated system of 35 g of sodium chloride with each 100 mL of water placed in the bottom of a desiccator maintains the specified humidity. Other methods may be employed to maintain these conditions.] After 24 h, and at each multiple thereof (see *Classification*), remove the packs from the chamber, and allow them to equilibrate for about 45 min. Record the weights of the individual packs, and return them to the chamber. Weigh the control packs as a unit, and divide the total weight by the number of control packs to obtain the average empty pack weight. [NOTE—If any indicating pellets turn pink during the procedure, or if the average pellet weight increase in any

[3] Suitable 4- to 8-mesh, anhydrous calcium chloride is available from VWR International.

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pack exceeds 10%, terminate the test, and regard only earlier determinations as valid.] The test and control containers are weighed after every 24 h and after suitable test intervals for the final weighings. $W_F$ and $C_F$ are as follows: 24 h for Class D, 48 h for Class C, 7 days for Class B, and NLT 28 days for Class A.

CALCULATIONS

**Methods ▲5 and 6:▲** (USP 1-Dec-2020) Calculate the rate of moisture vapor transmission, in mg/day/L:

$$(1000/14V)[(T_F - T_I) - (C_F - C_I)]$$

$V$ = volume of the container (mL)
$T_F$ = final weight of each test container (mg)
$T_I$ = initial weight of each test container (mg)
$C_F$ = average final weight of the two controls (mg)
$C_I$ = average initial weight of the two controls (mg)

**Method ▲7:▲** (USP 1-Dec-2020) Calculate, to two significant figures, the average rate of moisture vapor transmission, in mg/day, for each unit-dose container or blister in each pack taken:

$$[1/(N \times X)][(W_F - W_I) - (C_F - C_I)]$$

$N$ = number of days expired in the test period (beginning after the initial 24-h equilibration period)
$X$ = number of separately sealed units per pack
$W_F$ = final weight of each test pack (mg)
$W_I$ = initial weight of each test pack (mg)
$C_F$ = final weight of the control packs (mg)
$C_I$ = initial weight of the control packs (mg)

▲CLASSIFICATION▲ (USP 1-Dec-2020)

**Method ▲5:▲** (USP 1-Dec-2020) Packaging systems are ▲classified as▲ (USP 1-Dec-2020) "tight" if NMT 1 of the 10 test containers exceeds 100 mg/day/L in moisture vapor transmission, and none exceeds 200 mg/day/L. Packaging systems are ▲classified as▲ (USP 1-Dec-2020) "well-closed" if NMT 1 of the 10 test containers exceeds 2000 mg/day/L in moisture vapor transmission, and none exceeds 3000 mg/day/L.
**Method ▲6:▲** (USP 1-Dec-2020) Packaging systems are classified as ▲"tight"▲ (USP 1-Dec-2020) if the moisture vapor transmission exceeds 10 mg/day/L in NMT 1 of the 10 test containers, and none exceeds 25 mg/day/L.
**Method ▲7:▲** (USP 1-Dec-2020) Class A: No pack tested exceeds 0.5 mg/day/unit in average blister MVTR; Class B: No pack tested exceeds 5 mg/day/unit in average blister MVTR; Class C: No pack tested exceeds 20 mg/day/unit in average blister MVTR; and Class D: None of the packs tested meet any of the above average blister MVTR requirements.

## Classification Based on Water Method ▲for Liquid Oral Dosage Forms▲ (USP 1-Dec-2020)

The following procedure and classification scheme ▲(*Method 8*)▲ (USP 1-Dec-2020) is provided to ▲classify multiple-unit containers as "tight" for liquid oral dosage forms.▲ (USP 1-Dec-2020)

[NOTE—Determine the weights of individual container–closure systems (bottle; inner seal, if used; and closure), both as tare weights and fill weights, to the nearest 0.1 mg if the bottle capacity is less than 200 mL, to the nearest milligram if the bottle capacity is 200 mL or more but less than 1000 mL, or to the nearest ▲0.1 g▲ (USP 1-Dec-2020) if the bottle capacity is 1000 mL or more.]

PROCEDURE

**▲Method 8:▲** (USP 1-Dec-2020) Select 12 bottles of a uniform size and type, and clean the sealing surfaces with a lint-free cloth. Fit each bottle with a seal, closure liner (if applicable), and closure. Number each container–closure system, and record the tare weight.

Remove the closures and, using a pipet, fill 10 bottles with water to the fill capacity. Fill two containers with glass beads, to the same weight as the filled test containers. If using screw closures, apply a torque that is within the range specified in *Table 2*, and store the sealed containers at a temperature of 25 ± 2° and 40 ± 2% RH. After 336 ± 1 h (14 days), record the weight of the individual containers, and calculate the water weight loss rate, in %/year, for each bottle taken:

$$\frac{\left[\left(W_{1i} - W_T\right) - \left(W_{14i} - W_T\right) - \left(W_{C1} - W_{C14}\right)\right]}{\left(W_{1i} - W_T\right) \times 14} \times 365 \times 100$$

$W_{1i}$ = initial weight of each individual bottle ($i$) (mg)
$W_T$ = tare weight (mg)
$W_{14i}$ = weight of each individual bottle ($i$) at 14 days (mg)
$W_{C1}$ = initial weight of the control container at day 1 (mg)
$W_{C14}$ = weight of the control container at 14 days (mg)

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▲CLASSIFICATION

**Method 8:**▲ (USP 1-Dec-2020) The packaging systems are classified as "tight" ▲if NMT 1 of the 10 test containers exceeds 2.5%/year percentage of water weight loss and does not exceed 5.0%/year in any of them.▲ (USP 1-Dec-2020)

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## SPECTRAL TRANSMISSION ▲FOR LIGHT-RESISTANT PACKAGING COMPONENTS OR SYSTEMS

A packaging component or system intended to provide protection from light or offered as a light-resistant container meets the requirements for *Spectral Transmission for Light-Resistant Packaging Components or Systems*, where such protection or resistance is by virtue of the specific properties of the material of which the plastic component or system is composed, including any coating. A clear and colorless or a translucent packaging system that has light protection by means of an opaque enclosure by application of a suitable coating or wrapping (see *General Notices*) is exempt from the requirements for *Spectral Transmission for Light-Resistant Packaging Components or Systems*.▲ (USP 1-Dec-2020)

### Apparatus

▲If the suitability of the packaging system is demonstrated through stability testing, spectral transmission testing is not required; otherwise, use a UV-visible spectrophotometer of suitable sensitivity and accuracy (see ⟨857⟩), adapted for measuring the amount of light transmitted by plastic materials used for pharmaceutical containers.▲ (USP 1-Dec-2020)

### Procedure

Select a section representing the average ▲▲ (USP 1-Dec-2020) thickness. Cut a circular section from ▲the packaging component or system,▲ (USP 1-Dec-2020) and trim as necessary to get a segment convenient for mounting in the spectrophotometer. After cutting, wash and dry the specimen, taking care to avoid scratching the surfaces. If the specimen is too small to cover the opening in the specimen holder, mask the uncovered portion of the opening with opaque paper or masking tape, provided that the length of the specimen is greater than that of the slit in the spectrophotometer. Immediately before mounting in the specimen holder, wipe the specimen with lens tissue. Mount the specimen with the aid of a tacky wax, or by other convenient means, taking care to avoid leaving fingerprints or other marks on the surfaces through which light must pass. Place the section in the spectrophotometer with its cylindrical axis parallel to the plane of the slit and approximately centered with respect to the slit. When properly placed, the light beam is normal to the surface of the section and reflection losses are at a minimum.

Continuously measure the transmittance of the section with reference to air in the spectral region of interest with a recording instrument or at intervals of about 20 nm with a manual instrument, in the region of 290–450 nm.

### ▲Acceptance Criteria▲ (USP 1-Dec-2020)

The observed spectral transmission does not exceed the limits given in *Table 3* for ▲plastic components and systems▲ (USP 1-Dec-2020) for parenteral use. The observed spectral transmission for plastic containers for products intended for oral or topical administration does not exceed 10% at any wavelength in the range of 290–450 nm.

**Table 3. ▲Spectral Transmission Limits for Plastic Packaging Components or Systems▲** (USP 1-Dec-2020)

| Nominal Size (mL) | Maximum Percentage of Spectral Transmission at Any Wavelength Between 290 and 450 nm |
|---|---|
| 1 | ▲25▲ (USP 1-Dec-2020) |
| 2 | ▲20▲ (USP 1-Dec-2020) |
| 5 | ▲15▲ (USP 1-Dec-2020) |
| 10 | ▲13▲ (USP 1-Dec-2020) |
| 20 | ▲12▲ (USP 1-Dec-2020) |
| ▲50▲ (USP 1-Dec-2020) | ▲10▲ (USP 1-Dec-2020) |
| ▲>▲ (USP 1-Dec-2020)50 | ▲10▲ (USP 1-Dec-2020) |

***Delete the following:***

## ▲GLOSSARY

**Blister:** Formed, lidded, and sealed plastic or foil dome that contains the capsule or tablet (usually a single unit or unit dose).

**Low-barrier blister:** Blisters made from low-barrier materials, formed and sealed so that the moisture vapor transmission rate when tested at 40°/75% RH is greater than 1.0 mg/cavity/day.

**High-barrier blister:** Blisters made from high-barrier material, formed and sealed so that the moisture vapor transmission rate when tested at 40°/75% RH is less than 1.0 mg/cavity/day.

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**Ultra-high barrier blister:** Blisters made from ultra-high barrier material, formed and sealed so that the moisture vapor transmission rate when tested at 40°/75% RH is less than 0.01 mg/cavity/day.

**Blister card:** A contiguous group of blisters formed and sealed with lid in place. The number of blisters per card commonly ranges from 1 to 10 but may be more. The blister card may sometimes be referred to as a packaging system.

**Cavity:** Formed, lidded, and sealed plastic or foil dome (see *Blister*).

**Moisture vapor transmission rate:** The steady-state moisture vapor transmission (in unit time) through a packaging system, under specific conditions of temperature and humidity. These test methods use gravimetric measurement to determine the rate of weight gain as a result of moisture vapor transmission into the packaging system and subsequent uptake by a desiccant enclosed within the packaging system.

**Test specimen** (or specimen)**:** For multiple-unit containers, the bottle is the test specimen, and for single-unit or unit-dose containers, the blister card containing multiple blister cavities is the test specimen. For blisters, more than 1 card (or specimen) may be grouped into a test unit for conducting the test.

**Test unit:** For multiple-unit containers, the bottle is the test unit as well as being the test specimen, and for single-unit or unit-dose containers, the test unit is a group of test specimens (blister cards) processed together for temperature and humidity exposure and for weighing at each time point. The purpose of the test unit for single-unit or unit-dose containers is to gain the advantage of additive weight gain resulting from more blister cavities than are present on a single card. The test unit, when applied to bottles, is used to maintain naming congruence among test methods 1, 2, and 3.▲ (USP 1-Dec-2020)

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# ⟨695⟩ CRYSTALLINITY

This test is provided to determine compliance with the crystallinity requirement where stated in the individual monograph for a drug substance.

## PROCEDURE

A detailed test procedure is described under *Optical Microscopy* ⟨776⟩.

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# ⟨696⟩ CHARACTERIZATION OF CRYSTALLINE SOLIDS BY MICROCALORIMETRY AND SOLUTION CALORIMETRY

*For the purpose of this chapter, crystalline material, partially crystalline material, and amorphous material are considered as solids.*

## INTRODUCTION—THE CONCEPT OF CRYSTALLINITY

The perfectly ordered crystal lattice with every molecule in its expected lattice position is an ideal that is seldom, if ever, achieved. The other extreme is the amorphous state, in which a solid contains the maximum possible density of imperfections (defects of various dimensionalities), such that all long-range order is lost while only the short-range order, imposed by its nearest neighbors, remains. Real crystals lie somewhere between these two extremes. A crystal's position on a scale bounded by these two extremes is termed *crystallinity*.

All real crystals, even in the pure state, possess some lattice imperfections or defects, which increase both the energy (enthalpy under conditions of constant atmospheric pressure) and the disorder (expressed as the entropy) of the crystal lattice. A crystal with a relatively low density of imperfections is said to be highly crystalline and to possess a high crystallinity. By contrast, a particle with a relatively high density of imperfections is said to be partially amorphous and to possess a low crystallinity. In ideal terms, a totally amorphous particle corresponds to zero crystallinity. Amorphous particles may contain somewhat ordered domains that can act as nuclei for crystallization; such so-called amorphous particles are said to possess a low-level, but finite, crystallinity.

The ability to detect and quantify the amount of amorphous material within a highly crystalline substance is of great importance during the development and subsequent manufacture of a pharmaceutical preparation.

In reality, a powder probably contains particles with different degrees of crystallinity, just as it may contain particles with varying sizes and shapes. The lower the crystallinity of a solid, the greater its enthalpy and entropy. The increase in enthalpy is never totally compensated by the increase in entropy; therefore, the Gibbs free energy, which reflects the balance between them, actually increases. Hence, the lower the crystallinity of a material (powder), and consequently the greater its amorphous character, the greater its apparent intrinsic solubility and dissolution rate, but the lower its thermodynamic stability. Because of the great relevance of these properties, crystallinity is also an important property and requires measurement by a suitable method.

In the following chapter, the crystallinity or the content of amorphous parts of a powder are measured by calorimetric methods such as microcalorimetry or solution calorimetry, although other methods could be used (e.g., see general chapter *Characterization of Crystalline and Partially Crystalline Solids by X-ray Powder Diffraction (XRPD)* ⟨941⟩).

Many substances are capable of crystallizing in more than one type of crystal lattice, which is known as polymorphism. If water or a solvent is incorporated in the crystal lattice, the crystals are termed hydrates or solvates. Because of the different crystal packing, and/or molecular conformation and lattice energy, they usually exhibit different physical properties. For simplicity, calorimetry measurements for degree of crystallinity determination discussed here assume only one solid crystalline form present in the material of interest. The theory and experimental technique can be easily expanded to polymorphic systems with proper consideration of the enthalpy differences among the polymorphs.

## METHOD 1—MICROCALORIMETRY (DETERMINATION OF AMORPHOUS CONTENT)

Most chemical, physical, and biological processes are associated with the exchange of heat. Microcalorimetry is a highly sensitive technique to monitor and quantify both exothermic (heat producing) and endothermic (heat absorbing) changes associated with those processes. The technique allows the determination of the rate and extent of chemical reactions, changes of phase, or changes of structure.

Thermal events producing only a fraction of a microwatt can be observed using microcalorimetry. This means that temperature differences less than $10^{-6}$ K must be detectable. Microcalorimetry typically uses the heat flow (heat leakage) principle, where, in a thermally defined vessel, the heat produced (or absorbed) flows away from (or into) the vessel in an effort to re-establish thermal equilibrium with its surroundings. Exceptional thermal stability with its surrounding has to be achieved either by a heat sink or an electronically regulated surrounding.

Heat energy from an active sample in the reaction vessel is channeled typically through Peltier elements; they act as thermoelectric generators using the Seebeck effect. The heat energy is converted into a voltage signal proportional to the heat flow.

Results are typically presented as a measure of the thermal energy produced per unit of time (Watt) as a function of time.

### Apparatus

Microcalorimeters are typically designed as twin systems with a measuring vessel and a reference vessel. Vessels are typically made of glass or stainless steel. For certain applications, specially designed vessels that allow the addition of a gas, a liquid, or a solid material may be used.

### Calibration

The microcalorimeter is calibrated for heat flow (energy per time unit) using either calibrated external or internal electrical heat sources or a suitable standard reaction.

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## Sensitivity

The sensitivity of the microcalorimetric method can be assessed on the basis of an appropriate standard sample analyzed according to the corresponding method in conjunction with the determination of the instrument baseline noise.

## Procedure

Weigh an appropriate quantity of the material in a suitable vessel. Close the vessel carefully, to avoid any evaporation of solvents, and place the vessel in the sample holder. If appropriate, allow the vessel to equilibrate at the temperature of the measurement before placing it in the measuring position.

Begin the analysis, and record the heat flow with the time on the abscissa and the heat flow on the ordinate (specify the direction of exothermic or endothermic heat flow).

## Detection and Quantification of Amorphous Content in Powders

The amorphous state is metastable with respect to the crystalline state; recrystallization may therefore occur. The measurement of the heat of recrystallization enables the amorphous content to be determined by the area of the recrystallization peak. By relating the output from the microcalorimeter for a sample to that obtained from an amorphous standard, it is possible to quantify the amorphous content of the sample. The range of amorphous content covered by this method depends on the individual substance to be tested. In favorable cases, limits of detection below 1% can be reached.

Recrystallization can be initiated by subjecting the sample to higher relative humidity or an atmosphere containing organic vapor. The sample is typically placed in an ampul that also contains a small test tube containing an aqueous saturated salt solution, an organic solvent, or a solvent mixture.

The heat of recrystallization is typically measured using a fixed sample mass placed in a glass or steel vessel. The test tube containing a saturated salt solution or an organic solvent is chosen large enough to allow a full saturation of the atmosphere above the sample. The mass of the sample and the nature of the vapor atmosphere above the sample is chosen so that recrystallization occurs in such a way that a distinct peak is observed, clearly separated from initial thermal events caused by introduction of the sample.

The conditions under which the transition of the amorphous phase to a thermodynamically more stable crystalline state occurs will have a significant impact on the time of recrystallization. In particular, physical mixtures of purely amorphous and crystalline material will behave differently from a partially crystalline material. These effects should be considered when developing a method.

A typical response for the recrystallization of a mainly amorphous material is shown in *Figure 1*. The first part of the curve represents several concurrent processes taking place simultaneously, such as the absorption of water vapor into the amorphous parts of the powder and by the generation of water vapor from the test tube. After this initial response, there is a large exothermic response caused by the recrystallization of the amorphous material. Also included, but not seen, is the expulsion of excess water from the recrystallized parts and its condensation. Thus, the area under this exothermic recrystallization response is proportional to the heat of recrystallization.

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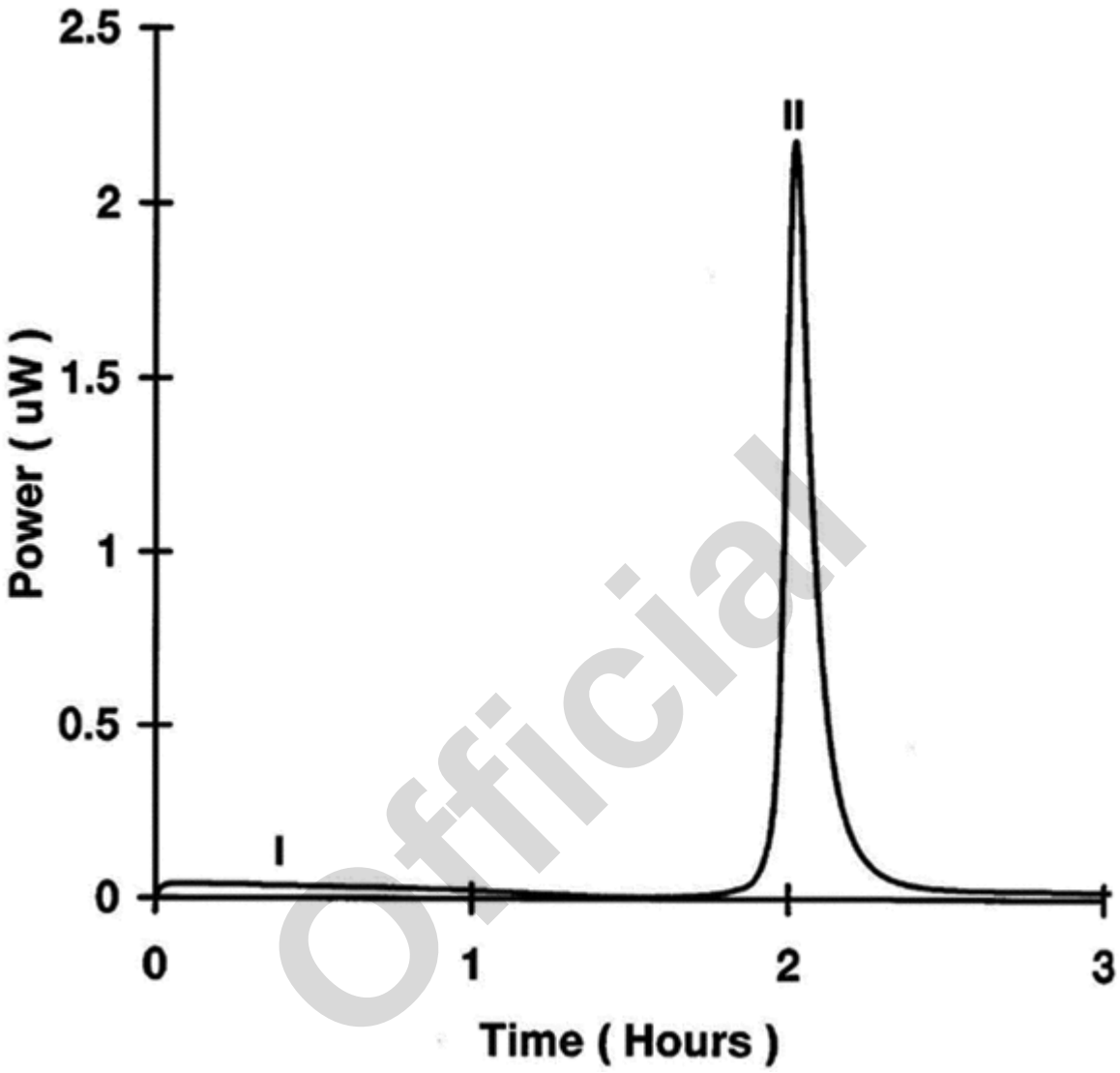


Figure 1. Typical microcalorimetric output of power (in µW) as a function of time (in h): amorphous collapse peak (I) and crystallization peak (II) for mainly amorphous lactose at 25° and 75% relative humidity.

## METHOD 2—SOLUTION CALORIMETRY (DETERMINATION OF CRYSTALLINITY)

Solution calorimetry provides a means of determining enthalpy of solution (i.e., heat of solution under constant atmospheric pressure) of a substance. Enthalpy of solution is defined as the enthalpy of the substance dissolved in the solution to a defined concentration minus the enthalpy of the original substance. The solvent for the dissolution process must be such that the mass of solid dissolves within a time frame that matches the response time of the calorimeter, as discussed below. The enthalpy of solution is proportional to the amount of solid being dissolved. This amount may be defined as one mole for molar enthalpy or as one gram for specific enthalpy. If the substance possesses adequate purity (as determined by the degree of accuracy required) and if its molecular mass is known, the molar enthalpy is preferred; otherwise the specific enthalpy must be used. The enthalpy of solution is weakly dependent on both the temperature, which is usually 25.0°, and the final concentration of the dissolved solute.

It is usually preferred to express the crystallinity, $P_c$, of a substance on a percentage scale. This procedure requires two reference standards, namely a highly crystalline sample assuming 100% crystallinity and having a measured enthalpy of solution of $\Delta H^s_c$, and an amorphous sample assuming 0% crystallinity and having a measured enthalpy of solution of $\Delta H^s_a$. From these values and from the measured enthalpy, $\Delta H^s_s$, of solution of the solid under study, the percentage crystallinity of the solid, $P_c$, may be calculated as follows:

$$P_c\,(\%) = 100(\Delta H^s_s - \Delta H^s_a)/(\Delta H^s_c - \Delta H^s_a)$$

Clearly, crystallinity expressed on a percentage scale depends on three measured values, and the enthalpies of solution may be replaced by other corresponding physical quantities that depend on crystallinity. The value of the percentage crystallinity

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of a sample, however, depends not only on the nature and method of preparation of the two reference standards, but also on the choice of the physical quantity that is measured.

The enthalpy of solution is measured either by an isoperibol (constant perimeter, i.e., jacket) solution calorimeter or by an isothermal (constant temperature) solution calorimeter. Typically, at least three measurements are made with each sample. The mean of these values is then calculated. The exact requirements will depend on the equipment capability and degree of accuracy needed.

## Isoperibol Solution Calorimetry

In the isoperibol solution calorimeter, the heat change during the solution process causes a corresponding change in temperature of the solvent–solute system (i.e., solution). This temperature change is measured by a temperature sensor, which is wired to an electrical circuit that records an electrical signal corresponding to the temperature change. Typically, this temperature change in an electronic form is measured at precisely defined time intervals to produce temperature–time data that are collected, analyzed by a computer, and then plotted. A blank run without addition of the solid solute to the solvent normally shows no discernible change in the slope of the temperature–time plot.

For isoperibol solution calorimeters, response is fairly rapid, but corrections must be made for any heat losses to or heat gains from the bath. Therefore, isoperibol solution calorimeters are more advantageous than isothermal solution calorimeters when the solution process is relatively fast. For all measurements of enthalpy of solution using isoperibol solution calorimeters, the choice of solvent is critical. The nature and mass of the solvent and the mass of sample allow the total heat change, corresponding to total dissolution of the solid, to proceed to completion within five min under vigorous stirring at a constant rotational speed within the range of 400–600 revolutions/min.

The effective heat capacity of the calorimeter cell and its contents is determined for every calorimeter run. This determination is accomplished by electrical heating of the contents of the calorimeter cell. The effective heat capacity is determined according to one of two protocols—either by making one determination after ampul breakage or by making one determination before and a second determination after ampul breakage, and then averaging the two results. The accuracy and reliability of the electrical heating are established by the accuracy and reliability of the aforementioned chemical calibrations.

## Isothermal Solution Calorimetry

In the isothermal (constant temperature) solution calorimeter, the heat change during the solution process is compensated for by an equal but opposite energy change, such that the temperature of the solvent–solute system (i.e., solution) remains essentially constant. This equal but opposite energy change is measured and, when its sign is reversed, provides the enthalpy of solution. For isothermal calorimeters, response is relatively slow, but the compensation process eliminates the effects of heat losses to or heat gains from the bath. Therefore, isothermal calorimeters are more advantageous than isoperibol calorimetry when the solution process is relatively slow.

## Solution Calorimeter Calibration

To ensure the accuracy of the calorimeter, chemical calibrations must be performed on a regular basis. For an endothermic solution process, the calibration of the calorimeter is checked by measuring the heat absorbed during the dissolution of potassium chloride in distilled water at 298.15 K (25.0°). The established enthalpy change in this endothermic process is 235.5 J/g (17.56 kJ/mol). For an exothermic solution process, the calorimeter is checked by measuring the heat evolved during the dissolution of 5 g/L of tromethamine [tris(hydroxymethyl)aminomethane, THAM] in a 0.1 mol/L aqueous hydrochloric acid solution at 298.15 K (25.0°). The established heat for the aforementioned process is –246.0 J/g (–29.80 kJ/mol).

## Sample Handling

The chemical and physical stability of solids may decrease with decreasing crystallinity. In particular, solids of low crystallinity, especially amorphous solids, tend to sorb water vapor from the atmosphere, leading to crystallization and a corresponding gain in crystallinity. For these reasons, anhydrous samples whose crystallinity is to be determined must be stored at zero humidity or below critical humidity levels in sealed chambers containing a desiccant, preferably containing an indicator of effectiveness. If crystallinity–humidity studies are to be carried out, the sample is stored in a sealed chamber containing a saturated salt solution to provide a defined relative humidity.

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# ⟨697⟩ CONTAINER CONTENT FOR INJECTIONS

Each container of an injection contains sufficient excess to allow withdrawal of the labeled quantity of drug (see *Pharmaceutical Dosage Forms* ⟨1151⟩*, Excess Volume in Injections*). Such withdrawal shall be performed according to labeled directions, if provided.

## DETERMINATION OF VOLUME OF INJECTION IN CONTAINERS

This section is harmonized with the corresponding texts of the *European Pharmacopoeia* and/or the *Japanese Pharmacopoeia*. These pharmacopeias have undertaken not to make any unilateral change to this harmonized section. A portion of the present text (see below) is national *USP* text, and therefore not part of the harmonized text; it is marked with symbols (♦,♦) to specify this fact.

Suspensions and emulsions must be shaken before withdrawal of the contents and before the determination of the density. Oily and viscous preparations may be warmed according to the instructions on the label, if necessary, and thoroughly shaken immediately before removing the contents. The contents are then cooled to 20°–25° before measuring the volume. ♦Sterile solid formulations must be constituted according to labeled directions before removing the contents. Contents are then to be measured following the procedures for suspensions, emulsions, or solutions, as appropriate.♦

### Single-Dose Containers

Select 1 container if the volume of the container is 10 mL or more, 3 containers if the nominal volume is more than 3 mL and less than 10 mL, or 5 containers if the nominal volume is 3 mL or less. Take up individually the total contents of each container selected into a dry syringe of a capacity not exceeding three times the volume to be measured and fitted with a 21-gauge needle NLT 2.5 cm (1 inch) in length. Expel any air bubbles from the syringe and needle, and then discharge the contents of the syringe, without emptying the needle, into a standardized, dry cylinder (graduated to contain rather than to deliver the designated volumes) of such size that the volume to be measured occupies at least 40% of its graduated volume. Alternatively, the volume of the contents in mL may be calculated as the mass, in g, divided by the density. For containers with a nominal volume of 2 mL or less, the contents of a sufficient number of containers may be pooled to obtain the volume required for the measurement, provided that a separate, dry syringe assembly is used for each container. The contents of containers holding 10 mL or more may be determined by means of opening them and emptying the contents directly into the graduated cylinder or tared beaker.

The volume is NLT the nominal volume in the case of containers examined individually or, in the case of containers with a nominal volume of 2 mL or less, is NLT the sum of the nominal volumes of the containers taken collectively.

### Multi-Dose Containers

For Injections in multiple-dose containers labeled to yield a specific number of doses of a stated volume, select 1 container, and proceed as directed for single-dose containers, using the same number of separate syringe assemblies as the number of doses specified. The volume is such that each syringe delivers NLT the stated dose.

### Injections in Cartridges or Prefilled Syringes

Select 1 container if the volume is 10 mL or more, 3 containers if the nominal volume is more than 3 mL and less than 10 mL, or 5 containers if the nominal volume is 3 mL or less. If necessary, fit the containers with the accessories required for their use (needle, piston, syringe) and transfer the entire contents of each container without emptying the needle into a dry tared beaker by slowly and constantly depressing the piston. Determine the volume, in mL, calculated as the mass, in g, divided by the density.

The volume measured for each of the containers is NLT the nominal volume.

### Large-Volume Intravenous Solutions

For intravenous solutions, select 1 container. Transfer the contents into a dry measuring cylinder of such a capacity that the volume to be determined occupies at least 40% of the nominal volume of the cylinder. Measure the volume transferred.

The volume is NLT the nominal volume.

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# ⟨698⟩ DELIVERABLE VOLUME

## PURPOSE

The following tests are designed to provide assurance that oral liquids will, when transferred from the original container, deliver the volume of dosage form that is declared on the label.

***Change to read:***

## SCOPE

These tests are applicable to products that are dispensed by pouring from the container. The tests apply whether the products are supplied as liquid preparations or liquid preparations that are constituted from solids upon the addition of a designated volume of a specific diluent. ▲When the container includes a flow restrictor or orifice reducer (e.g., a coupler for an oral syringe), the volume for this test should be discharged according to the labeling advice. These tests▲ (USP 1-Dec-2020) are not required for an article packaged in single-unit containers when the monograph includes the test for *Uniformity of Dosage Units* ⟨905⟩.

## DENSITY DETERMINATION

Because of the tendency of oral liquids to entrain air when shaken or transferred, a more accurate method for determining the delivered volume is to first determine the delivered mass, and then, using the density of the material, to convert the mass to delivered volume. In order to do that, a determination of the density of the material is required. The following is one method to determine density:

1. Tare a 100-mL volumetric flask containing 50.0 mL of water.
2. Add approximately 25 g of well-shaken product, and gently swirl the contents to mix.
3. Reweigh the flask.
4. From a buret, add an accurately measured amount of water to bring the flask contents to volume while gently swirling the contents of the flask. Record the volume taken from the buret.
5. Calculate the density of the sample:

$$\text{Result} = W/V$$

$W$ = weight of the material taken (g)
$V$ = 50.0 minus the volume, in mL, of the water necessary to adjust the contents of the flask to volume

Other methods of determining the density may be employed depending on the formulation (e.g., substantially nonaqueous formulations).

## TEST PREPARATIONS

For the determination of deliverable volume, select NLT 30 containers, and proceed as follows for the dosage form designated.

### Oral Solutions and Oral Suspensions

Shake the contents of 10 containers individually.

### Powders That Are Labeled to State the Volume of Oral Liquid That Results When the Powder Is Constituted with the Volume of Diluent Stated in the Labeling

Constitute 10 containers with the volume of diluent stated in the labeling, accurately measured, and shake individually.

***Change to read:***

## PROCEDURE

The deliverable volume can be determined by weight as follows:

1. Discharge the container contents into a suitable tared container (allowing drainage for NMT 5 s for single-▲unit▲ (USP 1-Dec-2020) containers and NMT 10 min for multiple-unit containers).
2. Determine the mass of the contents.
3. Calculate the volume using the density.

Alternatively, the following by-volume procedure may be used:

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1. Under conditions of use or as instructed in the labeling, carefully discharge the contents of each container into separate dry graduated cylinders of a rated capacity not exceeding 2.5 times the volume to be measured and calibrated "to contain" (see *Volumetric Apparatus* ⟨31⟩). Care must be taken to avoid the formation of air bubbles during the process. In the absence of labeling instructions, support the containers at about a 30° angle to the horizontal, and gently discharge the contents into the graduated cylinder.
2. Allow each container to drain for a period not to exceed 10 min for multiple-unit containers and 5 s for single-unit containers, unless otherwise specified in the monograph.
3. When free from bubbles, measure the volume of each mixture.

## ACCEPTANCE CRITERIA

Use the following criteria to determine compliance with this test.

### For Multiple-Unit Containers (see *Figure 1*)

The average volume of liquid obtained from the 10 containers is NLT 100%, and the volume of no container is less than 95% of the volume declared in the labeling. If (A), the average volume is less than 100% of that declared in the labeling, but the volume of no container is less than 95% of the labeled amount, or if (B), the average volume is NLT 100% and the volume of NMT 1 container is less than 95%, but is NLT 90% of the labeled volume, perform the test on 20 additional containers. The average volume of liquid obtained from the 30 containers is NLT 100% of the volume declared in the labeling; the volume of liquid obtained from NMT 1 of the 30 containers is less than 95%, but NLT 90% of that declared in the labeling.

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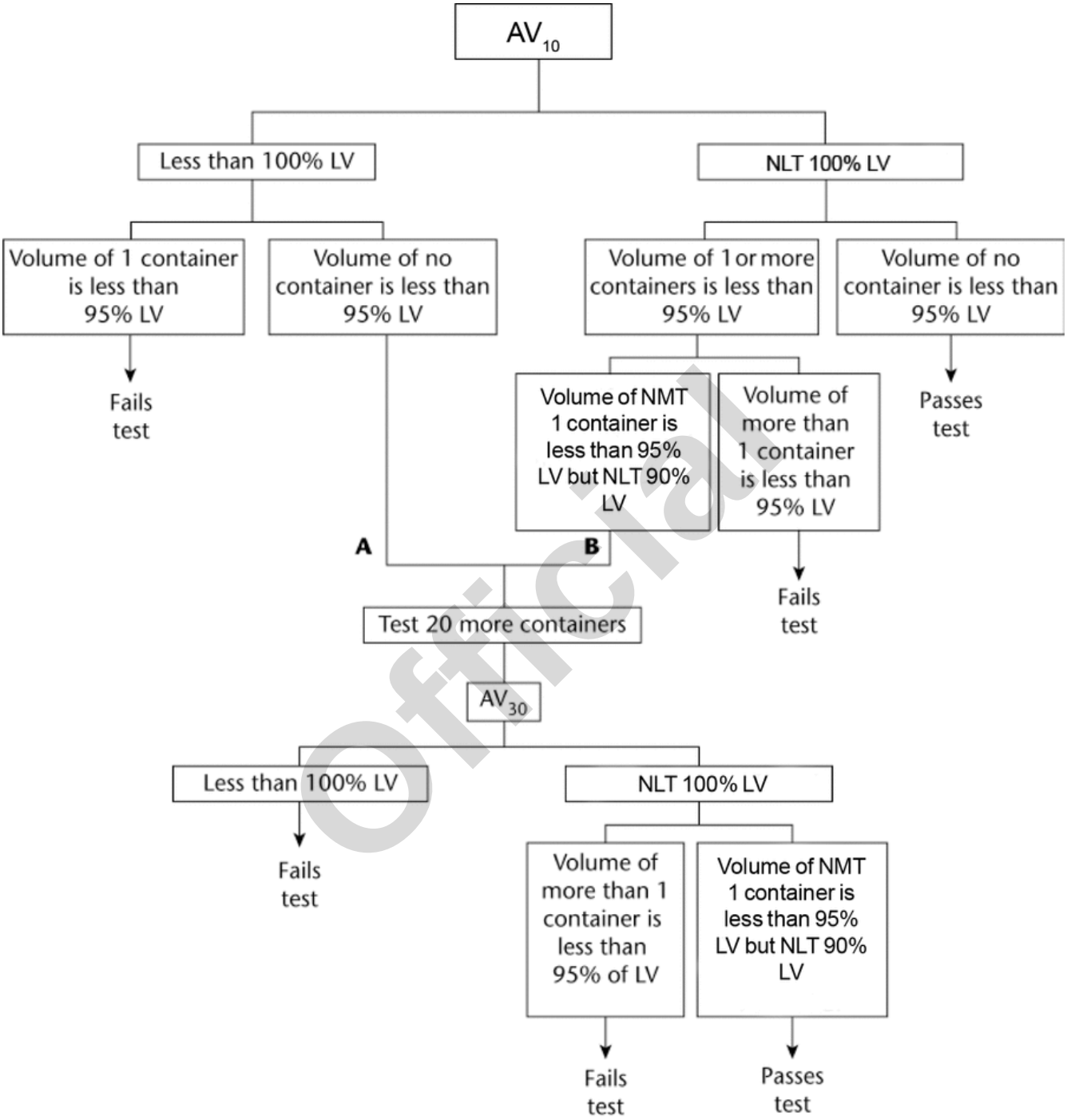


Figure 1. Decision scheme for multiple-unit containers (AV = average volume; LV = labeled volume).

## For Single-Unit Containers (see *Figure 2*)

The average volume of liquid obtained from the 10 containers is NLT 100%, and the volume of each of the 10 containers lies within the range of 95%–110% of the volume declared in the labeling. If (A), the average volume is less than 100% of that declared in the labeling, but the volume of no container is outside the range of 95%–110%, or if (B), the average volume is NLT 100% and the volume of NMT 1 container is outside the range of 95%–110%, but within the range of 90%–115%, perform the test on 20 additional containers. The average volume of liquid obtained from the 30 containers is NLT 100% of the volume declared in the labeling; the volume obtained from NMT 1 of the 30 containers is outside the range of 95%–110%, but within the range of 90%–115% of the volume declared on the labeling.

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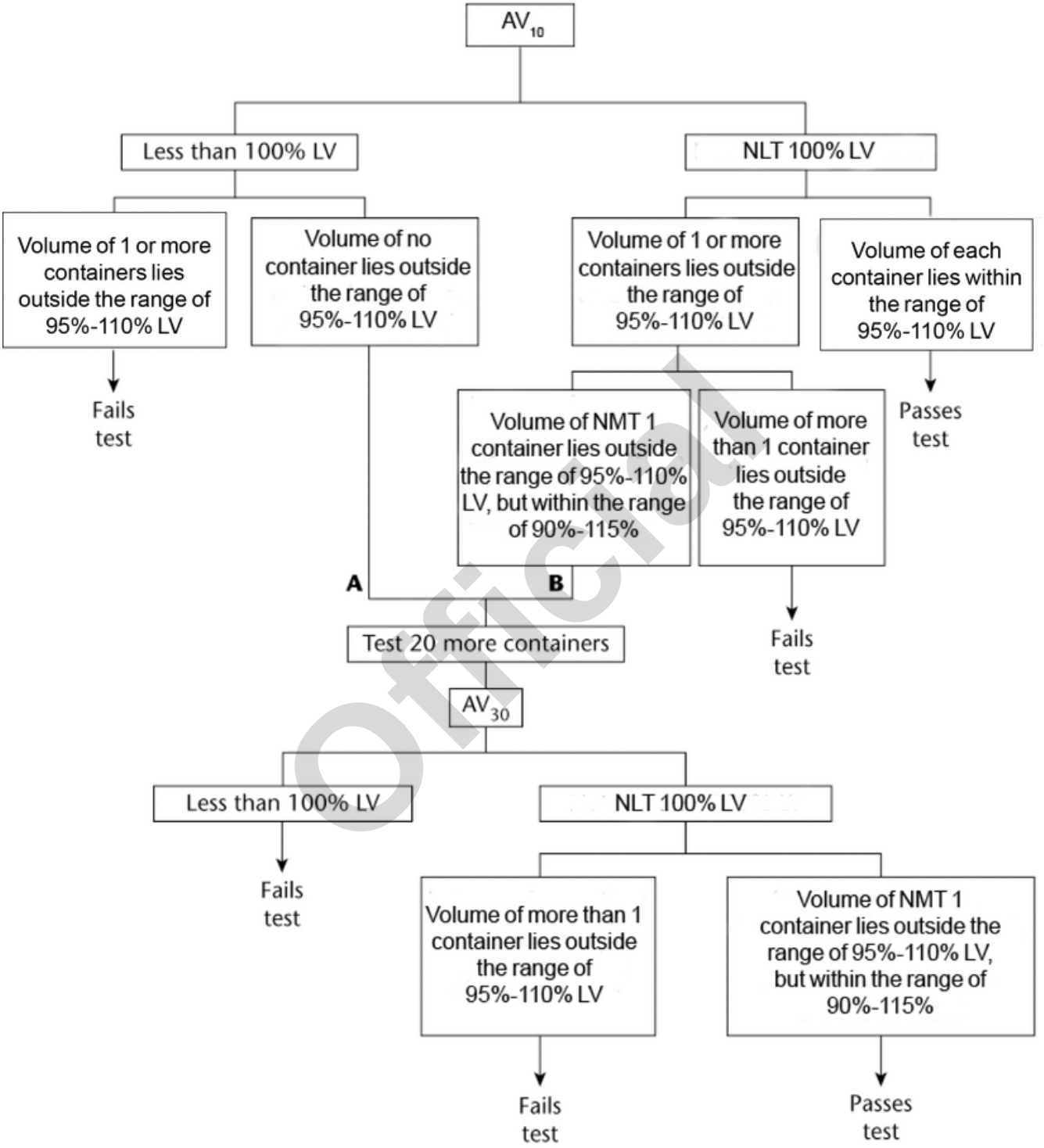


Figure 2. Decision scheme for single-unit containers (AV = average volume; LV = labeled volume).

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# ⟨699⟩ DENSITY OF SOLIDS

## TERMS AND DEFINITIONS

Density refers to the average spatial distribution of mass in a material. The density of solids typically is expressed in g per $cm^3$, in contrast to fluids, where the density is commonly expressed in g per mL at a stated reference temperature.

The density of a solid particle can assume different values depending on the method used to measure the volume of the particle. It is useful to distinguish among three different possibilities.

The *true density* of a substance is the average mass per unit volume, exclusive of all voids that are not a fundamental part of the molecular packing arrangement. It is a property of a particular material, and hence should be independent of the method of determination. The true density of a perfect crystal can be determined from the size and composition of the unit cell.

The *pycnometric density*, as measured by gas pycnometry, is a convenient density measurement for pharmaceutical powders. In a gas pycnometer, the volume occupied by a known mass of powder is determined by measuring the volume of gas displaced by the powder. The quotient of the mass and volume is the pycnometric density. The pycnometric density equals the true density unless the material contains impenetrable voids, or sealed pores, that are inaccessible to the gas used in the pycnometer.

The *granular density* includes contributions to particle volume from open pores smaller than some limiting size. The size limit depends on the method of measurement. A common measurement technique is mercury porosimetry, where the limiting pore size depends upon the maximum intrusion pressure. Because of the additional contribution from pore volume, the granular density will never be greater than the true density. A related concept is the *aerodynamic density*, which is the density of the particle with a volume defined by the aerodynamic envelope of the particle in a flowing stream. Both the closed and open pores contribute to this volume, but the open pores fill with the permeating fluid. The aerodynamic density, therefore, depends on the density of the test fluid if the particle is porous.

For brevity, the pycnometric density and the true density are both referred to as density. If needed, these quantities may be distinguished based on the method of measurement.

The density of a material depends on the molecular packing. For gases and liquids, the density will depend only on temperature and pressure. For solids, the density will also vary with the crystal structure and degree of crystallinity. If the solids are amorphous, the density may further depend upon the history of preparation and treatment. Therefore, unlike fluids, the densities of two chemically equivalent solids may be different, and this difference reflects a difference in solid-state structure. The density of constituent particles is an important physical characteristic of pharmaceutical powders.

Beyond these definitions of particle density, the *bulk density* of a powder includes the contribution of interparticulate void volume. Hence, the bulk density depends on both the density of powder particles and the packing of powder particles.

## GAS PYCNOMETRY FOR THE MEASUREMENT OF DENSITY

Gas pycnometry is a convenient and suitable method for the measurement of the density of powder particles. A simple schematic of one type of gas pycnometer is shown in *Figure 1.*

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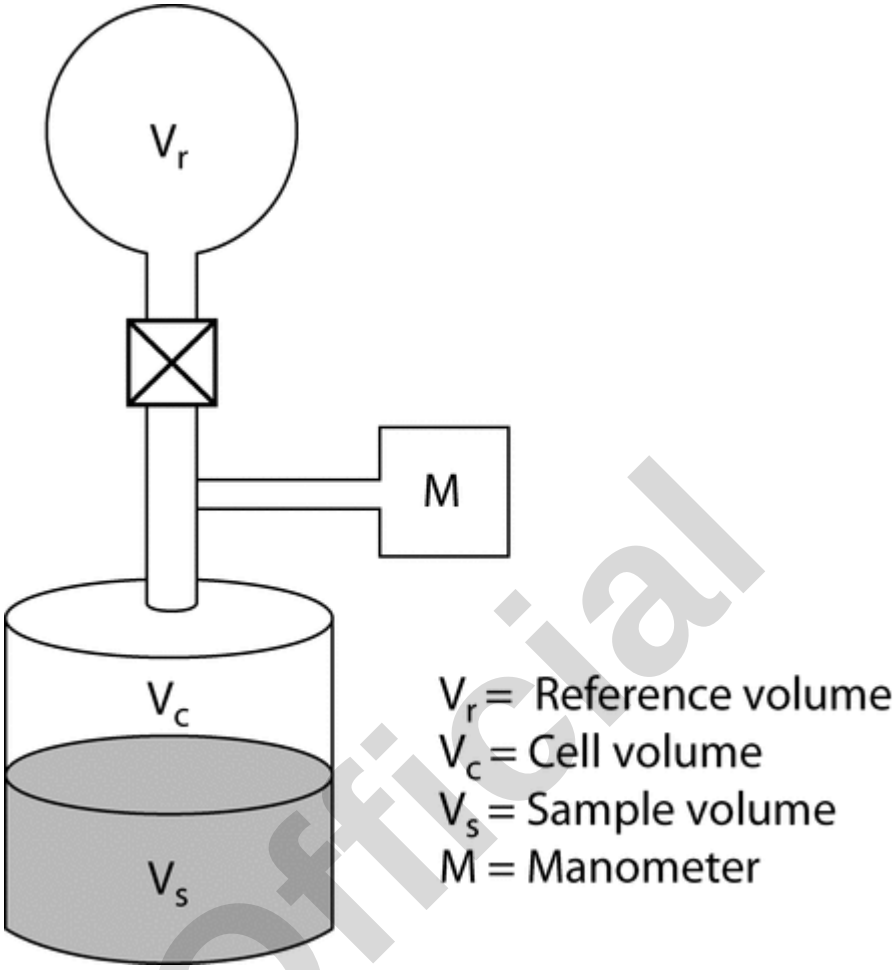


Figure 1. Schematic of gas pycnometer.

The sample, with mass *w* and volume $V_s$, is placed inside a sealed test cell with an empty cell volume of $V_c$. The system reference pressure, $P_r$, is determined at the manometer while the valve that connects the reference volume with the test cell is open. The valve is closed to separate the reference volume, $V_r$, from the test cell. The test cell is pressurized with the measurement gas to an initial pressure, $P_i$. Then the valve is opened to connect the reference volume, $V_r$, with the test cell, and the pressure drops to the final pressure, $P_f$. If the measurement gas behaves ideally under the conditions of measurement, the sample volume, $V_s$, is given by the following expression:

$$V_s = V_c - \frac{V_r}{\left[\frac{P_i - P_r}{P_f - P_r}\right] - 1} \quad (1)$$

The density, ρ, is given by the equation:

$$\rho = \frac{w}{V_S} \quad (2)$$

Details of the instrumental design may differ, but all gas pycnometers rely on the measurement of pressure changes as a reference volume is added to, or deleted from, the test cell.

The measured density is a volume-weighted average of the densities of individual powder particles. The density will be in error if the test gas sorbs onto the powder or if volatile contaminants are evolved from the powder during the measurement. Sorption is prevented by an appropriate choice of test gas. Helium is the common choice. Volatile contaminants in the powder are removed by degassing the powder under a constant purge of helium prior to the measurement. Occasionally, powders may have to be degassed under vacuum. Two consecutive readings should yield sample volumes that are equal within 0.2% if volatile contaminants are not interfering with the measurements. Because volatiles may be evolved during the measurement, the weight of the sample should be taken after the pycnometric measurement of volume.

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## Method

Ensure that the reference volume and the calibration volume have been determined for the gas pycnometer by an appropriate calibration procedure. The test gas is helium, unless another gas is specified in the individual monograph. The temperature of the gas pycnometer should be between 15° and 30° and should not vary by more than 2° during the course of the measurement. Load the test cell with the substance under examination that has been prepared according to the individual monograph. Where ⟨699D⟩ is indicated, dry the substance under examination as directed for *Loss on drying* in the monograph unless other drying conditions are specified in the monograph *Density of solids* test. Where ⟨699U⟩ is indicated, the substance under examination is used without drying. Use a quantity of powder recommended in the operating manual for the pycnometer. Seal the test cell in the pycnometer, and purge the pycnometer system with the test gas according to the procedure given in the manufacturer's operating instructions. If the sample must be degassed under vacuum, follow the recommendations in the individual monographs and the instructions in the operating manual for the pycnometer.

The measurement sequence above describes the procedure for the gas pycnometer shown in *Figure 1*. If the pycnometer differs in operation or in construction from the one shown in *Figure 1*, follow the operating procedure given in the manual for the pycnometer.

Repeat the measurement sequence for the same powder sample until consecutive measurements of the sample volume, $V_s$, agree to within 0.2%. Unload the test cell and measure the final powder weight, $w$. Calculate the pycnometric density, $\rho$, of the sample according to *Equation 2*.

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# ⟨701⟩ DISINTEGRATION

***Change to read:***

This general chapter is harmonized with the corresponding texts of the *European Pharmacopoeia* and/or the *Japanese Pharmacopoeia*. The texts of these pharmacopeias are therefore interchangeable, and the methods of the *European Pharmacopoeia* and/or the *Japanese Pharmacopoeia* may be used for demonstration of compliance instead of the present general chapter. These pharmacopeias have undertaken not to make any unilateral change to this harmonized chapter.

Portions of the present general chapter text that are national *USP* text, and therefore not part of the harmonized text, are marked with symbols (⬥⬥) to specify this fact.

This test is provided to determine whether tablets, capsules, ▲⬥or granules⬥▲ (USP 1-Aug-2019) disintegrate within the prescribed time when placed in a liquid medium at the experimental conditions presented below. ⬥Compliance with the limits on *Disintegration* stated in the individual monographs is required. ▲▲ (USP 1-Aug-2019) Determine the type of units under test from the labeling and from observation, and apply the appropriate procedure to 6 or more dosage units.⬥

For the purposes of this test, disintegration does not imply complete solution of the unit or even of its active constituent. Complete disintegration is defined as that state in which any residue of the unit, except fragments of insoluble coating or capsule shell, remaining on the screen of the test apparatus or adhering to the lower surface of the disk, if used, is a soft mass having no palpably firm core.

***Change to read:***

## APPARATUS

The apparatus consists of a basket-rack assembly, a 1000-mL low-form beaker 138–160 mm in height and having an inside diameter of 97–115 mm for the immersion fluid, a thermostatic arrangement for heating the fluid 35°–39°, and a device for raising and lowering the basket in the immersion fluid at a constant frequency rate 29–32 cycles/min through a distance of NLT 53 mm and NMT 57 mm. The volume of the fluid in the vessel is such that at the highest point of the upward stroke, the wire mesh remains at least 15 mm below the surface of the fluid and descends to NLT 25 mm from the bottom of the vessel on the downward stroke. At no time should the top of the basket-rack assembly become submerged. The time required for the upward stroke is equal to the time required for the downward stroke, and the change in stroke direction is a smooth transition, rather than an abrupt reversal of motion. The basket-rack assembly moves vertically along its axis. There is no appreciable horizontal motion or movement of the axis from the vertical.

### Basket-Rack Assembly

The basket-rack assembly consists of 6 open-ended transparent tubes, each ▲75.0–80.0▲ (USP 1-May-2020) mm long and having an inside diameter of 20.7–23 mm and a wall 1.0–2.8 mm thick; the tubes are held in a vertical position by two plates, each 88–92 mm in diameter and 5–8.5 mm in thickness, with 6 holes, each 22–26 mm in diameter, equidistant from the center of the plate and equally spaced from one another. Attached to the under surface of the lower plate is a woven stainless steel wire cloth, which has a plain square weave with 1.8–2.2-mm apertures and with a wire diameter of 0.57–0.66 mm. The parts of the apparatus are assembled and rigidly held by means of 3 bolts passing through the 2 plates. A suitable means is provided to suspend the basket-rack assembly from the raising and lowering device using a point on its axis.

The design of the basket-rack assembly may be varied somewhat, provided the specifications for the glass tubes and the screen mesh size are maintained. The basket-rack assembly conforms to the dimensions found in *Figure 1*.

### Disks

The use of disks is permitted only where specified or allowed ⬥in the monograph. If specified in the individual monograph,⬥ each tube is provided with a cylindrical disk ▲9.35–9.65▲ (USP 1-May-2020) mm thick and ▲20.55–20.85▲ (USP 1-May-2020) mm in diameter. The disk is made of a suitable transparent plastic material having a specific gravity of 1.18–1.20. Five parallel ▲1.9–2.1▲ (USP 1-May-2020)-mm holes extend between the ends of the cylinder. One of the holes is centered on the cylindrical axis. The other holes are ▲parallel to the cylindrical axis and▲ (USP 1-May-2020) centered ▲5.8–6.2▲ (USP 1-May-2020) mm from the axis on imaginary lines perpendicular to the axis and ▲▲ (USP 1-May-2020) to each other. Four identical trapezoidal-shaped planes are cut into the wall of the cylinder, nearly perpendicular to the ends of the cylinder. The trapezoidal shape is symmetrical; its parallel sides coincide with the ends of the cylinder and are parallel to an imaginary line connecting the centers of two adjacent holes 6 mm from the cylindrical axis. The parallel side of the trapezoid on the bottom of the cylinder has a length of ▲1.5–1.7▲ (USP 1-May-2020) mm, and its bottom edges lie at a depth of 1.5–1.8 mm from the cylinder's circumference. The parallel side of the trapezoid on the top of the cylinder has a length ▲9.2–9.6▲ (USP 1-May-2020) mm, and its center lies at a depth ▲2.5–2.7▲ (USP 1-May-2020) mm from the cylinder's circumference. All surfaces of the disk are smooth. If the use of disks is specified ⬥in the individual monograph,⬥ add a disk to each tube, and operate the apparatus as directed under *Procedure*. The disks conform to dimensions found in *Figure 1*.[1]

[1] The use of automatic detection employing modified disks is permitted where the use of disks is specified or allowed. Such disks must comply with the requirements for density and dimension given in this chapter.

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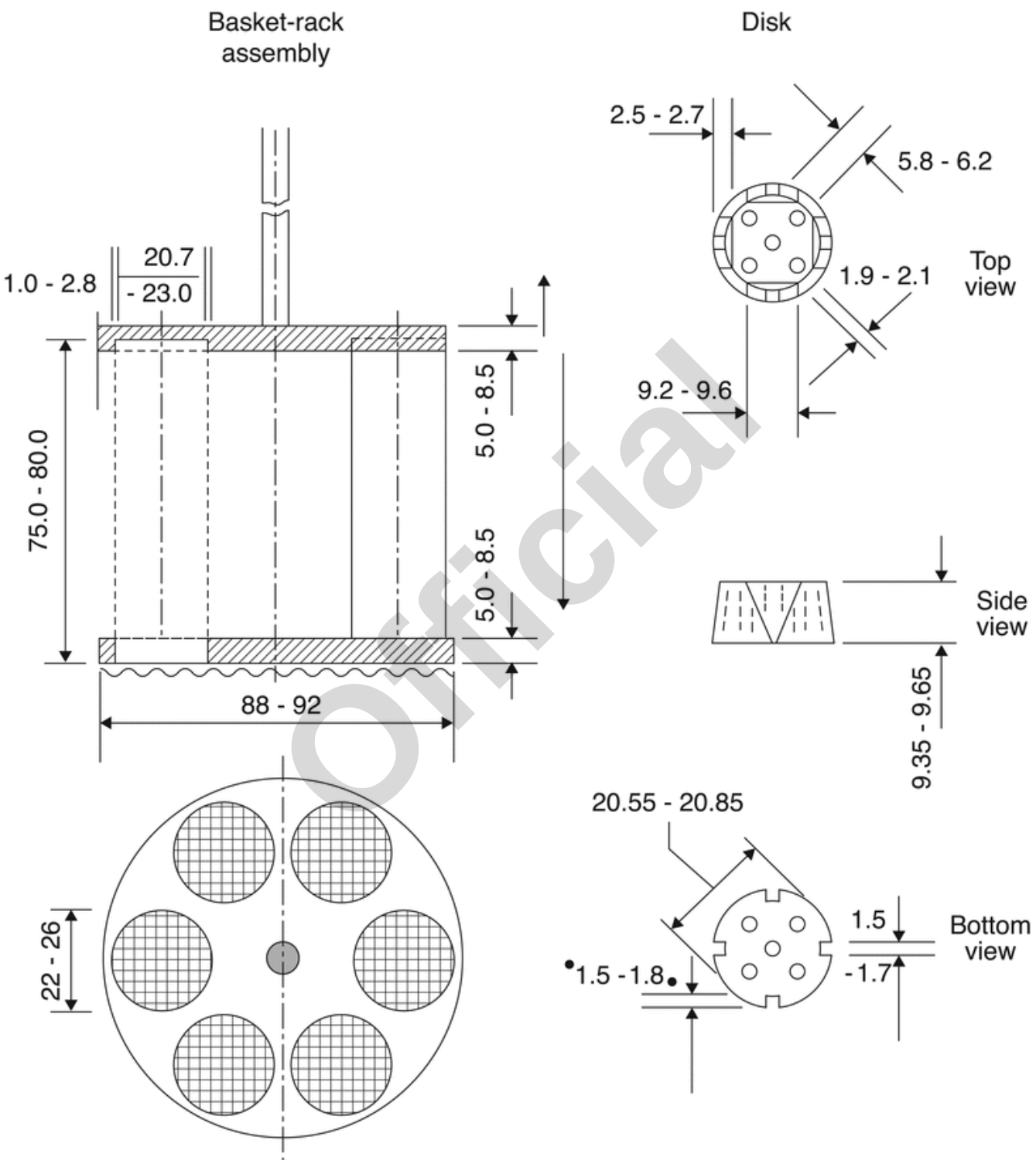


Figure 1. Disintegration apparatus. (All dimensions are expressed in mm.)

▲ (USP 1-May-2020)

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# PROCEDURE ◆▲AND CRITERIA

## Procedure and Criteria for▲ (USP 1-Aug-2019) Uncoated ▲or Plain-Coated▲ (USP 1-Aug-2019) Tablets

▲PROCEDURE FOR UNCOATED OR PLAIN-COATED TABLETS▲ (USP 1-Aug-2019)◆

Place 1 dosage unit in each of the 6 tubes of the basket▲◆-rack assembly◆▲ (USP 1-Aug-2019) and, if prescribed, add a disk. Operate the apparatus, using ◆water or◆ the specified medium as the immersion fluid, maintained at 37 ± 2°. At the end of the time limit specified ◆in the monograph,◆ lift the basket▲◆-rack assembly◆▲ (USP 1-Aug-2019) from the fluid, and observe the tablets. All of the tablets ▲◆should◆▲ (USP 1-Aug-2019) have disintegrated completely. If 1 or 2 tablets fail to disintegrate completely, repeat the test on 12 additional tablets.

▲◆CRITERIA FOR UNCOATED OR PLAIN-COATED TABLETS

1. If 6 tablets are tested, all 6 of the tablets are disintegrated.
2. If 18 tablets are tested,◆ the requirement is met if not fewer than 16 of the total of 18 tablets are disintegrated.▲ (USP 1-Aug-2019)

## ◆▲Procedure and Criteria for Delayed-Release Tablets and Capsules (tablets or capsules that are formulated with acid-resistant or enteric coatings)

PROCEDURE FOR DELAYED-RELEASE TABLETS AND CAPSULES▲ (USP 1-Aug-2019)

Place 1 ▲dosage unit▲ (USP 1-Aug-2019) in each of the 6 tubes of the basket. ▲If the dosage units are not sugar-coated, proceed to the *Acid stage*. If testing tablets that have a soluble external sugar coating, immerse the basket in water at room temperature for 5 min and then immediately proceed to the *Acid stage*. If specified in the monograph, add 1 disk to each tube.

**Acid stage**

**Immersion fluid:** 0.1 M hydrochloric acid, or simulated gastric fluid TS, or as specified in the monograph
**Temperature:** 37 ± 2°
**Time:** 1 h

If after 1 h no dosage unit shows evidence of disintegration, cracking, or softening, proceed with the *Buffer stage*.

**Buffer stage**

**Immersion fluid:** pH 6.8 phosphate buffer, or simulated intestinal fluid TS, or as specified in the monograph
**Temperature:** 37 ± 2°
**Time:** As specified in the individual monograph

CRITERIA FOR DELAYED-RELEASE TABLETS AND CAPSULES

**Acid stage:** No dosage unit shows evidence of disintegration, cracking, or softening.
**Buffer stage:** Apply the *Criteria for Uncoated or Plain-Coated Tablets*.

## Procedure and Criteria for Buccal Tablets, Sublingual Tablets, Capsules, Tablets for Oral Suspension, Tablets for Oral Solution, Tablets for Topical Solution, Orally Disintegrating Tablets, and Chewable Tablets

Apply the *Procedure and Criteria for Uncoated or Plain-Coated Tablets*.

## Procedure and Criteria for Effervescent Tablets for Oral Solution

PROCEDURE FOR EFFERVESCENT TABLETS FOR ORAL SOLUTION

Place 1 tablet in each of 6 beakers containing 200 mL of water. A suitable beaker will have a nominal volume of 250–400 mL.
**Time:** 5 min or as specified in the individual monograph

CRITERIA FOR EFFERVESCENT TABLETS FOR ORAL SOLUTION

Numerous bubbles of gas are evolved. After 5 min or as specified in the individual monograph, the evolution of gas around each tablet or its fragments has ceased. Each tablet has dissolved or disintegrated and has been dispersed in the water so that no appreciable agglomerates remain.

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# Procedure and Criteria for Effervescent Granules

## PROCEDURE FOR EFFERVESCENT GRANULES

In each of 6 beakers containing 200 mL of water place 1 dose of the effervescent granules. A suitable beaker will have a nominal volume of 250–400 mL.
**Time:** 5 min or as specified in the individual monograph

## CRITERIA FOR EFFERVESCENT GRANULES

Numerous bubbles of gas are evolved. After 5 min or as specified in the individual monograph, the evolution of gas around the granules in each beaker has ceased. The granules have dissolved or disintegrated and have been dispersed in the water. ▲ (USP 1-Aug-2019) ◆

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# ⟨705⟩ QUALITY ATTRIBUTES OF TABLETS LABELED AS HAVING A FUNCTIONAL SCORE

## PURPOSE

This chapter provides quality attributes for products with approved labeling indicating that the tablets can be split to produce multiple portions that have an accurate fractional dose (labeled as functionally scored). The label claim of the split portions should be a simple fractional part of the claim for the intact tablet based on the number of scores and the size of the split portion (e.g., one-half, one-third, or one-quarter). At the time of splitting, the intact tablets should conform to the monograph specification. With the exception of dose, each split portion from tablets labeled as having a functional score are expected to conform to the quality attributes of the whole tablets. The split portions resulting from subdividing a functionally scored tablet should conform to the tests for *Splitting Tablets with Functional Scoring* and *Dissolution* or *Disintegration* given in this chapter.

## SCOPE

This chapter applies to tablets labeled as having a functional score and to the split portions that represent any labeled fraction of the whole functionally scored tablet dose. Tablets should be split as part of the test procedure and the storage conditions for the split portions should be defined in the test procedure. For *Dissolution* or *Disintegration* testing, analysts should use only split portions from tablets determined to be acceptable by the *Splitting Tablets with Functional Scoring* test.

## SPLITTING TABLETS WITH FUNCTIONAL SCORING

### Test Procedure

1. Take a random sample of 30 intact tablets, and proceed as follows.
2. Accurately weigh each tablet, and record its weight.
3. For each intact tablet, determine the expected weight of the split portions by dividing the whole-tablet weight by the designated number of split portions indicated on the labeling.
4. Split each tablet by hand (without mechanical assistance) into the designed number of split portions, and weigh each split portion.
5. For each tablet, determine the percent of the expected weight in each split portion.

An acceptable tablet breaks into the designed number of segments, and each split portion has NLT 75% and NMT 125% of the expected weight of the split tablet portion. [NOTE—Set aside split tablet portions derived from acceptable tablets for subsequent testing for dissolution or disintegration.]

**Acceptance criteria:** NLT 28 of the 30 tablets are acceptable.

## DISSOLUTION

Use split portions from tablets that are acceptable according to the *Splitting Tablets with Functional Scoring* test.

### Immediate-Release Tablets

Dissolution for immediate-release tablets is performed at the $S_2$ stage (see *Dissolution* ⟨711⟩). Test 12 split tablet portions according to the specified *Medium, Apparatus, Times*, and *Analysis*. The average of the 12 results is NLT *Q,* and no result is less than $Q - 15\%$.

### Extended-Release Tablets

Perform dissolution testing of split tablet portions from extended-release tablets by one of the two alternative procedures. The procedure to be used is specified in the monograph.

**Procedure 1** (*Procedure for Extended-Release Dosage Forms, Dissolution* ⟨711⟩)**:** Individually test 12 split tablet portions and 12 intact tablets.

**Medium, Apparatus,** and **Analysis:** As given in the monograph following the appropriate test number found on the labeling. Dissolution profile test time points are determined as follows. From the appropriate dissolution test in the monograph, use the time points given. At a minimum, use three time points with no more than one time point where the results exceed 85% dissolved.

Calculate the similarity factor ($f_2$) for the intact-tablet results and the split-tablet portion results:

$$f_2 = 50 \cdot \log\left\{\left[1 + (1/n)\sum_{t=1}^{n}\left(R_t - T_t\right)^2\right]^{-0.5} \cdot 100\right\}$$

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$R_t$ = cumulative percentage of the labeled drug dissolved at each of the selected *n* time points of the intact tablets
$T_t$ = cumulative percentage of the labeled drug dissolved at each of the selected *n* time points of the split tablet portions

**Acceptance criteria:** The calculated $f_2$ is NLT 50 (acceptable range: 50–100).

**Procedure 2** (*Procedure for Extended-Release Dosage Forms, Dissolution* ⟨711⟩)**:** Use a split-tablet portion as the dosage unit. Individually test 12 dosage units.

**Medium, Apparatus, Times,** and **Analysis:** As given in the monograph following the appropriate test number found on the labeling.

**Acceptance criteria:** The percentages of the labeled amount released at the times specified conform to the $L_2$ level criteria of *Acceptance Table 2* in ⟨711⟩.

## DISINTEGRATION

Disintegration testing is necessary only when used as a surrogate for dissolution testing as specified in the monograph. Follow the procedure using split portions from tablets that are acceptable according to the *Splitting Tablets with Functional Scoring* test as the dosage unit (see *Disintegration* ⟨701⟩).

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# ⟨711⟩ DISSOLUTION

This general chapter is being harmonized with the corresponding texts of the *European Pharmacopoeia* and/or the *Japanese Pharmacopoeia*. These pharmacopeias have undertaken to not make any unilateral change to this harmonized chapter.

Portions of the present general chapter text that are national *USP* text, and therefore not part of the harmonized text, are marked with symbols (•,) to specify this fact.

This test is provided to determine compliance with the dissolution requirements •where stated in the individual monograph,• for dosage forms administered orally. In this general chapter, a dosage unit is defined as 1 tablet or 1 capsule or the amount specified. •Of the types of apparatus designs described herein, use the one specified in the individual monograph. Where the label states that an article is enteric coated and a dissolution or disintegration test does not specifically state that it is to be applied to delayed-release articles and is included in the individual monograph, the procedure and interpretation given for *Delayed-Release Dosage Forms* are applied, unless otherwise specified in the individual monograph.•

## •FOR DOSAGE FORMS CONTAINING OR COATED WITH GELATIN

If the dosage form containing gelatin does not meet the criteria in the appropriate *Acceptance Table* (see *Interpretation*, *Immediate-Release Dosage Forms*, *Extended-Release Dosage Forms*, or *Delayed-Release Dosage Forms*) because of evidence of the presence of cross-linking, the dissolution procedure should be repeated with the addition of enzymes to the medium, as described below, and the dissolution results should be evaluated starting at the first stage of the appropriate *Acceptance Table*. It is not necessary to continue testing through the last stage (up to 24 units) when criteria are not met during the first stage testing, and evidence of cross-linking is observed.

Gelatin, in the presence of certain compounds and/or in certain storage conditions, including but not restricted to high humidity and temperature, may present cross-linking. A pellicle may form on the external and/or internal surface of the gelatin capsule shell or on the dosage form that prevents the drug from being released during dissolution testing (see more information in *Capsules—Dissolution Testing and Related Quality Attributes* ⟨1094⟩).

[NOTE—All references to a chapter above ⟨1000⟩ are for information purposes only and for use as a helpful resource. These chapters are not mandatory unless explicitly called out for this application.]

### Dissolution Medium with pH ≤4.0

**Enzyme:** Pepsin, activity determined by the procedure in *purified pepsin*, in the *Reagent Specifications* section
**Amount:** A quantity of pepsin that results in an activity of not more than 750,000 units/L of dissolution medium

### Dissolution Medium with pH >4.0 and <6.8

**Enzyme:** Papain, activity determined by the *Assay* test in the monograph for *Papain*; or bromelain, activity determined by the procedure in *bromelain*, in the *Reagent Specifications* section
**Amount:** A quantity of papain that results in an activity of not more than 550,000 units/L of dissolution medium, or a quantity of bromelain that results in an activity of not more than 30 gelatin-digesting units (GDU)/L of dissolution medium

### Dissolution Medium with pH ≥6.8

**Enzyme:** Pancreatin, protease activity determined by the procedure in *Assay for protease activity* (*Casein digestive power*) in the monograph for *Pancreatin*
**Amount:** A quantity of pancreatin that results in a protease activity of not more than 2000 units/L of dissolution medium

## Dissolution Medium Containing Surfactant or Other Ingredients Known to Denature the Enzyme

If the dissolution medium contains surfactant or other ingredients that are known to denature the enzyme used, a pretreatment step in the dissolution testing of the dosage form may be applied. This pretreatment step is done using the specified dissolution medium without the surfactant or the ingredient and with the addition of the appropriate amount of enzyme according to the medium pH. The amount of enzyme added is appropriate to the volume of dissolution medium used in the pretreatment. To achieve the specified medium volume for the final dissolution testing, the pretreatment step may be conducted with a smaller volume of medium without the ingredient such that the final volume is obtained when the ingredient is added at the end of the pretreatment step. All of the other conditions of the test (apparatus, rotation, or flow rate) should remain as described in the method or monograph. Typically, the duration of the pretreatment step is not more than 15 min. The required pretreatment time should be evaluated on a case-by-case basis and should be scientifically justified. This time should be included in the total time of the test. As an example, if the total time of the test is 45 min and 15 min are used in the pretreatment step, the test will continue for 30 min after the addition of the ingredient.•

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# APPARATUS

## Apparatus 1 (Basket Apparatus)

The assembly consists of the following: a vessel, which may be covered, and made of glass or other inert, transparent material[1] ; a motor; a metallic drive shaft; and a cylindrical basket. The vessel is partially immersed in a suitable water bath of any convenient size or heated by a suitable device, such as a heating jacket. The water bath or heating device permits holding the temperature inside the vessel at 37 ± 0.5° during the test and keeps the bath fluid in constant, smooth motion. No part of the assembly, including the environment in which the assembly is placed, contributes significant motion, agitation, or vibration beyond that due to the smoothly rotating, stirring element. An apparatus that permits observation of the specimen and of the stirring element during the test is preferable. The vessel is cylindrical, with a hemispherical bottom and •with one of the following dimensions and capacities: for a nominal• capacity of 1 L, the height is 160–210 mm, and its inside diameter is 98–106 mm; •for a nominal capacity of 2 L, the height is 280–300 mm, and its inside diameter is 98–106 mm; and for a nominal capacity of 4 L, the height is 280–300 mm, and its inside diameter is 145–155 mm•. Its sides are flanged at the top. A fitted cover may be used to retard evaporation.[2] The shaft is positioned so that its axis is not more than 2 mm at any point from the vertical axis of the vessel and rotates smoothly and without significant wobble that could affect the results. A speed-regulating device is used that allows the shaft rotation speed to be selected and maintained at the specified rate •given in the individual monograph• within ±4%.

Shaft and basket components of the stirring element are fabricated of stainless steel, type 316, or other inert material, to the specifications shown in *Figure 1*. A basket having a gold coating of about 0.0001 inch (2.5 µm) thick may be used. A dosage unit is placed in a dry basket at the beginning of each test. The distance between the inside bottom of the vessel and the bottom of the basket is maintained at 25 ± 2 mm during the test.

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[1] The materials should not sorb, react, or interfere with the specimen being tested.
[2] If a cover is used, it provides sufficient openings to allow ready insertion of the thermometer and withdrawal of specimens.

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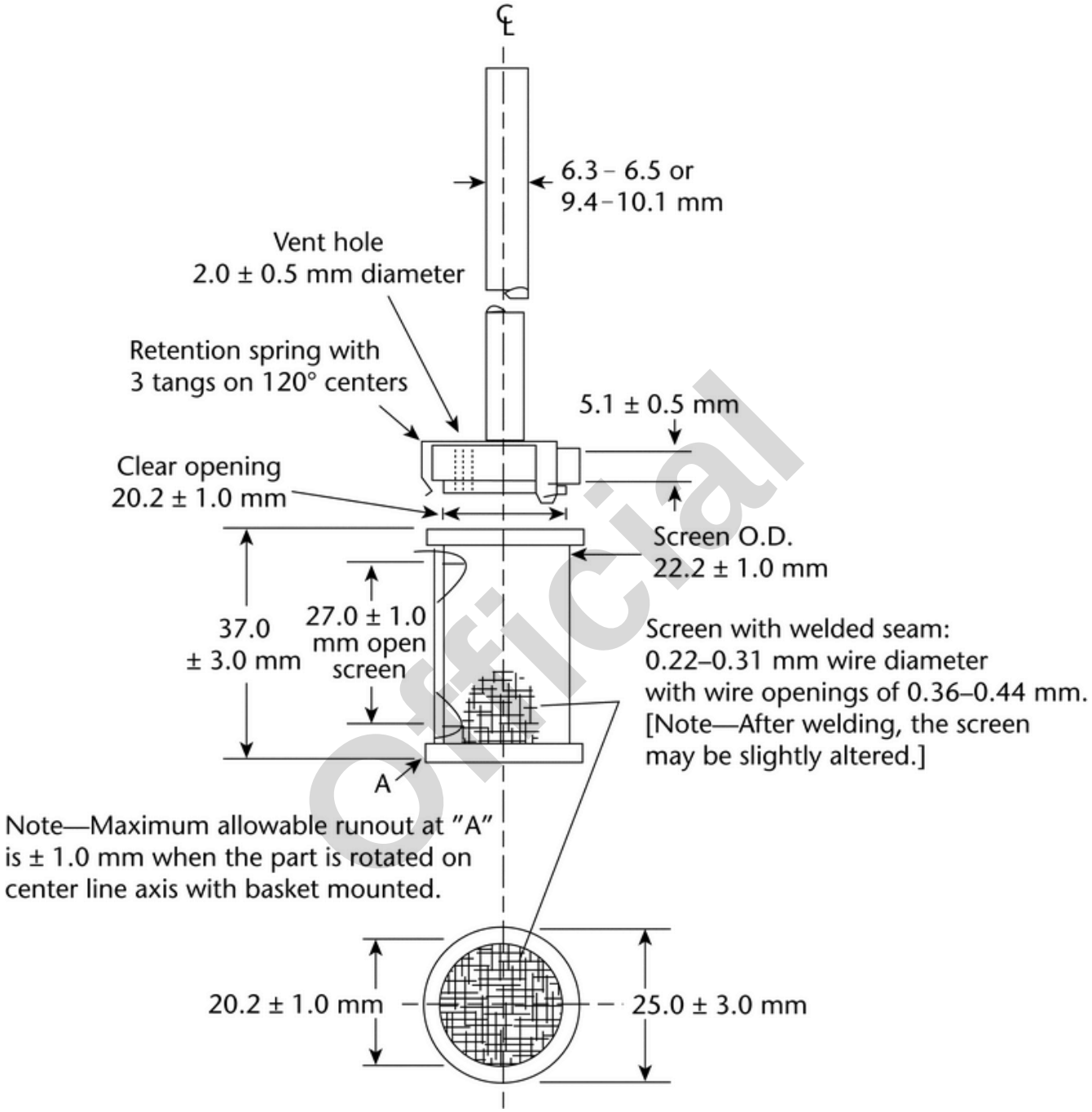


Figure 1. Basket stirring element.

## Apparatus 2 (Paddle Apparatus)

Use the assembly from *Apparatus 1*, except that a paddle formed from a blade and a shaft is used as the stirring element. The shaft is positioned so that its axis is not more than 2 mm from the vertical axis of the vessel at any point and rotates smoothly without significant wobble that could affect the results. The vertical center line of the blade passes through the axis of the shaft so that the bottom of the blade is flush with the bottom of the shaft. The paddle conforms to the specifications shown in *Figure 2*. The distance of 25 ± 2 mm between the bottom of the blade and the inside bottom of the vessel is maintained during the test. The metallic or suitably inert, rigid blade and shaft compose a single entity. A suitable two-part, detachable design may be used, provided that the assembly remains firmly engaged during the test. The paddle blade and shaft may be coated with a suitable coating so as to make both of them inert. The dosage unit is allowed to sink to the bottom of the vessel before rotation of the blade is started. A small, loose piece of nonreactive material, such as not more than a few turns of wire helix, may be attached to dosage units that would otherwise float. An alternative sinker device is shown in *Figure 2a*. Other validated sinker devices may be used.

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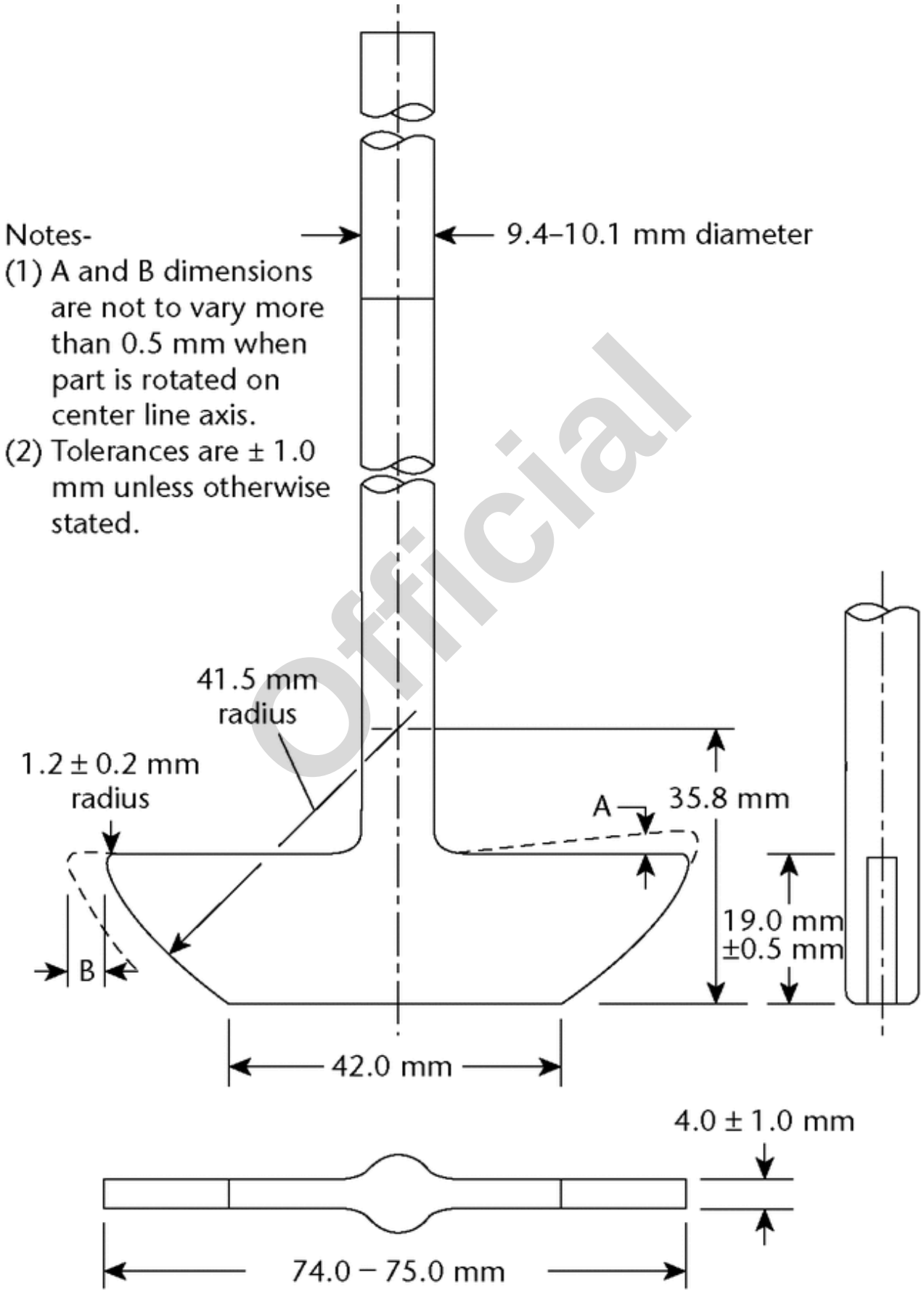


Figure 2. Paddle stirring element.

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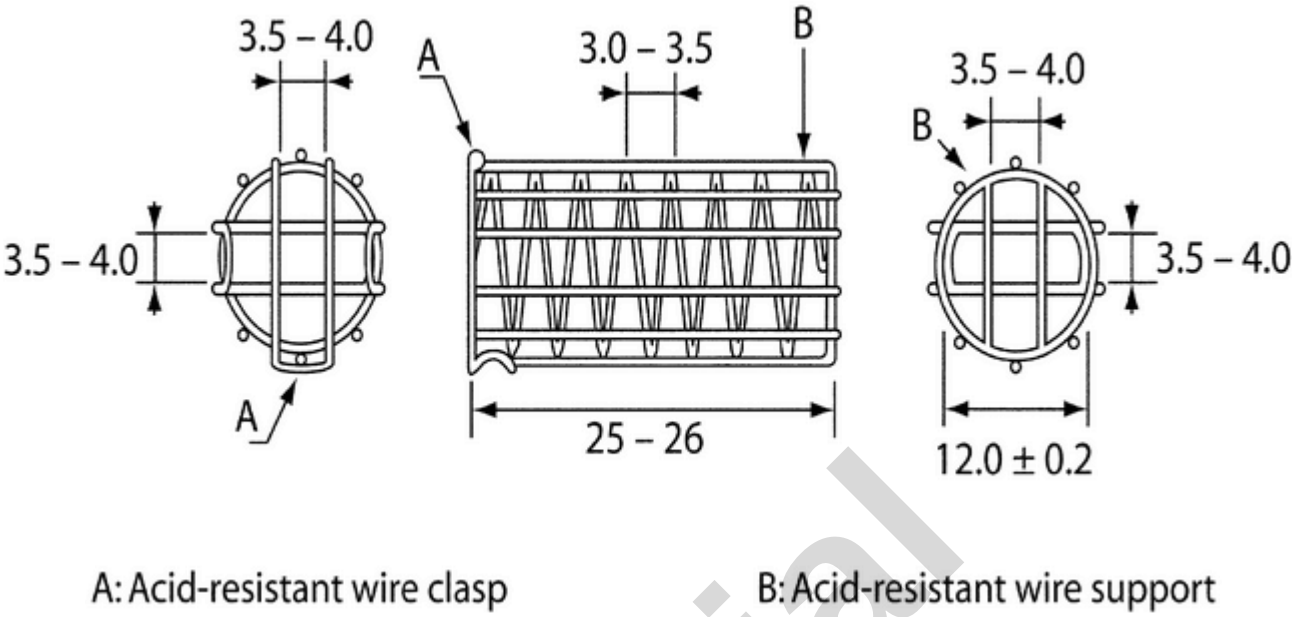


Figure 2a. Alternative sinker. All dimensions are expressed in millimeters.

•An alternative to sinkers is the stationary basket. The dosage form is placed in a quadrangular basket made of stainless steel wire mesh, soldered in one of its upper, narrow sides and attached to the end of a stainless steel connecting rod (see *Figure 2b*). The cover is placed in the horizontal diagonal of the basket. The rod assembly is attached to the cover of the dissolution vessel via an adjustable threaded steel rod, and is fixed by means of two Teflon nuts, about 3.2 cm from the center of the vessel, or by another appropriate means. The lower corner of the bottom of the basket is adjusted to about 1 cm above the top of the paddle blade (see *Figure 2c*). The axis of the connecting rod is parallel to the axis of the paddle shaft along the vertical length of the connecting rod and the largest face of the basket lies in a vertical plane perpendicular to the radius of the cylinder of the vessel.

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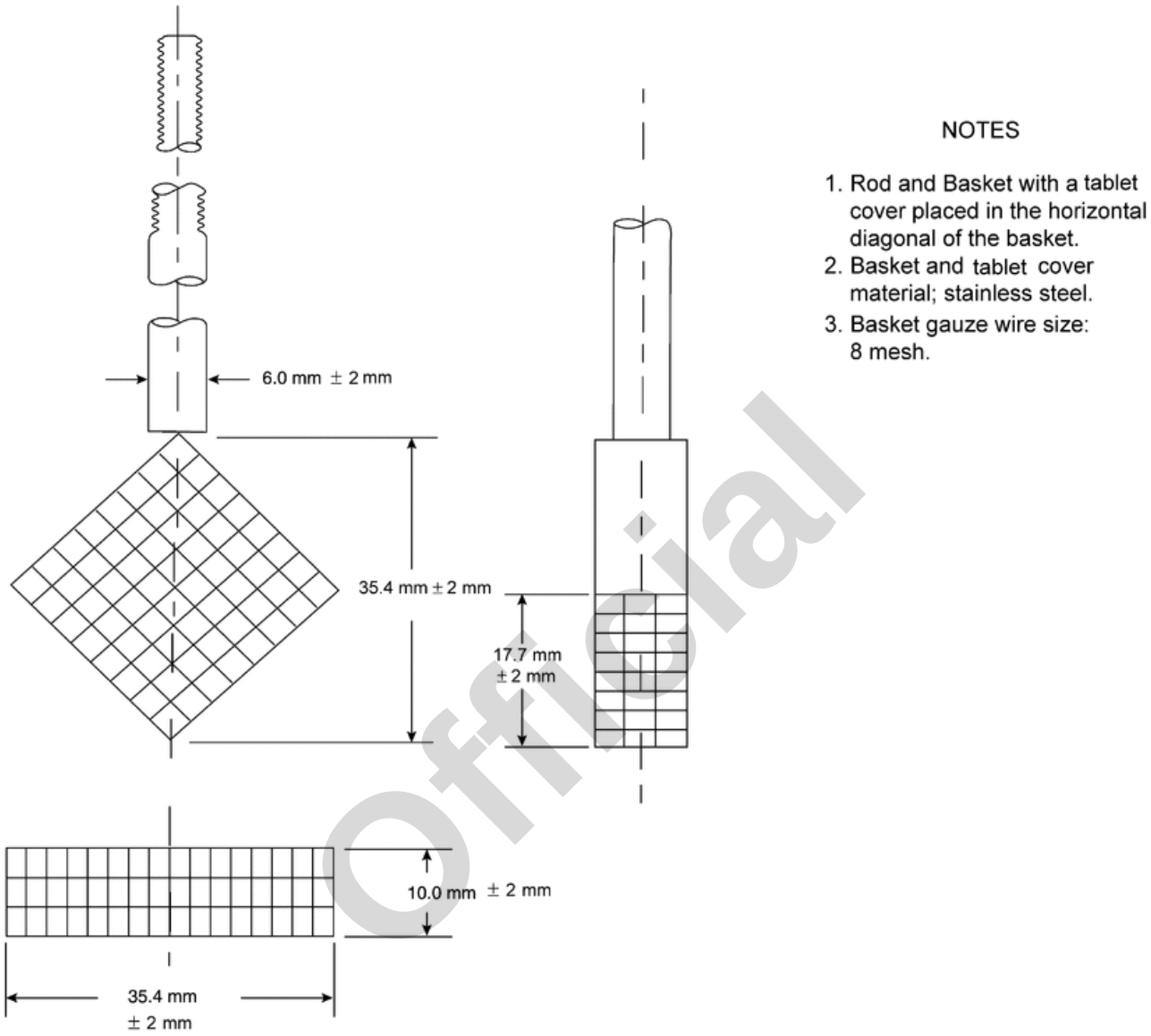


Figure 2b. Stationary basket.

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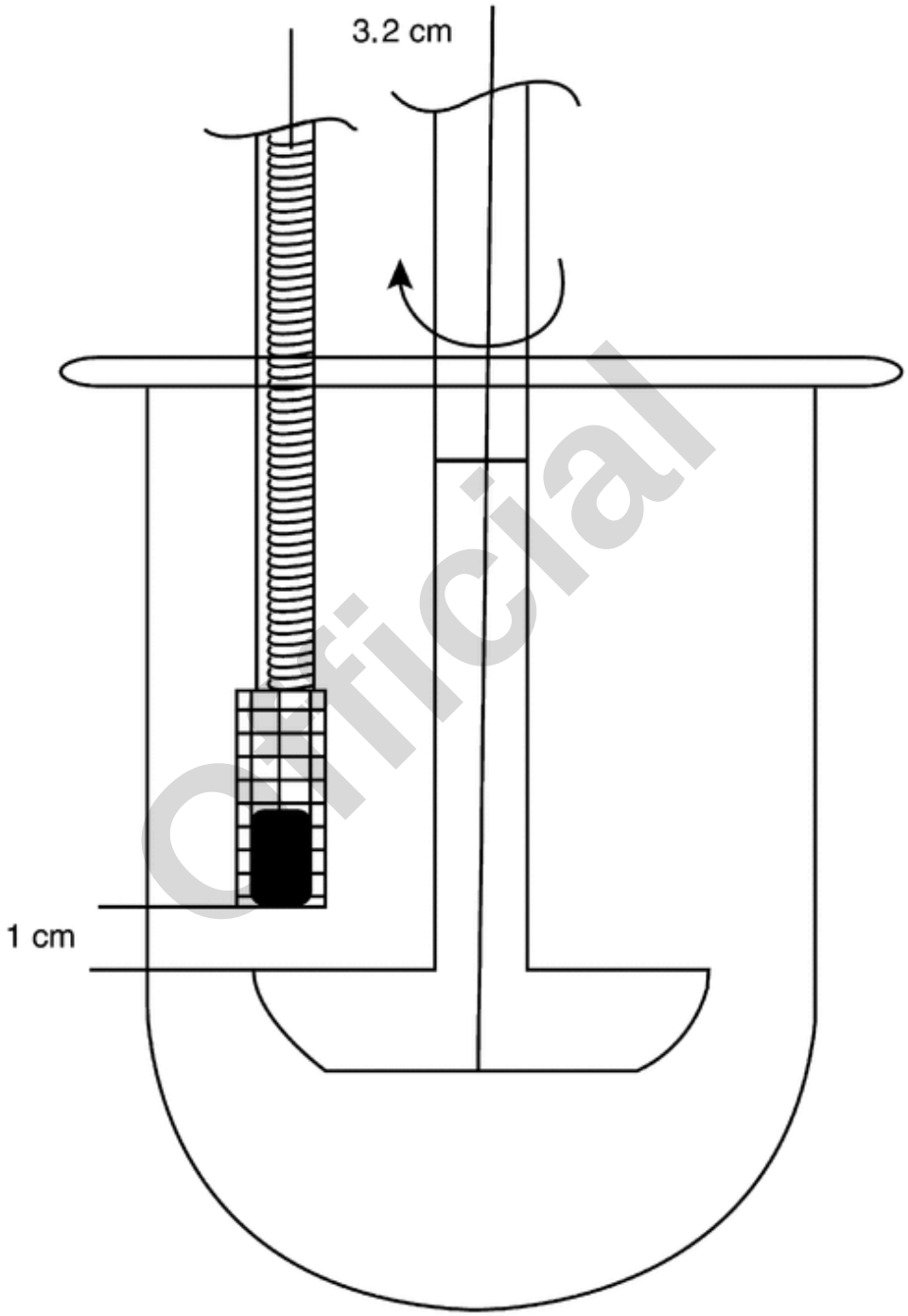


Figure 2c. Position of the stationary basket in the dissolution vessel.

## Apparatus 3 (Reciprocating Cylinder)[3]

The assembly consists of a set of cylindrical, flat-bottomed glass vessels; a set of glass reciprocating cylinders; inert fittings (stainless steel type 316 or other suitable material), and screens that are made of suitable nonsorbing and nonreactive material

[3] Not accepted by the *Japanese Pharmacopoeia*.

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and that are designed to fit the tops and bottoms of the reciprocating cylinders; and a motor and drive assembly to reciprocate the cylinders vertically inside the vessels; if desired, index the reciprocating cylinders horizontally to a different row of vessels. The vessels are partially immersed in a suitable water bath of any convenient size that permits holding the temperature at 37 ± 0.5° during the test. No part of the assembly, including the environment in which the assembly is placed, contributes significant motion, agitation, or vibration beyond that due to the smooth, vertically reciprocating cylinder. A device is used that allows the reciprocation rate to be selected and maintained at the specified dip rate ⧫given in the individual monograph⧫, within ±5%. An apparatus that permits observation of the specimens and reciprocating cylinders is preferable. The vessels are provided with evaporation caps that remain in place for the duration of the test. The components conform to the dimensions shown in *Figure 3*, unless otherwise specified ⧫in the individual monograph⧫.

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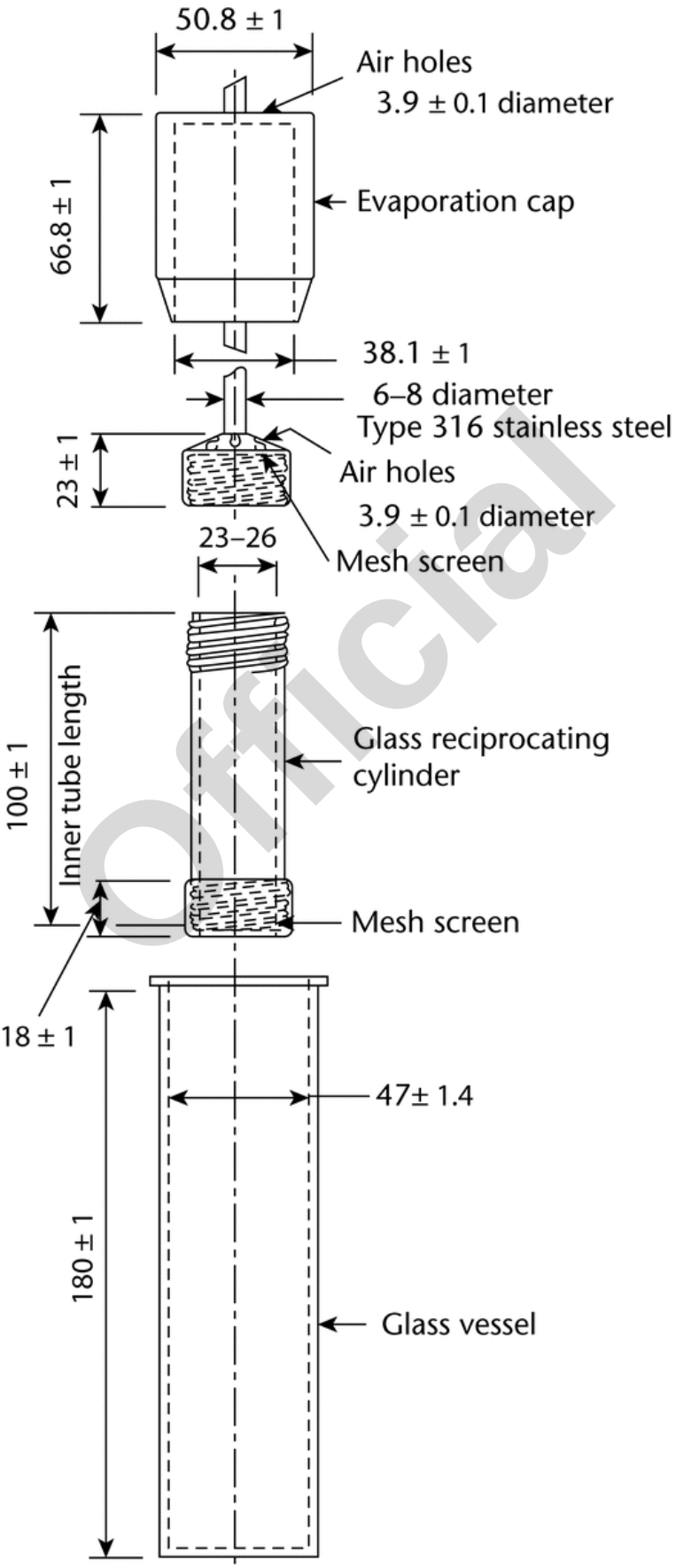


Figure 3. Apparatus 3 (reciprocating cylinder). (All measurements are expressed in millimeters unless noted otherwise.)

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## Apparatus 4 (Flow-Through Cell)

The assembly consists of a reservoir and a pump for the *Dissolution medium*; a flow-through cell; and a water bath that maintains the *Dissolution medium* at 37 ± 0.5°. Use the specified cell size •as given in the individual monograph•.

The pump forces the *Dissolution medium* upward through the flow-through cell. The pump has a delivery range between 240 and 960 mL/h, with standard flow rates of 4, 8, and 16 mL/min. It must deliver a constant flow (±5% of the nominal flow rate); the flow profile is sinusoidal with a pulsation of 120 ± 10 pulses/min. A pump without pulsation may also be used. Dissolution test procedures using a flow-through cell must be characterized with respect to rate and any pulsation.

The flow-through cell (see *Figure 4* and *Figure 5*), of transparent and inert material, is mounted vertically with a filter system (specified in the individual monograph) that prevents escape of undissolved particles from the top of the cell; standard cell diameters are 12 and 22.6 mm; the bottom cone is usually filled with small glass beads of about 1-mm diameter with one bead of about 5 mm, positioned at the apex to protect the fluid entry tube; and a tablet holder (see *Figure 4* and *Figure 5*) is available for positioning of special dosage forms, e.g., inlay tablets. The cell is immersed in a water bath, and the temperature is maintained at 37 ± 0.5°.

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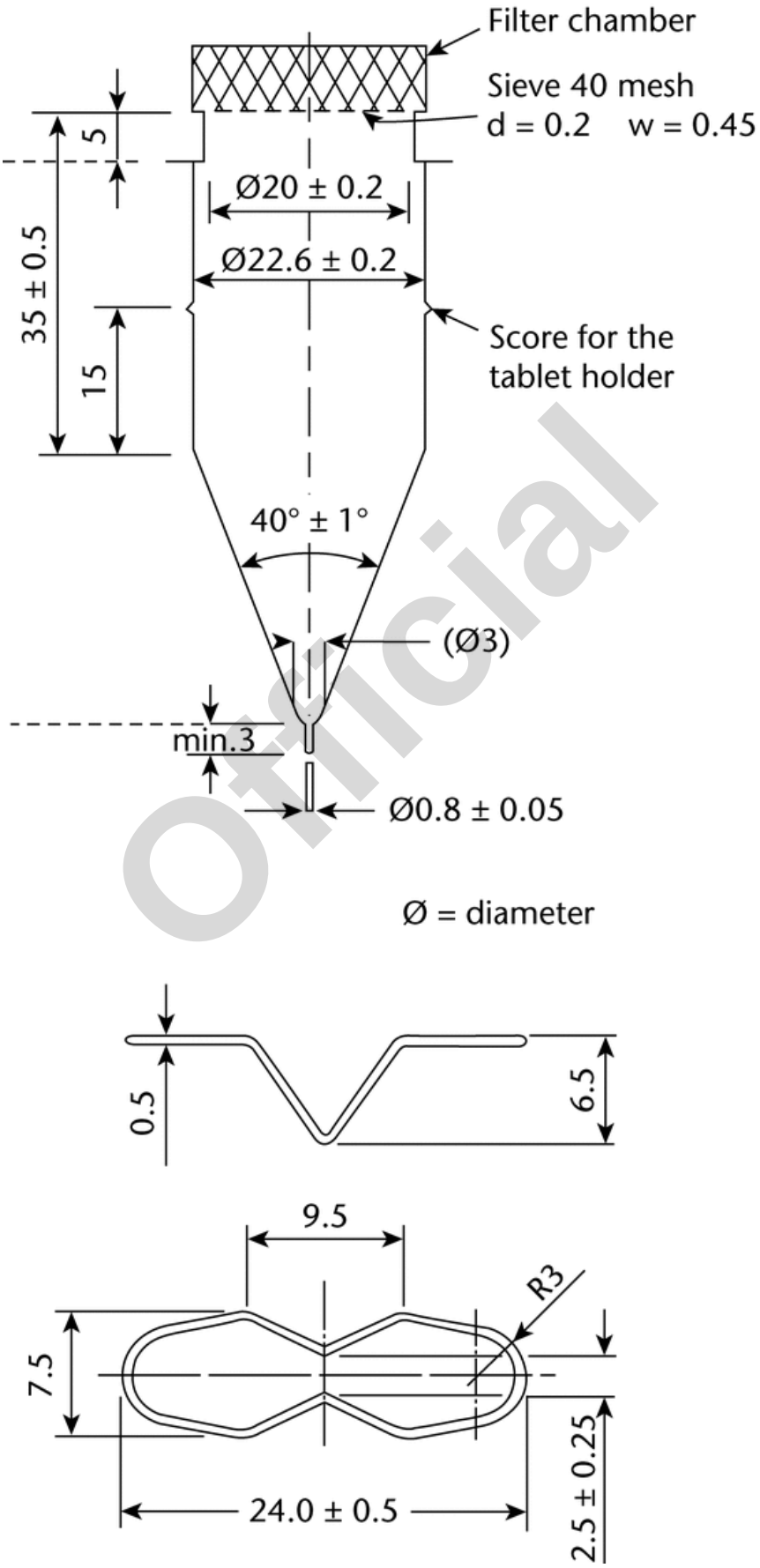


Figure 4. Apparatus 4: large cell for tablets and capsules (top); tablet holder for the large cell (bottom). (All measurements

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are expressed in millimeters unless noted otherwise.)

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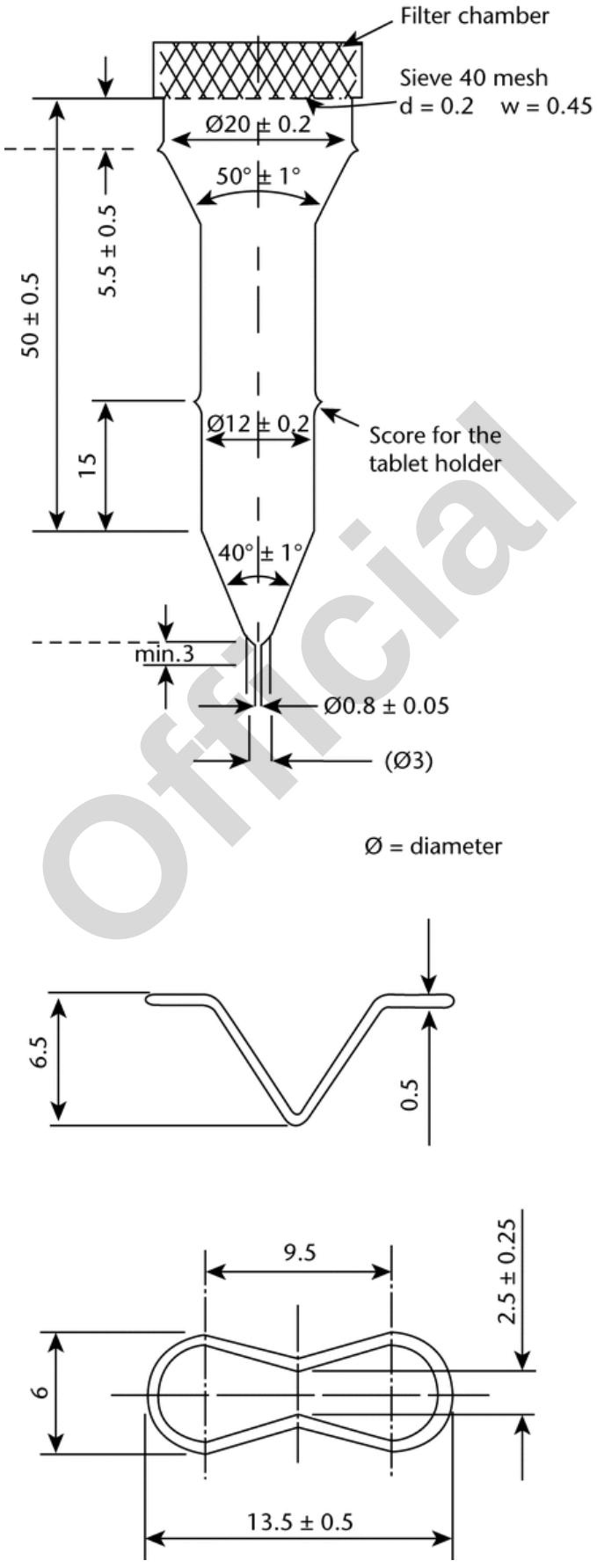


Figure 5. Apparatus 4: small cell for tablets and capsules (top); tablet holder for the small cell (bottom). (All measurements

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are expressed in millimeters unless noted otherwise.)

The apparatus uses a clamp mechanism and two O-rings to assemble the cell. The pump is separated from the dissolution unit to shield the latter against any vibrations originating from the pump. The position of the pump should not be on a level higher than the reservoir flasks. Tube connections are as short as possible. Use suitably inert tubing, such as polytef, with about a 1.6-mm inner diameter and chemically inert, flanged-end connections.

### APPARATUS SUITABILITY

The determination of suitability of a test assembly to perform dissolution testing must include conformance to the dimensions and tolerances of the apparatus as given above. In addition, critical test parameters that have to be monitored periodically during use include volume and temperature of the *Dissolution medium*, rotation speed (*Apparatus 1* and *Apparatus 2*), dip rate (*Apparatus 3*), and flow rate of medium (*Apparatus 4*).

Determine the acceptable performance of the dissolution test assembly periodically. •The suitability for the individual apparatus is demonstrated by the *Performance verification test*.

**USP Reference Standards** ⟨11⟩: ▲USP Dissolution Performance Verification Standard-Prednisone RS▲ (IRA 1-May-2023)

**Performance verification test, Apparatus 1 and Apparatus 2:** Test ▲USP Dissolution Performance Verification Standard-Prednisone RS▲ (IRA 1-May-2023) according to the operating conditions specified. The apparatus is suitable if the results obtained are within the acceptable range stated in the technical data sheet specific to the lot used and the apparatus tested.

**Performance verification test, Apparatus 3:** [To come]

**Performance verification test, Apparatus 4:** [To come]•

## PROCEDURE

## Apparatus 1 and Apparatus 2

### IMMEDIATE-RELEASE DOSAGE FORMS

Place the stated volume of the *Dissolution medium* (±1%) in the vessel of the specified apparatus •given in the individual monograph•, assemble the apparatus, equilibrate the *Dissolution medium* to 37 ± 0.5°, and remove the thermometer. Place 1 dosage unit in the apparatus, taking care to exclude air bubbles from the surface of the dosage unit, and immediately operate the apparatus at the specified rate •given in the individual monograph•. Within the time interval specified, or at each of the times stated, withdraw a specimen from a zone midway between the surface of the *Dissolution medium* and the top of the rotating basket or blade, not less than 1 cm from the vessel wall.

[NOTE—Where multiple sampling times are specified, replace the aliquots withdrawn for analysis with equal volumes of fresh *Dissolution medium* at 37° or, where it can be shown that replacement of the medium is not necessary, correct for the volume change in the calculation. Keep the vessel covered for the duration of the test, and verify the temperature of the mixture under test at suitable times.]

Perform the analysis •as directed in the individual monograph• using a suitable assay method.[4] Repeat the test with additional dosage form units.

If automated equipment is used for sampling or the apparatus is otherwise modified, verification that the modified apparatus will produce results equivalent to those obtained with the standard apparatus described in this general chapter is necessary.

**Dissolution medium:** A suitable dissolution medium is used. Use the solvent specified •in the individual monograph•. The volume specified refers to measurements made between 20° and 25°. If the *Dissolution medium* is a buffered solution, adjust the solution so that its pH is within 0.05 unit of the specified pH •given in the individual monograph•. [NOTE—Dissolved gases can cause bubbles to form, which may change the results of the test. If dissolved gases influence the dissolution results, dissolved gases should be removed before testing.[5]]

**Time:** Where a single time specification is given, the test may be concluded in a shorter period if the requirement for the minimum amount dissolved is met. Specimens are to be withdrawn only at the stated times, within a tolerance of ±2%.

**•Procedure for a pooled sample for immediate-release dosage forms:** Use this procedure where *Procedure for a Pooled Sample* is specified in the individual monograph. Proceed as directed for *Immediate-Release Dosage Forms* in *Apparatus 1 and Apparatus 2* in the *Procedure* section. Combine equal volumes of the filtered solutions of the 6 or 12 individual specimens withdrawn, and use the pooled sample as the test specimen. Determine the average amount of the active ingredient dissolved in the pooled sample.•

### EXTENDED-RELEASE DOSAGE FORMS

Proceed as directed for *Immediate-Release Dosage Forms*.

**Dissolution medium:** Prepare as directed for *Immediate-Release Dosage Forms*.

**Time:** The test time points, generally three, are expressed in hours.

[4] Test specimens are filtered immediately upon sampling unless filtration is demonstrated to be unnecessary. Use an inert filter that does not cause adsorption of the active ingredient or contain extractable substances that would interfere with the analysis.

[5] One method of deaeration is as follows: Heat the medium, while stirring gently, to about 41°, immediately filter under vacuum using a filter having a porosity of 0.45 µm or less, with vigorous stirring, and continue stirring under vacuum for about 5 min. Other validated deaeration techniques for removal of dissolved gases may be used.

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DELAYED-RELEASE DOSAGE FORMS[3]

Use *Method A Procedure* or *Method B Procedure* and the apparatus specified •in the individual monograph•. All test times stated are to be observed within a tolerance of ±2%, unless otherwise specified.

## Method A Procedure •(unless otherwise directed in the individual monograph)•

ACID STAGE

Place 750 mL of 0.1 N hydrochloric acid in the vessel, and assemble the apparatus. Allow the medium to equilibrate to a temperature of 37 ± 0.5°. Place 1 dosage unit in the apparatus, cover the vessel, and operate the apparatus at the specified rate •given in the monograph•.

After 2 h of operation in 0.1 N hydrochloric acid, withdraw an aliquot of the fluid, and proceed immediately as directed in the *Buffer Stage*.

Perform an analysis of the aliquot using a suitable assay method. •The procedure is specified in the individual monograph.•

BUFFER STAGE

[NOTE—Complete the operations of adding the buffer and adjusting the pH within 5 min.] With the apparatus operating at the rate specified •in the monograph•, add to the fluid in the vessel 250 mL of 0.20 M tribasic sodium phosphate that has been equilibrated to 37 ± 0.5°. Adjust, if necessary, with 2 N hydrochloric acid or 2 N sodium hydroxide to a pH of 6.8 ± 0.05. Continue to operate the apparatus for 45 min, or for the specified time •given in the individual monograph•. At the end of the time period, withdraw an aliquot of the fluid, and perform the analysis using a suitable assay method. •The procedure is specified in the individual monograph. The test may be concluded in a shorter time period than that specified for the *Buffer Stage* if the requirement for the minimum amount dissolved is met at an earlier time.•

## Method B Procedure •(unless otherwise directed in the individual monograph)•

ACID STAGE

Place 1000 mL of 0.1 N hydrochloric acid in the vessel, and assemble the apparatus. Allow the medium to equilibrate to a temperature of 37 ± 0.5°. Place 1 dosage unit in the apparatus, cover the vessel, and operate the apparatus at the rate specified •in the monograph•. After 2 h of operation in 0.1 N hydrochloric acid, withdraw an aliquot of the fluid, and proceed immediately as directed in the *Buffer Stage*.

Perform an analysis of the aliquot using a suitable assay method. •The procedure is specified in the individual monograph.•

BUFFER STAGE

[NOTE—For this stage of the procedure, use buffer that previously has been equilibrated to a temperature of 37 ± 0.5°.] Drain the acid from the vessel, and add to the vessel 1000 mL of pH 6.8 phosphate buffer, prepared by mixing 0.1 N hydrochloric acid with 0.20 M tribasic sodium phosphate (3:1) and adjusting, if necessary, with 2 N hydrochloric acid or 2 N sodium hydroxide to a pH of 6.8 ± 0.05. [NOTE—This may also be accomplished by removing from the apparatus the vessel containing the acid, then replacing it with another vessel containing the buffer, and transferring the dosage unit to the vessel containing the buffer.]

Continue to operate the apparatus for 45 min, or for the specified time •given in the individual monograph•. At the end of the time period, withdraw an aliquot of the fluid, and perform the analysis using a suitable assay method. •The procedure is specified in the individual monograph. The test may be concluded in a shorter time period than that specified for the *Buffer Stage* if the requirement for minimum amount dissolved is met at an earlier time.•

## Apparatus 3 (Reciprocating Cylinder)

IMMEDIATE-RELEASE DOSAGE FORMS[3]

Place the stated volume of the *Dissolution medium* in each vessel of the apparatus, assemble the apparatus, equilibrate the *Dissolution medium* to 37 ± 0.5°, and remove the thermometer. Place 1 dosage form unit in each of the six reciprocating cylinders, taking care to exclude air bubbles from the surface of each dosage unit, and immediately operate the apparatus as specified •in the individual monograph•. During the upward and downward strokes, the reciprocating cylinder moves through a total distance of 9.9–10.1 cm. Within the time interval specified, or at each of the times stated, raise the reciprocating cylinders and withdraw a portion of the solution under test from a zone midway between the surface of the *Dissolution medium* and the bottom of each vessel. Perform the analysis as directed •in the individual monograph•. If necessary, repeat the test with additional dosage-form units.

**Dissolution medium:** Prepare as directed for *Immediate-Release Dosage Forms* in *Apparatus 1 and Apparatus 2*.

**Time:** Proceed as directed for *Immediate-Release Dosage Forms* in *Apparatus 1 and Apparatus 2*.

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EXTENDED-RELEASE DOSAGE FORMS

Proceed as directed for *Immediate-Release Dosage Forms* in *Apparatus 3*.
**Dissolution medium:** Prepare as directed for *Extended-Release Dosage Forms* in *Apparatus 1 and Apparatus 2*.
**Time:** Proceed as directed for *Extended-Release Dosage Forms* in *Apparatus 1 and Apparatus 2*.

DELAYED-RELEASE DOSAGE FORMS

Proceed as directed for *Delayed-Release Dosage Forms, Method B Procedure* in *Apparatus 1 and Apparatus 2*, using one row of vessels for the acid stage media and the following row of vessels for the buffer stage media, and using the volume of medium specified (usually 300 mL).
**Time:** Proceed as directed for *Immediate-Release Dosage Forms* in *Apparatus 1 and Apparatus 2*.

## Apparatus 4 (Flow-Through Cell)

IMMEDIATE-RELEASE DOSAGE FORMS

Place the glass beads into the cell specified ♦in the monograph♦. Place 1 dosage unit on top of the beads or, if specified ♦in the monograph♦, on a wire carrier. Assemble the filter head, and fix the parts together by means of a suitable clamping device. Introduce by the pump the *Dissolution medium* warmed to 37 ± 0.5° through the bottom of the cell to obtain the flow rate specified ♦in the individual monograph♦ and measured with an accuracy of 5%. Collect the eluate by fractions at each of the times stated. Perform the analysis as directed ♦in the individual monograph♦. Repeat the test with additional dosage form units.
**Dissolution medium:** Prepare as directed for *Immediate-Release Dosage Forms* in *Apparatus 1 and Apparatus 2*.
**Time:** Proceed as directed for *Immediate-Release Dosage Forms* in *Apparatus 1 and Apparatus 2*.

EXTENDED-RELEASE DOSAGE FORMS

Proceed as directed for *Immediate-Release Dosage Forms* in *Apparatus 4*.
**Dissolution medium:** Prepare as directed for *Immediate-Release Dosage Forms* in *Apparatus 4*.
**Time:** Proceed as directed for *Immediate-Release Dosage Forms* in *Apparatus 4*.

DELAYED-RELEASE DOSAGE FORMS

Proceed as directed for *Delayed-Release Dosage Forms* in *Apparatus 1 and Apparatus 2*, using the specified media.
**Time:** Proceed as directed for *Delayed-Release Dosage Forms* in *Apparatus 1 and Apparatus 2*.

# INTERPRETATION

## Immediate-Release Dosage Forms

Unless otherwise specified ♦in the individual monograph♦, the requirements are met if the quantities of active ingredient dissolved from the dosage units tested conform to *Acceptance Table 1*. Continue testing through the three stages unless the results conform at either $S_1$ or $S_2$. The quantity ($Q$) is the amount of dissolved active ingredient ♦specified in the individual monograph♦, expressed as a percentage of the labeled content of the dosage unit; the 5%, 15%, and 25% values in *Acceptance Table 1* are percentages of the labeled content so that these values and $Q$ are in the same terms.

**Acceptance Table 1**

| Stage | Number Tested | Acceptance Criteria |
|---|---|---|
| $S_1$ | 6 | Each unit is NLT $Q$ + 5%. |
| $S_2$ | 6 | Average of 12 units ($S_1$ + $S_2$) is $\geq Q$, and no unit is $< Q$ − 15%. |
| $S_3$ | 12 | Average of 24 units ($S_1$ + $S_2$ + $S_3$) is $\geq Q$, NMT 2 units are $< Q$ − 15%, and no unit is $< Q$ − 25%. |

## ♦Immediate-Release Dosage Forms Pooled Sample

Unless otherwise specified in the individual monograph, the requirements are met if the quantities of active ingredient dissolved from the pooled sample conform to the accompanying *Acceptance Table for a Pooled Sample*. Continue testing through the three stages unless the results conform at either $S_1$ or $S_2$. The quantity ($Q$) is the amount of dissolved active ingredient specified in the individual monograph, expressed as a percentage of the labeled content.

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**Acceptance Table for a Pooled Sample**

| Stage | Number Tested | Acceptance Criteria |
|---|---|---|
| $S_1$ | 6 | Average amount dissolved is NLT $Q$ + 10%. |
| $S_2$ | 6 | Average amount dissolved ($S_1$ + $S_2$) is ≥$Q$ + 5%. |
| $S_3$ | 12 | Average amount dissolved ($S_1$ + $S_2$ + $S_3$) is ≥$Q$. |

## Extended-Release Dosage Forms

Unless otherwise specified in the individual monograph, the requirements are met if the quantities of active ingredient dissolved from the dosage units tested conform to *Acceptance Table 2*. Continue testing through the three levels unless the results conform at either $L_1$ or $L_2$. Limits on the amounts of active ingredient dissolved are expressed in terms of the percentage of labeled content. The limits embrace each value of $Q_i$, the amount dissolved at each specified fractional dosing interval. Where more than one range is specified in the individual monograph, the acceptance criteria apply individually to each range.

**Acceptance Table 2**

| Level | Number Tested | Acceptance Criteria |
|---|---|---|
| $L_1$ | 6 | No individual value lies outside each of the stated ranges, and no individual value is less than the stated amount at the final test time. |
| $L_2$ | 6 | The average value of the 12 units ($L_1$ + $L_2$) lies within each of the stated ranges and is not less than the stated amount at the final test time; none is >10% of labeled content outside each of the stated ranges; and none is >10% of the labeled content below the stated amount at the final test time. |
| $L_3$ | 12 | The average value of the 24 units ($L_1$ + $L_2$ + $L_3$) lies within each of the stated ranges and is not less than the stated amount at the final test time; NMT 2 of the 24 units are more than 10% of labeled content outside each of the stated ranges; NMT 2 of the 24 units are >10% of labeled content below the stated amount at the final test time; and none of the units are >20% of labeled content outside each of the stated ranges or >20% of the labeled content below the stated amount at the final test time. |

## Delayed-Release Dosage Forms[3]

### ACID STAGE

Unless otherwise specified in the individual monograph, the requirements of this portion of the test are met if the quantities, based on the percentage of the labeled content, of active ingredient dissolved from the units tested conform to *Acceptance Table 3*. Continue testing through all levels unless the results of both the *Acid Stage* and *Buffer Stage* conform at an earlier level.

**Acceptance Table 3**

| Level | Number Tested | Acceptance Criteria |
|---|---|---|
| $A_1$ | 6 | No individual value exceeds 10% dissolved. |
| $A_2$ | 6 | Average of the 12 units ($A_1$ + $A_2$) is NMT 10% dissolved, and no individual unit is >25% dissolved. |
| $A_3$ | 12 | Average of the 24 units ($A_1$ + $A_2$ + $A_3$) is NMT 10% dissolved, and no individual unit is >25% dissolved. |

### BUFFER STAGE

Unless otherwise specified in the individual monograph, the requirements are met if the quantities of active ingredient dissolved from the units tested conform to *Acceptance Table 4*. Continue testing through the three levels unless the results of both stages conform at an earlier level. The value of $Q$ in *Acceptance Table 4* is 75% dissolved unless otherwise specified in the individual monograph. The quantity ($Q$) specified in the individual monograph is the total amount of active ingredient dissolved in both the *Acid Stage* and the *Buffer Stage*, expressed as a percentage of the labeled content. The 5%, 15%, and 25% values in *Acceptance Table 4* are percentages of the labeled content so that these values and $Q$ are in the same terms.

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**Acceptance Table 4**

| Level | Number Tested | Acceptance Criteria |
|---|---|---|
| $B_1$ | 6 | Each unit is NLT $Q$ + 5%. |
| $B_2$ | 6 | Average of 12 units ($B_1$ + $B_2$) is $\geq Q$, and no unit is $< Q$ − 15%. |
| $B_3$ | 12 | Average of 24 units ($B_1$ + $B_2$ + $B_3$) is $\geq Q$, NMT 2 units are $< Q$ − 15%, and no unit is $< Q$ − 25%. |

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# ⟨721⟩ DISTILLING RANGE

To determine the range of temperatures within which an official liquid distills, or the percentage of the material that distills between two specified temperatures, use Method I or Method II as directed in the individual monograph. The *lower limit* of the range is the temperature indicated by the thermometer when the first drop of condensate leaves the tip of the condenser, and the *upper limit* is the Dry Point, i.e., the temperature at which the last drop of liquid evaporates from the lowest point in the distillation flask, without regard to any liquid remaining on the side of the flask, or the temperature observed when the proportion specified in the individual monograph has been collected.

[NOTE—Cool all liquids that distill below 80° to between 10° and 15° before measuring the sample to be distilled.]

## METHOD I

### Apparatus

Use apparatus similar to that specified for *Method II*, except that the distilling flask is of 50- to 60-mL capacity, and the neck of the flask is 10 to 12 cm long and 14 to 16 mm in internal diameter. The perforation in the upper insulating board, if one is used, should be such that when the flask is set into it, the portion of the flask below the upper surface of the insulating material has a capacity of 3 to 4 mL.

### Procedure

Proceed as directed for *Method II*, but place in the flask only 25 mL of the liquid to be tested.

## METHOD II

### Apparatus

Use an apparatus consisting of the following parts:

DISTILLING FLASK

A round-bottom distilling flask, of heat-resistant glass, of 200-mL capacity, and having a total length of 17 to 19 cm, and an inside neck diameter of 20 to 22 mm. Attached about midway on the neck, approximately 12 cm from the bottom of the flask, is a side-arm 10 to 12 cm long and 5 mm in internal diameter, which forms an angle of 70° to 75° with the lower portion of the neck.

CONDENSER

A straight glass condenser 55 to 60 cm in length with a water jacket about 40 cm in length, or a condenser of other design having equivalent condensing capacity. The lower end of the condenser may be bent to provide a delivery tube, or it may be connected to a bent adapter that serves as a delivery tube.

INSULATING BOARDS

Two pieces of insulating board, 5 to 7 mm thick and 14 to 16 cm square, suitable for confining the heat to the lower part of the flask. Each board has a hole in its center, and the two boards differ only with respect to the diameter of the hole, i.e., the diameters are 4 cm and 10 cm, respectively. In use, the boards are placed one upon the other, and rest on a tripod or other suitable support, with the board having the larger hole on top.

RECEIVER

A 100-mL cylinder graduated in 1-mL subdivisions.

THERMOMETER

In order to avoid the necessity for an emergent stem correction, an accurately standardized, partial-immersion thermometer having the smallest practical subdivisions (not greater than 0.2°) is recommended. Suitable thermometers are available as the ASTM E-1 series 37C through 41C, and 102C through 107C (see *General Notices, 6.80.30. Temperature Reading Devices*). When placed in position, the stem is located in the center of the neck, and the top of the contraction chamber (or bulb, if 37C or 38C is used) is level with the bottom of the outlet to the side-arm.

HEAT SOURCE

A small Bunsen burner or an electric heater or mantle capable of adjustment comparable to that possible with a Bunsen burner.

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## Procedure

Assemble the apparatus, and place in the flask 100 mL of the liquid to be tested, taking care not to allow any of the liquid to enter the side-arm. Insert the thermometer, shield the entire burner and flask assembly from external air currents, and apply heat, regulating it so that between 5 and 10 minutes elapse before the first drop of distillate falls from the condenser. Continue the distillation at a rate of 4 to 5 mL of distillate per minute, collecting the distillate in the receiver. Note the temperature when the first drop of distillate falls from the condenser, and again when the last drop of liquid evaporates from the bottom of the flask or when the specified percentage has distilled over. Unless otherwise specified in the individual monograph, apply when necessary the emergent stem correction and report the temperatures adjusting the barometric pressure by the following formula:

$$t = t_0 + [(t_0 10^{-4} + 0.033)(760 - p)]$$

in which t is the corrected boiling temperature, in Celsius scale; $t_0$ is the measured boiling temperature, in Celsius scale; and p is the barometric pressure at the time of measurement, in mm Hg.

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# ⟨724⟩ DRUG RELEASE

## SCOPE

This test is provided to determine compliance with drug release or dissolution requirements where stated in the individual monographs for transdermal systems (TDS) and other dosage forms. From the types of apparatus described herein, use the one specified in the individual monograph.

## GENERAL DRUG RELEASE STANDARDS

### Apparatus 5 (Paddle over Disk)

APPARATUS

Use the paddle and vessel assembly as described in *Dissolution* ⟨711⟩, *Apparatus, Apparatus 2*, with the addition of a disk assembly designed to hold the TDS at the bottom of the vessel. This disk assembly is designed to minimize any "dead" volume between the assembly and the bottom of the vessel. The disk assembly holds the TDS flat and is positioned such that the release surface is parallel with the bottom of the paddle blade (see *Figure 1a*, *Figure 1b*, and *Figure 1c*). A distance of 25 ± 2 mm between the paddle blade and the surface of the disk assembly is maintained during the test. Other appropriate devices may be used (see *Figure 2*), provided they do not sorb, react with, or interfere with the specimen being tested. [NOTE—A suitable device is catalog number TRANDE-SC from www.qla-llc.com.]

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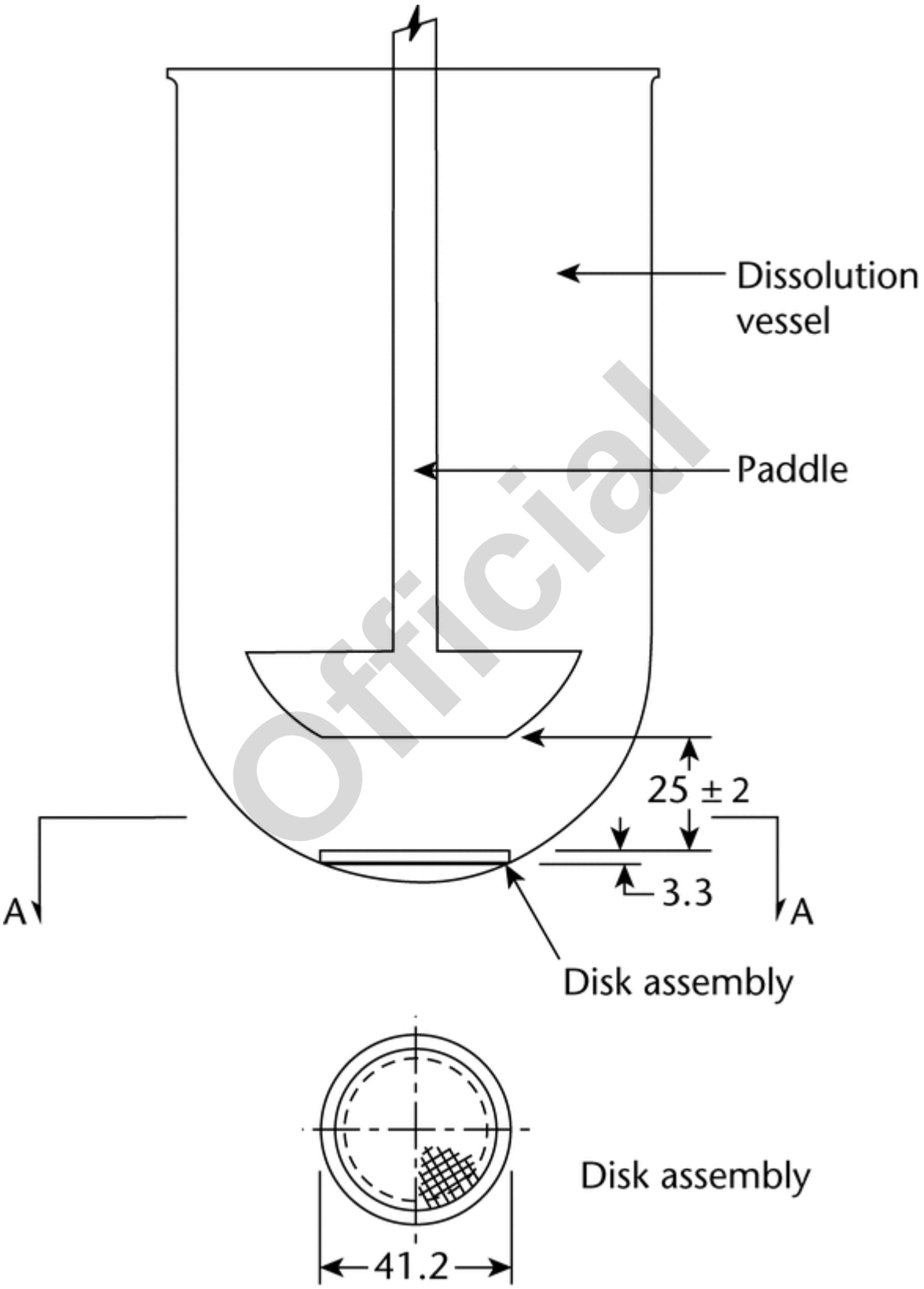


Figure 1a. Paddle over disk. (All measurements are expressed in mm unless noted otherwise.)

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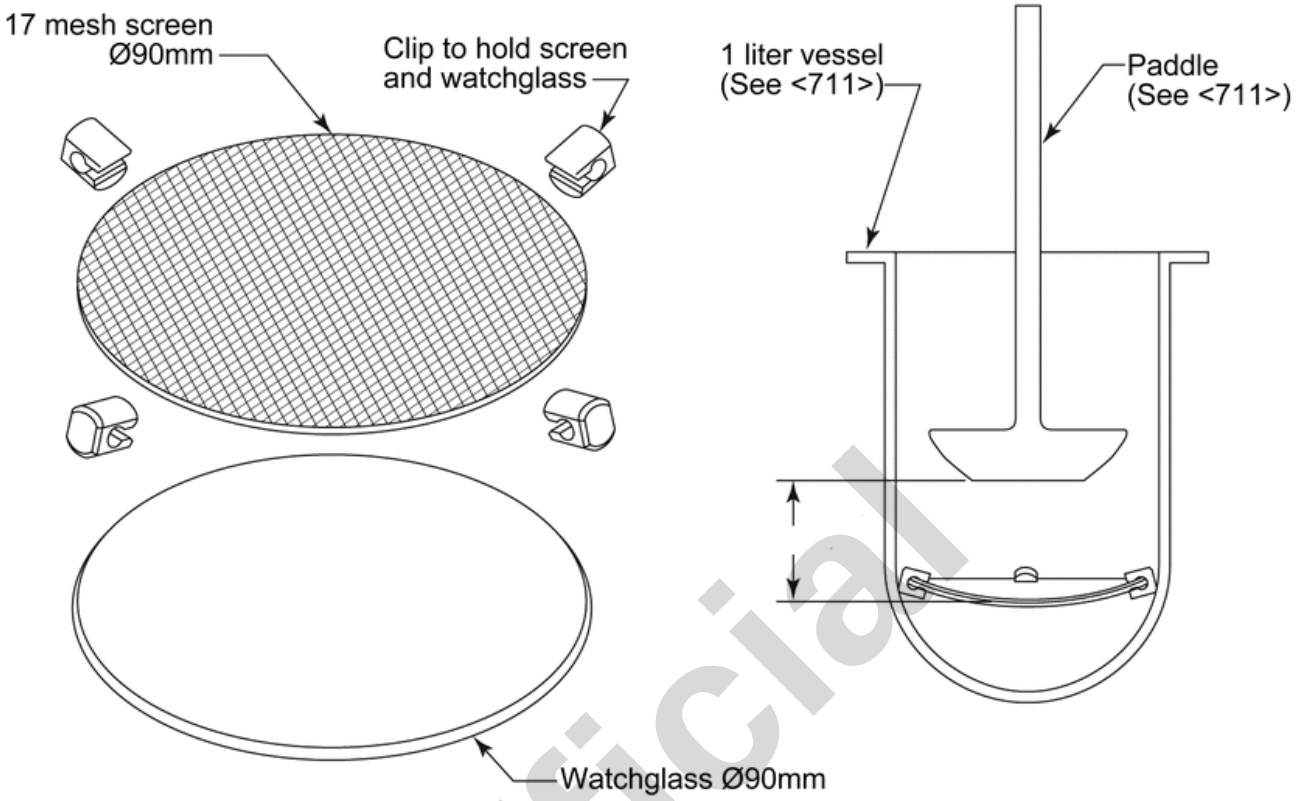


Figure 1b. Paddle over disk (screen and watchglass).

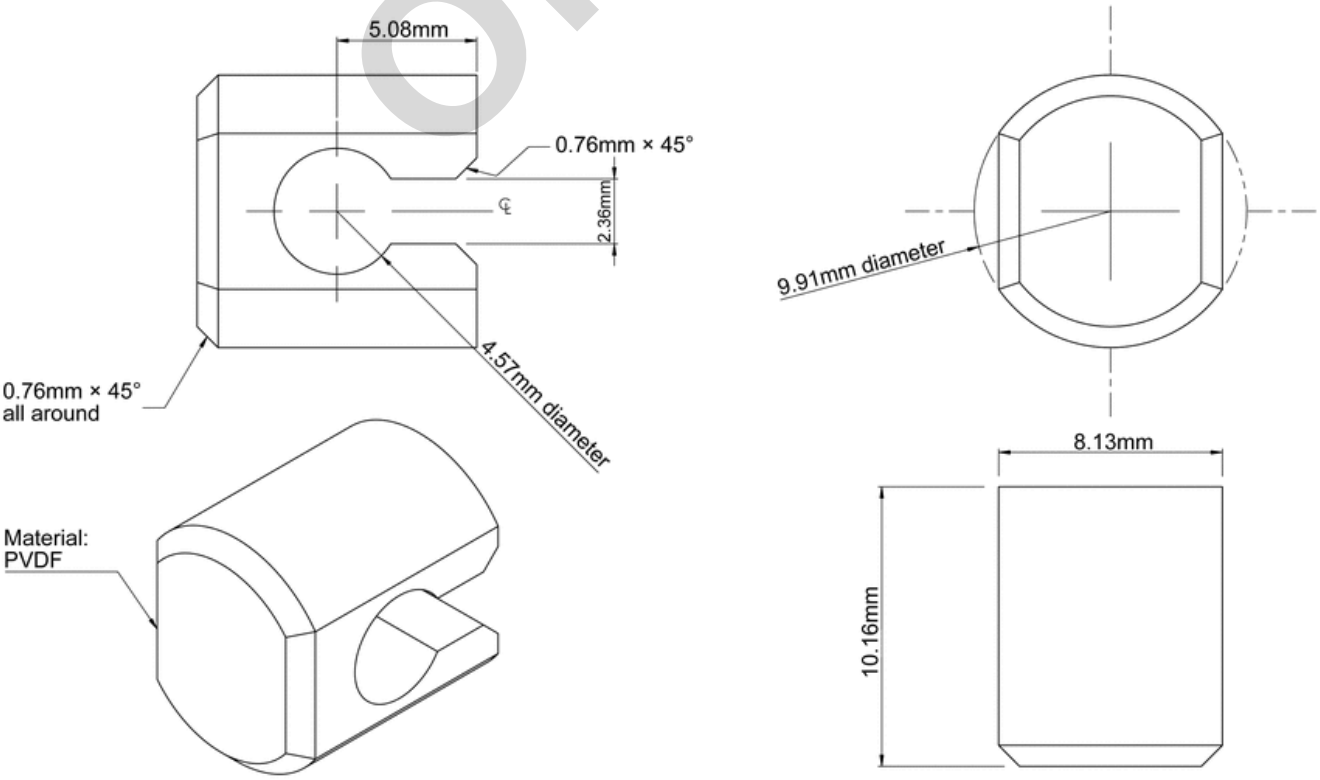


Figure 1c. Clip to hold screen and watchglass.

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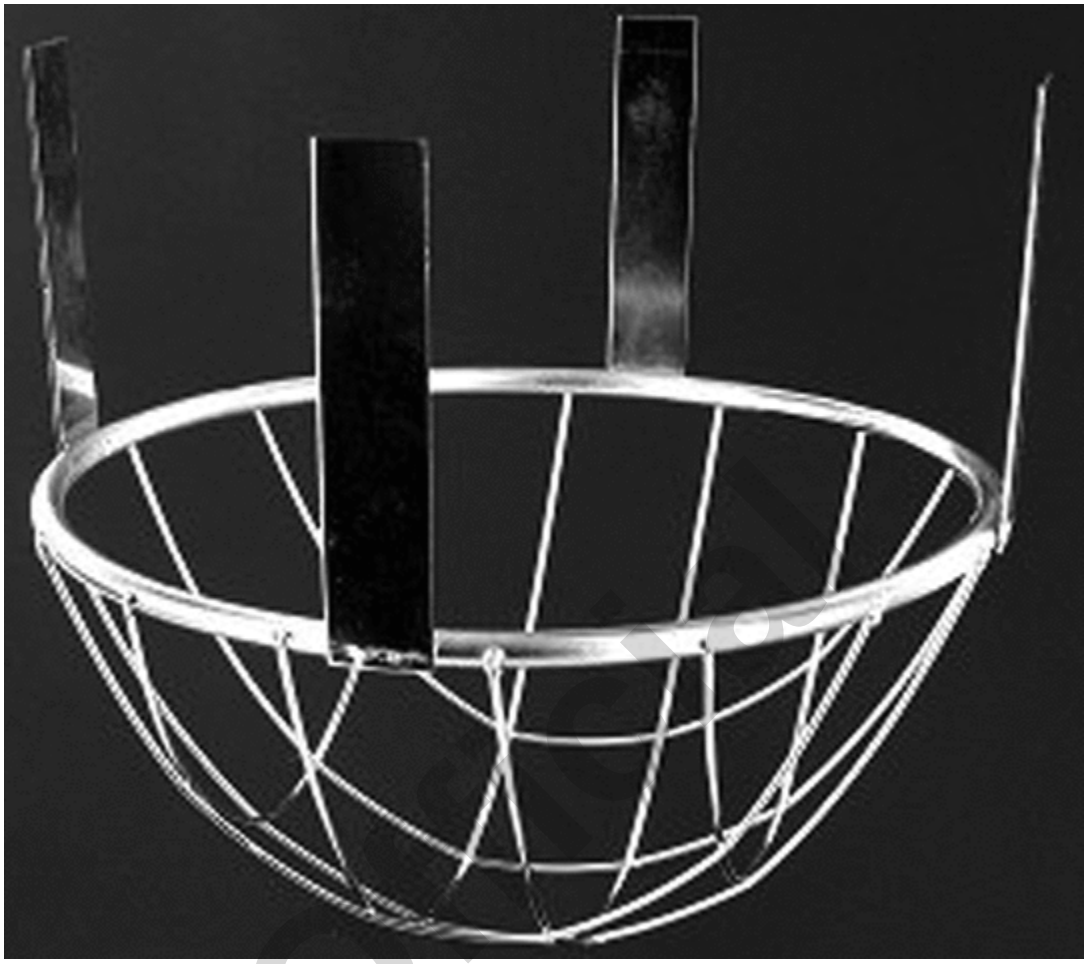

Figure 2. Holding wire screen.

## APPARATUS SUITABILITY

Proceed as directed for *Apparatus 2* in Dissolution ⟨711⟩, *Apparatus, Apparatus 4, Apparatus Suitability, Performance verification test, Apparatus 1 and Apparatus 2*.

## MEDIUM

Proceed as directed for *Dissolution medium* in *Dissolution* ⟨711⟩, *Procedure, Apparatus 1 and Apparatus 2, Immediate-Release Dosage Forms*.

## PROCEDURE

Place the stated volume of *Medium* in the vessel, and equilibrate to 32 ± 0.5°.

Apply the TDS to the disk assembly, assuring that the release surface is as smooth as possible and the TDS is completely and firmly attached to the disk. The TDS, with release surface side up, may be attached to the disk by an appropriate and validated procedure such as use of an adhesive, double-face adhesive tape, screen, or membrane. Care must be taken to avoid the presence of air bubbles between the membrane, if used, and the TDS, or the presence of wrinkles on the surface of the TDS. Carefully remove the protective liner from the TDS without causing damage to the surface of the TDS.

Place the disk assembly flat at the bottom of the vessel with the release surface facing up and parallel to the edge of the paddle blade and surface of the *Medium*. Ensure that the surface of the TDS is devoid of air bubbles. The bottom edge of the paddle is 25 ± 2 mm from the surface of the disk assembly. The vessel may be covered during the test to minimize evaporation.

Immediately operate the apparatus at the rate specified in the monograph.

At each sampling time interval, withdraw a specimen from a zone midway between the surface of the *Medium* and the top of the blade, not less than 1 cm from the vessel wall.

Perform the analysis on each sample aliquot as directed in the individual monograph, correcting for any volume losses, as necessary. Repeat the test with additional TDS as needed.

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### TIME

The test time points, at least three, are expressed in hours. Specimens are to be withdrawn within a tolerance of ±15 min or ±2% of the stated time, selecting the tolerance that results in the narrowest time interval.

### INTERPRETATION

Unless otherwise specified in the individual monograph, the requirements are met if the quantities of active ingredient released from the system conform to *Acceptance Table 1* below. Continue testing through the three levels unless the results conform at either $L_1$ or $L_2$.

**Acceptance Table 1**

| Level | Number Tested | Criteria |
|---|---|---|
| $L_1$ | 6 | No individual value lies outside the stated range. |
| $L_2$ | 6 | The average value of the 12 units ($L_1 + L_2$) lies within the stated range. No individual value is outside the stated range by more than 10% of the average of the stated range. |
| $L_3$ | 12 | The average value of the 24 units ($L_1 + L_2 + L_3$) lies within the stated range. Not more than 2 of the 24 units are outside the stated range by more than 10% of the average of the stated range; and none of the units is outside the stated range by more than 20% of the average of the stated range. |

## Apparatus 6 (Cylinder)

### APPARATUS

Use the vessel assembly as described in *Dissolution* ⟨711⟩, *Apparatus, Apparatus 1*, except replace the basket and shaft with a stainless steel cylinder stirring element with the specifications shown in *Figure 3*. This cylinder stirring element is composed of two parts, one comprising the shaft and the upper cylinder, and the other being the extension cylinder. Since both parts are serially numbered, it is recommended that they be kept paired because the friction fitting is done individually for each cylinder, allowing a very tight fit. The distance between the inside bottom of the vessel and the cylinder is maintained at 25 ± 2 mm during the test.

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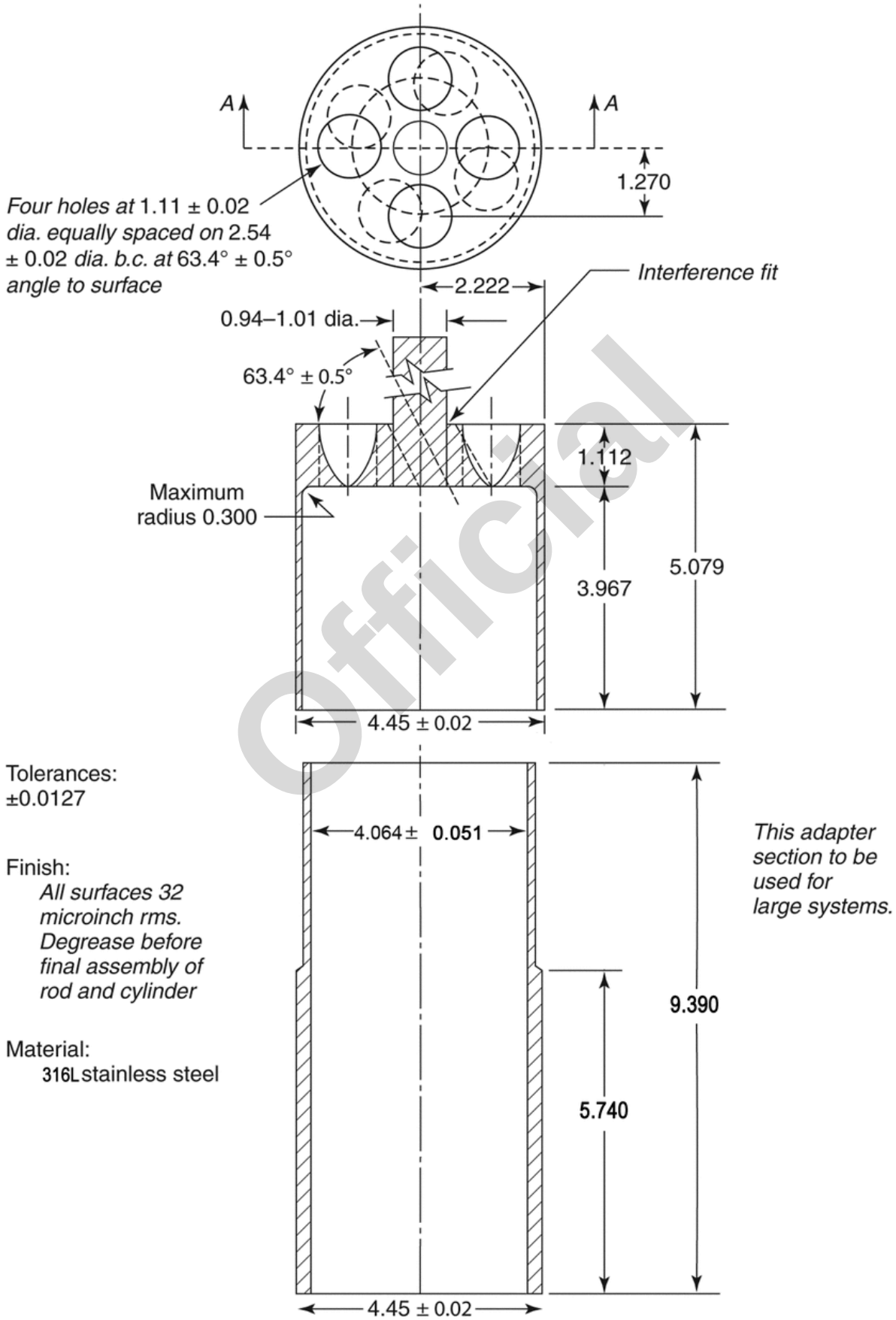


Figure 3. Cylinder stirring element. (All measurements are expressed in cm unless noted otherwise.)

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MEDIUM

See *Dissolution medium* in *Dissolution* ⟨711⟩*, Procedure, Apparatus 1 and Apparatus 2, Immediate-Release Dosage Forms*.

PROCEDURE

Place the stated volume of *Medium* in the vessel, and equilibrate to 32 ± 0.5°.

Apply the TDS to the cylinder, assuring that the release surface is as smooth as possible and the TDS is completely and firmly attached to the cylinder. The TDS, with release side facing the *Medium*, may be attached to the cylinder by an appropriate and validated procedure such as use of an adhesive, double-face adhesive tape, membrane, or nylon net. Care must be taken to avoid the presence of air bubbles between the membrane, if used, and the TDS, or the presence of wrinkles on the surface of the TDS. Carefully remove the protective liner from the TDS without causing damage to the surface. If additional reinforcement of the TDS to the cylinder is needed, inert metal wire or a polymer ring may be used.

Place the cylinder in the apparatus, and immediately rotate at the rate specified in the individual monograph. The vessel may be covered during the test to minimize evaporation.

At each sampling time interval, withdraw a specimen from a zone midway between the surface of the *Medium* and the top of the rotating cylinder, not less than 1 cm from the vessel wall.

Perform the analysis on each sample aliquot as directed in the individual monograph, correcting for any volume losses, as necessary. Repeat the test with additional TDS, as needed.

TIME

The test time points, at least three, are expressed in hours. Specimens are to be withdrawn within a tolerance of ±15 min or ±2% of the stated time, selecting the tolerance that results in the narrowest time interval.

INTERPRETATION

Unless otherwise specified in the individual monograph, the requirements are met if the quantities of active ingredient released from the system conform to *Acceptance Table 1* above. Continue testing through the three levels unless the results conform at either $L_1$ or $L_2$.

***Change to read:***

## Apparatus 7 (Reciprocating Holder)

APPARATUS

The assembly consists of (1) a set of volumetrically calibrated or tared solution containers made of glass or other suitable inert material that should not sorb, react with, or interfere with the specimen being tested; (2) a motor and drive assembly to reciprocate the system vertically and to index the system horizontally to a different row of vessels, automatically if desired; and (3) a set of suitable sample holders (see *Figure 4*, *Figure 5a*, *Figure 5b*, *Figure 5c*, and *Figure 5d*).

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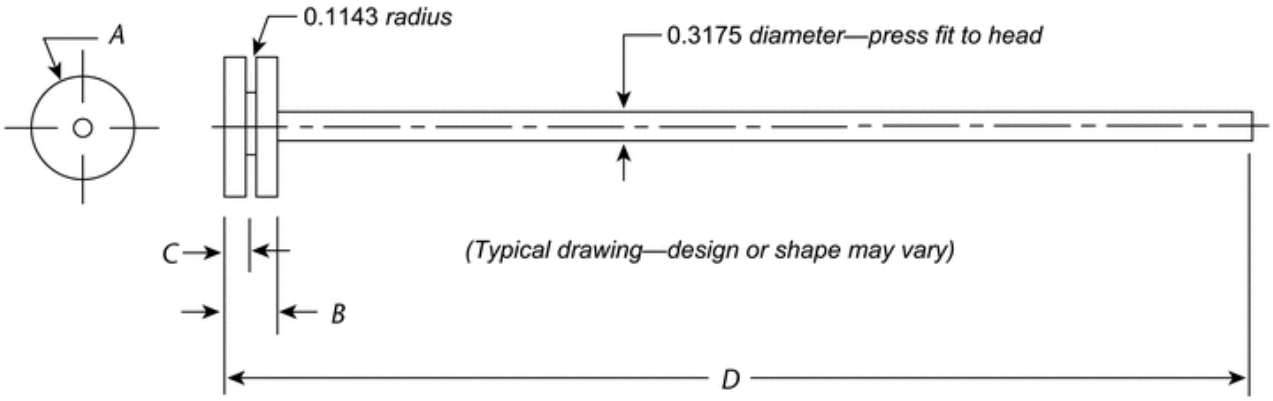


Dimensions are in centimeters.

| | HEAD | | | | ROD | | O-RING |
|---|---|---|---|---|---|---|---|
| System[a] | *A* (Diameter) | *B* | *C* | Material[b] | *D* | Material[c] | (not shown) |
| 1.6 $cm^2$ | 1.428 | 0.9525 | 0.4750 | SS/VT | 30.48 | SS/P | Parker 2–113–V884–75 |
| 2.5 $cm^2$ | 1.778 | 0.9525 | 0.4750 | SS/VT | 30.48 | SS/P | Parker 2–016–V884–75 |
| 5 $cm^2$ | 2.6924 | 0.7620 | 0.3810 | SS/VT | 8.890 | SS/P | Parker 2–022–V884–75 |
| 7 $cm^2$ | 3.1750 | 0.7620 | 0.3810 | SS/VT | 30.48 | SS/P | Parker 2–124–V884–75 |
| 10 $cm^2$ | 5.0292 | 0.6350 | 0.3505 | SS/VT | 31.01 | SS/P | Parker 2–225–V884–75 |

[a] Typical system sizes.
[b] SS/VT=Either stainless steel or virgin Teflon.
[c] SS/P=Either stainless steel or Plexiglas.

Figure 4. Reciprocating disk sample holder.

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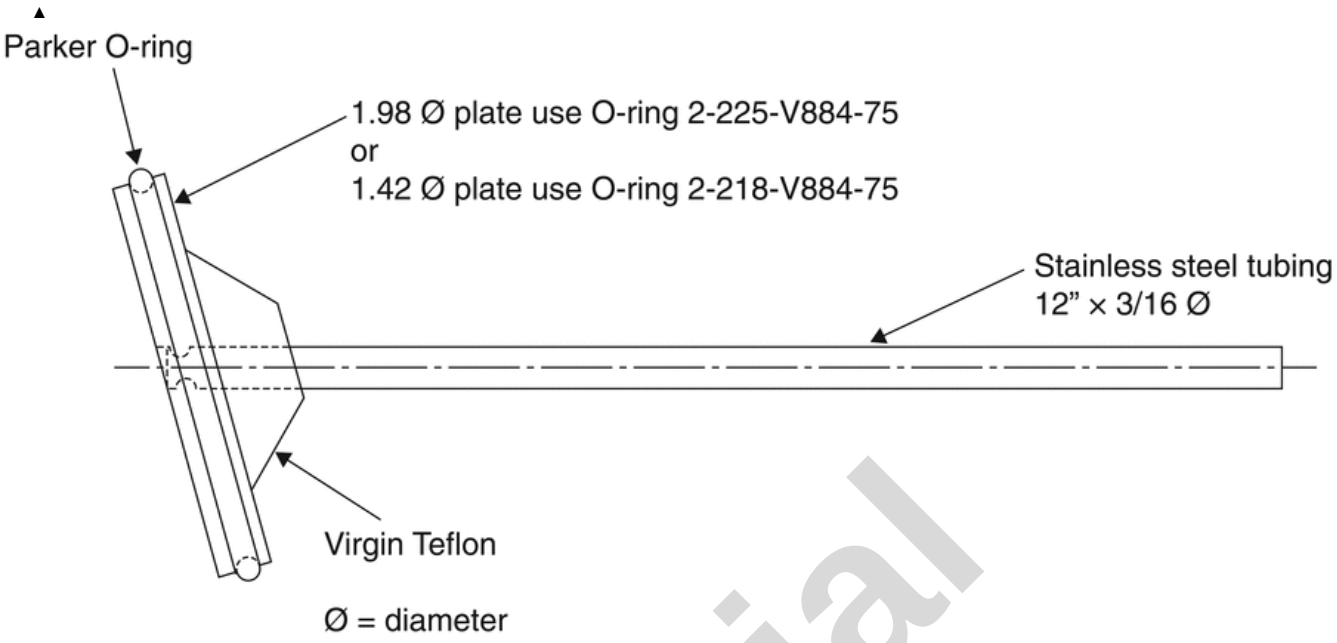


Figure 5a. Transdermal system holder—angled disk.

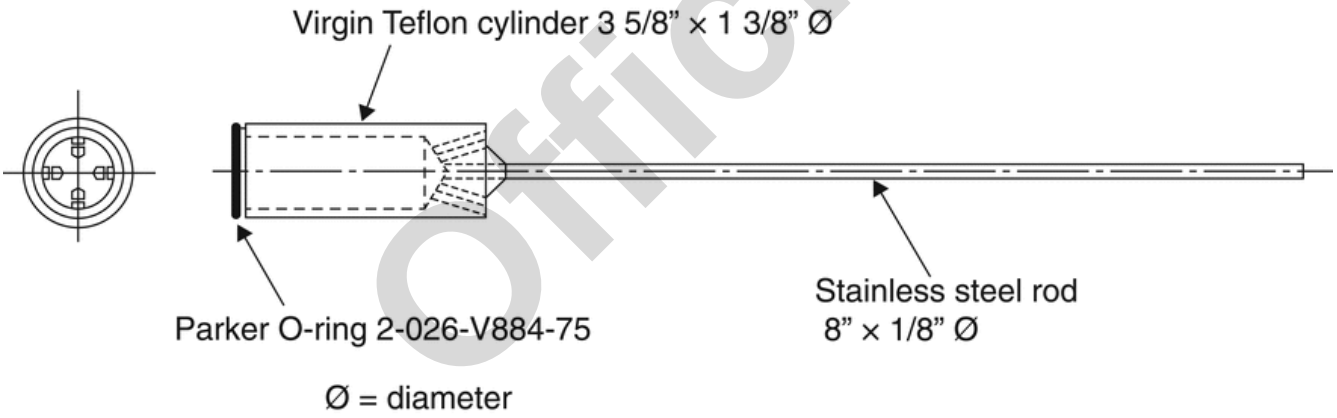


Figure 5b. Transdermal system holder—cylinder.

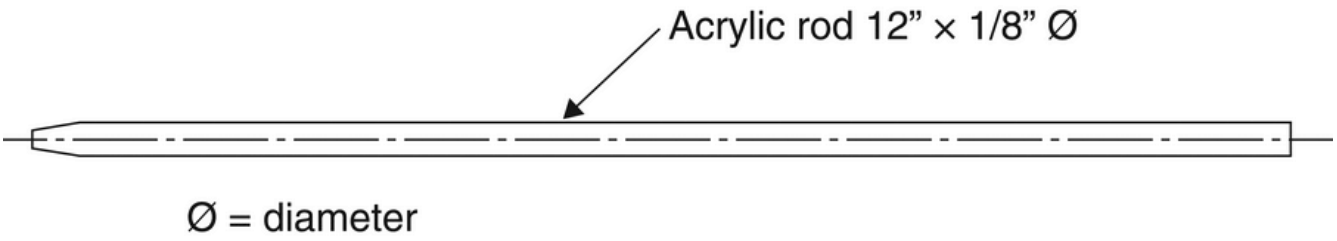


Figure 5c. Oral extended-release tablet holder—rod, pointed for gluing.

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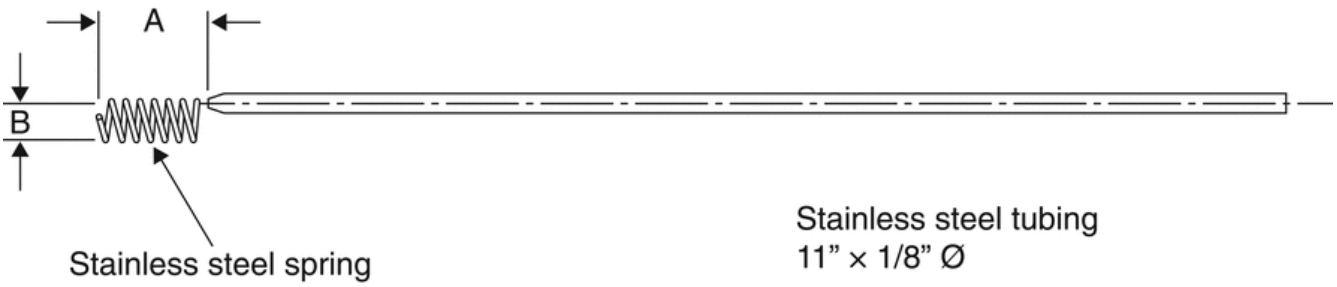


Stainless Steel Spring Dimensions

| A | B |
|---|---|
| 1.45 | .58 Ø |
| 1.40 | .31 Ø |
| .96 | .33 Ø |
| .60 | .25 Ø |

Ø = diameter

Figure 5d. Oral extended-release tablet holder—spring holder.

▲ (ERR 1-May-2018)

During the test, the solution containers are partially immersed in a suitable water bath of any convenient size that permits maintaining the temperature inside the containers at 32 ± 0.5° for TDS or at 37 ± 0.5° for other dosage forms during the test.

No part of the assembly, including the environment in which the assembly is placed, contributes significant motion, agitation, or vibration beyond that due to the smooth, vertically reciprocating sample holder.

Apparatus that permits observation of the system and holder during the test is preferable.

Use the size container and sample holder as specified in the individual monograph.

## MEDIUM

See *Dissolution medium* in *Dissolution* ⟨711⟩, *Procedure, Apparatus 1 and Apparatus 2, Immediate-Release Dosage Forms*.

## SAMPLE PREPARATION A (FOR OSMOTIC PUMP TABLETS)

Attach each unit to be tested to a suitable holder by an appropriate and validated procedure such as use of an adhesive to adhere the edge of the tablet to a holder (e.g., *Figure 5c*), the spring holder (*Figure 5d*), a small nylon net bag, or a membrane.

## SAMPLE PREPARATION B (FOR TDS)

Apply the TDS to the appropriate holder, assuring that the release surface is as smooth as possible and the TDS is completely and firmly attached to the holder. The TDS, with release side facing the *Medium*, may be attached to the holder by an appropriate and validated procedure such as use of an adhesive, double-face adhesive tape, membrane, or nylon net. Care must be taken to avoid the presence of air bubbles between the membrane, if used, and the TDS, or the presence of wrinkles on the surface of the TDS. Carefully remove the protective liner from the TDS without causing damage to the surface of the TDS. If additional reinforcement of the TDS to the holder is needed, inert metal wire or a polymer ring may be used.

## SAMPLE PREPARATION C (FOR OTHER DOSAGE FORMS)

Attach each dosage form to be tested to a suitable holder.

## PROCEDURE

Place the stated volume of *Medium* in the solution containers, and equilibrate to the test temperature.

Suspend each prepared sample holder from a vertically reciprocating shaker such that each system is continuously immersed in *Medium* during the entire test.

Reciprocate at a frequency of about 30 cycles/min with an amplitude of about 2 cm, or as specified in the individual monograph, for the specified time.

At each sampling time interval, remove the solution containers from the bath, cool to room temperature, and add sufficient solvent (i.e., water in most cases) to correct for evaporative losses.

Perform the analysis on each sample as directed in the individual monograph. Repeat the test with additional TDS, as needed.

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## TIME

The test time points, at least three, are expressed in hours. Specimens are to be withdrawn within a tolerance of ±15 min or ±2% of the stated time, selecting the tolerance that results in the narrowest time interval.

## INTERPRETATION

Unless otherwise specified in the individual monograph, the requirements are met if the quantities of active ingredient released from the TDS conform to *Acceptance Table 1* above, or the appropriate acceptance table in ⟨711⟩ for other dosage forms. Continue testing through the three levels unless the results conform at either $L_1$ or $L_2$.

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# ⟨729⟩ GLOBULE SIZE DISTRIBUTION IN LIPID INJECTABLE EMULSIONS

## INTRODUCTION

Lipid injectable emulsions for intravenous administration are sterile oil-in-water emulsions of soybean oil, used to provide an ample supply of essential fatty acids, linoleic and linolenic, dispersed with the aid of an emulsifying agent in *Water for Injection*. Alternatively, soybean oil can be mixed with other suitable oils (neutral triglycerides), such as safflower oil, medium-chain triglycerides (MCT) derived from coconut or palm kernel oils, olive oil, or a marine oil, such as menhaden oil. The size of the lipid droplets is critical: because of mechanical filtration, larger-size fat globules (>5 µm) can be trapped in the lungs. The essential size characteristics of a lipid injectable emulsion for intravenous use include the mean diameter of the lipid droplets and the range of the various droplet diameters distributed around the mean diameter, expressed as the standard deviation. In particular, the amounts of fat globules comprising the large-diameter tail of the globule size distribution are especially important with respect to infusion safety. These two regions of the globule size distribution (mean droplet size and large-diameter tail) must be controlled within specified limits.

The two methods described below are used for determination of the mean lipid droplet diameter and the distribution of large-diameter globule sizes in lipid injectable emulsions. *Method I* and *Method II* must be validated. The methods described below to assess the quality of lipid injectable emulsions are to be performed in two stages.

***Change to read:***

## METHOD I—▲MEASUREMENT OF MEAN DROPLET DIAMETER BY DYNAMIC LIGHT SCATTERING OR CLASSICAL LIGHT SCATTERING▲ (USP 1-AUG-2019)

For the determination of the mean droplet size of lipid injectable emulsions, either of two common light-scattering techniques may be employed: (1) dynamic light scattering (DLS), also known as photon correlation spectroscopy (PCS), or (2) classical light scattering ▲[laser diffraction (LD)],▲ (USP 1-Aug-2019) based on Mie scattering theory. The DLS, or PCS, technique is based on analyzing the rapid temporal fluctuations in the scattered light intensity that occur due to the random Brownian motion, or diffusion, of any particles, including lipid droplets, suspended in liquid. The intensity is measured at a given angle (usually 90°) by a suitable detector (e.g., photomultiplier tube) able to measure the rapidly fluctuating scattered light intensity produced by the suspended, diffusing droplets. These scattered intensity data are typically used to calculate the intensity autocorrelation function, which is a simple decaying exponential function in time for droplets of uniform size. A distribution of droplet sizes expresses itself by ▲the superposition, or summation, of▲ (USP 1-Aug-2019) exponential functions of different decay times, ▲each representative of a given droplet size.▲ (USP 1-Aug-2019) The autocorrelation function generated by the scattered intensity data obtained from a given emulsion can be "inverted" by means of an appropriate deconvolution algorithm in order to obtain the approximate distribution of intensity-weighted diffusion coefficients. From the latter, the distribution of small-diameter droplets is calculated, using the Stokes–Einstein equation and the rules of classical (Mie) light scattering.

By contrast, classical light scattering based on Mie theory analyzes the spatial ▲(angular),▲ (USP 1-Aug-2019) rather than temporal, variation of the scattered light intensity by measuring the latter as a function of the scattering angle, typically over a large range of detected angles. The temporal fluctuations in the scattering intensity due to Brownian motion are averaged out in time for each angular measurement. This angular variation occurs as a consequence of the mutual interference of individual scattered waves arriving at the detector with different phases from different points within ▲each of the▲ (USP 1-Aug-2019) lipid droplets, ▲summed over all of the▲ (USP 1-Aug-2019) different particles. The extent of the angular variation is significant whenever the droplet diameter is not small compared with the wavelength of the laser light (typically ▲633–▲ (USP 1-Aug-2019)635 nm). Droplets of a given size and refractive index yield a unique curve of scattering intensity versus angle. A distribution of droplet sizes gives rise to a final angular dependence that represents the superposition, or summation, of ▲all of the▲ (USP 1-Aug-2019) individual (different) intensity versus angle curves. The measured angular dependence of the scattering intensity obtained from a given emulsion sample, ▲in principle,▲ (USP 1-Aug-2019) can be inverted by means of an appropriate deconvolution algorithm ▲in combination with▲ (USP 1-Aug-2019) Mie scattering theory in order to obtain the approximate droplet size distribution.

Thus, light scattering, using either dynamic light scattering (i.e., ▲varying▲ (USP 1-Aug-2019) temporal fluctuations due to droplet diffusion) or classical light scattering/Mie theory (i.e., ▲varying,▲ (USP 1-Aug-2019) average intensity versus angle), can provide acceptable results for both the mean diameter and, ▲to a lesser degree, the▲ (USP 1-Aug-2019) standard deviation of the droplet size distribution. For purposes of illustrating the method used in *Method I*, a dynamic light-scattering technique is described. For guidance regarding instruments employing classical Mie-theory light scattering, see *Light Diffraction Measurement of Particle Size* ⟨429⟩.

### Apparatus

A suitable DLS/PCS instrument with or without the capability of automatic sample dilution is controlled by validated software and is used to perform the measurement, with the scattering angle typically set at 90°. The intensity-weighted results (mean diameter and standard deviation) are reported, provided it is clearly stated which values are given and that the necessary parameter values required for all requisite calculations are also given.

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## Water

Pass distilled water through a filter of 0.2-µm pore size, and degas by sonication, or use *Sterile Water for Injection* stored in a glass container.

## Standard Preparation

To a pre-established volume of *Water* add an appropriate amount of concentrated suspension, containing National Institute of Standards and Technology (NIST)-traceable polystyrene latex standard particles or other suitable nanospheres. Gently mix the fluids to achieve a homogeneous suspension. The diluted suspension will be slightly turbid in appearance. If the DLS/PCS instrument is equipped with an automatic dilution system, the starting concentrated sample can be analyzed by injection directly into the instrument via a syringe, with further dilution occurring automatically to optimize the droplet concentration for analysis. Alternatively, the sample would require greater manual dilution with *Water* (typically by at least a factor of 10 over the first dilution), and then this sample would be instilled into a "drop-in" cuvette. The optimum dilution scheme that achieves the proper scattering intensity for the cuvette-based analysis will be determined by the instrument specifications. Thus, the concentration of latex in the final sample must be optimized for the DLS/PCS instrument used. This should be performed separately for three different size standards of approximately 100, 250, and 400 nm (triplicate analyses per size), and the corresponding results of intensity-weighted mean diameter and standard deviation should coincide with the expected values within acceptable errors.

## Test Preparation

To a pre-established volume of *Water* add an appropriate volume of sample from the lipid injectable emulsion. Gently mix the fluids to achieve a homogeneous suspension. The diluted suspension will be slightly turbid in appearance. If the DLS/PCS instrument is equipped with an automatic dilution system, the starting concentrated sample can be analyzed by injection directly into the instrument via a syringe. Further dilution of the sample then occurs automatically to optimize the droplet concentration for analysis, ensuring that it is not so high as to cause artifacts due to multiple scattering or interdroplet interactions. Alternatively, the sample would require greater manual dilution with *Water* (typically by at least a factor of 10 over the first dilution), and then this sample would be instilled into a "drop-in" cuvette. The optimum dilution scheme that achieves the proper scattering intensity for the cuvette-based analysis will be determined by the instrument specifications. Thus, the concentration of lipid injectable emulsion in the final sample must be optimized for the DLS/PCS instrument used. ▲Testing should be done in triplicate or, as appropriate, based on method validation data.▲ (USP 1-Aug-2019)

## System Suitability

Using the *Standard Preparation,* measure the intensity-weighted mean particle diameter and the corresponding standard deviation. The system is suitable once the sample temperature has reached equilibration and the results have stabilized and triplicate mean droplet diameter measurements are obtained. The coefficient of variation (CV) should not exceed ▲15% (see below)▲ (USP 1-Aug-2019) of the NIST-traceable mean droplet diameter. A larger CV value indicates that the latex microspheres are not suitable as a standard because they either inherently lack uniformity or have become agglomerated to an unacceptable extent. In this case, another standard latex suspension must be selected and tested.

▲[NOTE—The recommended 15% CV limit is increased here because of the differences in the way the mean droplet size is determined by DLS versus classical light scattering.]▲ (USP 1-Aug-2019)

## Procedure and Interpretation

If the DLS/PCS instrument is equipped with an automatic dilution system, use a disposable syringe to load the *Standard preparation* or *Test preparation*. If no automatic dilution system is used, transfer the appropriately diluted preparation to a cuvette, and place the cuvette in the ▲temperature-regulated sample holder of the instrument.▲ (USP 1-Aug-2019) Allow the sample to equilibrate to a preset controlled temperature close to ambient (between 20° and 25°, as in the USP definition found in *Packaging and Storage Requirements* ⟨659⟩). Set the instrument scattering angle to 90°, and carry out the measurements. As long as the chi-square ($\chi^2$) goodness-of-fit parameter remains acceptably low (per instrument specifications), the results for the *Test preparation* are acceptable. Excessive values of the $\chi^2$ parameter suggest that the droplet distribution is not normal and may indicate an unstable emulsion. The intensity-weighted mean droplet diameter (MDD) for lipid injectable emulsions must be less than 500 nm or 0.5 µm, irrespective of the concentration of the dispersed lipid phase.

▲Submicron results from the DLS method and classical (Mie) light scattering method may vary due to the different physical principles of the mechanism of testing and the different mathematical procedures used to analyze the underlying data.▲ (USP 1-Aug-2019)

***Change to read:***

# METHOD II—MEASUREMENT OF LARGE GLOBULE CONTENT BY LIGHT OBSCURATION OR EXTINCTION METHOD

For determination of the extent of the large-diameter droplet tail (>5 µm) of lipid injectable emulsions, a light obscuration (LO) or light extinction (LE) method that employs a single-particle (globule) optical sizing (SPOS) technique is used. ▲DLS

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methodology is effective only for characterizing smaller particle sizes in the nominal size range of about 1–1000 nm; therefore, DLS is complimentary to LE and is particularly effective in sizing droplets smaller than 500 nm. Importantly, DLS is only a sizing method and provides only semi-quantitative information on the relative droplet size distribution. DLS does not count particles or droplets; thus, it is mainly a qualitative method. In contrast, LE both sizes (1.3–400 µm) and counts fat globules providing "reliable" count values at a size threshold of 5 µm.▲ (USP 1-Aug-2019)

During application of the LE/SPOS technique, passage of a droplet through a thin optical sensing zone results in ▲effective▲ (USP 1-Aug-2019) blockage of a portion of the incident light beam, causing a momentary decrease in the light intensity reaching the ▲distant▲ (USP 1-Aug-2019) "extinction" detector. The magnitude of this decrease in the signal is ▲related▲ (USP 1-Aug-2019) to the cross-sectional area of the droplet (assumed smaller than the sensing zone thickness), i.e., to the square of the droplet diameter. ▲The larger the droplet diameter, the larger the height (voltage) of the signal pulse produced by the momentary decrease in the detected light intensity.▲ (USP 1-Aug-2019) During optimization of the LE/SPOS instrument for a given emulsion sample, a series of dilutions should be tested to achieve consistency between samples. The goal is to identify a standard range of dilutions that yield consistent data and are most applicable to the formulation tested. Ideally, when comparing different emulsions, the same approximate number of globules are sized each time, and once a standard is achieved, it should be incorporated into the routine sampling plan for validation testing. As long as the fat globule concentration is below the "coincidence limit" of the sensor (determined by the flow cell and optical design, ▲verified experimentally),▲ (USP 1-Aug-2019) only one globule at most will pass through the sensing zone at any given time, allowing it to be counted and accurately sized (with less than 1% ▲particle or globule▲ (USP 1-Aug-2019) coincidence events). Both the coincidence ▲(concentration)▲ (USP 1-Aug-2019) limit and the optimal flow rate must be known for the LE/SPOS sensor used. Furthermore, it is prudent to perform the large-diameter measurements at a reduced emulsion concentration such that the measurable droplet concentration ▲from the▲ (USP 1-Aug-2019) threshold of detection (e.g., >1.8 µm) to an ▲appropriate▲ (USP 1-Aug-2019) upper ▲size▲ (USP 1-Aug-2019) limit (e.g., 50 µm) is only approximately one-third of the nominal coincidence limit for the sensor used. The resulting single pulse heights are converted to droplet diameters using a standard calibration curve previously constructed from NIST-traceable monosized polystyrene microspheres of known diameters. For additional guidance in the use of the light obscuration methodology, see *Particulate Matter in Injections* ⟨788⟩.

## Apparatus

A suitable light obscuration instrument with or without the capability of automatic sample dilution and controlled by a personal computer (PC) is used for the measurement. The number- and volume-weighted particle size distribution data are reported, provided that it is clearly stated which values are given and that the necessary parameter values required for all necessary calculations are also given.

## Water

Pass distilled water through a filter of 0.2-µm pore size ▲and degas by sonication,▲ (USP 1-Aug-2019) or use *Sterile Water for Injection* stored in a glass container.

## Standard Preparation

To a pre-established volume of *Water* add an appropriate amount of concentrated suspension, containing NIST-traceable polystyrene latex standard particles or other suitable microspheres. Gently mix the fluids to achieve a homogeneous suspension. If the light obscuration instrument is equipped with an automatic dilution system, the starting concentrated sample can be analyzed by injection directly into the instrument via a syringe or Teflon sample line. Further dilution of the sample then occurs automatically to optimize the particle concentration for analysis. Alternatively, the sample would require greater manual dilution with water (typically by at least a factor of 10 over the first dilution). The resulting diluted sample is then instilled in an appropriate, clean container, such as a sterile Type I glass container, before being passed through the sensor. In either case the final particle concentration is caused to lie below the coincidence limit of the sensor. The sizing and counting accuracy of the light obscuration instrument should be obtained using two different size standards of approximately 5 and 10 µm (triplicate analyses per size). For the standards after system calibration, set the instrument threshold of detection at 1.8 µm, extended to an upper limit of 50 µm. The corresponding results for the mean diameter should coincide with the expected values, within 10% of the relative standard deviation and 90%–110% size accuracy. In addition, the number of particle counts obtained per milliliter should also agree within ±10% with the concentration values certified in the documentation provided with each NIST-traceable size standard.

## Test Preparation

To a pre-established volume of *Water* add an appropriate volume of sample from the lipid injectable emulsion (triplicate analyses per sample). Gently mix the fluids to achieve a homogeneous suspension. The diluted emulsion will be slightly turbid in appearance. If the light obscuration instrument is equipped with an automatic dilution system, the starting concentrated sample can be analyzed by injection directly into the instrument via a syringe or nonreactive[1] Teflon sample line. Further dilution then occurs automatically to optimize the droplet/globule concentration for analysis. Alternatively, the sample would require greater manual dilution with water (typically by at least a factor of 10 over the first dilution). The resulting diluted sample is then instilled in an appropriate, clean container such as a sterile Type I glass container. In either case the final droplet/globule concentration is caused to lie below the coincidence limit of the sensor.

[1] Polyvinyl chloride (PVC) with diethylhexylphthalate (DEHP) has been shown to induce breakdown of lipid injectable emulsions (Drug Product Problem Reporting System. USP File Access No. 11173, May 15, 1991).

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## System Suitability

Perform prior to the test procedure, using the *Standard preparation* of a 5- and 10-µm NIST-traceable particle. Measure in triplicate the number-weighted particle diameter and the counts/mL of the standard. The system is suitable when the triplicate mean number-weighted particle diameter measurements are within 10% of the target value, both in terms of repeatability (CV) and closeness to the certified size on the label of the NIST-traceable standard.

## Procedure and Interpretation

If the light obscuration instrument is equipped with an automatic dilution system, use a disposable syringe or Teflon sample line to load the *Standard Preparation* or *Test Preparation.* If no automatic dilution system is used, transfer the sample to an appropriate large-volume, clean container such as a sterile Type I glass vessel containing an appropriate volume of water. Allow the sample and water to mix thoroughly to achieve a homogeneous suspension. Set the instrument threshold of detection at 1.8 µm, extended to an upper ▲size▲ (USP 1-Aug-2019) limit of 50 µm, and vary the concentration and/or data collection times such that there is at least a factor of two in the difference of the total number of globules that measure >5 µm between at least two sample runs. In any case, the number of globules that measure >5 µm should be large enough so that it represents an adequate number of globules that are statistically representative of the large-diameter tail population of the native emulsion. The volume-weighted, large-diameter fat globule limits of the dispersed phase, expressed as the percentage of fat residing in globules larger than 5 µm (PFAT5) for a given lipid injectable emulsion, must not exceed 0.05%. ▲Most commonly used instruments provide calculation in software (HIAC Royco, PSS AccuSizer, Klotz). However, the general equation applied is based on the sum of the number of droplets per milliliter of sample per channel multiplied by the volume of a sphere of the geometric mean of each channel, i.e., $\Sigma(\#/mL/channel)\ (\pi D3/6)$.▲ (USP 1-Aug-2019)

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# ⟨730⟩ PLASMA SPECTROCHEMISTRY

## INTRODUCTION

Plasma-based instrumental techniques, which are useful for pharmaceutical analyses, fall into two major categories: those based on the inductively coupled plasma, and those where a plasma is generated at or near the surface of the sample. The inductively coupled plasma (ICP) is a high-temperature excitation source that desolvates, vaporizes, and atomizes aerosol samples and ionizes the resulting atoms. The excited analyte ions and atoms can then subsequently be detected by observing their emission lines, a method termed inductively coupled plasma–optical emission spectroscopy (ICP–OES; also referred to as inductively coupled plasma–atomic emission spectroscopy), or the excited or ground state ions can be determined by a technique known as inductively coupled plasma–mass spectrometry (ICP–MS). ICP–OES and ICP–MS may be used for either single- or multi-element analysis and used for either sequential or simultaneous analyses with good sensitivity over an extended linear range.

For additional information and discussion of the theory and principles of measurements, see *Plasma Spectrochemistry—Theory and Practice* ⟨1730⟩.

## QUALIFICATION OF PLASMA SPECTROPHOTOMETERS

Qualification of the ICP–OES or the ICP–MS can be divided into three elements: installation qualification (IQ), operational qualification (OQ), and performance qualification (PQ)—see also the general chapter *Analytical Instrument Qualification* ⟨1058⟩.

### Installation Qualification

The IQ requirements provide evidence that the hardware and software are properly installed in the desired location, and that the environment, in which the instrument will be used, is adequate.

### Operational Qualification

The purpose of OQ is to demonstrate that instrument performance is suitable. In OQ, an instrument's performance is characterized using standards of known spectral properties to verify that the system operates within target specifications. OQ is a check of the key operational parameters performed after installation and after repairs and/or maintenance. Instrument vendors often have samples and test parameters available as part of the IQ/OQ package.

### Performance Qualification

PQ determines whether the instrument is capable of meeting the user's requirements for all the parameters that may affect the quality of the measurement. Depending on typical use, the specifications for PQ may be different from the manufacturer's specifications. For validated methods, specific PQ tests, also known as system suitability tests, can be used in lieu of PQ requirements. A solution used in the initial standard curve of an ICP–OES or ICP–MS instrument must be reassayed as a check standard at appropriate, pre-established intervals throughout the analysis of the sample set.

For single-element ICP–OES analyses, when analytical wavelengths are between 200 and 500 nm, or concentrations are >1 µg/mL, the reassayed standard should agree with its expected value to within ±10%, or as specified in an individual monograph. For multi-element ICP–OES analyses, when analytical wavelengths are <200 nm or >500 nm, or at concentrations of <1 µg/mL, the reassayed standard should agree with its theoretical value to within ±20%, or as specified in an individual monograph. For single-element ICP–MS analyses, when analytical masses that are free of interferences and when concentrations are >1 ng/mL, the reassayed standard should agree with its expected value to within ±10%, or as specified in an individual monograph.

For multi-element ICP–MS analyses, or when concentrations are <1 ng/mL, or for single-element analyses where interferences may be present, the reassayed standard should agree with its expected value to within ±20%, or as specified in an individual monograph.

In cases where an individual monograph provides different guidance regarding the reassayed check standard for ICP–OES or ICP–MS, the requirements of the monograph take precedence. Specific procedures, acceptance criteria, and time intervals for characterizing ICP–OES or ICP–MS performance depend on the instrument and intended application.

## PROCEDURE

Users must evaluate and select the type of material of construction, pretreatment, and cleaning of analytical labware used in ICP–OES and ICP–MS analyses. The material must be inert and, depending on the specific application, resistant to caustics, acids, and/or organic solvents. For some analyses, diligence must be exercised to prevent the adsorption of analytes onto the surface of a vessel; contamination of the sample solutions from metal and ions present in the container also can lead to inaccurate results. For the analysis of a ubiquitous element, it is often necessary to use the purist grade of reagent or solvent available. Check all solutions for elemental contamination before they are used in an analysis.

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### Standard Solution

Standard solutions are used to standardize the instrument at the time-of-use, and prepare these solutions as directed in the individual monograph. [NOTE—Commercially available single- or multi-element standard solutions, traceable to the National Institute of Standards and Technology or to an equivalent national metrology organization, can be used in the preparation of standard solutions.] Standard solutions, especially those used for ultra-trace analyses, may have limited shelf-life. Stability of standard solutions can vary depending on concentration, analyte of interest, storage container type, and storage conditions. For these reasons, standard solutions with concentrations <10 ppm (w/v) should be retained for NMT 24 h, unless stability is demonstrated experimentally.

The method of standard additions can be used for instrument standardization. This method involves adding a known concentration of the analyte element to the sample at NLT two concentration levels against an unspiked sample preparation. The instrument response is plotted against the concentration of the added analyte element, and a linear regression line is drawn through the data points. The absolute value of the *x*-intercept multiplied by any dilution factor is the concentration of the analyte in the sample, and many instruments will perform the analyte calculation automatically. Once a method has been developed and is in routine use, it is common practice to calibrate with a blank and a single standard. One-point standardizations are suitable for conducting limit tests on production materials and final products, provided that the methodology has been rigorously validated for sufficient specificity, sensitivity, linearity, accuracy, precision, ruggedness, and robustness.

### Sample Solution

Although solid samples and slurried samples may be analyzed, it is generally necessary to prepare solutions of samples for analyses as directed in the individual monograph. Samples may be dissolved in any solvent, including organic solvents or acids/bases, that are compatible with the instrument system. Samples that are not soluble in a solvent require digestion. A variety of digestion techniques could be used to dissolve a sample. These include hot-plate and microwave-assisted digestions, including open-vessel and closed-vessel approaches. Note that open-vessel digestion generally is not recommended for the analysis of volatile metals, e.g., selenium and mercury.

### Analysis

The instrument must be standardized for quantitation at the time of use, following the procedure as directed in the individual monograph for the instrumental parameters. The response of standard solutions that bracket the target concentration of an analyte in a sample is determined against an appropriate blank. The detector response is plotted as a function of the analyte concentration. When an analysis is performed at or near the detection limit, the analyst cannot always use a bracketing standard. In such a case, a standard prepared at or near (within ±10% of) the detection limit should be run in triplicate, with an acceptance criteria of ±10% of the theoretical value for the standard for single-element standards, and ±20% of the theoretical value for the standard for multi-element standards.

To demonstrate the stability of the system's initial standardization, the analyst must reassay a solution used in the initial standard curve as a check standard at appropriate, pre-established intervals throughout the analysis of the sample set. Unless otherwise indicated in the individual monograph, the reassayed standard should meet the same acceptance criteria as those outlined in the PQ section above. Sample concentrations are calculated versus the working curve generated by plotting the detector response versus the concentration of the analyte in the standard solutions, and most instruments perform this calculation automatically.

## VALIDATION AND VERIFICATION

### Validation

Validation is required when the ICP–OES or ICP–MS method is intended for use as an alternative to the official procedure for testing an official article. The objective of the ICP–OES or the ICP–MS procedure validation is to demonstrate that the measurement is suitable for its intended purpose, including quantitative determination of the main component in a drug substance or a drug product (Category I assays), quantitative determination of impurities or limit tests (Category II), and identification tests (Category IV). [NOTE—For definition of different categories, see *Validation of Compendial Procedures* ⟨1225⟩.] Depending on the category of the test, analytical procedure validation for the ICP–OES or ICP–MS requires the testing of linearity, range, accuracy, specificity, precision, detection limit, quantitation limit, and robustness. These analytical performance characteristics apply to externally standardized methods and to the method of standard additions.

General chapter ⟨1225⟩ provides definitions and general guidance on the procedures for analytical validation without indicating specific validation criteria for each characteristic. The intention of the following sections is to provide the user with specific validation criteria that represent the minimum expectations for this technology.

#### ACCURACY

For Category I assays or Category II tests, accuracy can be determined by conducting recovery studies with the appropriate matrix spiked with known concentrations of elements. It is also an acceptable practice to compare assay results obtained using the ICP–OES or ICP–MS procedure under validation to those of an established analytical procedure. In standard addition methods, accuracy assessments are based on the final intercept concentration, not the recovery calculated from the individual standard additions.

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**Validation criteria:** 95.0%–105.0% mean recovery for the assay, and 70.0%–150.0% mean recovery for the impurity analysis. These criteria apply throughout the intended range.

## Precision

### REPEATABILITY

The analytical procedure is assessed by measuring the concentrations of six independently prepared sample solutions at 100% of the assay test concentration.
**Validation criteria:** The relative standard deviation is NMT 5.0% for the drug substance assay, NMT 5.0% for the drug product assay, and NMT 20% for the impurity analysis.

### INTERMEDIATE PRECISION

The effect of random events on the analytical precision of the procedure should be established. Typical variables include performing the analysis on different days, using different instrumentation, or having the method performed by two or more analysts. As a minimum, any combination of these factors totaling three experiments will provide an estimation of intermediate precision. For example, this could be one analyst on each of 3 days, or one analyst on two sets of equipment on two different days for one instrument; three analysts could be on the same piece of equipment.
**Validation criteria:** The relative standard deviation is NMT 8.0% for the drug substance assay, NMT 8.0% for the drug product assay, and NMT 25.0% for the impurity analysis.

### SPECIFICITY

The procedure must be able to unequivocally assess each analyte element in the presence of components that are expected to be present, including any matrix components.
**Validation criteria:** Demonstrated by meeting the accuracy requirement

### QUANTITATION LIMIT

The limit of quantitation (QL) is estimated by calculating the standard deviation of NLT 10 replicate measurements of a blank solution and multiplying by 10. When validating a procedure using the method of standard additions, the slope of standards applied to a solution of the test material is used. Other suitable approaches can be used (see ⟨1225⟩).

A measurement of a test solution prepared from a representative sample matrix spiked at the estimated QL concentration must be performed to confirm accuracy. When validating a procedure using the method of standard additions, the validation criterion applies to the final experimental result, not the spike recovery of the individual standard addition levels.
**Validation criteria:** The analytical procedure should be capable of determining the analyte precisely and accurately at a level equivalent to 50% of the specification.

### LINEARITY

A response curve between the analyte concentration and absorbance is prepared from NLT two standard solutions and a blank (for a total of three data points), at concentrations that encompass the anticipated concentration of the test solution. The standard curve is then evaluated using appropriate statistical methods, such as a least-squares regression.

For experiments that do not yield a linear relationship between analyte concentration and the ICP–OES or ICP–MS response, appropriate statistical methods must be applied to describe the analytical response.
**Validation criteria:** Correlation coefficient (*R*), NLT 0.995 for Category I assays and NLT 0.99 for Category II quantitative tests

### RANGE

Range is the interval between the upper and lower concentrations (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy, and linearity that is demonstrated by meeting the precision, accuracy, and linearity requirements.
**Validation criteria:** For Category I tests, the validation range for 100.0% centered acceptance criteria is 80.0%–120.0%. For noncentered acceptance criteria, the validation range is 10.0% below the lower limit to 10.0% above the upper limit. For content uniformity, the validation range is 70.0%–130.0%. For Category II tests, the validation range covers 70.0%–130.0% of the acceptance criteria.

### ROBUSTNESS

The reliability of an analytical measurement is demonstrated by deliberate changes to experimental parameters. Because certain changes to experimental parameters could result in potential safety issues or damage to equipment, robustness is demonstrated by meeting the intermediate precision requirements set forth above.

## Verification

U.S. Current Good Manufacturing Practices regulations [21 CFR 211.194(a)(2)] indicate that users of the analytical procedures, as described in *USP–NF*, are not required to validate these procedures if they are provided in a monograph. Instead, users must simply verify their suitability under actual conditions of use.

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The objective of ICP–OES or ICP–MS procedure verification is to demonstrate that the procedure, as prescribed in a specific monograph, should be executed by the user with suitable accuracy, specificity, and precision using the instruments, analysts, and sample matrices available. According to *Verification of Compendial Procedures* ⟨1226⟩, if the verification of the compendial procedure by following the monograph is not successful, the procedure may not be suitable for use with the article under test. It may be necessary to develop and validate an alternative procedure as allowed in *General Notices, 6.30 Alternative and Harmonized Methods and Procedures*.

Verification of compendial ICP–OES or ICP–MS methods should, at a minimum, include the execution of the validation parameters for specificity, accuracy, precision, linearity, and limit of quantitation, when appropriate, as indicated in *Validation*.

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# ⟨731⟩ LOSS ON DRYING

***Change to read:***

The procedure set forth in this chapter determines the amount of volatile matter of any kind that is driven off under the conditions specified, ▲which is defined as Loss on Drying value.▲ (USP 1-Aug-2020) For substances appearing to contain water as the only volatile constituent, the procedure given in *Water Determination* ⟨921⟩ is appropriate, and is specified in the individual monograph.

Unless otherwise directed in the individual monograph, conduct the determination of Loss on Drying on a 1- to 2-g test specimen. Mix the substance to be tested and, if it is in the form of large particles, reduce the particle size to about 2 mm by ▲▲ (USP 1-Aug-2020) crushing before weighing ▲it▲ (USP 1-Aug-2020) out. ▲▲ (USP 1-Aug-2020) Tare an appropriate glass-stoppered▲▲ (USP 1-Aug-2020) weighing bottle that has been dried for about 30 min under the same conditions to be employed in the determination and cooled to room temperature in a desiccator. Put the test specimen in the bottle, replace the ▲stopper,▲ (USP 1-Aug-2020) and accurately weigh the ▲stoppered▲ (USP 1-Aug-2020) bottle and the contents. By gentle, sidewise shaking, distribute the test specimen as evenly as practicable to a depth of about 5 mm generally, and NMT 10 mm in the case of ▲low bulk density▲ (USP 1-Aug-2020) materials. Place the loaded bottle in the drying chamber, ▲remove▲ (USP 1-Aug-2020) the stopper and ▲leave▲ (USP 1-Aug-2020) it also in the chamber. Dry the test specimen at the▲specified▲ (USP 1-Aug-2020) temperature and time ▲conditions.

[NOTE—The Loss on Drying value is a function of both temperature and time. Therefore, these values must be identified and reported.▲ (USP 1-Aug-2020) The temperature specified in the monograph is to be regarded as being within the range of ±2° of the stated ▲value.▲ (USP 1-Aug-2020)]

When "dry to constant weight" is specified in a monograph, drying shall be continued until two consecutive weighings do not differ by more than 0.50 mg/g of ▲specimen taken, where the second weighing follows▲ (USP 1-Aug-2020) an additional hour of drying. Upon opening the chamber, ▲reapply the same stopper to the bottle,▲ (USP 1-Aug-2020) and allow it to come to room temperature in a desiccator before weighing ▲accurately.▲ (USP 1-Aug-2020)

If the substance melts at a lower temperature than that specified for the determination of Loss on Drying, maintain the bottle with its contents for 1–2 h at a temperature 5°–10° below the melting temperature, then dry at the specified temperature.

Where capsules are to be tested, use a ▲representative sample mixture, excluding the capsule shell, from not▲ (USP 1-Aug-2020) fewer than 4 capsules.

Where tablets are to be tested, use ▲a representative sample mixture▲ (USP 1-Aug-2020) from NLT 4 crushed tablets.

Where the individual monograph directs that Loss on Drying be determined by thermogravimetric analysis, a ▲suitable▲ (USP 1-Aug-2020) balance is to be used ▲(see *Balances* ⟨41⟩.▲ (USP 1-Aug-2020)

Where drying under vacuum over a desiccant is directed in the individual monograph, a vacuum desiccator or a vacuum drying pistol, or other suitable vacuum drying apparatus, is to be used.

Where drying in a desiccator is specified, exercise particular care to ensure that the desiccant is ▲▲ (USP 1-Aug-2020) fully effective. ▲▲ (USP 1-Aug-2020)

Where drying in a capillary-stoppered bottle[1] under vacuum is directed in the individual monograph, use a bottle or tube fitted with a stopper having a 225 ± 25-µm diameter capillary, and maintain the heating chamber at a pressure of 5 mm or less of mercury. At the end of the heating period, admit dry air to the heating chamber, remove the bottle, and with the capillary stopper still in place allow it to cool to room temperature in a desiccator before weighing.

[1] Available as an "antibiotic moisture content flask" from Kimble ▲Chase Life Science and Research Products,▲ (USP 1-Aug-2020) 1022 Spruce St., Vineland, NJ ▲08360.▲ (USP 1-Aug-2020)

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# ⟨733⟩ LOSS ON IGNITION

***Change to read:***

This procedure is provided for the purpose of determining the percentage of test material that is volatilized and driven off under the conditions specified.

Perform the test on finely powdered material, and break up lumps, if necessary, with the aid of a mortar and pestle before weighing the specimen. Weigh the specimen to be tested without further treatment, unless a preliminary drying at a lower temperature, or other special pretreatment, is specified in the individual monograph. Unless other equipment is designated in the individual monograph, conduct the ignition in a suitable muffle furnace or oven that is capable of maintaining a temperature within 25° of that required for the test, and use a suitable crucible, complete with cover, previously ignited for 1 h at the temperature specified for the test, cooled in a desiccator, and accurately weighed.

Unless otherwise directed in the individual monograph, transfer to the tared crucible an accurately weighed quantity, in g, of the substance to be tested, about equal to that calculated by the formula:

$$10/L$$

in which $L$ is the limit (or the mean value of the limits) for Loss on Ignition, in percentage. Ignite the loaded uncovered crucible, and cover at the temperature (±25°) and for the period of time designated in the individual monograph. When "ignition to constant weight" is specified in a monograph, ignite for successive 1-h periods until two consecutive weighings do not differ by more than 0.50 mg/g of specimen. Upon completion of each ignition, cover the crucible, and allow it to cool in a desiccator to room temperature before weighing ▲accurately▲ (ERR 1-Nov-2020).

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# ⟨735⟩ X-RAY FLUORESCENCE SPECTROMETRY

## INTRODUCTION

X-ray fluorescence (XRF) spectrometry is an instrumental method based on the measurement of characteristic X-ray photons caused by the excitation of atomic inner-shell electrons by a primary X-ray source. The XRF method can be used for both qualitative and quantitative analysis of liquids, powders, and solid materials. The X-rays produced by an X-ray tube include characteristic lines that correspond to the anode material and a continuum known as Bremsstrahlung radiation. Both types of X-rays can be used to excite atoms and thus induce X-rays. XRF instrumentation can be divided into one of two categories: Wavelength Dispersive X-ray Fluorescence (WDXRF) and Energy Dispersive X-ray Fluorescence (EDXRF). The main factor distinguishing these technologies is the method used to separate the spectrum emitted by the atoms in the sample. The energy of the X-ray photon is characteristic for a given electron transition in an atom and is *qualitative* in nature. The *intensity* of the emitted radiation is indicative of the number of atoms in the sample and constitutes the *quantitative* nature of the method.

## QUALIFICATION OF XRF SPECTROMETERS

### Installation Qualification

See also *USP* general information chapter *Analytical Instrument Qualification* ⟨1058⟩.

### Operational Qualification

The purpose of operational qualification (OQ) is to verify that the system operates within target tolerances using appropriate samples with known spectral properties. OQ is a check of the key operating parameters and should be performed following installation, repairs, or major maintenance that can affect the performance of the instruments. Note that all calibration samples must be handled with cotton or nitrile gloves and must be stored in sealed plastic containers. Alternatively, they may be fixed in the instrument.

The OQ tests and specifications in the following sections are typical examples only (see *Tables 1* and *3*). Other tests and standards can be used to establish tolerances for these purposes. The instrument vendor often makes samples and test parameters available as part of the IQ package.

**Table 1. EDXRF OQ Specifications**

| Test | Procedure | Acceptance Criteria |
|---|---|---|
| Peak position | Acquire a spectrum of the Al–Cu energy calibration sample. | The spectrum should have at least 3000 counts at the top of both the Al Kα and the Cu Kα peaks. The energy corresponding to the peaks of Al Kα and Cu Kα should differ less than 0.1% from the tabulated values. |
| Detector resolution | Calculate the resolution (full width, half maximum) at the same energy and at the same count rate that was used at IQ. | The resolution value should not change more than 10% from the value determined at IQ. |
| Count rate | Measure the count rate of Al and Cu Kα lines from the energy calibration sample. | <10% change from initial measurements at IQ, for each peak |

The Al–Cu energy calibration sample is a disc of Al–Cu alloy (EN AW-AlCu6BiPb; Alloy 2011, ASTM B211) that has been selected for use in EDXRF spectrometers. This alloy is generally available, is resistant to corrosion, and provides adequate intensities for both Al Kα and Cu Kα. These characteristic lines cover the typical energies used for XRF analysis. Also, sufficient information can be obtained from spectra recorded on this material to assess detector resolution. A more complete compositional specification for this calibration sample is given in *Table 2*.

**Table 2. Specification of the Concentration of the Alloying Elements in Al–Cu Alloy (the Remaining Balance Is Aluminum)**

| Element | Concentration Limits (in % by Weight) |
|---|---|
| Cu | 5–6 |
| Zn | 0.3 maximum |
| Fe | 0.7 maximum |
| Bi | 0.2–0.6 |
| Pb | 0.2–0.6 |
| Si | 0.4 maximum |

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When the energy range of the analytical lines for which the spectrometer will be used includes energies above 50 keV, a pure W metal (99.5% minimum) should be used instead of the Al–Cu alloy. This metal is stable, is generally available, and provides well-defined characteristic lines at 8.40 keV (L-lines) and 59.3 keV (K-lines).

**Table 3. WDXRF OQ Specifications**

| Test | Procedure | Acceptance Criteria |
|---|---|---|
| Peak angle | Perform according to the manufacturer's procedure. Repeat for each crystal. | The angle corresponding to the peak maximum should differ less than 0.10 degree 2θ of the angle measured at IQ. |
| Detector resolution | Full width half maximum at specified wavelengths and at the same measurement conditions at the time of IQ. Repeat for each detector available. | NMT 20% change |
| Count rate | Measure count rate from the specified monitor specimen at a specified wavelength at the same measurement conditions at the time of IQ. Repeat for each detector available. | <10% change from initial measurements at IQ |

Use Inconel 625 (Special Metals Corporation, New Hartford, NY) as a sample for WDXRF spectrometers. Other designations for this alloy are UNS N06625, DIN 2.4856, ASTM B443, ASME SB-443, and AMS 5599. This is a nickel-based alloy that includes chromium, molybdenum, and niobium as the most important alloying elements. A more complete compositional specification is given in *Table 4*.

**Table 4. Elemental Concentrations in Inconel 625**

| Element | Concentration Limits (in % by Weight) |
|---|---|
| Ni | 58.0 minimum |
| Cr | 20.0–23.0 |
| Fe | 5.0 maximum |
| Mo | 8.0–10.0 |
| Nb[a] | 3.15–4.15 |

[a] This could include Ta.

A polished piece of Inconel 625 should never require resurfacing when stored and used appropriately.

The radiation characteristics of nickel can be detected by all detectors that are used in sequential WDXRF, whether they are flow proportional, sealed gas, or scintillation detectors. In combination with Mo K (and L) radiation, the tests regarding peak position and detector response of WDXRF spectrometers can be completed readily. *Table 5* includes the typical wavelengths or energies that are used for XRF analysis.

**Table 5. Energies and Wavelengths[a] for Al, Ni, Cu, Mo, and W**

| | Al | Ni | Cu | Mo | W |
|---|---|---|---|---|---|
| $K\alpha_1$ Line transition | $K–L_{2,3}$ | $K–L_{2,3}$ | $K–L_{2,3}$ | $K–L_3$ | $K–L_3$ |
| $K\alpha_1$ Energy (eV) | 1487 | 7473 | 8041 | 17479 | 59310 |
| $K\alpha_1$ Wavelength (Å) | 8.340 | 1.659 | 1.542 | 0.709 | 0.209 |
| $L\alpha_1$ Line transition | N/A[b] | N/A | N/A | $L_3–M_5$ | $L_3–M_5$ |
| $L\alpha_1$ Energy (eV) | N/A | N/A | N/A | 2293.2 | 8398.2 |
| $L\alpha_1$ Wavelength (Å) | N/A | N/A | N/A | 5.4066 | 1.47632 |

[a] From: Deslattes RD, Kessler EG, Indelicato P, et al. X-ray transition energies: new approach to a comprehensive evaluation. *Rev Mod Phys*. 2003;75(1):35–99. For Al, Ni, and Cu, the $K\alpha_1$ and $K\alpha_2$ energies from *Table V* were averaged with 2:1 weighting. Wavelength conversion uses the *hc*/*E* value from page 94. Values for Mo and W are taken from *Table VI*.
[b] N/A = not applicable.

## Performance Qualification

The purpose of performance qualification (PQ) is to determine that the instrument is capable of meeting the user's requirements for all critical-to-quality measurements.

Depending on typical use, the specifications for PQ may be different from the manufacturer's specifications. Method-specific PQ tests, also known as system suitability tests, may be used in lieu of PQ requirements for validated methods.

Specific procedures, acceptance criteria, and time intervals for characterizing XRF performance depend on the instrument and its intended application. Demonstrating stable instrument performance over extended periods of time provides some assurance that reliable measurements can be obtained from sample spectra using previously validated XRF experiments.

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# PROCEDURE

## General Recommendations

Analysts should check the suitability of all reagents and materials for contamination before using depending on the method used. The analysis of a ubiquitous element often requires the use of the purest grade of reagent or material available. Sample holders and support windows should be appropriate for the analysis and the instrument configuration. Analysts should evaluate the cleaning of equipment used to prepare samples for XRF analysis in order to avoid cross-contamination of samples.

### SAMPLES

**Liquids:** Liquid samples can be introduced directly to the XRF spectrometer provided that the solution consists of a single phase and has sufficiently low volatility. Analysis of liquid samples requires use of a special liquid sample holder and a commercially available support window composed of a suitable polymer film. Alternatively, liquid samples can be transferred onto the surface of a disk and dried before analysis. The experiment typically is conducted using a purge gas. The liquid sample can be spiked directly with solution standards at appropriate concentrations to facilitate accuracy, precision, and specificity tests as required for method validation.

**Powders:** Prepared powders may be measured directly in a liquid sample holder. Alternatively, they may be pressed into pellets. If the powder has poor self-binding properties, it may require a binder such as a wax or ethyl cellulose.

Powders also can be prepared for XRF analysis by fusing the sample material into a glass using a flux, typically sodium tetraborate, lithium tetraborate, and lithium metaborate. Because the temperatures required to melt the flux and dissolve the sample are relatively high (800°–1300°), this procedure is not suitable for the analysis of volatile elements such as mercury and arsenic.

Powder samples can be mixed with appropriate quantities of a certified reference material to facilitate accuracy, precision, and specificity tests as required for method validation. Alternatively, powder samples can be spiked with appropriate quantities of solution-based standards and then dried, ground if necessary, and thoroughly mixed before analysis. Standard additions can be used in instances when physical or chemical properties of the powder may introduce an analyte response bias.

## Standards

Appropriate reference materials that are traceable to the National Institute of Standards and Technology, or equivalent, can be used in the preparation of XRF standards.

## Analysis

For the instrumental parameters (if applicable) follow the procedure in the individual monograph. Because of differences in manufacturers' equipment configurations, analysts can use the manufacturer's suggested default conditions. At the time of use, the instrument must be standardized for the intended use. Analysts should use calibration standards to bracket the expected range of typical analyte concentrations. When they perform an analysis at or near the detection limit, analysts cannot always use a bracketing standard, which is an acceptable strategy for limit tests. Analysts should use regression analysis of the standard plot to evaluate the linearity of detector response, and individual monographs may set criteria for the residual error of the regression line.

To demonstrate the stability of the system's initial standardization, at appropriate intervals throughout their tests on the sample set analysts must re-assay the calibration standard used in the initial standard curve as a check standard. The use of an independently prepared standard also is acceptable. Unless otherwise indicated in the individual monograph, the re-assayed standard should agree with its expected value to within ±2% for *Assay* or ±20% for an impurity analysis.

Sample concentrations are calculated versus the working curve generated by plotting the instrument response versus the concentration of the analyte in the standard solutions.

# VALIDATION AND VERIFICATION

Current Good Manufacturing Practice regulations [21 CFR 211.194(a)(2)] indicate that users of analytical methods described in *USP–NF* are not required to validate the accuracy and reliability of these methods but rather must verify their suitability under actual conditions of use. In this context, and according to these regulations, validation is required when an XRF procedure is used to test a nonofficial article and when this procedure is used as an alternative to the official procedure for testing an official article (see *USP–NF General Notices, 6.30 Alternative and Harmonized Methods and Procedures*). On the other hand, verification must be performed the first time an official article is tested using a *USP* procedure (for informational purposes only, refer to *Verification of Compendial Procedures* ⟨1226⟩).

## Validation

The objective of an XRF method validation is to demonstrate that the measurement procedure is suitable for its intended purpose, including quantitative determination of the main component in a drug substance or a drug product (Category I assays), quantitative determination of impurities or limit tests (Category II), and identification tests (Category IV). Depending on the category of the test (see *Table 2* in *Validation of Compendial Procedures* ⟨1225⟩), the analytical method validation process for XRF requires the testing of linearity, range, accuracy, specificity, precision, quantitation limit, and robustness.

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Performance characteristics that demonstrate the suitability of an XRF method are similar to those required for any analytical procedure. A discussion of the applicable general principles is found in ⟨1225⟩. Specific acceptance criteria for each validation parameter must be consistent with the intended use of the method. The samples for validation should be independent of the calibration set.

## ACCURACY

For Category I assays or Category II tests, analysts can determine accuracy by conducting recovery studies using the appropriate matrix spiked with known concentrations of elements. An appropriate certified standard material provided by USP also can be used. It also is an acceptable practice to compare assay results obtained using the XRF method under validation to those from an established analytical method. When analysts use the method of standard additions, accuracy assessments are based on the final intercept concentration, not the recovery calculated from the individual standard additions.
**Acceptance Criteria:** 98.0%–102.0% recovery for drug substances and drug product assay, 70.0%–150.0% recovery for impurity analysis. These acceptance criteria should be met throughout the validated range.

## PRECISION

**Repeatability:** Analysts should assess the analytical method by measuring the concentrations of six separate standards at 100% of the assay test concentration. Alternatively, they can measure the concentrations of three replicates of three separate samples at different concentrations. The three concentrations should be close enough so that the repeatability is constant across the concentration range. In this case, the repeatability at the three concentrations is pooled for comparison to the acceptance criteria.

*Acceptance Criteria:* The relative standard deviation is NMT 1.0% for drug substance assay, NMT 2.0% for drug product assay, and NMT 20.0% for impurity analysis.
**Intermediate Precision:** Analysts should establish the effect of random events on the method's analytical precision. Typical variables include performing the analysis on different days, using different instrumentation, or having two or more analysts perform the method. As a minimum, any combination of at least two of these factors totaling six experiments will provide an estimation of intermediate precision.

*Acceptance Criteria:* The relative standard deviation is NMT 1.0% for drug substance assay, NMT 3.0% for drug product assay, and NMT 25.0% for impurity analysis.

## SPECIFICITY

The procedure must unequivocally assess each analyte element in the presence of components that may be expected to be present, including any matrix components.
**Acceptance Criteria:** Demonstrated by meeting the *Accuracy* requirement.

## QUANTITATION LIMIT

The limit of quantitation (LOQ) can be estimated by calculating the standard deviation of NLT 6 replicate measurements of a blank and multiplying by 10. Other suitable approaches may be used (see ⟨1225⟩). A measurement of a test sample prepared from a representative sample matrix and spiked so that the concentration is similar to the estimated LOQ concentration must be performed to confirm accuracy.
**Acceptance Criteria:** The analytical procedure should be capable of determining the analyte precisely and accurately at a level equivalent to 50% of the specification.

## LINEARITY

Analysts should demonstrate a linear relationship between the analyte concentration and corrected XRF response by preparing no fewer than five standards at concentrations that encompass the anticipated concentration of the test sample. The standard curve then should be evaluated using appropriate statistical methods such as a least squares regression. The correlation coefficient ($R$), $y$-intercept, and slope of the regression line must be determined.

For experiments that do not have a linear relationship between analyte concentration and XRF response, appropriate statistical methods must be applied to describe the analytical response.
**Acceptance Criteria:** $R$ is NLT 0.995 for Category I assays and NLT 0.99 for Category II quantitative tests.

## RANGE

The range is the interval between the upper and lower concentration (amounts) of analyte in the sample (including the upper and lower concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy, and linearity. Range is demonstrated by meeting the linearity and accuracy requirement.
**Acceptance Criteria:** For 100.0% centered acceptance criteria: 80.0%–120.0%. For non-centered acceptance criteria: 10% below the lower limit of the specification to 10% above the upper limit of the specification. For content uniformity: 70.0–130.0%. For Category II the range requirements are 50.0%–120.0% of the acceptance criteria.

## ROBUSTNESS

The reliability of an analytical measurement should be demonstrated by deliberate changes to experimental parameters. For XRF this can include measuring the stability of the analyte under specified storage conditions.

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**Acceptance Criteria:** The measurement of a standard or sample response following a change in experimental parameters should differ from the same standard measured using established parameters by NMT ±2.0% for a drug product assay and NMT ±20.0% for an impurity analysis.

## Verification

The objective of an XRF method verification is to demonstrate that the procedure as prescribed in a specific monograph is being executed with suitable accuracy, sensitivity, and precision. According to ⟨1226⟩, if the verification of the compendial procedure according to the monograph is not successful, the procedure may not be suitable for use with the article under test. It may be necessary to develop and validate an alternative procedure as allowed in *USP–NF General Notices, 6.30 Alternative and Harmonized Methods and Procedures*.

Although complete revalidation of a compendial XRF method is not required, verification of compendial XRF methods should at minimum include the execution of the validation parameters for specificity, accuracy, precision, and limit of quantitation, when appropriate, as indicated under *Validation* (above).

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# ⟨736⟩ MASS SPECTROMETRY

## INTRODUCTION

Mass spectrometry (MS) is an analytical technique based on the measurement of the mass-to-charge ratio of ionic species related to the analyte under investigation. MS can be used to determine the molecular mass and elemental composition of an analyte as well as provide an in-depth structural elucidation of the analyte.

In addition to being recognized as a powerful structure-elucidation tool, MS is also extensively used for quantitative measurements. For additional information, see general information chapter *Applications of Mass Spectrometry* ⟨1736⟩, which provides a detailed discussion of MS.

Currently available MS instrumentation offers a wide range of capabilities for qualitative and quantitative analysis, which results in a wide range of potential MS experimental approaches for a given measurement need. Because of the diversity of approaches, this chapter does not present specific procedures but instead provides experimental and system suitability information for MS procedures.

## QUALITATIVE ANALYSIS

MS is a sensitive and highly specific technique for the identification of analytes. Identification or verification of structure (i.e., comparison against an authentic standard) by MS is particularly powerful when used in conjunction with a separation technique such as gas chromatography (GC) or high-performance liquid chromatography (HPLC). Additional degrees of specificity can be obtained by the use of tandem mass spectrometry (MS/MS) or high-resolution mass spectrometry (HRMS). Also, tighter tolerances on mass accuracy are also afforded using high-resolution mass spectrometry (HRMS).

### Experimental Parameters

The following MS experimental parameters should be defined for a qualitative (e.g., identification) procedure.

#### MASS RESOLUTION

Unit mass resolution is sufficient for most identification tests. When higher resolution is required, the resolution is specified in the procedure, and demonstration of adequate resolution is included in the system suitability tests for the procedure.

#### MASS ACCURACY

A mass accuracy or agreement of ±0.50 mass units for singly charged ions from a known standard should be sufficient for most applications. A mass accuracy or agreement of ±0.05% from a standard should be sufficient for the identification of large molecules (above 2000 m/z) when multiply charged ions are employed for the identification test.

When higher mass accuracy is required, the required mass accuracy is specified in the procedure. A demonstration of the mass accuracy then is included in the system suitability tests for the procedure or as part of the instrument's established performance qualification (PQ) procedure discussed later in this chapter.

#### MASS RANGE

The mass range to be scanned is also presented in the procedure. The mass range must encompass all ions used as part of the identification confirmation.

### System Suitability

The system suitability for the MS procedure should include demonstration of adequate performance for the following experimental attributes.

#### MASS RESOLUTION

A demonstration of the appropriate resolution is included in the system suitability tests for a procedure. The performance test in the instrument's established PQ, executed daily or prior to the time of use, may suffice. When resolutions greater than unit mass are required for a procedure, the system suitability test includes demonstration of adequate resolution along with acceptance criteria.

#### MASS ACCURACY

A demonstration of the mass accuracy is included in the system suitability tests for the procedure or is part of the instrument's established PQ procedure. A mass accuracy or agreement of ±0.50 mass units for singly charged ions from a known standard should be sufficient for most applications. If higher mass accuracy is required for a given procedure, the appropriate acceptance criteria are specified.

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## Interpretation Directions

An identification or verification experiment involves comparison of the compound of interest to an authentic standard (e.g., a USP Reference Standard). For this form of identification or verification, the standard and the sample are run under identical conditions and should have results that are within acceptable experimental error for the procedure. Applications of MS for identification purposes in a compendial monograph may incorporate the mass spectral data alone or may combine this spectral identification with the chromatographic retention time if more specificity is dictated by the particular monograph identification needs.

The procedure should also provide specific instructions that define a successful mass spectral match. If the procedure represents the only spectroscopic identification test or if no other identification tests (e.g., peptide mapping or amino acid analysis) provide structural information, the spectrum of the standard should closely resemble that of the sample and a minimum of three structurally relevant ions, preferably one of which is an ion representing the molecular mass of the analyte, should be used for the comparison. In the case where only the ion representing the intact molecule is produced, the accurate mass or MS/MS spectrum of the molecular ion may be used to strengthen the identification. If other structurally relevant identification tests are also conducted, the comparison can be conducted with fewer ions as long as one of the ions represents the molecular mass of the analyte. For example, the MS identification test for a protein may examine only an ion that represents the molecular mass if several other tests are conducted to confirm the protein structure.

# QUANTITATIVE ANALYSIS

The sensitivity and specificity of MS also make it a suitable analytical tool for the quantification of analytes. Quantification is particularly powerful when used in conjunction with a separation technique such as GC or HPLC. Further degrees of specificity can be obtained by the use of MS/MS or HRMS.

## Experimental Parameters

The following experimental parameters should be defined within a quantitative (e.g., identification) MS procedure.

### MASS RESOLUTION

Unit mass resolution is sufficient for most quantitative tests. When higher resolution is required, the resolution is specified in the procedure, and demonstration of the mass accuracy is included in the system suitability tests for the procedure.

### MASS ACCURACY

The mass accuracies listed in the previous qualitative section should be sufficient for most quantitative applications. When higher mass accuracy is required, it is specified in the procedure. A demonstration of the mass accuracy is included in the system suitability tests for the procedure or as part of the instrument's established PQ procedure.

### MASS SELECTION

The masses to be monitored (e.g., mass range, individual masses, or MS/MS transitions) are presented in the procedure.

## System Suitability

The system suitability for the MS procedure should include demonstration of adequate performance for the following experimental attributes, as appropriate for the procedure.

### MASS RESOLUTION

A demonstration of the appropriate resolution is included in the system suitability tests for the procedure or is part of the instrument's established PQ procedure. When resolutions greater than unit mass are required for a procedure, the system suitability test includes demonstration of adequate resolution along with acceptance criteria.

### MASS ACCURACY

A demonstration of the mass accuracy is included in the system suitability tests for the procedure or is part of the instrument's established PQ procedure. A mass accuracy or agreement of ±0.50 mass units for singly charged ions from a known standard should be sufficient for most applications. If higher mass accuracy is required for a given procedure, the appropriate acceptance criteria are specified.

### PRECISION

The system suitability includes a demonstration of adequate precision. See *Table 1* for maximum limits for precision. Typically, system suitability limits are set to tighter limits than those employed to ensure adequate precision for the validation experiments. (See also the section on *Validation and Verification of Mass Spectrometry Analytical Procedures*.)

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LINEARITY

The system suitability includes a demonstration of adequate linearity. See *Table 1* for appropriate linearity limits. (See also the section on *Validation and Verification of Mass Spectrometry Analytical Procedures*.)

ACCURACY

In certain situations, quality control (or check) samples may also be appropriate for inclusion in the procedure to ensure the quality of the measurement. Typically, these quality control samples are of known analyte concentration and are prepared identically to the test samples. If used, quality control (or check) samples are also prepared as a verification of time-of-application method accuracy. The procedure specifies the number or analysis order of quality control (or check) samples needed. Acceptance criteria for calibration and quality control (or check) sample results should be aligned with the validation requirements as required by the application type (i.e., Category I or II) as outlined in *Table 1*.

QUANTITATION LIMIT

In certain applications (e.g., limits tests), it may be necessary to include demonstration of the ability to detect the analyte at a prescribed level. For these applications, the procedure specifies the limit and success criteria (e.g., signal-to-noise ratio).

## QUALIFICATION OF MASS SPECTROMETRY INSTRUMENTS

Qualification of an MS instrument can be divided into three elements: installation qualification (IQ), operational qualification (OQ), and PQ. For additional information, see *Analytical Instrument Qualification* ⟨1058⟩, which may be a helpful, but not mandatory resource.

### Installation Qualification

IQ provides evidence that the hardware and software are installed to accommodate safe and effective use of the instrument at the desired location.

### Operational Qualification

In OQ, an instrument's performance is characterized using standards to verify that the system operates within target specifications. The purpose of OQ is to demonstrate that instrument performance is suitable for a given application. Because so many different approaches are available for measuring MS spectra, OQ using standards with known spectral properties is recommended. Because of the diversity of MS instrumentation, interfaces, and experimental approaches, MS instruments should be qualified against target specifications for the intended application, not simply the specifications supplied by the manufacturer.

### Performance Qualification

PQ helps to determine that the instrument is capable of meeting the user's requirements for all critical-to-quality measures. PQ documentation should describe the following:

- the definition of the specific performance criteria and detailed test procedures, including test samples and instrument parameters;
- the elements that will be measured to evaluate the criteria and the predefined specifications;
- the test interval, which may be daily or time-of-use measurements;
- the use of bracketing samples or groups of samples; and
- corrective actions that will be implemented if the spectrometer does not pass the specifications.

Periodic PQ should include a subset of the OQ tests to ensure that the instrument as supplied is performing at a level that produces data that are suitable for their intended use. Depending on typical use, the specifications for PQ may be higher or lower than the manufacturer's installation specifications. Method-specific PQ tests, also known as system suitability tests, may be used in lieu of PQ requirements for validated procedures.

Because of the diversity of MS instrumental configurations and experimental designs, a standard sample or experiment for all PQ assessments may not be available. Thus, method-specific PQ tests or system suitability tests often are needed. The PQ experimental design should be sufficiently robust to ensure proper instrument performance for the intended application, including the specifications associated with the measurement. At minimum, PQ experiments should include the following.

- For qualitative applications, the PQ experiment includes a check of the mass accuracy of the instrumentation. A mass accuracy or agreement of ±0.50 mass units for singly charged ions from a known standard should be sufficient for most applications.
- For quantitative applications, the PQ experiment includes checks of mass accuracy and precision. A mass accuracy or agreement of ±0.50 mass units for singly charged ions from a known standard should be sufficient for most applications. The success criteria for precision is established via consideration of the instrument and method capability, and provides sufficient controls relative to the specification for the measurement in question.

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## Characterizing Instrument Performance

Specific procedures, acceptance criteria, and time intervals for characterizing MS spectrometer performance depend on the instrument and its intended applications. Many MS applications use previously validated experiments that relate MS spectra to a chemical property of interest. Analysts typically demonstrate stable instrument performance over extended periods of time. This practice provides some assurance that reliable measurements can be taken from sample spectra using previously validated MS experiments.

# VALIDATION AND VERIFICATION OF MASS SPECTROMETRY ANALYTICAL PROCEDURES

Validation is required only when an MS procedure is an alternative to the official procedure for testing an official article.

The objective of validating an MS procedure is to demonstrate that the measurement is suitable for its intended purpose, including quantitative determination of the main component in a drug substance or a drug product (Category I assays), quantitative determination of impurities (Category II), and identification tests (Category IV). [NOTE—For additional information on the different category definitions, see *Validation of Compendial Procedures* ⟨1225⟩.] Depending on the category of the test, analytical procedure validation requires the testing of linearity, range, accuracy, specificity, precision, quantitation limit, and robustness. These analytical performance characteristics apply to externally standardized methods and to the method of standard additions.

Chapter ⟨1225⟩ provides definitions and general guidance about analytical procedures validation without indicating specific validation criteria for each characteristic. The intention of the following sections is to provide the user with specific validation criteria that represent the minimum expectations for this technology. For each particular application, tighter criteria may be needed in order to demonstrate suitability for the intended use.

## Measurement Categories for Mass Spectrometry Analytical Procedures

The required validation performance characteristics of an MS analytical procedure, assuming the typical Category I USP specifications of 98.0%–102.0% for drug substances and 95.0%–105.0% for drug products, are listed in *Table 1*. The actual validation performance characteristics would be dependent upon the specifications in place and should provide sufficient evidence that the measurement capability is sufficient for those specifications. A procedure validation protocol must specify the required validation experiments and validation criteria. These criteria are determined according to the intended purpose of the analytical procedure.

**Table 1. Analytical Measurement Requirements**

| Analytical Performance Characteristics | Category I | Category II Quantitative |
|---|---|---|
| Specificity | Ensured by use of a reference standard when possible and demonstrable lack of interference from other components | |
| Linearity | Correlation coefficient *(R)* NLT 0.995 | Correlation coefficient *(R)* NLT 0.99 |
| Range | For 100.0% centered acceptance criteria: 80.0%–120.0%. For noncentered acceptance criteria: 10.0% below the lower limit to 10.0% above the upper limit. For content uniformity: 70.0%–130.0% | 50%–120% |
| Accuracy | 98.0%–102.0% (drug substance) 95.0%–105.0% (drug product) | 80.0%–120.0% |
| Repeatability | NMT 1.0% (drug substance) NMT 2.0% (drug product) | NMT 20.0% |
| Intermediate precision | NMT 1.0% (drug substance) NMT 3.0% (drug product) | NMT 25.0% |
| Quantitation limit | — | The analytical procedure should be capable of determining the analyte precisely and accurately at a level equivalent to 50% of the specification. |
| Robustness | The reliability of an analytical measurement should be demonstrated by deliberate changes to experimental parameters. | |

## Analytical Procedure Validation

The objective of analytical procedure validation is to demonstrate that the analytical procedure is suitable for its intended purpose by conducting experiments and obtaining results that meet predefined acceptance criteria. MS analytical procedures can include quantitative tests for major component and impurities content, limit tests for the presence of impurities, quantification of a component in a product or formulation, or identification tests.

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VALIDATION PARAMETERS

Performance characteristics that demonstrate the suitability of an analytical procedure are similar to those required for any analytical procedure. For additional information on the applicable general principles, see ⟨1225⟩. Specific acceptance criteria for each validation parameter must be consistent with the intended use of the analytical procedure.

The performance characteristics that are required as part of a validation for each of the analytical procedure categories are given in *Table 1*.

**Specificity:** The purpose of a specificity test is to demonstrate that measurements of the intended analyte signals are free of interference from components and impurities in the test material. Specificity tests can be conducted to compare spectra of components and impurities that are known from synthetic processes, formulations, and test preparations. Specificity is also to be demonstrated for any materials added as part of the procedure (e.g., specificity versus isotope-labeled internal standards).

For an identification MS analytical procedure (Category I and II), validation experiments may include multidimensional MS experiments to validate correct assignments of an ion's structure or origin.

**Linearity:** A linear relationship is exhibited between the analyte concentration and instrument response. This is demonstrated by measuring analyte responses from NLT five standard solutions at concentrations that encompass the anticipated concentration range of analyte(s) in the test solution. For Category I, standard solutions can be prepared from reference materials in appropriate solvents. For Category II (MS analytical procedures that are used to quantitate impurities), linearity samples are prepared by spiking suitable test samples that contain low amounts of analyte or by spiking matrix samples at concentrations of the expected range. The standard curve then is constructed using appropriate statistical analytical procedures such as a least-squares regression. The correlation coefficient ($R$), $y$-intercept, slope of the regression line, and residual root mean square are then determined. Absolute values determined for these factors are appropriate for the procedure being validated.

**Range:** The range between the low and high concentrations of analyte is given by the quantitative MS analytical procedure. This typically is based on test article specifications in the *USP* monograph. It is the range within which the analytical procedure can demonstrate an acceptable degree of linearity, accuracy, and precision and can be obtained from an evaluation of that analytical procedure.

Recommended ranges for various MS analytical procedures are as follows.

- For Category I—assay of a drug substance (or a finished product): 80%–120% of the test concentration;
- For Category I—content uniformity: a minimum of 70%–130% of the test concentration;
- For Category II—determination of an impurity: 50%–120% of the acceptance criteria.

**Accuracy:** The accuracy of a quantitative MS analytical procedure is determined across the required analytical range. Typically, three levels of concentrations are evaluated using triplicate preparations at each level.

*Preparation of accuracy samples:* For drug substance assays (Category I), accuracy is determined by analyzing a reference standard of known purity. For drug product assays (Category I), a composite sample of reference standard and other components in a pharmaceutical finished product should be used for analytical procedure validation. The assay results are compared to the theoretical value of the reference standard to estimate errors or percent recovery. For the quantitation of impurities (Category II), the accuracy of the analytical procedure can be determined by conducting studies with drug substances or products spiked with known concentrations of the analyte under test.

Assay results from the analytical procedure being validated may be compared to those of an established alternative analytical procedure.

**Precision:**

*Repeatability:* The analytical procedure is assessed by measuring the concentrations of three replicates of separate standard solutions at three different concentrations that encompass the analytical range. Alternatively, the concentrations of six separate standard solutions at 100% of the test concentration can be measured. The relative standard deviation from the replicate measurements is then evaluated to determine if the solutions meet the acceptance criteria.

*Intermediate precision:* The effect of random events on the analytical precision of the analytical procedure is to be established. Typical variables include performing the analysis on different days, using different instruments that are suitable as specified in the analytical procedure, or having the analytical procedure performed by two or more analysts.

**Quantitation limit:** The quantitation limit is validated by measuring six replicates of test samples spiked with analyte at 50% of specification.

From these replicates, analysts are then able to determine accuracy and precision. Examples of specifications for Category II quantitative determinations are that the measured concentration is within 70%–130% of the spike concentration and the relative standard deviation is NMT 15%.

**Robustness:** The reliability of an analytical measurement is demonstrated with deliberate changes to critical experimental parameters. These can include measuring the stability of the analyte under specified storage, chromatographic, or ionization conditions.

## Analytical Procedure Verification

U.S. Current Good Manufacturing Practices regulations [21 CFR 211.194(a)(2)] indicate that users of analytical procedures described in *USP–NF* do not need to validate procedures that are provided in a monograph. Instead, they must simply verify the suitability of the procedures under actual conditions of use.

The objective of an MS procedure verification is to demonstrate that the procedure as prescribed in a specific monograph can be executed by the user with suitable accuracy, specificity, and precision using the instruments, analysts, and sample matrices available. According to the general information chapter *Verification of Compendial Procedures* ⟨1226⟩, if the verification of the compendial procedure by following the monograph is not successful, the procedure may not be suitable for use with the article under test. It may be necessary to develop and validate an alternative procedure as allowed in *General Notices, 6.30 Alternative and Harmonized Methods and Procedures*.

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Verification of a compendial MS procedure includes at minimum the execution of the validation parameters for specificity, accuracy, precision, and limit of quantitation, when appropriate, as indicated in the *Validation and Verification of Mass Spectrometry Analytical Procedures* section in this chapter.

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# ⟨741⟩ MELTING RANGE OR TEMPERATURE

The terms melting range, melting point, or melting temperature are all used in pharmacopeial contexts. Most substances exhibit a melting transition, spanning the temperatures at which the first detectable change of phase or liquid phase is detected to the temperature at which no solid phase is apparent. The transition may appear instantaneous for a highly pure material, but usually a range is observed from the beginning to the end of the process. Factors influencing this transition include the sample size, the particle size, the efficiency of heat diffusion within the sample, and the heating rate, among other variables, that are controlled by procedure instructions.

For pharmacopeial purposes, the temperatures of the beginning (onset temperature) and end of transition (clear temperature) represent the melting range, except as defined otherwise for *Procedure for Class II* and *Procedure for Class III* below. The terms melting point and melting temperature are considered to be equivalent.

Substances which melt with no decomposition or chemical change are known to melt congruently. In these cases, the melting point is taken to be the end of the melting range, i.e., the temperature at which no solid phase is apparent. In some articles, the melting process is accompanied by simultaneous decomposition, which is visually evidenced as a side event like darkening of the material, charring, bubbling, or other incident. These transitions are known to be non-congruent. The visual impact of this side reaction frequently obscures the end of the melting process, which may be impossible to accurately determine. In those circumstances, only the beginning of the melting can be accurately established, and it is to be reported as the melting point.

Since there may be a thermal lag between the heating medium and the sample within the capillary tube, in order to achieve consistency and repeatability, it is important to perform the melting determination at a heating rate, also referred to as ramp rate, of 1°/min.

The accuracy of the apparatus to be used as described below should be checked at suitable intervals by the use of one or more of the available USP Melting Point Standards, preferably those that melt nearest the melting temperatures of the compounds being tested (see *USP Reference Standards* ⟨11⟩). The USP Melting Point Standards are intended to check the accuracy of the device and may be suitable to calibrate.

Eight procedures for the determination of melting range or temperature are given herein, varying in accordance with the nature of the substance. When no class is designated in the monograph, use the *Procedure for Class Ia* for crystalline or amorphous substances and the *Procedure for Class II* for waxy substances.

The procedure known as the mixed-melting point determination, whereby the melting range or temperature of a solid under test is compared with that of an intimate mixture of equal parts of the solid and an authentic specimen of it, e.g., the corresponding USP Reference Standard, if available, may be used as a confirmatory identification test. Agreement of the observations on the original and the mixture constitutes reliable evidence of chemical identity.

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## APPARATUS

Apparatus with cameras or other computerized equipment to improve accuracy, sensitivity, or precision may be used provided that the apparatus is properly qualified.

### Apparatus I

An example of a suitable melting range *Apparatus I* consists of a glass container for a bath of transparent fluid, a suitable stirring device, an accurate thermometer, and a controlled source of heat. The bath fluid is selected with a view to the temperature required, but light paraffin is used generally and certain liquid silicones are well adapted to the higher temperature ranges. The fluid is deep enough to permit immersion of the thermometer to its specified immersion depth so that the bulb is still about 2 cm above the bottom of the bath. The heat may be supplied by an open flame or electrically. The capillary tube is about 10 cm long and 0.8–1.2 mm in internal diameter with walls 0.2–0.3 mm in thickness.

### Apparatus II

An instrument may be used in the *Procedure for Class I*, *Procedure for Class Ia*, and *Procedure for Class Ib*. An example of a suitable melting range *Apparatus II* consists of a block of metal that may be heated at a controlled rate, with its temperature being monitored by a sensor. The block accommodates the capillary tube containing the test substance and permits monitoring of the melting process, typically by means of a beam of light and a detector. The detector signal may be processed by a microcomputer to determine and display the melting point or range, or the detector signal may be plotted to allow visual estimation of the melting point or range. Some approaches broadly used by automated systems employ optical methods such as light absorption or bulk reflection.

## PROCEDURES

### Procedure for Class I, Apparatus I

Reduce the substance under test to a very fine powder, and, unless otherwise directed, render it anhydrous when it contains water of hydration by drying it at the temperature specified in the monograph, or, when the substance contains no water of hydration, dry it over a suitable desiccant for NLT 16 h (or at the conditions stated in *Loss on Drying* ⟨731⟩, if appropriate).

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Charge a capillary glass tube, one end of which is sealed, with a sufficient amount of the dry powder to form a column in the bottom of the tube to a nominal height of 3 mm high when packed down as closely as possible by moderate tapping on a solid surface. Due to the instrument design, alternative sample sizes may be used as instructed by the instrument manufacturer.

Heat the bath until the temperature is about 10° below the expected melting point. Remove the thermometer, and quickly attach the capillary tube to the thermometer by wetting both with a drop of the liquid of the bath or otherwise, and adjust its height so that the material in the capillary is level with the thermometer bulb. Replace the thermometer, and continue the heating, with constant stirring, sufficiently to achieve the ramp rate of about 3°/min. When the temperature is about 3° below the lower limit of the expected melting range, reduce the heating so that the ramp rate of about 1°/min is achieved. Continue heating until melting is complete.

The temperature at which the column of the substance under test is observed to collapse definitely against the side of the tube at any point indicates the beginning of melting, and the temperature at which the test substance becomes liquid throughout corresponds to the end of melting or the melting point. The two temperatures fall within the limits of the melting range. If melting occurs with decomposition, the melting temperature corresponding to the beginning of the melting (melting point) is within the range specified.

## Procedure for Class Ia, Apparatus I

Prepare the test substance and charge the capillary as directed in *Procedure for Class I*, *Apparatus I.* Heat the bath until the temperature is about 10° below the expected melting point and is rising at a rate of about 1°/min. Insert the capillary as directed in *Procedure for Class I*, *Apparatus I* when the temperature is about 5° below the lower limit of the expected melting range, and continue heating until melting is complete. Record the melting range as directed in *Procedure for Class I*, *Apparatus I*.

## Procedure for Class Ib, Apparatus I

Place the test substance in a closed container and cool to 10°, or lower, for at least 2 h. Without powdering, charge the cooled material into the capillary tube as directed in *Procedure for Class I*, *Apparatus I*, then immediately place the charged tube in a vacuum desiccator and dry at a pressure not exceeding 20 mm of mercury for 3 h. Immediately upon removal from the desiccator, fire-seal the open end of the tube, and as soon as practicable proceed with the determination of the melting range as follows. Heat the bath until the temperature is about 10° below the expected melting range, then introduce the charged tube, and heat at a rate of rise of about 1°/min until melting is complete. Record the melting range as directed in *Procedure for Class I*, *Apparatus I.*

If the particle size of the material is too large for the capillary, precool the test substance as directed above, then with as little pressure as possible gently crush the particles to fit the capillary, and immediately charge the tube.

## Procedure for Class I, Apparatus II

Prepare the substance under test and charge the capillary tube as directed in *Procedure for Class I, Apparatus I*. Operate the apparatus according to the manufacturer's instructions. Heat the block until the temperature is about 10° below the expected melting point. Insert the capillary tube into the heating block, and continue heating at a rate of temperature increase of about 1°/min until melting is complete.

The temperature at which the detector signal first leaves its initial value indicates the beginning of melting, and the temperature at which the detector signal reaches its final value corresponds to the end of melting, or the melting point. The two temperatures fall within the limits of the melting range. If melting occurs with decomposition, the melting temperature corresponding to the beginning of the melting (melting point) is within the range specified.

## Procedure for Class Ia, Apparatus II

Prepare the test substance and charge the capillary as directed in *Procedure for Class I*, *Apparatus I*. Operate the apparatus according to the manufacturer's instructions. Heat the block until the temperature is about 5° below the expected melting point and is rising at a rate of about 1°/min. Insert the capillary as directed in *Procedure for Class I*, *Apparatus I*, and continue heating until melting is complete. Record the melting range as directed in *Procedure for Class I*, *Apparatus I*. If melting occurs with decomposition, the melting temperature corresponding to the beginning of the melting (melting point) is within the range specified.

## Procedure for Class Ib, Apparatus II

Place the test substance in a closed container and cool to 10°, or lower, for at least 2 h. Without previous powdering, charge the cooled material into the capillary tube as directed in *Procedure for Class I*, *Apparatus I*, then immediately place the charged tube in a vacuum desiccator, and dry at a pressure not exceeding 20 mm of mercury for 3 h. Immediately upon removal from the desiccator, fire-seal the open end of the tube, and as soon as practicable proceed with the determination of the melting range as follows. Operate the apparatus according to the manufacturer's instructions. Heat the block until the temperature is about 10° below the expected melting range, then introduce the charged tube, and heat at a rate of rise of about 1°/min until melting is complete. Record the melting range as directed in *Procedure for Class I*, *Apparatus I*.

If the particle size of the material is too large for the capillary, precool the test substance as directed above, then with as little pressure as possible gently crush the particles to fit the capillary, and immediately charge the tube.

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## Procedure for Class II

Carefully melt the material to be tested at as low a temperature as possible, and draw it into a capillary tube, which is left open at both ends, to a depth of about 10 mm. Cool the charged tube at 10°, or lower, for 24 h, or in contact with ice for at least 2 h. Then attach the tube to the thermometer by suitable means, adjust it in a water bath so that the upper edge of the material is 10 mm below the water level, and heat as directed in *Procedure for Class I*, *Apparatus I* except, within 5° of the expected melting temperature, to regulate the rate of rise of temperature of about 1.0°/min. The temperature at which the material is observed to rise in the capillary tube is the melting temperature.

## Procedure for Class III

While stirring, melt a quantity of the test substance slowly until it reaches a temperature of 90°–92°. Remove the source of the heat, and allow the molten substance to cool to a temperature of 8°–10° above the expected melting point. Chill the bulb of a suitable thermometer to 5°, wipe it dry, and while it is still cold dip it into the molten substance so that approximately the lower half of the bulb is submerged. Withdraw it immediately, and hold it vertically away from the heat until the wax surface dulls, then dip it for 5 min into a water bath having a temperature NMT 16°.

Fix the thermometer securely in a test tube so that the lower point is 15 mm above the bottom of the test tube. Suspend the test tube in a water bath adjusted to about 16°, and raise the temperature of the bath at the rate of about 2°/min to 30°, then change to a rate of about 1°/min, and note the temperature at which the first drop of melted substance leaves the thermometer. Repeat the determination twice on a freshly melted portion of the test substance. If the variation of three determinations is less than 1°, take the average of the three as the melting point. If the variation of three determinations is 1° or greater than 1°, make two additional determinations and take the average of the five.

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# ⟨755⟩ MINIMUM FILL

***Change to read:***

## SCOPE

The following tests and ▲acceptance criteria▲ (USP 1-Dec-2020) apply to articles such as creams, gels, lotions, ointments, pastes, powders, aerosols, ▲foams,▲ (USP 1-Dec-2020) and sprays that are packaged in containers. To minimize the impact of entrained air for products labeled by volume, the fill determination is performed by mass from which the volume is calculated by use of the density of the preparation.

## PURPOSE

The test for minimum fill ensures that the amount of material filled into the product conforms to the labeled amount.

***Change to read:***

## PROCEDURE FOR DOSAGE FORMS OTHER THAN AEROSOLS,▲SPRAYS, AND FOAMS▲ (USP 1-DEC-2020)

### For Containers Labeled by Weight

Select a sample of 10 filled containers, and remove any labeling that might be altered in weight during the removal of the container contents. Thoroughly cleanse and dry the outside of the containers by a suitable means, and weigh individually. Quantitatively remove the contents from each container, cutting the latter open and washing with a suitable solvent, if necessary, taking care to retain the closure and other parts of each container that were present during the initial weighing. Dry and reweigh each empty container, together with its corresponding parts. Determine the net weight of the contents of the container by difference.

### For Containers Labeled by Volume

Proceed as indicated above for products labeled by weight, but convert the mass to volume using the density of the preparation. A suggested approach to determine the density of the materials is as follows:

1. Tare a 100-mL volumetric flask containing 50.0 mL of liquid that is miscible with the formulation.
2. Add approximately 25 mL of a representative sample of the product and gently swirl the contents to mix.
3. Reweigh the flask.
4. From a buret, add an accurately measured amount of the miscible liquid to bring the flask contents to volume while gently swirling the contents of the flask. Record the volume taken from the buret.
5. Calculate the density of the sample:

$$\text{Result} = W/V$$

$W$ = weight of the material taken (g)
$V$ = 50.0 minus the volume, in mL, of the miscible fluid necessary to adjust the contents of the flask to 100 mL

Other methods of determining the density may be employed depending on the formulation (e.g., substantially nonaqueous formulations). Similarly, if the container contents are less than 25 mL, smaller graduated vessels may be used, with the quantities of miscible liquid adjusted accordingly.

Alternatively, pour the contents of 10 containers into 10 suitable graduated cylinders, and allow to drain completely. Record the volume of the contents of each of the 10 containers.

### Acceptance Criteria

This test meets the acceptance criteria either at *Stage 1* or *Stage 2*:

Stage 1:

1. The average net content of the 10 containers is NLT the labeled amount, and the net content of any single container is NLT 90% of the labeled amount where the labeled amount is 60 g or 60 mL or less, or NLT 95% of the labeled amount where the labeled amount is more than 60 g or 60 mL. If these criteria are not met, and the net content of NMT 1 container is less than 90% of the labeled amount where the labeled amount is 60 g or 60 mL or less, or ▲less than▲ (USP 1-Dec-2020) 95% of the labeled amount where the labeled amount is more than 60 g or 60 mL, proceed to *Stage 2*.

Stage 2:

1. Determine the content of 20 additional containers.

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2. The average content of the 30 containers is NLT the labeled amount, and the net content of NMT 1 of the 30 containers is less than 90% of the labeled amount where the labeled amount is 60 g or 60 mL or less, or less than 95% of the labeled amount where the labeled amount is more than 60 g or 60 mL.

***Change to read:***

## PROCEDURE FOR AEROSOLS, SPRAYS, AND ▲FOAMS▲ (USP 1-DEC-2020)

Select a sample of 10 filled containers, and remove any labeling that might be altered in weight during the removal of the container contents. Thoroughly cleanse and dry the ▲outside▲ (USP 1-Dec-2020) of the containers by suitable means, and weigh individually. Remove the contents from each container by employing any safe technique (e.g., chill to reduce the internal pressure, remove the valve, and pour). Remove any residual contents with suitable solvents, then rinse with a few portions of methanol. Retain as a unit the container, the valve, and all associated parts, and heat them at 100° for 5 min. Cool, and again weigh each of the containers together with its corresponding parts. The difference between the original weight and the weight of the empty aerosol container is the net fill weight. Determine the net fill weight for each container tested.

### Acceptance Criteria

The requirements are met if the net weight of the contents of each of the 10 containers is NLT the labeled amount.

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# ⟨761⟩ NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

## INTRODUCTION

Nuclear magnetic resonance (NMR) spectroscopy is an analytical method based on the magnetic properties of certain atomic nuclei. As is the case with other types of spectroscopy, absorption or emission of electromagnetic energy at characteristic frequencies provides structural information. NMR differs from other types of spectroscopy because the discrete energy levels between which the transitions take place are present only when the nuclei are placed in a magnetic field.

Although widely recognized as one of the most powerful structure-elucidation tools available, with proper experimental design, it can also be used for accurate qualitative and quantitative measurements. See general information chapter *Applications of Nuclear Magnetic Resonance Spectroscopy* ⟨1761⟩. [NOTE—Above 1000 chapters are for informational purposes only.]

## QUALIFICATION OF NMR INSTRUMENTS

Qualification of an NMR instrument can be divided into three elements: Installation Qualification (IQ), Operational Qualification (OQ), and Performance Qualification (PQ). For further discussion, see general information chapter *Analytical Instrument Qualification* ⟨1058⟩.

### Installation Qualification

The IQ requirements provide evidence that the hardware and software are installed to accommodate safe and effective use of the instrument at the desired location.

### Operational Qualification

In OQ, an instrument's performance is characterized using standards to verify that the system operates within target specifications. The purpose of OQ is to demonstrate that instrument performance is suitable for a given application. Because so many different approaches are available for measuring NMR spectra, OQ using standards with known spectral properties is recommended. Generally, sealed NMR tubes are available as reference standards for measuring signal-to-noise (S/N) and lineshape.

### Performance Qualification

PQ helps to determine that the instrument is capable of meeting the user's requirements for all critical-to-quality (CTQ) measures. PQ documentation should describe the following:

1. The definition of the specific performance criteria and detailed test procedures including test samples and instrument parameters.
2. The elements that will be measured to evaluate the criteria and the predefined specifications.
3. The test interval, which may be time-of-use.
4. The use of bracketing samples or groups of samples.
5. The defined corrective actions that will be implemented if the spectrometer does not pass the specifications.

Periodic PQ should include a subset of the OQ tests to ensure that those aspects of the instrument that are being supplied are performing at a level that produces data that are suitable for its intended use. Depending on typical use, the specifications for PQ may be higher or lower than the manufacturer's installation specifications. Typical CTQs include S/N ratio and resolution tests for all nuclei of interest. Method-specific PQ tests, also known as system suitability tests, may be used in lieu of PQ requirements for validated procedures.

The PQ samples and tests in the following subsections are typical examples only. Other tests and samples may be used to establish specifications for specific purposes. Instrument vendors often provide samples and test parameters that can be used as part of the PQ package.

RESOLUTION AND LINESHAPE MEASUREMENT—$^{1}$H NMR (see *Figure 1*)

**Sample:** 1% chloroform in acetone-$d_6$ (≥ 500 MHz), 3% chloroform in acetone-$d_6$, degassed and sealed
**Spectral width:** < 1 KHz
**Data acquisition time:** NLT 10 s
**Tip angle:** 90°
**Relaxation time:** 60 s
**Spinning rate:** Static or 20 Hz
**Pulse sequence:** Delay-pulse-acquire with no decoupling
**Processing:** No line broadening, zero-filling to 128 k

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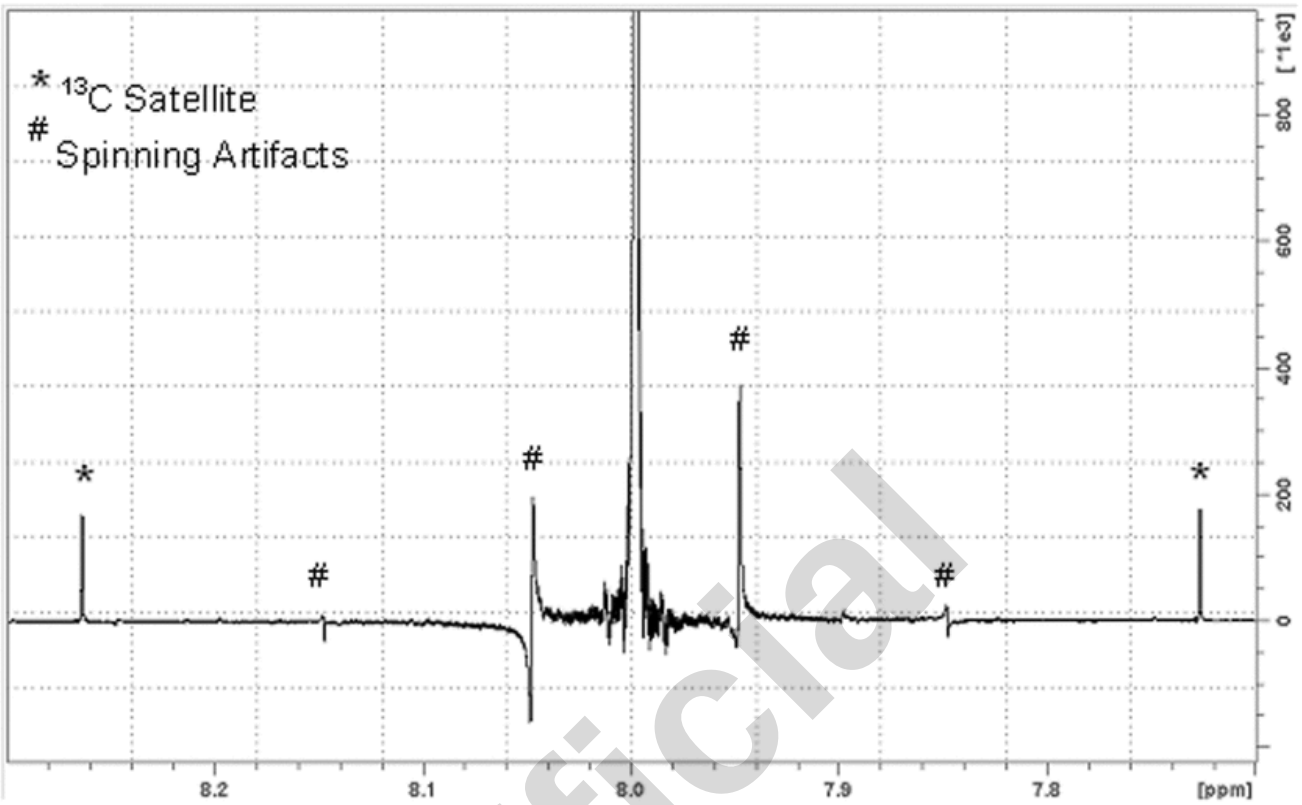


Figure 1. $^{1}$H NMR spectrum of chloroform in acetone-$d_6$ obtained at 400 MHz. The linewidth measured at 0.55% and 0.11% of the $^{13}$C satellites was 2.7 and 5.5 Hz, respectively.

Shim the magnet with special attention to the off-axis shims, acquire a single acquisition, phase to pure absorption, and measure the linewidth at 50%, 0.55%, and 0.11% maximum intensity. The linewidth should pass specifications at these positions, and, in addition, the lineshape should be Lorentzian. On modern NMR spectrometers, the lineshape is frequently obtained on a nonspinning sample because the off-axis shims can be set so well that there is essentially no difference between spectra obtained spinning and nonspinning. In addition, two-dimensional spectra should be obtained on a static sample.

S/N MEASUREMENTS—$^{1}$H NMR (see *Figure 2*)

**Sample:** 0.1% ethylbenzene in chloroform-d, 1% ethylbenzene in chloroform-d (< 200 MHz) degassed and sealed
**Spectral width:** 10 ppm
**Data acquisition time:** 400 ms
**Tip angle:** 90°
**Relaxation delay:** 60 s
**Spinning rate:** 0 or approximately 20 Hz
**Pulse sequence:** Delay-pulse-acquire with no decoupling
**Processing:** Exponential with 1-Hz line broadening
**Referencing:** Tetramethylsilane (TMS) = 0.0 ppm or the center of the quartet = 2.65 ppm

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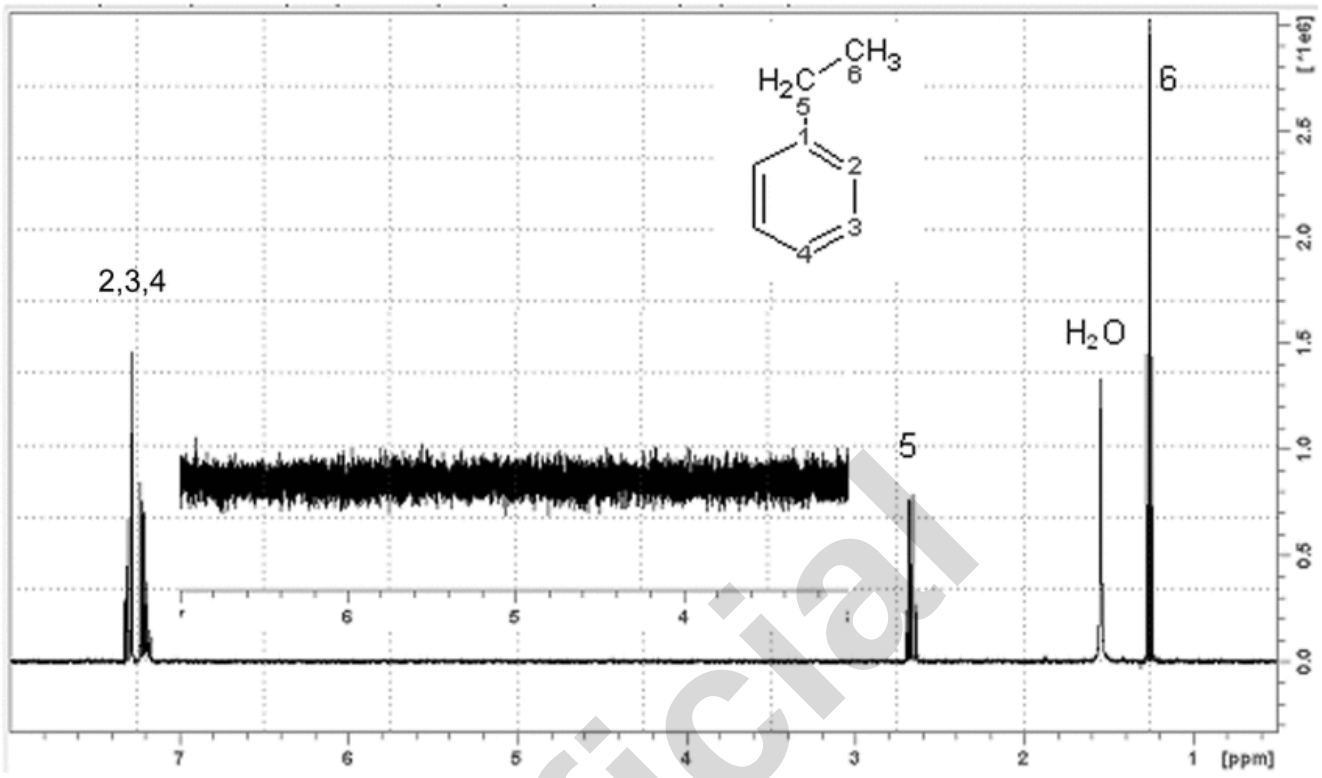


Figure 2. $^{1}$H NMR spectrum of 0.1% ethylbenzene obtained at 400 MHz with an S/N ratio of 550:1.

The concentration of ethylbenzene should be chosen to achieve S/N ratio specifications in the range of 20–1000. Concentrations that typically result in measurements outside that range are of limited utility in assessing the performance of the instrument. Nevertheless, established standard solutions are conventionally used. The magnet should be shimmed as well as possible. Ideally, this test should be run immediately after the lineshape test because most of the shims will be nearly maximized. Acquire a single acquisition, phase the spectrum in pure absorption mode, and measure the S/N of the ethylbenzene quartet. This experiment can be run with or without sample spinning. With a spinning sample, the S/N value that is measured should be only about 10% higher than that obtained with a nonspinning sample if the off-axis shims are well adjusted. A higher ratio would indicate that the determination would benefit from further shimming with the off-axis shims.

Most modern spectrometers have software that perform the S/N measurement after the operator has identified the signal and noise regions. Manual calculations can also be made. Measure the amplitude (*A*) from the center of the baseline to the peak of the highest of the central two lines in the quartet. Measure the peak-to-peak noise height (*H*) from the lowest noise peak to the highest noise peak in the 3–5 ppm region. The noise may be vertically multiplied by a factor for accurate measurement of high S/N spectra. Calculate the S/N as follows:

$$S/N = k \times 2.5 \times A/H \quad [1]$$

where *k* is the vertical expansion factor of the noise region used. The factor of 2.5 converts the peak-to-peak S/N to root-mean-squared (rms) noise, which is the standard convention for reporting S/N in NMR spectroscopy. Computerized S/N calculations can be used provided the specifications are set and tested by the same procedure. At the discretion of the spectroscopist, an S/N value lower than that specified by the manufacturer may be used if it is judged to be sufficient for the current application.

### S/N MEASUREMENTS $^{13}$C NMR (see *Figure 3*)

**Sample:** 40% *p*-dioxane in benzene-$d_6$ (v/v) (degassed and sealed)
**Spectral width:** Approximately 200 ppm
**Tip angle:** 90°
**Relaxation delay:** 300 s
**Spinning rate:** Approximately 20 Hz
**Pulse sequence:** Delay-pulse-acquire with no decoupling
**Processing:** Exponential with 3.5-Hz line broadening, zero-filling to 32k
**Referencing:** TMS = 0.0 ppm or the center of the benzene triplet = 128.4 ppm

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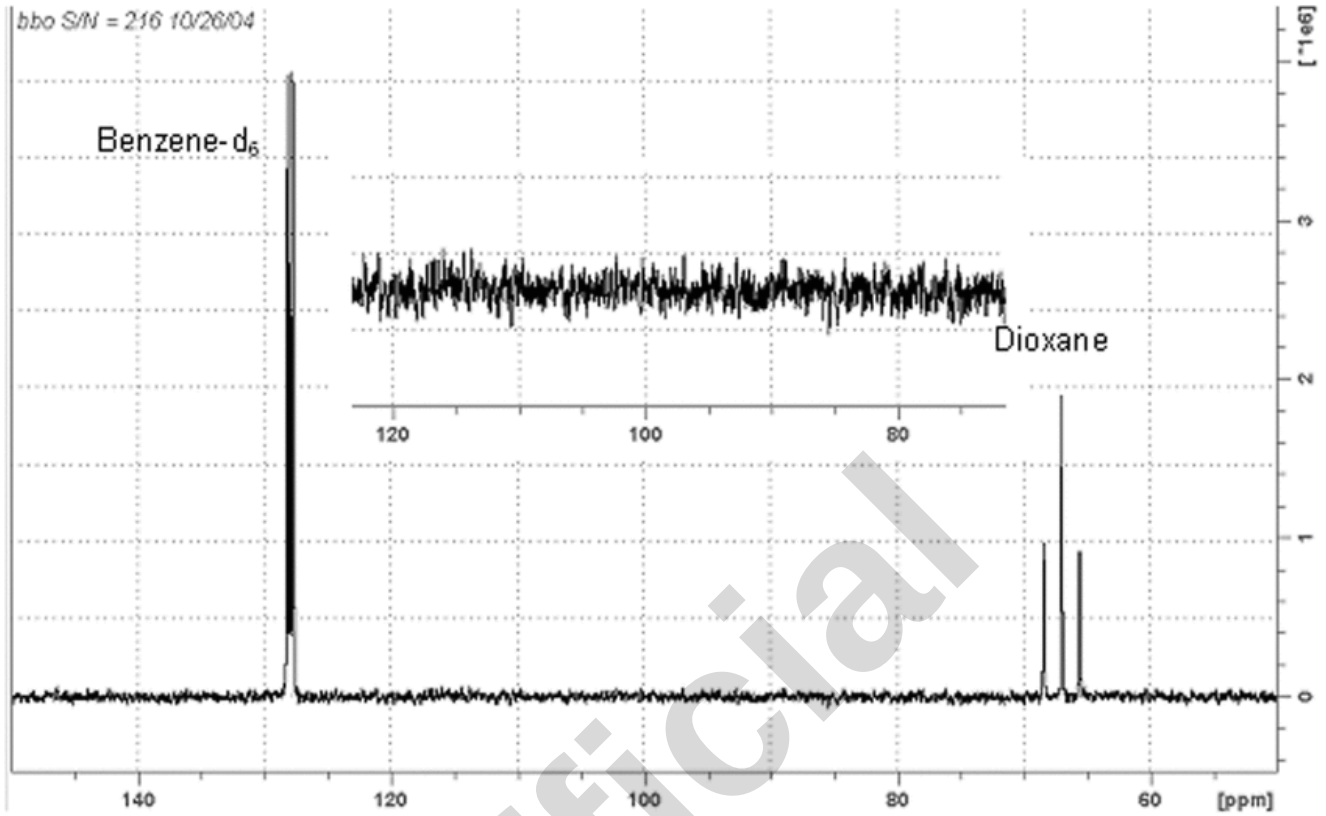


Figure 3. $^{13}C$ NMR spectrum of the ASTM standard 40% *p*-dioxane in benzene-$d_6$ (v/v) obtained at 100.6 MHz, with an S/N ratio of 140:1.

With a well-shimmed magnet, acquire a single acquisition following a minimum delay of 300 s, phase the spectrum in pure absorption mode, and measure the height of the benzene triplet at approximately 128.4 ppm from the center of the baseline. The peak-to-peak noise can be measured as above with appropriate vertical expansion of 80–120 ppm. S/N calculations can be made as in *Equation 1* or by computer calculation.

The benzene-$d_6$ triplet has no nuclear Overhauser enhancement (NOE). Consequently, this test verifies the performance of only the $^{13}C$ channel.

## PERFORMANCE OF BOTH OF THE $^{13}C$ AND $^{1}H$ CHANNELS (see *Figure 4*)

**Sample:** up to 10% ethylbenzene in chloroform-d (degassed and sealed)
**Spectral width:** 200 ppm
**Data acquisition length:** 64k points
**Tip angle:** 90°
**Relaxation delay:** 300 s
**Spinning rate:** Approximately 20 Hz
**Pulse sequence:** Delay-pulse-acquire with composite pulse decoupling set to the center of the $^{1}H$ spectrum
**Processing:** Exponential with 0.3-Hz line-broadening
**Referencing:** TMS = 0.0 ppm or the center of the chloroform-d, triplet = 77.23 ppm

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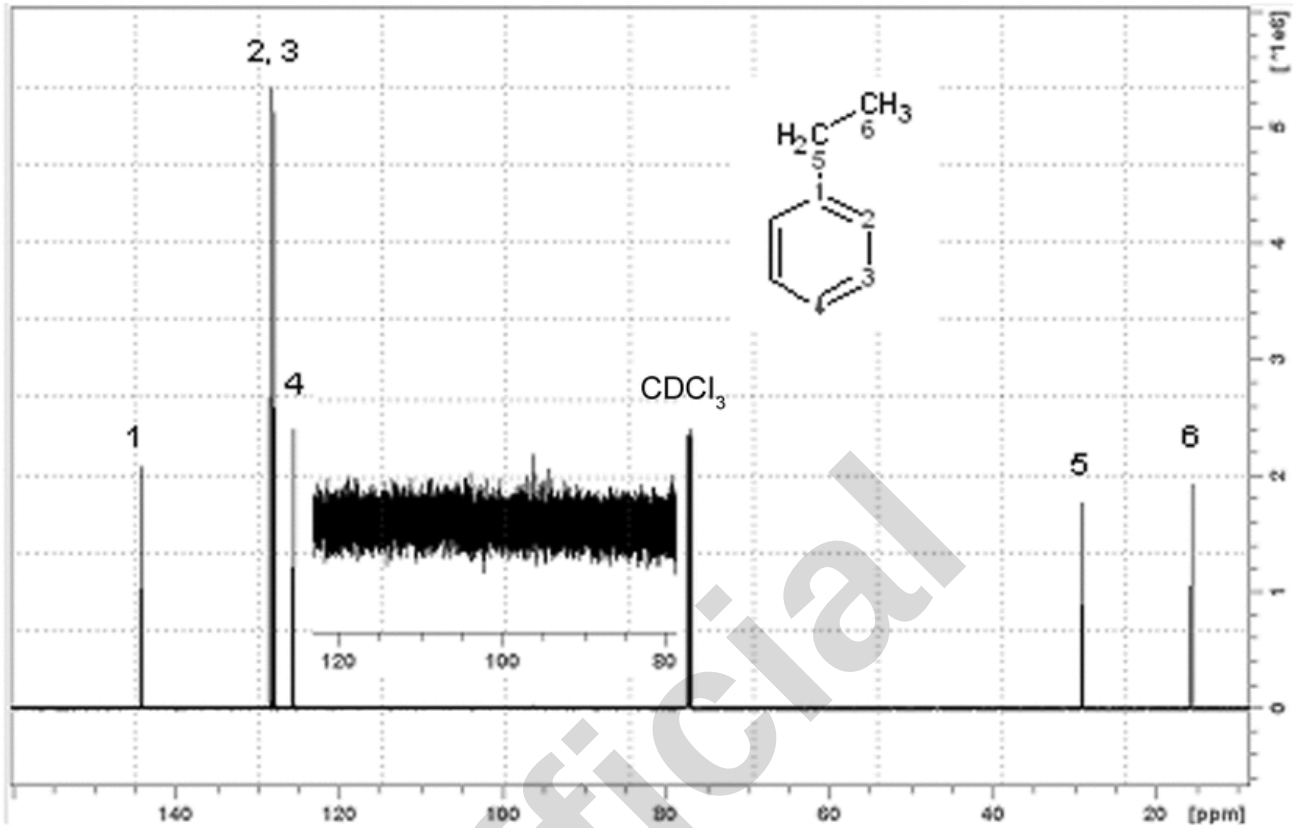


Figure 4. $^{13}C$ NMR spectrum of 10% ethylbenzene obtained using a cryogenically cooled dual $^{1}H/^{13}C$ probe at 150.9 MHz, with an S/N ratio of 640:1.

The shimming should be sufficient to pass the resolution and lineshape tests described above. The measurement of S/N is done from the peak height of the larger resonance of the two near 128 ppm. The noise is measured as above in the region of 80–120 ppm, with appropriate vertical expansion. S/N is calculated by the computer or as in *Equation 1*.

RELAXOMETRY MEASUREMENTS—LOW FIELD-NMR (LF-NMR)

The PQ should be performed before the collection of experimental data.

Dissolve an accurately weighed quantity of manganese (II) chloride tetrahydrate (MW 197.91) in water, and quantitatively dilute with water to obtain check solutions that have known concentrations of 0.9, 2.7, and 4.5 mM.

Place a portion of each of the solutions into sample holders suitable for the configuration of the specific model of the LF-NMR spectrometer. Warm to 40° for NLT 10 min, and measure the spin-lattice relaxation time ($T_1$) of water. The average $T_1$ for replicate measurements must be within 5% of 156, 52, and 32 ms for the 0.9, 2.7, and 4.5 mM solutions, respectively.

## Characterizing Instrument Performance

Specific procedures, acceptance criteria, and time intervals for characterizing NMR spectrometer performance depend on the instrument and its intended application. Many NMR applications use previously validated experiments that relate NMR spectra to a physical or chemical property of interest. Stable instrument performance over extended periods of time should be demonstrated. This practice provides some assurance that reliable measurements can be taken from sample spectra using previously validated NMR experiments.

# QUALITATIVE AND QUANTITATIVE NMR ANALYSIS

NMR spectroscopy has been used for a wide range of applications such as structure elucidation; thermodynamic, kinetic, and mechanistic studies; and quantitative analysis. Some of these applications are beyond the scope of compendial methods.

All characteristics of the signal—chemical shift, multiplicity, linewidth, coupling constants, relative intensity, and relaxation time—contribute analytical information.

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## Qualitative Applications

Comparison of a spectrum from the literature or from an authentic standard with that of a test sample may be used to confirm the identity of a compound and to detect the presence of impurities that generate extraneous signals. The NMR spectra of simple structures can be adequately described by the value of the chemical shifts and coupling constants, and by the relative number of nuclei represented by the integral of each signal. (The software of modern instruments have available programs that generate simulated spectra using these data.) Experimental details, such as the solvent used, and the chemical shift reference, must also be provided.

For unknown samples, NMR analysis, usually coupled with other analytical techniques, is a powerful tool for structure elucidation. Chemical shifts provide information on the chemical environment of the nuclei. Extensive literature is available with correlation charts and rules for predicting chemical shifts. The multiplicity of the signals provides important structural information. The magnitude of the scalar coupling constant, *J*, between residual protons on substituted aromatic, olefinic, or cycloalkyl structures is used to identify the relative position of the substituents. Routine $^{13}C$ spectra are obtained under proton decoupling conditions that remove all heteronuclear $^{13}C$-$^{1}H$ couplings. As a result of this decoupling, the carbon signals appear as singlets, unless other nuclei that are not decoupled are present (e.g., $^{19}F$, $^{31}P$).

*Chemical exchange* is an example of the effect of intermolecular and intramolecular rate processes on NMR spectra. If a proton can experience different environments by virtue of such a process (tautomerism, rotation about a bond, exchange equilibria, ring inversion, etc.), the appearance of the spectrum will be a function of the rate of the process. Slow processes (on an NMR time scale) result in more than one signal from the interconverting species; fast processes average these signals to one line; and intermediate processes produce broad signals, which sometimes cannot be easily found in the spectra.

The software of modern FT-NMR spectrometers allows for sequences of pulses much more complex than the repetitive accumulation of transients described above. Such experiments include homonuclear or heteronuclear *multidimensional analysis*, which determines the correlation of couplings and may simplify the interpretation of otherwise complex spectra.

See chapter ⟨1761⟩ for detailed descriptions of common two-dimensional experiments.

## Quantitative Applications

I. General considerations of quantitative NMR: see ⟨1761⟩.

II. The scope of this section is limited to quantitation by one-dimensional NMR. Although any of the NMR active nuclei can be used to obtain quantitative data, the discussion here will be limited to $^{1}H$. There are two kinds of quantitation by NMR: relative and absolute.

1. **Relative quantitation** involves measurement of relative amounts of species in a sample based on integration of peaks due to each of the components measured. The integrals are normalized by factor *N*, that is, the integral is divided by the number of equivalent nuclei represented by that peak to give the relative molar concentration of each component.
2. **Absolute quantitation** is the direct measurement of the actual amount of analyte independent of other components contained in that sample. There are two basic methods for absolute quantitation based on the kind of reference standard that is used to calibrate the NMR signal.
    A. **Internal reference standard**
        I. **Definition:** The reference standard is co-dissolved in the analyte test solution.
        II. **Procedure**

**Typical NMR solution preparation:** An NMR solution is prepared with exact weights of both the analyte and reference standard. The largest source of error in this quantitative NMR method is from weighing, so the use of larger weights is recommended to minimize this error. This quantitative method is based on a comparison of the reference standard and analyte NMR peaks and their respective concentrations. Since the analyte and reference standard are in the same solution, the analyte and reference standard are contained in the same volume, and only their masses are compared. Therefore, the exact volume is not required. Typically, at least three replicates are prepared.

**Data acquisition:** Data is acquired under quantitative conditions, see ⟨1761⟩. For example, the pulse repetition time should be at least 5 times the longest $T_1$ when a 90° pulse is used.

**Data processing:** Process the data, using zero-filling if necessary, such that a sufficient number of points define a peak. For example, experience has shown that at least 16 points gives a good quantitative representation of a peak.

**Analysis:** Integrate appropriate peaks. For example, avoid using peaks that are overlapped, or due to hydrogens capable of exchanging. The determination of the amount of analyte derives from the basic proportionality between the peak intensity and the concentration of the solute.

$$\frac{[A]_{1H}}{I_A/N_A} = \frac{[RS]_{1H}}{I_{RS}/N_{RS}} \qquad [2]$$

where *I* = integral; *N* = normalization factor; and $[\,]_{^1H} = {}^1H$ relative molar concentration, and the subscripts *A* and *RS* represent the analyte and reference standard, respectively. The mass of the analyte is thus calculated according to the following equation.

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$$M_A = \frac{I_A}{I_{RS}} \times \frac{N_{RS}}{N_A} \times \frac{MM_A}{MM_{RS}} \times M_{RS} \times P \qquad [3]$$

where $M_A$ = mass of the analyte, $MM$ = molar mass, and $P$ = purity of the reference standard.

III. A common application of absolute quantitation is the determination of the purity of a sample. The weight % purity is given by

$$weight\,\%\,purity = \frac{M_A}{M_S} \times 100\,\% \qquad [4]$$

where $M_S$ is the total mass of the sample with contributions from the analyte plus any contaminants that may be present in the sample such as water and salts. Combining *Equations 3* and *4*, the weight % purity is given by

$$weight\,\%\,purity = \frac{I_A}{I_{RS}} \times \frac{N_{RS}}{N_A} \times \frac{MM_A}{MM_{RS}} \times \frac{M_{RS}}{M_S} \times P \times 100 \qquad [5]$$

B. **External reference standard**

I. **Definition:** The classical external reference standard method consists of solutions of a reference standard and analyte that are each in separate NMR tubes. One variation of an external reference standard is the standard test solution contained in a coaxial tube and is inserted into an analyte test solution contained in an NMR tube. Another variation is the introduction of a computer-generated signal into the spectrum of a reference standard solution of known concentration to calibrate the signal's response (intensity per $^1$H molar concentration, in the case of $^1$H NMR), followed by insertion of that calibrated computer-generated signal into the spectrum of an analyte test solution. This section will address the use of an external reference in the classical sense.

II. **Procedure**

**NMR solution preparation:** NMR solutions of known concentrations of each of the analyte and reference standard are prepared using exact weights and volumes. Again, the use of larger weights is recommended to minimize the weighing error. Typically replicates of the analyte solutions and reference standard solutions are prepared. The analyte and reference must be prepared in the same solvent to minimize probe tuning differences.

**Data acquisition and processing:** Same as in II.B.1.b, internal reference standard. Apply the same acquisition and processing parameters to the analyte and reference standard spectra.

**Analysis:** Integrate appropriate peaks in the spectra of the analyte and reference standard. The amount of analyte is calculated according to the following equation:

$$M_A = \frac{I_A}{I_{RS}} \times \frac{N_{RS}}{N_A} \times \frac{V_A}{V_{RS}} \times \frac{MM_A}{MM_{RS}} \times M_{RS} \times P \qquad [6]$$

where $V$ = volume.

Application to weight % purity: Weight % purity values may be similarly calculated as in *Equation 5*.

3. The internal and external reference standard methods each have their own set of advantages and disadvantages.

A. **Chemical interactions:** Preparation of the reference standard and the test material in separate solutions avoids chemical interactions between the test sample and reference standard that may otherwise occur with an internal reference standard.

B. **Spectral overlap:** The use of an external reference standard also avoids potential overlap between peaks of the reference standard and test sample that can occur with an internal standard.

C. **Calibration:** Once an NMR response has been calibrated with external reference standard solutions, this calibration may be applied to any other sample in the same solvent given that i) the instrument has been demonstrated to be stable over the time between when the calibration is done and when data is acquired on the test material, ii) system suitability has been established on the day that the measurement on the test material is made, and iii) absolute integrals are compared. In the case of internal reference standards, the measurement on the reference standard and test sample is made under absolutely identical conditions.

D. **Accuracy and precision:** Multiple external reference standard solutions may be prepared to average the errors in the mass and volume measurements during sample preparation, thereby improving the accuracy of the calibrated NMR response. In the case of internal reference standards, single measurements of the reference standard and analyte are made for each replicate test solution. The combined errors from the mass measurements of the reference standard and test sample as well as instrumental electronic variations determine the standard deviation of the average $M_A$ or weight % purity values.

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# VALIDATION AND VERIFICATION OF NMR ANALYTICAL PROCEDURES

If an NMR procedure is provided in a monograph, verification of suitability (see ⟨1226⟩) under actual conditions of use is required. Validation is required only when an NMR method is an alternative to the official procedure for testing an official article. The objective of validation of a procedure relying on the NMR method is to demonstrate that the measurement is suitable for its intended purpose, including the following: quantitative determination of the main component in a drug substance or a drug product (Category I assays), quantitative determination of impurities (Category II), and identification tests (Category IV). [NOTE—For a definition of the different categories, see *Validation of Compendial Procedures* ⟨1225⟩.] Depending on the category of the test, analytical procedure validation requires the testing of specificity, linearity, range, accuracy, precision, quantitation limit, and robustness. These analytical performance characteristics apply to externally standardized methods and to the method of standard additions.

Chapter ⟨1225⟩ provides definitions and general guidance on analytical procedures validation without indicating specific validation criteria for each characteristic. The intention of the following sections is to provide the user with specific validation criteria that represent the minimum expectations for this technology. For each particular application, tighter criteria may be needed in order to demonstrate suitability for the intended use.

## Analytical Procedure Validation

The objective of an analytical procedure validation is to demonstrate that the analytical procedure is suitable for its intended purpose by conducting experiments and obtaining results that meet predefined acceptance criteria. NMR analytical procedures can include the following: quantitative tests for major component and impurities content, limit tests for the presence of impurities, quantification of component in a product or formulation, and/or identification tests.

Performance characteristics that demonstrate the suitability of an analytical procedure are similar to those required for any analytical procedure. A discussion of the applicable general principles is found in chapter ⟨1225⟩. Specific acceptance criteria for each validation parameter must be consistent with the intended use of the analytical procedure.

The performance characteristics that are required as part of a validation for each of the analytical procedure categories is given below.

### SPECIFICITY

The purpose of a specificity test is to demonstrate that measurements of the intended analyte signals are free of interference from components and impurities in the test material. Specificity may be applied to all categories and is a requirement for Category IV. Specificity tests can be conducted to compare NMR spectra of other components and impurities that are known from synthetic processes and formulations and test preparations. For an identification NMR analytical procedure (Category IV), validation experiments may include multidimensional NMR experiments to validate correct assignments of chemical shifts and to confirm the structure of the analyte.

**Validation criteria:** Specificity is ensured by use of a reference standard wherever possible and demonstrable lack of interference from other components.

### LINEARITY

A linear relationship is exhibited between the analyte concentration and instrument response; this should be demonstrated by measuring responses of analyte from NLT five standard solutions at concentrations encompassing the anticipated concentration range of analyte(s) of the test solution. For Category I, standard solutions can be prepared from reference materials in an appropriate NMR solvent. For Category II, NMR analytical procedures that are used to quantitate impurities, linearity samples can be prepared by spiking suitable test samples that contain low amounts of analyte or by spiking matrix samples at concentrations of the expected range. The standard curve should then be constructed using appropriate statistical analytical procedures such as a least squares regression. The correlation coefficient ($R$), $y$-intercept, and slope of the regression line should be determined. Absolute values determined for these factors should be appropriate for the procedure being validated.

**Validation criteria:** The correlation coefficient ($R$) must be NLT 0.995 for Category I assays and NLT 0.99 for Category II quantitative tests.

### RANGE

The range between the low and high concentrations of analyte is given by the quantitative NMR analytical procedure. This is normally based on test article specifications in the *USP* monograph. It is the range within which the analytical procedure can demonstrate an acceptable degree of linearity, accuracy, and precision, and may be obtained from an evaluation of that analytical procedure. Recommended ranges for various NMR analytical procedures are given below.

**Validation criteria:** For Category I tests, the validation range for 100.0% centered acceptance criteria is 80.0%–120.0%. For noncentered acceptance criteria, the validation range is 10.0% below the lower limit to 10.0% above the upper limit. For content uniformity, it is 70.0%–130.0%. For Category II quantitative tests, the validation range covers 50.0%–120.0% of the acceptance criteria.

### ACCURACY

The accuracy of a quantitative NMR analytical procedure should be determined across the required analytical range. Typically, three levels of concentrations are evaluated using triplicate preparations at each level.

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For drug substance assays (Category I), accuracy can be determined by analyzing a reference standard of known purity. For drug product (Category I), a composite sample of reference standard and other components in a pharmaceutical finished product should be used for analytical procedure validation. The assay results are compared to the theoretical value of the reference standard to estimate errors or percent recovery. For the quantitation of impurities (Category II), the accuracy of the analytical procedure can be determined by conducting studies with drug substances or products spiked with known concentrations of the analyte under test. It is also acceptable to compare assay results from the analytical procedure being validated to those of an established, alternative analytical procedure.
**Validation criteria:** 98.0%–102.0% recovery for drug substances, 95.0%–105.0% recovery for compounded pharmaceutical finished products assay, and 80.0%–120.0% recovery for the quantitative impurity analysis. These criteria should be met throughout the intended range.

PRECISION

**Repeatability:** The analytical procedure should be assessed by measuring the concentrations of six separate standard solutions at 100% of the test concentration. The relative standard deviation from the replicate measurements should be evaluated to meet acceptance criteria. Alternatively they can measure the concentrations of three replicates of three separate sample solutions at different concentrations. The three concentrations should be close enough so that the repeatability is constant across the concentration range. If this is done, the repeatability at the three concentrations is pooled for comparison to the acceptance criteria.

*Validation criteria:* The relative standard deviation is NMT 1.0% for drug substances, NMT 2.0% for compounded pharmaceutical finished products, and NMT 20.0% for the quantitative impurity analysis.

*Intermediate precision:* The effect of random events on the analytical precision of the analytical procedure should be established. Typical variables include performing the analysis on different days, using different instrumentation that are suitable as specified in the analytical procedure, and/or having the analytical procedure performed by two or more analysts. As a minimum, any combination of at least two of these factors totaling six experiments will provide an estimation of intermediate precision.

*Validation criteria:* The relative standard deviation is NMT 1.0% for drug substances, NMT 3.0% for compounded pharmaceutical finished products, and NMT 25.0% for quantitative impurity analysis.

QUANTITATION LIMIT (QL)

The QL can be validated by measuring six replicates of test samples spiked with analyte at 50% of specification.

From these replicates, accuracy and precision can be determined. Examples of specifications for Category II quantitative determinations are that the measured concentration is within 70.0%–130.0% of the spike concentration and the relative standard deviation is NMT 15%.

ROBUSTNESS

The reliability of an analytical measurement should be demonstrated with deliberate changes to critical experimental parameters. This can include measuring the stability of the analyte under specified storage conditions, slightly varied inter-pulse delay, probe temperature, and possible interfering species, to list a few examples. Robustness is required for Category I and Category II, quantitative methods.

## Analytical Procedure Verification

U.S. Current Good Manufacturing Practices regulations [21 CFR 211.194(a)(2)] indicate that users of analytical procedures described in *USP–NF* do not need to validate these procedures if provided in a monograph. Instead, they must simply verify their suitability under actual conditions of use.

The objective of an NMR procedure verification is to demonstrate that the procedure as prescribed in a specific monograph can be executed by the user with suitable accuracy, specificity, and precision using the instruments, analysts, and sample matrices available. According to general information chapter *Verification of Compendial Procedures* ⟨1226⟩, if the verification of the compendial procedure by following the monograph is not successful, the procedure may not be suitable for use with the article under test. It may be necessary to develop and validate an alternative procedure as allowed in *General Notices, 6.30 Alternative and Harmonized Methods and Procedures*.

Verification of a compendial NMR procedure should at minimum include the execution of the validation parameters for specificity, accuracy, precision, and limit of quantitation, when appropriate, as indicated in this section.

## GLOSSARY

**Internal standard:** An internal standard (IS) is a substance added to a sample solution at a known concentration. One should select an IS with at least a single NMR resonance that does not overlap with those of the analyte. The ratio of a specific internal standard peak area and that of an analyte peak area is used to determine the concentration of the analyte. The number of nuclei corresponding to the integrated peaks in the IS and analyte spectra must be known.

**NMR reference:** An NMR reference, also known as an NMR shift reference, is a substance added to a sample and from which the chemical shift for the δ scale is established. Common examples for proton and carbon NMR analyses are tetramethylsilane (TMS) for use in organic solvents and the sodium salt of 2,2-dimethyl-2-silapentane-5-sulfonic acid (DSS) or sodium-3-trimethylsilylpropionate (TMSP) for use in aqueous media. In both cases, the chemical shift of the methyl peaks is defined as 0.0 ppm.

**Reference standard:** A reference standard is a substance authenticated by appropriate experimental means to be of a specific chemical structure. In NMR spectroscopy, a reference standard is typically used for the qualitative analysis of a test

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material. Structure can be confirmed if one directly compares the chemical shifts and multiplicities of the peaks in the NMR spectrum of the test material against the spectrum of the reference standard acquired under comparable experimental conditions.

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# ⟨771⟩ OPHTHALMIC PRODUCTS—QUALITY TESTS

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## INTRODUCTION

Ophthalmic products are sterile products that are intended for application to any ocular structure, including any space adjacent to an ocular structure and its immediate surrounding spaces.

The routes of administration of ophthalmic products fall into three general categories: topical, intraocular injections, and extraocular injections. Topical drug products are intended to be administered to an ocular surface component, such as the eyelid, conjunctiva, or cornea, and can produce local or systemic effects. Intraocular and extraocular injections are administered through external boundary tissue. ▲Due to the potential sensitivity of some ocular tissues, injections have additional requirements for subvisible particulate matter content (see *Particulate and Foreign Matter*, *Subvisible Particulate Matter in Ophthalmic Solutions* ⟨789⟩, and *Visible Particulates in Injections* ⟨790⟩).▲ (USP 1-Dec-2022) The ophthalmic routes of administration include, but are not limited to: topical, subconjunctival, sub-Tenons, subretinal, subchoroidal, intracorneal, intrascleral, suprachoroidal, intravitreal, intracameral, juxtascleral, and retrobulbar routes (see *Figure 1*). Ophthalmic products are administered to the eye in a wide variety of dosage forms, including but not restricted to: solutions, suspensions, ointments, gels, emulsions, strips, injections, inserts, and implants.

▲

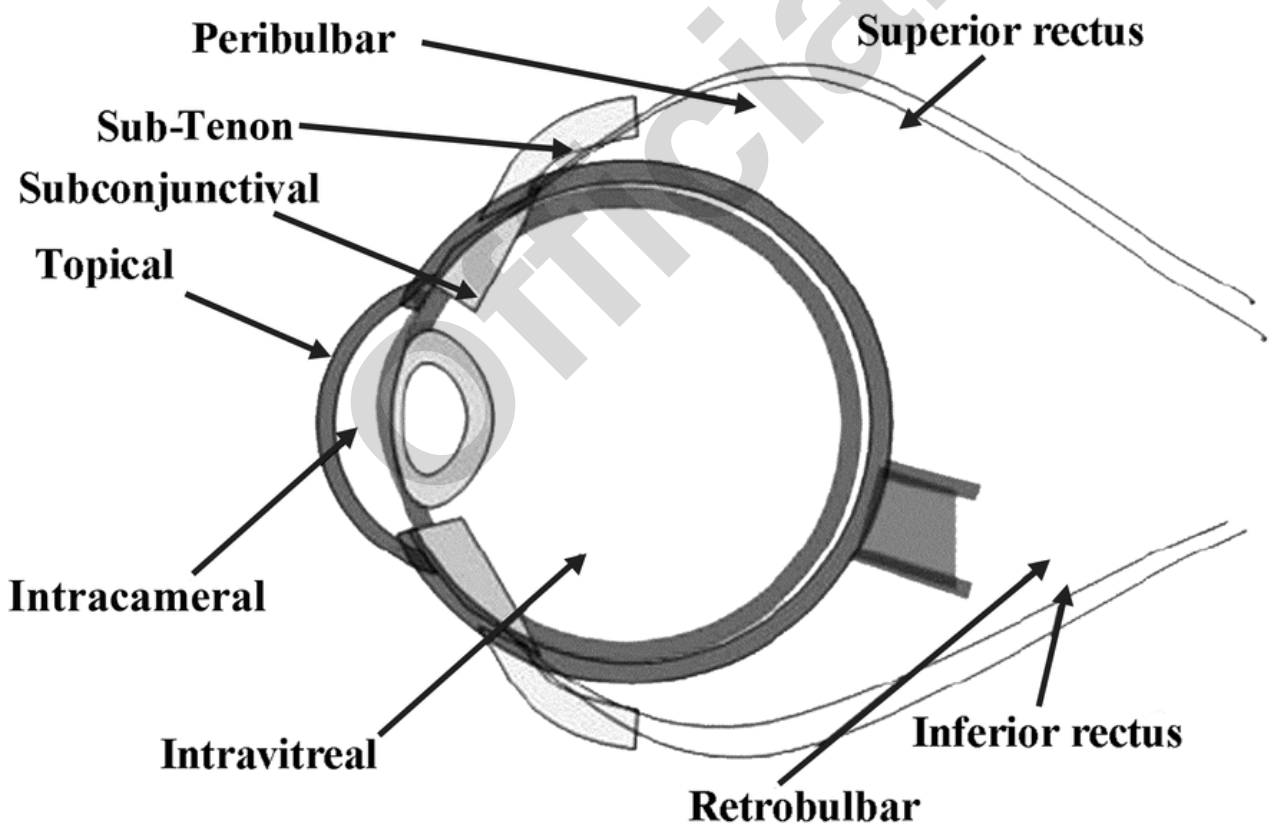


Figure 1. Examples of ocular routes of administration.

▲ (USP 1-Dec-2022)

This chapter provides lists of consolidated common product quality test requirements in a concise and coherent fashion. This chapter applies, in part or in its entirety, when referenced in a drug product monograph (see *General Notices, 3.10 Applicability of Standards*). This chapter includes the quality tests for the specific route of administration. The quality tests listed can be used, as appropriate, by manufacturers toward the development of new drug product monographs for submission to the *USP–NF*.▲▲ (USP 1-Dec-2022)

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# OPHTHALMIC DOSAGE FORMS

This chapter discusses the specific characteristics of dosage forms that are administered to any structure in the eye. See *Pharmaceutical Dosage Forms* ⟨1151⟩ for additional information pertaining to the description and manufacturing of pharmaceutical dosage forms.

Ophthalmic products have the same or similar requirements as injectables and implants. For more information on the requirements for these dosage forms, see *Injections and Implanted Drug Products* ⟨1⟩.

▲Degradation and/or morphological changes of the drug product can occur during the sterilization step of the manufacturing process depending on the formulation, container-closure system, and sterilization process. These changes should be minimized.▲ (USP 1-Dec-2022) Temperatures required for autoclaving can cause irreversible damage to certain dosage forms such as suspensions, semisolids, emulsions, and others, whereas filtration is applicable only to formulations containing particulates with particle sizes <0.2 µm. An alternative ▲approach▲ (USP 1-Dec-2022) is to manufacture the ▲drug▲ (USP 1-Dec-2022) product from sterile ingredients ▲maintained within▲ (USP 1-Dec-2022) an aseptic environment.

▲Multi-dose products shall contain a suitable antimicrobial preservative, and the effectiveness of the preservative over the shelf life of the product must be verified. Alternatively, preservative-free multi-dose products require a packaging system that has been demonstrated to maintain sterility over the shelf-life of the product.▲ (USP 1-Dec-2022)

## Solutions

[NOTE—Included in this section are ▲solutions and▲ (USP 1-Dec-2022) those solid products that, when reconstituted according to the ▲instructions in the labeling,▲ (USP 1-Dec-2022) result in a solution.]

The corneal contact time of topical ophthalmic solutions increases with the viscosity of the formulations. Several synthetic polymers, including but not limited to polyvinyl alcohol, polyvinylpyrrolidone, polyethylene glycol, poly(acrylic acid), and ▲▲ (USP 1-Dec-2022) cellulose derivatives such as hypromellose and hydroxyethyl cellulose, are commonly used as viscosity enhancers because of their physiologic compatibility and ▲desirable▲ (USP 1-Dec-2022) physicochemical properties.

Another approach to increase the corneal contact time involves the use of polymers that provide the liquid formulation with semisolid consistency only when ▲the liquid formulation▲ (USP 1-Dec-2022) is placed in the conjunctival or corneal area. In this way, ▲▲ (USP 1-Dec-2022) instillation of the solution is followed by prolonged permanence as a result of the viscoelastic properties of the formed hydrogel. "Hydrogel", or "water-based gels", are three-dimensional networks of polymer chains containing water within the network. ▲The structure of the polymer network can be physically crosslinked (e.g., via ionic or hydrogen bonds) or chemically crosslinked (via a covalent bond).▲ (USP 1-Dec-2022) The water content of hydrogels can be adjusted by modulating the composition and conformation of polymers, such as the hydrophilic/hydrophobic balance of polymer chains and pendant groups, and the degree of cross-linking. The in-situ gelling phenomenon is caused ▲upon delivery to the eye▲ (USP 1-Dec-2022) by a change in the conformation of the polymer(s) that can be triggered by external stimuli such as temperature, pH, ionic content, and lacrimal fluid. ▲▲ (USP 1-Dec-2022) Additionally, some polymers can interact, via noncovalent bonds, with conjunctival mucin and maintain the formulation in contact with corneal tissues.

Solutions can be injected not only by the intravitreal route but also by other routes, such as subconjunctival, sub-Tenons, retrobulbar, suprachoroidal, and subretinal routes.

## Suspensions

[NOTE—Included in this section are ▲suspensions and▲ (USP 1-Dec-2022) those solid products that, when reconstituted according to the ▲instructions in the labeling,▲ (USP 1-Dec-2022) result in a suspension.]

Use of aqueous or oily suspensions may be considered for a number of reasons such as the following: 1) ▲a drug is▲ (USP 1-Dec-2022) poorly ▲▲ (USP 1-Dec-2022) soluble ▲in water▲ (USP 1-Dec-2022), 2) ▲a drug has poor stability in water▲ (USP 1-Dec-2022), and 3) in cases where there is a need to increase contact time with the eye and to increase wetting of the eye. ▲A▲ (USP 1-Dec-2022) drug particle size ▲of <10 µm is desirable to reduce irritation.▲ (USP 1-Dec-2022)

After topical instillation, particles are expected to be retained in the cul-de-sac, and the drug slowly dissolves or is released from the polymeric structures by diffusion, dissolution, polymer degradation, or ion-exchange.

Suspensions can be injected not only by the intravitreal route but also through other routes, such as subconjunctival, sub-Tenons, retrobulbar, suprachoroidal, and subretinal.

## Ointments

Eye ointments are semisolid products usually intended for application to the conjunctiva, cornea, or eyelid. They have longer contact time when compared to many solutions. A suitable ophthalmic ointment base is nonirritating to the eye and permits diffusion of the drug substance throughout the secretions bathing the eye.

The majority of water-free, oleaginous eye ointment bases are composed of white petrolatum and liquid petrolatum (mineral oil), or the eye ointment base is a modification of the petrolatum-based formula. They are designed to melt at body temperature. Other types of lipophilic ointment bases can also be used. An anhydrous vehicle can be advantageous for moisture-sensitive drugs.

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## Gels

Semisolid ▲gel▲ (USP 1-Dec-2022) products are an alternative to traditional ointments and are based on the effect of increasing the viscosity to prolong the retention of the drug in the eye. Several types of gelling agents can be used, such as poly(acrylic acid) derivatives (e.g., carbomer) ▲or▲ (USP 1-Dec-2022) hypromellose.

## Emulsions

Topical ophthalmic emulsions are generally prepared by dissolving or dispersing the drug substance into an oil phase, adding suitable emulsifying and suspending agents, and ▲vigorously mixing these components with water▲ (USP 1-Dec-2022) to form a uniform oil-in-water emulsion. Each phase is typically sterilized before or during charging into the mixing vessel. High-shear homogenization may be used to reduce oil droplet size to submicron size, which may improve the physical stability of the oil micelles so that they do not coalesce. The resulting dosage form should contain small oil droplets, uniformly suspended.

Limited aqueous solubility of the drug substance(s) is the most common rationale for developing an ophthalmic emulsion. The drug substance(s) can be added to the phase in which it is soluble at the beginning of the manufacturing process, or the drug substance can be added after the emulsion is prepared by a suitable dispersion process.

The physical stability of the emulsion can be measured by light-scattering techniques that characterize oil-phase, globule-size distribution. Suitable surfactants may be added to improve emulsion stability.

## Strips

Ophthalmic strips can have different ophthalmic uses including, but not limited to, use as a diagnostic tool to visualize defects or aberrations in the corneal epithelium or to measure the amount of tear production. They are made of filter paper, and they can contain compounds such as fluorescein sodium. They are individually packed to preserve sterility until the moment of use.

## Injections

Although injections are considered a dosage form for nomenclature purposes, they are ▲referred to in this chapter by other dosage forms such as solution, suspension, emulsion, and other appropriate terminology based on physical form.▲ (USP 1-Dec-2022)

## Inserts

Ophthalmic inserts and ocular systems are solid dosage forms of appropriate size and shape that are placed in the conjunctival fornix, in the lacrimal punctum (*Figure 2*), or on the cornea. Inserts can usually be removed, if adverse effects develop, and ▲can▲ (USP 1-Dec-2022) provide extended release of the drug over a certain period of time. Inserts can be classified as ▲bioabsorbable▲ (USP 1-Dec-2022) (soluble) or ▲non-bioabsorbable▲ (USP 1-Dec-2022) (insoluble). Drug release from soluble inserts involves two steps: 1) fast release of a portion of the drug as the tear fluid penetrates into the system and 2) slow release as a gel layer is formed on the surface of the insert. Collagen shields made from porcine sclera collagen or bovine corium tissue—and devices obtained by molding, extrusion, or compression (minitablets) of gelling polymers—belong to this category of soluble inserts. ▲Bioabsorbable▲ (USP 1-Dec-2022) polymers (e.g., cross-linked gelatin derivatives and polyesters) can be used to prepare ▲bioabsorbable▲ (USP 1-Dec-2022) inserts; these matrices act as reservoirs or interact with the drug molecules through labile bonds.

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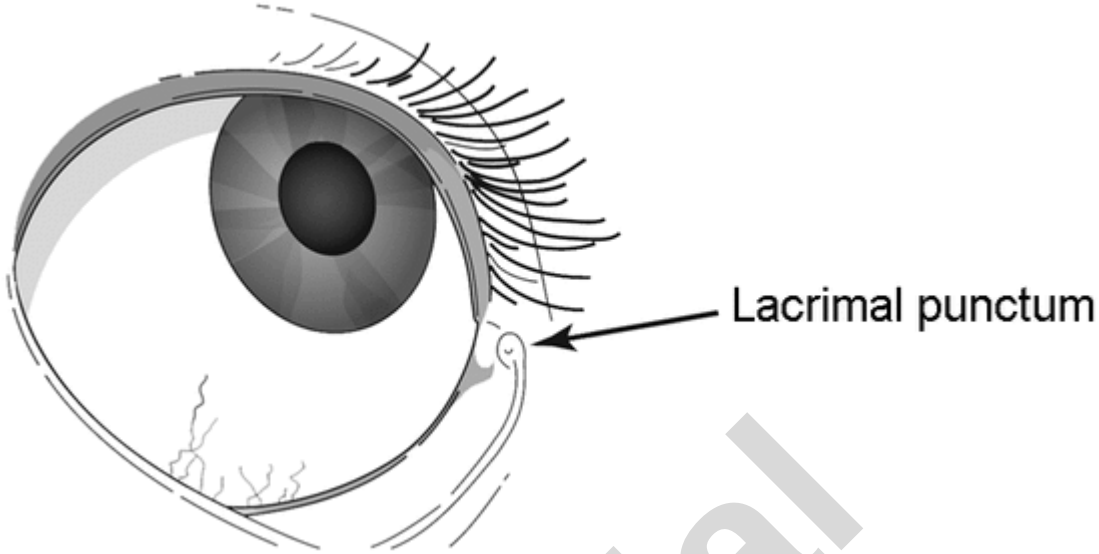


Figure 2. Lacrimal punctum.

▲ (USP 1-Dec-2022)

Soluble inserts can have the drug incorporated into ▲a bioabsorbable▲ (USP 1-Dec-2022) matrix, such as hydroxypropyl cellulose, hyaluronic acid, ▲or poly(acrylic acid) matrices.▲ (USP 1-Dec-2022) Soluble inserts may be placed in the lower cul-de-sac and generally dissolve within 12–24 h. ▲Bioabsorbable▲ (USP 1-Dec-2022) polymeric products undergo gradual dissolution while releasing the drug, and the patient does not have to remove the ▲bioabsorbable▲ (USP 1-Dec-2022) polymeric insert after use.

Insoluble inserts can have a reservoir or matrix structure. Their mechanism of action is based on diffusion of a fluid ▲from the eye▲ (USP 1-Dec-2022) into the device, ▲dissolution▲ (USP 1-Dec-2022) of the drug, and the creation of a saturated solution released to the medium by diffusion out of the insert. ▲▲ (USP 1-Dec-2022)Insoluble inserts need to be removed after a certain period of time.

## Implants

Implants are injected or implanted into the anterior segment (intracameral, subconjunctival, sub-Tenon, or other parts of the anterior segment) or intravitreal cavities, and the implants can be in different shapes, such as a disk or a thin rod. Implants can be made of ▲non-bioabsorbable▲ (USP 1-Dec-2022) or ▲bioabsorbable▲ (USP 1-Dec-2022) polymers.

Among the types of ▲non-bioabsorbable▲ (USP 1-Dec-2022) solid (polymer) implants are reservoir-type implants anchored to the sclera, in which the drug is released across a ▲non-bioabsorbable▲ (USP 1-Dec-2022), semipermeable polymer. The reservoir-type implants can be made of a variety of materials, including ▲poly(vinyl alcohol–ethylene vinyl acetate). Bioabsorbable▲ (USP 1-Dec-2022) polymers can be used to form injectable solid implants.

## Drug–Device Combination Products

An ophthalmic drug–device combination product is constituted, in most cases, of two components. One is a pharmaceutical dosage form containing the drug substance(s), and the other is a device that will activate or facilitate the penetration of the drug substance(s) from the dosage form into a particular region of the eye. Some examples of the devices are those that generate waveforms (heat or light). ▲Light sources can activate photoresponsive drugs.▲ (USP 1-Dec-2022)

This chapter is applicable only to the pharmaceutical dosage form component of the ophthalmic drug–device combination product. The appropriate FDA regulations regarding medical devices should be used for the device component.

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# DRUG PRODUCT QUALITY

Procedures and acceptance criteria for testing ophthalmic products are divided into two categories: 1) those that assess general quality attributes, e.g., identification, potency, purity (and impurities), sterility, and particulate matter; and 2) those that assess in vitro product performance, ▲i.e.▲ (USP 1-Dec-2022), dissolution or drug release of the drug substance from the drug product. Quality tests assess the integrity of the dosage form, whereas the performance tests assess drug release and other attributes that relate to in vivo drug performance. Taken together, quality and performance tests ensure the identity, strength, quality, purity, and efficacy of the drug product. This chapter addresses the quality tests for ophthalmic products. The performance tests (dissolution/drug release) are addressed by *Ophthalmic Products—Performance Tests* ⟨1771⟩.

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# Universal Tests

In this chapter, the division of the product quality tests into universal and specific tests does not strictly follow the ICH guidance *Q6A Specifications: Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Chemical Substances* (available at www.ich.org). Universal tests in this chapter mean the tests that are applicable to all ophthalmic products, regardless of the dosage form type.

## DESCRIPTION

A qualitative description of the drug product should be provided. The acceptance criteria should contain the final acceptable appearance, including clarity and color, of the dosage form and packaging. If the color changes during storage, a quantitative procedure may be appropriate. This is not a compendial test but is part of the manufacturer's specification of the drug product.

## IDENTIFICATION

Identification tests are discussed in *General Notices, 5.40 Identification*. Identification tests should establish the identity of the drug or drugs present in the article and should discriminate between compounds of closely related structures that are likely to be present. Identity tests should be specific for the drug substance(s). ▲▲ (USP 1-Dec-2022) If the test is nonspecific, at least two orthogonal nonspecific tests should be used. ▲▲ (USP 1-Dec-2022) Identification by use of a single chromatographic retention time does not constitute a specific test.

## ASSAY

A specific and stability-indicating test should be used to determine the strength (content) of the drug product. In cases where the use of a nonspecific assay test is justified, other supporting analytical procedures should be used to achieve overall specificity. A specific procedure should be used when there is evidence of excipient interference with the nonspecific assay test. Additional information on specific assays may be found in *Antibiotics—Microbial Assays* ⟨81⟩, *Chromatography* ⟨621⟩, and *Ion Chromatography* ⟨1065⟩.

## IMPURITIES

Process impurities, synthetic byproducts, and other inorganic and organic impurities may be present in the drug substance and excipients used in the manufacture of the drug product. These impurities are controlled by the drug substance and excipient monographs. Organic impurities arising from the degradation of the drug substance in the drug product and those impurities arising during the manufacturing process of the drug product should be monitored. All articles meet the requirements in *Elemental Impurities—Limits* ⟨232⟩ and *Residual Solvents* ⟨467⟩.

## pH

See *pH* ⟨791⟩. Normal tears have a pH of about 7.4. The eye can tolerate products over a range of pH values from about 3.0 to about 8.6, depending on the buffering capacity of the formulation. The pH value of the formulation should be the one where the drug product is the most stable. Formulations that target the extremes of the acceptable pH range will have better patient acceptability if the formulations have a low buffering capacity.

## OSMOLARITY

See *Osmolality and Osmolarity* ⟨785⟩. Ophthalmic products may be tolerated over a ▲▲ (USP 1-Dec-2022) wide range of tonicity (0.5%–5% sodium chloride, equivalent to about 171–1711 mOsm/kg). Hypotonic solutions are better tolerated than hypertonic solutions. Precautions should be taken to ensure that the product maintains its osmolarity during shelf life. Any possible contributions or interferences from the packaging system should be considered.

## PARTICULATE AND FOREIGN MATTER

▲As described in *Visual Inspection of Injections* ⟨1790⟩*, 4.1 Extrinsic, Intrinsic, or Inherent Particles*, unwanted particulate matter is categorized as extrinsic (foreign matter) and intrinsic (formulation-, process-, or container-related matter or particulate matter due to product instability or product package interactions). A third category, inherent, describes a physical state that is an expected attribute of the product, such as a suspension or emulsion. It is expected that the manufacturer has established an acceptable control level for any inherent attribute, such as solution color, haze, or drug/excipient-related particle content.

The term 100% unit inspection is defined in ⟨1790⟩*, 3.1 100% Inspection* as the complete nondestructive inspection of the container–closure system and its contents. Therefore, evaluation of package and fill acceptability is shown by using the methodology in *Visible Particulates in Injections* ⟨790⟩, and this methodology is applied to all ophthalmic products: topical, intraocular injections or ophthalmic products used in association with a surgical procedure, and extraocular injections.

Intraocular administration includes all ophthalmic products that cross (penetrate) boundary tissue such as the cornea and sclera. Extraocular administration of ophthalmic products includes all the other ocular components and spaces.

For all intraocular solutions, conformance to ⟨790⟩ and *Particulate Matter in Ophthalmic Solutions* ⟨789⟩ is required. Additionally, for extraocular (nontopical) solution products, conformance to ⟨790⟩ and evaluation by *Particulate Matter in Injections* ⟨788⟩ is required. For topical products, conformance to ⟨790⟩ is required. See the summary of this information in *Table 1*.

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**Table 1. Particulate and Foreign Matter Requirements for Ophthalmic Solutions**

| Route of Administration (See *Figure 1*) | Must comply with[a] |
|---|---|
| Topical | ⟨790⟩ |
| Peribulbar | ⟨790⟩, ⟨788⟩ |
| Superior rectus | ⟨790⟩, ⟨788⟩ |
| Sub-Tenon | ⟨790⟩, ⟨788⟩ |
| Subconjunctival | ⟨790⟩, ⟨788⟩ |
| Inferior rectus | ⟨790⟩, ⟨788⟩ |
| Retrobulbar | ⟨790⟩, ⟨788⟩ |
| Suprachoroidal | ⟨790⟩, ⟨789⟩ |
| Juxtascleral | ⟨790⟩, ⟨788⟩ |
| Intrascleral | ⟨790⟩, ⟨789⟩ |
| Intracorneal | ⟨790⟩, ⟨789⟩ |
| Subchoroidal | ⟨790⟩, ⟨789⟩ |
| Subretinal | ⟨790⟩, ⟨789⟩ |
| Intracameral | ⟨790⟩, ⟨789⟩ |
| Intravitreal | ⟨790⟩, ⟨789⟩ |

[a] Chapter ⟨787⟩ may be used instead of ⟨788⟩ for protein biotherapeutic formulas. For further information on difficult-to-inspect products, see ⟨1790⟩.

For nonsolution dosage forms (e.g., ointments, gels, suspensions, and emulsions) and dosage forms packaged in opaque containers (e.g., opaque bottles, metal tubes), the approach must be modified because these products are not directly amenable to the tests listed in *Table 1*. It is recommended to use 100% unit inspection per ⟨790⟩ for package acceptability (including but not limited to integrity, form, dimensions, cleanliness, label); whereas, an alternate testing strategy may be used for foreign particles where the dosage form hinders or precludes the use of these tests. For these products, a statistically relevant sample, examined by a destructive or other alternative method, will determine batch acceptability for visible particles as required by ⟨790⟩ and further described in ⟨1790⟩.

All ophthalmic products will benefit from evaluation of unwanted particulate matter content through the control strategy discussed in ⟨1790⟩ and in the FDA's current good manufacturing practices guidance. The final product, therefore, benefits from a control strategy including comprehensive evaluation of excipient and active ingredient quality, processing effects, package quality, and fill control. These concepts are discussed in ⟨1790⟩.▲ (USP 1-Dec-2022)

## STERILITY

Ophthalmic dosage forms must meet the requirements of *Sterility Tests* ⟨71⟩. If the specific ingredients used in the formulation do not lend themselves to routine sterilization techniques, ingredients that meet the sterility requirements described in ⟨71⟩, along with aseptic manufacture, may be used. The immediate container for ophthalmic products shall be sterile at the time of filling and closing. See also *Universal Tests, Container–Closure Integrity*.

## ANTIMICROBIAL PRESERVATIVES

Antimicrobial agents must be added to products that are packaged in containers that allow for the withdrawal or administration of multiple doses, unless one of the following conditions prevails: 1) there are different directions in the individual monograph; 2) the ▲radiopharmaceutical drug product▲ (USP 1-Dec-2022) contains a radionuclide with a physical half-life of <24 h; ▲▲ (USP 1-Dec-2022) 3) the drug product, without additional agents, is sufficiently microbicidal to meet the requirements of *Antimicrobial Effectiveness Testing* ⟨51⟩, ▲or 4) the container–closure system is capable of maintaining sterility of the product throughout its shelf life until opened or accessed. Once opened or accessed, it must maintain antimicrobial effectiveness during the intended use period. Antimicrobial agents▲ (USP 1-Dec-2022) must meet the requirements of ⟨51⟩ and *Antimicrobial Agents—Content* ⟨341⟩. Acceptance criteria for antimicrobial preservative content in ▲multi-dose▲ (USP 1-Dec-2022) products should be established.

## BACTERIAL ENDOTOXINS

All injected ophthalmic drug products shall be prepared in a manner designed to minimize bacterial endotoxins as defined in *Bacterial Endotoxins Test* ⟨85⟩. The limits are not more than 0.5 EU/mL for ophthalmic irrigation products and not more than 2.0 EU/dose/eye for injected or implanted drug products. Typically, this test is not required for topically applied ophthalmic products. This chapter does not address the endotoxin limits for devices that are injected or implanted.

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### UNIFORMITY OF DOSAGE UNITS

This test is applicable to dosage forms packaged in single-unit containers. It includes both the mass of the dosage form and the content of the drug substance(s) in the dosage form. The test can be performed by either content uniformity or weight variation (see *Uniformity of Dosage Units* ⟨905⟩).

### CONTAINER CONTENTS

Container contents of ophthalmic products should be determined (see *Minimum Fill* ⟨755⟩).

### LEACHABLES AND EXTRACTABLES

The packaging system should not interact physically or chemically with the product in any manner to alter the strength, quality, or purity of the drug product. The packaging system should meet the requirements in *Elastomeric Components in Injectable Pharmaceutical Product Packaging/Delivery Systems* ⟨381⟩, *Containers—Glass* ⟨660⟩, ▲and, depending on which standards the manufacturer has implemented, *Plastic Packaging Systems and Their Materials of Construction* ⟨661⟩ or ▲ (USP 1-Dec-2022) *Plastic Materials of Construction* ⟨661.1⟩ and *Plastic Packaging Systems for Pharmaceutical Use* ⟨661.2⟩. Further information regarding packaging systems testing can be found in *Assessment of Extractables Associated with Pharmaceutical Packaging/Delivery Systems* ⟨1663⟩ and *Assessment of Drug Product Leachables Associated with Pharmaceutical Packaging/Delivery Systems* ⟨1664⟩. The assessment of possible leachables and/or extractables and the establishment of any acceptance criteria for these compounds should ▲consider▲ (USP 1-Dec-2022) the risk assessment of the product, its indication, and its packaging system. This assessment is done in a case-by-case approach during product development and re-evaluated when any changes are made to the product including, but not restricted to, changes in manufacturing process, formulation, and packaging material.

Furthermore, it should be noted that a risk assessment of extractable/leachable impact on ▲the▲ (USP 1-Dec-2022) topical or intraocular route of administration is a challenging undertaking. Toxicological or safety assessments of primary or secondary packaging component extractables and leachables are not typically available for ophthalmic routes of administration. A risk assessment may include evaluation of toxicology and safety from other routes of administration and an assessment of the total daily intake of the extractable/leachable being evaluated. The preponderance of such assessments leads to an estimate of extractable/leachable risk via ocular delivery to the patient.

### CONTAINER–CLOSURE INTEGRITY

The packaging system should be closed or sealed in such a manner as to prevent contamination or loss of contents and should ▲incorporate a tamper-evident design.▲ (USP 1-Dec-2022) Validation of container integrity must demonstrate no penetration of microbial contamination or of chemical or physical impurities (see *Package Integrity Evaluation—Sterile Products* ⟨1207⟩).

## Specific Tests

### VISCOSITY

An increase in viscosity increases the residence time ▲of ocular formulations.▲ (USP 1-Dec-2022) However, drug diffusion out of ▲these formulations▲ (USP 1-Dec-2022) into the ▲ocular tissue▲ (USP 1-Dec-2022) may be ▲modified▲ (USP 1-Dec-2022) due to high product viscosity. Ophthalmic ointments are designed to be of very high viscosity to prolong the residence time ▲of the formulation▲ (USP 1-Dec-2022) in the eye. The inclusion of viscosity evaluation in the specification of the product should be based on the type of dosage form and whether changes in product viscosity will affect its performance. This is not a compendial test but is part of the manufacturer's specification of the drug product. See *Viscosity—Capillary Methods* ⟨911⟩, *Viscosity—Rotational Methods* ⟨912⟩, and *Viscosity—Rolling Ball Method* ⟨913⟩.

### ANTIOXIDANT CONTENT

If antioxidants are present in the drug product, tests of their content should be established, unless oxidative degradation can be detected by another test method such as impurity testing. Acceptance criteria for antioxidant content should be established. They should be based on the levels of antioxidant necessary to maintain the product's stability at all stages throughout its proposed usage and shelf life.

### RESUSPENDABILITY/REDISPERSIBILITY

Consideration must be given to establishing good physical stability of a suspension. If the particles settle and eventually produce a cake at the bottom of the container, they must redisperse readily at the time of use to achieve dosage uniformity. ▲Quantitative demonstration of the active ingredient's resuspendability/redispersibility during product development is also recommended.▲ (USP 1-Dec-2022)

### PARTICLE SIZE AND PARTICLE SIZE DISTRIBUTION

The potential for any changes in the particle size of ophthalmic suspensions and emulsions needs to be evaluated through stability testing (see *Light Diffraction Measurement of Particle Size* ⟨429⟩).

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## DROP SIZE

For ophthalmic drug products dispensed as drops, drop sizes may typically range from 20–70 µL. Drop size can be controlled by weight or by volume, and it is typically evaluated during product development. Drop size can be determined by any appropriate validated procedure.

## ADDED SUBSTANCES

Suitable substances may be added to ophthalmic products to increase stability, unless proscribed in the individual monograph, provided they are harmless in the amounts administered and do not interfere with therapeutic efficacy or with responses to the specified assays and tests. Typically, ▲these substances▲ (USP 1-Dec-2022) should be evaluated during product development. The use of ingredients solely to impart ▲▲ (USP 1-Dec-2022) an odor or flavor is prohibited. ▲The use of ingredients solely to impart a color is prohibited unless coloring is needed to visualize the administration location of the product.▲ (USP 1-Dec-2022)

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# ⟨776⟩ OPTICAL MICROSCOPY

Optical microscopy for particle characterization can generally be applied to particles 1 µm and greater. The lower limit is imposed by the resolving power of the microscope. The upper limit is less definite and is determined by the increased difficulty associated with the characterization of larger particles. Various alternative techniques are available for particle characterization outside the applicable range of optical microscopy. Optical microscopy is particularly useful for characterizing particles that are not spherical. This method may also serve as a base for the calibration of faster and more routine methods that may be developed.

## APPARATUS

Use a microscope that is stable and protected from vibration. The microscope magnification (product of the objective magnification, ocular magnification, and additional magnifying components) must be sufficient to allow adequate characterization of the smallest particles to be classified in the test specimen. The greatest numerical aperture of the objective should be sought for each magnification range. Polarizing filters may be used in conjunction with suitable analyzers and retardation plates. Color filters of relatively narrow spectral transmission should be used with achromatic objectives, are preferable with apochromats, and are required for appropriate color rendition in photomicrography. Condensers corrected at least for spherical aberration should be used in the microscope substage and with the lamp. The numerical aperture of the substage condenser should match that of the objective under the conditions of use and is affected by the actual aperture of the condenser diaphragm and by the presence of immersion oils.

## ADJUSTMENT

The precise alignment of all elements of the optical system and proper focusing are essential. The focusing of the elements should be done in accordance with the recommendations of the microscope manufacturer. Critical axial alignment is recommended.

## ILLUMINATION

A requirement for good illumination is a uniform and adjustable intensity of light over the entire field of view; Kohler illumination is preferred. With colored particles, choose the color of the filters used so as to control the contrast and detail of the image.

## VISUAL CHARACTERIZATION

The magnification and numerical aperture should be sufficiently high to allow adequate resolution of the images of the particles to be characterized. Determine the actual magnification using a calibrated stage micrometer to calibrate an ocular micrometer. Errors can be minimized if the magnification is sufficient that the image of the particle is at least 10 ocular divisions. Each objective must be calibrated separately. To calibrate the ocular scale, the stage micrometer scale and the ocular scale should be aligned. In this way, a precise determination of the distance between ocular stage divisions can be made. Several different magnifications may be necessary to characterize materials having a wide particle size distribution.

## PHOTOGRAPHIC CHARACTERIZATION

If particle size is to be determined by photographic methods, take care to ensure that the object is sharply focused at the plane of the photographic emulsion. Determine the actual magnification by photographing a calibrated stage micrometer, using photographic film of sufficient speed, resolving power, and contrast. Exposure and processing should be identical for photographs of both the test specimen and the determination of magnification. The apparent size of a photographic image is influenced by the exposure, development, and printing processes as well as by the resolving power of the microscope.

## PREPARATION OF THE MOUNT

The mounting medium will vary according to the physical properties of the test specimen. Sufficient but not excessive contrast between the specimen and the mounting medium is required to ensure adequate detail of the specimen edge. The particles should rest in one plane and be adequately dispersed to distinguish individual particles of interest. Furthermore, the particles must be representative of the distribution of sizes in the material and must not be altered during preparation of the mount. Care should be taken to ensure that this important requirement is met. Selection of the mounting medium must include a consideration of the analyte solubility.

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## CRYSTALLINITY CHARACTERIZATION

The crystallinity of a material may be characterized to determine compliance with the crystallinity requirement where stated in the individual monograph of a drug substance. Unless otherwise specified in the individual monograph, mount a few particles of the specimen in mineral oil on a clean glass slide. Examine the mixture using a polarizing microscope: the particles show birefringence (interference colors) and extinction positions when the microscope stage is revolved.

## LIMIT TEST OF PARTICLE SIZE BY MICROSCOPY

Weigh a suitable quantity of the powder to be examined (for example, 10 to 100 mg), and suspend it in 10 mL of a suitable medium in which the powder does not dissolve, adding, if necessary, a wetting agent. A homogeneous suspension of particles can be maintained by suspending the particles in a medium of similar or matching density and by providing adequate agitation. Introduce a portion of the homogeneous suspension into a suitable counting cell, and scan under a microscope an area corresponding to not less than 10 µg of the powder to be examined. Count all the particles having a maximum dimension greater than the prescribed size limit. The size limit and the permitted number of particles exceeding the limit are defined for each substance.

## PARTICLE SIZE CHARACTERIZATION

The measurement of particle size varies in complexity depending on the shape of the particle, and the number of particles characterized must be sufficient to ensure an acceptable level of uncertainty in the measured parameters. Additional information on particle size measurement, sample size, and data analysis is available, for example, in ISO 9276. For spherical particles, size is defined by the diameter. For irregular particles, a variety of definitions of particle size exist. In general, for irregularly shaped particles, characterization of particle size must also include information on the type of diameter measured as well as information on particle shape. Several commonly used measurements of particle size are defined below (see *Figure 1*):

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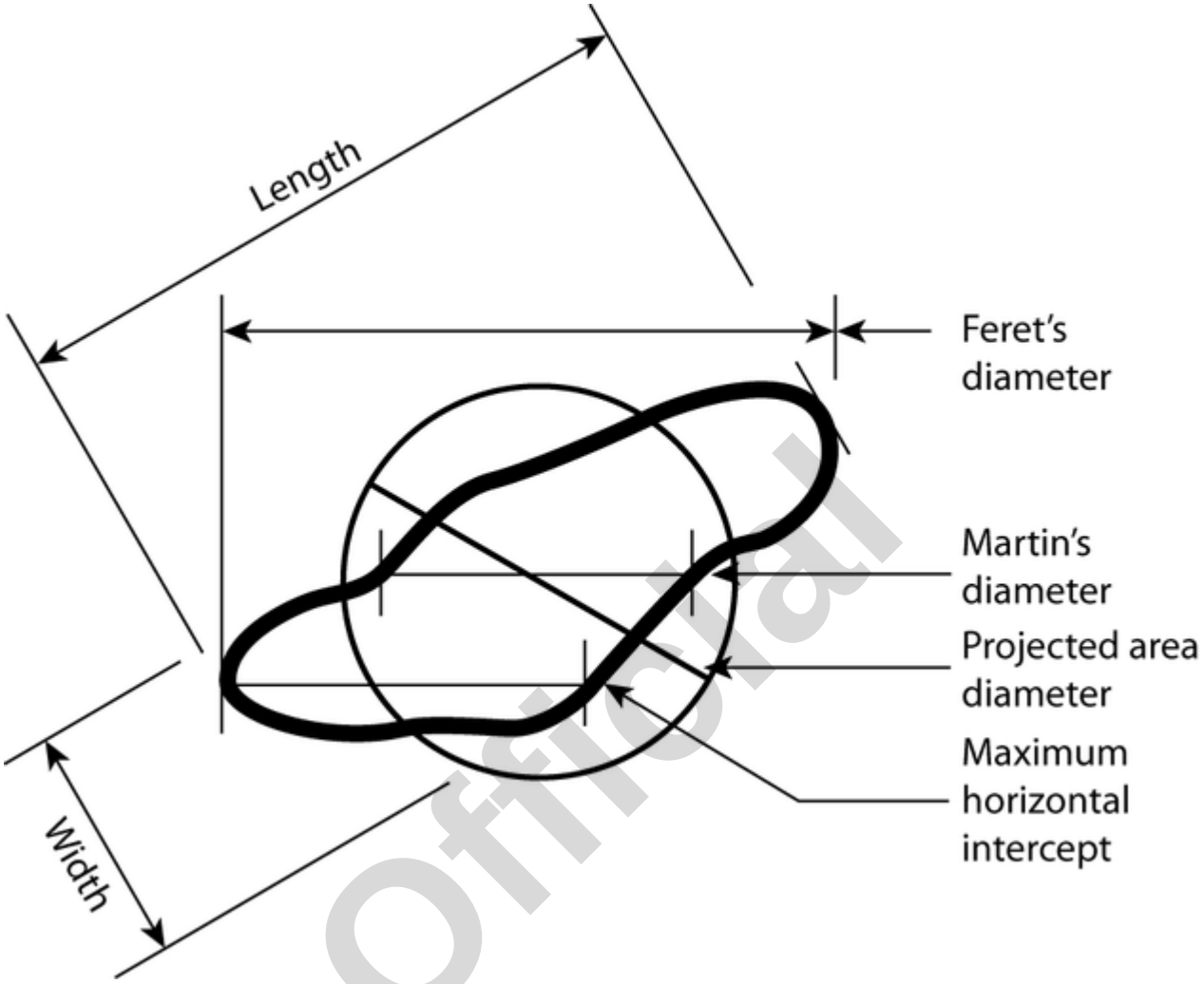


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Figure 1. Commonly used measurements of particle size.

*Feret's Diameter*—The distance between imaginary parallel lines tangent to a randomly oriented particle and perpendicular to the ocular scale.
*Martin's Diameter*—The diameter of the particle at the point that divides a randomly oriented particle into two equal projected areas.
*Projected Area Diameter*—The diameter of a circle that has the same projected area as the particle.
*Length*—The longest dimension from edge to edge of a particle oriented parallel to the ocular scale.
*Width*—The longest dimension of the particle measured at right angles to the length.

## PARTICLE SHAPE CHARACTERIZATION

For irregularly shaped particles, characterization of particle size must also include information on particle shape. The homogeneity of the powder should be checked using appropriate magnification. The following defines some commonly used descriptors of particle shape (see *Figure 2*):

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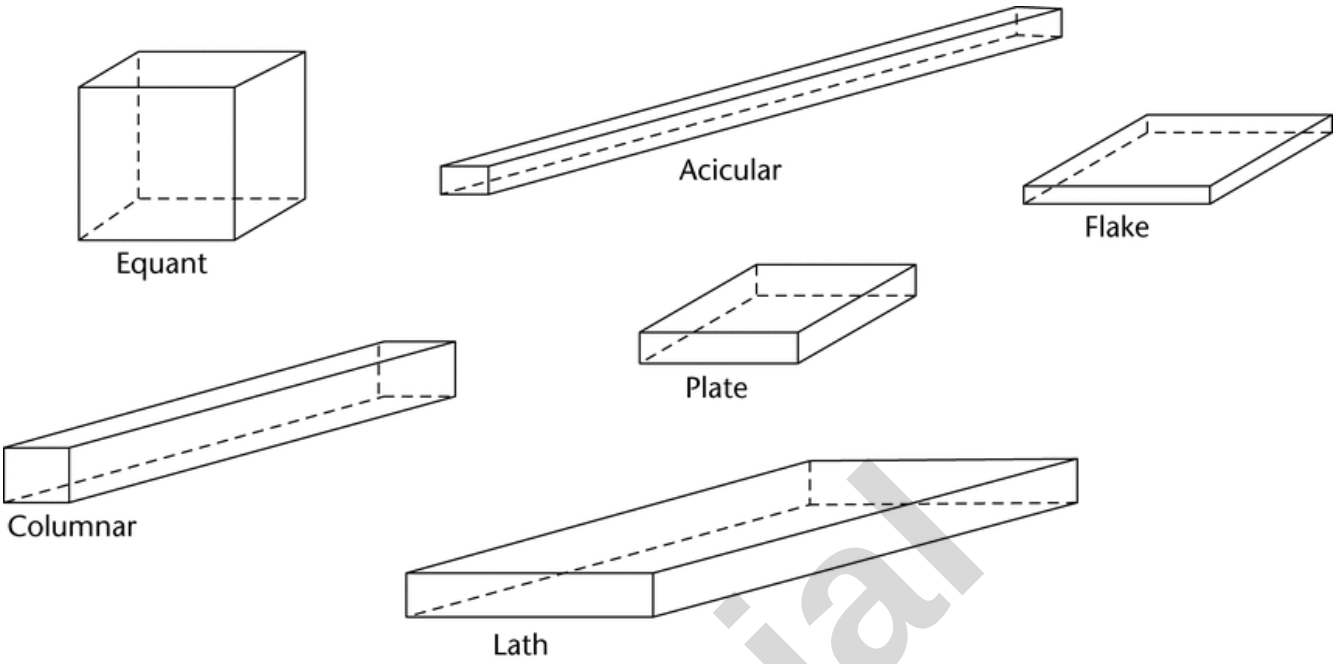


Figure 2. Commonly used descriptions of particle shape.

*Acicular*—Slender, needle-like particle of similar width and thickness.
*Columnar*—Long, thin particle with a width and thickness that are greater than those of an acicular particle.
*Flake*—Thin, flat particle of similar length and width.
*Plate*—Flat particles of similar length and width but with greater thickness than flakes.
*Lath*—Long, thin, and blade-like particle.
*Equant*—Particles of similar length, width, and thickness; both cubical and spherical particles are included.

## GENERAL OBSERVATIONS

A particle is generally considered to be the smallest discrete unit. A particle may be a liquid or semisolid droplet; a single crystal or polycrystalline; amorphous or an agglomerate. Particles may be associated. This degree of association may be described by the following terms:

*Lamellar*—Stacked plates.
*Aggregate*—Mass of adhered particles.
*Agglomerate*—Fused or cemented particles.
*Conglomerate*—Mixture of two or more types of particles.
*Spherulite*—Radial cluster.
*Drusy*—Particle covered with tiny particles.

Particle condition may be described by the following terms:

*Edges*—Angular, rounded, smooth, sharp, fractured.
*Optical*—Color (using proper color-balancing filters), transparent, translucent, opaque.
*Defects*—Occlusions, inclusions.

Surface characteristics may be described as:

*Cracked*—Partial split, break, or fissure.
*Smooth*—Free of irregularities, roughness, or projections.
*Porous*—Having openings or passageways.
*Rough*—Bumpy, uneven, not smooth.
*Pitted*—Small indentations.

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# ⟨781⟩ OPTICAL ROTATION

***Change to read:***

## INTRODUCTION

Many pharmaceutical substances are optically active in the sense that they rotate an incident plane of polarized light so that the transmitted light emerges at a measurable angle to the plane of the incident light. This property is characteristic of some crystals and of many pharmaceutical liquids or solutions of solids. Where the property is possessed by a liquid or by a solute in solution, it is generally the result of the presence of one or more asymmetric centers, usually a carbon atom with four different substituents. The number of optical isomers is $2^n$, where $n$ is the number of asymmetric centers. Polarimetry, the measurement of optical rotation, of a pharmaceutical ▲substance▲ (USP 1-Dec-2022) may be the only convenient means for distinguishing optically active isomers from each other and thus is an important criterion of identity and purity.

Substances that may show optical rotatory properties are "chiral". Those that rotate light in a clockwise direction ▲▲ (USP 1-Dec-2022) are "dextrorotatory" or "(+) optical isomers", and those that rotate light in a counterclockwise direction are called "levorotatory" or "(–) optical isomers". (The symbols *d*- and *l*-, formerly used to indicate dextrorotatory and levorotatory isomers, are no longer sanctioned owing to confusion with D- and L-, which refer to configuration relative to D-glyceraldehyde. The symbols *R* and *S*, or α and β, are also used to indicate configuration, the arrangement of atoms or groups of atoms in space.)

The physicochemical properties of nonsuperimposable chiral substances rotating plane-polarized light in opposite directions to the same extent, "enantiomers", are identical, except for this property and in their reactions with other chiral substances. Enantiomers often exhibit profound differences in pharmacology and toxicology, owing to the fact that biological receptors and enzymes themselves are chiral. Many ▲pharmaceutical substances▲ (USP 1-Dec-2022) from natural sources such as amino acids, proteins, alkaloids, antibiotics, glycosides, and sugars exist as chiral compounds. Synthesis of such compounds from nonchiral materials usually results in equal amounts of the enantiomers, i.e., "racemates". Racemates have a net null optical rotation, and their physical properties may differ from those of the component enantiomers. Use of stereoselective or stereospecific synthetic methods or separation of racemic mixtures can be used to obtain individual optical isomers.

Measurement of optical rotation is performed using a polarimeter. ▲For a neat, undiluted liquid, the specific rotation is a function of the density of the liquid at the temperature of interest:

$$[\alpha]_{\lambda}^{t} = \frac{\alpha}{l\rho}$$

When measuring the specific rotation of an analyte in solution, it may be convenient to prepare the *Sample solution* in a volume containing a mass of the analyte, with a concentration of the analyte per unit of volume (g analyte/mL solution), $c_v$, or per unit of mass (g analyte/g solution), $c_w$. The equations for determining the specific rotation of a *Sample solution* are:

For *Sample solution* (g/mL):

$$[\alpha]_{\lambda}^{t} = \frac{\alpha}{lc_v}$$

For *Sample solution* (g/g):

$$[\alpha]_{\lambda}^{t} = \frac{\alpha}{l\rho c_w}$$

$[\alpha]_{\lambda}^{t}$ = specific rotation at wavelength λ and temperature $t$ (°C). The units of specific rotation are $[(^\circ) \cdot mL \cdot dm^{-1} \cdot g^{-1}]$, which is typically expressed just as degrees (°).
α = observed rotation in degrees (°)
$l$ = path length (dm)
ρ = density of the solution or liquid at temperature $t$ (g/mL)
$c_v$ = concentration of the analyte per unit of volume (g analyte/mL solution)
$c_w$ = concentration of the analyte per unit of mass (g analyte/g solution)

For some pharmaceutical substances, especially liquids such as essential oils, the optical rotation requirement is expressed in terms of the observed angle of optical rotation, α, measured under conditions defined in the monograph.▲ (USP 1-Dec-2022)

Historically, polarimetry was performed using an instrument where the extent of optical rotation is estimated by visual matching of the intensity of split fields. For this reason, the D-line of the sodium lamp at the visible wavelength of 589 nm was most often employed.[1] Specific rotation determined at the D-line ▲at 25° C or 20° C▲ (USP 1-Dec-2022) is expressed by the symbol:

$$[\alpha]_{D}^{25} \text{ or } [\alpha]_{D}^{20}$$

Much of the data available are expressed in this form. Use of lower wavelengths, such as those available with the mercury lamp lines isolated by means of filters of maximum transmittance at approximately 546, 436, 405, and 365 ▲▲ (USP 1-Dec-2022) nm in a

[1] All references of wavelengths are in vacuum. The sodium D-line is 589.44 nm in vacuum and 589.3 nm in air.

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photoelectric polarimeter, has been found to provide advantages in sensitivity with a consequent reduction in the concentration of the test compound. In general, the observed optical rotation at 436 nm is about double, and at 365 nm, about 3 times that at 589 nm.[1] ▲ (USP 1-Dec-2022)

It is now common practice to use other light sources such as ▲light emitting diodes (LEDs)▲ (USP 1-Dec-2022) or xenon or tungsten halogen ▲lamps,▲ (USP 1-Dec-2022) with appropriate filters, instead of traditional light sources because these ▲contemporary light sources▲ (USP 1-Dec-2022) may offer advantages of cost, long life, and broad wavelength emission range.

***Add the following:***

## ▲QUALIFICATION OF POLARIMETERS

Qualification of polarimeters can be divided into three elements: installation qualification (IQ), operational qualification (OQ), and performance qualification (PQ). For a more detailed discussion, see *Analytical Instrument Qualification* ⟨1058⟩.

### Installation Qualification (IQ)

The IQ requirements provide evidence that the hardware and software are properly installed in the desired location and ambient conditions (temperature and humidity).

### Operational Qualification (OQ)

The OQ for the polarimeter should verify the temperature control of the sample, the wavelength accuracy and bandwidth of the light source, the accuracy and repeatability of the optical rotation measurements at the operating wavelengths, and for quantitative applications, the linearity of the instrument response.

#### TEMPERATURE CONTROL

The optical rotation is affected by the temperature of the sample. Therefore, proper temperature control of the sample will be critical for maintaining suitable accuracy and repeatability of the optical rotation measurements. The polarimeter should report the temperature of measurement to at least ±0.1° C or better and should be capable of maintaining constant temperature during the measurement. The OQ of a polarimeter's temperature control is performed by demonstrating that the operating temperature is within ±0.5° C to a temperature recorded on a temperature reading device that is traceable to a National Institute of Standards and Technology (NIST) standard or equivalent (see *General Notices, 6.80.30. Temperature Reading Devices*).

#### WAVELENGTH ACCURACY AND BANDWIDTH

For polarimeters that employ continuum light sources (e.g., LED or tungsten halogen lamps), bandpass filters are typically used to select the wavelength for measurement. It is recommended to record attributes of the wavelength and the bandwidth. If other light sources are employed (e.g., sodium emission lamps), it is recommended to record the wavelength.

#### ACCURACY OF OPTICAL ROTATION

To verify accuracy of the polarimeter, the polarimeter must be capable of reporting the optical rotation to ±0.01° or better and the certified optical rotation reference material must be certified to ±0.01° or better. Verify the accuracy of the polarimeter at the operating wavelength(s) by measuring a certified optical rotation reference material having an associated expanded uncertainty. Typically, quartz plate reference standards with certified optical rotation values are used.[2] Alternatively, other suitable certified reference materials or reference standards may be used.[3] The result should agree with the stated certified value within the limits of the expanded uncertainty of the reference material added to the accuracy specification of the instrument (as provided by the manufacturer).

#### REPEATABILITY

Perform at least five replicate measurements of a reference material at the operating wavelength. Calculate the relative standard deviation (% RSD) of the replicates. The result should be equal to or less than the stated repeatability performance specification of the instrument (as provided by the manufacturer).

#### LINEARITY

Linearity should be verified if the optical rotation measurement is used for quantitation of an enantiomer. For quantitative applications, linearity is verified if optical rotation at three or more values representing the operational range meet the criteria for accuracy.

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[2] Qualification may be performed using a polarization reference standard, which consists of a plate of quartz mounted in a holder perpendicular to the light path. Acceptable periodic calibrations may be made by a National Metrology Institute (NMI) that is signatory to the International Committee for Weights and Measures Mutual Recognition Arrangement (CIPM MRA) or an ISO/IEC 17025 accredited laboratory for calibration where the accreditation body is a signatory to the International Laboratory Accreditation Cooperation Mutual Recognition Arrangement (ILAC MRA).

[3] A certified reference material is available from the Office of Standard Reference Materials, National Institute of Standards and Technology (NIST), Gaithersburg, MD 20899, as a current lot of Standard Reference Material, Sucrose.

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## Performance Qualification (PQ)

PQ consists of periodic qualification of optical rotation accuracy and repeatability as well as temperature control, along with performance checks performed as needed by the instrument user.▲ (USP 1-Dec-2022)

***Change to read:***

## PROCEDURES

▲When polarimetry is used to differentiate an optically active material (e.g., a pure chiral isomer) from an optically inactive material (e.g., a racemic mixture of substances), the polarimeter may provide a small, measurable optical rotation that is close to 0°, which may require interpretation to determine whether the result indicates that a material is classified as dextrorotatory, levorotatory, or optically inactive. The term "dextrorotatory" should be interpreted to mean that the observed angle of optical rotation is a positive, optically active value of optical rotation. The term "levorotatory" should be interpreted to mean that the observed angle of optical rotation is a negative, optically active value of optical rotation. The term "optically inactive" should be interpreted to mean the observed angle of rotation is not significantly different from 0°.

For a neat, undiluted liquid, follow the procedure for *Angular Rotation* in this chapter unless otherwise indicated in the monograph. (In some cases, a drug product is treated as a neat, undiluted liquid.) For *Test solutions* or *Sample solutions* prepared by diluting the analyte in a solvent, follow the procedure for *Specific Rotation* in this chapter unless otherwise indicated in the monograph.

Temperature, which applies to the solution or the liquid under test, should be maintained to ±0.5° C of the stated value.▲ (USP 1-Dec-2022)

## Specific Rotation

The reference ▲"⟨781S⟩"▲ (USP 1-Dec-2022)in a monograph signifies that specific rotation is to be calculated from observed optical rotations in the *Test solution* or *Sample solution* obtained as directed therein. Unless otherwise directed, measurements of optical rotation are made in a 1.0-dm ▲measurement cell▲ (USP 1-Dec-2022) at 589 nm at 25° ▲C ± 0.5° C corrected for the reading of the solvent blank.▲ (USP 1-Dec-2022)[1] Unless otherwise specified, specific rotation is calculated on the dried basis where *Loss on Drying* ▲⟨731⟩▲ (USP 1-Dec-2022) is specified in the monograph or on the anhydrous basis where *Water Determination* ▲⟨921⟩▲ (USP 1-Dec-2022) is specified.

Optical rotation of solutions should be determined within 30 min of preparation. In the case of substances known to undergo racemization or mutarotation, care should be taken to standardize the time between adding the solute to the solvent and introduction of the solution into the polarimeter ▲measurement cell.▲ (USP 1-Dec-2022)

## Angular Rotation

The reference ▲"⟨781A⟩"▲ (USP 1-Dec-2022)in a monograph signifies, unless otherwise directed, that the optical rotation of the neat, ▲undiluted▲ (USP 1-Dec-2022) liquid is measured in a 1.0-dm ▲measurement cell▲ (USP 1-Dec-2022) at 589 nm at 25° ▲C ± 0.5° C▲ (USP 1-Dec-2022) corrected for the reading of the dry, empty ▲measurement cell blank.[1] The result is reported as the observed angle of optical rotation.▲ (USP 1-Dec-2022)

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# ⟨782⟩ VIBRATIONAL CIRCULAR DICHROISM SPECTROSCOPY

1. INTRODUCTION
2. QUALIFICATION OF VCD SPECTROMETERS
    2.1 Installation Qualification
    2.2 Operational Qualification
    2.3 Performance Qualification
3. PROCEDURE
    3.1 Sample
    3.2 Standard
    3.3 Analysis
4. VALIDATION AND VERIFICATION
    4.1 Validation
    4.2 Precision
    4.3 Verification

## 1. INTRODUCTION

Vibrational circular dichroism (VCD) is a form of chiroptical spectroscopy for which chiral molecules have non-zero spectra with intensities that are identical for mirror-image pairs (enantiomers) of chiral molecules but with signs that are opposite with respect to zero intensity. For a chiral molecular substance, two important properties are 1) absolute configuration (AC), which indicates which of two mirror-image forms of the molecule is present; and 2) enantiomeric excess (EE, also called enantiomeric purity), the fractional excess of one enantiomer of a chiral molecule over its mirror-image enantiomer. The principal chapter in *USP–NF* that addresses molecular chirality is *Optical Rotation* ⟨781⟩. Optical rotation (OR) and electronic circular dichroism (ECD) are the traditional forms of chiroptical spectroscopy, whereas VCD is a newer form of chiroptical spectroscopy that contains more molecular structure information and recently has been adopted more broadly for use in the pharmaceutical industry.

In this chapter, the required methodology for the use of VCD is described in terms of instrument qualification; sign and intensity calibration; procedures, validation, and verification of VCD measurement; calculation; and statistical analysis as needed for the determination of AC and EE of chiral pharmaceutical products. VCD is the extension of ECD from the region of electronic transitions in the UV and visible regions of the spectrum to vibrational transitions in the infrared (IR) region.

VCD is defined as:

$$\Delta A = A_L - A_R$$

$\Delta A$ = difference in the IR absorbance
$A_L$ = sample absorbance for left circularly polarized (LCP) radiation
$A_R$ = sample absorbance for right circularly polarized (RCP) radiation

Unpolarized IR intensity is defined as the average of LCP and RCP intensities:

$$A = (A_L + A_R)/2$$

$A$ = IR absorbance
$A_L$ = sample absorbance for left circularly polarized (LCP) radiation
$A_R$ = sample absorbance for right circularly polarized (RCP) radiation

The IR intensities of enantiomers are identical, whereas enantiomers have equal and opposite-signed VCD intensities.

For discussion of the theory and principles of VCD measurements, as well as a more detailed explanation of the application of VCD to problems of pharmaceutical interest, see *Vibrational Circular Dichroism Spectroscopy—Theory and Practice* ⟨1782⟩.

## 2. QUALIFICATION OF VCD SPECTROMETERS

This section on qualification is divided into three subsections: *2.1 Installation Qualification* (IQ), *2.2 Operational Qualification* (OQ), and *2.3 Performance Qualification* (PQ) of a Fourier transform (FT)-VCD instrument. Although a VCD instrument can be based on a scanning dispersive spectrometer, no such VCD spectrometers are commercially available, and hence they will not be considered. Additional details on the qualification of instruments can be found in *Analytical Instrument Qualification* ⟨1058⟩.

### 2.1 Installation Qualification

The IQ requirements provide evidence that the hardware and software are properly installed in the desired location. An FT-VCD instrument is a sensitive, circular-polarization-difference, FT-infrared (FT-IR) spectrometer. Its installation requires a stable bench-top surface, access to dry air or nitrogen for purging of water vapor, and standard electrical outlets. Evidence for IQ involves ensuring that the IR interferometer is scanning, that its photoelastic modulator(s) [PEM(s)] is operating, and that

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the electronics hardware is interacting with both the FT-IR main bench and the PEM. The final evidence for IQ is that the software display for acquisition of a VCD spectrum is ready for operator use.

## 2.2 Operational Qualification

In OQ, an instrument's performance is characterized using a qualification standard of known spectral properties to verify that the system operates within target specifications. The purpose of OQ is to demonstrate that instrument performance is suitable. OQ is a check of the key operational parameters and should be performed after installation and after repairs and/or maintenance.

The OQ tests described in the following sections are typical examples only. Other tests and samples can be used to establish specifications for OQ. Instrument vendors often have samples and test parameters available as part of the IQ/OQ package. The acceptance criteria provided in this section are applicable for general use, whereas specifications for particular instruments and applications may vary, depending on analytical methods used and desired accuracy of the final result.

### CHARACTERIZING INSTRUMENT PERFORMANCE

FT-VCD spectrometers measure simultaneously the VCD spectrum and the parent IR spectrum. The VCD spectrum is obtained from a VCD interferogram arising from the combined high-frequency PEM polarization modulation and the Fourier frequencies, whereas the parent IR spectrum arises from the conventional FT-IR interferogram based only on the instrument's Fourier frequencies. Part of the instrument performance is from the underlying FT-IR operation, and the remaining part of the instrument performance is from those components that are uniquely associated with the FT-VCD operation. The combined FT-IR and FT-VCD criteria are wavenumber accuracy and absorbance linearity, whereas those uniquely associated with the VCD operation are VCD sign and intensity calibration, empty-beam baseline accuracy, sample-associated baseline deviations and artifact levels, baseline stability and spectral reproducibility, and signal-to-noise ratio. As described in ⟨1782⟩, two standard samples are commonly used for specifying VCD performance. For sign and intensity calibration, neat α-pinene is used as a validation standard, whereas for baseline characteristics, a solution of camphor in carbon tetrachloride ($CCl_4$) is used as the qualification standard. VCD spectra presented in ⟨1782⟩ serve as examples of performance characteristics of typical spectra for VCD sign and intensity calibration, baseline stability, and signal-to-noise ratio.

### WAVENUMBER ACCURACY

The wavenumber accuracy of FT-VCD instruments is based on the corresponding wavenumber accuracy of the parent FT-IR operation. This accuracy is determined by fringe counting of the visible laser co-operating with the FT-IR interferometer. The frequency calibration is accurate to within the spectral resolution of the instrument and requires no calibration standard. The accuracy of wavenumber frequency should be checked by measuring the IR spectrum of a 35-µm-thick polystyrene film, as described in the *USP–NF* chapter on mid-IR spectroscopy, *Mid-Infrared Spectroscopy* ⟨854⟩, with additional information in *Mid-Infrared Spectroscopy—Theory and Practice* ⟨1854⟩. Because the FT-VCD and FT-IR spectra are simultaneously measured by the action of the same interferometer, the wavenumber accuracy of the VCD spectrum is the same as that of the FT-IR operation of the instrument.

### LINEARITY AND STABILITY

Commercial FT-IR instruments have excellent linearity, provided that the infrared detector remains unsaturated. FT-IR spectra are particularly prone to detector saturation, because the intensity of the entire spectrum is present simultaneously at the detector. Detector saturation can be avoided by the use of a long-wavelength pass filter or appropriate adjustment of the gain of the preamplifier to ensure that the maximum of the FT-IR interferogram lies within the range of preamplifier voltage capability. The hallmark of the onset of nonlinearity is distortion of the FT-IR interferogram that can lead to negative FT-IR absorbance values at the endpoint frequency of the spectral coverage, which is clearly incorrect for an absorbing sample. Aperture reduction, insertion of screens, or neutral density filters can be used to avoid detector saturation.

These same considerations apply to the linearity of FT-VCD measurements. The linearity of VCD is more sensitive to the onset of detector saturation than is the parent IR spectrum. VCD spectra are unstable and distorted when the parent FT-IR spectrum has any saturation, and in some cases linearity in the VCD is not restored until the FT-IR intensity is well below saturation. Saturation can be tested by varying the throughput of the instrument by changing the sample path length and making sure that all of the points across the spectrum are changed by the same factor, i.e., there are no relative shape changes in the spectrum. These relative shape changes should be less than the apparent noise level or, at most, 2% (1.00 ± 0.02), depending on the accuracy needed for the VCD measurements.

### PHASE CORRECTION, SIGN, AND INTENSITY CALIBRATION

The raw VCD spectrum is measured as the ratio of the FT of the VCD interferogram to that of the FT of the IR interferogram. Both of these interferograms must be phase-corrected before performing the FT. The phase correction for the IR is straightforward, but the phase correction of the VCD interferogram requires transfer of a phase file from a VCD interferogram representing only positive intensities. Most commercial FT-VCD instruments provide such phase files for the measurement of VCD.

Next, the measured raw VCD spectrum must be calibrated such that VCD spectra with the correct signs and intensities are produced. The signs can be determined by measuring the qualification standard of (–)-camphor or (+)-camphor in carbon tetrachloride solution and comparing it to a previously published standard VCD spectrum of this molecule, such as that provided in ⟨1782⟩. Either all of the signs agree, in which case the sign is correctly set, or all of the signs are opposite, in which

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case the phase of the VCD synchronous detection (lock-in amplifier or numerical processing) must be changed by 180° to reverse the signs of all VCD bands, positive-to-negative and negative-to-positive.

Calibration of VCD intensities is performed by dividing the raw VCD by a calibration curve representing the magnitude of unit VCD intensity for the VCD spectrometer. The shape of the calibration curve follows a first-order Bessel function, which in turn depends on the settings of the PEM generating LCP and RCP radiation. The maximum of the calibration curve corresponds to the maximum wavenumber value set on the PEM controller. The calibration curve for a given PEM setting can be obtained from the crossing points of curves generated by placing a multiple-wave plate followed by a polarizer in place of the sample and measuring the VCD spectrum in the usual manner. Commercial VCD instruments provide such curves, and the correct calibration curve must be used for each wavenumber setting of the PEM. For an example of a calibration curve for the PEM setting at 1200 $cm^{-1}$, see ⟨1782⟩.

After calibration of the intensities of the VCD instrument, a measurement of a 0.9 M solution of (+)-*R*-camphor in carbon tetrachloride measured in a 100-µm path-length cell should be approximately $2 \times 10^{-4}$. After baseline subtraction, described below and in ⟨1782⟩, this difference should be a positive peak $\Delta A$ value of $+1 \times 10^{-4}$ absorbance units at 1240 $cm^{-1}$ and a negative $\Delta A$ peak value of $-1 \times 10^{-4}$ at 1040 $cm^{-1}$.

### EMPTY BEAM BASELINE CHARACTERISTICS

To avoid significant levels of baseline deviations from the absorbance features of a sample, the empty beam baseline for a VCD instrument should be as close as possible to the true zero of the measurement. A minimum standard for FT-VCD spectrometers is to have empty beam baselines sufficiently close to zero so that deviations from true zero are <25% of the span of maximum positive-to-negative absorbance intensity of a 0.9 M solution of (+)-*R*-camphor in carbon tetrachloride, measured in a 100-µm path-length cell. This magnitude, noted above, is approximately $2 \times 10^{-4}$ as determined from a positive peak value of $+1 \times 10^{-4}$ at 1240 $cm^{-1}$ and a negative peak value of $-1 \times 10^{-4}$ at 1040 $cm^{-1}$. The value of 25% of this intensity range is approximately $5 \times 10^{-5}$. VCD empty beam baseline deviations (maximum to minimum), over the mid-IR range from 900 to 1800 $cm^{-1}$, should not exceed $\pm 5 \times 10^{-4}$ and should not deviate more than $+5 \times 10^{-4}$ or $-5 \times 10^{-4}$ from the zero of VCD intensity over this wavenumber range.

### SAMPLE BASELINES AND ABSORPTION ARTIFACTS

An important test for a VCD measurement is the VCD spectrum of a racemic sample relative to that of the baseline of the empty instrument or the VCD spectrum of a nonchiral solvent, such as carbon tetrachloride. Because of possible baseline changes due to the sample cell, it is preferable to compare the VCD of the solvent with that of a solution of the racemic sample of the chiral molecule in the same solvent. Both should have zero VCD, but the racemic solution may have baseline artifacts (offsets) at the location of the absorption bands. Absorption bands change the index of refraction of the sample and can cause baseline deviations that are particular to the sample molecule. If such absorption artifacts are present, the only way to recover the artifact-free VCD spectrum is by subtraction of the racemic mixture (equal quantities of both enantiomers) from that of the measured VCD spectrum. Alternatively, subtraction of the VCD spectrum of the opposite enantiomer is divided by 2, because the VCD intensity doubles if the VCD spectrum of one enantiomer is subtracted from that of the other. The qualification standard used for measuring the VCD baseline with an absorbing sample present is racemic camphor in carbon tetrachloride solution versus the VCD spectrum of neat carbon tetrachloride in the range 900–1800 $cm^{-1}$, where there are only absorption bands from camphor and none for carbon tetrachloride. Both samples should have zero VCD and a baseline that is featureless with respect to the camphor absorption bands.

### SIGNAL-TO-NOISE RATIO

The signal-to-noise ratio of a VCD measurement can be determined from the noise curve generated by subtracting one-half of the FT scans of a measurement from the other half, thus cancelling the signal and exposing the noise level. The noise level varies across the spectrum and increases when the light level reaching the detector decreases, either due to the overall throughput of the instrument in a particular spectral region or directly within the absorption bands of the sample. The level of noise in the noise curve can be reduced by increasing the measurement time of the spectrum. The noise level is inversely proportional to the square root of the measurement time. This is illustrated with a particular example in *Figures 9* and *10* of ⟨1782⟩.

Smoothing a VCD spectrum without increasing the measurement time will reduce the size of the peak-to-peak noise excursions but will not reduce the overall accuracy of a VCD measurement, because the VCD baseline, and hence the VCD intensity relative to the zero baseline, will undergo the same level of variations, although the VCD spectrum appears to have less noise after smoothing. To know the true level of the noise and hence the uncertainty level of a VCD spectrum, all smoothing functions, if any, should be turned off. Noise can legitimately be reduced by lowering the spectral resolution. In evaluating the noise level of the VCD measurement, the resolution and interferogram apodization must be specified.

## 2.3 Performance Qualification

PQ determines if the instrument is capable of meeting the user's requirements for all the parameters that may affect the quality of the measurement. Depending on typical use, the specifications for PQ may be different from the manufacturer's OQ specifications; however, for general use across a range of typical chiral molecules and sampling conditions, the specifications for OQ will be the same as those needed for PQ. For validated methods, specific PQ tests, also known as system suitability tests, can be used in lieu of PQ requirements.

Specific procedures, acceptance criteria, and time intervals for characterizing performance depend on the instrument and intended application. Demonstrating stable instrument performance over extended periods of time provides some assurance that reliable measurements can be taken from test sample spectra.

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Particular long-term stability tests that can be carried out for PQ evaluation beyond those carried out for IQ or OQ are as follows. It is recommended that a 60-min measurement of a 0.9 M solution of (+)-*R*-camphor in carbon tetrachloride, measured in a 100-µm path-length cell, be performed between different applications of the VCD spectrometer to ensure that the signs, intensities, and signal-to-noise ratio of the VCD spectrum established as a qualification test during OQ are maintained. This is particularly important if there has been an interruption in power or a long hiatus between measurements.

If averaging over long periods of time, such as overnight, it is important that the stability of both the VCD baseline and the sample VCD spectrum are stable over time and that the signal-to-noise ratio improves as expected with the square-root of measurement time or number of interferometer scans, as illustrated by the example in ⟨1782⟩. In general, stability should be maintained to within the noise level of the VCD measurement. Because VCD intensities are generally three to four orders of magnitude smaller than the parent IR intensity, the instrument stability for the FT-IR performance of the instrument is typically larger over time relative to the noise level of the VCD spectrum.

## 3. PROCEDURE

The measurement of the VCD spectrum of a sample follows the same general rules as the measurement of an IR spectrum as for mid-IR spectroscopy described in ⟨854⟩.

### 3.1 Sample

A VCD spectrum can be measured in all types of standard IR transmission cells, either for neat liquids or solutions. For solids, such as in potassium bromide (KBr) pellets, mulls, or thin films, it is recommended that additional steps be taken to ensure that artifacts arising from sample linear birefringence are not present as described in ⟨1782⟩ for advanced instrumentation methods, such as dual PEM modulation or rotating sample cells.

Because of the importance of noise control in a VCD measurement, the absorbance of a sample should not exceed $A = 1.0$ in the region of measurement interest. For absorbance >1.0, insufficient radiation is present at the detector, and noise dominates the VCD spectrum. The optimum level of absorbance relative to the sample reference (empty beam or solvent) is approximately 0.4. One should adjust the path length and/or concentration of the sample such that the average absorbance is approximately 0.4–0.5 over the region of interest, with limits of 0.1–1.0. For samples with a wide range of absorbance, more than one measurement can be carried out with differing average absorbance values to ensure that a VCD spectrum of adequate quality is obtained over the entire range of interest.

To perform a solution-state VCD measurement, first find a solvent that dissolves an adequate amount of solute to achieve a desired absorbance level. Solvents without CH bonds are the most desirable for this purpose; these include carbon tetrachloride, deuterated chloroform ($CDCl_3$), or deuterated dimethyl sulfoxide (DMSO-$d_6$). The IR cell should have windows that are transparent in the mid-IR region, with generally low levels of linear birefringence. Commonly used materials are barium fluoride ($BaF_2$), which has good transmission to 800 $cm^{-1}$, and calcium fluoride ($CaF_2$), which is used if coverage to only 1100–1200 $cm^{-1}$ is needed, such as for aqueous solutions.

### 3.2 Standard

To ensure that the analyst can use an IR cell for a transmission measurement of a solution, it is recommended that a standard OQ measurement of a 0.9 M solution of (+)-*R*-camphor in carbon tetrachloride using a 100-µm barium fluoride cell be performed. For different choices of concentration or path length, one can use the linear dependence of these quantities on the measured $\Delta A$ to ensure that an accurate measurement with the correct signs and intensities has been achieved.

### 3.3 Analysis

VCD spectra, once measured, can be analyzed in a variety of ways, depending on the intended application. The principal application of VCD in the pharmaceutical industry is the determination of absolute configuration (AC) in conjunction with a VCD calculation using a quantum chemistry computation software package. A second application is the determination of EE of a sample, either for the purpose of determining the EE of a sample or for monitoring the EE of one or more species during the course of the chemical reaction involving chiral species. Achievement of the highest level of accuracy in the determination of EE requires the use of a chemometric software package that uses the entire range of the VCD spectrum, as opposed to a single band or wavenumber frequency. Other applications of VCD are its use with a standard database of VCD and IR spectra to simultaneously ensure the correct identity, AC, and EE of a chiral substance, for example as a chiral raw material identification. VCD can also be used to specify the AC and EE of a drug product in the presence of either achiral or chiral excipients, such as carbohydrates. These applications are described in more detail in ⟨1782⟩.

## 4. VALIDATION AND VERIFICATION

### 4.1 Validation

The objective of VCD procedure validation, as with the validation of any analytical process, is to demonstrate that the measurement is suitable for its intended purpose. The application of VCD spectroscopy is somewhat different from that of conventional vibrational spectroscopic techniques because it is not a primary technique. Rather, it is the polarization difference spectroscopy of the parent IR spectroscopy that has been used by itself for decades as a primary identification and quantitation technique as described for mid-IR spectroscopy in ⟨854⟩. The differential VCD spectrum supplements the parent IR spectrum

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with a new set of intensities at each position of an IR absorption band. The new set of intensities then reveals stereospecific properties of the sample molecules that must be chiral to have a non-zero VCD spectrum. There are two primary properties of a sample of a chiral molecule: one is the AC of the dominant chiral species (dominant enantiomer), and the second property is the EE of the dominant enantiomer over its lesser (mirror-image) enantiomer. In addition, the VCD spectrum carries additional information about the solution-state conformation, or conformational distribution, of the molecule that may be available in only lesser detail, if any, using the parent IR spectrum alone. Applications that require validation for the use of VCD spectroscopy also exist for the combined use of the measurement of the VCD of a substance for which there exists a reference spectrum with known AC and EE. Any subsequent measurement of the VCD spectrum, together with the simultaneous measurement of its parent IR spectrum, confirms in one measurement the molecular identity of the sample as well as the AC and EE. Such applications are useful, for example, for chiral raw-material identification of AC and EE, for which there is currently no established methodology for a single measurement. VCD can also confirm, in a single measurement, the AC and EE of a final drug product, including the chirality (if any) of the excipients.

Before validation of VCD for AC determination, an overall instrument validation should be carried out by the measurement of the VCD spectrum of neat (–)-*S*-α-pinene or (+)-*R*-α-pinene using a 75-µm cell. The calibrated, baseline-corrected VCD spectrum should yield a positive peak maximum of $\Delta A$ equal to approximately $+5 \times 10^{-4}$ absorbance units at 1220 $cm^{-1}$ and a negative peak value of approximately $-4 \times 10^{-4}$ at 1130 $cm^{-1}$. The noise level should be comparable to that established for the instrument during the OQ phase of instrumental qualification described above. Because the VCD spectrum of α-pinene is relatively large, sufficient signal-to-noise ratio is available with a 20-min collection to verify that the instrument is operating with correct VCD intensities, signs, and noise levels for the applications of VCD described below.

Validation for AC determination by VCD can be achieved by the successful comparison of the measured VCD spectrum of the validation standard, neat (–)-*S*-α-pinene, to its quantum-chemistry-calculated VCD spectrum. Success is achieved by measuring the baseline-corrected VCD spectrum with sufficient signal-to-noise ratio that the signs, frequencies, and relative intensities of the major VCD bands can be compared clearly to the corresponding VCD signs, frequencies, and relative intensities of the calculated VCD spectrum. If the signs, frequencies, and relative intensities agree, then the AC of the measured sample of (–)-α-pinene is the same as that for the model of *S*-α-pinene that has been constructed for the calculation of this standard molecule. If the frequencies and relative VCD intensities are opposite after instrument sign calibration, then the AC of the sample is opposite (the mirror image) of the enantiomer used for the calculation. A successful comparison requires pretreatment of the VCD data for baseline subtraction and intensity calibration. It also requires a computational procedure that is known to be accurate in calculating VCD spectra. Finally, a program should be used to impartially assess the degree of agreement between measured and calculated VCD spectra such that one relies on more than an analyst's visual assessment of the agreement, or lack of agreement, between a measured spectrum and a calculated spectrum. An example of this procedure can be found in ⟨1782⟩ as well as in the references cited in ⟨1782⟩.

Validation of VCD spectroscopy for the determination of EE is achieved by plotting the EE predicted by VCD versus the EE for a sample of neat (–)-*S*-α-pinene for a series of samples with differing values of %EE over the range from 100% EE to 0% EE. The EE predicted by VCD involves measurement of the VCD spectra at a chosen spectral resolution and time of collection to achieve a desired level of signal-to-noise ratio, followed by pretreatment of the measured VCD spectra for baseline subtraction. The prediction of EE could be based on a size of a single VCD band for the samples with differing VCD, normalized for constant IR intensity; but more generally and with better accuracy, the entire range of VCD bands across the spectrum is used for each spectrum by means of a chemometrics software package. Validation is accompanied by a measure of the %EE accuracy through a cross-correlation analysis, yielding a root mean square (RMS). An example of such a determination of %EE by VCD for neat (–)-*S*-α-pinene is provided, along with literature references, in ⟨1782⟩.

More generally, validation is required when a method is intended for use as an alternative to the official procedure for testing an official article. The objective is to demonstrate that the measurement of VCD is suitable for its intended purpose; for examples of various types of measurements, see *Validation of Compendial Procedures* ⟨1225⟩. For %EE determination, creation of a linear plot of actual versus VCD-predicted values of %EE establishes that the magnitude of IR-normalized VCD spectra, as evaluated with chemometrics, exhibits sufficient linearity, range, accuracy, specificity, precision, detection limit, quantitation limit, and robustness with respect to the detection of %EE.

Chapter ⟨1225⟩ provides definitions and general guidance on analytical procedures validation without indicating specific validation criteria for each characteristic. The intention of the following sections is to provide the user with specific validation criteria that represent the minimum expectations for VCD technology. For each particular application, tighter criteria may be needed to demonstrate suitability for the intended use.

#### ACCURACY

Accuracy can be determined by the measurement of a VCD spectrum of the established validation standard of neat (–)-α-pinene or (+)-α-pinene, as presented, for example, in ⟨1782⟩, and verifying that the measured signs and intensities of the VCD spectrum agree with the standard VCD spectrum to within the noise level of the measurement. In particular, the VCD spectrum should yield a positive peak maximum of $\Delta A$ equal to approximately $+5 \times 10^{-4}$ absorbance units at 1220 $cm^{-1}$ and a negative peak value of approximately $-4 \times 10^{-4}$ at 1130 $cm^{-1}$. This validation ensures that the VCD spectrometer is accurately calibrated and has the correct signs for the VCD bands.

### 4.2 Precision

#### REPEATABILITY

**Validation criteria:** Measurement of VCD spectra of the validation standard sample, neat (+)-α-pinene or (–)-α-pinene, in 20-min blocks with variations in the VCD spectra at or less than the noise level of each 20-min block that is approximately $\Delta A$ equal to or less than $1 \times 10^{-5}$

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INTERMEDIATE PRECISION

The effect of random events on the analytical precision of the validation criteria of VCD measurement should be established. Typical variables include performing the analysis on different days, using different instrumentation, or having the method performed by two or more analysts. As a minimum, any combination of at least two of these factors totaling six experiments will provide an estimation of intermediate precision. (For example, this could be two analysts on each of 3 days, or two analysts on two sets of equipment on 2 days for each analyst–equipment combination.)
**Validation criteria:** The *Validation criteria* are the same as those for *Repeatability*.

SPECIFICITY

Specificity is not a required metric for the determination of AC. For the measurement of EE for more than one chiral species in a single set of measurements, the analytical procedure, including chemometric analysis, must be able to unequivocally assess the EE of chiral species in the presence of components that may be expected to be present, i.e., solvent and other nonchiral species.
**Validation criteria:** The *Validation criteria* are demonstrated by meeting the *Accuracy* requirement.

QUANTITATION LIMIT

For the determination of AC, there is no quantitation limit (QL). The QL for EE can be determined by carrying out a determination of quantitation of %EE for the VCD validation standard of neat α-pinene for a series of EE values, performing a chemometric analysis of VCD-predicted EE values and performing a determination of RMS error, as illustrated, for example, in ⟨1782⟩.
**Validation criteria:** The analytical procedure should be capable of determining the analyte precisely and accurately at a level equivalent to approximately 1% EE for neat α-pinene.

LINEARITY

A linear relationship between the analyte concentration and response should be demonstrated by preparing NLT 5 standard solutions at concentrations encompassing the anticipated concentration of the test solution. The standard curve should then be evaluated using appropriate statistical methods, such as a least-squares regression, for the area of peak height of a selected VCD band, or for the entire spectrum, using chemometric analysis such as partial least squares. For experiments that do not yield a linear relationship between analyte concentration and response, appropriate statistical methods must be applied to describe the analytical response.
**Validation criteria:** The *Validation criteria* find the correlation coefficient above 0.95 for a linear plot of actual EE versus VCD-predicted EE.

ROBUSTNESS

The reliability of an analytical measurement should be demonstrated by deliberate changes to experimental parameters, such as changes in spectral resolution, concentration, or path length for measurement of VCD.

## 4.3 Verification

U.S. Current Good Manufacturing Practices regulations [21 CFR 211.194(a)(2)] indicate that users of the analytical procedures, as described in *USP–NF*, are not required to validate these procedures if provided in a monograph. Instead, they must simply verify their suitability under actual conditions of use.

The objective of procedure verification is to demonstrate that the procedure, as prescribed in a specific monograph, can be executed by the analysts with suitable accuracy, specificity, and precision using the instruments, and sample solutions of chiral molecules available. If the verification of the compendial procedure by following the monograph is not successful, the procedure may not be suitable for use with the article under test (for additional information, see *Verification of Compendial Procedures* ⟨1226⟩). It may be necessary to develop and validate an alternative procedure as allowed in *General Notices, 6.30 Alternative and Harmonized Methods and Procedures*.

Verification of compendial methods should, at a minimum, include the execution of the validation parameters for specificity, accuracy, precision, and limit of quantitation, when appropriate, as indicated in *4.1 Validation*.

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# ⟨785⟩ OSMOLALITY AND OSMOLARITY

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## INTRODUCTION

Osmotic pressure plays a critical role in all biological processes that involve diffusion of solutes or transfer of fluids through membranes.

▲Osmosis occurs when the osmotic pressure drives the solvent, but not solute, to cross a semipermeable membrane from regions of lower to higher solute concentrations until equilibrium is reached. Osmotic pressure is a colligative property that depends on the number, but not the identity, of moles of solutes present in solution. Quantitative measurement of the number of moles of solutes in solution is necessary for predicting and controlling the potential damage to biological tissues due to fluid transfer across biological tissue membranes.

The terms osmolality, osmolarity, and tonicity are often used to describe the number of solutes in the solution, however, it is incorrect to use these terms interchangeably. The measurement of the number of solutes in the solution, using colligative properties, allows the direct determination of the osmolality of the solution with units of moles of solutes per kilogram of solvent. This result can be converted into a value for osmolarity with units of moles of solutes per liter of solvent, typically water. Tonicity is a medical term that relates to the osmotic pressure difference between the internal and external sides of a cell membrane.▲ (USP 1-Dec-2020)

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## ▲THEORETICAL BACKGROUND

The osmotic pressure of a solution depends on the number of solutes in a solution, and is therefore referred to as a colligative property. For an ideal solution with water as the solvent, the osmotic pressure, $\Pi$, is a function of the water activity, $a_W$:

$$\Pi = -\frac{RT}{V_m}\ln\ a_w$$
$$a_w = \gamma_w x_w$$

where $V_m$ is the molar volume of water at temperature $T$, $V_m$ = 18.018 $cm^3 mol^{-1}$ at 273.15 K (*1*); $R$ is the gas constant, $R$ = 8.3144598 J $mol^{-1}$ $K^{-1}$ (*2*); $T$ is the temperature in Kelvin; $\gamma_W$ is the activity coefficient of water; and $x_W$ is the mole fraction of water, $x_W = (1 - x_S)$, where $x_S$ is the mole fraction of solute. At low solute concentrations ($-\ln a_W \approx x_S \approx n_S/n_W$), the equation for osmotic pressure, above, can be simplified to the van't Hoff equation for osmotic pressure which states:

$$\Pi = RT\left(\frac{n_S}{V}\right) = RT\left(\frac{n_S}{n_W V_m}\right) = \frac{RTM_W}{V_m}\left(\frac{n_S}{n_W M_W}\right)$$

where

$$\left(\frac{n_S}{n_W M_W}\right) = v \times m = \xi_{m,\ ideal}$$

which yields

$$\Pi = \frac{RTM_W}{V_m}\xi_{m,\ ideal}$$

where $n_S$ is the moles of solute molecules; $n_W$ is the moles of water molecules; $V$ is the volume of water at temperature, $T$; $M_W$ is the molar mass of water, $M_W$ = 18.015268 g $mol^{-1}$ (*3*); $\xi_{m,ideal}$ is the osmolality of the solution in moles per kilogram of water; $v$ is the stoichiometric coefficient for the solute; and $m$ is the molal concentration of the solute in moles of solute per kilogram of water. For an ideal, non-dissociating solute, $v$ = 1 and the osmolality, $\xi_m$, is equal to the molal concentration of solute. For a dissociating solute, $v$ is equal to the stoichiometric number of moles into which one mole of solute will dissociate. For non-ideal solutes, an osmolality coefficient, $\Phi_m$, is introduced to correct for the difference between the predicted behavior of ideal solutions and the observed behavior of real solutions. The osmolality coefficient is defined as:

$$\Phi_m = \frac{-\ln a_W}{vmM_W} = \frac{\xi_{m,real}}{\xi_{m,ideal}}$$

The osmolality of a real solution is, therefore:

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$$\xi_{m,\,real} = \nu m \Phi_m$$

When multiple solutes are present, the osmolality may be approximated by summing the contributions from all individual solutes, *i*, as:

$$\xi_{m,real} = \sum_i \nu_i m_i \Phi_{m,i}$$

where $m_i$ is the molal concentration of the $i^{th}$ solute; $\nu_i$ is the number of ions into which the solute dissociates; and $\Phi_{m,i}$ is the osmolality coefficient for the $i^{th}$ solute at the molality, *m*. This simplified additive approach assumes that the osmolality coefficient for each solute in the mixture is not different from the osmolality coefficient determined for the individual solute (*4*).

In addition to osmotic pressure, other colligative properties can be used to measure the osmolality of solutions including dew-point depression, boiling-point elevation, and freezing-point depression. The most commonly used method for analysis of the osmolality of solutions is freezing-point depression. The freezing-point depression, $\Delta T_f$, of an aqueous solution relative to pure water is proportional to the change in the water activity, $a_W$:

$$\Delta T_f = \frac{RT_f^2}{\Delta H_{fus}} \Delta \ln a_W$$

where $\Delta H_{fus}$ is the enthalpy of fusion of water, and $T_f$ is the freezing point of the solution in Kelvin. At low solute concentrations, this can be simplified to express freezing-point depression in terms of osmolality as:

$$\Delta T_f = \frac{M_W RT_f^2}{\Delta H_{fus}} \xi_m = K_f \xi_m$$

where

$$K_f = \frac{M_W RT_f^2}{\Delta H_{fus}}$$

The value of $\Delta H_{fus}$ is temperature dependent (*3*) and so is $K_f$; however, $K_f$ is typically treated as a constant and set to the value for pure water at the freezing point. For water, the value of $\Delta H_{fus}$ at 273.15 K is 6006.78 J $mol^{-1}$ (*3*), which gives a value for $K_f$ of 1.86056 K kg $mol^{-1}$.

Vapor pressure osmometers measure the relative humidity of the air above the sample that is in equilibrium with the sample. The dew point is the temperature below which the water vapor in the air is above saturation and condensation occurs. The change in the dew point of the air, $\Delta T_D$, is given by the Clausius–Clapeyron equation:

$$\Delta T_D = \frac{RT_D^2}{\Delta H_{vap}} \frac{\left(p_{SW}^0 - p_{SW}\right)}{p_{SW}^0}$$

where $p_{sw}$ is the saturation vapor pressure of the solution; $p^0_{sw}$ is the vapor pressure of pure water; and $\Delta H_{vap}$ is the enthalpy of vaporization of water. The change in dew point can be expressed in terms of osmolality as:

$$\Delta T_D = \frac{M_W RT^2}{\Delta H_{vap}} \xi_m = K_D \xi_m$$

where

$$K_D = \frac{M_W RT^2}{\Delta H_{vap}}$$

The value of $\Delta H_{vap}$ is temperature dependent (*5*) and so is $K_D$. At 25° (298.15 K), the enthalpy of vaporization of water is $\Delta H_{vap}$ = 43.990 k J $mol^{-1}$ (*5*) and this results in a value of $K_D$ = 0.3027 K kg $mol^{-1}$. At the boiling point, 373.15 K, $\Delta H_{vap}$ = 40.657 k J $mol^{-1}$ (*5*) and $K_D$ = 0.5130 K kg $mol^{-1}$ and is equivalent to the ebullioscopic constant, $K_b$, for water which correlates the osmolality to boiling-point elevation.

To calibrate instruments for measurements of water activity and osmolality, it is necessary to have well-characterized solutions that are easy to prepare. The nonlinear relationship between the osmolality coefficient, $\Phi_m$, and the molality of the solute has been studied for many solutions of single salts and is most often represented by the Pitzer–Debye–Hückel equation (*1,4,6*) as a virial expansion in terms of molality as follows:

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$$\Phi_m - 1 = f_\Phi + B_\Phi m + C_\Phi m^2$$

where

$$f_\Phi = -A_\Phi\left(\frac{\sqrt{I}}{1 + b\sqrt{I}}\right)$$

and

$$B_\Phi = \beta_0 + \beta_1 e^{-\alpha\sqrt{I}}$$

and

$$I = \frac{1}{2}\sum_i m_i z_i$$

where $A_\Phi$ is the Debye–Hückel slope; $I$ is the ionic strength; $z_i$ is the charge of $i$; $b$ is the empirical ion size parameter and equal to 1.2; and $\alpha$ is the empirical ionic strength dependence parameter and equal to 2.0. The parameters $\beta_0$, $\beta_1$, and $C_\Phi$ are constants that are specific to the particular solute.

For a simple 1:1 electrolyte, like sodium chloride (NaCl), the ionic strength, $I$, is equal to the molality, and the relationship between molality and osmolality coefficient simplifies to:

$$\Phi_m = 1 - A_\Phi\left(\frac{\sqrt{m}}{1 + b\sqrt{m}}\right) + \left(\beta_0 + \beta_1 e^{-\alpha\sqrt{m}}\right)m + C_\Phi m^2$$

For sodium chloride (NaCl), the value of $A_\Phi$ has been calculated as a function of temperature and pressure (*7*), and at 273.15 K and standard pressure, $A_\Phi = 0.37642$. Using this value for $A_\Phi$ and keeping $\alpha = 2$ and $b = 1.2$, the remaining values for sodium chloride (NaCl) ($\beta_0$, $\beta_1$, and $C_\Phi$) can be fit to the experimental results for the freezing-point depression of sodium chloride (NaCl) solutions from Scatchard and Prentiss (*8*) and Gibbard and Gossmann (*9*). The entire set of experimental results span the range from 2–7300 mOsmol/kg. The resulting best-fit parameters of $\beta_0 = 0.040933$, $\beta_1 = 0.26955$, $C_\Phi = 0.004506$ enable the calculation of recipes for sodium chloride calibration standard solutions for osmolalities within this experimental range. The resulting recipes for *Standard Solutions* are listed in *Table 1*.▲ (USP 1-Dec-2020)

***Change to read:***

## ▲TYPES OF OSMOLALITY INSTRUMENTS

The osmolality of a solution is commonly determined by measurement of the freezing-point depression or dew-point depression. A freezing-point depression instrument measures the osmolality of a solution at the freezing-point of the solution, whereas a dew-point depression instrument measures the osmolality of a solution at ambient temperatures. Significant changes in the solubility, solvation, and ionization of solutes may occur as temperature changes resulting in a temperature dependence of activity and osmotic coefficients. These factors may lead to significant differences in the osmolality results obtained by different types of osmometers on the same test solutions. Volatile solutes will evaporate during a measurement with vapor pressure osmometers and result in erroneous values. Other techniques need to be used when measuring osmolality in solutions containing volatile solutes.

### Freezing-point Depression Osmometer

A freezing-point depression osmometer typically consists of the following: a means of cooling the container used for the measurement, a thermocouple for measuring the temperature of the sample, and a means of initiating crystallization. The liquid sample (generally about 0.02–2 mL) is typically placed into a cooling bath or a cooling pit while being mixed to facilitate uniform cooling. In small volume freezing-point osmometers (with sample sizes smaller than 100 µL), mixing the liquid is not required for uniform cooling. The sample is supercooled to below the expected freezing point and then a short “buzz” (a brief pulse or shock to initiate crystallization of the water) is applied to the sample. As the sample freezes, the temperature of the thermocouple rises asymptotically toward the freezing point until the sample is completely frozen and the temperature stabilizes. The freezing-point depression is the difference between the freezing point of pure water and the freezing point of the sample.

### Vapor Pressure Osmometer

Vapor pressure osmometers use hygrometric (dew-point temperature depression) measurements for determining water activity. They require a smaller volume of specimen (generally about 5–20 µL). The hygrometric technique is based on measuring the temperature of a wet thermocouple junction in a sealed chamber containing the sample. A current is applied to cool the thermocouple, by means of the Peltier effect, below the dew-point temperature, resulting in condensation of water from the air onto that junction. As water condenses on the thermocouple, the temperature of the thermocouple rises asymptotically

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toward the dew point. When the temperature of the thermocouple reaches the dew point, condensation ceases, causing the thermocouple temperature to stabilize. The dew-point temperature depression is the difference between the dew-point temperature for air in equilibrium with pure water and the dew-point temperature for air in equilibrium with the sample. In some instruments, the solution to be tested is pipetted onto a small, solute-free paper disk, which is then inserted into the sample chamber containing the thermocouple hygrometer. In other instruments, the solution is tested as a drop at the end of a micropipette inserted into the chamber containing the thermocouple hygrometer.▲ (USP 1-Dec-2020)

***Change to read:***

## ▲QUALIFICATION OF OSMOLALITY INSTRUMENTS

Osmometers are classified as Group B instruments as described in *Analytical Instrument Qualification* ⟨1058⟩. The qualification of an osmometer instrument should cover the intended operational range of the instrument, the required accuracy of the instrument, and the required precision of the instrument. For more details, see ⟨1058⟩. Acceptance criteria for fitness for purpose are based on the uncertainty of the reference material and the performance specification of the instrument. Any osmometer that is shown to be fit for the intended purpose, by meeting the qualification criteria, may be used to provide osmolality results according to the guidelines of this chapter.

### Installation Qualification (IQ)

The installation qualification (IQ) requirements provide evidence that the hardware and software are properly installed in the desired location.

### Operational Qualification (OQ)

The operational qualification (OQ) for the osmolality instrument should characterize the precision and accuracy of the osmolality measurements. Prior to characterizing the performance of the osmolality instrument, perform a full calibration of the instrument, as instructed by the instrument operation manual, and confirm the proper calibration with a calibration check (see *Procedure for Calibration Check*).

#### OPTIMIZATION OF ADJUSTABLE FREEZING PARAMETERS

Freezing-point depression osmometers may have user-adjustable parameters that affect the freezing of the *Test Solutions*. These parameters may include stirring amplitude (during cooling step), "buzz" point (amount of supercooling that precedes initiation of freezing), and "buzz" amplitude (amplitude used to initiate freezing). These test parameters may be optimized, following the manufacturer's recommendations, to reliably freeze the *Test Solutions*.

#### PRECISION/REPEATABILITY

The precision of the measurement system should be characterized as part of the operational qualification by performing repeated measurements on the mid-range osmolality standard solution that was used in the instrument calibration. Calculate the sample standard deviation using ($n$–1) degrees of freedom, σ, of six or more measurements, and express the repeatability as a percentage (%) relative to the mean value of the osmolality standard, $X_0$:

$$R_{\%} = \frac{100\sigma}{\overline{X}_0}$$

The precision of the instrument, in mOsmol/kg, at the osmolality of a test solution being analyzed is:

$$R_{osm} = \frac{R_{\%}}{100}\overline{X}$$

where $\overline{X}$ is the mean value for the test solution. After characterizing the precision of the measurement system, this result should be compared to the manufacturer's claimed repeatability. If the result is significantly greater than the claimed repeatability (e.g., more than twice the claimed repeatability), then the performance of the osmometer should be more fully investigated. The acceptance criterion is that the relative standard deviation should be NMT 2%.

#### ACCURACY

Verify the accuracy of the calibrated instrument by measurement of at least three replicates of the highest and lowest osmolality standard used during the calibration. The instrument reading should be within ±4 mOsmol/kg of the stated value for the standard solutions up to 400 mOsmol/kg and within ±1% of the stated value for the standard solutions >400 mOsmol/kg.

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## Performance Qualification (PQ)

The performance qualification (PQ) should be evaluated by periodic recalibration of the instrument and a day-of-use calibration check. It is recommended that the result for the day-of-use calibration check be monitored over time to track performance of the instrument. ▲ (USP 1-Dec-2020)

***Change to read:***

# ▲OPERATION AND CALIBRATION OF OSMOMETERS

## Standard Solutions

Solutions for instrument calibration may be purchased (*10*) or may be prepared as specified in *Table 1*. Other concentrations not listed in *Table 1* are acceptable. They may be stored at 2°–30°, but should not be frozen. When using purchased solutions, follow the manufacturer's expiry date and use instructions. Purchased solutions are typically provided in single-use ampules and should be discarded after use. Solutions prepared as specified in *Table 1* should be stored in nonpermeable (e.g., glass) containers to prevent evaporation. Fresh solutions should be prepared at least monthly as they are often nonsterile unpreserved solutions.

**Table 1. Standard Solutions (STD) for Freezing-Point Depression Osmometer Calibration[a, b]**

| mOsmol/kg of STD | g of Sodium Chloride/kg of Water | mOsmol/kg of STD | g of Sodium Chloride/kg of Water |
|---|---|---|---|
| 50 | 1.530 | 1600 | 51.39 |
| 100 | 3.098 | 1700 | 54.58 |
| 150 | 4.683 | 1800 | 57.76 |
| 200 | 6.278 | 1900 | 60.92 |
| 250 | 7.880 | 2000 | 64.08 |
| 290 | 9.165 | — | — |
| 300 | 9.487 | 2100 | 67.23 |
| 350 | 11.10 | 2200 | 70.36 |
| 400 | 12.71 | 2300 | 73.48 |
| 450 | 14.33 | 2400 | 76.59 |
| 500 | 15.94 | 2500 | 79.69 |
| 550 | 17.56 | 2600 | 82.78 |
| 600 | 19.18 | 2700 | 85.85 |
| 650 | 20.80 | 2800 | 88.91 |
| 700 | 22.41 | 2900 | 91.95 |
| 750 | 24.03 | 3000 | 94.98 |
| 800 | 25.65 | 3100 | 98.00 |
| 850 | 27.27 | 3200 | 101.00 |
| 900 | 28.89 | 3300 | 103.99 |
| 950 | 30.50 | 3400 | 106.96 |
| 1000 | 32.12 | 3500 | 109.92 |
| 1100 | 35.35 | 3600 | 112.86 |
| 1200 | 38.57 | 3700 | 115.79 |
| 1300 | 41.79 | 3800 | 118.70 |
| 1400 | 45.00 | 3900 | 121.60 |
| 1500 | 48.20 | 4000 | 124.48 |

[a] Calculated from experimental data from Scatchard and Prentiss (*8*) and Gibbard and Gossmann (*9*) with best-fit parameters of $A_\phi = 0.37642$; $\beta_0 = 0.040933$; $\beta_1 = 0.26955$; $C_\phi = 0.004506$; $\alpha = 2$, $b = 1.2$.

[b] The amount of sodium chloride (NaCl) specified is added to 1 kg of water (on weight basis). Weigh sodium chloride (NaCl), which has been previously dried at 110° for 2 h, to ±0.03 g and weigh water to ±0.2 g to ensure osmolality is accurate to correct whole number of mOsmol/kg. If correctly prepared, the accuracy of these *Standard Solutions* is ±1 mOsmol/kg. For purchased solutions, the accuracy is typically ±2 mOsmol/kg for concentrations up to 400 mOsmol/kg, and ±0.5% for concentrations >400 mOsmol/kg. Other *Standard Solution* concentrations can be prepared as needed.

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## Calibration

The osmometer should be calibrated as per the manufacturer's instructions using either purchased or prepared *Standard Solutions* (as described in *Table 1*).

### PROCEDURE FOR CALIBRATION CHECK

The calibration check should be performed in the same manner that measurements will be performed. If individual measurements will be reported, the calibration check should be performed with a single measurement of a standard solution. If the mean of three measurements will be reported, the calibration check should be performed with the mean of three measurements of a standard solution. Unless a different solution is specified as part of the manufacturer's instructions, select at least one solution from *Table 1* that lies near the center of the expected range of osmolality for the test solution. Two *Standard Solutions* bracketing the test sample within 300 mOsmol/kg are also acceptable. The instrument reading should be within ±4 mOsmol/kg from the stated value for solutions ≤400 mOsmol/kg and within ±1% from the stated value for solutions >400 mOsmol/kg. If the instrument fails to meet the acceptance criteria for the calibration check, then calibrate the instrument as per the manufacturer's instructions using either purchased or prepared *Standard Solutions* (as described in *Table 1*), and then repeat the calibration check of the instrument. After a successful check of the calibration, the instrument is ready for use.

## Test Solutions

For a solid for injection, reconstitute with the appropriate diluent as specified in the instructions on the labeling. For solutions, use the sample as is. [NOTE—A solution can be diluted to bring it within the range of measurement of the osmometer, if necessary, but the results must be expressed as those of the diluted solution and must NOT be multiplied by a dilution factor to calculate the osmolality of the original solution unless otherwise indicated in the monograph. The molal osmotic coefficient is a function of concentration and changes nonlinearly with dilution.] Unless otherwise directed in the monograph, use *Method 1*.

## Method 1: Freezing-Point Osmometry

### PROCEDURE

Verify that the instrument has been turned on for an appropriate amount of time (NLT 30 min unless the instrument contains an internal sensor) to enable the cooling bath to equilibrate to its normal operating temperature. Verify that the calibration of the instrument brackets the expected osmolality of the test solution. If not, perform a new calibration of the instrument such that the lower and upper calibration standards bracket the expected osmolality of the samples to be evaluated. At least each day of use, confirm the instrument calibration as described in *Procedure for Calibration Check*. Before each measurement, rinse the measurement cell with the solution to be tested, and verify that the thermocouple is clean and dry before immersing it into the test solution.

### TROUBLESHOOTING

Samples may sometimes fail to freeze if the sample viscosity is too high, if the sample is precooled too much or not enough, or if the mixing speed or the amplitude of the instrument is not optimally set. If test solutions occasionally fail to freeze, the osmolality measurements may not be accurate. The settings of the instrument should be optimized to reliably freeze the test solution, and then the instrument should be recalibrated with the optimized settings prior to re-evaluation of the test solutions.

Insoluble particles, emulsified droplets that are insoluble and entrained air, will not significantly affect the osmolality of the aqueous vehicle; however, these may affect the ability of the sample to freeze. If accurate measurement of the osmolality is affected by the presence of the insoluble phase, the insoluble phase should be removed by centrifugation and the osmolality of the clarified aqueous phase should be evaluated and reported.

## Method 2: Vapor Pressure Osmometry

### STANDARD SOLUTIONS

The osmolality reference standards in *Table 1* are based on freezing-point depression measurements (*8,9*) and represent the osmolality of the solution at the freezing point. However, manufacturers of dew point osmometers often typically design their instruments to be calibrated with the same standard solutions used for freezing-point depression instruments. Unless otherwise directed by the instrument manufacturer, it is permitted to use the same *Standard Solutions* that were defined above for use with the freezing-point depression instruments. When performing an instrument calibration and calibration check with these *Standard Solutions*, the stated value for the standard solution is considered to be the same as indicated for the freezing-point depression instruments.

### PROCEDURE

Ensure that the sample and instrument are located in a laboratory with good temperature control and that the sample is equilibrated to the temperature of the laboratory before performing the measurement. Verify that the instrument has been turned on for NLT 30 min to completely equilibrate to the lab temperature while operating. Verify that the calibration of the

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instrument brackets the expected osmolality of the test solution. If not, perform a new calibration of the instrument such that the lower and upper calibration standards bracket the expected osmolality of the samples to be evaluated. At least each day of use, confirm the instrument calibration as described in *Procedure for Calibration Check*. If the sample is dispensed for the measurement by a pipette, use a calibrated pipette to deliver the specified volume of liquid to the sample chamber. Consistent timing during sample loading is important for consistent repeatability. The procedure and timing for sample loading need to be equivalent to those used for the instrument calibration.

TROUBLESHOOTING

Volatile ingredients (e.g., ethanol) may evaporate rapidly during the sample loading and measurement resulting in inaccurate and variable results.▲ (USP 1-Dec-2020)

***Change to read:***

## ▲REPORTING OF RESULTS

### Units

The units of osmolality are osmole per kilogram of water (Osmol/kg) or milliosmole per kilogram of water (mOsmol/kg). The units of osmolarity are osmole per liter of solution (Osmol/L) or milliosmole per liter of solution (mOsmol/L). It is not necessary to state "of water" or "of solution" when using these units. The use of "Osmol" alone must be avoided because it is not clear whether it refers to the number (osmoles of solute) or the osmolality or the osmolarity.

### Measurement Result

If a single measurement result is reported, it should be reported with the precision given by the repeatability of the instrument. Preferably, the mean of three or more measurements on the same solution should be reported with a precision represented by the standard deviation of the averaged measurements. Most osmometers report results to the nearest whole number, therefore, the mean result should also be rounded to the nearest whole number and reported in units of mOsmol/kg.

### Conversion to Osmolarity

Freezing-point depression and vapor pressure measurements directly result in an assessment of the osmolality. However, it may be desirable to report the results in units of osmolarity. The measured osmolality $(\xi_m)$ can be converted to osmolarity $(\xi_c)$ using:

$$\xi_c = \xi_m(\rho - C)$$

where $\rho$ is the density of the solution and C is the total solute concentration, both expressed in grams per milliliter.

Example of conversion to osmolarity:

A 3% (w/v) solution of sodium bicarbonate contains 0.030 g/mL of anhydrous sodium bicarbonate. The density of this solution was found to be 1.0192 g/mL at 20°, and its measured osmolality $(\xi_m)$ was 615 mOsmol/kg.

[NOTE—1 g/mL = 1 kg/L.]

$$(p - C) = \left(1.0192\frac{\text{kg solution}}{\text{L solution}} - 0.030\frac{\text{kg solution}}{\text{L solution}}\right) = 0.9892\frac{\text{kg water}}{\text{L solution}}$$

$$\text{Osmolarity}\,(\xi_C) = (615\ \ \text{mOsmol/kg}\ \ H_2O) \times (0.9892\ \text{kg}\ \ H_2O/\text{L solution}) = 608\ \text{mOsmol/L solution}$$

### Tonicity

Tonicity is a medical term that relates to the osmotic pressure difference between the internal and external sides of a cell membrane. In many cases tonicity and osmolality are not the same because some solutes are able to rapidly pass through the cell membrane. Red blood cells, erythrocytes, are typically considered the standard for defining what is considered an isotonic solution. If red blood cells are immersed in a solution and the cells swell and become turgid or burst, the solution is hypotonic. If the red blood cells shrink and desiccate, the solution is hypertonic. An isotonic solution produces no change in the size of the red blood cells and the cell wall remains flaccid. Measurements of osmolality or osmolarity alone are not sufficient to characterize the tonicity of a liquid. It is preferable to use the terms hypoosmotic, hyperosmotic, and isoosmotic when discussing the relative osmolality of solutions. The standard for an isoosmotic solution is 0.9% sodium chloride (NaCl) solution, which has an osmolality of 285 mOsmol/kg and an osmolarity of 284 mOsmol/L.▲ (USP 1-Dec-2020)

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## ▲OSMOLARITY

Osmolarity of a solution is a theoretical quantity expressed in osmoles/L (Osmol/L) of a solution and is widely used in clinical practice because it expresses osmoles as a function of volume. Osmolarity cannot be measured but is calculated theoretically from the experimentally measured value of osmolality.

Sometimes, osmolarity ($\xi_c$) is calculated theoretically from the molar concentrations:

$$\xi_c = \Sigma \nu_i c_i$$

$\nu_i$ = number of particles formed by the dissociation of one molecule of the $i^{th}$ solute
$c_i$ = molar concentration of the $i^{th}$ solute in solution

For example, the osmolarity of a solution prepared by dissolving 1 g of vancomycin in 100 mL of 0.9% sodium chloride solution can be calculated as follows:

[3 × 10 g/L/1449.25(molecular weight of vancomycin) + 2 × 9 g/L/58.44(molecular weight of sodium chloride)] × 1000 = 329 mOsmol/L

The results suggest that the solution is slightly hyperosmotic because the osmolality of blood ranges between 285 and 310 mOsmol/kg. However, the solution is found to be hypoosmotic and has an experimentally determined osmolality of 255 mOsmol/kg.[1] The example illustrates that osmolarity values calculated theoretically from the concentration of a solution should be interpreted cautiously and may not represent the osmotic properties of infusion solutions.

The discrepancy between theoretical (osmolarity) and experimental (osmolality) results is, in part, due to the fact that the osmotic pressure of a real solution is less than that of an ideal solution because of interactions between solute molecules or between solute and solvent molecules in a solution. Such interactions reduce the pressure exerted by solute molecules on a semipermeable membrane, reducing experimental values of osmolality compared to theoretical values. This difference is related to the molal osmotic coefficient ($\Phi_{m,i}$). The example also illustrates the importance of determining the osmolality of a solution experimentally, rather than calculating the value theoretically.

To convert to osmolality, it is necessary to know the kilogram of water per liter of solution as the conversion factor. The density of the solution should be measured and the amount of (anhydrous) solute per unit of volume should be known based on the preparation of the solution. If working with an unknown solution, the value of a loss on drying measurement could also be used (assuming water is the only volatile component in the solution).

**Example 1:** A 3% (w/v) solution of sodium bicarbonate contains 0.030 g/mL of anhydrous sodium bicarbonate. The density of this solution was found to be 1.0192 g/mL at 20°, and its measured osmolality was 615 mOsmol/kg.

[NOTE—1 g/mL = 1 kg/L.]

$$\left(p - c\right) = \left(1.0192\ \frac{kg\ \ solution}{L\ \ solution} - 0.030\frac{kg\ \ solute}{L\ \ solution}\right)$$

$$\left(p - c\right) = 0.9892\frac{kg\ \ solution}{L\ \ solution}$$

$$Osmolarity\left(\xi_c\right) = \left(615\ mOsmol/kg\ H_2O\right) \times \left(0.9892\ \ kg\ H_2O/L\ \ solution\right)$$

$$Osmolarity\ \left(\xi_c\right) = 608\ \ \ mOsmol/L$$

**Example 2:** An aqueous solution sodium lauryl sulfate (SLS) as raw material has a content of 30% (on a w/v basis) and has a specific gravity of 1.08 g/mL at 20°. It also contains 0.5% of sodium sulfate ($Na_2SO_4$). Calculate the predicted osmolarity and convert this into expected osmolality, if it is evaluated (assume an ideal solute, $\Phi_m = 1.0$). The molecular weight of SLS is 288.37 g/mol. The molecular weight of sodium sulfate ($Na_2SO_4$) is 142.04 g/mol. The density of the material at 20° is 1.08/0.997 = 1.083 g/mL.

$$\left(p - c\right) = \left(1.083\frac{kg\ \ solution}{L\ \ solution} - 0.305\frac{kg\ \ solute}{L\ \ solution}\right)$$

$$\left(p - c\right) = 0.778\frac{kg\ \ water}{L\ \ solution}$$

$$Osmolarity\ \left(\xi_c\right) = 608\ \ mOsmol/L$$

The osmolarity contributions would be:

[1] Kastango ES, Hadaway L. New perspectives on vancomycin use in home care, part 1. *Int J Pharm Compd.* 2001;5(6):465–469.

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$$300\frac{g\ SLS}{1\ L\ \ solution}\times\frac{1\ mol\ SLS}{288.37\ g\ SLS}\times\frac{2\ mol\ solute}{mol\ SLS}=2.0806\ \ M=2080.6\,mOsmol/L\ \left(from\ SLS\right)$$

$$5\frac{g\ sodium\ sulfate}{1\ L\ solution}\times\frac{1\ mol\,sodium\,sulfate}{142.04\ g\ sodium\,sulfate}\times\frac{3\ mol\ solute}{1mol\ sodium\ sulfate}=0.1056\ M=105.6\,mOsmol/L\ \left(from\ \ sodium\ sulfate\right)$$

Total osmolarity = 2080.6 + 105.6 = 2186.2 mOsmol/L▲ (USP 1-Dec-2020)

***Delete the following:***

## ▲MEASUREMENT OF OSMOLALITY

The osmolality of a solution is commonly determined by the measurement of the freezing point depression of the solution.

### Apparatus

The apparatus, an osmometer for freezing point depression measurement, consists of the following: a means of cooling the container used for the measurement; a temperature measuring device with an appropriate current- or potential-difference measurement device that may be graduated in temperature change or in osmolality; and a means of mixing the sample.

Osmometers that measure the vapor pressures of solutions are less frequently employed. They require a smaller volume of specimen (generally about 5 µL), but the accuracy and precision of the resulting osmolality determination are comparable to those obtained by the use of osmometers that depend upon the observed freezing points of solutions.

### Standard Solutions

Prepare *Standard Solutions* as specified in *Table 1*, as necessary.[2]

Once prepared, the *Standard Solutions* may be stored in nonpermeable container–closure systems. They should be prepared fresh every week as they are nonsterile, unpreserved solutions. If using purchased solutions, follow the manufacturer's expiration date.

**Table 1. Standard Solutions for Osmometer Calibration[a]**

| Standard Solutions (Weight in g of sodium chloride per kg of water) | Osmolality (mOsmol/kg) ($\xi_m$) | Molal Osmotic Coefficient ($\Phi_{m,\,NaCl}$) | Freezing Point Depression (°) $\Delta T_f$ |
|---|---|---|---|
| 3.087 | 100 | 0.9463 | 0.186 |
| 6.260 | 200 | 0.9337 | 0.372 |
| 9.463 | 300 | 0.9264 | 0.558 |
| 12.684 | 400 | 0.9215 | 0.744 |
| 15.916 | 500 | 0.9180 | 0.930 |
| 19.147 | 600 | 0.9157 | 1.116 |
| 22.380 | 700 | 0.9140 | 1.302 |

[a] Adapted from the *European Pharmacopoeia,* 4th Edition, 2002, p. 50

### Test Solution

For a solid for injection, constitute with the appropriate diluent as specified in the instructions on the labeling. For solutions, use the sample as is.

[NOTE—A solution can be diluted to bring it within the range of measurement of the osmometer, if necessary, but the results must be expressed as that of the diluted solution and must NOT be multiplied by a dilution factor to calculate the osmolality of the original solution, unless otherwise indicated in the monograph. The molal osmotic coefficient is a function of concentration. Therefore, it changes with dilution.]

### Procedure

First, calibrate the instrument by the manufacturer's instructions. Confirm the instrument calibration with at least one solution from *Table 1* such that the osmolality of the *Standard Solution* lies within 50 mOsmol/kg of the expected value of the *Test Solution* or the center of the expected range of osmolality of the *Test Solution*. The instrument reading should be within ±4

[2] Commercially available solutions for osmometer calibration, with osmolalities equal to or different from those listed in *Table 1* and standardized by methods traceable to National Institute of Standards and Technology (NIST) (www.nist.gov), may be used.

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mOsmol per kg from the *Standard Solution.* Introduce an appropriate volume of each *Standard Solution* into the measurement cell as in the manufacturer's instructions, and start the cooling system. Usually, the mixing device is programmed to operate at a temperature below the lowest temperature expected from the freezing point depression. The apparatus indicates when the equilibrium is attained. If necessary, calibrate the osmometer, using an appropriate adjustment device such that the reading corresponds to either the osmolality or freezing point depression value of the *Standard Solution* shown in *Table 1*.

[NOTE—If the instrument reading indicates the freezing point depression, the osmolality can be derived by using the appropriate formula under *Osmolality*.]

Repeat the procedure with each *Test Solution*. Read the osmolality of the *Test Solution* directly, or calculate it from the measured freezing point depression.

Assuming that the value of the osmotic coefficient is essentially the same whether the concentration is expressed in molality or molarity, the experimentally determined osmolality of a solution can be converted to osmolarity in the same manner in which the concentration of a solution is converted from molality to molarity. Unless a solution is very concentrated, the osmolarity of a solution ($\xi_c$) can be calculated as follows:

$$\xi_c = 1000\xi_m/(1000/\rho + \Sigma w_i v_i)$$

$\xi_m$ = experimentally determined osmolality
$\rho$ = density of the solution
$w_i$ = weight (g)
$v_i$ = partial specific volume of the $i^{th}$ solute (mL/g)

The partial specific volume of a solute is the change in volume of a solution when an additional 1 g of solute is dissolved in the solution. This volume can be determined by the measurement of densities of the solution before and after the addition of the solute. The partial specific volumes of salts are generally very small, around 0.1 mL/g. However, those of other solutes are generally higher. For example, the partial specific volumes of amino acids are in the range of 0.6–0.9 mL/g. It can be shown from the above equation correlating osmolarity with osmolality that:

$$\xi_c = \xi_m (\rho - c)$$

$\xi_m$ = experimentally determined osmolality
$\rho$ = density of the solution (g/mL)
$c$ = total solute concentration (g/mL)

Thus, alternatively, the osmolarity can also be calculated from experimentally determined osmolality from the measurement of density of the solution by a suitable method and the total weight of the solute, after correction for water content, dissolved per milliliter of the solution. ▲ (USP 1-Dec-2020)

***Change to read:***

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# ⟨786⟩ PARTICLE SIZE DISTRIBUTION ESTIMATION BY ANALYTICAL SIEVING

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▲•Portions of the chapter text that are national *USP* text, and are not part of the harmonized text, are marked with symbols (• •) to specify this fact.•▲ (USP 1-May-2023)

Sieving is one of the oldest methods of classifying powders and granules by particle size distribution. When using a woven sieve cloth, the sieving will essentially sort the particles by their intermediate size dimension (i.e., breadth or width). Mechanical sieving is most suitable where the majority of the particles are larger than about 75 µm. For smaller particles, the light weight provides insufficient force during sieving to overcome the surface forces of cohesion and adhesion that cause the particles to stick to each other and to the sieve, and thus cause particles that would be expected to pass through the sieve to be retained. For such materials, other means of agitation such as air-jet sieving or sonic sifting may be more appropriate. Nevertheless, sieving can sometimes be used for some powders or granules having median particle sizes smaller than 75 µm where the method can be validated. In pharmaceutical terms, sieving is usually the method of choice for classification of the coarser grades of single powders or granules. It is a particularly attractive method in that powders and granules are classified only on the basis of particle size, and in most cases the analysis can be carried out in the dry state.

Among the limitations of the sieving method are the need for an appreciable amount of sample (normally at least 25 g, depending on the density of the powder or granule, and the diameter of test sieves) and difficulty in sieving oily or other cohesive powders or granules that tend to clog the sieve openings. The method is essentially a two-dimensional estimate of size because passage through the sieve aperture is frequently more dependent on maximum width and thickness than on length.

This method is intended for estimation of the total particle size distribution of a single material. It is not intended for determination of the proportion of particles passing or retained on one or two sieves.

Estimate the particle size distribution as described under *Dry Sieving Method,* unless otherwise specified in the individual monograph. Where difficulty is experienced in reaching the endpoint (i.e., material does not readily pass through the sieves) or when it is necessary to use the finer end of the sieving range (below 75 µm), serious consideration should be given to the use of an alternative particle-sizing method.

Sieving should be carried out under conditions that do not cause the test sample to gain or lose moisture. The relative humidity of the environment in which the sieving is carried out should be controlled to prevent moisture uptake or loss by the sample. In the absence of evidence to the contrary, analytical test sieving is normally carried out at ambient humidity. Any special conditions that apply to a particular material should be detailed in the individual monograph.

## PRINCIPLES OF ANALYTICAL SIEVING

Analytical test sieves are constructed from a woven-wire mesh, which is of simple weave that is assumed to give nearly square apertures and is sealed into the base of an open cylindrical container. The basic analytical method involves stacking the sieves on top of one another in ascending degrees of coarseness, and then placing the test powder on the top sieve.

The nest of sieves is subjected to a standardized period of agitation, and then the weight of material retained on each sieve is accurately determined. The test gives the weight percentage of powder in each sieve size range.

This sieving process for estimating the particle size distribution of a single pharmaceutical powder is generally intended for use where at least 80% of the particles are larger than 75 µm. The size parameter involved in determining particle size distribution by analytical sieving is the length of the side of the minimum square aperture through which the particle will pass.

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## TEST SIEVES

Test sieves suitable for pharmacopeial tests conform to the current edition of International Organization for Standardization ▲(ISO)▲ (USP 1-May-2023) ISO 3310-1 ▲specification▲ (USP 1-May-2023): Test Sieves—Technical Requirements and Testing▲— Part 1: Test sieves of metal wire cloth.▲ (USP 1-May-2023) Unless otherwise specified in the monograph, use those ISO sieves listed in *Table 1* as recommended in the particular region.

**Table 1. Sizes of Standard Sieve Series in Range of Interest**

| ISO Nominal Aperture | | | US Sieve No. | Recommended USP Sieves (microns) | European Sieve No. | Japan Sieve No. |
|---|---|---|---|---|---|---|
| Principal Sizes | Supplementary Sizes | | | | | |
| R 20/3 | R 20 | R 40/3 | | | | |
| 11.20 mm | 11.20 mm | 11.20 mm | | | 11200 | |
| | 10.00 mm | | | | | |
| | | 9.50 mm | | | | |
| | 9.00 mm | | | | | |
| 8.00 mm | 8.00 mm | 8.00 mm | | | | |

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**Table 1. Sizes of Standard Sieve Series in Range of Interest** *(continued)*

| ISO Nominal Aperture | | | US Sieve No. | Recommended USP Sieves (microns) | European Sieve No. | Japan Sieve No. |
|---|---|---|---|---|---|---|
| Principal Sizes | Supplementary Sizes | | | | | |
| R 20/3 | R 20 | R 40/3 | | | | |
| | 7.10 mm | | | | | |
| | | 6.70 mm | | | | |
| | 6.30 mm | | | | | |
| 5.60 mm | 5.60 mm | 5.60 mm | | | 5600 | 3.5 |
| | 5.00 mm | | | | | |
| | | 4.75 mm | | | | 4 |
| | 4.50 mm | | | | | |
| 4.00 mm | 4.00 mm | 4.00 mm | 5 | 4000 | 4000 | 4.7 |
| | 3.55 mm | | | | | |
| | | 3.35 mm | 6 | | | 5.5 |
| | 3.15 mm | | | | | |
| 2.80 mm | 2.80 mm | 2.80 mm | 7 | 2800 | 2800 | 6.5 |
| | 2.50 mm | | | | | |
| | | 2.36 mm | 8 | | | 7.5 |
| | 2.24 mm | | | | | |
| 2.00 mm | 2.00 mm | 2.00 mm | 10 | 2000 | 2000 | 8.6 |
| | 1.80 mm | | | | | |
| | | 1.70 mm | 12 | | | 10 |
| | 1.60 mm | | | | | |
| 1.40 mm | 1.40 mm | 1.40 mm | 14 | 1400 | 1400 | 12 |
| | 1.25 mm | | | | | |
| | | 1.18 mm | 16 | | | 14 |
| | 1.12 mm | | | | | |
| 1.00 mm | 1.00 mm | 1.00 mm | 18 | 1000 | 1000 | 16 |
| | 900 µm | | | | | |
| | | 850 µm | 20 | | | 18 |
| | 800 µm | | | | | |
| 710 µm | 710 µm | 710 µm | 25 | 710 | 710 | 22 |
| | 630 µm | | | | | |
| | | 600 µm | 30 | | | 26 |
| | 560 µm | | | | | |
| 500 µm | 500 µm | 500 µm | 35 | 500 | 500 | 30 |
| | 450 µm | | | | | |
| | | 425 µm | 40 | | | 36 |
| | 400 µm | | | | | |
| 355 µm | 355 µm | 355 µm | 45 | 355 | 355 | 42 |
| | 315 µm | | | | | |
| | | 300 µm | 50 | | | 50 |
| | 280 µm | | | | | |
| 250 µm | 250 µm | 250 µm | 60 | 250 | 250 | 60 |
| | 224 µm | | | | | |
| | | 212 µm | 70 | | | 70 |
| | 200 µm | | | | | |

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**Table 1. Sizes of Standard Sieve Series in Range of Interest** *(continued)*

| ISO Nominal Aperture | | | US Sieve No. | Recommended USP Sieves (microns) | European Sieve No. | Japan Sieve No. |
|---|---|---|---|---|---|---|
| Principal Sizes | Supplementary Sizes | | | | | |
| R 20/3 | R 20 | R 40/3 | | | | |
| 180 µm | 180 µm | 180 µm | 80 | 180 | 180 | 83 |
| | 160 µm | | | | | |
| | | 150 µm | 100 | | | 100 |
| | 140 µm | | | | | |
| 125 µm | 125 µm | 125 µm | 120 | 125 | 125 | 119 |
| | 112 µm | | | | | |
| | | 106 µm | 140 | | | 140 |
| | 100 µm | | | | | |
| 90 µm | 90 µm | 90 µm | 170 | 90 | 90 | 166 |
| | 80 µm | | | | | |
| | | 75 µm | 200 | | | 200 |
| | 71 µm | | | | | |
| 63 µm | 63 µm | 63 µm | 230 | 63 | 63 | 235 |
| | 56 µm | | | | | |
| | | 53 µm | 270 | | | 282 |
| | 50 µm | | | | | |
| 45 µm | 45 µm | 45 µm | 325 | 45 | 45 | 330 |
| | 40 µm | | | | | |
| | | 38 µm | | | 38 | 391 |

Sieves are selected to cover the entire range of particle sizes present in the test specimen. A nest of sieves having a $\sqrt{2}$ progression of the area of the sieve openings is recommended. The nest of sieves is assembled with the coarsest screen at the top and the finest at the bottom. Use micrometers or millimeters in denoting test sieve openings. [NOTE—▲Sieve▲ (USP 1-May-2023) numbers are provided in the table for conversion purposes only.] Test sieves are made from stainless steel or, less preferably, from brass or other suitable nonreactive wire.

Calibration and recalibration of test sieves is in accordance with the most current edition of ISO 3310-1. Sieves should be carefully examined for gross distortions and fractures, especially at their screen frame joints, before use. Sieves may be calibrated optically to estimate the average opening size, and opening variability, of the sieve mesh. Alternatively, for the evaluation of the effective opening of test sieves in the size range of 212 to 850 µm, Standard Glass Spheres are available. Unless otherwise specified in the individual monograph, perform the sieve analysis at controlled room temperature and at ambient relative humidity.

## Cleaning Test Sieves

Ideally, test sieves should be cleaned using only an air jet or a liquid stream. If some apertures remain blocked by test particles, careful gentle brushing may be used as a last resort.

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## Test Specimen

If the test specimen weight is not given in the monograph for a particular material, use a test specimen having a weight between 25 and 100 g, depending on the bulk density of the material, and test sieves having a ▲diameter of ▲ (USP 1-May-2023) 200-mm ▲or 203-mm (8-inch)▲ (USP 1-May-2023). For ▲sieves of 75-mm or▲ (USP 1-May-2023) 76-mm ▲(3-inch) diameter▲ (USP 1-May-2023), the amount of material that can be accommodated is approximately 1/7th▲ of ▲ (USP 1-May-2023) which can be accommodated on a 200-mm ▲or 203-mm ▲ (USP 1-May-2023)sieve. ▲♦ [NOTE—The 203-mm (8-inch) frame diameter sieve is equivalent to the 200-mm frame diameter sieve. The 76-mm (3-inch) frame diameter sieve is equivalent to the 75-mm frame diameter sieve.] ♦▲ (USP 1-May-2023) Determine the most appropriate weight for a given material by test sieving accurately weighed specimens of different weights, such as 25, 50, and 100 g, for the same time period on a mechanical shaker. [NOTE—If the test results are similar for the 25-g and 50-g specimens, but the 100-g specimen shows a lower percentage through the finest sieve, the 100-g specimen size is too large.] Where only a specimen of 10 to 25 g is available, smaller diameter test sieves conforming to the same mesh specifications may be substituted, but the endpoint must be redetermined. The use of test samples having a smaller mass (e.g., down to 5 g) may be needed. For materials with low apparent particle density, or for materials mainly comprising particles with a highly isodiametrical shape, specimen weights below 5 g for a 200-mm ▲or 203-mm sieve▲ (USP 1-May-2023) may

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be necessary to avoid excessive blocking of the sieve. During validation of a particular sieve analysis method, it is expected that the problem of sieve blocking will have been addressed.

If the test material is prone to picking up or losing significant amounts of water with varying humidity, the test must be carried out in an appropriately controlled environment. Similarly, if the test material is known to develop an electrostatic charge, careful observation must be made to ensure that such charging is not influencing the analysis. An antistatic agent, such as colloidal silicon dioxide and/or aluminum oxide, may be added at a 0.5 percent (m/m) level to minimize this effect. If both of the above effects cannot be eliminated, an alternative particle-sizing technique must be selected.

## Agitation Method

Several different sieve and powder agitation devices are commercially available, all of which may be used to perform sieve analyses. However, the different methods of agitation may give different results for sieve analyses and endpoint determinations because of the different types and magnitude of the forces acting on the individual particles under test. Methods using mechanical agitation or electromagnetic agitation, and that can induce either a vertical oscillation or a horizontal circular motion, or tapping or a combination of both tapping and horizontal circular motion are available. Entrainment of the particles in an air stream may also be used. The results must indicate which agitation method was used and the agitation parameters used (if they can be varied), because changes in the agitation conditions will give different results for the sieve analysis and endpoint determinations, and may be sufficiently different to give a failing result under some circumstances.

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## Endpoint Determination

The test sieving analysis is complete when the weight on any of the test sieves does not change by more than 5% or 0.1 g (10% in the case of ▲75-mm or▲ (USP 1-May-2023) 76-mm sieves) of the previous weight on that sieve. If less than 5% of the total specimen weight is present on a given sieve, the endpoint for that sieve is increased to a weight change of not more than 20% of the previous weight on that sieve.

If more than 50% of the total specimen weight is found on any one sieve, unless this is indicated in the monograph, the test should be repeated, but with the addition to the sieve nest of a more coarse sieve, intermediate between that carrying the excessive weight and the next coarsest sieve in the original nest, i.e., addition of the ISO series sieve omitted from the nest of sieves.

# SIEVING METHODS

## Mechanical Agitation

### DRY SIEVING METHOD

Tare each test sieve to the nearest 0.1 g. Place an accurately weighed quantity of test specimen on the top (coarsest) sieve, and replace the lid. Agitate the nest of sieves for 5 minutes. Then carefully remove each from the nest without loss of material. Reweigh each sieve, and determine the weight of material on each sieve. Determine the weight of material in the collecting pan in a similar manner. Reassemble the nest of sieves, and agitate for 5 minutes. Remove and weigh each sieve as previously described. Repeat these steps until the endpoint criteria are met (see *Endpoint Determination* under *Test Sieves*). Upon completion of the analysis, reconcile the weights of material. Total losses must not exceed 5% of the weight of the original test specimen.

Repeat the analysis with a fresh specimen, but using a single sieving time equal to that of the combined times used above. Confirm that this sieving time conforms to the requirements for endpoint determination. When this endpoint has been validated for a specific material, then a single fixed time of sieving may be used for future analyses, providing the particle size distribution falls within normal variation.

If there is evidence that the particles retained on any sieve are aggregates rather than single particles, the use of mechanical dry sieving is unlikely to give good reproducibility, and a different particle size analysis method should be used.

## Air Entrainment Methods

### AIR JET AND SONIC SIFTER SIEVING

Different types of commercial equipment that use a moving air current are available for sieving. A system that uses a single sieve at a time is referred to as air jet sieving. It uses the same general sieving methodology as that described under the *Dry Sieving Method,* but with a standardized air jet replacing the normal agitation mechanism. It requires sequential analyses on individual sieves starting with the finest sieve to obtain a particle size distribution. Air jet sieving often includes the use of finer test sieves than those used in ordinary dry sieving. This technique is more suitable where only oversize or undersize fractions are needed.

In the sonic sifting method, a nest of sieves is used, and the test specimen is carried in a vertically oscillating column of air that lifts the specimen and then carries it back against the mesh openings at a given number of pulses per minute. It may be necessary to lower the sample amount to 5 g, when sonic sifting is employed.

The air jet sieving and sonic sieving methods may be useful for powders or granules when mechanical sieving techniques are incapable of giving a meaningful analysis.

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These methods are highly dependent upon proper dispersion of the powder in the air current. This requirement may be hard to achieve if the method is used at the lower end of the sieving range (i.e., below 75 µm), when the particles tend to be more cohesive, and especially if there is any tendency for the material to develop an electrostatic charge. For the above reasons endpoint determination is particularly critical, and it is very important to confirm that the oversize material comprises single particles and is not composed of aggregates.

## INTERPRETATION

The raw data must include the weight of test specimen, the total sieving time, and the precise sieving methodology and the set values for any variable parameters, in addition to the weights retained on the individual sieves and in the pan. It may be convenient to convert the raw data into a cumulative weight distribution, and if it is desired to express the distribution in terms of a cumulative weight undersize, the range of sieves used should include a sieve through which all the material passes. If there is evidence on any of the test sieves that the material remaining on it is composed of aggregates formed during the sieving process, the analysis is invalid.

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# ⟨787⟩ SUBVISIBLE PARTICULATE MATTER IN THERAPEUTIC PROTEIN INJECTIONS

This chapter can be used as an alternative to *USP* general chapter *Particulate Matter in Injections* ⟨788⟩. It specifically addresses therapeutic protein injections and related preparations, allowing use of smaller test product volumes and smaller test aliquots to determine particulate matter content, with sample-handling instructions that take into account the issues associated with the analysis of these materials. Although the methodology and limits presented in this chapter are preferred for therapeutic protein injections, the use of regulatory-acceptable alternative analytical methods with adequately developed subvisible particulate limits is acceptable.

Therapeutic protein injections are biotechnology-derived products of protein or peptides and other therapeutic protein injections such as naturally sourced therapeutic proteins, including their final infusion preparations. Certain preparations that are not amenable to these tests, such as prophylactic vaccines, are not included in this guidance.

Particulate matter in therapeutic protein injections consists of mobile undissolved substances that may originate from various sources. The particles may be (a) truly foreign, or "extrinsic", e.g., unexpected foreign material, such as cellulose; (b) "intrinsic" resulting from addition or by insufficient cleaning during manufacturing, such as tank metals or gaskets, lubricants, filling hardware, or resulting from instability, e.g., changes over time, such as insoluble drug salt forms or package degradation; and (c) "inherent", such as particles of the protein or formulation components. All of these particle types may be detected and counted in the test method described in this chapter. In addition, for light obscuration (LO) methods, gas bubbles are commonly counted during particulate matter testing and are counting artifacts. For the determination of subvisible particles in therapeutic protein injections, the *Light Obscuration Particle Count Test* is the preferred method. The *Microscopic Particle Count Test* similar to that described in ⟨788⟩ may be useful in cases where the particles being analyzed are primarily nonproteinaceous, or in cases where examination of particle characteristics may be important. The results from this test are not equivalent to those from the LO test.

Not all therapeutic protein injections can be examined directly for subvisible particles by the LO method. When the test method is not directly applicable to specific test samples, as indicated by issues with method verification (reproducibility, linearity), a quantitative dilution with an appropriate diluent can be made to allow analysis by light obscuration. Method verification may be required to ensure the appropriateness of the sample handling procedures and the performance of the method for each drug product.

In the tests described below, the results obtained by examining a discrete unit or group of units for particulate matter cannot be extrapolated with certainty to other units that remain untested. Thus, sampling plans based on known operational factors must be developed to support valid inferences from observed data in order to characterize the level of particulate matter in a large group of units. Sampling plans should be based on considerations of product volume, particle numbers historically found in comparison to limits, particle size distribution, and variability of particle counts between units. Products that are used with a final filter during administration (in-line) are exempt from these requirements, providing that scientific data are available to justify the exemption.

***Change to read:***

## LIGHT OBSCURATION PARTICLE COUNT TEST

Analysts should use a suitable instrument that is based on the principle of light blockage and that allows an automatic determination of particle size and number.

The sensor selected must be appropriate for the intended particle size range and anticipated particle count. Several standardization steps such as sample volume accuracy, sample flow rate, sensor resolution, calibration, and particle count accuracy are important for the proper operation of the apparatus and are further reviewed in general information chapter ▲*Methods for the Determination of Subvisible Particulate Matter* ⟨1788⟩▲ (CN 1-May-2021).[1]

For equipment calibration purposes, use of NIST-traceable particle size standards or equivalent traceable standards, see ⟨1788⟩. These standards generally are between 2 and 100 µm. Analysts should verify the performance of the apparatus using the USP Particle Count RS dispersed in particle-free water at an appropriate volume. Care must be taken to avoid agglomeration of particles during dispersion in the calibration process. The definition for *Particle-Free Water* is provided in the introductory section of *Reagents, Indicators, and Solutions*.

### General Precautions

Carry out the test under conditions that limit ingress of particulate matter, preferably in a laminar-flow cabinet. The glassware and equipment used should be properly cleaned and handled to remain particle free. Care should be taken to minimize shaking and other stresses to the preparations (for liquids or reconstituted powders) and to prevent introduction of air bubbles into the preparation under examination. This is particularly important when preparations are combined or transferred to the container in which the determination will be carried out.

[1] For informational purposes only, analysts can refer to ⟨1788⟩, which may be a helpful but not mandatory resource.

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## Preparatory Steps

Glassware selected for the method should accommodate the volume of blank and sample aliquots, allow easy mixing, and be amenable to repeated cleaning used to prevent any significant contribution to particle counts. Typically, separate vessels are utilized and retained specifically for this test only. Cleaning and rinsing of the method glassware should be in close proximity to the counting area.

The work area and equipment should be separated from general lab traffic. A good supply of filtered water (to comply with *Particle-Free Water*) and support equipment should be maintained and available for the routine work. Refer to general information chapter ⟨1788⟩.[1]

Two types of system controls are recommended: a *Blank Test* and *System Suitability Verification*.

1. The *Blank Test* involves the following:
    A. Run at the start of the day or analysis to ensure that proper care has been used in preparing the site and apparatus for use (*Environmental Blank*).
    B. Any additional blanks run throughout the day (such as between product families) should also comply with the *Blank Test* requirements (*Procedural Blank*).
2. The *System Suitability Verification* is run to verify proper count/mL and ratio for the current USP Particle Count RS or equivalent commerical standard. See general information chapter ⟨1788⟩.[1]

## Blank Test

In order to check that (a) the environment is suitable for the test, (b) the glassware is properly cleaned, and (c) the water or suitable solvent that will be used is particle free (b and c constitute the "blank"), the following test is carried out. Determine the count in five aliquots of degassed particle-free water or suitable solvent, representative of the product test volume (but NLT 1 mL). If the number of particles 10 µm or greater in size exceeds 1 particle per mL for the combined aliquot volume, the precautions taken for the test are not sufficient. Repeat the preparatory steps until the environment, glassware, and water are suitable for the test determination. The *Blank Test* must be run initially each day (environment) and is recommended throughout the daily routine, especially between product families (procedural).

## System Suitability Verification

To verify system suitability, *Particle-Free Water* and the USP Particle Count RS or equivalent commercial standard aqueous suspension are tested from the same or similar type of container as product samples, are degassed in the same manner, and generally are handled like the test article. Ensure that the system can meet the *Blank Test* requirement. Determine the particulate matter in a sample of the USP Particle Count RS that corresponds to the product test volume, according to the method described below. Results from the Standard preparation should fall within the stated specifications of the Reference Standard.

## Method

Product samples are tested in the manner that most suitably represents delivery (e.g., expelled syringe contents). For parenterals that have a sufficient volume (i.e., volume large enough to facilitate testing), testing of individual units is often diagnostic. For parenteral products that do not have a sufficient volume, carefully and thoroughly mix each unit, then combine the contents of a suitable number of units in a separate container to obtain the volume required for a single test (generally 0.2–5.0 mL). If a dilution is performed, ensure that the blank container has sufficient volume for product and diluents and that few, if any, particles are introduced in the process. Open each unit cautiously, remove the sealing closure, and avoid contamination of the contents before sampling, diluting, or, if necessary, pooling.

When a product solvent is specified, e.g., for lyophilized solids or powders for parenteral use, the reconstitution or dilution must be performed with the appropriate amount of specified solvent. In this case, the solvent itself may be tested to ensure that it is not a significant source of particles. Subtraction of the solvent particle count from the total count is not allowed.

Eliminating gas bubbles is a key step, especially for proteinaceous products that readily entrain gas. Two methods are recommended: either allowing the product fluid to stand under ambient pressure or applying a gentle (e.g., 75 Torr) vacuum. Other methods may be used when demonstrated to be suitable. Sonication should be avoided.

Once the samples have been degassed, they must be remixed gently to suspend all particles by mixing the contents of the sample gently but thoroughly by an appropriate means, e.g., slow swirling of the container by hand. Inversion to mix the product fluid is not recommended at any time.

Immediately after mixing, withdraw NLT four aliquots, each of a volume appropriate for the instrument's capacity (generally 0.2–5.0 mL). Count the number of particles over the selected size range, including particles equal to or greater than 10 and 25 µm. Disregard the result obtained for the first aliquot, and calculate the mean number of particles at each size range for the remaining aliquots of the preparation being tested. A tare volume also can be applied to control sampling that should be representative of the sensor dynamic range and needle volume.

## General Considerations

Dilution is allowed as long as the diluent and methods are demonstrated to be appropriate and the smallest level of dilution that allows for reproducible testing is used. Under certain circumstances dilution of samples may be required to obtain reliable results. This may be the case with high-concentration products and/or high-viscosity products that cannot be drawn into the instrument properly; products that cause saturation of the instrument sensor due to high counts, especially in the smaller than 10-µm size ranges; and cases where the high concentration results in very small differences in refractive index between the

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solution and the protein aggregates, etc. In situations where sample dilution is conducted, the underlying rationale for the dilution as well as the suitability of the selected dilution scheme including choice of diluent must be demonstrated. Suitability of the dilution scheme and of the diluent may be assessed by demonstrating consistency of results over a dilution range. Bridging studies with and without dilution at size ranges intended for particle counting should be considered. Studies exploring the ability of dilution to decrease the number of aggregates, or to increase particles due to changes in the ratio of protein to excipients, should also be performed. An orthogonal technique may be used to justify the suitability of the method by verifying the impact of dilution.

## Evaluation

The values below are derived historically from general chapters ⟨788⟩ and ⟨789⟩. If specifications are different from those stated below, they will be indicated in an individual monograph where available.

For parenteral products that are therapeutic protein injections for infusion or injection supplied in containers with a nominal content of less than or equal to 100 mL:

> The average number of particles present in the units tested should not exceed 6000 per container equal to or greater than 10 µm and should not exceed 600 per container equal to or greater than 25 µm.

For therapeutic protein injections supplied in containers with a nominal content of more than 100 mL, and parenteral infusion preparations or injections with a nominal content of more than 100 mL:

> The average number of particles present in the units tested should not exceed 25/mL equal to or greater than 10 µm and should not exceed 3/mL equal to or greater than 25 µm. Also, total particle load should not exceed 6000 per container equal to or greater than 10 µm and should not exceed 600 per container equal to or greater than 25 µm.

Products that are used with a final filter during administration (in-line) are exempt from these requirements, providing that scientific data are available to justify the exemption. However, filtrates are expected to comply with the guideline. For products supplied or first reconstituted in <100 mL, and then diluted for infusion in a volume >100 mL, particle content should be assessed both before and after dilution and evaluated based on their final volume.

## MICROSCOPIC PARTICLE COUNT TEST

As noted, the LO method is the preferred method for therapeutic protein injections and parenteral infusions. However, the microscopic method may be used when appropriate, such as determination of extrinsic and intrinsic particle types only. It should be demonstrated, however, that particular classes of particles (e.g., inherent) are also being counted when using this method. For the determination of product acceptability, apply the limits for the membrane microscopic test in general chapter ⟨788⟩. Because of the interference of some protein particles and their physical characteristics (fragile or translucent), the results of the *Microscopic Particle Count Test* are not equivalent to those of the *Light Obscuration Particle Count Test*, and the two methods cannot be considered interchangeable.

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# ⟨788⟩ PARTICULATE MATTER IN INJECTIONS

This general chapter is harmonized with the corresponding texts of the *European Pharmacopoeia* and/or the *Japanese Pharmacopoeia*. These pharmacopeias have undertaken not to make any unilateral change to this harmonized chapter. Portions of the present general chapter text that are national USP text, and therefore not part of the harmonized text, are marked with symbols (⬥⬥) to specify this fact.

Particulate matter in injections and parenteral infusions consists of extraneous mobile undissolved particles, other than gas bubbles, unintentionally present in the solutions.

⬥As stated in *Injections and Implanted Drug Products* ⟨1⟩, solutions for injection administered by the intramuscular or subcutaneous route must meet the requirements of *Particulate Matter in Injections* ⟨788⟩. This requirement has been indefinitely postponed for products for veterinary use. Parenterals packaged and labeled exclusively for use as irrigating solutions are exempt from the requirements of *Particulate Matter in Injections* ⟨788⟩. Radiopharmaceutical preparations are exempt from the requirements of *Particulate Matter in Injections* ⟨788⟩. Parenteral products for which the labeling specifies use of a final filter prior to administration are exempt from the requirements of *Particulate Matter in Injections* ⟨788⟩, provided that scientific data are available to justify this exemption.⬥

For the determination of particulate matter, two procedures, *Method 1 (Light Obscuration Particle Count Test)* and *Method 2 (Microscopic Particle Count Test)*, are specified hereinafter. When examining injections and parenteral infusions for subvisible particles, *Method 1* is preferably applied. However, it may be necessary to test some preparations by the *Light Obscuration Particle Count Test* followed by the *Microscopic Particle Count Test* to reach a conclusion on conformance to the requirements.

Not all parenteral preparations can be examined for subvisible particles by one or both of these methods. When *Method 1* is not applicable, e.g., in the case of preparations having reduced clarity or increased viscosity, the test should be carried out according to *Method 2*. Emulsions, colloids, and liposomal preparations are examples. Similarly, products that produce air or gas bubbles when drawn into the sensor may also require microscopic particle count testing. If the viscosity of the preparation to be tested is sufficiently high so as to preclude its examination by either test method, a quantitative dilution with an appropriate diluent may be made to decrease viscosity, as necessary, to allow the analysis to be performed.

The results obtained in examining a discrete unit or group of units for particulate matter cannot be extrapolated with certainty to other units that remain untested. Thus, statistically sound sampling plans must be developed if valid inferences are to be drawn from observed data to characterize the level of particulate matter in a large group of units.

⬥For the purpose of this chapter, small-volume parenteral is synonymous with small-volume injection, and large-volume parenteral is synonymous with large-volume injection.⬥

## METHOD 1 LIGHT OBSCURATION PARTICLE COUNT TEST

Use a suitable apparatus based on the principle of light blockage that allows for an automatic determination of the size of particles and the number of particles according to size. The definition for *particle-free water* is provided in *Reagents, Indicators, and Solutions—Reagent Specifications*.

The apparatus is calibrated using dispersions of spherical particles of known sizes between 10 µm and 25 µm. These standard particles are dispersed in *particle-free water*. Care must be taken to avoid aggregation of particles during dispersion. ⬥System suitability can be verified by using the USP Particle Count RS.⬥

### General Precautions

The test is carried out under conditions limiting particulate matter, preferably in a laminar flow cabinet.

Very carefully wash the glassware and filtration equipment used, except for the membrane filters, with a warm detergent solution, and rinse with abundant amounts of water to remove all traces of detergent. Immediately before use, rinse the equipment from top to bottom, outside and then inside, with *particle-free water*.

Take care not to introduce air bubbles into the preparation to be examined, especially when fractions of the preparation are being transferred to the container in which the determination is to be carried out.

In order to check that the environment is suitable for the test, that the glassware is properly cleaned, and that the water to be used is particle-free, the following test is carried out. Determine the particulate matter in five samples of *particle-free water*, each of 5 mL, according to the method described below. If the number of particles of 10 µm or greater size exceeds 25 for the combined 25 mL, the precautions taken for the test are not sufficient. The preparatory steps must be repeated until the environment, glassware, and water are suitable for the test.

### Method

Mix the contents of the sample by slowly inverting the container 20 times successively. If necessary, cautiously remove the sealing closure. Clean the outer surfaces of the container opening using a jet of *particle-free water*, and remove the closure, avoiding any contamination of the contents. Eliminate gas bubbles by appropriate measures such as allowing to stand for 2 min or sonicating.

For large-volume parenterals, single units are tested. For small-volume parenterals less than 25 mL in volume, the contents of 10 or more units are combined in a cleaned container to obtain a volume of NLT 25 mL; the test solution may be prepared by mixing the contents of a suitable number of vials and diluting to 25 mL with *particle-free water* or with an appropriate particle-free solvent when *particle-free water* is not suitable. Small-volume parenterals having a volume of 25 mL or more may be tested individually.

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Powders for parenteral use are reconstituted with *particle-free water* or with an appropriate particle-free solvent when *particle-free water* is not suitable.

For pharmacy bulk packages for parenteral use labeled "Not for Direct Infusion", proceed as directed for small-volume parenterals when the volume is 25 mL or more. Calculate the test result on a portion that is equivalent to the maximum dose given in the labeling. For example, if the total bulk package volume is 100 mL and the maximum dose volume is 10 mL, then the average particle count per mL would be multiplied by 10 to obtain the test result based on the 10-mL maximum dose. [NOTE—For the calculation of test results, consider this maximum dose portion to be equivalent to the contents of one full container.]

Products packaged with dual compartments meant to hold a drug product and a solvent should be prepared and tested as directed for large-volume parenterals or small-volume parenterals, depending on container volume. Mix each unit as directed in the labeling, activating and agitating to ensure thorough mixing of the separate components and drug dissolution.

The number of test specimens must be adequate to provide a statistically sound assessment. For large-volume parenterals or for small-volume parenterals having a volume of 25 mL or more, fewer than 10 units may be tested, using an appropriate sampling plan.

Remove four portions, NLT 5 mL each, and count the number of particles equal to or greater than 10 µm and 25 µm. Disregard the result obtained for the first portion, and calculate the mean number of particles for the preparation to be examined.

### Evaluation

For preparations supplied in containers with a nominal volume of more than 100 mL, apply the criteria of *Test 1.A*.
For preparations supplied in containers with a nominal volume of less than 100 mL, apply the criteria of *Test 1.B*.
For preparations supplied in containers with a nominal volume of 100 mL, apply the criteria of *Test 1.B*.
[NOTE—*Test 1.A* is used in the *Japanese Pharmacopoeia*.]
If the average number of particles exceeds the limits, test the preparation by the *Microscopic Particle Count Test*.

TEST 1.A

**Solutions for parenteral infusion or solutions for injection supplied in containers with a nominal content of more than 100 mL:** The preparation complies with the test if the average number of particles present in the units tested does not exceed 25 per mL equal to or greater than 10 µm and does not exceed 3 per mL equal to or greater than 25 µm.

TEST 1.B

**Solutions for parenteral infusion or solutions for injection supplied in containers with a nominal content of less than 100 mL:** The preparation complies with the test if the average number of particles present in the units tested does not exceed 6000 per container equal to or greater than 10 µm and does not exceed 600 per container equal to or greater than 25 µm.

## METHOD 2 MICROSCOPIC PARTICLE COUNT TEST

Use a suitable binocular microscope, a filter assembly for retaining particulate matter, and a membrane filter for examination.

The microscope is adjusted to 100 ± 10 magnifications and is equipped with an ocular micrometer calibrated with an objective micrometer, a mechanical stage capable of holding and traversing the entire filtration area of the membrane filter, and two suitable illuminators to provide episcopic illumination in addition to oblique illumination.

The ocular micrometer is a circular diameter graticule (see *Figure 1*) and consists of a large circle divided by crosshairs into quadrants, transparent and black reference circles 10 µm and 25 µm in diameter at 100 magnifications, and a linear scale graduated in 10-µm increments. It is calibrated using a stage micrometer that is certified by either a domestic or international standard institution. A relative error of the linear scale of the graticule within ±2% is acceptable. The large circle is designated the graticule field of view (GFOV).

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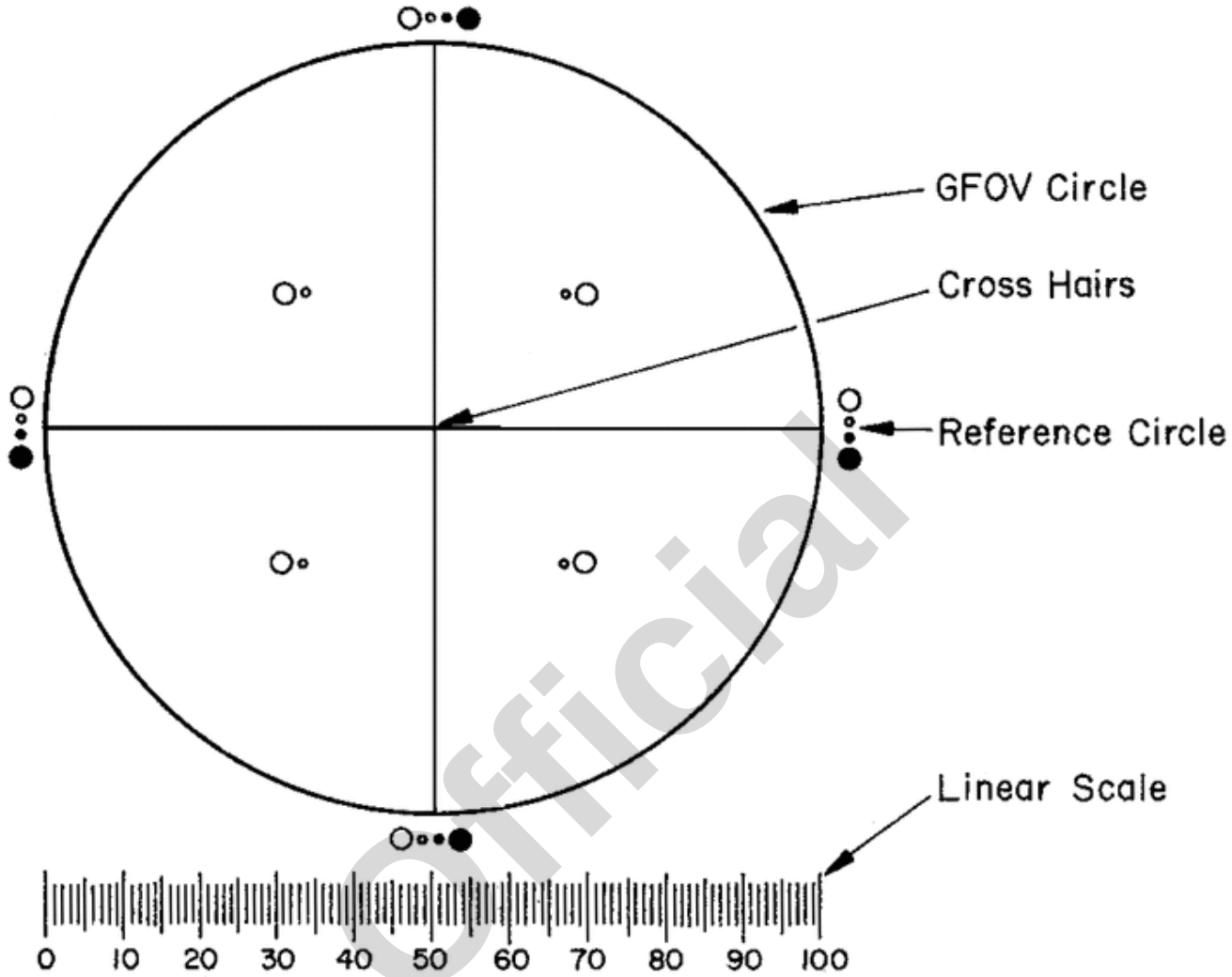


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Figure 1. Circular diameter graticule. The large circle divided by crosshairs into quadrants is designated the graticule field of view (GFOV). Transparent and black circles having 10-µm and 25-µm diameters at 100× are provided as comparison scales for particle sizing.

Two illuminators are required. One is an episcopic brightfield illuminator internal to the microscope, the other is an external, focusable auxiliary illuminator that can be adjusted to give reflected oblique illumination at an angle of 10°–20°.

The filter assembly for retaining particulate matter consists of a filter holder made of glass or other suitable material, and is equipped with a vacuum source and a suitable membrane filter.

The membrane filter is of suitable size, black or dark gray in color, nongridded or gridded, and 1.0 µm or finer in nominal pore size.

## General Precautions

The test is carried out under conditions limiting particulate matter, preferably in a laminar flow cabinet.

Very carefully wash the glassware and filter assembly used, except for the membrane filter, with a warm detergent solution, and rinse with abundant amounts of water to remove all traces of detergent. Immediately before use, rinse both sides of the membrane filter and the equipment from top to bottom, outside and then inside, with *particle-free water*.

In order to check that the environment is suitable for the test, that the glassware and the membrane filter are properly cleaned, and that the water to be used is particle-free, the following test is carried out. Determine the particulate matter of a 50-mL volume of *particle-free water* according to the method described below. If more than 20 particles 10 µm or larger in size or if more than five particles 25 µm or larger in size are present within the filtration area, the precautions taken for the test are not sufficient. The preparatory steps must be repeated until the environment, glassware, membrane filter, and water are suitable for the test.

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## Method

Mix the contents of the samples by slowly inverting the container 20 times successively. If necessary, cautiously remove the sealing closure. Clean the outer surfaces of the container opening using a jet of *particle-free water*, and remove the closure, avoiding any contamination of the contents.

For large-volume parenterals, single units are tested. For small-volume parenterals less than 25 mL in volume, the contents of 10 or more units are combined in a cleaned container; the test solution may be prepared by mixing the contents of a suitable number of vials and diluting to 25 mL with *particle-free water* or with an appropriate particle-free solvent when *particle-free water* is not suitable. Small-volume parenterals having a volume of 25 mL or more may be tested individually.

Powders for parenteral use are constituted with *particle-free water* or with an appropriate particle-free solvent when *particle-free water* is not suitable.

The number of test specimens must be adequate to provide a statistically sound assessment. For large-volume parenterals or for small-volume parenterals having a volume of 25 mL or more, fewer than 10 units may be tested, using an appropriate sampling plan.

Wet the inside of the filter holder fitted with the membrane filter with several mL of *particle-free water*. Transfer to the filtration funnel the total volume of a solution pool or of a single unit, and apply a vacuum. If needed, add stepwise a portion of the solution until the entire volume is filtered. After the last addition of solution, begin rinsing the inner walls of the filter holder by using a jet of *particle-free water*. Maintain the vacuum until the surface of the membrane filter is free from liquid. Place the membrane filter in a Petri dish, and allow the membrane filter to air-dry with the cover slightly ajar. After the membrane filter has been dried, place the Petri dish on the stage of the microscope, scan the entire membrane filter under the reflected light from the illuminating device, and count the number of particles that are equal to or greater than 10 µm and the number of particles that are equal to or greater than 25 µm. Alternatively, partial membrane filter count and determination of the total filter count by calculation is allowed. Calculate the mean number of particles for the preparation to be examined.

The particle sizing process with the use of the circular diameter graticule is carried out by estimating the equivalent diameter of the particle in comparison with the 10 µm and 25 µm reference circles on the graticule. Thereby the particles are not moved from their initial locations within the graticule field of view and are not superimposed on the reference circles for comparison. The inner diameter of the transparent graticule reference circles is used to size white and transparent particles, while dark particles are sized by using the outer diameter of the black opaque graticule reference circles.

In performing the *Microscopic Particle Count Test*, do not attempt to size or enumerate amorphous, semiliquid, or otherwise morphologically indistinct materials that have the appearance of a stain or discoloration on the membrane filter. These materials show little or no surface relief and present a gelatinous or film-like appearance. In such cases, the interpretation of enumeration may be aided by testing a sample of the solution by the *Light Obscuration Particle Count Test*.

## Evaluation

For preparations supplied in containers with a nominal volume of more than 100 mL, apply the criteria of *Test 2.A*.
For preparations supplied in containers with a nominal volume of less than 100 mL, apply the criteria of *Test 2.B*.
For preparations supplied in containers with a nominal volume of 100 mL, apply the criteria of *Test 2.B*.
[NOTE—*Test 2.A* is used in the *Japanese Pharmacopoeia*.]

TEST 2.A

**Solutions for parenteral infusion or solutions for injection supplied in containers with a nominal content of more than 100 mL:** The preparation complies with the test if the average number of particles present in the units tested does not exceed 12 per mL equal to or greater than 10 µm and does not exceed 2 per mL equal to or greater than 25 µm.

TEST 2.B

**Solutions for parenteral infusion or solutions for injection supplied in containers with a nominal content of less than 100 mL:** The preparation complies with the test if the average number of particles present in the units tested does not exceed 3000 per container equal to or greater than 10 µm and does not exceed 300 per container equal to or greater than 25 µm.

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# ⟨789⟩ PARTICULATE MATTER IN OPHTHALMIC SOLUTIONS

Particulate matter consists of mobile, randomly sourced, extraneous substances, other than gas bubbles, that cannot be quantitated by chemical analysis because of the small amount of material they represent and because of their heterogeneous composition. Ophthalmic solutions should be essentially free from particles that can be observed on visual inspection. The tests described herein are physical tests performed for the purpose of enumerating extraneous particles within specific size ranges.

Every ophthalmic solution is subject to the particulate matter limits set forth for the test being applied, unless otherwise specified in the individual monograph. When higher limits are appropriate, they will be specified in the individual monograph. Ophthalmic preparations that are suspensions, emulsions, or gels are exempt from these requirements, as are medical devices. Refer to the specific monograph when a question of test applicability occurs.

Light obscuration and microscopic procedures for the determination of particulate matter in ophthalmic solutions are identical to those for injections; therefore, where appropriate, *USP* general test chapter *Particulate Matter in Injections* ⟨788⟩ is cross-referenced. This chapter provides a test approach in two stages. The ophthalmic solution is first tested by the light obscuration procedure (stage 1). If it fails to meet the prescribed limits, it must pass the microscopic procedure (stage 2) with its own set of test limits. Where for technical reasons the ophthalmic solution cannot be tested by light obscuration, microscopic testing may be used exclusively. Documentation is required, demonstrating that the light obscuration procedure is incapable of testing the ophthalmic solution or that it produces invalid results.

It is expected that most articles will meet the requirements on the basis of the light obscuration test alone; however, it may be necessary to test some articles by the light obscuration test followed by the microscopic test to reach a conclusion on conformance to requirements. Any product that is not a pure solution having a clarity and a viscosity approximating those of water may provide erroneous data when analyzed by the light obscuration counting method. Such materials may be analyzed by the microscopic counting method. In some instances, the viscosity of a material to be tested may be sufficiently high so as to preclude its analysis by either test method. In this event, a quantitative dilution with an appropriate diluent may be made to decrease viscosity, as necessary, to allow the analysis to be performed.

In the tests described below, the results obtained by examining a discrete unit or group of units for particulate matter cannot be extrapolated with certainty to other units that remain untested. Thus, sampling plans based on known operational factors must be developed if valid inferences are to be drawn from observed data to characterize the level of particulate matter in a large group of units. Sampling plans need to be based on consideration of product volume, particle numbers historically found to be present in comparison to limits, size distribution of particles present, and variability of particle counts between units.

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## LIGHT OBSCURATION PARTICLE COUNT TEST

This test applies to ophthalmic solutions, including solutions constituted from sterile solids, for which a test for *Particulate matter* is specified in the individual monograph. The test counts suspended particles that are solid or liquid.

### Test Apparatus, Instrument Standardization, Test Environment, Test Procedure, and Calculations

Proceed as directed in *Particulate Matter in Injections* ⟨788⟩, *Method 1 Light Obscuration Particle Count Test*.

### Interpretation

The ophthalmic solution meets the requirements of the test if the average number of particles present in the units tested does not exceed the appropriate value listed in *Table 1*. If the average number of particles exceeds the limit, test the article by the *Microscopic Particle Count Test*.

**Table 1. Light Obscuration Particle Test Limits**

| | Diameter | | |
|---|---|---|---|
| | ≥10 µm | ≥25 µm | ≥50 µm |
| Number of particles | 50/mL | 5/mL | 2/mL |

## MICROSCOPIC PARTICLE COUNT TEST

Some articles cannot be tested meaningfully by light obscuration. In such cases, individual monographs clearly specify that only a microscopic particle count is to be performed. The microscopic particle count test enumerates subvisible, essentially solid, particulate matter in ophthalmic solutions after collection on a microporous membrane filter. Some ophthalmic solutions, such as solutions that do not filter readily because of their high viscosity, may be exempted from analysis using the microscopic test.

When performing the microscopic test, do not attempt to size or enumerate amorphous, semiliquid, or otherwise morphologically indistinct materials that have the appearance of a stain or discoloration on the membrane surface. These materials show little or no surface relief and present a gelatinous or film-like appearance. Because in solution this material consists of units on the order of 1 µm or less, which may be counted only after aggregation or deformation on an analytical membrane, interpretation of enumeration may be aided by testing a sample of the solution by the light obscuration particle count method.

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## Test Apparatus, Test Environment, Test Procedure, and Enumeration of Particles

Proceed as directed in *Particulate Matter in Injections* ⟨788⟩, *Method 2 Microscopic Particle Count Test*.

## Interpretation

The ophthalmic solution meets the requirements of the test if the average number of particles present in the units tested does not exceed the appropriate value listed in *Table 2*.

**Table 2. Microscopic Particle Test Limits**

| | Diameter | | |
|---|---|---|---|
| | **≥10 µm** | **≥25 µm** | **≥50 µm** |
| Number of particles | 50/mL | 5/mL | 2/mL |

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# ⟨790⟩ VISIBLE PARTICULATES IN INJECTIONS

## INTRODUCTION

All products intended for parenteral administration must be visually inspected for the presence of particulate matter as specified in *Injections and Implanted Drug Products* ⟨1⟩. Dry solids, from which constituted solutions are prepared for injection, meet the requirements for *Completeness and clarity of solutions* in *Injections and Implanted Drug Products* ⟨1⟩ when they are prepared just before use. Where used in this chapter, the term *essentially free* means that when injectable drug products are inspected as described herein, no more than the specified number of units may be observed to contain visible particulates. Particulate matter is defined in *Particulate Matter in Injections* ⟨788⟩ as extraneous mobile undissolved particles, other than gas bubbles, unintentionally present in solutions. See *Subvisible Particulate Matter in Therapeutic Protein Injections* ⟨787⟩ for additional information on inherent, intrinsic, and extrinsic particulates. Examples of such particulate matter include, but are not limited to, fibers, glass, metal, elastomeric materials, and precipitates. However, some products may contain inherent particles or agglomerates; in such cases, requirements for these particular visible particulates are specified in the individual monograph or in the approved regulatory application but the control and acceptance criteria for extraneous particles described in this chapter apply.

Where the nature of the contents or the container–closure system permits only limited capability for inspection of the total contents, the 100% inspection of a batch shall be supplemented with the inspection of constituted (e.g., dried) or withdrawn (e.g., dark amber container, suspensions, highly colored liquids) contents of a sample of containers from the batch. The destructive nature of these tests requires the use of a sample smaller[1] than those traditionally used for non-destructive acceptance sampling after 100% inspection. Although the tests described in this chapter may be useful during studies to examine product stability, this chapter is not intended to establish any new testing requirements for stability studies.

## INSPECTION PROCEDURE

Used along with 100% inspection during the manufacturing process, this procedure is sufficient to demonstrate that the batch is essentially free of visible particulates. A complete program for the control and monitoring of particulate matter remains an essential prerequisite.

Inspected units must be free of visible particulates when examined without magnification (except for optical correction as may be required to establish normal vision) against a black background and against a white background. Illumination at the inspection point is maintained at a minimum intensity between 2000 and 3750 lux. This can be achieved through the use of two 13-W or 15-W fluorescent lamps (e.g., F13/T5 or F15/T8). The use of a high-frequency ballast to reduce flicker from the fluorescent lamps is recommended. Alternative light sources (e.g., incandescent, LED) that provide illumination at the point of inspection within the specified minimum intensity range are acceptable. Higher illumination intensity is recommended for examination of colored solutions or product in containers other than clear glass.

Before performing the inspection, remove any adherent labels from the container, and wash and dry the outside. The unit under inspection should be gently swirled and/or inverted, ensuring that no air bubbles are produced, and inspected for approximately 5 s against each of the backgrounds. The presence of any particles should be recorded.

### Sampling at Batch Release (After 100% Manufacturing Inspection)

Sample and inspect the batch using ANSI/ASQ Z1.4 (or ISO 2859-1). General Inspection Level II, single sampling plans for normal inspection with an AQL of 0.65%. Alternative sampling plans with equivalent or better protection are acceptable. NMT the specified number of units contains visible particulates.

### Product in Distribution[2]

If it becomes necessary to evaluate product that has been shipped to customers (e.g., because of a complaint or regulatory concern), sample and inspect 20 units. If no particles are observed in the sample, the batch is considered essentially free of visible particulates. If available, additional units may be inspected to gain further information on the risk of particulates in the batch.

[1] The special level sampling plans described in ANSI/ASQ Z1.4–2008 or ISO 2859 are appropriate to guide the selection of sample size and acceptance criteria for this purpose.

[2] Testing outlined in *Product in Distribution* is permissible only if *Sampling at Batch Release (after 100% Manufacturing Inspection)* has been successfully completed.

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# ⟨791⟩ pH

## INTRODUCTION

For compendial purposes, pH is defined as the value given by a suitable, properly calibrated, potentiometric sensor and measuring system. [NOTE—The measuring system has traditionally been referred to as the "pH meter." While the pH meter is still in common use, the measuring system can also be embedded inside the pH sensor, and the pH signal can be transmitted digitally to an external device such as a computer, Programmable Logic Controller (PLC), Distributed Control System (DCS), data acquisition system, terminal, or other microprocessor-controlled device.] By definition, pH is equal to $-\log_{10}[a_{H^+}]$ where $a_{H^+}$ is the activity of the hydrogen ($H^+$) or hydronium ion ($H_3O^+$), and the hydrogen ion activity very closely approximates the hydrogen ion concentration.

The practical pH scale is defined:

$$pH = pH_S + [(E - E_S)/k]$$

$E$ = measured potential where the galvanic cell contains the solution under test (pH)
$E_S$ = measured potential where the galvanic cell contains the appropriate buffer solution for calibration ($pH_S$)
$k$ = change in potential/unit change in pH and is derived from the Nernst equation (as follows)

$$k = \log_e(10) \times (RT/nF)$$

$R$ = 8.314 *J*/mole/°K
$T$ = temperature (°K)
$n$ = moles/half-reaction
$F$ = Faraday constant, *96485 C*/mole

The resulting equation is [0.05916 + 0.0001984($T$ – 25°)] volts at temperature $T$. Values of $k$ from 15°–35° are provided in *Table 1*.

**Table 1. Values of *k* for Various Temperatures**

| Temperature (°C) | *k* (V) |
|---|---|
| 15.00 | 0.05718 |
| 20.00 | 0.05817 |
| 25.00 | 0.05916 |
| 30.00 | 0.06016 |
| 35.00 | 0.06115 |

Values of $k$ at other temperatures can be determined from the equation above. For practical purposes, values of $k$ are determined from pH sensor calibration.

## pH MEASUREMENT SYSTEM

The measurement system consists of 1) a measuring electrode sensitive to hydrogen-ion activity, typically a glass electrode, though other electrode types are possible; 2) a suitable reference electrode, for example, a silver–silver chloride electrode; and 3) a voltage measurement system with an input resistance capable of measuring at a high input impedance of the pH sensor. The measuring and reference electrode may be separated or combined. The voltage measurement system may be separated from the pH sensor or integrated into the sensor. For most applications, a temperature measurement will be necessary for compensation of the Nernst temperature influence described above. A temperature device may be embedded into the pH sensor, or an external temperature device may be used.

## INSTRUMENT REQUIREMENTS

The measurement system shall be capable of performing a two-point (or more) pH calibration (see below). The accuracy of the pH measurement system is described in the *Calibration* section. The resolution of the pH measurement system shall be at least 0.01 pH. The instrument shall be capable of temperature-compensating the pH sensor measurement to convert the millivolt signal to pH units at any temperature, either automatically using a temperature device built into the sensor system or by manual entry of the sample temperature into the measurement system. The accuracy of the temperature measurement system shall be ±1°. The resolution of the temperature measurement system shall be at least 0.1°. Lab-based pH measurements are typically performed at 25 ± 2° unless otherwise specified in the individual monograph or herein. However, temperatures outside this range are acceptable if samples are more conveniently prepared at alternative temperatures. Examples of non-lab-based measurements include test samples inside process pipes, vessels, tanks, and other non-standard processing conditions. [NOTE—The definitions of pH, the pH scale, and the values assigned to the buffer solutions for calibration are for the purpose

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of establishing a practical, operational system so that results may be compared between laboratories. The measured pH values do not necessarily correspond exactly to those obtained by the definition, $pH = -\log_{10} [a_{H^+}]$, rather the values obtained are closely related to the activity of the hydrogen-ion in aqueous solutions.] [NOTE—Where a pH measurement system is calibrated by use of an aqueous buffer and then used to measure the pH of nonaqueous systems, the ionization constant of the acid or base, the dielectric constant of the medium, the liquid-junction potential (which may give rise to errors of approximately 1 pH unit), and the hydrogen-ion response of the glass electrode are all changed. For these reasons, the values so obtained with solutions that are only partially aqueous in character can be regarded only as apparent pH values.]

## BUFFER SOLUTIONS FOR CALIBRATION OF THE pH MEASUREMENT SYSTEM

Buffer solutions for calibration are prepared as directed in *Table 2*.[1] Buffer salts of requisite purity can be obtained from the National Institute of Standards and Technology (NIST), other national authorities, or other suppliers. Buffer solutions should be stored in appropriate containers that ensure stability of the pH through the expiry date, and fitted with a tight closure. For buffer solutions greater than 11, the storage should be in containers that are resistant to or reduce carbon dioxide intrusion, which would lower the pH of the buffer. For buffer solutions lower than 11, they should typically be prepared at intervals not to exceed 3 months. For buffer solutions greater than 11, they should typically be prepared and used fresh unless carbon dioxide ingress is restricted. All buffer solutions should be prepared using *Purified Water*. *Table 2* indicates the pH of the buffer solutions as a function of temperature. The instructions presented here are intended for the preparation of solutions having the designated molal (m) concentrations. However, in order to facilitate their preparation, the instructions are given in molarity. The difference in concentration between molality and molarity preparations for these buffer solutions is less than 1%, and the pH difference is negligible. Calibration using buffer solutions shall be done in the temperature range of the buffers listed in *Table 2*. [NOTE—The Nernst temperature compensation corrects only for the electrode millivolt output change with temperature, not the actual pH change of the buffer solution with temperature, which is unique for each buffer.] Features such as automatic buffer recognition or buffer pH–temperature correction are available for convenience in accommodating the temperature influence on buffer solutions. The pH–temperature response can be determined from the values in *Table 2*.

**Table 2. pH Values of Buffer Solutions for Calibration**

| Temperature (°C) | Potassium Tetraoxalate, 0.05 m | Potassium Hydrogen Tartrate, Saturated at 25° | Potassium Dihydrogen Citrate, 0.05 M | Potassium Biphthalate, 0.05 m | Equimolal Phosphate, 0.05 m | Potassium Dihydrogen Phosphate, 0.0087 M and Disodium Hydrogen Phosphate, 0.0303 M | Sodium Tetraborate, 0.01 m | Sodium Carbonate, 0.025 M and Sodium Bicarbonate, 0.025 M | Calcium Hydroxide, Saturated at 25° |
|---|---|---|---|---|---|---|---|---|---|
| 10 | 1.67 | — | — | 4.00 | 6.92 | — | 9.33 | — | 13.00 |
| 15 | 1.67 | — | 3.80 | 4.00 | 6.90 | 7.45 | 9.28 | 10.12 | 12.81 |
| 20 | 1.68 | — | 3.79 | 4.00 | 6.88 | 7.43 | 9.23 | 10.06 | 12.63 |
| 25 | 1.68 | 3.56 | 3.78 | 4.01 | 6.86 | 7.41 | 9.18 | 10.01 | 12.45 |
| 30 | 1.68 | 3.55 | 3.77 | 4.02 | 6.85 | 7.40 | 9.14 | 9.97 | 12.29 |
| 35 | 1.69 | 3.55 | 3.76 | 4.02 | 6.84 | 7.39 | 9.10 | 9.93 | 12.13 |
| 40 | 1.69 | — | — | 4.04 | 6.84 | — | 9.07 | — | 11.98 |
| 45 | 1.70 | — | — | 4.05 | 6.83 | — | 9.04 | — | 11.84 |
| 50 | 1.71 | — | — | 4.06 | 6.83 | — | 9.01 | — | 11.71 |
| 55 | 1.72 | — | — | 4.08 | 6.83 | — | 8.99 | — | 11.57 |
| 60 | 1.72 | — | — | 4.09 | 6.84 | — | 8.96 | — | 11.45 |
| ΔpH/Δ°C | 0.0010 | –0.0014 | –0.0022 | 0.0018 | –0.0016 | –0.0028 | –0.0074 | –0.0096 | –0.0310 |

Preparation of alternative volumes at the same concentrations to those indicated below is acceptable.

**Potassium tetraoxalate, 0.05 m:** Dissolve 12.61 g of $KH_3(C_2O_4)_2 \cdot 2H_2O$, and dilute with water to make 1000.0 mL.

**Potassium hydrogen tartrate, saturated at 25°:** Add $C_4H_5KO_6$ to water until saturation is exceeded at 25°. Then filter or decant.

**Potassium dihydrogen citrate, 0.05 M:** Dissolve 11.41 g of $C_6H_7KO_4$, and dilute with water to make 1000.0 mL.

**Potassium biphthalate, 0.05 m:** Dissolve 10.12 g of $KHC_8H_4O_4$, previously dried at 110° for 1 h, and dilute with water to make 1000.0 mL.

[1] Commercially available buffer solutions for pH measurement system, calibrated by methods traceable to NIST or other national authorities, labeled with a pH value accurate to 0.02 pH units may be used. Solutions prepared from ACS reagent-grade materials or other suitable materials, may be used provided the pH of the resultant solution is the same as that of the solution prepared from the NIST (or other national authorities)-certified material. Buffer solutions that are greater than 12 should be used immediately or should be prepared using freshly boiled water, and they should be stored under conditions to minimize carbon dioxide absorption and ingress.

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**Equimolal phosphate, 0.05 m:** Dissolve 3.53 g of disodium hydrogen phosphate ($Na_2HPO_4$) and 3.39 g of potassium dihydrogen phosphate ($KH_2PO_4$), each previously dried at 120° for 2 h, and dilute with water to make 1000.0 mL.

**Potassium dihydrogen phosphate, 0.0087 M** and **disodium hydrogen phosphate, 0.0303 M:** Dissolve 1.18 g of $KH_2PO_4$ and 4.30 g of $Na_2HPO_4$, both dried for 2 h at 120 ± 2°, and dilute with water to make 1000.0 mL.

**Sodium tetraborate, 0.01 m:** Dissolve 3.80 g of $Na_2B_4O_7 \cdot 10H_2O$, and dilute with water to make 1000.0 mL. Protect from absorption of carbon dioxide.

**Sodium carbonate, 0.025 M** and **sodium bicarbonate, 0.025 M:** Dissolve 2.64 g of sodium carbonate ($Na_2CO_3$) and 2.09 g of sodium bicarbonate ($NaHCO_3$), and dilute with water to make 1000.0 mL.

**Calcium hydroxide, saturated at 25°:** Add $Ca(OH)_2$ to water until saturation is exceeded at 25°. Use water that has been recently boiled and protected from the atmosphere to limit carbon dioxide absorption. Then filter or decant.

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## CALIBRATION

Because of variations in the nature and operation of the available pH measurement systems, it is not practical to provide universal directions for the calibration of the measurement system. However, the general principles to be followed are set forth in the following paragraphs. Examine the electrodes, especially the reference electrode and electrolyte level, if a liquid electrolyte is used. If necessary, replenish electrolyte supply, and observe other precautions indicated by the instrument and electrode manufacturers.

The calibration or verification of the pH measurement system should be periodically executed. The historical performance of the measurement system, the criticality of the pH measurement, the maintenance of the pH sensor, and the frequency of measurement operation are used to determine the frequency of the calibration/verification. ▲The procedure below allows for several calibration methodologies (two-point calibration, multiple-point calibration, and multiple-segment calibration).▲ (USP 1-Aug-2020)

If the pH of the buffer is sensitive to ambient carbon dioxide, then use *Purified Water* that has been recently boiled, and subsequently stored in a container designed to minimize ingress of carbon dioxide.

1. To calibrate the pH measurement system, select three buffer solutions for calibration, preferably from those given in *Table 2*, such that the expected pH of the material under test falls within their range. Two of the buffers are used for the calibration process, and the third buffer is used for verification. The value of the verification buffer shall be between two of the calibration buffers. If the operational range of the pH sensor is beyond the pH range of the buffer solutions in *Table 2*, then either 1) select two nearby pH buffers from *Table 2* or 2) select one from *Table 2* and another documented prepared buffer that is outside the range.
2. Rinse the pH sensor several times with water, then with the first buffer solution.
3. Immerse the pH sensor in the first buffer solution at a temperature within the range of *Table 2*.
4. If automatic temperature measurement and compensation are not included in the measuring system, manually enter the temperature of the buffer and pH value of the buffer solution at that temperature into the instrument. For temperatures not listed in *Table 2*, use linear interpolation to determine the pH value as a function of temperature.
5. Initiate the two-point calibration sequence with the first buffer according to the manufacturer's instructions.
6. Remove the pH sensor from the first buffer and rinse the electrode(s) with water, and then with the second buffer solution.
7. Immerse the pH sensor in the second buffer at a temperature within the range of *Table 2*.
8. If automatic temperature measurement and compensation are not included in the measuring system, manually enter the temperature of the buffer and pH value of the buffer solution at that temperature into the instrument.
9. Continue the two-point calibration sequence with the second buffer according to the manufacturer's instructions.
10. After completion of the two-point calibration process, verify that the pH slope and offset are within acceptable parameters. Typical acceptable parameters are a slope of 90%–105% and an offset of 0 ± 30 mV (0.5 pH units at 25°). Depending on the pH instrumentation, the pH slope and offset may be determined in software or by manual methods. If using manual methods, follow supplier instructions to calculate the pH sensor slope/offset. If these parameters are not within acceptable parameters, the sensor should be properly cleaned, replenished, serviced, or replaced, and the two-point calibration process shall be repeated.
11. Remove the pH sensor from the second buffer, and rinse thoroughly with water, and then the verification buffer.
12. Immerse the pH sensor in the verification buffer at a temperature within the range of *Table 2*.
13. If automatic temperature measurement and compensation are not included in the measuring system, manually enter the temperature of the buffer and pH value of the buffer solution at that temperature into the instrument.
14. The pH reading shall be within ±0.05 pH of the value in *Table 2* at the buffer solution temperature.

▲NOTE—If a multiple-point calibration process (three or more calibration buffers) plus at least one verification buffer are used, then repeat steps 9–14, assuring that the pH sensor slope and offset criteria (see step 10) and the calibration accuracy (see step 14) of this range are met. The value of the verification buffers shall be between the highest and lowest calibration buffers of the range.

NOTE—If a multiple-segment calibration process (three or more calibration buffers with at least two slopes and offsets) plus at least one verification buffer for each segment are used, then repeat steps 9–14 for each segment, assuring that the pH sensor slope and offset criteria (see step 10) and the calibration accuracy (see step 14) of each segment are met. The value of each verification buffer shall be between the highest and lowest calibration buffers for each segment.▲ (USP 1-Aug-2020)

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## OPERATION

All test samples should be prepared using *Purified Water*, unless otherwise specified in the monograph. All test measurements should use manual or automated Nernst temperature compensation.

1. Prepare the test material according to requirements in the monograph or according to specific procedures. If the pH of the test sample is sensitive to ambient carbon dioxide, then use *Purified Water* that has been recently boiled, and subsequently stored in a container designed to minimize ingress of carbon dioxide.
2. Rinse the pH sensor with water, then with a few portions of the test material.
3. Immerse the pH sensor into the test material and record the pH value and temperature.

In all pH measurements, allow sufficient time for stabilization of the temperature and pH measurement.

Diagnostic functions such as glass or reference electrode resistance measurement may be available to determine equipment deficiencies. Refer to the electrode supplier for diagnostic tools to assure proper electrode function.

Where approximate pH values suffice, indicators and test papers (see *Reagents, Indicators, and Solutions—Indicators and Indicator Test Papers, Indicators* and *Indicator and Test Papers*) may be suitable.

For a discussion of buffers, and for the composition of standard buffer solutions called for in compendial tests and assays, see *Reagents, Indicators, and Solutions—Solutions, Buffer Solutions*. This referenced section is not intended to replace the use of the pH calibration buffers in *Table 2*.

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# ⟨795⟩ PHARMACEUTICAL COMPOUNDING—NONSTERILE PREPARATIONS

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## ▲1. INTRODUCTION AND SCOPE

This chapter describes the minimum standards to be followed for the preparation of compounded nonsterile preparations (CNSPs) for humans and animals. For purposes of this chapter, nonsterile compounding is defined as combining, admixing, diluting, pooling, reconstituting other than as provided in the manufacturer's labeling, or otherwise altering a drug product or bulk drug substance to create a nonsterile preparation.

The requirements in this chapter must be followed to minimize harm, including death, to human and animal patients that could result from 1) excessive microbial contamination, 2) variability from the intended strength of correct ingredients (e.g., ±10% of the labeled strength), 3) physical and chemical incompatibilities, 4) chemical and physical contaminants, and/or 5) use of ingredients of inappropriate quality.

Handling of nonsterile hazardous drugs (HDs) must additionally comply with *Hazardous Drugs—Handling in Healthcare Settings* ⟨800⟩.

### 1.1 Scope

**1.1.1 CNSPs subject to the requirements in this chapter:** CNSPs that must comply with this chapter include but are not limited to the following dosage forms:

- Solid oral preparations
- Liquid oral preparations
- Rectal preparations
- Vaginal preparations
- Topical preparations (i.e., creams, gels, and ointments)
- Nasal and sinus preparations intended for local application (i.e., nasal sprays and nasal irrigation)
- Otic preparations (excluding use in perforated eardrums)

**1.1.2 Practices not subject to the requirements in this chapter:** The following practices are not considered compounding and are not required to meet the requirements of this chapter. Handling of nonsterile HDs should additionally comply with ⟨800⟩. Refer to facility SOPs for additional safe practices (e.g., labeling).

- *Nonsterile radiopharmaceuticals:* Compounding of nonsterile radiopharmaceuticals is subject to the requirements in *Radiopharmaceuticals—Preparation, Compounding, Dispensing, and Repackaging* ⟨825⟩.
- *Reconstitution:* Reconstitution of a conventionally manufactured nonsterile product in accordance with the directions contained in the manufacturer approved labeling
- *Repackaging:* Repackaging of conventionally manufactured drug products (see *Good Repackaging Practices* ⟨1178⟩ for recommendations)
- *Splitting tablets:* Breaking or cutting a tablet into smaller portions
- *Administration:* Preparation of a single dose for a single patient when administration will begin within 4 h. This includes crushing a tablet(s) or opening a capsule(s) to mix with food or liquids to facilitate patient dosing.

**1.1.3 Personnel and settings affected:** This chapter applies to all persons who prepare CNSPs and all places where CNSPs are prepared. This includes but is not limited to pharmacists, technicians, nurses, physicians, veterinarians, dentists, naturopaths, and chiropractors in all places including but not limited to pharmacies, hospitals and other healthcare institutions, patient treatment sites, and physicians' or veterinarians' practice sites.

The compounding facility's leadership and all personnel involved in preparing, storing, packaging, dispensing, and transporting CNSPs are responsible for 1) ensuring that the applicable practices and quality standards in this chapter are continually and consistently applied to their operations, and 2) proactively identifying and remedying potential problems within their operations. Personnel engaged in the compounding and dispensing of CNSPs must also comply with laws and regulations of the applicable regulatory jurisdiction.

**1.1.4 Oversight by designated person(s):** The compounding facility must designate one or more individuals to be responsible and accountable for the performance and operation of the facility and personnel for the preparation of CNSPs. The responsibilities of the designated person(s) include but are not limited to:

- Overseeing a training program to ensure competency of personnel involved in compounding, handling, and preparing CNSPs
- Selecting components
- Monitoring and observing compounding activities and taking immediate corrective action if deficient practices are observed
- Ensuring that standard operating procedures (SOPs) are fully implemented. The designated person(s) must ensure that follow-up is carried out if problems, deviations, or errors are identified
- Establishing, monitoring, and documenting procedures for the handling and storage of CNSPs and/or components of CNSPs

The designated person(s) must be identified in the facility's SOPs. If the compounding facility has only one person responsible for all compounding in the facility, then that person is the designated person.

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## 2. PERSONNEL TRAINING AND EVALUATION

All personnel who compound or have direct oversight of compounding CNSPs must be initially trained and qualified by demonstrating knowledge and competency according to the requirements in this section (*2. Personnel Training and Evaluation*) before being allowed to perform their job functions independently.

Designated person(s) are responsible for creating and implementing a training program that describes the required training, the frequency of training, and the process for evaluating the competency of personnel. This program must equip personnel with knowledge and training in the required skills necessary to perform their assigned tasks. Personnel who compound or have direct oversight of compounding personnel must complete training initially and at least every 12 months in appropriate compounding principles and practices as described in this section. Other personnel, who do not compound and only perform functions such as in-process checks, final verification, or dispensing of CNSPs, must undergo training as required by the facility's SOPs.

Training and competency of personnel must be documented as described in *14. Documentation*.

Before beginning to compound CNSPs independently or have direct oversight of compounding personnel, personnel must complete training and be able to demonstrate knowledge of principles and competency of skills for performing nonsterile manipulations as applicable to their assigned tasks. Knowledge and competency must be demonstrated initially and at least every 12 months in at least the following core competencies:

- Hand hygiene
- Garbing
- Cleaning and sanitizing
- Handling and transporting components and CNSPs
- Measuring and mixing
- Proper use of equipment and devices selected to compound CNSPs
- Documentation of the compounding process (e.g., *7. Master Formulation and Compounding Records*)

Steps in the training procedure must include the following:

- Understand the requirements in this chapter
- Understand and interpret safety data sheets (SDSs) and, if applicable, certificates of analysis (COA)
- Read and understand procedures related to their compounding duties

Training and observation may be performed by the designated person(s) or an assigned trainer. Personnel must be observed and guided throughout the training process. The personnel will then be expected to repeat the procedures independently while under the direct supervision of the designated person(s) and/or assigned trainer. Personnel will be permitted to perform the procedure without direct supervision only after independently demonstrating understanding and competency. Upon completion of the training program, the designated person(s) and/or assigned trainer must document that personnel have been trained and successfully completed competency assessments (see *14. Documentation*).

In addition to the initial and annual competency training and evaluation described in this section, the designated person(s) should monitor and observe compounding activities and must take immediate corrective action if deficient practices are observed. Facility SOPs must describe procedures for monitoring and observing compounding activities and personnel.

If the facility has only one person in the compounding operation, that person must document that they have obtained training and demonstrated competency, and they must comply with the other requirements of this chapter.

## 3. PERSONAL HYGIENE AND GARBING

Individuals entering the compounding area must maintain appropriate personal hygiene. Individuals must evaluate whether they have a personal risk of potentially contaminating the compounding environment and CNSP (e.g., personnel with rashes, recent tattoos, oozing sores, conjunctivitis, or active respiratory infection). Individuals must report these conditions to the designated person(s). Because of the risk of contaminating the CNSP and the environment, the designated person(s) is responsible for evaluating whether these individuals should be excluded from working in compounding areas until their conditions have resolved.

### 3.1 Personnel Preparation

Personnel engaged in compounding must maintain appropriate hand hygiene and maintain appropriate cleanliness required for the type of compounding performed.

Before entering the compounding area, compounding personnel must remove any items that are not easily cleanable and that might interfere with garbing. At a minimum, personnel must:

- Remove personal outer garments (e.g., bandanas, coats, hats, and jackets)
- Remove all hand, wrist, and other exposed jewelry, including piercings that could interfere with the effectiveness of garbing or hand hygiene (e.g., watches or rings that may tear gloves)
- Remove earbuds or headphones

The designated person(s) may permit accommodations provided that the quality of the environment and CNSP will not be affected. All accommodations should be documented.

### 3.2 Hand Hygiene

Personnel must perform procedures necessary for appropriate hand hygiene when entering the compounding area to compound as described in *Box 1*.

The use of alcohol-based hand rub alone is not sufficient.

**Box 1. Hand Hygiene Procedures**

- Wash hands with soap and water for at least 30 s
- Dry hands completely with disposable towels or wipers
- Don gloves

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To minimize the risk of cross contaminating other CNSPs and contaminating other objects (e.g., pens and keyboards), gloves should be wiped or replaced before beginning a CNSP that has different components.

All gloves must be inspected for holes, punctures, or tears and must be replaced immediately if such defects are detected.

### 3.3 Garb and Glove Requirements

Gloves must be worn for all compounding activities. Other garb (e.g., shoe covers, head or hair covers, facial hair covers, face masks, and gowns) must be appropriate for the type of compounding performed and should be worn as needed for the protection of personnel from chemical exposures and for prevention of CNSP contamination. Garbing requirements and frequency of changing garb must be determined by the facility and documented in the facility's SOPs.

Garb must be replaced immediately if it becomes visibly soiled or if its integrity is compromised. All gloves must be inspected for holes, punctures, or tears and must be replaced immediately if such defects are detected. Garb must be stored in a manner that minimizes contamination (e.g., away from sinks to avoid splashing).

Garb should be removed when leaving the compounding area. When personnel exit the compounding area, garb, except for gowns, should be discarded. Disposable garb must not be laundered. If gowns are worn, they may be reused if not damaged or soiled. If gowns are to be reused, they must remain in the compounding area, and should only be reused during the same shift. The facility's SOPs must describe cleaning and sanitization procedures for reusing goggles, respirators, and other reusable equipment.

If compounding an HD, appropriate personal protective equipment (PPE) must be worn and disposed of in accordance with ⟨800⟩.

## 4. BUILDINGS AND FACILITIES

### 4.1 Compounding Area

An area must be designated for nonsterile compounding. The method of designation must be described in the facility's SOPs. Other activities must not be occurring in the compounding area at the same time as compounding. The compounding area must be well lit and must be maintained in a clean, orderly, sanitary condition and in a good state of repair. There should not be carpet in the compounding area.

The compounding area must provide for the orderly placement of equipment and materials to prevent mix-ups among components, containers, labels, in-process materials, and finished CNSPs. The area should be designed, arranged, and used in a way that minimizes cross contamination from noncompounding areas.

### 4.2 Storage Area

Compounding personnel must monitor temperatures in the storage area(s) either manually at least once daily on days that the facility is open, or continuously with a temperature recording device to ensure the temperature remains within the appropriate range for the CNSPs and components. The results of the temperature readings must be documented on a temperature log or stored in the continuous temperature recording device and must be retrievable. All temperature monitoring equipment must be calibrated or verified for accuracy as recommended by the manufacturer or every 12 months if not specified by the manufacturer.

The compounding facility must adhere to SOPs to detect and reduce the risk of temperature excursions within the storage area(s).

When it is known that a CNSP or component has been exposed to temperatures either below or above the storage temperature limits for the CNSP or component, personnel must determine whether the CNSP or component integrity or quality has been compromised, and, if so, the CNSP or component must be discarded.

All CNSPs, components, equipment, and containers must be stored off the floor in a manner that prevents contamination and permits inspection and cleaning of the storage area(s).

### 4.3 Water Sources

A source of hot and cold water and an easily accessible sink must be available. The sink must be emptied of all items unrelated to compounding and must be cleaned if visibly soiled before being used to clean any equipment used in nonsterile compounding. The plumbing system must be free of defects that may contribute to the contamination of any CNSP. *Purified Water* (see *Water for Pharmaceutical Purposes* ⟨1231⟩*, 3.1.1 Purified Water*), distilled water, or reverse osmosis water should be used for rinsing equipment and utensils.

## 5. CLEANING AND SANITIZING

Cleaning and sanitizing the surfaces in the nonsterile compounding area(s) must occur on a regular basis at the minimum frequencies specified in *Table 1* or, if compounding is not performed daily, cleaning and sanitizing must be completed before initiating compounding. Cleaning and sanitizing must be repeated when spills occur and when surfaces are visibly soiled. Applicable cleaning and sanitizing must be documented daily on days when compounding occurs.

Surfaces should be resistant to damage by cleaning and sanitizing agents. Floors in the compounding area should be easily cleanable and should not be porous or particle generating.

Cleaning and sanitizing agents must be selected and used with consideration of compatibilities, effectiveness, and minimal potential to leave residues.

If cleaning and sanitizing are performed as separate steps, cleaning must be performed first.

**Table 1. Minimum Frequency for Cleaning and Sanitizing in Nonsterile Compounding Area(s)—Surfaces**

| Site | Minimum Frequency |
|---|---|
| Work surfaces | • At the beginning and end of each shift on days when compounding occurs, after spills, and when surface contamination (e.g., from splashes) is known or suspected<br>• Between compounding CNSPs with different components |
| Floors | • Daily on days when compounding occurs, after spills, and when surface contamination (e.g., from splashes) is known or suspected |
| Walls | • When visibly soiled, after spills, and when surface contamination (e.g., from splashes) is known or suspected |
| Ceilings | • When visibly soiled and when surface contamination (e.g., from splashes) is known or suspected |

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**Table 1. Minimum Frequency for Cleaning and Sanitizing in Nonsterile Compounding Area(s)—Surfaces** *(continued)*

| | |
|---|---|
| Storage shelving | • Every 3 months, after spills, and when surface contamination (e.g., from splashes) is known or suspected |

## 6. EQUIPMENT AND COMPONENTS

### 6.1 Equipment

The equipment and components used for compounding a CNSP must be suitable for the specific compounding process.

Equipment surfaces that contact components must not be reactive, additive, or sorptive, and must not alter the quality of the CNSP. Disposable or dedicated equipment may be used to reduce the chance of bioburden and cross contamination.

Equipment must be stored in a manner that minimizes the risk of contamination and must be located to facilitate equipment use, maintenance, and cleaning. Equipment and devices used in the compounding or testing of compounded preparations must be inspected prior to use and, if appropriate, verified for accuracy as recommended by the manufacturer at the frequency recommended by the manufacturer or at least every 12 months, whichever is more frequent. After compounding, the equipment must be cleaned to prevent cross contamination of the next preparation.

Weighing, measuring, or otherwise manipulating components that could generate airborne chemical particles (e.g., active pharmaceutical ingredients [APIs], added substances, and conventionally manufactured products) must be evaluated to determine if these activities must be performed in a closed-system processing device to reduce the potential exposure to personnel or contamination of the facility or CNSPs. Examples of closed-system processing devices include containment ventilated enclosures (CVEs), biological safety cabinets (BSCs), and single-use containment glove bags. The process evaluation must be carried out in accordance with the facility's SOPs, and the assessment must be documented.

If a CVE or BSC is used, it must be certified at least every 12 months according to manufacturer specifications or other laws and regulations of the applicable regulatory jurisdiction.

If a BSC, CVE, or other nondisposable device is used, it must be cleaned as described in *Table 2*.

**Table 2. Minimum Frequency for Cleaning and Sanitizing in Nonsterile Compounding Area(s)—Equipment**

| Site | Minimum Frequency |
|---|---|
| CVE | • At the beginning and end of each shift on days when compounding occurs, after spills, and when surface contamination (e.g., from splashes) is known or suspected<br>• Clean and sanitize the horizontal work surface of the CVE between compounding CNSPs with different components |
| BSC | • At the beginning and end of each shift on days when compounding occurs, after spills, and when surface contamination (e.g., from splashes) is known or suspected<br>• Clean and sanitize the horizontal work surface of the BSC between compounding CNSPs with different components<br>• Clean and sanitize under the work surface at least monthly |
| Other devices and equipment used in compounding operations | • Before first use and thereafter in accordance with the manufacturer's recommendations<br>• If no recommendation is available, between compounding CNSPs with different components |

### 6.2 Components

The compounding facility must have written SOPs for the selection and inventory control of all components from receipt to use in a CNSP.

SDSs must be readily accessible to all personnel working with components located in the compounding facility. Personnel must be instructed on how to retrieve and interpret needed information.

**6.2.1 Component selection:** Designated person(s) must be responsible for selecting components to be used in compounding.

APIs:

- Must comply with the criteria in the *USP–NF* monograph, if one exists
- Must have a COA that includes specifications (e.g., compendial requirements for quality) and test results for the component that show the API meets expected quality
- In the United States, must be manufactured by an FDA-registered facility
- Outside of the United States, must comply with the laws and regulations of the applicable regulatory jurisdiction

All components other than APIs:

- Should be accompanied by a COA that verifies that the component meets the criteria in the *USP–NF* monograph, if one exists, and any additional specifications for the component
- In the United States, should be manufactured by an FDA-registered facility (If a component cannot be obtained from an FDA-registered facility, the designated person(s) must select a component that is suitable for the intended use.)
- Outside of the United States, must comply with the laws and regulations of the applicable regulatory jurisdiction

Water:

- *Purified Water* or better quality, e.g., *Sterile Water for Irrigation*, must be used for compounding nonsterile drug preparations when formulations indicate the inclusion of water

**6.2.2 Component receipt:** Upon receipt of components other than conventionally manufactured products, the COA must be reviewed to ensure that the component has met the acceptance criteria in an appropriate *USP–NF* monograph, if one exists. The following information must be documented (see *14. Documentation*) according to the facility's SOPs:

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receipt date, quantity received, supplier name, lot number, expiration date, and results of any in-house or third-party testing performed.

For all components that lack a vendor expiration date, the date of receipt by the compounding facility must be clearly and indelibly marked on each packaging system. Packaging systems of components (i.e., API and added substances) that lack a vendor's expiration date must not be used by the compounding facility after 3 years from the date of receipt. A shorter expiration date must be assigned according to *Pharmaceutical Compounding—Sterile Preparations* ⟨*797*⟩*, 9.3.2 Component receipt* if the same component container is also used in sterile compounding or if the ingredient is known to be susceptible to degradation.

Any component found to be of unacceptable quality must be promptly rejected, clearly labeled as rejected, and segregated from active stock to prevent use before appropriate disposal. Any other lots of that component from the same vendor must be examined to determine whether the other lots have the same defect.

**6.2.3 Component evaluation before use:** Before use, compounding personnel must visually re-inspect all components. Each packaging system must be inspected to detect any container breakage, looseness of the cap or closure, or deviation from the expected appearance or texture of the contents that might have occurred during storage.

Compounding personnel must ascertain before use that components are of the correct identity based on the labeling and have been stored under required conditions in the facility.

If the identity, strength, purity, and quality of components intended for preparation of CNSPs cannot be verified (e.g., containers with damaged or incomplete labeling), the components must be immediately rejected. Any component found to be of unacceptable quality must be promptly rejected, clearly labeled as rejected, and segregated from active stock to prevent use before appropriate disposal.

**6.2.4 Component handling:** All components must be handled in accordance with the manufacturer's instructions or per laws and regulations of the applicable regulatory jurisdiction. The handling must minimize the risk of contamination, mix-ups, and deterioration (e.g., loss of identity, strength, purity, or quality). For each use, the lot must be examined for evidence of deterioration and other aspects of unacceptable quality. Once removed from the original container, any component not used in compounding (e.g., excess after weighing) should be discarded and not returned to the original container to minimize the risk of contaminating the original container.

**6.2.5 Component spill and disposal:** The facility must maintain current chemical hazard and disposal information (e.g., SDSs). Such information must be made accessible to compounding personnel.

The management and documentation of nonhazardous component spills and disposal must be described in the facility's SOPs.

The facility must have a readily accessible spill kit in the compounding area.

All personnel who may be required to remediate a spill must receive training in spill management of chemicals used and stored at the compounding facility. Training must be conducted at least every 12 months and documented for all personnel who may be required to clean up a spill.

Waste of any component must be disposed of in accordance with laws and regulations of the applicable regulatory jurisdiction. For information on the handling of HDs, see ⟨800⟩.

## 7. MASTER FORMULATION AND COMPOUNDING RECORDS

### 7.1 Creating Master Formulation Records

A master formulation record (MFR) is a detailed record of procedures that describes how the CNSP is to be prepared. An MFR must be created for each unique formulation of a CNSP. CNSPs are prepared according to the MFR, and the details of each preparation are documented on a compounding record (see *7.2 Creating Compounding Records*). Any changes or alterations to the MFR must be approved and documented according to the facility's SOP. See *Box 2* for information that must be included in an MFR.

**Box 2. Master Formulation Record**

An MFR must include at least the following information:

- Name, strength or activity, and dosage form of the CNSP
- Identities and amounts of all components; if applicable, relevant characteristics of components (e.g., particle size, salt form, purity grade, solubility)
- Container closure system(s)
- Complete instructions for preparing the CNSP including equipment, supplies, and description of compounding steps
- Physical description of the final CNSP
- Beyond-use date (BUD) and storage requirements
- Reference source to support the assigned BUD
- If applicable, calculations to determine and verify quantities and/or concentrations of components and strength or activity of the API(s)
- Labeling requirements (e.g., shake well)
- Quality control (QC) procedures (e.g., pH testing, visual inspection) and expected results
- Other information needed to describe the compounding process and ensure repeatability (e.g., adjusting pH, temperature)

### 7.2 Creating Compounding Records

A compounding record (CR) documents the compounding of each CNSP. A CR must be created for all CNSPs. Each CR must be reviewed for completeness before the CNSP is released. The name or other unique identifier of the person completing the review and the date of the review must be documented on the CR. The CR must permit traceability of all components in the case of a recall or known quality issue. The MFR can be used as the basis for preparing the CR. For example, a duplicate can be made of the MFR with blank fields for recording the information necessary to complete the CR. See *Box 3* for information that must be included in a CR.

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**Box 3. Compounding Record**

A CR must include at least the following information:

- Name, strength or activity, and dosage form of the CNSP
- Date—or date and time—of preparation of the CNSP
- Assigned internal identification number (e.g., prescription, order, or lot number)
- A method to identify the individuals involved in the compounding process and individuals verifying the final CNSP
- Name, vendor or manufacturer, lot number, and expiration date of each component
- Weight or measurement of each component
- Total quantity of the CNSP compounded
- Assigned beyond-use date (BUD) and storage requirements
- If applicable, calculations to determine and verify quantities and/or concentrations of components and strength or activity of the API(s)
- Physical description of the final CNSP
- Results of quality control procedures (e.g., pH testing and visual inspection)
- MFR reference for the CNSP

## 8. RELEASE INSPECTIONS AND TESTING

All release inspections must be included in the facility's documentation (see *7. Master Formulation and Compounding Records* and *11. SOPs*). All checks, inspections, and any other required tests to ensure the quality of the CNSP must be detailed in the facility's MFR.

### 8.1 Visual Inspection

At the completion of compounding, before releasing and dispensing, the CNSP must be visually inspected to determine whether the physical appearance of the CNSP is as expected (e.g., color, texture, physical uniformity). Some CNSPs, as noted in their MFR, also must be visually checked for certain characteristics (e.g., emulsions must be checked for phase separation). The CNSP must be visually inspected to confirm that the CNSP and its labeling match the CR and the prescription or medication order. The inspection also must include a visual inspection of container closure integrity (e.g., checking for leakage, cracks in the container, or improper seals).

When a CNSP will not be released or dispensed on the day of preparation, a visual inspection must be conducted immediately before it is released or dispensed to make sure that the CNSP does not exhibit any defects (e.g., leakage) that could develop during storage. Any CNSP found to be of unacceptable quality (e.g., observed defects) must be promptly rejected, clearly labeled as rejected, and segregated from active stock to prevent use before appropriate disposal.

## 9. LABELING

Every CNSP must be labeled with appropriate, legible identifying information to prevent errors during storage, dispensing, and use. The term *labeling* designates all labels and other written, printed, or graphic matter on the immediate container or on or inside any packaging system or wrapper in which the article is enclosed, except any outer shipping container. The term *label* designates the part of the labeling on the immediate container. (See *Labeling* ⟨*7*⟩.)

All labeling must be in compliance with laws and regulations of the applicable regulatory jurisdiction.

The label on each container of the prepared CNSP must, at a minimum, display prominently and legibly the following information:

- Assigned internal identification number (e.g., barcode, prescription, order, or lot number)
- Active ingredient(s), and their amount(s), activity(ies), or concentration(s)
- Storage conditions if other than controlled room temperature
- BUD
- Dosage form
- Total amount or volume if it is not obvious from the container

The labeling on the dispensed CNSP should display the following information:

- Route of administration
- Indication that the preparation is compounded
- Any applicable special handling instructions
- Any applicable warning statements
- Compounding facility name, and contact information if the CNSP is to be sent outside of the facility or healthcare system in which it was compounded

Labeling procedures must be followed as described in the facility's SOPs to prevent labeling errors and CNSP mix-ups.

The label of the CNSP must be verified to ensure that it conforms with the following:

1. Prescription or medication order;
2. MFR (see *7.1 Creating Master Formulation Records*); and
3. CR (see *7.2 Creating Compounding Records*).

## 10. ESTABLISHING BEYOND-USE DATES

### 10.1 Terminology

Each CNSP label must state the date, or the hour and date, beyond which the preparation cannot be used and must be discarded (i.e., the BUD). BUDs for CNSPs are calculated in terms of hours, days, or months.

BUDs and expiration dates are not the same. An expiration date identifies the time during which a conventionally manufactured product, API, or added substance can be expected to meet the requirements of a compendial monograph, if one exists, or maintain expected quality, provided it is kept under the prescribed storage conditions. The expiration date limits the time during which a conventionally manufactured product, API, or added substance may be dispensed or used (see ⟨*7*⟩, *Labels and Labeling for Products in Other Categories, Expiration Date and Beyond-Use Date*).

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### 10.2 Parameters to Consider in Establishing a BUD

BUDs for CNSPs should be established conservatively to ensure that the preparation maintains its required characteristics to minimize the risk of contamination or degradation.

When establishing a BUD for a CNSP, compounders must consider parameters that may affect quality, including but not limited to the following:

- Chemical and physical stability properties of the API and any added substances in the preparation (e.g., if the API and added substances in the preparation are known to rapidly degrade over time and/or under certain storage conditions, reduce the strength of the preparation, or produce harmful impurities)
- Compatibility of the container closure system with the finished preparation (e.g., leachables, interactions, adsorption, and storage conditions)
- Degradation of the container closure system, which can lead to a reduction in integrity of the CNSP
- Potential for microbial proliferation in the CNSP
- Significant deviations from essential compounding steps and procedures; changes to essential compounding steps may have an impact on the stability of the formulation

### 10.3 Establishing a BUD for a CNSP

The BUDs in *Table 4* are based on the ability of the CNSP to maintain chemical and physical stability and to suppress microbial growth. These BUDs represent the limit for CNSPs that are packaged in tight, light-resistant containers unless conditions in *10.4 CNSPs Requiring Shorter BUDs* or *10.5 Extending BUDs for CNSPs* apply.

The aqueous and nonaqueous dosage forms in *Table 4* are defined based on the water activity ($a_w$) of the most similar drug preparations described in *Table 3* or *Application of Water Activity Determination to Nonsterile Pharmaceutical Products* ⟨1112⟩. In general, the use of $a_w$ aids in assessing the susceptibility of CNSPs to microbial contamination and the potential for API degradation due to hydrolysis. The $a_w$ is different from the water content and may be considered as the available water to support microbial growth and hydrolytic reactions. Nonaqueous dosage forms will not support spore germination or microbial growth due to their low $a_w$. Reduced $a_w$ greatly assists in the prevention of microbial proliferation in conventionally manufactured products and is expected to convey the same benefit to CNSPs.

Compounders are not required to measure $a_w$ for CNSPs. While the manufactured products in ⟨1112⟩, *Table 2* and compounded preparations in *Table 3* below are not exhaustive, they provide examples of dosage forms that have an $a_w < 0.6$ and those that have an $a_w \geq 0.6$ and can assist personnel in determining the BUD by dosage form using *Table 4*.

When preparing CNSPs, raw materials and equipment contribute a bioburden to the final preparation. CNSPs with an $a_w \geq 0.6$, and a BUD within the limits of *Table 4*, should contain suitable antimicrobial agents to protect against the proliferation of bacteria, yeast, and mold contamination that may be inadvertently introduced anytime during the compounding process or throughout the BUD under appropriate handling and storage conditions. All CNSPs with an extended BUD must follow *10.5 Extending BUDs for CNSPs*. Careful consideration should be taken when selecting a preservative to ensure microbiological effectiveness and stability. When antimicrobial preservatives are contraindicated in a CNSP, storage of the preparation in a refrigerator is required if such storage does not change the physical or chemical properties of the CNSP (i.e., precipitation).

**Table 3. Water Activity of Common Compounded Nonsterile Dosage Forms[a]**

| Nonaqueous Dosage Forms: $a_w < 0.6$ | | | Aqueous Dosage Forms: $a_w \geq 0.6$ | | |
|---|---|---|---|---|---|
| **Dosage Form** | **Description** | **$a_w$** | **Dosage Form** | **Description** | **$a_w$** |
| Animal treat | Animal treat (oil flavor) | 0.507 | Animal treat | Animal treat with 15%–18% aqueous flavor | 0.716 |
| Capsule (oil filled) | Olive oil encapsulated | 0.468 | Cream | Cream vehicle (oil in water emulsion, petrolatum free) | 0.968 |
| Capsule (powder filled) | Powder base encapsulated | 0.435 | Cream | Emollient cream (petrolatum and mineral oil) | 0.984 |
| Gel (glycol based) | Propylene glycol, ethoxy diglycol, hydroxypropyl cellulose gel | 0.056 | Cream | Cream (oil in water emulsion with natural oils) | 0.989 |
| Lollipop (sorbitol based) | Sorbitol-based lollipop | 0.460 | Foam | Foaming surfactant solution | 0.983 |
| Ointment | Hydrophilic petrolatum | 0.396 | Gel (water based) | Alcohol-free aqueous gel | 0.990 |
| Ointment | Polyethylene and mineral oil gel base | 0.459 | Gel (water based) | Hydroxypropyl methylcellulose (HPMC) gel | 1.000 |
| Oral solution (glycol based) | 20% Polyethylene glycol and 80% propylene glycol | 0.009 | Lotion | Lotion (oil in water emulsion) | 0.986 |
| Oral solution (oil based) | Medium chain triglycerides oil | 0.338 | Nasal spray | Nasal spray | 0.991 |
| Oral suspension (fixed oil) | Fixed oil with thickener | 0.403 | Oral solution (water based) | Low-sucrose syrup vehicle | 0.906 |

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**Table 3. Water Activity of Common Compounded Nonsterile Dosage Forms[a]** *(continued)*

| Nonaqueous Dosage Forms: $a_w < 0.6$ | | | Aqueous Dosage Forms: $a_w \geq 0.6$ | | |
|---|---|---|---|---|---|
| **Dosage Form** | **Description** | $a_w$ | **Dosage Form** | **Description** | $a_w$ |
| Powder for inhalation | Encapsulated powder for inhalation | 0.402 | Oral solution (water based) | 90% Water and 10% glycerin | 0.958 |
| Stick | Lip balm | 0.181 | Oral suspension (water based) | Oral suspension base | 0.992 |
| Suppository | Polyethylene glycol base | 0.374 | Rinse | Polymer gel with 30% water | 0.960 |
| Suppository | Fatty acid base | 0.385 | Shampoo | Shampoo | 0.976 |
| Tablet (compressed) | Compressed tablet | 0.465 | Simple syrup | Simple syrup | 0.831 |
| Tablet (triturate) | Tablet triturate (lactose and/or sucrose) | 0.427 | — | — | — |
| Troche or lozenge (gelatin based) | Gelatin troche or lozenge with NMT 3% aqueous flavor | 0.332 | — | — | — |
| Troche or lozenge (glycol based) | Polyethylene glycol troche or lozenge with NMT 3% aqueous flavor | 0.571 | — | — | — |

[a] The measured $a_w$ values in *Table 3* for the different dosage forms are intended to be representative examples. The descriptions listed are details about the tested formulation and are provided to assist personnel in determining whether their CNSPs are aqueous or nonaqueous.

**Table 4. BUD Limit by Type of Preparation in the Absence of a *USP–NF* Compounded Preparation Monograph or CNSP-Specific Stability Information[a]**

| Type of Preparation | BUD (days) | Storage Temperature[b] |
|---|---|---|
| **Aqueous Dosage Forms ($a_w \geq 0.60$)** | | |
| Nonpreserved aqueous dosage forms[c] | 14 | Refrigerator |
| Preserved aqueous dosage forms[c] | 35 | Controlled room temperature or refrigerator |
| **Nonaqueous Dosage Forms ($a_w < 0.60$)** | | |
| Oral liquids (nonaqueous)[d] | 90 | Controlled room temperature or refrigerator |
| Other nonaqueous dosage forms[e] | 180 | Controlled room temperature or refrigerator |

[a] A shorter BUD must be assigned when the physical and chemical stability of the CNSP is less than the BUD limit stated in the table (see *10.4 CNSPs Requiring Shorter BUDs*).
[b] See *Packaging and Storage Requirements* ⟨659⟩.
[c] An aqueous preparation is one that has an $a_w \geq 0.6$ (e.g., emulsions, gels, creams, solutions, sprays, or suspensions).
[d] A nonaqueous oral liquid is one that has an $a_w < 0.6$.
[e] Other nonaqueous dosage forms that have an $a_w < 0.6$ (e.g., capsules, tablets, granules, powders, nonaqueous topicals, suppositories, and troches or lozenges).

### 10.4 CNSPs Requiring Shorter BUDs

The BUDs in *Table 4* are the BUD limits for CNSPs in the absence of specific stability information. This does not absolve the designated person(s) from performing due diligence to determine if there is existing stability data that would require a shorter BUD.

Additionally,

- The BUD of the CNSP must not exceed the shortest remaining expiration date of any of the commercially available starting components.
- For CNSPs prepared from one or more compounded components, the BUD should generally not exceed the shortest BUD of any of the individual compounded components. However, there may be acceptable instances when the BUD of the final CNSP exceeds the BUD assigned to compounded components (e.g., pH-altering solutions). If the assigned BUD of the final CNSP exceeds the BUD of the compounded components, the physical, chemical, and microbiological quality of the final CNSP must not be negatively impacted.

### 10.5 Extending BUDs for CNSPs

- *CNSPs with a USP–NF monograph:* When compounding from a *USP–NF* compounded preparation monograph for the CNSP, the BUD must not exceed the BUD specified in the monograph.
- *CNSPs with stability information:* If there is a stability study using a stability-indicating analytical method for the API(s), CNSP formulation, and material of composition of the container closure that will be used, then the BUD indicated by the study may be used in lieu of the BUDs specified in *Table 4* for aqueous and nonaqueous dosage forms, up to a maximum of 180 days.

If the BUD of the CNSP is extended beyond the BUDs in *Table 4*, an aqueous CNSP must be tested for antimicrobial effectiveness (see *Antimicrobial Effectiveness Testing* ⟨51⟩). The designated person(s) may rely on antimicrobial effectiveness testing that is conducted (or contracted for) once for each formulation in the particular container closure system—including materials of composition of the container closure system—in which it will be packaged. Alternatively,

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the designated person(s) may rely on antimicrobial effectiveness testing results provided by an FDA-registered facility or published in peer-reviewed literature as long as the CNSP formulation (including any preservative) and container closure materials of composition are the same as those tested (unless a bracketing study is performed). When a bracketing study is performed, antimicrobial effectiveness testing may be performed on a low concentration and on a high concentration of the active ingredient in the formulation to establish preservative effectiveness across various strengths of the same formulation (e.g., bracketing). The concentration of all other ingredients (including preservatives) must fall within the bracketed range.

## 11. SOPS

Facilities preparing CNSPs must develop SOPs on all aspects of the compounding operation. All personnel who conduct or oversee compounding activities must be trained in the facility's SOPs and be responsible for ensuring that they are followed.

One or more person(s) must be designated to ensure that the facility's SOPs are fully implemented. The designated person(s) must ensure that follow-up occurs if problems, deviations, or errors are identified.

## 12. QUALITY ASSURANCE AND QUALITY CONTROL

Quality assurance (QA) is a system of procedures, activities, and oversight that ensures that the compounding process consistently meets quality standards. Quality control (QC) is the sampling, testing, and documentation of results that, taken together, ensure that specifications have been met before release of the CNSP. See *Quality Assurance in Pharmaceutical Compounding* ⟨1163⟩.

A facility's QA and QC programs must be formally established and documented in the facility's SOPs that ensure that all aspects of the preparation of CNSPs are conducted in accordance with the requirements in this chapter ( ⟨795⟩) and the laws and regulations of the applicable regulatory jurisdiction. Designated person(s) must ensure that the facility has formal, written QA and QC programs that establish a system of

1. Adherence to procedures,
2. Prevention and detection of errors and other quality problems,
3. Evaluation of complaints and adverse events, and
4. Appropriate investigations and corrective actions.

The facility's SOPs must describe the roles, duties, and training of the personnel responsible for each aspect of the QA program. Designated person(s) responsible for the QA program must have the training, experience, responsibility, and authority to perform these duties. The overall QA and QC program must be reviewed at least once every 12 months by the designated person(s). The results of the review must be documented, and appropriate action must be taken if needed.

### 12.1 Notification About and Recall of Dispensed CNSPs

The facility must have procedures in place to

- Determine when recalls must be initiated, which should include procedures to immediately notify the prescriber of a failure of specifications with the potential to cause patient harm (e.g., strength, purity, or other quality attributes)
- Recall any unused dispensed CNSPs and quarantine any stock remaining in the pharmacy
- Investigate if other lots are affected and recall if necessary

An SOP for recall of dispensed CNSPs must contain

- Procedures to determine the severity of the problem and the urgency for implementation and completion of the recall
- Procedures to determine the distribution of any affected CNSP, including the data and quantity of distribution
- Procedures to identify patients who have received the CNSP
- Procedures for disposal and documentation of the recalled CNSP
- Procedures to investigate and document the reason for recall

The nonsterile compounding facility must document the implementation of the recall procedures. The recall must be reported to appropriate regulatory bodies as required by the laws and regulations of the applicable regulatory jurisdiction.

### 12.2 Complaint Handling

Compounding facilities must develop and implement SOPs for handling complaints. Complaints may include but are not limited to concerns or reports on the quality, labeling, or possible adverse reactions related to a specific CNSP.

A designated person(s) must review all complaints to determine whether the complaint indicates a potential quality problem with the CNSP. If it does, a thorough investigation into the cause of the problem must be initiated and completed. The investigation must consider whether the quality problem extends to other CNSPs. Corrective action, if necessary, must be implemented for all potentially affected CNSPs.

Consider whether to initiate a recall of potentially affected CNSPs and whether to cease nonsterile compounding processes until all underlying problems have been identified and corrected.

A readily retrievable written or electronic record of each complaint must be kept by the facility, regardless of the source of the complaint (e.g., email, telephone, or mail). The record must contain the name of the complainant or other unique identifier, the date the complaint was received, the nature of the complaint, and the response to the complaint. In addition, to the extent that the information is known, the following should be recorded: the name and strength of the CNSP and the assigned internal identification number (e.g., prescription, order, or lot number).

The record must also include the findings of any investigation and any follow-up. Records of complaints must be easily retrievable for review and evaluation for possible trends and must be retained in accordance with the record-keeping requirements in *14. Documentation*. A CNSP that is returned in connection with a complaint must be quarantined until it is destroyed after completion of the investigation and in accordance with laws and regulations of the applicable regulatory jurisdiction.

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### 12.3 Adverse Event Reporting

Adverse events potentially associated with the quality of CNSPs must be reported in accordance with the facility's SOPs and all laws and regulations of the applicable regulatory jurisdiction. If the investigation into an adverse event reveals a quality problem with a CNSP that is likely to affect other patients, those patients and prescribers potentially affected must be informed.

## 13. CNSP PACKAGING AND TRANSPORTING

### 13.1 Packaging of CNSPs

The facility's SOPs must describe packaging of CNSPs. Personnel should select and use packaging materials that will maintain the physical and chemical integrity and stability of the CNSPs. Packaging materials must protect CNSPs from damage, leakage, contamination, and degradation, while simultaneously protecting personnel from exposure.

### 13.2 Transporting of CNSPs

If transporting CNSPs, the facility must have written SOPs to describe the mode of transportation, any special handling instructions, and whether temperature monitoring devices are needed.

## 14. DOCUMENTATION

All facilities where CNSPs are prepared must have and maintain written or electronic documentation to demonstrate compliance with the requirements in this chapter. This documentation must include, but is not limited to, the following:

- Personnel training, competency assessments, and qualification records including corrective actions for any failures
- Equipment records (e.g., calibration, verification, and maintenance reports)
- COAs and all documentation required for components not conventionally manufactured
- Receipt of components
- SOPs, MFRs, and CRs
- Release inspection and testing records
- Information related to complaints and adverse events including corrective actions taken
- Results of investigations and corrective actions
- Records of cleaning and sanitizing the designated compounding area
- Temperature logs
- Accommodations to personnel compounding CNSPs
- Any required routine review (e.g., yearly review of QA and QC programs, yearly review of chemical hazard and disposal information)

Documentation must comply with all laws and regulations of the applicable regulatory jurisdiction. Records must be legible and stored in a manner that prevents their deterioration and/or loss. All required CRs for a particular CNSP (e.g., MFR, CR, and release inspection and testing results) must be readily retrievable for at least 2 years after preparation or as required by the laws and regulations of the applicable regulatory jurisdiction, whichever is longer.

## GLOSSARY

**Active pharmaceutical ingredient (API):** Any substance or mixture of substances intended to be used in the compounding of a preparation, thereby becoming the active ingredient in that preparation and furnishing pharmacological activity or other direct effect in the diagnosis, cure, mitigation, treatment, or prevention of disease in humans or animals or affecting the structure and function of the body. Also referred to as *Bulk drug substance*. A conventionally manufactured drug product is not an API but is typically manufactured from an API(s).

**Added substance:** An ingredient that is necessary to compound a preparation but is not intended or expected to cause a pharmacologic response if administered alone in the amount or concentration contained in a single dose of the compounded preparation. The term is used synonymously with inactive ingredient, excipient, and pharmaceutical ingredient.

**Administration:** The preparation of a nonsterile pharmacologic or other therapeutic agent for ingesting, inserting, applying, or otherwise providing a nonsterile medication in its final form to a single patient. This includes crushing a tablet(s) or opening a capsule(s) to mix with food or liquids to facilitate patient dosing.

**Alcohol-based hand rub:** An alcohol-containing preparation (liquid, gel, or foam) designed for application to the hands of healthcare personnel to inactivate microorganisms and/or temporarily suppress their growth. Such preparations may contain one or more types of alcohol, other active ingredients, excipients, and humectants.

**Assigned trainer:** One or more individuals assigned by the designated person(s) to be responsible and accountable for directly providing the training, observation, and/or evaluation of personnel for the preparation of CNSPs.

**Beyond-use date (BUD):** The date, or hour and date, after which a CNSP must not be used, stored, or transported. The date is determined from the date or time the preparation is compounded.

**Biological safety cabinet (BSC):** A ventilated cabinet that may be used for compounding. These cabinets are divided into three general classes (Class I, Class II, and Class III). Class II BSCs are further divided into types (Type A1, Type A2, Type B1, Type B2, and Type C1).

**Bulk drug substance:** See the entry for *Active pharmaceutical ingredient*.

**Certificate of analysis (COA):** A report from the supplier of a component, container, or closure that accompanies the supplier's material and contains the specifications and results of all analyses and a description of the material.

**Cleaning:** The process of removing substances (e.g., organic and inorganic material) from objects and surfaces, normally accomplished by manually or mechanically using water with detergents or enzymatic products.

**Cleaning agent:** An agent, usually containing a surfactant, used for the removal of substances (e.g., dirt, debris, microbes, and residual drugs or chemicals) from surfaces.

**Closed-system processing device:** A device designed to reduce the potential exposure to personnel or contamination of the facility or CNSPs. Examples include CVEs, BSCs, or single-use containment glove bags.

**Component:** Any ingredient used in the compounding of a preparation, including any API, added substance, or conventionally manufactured product.

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**Compounded nonsterile preparation (CNSP):** A preparation not intended to be sterile that is created by combining, admixing, diluting, pooling, reconstituting other than as provided in the manufacturer's labeling, or otherwise altering a drug product or bulk drug substance.
**Compounding:** The process of combining, admixing, diluting, pooling, reconstituting other than as provided in the manufacturer's labeling, or otherwise altering a drug product or bulk drug substance to create a nonsterile preparation.
**Compounding area:** An area that is specifically designated for nonsterile compounding.
**Compounding personnel:** Personnel trained to compound or oversee compounding of preparations.
**Compounding record (CR):** Documents the compounding of each CNSP.
**Container closure system:** Packaging system components that together contain and protect the dosage form. This includes primary packaging system components and secondary packaging system components if the latter are intended to provide additional protection.
**Containment glove bag:** A single-use disposable glove bag that is capable of containing airborne chemical particles.
**Containment ventilated enclosure (CVE):** A full or partial enclosure that uses ventilation principles to capture, contain, and remove airborne contaminants through high-efficiency particulate air (HEPA) filtration and to prevent their release into the work environment.
**Conventionally manufactured product:** A pharmaceutical dosage form, usually the subject of an application approved by the applicable national regulatory agency, that is manufactured under current good manufacturing practice conditions.
**Designated person(s):** One or more individuals assigned to be responsible and accountable for the performance and operation of the facility and personnel as related to the preparation of CNSPs.
**FDA:** US Food and Drug Administration.
**Formulation:** The specific qualitative and quantitative composition of the final CNSP.
**Hazardous drug (HD):** Any drug identified by at least one of the following criteria: carcinogenicity, teratogenicity or developmental toxicity; reproductive toxicity in humans; organ toxicity at low dose in humans or animals; genotoxicity or new drugs that mimic existing HDs in structure or toxicity. See ⟨800⟩.
**Label:** The part of the labeling on the immediate container.
**Labeling:** All labels and other written, printed, or graphic matter on the immediate container or on or inside any packaging system or wrapper in which the article is enclosed, except for any outer shipping container.
**Master formulation record (MFR):** A detailed record of procedures that describes how the CNSP is to be prepared.
**Monograph:** A quality documentary standard within *USP–NF* that articulates the quality expectations for a medicine including for its identity, strength, purity, and performance. It also describes the tests to validate that a medicine and its ingredients meet these criteria.
**Oversight:** The review, monitoring, and supervision of actions taken by personnel; bearing responsibility for those actions; and being available if and when needed for consultation even if not physically present.
**Preservative:** A substance added to inhibit microbial growth.
**Purified water:** The minimal quality of source water for the production of *Purified Water* is drinking water whose attributes are prescribed by the US Environmental Protection Agency (EPA), the European Union, Japan, or the World Health Organization (WHO). This source water may be purified using unit operations that include deionization, distillation, ion exchange, reverse osmosis, filtration, or other suitable purification procedures. (See ⟨1231⟩*, 3.1.1 Purified Water*.)
**Quality assurance (QA):** A system of procedures, activities, and oversight that ensures that the compounding process consistently meets quality standards.
**Quality control (QC):** The sampling, testing, and documentation of results that, taken together, ensure that specifications have been met for the CNSP.
**Reconstitution:** The process of adding a diluent to a conventionally manufactured product to prepare a solution or suspension.
**Release inspection and testing:** Visual inspection and testing performed to ensure that a preparation meets appropriate quality characteristics.
**Sanitizing agent:** An agent for reducing, on inanimate surfaces, the number of microorganisms (e.g., 70% isopropyl alcohol).
**SDS:** Safety data sheet.
**SOP:** Standard operating procedure.
**Specification:** The tests, analytical methods, and acceptance criteria to which any components, CNSP, container closure system, equipment, or other material used in the compounding of CNSPs must conform to be considered acceptable for its intended use.
**Stability:** The extent to which a product or preparation retains physical and chemical properties and characteristics within specified limits throughout its expiration or BUD.
**Verify:** To confirm that a method, process, system, or equipment will perform as expected under the conditions of actual use.
**Water activity ($a_w$):** A measure of the fraction of total water that is unbound and freely available to participate in chemical, biochemical, or physicochemical reactions or provide an environment that can support microbial growth. Note that $a_w$ is not water content.▲ (Official 1-Nov-2023)

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# ⟨797⟩ PHARMACEUTICAL COMPOUNDING—STERILE PREPARATIONS

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## ▲1. INTRODUCTION AND SCOPE

This chapter describes the minimum standards to be followed for the preparation of compounded sterile preparations (CSPs) for human and animal drugs. Sterile compounding is defined as combining, admixing, diluting, pooling, reconstituting, repackaging, or otherwise altering a drug product or bulk drug substance to create a sterile preparation.

The requirements in this chapter must be followed to minimize harm, including death, to human and animal patients that could result from 1) microbial contamination [nonsterility], 2) excessive bacterial endotoxins, 3) variability from the intended strength of correct ingredients, 4) physical and chemical incompatibilities, 5) chemical and physical contaminants, and/or 6) use of ingredients of inappropriate quality.

Aseptic techniques, processes, and procedures must be followed for preparing any sterile medication. Processes and procedures must be in place to minimize the potential for contact with nonsterile surfaces, introduction of particulate matter or biological fluids, and mix-ups with other products or CSPs.

The use of technologies, techniques, materials, and procedures other than those described in this chapter is not prohibited as long as they are noninferior to those described herein and validated for the intended purpose (e.g., *Validation of Alternative Microbiological Methods* ⟨1223⟩ and *Validation of Compendial Procedures* ⟨1225⟩).

Unless otherwise specified in each section, the requirements of this chapter apply to compounding all categories of CSPs.

### 1.1 Scope

**1.1.1 CSPs affected:** The requirements in this chapter must be met to ensure the sterility of any CSP. Although the list below is not exhaustive, the following must be sterile:

- Injections, including infusions
- Irrigations for internal body cavities (i.e., any space that does not normally communicate with the environment outside of the body, such as the bladder cavity or peritoneal cavity). [NOTE—Irrigations for the mouth, rectal cavity, and sinus cavity are not required to be sterile.]
- Ophthalmic dosage forms
- Aqueous preparations for pulmonary inhalation. [NOTE—Nasal dosage forms intended for local application are not required to be sterile.]
- Baths and soaks for live organs and tissues
- Implants

**1.1.2 Specific practices**

**Allergenic extracts:** Licensed allergenic extracts are mixed and diluted to prepare prescription sets for administration to patients. A prescription set is a vial or set of vials of premixed licensed allergenic extracts for subcutaneous immunotherapy that have been diluted with an appropriate diluent for an individual patient. Because of certain characteristics of allergenic extracts and allergy practice, preparation of allergenic extract prescription sets is not subject to all of the requirements in this chapter that are applicable to other sterile CSPs. The standards for compounding allergenic extracts, which are described in *21. Compounding Allergenic Extracts*, are applicable only when

1. The compounding process involves transfer via sterile needles and syringes of conventionally manufactured sterile allergen products and appropriate conventionally manufactured sterile added substances; and
2. Manipulations are limited to penetrating stoppers on vials with sterile needles and syringes and transferring sterile liquids in sterile syringes to sterile vials.

**Blood-derived and other biological materials:** When compounding activities require the manipulation of a patient's blood-derived or other biological material (e.g., autologous serum), the manipulations must be clearly separated from other compounding activities and equipment used in CSP preparation activities, and they must be controlled by specific standard operating procedures (SOPs) to avoid any cross-contamination. Handling of blood components and other biological materials must additionally comply with laws and regulations of the applicable regulatory jurisdiction.

**Hazardous drugs:** Handling of sterile hazardous drugs (HDs) must additionally comply with *Hazardous Drugs—Handling in Healthcare Settings* ⟨800⟩.

**Repackaging:** Repackaging of a sterile product or preparation from its original container into another container must be performed in accordance with the requirements in this chapter.

**Sterile radiopharmaceuticals:** Compounding of radiopharmaceuticals is not required to meet the standards of this chapter as they are subject to the requirements in *Radiopharmaceuticals—Preparation, Compounding, Dispensing, and Repackaging* ⟨825⟩.

**1.1.3 Personnel and settings affected:** This chapter describes the minimum requirements that apply to all persons who prepare CSPs and all places where CSPs are prepared. This includes but is not limited to pharmacists, technicians, nurses, physicians, veterinarians, dentists, naturopaths, and chiropractors in all places including but not limited to hospitals and other healthcare institutions, medical and surgical patient treatment sites, infusion facilities, pharmacies, and physician or veterinarian practice sites. Any person entering a sterile compounding area, whether preparing a CSP or not, must meet the requirements in *3. Personal Hygiene and Garbing*.

The compounding facility must designate one or more individuals (i.e., the designated person(s)) to be responsible and accountable for the performance and operation of the facility and personnel in the preparation of CSPs and for performing other functions as described in this chapter.

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**1.2 Administration**

For the purposes of this chapter, "administration" means the direct application of a sterile product or preparation to a single patient by injecting, infusing, or otherwise providing a sterile product or preparation in its final form.

Administration of medication is out of the scope of this chapter. Standard precautions such as the Centers for Disease Control and Prevention (CDC) safe injection practices apply to administration. See ⟨800⟩ for additional recommendations for the administration of hazardous drugs.

**1.3 Immediate-Use CSPs**

When all of the following conditions are met, compounding of CSPs for direct and immediate administration is not subject to the requirements for Category 1, Category 2, or Category 3 CSPs:

1. Aseptic techniques, processes, and procedures are followed, and written SOPs are in place to minimize the potential for contact with nonsterile surfaces, introduction of particulate matter or biological fluids, and mix-ups with other conventionally manufactured products or CSPs.
2. Personnel are trained and demonstrate competency in aseptic processes as they relate to assigned tasks and the facility's SOPs.
3. The preparation is performed in accordance with evidence-based information for physical and chemical compatibility of the drugs (e.g., approved labeling, stability and compatibility studies).
4. The preparation involves not more than 3 different sterile products.
5. Any unused starting component from a single-dose container must be discarded after preparation is complete. Single-dose containers must not be used for more than one patient.
6. Administration begins within 4 h following the start of preparation. If administration has not begun within 4 h following the start of preparation, it must be promptly, appropriately, and safely discarded.
7. Unless directly administered by the person who prepared it or administration is witnessed by the preparer, the CSP must be labeled with the names and amounts of all active ingredients, the name or initials of the person who prepared the preparation, and the 4-h time period within which administration must begin.

Handling of sterile hazardous drugs (HDs) must additionally comply with ⟨800⟩.

**1.4 Preparation Per Approved Labeling**

Compounding does not include mixing, reconstituting, or other such acts that are performed in accordance with directions contained in approved labeling or supplemental materials provided by the product's manufacturer.

Preparing a conventionally manufactured sterile product in accordance with the directions in the manufacturer's approved labeling is out of scope of this chapter only if

1. The product is prepared as a single dose for an individual patient; and
2. The approved labeling includes information for the diluent, the resultant strength, the container closure system, and storage time.

See ⟨800⟩ for additional recommendations for the preparation of hazardous drugs.

**Proprietary bag and vial systems:** Docking and activation of proprietary bag and vial systems in accordance with the manufacturer's labeling for *immediate* administration to an individual patient is not considered compounding and may be performed outside of an International Organization for Standardization (ISO) Class 5 environment.

Docking of the proprietary bag and vial systems for *future activation* and administration is considered compounding and must be performed in an ISO Class 5 environment in accordance with this chapter, with the exception of *14. Establishing Beyond-Use Dates*. Beyond-use dates (BUDs) for proprietary bag and vial systems must not be longer than those specified in the manufacturer's labeling.

**1.5 CSP Categories**

This chapter distinguishes three categories of CSPs: Category 1, Category 2, and Category 3, primarily based on the state of environmental control under which they are compounded, the probability for microbial growth during the time they will be stored, and the time period within which they must be used.

Category 1 CSPs are compounded under the least controlled environmental conditions and therefore are assigned a BUD of 12 h or less at controlled room temperature or 24 h or less when refrigerated, if compounded in accordance with all of the applicable requirements for Category 1 CSPs in this chapter.

Category 2 CSPs require more environmental controls and testing than Category 1 CSPs and may be assigned a BUD of greater than 12 h at controlled room temperature or more than 24 h if refrigerated, but not exceed the limits established in *Table 13* (see *14. Establishing Beyond-Use Dates*), if compounded in accordance with all of the applicable requirements for Category 2 CSPs in this chapter.

Category 3 CSPs undergo sterility testing, supplemented by endotoxin testing when applicable, and have more requirements than Category 2 CSPs for personnel qualification, use of sterile garb, use of sporicidal disinfectants, frequency of environmental monitoring, and stability determination. Category 3 CSPs may be assigned longer BUDs than those set for Category 2 CSPs but not exceeding the limits in *Table 14* (see *14. Establishing Beyond-Use Dates*), if compounded in accordance with all applicable requirements for Category 3 CSPs in this chapter (see *14.4 Additional Requirements for Category 3 CSPs*).

The requirements that are not specifically described as applicable to Category 1, Category 2, or Category 3, are applicable to the compounding of all CSPs unless the CSP is otherwise described in *1.1 Scope*.

Category 1, Category 2, and Category 3 CSPs can be compounded by using only sterile starting ingredients, or by using some or all nonsterile starting ingredients. If all components used to compound are sterile from the start, the sterility of the components must be maintained during compounding to produce a CSP.

If one or more of the starting components being used to compound is not sterile, the sterility of the compounded preparation must be achieved through a sterilization process (e.g., terminal sterilization in the final sealed container) or sterilizing filtration, and then sterility must be maintained if the CSP is subsequently manipulated. When compounding with nonsterile starting components, supplies, or equipment, the quality of the components, the

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effectiveness of the sterilization step, and bacterial endotoxin mitigation strategies are critical to achieving a sterile preparation that is free from excessive bacterial endotoxins.

## 2. PERSONNEL TRAINING AND EVALUATION

All personnel who compound or have direct oversight of compounding personnel must be initially trained and qualified by demonstrating knowledge and competency in compounding CSPs according to the requirements in this section before being allowed to perform their job functions independently. Designated person(s) are responsible for creating and implementing a training program for personnel and for ensuring that compounders, personnel who have direct oversight of compounders, and personnel who perform restocking or cleaning and disinfection duties are initially trained and qualified by demonstrating knowledge and competency in maintaining the quality of the sterile compounding environment before being allowed to perform their job functions independently. Training and observation may be performed by the designated person(s) or an assigned trainer. Personnel who compound or have direct oversight of compounding personnel must complete training initially and at least every 12 months in appropriate sterile compounding principles and practices as described below (see *2.1 Demonstrating Knowledge and Competency of Core Skills*). Personnel who only perform restocking or cleaning and disinfecting duties outside of the primary engineering control (PEC) must complete ongoing training as required by the facility's SOPs. Personnel compounding only immediate-use CSPs must complete training as required by the facility's SOPs (see *1.3 Immediate-Use CSPs*).

Each compounding facility must develop a written training program that describes the required training, the frequency of training, and the process for evaluating the performance of individuals who compound, have direct oversight of compounding personnel, perform in-process checks, final verification, and dispensing of CSPs. This program must equip personnel with the appropriate knowledge and train them in the required skills necessary to perform their assigned tasks, and SOPs should specify the training required for such tasks.

Training and evaluation of personnel must be documented (see *20. Documentation*).

### 2.1 Demonstrating Knowledge and Competency of Core Skills

Before beginning to compound CSPs independently or have direct oversight of compounding personnel, personnel must complete training and be able to demonstrate knowledge of principles and competency of skills for performing sterile manipulations and achieving and maintaining appropriate environmental conditions as applicable to their assigned job functions. This must be completed initially and at least every 12 months in at least the following:

- Hand hygiene
- Garbing
- Cleaning and disinfection
- Calculations, measuring, and mixing
- Aseptic technique
- Achieving and/or maintaining sterility (and apyrogenicity if compounding with nonsterile components)
- Use of equipment
- Documentation of the compounding process (e.g., master formulation and compounding records)
- Principles of high-efficiency particulate air (HEPA)-filtered unidirectional airflow within the ISO Class 5 area
- Proper use of PECs
- Principles of movement of materials and personnel within the compounding area

If the facility has only one person in the compounding operation, that person must document that they have obtained training and demonstrated competency, and they must comply with the other requirements of this chapter.

### 2.2 Demonstrating Competency in Garbing and Hand Hygiene

Before beginning to compound Category 1, Category 2, or Category 3 CSPs or have direct oversight of compounding personnel, personnel must successfully complete an initial garbing competency evaluation no fewer than 3 separate times. The 3 successful completions must be in succession—failure of any of the 3 initial garbing competency evaluations requires repeat testing until personnel successfully complete 3 evaluations in a row. The garbing competency evaluation consists of a visual observation and gloved fingertip and thumb sampling (GFT) of both hands (see *Box 1*). Each of the 3 initial competency evaluations must occur after performing a separate and complete hand hygiene and full garbing procedure. All garbing competencies must be completed with gloved fingertip and thumb sampling after garbing (see *Box 1*) and a documented visual audit while performing hand hygiene and garbing procedures (see *3. Personal Hygiene and Garbing*). Gloved fingertip and thumb sampling after garbing, but before applying sterile 70% IPA to gloves, must be performed on donned sterile gloves on both hands in a classified area or segregated compounding area (SCA).

Failure is indicated by visual observation of improper hand hygiene and garbing procedures and/or gloved fingertip and thumb sampling results that exceed the action levels in *Table 1*. Results of the evaluation and corrective actions, in the event of failure, must be documented and the documentation maintained to provide a record and long-term assessment of personnel competency. Documentation must at a minimum include the name of the person evaluated; evaluation date and time; media and components used including manufacturer, expiration date, and lot number; starting temperature for each interval of incubation; dates of incubation; results and identification of the observer and personnel reading and documenting the results. Microbial identification of the colony-forming units (cfu) is not required for gloved fingertip and thumb sampling.

After the initial garbing competency evaluations, compounding personnel must successfully complete the garbing competency (see *Table 1*) at least one time every 6 months for personnel compounding Category 1 and Category 2 CSPs, and at least one time every 3 months for personnel compounding Category 3 CSPs. Personnel who have direct oversight of compounding personnel, but do not compound, must complete a garbing competency evaluation every 12 months. The evaluation should correspond to the type of garbing activities of the personnel they oversee. Personnel who have direct oversight of compounding personnel must not compound unless they successfully complete the garbing competency evaluation at the same intervals required for compounding personnel.

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**Box 1. Gloved Fingertip and Thumb Sampling Procedures**

- Use one sampling media device (e.g., plates, paddles, or slides) per hand, containing general microbial growth agar (e.g., trypticase soy agar [TSA]) supplemented with neutralizing additives (e.g., lecithin and polysorbate 80) as this agar supports both bacterial and fungal growth.
- Label each media device with a personnel identifier, right or left hand, and the date and time of sampling.
- Do not apply sterile 70% isopropyl alcohol (IPA) to gloves immediately before touching the media device because this could cause a false-negative result. Using a separate media device for each hand, collect samples from all gloved fingertips and thumbs from both hands by rolling fingertip pads and thumb pad over the agar surface.
- Incubate the media device at 30°–35° for no less than 48 h and then at 20°–25° for no less than 5 additional days. Samples must be incubated in an incubator. Handle and store media devices to avoid contamination and prevent condensate from dropping onto the agar during incubation and affecting the accuracy of the cfu reading (e.g., invert plates).
- Record the number of cfu per hand (left hand, right hand).
- Determine whether the cfu action level is exceeded by counting the total number of cfu from both hands.

### 2.3 Competency Testing in Aseptic Manipulation

Before beginning to compound Category 1, Category 2, or Category 3 CSPs independently or have direct oversight of compounding personnel, personnel must successfully complete an aseptic manipulation competency evaluation. The aseptic manipulation competency evaluation consists of a visual observation, media-fill testing, followed by a gloved fingertip and thumb sampling on both hands, and surface sampling of the direct compounding area to assess aseptic technique and related practices (see *Box 2*).

For personnel compounding Category 1 and Category 2 CSPs, the aseptic manipulation competency must occur initially and at least every 6 months thereafter. For personnel compounding Category 3 CSPs, the aseptic manipulation competency must occur initially and at least every 3 months thereafter. Personnel who have direct oversight of compounding personnel must complete an aseptic manipulation competency evaluation annually. The evaluation should correspond to the type of activities of the personnel they oversee but does not require the same quantities. Personnel who have direct oversight of compounding personnel must not compound unless they successfully complete the aseptic manipulation competency evaluation that simulates the most difficult and challenging aseptic compounding procedures encountered by the person at the same intervals required for compounding personnel.

When performing a media-fill test, simulate the most difficult and challenging aseptic compounding procedures encountered by the person replacing all the components used in the CSPs with soybean–casein digest media. The simulation must capture elements that could potentially affect the sterility of the CSP including but not limited to:

- Factors associated with the length of the process that can pose contamination risk (e.g., operator fatigue, quality of equipment)
- Number of aseptic additions or transfers
- Number, type, and complexity of manipulations
- Number of personnel in the buffer room or SCA

If using commercial sterile microbial growth media, a certificate of analysis (COA) must be obtained from the supplier stating that the lot of the growth media will support the growth of microorganisms. Store microbial growth media in accordance with manufacturer instructions and initiate the media-fill test by the expiration date of the media. If preparing sterile microbial growth media in-house for sterile-to-sterile media-fill testing, the growth promotion capability of the media must be demonstrated for each batch and documented as described in *Sterility Tests* ⟨71⟩, *Culture Media and Incubation Temperatures, Growth Promotion Test of Aerobes, Anaerobes, and Fungi*.

Failure is indicated by visible turbidity or other visual manifestations of growth in the media in one or more container closure unit(s) on or before the end of the incubation period. Microbial identification of the cfu is not required for media-fill testing.

Immediately following the media-fill test, gloved fingertip and thumb sampling must be performed on both hands and inside of an ISO Class 5 PEC. If conducting gloved fingertip and thumb sampling in a compounding aseptic isolator (CAI), compounding aseptic containment isolator (CACI), or a pharmaceutical isolator, samples must be taken from the sterile gloves placed over the gloves attached to the restricted-access barrier system (RABS) or pharmaceutical isolator sleeves.

Successful completion of the gloved fingertip and thumb sampling after media-fill testing is defined as ≤3 cfu as a total from both hands. See *Table 1* for action levels for gloved fingertip and thumb sampling results. Microbial identification of the cfu is not required for gloved fingertip and thumb sampling.

Surface sampling of the direct compounding area must occur in accordance with the requirements in *6.3 Monitoring Surfaces for Viable Particles*. A failure in the media fill, gloved fingertip and thumb sampling, or surface sample constitutes an overall failure of the aseptic manipulation competency.

Results of the evaluation and corrective actions must be documented and the documentation maintained to provide a record and long-term assessment of personnel competency. Documentation must at a minimum include 1) the name of the person evaluated, 2) evaluation date and time, 3) media and components used including their manufacturer or supplier, 4) expiration dates and lot numbers, 5) starting temperature for each interval of incubation, 6) dates of incubation, 7) the results, and 8) the names or other identification of the observer and the person who reads and documents the results.

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**Box 2. Media-Fill Testing Procedures**

- If all of the starting components are sterile to begin with, manipulate them in a manner that simulates sterile-to-sterile compounding activities, and transfer the sterile soybean–casein digest media into the same types of container closure systems commonly used at the facility. Do not further dilute the media unless specified by the manufacturer.
- If some of the starting components are nonsterile to begin with, dissolve a commercially available nonsterile soybean–casein digest powder in nonbacteriostatic water to make a 3% nonsterile solution. Manipulate it in a manner that simulates nonsterile-to-sterile compounding activities. Prepare at least 1 container as the positive control to demonstrate growth promotion, which is indicated by visible turbidity upon incubation.
- Once the compounding simulation is completed and the final containers are filled with the test media, perform a gloved fingertip and thumb sample on each hand and surface sample of the direct compounding area inside the PEC. Take the samples prior to disinfecting gloves and PEC. Handle and store samples to avoid contamination and prevent condensate from dropping onto the agar during incubation and affecting the accuracy of the cfu reading (e.g., invert containers).
- Incubate the final containers at 20°–25° and 30°–35° for a minimum of 7 days at each temperature band to detect a broad spectrum of microorganisms. The order of the incubation temperatures must be described in the facility's SOPs. Final containers must be incubated in an incubator.
- Failure is indicated by visible turbidity or other visual manifestations of growth in the media in one or more container closure unit(s) on or before 14 days.

**Table 1. Action Levels for Gloved Fingertip and Thumb Sampling[a]**

| Gloved Fingertip and Thumb Sampling | Action Levels (cfu, total from both hands) |
|---|---|
| After garbing | >0 |
| After media-fill testing | >3 |

[a] Action levels are based on the total cfu count from both hands.

**Table 2. Initial Training and Competency**

| Personnel Function | Defined by Facility SOPs | Required in ⟨797⟩ and Supplemented by Facility SOPs | | | |
|---|---|---|---|---|---|
| | | Training and Competency in Maintaining the Quality of the Sterile Compounding Environment | Training and Competency in Sterile Compounding Principles and Practices | Garbing Competency (Including GFT) | Media Fill with Post-GFT and Surface Sampling |
| Compounder | | X | X | X | X |
| Designated person and personnel with direct oversight of compounding personnel | | X | X | X | X |
| Personnel who restock or clean and disinfect the sterile compounding area[a] | X | | | | |
| Personnel who perform in-process checks or final verification of CSPs[a] | X | | | | |
| Personnel who only compound immediate-use CSPs | X | | | | |
| Others (e.g., maintenance personnel, certifiers, contractors, inspectors, surveyors)[a] | X | | | | |

[a] Personnel who do not compound nor have direct oversight of compounding personnel.

**Table 3. Ongoing Training and Competency**

| Personnel Function | Defined by Facility SOPs | Required ⟨797⟩ and Supplemented by Facility SOPs | | |
|---|---|---|---|---|
| | | Training and Competency in Sterile Compounding Principles and Practices | Garbing Competency (Including GFT) | Media Fill with Post-GFT and Surface Sampling |
| Compounder | | At least every 12 months | Category 1 and 2 at least every 6 months<br>Category 3 at least every 3 months | Category 1 and 2 at least every 6 months<br>Category 3 at least every 3 months |
| Designated person and personnel with direct oversight of compounding personnel | | At least every 12 months unless compounding[a] | At least every 12 months unless compounding[a] | At least every 12 months unless compounding[a] |
| Personnel who restock or clean and disinfect the sterile compounding area[b] | X | | | |

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**Table 3. Ongoing Training and Competency** *(continued)*

| Personnel Function | Defined by Facility SOPs | Required ⟨797⟩ and Supplemented by Facility SOPs | | |
|---|---|---|---|---|
| | | Training and Competency in Sterile Compounding Principles and Practices | Garbing Competency (Including GFT) | Media Fill with Post-GFT and Surface Sampling |
| Personnel who perform in-process checks or final verification of CSPs[b] | X | | | |
| Personnel who only compound immediate-use CSPs | X | | | |
| Others (e.g., maintenance personnel, certifiers, contractors, inspectors, surveyors)[b] | X | | | |

[a] If compounding, follow compounder requirements.
[b] Personnel who do not compound or have direct oversight of compounding personnel.

## 3. PERSONAL HYGIENE AND GARBING

Personal hygiene and garbing are essential to maintain microbial control of the environment. Most microorganisms detected in cleanrooms are transferred from individuals. Squamous cells are normally shed from the human body at a rate of $10^6$ or more per hour, and those skin particles are covered with microorganisms. Individuals entering a compounding area must be properly garbed and must maintain proper personal hygiene to minimize the risk of contamination to the environment and/or CSPs.

Individuals that may have a higher risk of contaminating the CSP and the environment (e.g., personnel with rashes, recent tattoos, oozing sores, conjunctivitis, or active respiratory infections) must report these conditions to the designated person(s). The designated person(s) is responsible for evaluating whether these individuals should be excluded from working in compounding areas before their conditions have resolved because of the risk of contaminating the CSPs and the environment.

### 3.1 Personnel Preparation

All personnel entering a compounding area where Category 1, Category 2, or Category 3 CSPs are prepared must take appropriate steps to minimize microbial contamination of the environment and of the CSPs, including hand hygiene (see *3.2 Hand Hygiene*), garbing (see *3.3 Garbing Requirements*), and consideration of needed materials to be brought into the compounding area.

Food (including mints, gum, etc.) and drinks must not enter anterooms, buffer rooms, or segregated compounding areas.

Before entering a compounding area, individuals must remove any items that are not easily cleanable or are not necessary for compounding. At a minimum, individuals must:

- Remove personal outer garments (e.g., bandanas, coats, hats, jackets, sweaters, vests)
- Remove all cosmetics because they shed flakes and particles
- Remove all hand, wrist, and other exposed jewelry, including piercings that could interfere with the effectiveness of garbing (e.g., the fit of gloves, cuffs of sleeves, and eye protection) or otherwise increase the risk of contamination of the CSP. Cover any jewelry that cannot be removed.
- Not wear earbuds or headphones
- Not bring electronic devices that are not necessary for compounding or other required tasks into the compounding area
- Keep nails clean and neatly trimmed to minimize particle shedding and avoid glove punctures. Nail products (e.g., polish, artificial nails, and extenders) must not be worn
- Wipe eyeglasses, if worn

The designated person(s) may permit accommodations to personnel preparation as long as the quality of the CSP and environment will not be affected. Accommodations must be documented.

### 3.2 Hand Hygiene

Any person entering a compounding area where Category 1, Category 2, or Category 3 CSPs are prepared must wash hands and forearms up to the elbows with soap and water before initiating compounding activities. Brushes must not be used for hand hygiene. Hand dryers must not be used. To minimize the risk of extrinsic contamination, disposable soap containers must not be refilled or topped off.

**Box 3. Hand Washing Procedures**

- Clean underneath fingernails under warm running water using a disposable nail cleaner.
- Wash hands and forearms up to the elbows with soap and water for at least 30 s.
- Dry hands and forearms up to the elbows completely with low-lint disposable towels or wipers.

The order of hand washing and garbing depends on the placement of the sink (see *4.4 Water Sources*). The order of garbing must be determined by the facility and documented in the facility's SOPs. Hands must be sanitized with alcohol-based hand rub before donning sterile gloves (see *Box 4*). Sterile gloves must be donned in a classified room or SCA.

Unofficial

**Box 4. Hand Sanitizing Procedures**

- Apply an alcohol-based hand rub to dry skin.
- Apply product to one hand and rub hands together, covering all surfaces of hands and fingers, until hands are dry.
- Allow hands to dry thoroughly before donning sterile gloves.

### 3.3 Garbing Requirements

Any person entering a compounding area where Category 1, Category 2, or Category 3 CSPs are prepared must be properly garbed. Garb must be donned and doffed in an order that reduces the risk of contamination. The required garb, manner of storage, and order of garbing must be determined by the facility and documented in the facility's SOPs. When preparing Category 2 or Category 3 CSPs, all garb should be donned in a classified area before entering the buffer room. If hand hygiene is completed outside of a classified area, alcohol-based hand rub must be used prior to donning garb. Skin must not be exposed inside the ISO Class 5 PEC (e.g., gloves must not be donned or doffed inside the ISO Class 5 PEC exposing bare hands). Donning and doffing garb should not occur in the same area at the same time. The minimum garbing requirements for preparing Category 1 and Category 2 CSPs include the following:

- Low-lint garment with sleeves that fit snugly around the wrists and an enclosed neck (e.g., gown or coverall)
- Low-lint covers for shoes
- Low-lint cover for head that covers the hair and ears, and if applicable, cover for facial hair
- Low-lint face mask
- Sterile powder-free gloves
- If using a RABS (i.e., a CAI or CACI), disposable gloves should be worn inside the gloves attached to the RABS sleeves. Sterile gloves must be worn over the gloves attached to the RABS sleeve

Garb must be replaced immediately if it becomes visibly soiled or if its integrity is compromised. Gowns and other garb must be stored in a manner that minimizes contamination (e.g., away from sinks to avoid splashing). If compounding Category 1 and Category 2 CSPs, gowns may be reused within the same shift by the same person if the gown is maintained in a classified area or adjacent to, or within, the SCA in a manner that prevents contamination. When personnel exit the compounding area, garb, except for gowns, cannot be reused and must be discarded or laundered before reuse. The facility's SOPs must describe disinfection procedures for reusing goggles, respirators, and other reusable equipment.

If the facility compounds Category 3 CSPs, additional garbing requirements must be continuously met in the buffer room in which Category 3 CSPs are prepared. The following additional garbing requirements must be followed in the buffer room where Category 3 CSPs are prepared for all personnel regardless of whether Category 3 CSPs are compounded on a given day:

1. Do not allow any exposed skin in the buffer room (i.e., face and neck must be covered).
2. All low-lint outer garb must be sterile, including the use of sterile sleeves over gauntlet sleeves when a RABS is used.
3. Disposable garbing items must not be reused, and laundered garb must not be reused without being laundered and resterilized with a validated cycle.
4. The facility's SOPs must describe disinfection procedures for reusing goggles, respirators, and other reusable equipment.

If compounding an HD, appropriate personal protective equipment (PPE) must be worn and disposed of in accordance with ⟨800⟩.

**Gloves:** Gloves must be sterile and powder free. Application of sterile 70% IPA to gloves must occur immediately before compounding and regularly throughout the compounding process.

All gloves must be inspected for holes, punctures, or tears and must be replaced immediately if such defects are detected.

The RABS sleeves and gloves and the pharmaceutical isolator sleeves and gloves should be changed per the manufacturer's recommendations and as defined in the facility's SOPs.

## 4. FACILITIES AND ENGINEERING CONTROLS

Sterile compounding facilities must be designed, outfitted, and maintained properly to minimize the risk of contamination of CSPs. The required air quality must be achieved and maintained through PECs and secondary engineering controls (SECs). The anteroom, buffer room, and SCA must be separated from areas not directly related to compounding. The anteroom and buffer room must be appropriately controlled to achieve and maintain the required air quality classifications. The design of the facility should take into account the number of personnel and their movements, and the impact the placement of equipment, supplies, and components could have on the maintenance of air quality. The number of operations being performed, the equipment (e.g., PECs, carts, computers), the personnel in the compounding area (and in adjacent areas), and the complexity of the compounding procedures are critical considerations for maintaining control of environmental conditions in the facility.

### 4.1 Protection from Airborne Contaminants

Sterile compounding facilities must be designed to minimize the risk of airborne contamination of the area in which sterile compounding occurs. Proper design and controls are required to minimize the risk of exposure of CSPs to airborne contaminants.

**4.1.1 Air quality standards:** The ISO standards for air quality in controlled environments are provided in *Table 4* and referenced throughout this chapter.

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**Table 4. ISO Classification of Particulate Matter in Room Air[a]**

| ISO Class | Particle Count per Cubic Meter[b] |
|---|---|
| 3 | 35.2 |
| 4 | 352 |
| 5 | 3520 |
| 6 | 35,200 |
| 7 | 352,000 |
| 8 | 3,520,000 |

[a] Adapted from ISO 14644-1, Cleanrooms and associated controlled environments—Part 1: Classification of air cleanliness by particle concentration.
[b] Limits for number of particles ≥0.5 µm measured under dynamic operating conditions.

**4.1.2 Design requirements to maintain air quality:** Facilities used for compounding CSPs must be designed so that air quality improves with movement through separate operational areas to the PEC. Classified areas in which the air quality is controlled (see *Table 4*) include anterooms, buffer rooms, and PECs.

- Anterooms providing access only to positive-pressure buffer rooms must meet at least ISO Class 8 classification. Anterooms providing access to negative-pressure buffer rooms must meet at least ISO Class 7 classification (see ⟨800⟩). Typically, personnel hand hygiene and garbing procedures, staging of components, and other activities that potentially generate higher levels of particulates are performed in the anteroom. Anterooms are also transition areas to ensure that proper air classification and pressure relationships are maintained between classified and unclassified areas.
- A buffer room must meet at least ISO Class 7 air quality. Activities in the buffer room must be controlled to minimize any effects on air quality in the area where CSPs are prepared.
- Category 1, Category 2, and Category 3 CSPs must be compounded in an ISO Class 5 or better PEC. If compounding only Category 1 CSPs, the PEC may be placed in an unclassified SCA.

**4.2 Facility Design and Environmental Controls**

In addition to minimizing airborne contamination, sterile compounding facilities must be designed and controlled to provide a well-lighted and comfortable working environment (see *Physical Environments That Promote Safe Medication Use* ⟨1066⟩). The cleanroom suite should be maintained at a temperature of 20° or cooler and a relative humidity of 60% or below to minimize the risk of microbial proliferation and to provide comfortable conditions for compounding personnel attired in the required garb. The temperature and humidity must be monitored in each room of the cleanroom suite each day that compounding is performed, either manually or by a continuous recording device. The results of the temperature and humidity readings must be documented at least once daily or stored in the continuous recording device and must be retrievable. The temperature and humidity readings must be reviewed as described in the facility's SOPs. Temperature and humidity in the cleanroom suite must be controlled through a heating, ventilation, and air conditioning (HVAC) system. Free-standing air conditioners, humidifiers, and dehumidifiers must not be used within the classified area or the SCA. Temperature and humidity monitoring devices must be verified for accuracy at least every 12 months or as required by the manufacturer.

The designated person(s) is responsible for ensuring that each area related to CSP preparation meets the classified air quality standard appropriate for the activities to be conducted in that area. The designated person(s) must also ensure that the ISO Class 5 areas are located, operated, maintained, monitored, and certified to have appropriate air quality.

**4.2.1 Types of SECs and design:** The PEC must be located in the buffer room of the cleanroom suite or the SCA in a manner that minimizes conditions that could increase the risk of microbial contamination. For example, strong air currents from opened doors, personnel traffic, or air streams from the HVAC system(s) can disrupt the unidirectional airflow of an open-faced PEC such as a laminar airflow workbench (LAFW). Access to the SEC must be restricted to authorized personnel and required materials.

**Cleanroom suite:** The ISO-classified anteroom and buffer room must be separated from the surrounding unclassified areas of the facility by fixed walls and doors, and controls must be in place to minimize the flow of lower-quality air into the more controlled areas. The classified rooms must be equipped with a pressure-differential monitoring system. Air supplied to the cleanroom suite must be introduced through HEPA filters that are located in the ceiling of the buffer room and anteroom.

Air returns in the cleanroom suite must be low on the wall unless a visual smoke study demonstrates an absence of stagnant airflow. This smoke study along with environmental monitoring must be repeated whenever a change is made to the placement of equipment within the room or any other alteration is performed within the cleanroom suite that affects the quality of the air (e.g., HVAC alterations, change of HEPA filter units).

The anteroom must have a line of demarcation to separate the clean side from the dirty side. The anteroom is entered through the dirty side, and the clean side is the area closest to the buffer room. Alternatively, facilities may be designed with two separate anterooms—a dirty anteroom and a clean anteroom. The anteroom is entered through the dirty anteroom, and the clean anteroom is the area closest to the buffer room.

It is also critical to control materials (e.g., supplies and equipment) as they move from classified areas of lower quality to those of higher quality (e.g., from an ISO Class 8 anteroom to an ISO Class 7 buffer room to an ISO Class 5 PEC) to minimize the influx of contaminants. Airlocks and interlocking doors may be used to facilitate better control of air balance between areas of differing ISO classification (e.g., between the buffer room and anteroom) or between a classified area and an unclassified area (e.g., between the anteroom and a hallway). If a pass-through chamber is used, both doors must never be opened at the same time, and doors should be interlocking.

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Due to the interdependence of the various rooms or areas that make up a sterile compounding facility, it is essential to carefully define and control the dynamic interactions permitted between areas and rooms. Consider the placement of door closures, door surfaces, and the movement of the doors, all of which can affect airflow. Seals and sweeps should not be installed at doors between buffer rooms and anterooms. Access doors should be hands-free. Tacky mats must not be placed within ISO-classified areas. If compounding both sterile and nonsterile preparations (e.g., presterilization procedures), the respective PECs must be placed in separate rooms unless those PECs are sufficiently effective that the room can continuously maintain ISO Class 7 classification. If the PECs used for sterile and nonsterile compounding are placed in the same room, they must be placed at least 1 m apart, and particle-generating activity must not be performed when sterile compounding is in process.

**Segregated compounding area (SCA):** A PEC may be located within an unclassified area without an anteroom or buffer room. This type of design is called an SCA. Only Category 1 CSPs may be compounded in an SCA. The SCA must be located away from unsealed windows, doors that connect to the outdoors, and traffic flow, all of which may adversely affect the air quality in the PEC. An SCA must not be located where environmental control challenges (e.g., restrooms, warehouses, or food preparation areas) could negatively affect the air quality of the PEC within the SCA. The impact of activities (e.g., patient care activities) that will be conducted around or adjacent to the SCA must be considered carefully when designing such an area. The area within 1 m of the PEC should be dedicated only for sterile compounding (e.g., not storage, hand hygiene, donning and doffing garb, or other highly particle-generating activities such as patient care).

**4.2.2 The CSP compounding environment:** The PEC must be certified to meet ISO Class 5 or better conditions (see *Table 4*) during dynamic operating conditions and must be designed to minimize the risk of contamination during compounding of CSPs.

Unidirectional airflow must be maintained in the PEC. HEPA-filtered air must be supplied by the PEC at a velocity sufficient to sweep particles away from critical sites and maintain unidirectional airflow during operations. Proper design, control, and use minimizes turbulence and creation of eddies or stagnant air in the PEC.

**4.2.3 Types of PECs and placement:** Proper placement of the PEC is critical to ensuring an ISO Class 5 environment for preparing CSPs. Placement of the PEC must allow for cleaning around the PEC. See *Table 5* for a summary of minimum requirements for the placement of PECs for preparing non-HD CSPs.

Types of PECs and their placement include the following:

**Laminar airflow system (LAFS):** An LAFS provides an ISO Class 5 or better environment for sterile compounding. The LAFS provides unidirectional HEPA-filtered airflow that is designed to minimize the risk of contamination of a sterile compounding environment. The unidirectional airflow within the LAFS helps protect the direct compounding area (DCA) from process-generated contamination (e.g., opening wrappings of sterile containers, compounder movement) as well as from outside sources.

Types of LAFS and their placement include the following:

**Laminar airflow workbench (LAFW):** An LAFW is a device that provides an ISO Class 5 or better environment for sterile compounding. The LAFW provides either horizontal or vertical unidirectional HEPA-filtered airflow. [NOTE—An LAFW must not be used for preparation of antineoplastic and/or active pharmaceutical ingredient (API) HDs (see ⟨800⟩).]

**Integrated vertical laminar flow zone (IVLFZ):** An IVLFZ is a designated ISO Class 5 area serving as the PEC within an ISO Class 7 or cleaner buffer room. In the IVLFZ, unidirectional airflow is created by placing HEPA filters over the entire surface of the worktables and by effective placement of air returns. The unidirectional HEPA-filtered zone must be separated from the ISO Class 7 area with a physical barrier to direct the airflow downward over the work area to separate the DCA from potential sources of contamination. Strategic location of air returns in addition to full coverage of HEPA filters above the work surface is required. Both static and dynamic smoke studies verifying a continuous flow of HEPA-filtered air void of turbulence, dead air zones, and refluxing from the HEPA filters to and across the entire work area and to the air returns must be documented (e.g., with video). [NOTE—Dynamic airflow smoke pattern tests have shown that it is difficult to achieve this type of design and also achieve and maintain unidirectional airflow under dynamic operating conditions.]

[NOTE—An IVLFZ must not be used for preparation of antineoplastic and/or API HDs (see ⟨800⟩).]

**Class II biological safety cabinet (BSC):** A Class II BSC is a ventilated cabinet with an open front and inward and downward unidirectional HEPA-filtered airflow and HEPA-filtered exhaust. The BSC is designed to provide worker protection from exposure to airborne drugs and to provide an ISO Class 5 or better environment for preparing CSPs. [NOTE—The exhaust air from the BSC must be externally vented for preparation of antineoplastic and/or API HDs (see ⟨800⟩).]

**Placement of LAFS:** The LAFS must be located out of traffic patterns and away from room air currents that could disrupt the intended airflow patterns inside the PEC. If used to prepare only Category 1 CSPs, the ISO Class 5 PEC may be located in an unclassified SCA. If used to prepare Category 2 or Category 3 CSPs, the LAFS must be located within a cleanroom suite with an ISO Class 7 or better buffer room with an ISO Class 8 or better anteroom. A dynamic airflow smoke pattern test must be performed in the PEC initially and at least every 6 months to ensure that 1) the LAFS is properly placed into the facility and 2) compounders understand how to utilize the unidirectional airflow to maintain first air in the DCA.

**Restricted-access barrier system (RABS):** A RABS is an enclosure that provides HEPA-filtered ISO Class 5 unidirectional air. It allows for the ingress and/or egress of materials through defined openings that have been designed and validated to preclude the transfer of environmental air contamination and are generally not to be opened during compounding operations. RABS include compounding aseptic isolators (CAIs) and compounding aseptic containment isolators (CACIs). In a CAI or CACI, glove ports are used to provide physical separation between the surrounding area and the aseptic manipulations.

**Compounding aseptic isolator:** A CAI is designed for compounding non-HD CSPs. It is designed to maintain an ISO Class 5 environment throughout the compounding and material transfer processes. Air exchange into the

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CAI from the surrounding environment must not occur unless the air has first passed through a HEPA filter. [NOTE—A CAI must not be used for preparation of antineoplastic and/or API HDs (see ⟨800⟩).]

**Compounding aseptic containment isolator:** A CACI is designed to provide worker protection from exposure to undesirable levels of airborne drug throughout the compounding and material transfer processes and to maintain an ISO Class 5 environment for compounding sterile HD preparations (see ⟨800⟩).

**Placement of RABS:** If used to prepare only Category 1 CSPs, the ISO Class 5 environment may be achieved by placing the RABS in an unclassified SCA. If used to prepare Category 2 or Category 3 CSPs, the RABS must be located within a cleanroom suite with an ISO Class 7 or better buffer room with an ISO Class 8 or better anteroom. For placement of a CACI used for the preparation of antineoplastic and/or API HDs, see ⟨800⟩.

When a RABS is used, the recovery time after opening the transfer chamber to achieve ISO Class 5 air quality must be documented (e.g., by the manufacturer), and internal procedures must be developed to ensure that adequate recovery time is allowed after opening and closing the RABS, both before and during compounding operations. A dynamic airflow smoke pattern test must be performed in the PEC under dynamic operating conditions initially and at least every 6 months to ensure that 1) the RABS is properly integrated into the facility and 2) compounders understand how to utilize the unidirectional airflow to maintain first air in the DCA.

**Pharmaceutical isolator:** A pharmaceutical isolator provides isolation from the surrounding area and maintains ISO Class 5 air quality during dynamic operating conditions. [NOTE—A CAI or CACI is not a pharmaceutical isolator.] A pharmaceutical isolator comprises four elements:

1. Controlled workspace
2. Transfer device(s)
3. Access device(s)
4. Integral decontamination system

**Placement of pharmaceutical isolators:** A pharmaceutical isolator used to prepare only Category 1 CSPs can be placed in an unclassified SCA. If the pharmaceutical isolator is used to prepare Category 2 or Category 3 CSPs, the pharmaceutical isolator must be placed in an ISO Class 8 or better room. [NOTE—An anteroom is not required when using a pharmaceutical isolator.] A dynamic airflow smoke pattern test must be performed in the PEC initially and at least every 6 months to ensure that 1) the pharmaceutical isolator is properly placed in the facility and 2) compounders understand how to utilize the unidirectional airflow to maintain first air in the work zone.

**Table 5. Summary of Minimum Requirements for Placement of PECs for Compounding Non-HD CSPs[a]**

| PEC Type | Device Type | Placement for Compounding Only Category 1 CSPs | Placement for Compounding Category 2 and 3 CSPs |
|---|---|---|---|
| LAFS | LAFW | Unclassified SCA | ISO Class 7 positive-pressure buffer room with an ISO Class 8 positive-pressure anteroom |
| | IVLFZ | N/A[b] | ISO Class 7 positive-pressure buffer room with an ISO Class 8 positive-pressure anteroom |
| | BSC | Unclassified SCA | ISO Class 7 positive-pressure buffer room with an ISO Class 8 positive-pressure anteroom |
| RABS | CAI or CACI | Unclassified SCA | ISO Class 7 positive-pressure buffer room with an ISO Class 8 positive-pressure anteroom |
| Pharmaceutical isolator | Pharmaceutical isolator | Unclassified SCA | ISO Class 8 positive-pressure room |

[a] For compounding HDs, see ⟨800⟩.
[b] An IVLFZ must not be used in an unclassified area.

If a robotic enclosure is used as the PEC, or placed within the PEC, a dynamic airflow smoke pattern test must be performed initially and at least every 6 months thereafter to ensure that 1) it is properly integrated into the facility, 2) there is no turbulence or refluxing at any critical site(s), 3) room air does not enter the PEC where sterile products and/or preparations may be exposed, and 4) all processes can be performed without introducing contamination to the DCA(s).

**4.2.4 Air exchange requirements:** For cleanroom suites, adequate HEPA-filtered airflow to the buffer room(s) and anteroom(s) is required to maintain the appropriate ISO classification during compounding activities. Airflow is measured in terms of the number of air changes per hour (ACPH). The ACPH may need to be higher to maintain the required ISO classification and microbial state of control depending on the following factors:

- Number of personnel permitted to work in the area
- Number of particles that may be generated from activities and processes in the area
- Equipment located in the room
- Room pressure

See *Table 6* for a summary of ACPH requirements for non-HD sterile compounding areas. Additional ACPH requirements include:

A minimum of 30 total HEPA-filtered ACPH must be supplied to ISO Class 7 rooms:

- The total HEPA-filtered air change rate must be adequate to maintain ISO Class 7 during dynamic operating conditions considering the factors listed above

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- At least 15 ACPH of the total air change rate in a room must come from the HVAC through HEPA filters located in the ceiling
- The HEPA-filtered air from the PEC, when added to the HVAC-supplied HEPA-filtered air, must increase the total HEPA-filtered ACPH to at least 30 ACPH
- If the PEC is used to meet the minimum total ACPH requirements, the PEC must not be turned off except for maintenance
- Rooms where activity levels are high may require more HEPA-filtered ACPH to maintain ISO Class 7 air quality under dynamic operating conditions
- The ACPH from HVAC, ACPH contributed from the PEC, and the total ACPH must be documented on the certification report

A minimum of 20 total HEPA-filtered ACPH must be supplied to ISO Class 8 rooms:

- The total HEPA-filtered air change rate must be adequate to maintain ISO Class 8 under dynamic operating conditions considering the factors listed above
- At least 15 ACPH of the total air change rate in a room must come from the HVAC through HEPA filters located in the ceiling
- Rooms where activity levels are high may require more HEPA-filtered ACPH to maintain ISO Class 8 air quality under dynamic operating conditions
- The total ACPH must be documented on the certification report

**Table 6. Summary of ACPH Requirements for Non-HD Sterile Compounding Areas**

| Compounding Area | ACPH Requirement |
|---|---|
| Unclassified SCA | No requirement |
| ISO Class 7 room(s) | ≥30 ACPH |
| ISO Class 8 room(s) | ≥20 ACPH |

**4.2.5 Establishing and maintaining pressure differentials:** Continuous differential positive pressure is required to minimize airflow from an area with lower air-quality classification to an area of higher air-quality classification. In a cleanroom suite, a minimum differential positive pressure of 0.020-inch water column is required between adjacent ISO-classified areas (e.g., between the buffer room and anteroom). The pressure differential between the anteroom and the unclassified area must not be less than 0.020-inch water column. No pressure differential is required between the SCA and the surrounding area. See ⟨800⟩ for pressure requirements for compounding HD CSPs.

Where pressure differentials are required, a pressure differential monitoring device must be used to continuously monitor the pressure differentials. The quantitative results from the pressure monitoring device must be reviewed and documented at least daily on the days when compounding is occurring.

**4.2.6 Facilities preparing Category 2 or Category 3 CSPs from nonsterile starting components:** Weighing, measuring, or otherwise manipulating components could generate airborne chemical particles (e.g., API or added substances). If preparing Category 2 or Category 3 CSP from nonsterile component(s), presterilization procedures, such as weighing and mixing, must be completed in an ISO Class 8 or better environment (e.g., anteroom or buffer room). Presterilization procedures must be performed in single-use containment glove bags, containment ventilated enclosures (CVEs), BSCs, or CACIs to minimize the risk of airborne contamination. CVEs, BSCs, or CACIs used for presterilization procedures must be certified at least every 6 months.

Presterilization procedures must not adversely affect the required air quality of the SEC as demonstrated during certification under dynamic operating conditions. Personnel must follow the hygiene and garbing requirements as described in *3. Personal Hygiene and Garbing* during presterilization procedures.

**4.3 Creating Areas to Achieve Easily Cleanable Conditions**

**4.3.1 Cleanroom suite:** The surfaces of ceilings, walls, floors, doors, door frames, fixtures, shelving, work surfaces, counters, and cabinets in the classified area must be smooth, impervious, free from cracks and crevices, and nonshedding so they can be cleaned and disinfected and to minimize spaces in which microorganisms and other contaminants can accumulate. Surfaces should be resistant to damage (e.g., rust) by cleaning agents, sporicidal and other types of disinfectants, and tools used to clean. Junctures between the ceiling and the walls and between the walls and the floor must be sealed to eliminate cracks and crevices where dirt can accumulate. If ceilings consist of inlaid panels, the panels must be caulked around each panel to seal them to the support frame.

Walls must be constructed of, or may be covered with, durable material (e.g., epoxy painted walls or heavy-gauge polymer) and the integrity of the surface must be maintained. Panels must be joined together and sealed to each other and the support structure. Floors must include coving to the sidewall, or the juncture between the floor and the wall must be caulked. Classified areas should minimize dust-collecting overhangs, such as utility pipes, and ledges, such as windowsills. If overhangs or ledges are present, they must be easily cleanable. The exterior lens surface of ceiling light fixtures must be smooth, mounted flush, and sealed. Any other penetrations through the ceiling or walls must be sealed.

**4.3.2 SCA:** The SCA and all surfaces (e.g., walls, floors, counters, and equipment) in the SCA must be clean, uncluttered, and dedicated to compounding. Surfaces in the SCA should be smooth, impervious, free from cracks and crevices, and non-shedding so they can be easily cleaned and disinfected and to minimize spaces in which microorganisms and other contaminants can accumulate. Surfaces should be resistant to damage (e.g., rust) by cleaning agents, sporicidal and other types of disinfectants, and tools used to clean. Dust-collecting overhangs, such as utility pipes, and ledges, such as windowsills, should be minimized. If overhangs or ledges are present, they must be easily cleanable.

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**4.4 Water Sources**

The facility where CSPs are prepared must be designed so that activities such as hand hygiene and garbing will not adversely affect the ability of the PEC to function as designed. Sinks should enable hands-free use. Surfaces of the sink(s) must be cleaned and disinfected each day of use, and a sporicidal disinfectant must be applied at least monthly (see *7.1 Agents and Supplies for Cleaning, Disinfecting, and Applying Sporicidal Disinfectants*).

In facilities with a cleanroom suite, the sink used for hand hygiene may be placed either inside or outside of the anteroom. If the sink is located outside of the anteroom, it must be located in a clean space to minimize the risk of bringing contaminants into the anteroom. If the sink is located inside the anteroom, it may be placed on either the clean side or the dirty side of the anteroom. [NOTE—The order of hand washing and garbing depends on the placement of the sink (see *3.2 Hand Hygiene* and *3.3 Garbing Requirements*)] . The buffer room must not contain plumbed water sources [e.g., sink(s), eyewash(es), shower(s), or floor drain(s)]. The anteroom must not contain floor drain(s). If installed, sprinkler systems should be recessed and covered, and the covers should be easily cleanable.

In a facility with an SCA design, a hand-washing sink must be placed not closer than 1 m to the PEC and may be either inside the SCA or in close proximity to the SCA.

**4.5 Placement and Movement of Materials**

Only furniture, equipment, and other materials necessary for performing compounding activities are permitted in a classified area or SCA, and they should be low-shedding and easily cleaned and disinfected. Their number, design, location, and manner of installation must not impact environmental air quality and must promote effective cleaning and disinfecting. No shipping carton(s) or other corrugated or uncoated cardboard are allowed in a classified area or SCA.

Carts used to transport components or equipment into classified areas must be constructed from nonporous materials with cleanable casters and wheels to promote mobility and ensure ease of cleaning and disinfection. In a cleanroom suite, carts must not be moved from the dirty side to the clean side of the anteroom unless the entire cart, including casters, is cleaned and disinfected.

Only equipment necessary for performing compounding activities is permitted in the PEC. Proper placement of equipment in a PEC must be initially verified by a dynamic airflow smoke pattern test to demonstrate minimal disruption in airflow. The dynamic airflow smoke pattern test must be repeated if equipment is placed in a different location. Equipment and other items used in a classified area or SCA should not be removed except for calibration, servicing, cleaning, or other activities associated with maintenance. If removed, these items must be cleaned and wiped with sterile 70% IPA or a suitable disinfectant before they are returned to the classified area or the SCA.

Materials necessary for performing compounding activities that have been exposed in patient care and treatment areas must not enter anterooms, buffer rooms, or segregated compounding areas unless thoroughly cleaned and disinfected. The designated person(s) is responsible for addressing other areas of risk in the facility's SOPs. The designated person(s) may permit accommodations as long as the quality of the CSP and environment will not be affected. Accommodations must be documented.

## 5. CERTIFICATION AND RECERTIFICATION

Before a compounding area is used to compound either Category 1, Category 2, or Category 3 CSPs, it must be independently certified using the requirements in this chapter and when applicable, manufacturer specifications. Certification indicates that the compounding area is meeting its design and air quality specifications (see *Table 4*).

Certification of the classified areas including the PEC must be performed initially, and recertification must be performed at least every 6 months and must include:

- *Airflow testing:* Airflow testing is performed to determine acceptability of the air velocity, the room air exchange rate, and the room pressure differential in doorways between adjacent rooms to ensure consistent airflow and that the appropriate quality of air is maintained under dynamic operating conditions. The ACPH from HVAC, ACPH contributed from the PEC, and the total ACPH must be documented on the certification report.
- *HEPA filter integrity testing:* HEPA filters must be leak tested at the factory and then leak tested again after installation and as part of recertification.
- *Total particle count testing:* (See *5.1 Total Airborne Particle Sampling*.) Total particle count testing must be performed under dynamic operating conditions using calibrated electronic equipment.
- *Dynamic airflow smoke pattern test:* Smoke pattern tests must be performed for each PEC during dynamic operating conditions to demonstrate unidirectional airflow and sweeping action over and away from the preparation(s).

Classified areas additionally must be recertified if there are changes to the area such as redesign, construction, replacement or relocation of any PEC, or alteration in the configuration of the room that could affect airflow or air quality.

All certification and recertification records must be reviewed by the designated person(s) to ensure that the classified environments meet the minimum requirements in this chapter. The number of personnel present in each PEC and SEC during total particle-count tests and dynamic airflow smoke-pattern tests must be documented. Records must be maintained in accordance with the requirements in *20. Documentation*.

A corrective action plan must be implemented and documented in response to any out-of-range results. Data collected in response to corrective actions must be reviewed to confirm that the actions taken have been effective.

**5.1 Total Airborne Particle Sampling**

The engineering control equipment function must function as designed to ensure that the levels of total airborne particles remain within acceptable limits during compounding (see *Table 4*). Total airborne particle count testing must be conducted in all classified areas during dynamic operating conditions at least every 6 months to measure the performance of the engineering controls that are being used to provide the specified levels of air cleanliness (e.g., in the ISO Class 5 PEC and ISO Class 7 and 8 rooms).

Total airborne particle sampling sites must be selected in all classified areas. Measurements of total airborne particles must be taken in each PEC at locations where there is greatest risk to the exposed CSPs, containers, and closures. When conducting sampling of the PEC, care should be taken to avoid disturbing the unidirectional airflow within the PEC. All sampling sites and procedures must be described in the facility's SOPs. Measurements of total airborne particles in other

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classified areas, including the buffer room(s) and anteroom(s), should be taken at representative locations that reflect the quality of air in the room(s).

**Data evaluation and action levels:** If levels measured during the total air sampling program exceed the criteria in *Table 4* for the ISO classification of the area sampled, the cause must be investigated and corrective action taken and documented. Data collected in response to corrective actions must be reviewed to confirm that the actions taken have been effective. Some examples of corrective action include process or facility improvements or HEPA filter replacement or repair. The extent of the investigation should be consistent with the deviation and should include an evaluation of trends.

## 6. MICROBIOLOGICAL AIR AND SURFACE MONITORING

An effective microbiological air and surface monitoring program provides information on the environmental quality of the compounding area. In addition, an effective microbiological air and surface monitoring program identifies environmental quality trends over time, identifies potential routes of contamination, and allows for implementation of corrective actions to minimize the risk of CSP contamination. Sterile compounding facilities must develop and implement written procedures for microbiological air and surface monitoring (see *17. SOPs*). All microbiological air and surface monitoring procedures, the test results, and the corrective actions must be documented, and the records must be maintained in accordance with the requirements in *20. Documentation*. Data collected in response to corrective actions must be reviewed to confirm that the actions taken have been effective.

### 6.1 General Monitoring Requirements

The microbiological air and surface monitoring program must include 1) viable impact volumetric airborne particulate sampling and 2) surface sampling. The goals of a microbiological air and surface monitoring program are to determine whether contamination is present at unacceptable levels and to assess whether proper personnel practices are being followed, cleaning and disinfecting agents are effective, and environmental quality is maintained.

The microbiological air and surface monitoring program involves the collection and evaluation of samples from various air and surface locations to detect airborne and surface contaminants. The data from microbiological airborne and surface sampling are then used to assess risks for contamination, potential routes of contamination, and the adequacy of cleaning and disinfecting agents and procedures. Regular review of the sampling data must be performed to detect trends and the results of the review must be documented.

In addition, results from microbiological air and surface sampling must be reviewed in conjunction with personnel data (i.e., training records, visual observations, competency assessments) to assess the state of control and to identify potential risks of contamination. Corrective action in response to any adverse findings is required to maintain the necessary environmental quality for preparation of CSPs. Data must also be reviewed following corrective actions to confirm that the actions taken have been effective in achieving the required microbiological air and surface quality levels (see *Table 4*, *Table 7*, and *Table 8*).

Microbiological air and surface monitoring must be performed initially for sterile compounding facilities to establish a baseline level of environmental quality. After initial sampling, the environment in which sterile compounding activities are performed must be monitored according to the minimum frequencies described in this section to ensure that the environment remains suitable for sterile compounding.

Evaluating results collected over a period of time can be useful in identifying trends or determining that a significant change has occurred, even when the results fall within the specified levels.

Microbiological air and/or surface monitoring must be conducted in all classified areas during dynamic operating conditions to confirm that the required environmental quality is maintained. In addition to the specific sampling frequencies described in this section, sampling must be performed in the following circumstances:

- In conjunction with the certification of new facilities and equipment
- After any servicing of facilities or equipment (see *4. Facilities and Engineering Controls*)
- In response to identified problems (e.g., positive growth in sterility tests of CSPs)
- In response to identified trends (e.g., repeated positive gloved fingertip and thumb sampling results, failed media fill testing, or repeated observations of air or surface contamination)
- In response to changes that could impact the sterile compounding environment (e.g., change in cleaning agents)

The microbiological air and surface monitoring program must be clearly described in the facility's SOPs, which must include a diagram of the sampling locations, procedures for collecting samples, frequency of sampling, size of samples (e.g., surface area, volume of air), time of day of sampling in relation to activities in the compounding area, and action levels that will trigger corrective action.

The times and locations of sampling should be carefully selected based on their relationship to the activities performed in the area. It is important to obtain samples from locations that pose the highest possible risk of contamination to the CSP and that are likely to be representative of the conditions throughout the area. To obtain air and surface samples that are representative of the typical compounding conditions at the facility, in all PECs and classified rooms, air sampling must be conducted during dynamic operating conditions and surface sampling should be performed at the end of a compounding activity or shift but before the area has been cleaned and disinfected. The monitoring program must be designed and conducted in a manner that minimizes the chance that the sampling itself will contribute to contamination of the CSP or the environment.

It is important that personnel are trained and competent in air and surface sampling procedures to ensure accurate and reproducible sampling. All impaction air samplers must be serviced and calibrated as recommended by the manufacturer.

### 6.2 Monitoring Air Quality for Viable Airborne Particles

A monitoring program for viable airborne particles must be developed and implemented to assess microbiological air quality in all classified areas.

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**6.2.1 Viable air sampling—timing and locations:** Volumetric active air sampling of all classified areas using an impaction air sampler must be conducted in each classified area [e.g., ISO Class 5 PEC and ISO Class 7 and 8 room(s)] during dynamic operating conditions. For entities compounding Category 1 and Category 2 CSPs, this must be completed at least every 6 months. For entities compounding any Category 3 CSPs, this must be completed within 30 days prior to the commencement of any Category 3 compounding and at least monthly thereafter regardless of the frequency of compounding Category 3 CSPs. Air sampling sites must be selected in all classified areas.

**6.2.2 Viable air sampling procedures:** When conducting sampling of the PEC, care should be taken to avoid disturbing unidirectional airflow. See *Box 5* for active air sampling procedures. A general microbiological growth media that supports the growth of bacteria and fungi must be used (e.g., TSA). COAs from the manufacturer must verify that the sampling media devices meet the expected growth promotion, pH, and sterilization requirements. Samples must be incubated in an incubator at the temperatures listed in *Box 5*. The incubator temperature must be monitored during incubation, either manually or by a continuous recording device, and the results must be reviewed and documented as described in the facility's SOPs. The incubator must be placed in a location outside of the sterile compounding area.

**Box 5. Active Air Sampling Procedures for Viable Airborne Monitoring**

1. Follow the manufacturer's instructions for operation of the impaction air sampler, including placement of media device(s).
2. Using the impaction air sampler, test at least 1 cubic meter or 1000 L of air from each location sampled.
3. At the end of each sampling period, retrieve the media device and cover it. Handle and store media devices to avoid contamination and prevent condensate from dropping onto the agar during incubation and affecting the accuracy of the cfu reading (e.g., invert plates).
4. Incubate the media device at 30°–35° for no less than 48 h. Examine for growth. Record the total number of discrete colonies of microorganisms on each media device as cfu per cubic meter of air on an environmental sampling form based on sample type (i.e., viable air), sample location, and sample date.
5. Then incubate the media device at 20°–25° for no less than 5 additional days. Examine for growth. Record the total number of discrete colonies of microorganisms on each media device as cfu per cubic meter of air on an environmental sampling form based on sample type (i.e., viable air), sample location, and sample date.
6. Alternatively, to shorten the overall incubation period, two sampling media devices may be collected for each sample location and incubated concurrently.
   A. Both media devices could be TSA or one media device could be TSA and the other fungal media (e.g., malt extract agar [MEA] or Sabouraud dextrose agar [SDA]).
   B. Incubate each media device in a separate incubator. Incubate one media device at 30°–35° for no less than 48 h, and incubate the other media device at 20°–25° for no less than 5 days. If fungal media are used as one of the samples, incubate the fungal media sample at 20°–25° for no less than 5 days.
   C. Count the total number of discrete colonies of microorganisms on each media device, and record these results as cfu per cubic meter of air.
   D. Record the results of the sampling on an environmental sampling form based on sample type (i.e., viable air), and include the sample location and sample date.

**6.2.3 Viable air sampling data evaluation and action levels:** Evaluate cfu counts against the action levels in *Table 7* and examine counts in relation to previous data to identify adverse results or trends. If two sampling media devices are collected at a single location, all recovered growth on each must be documented and action levels applied to each sampling media device separately. If levels measured during the viable air monitoring program exceed the levels in *Table 7* for the ISO classification levels of the area sampled, the cause must be investigated and corrective action must be taken. Data collected in response to corrective actions must be reviewed to confirm that the actions taken have been effective. The corrective action plan must be dependent on the cfu count and the microorganism recovered. Some examples of corrective action include process or facility improvements, personnel training, cleaning and disinfecting, or HEPA filter repair and/or replacement. The extent of the investigation should be consistent with the deviation and should include an evaluation of trends. The corrective action plan must be documented and should include resampling of failed areas to confirm corrective action was successful. If levels measured during viable air sampling exceed the levels in *Table 7*, an attempt must be made to identify any microorganisms recovered to the genus level (see *Microbial Characterization, Identification, and Strain Typing* ⟨1113⟩) with the assistance of a microbiologist.

**Table 7. Action Levels for Viable Airborne Particle Air Sampling**

| ISO Class | Air Sampling Action Levels [cfu/cubic meter (1000 liters) of air/media device] |
|---|---|
| 5 | >1 |
| 7 | >10 |
| 8 | >100 |

### 6.3 Monitoring Surfaces for Viable Particles

Surface sampling is an important tool used to assist in maintenance of a suitably controlled environment for compounding CSPs. Surface sampling is useful for evaluating facility cleaning and material handling procedures, work surface cleaning and disinfecting procedures, and personnel competency in work practices such as cleaning and disinfecting. All sampling sites and procedures must be described in the facility's SOPs.

**6.3.1 Surface sampling—timing and locations:** Each classified area, including each room and the interior of each ISO Class 5 PEC and pass-through chambers connecting to classified areas, must be sampled for microbial contamination using a risk-based approach. Samples should be taken from the following classified areas:

- Equipment contained within the PEC
- Staging or work area(s) near the PEC
- Frequently touched surfaces

Surface sampling in the DCA must also be conducted in conjunction with media-fill testing to assess aseptic manipulation competency (see *2.3 Competency Testing in Aseptic Manipulation*)

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When conducted, surface sampling should be performed at the end of a compounding activity or shift but before the area has been cleaned and disinfected.

For entities compounding Category 1 and Category 2 CSPs, surface sampling of all classified areas, and pass-through chambers connecting to classified areas, must be conducted at least monthly (see *Microbiological Control and Monitoring of Aseptic Processing Environments* ⟨1116⟩).

For entities compounding any Category 3 CSPs, surface sampling of all classified areas, and pass-through chambers connecting to classified areas, must be completed prior to assigning a BUD longer than the limits established in *Table 13*, and at least weekly (see ⟨1116⟩) on a regularly scheduled basis regardless of the frequency of compounding Category 3 CSPs. Additionally, surface sampling must be conducted within the PEC used to prepare Category 3 CSPs, at the end of each batch before cleaning and disinfection occurs, unless a self-enclosed robotic device is used. When a self-enclosed robotic device is used as the PEC to prepare Category 3 CSPs, surface sampling must be conducted at least once daily at the end of compounding operations, before cleaning and disinfection occurs.

**6.3.2 Surface sampling procedures:** See *Box 6* for the procedures for surface sampling on flat surfaces. Surface sampling media devices (e.g., plates, paddles, or slides) containing microbial growth media must be used for sampling flat surfaces. COAs from the manufacturer must verify that the sampling media devices meet the expected growth promotion, pH, and sterilization requirements. Surface sampling media devices must contain general microbial growth media (e.g., TSA) supplemented with neutralizing additives (e.g., lecithin and polysorbate 80) to neutralize the effects of any residual disinfecting agents. Surface sampling media devices must have a raised convex surface. Sterile swabs wetted with sterile water or a sterile neutralizing buffer may be used when sampling irregular surfaces and difficult-to-reach locations such as crevices, corners, and spaces between surfaces. After sampling, the sampled area must be thoroughly cleaned and disinfected (see *7. Cleaning, Disinfecting, and Applying Sporicidal Disinfectants and Sterile 70% IPA*).

Samples must be incubated in an incubator at the temperatures listed in *Box 6*. The incubator temperature must be monitored during incubation, either manually or by a continuous recording device, and the results must be reviewed and documented. The incubator must be placed in a location outside of the sterile compounding area.

**Box 6. Surface Sampling Procedures**

1. Remove the cover from the surface sampling media device. Using a rolling motion, firmly press the media surface onto the surface to be sampled. The media device will leave a residue of growth media on the sample site. After sampling, clean and disinfect the sampled area to remove the residue from the surface.
2. Cover each media device. Handle and store media devices to avoid contamination and prevent condensate from dropping onto the agar during incubation and affecting the accuracy of the cfu reading (e.g., invert plates).
3. Incubate media device(s) at 30°–35° for no less than 48 h. Examine for growth. Record the total number of discrete colonies of microorganisms on each media device as cfu per media device on an environmental sampling form based on sample type (i.e., surface), sample location, and sample date.
4. Incubate the media device at 20°–25° for no less than 5 additional days. Examine for growth. Record the total number of discrete colonies of microorganisms on each media device (cfu per sample) on the environmental sampling record based on sample type (i.e., surface), sample location, and sample date.
5. Alternatively, to shorten the overall incubation period, two surface sampling media devices may be collected for each sample location and incubated concurrently.
   A. Both media devices could be TSA or one media device could be TSA and the other fungal media (e.g., malt extract agar [MEA] or Sabouraud dextrose agar [SDA]). Media must be supplemented with neutralizing additives (e.g., lecithin and polysorbate 80).
   B. Incubate each media device in a separate incubator. Incubate one media device at 30°–35° for no less than 48 h, and incubate the other media device at 20°–25° for no less than 5 days. If fungal media are used, incubate the fungal media device at 20°–25° for no less than 5 days.
   C. Count the total number of discrete colonies of microorganisms on each media device, and record these results as cfu per media device.
   D. Record the results of the sampling on an environmental sampling form based on sample type (i.e., surface), and include the sample location and sample date.

**6.3.3 Surface sampling data evaluation and action levels:** Evaluate cfu counts against the action levels in *Table 8*, and examine counts in relation to previous data to identify adverse results or trends. If two sampling media devices are collected at a single location, all recovered growth on each must be documented and action levels applied to each sampling media device separately. If levels measured during surface sampling exceed the levels in *Table 8* for the ISO classification levels of the area sampled, the cause must be investigated and corrective action must be taken. Data collected in response to corrective actions must be reviewed to confirm that the actions taken have been effective. The corrective action plan must be dependent on the cfu count and the microorganism recovered. Some examples of corrective action include process or facility improvements, personnel training, cleaning and disinfecting, or HEPA filter replacement and/or repair. The extent of the investigation should be consistent with the deviation and should include an evaluation of trends. The corrective action plan must be documented. If levels measured during surface sampling exceed the levels in *Table 8*, an attempt must be made to identify any microorganism recovered to the genus level (see ⟨1113⟩) with the assistance of a microbiologist.

**Table 8. Action Levels for Surface Sampling**

| ISO Class | Surface Sampling Action Levels (cfu/media device) |
|---|---|
| 5 | >3 |
| 7 | >5 |
| 8 | >50 |

## 7. CLEANING, DISINFECTING, AND APPLYING SPORICIDAL DISINFECTANTS AND STERILE 70% IPA

Surfaces in classified areas used to prepare Category 1, Category 2, and Category 3 CSPs must be:

- Cleaned
- Disinfected

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- Sporicidal disinfectants applied

according to the frequencies described in *Table 10* for each CSP category.

Additionally, in a PEC, sterile 70% IPA must be applied after cleaning and disinfecting, or after the application of a one-step disinfectant cleaner or sporicidal disinfectant, to remove any residue. Sterile 70% IPA must also be applied immediately before initiating compounding. During the compounding process sterile 70% IPA must be applied to the horizontal work surface, including any removable work trays, of the PEC at least every 30 min if the compounding process takes 30 min or less. If the compounding process takes more than 30 min, compounding must not be disrupted, and the work surface of the PEC must be disinfected immediately after compounding.

These activities are important because surfaces in classified areas and SCAs are a potential source of microbial contamination of CSPs.

The process of cleaning involves removing organic and inorganic materials from surfaces, usually with a manual or mechanical process and a cleaning agent. The process of disinfecting involves destruction of microorganisms, usually with a chemical agent. The process of applying a sporicidal disinfectant involves the destruction of bacterial and fungal spores. See *Table 9* for a summary of the purposes of the cleaning, disinfectant, and sporicidal disinfectants.

**Table 9. Purpose of Cleaning, Disinfecting, and Sporicidal Disinfectants**

| Type of Agent | Purpose |
|---|---|
| Cleaning | An agent, usually containing a surfactant, used for the removal of substances (e.g., dirt, debris, microbes, and residual drugs or chemicals) from surfaces. |
| Disinfectant | A chemical or physical agent used on inanimate surfaces and objects to destroy fungi, viruses, and bacteria. |
| Sporicidal | A chemical or physical agent that destroys bacterial and fungal spores when used at a sufficient concentration for a specified contact time. It is expected to kill all vegetative microorganisms. |

Surfaces must be cleaned prior to being disinfected with an EPA-registered disinfectant (or equivalent for entities outside the US) unless an EPA-registered (or equivalent for entities outside the US) one-step disinfectant cleaner is used to accomplish both the cleaning and disinfection in one step. A sporicidal disinfectant must also be applied. Some EPA-registered (or equivalent) one-step disinfectant cleaners may have sporicidal properties. Cleaning and disinfecting surfaces and applying a sporicidal disinfectant must occur at the minimum frequencies specified in *Table 10*.

All cleaning and disinfecting activities must be performed by trained and appropriately garbed personnel using facility-approved agents and procedures, which must be described in written SOPs. Personnel must be trained if there are any changes in the cleaning and disinfecting procedures. Cleaning must be performed in the direction of clean to dirty areas. The same floor mop may be used in both the buffer and anteroom, but only in that order. Mops used in areas where HDs are compounded must be dedicated for use only in those areas.

The frequency, method(s), and location(s) of cleaning, disinfecting, and applying sporicidal disinfectants must be established in written SOPs, in accordance with the manufacturer's instructions and must be followed by all cleaning personnel. The manufacturer's directions or published data for the minimum contact time must be followed for each of the cleaning, disinfecting, and sporicidal disinfectants used. When sterile 70% IPA is used, it must be allowed to dry. All cleaning, disinfecting, and application of sporicidal disinfectants must be documented according to the facility's SOPs.

**Table 10. Minimum Frequency for Cleaning and Disinfecting Surfaces and Applying Sporicidal Disinfectants in Classified Areas and in the SCA[a]**

| Site | Cleaning | Disinfecting[b] | Applying Sporicidal Disinfectant |
|---|---|---|---|
| PEC(s) and equipment inside the PEC(s) | • Equipment and all interior surfaces of the PEC daily on days when compounding occurs and when surface contamination is known or suspected | • Equipment and all interior surfaces of the PEC daily on days when compounding occurs and when surface contamination is known or suspected | • Monthly for entities compounding Category 1 and/or Category 2 CSPs<br>• Weekly for entities compounding Category 3 CSPs |
| Removable work tray of the PEC, when applicable | • Work surface of the tray daily on days when compounding occurs<br>• All surfaces and the area underneath the work tray monthly | • Work surface of the tray on days when compounding occurs<br>• All surfaces and the area underneath the work tray monthly | • Work surfaces of the tray monthly<br>• All surfaces and the area underneath the work tray monthly |
| Pass-through chambers | • Daily on days when compounding occurs | • Daily on days when compounding occurs | • Monthly for entities compounding Category 1 and/or Category 2 CSPs<br>• Weekly for entities compounding Category 3 CSPs |
| Work surface(s) outside the PEC | • Daily on days when compounding occurs | • Daily on days when compounding occurs | |
| Floor(s) | • Daily on days when compounding occurs | • Daily on days when compounding occurs | |
| Wall(s), door(s), and door frame(s) | • Monthly | • Monthly | • Monthly |
| Ceiling(s)[c] | | | |
| Storage shelving and bin(s) | | | |
| Equipment outside the PEC(s) | | | |

[a] Cleaning of sinks is described in *4.4 Water Sources*.

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[b] Many disinfectants registered by the EPA are one-step cleaning and disinfecting agents, which means that the disinfectant has been formulated to be effective in the presence of light-to-moderate soiling without a separate cleaning step.
[c] Ceilings of the SCA are required to be cleaned, disinfected, and applied with sporicidal disinfectant only when visibly soiled and when surface contamination is known or suspected.

### 7.1 Agents and Supplies for Cleaning, Disinfecting, and Applying Sporicidal Disinfectants

**7.1.1 Agents:** Cleaning and disinfecting agents must be selected and used with careful consideration of compatibilities, effectiveness, and user safety. Considerations when selecting and using disinfectants include their antimicrobial activity, inactivation by organic matter, residue, shelf life, preparation requirements of the agent, and suitability for surfaces being disinfected. After the disinfectant or sporicidal disinfectant is applied to the surface, the agent must be allowed to dwell for the minimum contact time specified by the manufacturer.

Cleaning, disinfecting and sporicidal agents used within the PEC must be sterile. When diluting concentrated cleaning and disinfecting agents for use in the PEC, sterile water must be used. In classified areas outside of the PEC, sterile cleaning and disinfecting agents should be used. When diluting concentrated cleaning and disinfecting agents for use outside of the PEC, sterile water should be used.

**7.1.2 Supplies:** All cleaning and disinfecting supplies (e.g., wipers, sponges, pads, and mop heads) with the exception of tool handles and holders must be low lint. In addition, cleaning and disinfecting supplies used in the PEC must be sterile with the exception of tool handles and holders, which must be cleaned and disinfected prior to use in a PEC.

Wipers, sponges, pads, and mop heads should be disposable. If disposable cleaning supplies are used, they must be discarded after each cleaning activity. Reusable cleaning tools must be made of cleanable materials (e.g., handles should not be made of wood or any other porous material) and must be cleaned and disinfected before and after each use. Reusable cleaning tools must be dedicated for use in the classified areas or SCA and must not be removed from these areas except for disposal. They must be discarded as determined based on the condition of the tools. Cleaning supplies used in the classified areas and SCAs must be disposed of in a manner that minimizes the potential for dispersing contaminants into the air (e.g., with minimal agitation, away from work surfaces).

Once opened, sterile cleaning and disinfecting agents and supplies (e.g., closed containers of sterile wipers) and sterile 70% IPA may be reused for a time period specified as by the manufacturer and/or described in the facility written SOPs.

### 7.2 Procedures for Cleaning, Disinfecting, and Applying Sporicidal Disinfectants and Sterile 70% IPA in the PEC

Clean, disinfect, and apply a sporicidal disinfectant to equipment and all interior surfaces in the PEC at the minimum frequencies specified in *Table 10*. See *Box 7* and *Box 8* for procedures for cleaning, disinfecting, and applying a sporicidal disinfectant in the PEC.

**Box 7. Procedures for Cleaning and Disinfecting the PEC**

- If necessary, remove visible particles, debris, or residue with an appropriate solution (e.g., *Sterile Water for Injection* or *Sterile Water for Irrigation*) using sterile, low-lint wipers.
- Using a sterile low-lint wiper, apply a sterile cleaning agent followed by a sterile EPA-registered disinfectant or apply a sterile EPA-registered (or equivalent for entities outside the US) one-step disinfectant cleaner to equipment and all interior surfaces of the PEC.
- Ensure the contact time specified by the manufacturer is achieved.
- Using a sterile low-lint wiper, apply sterile 70% IPA to equipment and all interior surfaces in the PEC.
- Allow the surface to dry completely before beginning compounding.

**Box 8. Procedures for Applying a Sporicidal Disinfectant in the PEC**

- If necessary, remove visible particles, debris, or residue with an appropriate solution (e.g., *Sterile Water for Injection* or *Sterile Water for Irrigation*) using sterile, low-lint wipers.
- After cleaning and disinfecting (see *Box 7*), apply the sterile sporicidal disinfectant using a sterile low-lint wiper to all surfaces and the area underneath the work tray; if the sporicidal disinfectant is a sterile EPA-registered (or equivalent for entities outside the US) one-step disinfectant sporicidal cleaner, separate cleaning and disinfecting steps are not required.
- Ensure the contact time specified by the manufacturer is achieved.
- Using a sterile low-lint wiper, apply sterile 70% IPA to all interior surfaces, including underneath the work tray.
- Allow the surface to dry completely before beginning compounding.

## 8. INTRODUCING ITEMS INTO THE SEC AND PEC

### 8.1 Introducing Items into the SEC

Before any item is introduced into the clean side of anteroom(s), placed into pass-through chamber(s), or brought into the SCA, providing that packaging integrity will not be compromised, it must be wiped with a sporicidal disinfectant, EPA-registered disinfectant, or sterile 70% IPA using low-lint wipers by personnel wearing gloves. If an EPA-registered disinfectant or sporicidal disinfectant is used, the agent must be allowed to dwell for the minimum contact time specified by the manufacturer. If sterile 70% IPA is used, it must be allowed to dry. The wiping procedure should not compromise the packaging integrity or render the product label unreadable.

### 8.2 Introducing Items into the PEC

Just before any item is introduced into the PEC, it must be wiped with sterile 70% IPA using sterile low-lint wipers and allowed to dry before use. When sterile items are received in sealed containers designed to keep them sterile until opening, the sterile items may be removed from the covering as the supplies are introduced into the ISO Class 5 PEC without the need to wipe the individual sterile supply items with sterile 70% IPA. The wiping procedure must not render the product label unreadable.

### 8.3 Use of Sterile 70% IPA on Critical Sites within the PEC

Critical sites (e.g., vial stoppers, ampule necks, and intravenous bag septums) must be wiped with sterile 70% IPA in the PEC to provide both chemical and mechanical actions to remove contaminants. The sterile 70% IPA must be allowed to dry before personnel enter or puncture stoppers and septums or break the necks of ampules.

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## 9. EQUIPMENT, SUPPLIES, AND COMPONENTS

### 9.1 Equipment

PECs are described in *4.2.3 Types of PECs and placement*. Other equipment used in compounding CSPs (e.g., automated compounding devices [ACDs] and balances) should be of suitable composition such that the surfaces that contact components are not reactive or sorptive. Equipment that must be brought into classified areas must be wiped with a sporicidal disinfectant, EPA-registered disinfectant, or sterile 70% IPA using low-lint wipers.

Equipment must be placed in a manner that facilitates sterile compounding operations. The equipment must be capable of operating properly and within required performance parameters. Compounding personnel must follow established SOPs for the calibration, maintenance, cleaning, and use of the equipment based on the manufacturer's recommendations. Personnel must maintain records from equipment calibration, verification, and maintenance in accordance with the requirements in *20. Documentation*.

ACDs and other similar equipment are designed to assist in the compounding of preparations by delivering specific volumes of solution(s) automatically under computerized control.

Before using ACDs or other similar equipment, compounding personnel must conduct an accuracy assessment before the first use and again each day the equipment is used to compound CSPs. The precision of the equipment can be monitored based on an assessment of day-to-day variations in its accuracy measures. Compounding personnel must maintain a daily record of the accuracy measurements on the days the equipment is in use. Corrective actions must be implemented if accuracy measurements are outside the manufacturer's specification.

Weighing, measuring, or otherwise manipulating components that could generate airborne chemical particles (e.g., active pharmaceutical ingredients [APIs], added substances, conventionally manufactured products) must be evaluated to determine if these activities must be performed in a PEC or other closed system processing device (e.g., single use containment glove bag) to reduce the potential exposure to personnel or contamination of the facility or CSPs (See *4.2.6 Facilities preparing Category 2 or Category 3 CSPs from nonsterile starting component(s)*). The process evaluation must be carried out in accordance with the facility's SOPs and the assessment must be documented.

### 9.2 Supplies

Supplies (e.g., beakers, utensils, needles, syringes, filters, and tubing sets) should be of suitable composition such that the surfaces that contact components are not reactive or sorptive. Supplies in direct contact with the CSP must be sterile and depyrogenated.

### 9.3 Components

Compounding personnel must follow the facility's SOPs, which must address the selection, receipt, evaluation, handling, storage, and documentation of all CSP components, including all ingredients and container closures.

**9.3.1 Component selection:** Conventionally manufactured sterile products should be used when available and appropriate for the intended CSP.

When APIs are used:

- Must comply with the criteria in the *USP–NF* monograph, if one exists
- Must have a COA that includes the specifications (e.g., compendial requirements for quality) and that test results for the component show that the API meets expected quality
- In the United States, must be manufactured by an FDA-registered facility
- Outside of the United States, must comply with the laws and regulations of the applicable regulatory jurisdiction

For all components other than APIs:

- Must comply with the criteria in the *USP–NF* monograph, if one exists
- Must be accompanied by documentation (e.g., COA, labeling) that includes the specifications and test results and shows that the component meets the specifications
- In the US, should be manufactured by an FDA-registered facility
  - If a component cannot be obtained from an FDA-registered facility, the designated person(s) must select an acceptable and reliable source (see *Good Distribution Practices for Bulk Pharmaceutical Excipients* ⟨1197⟩). The compounding facility must establish the identity, strength, purity, and quality of the ingredients obtained from that supplier by reasonable means. Reasonable means may include but are not limited to visual inspections, evaluation of a COA supplied by the manufacturer, and/or verification by analytically testing a sample to determine conformance with the COA or other specifications.
- Outside of the US, must comply with the laws and regulations of the applicable regulatory jurisdiction

When CSPs are used as components, see *16. Use of CSPs as Components*. All APIs and other components used must be evaluated for suitability for use in sterile drug preparation. Components labeled with "not for pharmaceutical use", "not for injectable use", "not for human use" or an equivalent statement must not be used to compound for these purposes.

Each lot of commercially available sterile, depyrogenated containers and container closure systems must be accompanied by a COA or other documentation showing conformance with established specifications (i.e., sterility and depyrogenation requirements). If sterilization and depyrogenation of supplies or container closure systems are performed on site, the efficacy of each process must be established and documented (see *Sterilization of Compendial Articles* ⟨1229⟩).

**9.3.2 Component receipt:** Upon receipt of each lot of a component, the external packaging must be examined for evidence of deterioration and other aspects of unacceptable quality. Facility personnel must verify the labeling and condition of the component [e.g., whether the outer packaging is damaged and whether temperature-sensing indicators show that the component has been exposed to excessive temperature(s)].

Any component found to be of unacceptable quality must be promptly rejected, clearly labeled as rejected, and segregated from active stock to prevent use before appropriate disposal. Any other lots of that component from that vendor must be examined to determine whether other lots have the same defect.

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The date of receipt by the compounding facility must be clearly marked on each API or added substance package that lacks a vendor expiration date. Packages of components (i.e., API and added substances) that lack a vendor's expiration date must be assigned a conservative expiration date, not to exceed 1 year after receipt by the compounding facility.

**9.3.3 Component evaluation before use:** Compounding personnel must ascertain before use that components for CSPs are of the correct identity, appropriate quality, within expiry date and have been stored under appropriate conditions. The following information should be used to make this determination: prescription or medication order, compounding record (CR), master formulation record (if used), vendor label(s), COA(s) of API(s) and other component(s), product labeling of any conventionally manufactured sterile products, labeling of CSP(s), and documentation of the compounding facility's storage conditions and practices.

All components must be reinspected before use. All packages must be reinspected to detect container breaks, looseness of the cap or closure, and deviation from the expected appearance, aroma, and/or texture of the contents that might have occurred during storage. Sterile container closures must be visually reinspected to ensure that they are free from defects that could compromise sterility and that they are otherwise suitable for their intended use.

Any component found to be of unacceptable quality must be promptly rejected, clearly labeled as rejected, and segregated from active stock to prevent use before appropriate disposal. Any other lots of that component from that vendor must be examined to determine whether other lots have the same defect.

**9.3.4 Component handling and storage:** All components must be handled and stored in a manner that prevents contamination, mix-ups, and deterioration.

Components must be stored in closed containers under temperature, humidity, and lighting conditions consistent with those indicated in official monographs or specified by the suppliers and/or manufacturers.

Personnel must monitor temperature in the area(s) where components are stored either manually at least once daily on days that the facility is open or by a continuous temperature recording device to determine whether the temperature remains within the appropriate range. The results of the temperature readings must be documented on a temperature log or stored in the continuous recording device and must be retrievable. All monitoring equipment must be calibrated or verified for accuracy as recommended by the manufacturer or every 12 months if not specified by the manufacturer.

## 10. STERILIZATION AND DEPYROGENATION

When selecting the sterilization method for CSPs prepared from one or more nonsterile starting components or using nonsterile supplies or devices, personnel must take into consideration the nature of the component(s), their physical and chemical properties, and the intended container closure system.

The sterilization method used must sterilize the CSP without degrading its physical and chemical stability (e.g., affecting its strength, purity, or quality) or the packaging integrity. (See also the ⟨1229⟩ series of chapters.)

The following must be considered when selecting an appropriate sterilization method:

- Terminal sterilization (e.g., steam, dry heat, or irradiation) is the preferred method unless the specific CSP or container closure system cannot tolerate terminal sterilization
- Steam sterilization is not an option if moisture, pressure, or the temperatures used would degrade the CSP or if there is insufficient moisture to sterilize the CSP within the final, sealed, container closure system
- Filtration may not be an option for some compounded preparations, for example preparations with suspended drug particles or emulsions with a significant droplet size.

CSPs that are terminally sterilized (e.g., steam, dry heat, or irradiation) must use a process intended to achieve a probability of a nonsterile unit (PNSU) of $10^{-6}$. [NOTE—This is also called the sterility assurance level (SAL).] A PNSU of $10^{-6}$ is equivalent to a probability that 1 unit in a million is nonsterile. A PNSU value cannot be applied to CSPs that are aseptically filled into a sterile container following sterilization by filtration because sterilization by filtration is not terminal sterilization.

Injectable compounded preparations that contain nonsterile components or that come into contact with nonsterile devices (e.g., containers, tubing) during any phase of the compounding procedure must be sterilized within 6 h after completing the preparation to minimize the generation of bacterial endotoxins in CSPs.

A description of the terminal sterilization and depyrogenation process, including the temperature, pressure (if applicable), duration, permissible load conditions for each cycle, and the use of biological indicators and endotoxin challenge vials (ECVs) must be included in the facility's SOPs.

SOPs must include training and competency of personnel on all sterilization methods and equipment used by the facility. In addition, the SOPs must include a schedule and method for establishing and verifying the effectiveness of the terminal sterilization and depyrogenation methods selected, as well as the methods for maintaining and cleaning the sterilizing and depyrogenation equipment.

### 10.1 Depyrogenation

See *Dry Heat Depyrogenation* ⟨1228.1⟩. Dry heat depyrogenation must be used to render glassware, metal, and other thermostable containers and components pyrogen free. Depyrogenation processes typically operate at a range of temperatures, from approximately 170°–400°, depending on the exposure time (e.g., a cycle might hold the items at 250° for 30 min to achieve sterility and depyrogenation). The duration of the exposure period must include sufficient time for the items to reach the depyrogenation temperature. The items must remain at the depyrogenation temperature for the duration of the depyrogenation period.

The effectiveness of the dry heat depyrogenation cycle must be established initially and verified annually using ECVs to demonstrate that the cycle is capable of achieving a ≥3-log reduction in endotoxins (see *Bacterial Endotoxins Test* ⟨85⟩). The effectiveness of the depyrogenation cycle must be re-established if there are changes to the depyrogenation cycle described in SOPs (e.g., changes in load conditions, duration, or temperature). This verification must be documented.

Items that are not thermostable must be depyrogenated by multiple rinses with sterile, nonpyrogenic water (e.g., *Sterile Water for Injection* or *Sterile Water for Irrigation*) and then thoroughly drained or dried immediately before use in compounding See *Depyrogenation by Rinsing* ⟨1228.4⟩.

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**10.2 Sterilization by Filtration**

See *Sterilizing Filtration of Liquids* ⟨1229.4⟩. Sterilizing filters must be sterile, depyrogenated, have a nominal pore size of 0.22 µm or smaller, and be appropriate for pharmaceutical use. Sterilizing filters with labeling that states "for laboratory use only" or a similar statement must not be used for compounding CSPs. Sterilizing filters must be certified by the manufacturer to retain at least $10^7$ microorganisms of a strain of *Brevundimonas diminuta* per square centimeter of upstream filter surface area under conditions similar to those in which the CSPs will be filtered (i.e., pressure, flow rate, and volume filtered).

The designated person(s) must ensure—from available published information, from supplier documentation, or through direct challenge (e.g., filtering the CSP)—that the filters 1) are chemically and physically compatible with all ingredients in the CSP (e.g., water-miscible alcohols may damage filter integrity); 2) are chemically stable at the pressure and temperature conditions that will be used; and 3) have enough capacity to filter the required volumes. The filter dimensions and the CSP to be sterilized by filtration should permit the sterilization process to be completed without the need for replacement of the filter during the process. Filter units used to sterilize CSPs must be subjected to the manufacturers' recommended integrity testing, such as a post-use bubble point test. If multiple filters are required for the compounding process, each of the filters must pass a filter-integrity test.

When CSPs are known to contain excessive particulate matter, a prefiltration step must be performed using a filter of larger nominal pore size (e.g., 1.2 µm) or a separate filter of larger nominal pore size should be placed upstream of (i.e., prior to) the sterilizing filter to remove gross particulate contaminants before the CSP is passed through the sterilizing-grade filter. Excessive particulate matter requiring a prefiltration step could potentially be a signal of an inappropriate formulation, and therefore the formulation and the process should be assessed and modified if necessary. CSPs that were prepared using a filter that failed integrity tests must be discarded or, after investigating the cause of the failure and selection of an appropriate filter, refiltered for sterilization not more than one additional time.

**10.3 Sterilization by Steam Heat**

Temperatures used to achieve sterilization by steam heat are lower than those used to achieve depyrogenation. The process of thermal sterilization using saturated steam under pressure (i.e., autoclaving) is the preferred method for terminal sterilization of aqueous CSPs in their final, sealed container closure system (see *Steam Sterilization by Direct Contact* ⟨1229.1⟩). Steam sterilization is not an option if moisture, pressure, or the temperatures used would degrade the CSP.

To achieve sterility when steam sterilization is used, all materials must be directly exposed to steam under adequate pressure for the length of time necessary, as determined by use of appropriate biological indicators, to render the items sterile (e.g., 20–60 min at 121° saturated steam under a pressure of 15 psi, depending on the volume or size of the CSP being sterilized). The duration of the exposure period must include sufficient time for the entire contents of the CSP and other items to reach the sterilizing temperature. The CSP and other items must remain at the sterilizing temperature for the duration of the sterilization period.

CSPs must be placed in the autoclave to allow steam to reach the CSPs without entrapment of air. Flat, stainless-steel trays with low sides or ventilated bottoms will permit steam contact. When preparing items that must be wrapped for steam sterilization, wrap them in low-lint protective fabric or paper or seal in envelopes that will permit steam penetration and are designed to minimize the risk of post-sterilization microbial contamination. For CSPs, immediately before filling containers that will be steam sterilized, solutions must be passed through a filter with a nominal pore size of not larger than 1.2 µm for removal of particulate matter.

Sealed containers must be able to generate steam internally. Stoppered and crimped empty vials must contain a small amount of sterile water to generate steam.

The effectiveness of steam sterilization must be verified and documented with each sterilization run or load by using appropriate biological indicators, such as spores of *Geobacillus stearothermophilus* (ATCC 12980, ATCC 7953, or equivalent; see *Biological Indicators for Sterilization* ⟨1229.5⟩), and other confirmation methods such as physicochemical indicators (see *Physicochemical Integrators and Indicators for Sterilization* ⟨1229.9⟩).

The steam supplied must be generated using water per the manufacturer's recommendation. A calibrated data recorder or chart must be used to monitor each cycle and to examine for cycle irregularities (e.g., deviations in temperature or pressure). The date, run, and load numbers of the steam sterilizer used to sterilize a CSP must be documented in the CR.

**10.4 Sterilization by Dry Heat**

Dry heat may be used for those items that cannot be sterilized by steam or other means when the moisture would damage the material or the wrapping material is impermeable (see *Dry Heat Sterilization* ⟨1229.8⟩). Sterilization by dry heat requires higher temperatures and longer exposure times than sterilization by steam. The duration of the exposure period must include sufficient time for the entire contents of CSPs and other items to reach the sterilizing temperature. The CSP and other items must remain at the sterilizing temperature for the duration of the sterilization period. Immediately before filling ampules and vials that will be sterilized by dry heat, CSP solutions must be passed through a filter with a nominal pore size of not larger than 1.2 µm for removal of particulate matter.

Dry heat sterilization is usually performed in an oven designed for sterilization at 160° or higher. If lower temperatures are used, they must be shown to achieve effective sterilization (see ⟨1229.8⟩*, Validation of Dry Heat Sterilization, Biological Indicators*).

Heated air must be evenly distributed throughout the chamber, which is typically accomplished by an air blower. The calibrated oven must be equipped with temperature controls and a timer. During sterilization, sufficient space must be left between materials to allow for circulation of the hot air. A calibrated data recorder or chart must be used to monitor each cycle and the data must be reviewed to identify cycle irregularities (e.g., deviations in temperature or exposure time).

The effectiveness of the dry heat sterilization method must be verified and documented with each sterilization run or load using appropriate biological indicators such as spores of *Bacillus atrophaeus* (ATCC 9372; see ⟨1229.5⟩) and other confirmation methods (e.g., temperature-sensing devices). The date, run, and load numbers of the dry heat oven used to sterilize a CSP must be documented in the CR.

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## 11. MASTER FORMULATION AND COMPOUNDING RECORDS

### 11.1 Creating Master Formulation Records

A master formulation record (MFR) is a detailed record of procedures that describes how the CSP is to be prepared. An MFR must be created for all CSPs prepared from nonsterile ingredient(s) or CSPs prepared for more than one patient. Any changes or alterations to the MFR must be approved and documented according to the facility's SOPs. *Box 9* lists the information that must be included in an MFR.

**Box 9. Master Formulation Records**

An MFR must include at least the following information:

- Name, strength or activity, and dosage form of the CSP
- Identities and amounts of all ingredients; if applicable, relevant characteristics of components (e.g., particle size, salt form, purity grade, solubility)
- Type and size of container closure system(s)
- Complete instructions for preparing the CSP, including equipment, supplies, a description of the compounding steps, and any special precautions
- Physical description of the final CSP
- BUD and storage requirements
- Reference source to support the stability of the CSP
- Quality control (QC) procedures (e.g., pH testing, filter integrity testing)
- Other information as needed to describe the compounding process and ensure repeatability (e.g., adjusting pH and tonicity; sterilization method, such as steam, dry heat, irradiation, or filter)

### 11.2 Creating Compounding Records

A CR documents the compounding of each CSP. A CR must be created for all Category 1, Category 2, and Category 3 CSPs. A CR must also be created for immediate-use CSPs prepared for more than one patient. The CR must be created to document the compounding process. A prescription or medication order or label may serve as the CR. If an ACD, workflow management system, or other similar equipment is used, the required information in the CR may be stored electronically as long as it is retrievable and contains the required information (see *Box 10*). An MFR can serve as the basis for preparing the CR. For example, a copy of the MFR can be made that contains spaces for recording the information needed to complete the CR. *Box 10* lists the information that must be included in a CR.

**Box 10. Compounding Records**

CRs must include at least the following information:

- Name, strength or activity, and dosage form of the CSP
- Date and time of preparation of the CSP
- Assigned internal identification number (e.g., prescription, order, or lot number)
- A method to identify the individuals involved in the compounding process and individuals verifying the final CSP
- Name of each component
- Vendor, lot number, and expiration date for each component for CSPs prepared for more than one patient and for CSPs prepared from nonsterile ingredient(s)
- Weight or volume of each component
- Strength or activity of each component
- Total quantity compounded
- Final yield (e.g., quantity, containers, number of units)
- Assigned BUD and storage requirements
- Results of QC procedures (e.g., visual inspection, filter integrity testing, pH testing)

If applicable, the CR must also include:

- MFR reference for the CSP
- Calculations made to determine and verify quantities and/or concentrations of components

## 12. RELEASE INSPECTIONS AND TESTING

All release testing procedures (e.g., visual inspections and testing) must be included in the facility's documentation (see *11. Master Formulation and Compounding Records* and *17. SOPs*). Any out-of-specification results must be investigated, and a corrective action plan must be implemented and documented as part of the quality assurance (QA) and QC program (see *18. Quality Assurance and Quality Control*).

### 12.1 Visual Inspection

At the completion of compounding, before release and dispensing, the CSP must be visually inspected to determine whether the physical appearance of the CSP is as expected (e.g., free of inappropriate visible particulates or other foreign matter, discoloration, or other defects). The CSP label must be visually inspected to confirm that the CSP and its labeling match the prescription or medication order. The inspection also must include a visual inspection of container closure integrity (e.g., checking for leakage, cracks in the container, or improper seals). Any CSP found to be of unacceptable quality (e.g., observed defects) must be promptly rejected, clearly labeled as rejected, and segregated from active stock to prevent use before appropriate disposal.

When a CSP will not be released or dispensed on the day of preparation, a visual inspection must be conducted immediately before it is released or dispensed to make sure that the CSP does not exhibit any defects such as precipitation, cloudiness, or leakage, which could develop during storage. Any CSP found to be of unacceptable quality (e.g., observed defects) must be promptly rejected, clearly labeled as rejected, and segregated from active stock to prevent use before appropriate disposal. Defects that indicate sterility or stability problems must be investigated to determine the cause according to the facility's SOPs (see *18. Quality Assurance and Quality Control*).

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**12.2 Sterility Testing**

Sterility testing is not required for Category 1 CSPs (see *Table 12*). For Category 2 CSPs assigned a BUD that requires sterility testing (see *Table 13*) and all Category 3 CSPs, the testing must be performed according to ⟨71⟩ or a validated alternative method (see ⟨1223⟩) that is noninferior to ⟨71⟩ testing.

If sterility testing is performed, the minimum quantity of each container to be tested for each media is specified in ⟨71⟩, *Table 2*, and the number of containers required to be tested in relation to the batch size is specified in ⟨71⟩, *Table 3*, except as described below. The maximum batch size for all CSPs requiring sterility testing must be limited to 250 final yield units.

If the number of CSPs to be compounded in a single batch is less than the number of CSPs needed for testing as specified in ⟨71⟩, *Table 3*, additional units must be compounded to perform sterility testing as follows:

- If 1–39 CSPs are compounded in a single batch, the sterility testing must be performed on a number of units equal to 10% of the number of CSPs prepared, rounded up to the next whole number. For example:
  - If 1 CSP is compounded, 10% of 1 rounded up to the next whole number would indicate that 1 additional CSP must be prepared for sterility testing
  - If 39 CSPs are compounded, 10% of 39 rounded up to the next whole number would indicate that 4 additional CSPs must be prepared for sterility testing
- If more than 40 CSPs are prepared in a single batch, the sample sizes specified in ⟨71⟩, *Table 3* must be used.

If sterility testing is performed according to ⟨71⟩, the *Method Suitability Test* from that chapter must be performed to ensure that contamination can be recovered. If performing sterility testing according to ⟨71⟩, the *Membrane Filtration* method from that chapter is the method of choice when the CSP formulation permits. The preferred alternative is the ⟨71⟩, *Test for Sterility of the Product to Be Examined, Direct Inoculation of the Culture Medium* method. If an alternative method is used for sterility testing, the method must be validated (see ⟨1223⟩) and demonstrated to be suitable for that CSP formulation.

Sterility tests resulting in failures must prompt an investigation into the possible causes and must include identification of the microorganism, as well as an evaluation of the sterility testing procedure, compounding facility, process, and/or personnel that may have contributed to the failure. The source(s) of the contamination, if identified, must be corrected, and the facility must determine whether the conditions causing the sterility failure affect other CSPs. The investigation and resulting corrective actions must be documented.

**12.3 Bacterial Endotoxins Testing**

Category 1 injectable CSPs do not require testing for bacterial endotoxins. Category 2 injectable CSPs compounded from one or more nonsterile component(s) and assigned a BUD that requires sterility testing (see *Table 13*) and Category 3 injectable CSPs compounded from one or more nonsterile component(s) must be tested to ensure that they do not contain excessive bacterial endotoxins (see ⟨85⟩). Category 2 injectable CSPs compounded from one or more nonsterile component(s) and assigned a BUD that does not require sterility testing should be tested for bacterial endotoxins. In the absence of a bacterial endotoxin limit in an official *USP–NF* monograph or other CSP formula source, the CSP must not exceed the endotoxin limit calculated as described in ⟨85⟩ for the appropriate route of administration for humans. CSPs for nonhuman species must not exceed the endotoxin limit calculated as described in ⟨85⟩ based on the largest recommended dose and weight (or average weight for more than a single animal) of the target animal species unless a different limit is scientifically supported. CSPs administered epidurally should have the same endotoxin limit as that of intrathecally administered CSPs. See also *Guidelines on the Endotoxins Test* ⟨1085⟩.

## 13. LABELING

Category 1, Category 2, and Category 3 CSPs must be labeled with appropriate, legible identifying information to prevent errors during storage, dispensing, and use. The term *labeling* designates all labels and other written, printed, or graphic matter on the immediate container or on or inside any package or wrapper in which it is enclosed, except any outer shipping container. The term *label* designates that part of the labeling that is on the immediate container. See *Labeling* ⟨7⟩.

All labeling must be in compliance with laws and regulations of the applicable regulatory jurisdiction.

The label on each immediate container of the CSP must, at a minimum, display prominently and legibly the following information:

- Assigned internal identification number (e.g., barcode, prescription, order, or lot number)
- Active ingredient(s) and their amount(s), activity(ies), or concentration(s)
- Storage conditions if other than controlled room temperature
- BUD
- Dosage form
- Total amount or volume if it is not obvious from the container
- If it is a single-dose container, a statement stating such when space permits
- If it is a multiple-dose container, a statement stating such

The labeling on the CSP must display the following information, as applicable:

- Route(s) of administration
- Special handling instructions
- Warning statements
- Compounding facility name and contact information if the CSP is to be sent outside of the facility or healthcare system in which it was compounded

The labeling on the CSP should indicate that the preparation is compounded.

Labeling procedures must be followed as described in the facility's SOPs to prevent labeling errors and CSP mix-ups. The label of the CSP must be verified to ensure that it conforms with the

1. Prescription or medication order;

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2. MFR, if required (see *11.1 Creating Master Formulation Records*); and
3. CR, if required (see *11.2 Creating Compounding Records*)

## 14. ESTABLISHING BEYOND-USE DATES

### 14.1 Terminology

Each CSP label must state the date, or the hour and date, beyond which the preparation must not be used and must be discarded (i.e., the BUD). The BUD is determined from the date and time that preparation of the CSP is initiated. The BUD is not intended to limit the time during which the CSP is administered (e.g., infused).

BUDs and expiration dates are not the same. An expiration date identifies the time during which a conventionally manufactured product, API, or added substance can be expected to meet the requirements of a *USP–NF* monograph, if one exists, or maintain expected quality provided it is kept under the specified storage conditions. The expiration date limits the time during which a conventionally manufactured product, API, or added substance may be dispensed or used (see ⟨7⟩, *Labels and Labeling for Products in Other Categories, Expiration Date and Beyond-Use Date*). Expiration dates are assigned by manufacturers based on analytical and performance testing of the sterility, chemical and physical stability, and packaging integrity of the product. Expiration dates are specific to a particular formulation in its container and at stated exposure conditions of illumination and temperature. See *Table 11* for a summary of terms.

**Table 11. Summary of Terms**

| Term | Definition | Applicability |
|---|---|---|
| BUD | Either the date, or hour and date, after which a CSP must not be used. The BUD is determined from the date and time that preparation of the CSP is initiated | Applies to all CSPs |
| Expiration date | The time during which a product can be expected to meet the requirements of the *USP–NF* monograph, if one exists, or maintain expected quality provided it is kept under the specified storage conditions. | Applies to all conventionally manufactured products, APIs, and added substances |

### 14.2 Parameters to Consider in Establishing a BUD

BUDs for CSPs should be established conservatively to ensure that the drug maintains its required characteristics (i.e., stability and sterility) until its BUD.

When establishing a BUD for a CSP, compounders must consider parameters that may affect quality, including but not limited to:

- Chemical and physical stability properties of the drug and/or its formulation
- Materials of composition of the container closure system and compatibility of the container closure system with the final preparation (e.g., leachables, interactions, adsorption, and storage conditions)

The BUDs for CSPs are based primarily on factors that affect the achievement and maintenance of sterility, which include but are not limited to the following:

- Conditions of the environment in which the CSP is prepared
- Aseptic processing and sterilization method
- Starting components (e.g., sterile or nonsterile ingredients)
- Whether or not sterility testing is performed
- Storage conditions (e.g., packaging and temperature)

**14.2.1 Terminal sterilization methods and aseptic processing:** A CSP may be prepared by the following methods (see *10. Sterilization and Depyrogenation*):

- Terminal sterilization, which includes compounding with sterile and/or nonsterile starting ingredient(s) and subsequent sterilization with a process intended to achieve a PNSU of $10^{-6}$ (e.g., steam, dry heat, irradiation).
- Aseptic processing, which includes 1) compounding with only sterile starting ingredient(s) or 2) compounding with nonsterile ingredient(s) followed by sterilization by filtration. [NOTE—Sterilization by filtration is not a form of terminal sterilization.]

Terminal sterilization is the preferred method of sterilization, unless the specific CSP or container closure system cannot tolerate terminal sterilization. *Table 13* allows for longer BUDs for terminally sterilized CSPs than for aseptically processed CSPs because terminal sterilization using a verified method provides reasonable assurance that a CSP will be sterile.

**14.2.2 Starting components:** The use of one or more nonsterile starting component(s) is a risk factor to be considered when preparing a CSP. A longer BUD is permitted for CSPs that are aseptically processed from conventionally manufactured sterile starting component(s) than from one or more nonsterile starting component(s).

**14.2.3 Sterility testing:** Sterility testing (see *12.2 Sterility Testing*) of a CSP can provide additional assurance of the absence of contamination, although passing a sterility test does not guarantee that all units of a batch of CSPs are sterile because contamination may not be uniformly distributed throughout the batch. A longer BUD is permitted if sterility testing results are within acceptable limits. The maximum batch size for all CSPs requiring sterility testing must be limited to 250 final yield units.

**14.2.4 Storage conditions:** Storage in colder conditions (i.e., in a refrigerator or freezer [see *Packaging and Storage Requirements* ⟨659⟩]) has been shown to slow the growth of most microorganisms. However, the chemical and physical stability of the CSP and its components must be considered when storing in colder conditions (e.g., some formulations may precipitate when stored in a refrigerator or freezer). A longer BUD is permitted in *Table 12* and *Table 13* for CSPs stored in colder conditions than for CSPs stored at controlled room temperature.

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If the CSP will be stored in a frozen state, the container closure system must be able to withstand the physical stress (i.e., without breaking or cracking) during storage in a freezer. The CSP must be thawed in appropriate conditions to avoid compromising the physical and chemical stability of the preparation and its components (e.g., do not heat in a microwave). Once the CSP is thawed, the CSP must not be refrozen.

CSPs may be stored under different storage conditions before they are used (e.g., CSPs may first be frozen, then thawed in the refrigerator, and finally kept at controlled room temperature before administration). The storage time of a CSP must not exceed the original BUD placed on the CSP for its labeled storage condition, and BUDs must not be additive. For example, an aseptically processed CSP prepared from one or more nonsterile starting component(s) cannot be stored for 45 days in a freezer, then 4 days refrigerated, and then 24 h at controlled room temperature for a total of 50 days. Once a CSP has been stored under a condition that would require a shorter BUD (e.g., controlled room temperature), the CSP must be used within the time frame for that storage condition (in the previous example, 24 h).

### 14.3 Establishing a BUD for a CSP

BUDs for CSPs must be established in accordance with *Table 12* for Category 1 CSPs, *Table 13* for Category 2 CSPs and *Table 14* for Category 3 CSPs. One day is equivalent to 24 h.

The BUD limits in these tables are based on the risk of microbial contamination or not achieving and maintaining sterility despite implementation of the requirements in this chapter. The CSP formulation must remain chemically and physically stable, and its packaging must maintain its integrity for the duration of the BUD.

A shorter BUD must be assigned when the stability of the CSP or its components is less than the hours or days stated in the applicable table below. Additionally:

- The BUD must not exceed the shortest remaining expiration date of any of the commercially available starting components.
- For CSPs prepared from one or more compounded components, the BUD should generally not exceed the shortest BUD of any of the individual compounded components. However, there may be acceptable instances when the BUD of the final CSP exceeds the BUD assigned to compounded components (e.g., pH-altering solutions). If the assigned BUD of the final CSP exceeds the BUD of the compounded components, the physical, chemical, and microbiological quality of the final CSP must not be negatively impacted.

*Table 12* establishes the longest permitted BUDs for Category 1 CSPs. Category 1 CSPs may be prepared in an SCA or cleanroom suite (see *4.2 Facility Design and Environmental Controls*).

**Table 12. BUD Limits for Category 1 CSPs[a]**

| Storage Conditions | |
|---|---|
| **Controlled Room Temperature (20°–25°)** | **Refrigerator (2°–8°)** |
| ≤12 h | ≤24 h |

[a] A shorter BUD must be assigned when the physical and chemical stability of the CSP is less than the BUD limit stated in the table.

*Table 13* establishes the longest permitted BUDs for Category 2 CSPs. Category 2 CSPs must be prepared in a cleanroom suite (see *4.2 Facility Design and Environmental Controls*).

**Table 13. BUD Limits for Category 2 CSPs[a]**

| Preparation Characteristics | | Storage Conditions | | |
|---|---|---|---|---|
| **Compounding Method** | **Sterility Testing Performed and Passed** | **Controlled Room Temperature (20°–25°)** | **Refrigerator (2°–8°)** | **Freezer (–25° to –10°)** |
| Aseptically processed CSPs | No | Prepared from one or more nonsterile starting component(s): 1 day | Prepared from one or more nonsterile starting component(s): 4 days | Prepared from one or more nonsterile starting component(s): 45 days |
| | | Prepared from only sterile starting components: 4 days | Prepared from only sterile starting components: 10 days | Prepared from only sterile starting components: 45 days |
| | Yes | 30 days | 45 days | 60 days |
| Terminally sterilized CSPs | No | 14 days | 28 days | 45 days |
| | Yes | 45 days | 60 days | 90 days |

[a] A shorter BUD must be assigned when the physical and chemical stability of the CSP is less than the BUD limit stated in the table.

### 14.4 Additional Requirements for Category 3 CSPs

**14.4.1 Assigning Category 3 BUDs:** Increasing the storage time of a CSP introduces additional risk for chemical degradation, physical incompatibilities, the compromising of the container closure system, and microbial proliferation. To address these risks and maintain a higher state of environmental control, additional requirements must be met when assigning BUDs for Category 3 CSPs in accordance with *Table 14*. Category 3 CSPs must not be assigned a BUD longer than the limits in *Table 14*.

**14.4.2 Facility and Personnel Requirements for Category 3 CSPs:** In addition to the requirements in this section, other facility and personnel requirements related to compounding Category 3 CSPs are addressed throughout the chapter.

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- Category 3 personnel competency requirements apply to personnel who participate in or oversee the compounding of Category 3 CSPs (see *2.2 Demonstrating Competency in Garbing and Hand Hygiene* and *2.3 Competency Testing in Aseptic Manipulation*).
- Category 3 garbing requirements apply to all personnel entering the buffer room where Category 3 CSPs are compounded and apply at all times regardless of whether Category 3 CSPs are being compounded on a given day (see *3.3 Garbing Requirements*).
- Increased environmental monitoring requirements apply to all classified areas where Category 3 CSPs are compounded and apply at all times regardless of whether Category 3 CSPs are being compounded on a given day (see *6.2 Monitoring Air Quality for Viable Airborne Particles* and *6.3 Monitoring Surfaces for Viable Particles*).
- The frequency of application of sporicidal disinfectants applies to all classified areas where Category 3 CSPs are compounded and applies at all times regardless of whether Category 3 CSPs are being compounded on a given day (see *Table 10*).

**14.4.3 Stability Requirements for Category 3 CSPs:** The BUD assigned to a Category 3 CSP must be supported by stability data obtained using a stability-indicating analytical method that is able to distinguish the active ingredient from its degradants and impurities (e.g., by forced degradation studies) and quantify the amount of the active ingredient.

- The Category 3 CSP must be prepared according to the exact formulation (API and other ingredients of identical grade and procedures) from which the stability data are derived.
- The Category 3 CSP must be packaged and stored in a container closure of the same materials of composition as that used in the study.
- The analytical method must be validated based on characteristics such as those described in ⟨1225⟩.
- The compounding facility must have documentation of the stability study, including a description of the methodology (e.g., number of samples taken, storage conditions), validation of the method, the stability-indicating analytical method, and all of the results of the study.

If the Category 3 CSP is an injection (*Particulate Matter in Injections* ⟨788⟩) or if it is an ophthalmic solution (*Particulate Matter in Ophthalmic Solutions* ⟨789⟩), particulate-matter testing is conducted once per formulation with acceptable results.

Once for each formulation and for each container closure system in which it will be packaged, the container closure system used is evaluated for and conforms to container closure integrity to the end of the BUD (see *Package Integrity Evaluation—Sterile Products* ⟨1207⟩).

**14.4.4 Release testing for Category 3 CSPs**

- Each time the Category 3 CSP is prepared, it is sterility tested and meets the requirements of ⟨71⟩ or a validated alternative method (see *Table 14*) that is noninferior to ⟨71⟩ testing.
- Each time the Category 3 CSP is prepared, it is tested for endotoxins for acceptable results, if endotoxin testing is required under *12.3 Bacterial Endotoxins Testing*.

*Table 14* establishes the longest permitted BUDs for Category 3 CSPs. If all of the conditions described for Category 3 CSPs in this chapter are not met, the applicable BUD in *Table 13* must not be exceeded.

**Table 14: BUD Limits for Category 3 CSPs[a]**

| Preparation Characteristics | Storage Conditions | | |
|---|---|---|---|
| Compounding Method | Controlled Room Temperature (20°–25°) | Refrigerator (2°–8°) | Freezer (–25° to –10°) |
| Aseptically processed, sterility tested, and passing all applicable tests for Category 3 CSPs | 60 days | 90 days | 120 days |
| Terminally sterilized, sterility tested, and passing all applicable tests for Category 3 CSPs | 90 days | 120 days | 180 days |

[a] A shorter BUD must be assigned when the physical and chemical stability of the CSP is less than the BUD limit stated in the table.

### 14.5 Multiple-Dose CSPs

A compounded multiple-dose container is designed to contain more than one dose, intended to be entered or penetrated multiple times, and usually contains a preservative. A preservative is intended to inhibit the growth of microorganisms and minimize the risk of contamination. The use of preservatives must be appropriate for the CSP formulation and the route of administration. For example, the preservative must not be inactivated by any ingredients in the CSP, and some preservatives are not always appropriate for the patient (e.g., neonates) or route of administration (e.g., intrathecal or ophthalmic injection). The use of preservatives, however, must not be considered a substitute for aseptic technique.

A multiple-dose CSP must be prepared as a Category 2 or Category 3 CSP. An aqueous multiple-dose CSP must additionally pass antimicrobial effectiveness testing in accordance with *Antimicrobial Effectiveness Testing* ⟨51⟩. The compounder may rely on antimicrobial effectiveness testing 1) conducted (or contracted for) once for each formulation in the particular container closure system in which it will be packaged or 2) results from an FDA-registered facility or published in peer-reviewed literature sources, provided that the CSP formulation (including any preservative) and container closure system are exactly the same as those tested, unless a bracketing study is performed. Antimicrobial effectiveness testing may be performed on a low concentration and a high concentration of the active ingredient in the formulation to establish preservative effectiveness across various strengths of the same formulation (e.g., bracketing). The concentration of all other ingredients (including preservatives) must be the same throughout the bracketing study.

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After a multiple-dose CSP container is initially entered or punctured, the multiple-dose container must not be used for longer than the assigned BUD or 28 days if supported by antimicrobial effectiveness testing results (see ⟨51⟩) on the CSP, whichever is shorter.

The container closure system used to package the multiple-dose CSP must be evaluated for and conform to container closure integrity (see ⟨1207⟩). The container closure integrity test needs to be conducted only once on each formulation and on fill volume in the particular container closure system in which the multiple-dose CSP will be packaged.

**Multiple-dose, nonpreserved, aqueous topical, and topical ophthalmic, CSPs:** The beyond-use date of a multiple-dose, aqueous, nonpreserved CSP intended for topical, including topical ophthalmic, administration may be assigned in accordance with *14.5 Multiple-Dose CSPs*. However, unpreserved aqueous, topical, including topical ophthalmic, formulations, are at high risk of microbial proliferation if contaminated during preparation or use.

To minimize the risk of patient harm, the requirement for passing antimicrobial effectiveness testing in accordance with ⟨51⟩ is not required only if the preparation is:

- Prepared as a Category 2 or Category 3 CSP
- For use by a single patient
- Labeled (in the label or labeling) to indicate that once opened, it must be discarded after 24 h when stored at controlled room temperature and/or that once opened, it must be discarded after 72 h when stored under refrigeration

## 15. USE OF CONVENTIONALLY MANUFACTURED PRODUCTS AS COMPONENTS

This section addresses the time within which an entered or punctured conventionally manufactured product must be used.

### 15.1 Use of Conventionally Manufactured Single-Dose Containers

A conventionally manufactured single-dose container is a container closure system that holds a sterile product for parenteral administration (injection or infusion) that is not required to meet the antimicrobial effectiveness testing requirements. If a single-dose vial is entered or punctured only in an ISO Class 5 or cleaner air, it may be used up to 12 h after initial entry or puncture as long as the labeled storage requirements during that 12-h period are maintained. Opened single-dose ampules must not be stored for any time period.

### 15.2 Use of Conventionally Manufactured Multiple-Dose Containers

A conventionally manufactured product in a multiple-dose container is intended to contain more than one dose of a drug product (see ⟨659⟩*, General Definitions, Injection Packaging Systems*). Once initially entering or puncturing the multiple-dose container, the multiple-dose container must not be used for more than 28 days (see ⟨51⟩) unless otherwise specified by the manufacturer on the labeling.

### 15.3 Use of Conventionally Manufactured Pharmacy Bulk Packages

A conventionally manufactured pharmacy bulk package is a container of a sterile product for parenteral use that contains many single doses. The contents are intended for use in a pharmacy admixture program and are restricted to the sterile preparation of admixtures for infusion or, through a sterile transfer device, for the filling of empty sterile containers. The pharmacy bulk package must be used according to the manufacturer's labeling (see ⟨659⟩*, General Definitions, Injection Packaging Systems*). The pharmacy bulk package must be entered or punctured only in an ISO Class 5 PEC.

## 16. USE OF CSPs AS COMPONENTS

This section addresses the use of CSPs (e.g., multiple-dose CSPs, single-dose CSPs, and compounded stock solutions) as components to prepare final CSPs.

When a CSP is used as a component, care must be taken to minimize the risk of contamination of both the starting component CSP and the final CSP(s).

- *Component CSP*: The component CSP must be assigned a BUD consistent with *14. Establishing Beyond-Use Dates* and must be stored under conditions for its assigned BUD when not in use.
- *Final CSP*: The final CSP must be assigned a BUD consistent with *14. Establishing Beyond-Use Dates*.

### 16.1 Use of Compounded Multiple-Dose CSPs

A multiple-dose CSP is designed to contain more than one dose of sterile preparation, intended to be entered or punctured multiple times, and usually contains a preservative. Multiple-dose CSPs are required to meet the criteria for antimicrobial effectiveness testing (see ⟨51⟩) and the requirements in *14.5 Multiple-Dose CSPs*. Multiple-dose CSPs must be stored under the conditions upon which its BUD is based (e.g., refrigerator or controlled room temperature). After a multiple-dose CSP is initially entered or punctured, the multiple-dose CSP must not be used for longer than the assigned BUD or 28 days, whichever is shorter. This time limit for entering or puncturing is not intended to restrict the BUD of the final CSP. See *14. Establishing Beyond-Use Dates*

### 16.2 Use of Compounded Single-Dose CSPs and CSP Stock Solutions

When a compounded single-dose CSP or CSP stock solution is used as a component to compound additional CSPs, the original compounded single-dose CSP or CSP stock solution must be entered or punctured in ISO Class 5 or cleaner air and must be stored under the conditions upon which its BUD is based (e.g., refrigerator or controlled room temperature). The component CSP may be used for sterile compounding for up to 12 h or its assigned BUD, whichever is shorter, and any remainder must be discarded. This time limit for entering or puncturing is not intended to restrict the BUD of the final CSP. See *14. Establishing Beyond-Use Dates*.

## 17. SOPs

Facilities that prepare CSPs must develop SOPs for the compounding process and other support activities. SOPs must include the types of CSPs that are prepared (i.e., Category 1, Category 2, Category 3). A designated person(s) must ensure that SOPs are appropriate and are implemented, which includes ensuring that personnel demonstrate competency in performing every procedure that relates to their job function. A designated person(s) must follow up to ensure that corrective actions are taken if problems, deviations, failures, or errors are identified. The corrective action must be documented.

All personnel who perform or oversee compounding or support activities must be trained in the SOPs. All compounding personnel must be trained to:

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- Recognize potential problems, deviations, failures, or errors associated with preparing a CSP (e.g., those related to equipment, facilities, materials, personnel, the compounding process, or testing) that could potentially result in contamination or other adverse impact on CSP quality
- Report any problems, deviations, failures, or errors to the designated person(s)

SOPs must be reviewed initially and at least every 12 months by the designated person(s) to ensure that they reflect current practices, and the review must be documented. Any changes or alterations to an SOP must be made only by a designated person(s) and must be documented. Revisions to SOPs must be communicated to all personnel involved in these processes and procedures, and personnel should document acknowledgment of the communication.

## 18. QUALITY ASSURANCE AND QUALITY CONTROL

QA is a system of procedures, activities, and oversight that ensures that the compounding process consistently meets quality standards. QC is the sampling, testing, and documentation of results that, taken together, ensure that specifications have been met before release of the CSP. See *Quality Assurance in Pharmaceutical Compounding* ⟨1163⟩.

A facility's QA and QC programs must be formally established and documented in the facility's SOPs that ensure that all aspects of the preparation of CSPs are conducted in accordance with the requirements in this chapter ( ⟨797⟩) and the laws and regulations of the applicable regulatory jurisdiction. Designated person(s) must ensure that the facility has formal, written QA and QC programs that establish a system of:

1. Adherence to procedures
2. Prevention and detection of errors and other quality problems
3. Evaluation of complaints and adverse events
4. Appropriate investigations and corrective actions

The facility's SOPs must describe the roles, duties, and training of the personnel responsible for each aspect of the QA program. Designated person(s) responsible for the QA program must have the training, experience, responsibility, and authority to perform these duties. The overall QA and QC program must be reviewed at least once every 12 months by the designated person(s). The results of the review must be documented, and appropriate action must be taken if needed.

### 18.1 Notification About and Recall of Out-of-Specification Dispensed CSPs

If a CSP is dispensed or administered before the results of release testing are known, the facility must have procedures in place to:

- Immediately notify the prescriber of a failure of specifications with the potential to cause patient harm (e.g., sterility, strength, purity, bacterial endotoxin, or other quality attributes)
- Recall any unused dispensed CSPs and quarantine any stock remaining in the pharmacy
- Investigate if other lots are affected and recall if necessary

An SOP for recall of out-of-specification dispensed CSPs must contain:

- Procedures to determine the severity of the problem and the urgency for implementation and completion of the recall
- Procedures to determine the distribution of any affected CSP, including the date and quantity of distribution
- Procedures to identify patients who have received the CSP
- Procedures for disposal and documentation of the recalled CSP
- Procedures to investigate and document the reason for failure

The sterile compounding facility must document the implementation of the recall procedures. The recall must be reported to appropriate regulatory bodies as required by laws and regulations of the applicable regulatory jurisdiction.

### 18.2 Complaint Handling

Compounding facilities must develop and implement SOPs for handling complaints. Complaints may include but are not limited to concerns or reports on the quality, labeling, or possible adverse reactions related to a specific CSP.

A designated person(s) must review all complaints to determine whether the complaint indicates a potential quality problem with the CSP. If it does, a thorough investigation into the cause of the problem must be initiated and completed. The investigation must consider whether the quality problem extends to other CSPs. Corrective action, if necessary, must be implemented for all potentially affected CSPs.

Consider whether to initiate a recall of potentially affected CSPs and whether to cease sterile compounding processes until all underlying problems have been identified and corrected.

A readily retrievable written or electronic record of each complaint must be kept by the facility, regardless of the source of the complaint (e.g., email, telephone, or mail). The record must contain the name of the complainant or other unique identifier, the date the complaint was received, the nature of the complaint, and the response to the complaint. In addition, to the extent that the information is known, the following should be recorded: the name and strength of the CSP and the assigned internal identification number (e.g., prescription, order, or lot number).

The record must also include the findings of any investigation and any follow-up. Records of complaints must be easily retrievable for review and evaluation for possible trends and must be retained in accordance with the record-keeping requirements in *20. Documentation*. A CSP that is returned in connection with a complaint must be quarantined until it is destroyed after completion of the investigation and in accordance with laws and regulations of the applicable regulatory jurisdiction.

### 18.3 Adverse Event Reporting

Adverse events potentially associated with the quality of CSPs must be reported in accordance with the facility's SOPs and all laws and regulations of the applicable regulatory jurisdiction. If the investigation into an adverse event reveals a quality problem with a CSP that is likely to affect other patients, those patients and prescribers potentially affected must be informed.

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## 19. CSP HANDLING, STORAGE, PACKAGING, SHIPPING, AND TRANSPORT

Processes and techniques for handling, storing, packaging, and transporting CSPs must be outlined in the facility's SOPs. Personnel who will be handling, storing, packaging, and transporting CSPs within the facility must be trained in accordance with the relevant SOPs, and the training must be documented.

### 19.1 Handling and Storing CSPs

CSPs must be handled in a manner that maintains CSP quality and packaging integrity. To help ensure that CSP quality is maintained during storage at the compounding facility, personnel must monitor conditions in the storage areas. A controlled temperature area (see ⟨659⟩) must be established and monitored to ensure that the temperature remains within the appropriate range for the CSP. The temperature must be monitored each day, either manually or by a continuous recording device. The results of the temperature readings must be documented in a temperature log per facility SOPs or stored in the continuous temperature recording device and must be retrievable. Temperature monitoring devices must be verified for accuracy at least every 12 months or as required by the manufacturer.

The compounding facility must detect and minimize temperature excursions that are outside the temperature limits within the controlled temperature areas. When it is known that a CSP has been exposed to temperatures either below or above the storage temperature limits for the CSP, a designated person(s) must determine (e.g., by consulting literature or analytical testing) whether the CSP is expected to retain its integrity or quality. If this cannot be determined, it must be discarded.

### 19.2 Packaging of CSPs

Packaging materials should protect CSPs from damage, leakage, contamination, degradation, and adsorption while preventing inadvertent exposure to transport personnel. The facility must select appropriate shipping containers and packaging materials based on the product specifications, information from vendors, and the mode of transport.

Alternative modes of transport and/or special packaging (e.g., tamper-evident closures) may be needed to protect the quality of CSPs. If the CSP is sensitive to light, light-resistant packaging materials must be used. In some cases, the CSP must be packaged in a special container (e.g., a cooler) to protect it from temperature fluctuations.

### 19.3 Shipping and Transporting CSPs

Compounding personnel must select modes of transport that are expected to deliver properly packed CSPs in an undamaged, sterile, and stable condition. Inappropriate transport can adversely affect the quality of CSPs. For example, preparation-specific considerations should be given to physical shaking that might occur during pneumatic tube transport or undue exposure to heat, cold, or light. When shipping or transporting CSPs that require special handling (e.g., CSPs with stability concerns), personnel must include specific handling instructions on the exterior of the container.

## 20. DOCUMENTATION

All facilities where CSPs are prepared must have and maintain written or electronic documentation to demonstrate compliance with the requirements in this chapter. This documentation must include, but is not limited to, the following:

- Personnel training, competency assessments, and qualification records including corrective actions for any failures
- Certification reports, including corrective actions for any failures
- Environmental air and surface monitoring procedures and results
- Equipment records (e.g., calibration, verification, and maintenance reports)
- Receipt of components
- SOPs, MFRs (if required), and CRs (if required)
- Release inspection and testing records
- Information related to complaints and adverse events including corrective actions taken
- Results of investigations and corrective actions

Documentation must comply with all laws and regulations of the applicable regulatory jurisdiction. Records must be legible and stored in a manner that prevents their deterioration and/or loss. All required documentation for a particular CSP (e.g., MFR, CR, and release inspection and testing results) must be readily retrievable for at least 2 years after preparation or as required by laws and regulations of the applicable regulatory jurisdiction or accrediting organization(s), whichever is longer.

## 21. COMPOUNDING ALLERGENIC EXTRACTS

Licensed allergenic extracts are mixed and diluted into prescription sets for an individual patient, even though these allergenic extract combinations are not specified in the approved licenses for the licensed biological products (e.g., Biological License Applications [BLA]).

Allergenic extract prescription sets must follow standards at least as stringent as those in this section as follows:

### 21.1 Personnel Qualifications for Compounding Allergenic Extract Prescription Sets

- A designated person(s) with training and expertise in allergen immunotherapy is responsible for ensuring that personnel who will be preparing allergenic extract prescription sets are trained, evaluated, and supervised.
- Before beginning to independently prepare allergenic extracts, all compounding personnel must complete training and be able to demonstrate knowledge of principles and skills for sterile compounding.
- Annual personnel training and competency must be documented. Personnel must demonstrate knowledge and competency in these procedures by passing written or electronic testing before they can be allowed to compound allergenic extract prescription sets.
- Before being allowed to independently compound, all compounders must successfully complete gloved fingertip and thumb sampling on both hands (see *Box 1* and *Table 1*) no fewer than 3 separate times. Each fingertip and thumb evaluation must occur after performing separate and complete hand hygiene and garbing procedures. After the initial competency evaluation, compounding personnel must successfully complete gloved fingertip and thumb sampling on both hands at least every 12 months thereafter.

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- Compounding personnel must have their sterile technique and related practices evaluated at least every 12 months as demonstrated by successful completion of a media-fill test (see *Box 2*). If compounding outside of a PEC, the post-media-fill surface sample is not required.
- Personnel who fail competency evaluations must successfully pass reevaluations in the deficient area(s) before they can resume compounding of allergenic extract prescription sets. The designated person(s) must identify the cause of failure and determine appropriate retraining requirements.
- Personnel who have not compounded an allergenic extract prescription set in more than 6 months must be evaluated in all core competencies before resuming compounding duties.

**21.2 Personnel Hygiene and Garbing for Compounding Allergenic Extract Prescription Sets**

- Before beginning compounding of allergenic extract prescription sets, personnel must perform hand hygiene (see *Box 3*) and garbing procedures according to the facility's SOPs.
- The minimum garb requirements include:
  - A low-lint garment with sleeves that fit snugly around the wrists and an enclosed neck (e.g., gowns)
  - A low-lint, disposable head cover that covers the hair and ears and, if applicable, a disposable cover for facial hair
  - Face mask
  - Sterile powder-free gloves
- Throughout the compounding process, personnel must apply sterile 70% IPA onto all surfaces of the gloves and allow them to dry thoroughly.

**21.3 Facilities for Compounding Allergenic Extract Prescription Sets**

- The compounding process must occur in an ISO Class 5 PEC or in a dedicated allergenic extract compounding area (AECA). The PEC or AECA used to compound allergenic extract prescription sets must be located away from unsealed windows, doors that connect to the outdoors, and traffic flow, all of which may adversely affect the air quality. Neither a PEC nor an AECA may be located where environmental control challenges (e.g., restrooms, warehouses, or food preparation areas) could negatively affect the air quality. The PEC or the work surfaces in the AECA must be located at least 1 m away from a sink. The impact of activities that will be conducted around or adjacent to the PEC or AECA must be considered carefully when designing such an area.
- If used, the PEC must be certified at least every 6 months (see *5. Certification and Recertification*).
- If used, a visible perimeter must define the AECA.
  - Access to the AECA during compounding must be restricted to authorized personnel.
  - During compounding activities, no other activity is permitted in the AECA.
  - The surfaces of walls, floors, fixtures, shelving, counters, and cabinets in the AECA must be cleanable.
  - Carpet is not allowed in the AECA.
  - Surfaces should be resistant to damage by cleaning and disinfecting agents.
  - The surfaces in the AECA upon which the allergenic extract prescription sets are prepared must be smooth, impervious, free from cracks and crevices, and non-shedding to allow for easy cleaning and disinfecting.
  - Dust-collecting overhangs such as utility pipes, ledges, and windowsills should be minimized. If overhangs or ledges are present, they must be easily cleanable.
  - The AECA must be designed and controlled to provide a well-lighted working environment, with temperature and humidity controls for the comfort of compounding personnel wearing the required garb.

**21.4 Cleaning and Disinfecting for Compounding Allergenic Extract Prescription Sets**

- In a PEC, all interior surfaces of the PEC must be cleaned and disinfected each day of use before compounding begins and when surface contamination is known or suspected. Apply sterile 70% IPA to the horizontal work surface between each prescription set.
- In an AECA, all work surfaces in the AECA where direct compounding is occurring must be cleaned and disinfected each day of use before compounding begins and when surface contamination is known or suspected. Apply sterile 70% IPA to the horizontal work surface between each prescription set.
  - If present, walls, doors, and door frames within the perimeter of the AECA must be cleaned and disinfected monthly and when surface contamination is known or suspected.
  - Ceilings within the perimeter of the AECA must be cleaned and disinfected when visibly soiled and when surface contamination is known or suspected.
- Vial stoppers on packages of conventionally manufactured sterile ingredients must be wiped with sterile 70% IPA to ensure that the critical sites are wet and allowed to dry before they are used to compound allergenic extract prescription sets.

**21.5 Establishing BUDs for Allergenic Extract Prescription Sets**

- The BUD for the prescription set must be no later than the earliest expiration date of any allergenic extract or any diluent that is part of the prescription set, and the BUD must not exceed 1 year from the date the prescription set is mixed or diluted.

**21.6 Labeling for Allergenic Extract Prescription Sets**

- The label of each vial of an allergenic extract prescription set must display the following prominently and understandably:
  - Patient name
  - Type and fractional dilution of each vial, with a corresponding vial number
  - BUD
  - Storage conditions

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**21.7 Shipping and Transporting Allergenic Extract Prescription Sets**

- If shipping or transporting allergenic extract prescription sets, compounding personnel must select modes of transport that are expected to deliver properly packed prescription sets in an undamaged, sterile, and stable condition. Inappropriate transport can adversely affect the quality of allergenic extract prescription sets.
- When shipping or transporting allergenic extract prescription sets that require special handling, personnel must include specific handling instructions on the exterior of the container.

**21.8 Documentation for Compounding Allergenic Extract Prescription Sets**

- All facilities where allergenic extract prescription sets are prepared must have and maintain written or electronic documentation to include, but not limited to, the following:
  - SOPs describing all aspects of the compounding process
  - Personnel training records, competency assessments, and qualification records including corrective actions for any failures
  - Certification reports of the PEC, if used, including corrective actions for any failures
  - Temperature logs for refrigerator(s)
  - CRs for individual allergenic extract prescription sets (see *Box 10*)
  - Information related to complaints and adverse events including corrective actions taken
  - Investigations and corrective actions

## GLOSSARY

**ACD:** Automated compounding device.
**ACPH:** Air changes per hour.
**Active pharmaceutical ingredient (API):** Any substance or mixture of substances intended to be used in the compounding of a preparation, thereby becoming the active ingredient in that preparation and furnishing pharmacological activity or other direct effect in the diagnosis, cure, mitigation, treatment, or prevention of disease in humans and animals or affecting the structure and function of the body. Also referred to as *Bulk drug substance*. A conventionally manufactured drug product is not an API but is typically manufactured from an API(s).
**Added substance:** An ingredient that is necessary to compound a preparation but is not intended or expected to cause a pharmacologic response if administered alone in the amount or concentration contained in a single dose of the compounded preparation. The term is used synonymously with the terms *inactive ingredient*, *excipient*, and *pharmaceutical ingredient*.
**Administration:** The direct application of a sterile product or preparation to a single patient by injecting, infusing, or otherwise providing a sterile product or preparation in its final form.
**Airlock:** A space with interlocked doors, constructed to maintain air pressure control when items move between two adjoining areas (generally with different air cleanliness standards). The intent of an airlock is to prevent ingress of particulate matter and microbial contamination from a lesser-controlled area.
**Alcohol-based hand rub:** An alcohol-containing preparation (liquid, gel, or foam) designed for application to the hands of healthcare personnel to inactivate microorganisms and/or temporarily suppress their growth. Such preparations may contain one or more types of alcohol, other active ingredients, excipients, and humectants.
**Allergenic extract:** A biological substance used for the diagnosis and/or treatment of allergic diseases such as allergic rhinitis, allergic sinusitis, allergic conjunctivitis, bee venom allergy, and food allergy.
**Allergenic extract compounding area (AECA):** A designated space, area, or room that is not required to be classified, with a visible perimeter that is suitable for preparation of allergenic extract prescription sets.
**Allergenic extract prescription set:** Combinations of licensed allergenic extracts that would be mixed and diluted to provide subcutaneous immunotherapy to an individual patient, even though these allergenic extract combinations are not specified in the approved BLAs for the licensed biological products.
**Anteroom:** An ISO Class 8 or cleaner room with fixed walls and doors where personnel hand hygiene, garbing procedures, and other activities that generate high particulate levels may be performed. The anteroom is the transition room between the unclassified area of the facility and the buffer room.
**Aseptic processing:** A method by which separate, sterile components (e.g., drugs, containers, or closures) are brought together under conditions that maintain their sterility. The components can either be purchased as sterile or, when starting with nonsterile components, can be separately sterilized prior to combining (e.g., by membrane filtration or by autoclave).
**Aseptic technique:** A set of methods used to keep objects and areas free of microorganisms and thereby minimize infection risk to the patient. It is accomplished through practices that maintain the microbe count at an irreducible minimum.
**Assigned trainer:** One or more individuals assigned by the designated person(s) to be responsible and accountable for directly providing the training, observation, and/or evaluation of personnel for the preparation of CSPs.
**Batch:** More than one CSP prepared as described in the MFR in a single, discrete process, and expected to have uniform character and quality, within specified limits.
**Beyond-use date (BUD):** The date, or hour and the date, after which a CSP must not be used, stored, or transported. The date is determined from the date and time the preparation is compounded.
**Biological safety cabinet (BSC):** A ventilated cabinet that may be used for compounding. These cabinets are divided into three general classes (Class I, Class II, and Class III). Class II BSCs are further divided into types (Type A1, Type A2, Type B1, Type B2, and Type C1).
**Biological safety cabinet (BSC), Class II:** A ventilated cabinet with an open front and inward and downward unidirectional HEPA-filtered airflow and HEPA-filtered exhaust. A BSC used to prepare a CSP must be capable of providing an ISO Class 5 or better environment for preparation of the CSPs.
**BLA:** Biological license application.

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**Blood components:** Any therapeutic constituent of blood separated by physical or mechanical means (e.g., white cells, red cells, platelets, plasma, serum). It is not intended to include plasma-derived products (e.g., albumin, coagulation factors, immunoglobulins) manufactured under an approved BLA or equivalent.
**BMBL:** Biosafety in Microbiological and Biomedical Laboratories.
**Buffer room:** An ISO Class 7 or cleaner room with fixed walls and doors where PEC(s) that generate and maintain an ISO Class 5 environment are physically located. The buffer room may only be accessed through the anteroom or another buffer room.
**Bulk drug substance:** See the entry for *Active pharmaceutical ingredient*.
**Category 1 CSP:** A CSP that is assigned a BUD of 12 h or less at controlled room temperature or 24 h or less refrigerated that is compounded in accordance with all applicable requirements for Category 1 CSPs in this chapter.
**Category 2 CSP:** A CSP that may be assigned a BUD of greater than 12 h at controlled room temperature or greater than 24 h refrigerated that is compounded in accordance with all applicable requirements for Category 2 CSPs in this chapter.
**Category 3 CSP:** A CSP that may be assigned a BUD exceeding the limits in *Table 13* for Category 2 CSPs and is compounded in accordance with all applicable requirements for Category 3 CSPs in this chapter.
**CDC:** Centers for Disease Control and Prevention.
**Certificate of analysis (COA):** A report from the supplier of a component, container, or closure that accompanies the supplier's material and contains the specifications and results of all analyses and a description of the material.
**CFU:** Colony-forming units.
**Classified area:** An area that maintains an air quality classification based on the ISO standards required in this chapter (see also the definition for *ISO class*).
**Cleaning:** The process of removing substances (e.g., organic and inorganic material) from objects and surfaces, normally accomplished by manually or mechanically using water with detergents or enzymatic products.
**Cleaning agent:** An agent, usually containing a surfactant, used for the removal of substances (e.g., dirt, debris, microbes, and residual drugs or chemicals) from surfaces.
**Cleanroom suite:** A classified area that consists of both an anteroom and buffer room.
**Component:** Any ingredient used in the compounding of a preparation, including any active ingredient, added substance, or conventionally manufactured product.
**Compounded sterile preparation (CSP):** A preparation intended to be sterile that is created by combining, admixing, diluting, pooling, reconstituting, repackaging, or otherwise altering a drug product or bulk drug substance.
**Compounded stock solution:** A sterile mixture of components that is used to compound additional CSPs.
**Compounding:** The process of combining, admixing, diluting, pooling, reconstituting, repackaging, or otherwise altering a drug product or bulk drug substance to create a sterile preparation.
**Compounding area:** The area where compounding is occurring (i.e., a cleanroom suite, inside the perimeter of the SCA, or AECA).
**Compounding aseptic containment isolator (CACI):** A type of RABS that uses HEPA filtration to provide an ISO Class 5 unidirectional air environment designed for the compounding of sterile HDs.
**Compounding aseptic isolator (CAI):** A type of RABS that uses HEPA filtration to provide an ISO Class 5 unidirectional air environment designed for compounding of sterile non-HDs.
**Compounding record (CR):** Documents the compounding of each CSP.
**Container closure system:** Packaging components that together contain and protect the dosage form. This includes primary packaging components and secondary packaging components, if the latter are intended to provide additional protection.
**Containment glove bag:** A single-use disposable glove bag that is capable of containing airborne chemical particles.
**Containment ventilated enclosure (CVE):** A non-ISO classified full or partial enclosure that uses ventilation principles to capture, contain, and remove airborne contaminants through HEPA filtration and prevent their release into the work environment.
**Conventionally manufactured product:** A pharmaceutical dosage form, usually the subject of an application approved by the applicable national regulatory agency, that is manufactured under current good manufacturing practice conditions.
**Critical site:** A location that includes any component or fluid pathway surfaces (e.g., vial septa, injection ports, and beakers) or openings (e.g., opened ampules and needle hubs) that are exposed and at risk of direct contact with air (e.g., ambient room or HEPA filtered), moisture (e.g., oral and mucosal secretions), or touch contamination.
**Designated person(s):** One or more individuals assigned to be responsible and accountable for the performance and operation of the facility and personnel as related to the preparation of CSPs.
**Direct compounding area (DCA):** A critical area within the ISO Class 5 PEC where critical sites are exposed to unidirectional HEPA-filtered air, also known as first air.
**Disinfectant:** A chemical or physical agent used on inanimate surfaces and objects to destroy fungi, viruses, and bacteria. Sporicidal disinfectants are considered a special class of disinfectants that also are effective against bacterial and fungal spores.
**Dynamic airflow smoke pattern test:** A PEC test in which a visible source of smoke, which is neutrally buoyant, is used to observe air patterns within the unidirectional space (i.e., the DCA) under dynamic operating conditions (see the entry for *Dynamic operating conditions*). This test is not appropriate for ISO Class 7 or ISO Class 8 cleanrooms that do not have unidirectional airflow (see the entry for *Visual smoke study*).
**Dynamic operating conditions:** Conditions in the compounding area in which operating personnel are present and simulating or performing compounding. The conditions should reflect the largest number of personnel and highest complexity of compounding expected during routine operations as determined by the designated person(s).
**ECV:** Endotoxin challenge vial.
**EPA:** US Environmental Protection Agency.
**Excipient:** See the entry for *Added substance*.
**FDA:** US Food and Drug Administration.
**Filter integrity test:** A test (e.g., bubble point test) of the integrity of a sterilizing grade filter performed after the filtration process to detect whether the integrity of the filter has been compromised.
**Final yield:** The total number of containers actually prepared at the end of the compounding process prior to release testing.

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**First air:** The air exiting the HEPA filter in a unidirectional air stream.
**Formulation:** The specific qualitative and quantitative composition of the final CSP.
**Garb:** Items such as gloves, garments (e.g., gowns), shoe covers, head and facial hair covers, masks, and other items designed to reduce particle-shedding from personnel and minimize the risk of contamination of CSP(s).
**GFT:** Gloved fingertip and thumb sampling.
**Hazardous drug (HD):** Any drug identified by at least one of the following six criteria: carcinogenicity, teratogenicity or developmental toxicity, reproductive toxicity in humans, organ toxicity at low dose in humans or animals, genotoxicity, or new drugs that mimic existing HDs in structure or toxicity.
**High-efficiency particulate air (HEPA) filtration:** Being, using, or containing a filter designed to remove 99.97% of airborne particles measuring 0.3-micron or greater in diameter passing through it.
**HVAC:** Heating, ventilation, and air conditioning.
**Integrated vertical laminar flow zone (IVLFZ):** A designated ISO Class 5 area serving as the PEC within an ISO Class 7 or cleaner buffer room. In the IVLFZ, unidirectional airflow is created by placing HEPA filters over the entire surface of the worktables and by effective placement of air returns.
**IPA:** Isopropyl alcohol.
**ISO:** International Organization for Standardization.
**ISO class:** An air-quality classification from the International Organization for Standardization.
**Label:** The part of the labeling on the immediate container.
**Labeling:** All labels and other written, printed, or graphic matter on the immediate container or on or inside any packaging system or wrapper in which the article is enclosed, except any outer shipping container.
**Laminar airflow system (LAFS):** A device or zone within a buffer room that provides an ISO Class 5 or better air quality environment for sterile compounding. The system provides a unidirectional HEPA-filtered airflow.
**Laminar airflow workbench (LAFW):** A device that is a type of LAFS that provides an ISO Class 5 or better air quality environment for sterile compounding. The device provides a unidirectional HEPA-filtered airflow.
**Line of demarcation:** A visible line on the floor that separates the clean and dirty sides of the anteroom.
**Low-lint wiper:** A wiper exhibiting few, if any, fibers or other contamination, visible without magnification, which is separate from, or easily removed from, the wiper material in a dry condition.
**Master formulation record (MFR):** A detailed record of procedures that describes how the CSP is to be prepared.
**MEA:** Malt extract agar.
**Media-fill test:** A simulation used to qualify processes and personnel engaged in sterile compounding to ensure that the processes and personnel are able to prepare CSPs without contamination.
**Monograph:** A quality documentary standard within *USP–NF* that articulates the quality expectations for a medicine including for its identity, strength, purity, and performance. It also describes the tests to validate that a medicine and its ingredients meet these criteria.
**Multiple-dose container:** A container of sterile product for parenteral administration (e.g., injection or infusion) that is designed to contain more than one dose of the sterile product. A multiple-dose container is usually required to meet the antimicrobial effectiveness testing criteria. See ⟨659⟩, *Injection Packaging Systems, Multiple-dose container*.
**One-step disinfectant cleaner:** A product with an EPA-registered (or equivalent) claim that it can clean and disinfect a nonporous surface in the presence of light to moderate organic soiling without a separate cleaning step.
**Oversight:** The review, monitoring, and supervision of actions taken by personnel, bearing responsibility for those actions, and being available for consultation if and when needed even if not physically present.
**Pass-through chamber:** An enclosure with sealed doors on both sides that should be interlocked. The pass-through chamber is positioned between two spaces for the purpose of minimizing particulate transfer while moving materials from one space to another.
**Perimeter:** A visible demarcation (such as a door, walls, or visible marking on the floor) that defines the SCA or AECA.
**Pharmaceutical isolator:** An enclosure that provides HEPA-filtered ISO Class 5 unidirectional air operated at a continuously higher pressure than its surrounding environment and is decontaminated using an automated system. It uses only decontaminated interfaces or rapid transfer ports for materials transfer. [NOTE—A CAI or CACI is not a pharmaceutical isolator.]
**Pharmacy bulk package:** A conventionally manufactured sterile product for parenteral use that contains many single doses intended for use in a pharmacy admixture program. A pharmacy bulk package may either be used to prepare admixtures for infusion or, through a sterile transfer device, for filling sterile containers. See ⟨659⟩, *Injection Packaging Systems, Pharmacy bulk package*.
**Positive-pressure room:** A room that is maintained at higher pressure than the adjacent spaces, and therefore the net airflow is out of the room.
**PPE:** Personal protective equipment.
**Preservative:** A substance added to inhibit microbial growth.
**Primary engineering control (PEC):** A device or zone that provides an ISO Class 5 air quality environment for sterile compounding.
**Probability of a nonsterile unit (PNSU):** The probability of an item being nonsterile after it has been exposed to a verified sterilization process. A PNSU value can only be applied to terminal sterilization. [NOTE—This is also called the sterility assurance level (SAL).]
**Pyrogen:** A substance that induces a febrile reaction in a patient.
**Quality assurance (QA):** A system of procedures, activities, and oversight that ensures that the compounding process consistently meets quality standards.
**Quality control (QC):** The sampling, testing, and documentation of results that, taken together, ensure that specifications have been met before release of the CSP.
**Reconstitution:** The process of adding a diluent to a conventionally manufactured product to prepare a sterile solution or suspension.

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**Release inspection and testing:** Visual inspection and testing performed to ensure that a preparation meets appropriate quality characteristics.

**Repackaging:** The act of removing a sterile product or preparation from its original primary container and placing it into another primary container, usually of smaller size without further manipulation.

**Restricted-access barrier system (RABS):** An enclosure that provides HEPA-filtered ISO Class 5 unidirectional air that allows for the ingress and/or egress of materials through defined openings that have been designed and validated to preclude the transfer of contamination, and that generally are not to be opened during operations. Examples of RABS include CAIs and CACIs.

**SDA:** Sabouraud dextrose agar.

**Secondary engineering control (SEC):** The area where the PEC is placed (e.g., a cleanroom suite or an SCA). It incorporates specific design and operational parameters required to minimize the risk of contamination within the compounding area.

**Segregated compounding area (SCA):** A designated space, area, or room that is not required to be classified and is defined with a visible perimeter. The SCA must contain a PEC and is suitable for preparation of Category 1 CSPs only.

**Single-dose containers:** A container of sterile product for parenteral administration (e.g., injection or infusion) that is designed for use with a single patient as a single injection/infusion. A single-dose container usually does not contain a preservative. See ⟨659⟩, *Injection Packaging Systems, Single-dose container*.

**SOP:** Standard operating procedure.

**Specification:** The tests, analytical methods, and acceptance criteria to which any component, CSP, container closure system, equipment, or other material used in compounding CSPs must conform to be considered acceptable for its intended use.

**Sporicidal disinfectant:** A chemical or physical agent that destroys bacterial and fungal spores when used in sufficient concentration for a specified contact time. It is expected to kill all vegetative microorganisms.

**Stability:** The extent to which a product or preparation retains physical and chemical properties and characteristics within specified limits throughout its expiration or BUD.

**Sterility:** The absence of viable microorganisms.

**Sterility assurance level (SAL):** See the entry for *Probability of a nonsterile unit* (PNSU).

**Sterilization by filtration:** Passage of a gas or liquid through a sterilizing-grade membrane to yield filtrates that are sterile.

**Sterilizing-grade filter:** Filter membranes that are documented to retain 100% of a culture of $10^7$ microorganisms of a strain of *Brevundimonas diminuta* per square centimeters of membrane surface under a pressure of not less than 30 psi. Such filter membranes are nominally 0.22- or 0.2-µm pore size.

**Terminal sterilization:** The application of a lethal process (e.g., steam, dry heat, irradiation) to sealed containers for the purpose of achieving a predetermined PNSU of greater than $10^{-6}$ or a probability of less than one in one million of a nonsterile unit.

**TSA:** Trypticase soy agar.

**Unclassified space:** A space not required to meet any air cleanliness classification based on the ISO.

**Unidirectional airflow:** Air within a PEC moving in a single direction in a uniform manner and at sufficient velocity to sweep particles away from the DCA.

**Verify:** To confirm that a method, process, system, or equipment will perform as expected under the conditions of actual use.

**Visual smoke study:** A test, used in ISO Class 7 and ISO Class 8 rooms that do not have unidirectional airflow, in which a visible source of smoke, which is neutrally buoyant, is used to verify an absence of stagnant airflow. This test does not need to be performed under dynamic operating conditions and is not appropriate for PECs (see the entry for *Dynamic airflow smoke pattern test*).

**Workflow management system:** Technology comprised of hardware and/or software that allows for automation to assist in the verification of components of, and preparation of, CSPs and to document components and processes.▲ (Official 1-Nov-2023)

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# ⟨800⟩ HAZARDOUS DRUGS—HANDLING IN HEALTHCARE SETTINGS

## 1. INTRODUCTION AND SCOPE

This chapter describes practice and quality standards for handling hazardous drugs (HDs) to promote patient safety, worker safety, and environmental protection. Handling HDs includes, but is not limited to, the receipt, storage, compounding, dispensing, administration, and disposal of sterile and nonsterile products and preparations.

This chapter applies to all healthcare personnel who handle HD preparations and all entities that store, prepare, transport, or administer HDs (e.g., pharmacies, hospitals and other healthcare institutions, patient treatment clinics, physicians' practice facilities, or veterinarians' offices). Personnel who may potentially be exposed to HDs include, but are not limited to: pharmacists, pharmacy technicians, nurses, physicians, physician assistants, home healthcare workers, veterinarians, and veterinary technicians.

Entities that handle HDs must incorporate the standards in this chapter into their occupational safety plan. The entity's health and safety management system must, at a minimum, include:

- A list of HDs
- Facility and engineering controls
- Competent personnel
- Safe work practices
- Proper use of appropriate Personal Protective Equipment (PPE)
- Policies for HD waste segregation and disposal

The chapter is organized into the following main sections:

1. Introduction and Scope
2. List of Hazardous Drugs
3. Types of Exposure
4. Responsibilities of Personnel Handling Hazardous Drugs
5. Facilities and Engineering Controls
6. Environmental Quality and Control
7. Personal Protective Equipment
8. Hazard Communication Program
9. Personnel Training
10. Receiving
11. Labeling, Packaging, Transport, and Disposal
12. Dispensing Final Dosage Forms
13. Compounding
14. Administering
15. Deactivating, Decontaminating, Cleaning, and Disinfecting
16. Spill Control
17. Documentation and Standard Operating Procedures
18. Medical Surveillance

Glossary

Appendices

Appendix 1: Acronyms
Appendix 2: Examples of Designs for Hazardous Drug Compounding Areas
Appendix 3: Types of Biological Safety Cabinets

References

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## 2. LIST OF HAZARDOUS DRUGS

The National Institute for Occupational Safety and Health (NIOSH) maintains a list of antineoplastic and other HDs used in healthcare. ▲For the purposes of this chapter, the term antineoplastic only refers to antineoplastic drugs included in Table 1 of the most current NIOSH List.▲ (RB 1-Jul-2020) An entity must maintain a list of HDs, which must include any items on the current NIOSH list that the entity handles. The entity's list must be reviewed at least every 12 months. Whenever a new agent or dosage form is used, it should be reviewed against the entity's list.

The NIOSH list of antineoplastic and other HDs provides the criteria used to identify HDs. These criteria must be used to identify HDs that enter the market after the most recent version of the NIOSH list, or that the entity handles as an investigational drug. If the information available on a drug is deemed insufficient to make an informed decision, consider the drug hazardous until more information is available.

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**Box 1: Containment Requirements**

- Drugs on the NIOSH list that must follow the requirements in this chapter include:
  - Any HD API
  - Any antineoplastic requiring HD manipulation
- Drugs on the NIOSH list that do not have to follow all the containment requirements of this chapter if an assessment of risk is performed and implemented include:
  - Final dosage forms of compounded HD preparations and conventionally manufactured HD products, including antineoplastic dosage forms that do not require any further manipulation other than counting or repackaging (unless required by the manufacturer)
- For dosage forms of other HDs on the NIOSH list, the entity may perform an assessment of risk to determine alternative containment strategies and/work practices

Some dosage forms of drugs defined as hazardous may not pose a significant risk of direct occupational exposure because of their dosage formulation (e.g., tablets or capsules—solid, intact medications that are administered to patients without modifying the formulation). However, dust from tablets and capsules may present a risk of exposure by skin contact and/or inhalation. An assessment of risk may be performed for these dosage forms to determine alternative containment strategies and/or work practices. If an assessment of risk is not performed, all HDs must be handled with all containment strategies defined in this chapter.

The assessment of risk must, at a minimum, consider the following:

- Type of HD (e.g., antineoplastic, non-antineoplastic, reproductive risk only)
- Dosage form
- Risk of exposure
- Packaging
- Manipulation

If an assessment of risk approach is taken, the entity must document what alternative containment strategies and/or work practices are being employed for specific dosage forms to minimize occupational exposure. If used, the assessment of risk must be reviewed at least every 12 months and the review documented.

## 3. TYPES OF EXPOSURE

Routes of unintentional entry of HDs into the body include dermal and mucosal absorption, inhalation, injection, and ingestion (e.g., contaminated foodstuffs, spills, or mouth contact with contaminated hands). Containers of HDs have been shown to be contaminated upon receipt. Both clinical and nonclinical personnel may be exposed to HDs when they handle HDs or touch contaminated surfaces. *Table 1* lists examples of potential routes of exposure based on activity.

**Table 1. Examples of Potential Opportunities of Exposure Based on Activity**

| Activity | Potential Opportunity of Exposure |
|---|---|
| Receipt | • Contacting HD residues present on drug containers, individual dosage units, outer containers, work surfaces, or floors |
| Dispensing | • Counting or repackaging tablets and capsules |
| Compounding and other manipulations | • Crushing or splitting tablets or opening capsules<br>• Pouring oral or topical liquids from one container to another<br>• Weighing or mixing components<br>• Constituting or reconstituting powdered or lyophilized HDs<br>• Withdrawing or diluting injectable HDs from parenteral containers<br>• Expelling air or HDs from syringes<br>• Contacting HD residue present on PPE or other garments<br>• Deactivating, decontaminating, cleaning, and disinfecting areas contaminated with or suspected to be contaminated with HDs<br>• Maintenance activities for potentially contaminated equipment and devices |
| Administration | • Generating aerosols during administration of HDs by various routes (e.g., injection, irrigation, oral, inhalation, or topical application)<br>• Performing certain specialized procedures (e.g., intraoperative intraperitoneal injection or bladder instillation)<br>• Priming an IV administration set |
| Patient-care activities | • Handling body fluids (e.g., urine, feces, sweat, or vomit) or body-fluid-contaminated clothing, dressings, linens, and other materials |
| Spills | • Spill generation, management, and disposal |
| Transport | • Moving HDs within a healthcare setting |
| Waste | • Collection and disposal of hazardous waste and trace contaminated waste |

## 4. RESPONSIBILITIES OF PERSONNEL HANDLING HAZARDOUS DRUGS

Each entity must have a designated person who is qualified and trained to be responsible for developing and implementing appropriate procedures; overseeing entity compliance with this chapter and other applicable laws, regulations, and standards; ensuring competency of personnel; and ensuring environmental control of the storage and compounding areas. The designated

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person must thoroughly understand the rationale for risk-prevention policies, risks to themselves and others, risks of non-compliance that may compromise safety, and the responsibility to report potentially hazardous situations to the management team. The designated person must also be responsible for the oversight of monitoring the facility and maintaining reports of testing/sampling performed in facilities, and acting on the results.

All personnel who handle HDs are responsible for understanding the fundamental practices and precautions and for continually evaluating these procedures and the quality of final HDs to prevent harm to patients, minimize exposure to personnel, and minimize contamination of the work and patient-care environment.

## 5. FACILITIES AND ENGINEERING CONTROLS

HDs must be handled under conditions that promote patient safety, worker safety, and environmental protection. Signs designating the hazard must be prominently displayed before the entrance to the HD handling areas. Access to areas where HDs are handled must be restricted to authorized personnel to protect persons not involved in HD handling. HD handling areas must be located away from breakrooms and refreshment areas for personnel, patients, or visitors to reduce risk of exposure.

Designated areas must be available for:

- Receipt and unpacking
- Storage of HDs
- Nonsterile HD compounding (if performed by the entity)
- Sterile HD compounding (if performed by the entity)

Certain areas are required to have negative pressure from surrounding areas to contain HDs and minimize risk of exposure. Consideration should be given to uninterrupted power sources (UPS) for the ventilation systems to maintain negative pressure in the event of power loss.

### 5.1 Receipt

Antineoplastic HDs and all HD APIs must be unpacked (i.e., removal from external shipping containers) in an area that is neutral/normal or negative pressure relative to the surrounding areas. HDs must not be unpacked from their external shipping containers in sterile compounding areas or in positive pressure areas.

### 5.2 Storage

HDs must be stored in a manner that prevents spillage or breakage if the container falls. Do not store HDs on the floor. In areas prone to specific types of natural disasters (e.g., earthquakes) the manner of storage must meet applicable safety precautions, such as secure shelves with raised front lips.

Antineoplastic HDs requiring manipulation other than counting or repackaging of final dosage forms and any HD API must be stored separately from non-HDs in a manner that prevents contamination and personnel exposure. These HDs must be stored in an externally ventilated, negative-pressure room with at least 12 air changes per hour (ACPH). Non-antineoplastic, reproductive risk only, and final dosage forms of antineoplastic HDs may be stored with other inventory if permitted by entity policy.

Sterile and nonsterile HDs may be stored together, but HDs used for nonsterile compounding should not be stored in areas designated for sterile compounding to minimize traffic into the sterile compounding area.

Refrigerated antineoplastic HDs must be stored in a dedicated refrigerator in a negative pressure area with at least 12 ACPH [e.g., storage room, buffer room, or containment segregated compounding area (C-SCA)]. If a refrigerator is placed in a negative pressure buffer room, an exhaust located adjacent to the refrigerator's compressor and behind the refrigerator should be considered.

### 5.3 Compounding

Engineering controls are required to protect the preparation from cross-contamination and microbial contamination (if preparation is intended to be sterile) during all phases of the compounding process. Engineering controls for containment are divided into three categories representing primary, secondary, and supplementary levels of control. A containment primary engineering control (C-PEC) is a ventilated device designed to minimize worker and environmental HD exposure when directly handling HDs. The containment secondary engineering control (C-SEC) is the room in which the C-PEC is placed. Supplemental engineering controls [e.g., closed-system drug-transfer device (CSTD)] are adjunct controls to offer additional levels of protection. *Appendix 2* provides examples for designs of HD compounding areas.

Sterile and nonsterile HDs must be compounded within a C-PEC located in a C-SEC. The C-SEC used for sterile and nonsterile compounding must:

- Be externally vented
- Be physically separated (i.e., a different room from other preparation areas)
- Have an appropriate air exchange (e.g., ACPH)
- Have a negative pressure between 0.01 and 0.03 inches of water column relative to all adjacent areas

The C-PEC must operate continuously if it supplies some or all of the negative pressure in the C-SEC or if it is used for sterile compounding. If there is any loss of power to the C-PEC, or if repair or moving occurs, all activities occurring in the C-PEC must be suspended immediately. If necessary, protect the unit by covering it appropriately per the manufacturer's recommendations. Once the C-PEC can be powered on, decontaminate, clean, and disinfect (if used for sterile compounding) all surfaces and wait the manufacturer-specified recovery time before resuming compounding.

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A sink must be available for hand washing. An eyewash station and/or other emergency or safety precautions that meet applicable laws and regulations must be readily available. Care must be taken to locate water sources and drains in areas where their presence will not interfere with required ISO classifications. Water sources and drains must be located at least 1 meter away from the C-PEC.

For entities that compound both nonsterile and sterile HDs, the respective C-PECs must be placed in separate rooms, unless those C-PECs used for nonsterile compounding are sufficiently effective that the room can continuously maintain ISO 7 classification throughout the nonsterile compounding activity. If the C-PECs used for sterile and nonsterile compounding are placed in the same room, they must be placed at least 1 meter apart and particle-generating activity must not be performed when sterile compounding is in process.

### 5.3.1 NONSTERILE COMPOUNDING

In addition to this chapter, nonsterile compounding must follow standards in *Pharmaceutical Compounding—Nonsterile Preparations* ⟨795⟩. A C-PEC is not required if manipulations are limited to handling of final dosage forms (e.g., counting or repackaging of tablets and capsules) that do not produce particles, aerosols, or gasses.

The C-PECs used for manipulation of nonsterile HDs must be either externally vented (preferred) or have redundant–HEPA filters in series. Nonsterile HD compounding must be performed in a C-PEC that provides personnel and environmental protection, such as a Class I Biological Safety Cabinet (BSC) or Containment Ventilated Enclosure (CVE). A Class II BSC or a compounding aseptic containment isolator (CACI) may also be used. For occasional nonsterile HD compounding, a C-PEC used for sterile compounding (e.g., Class II BSC or CACI) may be used but must be decontaminated, cleaned, and disinfected before resuming sterile compounding in that C-PEC. A C-PEC used only for nonsterile compounding does not require unidirectional airflow because the critical environment does not need to be ISO classified.

The C-PEC must be placed in a C-SEC that has at least 12 ACPH. *Table 2* summarizes the engineering controls required for nonsterile HD compounding.

Due to the difficulty of cleaning HD contamination, surfaces of ceilings, walls, floors, fixtures, shelving, counters, and cabinets in the nonsterile compounding area must be smooth, impervious, free from cracks and crevices, and non-shedding.

**Table 2. Engineering Controls for Nonsterile HD Compounding**

| C-PEC | C-SEC Requirements |
|---|---|
| • Externally vented (preferred) or redundant–HEPA filtered in series<br>• Examples: CVE, Class I or II BSC, CACI | • Externally vented<br>• 12 ACPH<br>• Negative pressure between 0.01 and 0.03 inches of water column relative to adjacent areas |

### 5.3.2 STERILE COMPOUNDING

In addition to this chapter, sterile compounding must follow standards in ⟨797⟩.

All C-PECs used for manipulation of sterile HDs must be externally vented. Sterile HD compounding must be performed in a C-PEC that provides an ISO Class 5 or better air quality, such as a Class II or III BSC or CACI. Class II BSC types A2, B1, or B2 are acceptable. For most known HDs, type A2 cabinets offer a simple and reliable integration with the ventilation and pressurization requirements of the C-SEC. Class II type B2 BSCs are typically reserved for use with volatile components. *Appendix 3* describes the different types of BSCs.

A laminar airflow workbench (LAFW) or compounding aseptic isolator (CAI) must not be used for the compounding of an antineoplastic HD. A BSC or CACI used for the preparation of HDs must not be used for the preparation of a non-HD unless the non-HD preparation is placed into a protective outer wrapper during removal from the C-PEC and is labeled to require PPE handling precautions.

The C-PEC must be located in a C-SEC, which may either be an ISO Class 7 buffer room with an ISO Class 7 ante-room (preferred) or an unclassified containment segregated compounding area (C-SCA). If the C-PEC is placed in a C-SCA, the beyond-use date (BUD) of all compounded sterile preparations (CSPs) prepared must be limited as described in ⟨797⟩ for CSPs prepared in a segregated compounding area. *Table 3* summarizes the engineering controls required for sterile HD compounding.

**Table 3. Engineering Controls for Sterile HD Compounding**

| Configuration | C-PEC | C-SEC | Maximum BUD |
|---|---|---|---|
| ISO Class 7 buffer room with an ISO Class 7 ante-room | • Externally vented<br>• Examples: Class II BSC or CACI | • Externally vented<br>• 30 ACPH<br>• Negative pressure between 0.01 and 0.03 inches of water column relative to adjacent areas | As described in ⟨797⟩ |
| Unclassified C-SCA | • Externally vented<br>• Examples: Class II BSC or CACI | • Externally vented<br>• 12 ACPH<br>• Negative pressure between 0.01 and 0.03 inches of water column relative to adjacent areas | As described in ⟨797⟩ for CSPs prepared in a segregated compounding area |

**ISO Class 7 buffer room with an ISO class 7 ante-room:** The C-PEC is placed in an ISO Class 7 buffer room that has fixed walls, HEPA-filtered supply air, a negative pressure between 0.01 and 0.03 inches of water column relative to all adjacent areas and a minimum of 30 ACPH.

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The buffer room must be externally vented. Because the room through which entry into the HD buffer room (e.g., ante-room or non-HD buffer room) plays an important role in terms of total contamination control, the following is required:

- Minimum of 30 ACPH of HEPA-filtered supply air
- Maintain a positive pressure of at least 0.02 inches of water column relative to all adjacent unclassified areas
- Maintain an air quality of ISO Class 7 or better

An ISO Class 7 ante-room with fixed walls is necessary to provide inward air migration of equal cleanliness classified air into the negative pressure buffer room to contain any airborne HD. A hand-washing sink must be placed in the ante-room at least 1 meter from the entrance to the HD buffer room to avoid contamination migration into the negative pressure HD buffer room.

Although not a recommended facility design, if the negative-pressure HD buffer room is entered though the positive-pressure non-HD buffer room, the following is also required:

- A line of demarcation must be defined within the negative-pressure buffer room for donning and doffing PPE
- A method to transport HDs, HD CSPs, and HD waste into and out of the negative pressure buffer room to minimize the spread of HD contamination. This may be accomplished by use of a pass-through chamber between the negative-pressure buffer area and adjacent space. The pass-through chamber must be included in the facility's certification to ensure that particles are not compromising the air quality of the negative-pressure buffer room. A refrigerator pass-through must not be used. Other methods of containment (such as sealed containers) may be used.

HD CSPs prepared in an ISO Class 7 buffer room with an ISO Class 7 ante-room may use the BUDs described in ⟨797⟩, based on the categories of CSP, sterility testing, and storage temperature.

**Containment segregated compounding area (C-SCA):** The C-PEC is placed in an unclassified C-SCA that has fixed walls, a negative pressure between 0.01 and 0.03 inches of water column relative to all adjacent areas, and a minimum of 12 ACPH. The C-SCA must be externally vented. A hand-washing sink must be placed at least 1 meter from C-PEC and may be either inside the C-SCA or directly outside the C-SCA.

Only low- and medium-risk HD CSPs may be prepared in a C-SCA. HD CSPs prepared in the C-SCA must not exceed the BUDs described in ⟨797⟩ for CSPs prepared in a segregated compounding area.

### 5.4 Containment Supplemental Engineering Controls

Containment supplemental engineering controls, such as CSTDs, provide adjunct controls to offer an additional level of protection during compounding or administration. Some CSTDs have been shown to limit the potential of generating aerosols during compounding. However, there is no certainty that all CSTDs will perform adequately. Until a published universal performance standard for evaluation of CSTD containment is available, users should carefully evaluate the performance claims associated with available CSTDs based on independent, peer-reviewed studies and demonstrated contamination reduction.

A CSTD must not be used as a substitute for a C-PEC when compounding. CSTDs should be used when compounding HDs when the dosage form allows. CSTDs must be used when administering antineoplastic HDs when the dosage form allows. CSTDs known to be physically or chemically incompatible with a specific HD must not be used for that HD.

## 6. ENVIRONMENTAL QUALITY AND CONTROL

Environmental wipe sampling for HD surface residue should be performed routinely (e.g., initially as a benchmark and at least every 6 months, or more often as needed, to verify containment). Surface wipe sampling should include:

- Interior of the C-PEC and equipment contained in it
- Pass-through chambers
- Surfaces in staging or work areas near the C-PEC
- Areas adjacent to C-PECs (e.g., floors directly under C-PEC, staging, and dispensing area)
- Areas immediately outside the HD buffer room or the C-SCA
- Patient administration areas

There are currently no studies demonstrating the effectiveness of a specific number or size of wipe samples in determining levels of HD contamination. Wipe sampling kits should be verified before use to ensure the method and reagent used have been tested to recover a specific percentage of known marker drugs from various surface types found in the sampled area. There are currently no certifying agencies for vendors of wipe sample kits.

There is currently no standard for acceptable limits for HD surface contamination. Common marker HDs that can be assayed include cyclophosphamide, ifosfamide, methotrexate, fluorouracil, and platinum-containing drugs. An example of measurable contamination would be cyclophosphamide levels >1.00 ng/cm$^2$, which were shown in some studies to result in uptake of the drug in exposed workers. If any measurable contamination is found, the designated person must identify, document, and contain the cause of contamination. Such action may include reevaluating work practices, re-training personnel, performing thorough deactivation, decontamination, cleaning, and improving engineering controls. Repeat the wipe sampling to validate that the deactivation/decontamination and cleaning steps have been effective.

## 7. PERSONAL PROTECTIVE EQUIPMENT

Personal Protective Equipment (PPE) provides worker protection to reduce exposure to HD aerosols and residues. Additional PPE may be required to handle the HDs outside of a C-PEC, such as treating a patient or cleaning a spill. The NIOSH list of antineoplastic and other HDs provides general guidance on PPE for possible scenarios that may be encountered in healthcare settings. Disposable PPE must not be re-used. Reusable PPE must be decontaminated and cleaned after use.

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Gowns, head, hair, shoe covers, and two pairs of chemotherapy gloves are required for compounding sterile and nonsterile HDs. Two pairs of chemotherapy gloves are required for administering injectable antineoplastic HDs. Gowns shown to resist permeability by HDs are required when administering injectable antineoplastic HDs. For all other activities, the entity's SOP must describe the appropriate PPE to be worn based on its occupational safety plan and assessment of risk (if used). The entity must develop SOPs for PPE based on the risk of exposure (see *Types of Exposure*) and activities performed.

Appropriate PPE must be worn when handling HDs including during:

- Receipt
- Storage
- Transport
- Compounding (sterile and nonsterile)
- Administration
- Deactivation/decontamination, cleaning, and disinfecting
- Spill control
- Waste disposal

## 7.1 Gloves

When chemotherapy gloves are required, they must meet American Society for Testing and Materials (ASTM) standard D6978 (or its successor). Chemotherapy gloves should be worn for handling all HDs including non-antineoplastics and for reproductive risk only HDs. Chemotherapy gloves must be powder-free because powder can contaminate the work area and can adsorb and retain HDs. Gloves must be inspected for physical defects before use. Do not use gloves with pin holes or weak spots.

When used for sterile compounding, the outer chemotherapy gloves must be sterile. Chemotherapy gloves should be changed every 30 minutes unless otherwise recommended by the manufacturer's documentation and must be changed when torn, punctured, or contaminated. Hands must be washed with soap and water after removing gloves.

## 7.2 Gowns

When gowns are required, they must be disposable and shown to resist permeability by HDs. Gowns must be selected based on the HDs handled. Disposable gowns made of polyethylene-coated polypropylene or other laminate materials offer better protection than those made of uncoated materials. Gowns must close in the back (i.e., no open front), be long sleeved, and have closed cuffs that are elastic or knit. Gowns must not have seams or closures that could allow HDs to pass through.

Cloth laboratory coats, surgical scrubs, isolation gowns, or other absorbent materials are not appropriate protective outerwear when handling HDs because they permit the permeation of HDs and can hold spilled drugs against the skin, thereby increasing exposure. Clothing may also retain HD residue from contact, and may transfer to other healthcare workers or various surfaces. Washing of non-disposable clothing contaminated with HD residue should only be done according to facility policy as drug residue may be transferred to other clothing. Potentially contaminated clothing must not be taken home under any circumstances.

Gowns must be changed per the manufacturer's information for permeation of the gown. If no permeation information is available for the gowns used, change them every 2–3 hours or immediately after a spill or splash. Gowns worn in HD handling areas must not be worn to other areas in order to avoid spreading HD contamination and exposing other healthcare workers.

## 7.3 Head, Hair, Shoe, and Sleeve Covers

Head and hair covers (including beard and moustache, if applicable), shoe covers, and sleeve covers provide protection from contact with HD residue. When compounding HDs, a second pair of shoe covers must be donned before entering the C-SEC and doffed when exiting the C-SEC. Shoe covers worn in HD handling areas must not be worn to other areas to avoid spreading HD contamination and exposing other healthcare workers.

Disposable sleeve covers may be used to protect areas of the arm that may come in contact with HDs. Disposable sleeve covers made of polyethylene-coated polypropylene or other laminate materials offer better protection than those made of uncoated materials.

## 7.4 Eye and Face Protection

Many HDs are irritating to the eyes and mucous membranes. Appropriate eye and face protection must be worn when there is a risk for spills or splashes of HDs or HD waste materials when working outside of a C-PEC (e.g., administration in the surgical suite, working at or above eye level, or cleaning a spill). A full-facepiece respirator provides eye and face protection. Goggles must be used when eye protection is needed. Eye glasses alone or safety glasses with side shields do not protect the eyes adequately from splashes. Face shields in combination with goggles provide a full range of protection against splashes to the face and eyes. Face shields alone do not provide full eye and face protection.

## 7.5 Respiratory Protection

Personnel who are unpacking HDs that are not contained in plastic should wear an elastomeric half-mask with a multi-gas cartridge and P100-filter until assessment of the packaging integrity can be made to ensure no breakage or spillage occurred during transport. If the type of drug can be better defined, a more targeted cartridge can be used.

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Surgical masks do not provide respiratory protection from drug exposure and must not be used when respiratory protection from HD exposure is required. A surgical N95 respirator provides the respiratory protection of an N95 respirator, and like a surgical mask, provides a barrier to splashes, droplets, and sprays around the nose and mouth.

For most activities requiring respiratory protection, a fit-tested NIOSH-certified N95 or more protective respirator is sufficient to protect against airborne particles. However, N95 respirators offer no protection against gases and vapors and little protection against direct liquid splashes (see the Centers for Disease Control and Prevention's (CDC's) Respirator Trusted-Source Information).

Fit test the respirator and train workers to use respiratory protection. Follow all requirements in the Occupational Safety and Health Administration (OSHA) respiratory protection standard (29 CFR 1910.134). An appropriate full-facepiece, chemical cartridge-type respirator or powered air-purifying respirator (PAPR) should be worn when there is a risk of respiratory exposure to HDs, including when:

- Attending to HD spills larger than what can be contained with a spill kit
- Deactivating, decontaminating, and cleaning underneath the work surface of a C-PEC
- There is a known or suspected airborne exposure to powders or vapors

### 7.6 Disposal of Used Personal Protective Equipment

Consider all PPE worn when handling HDs to be contaminated with, at minimum, trace quantities of HDs. PPE must be placed in an appropriate waste container and further disposed of per local, state, and federal regulations. PPE worn during compounding should be disposed of in the proper waste container before leaving the C-SEC. Chemotherapy gloves and sleeve covers (if used) worn during compounding must be carefully removed and discarded immediately into a waste container approved for trace contaminated waste inside the C-PEC or contained in a sealable bag for discarding outside the C-PEC.

## 8. HAZARD COMMUNICATION PROGRAM

Entities are required to establish policies and procedures that ensure worker safety during all aspects of HD handling. The entity must develop SOPs to ensure effective training regarding proper labeling, transport, storage, and disposal of the HDs and use of Safety Data Sheets (SDS), based on the Globally Harmonized System of Classification and Labeling of Chemicals (GHS).

Elements of the hazard communication program plan must include:

- A written plan that describes how the standard will be implemented
- All containers of hazardous chemicals must be labeled, tagged, or marked with the identity of the material and appropriate hazard warnings
- Entities must have an SDS for each hazardous chemical they use (29 CFR 1910.1200)
- Entities must ensure that the SDSs for each hazardous chemical used are readily accessible to personnel during each work shift and when they are in their work areas
- Personnel who may be exposed to hazardous chemicals when working must be provided information and training before the initial assignment to work with a hazardous chemical, and also whenever the hazard changes
- Personnel of reproductive capability must confirm in writing that they understand the risks of handling HDs

## 9. PERSONNEL TRAINING

All personnel who handle HDs must be trained based on their job functions (e.g., in the receipt, storage, compounding, repackaging, dispensing, administrating, and disposing of HDs). Training must occur before the employee independently handles HDs. The effectiveness of training for HD handling competencies must be demonstrated by each employee. Personnel competency must be reassessed at least every 12 months. Personnel must be trained prior to the introduction of a new HD or new equipment and prior to a new or significant change in process or SOP. All training and competency assessment must be documented.

The training must include at least the following:

- Overview of entity's list of HDs and their risks
- Review of the entity's SOPs related to handling of HDs
- Proper use of PPE
- Proper use of equipment and devices (e.g., engineering controls)
- Response to known or suspected HD exposure
- Spill management
- Proper disposal of HDs and trace-contaminated materials

## 10. RECEIVING

The entity must establish SOPs for receiving HDs. HDs should be received from the supplier in impervious plastic to segregate them from other drugs and to allow for safety in the receiving and internal transfer process. HDs must be delivered to the HD storage area immediately after unpacking.

PPE, including chemotherapy gloves, must be worn when unpacking HDs (see *Personal Protective Equipment*). A spill kit must be accessible in the receiving area.

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The entity must enforce policies that include a tiered approach, starting with visual examination of the shipping container for signs of damage or breakage (e.g., visible stains from leakage, sounds of broken glass). *Table 4* summarizes the steps for receiving and handling of damaged shipping containers.

**Table 4. Summary of Requirements for Receiving and Handling Damaged HD Shipping Containers**

| | |
|---|---|
| If the shipping container appears damaged | • Seal container without opening and contact the supplier<br>• If the unopened package is to be returned to the supplier, enclose the package in an impervious container and label the outer container "Hazardous"<br>• If the supplier declines return, dispose of as hazardous waste |
| If a damaged shipping container must be opened | • Seal the container in plastic or an impervious container<br>• Transport it to a C-PEC and place on a plastic-backed preparation mat<br>• Open the package and remove undamaged items<br>• Wipe the outside of the undamaged items with a disposable wipe<br>• Enclose the damaged item(s) in an impervious container and label the outer container "Hazardous"<br>• If the supplier declines return, dispose of as hazardous waste<br>• Deactivate, decontaminate, and clean the C-PEC (see *Deactivating, Decontaminating, Cleaning, and Disinfecting*) and discard the mat and cleaning disposables as hazardous waste |

When opening damaged shipping containers, they should preferably be transported to a C-PEC designated for nonsterile compounding. If a C-PEC designated for sterile compounding is the only one available, it must be disinfected after the decontamination, deactivation, and cleaning step before returning to any sterile compounding activity.

Damaged packages or shipping cartons must be considered spills that must be reported to the designated person and managed according to the entity's SOPs. Segregate HDs waiting to be returned to the supplier in a designated negative pressure area. Clean-up must comply with established SOPs.

## 11. LABELING, PACKAGING, TRANSPORT AND DISPOSAL

The entity must establish SOPs for the labeling, packaging, transport, and disposal of HDs. The SOPs must address prevention of accidental exposures or spills, personnel training on response to exposure, and use of a spill kit. Examples of special exposure-reducing strategies include small-bore connectors (such as Luer Lock) and syringes, syringe caps, CSTDs, the capping of container ports, sealed impervious plastic bags, impact-resistant and/or water-tight containers, and cautionary labeling.

### 11.1 Labeling

HDs identified by the entity as requiring special HD handling precautions must be clearly labeled at all times during their transport. Personnel must ensure that the labeling processes for compounded preparations do not introduce contamination into the non-HD handling areas.

### 11.2 Packaging

Personnel must select and use packaging containers and materials that will maintain physical integrity, stability, and sterility (if needed) of the HDs during transport. Packaging materials must protect the HD from damage, leakage, contamination, and degradation, while protecting healthcare workers who transport HDs. The entity must have written SOPs to describe appropriate shipping containers and insulating materials, based on information from product specifications, vendors, and mode of transport.

### 11.3 Transport

HDs that need to be transported must be labeled, stored, and handled in accordance with applicable federal, state, and local regulations. HDs must be transported in containers that minimize the risk of breakage or leakage. Pneumatic tubes must not be used to transport any liquid HDs or any antineoplastic HDs because of the potential for breakage and contamination.

When shipping HDs to locations outside the entity, the entity must consult the Transport Information on the SDS. The entity must ensure that labels and accessory labeling for the HDs include storage instructions, disposal instructions, and HD category information in a format that is consistent with the carrier's policies.

### 11.4 Disposal

All personnel who perform routine custodial waste removal and cleaning activities in HD handling areas must be trained in appropriate procedures to protect themselves and the environment to prevent HD contamination. Disposal of all HD waste, including, but not limited to, unused HDs and trace-contaminated PPE and other materials, must comply with all applicable federal, state, and local regulations.

## 12. DISPENSING FINAL DOSAGE FORMS

HDs that do not require any further manipulation, other than counting or repackaging of final dosage forms, may be prepared for dispensing without any further requirements for containment unless required by the manufacturer or if visual indicators of HD exposure hazards are present (e.g., HD dust or leakage).

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Counting or repackaging of HDs must be done carefully. Clean equipment should be dedicated for use with HDs and should be decontaminated after every use. Tablet and capsule forms of antineoplastic HDs must not be placed in automated counting or packaging machines, which subject them to stress and may create powdered contaminants.

## 13. COMPOUNDING

Entities and personnel involved in compounding HDs must be compliant with the appropriate USP standards for compounding including ⟨795⟩ and ⟨797⟩. Compounding must be done in proper engineering controls as described in *Compounding*. When compounding HD preparations in a C-PEC, a plastic-backed preparation mat should be placed on the work surface of the C-PEC. The mat should be changed immediately if a spill occurs and regularly during use, and should be discarded at the end of the daily compounding activity. Disposable or clean equipment for compounding (such as mortars and pestles, and spatulas) must be dedicated for use with HDs.

Bulk containers of liquid and API HD must be handled carefully to avoid spills. If used, APIs or other powdered HDs must be handled in a C-PEC to protect against occupational exposure, especially during particle-generating activities (such as crushing tablets, opening capsules, and weighing powder).

## 14. ADMINISTERING

HDs must be administered safely using protective medical devices and techniques. Examples of protective medical devices include needleless and closed systems. Examples of protective techniques include spiking or priming of IV tubing with a non-HD solution in a C-PEC and crushing tablets in a plastic pouch.

Appropriate PPE must be worn when administering HDs. After use, PPE must be removed and disposed of in a waste container approved for trace-contaminated HD waste at the site of drug administration. Equipment (such as tubing and needles) and packaging materials must be disposed of properly, such as in HD waste containers, after administration.

CSTDs must be used for administration of antineoplastic HDs when the dosage form allows. Techniques and ancillary devices that minimize the risk posed by open systems must be used when administering HDs through certain routes. Administration into certain organs or body cavities (e.g., the bladder, eye, peritoneal cavity, or chest cavity) often requires equipment for which locking connections may not be readily available or possible.

Healthcare personnel should avoid manipulating HDs such as crushing tablets or opening capsules if possible. Liquid formulations are preferred if solid oral dosage forms are not appropriate for the patient. If HD dosage forms do require manipulation such as crushing tablet(s) or opening capsule(s) for a single dose, personnel must don appropriate PPE and use a plastic pouch to contain any dust or particles generated.

## 15. DEACTIVATING, DECONTAMINATING, CLEANING, AND DISINFECTING

All areas where HDs are handled and all reusable equipment and devices must be deactivated, decontaminated, and cleaned. Additionally, sterile compounding areas and devices must be subsequently disinfected.

The entity must establish written procedures for decontamination, deactivation, and cleaning, and for sterile compounding areas disinfection. Additionally, cleaning of nonsterile compounding areas must comply with ⟨795⟩ and cleaning of sterile compounding areas must comply with ⟨797⟩. Written procedures for cleaning must include procedures, agents used, dilutions (if used), frequency, and documentation requirements.

All personnel who perform deactivation, decontamination, cleaning, and disinfection activities in HD handling areas must be trained in appropriate procedures to protect themselves and the environment from contamination. All personnel performing these activities must wear appropriate PPE resistant to the cleaning agents used, including two pairs of chemotherapy gloves and impermeable disposable gowns (see *Personal Protective Equipment*). Additionally, eye protection and face shields must be used if splashing is likely. If warranted by the activity, respiratory protection must be used.

The deactivating, decontaminating, cleaning, and disinfecting agents selected must be appropriate for the type of HD contaminant(s), location, and surface materials. The products used must be compatible with the surface material. Consult manufacturer or supplier information for compatibility with cleaning agents used. Agents used for deactivation, decontamination, and cleaning should be applied through the use of wipes wetted with appropriate solution and not delivered by a spray bottle to avoid spreading HD residue. All disposable materials must be discarded to meet EPA regulations and the entity's policies. Perform cleaning in areas that are sufficiently ventilated. *Table 5* summarizes the purpose and example agents for each step.

**Table 5. Cleaning Steps**

| Cleaning Step | Purpose | Example Agents |
|---|---|---|
| Deactivation | Render compound inert or inactive | As listed in the HD labeling or other agents which may incorporate Environmental Protection Agency (EPA)-registered oxidizers (e.g., peroxide formulations, sodium hypochlorite, etc.) |
| Decontamination | Remove HD residue | Materials that have been validated to be effective for HD decontamination, or through other materials proven to be effective through testing, which may include alcohol, water, peroxide, or sodium hypochlorite |
| Cleaning | Remove organic and inorganic material | Germicidal detergent |

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**Table 5. Cleaning Steps** *(continued)*

| Cleaning Step | Purpose | Example Agents |
|---|---|---|
| Disinfection (for sterile manipulations) | Destroy microorganisms | EPA-registered disinfectant and/or sterile alcohol as appropriate for use |

## 15.1 Deactivation

Deactivation renders a compound inert or inactive. Residue from deactivation must be removed by decontaminating the surface.

There is no one proven method for deactivating all compounds. The ultimate goal should be complete surface decontamination. Products that have known deactivation properties (EPA-registered oxidizing agents that are appropriate for the intended use) should be used when possible. Care should be taken when selecting materials for deactivation due to potential adverse effects (hazardous byproducts, respiratory effects, and caustic damage to surfaces). Damage to surfaces is exhibited by corrosion to stainless steel surfaces caused by sodium hypochlorite if left untreated. To prevent corrosion, sodium hypochlorite must be neutralized with sodium thiosulfate or by following with an agent to remove the sodium hypochlorite (e.g., sterile alcohol, sterile water, germicidal detergent, or sporicidal agent).

## 15.2 Decontamination

Decontamination occurs by inactivating, neutralizing, or physically removing HD residue from non-disposable surfaces and transferring it to absorbent, disposable materials (e.g., wipes, pads, or towels) appropriate to the area being cleaned. When choosing among various products available for decontaminating HDs, consideration should be given to surface compatibility and facility requirements. It is imperative to adhere to manufacturer's use instructions. Because of the growing number of assays available for HDs, additional surface wipe sampling is now possible and should be done to document the effectiveness of any agent used for decontamination of HD residue from work surfaces (see *Environmental Quality and Control*).

The amount of HD contamination introduced into the C-PEC may be reduced by wiping down HD containers. The solution used for wiping HD packaging must not alter the product label. The work surface of the C-PEC must be decontaminated between compounding of different HDs. The C-PEC must be decontaminated at least daily (when used), any time a spill occurs, before and after certification, any time voluntary interruption occurs, and if the ventilation tool is moved.

C-PECs may have areas under the work tray where contamination can build up. These areas must be deactivated, decontaminated, and cleaned at least monthly to reduce the contamination level in the C-PEC. Accessing this area may be difficult. Deactivate, decontaminate, and clean as much as possible of the C-PEC surfaces before accessing the area under the work tray. When deactivating, decontaminating, and cleaning the area under the work tray of a C-PEC, the containment airflows are compromised by opening the cabinets. To provide protection to the worker performing this task, respiratory protection may be required.

## 15.3 Cleaning

Cleaning is a process that results in the removal of contaminants (e.g., soil, microbial contamination, HD residue) from objects and surfaces using water, detergents, surfactants, solvents, and/or other chemicals. Cleaning agents used on compounding equipment should not introduce microbial contamination. No cleaning step may be performed when compounding activities are occurring.

## 15.4 Disinfection

Disinfection is a process of inhibiting or destroying microorganisms. Before disinfection can be adequately performed, surfaces must be cleaned. Disinfection must be done for areas intended to be sterile, including the sterile compounding areas.

# 16. SPILL CONTROL

All personnel who may be required to clean up a spill of HDs must receive proper training in spill management and the use of PPE and NIOSH-certified respirators (see *Personal Protective Equipment*). Spills must be contained and cleaned immediately only by qualified personnel with appropriate PPE. Qualified personnel must be available at all times while HDs are being handled. Signs must be available for restricting access to the spill area. Spill kits containing all of the materials needed to clean HD spills must be readily available in all areas where HDs are routinely handled. If HDs are being prepared or administered in a non-routine healthcare area, a spill kit and respirator must be available. All spill materials must be disposed of as hazardous waste.

The circumstances and management of spills must be documented. Personnel who are potentially exposed during the spill or spill clean up or who have direct skin or eye contact with HDs require immediate evaluation. Non-employees exposed to an HD spill should follow entity policy, which may include reporting to the designated emergency service for initial evaluation and completion of an incident report or exposure form.

SOPs must be developed to prevent spills and to direct the clean up of HD spills. SOPs must address the size and scope of the spill and specify who is responsible for spill management and the type of PPE required. The management of the spill (e.g., decontamination, deactivation, and cleaning) may be dependent on the size and type of spill. The SOP must address the location of spill kits and clean-up materials as well as the capacity of the spill kit. Written procedures should address use of appropriate full-facepiece, chemical cartridge-type respirators if the capacity of the spill kit is exceeded or if there is known or suspected airborne exposure to vapors or gases.

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## 17. DOCUMENTATION AND STANDARD OPERATING PROCEDURES

The entity must maintain SOPs for the safe handling of HDs for all situations in which these HDs are used throughout a facility. The SOPs must be reviewed at least every 12 months by the designated person, and the review must be documented. Revisions in forms or records must be made as needed and communicated to all personnel handling HDs.

The SOPs for handling of HDs should include:

- Hazard communication program
- Occupational safety program
- Designation of HD areas
- Receipt
- Storage
- Compounding
- Use and maintenance of proper engineering controls (e.g., C-PECs, C-SECs, and CSTDs)
- Hand hygiene and use of PPE based on activity (e.g., receipt, transport, compounding, administration, spill, and disposal)
- Deactivation, decontamination, cleaning, and disinfection
- Dispensing
- Transport
- Administering
- Environmental monitoring (e.g., wipe sampling)
- Disposal
- Spill control
- Medical surveillance

Personnel who transport, compound, or administer HDs must document their training according to OSHA standards (see OSHA Standard 1910.120 Hazardous Waste Operations and Emergency Response) and other applicable laws and regulations.

## 18. MEDICAL SURVEILLANCE

Medical surveillance is part of a comprehensive exposure control program complementing engineering controls, safe work processes, and use of PPE. Healthcare workers who handle HDs as a regular part of their job assignment should be enrolled in a medical surveillance program. The general purpose of surveillance is to minimize adverse health effects in personnel potentially exposed to HDs. Medical surveillance programs involve assessment and documentation of symptom complaints, physical findings, and laboratory values (such as a blood count) to determine whether there is a deviation from the expected norms.

Medical surveillance can also be viewed as a secondary prevention tool that may provide a means of early detection if a health problem develops. Tracking personnel through medical surveillance allows the comparison of health variables over time in individual workers, which may facilitate early detection of a change in a laboratory value or health condition. Medical surveillance programs also look for trends in populations of workers. Examining grouped data compared with data from unexposed workers may reveal a small alteration or increase in the frequency of a health effect that would be obscured if individual workers' results alone were considered.

Medical surveillance evaluates the protection afforded by engineering controls, other administrative controls, safe work processes, PPE, and worker education about the hazards of the materials they work with in the course of their duties. The data-gathering elements of a medical surveillance program are used to establish a baseline of workers' health and then to monitor their future health for any changes that may result from exposure to HDs.

Elements of a medical surveillance program should be consistent with the entity's Human Resource policies and should include:

- Development of an organized approach to identify workers who are potentially exposed to HDs on the basis of their job duties
- Use of an entity-based or contracted employee health service to perform the medical surveillance while protecting the confidentiality of the employees' personal medical information
- Initial baseline assessment (pre-placement) of a worker's health status and medical history. Data elements collected include a medical (including reproductive) history and work history to assess exposure to HDs, physical examination, and laboratory testing. Methods used to assess exposure history include a review of:
  - Records of HDs handled, with quantities and dosage forms
  - Estimated number of HDs handled per week
  - Estimates of hours spent handling HDs per week and/or per month
  - Performance of a physical assessment and laboratory studies linked to target organs of commonly used HDs, such as a baseline complete blood count. Biological monitoring to determine blood or urine levels of specific HDs is not currently recommended in surveillance protocols, but may have a role in the follow-up of acute spills with a specific agent.
- Medical records of surveillance should be maintained according to OSHA regulation concerning access to employee exposure and medical records
- Monitoring workers' health prospectively through periodic surveillance using the elements of data gathering described above (updated health and exposure history, physical assessment, and laboratory measures, if appropriate)

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- Monitoring of the data to identify prevention failure leading to health effects; this monitoring may occur in collaboration with the employee health service
- Development of a follow-up plan for workers who have shown health changes suggesting toxicity or who have experienced an acute exposure. This follow-up should include evaluation of current engineering and administrative controls and equipment to ensure that all systems are appropriately and accurately implemented (see *Follow-Up Plan*)
- Completion of an exit examination when a worker's employment at the entity ends, to document the information on the employee's medical, reproductive, and exposure histories. Examination and laboratory evaluation should be guided by the individual's history of exposures and follow the outline of the periodic evaluation

## 18.1 Follow-Up Plan

The occurrence of exposure-related health changes should prompt immediate re-evaluation of primary preventive measures (e.g., administrative and engineering controls, PPE, and others). In this manner, medical surveillance acts as a check on the effectiveness of controls already in use.

The entity should take the following actions:

- Perform a post-exposure examination tailored to the type of exposure (e.g., spills or needle sticks from syringes containing HDs). An assessment of the extent of exposure should be conducted and included in a confidential database and in an incident report. The physical examination should focus on the involved area as well as other organ systems commonly affected (i.e., the skin and mucous membranes for direct contact or inhalation; the pulmonary system for aerosolized HDs). Treatment and laboratory studies will follow as indicated and be guided by emergency protocols
- Compare performance of controls with recommended standards; conduct environmental sampling when analytical methods are available
- Verify and document that all engineering controls are in proper operating condition
- Verify and document that the worker complied with existing policies. Review policies for the use of PPE and employee compliance with PPE use and policies. Review availability of appropriate PPE (see *Personal Protective Equipment*)
- Develop and document a plan of action that will prevent additional exposure of workers
- Ensure confidential, two-way communication between the worker and the employee health unit(s) regarding notification, discussions about a change in health condition, or detection of an adverse health effect
- Provide and document a follow-up medical survey to demonstrate that the plan implemented is effective
- Ensure that any exposed worker receives confidential notification of any adverse health effect. Offer alternative duty or temporary reassignment
- Provide ongoing medical surveillance of all workers at risk for exposure to HDs to determine whether the plan implemented is effective

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# GLOSSARY

**Active pharmaceutical ingredient (API):** Any substance or mixture of substances intended to be used in the compounding of a drug preparation, thereby becoming the active ingredient in that preparation and furnishing pharmacological activity or other direct effect in the diagnosis, cure, mitigation, treatment, or prevention of disease in humans and animals or affecting the structure and function of the body.

**Alternative duty:** Performance of other tasks that do not include the direct handling of HDs.

**Ante-room:** An ISO Class 7 or cleaner room where personnel hand hygiene, garbing procedures, and other activities that generate high particulate levels are performed. The ante-room is the transition room between the unclassified area of the facility and the buffer room.

**Assessment of risk:** Evaluation of risk to determine alternative containment strategies and/or work practices.

**Beyond-use date (BUD):** The date or time beyond which a compounded preparation cannot not be used and must be discarded (see ⟨795⟩ and ⟨797⟩). The date or time is determined from the date or time when the preparation was compounded.

**Biological safety cabinet (BSC):** A ventilated cabinet often used for preparation of hazardous drugs. These cabinets are divided into three general classes (Class I, Class II, and Class III). Class II BSCs are further divided into types (Type A1, Type A2, Type B1, and Type B2). See *Appendix 3* for details.

**Buffer room:** A type of C-SEC under negative pressure that meets ISO Class 7 or better air quality where the C-PEC that generates and maintains an ISO Class 5 environment is physically located. Activities that occur in this area are limited to the preparation and staging of components and supplies used when compounding HDs.

**Chemotherapy glove:** A medical glove that meets the ASTM Standard Practice for Assessment of Resistance of Medical Gloves to Permeation by Chemotherapy Drugs (D6978) or its successor.

**Classified space:** An area that maintains an air cleanliness classification based on the International Organization for Standardization (ISO).

**Cleaning:** The process of removing soil (e.g., organic and inorganic material) from objects and surfaces, normally accomplished by manually or mechanically using water with detergents or enzymatic products.

**Closed-system drug-transfer device (CSTD):** A drug-transfer device that mechanically prohibits the transfer of environmental contaminants into the system and the escape of HD or vapor concentrations outside the system.

**Compounded preparation:** A nonsterile or sterile drug or nutrient preparation that is compounded in a licensed pharmacy or other healthcare-related facility in response to or anticipation of a prescription or a medication order from a licensed prescriber.

**Compounding aseptic containment isolator (CACI):** A specific type of CAI that is designed for the compounding of sterile HDs. The CACI is designed to provide worker protection from exposure to undesirable levels of airborne drugs throughout the compounding and material transfer processes and to provide an aseptic environment with unidirectional airflow for compounding sterile preparations.

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**Compounding aseptic isolator (CAI):** An isolator specifically designed for compounding sterile, non-hazardous pharmaceutical ingredients or preparations. The CAI is designed to maintain an aseptic compounding environment throughout the compounding and material transfer processes.

**Compounding personnel:** Individuals who participate in the compounding process.

**Containment primary engineering control (C-PEC):** A ventilated device designed and operated to minimize worker and environmental exposures to HDs by controlling emissions of airborne contaminants through the following:

- The full or partial enclosure of a potential contaminant source
- The use of airflow capture velocities to trap and remove airborne contaminants near their point of generation
- The use of air pressure relationships that define the direction of airflow into the cabinet
- The use of HEPA filtration on all potentially contaminated exhaust streams

**Containment secondary engineering control (C-SEC):** The room with fixed walls in which the C-PEC is placed. It incorporates specific design and operational parameters required to contain the potential hazard within the compounding room.

**Containment segregated compounding area (C-SCA):** A type of C-SEC with nominal requirements for airflow and room pressurization as they pertain to HD compounding.

**Containment ventilated enclosure (CVE):** A full or partial enclosure that uses ventilation principles to capture, contain, and remove airborne contaminants through HEPA filtration and prevent their release into the work environment.

**Deactivation:** Treatment of an HD contaminant on surfaces with a chemical, heat, ultraviolet light, or another agent to transform the HD into a less hazardous agent.

**Decontamination:** Inactivation, neutralization, or removal of HD contaminants on surfaces, usually by chemical means.

**Doff:** To remove PPE.

**Don:** To put on PPE.

**Disinfection:** The process of inhibiting or destroying microorganisms.

**Engineering control:** Primary, secondary, and supplemental devices designed to eliminate or reduce worker exposure to HDs.

**EPA-registered disinfectant:** Antimicrobial products registered with the Environmental Protection Agency (EPA) for healthcare use against pathogens specified in the product labeling.

**Externally vented:** Exhausted to the outside.

**Final dosage form:** Any form of a medication that requires no further manipulation before administration.

**Globally Harmonized System of Classification and Labeling of Chemicals (GHS):** A system for standardizing and harmonizing the classification and labeling of chemicals.

**Goggles:** Tight-fitting eye protection that completely covers the eyes, eye sockets, and facial area that immediately surrounds the eyes. Goggles provide protection from impact, dust, and splashes. Some goggles fit over corrective lenses.

**Hazardous drug (HD):** Any drug identified by at least one of the following criteria:

- Carcinogenicity, teratogenicity, or developmental toxicity
- Reproductive toxicity in humans
- Organ toxicity at low dose in humans or animals
- Genotoxicity or new drugs that mimic existing HDs in structure or toxicity

**High-efficiency particulate air (HEPA) filtration:** An extended-medium, dry-type filter in a rigid frame, having a minimum particle collection efficiency of 99.97% for particles with a mass median diameter of 0.3 µm when tested at a rated airflow in accordance with MIL STD 282 using IEST Recommended Standard RP-CC001.5.

**Negative-pressure room:** A room that is maintained at a lower pressure than the adjacent areas; therefore the net flow of air is into the room.

**Pass-through:** An enclosure with interlocking doors that is positioned between two spaces for the purpose of reducing particulate transfer while moving materials from one space to another. A pass-through serving negative-pressure rooms needs to be equipped with sealed doors.

**Personal protective equipment (PPE):** Items such as gloves, gowns, respirators, goggles, faceshields, and others that protect individual workers from hazardous physical or chemical exposures.

**Positive-pressure room:** A room that is maintained at a higher pressure than the adjacent areas; therefore, the net flow of air is out of the room.

**Repackaging:** The act of removing a product from its original primary container and placing it into another primary container, usually of smaller size.

**Safety data sheet (SDS):** An informational document that provides written or printed material concerning a hazardous chemical. The SDS is prepared in accordance with the HCS [previously known as a Material Safety Data Sheet (MSDS)].

**Spill kit:** A container of supplies, warning signage, and related materials used to contain the spill of an HD.

**Standard operating procedure (SOP):** Written procedures describing operations, testing, sampling, interpretation of results, and corrective actions that relate to the operations that are taking place.

**Supplemental engineering control:** An adjunct control (e.g., CSTD) that may be used concurrently with primary and secondary engineering controls. Supplemental engineering controls offer additional levels of protection and may facilitate enhanced occupational protection, especially when handling HDs outside of primary and secondary engineering controls (e.g., during administering).

**Unclassified space:** A space not required to meet any air cleanliness classification based on the International Organization for Standardization (ISO).

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# APPENDICES

## Appendix 1: Acronyms

| | |
|---|---|
| ACPH | Air changes per hour |
| API | Active pharmaceutical ingredient |
| ASTM | American Society for Testing and Materials |
| BSC | Biological safety cabinet |
| BUD | Beyond-use date |
| CACI | Compounding aseptic containment isolator |
| CAI | Compounding aseptic isolator |
| CDC | Centers for Disease Control and Prevention |
| C-PEC | Containment primary engineering control |
| C-SCA | Containment segregated compounding area |
| C-SEC | Containment secondary engineering control |
| CSP | Compounded sterile preparation |
| CSTD | Closed-system drug-transfer device |
| CVE | Containment ventilated enclosure |
| EPA | Environmental Protection Agency |
| GHS | Globally Harmonized System of Classification and Labeling of Chemicals |
| HCS | Hazard Communication Standard |
| HD | Hazardous drug |
| HEPA | High-efficiency particulate air |
| IV | Intravenous |
| LAFW | Laminar airflow workbench |
| NIOSH | National Institute for Occupational Safety and Health |
| ONS | Oncology Nursing Society |
| OSHA | Occupational Safety and Health Administration |
| PAPR | Powered air-purified respirator |
| PPE | Personal protective equipment |
| SDS | Safety Data Sheet |
| SOP | Standard operating procedure |
| ULPA | Ultra-low particulate air |
| UPS | Uninterrupted power source |

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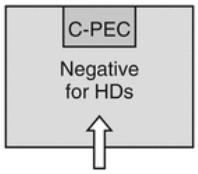
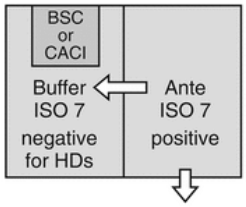
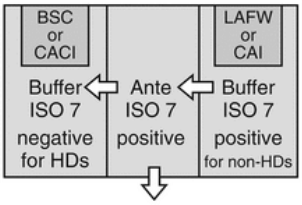
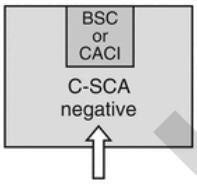
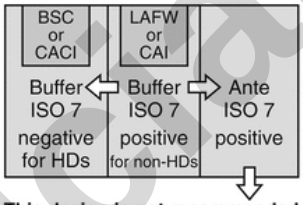
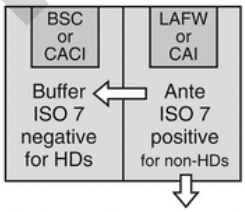
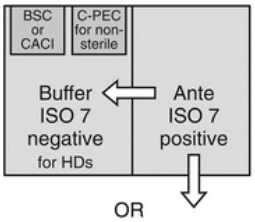
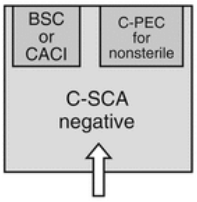
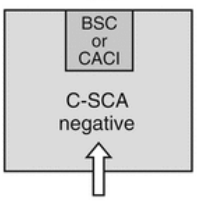
## Appendix 2: Examples of Designs for Hazardous Drug Compounding Areas[a]

| Use | Optimal Primary and Secondary Control | Minimum ACPH | Limitations Primary and Secondary Control | Minimum ACPH | Notes for limitations |
|---|---|---|---|---|---|
| Nonsterile HD compounding |  | 12 | | | |
| Sterile HD compounding |  <br>OR<br>  | 30 |  <br>OR<br>  <br>**This design is not recommended**<br>OR<br>  <br>Typically used in oncology clinic settings. | 12<br>30<br>30 | Maximum BUD as described in <797> for segregated compounding area.<br>If this design is in place, measures must be taken to avoid contamination of the positive-pressure buffer room.<br>Maximum BUD as described in <797>. |
| Both sterile HD and nonsterile HD compounding | A separate room for sterile and nonsterile compounding is recommended | |  <br>OR<br>  <br>OR<br>  | 30<br>12<br>12 | For rooms used for both sterile and nonsterile compounding, particle-generating activity must not be performed when sterile compounding is in process. C-PECs must be at least 1 meter apart.<br>Maximum BUD as described in <797> for segregated compounding area.<br>Maximum BUD as described in <797> for segregated compounding area. |

[a] The arrows indicate direction of airflow.

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## Appendix 3: Types of Biological Safety Cabinets

**Class I:** A BSC that protects personnel and the environment but does not protect the product/preparation. A minimum velocity of 75 linear feet/minute of unfiltered room air is drawn through the front opening and across the work surface, providing personnel protection. The air is then passed through a HEPA/ULPA (ultra-low particulate air) filter, either into the room or to the outside in the exhaust plenum, providing environmental protection.

**Class II:** Class II (Types A1, A2, B1, and B2) BSCs are partial barrier systems that rely on the movement of air to provide personnel, environmental, and product/preparation protection. Personnel and product/preparation protection are provided by the combination of inward and downward airflow captured by the front grille of the cabinet. Side-to-side cross-contamination of products/preparations is minimized by the internal downward flow of HEPA/ULPA filtered air moving toward the work surface and then drawn into the front and rear intake grilles. Environmental protection is provided when the cabinet exhaust air is passed through a HEPA/ULPA filter.

**Type A1 (formerly, Type A):** These Class II BSCs maintain a minimum inflow velocity of 75 feet/minute; have HEPA-filtered, down-flow air that is a portion of the mixed down-flow and inflow air from a common plenum; may exhaust HEPA-filtered air back into the laboratory or to the environment through an exhaust canopy; and may have positive-pressure contaminated ducts and plenums that are not surrounded by negative-pressure plenums. Type A1 BSCs are not suitable for use with volatile toxic chemicals and volatile radionuclides.

**Type A2 (formerly, Type B3):** These Class II BSCs maintain a minimum inflow velocity of 100 feet/minute; have HEPA-filtered, down-flow air that is a portion of the mixed down-flow and inflow air from a common exhaust plenum; may exhaust HEPA-filtered air back into the laboratory or to the environment through an exhaust canopy; and have all contaminated ducts and plenums under negative pressure or surrounded by negative-pressure ducts and plenums. If these cabinets are used for minute quantities of volatile toxic chemicals and trace amounts of radionuclides, they must be exhausted through properly functioning exhaust canopies.

**Type B1:** These Class II BSCs maintain a minimum inflow velocity of 100 feet/minute; have HEPA-filtered, down-flow air composed largely of uncontaminated, recirculated inflow air; exhaust most of the contaminated down-flow air through a dedicated duct exhausted to the atmosphere after passing it through a HEPA filter; and have all contaminated ducts and plenums under negative pressure or surrounded by negative-pressure ducts and plenums. If these cabinets are used for work involving minute quantities of volatile toxic chemicals and trace amounts of radionuclides, the work must be done in the directly exhausted portion of the cabinet.

**Type B2 (total exhaust):** These Class II BSCs maintain a minimum inflow velocity of 100 feet/minute; have HEPA-filtered, down-flow air drawn from the laboratory or the outside; exhaust all inflow and down-flow air to the atmosphere after filtration through a HEPA filter without recirculation inside the cabinet or return to the laboratory; and have all contaminated ducts and plenums under negative pressure or surrounded by directly exhausted negative-pressure ducts and plenums. These cabinets may be used with volatile toxic chemicals and radionuclides.

**Class III:** The Class III BSC is designed for work with highly infectious microbiological agents and other hazardous operations. It provides maximum protection for the environment and the worker. It is a gas-tight enclosure with a viewing window that is secured with locks and/or requires the use of tools to open. Both supply and exhaust air are HEPA/ULPA filtered. Exhaust air must pass through two HEPA/ULPA filters in series before discharge to the outdoors.

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# ⟨801⟩ POLAROGRAPHY

Polarography is an electrochemical method of analysis based on the measurement of the current flow resulting from the electrolysis of a solution at a polarizable microelectrode, as a function of an applied voltage. The polarogram (see *Figure 1*) obtained by this measurement provides qualitative and quantitative information on electro-reducible and electro-oxidizable substances. The normal concentration range for substances being analyzed is from $10^{-2}$ molar to $10^{-5}$ molar.

In direct current (dc) polarography, the microelectrode is a dropping mercury electrode (DME) consisting of small reproducible drops of mercury flowing from the orifice of a capillary tube connected to a mercury reservoir. A saturated calomel electrode (SCE) with a large surface area is the most commonly employed reference electrode. As the voltage applied to the cell increases, only a very small residual current flows until the substance under assay undergoes reduction or oxidation. Then the current increases, at first gradually, then almost linearly with voltage, and it gradually reaches a limiting value as is shown in *Figure 1*.

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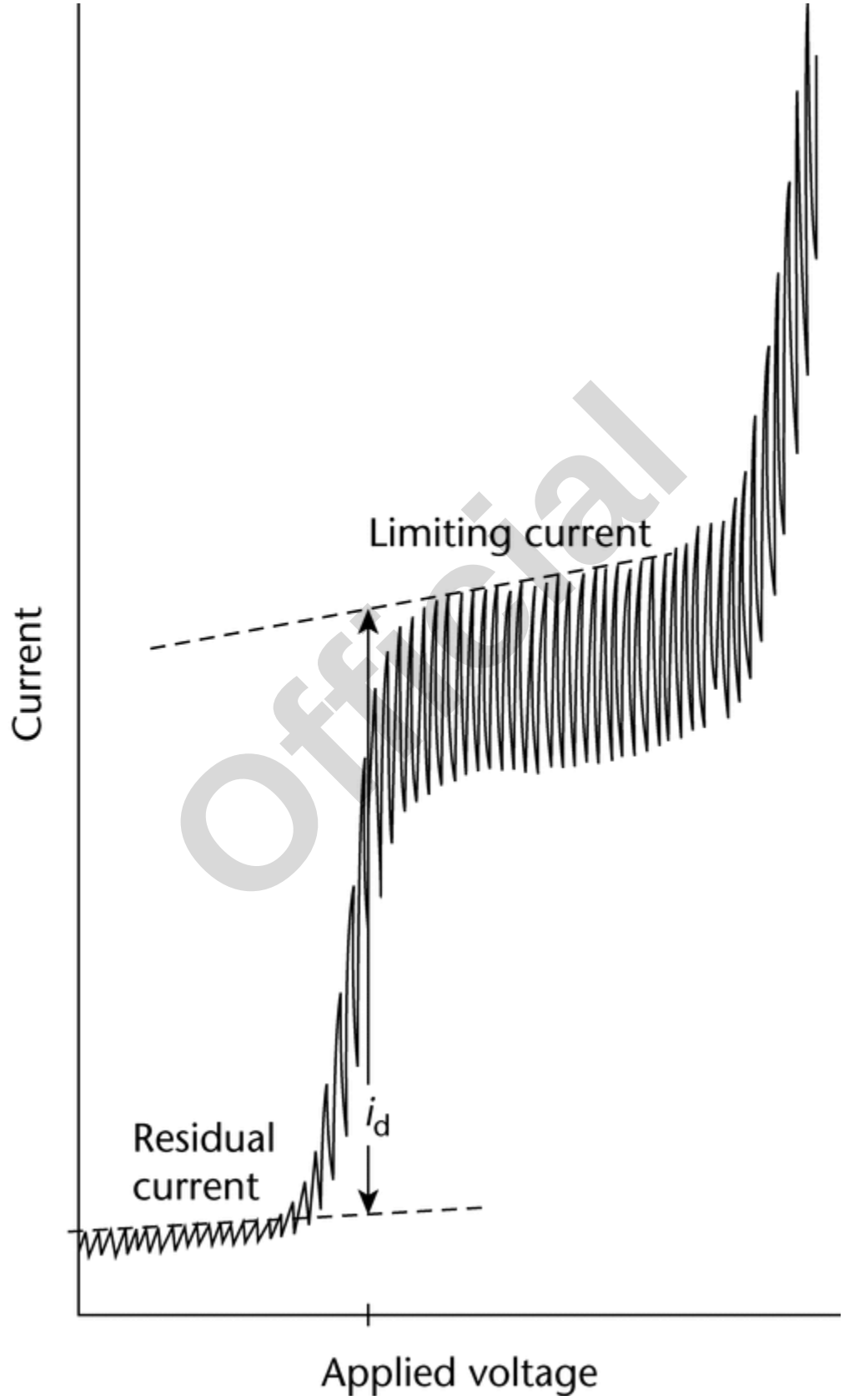


Fig. 1. Typical Polarogram Showing Change in Current Flow with Increasing Potential Applied to the Dropping Mercury Electrode.

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On the initial rising portion of the polarographic wave, the increased flow of current results in a decrease in the concentration of the electro-active species at the electrode surface. As the voltage and current increase, the concentration of the reactive species decreases further to a minimal value at the electrode surface. The current is then limited by the rate at which the reacting species can diffuse from the bulk of the solution to the surface of the microelectrode. The final current rise is caused by the reaction of the supporting electrolyte. This large concentration of electrolyte is inert within the potential range used in the analysis, and it prevents the reactive species from reaching the electrode by electrical migration, thus assuring that the limiting current is diffusion-controlled.

Since, in the case of the DME, the electrode surface is being constantly renewed in a cyclic fashion, the current increases from a small value as the drop begins to form to a maximum value as the drop falls. By the use of a suitable recorder to measure the current, the characteristic saw-toothed record is obtained. The limiting current is the sum of the residual and the diffusion currents. The residual current is subtracted from the limiting current to give the wave height.

## ILKOVIC EQUATION

The linear relationship between the diffusion current ($i_d$) and the concentration of electro-active species is shown by the Ilkovic equation:

$$i_d = 708nD^{1/2}Cm^{2/3}t^{1/6}$$

in which $i_d$ is the maximum current in microamperes; n is the number of electrons required per molecule of electro-active substance; D is its diffusion coefficient, in square cm per second; C is the concentration, in millimoles per L; m is the rate of mercury flow from the DME, in mg per second; and t is the drop time, in seconds.

Modern polarographs are equipped with recorders capable of following the current during the latter portion of the drop life; consequently, the maximum of the oscillations is the measure of the current. When the current is measured only at the end of the drop life, the technique is termed sampled dc polarography. In this case, only the maximum currents are recorded and oscillations due to drop growth are not observed.

For instruments equipped with galvanometers to measure the current or recorders operated in a damped mode, the saw-toothed waves correspond to oscillations about the average current. In the latter case, the average of the oscillations is the measure of the current. For polarograms obtained in this manner, the $i_d$ given by the Ilkovic equation is the average current in microamperes observed during the life of the drop, when the coefficient 708 is replaced by 607.

## CONTROL OF THE DIFFUSION CURRENT

The Ilkovic equation identifies the variables that must be controlled to ensure that the diffusion current is directly proportional to the concentration of electro-active material. At 25° the diffusion coefficients for aqueous solutions of many ions and organic molecules increase 1% to 2% per degree rise in temperature. Thus the temperature of the polarographic cell must be controlled to within ±0.5°. The quantities m and t depend upon the dimensions of the capillary and the height of the mercury column above the electrode. Although results obtained with different capillaries can be compared if the product $m^{2/3}t^{1/6}$ is known, it is advisable to use the same capillary with a constant head of mercury during a series of analyses. The diffusion current is proportional to the square root of the height of the mercury column. A mercury reservoir with a diameter greater than 4 cm prevents any significant drop in the mercury level during a series of runs.

The capillary for the DME has a bore of approximately 0.04 mm and a length of 6 cm to 15 cm. The height of the mercury column, measured from the tip of the capillary to the top of the mercury pool, ranges from 40 cm to 80 cm. The exact length of the capillary and the height of the mercury column are adjusted to give a drop-time of between 3 and 5 seconds at open circuit with the capillary immersed in the test solution.

Equipment is available that allows controlled drop-times of fractions of a second to several seconds. As detail within a polarogram is related to the number of drops delivered during a given potential change, such short drop-times allow more rapid recording of the polarogram.

The current flowing through the test solution during the recording of a polarogram is in the microampere range. Thus, the current flow produces negligible changes in the test solution and several polarograms can be run on the same test solution without significant differences.

## HALF-WAVE POTENTIAL

The half-wave potential ($E_{1/2}$) occurs at the point on the polarogram one-half the distance between the residual current and the limiting current plateau. This potential is characteristic of the electro-active species and is largely independent of its concentration or the capillary used to obtain the wave. It is dependent upon the solution composition and may change with variations in the pH or in the solvent system or with the addition of complexing agents. The half-wave potential thus serves as a criterion for the qualitative identification of a substance.

The potential of the DME is equal to the applied voltage versus the reference electrode after correction for the iR drop (that potential need to pass the current, i, through the solution with a resistance R). It is especially important to make this correction for nonaqueous solutions, which ordinarily possess high resistance, if an accurate potential for the DME is needed. Correction of the half-wave potential is not required for quantitative analysis. Unless otherwise indicated, it is to be understood that potentials represent measurements made against the SCE.

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## REMOVAL OF DISSOLVED OXYGEN

Inasmuch as oxygen is reduced at the DME in two steps, first to hydrogen peroxide and then to water, it interferes where polarograms are to be made at potentials more negative than about 0 volt versus SCE, and must be removed. This may be accomplished by bubbling oxygen-free nitrogen through the solution for 10 to 15 minutes immediately before recording the wave, the nitrogen first having been "conditioned" to minimize changes due to evaporation, by being passed through a separate portion of the solution.

It is necessary that the solution be quiet and vibration-free during the time the wave is recorded, to ensure that the current is diffusion-controlled. Therefore, the nitrogen aeration should be stopped and the gas be directed to flow over the surface of the solution before a polarogram is recorded.

In alkaline media, sodium bisulfite may be added to remove oxygen, provided the reagent does not react with other components of the system.

## MEASUREMENT OF WAVE HEIGHT

To use a polarogram quantitatively, it is necessary to measure the height of the wave. Since this is a measure of the magnitude of the diffusion current, it is measured vertically. To compensate for the residual current, the segment of the curve preceding the wave is extrapolated beyond the rise in the wave. For a well-formed wave where this extrapolation parallels the limiting current plateau, the measurement is unambiguous. For less well-defined waves, the following procedure may be used unless otherwise directed in the individual monograph. Both the residual current and the limiting current are extrapolated with straight lines, as shown by the graph (*Figure 1*). The wave height is taken as the vertical distance between these lines measured at the half-wave potential.

## PROCEDURE

[**Caution**—Mercury vapor is poisonous, and metallic mercury has a significant vapor pressure at room temperature. The work area in which mercury is used should be constructed in such a way that any spilled or spattered droplets of mercury can be completely recovered with relative ease. Scrupulously clean up mercury after each use of the instrument. Work in a well-ventilated laboratory, taking care to clean up any spilled mercury.]

Transfer a portion of the final dilution of the substance being assayed to a suitable polarographic cell immersed in a water bath regulated to 25 ± 0.5°. Pass a stream of nitrogen through the solution for 10 to 15 minutes to remove dissolved oxygen. Start the mercury dropping from the capillary, insert the capillary into the test solution, and adjust the height of the mercury reservoir. Switch the flow of nitrogen to pass over the surface of the solution, and record the polarogram over the potential range indicated in the individual monograph, using the appropriate recorder or galvanometer sensitivity to give a suitable wave. Measure the height of the wave, and unless otherwise directed in the monograph, compare this with the wave height obtained with the appropriate USP Reference Standard, measured under the same conditions.

## PULSE POLAROGRAPHY

In conventional dc polarography, the current is measured continuously as potential is applied as a linear ramp (see *Figure 2)*.

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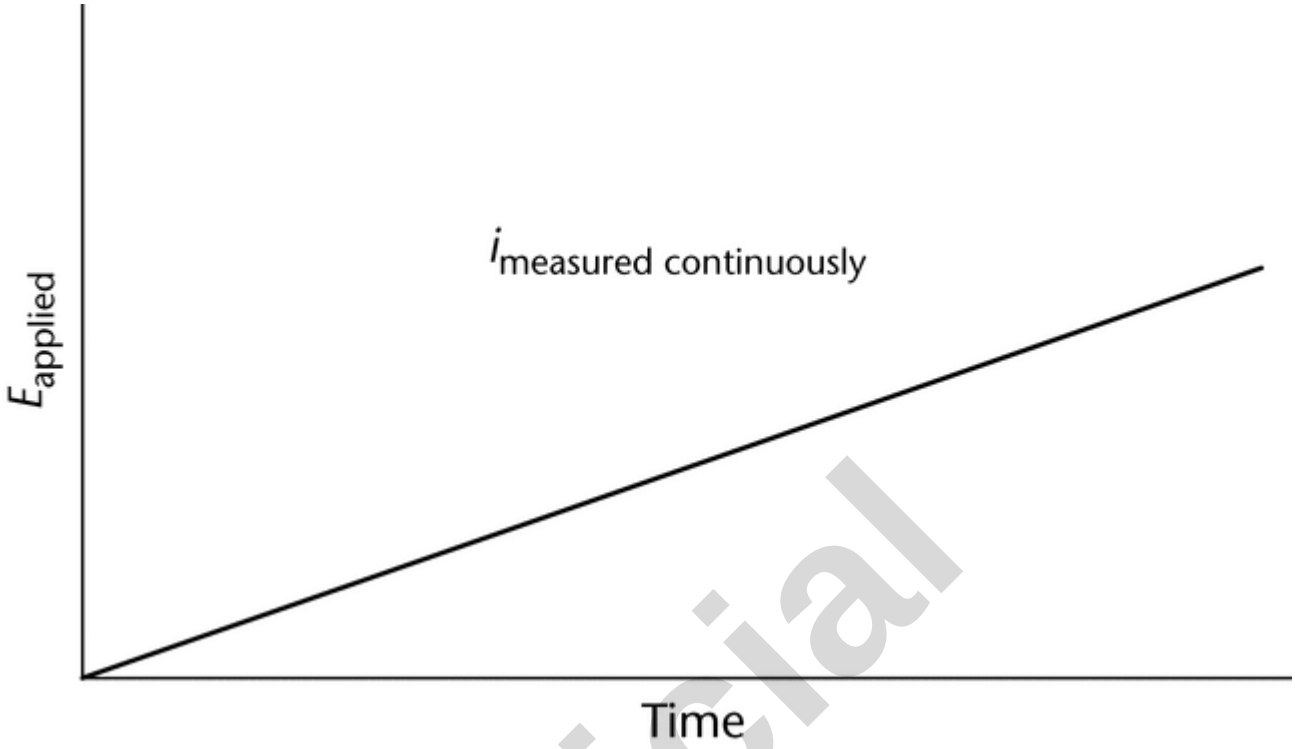


Fig. 2. Direct Current (dc) Polarography.

This current is composed of two elements. The first, the diffusion (faradaic) current, is produced by the substance undergoing reduction or oxidation at the working electrode, and is directly proportional to the concentration of this substance. The second element is the capacitative current (charging of the electrochemical double layer). The changes in these currents as the mercury drop varies in size produce the oscillations present in typical dc polarograms.

In normal pulse polarography, a potential pulse is applied to the mercury electrode near the end of the drop life, with the drop being held at the initial potential during growth period (see *Figure 3).*

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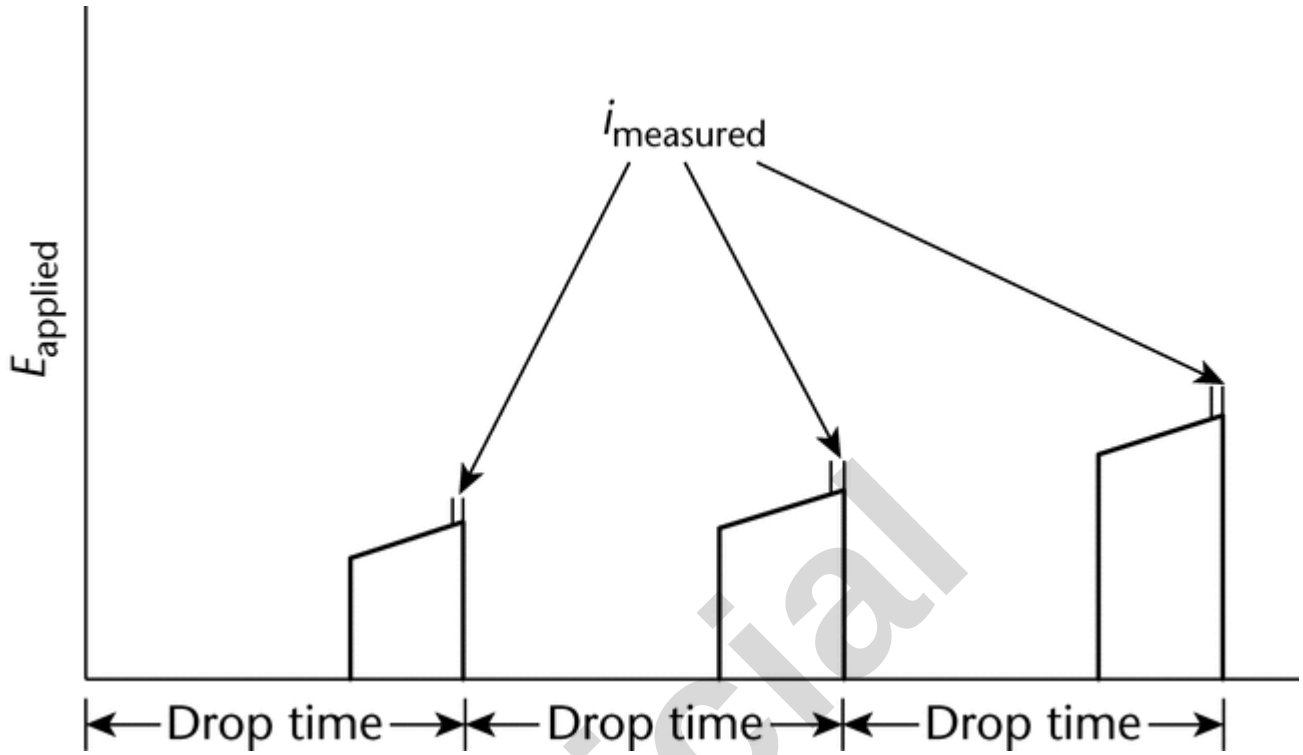


Fig. 3. Pulse Polarography.

Each succeeding drop has a slightly higher pulse applied to it, with the rate of increase being determined by the selected scan rate. The current is measured at the end of the pulse where the capacitative current is nearly zero, and thus primarily faradaic current is measured (see *Figure 4).*

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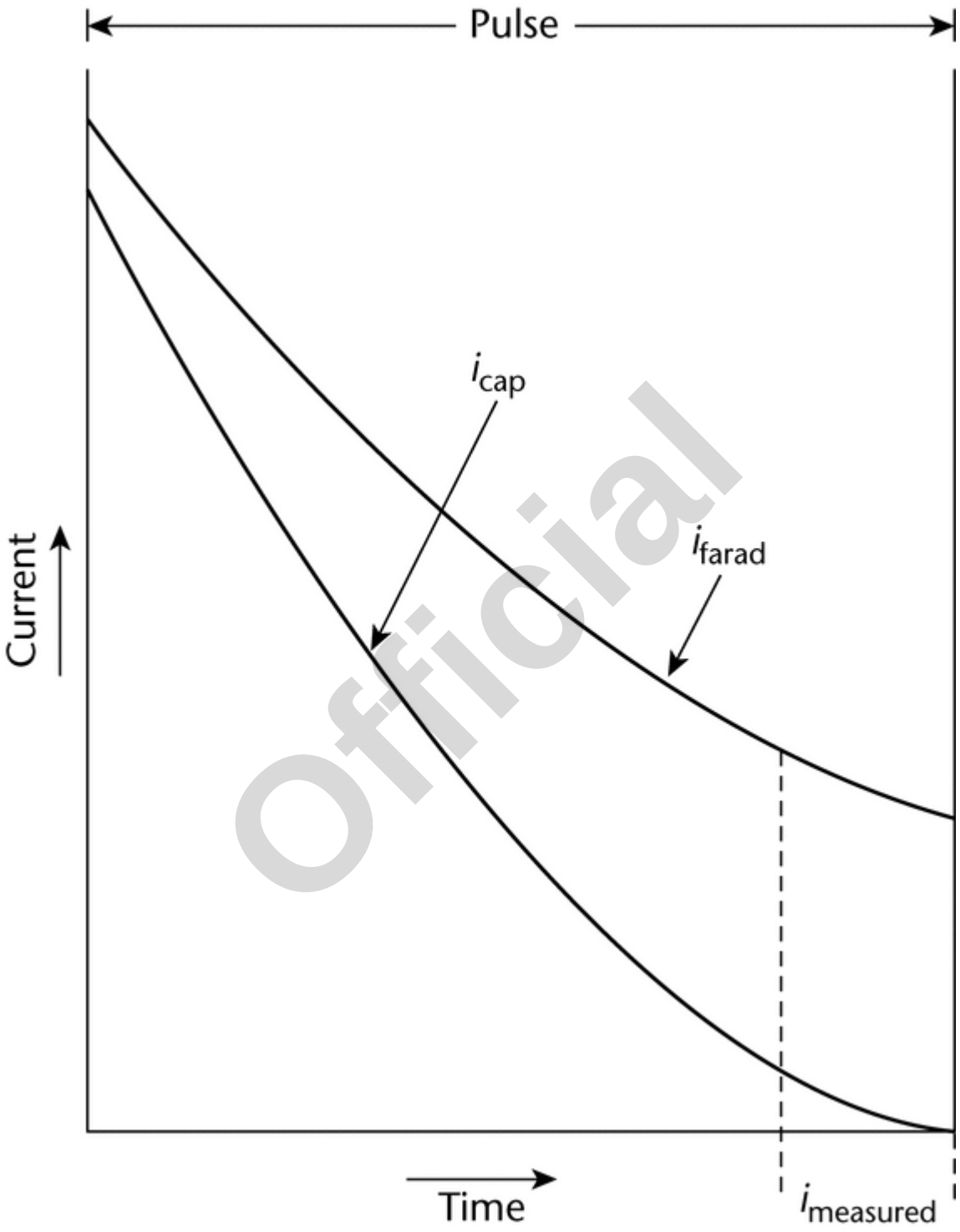


Fig. 4. Plot of Current Versus Time in Pulse Polarography.

In addition, since the pulse is applied for only a short duration, the diffusion layer is not depleted as extensively as in dc polarography and larger current levels are obtained for equivalent concentrations. Concentrations as low as $10^{-6}$ M can be measured, providing approximately a ten-fold increase in sensitivity over that with dc polarography. Limiting current values are more easily measured, since the waves are free from oscillations.

Differential pulse polarography is a technique whereby a fixed-height pulse applied at the end of the life of each drop is superimposed on a linear increasing dc ramp (see *Figure 5).*

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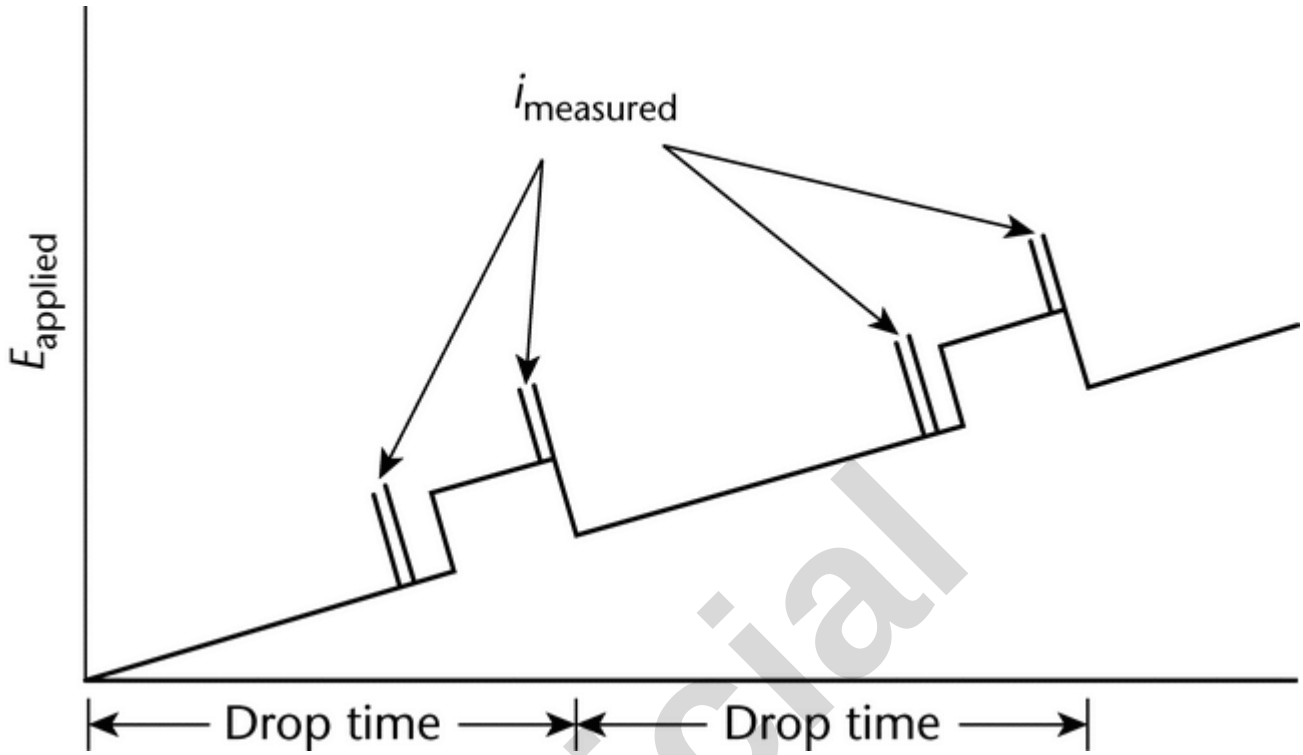


Fig. 5. Differential Pulse Polarography.

Current flow is measured just before application of the pulse and again at the end of the pulse. The difference between these two currents is measured and presented to the recorder. Such a differential signal provides a curve approximating the derivative of the polarographic wave, and gives a peak presentation. The peak potential is equivalent to:

$$E_{1/2} - \Delta E/2$$

where $\Delta E$ is the pulse height. The peak height is directly proportional to concentration at constant scan rates and constant pulse heights. This technique is especially sensitive (levels of $10^{-7}$M may be determined) and affords improved resolution between closely spaced waves.

## ANODIC STRIPPING VOLTAMMETRY

Anodic stripping voltammetry is an electrochemical technique whereby trace amounts of substances in solution are concentrated (reduced) onto an electrode and then stripped (oxidized) back into solution by scanning the applied voltage anodically. The measurement of the current flow as a function of this voltage and scanning rate provides qualitative and quantitative information on such substances. The concentration step permits analyses at $10^{-7}$M to $10^{-9}$M levels.

Basic instrumentation includes a voltage ramp generator; current-measuring circuitry; a cell with working, reference, and counter electrodes; and a recorder or other read-out device. Instruments having dc or pulse-polarographic capabilities are generally quite adequate for stripping application. The working electrode commonly used is the hanging mercury drop electrode (HMDE), although the mercury thin-film electrode (MTFE) has acquired acceptance. For analysis of metals such as silver, platinum, and gold, whose oxidation potentials are more positive than mercury, and mercury itself, the use of solid electrodes such as platinum, gold, or carbon is required. A saturated calomel electrode or a silver–silver chloride electrode serves as the reference except for the analysis of mercury or silver. A platinum wire is commonly employed as the counter electrode.

Test specimens containing suitable electrolyte are pipeted into the cell. Dissolved oxygen is removed by bubbling nitrogen through the cell for 5 to 10 minutes.

Generally, an electrolysis potential equivalent to 200 to 300 mV more negative than the half-wave potential of the material to be analyzed is applied (although this potential is to be determined experimentally), with stirring for 1 to 10 minutes. For reproducible results, maintain constant conditions (i.e., deposition time, stirring rate, temperature, specimen volume, and drop size if HMDE is used).

After deposition, the stirring is discontinued and the solution and electrode are allowed to equilibrate for a short period. The potential is then rapidly scanned anodically (10 mV/second or greater in dc polarography and 5 mV/second in differential pulse polarography). As in polarography, the limiting current is proportional to concentration of the species (wave height in dc and pulse; peak height in differential pulse), while the half-wave potential (dc, pulse) or peak potential (differential pulse) identifies the species. It is imperative that the choice of supporting electrolyte be made carefully in order to obtain satisfactory behavior. Quantitation is usually achieved by a standard addition or calibration method.

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This technique is appropriate for trace-metal analysis, but has limited use in organic determinations, since many of these reactions are irreversible. In analyzing substances such as chloride, cathodic stripping voltammetry may be used. The technique is the same as anodic stripping voltammetry, except that the substance is deposited anodically and then stripped by a cathodic voltage scan.

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# ⟨811⟩ POWDER FINENESS

The particle size distribution should be estimated by *Particle Size Distribution Estimation by Analytical Sieving* ⟨786⟩ or by application of other methods where practical. A simple descriptive classification of powder fineness is provided in this chapter. For practical reasons, sieves are commonly used to measure powder fineness. Sieving is most suitable where a majority of the particles are larger than about 75 µm, although it can be used for some powders having smaller particle sizes where the method can be validated. Light diffraction is also a widely used technique for measuring the size of a wide range of particles.

## CLASSIFICATION OF POWDER FINENESS

Where the cumulative distribution has been determined by analytical sieving or by application of other methods, powder fineness may be classified in the following manner:

$x_{90}$ = particle dimension corresponding to 90% of the cumulative undersize distribution

$x_{50}$ = median particle dimension (i.e., 50% of the particles are smaller and 50% of the particles are larger)

$x_{10}$ = particle dimension corresponding to 10% of the cumulative undersize distribution

It is recognized that the symbol $d$ is also widely used to designate these values. Therefore, the symbols $d_{90}$, $d_{50}$, and $d_{10}$ may be used.

The following parameters may be defined based on the cumulative distribution. $Q_R(x)$ = cumulative distribution of particles with a dimension less than or equal to $x$ where the subscript $R$ reflects the distribution type.

| *R* | Distribution Type |
|---|---|
| 0 | Number |
| 1 | Length |
| 2 | Area |
| 3 | Volume |

Therefore, by definition:

1. $Q_R(x) = 0.90$ when $x = x_{90}$
2. $Q_R(x) = 0.50$ when $x = x_{50}$
3. $Q_R(x) = 0.10$ when $x = x_{10}$

An alternative but less informative method of classifying powder fineness is by use of the terms in the following table.

**Classification of Powders by Fineness**

| Descriptive Term | $X_{50}$ (µm) | Cumulative Distribution by Volume Basis, $Q_3(x)$ |
|---|---|---|
| Coarse | >355 | $Q_3(355) < 0.50$ |
| Moderately Fine | 180–355 | $Q_3(180) < 0.50$ and $Q_3(355) \geq 0.50$ |
| Fine | 125–180 | $Q_3(125) < 0.50$ and $Q_3(180) \geq 0.50$ |
| Very Fine | ≤125 | $Q_3(125) \geq 0.50$ |

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# ⟨821⟩ RADIOACTIVITY

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## 1. INTRODUCTION

Radiopharmaceuticals and radioactive devices require specialized techniques in their production, testing, handling, dispensing, and administration in order to ensure optimal effectiveness and maintain safety for workers, patients, and the public. Hence, all operations involving these articles should be carried out by or under the supervision of personnel who have been appropriately trained in the handling of radioactive materials and appropriate instruments.

The facilities for the production, storage, and use of radioactive drugs and radioactive devices are generally subject to licensing by the U.S. Nuclear Regulatory Commission or Agreement State agency, or similar governmental agency outside the United States. Most radioactive drugs and radioactive devices are subject to other regulations relating to transportation, environmental release, and workplace safety.

*USP* monographs for radioactive articles include specifications for radionuclide identification and assay for radioactivity. Moreover, radioactivity counting assemblies or radiation detectors are required instruments for the determination of radionuclidic purity and radiochemical purity. The purpose of this general chapter is to provide standards for measurement of radioactivity, including instrument qualification, performance checks, identification of radionuclides and radionuclidic impurities, and assay of radionuclides.

Information regarding radioactivity is described in *Radioactivity—Theory and Practice* ⟨1821⟩, which includes definitions, types of decay, general considerations relating to radioactive decay, counting, radionuclide production, purity, labeling, and instrumentation for detection and measurement of radioactive emissions, as well as a glossary.

## 2. PERSONNEL TRAINING AND DOCUMENTATION

Personnel training should be completed in accordance with written policies and/or procedures. Documentation of the personnel training should be included in the Quality Assurance Program. The length of time to retain the documentation varies depending on the appropriate regulatory requirements.

## 3. QUALIFICATION OF INSTRUMENTS

Qualification of instruments should be completed in accordance with written policies and/or procedures. Documentation of the qualification of instruments should be included in the Quality Assurance Program. The length of time to retain the documentation varies depending on the applicable regulatory requirements.

### 3.1 Installation Qualification

Installation qualification (IQ) is to be performed upon installation of an instrument and after major repair. There are two categories in an IQ—visual inspection and operating environment. Visual inspection involves a check of the condition of the

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instrument to determine if it is ready to use. The inspection confirms that all cables are intact, the instrument is intact with no broken pieces/parts, and all components of the equipment are in working order. The operating environment for the device is checked for proper temperature, humidity, and power requirements. The operating environment should also be checked to avoid high background radioactivity. Temperatures are typically 10°–30° C and relative humidity ranges are generally 20%–90%. Shielding may be necessary to ensure that the background radiation has no negative effect on the instrument performance.

### 3.2 Operational Qualification

Operational qualification (OQ) is a check of operational specifications for the equipment, including equipment set-up, functional testing of subsystems, and proper overall operation. OQ should describe operational procedures for the equipment and is performed after IQ and after major repairs. OQ should include all appropriate tests to show that the instrument is capable of functioning properly. Typical examples of OQ include clock accuracy, high voltage, zero adjust, background response, and contamination check.

### 3.3 Performance Qualification

Performance qualification (PQ) demonstrates that the equipment is capable of performing tasks required in the operating environment and provides the intended results. PQ should describe the required performance tasks for the equipment and include all appropriate tests to demonstrate that the instrument is performing within its operating parameters. These PQ tests are done following the OQ tests. Additionally, some or all the PQ tests are performed routinely (e.g., daily, quarterly, semiannually, annually) to demonstrate the instruments continual acceptance for use. Typical PQ tests include constancy and relative response, accuracy, reproducibility (precision), system linearity, determination of “quench” curves in liquid scintillation counters, and supplier equivalence.

### 3.4 Geometry Testing

The purpose of the geometry test is to demonstrate correct readings of the test sample, accounting for differences in the container, volume, or position of the test sample compared to the radioactive calibration standard. Geometry testing should be performed at installation of the dose calibrator and for each type of vial and syringe used to contain the test sample. Testing should include the test sample holder. Corrections factors may be developed as needed.

## 4. ONGOING PERFORMANCE TESTS OF INSTRUMENTS

Performance and operational tests are completed periodically to ensure the instrument is capable of meeting specified criteria for accurate assays and that no changes have occurred since initial testing. Tests include physical inspection, measurement of system parameters, and operational tests with radioactive calibration sources. These tests should also be completed after repair. Regulatory agencies may require specific tests at a regular frequency. The following sections describe the performance tests using a dose calibrator as an example. Ongoing performance tests for other instruments used for radiation measurements should be designed as appropriate to each instrument, testing the parameters that are pertinent to ensuring the performance of the instrument, considering its application and the level of precision of measurement required. The same principles will apply to other instruments used for radiation measurements.

### 4.1 Physical Inspections

Ensure that the dose calibrator and associated accessories, such as source holders and well liners, are undamaged and that foreign objects are not present in the chamber. Prior to use, repair or replace any damaged components as required and remove all foreign objects.

### 4.2 System Parameters

The system electronics should be tested according to the manufacturer’s diagnostic testing procedures. The results should meet the acceptance criteria detailed in the manufacturer’s manual. These parameters typically include the high voltage on the chamber, the zero adjustment on the electrometer, verification of communication between components of the system, and the accuracy of the system clock.

If the manufacturer’s diagnostic procedures are not available, then at a minimum the user should check, record, and trend the chamber high voltage and the measured value for the electrometer zero check. Small changes in the high voltage will identify system malfunctions that might not be apparent with the use of radioactive calibration sources, but will begin impacting higher activity readings. The system clock should be checked and should be accurate within one minute.

### 4.3 Operational Tests

#### BACKGROUND AND CONTAMINATION TEST

The purpose of this test is to determine the background radiation level and to ensure the absence of contamination that may affect measurements on the dose calibrator. Background radiation may fluctuate due to movement of sources near the chamber,

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as well as by contamination in or near the chamber, the liner, or the source holder. For this reason, this test should be performed with the liner and the source holder in the chamber. All radioactive sources should be well shielded or at a sufficient distance from the chamber. The background radiation level should be determined and recorded at initial installation, and periodically verified. The background should be recorded at least once on each day of use, or more frequently if necessary, using the most common radionuclide setting. It is especially important to verify if the minimum assayed quantity of radioactivity decreases.

## CONSTANCY TEST

The purpose of this test is to ensure the constancy of the dose calibrator over time. This test should be performed on each day of use, and completed after the system tests outlined above. Typical radioactive calibration sources used in this test are Cesium–137 or Cobalt–60 contained within a solid matrix. Cobalt–57 might not be suitable because of its nominal half-life of less than one year. The radioactive calibration source is not required to have the same geometry as routine test samples. A single radioactive calibration source is sufficient for this check. The source is placed in the source holder and the reading recorded for the dedicated radionuclide setting, as well as for all other commonly used radionuclide settings. This ensures no drift in response for any setting. Results should be recorded and be within the appropriate range of the decay corrected value recorded during IQ. Control charts or other data representations should be used to identify and document trends.

## ACCURACY TEST

The purpose of this test is to demonstrate the stability of the dose calibrator over the energy range used. This test should be performed annually or after the dose calibrator is repaired or moved. If the dose calibrator is used to measure the quantity of multiple radionuclides, two or three different radioactive calibration sources with different energies should be included to cover the expected energy range of the radionuclides. These calibration sources are typically contained in a solid matrix and do not necessarily match the geometry of routine test samples. If the dose calibrator is only used for a single radionuclide, or only for positron emission tomography (PET) radionuclides, a single calibration source may be adequate. The quantity of radioactivity in the calibration source should be greater than 100 µCi ($3.7 \times 10^6$ Bq). Sources used for this test should be traceable to a National Metrology Institute (NMI). Measurements of the calibration source activity are recorded and the average measured value is compared to the expected value. The measured value should be within the appropriate range of the expected value.

## REPRODUCIBILITY TEST

The purpose of this test is to measure the precision of the dose calibrator and should be performed at least annually. The radioactive calibration source should have a half-life such that decay corrections are not required over the period of the test. The quantity of radioactivity in the calibration source should be greater than 100 µCi ($3.7 \times 10^6$ Bq). An appropriate number of consecutive readings are recorded using the same geometry. Each of the measurements taken should be within an appropriate range of the mean of all the measurements.

## LINEARITY TEST

The purpose of this test is to show that the response of the dose calibrator is linear across the range of radioactivity levels to be measured. This is especially critical at high levels of radioactivity where the measured radioactivity may differ from the true radioactivity due to saturation effects, and at low levels of radioactivity due to variations on the background radiation and changes in the source positioning. This test should be performed annually or after the dose calibrator has been repaired or moved.

The test is considered successful if the ratio of the measured radioactivity to the expected activity is within the appropriate range of the expected values. Three techniques may be employed for this test: decay, graded shield, and graded source. Use of graded shields is acceptable once an initial linearity test using the decay method is successfully completed. Refer to the manufacturer's recommendations on the use of the graded shields. The graded source method requires accurate measurements of volume or mass, and is not generally recommended.

*Table 1* is an example of ongoing performance checks for dose calibrators.

**Table 1. List of Performance Tests**

| Parameter | Daily[a] | Annually (at a minimum) |
|---|---|---|
| Physical inspection | Yes | N/A |
| System electronics | Yes | N/A |
| Clock accuracy | Yes | N/A |
| High voltage | Yes | N/A |
| Zero adjust | Yes | N/A |
| Background response/contamination check | Yes | N/A |
| Radioactive calibration source (constancy and relative response) | Yes | N/A |
| Accuracy | See footnote[b] | Yes |
| Reproducibility (precision) | See footnote[b] | Yes |
| System linearity | See footnote[b] | Yes |

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[a] At the beginning of each day of use.
[b] This is required after repair.

# 5. IDENTIFICATION OF RADIONUCLIDES

## 5.1 Half-Life Determination

The half-life is an identifying characteristic of a radionuclide. The half-life is determined by measuring the quantity of radioactivity in the test sample as a function of time. The approximate half-life may be used to confirm the radionuclidic identity. Perform the measurements using an appropriately calibrated instrument, such as a quantitative radioactivity detector, provided the quantity of radioactivity is within the linear range of the instrument throughout the measurements and the geometry of the test sample is not changed during the measurements.

The test sample may be used directly, or diluted and/or dried in a capsule as needed. The test sample is prepared in a manner that avoids losses during handling. If the test sample is a liquid or solution, the sample is held in a closed container. If the test sample is a residue from drying in a capsule, the sample is protected by a cover consisting of a sheet of adhesive cellulose acetate or other material. The quantity of radioactivity in the test sample should be sufficient to allow measurements over the time frame of the test. If necessary, correction for dead-time losses may be applied. The measured half-life should be within the range of the expected half-life as defined in each particular application of this test.

## 5.2 Gamma-Ray Spectrometry

The gamma-ray emission spectrum may be used to identify and quantify gamma-emitting radionuclides. The detector should be calibrated with a radioactive calibration source traceable to a NMI. The calibration source used to determine the efficiency of the detector should have a sufficient quantity of radioactivity to produce an adequate number of counts for each photopeak used in the calibration. The calibration source and the test sample should be measured with the same geometry and distance from the detector. It is critical that the geometry, including vial type and volume, be the same because these factors affect the amount of incident radiation on the detector. Photopeaks in the test sample should be comparable to those in the calibration source, both in terms of energy and intensity. The presence of unknown peaks or changes in peak abundance is indicative of impurities. Ensure that background radiation levels remain constant during the measurement, as changes may result in unknown peaks. Counting time or shielding should be optimized to achieve the necessary required minimum detectable activity to meet test specifications. It may be necessary to allow the radioactivity in the test sample to decay before it is possible to detect impurities at the required levels. There may also be short-lived impurities present, so an analysis should be completed to identify impurities and their half-lives to ensure the sample is assayed prior to their decay. Positron-emitting radionuclides typically cannot be differentiated because their emitted energy (511 keV) is the same for each radionuclide; thus, gamma-ray spectrometry is not a recommended prerelease test for radionuclidic identity for PET radionuclides. Some positron-emitting radionuclides have characteristic gamma-ray emissions in addition to 511 keV, which may be used for purposes of identification (e.g., Ge-69 and Na-22). Performance tests of the gamma-spectroscopy system should be completed on each day of use to ensure the functionality of the system.

## 5.3 Beta-Emitter Analysis and Identification

The purpose of the beta-emitter analysis and identification is to determine the maximum energy of the beta particle, which is unique and can be used for identification. Beta-emitting radionuclides can be identified either by evaluation of their spectrum or by measuring their mass attenuation coefficients in a series of absorption foils and constructing an attenuation curve. Performance tests of the beta counting system should be completed on each day of use to ensure the functionality of the system.

## 5.4 Alpha Spectrometry

The alpha emission spectrum may be used to identify and quantify alpha-emitting radionuclides. Pure alpha emitters present unique challenges due to their low penetration distance and their toxicity, especially if they are volatile compounds that could create airborne radioactivity and therefore internal radiation exposure to the experimenter or operator. Alpha particles can be detected either by the use of proportional counters, scintillation detection using a silver-activated zinc sulfide phosphor, or by the techniques of liquid scintillation counting (LSC). Performance tests of the alpha spectroscopy system should be completed on each day of use to ensure the functionality of the system.

# 6. ASSAY OF RADIONUCLIDES

## 6.1 Measurement of Radioactivity Using a Dose Calibrator

Using a holder, the test sample is placed in the chamber of the dose calibrator at a given position. Once the response is stable, the radioactivity reading is taken. Performing measurements on the test sample with the same geometry as the calibration source provides the most accurate results; however, geometry correction factors may be developed and used as necessary. Refer to the manufacturer's recommendations for the maximum radioactivity that can be determined in the dose calibrator.

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## 6.2 Measurement of Radioactivity Using a Solid-State Detector

The test sample is positioned in front of the detector or into the well of a well-type detector. The detector must be adequately shielded and calibrated using a radioactive calibration source traceable to an NMI.

## 6.3 Measurement of Radioactivity Using Chromatographic Systems

A scintillation detector can be used for dynamic radioactivity measurements (e.g., the eluate of a liquid chromatograph is directed over or through a detector). The quantity of radioactivity in the test sample should provide a count rate that is within the linear range of the detector.

## 6.4 Measurement of Radioactivity Using a Liquid Scintillation Counter

LSC is the standard laboratory method to quantify radioactivity of particle-emitting radionuclides, mostly beta- and alpha-emitting radionuclides. The sample to be analyzed may require a liquid scintillation fluid (cocktail) depending on the energy of the beta particle. The LSC detection method uses liquid scintillation cocktails to transform emitted radiation into detectable light photons. The sample to be analyzed is placed into a liquid scintillation vial and the cocktail is added in the required amount. While a sample that can be dissolved into the fluid is preferred, other samples can be assayed if the impact on the light output is quantified. The detector should be calibrated using an appropriate radioactive calibration source traceable to an NMI. The absorption of scintillation photons is called quench, and it is necessary to develop a "quench curve" during PQ and additional quench curves may be necessary for different types of samples to correct the counting efficiency. Performance tests of the LSC should be completed on each day of use to ensure the functionality of the system.

## GLOSSARY

The following definitions apply to commonly encountered words and phrases when dealing with radioactivity.

**Alpha particles** ($\alpha$)**:** Positively charged particles emitted from nuclei during radioactive decay. Alpha particles are Helium-4 nuclei, consisting of two protons and two neutrons, but no electrons.

**Beta particles ($\beta^-$):** Negatively charged particles that are emitted from nuclei during radioactive decay. Beta particles are electrons.

**Calibration factor:** The coefficient used to convert the measured ionization chamber current to a nominal radioactivity. This term is often referred to as the "calibration coefficient".

**Calibration time:** Arbitrary time and date, if appropriate at which the specified amount of radioactivity is present.

**Counting assembly:** Consists of a sensing unit and an electronic scaling device. The sensing unit may be a Geiger-Müller tube, a proportional counter, a scintillation detector in which a photomultiplier tube is employed in conjunction with a scintillator, or a solid-state semiconductor.

**Dose calibrator:** A well-type ionization chamber commonly used to assay radiopharmaceuticals. Display units are typically in curies (μCi/mCi/Ci) or becquerels (kBq/MBq/GBq).

**Gamma rays** ($\gamma$-rays)**:** Electromagnetic radiation emitted from nuclei during radioactive decay. Gamma rays have a wide range of energies. The gamma rays emitted from a given radionuclide are always at the same energy(ies) providing a unique signature that enables the identification of a gamma-emitting radionuclide.

**Geiger-Müller counter:** Often referred to as "G-M counter" or "Geiger counter". An instrument in which a high voltage potential is applied across a volume of inert gas for the purpose of collecting ions produced by a radiation field. The negative electrons are internally multiplied to produce a readily detectable current pulse. Display units are typically counts per minute (cpm) or milliroentgen per hour (mR/hr).

**Geometry:** The characteristics of a radioactive source (i.e., container type, container wall thickness, volume, and position of the container in the well chamber) that, along with the physical characteristics of the ionization chamber, affect the magnitude of the calibration coefficient for a specific radionuclide. The principal geometry considerations that may affect the accuracy of a source measurement in a dose calibrator are container configuration, source volume, position of the source in the chamber well, and the radionuclide itself.

[NOTE—It is customary to compare a standardized preparation and radiopharmaceutical drug or preparation using identical geometry conditions for assay, identification, and other parameters. The validity of the result is critically dependent upon the reproducibility of the spatial relationships of the source to the detector and its surroundings and upon the accuracy of the standardized preparation.]

**Ionization chamber:** An instrument in which an electric field is applied across a volume of inert gas for the purpose of collecting ions produced by a radiation field. The positive ions and negative electrons drift along the lines of force of the electric field, and are collected on electrodes, producing an ionization current. The most commonly used form of ionization chambers for measurement of the activities of radiopharmaceuticals is a well-type ionization chamber known as a dose calibrator.

**Isobars:** Nuclides with the same mass number (protons + neutrons).

**Isomers:** Atoms with the same number of protons and neutrons, but a different nuclear energy configuration. Short-lived radioactive isomers are generally referred to as metastable. Different isomers are different nuclides based on their nuclear energy configurations.

**Isotones:** Nuclides with the same number of neutrons and a different number of protons. Isotones are different elements with different atomic masses.

**Isotopes:** Nuclides with the same number of protons and a different number of neutrons. Isotopes are the same element with a different atomic mass.

**Isotopic carrier** (also referred to as carrier)**:** A stable isotope of the element concerned either present in or added to the radioactive preparation in the same chemical form as the radionuclide.

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**Liquid scintillation counter (LSC):** An instrument which detects scintillation light from the absorption of radiation energy in a scintillation liquid. This instrument is used primarily for beta-emitting radionuclides that do not also emit gamma photons. For best results, the radioactive sample should be soluble in the scintillation liquid.

**Minimum detectable activity:** Smallest quantity of radioactivity that can be detected above the background with a specified level of confidence.

**National Metrology Institute** (NMI)**:** A measurement standards body, which is a laboratory of metrology that establishes standards for a country or organization. For example, National Institute of Standards and Technology (NIST) is the NMI for the United States.

**Nuclide:** An atom with a specific number of protons and neutrons in a given nuclear energy state.

**Positrons** ($\beta^+$)**:** Positively charged particles emitted from a nucleus during radioactive decay. Positrons are anti-electrons.

**Radioactivity:** (1) The spontaneous transformation of nuclei characterized by the emission of particles or photons. Radioactivity is typically described as atoms undergoing radioactive decay per unit time (or disintegrations per unit time). (2) The quantity of radioactive material, as measured in units of curies (U.S. units) or becquerels (SI units). The quantity of radioactive material may also be referred to as activity.

**Radiochemical identity:** The molecular structure of the intended active radiopharmaceutical ingredient, which is present in the radiopharmaceutical preparation.

**Radiochemical purity:** The ratio, expressed as a percentage, of the radioactivity of the intended active radiopharmaceutical ingredient to the total radioactivity of all radioactive ingredients and impurities present in the radiopharmaceutical preparation.

**Radioisotope:** A radioactive atom, generally used in the context of an isotope of an element.

**Radionuclide:** An unstable nuclide that undergoes radioactive decay; a radioactive nucleus. The terms radionuclide and radioisotope are commonly used interchangeably.

**Radionuclidic identity:** The intended radionuclide in the radiopharmaceutical preparation.

**Radionuclidic purity:** The ratio, expressed as a percentage, of the radioactivity of the intended radionuclide to the total radioactivity of all radionuclides in the radiopharmaceutical preparation.

**Radiopharmaceutical** (radiopharmaceutical preparation/radioactive drug)**:** A finished dosage form that contains a radioactive substance in association with one or more other ingredients and that is intended to diagnose, stage a disease, monitor treatment, or provide therapy. A radiopharmaceutical includes any nonradioactive reagent kit or radionuclide generator that is intended to be used in the preparation of any such substance. The terms radiopharmaceutical and radioactive drug are commonly used interchangeably.

**Scintillation crystal counter:** An instrument consisting of a crystal scintillator, such as NaI (Tl), with attached photomultiplier tube and associated electronics. Scintillation light produced from the absorption of gamma and X-rays in the crystal is converted to electrons and amplified in the photomultiplier tube. The resultant current pulse may be further analyzed with regard to photon energy. A commonly used form of this instrument that has a hole in the crystal of sufficient size to allow placement of a test tube or similar container is known as a "well counter".

**Semiconductor detector:** An instrument consisting of a semiconductor material, such as silicon or germanium crystals, that detects ionizing radiation through generation of charge carriers (passage of electrons through holes). The current pulse produced by migration of these charge carriers, under the influence of a voltage potential across the material, can be further amplified and analyzed to determine the quantity and energy of the incident radiation.

**Solid-state detector:** A crystal-based detector, in contrast to a gas-based detector. Often is used as a synonym for a semiconductor detector.

**Strength:** The radioactivity concentration of the radiopharmaceutical at the calibration time. The unit of strength is the amount of radioactivity on a volume basis (e.g., mCi/mL or MBq/mL).

**Total radioactivity:** The radioactivity of the radionuclide, expressed per unit (e.g., vial, capsule, ampoule, generator, and others) at the calibration time.

**Validation:** Establishment of documented evidence that a method, process, or system meets its intended requirements.

**Verification:** Confirmation that an established method, process, or system meets predetermined acceptance criteria.

**X-rays:** A type of electromagnetic radiation emitted from the electron orbitals. While they do not arise from the nucleus they are often present immediately following a decay event if there are interactions between the emitted radiation and the orbital electrons.

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# ⟨823⟩ POSITRON EMISSION TOMOGRAPHY DRUGS FOR COMPOUNDING, INVESTIGATIONAL, AND RESEARCH USES

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## 1. INTRODUCTION

Radionuclides used in positron emission tomography (PET) typically possess short physical half-lives, $T_{1/2}$ (e.g., $T_{1/2}$ of $^{15}O$ = 2.03 min, $^{62}Cu$ = 9.67 min, $^{13}N$ = 9.96 min, $^{11}C$ = 20.4 min, $^{68}Ga$ = 67.7 min, $^{18}F$ = 109.8 min, $^{64}Cu$ = 12.7 h). As a result, these radionuclides usually are produced using particle acceleration techniques (e.g., cyclotrons) or from generators, and then are processed into the final PET drug product in close proximity to the site where the PET procedure will be conducted.

The short half-lives of PET radionuclides create unique constraints for the preparation and testing of PET drug products. This chapter describes guidelines for making and testing PET drug products based on the following constraints:

- It is not possible to complete all testing before the use of PET drug products.
- An entire batch or sub-batch of a PET drug product may be contained in a single vial. Samples withdrawn for quality control (QC) testing are representative of the entire batch or sub-batch.
- An entire batch or sub-batch may be administered to a single patient.
- The mass of the PET drug in a PET drug product usually ranges from nanogram to microgram quantities.
- PET drug products do not enter a traditional drug distribution chain. Instead, PET drug products are used in-house or are delivered to the point of use by dedicated couriers.
- Small-scale facilities for the preparation of PET drug products have limited personnel and resources, which require the following:
    - Allowance for multiple operations in one area with adequate controls;
    - Allowance for the making and testing of multiple PET drug products using shared equipment;
    - Appropriate requirements for aseptic operations;
    - Appropriate requirements for system suitability and other day-of-use activities;
    - QC requirements for components, materials, and supplies;
    - Self-verification of significant steps in radionuclide production, PET drug production, or compounding and testing; and
    - Single-person oversight of production and compounding, review of batch records, and release authorization.

The scope of this chapter includes the production and compounding of PET drug products for human administration as used (a) according to state-regulated practice of medicine and pharmacy, (b) according to an approved investigational new drug (IND) application (see 21 CFR 312), and (c) according to research uses under the supervision of a Radioactive Drug Research Committee (RDRC; see 21 CFR 361). The scope of this chapter does not include dispensing activities as defined in other USP general chapters.

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## 2. PERSONNEL

Sufficient numbers of personnel with the appropriate education, training, and experience are needed for the preparation and testing of PET drug products. The number depends on the size and complexity of the operations executed at each facility.

### 2.1 Training Requirements

Personnel should be trained before they begin to make and test PET drug products. Training can be performed by various methods, including live instruction, audio-video instruction, and study of publications. Training should address but is not limited to radionuclide production techniques, synthetic and purification methods, materials, components, reagents, stock solutions, automated and manual apparatus used to make PET drug products, and QC methods, including equipment, software, and documentation. Training must be documented.

### 2.2 Aseptic Operations Training

Training should address aseptic manipulations as well as the techniques and equipment used to achieve and maintain International Organization for Standardization (ISO) Class 5 environmental conditions. Training also should address all aseptic operations, including the assembly of sterile components, compounding, and filtration. Manipulations of sterile solutions should be performed by operators who are qualified to use aseptic techniques (see *Facilities and Equipment* below).

Personnel involved in aseptic operations should be evaluated periodically by aseptic simulations in which a microbiological growth medium is used to assess the quality of the aseptic operation. Aseptic simulations should provide the following:

- Include all manipulations required for the aseptic assembly of the PET drug product vial assembly (e.g., vial, filter, and syringe assembly, etc.).
- Represent worst-case scenarios for aseptic operations.
- Be performed in triplicate to qualify a new operator. Each operator should be requalified annually by conducting at least one media fill.
- Be performed any time procedures are changed significantly.

After the simulation process, the media should show the absence of contamination after incubation at a suitable temperature for 14 days. An operator who fails written assessments or whose aseptic simulations result in microbial growth should be immediately re-instructed and re-evaluated to ensure correction of aseptic practice deficiencies.

## 3. QUALITY ASSURANCE

QA is a broad concept that covers all matters that influence identity, strength, quality, and purity of a PET drug product. QC is a subset of QA that deals with testing of materials and PET drug products to determine if they meet acceptance criteria. The QA function typically consists of oversight activities, and the QC function consists of execution activities.

QC functions include the following.

- Evaluate each lot of incoming material to ensure that it meets its established specifications before use in the preparation or testing of PET drug products.
- Evaluate each batch of a PET drug product to ensure the batch meets its established specifications before authorizing the final release of the batch.

The oversight functions associated with QA include the following:

- Review completed batch records for accuracy and completeness.
- Approve procedures, specifications, processes, and methods.
- Ensure that personnel are properly trained and qualified, as appropriate.
- Ensure that PET drug products have adequately defined identity, strength, quality, and purity.
- Ensure that changes to component quality, suppliers, changes to production procedures, and changes to testing procedures and specifications are appropriate and implemented properly.
- Investigate errors and ensure that appropriate corrective and preventive actions are taken to prevent their recurrence.
- Handle complaints.
- Ensure that the PET drug products are produced, tested, labeled, released, and distributed according to the facility's established procedures and practices for PET drug products.
- Conduct periodic audits to monitor compliance with established procedures and practices for PET drug products.

Personnel at the facility may perform both QA and QC functions.

## 4. FACILITIES AND EQUIPMENT

Facilities should be adequate for the production, compounding, and testing of PET drug products. Work areas should be organized to prevent cross-contamination, mix-ups, and errors, especially in areas used for making multiple PET drug products. Work areas should be periodically cleaned to prevent the contamination of equipment, materials, components, or PET drug products by personnel or environmental conditions that could reasonably be expected to adversely affect PET drug product quality. These requirements should be described in written procedures, and their routine execution should be documented.

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## 4.1 Environmental Controls for Parenteral PET Drug Products

Because the sterility test results for parenteral PET drug products are obtained after release, facilities and equipment should ensure a sterile PET drug product.

### ASEPTIC WORKSTATION

The primary environmental control for aseptic operations is a high-efficiency particulate air (HEPA) filter that is capable of producing air with a cleanliness rating of ISO Class 5. This can be achieved with a laminar airflow workstation, aseptic isolator, biological safety cabinet, or other suitable device (generically, aseptic workstations). The aseptic workstation should be protected from sources of microbial contamination and should be located in an area where personnel traffic is limited. The area around the aseptic workstation should not be used for storage of materials that shed large quantities of particulate matter (e.g., corrugated boxes).

The proper operation of the aseptic workstation must be certified by measurement of airborne particles, HEPA filter integrity testing, pressure differential testing, or other means. The specific tests depend on the type of aseptic workstation. Certification should be performed at the inception of operation and at least annually thereafter or after repair or replacement of the HEPA filter. These requirements supersede those in other USP general information chapters (e.g., *Microbiological Control and Monitoring of Aseptic Processing Environments* ⟨1116⟩).

The work area inside the aseptic workstation should be clean. The internal surfaces should allow easy cleaning and disinfection. The internal surfaces should be cleaned and disinfected with appropriate disinfectants that are sterile filtered or certified sterile with a manufacturer's certification.

### MICROBIOLOGICAL TESTING

Microbiological testing of the environment should be performed to assess air quality and surface disinfection of the aseptic areas. This can be achieved by either settling plates or active air-sampling plates. Surface disinfection of critical surfaces (e.g., the work surface of the aseptic workstation or operators' fingers) should be assessed with swab or contact plates. For microbiological testing of the aseptic workstation, the air should be tested as part of the workstation qualification (e.g., every six months) and the surface (using swab or contact plates) should be assessed after use, each day of use. Nonviable particle counts may be determined less frequently following certification of the *Aseptic workstation* (see above).

Alert and action limits should be established for samples obtained during microbiological testing. Typical alert levels are set at less than three colony-forming units (cfu) per plate. More than three cfu require corrective actions that may include operator retraining, recertification of the aseptic workstation, or other actions. The results of microbiological testing also should be used in the investigation of positive sterility tests.

## 4.2 Equipment

Equipment used to make and test PET drug products should be appropriate for its intended purpose and should be installed, cleaned, and maintained in an appropriate manner. Equipment should be capable of producing consistent results.

The following requirements should be described in written procedures, and performance of these procedures should be documented.

1. *Installation of New Equipment*—Newly installed equipment should be qualified before it is used to make or test PET drug products at an appropriate level of detail based on complexity. All qualification activities should be properly documented, including the date and the name of the person who performed the qualification. For more complex equipment, qualification consists of three phases:
    - Installation Qualification (IQ)—IQ is a check of items required for proper installation of the equipment, including physical location, required utilities and supplies, communications, and environmental conditions. IQ should describe the installation procedure for the equipment.
    - Operational Qualification (OQ)—OQ is a check of operational specifications for the equipment, including equipment set-up, functional testing of subsystems, and proper overall operation. OQ should describe operational procedures for the equipment.
    - Performance Qualification (PQ)—PQ demonstrates that the equipment is capable of performing tasks required to make and test PET drug products in the operating environment and that the equipment provides the intended results. PQ should describe the required performance tasks for the equipment.
2. *Calibration of Equipment*—Analytical equipment calibration should be performed before use, as appropriate. A schedule should be developed for recalibration and should have a sufficient frequency to ensure accurate results. Calibration activities should be properly documented, including the date and the name of the person who performed the calibration.
3. *Preventive Maintenance of Equipment*—A preventive maintenance schedule should be developed for major production and testing equipment, including automated chemistry modules, gas chromatographs, high-performance liquid chromatographs, and others. The schedule should have a sufficient frequency to minimize equipment downtime. Major repairs may require recalibration and requalification. Preventative maintenance activities should be properly documented, including the date of such performance and the name of the person who performed them.

## 4.3 Cleaning Equipment and Components

Equipment used in production or compounding of PET drug products includes automated, computer-controlled devices, as well as manually operated apparatus. Before it is used in making PET drug products, equipment should be properly cleaned to

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ensure that the resulting PET drug product meets established specifications for identity, strength, quality, and purity (see *Controls and Acceptance Criteria for Finished PET Drug Products* below). Once cleaned, equipment should be maintained in a state of cleanliness before use.

Equipment may be used to make multiple batches of one or more PET drug products. Documented studies should demonstrate the effectiveness of the cleaning process between batches. All impurities should be controlled at levels that conform to established specifications for identity, strength, quality, and purity. Written procedures for line clearance between batches of different PET drug products should describe routine execution of cleaning processes.

### 4.4 Day-of-Use Checks

Day-of-use checks are necessary for processing equipment to ensure proper function. Written procedures for the day-of-use checks should be established and followed. These procedures should be designed to check key parameters at the beginning of each operational cycle (e.g., temperature, pressure integrity, gas supply, vacuum supply, proper delivery line selection, reagent delivery volumes, gas flow rates, radiation monitors, and other process sensors). Some parameters may be periodically checked as part of the calibration and preventive maintenance schedules as described above.

### 4.5 System Suitability for QC Equipment

System suitability tests are necessary for QC equipment to ensure that the equipment, components, and personnel (i.e., the system) function as a whole to execute the desired analytical method. System suitability tests should be performed prior to using the equipment according to established procedures. Written procedures should be established and followed for system suitability tests, and the test results should be documented.

The system suitability tests required for chromatographic methods include tailing factor, replicate injections, and resolution. When the test chromatogram used for system suitability contains only a single peak, then tailing factor, replicate injections, and column efficiency (theoretical plates) are adequate. The use of internal or external standards with a known concentration is necessary for these determinations. Standards should be prepared from well-characterized materials or from materials that have a manufacturer's certification. Two acceptable approaches that may be used for chromatographic methods are the following:

1. Create a calibration curve from a range of standards with known concentrations. The concentrations of the standards should bracket the conditions of use for the chromatographic method. The calibration curve should be used over a suitably specified period of time (e.g., six months), after which time a new one should be created. A new calibration curve should be created each time an alteration is made to the chromatographic system. Routine system suitability for replicate injections consists of a single injection of a known standard and a measurement of the concentration based on the calibration curve. If the measured concentration agrees with the known concentration within a predefined range (e.g., 10% for manual injections and 5% for automated injections), this demonstrates the suitability of the system for replicate injections and ensures that the calibration curve is appropriate for use in subsequent sample injections. The tailing factor and resolution (or column efficiency, as appropriate) should be determined from the same chromatogram.
2. At the beginning of each testing cycle, create a single-point calibration from two injections of a known standard. The measured area of the peaks for these injections should agree within a predefined range (e.g., 10% for manual injections and 5% for automated injections). Then the results are averaged and used with the standard concentration to provide a calibration factor that is used in subsequent sample injections for that day. The tailing factor and resolution (or column efficiency as appropriate) should be determined from one of the two chromatograms.

Other chromatographic parameters such as signal-to-noise ratio, limit of detection, and limit of quantitation can be determined as part of routine system suitability testing.

System suitability tests also may be appropriate for other QC equipment, including dose calibrators, scanners for radio–thin layer chromatography (radio–TLC), and multichannel analyzers. When used, these tests should be performed at installation, relocation, and appropriate intervals thereafter. These tests should use known standards to demonstrate the proper function of the equipment, for example:

1. *Dose Calibrator*—Accuracy, geometry, and linearity should be assessed at installation and at appropriate intervals thereafter. The instrument should be calibrated in accordance with nationally recognized standards or the manufacturer's instructions. Routine system suitability testing should include a constancy check with a suitable high-energy radionuclide source.
2. *Radio–TLC Scanner*—Uniformity, positional accuracy, detector linearity, and resolution should be assessed with a suitable radionuclide source. Routine system suitability testing should include checks for these parameters.
3. *Multichannel Analyzer*—Sensitivity and resolution should be assessed at installation and at appropriate intervals thereafter. Routine system suitability testing should include a constancy check with a suitable high-energy radionuclide source.

## 5. CONTROL OF COMPONENTS, MATERIALS, AND SUPPLIES

Components, materials, and supplies that are used in the preparation of PET drug products should be controlled to avoid contamination, mix-ups, and errors. A designated person should be responsible for ensuring that these activities are carried out and completed properly. Records of completed examinations and tests for components, materials, and supplies should be maintained for one year after their expiration or for one year after batch release, whichever is longer. The following activities should be established and performed:

1. Establish written specifications for the identity, strength, quality, and purity of ingredients, reagents, target materials, and gases.

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2. Establish written specifications for the identity and quality of sterile empty vials, transfer lines, sterile stopcocks, sterile needles, sterile membrane filters, and other components used in the PET drug product vial assembly.
3. Establish written specifications for the identity, strength, quality, and purity of analytical supplies (e.g., solvents, chromatography columns, and authentic standards), sterility test media, and endotoxin test reagents used in the testing of PET drug products.
4. Establish appropriate storage conditions (based on heat, light, humidity, and other factors) for components, materials, and supplies used to make and test PET drug products.
5. Store components, materials, and supplies in a controlled-access area according to established storage conditions. Segregate components, materials, and supplies as appropriate to avoid mix-ups and errors.
6. Log each lot of shipment of components, materials, and supplies, and record the date of receipt, quantity received, manufacturer, manufacturer's lot number, and expiration date. If no expiration date is designated by the manufacturer, assign one based on knowledge of its physical and chemical properties and previous experience with its use. For organic substrates and reagents that are potentially susceptible to degradation or to a change in composition, the expiration date should be based on the material's stability.
7. Determine that each lot of components, materials, and supplies complies with established written specifications. Compliance with specifications can be demonstrated by inspection of the labeling or inspection of the manufacturer's certification. The identity of each lot of components, materials, and supplies should be verified by defined procedures, tests, or documented manufacturer's certification, as appropriate. Perform an identity test for precursors (e.g., melting point determination or other appropriate tests). Alternatively, the manufacturer's certification can be used as the only acceptance criterion for a precursor if final testing of the PET drug product ensures that the correct precursor has been used. Reference standards used in chromatographic procedures should have suitable documentation of identity and purity. Other components can be accepted on the basis of a manufacturer's certification only.
8. Membrane filters used with parenteral PET drug products should have a manufacturer's certification. Examine the manufacturer's certification for each lot to ensure compliance with written specifications.
9. Media used in the sterility testing of PET drug products may be obtained from commercial sources. If the media is obtained from commercial sources, then growth-promotion testing that uses a suitable single species of organism should be performed on initial qualification of the supplier and periodically (e.g., quarterly) thereafter.

## 6. PROCESS AND OPERATIONAL CONTROLS

### 6.1 Process Controls

The following process controls should be established and summarized in a master formula for the PET drug product. A designated person should be responsible for ensuring that these activities are carried out and completed properly.

1. Written acceptance criteria for the identity, strength, quality, and purity of each PET drug product should be established. For PET drug products intended for parenteral administration, specifications should include sterility and bacterial endotoxins. If a *USP* monograph exists or if there are specifications that have been previously accepted by the appropriate regulatory agency (e.g., FDA), then these standards, if applicable, may be applied as the minimum acceptance criteria.
2. Written procedures for the preparation of each PET drug product should provide the following:
    - Incorporate, for each PET drug product intended for parenteral administration, sterile membrane filtration (0.22 µm) or steam sterilization;
    - Incorporate, for each PET drug product intended for inhalation, particulate filtration (0.45 µm);
    - Describe routine cleaning procedures for equipment and facilities;
    - Describe components, materials, and supplies used to make PET drug products, including precursors, standards, reagents, stock solutions, and related items;
    - Describe the process and the steps used to make the PET drug product;
    - Describe the formulation process, including the use of stabilizers, buffers, and other agents;
    - Describe calculations performed for quantitative parameters associated with making and QC testing the PET drug product (e.g., including radiochemical yield, radiochemical purity, specific activity, solvent amounts, etc.);
    - Describe QC tests for the final PET drug product (see *Controls and Acceptance Criteria for Finished PET Drug Products* below), including a schedule that defines whether or not each test should be performed on each batch and that states if the test results should be complete at the time of release.
3. The quality of each batch of a PET drug product should be verified by full finished product testing prior to use to ensure the product meets all specifications.
4. In cases where testing as described in the previous paragraph is not possible or impractical, the quality of a PET drug product may also be ensured by documented validation studies in lieu of prerelease tests. Such studies should provide the following:
    - Demonstrate a consistent process that is suitable for the intended preparation of the PET drug product;
    - Be completed on three batches made according to the master formula, and all three batches should meet all acceptance criteria;
    - Include evaluation of radiochemical identity and purity, radionuclidic identity and purity, specific activity, sterility (for parenteral PET drug products), bacterial endotoxins (for parenteral PET drug products), pH, appearance, stereochemical purity (for applicable compounds), residual solvents, other toxic chemicals that may have been used during the synthesis or purification procedure, effective concentration of a stabilizer (if any), chemical purity of the PET drug product, and equivalence of initial and final sub-batches (see *Definitions* above);

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- Be repeated if the process and steps described in the master formula have been altered in a way that could change the identity, strength, quality, or purity of the PET drug product;

5. The processes and steps described in the master formula should be updated as needed and should be reviewed annually to ensure they are current. Prior to the implementation of updates, appropriate validation and/or verification should be approved and performed.

Appropriate controls of computer-controlled equipment should ensure that process changes are instituted only by authorized personnel and that such changes are documented and verified. Production, compounding, and test methods should be backed up and controlled to avoid accidental use of outdated methods. In the case of processes or test methods from a vendor that are used without alteration, it is acceptable to rely on vendor certification for software verification and proper operation.

## 6.2 Operational Controls

The following operational controls should be established and summarized in a batch record that is a subset of the master formula for the PET drug product. The batch record should adequately document the routine process for making the PET drug product. A designated person should be responsible for ensuring that these activities are carried out and completed properly. Completed batch records and associated documentation should be maintained for one year after batch release.

1. Execute suitable line clearance procedures to avoid mix-ups and cross-contamination, including the inspection of areas used to make and test PET drug products, and the inspection of all equipment for cleanliness and suitability before use. Remove extraneous materials and labels from these areas and equipment.
2. Ensure the correct identity, strength, quality, and purity of components, materials, and supplies used in the preparation of the PET drug product. Label components as appropriate for identity and traceability purposes.
3. Execute routine cleaning procedures for equipment and facilities.
4. Prepare the PET drug product according to the current master formula, and for each batch maintain a batch record. Batch records may consist of paper documents, electronic records, or combinations thereof. Spreadsheets and other electronic recordkeeping tools should be verified to ensure traceability, data integrity, accuracy of results for calculations, and so on. The batch record should include the following:
    - Lot numbers or other unique identifiers for all components, materials, and supplies used to make the PET drug product;
    - A description of the individual procedures that were followed;
    - The initials, signature, or other identifier of the responsible individual indicating that critical steps and processes used to make and test the PET drug product were completed;
    - The percent yield calculated on the basis of the known or expected amount of the starting radionuclide that is synthetically incorporated into the PET drug product;
    - Raw analytical data on each batch of the PET drug product;
    - Labeling for the PET drug product (see *Labeling* below);
    - Calculations for key parameters defined in the master formula;
    - Results obtained from QC tests of the PET drug product, including chromatograms, print-outs, and other test data;
    - The initials of the analyst who performed each QC test;
    - A notation of the result for each QC test and whether or not the result meets the acceptance criteria;
    - The date and time of release and the signature of the individual who assumes overall responsibility for, and adherence to, the procedures used to make the batch and authorizes the release of the batch for human administration; and
    - Documentation on the batch record of process deviations, when applicable.

Entries in batch records should be made immediately after the activity is performed and should include the initials, signature, or other identifier for the person making the entry. Corrections to paper entries should be dated and initialed, signed, or noted with an identifier of the person making the corrections but leaving the original entry still readable.

## 6.3 Aseptic Operations for Parenteral PET Drug Products

Because the sterility test results for parenteral PET drug products are obtained after release for human administration, aseptic operations and procedures should adequately ensure a sterile PET drug product. All aseptically prepared PET drug products for parenteral administration should be filtered through a sterile membrane filter of 0.22-µm or finer pore size into a closed sterile vial or container or sterilized by steam sterilization. Although the chemical synthesis of a parenteral PET drug product may take place in an open or closed apparatus, the membrane filtration of the PET drug product should be a closed system downstream of the membrane filter. This system should be aseptically assembled from presterilized, commercially available components.

### COMPONENTS

The sterile components used in the aseptically assembled apparatus typically consist of an empty vial, needles, membrane filters, vent needles, syringes, tubing, stopcocks, and perhaps others. All components should be single-use, commercially available, presterilized items. If components in the aseptically assembled apparatus are sterilized by the PET facility, the sterilization processes should be verified. The exact configuration of the PET drug product vial assembly is process dependent. A typical example is a sterile, empty vial with a membrane filter of 0.22-µm pore size attached to a needle that is inserted through the vial septum for filtration, a membrane filter of 0.22-µm pore size attached to a needle that is inserted through the vial septum for venting the vial during filtration, and a syringe with needle inserted through the vial septum for removal of the QC sample after filtration is complete.

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PET DRUG PRODUCT VIAL ASSEMBLY

Aseptic techniques should be used in the preparation of the PET drug product vial assembly, especially the assembly of all components downstream from the membrane sterilizing filter. These operations should be performed in an ISO Class 5 environment (see *Facilities and Equipment* above).

Following the creation of the PET drug product vial assembly in the ISO Class 5 environment, the assembly can be removed to another location for filtration. The location can be a noncontrolled environment as long as the integrity of the PET drug product vial assembly is not compromised during the process. Any PET drug product vial assembly that is compromised during this process should be discarded.

ASEPTIC TECHNIQUES

Any sterile component downstream from the membrane filter that contacts the PET drug product should be handled using suitable aseptic techniques inside the aseptic workstation. During aseptic operations, operators should wear proper attire, including a clean laboratory jacket, forearm sleeves, hair cover, sanitized gloves that cover the wrist, and beard/moustache covers (as appropriate). Multiple PET drug product vial assemblies can be prepared in a single aseptic operational cycle. The storage conditions and time for assembled vials should be based on data from aseptic simulations.

STERILITY TEST INOCULATIONS

Sterility tests should be performed to assess the quality of PET drug products intended for parenteral administration. The inoculation of sterility test media should be performed in a manner that is consistent with personnel radiation exposure requirements but that also minimizes the risk of false positives caused by adventitious contamination during the inoculation process. For media tubes with a screw-cap opening, the inoculation should be performed in the aseptic workstation. Media tubes with a septum cap can be inoculated in a shielded area that does not contain a HEPA filter.

## 7. STABILITY

Written specifications for the expiration time and storage conditions should be established for each PET drug product. The expiration time should be based on the results of stability testing (and specific activity requirements, as appropriate). Stability testing of the PET drug product should be performed at the highest strength of the PET drug product and in the intended final vial or container. At least three batches of the PET drug product should be stored according to proposed conditions and should be examined after a time period equal to the proposed shelf life. In addition, the PET drug product should meet acceptance criteria for radiochemical purity, appearance (color and clarity), pH, and stabilizer effectiveness (as appropriate) and chemical purity at expiry. Analytical methods should be reliable, meaningful, and specific. Stability studies should be repeated if there is a change in strength, stabilizer (or preservative) content that has the potential to affect the stability, the final vial or container, storage conditions, or expiration time. The results of stability testing should be documented.

## 8. CONTROLS AND ACCEPTANCE CRITERIA FOR FINISHED PET DRUG PRODUCTS

Written specifications for identity, strength, quality, and purity should be established for each PET drug product. For PET drug products intended for parenteral administration, specifications should be included for sterility and bacterial endotoxins.

Written procedures should be developed for QC tests. QC and documentation requirements should be established for each batch or sub-batch of a PET drug product (see *Process and Operational Controls* above). All QC tests should be executed by qualified and trained personnel according to written procedures.

The short half-life of PET radionuclides frequently precludes the completion of all QC tests before shipment of the PET drug product. This effectively creates two levels of release, one for distribution and the other for human administration. This is acceptable as long as the QC tests required for release of the PET drug product for human administration (see below) are completed before administration. The controls used in the release for distribution should be previously established in writing and should be documented in routine practice. It is not necessary to retain reserve samples of PET drug products.

If a *USP* compendial test procedure is used, the procedure should be verified to demonstrate that the test works under the conditions of actual use. Noncompendial test procedures used in the testing of a PET drug product should be reliable and specific. Supporting data for use of all analytical methods should be documented. Data derived from process studies or from in-process controls can be used as a basis for the omission of some QC tests. An example of this approach is the chlorodeoxyglucose determination in the testing of [$^{18}$F]fludeoxyglucose. Supporting data from process studies or in-process controls should be documented.

### 8.1 Quality Control Tests

The following QC tests should be performed on each batch before release for administration:

1. Appearance by visual inspection for color and clarity (absence of particulate matter) for parenteral dosage forms.
2. Measurement of the pH for parenteral dosage forms.
3. Determination of the radiochemical purity and identity of all dosage forms.
4. Determination of the radionuclidic identity of all dosage forms by half-life measurement.
5. Determination of the strength.
6. Determination of the specific activity of PET drug products that have mass-dependent localization or toxicity concerns.

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7. Determination of residual solvents used in the synthesis or purification processes.
8. Determination of the chemical purity and residual compounds used in the synthesis or purification processes (e.g., cryptand [2.2.2]).
9. Determination of preservative or stabilizer, if present.

For PET drug products with very short-lived radionuclides, prepare an initial QC sub-batch that is representative of successive sub-batches prepared in a defined operational cycle. The QC tests described in the previous paragraph should be considered for the QC sub-batch before release of subsequent sub-batches for human administration. For subsequent sub-batches of parenteral and inhaled dosage forms, visual inspection should be performed before human administration. In certain cases, limited testing of each sub-batch before administration may be appropriate (e.g., for pH determination of [$^{13}$N]ammonia produced by Devarda's alloy).

## 8.2 Periodic Quality Indicating Tests

For all PET drug products, periodically measure the radionuclidic purity of decayed samples of the PET drug product to assess the presence of long-lived radionuclides that are produced in targetry associated with the particle accelerator. For PET drug products labeled with certain radionuclides (e.g., $^{94m}$Tc, $^{124}$I, $^{64}$Cu, $^{76}$Br, and others), consider the measurement of radionuclidic purity by gamma spectrometry. Periodic quality indicating tests for PET drug products also include low-level nontoxic impurities (e.g., Class 3 residual solvents). The periodic testing should be performed at predetermined intervals rather than on a batch-to-batch basis.

## 8.3 Microbiological Tests for Sterile PET Drug Products

For PET drug products intended for parenteral administration, perform the following QC tests in addition to those described previously:

1. Determine the integrity of the membrane filter. Filter units used to sterilize PET drug products should be subjected to manufacturers' recommended integrity tests such as the bubble point test. Perform the filter integrity test after completion of filtration and before release of the PET drug product for human administration. In the case of PET drug products with $T_{1/2} < 10$ min, the PET drug product can be released for human administration before completion of the filter integrity test. In this case, the test should be completed as soon as possible after release.
2. Perform a test for bacterial endotoxins on each batch or QC sub-batch of a PET drug product. The test can be performed using recognized procedures in *USP* (see *Bacterial Endotoxins Test* ⟨85⟩). Regardless of which test is used, it should be initiated before release of each batch for human administration. For PET drug products with very short-lived radionuclides, complete the test on the QC sub-batch before the release of subsequent sub-batches for human administration. After a record of successful bacterial endotoxin tests is established for a particular PET drug product, it is necessary only to test the first batch prepared each day for that PET drug product.
3. Perform a test for sterility on each batch or QC sub-batch. The sterility test consists of the inoculation and incubation of a sample into each of two media: tryptic soy broth and fluid thioglycollate. The inoculated volume may be adjusted to avoid excessive losses because of sterility testing (e.g., 0.1 mL inoculated into 10 mL of media). The incubation period for sterility tests should begin within 30 hours of the membrane filtration. The samples can be inoculated immediately after completion of the membrane filtration, or they can be allowed to decay in a shielded area for as long as 30 hours before inoculation. It is acceptable to exceed the 30-hour period because of weekends or holidays provided it is shown that the extended period does not significantly reduce the viability of a suitable indicator organism in the sample. The sterility test may be performed using other recognized procedures in *USP* (see *Sterility Tests* ⟨71⟩). Samples should be tested individually and may not be pooled. After a record of successful sterility tests is established for a particular PET drug product, it is only necessary to test the first batch prepared each day for that PET drug product.

## 8.4 Conditional Final Release Tests

When a required QC test for a PET drug product cannot be completed because of a malfunction of testing equipment, it may be appropriate to conditionally release the batch. PET drug products may not be released without determination of radiochemical identity and purity. The batch may be released if the following conditions are met:

1. Review historical QC data to assess the frequency of out-of-specification (OOS) results or failures associated with the QC test. A conditional release is appropriate only if the historical data reveal a record of successful completion of the QC test.
2. Confirm that the acceptance criteria are met for all other QC tests for the batch.
3. Retain a sample of the conditionally released batch.
4. Promptly correct the malfunction of the testing equipment.
5. Complete the omitted QC test on the sample as soon as possible after the malfunction has been corrected. This is not necessary if the omitted QC result is meaningless after decay of the PET drug product.
6. If the sample fails the omitted QC test, immediately notify the physician or receiving facility that ordered the PET drug product.
7. Document all actions regarding the conditional release of the PET drug product, including the justification for the release, results of completed testing, and any notifications and corrective and preventive actions resulting from the incident.

In addition to the finished QC testing, other appropriate laboratory determinations could involve in-process testing of an attribute that is equivalent to finished-product testing of that attribute; continuous statistical process monitoring; or some combination of these approaches with finished testing of each PET drug product.

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## 9. IF A PET DRUG PRODUCT DOES NOT CONFORM TO SPECIFICATIONS

When the result of a QC test for a PET drug product does not meet established acceptance criteria, the result is OOS. An OOS result does not necessarily mean that the final PET drug product is a failure and should be rejected. Instead, an OOS investigation should be performed to determine if the OOS result indicates a true failure or an analytical error.

If an OOS investigation concludes that the OOS result was caused by an analytical error, invalidate the original test. If a printout is associated with this test, mark the printout *invalid*, retain it for the batch record, and repeat the test.

If an OOS investigation concludes that the OOS result was a true failure, the batch should be rejected and cannot be released for human administration. Segregate the batch to avoid its potential use. Investigate all failures and document the results according to written procedures. The investigation should include, but is not limited to, the examination of processes, operations, and records from previous batches, as well as complaints and other relevant sources of information. If possible, assign an actual or probable cause to the failure, and document corrective actions undertaken as a result of the investigation. Depending on the nature of the failure, the PET drug product may be reprocessed according to pre-established written procedures (see *Reprocessing* below).

When a sterility test for a PET drug product shows signs of microbial growth, the test result is OOS and should be investigated. Upon completion of the investigation, immediately notify all receiving facilities if the product fails to meet the criterion for sterility, including the microbiological findings from the investigation.

## 10. REPROCESSING

If a PET drug product is rejected as a true failure, the batch may be reprocessed according to established procedures. It is not possible to describe all possible reprocessing operations, but some examples could include the following:

- pH adjustment;
- A second passage through a membrane filter in the event of a failed filter integrity test; and
- A second passage through a purification column to remove an impurity.

If a PET drug product is reprocessed, the reprocessed batch should be tested to ensure it meets the established acceptance criteria for the PET drug product before release for human administration.

## 11. LABELING

The following information should appear on the label attached to the final PET drug container:

- The name of the PET drug product, including the dosage form;
- The assigned batch number; and
- Any required warning statements or symbols (e.g., investigational use, radioactive).

The following information should appear on the shielding for the PET drug product:

- The name of the PET drug product, including the dosage form;
- The assigned batch number;
- The date and time of calibration;
- Any required warning statements or symbols (e.g., investigational use, radioactive);
- As appropriate, the total radioactivity in MBq (or mCi) or the strength in MBq/mL (or mCi/mL) at time of calibration;
- Expiration time and date;
- Added substance(s) (e.g., stabilizer inactive ingredients);
- The name of the producer where the PET drug product was made or the name of the distributor;
- Other applicable warning statement(s) (e.g., “Do not use if cloudy or if it contains particulate matter” or investigational use labeling); and
- Other pertinent information (if required), such as storage condition(s), half-life.

## GLOSSARY

**Batch:** A quantity of PET drug product that is intended to have uniform character and quality, within specified limits, and that is made in a single operational cycle produced according to one or more production order(s).

**Conditional final release:** A final release for patient administration before completion of required tests because of a malfunction of analytical equipment.

**Lot:** A quantity of materials (e.g., reagents, solvents, gases, purification columns, and other auxiliary materials) that have uniform character and quality within specified limits and are used to make a PET drug product.

**PET drug:** A radioactive substance (active pharmaceutical ingredient) that exhibits spontaneous disintegration of unstable nuclei by the emission of positrons and is incorporated into a PET drug product to furnish direct effect in the diagnosis or monitoring of a disease or a manifestation of a disease in humans, or monitoring treatment of disease or therapeutic procedures (e.g., tumor therapy).

**PET drug product:** A finished dosage form that contains a PET drug, whether or not in association with one or more other ingredients.

**Compounding:** The practice as described in the Food, Drug and Cosmetic Act (1997) Chapter II, Section 121 (a) (ii) (1) (B) of synthesizing or formulating a PET drug product, by or on the order of a practitioner who is licensed by a State to compound or order compounding for a PET drug product, and is compounded in accordance with that State’s law, for a patient or for research, teaching, or quality control.

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**Line clearance:** The segregation and cleaning of different processing and work areas to avoid cross-contamination and mix-ups between the production and/or compounding of different PET drug products.

**Manufacturer's certification:** Documentation, including, but not limited to, certificates of analysis, certificates of conformance, or certificates of quality obtained from the manufacturer, supplier, or vendor of a material or component that describes critical quality characteristics used to determine acceptability of use.

**Out of specification (OOS):** A quality control test result for a PET drug product that does not conform to established acceptance criteria.

**Production:** The process of synthesis or formulation of a PET drug product including processing, packaging, labeling, reprocessing, and testing for investigational or research use.

**Quality assurance (QA):** A planned system for ensuring that a PET drug product possesses defined identity, strength, quality, and purity required for its intended purpose by procedures, tests, and analytical methods.

**Quality control (QC):** A system for testing the quality of components, materials, supplies, and PET drug products by procedures, tests, analytical methods, and acceptance criteria.

**Specific Activity:** The radioactivity of a radionuclide per unit mass of the element or compound. The unit of specific activity is radioactivity per mass expressed on a gram or mole basis (e.g., mCi/µg [MBq/µg], Ci/mmol [GBq/mmol]).

**Strength:** The radioactivity concentration of the PET drug in the PET drug product on a volume basis at the time of calibration. The unit of strength is the amount of radioactivity per volume at the time of calibration (e.g., mCi/mL [MBq/mL]).

**Sub-batch:** A quantity of PET drug product having uniform character and quality, within specified limits, that is produced during one succession of multiple irradiations using a given synthesis or purification operation. A group of sub-batches collectively form a batch that is intended to have uniform character and quality, within specified limits. Sub-batches may be required for PET drug products with very short-lived radionuclides (e.g., $^{13}N$ and $^{15}O$) because QC tests cannot be completed before use.

**Validation:** Establishment of documented evidence that a method, process, or system meets its intended requirements.

**Verification:** Confirmation that an established method, process, or system meets predetermined acceptance criteria.

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# ⟨825⟩ RADIOPHARMACEUTICALS—PREPARATION, COMPOUNDING, DISPENSING, AND REPACKAGING

▲▲ (RB 1-Jan-2024)

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# 1. INTRODUCTION

Radiopharmaceuticals, as defined in this chapter (see *Glossary*), are a subset of radioactive materials (RAMs) falling under the control of the US Nuclear Regulatory Commission (NRC) or NRC-contracted agreement state agency. Radiopharmaceuticals are also a subset of prescription drugs falling under the control of the US FDA for manufacturing and marketing. Other federal regulatory authorities (e.g., Department of Transportation) have control over certain activities related to radiopharmaceuticals. Compliance with these regulations, as applicable, must be ensured in addition to compliance with the standards described in this chapter. [NOTE—Users outside the US must comply with equivalent regulations, as applicable, pertaining to radiopharmaceuticals.]

This chapter is intended to provide uniform minimum standards for the preparation, compounding, dispensing, and repackaging of sterile and nonsterile radiopharmaceuticals for humans and animals that occur as part of state-licensed activities (e.g., the practice of pharmacy and the practice of medicine). These standards apply to all radiopharmaceutical processing activities, including those with radionuclides that emit a single photon, a positron, or a therapeutic particle. Furthermore, these standards apply to sterile intravascular radioactive devices (e.g., radioactive microspheres for intravascular brachytherapy).

This chapter does not apply to the following activities:

- Manufacturing of approved radiopharmaceuticals (e.g., NDA, ANDA, BLA) in FDA-registered manufacturing establishments
- Manufacturing of radiopharmaceuticals as investigational agents (e.g., IND, RDRC)
- Compounding of radiopharmaceuticals in a registered FDCA §503B outsourcing facility
- Preparation/compounding of positron emission tomography (PET) drugs that are not manufactured as approved drug products (e.g., NDA, ANDA, BLA) and conforms with *Positron Emission Tomography Drugs for Compounding, Investigational, and Research Uses* ⟨823⟩
- Administration of radiopharmaceuticals to patients

In each of these scenarios except for patient administration, the further processing and manipulation of the drug product after release falls within the scope of this chapter.

This chapter does not apply to the preparation of non-radioactive drugs, including those used as pharmacologic adjuncts for certain nuclear medicine procedures. These drugs must be prepared following standards described in *Pharmaceutical Compounding—Nonsterile Preparations* ⟨795⟩ and *Pharmaceutical Compounding—Sterile Preparations* ⟨797⟩.

This chapter applies to all practice settings where radiopharmaceuticals are prepared, compounded, dispensed, or repackaged. Practice settings consist of state-licensed nuclear pharmacies, federal nuclear pharmacy facilities, and other healthcare facilities, including, but not limited to: nuclear medicine departments in hospitals and clinics, nuclear cardiology clinics (fixed site or mobile), and other specialty clinics.

This chapter applies to all individuals who prepare, compound, dispense, or repackage radiopharmaceuticals. Applicable individuals consist of authorized nuclear pharmacists (ANPs) and authorized user (AU) physicians, as well as individuals working under their supervision. This includes, but is not limited to, student pharmacists, nuclear pharmacy technicians, nuclear medicine technologists and students, and physician residents and trainees.

US federal and state radiation regulatory authorities require limiting radiation exposure to personnel who handle radiopharmaceuticals, which necessitates special provisions for radiation protection. The principles of radiation safety involve time, distance, shielding, and radioactive contamination control. Moreover, the use of radiation detection and measuring devices is a necessary component of radiopharmaceutical handling procedures. Strict adherence to all typical aseptic handling practices is not possible in many scenarios where radiopharmaceuticals are handled. Thus, it is necessary to balance aseptic handling practices (patient safety) with radiation protection practices (worker safety). This chapter describes appropriate strategies that provide assurance of maintaining patient safety, while also ensuring the safety of individuals performing these activities. Because radiopharmaceuticals represent a unique class of prescription drugs, the use of technologies, techniques, materials, and procedures other than those described in this chapter are not prohibited so long as they are documented to be equivalent or superior to those described herein.

## 1.1 Nonsterile Radiopharmaceuticals

Examples of nonsterile radiopharmaceuticals include oral capsules and oral solutions. For conventionally manufactured products or compounded preparations obtained from 503B-registered outsourcing facilities, dispensing can proceed as described in *12. Dispensing*. For prepared or compounded preparations, such preparations must comply with applicable identity, quality, and purity standards, as described in manufacturer labeling, *USP* monographs, or other appropriate sources (e.g., documented, peer-reviewed materials). They can then be dispensed as described in this chapter.

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### 1.2 Sterile Radiopharmaceuticals

Examples of sterile radiopharmaceuticals include injectables (e.g., intravenous, intrathecal, intraperitoneal, subcutaneous, and intradermal), inhalations, ophthalmics, and intra-organ instillations. For conventionally marketed products, see *12. Dispensing*. For prepared or compounded preparations, such preparations must comply with applicable identity, quality, and purity standards. For compounded preparations involving one or more nonsterile components, a sterilization procedure (e.g., filtration with bubble point testing) must be performed prior to dispensing. For injectable compounded preparations involving one or more components that are not certified to be pyrogen-free, bacterial endotoxin testing, as defined in *Bacterial Endotoxins Test* ⟨85⟩, must be performed prior to dispensing.

The most important factor for maintaining sterility is the avoidance of touch contamination. Wipe the vial septum with sterile 70% isopropyl alcohol (IPA) prior to initial needle puncture. If the vial shield top is then closed, the septum must be disinfected again with sterile 70% IPA prior to another needle puncture. Some vial shields are constructed such that the vial septum is recessed and difficult to access. One approach for disinfecting the vial septum in this type of vial shield is to use right-angle forceps to hold a sterile 70% IPA wipe and apply direct contact with the vial septum. It is also acknowledged that such vial shields disrupt first air contacting the vial septum during certain handling conditions. Wipe the septum with sterile 70% IPA frequently whenever multiple punctures are occurring (e.g., removing several individual doses from a multiple-dose container).

## 2. RADIATION SAFETY CONSIDERATIONS

The handling of radiopharmaceuticals necessitates meeting the radiation regulatory agency requirements for worker safety. This involves licensing commitments to keep all exposure levels for the workers involved as low as reasonably achievable (ALARA) practices. Principles of radiation safety involve time, distance, shielding, and contamination control. Moreover, radiation detection and measuring devices are necessary. Aseptic handling practices must be balanced with radiation safety considerations, based on the following:

- Knowledge, training, experience, and professional judgment related to the type, abundance, and energy of the radioactive emissions
- The quantity of radioactivity, volume, handling steps, and timing
- Other factors, which can vary on a case-by-case basis

### 2.1 Time

Radiation exposure to personnel is directly proportional to the quantity of radiation handled and the time handling the RAM; minimizing handling time will minimize radiation exposure. Personnel handling radiopharmaceuticals may work quickly in a controlled and safe manner, including multiple hand movements in and out of the ISO Class 5 primary engineering control (PEC) during aseptic processes.

### 2.2 Distance

Radiation exposure follows the inverse square law; increasing the distance between the operator and the RAM will decrease radiation exposure to personnel by the square of the distance. Handlers of radiopharmaceuticals may utilize techniques to increase distance, such as using remote handling tools, including within an ISO Class 5 PEC.

### 2.3 Shielding

Radiation exposure to personnel decreases with the use of shielding materials. Therefore, handlers of radiopharmaceuticals may use various shielding materials (e.g., lead, tungsten) in various configurations. The use of shielding, such as L-block, torso, vial, and syringe shields, is usually required throughout the radiopharmaceutical handling process, including within an ISO Class 5 PEC.

### 2.4 Radiation Contamination Control

RAM contamination (e.g., spills, drips, sprays, volatility) is an important concern for radiation protection. Therefore, various techniques and materials may be used by handlers of radiopharmaceuticals to minimize radioactive contaminations. For example, container contents are maintained at neutral or negative pressure, because positive pressure in a container is a common cause of radioactive contamination. Disposable absorbent pads are commonly used to contain such radioactive contamination and, when used in an ISO Class 5 PEC, the pads must be clean and low-lint. Vertical air flow, not horizontal, in a PEC is used to control contamination. When exposure to blood and other potentially infectious material is reasonably anticipated, some engineered needlestick prevention devices may pose a radiation hazard to employees. Policies must be implemented for handling biohazardous radioactive sharps while minimizing contamination.

#### RADIATION DETECTORS AND MEASURING DEVICES

Radiopharmaceuticals require measurement with a suitable radiation measuring device (e.g., dose calibrator). These and other necessary equipment, (e.g., monitors, bar code scanner, label printer) may be placed inside an ISO Class 5 PEC but should be placed in a manner that minimizes disruptions of airflow.

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As per RAM license requirements, individuals must wear body and, as required, extremity dosimeters (e.g., a ring worn on a finger) for long-term monitoring of personnel radiation exposure. The body dosimeter should be worn underneath the gown. Any extremity dosimeter must be worn underneath gloves and must not interfere with proper fit of gloves.

## 3. IMMEDIATE USE OF STERILE RADIOPHARMACEUTICALS

The preparation and dispensing of sterile radiopharmaceuticals in a patient care setting may be handled as an immediate use practice. The information below describes the appropriate handling requirements for immediate use sterile radiopharmaceuticals in an ambient environment that lacks primary and secondary engineering controls (SEC) when intended for a single patient. Strict aseptic technique and limited beyond-use date (BUD) must be adhered to given the lack of engineering controls.

- Appropriate for preparation (including minor deviations) and/or dispensing that is limited to use for a single patient.
- Preparation (including preparations with minor deviations) components must be sterile, conventionally manufactured drug products (e.g., NDA, ANDA).
- Dispensing of drug products produced under an approved IND or RDRC protocol is allowed.
- Manipulations for any unit doses (e.g., decreasing the dosage, needle changes) or dispensing for one patient (e.g., withdrawing a dose) is allowed.
- Must be administered within 1 hour of the first container puncture or exposure of any critical site involved (e.g., syringe tip, needle hub or needle) to ambient air, whichever is first.
- All components involved (e.g., Tc-99m sodium pertechnetate syringe or vial, final prepared radiopharmaceutical kit vial, diluent vial) must be discarded within 1 hour of being punctured or after use for a single patient administration, whichever is first.
- Dose pooling (combining doses from two or more syringes to meet one patient's need) may be performed as immediate use. Any residual activity that remains must be immediately discarded and not utilized for any other patient.
- Follow hand hygiene and garbing in *4.4 Hand Hygiene and Garbing for Immediate Use Preparations*.
- Follow *10.4 Preparation of Radiolabeled Red Blood Cells for Immediate Use* for red blood cell labeling.
- Follow *12.2 Labeling* for labeling.
- Area for sterile preparation and/or dispensing must be functionally separate from nonsterile compounding area (e.g., radiolabeling food) during the time of use.
- Does not require a segregated radiopharmaceutical processing area (SRPA), classified area, or PEC.
- The number of steps or punctures is not limited.
- Does not require personnel to complete the aseptic qualifications as detailed in *4.1 Aseptic Qualifications* (e.g., aseptic technique training with documented assessment, media fill challenge, gloved fingertip testing).
- While adding a non-radioactive, sterile and commercially manufactured pharmaceutical (e.g., lidocaine) to a unit dose is otherwise considered compounding, it is allowed for immediate use purposes as long as all of the above are adhered to.
- Dose splitting (splitting a unit dose for administration to more than one patient) may not be performed as immediate use; if performed, dose splitting must be done in an ISO class 5 PEC in either an SRPA or in an ISO class 8 or better buffer area.

## 4. PERSONNEL QUALIFICATIONS, TRAINING, AND HYGIENE

Personnel must be trained to work with radiopharmaceuticals per the policies and standard operating procedures (SOPs) authorized by an ANP or AU physician. These individuals (e.g., nuclear medicine technologists or nuclear pharmacy technicians) must follow these policies and SOPs of the ANP or AU physician and work under their supervision. As appropriate, this should include blood-borne pathogens training.

Individuals entering a compounding area must be properly garbed and must maintain proper personal hygiene to minimize the risk of contamination to the environment and/or radiopharmaceuticals. Individuals who have a condition that may pose a higher potential of contaminating the radiopharmaceutical and the environment with microorganisms (e.g., rashes, sunburn, recent tattoos, oozing sores, conjunctivitis, or active respiratory infection) must report these conditions to their supervisor. The designated person is responsible for evaluating whether these individuals should be excluded from working in sterile processing areas before their conditions are resolved.

### 4.1 Aseptic Qualifications

Personnel must prove competency, as applicable to their job functions, prior to performing radiopharmaceutical aseptic tasks that are beyond immediate use. These qualifications may be conducted at a different site if all SOPs are identical for the applicable job function. These qualifications must be completed and documented initially, and then successfully repeated at intervals described below in *Timing of Reevaluation and Requalification* under the observation of a designated person and include the following:

- Aseptic technique training with a documented assessment (written or electronic)
- Garbing and hand hygiene, as defined by the policies and SOPs
- PEC cleaning and disinfecting
- Gloved fingertip and thumb sampling
- Media-fill testing

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GLOVED FINGERTIP AND THUMB SAMPLING

Appropriate garbing, including sterile gloves, is necessary for personnel who enter and perform tasks in an ISO Class 5 PEC (e.g., aseptic manipulations, cleaning the PEC). Personnel that perform such functions must prove their competency in this process. Gloved fingertip and thumb sampling must be performed initially on both hands, immediately following hand-hygiene and garbing. Successful completion of initial gloved fingertip and thumb sampling is defined as zero colony-forming unis (cfu) and subsequent gloved fingertip and thumb sampling after media-fill testing is defined as ≤3 cfu (total for both hands).

- The gloved fingertip and thumb sampling must be performed with touch plates or other devices (e.g., plates, paddles, or slides) that contain a general microbial growth agar [e.g., trypticase soy agar (TSA) soybean–casein digest media] supplemented with neutralizing additives (e.g., lecithin and polysorbate 80) as this supports both bacterial and fungal growth
- Gloves must not be disinfected immediately before touching the sampling device, as this could cause a false-negative result
- Using a separate sampling device for each hand, a gloved fingertip and thumb sample from both hands must be collected by rolling finger pads and thumb pad over the agar surface
- The plates must be incubated in an incubator at 30°–35° for no less than 48 h, and then at 20°–25° for no less than 5 additional days

MEDIA-FILL TESTING

Media-fill testing is necessary for all personnel who prepare, compound, dispense, and repackage sterile radiopharmaceuticals. This testing must be reflective of the actual manipulations to be carried out by the individual and must simulate the most challenging and stressful conditions to be encountered in the worker's duties.

- Media-fill tests must be documented as defined by the facility's policies and SOPs.
- Media-fill tests should be performed at the end of a work session in the PEC.
- Media-fill tests must be performed with a commercial source of soybean–casein digest medium. Those performing sterile-to-sterile processing activities must start with sterile media. Those performing nonsterile-to-sterile compounding must use a nonsterile soybean–casein digest powder to make a solution. Dissolve nonsterile commercially available soybean–casein digest medium in nonbacteriostatic water to make a 3% nonsterile solution. Manipulate it in a manner that simulates nonsterile-to-sterile compounding activities. Prepare at least 1 container as the positive control to demonstrate growth promotion, which is indicated by visible turbidity upon incubation.
- The certificate of analysis (CoA) must include documentation of growth promotion testing for each lot of media used.
- Once the media-fill simulation is completed and the final containers are filled with the test medium, incubate media-filled containers in an incubator for 7 days at 20°–25° followed by 7 days at 30°–35° to detect a broad spectrum of microorganisms. Failure is indicated by visible turbidity or other visual manifestations of growth in the medium in 1 or more container–closure unit(s) on or before 14 days.
- In the event of failure, results of the evaluation and corrective actions must be documented and the documentation maintained to provide a record and long-term assessment of personnel competency. Documentation must at a minimum include the name of the person evaluated, evaluation date/time, media and components used including manufacturer, expiration date and lot number, starting temperature for each interval of incubation, dates of incubation, and the results.

## 4.2 Reevaluation, Retraining, and Requalification

REQUALIFICATION AFTER FAILURE

Personnel who fail visual observation of hand hygiene, garbing, and aseptic technique, gloved fingertip and thumb sampling, or media-fill testing must successfully pass reevaluations in the deficient area(s) before they can resume processing of sterile preparations. All failures, retraining, and reevaluations must be documented.

REQUALIFICATION PROGRAM

Personnel must successfully complete requalification in the core competencies listed in *4.1 Aseptic Qualifications*. Successful completion must be demonstrated through observation, written testing, and hands-on demonstration of skills.

TIMING OF REEVALUATION AND REQUALIFICATION

**Visual observation:** Personnel must be visually observed while performing hand hygiene, garbing SOPs, and aseptic technique procedures initially, and then at least once every 12 months.
**Gloved fingertip and thumb sampling:** Personnel must perform fingertip and thumb sampling 3 times initially, and then every 12 months (in conjunction with media-fill testing).
**Media-fill testing:** After initial qualification, conduct a media-fill test of all personnel engaged in sterile radiopharmaceutical processing at least every 12 months (in conjunction with gloved fingertip and thumb sampling).
**Cleaning and disinfecting:** Retrain and requalify personnel in the cleaning and disinfecting of sterile processing areas every 12 months or in conjunction with any change(s) in cleaning and disinfecting SOPs, whichever is sooner.
**After a pause in sterile radiopharmaceutical processing:** Personnel that have not performed radiopharmaceutical processing in more than 6 months must be requalified in all core competencies before resuming duties.

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**Sterile compounding using a nonsterile drug substance or components:** Personnel who perform sterile compounding using a nonsterile drug substance or components (see *11.3 Sterile Compounding Using a Nonsterile Drug Substance or Components*) must be requalified in all core competencies every 6 months.

### 4.3 Ancillary Personnel

Personnel who are authorized to be within the sterile processing area and do not handle sterile preparations are not required to complete training on media-fill testing but are required to complete all other training and testing. Other personnel or visitors (e.g., auditors, regulators, student observers) must comply with garbing and gloving SOPs but do not need to prove competency.

### 4.4 Hand Hygiene and Garbing for Immediate Use Preparations

Radiopharmaceuticals may be prepared and dispensed as immediate use, and the precautions related to personal hygiene to be followed must include the following:

- Hand hygiene: Wash hands and arms to the wrists with soap and water or use a suitable alcohol-based hand rub with a time based on institution policies to reduce bioburden on the hands.
- Garbing: Immediately after hand hygiene, don a clean coat/gown that has not been exposed to a patient or patient care area, and either don sterile gloves or don nonsterile disposable gloves and then disinfect the gloves with sterile 70% IPA. [NOTE—A different lab coat must be worn to care for a patient than the coat/gown used for radiopharmaceutical preparation.]

### 4.5 Hand Hygiene and Garbing for Buffer Areas and Segregated Radiopharmaceutical Processing Area

In situations involving repackaging, dispensing, preparation, preparation with minor deviations, or compounding of sterile radiopharmaceuticals in an ISO Class 5 PEC, the following precautions related to personal hygiene are to be followed:

- Before entering the SRPA or buffer area, personnel must remove outer garments (e.g., bandanas, coats, hats, jackets, sweaters, vests); all cosmetics; all hand, wrist, and other exposed jewelry including piercings that could interfere with the effectiveness of the garbing (e.g., the fit of gloves, cuffs of sleeves, and eye protection). Nail products (e.g., artificial nails, polish, extenders) are prohibited. Natural nails must be kept neat and trimmed. Remove ear buds and headphones. Radiation dosimetry devices are allowed, as required by the RAM license.
- Do not bring electronic devices that are not necessary for compounding or other required tasks.
- Immediately before entering the SRPA or buffer area, remove visible debris from underneath fingernails under warm running water using a disposable nail cleaner. Personnel must wash hands and arms up the elbows with soap and water for at least 30 s and then dry hands using low-lint towels. Alternatively, hand washing may be performed after donning shoe covers, head/hair covers, and face mask, as described below.
- Personnel must don the following garb—shoe covers, head/hair/facial hair covers, face mask—in an order that eliminates the greatest risk of contamination, as defined in facility SOPs.
- If not already performed, remove visible debris from underneath fingernails under warm running water using a disposable nail cleaner. Personnel must then wash hands and arms up to the elbows with soap and water for at least 30 s and then dry hands using low-lint towels. Electronic hand dryers are not permitted.
- Personnel must then perform hand antisepsis cleansing using a suitable alcohol-based hand rub.
- Personnel must then don a low-lint gown with sleeves that fit snugly around the wrists and enclosed at the neck. Disposable gowns are preferred. If reusable gowns are used, a clean gown must be donned daily.
- Personnel must then aseptically don sterile, powder-free gloves. Gloves must completely and snugly cover the ends of the gown cuffs so that skin on the wrists and upper hands is completely enveloped.
- Because gloves may not remain sterile due to touching or handling potentially nonsterile materials, personnel must periodically apply sterile 70% IPA to gloves while balancing the risk of radioactivity contamination.
- Personnel must also routinely inspect the gloves that they are wearing for holes, punctures, radioactivity contamination, or tears. If a defect, radioactivity contamination, or malfunction is detected, personnel must immediately remove the gloves, repeat antiseptic hand cleansing using an alcohol-based hand rub, and don new sterile gloves.
- Direct personnel touch contamination is the most common source of microorganisms, so personnel must avoid touch contamination of container septa, needles, syringe and needle hubs, and other critical sites.

When personnel exit the buffer area or SRPA, shoe covers, head/hair covers, face masks, and gloves must be properly disposed of and new ones donned for each reentry into the buffer area or SRPA. Gowns may be re-used within the same shift if the gown is maintained in a classified area or in (or immediately outside of) the SRPA that minimizes contamination (e.g., away from sinks).

## 5. FACILITIES AND ENGINEERING CONTROLS

### 5.1 Facility Design and Environmental Controls

In addition to minimizing airborne contamination, sterile radiopharmaceutical facilities must be designed and controlled to provide a well-lighted and comfortable working environment (see *Physical Environments That Promote Safe Medication Use* ⟨1066⟩). The classified areas and SRPA must be continuously maintained at a temperature of 25° or cooler and should be

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continuously maintained at a relative humidity (RH) below 60% to minimize the risk for microbial proliferation and provide comfortable conditions for personnel attired in the required garb. The temperature and humidity must be monitored in the classified areas each day that it is used, either manually or by a continuous recording device. The results of the temperature and humidity readings must be documented at least once daily or stored in the continuous recording device, and must be retrievable. The temperature and humidity readings must be reviewed as described in the facility's SOPs. Free-standing humidifiers/dehumidifiers and air conditioners must not be used within the classified area or SRPA. Temperature and humidity monitoring devices must be verified for accuracy at least every 12 months or as required by the manufacturer.

The designated person is responsible for ensuring that each area related to sterile radiopharmaceutical processes meets the classified air quality standard appropriate for the activities to be conducted in that area. They must also ensure that the ISO Class 5 PECs are located, operated, maintained, monitored, and certified to have appropriate air quality.

## TYPES OF SECONDARY ENGINEERING CONTROLS AND DESIGN

Due to the interdependence of the various areas or areas that make up a sterile radiopharmaceutical processing facility, it is essential to define and control the dynamic interactions permitted between areas. When designing doors, consider the placement of door closures, door surfaces, and the movement of the door, all of which can affect airflow. Tacky surfaces must not be used in ISO-classified areas.

The PEC must be located in a SEC, which may be either an ISO-classified buffer room with ante-room or an SRPA, in a manner that minimizes conditions that could increase the risk of microbial contamination. For example, strong air currents from opened doors, personnel traffic, or air streams from the HVAC system(s) can disrupt the unidirectional airflow of an open-faced PEC such as a laminar airflow workbench (LAFW) or biological safety cabinet (BSC). The ISO-classified ante-room and buffer area must be separated from the surrounding unclassified areas of the facility with fixed walls and doors. Facility design and controls must be in place to minimize the flow of lower-quality air into the more controlled areas. Air supplied to the classified areas must be introduced through HEPA filters that are located in the ceiling. Returns must be low on the wall unless a visual smoke study demonstrates an absence of stagnant airflow where particulate will accumulate. A smoke study of the PEC must be repeated whenever a change to the placement of the PEC within the area is made. The classified areas must be equipped with a pressure-differential monitoring system. The ante-room must have a line of demarcation to separate the clean side from the less clean side. The ante-room is entered through the less clean side, and the clean side is the area closest to the buffer area. Required garb must be worn prior to crossing the line of demarcation (see *4. Personnel Qualifications, Training, and Hygiene*).

A PEC may be located within an unclassified area, without an ante-room or buffer area. This type of design is called an SRPA. Only sterile radiopharmaceutical preparation, preparation with minor deviations, dispensing, and repackaging may be performed in an SRPA. If the SRPA meets ISO Class 8 total airborne particle count specifications, it can also be used for storage and elution of non-direct infusion radionuclide generators (e.g., Tc-99m). The SRPA must be located away from unsealed windows, doors that connect to the outdoors, and traffic flow which may adversely affect the air quality in the PEC. The impact of activities that will be conducted around or adjacent to the SRPA must be considered carefully when designing such an area. A visible perimeter must establish the boundaries of the SRPA. Access to the SRPA must be restricted to authorized personnel and required materials. An SRPA must not be located adjacent to environmental control challenges.

It is also critical to control materials (e.g., supplies and equipment) as they move from classified areas of lower quality to those of higher quality (e.g., ISO Class 8 ante-room to ISO Class 7 buffer area to ISO Class 5 PEC) to prevent the influx of contaminants. Airlocks and interlocking doors can be used to facilitate better control of air flow between areas of differing ISO classification (e.g., between the buffer area and ante-room), or between a classified area and an unclassified area (e.g., between the ante-room and an unclassified area such as a hallway) See *5.7 Environmental Controls* for a description of air pressure differentials. If a pass-through is used, both doors must never be opened at the same time, which may be achieved using interlocking mechanisms.

## THE RADIOPHARMACEUTICAL PROCESSING ENVIRONMENT

The PEC must be certified to meet ISO Class 5 or better conditions (see *Table 1*) and must be designed to minimize microbial contamination during processing of radiopharmaceuticals under dynamic operating conditions.

The airflow in the PEC must be unidirectional (laminar flow), and because of the particle collection efficiency of the filter, the "first air" at the face of the filter is, for the purpose of aseptic processing, free from airborne particulate contamination. HEPA-filtered air must be supplied in the direct processing area (DPA) (ISO Class 5; see *Table 1*) at a velocity sufficient to sweep particles away from aseptic processing areas and maintain unidirectional airflow as much as possible during operations, given the limitations added from the radiation shielding in the DPA. Proper design and control prevents turbulence and stagnant air in the DPA. In situ air pattern analysis via smoke studies must be conducted at the critical area to demonstrate unidirectional airflow and sweeping action under dynamic conditions.

**Table 1. ISO Classification of Particulate Matter in Area Air[a]**

| ISO Class | Particle Count[b] /m³ |
|---|---|
| 3 | 35.2 |
| 4 | 352 |
| 5 | 3520 |
| 6 | 35,200 |
| 7 | 352,000 |
| 8 | 3,520,000 |

[a] Adapted from ISO 14644-1, Clean areas and associated controlled environments—Part 1: Classification of air cleanliness by particle concentration.

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[b] Limits for number of particles ≥0.5 µm measured under dynamic operating conditions.

### TYPES OF PECS AND PLACEMENT

Proper placement of the PEC is critical to ensuring an ISO Class 5 environment for preparing radiopharmaceuticals. Placement of the PEC must allow for cleaning around the PEC.

A PEC provides an ISO Class 5 or better environment for sterile radiopharmaceuticals. The unidirectional airflow within the PEC helps protect the DPA from process-generated contamination of an aseptic processing environment. The unidirectional airflow within the PEC helps protect the DPA from process-generated contamination (e.g., opening wrappings of sterile containers, worker movement, etc.) as well as from outside sources.

**Laminar airflow workbench (LAFW):** An LAFW used for preparing radiopharmaceuticals must provide vertical unidirectional HEPA-filtered airflow. In cases where the LAFW is located within the segregated containment area of a hot-cell, it is acceptable for a horizontal unidirectional HEPA-filtered airflow pattern to be utilized.

**Biological safety cabinet (BSC) Class II:** A BSC Class II is a cabinet with an open front, inward airflow, downward unidirectional HEPA-filtered airflow, and HEPA-filtered exhaust. The BSC is designed to provide worker protection from exposure to biohazardous material and to provide an ISO Class 5 or better environment for preparing sterile radiopharmaceuticals.

**Placement of PEC:** The PEC must be located out of traffic patterns and away from area air currents that could disrupt the intended airflow patterns inside the PEC. If used only to prepare, prepare with minor deviations, dispense, or repackage sterile radiopharmaceuticals the ISO Class 5 PEC may be placed in an unclassified SRPA. If used to compound sterile radiopharmaceuticals, the PEC must be located within an ISO Class 7 or better buffer area with an ISO Class 8 or better ante-room. A dynamic airflow smoke pattern test must be performed initially and at least every 6 months to ensure that the PEC is properly placed into the facility and that workers understand how to utilize the unidirectional airflow to maintain first air as much as possible given the limitations added from the radiation shielding in the DPA.

### AIR-EXCHANGE REQUIREMENTS

For classified areas, adequate HEPA-filtered airflow to the buffer area(s) and ante-room(s) is required to maintain the appropriate ISO classification during processing activities. Airflow is measured in terms of the number of HEPA-filtered air changes per hour (ACPH). The ACPH may need to be higher to maintain the required ISO classification and microbial state of control depending on these factors: the number of personnel permitted to work in the area, the number of particulates that may be generated from activities and processes in the area, the equipment located in the area, the area pressure, and the effects of temperature. The summary of ACPH requirements is listed in *Table 2*.

A minimum of 30 total HEPA-filtered ACPH must be supplied to ISO Class 7 areas.

- The total HEPA-filtered air change rate must be adequate to maintain ISO Class 7 under dynamic operating conditions considering factors listed above
- At least 15 ACPH of the total air change rate in a room must come from the HVAC through HEPA filters located in the ceiling
- The HEPA-filtered air from the PEC, when added to the HVAC-supplied HEPA-filtered air, increases the total HEPA-filtered ACPH to at least 30 ACPH
- If the PEC is used to meet the minimum total ACPH requirements, the PEC must not be turned off except for maintenance
- The ACPH from HVAC, ACPH contributed from the PEC, and the total ACPH must be documented on certification reports

A minimum of 20 ACPH of HEPA-filtered air must be supplied to ISO Class 8 areas.

- The total HEPA-filtered air change rate must be adequate to maintain ISO Class 8 under dynamic operating conditions considering factors listed above
- At least 15 ACPH of the total air change rate in a room must come from the HVAC through HEPA filters located in the ceiling
- Ante-rooms where activity levels are high may require more HEPA-filtered ACPH to maintain ISO Class 8 under dynamic operating conditions
- The total ACPH must be documented on certification reports

**Table 2. Summary of ACPH Requirements for Sterile Radiopharmaceutical Processing**

| Processing Area | ACPH Requirement |
|---|---|
| Unclassified SRPA | No requirement |
| ISO Class 7 area | ≥30 ACPH |
| ISO Class 8 area | ≥20 ACPH |

## 5.2 Creating Areas to Achieve Easily Cleanable Conditions

### CLASSIFIED AREAS

The surfaces of ceilings, walls, floors, doors, door frames, fixtures, shelving, work surfaces, counters, and cabinets in the classified area must be smooth, impervious, free from cracks and crevices, and non-shedding, so they can be cleaned and disinfected, and to minimize spaces in which microorganisms and other contaminants can accumulate. Surfaces should be resistant to damage by cleaning agents, disinfectants, and tools used to clean. Junctures between the ceiling and the walls and between the wall and the floor must be sealed to eliminate cracks and crevices where dirt can accumulate. If ceilings consist of inlaid panels, each panel must be caulked or otherwise sealed and secured to seal them to the support frame.

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Walls must be constructed of or covered with a durable material (e.g., epoxy-painted walls or heavy-gauge polymer) and the integrity of the surface must be maintained. Panels must be joined together and sealed to each other and the support structure. Floors must include coving to the sidewall or the juncture between the floor and wall must be caulked. Floors must include coving to the sidewall. Classified areas should minimize dust-collecting overhangs such as utility pipes and ledges such as windowsills. If overhangs or ledges are present, they must be easily cleanable. The exterior lens surface of ceiling light fixtures must be smooth, mounted flush, and sealed. Any other penetrations through the ceiling or walls must be sealed.

SRPA

The SRPA and all surfaces (e.g., walls, floors, counters, equipment) within the SRPA must be clean, uncluttered, and dedicated to sterile radiopharmaceutical processing activities. Surfaces in the SRPA should be smooth, impervious, free from cracks and crevices, and non-shedding, so they can be easily cleaned and disinfected, and to minimize spaces in which microorganisms and other contaminants can accumulate. Surfaces should be resistant to damage by cleaning agents, disinfectants, and tools used to clean. Dust-collecting overhangs such as utility pipes and ledges such as windowsills should be minimized. If overhangs or ledges are present, they must be easily cleanable.

## 5.3 Water Sources

The facility where sterile radiopharmaceuticals are prepared must be designed so that activities such as hand hygiene and garbing should not adversely affect the ability of the PEC to function as designed. Sinks should enable hands-free use with a closed system of soap (i.e., non-refillable) to minimize the risk of extrinsic contamination. In facilities with an ante-room and buffer area, the sink used for hand hygiene may be placed either inside or outside of the ante-room. If the sink is located outside of the ante-room, it must be located in a clean space to minimize the risk of bringing in contaminants into the ante-room. If the sink is located inside the ante-room, it may be placed on either the clean side or the less-clean side of the ante-room. [NOTE—The order of hand washing and garbing would depend on the placement of the sink (see *4.5 Hand Hygiene and Garbing for Buffer Areas and Segregated Radiopharmaceutical Processing Area*).] The buffer area must not contain plumbed water sources [e.g., sink(s), eyewash(es), shower(s), or floor drain(s)]. The ante-room must not contain floor drain(s). If installed, sprinkler systems in classified areas should be recessed and covered, and should be easily cleanable. In a facility with an SRPA design, the sink must be accessible but located at least 1 m from the PEC and generators, if present. The sink must not be located inside the perimeter of the SRPA.

## 5.4 Placement and Movement of Materials

Only furniture, equipment, and other materials necessary are permitted in the classified area or SRPA and they should be low-shedding and easily cleaned and disinfected. Their number, design, location, and manner of installation must not adversely impact environmental air quality and must promote effective cleaning and disinfecting. No shipping carton(s) or other corrugated or uncoated cardboard are allowed in the classified area or SRPA.

Carts used to transport components or equipment into classified areas must be constructed from nonporous materials with cleanable casters and wheels. All items must be wiped with low-lint wipers and an appropriate disinfectant by personnel wearing gloves before they are brought into the clean side of ante-room(s), pass-through(s), into an SRPA or into an ISO 5 PEC. However, constraints that would lead to excessive radiation exposure to radiation for workers and thereby be contradictory to following ALARA safety principles (e.g., the wiping of unshielded sources of radioactive material) might preclude this from occurring. In a classified area, carts must not be moved from the dirty side to the clean side of the ante-room unless the entire cart, including casters, is cleaned and disinfected.

## 5.5 Classified Areas

Activities and tasks carried out within the buffer area must be limited to only those necessary. Food, drinks, and materials exposed in patient care and treatment areas must not enter ante-rooms or buffer areas. When processing activities require the manipulation of blood-derived or other biological material (e.g., radiolabeling patient's or donor's blood cells), the manipulations must be clearly separated from routine material-handling procedures and equipment used in radiopharmaceutical preparation activities, and they must be controlled by specific SOPs to avoid any cross-contamination.

## 5.6 Remote Aseptic Processing Involving a Hot-Cell

A hot-cell device provides an inherent physical segregation for the ISO Class 5 aseptic processing area. If the hot-cell is located in an ISO-classified space, personnel must garb according to requirements listed in *4.5 Hand Hygiene and Garbing for Buffer Areas and Segregated Radiopharmaceutical Processing Area*. In settings where tasks are carried out within the hot-cell enclosure not within an ISO-classified space by remote means (i.e., no direct intervention by personnel into the ISO Class 5 space), it is not necessary for personnel to don the garbing described in *4.5 Hand Hygiene and Garbing for Buffer Areas and Segregated Radiopharmaceutical Processing Area* to carry out these aseptic manipulations or to perform other routine tasks in the general area where the hot-cell is located. If hand and arm incursions into the interior of the hot-cell might be necessary for personnel to stage the required materials and supplies, the personnel must garb in relation to the contamination risk associated with the individual hot-cell/ISO Class 5 relationship.

For situations where a PEC device is located within a hot-cell, dynamic airflow smoke pattern tests must show that the staging of supplies and materials in the demarcated PEC area does not allow the influx of unclassified air into the PEC. Personnel may be garbed in nonsterile gloves and a low-particulate lab coat for interventions that are outside of the PEC. A failure of the airflow

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smoke pattern test requires personnel to garb in accordance with *4.5 Hand Hygiene and Garbing for Buffer Areas and Segregated Radiopharmaceutical Processing Area* for all incursions into the hot-cell.

For situations where the hot-cell is an integrated HEPA filtration system with a clear demarcated area that is a PEC, dynamic airflow smoke pattern tests must show that the staging of supplies and materials into the demarcated PEC area does not allow the influx of less than ISO Class 5 quality air into the PEC. Personnel may be garbed in nonsterile gloves and a low-particulate lab coat for interventions that are outside of the PEC. A failure of the airflow smoke pattern test requires personnel to garb in accordance with *4.5 Hand Hygiene and Garbing for Buffer Areas and Segregated Radiopharmaceutical Processing Area* for all incursions into the PEC.

Since other hot-cell/PEC configurations and technologies may exist, verification (either by airflow smoke pattern tests or other manufacturer specified methods) must ensure, upon each certification, that the staging of materials and supplies does not allow for the intrusion of less than ISO Class 5 air into the designated ISO Class 5 space. A failure of the airflow smoke pattern test requires personnel to garb in accordance with *4.5 Hand Hygiene and Garbing for Buffer Areas and Segregated Radiopharmaceutical Processing Area* for all incursions into the hot-cell.

## 5.7 Environmental Controls

All RAM users must comply with the conditions specified in their approved RAM license application and regulations, and RAM license conditions may supersede the following requirements for environmental controls described in this section. Pass-through enclosures for transferring radiopharmaceuticals from controlled handling areas (e.g., buffer area) should be designed to provide reasonable balance between maintenance of air quality and other worker safety concerns (e.g., radiation exposure, physical injury from lifting heavy shielded cases). At a minimum, there must be a mechanical system or SOP in place that ensures that both doors cannot be open at the same time. There may be both positive and negative air pressure within the facility; positive pressure to minimize the potential of microbial contamination in sterile drug preparation areas, and negative pressure to minimize potential radioactive contamination from volatile or airborne radiopharmaceuticals. Positive pressure environments must have a minimum differential positive pressure of 0.02-inch water column between each ISO-classified area (e.g., between the buffer area and ante-room). The pressure differential between the ante-room and the unclassified area must be no less than a positive 0.02-inch water column. Refer to the RAM license for negative pressure requirements. For preparation of sterile radiopharmaceuticals, consideration of both concerns could be addressed as follows:

1. Buffer area, if present, must be positive pressure compared to the ante-room
2. Ante-room, if present, must be positive pressure compared to unclassified portions of the restricted area
3. Restricted area, in the presence of volatile or airborne radiopharmaceuticals, must be negative pressure compared to the unrestricted area
4. SRPA must be negative pressure compared to unrestricted areas in the presence of volatile or airborne radiopharmaceuticals (e.g., I-131 sodium iodide and Xenon).

Various environmental controls for various preparation scenarios (see *Table 7* for maximum BUDs for differing environments) are described in the following sections. *Table 1* details the limits for particle counts for each specific ISO classification.

### ESTABLISHING AND MAINTAINING PRESSURE DIFFERENTIALS

Any time a pressure differential is required, a pressure monitoring device is required. In a classified area, a pressure differential monitoring system must be used to continuously monitor the pressure differential between the ante-room(s) and buffer area(s) and between the ante-room and the general environment outside the classified area(s) or area(s). The results from the pressure monitoring system must be reviewed and documented at least daily on days the area is used. All pressure monitoring devices must be tested for accuracy and required performance at least every 6 months.

### AMBIENT ATMOSPHERE FOR IMMEDIATE USE PREPARATIONS

The following requirements should be met in ambient atmosphere environments:

- Non-patient care space, functionally separate (not necessarily a different area) from the patient care area, such as a radiopharmaceutical handling space, or hot lab, in a hospital, clinic, or mobile coach
- A designated area for medication preparation that is clean and free from clutter
- Low traffic (i.e., limited number of people going in and out or moving around the area during times that radiopharmaceutical processing is being carried out)

### SRPA WITH VERTICAL FLOW ISO CLASS 5 PEC(S) FOR RADIOPHARMACEUTICAL PREPARATIONS

An SRPA with vertical ISO Class 5 PECs must meet the following requirements:

- Area surrounding the PEC may be ambient (unclassified) atmosphere
- Area must be clean, uncluttered, and dedicated to the processing of radiopharmaceuticals
- Appropriate for preparation, preparation with minor deviations, repackaging, and dispensing of radiopharmaceuticals

An area that meets ISO Class 8 total airborne particle-count specifications may be used to store and elute non-direct infusion radionuclide generators (e.g., Tc-99m).

### AN ISO CLASS 8 BUFFER AREA WITH VERTICAL FLOW ISO CLASS 5 PEC(S) WITH AN ADJACENT ISO CLASS 8 ANTE-ROOM

This environment is appropriate for all activities listed in *SRPA with Vertical Flow ISO Class 5 PEC(s) for Radiopharmaceutical Preparations*.

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AN ISO CLASS 7 BUFFER AREA WITH VERTICAL FLOW ISO CLASS 5 PEC(S) WITH AN ADJACENT ISO CLASS 8 OR BETTER ANTE-ROOM

This environment is appropriate for all activities listed in *An ISO Class 8 Buffer Area with Vertical Flow ISO Class 5 PEC(s) with an Adjacent ISO Class 8 Ante-Room* and sterile compounding.

HOT-CELL

This environment is appropriate for all activities listed in *SRPA with Vertical Flow ISO Class 5 PEC(s) for Radiopharmaceutical Preparations*.

CERTIFICATION OF PECS AND ENVIRONMENT IN WHICH THE PEC IS LOCATED

Certification of the classified areas, including the PEC, must be performed initially and recertification must be performed at least every 6 months using procedures outlined in the current Controlled Environment Testing Association (CETA) certification guide for *Sterile Compounding Facilities*, or an equivalent guideline, and must include the following:

- Airflow testing: To determine acceptability of the air velocity, the air exchange rate, and area pressure cascade to ensure that air consistently flows from most to least clean areas, and that the appropriate quality of air is maintained under dynamic operating conditions.
- HEPA filter integrity testing: HEPA filters must be leak tested after installation and as part of recertification.
- Total particle counts testing: Conducted under dynamic operating conditions using calibrated electronic equipment.
- Smoke visualization studies: Performed under either simulated or dynamic operating conditions to demonstrate unidirectional airflow and sweeping action over and away from the preparation(s).

In cases where technologies exist for hot-cell and PEC configurations that are not consistent for certification by the current CETA standards, other equivalent means for certifying the PEC may be performed and documented per facility SOPs. In this case, the PEC must maintain the environmental equivalent for total particle counts and the protection of the ISO Class 5 area from intrusions of lesser controlled air.

DAILY MONITORING OF ENVIRONMENT

The temperature and humidity must be monitored in the SRPA or area containing a hot-cell, and if in a classified area the pressure must monitored, each day that preparations are made, either manually or by a continuous recording device. These include:

- Relative humidity should be kept at 60% or lower
- Temperature and relative humidity continuous readings must be confirmed daily to have remained within the acceptable range
- Excursions must be documented and, if applicable, appropriate corrective actions taken
- Temperature monitoring devices must be verified for accuracy every 12 months or as required by the manufacturer
- Monitoring of pressure differentials must be performed

See *Packaging and Storage Requirements* ⟨659⟩ for information on controlled area temperature and allowable excursions.

## 6. MICROBIOLOGICAL AIR AND SURFACE MONITORING

An effective air and surface monitoring program provides information on the environmental quality of the classified areas where sterile radiopharmaceuticals are processed. The program identifies environmental quality trends over time, potential routes of microbiological contamination, and allows for implementation of corrective actions to prevent microbiological contamination of the radiopharmaceuticals. Facilities must develop and implement written air and surface monitoring procedures for all sterile radiopharmaceutical classified areas. Air and surface monitoring results and the corrective actions must be documented, and records must be readily retrievable as required by jurisdictional laws and regulations.

### 6.1 General Monitoring Requirements

The goals of an air and surface monitoring program are to determine whether microbiological contamination is present at unacceptable levels and to assess whether proper personnel practices are being followed, cleaning and disinfecting agents are effective, and environmental quality is maintained. The microbiological air and surface monitoring program must include viable impact volumetric airborne particulate sampling and surface sampling.

Air and surface sampling must be performed initially for classified areas in a facility to establish a baseline level of environmental quality. After initial sampling, the classified areas must be monitored according to the minimum frequencies described in this section to ensure that the environment remains in a suitable state for aseptic processing tasks.

The air and surface monitoring program involves the collection and evaluation of samples from various air and surface locations to detect viable microbiological contaminants. The data are then used to assess risks for contamination, potential routes of contamination, and the adequacy of cleaning and disinfection techniques and agents specified in the facility SOPs. Regular review of the sampling data must be performed to detect trends such as elevated levels of microbial bioburden, elevated levels of nonviable particulates, or other adverse changes within the environment. Evaluating results collected over a period of time can be useful in identifying trends or determining that a significant change has occurred, even when the results fall within the specified limits.

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In addition, results must be reviewed in conjunction with personnel data (i.e., training records, visual observations, competency assessments) to assess the state of control and to identify potential risks of contamination. Prompt corrective action in response to any adverse findings is required to maintain the necessary environmental quality for handling sterile radiopharmaceutical. Data must also be reviewed following corrective actions to confirm that the actions taken have been effective in achieving the required air and surface quality levels (see *Table 3* and *Table 4*).

Air and surface sampling must be conducted during actual or simulated dynamic operating conditions to confirm that the required environmental quality in classified areas is maintained. Due to radiation exposure concerns for the workers involved, it is permissible for sampling to be carried out at the conclusion of sterile radiopharmaceutical processing but prior to cleaning and disinfecting the surface area. In this case, simulated tasks that are reflective of the routine aseptic activities are performed. In addition to the specific sampling frequencies described in this section, sampling must be performed in any of the following circumstances:

- In conjunction with the certification of new facilities and equipment
- After any modification of facilities or equipment
- In response to identified problems (e.g., positive growth in sterility tests of compounded radiopharmaceuticals)
- In response to identified trends (e.g., repeated positive gloved fingertip sampling results or failed media-fill testing involving more than one operator where a review of the operator technique shows no reasonable flaws in process; repeated observations of air or surface contamination)
- In response to changes that could impact the controlled area environments (e.g., significant change in cleaning process or the agents involved)

To obtain an air and surface sample that is representative of the typical aseptic operating conditions at the facility, air and surface sampling must be conducted under dynamic or simulated dynamic operating conditions in all PECs and classified areas. If conducted during actual sterile processing, the monitoring program must be designed and conducted in a manner that minimizes the chance that the sampling itself will contribute to contamination of the sterile radiopharmaceutical(s) or the environment.

The air and surface monitoring program must be clearly described in the established SOPs of the facility and must include a diagram of the sampling locations, SOPs for collecting samples, frequency of sampling, size of samples (e.g., surface area, volume of air), time of day of sampling in relation to activities in the classified areas, and action levels that will trigger corrective action. The locations of sampling should be carefully selected based on their relationship to the activities performed in the area. It is important to obtain samples from locations that pose the highest possible contamination risk to the sterile radiopharmaceuticals involved with the operation's processes and that are likely to be representative of the conditions throughout the area.

Evaluating results collected over a period of time can be useful in identifying trends or determining that a significant change has occurred, even when the results fall within the specified limits.

It is important that personnel who operate the equipment be trained in the proper operation of the air and surface sampling equipment to ensure accurate and reproducible sampling. All air sampling devices must be serviced and calibrated as recommended by the manufacturer.

## 6.2 Monitoring Air Quality for Viable Airborne Particles

A monitoring program for viable airborne particles must be developed and implemented to assess microbiological air quality in all classified areas.

### VIABLE AIR SAMPLING: TIMING AND LOCATIONS

Volumetric active air sampling of all classified areas (e.g., ISO Class 5 PEC and ISO Class 7 and 8 areas) using an impaction device must be conducted during dynamic operating or simulated operating conditions at least every 6 months.

Air sampling sites must be selected in all classified areas. When conducting sampling of the PEC, care should be taken to avoid disturbing unidirectional airflow if taken during actual sterile processing activities. Viable air sampling must include:

1. Follow the manufacturer's instructions for operation of the air sampling device, including placement of media.
2. Using the sampling device, test at least 1 cubic meter or 1000 liters of air from each location sampled.
3. At the end of the sampling, retrieve the media plates/devices and cover.
4. Invert the media and incubate at 30°–35° for no less than 48 hours. Examine for growth. Record the total number of discrete colonies of microorganisms on each plate as cfu/$m^3$ of air on an environmental sampling form based on sample type (i.e., viable air). Include sample location and date.
5. Then incubate the inverted media at 20°–25° for no less than 5 additional days. Examine the media plates for growth. Record the total number of discrete colonies of microorganisms on each plate as cfu/$m^3$ of air on an environmental sampling form based on sample type (i.e., viable air). Include sample location and date.

Alternatively, to shorten the overall incubation period, two samples may be collected for each sample location and incubated concurrently. Both samples could be TSA or one sample could be TSA and the other fungal media [e.g., malt extract agar (MEA) or sabouraud dextrose agar (SDA)]. Incubate each sample in a separate incubator. Incubate one sample at 30°–35° for no less than 48 hours, and incubate the other sample at 20°–25° for no less than 5 days. Fungal media samples must be incubated at 20°–25° for no less than 5 days. Count the total number of discrete colonies of microorganisms on each sample, and record these results as cfu per sample.

Record the results of the sampling on an environmental sampling form based on sample type (i.e., viable air) and include the sample location, and sample date.

A general microbiological growth medium that supports the growth of bacteria and fungi must be used (e.g., TSA medium). CoA(s) from the manufacturer must verify that the medium meets the expected growth promotion, pH, and sterilization

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requirements. Samples must be incubated in a temperature monitored incubator with a calibrated measuring device. The incubator temperature must be monitored during incubation, either manually or by a continuous recording device, and the results must be reviewed and documented. Incubators used for microbiological testing must be placed in a location outside of any classified area or SRPA and kept away from areas where compounding or sterile processing activities are carried out. All sampling activities must be performed by trained individuals.

### DATA EVALUATION AND ACTION LEVELS

Evaluate cfu counts against the action levels in *Table 3* and in relation to previous data to identify adverse results and/or trends. If two pieces of media were collected at a single location, all recovered growth on each must be documented and action levels are applied individually to each plate/device (i.e., results from each cubic meter of air sampled must be compared to the action level for that area). If levels measured during the viable air monitoring program exceed the levels in *Table 3* for the ISO classification levels of the area sampled, the cause must be investigated and corrective action must be taken. The corrective action plan must be dependent on the cfu count and the microorganism recovered. Some examples of corrective action include process or facility improvements, personnel training, cleaning and disinfecting, or HEPA filter replacement and/or repair, or reducing the BUD of the radiopharmaceutical during investigation and while carrying out the corrective action plan. The extent of the investigation should be consistent with the deviation and should include an evaluation of trends. The corrective action plan must be documented. If levels measured during viable air sampling exceed the levels in *Table 3*, an attempt must be made to identify any microorganism recovered to the genus level (see *Microbial Characterization, Identification, and Strain Typing* ⟨1113⟩) with the assistance of a qualified individual (e.g., microbiologist or industrial hygienist).

**Table 3. Action Levels for Viable Airborne Particle Air Sampling[a]**

| ISO Class | Air Sampling Action Levels [cfu/m$^3$ (1000 L) of air per plate] |
|---|---|
| 5 | >1 |
| 7 | >10 |
| 8 | >100 |

[a] Adapted from *Guidance for Industry: Sterile Drug Products Produced by Aseptic Processing—Current Good Manufacturing Practice*. US Department of Health and Human Services, Food and Drug Administration (FDA), September 2004.

## 6.3 Monitoring Surfaces for Viable Particles

Surface sampling is an important component of the maintenance of a suitably controlled environment for sterile radiopharmaceutical processing, especially because transfer of microbial contamination from improperly disinfected work surfaces (e.g., via inadvertent touch contact by personnel) is a potential source of contamination of the radiopharmaceutical(s). Surface sampling is useful for evaluating facility cleaning and material handling procedures, work surface cleaning and disinfecting procedures, and personnel competency in work practices such as proper cleaning and disinfection. All sampling sites and procedures must be described in the facility's SOP.

### SURFACE SAMPLING: TIMING AND LOCATIONS

Surface sampling of all classified areas and all PECs must be conducted at least monthly for the detection of microbial contamination. Each classified area must be sampled (see *Microbiological Control and Monitoring of Aseptic Processing Environments* ⟨1116⟩). The DPA of the PEC, and any equipment permanently contained in the PEC, must be sampled. Staging or work surfaces in classified areas near the PEC, frequently touched surfaces in classified areas, and pass-through enclosure(s) for all classified areas are to be evaluated to determine the locations that pose the greatest risk of harboring microbial contamination.

Surface sampling must be performed at the end of the radiopharmaceutical aseptic activities or shift, but before the area has been cleaned and disinfected. However, radiopharmaceutical personnel must also consider the appropriate exposure and contamination prevention measures prior to and while collecting samples. If the worker assesses that the risk for exposure is not in conformance with ALARA safety standards, measures must be taken to eliminate the risk (e.g., implementation of appropriate shielding, performing the sampling at a later time or alternate day).

### SAMPLING PROCEDURES

Surface sampling devices (e.g., plates, paddles, or slides) containing microbial growth media must be used for sampling flat surfaces. CoAs from the manufacturer must verify that the media meet the expected growth promotion, pH, and sterilization requirements. Surface sampling devices must contain general microbial growth media (e.g., TSA) supplemented with neutralizing additives (e.g., lecithin and polysorbate 80) to neutralize the effects of any residual disinfecting agents. If used, contact plates must have a raised convex surface. Sterile swabs wetted with sterile water or a sterile neutralizing buffer may be used when sampling irregular surfaces and difficult-to-reach locations, such as crevices, corners, and spaces between surfaces. After sampling, the sampled area must be thoroughly cleaned and disinfected.

Use the following procedures for surface sampling on flat surfaces:

1. Remove the cover from the surface sampling device. Firmly press, using a rolling motion, if possible, the media surface onto the surface to be sampled. The surface sampling device will leave a residue of growth medium on the sample site. After sampling, use sterile 70% IPA to remove residue. Cover each surface sampling device.
2. If using plates, invert the plates.

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3. Incubate the surface sampling devices at 30°–35° for no less than 48 hours. Examine for growth. Record the total number of discrete colonies of microorganisms on each media device as cfu/sample on an environmental sampling form based on sample type (i.e., surface). Include sample location and date.
4. Incubate the device at 20°–25° for no less than 5 additional days. Examine the media plates for growth. Record the total number of discrete colonies of microorganisms (cfu/sample) on the environmental sampling record based on sample type (i.e., surface). Include sample location and date.

Alternatively, to shorten the overall incubation period, two samples may be collected for each sample location.

1. Both samples could be TSA or one sample could be TSA and the other fungal media (e.g., MEA or SDA).
2. Incubate each sample in a separate incubator. Incubate one sample at 30°–35° for no less than 48 hours, and incubate the other sample at 20°–25° for no less than 5 days.
3. If fungal media are used as one of the samples, incubate the fungal media sample at 20°–25° for no less than 5 days.
4. Count the total number of discrete colonies of microorganisms on each sample, and record these results as cfu per sample. Record the results of the sampling.
5. Record the results of the sampling.

### DATA EVALUATION AND ACTION LEVELS

Evaluate cfu counts against the action levels in *Table 4* and examine counts in relation to previous data to identify adverse results or trends. If two devices were collected at a single location, all recovered growth on each must be documented and action levels are applied to each piece of media individually (i.e., results from each sampling device must be compared to the action level for the area). If levels measured during surface sampling exceed the levels in *Table 4* for the ISO classification levels of the area sampled, the cause must be investigated and corrective action must be taken. Data collected in response to corrective actions must be reviewed to confirm that the actions taken have been effective. The corrective action plan must be dependent on the cfu count and the microorganism recovered. Examples of corrective action include process or facility improvements, personnel training, cleaning and disinfecting, or HEPA filter replacement and/or repair, or reducing the BUD of the radiopharmaceutical(s) during investigation and while carrying out the corrective action plan. The extent of the investigation should be consistent with the deviation and should include an evaluation of trends. The corrective action plan must be documented. If levels measured during surface sampling exceed the levels in *Table 4*, an attempt must be made to identify any microorganism recovered to the genus level (see ⟨1113⟩) with the assistance of a qualified individual (e.g., microbiologist or industrial hygienist).

**Table 4. Action Levels for Surface Sampling**

| ISO Class | Surface Sampling Action Levels (cfu/device or swab) |
|---|---|
| 5 | >3 |
| 7 | >5 |
| 8 | >50 |

## 7. CLEANING AND DISINFECTING

Cleaning and disinfecting are important because surfaces in classified areas and SRPAs are a potential source of microbial contamination of sterile radiopharmaceuticals. The process of cleaning involves removing organic and inorganic residues from surfaces, usually with a manual or mechanical process and a cleaning agent. The process of disinfecting involves destruction of microorganisms, usually with a chemical or physical agent. Surfaces must be cleaned prior to being disinfected unless an Environmental Protection Agency (EPA)-registered (or equivalent) one-step disinfectant cleaner is used to accomplish both the cleaning and disinfection in one step. After cleaning and disinfecting or the application of a one-step disinfectant cleaner in a PEC, apply sterile 70% IPA to remove any residue.

Cleaning and disinfecting surfaces should occur at the minimum frequencies specified in *Table 5* or if activities are not performed daily, cleaning and disinfecting must be completed before initiating activities. The act of reducing or removing radioactivity (radioactive decontamination) from an object or surface must be balanced with the risk of spreading radioactive contamination. At times the best approach may be to shield the area until the radiation exposure levels are lower. This balance must be specified in SOPs (e.g., trigger levels for safe cleaning). The PEC should be checked for radioactive contamination prior to cleaning and disinfecting to prevent spreading radioactive contamination in the PEC.

All cleaning and disinfecting activities must be performed by trained and appropriately garbed personnel using facility-approved agents and procedures that must be described in written SOPs. Cleaning must be performed in the direction of most to least clean areas. The frequency, method(s), and location(s) of cleaning, disinfecting, and sporicidal agent use must be established in written SOPs, in accordance with the manufacturer's instructions when available, or based on sound microbiological cleaning techniques when unavailable, and must be followed by all cleaning personnel. The manufacturer's direction or published data for the minimum contact time must be followed for the cleaning, disinfecting, and sporicidal agents used. When sterile 70% IPA is used, it must be allowed to dry. All cleaning, disinfecting, and application of sporicidal agents must be documented according to facility SOPs.

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**Table 5. Minimum Frequency for Cleaning and Disinfecting Surfaces in Classified Areas and within the Perimeter of the SRPA**

| Site | Cleaning | Disinfecting[a] | Applying Sporicidal |
|---|---|---|---|
| PEC(s) and equipment inside the PEC(s) | Prior to performing sterile processing of radiopharmaceuticals on each day that activities are carried out, the walls, bars, torso shield and any exposed surface of equipment inside the PEC must be cleaned to the extent possible as specified by the equipment manufacturer or the assessment of a qualified individual (e.g., microbiologist or industrial hygienist). Radioactive contamination may be shielded with appropriate temporary material, providing the material is covered with low-lint absorbent pads or has equivalent low-shedding properties. | Following cleaning on each day that activities are carried out, exposed surfaces of the equipment should be disinfected to the extent possible as specified by the equipment manufacturer or the assessment of a qualified individual (e.g., microbiologist or industrial hygienist).<br>When used, remove low-lint absorbent pads and survey the PEC for radioactive contamination prior to disinfecting. Replace with new pads after disinfecting or as required after spills. | Monthly |
| Surfaces of sink(s) | Daily | Daily | Monthly |
| Hot-cells (all interior surfaces, dependent on design, equipment, and shielding present) | Daily | Daily | Monthly |
| PEC and the equipment inside the PEC(s) located in a hot-cell | Prior to performing sterile processing of radiopharmaceuticals on each day that activities are carried out, the walls, bars, torso shield, and any exposed surface of equipment inside the PEC to the extent possible as specified by the equipment manufacturer or the assessment of a qualified individual (e.g., microbiologist or industrial hygienist). Radioactive contamination may be temporarily shielded with appropriate temporary material providing the material is covered with low-lint absorbent pads or has equivalent low-shedding properties. | Following cleaning on each day that activities are carried out, exposed surfaces of the equipment should be disinfected to the extent possible as specified by the equipment manufacturer or the assessment of a qualified individual (e.g., microbiologist or industrial hygienist) and should be specified by SOPs.<br>Remove low-lint absorbent pads and survey the PEC for radioactive contamination prior to disinfecting. Replace with new pads after disinfecting or as required after spills. | Monthly |
| Work surface(s) outside the PEC | Daily | Daily | Monthly |
| Ceiling(s) | Monthly | Monthly | Monthly |
| Wall(s),<br>door(s),<br>door frame(s), and other fixtures | Monthly | Monthly | Monthly |
| Floor(s) | Daily | Daily | Monthly |
| Storage shelving and storage bins | Monthly | Monthly | Monthly |

[a] Many disinfectants registered with the EPA are one-step cleaning and disinfecting agents, which means that the disinfectant has been formulated to be effective in the presence of light to moderate soiling without a separate cleaning step. Cleaning and disinfecting must be balanced with the risk of spreading radiation contamination. The best approach may be to shield the area until the radiation exposure levels are lower.

## 7.1 Cleaning, Disinfecting, and Sporicidal Agents

Cleaning and disinfecting agents must be selected and used with careful consideration of compatibilities, effectiveness, and user safety. Considerations when selecting and using disinfectants include their anti-microbial activity, inactivation by organic matter, residue, shelf life, preparation requirements of the agent, and suitability for surfaces being disinfected (see *Disinfectants and Antiseptics* ⟨1072⟩). After the disinfectant is applied on the surface to be disinfected, the disinfectant must be allowed to dwell for the minimum contact time specified by the manufacturer, during which time the surface cannot be disturbed. Only the 70% IPA used in the ISO Class 5 PEC must be sterile. Sporicidal agents must be used at least monthly on all surfaces in classified areas and SRPAs. Some EPA-registered (or equivalent) one-step disinfectant cleaners may have sporicidal properties. See *Table 6* for a summary of the purpose of the cleaning, disinfecting, and sporicidal agents.

**Table 6. Purpose of Cleaning, Disinfecting, and Sporicidal Agents**

| Type of Agent | Purpose |
|---|---|
| Cleaning agent | An agent for the removal of residues (e.g., dirt, debris, microbes, and residual drugs or chemicals) from surfaces. |
| Disinfecting agent | A chemical or physical agent used on inanimate surfaces and objects to destroy fungi, viruses, and bacteria. |
| Sporicidal agent | A chemical or physical agent that destroys bacterial and fungal spores when used in sufficient concentration for a specified contact time. It is expected to kill all vegetative microorganisms. |

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## 7.2 Cleaning Supplies

All cleaning supplies (e.g., wipers and mop heads), with the exception of tool handles and holders, must be low-lint and should be disposable. If disposable cleaning supplies are used, they must be discarded after each cleaning activity. Reusable cleaning tools must be made of cleanable materials (e.g., no wooden handles) and must be cleaned and disinfected before and after each use. Reusable cleaning tools must be dedicated for use in the classified areas or SRPAs and must not be removed from these areas except for disposal. They must be discarded after an appropriate amount of time, to be determined based on the condition of the tools. Cleaning supplies and solutions used in the classified areas and SRPAs should be monitored for radioactive contamination after use and prior to disposal, as per facility SOPs. Dispose of cleaning supplies used in the classified areas and SRPAs in a manner that minimizes the potential for dispersing particulates into the air (e.g., with minimal agitation, away from work surfaces).

## 7.3 Cleaning and Disinfecting the PEC

Clean and disinfect the PEC at the minimum frequencies specified in *Table 5*. If the PEC contains a removable work tray, all sides of the work tray and the area underneath the work tray must be cleaned and disinfected at least monthly.

1. Survey all surfaces of the PEC for radioactive contamination and follow facility SOPs to decontaminate, if necessary.
2. Remove, if necessary, any particles, debris, or residue with an appropriate solution (e.g., *Sterile Water for Injection* or *Sterile Water for Irrigation*) using sterile, low-lint wipers.
3. Apply a cleaning agent followed by a disinfecting agent or apply an EPA-registered (or equivalent) one-step disinfectant cleaner and ensure that the contact time specified per manufacturer instructions is achieved.
4. Apply sterile 70% IPA
5. Allow the surface to dry completely before beginning activities.
6. The PEC must be wiped with a sporicidal agent at least monthly.

## 7.4 Disinfecting Supplies for Classified Areas and SRPAs

No shipping carton(s) or other corrugated or uncoated cardboard are allowed in the classified area (e.g., clean side of ante-room) or within the perimeter of the SRPA. Before items are introduced into a classified area or SRPA, they must be wiped with a sporicidal agent, EPA-registered (or equivalent) one-step disinfectant cleaner, or sterile 70% IPA using low-lint wipers. After the sporicidal or sterile disinfectant is applied onto the surface, the agent must be allowed to dwell on the surface for the minimum contact time specified by the manufacturer (see *6.1 General Monitoring Requirements*). The agent used for disinfecting the packaging must be compatible with the packaging and must not render the product label unreadable.

Any item to be transferred into the PEC from the classified area or SRPA must be disinfected with a sterile disinfectant (e.g., sterile 70% IPA).

In the case of radiopharmaceuticals being processed by remote means in a hot-cell, the opening of sterile packages (e.g., syringes, luer lock caps) may not be possible by remote means within the ISO Class 5 area. In this case, the syringes may be opened and appropriately labeled outside of the ISO Class 5 environment and placed in disinfected shielding, immediately prior to the forthcoming dispensing cycle.

## 7.5 Disinfecting Critical Sites

Critical sites (e.g., vial stoppers) must be wiped with sterile 70% IPA. The critical site must be wiped ensuring that both chemical and mechanical actions are used to remove contaminants. The sterile 70% IPA must be allowed to dry before piercing critical sites.

## 7.6 Cleaning and Disinfecting Items from Patient Care Area

Radiation shielding and equipment used in the classified area/SRPA or PEC that is exposed to patient care areas during the process of administration must be cleaned and disinfected before returning to any classified area (e.g., buffer or ante-room) or SRPA in accordance with the Centers for Disease Control and Prevention guidelines[1] as noncritical equipment requiring low-risk disinfection. Syringes that have been used in a patient care area must not be brought back into the classified area (e.g., buffer or ante-room) or SRPA for re-assaying or disposal unless the syringe is sealed inside an impervious container (e.g., sealed plastic bag) that is disinfected prior to entry into the classified area or SRPA. Equipment that has been exposed to needles and syringes contaminated with blood-borne pathogens and RAMs are considered mixed waste (e.g., syringe shields and syringe carrying containers). This equipment must be cleaned and disinfected through actions regulated by the facilities' SOPs. Equipment that contained or was in contact with mixed waste must be cleaned and disinfected with an appropriate agent(s) for blood.

# 8. ASSIGNING BUD

BUDs are based on the risk of microbial contamination with the assumption that the radiopharmaceutical(s) should remain chemically and physically stable, and its container–closure system should maintain its integrity for the duration of the BUD (*Table 7*). The time starts at the moment of the first sterile vial puncture or exposure of a critical site (e.g., syringe tip, needle hub, or needle) to ambient air, whichever is first. The BUDs stated in *Table 7* are maximum values in the absence of sterility

[1] Centers for Disease Control and Prevention. *Guideline for Disinfection and Sterilization in Healthcare Facilities*, 2008.

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testing, and the assigned BUD may be shorter for a variety of reasons discussed below. The individual responsible for the manipulation assigns the BUD based on established testing data, either performed in-house or obtained from peer-reviewed literature.

**Table 7. Preparation Conditions for Sterile Radiopharmaceuticals**

| Preparation Conditions | | | |
|---|---|---|---|
| **Manipulation** | **PEC** | **SEC** | **BUD (hours)** |
| Immediate use | — | — | 1 |
| Direct infusion system, one puncture only (e.g., PET patient infusion system, Rb-82 generator) | — | — | 10 |
| Dispensing, repackaging, preparation, and preparation with minor deviations | ISO Class 5 | SRPA | 12 |
| Radionuclide generator storage/elution (e.g., non-direct infusion system; Tc-99m or Ga-68) | — | SRPA with ISO Class 8 total airborne particle count | 12 |
| Radionuclide generator storage/elution (e.g., non-direct infusion system; Tc-99m or Ga-68) | — | ISO Class 8 or better buffer area with ISO Class 8 or better ante-room | 24 |
| Dispensing, repackaging, preparation, and preparation with minor deviations | ISO Class 5 | ISO Class 8 or better buffer area with ISO Class 8 or better ante-room | 24 |
| Dispensing, repackaging, preparation, preparation with minor deviations, and compounding using sterile components | ISO Class 5 | ISO Class 7 or better buffer area with ISO Class 8 or better ante-room | 96 |
| Dispensing, repackaging, preparation, preparation with minor deviations, and compounding using a nonsterile component and performing sterilization procedure (e.g., filtration with bubble point testing) but without performing *Sterility Tests* ⟨71⟩ testing | ISO Class 5 | ISO Class 7 or better buffer area with ISO Class 8 or better ante-room | 24 |
| Radiolabeled blood components for immediate use [e.g., Tc 99m red blood cells (RBC)] | — | — | 1 |
| Radiolabeled blood components (e.g., radiolabeled leukocytes) | ISO Class 5 BSC | ISO Class 7 or better buffer area with ISO Class 8 or better ante-room | 6 h after the blood sample is obtained |

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For compounded preparations (sterile and nonsterile), the BUD is also dependent on maintenance of appropriate quality and purity, including radiochemical purity, radionuclidic purity, and other applicable parameters as specified in individual monographs or as clinically appropriate.

For preparations with minor deviations involving conventionally manufactured kits (sterile and nonsterile), the kit may state or suggest a use-by time in the package insert. For certain radiopharmaceuticals transportation time, radionuclide availability, and other factors may necessitate extending manufacturer-stated/suggested use-by time to meet patient needs. Assigning a BUD longer than the manufacturer-stated/suggested use-by time interval must be supported by evidence of the maintenance of appropriate quality and purity, including radiochemical purity and radionuclidic purity as specified in individual monographs, and other applicable parameters as clinically appropriate.

Assignment of a BUD for a radiopharmaceutical(s) must consider several factors, as applicable. Issues of concern include, but are not limited to, the following:

- Sterility: Maintenance of sterility is a major concern for any sterile preparation or product. Good aseptic handling practices in an appropriate environmentally-controlled area are the most critical factors in ensuring sterility. See *Table 7* for maximum BUD. The assigned BUD should not exceed the sterility-related times listed in *Table 7*, unless a longer time is justified by *Sterility Tests* ⟨71⟩.
- Radiochemical purity: Maintenance of radiochemical purity is affected by a variety of factors including, but not limited to, storage temperature, quantity of radioactivity, radioactivity concentration, presence or absence of antioxidants or other stabilizing agents, and container type (e.g., glass vial vs. plastic syringe). The assigned BUD must be based on stability studies in which these variables are controlled and are representative of the conditions of actual use. For factors that allow a range of values (e.g., storage temperature, quantity of radioactivity, radioactivity concentration), studies should be conducted at the extremes of the ranges.
- Radionuclidic purity: Because radionuclidic impurities may decay away more slowly than the primary radionuclide, the radionuclidic purity may decrease over time. For example, the ratio of Mo-99 (half-life of about 66 hours) to Tc-99m (half-life of about 6 hours) continuously increases over time. *USP* monographs for Tc-99m radiopharmaceuticals require that the radionuclidic impurity Mo-99 not exceed 0.15 μCi Mo-99 per mCi Tc-99m at the time of administration. Calculation of radionuclidic purity at future times is necessary to ensure compliance throughout the assigned BUD.

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- Age of generator eluate: As a generator eluate decays, the desired daughter radionuclide decays to form other nuclides and potential radiolytic products, which may interfere with radiolabeling of kits. For example, Tc-99m undergoes decay to Tc-99. More importantly, increasing amounts of peroxides formed as radiation interacts with water molecules. Increased amounts of Tc-99 and peroxides can interfere with the radiolabeling of many kits. Extension of the BUD for Tc-99m pertechnetate intended for radiolabeling of kits must take into account the build-up of Tc-99 and peroxides over time.
- Number of particles: For radiolabeled particulates, the number of particles per unit radioactivity increases over time as the radionuclide decays. For example, the BUD for Tc-99m albumin aggregated [macroaggregated albumin (MAA)] must take into account the increasing ratio over time of the number of particles per unit radioactivity. For example, if an MAA kit is prepared such that the radioactive patient dose is 200,000 particles at the time of calibration, the same patient dose will contain 700,000 particles at 10.85 hours after calibration. Calculation of the number of MAA particles in the patient dose is necessary to ensure compliance with the prescribed particle range throughout the assigned BUD.
- Specific activity: For some receptor-based radiopharmaceuticals, the mass quantity may influence uptake (i.e., too much mass may result in saturation of receptor sites and reduce target uptake of the radiopharmaceutical). As radioactivity decays over time, specific activity decreases resulting in more mass per unit radioactivity. In such situations, the assigned BUD must ensure that the patient dose contains no more than the specified maximum mass.
- Container type: Because radiochemical stability or other quality attributes of a radiopharmaceutical may be affected by its container characteristics, the BUD for a radiopharmaceutical dose dispensed in a plastic syringe may be different than the BUD of that same radiopharmaceutical maintained in a glass vial. The assigned BUD must be determined in the proper storage container.
- Cell viability: The viability of radiolabeled blood cells (e.g., leukocytes) decreases over time, and may also be affected by other factors such as the suspending medium, temperature, and agitation. The assigned BUD should be as short as circumstances reasonably allow so as to maximize cell viability.
- In the case of manufactured radiopharmaceuticals that are distributed to nuclear pharmacies or other healthcare facilities for terminal distribution/dispensing, the assigned BUD of the dispensed dose cannot exceed the expiration date/time of the manufactured radiopharmaceutical(s).
- In the case of radiopharmaceuticals prepared from kits, the BUD of a dispensed dose cannot exceed the assigned BUD of the finished kit preparation.
- A radiopharmaceutical may not exceed the shortest BUD of any of its components.

The facility must have policies and SOPs appropriate to the assignment of BUD and maintain documentation of applicable study results and calculations. Studies of radiolabeling efficiency and radiochemical stability should employ quality control (QC) testing methods described in the manufacturer's package insert, *USP* monographs and general chapters, or other equivalent testing methods and be sufficiently rigorous to allow statistical confidence in the results.

The facility must have SOPs to collect and evaluate complaints associated with the use of radiopharmaceuticals having assigned BUDs. Policies and SOPs should also be in place to reevaluate the assigned BUD based on complaints, which may include repeating studies and/or performing additional studies on radiolabeling efficiency and/or radiochemical stability.

## 9. DOCUMENTATION

Applicable records (hard-copy or electronic), including policies and SOPs, must be maintained for all activities involved in repackaging, preparing, preparing with minor deviations, compounding, and dispensing radiopharmaceuticals. Such records include, but are not limited to:

- Personnel training and testing, including visual assessment of aseptic technique competency, validation, garbing, hand hygiene, equipment/environment cleaning and disinfecting, gloved fingertip and thumb sampling, and media fill evaluation initially and follow up testing at specified intervals.
- Testing and monitoring of environmental controls, including ISO classification, ACPH, pressure differentials, temperature, humidity and viable air/surface and total airborne particle test results
- Equipment maintenance and cleaning/disinfecting
- End product radiochemical purity and other testing, as applicable, results of preparations, preparations with minor deviations, and compounded preparations
- Master Formulation Record (MFR) for preparation with minor deviation(s) and compounding
- Validation of stability testing to support the assigned BUD from SOPs by the compounder or derived from accepted literature
- Investigations and corrective actions and tracking of events to closure.

### 9.1 Master Formulation Record

A MFR is required only for a preparation with minor deviations or compounding, as described in *11. Compounding*. A MFR is not required for a preparation following the manufacturer's instructions.

Data that must be included in the MFR are as follows:

- Name of the radiopharmaceutical
- Name, identity, strength, purity, quality, and quantity of ingredients with validated documentation (e.g., CoA)
- Detailed procedure (e.g., heating, components, incubation time)
- Range of radioactivity
- Range of volume
- Equipment to be used

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- PEC and SEC to be used, if applicable
- Quality control tests to be performed for final release of the radiopharmaceutical (e.g., radiochemical purity, pH)
- Procedures for depyrogenation and sterility procedures and validations, as applicable, including limits
- Trained personnel
- Garbing procedure, if different than standard procedure
- Container(s)
- Reference source of the BUD assignment and storage conditions

### 9.2 Records for Preparation with Minor Deviations/Compounding

A record for preparation with minor deviation or compounding must include the following:

- Name of the radiopharmaceutical
- Physical form (e.g., capsule or solution)
- Name and quantity of ingredients including calibration time for radioactive ingredients (e.g., 100 mCi Tc 99m sodium pertechnetate @ 1300)
- Total volume
- Reference to the MFR
- Any deviation from the MFR, if applicable
- Name of vendor or manufacturer, lot numbers, and expiration dates of all ingredients and components
- Name of the person who prepared and name of the supervising personnel (e.g., ANP or AU physician)
- Date and time of preparation
- Assigned internal identification number (e.g., lot number)
- Unique reference [e.g., prescription, order number(s)]
- Assigned BUD and storage requirements
- Documentation of QC results

## 10. PREPARATION

The individual responsible for preparing the radiopharmaceutical(s) must ensure that the final preparation complies with quality and purity specifications throughout the assigned BUD. This includes, as appropriate for the preparation, radionuclidic purity, radiochemical purity, chemical purity, and physical and chemical properties.

### 10.1 Preparation Following Manufacturer Instructions

NONSTERILE PREPARATIONS

For nonsterile preparations, follow manufacturer preparation instructions (e.g., I-131 NaI capsules or solution), taking into account appropriate radiation safety considerations and environmental controls, if applicable (e.g., negative air pressure area, chemical fume hood, activated charcoal filters when handling a potentially volatile radionuclide). The area should be suitably cleaned and uncluttered to ensure the overall integrity and quality of the prepared radiopharmaceutical(s). There should be a documented process for activities (e.g., cleaning) between the preparation cycles of different nonsterile products, to decrease the likelihood of contamination from other prepared products.

STERILE PREPARATIONS

For sterile preparations (including intravascular devices), follow manufacturer preparation instructions, taking into account appropriate radiation safety considerations, appropriate environmental controls, and aseptic handling practices to maintain sterility. The minimum environmental standard for the preparation of sterile radiopharmaceuticals beyond immediate-use is within an ISO Classified area or device (see *Table 7*). Refer to *5. Facilities and Environmental Controls* and *Table 7* on the location of the PEC and the assignment of the BUD.

### 10.2 Preparation with Minor Deviations

In some cases, radiopharmaceuticals are prepared with minor deviations from manufacturer instructions that are necessary to accommodate circumstances not contemplated in the FDA-approved labeling. Note that *General Notices, 5.20.20.1 In Compounded Preparations* includes the statement: “Deviation from the specified processes or methods of compounding, although not from the ingredients or proportions thereof, may occur provided that the finished preparation conforms to the relevant standards and to preparations produced by following the specified process.” However, except for a few receptor-based radiopharmaceuticals where specific activity is an important parameter, there is a very broad range of acceptable values for specific activity and for proportions of ingredients. Deviations from manufacturer preparation instructions for radiopharmaceuticals must maintain the same ingredients but may differ in their proportions.

This requires appropriate in-house QC testing, designed to validate the radiochemical purity of the product for the entirety of the BUD or is supported by appropriate peer-reviewed publications for the minor deviation utilized.

Examples of minor deviations include, but are not limited to, the following:

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- Altering the quantity of radioactivity or volume added to the vial
- Changes in step-by-step operations (e.g., dilute Tc-99m sodium pertechnetate after rather than before addition to the vial)
- Using alternative devices or equipment (e.g., a heating block rather than a hot water bath, using a different sized needle, different shielding materials)
- Using QC test methods other than those described in the product labeling (e.g., radiochemical purity)
- Filtering Tc-99m sulfur colloid

## 10.3 Preparation of Radiolabeled Blood Components

Handling blood and radiolabeling of blood components requires special attention to biological risks and must be handled with standard precautions using aseptic technique to prevent the introduction of new microorganisms into the preparation that will be administered. Due to the potential presence of microorganisms in the original blood sample, the preparation must be administered as soon as possible but no later than 6 hours after the blood sample is obtained from the patient or blood bank.

The presence of microorganisms in a blood sample may present a risk to the individual performing the preparation as well as cross-contamination to other blood samples or other non-blood related radiopharmaceuticals. Equipment and supplies should never be shared with other activities unless they are first thoroughly cleaned and disinfected. Special precautions when radiolabeling of blood components for non-immediate use include:

- There must be complete physical separation (either fixed or non-fixed wall) of areas where blood products are handled from areas where non-blood products are handled. An ISO Class 5 BSC located in an ISO Class 7 buffer area is required for blood-labeling processes. If more than one ISO Class 5 PEC is located within the ISO Class 7 buffer area, policies and SOPs must be in place to include certification that the SEC meets conditions of air quality at maximum occupancy under dynamic operating conditions.
- One radiolabeling procedure per PEC at a time. Blood products from more than one patient must never be manipulated at the same workstation at the same time. Each area should have dedicated supplies, equipment (including dose calibrator), and waste disposal to eliminate sharing of these items or overlap in pathways.
- Thorough cleaning and disinfection of the ISO Class 5 BSC and all reusable equipment within, prior to starting another blood component radiolabeling procedure.
- If a dedicated dose calibrator is not available, then a means of preventing the blood container(s) from contaminating the dose calibrator must be used or the dose calibrator dipper and liner must be cleaned and disinfected following the radioassay.
- Centrifuge should be located within the ISO Class 7 buffer area that is dedicated for blood component radiolabeling processes.
- Dedicated (per each radiolabeling procedure) consumable products (e.g., 0.9% sodium chloride injection, diluent, tubes, syringes, and other supplies) necessary for each individual patient radiolabeling procedure.
- All tubes and syringes in contact with the patient's blood components must be clearly labeled with the patient's name and at least one additional identifier (e.g., date of birth, medical record number, barcode).
- Dedicated syringe shields and vial shields.
- Remove and replace any garb that enters the ISO Class 5 BSC before handling anything else not related to performing this procedure.
- Removal of all disposable items from the ISO Class 5 BSC utilized in each radiolabeling procedure.
- Cleaning and disinfection of all reusable equipment and components (e.g., BSC, centrifuge, dose calibrator, syringe shields, vial shields, syringe transport shields and delivery cases) after each radiolabeling procedure prior to any further use. Policies and SOPs must address cleaning and disinfection processes including the use of an EPA-registered (or equivalent) one-step disinfectant cleaner with activity against blood-borne pathogens followed by sterile 70% IPA. Sterile 70% IPA alone is not sufficient.
- After the completion of blood radiolabeling procedures, follow all requirements in *4.5 Hand Hygiene and Garbing for Buffer Areas and segregated Radiopharmaceutical Processing Area*.

## 10.4 Preparation of Radiolabeled Red Blood Cells for Immediate Use

In vitro red blood cell labeling must be prepared while following the conditions below:

- A dedicated space for blood handling must be designated through the entirety of the blood radiolabeling process. This area must be free from clutter and not used for any other radiopharmaceutical preparation or handling until the completion of cleaning and disinfection.
- Perform only one radiolabeling procedure at a time or have documented processes that maintain the integrity of samples and environment.
- Dedicated equipment must be used for blood radiolabeling procedure (e.g., L-block, syringe shield, vial shield, forceps, needle recapper).
- If a dedicated dose calibrator is not available, then a means of preventing the blood container(s) from contaminating the dose calibrator or a cleaning and disinfecting procedure with an appropriate product must be used to decontaminate the dipper and liner of the dose calibrator following the radioassay
- A cleaning and disinfecting procedure with an appropriate agent(s) must be used to decontaminate the area and equipment prior to and after the radiolabeling is complete and all disposable components have been discarded
- Follow all requirements in *4.4 Hand Hygiene and Garbing for Immediate Use Preparations*.

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- The start time of the preparation must begin with the initial container puncture or the exposure of a critical site (e.g., syringe tip, needle hub or needle) to ambient air, whichever is first.
- BUD of 1 hour (see *Table 7*).

# 11. COMPOUNDING

Each compounding activity must be based on a pre-established written procedure and must include maintenance of compounding records. The compounding record must provide traceability (see *9. Documentation*).

All sterile compounding, using aseptic technique, must be performed in an ISO 5 PEC. Refer to *5.7 Environmental Controls* and *Table 7* for further clarification on the location of the PEC and the applicability of the radiopharmaceutical BUD.

Compounding must not be performed for any radiopharmaceutical(s) that has been withdrawn from the market because of safety or lack of effectiveness, unless part of an institutional review board approved investigational study. Radiopharmaceuticals that are essentially copies of marketed FDA-approved radiopharmaceuticals must not be compounded unless there is a change that produces a clinical difference for an identified individual patient, as determined by a prescriber.

## 11.1 Compounding Nonsterile Radiopharmaceuticals

Compounding nonsterile radiopharmaceuticals is the combining, mixing, diluting, pooling, reconstituting or otherwise altering a drug or bulk drug substance other than as provided by the manufacturer's package insert to create a nonsterile radiopharmaceutical. Examples of compounding nonsterile radiopharmaceuticals include: changing the dosage form of a capsule to a solution, changing an intravenous dosage form to an oral dosage form, and radiolabeling a food for oral administration (e.g., Tc-99m sulfur colloid in eggs). Areas designated for nonsterile compounding must be cleaned and uncluttered and separated from areas designated for sterile radiopharmaceuticals. Compounding should take into account RAM licensing requirements for appropriate radiation safety considerations and utilize appropriate environmental controls, if applicable (e.g., chemical fume hood, activated charcoal filters when handling potentially volatile radionuclides). The placement of equipment and materials must take into account a design that prevents cross-contamination.

When feasible, disposable material should be used to reduce the chance of cross-contamination. Each compound must have a unique MFR (see *9.1 Master Formulation Record*). The preparation information is documented on a compounding record (see *9.2 Records for Preparation with Minor Deviations/Compounding*). The MFR details the selection of all components. The ingredients must be obtained from sources in this preferential order: FDA-approved product; FDA-registered facility; and lastly, if the ingredients for the compound are not available from either of these two sources, the MFR must detail the selection of a material that is suitable for the intended use. The MFR must establish the identity, strength, purity, and quality of the ingredients by validated means (e.g., CoA). Requirements for nonsterile oral meal components are limited to common food grade description and are not required to establish identity by validated means.

A BUD for the compounded radiopharmaceutical must be validated, taking into account the stability of the ingredients, any intermediate containers, the final container, and the storage conditions. A BUD cannot be extended past the labeled expiration date of any component in the compound. If the compounded radiopharmaceutical(s) includes components from other preparations or preparations with minor deviations, the BUD of the final compounded radiopharmaceutical must not exceed the shortest remaining BUD of any of those components.

## 11.2 Sterile Compounding

Some compounding activities involve only the addition of a conventionally manufactured drug product (e.g., *Ascorbic Acid Injection*, *Lidocaine Hydrochloride Injection*, *Sodium Bicarbonate Injection*) approved by the appropriate regulatory agency to a radiopharmaceutical.

Personnel responsible for compounding must consider all possible interactions between the components, such as altered chemical stability, radiochemical stability, solubility, or other parameters (e.g., osmolality) related to changes in pH, excipients, or other factors, in determining an appropriate BUD. In some cases, this may require systematic QC testing over time to validate the appropriateness of a particular BUD.

Another activity that is considered a compounding activity is the splitting of conventionally marketed kits. Kit-splitting (also referred to as "fractionation") may be used to meet patient need. For example, the contents of a kit vial can be reconstituted with 0.9% sodium chloride injection and aliquoted into other containers for storage and subsequent radiolabeling. The individual responsible must consider all possible interactions of kit components with these other containers (e.g., container walls, closures), as well as possible alterations in stability (e.g., physical stability, chemical stability) that may affect radiolabeling yields or performance parameters, when determining an appropriate BUD. Systematic QC testing is required to validate the appropriateness of a particular BUD.

## 11.3 Sterile Compounding Using a Nonsterile Drug Substance or Components

Some sterile compounding activities involve the use of materials other than commercially marketed products, such as drug substances and/or radionuclides. If one or more materials or components are not certified to be sterile and pyrogen-free, a sterilization procedure (e.g., filtration with bubble point testing) and testing described in ⟨85⟩ must be performed. The designated person for compounding is responsible for ensuring that the final preparation complies with pre-established standards or acceptance criteria for identity, quality, and purity, and must consider all possible interactions between the components, such as altered chemical stability, radiochemical stability, solubility, or other parameters (e.g., osmolality) related to changes in pH, excipients, or other factors, in determining an appropriate BUD. This may require testing to validate the appropriateness of a particular BUD.

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If compounding involves a bulk drug substance, the radiopharmaceutical must comply with standards of an applicable *USP* or *NF* monograph, if one exists, or be a component of an approved drug product. For this chapter, a bulk drug substance includes a radionuclide, a ligand, or other substance, such as a precursor that becomes an active ingredient in the final radiopharmaceutical. Each bulk drug substance should be manufactured by drug establishments registered with FDA and be accompanied by a valid CoA or equivalent testing procedures.

If compounding involves excipients or other inactive ingredients, the excipients or other inactive ingredients must comply with standards of an applicable *USP* or *NF* monograph, if one exists. It is also acceptable that any excipients or other inactive ingredients be approved products, manufactured by a drug establishment registered with the FDA.

# 12. DISPENSING

## 12.1 Dispensing and Radioassay

Dispensing refers to the manipulations necessary to transfer the prescribed or ordered amount of radiopharmaceutical into the final container (e.g., syringe or vial). Dispensing can take place from single-dose or multiple-dose containers of prepared, prepared with minor deviations, compounded, or manufactured radiopharmaceuticals, and may involve needle changes, affixing a sterile cap, or dilution (e.g., adding 0.9% sodium chloride injection) in the final container. For nonsterile radiopharmaceuticals, an example is obtaining 1 capsule from a container holding 1 or more capsules. For sterile radiopharmaceuticals, an example is withdrawing a volume of solution from a single-use or multiple-dose container into a syringe. Labeling of the final patient-ready dose or ordered amount of a radiopharmaceutical is also a component of the dispensing process.

Except for an unopened manufacturer container, the final dose or ordered amount must be radioassayed (i.e., in a dose calibrator). The measured activity should be mathematically corrected for radioactive decay to the time of scheduled administration (calibration time) (refer to *14. Quality Assurance and Quality Control*). The activity at calibration time must always be within federal, state, and local variance limits.

## 12.2 Labeling

The labeling of radiopharmaceuticals can fall under the jurisdiction of numerous regulatory agencies. Individual boards of pharmacy and other regulatory bodies may have very specific statutes and/or regulations concerning this process. The requirements specified in this chapter must be considered the minimum requirements for the labeling of the inner container (e.g., syringe, vial) and the outer shielding (e.g., syringe or vial shielding). Therefore, all personnel distributing and/or dispensing radiopharmaceuticals should verify that any labeling is in compliance with regulatory agencies.

The inner container must be labeled with the following:

- Standard radiation symbol
- The words “Caution—Radioactive Material”
- For all therapeutic and blood-products, the patient name/identifier
- Radionuclide and chemical form (generic name)
- Radioactivity at the date and time of calibration

The outer shielding must be labeled with the following:

- Standard radiation symbol
- The words “Caution—Radioactive Material”
- For all therapeutic and blood-products, the patient name/identifier
- Radionuclide and chemical form (generic name)
- Radioactivity at the date and time of calibration
- Volume or number of units dispensed (e.g., 2 capsules), as applicable
- Product expiration or BUD (see *Table 7*), as applicable, and any special storage and handling instructions for non-immediate use (e.g., refrigeration, resuspension)
- Route of administration

## 12.3 Direct Infusion Systems

The information in this section is limited to the sterility and aseptic technique for direct infusion systems. The described infusion systems are FDA-cleared medical devices or FDA-approved direct infusion generators without an ISO-5 environment. The manner in which all necessary solutions (e.g., radiopharmaceutical and eluant/diluent) are used in conjunction with the system was a consideration in the overall approval process for the system. Therefore, all operators of the direct infusion systems must follow the “Instructions for Use” in the device labeling.

- Direct infusion generators (e.g., rubidium chloride Rb 82 injection) may employ a container of eluant (e.g., bag of 0.9% sodium chloride injection) to allow administration of the eluate directly to patient(s).
- Direct infusion devices (e.g., portable PET patient-infusion system) provide a method for dispensing and administration from a multiple-dose container of the radiopharmaceutical (e.g., fludeoxyglucose F 18 injection) and the diluent (e.g., 0.9% sodium chloride injection) directly to patients to reduce the radiation exposure to personnel.

In each of these situations, the radiopharmaceutical container must be attached to or be needle-punctured by the respective direct infusion system. Given that such direct infusion systems are intended for multiple patients over the course of several

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hours, there could be a sterility concern if not operated properly. Therefore, the following parameters must be considered by the operator of the system:

- Setup attachment or needle-puncture should be performed in a defined environment
- Aseptic handling in ambient air with a maximum BUD of 10 hours is allowed for these direct infusion systems (see *Table 7*)
- The 0.9% sodium chloride bag attached to the device may only be punctured once and may be used for no more than 10 hours. The bag must be labeled with the date and time of puncture and the BUD
- Any nonsterile parts of the device that may encounter the septum of the radiopharmaceutical vial must be disinfected with sterile 70% IPA prior to puncturing the vial with the needle
- The septum of any vial and the ports of any diluent bag must be wiped with sterile 70% IPA prior to puncturing
- When puncturing the vial in ambient air, it must only be punctured once
- If there are problems with the infusion device, no sterile container(s) associated with the system can be repunctured or transferred to a PEC for further manipulations and the container, with contents, must be discarded

### 12.4 Transporting Generators Between Facilities

The following standards must be followed if transporting generators between facilities:

- The generator needle and/or ports must be capped in ISO Class 8 air or better with sterile protectors
- The generator must be packaged and transported in a manner to maintain the integrity and sterility of the generator system

## 13. REPACKAGING

Repackaging refers to the act of removing conventionally manufactured radiopharmaceutical(s) from the container in which it was distributed by the original manufacturer and placing it into a different container without further manipulation of the product. Repackaging also includes the act of placing the contents of multiple containers of the same finished drug product into one container, as long as the container does not include other ingredients. Repackaging may be performed for nonsterile radiopharmaceuticals (e.g., I-131 sodium iodide oral capsules) and for sterile radiopharmaceuticals (e.g., thallous chloride Tl 201 injection).

Except for unopened manufacturer dosage units (e.g., capsules, Xe-133 vials), the repackaged radiopharmaceutical must be radioassayed (i.e., in a dose calibrator). The inner container should be labeled with the following:

- Standard radiation symbol
- The words “Caution—Radioactive Material”
- The radionuclide and chemical form (generic name)
- Radioactivity with units at time of calibration and the calibration time

The outer shielding should be labeled with the following:

- Standard radiation symbol
- The words “Caution—Radioactive Material”
- The radionuclide and chemical form (generic name)
- Radioactivity with units at time of calibration and the calibration time
- Volume, or number of units (e.g., capsules), as applicable
- Product expiration or BUD (see *Table 7*), as applicable
- Special storage and handling instructions

## 14. QUALITY ASSURANCE AND QUALITY CONTROL

Quality assurance (QA) is a system of procedures, activities, and oversight that ensures that radiopharmaceutical processing consistently meets quality standards (see *Quality Assurance in Pharmaceutical Compounding* ⟨1163⟩). Quality control (QC) is the sampling, testing, and documentation of results that, taken together, ensure that specifications have been met before release of the radiopharmaceutical(s).

A facility’s QA and QC programs must be formally established and documented in SOPs that ensure that all aspects of the handling of radiopharmaceuticals are conducted in accordance with this chapter and applicable federal, state, and local laws and regulations. A designated person must ensure that the facility has formal, written QA and QC programs that establish a system of:

1. Adherence to procedures,
2. Prevention and detection of errors and other quality problems,
3. Evaluation of complaints and adverse events, and
4. Appropriate investigations and corrective actions.

The SOPs must describe the roles, duties, and training of the personnel responsible for each aspect of the QA program. The overall QA and QC program must be reviewed at least once every 12 months by the designated person. The results of the review must be documented and appropriate corrective action taken, if needed.

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## 14.1 Notification About and Recall of Out-of-Specification Dispensed Radiopharmaceuticals

If a radiopharmaceutical is dispensed or administered before the results of release testing are known, the facility must have SOPs in place to:

1. Immediately notify the prescriber of a failure of specifications with the potential to cause patient harm (e.g., sterility, strength, purity, bacterial endotoxin, or other quality attributes), and
2. Determine whether a recall is necessary.

The SOP for recall of out-of-specification dispensed radiopharmaceuticals must contain procedures to:

- Determine the severity of the problem and the urgency for the implementation and completion of the recall
- Determine the distribution of any affected radiopharmaceutical, including the date and quantity
- Identify patients who have received the radiopharmaceutical
- Outline the disposition and reconciliation of the recalled radiopharmaceutical

The facility must document the implementation of the recall procedures. The recall must be reported to appropriate regulatory bodies as required by laws and regulations of the applicable regulatory jurisdiction (e.g., state board of pharmacy, state health department).

## 14.2 Complaint Handling

Radiopharmaceutical facilities must develop and implement SOPs for handling complaints. Complaints may include concerns or reports on the quality and container labeling of, or possible adverse reactions to, a specific radiopharmaceutical.

A designated person must review all complaints to determine if they indicate potential quality problems with the radiopharmaceutical. If a complaint does, an investigation into the potential cause of the problem must be completed. The investigation must consider whether the quality problem could extend to other radiopharmaceuticals. Corrective action, if necessary, must be implemented for all potentially affected radiopharmaceuticals. Consider whether to initiate a recall of potentially affected radiopharmaceuticals and whether to cease sterile compounding until all underlying problems have been identified and corrected.

A readily retrievable record (written or electronic) of each complaint must be kept by the facility, regardless of the source of the complaint (e.g., e-mail, telephone, mail). The record must contain the name of the complainant, the date the complaint was received, the nature of the complaint, the response to the complaint, and, if known, the name and strength of the radiopharmaceutical and the assigned internal identification number (e.g., prescription, order, or lot number).

The record must also include the findings of any investigation and any follow-up. Records of complaints must be easily retrievable for review and evaluation for possible trends and must be retained in accordance with the record keeping requirements in *9. Documentation*. A radiopharmaceutical that is returned in connection with a complaint must be quarantined until it is destroyed after completion of the investigation and in accordance with applicable jurisdictional laws and regulations.

## 14.3 Adverse Event Reporting

Adverse events potentially associated with the quality of radiopharmaceuticals must be reported in accordance with the facility's SOPs and all applicable jurisdictional laws and regulations. In addition, adverse events potentially associated with the quality of the radiopharmaceutical preparation should be reported to the applicable jurisdictional regulatory body (e.g., state boards of pharmacy, state health departments, FDA's MedWatch program for human drugs).

## GLOSSARY

**Administration:** The direct and immediate application of a radiopharmaceutical to a patient by injecting, infusing, ingesting, or otherwise providing a radiopharmaceutical in its final form.

**Airlock:** A space with interlocked doors, constructed to maintain air pressure control when items move between two adjoining areas.

**Ante-room:** An ISO Class 8 or cleaner area with fixed walls and doors where personnel hand hygiene, garbing procedures, and other activities that generate high particulate levels are performed. The ante-room is the transition area between the unclassified area in a facility and the classified buffer area.

**Aseptic processing or preparation:** A process by which separate, sterile components (e.g., drugs, containers, or closures) are brought together under conditions that maintain their sterility.

**Aseptic technique:** Methods utilized during the processing of radiopharmaceuticals to keep objects and areas free of microorganisms and thereby minimize infection risk to the patient. It is accomplished through practices that maintain the microbe count at a nearly irreducible number.

**As low as (is) reasonably achievable (ALARA):** The effort to maintain exposures to ionizing radiation as far below the dose limits as practical. These efforts should be consistent with the purpose for which the licensed activity is undertaken, in relation to utilization of licensed materials in the public interest. Limiting exposure time, using adequate shielding, and maintaining the most distance possible from all radioactive sources (i.e., time, distance, shielding) are the basic principles for successfully following ALARA guidelines.

**Authorized nuclear pharmacist (ANP):** A pharmacist recognized by the U.S. Nuclear Regulatory Commission or an Agreement State agency as having met training and experience requirements for the practice of nuclear pharmacy.

**Authorized user (AU):** A physician recognized by the U.S. Nuclear Regulatory Commission or an Agreement State agency as meeting training and experience requirements for specified medical uses of radioactive material.

**Beyond-use date (BUD):** The assigned date and time beyond which the radiopharmaceutical must not be administered.

**Biological safety cabinet (BSC) Class II:** A ventilated cabinet with an open front and inward and downward unidirectional HEPA-filtered airflow and HEPA-filtered exhaust.

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**Blood components:** Any constituent of blood that is separated by physical or mechanical means (e.g., red cells, white cells, platelets)

**Buffer area:** An ISO Class 8 or cleaner area with fixed walls and doors where PEC(s) that generate and maintain an ISO Class 5 environment are physically located. The buffer area may only be accessed through the ante-room.

**Chemical purity:** The fraction of the total chemical species present in the radiopharmaceutical as the specified chemical component(s). A chemical impurity is the presence of an unwanted non-radioactive chemical.

**Classified area:** An area that maintains an air quality classification based on the ISO guidelines (i.e., ante-room, buffer area). See *ISO class*.

**Cleaning agent:** A material for the removal of residues (e.g., dirt, debris, microbes, and residual drugs or chemicals) from surfaces.

**Compounding:** The combining, mixing, pooling, or otherwise altering (excluding preparation with minor deviations) of a conventionally manufactured radiopharmaceutical or synthesizing/formulating a radiopharmaceutical from bulk drug substances and radionuclides. See *Preparation with minor deviations*.

**Container–closure system:** The packaging components that contain or come in contact with the radiopharmaceutical and maintain the integrity of the radiopharmaceutical contained within. Examples include (but are not limited to) vials, tubes and syringes.

**Critical site:** A location that includes any component or fluid pathway surface (e.g., vial septa, injection ports) or openings (e.g., needle hubs) that, when exposed is at risk for contamination by direct contact with air (e.g., ambient area or HEPA-filtered), moisture (e.g., oral and mucosal secretions), or touch.

**Designated person:** One or more individuals assigned to be responsible and accountable for the performance and operation of the radiopharmaceutical processing facility and for personnel who prepare, compound, dispense, and repackage radiopharmaceuticals.

**Direct infusion system:** An FDA-cleared medical device used to dispense and/or administer radiopharmaceuticals to multiple patients. The standards of this chapter pertain to devices with ambient air that lack and ISO Class 5 environment.

**Direct processing area (DPA):** An area within the ISO Class 5 PEC where critical sites are exposed to unidirectional HEPA-filtered air, also known as first air.

**Disinfectant:** A chemical or physical agent used on inanimate surfaces and objects to destroy microbiological contamination (e.g., fungi, viruses, and bacteria) when used in the appropriate concentrations and for the appropriate contact times. Sporicidal disinfectant agents are considered a special class of disinfectants that also are effective against bacterial endospores and fungal spores.

**Dispensing:** The manipulation or labeling of a radiopharmaceutical to render it in its final form for administration, typically obtained from a single-dose or multiple-dose container (e.g., withdrawing a volume of finished product or preparation from a vial into a syringe). Dispensing is performed under the supervision of a physician or pharmacist and for radiopharmaceuticals includes dilution with an appropriate diluent or adjusting the activity in an individual dosage.

**Dose pooling:** The combining of doses from two or more syringes to meet one patient's need, also see "repackaging".

**Dose splitting:** The splitting of a patient-ready unit dose for use with more than one patient.

**Dynamic operating conditions:** Conditions in the SRPA or classified area in which operating personnel are present and performing actual or simulated activities. The PEC should contain equipment and materials regularly used for radiopharmaceutical processing (e.g., low-lint absorbent pads, dose calibrator, syringe shields).

**Expiration date:** For conventionally manufactured radiopharmaceuticals, the specified date (and time) beyond which the product must not be administered. The expiration date is determined by the manufacturer.

**First air:** The air exiting the HEPA filter in a unidirectional air stream.

**Garb:** Gloves, gowns, shoe covers, head (covers ears and all hair) and facial hair covers, masks, and other items designed to reduce particle shedding from personnel and minimize the risk of microbiological contamination to radiopharmaceuticals.

**High efficiency particulate air (HEPA) filtration:** Using a tested and certified air filter designed to remove 99.97% of airborne particles measuring 0.3-micron or greater in diameter from the air passing through it.

**Hot-cell:** A device used for the shielding and containment of radioactive materials. The shielding material(s) (e.g., lead) is generally incorporated into the structure of the unit itself. Radiopharmaceutical personnel carry out the majority of the tasks within the hot-cell from the exterior of the unit. This is accomplished by the use of remote manipulation systems (e.g., manipulator arms, automated dispensing system) of various designs. Numerous air quality configurations of the hot-cell may exist, including integrated HEPA filtration systems to render all or a specified portion (DPA) of the device capable of certifying to a controlled ISO Class 5 environment. In other situations, the hot-cell offers only radiation protection and a laminar flow PEC, capable of achieving an ISO Class 5 environment, is placed within the enclosure to allow for safe aseptic manipulations. A hot-cell may also be referred to by other designations (e.g., shielded isolator with laminar flow, PET dispensing station, manipulator hot-cell, shielded isolators for dispensing, radiopharmaceutical dispensing isolator).

**Hot lab:** Unclassified radiopharmaceutical processing area located within a hospital or clinical site that is only appropriate for immediate use radiopharmaceuticals if there is not an ISO 5 PEC within SRPA located within the area.

**Immediate use:** A preparation (including preparations with minor deviations) and/or dispensing of a sterile radiopharmaceutical that is limited for a single patient. Only sterile conventionally manufactured drug products (e.g., NDA, ANDA) or drugs produced under an approved IND or RDRC protocol may be used. Administration must begin within 1 hour of the first container puncture or exposure of any critical site involved (e.g., syringe tip, needle hub or needle) to ambient air, whichever is first.

**Individual dose (unit dose):** A radiopharmaceutical in its final form ready for administration (e.g., capsule, sterile solution in a syringe) consisting of the amount (dose) prescribed, ordered, or other intended for an individual patient or research subject.

**Inverse square law:** The specified physical quantity or intensity of a radiation emission is inversely proportional to the square of the distance from the source of the emission.

**ISO class:** A quality classification from the International Organization for Standardization based on quantity and size of particles per volume of air.

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**Kit:** Conventionally manufactured package containing all ingredients required to prepare a radiopharmaceutical with the exception of the radionuclide.

**Kit-splitting (fractionation):** The act of dividing the contents of a kit vial and transferring aliquots into other containers for storage and subsequent radiolabeling.

**Ligand:** An ion or molecule that incorporates a metal atom to form a coordination complex.

**Line of demarcation:** A visible line on the floor that separates the clean and less clean sides of the ante-room.

**Low-lint wiper:** A wiper exhibiting few, if any, fibers or other particulates, visible without magnification, which are separate from, or easily removed from, the wiper material in a dry condition.

**Master Formulation Record (MFR):** A document or set of documents specifying the starting materials with their quantities and the packaging materials, together with a description of the procedures and precautions required to produce a specified quantity of a finished preparations as well as the processing instructions, including the in-process controls.

**Media-fill test:** A simulation used to qualify processes and personnel engaged in sterile radiopharmaceutical processing to ensure that the processes and personnel are able to prepare radiopharmaceuticals without microbiological contamination.

**Molar mass:** The measured mass that is attained from a molar amount of a given substance (e.g., element, compound). It is generally expressed with units such as g/mol and kg/mol.

**Multiple-dose container:** A container of a radiopharmaceutical for administration that is designed to contain more than one patient dose of the radiopharmaceutical.

**Negative-pressure area:** An area that is maintained at lower pressure than the adjacent spaces, and therefore the net airflow is into the area. This area is appropriate for volatile or gaseous radionuclides and radiopharmaceuticals (e.g., I-131 NaI, N-13 ammonia) and intended to lend a measure of protection for the radiation workers and the general public.

**One-step disinfectant cleaner:** A product with an EPA-registered claim (or equivalent) that it can clean and disinfect a nonporous surface in the presence of light to moderate organic soiling without a separate cleaning step.

**Pass-through:** An enclosure with sealed doors on both sides to ensure that both doors are not opened at the same time. The pass-through is positioned between two spaces creating an airlock for the purpose of minimizing particulate transfer while moving materials from one space to another.

**Perimeter:** A visible demarcation on the floor that defines the boundaries of the SRPA.

**Positive-pressure area:** An area that is maintained at higher pressure than the adjacent spaces, and therefore the net airflow is out of the area.

**Preparation:** The act of combining a conventionally manufactured kit with a conventionally manufactured radionuclide following manufacturer's recommended instructions. Mixing, reconstituting, combining, diluting, or repackaging of a radiopharmaceutical, or other such acts, performed in accordance with directions contained in the FDA-approved labeling.

**Preparation with minor deviations:** The act of preparing a conventionally manufactured kit with a conventionally manufactured radionuclide with volume, and/or radioactivity, and/or step-by-step deviations from the manufacturers recommended labeling while ensuring that the final preparation maintains appropriate radiochemical and radionuclidic purity for the entirety of the BUD. Examples of minor deviations include, but are not limited to, altering the amount of activity or volume added to the vial, changes in step-by-step operations (e.g., dilute Tc-99m solution after, rather than before, addition to the vial, use of a venting needle or filter), using alternative devices or equipment (e.g., a heating block rather than a hot water bath), and using alternative radiochemical purity testing methods.

**Primary engineering control (PEC):** A device or zone that provides an ISO Class 5 air quality environment for sterile processing.

**Pyrogen:** A substance that induces a febrile reaction in a patient.

**Quality assurance (QA):** The system of procedures, activities, and oversight that ensures that radiopharmaceutical processing consistently meets quality standards.

**Quality control (QC):** The sampling, testing, and documentation of results that, taken together, ensure that specifications have been met before release of the radiopharmaceutical.

**Radioactive materials (RAM) license:** A document(s) issued by the US NRC or an Agreement State agency that authorizes various activities involving the use of radioactive materials. These uses can include possession, research and development, distribution, medical use, and other purposes not included in this list. Only those activities specifically authorized are allowed.

**Radioassay:** Measurement of the quantity of radioactivity present in a container using a suitable and calibrated instrument, such as a well-type ionization chamber (i.e., dose calibrator).

**Radiochemical purity:** The ratio, expressed as a percentage, of the radioactivity of the intended active radiopharmaceutical ingredient to the total radioactivity of all radioactive ingredients and impurities present in the radiopharmaceutical preparation (see *Radioactivity* ⟨821⟩).

**Radionuclidic purity:** The ratio, expressed as a percentage, of the radioactivity of the intended radionuclide to the total radioactivity of all radionuclides in the radiopharmaceutical preparation (see ⟨821⟩).

**Radiopharmaceutical (radiopharmaceutical preparation/radioactive drug):** (See ⟨821⟩.) A finished dosage form that contains a radioactive substance in association with one or more other ingredients and that is intended to diagnose, stage a disease, monitor treatment, or provide therapy. A radiopharmaceutical includes any non-radioactive reagent kit or radionuclide generator that is intended to be used in the preparation of any such substance. The terms "radiopharmaceutical" and "radioactive drug" are commonly used interchangeably.

**Repackaging:** The act of removing a conventionally manufactured radiopharmaceutical from the container in which it was distributed by the original manufacturer and placing it into a different container without further manipulation of the product. Repackaging also includes the act of placing the contents of multiple containers (e.g., vials) of the same finished drug product into one container, as long as the container does not include other ingredients. Radiopharmaceutical manipulation in any other way, including reconstitution, dilution, mixing, or combination with another ingredient, is not considered repackaging.

**Restricted area:** Any area to which access is controlled for the protection of individuals from exposures to radiation and radioactive materials.

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**Secondary engineering control (SEC):** The area where the PEC is placed (e.g., a classified area or an SRPA). It incorporates specific design and operational parameters required to minimize the risk of microbial contamination.

**Segregated radiopharmaceutical processing area (SRPA):** A designated, unclassified space, area, or room with a defined (by facility procedures) perimeter that contains a PEC. An SRPA is only suitable for radiopharmaceutical preparation (with and without minor deviations), dispensing, and repackaging. If the SRPA is used to elute radionuclide generators it must have ISO Class 8 particle count non-viable particle count air quality.

**Shielding:** Barriers of appropriate radiation attenuating material, used for radiopharmaceuticals, to protect the personnel. These barriers can be general in nature (e.g., L-block, hot-cell), as to afford protection from a radiation field, or specific to a container used to hold a particular radiopharmaceutical (e.g., syringe shield, vial shields, "pigs").

**Single-dose container:** A container of a radiopharmaceutical for administration that is designed for use with a single patient as a single administration.

**Specific activity:** The radioactivity of a radionuclide per unit mass of the compound involved with the radionuclide (see *Radioactivity—Theory and Practice* ⟨1821⟩). The units of specific activity involve those for the activity (e.g., mCi, MBq, Ci, GBq) and those for the unit of mass (e.g., µg, mmol); expressed on an activity per mass basis (e.g., mCi/µg, MBq/µg, Ci/mmol, GBq/mmol).

**Sporicidal agent:** A chemical or physical agent that destroys bacterial and fungal spores when used in sufficient concentration for a specified contact time. It is expected to kill all vegetative microorganisms.

**Start of preparation:** Time at which a vial septum is punctured or a component container is opened (e.g., removal of cap on a pre-filled syringe), whichever comes first.

**Sterility:** The absence of viable microorganisms.

**Strength:** The radioactivity concentration of the radiopharmaceutical at the calibration time (see ⟨821⟩). Strength is expressed as the quantity of radioactivity on a volume basis (e.g., mCi/mL or MBq/mL).

**Unclassified space:** A space not required to meet any ISO air cleanliness classification.

**Unrestricted area:** An area in which a person should not be exposed to radiation levels in excess of 2 millirems in any 1 h from external sources.

**Use-by time:** For radiopharmaceuticals prepared from kits, the time period after preparation during which the radiopharmaceutical should be used or administered, as suggested or stated in the manufacturer's prescribing information.

## APPENDIX

### Abbreviations

| | |
|---|---|
| ACPH | Air changes per hour |
| ALARA | As low as reasonably achievable |
| ANDA | Abbreviated new drug application |
| ANP | Authorized nuclear pharmacist |
| AU | Authorized user |
| BLA | Biologics license application |
| BSC | Biological safety cabinet |
| BUD | Beyond-use date |
| CETA | Controlled Environment Testing Association |
| cfu | Colony-forming unit |
| CoA | Certificate of analysis |
| DPA | Direct processing area |
| EPA | Environmental Protection Agency |
| FDA | Food and Drug Administration |
| FDCA | Food, Drug, and Cosmetic Act |
| HEPA | High-efficiency particulate air |
| HVAC | Heating, ventilation, and air conditioning |
| IND | Investigational new drug |
| IPA | Isopropyl alcohol |
| ISO | International Organization for Standardization |
| LAFW | Laminar airflow workbench |
| MAA | Macroaggregated albumin |
| MEA | Malt extract agar |
| MFR | Master Formulation Record |
| NDA | New drug application |

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**Abbreviations** *(continued)*

| | |
|---|---|
| NRC | Nuclear Regulatory Commission |
| PEC | Primary engineering control |
| PET | Positron emission tomography |
| RAM | Radioactive material |
| RDRC | Radioactive drug research committee |
| RH | Relative humidity |
| SDA | Sabouraud dextrose agar |
| SEC | Secondary engineering control |
| SOP | Standard operating procedure |
| SRPA | Segregated radiopharmaceutical processing area |
| TSA | Trypticase soy agar |

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# ⟨831⟩ REFRACTIVE INDEX

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## ▲INTRODUCTION

Refractive index is a basic physical property of pharmaceutical materials. This general chapter only applies to the measurement of liquid pharmaceuticals, solutions, solvents, and liquid raw materials with stand-alone instruments and not to detectors of chromatographic systems. Refractive index measurements of liquid pharmaceuticals, solutions, solvents, and liquid raw materials are useful for the following:

- Identifying a pure substance
- Establishing the purity of some substances
- Determining the concentrations of some solutions such as alcohol solutions or sugar solutions (e.g., percentage of sugar in syrup)
- Determining the uniformity of final compounded preparations

The refractive index (*n*) of a substance, or medium, is defined as:

$$n = \frac{c}{v}$$

where *c* is the velocity of light in a vacuum and *v* is the velocity of light in the medium. For example, the refractive index of pure water at a measurement temperature and wavelength of 20° and 589 nm is 1.3330, meaning that light travels proportionately slower in water than in a vacuum.[1] The refractive index of a material is dependent on the temperature and the wavelength of light used to measure it; it is important to have precise control of both of these variables to accurately measure the refractive index. Refractive index is reported as $n_{\lambda}^{T}$, where *T* is the temperature, and λ is the wavelength of the incident beam (in nanometers). For example, the refractive index of water at 20° using the sodium D-line (at 589 nm) may be reported as either $n_{589}^{20}$ or $n_{D}^{20}$ = 1.3330.[1]

## QUALIFICATION OF REFRACTOMETERS

Qualification of refractometers can be divided into three elements: installation qualification (IQ), operational qualification (OQ), and performance qualification (PQ). For further discussion, see *Analytical Instrument Qualification* ⟨1058⟩.

### Installation Qualification

The IQ requirements provide evidence that the hardware and software are properly installed in the desired location.

### Operational Qualification

The OQ for the refractometer should verify the temperature control of the sample and the accuracy and repeatability of the refractive index measurements. The apparatus should provide refractive index readings to ±0.0001, and it should provide a means of operation at the prescribed temperature, with readings accurate to at least ±0.1°. The certified reference materials used must have an equal or better expanded uncertainty than ±0.001. Prior to verifying the operation of the refractometer, perform a full calibration of the instrument as instructed by the manufacturer.

#### ACCURACY

Verify the accuracy of the refractometer by measurement of certified reference materials that bracket the operational range of the instrument. The result should agree with the stated certified value within the limits of the expanded uncertainty of the reference material added to the accuracy specification of the instrument (as provided by the manufacturer). Additional uncertainty components may be included, according to the manufacturer's recommendation.

#### REPEATABILITY

Verify the repeatability of the refractometer by measuring at least two different certified reference materials covering the intended operational range of the instrument. Perform at least six replicate measurements of each certified reference material. The result should be equal to or less than the stated repeatability performance specification of the instrument (as provided by the manufacturer).

[1] The sodium D-line is identified as a doublet at 589.0 and 589.6 nm with a center wavelength at 589.3 nm in air.

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## Performance Qualification

PQ includes two stages: periodic instrument calibration and performance evaluation. Instrument calibration should be performed periodically according to the instrument manufacturer's instructions. The second stage of PQ evaluation should be conducted at regular intervals or on day of use by verifying the refractive index of suitable reference materials.

# PROCEDURES

## Sample Preparation

Samples should be kept in well-closed containers to protect them from any changes in composition due to differences in temperature and/or relative humidity at different locations. If the sample to be evaluated is not homogenous, is hazy, or is known to contain suspended particles or an emulsified phase—factors that influence the measurement—the sample should be clarified by filtration or centrifugation prior to measurement.

## Measurement Procedure

Determine the refractive index of the sample per the instrument manufacturer's instructions. Unless otherwise indicated in the monograph, the refractive index is measured at 25°, with reference to the wavelength of the sodium D-line (at 589 nm).[1] ▲ (USP 1-Dec-2023)

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# ⟨841⟩ SPECIFIC GRAVITY

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▲Specific gravity is the term for the relative density of a substance when water is used as the reference. The measurement of specific gravity necessarily involves the measurement of the density of the sample at a specified temperature as well as the measurement of the density of water.

The density $\rho$ (g/mL or g/cm$^3$) means the mass per unit volume, and the relative density means the ratio of the mass of a sample specimen to that of an equal volume of a standard substance. The relative density and specific gravity are unitless values.

The specific gravity ($d_t^{t'}$) means the ratio of the mass of the sample specimen at $t'$ to that of an equal volume of water ($H_2O$) at $t$.▲ (USP 1-Aug-2020)Unless otherwise stated in the individual monograph, the specific gravity determination is applicable only to liquids and ▲▲ (USP 1-Aug-2020) is based on the ratio of the weight of a liquid in air at 25° to that of an equal volume of water at the same temperature ▲(i.e., $d_{25}^{25}$).▲ (USP 1-Aug-2020) Where a temperature is specified in the individual monograph, the specific gravity is the ratio of the weight of the liquid in air at the specified temperature to that of an equal volume of water at the same temperature. ▲If▲ (USP 1-Aug-2020) the substance is a solid at 25°, determine the specific gravity ▲above the melting point▲ (USP 1-Aug-2020) (at the temperature directed in the individual monograph, ▲if specified).▲ (USP 1-Aug-2020)

Unless otherwise stated in the individual monograph, the density is defined as the mass of a unit volume of the substance at 25°, expressed in kilograms per cubic meter or grams per cubic centimeter (1 kg/m$^3$ = 10$^{-3}$ g/cm$^3$). Where the density is known, mass can be converted to volume, or volume converted to mass, by ▲using▲ (USP 1-Aug-2020) the formula, volume = mass/density.

▲▲ (USP 1-Aug-2020)

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## METHOD I

### Procedure

Select a ▲▲ (USP 1-Aug-2020)clean, dry pycnometer that has been previously calibrated by determining its weight and the weight of ▲the deaerated▲ (USP 1-Aug-2020) water contained in it at 25°. Adjust the temperature of the ▲sample▲ (USP 1-Aug-2020) liquid to about 20°, and fill the pycnometer with it. Adjust the temperature of the filled pycnometer to 25°, remove any excess liquid, and weigh. When the monograph specifies a temperature different from 25°, the filled pycnometers must be brought to the temperature of the balance before ▲weighing. The specific gravity ($d_t^{t'}$) can be calculated by use of the following equation:

$$d_t^{t'} = \frac{W_1 - W}{W_2 - W}$$

$W_1$ = mass of the pycnometer filled with the sample solution at $t'$
$W$ = mass of the clean, dry pycnometer
$W_2$ = mass of the pycnometer filled with water at $t$

The density of water can be used to calculate the density of the sample of the specimen according to the following equation:

$$\rho_{s,t'} = d_t^{t'} \rho_{w,t}$$

$\rho_{s,t'}$ = density of the sample at $t'$
$\rho_{w,t}$ = density of water at $t$▲ (USP 1-Aug-2020)

***Change to read:***

## METHOD II

The procedure ▲requires▲ (USP 1-Aug-2020) the use of the oscillating transducer density meter. The apparatus consists of the following:

- A U-shaped tube, usually of borosilicate glass, which contains the liquid to be examined
- A magneto-electrical or piezo-electrical excitation system that causes the tube to oscillate as a cantilever oscillator at a characteristic frequency depending on the density of the liquid to be examined
- A means of measuring the oscillation period ($T$), which may be converted by the apparatus to give a direct reading of density or used to calculate density by using the constants $A$ and $B$ described below
- A means to measure and/or control the temperature of the oscillating transducer containing the liquid to be tested

The oscillation period is a function of the spring constant ($c$) and the mass of the system:

$$T^2 = \{(M/c) + [(\rho \times V)/c]\} \times 4\pi^2$$

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where ρ is the density of the liquid to be tested, $M$ is the mass of the tube, and $V$ is the volume of the filled tube.

Introduction of two constants $A = c/(4\pi^2 \times V)$ and $B = (M/V)$ leads to the classical equation for the oscillating transducer:

$$\rho = A \times T^2 - B$$

The specific gravity of the liquid is given by the formula:

$$\rho_{(L)}/\rho_{(W)}$$

where $\rho_{(L)}$ and $\rho_{(W)}$ are the densities of the liquid and water, respectively, both determined at 25°, unless otherwise directed in the individual monograph.

▲Samples with high viscosity will dampen the oscillations in the instrument and will introduce an error in the density measurement. Some oscillating transducer density meters are able to provide a correction to the density based on the viscosity of the sample. Samples with viscosities <10 mPa · s can be accurately measured without a density correction. Samples with viscosities >10 mPa · s must be measured with a density correction. If a density correction is not possible for a sample with >10 mPa · s viscosity, use *Method I*.▲ (USP 1-Aug-2020)

## Calibration

The constants $A$ and $B$ are determined by operating the instrument with the U-shaped tube filled with two different samples of known density (e.g., degassed water and air). Perform the control measurements daily, using degassed water. The results displayed for the control measurement using degassed water ▲should▲ (USP 1-Aug-2020) not deviate from the reference value ($\rho_{25}$ = 0.997043 g/cm³) by more than its specified error. Precision is a function of the repeatability and stability of the oscillator frequency. Density meters are able to achieve measurements with an error on the order of $1 \times 10^{-3}$ g/cm³ to $1 \times 10^{-5}$ g/cm³ and a repeatability of $1 \times 10^{-4}$ g/cm³ to $1 \times 10^{-6}$ g/cm³. For example, an instrument specified to $\pm 1 \times 10^{-4}$ g/cm³ must display 0.9970 ± 0.0001 g/cm³ in order to be suitable for further measurement, otherwise a readjustment is necessary. Calibration with certified reference materials should be carried out regularly.

## Procedure

▲Verify the viscosity of the sample to be analyzed. If the viscosity is >10 mPa · s and the oscillating transducer density meter is unable to provide a density correction based on the viscosity, use *Method I*.▲ (USP 1-Aug-2020)

Using the manufacturer's instructions, perform the measurements using the same procedure as for *Calibration*. If necessary, equilibrate the liquid▲▲ (USP 1-Aug-2020) at 25° before introduction into the tube to avoid the formation of bubbles and to reduce the time required for measurement. Factors affecting accuracy include the following:

- Temperature uniformity throughout the tube
- Nonlinearity over a range of density
- Parasitic resonant effects
- Viscosity, if the oscillating transducer density meters used do not provide automatic compensation of sample viscosity influence

▲The oscillating transducer density meter provides the reportable value of the sample density at the selected temperature. The specific gravity of the sample is calculated from this measured density according to the following equation:

$$d_t^{t'} = \frac{\rho_{s,t'}}{\rho_{w,t}}$$

$\rho_{s,t'}$ = density of the sample at $t'$
$\rho_{w,t}$ = density of water at $t$

[NOTE—The density of water used in the calculation of the specific gravity should be the accepted value of the density of water at $t$ (e.g., at 25° use 0.9970 g/cm³) rather than the value reported by the instrument.▲ (USP 1-Aug-2020)]

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# ⟨846⟩ SPECIFIC SURFACE AREA

## INTRODUCTION

The specific surface area of a powder is determined by physical adsorption of a gas on the surface of the solid and by calculating the amount of adsorbate gas corresponding to a monomolecular layer on the surface. Physical adsorption results from relatively weak forces (van der Waals forces) between the adsorbate gas molecules and the adsorbent surface of the test powder. The determination is usually carried out at the temperature of liquid nitrogen. The amount of gas adsorbed can be measured by a volumetric or continuous flow procedure.

## BRUNAUER, EMMETT AND TELLER (BET) THEORY AND SPECIFIC SURFACE AREA DETERMINATION

### Multipoint Measurement

The data are treated according to the Brunauer, Emmett and Teller (BET) adsorption isotherm equation:

$$1/[V_a((P_O/P) - 1)] = [(C - 1)/(V_mC)] \times (P/P_O) + (1/V_mC) \quad (1)$$

| | | |
|---|---|---|
| $P$ | = | partial vapor pressure of adsorbate gas in equilibrium with the surface at 77.4 K (b.p. of liquid nitrogen), in Pa, |
| $P_o$ | = | saturated pressure of adsorbate gas, in Pa, |
| $V_a$ | = | volume of gas adsorbed at standard temperature and pressure (STP) [273.15 K and atmospheric pressure ($1.013 \times 10^5$ Pa)], in mL, |
| $V_m$ | = | volume of gas adsorbed at STP to produce an apparent monolayer on the sample surface, in mL, |
| $C$ | = | dimensionless constant that is related to the enthalpy of adsorption of the adsorbate gas on the powder sample. |

A value of $V_a$ is measured at each of not less than three values of $P/P_o$.
Then the BET value

$$1/[V_a((P_o/P) - 1)]$$

is plotted against $P/P_o$, according to equation (1). This plot should yield a straight line usually in the approximate relative pressure range 0.05 to 0.3. The data are considered acceptable if the correlation coefficient, r, of the linear regression is not less than 0.9975; that is, $r^2$ is not less than 0.995. From the resulting linear plot, the slope, which is equal to $(C - 1)/V_mC$, and the intercept, which is equal to $1/V_mC$, are evaluated by linear regression analysis. From these values, $V_m$ is calculated as $1/(slope + intercept)$, while C is calculated as $(slope/intercept) + 1$. From the value of $V_m$ so determined, the specific surface area, S, in $m^2 \cdot g^{-1}$, is calculated by the equation:

$$S = (V_mNa)/(m \times 22400) \quad (2)$$

| | | |
|---|---|---|
| $N$ | = | Avogadro constant ($6.022 \times 10^{23}$ $mol^{-1}$), |
| $a$ | = | effective cross-sectional area of one adsorbate molecule, in square meters (0.162 $nm^2$ for nitrogen and 0.195 $nm^2$ for krypton), |
| $m$ | = | mass of test powder, in g, |
| 22,400 | = | volume, in mL, occupied by one mole of the adsorbate gas at STP allowing for minor departures from the ideal. |

A minimum of three data points is required. Additional measurements may be carried out especially when nonlinearity is obtained at a $P/P_o$ value close to 0.3. Because nonlinearity is often obtained at a $P/P_o$ value below 0.05, values in this region are not recommended. The test for linearity, the treatment of the data, and the calculation of the specific surface area of the sample are described above.

### Single-Point Measurement

Normally, at least three measurements of $V_a$, each at different values of $P/P_o$, are required for the determination of specific surface area by the dynamic flow gas adsorption technique *(Method I)* or by volumetric gas adsorption *(Method II).* However, under certain circumstances described below, it may be acceptable to determine the specific surface area of a powder from a single value of $V_a$ measured at a single value of $P/P_o$ such as 0.300 (corresponding to 0.300 mole of nitrogen or 0.001038 mole fraction of krypton), using the following equation for calculating $V_m$:

$$V_m = V_a[1 - (P/P_o)] \quad (3)$$

The specific surface area is then calculated from the value of $V_m$ by equation (2) given above.

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The single-point method may be employed directly for a series of powder samples of a given material for which the material constant C is much greater than unity. These circumstances may be verified by comparing values of specific surface area determined by the single-point method with that determined by the multipoint method for the series of powder samples. Close similarity between the single-point values and multipoint values suggests that 1/C approaches zero.

The single-point method may be employed indirectly for a series of very similar powder samples of a given material for which the material constant C is not infinite but may be assumed to be invariant. Under these circumstances, the error associated with the single-point method can be reduced or eliminated by using the multipoint method to evaluate C for one of the samples of the series from the BET plot, from which C is calculated as (1 + *slope*/*intercept*). Then $V_m$ is calculated from the single value of $V_a$ measured at a single value of $P/P_o$, by the equation:

$$V_m = V_a[(P_o/P) - 1]\,[(1/C) + ((C - 1)/C) \times (P/P_o)] \quad (4)$$

The specific surface area is calculated from $V_m$ by equation (2) given above.

## EXPERIMENTAL TECHNIQUES

This section describes the methods to be used for the sample preparation, the dynamic flow gas adsorption technique *(Method I)* and the volumetric gas adsorption technique *(Method II).*

### Sample Preparation

#### OUTGASSING

Before the specific surface area of the sample can be determined, it is necessary to remove gases and vapors that may have become physically adsorbed onto the surface after manufacture and during treatment, handling, and storage. If outgassing is not achieved, the specific surface area may be reduced or may be variable because an intermediate area of the surface is covered with molecules of the previously adsorbed gases or vapors. The outgassing conditions are critical for obtaining the required precision and accuracy of specific surface area measurements on pharmaceuticals because of the sensitivity of the surface of the materials.

The outgassing conditions must be demonstrated to yield reproducible BET plots, a constant weight of test powder, and no detectable physical or chemical changes in the test powder.

The outgassing conditions defined by the temperature, pressure, and time are chosen so that the original surface of the solid is reproduced as closely as possible. Outgassing of many substances is often achieved by applying a vacuum by purging the sample in a flowing stream of a nonreactive, dry gas or by applying a desorption-adsorption cycling method. In either case, elevated temperatures are sometimes applied to increase the rate at which the contaminants leave the surface. Caution should be exercised when outgassing powder samples using elevated temperatures to avoid affecting the nature of the surface and the integrity of the sample.

If heating is employed, the recommended temperature and time of outgassing are as low as possible to achieve reproducible measurement of specific surface area in an acceptable time. For outgassing sensitive samples, other outgassing methods such as the desorption-adsorption cycling method may be employed.

#### ADSORBATE

The standard technique is the adsorption of nitrogen of analytical quality at liquid nitrogen temperature.

For powders of low specific surface area (< 0.2 $m^2g^{-1}$), the proportion adsorbed is low. In such cases, the use of krypton at the liquid nitrogen temperature is preferred because the low vapor pressure exerted by this gas greatly reduces error. The use of larger sample quantities, where feasible (equivalent to 1 $m^2$ or greater total surface area using nitrogen), may compensate for the errors in determining low surface areas.

All gases used must be free from moisture.

#### QUANTITY OF SAMPLE

A quantity of the test powder is accurately weighed such that the total surface of the sample is at least 1 $m^2$ when the adsorbate is nitrogen and 0.5 $m^2$ when the adsorbate is krypton.

Lower quantities of sample may be used after appropriate validation.

### Measurements

Because the amount of gas adsorbed under a given pressure tends to increase when the temperature is decreased, adsorption measurements are usually made at a low temperature. Measurement is performed at 77.4 K, the boiling point of liquid nitrogen.

### Method I: The Dynamic Flow Method

#### PRINCIPLE

In the dynamic flow method (see *Figure 1*), the recommended adsorbate gas is dry nitrogen or krypton, while helium is employed as a diluent gas, which is not adsorbed under the recommended conditions.

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A minimum of three mixtures of the appropriate adsorbate gas with helium are required within the $P/P_o$ range 0.05 to 0.30.

The gas detector-integrator should provide a signal that is approximately proportional to the volume of the gas passing through it under defined conditions of temperature and pressure. For this purpose, a thermal conductivity detector with an electronic integrator is one among various suitable types. A minimum of three data points within the recommended range of 0.05 to 0.30 for $P/P_o$ is determined.

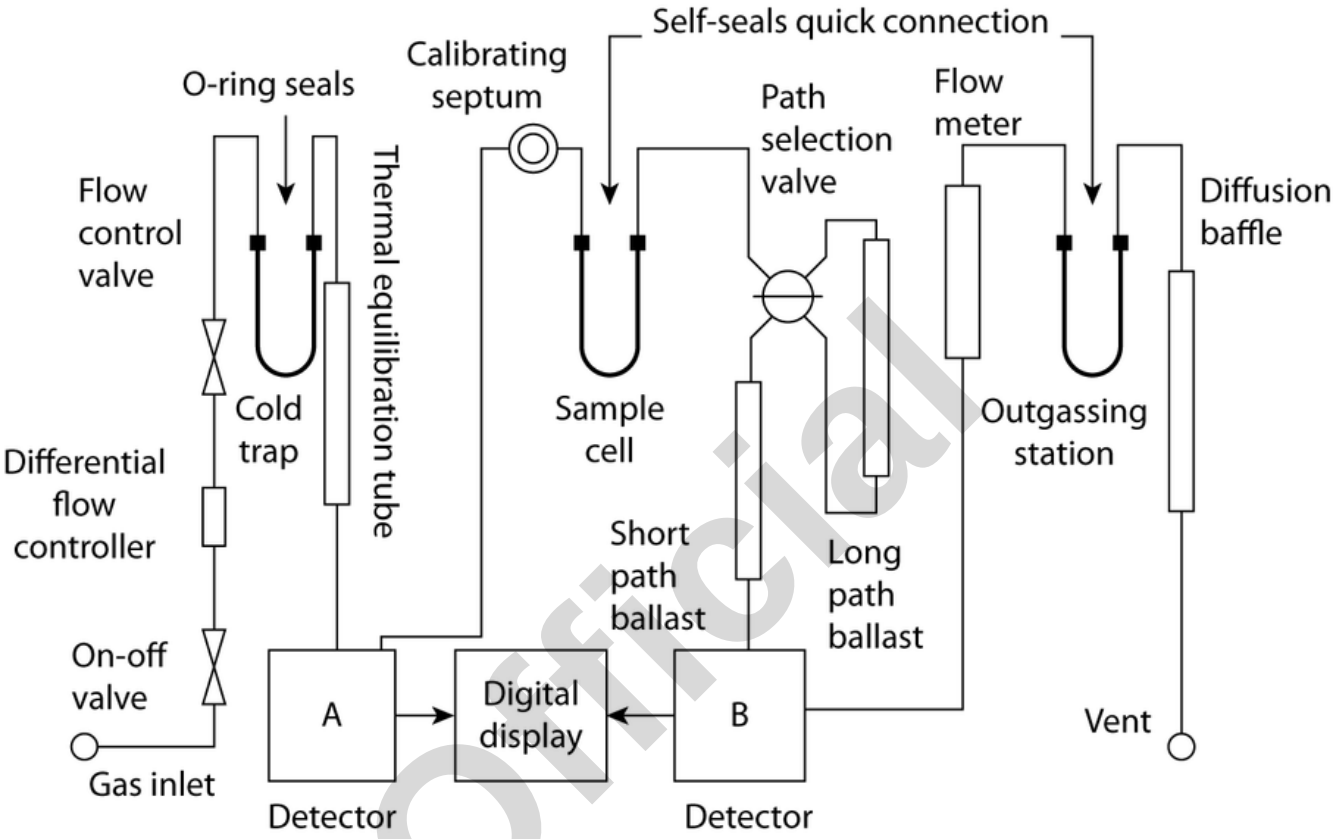


Fig. 1. Schematic diagram of the dynamic flow method apparatus.

PROCEDURE

A known mixture of the gases, usually nitrogen and helium, is passed through a thermal conductivity cell, through the sample again, through the thermal conductivity cell, and then to a recording potentiometer.

The sample cell is immersed in liquid nitrogen, and the sample adsorbs nitrogen from the mobile phase. This unbalances the thermal conductivity cell, and a pulse is generated on a recorder chart.

The sample is removed from the coolant; this gives a desorption peak equal in area and in the opposite direction to the adsorption peak. Because this is better defined than the adsorption peak, it is the one used for the determination.

To effect the calibration, a known quantity of adsorbate, sufficient to give a peak of similar magnitude to the desorption peak, is injected into the system, and the proportion of gas volume per unit peak area is obtained.

A mixture of nitrogen and helium is used for a single-point determination; and several such mixtures or premixing two streams of gas are used for a multipoint determination.

The calculation is the same as the volumetric method.

## Method II: The Volumetric Method

PRINCIPLE

In the volumetric method (see *Figure 2*), the recommended adsorbate gas is nitrogen, which is admitted into the evacuated space above the previously outgassed powder sample to give a defined equilibrium pressure, P, of the gas. The use of a diluent gas, such as helium, is therefore unnecessary, although helium may be employed for other purposes, such as to measure the dead volume.

Because only pure adsorbate gas, instead of a gas mixture, is employed, interfering effects of thermal diffusion are avoided in this method.

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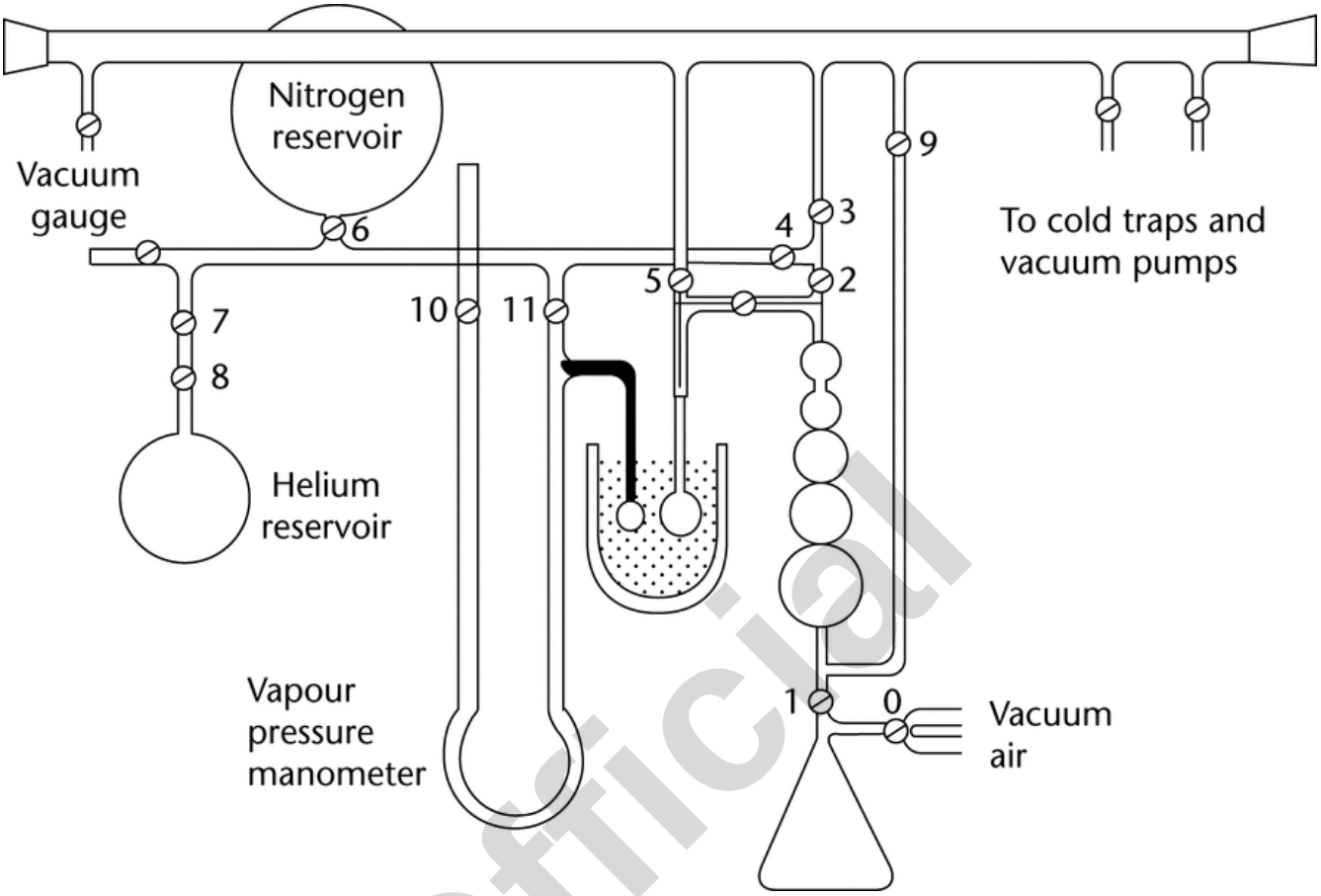


Fig. 2. Schematic diagram of the volumetric method apparatus.

PROCEDURE

A small amount of dry nitrogen is admitted into the sample tube to prevent contamination of the clean surface, the sample tube is removed, a stopper is inserted, the tube is weighed, and the weight of the sample is calculated. Then the sample tube is attached to the volumetric apparatus. The sample is cautiously evacuated down to the specified pressure (e.g., between 2 Pa and 10 Pa). Alternately, some instruments are operated by evacuating to a defined rate of pressure change (e.g., less than 13 Pa/30 s) and by holding for a defined period of time before commencing the next step.

If the principle of operation of the instrument requires the determination of the dead volume in the sample tube, for example, by the admission of a nonadsorbed gas, such as helium, this procedure is carried out at this point, followed by evacuation of the sample. The determination of dead volume may be avoided using difference measurements: that is, by means of reference and sample tubes connected by a differential transducer. The adsorption of nitrogen gas is then measured as described below.

Raise a Dewar vessel containing liquid nitrogen at 77.4 K up to a defined point on the sample cell. Admit a sufficient volume of adsorbate gas to give the lowest desired relative pressure. Measure the volume adsorbed, $V_a$. For multipoint measurements, repeat the measurement of $V_a$ at successively higher $P/P_o$ values. When nitrogen is used as the adsorbate gas, $P/P_o$ values of 0.10, 0.20, and 0.30 are often suitable.

## Reference Materials

Periodically verify the functioning of the apparatus using appropriate reference materials of known surface area that have a specific surface area similar to that of the sample to be examined.

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# ⟨852⟩ ATOMIC ABSORPTION SPECTROSCOPY

## INTRODUCTION

Atomic absorption (AA) spectroscopy is an analytical method that supports qualification and/or quantification of elements. In this use, the AA method supports procedures that measure the absorbance of radiation at a characteristic wavelength by a vapor composed of ground state atoms. A typical instrument consists of a primary energy source that produces the spectrum of the element under examination, a monochromator, and a suitable detector.

For discussion of the theory and principles of measurements, see *Atomic Absorption Spectroscopy—Theory and Practice* ⟨1852⟩, a resource that may be helpful but is not mandatory.

## QUALIFICATION OF ATOMIC ABSORPTION SPECTROPHOTOMETERS

Qualification of an atomic absorption spectrophotometer (AAS) can be divided into three elements: installation qualification (IQ), operational qualification (OQ), and performance qualification (PQ); see also *Analytical Instrument Qualification* ⟨1058⟩.

### Installation Qualification

The IQ requirements provide evidence that the hardware and software are properly installed in the desired location.

### Operational Qualification

In OQ, an instrument's performance is characterized using standards of known spectral properties to verify that the system operates within target specifications (see *Table 1* and *Table 2*). The purpose of OQ is to demonstrate that instrument performance is suitable. OQ is a check of the key operational parameters performed following installation and following repairs and/or maintenance.

The OQ tests in the following sections are typical examples only. Other tests and samples can be used to establish specifications for OQ. Instrument vendors often have samples and test parameters available as part of the IQ/OQ package.

**Table 1. OQ Test and Acceptance Criteria for Flame AAS**

| Test | Procedure | Acceptance Criteria |
|---|---|---|
| Sensitivity | Aspirate a 0.3-µg/mL zinc standard and record the absorbance. | ±20% of AU[a] specified by instrument manufacturer |
| Linearity | Aspirate a blank and 0.05-, 0.075-, 0.10-, 0.25-, and 0.50-µg/mL zinc standards. Generate a calibration curve and record the correlation coefficient (*R*). | Correlation coefficient NLT 0.995 |
| Precision | Assay 5 separate replicates of the 0.10-µg/mL zinc standard versus the standard curve generated for the *Linearity* test. Calculate the %RSD of the 5 results in µg/mL. | %RSD[b] NMT 3% |

[a] AU = absorption unit.
[b] %RSD = % relative standard deviation.

**Table 2. OQ Test and Acceptance Criteria for Graphite Furnace AAS**

| Test | Procedure | Acceptance Criteria |
|---|---|---|
| Sensitivity | Prepare a copper standard and measure the characteristic mass as described by the manufacturer. | ±20% of copper characteristic mass as specified by the manufacturer |
| Linearity | Generate a calibration curve from a blank and 25-, 50-, 75-, and 100-µg/L copper standards. Inject each standard in triplicate and record the %RSD. | Correlation coefficient NLT 0.995 |
| | | %RSD of triplicate injections of each copper standard NMT 5% (not including the blank) |
| Precision | Assay 5 separate replicates of the 50-µg/L copper standard versus the standard curve generated for the *Linearity* test. Inject each replicate in triplicate. | %RSD of 5 replicates NMT 3% |
| | | %RSD of triplicate injections NMT 5% |

### Performance Qualification

PQ determines that the instrument is capable of meeting the user's requirements for all the parameters that may affect the quality of the measurement.

Depending on typical use, the specifications for PQ may be different from the manufacturer's specifications. For validated methods, specific PQ tests, also known as system suitability tests, can be used in lieu of PQ requirements.

Specific procedures, acceptance criteria, and time intervals for characterizing AA spectrophotometer performance depend on the instrument and intended application. Demonstrating stable instrument performance over extended periods of time provides some assurance that reliable measurements can be taken from test sample spectra using validated AA procedures.

***Change to read:***

## PROCEDURE

Evaluate and select the type of material of construction, pretreatment, and cleaning of analytical labware used in AA analyses. The material must be inert and, depending on the specific application, resistant to caustics, acids, and/or organic solvents. For some analyses, diligence must be exercised to prevent the adsorption of analytes onto the surface of a vessel, particularly in ultra-trace analyses. Contamination of the sample solutions from metal and ions present in the container also can lead to inaccurate results.

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For the analysis of a ubiquitous element, it is often necessary to use the purist grade of reagent or solvent available. Check all solutions (diluents, matrix modifier solutions, ionization suppression solutions, reactants, and others) for elemental contamination before they are used in an analysis.

**Standard Solution**

Prepare as directed in the individual monograph. [NOTE—Commercially available single- or multi-element standard solutions, traceable to the National Institute of Standards and Technology or to an equivalent national metrology organization, can be used in the preparation of standard solutions.] Standard solutions, especially those used for ultra-trace analyses, may have limited shelf life. ▲Working standard solutions for ultra-trace analyses▲ (USP 1-Aug-2022) should be retained for not more than 24 h unless stability is demonstrated experimentally.

The method of standard additions also can be used. This method involves adding a known concentration of the analyte element to the sample at no fewer than 2 concentration levels against an unspiked sample preparation. The instrument response is plotted against the concentration of the added analyte element, and a linear regression line is drawn through the data points. The absolute value of the *x*-intercept multiplied by any dilution factor is the concentration of the analyte in the sample.

**Sample Solution**

Prepare as directed in the individual monograph.

A variety of digestion techniques may be indicated to dissolve the sample. These may include hot-plate and microwave-assisted digestions, including open-vessel and closed-vessel approaches. Note that open-vessel digestion generally is not recommended for the analysis of volatile metals, e.g., selenium and mercury.

**Analysis**

Follow the procedure as directed in the individual monograph for the instrumental parameters.

The instrument must be standardized for quantification at the time of use. ▲For a Category I assay, the absorbances of standard solutions that bracket the target concentration of the sample solutions are determined. For a Category II quantitative assay, the absorbances of standard solutions that bracket the specification limit concentration of the sample solutions are determined. For a Category II limit test or a Category IV identification test, only a single standard measurement may be required per the specific monograph. For a multiple standard curve, the detector response is plotted as a function of the analyte concentration to generate the standard plot.▲ (USP 1-Aug-2022) Regression analysis of the standard plot should be used to evaluate the linearity of detector response, and individual monographs may set criteria for the residual error of the regression line.

To demonstrate the stability of the system's initial standardization, the analyst must reassay a solution used in the initial standard curve as a check standard at appropriate intervals throughout the analysis of the sample set. Unless otherwise indicated in the individual monograph, the reassayed standard should agree with its expected value to within ±3% for an assay or ±20% for an impurity analysis.

▲When the method of standard additions is applied, a separate system suitability standard is prepared at a concentration within the analytical range of the method. The concentration of the system suitability standard is determined using the method of standard additions in the same manner as the test solutions. The measured concentration of the system suitability standard should agree with its expected value within ±3% for an assay or ±20% for an impurity analysis.▲ (USP 1-Aug-2022)

Sample concentrations are calculated versus the working curve generated by plotting the detector response versus the concentration of the analyte in the standard solutions.

***Change to read:***

## VALIDATION AND VERIFICATION

**Validation**

Validation is required when an AA method is intended for use as an alternative to the official procedure for testing an official article.

The objective of an AA procedure validation is to demonstrate that the measurement is suitable for its intended purpose, including quantitative determination of the main component in a drug substance or a drug product (Category I assays), quantitative determination of impurities or limit tests (Category II), and identification tests (Category IV). [NOTE—For definition of different categories, see *Validation of Compendial Procedures* ⟨1225⟩.] Depending on the category of the test, analytical procedure validation for AA may require the testing of linearity, range, accuracy, specificity, precision, detection limit, quantitation limit, and robustness. These analytical performance characteristics apply to externally standardized methods and to the method of standard additions.

General information chapter ⟨1225⟩ provides definitions and general guidance on analytical procedures validation without indicating specific validation criteria for each characteristic. The intention of the following sections is to provide the user with specific validation criteria that represent the minimum expectations for this technology. For each particular application, tighter criteria may be needed to demonstrate suitability for the intended use.

- **ACCURACY:** For Category I assays or Category II tests, accuracy can be determined by conducting recovery studies with the appropriate matrix spiked with known concentrations of elements. It is also an acceptable practice to compare assay results obtained using the AA procedure under validation to those of an established analytical procedure. In standard addition methods, accuracy assessments are based on the final intercept concentration, not the recovery calculated from the individual standard additions.

  **Validation criteria:** 95.0%–105.0% mean recovery for the drug substance assay and the drug product assay, and 70.0%–150.0% mean recovery for the impurity analysis. These criteria apply throughout the intended range.

- **PRECISION**

  **Repeatability:** The analytical procedure should be assessed ▲using 6 replicate measurements (▲ (USP 1-Aug-2022)measuring the concentrations of 6 independently prepared sample solutions▲)▲ (USP 1-Aug-2022) at 100% of the assay test concentration. Alternatively, ▲3 concentration levels with 3 independently prepared samples for each level▲ (USP 1-Aug-2022)

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can be used. The 3 concentrations should be close enough that the repeatability is constant across the concentration range. If this is done, the repeatability at the 3 concentrations is pooled for comparison to the acceptance criteria. If validating a procedure by the method of standard additions, the precision criterion applies to the final experimental result, not the accuracy of the individual standard addition levels.

**Validation criteria:** The relative standard deviation is not more than 5.0% for the drug substance assay, not more than 5.0% for the drug product assay, and not more than 20% for the impurity analysis.

**Intermediate precision:** The effect of random events on the analytical precision of the procedure should be established. Typical variables include performing the analysis on different days, using different instrumentation, or having the method performed by two or more analysts. As a minimum, the analytical procedure should be assessed by performing the repeatability test ▲(6 replicate measurements)▲ (USP 1-Aug-2022) in any combination of at least two of the conditions previously mentioned (totaling 12 measurements).

**Validation criteria:** The relative standard deviation is not more than 8.0% for the drug substance assay, not more than 8.0% for the drug product assay, and not more than 25.0% for the impurity analysis ▲based on the 12 measurements.▲ (USP 1-Aug-2022)

- **SPECIFICITY:** The procedure must be able to unequivocally assess each analyte element in the presence of components that may be expected to be present, including any matrix components.
  **Validation criteria:** Demonstrated by meeting the accuracy requirement.
- **QUANTITATION LIMIT:** The limit of quantitation (QL) can be estimated by calculating the standard deviation of no fewer than 6 replicate measurements of a blank solution, divided by the slope of a standard curve, and multiplying by 10. If validating a procedure using the method of standard additions, the slope of standards applied to a solution of the test material is used. Other suitable approaches can be used (see ⟨1225⟩).
  A measurement of a test solution prepared from a representative sample matrix spiked at the estimated QL concentration must be performed to confirm accuracy. If validating a procedure using the method of standard additions, the validation criterion applies to the final experimental result, not the spike recovery of the individual standard addition levels.
  **Validation criteria:** The analytical procedure should be capable of determining the analyte precisely and accurately at a level equivalent to 50% of the specification.
- **LINEARITY:** A response curve between the analyte concentration and absorbance is prepared from no fewer than 5 standard solutions at concentrations encompassing the anticipated concentration of the test solution. The standard curve is then evaluated using appropriate statistical methods, such as a least-squares regression.
  For experiments that do not yield a linear relationship between analyte concentration and AA response, appropriate statistical methods must be applied to describe the analytical response.
  **Validation criteria:** Correlation coefficient (*R*), not less than 0.995 for Category I assays and not less than 0.99 for Category II quantitative tests.
- **RANGE:** Range is the interval between the upper and lower concentrations (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy, and linearity. Range is demonstrated by meeting the linearity, precision, and accuracy requirements.
  **Validation criteria:** For Category I tests, the validation range for 100.0% centered acceptance criteria is 80.0%–120.0%. For noncentered acceptance criteria, the validation range is 10.0% below the lower limit to 10.0% above the upper limit. For content uniformity, the validation range is 70.0%–130.0%. For Category II tests, the validation range covers 50.0%–120.0% of the acceptance criteria.
- **ROBUSTNESS:** The reliability of an analytical measurement is demonstrated by deliberate changes to experimental parameters. For AA this can include but is not limited to sample preparation steps and heating programs, including atomization hold time or atomization temperature. Exercise caution when changing fuel and oxidant gas flows and burner hardware, because this could potentially create a flash-back condition.

**Verification**

US current good manufacturing practices regulations [21 CFR §211.194(a)(2)] indicate that users of the analytical procedures, as described in *USP–NF*, are not required to validate these procedures if provided in a monograph. Instead, they must simply verify their suitability under actual conditions of use.

The objective of an AA procedure verification is to demonstrate that the procedure, as prescribed in a specific monograph, can be executed by the user with suitable accuracy, specificity, linearity, and precision using the instruments, analysts, and sample matrices available. According to *Verification of Compendial Procedures* ⟨1226⟩, if the verification of the compendial procedure by following the monograph is not successful, the procedure may not be suitable for use with the article under test. It may be necessary to develop and validate an alternative procedure as allowed in *General Notices, 6.30 Alternative and Harmonized Methods and Procedures*.

▲▲ (USP 1-Aug-2022)

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# ⟨853⟩ FLUORESCENCE SPECTROSCOPY

## INTRODUCTION

Fluorescence is a two-step process that requires absorption of light at a specific wavelength (excitation) followed by emission of light, usually at a higher wavelength. The emission of light is termed *fluorescence*.

The most common type of fluorescent sample is a submicromolar transparent solution that absorbs light following the Beer–Lambert–Bouguer Law and fluoresces with an intensity that is directly proportional to the concentration, the absorptivity, and the fluorescence quantum yield of the fluorescent species or fluorophore.

Unlike absorption spectroscopy, where deviation from linearity is the exception, fluorescence linearity can be affected by a number of sample-related effects. For additional information, see *Fluorescence Spectroscopy—Theory and Practice* ⟨1853⟩.

Fluorescence methods also are termed *background-free* because little excitation light reaches the detector. This characteristic makes fluorescence detection highly sensitive, down to single-molecule detection in some cases. Fluorescence detection also can be highly specific because a fluorophore emits a characteristic emission pattern. Specificity and sensitivity are two of the more important strengths of fluorescence methods.

## QUALIFICATION OF FLUORESCENCE INSTRUMENTS

Analysts ensure the suitability of a specific instrument for a given procedure by using a stepwise evaluation for the desired application from selection to instrument retirement: design qualification (DQ); installation qualification (IQ); an initial performance-to-specification qualification, also known as operational qualification (OQ); and an ongoing performance qualification (PQ). For additional information, see general chapter *Analytical Instrument Qualification* ⟨1058⟩.

DQ and IQ are not further considered in this chapter. The purpose of this section is to provide test methods and acceptance criteria to ensure that the instrument is suitable for its intended use (OQ) and that it will continue to function properly over extended time periods (PQ).

As with any spectrometric device, analysts must qualify a spectrofluorometer for both wavelength (*x*-axis) and relative intensity (*y*-axis or signal axis) accuracy and precision. They also must establish sensitivity. OQ should span the operational ranges required within the laboratory for both intensity and wavelength scales.

### Instrument Operational Qualification

The tolerances given in both the instrument OQ and PQ are applicable for general use. Specifications for particular instruments and applications can vary depending on the analytical procedure used and the desired accuracy of the final result. Instrument vendors often have samples and test parameters available as part of the IQ/OQ package.

Wherever possible, analysts should use certified reference materials for purposes of calibration in the steps detailed below in preference to laboratory-prepared solutions. When certified reference materials are obtained from a recognized accredited source, they have independently verified traceable value assignments with associated calculated uncertainties.

Two general types of instrumental measurements are differentiated here: spectral (i.e., those that measure intensity versus wavelength) and fixed (i.e., those that measure intensity at a fixed wavelength and bandwidth).

#### CONTROL OF WAVELENGTHS

The level of confidence of measured peak positions is defined by wavelength accuracy for spectral measurements. Determination of the accuracy of many wavelengths across the desired wavelength range demonstrates if further calibration beyond a single point is needed. Multipoint calibration involves measuring wavelength biases at multiple wavelengths and correcting for the wavelength dependence of the bias. A single-point calibration often can be applied to the wavelength axis in an instrument's software before data are collected, but a multipoint calibration may require that the correction be applied to spectra after they are collected.

For fixed measurements, the wavelength position and bandwidth must be reproducible. For filter-based wavelength selection, this requires that only the same filter be used when analysts compare data over time. If a different filter must be used (e.g., when data are compared across instruments and laboratories), then the transmission curves of the filters must be compared.

Wavelength precision should be determined over the operational range using at least six replicate measurements. The standard deviation should not exceed ±1 nm.

#### ATOMIC LINE SPECTRA

This procedure is described as the primary application because the emission lines produced from a discharge lamp are characteristic of the source element, and, as a fundamental physical standard, these wavelengths have been measured with an uncertainty of NMT ± 0.01 nm. In solution spectrofluorometry the wavelength bias required rarely exceeds 1.0 nm. For these reasons, the atomic line standard values are cited without uncertainty. The lamp should be placed at the source position in the spectrofluorometer.

A commonly employed low-pressure mercury lamp has a number of intense lines that cover a large part of the UV and visible range. Manufacturers often use two Xenon lines from the source at 260.5 and 541.9 nm as an internal calibration check, because

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the accuracy of both the excitation and emission monochromators can be verified and can be used for diagnostic purposes (see *Table 1*).[1]

**Table 1. Elemental Line Spectra Wavelengths**

| Element | Wavelength (nm) |
|---|---|
| Hg | 253.7 |
| Xe | 260.5 |
| Hg | 296.7 |
| Hg | 365.0 |
| Hg | 404.7 |
| Hg | 435.8 |
| Xe | 541.9 |
| Hg | 546.1 |
| Hg | 577.0 |
| Hg | 579.1 |

## USE OF RARE EARTH OXIDE SOLUTIONS

This procedure uses a solution of a rare earth oxide prepared by dissolution in acid media. The most frequently used is holmium oxide in perchloric acid in combination with a diffuse reflector located at the sample position. Suitable certified reference materials are available commercially.[2] The wavelength selector not being scanned should be removed; if removal is not practicable, it should be set to zero order (in this position a grating behaves like a mirror reflecting all wavelengths). The diffuse reflector is scanned with and without the rare earth sample in place, and the ratio of the two intensities is calculated to obtain an effective transmittance spectrum. Minima in the intensity ratio correspond to absorption peaks of the sample. For a 4% (w/w) solution of holmium oxide in perchloric acid at 1.0 nm spectral bandwidth and a path length of 1 cm, these minima are in *Table 2*.[3]

**Table 2**

| Wavelength (nm) |
|---|
| 241.1 |
| 249.9 |
| 278.1 |
| 287.2 |
| 333.5 |
| 345.4 |
| 361.3 |
| 385.6 |
| 416.3 |
| 451.4 |
| 467.8 |
| 485.2 |
| 536.6 |
| 640.5 |

If the operational range of the spectrophotometer lies outside 240–650 nm, other certified rare earth oxides or other solutions are used.

Didymium (a mixture of neodymium and praseodymium) is available as a traceable standard in both solution and glass presentations. Didymium is similar in preparation to the holmium materials and has useful peak characteristics in the 730–870 nm region. Useful peaks are found in the didymium solution at approximately 731.6, 740.0, 794.1, 799.0, and 864.4 nm.

[1] The rounded values are taken from ASTM Standard E388-04 (2009).
[2] NIST SRM 2034 is no longer available.
[3] The rounded values are taken from the intrinsic wavelength standard absorption band data from Travis et al. *J Phys Chem Ref Data* 2005;34(1):41. The maximum 95% measurement uncertainty is ±0.06 nm.

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### USE OF POLYMETHYLMETHACRYLATE-DOPED REFERENCES

This procedure uses solid reference materials manufactured by polymerization of a variety of fluorescent active aromatic ring compounds into an inert polymethylmethacrylate (PMMA) matrix. These materials are supplied as polished blocks for use in a standard cuvette holder (see *Table 3*).

**Table 3. Dopant, Excitation, and Emission Data for Selected Reference Materials**

| Dopant | Excitation λ (nm) | Emission λ (nm) |
|---|---|---|
| *p*-Terphenyl | 295 | 338 |
| Ovalene | 342 | 482 |
| Tetraphenylbutadiene | 348 | 422 |
| Anthracene | 360 | 402 |

## Performance Verification

Results from day-to-day testing of photostable intensity standards are used to verify the performance of an instrument. If the measured intensity does not change from that observed when the instrument was qualified, then instrument performance has not changed and remains qualified. Using such standards to determine an artifact-based or quasi-absolute intensity scale potentially enables measured intensities and instrument sensitivity to be compared over time or between instruments. Intensity measurements should be within the linear range of the instrument's detection system before analysts attempt intensity comparisons, and will be affected by any instrumental effect directly related to the fluorescence signal, e.g., changes in source intensity, detector response, etc.

For instruments with filter-based wavelength selection, analysts use fluorescence standards for spectral correction to determine expected intensity differences caused by filters with different transmission profiles. By compensating for these intensity differences due to spectral mismatch, the analyst can determine a quasi-absolute intensity scale for these instruments. Analysts should approach instrument-to-instrument comparisons with particular caution because of the relatively large and difficult-to-quantify uncertainties involved.

### USE OF LOW-CONDUCTIVITY (18-MΩ) WATER

The Raman band of water is used to measure signal-to-noise ratios in fluorescent instruments. The Raman band of water is inherently reproducible and does not degrade with time. Water is convenient to obtain in a pure state and allows interlaboratory comparisons to be made with a high level of confidence. No preparation or dilution is required. The Raman band is a low-level signal that provides a good test for both the optics and the electronics of an instrumental system.

The Raman band of water is not caused by fluorescence but is a result of Raman scattering. For water, the Raman band is always red-shifted 3382 $cm^{-1}$ relative to the excitation.[4] This band usually is measured by excitation at 350 nm, resulting in a Raman peak at 397 nm, but radiation up to 500 nm also can be used as the excitation wavelength, and the corresponding emission peak is 602 nm.

### USE OF INTENSITY STANDARDS

Several solid-doped fluorescent materials are available.[5] These polymers or glasses enable the relative spectral correction and day-to-day performance qualification of fluorescence instruments across the UV, visible, and NIR regions from 320 to 830 nm. The high photostability of the materials makes them particularly useful as day-to-day intensity standards, even when spectral correction is not needed or when the excitation wavelength differs from that used for certification. A certified, steady-state emission spectrum is supplied with each certified reference material, along with the estimated total uncertainties. The reference is available in the form of a solid glass, standard-sized cuvette (12.5 mm × 12.5 mm × 45 mm) with three polished long faces for 90° detection and one frosted long face for front-face or epifluorescence detection.

Alternatively, analysts can use fluorophores in solutions that have been shown to be stable.[6, 7]

# QUALITATIVE AND QUANTITATIVE FLUORESCENCE MEASUREMENTS

Two general classes of procedural measurements commonly are performed by fluorescence spectrometry: qualitative and quantitative.

[4] The red-shift value is taken from Parker CA. Raman spectra in spectrofluorimetry. *Analyst*. 1959;84:446–453.
[5] Available from commercial vendors and from NIST as SRMs 2940 (orange emission), 2941 (green emission), 2942 (UV emission), 2943 (blue emission), and 2944 (red/NIR emission).
[6] Commercial vendors provide a 1-mg/L solution of quinine sulfate dehydrate in 0.105 M perchloric acid that has been fully characterized by NIST as SRM 936a.
[7] A series of day-to-day Intensity Standards is available from the German Federal Institute for Materials Research and Testing (BAM).

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### Qualitative Fluorescence Measurements

Qualitative fluorescence measurements are used to detect the presence of particular analytes and yield a positive or negative answer. The excitation and emission wavelengths often are selected at the peak maximum of the fluorophore to be detected. The minimum amount of analyte needed for a positive result should be considered by the analyst to ensure that the method is appropriate for the particular application. The observation of fluorescence at the peak position above the limit of detection (usually 3 times the noise level) indicates a positive result.

### Quantitative Fluorescence Measurements

Quantitative fluorescence measurements are used to determine amounts or concentrations of analytes in unknown samples. These quantities may be determined in absolute units, such as moles or moles per L, or in relative units, such as the ratio of the concentrations of two fluorescent analytes contained in a single unknown solution. These determinations use the following proportionality relating fluorescence signal ($S$) at a given pair of excitation and emission wavelengths ($\lambda_{ex}$, $\lambda_{em}$) to fluorescent analyte concentration ($c$):

$$S \propto I_0 \times \Omega \times R_d \times \alpha \times \Phi \times c$$

$I_0$ = intensity of the excitation beam
$\Omega$ = fraction of the fluorescence collected by the detection system
$R_d$ = responsivity of the detection system
$\alpha$ = absorption coefficient
$\Phi$ = fluorescence quantum yield
$c$ = concentration of the fluorescent analyte

This linear proportionality with concentration applies to optically dilute samples (e.g., solutions with an absorbance of less than 0.05 at a path length of 1 cm).

## GOOD SPECTROSCOPIC PRACTICE

Comparisons of a test specimen with a Reference Standard are best made at a peak of spectral emission for the compound of interest. Assays based on spectrofluorometry give the commonly accepted wavelengths for excitation and peak spectral emission of the substance in question. Different spectrofluorometers may show minor variation in the apparent wavelength of this peak. Comparisons should be made at the wavelength at which peak emission occurs. If this differs from the wavelength specified in the monograph by more than ±1 nm in the range of 200–400 nm or by more than ±2 nm in the range of 400–800 nm, recalibration of the instrument may be indicated.

### Use of Reference Standards

With few exceptions, pharmacopeial spectrofluorometric procedures provide results by comparison against a USP Reference Standard. This ensures measurement under identical conditions for the test specimen and the Reference Standard. These conditions could include wavelength setting, spectral bandwidth selection, cell placement and correction, and intensity levels. Cells that exhibit identical optical fluorescence characteristics at a specific wavelength may differ considerably at other wavelengths. Analysts should establish and use appropriate cell corrections where required.

The terms *similar preparation* and *similar solution* in tests and assays that involve spectrofluorometry indicate that the Reference Standard should be prepared and observed in a manner that is identical to that used for the sample under test. Usually when a solution of the specified Reference Standard is prepared at (i.e., within 10% of) the desired concentration, the fluorescence intensity is calculated on the basis of the exact amount weighed out. If analysts have not used a previously dried specimen of the Reference Standard, they should correct this intensity on the anhydrous basis.

The expressions *concomitantly determine* and *concomitantly measured* as used in procedures that involve spectrofluorometry indicate that the fluorescence of both the sample solution and the standard solution, relative to the specified test blank, are to be measured in immediate succession.

### Sample Solution Preparation

For determinations using UV or visible spectrofluorometry, the specimen generally is dissolved in a solvent. Unless otherwise directed in the monograph, determinations are made at room temperature by using a path length of 1 cm. Many solvents are suitable for these ranges, including water, alcohols, chloroform, lower hydrocarbons, ethers, and dilute solutions of strong acids and alkalis. Solvents should be free from contaminants that fluoresce in the spectral region under examination. For the solvent, water-free methanol or alcohol or alcohol that has been denatured by the addition of methanol but does not contain benzene or other interfering impurities should be used. Spectrophotometric-quality solvents that are guaranteed to be free from contaminants are available commercially from several sources, but some analytical reagent-grade organic solvents may contain traces of impurities that fluoresce strongly in the UV region. New lots of these solvents should be checked for their transparency, and analysts should take care to use the same lot of solvent for the preparation of the sample solution, the standard solution, and the blank. Solvents that do not have an interfering fluorescence signature at the wavelength(s) of interest should be used. In normal usage, the fluorescence baseline intensity should not be more than 2% of the expected measurement signal unless a larger value previously has been justified.

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Assays in the visible region usually call for comparing concomitantly the fluorescence intensities produced by the sample solution with that produced by a standard solution that contains approximately an equal quantity of a USP Reference Standard. In some situations, it may be permissible to omit the use of a Reference Standard. This is true when spectrofluorometric assays are made with routine frequency, when a suitable standard curve is available and is prepared with the appropriate USP Reference Standard, and when the substance assayed conforms to Beer–Lambert–Bouguer Law within the range of about 75%–125% of the final concentration used in the assay. Under these circumstances, the fluorescence intensity found in the assay can be interpolated on the standard curve, and the assay result can be calculated. Such standard curves should be confirmed frequently and always when a new spectrofluorometer or new lots of reagents are put into use.

## VALIDATION AND VERIFICATION

### Validation

Validation is required when a procedure based on fluorescence spectroscopy is intended for use as an alternative to the official procedure. The objective of validation is to demonstrate that the measurement is suitable for its intended purpose, including the following: quantitative determination of the main component in a drug substance or a drug product (Category I assays), quantitative determination of impurities or limit tests (Category II), and identification tests (Category IV). Depending on the category of the test (for additional information, see *Table 2* in *Validation of Compendial Procedures* ⟨1225⟩), the process for analytical procedure validation for fluorescence requires testing for linearity, range, accuracy, specificity, precision, detection limit, quantitation limit, and robustness. These analytical performance characteristics apply to externally standardized procedures and those that use standard additions. Chapter ⟨1225⟩ provides definitions and general guidance about analytical procedures validation without indicating specific validation criteria for each characteristic. The intention of the following sections is to provide the user with specific validation criteria that represent the minimum expectations for fluorescence technology. For each particular application tighter criteria may be needed in order to demonstrate suitability for the intended use.

#### ACCURACY

For Category I, Category II, and Category III procedures, accuracy is determined by conducting recovery studies with the appropriate matrix spiked with known concentrations of the analyte. Analysts also can compare assay results obtained using the fluorescence procedure under validation to those from an established analytical procedure.
**Validation criteria:** 98.0%–102.0% mean recovery for a drug substance, 95.0%–105.0% mean recovery for a drug product assay, and 80.0%–120.0% mean recovery for impurity analysis. These criteria must be met throughout the intended range.

### Precision

#### REPEATABILITY

Repeatability of the analytical procedure is assessed by measuring the concentrations of six independently prepared sample solutions at 100% of the assay test concentration. Alternatively, repeatability is assessed by measuring concentrations of three replicates of three separate sample solutions at different concentrations. The three concentrations should be sufficiently similar so that the repeatability is similar across the concentration range. If this is done, the repeatability at the three concentrations can be pooled for comparison to the acceptance criteria.
**Validation criteria:** The relative standard deviation is NMT 1.0% for a drug substance, NMT 2.0% for a drug product assay, and NMT 20.0% for impurity analysis.

#### INTERMEDIATE PRECISION

The effect of random events on the analytical precision of the procedure should be evaluated. Typical variables include performing the analysis on different days, using different instrumentation, and having the method performed by two or more analysts. As a minimum, any combination of at least two of these factors totaling six experiments will provide an estimation of intermediate precision.
**Validation criteria:** The relative standard deviation is NMT 1.0% for a drug substance, NMT 3.0% for a drug product assay, and NMT 25.0% for impurity analysis.

#### SPECIFICITY

In fluorescence measurements, specificity is ensured by the use of a Reference Standard wherever possible and is demonstrated by the lack of interference from other components present in the matrix.
**Validation criteria:** Demonstrated by meeting the accuracy requirement

#### DETECTION LIMIT

Analysts can estimate the detection limit (DL) by calculating the standard deviation of NLT six replicate measurements of a blank solution and multiplying by 3.3. Alternatively, the standard deviation can be determined from the error of the intercept from a calibration curve or by demonstration that the signal-to-noise ratio is >3.3. Analysts must confirm the estimated DL by analyzing samples at the calculated concentration.

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### QUANTITATION LIMIT

Analysts can estimate the quantitation limit (QL) by calculating the standard deviation of NLT six replicate measurements of a blank solution and multiplying by 10. Alternatively, the standard deviation can be determined from the error at the intercept from a calibration curve or by demonstration that the signal-to-noise ratio is >10.

A sample solution prepared from a representative sample matrix spiked at the required QL concentration is measured to confirm sufficient sensitivity and adequate precision. The observed signal-to-noise ratio at the required QL should be >10.

**Validation criteria:** For the estimated limit of quantitation to be considered valid, the measured concentration must be accurate and precise at a level equal to or less than 50% of the specification.

### LINEARITY

A response curve between the analyte concentration and the fluorescence signal is prepared from NLT five Standard solutions at concentrations that encompass the anticipated concentration of the sample solution. Analysts then should evaluate the standard curve for linearity using appropriate statistical methods such as a least-squares regression. Deviation from linearity can result from either instrumental or sample factors, or both, and can be reduced to acceptable levels by reduction of the analyte concentration and thereby the associated absorbance values.

**Validation criteria:** The correlation coefficient ($R$) must be NLT 0.995 for Category I assays and NLT 0.99 for Category II quantitative tests.

### RANGE

The operational range of an analytical instrument (and the analytical procedure as a whole) is the interval between the upper and lower concentrations (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the instrumental response function has a suitable level of precision, accuracy, and linearity.

**Validation criteria:** For Category I tests, the validation range for 100.0% centered acceptance criteria is 80.0%–120.0%. For noncentered acceptance criteria, the validation range is 10.0% below the lower limit to 10.0% above the upper limit. For content uniformity, the validation range is 70.0%–130.0%. For Category II tests, the validation range covers 50.0%–120.0% of the acceptance criteria.

### ROBUSTNESS

Analysts should demonstrate the reliability of an analytical measurement by deliberate changes to experimental parameters. For fluorescence these changes can include measuring the stability of the analyte under specified storage conditions, varying pH, removal of oxygen, and adding possible interfering species, to list a few examples. Analysts should determine robustness concurrently using a suitable design-of-experiments procedure.

## Verification

Analytical procedures described in *USP–NF* do not require validation. Instead, a verification is used to determine a procedure's suitability under actual conditions of use.

Thus the objective of fluorescence procedure verification is to demonstrate the suitability of a test procedure under actual conditions of use. Performance characteristics that verify the suitability of a fluorescence procedure are similar to those required for any analytical procedure. For additional information, see *Verification of Compendial Procedures* ⟨1226⟩ for a discussion of the applicable general principles. Verification should be performed using a reference material and a well-defined matrix. Verification of compendial fluorescence procedures should at a minimum include the execution of the validation parameters for specificity, accuracy, precision, and quantitation limit, when appropriate, as indicated in *Validation*.

## Indirect Measurement Requirements

Some fluorescence procedures employ chromogenic reactions. Generally the requirements for the analytical performance characteristics should be used. In some instances the required accuracy and precision for the direct measurements may not be achievable. Under these circumstances, the accuracy and precision requirements may be widened by as much as 50%. However, any such widening must be justified on scientific grounds and with documented evidence. Under these circumstances, the amount of replication required to produce a scientifically sound reportable value may be increased.

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# ⟨854⟩ MID-INFRARED SPECTROSCOPY

## INTRODUCTION

Mid-infrared (mid-IR) spectroscopy is an instrumental method used to measure the absorption of electromagnetic radiation over the wavenumber range between 4000 and 400 $cm^{-1}$ (corresponding to wavelengths between 2.5 and 25 µm). Unless otherwise specified in a monograph or other validated procedure, the region from 3800 to 650 $cm^{-1}$ (corresponding to wavelengths from 2.6 to 15 µm) should be used to ensure compliance with monograph specifications for IR absorption. The absorption of photons causes the promotion of molecules from a ground state of their vibrational mode to an excited vibrational state.

Vibrational modes are defined by the motion of all atoms in a molecule. When molecules contain certain functional groups, IR absorption often occurs in specific narrow spectral ranges. In these cases, the wavenumbers at which these transitions occur are known as group frequencies. When a vibrational mode involves atomic motions of more than just a few atoms, the frequencies occur over wider spectral ranges and are not characteristic of a particular functional group but are more characteristic of the molecules as a whole. Such bands are known as fingerprint bands. All strong bands that absorb at wavenumbers above 1500 $cm^{-1}$ are group frequencies. Strong bands that absorb below 1500 $cm^{-1}$ can be either group frequencies or fingerprint bands.

For discussion of the theory and principles of measurements, see *Mid-Infrared Spectroscopy—Theory and Practice* ⟨1854⟩, which may be a helpful, but not mandatory, resource.

## QUALIFICATION OF IR SPECTROPHOTOMETERS

Qualification of mid-IR spectrophotometers is divided into three components: *Installation Qualification* (IQ), *Operational Qualification* (OQ), and *Performance Qualification* (PQ). For further information, see *Analytical Instrument Qualification* ⟨1058⟩.

### Installation Qualification

The IQ requirements provide evidence that the hardware and software are properly installed in the desired location.

### Operational Qualification

Because the majority of mid-IR spectra are measured with Fourier-transform IR (FTIR) spectrophotometers, only these instruments will be discussed. [NOTE—No recommended values for signal-to-noise ratio or 100% line stability are included in this chapter because these vary with manufacturer, model, and age of the instrument.]

WAVENUMBER ACCURACY

The most commonly used wavenumber standard for IR spectrophotometry is an approximately 35 µm thick, matte polystyrene film. The spectrum of such a film has several sharp bands at 3060.0, 2849.5, 1942.9, 1601.2, 1583.0, 1154.5, and 1028.3 $cm^{-1}$. The most frequently chosen band for wavenumber accuracy determination is located at 1601.2 $cm^{-1}$. Using a suitable polystyrene film or other well-characterized wavenumber standard, scan from 3800 to 650 $cm^{-1}$ wavenumbers, and compare the wavenumber of maximum response of the chosen band using the center-of-gravity, polynomial spline procedure, or other peak-picking algorithms to the known absorption wavenumber of the standard. The acceptable tolerance for the measured wavenumber is ±1.0 $cm^{-1}$.

### Performance Qualification

The purpose of performance qualification (PQ) is to determine that the instrument is capable of meeting the user's requirements for all the parameters that may affect the quality of the measurement.

## PROCEDURE

Mid-IR spectra can be measured by transmission, external reflection, internal reflection (often called attenuated total reflection), diffuse reflection, and photoacoustic spectroscopy. Different sample preparation techniques are available for these options. The most common sample preparation techniques are presented below.

### Potassium Bromide (KBr) Disks

Certain powdered alkali halides such as potassium bromide, potassium chloride, and caesium iodide coalesce under high pressure and can be formed into self-supporting disks that are transparent to mid-IR radiation. The alkali halide most commonly used is powdered, dry, highly pure potassium bromide, which is transparent to mid-IR radiation above 400 $cm^{-1}$.

Commercial presses and dies in a range of diameters are available for the preparation of alkali halide and similar disks.

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## Mineral Oil Mulls

A typical procedure to prepare a mull is to place 10–20 mg of the sample into an agate mortar, and then grind the sample to a fine particle-size powder using a vigorous rotary motion of the pestle. A small drop of the mulling agent is added to the mortar. Rotary motion of the pestle is used to mix the components into a uniform paste, which is transferred to the center of a clean IR-transparent window (e.g., potassium bromide, sodium chloride, silver bromide, or caesium iodide). A second matching window is placed on top of the mull, and the mull is squeezed to form a thin, translucent film that is free from bubbles.

The most widely used mulling agent for the mid-IR region is a saturated hydrocarbon mineral oil (liquid paraffin, Nujol).

## Self-Supported Polymer Films

The mid-IR transmission spectrum of many polymers used as packaging materials is at times recorded from samples prepared as thin self-supporting films using hot compression molding or microtoming.

## Capillary Films

Nonvolatile liquids can be examined neat in the form of a thin layer sandwiched between two matching windows that are transparent to mid-IR radiation. The liquid layer must be free of bubbles and must completely cover the diameter of the IR beam focused on the sample.

## Liquids and Solutions in Transmission Cells

For the examination of liquid and solution samples, transmission cell assemblies that comprise a window pair, spacer, filling ports, and a holder are available commercially in both macro- and micro-sample configurations.

For laboratory applications, spacers typically are formed from lead, poly(tetrafluoroethylene), or poly(ethylene terephthalate) and can be supplied, depending on spacer materials, in standard thickness path lengths from approximately 6 µm to 1 mm or larger.

## Gases

Mid-IR transmission cells for static or flow-through gas and vapor sampling are available in a wide range of materials to suit the application, from laboratory to process scale. In the laboratory, the traditional gas cell has been a 10 cm long cylinder made from borosilicate glass or stainless steel with an approximately 40-mm aperture at each end. Each open end is covered with an end cap that contains one of a pair of mid-IR–transparent windows constructed from, e.g., potassium bromide, zinc selenium, or calcium fluoride.

## Attenuated Total Reflection

Attenuated total reflectance spectroscopy relies on the optical phenomenon of radiation passing through a medium of high refractive index at a certain angle of incidence entirely reflected internally at a boundary in contact with a material of lower refractive index. The medium of high refractive index is also known as the internal reflection element (IRE).

The sample under examination should be placed in close contact with the IRE such as diamond, germanium, zinc selenide, or another suitable material of high refractive index. Ensure close and uniform contact between the substance and the whole crystal surface by applying pressure in the case of solid samples or by dissolving the substance in an appropriate solvent and then covering the IRE with the solution and evaporating to dryness.

## Diffuse Reflection

The most important and commonly used form of sample preparation for diffuse reflection is to dilute the sample by intimately mixing it with 90%–99% of nonabsorbing diluents such as finely powdered potassium bromide or potassium chloride. The sample dilution has the added benefit of reducing absorption band intensities to an appropriate level.

## Microscope Sampling

Coupling a light microscope with a mid-IR spectrophotometer allows spectra to be obtained from very small samples. Generally applied in transmittance or reflectance modes, it provides, for example, a powerful tool for obtaining spectroscopic data of contaminants in pharmaceutical samples.

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# VALIDATION AND VERIFICATION

## Validation

Validation is required when an IR method is intended for use as an alternative to the official procedure for testing an official article.

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The objective of an IR method validation is to demonstrate that the measurement is suitable for its intended purpose including: quantitative determination of the main component in a drug substance or a drug product (Category I assays), quantitative determination of impurities or limit tests (Category II), and identification tests (Category IV, see *Table 2* in *Validation of Compendial Procedures* ⟨1225⟩). Depending on the category of the test, the validation process for IR may require the testing of linearity, range, accuracy, specificity, precision, detection limit, quantitation limit, and robustness. If the IR procedure employs a chemometrics model calculated against the response of another analytical technology (e.g., HPLC), then the principles of ▲*Near-Infrared Spectroscopy—Theory and Practice* ⟨1856⟩▲ (CN 1-May-2020), specifically the *Method Validation* section, is to be applied.

Chapter ⟨1225⟩ provides definitions and general guidance on analytical procedures validation without indicating specific validation for each characteristic. The following sections are intended to provide the user with specific validation criteria that represent the minimum expectations for this technology. For each particular application, tighter criteria may be needed in order to demonstrate suitability for the intended use.

## ACCURACY

For Category I and II procedures, accuracy can be determined by conducting recovery studies with the appropriate matrix spiked with known concentrations of the analyte. It is also an acceptable practice to compare assay results obtained using the IR procedure under validation with those obtained from an established alternative analytical method.

**Validation criteria:** 98.0%–102.0% mean recovery for drug substances, 95.0%–105.0% mean recovery for drug product assay, and 70.0%–150.0% mean recovery for impurity analysis. These criteria are met throughout the intended range.

## PRECISION

**Repeatability:** The analytical procedure is assessed by measuring the concentrations of six independently prepared sample preparations at 100% of the assay test concentration. Alternatively it can be based on measurements of three replicates of three separate sample solutions at different concentrations. The three concentrations should be close enough so that the repeatability is constant across the concentration range. If this is done, the repeatability at the three concentrations is pooled for comparison to the acceptance criteria.

*Validation criteria:* The relative standard deviation is NMT 1.0% for drug substance, NMT 2.0% for drug product, and NMT 20.0% for impurity analysis.

**Intermediate precision:** The effect on analytical precision caused by changes in variables such as performing the analysis on different days, using different instrumentation, or having the method performed by two or more analysts needs to be assessed. At a minimum, any combination of at least two of these factors totaling six experiments will provide an estimation of intermediate precision.

*Validation criteria:* The relative standard deviation is NMT 1.0% for drug substance, NMT 3.0% for drug product assay, and NMT 25.0% for impurity analysis.

## SPECIFICITY

For Category IV tests, the identity of the analyte should be ensured. Regarding Category I and II procedures, the accuracy requirement also demonstrates specificity for the targeted analytes.

In the case of identification tests, the ability to select between compounds of closely related structure that are likely to be present should be demonstrated. This should be confirmed by obtaining positive results (perhaps by comparison to a known reference material) from samples containing the analyte, coupled with negative results from samples that do not contain the analyte and by confirming that a positive response is not obtained from materials structurally similar to or closely related to the analyte.

## QUANTITATION LIMIT

The quantitation limit can be estimated by calculating the standard deviation of NLT 6 replicate measurements of a blank preparation divided by the slope of the calibration line and multiplying by 10. Other suitable approaches can be used (see ⟨1225⟩). A measurement of a representative sample matrix spiked at the estimated quantitation limit concentration must be performed to confirm accuracy.

**Validation criteria:** For the estimated quantitation limit to be considered valid, the measured concentration must be accurate and precise at a level equal to or less than 50% of the specification.

## LINEARITY

A linear relationship between the analyte concentration and the IR spectral response is demonstrated by preparing NLT 5 standard preparations at concentrations encompassing the anticipated concentration of the test preparation. The standard curve should then be evaluated using appropriate statistical methods such as a least squares regression. For experiments that do not have a linear relationship between analyte concentration and IR spectral response, appropriate statistical methods should be applied to describe the analytical response.

**Validation criteria:** Correlation coefficient ($R$), NLT 0.995 for Category I assays and NLT 0.99 for Category II quantitative tests.

## RANGE

This parameter is demonstrated by meeting linearity, precision, and accuracy requirements.

**Validation criteria:** For Category I tests, the validation range for 100.0% centered acceptance criteria is 80.0%–120.0%. For non-centered acceptance criteria, the validation range is 10.0% below the lower limit to 10.0% above the upper limit. For

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content uniformity, the validation range is 70.0%–130.0%. For Category II tests, the validation range covers 50.0%–120.0% of the acceptance criteria.

ROBUSTNESS

The reliability of an analytical measurement should be demonstrated by deliberate changes to experimental parameters. For mid-IR this can include but is not limited to changes in sample preparation procedure or changes in hardware settings.

## Verification

U.S. Current Good Manufacturing Practices regulations [21 CFR 211.194(a)(2)] indicate that users of analytical procedures described in *USP–NF* are not required to validate these procedures if provided in a monograph. Instead, they must simply verify their suitability under actual conditions of use.

The objective of an IR procedure verification is to demonstrate that the method, as prescribed in specific monographs, is being executed with suitable accuracy, sensitivity, and precision. *Verification of Compendial Procedures* ⟨1226⟩ notes that if the verification of the compendial procedure, according to the monograph, is not successful, the procedure is not suitable for use with the article under test. It may be necessary to develop and validate an alternative procedure as allowed in *General Notices, 6.30 Alternative and Harmonized Methods and Procedures*.

Although complete revalidation of a compendial procedure is not required, verification of the compendial Mid-IR procedure includes the execution of certain critical parameters. When the method being verified is for identification purposes, specificity is the only parameter required. For quantitative applications, additional validation parameters are studied. Typically these include accuracy, precision, and quantitation limit, as indicated in *Validation*.

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# ⟨855⟩ NEPHELOMETRY ▲AND▲ (USP 1-May-2019) TURBIDIMETRY

▲▲ (USP 1-May-2019)

***Add the following:***

▲1. INTRODUCTION
2. TERMS AND DEFINITIONS
3. APPLICATIONS
4. INSTRUMENTATION
5. FORMAZIN TURBIDITY STANDARDS
    5.1 Preparation of the Formazin Standards
6. QUALIFICATION OF TURBIDIMETERS AND NEPHELOMETERS
    6.1 Calibration
    6.2 Stray Light
    6.3 Range of Measuring Capability
    6.4 Resolution
    6.5 Accuracy
    6.6 Performance Qualification
7. PROCEDURES
    7.1 Turbidimetric Procedures
    7.2 Nephelometric Procedures
8. VALIDATION AND VERIFICATION
    8.1 Validation
    8.2 Verification▲ (USP 1-May-2019)

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## ▲1. INTRODUCTION

Nephelometry and turbidimetry are analytical techniques that are based on the principles of light-scattering phenomena. Light scattering is the physical phenomenon in which a beam of light changes its direction of propagation (known as deflection) as a result of interaction with sufficiently small matter particles. It has been established from the Maxwell electromagnetic theory that a prerequisite for scattering to occur is that the refractive indexes of the suspended particles must be different from those of the suspending liquid. The larger the difference, the more intense the scattering becomes. There are two types of light scattering: 1) elastic scattering, in which the wavelength of the scattered light and incident light are the same; and 2) inelastic light scattering, in which the wavelength of the scattered light and incident light are different. Only the first type of light scattering (elastic) is relevant to nephelometry and turbidimetry.

In turbidimetry, the intensity of the transmitted light is measured and the attenuation of the intensity of incident light as a result of scattering is measured at the direction of incident light (i.e., 0°) and compared to the intensity of incident light (blank measurement). The measured property is an indirect measurement of the scattering effect of the suspended particles and is referred to as turbidance. Any absorbance of light by the suspended sample will result in additional attenuation of light intensity (see *Ultraviolet-Visible Spectroscopy* ⟨857⟩ and *Ultraviolet-Visible Spectroscopy—Theory and Practice* ⟨1857⟩). Hence, it is important to ensure that the material being measured does not absorb light at the measurement wavelength. Indeed the equations governing absorption and turbidimetry are the same (albeit with different values for the attenuation constants). In nephelometric techniques, the intensity of the scattered light at a 90° angle from the propagation direction of the incident light is measured. Therefore, a nephelometric measurement is a direct measurement of the scattering effect of suspended matter.

## 2. TERMS AND DEFINITIONS

Terms commonly used in describing turbidimetric and nephelometric techniques are:

- Turbidance (symbol, $S$): A measure of the decrease of the transmitted incident light beam intensity as a result of the light-scattering effect of suspended particles. The amount of suspended matter may be measured by observation of either the transmitted light (turbidimetry) or the scattered light (nephelometry).

$$\log I_0/I_t = kbc = T$$

$I_0$ = intensity of incident light
$I_t$ = intensity of transmitted light
$k$ = molar turbidity coefficient
$b$ = cell path length
$c$ = concentration

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$T$ = turbidance

- Turbidity (symbol, $\tau$): In turbidimetric measurements, the turbidity is the measure of the decrease in incident beam intensity/unit length of a given suspension. The International Organization for Standardization defines turbidity as “the reduction of transparency of a liquid caused by the presence of undissolved matter”.
- Turbidity Measurement Units: The turbidity units are stated using a descriptor which indicates the method of measurement.
- Nephelometric Turbidity Units (NTUs): When the turbidity is measured using a nephelometer, which measures the scattered light at a 90° angle from the direction of propagation of incident light, the units of turbidity are called nephelometric turbidity units (NTUs). The magnitude of NTU is defined based on the turbidity generated by primary formazin standard (a suspension made by mixing solutions of hydrazine sulfate and hexamethylenetetramine in water). Safer polymer-bead suspensions are now commercially available and are recognized as an acceptable alternative. However, all those standards are traced to formazin. The primary formazin standard solution has been assigned a turbidity of 4000 NTUs.

Other recognized units for turbidity include the formazin turbidity unit (FTU) and the formazin nephelometric unit (FNU). These units are equivalent to NTU for the range from 0–40 NTUs.

## 3. APPLICATIONS

Turbidimetric and nephelometric techniques have applications that include 1) concentration determination of solutions and/or suspensions (determination of several cations and anions by precipitating and suspending the resulting precipitate at well-controlled reaction parameters); 2) measurement of the degree of turbidity of turbid solutions or suspensions; 3) determination of weight-average molecular weights and dimensions of polydisperse systems in the molecular weight range from 1000 to several hundred million; 4) measurement of immunoassays’ reaction kinetics or kinetics of immunoprecipitations (rate nephelometry); 5) monitoring of cell and bacteria growth; and 6) particle size distribution determination of suspended material, particle counting, etc.

Rate nephelometry is widely used for vaccine components assays and/or quantitation of components in blood serum. It is also used for host cell protein qualification in recombinant biopharmaceuticals. When using the technique, the measurement of the change in the light-scattering response by antigen–antiserum or antigen-purified antibody complexes is used to calculate the amount of antigen (Ag) or antibody (Ab) responsible for the immunological Ab-Ag precipitation reaction or agglutination reaction. Often the antigens under consideration are linked covalently or adsorbed to polymeric microspheres to increase the scattering efficiency; the resulting technique is known as "particle-enhanced immunoassay". Although the technique is described as nephelometry, usually both scattered and transmitted light are measured using the ratio instruments.

Nephelometric measurements are more reliable in low turbidity ranges (relatively low concentration of the scattering medium). In this range, a linear relationship is observed between the sample concentration and the detector’s signal intensity expressed as NTU. As the concentration increases, so does the incidence of multiple scattering that deviates the response from the linearity. The maximum NTU value, which supports a reliable linearity relationship, is in the range of 1750–2000 NTUs. Turbidimetry is preferred for higher turbidity ranges (concentrations of the scattering media). To achieve consistent results, all measurement variables must be carefully controlled. Where such control is possible, extremely dilute suspensions may be measured.

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## 4. INSTRUMENTATION

Instruments used for turbidimetric and nephelometric measurements are called turbidimeters and nephelometers, respectively. Generally, these instruments consist of a mercury lamp with filters for the strong green or blue lines, a shutter, a set of neutral filters with known transmittance, and a sensitive photomultiplier, which can be mounted fixed at 0° or at a 90° angle from the incident light propagation direction, or on an arm that can be rotated around the solution cell and set at any angle from –135° to 0° to +135° by a dial outside of the light-tight housing. Solution cells are of various shapes, such as square for measuring 90° scattering; semioctagonal for 45°, 90°, and 135° scattering; and cylindrical for scattering at all angles (see *Figure 1*).

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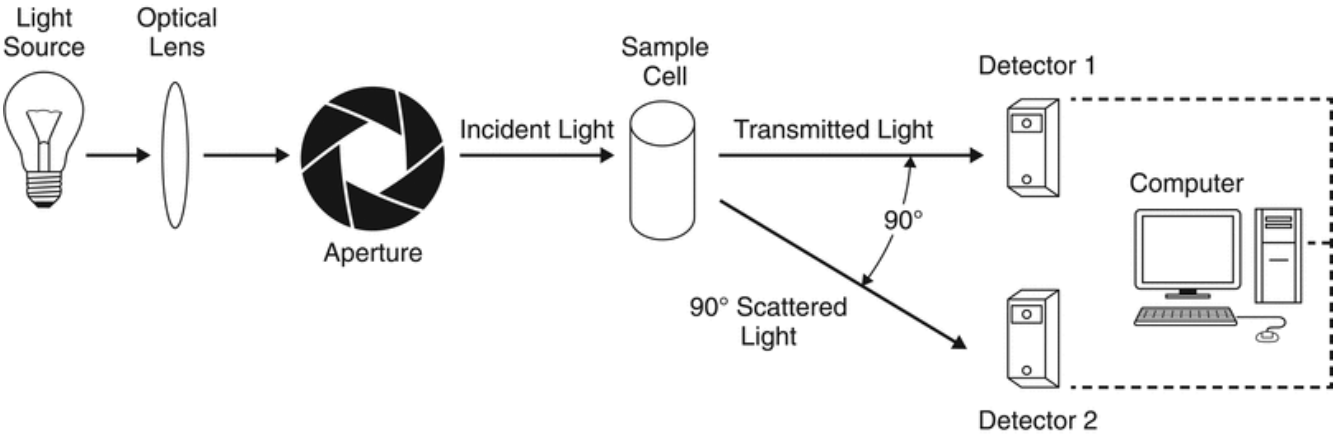


Figure 1. Representative nephelometric (turbidimetric) instrument. Note that Detector 2 may be mounted on a movable arm.

Turbidity also can be measured with a standard photoelectric filter photometer or spectrophotometer, preferably with illumination in the blue portion of the spectrum. Nephelometric measurements require an instrument with a photocell placed so as to receive scattered, rather than transmitted, light. Because this is the same geometry used in fluorometers, they can be used as nephelometers by proper selection of filters. A ratio turbidimeter combines the technology of 90° nephelometry and turbidimetry. It contains photocells that receive and measure scattered light at a 90° angle from the sample as well as receiving and measuring the forward scatter in front of the sample. It also measures light transmitted directly through the sample. Linearity is attained by calculating the ratio of the 90° angle scattered light measurement to the sum of the forward scattered light measurement and the transmitted light measurement. The benefit of using a ratio turbidimetric system is that the measurement of stray light becomes negligible. In addition, the determination of turbidity of colored suspensions is done exclusively using turbidimetric or nephelometric instruments with ratio mode because this procedure compensates for the attenuation of light as the result of the suspension color. Typically, the light source in these instruments is a tungsten lamp with most of the light intensity at about 550 nm operating at the filament temperature of 2700 K. Other suitable light sources are also available. Typically, the detectors are ▲silicon diodes▲ (ERR 1-May-2019) and photomultipliers. An alternative for eliminating the color effect involves using an infrared light-emitting diode as a light source, which yields an emission maximum centered at about 860 nm and a spectral bandwidth of 60 nm. When laser light sources are also used, especially in nephelometric instruments, the technique is commonly known as "laser nephelometry". The advantage of using laser nephelometers is the significant improvement in signal-to-noise ratio at very low detection levels. Usually the light source is a laser diode with a working wavelength at 660 nm. The high-power density of the laser beam gives rise to higher scattered intensity from smaller particles. Combined with a light trap, which absorbs the unscattered light, the system lowers the stray light significantly. When the use of a nephelometer or turbidimeter is indicated for a procedure in a monograph, instruments working in ratio mode may be used instead.

***Change to read:***

## 5. FORMAZIN TURBIDITY STANDARDS

Formazin is the only known primary turbidity standard. All other standards are secondary and must be traced to formazin. The primary standard is defined in the ▲*IUPAC Compendium of Chemical Terminology,*▲ (ERR 1-May-2019) 2nd ed. (the Gold Book) as one that is prepared by the user from traceable materials using well-defined methodologies and conditions.

Formazin suspension has many features that ensure its suitability as a primary standard. It can be consistently and accurately prepared from reagent-grade starting materials. The suspension consists of random polymers with different lengths and of random configurations, which result in moieties of varying shapes and sizes ranging from less than 0.1 μm to more than 10 μm. Although the polymer chain length distribution has been shown to vary from preparation to preparation, the overall resulting turbidity has been very reproducible.

### 5.1 Preparation of the Formazin Standards

[NOTE—All procedures described below must be performed at 20 ± 2° (see *Volumetric Apparatus* ⟨31⟩.]

- **Hydrazine sulfate solution:** Dissolve 1.000 g of ACS grade hydrazine sulfate ($N_2H_4 \cdot H_2SO_4$) in particle-free water in a 100-mL Class A volumetric flask and dilute with particle-free water to volume. Allow this solution to stand for 4–6 h.
- **Primary formazin standard:** Dissolve 2.50 g of analytical grade hexamethylenetetramine [$(CH_2)_6N_4$] in 25.0 mL of particle-free water in a 100-mL flask. Add 25.0 mL of hydrazine sulfate solution using a Class A 25-mL "to deliver" pipette and mix thoroughly. Allow the preparation to stand for 48 h at 25 ± 1° before using. The resulting suspension is stable for 2 months.
- **Formazin stock standard suspension 1:** Using a 15-mL Class A "to deliver" pipette, transfer 15 mL of the *Primary formazin standard* to a 1-L volumetric flask, and dilute with particle-free water to volume and mix. The resulting suspension has a turbidity of 60 NTU.

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- **Formazin stock standard suspension 2:** Using a 50-mL Class A "to deliver" pipette, transfer 50 mL of *Primary formazin standard* to a 200-mL volumetric flask, and dilute with particle-free water to volume and mix. The resulting suspension has a turbidity of 1000 NTUs.
- **Formazin reference suspensions:** Prepare by mixing in a 100-mL volumetric flask, portions of the respective *Formazin stock standard suspension* and particle-free water according to *Table 1*.

**Table 1. Formazin Reference Suspensions Preparations**

| Formazin Reference Suspensions | Formazin Stock Standard Suspension 1 Volume (mL) | Formazin Stock Standard Suspension 2 Volume (mL) | Particle-Free Water Volume (mL) | Turbidity Value (NTU) |
|---|---|---|---|---|
| Reference suspension I | 5.0 | — | 95.0 | 3 |
| Reference suspension II | 10.0 | — | 90.0 | 6 |
| Reference suspension III | 30.0 | — | 70.0 | 18 |
| Reference suspension IV | 50.0 | — | 50.0 | 30 |
| Stock standard suspension 1 | 100 | — | 0 | 60 |
| Reference suspension VI | — | 10.0 | 90.0 | 100 |
| Reference suspension VII | — | 20.0 | 80.0 | 200 |
| Reference suspension VIII | — | 40.0 | 60.0 | 400 |
| Reference suspension IX | — | 70.0 | 30.0 | 700 |
| Stock standard suspension 2 | — | 100 | 0 | 1000 |

## 6. QUALIFICATION OF TURBIDIMETERS AND NEPHELOMETERS

The suitability of a specific instrument for a given procedure is ensured by a stepwise life cycle evaluation for the desired application from selection to instrument retirement. The qualification comprises three components: 1) installation qualification (IQ), 2) operational qualification (OQ), and 3) performance qualification (PQ) (see *Analytical Instrument Qualification* ⟨1058⟩).

The purpose of this section is to provide test methods and acceptance criteria to ensure that the instrument is suitable for its intended use (OQ), and that it will continue to function properly over extended time periods (PQ). As with any spectrometric device, a turbidimetric and nephelometric spectrometer must be qualified for both wavelength (*x*-axis, if not fixed) and photometric (*y*-axis, or signal axis) accuracy and precision, and meet the requirements for the stray light. OQ is carried out across the operational ranges required within the laboratory for both the absorbance and wavelength scales.

Acceptance criteria for critical instrument parameters that establish "fitness for purpose" are verified during IQ and OQ. Specifications for particular instruments and applications can vary depending on the analytical procedure used and the desired accuracy of the final result. Instrument vendors often have samples and test parameters available as part of the IQ/OQ package.

Wherever possible in the procedures detailed as follows, primary reference standards or certified reference materials (CRMs) are to be used. Formazin is the only primary reference standard used in turbidimetry and nephelometry. All the other standards, including the CRMs, must be correlated to formazin. The CRMs should be obtained from a recognized accredited source and include independently verified traceable value assignments with associated calculated uncertainty. CRMs must be kept clean and free from dust. Recertification should be performed periodically to maintain the validity of the certification.

### 6.1 Calibration

All of the turbidimetric and nephelometric instruments are calibrated against standards of known turbidity. The instrument must be calibrated using formazin turbidity standards prior to its first time use and at least every 3 months or as specified by the vendor. Calibration is performed using at least four formazin turbidity standards whose turbidity proportionally covers the range of interest. Many instrument manufactures provide calibration verification standards. They usually consist of sealed sample cells filled with a latex suspension or with metal oxide particles in polymer gel. These standards must be used only for checking the calibration in the time intervals between the instrument recommended calibrations.

### 6.2 Stray Light

Stray light (stray radiant energy) is a very significant error source, especially for measurements in the range of the lower turbidity readings. It is defined as external light that reaches the detector without being scattered from the sample. There are several sources of stray light including the inherent cell surface imperfections, reflections from within the cell that are unaccounted for, optical system parts, light sources, and, to a smaller degree, the electronics fluctuations. Although there are many design features that instrument vendors use to minimize the stray light, a complete mitigation of the stray light cannot be achieved. Unlike spectrophotometric measurements, the stray light cannot be compensated for in turbidimetry. The stray light must be measured and the values should be within the specification range set by the vendor of the particular instrument or <0.15 NTUs for the measurement in the range of 0–10 NTUs and 0.5 NTUs for the measurements in the range of 10–1100 NTUs, whichever is smaller.

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## 6.3 Range of Measuring Capability

The instrument must be able to measure the turbidity in the range of 0.01–1100 NTUs or from 50%–200% of the target turbidity. To demonstrate the linearity for the intended measurements range, choose at least four appropriate reference suspensions from *Table 1*.

## 6.4 Resolution

Instrument resolution must be 0.01 NTU or less for the measurements range of 0–9.99 NTUs; 0.1 NTU or less for the measurements range of 10–99.9 NTUs; and 1 NTU for the measurements above 100 NTUs.

## 6.5 Accuracy

The instrument reading accuracy must be ±10% of the reading + 0.01 NTU for the measurement range from 0–19.9 NTUs, and ±7.5% of the reading for the measurement range from 20–1100 NTUs.

## 6.6 Performance Qualification

The instrument PQ is accomplished periodically or as needed between the calibrations. Primary turbidity standards (formazin) or secondary calibration verification standards (latex suspensions or metal oxide particles in polymer gels contained in sealed sample cells) supplied by instrument manufacturers may be used.

# 7. PROCEDURES

## 7.1 Turbidimetric Procedures

### SAMPLE CELL PREPARATION

The sample cells for sample measurements must be clean. Follow the sample cell or instrument manufacturer recommendations for cleaning the sample cells appropriately. For low turbidity measurements it is a good practice to use a single-indexed sample cell or a flow cell, which help ensure adequate precision and repeatability of the measurements. Using particle-free water, find the sample cell orientation in the sample cell holder that gives the lowest reading. For higher values of turbidity, different sample cells may be used. However, the sample cells must be matched (the difference in readings for a standard prepared at nominal sample concentration from two different sample cells must be within ±0.005 NTU or below the measurement precision requirement, whichever is lower).

### SAMPLE PREPARATION

Prepare the samples as prescribed in the individual monograph. Carefully mix the samples thoroughly by swirling or inverting the volumetric flask slowly several times. Avoid shaking or stirring since it may introduce bubbles. Degassing the samples helps to improve the measurements. For degassing, the samples could stand for several minutes or a vacuum could be applied, or they could be gently sonicated using an ultrasonic bath. After degassing, let the samples stand for several minutes and mix again by carefully inverting two to three times. Transfer the sample to the sample cell and take the readings.

### USE OF FLOW CELLS

Flow cells are mainly used for low turbidity measurements for samples with small particles. When such cells are used, the sample is introduced by carefully pouring it down the interior edge of the inlet reservoir.

In practice, it is advisable to ensure that settling of the particles being measured is negligible. This is usually accomplished by including a protective colloid in the liquid-suspending medium. It is important that results be interpreted by a comparison of readings with those representing known concentrations of suspended matter, produced under precisely the same conditions.

## 7.2 Nephelometric Procedures

Nephelometric procedures are performed similarly to turbidimetric procedures for both direct measurements and measurements in the ratio mode as described above.

### RATE NEPHELOMETRIC PROCEDURES

The overall procedure for monitoring the progress of the reaction consists of three well-defined steps: 1) record a baseline reading of the turbidity of the medium (blank); 2) record the turbidity after the first reagent (antigen) is added, which results in an increase of the turbidity until a plateau is reached; and 3) add the second reagent (antibody), which results in another turbidity increase and a second plateau followed by a final turbidity increase that continues until a third plateau is reached. The measurement zone is selected from the addition of the antibody until the third plateau, depending on the purpose of the assay and the respective component concentrations. *Kinetic nephelometry* and *Endpoint nephelometry* are two general procedures that are used for quantifying the immune complexes formed in the immunoassay methods (also known as immunonephelometry

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because the measured turbidity is due to immunocomplexes that are formed). For each procedure, there are several parameters that need to be optimized in each individual application. The main parameters are 1) with or without particle enhancement; 2) particle types, sizes, and respective optimum wavelength, if applicable; 3) monitoring reaction kinetic or endpoint; 4) antibody/antigen under consideration and, related to that, the optimum level of antigen loading; 5) buffers and other ionic species and respective optimal pH; 6) type and concentration of polymers used to modify the solubility of proteins; and 7) temperature and other environmental factors. Generally these parameters are optimized during the method development and the values are given in specific monograph(s) and/or chapter(s) as applicable.

**Kinetic nephelometry:** The kinetic nephelometry is advantageous compared to the endpoint nephelometry mainly because of the capability to take a sample blank reading in addition to a reagent blank reading. This procedure assesses the rate of the immunocomplex formation based on the increased intensity response of the scattered light of the chosen wavelength. The reaction kinetic may be monitored continuously or a certain number of data points may be taken, depending on the time response of the instrument used and the type of application. At times it may involve only two data points; however, proper care must be exercised because the choice of point selection can influence the overall accuracy in cases where differences in reaction kinetics exist between samples and calibrating standards. Careful consideration should be given to the appropriate choice of specificity control strategy.

**Endpoint nephelometry:** In this method, an initial measurement is performed before adding the reagent, which represents the blank reading. A second measurement is performed after the immune complex is formed after approximately 60 min. The difference between these two measurements is proportional to the content of the component being assayed.

## 8. VALIDATION AND VERIFICATION

### 8.1 Validation

Validation is required when a nephelometric/turbidimetric method is intended for use as an alternative to the official procedure for testing an official article. The objective of nephelometric/turbidimetric method validation is to demonstrate that the measurement is suitable for its intended purpose, including quantitative determination of the main component in a drug substance or a drug product (Category I assays), quantitative determination of impurities or limit tests (Category II), and identification tests (Category IV). Depending on the category of the test (see *Validation of Compendial Procedures* ⟨1225⟩, *Table 2*), the analytical method validation process for nephelometry/turbidimetry requires testing for accuracy, precision, specificity, detection limit (DL), quantitation limit (QL), linearity, range, and robustness. These analytical performance characteristics apply to externally standardized procedures and those that use standard additions.

*Validation of Compendial Procedures* ⟨1225⟩ provides definitions and general guidance on analytical procedures validation without indicating specific validation criteria for each characteristic. The intention of the following sections is to provide the user with specific validation criteria that represent the minimum expectations for this technology. For each particular application, tighter criteria may be needed in order to demonstrate suitability for the intended use.

#### ACCURACY

For Category I, II, and III procedures, accuracy can be determined by conducting recovery studies with the appropriate matrix spiked with known concentrations of the analyte. Analysts can also compare the assay results obtained using the nephelometric/turbidimetric procedure under validation to those from an established analytical procedure.

**Validation criteria:** 98.0%–102.0% mean recovery for the drug substances, 95.0%–105.0% mean recovery for the drug product assay, and 80.0%–120.0% mean recovery for the impurity analysis. These criteria are met throughout the specified range.

#### PRECISION

**Repeatability:** The repeatability of the analytical procedure is assessed by measuring the concentrations of six independently prepared sample solutions at 100% of the assay test concentration. Alternatively, it can be assessed by measuring the concentrations of three replicates of three separate sample solutions at different concentrations. The three concentrations should be close enough so that the repeatability is constant across the concentration range. If this is done, the repeatability at the three concentrations is pooled for comparison to the acceptance criteria.

*Validation criteria:* The relative standard deviation is NMT 1.0% for the drug substance, NMT 2.0% for the drug product assay, and NMT 20.0% for the impurity analysis.

**Intermediate precision:** The effect of random events on the analytical precision of the method must be established. Typical variables include performing the analysis on different days, using different instrumentation, and/or having the method performed by two or more analysts. At a minimum, any combination of at least two of these factors totaling six experiments will provide an estimation of intermediate precision.

*Validation criteria:* The relative standard deviation is NMT 1.5% for the drug substance, NMT 3.0% for the drug product assay, and NMT 25.0% for the impurity analysis.

#### SPECIFICITY

In nephelometric/turbidimetric measurements, specificity is demonstrated by the lack of interference from other components present in the matrix (other components of the matrix produce a true solution).

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DETECTION LIMIT

The DL can be estimated by calculating the concentration of a solution that would give the signal-to-noise ratio of ≥3.3. The estimated DL must be confirmed by analyzing samples at the calculated concentration.

QUANTITATION LIMIT

The QL can be estimated by calculating the concentration of a solution that would give the signal-to-noise ratio of ≥10.0. The estimated QL must be confirmed by analyzing samples at the calculated concentration. Measurement of a test solution prepared from a representative sample matrix spiked at the required QL concentration must be performed to confirm sufficient sensitivity and adequate precision. The observed signal-to-noise ratio at the required QL should be >10.
**Validation criteria:** For the estimated limit of quantitation to be considered valid, the measured concentration must be accurate and precise at a level ≤50% of the specification.

LINEARITY

A linear relationship between the analyte concentration and measured turbidity response must be demonstrated by preparation of at least four standard solutions at concentrations encompassing the anticipated concentration of the test solution. The standard curve is then evaluated using appropriate statistical methods such as a least-squares regression. Deviation from linearity results from instrumental or sample factors, or both, can be reduced to acceptable levels by reducing or increasing the analyte concentration, thereby respectively decreasing or increasing the turbidity readings to within the nephelometer/turbidimeter instrument linearity range.
**Validation criteria:** The correlation coefficient (*R*) must be NLT 0.995 for Category I assays and NLT 0.99 for Category II quantitative tests.

RANGE

The operational range of an analytical instrument (and the analytical procedure as a whole) is the interval between the upper and lower concentrations (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the instrumental response function has a suitable level of precision, accuracy, and linearity.
**Validation criteria:** For Category I tests, the validation range for 100.0% centered acceptance criteria is 80.0%–120.0%. For non-centered acceptance criteria, the validation range is 10.0% below the lower limit to 10.0% above the upper limit. For Category II tests, the validation range covers 50.0%–120.0% of the acceptance criteria.

ROBUSTNESS

The reliability of an analytical measurement is demonstrated by deliberate changes to experimental parameters. For nephelometry/turbidimetry this can include, for example, measuring the stability of the analyte under specified storage conditions, varying pH, and adding possible interfering species. Robustness is determined concurrently using a suitable design for the experimental procedure.

## 8.2 Verification

Current U.S. Good Manufacturing Practices regulations [21 CFR 211.194(a)(2)] indicate that users of analytical procedures described in the *U.S. Pharmacopeia* and *National Formulary* are not required to validate these procedures if provided in a monograph. Instead, they simply must verify their suitability under actual conditions of use.

The objective of nephelometric/turbidimetric procedure verification is to demonstrate the suitability of a test procedure under actual conditions of use. Performance characteristics that verify the suitability of a nephelometric/turbidimetric procedure are similar to those required for any analytical procedure. A discussion of the applicable general principles is found in *Verification of Compendial Procedures* ⟨1226⟩. Verification is usually performed using a reference material and a well-defined matrix. Verification of compendial nephelometric/turbidimetric procedures includes, at minimum, the execution of the validation parameters for specificity, accuracy, precision, and QL, when appropriate, as indicated in *8.1 Validation*.▲ (USP 1-May-2019)

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***Add the following:***

# ▲⟨856⟩ NEAR-INFRARED SPECTROSCOPY

1. INTRODUCTION
2. QUALIFICATION OF NIR SPECTROMETERS
    2.1 Installation Qualification
    2.2 Operational Qualification
    2.3 Performance Qualification
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## 1. INTRODUCTION

Near-infrared (NIR) spectroscopy covers the electromagnetic field wavelength range from 780 to 2500 nm, with the frequencies expressed in wavenumbers (about 12,821–4000 $cm^{-1}$). NIR spectroscopy is a vibrational spectroscopic technique that measures the absorption of radiation in this wavelength range that is resonant with frequencies from molecular dipole changes in the sample; thus, NIR spectroscopy is related to infrared (IR) spectroscopy. The NIR spectroscopic region is dominated by overtone and combination frequencies of the molecular fundamental harmonic frequencies in the mid-IR region. The molar absorptivities in the NIR region tend to be much lower than those in the IR region, and the radiation can penetrate several millimeters into the material, giving rise to a wavelength-dependent scattering signal overlapping the molecular absorbance signals.

Not all materials absorb NIR radiation, and materials such as glass are relatively transparent. These attributes of NIR spectroscopy have been shown to be useful in the area of pharmaceutical analysis for both qualitative and quantitative applications. For further discussion of the theory and applications, see *Near-Infrared Spectroscopy—Theory and Practice* ⟨1856⟩, which may be a helpful, but not mandatory, resource.

The instrument qualification tests and acceptance criteria provided in this chapter may not be appropriate for some instrument configurations. In such cases, alternative instrument qualification and performance checks should be scientifically justified and documented before use. In addition, validation parameters discussed in this chapter may not be appropriate for all applications of NIR spectroscopy. Validation parameters characterized for a specific NIR-spectroscopic application should demonstrate the suitability of the application for its intended use. For further discussion of the theory and applications of chemometrics, see *Chemometrics* ⟨1039⟩.

## 2. QUALIFICATION OF NIR SPECTROMETERS

Qualification of NIR spectrometers is divided into three components: 1) installation qualification (IQ), 2) operational qualification (OQ), and 3) performance qualification (PQ). For further discussion on qualification, see *Analytical Instrument Qualification* ⟨1058⟩.

### 2.1 Installation Qualification

The IQ requirements elicit evidence that the hardware and software are properly installed in the desired location.

### 2.2 Operational Qualification

In OQ, an instrument's performance is characterized using standards to verify that the system operates within target specifications. The purpose of OQ is to demonstrate that instrument performance is suitable. Because there are so many different approaches for measuring NIR spectra, OQ using standards with known spectral properties is recommended. This may be achieved by using the instrument performance checks, and external, traceable certified reference materials (CRMs) as appropriate. As is the case with any spectroscopic device, wavelength accuracy, photometric linearity, and noise characteristics of NIR instruments must be qualified against target specifications for the intended application.

The OQ tests described in the sections that follow are typical examples only. Other tests and samples can be used to establish specifications for OQ. Instrument vendors often have samples and test parameters available as part of the IQ/OQ package. The acceptance specifications given in this section are applicable for general use specifically, whereas specifications for particular instruments and applications can vary, depending on the analytical method used and the desired accuracy of the final result.

#### 2.2.1 CHARACTERIZING INSTRUMENT PERFORMANCE

Use the apparatus according to the manufacturer's instructions, and carry out the prescribed verification at regular intervals. For in-line and on-line applications, the use of alternative means of control of instrument performance must be scientifically justified. For example, use the manufacturer's internal standards or use separate channels/probes, with scientific justification,

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to demonstrate instrument performance. System suitability tests may be required prior to sample scanning, and the instrument attributes with the most variation (typically photometric noise and wavelength accuracy) must be tested. The frequency at which each performance test is conducted must be assessed for risk, depending on the instrument type, application, and its environment. For example, instruments placed in harsh environments, such as environments with changing temperature and humidity, will need frequent performance testing.

Wherever possible, in the procedures for determining wavelength accuracy and linearity, analysts must use CRMs rather than laboratory-prepared solutions. These CRMs should be obtained from a recognized, accredited source and should include independently verified, traceable value assignments with associated calculated uncertainty. CRMs are to be kept clean and free from dust. Recertification is to be performed periodically to maintain the validity of the certification.

#### 2.2.2 WAVELENGTH ACCURACY

NIR spectra from sample and/or reference standard materials can be used to demonstrate an instrument's suitable wavelength-dispersion performance against target specifications. Typically, wavelength accuracy is characterized from a single spectrum (collected with the same spectral resolution used to obtain the standard value) using a minimum of three peaks that cover a suitable spectral range of the instrument.

**Verification of wavelength scale (except for filter apparatus):** Verify the wavelength scale utilized, which is generally in the region between about 780 and about 2500 nm (about 12,800 to about 4000 $cm^{-1}$) or in the intended spectral range using one or more suitable wavelength standards that have characteristic maxima or minima within the range of methodology wavelengths to be used. Fourier transform (FT) instruments have a linear frequency range; therefore, wavelength certification at a single frequency is sufficient. The USP Near IR System Suitability RS can be used for wavelength verification. Suitable materials for demonstrating wavelength dispersion performance must be used. With appropriate justification, alternative standards may be used. Additional information on calibration considerations may be found in ⟨1856⟩.

Typical tolerances for agreement with standard values are:

- ±1.0 nm at 780 nm (±16 $cm^{-1}$ at 12,800 $cm^{-1}$)
- ±1.0 nm at 1200 nm (±8 $cm^{-1}$ at 8300 $cm^{-1}$)
- ±1.0 nm at 1600 nm (±6 $cm^{-1}$ at 6250 $cm^{-1}$)
- ±1.5 nm at 2000 nm (±4 $cm^{-1}$ at 5000 $cm^{-1}$)
- ±3.0 nm at 2500 nm (±2 $cm^{-1}$ at 4000 $cm^{-1}$)

For the reference material used, apply the tolerance for the nearest wavelength (or wavenumber) from the above information for each peak used. For diode array instruments, the pixel resolution (wavelength between pixels) can be as large as 10 nm. The pixel resolution must be consistent with the required spectral resolution. The peak-finding algorithms are critical to wavelength accuracy. From a practical standpoint, ±2 nm is appropriate for peak wavelength accuracy when using such instrumentation. Alternatively, refer to the manufacturer's specifications for acceptance.

#### 2.2.3 PHOTOMETRIC LINEARITY AND RESPONSE STABILITY

Photometric linearity is typically characterized in the range from 10% to 90% reflectance (or transmittance). NIR applications based on measuring an absorbance larger than 1.0 may require standards with reflectivity properties between 2% and 5% reflectance (or transmittance) for characterizing instrument performance at low reflectance. The purpose is to demonstrate a linear relationship between NIR reflectance and/or transmittance and instrument response across the scanning range of the instrument. Subsequent verifications of the photometric model can use the initial observed absorbance values as the established values.

Measure four photometric standards across the working method absorbance range. Typical tolerances for a linear relationship are 1.00 ± 0.05 for the slope and 0.00 ± 0.05 for the intercept of a plot of the measured photometric response versus the standard photometric response. Alternative tolerances may be used when justified for specific applications. A tolerance of ±2% is acceptable for long-term stability.

#### 2.2.4 PHOTOMETRIC NOISE

NIR instrument software may include built-in procedures to automatically determine system noise and to provide a statistical report of noise or signal-to-noise ratio (S/N) over the instrument's operating range. In addition, it may be desirable to supplement such checks with measurements that do not rely directly on manufacturer-supplied procedures. Typical procedures involve measuring spectra of traceable reference materials with both high and low reflectance. Determine the photometric noise using suitable reflectance standards. Tolerances for these procedures should demonstrate suitable S/N for the intended application. Follow the manufacturer's methodology and specifications.

**High-flux noise:** Instrument noise is evaluated at high-light flux by measuring reflectance or transmittance of the reference standard, with the reference material (e.g., 99% reflectance standard) acting as both the sample and the background reference.

**Low-flux noise:** The same procedure may be used with a lower-reflectivity reference material (e.g., 10% reflectance standard) to determine system noise at reduced-light flux. The source, optics, detector, and electronics make significant contributions to the noise under these conditions.

### 2.3 Performance Qualification

The objective of PQ is to ensure that the instrument is performing within specified limits with respect to critical operational parameters. In certain cases, when the instrument has been set up for a specific measurement, it might no longer be possible or desirable to measure the wavelength and photometric (signal) qualification reference standards used in the OQ. Provided that the instrument OQ has shown that the equipment is fit for use, a single external performance verification standard can be

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used on a continuing basis (e.g., daily or before use). The performance verification standard must match the format of the samples in the current analysis as closely as possible and must use similar spectral acquisition parameters. Quantitative measurements of an external performance verification standard can be used to check both the wavelength accuracy and the photometric precision. Favorable comparison of a series of performance verification spectra demonstrates proper continued operation of the instrument.

Specific procedures, acceptance criteria, and time intervals for characterizing and monitoring NIR instrument performance are selected based on the instrument and intended application. Many NIR applications use previously validated models that relate NIR spectral response to a physical or chemical property of interest. Demonstrating stable instrument performance over extended periods of time provides some assurance that reliable measurements can be taken from sample spectra using previously validated chemometric models.

## 3. PROCEDURE

NIR spectra can be obtained from several sample presentations, including solids, powders, slurries, gels, liquids, films, and gases. In addition, measurement can be performed through glass or plastic films that are normally used for containment. Typically, NIR spectroscopy does not require any sample preparation because the detectors have a wide dynamic range, and the collection time can be adjusted by the user. Acceptable acquisition parameters or ranges hereof, must be included as a part of the analytical test procedure. Equally important, data pretreatment must be properly implemented such that the intended measurement sensitivity is not subsequently degraded. Specific values for tests such as spectrometer noise and bandwidth will be dependent on the instrument chosen and the purpose required. For this reason, specific instrument tests for these parameters are not indicated here. Additional details can be found in ⟨1856⟩.

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## 4. VALIDATION AND VERIFICATION

The objective of NIR procedure validation, as is the case with validation of any analytical procedure, is to demonstrate that it is suitable for its intended purpose. NIR spectroscopy is somewhat different from conventional vibrational spectroscopic techniques, because it is not a primary technique. Validation can be achieved by the assessment of variability (precision) and comparison to a reference standard or the result of a validated primary method (accuracy). The procedure for validation is related to the fundamental validation characteristics required for any analytical procedure. Data pretreatment is often a vital step in the chemometric analysis of NIR spectral data. Many suitable data pretreatments exist; the selection should be based on sound scientific judgment and suitability for the intended application.

### 4.1 Validation

The validation criteria described below are only required when an NIR spectroscopic procedure is intended for use as an alternative to the monograph procedure for testing an official article. The objective of procedure validation is to demonstrate that the measurement is suitable for its intended purpose, including: quantitative determination of the main component in a drug substance or drug product (Category I assays); quantitative determination of impurities or limit tests (Category II); and identification tests (Category IV; see *Validation of Compendial Procedures* ⟨1225⟩, *Table 2*). Depending on the category of the test, the analytical procedure validation process for an NIR spectroscopic method requires the testing of accuracy, precision, specificity, detection limit, quantitation limit, linearity, range, and robustness as appropriate. Note that validated NIR spectroscopy procedures may not be transferrable to all configurations of NIR spectrometers because of differences in their inherent performance characteristics.

Chapter ⟨1225⟩ provides definitions and general guidance on analytical procedures validation without indicating specific validation criteria for each characteristic. The following sections are intended to provide the user with specific validation criteria that represent the minimum expectations for this technology, assuming use of the typical Category I USP specifications of 98.0%–102.0% for a drug substance and 90.0%–110.0% for a drug product. The actual validation performance characteristics will depend on the specifications in place and must provide adequate evidence that the measurement capability is sufficient for those specifications. For certain particular applications, tighter criteria may be needed in order to demonstrate suitability for the intended use.

#### 4.1.1 ACCURACY

Accuracy is demonstrated by showing the closeness of agreement between the value that is obtained and either a conventional true value or an accepted reference value from a reference procedure. Accuracy can be determined by direct comparison between validation results and actual or accepted reference values. Suitable agreement with reference values is based on required measurement capability for a specific application. The purpose is to demonstrate a linear relationship between the NIR spectroscopic results and actual values. If a chemometric model is used, accuracy can be determined by using the methodologies in ⟨1039⟩. The error of the reference procedure may be known 1) on the basis of historical data, 2) through validation results specific to the reference procedure, or 3) by calculating the standard error of the laboratory (SEL).

For Category I and Category II procedures, accuracy can be determined by conducting recovery studies with the appropriate matrix spiked with known concentrations of the analyte. It is also an acceptable practice to compare assay results obtained using the NIR spectroscopy procedure under validation with those obtained from an established alternative analytical method.

**Validation criteria**

*Criteria 1:* Suitable agreement between ▲the standard error of prediction (SEP)▲ (ERR 1-May-2020) and SEL is based on required measurement capability for a specific application. For more information, see ⟨1039⟩.

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*Criteria 2:* In addition to Category I and Category II procedures, 98.0%–102.0% recovery for a drug substance, 95.0%–105.0% recovery for a drug product assay, and 70.0%–150.0% recovery for an impurity analysis. These criteria must be met throughout the intended range.

### 4.1.2 PRECISION

The precision of an NIR spectroscopic procedure expresses the closeness of agreement among a series of measurements under prescribed conditions. Precision measurements are typically expressed as the relative standard deviation of a series of results and should be suitable for the intended application. Two levels of precision should be considered for NIR spectroscopic procedures: repeatability and intermediate precision.

**Repeatability:** Repeatability can be demonstrated by statistical evaluation of multiple NIR procedure results, with each result derived from a replicate analysis of a sample after repositioning between spectral acquisitions. System precision may be obtained directly from the analysis of variance (ANOVA) table. The analytical procedure can be assessed by measuring the concentrations of six independently prepared sample preparations at 100% of the assay test concentration. Alternatively, it can be based on measurements of three replicates of three separate samples at different analyte levels. The three levels should be close enough that the repeatability is constant across the concentration range. If this is done, the repeatability at the three concentrations may be pooled for comparison to the acceptance criteria.

*Validation criteria:* The relative standard deviation is NMT 1.0% for a drug substance assay, NMT 2.0% for a drug product assay, and NMT 20.0% for an impurity analysis.

**Intermediate precision:** Analysts must test the effects of changes in variables, such as performing the analysis on different days, using different instrumentation, or having the method performed by two or more analysts in the repeatability study. At a minimum, any scientifically justified combination of at least two of these factors totaling (based upon intended application) six experiments will provide an estimation of intermediate precision.

*Validation criteria:* The relative standard deviation is NMT 1.0% for a drug substance assay, NMT 3.0% for a drug product assay, and NMT 25.0% for an impurity analysis.

### 4.1.3 SPECIFICITY

The extent of specificity testing depends on the intended application. Specificity is typically demonstrated by using the following approaches:

**Qualitative:** Identification testing is a common application of qualitative NIR spectroscopy. Identification is achieved by comparing a sample spectrum to a reference spectrum or spectra, for instance, by establishing a mathematical relationship (chemometric model). The specificity of the NIR identification procedure is demonstrated by obtaining positive identification from samples, coupled with negative results from materials that should not meet the criteria for positive identification. Materials to demonstrate specificity should be selected on the basis of sound scientific judgment and risk specific to the application (e.g., materials handled in the same area).

**Quantitative:** Quantitative applications of NIR spectroscopy typically first establish a mathematical relationship (chemometric model) between NIR spectral response and a physical or chemical property of interest. Demonstration of specificity against a physical or chemical property of interest is based on interpreting both NIR spectral attributes and chemometric parameters in terms of the intended application. The demonstration of specificity may include the following:

- Spectral regions can be correlated to the property of interest
- Wavelengths used by regression analysis for the calibration (e.g., for multiple linear regression models) or the loading vector for each factor (e.g., for partial least squares or principal component regression models) can be examined to verify relevant spectroscopic information that is used for the mathematical model
- Variation in spectra from samples for calibration should be examined and interpreted
- Variation in material composition and sample matrix may be shown to have no significant effect on quantification of the property of interest within the specified procedure range

For Category I and Category II procedures, the specificity is demonstrated by meeting the accuracy requirements.

For Category IV tests, the identity of the analyte must be confirmed by comparison with appropriate reference substances.

### 4.1.4 QUANTITATION LIMIT

Given that NIR methods are usually macro techniques, there is normally no need for determining the quantitation limit. However, fitness for purpose must be demonstrated over the operational range of the analyte.

### 4.1.5 LINEARITY

Quantitative procedures generally attempt to demonstrate a linear relationship between NIR spectral response function and the property of interest. Although demonstrating a linear response is not required for all NIR applications, the model chosen should properly represent the relationship.

Linearity of NIR spectroscopic procedures depends on variables such as matrix effects and data pretreatment. Validation of linearity in NIR procedures may be accomplished by examining NLT 5 samples that span the operational range and by plotting either the NIR spectral response functions versus actual or accepted values for the property of interest. Many applications may require models of higher order, and various statistical procedures are available for evaluation of the goodness of fit. Applicable statistics and graphical procedures may be used as appropriate.

The Pearson correlation coefficient, $r$, measures the strength and direction of the association between two variables ($x$ and $y$), in this instance, concentration and absorbance. The coefficient of determination, $r^2$, is a measure of the fraction of the data's variation that is adequately modelled and not a measure of linearity. Linearity depends on the standard error of the calibration function (and hence the reference procedure) and on the range of the calibration data. Thus, although values very near 1.00,

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such as 0.99 or greater, typically indicate a linear relationship, lower values do not distinguish between nonlinearity and variability.

**Validation criteria:** The coefficient of determination, $r^2$, must be NLT 0.995 for Category I assays and NLT 0.99 for Category II quantitative tests. Visual inspection of the residual plots should reveal no significant pattern. For further guidance on multivariate procedures, see ⟨1039⟩.

#### 4.1.6 RANGE

The specified range of an NIR spectroscopic procedure depends on the specific application. The range typically is established by confirming suitable measurement capability (accuracy and precision) over the proposed operational range. Controls must be used to ensure that results outside of the validated range are not accepted. In certain circumstances, it may not be possible or desirable to extend the validated range to include sample variability outside of the validated range. Extending the range of an NIR spectroscopic procedure requires demonstration of suitable measurement capability within the limits of the expanded range. Examples of situations in which only a limited sample range may be available are samples from a controlled manufacturing process and in-process samples. A limited procedure range does not preclude the use of an NIR spectroscopic procedure.

**Validation criteria:** For Category I procedures, the validation range for 100.0% centered acceptance criteria is 80.0%–120.0%. For noncentered acceptance criteria, the validation range is 10.0% below the lower limit to 10.0% above the upper limit. For content uniformity, the range is 70.0%–130.0%. For Category II procedures, the validation range covers typically 50.0%–120.0% of the acceptance criteria.

#### 4.1.7 ROBUSTNESS

The robustness of an analytical measurement must be demonstrated during development by making deliberate changes to experimental parameters. NIR spectroscopic measurement parameters selected to demonstrate robustness will vary depending on the application and the sample's interface with the instrument. Critical measurement parameters associated with robustness often are identified and characterized during method development. Typical measurement parameters include the following:

- Effect of environmental conditions (e.g., temperature, humidity, and vibration)
- Effect of sample temperature
- Sample handling (e.g., fiber-optic probe depth, compression of material, sample depth/thickness)
- Influence of instrument changes (e.g., lamp change, warm-up time)

### 4.2 Verification

US Current Good Manufacturing Practices regulations [21 Code of Federal Regulations (CFR) 211.194(a)(2)] indicate that users of analytical procedures described in *USP–NF* are not required to validate these procedures if provided in a monograph. Instead, they must simply verify their suitability under actual conditions of use.

The objective of method verification is to demonstrate that the procedure, as prescribed in specific monographs, is being executed with suitable accuracy, sensitivity, and precision. *Verification of Compendial Procedures* ⟨1226⟩ notes that if the verification of the compendial procedure, according to the monograph, is not successful, the procedure may not be suitable for use with the article under test. It may be necessary to develop and validate an alternative procedure as allowed in *General Notices*.

Although complete revalidation of a compendial procedure is not required, verification of the compendial NIR spectroscopic procedure includes the determination of certain validation parameters. When the method being verified is for identification purposes, specificity is the only parameter required. For quantitative applications, additional validation parameters, such as accuracy and precision, must be studied as indicated in *Validation of Compendial Procedures* ⟨1225⟩*, Validation* as appropriate.

## USP Reference Standards ⟨11⟩

USP Near IR System Suitability RS▲ (USP 1-May-2020)

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# ⟨857⟩ ULTRAVIOLET-VISIBLE SPECTROSCOPY

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## INTRODUCTION

For the purposes of this chapter, an ultraviolet-visible (UV-Vis) spectrometer is defined as an optical system capable of producing monochromatic radiation in the range of 200–780 nm and as a device capable of detecting the optical transmittance, usually expressed in absorbance (*A*), whose primary function is to measure the stated absorbance, or transmittance, at defined wavelength(s).

[NOTE—A UV-Vis spectrometer may also be referred to as a spectrophotometer or absorption spectrometer.]

This functionality may be extended to incorporate multiple channel measurements, either by instrumental design or with time, e.g., in dissolution or kinetic studies.

Chromatographic detectors ▲and any type of multichannel plate reader systems (i.e., filter or monochromator systems)▲ (USP 1-Dec-2022) are specifically excluded from this chapter.

For essential supporting information on the best practices and principles of measurements, see *Ultraviolet-Visible Spectroscopy —Theory and Practice* ⟨1857⟩.

The suitability of a specific instrument for a given procedure is ensured by a stepwise life cycle evaluation for the desired application from selection to instrument retirement: design qualification (DQ); installation qualification (IQ); an initial performance-to-specification qualification, also known as operational qualification (OQ); and an ongoing performance qualification (PQ). For more details, see *Analytical Instrument Qualification* ⟨1058⟩. *Acceptance criteria* for "fitness for purpose" are based on the uncertainty of the reference material and the performance specification of the instrument.

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### PRINCIPLES OF MEASUREMENT

UV-Vis spectra are derived when the interaction between incident radiation and the electron cloud in a chromophore results in an electronic transition involving the promotion of one or more of the outer shell or the bonding electrons from a ground state into a state of higher energy. The UV and visible spectral bands of substances generally are broad and do not possess a high degree of specificity for compound identification. Nevertheless, they are suitable for quantitative assays and, for many substances, are useful as an additional means of identification.

In the Beer–Lambert law, the absorbance ($A_\lambda$) of a solution at a given wavelength, λ, is defined as the logarithm to base 10 of the reciprocal of the transmittance ($T_\lambda$):

$$A_\lambda = \log_{10}\left(\frac{1}{T_\lambda}\right) \text{ and } T_\lambda = \frac{I_\lambda}{I_{\lambda_0}}$$

$T_\lambda$ = transmittance ▲at wavelength $\lambda$▲ (USP 1-Dec-2022)
$I_\lambda$ = intensity of the transmitted radiation at the same wavelength λ
$I_{\lambda 0}$ = intensity of the incident radiation at wavelength λ

In the absence of any other physical or chemical factors, $A_\lambda$ is proportional to path length, *b*, through which the radiation passes, and to the concentration, *c*, of the substance in the solution in accordance with the following:

$$A_\lambda = \epsilon_\lambda c b$$

$\varepsilon_\lambda$ = molar absorptivity
*c* = solute concentration (mol/L)
*b* = path length (cm)

If the concentration, *c*, is expressed in g/L, the constant $\varepsilon_\lambda$ becomes $a_\lambda$, which is called the absorptivity.

The expression $A^{1\%}_{1\text{ cm}}$, which represents the specific absorbance of a dissolved substance, refers to the absorbance of a 10-g/L solution (1%, m/v) in a 1-cm ▲cuvette▲ (USP 1-Dec-2022) measured at a defined wavelength so that:

$$A^{1\%}_{1cm} = 10a_\lambda = \frac{10\epsilon_\lambda}{M}$$

$a_\lambda$ = absorptivity
$\varepsilon_\lambda$ = molar absorptivity
*M* = molar concentration of the solution

When solutions are observed in 1-cm ▲cuvettes,▲ (USP 1-Dec-2022) concentrations of about 10 µg/mL often will produce absorbances of 0.2–0.8 ▲AU▲ (USP 1-Dec-2022) in the UV or visible region.

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## QUALIFICATION OF UV-VIS SPECTROMETERS

The purpose of this section of the chapter is to provide test methodologies and acceptance criteria to ensure that the instrument is suitable for its intended use (OQ), and that it will continue to function properly over extended time periods as part of PQ. As with any spectrometric device, a UV-Vis spectrometer must be qualified for both wavelength (*x*-axis) and photometric (*y*-axis, or signal axis) accuracy and precision, and the fundamental parameters of stray light and resolution must be established. OQ is carried out across the operational ranges required within the laboratory for both the absorbance and wavelength scales.

### INSTALLATION QUALIFICATION

Documented proof of the IQ requirements provide evidence that the hardware and software are properly installed in the desired location.

### OPERATIONAL QUALIFICATION

Acceptance criteria for critical instrument parameters that establish "fitness for purpose" are verified during IQ and OQ. Specifications for particular instruments and applications can vary, depending on the analytical procedure used and the desired accuracy of the final result. OQ should establish suitable control over the operational range of wavelength, absorbance, and the evaluation of stray light and resolution, i.e., spectral bandwidth (SBW). Instrument vendors often have reference materials and protocols available as part of the IQ/OQ package.

Wherever possible in the procedures detailed as follows, certified reference materials (CRMs) are to be used in preference to laboratory-prepared solutions. These CRMs should be obtained from a recognized accredited source and include independently verified, traceable value assignments with associated calculated uncertainty. CRMs must be kept clean and free from dust. Recertification should be performed periodically to maintain the validity of the certification.

#### Control of wavelengths

Ensure that the accuracy of the wavelength axis (*x*-axis) over the intended operational range is correct within acceptable limits. The control of wavelength OQ must include at least one method in each operational range where it is intended for use▲, and the wavelengths selected for qualification must bracket the intended range for use.▲ (USP 1-Dec-2022) The options and acceptance criteria are summarized in *Table 1*.

**Table 1. Wavelength Ranges and Procedures for Control of Wavelengths**

| Method | UV (200–400 nm) | Vis (400–780 nm) | Vis/NIR (400–900 nm) |
|---|---|---|---|
| Mercury (Hg) emission lines | Accuracy ±1 nm<br>Precision ≤0.5 nm | Accuracy ±2 nm<br>Precision ≤0.5 nm | — |
| Deuterium ($D_2$) emission lines | — | Accuracy ±2 nm<br>Precision ≤0.5 nm | — |
| Cerium oxide solutions | Accuracy ±1 nm<br>Precision ≤0.5 nm | — | — |
| Holmium oxide solutions or glasses | Accuracy ±1 nm<br>Precision ≤0.5 nm | Accuracy ±2 nm<br>Precision ≤0.5 nm | — |
| Didymium solutions or glasses | — | — | Accuracy ±2 nm<br>Precision ≤0.5 nm |

[NOTE—Certified reference standards, traceable to the National Institute of Standards and Technology (NIST) (www.nist.gov) or to other ▲national metrological institutes▲ (USP 1-Dec-2022)and recognized standards-setting organizations, are commercially available, and should be used where possible.]

For non-diode array instruments, wavelength accuracy and precision are determined over the operational range using at least six replicate measurements. For wavelength accuracy, the difference of the mean measured value to the certified value of the CRM must be within ±1 nm in the UV region (200–400 nm), and in the visible region (400–780 nm) and visible/NIR (near-infrared) region (400–900 nm) must be within ±2 nm.

For wavelength precision, the standard deviation ▲▲ (USP 1-Dec-2022) of the six wavelength measurements must not exceed 0.5 nm.

For diode array instruments, only one wavelength accuracy measurement is required ▲(no replicate measurements are required)▲ (USP 1-Dec-2022), and no precision determination needs to be performed, due to the non-mechanical design of the monochromator.

#### Establishment of acceptance limits (wavelength)—choice of standards

For all *Control of Wavelengths* accuracy procedures, acceptance limits for adequate calibration are established by adding the expanded uncertainty of the CRM to the instrument vendor specification at the wavelength(s) required in a linear manner, and these values must lie within the values specified in *Table 1*. In the case where atomic line spectra are used, this expanded uncertainty of the CRM is deemed to be zero for this process, i.e., the limit simply becomes the instrument vendor specification (*1*).

**Atomic line spectra:** This procedure is described as the primary application because the emission lines produced from a discharge lamp are characteristic of the source element and, as a fundamental physical standard, these wavelengths have been measured with an uncertainty of not more than ±0.01 nm. In solution spectrometry, the wavelength accuracy required rarely exceeds 1.0 nm. For these reasons, the atomic line standard values are cited without uncertainty.

The arc of the atomic emission source, or its image, needs to be located on the same optical path as the image of the primary light source of the spectrometer; thus, it can be used only in spectrometers that can be operated

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in a single-beam intensity mode and practically should be implemented only on a system designed to accommodate these sources, e.g., as an accessory.

A commonly employed low-pressure mercury lamp has a number of intense lines that cover a large part of the UV and visible spectra. Two deuterium lines from the source at 486.0 and 656.1 nm often are also used by manufacturers as an internal calibration check.▲ In addition, where a xenon source is used as an alternative source, the line at 541.9 nm can be used.▲ (USP 1-Dec-2022) (See *Table 2*) (*2*).

**Table 2. Recommended Atomic Lines from Low-Pressure Mercury (Hg)▲,▲ (USP 1-Dec-2022)Deuterium ($D_2$)▲, and Xenon (Xe)▲ (USP 1-Dec-2022) Lamps for Wavelength Calibration Purposes**

| Element | nm |
|---|---|
| Hg | 253.7 |
| ▲▲ (USP 1-Dec-2022) | ▲▲ (USP 1-Dec-2022) |
| Hg | 296.7 |
| Hg | 313.2 |
| Hg | 365.0 |
| Hg | 404.7 |
| Hg | 435.8 |
| $D_2$ | 486.0 |
| ▲Xe | 541.9▲ (USP 1-Dec-2022) |
| Hg | 546.1 |
| Hg | 577.0 |
| Hg | 579.1 |
| $D_2$ | 656.1 |

[NOTE—Select the nearest wavelength(s) spanning the operational range required.]

**Rare earth oxide solutions:** This procedure uses solutions of rare earth oxides prepared by dissolution in acid media. The most frequently used is holmium oxide in perchloric acid (*3*). Holmium oxide solution has been internationally accepted as an intrinsic wavelength standard, and suitable CRMs are available commercially (*4*).

The observed peak maxima are determined using the normal scan mode on the spectrometer. The peak maxima for a 4% (m/v) solution of holmium oxide in perchloric acid at 1.0-nm SBW and a path length of 1 cm are shown in *Table 3*.

**Table 3. Recommended Peak Maxima from a 4% Solution of Holmium Oxide in Perchloric Acid for Wavelength Calibration Purposes**

| nm |
|---|
| 241.1 |
| 249.9 |
| 278.1 |
| 287.2 |
| 333.5 |
| 345.4 |
| 361.3 |
| 385.6 |
| 416.3 |
| 451.4 |
| 467.8 |
| 485.2 |
| 536.6 |
| 640.5 |

[NOTE—Select the nearest wavelength(s) spanning the operational range required.]

If the operational range of the spectrometer lies outside the range 240–650 nm, other certified rare earth oxides or other solutions can be used if they are traceable to a national or international standard.

Cerium is available as a traceable solution CRM in acidic media, e.g., in sulfuric acid. It has useful peak characteristics in the 200–300 nm region at approximately 201.1, 211.4, 222.6, 240.4, and 253.7 nm.

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Didymium (a mixture of neodymium and praseodymium) is available as a traceable CRM both in solution and as a glass. Didymium is similar in preparation to the holmium materials and has useful peak characteristics in the 730–870-nm region, which may vary from melt to melt of the glass. Useful peaks are found in the didymium solution at approximately 731.6, 740.0, 794.1, 800.0, and 864.4 nm.

**Rare earth glasses:** This procedure uses glasses manufactured by fusing the appropriate rare earth oxide in a base glass matrix. The most frequently used is holmium, for which the reference wavelengths have been well defined. Although manufacturing can cause batch variation in these glasses, traceable CRMs are commercially available and can be used. Typical values for a holmium glass using a 1.0-nm SBW are the following: 241.5, 279.2, 287.5, 333.8, 360.9, 418.8, 445.8, 453.7, 460.2, 536.5, and 637.7 nm.

**Control of absorbance**

▲To establish adequate accuracy, precision, and photometric performance of a given system, it is necessary to verify the absorbance accuracy of a system over its intended operational range by selection and use of the following procedures as appropriate for the wavelength and absorbance ranges required. Demonstrating absorbance accuracy over the system's intended operational range assures proper photometric response.▲ (USP 1-Dec-2022)

The control of absorbance OQ must include at least one assessment at each wavelength range in the 0–2.00 AU region. If absorbance >2.00 AU is to be used for quantitation, then control of absorbance must also be evaluated in the >2.00 AU range.

**Establishment of acceptance limits (absorbance)—choice of standards**

For all *Control of Absorbance* procedures, acceptance limits for adequate calibration are established by adding the expanded uncertainty of the CRM to the instrument vendor specification at the wavelength(s) and absorbance levels required in a linear manner, and these values must lie within the values specified in *Table 4*.

[NOTE—CRMs, traceable to NIST Standard Reference Materials (SRMs), or other international or national standards, are commercially available and should be used where possible.▲When measuring these CRMs, the control of temperature may be important. For more information, see ⟨1857⟩.]

**Table 4. Available CRMs for the Control of Absorbance**

| Nominal Absorbance Range (AU) | Standard | UV (200–400 nm) | Vis (400–780 nm) |
|---|---|---|---|
| 0–1 | Certified nicotinic acid solutions 6–24 mg/L | Evaluate at 213 and 261 nm<br>Accuracy ±0.010 AU<br>Precision ≤0.005 AU | — |
| 1–3 | Certified nicotinic acid solutions 36–60 mg/L | Evaluate at 213 and 261 nm<br>Accuracy ±0.010 × $A_\lambda$<br>Precision ≤0.50 × $A_\lambda$/100 | — |
| 0–1 | Certified potassium dichromate ($K_2Cr_2O_7$) solutions 20–60 mg/L | Evaluate at 235, 257, 313, and 350 nm<br>Accuracy ±0.010 AU<br>Precision ≤0.005 AU | Evaluate 600 mg/L at 430 nm<br>Accuracy ±0.010 AU<br>Precision ≤0.005 AU |
| 1–3 | Certified potassium dichromate ($K_2Cr_2O_7$) solutions 80–200 mg/L | Evaluate at 235, 257, 313, and 350 nm<br>Accuracy ±0.010 × $A_\lambda$<br>Precision ≤0.50 × $A_\lambda$/100 | — |
| 0–1 | Certified metal-on-fused-silica filters | Evaluate at 250, 280, 340, 360, and 400 nm<br>Accuracy ±0.010 AU<br>Precision ≤0.005 AU | Evaluate at 465, 500, 546.1, 590, and 635 nm<br>Accuracy ±0.008 AU<br>Precision ≤0.005 AU |
| 1–2 | Certified metal-on-fused-silica filters | Evaluate at 250, 280, 340, 360, and 400 nm<br>Accuracy ±0.010 × $A_\lambda$<br>Precision ≤0.50 × $A_\lambda$/100 | Evaluate at 465, 500, 546.1, 590, and 635 nm<br>Accuracy ±0.008 × $A_\lambda$<br>Precision ≤0.50 × $A_\lambda$/100 |
| 0–1 | Certified neutral density glass filters | — | Evaluate at 440, 465, 546.1, 590, and 635 nm<br>Accuracy ±0.008 AU<br>Precision ≤0.005 AU |
| 1–3 | Certified neutral density glass filters | — | Evaluate at 440, 465, 546.1, 590, and 635 nm<br>Accuracy ±0.008 × $A_\lambda$<br>Precision ≤0.50 × $A_\lambda$/100 |

▲ (USP 1-Dec-2022)

[NOTE—Select the appropriate absorbance CRMs spanning the operational range(s) required.]

**Acidic nicotinic acid solutions in 0.1 ▲N▲ (USP 1-Dec-2022) hydrochloric acid:** ▲In the 6–60 mg/L range, nicotinic acid solutions provide reference values of up to 2.5 AU at one of the certified values of 213 or 261 nm. These solutions are available as CRMs.

Acceptable absorbance accuracy and precision performance can be determined from the calculated mean and the standard deviation of six replicate absorbance measurements at a specified wavelength λ. Measurements should be performed at the upper and the lower absorbance of the operational range of the spectrometer at a specified wavelength λ.

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For absorbance accuracy, the mean of the six values must not deviate from the certified value by more than ±0.010 AU for values below 1.00 AU, or ±1.0% of this certified absorbance at wavelength λ, $A_\lambda$, for values above 1.00 AU.

For absorbance precision, the standard deviation of the six values must not exceed 0.005 AU for values below 1.00 AU, or 0.50% of $A_\lambda$ for values above 1.00 AU.▲ (USP 1-Dec-2022)

**Acidic potassium dichromate solutions in 0.001 ▲M▲ (USP 1-Dec-2022) perchloric acid:** ▲In the 20–200 mg/L range, potassium dichromate solutions provide reference values of up to 3.0 AU at one of the certified values of 235, 257, 313, or 350 nm. These solutions are available as CRMs or can be prepared according to NIST from appropriate available SRMs, e.g., SRM 935*x*, or SRM 136*x* (where *x* can be any letter from "a" to "z").

Acceptable absorbance accuracy and precision performance can be determined from the calculated mean and the standard deviation of six replicate absorbance measurements at a specified wavelength λ. Measurements should be performed at the upper and the lower absorbance of the operational range of the spectrometer at a specified wavelength λ.

For absorbance accuracy, the mean of the six values must not deviate from the certified value by more than ±0.010 AU for values below 1.00 AU, or ±1.0% of this certified absorbance at wavelength λ, $A_\lambda$, for values above 1.00 AU.

For absorbance precision, the standard deviation of the six values must not exceed 0.005 AU for values below 1.00 AU, or 0.50% of $A_\lambda$ for values above 1.00 AU.▲ (USP 1-Dec-2022)

**Neutral-density glass filters:** ▲These gray glass filters are manufactured from doped glass and have a nominally flat spectrum in the region of the calibration wavelengths. They provide reference values of up to 3.5 AU at the certified values of 440, 465, 546.1, 590, and 635 nm. These filters are available as CRMs that are traceable to NIST SRM 930e, 1930, and 2930. Other certified standard solutions or optical filters can be used if they are traceable to a national or international standard.

Acceptable absorbance accuracy and precision performance can be determined from the calculated mean and the standard deviation of six replicate absorbance measurements at a specified wavelength λ. Measurements should be performed at the upper and the lower absorbance of the operational range of the spectrometer at a specified wavelength λ.

For absorbance accuracy, the mean of the six values must not deviate from the certified value by more than ±0.008 AU for values below 1.00 AU, or ±0.80% of this certified absorbance at wavelength λ, $A_\lambda$, for values above 1.00 AU.

For absorbance precision, the standard deviation of the six values must not exceed 0.005 AU for values below 1.00 AU, or 0.50% of $A_\lambda$ for values above 1.00 AU.▲ (USP 1-Dec-2022)

**Metal-on-fused-silica filters:** ▲These neutral density filters are manufactured by deposition of a metallic film on a silica substrate and have a substantially flat spectrum in the region of the calibration wavelengths.

[NOTE—These filters may produce significant interreflection errors. They should only be used in spectrometers designed to minimize these optical effects. For more information, see ⟨1857⟩.]

The filters provide reference values of up to 2 AU at the usually certified values of 250, 280, 340, 360, and 400 nm in the UV range and reference values of 465, 500, 546.1, 590, and 635 nm in the visible range. These filters are available as CRMs that are traceable to NIST SRM 2031*x* (where *x* can be any letter from "a" to "z"), or to other national or international standards.

Acceptable absorbance accuracy and precision performance can be determined from the calculated mean and the standard deviation of six replicate absorbance measurements at a specified wavelength λ. Measurements should be performed at the upper and the lower absorbance of the operational range of the spectrometer at a specified wavelength λ.

For absorbance accuracy in the UV range, the mean of the six values must not deviate from the certified value by more than ±0.010 AU for values below 1.00 AU, or ±1.0 % of this certified absorbance at wavelength λ, $A_\lambda$, for values above 1.00 AU.

For absorbance accuracy in the visible range, the mean of the six values must not deviate from the certified value by more than ±0.008 AU for values below 1.00 AU, or ±0.80% of this certified absorbance at wavelength λ, $A_\lambda$, for values above 1.00 AU.

For absorbance precision, the standard deviation of the six values must not exceed 0.005 AU for values below 1.00 AU, or 0.50% of $A_\lambda$ for values above 1.00 AU.▲ (USP 1-Dec-2022)

**Estimation of the limit of stray light (stray radiant energy)**

Although the measurement of absorbance or transmittance is a ratio measurement of intensities and therefore theoretically is independent of monochromatic source intensity, practical measurements are affected by the presence of unwanted radiation called "stray radiant energy" or "stray light". In addition, the adverse effect of stray light increases with aging of optical components and lamps in a spectrometer. The effects are greater at the extremes of detector and lamp operational ranges. The limit of stray light OQ must include evaluation at one or more UV wavelengths, by selection of appropriate material(s) shown in *Table 5* to span the UV operational range required. In the visible region, i.e., above 400 nm, stray light does not need to be evaluated.

[NOTE—Published stray light specifications for a given spectrometer must be verified in the OQ.]

Analysts must choose and use the appropriate reference(s) to monitor the level of stray light as part of PQ. Stray light can be detected at a given wavelength with a suitable liquid filter by either of the two methods (*Procedure A* or *Procedure B*) detailed below.

[NOTE—If measurements are being performed in the 250–330 nm region, on a spectrometer using individual sources for the UV and visible regions of the spectrum, then an additional PQ verification using acetone should be performed▲, using air as reference.▲ (USP 1-Dec-2022)]

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These solutions are available as CRMs or can be prepared at the concentrations shown in *Table 5* by using reagent-grade materials.

**Table 5. Spectral Ranges of Selected Materials for Monitoring Stray Light**

| Recommended Wavelength ▲for Procedure B▲ (USP 1-Dec-2022) (nm) | Spectral Range ▲for Procedure B▲ (USP 1-Dec-2022) (nm) | Liquid or Solution ▲Used for Procedures A or B▲ (USP 1-Dec-2022) |
|---|---|---|
| 198 | 190–210 | Aqueous potassium chloride (12 g/L) |
| 220 | 210–270 | Aqueous sodium iodide or potassium iodide (10 g/L) |
| 300 | 250–330 | Acetone |
| 340 | 300–400 | Aqueous sodium nitrite (50 g/L) |

**Procedure A**

The aim of this procedure is to produce the differential spectrum resulting from the subtraction of a spectrum produced by a 5-mm path length ▲cuvette▲ (USP 1-Dec-2022) from that of a 10-mm ▲cuvette,▲ (USP 1-Dec-2022) both filled with the same filter solution ▲specified in *Table 5*.▲ (USP 1-Dec-2022)This spectrum will contain a peak maximum absorbance value ▲($\Delta A$),▲ (USP 1-Dec-2022) and analysts can calculate the stray light value from the observed maximum absorbance using the formula:

$$▲s_\lambda = 0.25 \times 10^{-2\Delta A}▲ \text{ (USP 1-Dec-2022)}$$

$s_\lambda$ = stray light value, in transmittance ($T$)
▲$\Delta A$▲ (USP 1-Dec-2022) = observed maximum absorbance

This procedure requires the ▲5▲ (USP 1-Dec-2022)-mm ▲cuvette▲ (USP 1-Dec-2022) measurement to be subtracted from the ▲10▲ (USP 1-Dec-2022)-mm ▲cuvette,▲ (USP 1-Dec-2022) both filled with the same cut-off solution filter. This measurement can be achieved by either using the physical capabilities of the spectrometer, i.e., using the double-beam capability, with the 5-mm ▲cuvette▲ (USP 1-Dec-2022) as the reference, or by mathematically subtracting the spectra of the 10-mm ▲cuvette▲ (USP 1-Dec-2022) from the 5-mm ▲cuvette,▲ (USP 1-Dec-2022) chronologically produced by sequential measurement of both the 5- and 10-mm ▲cuvettes,▲ (USP 1-Dec-2022) using the single-sample beam, with the spectrometer referenced to air (blank holder).

**Acceptance criteria:** $s_\lambda \leq 0.01$ or ▲$\Delta A$▲ (USP 1-Dec-2022) $\geq 0.7$ AU

▲See ⟨1857⟩ for additional discussion.▲ (USP 1-Dec-2022)

**Procedure B**

Analysts can measure the absorbance of the cut-off solution filters specified in *Table 5* against a 10-mm ▲cuvette▲ (USP 1-Dec-2022) filled with an appropriate reference, and record the maximum absorbance value ($A_{max}$) or the minimum %Transmittance ($\%T_{min}$) ▲within▲ (USP 1-Dec-2022) the recommended wavelength ▲range▲ (USP 1-Dec-2022) listed in *Table 5*.

**Acceptance criteria:** $A_{max} \geq 2.0$ AU or $\%T_{min} < 1\%T$

**Control of Resolution**

If accurate absorbance measurements must be made on benzenoid compounds or other compounds with sharp absorption bands (natural half-bandwidths of <15 nm), the SBW of the spectrometer used should not be greater than 1/8$^{th}$ the natural half-bandwidth of the compound's absorption; i.e., this equates to a spectrometer with a SBW of 2 nm or less.

Determine the resolution of the spectrometer in the UV region by using the following procedure. Using UV-grade *n*-hexane as the reference, measure the absorbance of a 0.020% (v/v) solution of toluene in UV-grade *n*-hexane at the maximum, about 269 nm, and minimum, about 266 nm. The ratio of the absorbance at the maximum to the absorbance at the minimum will typically fall in the 1.0–2.6 range.

For most compendial quantitative purposes, a SBW of 2 nm or less is sufficient, and the *Acceptance criteria* for the ratio is not less than 1.3.

**PERFORMANCE QUALIFICATION**

The purpose of PQ is to determine that the instrument is capable of meeting the user's requirements for all the ▲critical▲ (USP 1-Dec-2022) parameters that may affect the quality of the measurement and to ensure that it will function properly over extended periods of time. ▲Statistical evaluation is recommended, and trend analysis can be used to confirm ongoing fitness for purpose of the instrument. See *Analytical Data—Interpretation and Treatment* ⟨1010⟩, *Appendix 1: Control Charts*.▲ (USP 1-Dec-2022)

***Change to read:***

## PROCEDURE

With few exceptions, compendial spectrometric tests and assays call for comparison against a USP Reference Standard. This helps ensure measurement under identical conditions for the test specimen and the reference substance. These conditions could include wavelength setting, SBW selection, ▲cuvette▲ (USP 1-Dec-2022) placement and correction, and transmittance levels. ▲Cuvettes▲ (USP 1-Dec-2022) that exhibit identical transmittance at a given wavelength may differ considerably in transmittance at other wavelengths. Appropriate ▲cuvette▲ (USP 1-Dec-2022) corrections should be established and used where required.

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Comparisons of a test specimen with a reference standard are best made at a peak of spectral absorption for the compound concerned. Assays that prescribe spectrometry give the commonly accepted wavelength for peak spectral absorption of the substance in question. Different spectrometers may show minor variation in the apparent wavelength of this peak. Good practice demands that comparisons be made at the wavelength at which peak absorption occurs. Should this differ by > ±1 nm (in the range 200–400 nm) or ±2 nm (in the range 400–800 nm) from the wavelength specified in the individual monograph, recalibration of the instrument may be indicated.

The expressions "similar preparation" and "similar solution" as used in tests and assays involving spectrometry indicate that the reference comparator, generally a USP Reference Standard, should be prepared and observed in an identical manner for all practical purposes to that used for the test specimen. Usually when analysts make up the solution of the specified reference standard, they prepare a solution with a concentration of about the desired concentration (i.e., within 10%), and they calculate the absorptivity on the basis of the exact amount weighed out. If a previously dried specimen of the reference standard has not been used, the absorptivity is calculated on the anhydrous basis. The expressions "concomitantly determine" and "concomitantly measure" as used in tests and assays involving spectrometry indicate that the absorbances of both the solution containing the test specimen and the solution containing the reference specimen, relative to the specified test blank, must be measured in immediate succession.

#### SAMPLE SOLUTION PREPARATION

For determinations using UV or visible spectrometry, the specimen generally is dissolved in a solvent. Unless otherwise directed in the monograph, analysts make determinations at room temperature using a path length of 1 cm. Many solvents are suitable for these ranges, including water, alcohols, lower hydrocarbons, ethers, and dilute solutions of strong acids and alkalis. Precautions should be taken to use solvents that are free from contaminants that absorb in the spectral region under examination. For the solvent, analysts typically should use water-free methanol or alcohol or alcohol denatured by the addition of methanol but without benzene or other interfering impurities. Solvents of special spectrometric quality, guaranteed to be free from contaminants, are available commercially from several sources. Some other analytical reagent-grade organic solvents may contain traces of impurities that absorb strongly in the UV region. New lots of these solvents should be checked for their transparency, and analysts should take care to use the same lot of solvent for preparation of the test solution, the standard solution, and the blank. The best practice is to use solvents that have not less than 40% transmittance (39.9% $T = 0.399$ *AU*) at the wavelength of interest.

Assays in the visible region usually call for concomitantly comparing the absorbance produced by the assay preparation with that produced by a standard preparation containing approximately an equal quantity of a USP Reference Standard. In some situations, analysts can omit the use of a reference standard (e.g., when spectrometric assays are made with routine frequency) when a suitable standard curve is available and is prepared with the appropriate USP Reference Standard, and when the substance assayed conforms to the Beer–Lambert law within the range of about 75%–125% of the final concentration used in the assay. Under these circumstances, the absorbance found in the assay may be interpolated on the standard curve, and the assay result can be calculated. Such standard curves should be confirmed frequently and always when a new spectrometer or new lots of reagents are put into use.

## VERIFICATION AND VALIDATION

#### VERIFICATION

Current Good Manufacturing Practices regulations [21 CFR §211.194(a)(2)] indicate that users of analytical procedures described in *USP–NF* are not required to validate these procedures if provided in a monograph. Instead, they simply must verify their suitability under actual conditions of use.

The objective of a UV-Vis procedure verification is to demonstrate the suitability of a test procedure under actual conditions of use. Performance characteristics that verify the suitability of a UV-Vis procedure are similar to those required for any analytical procedure. A discussion of the applicable general principles is found in *Verification of Compendial Procedures* ⟨1226⟩. Verification is usually performed using a reference material and a well-defined matrix. Verification of compendial UV-Vis procedures includes at minimum the execution of the validation parameters for specificity, accuracy, precision, and quantitation limit, when appropriate, as indicated in *Validation*.

#### VALIDATION

Validation is required when a UV-Vis method is intended for use as an alternative to the official procedure for testing an official article or when no official procedure exists in the current *USP–NF*.

The objective of UV-Vis method validation is to demonstrate that the measurement is suitable for its intended purpose, including quantitative determination of the main component in a drug substance or a drug product (Category I assays), quantitative determination of impurities or limit tests (Category II), and identification tests (Category IV). For dissolution procedures, see *The Dissolution Procedure: Development and Validation* ⟨1092⟩. Depending on the category of the test (see *Validation of Compendial Procedures* ⟨1225⟩*, Table 2*), the analytical method validation process for UV-Vis requires testing for linearity, range, accuracy, specificity, precision, detection limit, or quantitation limit. These analytical performance characteristics apply to externally standardized procedures and those that use standard additions.

⟨1225⟩ provides definitions and general guidance on analytical procedures validation without indicating specific validation criteria for each characteristic. The intention of the following sections is to provide the user with specific validation criteria that represent the minimum expectations for this technology. For each particular application, tighter criteria may be needed in order to demonstrate suitability for the intended use.

**Accuracy**

For Category I, II, and III procedures, accuracy can be determined by conducting recovery studies with the appropriate matrix spiked with known concentrations of the analyte. Analysts also can compare assay results obtained using the UV-Vis procedure under validation to those from an established analytical procedure.

**Validation criteria:** 98.0%–102.0% mean recovery for the drug substances, 95.0%–105.0% mean recovery for the drug product assay, and 80.0%–120.0% mean recovery for the impurity analysis. These criteria are met throughout the intended range.

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**Precision/Repeatability**

The repeatability of the analytical procedure is assessed by measuring the concentrations of 6 independently prepared sample solutions at 100% of the assay test concentration. Alternatively, it can be assessed by measuring the concentrations of 3 replicates of 3 separate sample solutions at different concentrations. The three concentrations should be close enough so that the repeatability is constant across the concentration range. If this is done, the repeatability at the 3 concentrations is pooled for comparison to the acceptance criteria.

**Validation criteria:** The relative standard deviation is not more than 1.0% for the drug substance assay, not more than 2.0% for the drug product assay, and not more than 15.0%–20.0% for the impurity analysis

**Precision/Intermediate precision**

The effect of random events on the analytical precision of the method must be established. Typical variables include performing the analysis on different days, using different instrumentation, and/or having the method performed by two or more analysts. At a minimum, any combination of at least two of these factors totaling 6 experiments will provide an estimation of intermediate precision.

**Validation criteria:** The relative standard deviation is not more than 1.5% for the drug substance assay, not more than 3.0% for the drug product assay, and not more than 15.0%–25.0% for the impurity analysis.

**Specificity**

In UV-Vis measurements, specificity is ensured by the use of a reference standard wherever possible and is demonstrated by the lack of interference from other components present in the matrix.

**Detection limit**

The detection limit (DL) can be estimated by calculating the standard deviation of not less than 6 replicate measurements of a blank solution and multiplying by 3.3. Alternatively, the standard deviation can be determined from the error of the intercept from a calibration curve or by determining that the signal-to-noise ratio is >3.3. The estimated DL must be confirmed by analyzing samples at the calculated concentration.

**Quantitation limit**

The quantitation limit (QL) can be estimated by calculating the standard deviation of not less than 6 replicate measurements of a blank solution and multiplying by 10. Alternatively, the standard deviation can be determined from the error of the intercept from a calibration curve or by determining that the signal-to-noise ratio is >10.

Measurement of a test solution prepared from a representative sample matrix spiked at the required QL concentration must be performed to confirm sufficient sensitivity and adequate precision. The observed signal-to-noise ratio at the required QL should be >10. [NOTE—A suitable procedure for measuring the signal-to-noise ratio is given in ASTM E1657-98 (*5*).]

**Validation criteria:** For the estimated limit of quantitation to be considered valid, the measured concentration must be accurate and precise at a level ≤50% of the specification.

**Linearity**

A linear relationship between the analyte concentration and UV-Vis response must be demonstrated by preparation of not less than 5 standard solutions at concentrations encompassing the anticipated concentration of the test solution. The standard curve is then evaluated using appropriate statistical methods such as a least-squares regression. Deviation from linearity results from either instrumental or sample factors, or both, and can be reduced to acceptable levels by reducing the analyte concentration and thereby the associated absorbance values.

The (Pearson) correlation coefficient, $r$, measures the strength and direction of the association between two variables ($x$ and $y$), in this instance, concentration and absorbance.

The coefficient of determination, $r^2$, is a measure of the fraction of the data's variation that is adequately modeled and not a measure of linearity. Linearity depends on the standard error of the calibration equation (and hence the reference procedure) and on the range of the calibration data. Thus, although values very near 1.00, such as 0.99 or greater, typically indicate a linear relationship, lower values do not distinguish between nonlinearity and variability.

**Validation criteria:** The coefficient of determination, $r^2$, must be not less than 0.995 for Category I assays and not less than 0.99 for Category II quantitative tests. Visual inspection of the residual plots should not reveal any significant pattern.

**Range**

The operational range of an analytical instrument (and the analytical procedure as a whole) is the interval between the upper and lower concentrations (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the instrumental response function has a suitable level of precision, accuracy, and linearity.

**Validation criteria:** For Category I tests, the validation range for 100.0% centered acceptance criteria is 80.0%–120.0%. For noncentered acceptance criteria, the validation range is 10.0% below the lower limit to 10.0% above the upper limit. For content uniformity, the validation range is 70.0%–130.0%. For Category II tests, the validation range covers 50.0%–120.0% of the acceptance criteria.

**Robustness**

This parameter is evaluated during method development.

The reliability of an analytical measurement is demonstrated by deliberate changes to experimental parameters. For UV-Vis, this can include measuring the stability of the analyte under specified storage conditions, varying pH, and adding possible interfering species, to list a few examples. Robustness is determined concurrently using a suitable design for the experimental procedure.

## INDIRECT MEASUREMENT REQUIREMENTS

For certain UV-Vis procedures, chromogenic reactions are employed. Generally, the requirements for the analytical performance characteristics are used. In some instances, the required accuracy and precision criteria for the direct measurements may not be achievable. Under these circumstances, the accuracy and precision requirements can be widened by as much as 50%. Any such widening must be justified on scientific grounds and with documented evidence. It may be necessary to increase the amount of replication required to produce a scientifically sound reportable value.

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# ▲⟨858⟩ RAMAN SPECTROSCOPY

INTRODUCTION
QUALIFICATION OF RAMAN SPECTROMETERS
Installation Qualification
Operational Qualification
Performance Qualification
PROCEDURE
VALIDATION AND VERIFICATION
Validation
Verification

## INTRODUCTION

Raman is a vibrational spectroscopic technique and is complementary to infrared (IR) and near-infrared (NIR) spectroscopy. The Raman effect itself arises as a result of a change in the polarizability of molecular bonds during a given vibrational mode and is measured as inelastically scattered radiation. The appearance of a Raman spectrum is much like that of an IR absorbance spectrum. The intensities, or the number of Raman photons counted, are plotted against the shifted energies. The Raman shift is usually expressed in wavenumber and represents the energy difference between the incident photon energy and the inelastically scattered photon energy. Unlike IR or NIR spectra that arise when vibrational modes in a molecule have a dipole change, Raman spectra have frequencies and intensities that arise when these same modes cause a change in polarizability. The spectrum is interpreted in the same manner as the corresponding mid-infrared spectrum. The positions of the (Raman-shifted) wavenumbers for a given vibrational mode are theoretically identical to the wavenumbers of the corresponding bands in an IR absorption spectrum. However, the stronger peaks in a Raman spectrum are often weak, or not present in an IR spectrum, and vice versa. Similarly, wavenumbers above 1500 $cm^{-1}$ are group frequencies, and strong bands that absorb below 1500 $cm^{-1}$ can be either group frequencies or fingerprint bands for the molecule. Thus, the two spectroscopic techniques are often said to be complementary. For discussion of the theory and principles of measurements, see ▲*Raman Spectroscopy—Theory and Practice* ⟨1858⟩▲ (CN 1-Aug-2020), which may be a helpful, but not a mandatory, resource. For further discussion of the theory and applications of chemometrics, see *Chemometrics* ⟨1039⟩.

## QUALIFICATION OF RAMAN SPECTROMETERS

Qualification of Raman spectrometers is divided into three components: 1) installation qualification (IQ); 2) operational qualification (OQ); and 3) performance qualification (PQ). For further discussion, see *Analytical Instrument Qualification* ⟨1058⟩ and ▲⟨1858⟩▲ (CN 1-Aug-2020).

### Installation Qualification

The IQ requirements provide evidence that the hardware and software are properly installed in the desired location.

### Operational Qualification

The requirements for OQ are application and user dependent. Therefore the user needs to specify fitness for purpose requirements for that application and use selection from below as appropriate. For example, applications for establishing the identity of materials may have different requirements than those for quantitative purposes.

Acceptance criteria given in this section are applicable for general use. Acceptance criteria for particular instruments and applications can vary, depending on the analytical method used and the desired accuracy of the final result. American Society for Testing and Materials (ASTM) Standard Reference Materials (SRMs) are also specified, with the understanding that in some circumstances (specifically, remote online applications), calibration using one of these materials may be impractical and other suitably verified materials can be used.

#### WAVELENGTH ACCURACY

The accuracy of the wavelength axis is ensured via calibration to maintain the integrity of Raman peak positions, see ▲⟨1858⟩▲ (CN 1-Aug-2020). The instrument wavelength accuracy can be determined by using a Raman-shift standard or other suitably high-purity material (e.g., acetaminophen, cyclohexane, or polystyrene). Selection of a standard with bands present across the full Raman spectral range is recommended so that instrument wavelength accuracy can be evaluated at multiple locations within the spectrum. Using a suitable, well-characterized wavenumber standard, the analyst conducts the calibration scans over the spectral range of the analytical system wavenumbers and compares the wavenumber of maximum response of several chosen bands using the appropriate peak-picking algorithms to the known Raman shift of the standard (see *Table 1*). Instrumentation for quantitative purposes must perform to these requirements. For qualitative requirements, the maximum allowable acceptance

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criteria for all wavelengths contained in *Table 1* is ±3.0 $cm^{-1}$. Under these circumstances the user is required to demonstrate fitness for purpose of the derived calibration model over the operational range.

**Table 1. Wavenumber Shifts of Polystyrene, Acetaminophen (paracetamol), and Cyclohexane with Acceptance Criteria[a]**

| Wavenumber Shifts[b] ($cm^{-1}$) | | Acceptance Criteria ($cm^{-1}$) |
|---|---|---|
| Polystyrene[c] | 620.9 | ±1.5 |
| | 1001.4 | ±1.5 |
| | 1031.8 | ±1.5 |
| | 1602.3 | ±1.5 |
| | 3054.3 | ±3.0 |
| Acetaminophen[d] | 797.2 | ±1.5 |
| | 857.9 | ±1.5 |
| | 1168.5 | ±1.5 |
| | 1236.8 | ±1.5 |
| | 1323.9 | ±1.5 |
| | 1648.4 | ±1.5 |
| | 2931.1 | ±2.0 |
| Cyclohexane[e] | 801.3 | ±1.5 |
| | 1028.3 | ±1.0 |
| | 1266.4 | ±1.0 |
| | 1444.4 | ±1.0 |
| | 2852.9 | ±2.0 |

[a] Range testing during wavelength accuracy can be adjusted to the application for which instrument is being used. For instance, if the method does not use wavelengths above 2900 $cm^{-1}$, the wavelength accuracy test may only consider reference peaks below that value.
[b] *Standard Guide for Raman Shift Standards for Spectrometer Calibration* (ASTM E1840).
[c] Polystyrene foil (30 mil, 76 µm) or polystyrene pellets [National Institute of Standards and Technology (NIST) 706a], or Certified Reference Materials (CRMs), traceable to NIST SRMs, or other international or national standards, are commercially available and should be used where possible.
[d] Acetaminophen for equipment qualification CRS.
[e] Cyclohexane reference material.

[NOTE—For scanning dispersive instruments, calibration might need to be performed more frequently, and precision in both a scanning and static operation mode may need to be verified.]

PHOTOMETRIC PRECISION

Laser variation in terms of the total emitted photons occurring between two measurements can give rise to changes in the photometric precision of the instrument. Unfortunately, it is very difficult to separate changes in the photometric response associated with variations in the total emitted laser photons from the sample and sampling-induced perturbations. This is one of the reasons why the photometric precision acceptance criteria are set relatively loosely; a maximum tolerance of 10% from reference measurements made from the reference material is applied.

## Performance Qualification

The objective of PQ is to ensure that the instrument is performing within specified limits with respect to wavelength accuracy and photometric precision. In certain cases, when the instrument has been set up for a specific measurement, it might no longer be possible or desirable to measure the wavelength and photometric (signal) qualification reference standards used in the OQ. Provided that instrument OQ has shown that the system is fit for use, a single external performance verification standard may be used on a continuing basis (e.g., daily or before use). The performance verification standard must match the format of the samples in the current analysis as closely as possible and must use similar spectral acquisition parameters. Quantitative measurements of an external performance verification standard can be used to check both the wavelength accuracy and the photometric precision.

# PROCEDURE

Raman spectra can be obtained from several sample presentations, including solids, powders, slurries, gels, liquids, films, and gases. In addition, measurement can be performed through glass or plastic films that are normally used for containment. Typically, Raman spectroscopy does not require any sample preparation because it is relatively insensitive to physical state solid or emulsion, slurry, etc. Furthermore, Raman uses back scattering geometry, right angle geometry, or transmission geometry, so the frequency and intensity are not affected much. Any intensity difference may arise from sample non-homogeneity and sampling area difference. The detectors have a wide dynamic range, and the collection time can be adjusted by the user.

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Considerations about exposure of the sample to the laser must be taken into account because high-intensity lasers and small measurement spots may result in burning of the sample.

Acceptable acquisition parameters and a summary of the validation results must be included as part of the respective analytical test procedure. Specific values for tests will depend on the instrument chosen and the required purpose. For this reason, specific instrument tests for these parameters are not indicated here. For additional details, see ▲⟨1858⟩▲ (CN 1-Aug-2020), which provides a detailed discussion of Raman spectroscopy.

## VALIDATION AND VERIFICATION

The objective of Raman procedure validation, as is the case with validation of any analytical process, is to demonstrate that the measurement is suitable for its intended purpose. The procedure for validation is related to the fundamental validation characteristics required for any analytical procedure. Data pretreatment is often a vital step in the chemometric analysis of Raman spectral data. Many suitable data pretreatments exist; the selection should be based on sound scientific judgment and suitability for the intended application. See ⟨1039⟩ and *Mid-Infrared Spectroscopy* ⟨854⟩.

### Validation

The validation criteria described below are only required when a Raman procedure is intended for use as an alternative to the official procedure for testing an official article. The objective of a Raman procedure validation is to demonstrate that the measurement is suitable for its intended purpose, including the following: quantitative determination of the main component in a drug substance or a drug product (Category I assays); quantitative determination of impurities or limit tests (Category II); and identification tests (Category IV); see *Validation of Compendial Procedures* ⟨1225⟩, *Table 2*. Depending on the category of the test, the process for analytical procedure validation for Raman spectroscopy may require the testing of accuracy, precision, specificity, detection limit, quantitation limit, linearity, range, and robustness. Validated Raman procedures may not be transferable to all models and configurations of Raman spectrometers because of differences in their inherent performance characteristics.

⟨1225⟩ provides definitions and general guidance on analytical procedures validation without indicating specific validation criteria for each characteristic. The sections that follow are intended to provide the user with specific validation criteria that represent the minimum expectations for this technology, assuming the typical Category I USP acceptance criteria of 98.0%–102.0% for a drug substance and 90.0%–110.0% for a drug product. The actual validation performance characteristics would depend on the acceptance criteria in place and must provide sufficient evidence that the measurement capability is sufficient for these acceptance criteria. For each particular application, tighter criteria may be needed to demonstrate suitability for the intended use.

#### ACCURACY

For Category I and II procedures, accuracy can be determined by conducting recovery studies with prepared samples or appropriate reference materials over the operational concentration range taking into account matrix effects. Comparison of assay results obtained using the Raman procedure under validation with those obtained from an established alternative analytical procedure may also be used to determine accuracy. See ⟨1039⟩ for more information.

**Validation criteria:** 98.0%–102.0% recovery for drug substances, 95.0%–105.0% recovery for drug product assays, and 70.0%–150.0% recovery for impurity analyses. These criteria should be met throughout the intended range.

#### PRECISION

**Repeatability:** The analytical procedure can be assessed by measuring the concentrations of six independently prepared sample preparations at 100% of the assay test concentration. Alternatively, this assessment can be based on measurements of three replicates of three separate samples at different concentrations. The three concentrations should be close enough that the repeatability is constant across the concentration range. If this is done, the repeatability at the three concentrations is pooled for comparison to the acceptance criteria.

*Validation criteria:* The relative standard deviation is NMT 1.0% for a drug substance, NMT 2.0% for a drug product, and NMT 20.0% for an impurity analysis.

**Intermediate precision:** Analysts test the effect on analytical precision of changes in variables, such as performing the analysis on different days, using different instrumentation, or having the procedure performed by two or more analysts. At a minimum, any scientifically justified combination of at least two of these factors based upon intended application, totaling at least six experiments, will provide an estimate of intermediate precision.

*Validation criteria:* The relative standard deviation is NMT 1.0% for a drug substance, NMT 3.0% for a drug product assay, and NMT 25.0% for an impurity analysis.

#### SPECIFICITY

The extent of specificity testing depends on the intended application. Specificity is typically demonstrated by using the following approaches:

**Qualitative:** Identification testing is a common application of qualitative Raman spectroscopy. Identification is achieved by comparing a sample spectrum to a reference spectrum or spectra either visually or by, for instance, establishing a "goodness of fit" criterion or use of a chemometric model. The specificity of the Raman identification procedure is demonstrated by obtaining positive identification from samples, coupled with negative results from materials that should not meet the criteria

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for positive identification. Materials to demonstrate specificity should be selected on the basis of sound scientific judgment and risk specific to the application (e.g., materials handled in the same area).

**Quantitative:** Quantitative applications of Raman spectroscopy typically first establish a mathematical relationship (chemometric model) between Raman spectral response and a physical or chemical property of interest. Demonstration of specificity against a physical or chemical property of interest is based on interpreting both Raman spectral attributes and chemometric parameters in terms of the intended application. The demonstration of specificity may include the following:

- Spectral regions can be correlated to the property of interest
- Wavelengths used by regression analysis for the calibration (e.g., for multiple linear regression models) or the loading vector for each factor (e.g., for partial least squares or principal component regression models) can be examined to verify relevant spectroscopic information that is used for the mathematical model
- Variation in spectra from samples for calibration should be examined and interpreted
- Variation in material composition and sample matrix should be shown to have no significant effect on quantification of the property of interest within the specified procedure range as part of robustness studies

For Category I and Category II procedures, the specificity is demonstrated by meeting the accuracy requirements.

For Category IV tests, the identity of the analyte must be confirmed by comparison with appropriate reference substances.

### QUANTITATION LIMIT

Given that Raman methods are typically multivariate, there is no need for determining a defined quantitation limit. However, fitness for purpose is demonstrated over the operational range of the analyte.

### LINEARITY AND CALIBRATION MODELS

Quantitative procedures generally attempt to demonstrate a linear relationship between Raman spectral response function and the property of interest. Although demonstrating a linear response is not required for all Raman applications, the model chosen should properly represent the relationship.

Linearity of Raman spectroscopic procedures depends on variables such as matrix effects and data pretreatment. Validation of linearity in Raman procedures may be accomplished by examining NLT 5 samples that span the expected concentration range and by plotting either the Raman spectral responses versus actual or accepted values for the property of interest. Many applications may require models of higher order, and various statistical procedures are available for evaluation of the goodness of fit. Applicable statistics and graphical procedures may be used as appropriate.

The Pearson correlation coefficient, $r$, measures the strength and direction of the association between two variables ($x$ and $y$), in this instance, concentration and absorbance. The coefficient of determination, $r^2$, is a measure of the fraction of the data's variation that is adequately modeled and not a measure of linearity. Linearity depends on the standard error of the calibration function (and hence the reference procedure) and on the range of the calibration data. Thus, although values very near 1.00, such as 0.99 or greater, typically indicate a linear relationship, lower values do not distinguish between nonlinearity and variability.

**Validation criteria:** Visual inspection of the residual plots should reveal no significant pattern. For further guidance on multivariate procedures, see ⟨1039⟩.

### RANGE

The specified range of a Raman spectroscopic procedure depends on the specific application. The range typically is established by confirming suitable measurement capability (accuracy and precision) over the proposed operational range. Controls must be used to ensure that results outside of the validated range are not accepted. In certain circumstances, it may not be possible or desirable to extend the validated range to include sample variability outside of the validated range. Extending the range of a Raman spectroscopic procedure requires demonstration of suitable measurement capability within the limits of the expanded range. Examples of situations in which only a limited sample range may be available are samples from a controlled manufacturing process and in-process samples. A limited procedure range does not preclude the use of a Raman spectroscopic procedure.

**Validation criteria:** For Category I procedures, the validation range for 100.0% centered acceptance criteria is 80.0%–120.0%. For non-centered acceptance criteria, the validation range is 10.0% below the lower limit to 10.0% above the upper limit. For content uniformity, the range is 70.0%–130.0%. For Category II procedures, the validation range typically covers 50.0%–120.0% of the acceptance criteria.

### ROBUSTNESS

The robustness of an analytical measurement must be demonstrated during development by making deliberate changes to experimental parameters. For Raman spectroscopy, this can include, but is not limited to, changes in sample position, laser–sample interaction, or hardware settings. See ▲ ⟨1858⟩▲ (CN 1-Aug-2020) for additional factors impacting measurement.

## Verification

United States Current Good Manufacturing Practice regulations [21 CFR §211.194(a)(2)] indicate that users of analytical procedures described in *USP–NF* are not required to validate these procedures if provided in a monograph. Instead, the users must verify suitability under actual conditions of use.

The objective of a Raman spectroscopic procedure verification is to demonstrate that the procedure, as prescribed in specific monographs, is being executed with suitable accuracy, sensitivity, and precision. *Verification of Compendial Procedures* ⟨1226⟩

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notes that if the verification of the compendial procedure, according to the monograph, is not successful, the procedure may not be suitable for use with the article under test. It may be necessary to develop and validate an alternative procedure as allowed in *General Notices and Requirements, 6.30 Alternative and Harmonized Methods and Procedures*.

Although complete revalidation of a compendial procedure is not required, verification of the compendial Raman spectroscopic procedure includes the demonstration of certain validation parameters. When the procedure being verified is for identification purposes, specificity is the only parameter required. However, for quantitative applications, additional validation parameters must be studied as appropriate; these may include accuracy and precision over the expected concentration range.

▲ (USP 1-Aug-2020)

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# ⟨861⟩ SUTURES—DIAMETER

***Change to read:***

▲Diameters of surgical sutures can be determined by either contact or noncontact methods.

## CONTACT METHODS

The gauge for determining suture diameter shall be mechanical or electrical, equipped with a direct-reading dial, a digital readout, or a printed readout. The gauge shall have a minimum accuracy of 0.002 mm, an anvil 50 ± 5 mm in diameter, and a flat contact point (presser foot) 12.70 ± 0.02 mm in diameter. The flat contact point and anvil surfaces shall be plane to within 0.005 mm and parallel to each other to within 0.005 mm. For suture sizes 7-0 and larger, the presser foot and moving parts attached to it shall apply a force of 210 ± 3 g to the specimen. For sutures of size 8-0 and smaller, the presser foot and moving parts attached to it shall apply a force of 60 ± 3 g to the specimen.

Measure sutures, whether packaged in dry or fluid form, immediately after removal from the container and without prior drying or conditioning. Do not stretch the suture before measuring.

## MONOFILAMENT SUTURES (ABSORBABLE AND NONABSORBABLE)

For monofilament sutures, lay the strand across the center of the anvil (see *Figure 1*). Apply tension to the suture NMT required to straighten the suture. Measure the diameter of the suture by slowly lowering the presser foot to avoid crushing the suture. Measure the suture diameter at 3 locations corresponding to approximately one-fourth, one-half, and three-fourths of its labeled length. Each location is considered an individual measurement.

Repeat the test on a total of 10 suture strands. Compare each individual measurement to the individual specifications in the appropriate monograph. Compare the average of the 30 individual measurements to the average specification in the appropriate monograph.

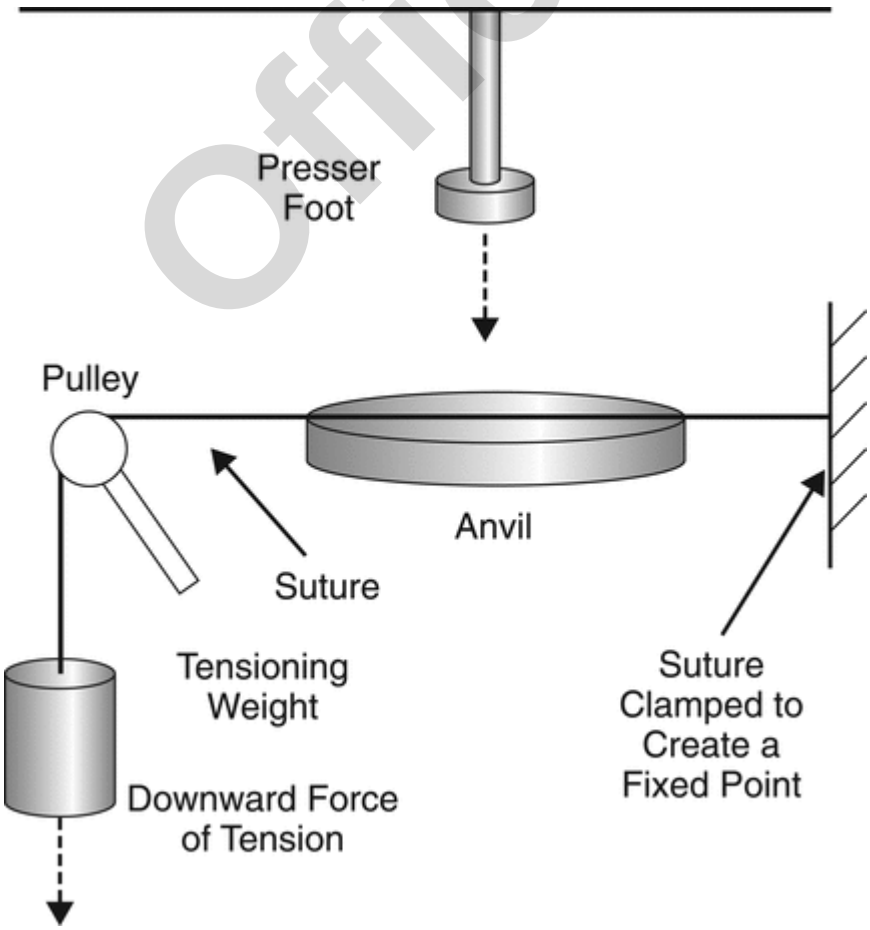


Figure 1. Equipment Setup.

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# MULTIFILAMENT SUTURES (ABSORBABLE AND NONABSORBABLE)

## Diameter Measurement Strategy

Refer to *Table 1* for the diameter measurement strategy for various suture lengths.

**Table 1. Diameter Measurement Strategy**

| Condition | Suture Length as Noted on Package (inches) | Number of Data Points/Strand | Number of Suture Strands | Sample Type |
|---|---|---|---|---|
| 1 | <4 | 1 | 30 | Use a noncontact method |
| 2 | 4 to ≤12 | 1 | 30 | Strand |
| 3 | >12 to ≤48 | 3 | 10 | Strand |
| 4 | >48 | 3 | 10 | Random subsection |

For Condition 1, where the suture length is less than 4 inches during diameter testing, use a noncontact method. For Condition 2, measure the suture diameter at a single location on 30 strands for a total of 30 individual diameter measurements. For Condition 3, measure the suture diameter at 3 locations corresponding to approximately one-fourth, one-half, and three-fourths of the labeled length. Measure 10 strands for a total of 30 individual diameter measurements. For Condition 4, measure the suture diameter at 3 locations corresponding to approximately one-fourth, one-half, and three-fourths on a random subsection NLT 48 inches in length from the total suture product. Measure 10 strands for a total of 30 individual diameter measurements.

## Preparation

Mount the suture on a tensioning board by clamping one end of the suture so that the strand lies across the center of the anvil in the same plane as the surface of the anvil and routing the free end of the suture to be tensioned, such as by passing the suture around a cylinder or a pulley and attaching a weight to the free end, ensuring the strand does not twist or untwist. Apply a load of about one-half of the knotted tensile strength for the nonsterilized Class I suture as given in *Table 2*. Class III sutures do not require tension.

## Measurement

Measure the diameter of the suture by slowly lowering the presser foot to avoid crushing the suture. Measure the suture diameter as specified in *Diameter Measurement Strategy*.

For suture sizes 2-0 and larger: Take 2 measurements at right angles to each other at each location. Average the 2 measurements taken at each location and record as the observed diameter at that location for comparison to the individual specifications. For suture sizes 3-0 and smaller, take a single measurement at each location for comparison to the individual specifications. Each location is considered an individual measurement. The average diameter of the samples are within the limits on average diameter (for all 30 measurements) per the monograph for the size stated on the label. None of the observed individual measurements should be less than or greater than the limits on individual diameter in the monograph. None of the individual observed measurements should be less or greater than the tolerances in the monograph.

## Data Analysis

Compare each individual measurement to the individual specifications in the appropriate monograph. Compare the average of the 30 individual measurements to the average specification in the appropriate monograph.

# NONCONTACT METHODS

The laser micrometer (optical micrometer) for determining suture diameter shall be equipped with a digital or printed readout. The laser micrometer shall have a minimum accuracy of 0.002 mm, and be capable of dual axis measurements. The test method should be correlated with the contact method and the appropriate specifications should be established based on the correlation.

Place the suture without any preconditioning into the path of the laser beam, ensuring the suture is appropriately aligned. For a monofilament suture, hold the suture with minimal applied tension. For a multifilament suture, apply a tensile load of about one-half of the knotted tensile strength (see *Tensile Strength* ⟨881⟩) for the nonsterilized Class I suture of the given size. Measure each suture at 3 points corresponding roughly to one-fourth, one-half, and three-fourths of its length.

# TENSIONS

Tensions for both contact and noncontact methods are calculated to represent approximately one-half of the knot-pull limit for nonsterile Class I sutures of the designated size in *Table 2*.

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**Table 2. Tensioning Weights (in grams) for Measuring Multifilament Strand Diameters**

| Size | Nonabsorbable | Absorbable |
|---|---|---|
| 12-0 | 0.6 | N/A |
| 11-0 | 3.8 | N/A |
| 10-0 | 12 | 12 |
| 9-0 | 27 | 25 |
| 8-0 | 38 | 35 |
| 7-0 | 69 | 70 |
| 6-0 | 125 | 125 |
| 5-0 | 250 | 340 |
| 4-0 | 375 | 475 |
| 3-0 | 600 | 887 |
| 2-0 | 899 | 1341 |
| 0 | 1351 | 1948 |
| 1 | 1702 | 2539 |
| 2 | 2199 | 3176 |
| 3 and 4 | 3046 | 3645 |
| 5 | 3849 | N/A |
| 6 | 4550 | N/A |
| 7 | 5647 | N/A▲ (USP 1-Dec-2019) |

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# ⟨871⟩ SUTURES—NEEDLE ATTACHMENT

Absorbable and nonabsorbable surgical sutures with standard needle attachment are such that the needles are firmly attached and are not intended to be separated. Sutures supplied with eyeless needles attached fall into either the category of standard needle attachment or the category of removable needle attachment. Removable needle attachment of both absorbable and nonabsorbable surgical sutures is such that the needle may be deliberately separated from the suture by means of a quick tug. Both types of attachments are tested on equipment as specified in *Tensile Strength* ⟨881⟩.

## PROCEDURE

Clamp each of 5 sutures in the tensile testing device so that the needle is in the fixed clamp with all of the swaged portion exposed and in line with the direction of force applied to the suture by the moving clamp. Determine the force required to detach the suture from the needle. In the case of standard needle attachment, the suture may break without needle detachment.

### Standard Needle Attachment

The requirements are met if neither the average of the 5 values nor any individual value is less than the limits given for the designated size in *Table 1*.

**Table 1. Standard Needle Attachment for Absorbable and Nonabsorbable Sutures**

| Metric Size (gauge no.) | | Limits on Needle Attachment | | | | |
|---|---|---|---|---|---|---|
| Absorbable Collagen Suture | Nonabsorbable and Synthetic Absorbable Sutures | USP Size | Average (in kgf) (min.) | Individual (in kgf) (min.) | Average (in N) (min.) | Individual (in N) (min.) |
| — | 0.1 | 11-0 and smaller | 0.007 | 0.005 | 0.069 | 0.049 |
| — | 0.2 | 10-0 | 0.014 | 0.010 | 0.137 | 0.098 |
| 0.4 | 0.3 | 9-0 | 0.021 | 0.015 | 0.206 | 0.147 |
| 0.5 | 0.4 | 8-0 | 0.050 | 0.025 | 0.490 | 0.245 |
| 0.7 | 0.5 | 7-0 | 0.080 | 0.040 | 0.785 | 0.392 |
| 1 | 0.7 | 6-0 | 0.17 | 0.08 | 1.67 | 0.784 |
| 1.5 | 1 | 5-0 | 0.23 | 0.11 | 2.26 | 1.08 |
| 2 | 1.5 | 4-0 | 0.45 | 0.23 | 4.41 | 2.26 |
| 3 | 2 | 3-0 | 0.68 | 0.34 | 6.67 | 3.33 |
| 3.5 | 3 | 2-0 | 1.10 | 0.45 | 10.8 | 4.41 |
| 4 | 3.5 | 0 | 1.50 | 0.45 | 14.7 | 4.41 |
| 5 | 4 | 1 | 1.80 | 0.60 | 17.7 | 5.88 |
| 6 and larger | 5 and larger | 2 and larger | 1.80 | 0.70 | 17.7 | 6.86 |

***Change to read:***

### Removable Needle Attachment

For USP sizes 5-0 through 2▲▲ (ERR 1-Aug-2022), the requirements are met if the individual values of the 5 sutures are NLT 0.028 kgf (0.275 N) and NMT 1.59 kgf (15.6 N).

[NOTE—For either type of attachment, if NMT one of the individual values falls outside the prescribed limits, repeat the test on an additional 10 sutures. The requirements of the test are met if none of the 10 additional values falls outside the individual limit requirements.]

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# ⟨881⟩ TENSILE STRENGTH

***Change to read:***

▲Testing devices▲ (USP 1-Dec-2019) for measurement of tensile strength used in the United States may be calibrated in the English units of measure. The following directions are given in metric units with the understanding that the corresponding English equivalents may be used.

***Change to read:***

## SURGICAL SUTURE

Determine the tensile strength of surgical suture on a motor-driven tensile strength testing machine having suitable clamps for holding the specimen firmly and using ▲▲ (USP 1-Dec-2019) the principle of constant rate of elongation of specimen, as described below. The apparatus has two clamps for holding the strand. One of these clamps is mobile. The clamps are designed so that the strand being tested can be attached without any possibility of slipping. Gauge length is defined as the interior distance between the two clamps. For gauge lengths of 125–200 mm, the mobile clamp is driven at a constant rate of elongation of 30 ± 5 cm/min. For gauge lengths of less than 125 mm, the rate of elongation per minute is adjusted to equal 2 times the gauge length per minute. For example, a 5-cm gauge length has a rate of elongation of 10 cm/min.

Determine the tensile strength of the suture, whether packaged in dry form or in fluid, promptly after removal from the container, without prior drying or conditioning. ▲Except where straight pull (no knot required) is indicated in the suture monographs, tie the test suture into a simple knot formed by placing one end of a strand held in the right hand over the other end held in the left hand. Then pass one end over the strand and through the loop that is formed (see *Figure 1*), and pull the knot tight so as not to affect the integrity of the suture.

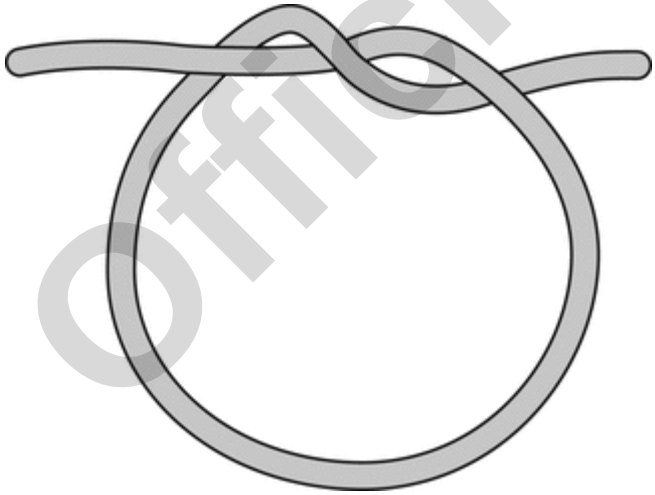

Figure 1. Knot for test suture.

Place the specimen in the testing device with the knot approximately midway between the clamps.▲ (USP 1-Dec-2019) Attach one ▲free▲ (USP 1-Dec-2019) end of the suture to the clamp at the load end of the machine, pass the other ▲free▲ (USP 1-Dec-2019) end through the opposite clamp, applying sufficient tension so that the specimen is taut between the clamps, and engage the second clamp. Perform as many breaks as are specified in the individual monograph. If the break occurs at ▲or in▲ (USP 1-Dec-2019) the clamp, discard ▲and replace with an additional pull with a new test suture. The discarded result should be noted and recorded, but not included in the averaging of the results.

For suture devices that are not knotted, a straight pull should be used for testing. Strength should be compared to the knot tie values for labeled sizes in the monograph table.▲ (USP 1-Dec-2019)

***Add the following:***

## ▲BARBED SUTURES

Barbed suture type devices that do not require the use of knots in their application should not be tested for USP knot-pull tensile strength by the use of knots. The alterations to the surface (i.e., barbing) of these devices would lead to testing bias were a USP knot pull be applied to the device. For these types of devices, refer to the manufacturer's specifications with respect to the USP size designation identified and perform straight pull testing accordingly. Compare results generated to the identified USP size knot-pull specifications.▲ (USP 1-Dec-2019)

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***Change to read:***

## TEXTILE FABRICS AND FILMS

Determine the tensile strength of textile fabrics, including adhesive tape, on a constant-speed or pendulum type of testing ▲device▲ (USP 1-Dec-2019) of the following general description.

The clamps for holding the specimen are smooth, flat, parallel jaws that are NLT 25 mm in length in the dimension parallel to the direction of application of the load. When the width of the strip being tested does not exceed 19 mm, the jaws of the clamp should be at least 25 mm wide. If the width of the strip is greater than 19 mm and NMT 44 mm, the width of the jaws of the clamp should be at least 50 mm. If the width of the specimen is greater than 44 mm, cut a 25-mm strip, and use a clamp with jaws NLT 50 mm wide. Round all edges that might have a cutting action on the specimen to a radius of 0.4 mm. The jaws are 76.2 mm apart at the beginning of the test, and they separate at the rate of 30.5 cm ± 13 mm/min. The machine is of such capacity that when the break occurs, the deviation of the pendulum from the vertical is 9°–45°.

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# ⟨891⟩ THERMAL ANALYSIS

## INTRODUCTION

Precisely determined thermodynamic events, such as a change of state, can indicate the identity and purity of drugs. Compendial standards have long been established for the melting or boiling temperatures of substances. These transitions occur at characteristic temperatures, and the compendial standards therefore contribute to the identification of the substances. Because impurities affect these changes in predictable ways, the same compendial standards contribute to the control of the purity of the substances.

Thermal analysis in the broadest sense is the measurement of physical–chemical properties of materials as a function of temperature. Instrumental methods have largely supplanted older methods dependent on visual inspection and measurements under fixed or arbitrary conditions, because they are objective, provide more information, afford permanent records, and are generally more sensitive, precise, and accurate. Furthermore, they may provide information on desolvation, dehydration, decomposition, crystal perfection, polymorphism, melting temperature, sublimation, glass transitions, evaporation, pyrolysis, solid-solid interactions, and purity. Such data are useful in the characterization of substances with respect to compatibility, stability, packaging, and quality control. The measurements used most often in thermal analysis, i.e., transition and melting point temperatures by differential scanning calorimetry (DSC), thermogravimetric analysis, hot-stage microscopy, and eutectic impurity analysis, are described here.

## TRANSITION AND MELTING POINT TEMPERATURES

As a specimen is heated, transitions can be observed using differential scanning calorimetry (DSC), differential thermal analysis (DTA), or hot-stage microscopy. In heat-flux DSC, the heat differential between the sample and reference material is determined. In power compensation DSC, the sample and reference materials are maintained at the same temperature, using individual heating elements, and the difference in power input to the two heaters is recorded. DTA monitors the difference in temperatures between the sample and the reference. The transitions that may be observed include those shown in *Table 1* below. In the case of melting, both an “onset” and a “peak” temperature can be determined objectively and reproducibly, often to within a few tenths of a degree. Although these temperatures are useful for characterizing substances, and the difference between the two temperatures is indicative of purity, the values cannot be directly compared to visual “melting-range” or “melting-point” values or with constants such as the triple point of the pure material.

Furthermore, caution should be used when comparing results obtained by different methods of analysis. Optical methods may measure the melting point as the temperature where the last trace of solid coalesces. In contrast, melting points measured by DSC may refer to the onset temperature or the temperature where the maximum melting rate (vertex) was observed. However, the vertex is sensitive to sample weight, heating rate, and other factors, whereas the onset temperature is less affected by these factors. With thermal techniques, it is necessary to consider the limitations of solid solution formation, insolubility in the melt, polymorphism, and decomposition during the analysis.

**Table 1**

| | | |
|---|---|---|
| Solid to liquid | Melting | Endothermic |
| Liquid to gas | Vaporization | Endothermic |
| Liquid to solid | Freezing | Exothermic |
| | Crystallization | Exothermic |
| Solid to gas | Sublimation | Endothermic |
| Solid to solid | Glass transition | Second order event |
| | Desolvation | Endothermic |
| | Amorphous to crystalline | Exothermic |
| | Polymorphic | Endothermic or Exothermic |

### Reporting Results of Instrumental Methods

A complete description of the conditions employed should accompany each thermogram, including make and model of instrument; record of last calibration; specimen size and identification (including previous thermal history); container; identity, flow rate, and pressure of gaseous atmosphere; direction and rate of temperature change; and instrument and recorder sensitivity.

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# DETERMINATION OF TRANSITION TEMPERATURE (MELT ONSET TEMPERATURE) AND MELTING POINT TEMPERATURE

## Apparatus

Use DTA or DSC instrumentation equipped with a temperature-programming device, thermal detector(s), and a recording system that can be connected to a computer, unless otherwise prescribed by the specific monograph for which this chapter is being used.

## Calibration

Calibrate instrumentation for temperature and enthalpy changes, using indium or another suitable certified material. Temperature calibration is performed by heating a standard through the melting transition and comparing the extrapolated onset of melting point of the standard to the certified onset of melting point. The temperature calibration should be conducted at the same heating rate as the experiment. Enthalpy calibration is performed by heating a standard through the melting transition and comparing the calculated heat of fusion to the theoretical value.

## Procedure

Accurately weigh an appropriate quantity of the substance to be examined in the sample pan, as described in the specific monograph. Set initial temperature, heating rate, direction of temperature change, and final temperature as specified in the monograph. If not specified in the monograph, these parameters are determined as follows: make a preliminary examination over a wide range of temperatures (typically, room temperature to decomposition temperature, or about 10° to 20° above the melting point) and over a wide range of heating rates (1° to 20° per minute) to reveal any unexpected effects. Then determine a lower heating rate such that decomposition is minimized and the transition temperature is not compromised. Determine a temperature range bracketing the transition of interest such that the baseline can be extended to intersect with the tangent of the melt (see *Figure 1*).

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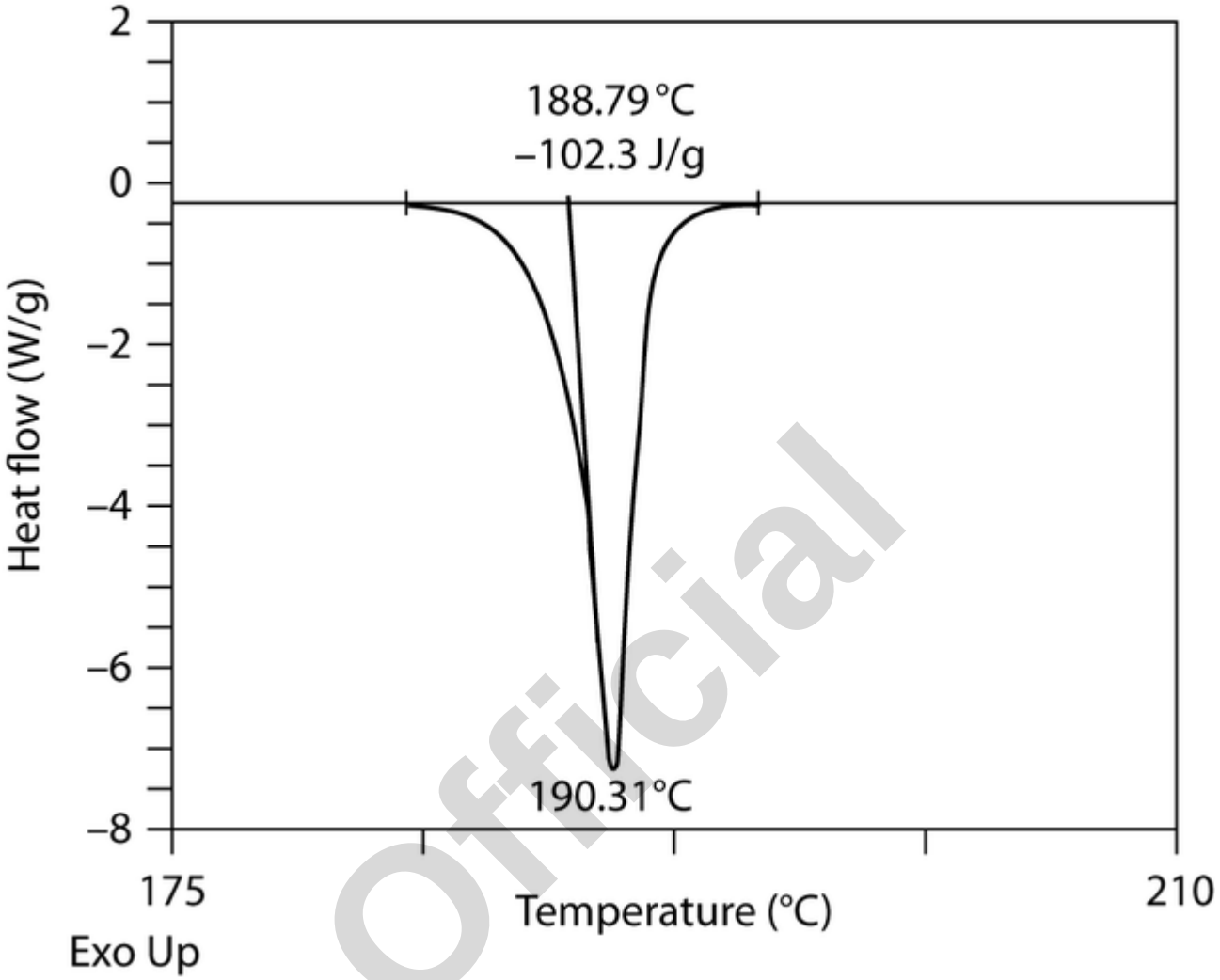


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Figure 1. Thermogram.

In examining pure crystalline materials, rates as low as 1° per minute may be appropriate, whereas rates of up to 20° per minute are more appropriate for polymeric and other semicrystalline materials. Begin the analysis, and record the differential thermal analysis curve with the temperature on the *x*-axis and the energy change on the *y*-axis. The melting temperature (melt onset temperature) is the intersection (188.79°) of the extension of the baseline with the tangent at the point of greatest slope (inflexion point) of the curve (see *Figure 1*). The vertex is the temperature at the peak of the curve (190.31°). The enthalpy of the event is proportional to the area under the curve after application of a baseline correction.

## THERMOGRAVIMETRIC ANALYSIS

Thermogravimetric analysis involves the determination of the mass of a specimen as a function of temperature, or time of heating, or both. It is often used to investigate dehydration/desolvation processes and compound decomposition. When thermogravimetry is properly applied, it provides more useful information than loss on drying at fixed temperature, often for a fixed time and in what is usually an ill-defined atmosphere. Usually, loss of surface-absorbed solvent can be distinguished from solvent in the crystal lattice and from degradation losses. The measurements can be carried out in atmospheres having controlled humidity and oxygen concentration to reveal interactions with the drug substance, between drug substances, and between active substances and excipients or packaging materials.

### Apparatus

While the details depend on the manufacturer, the essential features of the equipment are a recording balance and a programmable heat source. Equipment differs in the ability to handle specimens of various sizes, the means of sensing specimen temperature, and the range of atmosphere control.

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## Calibration

Calibration is required with all systems: that is, the mass scale is calibrated by the use of standard weights, and calibration of the temperature scale involves the use of standard materials, because it is assumed that the specimen temperature is the furnace temperature. Weight calibration is conducted by measuring the mass of a certified or standard weight and comparing the measured mass with the certified value. Temperature calibration is performed by analyzing a high-purity magnetic standard such as nickel for its curie temperature and comparing the measured value to the theoretical value.

## Procedure

Apply the method to the sample, using the conditions described in the monograph, and calculate the mass gain or loss, expressing the change in mass as percentage. Alternatively, place a suitable quantity of material in the sample holder, and record the mass. Because the test atmosphere is critical, the pressure or flow rate and the composition of the gas are specified. Set the initial temperature, heating rate, and final temperature according to the manufacturer's instructions, and initiate the temperature increase. Alternatively, conduct an examination of the thermogram over a wide range of temperatures (typically, from room temperature to the decomposition temperature, or 10° to 20° above the melting point at a heating rate of 1° to 20° per minute). Calculate the mass gain or loss, expressing the change in mass as percentage.

# HOT-STAGE MICROSCOPY

Hot-stage microscopy is an analytical technique that involves monitoring the optical properties of the sample using a microscope as a function of temperature. Hot-stage microscopy may be used as a complementary technique to other thermal analysis techniques such as DSC, DTA, and variable temperature X-ray powder diffraction for the solid-state characterization of pharmaceutical compounds. It is useful to confirm transitions such as melts, recrystallizations, and solid-state transformations using a visual technique. The hot-stage microscope must be calibrated for temperature.

# EUTECTIC IMPURITY ANALYSIS

The basis of any calorimetric purity method is the relationship between the melting and freezing point depression and the level of impurity. The melting of a compound is characterized by the absorption of latent heat of fusion, $\Delta H_f$, at a specific temperature, $T_o$. In theory, a melting transition for an absolutely pure crystalline compound should occur within an infinitely narrow range. A broadening of the melting range, due to impurities, provides a sensitive criterion of purity. The effect is apparent visually by examination of thermograms of specimens differing by a few tenths percent in impurity content. A material that is 99% pure is about 20% molten at 3° below the melting point of the pure material (see *Figure 2*).

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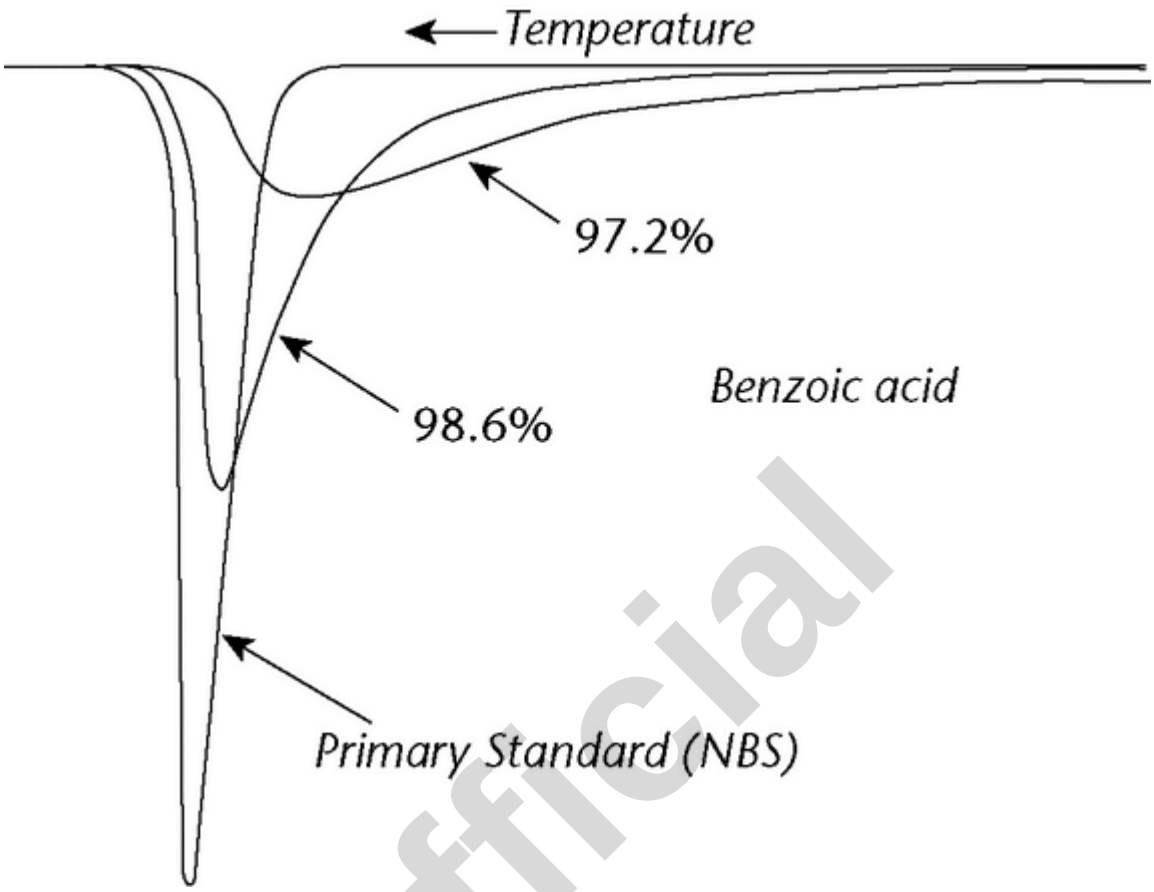


Figure 2. Superimposed thermograms illustrating the effect of impurities on DSC melting peak shape.

The parameters of melting (melting range, $\Delta H_f$, and calculated eutectic purity) are readily obtained from the thermogram of a single melting event using a small test specimen, and the method does not require multiple, precise actual temperature measurements. Thermogram units are directly convertible to heat transfer, millicalories per second.

The lowering of the freezing point in *dilute solutions* by molecules of nearly equal size is expressed by a modified van't Hoff equation:

$$\frac{dT}{dX_2} = \frac{RT^2}{\Delta H_f} \cdot \left(K_D - 1\right) \quad (1)$$

in which T = absolute temperature in kelvins; $X_2$ = mole fraction of minor component (solute, impurity); $\Delta H_f$ = molar heat of fusion of the major component in Joules per mol: R = gas constant in Joules per mol × kelvins; and $K_D$ = distribution ratio of solute between the solid and liquid phases.

Assuming that the temperature range is small and that no solid solutions are formed ($K_D = 0$).

Integration of the van't Hoff equation yields the following relationship between the mole fraction of impurity and the melting-point depression:

$$X_2 = \frac{\left(T_o - T_m\right)\Delta H_f}{RT_o^2} \quad (2)$$

in which $T_o$ = melting point of the pure compound, in kelvins, and $T_m$ = melting point of the test specimen, in kelvins.

With no solid solution formation, the concentration of impurity in the liquid phase at any temperature during the melting is inversely proportional to the fraction melted at that temperature, and the melting-point depression is directly proportional to the mole fraction of impurity. A plot of the observed test specimen temperature, $T_s$, versus the reciprocal of the fraction melted, 1/F, at temperature $T_s$, should yield a straight line with the slope equal to the melting-point depression ($T_o - T_m$). The theoretical melting point of the pure compound is obtained by extrapolation to 1/F = 0:

$$T_S = T_o - \frac{RT_o^2 X_2 (1/F)}{\Delta H_f} \quad (3)$$

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Substituting the experimentally obtained values for $T_o - T_m$, $\Delta H_f$, and $T_o$ in equation (2) yields the mole fraction of the total eutectic impurity, which, when multiplied by 100, gives the mole percentage of total eutectic impurities.

Deviations from the theoretical linear plot also may be due to solid solution formation ($K_D \neq 0$), so that care must be taken in interpreting the data.

To observe the linear effect of the impurity concentration on the melting-point depression, the impurity must be *soluble* in the liquid phase or melt of the compound, but *insoluble* in the solid phase, i.e., no solid solutions are formed. Some chemical similarities are necessary for solubility in the melt. For example, the presence of ionic compounds in neutral organic compounds and the occurrence of thermal decomposition may not be reflected in purity estimates. The extent of these theoretical limitations has been only partially explored.

Impurities present from the synthetic route often are similar to the end product, hence there usually is no problem of solubility in the melt. Impurities consisting of molecules of the same shape, size, and character as those of the major component can fit into the matrix of the major component without disruption of the lattice, forming solid solutions or inclusions; such impurities are not detectable by DSC. Purity estimates are too high in such cases. This is more common with less-ordered crystals as indicated by low heats of fusion.

In addition, the method is reliable when the purity of the major component is greater than 98.5 mol% and the materials are not decomposed during the melting phase.

Impurity levels calculated from thermograms are reproducible and generally reliable within 0.1% for ideal compounds.

Compounds that exist in polymorphic form cannot be used in purity determination unless the compound is completely converted to one form. On the other hand, DSC and DTA are inherently useful for detecting, and therefore monitoring, polymorphism.

## Procedure

The actual procedure and the calculations to be employed for eutectic impurity analysis are dependent on the particular instrument used. Consult the manufacturer's literature and/or the thermal analysis literature for the most appropriate technique for a given instrument. In any event, it is imperative to keep in mind the limitations of solid solution formation, insolubility in the melt, polymorphism, and decomposition during the analysis.

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***Add the following:***

# ▲⟨901⟩ DETECTION OF ASBESTOS IN PHARMACEUTICAL TALC

To view the Notice from the Expert Committee that posted in conjunction with this chapter, please click https://www.uspnf.com/notices/talc-official-dates-20230601.

This chapter provides procedures to detect the presence of asbestos in pharmaceutical talc using the analytical procedures of X-ray diffraction (XRD)—also termed X-ray powder diffraction (XRPD)—coupled with polarized light microscopy (PLM). Both *Procedure 1: X-Ray Diffraction* and *Procedure 2: Polarized Light Microscopy* are mandatory. For supporting information on the best practices and principles of measurements, see *Theory and Practice of Asbestos Detection in Pharmaceutical Talc* ⟨1901⟩.

## Procedure 1: X-Ray Diffraction

### INTRODUCTION

XRD is used to determine whether amphibole and/or serpentine minerals are present in a talc powder matrix and, when applicable, to estimate the percentage present. It is intended to be used in combination with a microscopy technique capable of determining whether asbestos is present. [NOTE—For additional information, see *Characterization of Crystalline and Partially Crystalline Solids by X-Ray Powder Diffraction (XRPD)* ⟨941⟩.]

With regard to sampling and subsampling, see *Bulk Powder Sampling Procedures* ⟨1097⟩.

### SAMPLE PREPARATION

Using the sample holders provided by the XRD manufacturer, fill the cavity using back-, front-, or side-filling techniques depending on the specific holder used. Be sure to use the same sample filling technique for reference standards (if used) and the samples being tested.

### SAMPLE ANALYSIS (QUALITATIVE AND QUANTITATIVE SCANS)

Qualitative scans are used to assess overall mineralogy (see ⟨1901⟩ for a list of peak positions for common minerals that may occur with talc). Quantitative scans are used to determine the presence of trace levels of amphibole and serpentine minerals (above minimum detection limits).

The following conventional XRD instrument conditions and parameters can be used to determine the detection limits outlined below.

- *X-ray tube voltage:* 45–50 kilovolts
- *X-ray tube current:* 30–45 milliamperes (consult tube manufacturer recommendations; do not exceed tube rating)
- *Target:* copper
- *Monochromator:* graphite or multilayer (tungsten/silicon, nickel/carbon, or similar); curved or flat diffracted or primary beam
- *Detector:* scintillation counter or alternative high-speed detector
- *Receiving slit:* 0.6 or 0.3 mm, none if using a high-speed detector
- *Scintillation or anti-scatter slit:* 1° or matched to the size of the high-speed detector
- *Divergent height-limiting slit or beam mask:* 10 mm
- *Divergent slit:* 1°, or smaller to ensure no sample overshoot
- *Scan mode:* fixed time (FT)
- *Single-stage or multi-position sample changer attachment*
- *Spin speed of sample:* 30 or 60 rpm

Any variation due to vendor limitations should be noted and used for samples, references, and standards. Additionally, use of high-speed detectors can permit much less analysis time for qualitative and quantitative testing. For example, a full qualitative scan from 3°–85° 2-theta (2θ) can be done in fewer than 35–40 min, and a quantitative evaluation for amphibole and serpentine can be accomplished within 2–3 h. *Table 1* outlines the target peak positions and scan ranges for XRD scans.

**Table 1. Target Peak Positions and Scan Ranges for Qualitative and Quantitative XRD Scans**

| Scan Type | Target Phase(s) | Target Peak(s) (2-Theta) | Start Angle (2-Theta) | End Angle (2-Theta) | Step Size (2-Theta) | Count Time per Step(s) |
|---|---|---|---|---|---|---|
| Qualitative scan | All (full scan) | All | 3 | 65 (minimum) | 0.02 | 2 |
| Quantitative scans | Amphibole[a] | 10.5<br>10.7 | 9.8 | 11.2 | 0.02 | 100 |
| | Serpentine<br>Chlorite | 12.1<br>12.5 | 11 | 13.2 | 0.02 | 100 |
| | Serpentine<br>Chlorite | 24.4<br>25.1 | 23.5 | 25.6 | 0.02 | 100 |
| | Orthorhombic amphibole (optional) | 29.3 | 28.8 | 29.8 | 0.02 | 100 |

[a] Both monoclinic (e.g., tremolite) and orthorhombic (e.g., anthophyllite) amphiboles are included in this scan and can be distinguished from each other. Monoclinic amphibole species may not necessarily be distinguished from each other at the trace level.

### LIMIT OF DETECTION AND QUANTIFICATION

Detection limits were verified using 0.25% spiked standards and the instrument conditions outlined above.

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Limit of detection for:

- Amphiboles (tremolite and anthophyllite): 0.2%
- Serpentine with no chlorite interference: 0.2%
- Serpentine with 1% chlorite interference: 0.5%
- Serpentine with >1% to 15%–20% chlorite interference: 1%, with resolution of the two phases confirmed using a 2θ range of 23.5–25.6

A detection limit of 0.1% has also been achieved using this method; however, use of lower limits will need to be verified by individual labs using a 0.1% spiked standard.

[NOTE—See more information in ⟨1901⟩.]

**REPORTING RESULTS**

- If no detectable peak is present for monoclinic or orthorhombic amphiboles (tremolite or anthophyllite), then report "amphibole not detected (<0.2%)".
- If no detectable peak is present for serpentine without a chlorite interference, then report "serpentine not detected (<0.2%)".
- If no detectable peak is present for serpentine with 1%–15% chlorite interference, then report "serpentine not detected (<1%)".
- If a peak is present for serpentine, then report "serpentine detected".
- If a peak is present for an amphibole, then report the type, i.e., "monoclinic amphibole detected" or "orthorhombic amphibole detected".

[NOTE—Alternative detection limits can be determined and reported using operating parameters at individual laboratories.]

Only report the detected and not detected result limit, not the quantitative result.

[NOTE—The limits are based on limits of detection.]

## Procedure 2: Polarized Light Microscopy

**INTRODUCTION**

This procedure uses PLM to identify asbestos particles in a talc powder matrix. It is intended to be used with *Procedure 1* as a complementary microscopy technique. Both *Procedure 1* and *Procedure 2* are mandatory.

**CONCENTRATION USING WET SIEVING**

Concentration of asbestos using the wet sieving technique has been found to be effective for PLM analysis (see supporting information in ⟨1901⟩). Use 76-mm (3-inch) sieves, precleaned in a beaker filled with detergent and water in a sonic bath for 15 min. Rinse thoroughly with deionized water.

1. Measure approximately 2 g of talc powder into a 100-mL plastic bottle with a removable lid.
2. Because talc is hydrophobic, wet the powder completely with isopropyl alcohol (IPA), then fill three quarters full of deionized water (approximately 75 mL total); shake vigorously for 30 s.
3. Slowly pour the suspension onto a 400-mesh (37-µm) sieve. The passing fraction should be collected and disposed of properly according to local regulations. Work in batches, alternately rinsing with water or IPA (if foaming) to avoid screen blinding. Optimal rinsing is accomplished by using a standard kitchen sink sprayer or by squeezing the end of a flexible hose attached to the faucet. In either case, water should be set to deliver a gentle spray to avoid splash back.
4. Repeatedly fill the plastic suspension bottle with water and IPA (if foaming) and replace the lid. Shake and pour onto the sieve until the bottle is washed clean.
5. Continue rinsing the sieve under gentle pressure until the water passing through appears clear. [NOTE—A very small amount of residue should be visible by eye, but continuously sprayed water should pass through the screen unimpeded. The optimum amount of residue retained on the screen can be determined by eye with experience.]
6. Rinse the residue from the screen using a squirt bottle with particle-free water into a tared small aluminum pan or glass dish for drying. Dry the sample by air or use an oven at 105°–110°.
7. Once dry, weigh the residue. The objective is to obtain 0.05–0.1 g of residue for subsequent sampling by PLM.
8. If less than 0.05 g is collected from the 400-mesh (37-µm) sieve using 2 g of starting material, one of the following procedures should be used:
   A. Repeat the above until at least 0.05 g is collected for analysis.
   B. Repeat the above using a greater amount of starting material. [NOTE—A 254-mm (10-inch) sieve could be used with up to 100 g of starting material weighed into a 1000-mL bottle.]
   C. Repeat the above using a finer mesh sieve.
9. Sieves should be cleaned and maintained in accordance with manufacturer's recommendations and/or instructions, or disposable sieves can be used. Use of any sieve size other than aforementioned should be validated following *General Notices 6.30. Alternative and Harmonized Methods and Procedures*.

**SAMPLE PREPARATION OF SIEVED RESIDUE**

1. Place a known amount of talc from the sieved residue on a glass slide so that particles cover 30%–50% of the area under the slide. This can be accomplished by taring a lab scale with the glass slide before adding an appropriate amount of sample.
2. Add 1 or 2 drops of refractive index (RI) liquid to the powder (see *Sample Analysis* for use of RI liquids). Swirl with a cleaned metal probe to distribute particles.
3. Place a coverslip on the sample, and, if needed, use a pencil eraser (or equivalent soft-tipped tool) to press gently on the coverslip to remove air bubbles and better disperse the particles.

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[NOTE—It may take some trial and error to obtain the optimal loading and starting sample weight. A minimum of 5 mg should be analyzed in total for each RI liquid used. This typically equates to 3–5 slide preparations with 1–2 mg each, using 22-mm × 22-mm square coverslips. Adjust as necessary.]

**SAMPLE ANALYSIS**

**Screen particles using the dispersion staining technique**

1. Configure the microscope in dispersion staining mode [i.e., plane-polarized light (PPL), no condenser lens, centered dispersion staining objective].
   - Confirm that the microscope is set up and aligned properly by obtaining dispersion staining colors on prepared standards of amphibole and chrysotile asbestos before analyzing unknown material.
2. Prepare microscope slides (number determined by the weight of sample on each) in 1.550 and 1.605 RI liquids.
3. Scan each slide in its entirety in an orthogonal fashion in dispersion staining mode, characterizing any suspect particles (see explanation below).
   - Use a mechanical stage to aid in the scanning process.
   - If particles are brought to the center of the field of view for characterization or the stage is rotated during the course of documentation, be sure to return the particle to its original location and orientation before resuming the scan.
4. Observe any suspect particles of amphibole and chrysotile, which will display low relief and dispersion staining colors, as referenced in the International Organization for Standardization's ISO 22262-1 (2012), when immersed in 1.605 and 1.550 RI liquids, respectively (summarized below and in *Table 2*). The appropriate dispersion staining colors are determined when the axis of elongation of the particle is oriented parallel to the lower polarizer (*n*-parallel) and perpendicular to the lower polarizer (*n*-perpendicular) in a standard PLM configuration.
   - 1.550 RI liquid
     - Talc plates lying flat (with their plate surface parallel to the microscope slide) will appear pale yellow during the full rotation of the stage.
     - Chrysotile particles will appear magenta (*n*-parallel) and blue (*n*-perpendicular).
     - Elongate talc particles can appear yellow (*n*-parallel) to blue (*n*-perpendicular). Rotating the stage to observe the yellow color in 1.550 RI liquid will distinguish elongate talc from chrysotile.
   - 1.605 RI liquid
     - Talc plates lying flat (with their plate surface parallel to the microscope slide) will appear pale blue during the full rotation of the stage.
     - Elongate amphibole particles will appear characteristically yellow to pale yellow (*n*-parallel).
     - For *n*-perpendicular, amphiboles can range in color from blue or magenta (tremolite) to golden yellow or yellow (actinolite or anthophyllite).
     - Amphibole particles have a lower relief in the *n*-perpendicular orientation, and the darker dispersion staining colors may be more difficult to see. If this is the case, rotating the stage should reveal the characteristic yellow color (*n*-parallel).
     - Carbonate particles in 1.605 RI liquid can also appear yellow in certain orientations. These particles can be distinguished by their high birefringence. Alternatively, pretreating the sample with 2 M hydrochloric acid will remove carbonate particles with interfering yellow color (refer to ISO 22262-2 for details).

   Typical central stop dispersion staining colors with corresponding $\lambda_0$ in nanometers are observed for talc and amphibole minerals in various Cargille Series E liquids for both parallel and perpendicular directions. The range of colors and $\lambda_0$ are a function of compositional variation for amphiboles in the compositional space for the stated amphibole (e.g., anthophyllite) taken from reported ranges of refractive indices. See ⟨1901⟩.

**Table 2. Typical Color of Particles in Dispersion Staining Mode**

| Name | RI Liquid for Screening | Dispersion Color[a] | |
|---|---|---|---|
| | | ***n*-parallel (approximate wavelengths, nm)** | ***n*-perpendicular (approximate wavelengths, nm)** |
| Talc plates | 1.550 | Pale yellow (≤400) | Pale yellow (≤400) |
| Talc plates (edge view) | 1.550 | Pale yellow (≤400) | Blue–green to light blue–green (≥620) |
| Chrysotile | 1.550 | Orange to blue–green (480–620) | Blue to light blue–green (580–680) |
| Talc plates | 1.605 | Pale blue (≥800) | Pale blue (≥800) |
| Talc plates (edge view) | 1.605 | Pale blue (≥800) | White (≥1000) |
| Tremolite | 1.605 | Pale yellow to yellow (≤400) | Magenta to blue–green (520–620) |

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**Table 2. Typical Color of Particles in Dispersion Staining Mode** *(continued)*

| Name | RI Liquid for Screening | Dispersion Color[a] | |
|---|---|---|---|
| | | ***n*-parallel (approximate wavelengths, nm)** | ***n*-perpendicular (approximate wavelengths, nm)** |
| Actinolite | 1.605 | White to pale yellow (≤400) | Pale yellow to yellow (≤400–450) |
| Anthophyllite | 1.605 | White to orange–magenta (≤300–500) | White to light blue–green (≥640) |
| Tremolite | 1.615 | Pale yellow to golden yellow (400–460) | Blue–green to pale blue–green (620–780) |
| Actinolite | 1.615 | White to pale yellow (≤400) | Pale yellow to magenta–purple (400–540) |
| Anthophyllite | 1.615 | White to blue–green (≤620) | Yellow to white (≥420) |
| Tremolite | 1.620 | Yellow to orange–magenta (440–500) | Pale blue–green (≥700) |
| Actinolite | 1.620 | Pale yellow to yellow (≤420) | Yellow to blue (420–590) |
| Anthophyllite | 1.620 | Pale yellow to blue–green (≤620) | Pale yellow to pale blue–green (≤400 to ≥800) |
| Tremolite | 1.635 | Magenta–purple to light blue–green (540–660) | Pale blue–green to white (≥800) |
| Actinolite | 1.635 | Pale yellow to magenta (≤540) | Magenta to pale blue–green (520–800) |
| Anthophyllite | 1.635 | Pale yellow to pale blue–green (≤400 to ≥1000) | Pale yellow to white (≤400 to ≥1000) |
| Tremolite | 1.640 | Blue to pale blue–green (580–760) | White (≥1000) |
| Actinolite | 1.640 | Pale yellow to blue–green (400–600) | Blue to pale blue–green (580 to ≥800 ) |
| Anthophyllite | 1.640 | Pale yellow to pale blue–green (≤400 to ≥800) | Yellow to pale blue–green (420 to ≥800) |

[a] Dispersion colors may vary between microscopes and microscopists. Colors given in printed lists may not correspond to those seen in a particular microscope system. Use of specific matching liquids with freshly mounted standards by the analyst should be used for direct color reference.

5. Rotate the slide 90° and scan the entire slide again, so that any amphibole particles showing colors that are more difficult to see (and possibly missed) in the perpendicular orientation may be more visible in the opposite orientation.
6. For any suspect amphibole particle encountered, confirm that RIs in both *n*-parallel and *n*-perpendicular orientations are greater than the 1.605 RI liquid using the Becke line technique. With the following exception, all amphibole RIs are higher than 1.605, while all talc RIs are lower than 1.605.
    - For the rare case where a specific orientation of low-iron anthophyllite indicates an RI slightly below 1.605, rotating the microscope stage or "tapping" the coverslip with a probe so that the particle rolls around its longitudinal axis will confirm the presence of RIs higher than 1.605, thereby distinguishing a possible amphibole particle from a talc particle. Also, the difference in the alpha prime (α′) RI and the 1.605 liquid will still be close enough to provide a measurable dispersion staining color for determination of $n_D$ (the standard refractive index at the "D" Fraunhofer line of approximately 589 nm).
    - With the Becke line technique, the bright white (or light) Becke line moves into the material (either the particle or the surrounding liquid) that has the higher relative RI during defocusing in the down direction (i.e., when increasing the distance between the objective and the sample). The opposite is true for defocusing in the up direction (i.e., the bright white or lighter colored Becke line moves into the material that has the lower relative RI when decreasing the distance between the objective and the sample). It is often helpful to observe both defocusing conditions to confirm the movement of the Becke lines. Where the RI of the particle and liquid are close enough to observe dispersion, the Becke lines become colored.
7. Document the following optical properties of any confirmed suspect particles (determined using a combination of illumination conditions and magnifications between 100× and 400×):
    - *Morphology and aspect ratio*—observed in all illumination conditions
        - Examples: straight, curved, bundle, wavy, splayed ends, polyfilamentous, etc.
    - *Color and pleochroism*—observed in PPL
    - *Extinction angle and characteristics*—observed in cross-polarized light (XPL)

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- Inclined extinction may be indicative of monoclinic amphibole. Anthophyllite (orthorhombic amphibole) always has parallel extinction; however, asbestiform amphibole can have anomalous parallel extinction for all types.

- *Sign of elongation*—observed in XPL with first-order wave plate (RPL) inserted
  - Typically, positive for amphibole and chrysotile (blue in orientation parallel to the slow direction of the first-order wave plate and yellow in orientation perpendicular to the slow direction of the first-order wave plate). Crocidolite/riebeckite (not commonly associated with talc) has a negative sign of elongation, where these colors occur in opposite directions.
- *Refractive index (RI)*—observed in dispersion staining mode and in PPL using the Becke line method. In 1.550 and 1.605 RI liquids, indicate if the RI of the particle is higher, lower, or a close match in both *n*-parallel and *n*-perpendicular orientations. For a more precise match, use additional RI liquids. For determination of RIs, proceed as follows:
  A. Use the appropriate RI liquids to determine the RIs of suspect particles encountered and use *Table 2* as a guide.
  B. Qualitatively determine a match of RI using dispersion staining colors and/or Becke lines. (See ⟨1901⟩ for more information about quantitatively determining wavelength of match). The following conditions indicate a close match between particle and liquid: deep blue and magenta dispersion staining colors, colored Becke lines, and low particle relief (particles appear to disappear in plane-polarized light). Light blue and yellow colors in dispersion staining mode can indicate a greater difference between particle and liquid than colored Becke lines. Additional RI liquids may be needed to evaluate a match.
  C. Correlating RI to wavelength of match can also be completed (see ⟨1901⟩). [NOTE—Confirm observed colors with known minerals under the operational conditions at the observation temperature.]
  D. Where monoclinic amphibole is suspected from optical properties, document "monoclinic amphibole". Where orthorhombic amphibole is probable, document "orthorhombic amphibole".
  E. If ambiguity still exists after this analysis, scanning electron microscopy (SEM) with energy dispersive X-ray spectroscopy (EDS) or transmission electron microscopy (TEM) with EDS should be used to determine the elemental composition; TEM with selected area electron diffraction can be used to confirm if a phase is amphibole and establish if it is orthorhombic or monoclinic. Optionally, a heating and cooling stage can be used to vary the RI liquid values to more precisely determine matching indices of the phases analyzed. For more information, see ⟨1901⟩.
- *Birefringence*—Report the difference in the maximum and minimum observed RI of a suspect particle type.

8. Use the definition below to evaluate all of the particles documented to determine if presence of asbestiform amphibole or serpentine is indicated. [The definition of "asbestiform" for this method is taken from the Glossary section of EPA 600/R-93/116 (emphasis in original).]
"Asbestiform (morphology)—Said of a mineral that is like asbestos, i.e., crystallized with the habit of asbestos. Some asbestiform minerals may lack the properties which make asbestos commercially valuable, such as long fiber length and high tensile strength. With the light microscope, the asbestiform habit is generally recognized by the following characteristics:
   - Mean aspect ratios ranging from 20:1 to 100:1 or higher for fibers longer than 5 µm. Aspect ratios should be determined for fibers, not bundles.
   - Very thin fibrils, usually less than 0.5 micrometers in width, and
   - Two or more of the following:
     - Parallel fibers occurring in bundles,
     - Fiber bundles displaying splayed ends,
     - Matted masses of individual fibers, and/or
     - Fibers showing curvature

   These characteristics refer to the population of fibers as observed in a bulk sample. It is not unusual to observe occasional particles having aspect ratios of 10:1 or less, but it is unlikely that the asbestos component(s) would be dominated by particles (individual fibers) having aspect ratios of <20:1 for fibers longer than 5 µm. If a sample contains a fibrous component of which most of the fibers have aspect ratios of <20:1 and that do not display the additional asbestiform characteristics, by definition the component should not be considered asbestos."

[NOTE—This definition of asbestiform is appropriate for light microscopy, including phase contrast and polarized light microscopy. However, it is not generally extendable to fibrils visible in the transmission electron microscope.]

**LIMIT OF DETECTION AND QUANTIFICATION**

The working detection limit of this method is 0.01% weight percentage, 100 ppm by weight (µg/g) established via a round robin study of spiked reference materials.

[NOTE—See more information in ⟨1901⟩.]

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**REPORTING RESULTS**

1. All amphibole or serpentine particles must be documented via a digital image capturing system attached to the microscope. The following images should be collected for each particle (if an image cannot be obtained, note this as well):
    - Dispersion staining and/or Becke line color with the fiber oriented parallel to the polarizer
    - Dispersion staining and/or Becke line color with the fiber oriented perpendicular to the polarizer
    - Sign of elongation with the fiber oriented parallel to the slow vibration direction of the first-order wave plate under crossed polarizers
    - Optional: additional images to show morphology (microscope in any configuration)
    - Optional: image(s) showing the extinction angle (inclined versus parallel) to supplement the documented angle (a cross-polarized image taken with first-order wave plate at extinction)
2. The final report should include:
    - Number of particles found
    - Length and width for the individual particles
    - Digital images or photographs of each particle (especially those of bundles or other unique features of asbestos)
    - Either an assessment of “detected” or “not detected” of asbestos with the detection limit of 0.01% weight percentage or 100 ppm by weight (µg/g)
3. If a single particle is observed in an analysis of 5 mg, analysis of an additional 5 mg of the originally received material is required. If the additional analysis results in 1 or fewer particles, report the result as below the detection limit of <0.01% weight percentage or <100 ppm by weight (µg/g). [NOTE—For this method, the typical number of particles found at 100 ppm is 8–16 particles when approximately 2 mg of material is tested.]
4. A single particle, even if conclusively identified as asbestos, is not sufficient to constitute a positive finding as it cannot be proven to be different from contamination. More than 1 particle with conclusive identification (e.g., 2 particles) is necessary to constitute a positive identification. All results with fewer than 2 confirmed asbestos fibers are reported as below the detection limit of <0.01% weight percentage or <100 ppm by weight (µg/g).
5. Determination of the type of amphibole asbestos (i.e., tremolite, actinolite, or anthophyllite) is not required; results will be reported simply as “chrysotile detected” or “amphibole asbestos detected”. Further clarification of the amphibole asbestos type can be achieved using EDS elemental analysis with an electron microscope.

[NOTE—The limits are based on limits of detection.▲ (USP 1-Dec-2023)]

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# ⟨905⟩ UNIFORMITY OF DOSAGE UNITS

***Change to read:***

This general chapter is harmonized with the corresponding texts of the *European Pharmacopoeia* and the *Japanese Pharmacopoeia*. Portions of the general chapter text that are national *USP* text, and are not part of the harmonized text, are marked with symbols (•,) to specify this fact.

[NOTE—In this chapter, *unit* and *dosage unit* are synonymous.•,]

To ensure the consistency of dosage units, each unit in a batch should have a drug substance content within a narrow range around the label claim. Dosage units are defined as dosage forms containing a single dose or a part of a dose of drug substance in each unit. The uniformity of dosage units specification is not intended to apply to ▲solutions,▲ (USP 1-Aug-2023) suspensions, emulsions, or gels in unit-dose containers intended for ▲local action following▲ (USP 1-Aug-2023) external, cutaneous administration.

The term "uniformity of dosage unit" is defined as the degree of uniformity in the amount of the drug substance among dosage units. Therefore, the requirements of this chapter apply to each drug substance being comprised in dosage units containing one or more drug substances, unless otherwise specified elsewhere in this Pharmacopeia.

The uniformity of dosage units can be demonstrated by either of two methods, *Content Uniformity* or •*Weight*•,*Variation* (see *Table 1*). The test for *Content Uniformity* of preparations presented in dosage units is based on the assay of the individual content of drug substance(s) in a number of dosage units to determine whether the individual content is within the limits set. The *Content Uniformity* method may be applied in all cases.

The test for •*Weight*•,*Variation* is applicable for the following dosage forms:

| | |
|---|---|
| (W1) | Solutions enclosed in unit-dose containers and into soft capsules; |
| (W2) | Solids (including powders, granules, and sterile solids) that are packaged in single-unit containers and contain no active or inactive added substances; |
| (W3) | Solids (including sterile solids) that are packaged in single-unit containers, with or without active or inactive added substances, that have been prepared from true solutions and freeze-dried in the final containers and are labeled to indicate this method of preparation; and |
| (W4) | Hard capsules, uncoated tablets, or film-coated tablets, containing 25 mg or more of a drug substance comprising 25% or more, by weight, of the dosage unit or, in the case of hard capsules, the capsule contents, except that uniformity of other drug substances present in lesser proportions is demonstrated by meeting the requirements for *Content Uniformity*. |

The test for *Content Uniformity* is required for all dosage forms not meeting the above conditions for the •*Weight*•,*Variation* test.[1]

**Table 1. Application of Content Uniformity (CU) and Weight Variation (WV) Tests for Dosage Forms**

| Dosage Form | Type | Subtype | Dose & Ratio of Drug Substance | |
|---|---|---|---|---|
| | | | ≥25 mg and ≥25% | <25 mg or <25% |
| Tablets | Uncoated | — | WV | CU |
| | Coated | Film | WV | CU |
| | | Others | CU | CU |
| Capsules | Hard | — | WV | CU |
| | Soft | Suspension, emulsion, or gel | CU | CU |
| | | Solutions | WV | WV |
| Solids in single-unit containers | Single component | — | WV | WV |
| | Multiple components | Solution freeze-dried in final container | WV | WV |
| | | Others | CU | CU |
| Solutions in unit-dose containers •and into soft capsules• | — | — | WV | WV |
| Others▲[a] ▲ (USP 1-Aug-2023) | — | — | CU | CU |

[1] •European Pharmacopoeia and Japanese Pharmacopoeia text not accepted by the United States Pharmacopeia: Alternatively, products listed in item (4) above that do not meet the 25 mg/25% threshold limit may be tested for uniformity of dosage units by Mass Variation instead of the Content Uniformity test if the concentration relative standard deviation (RSD) of the drug substance in the final dosage units is not more than 2%, based on process validation data and development data, and if there has been regulatory approval of such a change. The concentration RSD is the RSD of the concentration per dosage unit (w/w or w/v), where concentration per dosage unit equals the assay result per dosage unit divided by the individual dosage unit weight. See the RSD formula in *Table 2*.•,

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[a] Dosage forms not addressed by the other categories in this table including but not limited to suppositories, transdermal systems (patches), and semisolid preparations applied cutaneously and intended for systemic distribution of the drug substance.

## CONTENT UNIFORMITY

Select not fewer than 30 units, and proceed as follows for the dosage form designated.

Where different procedures are used for assay of the preparation and for the *Content Uniformity* test, it may be necessary to establish a correction factor to be applied to the results of the latter.

### Solid Dosage Forms

Assay 10 units individually using an appropriate analytical method. Calculate the acceptance value (see *Table 2*).

### Liquid or Semi-Solid Dosage Forms

Assay 10 units individually using an appropriate analytical method. Carry out the assay on the amount of well-mixed material that is removed from an individual container in conditions of normal use, and express the results as delivered dose. Calculate the acceptance value (see *Table 2*).

### Calculation of Acceptance Value

Calculate the acceptance value by the formula:

$$|M - \overline{X}| + ks$$

in which the terms are as defined in *Table 2*.

**Table 2**

| Variable | Definition | Conditions | Value |
|---|---|---|---|
| $\overline{X}$ | Mean of individual contents ($\chi_1$, $\chi_2$, …, $\chi_n$), expressed as a percentage of the label claim | — | — |
| $\chi_1$, $\chi_2$, …, $\chi_n$ | Individual contents of the units tested, expressed as a percentage of the label claim | — | — |
| n | Sample size (number of units in a sample) | — | — |
| k | Acceptability constant | If n = 10, then k = | 2.4 |
| | | If n = 30, then k = | 2.0 |
| s | Sample standard deviation | — | $\left[\frac{\sum_{i=1}^{n}(\chi_i - \overline{X})^2}{n-1}\right]^{\frac{1}{2}}$ |
| RSD | Relative standard deviation (the sample standard deviation expressed as a percentage of the mean) | — | $100s/\overline{X}$ |
| M (case 1) to be applied when T ≤101.5 | Reference value | If 98.5% ≤$\overline{X}$ ≤101.5%, then | M = $\overline{X}$ (AV = ks) |
| | | If $\overline{X}$ <98.5%, then | M = 98.5% (AV = 98.5 – $\overline{X}$ + ks) |
| | | If $\overline{X}$ >101.5%, then | M = 101.5% (AV = $\overline{X}$ – 101.5 + ks) |
| M (case 2) to be applied when T >101.5 | Reference value | If 98.5 ≤$\overline{X}$ ≤T, then | M = $\overline{X}$ (AV = ks) |
| | | If $\overline{X}$ <98.5%, then | M = 98.5% (AV = 98.5 – $\overline{X}$ + ks) |
| | | If $\overline{X}$ >T, then | M = T% (AV = $\overline{X}$ – T + ks) |

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**Table 2** *(continued)*

| Variable | Definition | Conditions | Value |
|---|---|---|---|
| Acceptance value (AV) | — | — | General formula: $\lvert M - \bar{X} \rvert + ks$ (Calculations are specified above for the different cases.) |
| L1 | Maximum allowed acceptance value | — | L1 = 15.0 unless otherwise specified |
| L2 | Maximum allowed range for deviation of each dosage unit tested from the calculated value of M | On the low side, no dosage unit result can be less than [1–(0.01)(L2)]M, while on the high side, no dosage unit result can be greater than [1 + (0.01)(L2)]M. (This is based on an L2 value of 25.0.) | L2 = 25.0 unless otherwise specified |
| T | Target content per dosage unit at the time of manufacture, expressed as a percentage of the label claim. Unless otherwise stated, T is 100.0%, or T is the manufacturer's approved target content per dosage unit. | — | — |

## ⋆WEIGHT⋆ VARIATION

Carry out an assay for the drug substance(s) on a representative sample of the batch using an appropriate analytical method. This value is result A, expressed as percentage of label claim (see *Calculation of Acceptance Value*). Assume that the concentration (weight of drug substance per weight of dosage unit) is uniform. Select not fewer than 30 dosage units, and proceed as follows for the dosage form designated.

### Uncoated or Film-Coated Tablets

Accurately weigh 10 tablets individually. Calculate the content, expressed as percentage of label claim, of each tablet from the ⋆weight⋆ of the individual tablet and the result of the *Assay*. Calculate the acceptance value.

### Hard Capsules

Accurately weigh 10 capsules individually, taking care to preserve the identity of each capsule. Remove the contents of each capsule by a suitable means. Accurately weigh the emptied shells individually, and calculate for each capsule the net ⋆weight⋆ of its contents by subtracting the ⋆weight⋆ of the shell from the respective gross ⋆weight⋆. Calculate the drug substance content of each capsule from the ⋆net weight⋆ of the individual capsule ⋆content⋆ and the result of the *Assay*. Calculate the acceptance value.

### Soft Capsules

Accurately weigh 10 intact capsules individually to obtain their gross ⋆weights⋆, taking care to preserve the identity of each capsule. Then cut open the capsules by means of a suitable clean, dry cutting instrument such as scissors or a sharp open blade, and remove the contents by washing with a suitable solvent. Allow the occluded solvent to evaporate from the shells at room temperature over a period of about 30 minutes, taking precautions to avoid uptake or loss of moisture. Weigh the individual shells, and calculate the net contents. Calculate the drug substance content in each capsule from the ⋆weight⋆ of product removed from the individual capsules and the result of the *Assay*. Calculate the acceptance value.

### Solid Dosage Forms Other Than Tablets and Capsules

Proceed as directed for *Hard Capsules*, treating each unit as described therein. Calculate the acceptance value.

### Liquid Dosage Forms

Accurately weigh the amount of liquid that is removed from each of 10 individual containers in conditions of normal use. If necessary, compute the equivalent volume after determining the density. Calculate the drug substance content in each container from the mass of product removed from the individual containers and the result of the *Assay*. Calculate the acceptance value.

### Calculation of Acceptance Value

Calculate the acceptance value as shown in *Content Uniformity*, except that the individual contents of the units are replaced with the individual estimated contents defined below.

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$\chi_1, \chi_2, \ldots, \chi_n$ = individual estimated contents of the units tested, where $\chi_i = w_i \times A/\overline{W}$
$w_1, w_2, \ldots, w_n$ = individual ⋆weights⋆ of the units tested
$A$ = content of drug substance (% of label claim) obtained using an appropriate analytical method
$\overline{W}$ = mean of individual ⋆weights⋆ $(w_1, w_2, \ldots, w_n)$

## CRITERIA

Apply the following criteria, unless otherwise specified.

### Solid, Semi-Solid, and Liquid Dosage Forms

The requirements for dosage uniformity are met if the acceptance value of the first 10 dosage units is less than or equal to L1%. If the acceptance value is > L1%, test the next 20 units, and calculate the acceptance value. The requirements are met if the final acceptance value of the 30 dosage units is ≤ L1%, and no individual content of ⋆any⋆ dosage unit is less than [1 – (0.01)(L2)]M nor more than [1 + (0.01)(L2)]M ⋆as specified⋆ in the *Calculation of Acceptance Value* under *Content Uniformity* or under ⋆*Weight⋆ *Variation*. Unless otherwise specified, L1 is 15.0 and L2 is 25.0.

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# ⟨911⟩ VISCOSITY—CAPILLARY METHODS

The following procedures are used to determine the viscosity of a Newtonian fluid, i.e., a fluid having a viscosity that is independent of the rate of shear. [NOTE—For additional information, see *Rheometry* ⟨1911⟩.]

- **METHOD I. SUSPENDED-LEVEL (OR UBBELOHDE-TYPE) CAPILLARY VISCOMETER**

**Apparatus:** The determination may be carried out with a suspended-level capillary viscometer (*Figure 1*).

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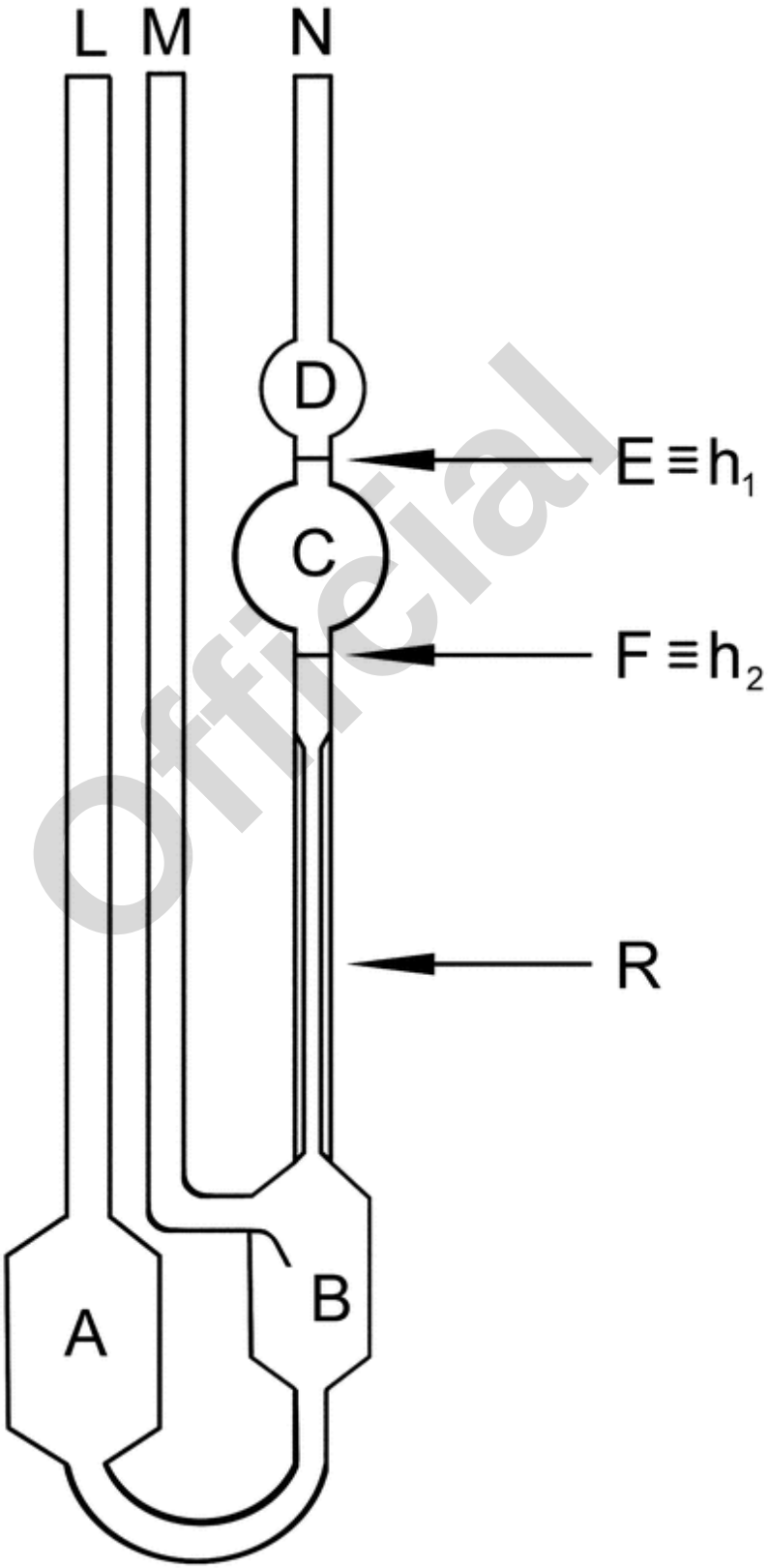


Figure 1. Suspended-level (or Ubbelohde-type) capillary viscometer.

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Other viscometers may be used provided that the accuracy and precision is NLT that obtained with the viscometers described in this chapter.

**Procedure:** Fill the viscometer through tube (L) with a sufficient quantity of the sample liquid that is appropriate for the viscometer being used or by following the manufacturer's instructions. Carry out the experiment with the tube in a vertical position. Fill bulb (A) with the liquid, and also ensure that the level of liquid in bulb (B) is below the exit to the ventilation tube (M). Immerse the viscometer in a water or oil bath stabilized at the temperature specified in the individual monograph, and control the temperature to ±0.1°, unless otherwise specified in the individual monograph. Maintain the viscometer in a vertical position for a time period of NLT 30 min to allow the sample temperature to reach equilibrium. Close tube (M), and raise the level of the liquid in tube (N) to a level about 8 mm above mark ($E \equiv h_1$). Keep the liquid at this level by closing tube (N) and opening tube (M). Open tube (N), and measure the time required for the level of the liquid to drop from mark ($E \equiv h_1$) to mark ($F \equiv h_2$), using an appropriate accurate timing device. [NOTE—The minimum flow time should be 200 s.]

**Calibration:** Calibrate each viscometer at the test temperature by using fluids of known viscosities of appropriate viscosity standards to determine the viscometer constant, $k$. The viscosity values of the calibration standards should bracket the expected viscosity value of the sample liquid.

Calculate the viscometer constant, $k$, in $mm^2/s^2$:

$$k = \eta/(\rho \times t)$$

$\eta$ = known viscosity of the liquid (mPa · s)
$\rho$ = density of the liquid (g/mL)
$t$ = flow time for the liquid to pass from the upper mark to the lower mark (s)

**Calculation of kinematic and Newtonian viscosities of sample fluid:** A capillary viscometer is chosen so that the flow time, $t$, is NLT 200 s, and the kinematic energy correction is typically less than 1%. If the viscosity constant, $k$, is known, use the following equation to calculate the kinematic viscosity, $v$, in $mm^2/s$, from the flow time, $t$, in s.

$$v = k \times t$$

If the density of the fluid is known at the temperature of the viscosity measurement, then the Newtonian viscosity, $\eta$, in mPa · s, is calculated:

$$\eta = v \times \rho$$

$\rho$ = density of the fluid (g/mL)

The flow time of the fluid under examination is the mean of NLT three consecutive determinations. The result is valid if the percentage of the relative standard deviation (%RSD) for the three readings is NMT 2.0%.

- **METHOD II. SIMPLE U-TUBE (OR OSTWALD-TYPE) CAPILLARY VISCOMETER**

**Apparatus:** The determination may be carried out with a simple U-tube capillary viscometer (*Figure 2*).

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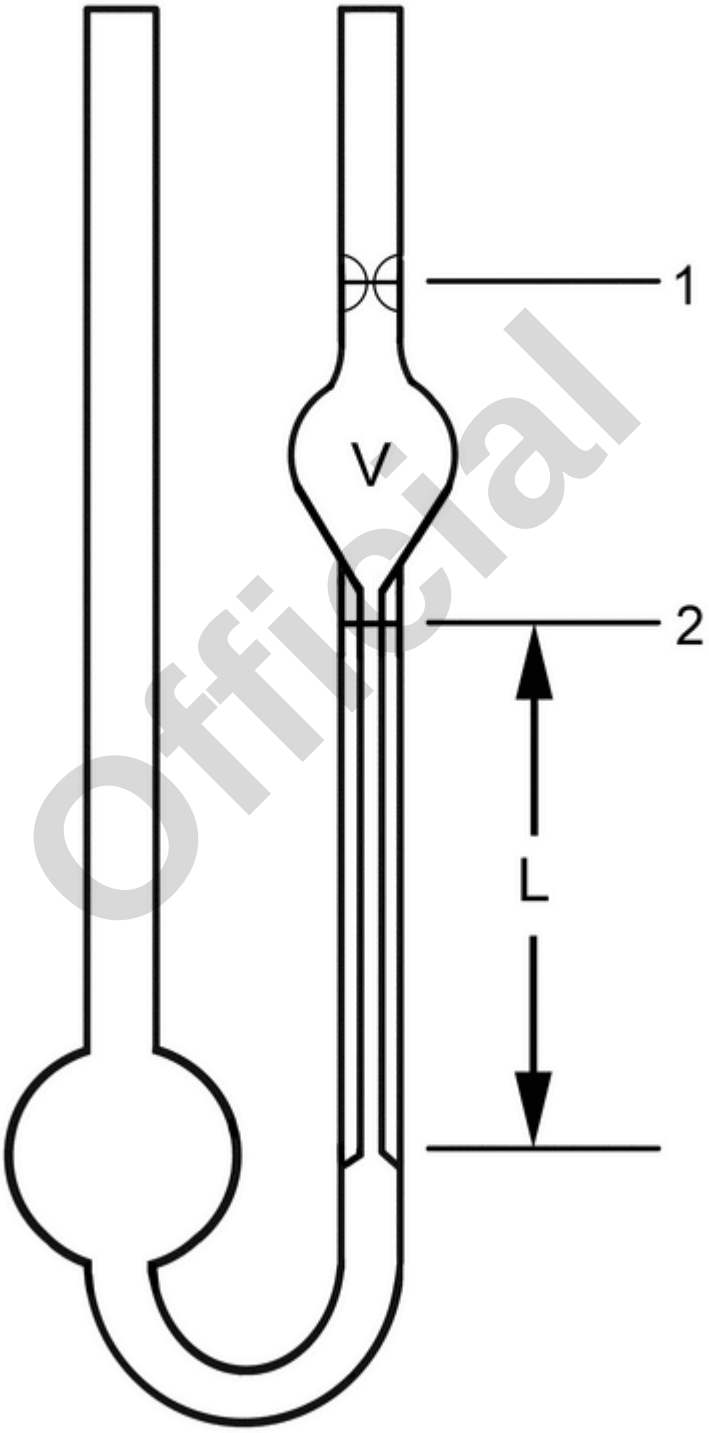


Figure 2. Simple U-tube (or Ostwald-type) capillary viscometer.

Variables and line numbers in *Figure 2* are defined as:

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| | |
|---|---|
| Line 1 | = upper mark in the tube |
| $V$ | = given volume of fluid ($m^3$) |
| Line 2 | = lower mark in the tube |
| $L$ | = capillary tube length (m) |

Other viscometers, such as modified Ostwald-type capillary viscometers,[1] may be used provided that the accuracy and precision is NLT that obtained with the viscometers described in this chapter.

**Procedure:** Fill the tube with an amount of the sample that is appropriate for the viscometer being used or by following the manufacturer's instructions. The volume of fluid used should be such that the lower bulb is not entirely emptied when the fluid is drawn up through the capillary tube to the uppermost graduation mark. Carry out the experiment with the tube in a vertical position. Immerse the viscometer in a water or oil bath stabilized at the temperature specified in the individual monograph, and control the temperature to ±0.1°, unless otherwise specified in the individual monograph. Maintain the viscometer in a vertical position for a time period of NLT 30 min to allow the sample temperature to reach equilibrium. Using suction, draw the fluid up through the capillary tube until the meniscus is at the level of the uppermost graduation. With both the filling and capillary tubes open to atmospheric pressure, record the time, in s, required for the liquid to flow from the upper mark to the lower mark in the capillary tube. [NOTE—The minimum flow time should be 200 s.]

**Calibration** and **Calculation of kinematic and Newtonian viscosities of sample fluid:** Proceed as directed in *Method I*. For certain simple U-tube capillary viscometers, determine the viscometer constant at the same temperature as the sample liquid under test.

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[1] For example, the Cannon-Fenske capillary viscometer is one of the simple U-tube capillary viscometers and is also called a modified Ostwald-type capillary viscometer.

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# ⟨912⟩ VISCOSITY—ROTATIONAL METHODS

The principle of the method is to measure the force (torque) acting on a rotor when it rotates at a constant angular velocity or rotational speed in a liquid. Rotational rheometers/viscometers are used for measuring the viscosity of fluids, both Newtonian and non-Newtonian. The following procedures are used to determine the viscosity of Newtonian fluids or the apparent viscosity of non-Newtonian fluids. The calculated viscosity of Newtonian fluids should be the same (within experimental error), regardless of the rate of shear (or rotational speed). Given the dependence of viscosity on temperature, the temperature of the substance being measured should be controlled to within ±0.1°, unless otherwise specified in the individual monograph. [NOTE—For additional information, see *Rheometry* ⟨1911⟩.]

• **METHOD I. SPINDLE VISCOMETERS**

**Apparatus:** In the spindle viscometer, the apparent viscosity is determined by rotating a cylinder- or disk (or disc)-shaped spindle, as shown in *Figure 1* and *Figure 2*, respectively, immersed in a large volume of liquid.

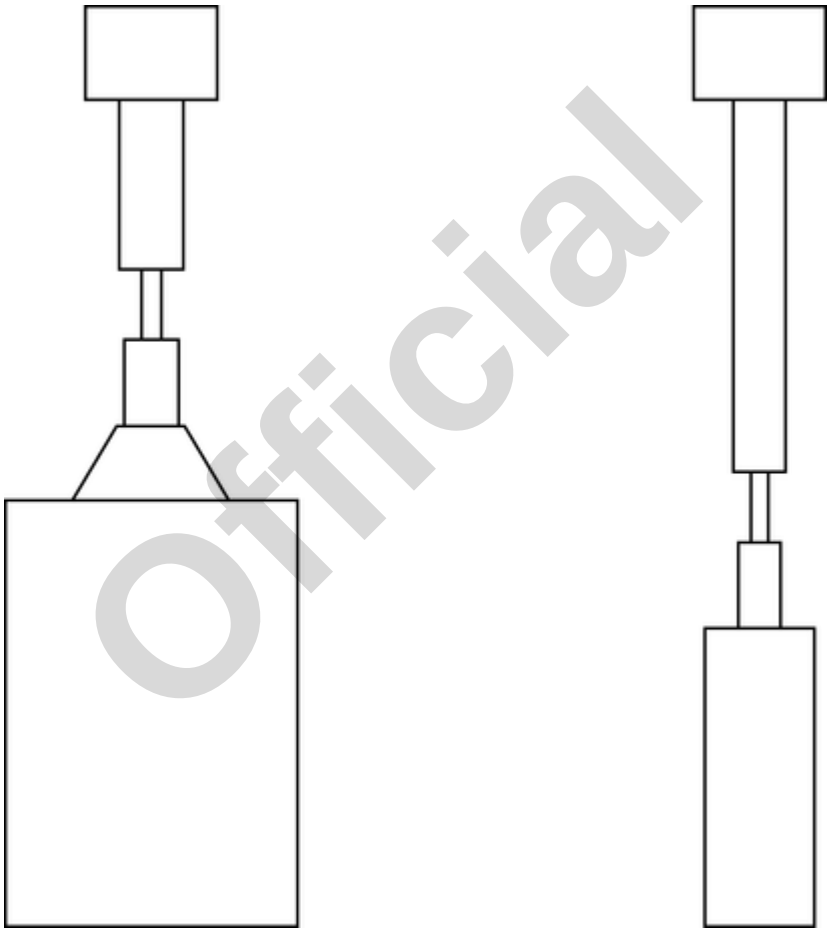

Figure 1. Cylinder-shaped spindles.

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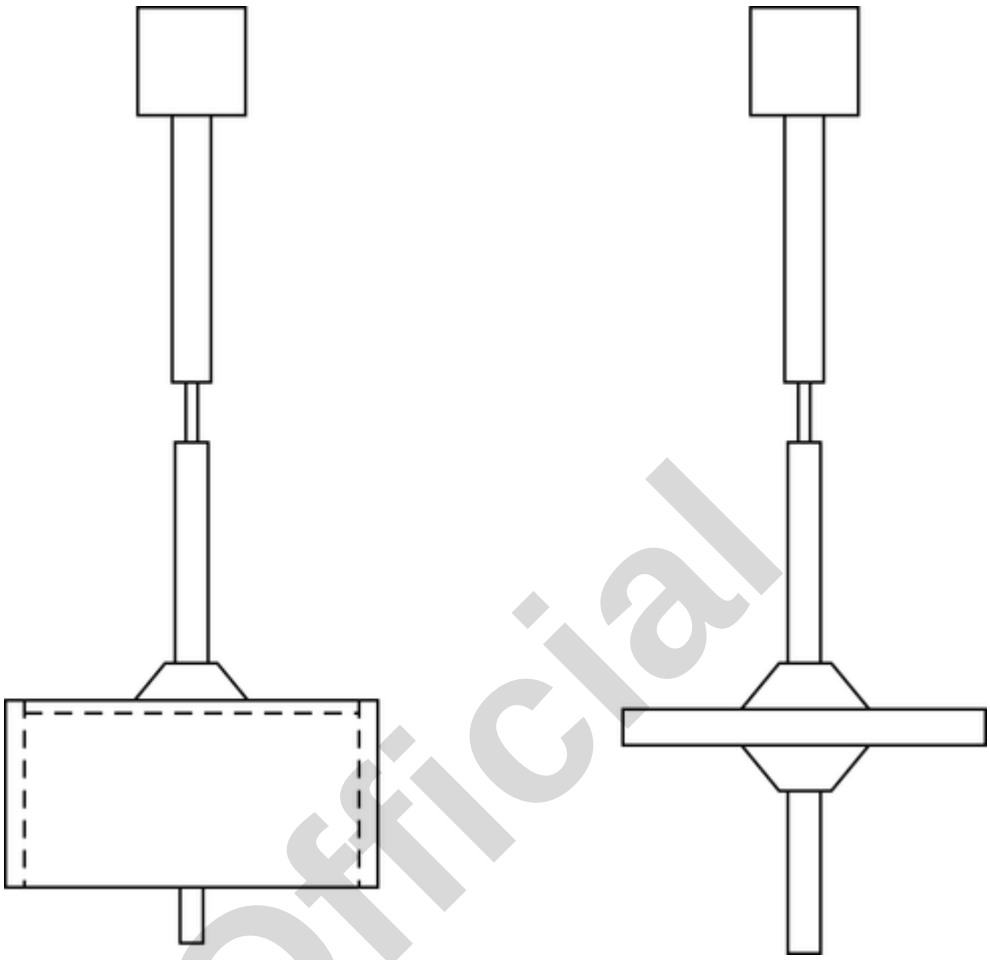

Figure 2. Disk-shaped spindles.

An absolute viscosity cannot be calculated due to the large gap between the spindle and the container wall, or the geometry of the spindle. The torque to maintain a given angular velocity does give a measure of the liquid resistance to flow but is often described as an apparent viscosity.

Other spindle type viscometers may be used provided that the accuracy and precision is NLT that obtained with the viscometers described in this chapter.

**Procedure:** When viscosity measurement is performed in a beaker or cup, as the shear rate is unknown, in order to enable reproducibility among labs that measure viscosity using different instrumentation, these parameters must be reported along with the measured viscosity:

1. Size and geometry of spindle
2. Angular velocity or rotational speed of the spindle
3. Temperature of the test substance

The spindle should be immersed to the recommended depth maintaining at least 1 cm clearance from the bottom and side of the container.

The preparation of the test specimen, including its temperature equilibration, is specified in each individual monograph. Follow the instrument manufacturer's recommendations regarding sample loading, spindle selection, and viscometer operation.

**Calibration check:** Check the calibration of a particular viscometer configuration at the test temperature using one or more fluids of known viscosities (Newtonian viscosity standards). The viscosity values of the calibration standards should bracket the expected viscosity value of the sample liquid. [NOTE—To help verify the linearity of the apparatus, it is suggested to perform measurements of a Newtonian viscosity standard at multiple rotational speeds at the test temperature.]

A viscometer is deemed to be calibrated if the measured apparent viscosities are within ±5% of the stated values.

Generally, calibration, operation, and cleaning of the viscometer should be performed according to the recommendations of the instrument manufacturer.

- **METHOD II. CONCENTRIC CYLINDER RHEOMETERS**

**Apparatus:** In the concentric cylinder rheometer, the apparent viscosity is determined by placing the liquid in the gap between the inner cylinder and the outer cylinder. Both controlled-stress and controlled-rate rotational rheometers are available commercially in configurations with absolute geometries (e.g., very small annular gaps between concentric cylinders) that allow calculation of apparent viscosities for non-Newtonian fluids. Controlled shear stress rheometers

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measure the shear rates resulting from the application of a given force or torque (the stress). Controlled shear rate rheometers measure the shear stress (from the torque on the rotor axis) resulting from a given shear rate (or rotational speed). Concentric cylinder rotational rheometers are sometimes referred to as cup-and-bob rheometers. These rheometers involve an additional design consideration depending on whether the outer cylinder (the cup) or the inner cylinder (the bob) rotates. Rotating-cup rheometers are called Couette systems, while rotating-bob rheometers are called Searle systems, as shown in *Figure 3* and *Figure 4*, respectively.

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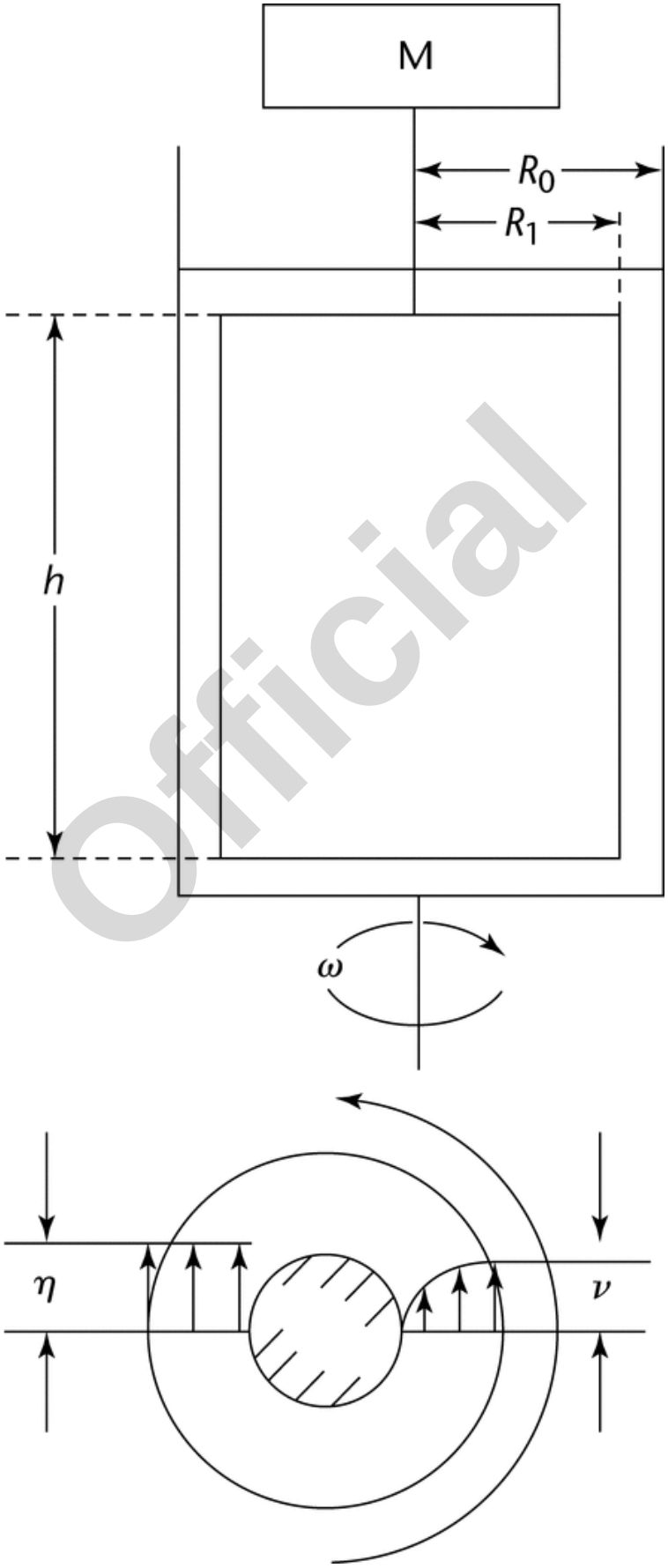


Figure 3. Couette concentric cylinder system for rotational rheometry.

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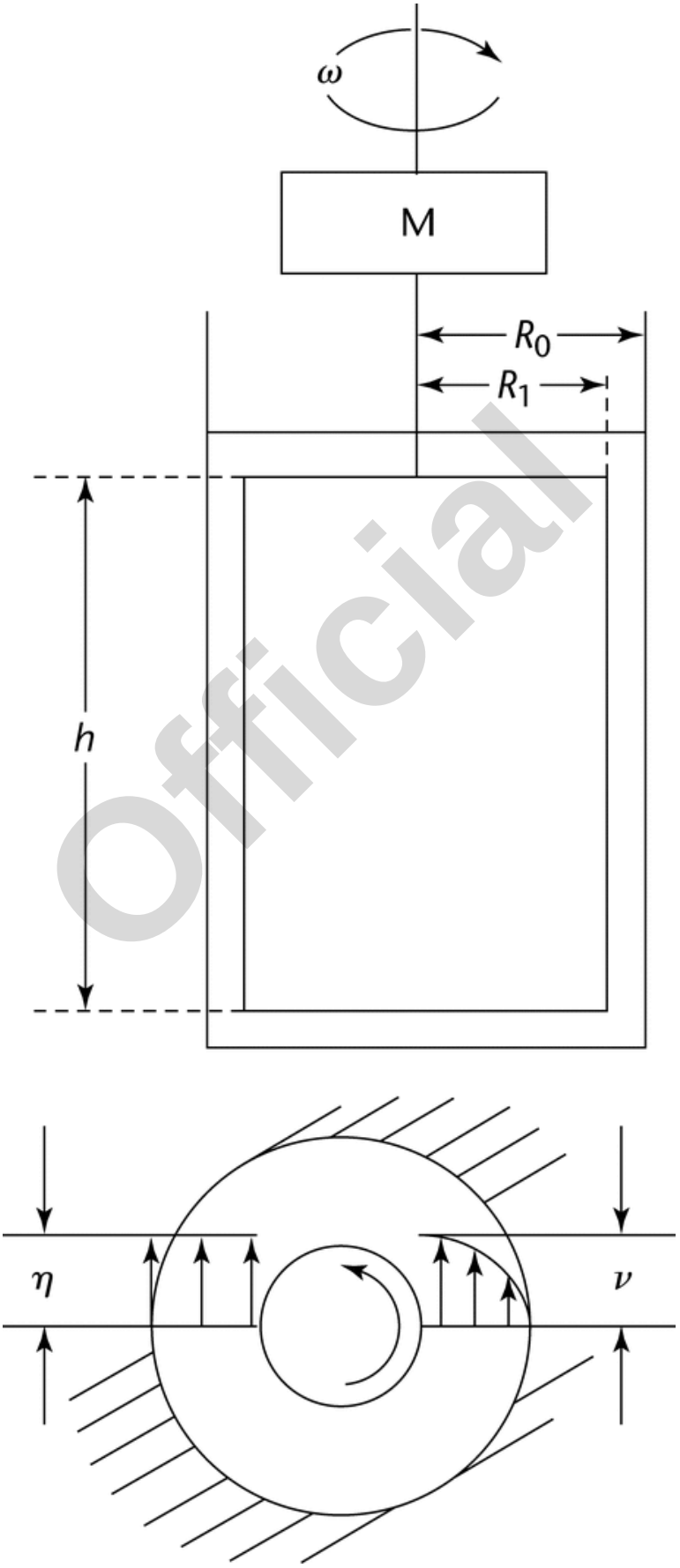


Figure 4. Searle concentric cylinder system for rotational rheometry.

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Variables in *Figure 3* and *Figure 4* are defined as:

$M$ = torque acting on the cylinder surface ($N \cdot m$)
$R_0$ = radius of the outer cylinder (m)
$R_1$ = radius of the inner cylinder (m)
$h$ = height of immersion of the inner cylinder in the liquid medium (m)
$\omega$ = angular velocity (radians/s)
$\eta$ = viscosity ($Pa \cdot s$)
$v$ = velocity (m/s)

**Procedure:** Place a sufficient quantity of test fluid in the rheometer, and allow the sample to reach thermal equilibrium, as indicated in the individual monograph. Operate the rheometer following the procedure recommended by the instrument manufacturer. For non-Newtonian systems, the monograph indicates the type of rheometer that should be used and the shear rate(s) at which the measurements should be made. Determine apparent viscosities by changing the shear rate (or shear stress, if using a controlled shear stress rheometer) over a range appropriate to the use of the material under test. From a series of such viscosity measurements, the relationship between the shear rate and the shear stress of a non-Newtonian liquid can be obtained.

- **Method III. Cone-and-Plate Rheometers**

**Apparatus:** In the cone-and-plate rheometer, the liquid is introduced into the fixed gap between a flat disk or plate and a cone forming a defined angle. The angle of the cone ensures a constant shear rate due to the increase in both the gap and the linear speed as the distance increases from the origin. Viscosity measurement can be performed by rotating the cone or the plate, as shown in *Figure 5* and *Figure 6*, respectively. [Note—Because the volume of sample is small, even a small absolute loss of solvents can cause a large percentage change in viscosity. Such a loss is particularly relevant for volatile solvents but could be significant even for nonvolatile solvents such as water.]

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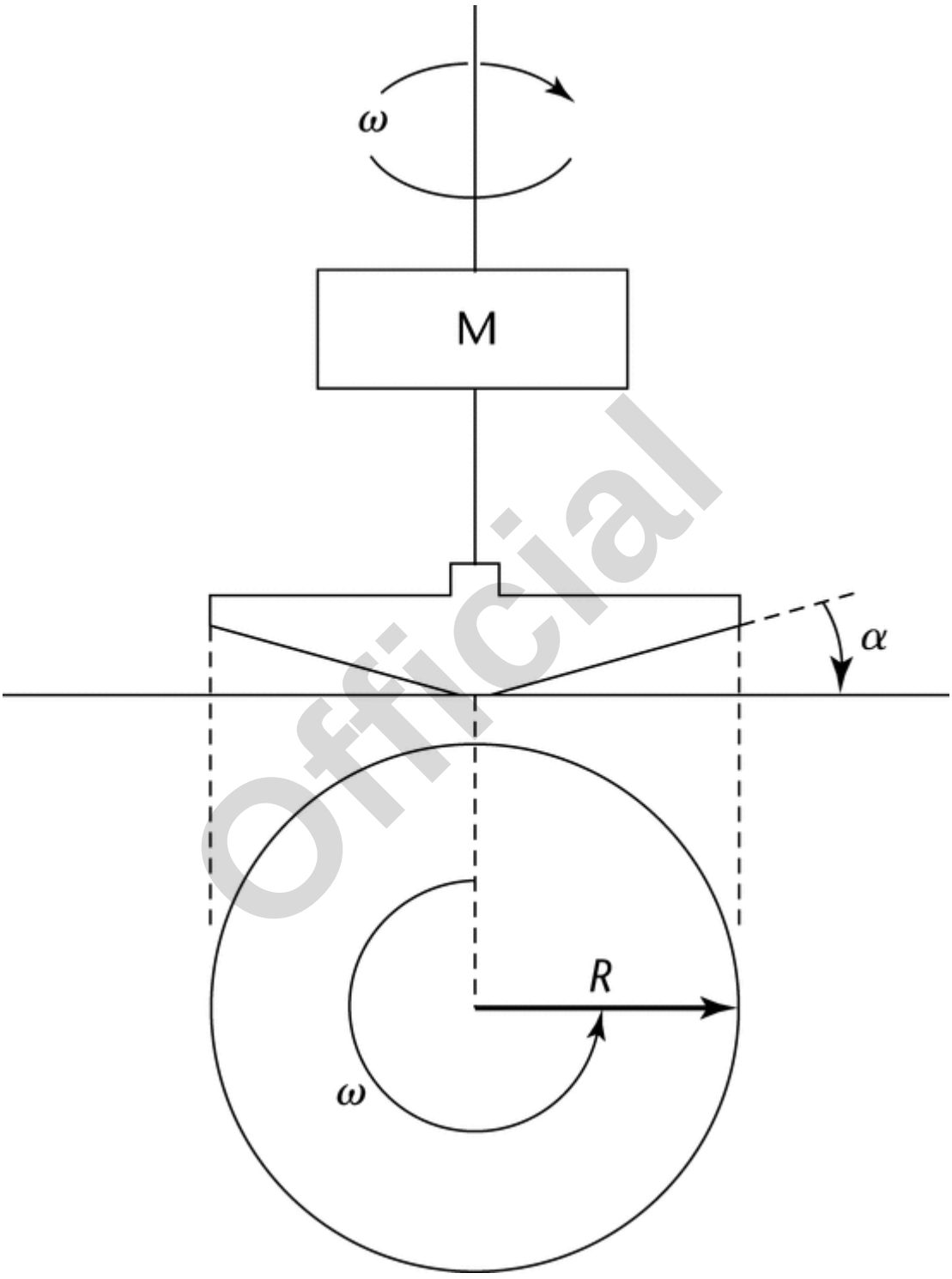


Figure 5. Cone-and-plate rotational rheometer with rotating cone.

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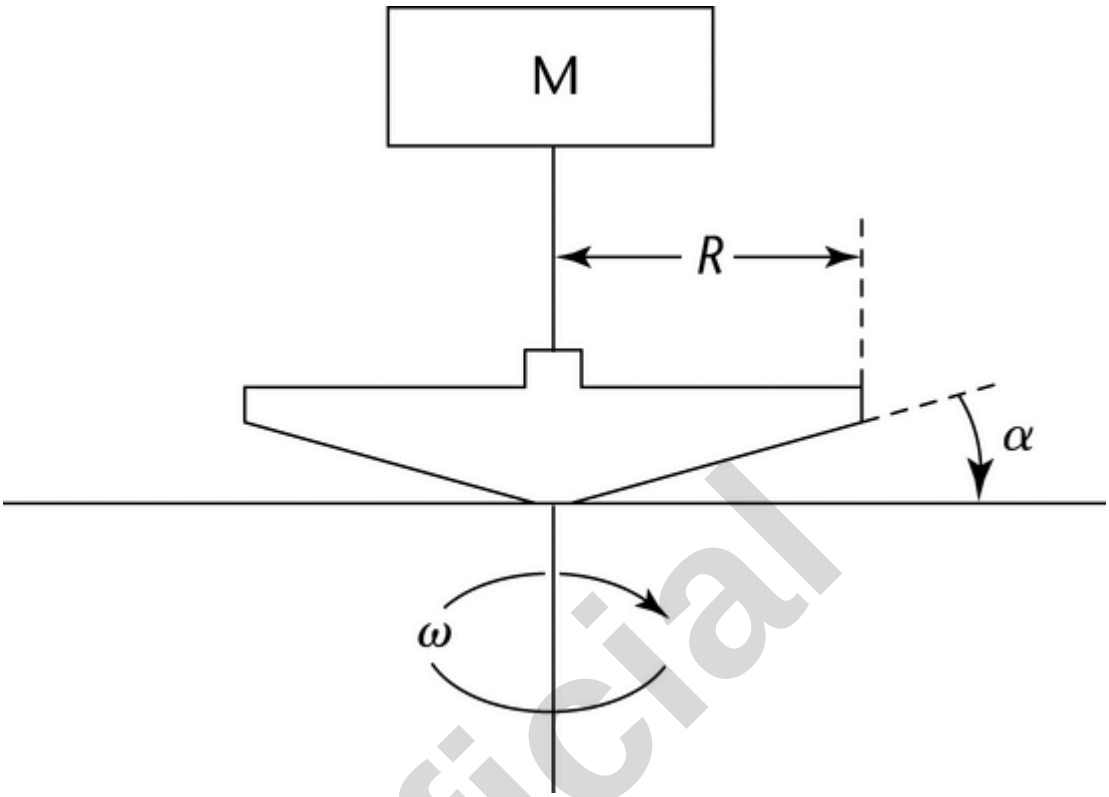


Figure 6. Cone-and-plate rotational rheometer with rotating plate.

Variables in *Figure 5* and *Figure 6* are defined as:

$\omega$ = angular velocity (radians/s)
$M$ = torque acting on the flat plate or cone surface (N · m)
$\alpha$ = angle between the flat plate and cone (radians)
$R$ = radius of the cone (m)

**Procedure:** Proceed as directed for *Method II. Concentric Cylinder Rheometers*.

- **Method IV. Parallel Plate (or Parallel Disk) Rheometers**

**Apparatus:** Parallel plate rheometers are similar to cone-and-plate rheometers except that the sample to be measured is introduced into the gap between a flat plate or disk (or disc) and another parallel flat plate or disk. Measurements are made typically with the lower plate or disk remaining stationary as the upper plate or disk is rotated at a constant angular velocity, $\omega$ (*Figure 7*).

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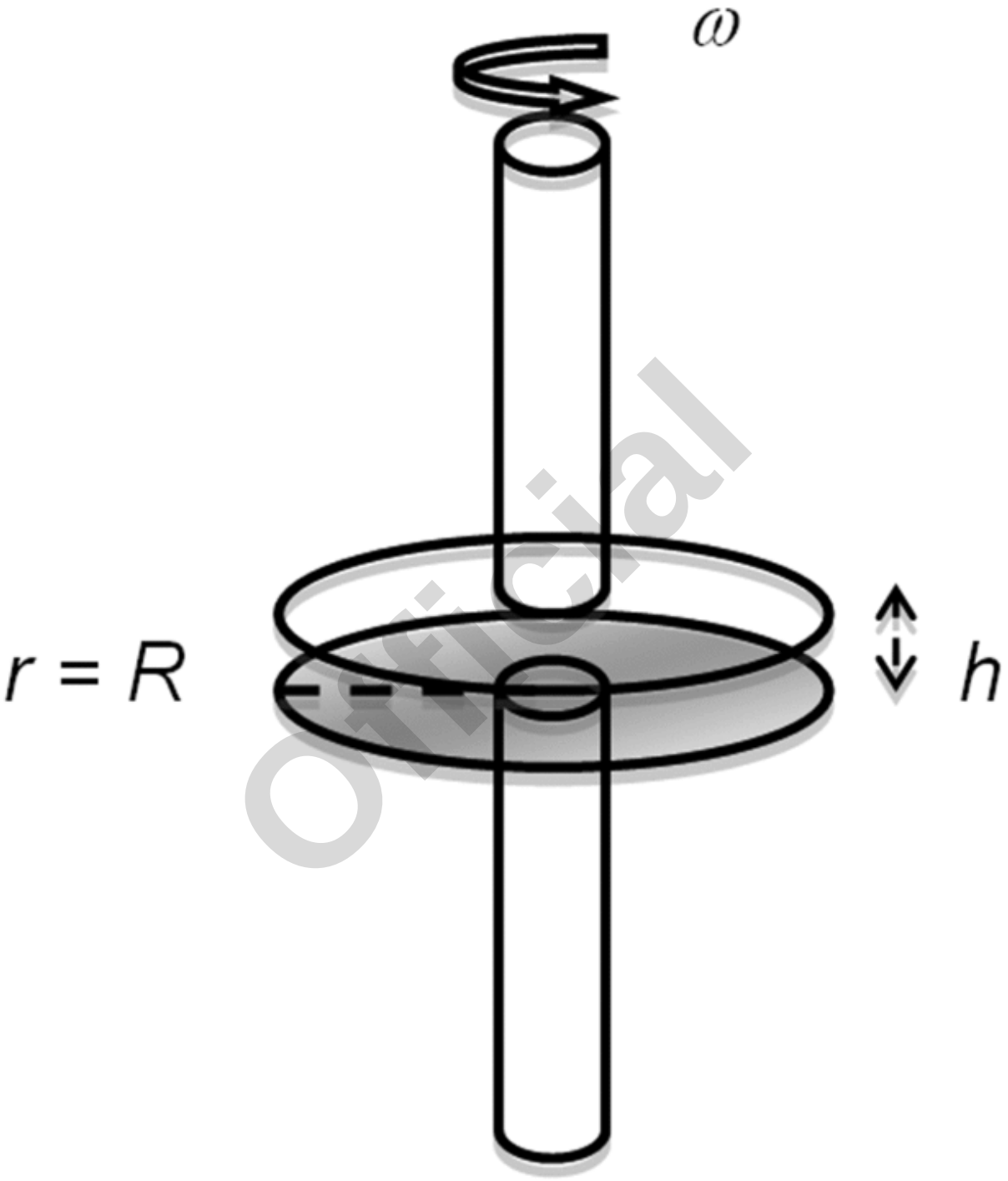


Figure 7. Parallel plate rotational rheometer.

Variables in *Figure 7* are defined as:

ω = angular velocity (radians/s)

*R* = radius of the plate (m)

*h* = distance between the two parallel plates (m)

In contrast to the cone-and-plate rheometer, the shear rate between parallel plates increases with distance from the origin of the axis of rotation due to the increasing linear speeds for a given angular velocity with a constant gap. Thus, an average shear rate is obtained. Nonetheless, some advantages of the parallel plate rheometer include ease of sample loading (especially for very viscous liquids and soft semisolids), and suitability for suspensions of particulates. For

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suspensions, the gap should be set high enough to avoid grinding particles between the plates. Parallel plates have a user-definable gap (within practical limits) and therefore, in the absence of large particles, may be used at narrower gaps. As with cone-and-plate rheometers, evaporative loss of solvent can markedly affect the viscosity of the sample, so adequate precautions need to be taken to minimize solvent loss.

**Procedure:** Proceed as directed for *Method II. Concentric Cylinder Rheometers*.

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# ⟨913⟩ VISCOSITY—ROLLING BALL METHOD

The following procedure is used to determine the viscosity of a Newtonian fluid, i.e., a fluid having a viscosity that is independent of the shearing stress or rate of shear.

[NOTE—For additional information, see *Rheometry* ⟨1911⟩.]

## APPARATUS

See *Figure 1*.

The basic design of a rolling ball viscometer consists of a tube (or capillary) that contains the sample liquid under test and a ball chosen so that it will require a minimum rolling time of 20 s at the measuring angle in the sample liquid.

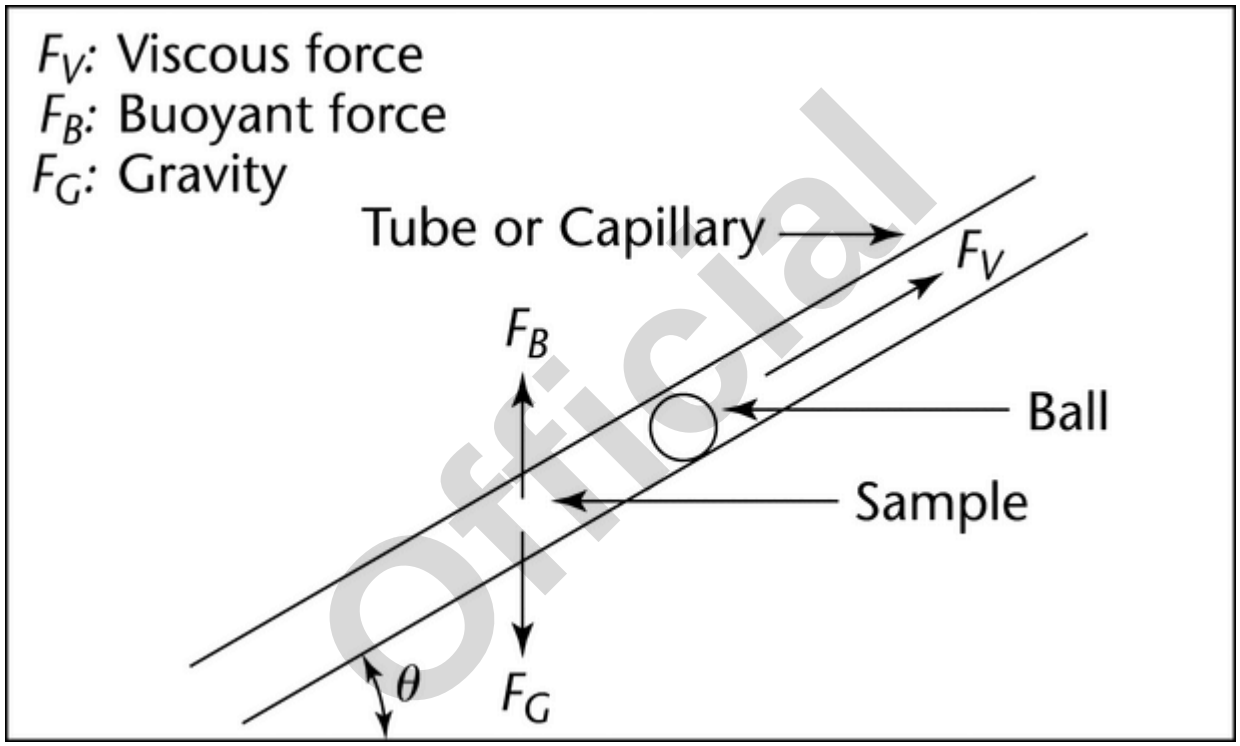


Figure 1. Basic design for rolling ball viscometer.

## MEASURING PRINCIPLE

The rolling ball measurement is based on Stokes's Law, as influenced by the angle of inclination of the tube (or capillary). Calculate the Newtonian viscosity, $\eta$, in mPa · s:

$$\eta = [(\rho_1 - \rho_2) \times g \times r^2 \times \sin\theta]/v_\infty$$

$\rho_1$ = density of the ball used (g/mL)
$\rho_2$ = density of the sample liquid (g/mL)
$g$ = gravitational constant (mm/s$^2$)
$r$ = radius of the ball (mm)
$\theta$ = angle of inclination of the tube (or capillary)
$v_\infty$ = terminal velocity of the ball (mm/s)

Determine the viscosity of a liquid by observing the rolling time of a solid sphere (ball) under the influence of gravity in an inclined cylindrical tube filled with the sample liquid. Measure the time taken by the ball to travel the fixed distance between two ring marks or measuring sensors. For each single measured rolling time, the resulting viscosity can be expressed as dynamic viscosity (mPa · s) as well as kinematic viscosity (mm$^2$/s) for a sample of known density.

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## PROCEDURE

Select a measuring system [tube (or capillary) and ball combination] within the anticipated range of viscosity of the sample liquid. As necessary, heat the clean and dry tube and ball of the viscometer to the temperature specified in the individual monograph, and control the temperature to ±0.1°, unless otherwise specified in the individual monograph. Select a measuring angle to obtain a minimum rolling time of 20 s. Fill the tube with the sample liquid, being careful to avoid bubble formation. Close the tube, and insert it in the instrument. Allow to equilibrate at the specified temperature for NLT 15 min for a rolling ball viscometer with a tube of large diameter. For a micro rolling ball viscometer, follow the instrument manufacturer's instructions regarding temperature equilibration. Release the ball, and record the time required for the ball to roll from the upper to the lower ring mark (or measuring sensor). Repeat the test run at least four times.

The rolling time in the fluid under examination is the mean of NLT four consecutive determinations. The result is valid if the percent relative standard deviation (%RSD) for the four readings is NMT 2.0%.

## CALCULATION AND CALIBRATION

Calculate the Newtonian viscosity, $\eta$, in mPa · s:

$$\eta = k \times (\rho_1 - \rho_2) \times t$$

$k$ = calibration constant of the instrument ($mm^2/s^2$) at a specified measuring angle and temperature
$\rho_1$ = density of the ball used (g/mL)
$\rho_2$ = density of the sample liquid (g/mL)
$t$ = rolling time of the ball (s)

Calibrate each tube (or capillary) and ball combination at the test temperature and test angle using fluids of known viscosities and densities (viscosity standards) to determine the measuring system constant, $k$.

[NOTE—Some automated viscometers use a polynomial function to determine the calibration for different angles and temperatures.]

The viscosity values of the calibration standards should bracket the expected viscosity value of the sample liquid.

Calibrations are specific to the ball radius, ball density, temperature, and test angle. Recalibration is necessary when any of these parameters is changed.

Where no reference values at the required test temperature are available, follow the manufacturer's instructions for mathematical corrections to the calibration function. When the materials of the ball and tube are dissimilar, apply corrections calculated using the linear thermal expansion coefficients of the materials.

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# ⟨914⟩ VISCOSITY—PRESSURE DRIVEN METHODS

## INTRODUCTION

The viscometers/rheometers that are included in this chapter can be used to measure the viscosity of both Newtonian and non-Newtonian fluids. The devices include slit viscometers/rheometers and capillary-extrusion viscometers.

Capillary viscometers that are described in *Viscosity—Capillary Methods* ⟨911⟩ are based on the principle that the flow is driven by gravity, and these viscometers can be classified under one of the pressure driven methods. However, limitations of glass capillary viscometers render them inherently unsuitable for use in characterizing non-Newtonian fluids, and their use in measurements of high-viscosity fluids is impractical as well. Chapter ⟨914⟩ does not address such viscometers.

[NOTE—For additional information, see *Rheometry* ⟨1911⟩.]

• **METHOD I. SLIT VISCOMETERS/RHEOMETERS**

**Apparatus:** See *Figure 1*.

The basic design of a slit viscometer/rheometer consists of a rectangular, slit channel with a uniform cross-sectional area. A test liquid is pumped at a constant flow rate through this channel. Multiple pressure sensors that are flush mounted at linear distances along the streamwise direction measure the pressure drop, as depicted in *Figure 1*.

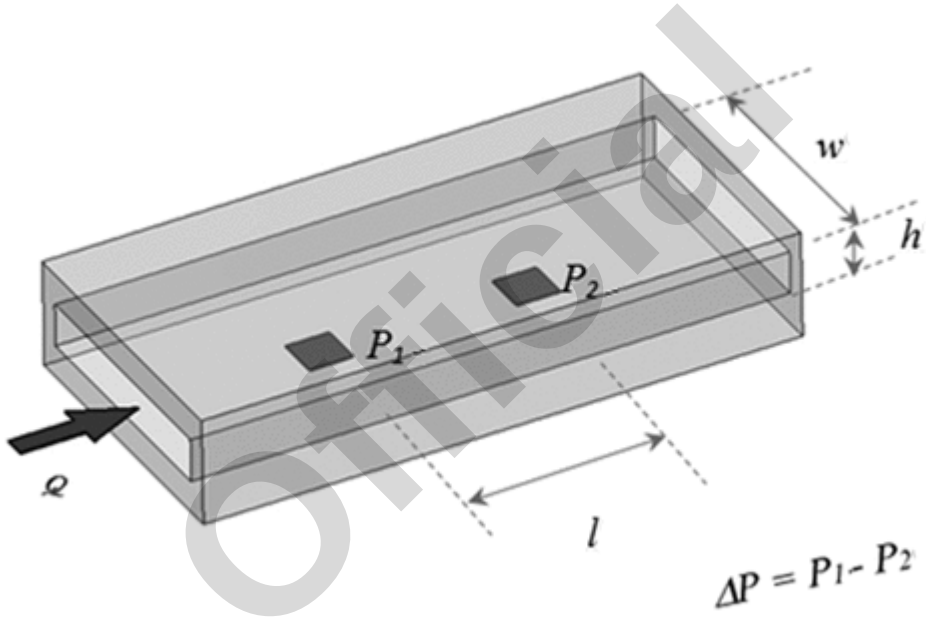


Figure 1. Basic design of the slit viscometer/rheometer.

**Measuring principle:** The slit viscometer/rheometer is based on the fundamental principle that a viscous liquid resists flow, exhibiting a decreasing pressure along the length of the slit. The pressure decrease or drop ($\Delta P$) is correlated with the shear stress at the wall boundary. The apparent shear rate is directly related to the flow rate and the dimension of the slit. The apparent shear rate ($\dot{\gamma}_a$), the shear stress ($\sigma$), and the apparent viscosity ($\eta_a$), respectively, are calculated as follows:

$$\dot{\gamma}_a = \frac{6 \times Q}{w \times h^2}$$

$$\sigma = \frac{h \times w}{2 \times (w + h)} \times \frac{\Delta P}{l}$$

$$\eta_a = \frac{\sigma}{\dot{\gamma}_a}$$

$\dot{\gamma}_a$ = apparent shear rate ($s^{-1}$)
$Q$ = flow rate (µL/s)
$w$ = width of the flow channel (mm)
$h$ = depth of the flow channel (mm)
$\sigma$ = shear stress (Pa)
$\Delta P$ = pressure difference between the leading pressure sensor and the last pressure sensor (Pa)
$l$ = distance between the leading pressure sensor and the last pressure sensor (mm)
$\eta_a$ = apparent viscosity (Pa · s)

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To determine the viscosity of a liquid, pump the liquid sample through the slit channel at a constant flow rate and measure the pressure drop. Using the equations shown above, calculate the apparent viscosity for the apparent shear rate. For a Newtonian liquid, the apparent viscosity is the same as the true viscosity, and the single shear-rate measurement is sufficient. For non-Newtonian liquids, the apparent viscosity is not true viscosity. To obtain true viscosity, measure the apparent viscosities at multiple apparent shear rates. Calculate true viscosities, $\eta$, at various shear rates using the Weissenberg–Rabinowitsch–Mooney correction factor:

$$\frac{1}{\eta} = \frac{1}{2 \times \eta_a} \times \left(2 + \frac{d\ln\dot{\gamma}_a}{d\ln\sigma}\right)$$

The calculated true viscosity should be the same as the value that is obtained using a cone-and-plate rheometer at the same shear rate.

**Procedure:** Select slit dimensions and full-scale pressure of pressure sensors to attain the desired range of viscosity and shear rates. With a syringe pump to control the flow rate—or interchangeably, the shear rate—load a test sample into a glass syringe. As necessary, control the temperature of the sample with the syringe and the slit viscometer specified in the individual procedure to ±0.1°, unless otherwise specified. Allow to equilibrate at the specified temperature for about 3–5 min. Set each pressure sensor to zero. Select the first flow rate or shear rate. Pump the sample at the flow rate, and measure the pressure readings once the steady-state values are reached. Increase the flow rate (or shear rate) until the pressure reading of the leading sensor is >5% of the full-scale value. Take the average of each pressure sensor over time, and calculate the pressure drops. Calculate the apparent viscosity. If required for the procedure, repeat the measurements four times. If the test sample is non-Newtonian, vary the shear rate and calculate the corresponding apparent viscosities.

**Calculation and calibration:** Calculate the apparent viscosity by dividing the shear stress by the apparent shear rate. For non-Newtonian liquids, apply the Weissenberg–Rabinowitsch–Mooney corrections to obtain the true viscosity curve. Commercial viscometers are provided with a built-in correction.

Calibrate individual pressure sensors by following the recommendations from the manufacturer. The slit viscometer, however, can be calibrated with a Newtonian standard of known viscosity. Choose a standard that covers the viscosity range of interest for the selected slit and pressure sensors. Measure the viscosities at shear rates for 10%, 50%, and 90% of full scale of the leading pressure sensor, and take the average. Calculate a constant ($k$) for the ratio of the reference value ($\eta_{ref}$) to the measured value ($\eta_{meas}$) and record.

$$k = \frac{\eta_{ref}}{\eta_{meas}}$$

$\eta_{ref}$ = reference viscosity (Pa · s)
$\eta_{meas}$ = measured viscosity (Pa · s)

Multiply new viscosity data by $k$, because the value of $k$ will correct for the uncertainty and variation of the slit geometry. Ideally, the value of $k$ should remain constant within a temperature change of 100°. If the $k$ value varies by >1%, consult with the manufacturer regarding the temperature compensation of the pressure sensors.

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# ⟨915⟩ MEASUREMENT OF STRUCTURAL STRENGTH OF SEMISOLIDS BY PENETROMETRY

***Change to read:***

## INTRODUCTION

This chapter describes the empirical methods of measuring the structural strength, or consistency, of a semisolid raw material or dosage form with a penetrometer. Viscosity represents the proportionality of the shear stress to the shear rate for a Newtonian fluid, whereas consistency is the term for this proportionality for semisolids that exhibit non-Newtonian rheological behavior. Because the term "consistency" may be confused with "uniformity" or "homogeneity", the term "structural strength" is now the preferred term. In the remainder of this chapter we will use the term structural strength to refer to this property of semisolids. One component of the structural strength of a semisolid is its hardness (yield stress). Penetrometry is one method for quantifying the hardness (yield stress) of semisolid materials.

Penetrometry allows a metal cone, with standardized dimensions and weight, to penetrate into a semisolid until the buoyancy of the cone and the yield stress of the semisolid exactly balance the gravity-applied force driving the penetrating object into the semisolid. This observed yield stress of the semisolid is a measure of the semisolid hardness. The yield stress (hardness) of the semisolid will be inversely proportional to the penetration depth of the cone.

This chapter outlines the method for performing the gravity-driven penetrometry measurements of semisolids. Although the results of the penetrometry measurements may be used to calculate the yield stress of the semisolid (see *Measurement of* ▲*Yield Stress*▲ (CN 1-Dec-2023) *of Semisolids* ⟨1912⟩ for more information), the results of the penetrometry experiment are reported as the observed penetration depth in tenths of millimeters [decimillimeters (dmm)].[1,2,3]

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## APPARATUS

The apparatus consists of a penetrometer made up of a stand and a penetrating object. An example of a suitable stand is shown in *Figure 1*, and a suitable penetrating object (cone) is shown in *Figure 2*.

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[1] ASTM D937-97. Standard test method for cone penetration of petrolatum. West Conshohocken, PA: ASTM; 1997.
[2] ASTM D217. Standard test methods for cone penetration of lubricating grease. West Conshohocken, PA: ASTM; 1997.
[3] *European Pharmacopoeia* 5.0, 2.9.9. Measurement of consistency by penetrometry. Strasbourg, France: European Directorate for the Quality of Medicine and Healthcare; 2014.

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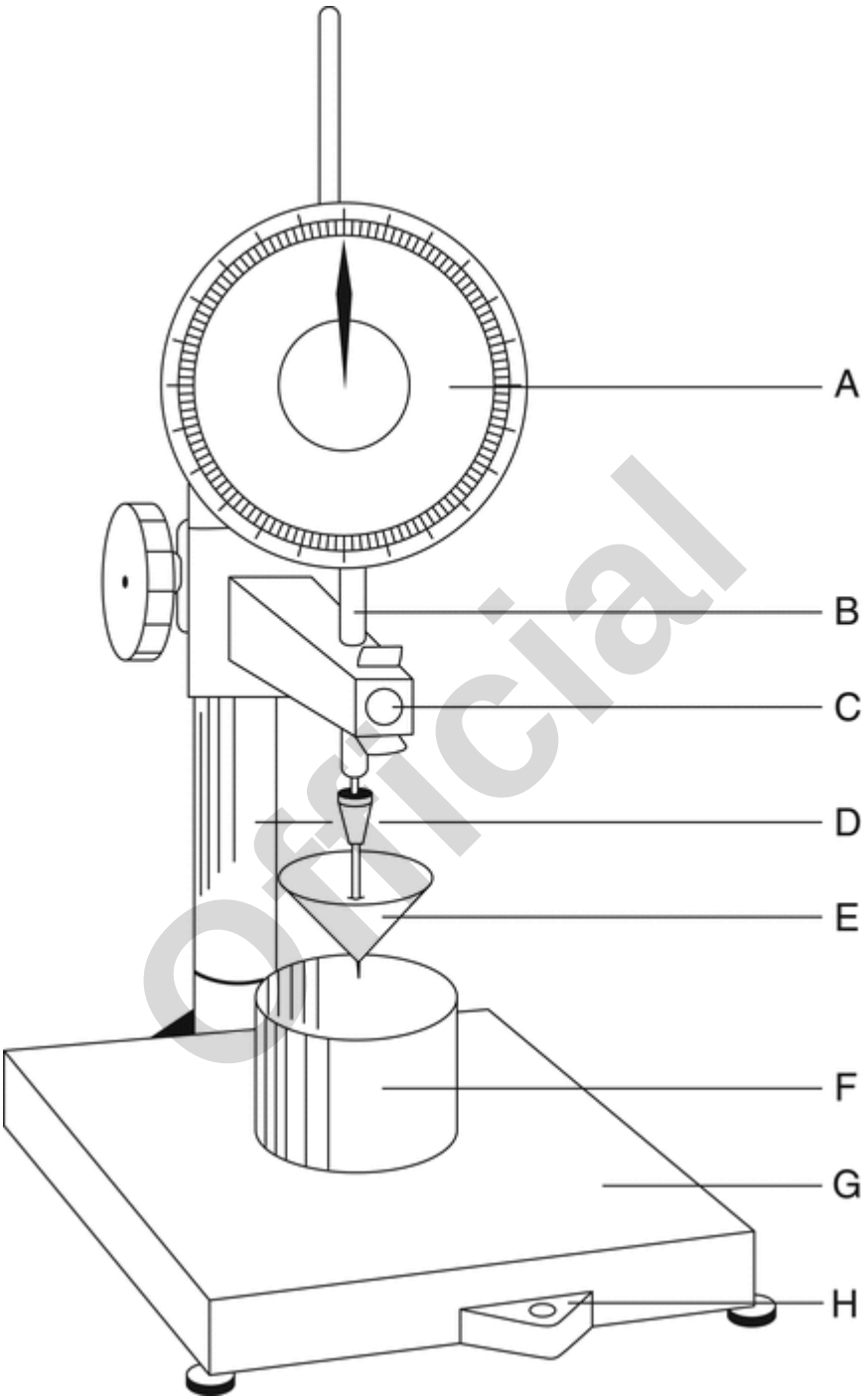


Figure 1. Penetrometer apparatus (gravity-driven). A) Scale showing the depth of penetration, graduated in dmm. B) Vertical shaft to maintain and guide the penetrating object. C) Device to retain and release the penetrating object. D) Device to ensure that the penetrating object is vertical and that the base is horizontal. E) Penetrating object made of a suitable material, has a smooth surface, and is characterized by its shape, size, and mass (see *Figure 2*). F) Sample container. G) Horizontal base. H) Control for the horizontal base.

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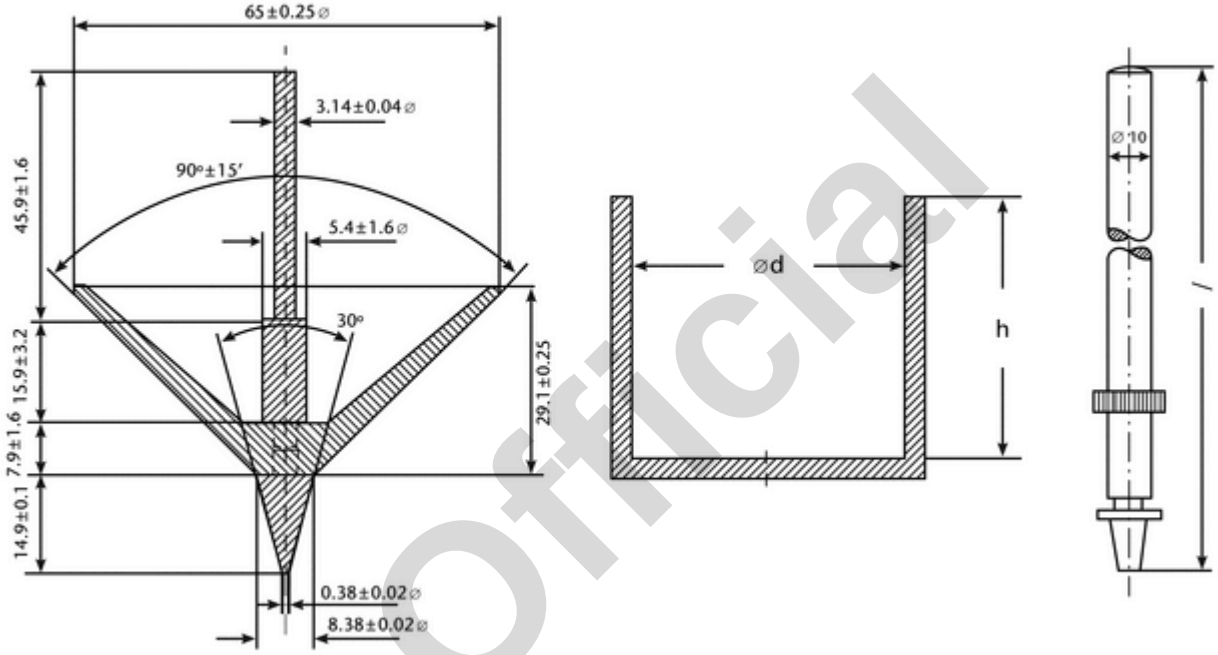


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Figure 2. Penetrometer standard cone (mass = 102.5 ± 0.05 g), suitable container ($d$ = 102 or 75 mm; $h \geq 62$ mm) and shaft ($l \approx 162$ mm; mass = 47.5 ± 0.05 g). Dimensions in millimeters. (Adapted with permission from *Pharmeuropa*, Vol. 9 No. 8, July 2019. © Council of Europe, 2019.)

## PROCEDURE

### Sample Preparation

With samples having penetrations <200 dmm, three tests (and sometimes more) may be made in one container by proper spacing. To prevent one test from being affected by the disturbed area of a previous test, the tip of the cone must not be placed nearer the edge of a previous test than the penetration distance of the sample. Some harder samples tend to form a marked depression in the center upon solidifying; such samples should not be tested in this depression because the results obtained may be different from those obtained in off-center positions on the level surface.

With samples having penetrations >200 dmm, only one test may be made in a container by placing the cone tip near the center of the container.

Fill the required number of containers to within 6 mm of their rims.

Prepare the test samples using one of the following procedures:

1. Carefully fill containers without forming air bubbles. Level, if necessary, to obtain a flat surface. Store the samples at 25 ± 0.5° for 24 h, unless otherwise prescribed.
2. Liquefy (i.e., heat to above the melting point) samples, and carefully fill containers without forming air bubbles. Store the samples at 25 ± 0.5° for 24 h, unless otherwise prescribed.

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3. If worked (sheared) samples are to be tested, first store samples at 25 ± 0.5° for 24 h. Apply a suitable shear to the samples for 5 min. Carefully and completely fill containers without forming air bubbles, and level, if necessary, to obtain a flat surface.

## Determination of Penetration

Penetration experiments use a gravity-driven apparatus (as shown in *Figure 1* or another functionally equivalent apparatus). *Method I* uses a standard cone as a penetrating object, and *Method II* uses a standard needle as a penetrating object. Unless otherwise directed in the individual monograph, use *Method I*.

### METHOD I (GRAVITY-DRIVEN MEASUREMENT OF STRUCTURAL STRENGTH WITH STANDARD CONE)

1. This method requires the use of a penetrating object that complies with the dimensions of *Figure 2*. The penetrating object should have a total effective mass of 150.0 ± 0.1 g (including the weight of the cone and additional masses firmly attached during the penetration experiment).
2. Place the test sample on the base of the penetrometer, and verify that its surface is perpendicular to the vertical axis of the penetrating object.
3. Bring the temperature of the penetrating object to 23.5 ± 2.0°, and then adjust its position such that its tip just touches the surface of the sample. [NOTE—Watching the shadow of the tip is an aid in accurate setting.]
4. Release the penetrating object and allow it to descend, without restraint, for 5 s.
5. Clamp the penetrating object and measure the depth of penetration.
6. Repeat the test at least two more times to obtain three or more values.

### METHOD II (GRAVITY-DRIVEN MEASUREMENT OF STRUCTURAL STRENGTH WITH NEEDLE)

1. This method requires the use of a polished metal needle weighing 2.50 ± 0.05 g and having a truncated symmetric tapered angle of 9°0′ ± 15′. The needle is tapered, with a length of 25.4 mm, and the shaft attached to the needle is 58 mm in length and 3.17 mm in diameter. The penetrating object should have a total effective mass of 100.0 ± 0.1 g (including the weight of the needle and additional masses firmly attached during the penetration experiment).
2. Place the test sample on the base of the penetrometer, and verify that its surface is perpendicular to the vertical axis of the penetrating object.
3. Bring the temperature of the penetrating object to 23.5 ± 2.0°, and then adjust its position such that its tip just touches the surface of the sample. [NOTE—Watching the shadow of the tip is an aid in accurate setting.]
4. Release the penetrating object and allow it to descend, without restraint, for 5 s.
5. Clamp the penetrating object and measure the depth of penetration.
6. Repeat the test at least two more times to obtain three or more values.

## EXPRESSION OF THE RESULTS

Report the mean and standard deviation of at least three replicate measurements in units of penetration depth. The penetration depth is expressed in dmm. If any of the individual results differ from the mean by >3%, repeat the test and report the mean and standard deviation of six replicate measurements in units of penetration depth.

## GLOSSARY

**Structural strength (Consistency):** The term "consistency" is sometimes used synonymously with "viscosity", albeit incorrectly. Viscosity represents the proportionality of the shear stress to the shear rate for a Newtonian fluid, whereas consistency is the term for this proportionality for semisolids that exhibit non-Newtonian rheological behavior. Because the term consistency may be confused with uniformity or homogeneity the term structural strength is now the preferred term. See ⟨1912⟩ for more information.

**Hardness:** "Hardness" is a term used synonymously with "tensile stress", and yield stress is one type of tensile stress. Hardness is proportional to yield stress—a harder semisolid also exhibits a larger apparent yield stress. In penetrometry, hardness has been more specifically defined as $H = C \times W/p^n$, where $C$ is a constant dependent on the cone geometry, $W$ is the weight of the penetrating cone, $p$ is the depth of penetration, and $n$ is an exponent. When $n = 2$, hardness has the same units as yield stress [pascal (Pa)]. See ⟨1912⟩ for more information.

**Penetrometer:** An instrument that measures the structural strength of semisolid materials by measuring the depth to which a specified cone or needle under a given force falls into the material.

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# ⟨921⟩ WATER DETERMINATION

***Change to read:***

Many Pharmacopeial articles either are hydrates or contain water in adsorbed form. As a result, the determination of the water content is important in demonstrating compliance with the Pharmacopeial standards. Generally one of the methods given below is called for in the individual monograph, depending upon the nature of the article. In rare cases, a choice is allowed between two methods. When the article contains water of hydration, *Method I (Titrimetric)*, *Method II (Azeotropic)*, or *Method III (Gravimetric)* is employed, as directed in the individual monograph, and the requirement is given under the heading *Water*. ▲Unless otherwise specified in the monograph, determine the water by *Method Ia* .▲ (USP 1-May-2022)

The heading *Loss on Drying* (see *Loss on Drying* ⟨731⟩) is used in those cases where the loss sustained on heating may be not entirely water.

***Change to read:***

## METHOD I (TITRIMETRIC)

▲▲ (USP 1-May-2022)

### Method Ia (Direct Titration)

PRINCIPLE

The titrimetric determination of water is based upon the quantitative reaction of water with an anhydrous solution of sulfur dioxide and iodine in the presence of a buffer that reacts with hydrogen ions.

In the original titrimetric solution, known as Karl Fischer Reagent, the sulfur dioxide and iodine are dissolved in pyridine and methanol. The test specimen may be titrated with the *Reagent* directly, or the analysis may be carried out by a residual titration procedure. The stoichiometry of the reaction is not exact, and the reproducibility of a determination depends upon such factors as the relative concentrations of the *Reagent* ingredients, the nature of the inert solvent used to dissolve the test specimen, and the technique used in the particular determination. Therefore, an empirically standardized technique is used in order to achieve the desired accuracy. Precision in the method is governed largely by the extent to which atmospheric moisture is excluded from the system. The titration of water is usually carried out with the use of anhydrous methanol as the solvent for the test specimen. In some cases, other suitable solvents may be used for special or unusual test specimens. In these cases, the addition of at least 20% of methanol or other primary alcohol is recommended.

APPARATUS

Any apparatus may be used that provides for adequate exclusion of atmospheric moisture and determination of the endpoint. In the case of a colorless solution that is titrated directly, the endpoint may be observed visually as a change in color from canary yellow to amber. The reverse is observed in the case of a test specimen that is titrated residually. More commonly, however, the endpoint is determined electrometrically with an apparatus employing ▲an▲ (USP 1-May-2022) electrical circuit ▲and▲ (USP 1-May-2022) a pair of platinum electrodes immersed in the solution to be titrated. At the endpoint of the titration a slight excess of the reagent increases the flow of current, ▲which is detected by the electrodes for not less than 30 s.▲ (USP 1-May-2022) The time is shortest for substances that dissolve in the reagent. With some automatic titrators, the abrupt change in current or potential at the endpoint serves to close a solenoid-operated valve that controls the buret delivering the titrant. Commercially available apparatus generally comprises a closed system consisting of one or two automatic burets and a tightly covered titration vessel fitted with the necessary electrodes and a magnetic stirrer. The air in the system is kept dry with a suitable desiccant, and the titration vessel may be purged by means of a stream of dry nitrogen or current of dry air.

REAGENT

Prepare the Karl Fischer Reagent as follows. Add 125 g of iodine to a solution containing 670 mL of methanol and 170 mL of pyridine, and cool. Place 100 mL of pyridine in a 250-mL graduated cylinder, and, keeping the pyridine cold in an ice bath, pass in dry sulfur dioxide until the volume reaches 200 mL. Slowly add this solution, with shaking, to the cooled iodine mixture. Shake to dissolve the iodine, transfer the solution to the apparatus, and allow the solution to stand overnight before standardizing. When freshly prepared, 1 mL of this solution is equivalent to approximately 5 mg of water, but it deteriorates gradually; therefore, standardize it within 1 h before use, or daily if in continuous use. Protect from light while in use. Store any bulk stock of the reagent in a suitably sealed, glass-stoppered container, fully protected from light, and under refrigeration. For determination of trace amounts of water (less than 1%), it is preferable to use a *Reagent* with a water equivalency factor of not more than 2.0, which will lead to the consumption of a more significant volume of titrant.

A commercially available, stabilized solution of Karl Fischer type reagent may be used. Commercially available reagents containing solvents or bases other than pyridine or alcohols other than methanol may be used also. These may be single solutions or reagents formed in situ by combining the components of the reagents present in two discrete solutions. The diluted *Reagent* called for in some monographs should be diluted as directed by the manufacturer. Either methanol or other suitable solvent, such as ethylene glycol monomethyl ether, may be used as the diluent.

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## TEST PREPARATION

Unless otherwise specified in the individual monograph, use an accurately weighed or measured amount of the specimen under test estimated to contain 2–250 mg of water. The amount of water depends on the water equivalency factor of the *Reagent* and on the method of endpoint determination. In most cases, the minimum amount of specimen, in milligrams, can be estimated using the formula:

$$\text{Result} = FCV/KF$$

in which $F$ is the water equivalency factor of the *Reagent*, in milligrams per milliliter; $C$ is the used volume, in percent, of the capacity of the buret; $V$ is the buret volume, in milliliters; and $KF$ is the limit or reasonable expected water content in the sample, in percent. $C$ is generally between 30% and 100% for manual titration, and between 10% and 100% for the instrumental method endpoint determination.

[NOTE—It is recommended that the product of $FCV$ be greater than or equal to 200 for the calculation to ensure that the minimum amount of water titrated is greater than or equal to 2 mg.]

Where the specimen under test is an aerosol with propellant, store it in a freezer for not less than 2 h, open the container, and test 10.0 mL of the well-mixed specimen. In titrating the specimen, determine the endpoint at a temperature of 10° or higher.

Where the specimen under test is capsules, use a portion of the mixed contents of not fewer than 4 capsules.

Where the specimen under test is tablets, use powder from not fewer than 4 tablets ground to a fine powder in an atmosphere of temperature and relative humidity known not to influence the results.

Where the monograph specifies that the specimen under test is hygroscopic, take an accurately weighed portion of the solid into the titration vessel, proceeding as soon as possible and taking care to avoid moisture uptake from the atmosphere. If the sample is constituted by a finite amount of solid as a lyophilized product or a powder inside a vial, use a dry syringe to inject an appropriate volume of methanol, or other suitable solvent, accurately measured, into a tared container, and shake to dissolve the specimen. Using the same syringe, remove the solution from the container and transfer it to a titration vessel prepared as directed for *Procedure*. Repeat the procedure with a second portion of methanol, or other suitable solvent, accurately measured; add this washing to the titration vessel; and immediately titrate. Determine the water content, in milligrams, of a portion of solvent of the same total volume as that used to dissolve the specimen and to wash the container and syringe, as directed for *Standardization of Water Solution for Residual Titration* in *Method IB*, and subtract this value from the water content, in milligrams, obtained in the titration of the specimen under test. Dry the container and its closure at 100° for 3 h, allow to cool in a desiccator, and weigh. Determine the weight of specimen tested from the difference in weight from the initial weight of the container.

When appropriate, the water may be desorbed or released from the sample by heat in an external oven connected with the vessel, to where it is transferred with the aid of an inert and dried gas such as pure nitrogen. Any drift due to the transport gas should be considered and corrected. Care should be taken in the selection of the heating conditions to avoid the formation of water coming from dehydration due to decomposition of the sample constituents, which may invalidate this approach.

## STANDARDIZATION OF THE REAGENT

Place enough methanol or other suitable solvent in the titration vessel to cover the electrodes, and add sufficient *Reagent* to give the characteristic endpoint color or ▲the appropriate electrode response.

Either▲ (USP 1-May-2022) *Purified Water*, ▲USP Sodium Tartrate Dihydrate RS, ▲ (USP 1-May-2022)or commercial standards with a certificate of analysis traceable to a national standard may be used to standardize the *Reagent*. The reagent equivalency factor, the recommended titration volume, buret size, and amount of standard to measure are factors to consider when deciding which standard and how much to use.[1] For *Purified Water* or water standards, quickly add the equivalent of between 2 and 250 mg of water. Calculate the water equivalency factor, $F$, in milligrams of water per milliliter of reagent:

$$\text{Result} = W/V$$

in which $W$ is the weight, in milligrams, of the water contained in the aliquot of standard used; and $V$ is the volume, in milliliters, of the *Reagent* used in the titration. For sodium tartrate dihydrate, quickly add 20–125 mg of sodium tartrate dihydrate ($C_4H_4Na_2O_6 \cdot 2H_2O$), accurately weighed by difference, and titrate to the endpoint. The water equivalence factor $F$, in milligrams of water per milliliter of reagent, is given by the formula:

$$\text{Result} = W/V\,(36.04/230.08)$$

in which 36.04 is 2 times the molecular weight of water and 230.08 is the molecular weight of sodium tartrate dihydrate; $W$ is the weight, in milligrams, of sodium tartrate dihydrate; and $V$ is the volume, in milliliters, of the *Reagent* consumed in the second titration. Note that the solubility of sodium tartrate dihydrate in methanol is such that fresh methanol may be needed for additional titrations of the sodium tartrate dihydrate standard.

## PROCEDURE

Unless otherwise specified, transfer enough methanol or other suitable solvent to the titration vessel, ensuring that the volume is sufficient to cover the electrodes (approximately 30–40 mL), and titrate with the *Reagent* to the electrometric or visual

[1] Consider a setup in which the reagent equivalency factor is 5 mg/mL, and the buret volume is 5 mL and an instrumental endpoint. Standard amounts equivalent to between 2.5 and 22.5 mg of water (10%–90% of buret capacity) could be used based on the buret and the reagent equivalency factor. The upper end of this range would involve an excessive amount of sodium tartrate dihydrate. If *Purified Water* or a standard is weighed, an analytical balance appropriate to the amount weighed is required.

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endpoint to consume any moisture that may be present. (Disregard the volume consumed because it does not enter into the calculations.) Quickly add the *Test Preparation*, mix, and again titrate with the *Reagent* to the electrometric or visual endpoint. Calculate the water content of the specimen taken, in milligrams:

$$\text{Result} = SF$$

in which $S$ is the volume, in milliliters, of the *Reagent* consumed in the second titration; and $F$ is the water equivalence factor of the *Reagent*.

## Method Ib (Residual Titration)

### PRINCIPLE

See the information given in the section *Principle* under *Method Ia*. In the residual titration, excess *Reagent* is added to the test specimen, sufficient time is allowed for the reaction to reach completion, and the unconsumed *Reagent* is titrated with a standard solution of water in a solvent such as methanol. The residual titration procedure is applicable generally and avoids the difficulties that may be encountered in the direct titration of substances from which the bound water is released slowly.

### APPARATUS, REAGENT, AND TEST PREPARATION

Use *Method Ia*.

### STANDARDIZATION OF WATER SOLUTION FOR RESIDUAL TITRATION

Prepare a *Water Solution* by diluting 2 mL of water with methanol or other suitable solvent to 1000 mL. Standardize this solution by titrating 25.0 mL with the *Reagent*, previously standardized as directed under *Standardization of the Reagent*. Calculate the water content, in milligrams per milliliter, of the *Water Solution* taken:

$$\text{Result} = V'F/25$$

in which $V'$ is the volume, in milliliters, of the *Reagent* consumed, and $F$ is the water equivalence factor of the *Reagent*. Determine the water content of the *Water Solution* weekly, and standardize the *Reagent* against it periodically as needed.

### PROCEDURE

Where the individual monograph specifies that the water content is to be determined by *Method Ib,* transfer enough methanol or other suitable solvent to the titration vessel, ensuring that the volume is sufficient to cover the electrodes (approximately 30–40 mL), and titrate with the *Reagent* to the electrometric or visual endpoint. Quickly add the *Test Preparation*, mix, and add an accurately measured excess of the *Reagent*. Allow sufficient time for the reaction to reach completion, and titrate the unconsumed *Reagent* with standardized *Water Solution* to the electrometric or visual endpoint. Calculate the water content of the specimen, in milligrams, taken:

$$\text{Result} = F(X' - XR)$$

in which $F$ is the water equivalence factor of the *Reagent*; $X'$ is the volume, in milliliters, of the *Reagent* added after introduction of the specimen; $X$ is the volume, in milliliters, of standardized *Water Solution* required to neutralize the unconsumed *Reagent*; and $R$ is the ratio, $V'/25$ (mL *Reagent*/mL *Water Solution*), determined from the *Standardization of Water Solution for Residual Titration*.

## Method Ic (Coulometric Titration)

### PRINCIPLE

The Karl Fischer reaction is used in the coulometric determination of water. Iodine, however, is not added in the form of a volumetric solution but is produced in an iodide-containing solution by anodic oxidation. The reaction cell usually consists of a large anode compartment and a small cathode compartment that are separated by a diaphragm. Other suitable types of reaction cells (e.g., without diaphragms) may also be used. Each compartment has a platinum electrode that conducts current through the cell. Iodine, which is produced at the anode electrode, immediately reacts with water present in the compartment. When all the water has been consumed, an excess of iodine occurs, which usually is detected electrometrically, thus indicating the endpoint. Moisture is eliminated from the system by pre-electrolysis. Changing the Karl Fischer solution after each determination is not necessary because individual determinations can be carried out in succession in the same reagent solution. A requirement for this method is that each component of the test specimen is compatible with the other components, and no side reactions take place. Samples are usually transferred into the vessel as solutions by means of injection through a septum. Gases can be introduced into the cell by means of a suitable gas inlet tube. Precision in the method is predominantly governed by the extent to which atmospheric moisture is excluded from the system; thus, the introduction of solids into the cell may require precautions, such as working in a glove box in an atmosphere of dry inert gas. Control of the system may be monitored by measuring the amount of baseline drift, which does not preclude the need of any blank correction when used as a vehicle for sample introduction. This method is particularly suited to chemically inert substances like hydrocarbons, alcohols, and ethers. In comparison with the volumetric Karl Fischer titration, coulometry is a micro-method.

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When appropriate, the water may be desorbed or released from the sample by heat in an external oven connected with the vessel, to where it is transferred with the aid of an inert and dried gas such as pure nitrogen. Any drift due to the transport gas should be considered and corrected. Care should be taken in the selection of the heating conditions to avoid the formation of water coming from dehydration due to decomposition of the sample constituents, which may invalidate this approach.

### APPARATUS

Any commercially available apparatus consisting of an absolutely tight system fitted with the necessary electrodes and a magnetic stirrer is appropriate. The instrument's microprocessor controls the analytical procedure and displays the results. Calibration of the instrument is not necessary, as the current consumed can be measured absolutely.

### REAGENT

See the manufacturer's recommendations.

### TEST PREPARATION

Where the specimen is a soluble solid, an appropriate quantity, accurately weighed, may be dissolved in anhydrous methanol or other suitable solvents.

Where the specimen is an insoluble solid, an appropriate quantity, accurately weighed, may be extracted using a suitable anhydrous solvent and may be injected into the anolyte solution. Alternatively, an evaporation technique may be used in which water is released and evaporated by heating the specimen in a tube in a stream of dry inert gas. The gas is then passed into the cell.

Where the specimen is to be used directly without dissolving in a suitable anhydrous solvent, an appropriate quantity, accurately weighed, may be introduced into the chamber directly.

Where the specimen is a liquid, and is miscible with anhydrous methanol or other suitable solvents, an appropriate quantity, accurately weighed, may be added to anhydrous methanol or other suitable solvents.

### PROCEDURE

Using a dry device, inject or add directly an accurately measured amount of the sample or sample preparation estimated to contain between 0.5 and 5 mg of water, or an amount recommended by the instrument manufacturer into the anolyte, mix, and perform the coulometric titration to the electrometric endpoint. Read the water content of the liquid *Test Preparation* directly from the instrument's display, and calculate the percentage that is present in the substance. Perform a blank determination, as needed, and make any necessary corrections.

## METHOD II (AZEOTROPIC—TOLUENE DISTILLATION)

### Apparatus

Use a 500-mL glass flask A connected by means of a trap B to a reflux condenser C by ground glass joints (see *Figure 1*).

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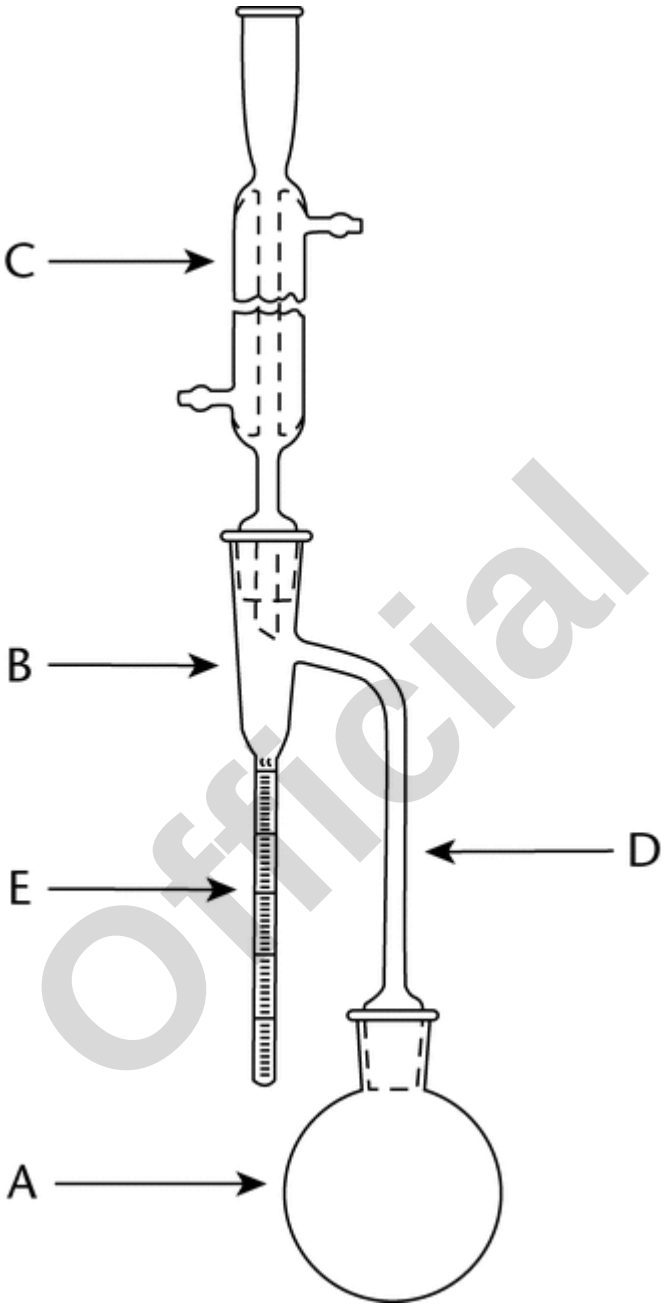


Figure 1. Toluene moisture apparatus.

The critical dimensions of the parts of the apparatus are as follows. The connecting tube D is 9–11 mm in internal diameter. The trap is 235–240 mm in length. The condenser, if of the straight-tube type, is approximately 400 mm in length and not less than 8 mm in bore diameter. The receiving tube E has a 5-mL capacity, and its cylindrical portion, 146–156 mm in length, is graduated in 0.1-mL subdivisions, so that the error of reading is not greater than 0.05 mL for any indicated volume. The source of heat is preferably an electric heater with rheostat control or an oil bath. The upper portion of the flask and the connecting tube may be insulated.

Clean the receiving tube and the condenser with a suitable cleanser, thoroughly rinse with water, and dry in an oven. Prepare the toluene to be used by first shaking with a small quantity of water, separating the excess water, and distilling the toluene.

## Procedure

Place in the dry flask a quantity of the substance, weighed accurately to the nearest centigram, which is expected to yield 2–4 mL of water. If the substance is of a pasty character, weigh it in a boat of metal foil of a size that will just pass through the neck of the flask. If the substance is likely to cause bumping, add enough dry, washed sand to cover the bottom of the flask, or a number of capillary melting-point tubes, about 100 mm in length, sealed at the upper end. Place about 200 mL of toluene

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in the flask, connect the apparatus, and fill the receiving tube E with toluene poured through the top of the condenser. Heat the flask gently for 15 min and, when the toluene begins to boil, distill at the rate of about 2 drops/s until most of the water has passed over, then increase the rate of distillation to about 4 drops/s. When the water has apparently all distilled over, rinse the inside of the condenser tube with toluene while brushing down the tube with a tube brush attached to a copper wire and saturated with toluene. Continue the distillation for 5 min, then remove the heat, and allow the receiving tube to cool to room temperature. If any droplets of water adhere to the walls of the receiving tube, scrub them down with a brush consisting of a rubber band wrapped around a copper wire and wetted with toluene. When the water and toluene have separated completely, read the volume of water, and calculate the percentage that was present in the substance.

## METHOD III (GRAVIMETRIC)

### Procedure for Chemicals

Proceed as directed in the individual monograph preparing the chemical as directed under ⟨731⟩.

### Procedure for Biologics

Proceed as directed in the individual monograph.

### Procedure for Articles of Botanical Origin

Place about 10 g of the drug, prepared as directed (see *Articles of Botanical Origin* ⟨561⟩*, Methods of Analysis*) and accurately weighed, in a tared evaporating dish. Dry at 105° for 5 h, and weigh. Continue the drying and weighing at 1-h intervals until the difference between two successive weighings corresponds to not more than 0.25%.

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***Add the following:***

# ▲⟨922⟩ WATER ACTIVITY

## INTRODUCTION

Many pharmacopeial articles include water within their matrices and are also susceptible to water exchange with the surrounding environment. Total water content is an important quality attribute, and several methods for determining water content are provided in *Water Determination* ⟨921⟩. Some of the water may be tightly bound and not available to participate in chemical, biochemical, or physicochemical reactions (e.g., as hydrate salts), whereas some of the water may be more freely available to participate in reactions such as hydrolysis or may provide an environment that can support microbiological growth. It is important to establish what fraction of the total water is available (active), and the determination of water activity ($a_w$) provides this information. This chapter outlines the recommended methods to qualify, calibrate, and use water activity meters to accurately measure the water activity ($a_w$) of raw materials and products.

## THEORETICAL BACKGROUND

Water activity ($a_w$) is equal to the vapor pressure of water in the sample ($p$) relative to the vapor pressure of pure water ($p_o$) at the same temperature (*1,2*):

$$a_w \equiv \left(\frac{p}{p_o}\right)_T = \left(\frac{RH_{eq}(\%)_{\text{sample}}}{RH_{eq}(\%)_{\text{water}}}\right)_T$$

$p$ = partial vapor pressure of water in the sample at the temperature of the measurement
$p_o$ = saturation vapor pressure of water at the temperature of the measurement
$T$ = temperature of the measurement
$RH_{eq}$ = the equilibrium relative humidity above the sample or pure water

The saturation vapor pressure of pure water depends only on its temperature, so, if $p$ and sample temperature are known, $a_w$ can be readily determined.

Measurement of $p$ is performed by allowing a sample to equilibrate with the moisture in the headspace above the sample at ambient or a standardized temperature and then measuring the vapor pressure of water in the headspace. Water activity meters may measure the relative humidity of the headspace with a moisture-dependent resistor or capacitor, or they may use psychrometry (wet bulb temperature), optical absorption, or dew point temperature.

The relative humidity of the headspace of the instrument cell is defined as:

$$RH = \left(\frac{p_a}{p_{o,a}}\right)_T$$
$$\%RH = RH \times 100$$

$p_a$ = partial vapor pressure of water in the air at the temperature of the measurement
$p_{o,a}$ = saturation vapor pressure of pure water in the air at the temperature of the measurement

When the water vapor in the sample and the headspace are in equilibrium ($p = p_a$), combine the two equations:

$$a_w \equiv \left(\frac{p}{p_o}\right)_T = \frac{\%RH}{100}\left(\frac{Po,a}{Po}\right)_T$$

Use this equation to determine the water activity of a sample from a relative humidity measurement of the headspace. If the air in the headspace is also in temperature equilibrium with the sample, the saturation vapor pressures divide out so that $a_w = RH$. If the temperatures are not equal, and one assumes that water activity equals the air relative humidity, errors can be significant.

If a wet bulb temperature is used to determine the headspace vapor pressure, the psychrometer equation is used, and vapor pressure of the sample is calculated as:

$$p = p_{o,Twb} - \gamma P(T_a - T_{wb})$$

$p_{o,Twb}$ = saturation vapor pressure of pure water at wet bulb temperature
$\gamma$ = psychrometer constant
$P$ = atmospheric pressure
$T_a$ = ambient temperature (temperature of the air in headspace)
$T_{wb}$ = wet bulb temperature

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The wet bulb temperature is lower than the ambient temperature as a result of the cooling provided by the evaporation rate of water. The psychrometric constant has a value of about 0.0006/degree but is reduced as the wet bulb temperature approaches the ambient temperature (*3*).

Optical absorption methods measure water vapor pressure via attenuation of ultraviolet or infrared light by the water vapor molecules in the headspace. The relationship between absorption and vapor pressure of the sample is given by Beer-Lambert law:

$$p = \frac{1}{\kappa x}\ln\left(\frac{I_o}{I}\right)$$

$k$ = extinction coefficient
$x$ = path length (cm)
$I_o$ = unattenuated beam strength
$I$ = attenuated beam strength

The vapor pressure from this equation can be directly used, along with sample temperature, to calculate $a_w$.

Dew point temperature can be used to determine water vapor pressure because the saturation vapor pressure of water at the dew point temperature is equal to the water vapor pressure of the air ($p_{o,Td} = p_a$) at the measurement temperature. For the dew point measurement, calculate the water activity as:

$$a_w = \left(\frac{p_{o,\,Td}}{p_o}\right)$$

$p_{o,Td}$ = saturation vapor pressure of pure water at dew point temperature

If the sample temperature and dew point temperature of the headspace air are known, the water activity can be calculated directly. Water vapor equilibration is still required, but temperatures do not need to be in equilibrium for this measurement to be accurate.

To accurately measure the relative humidity of the headspace above the sample, the temperature of the sample needs to be constant and the headspace being sampled needs to be sealed off from the rest of the laboratory environment. Some instruments only perform measurements at ambient temperature, but other instruments control the temperature in the sample chamber and may perform $a_w$ measurements over a wide range of temperatures. Methods that rely on dew point temperature or wet bulb temperature may not be able to accurately determine these lower temperatures if they are close to (or below) the freezing point of water. Therefore, these instruments may have difficulty accurately measuring samples with very low water activity. Verify the minimum water activity that the instrument manufacturer claims can be accurately measured with these instruments.

Errors in measurement may result from attempting to use the meter in a location where the temperature is not sufficiently stable or with a poor headspace seal (e.g., due to a worn O-ring or sample contamination of the O-ring). Presence of other volatiles (e.g., organic acids, glycerin, alcohol) may adversely affect the measurement unless the sensor is protected appropriately. Contamination of the probe or the sample cup with extraneous water or hygroscopic solids also can affect the accuracy of the measurements.

## FACTORS AFFECTING WATER ACTIVITY

When water is strongly bound to a solute or surface, the $a_w$ is reduced. In solution, this relationship between the $a_w$ and the solute concentration is given by:

$$\ln a_w = -\varphi_m v m M_w$$

$\varphi_m$ = osmolality coefficient
$v$ = stoichiometric coefficient for the solute
$m$ = concentration of the solute (mol of solute/kg of water)
$M_w$ = molar mass of water, 18.01528 g/mol

This relationship between solution osmolality and water activity allows the use of well-defined salt solutions for calibration of instruments. Specific water activity values can be produced by precise preparation of unsaturated salt solutions or by using saturated salt solutions at specific temperatures. Solutions with high osmolality will exhibit low water activity and will not support microbial growth.

When water is adsorbed to the surface of a solid, the relationship between the $a_w$ and the enthalpy of adsorption is given by:

$$\frac{\partial}{\partial T}\ln a_w = \frac{q_{st}}{RT^2} = \frac{\left(Q_{st} - \Delta H_{vap}\right)}{RT^2}$$

$q_{st}$ = net isosteric heat of sorption

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$R$ = universal gas constant
$T$ = temperature (°K)
$Q_{st}$ = isosteric heat of sorption
$\Delta H_{vap}$ = enthalpy of the transition from vapor to adsorption in the sample

Hygroscopic solids may have multiple binding sites for water on a given surface; some water may be tightly bound and unavailable for chemical reactions or to support microbial growth, whereas some water may be more loosely bound and available. At low relative humidities, the stronger binding sites will be occupied first, $q_{st}$ will be >44 kJ/mol, and the water activity will remain low. However, as relative humidity increases and once all the strong binding sites are occupied, additional adsorbed water will be associated with previously adsorbed water, $q_{st}$ will approach $\Delta H_{vap}$ of water (43.99 kJ/mol at 25° C), and the water activity of the solid will increase significantly.

Water activity is measured at a specific temperature, but the temperature to which products are exposed varies greatly. The temperature dependence of the water activity is described by the Clausius–Clapeyron equation:

$$\ln\left(a_{w2}\right) = \ln\left(a_{w1}\right) - \frac{q_{st}}{R}\left(\frac{1}{T_2} - \frac{1}{T_1}\right)$$

$a_{w1}$, $a_{w2}$ = water activity at $T_1$ and $T_2$, respectively
$q_{st}$ = isosteric heat of sorption
$T_1, T_2$ = temperature (°K)
$R$ = universal gas constant (8.314 J/mol · K)

If the natural logarithm of water activity is plotted as a function of reciprocal Kelvin temperature, a straight line will result, with slope $q_{st}/R$. In hygroscopic solids at low water content, $q_{st}$ will be larger but will approach $\Delta H_{vap}$ of water as the water content increases. As temperature increases, the water activity will also increase, but it will increase more significantly for hygroscopic solids with low water content. Therefore, if the water activity of a product or raw material is an important attribute (i.e., for risk assessments regarding the potential for supporting microbial growth) the risk assessment should be based on the worst-case (i.e., maximum) water activity, which is typically at the highest temperature.

Regardless of the principle of measurement, water activity meters can be calibrated and qualified using standard solutions (either saturated or unsaturated salt solutions) that provide a known and constant $RH_{eq}$. Alternatively, a relative humidity generator may also be used for calibration.

## APPLICATION OF WATER ACTIVITY MEASUREMENTS

The determination of $a_w$ aids in decisions during ingredient and product processes design, ingredient selection, packaging selection, and product storage conditions.[1] These include:

- Selecting ingredient isolation and product manufacturing processes conditions in terms of maintaining $a_w$ below the critical threshold to obtain thermodynamic control of the desired solid form (e.g., hydrate versus anhydrate)
- Selecting excipients for which $a_w$ may impact their material flow, compression characteristics, hardness, and performance characteristics (e.g., disintegration and dissolution) of dosage forms
- Optimizing fluidized bed drying processes
- Reducing the degradation of active ingredients within product formulations (e.g., those susceptible to chemical hydrolysis)
- Establishing the level of protection to product formulations to moisture by primary packaging materials during their shelf life
- Optimizing the shelf-life stability of probiotics
- Providing a complementary method for monitoring changes in water content
- Controlling and monitoring physical, chemical, and microbial product stability
- Optimizing formulations to improve the antimicrobial effectiveness of preservative systems
- Reducing the susceptibility of formulations to microbial contamination
- Providing a tool to justify the reduction of microbial testing of nonsterile drug and dietary supplements formulations (see *Application of Water Activity Determination to Nonsterile Pharmaceutical Products* ⟨1112⟩)

As per *Validation of Compendial Procedures* ⟨1225⟩, water activity methods will typically fall into Category III tests in which precision is a validation requirement; however, accuracy and specificity also may be relevant for fitness of purpose. Specificity may be a concern when other volatiles may affect the result of the water activity measurement and may be addressed by appropriate selection of the instrument type or use of organic filters to protect the water activity sensor. Optimal selection of the type of water activity instrument and the qualification requirements of that instrument will depend on the specific application(s).

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## TYPES OF WATER ACTIVITY INSTRUMENTS

### Dew Point Temperature—Chilled Mirror

At the dew point temperature, the relative humidity will be 100%, the evaporation and condensation rates will be equal, and there will not be any net condensation. When the temperature of a surface drops below the dew point temperature, water will condense on it. The dew point temperature can be determined by cooling a mirror in the sample chamber until condensation begins to occur and recording the mirror temperature at that point. The condensation on the chilled mirror is detected by an optical sensor. The difference between the dew point temperature and the temperature of the sample chamber is used to determine the relative humidity. The mirror must be kept clean and protected from contamination by dust or volatiles that may condensate on the chilled mirror. Therefore, measurements on micronized powders may increase the likelihood of mirror contamination issues. The measurement of dew point temperature is a primary method of determining the vapor pressure of the headspace.

### Electronic Hygrometers—Resistive (Electrolytic) Hygrometer

In this instrument, the relative humidity in the sample chamber is measured by a hygroscopic resistor. The resistor is a liquid electrolyte that absorbs or loses water to the air in the sample chamber, and the resulting resistance is proportional to the relative humidity. These sensors provide good precision and typically function over the $a_w$ range of 0.04–1.00. Volatile substances, which are soluble in the liquid electrolyte, may affect the resistance and interfere with the relative humidity measurement. Chemical filters may be used to absorb volatiles and prevent this interference. The response of these instruments is not necessarily linear, so sensors should be calibrated using multiple reference solutions over the entire operating range. Alternatively, a two-point linear calibration over a narrower range that brackets the sample may be used.

### Electronic Hygrometers—Capacitive Hygrometer

In this instrument, the relative humidity in the sample chamber is measured by a hygroscopic capacitor. The capacitor consists of a hygroscopic dielectric polymer held between conductive plates. The polymer absorbs or loses water to the air in the sample chamber, and the resulting capacitance is proportional to the relative humidity. These sensors may become saturated at high $a_w$ and may exhibit hysteresis after measurement of high $a_w$ samples. Capacitive hygrometers are typically less affected by other volatiles than resistive or dew point sensors; however, care should still be used when performing measurements in the presence of other volatile compounds. The response of these instruments is not necessarily linear, so sensors should be calibrated using multiple reference solutions over the entire operating range. Alternatively, a two-point linear calibration over a narrower range that brackets the sample may be used.

### Optical Hygrometer—Tunable Diode Laser

Water molecules in the gas phase above a product absorb UV and NIR radiation. Strong and broad absorption bands exist that can be used to measure the vapor pressure of air, but many of these are near absorption bands of other volatiles that may interfere. Therefore, broadband optical hygrometers are not useful for water activity measurements. The absorption bands are comprised of individual transitions are unique not only to a specific molecule but to a particular isotope of water. A diode laser can produce light at a specific wavelength, with a narrow bandwidth of <1 nm. The wavelength of a tunable diode laser (TDL) can be tuned to a specific isotopic absorption line in the water vapor spectrum by controlling the laser temperature and can be scanned across that line by modulating the laser current (and, therefore, the laser's temperature). This modulation provides both the attenuated and unattenuated laser intensities in the headspace, from which the vapor pressure of the headspace can be calculated. Because the TDL is capable of measuring vapor pressure in the presence of even high concentrations of other volatiles, it is the only method that can correctly measure water activity in these situations. Electronic and dew point hygrometers can sometimes function in the presence of low concentrations of volatiles, but readings are always adversely affected by the volatile. The TDL also can be used to measure water activity non-invasively in transparent sealed containers.

### Near-Infrared Spectroscopy

Near-infrared spectroscopy (NIR) does not measure $a_w$ directly but can be used to infer the $a_w$ simultaneously with the water content. The use of NIR to measure $a_w$ is based on first generating a prediction model for a particular official article (product or raw material) to correlate NIR absorbance spectra to the $a_w$ (measured with one of the previously mentioned direct methods). This prediction model then may be used to rapidly predict the $a_w$ of additional samples. When additional prediction models are available, NIR can be used for simultaneous determination of other components such as the content of active ingredients, additives, or excipients. The NIR method is ideally suited for rapid analysis of raw materials, in-process materials, and end products.

### Other Methods

Other methods for measurement of $a_w$ may be useful but are more rarely employed for routine measurements of pharmaceutical products, dietary supplements, and raw materials. These methods include hair hygrometry, freezing point depression, thermocouple psychrometry, and isopiestic methods. Hair hygrometers may be useful for approximate $a_w$ measurements but not for routine work due to long equilibration times and low accuracy. The freezing-point and thermocouple

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psychometry instruments are only useful for measurements at high $a_w$ (>0.8) and, therefore, have limited applicability. The isopiestic methods rely on the measurement of the moisture sorption isotherm and, therefore, entail lengthy procedures that gather information on the $a_w$ over a range of water content.

## QUALIFICATION OF WATER ACTIVITY METERS

Most water activity instruments, namely electronic hygrometers, dew point instruments, and others, are classified as Group B instruments as described in *Analytical Instrument Qualification* ⟨1058⟩. The qualification of a water activity instrument should cover the intended operational range of the instrument, the required accuracy of the instrument, and the required precision of the instrument (for more details, see ⟨1058⟩). Acceptance criteria for fitness for purpose are based on the uncertainty of $a_w$ generated by the reference material and the performance specification of the instrument.

Any type of water activity instrument that is shown to be fit for the intended purpose, by meeting the qualification criteria, may be used to provide $a_w$ results according to the guidelines of this chapter.

In contrast, NIR instruments are classified as Group C instruments as described in ⟨1058⟩. Therefore, the "analytical instrument qualification" described in ⟨1058⟩ and *Near-Infrared Spectroscopy* ⟨856⟩ is not applicable to these instruments.

## OPERATION AND CALIBRATION OF WATER ACTIVITY METERS

### Procedure for Standard Solutions

Water activity meters should be calibrated by using either prepared or purchased standard solutions or by using a calibrated humidity generator. Standard solutions may be prepared or purchased from the instrument manufacturer.

When using purchased solutions, follow the provided use instructions for these standard solutions, including any handling recommendations, storage recommendations, and expiry dating.

*Table 1* lists common saturated salt solutions that may be prepared by the user for calibration or checking the calibration of water activity instruments. Saturated salt solutions may be prepared using *Purified Water* and pure salts (≥99% purity). The salts do not need to be dried prior to use. Standard solutions should be prepared well before use to allow for equilibration of the salt solubility.[1] For previously prepared saturated salt solutions, it should be evident that the solution is still saturated due to the presence of additional undissolved salt, and liquid should completely cover the bottom of the sample container holding the saturated salt solution. Most water activity instrument sample cups are specifically designed to fit that instrument. The saturated salt solution may be prepared in the sample cup or may be prepared in a separate container and transferred to the sample cup for use. In either case, the standard solution should be placed into the sample cup according to the recommendations of the instrument manufacturer with regard to the amount of sample that should be present in the cup during the measurement. In addition to the saturated salt solutions listed in *Table 1*, *Purified Water* may be used as a 1.00 $a_w$ standard solution. It is not required to include a standard at zero water activity; rather, it is acceptable to use an appropriate low $a_w$ standard solution (e.g., lithium bromide or lithium chloride) for samples that have $a_w$ values up to 0.05 $a_w$ below the low $a_w$ standard solution.

**Table 1. Common Saturated Salt Solutions Routinely Used to Calibrate Water Activity Determination Instruments**

| Salt | Approximate Solubility at Saturation (g/100 mL) | $RH_{eq}$ (%) at 25° C[a] | $a_w$ at 25° C[a] |
|---|---|---|---|
| Potassium sulfate ($K_2SO_4$) | 11 | 97.30 | 0.973 ± 0.005 |
| Potassium chloride (KCl) | 34 | 84.34 | 0.843 ± 0.003 |
| Sodium chloride (NaCl) | 36 | 75.29 | 0.753 ± 0.001 |
| Sodium bromide (NaBr) | 91 | 57.57 | 0.576 ± 0.004 |
| Magnesium nitrate [$Mg(NO_3)_2$] | 70 | 52.89 | 0.529 ± 0.002 |
| Potassium carbonate ($K_2CO_3$) | 111 | 43.16 | 0.432 ± 0.004 |
| Magnesium chloride ($MgCl_2$) | 55 | 32.78 | 0.328 ± 0.002 |
| Potassium acetate ($KCH_3CO_2$) | 256 | 22.51 | 0.225 ± 0.003 |
| Lithium chloride (LiCl) | 84 | 11.30 | 0.113 ± 0.003 |
| Lithium bromide (LiBr) | 160 | 6.38 | 0.064 ± 0.005 |

[a] The equilibrium relative humidity for these salts at other temperatures, as well as humidities for other salts, are in Greenspan 1977 (*2*).

#### CALIBRATION OF WATER ACTIVITY METERS

Calibration should be performed annually, as well as any time the calibration check fails.

[1] A default equilibration time of 48 h is recommended for the initial preparation of saturated salt solutions; however, less time may be required if the water activity is confirmed to be stable experimentally. After preparation, the solution will thermally equilibrate to the temperature of use relatively quickly (i.e., 1–2 h).

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As described in *Types of Water Activity Instruments*, the sensors in many water activity meters may not provide a linear response over the entire usable water activity range. For instruments with significant non-linearity, a multi-point calibration may be required. However, it is also acceptable to use a two-point, linear calibration over a narrower range that brackets the range of anticipated use. Any time the range of intended use changes, a re-calibration of the instrument should be performed.

Water activity instruments may be adversely affected by significant changes in the atmospheric pressure in the laboratory. Some instruments may be able to correct for changes in the atmospheric pressure while others may require re-calibration. If the instrument includes a correction for atmospheric pressure, verify that the correct atmospheric pressure is used during the calibration, calibration check, and sample measurement procedures.

Perform the instrument calibration according to the directions of the instrument manufacturer using either prepared or purchased standard solutions or using a calibrated humidity generator.

Ideally, the calibration of the water activity instrument should be performed at the temperatures at which it will be used; however, this may not always be feasible. The calibration must be performed at a temperature for which the water activities of the standard solutions are accurately known. (Regardless of the temperature used for the calibration of the instrument, the calibration check should be performed at the temperature of use.)

Following successful calibration, proceed with the *Procedure for Calibration Check*.

## PROCEDURE FOR CALIBRATION CHECK

The purpose of the calibration check is to confirm that the instrument provides a measurement result that is statistically equivalent to the true value of the standard solution. It is acceptable to use either the instrument manufacturer's recommended acceptance criterion for a calibration check or the acceptance criterion described here. A calibration check should be performed each day the instrument is used to perform $a_w$ measurements (e.g., every 24 h during continual use). The results of the calibration check should be recorded so that the performance of the instrument can be monitored over time.

Any purchased or prepared standard solutions may be used to check the calibration of the instrument. It is recommended to use one or more standard solutions on a regular basis as a calibration check so that the performance of the instrument may be followed over time. Use of a low $a_w$ and high $a_w$ standard solution in a calibration check is recommended.

Preferably select a low $a_w$ and high $a_w$ standard solution that brackets the $a_w$ range of the samples to be evaluated or, alternatively, a single standard solution that is close to the expected $a_w$ of the samples. Perform the calibration check at the same temperature as the sample measurements. A successful calibration check may be performed either before or after the sample analysis. If there is a significant concern that sample measurements may adversely affect the water activity sensor (e.g., presence of volatiles or powders in the sample), it is recommended that a calibration check be performed after the sample analysis.

The acceptance criterion is that the absolute error (ABS ERR) must be less than the sum of the instrument repeatability and the uncertainty in the standard solution:

$$\text{ABS ERR} = \left| a_{w^\circ} - a_w \right| \leq (\text{Repeatability of the instrument}) + \left(\text{Uncertainty of } a_{w^\circ}\right)$$

$a_{w^\circ}$ = expected water activity for the standard salt solution at the temperature of the measurement (see *Table 1* or documentation for standard), repeatability of the instrument is twice the standard deviation of the instrument at that water activity,[2] and uncertainty of $a_{w^\circ}$ is either taken from *Table 1* or is provided by the supplier of the purchased standard solution. Because the $a_w$ result will be usually reported to two or three significant digits depending on the precision of the instrument, if the calculated acceptance criterion is calculated to be $\leq 0.01$, then the acceptance criterion of 0.01 should be used.

It is recommended to perform the calibration check with the same number of replicates as will be performed on the samples. For example, if single measurements will be tested and reported, then the calibration check should be performed with a single measurement. If replicate measurements will be performed, then the calibration check should use the same number of replicates and the acceptance criterion applied to the mean result. If the reading falls outside the acceptance range, clean the system and check the age of the standard solution. If the acceptance criterion is still not met, perform a new calibration.

In some use cases (e.g., use of water activity for process analytical measurements), it may be acceptable to allow for some instrument drift over time without requiring the re-calibration of the instrument. In these cases, the ABS ERR acceptance criterion should still be used to initially verify acceptable calibration of the instrument, but an alternative acceptance criterion can be used on subsequent calibration checks to verify acceptable calibration. As an example of a modified criterion:

$$\text{ABS ERR} = \left| a_{w^\circ} - a_w \right| \leq (\text{Repeatability of the instrument}) + \left(\text{Uncertainty of } a_{w^\circ}\right) + (\text{Acceptable drift})$$

Following a successful calibration check, proceed with the *Procedure for Sample Measurement*.

[2] The repeatability of the instrument may be based on the instrument manufacturer's characterization of the instrument capabilities or determined by performing replicate measurements on the standard solution as described in Precision (Repeatability).

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## Procedure for Sample Measurement

### SAMPLE PREPARATION

It is very important to ensure that after sampling, during transportation to the laboratory where $a_w$ determinations will take place, samples are kept in well-sealed, water-impermeable containers to protect them from any changes in water composition due to differences in temperature and/or *%RH* at different locations. It is recommended that the sample containers have minimal headspace or a larger sample mass to avoid significant changes in water activity due to re-equilibration of the sample to the environment in the sampling container. Large measurement errors can potentially result from 1) condensation of water on container walls due to temperature gradients in containers with samples that contain high water activity and 2) permeable sample containers exposed to high relative humidity. To prevent variability in the test results due to the exposure of the material to (and rapid re-equilibration with) the surrounding *%RH*, the analyst must be consistent with sample preparation. Performing the water activity test immediately after opening the sealed package is recommended.

To prepare a sample for testing, add the material to the sample container designed for use in the water activity meter. For uniform powders or liquids, it is recommended that the sample material should cover the bottom surface of the sample container entirely unless alternate methods have been verified. Avoid getting testing material on the outside, rim, or above the advised range as per manufacturer's instructions. For non-uniform samples, sample preparation details should be provided within monographs for products that specify the application of $a_w$ determinations. Details should include how the sample should be presented in the instrument (i.e., tablets could be presented as whole units or ground tablets, chewable gels could be sliced in thin pieces, and capsules could also be presented as whole units or capsule contents). The required approach should be specified together with the number of whole units or mass of ground-tablet powder/sliced gels/capsule contents.

### CALIBRATION CHECK

A calibration check should be performed every day the instrument is used to perform measurements. In addition, verify that the instrument is not due for its periodic calibration prior to performing measurements.

### MEASUREMENT PROCEDURE

Analyze the sample per the instrument manufacturer's instructions.

### REPORTING RESULTS

Record the equilibrium $a_w$ and the equilibrium temperature. Report the $a_w$ value or mean value of replicate measurements to two or more significant digits, depending on the application. Report the temperature rounded to the nearest 0.1° C.

## PROCEDURE FOR VERIFICATION AND VALIDATION

It is recommended that the testing laboratory conducts some verification of the $a_w$ method because $a_w$ determinations may be sensitive to variations in sampling procedures, the presence of other volatile components, variations in instrument types and option settings. The recommended parameters to evaluate for verification of the method are *Precision (Repeatability)* and *Intermediate Precision*.

## Precision (Repeatability)

Repeatability is assessed by conducting the $a_w$ determination on six separate aliquots of the same sample while applying the same sample preparation procedure and instrument settings intended for routine sample testing. Repeatability of the instrument can be assessed by repeated measurements of a salt standard solution in the same manner. If repeatability of the $a_w$ measurement on a sample is significantly greater than the repeatability of the instrument, then the instrument may not be suitable for routine measurements of that sample due to the presence of other volatile components interfering with the precision of the instrument.

The acceptance criterion is that the relative standard deviation is NMT 5.0% for $a_w > 0.4$, NMT 15% for $0.15 < a_w \leq 0.4$, and NMT 20% for $a_w \leq 0.15$.

## Intermediate Precision

The effect of random events on the analytical precision of the procedure should be evaluated. Typical variables include conducting the test on different days, using different instrumentation, and having different analysts perform the test. A combination of at least two of these factors totaling six determinations of aliquots taken from the same batch will provide an estimation of intermediate precision. The acceptance criterion is that the relative standard deviation is NMT 6.0% for $a_w > 0.4$, NMT 15% for $0.15 < a_w \leq 0.4$, and NMT 20% for $a_w \leq 0.15$.

## OPERATION, CALIBRATION, AND VALIDATION OF NIR INSTRUMENTS

As described in *Near-Infrared Spectroscopy*, NIR is based on a different physical principle than typical water activity meters and cannot be used for the measurement of the relative humidity of the air above the product. Therefore, the procedure for operation, calibration, and validation described above is not suitable for NIR instruments. More details about operation and

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calibration of NIR can be found in ⟨856⟩. Recommendations for the method development and validation of NIR instruments can be found in *Chemometrics* ⟨1039⟩ and ⟨1225⟩.

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# ⟨941⟩ CHARACTERIZATION OF CRYSTALLINE AND PARTIALLY CRYSTALLINE SOLIDS BY X-RAY POWDER DIFFRACTION (XRPD)

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▲Portions of the chapter text that are national *USP* text, and are not part of the harmonized text, are marked with symbols (◆) to specify this fact.▲ (USP 1-May-2022)

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## INTRODUCTION

Every crystalline phase of a given substance produces a characteristic X-ray diffraction pattern. Diffraction patterns can be obtained from a randomly oriented crystalline powder composed of crystallites ▲(crystalline regions within a particle)▲ (USP 1-May-2022) or crystal fragments of finite size. Essentially three types of information can be derived from a powder diffraction pattern: the angular position of diffraction lines (depending on geometry and size of the unit cell), the intensities of diffraction lines (depending mainly on atom type and arrangement and ▲preferred▲ (USP 1-May-2022) orientation within the sample), and diffraction line profiles (depending on instrumental resolution, crystallite size, strain, and specimen thickness).

Experiments giving angular positions and intensities of lines can be used for applications such as qualitative phase analysis (e.g., identification of crystalline phases) and quantitative phase analysis of crystalline materials. An estimate of the amorphous and crystalline fractions[1] can also be made.

The X-ray powder diffraction (XRPD) method provides an advantage over other means of analysis in that it is usually nondestructive in nature (to ensure a randomly oriented sample, specimen preparation is usually limited to grinding). XRPD investigations can also be carried out under in situ conditions on specimens exposed to nonambient conditions such as low or high temperature and humidity.

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## PRINCIPLE

X-ray diffraction results from the interaction between X-rays and electron clouds of atoms. Depending on atomic arrangement, interferences arise from the ▲elastically▲ (USP 1-May-2022) scattered X-rays. These interferences are constructive when the path difference between two diffracted X-ray waves differs by an integral number of wavelengths. This selective condition is described by the Bragg equation, also called Bragg's law (see *Figure 1*).

$$2d_{hkl}\sin\theta_{hkl} = n\lambda$$

[1] There are many other applications of the X-ray powder diffraction technique that can be applied to crystalline pharmaceutical substances, such as determination of crystal structures, refinement of crystal structures, determination of the crystallographic purity of crystalline phases, and characterization of crystallographic texture. These applications are not described in this chapter.

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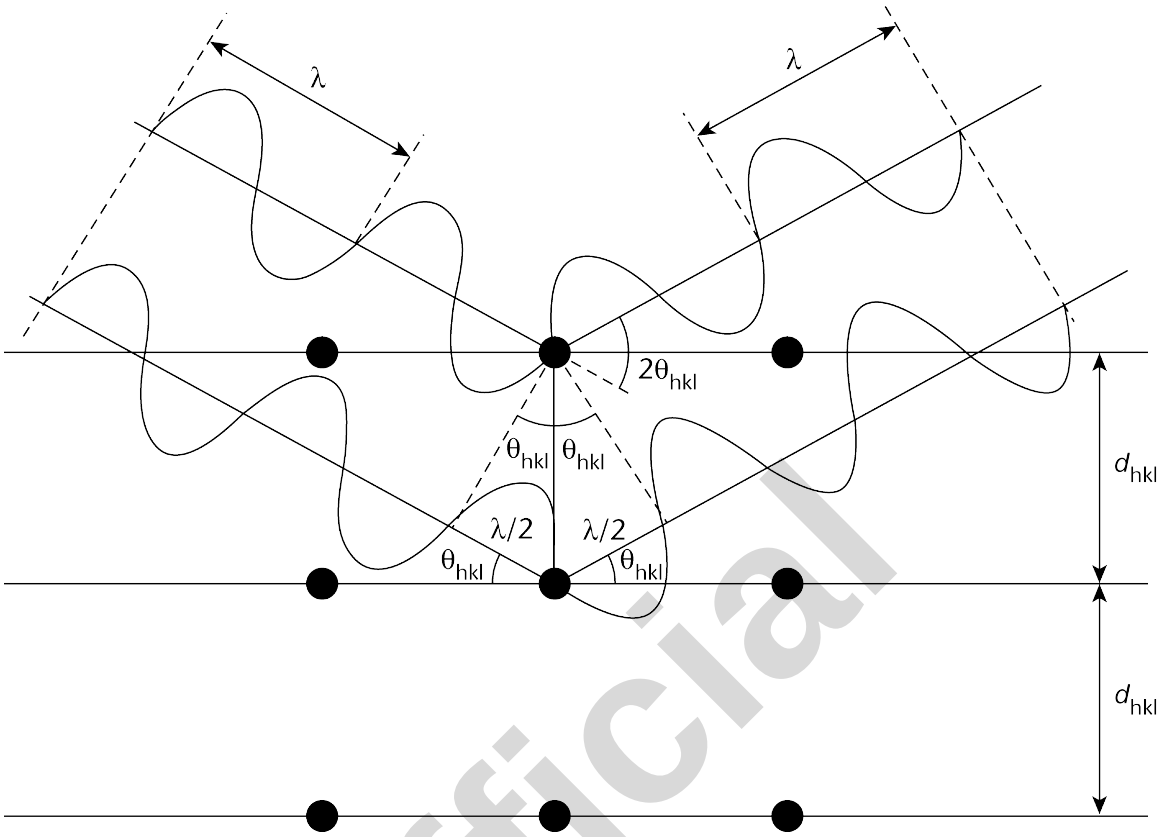


Figure 1. Diffraction of X-rays by a crystal according to Bragg's law.

The wavelength, λ, of the X-rays is of the same order of magnitude as the distance between successive crystal lattice planes, or $d_{hkl}$ (also called d-spacings). $\theta_{hkl}$ is the angle between the incident ray and the family of lattice planes, and sin $\theta_{hkl}$ is inversely proportional to the distance between successive crystal planes or d-spacings.

The direction and spacing of the planes with reference to the unit cell axes are defined by the Miller indices {hkl}. These indices are the reciprocals, reduced to the next-lower integer, of the intercepts that a plane makes with the unit cell axes. The unit cell dimensions are given by the spacings *a*, *b*, and *c*, and the angles between them α, β, and γ.

The interplanar spacing for a specified set of parallel hkl planes is denoted by $d_{hkl}$. Each such family of planes may show higher orders of diffraction where the *d* values for the related families of planes n*h*, n*k*, n*l* are diminished by the factor 1/n (*n* being an integer: 2, 3, 4, etc.).

Every set of planes throughout a crystal has a corresponding Bragg diffraction angle, $\theta_{hkl}$, associated with it (for a specific λ).

▲For a polycrystalline▲ (USP 1-May-2022) powder specimen ▲▲ (USP 1-May-2022) at any angle $\theta_{hkl}$ there are always crystallites in an orientation allowing diffraction according to Bragg's law.[2] For a given X-ray wavelength, the positions of the diffraction peaks (also referred to as "lines", "reflections", or "Bragg reflections") are characteristic of the crystal lattice (d-spacings), their theoretical intensities depend on the crystallographic unit cell content (nature and positions of atoms), and the line profiles depend on the perfection and extent of the crystal lattice. Under these conditions, the diffraction peak has a finite intensity arising from atomic arrangement, type of atoms, thermal motion, and structural imperfections, as well as from instrument characteristics.

The intensity is also dependent upon many factors such as structure factor, temperature factor, ▲▲ (USP 1-May-2022) polarization factor, multiplicity, ▲and Lorentz and microabsorption factors.▲ (USP 1-May-2022)

The main characteristics of diffraction line profiles are 2θ position, peak height, peak area, and shape (characterized by, e.g., peak width, or asymmetry, analytical function, and empirical representation). An example of ▲▲ (USP 1-May-2022) powder patterns obtained for five different solid phases of a substance ▲is▲ (USP 1-May-2022) shown in *Figure 2*.

[2] An ideal powder for diffraction experiments consists of a large number of small, randomly oriented spherical ▲particles▲ (USP 1-May-2022) (coherently diffracting crystalline domains). If this number is sufficiently large, there are always enough crystallites in any diffracting orientation to give reproducible diffraction patterns.

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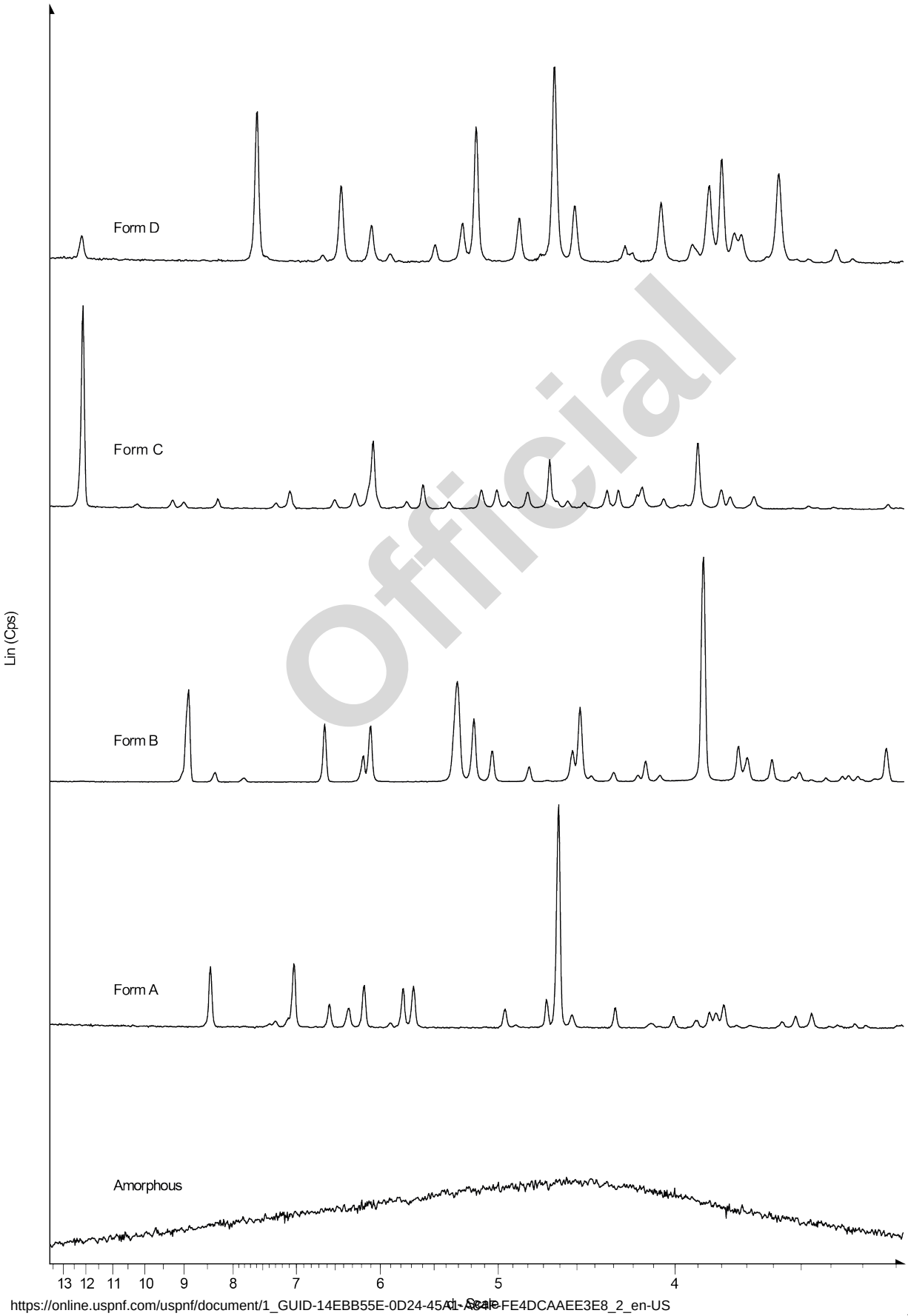


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Figure 2. X-ray powder diffraction patterns collected for five different solid phases of a substance (the intensities ▲of crystalline

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forms A–D▲ (USP 1-May-2022) are normalized).

In addition to the diffraction peaks, an X-ray diffraction experiment also generates a more or less uniform background, upon which the peaks are superimposed. Besides specimen preparation, other factors contribute to the background—for example, sample holder, diffuse scattering from air, ▲the sample▲ (USP 1-May-2022) and▲/or the▲ (USP 1-May-2022) equipment, and other instrumental parameters such as detector noise and general radiation from the X-ray tube. The peak-to-background ratio can be increased by minimizing background and by choosing prolonged exposure times.

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## INSTRUMENT

### Instrument Setup

X-ray diffraction experiments are usually performed using powder diffractometers or powder cameras.

A powder diffractometer generally comprises five main parts: an X-ray source; the incident beam optics, which may perform monochromatization, filtering, collimation, and/or focusing of the beam; a goniometer; the ▲diffracted▲ (USP 1-May-2022) beam optics, which may include monochromatization, ▲filtration,▲ (USP 1-May-2022) collimation, and▲/or▲ (USP 1-May-2022) focusing ▲▲ (USP 1-May-2022) of the beam; and a detector. Data collection and data processing systems are also required and are generally included ▲with▲ (USP 1-May-2022) current diffraction measurement equipment.

Depending on the type of analysis to be performed (phase identification, quantitative analysis, lattice parameters determination, etc.), different XRPD instrument configurations and performance levels are required. The simplest instruments used to measure powder patterns are powder cameras. Replacement of photographic film as the detection method by photon detectors has led to the design of diffractometers in which the geometric arrangement of the optics is not truly focusing, but parafocusing, such as in Bragg-Brentano geometry. The Bragg-Brentano parafocusing configuration is currently the most widely used and is therefore briefly described here.

A given instrument may provide a horizontal or vertical $\theta/2\theta$ geometry or a vertical $\theta/\theta$ geometry. For both geometries, the incident X-ray beam forms an angle $\theta$ with the specimen surface plane, and the diffracted X-ray beam forms an angle $2\theta$ with the direction of the incident X-ray beam (an angle $\theta$ with the specimen surface plane). ▲One example of a▲ (USP 1-May-2022) basic geometric arrangement is represented in *Figure 3*. The divergent beam of radiation from the X-ray tube (the so-called primary beam) passes through the ▲Soller slit▲ (USP 1-May-2022) and a divergence slit assembly and illuminates the flat surface of the specimen. All the rays diffracted by suitably oriented crystallites in the specimen at an angle $2\theta$ converge to a line at the receiving slit. A second set of ▲Soller slit▲ (USP 1-May-2022) and a scatter slit may be placed either behind or before the receiving slit; ▲the receiving slit is normally only used when a 0D detector is present.▲ (USP 1-May-2022) The axes of the line focus and of the receiving slit are at equal distances from the axis of the goniometer. The X-▲rays▲ (USP 1-May-2022) are counted by a ▲▲ (USP 1-May-2022) detector, usually a scintillation counter, ▲or▲ (USP 1-May-2022) a sealed-gas proportional counter. ▲However, currently,▲ (USP 1-May-2022) position-sensitive solid-state detectors ▲or hybrid photon counting detectors are more common.▲ (USP 1-May-2022) The receiving slit assembly and detector are coupled together and move tangentially to the focusing circle. For $\theta/2\theta$ scans, the goniometer rotates the specimen around the same axis as that of the detector, but at half the rotational speed, in a $\theta/2\theta$ motion. The surface of the specimen thus remains tangential to the focusing circle. The ▲Soller slit▲ (USP 1-May-2022) limits the axial divergence of the beam and hence partially controls the shape of the diffracted line profile.

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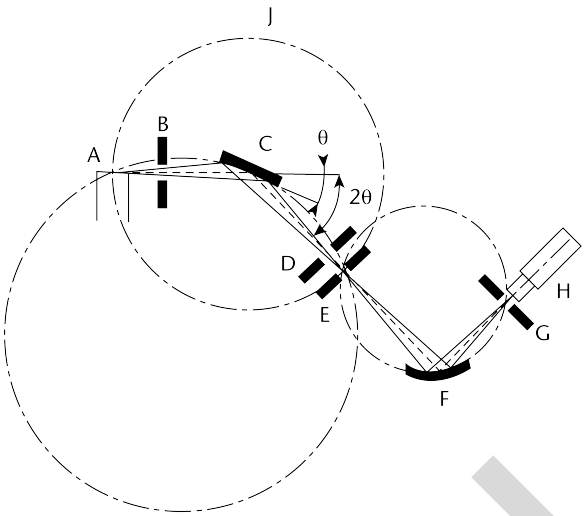


A. X-ray tube
B. Divergence slit
C. Sample
D. Anti-diffusion slit
E. Receiving slit
F. Monochromator
G. Detector receiving slit
H. Detector
J. Focusing circle

Figure 3. Geometric arrangement of the Bragg-Brentano parafocusing geometry.

A diffractometer may also be used in transmission mode. The advantage with this technology is to lessen the effects due to preferred orientation. A capillary of about 0.5- to 2-mm thickness can also be used for small sample amounts.

## X-Ray Radiation

In the laboratory, X-rays are obtained by bombarding a metal anode with electrons emitted by the thermionic effect and accelerated in a strong electric field (using a high-voltage generator). Most of the kinetic energy of the electrons is converted to heat, which limits the power of the tubes and requires efficient anode cooling. A 20- to 30-fold increase in brilliance can be obtained by using rotating anodes and by using X-ray optics. Alternatively, X-ray photons may be produced in a large-scale facility (synchrotron).

The spectrum emitted by an X-ray tube operating at sufficient voltage consists of a continuous background of polychromatic radiation ▲(Bremsstrahlung or white radiation)▲ (USP 1-May-2022) and additional characteristic radiation that depends on the type of anode. ▲Normally▲ (USP 1-May-2022) only this characteristic radiation is used in X-ray diffraction experiments. The principal radiation sources used for X-ray diffraction are vacuum tubes using copper, molybdenum, iron, cobalt, ▲silver,▲ (USP 1-May-2022) or chromium as anodes; copper ▲and▲ (USP 1-May-2022) molybdenum ▲▲ (USP 1-May-2022) X-rays are employed most commonly for organic substances▲▲ (USP 1-May-2022). The choice of radiation to be used depends on the absorption characteristics of the specimen and possible fluorescence by atoms present in the specimen. The wavelengths used in powder diffraction generally correspond to the $K_\alpha$ radiation from the anode. Consequently, it is advantageous to make the X-ray beam "monochromatic" by eliminating all the other components of the emission spectrum. This can be partly achieved using $K_\beta$ filters—that is, metal filters selected as having an absorption edge between the $K_\alpha$ and $K_\beta$ wavelengths emitted by the tube. Such a filter is usually inserted between the X-ray tube and the specimen. Another more commonly used way to obtain a monochromatic X-ray beam is via a large monochromator crystal (usually referred to as a "monochromator"). This crystal is placed before or behind the specimen and diffracts the different characteristic peaks of the X-ray beam (i.e., $K_\alpha$ and $K_\beta$) at different angles so that only one of them may be selected to enter into the detector. It is even possible to separate $K_{\alpha1}$ and $K_{\alpha2}$ radiations by using a specialized monochromator. Unfortunately, the gain in getting a monochromatic beam by using a filter or a monochromator is counteracted by a loss in intensity. Another way of separating $K_\alpha$ and $K_\beta$ wavelengths is by using curved X-ray mirrors that can simultaneously monochromate and focus or parallelize the X-ray beam.

### RADIATION PROTECTION

Exposure of any part of the human body to X-rays can be injurious to health. It is therefore essential that whenever X-ray equipment is used, adequate precautions be taken to protect the operator and any other person in the vicinity. Recommended practice for radiation protection as well as limits for the levels of X-radiation exposure are those established by national legislation in each country. If there are no official regulations or recommendations in a country, the latest recommendations of the International Commission on Radiological Protection should be applied.

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## SPECIMEN PREPARATION AND MOUNTING

The preparation of the powdered material and the mounting of the specimen in a suitable holder are critical steps in many analytical methods, particularly for X-ray powder diffraction analysis, since they can greatly affect the quality of the data to be collected.[3] The main sources of errors due to specimen preparation and mounting are briefly discussed in the following section for instruments in Bragg-Brentano parafocusing geometry.

### Specimen Preparation

In general, the morphology of many crystalline particles tends to give a specimen that exhibits some degree of preferred orientation in the specimen holder. This is particularly evident for needle-like or plate-like crystals when size reduction yields finer needles or platelets. Preferred orientation in the specimen influences the intensities of various reflections so that some are more intense and others less intense, compared to what would be expected from a completely random specimen. Several techniques can be employed to improve randomness in the orientation of crystallites (and therefore to minimize preferred orientation), but further reduction of particle size is often the best and simplest approach. The optimum number of crystallites depends on the diffractometer geometry, the required resolution, and the specimen attenuation of the X-ray beam. In some cases, particle sizes as large as 50 µm will provide satisfactory results in phase identification. However, excessive milling (▲particle▲ (USP 1-May-2022) sizes less than approximately 0.5 µm) may cause line broadening and significant changes to the sample itself, such as:

- Specimen contamination by particles abraded from the milling instruments (mortar, pestle, balls, etc.)
- Reduced degree of crystallinity
- Solid-state transition to another polymorph
- Chemical decomposition
- Introduction of internal stress
- Solid-state reactions

Therefore, it is advisable to compare the diffraction pattern of the nonground specimen with that corresponding to a specimen of smaller particle size (e.g., a milled specimen). If the X-ray powder diffraction pattern obtained is of adequate quality considering its intended use, then grinding may not be required.

It should be noted that if a sample contains more than one phase and if sieving is used to isolate particles to a specific size, the initial composition may be altered.

### Specimen Mounting

#### EFFECT OF SPECIMEN DISPLACEMENT

A specimen surface that is offset by $D$ with reference to the diffractometer rotation axis causes systematic errors that are very difficult to avoid entirely; for the reflection mode, this results in absolute $D \cdot \cos\theta$ shifts[4] in $2\theta$ positions (typically of the order of 0.01° in $2\theta$ at low angles $[\cos\ \theta\ \simeq 1]$ for a displacement $D = 15$ µm) and asymmetric broadening of the profile toward low $2\theta$ values. Use of an appropriate internal standard allows the detection and correction of this effect simultaneously with that arising from specimen transparency. This effect is by far the largest source of errors in data collected on well aligned diffractometers.

#### EFFECT OF SPECIMEN THICKNESS AND TRANSPARENCY

When the XRPD method in reflection mode is applied, it is often preferable to work with specimens of “infinite thickness”. To minimize the transparency effect, it is advisable to use a nondiffracting substrate (zero background holder)—for example, a plate of single crystalline silicon cut parallel to the 510 lattice planes.[5] One advantage of the transmission mode is that problems with sample height and specimen transparency are less important.

The use of an appropriate internal standard allows the detection and correction of this effect simultaneously with that arising from specimen displacement.

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## CONTROL OF THE INSTRUMENT PERFORMANCE

The goniometer and the corresponding incident and diffracted X-ray beam optics have many mechanical parts that need adjustment. The degree of alignment or misalignment directly influences the quality of the results of an XRPD investigation. Therefore, the different components of the diffractometer must be carefully adjusted (optical and mechanical systems, etc.) to

[3] Similarly, changes in the specimen can occur during data collection in the case of a nonequilibrium specimen (temperature, humidity).

[4] Note that a goniometer zero alignment shift would result in a constant shift on all observed $2\theta$-line positions; in other words, the whole diffraction pattern is, in this case, translated by an offset of Z° in $2\theta$.

[5] In the case of a thin specimen with low attenuation, accurate measurements of line positions can be made with focusing diffractometer configurations in either transmission or reflection geometry. Accurate measurements of line positions on specimens with low attenuation are preferably made using diffractometers with parallel beam optics. This helps to reduce the effects of specimen thickness.

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adequately minimize systematic errors, while optimizing the intensities received by the detector. The search for maximum intensity and maximum resolution is always antagonistic when aligning a diffractometer. Hence, the best compromise must be sought while performing the alignment procedure. There are many different configurations, and each supplier's equipment requires specific alignment procedures.

The overall diffractometer performance must be tested and monitored periodically, using suitable certified reference materials, ▲e.g., silicon powder or α-alumina (corundum).▲ (USP 1-May-2022) Depending on the type of analysis, other well defined reference materials may also be employed, although the use of certified reference materials is preferred.

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## QUALITATIVE PHASE ANALYSIS (IDENTIFICATION OF PHASES)

The identification of the phase composition of an unknown sample by XRPD is usually based on the visual or computer-assisted comparison of a portion of its X-ray powder pattern to the experimental or calculated pattern of a reference material. Ideally, these reference patterns are collected on well characterized single-phase specimens. This approach makes it possible in most cases to identify a crystalline substance by its 2θ diffraction angles or d-spacings and by its relative intensities. The computer-aided comparison of the diffraction pattern of the unknown sample to the ▲reference▲ (USP 1-May-2022) data can be based on either a more or less extended 2θ range of the whole diffraction pattern or on a set of reduced data derived from the pattern. For example, the list of d-spacings and normalized intensities, $I_{norm}$, a so-called ($d$, $I_{norm}$) list extracted from the pattern, is the crystallographic fingerprint of the material and can be compared to ($d$, $I_{norm}$) lists of single-phase samples compiled in databases.

For most organic crystals, when using Cu $K_\alpha$ radiation, it is appropriate to record the diffraction pattern in a 2θ range from as near 0° as possible to at least ▲30°.▲ (USP 1-May-2022) The agreement in the 2θ diffraction angles between specimen and reference is ▲expected to be▲ (USP 1-May-2022) within 0.2° for the same crystal form, whereas relative intensities between specimen and reference may vary considerably due to preferred orientation effects. By their very nature, variable hydrates and solvates are recognized to have varying unit cell dimensions, and as such, shifting occurs in peak positions of the measured XRPD patterns for these materials. In these unique materials, variance in 2θ positions of greater than 0.2° is not unexpected. As such, peak position variances such as 0.2° are not applicable to these materials. For other types of samples (e.g., inorganic salts), it may be necessary to extend the 2θ region scanned to well beyond ▲30°.▲ (USP 1-May-2022) It is generally sufficient to scan past the 10 strongest reflections identified in single-phase X-ray powder diffraction database files.

It is sometimes difficult or even impossible to identify phases in the following cases:

- Noncrystallized or amorphous substances
- The components to be identified are present in low mass fractions of the analyte amounts (generally less than 10% m/m)
- Pronounced preferred orientation effects
- The phase has not been filed in the database used
- The formation of solid solutions
- The presence of disordered structures that alter the unit cell
- The specimen comprises too many phases
- The presence of lattice deformations
- The structural similarity of different phases

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## QUANTITATIVE PHASE ANALYSIS

If the sample under investigation is a mixture of two or more known phases, of which not more than one is amorphous, the percentage (by volume or by mass) of each crystalline phase and of the amorphous phase can in many cases be determined. Quantitative phase analysis can be based on the integrated intensities, on the peak heights of several individual diffraction lines,[6] or on the full pattern. These integrated intensities, peak heights, or full-pattern data points are compared to the corresponding values of reference materials. These reference materials must be single phase or a mixture of known phases. The difficulties encountered during quantitative analysis are due to specimen preparation (the accuracy and precision of the results require, in particular, homogeneity of all phases and a suitable particle size distribution in each phase) and to matrix effects.

▲Amounts▲ (USP 1-May-2022) of crystalline phases as small as 10% may be determined in solid matrices, ▲and in favorable cases amounts of crystalline phases less than 10% may be determined.▲ (USP 1-May-2022)

### Polymorphic Samples

For a sample composed of two polymorphic phases $a$ and $b$, the following expression may be used to quantify the fraction $F_\alpha$ of phase $a$:

$$F_\alpha = 1/[1 + K(I_b/I_a)]$$

[6] If the crystal structures of all components are known, the Rietveld method can be used to quantify them with good accuracy. If the crystal structures of the components are not known, the Pawley method or the partial least-squares (PLS) method can be used.

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The fraction is derived by measuring the intensity ratio between the two phases, knowing the value of the constant $K$. $K$ is the ratio of the absolute intensities of the two pure polymorphic phases $I_{oa}/I_{ob}$. Its value can be determined by measuring standard samples.

## Methods Using a Standard

The most commonly used methods for quantitative analysis are as follows:

- The external standard method
- The internal standard method
- The spiking method (also often called the standard addition method)

The external standard method is the most general method and consists of comparing the X-ray diffraction pattern of the mixture, or the respective line intensities, with those measured in a reference mixture or with the theoretical intensities of a structural model, if it is fully known.

To limit errors due to matrix effects, an internal reference material can be used that has a crystallite size and X-ray absorption coefficient comparable to those of the components of the sample and with a diffraction pattern that does not overlap at all that of the sample to be analyzed. A known quantity of this reference material is added to the sample to be analyzed and to each of the reference mixtures. Under these conditions, a linear relationship between line intensity and concentration exists. This application, called the internal standard method, requires precise measurement of diffraction intensities.

In the spiking method (or standard addition method), some of the pure phase *a* is added to the mixture containing the unknown concentration of ▲phase▲ (USP 1-May-2022) *a*. Multiple additions are made to prepare an intensity-versus-concentration plot in which the negative *x*-intercept is the concentration of the phase *a* in the original sample.

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## ESTIMATE OF THE AMORPHOUS AND CRYSTALLINE FRACTIONS

In a mixture of crystalline and amorphous phases, the crystalline and amorphous fractions can be estimated in several ways. The choice of the method used depends on the nature of the sample:

- If the sample consists of crystalline fractions and an amorphous fraction of different chemical compositions, the amounts of each of the individual crystalline phases may be estimated using appropriate standard substances, as described above. The amorphous fraction is then deduced indirectly by subtraction.
- If the sample consists of one amorphous and one crystalline fraction, either as a 1- or a 2-phase mixture, with the same elemental composition, the amount of the crystalline phase (the "degree of crystallinity") can be estimated by measuring three areas of the diffractogram:

  $A$ = ▲sum of the▲ (USP 1-May-2022) area of ▲all▲ (USP 1-May-2022) the peaks arising from diffraction ▲of▲ (USP 1-May-2022) the crystalline fraction of the sample

  $B$ = ▲▲ (USP 1-May-2022) area ▲under the diffractogram generated by the sample (excluding▲ (USP 1-May-2022) area $A$)

  $C$ = ▲area of the▲ (USP 1-May-2022) background ▲noise▲ (USP 1-May-2022) (due to air scattering, fluorescence, equipment, etc.)

When these areas have been measured, the degree of crystallinity can be roughly estimated as:

$$\% \text{ Crystallinity} = 100A/(A + B - C)$$

It is noteworthy that this method does not yield an absolute degree of crystallinity values and hence is generally used for comparative purposes only. More sophisticated methods are also available, such as the Ruland method.

## SINGLE CRYSTAL STRUCTURE

In general, the determination of crystal structures is performed from X-ray diffraction data obtained using single crystals. However, crystal structure analysis of organic crystals is a challenging task, since the lattice parameters are comparatively large, the symmetry is low, and the scattering properties are normally very low. For any given crystalline form of a substance, the knowledge of the crystal structure allows for calculating the corresponding XRPD pattern, thereby providing a preferred orientation-free reference XRPD pattern, which may be used for phase identification.

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# ▲⟨1001⟩ IN VITRO RELEASE TEST METHODS FOR PARENTERAL DRUG PREPARATIONS

## INTRODUCTION

Parenterally administered drugs include injections that are administered through the external boundary tissue with subsequent immediate release or extended release of the drug substance into the circulatory system. Parenteral drugs also include drug preparations that are administered by placement within the body to achieve, for instance, extended release of drug substance at a local area. Immediate and modified-release parenteral drug products exist as injections (solutions, suspensions, emulsions) and implants. Immediately prior to administration, the parenteral drug preparation may be reconstituted from sterile powders to form solutions, suspensions, or emulsions. Such parenteral drug preparations also include drug/device combinations such as stents. Generally, performance tests, as discussed in this chapter, are not required for aqueous solutions.

The definitions and descriptions of dosage forms and drug preparations discussed in this chapter, as well as information about their composition and manufacturing processes, can be found in *Pharmaceutical Dosage Forms* ⟨1151⟩.

The intention of ⟨1001⟩ is to provide guidance on methods that have been demonstrated as useful for the evaluation of product release performance testing of parenteral drug preparations. When reference is made to "dissolution", "in vitro release", "in vitro drug release", "drug release", and "performance" in this chapter, these terms are intended to have the same meaning. This chapter is not intended to establish new testing requirements for parenteral drug preparations.

## COMMON PRINCIPLES

For parenteral dosage forms and parenteral drug preparations, the purpose of both the product quality tests and product performance tests is to assure batch-to-batch quality, reproducibility, reliability, and performance. Product quality tests are performed to assess attributes such as assay, identification, impurities, foreign particulate matter, sterility, bacterial endotoxins, water content, aluminum content, and content uniformity. These tests are part of the compendial monograph (see *Injections and Implanted Drug Products* ⟨1⟩). Method selection can be challenging for many reasons, and each preparation carries its own unique obstacles. *The Dissolution Procedure: Development and Validation* ⟨1092⟩ is an important reference to use when developing an in vitro release test procedure. It is advisable to consult with the appropriate regulatory agency to confirm the adequacy of an in-house in vitro drug release test for a drug product before filing the application.

The drug release test method should be developed early in product development so it can be used to characterize the preparations utilized in safety and efficacy trials. The drug release data for the batches used in these studies support the specifications for the drug release test at product release and expiry. Furthermore, it is important to coordinate the in vitro drug release method strategy for product development with the quality control (QC) method for release of the product. This strategy is useful in product life-cycle management to support possible manufacturing changes.

Each type of parenteral dosage form or preparation is discussed in this chapter, including highlights of the preparation's unique challenges. Examples of methods for current preparations are provided; these examples come from multiple sources including, but not limited to, the Food and Drug Administration's Dissolution Methods Database (*1*), workshop reports (*2–3*), *Pharmacopeial Forum* articles (*4–6*), and other scientific literature. For several parenteral drug preparations, procedures described in *Dissolution* ⟨711⟩, *Drug Release* ⟨724⟩, *Mucosal Drug Products—Performance Tests* ⟨1004⟩, or *Semisolid Drug Products—Performance Tests* ⟨1724⟩ may be applicable.

The FDA's Dissolution Methods Database currently includes several injections and performance test methods for injectable suspensions. When a specific method is not given in this database, the instructions state, "Develop a dissolution method using USP 4 (Flow-Through Cell), and, if applicable, Apparatus 2 (Paddle) or any other appropriate method, for comparative evaluation by the Agency." The injectable suspensions with methods provided use USP Apparatus 4 and 2, and the test media may include sodium lauryl sulfate (SLS), polysorbate, saline, water, and a small amount of ethanol. The testing time ranges from 45 min to as long as 168 h. Accelerated in vitro release test conditions may be necessary if a performance test method serves as a drug product QC tool as long time frames are generally not practical.

There are many types of extended-release parenteral drug products commercialized in the veterinary industry. The FDA's Center for Veterinary Medicine (CVM) Guidance for Industry #238, provides guidance and strategies for consideration as part of the development program for drug release testing and product specifications (*7*).

Because of the complex nature of some parenterals, in vitro drug release test methods should be applied on a case-by-case rather than one-size-fits-all basis; however, the inclusion of general approaches and the presentation of options can be useful. Some parenteral drug preparations provide continuous release of the drug substance(s) for weeks, months, or years. The in vitro release test conditions can, although they are not required to, mimic specific aspects of the intended physiological environment such as the osmolarity, pH, or buffer capacity. Non-sink conditions may be informative in some instances (sink conditions are defined in ⟨1092⟩). The composition of the drug-release media may be kept constant with regard to the physiological parameters for pH (e.g., pH 7.4) and osmotic pressure (e.g., 285 mOsm/kg). Because of the solubility of the drug substance and the requirement for an accelerated test, the addition of surfactants (e.g., polysorbate 80 or SLS) and/or organic solvents may be needed. Accelerated in vitro release testing is a practical solution for QC testing; however, it should be justified as having relevance. This is historically established through clinical trial data along with patient use and may be tied to product attributes that are controlled through validated manufacturing parameters (e.g., excipient quality and preparation, mixing, and time conditions) and batch release testing.

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It is well known that the in vitro release of a drug from a drug product is preparation dependent. Therefore, it is unlikely a universal in vitro release test method can be designed to characterize drug release for the wide variety of existing parenteral dosage forms and preparations that exist. Assessing the suitability of a method for a preparation is therefore crucial.

Characteristics of an in vitro drug release test for parenteral drug preparations may include, but are not limited to, the following:

- Distinguishes a compliant batch [where a compliant batch is bioequivalent (BE) to the pivotal batches]
- Mimics the drug release conditions in vivo
- Utilizes early sampling to characterize the initial release

The performance test method may need to discriminate the critical quality attributes, which may include initial release; and any material, process, or product attribute that affects the subsequent release rate. In vitro release testing should be conducted with parenteral drug preparations that are manufactured under target conditions then compared to drug products that are intentionally manufactured with critical attribute variations in preparation and manufacturing parameters (*8*–*9*).

Multiple components/manipulations make up the drug release methods and are reported in the literature, and their associated equipment (or apparatus), are summarized in *Table 1* (*10*).

**Table 1. Examples of Equipment and Techniques that Could be Incorporated into a Drug Release Method**

| Techniques | Continuous Sampling | Unique Advantages | Limitations |
|---|---|---|---|
| Dialysis | Yes | Filtration usually not required | Dialysis membrane, rather than the preparation, may be rate limiting; nonspecific membrane adsorption |
| Reverse dialysis/microdialysis | Yes | Filtration usually not required; direct measurement with microdialysis | Dialysis membrane, rather than the preparation, may be rate limiting; nonspecific membrane adsorption |
| Centrifugation | No | Filtration not required | Preparation dependent; sample may not be resuspendable; nonspecific membrane adsorption |
| Centrifugal filtration | No | Low centrifugal force possible | Filter clogging/particle deformation |
| Pressure ultrafiltration | No | Gentle separation at low pressure | Filter clogging/particle deformation |
| Size-exclusion chromatography | Yes | Selection of filter media | Column presaturation needed |
| In situ method (fiber optics) | Yes | Direct measurement | Polarography and UV/Vis limited |
| Continuous flow method | Yes | Flexible media volume | Filter clogging |
| USP Apparatus 4 | Yes | Flexible media volume | Filter clogging/potentially high volume of release media |
| USP Apparatus 1 | Yes | Well-characterized equipment | Defined maximum and minimum volume |
| USP Apparatus 2 | Yes | Well-characterized equipment | Defined maximum and minimum volume |

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The drug release test for some parenteral dosage forms may require the use of modified compendial or noncompendial equipment. For example, various volumes of dissolution media with or without agitation may be appropriate. The use of a dialysis membrane as well as the use of incubation methods have been shown to be beneficial for some preparations. *Table 2* provides known examples of suitable apparatus for types of preparations.

**Table 2. Known Examples of Apparatus Used for In Vitro Release for Parenteral Preparations**

| Preparations | Apparatus |
|---|---|
| Nonaqueous solutions/oily preparations | Apparatus 2 |
| Suspensions | Apparatus 2, Apparatus 4, reduced volume apparatus, dialysis cell |
| Nanosuspensions | Apparatus 2, Apparatus 4, dialysis cell, reduced volume |
| Liposomes | Apparatus 1, Apparatus 2, Apparatus 4, dialysis cell |
| Microparticles | Apparatus 2, Apparatus 4, incubation jar, dialysis cell |
| Powders for suspension | Apparatus 2, Apparatus 4, reduced volume apparatus, dialysis cell |
| Emulsions | Apparatus 2, reduced volume apparatus, vertical diffusion cell |
| Implants | Apparatus 2, Apparatus 4, Apparatus 7, incubation jar |
| Drug-eluting stents (DES) | Apparatus 7, Apparatus 4, modified flow-through cell |
| In-situ forming preparations | Apparatus 2, Apparatus 4, Apparatus 7, incubation jar |

When long-term in vitro release tests are needed, special attention should be paid to the evaporation of the test media, microbial growth, and chemical degradation of the released/dissolved drug as discussed below:

- **Evaporation:** Sealed containers (e.g., jars or vials) and Apparatus 4 help prevent test media evaporation. Where justified, it may be possible to include an internal standard to correct for test media volume changes due to evaporation. An

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appropriate internal standard would be chemically stable, nonvolatile, and possess suitable chromatographic or spectral properties so that it can be quickly and reliably quantified independently of the active ingredient. If a poorly sealed apparatus is used for real-time tests (e.g., Apparatus 2) with an internal standard, consideration should be given to whether the reduction in volume over time will affect the sink conditions of the test media.

- **Microbial growth:** Microbial growth may be limited by using preservatives in the test media. Concentrations between 0.1% and 0.01% w/v sodium azide are reported in the literature (*11–12*). It should be noted that sodium azide is toxic to humans and may be a carcinogen, so alternative antimicrobials should be considered.
- **Chemical degradation:** Degradation of the released or dissolved drug in the test media may be partially or wholly addressed by using a nonspecific measurement technique such as UV-Vis spectroscopy, HPLC with class separation (the drug and all solution-phase degradants coelute), or summing of all drug-related peaks (with appropriate justification). The latter approach is predicated on the common observation that solution-phase chemistry tends to be much faster than solid-phase. In other words, the drug likely degrades after dissolution, so summing all degradation products and the parent compound in solution is likely a good indication of the quantity of drug that dissolved. In some cases, the addition of an antioxidant to the dissolution media or the use of an alternate pH where the drug is more stable may also be useful. Lastly, the use of Apparatus 4 in open loop mode may help mitigate degradation, because a dissolved drug does not remain in the system for the duration of the test, but open loop may create challenges for detection sensitivity.

In the case of accelerated in vitro release methods, many of the same considerations described above apply, i.e., elevated temperature can promote evaporation and chemical degradation.

Although desirable, in vitro and in vivo correlations may not be possible for modified-release parenteral dosage forms due to the complexity of the release mechanisms and the lack of knowledge about in vivo release conditions. However, the in vitro release method should reflect the route of administration. An in vitro drug release method with proven IVIVC potential would have a long-term advantage in terms of serving as a surrogate for in vivo BE testing required to qualify higher level scale-up and post approval changes for modified release (SUPAC-MR).

The following sections provide performance test guidance that is unique to parenteral dosage forms and parenteral preparations discussed in this chapter.

## SOLUTIONS

One recent example reported by Priddy et al. describes the work conducted for a veterinary drug product consisting of a lipophilic drug dissolved in a low-viscosity oily matrix (*13*). This publication describes important considerations such as the identification of a suitable medium that dissolves the drug over time without degrading it and the development of a system and methodology that can be used as a QC lab tool and as a way to discriminate a quality drug product from one that was formulated or processed incorrectly.

## SUSPENSIONS

Suspension dosage forms are solids suspended in a suitable liquid. Examples of suspensions include liposomes, microparticles, poorly water-soluble drug substances, and drug substances deliberately formulated to achieve prolongation of their release compared to that observed or anticipated for an immediate-release dosage form. Nanosuspensions consist of solids with any dimension inside and outside the nanoscale range of 1–100 nm but with properties attributed to its dimension (less than 1000 nm), suspended in a suitable liquid, typically water with other components (*6,14–15* and *Drug Products Containing Nanomaterials* ⟨1153⟩).

The FDA provides guidance on in vitro performance testing of modified-release parenteral dosage forms and drug preparations containing nanomaterials (*14*). Ideally in vitro drug release testing should be performed using compendial instruments described in ⟨711⟩ and ⟨724⟩. However, use of compendial instruments is not required.

Determination of the test media composition is the first key step to setting up an in vitro performance test (e.g., choice of organic solvent(s), surfactant, buffer, and pH).

Addition of the suspension test article to the test vessel or cell is another key step. For example, addition of suspension test articles into the test media may significantly alter the inherent drug release and/or dissolution characteristics of the suspension depending on the composition and volume of the test media and how the suspension test article is diluted prior to dissolution.

Another key step is sampling from the test media, including separating the test article from the dissolved drug (*16*). Many isolation techniques are employed to separate the test article from the released drug. Two major filtration technologies are used to isolate test samples. One is dead-end filtration, where particles are collected on the surface of the filter device. The second type of filtration is cross-flow filtration, where the suspension flows across the surface of a membrane filter with minimal particle buildup on the filter. Filters with a particle pore size smaller than 100 nm may be considered. In situ fiber optics combined with derivative spectroscopy allow quantification of the dissolved drug substance in the presence of the bound or encapsulated drug substance associated with the nanoparticle (*17–18*).

Compendial instruments with a basket, paddle, or flow-through cell can accommodate a bag or pouch (e.g., dialysis tubing clamped at both ends) that contains the suspension test article. The bag or pouch can consist of a dialysis membrane with a defined molecular weight cut-off. Another dialysis technique (known as reverse dialysis) uses a dialysis bag or pouch with only test media inside. This pouch is placed in the test media containing the suspension test article. Samples of released drug are taken from inside the pouch. This technique allows for placement of test article in the bulk media to, for example, minimize the agglomeration and aggregation effects.

Nanosuspensions or suspensions can be deliberately formulated to prolong or accelerate the rate of release of a drug based on the poor water solubility of the drug and on the in vivo diffusion dynamics of the drug in the biological fluid or matrix. In such cases, dissolution of the suspension in a typical performance testing apparatus may be very rapid. The provision of full solubility is typically required in order to meet regulatory expectations (able to meet $Q = 80$, for example), and biologically

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relevant diffusion barriers are difficult to construct. For such suspensions, the most likely product attribute for which the performance test must discriminate (most likely to affect pharmacokinetics) may be the specific surface area (SSA) of the drug substance particles, for which drug substance particle size is often a good surrogate. As such, any in vitro release test that measures the impact of variations in SSA or particle-size distribution may be appropriate for performance control, regardless of the speed of dissolution. Consideration should also be given as to whether a direct measurement of particle size (e.g., via light scattering) may be a better measurement of likely in vivo product performance.

## LIPOSOMES

Liposomes are submicron to micron-sized vesicles composed of a single lipid bilayer surrounding an aqueous core or a concentric series of multiple bilayers separated by aqueous compartments. The bilayers are formed by amphipathic molecules, such as phospholipids and cholesterol. Generally, liposome drug products comprise the drug substance and lipid components, as well as non-lipid inactive ingredients.

In liposomal drug products, the drug substance can be encapsulated within the aqueous compartment(s) [hydrophilic drugs] , intercalated within the lipid bilayer(s) [hydrophobic drugs], or associated with the surface(s) of lipid bilayer(s) [charged and/ or amphiphilic drugs]. Drug release is not a consequence of dissolution of the liposomes. It is dependent on a combination of factors including the following:

- Lipid composition
- Nature of the drug (hydrophilic, hydrophobic, charged or uncharged)
- Location in the liposomes
- Molecular state (e.g., precipitated in the aqueous compartment or independent from environmental factors such as pH, counter ions, etc.)
- Liposome manufacturing/drug loading process (can affect the molecular state of the drug as well as the state of the bilayer)

In vitro drug release testing is performed to characterize the functionality of the lipid bilayer. Such drug release testing can also test for uncontrolled leakage (“dose-dumping”). In vitro release test conditions are chosen to discriminate changes that can occur in the drug product (the state of the lipid bilayer or the associated drug) during manufacturing or storage or in vivo. In vitro drug release tests are used for lot release and stability testing. Examples of in vitro release techniques include flow-through cells with dialysis membranes (USP Apparatus 4) and other dialysis methods. Furthermore, separation methods, such as ultracentrifugation or filtration, can be employed.

Development of in vitro drug release assays can be challenging due to the complex dependence of release on the specific nature of the liposome preparation and drug. Standardized in vitro release techniques do not exist. Existing guidance documents refer to specific drug products on the market. An FDA guidance document on chemistry, manufacturing, and control (CMC) aspects of liposomes is available (*19*). At present, developers of liposomal drug preparations have proprietary in vitro drug release methods for use in the QC of their preparations. More collaborative efforts among academia, industry, and regulators are needed to standardize in vitro drug release testing of liposomes (*20*).

## MICROPARTICLES

Microparticles, or microspheres, (⟨1151⟩) are larger than 100 nm and are typically incorporated into suspensions for injection. Often microparticles are powders containing extended-release particles that just prior to administration are suspended in a suitable liquid. There are two distinct challenges when performing an in vitro drug release test on microparticles. First, if microparticles do not wet well, as observed by floating or aggregating, then the test media can be modified by adding a small quantity of surfactant to the media to reduce the surface tension. Secondly, microparticles may require a prolonged testing time, which could cause extensive evaporation of the dissolution media and chemical degradation of the released drug in the media. See the *Common Principles* section on chemical degradation for additional recommendations. Methods that use dialysis membranes with microparticles have been reported in the literature (*21*).

## EMULSIONS

For emulsions, the drug substance is typically trapped within a water-in-oil or oil-in-water dispersed phase; however, the majority of parenteral emulsions are oil-in-water emulsions. When assessing the drug release of these type of emulsions, the apparatus described in ⟨711⟩, ⟨1004⟩, and ⟨724⟩ may be appropriate; these apparatus include USP Apparatus 2, dialysis cell, USP Apparatus 4, a vertical diffusion cell, and reduced-volume equipment.

## IMPLANTS

For extended-release implants, the in vitro drug release test should include a procedure to ascertain whether the drug releases as intended and to prove the robustness of the drug product. There may be a regulatory expectation for real-time (long-term) in vitro drug release tests used along with accelerated in vitro drug release tests. Some acceleration strategies include elevated temperatures, non-neutral pH to increase the hydrolysis of rate-controlling polymers and/or to increase the drug solubility, and organic cosolvents used to increase drug solubility and/or to increase the permeability of rate-controlling polymers. Both real-time and accelerated performance tests should be well studied and justified in their predictive ability (clinical relevance) and/or discriminatory power for QC.

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In the *USP* monograph *Goserelin Implants*, the apparatus is a sealed jar that is incubated. Apparatus 4 and Apparatus 7 are also used (*5*–*8*). The FDA database provides three implant methods: the goserelin implant uses an incubation method, the dexamethasone implant uses Apparatus 7, and the buprenorphine hydrochloride implant uses Apparatus 2.

## DRUG-ELUTING STENTS

Drug-eluting stents (DES) are a combination of a device and a drug, where the drug is usually within a polymeric matrix that coats the stent. As with implants and extended-release microparticle suspensions, the long-acting nature of the DES makes long in vitro release tests (up to 6 months in some cases) necessary to fully characterize the drug release profile. Long tests come with the challenges of media evaporation and stability of drug substances within the media. The possibility of accelerated conditions is an important consideration, as the in vivo release may occur over a long period of time. Other considerations are the positioning of the DES in the apparatus for adequate mixing and the use of a small volume of media due to the low drug concentration.

There are performance test methods suggesting the use of a reduced-volume paddle apparatus—USP Apparatus 4 or USP Apparatus 7 with stent holders—where small volumes of media are used (*4*). For the approved DES, sirolimus, USP Apparatus 4 (flow rate of 25 mL/min) was used with an elution media (50 mL) of 2% SLS and 10% acetonitrile at a pH of 4.5 and a temperature of 37° (*22*). This is a closed-loop configuration.

The method needs to reflect the transport forces that are predominant in vivo (*23*). This has been demonstrated in methods using a blood vessel-simulating flow-through cell apparatus (*24*). Other apparatus that have been used for in vitro drug release testing of DES are as follows: incubation cells of different volumes, agitation at 250–300 rpm at 37°, and USP Apparatus 7 with small-volume reciprocating holders in 10-mL glass cells at dip rates of 5 or 40 DPM (*25*).

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# ⟨1004⟩ MUCOSAL DRUG PRODUCTS—PERFORMANCE TESTS

## INTRODUCTION

Mucosal drug products deliver drug substances to the body via the mucosal route. For the purposes of this chapter, the mucosal route of drug administration is divided into seven membrane surfaces characterized as otic, ophthalmic, nasal, oropharyngeal, urethral, vaginal, and rectal. Mucosal drug products include a wide variety of dosage forms such as solutions, suspensions, emulsions, creams, ointments, gels, inserts, strips, aerosols, sprays, films, medicated chewing gums, lozenges, tablets, and suppositories. Some of these dosage forms are also administered by other routes. For example, creams can be administered by the mucosal route (vaginal) and also by the topical route. Two categories of tests—product quality and product performance—are performed on these products. These tests provide assurances of batch-to-batch quality, reproducibility, reliability, and performance of a drug product. Product quality tests are performed to assess attributes such as assay, identification, and content uniformity and are part of the compendial monograph (see *Mucosal Drug Products—Product Quality Tests* ⟨4⟩). Product performance tests are conducted to assess the drug release from the dosage form. For certain mucosal drug products, determination of aerodynamic particle size or globule size may serve as a product performance test.

Where a compendial performance test exists for a dosage form administered by a nonmucosal route, such as a dissolution test for an oral tablet, that test may have application for the dosage form administered by a mucosal route (e.g., buccal tablets or sublingual tablets).

## PERFORMANCE TESTS FOR MUCOSAL DRUG PRODUCTS

The performance tests for the various mucosal drug products can be broadly divided into two categories: 1) test procedures that use or can adopt methodology in existing general chapters, and 2) tests that need additional developmental work before they can be recommended.

*The Dissolution Procedure: Development and Validation* ⟨1092⟩ should be a reference when developing a drug release test (e.g., selecting the drug release medium, apparatus/procedure, or analytical method). For several mucosal drug products, drug release procedures described in *Dissolution* ⟨711⟩, *Drug Release* ⟨724⟩, and *Semisolid Drug Products—Performance Tests* ⟨1724⟩ may be applicable.

In some instances, mini-basket or mini-paddle apparatuses may be suitable. These apparatuses resemble *Dissolution* ⟨711⟩, *Apparatus 1 (Basket Apparatus)* and *Dissolution* ⟨711⟩, *Apparatus 2 (Paddle Apparatus)*, with dimensions scaled down to accommodate medium volumes of <500 mL (*1*,*2*). Several designs are commercially available. However, as of now, these apparatuses are not standardized.

Because of the varied and specific environments characterizing the mucosal route of administration, researchers may be inclined to use "physiological medium" for the drug release of the specific dosage form. It may not be essential to use such a medium for the performance test of the product, and in many instances a simple buffer may suffice. A reference (*3*) for the composition of such media is provided for additional information.

### Aerosols and Nasal Sprays

Performance tests for nasal and lingual aerosols and nasal sprays are largely concerned with droplet or particle size distribution and aerodynamic size distribution. The procedures in *Inhalation and Nasal Drug Products: Aerosols, Sprays, and Powders—Performance Quality Tests* ⟨601⟩ can be applied to products administered by mucosal routes. When the drug substance is present as a solid with a modified-release mechanism in the administered dose, attempts should be made to determine the dissolution of the particles (*4*).

### Creams, Gels, and Ointments

Drug release tests for creams, gels, and ointments can be performed using a procedure described in ⟨1724⟩.

***Change to read:***

### Emulsions

Performance tests for emulsions include globule size determination and dissolution/drug release testing. Globule size can be determined using a procedure described in *Globule Size Distribution in Lipid Injectable Emulsions* ⟨729⟩. The drug release test can be performed using *Dissolution* ⟨711⟩, *Apparatus 2 (Paddle Apparatus)* or a vertical diffusion cell as described in ▲*Semisolid Drug Products—Performance Tests* ⟨1724⟩, *In Vitro Release Test (IVRT)*▲ (CN 1-Dec-2023).

### Films

*Drug Release* ⟨724⟩, *Apparatus 5 (Paddle over Disk)* can be used to determine the drug release from film dosage forms. A mini-basket can be used for drug release testing of films.

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## Gums

For gum products, the performance test includes drug release from the formulation. A device is described in the *European Pharmacopoeia* (*5*). The release of drug from the formulation requires masticatory activity that renews the surface exposed to the medium. The rate of release will be a function in part of the frequency of chewing that is simulated by the test apparatus. Gums can require conditioning at the temperature of the mouth to deform plastically under the action of the oscillating platens of the test apparatus.

Important parameters for the apparatus include: dissolution medium volume, distance between upper and lower chewing surfaces, recommended rotation angle, temperature, and chewing frequency. The dissolution medium chosen, the test time(s), and the volume sampled are also important considerations.

## Inserts

Drug release testing for inserts can be performed using *Dissolution* ⟨711⟩*, Apparatus 1 (Basket Apparatus)* or *Dissolution* ⟨711⟩*, Apparatus 2 (Paddle Apparatus)*.

## Lozenges

Drug release testing of lozenges can be performed using either *Dissolution* ⟨711⟩*, Apparatus 1 (Basket Apparatus)*; *Dissolution* ⟨711⟩*, Apparatus 2 (Paddle Apparatus)* at high agitation (175 rpm); or *Dissolution* ⟨711⟩*, Apparatus 3 (Reciprocating Cylinder)*.

## Suppositories

There are two types of suppositories: 1) hydrophilic (water soluble), and 2) lipophilic (oil soluble or melting). Drug release (dissolution) for water-soluble suppositories can be performed using *Dissolution* ⟨711⟩*, Apparatus 1 (Basket Apparatus)*; *Dissolution* ⟨711⟩*, Apparatus 2 (Paddle Apparatus)*; or *Dissolution* ⟨711⟩*, Apparatus 4 (Flow-Through Cell)*. Drug release testing for lipophilic suppositories may need modification of the dissolution procedure to avoid analytical interference from the oil globules. Several alternative methods have been proposed (*6*–*8*). The flow-through cell apparatus using the cell for suppositories may be useful. The selection of the method will be dependent on the nature of the formulation. *Figure 1* shows the schematic view of a flow-through cell [*Dissolution* ⟨711⟩*, Apparatus 4 (Flow-Through Cell)*] specifically intended for dissolution of suppositories. The lower part (1) is made up of two adjacent chambers connected to an overflow device. The dissolution medium passes through chamber A and is subjected to an upward flow. The flow in chamber B is directed downward to a small-size bore exit that leads upward to a filter assembly. The middle part (2) of the cell has a cavity designed to collect lipophilic excipients that float on the dissolution medium. A metal grid serves as a rough filter. The upper part (3) holds a filter unit for paper, glass fiber, or cellulose filters.

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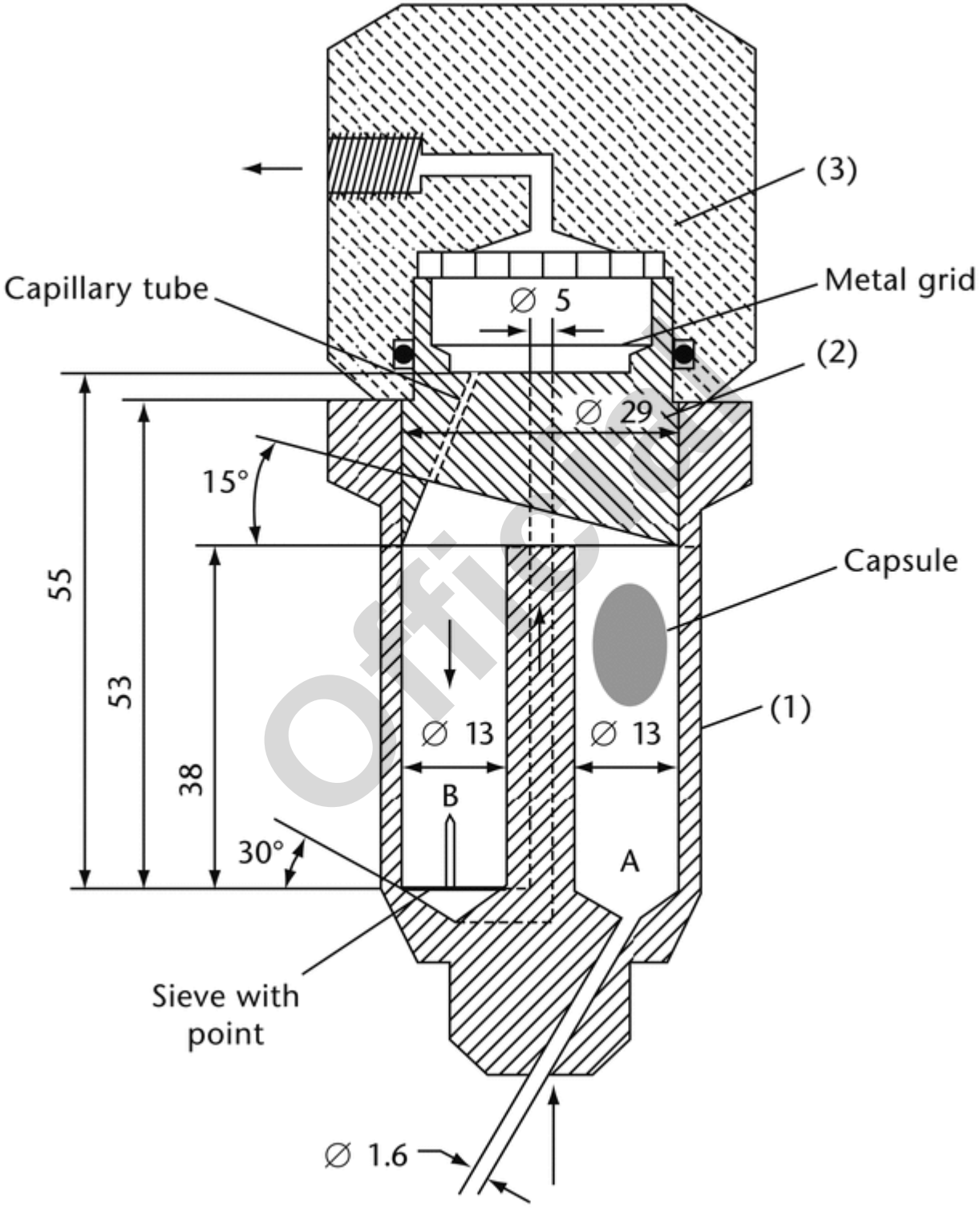


Figure 1. Flow-through cell designed for suppositories (dimensions in mm).

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## Suspensions

The dissolution test for suspensions can be performed using *Dissolution* ⟨711⟩*, Apparatus 2 (Paddle Apparatus)*. A small-volume, mini-paddle apparatus may be used.

## Sublingual Tablets and Buccal Tablets

Drug release for these dosage forms can be performed using *Dissolution* ⟨711⟩*, Apparatus 1 (Basket Apparatus)* or *Dissolution* ⟨711⟩*, Apparatus 2 (Paddle Apparatus)*. Mini-baskets or the mini-paddles can also be used for drug release testing of buccal and sublingual tablets.

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# ⟨1005⟩ ACOUSTIC EMISSION

## INTRODUCTION

Ultrasound techniques can be categorized into two distinct types: acoustic emission (passive mode) and ultrasound spectroscopy (active mode). Both of these techniques have many applications.

The technique of acoustic emission is based on the detection and analysis of sound produced by a process or system. This is essentially equivalent to listening to the process or system, although these sounds are often well above the frequencies that can be detected by the human ear. Generally, frequencies up to about 15 kHz are audible.

In the case of ultrasound spectroscopy, the instrument is designed to generate ultrasound waves across a defined frequency range. These waves travel through the sample and are measured using a receiver. An analogy can be drawn with UV-visible or IR spectroscopy in that the detected ultrasound spectrum reflects changes in velocity or sound attenuation due to the interaction with a sample across a range of frequencies. However, as the scope of this chapter is limited to acoustic emission, ultrasound spectroscopy will not be discussed further.

Acoustic emission is well-known in the study of fracture mechanics and therefore is used extensively by material scientists. It is also widely used as a nondestructive testing technique and is applied routinely for the inspection of aircraft wings, pressure vessels, load-bearing structures, and components. Acoustic emission is also used in the engineering industry for the monitoring of machine tool wear.

In terms of pharmaceutical applications, the dependence of the acoustic emission measurement on physical properties such as particle size, mechanical strength, and cohesivity of solid materials allows the technique to be used for the control and endpoint detection of processes such as high shear granulation, fluid bed drying, milling, and micronization.

### General Principles

Acoustic emissions can propagate by a number of modes. In solids, compressional and shear or transverse modes are important. Compressional modes have the highest velocity and thus reach the acoustic detector (or acoustic emission transducer) first. However, in most process applications of acoustic emission, there are many sources—each producing short bursts of energy—and, consequently, the different modes cannot easily be resolved. The detected signal, for example on the wall of a vessel, is a complex mixture of many overlapping waveforms resulting from many sources and many propagation modes.

At interfaces, depending on the relative acoustic impedance of the two materials, much of the energy is reflected back towards the source. In a fluidized bed, for example, acoustic emissions will only be detected from particles directly impacting the walls of the bed close to the transducer.

A convenient method of studying acoustic emission from processes is to use the “average signal level”. A root mean square-to-direct current (RMS-to-DC) converter may be used to convert the amplitude-modulated (AM) carrier into a more slowly varying DC signal. This is referred to as the average signal level (ASL). The ASL can then be digitally sampled (typically at a sampling frequency of about 50 Hz) and stored electronically for further signal processing.

The simplest way of studying the acoustic data is to examine changes in the ASL. However, other information can be derived from examining the power spectrum of the ASL. The power spectrum is calculated by taking the complex square of the amplitude spectrum and can be obtained by performing a Fast Fourier Transform (FFT) on the digitized raw data record. Power spectra may be averaged to produce a reliable estimate of power spectral density or to give a “fingerprint” of a particular process regime. Interpretation of the power spectrum is complicated by the fact that the acoustic signal originating in the system is distorted by several factors including transmission, reflection, and signal transfer characteristics.

The shape of the power spectrum of the ASL record is a function of the process dynamics. Periodic processes (e.g., mechanical stirring or periodic bubbling of a fluidized bed) show high power at certain discrete frequencies. Random processes show either flicker type properties, where power is inversely proportional to frequency, or white noise type properties in which power is independent of frequency. The amplitude of the power spectrum is also affected by the energy of the acoustic emissions produced by the process. For example, if hard material is being processed, the acoustic emission produced by particle impact will be greater than that produced by soft material.

## INSTRUMENTATION

Generally, piezoelectric sensors are used to detect and quantify the acoustic signals produced by a process. Piezoelectric transducers are constructed from piezoelectric crystalline solids connected to transducer control circuitry by electrical leads. When configured as a detector, an acoustic wave that impinges on the piezoelectric element is transformed into an electrical signal in the transducer control circuitry. When configured as an acoustic generator, an electrical signal applied to the piezoelectric element by the control circuitry creates an acoustic wave that can propagate into the medium to which the transducer is attached. Typically, this means that acoustic emission detectors can also be operated as acoustic wave generators and this feature is used to ensure good sensor performance as described later (see *Qualification and Verification of Acoustic Emission Instruments*).

In general acoustic emission applications, sensors with different resonance frequencies are often used (e.g., 70 and 190 kHz, although higher frequencies may be more appropriate at smaller scales of operation), incorporating various band-passes. As sound (ultrasound) of the appropriate frequency range reaches these sensors, an electrical signal is generated, the amplitude of which is directly proportional to the energy (amplitude) of the incident sound waves.

These signals are processed through the following:

1. a pre-amplifier (which incorporates signal filtering),

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2. an RMS-to-DC converter,
3. a variable gain amplifier, and
4. a PC-based data acquisition board.

The controlling software is also incorporated into the PC.

Acoustic emission equipment generally allows several sensors to be used simultaneously by incorporating multiple electronic channels into a single instrument.

### Signal Processing

The signal from a resonant transducer resembles an AM radio signal. At the resonance frequency of the transducer, the signal consists of a carrier wave that is modulated in amplitude by the process. An RMS-to-DC converter is used to demodulate the signal. The output of this device is the modulation signal or envelope.

The envelope is digitally resampled at a frequency appropriate for the process. For example, 50 Hz is a typical digital sampling rate for a fluid bed drier or high shear granulator.

## FACTORS AFFECTING MEASUREMENT

The following factors can affect the acoustic data obtained and should be considered when installing an acoustic emission system.

1. *Failure or Physical Damage*—As with any other type of sensor, acoustic emission sensors can fail with time or as a result of physical damage. It is important to check the sensor function as part of routine maintenance of the instrument. If multiple sensors are installed on the same vessel, an active signal can be generated from one sensor and this can be used to check the detection on another sensor. This exercise would ensure that the sensors are detecting the acoustic signals generated by the process. A statistically valid "minimum acceptable acoustic signal" for the sensor(s) should also be determined and monitored at the start, middle, and end of a process to ensure the performance of the sensor(s) during a process run. This may be established from the routine maintenance signal experiments or on the basis of historical data for the sensors.
2. *Issues of Sensor Interfacing*—Sensors are typically installed on the outer wall of the process vessel. Several types of adhesives (temporary or permanent) can be used to attach the sensor to the vessel wall. Through repeated cleaning and vessel movement, it is possible for the bonding between the sensor and vessel to be compromised. Checking the integrity of the installation should be part of routine maintenance. Similar to item 1 above, an active signal can be used to ensure proper bonding between sensor and vessel and helps to confirm the matching of acoustic impedance.
3. *Influence of Mechanical Noise*—The use of high frequencies significantly reduces the contribution of mechanical noise to the acoustic signal detected, especially at smaller scales of operation, although it does not eliminate it completely. Testing the effect of various motor settings, for example, can determine if the acoustic signal detected is a function of mechanical noise. If the effect is significant, using higher frequencies may be necessary. Awareness of the contribution of the mechanical noise, no matter how small, is important to consider as the motors age or are replaced.
4. *Influence of Vessel Wall Characteristics*—Because the sensors are often placed on the outer vessel wall, wall thickness can affect the quality of the signal detected. If the vessel is jacketed, the amplitude of the acoustic signal may be reduced. Adding more sensors on the vessel can improve signal quality. Alternatively, an increase in signal may be obtained by positioning sensor(s) at a location where contact exists between the inner and outer walls, essentially providing a waveguide between the sensor and sound source. Waveguides may also be incorporated into the design of manufacturing equipment to enable utilization of acoustic emission monitoring. Appropriate validation is required to ensure that this does not adversely affect the performance of equipment.
5. *Effect of Material Properties*—During operation, the acoustic signal collected is a summation of various events occurring within the process. For example, the acoustic signal generated as particles hit the wall in a granulator is a function of the material properties of the granules (i.e., density, size, porosity). Therefore, significant changes to any of these parameters can affect the acoustic signal and the quality of the ensuing prediction.
6. *Influence of Process-Related Factors*—Similar to item 5 above, the process-related properties (i.e., force of impact, frequency of impact, amount of material) can also affect the acoustic signal and the quality of the ensuing prediction.
7. *Impact of Environmental Conditions*—Finally, the influence of environmental factors (i.e., temperature, humidity) must also be considered.

The acoustic emission data collected is vessel/equipment specific. It is not advisable to apply a model generated on one piece of equipment to another because the acoustic information can differ as a result of the issues discussed in items 3, 4, and 5 above.

### Qualification and Verification of Acoustic Emission Instruments

A system suitability approach should be taken around instrument performance, establishing optimum measurement configuration, then comparing the instrument performance to the values obtained during routine use to those obtained during installation qualification (IQ).

This approach effectively answers the issues related to sampling because, unlike other on-line analytical systems, the transducers can be optimally positioned and attached to receive the maximum signal without vessel modification.

Sample rates need to comply with the Nyquist sampling theorem, which states that a signal must be sampled at a rate that is twice the highest frequency component in the signal. A low-pass filter should be used to remove the frequency components greater than half the sampling frequency (Nyquist frequency). Failure to comply with this criterion will result in aliasing.

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Owing to the nature of the piezoelectric transducers and because resonance frequencies are natural properties of the crystals, it is not necessary to test the variation (reproducibility) or drift in the frequency domain. If other types of transducers are used, this may be necessary. Any gross change in the frequency domain will be recorded as a drop in the power intensity at the resonance frequency, and therefore is covered by the power intensity tests.

The two main areas for instrument performance verification are power intensity and timings. Any change in the signal intensity will affect the raw signal and the ASL and, therefore, will also affect the power spectrum. Changes in power intensity can occur as a result of changes in the process (e.g., variation in hardness or moisture in the particles impacting the vessel wall) or changes in the acoustic conduit from the process to transducer.

Reproducibility of the acoustic conduit should be tested using a second transducer to input a pulse or "ping" at the resonance frequency of the receiving sensor. This reproducibility value represents the noise of the signal and can be used in calculations of limit of detection (LOD) and limit of quantitation (LOQ), where LOD is defined as three times the noise of the signal and LOQ is ten times the noise of the signal. The noise on the background signal level (in acoustic emission this background signal is mainly due to amplifier noise) should be calculated from twenty sequential ASL values acquired at the sampling frequency used for normal operation. This test should be repeated in reverse in order to establish that statistically similar intensity values can be obtained on both channels.

Short term reproducibility allows the calculation of noise. However, it does not give a measure of integrity of acoustic conduit over time or, more specifically, of changes caused by the process (e.g., variations in adhesive properties with process changes such as heating/cooling). The noise test should be repeated while executing the normal processing parameters (using an empty vessel) and the drift in the ASL should be calculated. Care should be taken to make sure that signal drift (due to normal variation in processing parameters) does not impact chemometric models used for endpoint determination. For trend plots, it should be shown that drift is not statistically significant; otherwise, drift correction will need to be applied. Values for noise, drift, and absolute ASL should be recorded and logged, and the tests re-executed if changes are made to the processing equipment or to the acoustic emission system. If no changes are made, then the tests should be re-executed every month. In this way the quality of the acoustic conduit can be shown to be intact and any changes to the signal intensity isolated and attributed to the process itself.

During routine use, it is recommended that the noise test be executed (as above) before each process run, and that power intensity and noise be calculated. These values should be logged and compared to those generated both during previous use and during installation. Impact of the deviation from previous values will be a function of the prediction model and should be addressed by method validation.

The noise data (from above) can also be used to calculate the time of flight of the pulse. If the pulse activation and signal reception are synchronized, the time taken for the pulse to transmit across the vessel can be measured. This is a good indication of the measurement electronics as well as the overall condition of the acoustic conduit. However, this test should be regarded as a measure of the "system" condition and needs to be executed only if changes have been made to the process equipment or the acoustic emission system, or every 6 months. Correlation of the measured timings with the historical ones should be statistically valid. If not, it is an indicator that the acoustic emission system may need requalification by the instrument manufacturer or supplier, or that there are changes in the acoustic conduit.

All of these tests require the use of an acoustic pulse generated electrically. Failure in any of the above tests could be attributed to the signal generation itself. It is recommended that the electrical pulse generation system be requalified and certified against National Institute of Standards and Technology (NIST) traceable standards every 12 months.

## DATA ANALYSIS

Acoustic emission from granulators and fluid bed driers is known as continuous acoustic emission. Continuous acoustic emission is aphasic (i.e., there are no starts or stops to the signal). This means that it is unnecessary to use signal processing techniques that preserve phase. Power spectral analysis is a useful technique in processing acoustic emission signals. The information in the power spectra, unlike the raw acoustic emission signals, is coherent in the short term, allowing signal averaging to be performed. This provides a better estimate of power spectral density than that provided by a single power spectrum.

To detect endpoints in batch processes (e.g., granulation or drying endpoint), a qualitative multivariate model is appropriate (e.g., PCA or SIMCA). The following sequence of operations is performed:

1. *Training/Calibration*—Acoustic emission spectra that are representative of the endpoint condition are obtained.
2. *Modeling*—A multivariate model describing the distribution of acoustic emission signals at the endpoint condition is created.
3. *Prediction*—Acoustic emission spectra are compared against the model. The fit to the model (usually expressed in terms of a number of standard deviations) is monitored. As the system approaches the endpoint, the fit improves and completion of the process is established once the model fits predefined criteria. The prediction model is generated from acoustic emission spectra obtained from the process operating under normal conditions. Upsets (e.g., unwanted agglomeration in coaters) are detected by observing statistically valid deviations from the model.
   Adaptive modeling has also been proposed for upset detection. This involves generating multivariate models continuously as the acoustic emission signals are acquired. Unusual deviation of the acoustic emission signal indicates the occurrence of a process upset. The advantage of adaptive modeling is that it is not necessary to perform a separate calibration step.

## GLOSSARY

**Acoustic Emission Transducer:** A solid state device usually incorporating a piezoelectric element to convert the acoustic emission wave to an electrical signal.

**Acoustic Impedance:** Acoustic impedance (Z) is defined as $Z = \rho v$ (where $\rho$ is density and v is the sound velocity). It is an important quantity and gives the proportion of sound energy transmitted from one medium to another and the amount of energy reflected at the interface.

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**Adaptive Modeling:** A method that predicts the state of a process without the use of a previously generated model (i.e., there is no prior training or calibration step).

**Aliasing:** Spurious low frequency components, appearing in the signal, that are really frequencies above the Nyquist frequency.

**Amplitude:** The magnitude or strength of a varying waveform.

**Average Signal Level (ASL):** A measure of the average power in an acoustic emission signal.

**Band-Pass:** The range of frequencies within which a component operates.

**Compressional Mode:** A longitudinal mode of acoustic transmission encountered in solids, liquids, and gases.

**Continuous Acoustic Emission:** Acoustic emission signals that cannot be separated in time and are typical of pharmaceutical processes such as granulation and fluid bed drying.

**Flicker Type Properties:** A type of signal associated with many natural processes. The characteristics of flicker noise are that the power of the noise is directly proportional to the signal and has approximately a 1/f (f = frequency) spectral density distribution.

**Gain:** The amplification factor for a component usually expressed in terms of decibels (dB).

Gain in dB = 20 $\log_{10}$ ($Voltage_{out}$$Voltage_{in}$).

**Nyquist Frequency:** The Nyquist frequency is defined as half the digital sampling rate and is the highest frequency that can be reproduced faithfully.

**Piezoelectric:** A material which generates an electric field when compressed. Piezoelectric materials are used in the construction of acoustic emission sensors. A common material is PZT (lead zirconium titanate).

**Power Spectrum:** A power spectrum of a signal is a representation of the signal power as a function of frequency. A power spectrum is calculated from the time domain signal by means of the Fast Fourier Transform (FFT) algorithm. It is useful to study acoustic emission signals in the frequency or spectral domain, as the spectrum is often characteristic of the mechanism. Improvements in signal-to-noise ratio can be obtained by averaging a number of power spectra, as they are coherent.

**Power Spectral Density:** The measure of acoustic emission power in each resolution element of the power spectrum.

**Resonance Frequency:** The frequency at which an acoustic emission sensor is most sensitive. Resonant acoustic emission sensors have a clearly defined resonance frequency, but are usually sensitive to other frequencies.

**RMS-to-DC Converter:** An electronic device that converts an alternating signal to a voltage level proportional to the average power in the signal.

**Shear Mode:** A transverse mode of acoustic transmission, encountered only in solids.

**Signal Filtering:** Filtering a signal means attenuating frequencies outside a prescribed range. In acoustic emission work, band-pass filtering is used to improve the signal-to-noise ratio by attenuating noise outside the bandwidth of the sensor. Low-pass filtering is used to remove frequencies higher than the Nyquist frequency in order to prevent aliasing.

**Transverse Mode:** A mode of wave propagation where the displacement of the material is perpendicular to the direction of propagation. These modes are only encountered in solid materials.

**White Noise:** The characteristic of white noise is a power spectrum of uniform spectral density and is associated with purely random processes.

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# ⟨1010⟩ ANALYTICAL DATA—INTERPRETATION AND TREATMENT

## 1. INTRODUCTION

This chapter provides information regarding acceptable practices for the use of analytical procedures to make decisions about pharmaceutical processes and products. Basic statistical approaches for decision making are described, and the comparison of analytical procedures is discussed in some detail.

[NOTE—It should not be inferred that the analysis tools mentioned in this chapter form an exhaustive list. Other, equally valid, statistical methods may be used at the discretion of the manufacturer and other users of this chapter.]

Assurance of the quality of pharmaceuticals is accomplished by combining a number of practices, including rigorous process and formulation design, development, validation, and execution of a robust control strategy. Each of these is dependent on reliable analytical procedures. In the development process, analytical procedures are utilized to ensure that the manufactured products are thoroughly characterized and to optimize the commercial manufacturing process. Final-product testing provides assurance that a product is consistently safe, efficacious, and in compliance with its specifications. Sound statistical approaches can be included in the commercial control strategy to further ensure that quality is preserved throughout the product life cycle.

While not meant to be a complete account of statistical methodology, this chapter will rely upon some fundamental statistical paradigms. Key among these are *population parameters*, *statistical design and sampling*, and *parameter uncertainty*. Population parameters are the true but unknown values of a scientific characteristic of interest. While unknown, these can be estimated using statistical design and sampling. Statistical design is used to fully represent the population of interest and to manage the uncertainty of a result, while the random acquisition of test samples as well as their introduction into the measurement process helps to mitigate bias. Lastly uncertainty should be acknowledged between the true population parameter and the estimation process. Uncertainty can be expressed as either a probabilistic margin between the true and estimated value of a population parameter (e.g., a 95% confidence interval) or as the certainty that the population parameter is compliant with some expectation or acceptance criterion (predictive probability).

This chapter provides direction for scientifically acceptable administration of pharmaceutical studies using analytical data. Focus is on investigational studies where analytical data are generated from carefully planned and executed experiments, as well as confirmatory studies which are strictly regulated with limited flexibility in design and evaluation. This is in contrast to exploratory studies where historical data are utilized to identify trends or effects which are subject to further investigation. The quality of decisions made from either investigational or confirmatory studies is enhanced through adherence to the scientific method, and to the application of sound statistical principles. The steps of the scientific method can be summarized as follows.

**Study objective.** A pharmaceutical study can be as simple as testing and releasing a batch of commercial material or as complex as a comparison of analytical procedures. The same considerations apply to the simple study as they do to the complex study. Each study is associated with a population parameter which is used to address the study objective. For release, the parameter might be the batch mean. For the analytical procedure comparison study, the parameter might be the difference in means produced by the analytical procedures. In each case an appropriate acceptance criterion on the population parameter is used to make a decision from the study.

**Study design.** The study should be designed with a structure and replication strategy which ensures representative consideration of the study objective, and which manages the risks associated with making an incorrect decision. Representative consideration of the study objective entails inclusion of samples and conditions which span the population being studied. Thus in release of a manufactured lot, samples across the range of manufacture might be included, while in a procedure comparison, each type and level of test sample might be considered. Similar consideration should be given to sample testing, where appropriate factors should be included in the procedure. The design should also acknowledge the study risks. The statistical basis for managing study risk is the reduction of the uncertainty in the estimation of the population parameter.

**Study conduct.** Once the study has been designed, samples are collected and data are generated using the analytical procedure. Effective use of randomization should be considered to minimize the impact of systematic variability or bias. Care should be taken during data collection to properly control the analytical procedure and to ensure accurate transcription and preservation of information. An adequate number of significant digits or decimal places should be saved and used throughout the calculations. Deviations from the study plan should be captured and assessed for their potential to impact study decisions.

**Study analysis and decision.** Prior to the final analysis, the data should be explored for data transformation and potential outliers. The analysis of the data should proceed according to the statistical methods considered during the study design. The analysis of the data and the reporting of study results should include proper consideration of uncertainty. Where appropriate, interval estimates should be used to communicate the robustness of the results (viz., the width of the interval) as well as facilitate communication of the study decision. A decision can be made when the objective of the study has been preformulated to make such a decision (e.g., as in an investigational or confirmatory study). The study may otherwise have been performed to estimate or describe some characteristic of a population. Caution should be taken in making decisions from post-hoc analyses of the data. This is called "data snooping" and can lead to inappropriate decisions.

This chapter has been written for the laboratory scientist and the statistician alike. The laboratory scientist is primarily skilled in the analytical procedures and the uses made of those procedures and should be aware of the value of statistical design and analysis in their practices. The statistician is primarily skilled in the design of empirical studies and the analysis which will return reliable decisions and should appreciate the science and constraints within the laboratories. While variously knowledgeable in their understanding across specialties, both disciplines should value the essential components that comprise uses of analytical data.

More detailed discussion related to the steps of the scientific method will be given in *4. Study Considerations* and will be illustrated with an example in *5. Comparison of Analytical Procedures*. Prior to this, there will be a section to review *2. Prerequisite Laboratory Practices and Principles* and a section to describe and illustrate *3. Basic Statistical Principles and Uncertainty*. A series of appendices is provided to illustrate topics related to the generation and use of analytical data. Control charts, equivalence and noninferiority testing, the principle of uncertainty, and Bayesian statistics are briefly discussed. The framework within which

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the results from a compendial test are interpreted is clearly outlined in *General Notices, 7. Test Results*. Selected references that might be helpful in obtaining additional information on the statistical tools discussed in this chapter are listed in *References* at the end of the chapter. USP does not endorse these citations, and they do not represent an exhaustive list. Further information about many of the methods cited in this chapter may also be found in most statistical textbooks.

## 2. PREREQUISITE LABORATORY PRACTICES AND PRINCIPLES

The sound application of statistical principles to analytical data requires the assumption that such data have been collected in a traceable (i.e., documented) and unbiased manner. To ensure this, the following practices are beneficial.

### Sound Record Keeping

Laboratory records are maintained with sufficient detail, so that other equally qualified analysts can reconstruct the experimental conditions and review the results obtained. When collecting data, the data should be obtained with more decimal places than the specification or study acceptance criterion requires. Rounding of results from uses of analytical data should occur only after final calculations are completed as per the *General Notices*. Study protocols and data analyses should be adequately documented so that a reviewer can understand the bases of the study design and the pathway to study decisions.

### Procedure Validation

Analytical procedures used to release and monitor stability of clinical and commercial materials are appropriately validated as specified in *Validation of Compendial Procedures* ⟨1225⟩ or verified as noted in *Verification of Compendial Procedures* ⟨1226⟩. Further guidance is given in *Statistical Tools for Procedure Validation* ⟨1210⟩ and *Biological Assay Validation* ⟨1033⟩. Analytical procedures published in the *USP–NF* should be validated and meet the current good manufacturing practices (GMP) regulatory requirement for validation as established in the United States Code of Federal Regulations. When an analytical procedure is used in a non-GMP study, it's good practice to ensure that the analytical procedure is adequately fit for use to support the study objective.

### Analytical Procedure and Sample Performance Verification

Verifying an acceptable level of performance for an analytical procedure in routine or continuous use is a valuable practice. This may be accomplished by analyzing a control sample at appropriate intervals or locations, or using other means, such as determining and monitoring variation among the standards, background signal-to-noise ratios, etc. This is commonly called *system suitability*. Attention to the measured performance attribute, such as charting the results obtained by testing of a control sample, can signal a change in performance that requires adjustment of the analytical system. Examples of control charts used to monitor analytical procedure performance are provided in *Appendix 1: Control Charts*.

Sample performance should also be verified during routine use of an analytical procedure. Variability among replicates as well as other sample specific performance attributes are used to ensure the reliability of sample measurement. A failure to meet a sample performance requirement can result in a retest of the sample after an appropriate investigation, versus a complete repeat of an analytical procedure run.

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## 3. BASIC STATISTICAL PRINCIPLES AND UNCERTAINTY

This section introduces the concept of uncertainty, and couples this with familiar statistical tools which facilitate decisions made from analytical data. At the core of these principles and tools is an understanding of risk; more specifically the risks of making incorrect decisions based on analyses using measurement data. The consequences of these risks can be minor or significant, and should be factored into considerations related to both design of a study, and the interpretation of the results. The understanding of uncertainty is not new to the pharmaceutical industry, or more broadly throughout industries that make decisions from analytical data. The study of measurement and measurement uncertainty falls formally into the field of metrology (see *Appendix 4: The Principle of Uncertainty*). This section will frame the concept of uncertainty and illustrate some well-known statistical tools.

### Uncertainty

A study is designed to reduce uncertainty in order to make more reliable decisions.

Uncertainty is associated with variability and communicates the closeness of a result to its true value. A fundamental aspect of uncertainty is probability which is sometimes expressed as confidence. The combination of the variability of the result from a study and confidence provides a powerful means to manage pharmaceutical decisions.

Uncertainty is directly related to risk. Risk may be expressed as a probability, but is more formally translated into cost, where cost is the opportunity loss due to making an incorrect decision times the probability of that loss. Here a loss may be quantifiable outcome such as the value of a lot of manufactured material, or less quantifiable such as the loss of patient benefit from a drug or biological.

Key to the concept of uncertainty is its relationship to the structure of variability. The overall variability of the result is a composition of many individual sources of variability. In a general sense one can manage the overall variability through

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refinement in one or some of those sources, or through strategic design (e.g., replication and blocking). In either case the effort results in higher certainty and lower risk.

## Basic Statistical Principles

All results from studies using analytical data are, at best, estimates of the true value because they contain uncertainty. Basic statistical principles related to estimation and uncertainty will be illustrated for the population mean of a manufactured lot.

### STATISTICAL MEASURES

Statistical measures used to estimate the center and dispersion of a population include the mean, standard deviation, and expressions derived there from, such as the percent coefficient of variation (%CV), sometimes referred to as percent relative standard deviation (%RSD). Such statistical measures can be used to calculate confidence intervals for summary parameters of the process generating the data, prediction intervals for capturing a single future measurement with specified confidence, or tolerance intervals capturing a specified proportion of the individual measurements with specified confidence.

### STATISTICAL ASSUMPTIONS

Statistical assumptions should be justified with respect to the underlying data generation process and verified using appropriate graphical or statistical tools. If one or more of these assumptions appear to be violated, alternative methods may be required in the evaluation of the data. In particular, most of the statistical measures and tests cited in this chapter rely on the assumptions that the underlying population of measurements is normally distributed and that the measurement results are independent and free from aberrant values or outliers. Assessment of the statistical assumptions and alternatives methods of analysis are discussed in *Appendix 2: Models and Data Considerations*.

### AVERAGING

A single analytical measurement may be useful in decision making if the sample has been prepared using a well-validated documented process, if the sample is representative of the population of interest, if the analytical errors are well known, and the measurement uncertainty associated with the single measurement is suitable to make the appropriate decision. The obtained analytical result may be qualified by including an estimate of the associated measurement uncertainty. For a single measurement this might come from the procedure validation or another source of prior knowledge.

There may be instances when one might consider averaging multiple measurements because the variability associated with the average value better meets the target measurement uncertainty requirement for its use. Thus, the choice of whether to use individual measurements or averages will depend upon the use of the measurement and the risks associated with making decisions from the measurement. For example, when multiple measurements are obtained on the same sample aliquot (e.g., from multiple injections of the sample in an HPLC procedure), it is generally advisable to average the individual values to represent the sample value. This should be supported by some routine suitability check on the variability amongst the individual measures. A decision rule, which defines and describes how a decision will be made, should be explicit to the population parameter of interest. When this is the center or the mean, then the average should be the basis of the rule. When this is variability amongst the individual measurements, then it should be the standard deviation, %CV, or range. Except in special cases (e.g., content uniformity), care should be taken in making decisions from individual measurements.

### ESTIMATING THE CENTER AND DISPERSION FROM A SAMPLE

Let $Y_1, Y_2, \ldots, Y_n$ represent a sample of ($n$) observations from a population of interest. When the appropriate assumptions are met the most commonly used statistic to describe the center of the ($n$) observations is the sample or arithmetic mean ($\overline{Y}$):

$$\overline{Y} = \frac{\sum_{i=1}^{n} Y_i}{n} = \frac{Y_1 + Y_2 + \ldots + Y_n}{n} \qquad \text{Equation 1}$$

The dispersion can be estimated from the observations in various ways. The most common and useful assessment of the dispersion is the determination of the sample standard deviation. The sample standard deviation is calculated as

$$S = \sqrt{\frac{\sum_{i=1}^{n} \left(Y_i - \overline{Y}\right)^2}{n-1}} \qquad \text{Equation 2}$$

The sample %CV is calculated as

$$\%\,\text{CV} = \frac{S}{\overline{Y}} \times 100\,\% \qquad \text{Equation 3}$$

It should be noted that %CV is an appropriate measure of variability only if the property being measured is an absolute quantity such as mass. It is incorrect to report %CV for estimates reported as a percentage (e.g., percent purity) or which are in transformed units (e.g., pH or other logarithmic units; see Torbeck [*26*]).

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STATISTICAL INTERVALS

Statistical intervals are used to describe or make decisions concerning population parameters or behavior of individual values. Three useful statistical intervals are prediction intervals, tolerance intervals, and confidence intervals. Prediction and tolerance intervals describe behavior of individual values and are discussed in ⟨1210⟩.

Confidence intervals are the basis for incorporating uncertainty into the estimate of a population parameter. A two-sided interval is composed of a lower bound (LB) and an upper bound (UB). For a confidence interval on a population parameter $\theta$ these bounds are functions of the sample values such that

$$\Pr[LB \leq \theta \leq UB] = 100 \times (1 - \alpha)\% \quad \text{Equation 4}$$

This leads to the construction of a $100 \times (1 - \alpha)\%$ two-sided confidence interval on a population mean

$$LB = \overline{Y} - t_{1-\alpha/2:n-1}\frac{S}{\sqrt{n}}$$
$$\blacktriangle UB = \overline{Y} + t_{1-\alpha/2:n-1}\frac{s}{\sqrt{n}}\blacktriangle \text{ (USP 1-Dec-2021)} \quad \text{Equation 5}$$

where $n$ is the sample size and $t_{1-\alpha/2:n-1}$ is the $1 - \alpha/2^{th}$ quantile of the cumulative Student $t$-distribution having area $1 - \alpha/2$ to the left and $n - 1$ degrees of freedom. One-sided intervals based on the individual bounds can be similarly defined.

The sampling and calculation process described above will provide a confidence interval that contains the true parametric value $100 \times (1 - \alpha)\%$ of the time. Alternatively one can utilize a Bayesian approach to derive an interval which contains, with probability $100 \times (1 - \alpha)\%$ the true value of the mean (*12*).

## 4. STUDY CONSIDERATIONS

There are a number of scientific and statistical considerations in conducting a study. These will be discussed in the context of the stages of the scientific method (see *Introduction*).

### Study Objective

The study objective is a statement of the goal(s) of the study. Generally, the goals are placed into two categories: (1) estimation, and (2) inference. Estimation is the goal when the investigator wishes to report results that estimate true quantities that underlie the data generating process and are the subject of the study. In statistics these true quantities are called *population parameters*. Inference includes the additional step of using these estimates to make a decision about the unknown true value of the population parameter.

Numerical estimates can either be single numbers (point estimates), a range of numbers (interval estimates), or distributions (distributional estimates). A point estimate is a single number that "best" represents the unknown true value of a population parameter. The computed average or standard deviation of a data set sampled from the study population are examples of point estimates. "Best" in this context means the estimate is in some sense close to the unknown parameter value, although the difference between the estimate and the parameter will vary from sample to sample.

A point estimate reported alone has little utility because it doesn't reflect the uncertainty manifested by the magnitude of the difference between the estimate and the true value. Statistical intervals can be used for this purpose. A discussion of statistical intervals can be found in *3. Basic Statistical Principles and Uncertainty, Basic Statistical Principles, Statistical Intervals*. Interval estimates provide additional details that may be useful for risk based decision making.

Distributional estimates are used in Bayesian analysis to define expectations when the population parameter is viewed as a random variable. In particular, posterior distributions formed by combining prior and sample information are used to assign probabilities that the unknown parameter will fall in a given range. *Appendix 5: Bayesian Inference* describes the utility of distributional estimates in more detail.

A statistical paradigm used to express the objective of an inferential study is a statistical hypothesis test. A hypothesis test is expressed as a pair of statements called the *null hypothesis* ($H_0$) and the *alternative hypothesis* ($H_a$). Both are expressed concerning some unknown population parameter. Population parameters are often denoted with Greek letters. The Greek letter theta ($\theta$) will be used for illustration. A two-sided hypothesis test can be written as

$$H_0: \theta = \theta_0$$
$$H_a: \theta \neq \theta_0 \quad \text{Equation 6}$$

where $\theta_0$ represents the hypothesized value for $\theta$. The alternative hypothesis is sometimes called the *research hypothesis* because it represents the objective of the study. As an example, consider the true slope of a linear model representing the average change in the purity of a compound over time. Traditionally, this parameter is represented with the Greek letter beta ($\beta$). An investigator intends to determine if there is evidence that the average change in purity is a function of time. That is, if it can be shown that the true value of the slope is non-zero. Accordingly, *Equation 6* is rewritten as

$$H_0: \beta = 0$$
$$H_a: \beta \neq 0 \quad \text{Equation 7}$$

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It should be noted that this is called a *two-sided hypothesis* because the direction of the difference is unspecified. This would be the case if the study sought to determine either a positive change (increase in purity) or a negative change (decrease in purity). But this is unlikely to be the desired objective of the study. It's more plausible that the study would strictly seek to determine if there is evidence that average purity decreases over time. This would be expressed as a one-sided hypothesis test as follows

$$H_0: \beta \geq 0$$
$$H_a: \beta < 0 \quad \text{Equation 8}$$

The choice of two-sided or one-sided hypothesis test should be made when formulating the study objective, and prior to design and execution of the study. It should be based on a plausible scientific objective and should never be decided on the basis of the study results. Examples of two-sided and one-sided hypothesis tests will be given in *5. Comparison of Analytical Procedures*.

An additional consideration in formulating a study objective is the use of equivalence or noninferiority testing. These procedures require that the investigator formulate their hypotheses with a scientifically or practically meaningful objective. These will be illustrated in *5. Comparison of Analytical Procedures* and is discussed in detail in *Appendix 3: Equivalence and Noninferiority Testing*.

## Study Design

Study design should ensure an acceptable level of uncertainty in an estimation study or an acceptable risk for drawing the wrong conclusion in a test of inference. This can be managed through use of statistical design tools, including blocking and replication. As discussed previously, the design should also consider strategic selection of samples and study conditions which are associated with experiences in normal practice.

### DESIGN OF AN ESTIMATION STUDY

The design of an estimation study may use sufficient replication (sample size) and blocking to ensure desired control of the uncertainty in the result. To illustrate, consider estimation of a mean based on a simple random sample of $n$ units from a study population. The half width of the confidence interval (also called the *margin of error*) in *Equation 5* represents the uncertainty in the estimation of the mean. In planning the study, the margin of error can be defined to be no greater than a maximum allowable value $H$. Selecting the confidence level, $(1 - \alpha)$, and providing a preliminary estimate for the standard deviation ($S$), one can solve for a required sample size using the equation

$$t_{1-\alpha/2:n-1} \times \frac{S}{\sqrt{n}} \leq H$$
$$n \geq \frac{t^2_{1-\alpha/2:n-1} \times S^2}{H^2} \quad \text{Equation 9}$$

Since the degrees of freedom of the $t$-value are a function of $n$, one must either solve *Equation 9* iteratively, or use an approximation by replacing the $t$-value with the associated $Z$-value. Preliminary estimates for $S$ are obtained from similar studies or through the advice of subject matter experts. Scale of the data (e.g., transformed or original scale) should be defined prior to obtaining the preliminary estimate of the standard deviation or defining $H$ (see *Appendix 2: Models and Data Considerations* for more on data transformation).

### DESIGN OF AN INFERENTIAL STUDY

The design of an inferential study is based on controlling the risks of drawing the wrong conclusion. Following the paradigm of a hypothesis test, these risks are illustrated in *Table 1* and *Table 2*.

**Table 1. Conclusions in a Statistical Test**

| | If $H_0$ is true | If $H_0$ is false |
|---|---|---|
| **Reject $H_0$** | Wrong conclusion (Type I error) | Correct conclusion |
| **Do not reject $H_0$** | Correct conclusion | Wrong conclusion (Type II error) |

**Table 2. Probabilities of a Wrong Conclusion**

| Wrong Conclusion | Probability of Occurrence |
|---|---|
| Type I error | $\alpha$ (called the *level of significance*) |
| Type II error | $\beta$ ($1 - \beta$ is called the *power*) |

It is important to determine the required sample size to control the Type I error ($\alpha$) and Type II error ($\beta$) simultaneously. Formulas for sample sizes supporting an inferential study that depend on selected values of ($\alpha$) and ($\beta$) are available in many textbooks and software packages. These formulas become more complex when the design includes blocking or experimental

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factors such as analyst or day. Computer simulation is a useful tool in these more complex situations, and support of a statistician can be useful.

While replication is an effective strategy for reducing the impact of random variability on uncertainty and risk, blocking can be used to remove known sources of variability. For example, in a study to compare two analytical procedures, each procedure might be used to measure each sample unit of material. This results in the removal of the variability between sample units of material, which provides a reduced error term used to compare differences between the two procedures. By reducing the error term in this manner, the power of the experiment is increased for a fixed number of sample units. A numerical example is provided in *5. Comparison of Analytical Procedures*.

## Study Conduct

It is important to avoid introducing systematic error or bias into the study results. Bias can be introduced through unintentional changes in experimental conditions, due to either known or unknown factors. Effective sampling and randomization are important considerations in mitigating the impact of bias. Sampling is performed after the study has been designed and constitutes the selection of test articles within the structure of the design. How to attain such a sample depends entirely on the question that is to be answered by the data. When possible, use of a random process is considered the most appropriate way of selecting samples.

The most straightforward type of random sampling is called *simple random sampling*. However, sometimes this method of selecting a random sample is not desirable because it cannot guarantee equal representation across study factors. The design of a study to release manufactured lots might incorporate factors such as selected times, locations, or parallel manufacturing streams (e.g., multiple filling lines). In this case a stratified sample whereby units are randomly selected from within each factor can be utilized. Regardless of the reason for taking a sample, a sampling plan should be established to provide details on how the sample is to be obtained to ensure that it is representative of the entirety of the population of interest.

Randomization should not be restricted to sampling. Study samples should be strategically entered into an analytical procedure using randomization, while blocking can be utilized to avoid confounding of the study objective with assay related factors.

Sometimes it's impossible to utilize sampling plans which are random or systematic in nature. This is especially true when the population is infinite. In this case representativeness is addressed through study design including blocking, where factors which are known to be the key structural components of the population are used to represent the infinite population.

The optimal sampling and analytical testing strategy will depend on knowledge of the manufacturing, analytical measurement, and/or study related processes. In the case of sampling to measure a property of a manufactured lot, it is likely that the sampling will include some element of random selection. There should be sufficient samples collected for the original analysis, subsequent verification analyses, and other supporting analyses. In the case of sampling to address a more complex study, representativeness should be addressed through strategic design. It is recommended that the subject matter expert work with a statistician to help select the most appropriate sampling plan and design for the specified objective.

An additional consideration in the conduct of a study is data recording. Many institutions store data in a laboratory information management system (LIMS). That data may be entered to the number of significant digits (decimals) of the reportable value for the test procedure. While this practice is appropriate for the purpose of reporting test data (such as in a certificate of analysis or in a regulatory dossier), it is inappropriate for data which may be used for subsequent analysis. This is noted in ASTM E29 where it is stated "As far as is practicable with the calculating device or form used, carry out calculations with the test data exactly and round only the final result" (*1*). Rounding intermediate calculated results contributes to the overall error in the final result. More on rounding is included in *General Notices, 7.20 Rounding Rules* and in *Appendix 2: Models and Data Considerations*.

## Study Analysis

The culmination of a study is a statistical analysis of the data, and a decision in the case of an inferential study. Simple summaries such as group averages and appropriate measures of variability, as well as plots of the data and summary results facilitate the analysis and communication of the study results and decision. Summaries should be supplemented with confidence intervals or bounds, which express the uncertainty in the summary result (see *3. Basic Statistical Principles and Uncertainty*). Transformations based on either scientific information or empirical evidence can be considered, and screening for outlying values and subsequent investigations completed (see *Appendix 2: Models and Data Considerations*).

Many common statistical analysis tools are found in calculation programs such as spreadsheets and instrument software. Software which is dedicated to statistical analysis and modeling contain additional tools to evaluate assumptions associated with the analysis tools, such as normality, homogeneity of variance, and independence. Those with limited or no statistical training should consult a statistician throughout the process of conducting a study, including study design and analysis. Their statistical skills complement the laboratory skills in ensuring appropriate study design, analysis, and decisions.

The study considerations outlined in this section will be illustrated hereafter.

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# 5. COMPARISON OF ANALYTICAL PROCEDURES

It is often necessary to compare two analytical procedures to determine if differences in accuracy and precision are less than an amount deemed practically important. For example, *General Notices, 6.30 Alternative and Harmonized Methods and Procedures* describes the need to produce comparable results to the compendial method. Transfer of analytical procedures as described in *Transfer of Analytical Procedures* ⟨1224⟩ allows for comparative testing as an acceptable process. A change in a

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procedure includes a change in technology, a change in laboratory (called *transfer*), or a change in the reference standard in the procedure.

For purposes of this section, the terms "old procedure" and "new procedure" are used to represent a procedure before and after a change. Procedures with differences less than the practically important criterion are said to be equivalent or better (see *Appendix 3: Equivalence and Noninferiority Testing*). This section follows the outline described in *4. Study Considerations* highlighting the scientific method of (1) study objective, (2) study design, (3) study conduct, and (4) study analysis.

## Study Objective of a Procedure Comparison

The study objective of a procedure comparison is to demonstrate that a new procedure performs equivalent to or better than an old procedure. There are two conceptual study populations: All future measurements made with the old procedure on a particular process, and all future measurements made with the new procedure on the same process. Each procedure is described in terms of the mean and standard deviation of the population of measurements. The mean and standard deviation of the reportable value of the new procedure are denoted by the Greek symbols $\mu_N$ and $\sigma_N$ respectively. The subscript *N* denotes the "new" procedure population. The mean and standard deviation of measurements using the "old" procedure are denoted $\mu_O$ and $\sigma_O$ respectively. These means and standard deviations are unknown, but conclusions concerning their potential equivalence or noninferiority (the new procedure is not inferior to the old procedure) are informed by estimates resulting from the experiment. Characteristics for comparison are most generally accuracy and precision across the range of the assay, and across conditions experienced during long term routine analysis. A risk analysis should be performed to identify such conditions. Discussion of accuracy and precision are found in ⟨1225⟩.

### ACCURACY

To compare accuracy of two procedures, one compares the procedure means. In particular, accuracy is compared using the absolute value of the true difference in means,

$$|\mu_D| = |\mu_N - \mu_O|. \quad \text{Equation 10}$$

The objective of such a study is to demonstrate that $|\mu_D|$ is less than a value deemed to be practically important, *d*. As an example, *d* may represent a numerical value that is small enough so that an increase in bias of this magnitude does not negatively impact decisions concerning lot disposition (i.e., conformance to specifications). The hypotheses used in an equivalence test are

$$\begin{aligned} H_0&: |\mu_D| \geq d \\ H_a&: |\mu_D| < d \end{aligned} \quad \text{Equation 11}$$

(see *Appendix 3: Equivalence and Noninferiority Testing*).

Probably the most difficult aspect of conducting an equivalence test is determination of *d*. Typically, *d* is determined in partnership between the analytical chemist and the statistician based on combined manufacturing and scientific knowledge. Definitions of *d* vary across companies based on differing risk profiles and experience. In some cases there exists a large amount of legacy data that may inform the decision, while in other cases there may be only limited data. An example where *d* is based on requirements of a manufacturing process follows in the section *Determination of d and k*.

### PRECISION

To compare precision of two procedures, one compares the procedure standard deviations. Whereas a comparison of means involves a difference, a comparison of standard deviations involves the ratio

$$\frac{\sigma_N}{\sigma_O} \quad \text{Equation 12}$$

The study objective is to demonstrate that the ratio in *Equation 12* is less than a practically important value *k*. The noninferiority hypotheses are

$$\begin{aligned} H_0&: \frac{\sigma_N}{\sigma_O} \geq k \\ H_a&: \frac{\sigma_N}{\sigma_O} < k \end{aligned} \quad \text{Equation 13}$$

(see *Appendix 3: Equivalence and Noninferiority Testing*). The selection of *k* should be in alignment with the selection of *d* for the accuracy assessment. This process is demonstrated in the following section.

### DETERMINATION OF *d* AND *k*

Values of *d* and *k* for the tests of accuracy and precision should be internally consistent. To demonstrate, consider a case where historical measurements using an old procedure for a monitored process have a process mean of $\mu_O = 100$ units and a

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combined process and analytical variance of $\sigma_L^2 + \sigma_O^2 = 0.80$ where $\sigma_L^2$ represents lot-to-lot variability of the manufacturing process. Historic measurements of a reference standard provide the estimate $\sigma_O^2 = 0.16$ so that the assumed value of the lot variance is $\sigma_L^2 = 0.80 - 0.16 = 0.64$. The process specifications are the lower specification limit (LSL) = 96 units and the upper specification limit (USL) = 104 units. The same manufacturing process measured with the new procedure can be represented as having mean $\mu_N = \mu_O + d$ and total process and analytical variance $\sigma_L^2 + \sigma_N^2 = \sigma_L^2 + k^2\sigma_O^2$.

Kringle et al. (*18*) recommend selecting values of *d* and *k* consistent with a rule that states the proportion of product that falls outside of specification (OOS) when measured with the new procedure is acceptable. *Table 3* reports the OOS rate when the process is in control and measured with the new procedure for several values of *d* and *k*. (Since the specifications are symmetric around $\mu_O$, negative values of *d* provide the same OOS rates as the positive values shown in the *Table 3*).

**Table 3. OOS Rate with New Procedure for Values of *d* and *k***

| *d* | *k* = 1 | *k* = 1.5 | *k* = 2 |
|---|---|---|---|
| 0 | 0.001% | 0.01% | 0.04% |
| 1 | 0.04% | 0.14% | 0.40% |
| 2 | 1.27% | 2.28% | 3.85% |

*Table 3* assumes the process is normal and the probability (Pr) in any cell is given by the equation

$$\text{▲Pr(OOS)} = 1 - \Pr(96 \le \text{Sampled Process Value} \le 104)$$
$$= 1 - \Phi\left(\frac{104 - (100 + d)}{\sqrt{0.64 + k^2 \times 0.16}}\right) + \Phi\left(\frac{96 - (100 + d)}{\sqrt{0.64 + k^2 \times 0.16}}\right) \text{▲ (USP 1-Dec-2021)} \quad \text{Equation 14}$$

where $\Phi(\bullet)$ represents the cumulative probability function of the standard normal distribution. ▲The choice of OOS rate with the new procedure should be a business decision.▲ (USP 1-Dec-2021)

## Study Design of a Procedure Comparison

The study design for comparing the old and new analytical procedures is comprised of the selection of test materials, experimental design, and sample size determination (the so-called "power calculation"). Results for two scenarios are provided in this section. The first scenario considers samples from homogeneous test material, and the second scenario considers test material with variation across sample units.

### SCENARIO 1: HOMOGENEOUS TEST MATERIAL

In this scenario, test samples of homogeneous material are selected and measured using one of the procedures on each test sample. There are $n_O$ samples measured with the old procedure and $n_N$ samples measured with the new procedure. It is recommended to design the study so that $n_O = n_N$. *Table 4* presents this design, which is referred to as an independent two-sample design.

**Table 4. Independent Two-Sample Design**

| Sample ID | New Procedure | Old Procedure |
|---|---|---|
| 1 | $Y_{N1}$ | — |
| 2 | $Y_{N2}$ | |
| ⋮ | ⋮ | |
| $n_N$ | $Y_{Nn_N}$ | |
| $n_N + 1$ | — | $Y_{O1}$ |
| $n_N + 2$ | | $Y_{O2}$ |
| ⋮ | | ⋮ |
| $n_N + n_O$ | | $Y_{On_O}$ |
| Sample Mean | $\overline{Y}_N = \frac{\sum_{j=1}^{n_N} Y_{Nj}}{n_N}$ | $\overline{Y}_O = \frac{\sum_{j=1}^{n_O} Y_{Oj}}{n_O}$ |

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**Table 4. Independent Two-Sample Design** *(continued)*

| Sample ID | New Procedure | Old Procedure |
|---|---|---|
| Sample Variance | $s_N^2 = \frac{\sum_{j=1}^{n_N}\left(Y_{Nj} - \overline{Y}_N\right)^2}{n_N - 1}$ | $s_O^2 = \frac{\sum_{j=1}^{n_O}\left(Y_{Oj} - \overline{Y}_O\right)^2}{n_O - 1}$ |

For the comparison of means the estimator of interest is the difference of sample means, $\overline{Y}_N - \overline{Y}_O$ which has variance

$$\text{Var}\left(\overline{Y}_N - \overline{Y}_O\right) = \sigma_N^2/n_N + \sigma_O^2/n_O \qquad \text{Equation 15}$$

Power calculations are needed to ensure the sample size is great enough to find evidence that $H$▲$_{\alpha}$▲ (USP 1-Dec-2021) is true when such is the case. For testing the equivalence hypotheses in *Equation 11* assuming $\sigma_N = \sigma_O$, Bristol (*8*) recommends the sample size formula

$$n_N = n_O = 2 \times \left(\frac{\left(Z_{1-\alpha} + Z_{1-\beta}\right) \times \sigma_O}{d - |\mu_D|}\right)^2 + 1 \qquad \text{Equation 16}$$

where $Z_{1-\alpha}$ and $Z_{1-\beta}$ are standard normal percentiles with area 1–α and 1–β respectively, to the left. The Type I error rate is α and the Type II error rate is β. ▲When $|\mu_D|$ is equal to zero, replace $Z_{1-\beta}$ with $Z_{1-\frac{\beta}{2}}$.

If it is desired to have a probability of passing of 85% (β = 0.15) with α = 0.05 when means differ by one standard deviation, i.e., with $|\mu_D| = \sigma_O = \sqrt{0.16} = 0.4$, and $d$=1, the required sample size for both the new and old procedures is given by the equation

$$n_N = n_O = 2 \times \left(\frac{(1.645 + 1.036) \times 0.4}{1 - |0.4|}\right)^2 + 1 = 7.4\text{▲ (USP 1-Dec-2021)} \qquad \text{Equation 17}$$

which is rounded up to 8 for each procedure (for 16 total test samples).

To test the noninferiority hypotheses in *Equation 13*, it is desired to have a high power when $\sigma_N = \sigma_O$.

The required sample size is obtained by solving for $n_N$ and $n_O$ iteratively using the equation

$$1 - \beta = \Pr\left(F < \frac{\sigma_O^2 k^2}{\sigma_N^2} \times F_{\alpha: n_N - 1, n_O - 1}\right) \qquad \text{Equation 18}$$

where $F$ is a random variable following the $F$-distribution with degrees of freedom $n_N - 1$ and $n_O - 1$. As noted earlier, it is recommended that $n_N = n_O$▲▲ (USP 1-Dec-2021). *Table 5* reports the power for sample size combinations ▲with $k = 2$,▲ (USP 1-Dec-2021) α = 0.05, and $\sigma_N = \sigma_O = 0.4$.

**Table 5. Power Calculation for Noninferiority Test with α = 0.05**

| $n_N$ | $n_O$ | $\frac{\sigma_O^2 k^2}{\sigma_N^2}$ | $F_{\alpha: n_N - 1, n_O - 1}$ | $\frac{\sigma_O^2 k^2}{\sigma_N^2} \times F_{\alpha: n_N - 1, n_O - 1}$ | Power when $\sigma_N = \sigma_O$ |
|---|---|---|---|---|---|
| 8 | 8 | 4 | 0.264 | 1.056 | 0.528 |
| 14 | 14 | 4 | 0.388 | 1.552 | 0.781 |
| 15 | 15 | 4 | 0.403 | 1.610 | 0.808 |
| 19 | 19 | 4 | 0.451 | 1.804 | 0.890 |
| 20 | 20 | 4 | 0.461 | 1.845 | 0.904 |

From *Table 5* it is seen that the sample of size 8 required for the test of equivalence of means does not provide acceptable power for the noninferiority test (power = 0.528). This is because estimates of standard deviations have greater uncertainty than estimates of means. Practicality often dictates that one select a greater value for β in a test of noninferiority than in a test for equivalence of means. In the present example, β is selected as 0.20 ▲in the test for noninferiority, and▲ (USP 1-Dec-2021) a sample size of 15 per procedure (30 test samples in total) is selected for the design.

When a comparison is made between laboratories (as during procedure transfer) it's important to keep in mind that in order to be representative of future testing, the study design should include factors which have significant impact on the long term

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performance of the procedure. As noted previously, this may include analyst, but may also require that multiple instruments and batches of key reagents be included in the design. These may be nested or crossed. Failing to do so may underestimate the variability or confound the effects of some factors with the difference between labs. In general factors such as analysts where levels are unique within each laboratory might be nested within each lab, while factors such as reagent lots which might be routinely shared across laboratories could be crossed with laboratory. As such, the estimates of variability used in these equations should be representative of the variability induced by these factors. The best estimates of variability come from data collected on samples tested across a broad period of time, such as stability samples and an assay control. More considerations of this nature are described in ⟨1210⟩.

## SCENARIO 2: VARIATION ACROSS TEST SAMPLES

It is often desirable to compare procedures across manufactured lots or use different manufactured levels of an analyte. This is important if the study objective is to ensure the range of the procedure in the new laboratory, or when the procedure is intended to measure degraded samples. This selection of test material introduces a new source of variation to *Scenario 1* that must be considered during the study design in order to most efficiently compare the two procedures.

The recommended design in *Scenario 2* is a paired design in which each test sample is measured independently by both procedures, instead of having each test sample randomly measured by only one procedure as in *Scenario 1*. The term "test sample" is referred to as a blocking factor because observations within the same block are differenced (see *4. Study Considerations*). This has the effect of removing the variation across test samples from the analysis. *Table 6* presents a schematic illustration of the paired design using *n* test samples.

**Table 6. Paired Design**

| Test Sample | New Procedure | Old Procedure | Difference |
|---|---|---|---|
| *1* | $Y_{N,1}$ | $Y_{O,1}$ | $D_1 = Y_{N,1} - Y_{O,1}$ |
| *2* | $Y_{N,2}$ | $Y_{O,2}$ | $D_2 = Y_{N,2} - Y_{O,2}$ |
| ⋮ | ⋮ | ⋮ | ⋮ |
| *n* | $Y_{N,n}$ | $Y_{O,n}$ | $D_n = Y_{N,n} - Y_{O,n}$ |
| Sample Mean | $\overline{Y}_N$ | $\overline{Y}_O$ | $\overline{D} = \overline{Y}_N - \overline{Y}_O = \frac{\sum_{j=1}^{n} D_j}{n}$ |
| Sample Variance | NA | NA | $S_D^2 = \frac{\sum_{j=1}^{n} \left(D_j - \overline{D}\right)^2}{n-1}$ |

Using the paired design with *n* lots, the variance of $\overline{D}$ is $(\sigma_N^2 + \sigma_O^2)/n$ because the variability due to lots disappears when results on the same lot are differenced. The unbiased estimator of $\sigma_N^2 + \sigma_O^2$ is $S_D^2$.

The sample size formula for satisfying the mean test requirements for a paired design ▲▲ (USP 1-Dec-2021) is

$$n = \left( \frac{\left(Z_{1-\alpha} + Z_{1-\beta}\right) \times \sqrt{\sigma_N^2 + \sigma_O^2}}{d - |\mu_D|} \right)^2 + 1 \text{ ▲▲ (USP 1-Dec-2021)}$$

Equation 19

▲▲ (USP 1-Dec-2021)Using the same planning data from *Scenario 1*, ▲$d = 1$, $|\mu_D| = 0.4$, $\sigma_N^2 = \sigma_O^2 = 0.16$, $\beta = 0.15$, and $\alpha = 0.05$, the required sample size is

$$n = \left( \frac{(1.645 + 1.036) \times \sqrt{0.16 + 0.16}}{1 - 0.4} \right)^2 + 1 = 7.4 \text{ ▲▲ (USP 1-Dec-2021)}$$ Equation 20

which is rounded up to 8 test samples (which are each measured once by each procedure). When using a paired design for the test of non-inferiority, the ability to find a good estimate of $\sigma_O^2$ is critical. Good estimates of $\sigma_O^2$ are often available from previous method validation studies or repeated measurements of an assay control. If no such estimate exists, it is necessary to modify the design in *Table 6* and record two independent measurements with each procedure on each test sample. Independent estimates of both $\sigma_O^2$ and $\sigma_N^2$ can then be computed from the differences of the two paired values as shown in the section *Study Analysis of a Procedure Comparison* that follows.

If a good estimate for $\sigma_O^2$ is available, the required sample size for the noninferiority test is derived iteratively from the equation

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$$1 - \beta = \Pr\left(W < \frac{\left(k^2+1\right)\sigma_O^2 \times \chi^2_{\alpha:n-1}}{\sigma_N^2 + \sigma_O^2}\right) \quad \text{Equation 21}$$

where $W$ is a chi-squared random variable with $n - 1$ degrees of freedom.

*Table 7* reports the power for sample size combinations when $\alpha = 0.05$, ▲$k$=2,▲ (USP 1-Dec-2021) and $\sigma_N = \sigma_O = 0.4$.

**Table 7. Power Calculation for Noninferiority Test with $\alpha = 0.05$**

| $n$ | $\frac{\left(k^2+1\right)\sigma_O^2}{\sigma_N^2+\sigma_O^2}$ | $\chi^2_{\alpha:n-1}$ | $\frac{\left(k^2+1\right)\sigma_O^2}{\sigma_N^2+\sigma_O^2} \times \chi^2_{\alpha:n-1}$ | Power when $\sigma_N = \sigma_O$ |
|---|---|---|---|---|
| 8 | 2.5 | 2.167 | 5.418 | 0.391 |
| 17 | 2.5 | 7.962 | 19.904 | 0.775 |
| 18 | 2.5 | 8.672 | 21.679 | 0.803 |
| 22 | 2.5 | 11.591 | 28.978 | 0.885 |
| 23 | 2.5 | 12.338 | 30.845 | 0.901 |

To obtain a power of 0.80 when the two standard deviations are equal, a sample of 18 test samples is required. Note that each test sample need not be unique. For example, if samples are being selected from three lots of product, one could select 6 test samples from each lot.

## Study Conduct of a Procedure Comparison

When conducting the study, it is important to observe the random assignment of test samples to procedures in *Scenario 1* in order to guard against possible bias. If repeated measurements are used in *Scenario 2* to provide individual estimates of $\sigma_O^2$ and $\sigma_N^2$, then independent measurements are needed. This will require independent preparations for each portion of the test sample.

## Study Analysis of a Procedure Comparison

Two examples are provided to demonstrate the described formulas. Data in the examples were simulated from a population where $\mu_N = \mu_O = 100$ and $\sigma_N^2 = \sigma_O^2$. These values were selected to demonstrate the computed sample sizes are sufficient under the assumed conditions.

### SCENARIO 1: HOMOGENEOUS TEST MATERIAL

*Table 8* reports a sample data set with $n_N = n_O = 15$.

**Table 8. Data from Simulated Two-Sample Independent Design**

| Procedure | Sample Mean | Sample Variance |
|---|---|---|
| New | $\bar{Y}_N = 100.08$ | $S_N^2 = 0.214$ |
| Old | $\bar{Y}_O = 99.85$ | $S_O^2 = 0.159$ |

Accuracy is tested using the hypotheses in *Equation 11* by constructing a 100(1 – 2α)% confidence interval on $\mu_D$ using the equation

$$\text{▲}\bar{Y}_N - \bar{Y}_O \pm t_{1-\alpha:df}\sqrt{\frac{S_N^2}{n_N} + \frac{S_O^2}{n_O}}\text{▲ (USP 1-Dec-2021)}$$

$$df = \frac{\left(\frac{S_N^2}{n_N} + \frac{S_O^2}{n_O}\right)^2}{\frac{S_N^4}{n_N^2\left(n_N-1\right)} + \frac{S_O^4}{n_O^2\left(n_O-1\right)}} \quad \text{Equation 22}$$

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where $t_{1-\alpha:df}$ is a quantile from a central *t*-distribution with area 1 – α to the left and degrees of freedom (*df*). The null hypothesis in *Equation 11* is rejected, and equivalence demonstrated if the entire confidence interval computed from *Equation 22* falls in the range from –*d* to +*d*. This is the TOST described in *Appendix 3: Equivalence and Noninferiority Testing* and has a Type I error rate of α. With some software packages such as Excel, non-integer *df*-values are not accepted when determining the *t*-value. In this case, simply round to the nearest integer.

The 90% two-sided confidence interval that provides a Type I error rate of 0.05 computed from *Equation 22* is

$$df = \frac{\left(\frac{0.214}{15} + \frac{0.159}{15}\right)^2}{\frac{0.214^2}{15^2(15-1)} + \frac{0.159^2}{15^2(15-1)}} = 27.4 = 27 \text{ (rounded)}$$

$$\overline{Y}_N - \overline{Y}_O \pm t_{1-\alpha:df}\sqrt{\frac{S_N^2}{n_N} + \frac{S_O^2}{n_O}}$$

▲100.08 – 99.85 ± 1.703$\sqrt{\frac{0.214}{15} + \frac{0.159}{15}}$ = [–0.04 ; 0.50] ▲ (USP 1-Dec-2021) Equation 23

Since the computed confidence interval falls entirely in the range between –1 and +1 (i.e., –*d* to +*d*) equivalence of means has been demonstrated.

Precision is tested using the hypotheses in *Equation 13* by constructing a 100(1 – α)% one-sided upper confidence bound on the ratio $\sigma_N/\sigma_O$ using the formula

$$\frac{S_N}{S_O}\sqrt{\frac{1}{F_{\alpha, n_N - 1, n_O - 1}}}$$ Equation 24

where $F_{\alpha, n_N - 1, n_O - 1}$ is the *F*-quantile with area α to the left and degrees of freedom $n_N - 1$ and $n_O$▲ – 1▲ (USP 1-Dec-2021). If the upper bound computed with *Equation 24* is less than *k*, the null hypothesis is rejected and one concludes noninferiority of the standard deviation of the new procedure. This test has a Type I error rate of α.

The 95% upper bound on $\sigma_N/\sigma_O$ computed from *Equation 24* is

$$U = \frac{\sqrt{0.214}}{\sqrt{0.159}}\sqrt{\frac{1}{0.402}} = 1.83$$ Equation 25

Since this upper bound is less than *k* = 2, noninferiority of the standard deviation of the new procedure has been demonstrated.

## SCENARIO 2: VARIATION ACROSS TEST SAMPLES

*Table 9* provides summary results for 18 test samples in a paired design with $\overline{D} = \overline{Y}_N - \overline{Y}_O$.

**Table 9. Data from Simulated Paired Design with *n*=18**

| Sample Mean | Sample Variance |
|---|---|
| $\overline{D}$ = 0.39 | $S_D^2$ = 0.350 |

The 90% confidence interval on the difference in means for a paired design used to test equivalence of means with the data from *Table 9* is

$$\overline{D} \pm t_{0.95;\, n-1}\sqrt{\frac{S_D^2}{n}}$$

▲0.39 ± 1.74$\sqrt{\frac{0.350}{18}}$ = [0.15 to 0.63] ▲ (USP 1-Dec-2021) Equation 26

Since the computed confidence interval falls entirely in the range between –1 and +1 equivalence of means has been demonstrated.

The noninferiority hypotheses in *Equation 13* can be tested by constructing a 100(1 – α)% upper confidence bound on $\sigma_N/\sigma_O$ using the formula

$$\sqrt{\frac{(n-1)S_D^2}{\sigma_O^2 \times \chi^2_{\alpha:n-1}}} - 1$$ Equation 27

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where $\chi^2_{\alpha:n-1}$ is a percentile from the chi-squared distribution with area α to the left and degrees of freedom $n - 1$. If this upper bound is less than $k$, the null hypothesis is rejected and noninferiority has been demonstrated.

From historical data used to plan the sample size, a good estimate of the old procedure variance is $\sigma_0^2 = 0.16$. Using the confidence bound in *Equation 27*, the 95% upper confidence bound on $\sigma_N/\sigma_O$ is

$$U = \sqrt{\frac{(18-1)0.350}{0.16 \times 8.67} - 1}$$

$$U = 1.81 \quad \text{Equation 28}$$

Since this upper bound is less than $k$=2, noninferiority of the standard deviation of the new procedure has been demonstrated.

If a good estimate of $\sigma_0^2$is not available, the design requires replicate measures for each procedure on each test sample. Independent estimates of the analytical variances are computed using the formulas

$$S_{DN}^2 = \frac{\sum_{j=1}^{n}\left(\frac{Y_{jN1} - Y_{jN2}}{\sqrt{2}} - \overline{D}_N\right)^2}{n-1}$$

$$S_{DO}^2 = \frac{\sum_{j=1}^{n}\left(\frac{Y_{jO1} - Y_{jO2}}{\sqrt{2}} - \overline{D}_O\right)^2}{n-1} \quad \text{Equation 29}$$

where $Y_{jN1}$ is the first measurement on test sample $j$ with method $N$, $Y_{jN2}$ is the second measurement on test sample $j$ with method $N$, $Y_{jO1}$ is the first measurement on test sample $j$ with method $O$, and $Y_{jO2}$ is the second measurement on test sample $j$ with method $O$. The resulting 100(1 − α)% one-sided upper confidence bound on the ratio $\sigma_N/\sigma_O$ is

$$\frac{S_{DN}}{S_{DO}}\sqrt{\frac{1}{F_{\alpha,n-1,n-1}}} \quad \text{Equation 30}$$

where $F_{\alpha,n-1,n-1}$ is the $F$-quantile with area α to the left and degrees of freedom $n - 1$ and $n - 1$, and $n$ is the number of test samples (each with four independent measures). If this formulation is needed, then define $D_j = [(Y_{jN1} + Y_{jN2}) - (Y_{jO1} + Y_{jO2})]/\sqrt{2}$ in the test for mean equivalence.

## APPENDIX 1: CONTROL CHARTS

Control charts are used in the pharmaceutical industry to monitor the performance of manufacturing processes and analytical procedures. Using the vernacular of the scientific method, control charts are a tool to study these process populations, requiring a carefully developed objective, a strategic design, plans for implementation, and appropriate analysis. This appendix will discuss and illustrate the design and analysis of various control chart tools, as well as provide rules which are commonly used to make decisions.

Through its life cycle a process or a procedure can be influenced by known changes or unforeseen variability. For a manufacturing process this might impact the quality of the product or indicate the need to take action. For an analytical procedure which is routinely used to aid decision-making, this might increase the risk of drawing the wrong conclusion from a study or likewise indicate the need for action. Thus, it is important to continuously verify performance and provide ongoing assurance of a state of control. To this end, data from a manufacturing process or that relate to procedure performance are collected and analyzed. For a manufacturing process these may include process parameters and test results on manufactured materials. For an analytical procedure they can include analytical results for controls, standards used during the analysis, and system suitability data. It's important to note that the control samples are used to monitor the performance of the procedure and are not an indicator of the product performance or characteristics (FDA ISO/IEC 17025 [*21–23*]). For purposes of this appendix the term "process" will be used to refer to both a manufacturing process and an analytical procedure.

Although various trending methods exist, control charts are one of the most simple and effective graphical tools for such analysis. There are many types of control charts including the following:

- Individual (I) chart for plotting individual values over time
- X-bar chart for plotting sample means over time
- Range (R) chart for plotting sample ranges over time
- Moving range (MR) chart for plotting moving ranges over time
- S-chart for plotting sample standard deviations over time
- Exponentially weighted moving average (EWMA) and cumulative sum (CUSUM) charts which are used when small shifts in the mean of the procedure are of interest

A typical control chart consists of a centerline and lower and upper control limits. The centerline represents the center of the distribution of a variable measured in the process. The two control limits are determined such that if the process performs as intended, nearly all results will fall within the two limits. Observations outside the limits or points within the limits that indicate a systematic or non-random pattern are indicative of a potential performance issue. Non-systematic patterns have been defined by WECO (which stands for Western Electric Company) and Nelson (*20*) that can be used in evaluating a control chart. Historical

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data (the “control data”) are typically used to obtain the centerline and lower and upper control limits. The control chart provides a visual means for identifying shifts, trends, and variability indicative of potential performance issues. A clarifying example is presented in the next section based on the Individual or I-chart.

## Shewhart I-Chart

To develop a control chart for individual observations, it is customary to set control limits at

Process Mean ± 3 × Process Standard Deviation Equation 31

These limits are based on assuming the process data follow a normal probability distribution and that a range of 3 standard deviations about the mean contains roughly 99.7% of all the data. Given a sample of $Y_1$, $Y_2$, … , $Y_n$ observations from a controlled process, the process mean (average) is estimated using the formula

$$\overline{Y} = \frac{\sum_{i=1}^{n} Y_i}{n} \qquad \text{Equation 32}$$

The standard deviation can be estimated in a couple of ways, but for an I-chart, best practice is to base the estimate on the moving range statistic (MR). This estimator considers the “short term” variability of the process and guards against limits that are too wide if an unexpected trend exists in the data. Specifically, the MR represents the average difference of successive observations and is defined as

$$\overline{\text{MR}} = \frac{\sum_{i=2}^{n} \left| Y_i - Y_{i-1} \right|}{n-1} \qquad \text{Equation 33}$$

and the estimator for the process standard deviation is

$$\frac{\overline{\text{MR}}}{d_2} \qquad \text{Equation 34}$$

where $d_2$ is a constant that depends on the number of observations associated with the moving range calculation ($m$). In *Equation 33*, $m = 2$ since the range is based on adjacent observations. The value of $d_2$ when $m = 2$ is 1.128. The upper control limit (UCL) and lower control limit (LCL) for the I-chart are then

$$\text{LCL} = \overline{Y} - 3 \times \frac{\overline{\text{MR}}}{d_2}$$
$$\text{UCL} = \overline{Y} + 3\frac{\overline{\text{MR}}}{d_2} \qquad \text{Equation 35}$$

To demonstrate, consider a sample of 20 observations with $\overline{Y} = 31.2$ and $\overline{\text{MR}} = 2.18$. From *Equation 35* the computed control limits are

$$\text{LCL} = 31.2 - 3 \times \frac{2.18}{1.128} = 25.4$$
$$\text{UCL} = 31.2 + 3 \times \frac{2.18}{1.128} = 37.0 \qquad \text{Equation 36}$$

The associated I-chart is shown in *Figure 1*.

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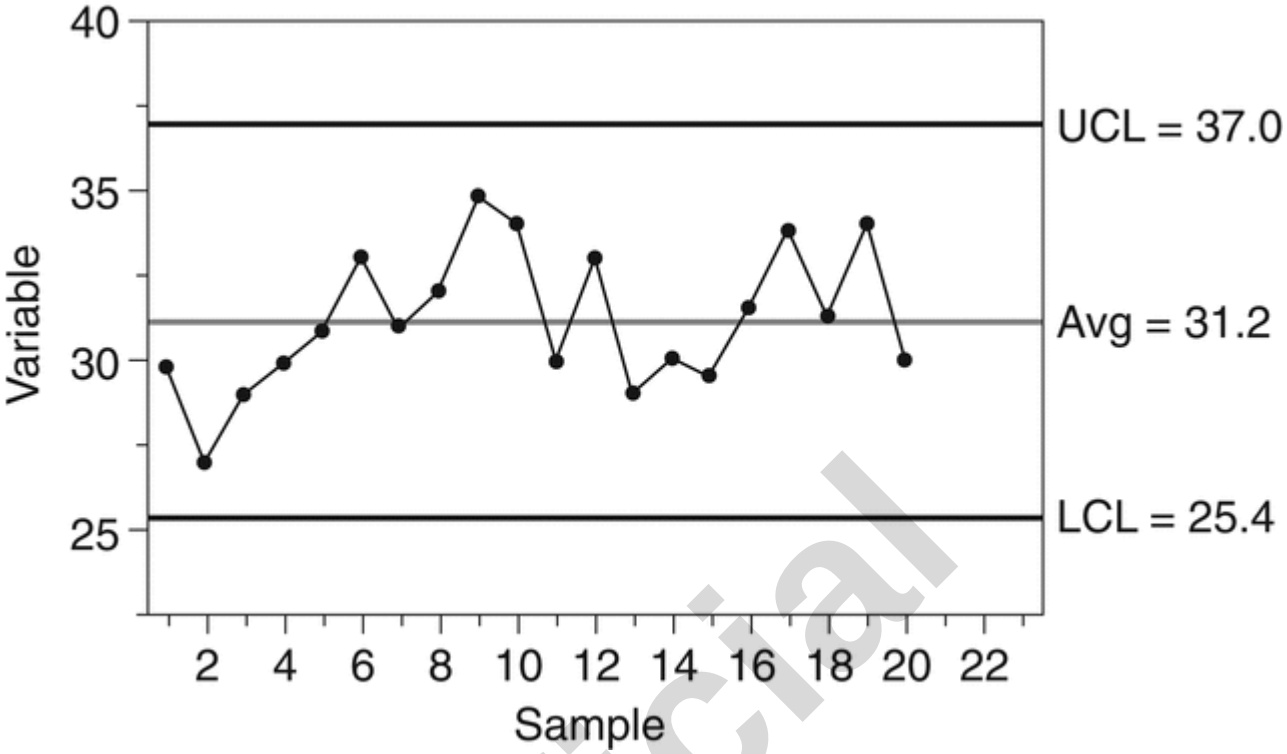


Figure 1. I-chart for example data set.

## Detection of Out-Of-Control Results

After a control chart is constructed, out-of-control results are detected using either WECO or Nelson rules. The Nelson rules are provided in *Table 10*. The relevance of these rules depends on the type of control chart. All eight rules can be applied to an I-chart, and selection of the particular rules depends on the desired sensitivity of the control process.

**Table 10. Nelson Rules for Detection of Out-of-Control Results**

| Rule | Description | Indication in an I-chart |
|---|---|---|
| 1 | 1 point exceeds either the LCL or UCL | 1 point is out of control |
| 2 | 9 points in a row on the same side of the center line | There is a mean shift in performance |
| 3 | 6 points in a row steadily increasing or decreasing | A trend exists |
| 4 | 14 points in a row alternating up and down | There is a negative correlation between neighboring points |
| 5 | 2 out of 3 points on the same side of the mean and greater than two standard deviations away from the mean | A possible increase in assay variability |
| 6 | 4 out of 5 points on the same side of the mean and greater than one standard deviation away from the mean | A possible increase in assay variability |
| 7 | 15 points in a row within one standard deviation of the mean | A possible decrease in assay variability |
| 8 | 8 points in a row on both sides of the mean with none within one standard deviation of the mean | Non-random sample |

*Figure 2* presents an I-chart for which a rule 2 violation is observed because the last 9 observations are all greater than the mean.

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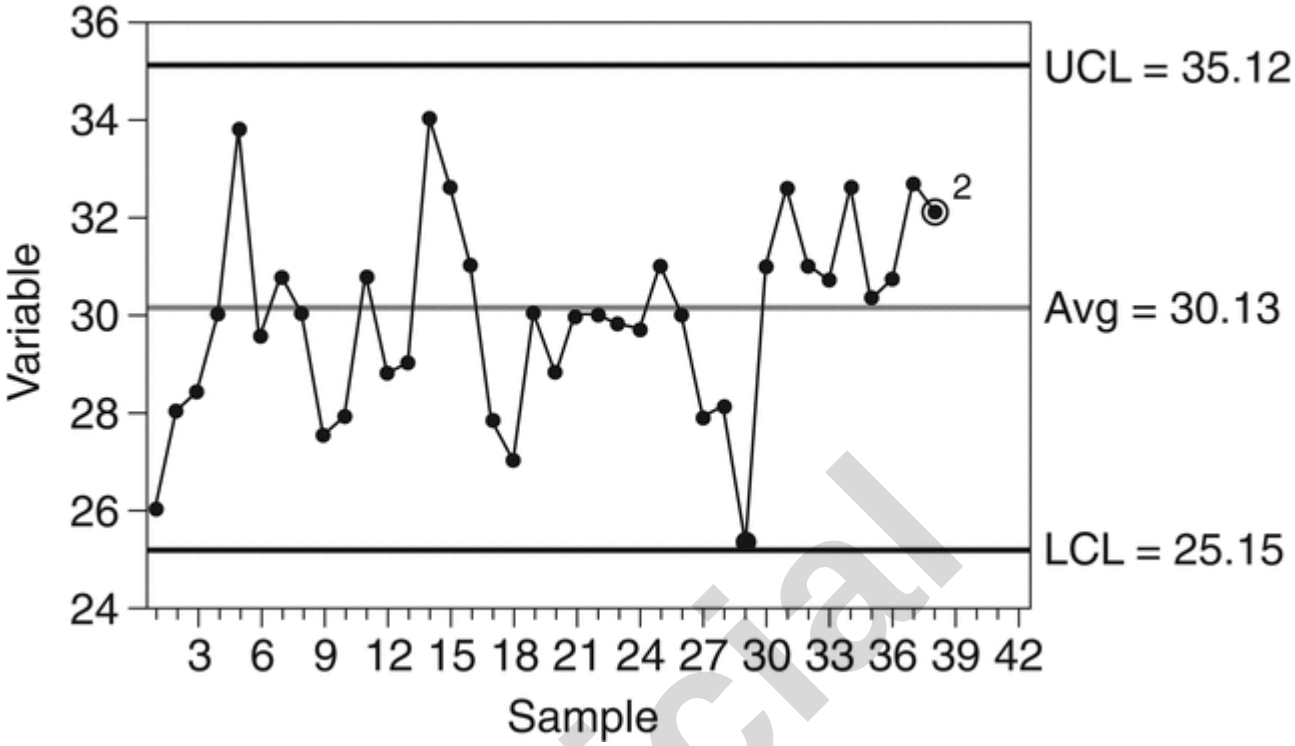


Figure 2. Individual control chart with mean shift detected using Nelson rule 2.

ASTM E2587 (*3*), Montgomery (*19*), and Wheeler (*29*) provide references for numerous control charts and example applications.

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## APPENDIX 2: MODELS AND DATA CONSIDERATIONS

Statistical analysis involves models and assumptions associated with the reliability of fitting models to data. Models can be simple (e.g., a means model associated with a reportable value) or complicated (e.g., a nonlinear mixed effects model common in complex pharmaceutical settings). Assumptions monitored with residuals from the model fit include normality, constant variance, and independence. This appendix focuses on adequacy of models that are fit to analytical data, as well as data considerations such as significant digits, transformations, and outliers.

### Models

In statistics, a model represents a functional description of some property(s) of a population. The term "population" refers to the set of all possible values of an attribute. A model parameter, also referred to as a population parameter, is the true but unknown value of a property, which is typically the subject of the statistical inquiry.

A means model characterizes the center of a univariate population, and can be written as

$$Y_i = \mu + E_i \quad \text{Equation 37}$$

where $Y_i$ is the $i^{th}$ observation in a sample of size $n$ from the population, $\mu$ is a model parameter representing the population mean, and $E_i$ the error. This error represents the effect of all factors that explain why the measured value is not always equal to $\mu$. Such factors typically include lot-to-lot variation in product or analytical method error. The means model is the basis of statistical inquiries related to a population mean, usually estimated by the sample mean

$$\overline{Y} = \frac{\sum_{i=1}^{n} Y_i}{n} \quad \text{Equation 38}$$

with errors estimated by residuals $R_i = Y_i - \overline{Y}$.

Another familiar model is the simple linear regression model. This model characterizes the linear trend in the population mean with some covariate $X_i$ (e.g., time or dose), and can be written as

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$$Y_i = \alpha + \beta X_i + E_i \quad \text{Equation 39}$$

where ($X_i$, $Y_i$) is the $i^{th}$ observation in a sample of size $n$ from the bivariate population, the parameters $\alpha$ and $\beta$ are the intercept and the slope, respectively, that defines the functional relationship and $E_i$ the error. Note that $\mu$ in *Equation 37* has been replaced with $\alpha + \beta X_i$ in *Equation 39* to allow the mean to change as a function of $X_i$. The parameters $\alpha$ and $\beta$ are estimated from sample data as was in *Equation 37*.

More complex models might be nonlinear, can include qualitative factors (e.g., analysts in a validation), or might include covariables which are random rather than fixed values (e.g., another measurement $Z_i$ made together with $X_i$).

## Significant Digits

The number of digits used for calculations and the number of digits appearing in a reportable value should be considered separately. It's important to record and carry more digits during calculation than will be reported. It is a good practice to perform all statistical calculations with as many digits as practical. Rounding should be used only as a final step before reporting the result. Automation facilitates the acquisition of numerous digits, while databases should be designed to store data with enough digits in anticipation of further calculations from the data.

The number of digits reported can sensibly be based on the standard deviation of the reportable value. ASTM E29, *USP General Notices, 7.20 Rounding Rules*, and the *FDA Laboratory Manual of Quality Policies* (*21–23*) provide guidance on rounding and determination of significant digits in a reported value.

## Transformation

A transformation is a functional re-expression of a measurement in order to better represent a known scientific relationship or to satisfy the assumptions of a statistical model. Transformations can also be discovered empirically with a representative set of the data using residual plots. One particularly useful transformation with analytical data is the logarithmic (log) transformation described in the next section.

### LOG TRANSFORMATION

Examples of transformations using scientific knowledge of the measurement system come from many biological systems. In particular, variation around the responses predicted by a means model is often proportional to the response. For these systems, it is useful to work with the log of the original response which will have nearly constant variance across the range of the response. The shape of the transformed distribution will also be more symmetric as shown in the lower panel of *Figure 3*. A log transformation can be conducted using any base including Napierian (base e), common (base 10), or base 2.

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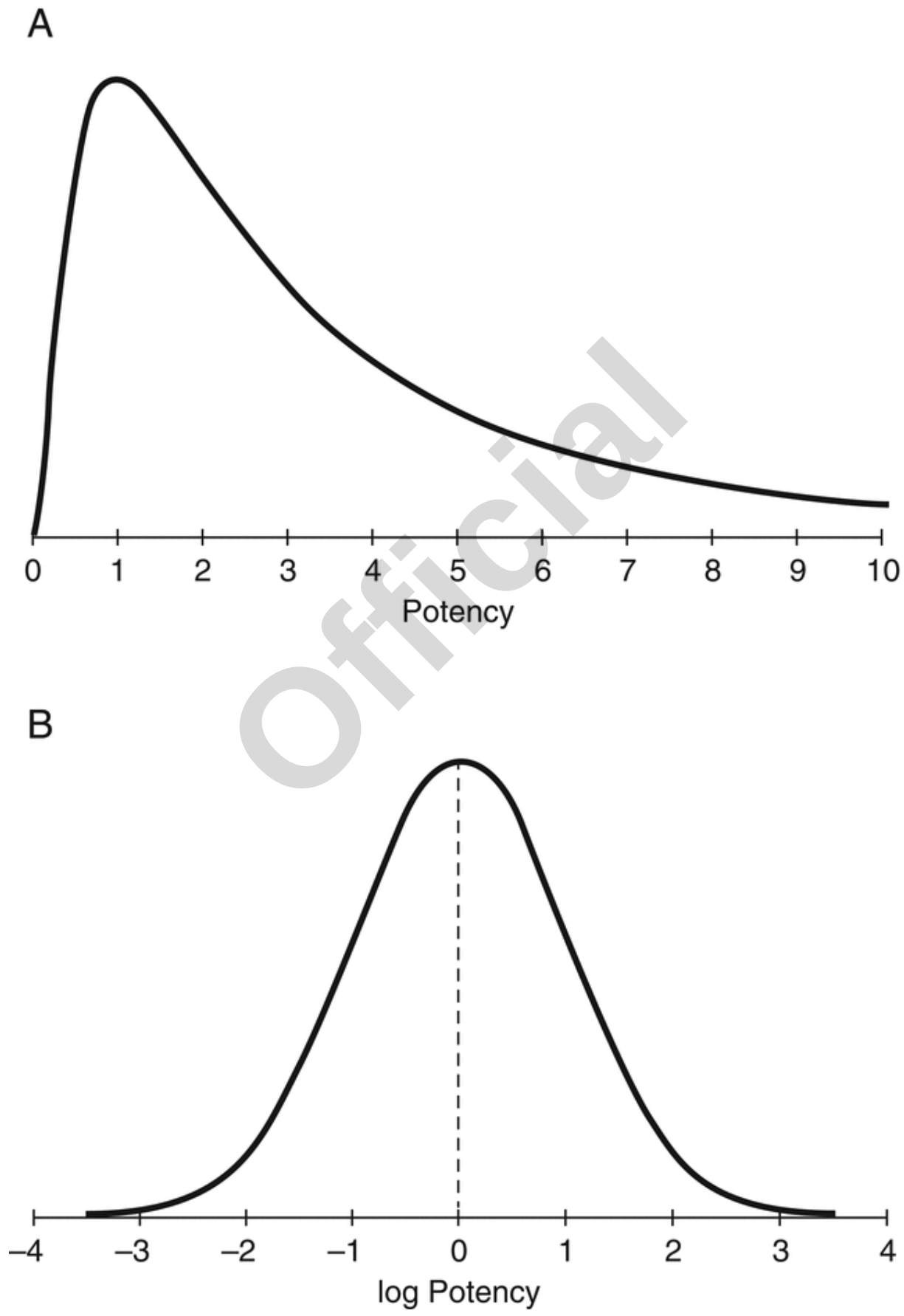


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Figure 3. A skewed log-normal distribution of potency (upper panel) and a symmetric normal distribution of log potency

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(lower panel).

Another reason for using a log transformation is that it can change a nonlinear functional form in the original scale to something more easily modeled in the log scale. For example, a log transformation can be used to re-express a nonlinear first order kinetics model as a linear model.

Statistical measures associated with the center and the dispersion from a sample are described in *3. Basic Statistical Principles and Uncertainty*. These include the sample mean ($\overline{Y}$) the sample standard deviation ($S$). These measures are meaningful when the data are approximately normally distributed and free of outliers. These measures may not be as meaningful when the normal distribution is not a good description of the data. To demonstrate, the top distribution in *Figure 3* is skewed to the right. The greater values in the tail have the effect of pulling the mean to the right of where some would deem to be the "center" of the data. The lower distribution in *Figure 3* shows the log-transformed responses of the top distribution. The top distribution is called a *log-normal distribution* because the distribution of its log values is normal. Because of the symmetry of the normal curve, the sample mean and sample standard deviation are meaningful estimates of the center and dispersion of the transformed distribution.

The sample mean of log-transformed responses can be transformed back to the original scale. This back-transformation results in what is called the *geometric mean* (GM) on the original scale. More formally, let $Y_i$ represent a measured response on the original scale and $T_i$ the transformed value of $Y_i$. Then

$$T_i = \ln\left(Y_i\right)$$

$$\overline{T} = \frac{\sum_{i=1}^{n} T_i}{n}$$

$$\mathrm{GM} = \exp\left(\overline{T}\right) = \left(\prod_{i=1}^{n} Y_i\right)^{\frac{1}{n}} \qquad \text{Equation 40}$$

The standard deviation of log-transformed responses ($S_T$) can likewise be back-transformed as $\exp(S_T)$. This term is referred to as the *geometric standard deviation* (GSD) by Kirkwood (*17*). That is,

$$\mathrm{GSD} = \exp(S_T) \qquad \text{Equation 41}$$

Because $S_T$ is non-negative, GSD $\geq 1$ and represents a fold-variation in the response scale. While a summary for arithmetically scaled responses can be written as $\pm S$, this might be summarized as GM ×/÷ GSD, or GM/GSD to GM × GSD for geometrically scaled responses. If for example GSD = 1.25 and GM = 1.0, a range might be summarized as 1.0/1.25 = 0.80 to 1.0 × 1.25 = 1.25. It should be noted that this represents a 1-standard deviation range. A more appropriate range might be calculated in the log transformed scale (see below).

Kirkwood also defines the percent geometric coefficient of variation as

$$\%\mathrm{GCV} = 100 \times (\mathrm{GSD} - 1)\% \qquad \text{Equation 42}$$

An alternative measure of variability derived from the arithmetic moments of the log-normal distribution in the original scale is

$$\%\mathrm{CV} = \sqrt{\exp\left(S_T^2\right) - 1} \times 100\% \qquad \text{Equation 43}$$

Numerically, %GCV and %CV of the log-normal distribution are close to each other when both are less than 20% (see Tan [*25*]). Their use along with GSD should be clearly specified when reporting the measure of variability or intervals for log-normal data. Interpretation of these measures are described more fully in *Biological Assay Validation* ⟨1033⟩*, Appendices, Appendix 1: Measures of Location and Spread for Log Normally Distributed Variables*.

From *Equation 5* in *3. Basic Statistical Principles and Uncertainty*, a 100(1 − α)% two-sided confidence interval on the mean in the log scale is

$$\mathrm{LB}(T) = \overline{T} - t_{1-\alpha/2:n-1}\frac{S_T}{\sqrt{n}}$$

$$\mathrm{UB}(T) = \overline{T} + t_{1-\alpha/2:n-1}\frac{S_T}{\sqrt{n}} \qquad \text{Equation 44}$$

where $n$ is the sample size and $t_{1-\alpha/2:n-1}$ is the $1 - \alpha/2^{\text{th}}$ quantile of the cumulative Student $t$-distribution having area $1 - \alpha/2$ to the left and $n - 1$ degrees of freedom.

The confidence interval on the geometric mean in the original scale is obtained from the bounds in *Equation 44* as

$$\mathrm{LB}(Y) = \exp(\mathrm{LB}(T))$$
$$\mathrm{UB}(Y) = \exp(\mathrm{UB}(T)). \qquad \text{Equation 45}$$

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Transformations other than logarithms may be considered for other types of data. For example, when working with proportions between 0 and 1 (or percentages between 0% and 100%), either the arcsine or logit transformation is useful. The arcsine transformation where $Y$ is represented as a proportion is

$$T = 2 \times \sin^{-1}(\sqrt{Y}) \quad \text{Equation 46}$$

and the logit transformation is

$$T = \ln\left(\frac{Y}{1-Y}\right) \quad \text{Equation 47}$$

These transformations are particularly useful when a majority of the data are pushed against the upper bound of 1.0 or the lower bound of 0.0. Count data may be transformed using a square root or a log transformation of the count.

Power transformations, the most common of which are Box-Cox transformations, are also useful re-expressions. These transformations are of the form

$$T = \frac{Y^{\lambda} - 1}{\lambda} \quad \lambda \neq 0$$

$$= \ln(Y) \quad \lambda \text{ ▲=▲ (USP 1-Dec-2021) } 0 \quad \text{Equation 48}$$

where $\lambda$ is selected to best transform the data set to normality. Information on Box-Cox transformations is provided in Section 6.5.2 of the *NIST/SEMATECH e-Handbook of Statistical Methods* (*24*).

Regardless of the transformation, summary measures and intervals calculated in the transformed scale can be back-transformed to the original scale. In all cases the data should be examined to establish if the transformed measurements exhibit almost uniform variability and are approximately normally distributed.

## Assessing Model Adequacy

All models involve assumptions about the processes that generate the data and the data itself. In addition to the assumed functional form, the distribution of the error term in *Equation 37* and *Equation 39* is of primary importance. Typical assumptions are that the error terms are independent, normally distributed, and have constant variance across the range of responses. When these assumptions are reasonable, statistical models are usually readily interpretable and powerful (i.e., able to measure subtle effects with good precision and discrimination between groups). As attractive as any model might be, it is imperative to check for and address violations of the assumptions upon which these models rely. Assessing model adequacy is the process of verifying these assumptions.

There are both graphical and quantitative methods for assessing model adequacy. In many data analysis projects, there are multiple iterations of conversations between researchers and statisticians before selecting a final model. Topics to consider include appropriate transformations of the data, the treatment and design factors of interest, potential candidate models, and assessment of model fit.

Useful tools for assessing model fit include residual plots with both raw and studentized residuals, model-based outlier detection methods, and regression leverage and influence measures. Plots of residuals can be generated in several ways. The most common format is a plot of the residuals on the vertical axis, and the predicted response on the horizontal axis. When the observations on a residual plot increase or decrease in spread along the horizontal axis, this indicates violation of the assumption of constant variance. Any linear or nonlinear trend in the residuals suggests the functional form of the model may not be correct, or that an important treatment factor is missing from the model. For example, a curved residual pattern may indicate the need for a quadratic term in the model. Additionally, residuals that fall outside the general cluster of points may be an indication of an outlier. As noted previously, some of these problems may be mitigated with an appropriate transformation.

Normality of the error terms is an especially important assumption if the model is used to predict future behavior. Graphical methods that can be used to monitor this assumption include dot plots, box and whisker plots, and normal probability plots (sometimes called *quantile-quantile plots* or *QQ plots*). These graphical tools are available in many common statistical software packages. Statistical tests of normality are described in Section 1.3.5 of the previously referenced NIST handbook and available in statistical software packages.

Lack of independence typically occurs when data are in some manner “batched” in groups. For example, measurements that are taken from the same plates on an assay are more similar than measurements recorded on other plates. This so-called "intragroup correlation" can be properly modeled by including a “batch” factor in the model to account for the correlation.

Care should be taken in the assessment of model assumptions. Statistical tests in particular are impacted by the size of the sample. For small samples such tests may be insensitive for detecting departures from the model assumptions. In contrast for large samples, they may detect an assumption violation even though visual assessment suggests the assumptions are reasonable. A combination of scientific understanding of the measurement process generating the data, graphical analyses and statistical tests can be used together to address model adequacy.

## Outliers

Occasionally, observed analytical results are very different from expected analytical results. Aberrant observations are properly called *outlying results*. These outlying results should be documented, interpreted, and managed. Such results may be accurate measurements of the property being measured but are very different from what is expected. Alternatively, due to an error in the analytical system, the results may not be typical, even though the property being measured is typical. A first defense

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against obtaining an outlying analytical result is application of an appropriate set of system suitability and control rules (see *Appendix 1: Control Charts*).

When an outlying result is obtained, systematic laboratory and process investigations are conducted to determine if an assignable cause can be established to explain the result. Factors to be considered when investigating an outlying result include human error, instrumentation error, calculation error, and product or component deficiency. A thorough investigation should consider the precision and accuracy of the procedure, the USP or in-house Reference Standard and controls, process and analytical trends, and the specification limits. If an assignable cause due to the analytical procedure can be identified, then retesting may be performed on the same sample, if appropriate, or on a new sample. Based on the documented investigation, data may be invalidated and eliminated from subsequent calculations.

"Outlier labeling" is informal recognition of outlying results that should be further investigated with more formal methods. Outlier labeling is most often performed visually with graphical techniques such as residual plots, standardized residual plots, or box and whisker plots. "Outlier identification" is the use of statistical significance tests to confirm that the values are inconsistent with the known or assumed data distribution. The selection of the correct outlier identification technique often depends on the initial recognition of the number and location of the values.

A simple example is presented to demonstrate this process. An analytical procedure requires measurements from 3 vials of liquid drug product which are used to provide a reportable concentration value (mg/ml) for the lot from which the vials were selected. When measuring the third vial, the analyst noted a slight deviation in the sample preparation which was not discussed in the protocol. The three measurements are reported in *Table 11*. Vial 3 is the vial in question.

**Table 11. Concentrations for 3 Vials of Drug Product**

| Vial | Concentration (mg/ml) |
|---|---|
| 1 | 49.9 |
| 2 | 49.8 |
| 3 | 51.8 |

The residual plot for the mean model described in *Equation 37* is shown in *Figure 4*. Here the residual is the measured value minus the sample mean of the 3 vials (50.5 mg/ml).

Figure 4. Residual plot of the data.

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The residual for vial 3 visually resides far from the other two values and is accordingly *labeled* as an outlier.

One statistical test that can be used to determine if vial 3 can be *identified* as an outlier is due to Dixon (*10*–*11*). This test is based on a ratio of differences between the observations. For this particular application where interest is in determining if the maximum value is an outlier, a single test statistic is computed and compared to a critical value based on a normal probability distribution. The minimum value in the data set is 49.8 mg/ml, the middle value is 49.9 mg/ml, and the maximum value is 51.8 mg/ml. The test statistic is defined as

$$\frac{\text{Maximum}-\text{Middle}}{\text{Maximum}-\text{Minimum}} = \frac{51.8-49.9}{51.8-49.8} = 0.95 \quad \text{Equation 49}$$

The calculated value in *Equation 49* is then compared to a table of values based on the distribution of order statistics for a normal probability distribution. The critical value that must be exceeded to be identified as an outlier with three values using a type 1 error rate of 0.05 and assuming a normal distribution is 0.941. Since the computed value of 0.95 exceeds 0.941, the measurement of vial 3 is identified as an outlier. Actions to be taken will depend on results of further investigations.

As noted, this particular version of the Dixon test requires an assumption of normality which cannot be verified with such a small sample. Rather, one would need to rely on previous measurements made with the procedure on previous process lots to support this argument. In general, the critical value as well as the ratio that one constructs for the Dixon test depends on the number of measurements in the data set and the type 1 error rate. A complete set of critical values for sample sizes less than 30 are available in Böhrer (*7*).

As noted previously, the process of identifying a statistical outlier generally requires scientific support for an assignable cause. For the applications performed in an analytical lab, candidate outlier tests are typically univariate. Two questions to consider when selecting a method are

1. Can the distribution be assumed to be normal, or should a test be applied that does not require this particular distributional form?
2. Do we suspect more than one outlier, and which observation(s) have been labeled?

With regard to question 1, outlier tests can be categorized as either parametric (model-based) or non-parametric. The parametric structure selected by such methods is typically the normal distribution. Question 2 considers whether there is one or more labeled outliers, and the relative location (i.e. greater or less than the bulk of the measurements). If more than one outlier is suspected, then sequential approaches may be needed to perform the test.

Useful references on this topic include Barnett and Lewis (*5*), Hawkins (*14*), ASTM E178 (*2*), and a literature review by Beckman and Cook (*6*).

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## APPENDIX 3: EQUIVALENCE AND NONINFERIORITY TESTING

*General Notices* describes the need to produce comparable results to the compendial method. Several options were identified to address this as noted in Hauck et al. (*13*). Among these was performance equivalence. Performance equivalence is used to establish the equivalence of the two procedure means, and noninferiority of the new procedure variability to that of the old procedure, as the basis for demonstrating comparability between two procedures.

The article goes on to describe an approach for demonstrating comparability using statistical hypothesis testing. This appendix describes the general principles of statistical hypothesis testing, as applied to equivalence testing of procedure means and noninferiority testing of procedure variabilities.

In classical statistical hypothesis testing, there are two hypotheses, the null and the alternative. For example, when comparing a new and an old procedure, the null may be that two means are equal and the alternative that they differ. This may be expressed as

$$H_0: \mu_N = \mu_O$$
$$H_a: \mu_N \neq \mu_O \quad \text{Equation 50}$$

or equivalently

$$\blacktriangle H_0: \mu_N - \mu_O = 0 \blacktriangle \text{ (USP 1-Dec-2021)}$$
$$H_a: \mu_N - \mu_O \neq 0 \quad \text{Equation 51}$$

where $\mu_N$ and $\mu_O$ are the means for the new and old procedures, respectively.

With this classical approach, one rejects the null hypothesis in favor of the alternative if the evidence is sufficient against the null. In such a case we accept the alternative hypothesis that the means are different. Because of this interpretation, this is sometimes called a *difference test*.

A common misinterpretation is to conclude that failure to reject the null hypothesis in a difference test is evidence that the null is true (i.e., the means are equal). Actually, failure to reject the null just means the evidence against the null was not sufficient to claim the means are different. This might occur if the variability is large, or the number of determinations too small to detect a difference in the means.

Thus, when one seeks to demonstrate equivalence of procedure means, it is necessary to place the claim of equivalence in the alternative hypothesis. A statistical test for an alternative hypothesis of equivalence is referred to as an *equivalence test*. It is important to understand that “equivalence” does not mean “equality.” Equivalence should be understood as “sufficiently similar” for the use of the new procedure. The definition of “sufficiently similar” is something to be decided a priori based on

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scientific considerations, and becomes the basis of the alternative hypothesis. Chatfield and Borman (*9*) offer some helpful suggestions for this process.

As a specific example, suppose it is decided a priori that to be considered equivalent, the means of two procedures can differ by no more than some positive value, *d*. This value is commonly called the *equivalence margin*. The hypotheses for the equivalence test are then

$$H_0: |\mu_N - \mu_O| \geq d$$
$$H_a: |\mu_N - \mu_O| < d \quad \text{Equation 52}$$

Note the alternative hypothesis is actually two individual one-sided hypotheses:

1. $H_{a1}: \mu_N - \mu_O < d$
2. $H_{a2}: \mu_N - \mu_O > -d$

For this reason, this testing procedure is referred to as two one-sided tests (TOST). As one-sided tests, each can be addressed with a type I error rate of $\alpha$ (typically, but not necessarily, 0.05). The TOST is often conducted by rejecting the null hypothesis in favor of the alternative hypothesis if the $100(1 - 2\alpha)\%$ two-sided confidence interval (typically, but not necessarily 90%) is entirely contained in the range $(-d, +d)$. When the null is rejected, we conclude that the two procedures are equivalent in their means.

Performance equivalence is not restricted to demonstrating equivalence of procedure means. A laboratory might want a new procedure to have equivalent or better variability as the old procedure. This requires a one-sided test because if the new procedure were to have a lesser variability, this would clearly be acceptable. What one needs to ensure is that the new procedure does not result in an important increase in variability. Thus, variability comparisons are conducted as one-sided *noninferiority tests*.

Similar to an equivalence test for means, a noninferiority test for variabilities places the desired relationship between procedure variabilities in the alternative hypothesis. Due to the statistical properties of standard deviations, an appropriate parameter for comparison is the ratio, $\sigma_N/\sigma_O$, where $\sigma_N$ and $\sigma_O$ represent the standard deviations of the new and the old procedures, respectively.

Suppose it is determined a priori that for the procedure to be fit for use, the standard deviation of the new procedure can exceed that of the old procedure by no more than a factor $k \geq 1$. The factor $k$ is called the *noninferiority margin*. The hypotheses associated with the noninferiority test are

$$H_0: \frac{\sigma_N}{\sigma_O} \geq k$$
$$H_a: \frac{\sigma_N}{\sigma_O} < k \quad \text{Equation 53}$$

Unlike the equivalence test of means, the noninferiority hypothesis is a single hypothesis which can be addressed with a level $\alpha$ (typically, but not necessarily, 0.05). In order to perform the test, the null hypothesis is rejected in favor of the alternative hypothesis if the $100(1 - \alpha)\%$ upper one-sided confidence bound on $\sigma_N/\sigma_O$ is less than $k$. When the null hypothesis is rejected, it is concluded that the variability of the new procedure is noninferior to that of the old procedure.

Hauck et al. offers other options to address the standard of "equivalent or better":

1. Minimum performance requirements for acceptable procedures
2. Results equivalence
3. Decision equivalence

The option of minimum performance requirements has evolved into the concept of the analytical target profile (ATP) which has been introduced in *Pharmacopeial Forum* (*4*). Results equivalence is addressed using the intraclass correlation coefficient or the concordance correlation coefficient. A tolerance interval approach using total variability is likewise used to address results. Decision equivalence relates to dichotomous outcomes such as pass/fail, and can be addressed through the kappa coefficient or receiver operating characteristic curves. Using these options (as with performance equivalence), care must be taken to properly formulate the statistical hypotheses and to address the comparison through meaningful acceptance criteria.

While this appendix has highlighted approaches for establishing procedure comparability, these apply to other scenarios involving comparisons of two groups; e.g., procedure transfer or standard qualification. Placement of the claim one desires to support into the alternative hypothesis results in an appropriate statistical conclusion.

Although the benefits of equivalence testing are apparent, in some situations one may not be able to collect a sufficient sample size to provide the necessary power to establish equivalence. In such a situation, use of the difference test may be the only option. However, one is reminded that failure to reject the null hypothesis of equality is not evidence that the procedure means are equal. A confidence interval should nonetheless be reported to communicate the difference of means between the two procedures.

## APPENDIX 4: THE PRINCIPLE OF UNCERTAINTY

While this chapter has concentrated on statistical studies which are performed using measurement data, the principles and practices are identical to those in the field of metrology. These are unified by a common understanding of the concept of uncertainty. This appendix introduces concepts related to the metrological principle of measurement uncertainty and unify these with the practices described for the scientific method.

The understanding of study uncertainty is not new to the pharmaceutical industry and has been employed more broadly throughout industries that make decisions from studies using measurements. The study of measurement uncertainty falls

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formally into the field of metrology. A measurement process like a study is designed to reduce uncertainty in order to make a more informed decision. No measurement or study result can provide exact knowledge. Proper interpretation and treatment of analytical data requires an understanding of the inherent sources of uncertainty in measurement outcomes and their impact on the information they provide. Recognition of the principles of uncertainty facilitates this understanding, as described by the Joint Committee for Guides in Metrology in the *Guide to the Expression of Uncertainty in Measurement* (GUM) (*16*).

Results from all studies, including quality control testing are uncertain. Uncertainty arises from sources of variability inherent in the measurement process, as well as from statistical sampling and study factors. The principles from the field of metrology are consistent with the statistical principles described in this chapter and provide further insight into the quantification of uncertainty from studies supported by measurements.

At the core of these principles is an understanding of risk. More specifically, this understanding considers the risks of making incorrect decisions based on studies utilizing measurements. The consequences of these risks can be minor or significant, and thus should be factored into considerations related to the design of a measurement system, the design of studies using the measurement system, and the interpretation of study results. The concepts of target measurement uncertainty (TMU) and the study objective can be unified as a basis for managing the risks associated with making decisions from studies. In fact, TMU is a special case of a study hypothesis which drives the design of all studies using analytical measurements.

To increase knowledge, two of the fundamental forces of metrological and statistical thinking are the desire to minimize the uncertainty in the measured value (an indication of the quantity being measured) and to ensure all sources of uncertainty have been evaluated and mitigated. In metrology the quantity intended to be measured is termed the *measurand*. This is called a *population parameter* in the broader sense of a study. Measurement or parameter uncertainty quantifies one's doubt about the true value that remains after making a measurement or estimating a parameter.

While the metrological concept of measurement uncertainty applies exclusively to a reportable value, this can be aligned with the concept of study uncertainty by viewing the quality control process as a study of a commercial lot. Employing the steps of the scientific method, the study of the commercial lot has an objective which can be formulated as a hypothesis test

$$H_0: \mu \leq \text{LSL or } \mu \geq \text{USL}$$
$$H_a: \text{LSL} < \mu < \text{USL} \quad \text{Equation 54}$$

where $\mu$ is the commercial lot mean and LSL and USL are the lower and upper specification limits respectively. The study can be designed using blocking and replication to satisfy the TMU, which should be such as to minimize the risks associated with the object of the testing (i.e., to support the alternative hypothesis, $H_a$). As part of study conduct, sampling and randomization can be utilized to mitigate the risks due to the introduction of bias. Finally, and perhaps most importantly, the data should be analyzed and reported with acknowledgment of the uncertainty in the reportable value.

## Metrological Principles Specific to Measurement Uncertainty

The reliability of study results are only as good as the fitness for use of the measurement process used to generate data for the study. The metrological concept of measurement uncertainty helps to ensure fitness for use. This and other principles are worth noting as a fundamental way to view a measurement process.

*Figure 5* represents several potential sources of random variation in a measurement process, which result in the combined standard uncertainty (the estimated standard deviation of the measurement). An example of inherent random variation is when the same chromatogram is given to several different analysts for peak integration. Slightly different values will be obtained which might also be affected by a laboratory's choice of software. In addition, the definition of the measurand can never be complete. This is known as definitional uncertainty or uncertainty of knowledge. Ideally the measurand is defined sufficiently so that the definitional uncertainty is relatively small when compared to the combined standard uncertainty. An example of lack of knowledge is when a component of the measurement process has associated uncertainty. For example, one might purchase a pH standard solution that is certified as pH = 7.00 ± 0.02 where the 0.02 is the expanded uncertainty in the assigned value of the standard solution. Expanded uncertainty is a measure of uncertainty that defines an interval about the measurement result y within which the value of the measurand Y can be confidently asserted to lie.

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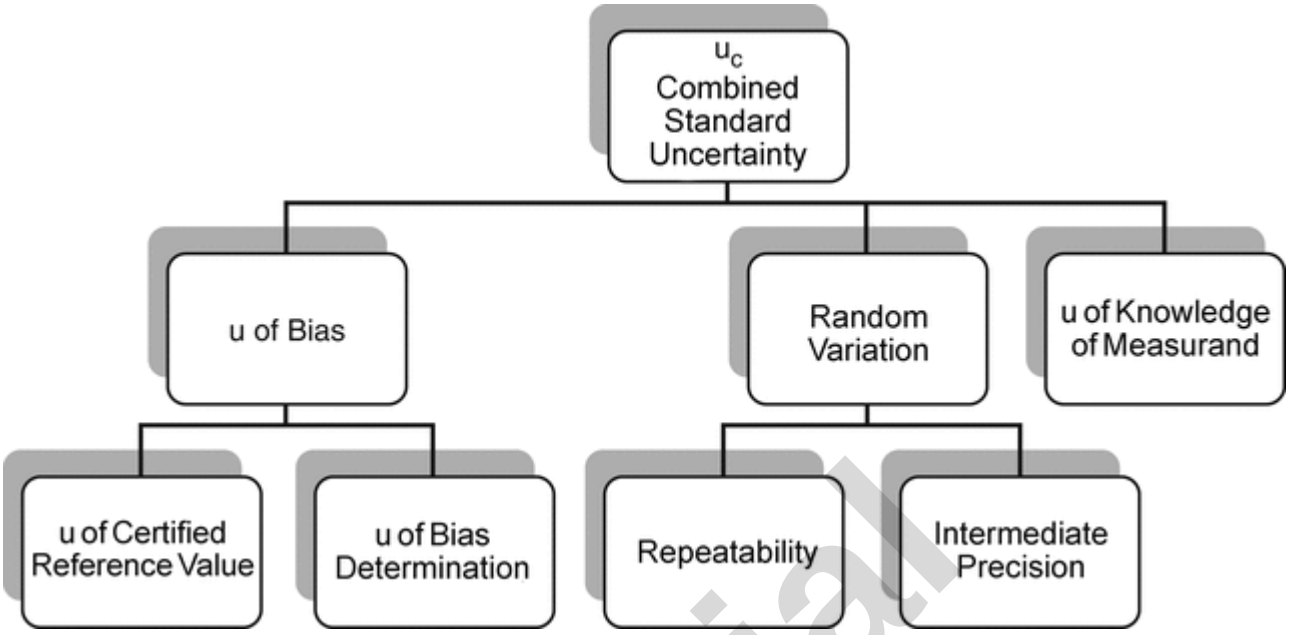


Figure 5. Various components of uncertainty (u) comprise the combined standard uncertainty. The figure is not comprehensive and is meant to illustrate the major uncertainty components.

GUM notes the evaluation of measurement uncertainty is neither a routine task nor a purely mathematical one. Judgment is essential in choosing which uncertainty components (i.e., potential causes of measurement uncertainty) to consider in procedure development, qualification, and measurement uncertainty evaluation. For example, when preparing a 1 mg/L solution, the process by which it is prepared can influence the final concentration. The analyst knows it would not be wise to weigh 1 mg of the substance followed by dilution to 1 L. Instead, recognizing the limits of typical analytical balances, a more precise result would be achieved by weighing 100 mg of substance and then serially diluting to the required concentration.

Measurement uncertainty arises from many sources including differences in instruments, mathematical algorithms, and analysts. A tutorial sampling of typical uncertainty components is provided in *Table 12*.

**Table 12. A Tutorial List of Uncertainty Components in Analytical Laboratory Practice**

| | |
|---|---|
| Variability due to analytical procedure design | Effect of the sample amount or volume<br>Between-unit variation of measurand<br>Purity of the primary standard<br>Effect of the sample storage conditions<br>Failure to recognize ruggedness factors |
| Variability due to measurement process | Carry-over effects in the auto sampler<br>Effect of static electricity on weighings<br>Incomplete recovery of the analyte<br>The effect of the sample matrix on calibration slope<br>Effect of the sample temperature on the volume<br>Effect of the blank correction |
| Variability due to analysts | Effect of the manual peak integration |
| Variability due to algorithms | Linear calibration forced through zero<br>Line fitting using weighted or unweighted algorithms |
| Variability due to sample | Effect of taking a sub-sample from a laboratory sample |

A detailed discussion of measurement uncertainty in the pharmaceutical industry that expands upon the metrological principles introduced here and provides detailed definitions is provided in Weitzel et al. (*27*). In addition, a worked example for a drug substance is provided in Weitzel et al. (*28*).

## APPENDIX 5: BAYESIAN INFERENCE

When describing statistical intervals in *3. Basic Statistical Principles and Uncertainty*, it was noted that one can utilize a Bayesian approach to derive an interval which contains, with probability $100 \times (1 - \alpha)\%$ the true value of the population mean. This is important because it returns a statement that the laboratory frequently wishes to make. This section will describe Bayesian inference and contrast it with frequentist inference which is more commonly understood throughout the pharmaceutical industry. Frequentist theory bases inferences on probability statements about statistics, while Bayesian inference is based on probability statements about population parameters. Population parameters are the unknowns that appear in statistical models (e.g., means, variances, difference of means) and statistics are summary measures or estimates based on data (e.g., parameter estimates). Frequentist inference regards parameter values as fixed and unknowable whereas Bayesian inference models their

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uncertainty using probability distributions. For instance the statement "there is a 95% probability that the difference in population means is between –0.1 to 0.1" is meaningless from a frequentist viewpoint, but reasonable from a Bayesian perspective. The Bayesian formulation offers a way for scientists needing to make risk based decisions. Bayesian inference can also incorporate prior information about statistical parameters together with the sample data to update what is known about a parameter. The ability to incorporate justified prior information potentially leads to better decisions when a study size is small, or when a factor is not adequately represented in the study design.

The purpose of this appendix is to provide a basic introduction to Bayesian inference applied to statistical studies and to analytical measurements. Gelman et al. (*12*) provides a source for more information.

## Parameter Uncertainty versus Sampling Variability

*Parameters* are unknown hypothetical or population quantities, such as the mean or standard deviation of a population, or the difference in means between procedures. While unknown a parameter can be estimated. The estimation of a parameter and the inherent uncertainty of that estimation is the basis of Bayesian thinking.

*Statistics* are observed quantities or summaries of observed quantities in a sample taken from a population or process of interest. Examples of statistics include an analytical result (a measurement), a sample mean, a sample standard deviation, a difference in observed means between procedures, or their estimated confidence bounds. On repeated sampling of the population, the observed values of *statistics* will differ because of *sampling variability*.

Frequentist statistical methodology considers parameters to be fixed values that do not change. It employs probability theory to model the *sampling variability* of *statistics* randomly obtained from the population. These sampling distributions are then used to make inferences about the fixed value of the *parameter*. A common frequentist methodology is the calculation of a confidence interval. The process of computing a 95% confidence interval ensures that the realized interval will contain (or cover) the unknown parameter 95% of the time on repeated use.

The 95% refers to the reliability of the methodology (i.e., its coverage), and not the probability that the parameter falls within the interval.

For example, suppose a computed 95% confidence interval on a mean is from 980 to 990 mg/g. It is not correct to state there is a 95% probability that the population mean is between 980 and 990 mg/g. To associate a probability with a fixed interval such as 980 to 990 mg/g, one must assume uncertainty is associated with the underlying parameter (i.e., it is not a fixed quantity). Rather, the 95% description of the confidence interval means that the interval will correctly contain the true parameter value in 95% of repeated sampling applications from the population. The 95% refers to the success rate of the sampling process and not the parameter (which is assumed fixed).

Bayesian statistical methodology considers a parameter value to be uncertain (not fixed), and models its likely levels using a probability distribution. It extends frequentist statistical methodology, using probability theory to model both the sampling variability of statistics and the decision maker's uncertainty associated with parameters. Bayesian models are sometimes called "complete" probability models because they quantify the uncertainty associated with the parameters of interest, given the assumed sampling variability of the observed statistics, any relevant prior information, and the observed data. For instance, it is correct to say that a given Bayesian 95% credible interval (Bayesian analog of the confidence interval) contains the value of a specific parameter of interest with 95% probability (conditional on the observed data and other modeling assumptions). The same principles apply to the Bayesian analogs of frequentist tolerance and prediction intervals. Unlike frequentist interval methodology in which the probability level must be fixed in advance (e.g., 95%) and the resulting interval is random, Bayesian methodology offers the opportunity to fix the interval in advance, and estimate the probability that the parameter value lies within that interval. Such an application is extremely useful for determining the probability that an analytical procedure will provide a signal outside a given range.

## Prior and Posterior Distributions

Both frequentist and Bayesian methodologies express models using probability distributions. Both use the same model for sampling variability known as the likelihood. The particular likelihood model choice is based on prior knowledge concerning statistical variability.

Bayesian inference also requires a probability model for parameter uncertainty, prior to observing the data, called the *prior distribution*. As with the likelihood, the prior distribution is a choice based on prior data, reliable knowledge, or common sense (e.g., the values of many parameters, such as a standard deviation, must be positive). Bayesian methodology requires care to assure that the chosen prior distributions are scientifically justified and do not unduly influence the inference. Use of appropriately justified knowledge of a prior distribution can potentially reduce sample size requirements for decision making. However, when there is little available theory, historical data, or expert knowledge available, prior distributions can be constructed that give minimal preference to any particular parameter value, and thus have minimal impact on the inference. Such prior distributions are often referred to as "non-informative". When non-informative prior distributions are employed, inferences typically agree with the frequentist counterparts since both are solely dependent on the likelihood.

Bayesian methodology combines likelihood and prior distributional models with observed data to produce an updated distributional model for parameter uncertainty called the *posterior distribution*. The posterior distribution provides the probability that the population parameter value lies within any interval of interest. Such intervals are called *credible intervals*. When certain classes of non-informative prior distributions (e.g., a Jeffrey prior used with a normal likelihood) are employed, a Bayesian credible interval can be calculated from the posterior distribution, and may sometimes be numerically equal to the corresponding traditional confidence interval. However, as previously noted, the interpretations of these intervals are different. The probability associated with the credible interval quantifies uncertainty in an estimated parameter value conditional on observed data, while the probability associated with the confidence interval quantifies the probability of coverage of the estimated parameter on repeated estimation over many data sets.

From the Bayesian perspective, all knowledge about the parameter of interest is based on the posterior distribution. The posterior distribution from a previous study can inform the prior distribution for a subsequent study. Updating the prior

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distribution in this manner as new data become available, provides a paradigm for knowledge building, and thus a statistical basis for applying *prior knowledge* during pharmaceutical development (see ICH Q8(R2), *Pharmaceutical Development* [*15*]).

The posterior distribution of parameters may also be re-combined with the likelihood to obtain a *posterior predictive distribution* of future observed data or statistics. As with the posterior distribution, the Bayesian perspective bases all knowledge about future values on this posterior predictive distribution, which can be used to construct Bayesian analogs of frequentist tolerance and prediction intervals. Unlike the frequentist analogs, the Bayesian intervals do not require a prespecified fixed probability level. A posterior predictive distribution can be used, for example, in estimating the probability of occurrence of future out-of-specification results.

## An Illustrative Example

Consider an analytical procedure for strength of drug product. The output of the procedure is a reportable value (mg/g) that estimates the mean strength, $\mu$, for the tested lot of drug product. For the lot to be considered safe and effective, $\mu$ must be between 980 and 1020 mg/g. The observed reportable result, $Y$, is 1010 mg/g.

A typical rule used for disposition is to release the lot if $980 \le Y \le 1020$. However, this rule is based on an observed reportable result that includes measurement error from the analytical procedure. What we really want to know is whether $\mu$ falls within the specification limits. This question can be informed using a Bayesian rule that releases the lot if the posterior probability that $980 \le \mu \le 1020$ is above some lower limit (e.g., 0.95). That is, we release the lot if the probability that the true value is within specifications is at least 0.95. Such a rule might be called a *minimum posterior probability* (MPP) rule. The MPP rule provides a probability based metric for acceptance of the lot under test.

The estimation of this posterior probability requires definitions of the likelihood model and its parameters, the prior distributions of these parameters, and the data. For this illustration, the following are assumed:

- *Likelihood*: Reportable results follow a normal distribution with two unknown parameters: the population mean ($\mu$) and intermediate precision standard deviation ($\sigma$). [NOTE—It is assumed the lot is homogeneous.]
- *Prior distributions*
  - *Prior distribution of $\mu$*: There is no prior information on the strength of this lot. To represent this lack of knowledge assume a wide uniform distribution over the analytical range. The uniform distribution gives equal probability to any range of a given length regardless of location.
  - *Prior distribution of $\sigma$*: Data collected during validation resulted in an estimated intermediate precision variance ($\sigma_0^2$) of 25 based on a sample of 10 independent reportable values. Based on this information, assume that $\sigma^2$ follows a scaled-inverse-chi-squared distribution (a common prior distributional choice for variances) having $df_0 = 10 - 1 = 9$ prior degrees of freedom and a prior scale parameter of $\sigma_O = \sqrt{25} = 5$ mg/g.
- *Data*: A reportable value, $Y = 1010$ mg/g

Given the above information, the Bayes rule leads to a Student-t posterior distribution for $(\mu - Y)/\sigma_O$ with $df_O = 9$ degrees of freedom. The integration of this posterior distribution over the fixed range for $\mu$ of 980 to 1020 mg/g can be conveniently obtained using commonly available spreadsheet functions. For example, in Excel this is computed using the formulas, =T.DIST((1020–1010)/5,9,TRUE) – T.DIST((980–1010)/5,9,TRUE). The resulting posterior probability that $980 \le \mu \le 1020$ for the tested lot is 0.96. That is, there is a $100 \times 0.96 = 96\%$ chance that the true mean of the lot falls within the specification limits. Because $0.96 > 0.95$, the lot would be accepted based upon the MPP rule. The estimated posterior probability of 0.96 serves as a quantitative risk-based measure of the quality of the lot.

In this example, the parameter of interest is $\mu$, a measurand quantity value. An analogous approach is used for Bayesian inference of other model parameters, such as the estimation of the difference in population means for two procedures, the underlying slope and intercept in a simple linear regression model, or performing tests of statistical equivalence.

In more complex situations (e.g., for complex, non-normal, or nonlinear models), Bayesian inference utilizes a form of computer simulation referred to as Markov-Chain Monte-Carlo (MCMC) simulation which is conducted using specialized software. MCMC technology requires care to assure that the MCMC iterations converge properly to the population posterior distribution.

## A Comparison of Frequentist and Bayesian Methods

Both frequentist and Bayesian approaches to inference are useful. Frequentist approaches are widely available, straightforward, and offer the reliability of known coverage probability. Bayesian approaches can be used to quantify the uncertainty in parameters of interest which can support quantitative risk based decision making. While often more technically challenging to apply, Bayesian MCMC methodology can often be applied to problems that are intractable by frequentist approaches. When informative prior distributions can be justified, Bayesian methods may require smaller samples sizes for decision making than frequentist statistical methods. *Table 13* provides a comparison of some characteristics from both frequentist and Bayesian perspectives.

**Table 13. Characteristic Differences Between Frequentist and Bayesian Inference**

| Characteristic | Frequentist Inference | Bayesian Inference |
|---|---|---|
| Statistics | Sampling variability modeled probabilistically | |
| Parameters | Treated as fixed and unknown | Uncertainty modeled probabilistically |
| Coverage probability | Known from theory (usually) | Must be determined via computer simulation (usually) |

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**Table 13. Characteristic Differences Between Frequentist and Bayesian Inference** *(continued)*

| Characteristic | Frequentist Inference | Bayesian Inference |
|---|---|---|
| Prior information | Introduced via sampling variability model | Introduced via sampling variability model and prior distributions of parameters |
| Types of estimates | Point and interval | Posterior distribution (from which point and interval estimates can be derived) |
| Observed data | Treated as one realization of a hypothetical series of repeated samples | Treated as fixed values on which inference is based |
| Parametric inference | Fixed probabilities based on repeated sampling coverage probability | Parameter values quantified probabilistically from posterior distribution |
| Impact of statistical design | May impact repeated sampling coverage probability | Less critical |
| Multiple comparisons | May impact repeated sampling coverage probability | Less critical |
| Risk assessment | Indirect risk assessment | Estimated probabilities appropriate for quantifying risk |
| Prior knowledge of parameter values | Excluded from the inference | A prior distribution for model parameters is required |
| Continuous knowledge building | Informal assessment of historical studies | Posterior distribution from historical study informs prior distribution for subsequent study |
| Prediction of future observed values | Indirect inference based on tolerance or prediction intervals | Direct probabilistic inference based on the posterior predictive distribution |
| Software | Widely available routines | Specialized expertise required |

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# ⟨1024⟩ BOVINE SERUM

## INTRODUCTION

Bovine serum is the liquid fraction of clotted blood, obtained from an ox (*Bos taurus,* among others), that has been depleted of cells, fibrin, and clotting factors. Since the advent of modern cell culture, manufacturers of biological products have used bovine serum extensively as a cell culture growth supplement. Its rich nutritional composition of proteins, growth factors, hormones, amino acids, vitamins, sugars, lipids, trace elements, and other components supports a broad range of cell culture applications in research and commercial manufacture of vaccines, natural source and recombinant biologics (hereafter biologics), engineered tissues, and other emerging cell-based therapeutic products intended for human or veterinary use. The predominant type of serum used in research applications is Fetal Bovine Serum (FBS). Calf serum (from newborn and older animals) is used much less frequently, but because of its lower cost it may be widely used in commercial manufacturing.

As is the case with other animal-derived products, bovine serum carries a potential risk of introducing extraneous agents into cell culture. Serum manufacturers and regulators must adopt rigorous sourcing and testing procedures and strict processing and production guidelines to ensure the quality of bovine serum.

The objective of increasing the quality and safety of biologics produced with bovine serum, coupled with attempts to mitigate regulatory burden, have caused developers to investigate alternatives to serum supplementation, resulting in application-specific serum-free medium formulations. Although it is recognized that bovine serum should be avoided when there is an option to use serum-free medium, there are cases where this is technically impossible or impractical.

This chapter describes issues related to sourcing, production, and characterization of bovine serum to ensure its safe use. A list of relevant regulatory and guidance documents is presented in *Appendix 1.* Serum manufacturers and serum end users (manufacturers of biological products) should consider and apply as needed the controls and procedures outlined in this chapter to ensure the safe use of bovine serum components in research and pharmaceutical manufacturing.

### Types of Bovine Serum

- FBS is obtained from the fetuses of healthy, prepartum bovine dams that had been deemed fit for human consumption through ante- and postmortem inspection by licensed veterinarians. It is collected in government-inspected and -registered slaughterhouses.
- Newborn calf serum (also known as newborn bovine serum) is obtained in government-inspected and -registered slaughterhouses from animals aged less than 20 days.
- Calf serum is obtained in government-inspected and -registered slaughterhouses from animals aged between 20 days and 12 months.
- Donor bovine serum (also known as donor calf serum) is obtained by the repeated bleeding of donor animals from controlled government-inspected and -registered donor herds. The animals are 12–36 months old.
- Adult bovine serum is obtained in government inspected and -registered slaughterhouses from cattle older than 12 months that are declared fit for human consumption.

## BOVINE SERUM: HISTORY AND TYPES OF USE

### History of Bovine Serum Use

Animal serum and other complex biological materials have been employed in the cultivation of mammalian cells for approximately 100 years. Several factors led to the wide adoption of bovine serum as a standard tissue culture supplement. In comparison to serum from other animal species (horse, goat), bovine serum is easily sourced, and thereby more affordable. Many investigators choose to use fetal serum in their experimental systems because of concerns associated with antibodies present in newborn and adult serum that could cross-react with cells in culture and cause cell lysis through complement-mediated pathways. To eliminate that concern, heat was introduced to inactivate complement that was potentially present in the serum. Studies of FBS undertaken in the 1950s on the cultivation of low-density human cells to elucidate mechanisms of cell growth found that (1) the albumin component may serve as a carrier of essential small molecules; (2) fetuin, a glycoprotein present at high levels in the alpha globulin fraction, facilitates cell attachment and stretching; and (3) fetuin markedly inhibits trypsin, and this antiproteolytic activity may play a role in the ability of fetuin to stimulate cell growth.

In the 1960s and 1970s, serum supplementation of tissue culture media became the norm, thus facilitating biomedical research as well as the first large-scale vaccine manufacturing processes. Serum supplementation reduced the requirement for optimizing medium formulations for different cell types. FBS was shown to provide a variety of polypeptide growth factors. Albumin promoted cell growth presumably because of its abilities to function as a carrier protein for small molecules or lipids, to bind metal ions, to serve as a pH buffer, and to protect cells against shear. Similar functions were found for other serum components such as transferrin, hormones, and other serum-derived attachment factors such as fibronectin, vitronectin, and laminin.

### Uses of Bovine Serum

Serum is a complex mixture of macromolecules that is required for cell growth and virus production, and its use as a raw material presents a number of challenges. These include its batch-to-batch composition and the risk of contamination by

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adventitious agents. The development of serum-free media has replaced serum in some new biotechnology manufacturing applications, but many cell lines used in manufacturing have not been adapted to these serum-free media. Regulatory constraints and scientific challenges generally make it impractical to alter existing manufacturing processes in which serum is used as a raw material.

FBS sometimes is required in cell and tissue bioprocessing, which often involves the cultivation of cells from tissue explants and biopsies. Some bioprocesses may also require the maintenance of specific cellular characteristics during cultivation. FBS often appears to facilitate such procedures and may affect the biological behavior of fastidious cell types. FBS has been shown to affect the transcription of developmentally important genes, apoptosis, and apoptosis-related gene expression, and to provide neuroprotective and antioxidative factors, all of which may be beneficial to the survival and development of cells in culture. Therefore, FBS will continue to play an important role as a cell culture supplement for production of cell- and tissue-based therapies.

In most viral vaccine manufacturing processes the media used for cell culture expansion and virus infection/production are supplemented with different types of serum at different concentrations. In these processes, bovine serum helps generate a mass of cells in an optimal physiological state for efficient viral replication.

## BOVINE SERUM HARVESTING AND PRODUCTION

### Blood Collection

For all types of bovine sera, blood should be collected in government-inspected and -registered premises (slaughterhouses, abattoirs, and donor farms). Blood should be collected by trained operators following the written procedures approved by the serum manufacturer and using either single-use disposable collection devices or reusable collection equipment for which cleaning procedures have been validated.

#### DONOR BOVINE SERUM

For each lot of serum from donor animals, serum manufacturers should ensure traceability to the donor herd of origin via production records and animal health and origin certificates. Donor animals are subjected to regular veterinary inspections and are bled multiple times following established procedures. Animals introduced into the herd should be traceable by source, breeding, and rearing history. Collectors should introduce new animals into the herd following specified and approved procedures that include prepurchase animal inspection and testing, proper transportation, a quarantine period, veterinary examination and testing during the quarantine period, and animal release criteria from quarantine to serum production. The collectors should not vaccinate donor animals for bovine viral diarrhea (BVD). Collectors should test animals for any agent and antibody from which the herd is claimed to be free.

#### NEWBORN CALF SERUM, CALF SERUM, AND ADULT BOVINE SERUM

Certificates of animal health and origin and/or serum production records should ensure that serum manufacturers can trace bovine serum derived from slaughtered animals back to the abattoir. Serum manufacturers should require abattoirs to maintain documentation of the origin of animals for slaughter. Blood should be collected from animals that have been slaughtered, for human consumption, in abattoirs inspected by the competent authority of the country of origin. Inspectors should routinely inspect animals both antemortem and postmortem to check for the clinical appearance of infections and parasitic diseases and other animal health-related problems or conditions. The animals must be free of clinical evidence of infectious diseases at the time of slaughter. Blood collection procedures must be in place to prevent cross-contamination with other tissues and body fluids and the surrounding environment. The standard procedure of slaughter consists of an approved method of animal stunning followed by exsanguination.

#### FETAL BOVINE SERUM

FBS product specifications and test procedures are presented in the proposed general chapter *Fetal Bovine Serum—Quality Attributes and Functionality Tests* ⟨90⟩. Serum manufacturers should collect fetal bovine blood from bovine fetuses whose dams have been slaughtered. The dams must have been deemed fit for human consumption and must have been slaughtered in abattoirs that were inspected by the competent authority of the country of origin. Inspectors should examine all animals both antemortem and postmortem to check for the clinical appearance of infections and parasitic diseases and other animal health-related problems or conditions. The animals must be free of clinical evidence of infectious diseases at the time of slaughter. The uterus is removed and transported to a dedicated space for fetal bovine blood harvest, where blood collection personnel evaluate the fetus for signs of fetal death, including bloating, skin discoloration, odor, deformation, and hair sloughing. Collectors also should check the amniotic fluid for color, quantity, and clarity. Serum manufacturers should collect blood from acceptable fetuses by cardiac puncture into a closed collection system under conditions designed to minimize microbial contamination. Manufacturers should have in place procedures that will prevent cross-contamination with other fetal tissues and bodily fluids and the surrounding environment.

### Serum Harvesting and Processing

Trained personnel should perform serum separation (harvesting) and further processing activities following written and approved procedures. Serum is first separated and pooled, followed by filtration and filling into clean and disinfected containers. If the serum is subjected to one or more virus inactivation treatments in the production process, serum manufacturers should

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validate the virus inactivation processes against a range of relevant viruses. It is recommended that bovine viral diarrhea virus (BVDV) be included in any virus validation study because it is ubiquitous.

## SERUM SEPARATION AND HARVESTING

Bovine blood should be processed and serum separated (harvested) in such a way as to minimize bacterial and mycoplasmal contamination, which in turn minimizes endotoxin levels in serum product. Gentle, quick blood processing helps to minimize hemolysis, further enhancing the quality of the serum product. After collection, blood is first allowed to clot for a specified period of time and under controlled conditions, then centrifuged in a refrigerated centrifuge. Serum is then removed from the clot, typically by centrifugation; pooled and mixed in a pooling vessel; transferred to labeled containers; and frozen, unless it is filter-sterilized immediately. Serum manufacturers should describe each process step and carry out serum processing activities, including sample collection and in-process quality control testing, following the manufacturer's approved procedures.

## POOLING BEFORE FILTRATION

Because limited amounts of blood can be collected from individual animals, serum manufacturers pool the raw serum from many animals in order to create commercial-sized lots. Serum is pooled, after raw serum thawing and before filtration, in a pooling vessel and mixed at a controlled mixing rate and temperature. Pools or lots of donor bovine serum may consist of many separate collections from individual members of the herd. Lots of FBS may consist of pooled serum from thousands of animals. Serum manufacturers should describe each prefiltration pooling process step and should carry out serum thawing, prefiltration pooling, and mixing activities following the manufacturer's approved procedures.

## FILTRATION

Pooled serum is mixed and aseptically passed through filters of pore size 0.2 µm or smaller, depending on the intended application. Filtration processes should be validated. Triple filtration using filters of pore size 0.1 µm has been shown to result in a high degree of mycoplasma removal. Although filtration may remove some large viruses and viral aggregates from the serum, generally viruses cannot be completely eliminated in this manner. Furthermore, the filters are not known to eliminate the causative agent of bovine spongiform encephalopathy (BSE). Following filtration, serum manufacturers fill filtered serum into sterile containers by aseptic processing in a suitably controlled environment. Serum manufacturers should describe each filtration process step and should perform serum filtration, filling, and sample collection activities following the manufacturer's approved procedures.

## IRRADIATION

Serum treatment by gamma irradiation is very common and one of the most effective methods of virus inactivation. The most frequently used minimum dose is 25 kilograys (kGy). Some countries specify higher dose requirements (>30 kGy) for imported serum. Gamma irradiation may inactivate viruses, mycoplasma, and bacteria, but serum end users should ensure that the gamma irradiation process does not negatively affect their specific applications. Irradiation may have adverse effects on serum quality, and these adverse effects tend to increase with higher doses.

Validation of gamma irradiation has two aspects: (1) dose delivery in a defined load configuration and (2) inactivation capacity. Critical irradiation process parameters include product (serum) temperature, packaging size and configuration, dosimeter distribution, and defined minimum/maximum dose received. Dosimeters should be used to monitor the established high-dose and low-dose positions in each irradiation run. If the serum manufacturer makes inactivation claims, these should be supported by the manufacturer's own well-designed viral inactivation studies.

## ULTRAVIOLET (UV) TREATMENT

Serum manufacturers may use UV treatment to inactivate viruses, mycoplasma, and bacteria, but manufacturers must validate the process to demonstrate its efficacy. In addition, manufacturers must be aware that UV treatment may have an adverse effect on serum quality and accordingly should consider the effects of UV treatment for each application, as should serum end users.

## HEAT INACTIVATION

Heat inactivation involves elevating the temperature of the serum to >56° for at least 30 minutes to inactivate complement. Heat inactivation may also inactivate viruses, mycoplasma, and bacteria; but it may have an adverse affect on serum quality, and manufacturers must validate the procedure's suitability for specific applications. Heat inactivation provides significantly less assurance of virus inactivation than does irradiation.

## VIRAL CLEARANCE STUDIES

*Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin* ⟨1050⟩ and other regulatory documents give guidance about conducting viral clearance studies that help validate removal/inactivation processes. Serum manufacturers should also perform formal spiking studies with relevant and representative (model) viruses, and should test and compare inactivated spiked serum samples, negative controls, and positive controls.

## CHARCOAL STRIPPING

Some serum manufacturers use charcoal/dextran treatment to reduce the levels of hormones in serum.

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### DIALYSIS

Some manufacturers use dialysis or diafiltration to remove low molecular weight components from serum.

### CLEANING AND STERILITY OF EQUIPMENT

Stainless steel systems and tubing used in the manufacture of bovine serum must be cleaned and sterilized to prevent cross-contamination and growth of adventitious agents. Serum manufacturers must validate their cleaning processes for removing and inactivating agents of concern. Thereafter, manufacturers should implement process controls that routinely verify cleaning cycles. Steam sterilization-in-place is a common and effective sterilization technique. Serum manufacturers that use this technology must validate steam cycles to demonstrate their uniformity and ability to destroy heat-resistant bacterial spores. Alternatively, manufacturers can use sterile disposable systems that do not require cleaning validation.

## Quality Control

### TRACEABILITY

**Abattoir Collection:** Materials collected in the U.S. should originate from U.S. Department of Agriculture (USDA)-registered facilities. Serum manufacturers should maintain documentation that traces a given serum sub-lot to the abattoir where it was collected. Slaughterhouses maintain records of animal source. General industry practice is to keep this information as part of the Device Master Record. General record-keeping requirements at USDA-licensed abattoir facilities are outlined in 9 Code of Federal Regulations (CFR) 320.

Materials collected from countries approved by the USDA for importation of bovine products into the U.S. should meet the requirements of the competent authority of the country of origin. In addition, serum manufacturers should keep USDA-required safety testing records of imported materials (if applicable) as part of their Device History Record.

Serum manufacturers should consult 9 CFR 309 and 9 CFR 310 about requirements for inspection of animals for various diseases pre- and post-slaughter. These requirements are recommended for materials collected outside the U.S.

**Donor Herd Collection:** Serum manufacturers should maintain traceability to the donor animal farm where blood was collected from donor animals. In most cases, manufacturers individually identify farm animals and keep records for bleed and processing dates, making it possible to trace blood collection to an individual animal. A licensed veterinarian or a designee under the guidance of a veterinarian should inspect animals regularly and should certify that the animals are free of disease and fit for human consumption, consistent with 9 CFR 309.

### PRODUCT STORAGE AND STABILITY

Serum should be stored in the frozen state at –10° or below. Serum is frozen as quickly as possible to preserve product quality and is stored under controlled storage conditions. Serum manufacturers should establish serum product stability in support of a proposed expiration date. Typical expiration dating for bovine serum is 5 years from the date of filtration and filling. Use of any type of bovine serum beyond the stated expiration date depends on the application, and the serum user must establish the product's continued suitability for use.

## Labeling

Finished product labels must contain the following information: product description, lot number, storage conditions, name and address of manufacturer, and a statement indicating the intended use. Materials intended for research purposes are exempt from labeling regulations (21 CFR 801). Typically, serum manufacturers supply a lot-specific Certificate of Analysis (COA) that is classified as part of the product's labeling. See COA requirements in the following section.

## Certification/Documentation

### CERTIFICATE OF ANALYSIS

The COA should provide information about a specific lot of serum, including tests performed and test results (according to the serum manufacturer's specifications for release), as well as critical labeling identifiers such as lot number, catalog number, description of type of bovine serum, country of origin, and either or both dates of manufacture and expiration. This document is distinct from the certificate of health issued by the competent authority of the country of origin.

### CERTIFICATE OF ORIGIN AND CERTIFICATION OF ANIMAL STATE OF HEALTH

The Certificate of Origin establishes the country in which the bovine blood was collected and veterinary certification of the health of the animals pre- and postcollection (9 CFR 327.4).

### IMPORT/EXPORT DOCUMENTS

Import/export documents contain formal certification of animal disease status of the country of origin and negotiated/agreed certification statements. These vary from country to country. Each country defines import/export requirements in order to control introduction of exotic animal diseases and their economic impact as well as product safety assessments (risk vs. research, diagnostic, and/or therapeutic benefits).

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### PRODUCTION REPORTS

Production reports typically are batch records that document the raw materials in identifiable and traceable ways, production methods (centrifugation or filtration) used in manufacturing, equipment and facility cleaning, quality control testing, and personnel performing required activities. Raw material with Certificates of Origin or serum production records facilitates traceability to the source of the blood that was used to create the serum. When serum is used as a raw material for further manufacturing, process documentation also helps demonstrate controlled manufacture of the bovine serum.

## BSE RISK ASSESSMENT

Despite the low risk potential of transmissible spongiform encephalopathies (TSEs) in bovine serum, various U.S. and international regulatory agencies have developed guidance to help manage and further reduce the potential risks of transmission. In the absence of appropriate test methods of detecting the infectious agent in fluids such as blood, the consensus recommendation from various regulatory agencies is to adopt good risk assessment strategies. This section of the chapter provides some background information on the disease and current methods of detection; it also highlights risk assessment and risk reduction strategies to potentially prevent transmission of the disease through the use of serum in the manufacturing of medicinal products.

### Description of the Disease

TSEs are transmissible animal and human diseases that are characterized by degeneration of the brain, associated with severe neurological signs and symptoms. Since the outbreaks of TSE in cattle, termed BSE, which were transmitted to other species, public health officials have been concerned about the risk of TSE infection, including the possibility of TSE transmission by the use of therapeutic products manufactured using bovine serum. In cattle infected with BSE, lower titers have been found in the cerebrospinal fluid, lung, lymph tissue, spleen, kidney, liver, and ileum. Studies have shown that transfusion of blood from sheep infected with either BSE or scrapie but without evident disease can infect naive sheep. Although the risk of cross-contamination is always present, to date no studies have shown that blood can transmit disease from cattle with BSE. Embryos from BSE-affected cattle have not transmitted diseases to mice. Calves born of dams that received embryos from BSE-affected cattle have survived for up to 7 years, and examination of the brains of both the unaffected dams and their offspring revealed no spongiform encephalopathy.

### Detection Strategies

No currently available procedures have been validated as being sufficiently sensitive for routine antemortem screening of asymptomatic animals, although analytical methods are under development for detection and quantitation from low-infectivity materials such as blood. The classic diagnostic test for TSEs is postmortem histological examination of brain tissue to confirm characteristic vacuolar degeneration. Other testing options include immunohistochemical tests that can confirm the presence of PrPSc, the abnormal disease-specific conformation of prion-related protein (PrP), in the vacuolated regions of the brain; and immunochemical tests such as Western blots and enzyme-linked immunosorbent assays that can detect PrPSc in tissues with high or moderately high titers. These tests typically take less time to perform than histological examination (6–8 hours vs. weeks, respectively) and can be partially or fully automated. Although most of these are postmortem tests, studies have demonstrated the feasibility of antemortem testing of lymphoid tissue samples from the tonsils or from the third eyelid of infected animals. Immunochemical tests require extensive sample collection and preparation and can be cost prohibitive for routine testing and monitoring the disease state of large herds. Diagnostic strategies must consider the sensitivity of testing in certain tissues as well as the test's ability to detect infectivity in animals before the development of clinical signs of disease. Negative results do not ensure the absence of infectivity. Detection of infectivity is possible if suspect tissue is inoculated into experimental animals intracranially where the causative agent can amplify. This approach for detection of low infectivity can take months to years to yield a positive result.

### Risk Assessment and Risk Reduction Strategies

Serum manufacturers should employ risk reduction strategies to eliminate the danger of cross-contamination of fetal blood with other tissues, including appropriate sourcing of animal-derived articles and using practices that have been shown to eliminate or minimize the risk of transmitting TSE, via either foods or health care products. Serum end users should perform a risk assessment of their sourcing strategy that takes into account the amount of bovine serum used in their application and should conduct supplier audits to ensure traceability of sourcing, handling, and appropriate quality control systems.

#### SOURCE AND AGE OF ANIMALS

Serum manufacturers should monitor the traceability of each lot of serum to ensure the qualification of bovine serum, as described previously in the two sections *Serum Harvesting and Processing* and *Quality Control*. In addition to traceability, careful selection of source materials is the most important criterion for the safety of medicinal products. Certification of the origin must be available from the supplier, and manufacturers should keep this information on file. The U.S. Food and Drug Administration (FDA) recommendations prohibit the use in FDA-regulated products (except gelatin) of any bovine-derived materials that originate from countries that report indigenous cases of BSE. The current proposed rule qualifies FBS as an unlikely source of BSE infectious material, because current evidence suggests that cow-to-calf transmission of BSE is unlikely. The proposed rule also states that prohibited cattle materials do not include materials sourced from fetal calves of cows that were inspected and

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passed, as long as the materials were obtained by procedures that can prevent contamination with specified risk materials. For veterinary biologics, current regulations enforced by the USDA's Center for Veterinary Biologics (CVB) indicate that ingredients of animal origin should be sourced from countries with no or low BSE risk, as defined by the U.S. National Center for Import and Export and 9 CFR 94.18.

The most satisfactory sources of materials are from countries with the following:

- No reported cases of indigenous BSE
- Compulsory notification of positive tests
- Compulsory clinical and laboratory verification of suspected cases
- Prohibition of the use in ruminant feed of meat and bone meal containing any ruminant protein
- No importation of cattle from countries where a high incidence of BSE has occurred
- No importation of progeny of affected females

BSE infectivity may increase with animal age. Although bovine serum is considered a low-risk material for TSE transmission, some end users consider it prudent to source serum from dams below a set maximum age. If manufacturers cannot determine the date of the dam's birth, they should consider both the implementation date of the feed ban in the country of origin and the incubation period of BSE in order to determine the safety of the source. A ruminant feed ban was imposed in the United Kingdom in July of 1988. These considerations are lot specific, so audits of the raw material supplier should include a review of records.

PRODUCTION PROCESS

End user manufacturing systems should be in place for monitoring the production process and for batch delineation (definition of batch, separation of batches, and cleaning between batches). Of primary importance is control of the potential for cross-contamination with possible infectious material. Because of the documented resistance of TSE agents to most inactivation procedures, controlled sourcing is the most important criterion in achieving acceptable product safety.

Whenever possible, manufacturers should identify steps that theoretically or demonstratively remove or inactivate agents during the manufacture of the material. Manufacturers should continue their investigations into removal and inactivation methods to identify steps/processes that will help ensure the removal or inactivation of TSE agents. Manufacturers should design production processes using available methods that have the greatest likelihood of inactivating or removing TSE agents. For example, prolonged exposure of tissues to high moist heat and high pH inactivates the BSE agent. Such treatments, however, are inappropriate for the extraction of many other types of bovine-derived articles, such as serum, because these treatments lead to the destruction of the material. Conventional chemical and biochemical extraction and isolation procedures may be sufficient to remove the infectious agent. Similar techniques may be effective for other bovine-derived articles. Further research will help to develop an understanding of the most appropriate methodology for validation studies. Issues to consider during validation of a process for removal of TSE agents include the following:

- The nature of the spiked material and its relevance to the natural situation
- Design of the study (including scale-down approaches)
- Method of detecting the agent (in vitro or in vivo assay) after spiking and after the treatment
- Characterization and standardization of reference materials for spiking
- Data treatment and analysis (see *Design and Analysis of Biological Assays* ⟨111⟩)

Because no studies have successfully validated analytical methods for the detection of small amounts of the TSE agent, TSE clearance validation studies typically employ the intracranial injection of in-process material into rodents for amplification and detection of potential residual infectivity.

## TESTING AND CONTROL OF ADVENTITIOUS AGENTS

### Introduction

Rigorous testing procedures, strict processing and production guidelines, and appropriate risk assessments help ensure the safety of the different types of bovine serum. This section discusses specific tests that can detect and control adventitious agents.

### Adventitious Agents Testing

The adventitious agents testing required for the evaluation of master seeds, master cells, and bulk and final products is described in 9 CFR 113.53 and by directives from the European Agency for the Evaluation of Medicinal Products (EMEA) (EMEA/CVMP/743/00 and EMEA/CPMP/BWP/1793/02). The testing methods outlined in these documents can detect a wide range of bovine microbial agents in serum products. These testing methods meet the requirements for most of the world's regulatory agencies. Serum manufacturers should test a representative sample of each batch of serum to determine the presence of adventitious agents. Testing is performed after filtration but before any further processing that is intended to inactivate or remove viruses.

Filtration with 100-nm (0.1-µm) pore size filters is an accepted method for removing mycoplasmas and gamma irradiation (> 25 kGy while frozen), and chemical treatments (e.g., with betapropiolactone) are accepted methods of inactivating viruses and mycoplasmas; serum manufacturers routinely use these tools in both production and testing facilities. These treatments do not remove antibodies that may interfere with some applications. Additionally, the treatments do not ensure complete viral removal or inactivation, but can significantly reduce the risk of viral activity. The testing series to screen bovine serum for the absence of adventitious agents typically includes the following:

- Bacterial and fungal sterility testing as described in 9 CFR 113.26

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- Mycoplasma testing as described in 9 CFR 113.28
- Viral testing as described in 9 CFR 113.53

The procedures described in *Sterility Tests* ⟨71⟩ confirm the absence of bacterial and fungal infection. For viruses, only cultivation using suitable substrate cells can indicate viral infectivity and replication. Those who use serum for research or production should test the serum for the absence of adventitious agents in a manner that is consistent with the product's intended application, bearing in mind that testing indicates only presence or absence of adventitious agents within the limits of the test procedures used.

## Mycoplasma Testing

Mycoplasma contamination in tissue culture can arise from many animal origin sources, including serum, but more commonly it results from cross-contamination of infected cultures. Mycoplasmas are particularly insidious contaminants in cell culture because they

- cannot be visualized by light microscopy even at high density (>$10^7$ colony-forming units/mL);
- cause no observable change in turbidity or pH of the culture fluid;
- cannot routinely be removed by single sterilizing filters, although removal can be obtained through a triple series of 0.1-µm filters;
- are unaffected by traditional antibiotics used in cell culture; and
- exert an extremely wide variety of adverse effects in tissue culture.

Classical mycoplasma detection is described in *Mycoplasma Tests* ⟨63⟩.

In addition to these methods, more recent detection procedures include luminescent and polymerase chain reaction (PCR) assay procedures. *Nucleic Acid-Based Techniques—Amplification* ⟨1127⟩ describes the general principles of PCR assays. The sensitive 20-minute luminescent assay measures a specific enzyme activity of mollicutes that converts adenosine diphosphate to adenosine triphosphate via a luciferase/luciferin reaction. Results are unequivocal and semiquantitative. PCR methods are quick and sensitive and display with good reliability, but occasional false positive results are a source of concern with commercial testing service labs. PCR may detect mycoplasmal DNA fragments that are non-infectious.

## Viral Testing

The virus testing procedures for serum products are outlined in 9 CFR 113.52 and 9 CFR 113.53. In addition, there are other documents that may include equivalent or relevant testing such as EMEA/CVMP/743/00-Rev.2 from the Committee for Veterinary Medicinal Products (CVMP) *Revised Guideline on Requirements and Controls Applied to Bovine Serum Used in the Production of Immunological Veterinary Medicinal Products* and EMEA/CPMP/BWP/1793/02 from the Committee for Proprietary Medicinal Products (CPMP) *Note for Guidance on the Use of Bovine Serum in the Manufacture of Human Biological Medicinal Products*. Serum manufacturers should perform virus testing in compliance with this regulation, using at least two different and sensitive detector cell lines, one of which should be of bovine origin. The tests include cultivation of detector cells in cell culture media supplemented with 15% test serum for at least 21 days. Cells are subcultured at least twice during this period, usually 7 and 14 days post inoculation. At the conclusion of the last subculture (after a total of at least 21 days of incubation), cells are examined for general signs of virus amplification. The following end points are used for general virus detection: microscopic cell examination for cytopathogenic agents such as infectious bovine rhinotracheitis virus, cell staining and microscopic examination for inclusion bodies, and hemadsorption test to detect hemadsorbing agents such as PI-3. In addition to this series of testing and at the conclusion of the last subculture (after a total of at least 21 days of incubation), cells are stained with specific fluorescent antibodies against the following specific viral agents:

- BVDV
- Bovine parvovirus
- Bovine adenovirus
- Bluetongue virus
- Bovine respiratory syncytial virus
- Reovirus
- Rabies virus

In addition to the viruses listed above, other viruses can be causative agents of disease and may require testing in various bovine serum applications. The serum end user is responsible for determining whether full 9 CFR testing is sufficient, and if other specific viral agents should be tested for. Examples of specific viruses not covered by the current virus testing guide may include akabane, bovine herpesvirus 1 (BHV-1), Parainfluenza-3 virus (PI-3), bovine leukemia, bovine rotavirus, bovine circovirus, bovine polyomavirus, coronavirus, torovirus, bovine enterovirus, bovine astrovirus, foot-and-mouth disease virus (FMDV), and rinderpest. *Appendix 2* provides a general description of some of these viruses as well as the ones for which testing is required. A serum end user's thorough risk analysis should determine the scope of testing and serum treatment options.

## Risk Assessment and Detection Strategies

Serum manufacturers and serum end users should carry out a comprehensive, science-based risk assessment (e.g. Failure Modes and Effects Analysis) in order to better understand the safety profile of the serum product. The following risk assessment elements can be taken into consideration, but other elements can be included as appropriate: country of origin, region of the country, animal disease status of the country/region of origin, animal age, blood collection process, animal stunning method and exsanguination method, serum manufacturing process, type of production quality system, production in-process controls,

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final product testing, virus inactivation, equipment segregation, equipment cleaning procedure, personnel training, serum use/application, pharmaceutical product type, and intended use.

The species barrier provides a degree of protection against infection by some animal etiologic agents. This barrier is not an alternative to proactively ensuring that pharmaceutical products are manufactured only from raw materials of animal origin that have undetectable levels of adventitious agents. Inoculation of viable organisms into a nonhost species carries a risk that the organisms could cross the species barrier. An appropriate test regimen of serum material should therefore include examination for potential contaminants associated with the species of origin and the species of intent. Serum treatments to inactivate viral agents are a factor in establishing the appropriate test regimen for a particular material. Lowest risk of contamination is associated with biological materials that are terminally sterilized.

Zero risk is neither possible nor reasonable. The serum manufacturers should fully describe specific testing regimens in the product specifications, and these will vary depending on the type and source of the serum. Therefore, the guidelines for screening described in this chapter are examples only, and screening for all viruses listed may not be required for a particular material. Some manufacturers may perform certain tests on the finished product or on in-process materials rather than on individual component(s). Manufacturers must also evaluate the dilution effect in relation to the limit of detection of the test procedure. Interference with growth or neutralization of viral activity by serum may be an indication of a specific antibody or certain nonspecific factors in serum masking the viral agent. It is recommended that serum manufacturers consider this possibility when determining an adequate level of treatment in their viral inactivation studies or in virus testing applications.

Serum manufacturers should confirm that the species of origin is bovine to ensure that no other nonbovine agents may be present. Manufacturers should perform extraneous virus testing in appropriate cell cultures (see *Virology Test Methods* ⟨1237⟩ for appropriate cell line choices dependent on assay and targeted agent). If necessary, seroconversion studies should be conducted in susceptible animal species using a host species immune antibody response as the method of detection. Studies should use this procedure following an inactivation step to detect whether the virus was present before the virus inactivation process.

Serum manufacturing processes should be conducted in a consistent manner, following the established manufacturing procedures, with adequate quality systems built into the production process. Furthermore, equipment segregation (by species of origin), equipment and facility cleaning procedures, and personnel training are important elements in the risk assessment of the process.

### Safety Considerations

End users of donor bovine serum may require serum that does not have detectable antibodies against BVDV or other specific agents so that the users can propagate cell cultures used in vaccine production, diagnostic testing, and test kit preparation, especially for the maintenance of master seed and master cell stocks. More than 40 cell types are available for the production of veterinary biologicals, but fewer than 10 media types are available for their propagation. Some researchers have proposed serum-free media as an alternative in propagating certain cells and viruses; but this means adapting culture procedures, which may alter the cells and change production results. If new or different sera are imported into the U.S., serum end users will require confirmation of source, species, and documentation of the origin of the sera in countries that are free of FMD and rinderpest.

## CHARACTERIZATION OF BOVINE SERUM

### Introduction

In the absence of end product-specific requirements, each lot of FBS should be tested to confirm that the serum meets the requirements of the proposed general chapter *Fetal Bovine Serum—Quality Attributes and Functionality Tests* ⟨90⟩. For all other types of bovine sera, this section describes several key procedures for characterization. These procedures are not mandatory but are guidelines that manufacturers may consider for their individual applications. The table in the *Hemoglobin* section shows samples of specifications for the different types of bovine sera.

### Species Identification

Both inter- and intraspecies identification assays should be performed on bovine sera to confirm species identity and the integrity of the serum products, and to ensure that nonbovine agents are not present. The most commonly used assay for the identification of bovine species identity is based on the electrophoretic profile of specific serum proteins. With electrophoresis, the serum proteins usually separate into as many as six fractions: albumin, alpha 1, alpha 2, beta 1, beta 2, and gamma globulins.

Other procedures used for bovine speciation include radial immunodiffusion (RID) and the double diffusion Ouchterlony method. These procedures allow either qualitative or quantitative measurements of the immunoglobulin G levels in serum. The RID method is based on the diffusion of an antigen from a circular well into a homogeneous gel that contains specific antiserum for each particular antigen. A circle of precipitated antigen and antibody forms and continues to grow until it reaches equilibrium. The diameters of the rings are a function of antigen concentration. The Ouchterlony method is a double gel diffusion test wherein antigen and antibody diffuse toward each other in a semisolid medium to a point in the medium where optimum concentration of each is reached, forming a precipitate. The Ouchterlony plates contain cylindrical wells—a central 8-mm diameter antigen well, surrounded by six 3-mm antisera wells—which make possible the simultaneous monitoring of multiple antigen–antibody systems and the identification of particular antigens in a preparation. The proposed general chapter *Fetal Bovine Serum—Quality Attributes and Functionality Tests* ⟨90⟩ describes the accepted procedure.

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## Hemoglobin

Hemoglobin is a multi-subunit protein that forms an unstable reversible bond with oxygen in the red blood cells. The oxygen-loaded form is called oxyhemoglobin and is bright red. The oxygen-unloaded form is called deoxyhemoglobin and is purple-blue. Oxyhemoglobin is the predominant form in red blood cells.

Low hemoglobin content in sera is widely accepted as a good general indication of rapid and careful processing of blood that will be used for serum. Red blood cells are fragile and rupture easily, releasing hemoglobin into the serum. Rough handling of the harvested blood, poor temperature control, or delayed processing elevates hemoglobin content in serum. Acceptable levels of hemoglobin may vary with intended application. The hemoglobin levels are measured using spectrophotometric procedures (see *Ultraviolet-Visible Spectroscopy* ⟨857⟩) as described in the proposed general chapter *Fetal Bovine Serum—Quality Attributes and Functionality Tests* ⟨90⟩.

| | FBS | Newborn Calf Serum | Calf Serum | Donor Bovine Serum | Adult Bovine Serum |
|---|---|---|---|---|---|
| Sterility test | No growth detected | No growth detected | No growth detected | No growth detected | No growth detected |
| Mycoplasma | Not detected | Not detected | Not detected | Not detected | Not detected |
| Virus testing | Not detected | Not detected | Not detected | Not detected | Not detected |
| Hemoglobin (mg/dL) | <30 | <30 | <30 | <30 | <30 |
| Total protein (g/dL) | 3.0–4.5 | 3.5–6.0 | 5.0–8.0 | 5.0–8.0 | 6.0–10.0 |
| pH | 7.00–8.00 | 7.00–8.00 | 7.00–8.00 | 7.00–8.00 | 7.00–8.00 |
| Osmolality (mOsmol/Kg) | 280–360 | 240–340 | 240–340 | 240–340 | 240–340 |

## Chemical Profile

The testing of components such as cholesterol, alpha globulin, beta globulin, gamma globulin, albumin, creatinine, bilirubin, glucose, alanine aminotransferase, aspartate aminotransferase, phosphorus, potassium, calcium, and sodium usually is not considered a criterion for bovine serum lot release. Some manufacturers do not perform the tests on a routine basis but only as auxiliary tests. In some instances hospital clinical laboratories may run the tests. The levels of these chemicals in serum are important to end users and may also be used to assess lot-to-lot variability.

## Endotoxin Levels

Although high endotoxin levels are not suitable for applications involving injectables, acceptable levels in bovine sera vary depending on the intended application. Some manufacturers may overlook the importance of low endotoxin levels in bovine sera used in cell culture applications. Endotoxin influences more than 30 biological activities. Some of these are macrophage activation, mitogenic stimulation, and induction of interferon and colony-stimulating factor (for some applications, these may be positive activities). Endotoxin can also lead to cytotoxicity by initiating complement activation. The most commonly used methods for endotoxin detection are the semiquantitative gel clot Limulus amebocyte lysate procedure and the quantitative kinetic chromogenic method described in *Bacterial Endotoxins Test* ⟨85⟩. For both the gel clot and the kinetic chromogenic assays, valid endotoxin assays require appropriate treatment by heat or dilution in order to avoid adverse effects of interfering substances in serum. Researchers should include a positive product control in each assay to confirm that any interference has been overcome by the heat or dilution treatment.

## Osmolality

The osmolality test is designed to evaluate the electrolyte concentration in bovine serum. Chemicals that affect serum osmolality include sodium, chloride, bicarbonate, potassium, proteins, and glucose. Serum manufacturers should measure the osmolality of each serum batch to verify compliance with product specifications, using equipment calibrated with standards that are traceable to the National Institute of Standards and Technology. *Osmolality and Osmolarity* ⟨785⟩ describes how osmolality is determined by freezing-point depression of the bovine serum solution. Scientists use at least two standards to calibrate the instrument. The osmolality of each sample is calculated and related to the serum water content and is expressed as mOsmol/kg $H_2O$.

## Total Protein Level

The total protein level in serum is measured to verify animal age and compliance with product specifications. *Biotechnology-Derived Articles—Total Protein Assay* ⟨1057⟩ describes two procedures, the Biuret and Bradford methods, for determining protein concentration. The acceptable level of protein in serum should be assessed by the end user based on the intended application.

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## Cell Growth Properties

Each lot of serum should be tested for its ability to support in vitro growth of specific cell lines. Bovine sera are highly variable, and different lots may yield different results. Because of this variability, end users should characterize and standardize the cell lines that they will use for this type of testing. End users should design cell growth procedures that will help them check the efficacy of bovine serum in promoting cell growth. Serum manufacturers will benefit from monitoring growth promotion over several generations of subcultures to detect any evidence of cytotoxicity or changes in cell morphology. Different serum manufacturers use different cell types, and the growth studies and cell lines used by serum manufacturers also may differ from those applied by serum end users. When serum manufacturers evaluate the growth properties of a specific cell line in response to a specific lot of serum, they should take into account plating efficiency and/or growth promotion or some other functionality tests that qualify the serum lot for its intended use.

Plating efficiency at low cell density is a preferred method for analyzing the proliferative capacity and survival of single cells under optimal growth conditions. This procedure can reveal differences in the growth rate within the population and is capable of distinguishing between changes in growth rate (colony size) and cell survival (colony number). The growth kinetic is another important aspect in the design of cell-based experiments. Determining the growth curve of each cell line helps define optimal culture conditions, because variation in serum and other growth additives may influence growth parameters, which may affect the experimental outcome.

In the absence of specific tests designed for their particular products, serum users can refer to the functionality tests described in the proposed general chapter *Fetal Bovine Serum—Quality Attributes and Functionality Tests* ⟨90⟩ to determine whether a lot of serum is suitable for their application. This chapter provides guidance about how to perform growth promotion and plating efficiency tests.

## In Vitro Cytotoxicity

Serum manufacturers should use an appropriate cell line for testing each lot of serum, and should perform growth studies through several subcultured generations to ensure that the serum has no cytotoxic effect on the cells. The choice of cell line depends on the intended use of serum. The cell growth and cytotoxicity assays should be performed on the final batch of serum after any viral inactivation step or any further processing.

# CONCLUSION

Bovine serum is likely to remain an important component in the manufacture of many biologics, particularly those relying on cell culture. As with similar materials, bovine serum displays inherently variable quality. As a result, serum end users must establish suitable tests, procedures, and acceptance criteria for introduction of materials into a particular application process that uses serum. This may mean screening multiple lots of bovine serum to determine which lots meet the specification (see the section *Characterization of Bovine Serum*).

Manufacturers of therapeutic products using bovine serum are responsible for ensuring and documenting its quality and its impact on the quality, safety, and efficacy of the final product. In addition, it is important to ensure that each lot of serum performs in an equivalent manner during manufacturing. Serum can also interfere with final product purification; therefore it is important to understand the effect of bovine serum on the manufacturing process in order to understand the effect that various processes might have on the final product. Finally, risks can also be mitigated through the design of processes to include steps to adequately remove the bovine material through dilution, separation, or inactivation as well as the development of analytical assays to assess the bovine-derived residual content during processes and in the final therapeutic product. A number of sensitive assays can provide a quantitative means of detecting bovine material at picogram levels.

# APPENDICES

## Appendix 1: Relevant Regulatory References

Bovine sera and serum-related products used in the manufacture of biological products are regulated in the context of *Requirements for Ingredients of Animal Origin Used for the Production of Biologics*, 9 CFR 113.53. Currently, individual serum manufacturers perform detection studies to identify contaminating viruses. Because of the potential international market for serum, serum manufacturers need to be mindful of other regulatory requirements. Manufacturers can use the documents listed here as guidance for screening bovine sera for contamination by adventitious agents. Because of the risk carried by animal-derived serum products, serum manufacturers and end users should ensure that the country of origin of the material complies with applicable regulatory requirements. Although no cell performance assays currently demonstrate lack of BSE in serum, serum manufacturers must comply with the regulatory requirements of countries where the serum is sourced and marketed to ensure a minimal risk of infection with BSE/TSE.

Beyond relevant *USP* chapters referenced in this chapter, the following list of documents includes regulatory documents as well as best practices in product and process development, manufacturing, quality control, and quality assurance.

CFR

- 9 CFR 94.18 (CVB, 2001)
- 9 CFR 113.46
- 9 CFR 113.47
- 9 CFR 113.52
- 9 CFR 113.53

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- 9 CFR 113.55
- 9 CFR 320
- 9 CFR 327.4
- 21 CFR 211 Subpart E
- 21 CFR 801.1
- 21 CFR 809.10

FDA

- FDA. Center for Biologics Evaluation and Research (CBER). 2000. Letter to manufacturers of biological products. Available at: http://www.fda.gov/BiologicsBloodVaccines/SafetyAvailability/ucm105877.htm
- Use of materials derived from cattle in medicinal products intended for use in humans and drugs intended for use in ruminants (Proposed Rule). *Federal Register*. 2007; *72*(8): 1582–1619. Available at: http://www.reginfo.gov/public/

International Regulations and Guidance Documents

- CPMP/Biotechnology Working Party/EMEA (CPMP/BWP/EMEA). 1996. *Note for guidance on virus validation studies: the design, contribution and interpretation of studies validating the inactivation and removal of viruses*. Available at http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003684.pdf.
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- CPMP/CVMP. *Note for guidance on minimising the risk of transmitting animal spongiform encephalopathy agents via human and veterinary medicinal products*. Available at http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003712.pdf.
- World Health Organization (WHO), Office International des Epizooties. *Terrestrial animal health code.* Available at http://www.oie.int/doc/ged/D10905.pdf.
- WHO. 2006. *WHO guidelines on tissue infectivity distribution in transmissible spongiform encephalopathies*. http://www.who.int/bloodproducts/cs/TSEPUBLISHEDREPORT.pdf.

## Appendix 2: Viruses to Consider when testing Bovine Serum

Following is a general description of viruses that manufacturers can consider when testing bovine serum for the absence of adventitious agents. The list is intended only to provide general information. The list of required testing is described in this chapter in the section *Viral Testing*.

### Akabane

An insect-transmitted virus that causes congenital abnormalities of the central nervous system in ruminants. Disease due to *Akabane* virus has been recognized in Australia, Israel, Japan, and Korea. Antibodies to it have been found in a number of countries in Southeast Asia, the Middle East, and Africa. The disease affects fetuses of cattle, sheep, and goats. Asymptomatic infection has been demonstrated serologically in horses, buffalo, and deer (but not in humans or pigs) in endemic areas.

### Bluetongue

An infectious, noncontagious arthropod-borne viral disease primarily of domestic and wild ruminants. Infection with bluetongue virus is common worldwide but is usually subclinical or mild. *Bluetongue* virus is the type-species of the genus *Orbivirus* in the family Reoviridae. Worldwide, 24 serotypes have been identified, although not all serotypes exist in any one geographic area: e.g., only 5 serotypes (2, 10, 11, 13, and 17) have been reported in the U.S. Distribution throughout the world parallels the spatial and temporal distribution of vector species of *Culicoides* biting midges, which are the only significant natural transmitters of the virus.

### Bovine adenovirus

Associated with a wide spectrum of diseases. *Bovine adenovirus* type 3 is the serotype most often associated with bovine respiratory disease. *Bovine adenoviruses* are DNA viruses that have been separated into two genera: the *Mastadenovirus,* or *mammalian adenoviruses,* and the *Aviadenovirus,* or *avian adenoviruses.* Within the genus *Mastadenovirus* are numerous species-specific serotypes, nine of which have been identified in cattle. *Epitheliotrophic adenoviruses* have also been isolated from ruminants, and usually are clinically unapparent. Clinical disease is dictated by various factors, including the strain of virus, concurrent infection, stress, environmental conditions, and management practices.

### Bovine herpesvirus 1 (BHV-1)

Associated with several diseases in cattle, including infectious bovine rhinotracheitis, infectious pustular vulvovaginitis, balanoposthitis, conjunctivitis, abortion, encephalomyelitis, and mastitis. BHV-1 infections are widespread in the cattle population. In feedlot cattle the respiratory form is most common.

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## Bovine leukemia

An exogenous C-type oncovirus in the family Retroviridae. Bovine leukemia is a viral disease of adult cattle characterized by neoplasia of lymphocytes and lymph nodes. Infection occurs by iatrogenic transfer of infected lymphocytes and is followed by a permanent antibody response. The prevalence of infection in a herd may be high, but only a few animals develop fatal lymphosarcoma. Infection is spread by contact with contaminated blood from an infected animal.

## Bovine reovirus

Double-stranded ribonucleic acid (RNA) (dsRNA) viruses with nonenveloped spherical virions 60–80 nm in diameter. They cause bovine respiratory diseases.

## Bovine respiratory syncytial virus (BRSV)

An RNA virus classified as a pneumovirus in the Paramyxovirus family. This virus was named for its characteristic cytopathic effect—the formation of syncytial cells. In addition to cattle, sheep and goats can also be infected by respiratory syncytial viruses. Human respiratory syncytial virus (HRSV) is an important respiratory pathogen in infants and young children. HRSV has antigenic subtypes, and preliminary evidence suggests the existence of antigenic subtypes of BRSV. BRSV is distributed worldwide, and the virus is indigenous in the cattle population. BRSV infections associated with respiratory disease occur predominantly in young beef and dairy cattle.

## Bovine rotavirus

A dsRNA spherical virion 60–80 nm in diameter without an envelope. It is the most common viral cause of diarrhea in calves and lambs.

## Bovine viral diarrhea virus (BVDV)

An RNA virus classified as a *Pestivirus* in the family Flaviviridae. BVDV can cross the placenta and appears to be capable of inducing immunosuppression, which allows the development of secondary bacterial pneumonia. BVDV has been reported to be the virus most frequently associated with multiple viral infections of the respiratory tract of calves.

## Foot-and-mouth disease (FMD)

A highly infectious viral disease of cattle, pigs, sheep, goats, buffalo, and artiodactyl wildlife species. In a susceptible population, morbidity approaches 100%. The disease is rarely fatal except in young animals. FMD is caused by an *Aphthovirus* of the family Picornaviridae. Seven immunologically distinct serotypes are known, and within each serotype exist a large number of strains that exhibit a spectrum of antigenic characteristics.

## Parainfluenza-3 virus (PI-3)

An RNA virus classified in the Paramyxovirus family. Although PI-3 is capable of causing disease, the virus usually is associated with mild to subclinical infection. The most important role of PI-3 is to serve as an initiator that can lead to the development of secondary bacterial pneumonia. Infections caused by PI-3 are common in cattle.

## Parvovirus

A relatively heat-stabile single-stranded DNA virus approximately 20 nm in diameter that has been recovered from cattle but under natural conditions is not known to cause disease.

## Rabies

An acute viral encephalomyelitis that principally affects carnivores and bats, although it can affect any mammal. Rabies is caused by *Lyssaviruses* in the Rhabdovirus family. Although they are usually confined to one major reservoir species in a given geographic area, spillover to other species is common.

## Rinderpest

A *Morbillivirus,* closely related to the viruses that cause canine distemper and measles. Strains may vary markedly in host range and virulence. Sera from recovered or vaccinated cattle cross-react with all strains in neutralization tests, but minor antigenic differences have been demonstrated. The virus is fragile and becomes rapidly inactivated by heat and light but remains viable for long periods in chilled or frozen tissues. Rinderpest is endemic in many countries in Asia and Africa. Historically, the virus has been widely distributed throughout Europe and Africa but to date has not established itself in North America, Central America, the Caribbean Islands, South America, Australia, or New Zealand. Rinderpest is included in the WHO's Office International des Epizooties list of communicable diseases that have the potential for very serious and rapid spread, irrespective of national borders; that are of serious socioeconomic or public health consequence; and that are of major importance in the international trade of livestock and livestock products.

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# ⟨1025⟩ PANCREATIN

## OUTLINE

1. Introduction
2. Pancreas Collection and Pancreatin Production
3. Quality Control
4. Labeling
5. Certification and Documentation
6. Testing and Control of Adventitious Infectious Agents
    - 6.1 Overview
    - 6.2 Risk Assessment Strategies
    - 6.3 Identification of Relevant Virus Panel Test
    - 6.4 Virus Clearance by Manufacturing Process Steps
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7. Characterization of Pancreatin
    - 7.1 Description and Physico-Chemical Properties
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## 1. INTRODUCTION

Pancreatin is a pancreatic enzyme preparation containing amylase, protease, and lipase enzymes isolated from the pancreas gland of the hog, *Sus scrofa* L. var. *domesticus* Gray (Fam. Suidae). The pancreas is a secretory organ that plays a crucial role in the digestive process by producing bicarbonate to neutralize the acidic environment in the duodenum, hormones to regulate various catabolic functions, and a variety of digestive enzymes to degrade food in the small intestine. Pancreatin and pancreatin-containing medicinal products are used to aid digestion and absorption of food (carbohydrates, fat, and proteins) in patients with exocrine pancreatic insufficiency (EPI) caused by cystic fibrosis, chronic pancreatitis, and other conditions that might cause a deficiency in the secretion of pancreatic enzymes.

Pancreatic enzyme products (PEPs) of porcine origin have been marketed in the United States for the treatment of EPI before the enactment of the Federal Food, Drug, and Cosmetic Act of 1938. Supplemental pancreatic enzymes are available in prescription and non-prescription forms. Since 2004, the U.S. Food and Drug Administration (FDA) has required that all pancreatic enzyme drug products marketed in the United States obtain FDA approval via a New Drug Application (NDA). Although over-the-counter pancreatic enzyme products are available without a prescription, they are classified as dietary supplements rather than drugs. Pancreatin and pancrelipase share similar functions and indications; however, pancrelipase, which is available only as a prescription drug, contains more of the active lipase enzyme and also more purified pancreatic extract than pancreatin does.

This chapter describes best practices related to the sourcing and manufacturing of pancreatin raw materials used in both pancreatin and pancrelipase drug products; these best practices help to ensure the safety and efficacy of the drug products made from this active pharmaceutical ingredient (API). A list of applicable regulatory guidance documents is provided in *Appendix 1: Regulatory Bibliography*.

## 2. PANCREAS COLLECTION AND PANCREATIN PRODUCTION

The animal-sourced raw material (pancreas glands) intended for pharmaceutical processing is a by-product of meat production and is collected in slaughterhouses that are approved by the national competent authority and inspected by the relevant veterinary authority. The animals from which pancreatin is derived must fulfill the requirements for the health of animals suitable for human consumption.

An example of a typical manufacturing process is summarized in the description and flow chart (*Figure 1*) below.

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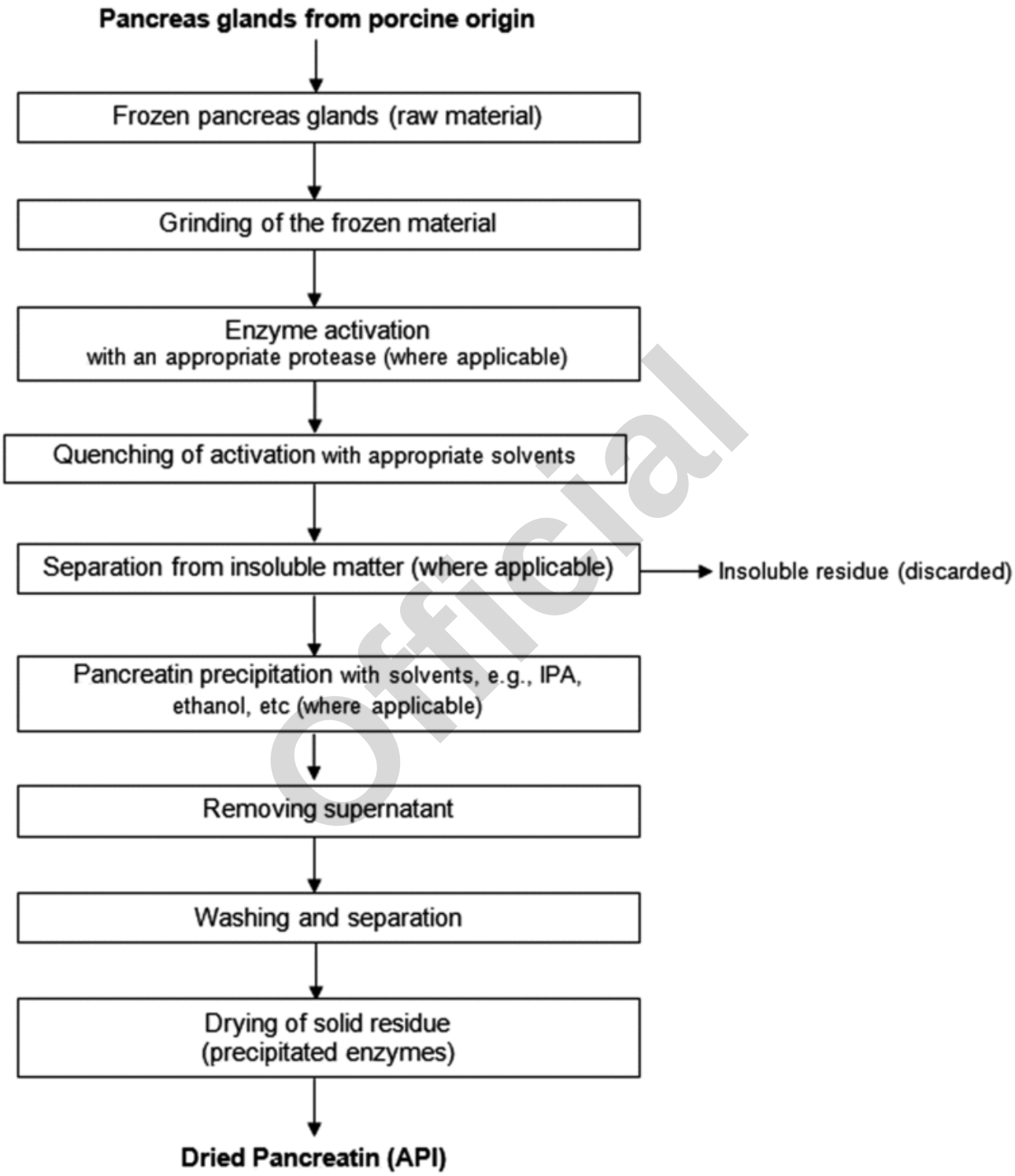


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Figure 1. Flow chart showing the steps in the manufacturing process for pancreatin.

The pancreatic glands should be kept frozen to prevent a loss of enzymatic activity during holding and transport. After the manufacturer accepts the frozen materials, the pancreatin is extracted and purified under conditions that reduce microbiological load and other potential impurities. To accomplish this, the frozen pancreatic glands are macerated into fine slurry and treated with activators to convert the inactive pancreatic enzyme precursors (zymogens) into active enzymes. The activation is carried out under controlled conditions; factors such as time, temperature, ionic strength, or concentration are controlled as defined

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by each manufacturer's process. Once the activation is complete, the activation step is stopped by the addition of solvents such as acetone, isopropyl alcohol, or other enzyme-compatible solvents. After separation from insoluble matter (where applicable), the mixture is combined with solvent to precipitate the pancreatin. The supernatant is separated, and the solid residue is washed with solvent. Finally, the pancreatin is dried at appropriate temperature and vacuum. The biological activity may be adjusted and/or stabilized by adding suitable fillers, such as lactose; sucrose containing not more than 3.25% starch; pancreatin of lower digestive power; microcrystalline cellulose; maltodextrin; or sodium chloride.

## 3. QUALITY CONTROL

Each lot of pancreatin is subject to appropriate quality control testing. Quality control testing needs to address the requirements of the applicable monograph, as well as other identified quality parameters. Such tests should include appearance, identification, purity, and activity of the pancreatin. Process- and product-related impurities, such as residual solvents from extraction and precipitation and fat, should be considered.

Water is critical for enzyme activity and stability, and thus the limit of water content should be specified and monitored by appropriate analytical methods such as loss on drying (LOD) (see chapter ⟨731⟩ *Loss on Drying*). The activity assays are applied to confirm that pancreatin meets predefined limits for activity levels of lipase, amylase, and proteases, which are considered critical quality attributes.

Because pancreatin is of biological origin, quality control includes microbial testing as well as testing for the absence of certain adventitious agents that are pathogenic to humans.

## 4. LABELING

Product should be labeled in conformance to monograph and regulatory requirements but also should conform to customer requirements as applicable. The label contains information such as the name and address of the manufacturer, manufacturing date, retesting date, expiration date, lot number, storage conditions and specific precautions (e.g., "protect from moisture"), and a statement indicating the intended use.

## 5. CERTIFICATION AND DOCUMENTATION

The product must be accompanied by required certificates and documentation, as applicable. This documentation should include a certificate of analysis (COA) or other certificate documenting conformance to monograph requirements and quality control test results; a certificate of origin indicating the animal species; and certifications related to adventitious agents, including, where applicable, Transmissible Spongiform Encephalopathy/Bovine Spongiform Encephalopathy statements. Other pertinent and required information, such as retest or expiration date, storage, and packaging recommendations, should also be included as applicable.

The manufacturer/supplier should also be able to provide documentation for regulatory purposes containing the following information:

- Information on the regulatory agency's approval/license number and the full address of the pancreatin manufacturing site.
- A statement that the animal-sourced raw materials intended for pharmaceutical processing were collected and delivered under oversight of the responsible/competent authorities.
- A list of the countries of origin of the collected raw materials.
- A statement that during transportation, the pancreas glands can be identified for animal by-products intended for pharmaceutical purposes by appropriate documentation according to the corresponding legal regulations (such as animal health certificates from official veterinarians or a technical trade documentation).
- A statement that the pancreas glands are collected in approved slaughterhouses, and the animals from which pancreas glands are collected have undergone inspection in compliance with the current applicable legislation and were declared as suitable for human consumption, or no visible signs of diseases transmittable to humans or animals were detected at the time of slaughter.

## 6. TESTING AND CONTROL OF ADVENTITIOUS INFECTIOUS AGENTS

### 6.1 Overview

Although there have been no reports documenting any infectious illness subsequent to the use of pancreatin-derived medicinal products, there is a theoretical risk of porcine pathogen transmission from pancreatin.

The safety of porcine-derived PEPs should be enhanced by implementation of multiple complementary and/or overlapping strategies for adventitious agent containment, clearance, and control. Manufacturers should take an ongoing, risk-based approach to enhancing the safety of these products with regard to adventitious agents that includes incorporation of risk assessment, risk mitigation, and process materials management strategies. Testing should be included, when appropriate, to provide maximum possible assurance of a sufficiently low risk of harm that is significantly outweighed by the therapeutic benefits and concurrent assurance of availability of the drug to patients.

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## 6.2 Risk Assessment Strategies

The FDA's "Guidance for Industry: Exocrine Pancreatic Insufficiency Drug Products" encourages a risk-based approach to the potential for viral contamination of pancreatic enzyme products, in agreement with ICH Q5A(R1) and chapter ⟨1050⟩ *Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin*. The adventitious-agent risk profile of biological products in general is contingent upon a variety of factors including the origin of the biological, the type of raw materials used, manufacturing processes, and the route of administration. As sourcing and manufacturing may vary between manufacturers, each manufacturer should establish and implement an individual, full adventitious-agent risk assessment.

Although the scope of ICH Q5A(R1) covers the viral safety evaluation of biotechnology products derived from cell lines of human and animal origin, the principles and risk assessment approaches can provide the basis for a risk evaluation strategy for the pancreatin products. Applying the principles of ICH Q5A(R1), the risk minimization strategy to protect patients against inadvertent adventitious agent exposure should reflect a combination of three components:

1. **Sourcing:** Use of diligent sourcing to limit adventitious agents' access to the manufacturing process. Because pancreatin material is a by-product of the meat industry, it is important to ensure that pancreatin API is produced only from animals suitable for human consumption.
2. **Clearance:** Incorporation of robust clearance steps into the manufacturing process. The efficacy of these strategies depends on the adventitious agents' resistance to the type of physical and chemical inactivation used.
3. **Testing:** The control and testing of adventitious agents at suitable stages of the manufacturing process to provide assurance that any remaining load of potentially harmful adventitious agent is at sufficiently low levels. This is accomplished by using suitable screening assays against a relevant test virus panel. In this part of the risk assessment, the potential of porcine viruses to pose a risk to humans should be taken into account.

To identify the potential adventitious agents that might be present in a pancreatin preparation, manufacturers should identify adventitious agents that are present in the pig and assess the probability of their presence in the starting materials and API. Chapters ⟨61⟩ *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* and ⟨62⟩ *Microbiological Examination of Nonsterile Products: Tests for Specified Microorganisms* address testing for specific microbiological contamination, as defined in the relevant pancreatin-related product monographs. Additionally, manufacturers should specifically evaluate the potential presence of porcine viruses. In this evaluation, the manufacturing steps capable of removing or inactivating viruses should be identified, and the efficiency of the process for removing and/or inactivating viruses should be demonstrated by viral validation studies that follow applicable guidelines. Once a list of critical viruses potentially present in the starting material and/or in the API is established, manufacturers will decide on the appropriate process stages for viral testing and the level of viral testing to be performed on a batch-to-batch basis. Tests for specific viruses should be developed and validated, and acceptance criteria should be established and used in making accept/reject decisions about pancreatin API batches.

The potential zoonotic or non-zoonotic character of the virus should be taken into account when setting acceptance criteria.

## 6.3 Identification of Relevant Virus Panel Test

The risk assessment strategy described above requires the identification of potential viral contaminants of porcine-derived starting materials. *Table 1* gives an overview of enveloped and non-enveloped viruses known to be present in pigs that may present a contamination risk when using pigs deemed fit for human consumption as source animals. The risk assessment should address at least the viruses listed; however, depending on the origin of animals and the manufacturing process capability, the list may be adapted, and additional viruses may be considered (see the example in *Figure 2*). Manufacturers should implement systems to identify emergence of potentially relevant new viruses.

**Table 1. Hazard Identification: Viruses Known to be Present in Pigs**

| Enveloped Viruses | Non-Enveloped Viruses |
|---|---|
| Classical swine fever virus | Encephalomyocarditis virus (EMCV) |
| African swine fever virus (ASFV) | Swine vesicular disease virus (SVDV) |
| Influenza virus | Foot and mouth disease virus (FMDV) |
| West Nile virus (WNV) | Reoviruses (REO) |
| Vesicular stomatitis virus (VSV) | Swine hepatitis E virus (swHEV) |
| Eastern equine encephalitis virus (EEEV) | Porcine rotaviruses (pRotaV) |
| Rabies virus (RABV) | Porcine enteroviruses (PEVs) |
| Porcine reproductive and respiratory syndrome virus (PRRSV) | Porcine parvoviruses (PPV) |
| Transmissible gastroenteritis virus (TGEV) | Porcine circovirus types 1 and 2 (PCV1/2) |
| Pseudorabies virus (SuHV-1) | |
| Nipah virus | |
| Porcine endogenous retroviruses (PERVs) | |

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## 6.4 Virus Clearance by Manufacturing Process Steps

Demonstration of viral clearance is a critical component of ensuring the overall safety of pancreatin-derived products. The objective of virus-clearance evaluation studies is not only to evaluate the ability of the manufacturing process to clear known viral contaminants and to estimate quantitatively the overall level of virus reduction obtained by the manufacturing process but also to estimate the capability of the manufacturing process steps to clear viruses in general. Viral clearance studies for the manufacturing process of pancreatin should be performed in accordance with the applicable, current guideline, ICH Q5A(R1).

Current pancreatin production processes are considered to be effective for inactivating enveloped viruses, following the results of virus clearance studies. However, non-enveloped viruses are more resistant to physico-chemical inactivation, making their inactivation more variable. Examples of viruses that are classified as having a high viral resistance toward treatment and that show moderate to limited inactivation include PPV and PCV1/2 (see *Figure 2*).

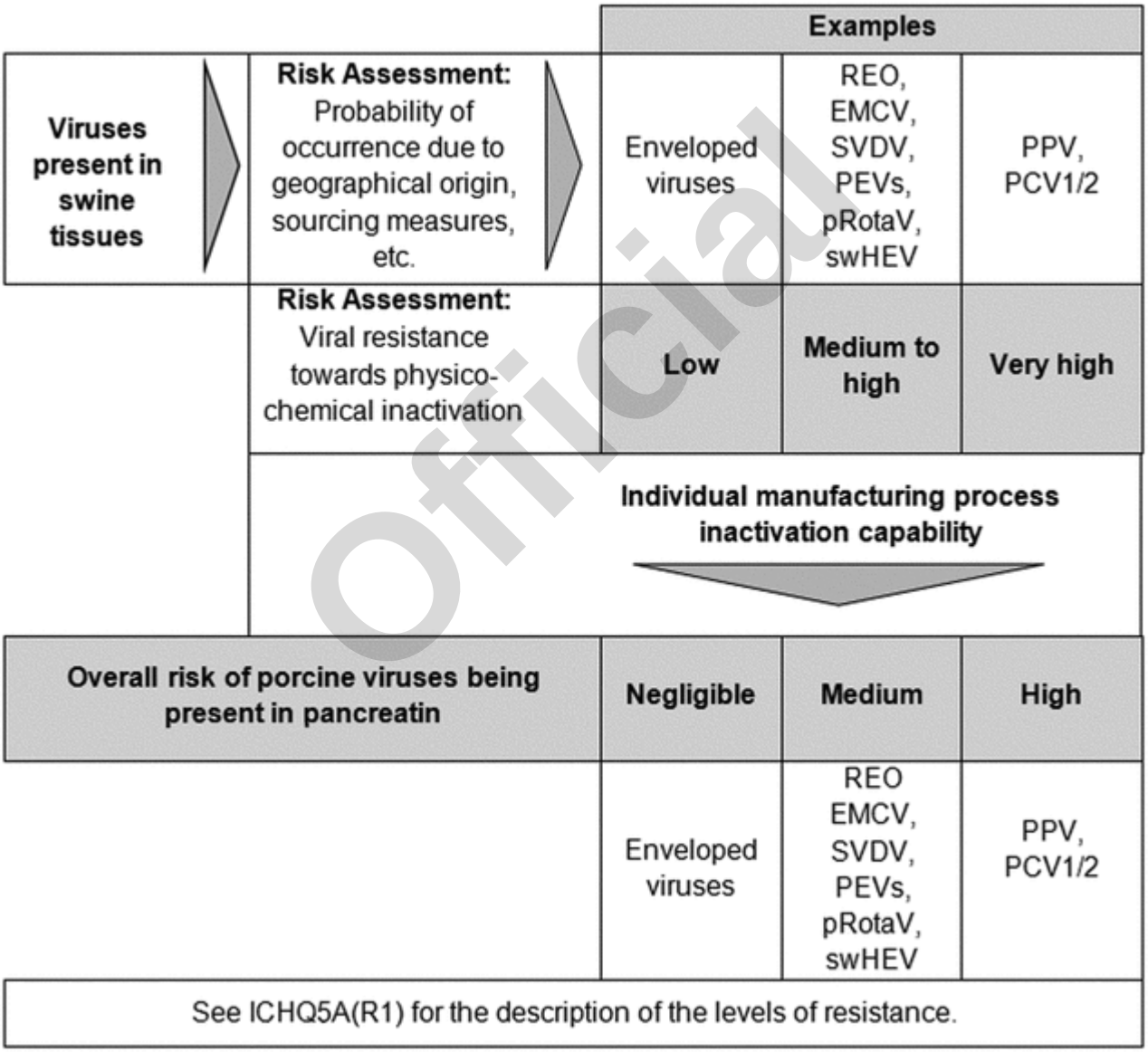


Figure 2. Example of a risk-assessment approach to virus identification for the test panel.

## 6.5 Viral Testing

A testing strategy is needed when the ability of the process to remove or inactivate a specific virus to appropriate levels has not been demonstrated. The potential zoonotic or non-zoonotic character of the virus should be taken into account when setting acceptance criteria, in terms of both assay sensitivity and specification limit setting. API batches that test positive for zoonotic viruses should be rejected. As a part of quality control, viral testing should be performed on each lot of API. The tests used should allow exclusion of any detectable load of potentially harmful levels of adventitious agent. The 9 CFR describes

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testing requirements for porcine biological products including live virus vaccines and antibody products but does not specifically address pancreatin. Applying the principles of 9 CFR, assays to detect porcine viruses can, for example, be based on cell culture monitoring for cytopathogenic effects for an appropriate incubation time, hemadsorption testing, virus-specific staining techniques, or appropriate combinations thereof (also see chapter ⟨1237⟩ *Virology Test Methods*). In addition to the technologies and viruses covered by 9 CFR, new molecular biology-based technologies may be used, and other viruses with zoonotic potential that are identified may require testing. Examples of specific viruses not covered by the current virus testing guide may include swHEV.

The definition of the test virus panel, selection of the appropriate process stage, suitability of the test method, and test method sensitivity should be justified by the manufacturer. All tests for specific viruses should be developed and validated in compliance with current guidance, for example, chapters ⟨1225⟩ *Validation of Compendial Procedures* and ⟨1033⟩ *Biological Assay Validation*, as applicable, and acceptance criteria should be established and used for accept/reject decisions of pancreatin API batches.

# 7. CHARACTERIZATION OF PANCREATIN

## 7.1 Description and Physico-Chemical Properties

Pancreatin is a slightly brownish to tan amorphous powder with a raw meat odor and taste. Pancreatin is partly soluble in water, forming a weak turbid solution, and insoluble in alcohol and ether. The following conditions may degrade pancreatin: mineral acids, alkali hydroxides, oxidizing agents, many metallic salts, and high humidity and temperatures.
Pancreatin-containing solutions should be filtered with caution, due to potential retention of the lipase and proteases on the filter. The enzymatic activity reaches a maximum in neutral-to-weakly alkaline solutions. The activity decreases quickly in acidic or strong alkaline solutions. The same applies to boiling of pancreatin-containing aqueous solutions. In non-enteric-coated formulations, exposing the product at a pH of 4.5 or less is not recommended, because a nearly complete loss of lipolytic activity has been observed.

Because pancreatin is of biological origin, other components are present in addition to enzymatically active proteins. These other components include proteins, amino acids, peptides, nucleic acids and fragments thereof, tissue components, fat, and inorganic substances. These components may have an impact on the quality of the final material.

## 7.2 Protein and Enzyme Contents

Pancreatin contains different digestive enzymes, most of which are produced and stored as zymogens (inactive precursors) in the pancreatic acinar cells. Under physiological conditions, pancreatic zymogens are transformed into active enzymes once the pancreatic secretion reaches the upper small intestine. An intestinal protease, enterokinase, triggers the activation process by cleaving the zymogen of trypsin, which further activates the other proteases. During the production process, the enzymes present in pancreatin are activated by trypsin (see *Figure 1*).

*Table 2* summarizes some of the important characteristics of the main pancreatic enzymes found in pancreatin.

**Table 2. Characteristics of Known Enzymes Present in Porcine Pancreatin**

| Name | E.C. Number | UniProtKB/ Swiss-Prot Accession Number | Substrates | Produced as a Precursor | Molecular Mass (kDa) | Sequence Length | Isoelectric Point |
|---|---|---|---|---|---|---|---|
| Trypsin | 3.4.21.4 | P00761.1 | Proteins | Yes | 23.8 (zymogen), 23.5 (activated) | 231 a.a. (zymogen), 223 a.a. (activated) | 9.3 (zymogen), 10.5 (activated) |
| Chymotrypsin | 3.4.21.1 | G1ARD6_PIG | Proteins | Yes | 29.1 (zymogen), 25.6 (activated) | 268 a.a. (zymogen), 221 a.a. (activated) | 8.7 |
| Elastase | 3.4.21.36 | P00772.1 | Proteins | Yes | 25.9 (activated) | 250 a.a. (zymogen), 240 a.a. (activated) | 8.5 |
| Carboxypeptidase A1 | 3.4.17.1 | P09954 | Proteins | Yes | 34.7 (activated) | 308 a.a. (activated) | Not known |
| Carboxypeptidase B | 3.4.17.2 | P09955.5 | Proteins | Yes | 34.7 (activated) | 305 a.a. (activated) | 6.0 |
| Kallikrein, glandular | 3.4.21.35 | P00752.4 | Proteins | Yes | 25–28 kDa | 246 a.a. (zymogen), 239 a.a. (activated) | 4.2–4.3 |
| Triacylglycerol lipase | 3.1.1.3 | P00591.2 | Triglycerides, diglycerides | No | 50.1 (two glycosylation isoforms, lipases A and B) | 450 a.a. (mature) | 4.9 (lipase A), 5.0 (lipase B) |
| Colipase | | P02703.3 | No enzyme activity, cofactor of pancreatic lipase | Yes | 10.3 (porcine procolipase A), 10.1 (porcine procolipase B) | 93 a.a. (procolipase A), 95 a.a. (procolipase B) | Not known |

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**Table 2. Characteristics of Known Enzymes Present in Porcine Pancreatin** *(continued)*

| Name | E.C. Number | UniProtKB/ Swiss-Prot Accession Number | Substrates | Produced as a Precursor | Molecular Mass (kDa) | Sequence Length | Isoelectric Point |
|---|---|---|---|---|---|---|---|
| Phospholipase A2 | 3.1.1.4 | P00592 | Phospholipids | Yes | 14.7 (zymogen), 14 (activated) | 146 a.a. (zymogen), 123 a.a. (activated) | 4.4–4.5 |
| Cholesterol esterase, also named carboxyl ester lipase (CEL), carboxyl ester hydrolase (CEH) or bile-salt stimulated lipase (BSSL) | 3.1.1.13, 3.1.1.1 | Complete amino acid sequence still unknown in pig | Cholesterol esters, vitamin esters, monoglycerides, phospholipids, galactolipids, some activity on triglycerides | No | Inconsistent values in the literature ranging from 65 to 98 kDa. While proteolytic forms have been identified, the exact mass is still unknown. | Complete amino acid composition still unknown in pig | 4.2–4.8 |
| α-Amylase | 3.2.1.1 | P00690.3 | Polysaccharides | No | 55.3 | 496 a.a. (mature) | 5.95 (amylase I), 5.45 (amylase II) |

### 7.2.1 PANCREATIC PROTEASES

Proteases are enzymes that digest proteins into smaller peptide fragments and amino acids by hydrolyzing the peptide bonds. Pancreatin contains five major proteases: trypsin, elastase, chymotrypsin, carboxypeptidase A1, and carboxypeptidase B. Trypsin, chymotrypsin, and elastase are classified as both serine proteases and endopeptidases because they cleave peptide bonds at the C-terminal side of an amino acid and also have a catalytically important serine residue in their active sites. Carboxypeptidases catalyze hydrolysis of the amino acids from the C-terminal end position in polypeptides and thus are classified as exopeptidases. Carboxypeptidases sequentially release residues from the C-terminus of proteins and peptides with a well-defined specificity.

Trypsin acts specifically on the C-terminal side of the positively charged amino acid residues lysine and arginine. Trypsinogen is secreted by the pancreas as an inactive precursor and discharged into the duodenum where enterokinase converts trypsinogen to active trypsin. Enterokinase is secreted in the duodenum by cells of the duodenal mucosa. Enterokinase removes a terminal octapeptide from trypsinogen and yields a polypeptide chain of active trypsin cross-linked by six disulfide bridges. Trypsin contains one high-affinity calcium binding site that is required for enzyme stability.

Chymotrypsin acts preferentially on the C-terminal side of tyrosine, phenylalanine, and tryptophan residues. It is secreted as an inactive zymogen, chymotrypsinogen, which undergoes proteolytic processing by trypsin to form the active enzyme. Chymotrypsin binds one calcium ion per molecule.

Elastase acts on small, neutral amino acid residues, such as glycine and alanine, but also hydrolyses amides and esters and is distinctive in that it acts upon elastin. Elastase is produced as a zymogen and the activated form contains four disulfide bridges. Elastase binds one calcium ion per molecule.

Carboxypeptidase A1 is an exopeptidase hydrolyzing the peptide bond adjacent to the C-terminal end of a polypeptide chain, thus releasing the C-terminal amino acid. It cleaves aromatic and bulky aliphatic amino acid residues and shows little or no action with aspartic acid, glutamic acid, arginine, lysine, and proline amino acid residues. It contains one zinc ion per molecule. The zinc ion is essential for activity; if removed during dialysis it must be replaced. Thus, carboxypeptidase A1 is also classified as a metalloprotease.

Carboxypeptidase B catalyzes the hydrolysis of the basic amino acids lysine, arginine, and ornithine from the C-terminal end of a polypeptide chain. It may also have a function in the further degradation of products of tryptic digestion. The enzyme binds one zinc ion per molecule, which is a necessary functional part of the enzyme for activity, and thus carboxypeptidase B is also classified as a metalloprotease.

### 7.2.2 PANCREATIC LIPASE AND COLIPASE

Pancreatic lipase (PL, also known as triacylglycerol acyl hydrolase) is a glycoprotein and produced directly as an active enzyme by the pancreas. Two glycosylation isoforms of PL, lipase A and lipase B, are present in pancreatin. These two isoforms have identical amino acid compositions but differ slightly in their glycosylation patterns, with lipase A as more acidic than lipase B. PL is a water-soluble enzyme that acts on insoluble lipid substrates, triglycerides, at the lipid–water interface. Its activity is dependent on the substrate-specific surface accessible to the enzyme, and it increases with the state of emulsification of the lipids. PL preferentially hydrolyzes ester bonds at the C-1 and C-3 positions of triglycerides and exhibits a broad spectrum of fatty acid chain length specificity. Therefore, PL is active against a wide variety of the triglycerides that are typically present in the diet. It also acts on diglycerides, but its activity on monoglycerides is very weak. It mainly converts triglycerides into monoglycerides and free fatty acids; the more polar lipolysis products are absorbed in the small intestine. In the presence of various amphiphiles such as bile salts at micellar concentrations, the PL adsorption at the lipid–water interface can be hindered, thus decreasing lipolytic activity.

A small protein cofactor also produced by the pancreas, colipase, helps PL to anchor to interfaces in the presence of competitive amphiphiles, and thus restores PL activity. Colipase is also produced by the pancreas as a precursor, procolipase. Procolipase is activated by trypsin.

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#### 7.2.3 PANCREATIC PHOSPHOLIPASE A2

Phospholipase A2 (also known as type IB secretory PLA2) is a heat-stable, water-soluble enzyme that catalyzes the calcium-dependent hydrolysis of the 2-acyl groups in 3-sn-phosphoglycerides. It is produced as a precursor that is activated by trypsin.

#### 7.2.4 PANCREATIC AMYLASE

Porcine pancreatic α-amylase (1,4-α-D-glucan glucanohydrolase) catalyzes the hydrolysis of internal 1,4-α-D glucosidic linkages in polysaccharides containing three or more 1,4-α-linked D-glucose units to yield a mixture of dextrins, maltose, and glucose. Amylase exists in two forms (I and II) that have similar enzymatic properties but that differ in their isoelectric points. Both molecular forms of amylase are glycoproteins that contain fucose, galactose, mannose, and different contents of glucosamine. Both amylases consist of a single polypeptide chain with four disulfide bridges and contain a tightly bound calcium ion.

## 8. ENZYME ACTIVITY MEASUREMENTS

Although pancreatin contains a variety of enzymes, it is usually characterized by measuring the activities of the three main enzyme classes, lipase, protease, and amylase.

### 8.1 Lipase Activity

The lipase activity of pancreatin on triglycerides with long-chain fatty acids is mainly due to PL, which requires the presence of its specific cofactor, colipase, to act on triglyceride emulsions in the presence of bile salts that are competitors for lipase adsorption at the surface of lipid droplets. Colipase and lipase form an active complex with a 1-to-1 stoichiometry. Commercial preparations of pancreatin usually contain colipase; however, it is recommended to check that the API manufacturing process provides enough colipase for the lipase activity. This control should be part of a proper characterization to be performed during process validation of the API.

Besides PL, pancreatin also contains pancreatic carboxyl ester lipase (CEL), which hydrolyzes several lipid substrates. CEL activity on triglycerides is usually considered very low, compared with that of PL, and its contribution to pancreatin lipase activity is negligible, as measured by the USP lipase assay.

The lipase assay described in the USP *Pancreatin* monograph is based on the enzyme's rate of digestion of olive oil emulsified with acacia (also known as gum arabic). The activity of the test sample is calculated by comparing it to a standard preparation of enzyme with known activity. Olive oil contains triglycerides with long-chain fatty acids that are representative of dietary triglycerides. Lipase activity is measured on the basis of the titration of the free fatty acids released from olive oil upon lipolysis by the lipase present in pancreatin. This titration by sodium hydroxide is performed at a constant pH of 9.0 by pH- or potentiostatic titration, at which long-chain fatty acids are totally ionized. It is worth noting that this pH value does not correspond to physiological conditions (the mean pH of the small intestine contents is close to 6.0 during a meal), but it allows measurement of optimum lipase activity *in vitro*. Because the USP lipase assay solution contains USP Bile Salts Reference Standard (RS), the detection of enzyme activity requires that both lipase and colipase are present in pancreatin. One USP Unit of lipase is defined as the amount of enzyme that, under the defined conditions with the defined substrate, liberates 1 µmole of fatty acid per minute.

Other lipase assays are available for monitoring lipase activity in pancreatin. When using lipase assays with non-natural substrates, such as tributyrin, it is recommended to confirm that the pancreatin sample tested is also active on long-chain triglycerides, particularly using the USP lipase assay with olive oil–acacia emulsion.

### 8.2 Protease Activity

The proteolytic activity of pancreatin on polypeptides and proteins is due to the inherent enzymes trypsin, chymotrypsin, elastase, carboxypeptidase A1, and carboxypeptidase B. The two major pancreatic proteases are trypsin and chymotrypsin, and along with elastase, these endopeptidases generate small polypeptides from larger proteins. The further action of the exopeptidases carboxypeptidase A1 and B leads to single amino acids during digestion.

The USP spectrophotometric assay for protease activity from pancreatin is based on the enzyme's rate of digestion of casein under test conditions. The activity of the test sample is calculated by comparing it to a standard preparation of enzyme with known activity. Casein is composed of α (s1) and α (s2)-caseins, β-casein, and κ-casein, which are phosphorylated on serine residues and lack disulfide bridges. The conformation of casein is similar to that of denatured globular proteins with little or no tertiary structure. It is recommended that users evaluate suppliers of the casein substrate for consistency of dispersion.

The hydrolysis of casein by pancreatic proteases generates single amino acids and small peptides, and their release can be quantified by measuring their absorption at 280 nm. Before this measurement, non-hydrolyzed proteins and large peptides have to be separated by a selective precipitation with trichloroacetic acid, followed by filtration. The filtrate is then used for the spectrophotometric assay of protease activity, using tyrosine as a calibrant. One USP Unit of protease activity is contained in the amount of pancreatin that hydrolyzes casein at an initial rate such that the amount of peptides liberated per minute and not precipitated by trichloroacetic acid gives the same absorbance at 280 nm as 15 nmol of tyrosine.

Other assays are available for monitoring the activity of individual and total proteases in pancreatin, and the unit assignments are specific for each substrate. *N*-Acetyl-L-tyrosine ethyl ester is commonly used for both titrimetric and spectrophotometric (λ = 237 nm) assays of chymotrypsin activity. Similarly, *N*-benzoyl-L-arginine ethyl ester is commonly used for both titrimetric and spectrophotometric (λ = 253 nm) assays of trypsin activity. In both cases, the titrimetric assay is based on ester hydrolysis by proteases and the release of acid groups, whereas the spectrophotometric assay is based on the chromogenic properties of

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these acids. Toluene-sulfonyl-L-arginine methyl ester is another chromogenic substrate (λ = 247 nm) used for measuring trypsin activity. These substrates are, however, not proteins, and the assays involve the cleavage of a carboxylic ester bond instead of a peptide bond.

Other assays using chromogenic peptides as substrates have been developed for improving specificity and sensitivity. More specific and sensitive protease assays have been developed using short synthetic peptide substrates (3–5 amino acid residues) with a chromogenic group (4-nitroaniline) coupled to the C-terminal end by an amide bond. The chromogenic group is specifically removed by proteases and is measured photometrically. The change in the absorbance at 405 nm is directly proportional to the protease activity. Specific substrates are commercially available for trypsin (carbobenzoxy-valyl-glycyl-arginine-4-nitril-anilide acetate) and chymotrypsin (methyl-*O*-succinoyl-arginyl-prolyl-tyrosine-4-nitril-anilide chlorhydrate). Fluorescein isothiocyanate (FITC)-labeled casein is also used as a general protease substrate. The assay is based on the quenching of the fluorescein label bound to casein. When FITC-casein is digested into smaller peptides by proteases, fluorescence at 530 nm (excitation at 485 nm) is increased and can be measured to determine protease activity.

### 8.3 Amylase Activity

The glycolytic activity of pancreatin is due to α-type amylases. Such enzymes catalyze the hydrolysis on internal α-1,4-glucan links in polysaccharides containing three or more α-1,4-linked D-glucose units, yielding a mixture of maltose and glucose. The two major isoforms (I and II) of porcine amylases have identical enzymatic properties. In the last decades, several methods have been developed for assaying amylase activity; many of these are based on the detection of starch hydrolysis, as this polymer is the natural amylase substrate. Starch consists of two types of molecules, amylose (usually 20%–30%) and amylopectin (usually 70%–80%). Both polymers result from the assembly of glucose units connected via α-1,4-glucan links; in addition, in amylopectin, about 1 residue in every 20 or so, is also linked α-(1→6), forming branch-points.

The USP Amylase assay in the *Pancreatin* monograph is based on the enzyme's rate of digestion of starch, and the activity of the test sample is calculated by comparing it to a standard preparation of enzyme with known activity. Starch is hydrolyzed by amylase; the reducing groups resulting from the hydrolysis react with iodine in alkaline solution; and the excess iodine is titrated with thiosulphate. One USP Unit of amylase activity is defined as the amount of pancreatin that decomposes starch at an initial rate such that 0.16 µEq of glycosidic linkage is hydrolyzed per minute under the conditions of the assay.

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# ⟨1027⟩ FLOW CYTOMETRY

## INTRODUCTION

Flow cytometry is an analytical method that plays a critical role in the quantitative and qualitative assessment of cell populations in patient and cellular product samples. The power of flow cytometry lies in its ability to rapidly and reliably analyze multiple attributes of individual cells within heterogeneous cell populations. Despite the value of flow cytometry data, method validation is challenging—perhaps more so than for other analytical methods—because of errors and artifacts from multiple sources.

Although flow cytometric methods can also be used to sort and isolate cells as part of the manufacturing process for cell- and tissue-based biological products, the scope of this chapter is limited to the use of flow cytometry as an analytical method. This chapter presents the technical aspects of the method, including instrumentation, sample handling and staining, and data analysis. Sources of error are considered in the context of technical features, as well as in the discussion of quality control, quality assurance, and standardization. Finally, current applications and assay troubleshooting principles are presented. For additional information on the basics and practical aspects of flow cytometry, see the current edition of *Practical Flow Cytometry* (Shapiro, 2003).

Flow cytometry is widely used to characterize cell and tissue-based products, but most assay methods are not yet standardized. In addition to issues related to technical complexity, there are also challenges to standardization of flow cytometric methods for specific product classes or types related to the heterogeneous nature of these products, even among those with similar manufacturing processes and clinical uses. Current and future innovations in instrumentation, analytic reagents, analytic algorithms, and automation are likely to improve the technology's capabilities but are unlikely to eliminate challenges (e.g., bioassay, identification tests, and other applications).

## PRINCIPLES OF OPERATION, METHODS, QUALITY, AND STANDARDIZATION

The process of flow cytometry requires that cells move past a fixed light source consisting of one or more lasers so that individual cells can be observed or interrogated for characteristics such as size, granularity, and presence of surface membrane or intracellular antigens or molecules. The cells are suspended in fluid in which movement is controlled by the size and configuration of tubing, chambers, and pumps specific to the flow cytometry instrument. The pattern of light signals produced from the laser light's interaction with the cells is captured by a detection system, also specific to the instrument, and the detected signals are transformed into data elements that can be analyzed and combined with data from other cells in a given sample. Data from a cell suspension can then be expressed and presented in one-, two-, or three-dimensional visual formats, or in numerical formats, to characterize the cellular sample and its subpopulations both qualitatively and quantitatively.

### Flow Cytometry Instrumentation

Flow cytometers, which incorporate fluidic, optical, and electronic signal processing elements, are described below.

#### FLUIDICS

The fluidics system moves a bulk mixture of cells so that a stream of single cells is formed. Within the flow cytometer, the single-cell suspension passes through a confined region where each cell is sequentially illuminated by a uniform light source at the observation point (interrogation point). Most instruments use a flow chamber (flow cell) that, after the cell sample is drawn into the sample injection tip, combines the cells with isotonic sheath fluid, using a conical nozzle assembly that is geometrically designed to produce a laminar flow of fluid (*Figure 1*). The fluid outlet nozzle typically has an orifice of 50–250 µm in diameter through which fluid exits at a high flow rate. Differential pressures between the sample stream of cells (lower pressure) and the sheath stream (higher pressure) draw the cells/particles out into a confined stream. The resulting coaxial stream within a stream is highly efficient, and the sample stream at the observation point is typically only slightly larger than the cells or particles contained within. At least one manufacturer uses an alternative approach in which the coaxial stream strategy is replaced by the use of microcapillaries to focus and direct the cells. The light signal deflected or emitted by the cell is then measured and analyzed.

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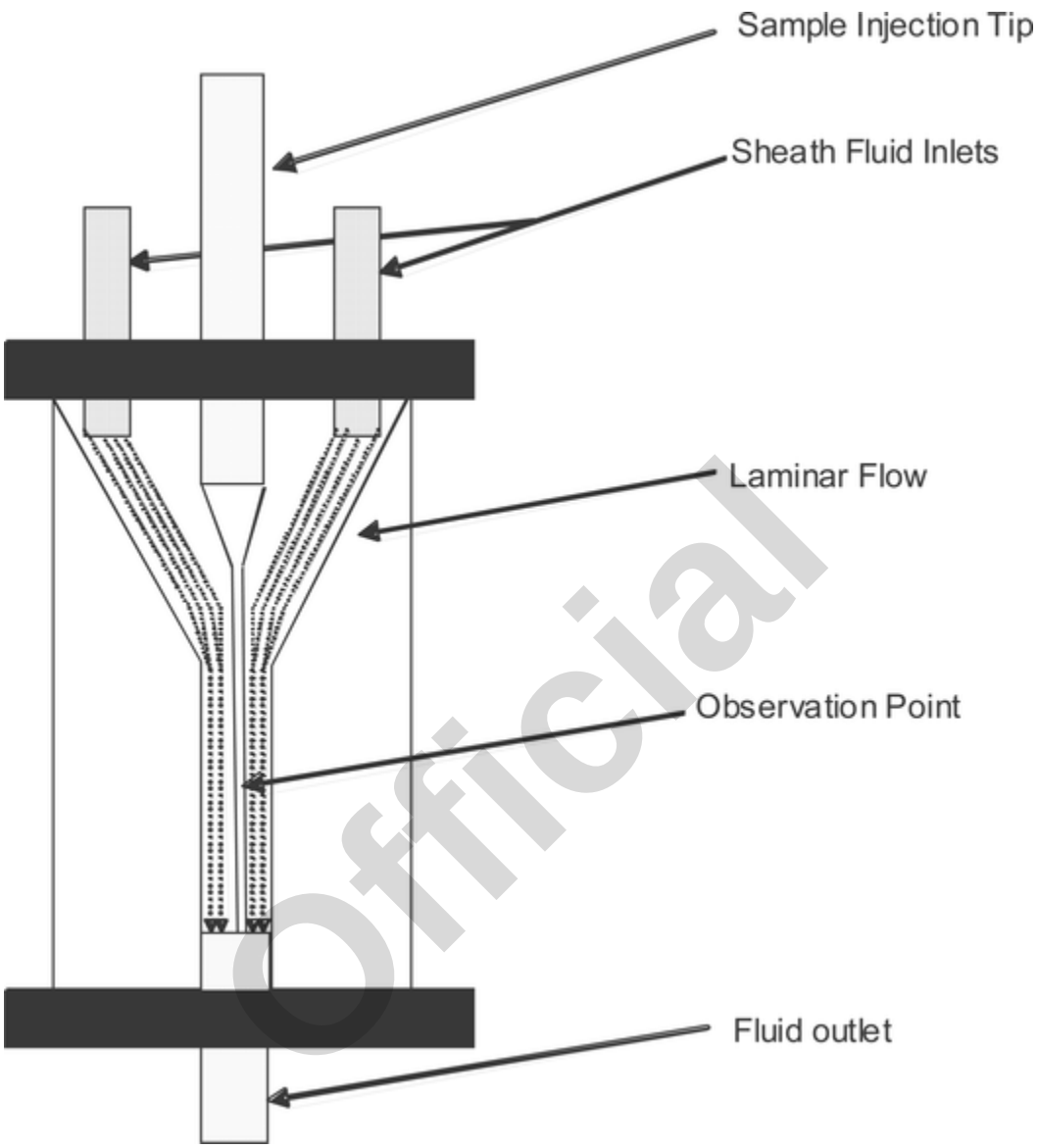


Figure 1. Schematic diagram of a flow cell.

## OPTICS

When cells are stained with fluorescent dyes or with fluorescent-labeled antibody reagents, light emitted from the laser interacts with the fluorescent dye to produce a stimulated emission that has coherent (parallel) waves of light of uniform wavelength, phase, and polarization. Fluorescent light signals generated from the interaction of the laser light with the cells are collected by an array of detectors oriented in direct line with, and at 90° to, the incoming laser beam. The most common commercially available flow cytometer lasers (with corresponding wavelengths) are the blue argon laser (488 nm), the red diode laser (635 nm), and the violet laser (405 nm).

## ELECTRONIC SIGNAL PROCESSING AND DATA OUTPUT

When a cell passes through the optical system of a flow cytometer, the light-scattering patterns or fluorescence from any fluorochrome on or in the cell are detected by various types of photodetectors or photomultiplier tubes (PMT) that transform the information about the characteristics of the cell into a computerized readout. Each analyzed cell generates an event in each parameter (forward scatter, side scatter, fluorescence) for which it is measured. *Figure 2* shows an example of a typical two-color flow cytometer configuration. Different cell types have distinctive sets of signals in the various parameters. For example, when the cell passes through a beam of light, the light deflected in the forward direction (usually about 20° from the forward direction of the laser) is called forward scatter and is collected by a detector known as the forward scatter channel (FSC). The amount of deflection in the FSC is proportional to cell size. Light deflected at a 90° angle is known as side scatter and is collected by the side scatter channel (SSC). This provides a measure of the cell's structural complexity caused by granules, membrane roughness, or nucleus, all of which are associated with higher SSC. The light deflected by other PMTs using a specific band-pass filter is

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collected by specific fluorescence channels (FL1 and FL2 in *Figure 2*). The electrical pulses, originating from light detected by the PMTs, are processed by a series of linear and log amplifiers. Logarithmic amplification is often used to measure fluorescence in cells. *Figures 3–7* show histograms for cells stained with antibodies conjugated with specific fluorochromes (see *Table 1*). The antibodies are specific to some of the cluster of differentiation (CD) markers discussed in *Immunophenotyping* (see below).

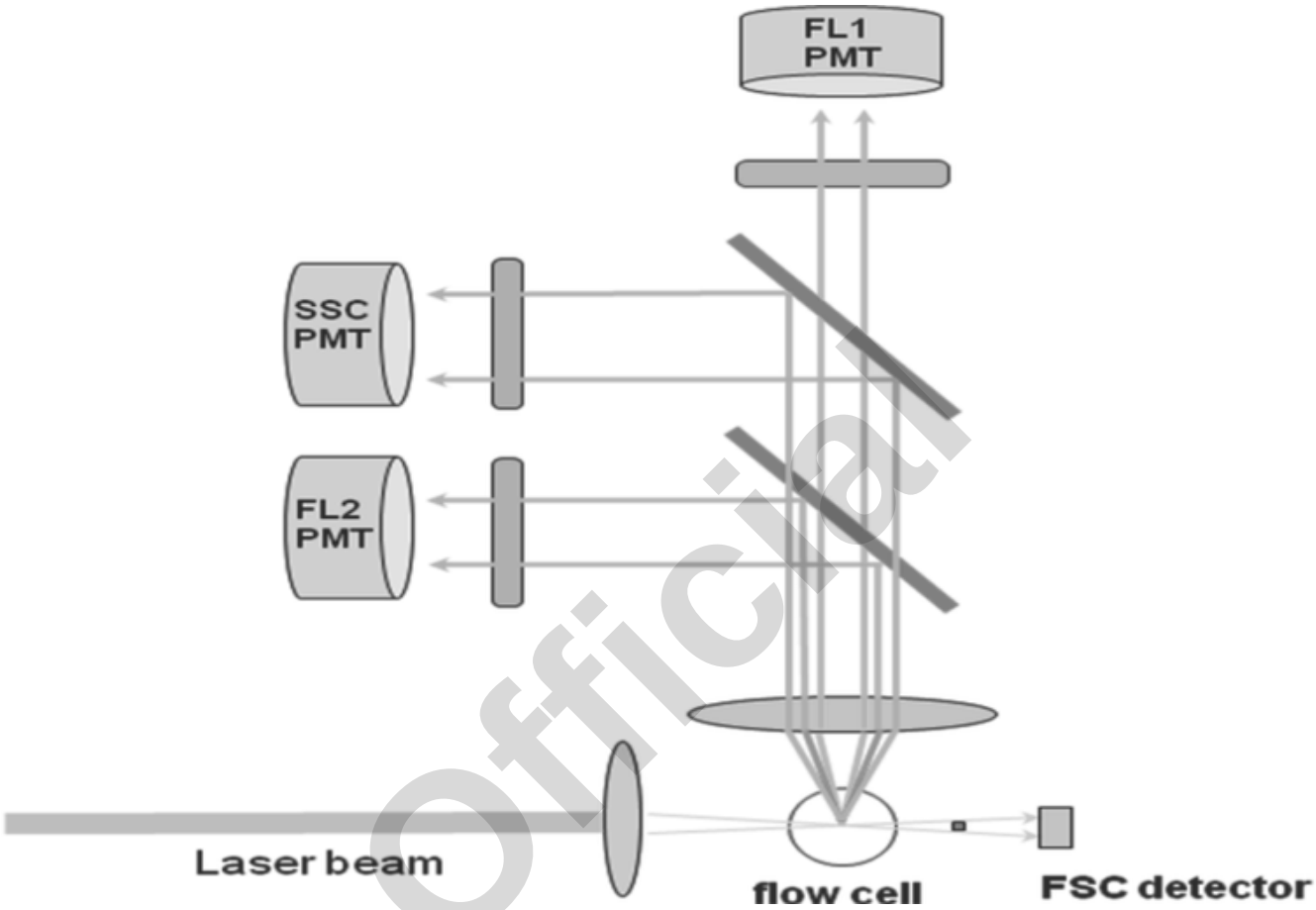


Figure 2. Typical 2-color flow cytometer with detectors for FSC, SCC, and fluorescence.

**Table 1. Fluorochromes Commonly Used in Flow Cytometry**

| Fluorochrome | Typical Excitation Laser (nm) | Emission Peak (nm) |
|---|---|---|
| Cascade Blue | 375; 401 | 423 |
| Pacific Blue | 403 | 455 |
| R-Phycoerythrin (R-PE) | 480; 565 | 578 |
| PE-Cy5 conjugates | 480; 565; 650 | 670 |
| PE-Cy7 conjugates | 480; 565; 743 | 767 |
| Red 613 (Texas Red) | 480; 565 | 613 |
| Peridinin Chlorophyll (PerCP) | 490 | 675 |
| Fluorescein (FITC) | 495 | 519 |
| Allophycocyanin (APC) | 650 | 660 |
| APC-Cy7 conjugates | 650; 755 | 767 |

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The versatility of flow cytometry comes from the ability to attach fluorescent tags to the cell's surface, cytoplasm, or nucleus or to products of the cell. Fluorescent markers attached to the cell can be excited by lasers to emit light of specific wavelengths, and this light is then detected and analyzed in the manner described above. The type and amount of fluorescence detected provide both quantitative and qualitative information about the cell.

The photodetectors convert light into an analyzable output by generating a small current of which the voltage has amplitude proportional to the amount of light. The voltage is amplified and converted into electrical signals large enough to be plotted by the computer in several different ways. Thus, the FSC, SSC, and fluorescent detectors collect the light and convert it into electrical signals that can be analyzed by the computer. In this way, the signals coming from each photodetector can be measured for their intensity (low to high) and sorted into channels. The channels are arranged as a continuum so that a cell population with many large cells will have many events in the higher channels, and one with many small cells will have many events in the lower channels.

## DATA ANALYSIS

Data output from the flow cytometer can be represented in several ways, the most basic of which is the single-parameter histogram (*Figure 3*), in which events with similar intensity of light (forward scatter, side scatter, or fluorescence) are collected in channels and then plotted. This plot demonstrates the number of cells with similar optical characteristics. *Figure 4* is an example of graphs that display two measurement parameters, one on the *x*-axis and one on the *y*-axis, and the cell count as a density (dot) plot or contour map. The parameters could be SSC, FSC, or fluorescence. These parameters can be collected in one channel.

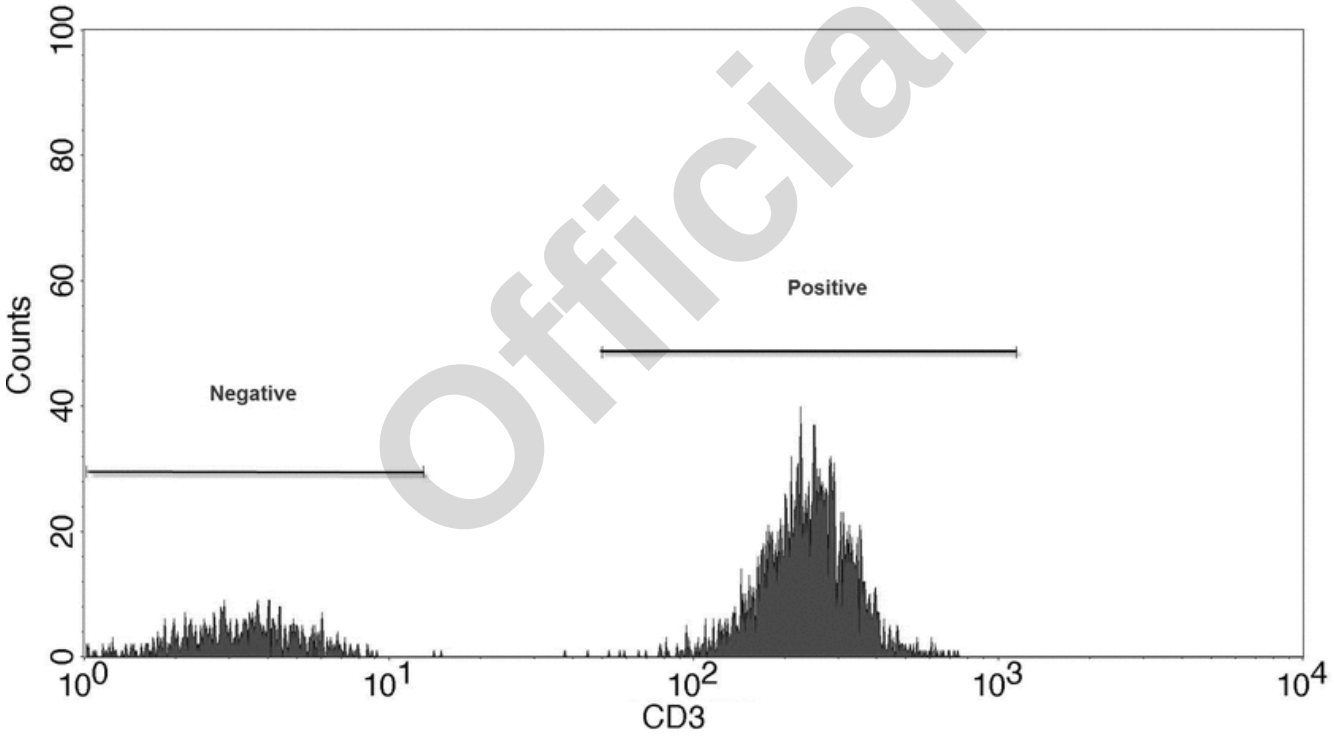


Figure 3. Single-parameter histogram showing expression of the cellular antigen CD3 in a mixture of cells.

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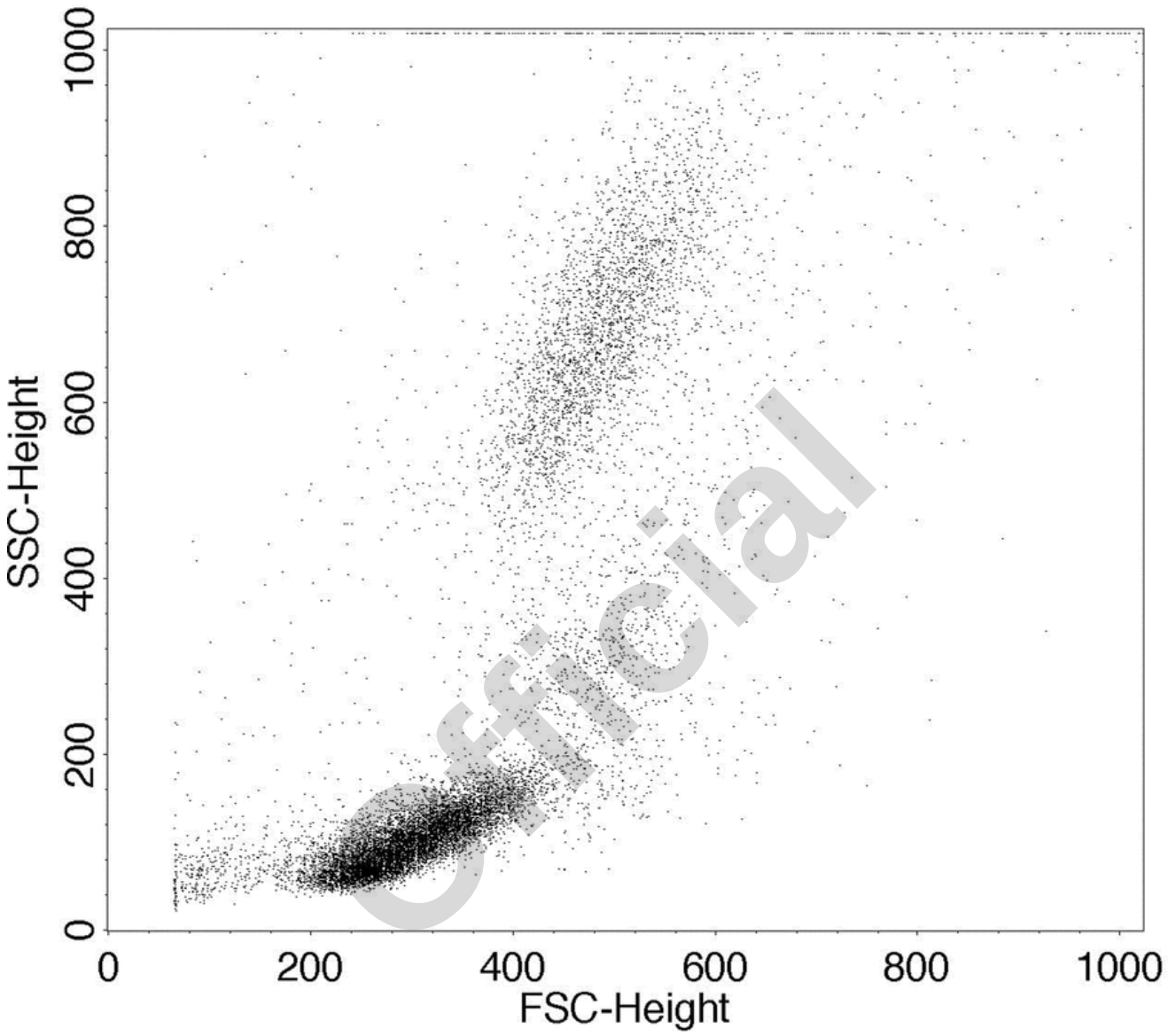


Figure 4. Bivariate dot plot of cells displayed by FSC and SSC.

A dot plot displays a dot for each cell, and density plots and contour plots show a heat map or a topographical linear map, respectively, based on the relative number of cells in each channel. The forward versus side scatter histogram is the most common method of identifying different hematopoietic cell types. *Figure 5* shows a contour plot that is a 3-dimensional representation of the relative number of cells in the various channels.

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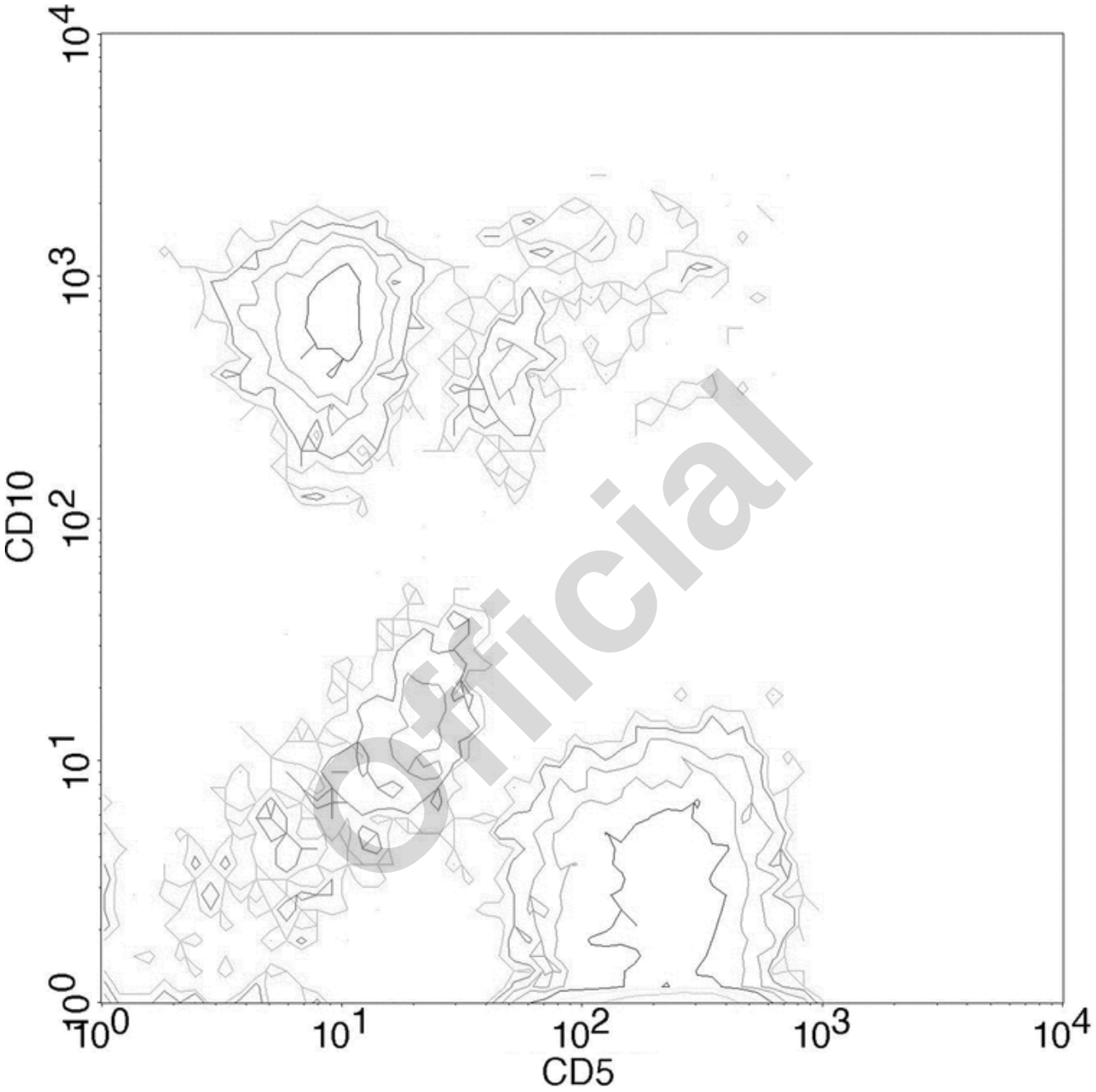


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Figure 5. Bivariate contour plot showing relative numbers of cells present in each channel that co-express 2 CD markers.

When cells are stained with antibodies for different epitopes carrying two different fluorochromes, the data are presented as a plot of the two parameters plotted against each other. Cursors can be set on each axis to separate positive populations from negative populations for each of the attributes. This results in a graphic representation of cells that are positive for both markers, negative for both markers, and positive for only one of the two markers (*Figure 6*).

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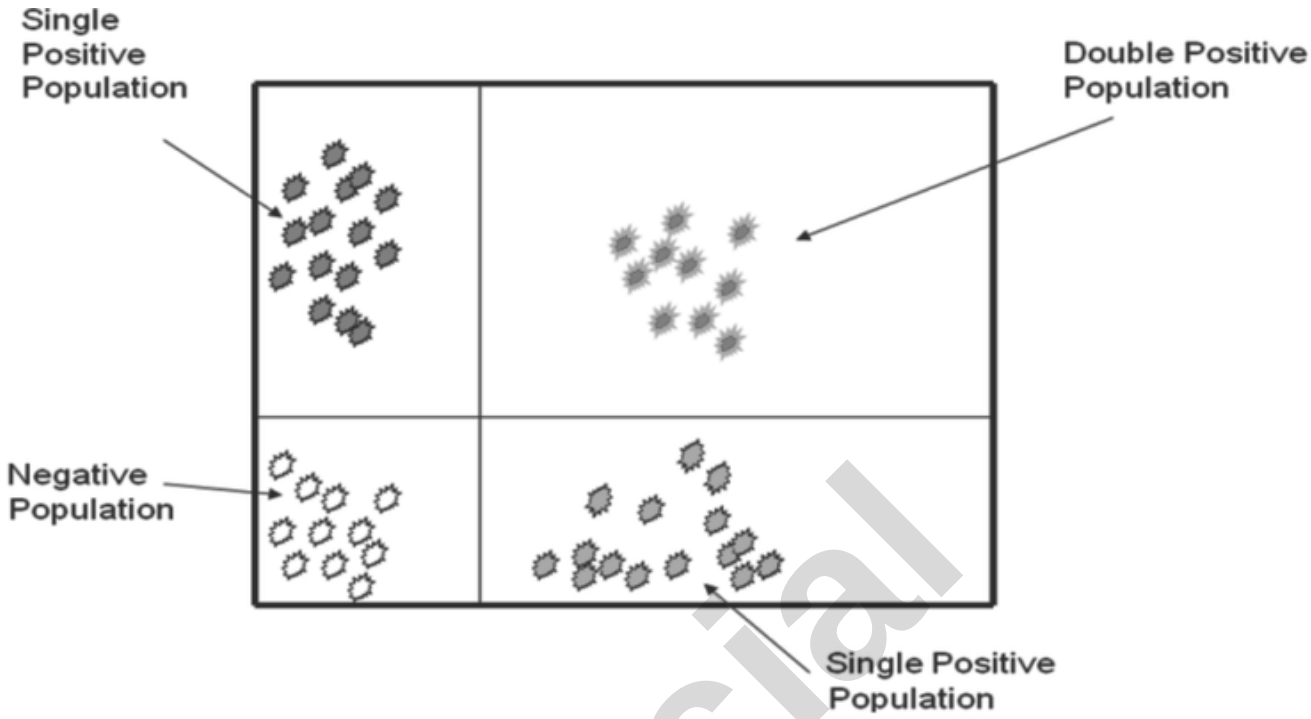


Figure 6. Schematic presentation of a 2-parameter histogram.

The flow cytometer allows the user to set the limits of positive and negative for each marker. Flow cytometric data are collected in list mode, where each electronic signal from a respective cell is displayed in the sequence in which it was acquired. List mode files can be edited at a later time to include or exclude any event. A basic advantage of flow cytometry is the ability to separate the data about the cells of interest from both the background and dead cells (e.g., noncellular particles or debris) when dealing with forward and side scatter and cells of other populations. The user must decide which signals are the actual light outputs from the cells and must construct an electronic gate to tell the computer to count as positive only the events that fall within the gate. Cell populations can vary widely depending on the tissue or cell source and the characteristics of the flow cytometer used. Gating allows the user to determine which outputs to consider actual events, so this process is of prime importance in standardizing flow cytometry data (*Figure 7*).

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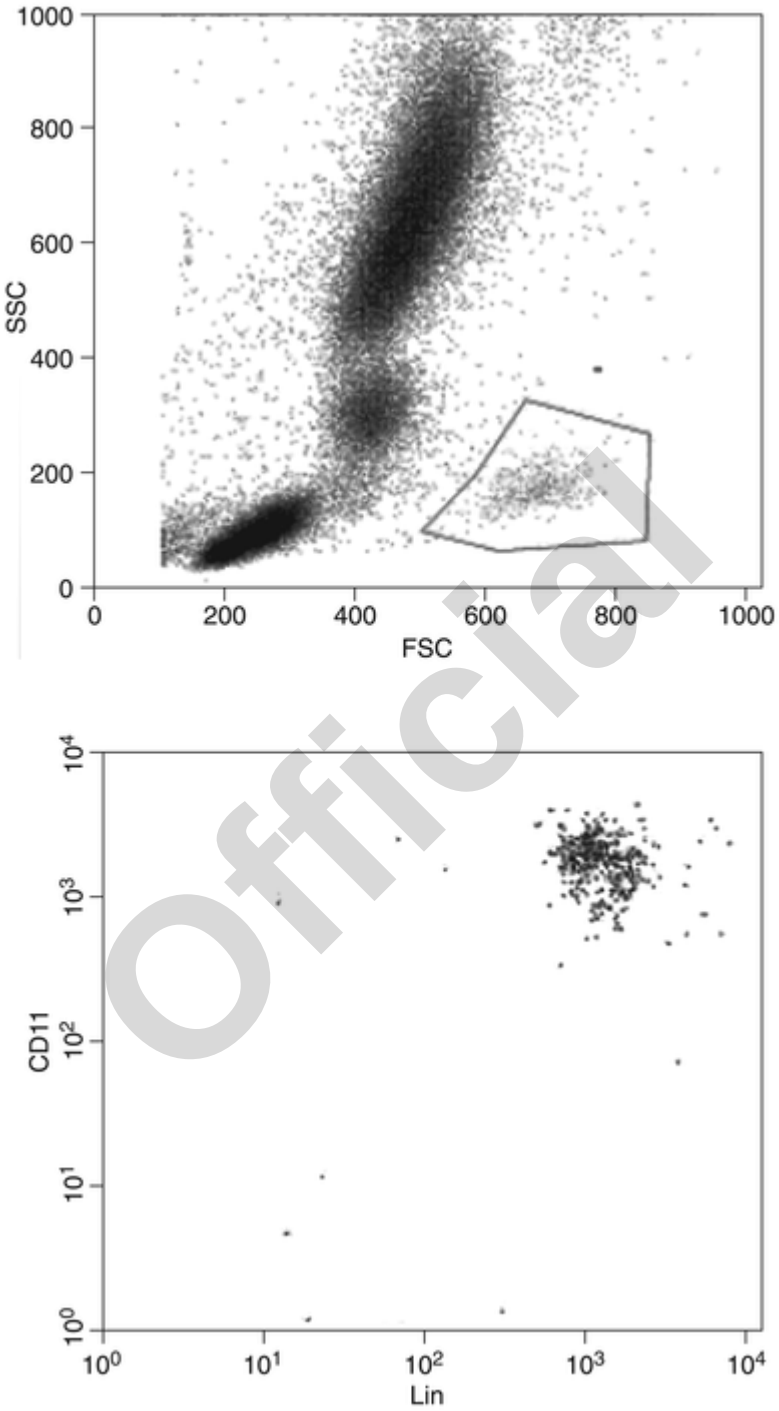


Figure 7. Gating of a cell population with low side scatter and high forward scatter, which is distinct from other cell populations in the sample.

A technique known as compensation can be used to separate spectral overlap of fluorochromes that have similar emission wavelengths. For analog-style flow cytometers manufactured before the late 1990s, compensation must be set before data acquisition. In modern digital instruments, compensation can be set either before or after data acquisition. The adjustment of compensation can be more of an art than a science, and considerable literature has been focused on the relative merits of various methods to determine the correct compensation for cell types or experimental conditions. The analyst should have considerable knowledge of the cell type under analysis in order to prevent errors in phenotyping that can result from improper adjustment of compensation.

The number of events counted should be adequate for statistical confidence in the results. The instrument can be set so that data are collected until a certain number of events in a channel have been measured. This feature allows the operator to vary the length of time or number of events from the sample so that statistically reliable data are generated. Thus, a sample that

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measures a rare event will analyze more total cells than one that measures a common event. Use of list mode files, the electronic data files that represent the most uncensored data, provides an advantage because these files can be further analyzed after data acquisition. Investigators need to ensure that all raw data, documentation, protocols, specimens, and final reports are archived at the close of the study. To ensure the integrity and quality of raw data collected, researchers need to abide by U.S. FDA Regulations for Good Laboratory Practices (GLP) as prescribed in 21 CFR Part 58, Good Laboratory Practice for Nonclinical Laboratory Studies; and Part 11, Electronic Records and Electronic Signatures.

## Flow Cytometry: Elements of a Procedure

Flow cytometric methods incorporate sample handling, preparation, and staining; instrument setup and operation; data collection, analysis, and storage; and associated quality control measures.

### SAMPLE HANDLING AND STAINING

**Sample Collection, Handling, and Anticoagulation**

In order to make accurate conclusions about the cell-based drug product, the analyst should ensure that samples from cellular products are as representative as possible of the whole product. Blood, apheresis samples, and cell suspension products should be well mixed before sampling, and care should be taken to obtain adequate sample volumes.

Cellular samples containing human blood/plasma must be anticoagulated. Citrate-based anticoagulants (e.g., Anticoagulant Citrate Dextrose Solution A) or heparin are recommended more highly than EDTA because they will optimally preserve samples being held for more than a few hours. For longer-term samples, specific transport/storage media may be required, and validation studies should be performed to ensure that those samples are equivalent to fresh samples at the time of flow cytometric analysis.

Samples intended for whole blood lysis and surface antigen staining should be transported and stored, preferably on a slow oscillating mixer, at room temperature. Fixed samples or live cell preparations should be stored at 4°. When the sample may be exposed to extreme temperatures, temperature-control materials (room temperature packs, wet ice/cold packs, and insulation) may be necessary, and validation studies should be performed to ensure sample integrity. For critical or high-value samples, temperature-monitoring devices may be needed during transport.

After acquisition, specimens should be analyzed as soon as possible. Special attention should be given to situations in which cellular proliferation and metabolic depletion of energy sources within the transport/storage media can lead to apoptosis. When accurate counting is not required or if infectious agents are suspected, a commercial lyse/fix solution can increase storage time and reduce the risk of disease transmission. For specimens separated by density-gradient centrifugation, storage in a solution of buffered paraformaldehyde (0.1% to 2.0%) is recommended after cell labeling has occurred.

**Sample Processing, Staining, and Fixation**

Reagents used in sample processing, staining, and fixation should be qualified for their intended use. Further guidance is available in ICH Q6B, *Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products*. When using reagent kits, follow the manufacturer's sample processing instructions.

Sample processing that involves centrifugation, washing, red blood cell (RBC) removal or lysis, or density-gradient separation is commonly done during many flow cytometric applications but can introduce error and artifact. Several techniques and reagents are available for RBC removal and lysis. Clinical grade [in vitro diagnostics (IVD) or analyte-specific reagents (ASR)] reagents are recommended for optimal quality, but artifacts can still occur. Density-gradient centrifugation can introduce error associated with variable cell losses among subpopulations that are being measured. These sources of error and artifact can be avoided by analyzing live whole blood whenever possible.

Most whole blood lysate instructions recommend staining at room temperature and in the dark. Many methods include a dilute fixative to prevent capping and internalization of fluorochrome. In contrast, cell preparations (density-gradient cell preparations, apheresis specimens, tissue culture) should be stained at 4°, washed with cold buffer, and stored cold until analyzed.

Fixation that also preserves cell surface antigens can be accomplished using commercial leukocyte preservatives or with buffered formaldehyde or paraformaldehyde. Very little validation of storage times, antibody binding, or fluorochrome intensity has been reported. Any laboratory that considers batch analysis of fixed specimens should validate these techniques thoroughly before implementing.

### USE AND CHOICE OF FLUOROCHROMES

**Fluorochromes**

Fluorochromes are used for direct staining of cells or as agents bound to antibody or other reagents to stain cellular antigens or other structures. *Table 1* lists examples of common fluorochromes used for flow cytometry and their excitation and emission wavelengths. Wavelengths (nm) may vary slightly depending on the environment. Synthetic probes from specific manufacturers are also available.[1] Fluorochromes must match the spectral range for the lasers and filter sets specific to the user's flow cytometer.

When choosing fluorochromes for multicolor phenotyping, the operator should refer to established methods for the particular instrument. In general, the brightest fluorochromes should be matched with the antigens that are expected to have the lowest expression on the cell surface. The brightness of tandem dyes can also be reduced by the use of certain fixatives, some of which are less problematic than others. When designing a multicolor flow and tandem dye procedure that has not been previously validated, the operator should consult the manufacturer's technical service, compare tandem dye/fixative combinations, and validate the final fluorochrome combination to ensure sample-to-sample consistency.

Tandem fluorescent dyes are dual-conjugated fluorescent molecules. When the two labels are in close proximity, energy produced by the laser exciting the donor fluor is transferred to the acceptor fluor, releasing a photon at the emission wavelength

[1] AlexaFluor series (InVitrogen/Molecular Probes) or the Cy series (GE Healthcare).

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of the acceptor fluor (also known as fluorescence resonance energy transfer, or FRET). For example, PECy5 will excite at the excitation wavelength for PE (565 nm), transfer energy to Cy5, and emit at the emission wavelength for Cy5 (670 nm).

**Fluorescently Labeled Antibodies**

Most commercially available antibody reagents are monoclonal, but polyclonal reagents may be available, and desirable, for some applications. The quality and specificity of an antibody can vary widely. Antibodies directed at a given antigen may differ in their binding specificity for different antigenic epitopes or in the strength of binding to the same epitope. If possible, use directly conjugated fluorochrome–antibody combinations that are IVD or ASR grade. Optimization of antibody concentration for the desired cell population is protocol specific but is generally accomplished by using increasing concentrations of antibody with a fixed number of cells to bracket the optimal brightness between autofluorescence and quenching. Quenching is caused by the prozone phenomenon, which occurs when excess antibody leads to immunoprecipitation and loss of fluorescence intensity. Further details are available in methods manuals such as *Current Protocols in Immunology* (Coligan et al. 1994).

**Cell Surface Antigen Staining**

Techniques for surface antigen staining vary with the type of specimen. Whole blood lysis techniques generally require surface labeling at room temperature in darkness for 15–30 min, followed by RBC lysis and, if desired, fixation. Published techniques use ammonium chloride ($NH_4Cl$) lysis of whole blood or marrow specimens followed by washing before antibody labeling for leukemia immunophenotyping. Mononuclear cell or cultured cell samples that are stained live should be kept at 4° or in an azide-containing buffer to prevent capping and internalization of the antibodies.

**Intracellular Staining**

Several standardized procedures also exist for labeling intracellular antigens and cytokines. The operator should consult the manufacturer's protocol and standardized reagents for these procedures. Because permeabilizing reagents vary among procedures and manufacturers, do not mix and match reagents. For cytokine labeling it is often necessary to use an activating step and a Golgi block to allow a sufficient amount of cytokine to accumulate for detection. If standardized reagents or procedures are not available from the manufacturer or if analysis of specialized functions is required, many common procedures and techniques can be found in sources such as *Current Protocols in Immunology* (Coligan et al., 1994).

**Quantitation of Antigens**

Some applications require quantitation of the average number and density of antigen molecules per cell in order to give a more complete picture of the immunological behavior of cells (e.g., in studies where extracellular antigens are expressed differentially in relation to activating stimuli). The intensity of an antibody/fluorochrome labeled cell preparation is compared to the intensity of a set of microbead fluorescence standards collected at the same PMT voltage settings. The standards are calibrated in molecules of soluble fluorescence (MESF) units, from which one can determine effective fluorescence to antibody (F/P) ratio, the number of antigen molecules per cell, and the density of available sites per cell.

## INSTRUMENT SETUP AND OPERATION

**Compensation**

Most instrument manufacturers supply software and test reagents (usually fluorescent beads) to set PMTs and compensation in order to target values found with the most common clinical tests (e.g., lymphocyte phenotyping, CD34 analysis). The operator should also use a biological control such as preserved blood or mononuclear cells, which are commercially available. Compensation must be set before acquisition on analog instruments. Digital instruments' PMTs must be set correctly because the values for these settings cannot be changed once the list mode file has been generated. When rare events are examined and/or intracellular dyes (e.g., 7-amino actinomycin D [7-AAD], propidium iodide [PI], Syto-16, etc.) are used in conjunction with fluorescently labeled antibodies, the balance of PMT voltage and spectral overlap must be closely monitored.

Autofluorescence (AF) is fluorescence above baseline in the absence of fluorochrome staining. This occurs in some cells, typically myeloid cells (especially alveolar macrophages) and cultured primary cells. If desired or necessary, AF can be measured directly on a fixed PMT voltage or can be calculated from a reference standard of fluorescent reference bead preparations (see *Quantitation of Antigens*, above). Avoid use of the 488 or 532 nm excitation wavelength and subsequent spectral compensation of the AF as an additional fluorochrome.

**Data Acquisition and Gating Strategies**

When possible, all events should be acquired in list mode, i.e., without selective gating of events. *Live gating*, defined as selective gating of events during acquisition, should be employed only when the desired subset is sufficiently rare that >2 million total events must be analyzed in order to count a significant (100 or greater) number of events of the desired population. List mode data can be acquired uncompensated when digital instrumentation is used, but most operators find that analysis is much less difficult and time-consuming if the data are in the range of proper compensation before acquisition. In addition, it is often desirable to set thresholds for exclusion of debris. Setting a forward scatter threshold, for instance, excludes events below a predetermined size in order to prevent the large list mode file size that can occur when these events are counted.

**Use of Controls**

Fluorochrome-conjugated bead preparations are used for standardizing PMTs and compensation and for quantifying the expression of specific markers. The use of biologic controls is also highly recommended. Stain the cell samples with an isotype control and primary and secondary antibodies to assess nonspecific binding unless the laboratory has ascertained by rigorous validation procedures that nonspecific binding does not interfere with assay results.

Antigen-positive and -negative cell populations (prepared and stained in a manner identical to that for the test articles) provide internal system suitability standards. Such control cell populations also allow the laboratory to assess lot-to-lot variations in antibody preparations and staining reagents.

**Use of Dyes and Gating for Cell Viability**

Cell viability dyes such as 7-AAD, PI, and TO-PRO iodide are commonly used to determine the proportion of dead cells in a cell therapy product. These dyes are typically excluded from live cells but pass through the cell membranes of dead cells, staining their DNA. Cell viability staining can be combined with surface membrane or intracellular staining to evaluate subpopulations and the proportion of live and dead cells stained with a given marker. Viability staining can also be used in conjunction with a membrane dye in flow cytometry-based cytotoxicity assays. These viability dyes should not be confused with the many

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apoptosis-detection reagents now available. Validation techniques for non-IVD viability dyes involve the preparation of a dead cell population that is added in serial dilution to a live cell product, and the cell mixture is then assessed for fidelity to the known proportion by staining with the dye of interest.

**Cell Enumeration**

Absolute cell count, expressed as the number of cells in a given sample volume, can be determined by dual- and single-platform methods. The dual-platform method relies on a separate automated cell-counting instrument or manual counting method to first enumerate the cell population. The percentage of a subset(s) of interest is then determined by flow cytometry, that percentage is multiplied by the cell count, and the result is divided by 100. Single-platform methods enumerate the cell population and subset counts directly by counting the cells in the sample simultaneously while counting reference beads that have been added to that sample volume in a known concentration. Reference beads are often provided as a bead suspension that is added to the specimen. Alternatively, a given volume of sample may be added to a known number of reference beads provided as a solid phase matrix in polystyrene tubes. These approaches are subject to pipetting error, so extra care must be taken to ensure accuracy and reproducibility.

**Instrument Setup and Quality Assurance**

Each laboratory should have a quality plan that defines the standard operating procedures for instrument setup and calibration, as well as regular instrument monitoring, maintenance, and cleaning. Instrument logs should document these activities and operators. In general, the instrument manufacturer's quality program should be followed unless a suitable alternative has been established.

Instrument parameters such as laser current, voltage, output, and PMT voltages during calibration should be monitored and recorded whenever the instrument is in use. Careful monitoring of instrument setup parameters can be helpful in detecting trends and predicting laser or PMT failure. Biological control testing results should also be monitored and recorded to detect and prevent analytical method drift.

The laboratory should also participate in a proficiency-testing program that reflects the test menu. Depending on need, this could range from a formal program such as the one administered by the College of American Pathologists (CAP) to simple sharing of specimens and analysis with another laboratory. Ensuring that operators are trained, qualified, and periodically evaluated for proficiency to perform specific procedures will also help ensure the consistency of techniques and controls.

### DATA MANAGEMENT AND STATISTICAL CONSIDERATIONS

**Data Management and Storage**

Quality control assays and sample test assays (in list mode) should be stored in a manner that complies with regulatory requirements applicable to the laboratory. This can be accomplished by transfer to fixed drives, removable media, or to a server such as a commercial laboratory information system. Storage of results should always be traceable to the original FCS list mode file, instrument settings, and quality control parameters for that particular specimen. Data should be backed up to avoid loss of files. Storage and backup procedures should also be established for manual (paper) records that may be used for calculations and summary data.

**Data Analysis and Statistical Considerations**

For most flow cytometry applications, data analysis involves displaying the data from list mode files or live gating in a plot (single-parameter histogram plot, two-parameter dot plot with regions, or three-dimensional plot), and measuring the distribution of events within that plot. Further analysis of data within selected populations can be done by gating on specific cell populations. Description of the data typically includes the percentage of events within the population with a given characteristic (forward scatter, side scatter, fluorescent marker). The numerator is the number of events with the characteristic, and the denominator is either the number of total events counted or the number of gated events counted. For two-dimensional plots, analysis is typically done using computer software that analyzes and reports regional (e.g., quadrant) statistics. Because cell population clusters may shift their positions from one data file to the next, software has been developed for cluster analysis.

Statistical analysis of quantitative flow cytometry applications differs from qualitative applications in which a cell is considered either positive or negative for a given marker. For a typical quantitative application in which the number of molecules on the cell surface is estimated, the mean or median fluorescence intensity of the sample cells labeled with a fluorescent antibody bound to the molecule of interest can be compared to appropriate controls, including standard curves of cells or particles with known quantities of that molecule/antibody.

A common practical consideration for flow cytometric analysis of cell therapy products, especially autologous and related donor allogeneic products, is that sample size for analytical testing is often limited because of the limited cell content of the therapeutic product itself. This creates special challenges if cells containing the flow marker of interest are rare events. In these cases, before making a decision about sample size, the user must consider the expectations for detecting the rare events within a given number of total cells (i.e., prevalence, variability, sampling error) in relationship to the desired precision of the estimate.

## Quality and Standardization

Standardization of flow cytometry practices and equipment requires validation, quality assurance (QA), and quality control (QC) practices. Although flow cytometry is used widely in both research and clinical laboratories, testing of cells for the development of clinical diagnostic and therapeutic applications is increasing, leading to more comprehensive regulatory requirements and attention to standardization. As an example, flow cytometry operators have traditionally used fluorescent microspheres (beads) or cells for instrument setup and QC, frequently based upon manufacturers' recommendations. However, consistent instructions about how these control standards should be applied to instrument setup and QC remain elusive.

Properly applied, validation provides documented evidence that the manufacturing or testing process consistently produces product that meets predetermined specifications. Based on a thorough understanding of critical process parameters, validation helps to define product quality and helps to ensure a consistent and well-controlled manufacturing or testing process. Validation of flow cytometric methods should incorporate instrument qualification, analytical method validation, and operator qualification.

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## DOCUMENTATION

GLP and GMP processes require appropriate documentation such as standard operating procedures (SOPs) for all lab processes. SOPs must also be periodically updated and approved to reflect current practices. Training and qualification are required so that laboratory staff have the appropriate level of competence for their assigned responsibility. Operator competencies must be continually reviewed and assessed in relationship to SOPs and policies.

Integrating both internal and external quality processes is an important element of quality assurance. These involve equipment validation, manufacturing controls and limits, and product specifications. Process and equipment validation processes generally require installation qualification (IQ), operational qualification (OQ), and performance qualification (PQ).

## EQUIPMENT AND ASSAY QUALIFICATION

IQ establishes that the instrument is received as designed and specified, and that it is properly installed in a suitable environment. This generally means checking physical and facility requirements to determine whether the flow cytometer can be suitably installed. Qualification factors verified typically include temperature, humidity, space, and electrical facility capabilities in relationship to the instrument manufacturer's requirements. IQ procedures also ensure that all hardware and software components purchased are installed properly by the instrument manufacturer's representative.

OQ demonstrates that an instrument will function according to the manufacturer's specifications. This generally means component-level testing by the instrument manufacturer's representative or using a manufacturer's validation package that guides the end user to perform this function. Where possible, these tests should have specifications with corresponding quantitative control limits. This testing ensures that the instrument hardware and software are operational by comparison with the manufacturer's specifications.

PQ demonstrates that both the instrument and the assay consistently perform according to specifications. For flow cytometry, these specifications are generally determined by the laboratory performing the flow cytometric testing and usually include daily instrument and assay control test specifications. For specific assays, PQ should incorporate standardized methodology, application-specific setup and compensation, and specifications for linearity, precision, and accuracy of reported assay results. On some digital flow cytometers, a baseline instrument setup may be necessary in order to determine the optimal instrument settings for a given assay.

## INSTRUMENT PERFORMANCE

Performance specifications may be identified by the flow cytometer manufacturer and may not address all of the operator's specification requirements. After identifying the manufacturer's specifications, the laboratory must establish specifications that are appropriate for the hardware configuration(s) that will be used, and the specifications must be standardized. As an example, the manufacturer may have a base specification for a four-color dual-laser flow cytometer. If the base specification calls for the standard red diode 635-nm laser but a helium–neon air-cooled 633-nm laser is substituted, the base specifications are no longer valid for the system. Similarly, if the specifications are based on the use of a 660-nm band-pass filter but a 675-nm long-pass filter is substituted, the base specifications are not valid because of differences in the emission filter characteristics.

## PERFORMANCE MONITORING

Instrument performance can be monitored on a daily basis, using commercially available fluorescent beads. Light detectors such as PMTs, photodiodes (PD), and avalanche photodiodes (APDs) are used in most systems to detect signals, and their gains can be changed to increase or decrease the sensitivity of the detectors. Therefore, monitoring the settings of these detectors is as important as monitoring the signals, and these settings should be associated with each raw data file. The easiest approach is to maintain the same instrument detector settings from day to day and to measure the intensity of the fluorescence signals. This approach should be implemented for all parameters that must be validated using the appropriate beads. Instrument sensitivity is based on the ability of the detection system to resolve dim cell or bead populations. For this reason, measuring the coefficient of variation (CV) of dim to moderately intense fluorescent bead populations is a means to monitor fluorescence sensitivity on a daily basis. The instrument manufacturer's recommendations should be used to monitor performance.

Ambient high temperature can affect laser and PMT performance and should also be monitored on a daily basis.

Incorrect compensation for spectral overlap can strongly affect data during multicolor analysis. Many approaches have been established for this purpose, and recently mathematical algorithms have been used rather than analog circuitry. Algorithms, such as those that use matrix algebra, enable the operator or investigators to apply objective criteria to compensate for spectral overlap after all data have been collected. On older systems, the standard approach has been to compensate using a subtractive hardware adjustment to the observed preliminary data before all data have been collected. This approach can be subjective and is not as likely to produce accurate results as compensation by newer methods. Antibody-bound capture beads are valuable compensation tools because they can be used with the same antibody and tandem dyes for all fluorophores that will be used on cells. Validate the use of beads in place of cells for compensation purposes.

## STANDARDS

Microsphere-based fluorescence standards for flow cytometry have been categorized by their purpose:

- Type I standards are alignment standards that are used to make adjustments to the instrument's optical alignment. These are typically used by field service engineers and by users of operator-adjusted systems to check the optical signal alignment in order to improve instrument sensitivity. These particles are typically small (≈ 2 µm) and bright, and they provide the most uniform illumination.

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- Type II standards are reference beads and are the most commonly used bead standards. These typically are used on a daily basis, have dim to moderate fluorescence intensity, and can be obtained with various attached fluorophores. These can be used to mimic cells and, with dedicated software, to determine relative instrument sensitivity.
- Type III standards are used for fluorescence calibration. These are used for specialized applications that require calibration of one or more fluorescence detectors for quantitation of molecules of fluorochrome. Determination of the ratio of fluorophores to antibody (F/P ratio) allows subsequent calculation of the number of antibodies bound per cell.

## INSTRUMENT SETUP AND QUALITY ASSURANCE

Instrument setup and quality assurance should be activities independent of the biological assay. Many variables related to instrumentation can lead to artifacts in the biological assay results. Two activities can be used for instrument quality assurance: baseline setup and daily setup.

**Baseline Setup**

On newer digital instruments it is desirable to establish a baseline setup of instrument settings that provide optimal sensitivity. This setup is not a daily procedure but should be performed if the instrument configuration is changed or if the instrument is serviced. Because PMT voltages and instrument configuration can strongly affect instrument sensitivity, this method should be used to provide objectivity as well as improved sensitivity. PMT voltages can be increased to a range that provides a lower CV (*Figure 8*). These settings can be, but may not always be, used for the biological assay.

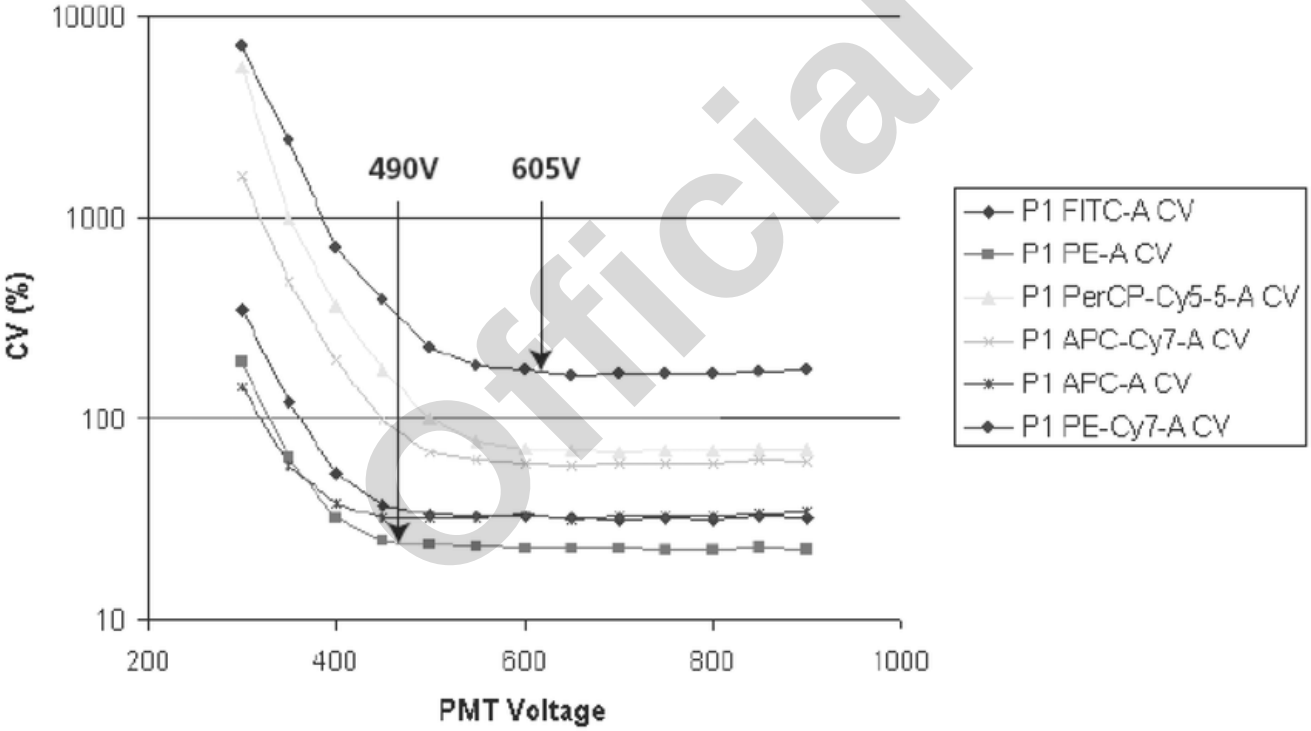


Figure 8. PMT voltages can be increased to a range that provides a lower CV.

**Daily Setup**

Type II standards, as reference particles, can be used to monitor signal intensity, separation of moderately bright and dim particles, and signal resolution. Fluorophore-matched beads provide a compensation tool as well as a means of knowing that the instrument is able to detect those wavelengths. Fluorophore-matched beads, however, do not have the fluorescence uniformity required for measuring CVs. Based on the optical performance of the instrument, CVs are best measured using dim and moderately bright hard-dyed beads such as coumarin-dyed microparticles that fluoresce in a broad spectral range.

It is easy to confuse assay controls and instrument controls. Beads provide a fluorescence uniformity and consistency that cannot be obtained with cells, and, accordingly, beads are useful for monitoring instrument performance. For this reason, it is better to use a process control cell preparation to verify compensation and fluorophore acceptability.

Instrument settings for daily setup are generally the same settings used for assays. Not all assays can be used with the same instrument settings, and it is not always necessary to perform these activities for every instrument setting unless it is required by validation.

Daily activities include consistent instrument setting from day to day, use of a broad range of dim to moderate intensity beads, and monitoring key parameters, including bead fluorescence intensity (absolute and % CV), PMT values, temperature, laser power, and laser wavelength.

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### ASSAY QUALITY CONTROL AND QUALITY ASSURANCE

Assay-specific instrument settings should be established to demonstrate that all cell populations can be identified in bivariate plots for fluorescence and light scatter. Most importantly, positive populations must be on scale and properly compensated. This is critical when exciting cells with red lasers, which do not cause cells to generate significant autofluorescence. For this reason, it is important to verify appropriate PMT settings so that the positive population is in the upper part of its fluorescence scale, because it may be extremely difficult to identify the negative cells.

Fluorescence compensation is a critical adjustment. Digital instruments provide objective offline adjustments during analysis, and detailed instructions for proper compensation settings are available. Using cells or capture beads stained with a single antibody-fluorochrome is generally the best approach, but specialized fluorochrome-labeled bead mixtures can also work well to compensate for multicolor acquisition and analysis.

### ISOTYPE CONTROLS

An isotype control is a negative-control antibody that should not react with the antigen of interest and is the same isotype as the test antibody. Myeloma protein or immunoglobulin that has no specificity to the species being tested and has the same Ig chain class and subclass as the test antibody is conjugated to a fluorochrome identical to that on the test antibody. Ideally, very little or no binding occurs when the isotype control is used in parallel with the test. Idiotypic nonspecific binding frequently occurs, however, and is independent of the isotype of the antibody. This is most likely related to other differences in antibody chemistry and can be especially problematic with rare-event detection assays, such as those for hematopoietic stem cell assays in peripheral blood.

### FLUORESCENCE MINUS ONE CONTROLS

Fluorescence minus one (FMO) controls are used to control nonspecific staining during a multicolor assay. After compensation has been set, a tube containing all of the fluorochrome-labeled antibodies, except one, is run. If the compensation has been properly set, any positive fluorescence in the parameter corresponding to the missing fluorochrome-labeled antibody is caused by nonspecific staining and can be an indication of antibody excess or degradation of related tandem dyes. Although FMO controls are very useful for estimating the sensitivity of a particular detector in the context of other reagents, the controls do not take into account nonspecific binding that can occur with the addition of the test antibody. FMO control tubes are most appropriately used for troubleshooting or when establishing a new multicolor reagent cocktail.

### PROCESS CONTROLS

Process controls, also known as system suitability standards, account for sample preparation and data acquisition. They can include commercially available preserved control cells, cell lines, or primary cells such as normal peripheral blood. Process controls can also be used to test new lots of antibody reagent against old lots.

### BIOLOGICAL CONTROLS

When treated or stimulated cells are compared to untreated or unstimulated cells, the untreated or unstimulated cells may in some cases be the most useful control for setting a positive/negative boundary. However, use of isotype controls may also apply to these situations, because stimulation may lead to Fc receptor upregulation, leading in turn to increased background staining, the presence of which can be elucidated by an isotype control.

## FLOW CYTOMETRY APPLICATIONS FOR CELLULAR SAMPLES AND CELL THERAPY PRODUCTS

A wide variety of flow cytometry applications have been developed for research, clinical diagnosis and monitoring, drug development, and cellular product characterization and quality assessment (i.e., control to allow batch release). Traditional clinical applications include monitoring HIV disease and diagnosis and monitoring leukemia and lymphoma. Both pharmaceutical and academic research laboratories have increasingly broadened the application of flow cytometry from immunophenotyping to functional cellular assays, as well as microsphere-based multiplex assays capable of measuring multiple functional parameters on individual cells. Current functional assays include those that allow direct study of cellular activation status by measuring intracellular cytokine production or secretion of chemokines or cytokines, using a ligand-binding sandwich assay on microspheres.

### Immunophenotyping

Flow cytometry allows the characterization of leukocyte subtypes by labeling cells with fluorochrome-conjugated monoclonal antibodies. The CD system defines monoclonal antibodies that recognize unique cell-surface antigens. Many clinical applications take advantage of flow cytometry's unique capabilities to measure multiple CD antigens on thousands of individual cells.

### CD4 ENUMERATION

In the early 1980s, investigators discovered that HIV infects CD4 T cells and that a patient's peripheral blood CD4 T cell count is a useful indicator of immune status. CD4 enumeration has become the most commonly used diagnostic test in HIV-infected patients to determine the need for anti-retroviral (ARV) therapy and for monitoring the effectiveness of ARV drugs. T cell subset

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counts are typically expressed in terms of cells per microliter and as a percentage of lymphocytes, using a standardized reagent, software, and instrument system.

### LEUKEMIA AND LYMPHOMA

Multidimensional flow cytometric analysis enables identification of aberrant cell populations in bone marrow, lymphatic tissue, and peripheral blood of patients with leukemia or lymphoma. This is accomplished with oncology-relevant and lineage-specific cocktails of monoclonal antibodies. With optimal fluorophores and improved optical/electronic configurations in flow cytometry instrumentation, additional cell markers can be detected to more precisely identify leukemia or lymphoma cell phenotypes and to improve the physician's assessment of patient status. Rare-event detection methods have improved the ability to detect minimal residual disease.

### DENDRITIC CELLS

Dendritic cells (DCs) act as antigen-presenting cells that can influence the nature and strength of the immune response to specific antigens. This finding has led to the development of DCs as cell-based therapies for cancer, infectious disease, and autoimmune disease. DCs are morphologically and phenotypically diverse and can be derived from several cell types. Two major DC lineages, known as myeloid and plasmacytoid DCs, can be segregated on the basis of their expression of CD11c and CD123, respectively. Additionally, the expression of the costimulatory molecules CD80 and CD86 can be monitored to determine DC maturation state.

### STEM AND PROGENITOR CELLS

CD34 expression is commonly used to characterize hematopoietic stem cells (HSCs) in peripheral blood, cord blood, bone marrow, and purified HSC preparations from these sources. Flow cytometric identification and enumeration of HSCs is possible by using monoclonal antibodies specific to the CD34 class III epitope, along with other well-characterized reagents, analysis software, and protocols. The reagent combination of anti-CD45, anti-CD34, and a viability dye such as 7-AAD is widely used for clinical applications. Increasing interest in developing cell-based therapies from embryonic, fetal, and adult tissue sources has led to the use of a wide variety of conventional and novel phenotypic markers for characterization of source cells and their more differentiated progeny. Flow cytometric assays are being developed as part of assay batteries to assess differentiated cellular products derived from pluripotent stem cell sources. These assays will help define appropriate numbers and types of desired cell populations, as well as help detect undesired cells such as residual pluripotent cells that could prove tumorigenic in the recipient.

### LEUKOCYTES

Leukoreduction of blood products is a process used to produce blood products with a residual leukocyte content of less than $5 \times 10^6$ per unit. Clinical data suggest that nonhemolytic febrile transfusion reactions can be prevented by leukodepletion. Leukodepletion also prevents alloimmunization to HLA antigens in patients who will repeatedly require transfusion of blood products. Flow cytometry is routinely used to quantitate leukocyte contamination in leukocyte-depleted blood products.

### PLATELETS

Flow cytometry is a rapid and useful method for diagnosing many primary thrombocytopathies related to defects in structural or functional glycoproteins (e.g., abnormal expression of gpIIb/IIIa in Glanzmann thrombasthenia or gpIb in Bernard-Soulier disease). The use of thiazole orange, a fluorescent dye that binds RNA, allows immature platelets (reticulated platelets) to be quantified. The reticulated platelet count can be used to determine the rate of thrombopoiesis. This measurement can separate unexplained thrombocytopenias into those with increased destruction and those with defects in platelet production.

### ERYTHROCYTES

Rhesus D-negative women receive prophylactic Rh-immunoglobulin to prevent alloimmunization from $Rh(D)^+$ erythrocytes (RBCs). If fetomaternal hemorrhage is suspected, the mother's blood is tested for the presence and quantity of fetal RBCs, using fluorescently labeled antibodies to the Rh(D) antigen or to hemoglobin F.

The reticulocyte count is used to help determine whether the bone marrow is responding adequately to the body's need for RBCs and to help classify different types of anemia. Reticulocyte counts are based on the identification of residual ribosomes and RNA in immature nonnucleated RBCs. Flow cytometric enumeration of reticulocytes and their discrimination from mature RBCs uses fluorescent dyes that bind the residual RNA (e.g., thiazole orange).

## Bead-Based Immunoassays

Multiplex microsphere-based flow cytometric assays combine a series of particles of discrete size and/or fluorescence intensity with matched antibody pairs to allow simultaneous detection of multiple soluble analytes on a flow cytometer. The flow cytometer's capacity to discriminate particles on the basis of size and color enables determination of multiple results from a single tube or well. Many investigators use such assays to measure secreted chemokines or cytokines, kinases, and anti-HLA antibodies.

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## Proliferation Assays

### DYE INCORPORATION INTO DNA

Bromodeoxyuridine (BrdU) is a thymidine analog that can be incorporated into the DNA of cells during S phase, and then can be detected using specific, labeled monoclonal antibodies. By pulsing a stimulated cell culture with BrdU, cells can be identified that have proliferated (passed through S phase) during the time of the pulse. This assay has become a useful alternative to $^{3}$H-thymidine incorporation as a measure of proliferation because it is nonradioactive and can identify phenotypes of proliferating cells by the use of multiple markers and flow cytometry.

### DYE INCORPORATION INTO CELLULAR PROTEINS OR CELL MEMBRANE

Cell-tracking dyes such as carboxyfluorescein succinimidyl ester (CFSE) and PKH26 have proven useful in the assessment of cell proliferation. CFSE binds covalently to cytosol and membrane proteins, and PKH26 binds non-covalently to cell membranes. When cells divide, CFSE/PKH26 labeling is partitioned equally between the daughter cells, which are therefore half as fluorescent as the parents. The fluorescence of each cell is further halved with each succeeding generation. This property makes CFSE/PKH26 assays useful not only for determining the fraction of cells that have proliferated in a stimulated culture but also, under ideal conditions, the number of generations that have elapsed. In this manner, the precursor frequencies of small populations that have proliferated over several days in culture can be calculated.

## Functional Assays

### INTRACELLULAR CYTOKINE EXPRESSION

Cell surface and intracellular labeling techniques have been applied to the identification of cell subsets with specific functional characteristics. For example, brief stimulation of cells such as PBMC with protein or peptide antigens can result in the expression of activation markers and cytokines that can then be measured along with other phenotypic markers on the surface of the responding cells. The use of a secretion inhibitor such as brefeldin A or monensin allows the intracellular accumulation of cytokines. The cells are then fixed, permeabilized, and detected by a flow cytometric method. Such assays are useful for monitoring T cell subpopulations that respond to vaccines, infectious disease agents, or cancer. Functional properties of other cell types, including monocytes, DCs, and NK cells, can also be monitored using functional assays with appropriate stimuli.

### KINASES

Phosphorylation-specific cell activation intermediates can be identified using phospho-specific antibodies and flow cytometry. These reagents are useful for mapping intracellular signaling mechanisms, often in the context of other cell-surface phenotypic markers. Thus, multicolor flow cytometry can provide single-cell assessment of intracellular activation states in complex cell populations. These assays may have utility in detecting altered signaling states in cancer cells or in directing appropriate therapies based on the signaling properties of a patient's tumor cells.

### APOPTOSIS

Apoptosis, commonly described as programmed cell death, is the process of cell death caused by regulated, physiologic processes. The apoptotic process manifests itself as a series of morphological, biochemical, and molecular changes to the cell and can be initiated by external or internal stimuli. A central event during apoptosis is the activation of caspases, a family of proteolytic enzymes. Caspases are synthesized as inactive proenzymes and are activated by other caspases or by similar molecules. They form a cascade that can lead to the cleavage of various cytoplasmic or nuclear proteins. One of the caspases that is reported to be crucial for the apoptotic process is caspase-3, which is activated during the early stages of apoptosis.

Flow cytometric methods for detecting apoptotic cells include measuring morphology, changes in membrane structure, DNA cleavage by endonucleases, and mitochondrial membrane potential. Natural or artificial caspase substrates or antibodies against the activated form of the enzyme have also been used for this purpose.

### CELL VIABILITY

Flow cytometry is often used to discriminate live cells from dead cells. The principle of nucleic acid dye exclusion is the basis of this application. A nucleic acid dye such as PI or 7-AAD is added to cells in suspension. During flow cytometric analysis, cells that fluoresce above background are considered nonviable because they cannot exclude the dye, which fluoresces when it binds to cellular DNA.

## Flow Cytometry Immunoglobulin Assays

### FLOW CYTOMETRY CROSS-MATCHING

Before organ transplantation, flow cytometry cross-matching (FCXM) is performed on recipients to screen for anti-HLA antibodies that can cause rejection. Anti-HLA antibodies are detected by incubating HLA-defined leukocytes, B-cell lines, or HLA antigen-coated beads with the serum sample, followed by anti-human immunoglobulin fluorescently labeled antibodies. Leukocytes are immunostained to identify T and B cells in order to distinguish between anti-HLA class I and II activity,

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respectively. In addition, screening of blood donations for anti-HLA antibodies is also increasingly employed to identify donors whose blood products may have increased risk of causing transfusion-related acute lung injury (TRALI) in recipients.

### ANTI-HUMAN NEUTROPHIL ANTIBODIES

Anti-human neutrophil antibodies (HNA) can cause neutropenia and have been implicated in TRALI. Autoimmune neutropenias may develop in patients who have autoimmune disorders such as Felty syndrome, systemic lupus erythematosus, and Hashimoto thyroiditis. The absence of anti-HNA antibodies narrows the differential diagnosis to nonimmune causes such as bone marrow failure, myelodysplasia, or marrow-infiltrative processes. Flow cytometry can detect anti-neutrophil antibodies and can confirm the origin of neutropenia or TRALI.

### ANTI-HUMAN PLATELET ANTIBODIES

Anti-human platelet antibodies (HPA) are detected by both indirect and direct flow cytometry-based platelet-associated immunoglobulin assays. In autoimmune thrombocytopenic purpura, free serum antibodies are not found as frequently as are platelet-bound antibodies. In cases of alloantibody formation, serum antibodies may be detected without evidence of platelet-associated antibodies.

## FLOW CYTOMETRY ASSAY TROUBLESHOOTING

When developing a flow cytometry method, first determine the ultimate purpose of the assay. For assays intended for research, the cell samples, reagents, and protocols may be difficult to standardize. Assays intended for patient diagnosis or to qualify a cellular product for release before administration to a patient demand more stringent assay and sample standardization. Regulatory guidelines, the type and stage of clinical investigation, and the ultimate purpose of the assay determine the level of assay rigor required.

Flow cytometry assay development should include the establishment and qualification of staining, handling, instrument, and analysis parameters and limits. Assuming that the method has been well developed, the operators are properly trained, the instrument has been properly set up, appropriate assay and instrument controls have been applied, and, if necessary, the instrument and method validations have been performed, operators may encounter and address instances when troubleshooting is necessary.

The most common flow cytometry challenges are high fluorescence or side scatter background, abnormal event rates, high fluorescence intensity, and low fluorescence signal. Approaches to alleviating these issues are described below.

### High Particulate Background

Excessive cell handling (e.g., vortexing), improper fixation, and bacterial contamination of the cells can all increase the particulate background. In addition, if the instrument's forward threshold is set too low, cell debris will be detected as events. Gentle cell handling, fresh reagents, and appropriate instrument settings help ensure consistent side scatter profiles.

### High Fluorescence Background

High fluorescence intensity can be attributed to excessive antibody concentration, inadequate cell washing, or inadequate Fc receptor blocking. In addition, improperly high instrument PMT gain can also result in a high background. Consistent antibody concentration and cell density, adequate washing and blocking, and appropriate instrument settings will help avoid abnormally high fluorescence background.

### High Event Rate

Abnormally high event rates are often attributed to high cell densities during antibody staining or in the final cell sample. Inadequate mixing and settling of the cell sample can result in high cell event rates, as can improper or inconsistent gating.

### Low Event Rate

Cell clumping, low final sample cell densities, blockages in the instrument fluidics, or improper gating can often result in abnormally low event detection. Proper cleaning, maintenance, and setup of the instrument, as well as consistent staining protocols, can help achieve consistent results with sufficient sensitivity.

### High Fluorescence Intensity

As in the case for high fluorescence background, high mean cell fluorescence can result from too much labeled antibody, inadequate or inconsistent cell washing, or inadequate blocking. Including detergent in the wash buffer, especially during intracellular staining, can help prevent nonspecific antibody binding.

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## Weak Fluorescence Intensity

Many factors can result in weak fluorescence intensity. Instrument parameters such as poor laser alignment, improper compensation, improper setup, inconsistent gain settings, and weak laser output can all negatively affect fluorescence intensity. In addition, cell physiology or reagent preparation issues, such as insufficient antibody concentration, labile or secreted target antigen, poor-quality or improperly stored reagents (resulting in fluorochrome fading), or inaccessible target antigen, can all result in a weak signal. Adequate assay development, proper instrument maintenance, and adherence to qualified protocols can all improve the fluorescence signal intensity.

Flow cytometry enables investigators to analyze cells for many different applications. Types of immunophenotypic and functional assays are increasing in number and in scope. The presence of proteins and cellular processes and detection of rare or abnormal cell populations can be studied. The reader is referred to the technical literature for application and method details.

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## INTRODUCTION

### Purpose

Documentation can be viewed as the foundation of all quality systems because clear, complete, accurate records are essential to all operations and procedures. This general chapter provides guidelines on good documentation practices for the Good Manufacturing Practice (GMP)-regulated industries, to be used in the production and control of pharmaceutical products, active pharmaceutical ingredients (APIs), excipients, dietary supplements, food ingredients, and medical devices. This chapter describes the underlying principles of proper documentation for GMP operations to assist the user while working with GMP activities. These guidelines should be helpful for building the basic foundation of a quality system that will ensure proper documentation as well as record integrity and control.

### Scope

This chapter covers different levels and types of documentation, including paper and electronic records that consist of raw data, reports, protocols, and procedures related to manufacturing controls and analytical data. The chapter also includes recommendations on information that should be recorded for various types of GMP documents. Electronic systems should be developed to meet guidelines described in this chapter.

This chapter does not provide information about all applicable current legal requirements, nor does it affect any applicable current requirements under GMP regulations.

## PRINCIPLES OF GOOD DOCUMENTATION

All steps related to the manufacturing, testing, packing, or holding of pharmaceutical products, APIs, excipients, dietary supplements, food ingredients, and medical devices should be documented.

Good documentation principles for manual or electronic records include the following, as applicable:

- Records should be clear, concise, accurate, and legible.
- Data entries should be recorded promptly when actions are performed.
- Backdating and postdating are not allowed.
- All corrections to the original entries should be initialed and dated (or captured within an electronic audit trail), with an explanation included in cases where the reason for the change is not obvious.
- Data entries should be traceable to the person who made the entry.
- Uncommon abbreviations and acronyms should be defined.
- Controls should be in place to protect the integrity of the records.
- In the event that ink may have faded over time (e.g., thermal paper), a copy can be used with verification of its accuracy; the copy should be initialed and dated.
- Notebooks, data sheets, and worksheets should be traceable.
- An adequate documentation system is needed to ensure data integrity and availability of current and archived records.
- Records should be retained per regulatory requirements and be readable during the retention period.
- All pages should be paginated. Attachments (supporting documents) should be paginated with a reference to the parent document.

## DATA COLLECTION AND RECORDING

Formats for data collection and recording include, but are not limited to, the following:

- Paper forms, data sheets, and worksheets
- Notebooks and logbooks
- Instrument printouts
- Electronic data obtained with a system such as an electronic data system, laboratory information management system (LIMS), or electronic laboratory notebook (ELN)

All data should be permanently recorded directly and legibly when the activity is performed. If it is paper record, then it should be recorded in indelible ink. All data entries should be traceable to who made the entry and when. Additionally, electronic records must meet the requirements of the *Code of Federal Regulations Title 21* (21 CFR), Part 11.

Any change to an entry should be made in a way that does not obscure the original entry, with an explanation in cases where the reason for the change is not obvious. Changes should be traceable to who made the change and when the change was made. For clarity, predefined correction codes may be used, for example, WD = wrong date.

Notebook pages and worksheets should be used consecutively, and information should be recorded chronologically. GMP records such as batch records, test methods, and specifications should be given unique identifiers and use version control for the documents.

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All data entry fields should be completed. A single line and/or "N/A" should be drawn through portion(s) of a page that are not used. If the record is in an electronic system and the system provides traceability of who filled each field and when, the field can be left blank.

Decimals less than one should be preceded by a zero. Rounding rules and guidelines on significant figures are described in *General Notices, 7.20 Rounding Rules*.

All dates should be expressed in a format that clearly indicates the day, month, and year.

All GMP records for data collection should undergo appropriate review and signature by a second person to confirm the accuracy, compliance, and completeness of the entries. Additional signatures may be required on the basis of local Standard Operating Procedures (SOPs) for different levels of review (e.g., performed by, verified by, checked by, reviewed by, approved by) as accountability steps.

An official record of signature and initials should exist for each employee or can be contained within the document. Controls should be in place for assigning signature approval requirements and delegation of signature authority, when needed.

In the event that verified copies of raw data have been prepared, the verified copy may be substituted for the original source as raw data.

All multiple-page data sheets or instrument printouts in paper form should be signed/initialed on the first or last page with a note indicating the total number of pages. The first page and all subsequent pages should be uniquely identified to the activity being performed, such as the notebook reference, study number, or worksheet reference.

## DIFFERENT TYPES OF GMP DOCUMENTS

The following or similar documents should include the following information, but are not limited to these items.

### Laboratory Records

Laboratory records should be organized to ensure that the records are concise, clear, legible, and accurate, and detail the following:

- Description of materials, such as reagents. This information typically includes the material name, manufacturer and lot number, titer or concentration, expiration date, grade (if known), and a reference to the lab notebook if prepared in the lab
- Identification of equipment used. This information typically includes equipment name, unique control number, and calibration expiration date, as applicable
- Procedures used
- Measurements
- Formulae and calculations
- Results and conclusions

### Equipment-Related Documentation

All equipment used in manufacturing, testing, packing, or holding of a raw material, component, API, finished product, or other similar item should be maintained and qualified for its intended use. The documentation related to equipment includes:

- Policies and procedures for operation and maintenance
- Equipment use
- Maintenance records
- Calibration or qualification records
- Instrument labeling

### Deviations and Investigations

All aberrations, anomalies, and exceptions related to manufacturing, testing, packing, or holding of a raw material, component, API, finished product, or other similar item should be documented. Once documented, the deviation should be evaluated and investigated, as appropriate. Procedures should be in place for documenting, evaluating, and investigating such events. Documentation of the investigation should include the following:

- Description of the event
- Root-cause investigation
- Evaluation of data trend
- Responsibilities of people involved in the investigation or deviations
- Impact assessment
- Corrective Action and Preventive Action (CAPA) with timelines
- Review and approval

### Batch Records

A Master Batch Record (MBR) is created as a template for the manufacture of a specific product. An Executed Batch Record, based on the MBR, is used to document the steps and materials involved in the production of a specific batch of a raw material,

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component, API, finished product, or other similar item. Typically, the following sections are included in a Batch Record, and should be approved by an appropriate representative from the manufacturing site or packaging site:

- Header information (e.g., product name, batch number, manufacturing site)
- Unit of operation (e.g., blending, coating, filling)
- Manufacturing process
  - Target weights (raw materials)
  - Conditions (time, temperature)
  - Deviations and investigations
- In-process sampling or testing
- Other critical information, as applicable
- Sampling plan for release, stability, and retention
- Review and approval

## Certificate of Analysis

The purpose of the Certificate of Analysis ("C of A" or CoA) is to report analytical results for a specific batch of a raw material, component, API, finished product, or other similar item. Typically, the following sections are included on a C of A and should be approved by an appropriate representative from the testing site:

- Vendor, supplier, or manufacturer information (as applicable)
- Product information (name and strength)
- Results for the specific batch, with name of test, acceptance criteria, and result for each test
- Conformance statement or equivalent
- Reference to procedure and specification document
- Reference of data source
- Approval and date
- Expiration date or retest information

## Standard Operating Procedures

The purpose of an SOP is to provide directions to trained personnel regarding a given set of activities. SOPs should be clear and concise. The following sections are typically included in an SOP:

- Purpose and scope
- Instructions and procedure
- Responsibilities and roles
- Materials or equipment, as appropriate
- Definitions or references, as needed
- Review and approval
- Revision history

## Protocols and Reports

Many tasks and activities are executed on the basis of a predefined, preapproved protocol. The results of these activities are then documented in a final report with conclusions. Examples of such activities are as follows:

- Equipment qualification
- Analytical method validation or verification
- Manufacturing process validation
- Analytical method or manufacturing technology transfer
- Cleaning validation
- Stability study or testing
- Comparability study

Both the protocol and the report should typically include the following sections:

- Purpose
- Plan or instructions
- Predetermined acceptance criteria
- Deviations or investigations, or a reference to (for report only)
- Assessment or evaluation (for report only)
- Data reference (for report only)
- Review and approval
- Revision history

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## Analytical Procedures

Analytical procedures provide direction to an operator on how to perform a given analytical test. The following sections will typically be included in the analytical procedure:

- Purpose
  - Test information
  - Product information
- Safety information, if applicable
- Materials and equipment
- Procedure, as applicable
  - System suitability
  - Preparation of solutions and reagents
  - Preparation of standards and samples
  - Instrument parameters
  - Calculations and reporting
- Review and approval with approval dates
- Revision history

## Training Documentation

Personnel should be trained to perform their assigned tasks. The training should be documented, and the training records should be retained and kept readily accessible. In general, training documentation should include:

- Training description including name of training, version, and mode (self-training or instructor led)
- Completion date
- Information on the trainer, as applicable

## Retention of Documents

An adequate policy for record retention and archiving should be established for the above records. The required length of time depends on the regulatory requirements or company procedures; however, it should be at least 1 year after the batch expiration date.

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# ⟨1030⟩ BIOLOGICAL ASSAY CHAPTERS—OVERVIEW AND GLOSSARY

*USP–NF* contains four general chapters regarding the development, validation, and analysis of bioassays (biological assays): *Design and Analysis of Biological Assays* ⟨111⟩, *Design and Development of Biological Assays* ⟨1032⟩, *Biological Assay Validation* ⟨1033⟩, and *Analysis of Biological Assays* ⟨1034⟩. This proposed new chapter, *Biological Assay Chapters—Overview and Glossary* ⟨1030⟩, provides an overview and some material common to chapters ⟨1032⟩, ⟨1033⟩, and ⟨1034⟩, including a glossary of bioassay-related terms.

The suite of *USP* bioassay chapters focuses on relative potency assays. These assays recognize the inherent variability in biological test systems (whether animals or cells) that may be seen from laboratory to laboratory and from day to day. That inherent variability compromises the reliability of an absolute measure of potency. In relative potency assays, the biological activity of a Test material is compared to the activity of a Standard in an assay system wherein the use of a Standard reduces the influence of the inherent variability of the system on the estimation of relative potency. Relative potency assays also provide focus on important variability in response because of differences between the Test and Standard materials (if such a difference exists). The Test is expected to behave as a dilution or concentration of the Standard and should exhibit the property of *similarity*. Although they are intended for relative potency bioassays, the principles and practices developed in these chapters may have wider application—for example, to immunoassays and receptor-ligand–binding assays used to determine relative potency.

Chapter ⟨1032⟩ provides information for scientists developing a new biological assay. As seen in *Table 1*, the chapter covers a range of activities across the life cycle of the assay, with emphasis on development leading to validation, including the choice of test system and design considerations (e.g., plate layout). It also addresses data analysis strategies that should be considered during development (before validation) but that are not routinely addressed later. Among these strategies are the choice of weighting scheme, data transformation, if any, and choice of statistical model. Statistical details in support of these sections of chapter ⟨1032⟩ are found in chapter ⟨1034⟩.

**Table 1. Primary Sections of** *Design and Development of Biological Assays* ⟨1032⟩

| Section | Section Title |
|---|---|
| 1 | Introduction |
| 1.1 | Purpose and Scope |
| 1.2 | Audience |
| 2 | Bioassay Fitness for Use |
| 2.1 | Process Development |
| 2.2 | Process Characterization |
| 2.3 | Product Release |
| 2.4 | Process Intermediates |
| 2.5 | Stability |
| 2.6 | Qualification of Reagents |
| 2.7 | Product Integrity |
| 3 | Bioassay Fundamentals |
| 3.1 | In Vivo Bioassays |
| 3.2 | Ex Vivo Bioassays |
| 3.3 | In Vitro (Cell-Based) Bioassays |
| 3.4 | Standard |
| 4 | Statistical Aspects of Bioassay Fundamentals |
| 4.1 | Data |
| 4.2 | Assumptions |
| 4.3 | Variance Heterogeneity, Weighting, and Transformation |
| 4.4 | Normality |
| 4.5 | Linearity of Concentration–Response Data |
| 4.6 | Common Bioassay Models |
| 4.7 | Suitability Testing |
| 4.8 | Outliers |
| 4.9 | Fixed and Random Effects in Models of Bioassay Response |
| 5 | Stages in the Bioassay Development Process |

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**Table 1. Primary Sections of** *Design and Development of Biological Assays* ⟨1032⟩ *(continued)*

| Section | Section Title |
|---|---|
| 5.1 | Design: Assay Layout, Blocking, and Randomization |
| 5.2 | Development |
| 5.3 | Data Analysis during Assay Development |
| 5.4 | Bioassay Validation |
| 5.5 | Bioassay Maintenance |

Chapter ⟨1034⟩ provides information about the data analyses appropriate for common relative potency bioassays, including parallel-line, slope-ratio, parallel-curve, and quantal models. The chapter also includes analyses supporting system and sample suitability assessment and methods for combining results from independent assays (see *Table 2*). This chapter is the most statistically advanced of the three chapters but is designed to be suitable for both biologists and statisticians. The conceptual material requires only a minimal statistical background. Methods sections require a statistical background at the level of *Analytical Data—Interpretation and Treatment* ⟨1010⟩ and familiarity with linear regression.

**Table 2. Primary Sections of** *Analysis of Biological Assays* ⟨1034⟩

| Section | Section Title |
|---|---|
| 1 | Introduction |
| 2 | Overview of Analysis of Bioassay Data |
| 3 | Analysis Models |
| 3.1 | Quantitative and Qualitative Assay Responses |
| 3.2 | Overview of Models for Quantitative Responses |
| 3.3 | Parallel-Line Models for Quantitative Responses |
| 3.4 | Nonlinear Models for Quantitative Responses |
| 3.5 | Slope–Ratio Concentration–Response Models |
| 3.6 | Dichotomous (Quantal) Assays |
| 4 | Confidence Intervals |
| 4.1 | Combining Results from Multiple Assays |
| 4.2 | Combining Independent Assays (Sample-Based Confidence Interval Methods) |
| 4.3 | Model-Based Methods |
| 5 | Additional Sources of Information |

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Chapter ⟨1033⟩ is intended to follow chapters ⟨1032⟩ (assay development) and ⟨1034⟩ (development of data analysis plans). That is, chapter ⟨1033⟩ assumes a fully developed bioassay (including a data analysis plan and at least tentative values for system and sample suitability criteria and the bioassay format) and provides guidance about the validation of that assay. The chapter addresses the validation characteristics relevant to relative potency bioassays and provides more detail regarding the statistical methods used in validation than does *Validation of Compendial Procedures* ⟨1225⟩. Principles and practices developed in chapter ⟨1033⟩, although they are intended for relative potency assays, may have wider application. The chapter emphasizes validation approaches that provide flexibility in adopting new bioassay methods, new biological drug products, or both (see *Table 3*).

**Table 3. Primary Sections of** *Biological Assay Validation* ⟨1033⟩

| Section | Section Title |
|---|---|
| 1 | Introduction |
| 2 | Fundamentals of Bioassay Validation |
| 2.1 | Bioassay Validation Protocol |
| 2.2 | Documentation of Bioassay Validation Results |
| 2.3 | Bioassay Validation Design |
| 2.4 | Validation Strategies for Bioassay Performance Characteristics |
| 2.5 | Validation Target Acceptance Criteria |
| 2.6 | Assay Maintenance |
| 2.7 | Statistical Considerations |
| 3 | A Bioassay Validation Example |

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**Table 3. Primary Sections of** *Biological Assay Validation* ⟨1033⟩ *(continued)*

| Section | Section Title |
|---|---|
| 3.1 | Intermediate Precision |
| 3.2 | Relative Accuracy |
| 3.3 | Range |
| 3.4 | Use of Validation Results for Bioassay Characterization |
| 3.5 | Confirmation of Intermediate Precision and Revalidation |
| 4 | Additional Sources of Information |
| Appendix | Measures of Location and Spread for Lognormally Distributed Variables |

## GLOSSARY

This glossary pertains to biological assays and provides a compendial perspective that is consistent across *USP–NF*'s suite of bioassay chapters, is complementary to previous authoritative usage, and provides a useful focus on the bioassay context. In many cases the terms cited here have common, though undocumented, usages or are defined in *Validation of Compendial Procedures* ⟨1225⟩ and in the International Conference on Harmonization (ICH) Guideline Q2(R1), *Validation of Analytical Procedures: Text and Methodology*.[1] (Chapter ⟨1225⟩ and ICH Q2(R1) agree on definitions.) The *Glossary* is intended to be consistent with these precedent usages, and notes are provided when a difference arises because of the bioassay context. Definitions from ⟨1225⟩ and ICH Q2(R1) are identified as, for example, " ⟨1225⟩" if taken without modification, or "adapted from ⟨1225⟩" if taken with minor modification for application to bioassay. Most definitions are accompanied by notes that elaborate on the bioassay context.

The terms are organized alphabetically within five topic sections:

I. General terms related to bioassays
II. Terms related to performing a bioassay
III. Terms related to precision and accuracy
IV. Terms related to validation
V. Terms related to statistical design and analysis

*Table 4* shows each term and the *Glossary* section in which it can be found.

**Table 4. Terms Listed in the *Glossary***

| Term | Section | Term | Section |
|---|---|---|---|
| Accuracy | III | Mixed-effects model | V |
| Analysis of variance (ANOVA) | V | Modeling, statistical | V |
| Analytical procedure | I | Nested | V |
| Assay | I | Out of specification | II |
| Assay data set | I | Parallelism | V |
| Bioassay | I | Partially crossed | V |
| Biological assay | I | Point estimate | V |
| Blocking | V | Potency | I |
| Complete block design | V | Precision | III |
| Confidence interval | V | Pseudoreplication | V |
| Crossed | V | *P* value | V |
| Design of experiments (DOE) | V | Quantitation limit | IV |
| Dilutional linearity | IV | Random effect | V |
| Direct bioassays | I | Random error | III |
| Equivalence test | V | Random factor | V |
| Errors, types of | III | Randomization | V |
| Expected mean square | V | Range | IV |
| Experimental design | V | Relative bias | III |
| Experimental unit | V | Relative potency | I |

[1] Available at: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf. Accessed 29 March 2012.

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**Table 4. Terms Listed in the *Glossary*** *(continued)*

| Term | Section | Term | Section |
|---|---|---|---|
| Factor | V | Repeatability | III |
| Factorial design | V | Replication | V |
| Fixed effect | V | Reportable value | I |
| Fixed factor | V | Reproducibility | III |
| Format variability | III | Robustness | IV |
| Format, bioassay | II | Run | I |
| Fractional factorial design | V | Sample suitability | II |
| Full factorial design | V | Significance probability | V |
| General linear model | V | Similar preparations | I |
| Geometric coefficient of variation | III | Similarity (algebraic) | I |
| Geometric standard deviation | III | Specificity | III |
| Incomplete block design | V | Standard error of estimate | V |
| Independence | V | Statistical process control (SPC) | V |
| Indirect bioassays | I | System suitability | II |
| Interaction | V | Systematic error | III |
| Intermediate precision | III | True replicates | V |
| Level | V | Truncation bias | III |
| Linearity, dilutional | IV | Type I error | V |
| Lognormal distribution | V | Type II error | V |
| Lower limit of quantitation | IV | Validation, assay | IV |
| Mean square | V | Variance component analysis | V |

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## I. General Terms Related to Bioassays

### ANALYTICAL PROCEDURE [ADAPTED FROM Q2(R1)]

Detailed description of the steps necessary to perform the analysis.

[NOTE—1. The procedure may include but is not limited to the sample preparation, the Reference Standard, and the reagents; use of equipment; generation of the standard curve; use of the formulae for the calculation; etc. 2. An FDA Guidance[2] provides a list of information that typically should be included in the description of an analytical procedure.]

### ASSAY

Analytical procedure to determine the quantity of one or more components or the presence or absence of one or more components.

[NOTE—1. *Assay* often is used as a verb synonymous with *test* or *evaluate*, as in "I will assay the material for impurities." In this glossary, *assay* is a noun and is synonymous with *analytical procedure* (q.v.). 2. The phrase *to run the assay* means to perform the analytical procedure as specified. 3. In common practice, *assay* and *run* (q.v.) often are used interchangeably. In this glossary, they are different. Also see *bioassay* and *bioassay data set*.]

### ASSAY DATA SET

The set of data used to determine a single potency or relative potency for all samples included in the bioassay.

[NOTE—1. The definition of an assay data set can be subject to interpretation as necessarily a minimal set. It may be possible to determine a potency or relative potency from a set of data but not do this well. It is not the intent of this definition to mean that an assay data set is the minimal set of data that can be used to determine a relative potency. In practice, an assay data set should include, at least, sufficient data to assess similarity (q.v.). It also may include sufficient data to assess other assumptions. 2. It is also not an implication of this definition that assay data sets used together in determining a reportable value (q.v.) are necessarily independent from one another, although it may be desirable that they be so. When a run (q.v.) consists of multiple assay data sets, independence of assay sets within the run must be evaluated.]

[2] Analytical Procedures and Methods Validation for Drugs and Biologics, Guidance for Industry. 2015. http://www.fda.gov/ucm/groups/fdagov-public/@fdagov-drugs-gen/documents/document/ucm386366.pdf. Accessed 20 April 2016.

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## BIOASSAY, BIOLOGICAL ASSAY (these terms are interchangeable)

Analysis (as of a drug) to quantify the biological activity or activities of one or more components by determining its capacity for producing an expected biological activity on a culture of living cells (in vitro) or on test organisms (in vivo), expressed in terms of units.

[NOTE—1. The components of a bioassay include the analytical procedure, the statistical design for collecting data, and the method of statistical analysis that eventually yields the estimated potency or relative potency. 2. Bioassays can be either direct or indirect.

**Direct bioassays—**Bioassays that measure the concentration of a substance that is required in order to elicit a specific response. For example, the potency of digitalis can be directly estimated from the concentration required to stop a cat's heart. In a direct assay, the response must be distinct and unambiguous. The substance must be administered in such a manner that the exact amount (threshold concentration) needed to elicit a response can be readily measured and recorded.

**Indirect bioassays—**Bioassays that compare the magnitude of responses for nominally equal concentrations of reference and test preparations rather than test and reference concentrations that are required to achieve a specified response. Most biological assays in *USP–NF* are indirect assays that are based on either quantitative or quantal (yes/no) responses.]

## POTENCY [21 CFR 600.3(S)]

The specific ability or capacity of the product, as indicated by appropriate laboratory tests or by adequately controlled clinical data obtained through the administration of the product in the manner intended, to effect a given result.

[NOTE—1. A wholly impotent sample has no capacity to produce the expected specific response, as a potent sample would. Equipotent samples produce equal responses at equal dosages. Potency typically is measured relative to a Reference Standard or preparation that has been assigned a single unique value (e.g., 100.0) for the assay; see *relative potency.* At times, additional qualifiers are used to indicate the physical standard employed (e.g., "international units"). 2. Some biological products have multiple uses and multiple assays. For such products there may be different reference lots that do not have consistently ordered responses across a collection of different relevant assays. 3. [21 CFR 610.10] Tests for potency shall consist of either in vitro or in vivo tests, or both, which have been specifically designed for each product so as to indicate its potency in a manner adequate to satisfy the interpretation of potency given by the definition in 21 CFR 600.3(s).]

## RELATIVE POTENCY

A measure obtained from the comparison of a Test to a Standard on the basis of capacity to produce the expected potency.

[NOTE—1. A frequently invoked perspective is that relative potency is the degree to which the Test preparation is diluted or concentrated relative to the Standard. 2. Relative potency is unitless and is given definition, for any test material, solely in relation to the reference material and the assay.]

## REPORTABLE VALUE

The value that will be compared to an acceptance criterion.

[NOTE—1. The acceptance criterion for comparison may be in the *USP* monograph, or it may be set by the company, e.g., for product release. 2. The term *reportable value* is inextricably linked to the "intended use" of an analytical procedure. Assays are performed on samples in order to yield results that can be used to evaluate some parameter. Assays may have different summary values or formats for different purposes (e.g., lot release vs. calibration of a new reference standard). The reportable value may be different even if the mechanics of the test itself are identical. Validation is required in order to support the properties of each choice of reportable value. In practice there may be one physical document that is the analytical procedure used for more than one application, but each application must be detailed separately within that document. Alternatively, there may be separate documents for each application. 3. When the inherent variability of a biological response, or that of the log potency, precludes a single assay data set's attaining a value sufficiently accurate and precise to meet a specification, the assay format may be changed as necessary. The number of blocks or complete replicates needed depends on the assay's inherent accuracy and precision and on the intended use of the reported value. It is practical to improve the precision of a reported value by reporting the geometric mean potency from multiple assays. The number of assays used is determined by the relationship between the precision required for the intended use and the inherent precision of the assay system.]

## RUN

The performance of the analytical procedure that can be expected to have consistent precision and trueness; usually, the assay work that can be accomplished by a single analyst in a set time with a given unique set of assay factors (e.g., standard preparations).

[NOTE—1. There is no necessary relationship of run to assay data set (q.v.). The term *run* is laboratory specific and relates to the laboratory's physical capability and environment for performing the work of an assay. An example of a run is given by one analyst's simultaneous assay of several samples in one day's bench work. During the course of a single run, it may be possible to determine multiple reportable values. Conversely, a single assay data set may include data from multiple runs. 2. From a statistical viewpoint, a run is one realization of the factors associated with intermediate precision (q.v.). Within-run variability is thus repeatability. It is good practice to associate runs with factors that are significant sources of variation in the assay. For example, if cell passage number is an important source of variation in the assay response obtained, then each change in cell passage number initiates a new run. If the variance associated with all factors that could be assigned to runs is negligible, then the influence of runs can be ignored in the analysis, and the analysis can focus on combining independent analysis data sets. 3. When a run contains multiple assays, caution is required regarding the independence of the assay results. Factors that typically

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are associated with runs and that cause lack of independence include cell preparations, groups of animals, analyst, day, a common preparation of reference material, and analysis with other data from the same run. Even though a strict sense of independence may be violated because some elements are shared among the assay sets within a run, the degree to which independence is compromised may have negligible influence on the reportable values obtained and should be verified and monitored.]

### Similar preparations

The property that the Test and the Standard contain the same effective constituent, or the same effective constituents in fixed proportions, and all other constituents are without effect in some specific assay context.

[Note—1. Having similar preparations is often summarized as the property that the Test behaves as a dilution (or concentration) of the Standard. 2. Similar preparations are fundamental to methods for determination of relative potency. Given similar preparations, a relative potency can be calculated, reported, and interpreted. In the absence of similar preparations, a meaningful relative potency cannot be reported or interpreted. 3. The practical consequence of similar preparations is algebraic similarity (q.v.). (Also see *Parallelism*, *section V.*)]

### Similarity (algebraic)

The Test and Standard concentration-response curves are algebraically related in a manner consistent with similar preparations.

[Note—1. Examples of similarity are parallelism (q.v.) of concentration–response curves and equality of intercepts in slope ratio models. 2. Failure to statistically demonstrate dissimilarity between a Reference and a Test does not amount to demonstration of similarity. To demonstrate similarity an equivalence approach is appropriate; see ⟨1032⟩ and ⟨1034⟩. 3. Similarity is typically a sample suitability (q.v.) criterion. Note, however, that suitability is a necessary but not sufficient condition for preparations to be similar. In practice, absent knowledge of differences between the Test and Standard materials, demonstration of similarity is accepted as demonstrating similar preparations.]

## II. Terms Related to Performing a Bioassay

### Format, bioassay

The intra- and inter-run replication strategy for replication of assay data sets that has been determined by variance analysis to support the use of the bioassay.

[Note—1. Modifications to bioassay format may occur as new information regarding sources of variability becomes available. Such modifications do not include changes to the dilution scheme of Test samples or Standard, or the replication strategy (part of what is sometimes called bioassay configuration). Assay configuration can include nested dimensions like plate design, multiple plates per day, single plates on multiple days, etc. 2. The geometric mean relative potency determined from the bioassay format is the reportable value, which may be used to assess conformance to specifications or as a component of subsequent analysis (e.g., stability evaluation).]

### Out of specification (OOS)

The property of a reportable value that falls outside its specification acceptance criterion.

[Note—*Out of specification* is not a property of the bioassay but rather a property of Test samples. The term is introduced into ⟨1033⟩ in conjunction with setting validation acceptance criteria which limit the risk of producing out-of-specification test results because of bioassay performance characteristics.]

### Sample suitability

A sample is suitable (may be used in the estimation of potency) if its response curve satisfies limits on critical properties that are stated in the assay procedure.

[Note—Response curve properties are to be taken generally; i.e., includes outliers and variability. The most significant of these properties for bioassays is similarity (q.v.) to the standard response curve. In addition, all assay systems have limits on the range of values they can report. For samples that fail one or more sample suitability criteria in a bioassay, the potency estimate from those samples should not be used as a reportable value or as a contributor to a reportable value. Also see *truncation bias* in this *Glossary* and the sections *Sample Suitability* and *Range* in general chapter ⟨1032⟩.]

### System suitability

An assay system is suitable for its intended purpose if it is capable of providing legitimate measurements as defined in the assay protocol.

[Note—System suitability may be thought of as an assessment of whether there is any evidence of a problem in the assay system. An example is provided by positive and negative controls, where values outside their normal ranges suggest that the assay system is not working properly.]

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## III. Terms Related to Precision and Accuracy

### ACCURACY ⟨1225⟩

The closeness of test results obtained by the procedure and the true value.

[NOTE—1. ICH and USP give the same definition of accuracy. However, ISO specifically regards accuracy as having two components, bias and precision.[3] That is, to be *accurate* as used by ISO, a measurement must both be on target (have low bias) and be precise. In contrast, ICH Q2(R1) states that accuracy is sometimes termed "trueness" but does not define *trueness*. ISO defines *trueness* as the "closeness of agreement between the average value obtained from a large series of test results and an accepted reference value" and indicates that "trueness is usually expressed in terms of bias." The 2001 FDA Guidance on Bioanalytical Method Validation[4] defines *accuracy* in terms of "closeness of *mean* test results obtained by the method to the true value (concentration) of the analyte" (emphasis added) and thus is consistent with the ICH usage. This glossary adopts the USP/ICH approach. That is, *accuracy* is defined as the agreement between the mean (or expected results) from an assay and the true value, and uses the phrase *accurate and precise* to indicate low bias (accurate) and low variability (precise). 2. Considerable caution is needed when using or reading the term *accuracy*. In addition to the inconsistency between USP/ICH and ISO, common usage is not consistent. 3. For purposes of bioassay validation, the terms *accuracy* and *bias* have been replaced by *relative accuracy* and *relative bias*.]

### ERROR, TYPES OF

Two sources of errors that affect the uncertainty of results of a biological assay are systematic error and random error.

A **systematic error** is one that happens with similar magnitude and consistent direction repeatedly. This introduces a bias in the determination. Effective experimental design, including randomization and/or blocking, can reduce systematic error.

A **random error** is one whose magnitude and direction vary without pattern. Random error is an inherent variability or uncertainty of the determination. Conversion of systematic into random error, through experimental design or randomization, increases the robustness of a biological assay and allows a comparatively simple analysis of assay data but may require a larger sample size.

### FORMAT VARIABILITY

Predicted variability for a particular bioassay format.

### GEOMETRIC COEFFICIENT OF VARIATION

Found as antilog($S$)−1, where $S$ is the standard deviation determined in the log scale.

[NOTE—The geometric coefficient of variation is usually reported as a percentage (%GCV). It is important not to confuse the %GCV with the %CV. The %GCV is a measure of spread relevant to data analyzed in the log-transformed [$Y = \log(X)$] scale, and the %CV is a measure relevant to data analyzed in the original ($X$) scale.]

### GEOMETRIC STANDARD DEVIATION (GSD)

The variability of the log-transformed values of a lognormal response expressed as a percentage in the untransformed scale. It is found as antilog($S$), where $S$ is the standard deviation determined in the log scale.

[NOTE—For example, if the standard deviation of log potency is $S$ using log base 2, the GSD of potency is $100 * 2^S$.]

### INTERMEDIATE PRECISION (ADAPTED FROM ⟨1225⟩)

Within-laboratory precision associated with changes in operating conditions.

[NOTE—1. Factors contributing to intermediate precision involve anything that can change within a given laboratory and that may affect the assay, including different days, different analysts, different equipment, etc. Intermediate precision is thus "intermediate" in scope between the extremes of repeatability (intra-assay) and reproducibility (inter-laboratory). 2. Any statement of intermediate precision should identify the factors that varied. For example, "The intermediate precision associated with changing equipment and operators is…." 3. Investigators can benefit from separately identifying the precision associated with each source (e.g., inter-analyst precision). This may be part of assay development and validation when there is value in identifying the important contributors to intermediate precision. 4. When reporting intermediate precision, particularly for individual sources, care should be taken to distinguish between intermediate precision variance and components of that variance. The intermediate precision *variance* includes repeatability and thus must be at least as large as the repeatability variance. A variance *component*, e.g., associated with analyst, also is a part of the intermediate precision variance for analyst, but it could be negligible and need not be larger in magnitude than the repeatability variance.]

[3] ISO. International Standard 5725-1. Accuracy (Trueness and Precision) of Measurement Methods and Results—Part 1: General Principles and Definitions. Geneva, Switzerland; 1994.

[4] FDA. Guidance for Industry. Bioanalytical Method Validation. May 2001. http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070107.pdf. Accessed 7 December 2011.

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### PRECISION ( ⟨1225⟩)

Measure of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of a homogeneous sample.

[NOTE—1. Precision may be considered at three levels: repeatability (q.v.), intermediate precision (q.v.), and reproducibility (q.v.). 2. Precision should be investigated using homogeneous, authentic samples. However, if it is not possible to obtain a homogeneous sample, precision can be investigated using spiked samples that mimic a true sample or a sample solution. 3. Precision may be expressed as the variance, standard deviation, coefficient of variation, or geometric coefficient of variation (q.v.).]

### RELATIVE BIAS

Measure of difference between the expected (or mean) value and the true value, expressed as a percentage of the true value.

### REPEATABILITY ( ⟨1225⟩)

The precision within a laboratory over a short interval of time, using the same analyst with the same equipment.

[NOTE—1. ICH Q2(R1) says that repeatability is also termed "intra-assay" precision. In the bioassay context, the better term is *intra-run*, and a "short interval of time" connotes *within-run*. 2. The idea of a "short interval of time" can be problematic with bioassays. If a run requires multiple weeks and consists of a single assay set, then intra-run precision cannot be determined. Alternatively, if a run consists of two assay data sets and a run can be done in a single day, repeatability of the relative potency determination can be assessed.]

### REPRODUCIBILITY ⟨1225⟩

The precision between laboratories.

[NOTE—1. Reproducibility includes contributions from repeatability and all factors that contribute to intermediate precision, as well as any additional contributions from inter-laboratory differences. 2. Reproducibility applies to collaborative studies such as those for standardization or portability of methodology. Depending on the design of the collaborative study, it may be possible to separately describe variance components associated with intra- and inter-laboratory sources of variability.]

### SPECIFICITY ⟨1225⟩

The ability to assess unequivocally the analyte in the presence of components that may be expected to be present.

[NOTE—1. Typically these components may include impurities, degradants, matrix, etc. See chapter ⟨1225⟩ for further discussion. 2. This definition is also associated with selectivity in other guidances for analytical methods. 3. Specificity can mean the measurement of the specific analyte of interest and no other similar analyte.]

### TRUNCATION BIAS

Bias that occurs when some portion of the distribution of responses is not observed or recorded.

[NOTE—1. When there is truncation bias, the distribution of recorded observations does not match the true distribution of responses. 2. Truncation bias may occur in a bioassay that does not report estimates of log potency outside a set potency range. For example, a sample with a true potency at an edge of this range is expected to fail to yield (report) a potency estimate in approximately half of the assays in which it appears. In this example, the mean of the observed potencies will be biased toward log potency 0.]

## IV. Terms Related to Validation

### DILUTIONAL LINEARITY (ADAPTED FROM ⟨1225⟩)

The ability (within a given range) of a bioassay to obtain measured relative potencies that are directly proportional to the true relative potency of the samples.

[NOTE— 1. To determine dilutional linearity, sometimes called bioassay analytical linearity, across a range of known relative potency values, analysts examine the relationship between known log potency and mean observed log potency. If that relationship yields an essentially straight line with a *y*-intercept of 0 and a slope of 1, the assay has direct proportionality. If that plot yields an essentially straight line but either the *y*-intercept is not 0 or the slope is not 1, the assay has a proportional linear response. 2. To assess whether the slope is (near) 1.0 requires an a priori equivalence or indifference interval. It is not proper statistical practice to test the null hypothesis that the slope is 1.0 against the alternative that it is not 1.0 and then to conclude a slope of 1.0 if this is not rejected. Bioassay analytical linearity is separate from consideration of the shape of the concentration–response curve. Linearity of concentration–response is not a requirement of bioassay analytical linearity since bioassay analytical linearity is possible regardless of the form of the concentration–response curve. 3. Dilutional linearity is further addressed in ⟨1033⟩.]

### QUANTITATION LIMIT (LOWER LIMIT OF QUANTITATION; ADAPTED FROM ⟨1225⟩)

The lowest known relative potency for which the assay has suitable precision and accuracy.

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[NOTE—1. This applies to assay results (log potency) rather than the reportable value. 2. The quantitation limit is not commonly determined for relative potency bioassays. Animal assays with serologic endpoints are examples of the use of this term.]

### RANGE (ADAPTED FROM ⟨1225⟩)

The interval between the upper and lower known relative potencies (and including those relative potencies) for which the bioassay is demonstrated to have a suitable level of precision, accuracy, and bioassay analytical linearity.

[NOTE—This applies to reportable values (typically a geometric mean) rather than the individual assay results.]

### ROBUSTNESS ( ⟨1225⟩)

A measure of an analytical procedure's capacity to remain unaffected by small but deliberate variations in method parameters listed in the procedure documentation.

[NOTE—1. Robustness is an indication of a bioassay's reliability during normal usage. For example, a cell culture assay system that is robust to the passage number of the cells can provide potency values with acceptable accuracy and precision across a consistent range of passage numbers. 2. ICH Q2(R1) states:

> The evaluation of robustness should be considered during the development phase and depends on the type of procedure under study. It should show the reliability of an analysis with respect to deliberate variations in method parameters. If measurements are susceptible to variations in analytical conditions, the conditions should be suitably controlled or a precautionary statement should be included in the procedure. One consequence of the evaluation of robustness should be that a series of system suitability [q.v.] parameters (e.g., resolution test) is established to ensure that the validity of the analytical procedure is maintained whenever used.[1] ]

### VALIDATION, ASSAY

Assay validation is the process of demonstrating and documenting that the performance characteristics of the procedure and its underlying method meet the requirements for the intended application and that the assay is thereby suitable for its intended use.

[NOTE—Formal validations are conducted prospectively according to a written plan that includes justifiable acceptance criteria on validation parameters. See ⟨1033⟩.]

## V. Terms Related to Statistical Design and Analysis

### ANALYSIS OF VARIANCE (ANOVA)

A statistical tool used to assess contributions to variability from experimental factors.

### BLOCKING

The grouping of related experimental units in experimental designs.

[NOTE—1. Blocking often is used to reduce the contribution to variability associated with a factor not of primary interest. 2. Blocks may consist, for example, of groups of animals (a cage, a litter, or a shipment), individual 96-well plates, sections of 96-well plates, or whole 96-well plates grouped by analyst, day, or batches of cells. 3. The goal is to isolate, by statistical design and analysis, a systemic effect, such as cage, so that it does not obscure the comparisons of interest.

> A **complete block design** occurs when all levels of a treatment factor (in a bioassay, the primary treatment factors are sample and concentration) can be applied to experimental units for that factor within a single block. Note that the two treatment factors *sample* and *concentration* may have different experimental units. For example, if the animals within a cage are all assigned the same concentration but are assigned unique samples, then the experimental unit for concentration is cage and the experimental unit for sample is animal, and cage is a blocking factor for sample.
>
> An **incomplete block design** occurs when the number of levels of a treatment factor exceeds the number of experimental units for that factor within the block.]

### CONFIDENCE INTERVAL

A random interval produced by a statistical method that contains the true (fixed, but unknown) parameter value with a stated confidence level on repeated application of the statistical method.

[NOTE—See chapter ⟨1010⟩ for more information.]

### CROSSED (AND PARTIALLY CROSSED)

Two factors are crossed (or fully crossed) if each level of each factor appears with each level of the other factor. Two factors are partially crossed when they are not fully crossed but multiple levels of one factor appear with a common level of the other factor.

[NOTE—1. For example, in a bioassay in which all samples appear at all dilutions, samples and dilutions are (fully) crossed. In a bioassay validation experiment in which two of four analysts each perform assays on the same set of samples on each of six days and a different pair of analysts is used on each day, the analysts are partially crossed with days. 2. Each factor may be applied to different experimental units, and the factors may be both fully crossed and nested (q.v.), creating a split-unit or split-plot design (q.v.). 3. Experiments with factors that are partially crossed require particular care for proper analysis. 4. A

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randomized complete block design (q.v.) is a design in which the block factor (which often is treated as a random effect) is crossed with the treatment factor (which usually is treated as a fixed effect).]

## Design of experiments (DOE) [ICH Q8(R2)][5]

A structured, organized method for determining the relationship between factors that affect a process and the output of that process.

[Note—DOE is used in bioassay development and validation; see ⟨1032⟩ and ⟨1033⟩.]

## Equivalence test

A test to demonstrate equivalence (e.g., similarity or conformance to validation acceptance criteria) of two quantities by conformance to an interval acceptance criterion.

[Note—1. An equivalence test differs from most common statistical tests in the nature of the statistical hypotheses. Most common statistical tests are difference tests—that is, the statistical null hypothesis is that of no difference, and the alternative is that there is some difference, without regard to the magnitude or importance of the difference. The difference may be between a characteristic of two populations or between a characteristic of a single population and an accepted value. In equivalence testing the null hypothesis is that the difference is not sufficiently small, and the alternative hypothesis is that the difference is sufficiently small that there is no important difference. In a common statistical difference test one concludes that there is insufficient evidence to establish nonconformance to an acceptance criterion. This may be the result of excess variability and/or an inadequate design. In an equivalence test the conclusion is that the data conform to the acceptance criterion (e.g., slopes are parallel). 2. A common statistical procedure used for equivalence tests is the two one-sided tests (TOST) procedure. 3. The interval acceptance criterion may be one- or two-sided. An example of a one-sided interval is a validation acceptance criterion for a %GCV of not more than XX%]

## Expected mean square

A mathematical expression of variances estimated by an ANOVA mean square.

## Experimental design

The structure of assigning treatments to experimental units.

[Note—1. Some aspects of experimental design are blocking (q.v.), randomization (q.v.), replication (q.v.), and specific choice of design (cf. ⟨1032⟩). 2. Important components of experimental design include the number of samples, the number of concentrations, and how samples and concentrations are assigned to experimental units and are grouped into blocks. 3. The experimental design influences which statistical methodology should be used to achieve the analytical objective.]

## Experimental unit

The smallest unit to which a distinct level of a treatment is randomly allocated.

[Note—1. Randomization of treatment factors to experimental units is essential in bioassays. 2. Different treatment factors can be applied to different experimental units. For example, samples may be assigned to rows on a 96-well plate, and dilutions may be assigned to columns on the plate. In this case, rows are the experimental units for samples, columns are the experimental units for concentrations, and wells are the experimental units for the interaction of sample and concentration. 3. An experimental unit must be distinguished from a sampling unit, the smallest unit on which a distinct measurement is recorded (e.g., a well). Because the sampling unit is often smaller than the experimental unit, it is an easy mistake to treat sampling units as if they are experimental units. This mistake is called pseudoreplication (q.v.).]

## Factor

An assay parameter or operational element that may affect assay response and that varies either within or across assay runs.

[Note—In a bioassay there are at least two treatment factors: sample and concentration.

A **fixed factor (fixed effect)** is a factor that is controllable and deliberately set at specific levels in a bioassay. Inference is made to the levels used in the experiment or intermediate values. Sample and concentration are examples of fixed factors in bioassays.

A **random factor (random effect)** is one which is generally not controllable and for which its levels represent a sample of ways in which that factor might vary. In a bioassay, the test organisms, plate, and day are often considered random factors. Whether a factor is treated as random or fixed may depend on the experiment and questions asked.]

## Factorial design

An experimental design in which there are multiple factors and the factors are partially or fully crossed.

In a **full factorial design**, each level of a factor appears with all combinations of levels of all other factors. For example, if factors are reagent batch and incubation time, for a full factorial design all combinations of incubation time and reagent batch must be included.

[5] ICH. Guidance Q8(R2) Pharmaceutical Development. November 2009. Available at http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM073507.pdf. Accessed 27 December 2011.

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A **fractional factorial design** is a reduced design in which each level of a factor appears with only a subset of combinations of levels of all other factors and some factor effects (main effects and/or interactions) are deliberately confounded with other combinations of factor effects. Fractional factorial designs should be carefully considered for screening and optimization purposes. This design can be considered without risk of information loss for validation.

## General linear model

A statistical linear model that relates study factors, which can be continuous or discrete, to experimental responses.

## Independence

For two measurements or observations *A* and *B* (raw data, assay sets, or relative potencies) to be independent, values for *A* must be unaffected by *B*'s responses and vice versa.

[Note—A consequence of the failure to recognize lack of independence is poor characterization of variance. In practice this means that if two potency or relative potency measurements share a common factor that might influence assay outcome (e.g., analyst, cell preparation, incubator, group of animals, or aliquot of Standard samples), then the correct initial assumption is that these relative potency measurements are not independent. The same concern for lack of independence holds if the two potency or relative potency measurements are estimated together from the same model or are in any way associated without including in the model some term that captures the fact that there are two or more potency measurements. As assay experience is gained, an empirical basis may be established (and monitored) so that it is reasonable to treat potency measurements as independent even if the measurements share a common level of a factor. This is the case when it has been demonstrated that a factor does not have a practically significant effect on long-term bioassay results.]

## Interaction

Two factors are said to interact if the response to one factor depends on the level of the other factor.

## Level

A location on the scale of measurement of a factor.

[Note—1. Factors have two or more distinct levels. For example, if a bioassay validation experiment employs three values of incubation time and two batches of a key reagent, the levels are the three times for the factor *incubation time* and the two batches for the factor *batch*. 2. Levels of a factor in a bioassay may be quantitative, such as concentration, or categorical, such as sample (i.e., test and reference).]

## Lognormal distribution

A distribution of values (assay responses or potencies) where the logarithms of the values have a normal distribution.

[Note—1. Most relative potency bioassay measurements are lognormally distributed. 2. The lognormal is a skewed distribution characterized by increased variability with increased level of response.]

## Mean square

A calculation in ANOVA representing the variability associated with an experimental factor.

## Mixed-effects model

A statistical model that includes both fixed and random effects.

## Modeling, statistical

The mathematical specification of the relationship between inputs (*X*s) and outputs (*Y*s) of a process, e.g., the concentration–response relationship in bioassay or the modeling of the effects of important sources of variation on potency measurement.

[Note—1. Modeling includes methods to capture the dependence of the response on the samples, concentration, experimental units, and groups or blocking factors in the assay configuration. 2. Modeling of bioassay data includes making many choices, some of which are driven by the assay design and data. For continuous data there is a choice between linear and nonlinear models. For discrete data there is a choice among logit/log models within a larger family of generalized linear models. In limiting dilution assays, published literature advocates Poisson models and Markov chain binomial models. One can use either fixed-effects models or mixed-effects models for bioassay data. On the one hand, the fixed-effects models are more widely available in software and are somewhat less demanding for statisticians to set up. On the other hand, mixed models have advantages over fixed ones: they are more accommodating of missing data and, more importantly, can allow each block to have different slopes, asymptotes, median effective concentrations required to induce a 50% effect ($EC_{50}$), or relative potencies. Particularly when the analyst is using straight-line models fitted to nonlinear responses or assay systems in which the concentration–response curve varies from block to block, the mixed model captures the behavior of the assay system in a much more realistic and interpretable way. 3. It is essential that any modeling approach for bioassay data should use all available data simultaneously to estimate the variation (or, in a mixed model, each of several sources of variation). It may be necessary to transform the observations before this modeling to include a variance model or to fit a means model (in which there is a predicted effect for each combination of sample and concentration) to get pooled estimate(s) of variation.]

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## NESTED

A factor *A* is nested within another factor *B* if the levels of *A* are different for every level of *B*.

[NOTE—1. For example, in a bioassay validation experiment two analysts may perform assays on five days each. If the calendar days for the first analyst are distinct from those of the second analyst, days are nested within analyst. 2. Nested factors have a hierarchical relationship. 3. For two factors to be nested they must satisfy the following: (a) they are applied to different-sized experimental units; (b) the larger experimental unit contains more than one of the smaller experimental units; and (c) the factor applied to the smaller experimental unit is not fully crossed (q.v.) with the factor applied to the larger experimental unit. When conditions (a) and (b) are satisfied and the factors are partially crossed, then the experiment is partially crossed and partially nested. Experiments with this structure require particular care for proper analysis.]

## PARALLELISM (OF CONCENTRATION–RESPONSE CURVES)

A quality in which the concentration–response curves of the Test sample and the Reference Standard are identical in shape and differ only by a horizontal difference that is a constant function of relative potency.

[NOTE—1. When Test and Reference preparations are similar (q.v.) and assay responses are plotted against log concentrations, the resulting curve for the Test preparation will be the same as that for the Standard but will be shifted horizontally by an amount that is the logarithm of the relative potency. Because of this relationship, similarity (q.v.) is often equated with *parallelism* but they are not the same. See section 3.5, *Slope-Ratio Concentration–Response Models*, in chapter ⟨1034⟩, in which similar samples have concentration–response relationships with a common (or nearly common) *y*-intercept but may differ in their slopes. 2. In practice, it is not possible to demonstrate that the shapes of two curves are identical. Instead, the two curves are shown to be sufficiently algebraically similar (equivalent) in shape. Note that *similar* should be interpreted as "we have evidence that the two curves are close enough in shape" rather than "we do not have evidence that the two curves differ in shape." 3. The assessment of parallelism depends on the type of function used to fit the response curve. Parallelism for a nonlinear assay using a four-parameter logistic fit means that (a) the slopes of the rapidly changing parts of the Test and Reference Standard curves (that is, slope at a tangent to the curve where the first derivative is at a maximum) should be similar; and (b) the upper and lower asymptotes of the response curves (plateaus) should be similar. For straight-line analysis, the slopes of the lines should be similar.]

## POINT ESTIMATE

A single-value estimate obtained from statistical calculations.

[NOTE—1. Examples are the average relative bias, the %GCV, and relative potency. 2. The point estimate may be augmented with an *interval estimate* (confidence interval; q.v.) that employs an interval to express the uncertainty in the determination of the point estimate.]

## PSEUDOREPLICATION

The misidentification of samples from experimental units as independent and thus true replicates when they actually are not independent.

[NOTE—1. Pseudoreplication results in incorrect inferences because of the incorrect assignment of variability and the appearance of more replicates than are actually present. 2. Lack of recognition of pseudoreplication is critical because it is an easy mistake to make, and the consequences can be serious. For example, pseudoreplicates commonly arise when analysts make a dilution series for each sample in tubes (the dilution series can be made with serial dilutions, single-point dilutions, or any convenient dilution scheme). The analyst then transfers each dilution of each sample to several wells on one or more assay plates. The wells are then pseudoreplicates because they are simply aliquots of a single dilution process and thus are not representative of independent preparations. 3. A simple way to analyze data from pseudoreplicates is to average over the pseudoreplicates (if a transformation of the observed data is used, the transformation should be applied before averaging over pseudoreplicates) before fitting any concentration–response model. In many assay systems, averaging over pseudoreplicates leaves the assay without any replication. A more complex way to use data containing pseudoreplicates is to use a mixed model that treats the pseudoreplicates as a separate random effect. Although pseudoreplication normally is of little value, it can be advantageous when two conditions are satisfied: (a) the pseudoreplicate (e.g., well-to-well) variation is very large compared to the variation associated with replicates; and (b) the cost of pseudoreplicates is much lower than the cost of replicate experimental units.]

## *P* VALUE (SIGNIFICANCE PROBABILITY)

The probability of observing, in repeated trials, that an experimental outcome is as different or more different than that observed if the null hypothesis is true.

[NOTE—1. *More different* means further from the null hypothesis. 2. Commonly, $P<0.05$ is taken as a threshold for indicating statistically significant differences, although any value for the threshold may be used. Bases for choosing the threshold are the risks (costs) of making a wrong decision; see *type I error* and *type II error*.]

## RANDOMIZATION

A process of assignment of treatment to experimental units based on chance so that all equal-sized subgroups of units have an equal chance of receiving a given treatment.

[NOTE—1. The chance mechanism may be an unbiased physical process (rolling unbiased dice, flipping coins, drawing from a well-mixed urn), random-number tables, or computer-generated randomized numbers. Care must be taken in the choice and

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use of method. Good practice is to use a validated computerized random-number generator. 2. The use of randomization can help to prevent systematic error from becoming associated with particular samples or a dilution pattern and causing bias. For example, in 96-well bioassays, plate effects can be substantial and can cause bias in observed responses or summary measures. In animal studies, a variety of factors associated with individual animals can influence responses. If extraneous factors that influence either plate assays or animal assays are not routinely demonstrated to have been eliminated or minimized so as to be negligible, randomization is essential to obtaining unbiased data required for the calculation of true potency. 3. Randomization is a good practice even when there is evidence that operational factors (e.g., location, time, reagent lot) have little or no effect on the assay system. While randomization may not protect an individual assay (or perhaps a block of an assay) from a (perhaps newly) important operational factor, randomization provides assurance that results from a collection of assays are not biased due to operational factors.]

## Replication

A process in which multiple independent experimental units receive the same level of a treatment factor.
[Note—1. The purpose of replication is to minimize the effects of uncontrollable sources of random variability. 2. Replication can occur either completely at random or across blocks. Generally, replication within blocks is pseudoreplication (q.v.). 3. Replication of factors that contribute most greatly to variability, or factors that are at the highest levels in a nested layout, usually result in the most effective reduction of random variability.]

## True replicates

Samples based on independent experimental units.

## Standard error of estimate

A measure of uncertainty of an estimate of a reportable value or other parameter estimate because of sampling variation.
[Note—1. In bioassay the focus is on the precision (standard error) of the relative potency. 2. Standard errors can be made smaller with additional replication. 3. Technically, the standard error of an estimate is the standard deviation of the sampling distribution of the estimate. The term *standard error* is used to distinguish between this usage of standard deviation (that depends on sample size) and the common laboratory usage in which standard deviation (or coefficient of variation) is used to characterize the precision of individual measurements obtained from a procedure. This latter precision does not depend on sample size.]

## Statistical process control

A set of statistical methods used to monitor shifts and trends in a process.

## Type I error

The error in statistical hypothesis testing that the alternative hypothesis is accepted when it is false.
[Note—The probability of a type I error usually is denoted by $\alpha$.]

## Type II error

The error in statistical hypothesis testing that the alternative hypothesis is rejected when it is true.
[Note—The probability of a type II error usually is denoted by $\beta$.]

## Variance component analysis

A statistical analysis that partitions contributions made to total variability by components associated with influential assay factors, e.g., analyst, day, or instrument.

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# ⟨1031⟩ THE BIOCOMPATIBILITY OF MATERIALS USED IN DRUG CONTAINERS, MEDICAL DEVICES, AND IMPLANTS

***Change to read:***

This chapter provides guidance on the identification and performance of procedures for evaluating the biocompatibility of drug containers, elastomeric closures, medical devices, and implants. Biocompatibility refers to the tendency of these products to remain biologically inert throughout the duration of their contact with the body. The biocompatibility testing procedures referenced in this chapter are designed to detect the nonspecific, biologically reactive, physical or chemical characteristics of medical products or the materials used in their construction. In combination with chemical assays, these biological procedures can be used to detect and identify the inherent or acquired toxicity of medical products prior to or during their manufacturing and processing.

Preclinical testing procedures to evaluate the safety of the elastomers, plastics, or other polymers used in the construction of medical products are referenced or described in the following general chapters: *Injections and Implanted Drug Products* ⟨1⟩, *Biological Reactivity Tests, In Vitro* ⟨87⟩, *Biological Reactivity Tests, In Vivo* ⟨88⟩, *Medical Devices—Bacterial Endotoxin and Pyrogen Tests* ⟨161⟩, ▲*Elastomeric Components Used in Injectable Pharmaceutical Packaging/Delivery Systems* ⟨381⟩▲ (CN 1-Dec-2020), *Plastic Packaging Systems and Their Materials of Construction* ⟨661⟩, *Plastic Materials of Construction* ⟨661.1⟩, and *Plastic Packaging Systems for Pharmaceutical Use* ⟨661.2⟩. Specific in vitro and in vivo testing procedures to evaluate the biocompatibility of medical products in patients are described under *Biological Reactivity Tests, In Vitro* ⟨87⟩ and under *Biological Reactivity Tests, In Vivo* ⟨88⟩.

The procedures used to evaluate the biocompatibility of a medical product or its construction materials have been categorized as a panel of biological effects (toxicity procedures): cytotoxicity, sensitization, irritation or intracutaneous reactivity, acute systemic toxicity, subchronic toxicity (repeated), genotoxicity, implantation, hemocompatibility, chronic toxicity (extending beyond 10% of the life span of the test animal or beyond 90 days), carcinogenicity, reproductive or developmental toxicity, and biodegradation.[1] The USP general chapters referring to the toxicity procedures for these categories are indicated in *Table 1*. In addition, pyrogenicity, an area of special toxicity, is evaluated under *Pyrogen Test* ⟨151⟩ and under *Bacterial Endotoxins Test* ⟨85⟩. There are currently no general chapters that detail subchronic toxicity, genotoxicity, chronic toxicity, carcinogenicity, hemotoxicity, reproductive toxicity, or biodegradation testing[2] requirements.

**Table 1. Toxicity Procedures in the USP General Chapters**

| Biological Effect | USP General Chapter |
|---|---|
| Cytotoxicity | *Biological Reactivity Tests,In Vitro* ⟨87⟩* |
| Sensitization | *Sensitization Testing* ⟨1184⟩ |
| Irritation or intracutaneous reactivity | *Biological Reactivity Tests, In Vivo* ⟨88⟩† |
| Systemic toxicity (acute toxicity) | *Biological Reactivity Tests, In Vivo* ⟨88⟩ |
| Implantation | *Biological Reactivity Tests, In Vivo* ⟨88⟩ |

* Additional general chapters referring to this biological effect include *Medical Devices—Bacterial Endotoxin and Pyrogen Tests* ⟨161⟩, ▲*Elastomeric Components Used in Injectable Pharmaceutical Packaging/Delivery Systems* ⟨381⟩▲ (CN 1-Dec-2020), *Plastic Packaging Systems and Their Materials of Construction* ⟨661⟩, *Plastic Materials of Construction* ⟨661.1⟩, and *Plastic Packaging Systems for Pharmaceutical Use* ⟨661.2⟩.

† Additional general chapters referring to this biological effect include ⟨1⟩, *Medical Devices—Bacterial Endotoxin and Pyrogen Tests* ⟨161⟩, ▲*Elastomeric Components Used in Injectable Pharmaceutical Packaging/Delivery Systems* ⟨381⟩▲ (CN 1-Dec-2020), *Plastic Packaging Systems and Their Materials of Construction* ⟨661⟩, *Plastic Materials of Construction* ⟨661.1⟩, and *Plastic Packaging Systems for Pharmaceutical Use* ⟨661.2⟩.

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## DRUG CONTAINERS

### Biocompatibility of Plastic and Other Polymeric Drug Containers

Pharmaceutical containers consist of a container and a closure. Plastic containers may consist of polymers that upon extraction do not alter the stability of the contained product or do not exhibit toxicity. The biocompatibility testing requirements for drug containers are stated under ⟨1⟩, *Plastic Packaging Systems and Their Materials of Construction* ⟨661⟩, *Plastic Materials of Construction* ⟨661.1⟩, and *Plastic Packaging Systems for Pharmaceutical Use* ⟨661.2⟩. As directed in these chapters, the plastic or other polymeric portions of these products are tested according to the procedures set forth under *Biological Reactivity Tests, In Vitro* ⟨87⟩. A plastic or other polymer that does not meet the requirements of *Biological Reactivity Tests, In Vitro* ⟨87⟩ is not a suitable material for a drug container. Materials that meet the in vitro requirements qualify as biocompatible materials without the need for further testing and may be used in the construction of a drug container. If a class designation (classes I–VI) for plastics or other polymers is desired, the appropriate testing procedures are performed as discussed in the section *In Vivo Testing and Class Designation*.

[1] ISO document 10993-1:1997 (or latest version) entitled *Biological Evaluation of Medical Devices—Part 1: Evaluation and Testing.*
[2] See OECD Guidelines for Testing of Chemicals at www.oecd.org.

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## Elastomeric Closures

Elastomeric closures are closures that can be pierced by a syringe and maintain their integrity because of their elastic properties. Elastomeric materials may be composed of several chemical entities including fillers, pigments, plasticizers, stabilizers, accelerators, vulcanizing agents, and a natural or a synthetic polymer. These materials are used for manufacturing a product with the desired elastomeric physical properties, and they frequently demonstrate biological reactivity—cellular degeneration and malformation—when tested with in vitro cell cultures.

The biocompatibility of an elastomeric material is evaluated according to the two-stage testing protocol specified in the *Biological Test Procedures* under ▲*Elastomeric Components Used in Injectable Pharmaceutical Packaging/Delivery Systems* ⟨381⟩▲ (CN 1-Dec-2020). Unlike plastics or other polymers, an elastomeric material that does not meet the requirements of the first-stage (in vitro) testing may qualify as a biocompatible material by passing the second-stage (in vivo) testing, which consists of the *Systemic Injection Test* and the *Intracutaneous Test* described under *Biological Reactivity Tests, In Vivo* ⟨88⟩. No class or type distinction is made between elastomeric materials that meet the requirements of the first stage of testing and those that qualify as biocompatible materials by meeting the second-stage requirements. Elastomeric materials are not assigned class I–VI designation.

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# MEDICAL DEVICES AND IMPLANTS

Medical devices and implants, labeled nonpyrogenic, in direct or indirect contact with the cardiovascular system or other soft body tissues, meet the requirements described under *Medical Devices—Bacterial Endotoxin and Pyrogen Tests* ⟨161⟩. The products listed in this chapter that meet the criteria are solution administration sets, extension sets, transfer sets, blood administration sets, intravenous catheters, dialyzers and dialysis tubing and accessories, transfusion and infusion assemblies, and intramuscular drug delivery catheters. The outlined criteria do not apply to medical products such as orthopedic products, latex gloves, and wound dressings.

The testing requirements described or referenced under *Medical Devices—Bacterial Endotoxin and Pyrogen Tests* ⟨161⟩ include *Sterility*, *Bacterial endotoxins*, *Pyrogen*, and *Other requirements*. A procedure to evaluate the presence of bacterial endotoxins is set forth under *Bacterial Endotoxins Test* ⟨85⟩, and the limits are set in *Bacterial Endotoxins* under *Medical Devices—Bacterial Endotoxin and Pyrogen Tests* ⟨161⟩. For devices that cannot be tested by the *Bacterial Endotoxins Test* ⟨85⟩ because of nonremovable inhibition or enhancement, the *Pyrogen Test* ⟨151⟩ is applied. The procedures for evaluating medical devices purported to contain sterile pathways are set forth in *Sterile Devices* under *Sterility Tests* ⟨71⟩. A procedure for evaluating the safety of medical devices is set forth in the *Safety Test* under *Biological Reactivity Tests, In Vivo* ⟨88⟩.

The plastic or other polymer components of medical devices meet the requirements specified for plastics and other polymers under *Plastic Packaging Systems and Their Materials of Construction* ⟨661⟩, *Plastic Materials of Construction* ⟨661.1⟩, and *Plastic Packaging Systems for Pharmaceutical Use* ⟨661.2⟩; those made of elastomers meet the requirements under ▲*Elastomeric Components Used in Injectable Pharmaceutical Packaging/Delivery Systems* ⟨381⟩▲ (CN 1-Dec-2020). As directed in these chapters, the biocompatibility of the plastic, other polymeric, and elastomeric portions of these products are tested according to the procedures described under *Biological Reactivity Tests, In Vitro* ⟨87⟩.If a class designation for a plastic or other polymer is also required, the appropriate testing procedures described under *Biological Reactivity Tests, In Vivo* ⟨88⟩ are performed.

As required for elastomeric closures, elastomeric materials that do not meet the in vitro requirements may qualify as biocompatible materials and may be used in the construction of medical devices if they meet the requirements of the *Systemic Injection Test* and the *Intracutaneous Test* under *Biological Reactivity Tests, In Vivo* ⟨88⟩. As required for drug containers, plastics and other polymers that do not meet the in vitro testing requirements are not suitable materials for use in medical devices.

## IN VITRO TESTING, IN VIVO TESTING, AND CLASS DESIGNATION FOR PLASTICS AND OTHER POLYMERS

The testing requirements specified under *Biological Reactivity Tests, In Vitro* ⟨87⟩ and *Biological Reactivity Tests, In Vivo* ⟨88⟩ are designed to determine the biological reactivity of mammalian cell cultures and the biological response of animals to elastomeric, plastic, and other polymer materials with direct or indirect patient contact. The biological reactivity of these materials may depend on both their surface characteristics and their extractable chemical components. The testing procedures set forth in these chapters can often be performed with the material or an extract of the material under test, unless otherwise specified.

### Preparation of Extracts

Evaluation of the biocompatibility of a whole medical product is often not realistic; thus, the use of representative portions or extracts of selected materials may be the only practical alternative for performing the assays. When representative portions of the materials or extracts of the materials under test are used, it is important to consider that raw materials may undergo chemical changes during the manufacturing, processing, and sterilization of a medical product. Although in vitro testing of raw materials can serve as an important screening procedure, a final evaluation of the biocompatibility of a medical product is performed with portions of the finished and sterilized product.

The preparation of extracts is performed according to the procedures set forth under *Biological Reactivity Tests, In Vitro* ⟨87⟩ and under *Biological Reactivity Tests, In Vivo* ⟨88⟩. Extractions may be performed at various temperatures (121°, 70°, 50°, or 37°), for various time intervals (1 hour, 24 hours, or 72 hours), and in different extraction media. The choice of extraction medium for the procedures under *Biological Reactivity Tests, In Vitro* ⟨87⟩ includes *Sodium Chloride Injection* (0.9% NaCl) or tissue culture medium with or without serum. When medium with serum is used, the extraction temperature cannot exceed 37°. In vivo

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extraction medium includes the choices described under *Biological Reactivity Tests, In Vivo* ⟨88⟩ or the solvent to which the drug or medical device is exposed.

When choosing extraction conditions, select the temperature, solvent, and time variables that best mimic the "in use" conditions of the product. The performance of multiple tests at various conditions can be used to simulate variations in the "in use" conditions. Although careful selection of extraction conditions allows the simulation of manufacturing and processing conditions in the testing of raw materials, an evaluation of the biocompatibility of the product is performed with the finished and sterilized product.

## In Vitro Testing

The procedures described under *Biological Reactivity Tests, In Vitro* ⟨87⟩ include an *Agar Diffusion Test* (indirect contact test), a *Direct Contact Test*, and an *Elution Test* (extraction test). The sample is biocompatible if the cell cultures do not exhibit greater than a mild reactivity (Grade 2) to the material under test, as described under *Biological Reactivity Tests, In Vitro* ⟨87⟩. The *Agar Diffusion Test* is designed to evaluate the biocompatibility of elastomeric materials. The material is placed on the agar overlay of the cell monolayer, which cushions the cells from physical damage by the material and allows leachable chemicals or materials to diffuse from the elastomer and contact the cell monolayer. Extracts of elastomeric materials are tested by placing the filter paper saturated with an extract of the elastomer on the solidified surface of the agar. The *Direct Contact Test* is designed for elastomeric or plastic materials that will not physically damage cells with which they are in direct contact. Any leachable chemicals diffuse from the material into the serum-supplemented growth medium and directly contact the cell monolayer. The *Elution Test* is designed to evaluate the extracts of polymeric materials. The material may be applied directly to the tissue culture media.

The performance of either the *Agar Diffusion Test* or the *Direct Contact Test* in combination with the *Elution Test* is the preferred testing protocol. Extraction of the product or materials for the *Agar Diffusion Test* or the *Elution Test* is performed as described in the *Preparation of Extracts*.

## In Vivo Testing and Class Designation

According to the injection and implantation requirements specified in *Table 1* under *Biological Reactivity Tests, In Vivo* ⟨88⟩, plastics and other polymers are assigned a class designation between class I and class VI. To obtain a plastic or other polymer class designation, extracts of the test material are produced according to the specified procedures in various media.

To evaluate biocompatibility, the extracts are injected systemically and intracutaneously into mice and rabbits or guinea pigs. According to the specified injection requirements, a plastic or other polymer may initially be graded as class I, II, III, or V. If in addition to injection testing, implantation testing using the material itself is performed, the plastic or other polymer may be classified as class IV or class VI.

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# BIOCOMPATIBILITY OF MEDICAL DEVICES AND IMPLANTS

In addition to evaluating medical products for compendial purposes according to the procedures specified under ⟨1⟩, *Sterility Tests* ⟨71⟩, *Biological Reactivity Tests, In Vitro* ⟨87⟩, *Biological Reactivity Tests, In Vivo* ⟨88⟩, *Medical Devices—Bacterial Endotoxin and Pyrogen Tests* ⟨161⟩, ▲*Elastomeric Components Used in Injectable Pharmaceutical Packaging/Delivery Systems* ⟨381⟩▲ (CN 1-Dec-2020), *Plastic Packaging Systems and Their Materials of Construction* ⟨661⟩, *Plastic Materials of Construction* ⟨661.1⟩, and *Plastic Packaging Systems for Pharmaceutical Use* ⟨661.2⟩, medical devices and implants are evaluated for sensitization, subchronic toxicity, genotoxicity, hemocompatibility, chronic toxicity, carcinogenicity, reproductive or developmental toxicity, and biodegradation as required by the regulatory agencies.

The guidance provided by the regulatory agencies indicates that the extent of testing that is performed for a medical device or an implant depends on the following factors: (1) the similarity and uniqueness of the product relative to previously marketed ("predicate") products as considered in the *Decision Flowchart*; (2) the extent and duration of the contact between the product and the patient as described in the *Categorization of Medical Devices*; and (3) the material composition of the product as considered in the sections *Decision Flowchart* and *In Vivo Testing and Class Designation*.

## Decision Flowchart

Guidance on comparing a medical device or an implant to previously marketed products is provided by the Biocompatibility Decision Flowchart (see *Figure 1*[3]) as adapted from the FDA's Blue Book Memorandum #G95-1. The purpose of the flowchart is to determine whether the available data from previously marketed devices are sufficient to ensure the safety of the device under consideration. As indicated by the flowchart, the material composition and the manufacturing techniques of a product are compared to those of the previously marketed products for the devices that come in direct contact with the body. In addition, the flowchart requires an evaluation of the toxicity of any unique material that has not been used in predicate devices. Responses to the questions posed in the flowchart lead to the conclusion that either the available data are sufficient or additional testing is required to ensure the safety of the product. When additional testing is required, guidance on the identification of appropriate testing procedures is provided in the section *Test Selection Matrix*.

[3] Adapted from the FDA Blue Book Memorandum #G95-1 ("Use of International Standard ISO-10993. 'Biological Evaluation of Medical Devices-Part 1: Evaluation and Testing.'")

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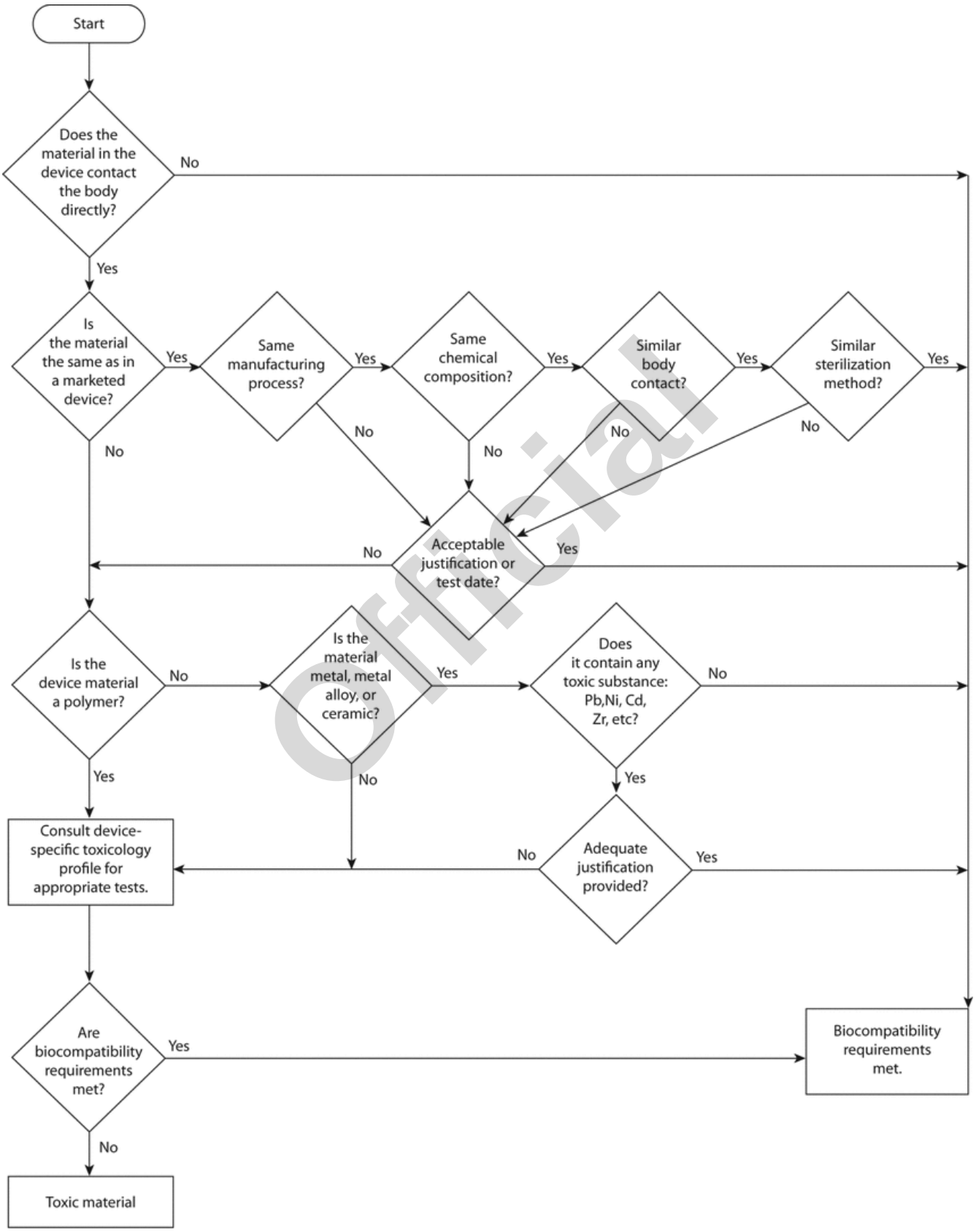


Figure 1. Biocompatibility flowchart.

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## Categorization of Medical Devices

To facilitate the identification of appropriate testing procedures, medical devices are divided and subdivided, as shown in *Table 2*, according to the nature and extent of their contact with the body. Major categories of medical devices are surface devices, external communicating devices, and implant devices. These are then further subcategorized. Some examples of medical devices and implants belonging to each of the subcategories are also presented in *Table 2*.

**Table 2. Classification and Examples of Medical Devices**

| Device Category | Device Subcategory | Nature or Extent of Contact | Some Examples |
|---|---|---|---|
| Surface Devices | Skin | Devices that contact intact skin surfaces only | Electrodes, external prostheses, fixation tapes, compression bandages, and monitors of various types |
| | Mucosal Membrane | Devices communicating with intact mucosal membranes | Contact lenses, urinary catheters, intravaginal and intraintestinal devices (stomach tubes, sigmoidoscopes, colonoscopes, gas troscopes), endotracheal tubes, bronchoscopes, dental prostheses, orthodontic devices, and intrauterine devices |
| | Breached or Compromised Surfaces | Devices that contact breached or otherwise compromised body surfaces | Ulcer, burn, and granulation tissue dressings or healing devices and occlusive patches |
| External Communicating Devices | Blood Path, Indirect | Devices that contact the blood path at one point and serve as a conduit for entry into the vascular system | Solution administration sets, extension sets, transfer sets, and blood administration sets |
| | Tissue, Bone, or Dentin Communicating | Devices and materials communicating with tissue, bone, or pulp and dentin system | Laparoscopes, arthroscopes, draining systems, dental cements, dental filling materials, and skin staples |
| | Circulating blood | Devices that contact circulating blood | Intravascular catheters, temporary pacemaker electrodes, oxygenators, extracorporeal oxygenator tubing and accessories, dialyzers, dialysis tubing and accessories, hemoadsorbents, and immunoadsorbents |
| Implant Devices | Tissue or Bone | Devices principally contacting bone or principally contacting tissue and tissue fluid | Examples of the former are orthopedic pins, plates, replacement joints, bone prostheses, cements, and intraosseous devices; examples of the latter are pacemakers, drug supply devices, neuromuscular sensors and simulators, replacement tendons, breast implants, artificial larynxes, subperiosteal implants, and ligation clips |
| | Blood | Devices principally contacting blood | Pacemaker electrodes, artificial arteriovenous fistulae, heart valves, vascular grafts, internal drug delivery catheters, and ventricular-assist devices |

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## Test Selection Matrix

The matrix provides guidance on the identification of appropriate biological testing procedures for the three categories of medical devices: tests for *Surface Devices* (see *Table 3*), tests for *External Communicating Devices* (see *Table 4*), and tests for *Implant Devices* (see *Table 5*). Each category of devices is subcategorized and then even further subdivided according to the duration of the contact between the device and the body. The duration of contact is defined as (A) limited (less than 24 hours); (B) prolonged (24 hours to 30 days); or (C) permanent (more than 30 days). The biological effects that are included in the matrix are cytotoxicity, sensitization, irritation or intracutaneous reactivity, systemic toxicity, subchronic toxicity, genotoxicity, implantation, hemocompatibility, chronic toxicity, carcinogenicity, reproductive or developmental toxicity, and biodegradation. The general chapters that contain toxicity testing procedures for these biological effects are indicated in *Table 1*.

Each subcategory in the matrix has an associated panel of testing requirements. Generally, the number of tests in the panel increases as the duration of the contact between the device and the body is extended and as the device or implant comes in closer contact with the circulatory system. Within several subcategories, the option of performing additional tests beyond those required should be considered on a case-by-case basis. Specific situations such as use of permanent implant devices or external communicating devices for pregnant women and children have to be taken into consideration in the manufacturer's decision to include reproductive or developmental testing. Guidance on the identification of possible additional testing procedures is provided in the matrix for each subcategory of medical devices.

# GUIDANCE IN SELECTING THE PLASTIC OR OTHER POLYMER CLASS DESIGNATION FOR A MEDICAL DEVICE

To provide guidance on selecting the appropriate plastic or other polymer class designation for a medical device, each subcategory of *Surface Devices* (see *Figure 2*)

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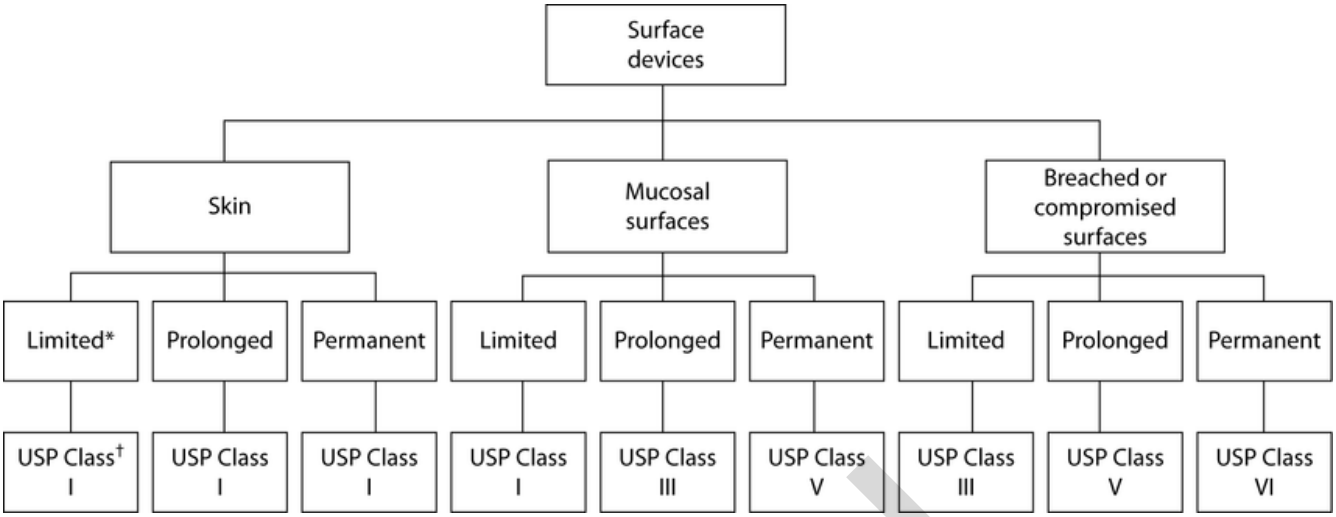


Figure 2. USP plastic and other polymer class requirements for surface devices. *Categorization based on duration of contact: limited—less than 24 hours; prolonged—24 hours to 30 days; permanent—more than 30 days. †USP Plastic Class designation.

and *External Communicating Devices* (see *Figure 3*)

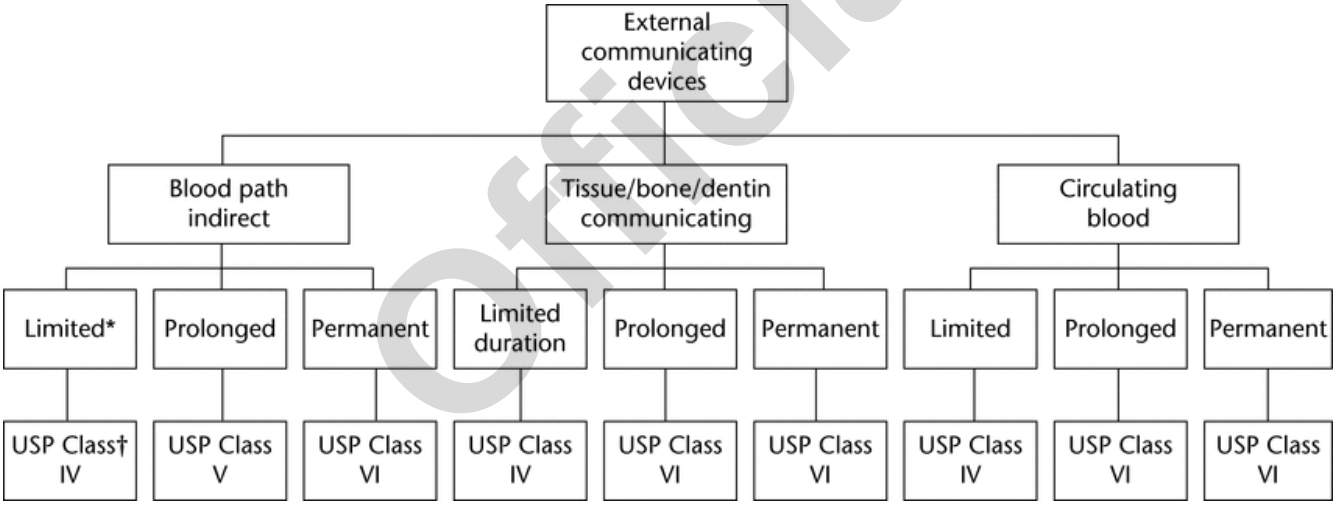


Figure 3. USP plastic and other polymer class requirements for external communicating devices. *Categorization based on duration of contact: limited—less than 24 hours; prolonged—24 hours to 30 days; permanent—more than 30 days.†USP Plastic Class designation.

is assigned a USP Plastic Class designation (see *Biological Reactivity Tests, In Vivo* ⟨88⟩). If the tests for each USP class designation are not sufficient for a specific device, the manufacturer or the practitioner must develop an appropriate set of tests. The indicated numerical class number increases relative to the duration (risk) of contact between the device and the body. In the category of *Implant Devices,* the exclusive use of class VI is mandatory. The assignment of USP Plastic Class designation is based on the test selection matrices illustrated in *Tables 3*, *4*, and *5*.

The assignment of a plastic or other polymer class designation to a subcategory is not intended to restrict the use of higher classes of plastics or other polymers. Although the assigned class defines the lowest numerical class of plastic or other polymer that may be used in the corresponding device, the use of a numerically higher class of plastic is optional. When a device can be defined as belonging to more than one device category, the plastic or other polymer should meet the requirements of the highest numerical class.

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**Table 3. Test Selection Matrix for Surface Devices***

| Device Categories | | | Biological Effect[b] | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Body Contact | | Contact Duration[a] | Cytotoxicity | Sensitization | Irritation or Intracutaneous Reactivity | Systemic Toxicity (Acute) | Subchronic Toxicity | Genotoxicity | Implantation | Hemocompatability | Chronic Toxicity | Carcinogenicity | Reproductive or Development Toxicity | Biodegradation |
| Surface Devices | Skin | A | X | X | X | — | — | — | — | — | — | — | — | — |
| | | B | X | X | X | — | — | — | — | — | — | — | — | — |
| | | C | X | X | X | — | — | — | — | — | — | — | — | — |
| | Mucosal Membrane | A | X | X | X | — | — | — | — | — | — | — | — | — |
| | | B | X | X | X | O | O | — | O | — | — | — | — | — |
| | | C | X | X | X | O | X | X | O | — | O | — | — | — |
| | Breached or Compromised Surfaces | A | X | X | X | O | — | — | — | — | — | — | — | — |
| | | B | X | X | X | O | O | — | O | — | — | — | — | — |
| | | C | X | X | X | O | X | X | O | — | O | — | — | — |

* Adapted from the FDA's Blue Book Memorandum #G95-1 (*Table 1. Initial Evaluation Tests for Consideration* and *Table 2. Supplementary Evaluation Tests for Consideration*).
[a] Legend A—limited (less than 24 hours); B—prolonged (24 hours to 30 days); C—permanent (more than 30 days).
[b] Legend X—ISO evaluation tests for consideration; O—additional tests that may be applicable.

**Table 4. Test Selection Matrix for External Communicating Devices***

| Device Categories | | | Biological Effect[b] | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Body Contact | | Contact Duration[a] | Cytotoxicity | Sensitization | Irritation or Intracutaneous Reactivity | Systemic Toxicity (Acute) | Subchronic Toxicity | Genotoxicity | Implantation | Hemocompatability | Chronic Toxicity | Carcinogenicity | Reproductive or Development Toxicity | Biodegradation |
| External Communicating Devices | Blood Path, Indirect | A | X | X | X | X | — | — | — | X | — | — | — | — |
| | | B | X | X | X | X | O | — | — | X | — | — | — | — |
| | | C | X | X | O | X | X | X | O | X | X | X | — | — |
| | Tissue, Bone, or Dentin Communicating | A | X | X | X | O | — | — | — | — | — | — | — | — |
| | | B | X | X | O | O | O | X | X | — | — | — | — | — |
| | | C | X | X | O | O | O | X | X | — | X | X | — | — |
| | Circulating Blood | A | X | X | X | X | — | O | — | X | — | — | — | — |
| | | B | X | X | X | X | O | X | O | X | — | — | — | — |
| | | C | X | X | X | X | X | X | O | X | X | X | — | — |

* Adapted from the FDA's Blue Book Memorandum #G95-1 (*Table 1. Initial Evaluation Tests for Consideration* and *Table 2. Supplementary Evaluation Tests for Consideration*).
[a] Legend A—limited (less than 24 hours); B—prolonged (24 hours to 30 days); C—permanent (more than 30 days).
[b] Legend X—ISO evaluation tests for consideration; O—additional tests that may be applicable.

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**Table 5. Test Selection Matrix for Implant Devices***

| Device Categories | | | Biological Effect[b] | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Body Contact | | Contact Duration[a] | Cytotoxicity | Sensitization | Irritation or Intracutaneous Reactivity | Systemic Toxicity (Acute) | Subchronic Toxicity | Genotoxicity | Implantation | Hemocompatability | Chronic Toxicity | Carcinogenicity | Reproductive or Developmental Toxicity | Biodegradation |
| Implant Devices | Tissue or Bone | A | X | X | X | O | — | — | — | — | — | — | — | — |
| | | B | X | X | O | O | O | X | X | — | — | — | — | — |
| | | C | X | X | O | O | O | X | X | — | X | X | — | — |
| | Blood | A | X | X | X | X | — | — | X | X | — | — | — | — |
| | | B | X | X | X | X | O | X | X | X | — | — | — | — |
| | | C | X | X | X | X | X | X | X | X | X | X | — | — |

* Adapted from the FDA's Blue Book Memorandum #G95-1 (*Table 1. Initial Evaluation Tests for Consideration* and *Table 2. Supplementary Evaluation Tests for Consideration*).
[a] Legend A—limited (less than 24 hours); B—prolonged (24 hours to 30 days); C—permanent (more than 30 days).
[b] Legend X—ISO evaluation tests for consideration; O—additional tests that may be applicable.

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# ⟨1032⟩ DESIGN AND DEVELOPMENT OF BIOLOGICAL ASSAYS

## 1. INTRODUCTION

### 1.1 Purpose and Scope

General chapter *Design and Development of Biological Assays* ⟨1032⟩ presents methodology for the development of bioassay procedures that have sound experimental design, that provide data that can be analyzed using well-founded statistical principles, and that are fit for their specific use.

General chapter ⟨1032⟩ is one of a group of five general chapters that focus on relative potency assays, in which the activity of a Test material is quantified by comparison to the activity of a Standard material. However, many of the principles can be applied to other assay systems.

This general chapter is intended to guide the design and development of a bioassay for a drug substance or product intended for commercial distribution. Although adoption of this chapter's recommended methods may be resource intensive during assay development, early implementation can yield benefits. Lastly, the perspectives and methods described herein are those recommended from among the many alternatives which contemporary bioassay theory and practice offers.

#### Focus on Relative Potency

Because of the inherent variability in biological test systems (including that from animals, cells, instruments, reagents, and day-to-day and between-lab), an absolute measure of potency is more variable than a measure of activity relative to a Standard. This has led to the adoption of the relative potency methodology. Assuming that the Standard and Test materials are biologically *similar*, *statistical similarity* (a consequence of the Test and Standard similarity) should be present, and the Test sample can be expected to behave like a concentration or dilution of the Standard. Relative potency is a unitless measure obtained from a comparison of the dose-response relationships of Test and Standard drug preparations. For the purpose of the relative comparison of Test to Standard, the potency of the Standard is usually assigned a value of 1 (or 100%). The Standard can be a material established as such by a national (e.g., USP) or international (e.g., WHO) organization, or it could be an internal Standard.

### 1.2 Audience

This chapter is intended for both the practicing bioassay analyst and the statistician who are engaged in developing a bioassay. The former will find guidance for implementing bioassay structure and methodology to achieve analytical goals while reliably demonstrating the biological activity of interest, and the latter will gain insights regarding the constraints of biology that can prove challenging to balance with a rigorous practice of statistics.

## 2. BIOASSAY FITNESS FOR USE

To evaluate whether an assay is fit for use, analysts must specify clearly the purpose(s) for performing the bioassay. Common uses for a bioassay include lot release of drug substance (active pharmaceutical ingredient) and drug product; assessment of stability; qualification of Standard and other critical reagents; characterization of process intermediates and formulations; characterization of contaminants and degradation products; and support of changes in the product production process. The relative accuracy, specificity, precision, and robustness requirements may be different for each of these potential uses. It is a good strategy to develop and validate a bioassay to support multiple intended uses; for example, a bioassay primarily developed for batch release may serve other purposes. Decisions about fitness for use are based on scientific and statistical considerations, as well as practical considerations such as cost, turnaround time, and throughput requirements for the assay.

When assays are used for lot release, a linear-model bioassay may allow sufficient assessment of similarity. For bioassays used to support stability, comparability, to qualify reference materials or critical reagents, or in association with changes in the production or assay processes, it is generally useful to assess similarity using the entire concentration–response curve, including the asymptotes (if present).

### 2.1 Process Development

Bioassays are generally required in the development and optimization of product manufacturing, including formulation and scale-up processes. Bioassays can be used to evaluate purification strategies, optimize product yield, and measure product stability. Because samples taken throughout the process are often analyzed and compared, sample matrix effects that may affect assay response should be carefully studied to determine an assay's fitness for use. For relative potency measures, the Standard material may require dilution into a suitable matrix for quantitation. The bioassay's precision and accuracy should be sufficient for measuring process performance or for assessing and comparing the stability of candidate formulations.

### 2.2 Process Characterization

Bioassays may be performed to assess the effect on drug potency associated with different stages of drug manufacture or with changes in the manufacturing process (e.g., to demonstrate product equivalence before and after process changes are made). Bioassays used in this type of application may be qualitative or quantitative.

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### 2.3 Product Release

Bioassays are used to evaluate the potency of the drug before commercial product release. To the extent possible, the assay should reflect or mimic the product's known or intended mechanism of action. If the bioassay does not include the functional biology directly associated with the mechanism of action, it may be necessary to demonstrate a relationship between the bioassay's estimated potency determinations and those of some other assay that better or otherwise reflects putative functional activity.

For product-release testing, product specifications are established to define a minimum or range of potency values that are acceptable for product. The precision of the reportable value from the bioassay must support the number of significant digits listed in the specification (see general chapter *Biological Assay Validation* ⟨1033⟩), and, in conjunction with relative accuracy, support the specification range. In order to meet these specifications, manufacturing quality control will have sufficiently narrow product release specifications in order to accommodate any loss of activity due to instability and uncertainty in the release assay.

### 2.4 Process Intermediates

Bioassay assessment of process intermediates can provide information regarding specificity. Formulation and fill strategies may rely on bioassays in order to ensure that drug product, including that in final container, will meet its established specifications. For example, unformulated bulk materials may be held and evaluated for potency. Bulks may be pooled with other bulk lots, diluted, or reworked based on the potency results. For these types of applications, the bioassay must be capable of measuring product activity in different matrices. In some cases, a separate Standard material is made and is used to calculate relative potency for the process intermediate.

### 2.5 Stability

The potency assay may be used to assess biotechnology and vaccine product stability. Information from stability studies, performed during development under actual and/or accelerated or stressed storage conditions, may be used to establish shelf life duration as well as to identify and estimate degradation products and degradation rates. Post licensure stability studies may be used to monitor product stability. Knowledge of both short-term and long-term variability of the bioassay is important to assure an acceptable level of uncertainty in potency measures obtained.

### 2.6 Qualification of Reagents

The quantitative characterization of a new Standard requires an accurate and precise measurement of the new Standard's biological activity. This measurement is used either to establish that the new Standard lot is equivalent to the previous lot or to assign it a label potency to which Test samples can be compared. Additional replication (beyond routine testing) may be required to achieve greater precision in the potency measurement of the new Standard material. Additionally, the bioassay may be used to qualify a cell culture reagent such as fetal bovine serum. The fitness for use in such cases is tied to the ability of the assay to screen reagent lots and to ensure that lots that may bias or compromise the relative potency measurements are not accepted.

### 2.7 Product Integrity

Biotechnology, biological, and vaccine products may contain a population of heterogeneous material, including the intended predominant product material. Some process impurities and degradation products may be active, partially active, inactive in, or antagonistic to, the response measured in the bioassay. For product variants or derivatives for which changes in structure or relative composition may be associated with subtle yet characteristic changes in the bioassay response (e.g., change in slope or asymptote), the bioassay may be useful in the detection and measurement of these variants or derivatives. Studies that identify characteristic changes associated with variants of the intended product help ensure consistent product performance. Whenever practical, the bioassay should be accompanied by orthogonal methods that are sensitive to product variants, process impurities, and/or degradation products.

## 3. BIOASSAY FUNDAMENTALS

### 3.1 In Vivo Bioassays

In vivo potency assays are bioassays in which sets of dilutions of the Standard and Test materials are administered to animals and the concentration–response relationships are used to estimate potency. For some animal assays, the endpoint is simple (e.g., rat body weight gain assay for human growth hormone or rat ovarian weight assay for follicle stimulating hormone), but others require further processing of samples collected from treated animals (e.g., reticulocyte count for erythropoeitin, steroidogenesis for gonadotropins, neutrophil count for granulocyte colony stimulating factor, or antibody titer after administration of vaccines). With the advent of cell lines specific for the putative physiological mechanism of action (MOA), the use of animals for the measurement of potency has substantially diminished. Cost, low throughput, ethical, and other practical issues argue against the use of animal bioassays. Regulatory agencies have encouraged the responsible limitation of animal use whenever possible (see The Interagency Coordinating Committee on the Validation of Alternative Methods, Mission, Vision, and Strategic Priorities; February 2004). When in vitro activity is not strongly associated with in vivo activity (e.g., EPO), the combination of an in vitro cell-based assay and a suitable physicochemical method (e.g., IEF, glycan analysis) may substitute

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for in vivo assays. However, a need for in vivo assays may remain when in vitro assays cannot detect differences that are critical in regard to a drug's intended biological function.

Animals' physiological responses to biological drugs (including vaccines) may predict patients' responses. Selection of animal test subjects by species, strain, gender, and maturity or weight range is guided by the goal of developing a representative and sensitive model with which to assess the activity of Test samples.

Some assay methods lend themselves to the use of colony versus naive animals. For example, pyrogen and insulin testing benefit from using experienced colony rabbits that provide a reliable response capacity. If animals recently introduced to the colony fail to respond as expected after several administrations of a compound, they should be culled from the colony so they do not cause future invalid or indeterminate assay results. In the case of assaying highly antigenic compounds for pyrogens, however, naive animals should be used to avoid generating inaccurate or confounded results. Other colony advantages include common controlled environmental conditions (macro/room, and micro/rack), consistent feeding schedule, provision of water, and husbandry routine.

Historical data including colony records and assay data can be used to identify factors that influence assay performance. The influence of biasing factors can be reduced by applying randomization principles such as distribution of weight ranges across dose groups, group assignments from shipping containers to different cages, or use of computer-generated or deck patterns for injection/dosing. A test animal must be healthy and have time to stabilize in its environment to be suitable for use in a bioassay. Factors that combine to influence an animal's state of health include proper nutrition, hydration, freedom from physical and psychological stressors, adequate housing sanitization, controlled light cycle (diurnal/nocturnal), experienced handling, skillful injections and bleedings, and absence of noise or vibration. Daily observation of test animals is essential for maintenance of health, and veterinary care must be available to evaluate issues that have the potential to compromise the validity of bioassay results.

## 3.2 Ex Vivo Bioassays

Cells or tissues from human or animal donors can be cultured in the laboratory and used to assess the activity of a Test sample. In the case of cytokines, the majority of assays use cells from the hematopoietic system or subsets of hematopoietic cells from peripheral blood such as peripheral blood mononuclear cells or peripheral blood lymphocytes. For proteins that act on solid tissues, such as growth factors and hormones, specific tissue on which they act can be removed from animals, dissociated, and cultured for a limited period either as adherent or semi-adherent cells. Although an ex vivo assay system has the advantage of similarity to the natural milieu, it may also suffer from substantial donor-to-donor variability, as well as challenging availability of appropriate cells.

Bioassays that involve live tissues or cells from an animal (e.g., rat hepatocyte glucagon method) require process management similar to that of in vivo assays to minimize assay variability and bias. The level of effort to manage bias (e.g., via randomization) should be appropriate for the purpose of the assay. Additional factors that may affect assay results include time of day, weight or maturity of animal, anesthetic used, buffer components/reagents, incubation bath temperature and position, and cell viability.

## 3.3 In Vitro (Cell-Based) Bioassays

Bioassays using cell lines that respond to specific ligands or infectious agents can be used for lot-release assays. These cell lines can be derived from tumors, immortalized as factor-dependent cell lines, or engineered cell lines transfected with appropriate receptors. Additionally, nontransformed cell lines which can be maintained over a sufficient number of passages (e.g., fibroblasts) may also be used. Regardless of cell line, there is an expectation of adequately equivalent potency response through some number of continuous passages. Advances in recombinant DNA technology and the understanding of cellular signaling mechanisms have allowed the generation of engineered cell lines with improved response, stable expression of receptors and signaling mechanisms, and longer stability. The cellular responses to the protein of interest depend on the drug's MOA and the duration of exposure. Such responses include cell proliferation, cell killing, antiviral activity, differentiation, cytokine/mediator secretion, and enzyme activation. Assays involving these responses may require incubation of the cells over several days, during which time contamination, uneven evaporation, or other location effects may arise. Comparatively rapid responses based on an intracellular signaling mechanism—such as second messengers, protein kinase activation, or reporter gene expression—have proven acceptable to regulatory authorities. Lastly, most cell lines used for bioassays express receptors for multiple cytokines and growth factors. This lack of specificity may not be detrimental if the Test sample's specificity is demonstrated.

Cell-based bioassay design should reflect knowledge of the factors that influence the response of the cells to the active analyte. Response variability is often reflected in parameters such as slope, $EC_{50}$ of the concentration–response curve, or the response range (maximum minus minimum response). Even though relative potency methodology minimizes the effects on potency estimates of variation in these parameters among assays, and among blocks within an assay, such response variability can make an assay difficult to manage (i.e., it may be difficult to assess system suitability). Hence, while assay development should be focused primarily on the properties of potency, efforts to identify and control variation in the concentration–response relationship are also appropriate. For blocked assays (e.g., multiple cell culture plates in an assay) with appreciable variation in curve shape among blocks, an analysis that does not properly include blocks will yield inflated estimates of within-assay variation, making similarity assessment particularly difficult. Two strategies are available for addressing variation among blocks: one, a laboratory effort to identify and control sources of variation and two, a statistical effort to build and use a blocked design and analysis. Combining these strategies can be particularly effective.

The development of a cell-based bioassay begins with the selection or generation of a cell line. An important first step when developing a cell-based assay to assess a commercial product is to verify that the cell line of interest is not restricted to research use only. To ensure an adequate and consistent supply of cells for product testing, a cell bank should be generated if possible. To the extent possible, information regarding functional and genetic characteristics of the bioassay's cell line should be

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documented, including details of the cell line's history from origin to banking. For example, for a recombinant cell line this might include the identification of the source of the parental cell line (internal cell bank, external repository, etc.), of the DNA sequences used for transfection, and of the subsequent selection and functional testing regimen that resulted in selection of the cell line. Ideally, though not always practical, sufficient information is available to permit recreation of a similar cell line if necessary. Pertinent information may include identity (e.g., isoenzyme, phenotypic markers, genetic analysis); morphology (e.g., archived photographic images); purity (e.g., mycoplasma, bacteria, fungus and virus testing); cryopreservation; thaw and culture conditions (e.g., media components, thaw temperature and method, methods of propagation, seeding densities, harvest conditions); thaw viability (immediately after being frozen and after time in storage); growth characteristics (e.g., cell doubling times); and functional stability (e.g., ploidy).

Cell characterization and vigilance regarding aspects of assay performance that reflect on cell status are necessary to ensure the quality and longevity of cell banks for use in the QC environment. The general health and metabolic state of the cells at the time of bioassay can substantially influence the test results. After a cell line has been characterized and is ready for banking, analysts typically prepare a two-tiered bank (Master and Working). A Master Cell Bank is created as the source for the Working Cell Bank. The Working Cell Bank is derived by expansion of one or more vials of the Master Cell Bank. The size of the banks depends on the growth characteristics of the cells, the number of cells required for each assay, and how often the assay will be performed. Some cells may be sensitive to cryopreservation, thawing, and culture conditions, and the banks must be carefully prepared and characterized before being used for validation studies and for regular use in the QC laboratory.

There follow factors that may affect bioassay response and the assessment of potency, that are common to many cell-based bioassays: cell type (adherent or nonadherent); cell thawing; plating density (at thaw and during seed train maintenance) and confluence (adherent cells); culture vessels; growth, staging, and assay media; serum requirements (source, heat inactivation, gamma irradiation); incubation conditions (temperature, $CO_2$, humidity, culture times from thaw); cell harvesting reagents and techniques (for adherent cells, method of dissociation); cell sorting; cell counting; determination of cell health (growth rate, viability, yield); cell passage number and passaging schedule; cell line stability (genetic, receptor, marker, gene expression level); and starvation or stimulation steps. This list is not exhaustive, and analysts with comprehensive understanding and experience with the cell line should be involved during assay development. These experienced individuals should identify factors that might influence assay outcomes and establish strategies for an appropriate level of control whenever possible.

### 3.4 Standard

The Standard is a critical reagent in bioassays because of the necessity to have a reliable material to which a Test preparation can be quantitatively compared. The Standard may be assigned a unitage or specific activity that represents fully (100%) potent material. Where possible, a Standard should be representative of the samples to be tested in the bioassay. Testing performed to qualify a Standard may be more rigorous than the routine testing used for lot release.

A Standard must be stored under conditions that preserve its full potency for the intended duration of its use. To this end, the Standard may be stored under conditions that are different from the normal storage of the drug substance or drug product. These could include a different temperature (e.g., –70° or –20°, instead of 2°–8°), a different container (e.g., plastic vials instead of syringes), a different formulation (e.g., lyophilizable formulation or the addition of carrier proteins such as human serum albumin, stabilizers, etc.). The Standard material should be tested for stability at appropriate intervals. System suitability criteria of the bioassay such as maximum or background response, $EC_{50}$ slope, or potency of assay control may be used to detect change in the activity of the Standard. Accelerated stability studies can be performed to estimate degradation rates and establish recognizable characteristics of Standard instability.

At later stages in clinical development, the Standard may be prepared using the manufacturing process employed in pivotal clinical trials. If the Standard formulation is different from that used in the drug product process, it is important to demonstrate that the assay's assessment of similarity and estimate of potency is not sensitive to the differences in formulation. An initial Standard may be referred to as the *Primary Standard*. Subsequent Standards can be prepared using current manufacturing processes and can be designated *Working Standards*. Separate SOPs may be required for establishing these standards for each product. Bias in potency measurements sometimes can arise if the activity of the Standard gradually changes. Also, loss of similarity may be observed if, with time, the Standard undergoes changes in glycosylation. It is prudent to archive aliquots of each Standard lot for assessment of comparability with later Standards and for the investigation of assay drift.

## 4. STATISTICAL ASPECTS OF BIOASSAY FUNDAMENTALS

The statistical elements of bioassay development include the type of data, the measure of response at varying concentration, the assay design, the statistical model, pre-analysis treatment of the data, methods of data analysis, suitability testing, and outlier analysis. These form the constituents of the bioassay system that will be used to estimate the potency of a Test sample.

### 4.1 Data

Fundamentally, there are two bioassay data types: quantitative and quantal (categorical). Quantitative data can be either continuous (not limited to discrete observations; e.g., collected from an instrument), count (e.g., plaque-forming units), or discrete (e.g., endpoint dilution titers). Quantal data are often dichotomous; for example, life/death in an animal response model or positivity/negativity in a plate-based infectivity assay that results in destruction of a cell monolayer following administration of an infectious agent. Quantitative data can be transformed to quantal data by selecting a threshold that distinguishes a positive response from a negative response. Such a threshold can be calculated from data acquired from a negative control, as by adding (or subtracting) a measure of uncertainty (such as two or three times the standard deviation of negative control responses) to the negative control average. Analysts should be cautious about transforming quantitative data to quantal data because this results in a loss of information.

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## 4.2 Assumptions

A key assumption for the analysis of most bioassays is that the Standard and Test samples contain the same effective analyte or population of analytes and thus may be expected to behave similarly in the bioassay. This is termed *similarity*. As will be shown in more detail in the general chapter *Analysis of Biological Assays* ⟨1034⟩ for specific statistical models, biological similarity implies that statistical similarity is present (for parallel-line and parallel-curve models, the Standard and Test curves are parallel; for slope-ratio models, the Standard and Test lines have a common intercept). The reverse is not true. Statistical similarity (parallel lines, parallel curves, or common intercept, as appropriate) does not ensure biological similarity. However, failure to satisfy statistical similarity may be taken as evidence against biological similarity. The existence of a Standard–Test sample pair that passes the assessment of statistical similarity is thus a necessary but not sufficient condition for the satisfaction of the key assumption of biological similarity. Biological similarity thus remains, unavoidably, an assumption. Departures from statistical similarity that are consistent in value across replicate assays may be indicative of matrix effects or of real differences between Test and Standard materials. This is true even if the departure from statistical similarity is sufficiently small to support determination of a relative potency.

In many assays multiple compounds will yield similar concentration–response curves. It may be reasonable to use a biological assay system to describe or even compare response curves from different compounds. But it is not appropriate to report relative potency unless the Standard and Test samples contain only the same active analyte or population of analytes. Biological products typically exhibit lot-to-lot variation in the distribution of analytes (i.e., most biological products contain an intended product and, at acceptably low levels, some process contaminants that may be active in the bioassay). Assessment of similarity is then, at least partially, an assessment of whether the distribution of analytes in the Test sample is close enough to that of the distribution in the Standard sample for relative potency to be meaningful; that is, the assay is a comparison of like to like. When there is evidence (from methods other than the bioassay) that the Standard and Test samples do not contain the same active compound(s), the assumption of biological similarity is not satisfied, and it is not appropriate to report relative potency.

Other common statistical assumptions in the analysis of quantitative bioassays are constant variance of the responses around the fitted model (see section 4.3 *Variance Heterogeneity, Weighting, and Transformation* for further discussion), normally distributed residuals (a residual is the difference between an observed response and the response predicted by the model), and independence of the residuals.

Constant variance, normality, and independence are interrelated in the practice of bioassay. For bioassays with a quantitative response, a well-chosen data transformation may be used to obtain approximately constant variance and a nearly normal distribution of residuals. Once such transformation has been imposed, the remaining assumption of independence then remains to be addressed via reflection of the assay design structure in the analysis model. Independence of residuals is important for assessing system and sample suitability.

## 4.3 Variance Heterogeneity, Weighting, and Transformation

Simple analysis of quantitative bioassay data requires that the data be approximately normally distributed with near constant variance across the range of the data. For linear and nonlinear regression models, the variance referred to here is the residual variance from the fit of the model. Constant variance is often not observed; *variance heterogeneity* may manifest as an increase in variability with increase in response. If the variances are not equal but the data are analyzed as though they are, the estimate of relative potency may still be reasonable; however, failure to address nonconstant variance around the fitted concentration–response model results in an unreliable estimate of within-assay variance. Further, the assessment of statistical similarity may not be accurate, and standard errors and confidence intervals for all parameters (including a Fieller's Theorem-based interval for the relative potency) should not be used. Confidence intervals for relative potency that combine potency estimates from multiple assays may be erroneous if within-assay error is used for confidence interval calculation.

Constancy of variance may be assessed by means of residual plots, Box-Cox (or power law) analysis, or Levene's test. With Levene's test, rather than relying on the p value, change in the statistic obtained is useful as a basis for judging whether homogeneity is improved or worsened. Variance is best assessed on a large body of assay data. Using only the variance among replicates from the current assay is not appropriate, because there are too few data to properly determine truly representative variances specific to each concentration. Data on variance is sparse during development; it is prudent to re-assess variance during validation and to monitor it periodically during ongoing use of the assay.

Two methods used to mitigate variance heterogeneity are transformation and weighting. Lack of constant variance can be addressed with a suitable transformation. Additionally, transformation can improve the normality of residuals and the fit of some statistical models to the data. A transformation should be chosen for an assay system during development, checked during validation, used consistently in routine assay practice, and checked periodically. Bioassay data are commonly displayed with log-transformed concentration; slope-ratio assays are displayed with concentration on the original scale.

Transformation may be performed to the response data as well as to the concentration data. Common choices for a transformation of the response include log, square root (for counts), reciprocal, and, for count data with known asymptotes, logit of the percent of maximum response. Log transformations are commonly used, as they may make nearly linear a useful segment of the concentration–response relationship, and because of the ease of transforming back to the original scale for interpretation. A log–log fit may be performed on data exhibiting nonlinear behavior. Other alternatives are available; i.e., data may be transformed by the inverse of the *Power of the Mean* (POM) function. A POM coefficient of k = 2 corresponds to a log transformation of the data. For further discussion of relationships between log-transformed and untransformed data, see *Appendix* in the general chapter *Biological Assay Validation* ⟨1033⟩.

Note that transformation of the data requires re-evaluation of the model used to fit the data. From a statistical perspective there is nothing special about the original scale of measurement; any transformation that improves accordance with assumptions is acceptable. Analysts should recognize, however, that transformations, choice of statistical model, and choice of weighting scheme are interrelated. If a transformation is used, that may affect the choice of model. That is, transforming the response by a log or square root, for example, may change the shape of the response curve, and, for a linear model, may change the range of concentrations for which the responses are nearly straight and nearly parallel.

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For assays with non-constant variance, a weighted analysis may be a reasonable option. Though weighting cannot address lack of residual normality, it is a valid statistical approach to placing emphasis on more precise data. Ideally, weights may be based on the inverse of the predicted within-assay (or within-block) variance of each response where the predictors of variance are independent of responses observed in a specific assay.

In practice, many bioassays have relatively large variation in log $EC_{50}$ (compared to the variation in log relative potency) among assays (and sometimes among blocks within assay). If not addressed in the variance model, this variation in log $EC_{50}$ induces what appears to be large variation in response near the mean log $EC_{50}$, often yielding too-low weights for observations near the $EC_{50}$.

If the assay is fairly stable (low variability in $EC_{50}$), an alternative is to look at variance as a function of concentration. While not ideal, an approach using concentration-dependent variances may be reasonable when the weights are estimated from a large number of assays, the variances are small, any imbalance in the number of observations across concentrations is addressed in the variance model, and there are no unusual observations (outliers). This possibility can be examined by plotting the response variance at each concentration (preferably pooled across multiple assays) against concentration and then against a function of concentration (e.g., concentration squared). Variance will be proportional to the function of concentration where this plot approximates a straight line. The apparent slope of this line is informative, in that a horizontal line indicates no weighting is needed. If a function that yields a linear plot can be found, then the weights are taken as proportional to the reciprocal of that function. There may be no such function, particularly if the variation is higher (or lower) at both extremes of the concentration range studied.

Whether a model or historical data are used, the goal is to capture the relative variability at each concentration. It is not necessary to assume that the absolute level of variability of the current assay is identical to that of the data used to determine the weighting, but only that the ratios of variances among concentrations are consistent with the historical data or the data used to determine the variance function.

Appropriate training and experience in statistical methods are essential in determining an appropriate variance-modeling strategy. Sources of variability may be misidentified if the wrong variance model is used. For example, data may have constant variation throughout a four-parameter logistic concentration–response curve but can also have appreciable variation in the $EC_{50}$ parameter from block to block within the assay, or from assay to assay. If the between-block or between-assay variability is not recognized, this assay can appear to have large variation in the response for concentrations near the long-term average value of the $EC_{50}$. A weighted model with low weights for concentrations near the $EC_{50}$ would misrepresent a major feature of such an assay system.

## 4.4 Normality

Many statistical methods for the analysis of quantitative responses assume normality of the residuals. If the normality assumption is not met, the estimate of relative potency and its standard error may be reasonable, but suitability tests and a confidence interval for the relative potency estimate may be invalid. Most methods used in this chapter are reasonably robust to departures from normality, so the goal is to detect substantial nonnormality. During assay development, in order to discover substantial departure from normality, graphical tools such as a normal probability plot or a histogram (or something similar like stem-and-leaf or box plots) of the residuals from the model fit may be used. The histogram should appear unimodal and symmetric. The normal probability plot should approximate a straight line; a normal probability plot that is not straight (e.g., curved at one end, both ends, or in the middle) indicates the presence of nonnormality. A pattern about a straight line is an indication of nonnormality. Nonnormal behavior may be due to measurements that are log normal and show greater variability at higher levels of response. This may be seen as a concave pattern in the residuals in a normal plot.

Statistical tests of normality may not be useful. As per the previous discussion of statistical testing of constancy of variance, change of the value of a normality test statistic, rather than reliance on a p value, is useful for judging whether normality is improved or worsened. As for variance assessment, evaluate normality on as large a body of assay data as possible during development, re-assess during validation, and monitor periodically during ongoing use of the assay. Important departures from normality can often be mitigated with a suitable transformation. Failure to assess and mitigate important departure from normality carries the risks of disabling appropriate outlier detection and losing capacity to obtain reliable estimates of variation.

## 4.5 Linearity of Concentration–Response Data

Some bioassay analyses assume that the shape of the concentration–response curve is a straight line or approximates a straight line over a limited range of concentrations. In those cases, a linear-response model may be assessed to determine if it is justified for the data in hand. Difference testing methods for assessing linearity face the same problems as do difference testing methods applied to parallelism—more data and better precision make it more likely to detect nonlinearity. Because instances in which lack of linearity does not affect the potency estimate are rare, analysts should routinely assess departure from linearity if they wish to use a linear-response model to estimate potency.

If an examination of a data plot clearly reveals departure from linearity, this is sufficient to support a conclusion that linearity is not present. High data variability, however, may mask departure from linearity. Thus a general approach for linearity can conform to that for similarity, developed more elaborately in section 4.7 *Suitability Testing, Implementing Equivalence Testing for Similarity (parallelism)*.

1. Specify a measure of departure from linearity which can either combine across samples or be sample specific. Possibilities include the nonlinearity sum of squares or quadratic coefficients.
2. Use one of the four approaches in *Step 2* of *Implementing Equivalence Testing for Similarity (parallelism)* to determine, during development, a range of acceptable values (acceptance interval) for the measure of nonlinearity.
3. Determine a 90% two-sided confidence interval on the measure on nonlinearity, following the Two One-Sided Test (TOST) procedure, and compare the result to the acceptance interval as determined in (2).

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Often a subset of the concentrations measured in the assay will be selected in order to establish a linear concentration–response curve. The subset may be identified graphically. The concentrations at the extreme ends of the range should be examined carefully as these often have a large impact on the slope and calculations derived from the slope. If, in the final assay, the intent is to use only concentrations in the linear range, choose a range of concentrations that will yield parallel straight lines for the relative potencies expected during routine use of the assay; otherwise, the assay will fail parallelism tests when the potency produces assay response values outside the linear range of response. When potency is outside the linear range, it may be appropriate to adjust the sample concentration based on this estimated potency and test again in order to obtain a valid potency result. The repeat assays together with the valid assays may generate a biased estimate of potency because of the selective process of repeating assays when the response is in the extremes of the concentration–response curve.

The problem is more complex in assays where there is even modest variation in the shape or location of the concentration–response curve from run to run or from block to block within an assay. In such assays it may be appropriate to choose subsets for each sample in each assay or even in each block within an assay. Note that a fixed-effects model will mask any need for different subsets in different blocks, but a mixed-effects model may reveal and accommodate different subsets in different blocks (see section 4.9 *Fixed and Random Effects in Models of Bioassay Response*).

Additional guidance about selection of data subset(s) for linear model estimation of relative potency includes the following: use at least three, and preferably four, adjacent concentrations; require that the slope of the linear segment is sufficiently steep; require that the lines fit to Standard and Test samples are straight; and require that the fit regression lines are parallel. One way to derive a steepness criterion is to compute a t-statistic on the slope difference from zero. If the slope is not significant the bioassay is likely to have poor performance; this may be observed as increased variation in the potency results. Another aspect that supports requiring adequate steepness of slope is the use of subset selection algorithms. Without a slope steepness criterion, a subset selection algorithm that seeks to identify subsets of three or more contiguous data points that are straight and parallel might select concentrations on an asymptote. Such subsets are obviously inappropriate to use for potency estimation. How steep or how significant the steepness of the slope should be depends on the assay. This criterion should be set during assay development and possibly refined during assay validation.

## 4.6 Common Bioassay Models

Most bioassays consist of a series of concentrations or dilutions of both a Test sample and a Standard material. A mathematical model is fit to the concentration–response data, and a relative potency may then be calculated from the parameters of the model. Choice of model may depend on whether quantitative or qualitative data are being analyzed.

For quantitative data, models using parallel response profiles which support comparative evaluation for determining relative potency may provide statistical advantages. If such a model is used, concentrations or dilutions are usually scaled geometrically, e.g., usually in two-fold, log, or half-log increments. If a slope-ratio model is used, concentrations or dilutions can be equally spaced on concentration, rather than log concentration. Several functions may be used for fitting a parallel response model to quantitative data, including a linear function, a higher-order polynomial function, a four-parameter logistic (symmetric sigmoid) function, and a five-parameter logistic function for asymmetric sigmoids. Such functions require a sufficient number of concentrations or dilutions to fit the model. To assess lack of fit of any model it is necessary to have at least one, and preferably several, more concentrations (or dilutions) than the number of parameters that will be estimated in the model. Also, at least one, and better, two, concentrations are commonly used to support each asymptote.

A linear model is sometimes selected because of apparent efficiency and ease of processing. Because bioassay response profiles are usually nonlinear, the laboratory might perform an experiment with a wide range of concentrations in order to identify the approximately linear region of the concentration–response profile. For data that follow a four-parameter logistic model, these are the concentrations near the center of the response region, often from 20% to 80% response when the data are rescaled to the asymptotes. Caution is appropriate in using a linear model because for a variety of reasons the apparently linear region may shift. A stable linear region may be identified after sufficient experience with the assay and with the variety of samples that are expected to be tested in the assay. Data following the four-parameter logistic function may also be linearized by transformation. The lower region of the function is approximately linear when the data are log transformed (log–log fit).

Quantal data are typically fit using more complex mathematical models. A probit or logit model may be used to estimate a percentile of the response curve (usually the 50th percentile) or, more directly, the relative potency of the Test to the Standard. Spearman-Kärber analysis is a non-modeling method that may be employed for determining the 50th percentile of a quantal concentration–response curve.

## 4.7 Suitability Testing

System suitability and sample suitability assessment should be performed to ensure the quality of bioassay results. System suitability in bioassay, as in other analytical methods, consists of pre-specified criteria by which the validity of an assay (or, perhaps, a run containing several assays) is assessed. Analysts may assess system suitability by determining that some of the parameters of the Standard response are in their usual ranges and that some properties (e.g., residual variation) of the data are in their usual range. To achieve high assay acceptance rates, it is advisable to accept large fractions of these usual ranges (99% or more) and to assess system suitability using only a few uncorrelated Standard response parameters. The choice of system suitability parameters and their ranges may also be informed by empirical or simulation studies that measure the influence of changes in a parameter on potency estimation.

Sample suitability in bioassay is evaluated using pre-specified criteria for the validity of the potency estimate of an individual Test sample, and usually focuses on similarity assessment. System and sample suitability criteria should be established during bioassay development and before bioassay validation. Where there is limited experience with the bioassay, these criteria may be considered provisional.

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## SYSTEM SUITABILITY

System suitability parameters may be selected based on the design and the statistical model. Regardless of the design and model, however, system suitability parameters should be directly related to the quality of the bioassay. These parameters are generally based on standard and control samples. In parallel-line assays, for example, low values of the Standard slope typically yield estimates of potency with low precision. Rather than reject assays with low slope, analysts may find it more effective to use additional replicate assays until the assay system can be improved to consistently yield higher-precision estimates of potency. It may be particularly relevant to monitor the range of response levels and location of asymptotes associated with controls or Standard sample to establish appropriate levels of response. A drift or a trend in some of the criteria may indicate the degradation of a critical reagent or Standard material. Statistical process control (SPC) methods should be implemented to detect trends in system suitability parameters.

Two common measures of system suitability are assessment of the adequacy of the model (goodness of fit) and of precision. With replicates in a completely randomized design, a pure error term may be separated from the assessment of lack of fit. Care should be taken in deriving a criterion for lack of fit; the use of the wrong error term may result in an artificial assessment. The lack of fit sum of squares from the model fit to the Standard may, depending on the concentrations used and the way in which the data differ from the model, be a useful measure of model adequacy. A threshold may be established, based on *sensitivity analysis* (assessment of assay sensitivity to changes in the analyte) and/or historical data, beyond which the lack of fit value indicates that the data are not suitable. Note that the Test data are not used here; adequacy of the model for the Test is part of sample suitability.

For assessment of precision, two alternatives may be considered. One approach uses the mean squared error (residual variance) from the model fit to the Standard alone. Because this approach may have few degrees of freedom for the variance estimate, it may be more useful to use a pooled mean squared error from separate model fits to Standard and Test. Once the measure is selected, use historical data and sensitivity analysis to determine a threshold for acceptance.

## SAMPLE SUITABILITY

Sample suitability in bioassay generally consists of the assessment of similarity, which can only be done within the assay range. Relative potency may be reported only from samples that both show similarity to Standard, exhibit requisite quality of model fit, and have been diluted to yield an $EC_{50}$ (and potency) within the range of the assay system.

## SIMILARITY

In the context of similarity assessment, classical hypothesis (*difference*) testing evaluates a null hypothesis that a measure (a nonsimilarity parameter measuring the difference between Standard and Test concentration–response curves) is zero, with an implicit alternative hypothesis that the measure is non-zero or the statistical assumptions are not satisfied. The usual ("difference test") criterion that the p-value must be larger than a certain critical value in order to declare the sample similar to reference controls the probability that samples are falsely declared nonsimilar; this is the producer's risk of failing good samples. The consumer's risk (the risk that nonsimilar samples are declared similar) is controlled via the precision in the nonsimilarity measure and amount of replication in the assay; typically these are poorly assessed, leaving consumer risk uncontrolled.

In contrast to difference testing, equivalence testing for similarity (assessing whether a 90% confidence interval for a measure of nonsimilarity is contained within specified equivalence bounds) allows only a 5% probability that samples with nonsimilarity measures outside the equivalence boundaries will be declared similar (controlling the consumer's risk). With equivalence testing it is practical to examine and manage the producer's risk by ensuring that there is enough replication in the assay to have good precision in estimating the nonsimilarity measure(s).

For the comparison of slopes, difference tests have traditionally been used to establish parallelism between a Test sample and the Standard sample. Using this approach the laboratory cannot conclude that the slopes are equal. The data may be too variable, or the assay design may be too weak to establish a difference. The laboratory can, however, conclude that the slopes are sufficiently similar using the equivalence testing approach.

Equivalence testing has practical advantages compared to difference testing, including that increased replication yielding improved assay precision will increase the chances that samples will pass the similarity criteria; that decreased assay replication or precision will decrease the chances that samples will pass the similarity criteria; and that sound approaches to combining data from multiple assays of the same sample to better understand whether a sample is truly similar to Standard or not are obtained.

Because of the advantages associated with the use of equivalence testing in the assessment of similarity, analysts may transition existing assays to equivalence testing or may implement equivalence testing methods when changes are made to existing assays. In this effort, it is informative to examine the risk that the assay will fail good samples. This risk depends on the precision of the assay system, the replication strategy in the assay system, and the critical values of the similarity parameters (this constitutes a *process capability analysis*). One approach to transitioning an established assay from difference testing to equivalence testing (for similarity) is to use the process capability of the assay to set critical values for similarity parameters. This approach is reasonable for an established assay because the risks (of falsely declaring samples similar and falsely declaring samples nonsimilar) are implicitly acceptable, given the assay's history of successful use.

Similarity measures may be based on the parameters of the concentration–response curve and may include the slope for a straight parallel-line assay; intercept for a slope-ratio assay; the slope and asymptotes for a four-parameter logistic parallel-line assay; or the slope, asymptotes, and nonsymmetry parameter in a five-parameter sigmoid model. In some cases, these similarity measures have interpretable, practical meaning in the assay; certain changes in curve shape, for example, may be associated with specific changes (e.g., the presence of a specific active contaminant) in the product. When possible, discussion of these changes and their likely effects is a valuable part of setting appropriate equivalence boundaries.

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## IMPLEMENTING EQUIVALENCE TESTING FOR SIMILARITY (PARALLELISM)

As previously stated, many statistical procedures for assessing similarity are based on a null hypothesis stating that similarity is present and the alternative hypothesis of there being a state of nonsimilarity. Failure to find that similarity is statistically improbable is then taken as a conclusion of similarity. In fact, however, this failure to establish a probabilistic basis for nonsimilarity does not prove similarity. Equivalence testing provides a method for the analyst to proceed to a conclusion (if warranted by the data) of *sufficiently similar* while controlling the risk of doing so inappropriately. The following provides a sequence for this process of implementing equivalence testing.

*Step 1: Choose a measure of nonsimilarity.*

For the parallel-line case, this could be the difference or ratio of slopes. (The ratio of slopes can be less sensitive to the value of the slope. Framing the slope difference as a proportional change from Standard rather than in absolute slope units has an advantage because it is invariant to the units on the concentration and response axes.) For a slope-ratio assay, the measure of nonsimilarity can be the difference in y-intercepts between Test and Standard samples. Again, it can be advantageous to frame this difference as a proportion of the (possibly transformed) response range of Standard to make the measure invariant to the units of the response.

The determination of similarity could be based on the individual parameters, one at a time; for the four-parameter logistic model, similarity between Standard and Test samples can be assessed discretely for the upper asymptote, the slope, and the lower asymptote. If sigmoid curves with additional parameters are used to fit bioassay data, it is also important to consider addressing similarity between Standard and Test preparations of the additional curve parameters (e.g., asymmetry parameter of the five-parameter model). Alternatively, evaluation of similarity can be based on a single composite measure of nonparallelism, such as the *parallelism sum of squares*. This is found as the difference in residual sum of squared errors (RSSE) between the value obtained from fitting the Standard and Test curves separately and the value obtained from imposing parallelism:

$$\text{Parallelism sum of squares} = RSSE_p - RSSE_s - RSSE_t$$

where the subscripts P, S, and T denote Parallel model, Standard model, and Test model, respectively. With any composite measure, the analyst must consider the implicit relative weighting of the importance of the three (or more) curve regions and whether the weighting is appropriate for the problem at hand. For the parallelism sum of squares, for example, with nonlinear models, the weighting given to the comparison of the asymptotes depends on the amount of data in the current assay on and near the asymptotes.

*Step 2: Specify a range of acceptable values, typically termed an equivalence interval or "indifference zone," for the measure of nonsimilarity.*

The challenge in implementing equivalence testing is in setting appropriate equivalence bounds for the nonsimilarity measures. Ideally, information is available to link variation in similarity measures to meaningful differences in biological function (as measured by the bioassay). Information may be available from evaluation of orthogonal assays. The following four approaches can be used to determine this interval. If pharmacopeial limits have been specified for a defined measure of nonsimilarity, then the assay should satisfy those requirements.

a. The first approach is to compile historical data that compare the Standard to itself and using these data to determine the equivalence interval as a tolerance interval for the measure of nonparallelism. The advantage of using historical data is that they give the laboratory control of the false failure rate (the rate of failing a sample that is in fact acceptable). The disadvantage is that there is no control of the false pass rate (the rate of passing a sample that may have an unacceptable difference in performance relative to the Standard). The equivalence interval specification developed in this way is based solely on assay capability. Laboratories that use this approach should take caution that an imprecise assay in need of improvement may yield such a wide equivalence interval that no useful discrimination of nonsimilarity is possible.

b. Approach (a) is simple to implement in routine use and can be used with assay designs that do not provide reliable estimates of within-assay variation and hence confidence intervals. However, there is a risk that assays with larger than usual amounts of within-assay variation can pass inappropriately. The preferable alternative to (a) is therefore to determine a tolerance interval for the confidence interval for the measure of nonparallelism. The following is particularly appropriate to transition an existing assay with a substantial body of historical data on both Standard and Test samples from a difference testing approach to an equivalence approach:

i. For each value of the measure of nonparallelism from the historical data, determine a 95% confidence interval, (m,n).

ii. For each confidence interval, determine its maximum departure from perfect parallelism. This is max(|m|, |n|) for differences, max(1/m,n) for ratios, and simply n for quantities that must be positive, such as a sum of squares.

iii. Determine a tolerance interval for the maximum departures obtained in (ii). This will be a one-sided tolerance interval for these necessarily positive quantities. A nonparametric tolerance interval approach is preferred.

iv. "Sufficiently parallel" is concluded for new data if the confidence interval for the measure of nonparallelism falls completely within the interval determined in (iii).

Approaches (a) and (b), through their reliance on assay capability, control only the false fail rate, and neglect the false pass rate. Incorporating information from sources other than the evaluation of assay capability provides control of the false pass rate. Approaches (c) and (d) are means to this end.

c. The third approach starts with historical data comparing the Standard to itself and adds data comparing the Standard to known failures, e.g., to degraded samples. Compare values of the measure of nonsimilarity for data for which a conclusion of similarity is appropriate (Standard against itself) and data for which a conclusion of similarity is not appropriate, e.g., degraded samples. Based on this comparison, determine a value of the measure of nonsimilarity that discriminates between the two cases. If this approach is employed, a range of samples for which a conclusion of similarity is not appropriate should be utilized, including samples with the minimal important nonsimilarity. For nonlinear models,

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this comparison also can be used to determine which parameters should be assessed; some may not be sensitive to the failures that can occur with the specific assay or collection of nonsimilar samples.

d. The fourth approach is based on combining a sensitivity analysis of the assay curve to nonsimilarity parameters with what is known about the product and the assay. It is particularly helpful if information is available that links a shift in one or more nonsimilarity measures to properties of the product. These measures may be direct (e.g., conformational changes in a protein) or indirect (e.g., changes in efficacy or safety in an animal model). A complementary approach is provided by a limited sensitivity analysis that combines analyst and biologist judgment regarding the magnitude of shifts in a nonsimilarity parameter that are meaningful, with simulation and/or laboratory experiments, to demonstrate thresholds for similarity parameters that provide protection against important nonsimilarity. Additionally, risk analysis may be informed by the therapeutic index of the drug.

*Step 3. Examine whether the value of the nonsimilarity measure is found within the equivalence interval of acceptable values.*

For approaches (a) and (b), compare the obtained value of the measure of nonparallelism (a) or its confidence interval (b) to the interval obtained at the beginning of Step 2. The value must be within the limits if one uses (a), or the confidence interval must be completely within the limits if one uses (b).

An alternative to the approach described above [for (a)] is to use an average (historical) value for the variance of the ratio or difference in a similarity parameter—obtained from some number of individual assays—to compute an acceptance interval for a point estimate of the similarity parameter. This approach is simpler to implement in routine use and can be used with assay designs that are unable to provide reliable estimates of within-assay variation. However, there is a price. The equivalence testing approach that relies on assay-specific (within-assay) measure(s) of variation (i.e., the confidence intervals) is conservative in the sense that it will fail to pass similarity for samples from assays that have larger than usual amounts of within-assay variation. Using an acceptance region for a similarity parameter—rather than an acceptance region for confidence intervals for the similarity parameter—loses this conservative property and hence is not preferred where alternatives exist.

For approach (c), an approach that essentially treats the parallelism as a discrimination problem may be used. The choice of the cut point in (c) should take into account the rates of false positive and false negative decisions (and the acceptable risks to the laboratory) and should reflect the between-assay variability in precision. Thus it is reasonable to compare the point estimate of the measure of nonparallelism to the cut point and to not use confidence intervals. This approach is simpler to implement in routine use and can be used with assay designs that cannot provide reliable estimates of within-assay variation.

For approach (d), demonstrate that the measure of nonsimilarity is significantly greater than the lower endpoint of the acceptance interval and significantly less than the upper endpoint. (If the acceptance interval is one-sided, then apply only the single applicable test.) This is use of the TOST approach. For most situations, TOST can be most simply implemented by calculating a 90% two-sided confidence interval, which corresponds to a 5% equivalence test. If this confidence interval lies entirely within the equivalence interval specified at the beginning of Step 2, then similarity is sufficiently demonstrated. For parallel-line models, one can use either (1) a confidence interval based on the value of the difference of the slopes ±k times the standard error of that value, or (2) Fieller's Theorem for the ratio of slopes may be used. For slope ratio models use the confidence interval for the difference of intercepts. For nonlinear models, there is evidence that these simple confidence interval methods do not attain the stated level of confidence, and methods based on likelihood profile or resampling are more appropriate.

RANGE

The range for a relative potency bioassay is the interval between the upper and lower relative potencies for which the bioassay is shown to have suitable levels of precision, relative accuracy, linearity of log potency, and success rates for system and sample suitability. It is straightforward to determine whether or not a sample that is similar to a Standard has a relative potency within the (validated) range of the assay system. For samples that are not similar according to established criteria, it is more challenging to determine whether a relative potency estimate for the sample might be obtained. In a nonlinear parallel-line assay a sample that does not have data on one asymptote might be assumed to be out of the potency range of the assay. In a parallel straight-line assay a sample that does not have three or more points on the steep portion of the response curve may be out of the potency range of the assay. For samples that have not been shown to be similar to reference it is not appropriate to report potency or to construct a ratio of $EC_{50}$s from unrestricted fits. As such samples may be out of the assay range, it may be useful to shift the dilution of the test sample for a subsequent assay on the basis of an estimate of relative activity. This estimated relative activity may be obtained via the ratio of the concentrations of Standard and Test that yields responses that match the reference response at the reference $EC_{50}$.

## 4.8 Outliers

Bioassay data should be screened for outliers before relative potency analysis. Outliers may be simple random events or a signal of a systematic problem in the bioassay. Systematic error that generates outliers may be due to a dilution error at one or more concentrations of a Test sample or the Standard or due to a mechanical error (e.g., system malfunction). Several approaches for outlier detection can be considered. Visual inspection is frequently utilized but should be augmented with a more objective approach to avoid potential bias.

An outlier is a datum that appears not to belong among the other data present. An outlier may have a distinct, identifiable cause, such as a mistake in the bench work, equipment malfunction, or a data recording error, or it could just be an unusual value relative to the variability typically seen and may appear without an identifiable cause. The essential question pertaining to an outlier becomes: Is the apparent outlier sampled from the same population as the other, less discordant, data, or is it from another population? If it comes from the same population and the datum is, therefore, an unusual (yet still legitimate) value obtained by chance, then the datum should stand. If it comes from another population and the datum's excursive value is due to human error or instrument malfunction, then the datum should be omitted from calculations. In practice, the answer to this essential question is often unknown, and investigations into causes are often inconclusive. Outlier management relies on procedures and practices to yield the best answer possible to that essential question and to guide response accordingly.

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General chapter *Analytical Data—Interpretation and Treatment* ⟨1010⟩ addresses outlier labeling, identification, and rejection; statistical methods are included. General chapter ⟨1010⟩ also lists additional sources of information that can provide a comprehensive review of the relevant statistical methodology. General chapter ⟨1010⟩ makes no explicit remarks regarding outlier analysis in linear or nonlinear regression. Outlier analysis techniques appropriate for data obtained from regression of response on concentration can be used. Some remarks about outliers are provided here in the context of bioassays to emphasize or complement the information in ⟨1010⟩.

Of the procedures employed for analysis of drug compounds and biological drugs, the bioassay may be expected to be the most prone to outlying data. The management of outliers is appropriate with bioassay data on at least two levels: where an individual datum or a group of data (e.g., data at a concentration) can be checked against expected responses for the sample and concentration; and, separately, when estimates of relative potency from an assay can be checked for consistency with other independent estimates of the potency of the same material.

Three important aspects of outlier management are prevention, labeling, and identification.

Outlier prevention is preferred for obvious reasons, and is facilitated by procedures that are less subject to error and by checks that are sensitive to the sorts of errors that, given the experience gained in assay development, may be expected to occur. In effect, the error never becomes an outlier because it is prevented from occurring.

Good practice calls for the examination of data for outliers and labeling ("flagging") of the apparently outlying observation(s) for investigation. If investigation finds a cause, then the outlying datum may be excluded from analysis. Because of the ordinary occurrence of substantial variability in bioassay response, a laboratory's investigation into the outlying observation is likely to yield no determinable cause. However, the lack of evidence regarding an outlier's cause is not a clear indication that statistical outlier testing is warranted. Knowledge of the typical range of assay response variability should be the justification for the use of statistical outlier tests.

Outlier identification is the use of rules to confirm that the values are inconsistent with the known or assumed statistical model. For outliers with no determined cause, it is tempting to use statistical outlier identification procedures to discard unusual values. Discarding data solely because of statistical considerations should be a rare event. Falsely discarding data leads to overly optimistic estimates of variability and can bias potency estimates. The laboratory should monitor the failure rate for its outlier procedure and should take action when this is significantly higher than expected.

Statistical procedures for outlier identification depend on assumptions about the distribution of the data without outliers. Identification of data as outliers may mean only that the assumption about distribution is not correct. If dropping outliers because of statistical considerations is common, particularly if outliers tend to occur more often at high values or at high responses, then this may be an indication that the data require some adjustment, such as log transformation, as part of the assay procedure. Two approaches to statistical assessment of outlying data are replication-based and model-based.

### REPLICATION-BASED APPROACHES

When replicates are performed at concentrations of a Test sample and the Standard, an "extra variability" (EV) criterion may be employed to detect outliers. Historical data can be analyzed to determine the range in variability commonly observed among replicates, and this distribution of ranges can be used to establish an extreme in the range that might signal an outlier. Metrics that can be utilized are the simple range (maximum replicate minus minimum replicate), the standard deviation, or the CV or RSD among replicates. However, if the bioassay exhibits heterogeneity of variability, assumptions about uniform scatter of data are unsupported. Analysts can use a variable criterion across levels in the bioassay, or they can perform a transformation of the data to a scale that yields homogeneity of variability. Transformation can be performed with a POM approach as discussed previously. Where heterogeneity exists nonnormality is likely present, and the range rather than standard deviation or RSD should be used.

The actions taken upon detection of a potential outlier depend in part on the number of replicates. If EV is detected within a pair (n = 2) at a concentration of a Test sample or the Standard, it will not always be clear which of the replicates is aberrant, and the laboratory should eliminate the concentration from further processing. If more than two replicates are performed at each dilution the laboratory may choose to adopt a strategy that identifies which of the extremes may be the outlier. Alternatively, the laboratory may choose to eliminate the dilution from further processing.

### MODEL-BASED APPROACHES

Model-based approaches may be used to detect outliers within bioassay data. These approaches use the residuals from the fit of an appropriate model. In general, if using model-based methods to identify potential outliers, the models used may make fewer assumptions about the data than the models used to assess suitability and estimate potency. For example, a non-parametric regression (smoothing) model may be useful.

Lastly, an alternative to discarding outlying data is to use robust methods that are less sensitive to influence by outlying observations. Use of the median rather than the mean to describe the data's center exemplifies a robust perspective. Also, regression using the method of least squares, which underlies many of the methods in this chapter, is not robust in the presence of outliers. The use of methods such as robust regression may be appropriate but is not covered in the USP bioassay chapters.

## 4.9 Fixed and Random Effects in Models of Bioassay Response

The choice of treating factors as fixed or random is important for the bioassay design, the development experiments, the statistical analysis of data, and the bioassay validation. Fixed effects are factors for which all levels, or all levels of interest, are discretely present, like sample, concentration, temperature and duration of thaw, and incubation time. Data for a response at some level, or combination of levels, of a fixed factor, can predict future responses. Fixed effects are expected to cause a consistent shift in responses. Analysts study fixed effects by controlling them in the design and examining changes in means across levels of the factor.

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Random effects are factors of which the levels in a particular run of an assay are considered representative of levels that could be present. That is, there is no expectation that any specific value of the random factor will influence response. Rather, that value may vary subject to some expected distribution of values and thus may be a source of variability. For example, there is no desire to predict assay response for a specific day, but there is interest in predicting the variation in response associated with the factor "day". Examples of random effects include reagent lot, operator, or day if there is no interest in *specific* reagent lots, operators, or day as sources of variability. Analysts may study random effects by measuring the variance components corresponding to each random effect. Variance components can be estimated well only if there are an appreciable number of levels of each random effect. If there are, for example, only two or three reagent lots or analysts present, the variation associated with these factors will be poorly estimated.

Making a correct choice regarding treating a factor as fixed or random is important to the design of the assay and to proper reporting of its precision. Treating all factors as fixed, for example, leads to an understatement of assay variability because it ignores all sources of variability other than replication. The goal is to identify specific sources of variability that can be controlled, to properly include those factors in the design, and then to include other factors as random.

If the factor may switch from random to fixed effect or vice versa, the factor should normally be modeled as a random effect. For example, reagent lots cannot be controlled, so different lots are typically considered to cause variability, and reagent lot would be considered a random effect. However, if a large shift in response values has been traced to a particular lot, a comparison among a set of lots could be performed using reagent lot as a fixed effect. Similarly, within-assay location (e.g., block, plate, plate row, plate column, or well) or sequence may be considered a source of random variation or a source of a consistent (fixed) effect.

Assay designs that consist of multiple factors are efficient, but require corresponding statistical techniques that incorporate the factors as fixed or random effects in the analysis. If all factors are fixed, the statistical model is termed a fixed-effects model. If all are random, it is termed a random-effects model. If some factors are fixed and some random, the model is a mixed-effects model. Note that the concepts of fixed and random effects apply to models for quantitative, qualitative and integer responses. For assay designs that include multiple experimental units (e.g., samples assigned to sets of tubes and concentrations assigned to pre-plate tubes) a mixed-effects model in which the experimental units are treated as random effects is particularly effective. Additional complexity is added by the presence of designs with crossed random effects (e.g., each operator used material from one or more reagent batches, but many reagent batches were used by multiple operators). This can cause methodological and computational challenges for model fitting, especially when the designs are unbalanced.

## 5. STAGES IN THE BIOASSAY DEVELOPMENT PROCESS

Given the ubiquity of cell-based assays and the motivation to use one bioassay system to provide context for discussion, the development of a cell-based bioassay will be used to illustrate the stages in the bioassay development continuum.

### 5.1 Design: Assay Layout, Blocking, and Randomization

Most cell-based assays are performed using a cell culture plate (6-, 12-, 96-, or 384-well micro titer plate). Ideally, a plate is able to provide a uniform substrate for experimental treatments in all wells, including after wash steps and incubations. However, regardless of assay conditions intended to minimize the potential for bias (e.g., good analyst technique, careful calibration of pipets, controlled incubation time, and temperature), systematic gradients on the plate, independent of experimental treatments, may be observed. These gradients may occur across rows, across columns, or from the edge to the center of the plate and are often called *plate effects.* Even moderate or inconsistent plate effects should be addressed during assay development, by means of plate layout strategies, blocking, randomization, and replication.

Plate effects can be evaluated in a *uniformity trial* in which a single experimental treatment, such as an assay concentration chosen from the middle section of the concentration–response curve, is used in all wells of the plate. *Figure 1* provides an example of what may be observed; a trend of decreasing signal is evident from right to left. In this case, it was discovered that the plate washer was washing more briskly on the left side of the plate, and required adjustment to provide uniform washing intensity and eliminate the gradient. Another common plate effect is a differential cell-growth pattern in which the outer wells of the plate grow cells in such a way that the assay signal is attenuated. This is such a persistent problem that the choice is often made to not use the outer wells of the assay plate. Because location effects are so common, designs that place replicates (e.g., of sample by concentration combinations) in adjacent wells should be avoided.

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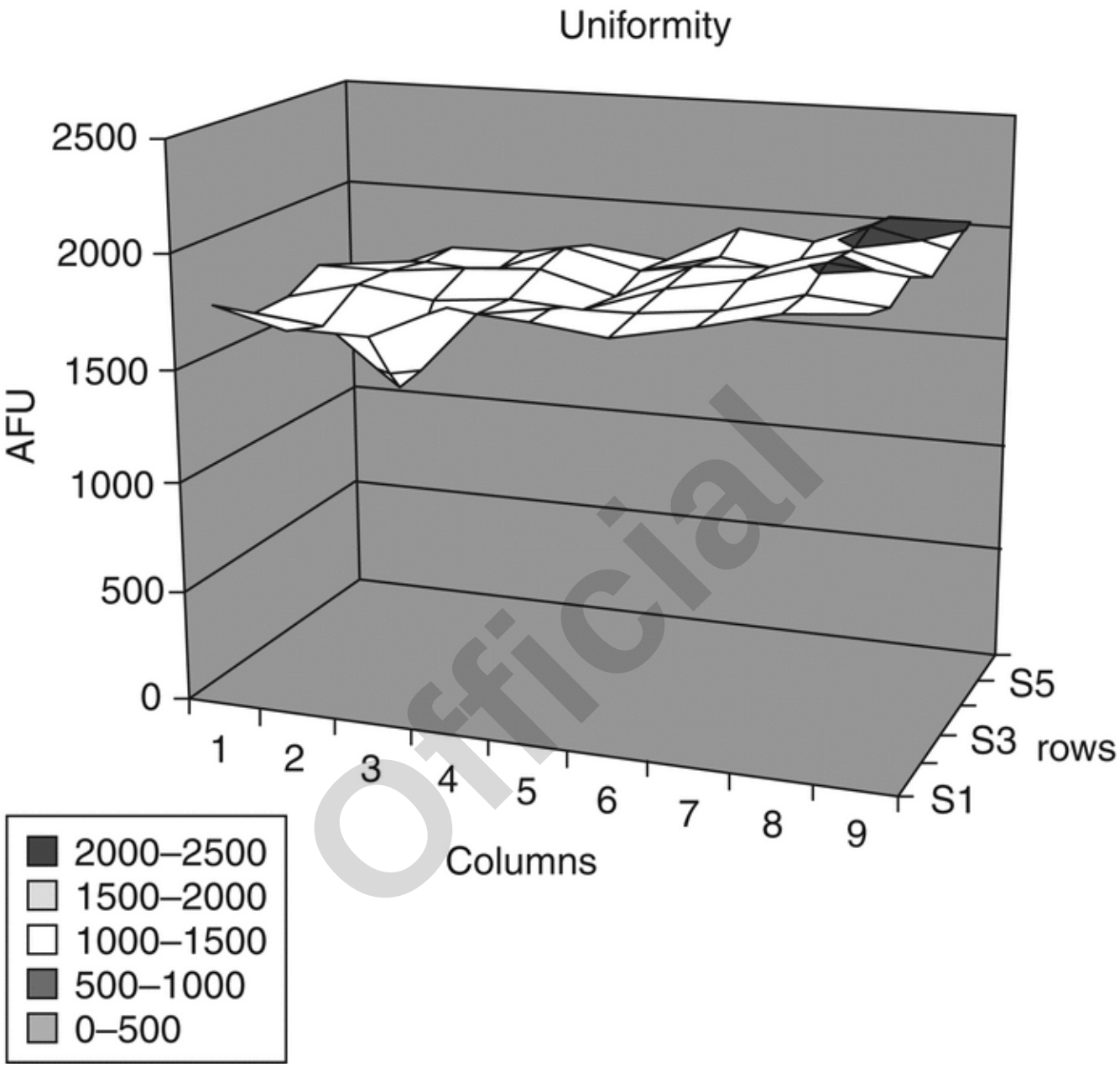


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Figure 1. Plot of change in assay response across a plate.

*Blocking* is the grouping of related experimental units in experimental designs. Blocks may consist of individual 96-well plates, sections of 96-well plates, or 96-well plates grouped by analyst, day, or batch of cells. The goal is to isolate any systematic effects so that they do not obscure the effects of interest. A *complete block design* occurs when all levels of a treatment factor (in a bioassay, the primary treatment factors are sample and concentration) are applied to experimental units for that factor within a single block. An *incomplete block design* occurs when the number of levels of a treatment factor exceeds the number of experimental units for that factor within the block.

*Randomization* is a process of assignment of treatment to experimental units based on chance so that all such experimental units have an equal chance of receiving a given treatment. Although challenging in practice, randomization of experimental treatments has been advocated as the best approach to minimizing assay bias or, more accurately, to protecting the assay results from known and unknown sources of bias by converting bias into variance. While randomization of samples and concentrations to individual plate wells may not be practical, a plate layout can be designed to minimize plate effects by alternating sample positions across plates and the pattern of dilutions within and across plates. Where multiple plates are required in an assay, the plate layout design should, at a minimum, alternate sample positions across plates within an assay run to accommodate possible bias introduced by the analyst or equipment on a given day. It is prudent to use a balanced rotation of layouts on plates so that the collection of replicates (each of which uses a different layout) provides some protection against likely sources of bias.

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*Figure 2* illustrates a patterned assay design that lacks randomization and is susceptible to bias. Dilutions and replicates of the Test preparations (A and B) and the Standard (R) are placed together sequentially on the plate. Bias due to a plate or incubator effect can influence some or all of the concentrations of one of the samples. Note that in *Figures 2* through *5* all outer plate wells are left as blanks to protect against *edge effect.*

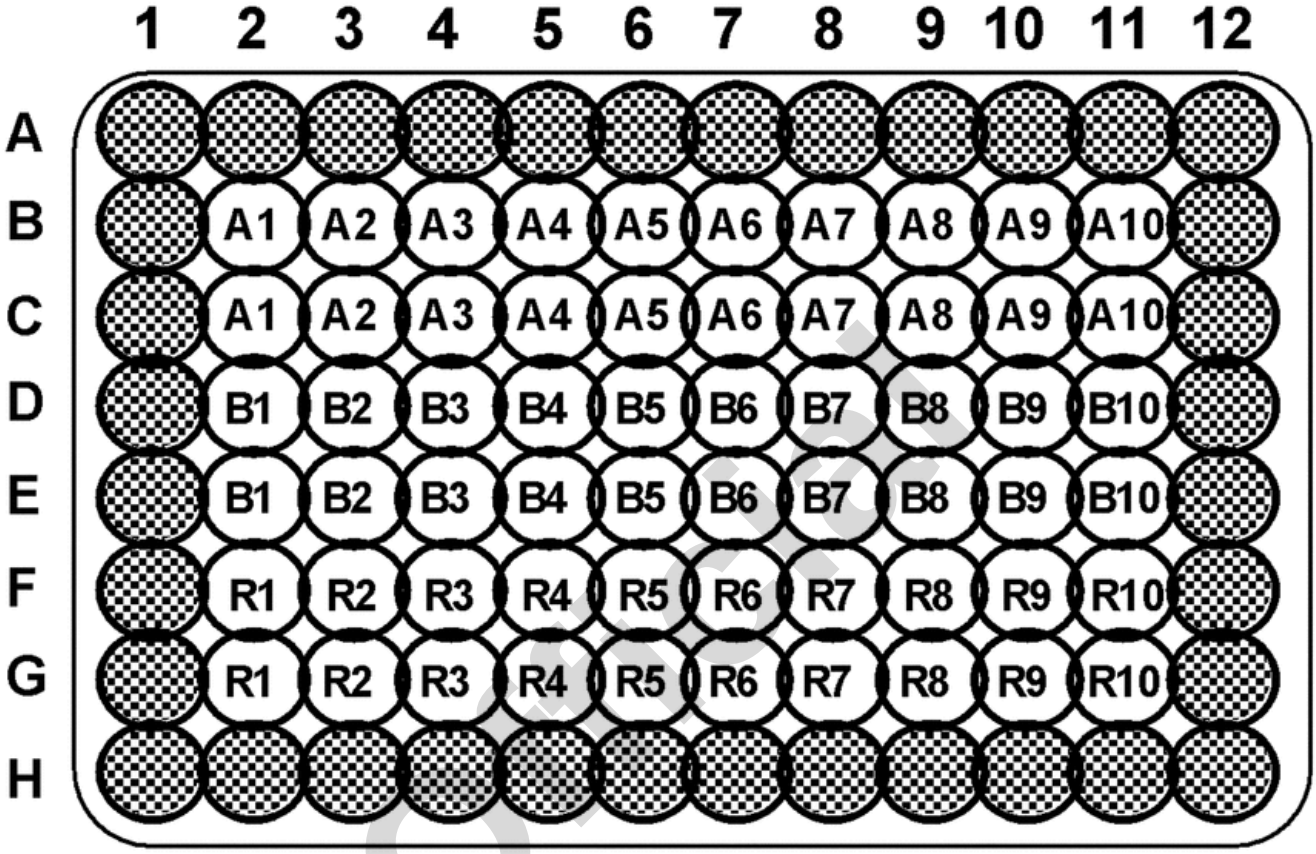


Figure 2. A highly patterned plate.

A layout that provides some protection from plate effects and can be performed manually is a *strip-plot design,* shown in *Figure 3*. Here samples are randomized to rows of a plate and dilution series are performed in different directions in different sections (blocks) on the plate to mitigate bias across columns of the plate. An added advantage of the strip-plot design is the ability to detect location effects by the interaction of sample and dilution direction (left-to-right or right-to-left).

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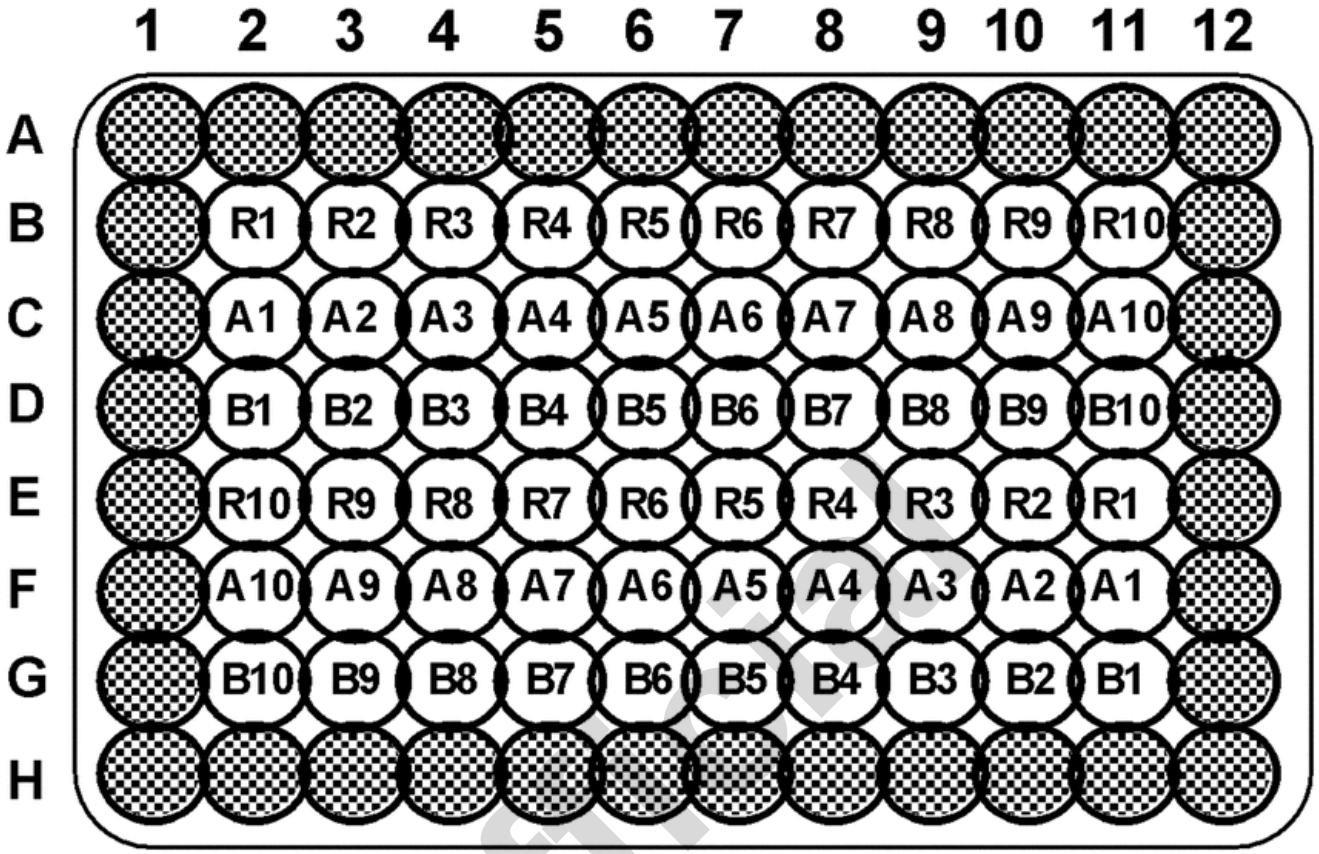


Figure 3. A strip-plot design.

*Figure 4* illustrates an alternation of Test (Test sample 1 = "1"; Test sample 2 = "2") and Standard ("R") positions on multiple plates, within a single assay run; this protects against plate row effects. Combining the two methods illustrated if *Figures 3* and *4* can effectively help convert plate bias into assay variance. Assay variance may then be addressed, as necessary, by increased assay replication (increased number of plates in an assay).

| Plate Row | Plate 1 | Plate 2 | Plate 3 |
|---|---|---|---|
| B | R | 2 | 1 |
| C | 1 | R | 2 |
| D | 2 | 1 | R |
| E | R | 2 | 1 |
| F | 1 | R | 2 |
| G | 2 | 1 | R |

Figure 4. A multi-plate assay with varied Test and Reference positions.

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A *split-plot design,* an alternative that assigns samples to plate rows randomly and randomizes dilutions (concentrations) within each row, is seen in *Figure 5*. Such a strategy may be difficult to implement even with the use of robotics.

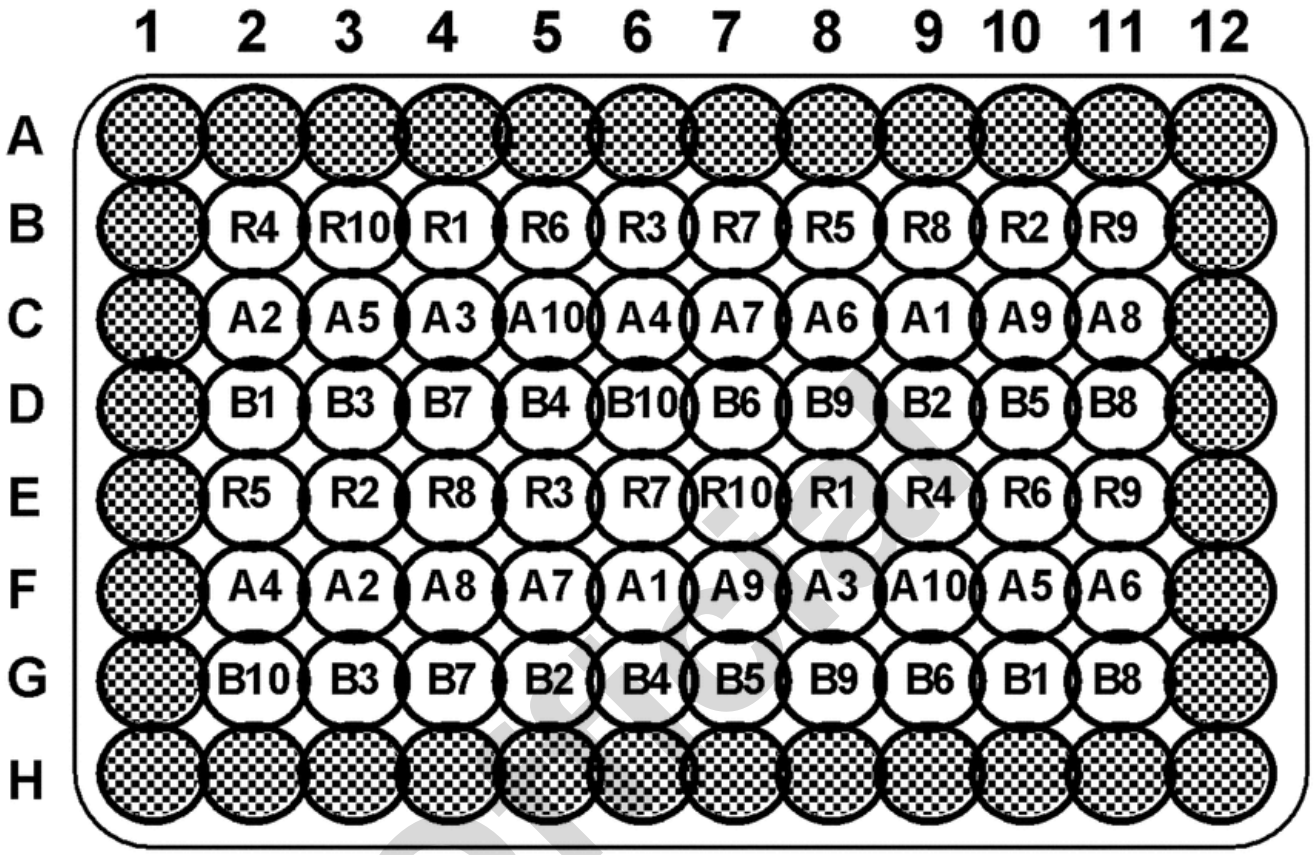


Figure 5. A split-plot design.

### Dilution Strategy

Assay concentrations of a Test sample and the Standard can be obtained in different ways. Laboratories often perform serial dilutions, in which each dilution is prepared from the previous one, in succession. Alternatively, the laboratory may prepare wholly independent dilutions from the Test sample and Standard to obtain independent concentration series. These two strategies result in the same nominal concentrations, but they have different properties related to error. Serial dilutions are subject to propagation of error across the dilution series, and a dilution error made at an early dilution will result in correlated, non-independent observations. Correlations may also be introduced by use of multichannel pipets. Independent dilutions help mitigate the bias resulting from dilution errors.

It is noteworthy that when working to improve precision, the biggest reductions in variance come when replicating at the highest possible levels of nested random effects. This is particularly effective when these highest levels are sources of variability. To illustrate: replicating extensively within a day for an assay known to have great day-to-day variation is not effective in improving precision of reportable values.

## 5.2 Development

A goal of bioassay development is to achieve optimal bioassay relative accuracy and precision of the potency estimate. An endpoint of assay development is the completed development of the assay procedure, a protocol for the performance of the bioassay. The procedure should include enough detail so that a qualified laboratory with a trained analyst can perform the procedure in a routine manner. A strategic part of development is a look forward toward performance maintenance. Standard operating procedures for reagent and technician qualification, as well as for calibration of the working Standard, help complete the bioassay development package.

### ONE FACTOR AT A TIME VERSUS DESIGN OF EXPERIMENTS

Bioassay development proceeds through a series of experiments in which conditions and levels of assay factors are varied to identify those that support a reliable and robust bioassay qualified for routine use. Those experiments may be conducted one factor at a time (OFAT), studying each parameter separately to identify ideal conditions, or through the use of multi-factor design of experiments (DOE). DOE is an efficient and effective strategy for developing a bioassay and improving bioassay

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performance, thus helping to obtain a measurement system that meets its requirements. In comparison to OFAT, DOE generally requires fewer experiments and also provides insight into interactions of factors that affect bioassay performance. Assay development using DOE may proceed through a series of steps: process mapping and risk analysis; screening; response optimization; and confirmation.

#### PROCESS MAPPING AND RISK ANALYSIS

Bioassay optimization may begin with a systematic examination and risk assessment to identify those factors that may influence bioassay response. It is useful to visualize bioassay factors using a bioassay process map such as a cause-and-effect or fishbone diagram. Using the process map as a guide, the laboratory can examine assay factors that might affect assay performance, such as buffer pH, incubation temperature, and incubation time. Historical experience with one or several of the bioassay steps, along with sound scientific judgment, can identify key factors that require further evaluation. One tool that may be used to prioritize factors is a failure mode and effects analysis. Factors are typically scored by the combination of their potential to influence assay response and the likelihood that they will occur. The laboratory must be careful to recognize potential interactions between assay factors.

#### SCREENING

Once potential key factors have been identified from process mapping and risk analysis, the laboratory may conduct an initial screening experiment to probe for effects that may require control. Screening designs such as factorial and fractional factorial designs are commonly used for this purpose. Software is available to assist the practitioner in the selection of the design and in subsequent analysis. Analysts should take care, however, to understand their assumptions about design selection and analysis to ensure accurate identification of experimental factors.

#### RESPONSE OPTIMIZATION

A screening design will usually detect a few important factors from among those studied. Such factors can be further studied in a response-optimization design. Response-optimization designs such as central composite designs are performed to determine optimal settings for combinations of bioassay factors for achieving desired response. The information obtained from response optimization may be depicted as a response surface and can be used to establish ranges that yield acceptable assay performance and will be incorporated into the bioassay procedure.

In the parlance of Quality by Design (QbD), the "region" where the combined levels of input variables and process parameters have been demonstrated to provide acceptable assay performance is described as the *design space* for the bioassay. Establishing a true design space for a bioassay is challenging; some but not all factors and levels of random factors will be included in the development DOE, and there is no assurance that the design space is not sensitive to unstudied random factors. Similarly, there is little assurance that the assay (design space) is robust to random factors that are studied using small samples (or non-random samples of levels). Elements of DOE that may be considered include the use of blocks; deliberate confounding among interactions that are of lower interest, or known to be unimportant; robust design (response surface designs with random effects); and use of split-plot, strip-plot, or split-lot designs.

#### CONFIRMATION

The mathematical model depicting assay performance as a function of changes in key assay factors is an approximation; thus, it is customary to confirm performance at the ideal settings of the bioassay. Confirmation can take the form of a qualification trial in which the assay is performed, preferably multiple independent times using optimal values for factors. Alternatively, the laboratory may determine that the bioassay has been adequately developed and may move to validation. Qualification is a good practice, not a regulatory requirement. The decision to perform confirmatory qualifying runs or to proceed to validation depends upon the strength of the accumulated information obtained throughout development.

## 5.3 Data Analysis during Assay Development

Analysis of bioassay data during assay development enables analysts to make decisions regarding the statistical model that will be used for routine analysis, including transformation and/or weighting of data, and the development of system and sample suitability criteria. The analysis also provides information regarding which elements of design structure should be used during outlier detection and the fitting of a full model. This may also include a plan for choosing subsets of data, such as a linear portion, for analysis or, for nonlinear bioassays, a model reduction strategy for samples similar to Standard. Once these decisions are made and proven sound during validation, they don't need to be reassessed with each performance of the assay. A process approach to enabling these decisions follows.

*Step 1: Choose an appropriate statistical model (also see section 4.6 Common Bioassay Models).*
Given the complexity of bioassays and the motivation to use an approach proven reliable, fairly standardized analytical models are common in the field of bioassay analysis. Nonetheless, many considerations are involved in choosing the most appropriate statistical model. First, the model should be appropriate for the type of assay endpoint—continuous, count, or dichotomous. Second, the model should incorporate the structure of the assay design. For any design other than completely randomized, there will be terms in the model for the structural elements. These could be, for example, within-plate blocking, location of cage in the animal facility, day, etc. A third consideration, applicable to continuous endpoints, involves whether to use a regression model or a means model (an analysis of variance model that fits a separate mean at each dilution level of each sample tested), with appropriate error terms. A means model can be appropriate at this stage because it makes no assumptions about the shape of the concentration–response curve.

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*Step 2: Fit the chosen statistical model to the data without the assumption of parallelism, and then assess the distribution of the residuals, specifically examining them for departures from normality and constant variance.*
Transform the data as necessary or, if needed, choose a weighting scheme (see section 4.3 *Variance Heterogeneity, Weighting, and Transformation*). Use as large a body of assay data, from independent assays, as possible. The primary goal is to address any departure from normality and from constant variance of responses across the range of concentrations in the assay. Step 2 will likely alternate between imposing a transformation and assessing the distribution of the residuals.

*Step 3: Screen for outliers, and remove as is appropriate.*
This step normally follows the initial choice of a suitable transformation and/or weighting method. Ideally the model used for outlier detection contains the important elements of the assay design structure, allows nonsimilar curves, and makes fewer assumptions about the functional shape of the concentration–response curve than did the model used to assess similarity. See section 4.8 *Outliers* and general chapter ⟨1010⟩ for discussion of outlier detection and removal. In some cases, outliers may be so severe that a reasonable model cannot be fit, and thus residuals will not be available. In such cases, it is necessary to screen the raw data for outliers before attempting to fit the model.
During assay development, a strategy should be developed for the investigation and treatment of an outlier observation, including any limits on how many outliers are acceptable. Include these instructions in the assay SOP. Good practice includes recording the process of an investigation, outlier test(s) applied, and results therefrom. Note that outlier procedures must be considered apart from the investigation and treatment of an out-of-specification (OOS) result (reportable value). Decisions to remove an outlier from data analysis should not be made on the basis of how the reportable value will be affected (e.g., a potential OOS result). Removing data as outliers should be rare. If many values from a run are removed as outliers, that run should be considered suspect.

*Step 4: Refit the model with the transformation and/or weighting previously imposed (Step 2) without the observations identified as outliers (Step 3) and re-assess the appropriateness of the model.*

*Step 5: If necessary or desired, choose a scheme for identifying subsets of data to use for potency estimation, whether the model is linear or nonlinear (see section 4.5 Linearity of Concentration–Response Data).*

*Step 6: Calculate a relative potency estimate by analyzing the Test and Standard data together using a model constrained to have parallel lines or curves, or equal intercepts.*

## 5.4 Bioassay Validation

The bioassay validation is a protocol-driven study that demonstrates that the procedure is fit for use. A stage-wise approach to validation may be considered, as in a "suitable for intended use" validation to support release of clinical trial material, and a final, comprehensive validation prior to BLA or MAA filing. Preliminary system and sample suitability controls should be established and clearly described in the assay procedure; these may be finalized based on additional experience gained in the validation exercise. Chapter ⟨1033⟩ provides validation comprehensive discussion of bioassay validation.

## 5.5 Bioassay Maintenance

The development and validation of a bioassay, though discrete operations, lead to ongoing activities. Assay improvements may be implemented as technologies change, as the laboratory becomes more skilled with the procedure, and as changes to bioassay methodology require re-evaluation of bioassay performance. Some of these changes may be responses to unexpected performance during routine processing. Corrective action should be monitored using routine control procedures. Substantial changes may require a study verifying that the bioassay remains fit for use. An equivalence testing approach can be used to show that the change has resulted in acceptable performance. A statistically-oriented study can be performed to demonstrate that the change does not compromise the previously acceptable performance characteristics of the assay.

### ASSAY TRANSFER

Assay transfer assumes both a known intended use of the bioassay in the recipient lab and the associated required capability for the assay system. These implicitly, though perhaps not precisely, demarcate the limits on the amount of bias and loss of precision allowed between labs. Using two laboratories interchangeably to support one product will require considering the variation between labs in addition to intermediate precision for sample size requirements to determine process capability. For a discussion and example pertaining to the interrelationship of bias, process capability, and validation, see *A Bioassay Validation Example* in ⟨1033⟩.

### IMPROVING OR UPDATING A BIOASSAY SYSTEM

A new version of a bioassay may improve the quality of bias, precision, range, robustness, specificity, lower the operating costs or offer other compelling advantages. When improving or updating a bioassay system a bridging study may be used to compare the performance of the new to the established assay. A wide variety of samples (e.g., lot release, stability, stressed, critical isoforms) can be used for demonstrating equivalence of estimated potencies. Even though the assay systems may be quite different (e.g., an animal bioassay versus a cell-based bioassay), if the assays use the same Standard and mechanism of action, comparable potencies may reasonably be expected. If the new assay uses a different Standard, the minimum requirement for an acceptable comparison is a unit slope of the log linear relationship between the estimated potencies. An important implication of this recommendation is that poor precision or biased assays used early can have lasting impact on the replication requirements, even if the assay is later replaced by an improved assay.

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# ⟨1033⟩ BIOLOGICAL ASSAY VALIDATION

## 1. INTRODUCTION

*Biological assays* (also called bioassays) are an integral part of the quality assessment required for the manufacturing and marketing of many biological and some non-biological drug products. Bioassays commonly used for drug potency estimation can be distinguished from chemical tests by their reliance on a biological substrate (e.g., animals, living cells, or functional complexes of target receptors). Because of multiple operational and biological factors arising from this reliance on biology, they typically exhibit a greater variability than do chemically-based tests.

Bioassays are one of several physicochemical and biologic tests with procedures and acceptance criteria that control critical quality attributes of a biological drug product. As described in the ICH Guideline entitled Specifications: Test Procedures And Acceptance Criteria For Biotechnological/Biological Products (Q6B), section 2.1.2, bioassay techniques may measure an organism's biological response to the product; a biochemical or physiological response at the cellular level; enzymatic reaction rates or biological responses induced by immunological interactions; or ligand- and receptor-binding. As new biological drug products and new technologies emerge, the scope of bioassay approaches is likely to expand. Therefore, general chapter *Biological Assay Validation* ⟨1033⟩ emphasizes validation approaches that provide flexibility to adopt new bioassay methods, new biological drug products, or both in conjunction for the assessment of drug potency.

Good manufacturing practice requires that test methods used for assessing compliance of pharmaceutical products with quality requirements should meet appropriate standards for accuracy and reliability. Assay validation is the process of demonstrating and documenting that the performance characteristics of the procedure and its underlying method meet the requirements for the intended application and that the assay is thereby suitable for its intended use. *USP* general chapter *Validation of Compendial Procedures* ⟨1225⟩ and ICH Q2(R1) describe the assay performance characteristics (parameters) that should be evaluated for procedures supporting small-molecule pharmaceuticals. Although evaluation of these validation parameters is straightforward for many types of analytical procedures for well-characterized, chemically-based drug products, their interpretation and applicability for some types of bioassays has not been clearly delineated. This chapter addresses bioassay validation from the point of view of the measurement of activity rather than mass or other physicochemical measurements, with the purpose of aligning bioassay performance characteristics with uses of bioassays in practice.

Assessment of bioassay performance is a continuous process, but bioassay validation should be performed when development has been completed. Bioassay validation is guided by a validation protocol describing the goals and design of the validation study. General chapter ⟨1033⟩ provides validation goals pertaining to *relative potency* bioassays. Relative potency bioassays are based on a comparison of bioassay responses for a Test sample to those of a designated Standard that provides a quantitative measure of the Test bioactivity relative to that of the Standard.

Validation parameters discussed include *relative accuracy, specificity, intermediate precision,* and *range.* Laboratories may use *dilutional linearity* to verify the *relative accuracy* and *range* of the method. Although robustness is not a requirement for validation, general chapter ⟨1033⟩ recommends that a bioassay's robustness be assessed prior to validation. In addition, ⟨1033⟩ describes approaches for validation design (sample selection and replication strategy), validation acceptance criteria, data analysis and interpretation, and finally bioassay performance monitoring through quality control. Documentation of bioassay validation results is also discussed, with reference to pre-validation experiments performed to optimize bioassay performance. In the remainder of general chapter ⟨1033⟩ the term "bioassay" should be interpreted as meaning "relative potency bioassay".

## 2. FUNDAMENTALS OF BIOASSAY VALIDATION

The goal of bioassay validation is to confirm that the operating characteristics of the procedure are such that the procedure is suitable for its intended use. The issues involved in developing a bioassay are described in greater detail in general chapter ⟨1032⟩ and are assumed resolved by the time the bioassay is in validation. Included in those decisions will be identification of what constitutes an assay and a run for the bioassay. Multiple dilutions (concentrations) of the Standard and one or more Test samples constitute a *replicate set* (also known as a minimal set), which contain a test substrate (e.g., group of animals or vessel of cells) at each dilution for each sample [Test(s) and Standard]. A *run* is defined as work performed during a period when the accuracy (trueness) and precision in the assay system can reasonably be expected to be stable. In practice, a run frequently consists of the work performed by a single analyst in one lab, with one set of equipment, in a short period of time (typically a day). An assay is the body of data used to assess similarity and estimate potency relative to a Standard for each Test sample in the assay. A run may contain multiple assays, a single assay, or part of an assay. Multiple assays may be combined to yield a reportable value for a sample. The reportable value is the value that is compared to a product specification.

In assays that involve groups at each dilution (e.g., 6 samples, each at 10 dilutions, in the non-edge wells of each of several 96-well cell culture plates) the groups (plates) constitute statistical *blocks* that should be elements in the assay and validation analyses (blocks are discussed in ⟨1032⟩). Within-block replicates for Test samples are rarely cost-effective. Blocks will not be further discussed in this chapter; more detailed discussion is found in ⟨1032⟩.

The amount of activity (potency) of the Standard is initially assigned a value of 1.0 or 100%, and the potency of the Test sample is calculated by comparing the concentration–response curves for the Test and Standard pair. This results in a unitless measure, which is the relative potency of the Test sample in reference to the potency of the Standard. In some cases the Standard is assigned a value according to another property such as protein concentration. In that case the potency of the Test sample is the relative potency times the assigned value of the Standard. An assumption of parallel-line or parallel-curve (e.g., four-parameter logistic) bioassays is that the dose–response curves that are generated using a Standard and a Test sample have similar (parallel) curve shape distinguished only by a horizontal shift in the log dose. For slope-ratio bioassays, curves generated for Standard and Test samples should be linear, pass through a common intercept, and differ only by their slopes. Information about how to assess parallelism is provided in general chapters ⟨1032⟩ and ⟨1034⟩.

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In order to establish the *relative accuracy* and *range* of the bioassay, validation Test samples may be constructed using a dilution series of the Standard to assess dilutional linearity (linearity of the relationship between known and measured relative potency). In addition, the validation study should yield a representative estimate of the variability of the relative potency determination. Although robustness studies are usually performed during bioassay development, key factors in these studies such as incubation time and temperature and, for cell-based bioassays, cell passage number and cell number may be included in the validation, particularly if they interact with another factor that is introduced during the validation (e.g., a temperature sensitive reagent that varies in its sensitivity from lot-to-lot). Because of potential influences on the bioassay from inter-run factors such as multiple analysts, instruments, or reagent sources, the design of the bioassay validation should include consideration of these factors. The variability of potency from these combined elements defines the *intermediate precision* (IP) of the bioassay. An appropriate study of the variability of the potency values obtained, including the impact of intra-assay and inter-run factors, can help the laboratory confirm an adequate testing strategy and forecast the inherent variability of the *reportable value* (which may be the average of multiple potency determinations). Variability estimates can also be utilized to establish the sizes of differences (fold difference) that can be distinguished between samples tested in the bioassay. (See section 3.4 *Use of Validation Results for Bioassay Characterization*.)

Demonstrating specificity (also known as selectivity) requires evidence of lack of influence from matrix components such as manufacturing process components or degradation products so that measurements quantify the target molecule only. Other analytical methods may complement a bioassay in measuring or identifying other components in a sample.

## 2.1 Bioassay Validation Protocol

A bioassay validation protocol should include the number and types of samples that will be studied in the validation; the study design, including inter-run and intra-run factors; the replication strategy; the intended validation parameters and justified target acceptance criteria for each parameter; and a proposed data-analysis plan. Note that in regard to satisfying acceptance criteria, failure to find a statistically significant effect is not an appropriate basis for defining acceptable performance in a bioassay; conformance to acceptance criteria may be better evaluated using an equivalence approach.

In addition, assay, run, and sample acceptance criteria such as system suitability and similarity should be specified before performing the validation. Depending on the extent of development of the bioassay, these may be proposed as tentative and can be updated with data from the validation. Assay, run, or sample failures may be reassessed according to criteria which have been defined in the validation protocol and, with sound justification, included in the overall validation assessment. Additional validation trials may be required in order to support changes to the method.

The bioassay validation protocol should include target acceptance criteria for the proposed validation parameters. Steps to be taken upon failure to meet a target acceptance criterion should be specified in the validation protocol, and may result in a limit on the range of potencies that can be measured in the bioassay or a modification to the replication strategy in the bioassay procedure.

## 2.2 Documentation of Bioassay Validation Results

Bioassay validation results should be documented in a bioassay validation report. The validation report should support the conclusion that the method is fit for use or should indicate corrective action (such as an increase in the replication strategy) that will be undertaken to generate sufficiently reliable results to achieve fitness for use. The report could include the raw data and intermediate results (e.g., variance component estimates should be provided in addition to overall intermediate precision) which would facilitate reproduction of the bioassay validation analysis by an independent reviewer. Estimates of validation parameters should be reported at each level and overall as appropriate. Deviations from the validation protocol should be documented with justification. The conclusions from the study should be clearly described with references to follow-up action as necessary. Follow-up action can include amendment of system or sample suitability criteria or modification of the bioassay replication strategy. Reference to prevalidation experiments may be included as part of the validation study report. Prevalidation experiments may include robustness experiments, where bioassay parameters have been identified and ranges have been established for significant parameters, and also may include qualification experiments, where the final procedure has been performed to confirm satisfactory performance in routine operation. Conclusions from prevalidation and qualification experiments performed during development contribute to the description of the operating characteristics of the bioassay procedure.

## 2.3 Bioassay Validation Design

The biological assay validation should include samples that are representative of materials that will be tested in the bioassay and should effectively establish the performance characteristics of the procedure. For *relative accuracy,* sample relative potency levels that bracket the range of potencies that may be tested in the bioassay should be used. Thus samples that span a wide range of potencies might be studied for a drug or biological with a wide specification range or for a product that is inherently unstable, but a narrower range can be used for a more durable product. A minimum of three potency levels is required, and five are recommended for a reliable assessment. If the validation criteria for relative accuracy and IP are satisfied, the potency levels chosen will constitute the *range* of the bioassay. A limited range will result from levels that fail to meet their target acceptance criteria. Samples may also be generated for the bioassay validation by stressing a sample to a level that might be observed in routine practice (i.e., stability investigations). Additionally, the influences of the sample matrix (excipients, process constituents, or combination components) can be studied strategically by intentionally varying these together with the target *analyte*, using a multifactorial approach. Often this will have been done during development, prior to generating release and stability data.

The bioassay validation design should consider all facets of the measurement process. Sources of bioassay measurement variability include sample preparation, intra-run factors, and inter-run factors. Representative estimation of bioassay variability

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necessitates consideration of these factors. Test sample and Standard preparation should be performed independently during each validation run.

The replication strategy used in the validation should reflect knowledge of the factors that might influence the measurement of potency. Intra-run variability may be affected by bioassay operating factors that are usually set during development (temperature, pH, incubation times, etc.); by the bioassay design (number of animals, number of dilutions, replicates per dilution, dilution spacing, etc.); by the assay acceptance and sample acceptance criteria; and by the statistical analysis (where the primary endpoints are the similarity assessment for each sample and potency estimates for the reference samples). Operating restrictions and bioassay design (intra- and inter-run formulae that result in a *reportable value* for a test material) are usually specified during development and may become a part of the bioassay operating procedure. IP is studied by independent *runs* of the procedure, perhaps using an experimental design that alters those factors that may have an impact on the performance of the procedure. Experiments (including those that implement formalized design of experiments [DOE]) with nested or crossed design structure can reveal important sources of variability in the procedure, as well as ensure a representative estimate of long-term variability. During the validation it is not necessary to employ the format required to achieve the reportable value for a Test sample. A well-designed validation experiment that combines both intra-run and inter-run sources of variability provides estimates of independent components of the bioassay variability. These components can be used to verify or forecast the variability of the bioassay format.

A thorough analysis of the validation data should include graphical and statistical summaries of the validation parameters' results and their conformance to target acceptance criteria. The analysis should follow the specifics of the data-analysis plan outlined in the validation protocol. In most cases, log relative potency should be analyzed in order to satisfy the assumptions of the statistical methods (see section 2.7 *Statistical Considerations, Scale of Analysis*,). Those assumptions include *normality* of the distribution from which the data were sampled and *homogeneity of variability* across the range of results observed in the validation. These assumptions can be explored using graphical techniques such as box plots and probability plots. The assumption of normality can be investigated using statistical tests of normality across a suitably sized collection of historical results. Alternative methods of analysis should be sought when the assumptions can be challenged. *Confidence intervals* should be calculated for the validation parameters, using methods described here and in general chapter *Analytical Data—Interpretation and Treatment* ⟨1010⟩.

## 2.4 Validation Strategies for Bioassay Performance Characteristics

Parameters that should be verified in a bioassay are *relative accuracy, specificity,* IP (which incorporates repeatability), and *range*. Other parameters discussed in general chapter ⟨1225⟩ and ICH Q2(R1) such as detection limit and quantitation limit have not been included because they are usually not relevant to a bioassay that reports relative potency. These may be relevant, however, to the validation of an ancillary assay such as one used to score responders or measure response in conjunction with an *in vivo* potency assay. Likewise linearity is not part of bioassay validation, except as it relates to relative accuracy (dilutional linearity). There follow strategies for addressing bioassay validation parameters.

### Relative Accuracy

The *relative accuracy* of a relative potency bioassay is the relationship between measured relative potency and known relative potency. Relative accuracy in bioassay refers to a unit slope (slope = 1) between log measured relative potency and log known relative potency. The most common approach to demonstrating relative accuracy for relative potency bioassays is by construction of target potencies by dilution of the standard material or a Test sample with known potency. This type of study is often referred to as a *dilutional linearity study*. The results from a dilutional linearity study should be assessed using the estimated relative bias at individual levels and via a trend in *relative bias* across levels. The *relative bias* at individual levels is calculated as follows:

$$\text{Relative Bias} = 100 \cdot \left( \frac{\text{Measured Potency}}{\text{Target Potency}} - 1 \right) \%$$

The trend in bias is measured by the estimated slope of log measured potency versus log target potency, which should be held to a target acceptance criterion. If there is no trend in *relative bias* across levels, the estimated *relative bias* at each level can be held to a prespecified target acceptance criterion that has been defined in the validation protocol (see section 3 *A Bioassay Validation Example*).

**Specificity—**For products or intermediates associated with complex matrices, specificity involves demonstrating lack of interference from matrix components or product-related components that can be expected to be present. This can be assessed via parallel dilution of the Standard with and without a spike addition of the potentially interfering compound. If the curves are similar and the potency conforms to expectations of a Standard-to-Standard comparison, the bioassay is specific against the compound. For these assessments both similarity and potency may be assessed using appropriate equivalence tests.

Specificity may also refer to the capacity of the bioassay to distinguish between different but related biopharmaceutical molecules. An understanding should be sought of the molecule and any related forms, and of opportunities for related molecules to be introduced into the bioassay.

**Intermediate Precision—**Because of potential influences on the bioassay by factors such as analysts, instruments, or reagent lots, the design of the bioassay validation should include evaluation of these factors. The overall variability from measurements taken under a variety of normal test conditions within one laboratory defines the IP of the bioassay. IP is the ICH and USP term for what is also commonly referred to as inter-run variability. IP measures the influence of factors that will vary over time after

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the bioassay is implemented. These influences are generally unavoidable and include factors like change in personnel (new analysts), receipt of new reagent lots, etc.

When the validation has been planned using multifactor DOE, the impact of each factor can first be explored graphically to establish important contributions to potency variability. The identification of important factors should lead to procedures that seek to control their effects, such as further restrictions on intra-assay operating conditions or strategic qualification procedures on inter-run factors such as analysts, instruments, and reagent lots.

Contributions of validation study factors to the overall IP of the bioassay can be determined by performing a *variance component analysis* on the validation results. *Variance component analysis* is best carried out using a statistical software package that is capable of performing a mixed-model analysis with restricted maximum likelihood estimation (REML).

A variance component analysis yields variance component estimates such as

$$\hat{\sigma}^2_{\text{Intra}}$$

and

$$\hat{\sigma}^2_{\text{Inter}}$$

corresponding to intra-run and inter-run variation. These can be used to estimate the IP of the bioassay, as well as the variability of the *reportable value* for different bioassay formats (format variability). IP expressed as percent *geometric* coefficient of variation (%GCV) is given by the following formula, in this case using the natural log of the relative potency in the analysis (see section 2.7 *Statistical Considerations, Scale of Analysis*):

$$\text{Intermediate Precision} = 100 \cdot \left(e^{\sqrt{\sigma^2_{\text{Inter}} + \sigma^2_{\text{Intra}}}} - 1\right)\%$$

The variability of the *reportable value* from testing performed with n replicate sets in each of k runs (*format variability*) is equal to:

$$\text{Format Variability} = 100 \cdot \left(e^{\sqrt{\sigma^2_{\text{Inter}}/k + \sigma^2_{\text{Intra}}/(nk)}} - 1\right)\%$$

This formula can be used to determine a testing format suitable for various uses of the bioassay (e.g., release testing and stability evaluation).

**Range**—The *range* of the bioassay is defined as the true or known potencies for which it has been demonstrated that the analytical procedure has a suitable level of relative accuracy and IP. The range is normally derived from the dilutional linearity study and minimally should cover the product specification range for potency. For stability testing and to minimize having to dilute or concentrate hyper- or hypo-potent Test samples into the bioassay range, there is value in validating the bioassay over a broader range.

## 2.5 Validation Target Acceptance Criteria

The validation target acceptance criteria should be chosen to minimize the risks inherent in making decisions from bioassay measurements and to be reasonable in terms of the capability of the art. When there is an existing product specification, acceptance criteria can be justified on the basis of the risk that measurements may fall outside of the product specification. Considerations from a process capability (Cp) index can be used to inform bounds on the *relative bias* (RB) and the IP of the bioassay. This chapter uses the following Cpm index:

$$\text{Cpm} = \frac{\text{USL} - \text{LSL}}{6 \cdot \sqrt{\sigma^2_{\text{Product}} + \text{RB}^2 + \sigma^2_{\text{RA}}}}$$

where USL and LSL are the upper and lower release specification, RB is a bound on the degree of relative bias in the bioassay, and

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$$\sigma^2_{Product}$$

and

$$\sigma^2_{RA}$$

are target product variance (i.e., lot-to-lot variability) and release assay variance (with associated format) respectively. (See section 3 *A Bioassay Validation Example* for an example of determination of

$$\sigma^2_{RA}$$

and Cpm.) This formulation requires prior knowledge regarding target product variability, or the inclusion of a random selection of lots to estimate this characteristic as part of the validation. Given limited understanding of assay performance, manufacturing history, and final specifications during development, this approach may be used simply as a guide for defining validation acceptance criteria.

The choice of a bound on Cpm is a business decision. The proportion of lots that are predicted to be outside their specification limits is a function of Cpm. Some laboratories require process capability corresponding to Cpm greater than or equal to 1.3. This corresponds to approximately a 1 in 10,000 chance that a lot with potency at the center of the specification range will be outside the specification limits.

When specifications have yet to be established for a product, a restriction on *relative bias* or IP can be formulated on the basis of the capability of the art of the bioassay methodology. For example, although chemical assays and immunoassays are often capable of achieving near single digit *percent coefficient of variation* (%CV, or percent relative standard deviation, %RSD), a more liberal restriction might be placed on bioassays, such as animal potency bioassays, that operate with much larger variability (measured as %GCV which can be compared to %CV; see *Appendix 1*). In this case the validation goal might be to *characterize* the method, using the validation results to establish an assay format that is predicted to yield reliable product measurements. A sound justification for target acceptance criteria or use of *characterization* should be included in the validation protocol.

## 2.6 Assay Maintenance

Once a bioassay has been validated it can be implemented. However, it is important to monitor its behavior over time. This is most easily accomplished by maintaining *statistical process control* (SPC) charts for suitable parameters of the Standard response curve and potency of assay QC samples. The purpose of these charts is to identify at an early stage any shift or drift in the bioassay. If a trend is observed in any SPC chart, the reason for the trend should be identified. If the resolution requires a modification to the bioassay or if a serious modification of the bioassay has occurred for other reasons (for example, a major technology change), the modified bioassay should be revalidated or linked to the original bioassay by an adequately designed bridging study with acceptance criteria that use equivalence testing.

## 2.7 Statistical Considerations

Several statistical considerations are associated with designing a bioassay validation and analyzing the data. These relate to the properties of bioassay measurements as well as the statistical tools that can be used to summarize and interpret bioassay validation results.

### SCALE OF ANALYSIS

The scale of analysis of bioassay validation, where data are the relative potencies of samples in the validation study, must be considered in order to obtain reliable conclusions from the study. This chapter assumes that appropriate methods are already in place to reduce the raw bioassay response data to relative potency (as described in general chapter ⟨1034⟩). Relative potency measurements are typically nearly *log normally distributed.Log normally distributed* measurements are skewed and are characterized by *heterogeneity of variability,* where the standard deviation is proportional to the level of response. The statistical methods outlined in this chapter require that the data be symmetric, approximating a normal distribution, but some of the procedures require *homogeneity of variability* in measurements across the potency range. Typically, analysis of potency after log *transformation* generates data that more closely fulfill both of these requirements. The base of the log transformation does not matter as long as a consistent base is maintained throughout the analysis. Thus, for example, if the natural log (log to the base e) is used to transform relative potency measurements, summary results are converted back to the bioassay scale utilizing base e.

The distribution of potency measurements should be assessed as part of bioassay development (as described in ⟨1032⟩). If it is determined that potency measurements are normally distributed, the validation can be carried out using methods described in the general chapter *Validation of Compendial Procedures* ⟨1225⟩.

As a consequence of the usual (for parallel-line assays) log transformation of relative potency measurements, there are advantages if the levels selected for the validation study are evenly spaced on the log scale. An example with five levels would be 0.50, 0.71, 1.00, 1.41, and 2.00. Intermediate levels are obtained as the *geometric mean* of two adjacent levels. Thus for example, the mid-level between 0.50 and 1.0 is derived as follows:

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$$GM = \sqrt{0.50 \cdot 1.0} = 0.71$$

Likewise, summary measures of the validation are influenced by the log normal scale. Predicted response should be reported as the *geometric mean* of individual relative potency measurements, and variability expressed as %GCV. GCV is calculated as the anti-log of the standard deviation, $S_{log}$, of log transformed relative potency measurements. The formula is given by:

$$GCV = \text{antilog}(S_{log}) - 1$$

Variability is expressed as GCV rather than RSD of the log normal distribution in order to preserve continuity using the log transformation (see additional discussion in the *Appendix 1* to this chapter). Intervals that might be calculated from GCV will be consistent with intervals calculated from mean and standard deviation of log transformed data. *Table 1* presents an example of the calculation of geometric mean (GM) and associated RB, with %GCV for a series of relative potency measurements performed on samples tested at the 1.00 level. The log base e is used in the illustration.

**Table 1. Illustration of calculations of GM and %GCV**

| RP[1] | ln RP | |
|---|---|---|
| 1.1299 | 0.1221 | |
| 0.9261 | –0.0768 | |
| 1.1299 | 0.1221 | |
| 1.0143 | 0.0142 | |
| 1.0027 | 0.0027 | |
| 1.0316 | 0.0311 | |
| 1.1321 | 0.1241 | |
| 1.0499 | 0.0487 | |
| | | |
| Average | 0.0485 | GM = 1.0497 |
| | | RB = 4.97% |
| SD | 0.0715 | %GCV = 7.4% |

[1] Relative potency (RP) is the geometric mean of duplicate potencies measured in the eight runs of the example given in *Table 4*.

Here the GM of the relative potency measurements is calculated as the anti-log of the average log relative potency measurements and then expressed as relative bias, the percent deviation from the target potency:

$$GM = e^{Average} = e^{0.0485} = 1.0497$$

$$RB = 100 \cdot \left( \frac{GM}{Target} - 1 \right)\% = 100 \cdot \left( \frac{1.0497}{1.00} - 1 \right)\% = 4.97\%$$

and the percent *geometric coefficient of variation* (%GCV) is calculated as:

$$\%GCV = 100 \cdot (e^{SD} - 1)\% = 100 \cdot \ (e^{0.0715} - 1)\% = 7.4\%$$

Note that the %GCV calculated for this illustration is not equal to the IP determined in the bioassay validation example for the 1.00 level (8.5%); see *Table 6*. This illustration utilizes the average of within-run replicates, while the IP in the validation example represents the variability of individual replicates.

**Reporting Validation Results Using Confidence Intervals**—Estimates of bioassay validation parameters should be presented as a *point estimate* together with a *confidence interval.* A *point estimate* is the numerical value obtained for the parameter, such as the GM or %GCV. A *confidence interval's* most common interpretation is as the likely range of the true value of the parameter. The previous example determines a 90% *confidence interval* for average log relative potency, $CI_{ln}$, as follows:

$$CI_{ln} = Average \pm t_{df} \cdot SD / \sqrt{n}$$

$$= 0.0485 \pm 1.89 \cdot 0.0715 / \sqrt{8} = (0.0007, 0.0963)$$

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For percent relative bias this is:

$$CI_{RB} = 100 \cdot \left( \frac{e^{0.0007}}{1.00} - 1 \right)\%, 100 \cdot \left( \frac{e^{0.0963}}{1.00} - 1 \right)\% = (0.07\%, 10.1\%)$$

The statistical constant (1.89) is from a t-table, with degrees of freedom (df) equal to the number of measurements minus one (df = 8 – 1 = 7). A confidence interval for IP or format variability can be formulated using methods for variance components; these methods are not covered in this general chapter.

**Assessing Conformance to Acceptance Criteria**—Bioassay validation results are compared to target acceptance criteria in order to demonstrate that the bioassay is fit for use. The process of establishing conformance of validation parameters to validation acceptance criteria should not be confused with establishing conformance of relative potency measurements to product specifications. Product specifications should inform the process of setting validation acceptance criteria.

A common practice is to apply acceptance criteria to the estimated validation parameter. This does not account, however, for the uncertainty in the estimated validation parameter. A solution is to hold the *confidence interval* on the validation parameter to the acceptance criterion. This is a standard statistical approach used to demonstrate conformance to expectation and is called an *equivalence test.* It should not be confused with the practice of performing a significance test, such as a t-test, which seeks to establish a difference from some target value (e.g., 0% relative bias). A significance test associated with a P-value > 0.05 (equivalent to a confidence interval that includes the target value for the parameter) indicates that there is insufficient evidence to conclude that the parameter is different from the target value. This is not the same as concluding that the parameter conforms to its target value. The study design may have too few *replicates,* or the validation data may be too variable to discover a meaningful difference from target. Additionally, a significance test may detect a small deviation from target that is of negligible importance. These scenarios are illustrated in *Figure 1*.

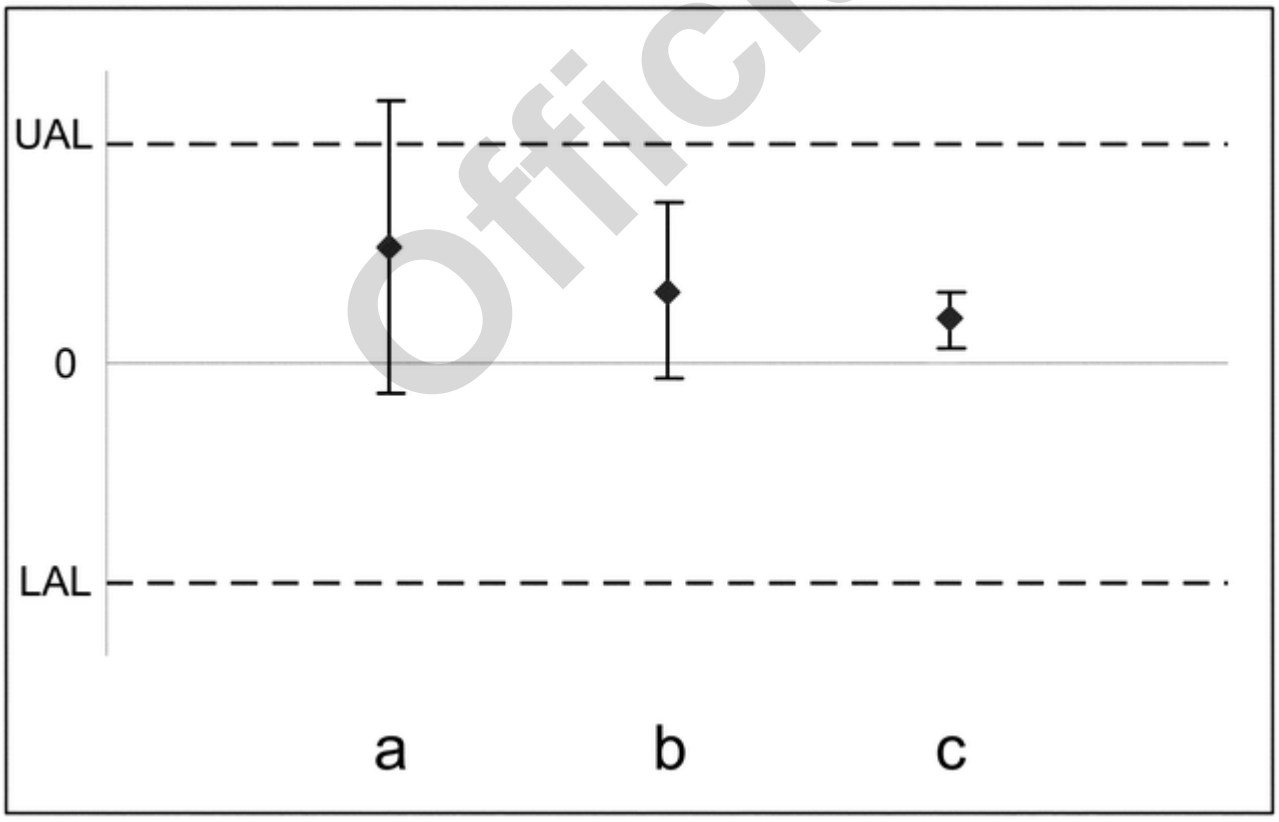


Figure 1. Use of confidence intervals to establish that validation results conform to an acceptance criterion.

The solid horizontal line represents the target value (perhaps 0% relative bias), and the dashed lines form the lower (LAL) and upper (UAL) acceptance limits. In scenario a, the confidence bound includes the target, and thus one could conclude there is insufficient evidence to conclude a difference from target (the significance test approach). However, although the *point estimate* (the solid diamond) falls within the acceptance range, the interval extends outside the range, which signifies that the true relative bias may be outside the acceptable range. In scenario b, the interval falls within the acceptance range, signifying conformance to the acceptance criterion. The interval in scenario c also falls within the acceptance range but excludes the target. Thus, for scenario c, although the difference of the point estimate from the target is statistically significant, c is acceptable because the confidence interval falls within the target acceptance limits.

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Using the 90% confidence interval calculated previously, we can establish whether the bioassay has acceptable relative bias at the 1.00 level compared to a target acceptance criterion of no greater than +12%, for example. Because the 90% confidence interval for percent relative bias (0.07%, 10.1%) falls within the interval (100*[(1/1.12) – 1]%, 100*[(1.12/1) – 1]%) = (– 11%, 12%), we conclude that there is acceptable relative bias at the 1.00 level. Note that a 90% confidence interval is used in an equivalence test rather than a conventional 95% confidence interval. This is common practice and is the same as the *two one-sided tests* (TOST) approach used in pharmaceutical bioequivalence testing.

**Risks in Decision-Making and Number of Validation Runs**—The application of statistical tests, including the assessment of conformance of a validation parameter to its acceptance criteria, involves risks. One risk is that the parameter does not meet its acceptance criterion although the property associated with that parameter is satisfactory; another, the converse, is that the parameter meets its acceptance criterion although the parameter is truly unsatisfactory. A consideration related to these risks is sample size.

The two types of risk can be simultaneously controlled via strategic design, including choice of the number of *runs* that will be conducted in the validation. Specifically, the minimum number of *runs* needed to establish conformance to an acceptance criterion for relative bias is given by:

$$n \geq \frac{\left(t_{\alpha,df} + t_{\beta/2,df}\right)^2 \hat{\sigma}_{IP}^2}{\theta^2}$$

where $t_{\alpha,df}$ and $t_{\beta,df}$ are distributional points from a Student's t-distribution; $\alpha$ and $\beta$ are the one-sided type I and type II errors, and represent the risks associated with drawing the wrong conclusion in the validation; df is the degrees of freedom associated with the study design (usually n – 1);

$$\hat{\sigma}_{IP}^2$$

is a preliminary estimate of IP; and $\theta$ is the acceptable deviation (target acceptance criterion).

For example, if the acceptance criterion for relative bias is ± 0.11 log (i.e., $\theta = 0.11$), the bioassay variability is

$$\hat{\sigma}_{IP} = 0.076 \text{ log}$$

and $\alpha = \beta = 0.05$,

$$n \geq \frac{(1.89+2.36)^2\, 0.076^2}{0.11^2} \approx 8 \text{ runs}$$

Note that this formulation of sample size assumes no intrinsic bias in the bioassay. A more conservative solution includes some nonzero bias in the determination of a sample size. This results in a greater sample size to offset the impact of the bias on the conclusions of the validation. In the current example the sample size increases to 10 runs if one assumes an intrinsic bias equal to 2%. Note also that this calculation represents a recursive solution (because the degrees of freedom depend on n) requiring statistical software or an algorithm that employs iterative methodology.

Note further that the selection of $\alpha$ and $\beta$ should be justified on the basis of the corresponding risks of drawing the wrong conclusion from the validation.

**Modeling Validation Results Using Mixed Effects Models**—Many analyses associated with bioassay validation must account for multiple design factors such as *fixed effects* (e.g., potency level), as well as *random effects* (e.g., analyst, run, and replicate). Statistical models composed of both *fixed* and *random effects* are called *mixed effects models* and usually require sophisticated statistical software for analysis. The results of the analysis may be summarized in an *analysis of variance* (ANOVA) table or a table of variance component estimates. The primary goal of the analysis is to estimate critical parameters rather than establish the significance of an effect. The modeling output provides parameter estimates together with their *standard errors* of estimates that can be utilized to establish conformance of a validation parameter to its acceptance criterion. Thus the average *relative bias* at each level is obtained as a portion of the analysis together with its associated variability. These compose a *confidence interval* that is compared to the acceptance criterion as described above. If variances across levels can be pooled, statistical modeling can also determine the overall *relative bias* and IP by combining information across levels performed in the validation. Similarly, mixed effects models can be used to obtain variance components for validation study factors and to combine results across validation study samples and levels.

**Statistical Design**—Statistical designs, such as multifactor DOE or *nesting,* can be used to organize assay and runs in a bioassay validation. It is useful to incorporate factors that are believed to influence the bioassay response and that vary during long-term

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use of the procedure into these designs. Using these methods of design, the sources of variability may be characterized and a strategic test plan to manage the variability of the bioassay may be developed.

*Table 2* shows an example of a multifactor DOE that incorporates multiple analysts, multiple cell culture preparations, and multiple reagent lots into the validation plan.

**Table 2. Example of a Multifactor DOE with 3 Factors**

| Run | Analyst | Cell Prep | Reagent Lot |
|---|---|---|---|
| 1 | 1 | 1 | 1 |
| 2 | 1 | 1 | 2 |
| 3 | 1 | 2 | 1 |
| 4 | 1 | 2 | 2 |
| 5 | 2 | 1 | 1 |
| 6 | 2 | 1 | 2 |
| 7 | 2 | 2 | 1 |
| 8 | 2 | 2 | 2 |

In this design each analyst performs the bioassay with both cell preparations and both reagent lots. This is an example of a *full factorial* design because all combinations of the factors are performed in the validation study. To reduce the number of runs in the study, *fractional factorial* designs may be employed when more than three factors have been identified. For example, if it is practical for an analyst to perform four assays in a run, a split-unit design could be used with analysts as the whole-plot factor and cell preparation and reagent lot as sub-plot factors. Unlike screening experiments, the validation design should incorporate as many factors at as many levels as possible in order to obtain a representative estimate of IP. More than two levels of a factor should be employed in the design whenever possible. This may be accomplished in a less structured manner, without regard to strict factorial layout. Validation runs should be randomized whenever possible to mitigate the potential influences of run order or time.

*Figure 2* illustrates an example of a validation using *nesting* (replicates nested within plate, plate nested within analyst).

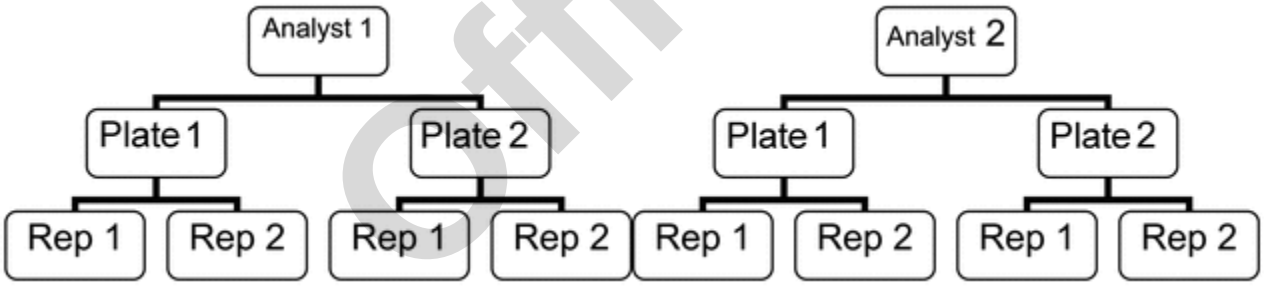


Figure 2. Example of a nested design using two analysts.

For both of these types of design as well as combinations of the two, components of variability can be estimated from the validation results. These components of variability can be used to identify significant sources of variability as well as to derive a bioassay *format* that meets the procedure's requirements for precision. It should be noted that significant sources of variability may have been identified during bioassay development. In this case the validation should confirm both the impact of these factors and the assay format that meets the requirement for precision.

**Significant Figures**—The number of significant figures in a reported result from a bioassay is related to the latter's precision. In general, a bioassay with %GCV between 2% and 20% will support two significant figures. The number of significant figures should not be confused with the number of decimal places—reported values equal to 1.2 and 0.12 have the same number (two) of significant figures. This standard of rounding is appropriate for log scaled measurements that have constant variation on the log scale and proportional rather than additive variability on the original scale (or the scale commonly used for interpretation). Note that rounding occurs at the end of a series of calculations when the final measurement is reported and used for decision making such as conformance to specifications. Thus if the final measurement is a reportable value from multiple assays, rounding should not occur prior to determination of the reportable value. Likewise, specifications should be stated with the appropriate number of significant figures.

## 3. A BIOASSAY VALIDATION EXAMPLE

An example illustrates the principles described in this chapter. The bioassay will be used to support a specification range of 0.71 to 1.41 for the product. Using the Cpm described in section 2.5 *Validation Target Acceptance Criteria*, a table is derived showing the projected rate of OOS results for various restrictions on RB and IP. Cpm is calculated on the basis of the variability of a reportable value using three independent runs of the bioassay (see discussion of format variability, above). Product variability

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is assumed to be equal to 0 in the calculations. The laboratory may wish to include target product variability. An estimate of target product variability can be obtained from data from a product, for example, manufactured by a similar process.

**Table 3. Cpm and Probability of OOS for Various Restrictions on RB and IP**

| LSL-USL | IP (%) | RB (%) | Cpm | Prob(OOS) (%) |
|---|---|---|---|---|
| 0.71–1.41 | 20 | 20 | 0.54 | 10.5 |
| 0.71–1.41 | 8 | 12 | 0.94 | 0.48 |
| 0.71–1.41 | 10 | 5 | 1.55 | 0.0003 |

The calculation is illustrated for IP equal to 8% and relative bias equal to 12% (n = 3 runs):

$$\text{Cpm} = \frac{\ln(1.41) - \ln(0.71)}{6 \cdot \sqrt{[\ln(1.08)]^2/3 + [\ln(1.12)]^2}} = 0.94$$

$$\text{Prob(OOS)} = 2 \cdot \Phi(-3 \cdot 0.94) = 0.0048\ (0.48\%),$$

where $\Phi$ represents the standard normal cumulative distribution function.

From *Table 3*, acceptable performance (less than 1% chance of obtaining an OOS result due to bias and variability of the bioassay) can be expected if the IP is ≤8% and relative bias is ≤12%. The sample size formula given in section 2.7 *Statistical Considerations, Risks in Decision-Making and Number of Validation Runs* can be used to derive the number of runs required to establish conformance to an acceptance criterion for relative bias equal to 12% (using $\%GCV_{IP}$ = 8%; $\alpha = \beta = 0.05$):

$$n \geq \frac{(1.89 + 2.36)^2 \cdot [\ln(1.08)]^2}{[\ln(1.12)]^2} \approx 8 \text{ runs}$$

Thus eight runs would be needed in order to have a 95% chance of passing the target acceptance criterion for relative bias if the true relative bias is zero. Note that the calculation of sample size assumes that a singlet of the validation samples will be performed in each validation run. The use of multiple replication sets and/or multiple assays will provide valuable information that allows separate estimates for intra-run and inter-run variability, and will decrease the risk of failing to meet the validation target acceptance criteria.

Five levels of the target analyte are studied in the validation: 0.50, 0.71, 1.00, 1.41, and 2.00. Two runs at each level are generated by two trained analysts using two media lots. Other factors may be considered and incorporated into the design using a fractional factorial layout. The laboratory should strive to design the validation with as many levels of each factor as possible in order to best model the long-term performance of the bioassay. In this example each analyst performs two runs at each level using each media lot. A run consists of a full dilution series of the Standard as described in the bioassay's operating procedure, together with two independent dilution series of the Test sample. This yields duplicate measurements of relative potency in each run; see *Table 4* for all relative potency observations. Note that the two potency estimates at each level of potency in a run are not independent due to common analysts and media lots.

**Table 4. Example of Bioassay Validation with Two Analysts, Two Media Lots, and Runs per Level for Each Combination of Analyst and Lot**

| Media Lot/Analyst | 1/1 | | 1/2 | | 2/1 | | 2/2 | |
|---|---|---|---|---|---|---|---|---|
| Run | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 |
| 0.50 | 0.5215 | 0.4532 | 0.5667 | 0.5054 | 0.5222 | 0.5179 | 0.5314 | 0.5112 |
| 0.50 | 0.5026 | 0.4497 | 0.5581 | 0.5350 | 0.5017 | 0.5077 | 0.5411 | 0.5488 |
| 0.71 | 0.7558 | 0.6689 | 0.6843 | 0.7050 | 0.6991 | 0.7463 | 0.6928 | 0.7400 |
| 0.71 | 0.7082 | 0.6182 | 0.8217 | 0.7143 | 0.6421 | 0.6877 | 0.7688 | 0.7399 |
| 1.00 | 1.1052 | 0.9774 | 1.1527 | 0.9901 | 1.0890 | 1.0314 | 1.1459 | 1.0273 |
| 1.00 | 1.1551 | 0.8774 | 1.1074 | 1.0391 | 0.9233 | 1.0318 | 1.1184 | 1.0730 |
| 1.41 | 1.5220 | 1.2811 | 1.5262 | 1.4476 | 1.4199 | 1.3471 | 1.4662 | 1.5035 |
| 1.41 | 1.5164 | 1.3285 | 1.5584 | 1.4184 | 1.4025 | 1.4255 | 1.5495 | 1.5422 |
| 2.00 | 2.3529 | 1.8883 | 2.3501 | 2.2906 | 2.2402 | 2.1364 | 2.3711 | 2.0420 |

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**Table 4. Example of Bioassay Validation with Two Analysts, Two Media Lots, and Runs per Level for Each Combination of Analyst and Lot** *(continued)*

| Media Lot/Analyst | 1/1 | | 1/2 | | 2/1 | | 2/2 | |
|---|---|---|---|---|---|---|---|---|
| 2.00 | 2.2307 | 1.9813 | 2.4013 | 2.1725 | 2.0966 | 2.1497 | 2.1708 | 2.3126 |

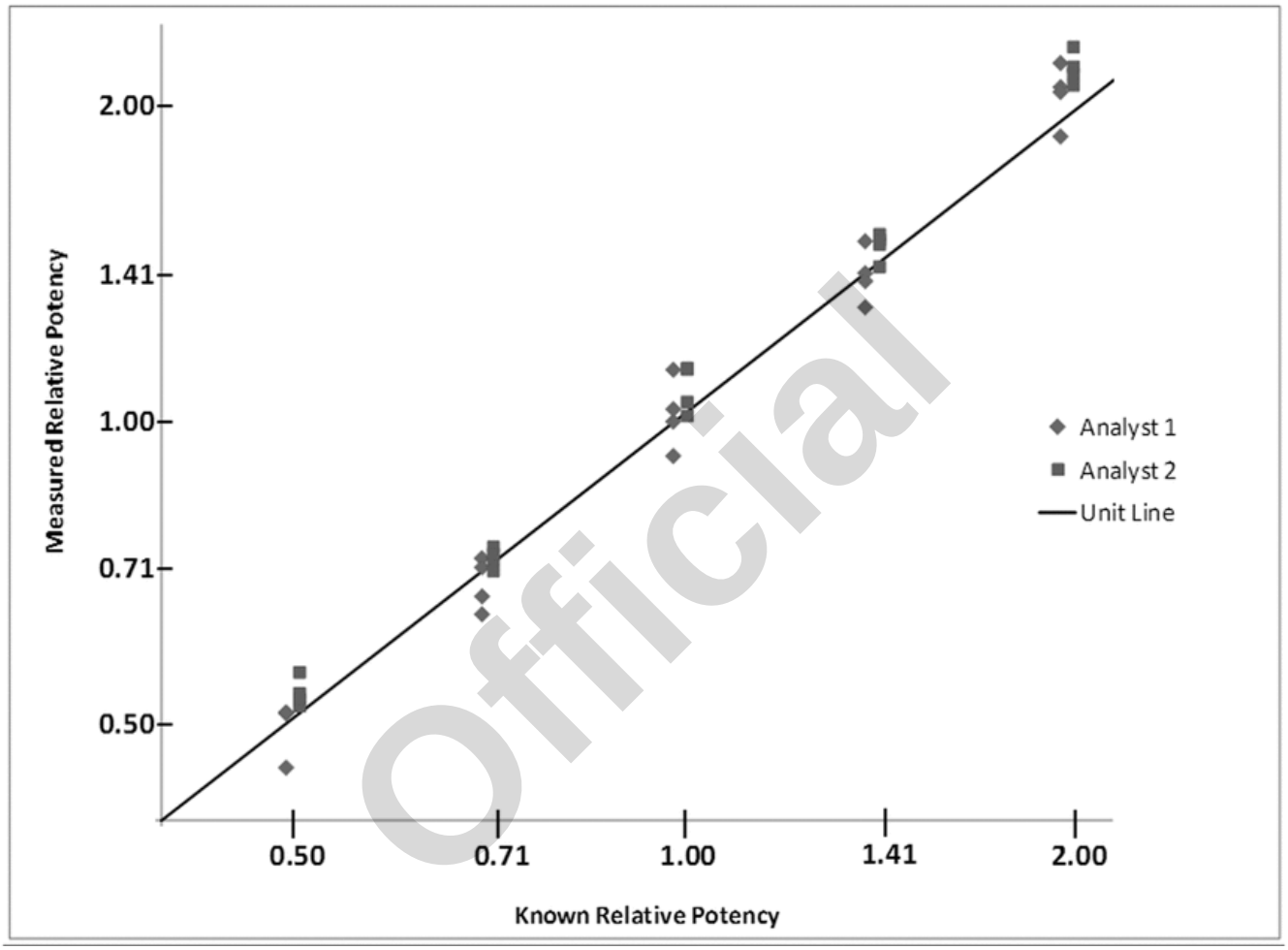


Figure 3. A plot of the validation results versus the sample levels.

A plot is used to reveal irregularities in the experimental results. In particular, a properly prepared plot can reveal a failure in agreement of validation results with validation levels, as well as *heterogeneity of variability* across levels (see discussion of the log transformation in section *2.7 Statistical Considerations*). The example plot in *Figure 3* includes the unit line (line with slope equal to 1, passing through the origin). The analyst 1 and analyst 2 data are deliberately offset with respect to the expected potency to allow clear visualization and comparison of the data sets from each analyst.

A formal analysis of the validation data might be undertaken in the following steps: (1) an assessment of variability (IP) should precede an assessment of relative accuracy or specificity in order to establish conformance to the assumption that variances across sample levels can be pooled; and (2) *relative accuracy* is assessed either at separate levels or by a combined analysis, depending on how well the data across levels can be pooled. These steps are demonstrated using the example validation data, along with some details of the calculations for illustrative purposes. Note that the calculations illustrated in the following sections are appropriate only with a balanced dataset. Imbalanced designs or datasets with missing relative potency measurements should be analyzed using a mixed model analysis with restricted maximum likelihood estimation (REML).

## 3.1 Intermediate Precision

Data at each level can be analyzed using *variance component analysis.* With balanced data, as in this example, variance components can be determined from a standard one-way ANOVA. An example of the calculation performed at a single level (0.50) is presented in *Table 5*.

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**Table 5. Variance Component Analysis Performed on Log Relative Potency Measurements at the 0.5 Level**

| Source | df | Sum of Squares | Mean Square | Expected Mean Square |
|---|---|---|---|---|
| Run | 7 | 0.055317 | 0.007902 | Var(Error) + 2 Var(Run) |
| Error | 8 | 0.006130 | 0.000766 | Var(Error) |
| Corrected total | 15 | 0.061447 | | |
| **Variance Component Estimates** | | | | |
| Var(Run) = 0.003568 | | | | |
| Var(Error) = 0.000766 | | | | |

The top of the table represents a standard ANOVA analysis. Analyst and media lot have not been included because of the small number of levels (2 levels) for each factor. The factor "Run" in this analysis represents the combined runs across the analyst by media lot combinations. The Expected Mean Square is the linear combination of variance components that generates the measured *mean square* for each source. The variance component estimates are derived by solving the equation "Expected Mean Square = Mean Square" for each component. To start, the *mean square* for Error estimates Var(Error), the within-run component of variability, is

$$\text{Var(Error)} = \text{MS(Error)} = 0.000766$$

The between-run component of variability, Var(Run), is subsequently calculated by setting the mean square for Run to the mathematical expression for the expected mean square, then solving the equation for Var(Run) as follows:

$$\text{MS(Run)} = \text{Var(Error)} + 2 \cdot \text{Var(Run)}$$

$$\text{Var(Run)} = \frac{\text{MS(Run)} - \text{MS(Error)}}{2}$$

$$= \frac{0.007902 - 0.000766}{2} = 0.003568$$

These *variance component estimates* are combined to establish the overall IP of the bioassay at 0.50:

$$\text{IP} = 100 \cdot \left( e^{\sqrt{\text{Var(Run)} + \text{Var(Error)}}} - 1 \right)\%$$

$$= 100 \cdot \left( e^{\sqrt{0.003568 + 0.000766}} - 1 \right)\% = 6.8\%$$

The same analysis was performed at each level of the validation, and is presented in *Table 6*.

**Table 6. Variance Component Estimates and Overall Variability for Each Validation Level and the Average**

| | Level | | | | | |
|---|---|---|---|---|---|---|
| Component | 0.50 | 0.71 | 1.00 | 1.41 | 2.00 | Average |
| Var(Run) | 0.003568 | 0.000648 | 0.003639 | 0.003135 | 0.002623 | 0.002723 |
| Var(Error) | 0.000766 | 0.004303 | 0.002954 | 0.000577 | 0.002258 | 0.002172 |
| Overall | 6.8% | 7.3% | 8.5% | 6.3% | 7.2% | 7.2% |

A combined analysis can be performed if the variance components are similar across levels. Typically a heuristic method is used for this assessment. One might hold the ratio of the maximum variance to the minimum variance to no greater than 10 (10 is used because of the limited number of runs performed in the validation). Here the ratios associated with the between-run variance component, 0.003639/0.000648 = 5.6, and the within-run component, 0.004303/0.000577 = 7.5, meet the 10-fold

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criterion. Had the ratio exceeded 10 and if this was due to excess variability in one or the other of the extremes in the levels tested, that extreme would be eliminated from further analysis and the range would be limited to exclude that level.

The analysis might proceed using statistical software that is capable of applying a *mixed effects model* to the validation results. That analysis should account for any imbalance in the design, random effects such as analyst and media lot, and fixed effects such as level (see section 2.7 *Statistical Considerations, Modeling Validation Results Using Mixed Effects Models*). Variance components can be determined for analyst and media lot separately in order to characterize their contributions to the overall variability of the bioassay.

In the example, variance components can be averaged across levels to report the IP of the bioassay. This method of combining estimates is exact only if a balanced design has been employed in the validation (i.e., the same replication strategy at each level). A balanced design was employed for the example validation, so the IP can be reported as 7.2% GCV.

Because of the recommendation to report validation results with some measure of uncertainty, a one-sided 95% upper confidence bound can be calculated for the IP of the bioassay. The literature contains methods for calculating confidence bounds for variance components. The upper bound on IP for the bioassay example is 11.8% GCV. The upper confidence bound was not calculated at each level separately because of the limited data at an individual level relative to the overall study design.

## 3.2 Relative Accuracy

The analysis might proceed with an assessment of relative accuracy at each level. *Table 7* shows the average and 90% confidence interval of validation results in the log scale, as well as corresponding potency and relative bias.

**Table 7. Average Potency and Relative Bias at Individual Levels**

| | | Log Potency | | Potency | | Relative Bias | |
|---|---|---|---|---|---|---|---|
| Level | n [a] | Average | (90% CI) | Average | (90% CI) | Average | (90% CI) |
| 0.50 | 8 | –0.6613 | (–0.7034, –0.6192) | 0.52 | (0.49, 0.54) | 3.23% | (–1.02, 7.67) |
| 0.71 | 8 | –0.3419 | (–0.3773, –0.3064) | 0.71 | (0.69, 0.74) | 0.06% | (–3.42, 3.67) |
| 1.00[b] | 8 | 0.0485 | (0.0006, 0.0964) | 1.05 | (1.00, 1.10) | 4.97% | (0.06, 10.12) |
| 1.41 | 8 | 0.3723 | (0.3331, 0.4115) | 1.45 | (1.40, 1.51) | 2.91% | (–1.04, 7.03) |
| 2.00 | 8 | 0.7859 | (0.7449, 0.8269) | 2.19 | (2.11, 2.29) | 9.72% | (5.31, 14.32) |

[a] Analysis performed on averages of duplicates from each run.
[b] Calculation illustrated in section 2.7 *Statistical Considerations, Scale of Analysis*.

The analysis has been performed on the average of the duplicates from each run (n = 8 runs) because duplicate measurements are correlated within a run by shared IP factors (analyst, media lot, and run in this case). A plot of relative bias versus level can be used to examine patterns in the experimental results and to establish conformance to the target acceptance criterion for relative bias (12%).

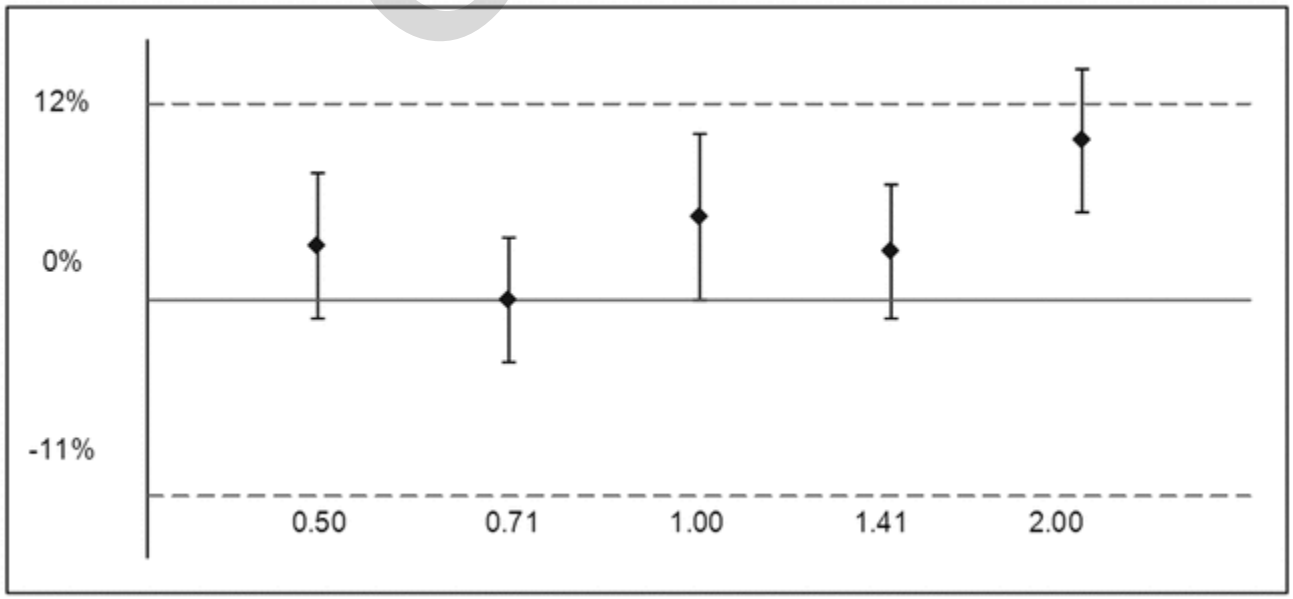


Figure 4. Plot of 90% confidence intervals for relative bias versus the acceptance criterion. Note lower acceptance criterion is equal to $100 \cdot [(1/1.12) - 1] = -11\%$.

*Figure 4* shows an average positive bias across sample levels (i.e., the average relative bias is positive at all levels). This consistency is due in part to the lack of independence of bioassay results across levels. In addition there does not appear to be a

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trend in relative bias across levels. The latter would indicate that a comparison of samples with different measured relative potency (such as stability samples) is biased, resulting perhaps in an erroneous conclusion. Trend analysis can be performed using a regression of log relative potency versus log level. Introduction during the development of the bioassay validation protocol of an acceptance criterion on a trend in relative accuracy across the range can be considered.

After establishing that there is no meaningful trend across levels, the analysis proceeds with an assessment of the relative accuracy at each level. The bioassay has acceptable relative bias at levels from 0.50 to 1.41, yielding 90% confidence bounds (equivalent to a *two one-sided t-test*) that fall within the acceptance region of –11% to 12% relative bias. The 90% confidence interval at 2.0 falls outside the acceptance region, indicating that the relative bias may exceed 12%.

A combined analysis can be performed utilizing statistical software that is capable of applying a *mixed effects model* to the validation results. That analysis accurately accounts for the validation study design. The analysis also accommodates *random effects* such as analyst, media lot, and run (see section 2.7 *Statistical Considerations, Modeling Validation Results Using Mixed Effects Models*).

## 3.3 Range

The conclusions derived from the assessment of IP and *relative accuracy* can be used to establish the bioassay's range that demonstrates satisfactory performance. Based on the acceptance criterion for IP equal to 8% GCV (see *Table 6*) and for relative bias equal to 12% (see *Table 7*), the range of the bioassay is 0.50 to 1.41. In this range, level 1.0 has a slightly higher than acceptable estimate of IP (8.5% versus the target acceptance criterion ≤8.0%), which may be due to the variability of the estimate that results from a small dataset. Because of this and other results in *Table 6*, one may conclude that satisfactory IP was demonstrated across the range.

## 3.4 Use of Validation Results for Bioassay Characterization

When the study has been performed to estimate the characteristics of the bioassay (characterization), the *variance component estimates* can also be used to predict the variability for different bioassay *formats* and thereby can determine a format that has a desired level of precision. The predicted variability for k independent *runs,* with n individual dilution series of the test preparation within a run, is given by the following formula for *format variability:*

$$\text{Format Variability} = 100 \cdot \left(e^{\sqrt{\text{Var(Run)}/k + \text{Var(Error)}/(nk)}} - 1\right)$$

Using estimates of intra-run and inter-run variance components from *Table 6* [Var(Run) = 0.002723 and Var(Error) = 0.002172] , if the bioassay is performed in three independent *runs*, the predicted variability of the *reportable value* (geometric mean of the relative potency results) is equal to:

$$\text{Format Variability} = 100 \cdot \left(e^{\sqrt{0.002723/3 + 0.002172/(1 \cdot 3)}} - 1\right) = 4.1\%$$

This calculation can be expanded to include various combinations of *runs* and *minimal* sets (assuming that the numbers of samples, dilutions, and replicates in the minimal sets are held constant) within *runs* as shown in *Table 8*.

**Table 8. Format Variability for Different Combinations of Number of Runs (k) and Number of Minimal Sets within Run (n)**

| | Number of Runs (k) | | | |
|---|---|---|---|---|
| **Reps (n)** | **1** | **2** | **3** | **6** |
| 1 | 7.2% | 5.1% | 4.1% | 2.9% |
| 2 | 6.4% | 4.5% | 3.6% | 2.6% |
| 3 | 6.0% | 4.2% | 3.4% | 2.4% |
| 6 | 5.7% | 4.0% | 3.3% | 2.3% |

Clearly the most effective means of reducing the variability of the *reportable value* (the geometric mean potency across runs and minimal sets) is by independent runs of the bioassay procedure. In addition, confidence bounds on the variance components used to derive IP can be utilized to establish the bioassay's format variability.

Significant sources of variability must be incorporated into runs in order to effect variance reduction. A more thorough analysis of the bioassay validation example would include analyst and media lot as factors in the statistical model. Variance component estimates obtained from such an analysis are presented in *Table 9*.

**Table 9. REML Estimates of Variance Components Associated with Analyst, Media Lot, and Run**

| Variance | Component Estimate |
|---|---|
| Var(Media Lot) | 0.0000 |
| Var(Analyst) | 0.0014 |
| Var(Analyst*Media Lot) | 0.0000 |
| Var(Run (Analyst*Media Lot)) | 0.0019 |
| Var(Error) | 0.0022 |

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Identification of analyst as a significant bioassay factor should ideally be addressed during bioassay development. Nonetheless the laboratory may choose to address the apparent contribution of analyst-to-analyst variability through improved training or by using multiple analysts in formatting the assay for routine performance of the bioassay.

Estimates of intra-run and inter-run variability can also be used to determine the sizes of differences (fold difference) that can be distinguished between samples tested in the bioassay. For k runs, with n minimal sets within each run, using an approximate two-sided critical value from the standard normal distribution with z = 2, the critical fold difference between reportable values for two samples that are tested in the same runs of the bioassay is given by:

$$\text{Critical Fold Difference} = e^{2 \cdot \sqrt{\text{Var(Run)}/k+\text{Var(Error)}/(nk)}}$$

When samples have been tested in different runs of the bioassay (such as long-term stability samples), the critical fold difference is given by (assuming the same format is used to test the two series of samples):

$$\text{Critical Fold Difference} = e^{2 \cdot \sqrt{2 \cdot [\text{Var(Run)}/k+\text{Var(Error)}/(nk)]}}$$

For comparison of samples the laboratory can choose a design (bioassay format) that has suitable precision to detect a practically meaningful fold difference between samples.

## 3.5 Confirmation of Intermediate Precision and Revalidation

The estimate of IP from the validation is highly uncertain because of the small number of runs performed. After the laboratory gains suitable experience with the bioassay, the estimate can be confirmed or updated by analysis of control sample measurements such as the variability of a positive control. This analysis can be done with the control prepared and tested like a Test sample (i.e., same or similar dilution series and replication strategy). This assessment should be made after sufficient assays have been performed to obtain an alternative estimate of the bioassay's intermediate precision, including implementation of changes (e.g., different analysts, different key reagent lots, and different cell preparations) associated with the standardized assay protocol. The reported IP of the bioassay should be modified as an amendment to the validation report if the assessment reveals a substantial disparity of results.

The bioassay should be revalidated whenever a substantial change is made to the method. This includes but is not limited to a change in technology or a change in readout. The revalidation may consist of a complete re-enactment of the bioassay validation or a bridging study that compares the current and the modified methods.

# APPENDICES

## Appendix 1: Measures of Location and Spread for Log Normally Distributed Variables

Two assumptions of common statistical procedures, such as ANOVA or confidence interval estimation, are (1) the variation in the bioassay response about its mean is normally distributed and (2) the standard deviation of the observed response values is constant over the range of responses that are of interest. Such responses are said to have a "normal distribution" and an "additive error structure". When these two conditions are not met, it may be useful to consider a transformation before using common statistical procedures.

The variation in bioassay responses is often found to be non-normal (skewed toward higher values) with a standard deviation approximately proportional (or nearly so) to the mean response. Such responses often have a "multiplicative error structure" and follow a "log normal distribution" with a percent coefficient of variation (%CV) that is constant across the response range of interest. In such cases, a log transformation of the bioassay response will be found to be approximately normal with a nearly constant standard deviation over the response range. After log transformation, then, the two assumptions are met, and common statistical procedures can be performed on the log transformed response. The following discussion presumes a log normal distribution for the bioassay response.

We refer to an observed bioassay response value, X, as being on the "original scale of measurement" and to the log transformed response, Y = log(X), as being on the "log transformed scale". Although common statistical procedures may be appropriate only on the log transformed scale, we can summarize bioassay response results by estimating measures of location (e.g., mean or median), measures of spread (e.g., standard deviation), or confidence intervals on either scale of measurement, as long as the scale being used is indicated. The %CV is useful on the original scale where it is constant over the response range. For the same reason, the standard deviation (SD) is relevant on the log transformed scale. There may be advantages to reporting statistical summaries on the basis of the log transformed (Y) scale. However, it is often informative to back transform the reported measures to the original scale of measurement (X).

For any given value of X, there is only one unique value of Y = log(X), and vice versa. Similarly for measures of location and spread, there is a unique one-to-one correspondence between measures of location and spread obtained on the original and log transformed scales. Further, just as there is a simple relationship between X and Y = log(X), there are relatively simple relationships that allow conversion between the corresponding measures on each scale, as indicated in *Table A-1* below. In the table, "Average" and "SD", wherever they appear, refer to measures calculated on the log transformed (Y) scale.

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**Table A-1. Comparison of Measures of Location and Spread**

| Measure | | Scale of Measurement | |
|---|---|---|---|
| | | Log Transformed (V) | Original (X) |
| Location | | Mean (average) | Geometric mean (GM) $= \sqrt[n]{\prod_{i=1}^{n} x_i} = e^{\text{Average}}$ |
| Spread | | Standard deviation (SD) | Geometric standard deviation (GSD) = $e^{SD}$ |
| Confidence intervals (k is an appropriate constant based on the t-distribution or large sample z approximation) | Lower | Average – k · SD/$\sqrt{n}$ | GM/$GSD^{k/\sqrt{n}}$ |
| | Upper | Average + k · SD/$\sqrt{n}$ | GM · $GSD^{k/\sqrt{n}}$ |
| | Size | Width (upper – lower) = 2 · k · SD/$\sqrt{n}$ | Ratio(upper/lower) = $GSD^{2k/\sqrt{n}}$ |
| Percent coefficient of variation (%CV) | | %GCV = 100 · (GSD – 1) | $\%CV = 100\sqrt{e^{SD^2} - 1} \approx \%GCV$ |

The geometric mean (GM) should not be misinterpreted as an estimate of the mean of the original scale (X) variable, but is instead an estimate of the median of X. The median is a more appropriate measure of location for variables with skewed error distributions such as the log normal, as well as symmetric error distributions where the median is equal to the mean.

Similarly, the geometric standard deviation (GSD) should not be misinterpreted as the standard deviation of the original scale (X) variable. GSD is, however, a useful multiplicative factor for obtaining confidence intervals on the original (X) scale that correspond to those on the log transformed (Y) scale, as shown in the above table. A GSD of 1 corresponds to no variation (SD of Y = 0). The ratio of the Upper to the Lower confidence bounds, on the untransformed (X) scale, will be equal to $GSD^{2k/\sqrt{n}}$, as can be seen from *Table A-1*.

The geometric coefficient of variation (%GCV) approximates the %CV on the original (X) scale when the %CV is below 20%. It is important not to confuse these different measures of spread. The %GCV is a measure relevant to the log transformed (Y) scale, and the %CV is a measure relevant to the original (X) scale. Depending on the preferred frame of reference, either or both measures may be useful.

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## Appendix 3: Additional Sources of Information

Additional information and alternative methods can be found in the references listed below.

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# ⟨1034⟩ ANALYSIS OF BIOLOGICAL ASSAYS

## 1. INTRODUCTION

Although advances in chemical characterization have reduced the reliance on bioassays for many products, bioassays are still essential for the determination of potency and the assurance of activity of many proteins, vaccines, complex mixtures, and products for cell and gene therapy, as well as for their role in monitoring the stability of biological products. The intended scope of general chapter *Analysis of Biological Assays* ⟨1034⟩ includes guidance for the analysis of results both of bioassays described in the *United States Pharmacopeia* (*USP*), and of non-USP bioassays that seek to conform to the qualities of bioassay analysis recommended by USP. Note the emphasis on analysis—design and validation are addressed in complementary chapters (*Development and Design of Bioassays* ⟨1032⟩ and *Biological Assay Validation* ⟨1033⟩, respectively).

Topics addressed in ⟨1034⟩ include statistical concepts and methods of analysis for the calculation of potency and confidence intervals for a variety of relative potency bioassays, including those referenced in *USP*. Chapter ⟨1034⟩ is intended for use primarily by those who do not have extensive training or experience in statistics and by statisticians who are not experienced in the analysis of bioassays. Sections that are primarily conceptual require only minimal statistics background. Most of the chapter and all the methods sections require that the nonstatistician be comfortable with statistics at least at the level of *USP* general chapter *Analytical Data—Interpretation and Treatment* ⟨1010⟩ and with linear regression. Most of sections 3.4 *Nonlinear Models for Quantitative Response* and 3.6 *Dichotomous (Quantal) Assays* require more extensive statistics background and thus are intended primarily for statisticians. In addition, ⟨1034⟩ introduces selected complex methods, the implementation of which requires the guidance of an experienced statistician.

Approaches in ⟨1034⟩ are recommended, recognizing the possibility that alternative procedures may be employed. Additionally, the information in ⟨1034⟩ is presented assuming that computers and suitable software will be used for data analysis. This view does not relieve the analyst of responsibility for the consequences of choices pertaining to bioassay design and analysis.

## 2. OVERVIEW OF ANALYSIS OF BIOASSAY DATA

Following is a set of steps that will help guide the analysis of a bioassay. This section presumes that decisions were made following a similar set of steps during development, checked during validation, and then not required routinely. Those steps and decisions are covered in general information chapter *Design and Development of Biological Assays* ⟨1032⟩. Section 3 *Analysis Models* provides details for the various models considered.

1. As a part of the chosen analysis, select the subset of data to be used in the determination of the relative potency using the prespecified scheme. Exclude only data known to result from technical problems such as contaminated wells, non-monotonic concentration–response curves, etc.
2. Fit the statistical model for detection of potential outliers, as chosen during development, including any weighting and transformation. This is done first without assuming similarity of the Test and Standard curves but should include important elements of the design structure, ideally using a model that makes fewer assumptions about the functional form of the response than the model used to assess similarity.
3. Determine which potential outliers are to be removed and fit the model to be used for suitability assessment. Usually, an investigation of outlier cause takes place before outlier removal. Some assay systems can make use of a statistical (noninvestigative) outlier removal rule, but removal on this basis should be rare. One approach to "rare" is to choose the outlier rule so that the expected number of false positive outlier identifications is no more than one; e.g., use a 1% test if the sample size is about 100. If a large number of outliers are found above that expected from the rule used, that calls into question the assay.
4. Assess system suitability. System suitability assesses whether the assay Standard preparation and any controls behaved in a manner consistent with past performance of the assay. If an assay (or a run) fails system suitability, the entire assay (or run) is discarded and no results are reported other than that the assay (or run) failed. Assessment of system suitability usually includes adequacy of the fit of the model used to assess similarity. For linear models, adequacy of the model may include assessment of the linearity of the Standard curve. If the suitability criterion for linearity of the Standard is not met, the exclusion of one or more extreme concentrations may result in the criterion being met. Examples of other possible system suitability criteria include background, positive controls, max/min, max/background, slope, $IC_{50}$ (or $EC_{50}$), and variation around the fitted model.
5. Assess sample suitability for each Test sample. This is done to confirm that the data for each Test sample satisfy necessary assumptions. If a Test sample fails sample suitability, results for that sample are reported as "Fails Sample Suitability." Relative potencies for other Test samples in the assay may still be reported. Most prominent of sample suitability criteria is similarity, whether parallelism for parallel models or equivalence of intercepts for slope-ratio models. For nonlinear models, similarity assessment involves all curve parameters other than $EC_{50}$ (or $IC_{50}$).
6. For those Test samples in the assay that meet the criterion for similarity to the Standard (i.e., sufficiently similar concentration–response curves or similar straight-line subsets of concentrations), calculate relative potency estimates assuming similarity between Test and Standard, i.e., by analyzing the Test and Standard data together using a model constrained to have exactly parallel lines or curves, or equal intercepts.
7. A single assay is often not sufficient to achieve a reportable value, and potency results from multiple assays can be combined into a single potency estimate. Repeat steps 1–6 multiple times, as specified in the assay protocol or monograph, before determining a final estimate of potency and a confidence interval.
8. Construct a variance estimate and a measure of uncertainty of the potency estimate (e.g., confidence interval). See section 4 *Confidence Intervals*.

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A step not shown concerns replacement of missing data. Most modern statistical methodology and software do not require equal numbers at each combination of concentration and sample. Thus, unless otherwise directed by a specific monograph, analysts generally do not need to replace missing values.

## 3. ANALYSIS MODELS

A number of mathematical functions can be successfully used to describe a concentration–response relationship. The first consideration in choosing a model is the form of the assay response. Is it a number, a count, or a category such as Dead/Alive? The form will identify the possible models that can be considered.

Other considerations in choosing a model include the need to incorporate design elements in the model and the possible benefits of means models compared to regression models. For purposes of presenting the essentials of the model choices, section 3 *Analysis Models* assumes a completely randomized design so that there are no design elements to consider and presents the models in their regression form.

### 3.1 Quantitative and Qualitative Assay Responses

The terms *quantitative* and *qualitative* refer to the nature of the response of the assay used in constructing the concentration–response model. Assays with either quantitative or qualitative responses can be used to quantify product potency. Note that the *responses* of the assay at the concentrations measured are not the relative potency of the bioassay. Analysts should understand the differences among responses, concentration–response functions, and relative potency.

A quantitative response results in a number on a continuous scale. Common examples include spectrophotometric and luminescence responses, body weights and measurements, and data calculated relative to a standard curve (e.g., cytokine concentration). Models for quantitative responses can be linear or nonlinear (see sections 3.2–3.5).

A qualitative measurement results in a categorical response. For bioassay, qualitative responses are most often quantal, meaning they entail two possible categories such as Positive/Negative, 0/1, or Dead/Alive. Quantal responses may be reported as proportions (e.g., the proportion of animals in a group displaying a property). Quantal models are presented in section 3.6. Qualitative responses can have more than two possible categories, such as end-point titer assays. Models for more than two categories are not considered in this general chapter.

Assay responses can also be counts, such as number of plaques or colonies. Count responses are sometimes treated as quantitative, sometimes as qualitative, and sometimes models specific to integers are used. The choice is often based on the range of counts. If the count is mostly 0 and rarely greater than 1, the assay may be analyzed as quantal and the response is Any/None. If the counts are large and cover a wide range, such as 500 to 2500, then the assay may be analyzed as quantitative, possibly after transformation of the counts. A square root transformation of the count is often helpful in such analyses to better satisfy homogeneity of variances. If the range of counts includes or is near 0 but 0 is not the preponderant value, it may be preferable to use a model specific for integer responses. Poisson regression and negative binomial regression models are often good options. Models specific to integers will not be discussed further in this general chapter.

Assays with quantitative responses may be converted to quantal responses. For example, what may matter is whether some defined threshold is exceeded. The model could then be quantal—threshold exceeded or not. In general, assay systems have more precise estimates of potency if the model uses all the information in the response. Using above or below a threshold, rather than the measured quantitative responses, is likely to degrade the performance of an assay.

### 3.2 Overview of Models for Quantitative Responses

In quantitative assays, the measurement is a number on a continuous scale. Optical density values from plate-based assays are such measurements. Models for quantitative assays can be linear or nonlinear. Although the two display an apparent difference in levels of complexity, parallel-line (linear) and parallel-curve (nonlinear) models share many commonalities. Because of the different form of the equations, slope-ratio assays are considered separately (section 3.5 *Slope-Ratio Concentration–Response Models*).

ASSUMPTIONS—The basic parallel-line, parallel-curve, and slope-ratio models share some assumptions. All include a residual term, e, that represents error (variability) which is assumed to be independent from measurement to measurement and to have constant variance from concentration to concentration and sample to sample. Often the residual term is assumed to have a normal distribution as well. The assumptions of independence and equal variances are commonly violated, so the goal in analysis is to incorporate the lack of independence and the unequal variances into the statistical model or the method of estimation.

Lack of independence often arises because of the design or conduct of the assay. For example, if the assay consists of responses from multiple plates, observations from the same plate are likely to share some common influence that is not shared with observations from other plates. This is an example of intraplate correlation. A simple approach for dealing with this lack of independence is to include a block term in the statistical model for plate. With three or more plates this should be a random effects term so that we obtain an estimate of plate-to-plate variability.

In general, the model needs to closely reflect the design. The basic model equations given in sections 3.3–3.5 apply only to completely randomized designs. Any other design will mean additional terms in the statistical model. For example, if plates or portions of plates are used as blocks, one will need terms for blocks.

CALCULATION OF POTENCY—A primary assumption underlying methods used for the calculation of relative potency is that of similarity. Two preparations are similar if they contain the same effective constituent or same effective constituents in the same proportions. If this condition holds, the Test preparation behaves as a dilution (or concentration) of the Standard preparation. Similarity can be represented mathematically as follows. Let $F_T$ be the concentration–response function for the Test, and let $F_S$ be the concentration–response function for the Standard. The underlying mathematical model for similarity is:

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$$F_T(z) = F_S(\rho\, z), \quad [3.1]$$

where z represents the concentration and ρ represents the relative potency of the Test sample relative to the Standard sample.

Methods for estimating ρ in some common concentration–response models are discussed below. For linear models, the distinction between parallel-line models (section 3.3 *Parallel-Line Models for Quantitative Response*) and slope-ratio models (section 3.5 *Slope-Ratio Concentration–Response Models*) is based on whether a straight-line fit to log concentration or concentration yields better agreement between the model and the data over the range of concentrations of interest.

## 3.3 Parallel-Line Models for Quantitative Responses

In this section, a linear model refers to a concentration–response relationship, which is a straight-line (linear) function between the logarithm of concentration, x, and the response, y. y may be the response in the scale as measured or a transformation of the response. The functional form of this relationship is $y = a + bx$. Straight-line fits may be used for portions of nonlinear concentration–response curves, although doing so requires a method for selecting the concentrations to use for each of the Standard and Test samples (see ⟨1032⟩).

MEANS MODEL VERSUS REGRESSION—A linear concentration–response model is most often analyzed with least squares regression. Such an analysis results in estimates of the unknown coefficients (intercepts and slope) and their standard errors, as well as measures of the goodness of fit [e.g., $R^2$ and root-mean-square error (RMSE)].

Linear regression works best where all concentrations can be used and there is negligible curvature in the concentration–response data. Another statistical method for analyzing linear concentration–response curves is the *means model*. This is an analysis of variance (ANOVA) method that offers some advantages, particularly when one or more concentrations from one or more samples are not used to estimate potency. Because a means model includes a separate mean for each unique combination of sample and dose (as well as block or other effects associated with the design structure) it is equivalent to a saturated polynomial regression model. Hence, a means model provides an estimate of error that is independent of regression lack of fit. In contrast, a regression residual based estimate of error is a mixture of the assay error, as estimated by the means model, combined with lack of fit of the regression model. At least in this sense, the means model error is a better estimate of the residual error variation in an assay system.

PARALLEL-LINE CONCENTRATION–RESPONSE MODELS—If the general concentration–response model (3.1 *Quantitative and Qualitative Assay Responses*) can be made linear in $x = \log(z)$, the resulting equation is then:

$$y = \alpha + \beta\log(z) + e = \alpha + \beta x + e,$$

where e is the residual or error term, and the intercept, α, and slope, β, will differ between Test and Standard. With the parallelism (equal slopes) assumption, the model becomes

$$\begin{aligned} y_S &= \alpha + \beta\log(z) + e = \alpha_S + \beta x + e \\ y_T &= \alpha + \beta\log(\rho z) + e = [\alpha + \beta\log(\rho)] + \beta x + e = \alpha_T + \beta x + e, \end{aligned} \quad [3.2]$$

where S denotes Standard, T denotes Test, $\alpha_S = \alpha$ is the y-intercept for the Standard, and $\alpha_T = \alpha + \beta\log(\rho)$ is the y-intercept for the Test (see *Figure 3.1*).

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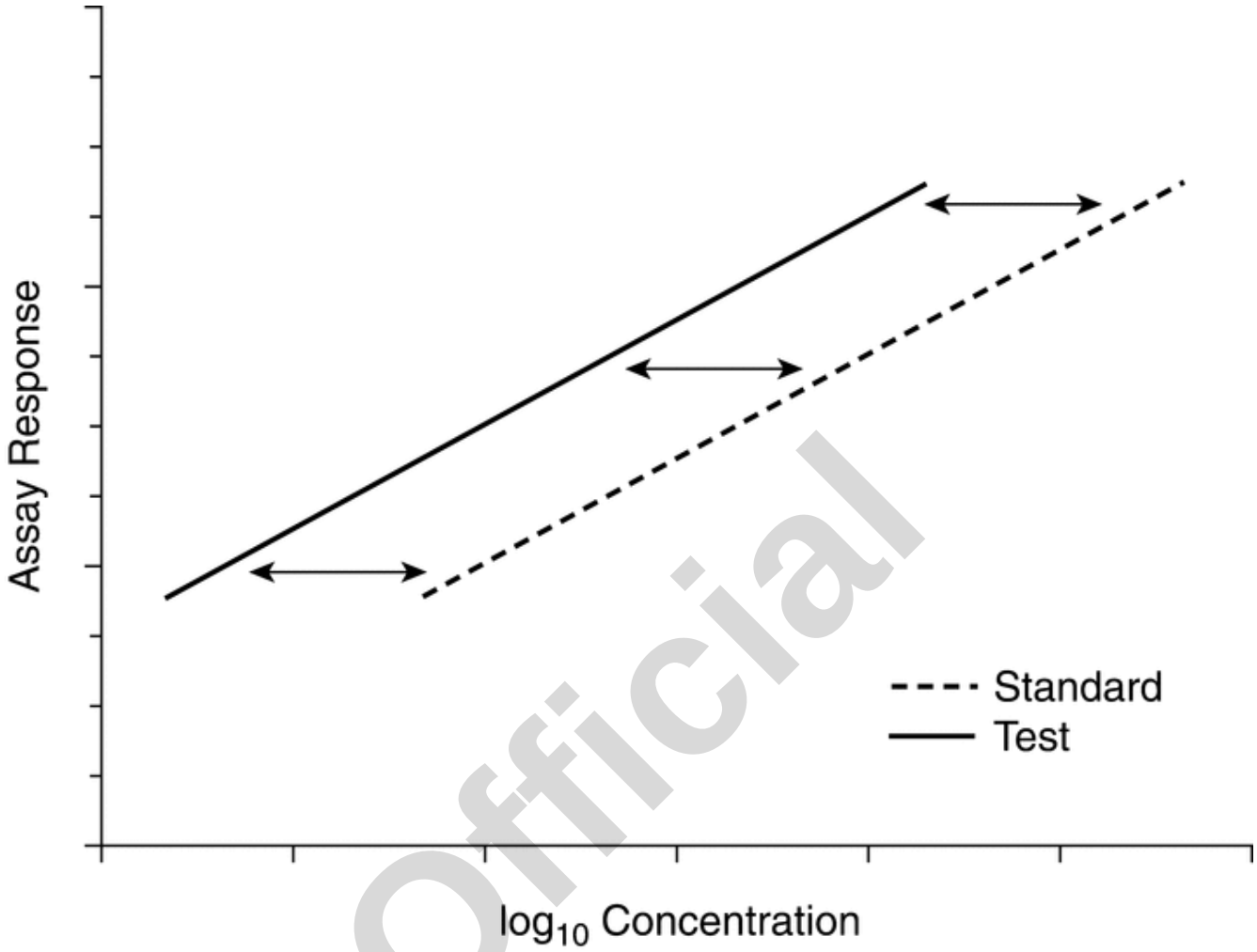


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Figure 3.1. Example of parallel-line model.

Where concentration–response lines are parallel, as shown in *Figure 3.1*, a separation or horizontal shift indicates a difference in the level of biological activity being assayed. This horizontal difference is numerically log(ρ), the logarithm of the relative potency, and is found as the vertical distance between the lines $\alpha_T$ and $\alpha_S$ divided by the slope, β. The relative potency is then

$$\rho = \text{antilog}\left(\frac{\alpha_T - \alpha_S}{\beta}\right)$$

ESTIMATION OF PARALLEL-LINE MODELS—Parallel-line models are fit by the method of least squares. If the equal variance assumption holds, the parameters of equation [3.2] are chosen to minimize

$$\Sigma\left(y - \hat{\alpha}_S - \hat{\delta}T - \hat{\beta}x\right)^2 \qquad [3.3]$$

where the carets denote estimates. This is a linear regression with two independent variables, T and x, where T is a variable that equals 1 for observations from the Test and 0 for observations from the Standard. The summation in equation [3.3] is over all observations of the Test and Standard. If the equal variance assumption does not hold but the variance is known to be inversely proportional to a value, w, that does not depend on the current responses, the y's, and can be determined for each observation, then the method is weighted least squares

$$\Sigma w\left(y - \hat{\alpha}_S - \hat{\delta}T - \hat{\beta}x\right)^2 \qquad [3.4]$$

Equation 3.4 is appropriate only if the weights are determined without using the response, the y's, from the current data (see ⟨1032⟩ for guidance in determining weights). In equations [3.3] and [3.4] β is the same as the β in equation [3.2] and $\delta = \alpha_T - \alpha_S = \beta\log\rho$. So, the estimate of the relative potency, ρ, is

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$$\hat{\rho} = \text{antilog}\left(\hat{\delta}/\hat{\beta}\right)$$

Commonly available statistical software and spreadsheets provide routines for least squares. Not all software can provide weighted analyses.

See section 4 for methods to obtain a confidence interval for the estimated relative potency. For a confidence interval based on combining relative potency estimates from multiple assays, use the methods of section 4.2. For a confidence interval from a single assay, use Fieller's Theorem (section 4.3) applied to $\hat{\delta}/\hat{\beta}$.

MEASUREMENT OF NONPARALLELISM—Parallelism for linear models is assessed by considering the difference or ratio of the two slopes. For the difference, this can be done by fitting the regression model,

$$y = \alpha_S + \delta T + \beta_S x + \gamma x T + e$$

where $\delta = \alpha_T - \alpha_S$, $\gamma = \beta_T - \beta_S$, and T = 1 for Test data and T = 0 for Standard data. Then use the standard t-distribution confidence interval for $\gamma$. For the ratio of slopes, fit

$$y = \alpha_S + \delta T + \beta_S x(1 - T) + \beta_T x T + e$$

and use Fieller's Theorem, equation [4.3], to obtain a confidence interval for $\beta_T/\beta_S$.

## 3.4 Nonlinear Models for Quantitative Responses

Nonlinear concentration–response models are typically S-shaped functions. They occur when the range of concentrations is wide enough so that responses are constrained by upper and lower asymptotes. The most common of these models is the four-parameter logistic function as given below.

Let y denote the observed response and z the concentration. One form of the four-parameter logistic model is

$$y = D + \frac{A - D}{1 + \left(\frac{z}{C}\right)^B} + e \qquad [3.5]$$

One alternative, but equivalent, form is

$$y = a_0 + \frac{d}{1 + \text{antilog}[M(\log z - b)]} + e$$

The two forms correspond as follows:

Lower asymptote: $D = a_0$

Upper asymptote: $A = a_0 + d$

Steepness: B = M (related to the slope of the curve at the $EC_{50}$)

Effective concentration 50% ($EC_{50}$): C = antilog(b) (may also be termed $ED_{50}$).

Any convenient base for logarithms is suitable; it is often convenient to work in log base 2, particularly when concentrations are twofold apart.

The four-parameter logistic curve is symmetric around the $EC_{50}$ when plotted against log concentration because the rates of approach to the upper and lower asymptotes are the same (see *Figure 3.2*). For assays where this symmetry does not hold, asymmetrical model functions may be applied. These models are not considered further in this general chapter.

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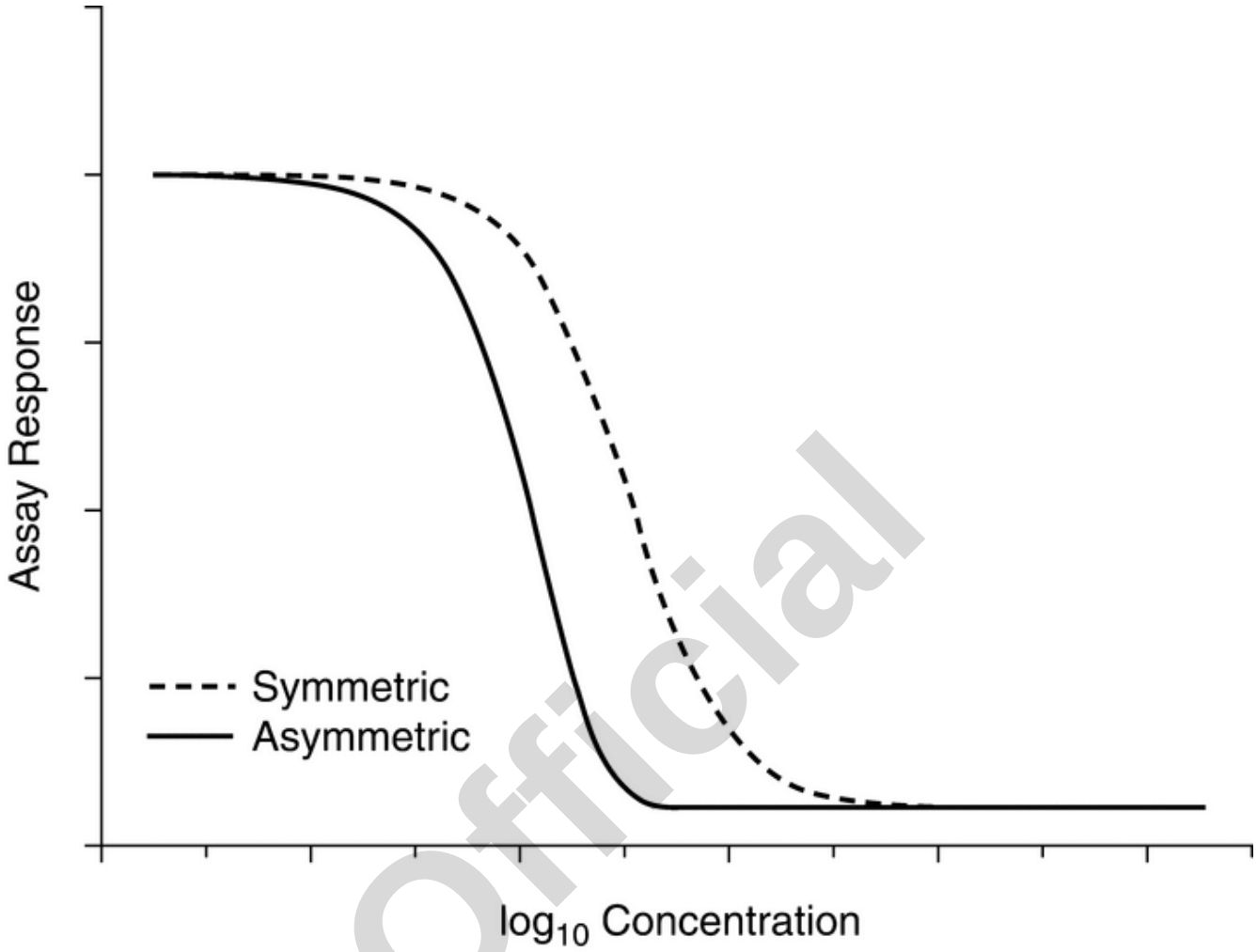


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Figure 3.2. Examples of symmetric (four-parameter logistic) and asymmetric sigmoids.

In many assays the analyst has a number of strategic choices to make during assay development (see *Development and Design of Biological Assays* ⟨1032⟩). For example, the responses could be modeled using a transformed response to a four-parameter logistic curve, or the responses could be weighted and fit to an asymmetric sigmoid curve. Also, it is often important to include terms in the model (often random effects) to address variation in the responses (or parameters of the response) associated with blocks or experimental units in the design of the assay. For simple assays where observations are independent, these strategic choices are fairly straightforward. For assays performed with grouped dilutions (as with multichannel pipets), assays with serial dilutions, or assay designs that include blocks (as with multiple plates per assay), it is usually a serious violation of the statistical assumptions to ignore the design structure. For such assays, a good approach involves a transformation that approximates a solution to non-constant variance, non-normality, and asymmetry combined with a model that captures the important parts of the design structure.

PARALLEL-CURVE CONCENTRATION–RESPONSE MODELS—The concept of parallelism is not restricted to linear models. For nonlinear curves, parallel or similar means the concentration–response curves can be superimposed following a horizontal displacement of one of the curves, as shown in *Figure 3.3* for four-parameter logistic curves. In terms of the parameters of equation [3.5], this means the values of A, D, and B for the Test are the same as for the Standard.

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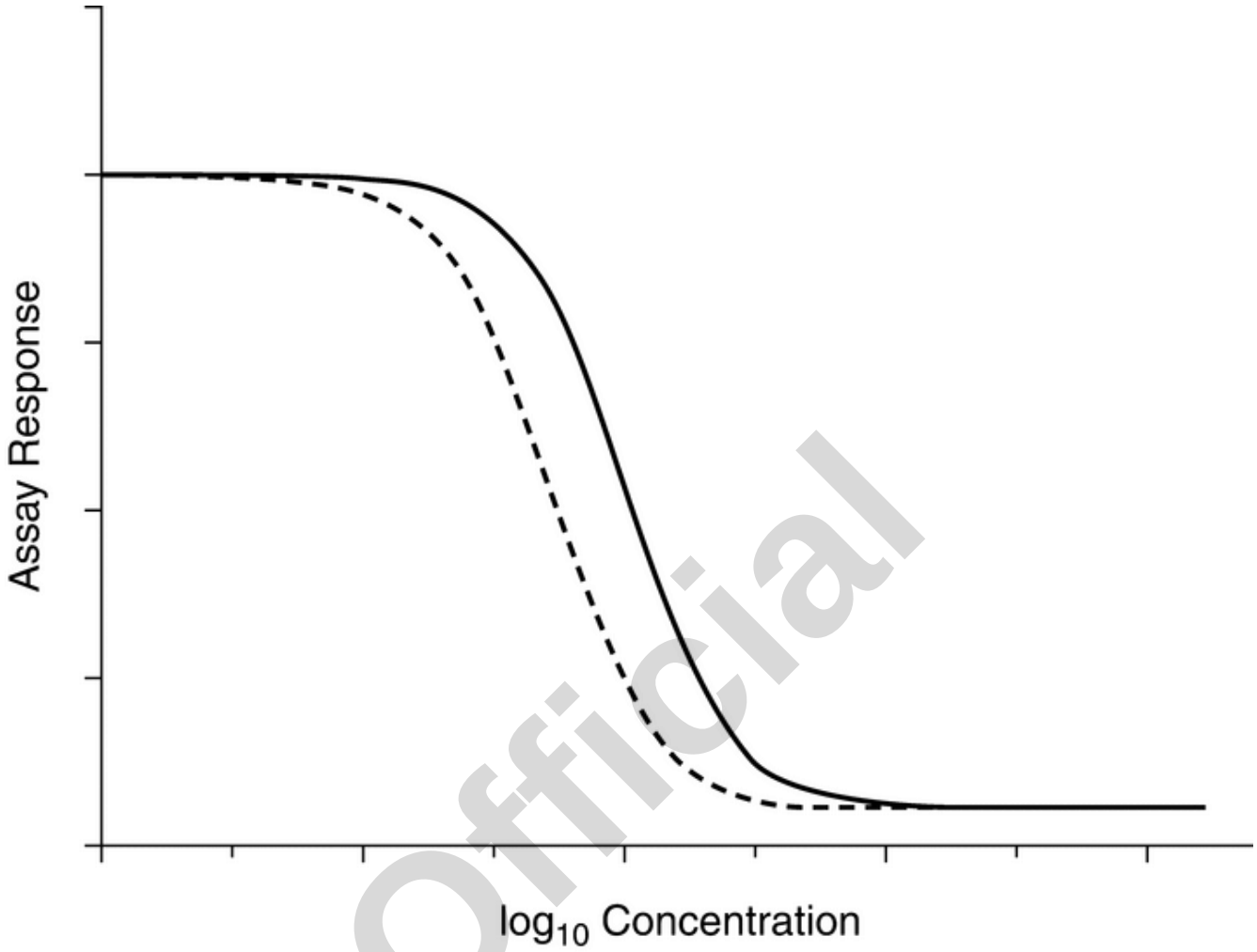


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Figure 3.3. Example of parallel curves from a nonlinear model.

The equations corresponding to the figure (with error term, e, added) are

$$y_S = D + \frac{A - D}{1 + \left(\frac{z}{C}\right)^B} + e$$

$$y_T = D + \frac{A - D}{1 + \left(\frac{\rho z}{C}\right)^B} + e$$

or

$$y_S = D + \frac{A - D}{1 + \text{antilog}\ [M\,(\log z - b)]} + e$$

$$y_T = D + \frac{A - D}{1 + \text{antilog}\ [M(\log z - b + \log \rho)]} + e$$

Log $\rho$ is the log of the relative potency and the horizontal distance between the two curves, just as for the parallel-line model. Because the $EC_{50}$ of the standard is antilog(b) and that of the Test is antilog(b – log $\rho$) = antilog(b)/$\rho$, the relative potency is the ratio of $EC_{50}$'s (standard over Test) when the parallel-curve model holds.

ESTIMATION OF PARALLEL-CURVE MODELS—Estimation of nonlinear, parallel-curve models is similar to that for parallel-line models, possibly after transformation of the response and possibly with weighting. For the four-parameter logistic model, the parameter estimates are found by minimizing:

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$$\Sigma\left(y - \widehat{D} - \frac{\widehat{A} - \widehat{D}}{1 + \text{antilog}\left[\widehat{M}\left(\log z - \widehat{b} + \widehat{r}T\right)\right]}\right)^2$$

without weighting, or

$$\Sigma w\left(y - \widehat{D} - \frac{\widehat{A} - \widehat{D}}{1 + \text{antilog}\left[\widehat{M}\left(\log z - \widehat{b} + \widehat{r}T\right)\right]}\right)^2$$

with weighting. (As for equation [3.4], equation [3.6] is appropriate only if the weights are determined without using the responses, y's, from the current data.) In either case, the estimate of r is the estimate of the log of the relative potency. For some software, it may be easier to work with d = A – D.

The parameters of the four-parameter logistic function and those of the asymmetric sigmoid models cannot be found with ordinary (linear) least squares regression routines. Computer programs with nonlinear estimation techniques must be used.

Analysts should not use the nonlinear regression fit to assess parallelism or estimate potency if any of the following are present: a) inadequate asymptote information is available; or b) a comparison of pooled error(s) from nonlinear regression to pooled error(s) from a means model shows that the nonlinear model does not fit well; or c) other appropriate measures of goodness of fit show that the nonlinear model is not appropriate (e.g., residual plots show evidence of a "hook").

See section 4 for methods to obtain a confidence interval for the estimated relative potency. For a confidence interval based on combining relative potency estimates from multiple assays, use the methods of section 4.2. For a confidence interval from a single assay, advanced techniques, such as likelihood profiles or bootstrapping are needed to obtain a confidence interval for the log relative potency, r.

MEASUREMENT OF NONPARALLELISM—Assessment of parallelism for a four-parameter logistic model means assessing the slope parameter and the two asymptotes. During development (see ⟨1032⟩), a decision should be made regarding which parameters are important and how to measure nonparallelism. As discussed in ⟨1032⟩, the measure of nonsimilarity may be a composite measure that considers all parameters together in a single measure, such as the parallelism sum of squares (see ⟨1032⟩), or may consider each parameter separately. In the latter case, the measure may be functions of the parameters, such as an asymptote divided by the difference of asymptotes or the ratio of the asymptotes. For each parameter (or function of parameters), confidence intervals can be computed by bootstrap or likelihood profile methods. These methods are not presented in this general chapter.

## 3.5 Slope-Ratio Concentration–Response Models

If a straight-line regression fits the nontransformed concentration–response data well, a slope-ratio model may be used. The equations for the slope-ratio model assuming similarity are then:

$$\begin{aligned} y_S &= \alpha + \beta z + e = \alpha + \beta_S z + e \\ y_T &= \alpha + \beta(\rho z) + e = \alpha + \beta_S \rho z + e = \alpha + \beta_T z + e \end{aligned} \qquad [3.7]$$

An identifying characteristic of a slope-ratio concentration–response model that can be seen in the results of a ranging study is that the lines for different potencies from a ranging study have the same intercept and different slopes. Thus, a graph of the ranging study resembles a fan. *Figure 3.4* shows an example of a slope-ratio concentration–response model. Note that the common intercept need not be at the origin.

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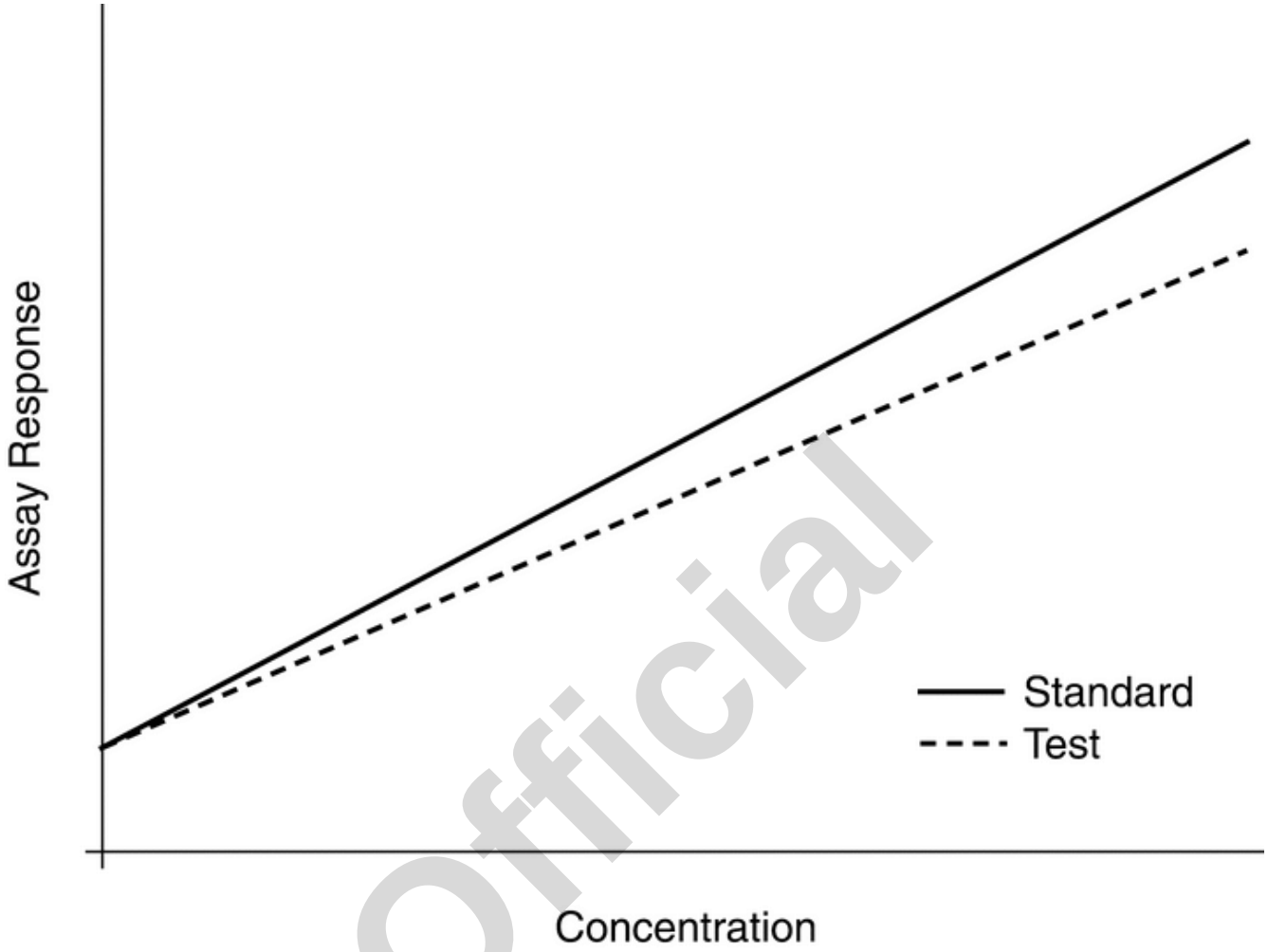


Figure 3.4. Example of slope-ratio model.

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An assay with a slope-ratio concentration–response model for measuring relative potency consists, at a minimum, of one Standard sample and one Test sample, each measured at one or more concentrations and, usually, a measured response with no sample (zero concentration). Because the concentrations are not log transformed, they are typically equally spaced on the original, rather than log, scale. The model consists of one common intercept, a slope for the Test sample results, and a slope for the Standard sample results as in equation [3.7]. The relative potency is then found from the ratio of the slopes:

$$\text{Relative Potency} = \text{Test sample slope/Standard sample slope} = \beta\rho/\beta = \rho$$

ASSUMPTIONS FOR AND ESTIMATION OF SLOPE-RATIO MODELS—The assumptions for the slope-ratio model are the same as for parallel-line models: The residual terms are independent, have constant variance, and may need to have a normal distribution. The method of estimation is also least squares. This may be implemented either with or without weighting, as demonstrated in equations [3.8] and [3.9], respectively.

$$\Sigma\left(y - \hat{\alpha} - \hat{\beta}_S z(1 - T) - \hat{\beta}_T zT\right)^2 \quad [3.8]$$

$$\Sigma w\left(y - \hat{\alpha} - \hat{\beta}_S z(1 - T) - \hat{\beta}_T zT\right)^2 \quad [3.9]$$

Equation [3.9] is appropriate only if the weights are determined without using the response, the y's, from the current data. This is a linear regression with two independent variables, $z(1 - T)$ and $zT$, where $T = 1$ for Test data and $T = 0$ for Standard data. $\beta_T$ is the estimated slope for the Test, $\beta_S$ the estimated slope for the Standard, and then the estimate of relative potency is

$$R = \hat{\beta}_T/\hat{\beta}_S$$

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Because the slope-ratio model is a linear regression model, most statistical packages and spreadsheets can be used to obtain the relative potency estimate. In some assay systems, it is sometimes appropriate to omit the zero concentration (e.g., if the no-dose controls are handled differently in the assay) and at times one or more of the high concentrations (e.g., if there is a hook effect where the highest concentrations do not have the highest responses). The discussion about using a means model and selecting subsets of concentrations for straight parallel-line bioassays applies to slope-ratio assays as well.

See section 4 for methods to obtain a confidence interval for the estimated relative potency. For a confidence interval based on combining relative potency estimates from multiple assays, use the methods of section 4.2. For a confidence interval from a single assay, use Fieller's Theorem (section 4.3) applied to

$$\hat{\beta}_T/\hat{\beta}_S$$

MEASUREMENT OF NONSIMILARITY—For slope-ratio models, statistical similarity corresponds to equal intercepts for the Standard and Test. To assess the similarity assumption it is necessary to have at least two nonzero concentrations for each sample. If the intercepts are not equal, equation [3.7] becomes

$$y_S = \alpha_S + \beta_S z + e$$

$$y_T = \alpha_T + \beta_T z + e$$

Departure from similarity is typically measured by the difference of intercepts, $\alpha_T - \alpha_S$. An easy way to obtain a confidence interval is to fit the model,

$$y = \alpha_S + \delta T + \beta_S z(1 - T) + \beta_T z T + e,$$

where $\delta = \alpha T - \alpha_S$ and use the standard t-distribution-based confidence interval for $\delta$.

## 3.6 Dichotomous (Quantal) Assays

For quantal assays the assay measurement has a dichotomous or binary outcome, e.g., in animal assays the animal is dead or alive or a certain physiologic response is or is not observed. For cellular assays, the quantal response may be whether there is or is not a response beyond some threshold in the cell. In cell-based viral titer or colony-forming assays, the quantal response may be a limit of integer response such as an integer number of particles or colonies. When one can readily determine if any particles are present—but not their actual number—then the assay can be analyzed as quantal. Note that if the reaction can be quantitated on a continuous scale, as with an optical density, then the assay is not quantal.

MODELS FOR QUANTAL ANALYSES—The key to models for quantal responses is to work with the probability of a response (e.g., probability of death), in contrast to quantitative responses for which the model is for the response itself. For each concentration, z, a treated animal, as an example, has a probability of responding to that concentration, P(z). Often the curve P(z) can be approximated by a sigmoid when plotted against the logarithm of concentration, as shown in *Figure 3.5*. This curve shows that the probability of responding increases with concentration. The concentration that corresponds to a probability of 0.5 is the $EC_{50}$.

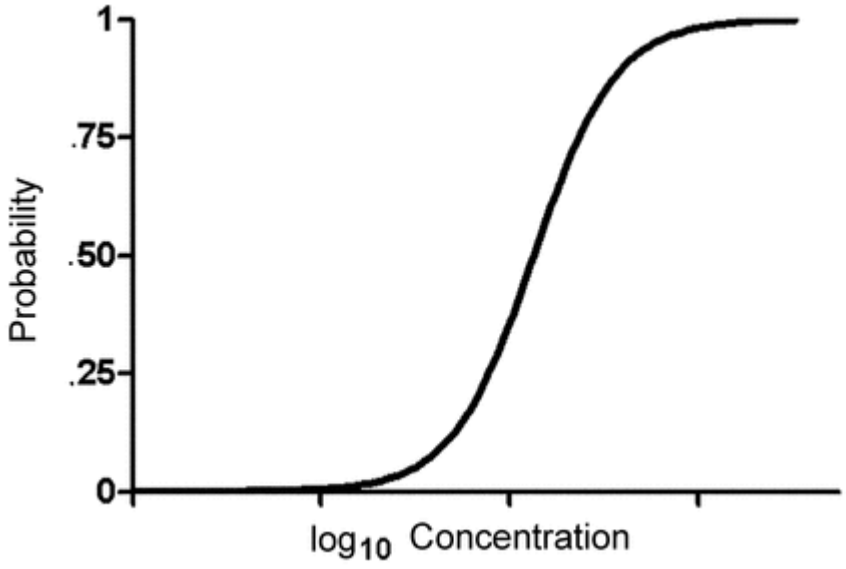


Figure 3.5. Example of sigmoid for P(z).

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The sigmoid curve is usually modeled based on the normal or logistic distribution. If the normal distribution is used, the resulting analysis is termed probit analysis, and if the logistic is used the analysis is termed logit or logistic analysis. The probit and logit models are practically indistinguishable, and either is an acceptable choice. The choice may be based on the availability of software that meets the laboratory's analysis and reporting needs. Because software is more commonly available for logistic models (often under the term logistic regression) this discussion will focus on the use and interpretation of logit analysis. The considerations discussed in this section for logit analysis (using a logit transformation) apply as well to probit analysis (using a probit transformation).

LOGIT MODEL—The logit model for the probability of response, P(z), can be expressed in two equivalent forms. For the sigmoid,

$$P(z) = \frac{1}{1 + \text{antilog}\left[-\beta_0 - \beta_1 \log(z)\right]} = \frac{1}{1 + \left(z/ED_{50}\right)^{-\beta_1}}$$

where $\log(ED_{50}) = -\beta_0/\beta_1$. An alternative form shows the relationship to linear models:

$$\text{logit transform of } P = \log\left(\frac{P}{1-P}\right) = \beta_0 + \beta_1 \log(z) \qquad [3.10]$$

The linear form is usually shown using natural logs and is a useful reminder that many of the considerations, in particular linearity and parallelism, discussed for parallel-line models in section 3.3 *Parallel-Line Models for Quantitative Responses* apply to quantal models as well.

For a logit analysis with Standard and Test preparations, let T be a variable that takes the value 1 for animals receiving the Test preparation and 0 for animals receiving the Standard. Assuming parallelism of the Test and Standard curves, the logit model for estimating relative potency is then:

$$\log\left(\frac{P}{1-P}\right) = \beta_0 + \beta_1 \log(z) + \beta_2 T$$

The log of the relative potency of the Test compared to the Standard preparation is then $\beta_2/\beta_1$. The two curves in *Figure 3.6* show parallel Standard and Test sigmoids. (If the corresponding linear forms equation [3.10] were shown, they would be two parallel straight lines.) The log of the relative potency is the horizontal distance between the two curves, in the same way as for the linear and four-parameter logistic models given for quantitative responses (sections 3.3 *Parallel-Line Models for Quantitative Responses* and 3.4 *Nonlinear Models for Quantitative Responses*).

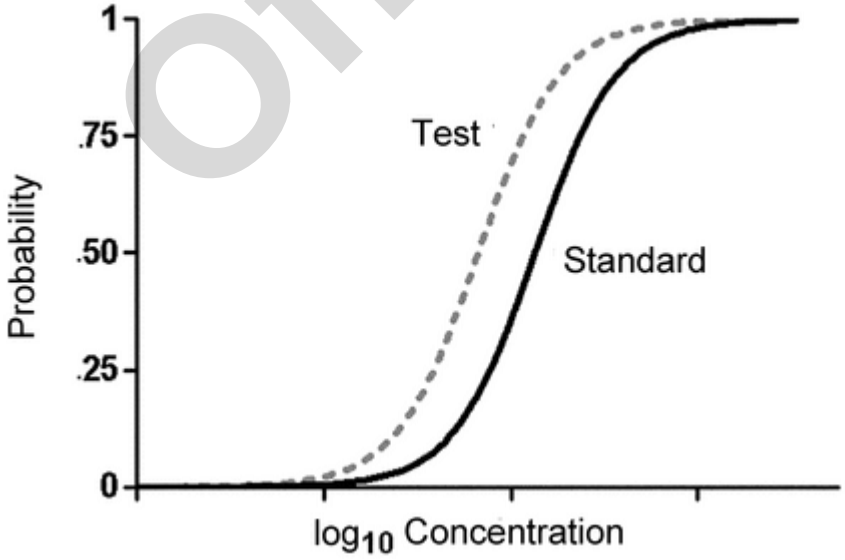


Figure 3.6. Example of Parallel Sigmoid Curves.

ESTIMATING THE MODEL PARAMETERS AND RELATIVE POTENCY—Two methods are available for estimating the parameters of logit and probit models: maximum likelihood and weighted least squares. The difference is not practically important, and the laboratory can accept the choice made by its software. The following assumes a general logistic regression software program. Specialized software should be similar.

Considering the form of equation [3.10], one observes a resemblance to linear regression. There are two independent variables, x = log(z) and T. For each animal, there is a yes/no dependent variable, often coded as 1 for yes or *response* and 0 for *no* or *no response*. Although bioassays are often designed with equal numbers of animals per concentration, that is not a requirement of analysis. Utilizing the parameters estimated by software, which include $\beta_0$, $\beta_1$, and $\beta_2$ and their standard errors, one obtains the estimate of the natural log of the relative potency:

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$$\text{Estimate of log of relative potency} = \frac{\hat{\beta}_2}{\hat{\beta}_1}$$

See section 4 for methods to obtain a confidence interval for the estimated relative potency. For a confidence interval based on combining relative potency estimates from multiple assays, use the methods of section 4.2. For a confidence interval from a single assay, use Fieller's Theorem (section 4.3) applied to $\hat{\beta}_2/\hat{\beta}_1$. The confidence interval for the relative potency is then [antilog(L), antilog(U)], where [L, U] is the confidence interval for the log relative potency.

ASSUMPTIONS—Assumptions for quantal models have two parts. The first concerns underlying assumptions related to the probability of response of each animal or unit in the bioassay. These are difficult to verify assumptions that depend on the design of the assay. The second part concerns assumptions for the statistical model for P(z). Most important of these are parallelism and linearity. These assumptions can be checked much as for parallel-line analyses for quantitative responses.

In most cases, quantal analyses assume a standard binomial probability model, a common choice of distribution for dichotomous data. The key assumptions of the binomial are that at a given concentration each animal treated at that concentration has the same probability of responding and the results for any animal are independent from those of all other animals. This basic set of assumptions can be violated in many ways. Foremost among them is the presence of litter effects, where animals from the same litter tend to respond more alike than do animals from different litters. Cage effects, in which the environmental conditions or care rendered to any specific cage makes the animals from that cage more or less likely to respond to experimental treatment, violates the equal-probability and independence assumptions. These assumption violations and others like them (that could be a deliberate design choice) do not preclude the use of logit or probit models. Still, they are indications that a more complex approach to analysis than that presented here may be required (see ⟨1032⟩).

CHECKING ASSUMPTIONS—The statistical model for P(z) assumes linearity and parallelism. To assess parallelism, equation [3.10] may be modified as follows:

$$\log\left(\frac{P}{1-P}\right) = \beta_0 + \beta_1 \log(z) + \beta_2 T + \beta_3 T * \log(z)$$

Here, $\beta_3$ is the difference of slopes between Test and Standard and should be sufficiently small. [The T*log(z) term is known as an *interaction term* in statistical terminology.] The measure of nonparallelism may also be expressed in terms of the ratio of slopes, $(\beta_1 + \beta_3)/\beta_1$. For model-based confidence intervals for these measures of nonparallelism, bootstrap or profile likelihood methods are recommended. These methods are not covered in this general chapter.

To assess linearity, it is good practice to start with a graphical examination. In accordance with equation [3.10], this would be a plot of log[(y + 0.5)/(n – y + 0.5)] against log(concentration), where y is the total number of responses at the concentration and n is the number of animals at that concentration. (The 0.5 corrections improve the properties of this calculation as an estimate of log[P/(1 – P)].) The lines for Test and Standard should be parallel straight lines as for the linear model in quantitative assays. If the relationship is monotonic but does not appear to be linear, then the model in [3.10] can be extended with other terms. For example, a quadratic term in log(concentration) could be added: $[\log(\text{concentration})]^2$. If concentration needs to be transformed to something other than log concentration, then the quantal model analogue of slope-ratio assays is an option. The latter is possible but sufficiently unusual that it will not be discussed further in this general chapter.

OUTLIERS—Assessment of outliers is more difficult for quantal assays than for quantitative assays. Because the assay response can be only *yes* or *no*, no individual response can be unusual. What may appear to fall into the outlier category is a single response at a low concentration or a single no-response at a high concentration. Assuming that there has been no cause found (e.g., failure to properly administer the drug to the animal), there is no statistical basis for distinguishing an outlier from a rare event.

ALTERNATIVE METHODS—Alternatives to the simple quantal analyses outlined here may be acceptable, depending on the nature of the analytical challenge. One such challenge is a lack of independence among experimental units, as may be seen in litter effects in animal assays. Some of the possible approaches that may be employed are Generalized Estimating Equations (GEE), generalized linear models, and generalized linear mixed-effects models. A GEE analysis will yield standard errors and confidence intervals whose validity does not depend on the satisfaction of the independence assumption.

There are also methods that make no particular choice of the model equation for the sigmoid. A commonly seen example is the Spearman–Kärber method.

## 4. CONFIDENCE INTERVALS

A report of an assay result should include a measure of the uncertainty of that result. This is often a standard error or a confidence interval. An interval (c, d), where c is the lower confidence limit and d is the upper confidence limit, is a 95% confidence interval for a parameter (e.g., relative potency) if 95% of such intervals upon repetition of the experiment would include the actual value of the parameter. A confidence interval may be interpreted as indicating values of the parameter that are consistent with the data. This interpretation of a confidence interval requires that various assumptions be satisfied. Assumptions also need to be satisfied when the width or half width [(d-c)/2] is used in a monograph as a measure of whether there is adequate precision to report a potency. The interval width is sometimes used as a suitability criterion without the confidence interpretation. In such cases the assumptions need not be satisfied.

Confidence intervals can either be *model-based* or *sample-based*. A model-based interval is based on the standard errors for each of the one or more estimates of log relative potency that come from the analysis of a particular statistical model. Model-based intervals should be avoided if sample-based intervals are possible. Model-based intervals require that the statistical model correctly incorporate all the effects and correlations that influence the model's estimate of precision. These include but

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are not be limited to serial dilution and plate effects. Section 4.3 *Model-Based Methods* describes Fieller's Theorem, a commonly used model-based interval.

Sample-based methods combine independent estimates of log relative potency. Multiple assays may arise because this was determined to be required during development and validation or because the assay procedure fixes a maximum acceptable width of the confidence interval and two or more independent assays may be needed to meet the specified width requirement. Some sample-based methods do not require that the statistical model correctly incorporate all effects and correlations. However, this should not be interpreted as dismissing the value of addressing correlations and other factors that influence within-assay precision. The within-assay precision is used in similarity assessment and is a portion of the variability that is the basis for the sample-based intervals. Thus minimizing within-assay variability to the extent practical is important. Sample-based intervals are covered in section 4.2 *Combining Independent Assays (Sample-Based Confidence Interval Methods).*

## 4.1 Combining Results from Multiple Assays

In order to mitigate the effects of variability, it is appropriate to replicate independent bioassays and combine their results to obtain a single reportable value. That single reportable value (and not the individual assay results) is then compared to any applicable acceptance criteria. During assay development and validation, analysts should evaluate whether it is useful to combine the results of such assays and, if so, in what way to proceed.

There are two primary questions to address when considering how to combine results from multiple assays:

*Are the assays mutually independent?*

A set of assays may be regarded as mutually independent when the responses of one do not in any way depend on the distribution of responses of any of the others. This implies that the random errors in all essential factors influencing the result (for example, dilutions of the standard and of the preparation to be examined or the sensitivity of the biological indicator) in one assay must be independent of the corresponding random errors in the other assays. Assays on successive days using the original and retained dilutions of the Standard, therefore, are not independent assays. Similarly, if the responses, particularly the potency, depend on other reagents that are shared by assays (e.g., cell preparations), the assays may not be independent.

Assays need not be independent in order for analysts to combine results. However, methods for independent assays are much simpler. Also, combining dependent assay results may require assumptions about the form of the correlation between assay results that may be, at best, difficult to verify. Statistical methods are available for dependent assays, but they are not presented in this general chapter.

*Are the results of the assays homogeneous?*

Homogeneous results differ only because of random within-assay errors. Any contribution from factors associated with intermediate precision precludes homogeneity of results. Intermediate precision factors are those that vary between assays within a laboratory and can include analyst, equipment, and environmental conditions. There are statistical tests for heterogeneity, but lack of statistically significant heterogeneity is not properly taken as assurance of homogeneity and so no test is recommended. If analysts use a method that assumes homogeneity, homogeneity should be assessed during development, documented during validation, and monitored during ongoing use of the assay.

Additionally, before results from assays can be combined, analysts should consider the scale on which that combination is to be made. In general, the combination should be done on the scale for which the parameter estimates are approximately normally distributed. Thus, for relative potencies based on a parallel-line, parallel-curve, or quantal method, the relative potencies are combined in the logarithm scale.

## 4.2 Combining Independent Assays (Sample-Based Confidence Interval Methods)

Analysts can use several methods for combining the results of independent assays. A simple method described below (Method 1) assumes a common distribution of relative potencies across the assays and is recommended. A second procedure is provided and may be useful if homogeneity of relative potency across assays can be documented. A third alternative is useful if the assumptions for Methods 1 and 2 are not satisfied. Another alternative, analyzing all assays together using a linear or nonlinear mixed-effects model, is not discussed in this general chapter.

METHOD 1—INDEPENDENT ASSAY RESULTS FROM A COMMON ASSAY DISTRIBUTION—The following is a simple method that assumes independence of assays. It is assumed that the individual assay results (logarithms of relative potencies) are from a common normal distribution with some nonzero variance. This common distribution assumption requires that all assays to be combined used the same design and laboratory procedures. Implicit is that the relative potencies may differ between the assays. This method thus captures interassay variability in relative potency. Note that the individual relative potencies should not be rounded before combining results.

Let $R_i$ denote the logarithm of the relative potency of the $i^{th}$ assay of N assay results to be combined. To combine the N results, the mean, standard deviation, and standard error of the $R_i$ are calculated in the usual way:

$$\text{Mean } \bar{R} = \sum_{i=1}^{N} R_i/N$$

$$\text{Standard Deviation } S = \sqrt{\frac{1}{N-1}\sum_{i=1}^{N}\left(R_i - \bar{R}\right)^2}$$

$$\text{Standard Error } SE = S/\sqrt{N}$$

A 100(1 – α)% confidence interval is then found as

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$$\bar{R} \pm t_{N-1,\alpha/2}SE,$$

where $t_{N-1,\alpha/2}$ is the upper $\alpha/2$ percentage point of a t-distribution with N – 1 degrees of freedom. The quantity $t_{N-1,\alpha/2}SE$ is the expanded uncertainty of $\bar{R}$. The number, N, of assays to be combined is usually small, and hence the value of t is usually large.

Because the results are combined in the logarithm scale, the combined result can be reported in the untransformed scale as a confidence interval for the geometric mean potency, estimated by antilog($\bar{R}$),

$$\text{antilog}\left(\bar{R} - t_{N-1,\alpha/2}SE\right), \ \text{antilog}\left(\bar{R} \pm t_{N-1,\alpha/2}SE\right)$$

METHOD 2—INDEPENDENT ASSAY RESULTS, HOMOGENEITY ASSUMED—This method can be used provided the following conditions are fulfilled:

1. The individual potency estimates form a homogeneous set with regard to the potency being estimated. Note that this means documenting (usually during development and validation) that there are no contributions to between-assay variability from intermediate precision factors. The individual results should appear to be consistent with homogeneity. In particular, differences between them should be consistent with their standard errors.
2. The potency estimates are derived from independent assays.
3. The number of degrees of freedom of the individual residual errors is not small. This is required so that the weights are well determined.

When these conditions are not fulfilled, this method cannot be applied and Method 1, Method 3, or some other method should be used. Further note that Method 2 (because it assumes no inter-assay variability) often results in narrower confidence intervals than Method 1, but this is not sufficient justification for using Method 2 absent satisfaction of the conditions listed above.

CALCULATION OF WEIGHTING COEFFICIENTS—It is assumed that the results of each of the N assays have been analyzed to give N estimates of log potency with associated confidence limits. For each assay, i, the logarithmic confidence interval for the log potency or log relative potency and a value $L_i$ are obtained by subtracting the lower confidence limit from the upper. (This formula, using the $L_i$, accommodates asymmetric confidence intervals such as from Fieller's Theorem, section 4.3 *Model-Based Methods*). A weight $W_i$ for each value of the log relative potency, $R_i$, is calculated as follows, where $t_i$ has the same value as that used in the calculation of confidence limits in the $i^{th}$ assay:

$$W_i = \frac{4t_i^2}{L_i^2} \qquad [4.1]$$

CALCULATION OF THE WEIGHTED MEAN AND CONFIDENCE LIMITS—The products $W_iR_i$ are formed for each assay, and their sum is divided by the total weight for all assays to give the weighted mean log relative potency and its standard error as follows:

$$\text{Mean } \bar{R} = \sum_{i=1}^{N} W_iR_i \Big/ \sum_{i=1}^{N} W_i$$

$$\text{Standard Error } SE = 1\Big/\sqrt{\sum_{i=1}^{N} W_i}$$

A 100(1 – α)% confidence interval in the log scale is then found as

$$\bar{R} \pm t_{k,\alpha/2}SE \quad [4.2]$$

where $t_{k,\alpha/2}$ is the upper $\alpha/2$ percentage point of a t-distribution with degrees of freedom, k, equal to the sum of the number of degrees of freedom for the error mean squares in the individual assays. This confidence interval can then be transformed back to the original scale as for Method 1.

METHOD 3—INDEPENDENT ASSAY RESULTS, COMMON ASSAY DISTRIBUTION NOT ASSUMED—Method 3 is an approximate method that may be considered if the conditions for Method 1 (common assay distribution) or Method 2 (homogeneity) are not met.

The observed variation then has two components:

- the intra-assay variation for assay i:

$$S_i^2 = 1/W_i$$

- the inter-assay variation:

$$S_B^2 = \frac{1}{N-1}\sum_{i=1}^{N}\left(R_i - \bar{R}\right)^2 - \frac{1}{N}\sum_{i=1}^{N} S_i^2$$

For each assay, a weighting coefficient is then calculated as

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$$W_i' = \frac{1}{s_i^2 + s_B^2}$$

which replaces $W_i$ in equation [4.1] and where t in equation [4.2] is often approximated by the value 2.

## 4.3 Model-Based Methods

Many confidence intervals are of the form:

Confidence interval = value ± k times the standard error of that value.

For such cases, as long as the multiplier k can be easily determined (e.g., from a table of the t-distribution), reporting the standard error and the confidence interval are largely equivalent because the confidence interval is then easily determined from the standard error. However, the logarithms of relative potencies for parallel-line models and some parameterizations of nonlinear models and the relative potencies from slope-ratio models are ratios. In such cases, the confidence intervals are not symmetric around the estimated log relative potency or potency, and Fieller's Theorem is needed. For these asymmetric cases the confidence interval should be reported because t±he standard error by itself does not capture the asymmetry.

Fieller's Theorem is the formula for the confidence interval for a ratio. Let R = a/b be the ratio for which we need a confidence interval. For the estimates of a and b, we have their respective standard errors, $SE_a$ and $SE_b$, and a covariance between them, denoted Cov. (The covariance is a measure of the degree to which the estimates of a and b are related and is proportional to the correlation between the estimates of a and b.) The covariance may be 0, as for some parameterizations of standard parallel-line analyses, but it need not be. The confidence interval for R then is as follows:

$$\left(R_L, R_U\right) = \frac{\left\{\hat{R} - \frac{g\mathrm{Cov}}{SE_b^2} \pm \frac{t}{\hat{b}}\sqrt{\left(1-g\right)SE_a^2 + \hat{R}^2 SE_b^2 - + 2\hat{R}\mathrm{Cov} + \frac{g\mathrm{Cov}^2}{SE_b^2}}\right\}}{1-g}$$

where

$$g = \frac{t^2 SE_b^2}{\hat{b}^2}$$

and t is the appropriate t deviate value that will depend on the sample size and confidence level chosen (usually 95%). If g > 1, it means that the denominator, $\hat{b}$, is not statistically significantly different from 0 and the use of the ratio is not sensible for those data.

For those cases where the estimates of a and b are statistically uncorrelated (Cov = 0), the confidence interval formula simplifies to

$$\left(R_L, R_U\right) = \frac{\left\{\hat{R} \pm \frac{t}{\hat{b}}\sqrt{\left(1-g\right)SE_a^2 + \hat{R}^2 SE_b^2}\right\}}{1-g} \quad [4.3]$$

# 5. ADDITIONAL SOURCES OF INFORMATION

A variety of statistical methods can be used to analyze bioassay data. This chapter presents several methods, but many other similar methods could also be employed. Additional information and alternative procedures can be found in the references listed below and other sources.

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# ⟨1039⟩ CHEMOMETRICS

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- 1.2 Content Summary of Document
- 1.3 Audience

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## 1. INTRODUCTION

### 1.1 Scope and Purpose

This chapter provides guidance regarding scientifically sound practices for the chemometric analysis and interpretation of typical multivariate data for compendial and industrial applications. Established chemometric practices, including calibration and validation, for applications using different analytical technologies (e.g., spectroscopic, chromatographic, and others) and for different purposes (e.g., fingerprinting, identification, classification, properties prediction, and others) are discussed under a lifecycle approach. Both qualitative and quantitative applications are described.

The chapter discusses how method quality and performance are ensured through the proper lifecycle management of a chemometrics-based model, including the selection of appropriate algorithms, calibration, validation, verification, transfer, and ongoing maintenance steps.

This chapter may be viewed as a supplement to other guidance chapters such as *Analytical Data—Interpretation and Treatment* ⟨1010⟩, which are mainly concerned with the analysis and interpretation of univariate data.

[NOTE—It should not be inferred that the multivariate analysis tools mentioned in this chapter form an exhaustive list. Other equally valid models may be used at the discretion of the manufacturer and other users of this chapter.]

### 1.2 Content Summary of Document

The mind map below (*Figure 1*) provides a visual representation of the content of this chapter. This diagram is meant to assist the reader by showing how the various concepts and practices of chemometrics relate to each other.

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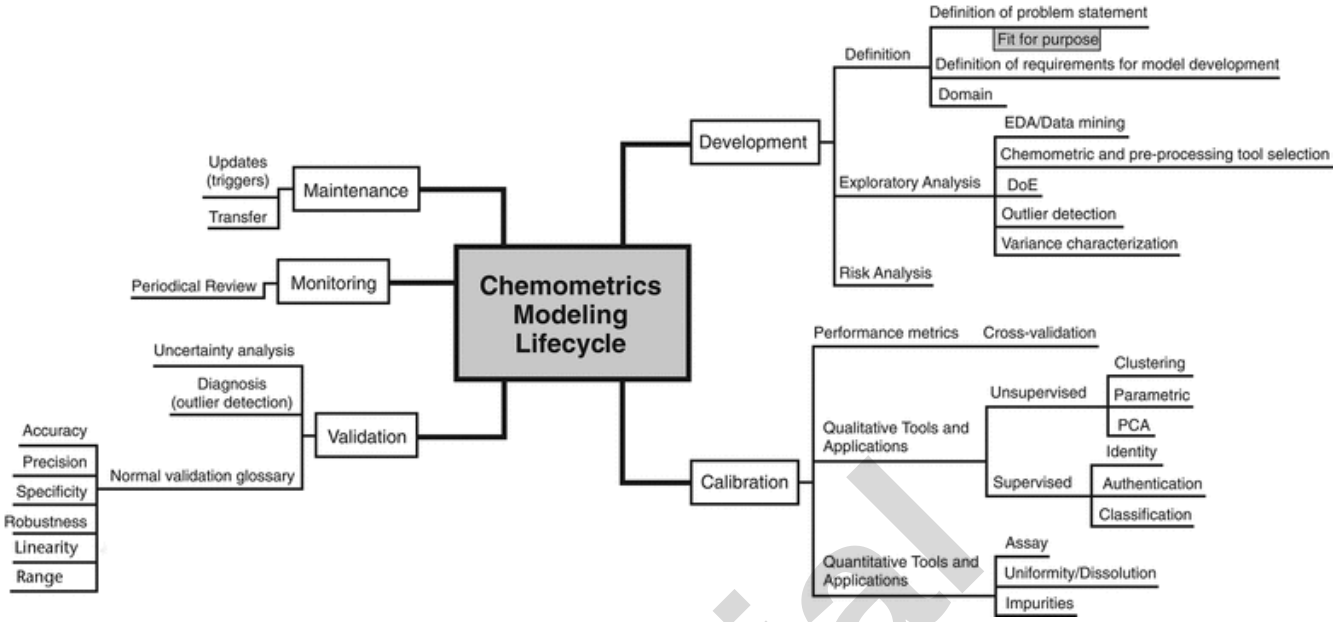


Figure 1. Content summary of document.

### 1.3 Audience

This chapter provides direction for both the chemometrician applying chemometric techniques to develop a model for a given application and the analyst that runs the model within an analytical procedure. The chemometrician will find support regarding algorithm selection for a given application and guidance for ensuring model performance throughout its lifecycle. The analyst will gain insight regarding the strengths and limitations of the chemometrics techniques as applied to support their applications.

## 2. WHAT IS CHEMOMETRICS?

Chemometrics was originally defined as the chemical discipline that uses mathematical, statistical, and other methods that employ formal logic to accomplish two objectives: 1) to design or select optimal measurement procedures and experiments, and 2) to provide the maximum amount of relevant chemical information by analyzing chemical data. (*1*,*2*) More specifically, "chemometrics" has come to mean the application of multivariate methods for the analysis of chemical or related data, although the algorithms in question may be used to extract information out of almost any measured data, regardless of origin—chemical, physical, biological, pharmaceutical, or others. This chapter does not focus on the development of optimal procedures or methods and the applied design of experiments (DoE), but rather on the analysis of multidimensional data collected from an analytical instrument, such as spectroscopic and chromatographic data. A multivariate data set (i.e., multivariate data obtained for a number of samples or objects) forms an $m \times n$ data table or matrix. The matrix is represented by **X**, with $m$ as the number of samples or objects and $n$ as the number of variables measured for each sample (*Figure 2*).

Consequently, the data analysis techniques considered in this chapter will be multivariate in nature. Depending on the purpose of the data treatment, different tools will be applied. Initially, the data handling techniques can be divided into two categories: unsupervised and supervised. The unsupervised tools use only the **X** matrix to extract information, but in supervised data analysis, in addition to the **X** matrix the samples are also described by a **y** vector. This is an $m \times 1$ table containing property information for each sample (e.g., concentration, enzyme inhibition activity). The supervised data analysis techniques are used to build a model between the **X** matrix and the **y** vector. In chemometric modeling, the equations provided are data driven to empirically describe the underlying variance in the data for either unsupervised or supervised purposes. The different techniques or tools applied for the different purposes are discussed in more detail in *4. Applications of Chemometrics*.

The most commonly used unsupervised technique (principal component analysis, or PCA) and supervised technique (partial least squares regression, or PLS) are by nature latent projection approaches (see *Figure 2*), which transform a large number of potentially correlated **X** variables, such as intensities at different retention times or wavelengths, into a possibly smaller number of uncorrelated variables (principal components, or PCs; latent variables). As can be seen in *Figure 2*, the original $n$-dimensional space was transformed to a two-PC space. When samples from the original data space are projected onto this lower-dimensionality space, the resulting sample coordinates are called scores (**T**). Visualization of the scores along two or more PCs forms a score plot that contains information on the relationships among different samples. The loadings (**P**) are a linear combination of the original variables and the coefficients or weights used in this linear combination. The loadings for specific latent variables also can be plotted in what is called a loading plot. The loading plot contains information on the relative importance among the original variables. Both scores and loading plots enable visualization and understanding of the underlying data structure (i.e., the presence of groups/clusters and/or outliers) within a reduced dimensional space. The variable matrix (**E**) produced by the model is defined as the residual error. The sample information along axes of common variance is

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captured by the model's PCs. Variance unaccounted for by these PCs (residual error) is left for each sample at each variable, forming the residual matrix (**E**).

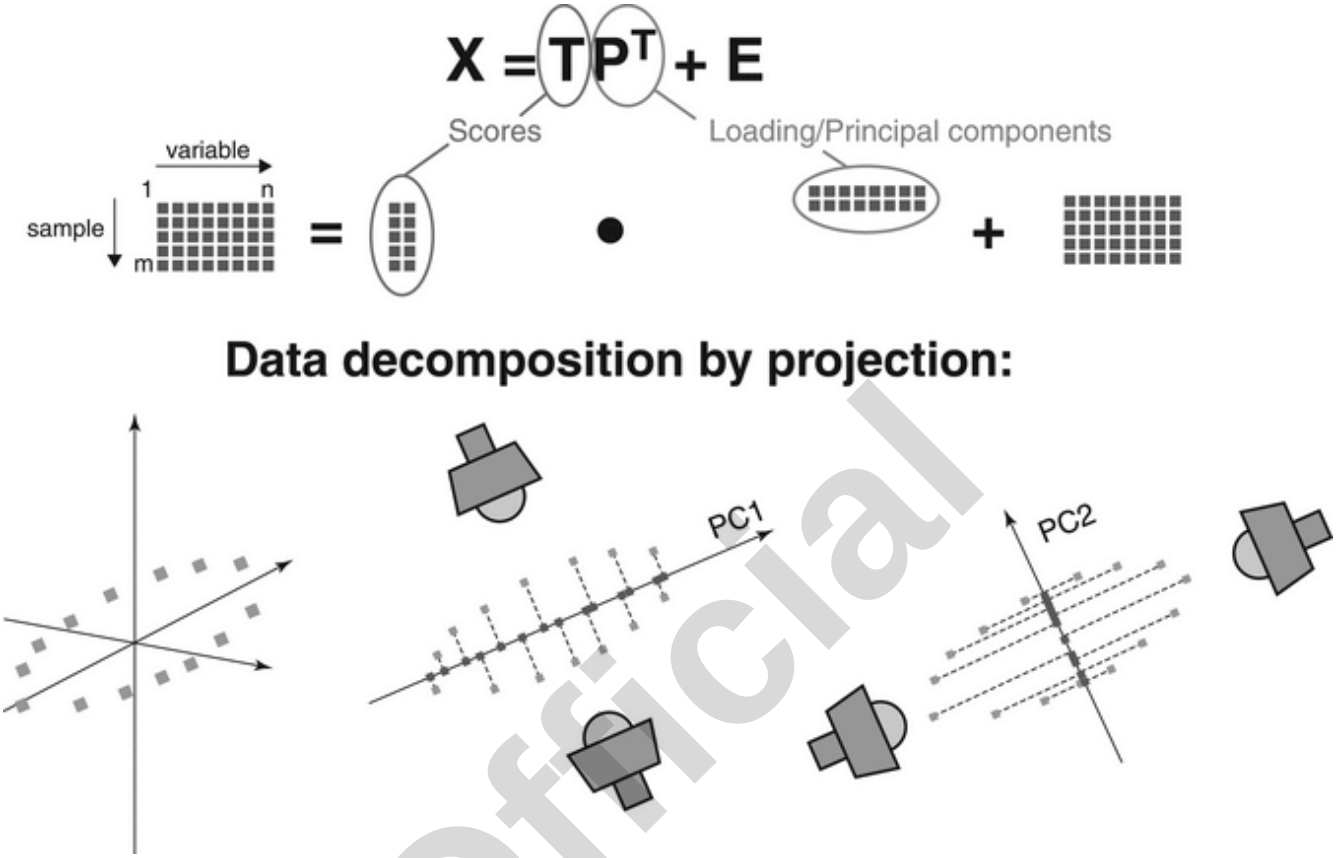


Figure 2. Schematic representation of latent projection techniques.

## 3. MODEL LIFECYCLE

The development of a chemometric model, as part of an analytical procedure, aims to fulfill a predefined, intended purpose. The intended purpose is typically a statement describing what to measure, the output format, and the level of performance needed with the result, and should be in accordance with the analytical target profile (ATP). The ATP may specify performance criteria for key characteristics of the analytical procedure output and/or decision risk probabilities expected in routine use of the analytical procedure.

Calibration of the model encompasses an iterative process that involves selection of the sample set to be used to develop the chemometric model, tuning of an appropriate chemometric algorithm with the necessary preprocessing algorithm, and evaluation of model performance according to predefined metrics for the ATP. During the validation stage, the method performance is demonstrated to fulfill the intended purpose and ATP. Both the knowledge gained during calibration and the assessment of specific metrics and corresponding limits are used to evaluate performance and define a method maintenance protocol that will be used for the monitoring stage, before deployment to routine use. Changes that may have an impact on model performance should trigger a defined set of activities to update it. Model update may also be triggered by the necessity of performing method transfer, in the course of its lifecycle, to different equipment or another site. The extent of the revalidation will be defined by the magnitude of the model update. *Figure 3* shows a schematic representation of the described workflow.

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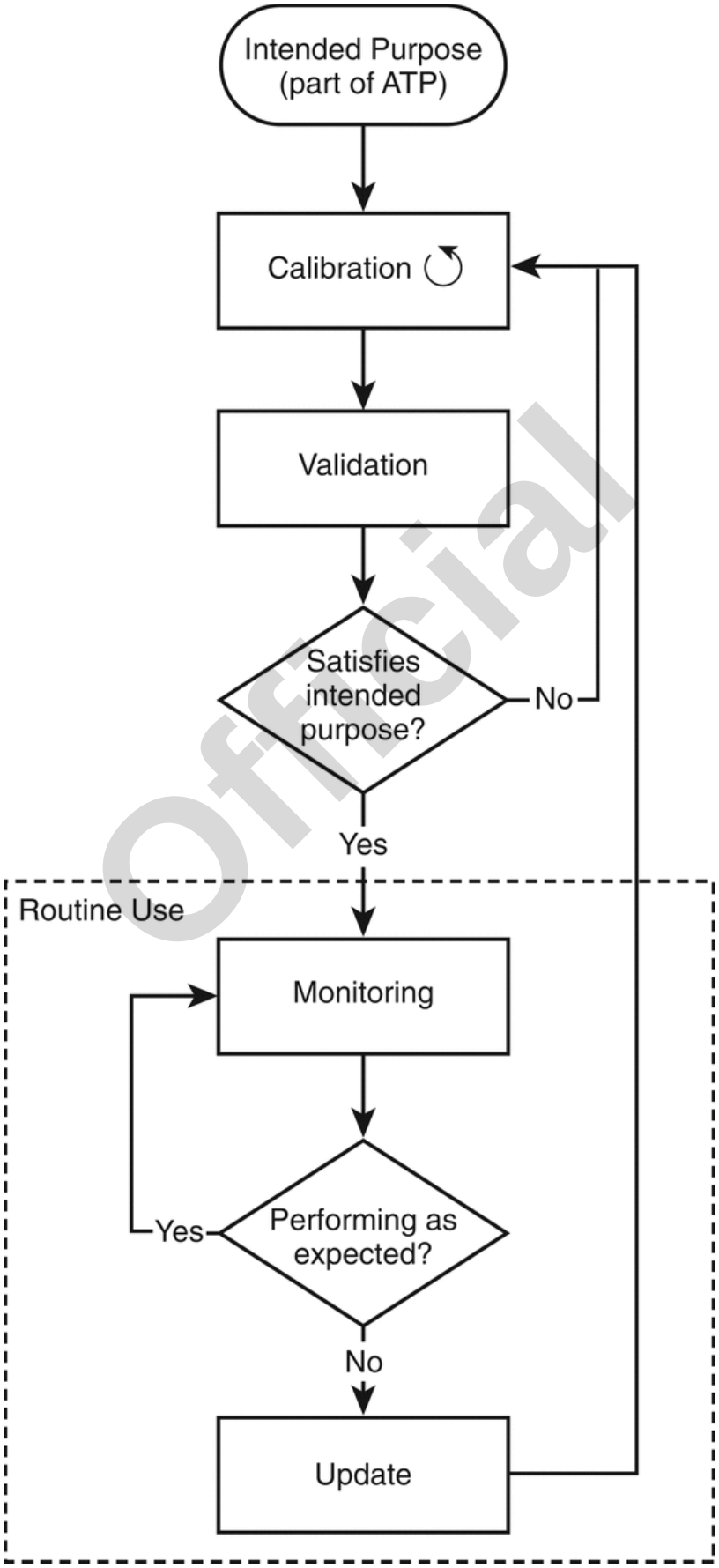


Figure 3. Schematic representation of the lifecycle workflow of a chemometric model.

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## 3.1 Model Development: Calibration

The goal of multivariate calibration is to develop a model that links predictor variables (e.g., absorbance values for the range of spectral wavelengths) with response variables such as concentrations of certain chemical compounds. Response variables are typically more difficult or costly to obtain than predictor variables. Calibration is a critical step because details of the final model are influenced by many factors including the selection of samples, variables, an algorithm, and preprocessing options, as well as the quality of the reference method initially used (where applicable) to measure the response variable(s). Thus, the order of individual components of model calibration presented in this section does not always represent the order of operation, and individual steps might be repeated in an iterative fashion depending upon the outcome of subsequent steps. To perform well, the calibration model should be robust to intended variations.

### SAMPLE SELECTION

Selection of samples for the calibration model is a critical step in model development. Scientifically sound rationales must be used to select representative calibration samples with respect to two criteria: the type/range of variability, and the number of samples. The range of response values in selected samples should cover the entire expected range of response variation for the intended application. The range and type of variation in predictors may include relevant variability in factors other than the property being predicted, such as particle size, different lots of ingredients used for sample preparation, analyst-to-analyst and day-to-day variation, and other sources of variation. In the manufacturing setting, batches of samples representing within and outside specification should be included. A risk-based approach is recommended for deciding which factors and variability will be included in the calibration samples. Performing a failure mode and effect analysis (FMEA) or a feasibility study to evaluate the effects of such factors is one of the commonly used approaches.

The required number of samples increases as the complexity of the calibration model (i.e., the type and the range of variability in both response and predictor variables) increases. In general, the larger the number of samples, the higher the probability that correct results can be achieved throughout the range of calibration. Meanwhile, the distribution of the calibration samples also deserves attention. A uniform distribution of the samples is preferred, although it is not required in all cases, depending on the specifics of the application. Scientifically sound rationales must be in place to justify the number and the distribution of calibration samples. When obtaining new calibration samples becomes costly, the DoE and/or historical database approaches are commonly used alternatives to build calibration models.

Data obtained from selected samples (i.e., a selected number of rows of the **X** matrix in *Figure 2*) should undergo further triage. Exploratory data analysis (see *Qualitative Application Examples*) may be valuable for understanding data structure, detecting potential clusters or groups of samples, identifying outliers, and selecting representative samples. Outliers may be detected using metrics that describe how well a given observation fits within model space (e.g., Hotelling's $T^2$ for latent variable methods) and distance to the model subspace (e.g., residual for latent variable methods).

### PREPROCESSING

The goal of preprocessing is to deploy mathematical transformations on the column of the **X** matrix (*Figure 2*) to amplify variation of interest within the data set and attenuate extraneous variation for both variables and observations. Preprocessing is an important initial step for most data analyses, and can be applied iteratively with variable selection. The initial selection of preprocessing options should be guided by an understanding of the sample data and/or the underlying analytical technique. Guidance may also be obtained through exploratory multivariate data analyses (see *Qualitative Application Examples*). Initial selections can be modified or refined within the context of subsequent model performance optimization. This process is almost always cyclic in practice; comparison across different preprocessing strategies leads to a better understanding of the data, which can further refine the preprocessing to maximize the signal-to-noise ratio.

However, there are two points of caution. Preprocessing should be used judiciously because: 1) overprocessing can attenuate the signal and inflate undesirable variations; and 2) many chemometric methods can accommodate noisy and visually unappealing data. Furthermore, in some instances the preprocessing has been accomplished on the instrument; many algorithms used by instrument vendors are proprietary, and it may be impossible to know exactly what modifications have been made to the data. The chemometrician must be aware of, and cautious about, any preprocessing already applied in the reported raw data.

Preprocessing may consist of transformation, normalization, and/or other mathematical treatment of data. Preprocessing of samples (rows of data matrix) may include mean or median centering, scaling, and other procedures. Variables (columns of data matrix) can also be transformed, aligned (e.g., time warping), or scaled (e.g., autoscaling). Mean centering is probably the most common preprocessing method. For example, the use of mean-centered data in the regression model removes the intercept term, resulting in a potentially parsimonious model. It is also common to remove the linear baseline (e.g., bias/offset correction) or polynomial interferences or to apply digital filtering techniques such as derivatives, wavelets, Fourier filter, or a Savitzky-Golay filter to remove or attenuate linear offsets and noise contribution. Normalization is the scaling of observations, giving objects similar impact on model creation by: 1) accounting for factors other than the analyte of interest that impact measurement (e.g., sample path length variability caused by particle size, sample volume, or tablet thickness); 2) correcting for variation in concentration or mass of an analyte for qualitative models; and 3) attenuating measurement artifacts (e.g., instrument performance drift). When variables are highly correlated, such as in spectroscopic measurements, normalization is typically performed via standard normal variate (SNV) and multiplicative scatter correction (MSC). These normalizations are typically conducted on each sample (row).

In cases where variables have different units, sensitivities, or signal-to-noise ratios, variable scaling is typically applied to correct for that; autoscaling is typically used, but the analyst may consider other alternatives, such as using the pooled standard deviations of measurement replicates as scaling/weight parameters. These normalizations are typically conducted on each variable (column). No guidelines exist for deciding which preprocessing approach is the best for a given data set. It is often a

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trial-and-error process as the analyst applies different preprocessing techniques to find the best one. Combinations of preprocessing techniques can be applied to a given data set as well.

## ALGORITHM SELECTION

Many different algorithms have been tried with varying degrees of success in chemometrics applications. The methods include relatively simple ones such as multivariate linear regression and its modifications (robust or weighted regression); the widely used latent variables approaches such as principal components regression (PCR) and PLS; local methods such as k-nearest neighbors (kNN); and the more sophisticated methods such as support vector machines (SVM) and artificial neural networks. More details on commonly used algorithmic tools can be found in *4. Applications of Chemometrics*. In general, it is difficult to predict which algorithm will produce the best results for a particular data set. The multitude of choices for sample selection, variable selection, and data normalization and preprocessing—as well as the combination of tuning parameters for each algorithm—could significantly affect the performance of the calibration model. Sometimes, the choice of algorithm even iterates with the steps of sample selection, variable selection, and preprocessing. Thus, the choice of algorithm may depend on the task at hand, software availability, and the subject matter expert's familiarity with the method. Another consideration is that some algorithms provide useful tools for diagnostics and interpretation—such as PLS scores, loadings, and coefficients plots, whereas others are sometimes referred to as "black boxes" because their inner workings can be difficult to interpret (e.g., neural networks).

It is important to keep in mind that many algorithms are empirical in nature. Almost always, some kind of model can be developed. Thus, the results should be evaluated critically to ensure that results generated by the model are relevant and correct, and that model performance meets the requirements of the ATP. This can be accomplished by cross-validation, and ultimately, by validation with the independent test data set that was not used to develop the model.

## VARIABLE SELECTION

The intended purpose of variable selection is to identify a subset of predictor variables (i.e., the column of the **X** matrix in *Figure 2*) that can improve the performance of the model. The underlying rationale for variable selection in chemometrics is twofold. First, certain predictor variables could be irrelevant to the intended purpose, and ideally, these variables should be minimized or removed from the model. For example, only specific wavelengths of the whole range generated by the spectrometer may bear information relevant to the variation in levels of the response variable. Variable selection for this purpose should be based on first principles and experience. Inclusion of unrelated predictors, such as irrelevant spectral regions, could potentially degrade performance of the model. A smaller number of variables from the preprocessed data can be used to achieve superior performance, such as accuracy, precision, and robustness.

The second part of the rationale for variable selection is to avoid overfitting. Overfitting is the situation where the model is not only describing the intended variability in the data but also includes the modeling of the noise. The latter has a negative influence on the predictive properties of the model. Typically in chemometrics, the number of predictor variables (hundreds or thousands) is larger than the number of observations (dozens to hundreds). There are two general strategies for handling the issue of overfitting. One strategy is to manually or computationally select only a subset of predictor variables and use them for model development. Predictor variables can be selected manually (e.g., choosing certain spectral wavelengths characteristic of a given compound) or by using a variety of statistical selection methods such as stepwise regression and using simple univariate statistical measures such as the *F*-test, *t*-test, correlation coefficient, signal-to-noise ratios, intensity thresholds, variable importance in projection metric, or genetic algorithms. Multivariate approaches may use PC variable loadings, linear discriminants, or regression coefficients to define the key features.

In the second strategy for avoiding overfitting, variables are not selected at all. The latent variable methods—PLS and PCR—by their nature have the capability to selectively give more weight to the important predictor variables and de-weight the less important ones. These new latent variables, or components, are constructed as linear combinations of the observed variables. If combined with variable selection, the performance of latent variable models could potentially be improved, because some predictor variables contain only noise, thus perturbing the model-building process.

Selecting a subset of the predictor variables that are informative will reduce the size of the data set, provide computational efficiency, and obtain better models for postprocessing. One caveat for applications such as authentication is that eliminating predictor variables that are devoid of information may prevent the recognition of adulterants or novel objects that have features in these excluded predictor variables.

## CROSS-VALIDATION

In practice, cross-validation is used to obtain an estimate of the model performance and to fine-tune an algorithm's parameters (e.g., the number of components for PLS). This is accomplished by repetitive splitting of the data (i.e., the number of rows of the **X** matrix in *Figure 2*) into a calibration set, which is used to develop the model, and a testing set (or internal validation set), which is used to make predictions and to compare actual and predicted values.

The approach of *n*-fold cross-validation is commonly used to split the data into *n* subsets to perform cross-validation. In each of the *n* iterations, *n*–1 subsets are used to develop the model, which is used to predict the remaining $n^{th}$ data split. The procedure is repeated until all subsets are predicted. The error between reference values and predicted values during cross-validation is then recorded as root-mean-squared error of cross-validation (RMSECV). Multiple approaches exist for splitting the data into *n* segments. Regardless of how one decides to split the original calibration data, one aspect that deserves attention is that any batches used in calibration and cross-validation must not be considered or reused as an independent dataset for method validation. The most straightforward form is called leave-one-out cross-validation (LOOCV), where samples are removed one at a time. The LOOCV can involve intensive computations; its results can be heavily affected by outliers and are less consistent than the results of other forms of cross-validation. It is also possible to split the data according to prior information, such as one subset per batch given the available historical data set across multiple batches. Another option for

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cross-validation is bootstrapping, where in each iteration a certain proportion of data is randomly sampled to create a calibration set, while the remainder of the data are used as a testing set. The procedure is repeated many times (typically 100 or more cycles) to obtain a stable estimate of prediction error.

The measure of error is the most common figure of merit used to characterize model performance during cross-validation. The intended purpose of the method determines the nature of the errors, such as the misclassification rate for qualitative methods and prediction error for quantitative methods. Irrespective of qualitative or quantitative applications, two metrics are commonly used to characterize error within cross-validation: root-mean-squared error of calibration (RMSEC) and RMSECV. The RMSEC is calculated for the samples when left in the calibration, which monotonically decreases with each additional factor (i.e., PC) added into the model. In comparison, the RMSECV that is calculated during cross-validation will decrease until the last meaningful (i.e., relevant signal-containing) factor is added. Then, as each additional factor is incorporated into the model, the RMSECV will increase, indicating that the calibration data are being overfit. The plot of the RMSEC and/or the RMSECV versus the number of factors in the model is referred to as a predicted residual error sum-of-squares plot. In general, the best practice is to avoid inclusion of factors beyond where the minimum of the RMSECV plot line occurs. In addition, correlation between observed and predicted values (e.g., $R^2$) is also commonly used to assess performance of quantitative methods. Finally, the cross-validating results are only meaningful when the calibration and testing sets are comparable (i.e., drawn from the same population). Otherwise, extrapolation may lead to incorrect predictions.

## 3.2 Method Validation

The objective of validation is to demonstrate that the performance of a method is suitable for its intended purpose. Validation of a model and validation of a method are two different activities. Model validation routinely involves the use of an internal validation set or cross-validation to assess the appropriate parameters of a model via identification or quantification error and uncertainty. Parameters often include the range of variables, the type of preprocessing, the model rank, the choice of the algorithm, and others. These activities have been addressed in detail in *3.1 Model Development: Calibration*. In comparison, method validation must be based upon a fully independent external validation set and must follow the validation requirement described in *Validation of Compendial Procedures* ⟨1225⟩, according to the method type category. The acceptance criteria should be justified for the intended purpose. During the lifecycle, method revalidation is necessary after a model transfer or model update. The method validation and revalidation strategy should be risk- and science-based and appropriate to its impact level.

The typical performance characteristics for method validation are specificity, accuracy, precision, linearity, range, and robustness for quantitative models, and specificity and robustness for qualitative models. The metrics and their descriptions are discussed below. In addition to those typical performance metrics, metrics such as limit of detection (LOD), limit of quantitation (LOQ), sensitivity, analytical sensitivity, and effective resolution may not be required for validation purposes but could be useful for understanding the boundary of the method performance for a specific analytical application and the analytical technique that a model is associated with.

PERFORMANCE CHARACTERISTICS FOR METHOD VALIDATION

The sections that follow provide method validation attribute descriptions and metrics.

**Accuracy:** Statistical comparison between predicted and reference values is recommended. For quantitative applications, root mean-squared error of prediction (RMSEP), squared error of prediction (SEP), and bias are the typical measures of method accuracy. For qualitative applications such as classification, sometimes misclassification rate or positive prediction rate could be used to characterize method accuracy. To ensure that a model is accurate enough when tested with an independent test set, RMSEP is often found to be comparable to RMSEC and RMSECV and to meet the requirements of ATP.

**Precision:** The routine metrics of RMSEP and SEP encompass both accuracy and precision. Assessment of precision could involve a determination of the uncertainty associated with the reportable result and a variance component analysis that determines an "error budget" that quantifies the sources of variation that contribute to the uncertainty. For an estimate of precision alone, the standard deviation across results for replicate and independent analyses on the same sample within method, across days, across analysts, across instruments, and across laboratories could be used.

**Specificity:** For both qualitative and quantitative methods, whenever possible the underlying chemical or physical meaning of the chemometric model should be demonstrated and validated. For example, the scientific meaning of the variables (such as spectral range) used for model construction, data preprocessing, regression vectors, and loading vectors [for PLS, PCR, PLS discriminant analysis (PLS-DA), and PCA] should be demonstrated. Specificity could also be validated by the tendency of sample components (matrix or other nonanalyte compounds present in the sample) to adversely affect the ability of the chemometric method to report results accurately and/or precisely. The level of specificity may be assessed by accuracy and precision in the presence of varying amounts of potentially interfering substances. Substances to be tested can be identified by considering the underlying physical/chemical methodology and modeling approach.

Specificity may be evaluated by including adulterated, substandard, or nonauthentic samples. Authentic samples, both within and out of the target criteria, may not be available for practical or economic reasons. For example, it may not be possible, due to cost, to obtain out-of-target samples for a controlled, large-scale process. Where possible, an exclusion panel of out-of-target samples that may be closely related to the target samples should be considered to increase the confidence in the specificity of the measurement. Simulated off-target samples (such as small-scale samples) may be used to validate the procedure's suitability for the intended purpose and range. In all cases, the suitability of validation samples must be justified with appropriate inclusion and exclusion criteria.

For qualitative methods, the method should demonstrate the capability to correctly identify or classify the samples. The receiver operating characteristic (ROC) curve and/or the probability of identification (POI) are commonly used metrics (detailed information on ROC curves can be found in *4.1 Qualitative* and *Figure 6*). The ROC approach is intended to illustrate true-positive rate (TPR) and false-positive rate (FPR) over a range of decision thresholds. A good identification method should generate a ROC curve with the area under the curve (AUC) close to 1. For most well-designed identification methods, the probability of a

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positive identification is near zero when the analyte is not present, and the probability should approach 1 as the analyte concentration or mass increases.

**Linearity:** The algorithm used for chemometric method construction can be linear or nonlinear, as long as it is appropriate for modeling the relationship between the analytical signal and the analyte. The measures commonly used to assess either the model fit or its predictive properties are the correlation coefficient, slope, *y*-intercept, and residual sum of squares of the plot between the predicted versus observed results. Note that the plot between the residual versus observed results across the analytical range is expected to show no pattern.

**Range:** The range of a method should be appropriate for its intended use (e.g., specification).

**Robustness:** Typical factors to be considered include the normal variability of materials (e.g., lot-to-lot variability), operating environment variability, instrument variability, such as minor instrument maintenance, and method parameter variability, such as the number of spectra averaged in a spectrometer. In conjunction with the validation results, the method development strategy—such as the design of the calibration set or library and the choice of model parameters—can be taken into account to demonstrate the method robustness.

### VALIDATION SAMPLES

Other general aspects to be considered for chemometric method validation include the validation samples. The validation samples should be independent of the calibration samples to demonstrate the ability of the model to predict. Being independent means that the validation samples were not used in the calibration set or used for model optimization. Internal validation or cross-validation is typically used during calibration for model parameter optimization, but is not considered sufficient for final method validation. The validation samples should be selected based upon the ATP and the desired performance characteristics of the model. Method robustness is based on the evaluation of authentic samples with typical sources of variance. For pharmaceutical or dietary supplement products, validation samples of nominal production scale, such as those routinely manufactured for in-process, release, and/or stability testing, may be included. For botanical articles, taxonomic identity, geographic origin, season of collection, and other variants may be included. For naturally sourced materials such as many food ingredients and excipients, variables such as the geographic or microbiological source of the material, processing conditions, impurity composition, and other relevant attributes may be included in the validation sample set.

### ACCEPTANCE AND DIAGNOSTIC CRITERIA

As with any other analytical procedure, the acceptance criteria for a chemometric method should be defined before execution of the validation protocol. If the chemometric model was developed using data from a secondary technique [e.g., near-infrared (NIR) or Raman] with reference values from a primary analytical procedure [e.g., gravimetric, nuclear magnetic resonance (NMR) spectroscopy, or high-performance liquid chromatography (HPLC)], the ATP for validation may not exceed that which is obtainable by the primary method. Some exceptions may occur, for example, it may be possible to achieve superior precision using a secondary procedure, although the accuracy will be limited to that of the reference technique.

In addition to those attributes addressed in *Performance Characteristics for Method Validation*, method validation must also take into consideration the setting of diagnostics limits for a multivariate model before deployment for routine use. Samples that are out of model space are considered outliers and not suitable for obtaining a reportable model prediction. The model diagnostic should have a demonstrated capability to flag any of the out-of-model-space samples. To set up the diagnostics limits, two cases must be considered. The first is to determine the statistical distribution of leverage and residual within-the-calibration data set. The second is to prepare intended in- and out-of-model-space validation samples to test the limit. For instance, for an NIR spectroscopy-based content uniformity method, the in- versus out-of-model-space samples could be samples at target label claim versus samples containing active pharmaceutical ingredient (API) concentrations outside of the intended range for the method.

## 3.3 Model Monitoring

Throughout the model lifecycle, changes that can affect model performance may occur. Procedures should be in place for continuous performance verification of the model and for model update and procedure revalidation if necessary. A specific order among model monitoring, model update, and model transfer does not exist. Scientifically sound rationales must be applied to determine an appropriate sequence for the intended application.

A control strategy for checking model performance over its lifecycle should be developed and documented as part of model development and procedure validation. The strategy should identify the necessary elements for ongoing monitoring and evaluation of model performance. In addition, a plan for monitoring, analyzing, and adjusting the model should be in place with a measurement frequency that allows identification of excursions related to critical aspects of the model. The level of maintenance should be considered part of the risk assessment activities and should be adequate for the model criticality. If applicable, analytical instrumentation used to generate the inputs for the model should be qualified and also subjected to a continuous verification plan (for relevant guidance, see general chapters related to the applicable analytical instrumentation).

Ongoing assurance of performance of the model throughout its lifecycle should include:

- Ongoing monitoring and review of the model
- Evaluation of risk assessment
- Evaluation of post-implementation changes and predefined model maintenance
- Model update and procedure revalidation as needed

The ongoing review of a model should occur at predefined intervals and also should be triggered by events that may have an impact on model performance. Examples of such events include changes in raw materials variability or manufacturer; changes in the upstream process that may alter the sample matrix (e.g., process equipment or operation settings); drifts in model prediction; and out-of-specification (OOS) or out-of-trend (OOT) results given by the model (dependent upon the root cause

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of the OOS or OOT). In addition to triggers that are based on the prediction output of the model, triggers based on model diagnostics metrics and corresponding action rules should be included. Multivariate models may be more strongly affected by aberrant data signatures than are univariate models. Special care is needed to: 1) justify the multivariate model diagnostics statistically, 2) verify with data from the model development and procedure validation process, and 3) implement multivariate diagnostics for monitoring as part of the control strategy. Comparison of model predictions and reference or orthogonal procedures should take place on a periodic basis or as part of the investigation triggered by the review process.

The use of model diagnostics when applied to a new sample ensures that the model prediction is valid with regard to the calibration and validation sets used during model development and procedure validation, and also ensures that the result does not constitute an outlier. The observation of an outlier means that the result is invalid, but it is not a reliable indication of an OOS result; an OOS result is an observation produced by a model when the prediction falls outside the acceptance criteria and the model diagnostics are within the thresholds. In the case of qualitative models, nonconforming results should be treated as outliers and should trigger an investigation; the output of such an investigation will indicate whether the result is OOS.

The review process for a model should produce a decision regarding the need for an extension of model maintenance; such a decision may be the result of a risk-assessment exercise or the outcome of a predefined decision workflow. Model criticality and usage will define the extension of model maintenance, which can include restraining the model conditions; adjusting the calibration set (samples can be added, replaced, or removed); or even completely rebuilding the model. The decision, and corresponding rationale, must be scientifically sound and documented.

## 3.4 Model Update and Method Transfer

Model updating must be considered part of the analytical procedure verification, and both the justification and the activities must be documented as a part of the analytical procedure lifecycle. Before performing a model update, it is critical to understand the underlying causal factor that has prompted the update. The reasons for model updating can be roughly divided into two categories.

The first category is when the calibration set simply needs to be expanded. In this case, nothing has actually changed in terms of the response of the instrument to specific analytes. Instead, the original model is no longer valid because of the expanded range of original calibration, the addition of new analytes, or the occurrence of other, previously unseen variations (e.g., changes in particle size distribution, or the drift of a chemical process to a new steady state). Thus, the calibration space must be expanded with samples exhibiting this variation.

The second category is when the samples are the same but the measurement system response function has changed. This is often due to changes in the measurement components (new light source, clouding of optics, wavelength registration shift) or can be due to method transfer across different instruments. This, in essence, is an instrument standardization problem. Changes in instrument or measurement procedures over time can render a calibration model unusable, in which case a model update becomes necessary or a new model should be developed.

In practice, there are multiple model updating techniques that could be applied to each category of model update. Some updating techniques are relatively straightforward and simple to implement, whereas others are technically complex. Selection of an appropriate model updating approach must be based on a full understanding of the underlying causal factor. As a general rule, simple updating methods (e.g., slope and bias adjustment, as described below) should be considered first.

Before any model maintenance work is initiated, it is good practice to confirm that the fundamental construct of the original model—such as data preprocessing and variable selection—remains sound. For both qualitative and quantitative applications-based model updates, a straightforward preprocessing approach and a scientifically sound variable selection approach are recommended as the initial attempt to address challenges associated with updating the model. These approaches are equally applicable to differences caused by instruments, measurement conditions, or sample matrices.

### SLOPE AND BIAS ADJUSTMENT

One of the simplest model-updating methods is to postprocess the predictions with a bias adjustment, a slope adjustment, or both. This approach is often used for quantitative applications. For some qualitative applications, this approach may also be useful, depending on the nature of the method. In a limited set of circumstances, bias/slope adjustments are expected to work. For example, if the matrix of the samples being predicted were systematically different from the calibration/validation sample set, the model predictions would be in error by a constant value. Bias/slope adjustments, however, would not correct for any new variation in the data, such as variation that would result from a different matrix (e.g., new analytes/interferents). Therefore, applying slope/bias adjustments without a full understanding of the underlying causal factor is not recommended. Guidance may be obtained via inspection of residuals corresponding to the new data obtained using the existing model or, alternately, from supplemental exploratory data analyses using techniques such as PCA (see *4. Applications of Chemometrics*).

### CALIBRATION EXPANSION

When expanding the original calibration set, one should consider the number of new samples to be added, the impact (leverage) of the added samples on the overall composition of the new calibration set, and how to partition any new samples between the calibration and validation sets. It may be appropriate to simply augment the original calibration and validation sets with all the new samples, or it may be advisable to use a subset of the new and original samples. Multiple approaches, such as the Kennard-Stone algorithm and nearest-neighbor approach, are available to aid in the selection of new samples to add to an existing calibration set, but in general, all the approaches use methods to identify new samples on the basis of high leverage (i.e., model influence). Hotelling's $T^2$, multivariate score plots, and their corresponding limits are also effective approaches for accomplishing this goal.

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CALIBRATION TRANSFER

Instrument standardization and calibration transfer methods are used to transform the response function of a measurement system so that it matches that of a reference measurement system. The reference measurement system could consist of a completely different analyzer, or it could be the same analyzer before it experienced a response-function shift. The vast majority of these methods generally requires the use of stable transfer samples that are measured on the original instrument and require the instrument to be standardized at the same time. In addition, the approaches commonly used for instrument standardization could also be applied effectively to address the challenges resulting from changes in sample matrix and measurement conditions.

### 3.5 Revalidation

Before the redeployment of a multivariate model, appropriate procedure revalidation should be established using criteria equivalent to those used in the original validation protocol. This revalidation is necessary to document the validity of the model as part of the analytical procedure verification. The nature and extent of the revalidation procedure, including aspects such as scientific justification and experimental approaches, must be based on the cause of the update and the nature of the corrective action required for establishing suitable performance. Revalidation should be documented as part of the analytical procedure lifecycle.

## 4. APPLICATIONS OF CHEMOMETRICS

As discussed in *2. What is Chemometrics?*, chemometric analyses may be performed in either a supervised or unsupervised manner depending on the availability of data and the specifics of a given application. This section provides an explanation of these different analysis scenarios, as well as the chemometric tools (i.e., algorithms) that are commonly used. Additionally, several specific applications will be described in detail.

### 4.1 Qualitative

GENERAL ASPECTS

Qualitative chemometric analyses may be performed by using supervised and/or unsupervised approaches. However, to be incorporated into analytical procedures that are alternatives to compendial methods, the performance of any chemometric model must be verified as described in *3. Model Lifecycle*. This may not be possible if very little is known about the samples being analyzed. Nevertheless, unsupervised analyses play an important supporting role during the development of chemometric alternatives to compendial procedures within the lifecycle framework, and are recommended for use before the development of subsequent supervised approaches (examples are provided in *Qualitative Application Examples*).

Qualitative compendial procedures are those that seek to supply the value of some categorical descriptor for an unknown sample. Examples of categorical properties include (among others):

- Chemical identity: microcrystalline cellulose versus API
- Morphology: polymorph A versus B; monoclinic versus triclinic
- Sample authenticity: authentic versus nonauthentic and/or adulterated
- Sample origin: facility A versus B; lot ABC-001 versus lot XYZ-002

Categorical properties can be modeled effectively using supervised chemometric algorithms that leverage either proximity or distance in multivariate space, resulting in a qualitative assignment of samples to one or more classes depending on the application and the technique used. According to ⟨1225⟩, procedures based on models of this type are suitable for incorporation into Category IV methods for identification and should be validated to demonstrate adequate specificity for the intended use. “Identification” is a term that is generally used to describe a range of analysis scenarios; several common ones are described in *Qualitative Tools*.

Supervised techniques used for classification purposes, besides the analytical sensor information, make use of discrete information related to the samples in the data set (e.g., class labels). It is intended to define the borders of the clusters (or classes), providing statistical criteria, in addition to the visualization techniques (e.g., scores plot of PCs from a PCA). The borders then can be used as acceptance criteria for inclusion or exclusion of new samples into a given class. Several useful tools are available to the analyst for building the model, such as linear discriminant analysis (LDA) and quadratic discriminant analysis (QDA), soft independent modeling of class analogy (SIMCA), kNN, and PLS-DA. More complex techniques also may be used with justification. Each type of model has its own strengths and weaknesses. If a particular algorithm does not give the desired level of performance, a different algorithm may be selected. These aspects are discussed further in *Qualitative Tools*.

For model development, optimization, and validation, the classification and discrimination techniques follow the same process described in *3. Model Lifecycle*. These techniques develop a threshold or limits to make class assignments. The simplest form of classification is a two-class system (e.g., good/bad or A/B). When a new sample is classified as good or bad (A or B) by the model, this results from what is called a discrimination technique. Classification is the technique of assigning a new sample as A, B, either A or B, or neither A nor B. The error rate of the model is the total number of incorrect classifications divided by the total number of samples. Depending on the requirements of the application and the associated risk levels, the error rate(s) might be class-specific or pooled across classes. The specific details of the application will determine whether the assignment of a sample to more than one class would be an acceptable result. Where this must be avoided, classification thresholds should be set appropriately during model development to guarantee single-class results.

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**PCA:** PCA is a commonly used exploratory analysis tool that was briefly introduced in *2. What is Chemometrics?*. PCA is a variable reduction technique and acts on the data by defining new variables, so-called principal components (PCs, see *Figure 2*). PCs are orthogonal and are defined in the direction of the largest (remaining) variance in the data. The results of PCA are discussed in terms of PC scores and loadings, which may be plotted graphically in two (and sometimes three) dimensions for visualizing clusters of samples or outlying samples (score plots, see *Figure 4*), whereas the loadings plots provide information on the original variables. The scores are the projections of the samples on the PCs, whereas the loadings provide the weights (coefficients) by which the values of the original variables are multiplied to generate the component score for a particular sample. Loadings are rich in information regarding which variables in the matrix are prominent and can be used to "understand" the information captured by the latent variables that provide the basis for the observed sample distribution in the scores plot (*Figure 4*). The PCs may also be used as the basis for quantitative models via PCR (see *4.2 Quantitative*).

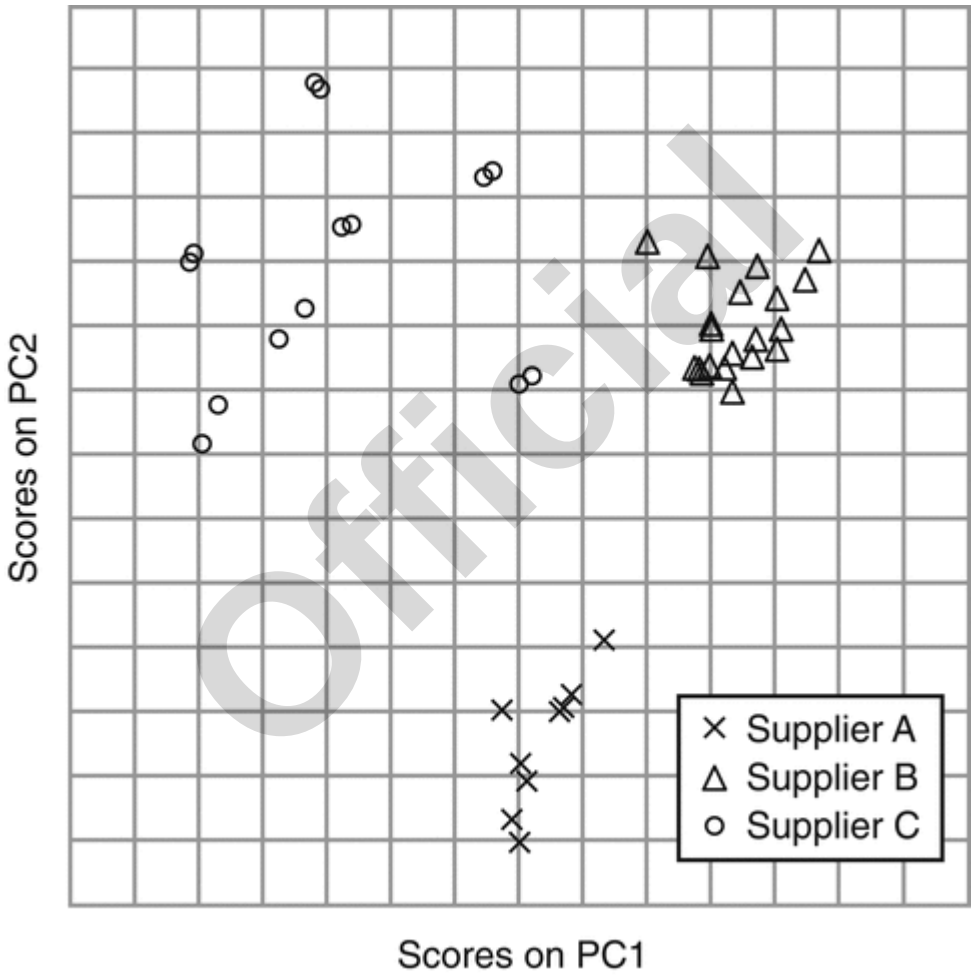


Figure 4. PCA scores plot: projection of PC1 versus PC2.

**Clustering algorithms:** The aim of clustering analyses is to partition data into different groups. Some methods, such as hierarchical cluster analysis (HCA), result in tree-like structures called dendrograms (*Figure 5*) that provide information on groups of samples or outlying objects occurring in the data set. Dendrograms can be built in many different ways and thus may show different aspects of the data set. A first possibility, called divisive or top-down methods, starts from the entire data set, which is split in consecutive steps until each object is in an individual cluster or all elements of a cluster fulfill a given similarity criterion. In the second scenario, called agglomerative or bottom-up methods, the opposite is done. Starting from individual objects/clusters, those most similar are merged until everything is in one cluster. Of the two options, bottom-up approaches tend to be computationally less intensive, are part of most computer packages, and are more frequently used.

The parameters used to express (dis)similarity between objects or clusters (see *y*-axis on *Figure 5*) can be either correlation-based (e.g., correlation coefficient, $r$ for similarity or $1-|r|$ for dissimilarity), distance-based (e.g., Euclidian distance) measures, or a combination of both (e.g., Mahalanobis distance). Two clusters are linked in the dendrogram at the height related to this (dis)similarity measure. The parameter applied is expressed in such a way that clusters/objects linked low (i.e., close to the *x*-axis) are similar, whereas those linked high are dissimilar. Many different methods or criteria exist for use in deciding which objects/clusters are consecutively merged [e.g., single linkage, complete linkage, (weighted) average linkage, and centroid linkage]. Depending on the applied criterion, the dendrograms may look very different. Drawing arbitrary

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horizontal lines (see *Figure 5*) splits the data set into different groups, which occasionally may be linked to sample properties. Outlying samples are linked very high in the dendrograms, most often as one-object clusters. The stability of clustering results can be affected by many factors, such as noise in the data, sample size, choice of algorithm, distance measure, and others. Divisive methods tend to produce more stable results than agglomerative methods, even though they are hardly ever used.

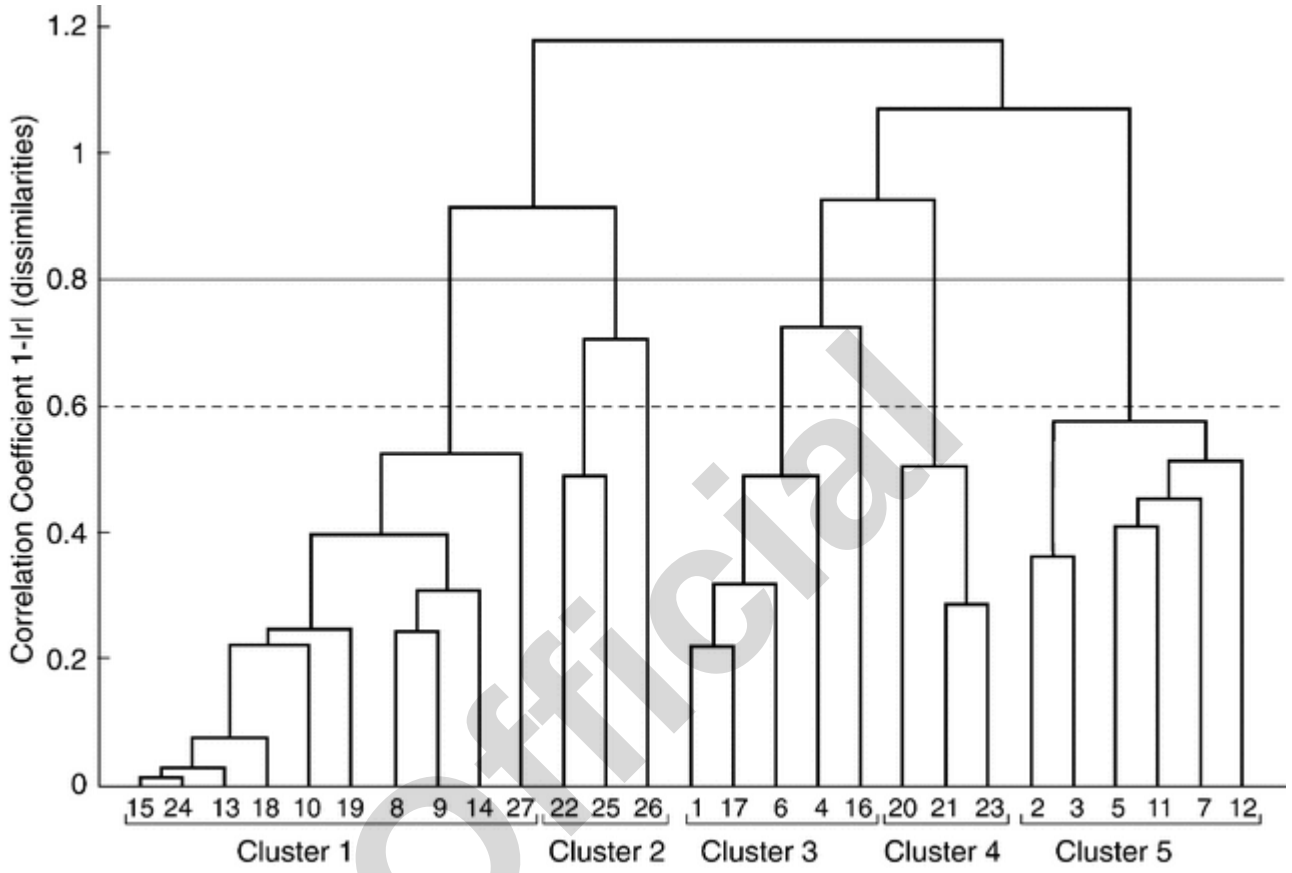


Figure 5. Hierarchical clustering based dendrogram for 27 objects. Abscissa: object numbers.

**Parametric modeling:** Authentication of a material or formulated product for quality assurance purposes may be achieved via multivariate parametric modeling of the chemical profile (e.g., spectrum or chromatogram). These methods are sometimes referred to as "one-class classifiers". Basically, a parametric model is developed statistically or empirically with a confidence boundary around the data from known authentic samples. Parametric models describe the behavior or structure of the majority of the data from a single class, assuming that a distribution (typically, a multivariate normal one) exists in those data. Predictions against a parametric model identify deviating points outside the significant boundaries of that distribution. Whereas classification or discrimination approaches provide identification (as previously described) of the unknown sample, parametric models will designate the unknown sample as either belonging to the authentic class or as an outlier, or exhibiting features other than those defined by the distribution. Some discrimination techniques will also optimize class separation, and may have greater selectivity, but will tend to fail when presented with an object from none of the classes. In contrast, parametric modeling methods will reject any sample that does not fall within the confidence boundary established for the model. Perhaps the most commonly used parametric modeling algorithm is SIMCA, where PCA is used to model the data and Hotelling's $T^2$ and $Q$ residuals (DmodX) are used to define the limits. In some instances, parametric modeling approaches will result in "soft classifications" wherein a sample may be assigned to more than one class. To satisfy compendial usage requirements, appropriate action must be predefined for cases of ambiguous class assignment, and/or the $\alpha$ and $\beta$ error rates must be established and justified according to risk.

## QUALITATIVE APPLICATION EXAMPLES

**Exploratory analysis:** Unsupervised algorithms are routinely used for initial exploratory data analyses, which precede formal calibration and validation exercises. Unsupervised data exploration can rapidly indicate, in the absence of any prior knowledge, whether distinct classes or outliers exist within the pool of available data. For instance, in *Figure 4* a graphical example is shown in which PCA applied to spectroscopic data was used to differentiate between samples from different suppliers. Exploratory models yield latent variables that may be inspected to identify the original variables with the greatest amounts of relevant analyte signal to carry forward into subsequent models or to guide and/or verify the results of variable selection approaches. This might occur during the analysis phase of a DoE study to assess which factors to include in subsequent designs and/or calibrations. Further, exploratory analyses may be used to test and empirically optimize various signal preprocessing options to

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maximize the relative contribution of desired analyte signal versus signals from sources of interference (e.g., scattering effects, optical path length differences, sample physical properties, and instrumental drift).

***Change to read:***

**Material identity testing:** Identification testing is routinely performed for all pharmaceutical active ingredients, excipients, drug products, and packaging materials to authenticate them and verify that the correct materials are used in manufacturing, release testing, and packaging operations. ▲*Spectroscopic Identification Tests* ⟨197⟩ ▲ (CN 1-May-2020) illustrates one of the most widely used analytical techniques for identification, Fourier transform IR spectroscopy. Typically, identification testing involves comparing the sample spectrum to the spectrum of a reference sample and assessing the level of agreement between the two. Different chemometric algorithms can be applied for identification, and the general model development and maintenance should follow the guidance in *3. Model Lifecycle*. All methods for identity testing are supervised classification methods because either a single reference spectrum or a set of reference spectra from material(s) with known identity is utilized by these algorithms. Algorithms can be applied in either original variable space or transformed variable space (such as latent variable space) after data preprocessing.

According to ⟨1225⟩, models of this type are suitable for incorporation into Category IV methods for identification and should, at a minimum, be validated to demonstrate adequate specificity for the intended use. An example approach for performance verification is the documentation of TPR and FPR. TPR is the percentage of samples that are correctly identified, whereas FPR is the percentage of samples that are incorrectly identified. TPR is also referred to as sensitivity, and FPR is referred to as specificity. Regardless of which approach is taken, there is always a trade-off between FPR and TPR, and this trade-off behavior and overall procedure performance are best visualized in a ROC curve plot (see *Figure 6*).

A classical ROC curve is generated by plotting the TPR on the ordinate and the FPR on the abscissa. Each point on a ROC curve represents a specific TPR and FPR pair obtained at a specific threshold. The threshold is typically a number generated by the algorithm, and when it is exceeded, this corresponds to a positive identification. This threshold value is determined during the procedure development process. The threshold could be the *P* value from hypothesis testing, hit quality index (HQI) threshold, Mahalanobis distance threshold, PLS discriminant score threshold, or some other threshold.

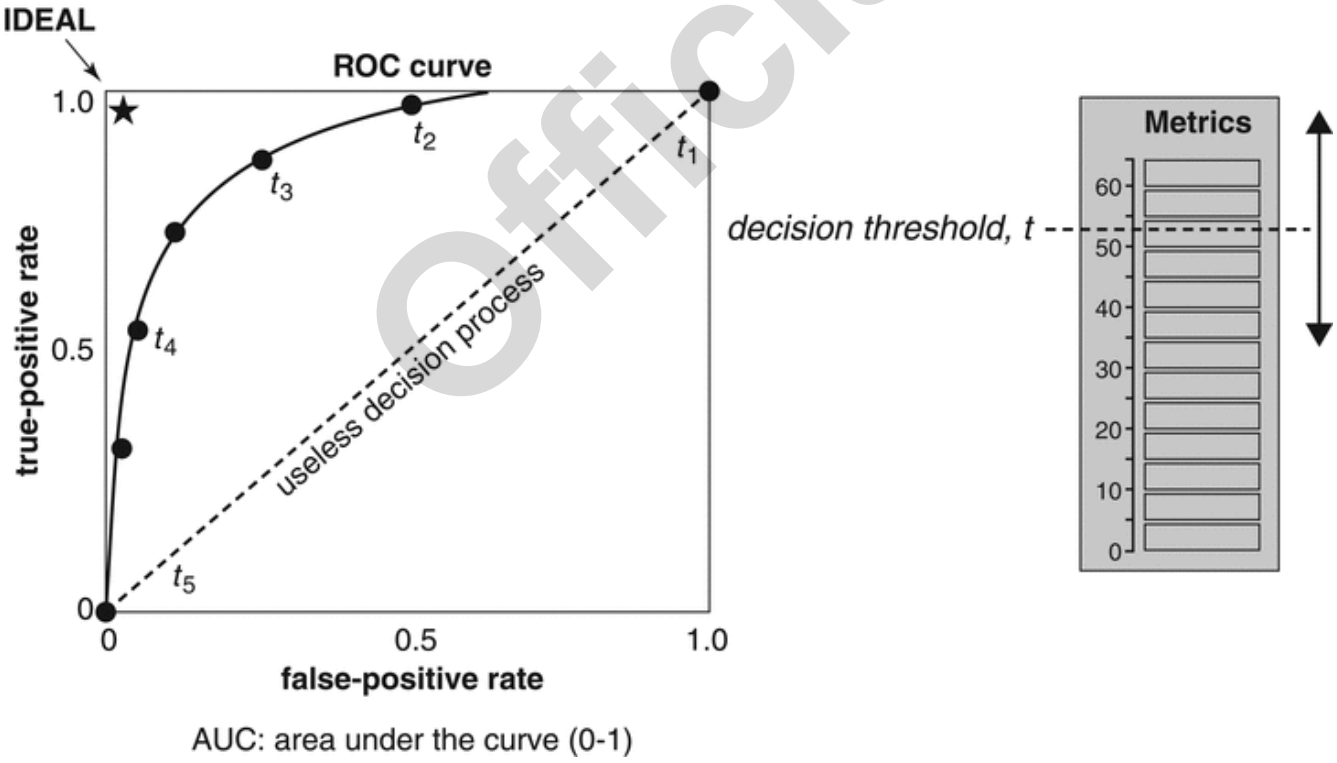


Figure 6. ROC curve plot showing the AUC (*3*).

During procedure development, a number of known positive and negative samples are examined, and the algorithm output for each sample is recorded. The performance of the method when operating at a particular threshold *t* is characterized by a single TPR/FPR pair, and the full operating characteristic curve can be produced by plotting TPR/FPR pairs over a range of decision thresholds. A false-positive identification corresponds to a decision threshold that is too low. False positives can be minimized by raising the decision threshold. However, excessively high decision thresholds can produce a false-negative identification.

A good identification method should generate a ROC curve that produces one or more TPR/FPR pairs in the top-left corner of the plot, as shown in *Figure 6*. This characteristic is best captured by the AUC. A random decision method is represented by the diagonal line with an AUC of 0.5, whereas a good identification method should have an AUC closer to 1. The shape of ROC

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curve depends upon a combination of spectral properties of the sample and the chemometric algorithm used for identification. Characterization of the ROC curve is a fundamental task associated with method development and validation.

For a two-class model, false positives and false negatives are the incorrectly assigned samples. Together with the true positives and true negatives, they provide the basis for the sensitivity and specificity of the model. The analyst may wish to care about one type of error (α or β) more than another, and therefore could change the model to be more conservative when choosing a threshold. Thresholds can be used as action criteria or evaluation criteria.

**Complex material authentication testing:** Nutraceuticals and samples of herbal origin may be complex mixtures of numerous components. Authentication relies upon assessment of the presence of multiple analytes and their relative concentrations as well as potentially demonstrating the absence of adulterants and/or related materials. Thus, authentication in this scenario is a multivariate decision process that is appropriate for chemometric approaches. Typically, a characteristic "fingerprint" pattern incorporating information from the various analytes (and adulterants, potentially) that are present in the sample is obtained by applying a combination of separation and characterization techniques or spectroscopic measurements. The chemical composition information in the sample fingerprint provides a basis for multivariate pattern recognition. In particular, techniques with exceptional analytical selectivity, such as mass spectrometry and NMR spectroscopy, have emerged as powerful fingerprinting tools, especially when operated as detectors for chromatographic separations of multicomponent samples. Chromatographic fingerprinting is recognized as a viable identification procedure for herbal medicines by both the World Health Organization and the U.S. Food and Drug Administration. (*4*,*5*)

Depending on the origin and purity of the sample, it may be appropriate to develop a multivariate classification model using either the entire fingerprint or a selected subset of peaks. Variable selection approaches may be useful in determining the optimal approach, depending on the nature of the samples and/or the ATP. It is crucial that the samples have an associated class label or quantitative property (activity) obtained by an objective reference technique (separate from that used to generate the fingerprint) to enable supervised modeling and subsequent model performance verification. Care should be taken to ensure that appropriate sources and levels of variability are included in model development and performance verification exercises based on the intended use of the test.

Several useful tools are available to the analyst for building the model, such as LDA and QDA, SIMCA, kNN, and PLS-DA. More complex techniques may also be used with justification (e.g., SVM).

Chemometric methods for multicomponent sample authentication are consistent with *Validation of Compendial Procedures* ⟨1225⟩*, Validation, Data Elements Required for Validation, Category IV*. Various numerical methods may be used to verify predictive performance. As discussed in *Qualitative Tools* for pure material identification, predictive performance may be verified by comparison of the TPR and FPR once a classification threshold appropriate to the application has been established. It is crucial to finalize this threshold before formal validation of the overall analytical method.

**Classification:** This is a supervised learning technique. The goal of calibration is to construct a classification rule based on a calibration set where both **X** and **Y** (class labels) are known. Once obtained, the classification rule can then be used for the prediction of new objects whose **X** data are available.

Classification models may be divided into two main groups: hard and soft. Hard classification directly targets the classification decision boundary without producing a probability estimation. Examples of hard classification techniques are kNN and expert systems. Soft classification estimates the class conditional likelihood explicitly and then makes the class assignment based on the largest estimated likelihood. SIMCA is a commonly used soft classification technique. In either scenario, the thresholds to determine the class boundaries are important and should be established during method development based on the resulting TPR and FPR, the requirements for which are determined by the ATP. These rates should also be verified as part of the full procedure validation.

In many applications, it is unacceptable to have a sample classified into two classes. Further, if a sample is not classified into any class, the sample is deemed an outlier to the calibration set. For example, a classification model built from NIR spectra of four materials (A, B, C, D) has four classes. Any material that has a source of variance (e.g., different water content) not previously seen and included in the model calibration may not be classified, and therefore should be identified as an outlier and investigated. Similarly, when a spectrum of another material (E) is applied to the model, the model should not be able to classify it into a given class. If similar samples are expected in the future, then the model should be revised to include the outlier samples in the calibration set, either to supplement the calibration data for existing classes or to create an entirely new class, depending on the nature of the outlier(s) and the specific requirements of the application.

## 4.2 Quantitative

### GENERAL ASPECTS

When the intended use of an analytical procedure is the determination of one or more sample components or properties that have values that can vary continuously rather than categorically, then a quantitative model is required. Similar to the process for qualitative modeling, quantitative chemometric models are produced in a supervised manner using both the independent **X**-block and dependent **y** variables to ensure optimal predictive performance. The difference arises in the nature of the **y** values, which are continuous/numerical (rather than categorical) for quantitative applications. Per ⟨1225⟩, quantitative chemometric models are suitable for incorporation into Category I, II, or III methods for determination of the content of drug substance or finished drug product, impurity levels, or performance characteristics, respectively. In-process tests applied in an offline manner or an in/on/at-line manner that produce the value for a continuous variable would also represent appropriate uses of quantitative chemometric models. The specific requirements of the analysis per the ATP (see *3. Model Lifecycle*) should be used to guide the validation metrics used to demonstrate performance.

### QUANTITATIVE TOOLS

The algorithmic tools needed for quantitative applications are wide ranging; for more complete descriptions see *Appendix, Additional Sources of Information*. Tools that are commonly used are briefly described here. As is the case with any chemometric

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model, the tool must be matched to the ATP, and vice versa. Certain advantages and disadvantages are characteristic of each tool, and the chemometrician must be mindful of these when justifying their use.

**Multiple Linear Regression (MLR):** This tool establishes a correlation between a dependent variable (or **y**, response variable) and multiple independent variables (or **x**, explanatory variables) by fitting a linear equation to observed data. MLR has two broad applications, as follows.

The first broad application of MLR is to establish a model that explains the importance or magnitude of effect on the response variable, and this is typically used in the DoE screening or response analysis. Typically the input variables are mutually independent and by design represent large magnitudes. Often, the higher order of terms such as square terms and interaction terms are included, and a step-wise procedure is used to exclude those that make a smaller contribution to the model. Caution is advised to avoid having too many variables in the model, because including too many terms will overfit the model. The adjusted $R^2$ statistic is typically used to estimate whether there are too many factors in the model, although other metrics may also be appropriate. Adjusted $R^2$ is a modification of the standard coefficient of determination that takes into account the number of model terms and the number of samples, resulting in a plot of $R^2$ (adjusted) that has a maximum value beyond which additional terms result in overfitting.

The second broad application of MLR is to establish a prediction model for analytical purpose. MLR can be used to fit a predictive model to an observed data set of **y** and **x** values. The established model can be used to predict the value **y** from the **x** of new samples. The model for prediction purpose created from correlated **x** inputs is suitable to explore the relationship between **y** and individual **x**'s.

The prediction MLR model can be used in a procedure or method for a pharmaceutical compendial test. For example, a filter NIR, which is designed for a specific use such as water testing in the product, produces a "spectrum" of only a few data points.

The development and validation can follow the same procedures as are used with latent variable models such as a PLS. During the development and validation of an MLR model: 1) appropriate data preprocessing needs to be used; 2) cross-validation is used to estimate the model performance; and 3) an independent data set is used to assess the model.

A limitation of MLR is that model predictive performance may be negatively impacted when the modeled variables are collinear. The issue of collinearity is not unique to MLR. It is mitigated in PLS modeling via generation of a new basis set of orthogonal (noncollinear) latent variables that are linear combinations of the original variables. However, this is not relevant for MLR models. In MLR models, variable selection or transformation (e.g., mean centering) may be needed to avoid or reduce collinearity in the calibration set. Even though MLR models have some diagnostic metrics to identify outliers and highly leveraged samples in the calibration, they do not have diagnostic metrics (such as Hotelling's $T^2$ and **X** residual test) that PLS models offer during the prediction of new samples.

**PCR:** This is a two-step modeling approach in which a PCA is first performed on the **X**-block data to obtain a basis set of PCs. The PCs are then used in combination with the **y** data to develop a regression model to predict the **y** values.

**PLS:** This is one of the most commonly used chemometric algorithms for both quantitative and qualitative (PLS-DA, see *Qualitative Tools*) modeling scenarios. The computational advantage that PLS offers over MLR and PCR is that the PCs are derived in the PLS algorithm using the **X** and **y** data simultaneously. This often results in predictive models that require fewer latent variables compared to PCR, thereby improving robustness. PLS is capable of handling mildly nonlinear relationships, whereas PCR and MLR are linear modeling techniques. Further, more than one **y** variable can be modeled using the same **X**-block data via different algorithmic approaches. PLS-1 results in a separate model for each **y** variable. PLS-2 will produce a single model capable of simultaneously predicting the values of two or more **y** variables. The relative advantages of each approach may vary from one application to another. For instance, if different mathematical preprocessing is necessary to resolve each component to be predicted, separate PLS-1 models may provide improved performance over a single PLS-2 model.

## QUANTITATIVE APPLICATION EXAMPLES

**Pharmaceutical dosage form assay and/or uniformity of content:** Assay of the API content in the final dosage form is commonly performed via measurement of a sample drawn from a homogenous composite of a number of individual dosage units (e.g., tablets or capsules). The quantitative result is then reported as an "average content" (expressed as percentage of target API amount) for the batch tested. Uniformity of content may be tested using a method similar to the assay, but measurements are performed on individual samples. The reported result is primarily based on the variability in assay results across the individual dosage units analyzed. Assay and uniformity of content methods are intended to characterize the "major" component in the sample(s) in question and are considered Category I tests per the definitions established in ⟨1225⟩. In the majority of cases, the target analyte will be the active ingredient in the sample, although quantitative assays for other components exist throughout the compendia as well.

For pharmaceutical solid dosage forms, the majority of assay procedures are based upon HPLC. HPLC analysis is time consuming and often involves the use of large volumes of solvent for the mobile phase. However, HPLC is highly linear and is typically calibrated using univariate mathematics (i.e., one measured variable is directly and uniquely proportional to sample concentration).

In contrast, alternative methods for assay that are based on spectroscopy (e.g., NIR and transmission Raman spectroscopies) offer the advantage of increased analysis speed and are nondestructive. However, in many cases the analyte signal may not display the same degree of linearity or signal-to-noise as with the corresponding HPLC method. Moreover, no single variable is directly and uniquely related to the concentration of interest. For this reason, multivariate models, often based on PLS regression, are commonly used in spectroscopic procedures for assay and uniformity of content.

For Category I tests, it is crucial to demonstrate the accuracy, precision, specificity, linearity, and range of results during the validation of the overall analytical procedure. Each of these performance aspects involves considerations during multivariate model development or validation that are distinct from those related to univariate techniques.

Most spectroscopic methods are not quantitative in an absolute sense. For example, models developed using NIR or Raman data must be calibrated relative to a primary reference technique (often HPLC or NMR spectroscopy). Given the nondestructive nature of NIR and Raman, it is straightforward to analyze a given set of samples using one of these techniques and then subsequently analyze the same set of samples via HPLC or NMR spectroscopy to obtain the reference content values. This

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approach is typical for assays of solid dosage forms, however, it is worth noting that reference values for assays of some dosage forms, APIs, polymorphs, or excipients might be derived from other techniques such as gravimetric data recorded during the calibration sample preparation. The chemometric models are developed via a multivariate calibration using the NIR or Raman spectral data and the content values from the reference technique. Accuracy is determined by using the absolute difference between the model predictions and the reference values. Precision is determined by using the standard deviation of procedure results alone, such as from replicate analyses of the same sample. Thus, precision is a measure of performance for the analytical procedure as a whole. The model itself will be absolutely precise. That is, given the same spectral input, the same numeric output will always be generated. That said, precision may be influenced by the specifics of the chemometric modeling approach (e.g., preprocessing, modeling algorithm, number of latent variables) and therefore precision should be evaluated during model development as well as during validation. It is important to note that the accuracy of chemometric models will not be able to exceed the accuracy of the reference analytical technique used to calibrate the model. However, it may in certain cases be possible to exceed the precision of the reference technique.

The range of the method will be determined by the calibration samples that are used to develop the multivariate model. According to ⟨1225⟩, it is recommended that assay calibration samples should have analyte contents ranging from 80%–120% of the target amount. For evaluating uniformity of content, the recommended range is 70%–130% of the target amount. In some cases, both the assay results and uniformity results may be obtained from the same set of sample measurements. Averaging of a requisite number of individual dosage-unit assay results will provide a value equivalent to the "average content" parameter. Calculation of the "acceptance value" (see *Uniformity of Dosage Units* ⟨905⟩) using this mean result combined with the standard deviation for the same set of individual dosage unit results will provide a uniformity of content result. In this scenario, a single calibration set that spans the wider of the two recommended ranges (70%–130%) should be used.

If it is not practical to obtain a sufficiently wide range of content values in calibration samples produced at commercial manufacturing scale, calibration samples may also be produced in a smaller scale or in the laboratory. In this scenario, all attempts should be made to replicate the physical properties representative of the commercial scale. Additionally, care should be taken to verify that minimal bias exists between samples from different scales. Commercial-scale samples may need to be incorporated into the model (and/or procedure) validation process to verify the accuracy and precision of the model (and/or procedure) results against any differences and/or variability in physical properties. Mathematical processing algorithms (e.g., normalization, second derivatives) should be optimized as much as possible during initial exploratory data analysis (see *Qualitative Application Examples*) to mitigate prediction bias resulting from these factors. For instance, it is known that thickness, particle size, and density differences can lead to spectral slope changes and baseline offsets in NIR spectra. Depending on which specific effect is present, improperly selected preprocessing may not fully correct for spectral differences, leading to errors in prediction.

Fit and predictive performance of chemometric models should be demonstrated via the linearity of a plot of the model outputs versus the reference or nominal values of the target analyte(s), ideally using results from a set of independent test samples. The raw analytical signal may demonstrate a nonlinear relationship with the reference analyte concentrations. Likewise, multiple latent variables may be utilized for the chemometric model building. However, the key aspect of the model that must be demonstrated to vary linearly with analyte concentration is the model outputs, not the inputs. A plot of the prediction residuals versus concentration may assist in revealing any systematic lack of fit. Any observations of patterns in this residuals plot may indicate a need for revision of either 1) the number of latent variables included in the model, 2) the preprocessing mathematics, or 3) the algorithm type used for modeling.

Demonstration of the specificity of the procedure for assay may be based on likely sources of interference or material substitution based on an understanding of the material properties of the sample components and the manufacturing process and/or supply chain. One approach might be to verify that low assay results that are OOS are consistently obtained for a placebo version of the dosage unit. Another approach might be to verify that dosage units of a different product (especially one manufactured in the same facility and/or tested in the same laboratory) result in assay values that are consistently OOS.

**Impurity limit tests:** Routine testing is required for determination of impurities and/or degradants in intermediates, bulk drug substances, and finished pharmaceutical products. Testing for this purpose may take the form of a limit test (Category II, ⟨1225⟩).

Typically, these impurities tests are based upon HPLC or other methods (e.g., Karl Fischer titration for water). These analytical procedures are highly linear and are typically calibrated using univariate mathematics. However, these methods are often time consuming and involve manual sampling, which is disadvantageous, especially for products having high potency or for those with toxic intermediates produced during API synthesis.

In contrast, alternative methods for assay based on spectroscopy (e.g., NIR and Raman spectroscopy) offer the advantage of increased analysis speed, and more importantly, total elimination of human sampling due to the noninvasive nature of the measurement. However, these advantages are typically offset by decreased sensitivity (LOD) and potentially nonlinear sensor response across the required analyte concentration range as the corresponding reference method. For this reason, multivariate approaches combining spectral preprocessing and latent variable models are often employed in spectroscopic methods for these limit tests. (*6*) However, due to the potential challenges involved in achieving adequate sensitivity for a spectroscopy-based limit test, careful development and feasibility studies must be employed.

For a limit test in Category II procedures, a calibration set containing varied concentrations of the analyte of interest is often used to characterize the performance of such an analytical procedure. Although it is not required, these performance metrics could include accuracy, range, linearity, and others. The use of the performance metrics depends on the nature of the limit test.

In contrast, it is critical to illustrate specificity and LOD during validation of a limit test according to ⟨1225⟩. For specificity, a comparison between loading/regression vector and a spectrum representative of the pure component of interest is often used. Additionally, measuring matrix effects on the determination of the analyte within the specified range is another useful approach to demonstrate that the analytical procedure is specific to the analyte of interest without impacts from other variables introduced in the calibration set. Moreover, evidence of method specificity can be demonstrated by calculation of a selectivity ratio from the calibration data set, defined as the variance of model-reconstructed spectra divided by that of residual spectra. The selectivity ratio at the analyte absorption band is expected to be higher relative to other spectral regions.

Regarding LOD, there is no generally accepted estimator for PLS models. A common practice to determine LOD involves calculating a spectral signal-to-noise ratio. The model results for replicate analyses of a blank sample(s) may be used to represent

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the noise. Alternatively, the noise may be approximated using the standard error of a multivariate model. Other more elaborate approaches were suggested to address the multivariate nature of PLS models. (*7*) With any approach, the estimated LOD should be verified experimentally using samples with analyte levels at or near that concentration. Because the LOD obtainable via a spectroscopic method will likely be higher than that obtainable via a chromatographic approach, it becomes crucial to demonstrate that the LOD based on the chemometric model meets the requirements of the ATP.

**Dissolution testing:** In vitro dissolution is a crucial performance test for solid dosage forms that is used to predict in vivo drug-release profiles during drug development and to assess batch-to-batch consistency for quality control purposes. Chemometric models can be used to predict the drug product dissolution from relevant measured product properties, and the method that uses such a model may be established as an alternative procedure. Alternatively, the dissolution profile can be modeled via several responses from different time points on the dissolution curve using individual models.

Dissolution data may be collected as a profile (i.e., a series of values obtained at various time points), even though the acceptance criteria may rely on a single time point. When building a chemometric model, it is essential for the model to be able to predict the entire profile. The number, and spacing in time, of points in the dissolution profile will vary depending upon the product type (e.g., immediate versus modified/extended release). A common approach is to transform the profile into a single value, which then serves as a dependent variable (or **y**, response variable). This variable, combined with another measured variable, can be used to restore the dissolution profile. The transformation can be accomplished by fitting the dissolution profile data into a mechanistic model (such as a first-order rate equation or its variants) or an empirical model (such as a Weibull function). After transformation, a few variables (typically, two or three variables) can be used to represent the dissolution profile. Generally, the two variables are a dissolution rate factor and a plateau factor; a third variable such as lag time may be necessary. The dissolution rate factor, which represents the rate of dissolution, will be the dependent variable for the modeling. The plateau factor represents the final amount of the drug in the solution, which should be equal to the drug content for most products. These two factors are used to restore the profile. Therefore, the model for dissolution prediction is used to predict the dissolution rate.

The key to designing a successful model is defining the input variables. The plateau factor may be obtained from drug content data, whereas the dissolution rate factor may be modeled from a data set of chemical and physical attributes of the product or intermediate product. The necessary knowledge of physicochemical properties and engineering concepts should be used to identify and justify the relevant inputs that have potential impact on the dissolution of the product. This typically involves risk assessment and an additional data collection step such as a small-scale DoE study. The inputs typically include the measured material attributes at various processing stages (such as particle size data for granules or blend; NIR measurements for blend or tablets; and physical tablet properties).

There are two approaches for defining samples in the model calibration: batch samples and individual unit dose samples. For the batch-sample approach, one product batch is treated as a sample, and the input variables are the attribute means of the batch. The individual unit dose approach is to measure the attributes of individual unit doses (such as NIR measurement on the individual tablets). The input variables from both approaches may be supplemented with other raw material properties as justified by the critical quality attributes.

The validation of the model-based dissolution prediction method could be different from the typical HPLC dissolution method validation, depending on the specific approach used. The HPLC dissolution method puts more emphasis on precision, as the dissolution is treated as a Category III method in ⟨1225⟩.

In the batch-sample approach, the model-based method treats dissolution as a batch property instead of a property of individual tablets, and accuracy is the focus of method validation or verification. For example, each batch forms a sample, and it is not realistic to have a large validation sample set. This approach generates a single profile for a batch, and the result at a certain time point can be used to assess the product batch quality. Evaluation of variability must be aligned with the criteria in *Dissolution* ⟨711⟩. The variability of the final dissolution result can be evaluated by analyzing the variations of inputs. Possible ways to evaluate the variation of the sample (batch) include the analysis of variation of inputs, simulations, and dividing a batch into multiple sub-batches.

In the individual unit dose approach, the model-based method evaluates individual dosage units. If many tablets are tested, this becomes a large *n* situation in which the acceptance criteria in ⟨711⟩ should be used with caution. The chemometrician may propose acceptance criteria as long as these criteria are demonstrated (by simulation or other means) to have equivalent or tighter passing criteria than those in ⟨711⟩. In the method validation or verification, it is desirable to have at least one sample that shows low dissolution (near or below the specification), and the chemometric model-based method should demonstrate the capability to distinguish this low-dissolution sample from normal samples.

## GLOSSARY

Many common chemometric terms have been defined within the text of this chapter. Some additional terms are defined here and serve as a reference to their usage in the text. For a more complete description, consult the texts listed in *Appendix, Additional Sources of Information*.

**Calibration model:** A mathematical expression used to relate the response from an analytical instrument to the properties of samples, or to capture the underlying structure of a calibration data set.

**Calibration set:** Collection of data used to develop a chemometric classification or model.

**Derivatives:** The change in intensity with respect to the measurement variable (i.e., abscissa). Derivatives are useful for removing baseline offsets (constant or linear) due to sample properties, or for highlighting small changes in a signal, helping to enhance selectivity and sensitivity (e.g., one spectroscopy and chromatographic peak with a shoulder peak adjacent).

**Internal validation:** The application of resampling statistics such as cross-validation. Subsets of the calibration data set are subjected to a variety of statistical processes to identify which calibration model best fits the available data. Each model is characterized by a statistical parameter. For cross-validation, the entire data set of samples is split into individual samples or groups of samples, which are removed individually from the rest of the samples and tested as unknowns against a calibration model constructed using the rest of the samples. The characteristic statistic is the standard error of cross validation (SECV).

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**Matrix:** A two-dimensional data structure comprised of columns and rows used to organize inputs or outputs during chemometric analyses. Common practice is for each row to correspond to an individual sample and each column to correspond to an individual variable.

**Validation set:** The data set that challenges the performance attributes of the model. The validation data set is independent of the training data set, although testing on the training data set gives an optimistic view of performance and thus allows for iteration of preprocessing and model tweaking until this optimistic view meets the expectations. Validation or cross-validation is then necessary to adequately gauge the performance of the model. The model performance with the validation data set will always be equal to or worse than its performance with the training data set. If the performance attributes are not met, then the chemometrician must assess whether the model underfits or overfits the data and whether iterations of the model are needed to meet the acceptable error rate. If these efforts fail, it is recommended to back up one step, alter the preprocessing conditions, and perform the same task again.

## APPENDIX

### Additional Sources of Information

Many books have been written on the subject of chemometrics and multivariate analysis. Various terms not found in the glossary may be found in this short list of additional sources of information.

- Massart DL, Vandeginste BGM, Buydens LMC, De Jong P, Lewi PJ, Smeyers-Verbeke J, eds. *Handbook of Chemometrics and Qualimetrics: Part A.* 1st ed. Amsterdam: Elsevier Science B.V.; 1997.
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# ⟨1041⟩ BIOLOGICS

Products such as antitoxins, antivenins, blood, blood derivatives, immune serums, immunologic diagnostic aids, toxoids, vaccines, and related articles that are produced under license in accordance with the terms of the federal Public Health Service Act (58 Stat. 682) approved July 1, 1944, as amended, have long been known as "biologics." However, in Table III, Part F, of the Act, the term "biological products" is applied to the group of licensed products as a whole. For Pharmacopeial purposes, the term "biologics" refers to those products that must be licensed under the Act and comply with Food and Drug Regulations —Code of Federal Regulations, Title 21 Parts 600-680, pertaining to federal control of these products (other than certain diagnostic aids), as administered by the Center for Biologics Evaluation and Research or, in the case of the relevant diagnostic aids, by the Center for Devices and Radiological Health of the federal Food and Drug Administration.

Each lot of a licensed biologic is approved for distribution when it has been determined that the lot meets the specific control requirements for that product as set forth by the Office. Licensing includes approval of a specific series of production steps and in-process control tests as well as end-product specifications that must be met on a lot-by-lot basis. These can be altered only upon approval by the Center for Biologics Evaluation and Research and with the support of appropriate data demonstrating that the change will yield a final product having equal or superior safety, purity, potency, and efficacy. No lot of any licensed biological product is to be distributed by the manufacturer prior to the completion of the specified tests. Provisions generally applicable to biologic products include tests for potency, general safety, sterility, purity, water (residual moisture), pyrogens, identity, and constituent materials (Sections 610.10 to 610.15 and see *Safety Tests—Biologicals* under *Biological Reactivity Tests, In Vivo* ⟨88⟩, *Sterility Tests* ⟨71⟩, *Water Determination* ⟨921⟩, and *Pyrogen Test* ⟨151⟩, as well as *Bacterial Endotoxins Test* ⟨85⟩). Constituent materials include ingredients, preservatives, diluents and adjuvants (which generally should meet compendial standards), extraneous protein in cell-culture produced vaccines (which, if other than serum-originating, is excluded) and antibiotics other than penicillin added to the production substrate of viral vaccines (for which compendial monographs on antibiotics and antibiotic substances are available). Additional specific safety tests are also required to be performed on live vaccines and certain other items. Where standard preparations are made available by the Center for Biologics Evaluation and Research (Section 610.20), such preparations are specified for comparison in potency or virulence testing. The U.S. Opacity Standard is used in estimating the bacterial concentration of certain bacterial vaccines and/or evaluating challenge cultures used in tests of them. ( *General Notices, 5.50.10 Units of Potency (Biological)*.)

The Pharmacopeial monographs conform to the Food and Drug Regulations in covering those aspects of identity, quality, purity, potency, and packaging and storage that are of particular interest to pharmacists and physicians responsible for the purchase, storage, and use of biologics. Revisions of the federal requirements affecting the USP monographs will be made the subjects of *USP Supplements* as promptly as practicable.

## VEHICLES AND ADDED SUBSTANCES

Vehicles and added substances suitable for biologics are those named in the Food and Drug Regulations.

## CONTAINERS FOR INJECTIONS

Containers for biologics intended to be administered by injection meet the requirements for *Packaging and Storage Requirements* ⟨659⟩*, Injection Packaging*.

## CONTAINER CONTENT

The volumes in containers of biologics intended to be administered by injection meet the requirements for *Container Content for Injections* ⟨697⟩.

## LABELING

Biologics intended to be administered by injection comply with the requirements for *Labeling* ⟨7⟩*, Labels and Labeling for Injectable Products*. In addition, the label on the final container for each biologic states the following: the title or proper name (the name under which the product is licensed under the Public Health Service Act); the name, address, and license number of the manufacturer; the lot number; the expiration date; and the recommended individual dose for multiple-dose containers. The package label includes all of the above, with the addition of the following: the preservative used and its amount; the number of containers, if more than one; the amount of product in the container; the recommended storage temperature; a statement, if necessary, that freezing is to be avoided; and such other information as the Food and Drug regulations may require.

## PACKAGING AND STORAGE

The labeling gives the recommended storage temperature (see *Packaging and Storage Requirements* ⟨659⟩). Precautions should be taken where products labeled to be stored at a temperature between 2° and 8° are stored in a refrigerator, in order to assure that they will not be frozen. Diluents packaged with biologics should not be frozen. Some products (as defined in Section 600.15) are to be maintained during shipment at specified temperatures.

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## EXPIRATION DATE

For compendial articles the expiration date identifies the time during which the article may be expected to meet the requirements of the Pharmacopeial monograph, provided it is kept under the prescribed storage conditions. This date limits the time during which the product may be dispensed or used ( *General Notices, 3.10 Applicability of Standards*). However, for biological products, the stated date on each lot determines the dating period, which begins on the date of manufacture (Section 610.50) and beyond which the product cannot be expected beyond reasonable doubt to yield its specific results and to retain the required safety, purity, and potency (Section 300.3 (1) and (m)). Such a dating period may comprise an in-house storage period during which it is permitted to be held under prescribed conditions in the manufacturer's storage, followed by a period after issue therefrom. The individual monographs usually indicate both the latter period and (in parentheses) the permissible in-house storage period. If the product is held in the manufacturer's storage for a longer period than that indicated (in parentheses), the expiration date is set so as to reduce the dating period after issue from the manufacturer's storage by a corresponding amount.

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***Add the following:***

# ▲⟨1042⟩ CELL BANKING PRACTICES FOR RECOMBINANT BIOLOGICS

## 1. INTRODUCTION

This general chapter is intended to provide users with practical guidelines regarding the derivation, production, storage, characterization, and qualification of bacterial and mammalian cell banks used in the manufacture of biological drug products. The chapter is primarily focused on the characterization of cell banks used to produce biological products that are subject to approval by the US FDA. Cell banking characterization and qualification should be based on sound scientific principles and current regulatory guidance. The relevant requirements and guidances for generating cell banks for biotherapeutics seeking FDA approval include the US Code of Federal Regulations (21 CFR Part 610.18) (*1*), FDA Points to Consider documents (*2–4*), FDA Guidance for Industry documents, and International Council for Harmonisation (ICH) guidelines (*5–8*). These guidances state that the final biological product and production should be consistent, uniform, and free from cellular and adventitious contaminants. [NOTE—It is recognized that there are also relevant guidances from other international organizations such as the World Health Organization (WHO) and European Medicines Agency (EMA); however, this chapter focuses on US FDA, USP, and ICH regulations and guidances.]

Therefore, the intent of this chapter is to elaborate on principles noted in FDA and ICH guidances and in *USP* general chapters for the establishment and characterization of mammalian and bacterial cell banks used in the manufacture of biological products. This chapter represents current best practices for the production, characterization, and maintenance of controlled and consistent cell sources using risk-based and product-phase-appropriate quality considerations.

The evaluation of the risk to quality (*9*) should be based on scientific knowledge and ultimately link to the protection of the patient; the level of effort, formality, and documentation of the quality risk management process should be commensurate with the level of risk. Regarding good cell line banking practice, risk analysis[1] should be conducted on the basis of existing experience with the type of cell line and should be updated throughout the cell line's product life cycle as further knowledge is gained to characterize the associated risks. The risk criteria for good cell line banking practices should include such quality risk factors as:

- Cell line history and origin
- Cell line growth and characteristics (relevant phenotypic and genotypic markers)
- Cell count
- Viability
- Authentication
- Absence of adventitious agents (e.g., microbial, fungal, viral, and mycoplasma contaminants)
- Genetic drift (e.g., transgene stability)
- Loss of cell line characteristics of interest (such as the host cell transformations, surface antigens, or monoclonal antibody expression)
- Process validation and capability
- In-process controls
- Change controls
- Cross contamination
- Testing
- Stability
- Storage
- Product and/or process trending that demonstrates the original cell line characteristics and resultant quality characteristics of the final product that are retained throughout the cell line and product's life cycle

Over time and experience—through feedback from the quality systems, trending, post-marketing surveillance, and pharmacovigilance systems—the risk assessment should continue to evolve to minimize the risks associated with the cell line's performance during the product's life cycle.[1]

### 1.1 Scope

Cell banks are the starting materials for manufacturing biological drug substances and therefore have a direct impact on the characteristics and safety of the resultant biological products. Cell banks preserve characteristics in a contamination-free process and ensure consistency of the quality attributes of the final drug substance and drug product (DP). This chapter covers cell banking for biotechnology products for human use that are derived from cell lines of mammalian and microbial origin. In many instances, biological product development involves developing new recombinant prokaryote [e.g., *Escherichia coli* (*E. coli*)], eukaryote [e.g., Chinese hamster ovary (CHO)] or transgenic cell lines, and requirements may differ depending on the strain or cell line. Each new strain/cell line needs to be evaluated with respect to its own particular cellular characteristics and its associated master cell banks (MCBs) and working cell banks (WCBs) need to be manufactured under conditions consistent with good manufacturing practices (GMPs). Each GMP cell bank should be tested and characterized to satisfy quality standards for identity, purity, and genetic stability and ensured to be free from adventitious contaminants for use in drug substances for commercial applications. If possible, storage stability should also be performed. A risk-based approach should be applied for

[1] See ICH and appropriate FDA guidelines for more specific information.

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manufacturing. The bank can be conditionally released at risk before it is fully tested to start drug substance manufacturing; however, the bank has to be fully tested, especially for safety prior to a drug substance's release for clinical applications.

This chapter does not cover cell banking for antibiotics, viral seed banks, viruses that express vaccine antigens, bacterial seed banks, avian or insect cells, or cell banks for cell and gene therapies. For specific information on cell banking for vaccines, see *Vaccines for Human Use—Bacterial Vaccines* ⟨1238⟩ and *Vaccines for Human Use—General Considerations* ⟨1235⟩.

This chapter primarily focuses on cell banks derived from recombinant *E. coli* strains and CHO cell lines as they are the most common substrates for production of therapeutic proteins and centers on the cell line or strain history, GMP manufacturing and testing, and qualification of cell banks. Most, if not all, of the techniques and practices discussed are also applicable to other cell strains besides *E. coli* and CHO cell substrates. Based on the organism, suitable approaches can be selected to cover the cell line/strain history, GMP manufacturing and testing, as well as qualification of cell banks.

## 2. GENERAL PROCESSES AND CONSIDERATIONS OF CELL BANKING

Cell banks used for manufacturing biological drug substances are usually prepared from research cell banks (RCBs) and use a two-tiered bank system, MCB and WCB or working seed lot. The MCB is generated as the source for the WCB, which is derived by expanding 1 or more vials of the MCB. Thus, the MCB is considered the starting raw material for the GMP manufacturing of drug substances. RCBs are usually generated under non-GMP conditions, whereas MCBs and WCBs are required to be produced in GMP facilities. Documentation of suitability of the RCB for GMP manufacture is needed. *Figure 1* outlines the general practices and considerations of cell banking processes and how they evolve through development into GMP manufacturing. The critical steps shown in the figure are applicable to recombinant and non-recombinant cell lines. More detailed best practices and recommendations are described in the sections that follow. For more detailed recommendations about preparation and preservation of cell banks, see ICH Q5D (*7*).

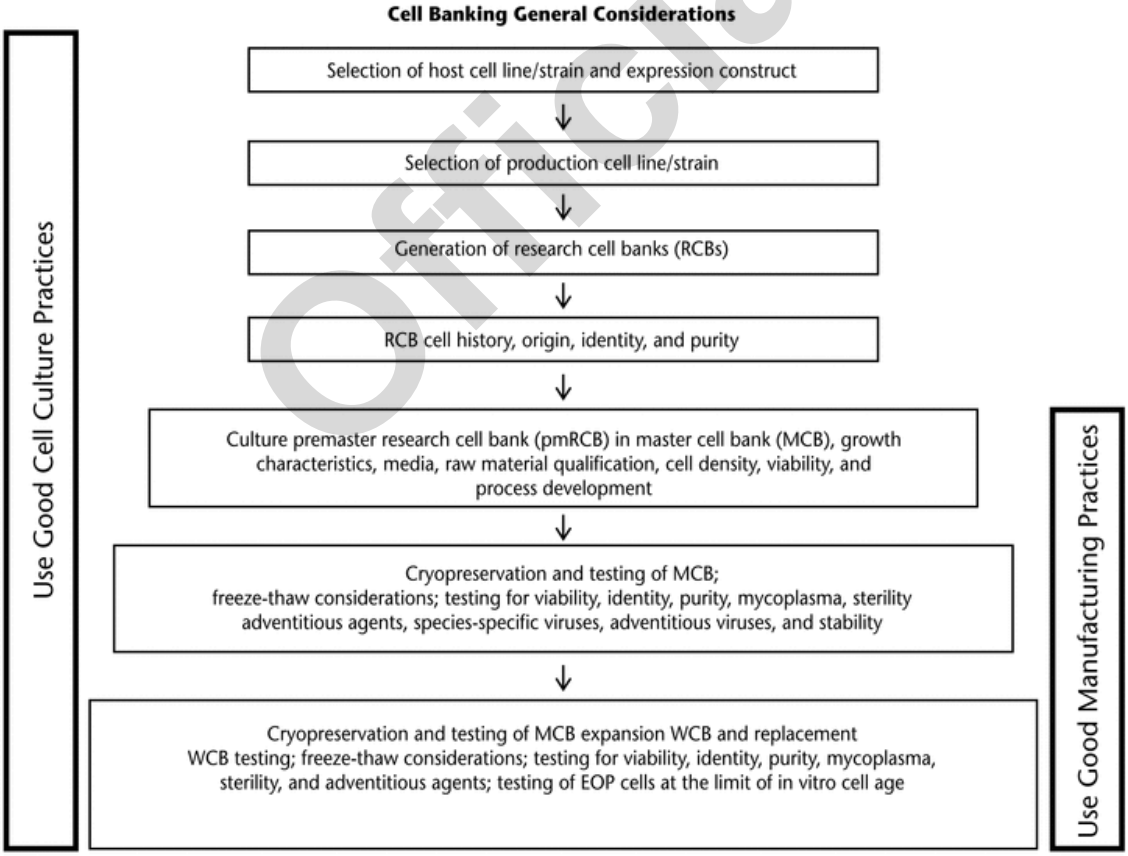


Figure 1. Quality aspects of cell banks for biologics.

The generation of an RCB starts with choosing a suitable expression system that consists of host cells and expression constructs carrying the genes of interest (GOI).

Any facility including a research laboratory should be regarded as a potential source of contamination by viruses, bacteria, fungi, and mycoplasma, so the introduction of a new cell line should be handled under quarantine conditions until proven negative for such contaminants. The history, identity, and purity, as well as associated testing of the host cells, should be well documented (*10*).

An expression construct consists of the GOI and an expression vector carrying the genetic elements such as a promoter, signal sequences, selection markers, and other elements needed for the stable expression of the GOI in the host cells. Detailed

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descriptions of the GOI and those genetic elements carried by the vectors, as well as the steps in the assembly of the expression construct, should be documented.

The next step for RCB is the generation of the candidate production cell lines/strains. It is critical to document the details of the cell-line generation history, including:

- Description of the transfection/transformation of the GOI
- Screening and selection of the production cell lines/strains
- Demonstration of clonal derivation
- Information about the raw materials used for the generation of candidate production cell lines

The source, origin, generation, propagation, and selection of the cell line must be documented and described in detail.

Multiple RCBs may be generated in relatively small quantities during the screening/selection processes. Only the final, chosen production cell lines/strains and backup cell lines/strains are made in larger quantities. The RCBs chosen for the production cell lines/strains and backup cell lines/strains are sometimes referred to as premaster research cell banks (pmRCBs), which need to go through purity testing prior to the production of MCBs and subsequent WCBs.

Cell substrate for manufacturing biological drug substances, MCBs, and WCBs must be produced in compliance with current GMPs (cGMPs). As illustrated in *Figure 2*, MCBs are often used for manufacturing drug substances for early clinical studies and as the source of the WCBs, which may not be available during the early phase of the development life cycle. Drug substances used for late-phase development and commercial applications are usually manufactured from WCBs. To ensure a consistent supply, it is generally recommended that additional replacement WCBs be produced as needed once a biologic is approved for marketing. Section *5. Cell Bank Characterization* describes in detail cell bank characterization, testing and qualifications, as well as general considerations on raw materials, cell bank cryopreservation, storage and stability, and transportation.

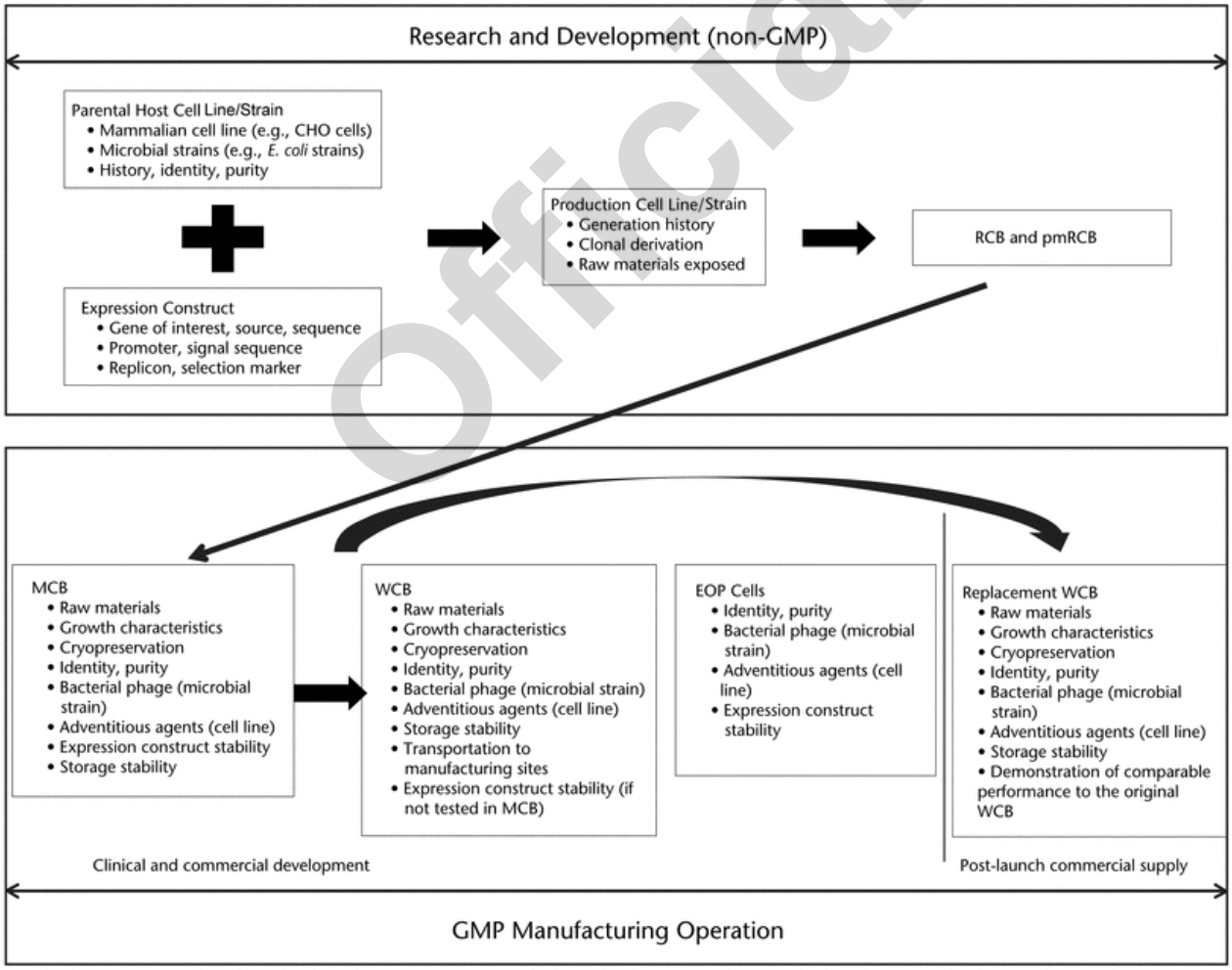


Figure 2. General processes and considerations for cell banking.

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# 3. PRODUCTION CELL LINE/STRAIN DEVELOPMENT AND RCB GENERATION

## 3.1 Common Cell Line Types and Associated Expression Constructs

Production cell line/strain development starts with choosing a suitable expression system including a host cell and an associated expression construct carrying the GOI. The choice of the host cell type for production of a recombinant protein is often a function of the molecular complexity of that protein. Prokaryotic production systems are often dedicated for simple, small proteins whereas eukaryotic expression systems are preferred for larger, more complex molecules because eukaryotic expression systems can perform necessary post-translational modifications for a recombinant protein to be functional in vivo. In addition, eukaryotic expression systems can secrete the recombinant protein outside the cell, which makes purification easier than, for instance, isolation of recombinant proteins from inclusion bodies formed in prokaryotic systems. Mammalian cell lines such as CHO cells, non-secreting (NS0) mouse myeloma cells, and human embryonic kidney (HEK) cells and microbial strains such as *E. coli* and *Bacillus* are examples of cell types that have been used to generate production cell lines/strains. This chapter focuses on mammalian and bacterial cells because they are currently the predominant cell substrate types used for recombinant therapeutic production.

### CHINESE HAMSTER OVARY CELLS

**General information:** CHO cell lines are several related, but unique, cell lines originally derived from a clonal and spontaneously immortalized CHO cell line isolated in 1957. CHO cells were widely used in molecular and classical cell genetics studies until the 1980s. The knowledge derived from those studies facilitated the migration of CHO cells from research laboratories to industrial bioreactors. In 1986, CHO cells became the first mammalian cell-derived cell substrate used to produce complex proteins for human use and since then have remained the most common cell line for the production of therapeutic glycoproteins for the following reasons:

1. CHO cells pose a lower adventitious virus safety risk as only a few human pathogenic viruses are able to propagate in CHO cells (*11*).
2. CHO cells grow well in animal-component-free, chemically defined media, which further minimizes adventitious virus safety concerns.
3. As immortal cells, CHO cells can be propagated for a long time in vitro. This is an important characteristic for commercial production of recombinant proteins because it takes many cell passages to progress from cell line generation through cell banking to the final, large-scale commercial production process. A non-immortalized cell line cannot be propagated long enough to generate a profitable and sustained commercial production process.
4. CHO cells are able to adapt and grow in suspension culture, which is ideal for large-scale manufacturing of biologics.
5. CHO cells have the capacity for efficient, post-translational modifications, such as glycosylation, which enable the CHO-derived therapeutic proteins to be both compatible with and bioactive in humans.
6. CHO cells are amenable to genetic manipulation, which allows for easy introduction of recombinant DNA resulting in expression/secretion of large quantities of the desired protein.

See *Table 1* for a comparison of two common cell substrates for recombinant therapeutic products.

**Table 1. Comparison of Two Common Cell Substrates for Recombinant Therapeutic Products**

| Cell Substrate | Mammalian Cell Lines (e.g., CHO Cell Lines) | Microbial Strains (e.g., *E. coli* Strains) |
|---|---|---|
| Advantages | • Recombinant proteins are soluble and are secreted in cell culture medium<br>• Secreted recombinant proteins are properly folded and are identical in higher order structure to the natural human protein<br>• Conformational and post-translational modifications in these proteins are similar to the types found in humans | • Fast growing<br>• High protein yield/low cost<br>• Easy transformation and fermentation<br>• Do not harbor mammalian viruses<br>• Simpler cell bank characterization |
| Disadvantages | • Slow growing<br>• Degree of downstream processing and validation<br>• Higher cost (complex media; testing of cell banks; downstream processing and validation, including virus removal)<br>• Safety concerns (adventitious agents from cells and media components)<br>• Concerns about genetic stability, protein folding, and culture conditions including cell viability and growth rates | • Recombinant protein may be insoluble and may aggregate in inclusion bodies<br>• Specific issues with solubilization, refolding, and appropriate disulfide bonding in inclusion bodies<br>• No cellular machinery for mammalian protein processing and post-translational modifications<br>• Potential for product degradation due to trace protease impurities<br>• Lysis of bacteria is often necessary for recovery of desired protein<br>• Require endotoxin removal during purification<br>• Expressed proteins may be toxic to *E. coli*<br>• Require N-terminal methionine for optimal translation that may not always be removed by *E. coli* proteolytic systems |

Because CHO cells are immortalized, they can pose a significant challenge, though, for the cell-line development process with respect to finding genetically stable cell lines needed for consistent commercial manufacturing processes. CHO cells have varied ploidy, and a culture of any CHO cell line, with or without any transgenes, consists of a population of cells that exhibit both genetic and phenotypic diversity and instability under continuous cultivation. Random integration of recombinant genes into the CHO genome adds new genetic variability to the CHO cell population. After transfection, implementation of a single-cell

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cloning step helps to minimize cell population heterogeneity by removing genetic variations that either existed prior to or were introduced during transfection.

All CHO cell substrates used for commercial recombinant protein production are expected to be single-cell derived. However, incorporation of the single-cell cloning step alone cannot prevent genetic and phenotypic heterogeneity within the population of cells that arises post-cloning, as these events are inherent to the use of immortalized mammalian cell lines (*12*). Thus, identifying an appropriate production cell line ultimately depends on the genotypic and phenotypic characterization of potential cell lines post-cloning at the limit of in vitro cell growth and the evaluation of the expressed product.

**Common CHO cell lines:** Some commonly used CHO cell lines include: CHO-DXB11, CHO-DG44, CHO-K1, and CHO-K1SV. These cell lines are transfected with expression plasmids carrying the GOI and corresponding selection markers specific to the CHO host cells. Their characteristics are briefly summarized below.

*CHO-DXB11 and CHO-DG44:* CHO-DXB11 (also known as CHO-DUK-XB11 or CHO-DUKX) and CHO-DG44 are dihydrofolate reductase (DHFR)-deficient mutants established in the early 1980s. CHO-DXB11 cells carry a deletion of one locus for DHFR and a missense mutation (T137R) of the second DHFR locus, rendering the cells incapable of reducing folate, a precursor for thymidine and hypoxanthine synthesis; CHO-DG44 cells carry the full deletion of both DHFR loci.

The DHFR-deficient cells are triple auxotrophs for glycine, hypoxanthine (a proline derivative), and thymidine. Introduction of the GOI into CHO cells can be accomplished by cotransfection with a functional DHFR gene, which obviates the need for these nutrients. Recombinant cell lines carrying the integrated GOI are selected in media devoid of those three nutrients. The GOI expression level can be further enhanced by gene amplification with methotrexate (MTX), an antagonist of DHFR. Selection of recombinant cell lines can be performed using stepwise increases in the media concentration of MTX, which results in amplification of the transfected DHFR gene together with the GOI, possibly increasing the productivity of the GOI.

Sometimes MTX-induced gene amplification can result in large genomic reorganizations. New chromosomal structures, known as homogeneously staining regions (HSRs), can be found in the metaphase chromosome of MTX-selected CHO cells. These regions show multiple repetitions—up to thousands of smaller chromosomal regions (amplicons)—all containing DNA encoding at least in part sequences with DHFR activity as well as hundreds and even up to thousands of copies of the GOI. Gene amplification and high-level expression of a transgene product can challenge the cells with potential toxicity and metabolic burden. Additional DNA rearrangements within the GOI region may also occur during long-term subculturing in the absence of MTX, which may eventually lead to instability of the recombinant protein production. Therefore, genetic stability evaluation of the chosen CHO production cell line is important to ensure that the desired characteristics for the commercial production are acquired.

*CHO-K1 and CHO-K1SV:* CHO-K1 was derived as a sub-clone of a parental CHO cell line lacking the proline synthesis gene. CHO-K1 was adapted to grow in suspension culture in protein-free medium, resulting in the cell line CHO-K1SV. Introduction of a GOI into the CHO-K1SV cells can be accomplished by cotransfection of the GOI with a functional glutamine synthetase (GS) gene followed by selection in the absence of glutamine. In addition, application of a GS inhibitor [methionine sulfoximine (MSX)] allows an increase in the stringency of selection. Although the copy number of the GOI and GS is significantly lower in the GS-based selection system compared to that seen in the DHFR-based system, instability of the GS-based CHO cell lines is still often observed during long-term cultivation. Therefore, it is important to evaluate genetic stability of the chosen CHO production cell line over the proposed in vitro cell age needed for commercial manufacturing.

**Expression vectors for CHO cell substrate:** Expression vectors used for CHO-based cell substrates consist of multiple components that contribute to the successful expression of the therapeutic proteins. These components include:

- The GOI and associated transcriptional and translational genetic elements (e.g., promoters, enhancers, Kozak translation initiation sequences, leader sequences, and polyadenylation signals)
- Selection markers such as the commonly used DHFR or GS for recombinant cell line selection
- Selection markers such as the β-lactamase gene (*bla*) and replicons such as ColE1 for propagation of the plasmid in *E. coli*

Expression plasmids are often linearized prior to transfection and are generally integrated into the CHO host cell genome by random integration. Resulting transfectants are a mixture of CHO host cells carrying variable copy numbers of expression plasmids randomly inserted in single or multiple loci in the host genome. A single-cell cloning process enables the clonal derivation of future production cell lines. Significant effort is spent on screening the clonally derived transfectants to identify candidates for final production cell lines that possess high productivity. Furthermore, the identified cell lines must be capable of maintaining the integrity and stability of the expression construct integrated into the host genome in order to be used for large-scale manufacturing processes.

To reduce the heterogeneity associated with traditional random integration approaches, new expression systems have been developed to facilitate targeted integration of the GOI into a specific desirable locus within the CHO genome. Targeted integration systems described in the literature include site-specific recombinases, integrases (ØC31), transposases (piggyBac), zinc finger nucleases, transcription activator-like effector nucleases (TALENs), and CRISPR-Cas RNA-guided nucleases. Detailed descriptions of the genetic components carried by the expression plasmids, the specific integration method, and identification of the preferred production cell line should be included in the history of the final production cell line.

**CHO host cell engineering:** In addition to expressing the GOI, recombinant CHO cell lines can be engineered to further enhance productivity and quality of the recombinant product as well as the robustness of the cell culture performance. Host engineering includes overexpression or knock-out of specific genes that may impact the desired recombinant product characteristics and thereby the performance of the production cell line. Examples of overexpressed genes are those involved in:

- Glycosylation [e.g., α-2,6-sialyltransferase (ST6Gal)]
- Cellular metabolism [e.g., vitreoscilla hemoglobin (VHb)]
- Anti-apoptotic pathways [e.g., B-cell lymphoma 2 (BCL2)]
- Pro-proliferative pathways [e.g., valosin-containing protein (VCP)], as well as genes that may enhance cellular protein biosynthesis machinery and secretion [e.g., transcription factor ATF4, heat shock 70 kDa protein 5, binding immunoglobulin protein (BiP), etc.]

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Alternatively, there are some host cell proteins that can decrease the quality or yield of recombinant proteins [e.g., α-1,6-fucosyltransferase (FUT8) and peptidylglycine α-amidating monooxygenase]. In these cases, genomic knockouts of the host cell genes can yield a more homogeneous product.

Although the overexpressed or knocked out genes do not encode sequences for the final therapeutic protein, they play critical roles in the productivity and quality of the expressed therapeutic proteins. Therefore, evaluation of the integrity and stability of the overexpressed genes for the intended purposes should be included as a part of the cell substrate genetic stability characterization. In addition, knocked out genes should be characterized.

## *ESCHERICHIA COLI*

**General information:** *E. coli* is one of the best-understood organisms in terms of genetics, physiology, and biochemistry.

The key advantages of the *E. coli* system include:

- The GOI can be easily inserted into an expression plasmid using recombinant DNA technology. The resulting recombinant plasmid, which is usually small and simple, can be inserted into the *E. coli* host by transformation and maintained as an extrachromosomal segment of the host DNA or a piece of the host genome after integration. Numerous *E. coli* expression systems are commercially available, making this organism easy to test for production of a new GOI.
- *E. coli* is well-characterized genetically with versatile comprehensive engineering tools.
- *E. coli* strains are capable of rapid growth in inexpensive media in small shake flasks as well as in large-scale fermenters used to produce therapeutics with high yield and quality. *E. coli* expression systems continue to evolve, and the advances in biotechnology and bioengineering as well as high-density fermentation technology have further enabled *E. coli* systems to produce a variety of complex proteins, including antibody fragments and Fc-fusion proteins.

However, *E. coli* strains also have limitations as cell substrates for therapeutics production, including their inability to perform certain post-translational modifications. In addition, endotoxins need to be thoroughly removed during purification of the recombinant protein produced by an *E. coli* host (also see *Table 1*).

Sometimes the recombinant protein itself can be an obstacle for production in *E. coli* systems because it can be toxic to the host. In such cases, a well-designed but more sophisticated expression system may be required, for instance, an inducible promoter to produce the recombinant protein at the end of cell expansions. In the following sections, the characteristics of some *E. coli*-based cell substrates consisting of *E. coli* host strains and vectors carrying the genes encoded for the therapeutic proteins are briefly summarized.

***E. coli* host strains:** The most commonly utilized strains are the K12 strain and its derivatives including *E. coli* RV308 and W3110, which are all genetically modified so they cannot colonize a human host or produce toxins. These strains may qualify for an exempt status according to the requirements of the National Institutes of Health (NIH) guidelines for research involving recombinant DNA molecules.

The other commonly used *E. coli* hosts are the B strain and its derivatives, such as BL21, C41, and C43. Although these strains do not qualify for exempt status and require full Institutional Biosafety Committee (IBC) review, they are still desirable hosts for therapeutic protein production in the industry because they are able to express various types of proteins. Advantages of the B strain and its derivatives are the low levels of acetate accumulation under high glucose concentrations, the specific protease deficiencies, and the high outer membrane permeability.

**Expression vectors for *E. coli* strains:** The expression vectors often used are the result of multiple combinations of replicons, promoters, selection markers, and other genetic elements that are essential or beneficial for the desired efficiency of transcription, translation, and protein secretion, as well as the stability of the plasmid.

A replicon in a vector consists of one origin of replication together with its associated cis-acting control elements. It controls the copy number of a plasmid replicated in an *E. coli* host. Commonly used replicons include:

- pMB1 and its derivative origins used in pUC-based vectors (500–700 copies/genome)
- pBR322-based vectors (10–20 copies/genome)
- p15A origin used in pACYC and pSC101, which have low copy numbers (about 5 copies/genome)

For large-scale therapeutic protein production, an optimal copy number should be identified because too few gene copies may result in low levels of mRNA leading to low protein productivity while too many copies may impose metabolic burdens on cells. In addition, high-copy-number plasmids have been shown to possess higher segregation instability, especially in the absence of selective markers. This can lead to the overgrowth of plasmid-free cells, resulting in a significant loss of protein productivity. Therefore, it is usually not desirable to use high-copy-number plasmids, and it is critical to monitor plasmid retention during large-scale production. Testing to qualify the microbial cell bank includes testing to demonstrate purity and freedom from phage contamination and to confirm strain identity. Additional testing includes viability, plasmid copy number, plasmid retention, and restriction enzyme mapping.

Promoters control and regulate the efficiency of transcription. Commonly used promoters include:

- *lac* and its derivatives such as *lac* UV5, *tat* and *trc*, which are induced by isopropyl-β-D-thiogalactopyranoside (IPTG)
- Bacteriophage λ $p_L$ and/or $p_R$ promoters, which are induced by temperature shift via the regulation of the temperature-sensitive cI857 repressor
- T7 promoter, specifically recognized by T7 bacteriophage RNA polymerase from T7 bacteriophage
- *phoA*, *trp*, and *ara* $P_{BAD}$, which are nutritionally inducible promoters

Selection markers, usually antibiotic-resistant genes such as TetR, are included in the plasmid to determine the growth of plasmid-free cells. Therefore, antibiotics are usually included in the growth media. Some common examples include neomycin, polymyxin B, streptomycin, and gentamicin. However, the use of antibiotics in clinical manufacturing can lead to adverse events in some patients and raises concerns over the development of drug-resistant pathogens, so it is desirable to develop antibiotic-free plasmid systems, if possible.

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In addition to the coding sequence encoding the protein, genetic elements in expression vectors that impact protein expression include the sequence and structure of the translation initiation region (TIR) at the 5′ end of each mRNA and the stability (half-life) of mRNA.

Another genetic element used in some expression vectors is the signal sequence fused to the N-terminus of the protein, which leads the protein to the periplasmic space of the host or the cell culture medium. Without signal sequences, recombinant proteins mainly accumulate in the bacterial cytoplasm. Due to the lack of glycosylation and disulfide-bond formation, many human therapeutic proteins expressed in *E. coli* tend to aggregate and form inclusion bodies due to misfolding. Correct folding of eukaryotic proteins containing multiple disulfide bonds is more likely to occur in the oxidative environment of the periplasm. The secretory process allows removal of the N-terminus signal peptide and yield of the authentic N-terminus of the expressed protein. Co-expression of periplasmic chaperones, fusion with secretory partners, and modification of the outer membrane structure by host engineering may further promote release of periplasmic proteins.

Overall, *E. coli* expression systems have been preferred choices as cell substrates for the expression of non-glycosylated therapeutic proteins due to their well-characterized genetics, rapid growth, and high-yield production capability. Both the host strains and the genetic elements carried by the expression vectors should be well described in the strain development histories and documented for the cGMP cell bank generation. Together with the therapeutic protein-coding sequence, the key genetic elements that may potentially impact the drug substance quality and the consistency of drug substance production should be evaluated during cell bank characterization and qualification.

**Clonal selection of *E. coli* host strains:** As with CHO-derived cell lines, it is expected that *E. coli* strains used for therapeutics production be clonally derived. Microbial clonality is easily achieved and accepted by picking transformants from well-isolated single colonies grown on solid media. Additional rounds (usually two) of single-colony isolation and phenotypic confirmation may be performed to ensure the clonality.

*Table 1* compares two commonly used mammalian cell and microbial strain substrates for generating cell banks for biological products.

*BACILLUS*

Other useful bacterial systems are Gram-positive bacilli, including *Bacillus megaterium*, *B. subtilis*, *B. licheniformis*, and *B. brevis*. *Bacillus* is very often considered an attractive alternative to *E. coli* for the following three reasons:

1. *Bacillus* strains usually secrete proteins
2. *Bacillus* strains are suitable for high-density cultures in fermenters
3. Most *Bacillus* strains lack membrane proteases

In addition, the genomes of *B. subtilis* and *B. licheniformis* have been sequenced, and there is no production of harmful exotoxins or endotoxins. With these bacterial systems, secretion of the desired proteins into the fermentation medium results in easy downstream processing, thereby eliminating the need for cell disruption or chemical processing techniques. Thus, the recovery is relatively efficient and cost effective. Host strains used for successful expression of recombinant proteins are often deleted for the genes *amyE*, *aprE*, *nprE*, *spoIIAC*, and *srfC* and are easily transformed. These bacteria are often preferred for homologous expression of enzymes such as proteases and amylases.

## 3.2 Production Cell Lines/Strains and Recommended Documentation

A production strain/cell line refers to the transformed strain or transfected cell line with the expression plasmid. The history of the production strain/cell line generation is an essential part of cell banking documentation and should include the following:

- Raw materials used
- Expression plasmid
- Host strain/cell line
- Generation of the production strains/cell lines
- Generation of RCB/pmRCB

This information should enable an overall evaluation of the risks associated with the use of the cell substrate derived from the strain/cell line and a detailed analysis of potential entry routes for adventitious agents into the MCBs.

DESCRIPTION OF EXPRESSION CONSTRUCT

The description of the expression plasmid used to generate the production cell line should include details on the origin of the coding genes used for the recombinant protein as well as all other genetic components carried by the plasmid. Additionally, the steps in the assembly of the expression construct should be provided. The recommended details are described below.

The name and the origin of the nucleotide sequence coding for the protein should be provided with a brief summary of the rationale for the development efforts. It is important to include descriptions of the engineering methods used to optimize the protein functions and biochemical/biophysical properties along with figures displaying the engineered residuals compared to the original sequences. For example, if the gene is for a therapeutic antibody (including bi-specific antibodies and Fc-fusion proteins), literature references to the constant regions/Fc regions such as the publicly known gene identification should be provided. In addition, if any particular mutations or residual substitutions in the constant regions have been performed, then these should also be provided with a rationale for such modifications. It is recommended that the well-annotated DNA sequence as well as the deduced amino acid sequence of the GOI be provided.

For example, for a therapeutic antibody, the annotations include the indications of the complementarity-determining regions (CDRs), variable and constant regions, linkers, signal sequences, and the sequence modifications applied to the molecule.

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The steps in the GOI assembly from the original sources to the final expression plasmid should be described in detail and are often summarized using a flow diagram. In addition, the source and function of all genetic components carried by the expression plasmid should be provided. The components include:

- The GOI and associated transcriptional and translational genetic elements (promoters, enhancers, Kozak translation initiation sequences, leader sequences, and polyadenylation signals)
- Selection markers such as DHFR or GS for recombinant cell line selection, or antibiotic resistance genes for recombinant strain selection
- Selection markers such as the *bla* gene and the replicon such as ColE1 for propagation of the plasmid in *E. coli*
- Any other expressed proteins encoded by the plasmid

Furthermore, a well-annotated plasmid map clearly indicating the size of the plasmid, all genetic components described above, and the restriction enzymes used for major cloning steps and for genetic stability characterization should be provided. The DNA sequence of the whole construct/plasmid, or at least the sequences encoding the recombinant protein that have been confirmed by DNA sequencing, should be included.

## PARENTAL *e. coli* STRAIN/CHO CELL LINE

A parental strain/cell line is a cell line from which the MCB will be derived following introduction of the expression construct. The origin of the host cells should be stated, together with a detailed record of their history, with relevant references from the scientific literature. This includes classifications such as genus, species, and strain designation as well as specific phenotypic and/or genotypic traits. Three key aspects of information should be provided:

1. Any biohazardous components that are produced by the parental cells
2. Any exposure of the parental cells to materials of human or animal origin such as serum, enzymes, hydrolysates, or other living cells
3. Genetic and phenotypic characteristics of the parental cells that may enhance the evaluation of the safety and characteristics of the subsequently derived cell banks

The lineage of the parental cells traced back to the original source or related literature references should be provided. If the parental cells undergo additional genetic modifications (e.g., targeted gene knockout or overexpression) that are aimed at impacting the quality of the future recombinant proteins, then the methodology and characterization results should be described in detail.

Characterization of the initial cell line should take risk into consideration. Use of low-risk cells (e.g., cells shown to be sterile and phage- or mycoplasma-free) is recommended. Cell line strain ID can be assessed with polymerase chain reaction (PCR) or using short tandem repeat (STR) profiling or DNA fingerprinting to verify the identity of the host cells. When higher-risk cells (e.g., a mixture of cells with varying degrees of genetic instability) are used, a more thorough screening is necessary, unless the supplier of the cells can provide documentation that minimizes the associated risk.

## GENERATION OF THE PRODUCTION CELL LINES/STRAINS

Generation of the production cell lines/strains usually consists of three steps:

1. Transferring the expression plasmid to the parental cells and selecting recombinant strains/cell lines
2. Single-cell cloning to produce clonally derived recombinant strains/cell lines
3. Choosing the production strains/cell lines through screening/characterization

Recommendations for documentation to include in regulatory submissions are as follows:

- Details on transformation/transfection conditions
- Manipulations of the plasmid DNA prior to the transfer (e.g., linearization by restriction enzymes often applied prior to transfection)
- Procedures/methods used for the selection of recombinant strains/cell lines
- Detailed description demonstrating the single-cell derivation of the production strain/cell line
    - For an *E. coli* strain, 2 or more rounds of single-colony streaking are commonly applied to ensure the clonal derivation of the production strains
    - For CHO cell lines, numerous approaches can be used to achieve clonal derivation (see *4. Mammalian Cell Line Clonality*)
- Description of how the production strains/cell lines were screened and identified including the following three parts:
    1. Detailed cell culture conditions
    2. Selection criteria
    3. All raw materials and reagents used throughout the generation process

It is helpful to provide a flow diagram (see *Figure 3*) of the production strain/cell line generation process, which includes the above three parts and ends with the MCB and WCB production.

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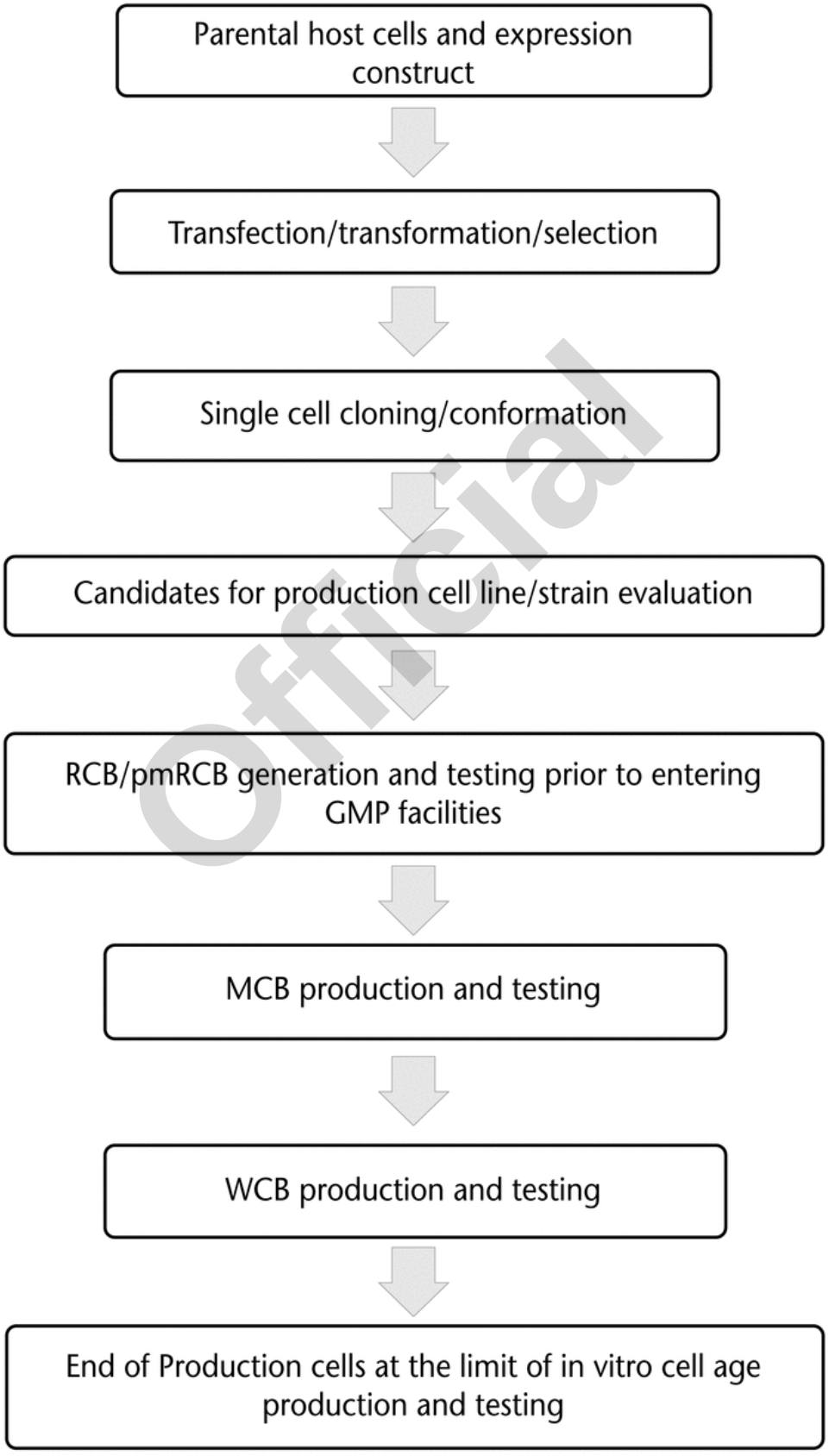


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Figure 3. Cell line production flow diagram.

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## GENERATION AND CONSIDERATIONS OF RCB/PMRCB

A common practice is to bank multiple potential production strains/cell lines in small quantities to generate RCBs or MCB as appropriate. Once the final production strains/cell lines are identified, the corresponding RCBs are thawed and expanded to generate the pmRCB. Descriptions of the cell culture medium/cryopreservation processes used to generate the pmRCB should be provided.

Because the RCB or pmRCB is usually generated in a non-GMP facility, it is recommended that RCBs/pmRCBs from mammalian cell lines should be tested and found sterile for contaminants and negative for mycoplasma before transfer to a GMP facility for the generation of the MCB. Appropriate qualification testing of RCB/pmRCB is recommended to be performed prior to moving into GMP. For an *E. coli*-derived RCB/pmRCB, testing for purity, identity, and bacteriophages is recommended to be performed before entry to a GMP facility. In addition, post-banking evaluation of the RCB/pmRCB for growth, viability, and viable cell density should be provided to aid MCB production and characterization. The MCB could be prepared by expansion of the production cell line over a suitable number of passages, dependent on the cell line. When the cell culture has reached an adequate cell density and total number of viable cells, the culture is prepared for freezing.

Numerous considerations for cell cryopreservation and management can be found in *Cryopreservation of Cells* ⟨1044⟩. Mammalian cells can be propagated in fermenters, roller bottles, shaker flasks, or wave bags and are frozen in a controlled process to optimize viability upon thawing. Typically, the cells are carefully mixed with a low concentration of dimethyl sulfoxide (DMSO) in cell culture media and/or other ingredients appropriate for that specific cell strain. The mixture is frozen by lowering the temperature over an appropriate time period to avoid cell osmotic shock, which can lead to disruption of the cell membrane. The vials must be labeled in advance with the cell bank lot number, tube number, and production date. After the carefully controlled freezing process, the new cell bank is moved to appropriate storage conditions. To avoid any unexpected loss of the cell bank, the containers are typically kept in at least two different geographical locations.

Post-banking controls after thawing include measuring cell number and viability. Because these parameters vary between individual cell banks, appropriate post-banking quality control for viability can be established and justified for each bank.

After the production of an MCB or WCB, each bank must be tested against specific acceptance criteria before that specific cell bank can be released for use. Mammalian cell banks should be stored in multiple geographical locations. *Figure 4* shows an example of a cell process for a mammalian (CHO) cell bank.

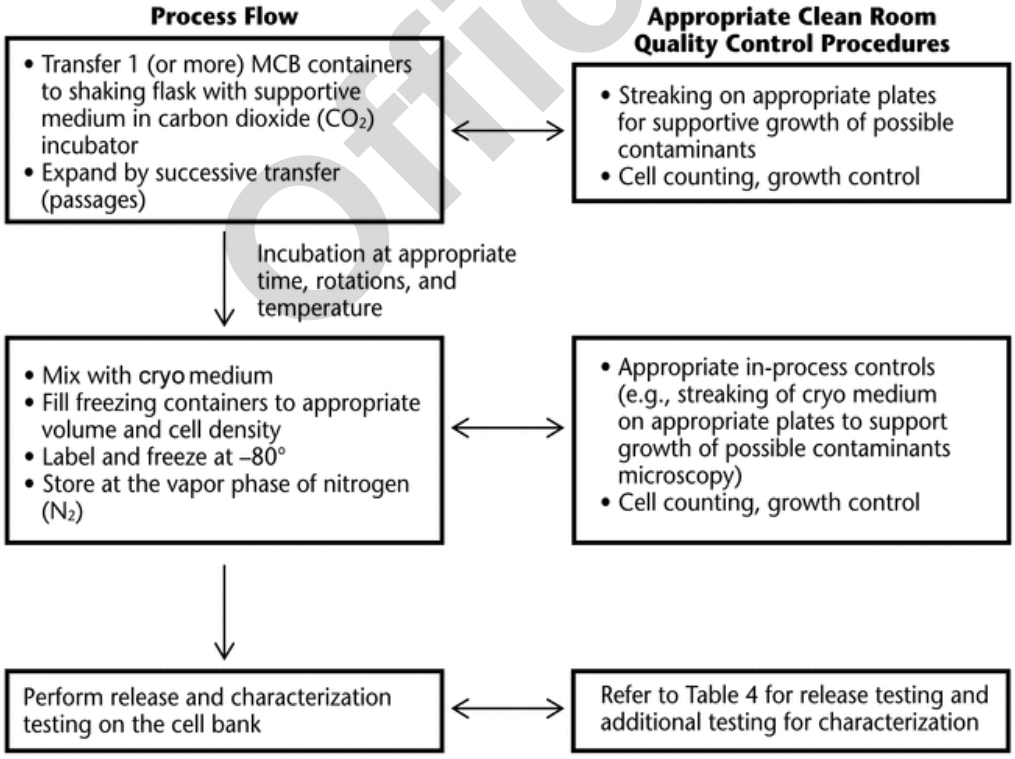


Figure 4. Example of mammalian (CHO) cell bank process.

Similarly, for bacterial cell lines used for therapeutic protein manufacturing, the chosen clone is subjected to growth propagation experiments to ensure that the best growth conditions are chosen for this specific clone. The growth conditions for an *E. coli* clone can be evaluated using shake-flask culturing. The optimal cell density is established by following growth

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curves in order to harvest the cells at an exponential growth. The cells are harvested in vials containing sterile 20%–30% glycerol under sterile conditions. *E. coli* cells harvested at a living cell density of $10^6$ to $10^{10}$ are adequate for production; however, this depends on the specific strains and selection pressure chosen.

All viability tests should be done with care, keeping in mind the actual selection system as well as how the harvest of any produced protein will be performed (e.g., from inclusion bodies). This is not a concern for the frozen *E. coli* cells after thoroughly mixing with glycerol; however, without adequate knowledge of the optimal growth point for harvesting and freezing as well as the actual needed cell density, the viability and expected growth rate can create problems at the production site.

The optimization process shall be described and followed in detail each time a new growth of the strain is necessary. From these optimized conditions, the first cell bank will be produced, which can be used for research purposes, followed by the production of an MCB. For the general process flow or production of a microbial WCB, see *Figure 5*.

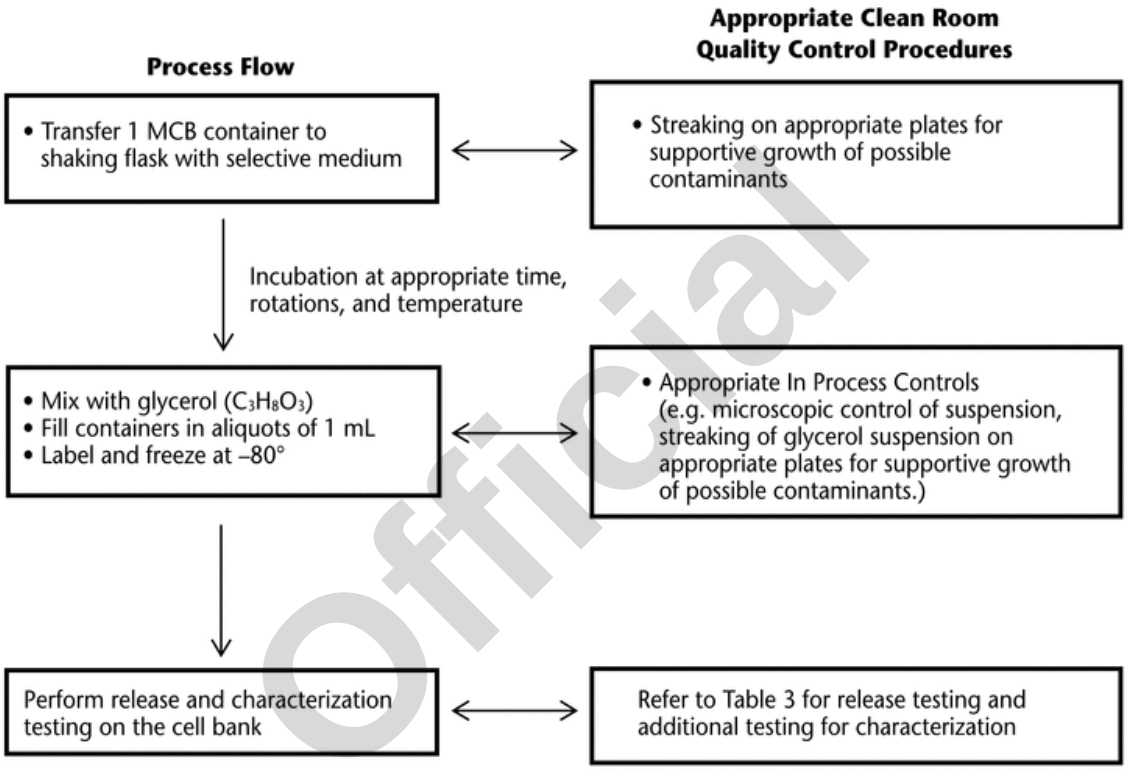


Figure 5. Example of an *E. coli* WCB batch production process.

As illustrated in *Figure 5*, in-process control testing is performed by checking the culture broth under a microscope before the filling of containers. After filling, the suspension of culture broth and glycerol (or DMSO) is checked for contamination by monitoring growth on appropriate plates. The containers are labeled individually with batch number, cell bank identification, and container number and then filled. The WCB containers are kept frozen under well-defined and controlled conditions.

## ADDITIONAL CONSIDERATIONS

During development, the production strains/cell lines used for manufacturing clinical materials may continue to evolve to meet the commercial manufacturing needs. For example, new expression plasmids or new parental cells (or both) may be used to generate higher-yielding production strains/cell lines for commercial applications. Additional testing considerations may be needed when retroviruses (e.g., lentiviruses) are used to insert the gene of interest. The new cell lines may be from a new species (e.g., a change from NS0 to CHO) or from the same species with additional genetic modifications to improve the cell culture performance and/or the quality/productivity of the therapeutic proteins. In such cases, it may be helpful to provide a summary of the linkage among the expression plasmids/parental cells used throughout product development. In addition, comparability and other product impacts should be evaluated. To avoid any unexpected loss of the cell bank, the containers are typically kept in at least two different geographical locations. During transportation, the containers are shipped and are continuously monitored for temperature to confirm that they remain acceptably frozen.

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# 4. MAMMALIAN CELL LINE CLONALITY

## 4.1 Overview

This section discusses clonality of production cell lines derived from mammalian cells. Obtaining a stable cell line that maintains high levels of recombinant therapeutic protein expression and consistent product quality is critical for commercial development. Typically, production cell lines are derived from immortalized cells, such as CHO cells, which exhibit a relatively high degree of genetic plasticity (*6*). Therefore, the addition of a cloning step is important to the overall cell line generation process. Incorporation of a cloning step serves two core purposes:

1. Minimizing cell population heterogeneity
2. Isolating a highly productive, stable cell population

It is recognized that incorporation of a cloning step does not prevent genetic and phenotypic heterogeneity within the population of cells that arises post-cloning due to genetic features of immortalized mammalian cell lines. However, non-clonally derived cell lines have been used for toxicology studies. It is important to demonstrate that critical quality attributes (CQAs) of the preclinical and clinical proteins are similar enough to assure safety based upon the toxicology studies.

This section describes relevant regulatory guidances regarding clonality as well as practices to achieve clonally derived production cell lines. These practices include limiting dilution, fluorescence-activated cell sorting (FACS), semisolid plating, and single-cell printing (see *Table 2*). In addition, this section describes principles and types of data used to support clonal derivation of a cell line.

The concept of clonality is recognized in multiple regulatory guidance documents. For instance, ICH Q5D (*7*) states: "For recombinant protein products, the cell substrate is the transfected cell containing the desired sequences, which has been cloned from a single cell progenitor." ICH Q5B (*6*) defines the MCB as: "An aliquot of a single pool of cells which generally has been prepared from the selected cell clone." The guidance goes on to indicate that "In addition, methods used to amplify the expression construct and criteria used to select the cell clone for production should be described in detail." Likewise, the FDA's *Points to Consider in the Manufacture and Testing of Monoclonal Antibody Products for Human Use* (*4*) indicates that a "description of the cell cloning procedures" should be captured in documentation.

The aforementioned guidance documents indicate a general expectation that a clonally derived cell line be used to produce recombinant therapeutic products. Using a cell line derived from a single progenitor minimizes the variability of the starting cryopreserved cell population, which would be anticipated to have the effect of minimizing product heterogeneity. However, sustained culturing of immortalized cells can result in genetic alterations, which may be further exacerbated by amplification procedures. Therefore, it is not feasible to term a population as truly genetically homogeneous following sustained culture. Despite this, the reason for generating a clonally derived cell line relates to the ability of a controlled process to produce a consistent product with minimal heterogeneity. Thus, for these reasons, any adaptations (e.g., switching to serum-free conditions) should be considered prior to cloning. In contrast, use of an entirely non-clonal cell population as a starting point may give rise to outgrowth of a subpopulation of cells that generate products with different CQAs. For instance, this could affect glycosylation, which could then impact the mechanism of action if the product is an antibody that functions by antibody-dependent cell-mediated cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC). Likewise, a different population with a different integration site might have altered expression levels, growth metrics, and stability, which could have the potential to lead to drug shortages if a cell bank is no longer performing as expected. Such adverse end points could be exacerbated in conditions where cell culture parameters or raw materials have been altered in a way that places selective pressure on the system.

## 4.2 Methodology

Several methodologies used to generate clonally derived cell lines are described below.

### LIMITING DILUTION CLONING

Limiting dilution cloning (LDC) is a procedure whereby cells are plated at an appropriately justified low cell density. Some wells will be devoid of cells. This is achieved by preparing a set of increasingly greater dilutions of the non-clonal starting population and visually verifying the number of cells initially deposited per well. Clones that grow out are screened for desirable growth metrics as well as product titer. Clones that appear to be good performers can be expanded through sequentially larger wells until sufficient cells are generated to establish a cell bank. Two rounds of LDC are recommended if manufacturers want to establish a clonal cell line, particularly in the absence of additional supporting technology, to ensure monoclonality (e.g., imaging). The appropriate number of rounds (e.g., 2 rounds) at an appropriate seeding density of LDC provide an approximately 99% probability that the cell line will be monoclonal. However, it is a time-consuming process and can take up to 12 months to complete.

### SINGLE-ROUND LDC WITH IMAGING

The supplemental evidence for the single-cell derivation of the cell line can be based on the use of a qualified imaging system, acquiring images of the single cell at day 0 and on subsequent days to follow the outgrowth of the clones. After LDC, the stained cells are deposited into imaging-quality well plates where day 0 images are acquired in both fluorescence and brightfield imaging. Fluorescence imaging may also be used if necessary. Image verification is reviewed at day 0. Fluorescence and brightfield images are evaluated for quality—based on criteria including image focus and clarity, cell shape, lack of debris or artifacts in the well—as well as for confirmation that the entire well was captured in the image.

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## FLUORESCENCE-ACTIVATED CELL SORTING

This technology can be used alone or in combination with a single round of LDC. The methods used for selection may rely on the detection of fluorescent intracellular reporter(s), cell-surface protein-expression target, or an unrelated marker. The method selection can also be based simply on the sorting of single cells from a preselected population. As with other cloning methods that rely on dilution to separate cells, it is important to verify that only 1 cell was sorted and deposited per well. This could be done by analysts or by an automated imaging machine starting at day 0. Some considerations include verifying that the parameters used for flow are able to distinguish single cells from doublets and that precautions are taken to make sure cell samples are not contaminated with samples previously sorted on the machine (i.e., sanitization). Any biologically derived agent should be tested for adventitious agents. For example, if antibodies are used in the selection process, they should be tested for adventitious agents.

## AUTOMATED COLONY PICKING FROM SEMISOLID MEDIUM

Automated colony-picking machines are increasingly being used for cell line development due to their high throughput capacities. Since the occurrence of high-producing transfectants with desirable growth metrics is relatively rare, screening a larger number of colonies increases the likelihood of identifying good candidates. The process in some cases can be completed in as little as 7 days, with the identification of stable clones by 4 weeks. These systems rely on the viscosity of a semisolid matrix in which the preselected cell population of transfectants is dispersed to separate the individual cells and, subsequently, the colonies and their products from one another. In addition, this semisolid matrix serves to retain product that is produced by the cell colony locally. Production of accumulated recombinant protein is detected by incorporation into a semisolid medium of a fluorescein isothiocyanate (FITC)-conjugated IgG antibody raised against the species of interest. As more recombinant protein is secreted into the medium, a halo of fluorescence builds up around the growing clone of interest. The system is able to image the cells under both white and fluorescent light; thus, both the cell colony that is growing and the amount of fluorescence that is being produced can be monitored. Up to a threshold level, mean exterior fluorescence has been shown to correlate with antibody productivity; however, this relationship was not maintained at levels above the threshold. On the basis of preset thresholds, the machine will automatically pick clones of interest and place them into microtiter plates for expansion. Optimization of the parameters used may be necessary. As with FACS, it is important to verify that the apparatus has been sanitized since its last use to avoid cross-contamination. Also, potential adventitious agents arising from the antibody used in the selection process are a concern; therefore, appropriate testing should be performed. As with other methods, it is important to keep in mind cellular behavior such as clumping. In addition, the plating density can impact the ability of the machine to select only 1 colony. Thus, such machines are often used in combination with a single round of LDC to support assurance of clonality.

## OTHER AUTOMATED CLONE SELECTION TECHNOLOGY

There are many methods that can be used for clone selection, and other methods may be developed. One example of clone selection methods is to combine high-speed image scanning and quantitation with high-speed laser manipulation, which allows for in situ measurement of recombinant protein secretion of single cells, followed by laser-mediated destruction of the low-producing cells in a population. This can enable higher throughput by using 384-well plates instead of 96-well plates. Image documentation of a clone begins on day 0, immediately after plating, with the aid of a fluorescent viable cell dye and continues throughout the entire expansion process. Multicolor live cell imaging is also possible through the simultaneous use of fluorescence detection reagents that associate specifically with either the cell clones or the expressed recombinant protein. The sample wells are coated with capture matrix to mediate in situ capture of expressed recombinant protein as most of the recombinant proteins are secreted out of the cell. By measuring the fluorescent intensity of the respective detection reagents, each cell in the same well is ranked based on the amount of secreted recombinant protein. Other criteria such as cell growth rate, area, and proximity to other cells also affect the ranking. The highest-ranking cell in a well is identified as the most suitable high-producer cell clone. Subsequently, a laser beam is directed to the lower-ranking cell clones in the same well to induce photomechanical lysis. This allows the high-producing cell remaining in the well to grow and then be transferred to a larger well for expansion, clone characterization, and cell banking. The platform could be used alone or in combination with a single round of LDC.

See *Table 2* for a comparison of the above methods.

**Table 2**

| Method Comparison | Advantages | Disadvantages |
|---|---|---|
| Limiting dilution cloning | • Statistically powered with two rounds<br>• Least expensive | • One round insufficient to provide monoclonality assurance; requires additional supplemental data<br>• Labor and time intensive<br>• Requires accurate initial cell counting |
| Single-round LDC with imaging | • Day 0 image confirmation of single cell in well provides high level of monoclonality assurance | • Requires expensive equipment<br>• Requires equipment qualification<br>• Requires highly trained operators |
| Fluorescence-activated cell sorting | • Single cell deposition<br>• High throughput<br>• Can be combined with imaging to provide monoclonality assurance<br>• Can be used to isolate high-producing cells | • Requires expensive equipment<br>• Requires equipment qualification/validation<br>• Requires highly trained operators |

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**Table 2** *(continued)*

| Method Comparison | Advantages | Disadvantages |
|---|---|---|
| Automated clone picking from semisolid medium | • Identification of isolated individual cells<br>• Can be coupled with LDC<br>• Can be used to identify high-producing cells | • Use of semisolid media may result in stacked cells in *z*-axis<br>• May require an additional round of LDC<br>• Does not allow imaging of single cells<br>• Requires expensive equipment<br>• Requires equipment qualification<br>• Requires highly trained operators |
| Other automated clone selection technology | • Day 0 image confirmation of single cell in well provides high level of monoclonality assurance<br>• High-throughput image scanning of high-producing cells with elimination of low-producing cells | • Requires expensive equipment<br>• Requires equipment qualification<br>• Requires highly trained operators |

## 4.3 Assurance of Clonality

It is important to appropriately document and provide data to support a claim of clonality. The analysis may include:

- Mathematical probability models, as well as detailed descriptions of the methodologies used and demonstrations of how they achieve single-cell isolation (e.g., experiments with mixed cell lines that have been cultured together)
- Images, especially day 0 and over time; genetic data; and consistent process data and product quality

When probability calculations are included, they should be done so prospectively. Back calculation from the number of wells in which the colonies grew does not take into account the number of wells in which cells failed to grow, resulting in an overestimate of monoclonality.

### IMAGERS

Imagers that can document the contents of an entire microplate well[2] from day 0 through colony growth can be powerful tools. An image of a single expanded colony would not be enough evidence of monoclonality, but a time lapse showing the development of the colony from a single cell would provide supportive evidence. In this case, it would be important that the entire well was captured to prove that only 1 cell was in the well from the beginning. Furthermore, day 0 fluorescence imaging as applicable allows for the detection of living cells and for differentiation from cell-like artifacts that can be observed in the brightfield image.

### GENETIC SUPPORT

For clones with random (non-site-specific) integration, genetic information supporting the location of the integration site provides evidence that the cell line was clonal. Information about the sites of integration can help to identify the number and location of genomic integration sites. Integration site identification does not prove that the cell line was clonally derived.

***Change to read:***

# 5. CELL BANK CHARACTERIZATION

Depending on the type of cells used, the biological production processes for cell banking can be variable, which has important consequences for the safety and efficacy of the resulting product. In order to introduce these cell-derived products into routine clinical use, the manufacturer must ensure that robust cell banking procedures are developed on the basis of process understanding and characterization, which should result in consistent production and quality of cell banks. Process understanding and consistency are critical because slight changes in cell growth conditions can occasionally lead to changes (e.g., in glycosylation) that can have major adverse effects later on (e.g., changes in immunogenicity), which may be a critical attribute for the expressed protein or polysaccharide. Validated and/or appropriately qualified-for-use test methods for characterization of the cell bank (e.g., host cell quality, purity, freedom from adventitious agents, in-process testing) are quality recommendations for any MCB or WCB.

## 5.1 Cell Bank Qualification and Characterization

Cell banks used in the manufacturing process must undergo extensive safety testing to demonstrate identity, stability, and purity. See *Table 3* for a description of microbial cell bank characterization and testing possibilities. See *Table 4* for a description of cell bank characterization of a mammalian cell line (CHO) and testing possibilities. Use of each test should be agreed upon with the applicable health authority.

Cell bank qualification is defined as any testing deemed appropriate to demonstrate that the bank is fit for its intended use and the environmental conditions were appropriate. Testing to qualify the mammalian cell bank includes testing to demonstrate freedom from adventitious agents such as bacteria, fungi, mycoplasmas, and viruses and to confirm species identity. Testing is also required to determine if cells produce retroviruses or retrovirus particles.

[2] For example, CloneSelect Imager.

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**Table 3. Microbial Cell Bank Characterization [a]**

| Assay Type | MCB | WCB | EOP[b] |
|---|---|---|---|
| **Specification Analysis** | | | |
| Microbial purity | + | + | — |
| Phage contamination | + | + | — |
| DNA sequencing | + | + | — |
| Strain identity | + | – | — |
| **Characterization Tests** | | | |
| Viability | + | + | + |
| Microbial purity[c] | — | — | + |
| DNA sequence | — | — | + |
| Plasmid copy number | + | + | + |
| Plasmid frequency or loss of plasmid[d] | + | + | + |
| Restriction enzyme mapping | + | + | + |
| Phage contamination | — | — | + |
| Nucleic acid fingerprinting | + | + | + |
| Nucleic acid profiling | + | + | + |

[a] ICH Q5A was under revision during development of this chapter. The table is aligned with the current R1 version of ICH Q5A. For more information see current version of ICH Q5A.
[b] EOP, end of production.
[c] Purity needs to be performed on EOP if not done in MCB and WCB.
[d] This test may be more appropriate to perform on the end-of-growth sample.

**Table 4. Biological Safety Characterization of Mammalian Cell Banks (CHO Cells)[a]**

| Assay Type | MCB | WCB | EOP[b] |
|---|---|---|---|
| **Microbial Contaminants** | | | |
| Sterility: *USP*/*European Pharmacopoeia*/ICH requirements | + | + | + |
| Mycoplasma: *European Pharmacopoeia 2.6.7* (*15*)/*Mycoplasma Tests* ⟨63⟩ | + | + | + |
| **Adventitious Virus Detection[c]** | | | |
| In vitro assay[d, e] | + | ▲–[f]▲ (ERR 1-May-2023) | +▲▲ (ERR 1-May-2023) |
| Antibody production tests[g, h] | + | — | – |
| Other species specific tests[i] | If applicable | If applicable | If applicable |
| In vivo assay[j] | + | —[e] | + |
| Bovine and porcine virus in vitro assay (*16–17*)[k] | – | – | – |
| **Detection of Retroviruses and Other Endogenous Elements** | | | |
| Electron microscopy | + | — | + |
| Infectivity | + | – | + |
| Reverse transcriptase assay[l] | + | – | + |
| **Cell Line Identity Tests** | | | |
| Nucleic acid fingerprinting or other | + | + | + |

[a] ICH Q5A was under revision during development of this chapter. The table is aligned with the current R1 version of ICH Q5A. For more information see current version of ICH Q5A.
[b] EOP, end of production.
[c] The strategy for testing extraneous agents should be developed based on a risk assessment following the principles of viral contamination risk (see *Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin* ⟨1050⟩).
[d] The nature of the assay and choice of cells used are governed by the species of origin of the cell bank and the type of virus that may be present based on the handling of the cells. The assay should include a human and/or a non-human primate cell line susceptible to human viruses. Both cytopathic and hemadsorbing viruses should be sought.
[e] The strategy should include suitable tests that are able to detect different families of extraneous agents that may infect the source materials, including cell substrates and raw materials of animal or plant origin. The strategy also considers the capacity of the manufacturing process to remove or inactivate viruses (see *Design, Evaluation, and Characterization of Viral Clearance Procedures* ⟨1050.1⟩).
[f] Performed on cells generated from the first WCB at the limit of in vitro cell age; for subsequent WCBs, a single in vitro and in vivo test can be done either directly on the WCB or on the cells at the limit of in vitro cell age (LIVCA). On the basis of a risk assessment, these tests may not be required.

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[g] Species-specific viruses present in rodent cell lines may be detected by mouse, rat, or hamster antibody production tests (MAP or HAP).
[h] Alternative validated method (e.g., NAT) agreed upon with the regulatory authority may be used.
[i] Selection of viruses to be screened should take into account cell lines that are known to have been infected by such agents and the source from which the cell line was derived [e.g., mouse minute virus (MMV), *Vesivirus*].
[j] Includes adult and suckling mice and embyronated eggs. Additional animal species may be used depending on the nature and source of the cell lines being tested.
[k] When bovine and porcine raw materials are used, these tests should be performed. Bovine and porcine virus in vitro assay can be omitted for MCB, WCB, and EOP if the parental cell line used to generate MCB had been tested negative for bovine and porcine viruses and no animal-derived raw material were used in transfection and subsequent steps leading to the generation of MCB and WCB as well as in the production of EOP.
[l] Not required if infectivity assay is positive.

## 5.2 Mammalian Cell Bank Testing

A new cell bank (MCB or WCB) needs to be qualified to demonstrate its suitability for biologic production. The newly prepared cell bank should be characterized extensively using appropriate release tests on an aliquot of the cell bank or on cultures derived from it. The testing on limit of in vitro cell age (LIVCA) cells should be conducted for the initial MCB or WCB. When new WCB are expanded to or beyond the LIVCA, testing should be conducted to assess against the existing MCB. However, extensive characterization and testing is not necessary for a WCB generated from a fully tested MCB. The characterization of the cell bank is dependent on its use, and the specific use should guide decisions about the required recommended testing and whether additional testing might be necessary.

Though rarely banked, primary cells are more likely to contain adventitious agents than banked, well-characterized cell lines. The genetic stability of diploid cell strains should be monitored throughout production.

The tumorigenicity study procedure and suitable animal models are described in the WHO's Technical Report Series No. 978, Annex 3 (*13*) and the FDA's *Guidance for Industry: Characterization and Qualification of Cell Substrates and other Biological Materials Used in the Production of Viral Vaccines for Infections Disease Indications* (*14*). If the cell substrate under evaluation is found to be tumorigenic, it must be evaluated for oncogenicity in suitable animal models (newborn nude mice, newborn hamsters, and newborn rats) using an appropriate positive control.

For an engineered cell line, the inserted GOI should remain intact and the copy number should be determined to support consistent production.

The cell bank should comply with the appropriate test for sterility given in *Sterility Tests* ⟨71⟩ and carried out using an appropriate volume from cell cultures. The test should be carried out on 1% of containers with a minimum of 2 containers. The cell bank should also comply with the test for mycoplasma in *Mycoplasma Tests* ⟨63⟩ performed on 1 or more containers.

Electron microscopy (EM) of the MCB should be done to detect the presence of any extraneous agents. The MCB should also be assessed by the vitro virus assay with the two endpoints of cytopathic effect and hemadsorption. The end of production (EOP) cells are also characterized to demonstrate absence of extraneous agents that might be present in the MCB.

Additionally, the cell bank should be tested for possible virus contaminants, depending on the origin of the cells, using appropriate specific tests (e.g., mouse, rat, or hamster antibody production tests or a nucleic acid amplification test may be used for specific contaminants). See *Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin* ⟨1050⟩ and *Design, Evaluation, and Characterization of Viral Clearance Procedures* ⟨1050.1⟩ for more details.

The MCB should be examined for retroviruses using:

- Infectivity assay
- Transmission electron microscopy (TEM)
- Reverse transcriptase (RT) or other appropriate assays should be performed to detect retroviruses that may be noninfectious if infectivity is not detected and no retrovirus or retrovirus-like particles have been observed by EM

Detection for retroviruses in the MCB is governed by the host species and can include detection methods such as reverse transcriptase activity, infectivity in sensitive cell cultures or electron microscopy. It is recommended to continue to follow advances in new technology to supplement cell bank characterization where appropriate.

## 5.3 Genetic Characterization

Genetic characterization to support the use of the production cell line for the MCB and WCB and a proposed LIVCA at the EOP stage are essential for any development program. The purpose of genetic characterization is to demonstrate the integrity of the expression construct carrying the GOI throughout the intended commercial manufacturing process. The manufacturing cell culture duration starts with the MCB and/or WCB and continues to the proposed LIVCA for the drug substance production. It is recommended that LIVCA be determined based on the cell age of the EOP cells for a defined duration beyond the routine commercial drug substance manufacturing process. At a minimum, LIVCA should have population doubling levels (PDLs) beyond the typical manufacturing window as appropriate. The additional generations are added to allow for future changes to the manufacturing process and to ensure that the LIVCA is not exceeded in future manufacturing operations. The EOP cells should be harvested from a representative commercial process, either at a pilot or commercial scale.

Genetic characterization is performed on samples taken at the EOP and compared to the MCB. If necessary, WCB could be tested with bridging to the MCB. Based on ICH Q5B, the genetic characterization consists of four aspects:

1. Verification of the GOI coding sequence
2. Assessment of the integration sites
3. Assessment of the integrity of the gross structure of the expression construct, which provides insight into potential insertions and/or deletions of the expression construct
4. Determination of a consistent GOI copy number over the length of time required to perform a biopharmaceutical manufacturing run

For microbial strain-derived cell banks, the GOI is usually carried by extrachromosomal expression systems such as a plasmid expression vector. The nucleotide sequence encoding the product can be directly sequenced from the plasmid isolated from

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the cell banks without further cloning. For cell banks carrying chromosomal copies of the GOI, the sequence encoding the product can be verified. This is commonly performed by sequencing of the coding regions amplified by the PCR from cDNA. The nucleic acid sequence should be identical, within the limits of detection method, to the sequence determined for the expression construct and should correspond to the expected protein sequence.

The most common methodology for the assessment of gross structure of the expression construct is restriction endonuclease (RE) mapping analysis. New technologies [e.g., next generation DNA sequencing (NGS)] are currently being explored for this purpose. For extrachromosomal expression systems, the RE analysis can be viewed through gel electrophoresis; for chromosomally integrated expression systems, the RE analysis is usually accomplished by Southern blotting or full sequencing by NGS. The RE digested patterns as well as the DNA sequence should be consistent for DNA derived from either the cell banks or the cells at the LIVCA.

Although not required by ICH Q5B, transcript analysis of GOI can be performed if needed to further evaluate the integrity of the GOI expression. A number of techniques can be used for transcript analysis, including Northern blotting, reverse transcription–polymerase chain reaction (RT–PCR) and transcriptome analysis by NGS.

The most common methodology for the evaluation of GOI copy number is quantitative PCR (qPCR). GOI-specific qPCR assays and a normalizer qPCR assay targeting a host genome region are usually applied. In some cases, there is no strong correlation between the stability and integrity of the therapeutic product. The GOI copy number analysis could be considered for the cell bank characterization purposes only. The genetic determination is part of the quality control. New DNA sequencing technology such as NGS may further enhance the capability for genetic characterization.

The impact of a change in cell culture process after the commercial process has been defined should be evaluated to determine if retesting of the cells at LIVCA is necessary. Changes in cell culture scale and/or manufacturing site may not require retesting of cells at LIVCA if the defined cell age covers any increase in population doubling resulting from the change and if cell culture performance meets the predetermined acceptance criteria. However, changes in media components (especially if animal-derived components are used) and growth conditions often result in changes in cell culture profiles, so one should consider retesting the integrity of the expression construct at LIVCA to ensure suitability of the cell line for commercial production.

Although a two-tier cell-banking system (MCB and WCB) is commonly established for the commercial manufacturing of a drug substance, the characterization of the expression construct for each WCB is considered unnecessary if the following criteria are met:

- The MCB has been fully characterized and the expression construct is confirmed to be stable
- The cell age of the current and future WCB, as well as the EOP cells derived from the future WCB, will be controlled within the previously determined LIVCA

If the characterization cannot be carried out on the MCB, it should be carried out on each WCB.

## 5.4 General Considerations for Qualification of Replacement WCBs

Replacement WCBs prepared using procedures equivalent (as described in the license) to those used to generate the previously approved WCB must meet all specified requirements [e.g., certificate of analysis (CoA) testing] but require no further evaluation under a validation protocol. When the new WCB is a “like-for-like” replacement, the WCB can be implemented after meeting the following criteria:

1. The new WCB must meet all cell bank release testing criteria, including tests for freedom from adventitious agents.
2. Prior to at-scale manufacturing, the WCB may be evaluated using either scale-down or industrial-scale cell culture tests from thaw through production culture to confirm cell culture performance. A minimum number of independent thaws should be included in the evaluation.
3. There is an option to use scale-down cell culture evaluation criteria including cell culture process key performance indicators (KPIs) and relevant product attributes and/or CQAs, but at least one full scale with expanded analytical characterization should be evaluated. For example, the KPI assessment may include specific growth rate and final viabilities for seed and inoculum train passages, final production culture viability, and final product titer. Product quality assessments may include purity, size-exclusion chromatography (SEC), and ion exchange chromatography (IEC) assays. The evaluation criteria can be based on 95% confidence/99% probability tolerance intervals (95/99 TIs) generated using representative data available at the time the evaluation is performed (where appropriate). Results outside the evaluation criteria should be justified or further assessed using additional cell culture studies and/or product attribute testing.
4. The new WCB should produce manufacturing-scale material that meets all specified drug substance release testing requirements. A drug substance manufactured from a replacement bank may not need to be on stability protocol, but does require a CoA.

The release of batches derived from the new WCB would be predicated on successfully completing all the above-mentioned criteria.

For WCBs prepared using a manufacturing process that differs significantly from the process used to produce previously approved WCBs, the same evaluation procedure described above (criteria 1–3) applies. However, additional evaluations may be performed and could include further KPI assessments and the testing of additional CQAs and non-CQAs. For cell culture-based infectivity assays (in vitro adventitious agent assays), refer to ⟨1050⟩ and ⟨1050.1⟩.

## 5.5 Other General Considerations for Cell Banks

### CELL BANK CRYOPRESERVATION

It is critical that cells are characterized and a feasibility assessment is performed to verify that cell performance is achieved when expanded at a defined concentration and duration using a specific technology platform. Containers should be

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appropriately labeled, and cryolabels must be able to withstand cryopreservation. Cryology considerations for cells are addressed in ⟨1044⟩. Additional considerations for cell banking are as follows:

- Cells are frozen at early passage (MCB), intermediate passage (WCB), or late passage (EOP).
- Frozen cell volumes can vary from 0.5 to >100 mL depending on the number of cells required upon thawing.
- Cell banks can be stored in cryovials or cryobags.
- Cell density can range from $10^6$ to $10^8$ cells/mL.
- Cell bank storage containers should be filled with an excess to accommodate loss of cells upon thaw.
- For MCB or WCB, cells should be banked at a density that allows for direct inoculation into vessels for expansion upon thawing.
- Mammalian cells should be preserved in GMP-grade cryopreservation solution with appropriate cryoprotectant (e.g., DMSO or glycerol) to support cell recovery consistent with established in-process viability limits.
- Post-bank thaw testing with assessment of cell viability should be performed and documented. A viability limit should be set and justified to demonstrate that the cells survived the cryopreservation process. The viability limit could be set based on historical data.
- Each cell bank vial or bag should be labeled in advance with the appropriate information (e.g., cell bank lot number, tube number, and production date) on liquid nitrogen-resistant cryolabels; use appropriate controls and limits.

If different cell banks are stored in the same facility, appropriate measures and controls (for example segregation or cryolabels) should be in place to prevent errors due to switching and cross contamination. Different freezers or storage areas within freezers should be used to separate the cell banks prior to release testing from the cell banks to be used for production.

## CELL BANK STORAGE STABILITY AND MONITORING

A cell bank is considered stable if, upon thaw and subsequent passage, it can consistently meet growth and viability criteria based on historical performance. Bank-specific limits for viability and growth rates can be set after thaw studies have been completed. Cell bank stability can be equally ensured through trending of thaw performance data across a more substantive data set such as WCB vial thaw and/or batch.

Stability limits for mammalian cell banks should be site independent, product specific, cell bank specific, and determined on the basis of thaw performance. Stability limits can also be vial-size specific due to the operational differences between seed strain sources from different-sized vials. Data used to establish final viability and specific growth rate limits should be documented.

Data for MCB stability monitoring should include data from when 1 or more vials of the cryopreserved MCB are thawed for:

- Initial characterization
- Production of clinical or commercial material
- Preparation of a new WCB

Data for WCB stability monitoring can include data from when 1 or more vials of the cryopreserved WCB are thawed for a clinical or commercial production campaign.

Data for WCB stability monitoring may also include data from appropriately documented scale-down or pilot-scale processes, such as those performed during process characterization and validation studies. WCB stability should be periodically monitored unless the bank is no longer in use for commercial production or the product has been removed from the market.

## CELL BANK TRANSPORTATION

A temperature-cycling study can be done to determine the effects of repetitive temperature fluctuations on frozen-cell stability and shelf life. Chapter ⟨1044⟩ outlines good storage and distribution practices for cells, cell therapy products, and cell banks to maintain the integrity of frozen cells during movement or transportation.

It is crucial to ensure that the facility maintaining the cell bank provides storage management systems, which should include an inventory of all stored material and routine documentation of entries and withdrawals. Transportation controls should be in place as per ⟨1044⟩. Distribution of cell bank vials should be handled carefully to ensure that integrity and cell viability are maintained throughout the transfer. Cells can be shipped on dry ice in containers, with appropriate procedures for replenishment of dry ice as needed. Dry shippers (well-insulated Dewars that contain a cryoabsorbent material) charged by addition of liquid nitrogen provide an optimal method for the shipping and handling of cell banks.

## RAW MATERIALS

Control of raw materials is necessary to reduce the risk of contamination due to adventitious agents and possible cross contamination from incoming raw materials. Contamination risk for all raw materials should be assessed; the assessment can then be used to support product exposure history.

**Qualification of raw materials:** Raw materials should be qualified and characterized as to their identity, purity, functionality, and safety. Appropriate testing for raw materials should be performed; examples include testing for endotoxins, mycoplasma, adventitious agents, and/or sterility (see *Ancillary Materials for Cell, Gene, and Tissue-Engineered Products* ⟨1043⟩). Any raw material used in the manufacture of a cell substrate (including media components) that is obtained from suppliers should be controlled appropriately by the cell substrate manufacturer (see ⟨1043⟩).

The qualification program should evaluate the source, identity, purity, and safety of the raw material relative to the established specifications for raw materials. Identification and selection of raw materials should be performed on the basis of suitability for use in the intended process and evaluation of toxicity, availability, consistency, traceability, and potential to be sources of contamination. If the raw materials are human derived, the certificate of origin (CoO) and donor infectious disease status should

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be evaluated. If the raw materials are animal-derived, the herd qualification and country of origin certificates, should be evaluated.

For some human- and animal-derived raw materials used in cell culture medium (e.g., insulin, transferrin), a validated production process used for elimination of viruses may substitute for virus detection tests, when justified. The evaluation of the suitability for use should encompass any effects on safety, potency, and purity. It should also include assessment of the potential risk of toxicity and adventitious agents and their removal if such raw materials are used in large amounts in manufacturing.

Drug manufacturers should develop a raw materials management system as part of their quality systems that would include a raw materials qualification program. This program should include:

1. Incoming receipt, segregation, inspection, and quality release of the raw materials prior to use
2. Vendor audits and certification
3. CoA verification testing or identity testing, as applicable
4. CoO
5. Formal procedures and policies for out of specification results
6. Stability testing
7. Archival sample storage

Any raw material supporting the cell bank growth will be an ancillary product; therefore, supplier material should be controlled by the manufacturer. Usually a CoA and/or a risk statement addressing the likelihood of the presence of animal-derived materials [e.g., bovine material, which carries the risk of bovine spongiform encephalopathy (BSE)] should be provided for all media components. In addition, contracts ensuring a steady supply of a material of the proper purity level should be secured.

## 6. CONCLUSION

The production of cell banks needs to satisfy quality standards for identity, purity, clonality, stability, and the suitability of cells for use in manufacturing. In addition to the appropriate testing for identity, purity, and adventitious agents, the homogeneity, reproducibility, history, cell bank generation and qualification, genetic stability, storage, raw materials, and reagent qualification, as well as good manufacturing practices, must be demonstrated.

## GLOSSARY

**Adventitious agent:** A microorganism [including bacteria, fungi, *Mycoplasma*/*Spiroplasma*, *Mycobacteria*, *Rickettsia*, viruses, protozoa, parasites, or transmissible spongiform encephalopathy (TSE) agents] that is inadvertently introduced into the production of a biological product (*15*).

**Cell bank:** A collection of appropriate containers, whose contents are of uniform composition, stored under defined conditions. Each container represents an aliquot of a single pool of cells (*7*).

**Cell bank qualification:** Any testing deemed appropriate to demonstrate fitness of use of the bank for its intended purpose and the environmental conditions where appropriate.

**Cell line:** Type of cell population that originates by serial subculture of a primary cell population that can be banked (*7*).

**Cell substrate:** Microbial cells or cell lines derived from human or animal sources that possess the full potential for generation of the desired biotechnological/biological products for human in vivo or ex vivo use (*7*).

**Clonality:** A group of cells derived from a single progenitor sharing common ancestry (*7*).

**Diploid cell line:** Type of cell population having a finite in vitro lifespan in which the chromosomes are paired (euploid) and are structurally identical to those of the species from which they were derived (ICH Q5D).

**End of production cells** (EOP cells)**:** Cells cultured (under conditions comparable to those used in production) from the MCB or WCB to a passage level or population doubling level comparable to or beyond the highest level reached in production (*15*).

**Expression construct:** The expression vector that contains the coding sequence of the recombinant protein and the elements necessary for its expression (*6*).

**Host cells:** See *Parental cells* below (*7*).

**Genetic integrity:** Test to establish that the gene of interest is intact, sequence of coding sequence is correct, and there are no insertions or deletions.

**Integration site:** The site where 1 or more copies of the expression construct are integrated into the host cell genome (*6*).

**Limit of in vitro cell age** (LIVCA)**:** Time between thaw of the MCB vial(s) and harvest of the production vessel as measured by elapsed chronological time, by population doubling level of the cells, or by passage level of the cells when subcultivated by a defined procedure for dilution of the culture (*6–7*).

**Master cell bank** (MCB)**:** An aliquot of a single pool of cells that generally has been prepared from the selected cell clone under defined conditions, dispensed into multiple containers, and stored under defined conditions (*6–7, 16*).

**Parental cells:** Cells to be manipulated to give rise to a cell substrate or an intermediate cell line. For microbial expression systems, it is typical to also describe the parental cells as the host cell (*7*).

**Passage level:** The number of times that a cell population has been removed from the culture vessel and has undergone a subculture (passage) process (*15*).

**Pilot plant scale:** The production of a recombinant protein by using a procedure fully representative of and simulating that to be applied on a full commercial manufacturing scale. The methods of cell expansion, harvest, and product purification should be identical except for the scale of production (*6*).

**Research cell bank** (RCB)**/Premaster research cell bank** (pmRCB)**:** Cell bank(s) developed as a precursor to the master cell bank.

**Recombinant biologic:** Biological substance produced by genetic engineering techniques.

**Tumorigenicity:** Process by which immortalized cells form tumors when inoculated into animals (*15*).

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**Viral clearance:** Elimination of target virus by removal of viral particles or inactivation of viral infectivity (*5*; ⟨1050⟩, ⟨1050.1⟩).

**Virus-like particles:** Structures visible by electron microscopy that morphologically appear to be related to known viruses (*5*).

**Working cell bank** (WCB)**:** A collection of cells prepared from aliquots of a homogeneous suspension of cells obtained by culturing the MCB under defined culture conditions (*6–7*).

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# ⟨1043⟩ ANCILLARY MATERIALS FOR CELL, GENE, AND TISSUE-ENGINEERED PRODUCTS

***Change to read:***

## INTRODUCTION

A wide variety of reagents and materials, many of which are unique or complex, are ▲used in▲ (USP 1-May-2020) the manufacture of cell, gene, and tissue-engineered ▲(CGT)▲ (USP 1-May-2020) products.

▲The purpose of this chapter is to provide guidance on the development of appropriate material qualification programs for CGT products. Because CGT products are not usually amenable to extensive purification, filtration, and terminal sterilization procedures, reagents and material qualification are critically important to ensuring CGT product quality.

The term "ancillary materials" (AMs) refers to materials that come into contact with the cellular starting material, CGT product intermediates, or final CGT product during manufacturing, but are not intended to be present in the final CGT product. AMs are therefore distinct from the cellular starting material, the intermediates, and final CGT products. Excipients, which are intended to be present in the final product, are therefore not AMs. Processing devices such as vessels and transfer tubing sets may be considered AMs if they have surfaces and materials that contact the cells during production. Processing devices and containers that are considered AMs typically incorporate natural or synthetic biomaterials. Scaffolds or delivery devices that are intended to be part of the final product are not AMs. Cell banks and virus banks are generally not considered AMs; feeder layer cells that are not intended to be incorporated in the final product may be considered an AM or an impurity. Guidance documents (i.e., ICH Q5D) describe specific requirements for cell and virus bank certification and regulatory approval. However, "helper" viruses and "helper" plasmids may be considered AMs if they are not intended to be part of the final product.

Reagents that fall under the AM classification include well-characterized chemicals, complex compounds (antibiotics, anticoagulants, density gradients, toxins), multi-component mixtures (buffers, culture media), and complex biological compounds or mixtures (enzymes; blood-, plasma-, or serum-derived products; biological extracts; cytokines; antibodies; and conditioned media from cultured cells).▲ (USP 1-May-2020)

***Add the following:***

## ▲REGULATORY CONSIDERATIONS

Regulatory agencies have referred to AMs (as defined within this chapter) as ancillary products, ancillary reagents, processing materials, processing aids, and process reagents. The US FDA first introduced the AM concept with the term "ancillary product" in a *Federal Register* notice, *Application of Current Statutory Authorities to Human Somatic Cell Therapy Products and Gene Therapy Products* [*Federal Register* 58(197), October 14, 1993, pp. 53248–53251], which established the FDA's authority to regulate human somatic cell therapy products and gene therapy products. In a subsequent guidance, the FDA defined the term "ancillary reagent" as a material "used in the manufacture or production of a biological product that may or may not end up as part of the final product," but the definition was not formalized or used consistently in other FDA guidance (see *Guidance for Industry: Characterization and Qualification of Cell Substrates and Other Biological Materials Used in the Production of Viral Vaccines for Infectious Disease Indications*, February 2010). The term "AM" is also synonymous with "processing material", as defined in 21 CFR 1271, *Current Good Tissue Practice for Manufacturers of Human Cellular and Tissue-Based Products; Inspection and Enforcement; Proposed Rule* [*Federal Register* 66(5), January 8, 2001, pp. 1508–1559]. The US FDA also provides information to cell and tissue product manufacturers in 21 CFR 876.5885, *Tissue Culture Media for Human Ex Vivo Tissue and Cell Culture Processing Applications*.

Various labeling terminology has been used by AM manufacturers. The labeling terms "Research Use Only" (RUO) and "Investigational Use Only" (IUO) originated in the medical device labeling regulations and apply to materials intended for in vitro diagnostic use (see *Distribution of In Vitro Diagnostic Products Labeled for Research Use Only or Investigational Use Only: Guidance for Industry and FDA Staff*, November 2013). Because a given AM's label may or may not be relevant to its intended use in CGT product manufacturing, it is ultimately the responsibility of the CGT product manufacturer to define and qualify the suitability of a given AM in the CGT product manufacturing process.

Global and regional differences in AM labeling and documentation may dictate CGT product regulatory submission requirements. In the US, the CGT product sponsor can sometimes cross-reference an AM manufacturer's Drug Master File (DMF), which allows proprietary AM information to be reviewed by regulators, but not necessarily by the CGT product manufacturer. Other countries and regulatory authorities manage the submission of AM information differently, and it is critically important for the CGT product manufacturer to be aware of these differences if the same CGT product will be clinically tested or marketed in more than one regulatory jurisdiction. In any case, the CGT product manufacturer is responsible for ascertaining the AM's labeling, essential features, quality characteristics, and suitability for use.▲ (USP 1-May-2020)

***Add the following:***

## ▲IMPACT OF ANCILLARY MATERIAL QUALITY ON PRODUCT QUALITY

AM quality can pose risks to the stability, safety, potency, and purity of any product. These risks are often heightened with CGT products, whether due to their short shelf lives, limited ability to conduct extensive in-process and release testing, or the fact that many CGT products expand in vivo after their administration to the patient. To minimize the risks associated with

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these commonly occurring scenarios, rigorous AM qualification and prudent application of manufacturing process controls are necessary.

## Sourcing of Ancillary Materials

AMs used in the manufacture of CGT products are often selected for their unique functional or biological effects, and not necessarily on their quality attributes. Many AMs are only intended for research use but may not be readily available in a grade comparable to that of well-characterized licensed therapeutic products manufactured by controlled and documented methods. For this reason, many CGT product manufacturers source AMs that are approved or licensed therapeutic products. More recently, many AM suppliers have committed to providing higher grade AMs, using high-quality manufacturing and testing approaches with extensive documentation. Although the terms “clinical grade” and “good manufacturing practices” (GMP) are often applied to these higher quality AMs, their documentation may not be fully compliant with regulatory requirements for finished pharmaceutical products and excipients. Regardless of the AM labeling claims or descriptions, CGT product manufacturers are ultimately responsible for ensuring that the AM meets the necessary functional, quality, and documentation requirements demanded by the relevant regulatory authorities.

Specific safeguards are necessary in order to minimize or eliminate the risk of transmitting adventitious agents (viruses, bacteria, mycoplasma, protozoa, fungi, prions, etc.) when sourcing AMs using human- or animal-derived components such as sera, antibodies, or growth factors. In general, manufacturers of human- or animal-derived components and AMs should practice the following procedures to ensure CGT product safety:

1. Screening and qualifying (e.g., through bacterial or viral testing) and documenting (e.g., through herd certification and traceability) the sources of human- or animal-derived components as being free of suspected adventitious agents
2. Validating the inactivation (e.g., through chemical, thermal, or irradiation treatments) and removal (e.g., through chromatographic or filtration methods) of suspected adventitious agents during the processing of human- or animal-derived AM components
3. Testing (e.g., through polymerase chain reaction or virally-responsive cell lines) the initial raw material and final purified human- or animal-derived AM components for the presence of suspected adventitious agents

Industry and regulators have not yet established standard terminology to fully describe the presence or absence of human- or animal-derived components in AMs. Nevertheless, CGT product manufacturers should strive to eliminate all possible risk of adventitious agents in their final CGT products. The most direct way to achieve this goal is to eliminate AMs containing materials of human or animal origin. If AMs containing human- or animal-derived components are used, CGT product manufacturers should require AM suppliers to verify and document the absence of adventitious agents through the proper sourcing, processing, and testing of their human- or animal-derived components.

Regardless of the stated grade of the AM, the CGT product manufacturer is responsible for developing comprehensive and scientifically sound qualification plans to ensure the traceability, consistency, suitability, purity, and safety of the AM. Developers would be well served by referring to the International Council for Harmonisation (ICH)Q8(R2) guideline on *Pharmaceutical Development*, which provides internationally harmonized methodology for incorporating a quality-by-design (QbD) approach into development processes. This guidance allows systematic consideration of the critical quality attributes of a manufacturing process, including materials, resulting in the design of a high quality product.▲ (USP 1-May-2020)

***Change to read:***

## QUALIFICATION OF ANCILLARY MATERIALS

▲AM qualification is the process of acquiring and evaluating data to establish the source, identity, purity, safety, and overall suitability of a specific AM used in the manufacturing process. CGT product developers and manufacturers are responsible for establishing and periodically reevaluating rational and scientifically sound AM qualification programs. These programs should include the characterization and quantification of each AM throughout the manufacturing process (i.e., measuring its tendency to be removed, degraded, or taken up by cells during manufacturing). Although the heterogeneity of many CGT products and AMs makes it difficult to recommend specific tests or protocols for a qualification program, the following provides a practical approach applicable to most AMs.

Thorough documentation is the cornerstone of any qualification program. A well-designed and well-documented AM qualification program becomes more comprehensive as product development progresses. Development of progressive specifications for AMs typically runs in parallel to the development of progressive specifications for the CGT product, including analytical testing. In early stages of CGT product development, safety is the primary focus; a complete description of AM characteristics, and precise quantitation of residual AM in the CGT product, may be limited by a lack of validated assays. In later CGT product stages, AM quality and residuals specifications, along with analytical test methods, should be more comprehensively elaborated to support eventual licensure of the CGT product.

In cases where AMs are products licensed for therapeutic use and manufactured under current good manufacturing practices (cGMP), the extent of qualification is typically less than that for a material intended for research purposes. While use of licensed therapeutic products as AMs may ensure a higher level of quality and safety, it is outside the scope of the licensed product’s intended use and labeling; therefore, the AM’s suitability for use in the CGT product manufacturing process still requires qualification. Modification of the licensed product, such as dilution, reformulation, mixture with another agent, aliquotting, or repackaging, could trigger additional qualification. Modification of the licensed product’s container–closure system may also be necessary if it will be used as an AM.

In many situations, complex or unique substances essential for CGT product process control or production will not be available in pharmaceutical grade. Even if an AM manufactured in compliance with cGMP is used, the CGT product manufacturer must develop a scientifically sound strategy for AM qualification based on assessments of criticality and principles of risk management. This assessment takes into account major risks to the CGT product, AM or CGT product failure modes,

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and impacts to the product or patient in the event of an AM or CGT product failure. A qualification program for AMs used in CGT product manufacturing should address each of the following areas: 1) identification and sourcing, 2) selection and suitability for use in manufacturing, 3) characterization, 4) vendor qualification, and 5) quality assurance and control.▲ (USP 1-May-2020)

## Identification ▲and Sourcing▲ (USP 1-May-2020)

The first step in any qualification program is the listing of all of the AMs used in a given ▲CGT▲ (USP 1-May-2020) product manufacturing ▲process, including details on▲ (USP 1-May-2020) where in the manufacturing process they are ▲▲ (USP 1-May-2020) employed. The source and intended use for each material should be established, and the necessary quantity or concentration of each material should be determined. Alternate sources for each material should be identified ▲and qualified.▲ (USP 1-May-2020)

## Selection and Suitability for Use

Developers of CGT products should establish and document selection criteria for AMs and qualification criteria for each vendor early in the design phase of ▲CGT▲ (USP 1-May-2020) product development. Selection criteria should include assessments of microbiological and chemical purity, identity, and biological activity pertinent to the specific ▲CGT product▲ (USP 1-May-2020) manufacturing process. It is important to address these issues early in product development because certain AMs that are initially considered necessary may be impossible or prohibitively expensive to qualify, thereby justifying the investigation of alternatives or replacements. Examples include some animal- or human-derived materials that ▲may▲ (USP 1-May-2020) have alternate (i.e., plant or chemically synthesized) sources.

AMs ▲containing components▲ (USP 1-May-2020) of animal or human origin ▲require careful selection because of the▲ (USP 1-May-2020) potential ▲risk of▲ (USP 1-May-2020) infectious or zoonotic disease, ▲and the associated regulatory scrutiny. Vendors should provide documentation on the country of origin for animal-derived AMs to address concerns regarding transmissible spongiform encephalopathies and other diseases of agricultural concern (e.g., tuberculosis, brucellosis, etc.).▲ (USP 1-May-2020) In many cases, the chain of custody for animal-derived AMs (i.e., abattoir → intermediate processing center → final processing center) ▲must▲ (USP 1-May-2020) be documented. Vendors of human-derived AMs should ▲▲ (USP 1-May-2020) supply documentation regarding material traceability. For instance, human plasma-derived AMs should be sourced from licensed facilities that control the donor pool and appropriately screen the individual donors for relevant human infectious diseases. ▲▲ (USP 1-May-2020) Vendors of animal- and human-derived AMs ▲may supply different grades of materials, some more suitable for use in CGT product manufacturing than others.▲ (USP 1-May-2020) Many animal- and human plasma-derived components are subjected to ▲validated▲ (USP 1-May-2020) chemical (detergent or solvent treatment) or physical (heat, ▲irradiation, or filtration)▲ (USP 1-May-2020) treatments that have been shown ▲▲ (USP 1-May-2020) to significantly reduce the risk of ▲contamination by▲ (USP 1-May-2020) adventitious microbial or viral ▲agents.▲ (USP 1-May-2020)

## Characterization

▲Quality control and characterization tests for each AM need to be developed (or adopted) and implemented. AM testing should assess a variety of quality attributes, including identity, purity, functionality, and freedom from microbial or viral contamination. The appropriate level of testing for each AM is derived from its risk assessment profile and the knowledge gained during CGT product development. AM test specifications should be established and justified to ensure consistency and performance in the CGT product manufacturing process. Acceptance criteria can be based on data from lots used in preclinical and early clinical studies, lots used for demonstration of manufacturing consistency, and relevant development data, such as those arising from analytical method development and stability studies. AM testing data should be tracked over time for consistency and retained samples should be archived for later analysis, if necessary.▲ (USP 1-May-2020)

Some AMs that are biological in nature may be difficult to fully characterize. Because these materials exert their effects through complex biological activities, and ▲because physicochemical▲ (USP 1-May-2020) testing may not be predictive of the AM's process performance, ▲it is frequently necessary to include▲ (USP 1-May-2020) functional or performance testing. ▲▲ (USP 1-May-2020) Performance variability of ▲AMs▲ (USP 1-May-2020) may have a detrimental impact on the potency and consistency of the ▲CGT▲ (USP 1-May-2020) product. Examples of complex ▲functional▲ (USP 1-May-2020) testing for AMs include growth promotion testing ▲▲ (USP 1-May-2020) of fetal bovine serum (FBS) ▲or human plasma/serum supplements,▲ (USP 1-May-2020) performance testing of digestive enzyme preparations, and in vitro tissue culture cytotoxicity assays (see *Performance Testing*).

## Vendor Qualification

Vendors ▲▲ (USP 1-May-2020) of AM should be qualified at the earliest opportunity. Auditing the vendor's manufacturing facility, including their GMP ▲capabilities▲ (USP 1-May-2020) and AM testing program, are basic elements of a vendor qualification program. A review of the vendor's processing procedures and documentation program is essential in establishing confidence in the vendor as a reliable supplier. Vendors certified through an ISO inspection program or audited by ▲▲ (USP 1-May-2020) governmental agencies ▲typically▲ (USP 1-May-2020) have robust quality systems in place. Reports of past audits of US suppliers obtained through the Freedom of Information (FOI) Act may augment the qualification process.

▲The CGT product developer should establish a written quality agreement with each AM vendor in order to clearly define the roles and responsibilities, including communication channels, change control, audit procedure, and dispute resolution.▲ (USP 1-May-2020) It is important to develop a good working relationship with vendors. In some cases, the vendor may provide higher manufacturing standards, custom formulation services, or replacement of substandard components upon request, with or without additional costs. ▲▲ (USP 1-May-2020) Vendors should be familiar with the principles of validation, especially

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cleaning, viral inactivation, and sterilization validation. It is also critical to ensure that the ▲AM▲ (USP 1-May-2020) vendor takes appropriate steps to prevent cross contamination between ▲different AMs▲ (USP 1-May-2020) during manufacture. Finally, ▲AM vendors should▲ (USP 1-May-2020) supply written certification of processing or sourcing changes to customers, well in advance of the implementation of the changes so that customers can evaluate the potential impact of such changes.

## Quality Control and Quality Assurance

Because the components of the ▲AM▲ (USP 1-May-2020) qualification program are multifaceted and need to be in compliance with cGMP, they should be monitored by a quality assurance/quality control unit (QAU). Typical QAU activities include the following systems or programs: 1) incoming receipt, segregation, inspection, and release of materials prior to use in manufacturing, 2) vendor auditing and certification, 3) certificate of analysis verification testing, 4) formal procedures and policies for out-of-specification materials, 5) stability testing, and 6) archival sample storage.

***Change to read:***

## RISK ▲MANAGEMENT

The evaluation of AM risk to CGT product quality should be based on scientific knowledge, with the ultimate goal of patient protection. ICH Q9 (step 4) "Quality Risk Management" provides a useful approach to risk management principles, process, methods, and definitions. Risk management processes include identifying, analyzing, and evaluating risks, followed by controlling potential risks. Tools or methods, such as a risk evaluation matrix (REM), can quantify risk and facilitate appropriate decision-making and risk acceptance.

Each AM is potentially subject to the risk management process, from selection and qualification to storage and distribution, and use in manufacturing. For reasons described earlier in this chapter, and especially because of the inability to thoroughly remove some AM residuals from the final CGT product, AM selection should incorporate a risk-based approach. Before conducting an AM risk assessment the CGT product manufacturing process must be understood, including identification of the AM's relationship to the CGT product's critical quality attributes (CQAs); these considerations are described in ICH Q11 (step 4) "Development and Manufacture of Drug Substances". Assessment tools can then be used to quantitate each critical parameter, resulting in an assessment of overall risk. For example, if an AM had a suitable safety profile, was used in minimal amounts in upstream steps of the CGT product manufacturing process, and was thoroughly removed from the final CGT product, it would earn a low risk category since both the likelihood of failure (toxicity) and the occurrence severity (consequence to the patient) are low. Conversely, an AM with known toxicity which is introduced downstream would have higher potential to appear as a toxic residual in the final product, and would therefore be assigned to a medium or high risk category. Regardless of the risk category, assessment of AM removal should be completed during CGT product manufacturing process characterization and validation.

As part of the risk assessment process a rational and scientific qualification program should be designed for each AM, taking into account sources and manufacturing processes. Whenever available, AMs that are approved or licensed therapeutic products are preferable because they are well-characterized, possess an established toxicological profile, and are manufactured according to controlled and documented procedures. Incorporating licensed or approved biologics, small molecule drugs, and medical devices or implantable materials into CGT product manufacturing processes presents a more favorable patient safety profile, compared to unapproved versions. Qualification programs for these AMs should reflect the extensive scrutiny to which they were subjected during development and manufacture. Consequently, greater emphasis may then be placed on the impact of inherent AM variability on final CGT product function. For example, if a manufacturer selects approved human serum albumin (HSA) as a cell culture medium supplement for CGT product manufacturing, it may be prudent to assess the impact of the HSA's lot-to-lot variability on cell growth rate and differentiation, its stability during manufacturing, or its interaction with other processing components. Such qualification approaches focus on the AM's variability as it influences final product potency and safety. AM qualification programs should be comprehensive to minimize risks to the consumer and the CGT product.

To aid CGT manufacturers and developers in the design of their AM risk management and qualification programs, potential risk categories are presented as Tiers 1–4 in *Table 1*, *Table 2*, *Table 3*, and *Table 4*, respectively; these are provided as a guide to create an REM. The REM should also consider the amount of the AM used, the stage at which the AM is used in the manufacturing process, and the residual AM remaining in the final CGT product.▲ (USP 1-May-2020)

### Tier 1

▲The AM is a highly qualified material that is well-suited for use in manufacturing of CGT products, such as a licensed biologic, drug, or medical device. Tier 1 AMs generally come with sterile packaging systems or dosage forms labeled with their intended use, and the CGT product developer clearly defines and documents their use within the manufacturing process. Changes to the packaging, dosage form, formulation, or storage conditions must be qualified and documented; these changes may also trigger further AM release testing and stability studies.▲ (USP 1-May-2020)

### Tier 2

▲The AM is a well-characterized material produced under an established quality system well-suited for CGT product manufacturing, but the AM is not a licensed or approved medical product. Many Tier 2 AMs are produced specifically for the manufacture of CGT products. Most animal-derived materials are excluded from this category.▲ (USP 1-May-2020)

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## Tier 3

▲The AM is either intended for research use, locally produced under laboratory conditions, or not intended for use in CGT product manufacturing; it may be approved by regulatory agencies as part of an in vitro diagnostic device. Tier 3 AMs require more qualification than Tier 1 or Tier 2 materials. Using a risk-based approach, an AM manufacturing process upgrade may be necessary to fully qualify the AM for CGT product manufacturing. For example, robust viral inactivation and removal steps may need to be added to the production process for a diagnostic-grade monoclonal antibody in order to produce a reagent suitable for CGT product manufacturing. The manufacturing process for an AM produced in an academic or pilot-stage manufacturing facility will likely need to be upgraded for late-stage clinical trials and eventual commercialization of the CGT product.▲ (USP 1-May-2020)

## Tier 4

▲The AM is produced for industrial or research use and may contain harmful impurities, and/or may contain animal- or human-derived components harboring adventitious agents. High-risk Tier 4 AMs require extensive qualification before they may be used in CGT product manufacturing. These materials may require one or more of the following measures:

1. An upgrade of the AM manufacturing process
2. Selection, screening, and testing of the animal or human sources
3. Testing of each AM lot to ensure that it is free of adventitious agents or specific contaminants
4. Treatment of the AM to inactivate or remove adventitious agents or specific contaminants
5. Validation of the CGT product manufacturing process to assess consistency of removal of a known toxic substance, and/or lot-release testing to demonstrate safe residual levels
6. Validation of the CGT product manufacturing process to demonstrate removal or inactivation of adventitious agents or specific contaminants

For Tier 4 AMs deemed critical to the CGT product manufacturing process, CGT product developers should explore alternative AMs or sources as early as possible in development.▲ (USP 1-May-2020)

**Table 1. AM Risk Tier 1 ▲Materials Intended for Use as Approved Biologics, Drugs, or Medical Devices▲** (USP 1-May-2020)

| Example | Typical Use in CGT Product Manufacturing | Qualification or Risk Reduction ▲▲ (USP 1-May-2020) |
|---|---|---|
| Recombinant insulin for injection | ▲Chemically defined basal cell culture medium▲ (USP 1-May-2020) | • DMF cross reference (when possible or practical)<br>• Certificate of analysis<br>• Assess lot-to-lot effect on process performance[b]<br>• Assess removal from final product<br>• ▲Assess AM stability as stored and used in CGT product manufacturing[c]<br>• Visual inspections<br>• Assess particulates and extractables▲ (USP 1-May-2020) |
| Organ ▲transport/▲ (USP 1-May-2020) preservation fluid | Process biological fluid employed in tissue transport or processing | |
| Human serum albumin for injection | Cell culture ▲additive▲ (USP 1-May-2020) | |
| Sterile fluids for injection | Process biological fluid employed in tissue transport, cell processing, purification | |
| Implantable ▲materials▲ (USP 1-May-2020) (collagen, silicone, polyurethane constructs intended for surgical implantation) | Scaffolds, matrices for immobilized cellular cultivation | |
| Recombinant deoxyribonuclease for inhalation or injection | Process enzyme employed in viral vector manufacturing, stem cell processing | |
| Injectable antibiotics[a] | ▲▲ (USP 1-May-2020) Biopsy transport fluid additive to reduce risk of bacterial contamination | |
| Injectable monoclonal antibodies | Immunologically targeting specific cell populations for selection or removal | |
| Injectable cytokines, ▲vitamins, chemicals, nutrients▲ (USP 1-May-2020) | Cell culture ▲additive▲ (USP 1-May-2020) | |
| ▲▲ (USP 1-May-2020) | ▲▲ (USP 1-May-2020) | |
| IV bags, transfer sets and tubing, cryopreservation bags, syringes, needles | Storage vessels or container–closure systems, closed aseptic transfer systems | |

[a] Beta lactam antibiotics should not be used as AMs due to the risk of patient hypersensitivity.
[b] Beta lactam antibiotics should not be used as AMs due to the risk of patient hypersensitivity. See *Performance Testing*.
[c] Often AMs are aliquoted or stored at different concentrations, in different buffers, or under conditions that are different from those stated on the label or previously validated. Data should be generated that demonstrate the stability and preservation of activity of the AM under the conditions that are specific to the manufacturing application.

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**Table 2. AM Risk Tier 2 ▲Well-Characterized Materials with Intended Use as AMs▲ (USP 1-May-2020)**

| Example | Typical Use in CGT Product Manufacturing | Qualification or Risk Reduction ▲ (USP 1-May-2020) |
|---|---|---|
| Recombinant growth factors, cytokines[a] | Cell culture ▲ (USP 1-May-2020) additive | ▲All of the qualification and risk mitigation activities in Tier 1, plus the following: ▲ (USP 1-May-2020)<br>• DMF cross reference (when possible or practical)<br>• ▲Confirm certificate of analysis test results that are critical to CGT product identity, purity, or potency<br>• Verify AMs containing animal- or human-derived materials have been purified, tested, and certified to be free of adventitious agents▲ (USP 1-May-2020)<br>• Vendor audit |
| Immunomagnetic beads | ▲Cell selection▲ (USP 1-May-2020) | |
| Human AB serum | Cell culture ▲ (USP 1-May-2020) additive | |
| Progesterone, estrogen, vitamins, purified chemicals (USP-grade) | Cell culture ▲ (USP 1-May-2020) additives, induction agents, buffer components | |
| Sterile process buffers | Process biological fluid employed in tissue transport, cell processing, purification | |
| Biocompatible polymers, scaffolds, hydrogels | Scaffolds, matrices for immobilized cellular cultivation | |
| Proteolytic enzymes | ▲Digest tissue or protein▲ (USP 1-May-2020) | |
| Tissue culture media | Cell culture ▲ (USP 1-May-2020) additive | |
| ▲ (USP 1-May-2020) | ▲ (USP 1-May-2020) | |
| Density gradient media | Cell separation via centrifugation | |

[a] ▲AMs▲ (USP 1-May-2020) produced from nonmammalian, recombinant sources (i.e., microbially grown in the absence of animal-derived growth medium components).

▲ ▲ (USP 1-May-2020)

**Table 3. AM Risk Tier 3 Moderate-Risk Materials Not Intended for Use as AMs▲ ▲ (USP 1-May-2020)**

| Example | Typical Use in CGT Product Manufacturing | Qualification or Risk Reduction ▲ (USP 1-May-2020) |
|---|---|---|
| Recombinant growth factors, cytokines | Cell culture ▲ (USP 1-May-2020) additive | ▲All of the qualification and risk mitigation activities in Tier 2, plus the following: ▲ (USP 1-May-2020)<br>• DMF cross reference (when possible or practical)<br>• ▲Confirm certificate of analysis test results▲ (USP 1-May-2020)<br>• Upgrade manufacturing process ▲and/or testing▲ (USP 1-May-2020) for material to ▲be suitable for therapeutic use▲ (USP 1-May-2020) |
| Tissue culture media | Cell culture ▲ (USP 1-May-2020) additive | |
| Monoclonal antibodies ▲(for diagnostic use▲ (USP 1-May-2020) produced in cell culture) | ▲Targeting cells for selection, activation, or removal▲ (USP 1-May-2020) | |
| Process buffers | Process biological fluid employed in tissue transport, cell processing, purification | |
| Novel polymers, scaffolds, hydrogels | Scaffolds, matrices for immobilized cellular cultivation | |
| Proteolytic enzymes | ▲Digest tissue or protein▲ (USP 1-May-2020) | |
| Purified chemicals (reagent-grade) | Culture medium additives, induction agents, buffer components | |

▲ ▲ (USP 1-May-2020)

**Table 4. AM Risk Tier 4 High-Risk Materials**

| Example | Typical Use in CGT Product Manufacturing | Qualification or Risk Reduction |
|---|---|---|
| ▲Animal- and human-derived materials▲ (USP 1-May-2020) | Cell culture ▲ (USP 1-May-2020) additives; ▲targeting moieties for cell selection, activation, or removal▲ (USP 1-May-2020) | ▲All of the qualification and risk mitigation activities in Tier 3, plus the following:<br>• Safety testing for residuals in CGT product<br>• Recombination-competent retroviral testing for relevant gene therapy AMs▲ (USP 1-May-2020)<br>• Adventitious agent testing for animal source-relevant viruses |
| ▲ ▲ (USP 1-May-2020) | ▲ ▲ (USP 1-May-2020) | |
| Animal-derived polymers, scaffolds, hydrogels | Scaffolds, matrices for immobilized cellular cultivation | |
| Purified enzymes | Process ▲additives such as trypsin or collagenase for digesting protein or tissue▲ (USP 1-May-2020) | |
| ▲ ▲ (USP 1-May-2020) | ▲ ▲ (USP 1-May-2020) | |
| Animal or human cells used as feeder layers | Cell culture substratum or ▲conditioned medium▲ (USP 1-May-2020) | |
| ▲Toxic entities (i.e., methotrexate, bacterial toxins, etc.) | Cell culture additives to maintain transgene expression, enhance proliferation, or improve cell survival upon cryopreservation▲ (USP 1-May-2020) | |

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## PERFORMANCE TESTING

▲In cases where an AM is chosen for its effect on a particular biological function of an intermediate or final CGT product, performance testing is an essential element of its overall qualification. This is especially true when the AM plays a critical role in modulating a complex biochemical effect or has a large impact on yield, purity, and/or final product potency. Performance testing is particularly critical for AMs that are complex substances, mixtures, or biologically sourced; these AMs are more likely to show significant lot-to-lot variability, lack a simple identity test, and be difficult to characterize. The development of well-defined performance assays for complex AMs will not only ensure process reproducibility and final product quality, but in many cases will satisfy regulatory requirements for identity testing.

Often, the initial qualification of an AM for use in CGT product manufacturing investigates the dose-response effect of the AM on yield, purity, or potency of the therapeutic product. The optimal amount of AM used in CGT product manufacturing should consistently yield the desired effect, while minimizing residuals. Performance testing frequently utilizes a scaled-down or simulated manufacturing process.

Some examples include:

- For a cellular proliferation AM added to culture medium, the assay could demonstrate that each lot of AM produces the expected rate and degree of cellular proliferation
- For a monoclonal antibody used to purify a particular cell type, the assay could demonstrate that each new lot of monoclonal antibody purifies a standard cell population with the expected recovery and purity
- For a density gradient material used to purify a vector or cell, the assay could demonstrate that each new lot of material purifies the vector or cell with the expected recovery and purity
- For a helper viral vector used to produce a gene therapy vector, the assay could demonstrate that new lots of the helper vector produce the expected amounts of active gene therapy vector

Functional assays may evolve as CGT product manufacturing processes develop, and the critical relationships between AM and final products are better understood.

When performance tests yield relative results, it is helpful to compare each new lot of AM side by side with a reference standard AM, if available. This simultaneous comparison reduces the variability due to different lots of cells or vectors used in testing.▲ (USP 1-May-2020)

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## ▲RESIDUAL▲ (USP 1-MAY-2020) ASSESSMENT AND REMOVAL

▲Though AMs are not intended to be present in the final CGT product, it is often challenging to completely remove AMs without impairing the CGT product yield or quality. To establish appropriate specifications for AM residuals, developers should address both AM removal procedures and AM analytical methods early in development. Developing appropriate residuals specifications and detection methods ensures CGT product and process consistency, allows characterization of the final CGT product, and reduces negative effects on product quality, clinical efficacy, and safety.

Thresholds for AM residuals are established, and may evolve, throughout product development. In early development, the fold reduction in AM concentration is often estimated for each manufacturing unit operation, and AM removal is typically expressed as a dilution factor or log reduction factor. In late development, direct residuals measurements utilize validated assays. However, all AM residuals measurements must be interpreted with some caution, because intracellular and extracellular AM concentrations may differ.

As a part of CGT product manufacturing process validation, spiking experiments with high AM concentrations may be required to mimic a worst case scenario and demonstrate manufacturing process robustness. Validation studies should incorporate the following considerations:

1. Residuals assay should accurately quantitate the AM in each sample matrix
2. If a scaled-down validation is conducted, the comparability of the small-scale and full-scale processes must be demonstrated
3. In spiking studies, the higher AM levels must not interfere with the purification process

Specifications for residual AM in the final CGT product are typically based on the residual AM in the CGT product lots used in toxicological or clinical studies, or on known toxicological data.▲ (USP 1-May-2020)

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## CONCLUSION

▲AM quality is critical to CGT product quality. AMs must be carefully selected, characterized, and qualified, so that they perform consistently and as intended in the CGT product manufacturing process. AM qualification programs should focus on developing appropriate AM specifications, robust CGT product manufacturing processes, and AM risk assessments and mitigations, in order to prevent negative impacts on CGT product quality, clinical efficacy, and patient safety.▲ (USP 1-May-2020)

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# APPENDIX

## Lists of Relevant *USP* Chapters and Regulatory References

AMs used in CGT products will be regulated in the context of the manufacturing process of the CGT products. ▲▲ (USP 1-May-2020) The following lists of documents include relevant regulatory ▲and technical guidances on▲ (USP 1-May-2020) product and process development, manufacturing, quality control, and quality assurance.

***USP* Chapters**

- *Biological Reactivity Tests, In Vitro* ⟨87⟩
- *Biological Reactivity Tests, In Vivo* ⟨88⟩
- ▲*Cell-based Advanced Therapies and Tissue-based Products* ⟨1046⟩▲ (CN 1-May-2020)
- ▲*Gene Therapy Products* ⟨1047⟩▲ (USP 1-May-2020)
- *Biotechnology-Derived Articles—Amino Acid Analysis* ⟨1052⟩
- *Capillary Electrophoresis* ⟨1053⟩
- *Biotechnology-Derived Articles—Isoelectric Focusing* ⟨1054⟩
- *Biotechnology-Derived Articles—Peptide Mapping* ⟨1055⟩
- *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* ⟨1056⟩
- *Biotechnology-Derived Articles—Total Protein Assay* ⟨1057⟩

**Code of Federal Regulations (CFR)**

| ▲Regulations | Title |
|---|---|
| 21 CFR 211 Subpart E, 211.80 through 211.94 and 211.101 | Control of Components and Drug Product Containers and Closures |
| 21 CFR 312 | Investigational New Drug Application |
| 21 CFR 314 | Applications for FDA Approval to Market a New Drug |
| 21 CFR 801.109(b)(1) | Labeling—Prescription Devices |
| 21 CFR 807 Subpart E, 807.81 through 807.97 | Premarket Notification Procedures |
| 21 CFR 812 | Investigational Device Exemptions |
| 21 CFR 814 | Premarket Approval of Medical Devices |
| 21 CFR 876.5885 | Tissue Culture Media for Human Ex Vivo Tissue and Cell Culture Processing Applications▲ (USP 1-May-2020) |

**FDA Guidance Documents**

- ▲▲ (USP 1-May-2020)*Guidance for Industry: Monoclonal Antibodies Used as Reagents in Drug Manufacturing*, ▲March 2001. https://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Blood/ucm080417.pdf.▲ (USP 1-May-2020)
- *Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals*, ▲May 1993. https://www.fda.gov/downloads/biologicsbloodvaccines/safetyavailability/ucm162863.pdf.▲ (USP 1-May-2020)
- *Class II Special Controls Guidance Document: Tissue Culture Media for Human ex vivo Tissue and Cell Culture Processing Applications—Final Guidance for Industry and FDA Reviewers*, May 16, 2001. ▲https://www.fda.gov/RegulatoryInformation/Guidances/ucm073567.htm.
- *Guidance for Industry: Contract Manufacturing Arrangements for Drugs: Quality Agreements*, November 2016. https://www.fda.gov/downloads/drugs/guidances/ucm353925.pdf.
- *Guidance for Industry and Food and Drug Administration Staff: Use of International Standard ISO 10993-1, "Biological evaluation of medical devices—Part 1: Evaluation and testing within a risk management process"*, June 16, 2016. https://www.fda.gov/downloads/medicaldevices/deviceregulationandguidance/guidancedocuments/ucm348890.pdf.▲ (USP 1-May-2020)

**National and International Regulatory Documents**

- ICH Q5A: Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human and Animal Origin. ▲Available at: http://www.ich.org.▲ (USP 1-May-2020)
- ICH Q5D: Derivation and Characterisation of Cell Substrates Used for Production of Biotechnological/Biological Products. ▲Available at: http://www.ich.org.
- ICH Q8(R2): Pharmaceutical Development. Available at: http://www.ich.org.
- ICH Q9: Quality Risk Management. Available at: http://www.ich.org.▲ (USP 1-May-2020)
- Public Health Service Guideline on Infectious Diseases Issues in Xenotransplantation. ▲Available at: https://www.cdc.gov/.
- Health Canada "Guidance Document: Preparation of Clinical Trial Applications for use of Cell Therapy Products in Humans". Available at: https://www.canada.ca/en/health-canada.html.▲ (USP 1-May-2020)

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# ⟨1044⟩ CRYOPRESERVATION OF CELLS

## INTRODUCTION

Cryopreservation is the process of cooling and storing cells, tissues, or organs at very low temperatures to maintain their viability. The purpose of cryopreservation is to bank the cells and allow their future use in in vitro or in vivo applications for which post-thaw function is sufficiently representative of the cells' prefreeze function. Cryopreservation also minimizes the risk of genetic mutation or development of subpopulations due to cell replication. Depending on the application, sufficient postcryopreservation function may be assessed by the ability to divide, proliferate, differentiate, express genes, or to produce proteins, or by another specific functional property.

This chapter presents best practices for cryopreservation, maintenance, and use of a wide range of cells, cell therapy products, and cell banks derived from a variety of sources including human, animal, and microbial cultures (the chapter also contains an *Appendix* with additional guidance documents that are useful for particular cell types and applications). Cryopreserved cells provide a ready source of viable cells that can be used, either directly or indirectly, for the purposes of diagnostic tests, therapy, manufacture of drug products and vaccines, and for bioassays used to evaluate the potency of therapeutic drugs and vaccines. In some cases the cells themselves, after cryopreservation and thaw, constitute the patient therapy, and in other cases the cells are propagated or otherwise manipulated ex vivo in order to generate the product (e.g., a culture-expanded cellular therapy, a therapeutic protein, a monoclonal antibody, or a vaccine). In all cases, proper cryopreservation is essential for retention of required cellular properties and, ultimately, for application toward the advancement of patient therapies.

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## PRINCIPLES OF CRYOPRESERVATION

### Overview

Understanding the role of water and the need to adequately remove it from cells or abrogate its ability to form ice crystals, which damage the cell membrane, is critical to successful cryopreservation. When cells are frozen in aqueous suspension, often they are destroyed. However, in the 1940s Polge and others discovered the cryoprotective properties of glycerol. Since then several chemicals, generically called *cryoprotectant agents* (CPAs), have been identified. The mechanism of action of CPAs is complex and is not fully understood. However, according to the commonly accepted theory of *colligative* action, CPAs increase solute concentration both within the cell and extracellularly, thereby suppressing ice formation. For this purpose, the so-called penetrating (or intracellular) CPAs [e.g., dimethylsulfoxide (DMSO), glycerol, propanediol, and methanol] must be able to cross the cell membrane readily and penetrate the cell without significant toxicity. There also is a group of nonpenetrating (or extracellular) CPAs (e.g., sucrose and trehalose) whose mechanism of action is thought to be related at least in part to their stabilizing interaction with cell membranes. This property also may explain the cryoprotective activities of certain large molecular weight compounds such as hydroxyethyl starch and polyvinylpropylene. Theoretical models of cryoprotection typically evoke the colligative theory, but full explanation of CPA action is yet to be established.

An alternative form of cell preservation, commonly called *vitrification,* whereby the cell suspension is loaded with high levels of penetrating CPAs (often several in combination), induces a glass-like state in which cellular and extracellular water cannot readily form ice crystals. When cell suspensions prepared in this way then are cooled very rapidly (cooling rates of 100°–1000°/min or more) the extreme viscosity prevents osmosis, and the water molecules are unable to form ice. This procedure has been widely used for complex structures including a variety of human, plant, and animal tissues and may help preserve those cell preparations that have variable degrees of cellular permeability or when standard cryoprotection cannot deliver the range of conditions required to optimally preserve viability in all the tissues' component cell types.

CPAs have biological activities beyond their cryoprotective properties. Some, like DMSO, can affect the cell membrane, cytoskeleton, and gene expression and may be toxic to cells following prolonged exposure. Therefore, during development of new cryopreservation protocols analysts should perform a toxicity assay in which the cells are exposed to the CPA over a range of time intervals to evaluate loss of viability or alteration of functionality.

### Key Elements of Cryopreservation Practice

For any cellular sample or therapeutic product being cryopreserved, method development should address the following elements:

#### PREFREEZE PROCESSING AND CHARACTERIZATION

Optimizing the condition of the cells immediately before cryopreservation is critical to a successful outcome. The nature and extent of prefreeze processing depends on the state of the original cells harvested for preservation, the composition of the cell suspension, and the specific processing steps leading into cryopreservation. Prefreeze processing may include selection of subpopulations, ex vivo expansion, or incubation with activating or priming factors.

Precryopreservation characteristics and identity should be established during early process development. For cell banks in particular, the cell status and optimal growth conditions, as well as documented history (with traceability to a qualified cell bank or acceptable source), characteristics, and authenticity should be documented. Cell status and history typically are described in terms of the nature and number of manipulations and culture passages from the primary cells or original isolate. Finite or primary cells usually are cryopreserved at an early passage to maintain integrity of the original tissue, but continuous

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cell lines may be cloned and expanded, ensuring a homogeneous cell population. It is recommended to prepare cell banks from a single preparation or expanded population of cells since it is often necessary to pool cells for freezing from multiple culture vessels. Cells from cultures with different passage histories and certainly from different donors should not be pooled. In both cases, analysts should maintain detailed records of the procedures.

To prepare for cryopreservation of cultured cells, cells should be harvested during exponential or the most rapid phase of growth and before the culture enters stationary phase. Harvesting cells during this phase ensures that the cells are most viable and uniform. The optimal concentration of cells will depend on the cell type, purpose, and best recovery. Typically this lies between $10^6$ and $10^7$/mL for manufacturing cell banks but may be different for other purposes. Complete growth medium renewal a day before cell harvest also can be beneficial. Additionally, most cell suspensions benefit from washing by centrifugation and resuspension in an isotonic medium to a specific cell concentration. Prefreeze processing should not result in cells that are stressed before the start of the freezing process, or cell losses during freezing or after thaw will be higher than expected.

Optimizing the growth conditions of a cell line or primary cells is important to maintain high viability of the cells in culture. Typically, cells growing actively and in exponential phase have a low cytoplasm to nuclear volume ratio, which is conducive to successful cryopreservation with penetrating cryoprotectants. Suboptimal or improper culture conditions may result in lower viability and cell states that will be less robust for preservation and recovery. The culture medium should be optimized and the same medium should be used throughout all experiments, and each batch of animal-derived materials (e.g., serum) and other culture reagents should be qualified (e.g., see the 2010 WHO guidance and the FDA 2010 guidance referenced in the *Appendix*). If possible, it is recommended to not use animal-derived components in the culture medium particularly for cells used for therapy or as manufacturing substrates.

Per the WHO 2010 guidance and based on a risk assessment, either the Master Cell Bank (MCB) or the Working Cell Bank (WCB) must be tested for adventitious agents. Ideally, samples of cells should be tested for adventitious agents before freezing. The specific testing regimen for potential microbial or viral contamination of cells depends on the donor source, the culture history, and the intended use. Detailed records of the cell history should be maintained as a basis for appropriate risk assessment to direct supplementary testing that may be required (e.g., exposure to bovine viruses in bovine serum albumin). Specific regulatory requirements for testing of cells, or donors of the cells, for products intended for a particular use (e.g., cell therapy or vaccine manufacture) are based on past experience regarding key agents that must be included or considered. *USP* general chapter ▲*Cell-based Advanced Therapies and Tissue-based Products* ⟨1046⟩▲ (CN 1-May-2020) contains guidance about sterility and safety testing requirements for cell therapy products.

## REAGENTS AND CONTAINERS

All cryoprotectants, containers, etc., should be fit for purpose as indicated in relevant regulatory guidances. Sterile, single-use, disposable plastic bags, cryovials, or straws are customarily used for cryopreservation. Manufacturers' specifications should be carefully reviewed to ensure that the material used to manufacture the cryocontainer is appropriate for use at the storage temperature, is chemically compatible with the contents, minimizes the potential for leachables and extractables, and assures container closure integrity. If straws are used, then primary or secondary containment during storage is important to prevent direct contact of the preserved cells with liquid nitrogen. Cryovials should be selected based on their ability to provide adequate cell bank integrity.

Preservation of cells typically requires the use of specialized solutions that contain a base (typically an isotonic saline-based solution) with CPAs (most commonly DMSO but sometimes glycerol) and sometimes proteins (fetal bovine serum, human serum or plasma, conditioned medium, or human albumin). The optimum composition for different cell types may need to be determined.

The types of vials, labels, ink, or markers used should withstand extreme liquid nitrogen temperatures. The markings on the label should be legible and barcoded if possible. The minimum information on the label should include name or description of cell population, date of cryopreservation, lot number, and passage number if needed. Since most cryolabels are very small, additional information can be included on associated documentation. In certain applications it may also be necessary to sequentially number vials within a single lot as part of the minimum information on the label, to enable better control over movement of vials from a single bank, and to identify sectors of the bank which may have received different cryopreservation conditions.

## ADDITION OF CRYOPROTECTANT SOLUTION

Cryoprotectant solutions typically are hypertonic and are not physiological. For example, a 10% DMSO solution used commonly in cell preservation has a concentration of approximately 1.4 osmolarity (Osm/L). Cells introduced into this type of solution rapidly dehydrate as water leaves the cell in order to reduce the difference in osmotic potential between the inside and outside of the cell. DMSO slowly permeates the cell to re-equilibrate. This may cause excursions in volume that can result in a loss of cell viability. Therefore, cryopreservation solutions commonly are added to a cell suspension in stepwise additions or gradually (e.g., using a syringe pump) or slowly dispensing down the side of the container to prevent cell losses resulting from osmotic stress. The method for introducing or removing a cryopreservation solution should be developed and evaluated for its impact on cell viability and functionality.

In the case of DMSO, a large latent enthalpy of mixing results in sample heating when the two solutions are mixed. This heating can be high enough to damage the cells, so solutions that contain DMSO commonly are precooled before mixing. Prechilling the solution reduces heating associated with mixing of the solution, reduces the osmotic volume changes that the cells experience, and reduces cell losses associated with exposure to DMSO. The time that cells are exposed to the cryoprotectant, prior to freezing, should be limited and the maximum time allowable, without deleterious effects, should be determined during development work for routine use.

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## COOLING

Two different types of freezing typically are used for cells: controlled-rate cooling (using programmable freezers) and passive cooling (including use of insulated containers). Controlled-rate freezers are attached to liquid nitrogen supplies. The temperature of the chamber should be controlled by increasing or decreasing the flow of cold nitrogen gas into the chamber according to a preprogrammed step. Controlled-rate freezing protocols typically involve several steps, each of which should be evaluated and qualified for a specific cell type.

The use of controlled-rate freezing provides more precise control of the freezing environment and therefore may provide more consistent (and higher) post-thaw recovery for cells that may have a narrow range of cooling rates associated with maximum survival or cells that are sensitive to the temperature at which ice forms in the extracellular solution. Temperature probes placed near the cells being frozen, or in a mock cell suspension that undergoes cryopreservation simultaneously, are used to monitor the freezing process and to provide process control. If release of the latent heat of fusion is delayed or poorly controlled, cells undergoing cryopreservation may be damaged and may have diminished viability after thaw.

Disruption of the controlled-rate freezing during the protocol may occur and typically is caused by failure of a valve in the controlled-rate freezer or cryogen. Protocols for handling disruption of the freezing process and backup plans should be prepared.

Passive freezing involves placing a product in a freezer (about –80° or –150°) and permitting the sample to cool in an uncontrolled fashion. Insulation or specially designed boxes are used to slow the cooling rate for the sample. The average cooling rate achieved for the majority of the process and the consistency of freezing curves should be evaluated and qualified for purpose. In general, control of the thermal environment during freezing results in improved post-thaw recovery, but certain cells exhibit comparable post-thaw recovery when they are passively cooled.

## CRYOGENIC STORAGE, SAFETY, AND TRANSPORT

After the freezing process has been completed, products are transferred from controlled-rate or mechanical freezers to cryogenic storage units. Some microbial cell cultures can be suitably maintained in mechanical freezers but this should be demonstrated. Sample warming should be minimized during transfer of the cell product from the freezing device to storage. Cold tables or insulated transfer devices can be used to minimize warming during transfer. Newly cryopreserved cells commonly are placed in a quarantine cryogenic storage unit before completion of testing for adventitious agents. After testing, cells that test negative for adventitious agents can be released for transfer into long-term cryogenic storage units.

The inventory system (or repository) for the maintenance of the cryopreserved cells should be designed for easy access to minimize specimen handling, and the number of times per day that a repository is accessed should be limited because exposure to warmer temperatures may compromise cell viability and, consequently, longer-term stability. Cell banks (e.g., MCBs) or other cell cultures that are accessed infrequently should be stored separately from WCBs or other cell cultures that are accessed more often. Frequent retrieval from the cell bank/culture may cause shifts in temperature. This activity must not compromise the long-term stability and performance of the infrequently used cell bank/culture. It is also valuable to divide a bank and store it in multiple locations to decrease risks due to a catastrophic event at a particular site.

When storing cryopreserved cells, analysts should ensure that the storage temperature does not rise above a critical temperature called the glass transition temperature. For long-term storage of fastidious specimens such as cell lines and primary cell cultures, this critical temperature is not warmer than –130° for nonclinical specimens or not warmer than –150° for clinical material (to give an adequate margin of error) in the vapor phase of the liquid-nitrogen freezer. Liquid-nitrogen freezers are prone to temperature gradients in the vapor phase based on the shape and design of the freezer and the level of liquid nitrogen. Although storing cryopreserved cells in liquid nitrogen prolongs longevity, hazards associated with unsuitable containers or container use (e.g., exploding vials and rupturing bags) have prompted greater use of nitrogen vapor phase storage. Liquid-nitrogen vapor phase provides a more convenient and safe environment for vial retrieval. If the liquid-nitrogen freezer is suitably configured, the working temperature in the vapor phase is commonly –150° or colder. The liquid-nitrogen freezer should be qualified, and the temperature of the vapor phase should be routinely checked to ensure that the temperature does not become warmer than –130° for cell lines or other frozen material or warmer than –150° for material used for clinical applications (e.g., cell therapies).

Temperature-monitoring systems should permit recording and storage of temperature history for quality control purposes. Storage units should be attached to alarms and facility monitoring systems. Critical storage units should be equipped with a multilevel alarm system to ensure backup in the monitoring and response. The storage units should be routinely monitored for temperature failure caused by power disruption and any other potential malfunctions. In the event of equipment or power failure, backup refrigeration should be available.

Proper operation of a repository requires monitoring of temperature and liquid-nitrogen levels and automatic filling. In addition, it is recommended to have a backup for emergency cooling (e.g., empty backup cryogenic storage) in case of freezer failure.

Only individuals who are trained for this purpose should access cryopreserved products or samples. In some cases, verification by a second person is required for source traceability. Personnel assigned to the implementation of the protocols should be trained in standard operating procedures (SOPs). Sample tracking systems that incorporate computer software and sometimes barcoding for identification, logging, and tracking of frozen samples are particularly useful for large sample repositories and may facilitate rapid retrieval of samples and minimize time that the entire repository is exposed to the risk of temperature excursions. In addition, it is recommended that all changes to cryostorage inventories be recorded in log books near the storage unit.

Products and samples such as primary cells, cell lines, and cell therapy products routinely are shipped among sites of collection, processing, storage, and use. Cryopreserved cells typically are shipped in liquid-nitrogen vapor shippers with temperature-monitoring systems to ensure that the unit does not become warmer than –130° for cell lines and –150° for clinical material during the shipping process. Shipping containers are subjected to significant vibrations and mechanical stresses during

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shipment and should be evaluated on a regular basis for proper function. Shipping validation studies should cover worst case scenarios and include temperature monitors for critical materials.

Cryopreserved cells, whether shipped nationally or internationally, should be transported using local postal, US Department of Transportation, and International Air Transport Association guidelines. Packages also should meet other regulatory requirements for quarantine, biosafety, and biosecurity. Cryopreserved cells should be retrieved, packed, and shipped in a manner that does not interfere with the integrity of the cells. For most cryopreserved cells, shipping in dry ice for short duration may be adequate, but the shipping process should be validated, shown to have no adverse impact on the cells, and temperature monitors should be included. However, some cells may require shipping in liquid-nitrogen vapor phase (Dewars). Prevalidation of the shipping methods may be required to determine the best option and prevalidation risk assessment should be performed even if only one option for transport is being considered. With the shipment, shippers should include instructions for proper storage upon cell receipt.

## THAWING

Cells frozen using conventional methods (controlled rate or passive freezing) or by vitrification should be thawed as rapidly as possible, and the thawing process starts as soon as the frozen sample is removed from storage. Slow warming rates result in recrystallization damage or exposure of the cells to high extracellular concentrations of CPAs, either of which can result in cell death. For each cell therapy product and cell line the most appropriate thawing procedure (temperature, gradient, and time) needs to be developed. These products and cell lines typically are thawed in a warm-water bath or for therapeutic cell preparations in a bead bath or thermoblock. The water bath should be cleaned regularly and should contain sterile water or *Water for Injection*. The temperature of the bath also should be monitored. Many clinical labs use plastic overwrap bags to hold the primary container during rapid thaw to reduce the risk of product contamination in case the integrity of the inner container is impaired. Alternatively, warm bead baths (usually approximately 37°) can be used to reduce contamination risks. Thawing rates should be as rapid as possible (>1°/s for most mammalian cells). Increasing bath temperatures above 42° to increase the warming rate must be done with extreme caution because hyperthermic temperatures can damage cells, inducing necrosis or apoptosis.

## POST-THAW PROCESSING AND EVALUATION

Because cryopreservation solutions are not physiological, it is not uncommon for some post-thaw processing to be performed. For cells preserved in DMSO, cells typically are washed or diluted immediately post-thaw because this CPA is harmful in particular to frozen and thawed cells. Cells are more sensitive to expansion than contraction, so CPA removal or dilution protocols must be carefully optimized to prevent cell losses from dilution or removal.

Quantifying the viability of cells post-thaw is important and may be performed by a variety of methods depending on the post-thaw requirements of the cells. Minimum viability limits should be set based on experience and thawed products with viabilities below the set limits should be discarded. The process of cryopreservation subjects the cells to significant stresses that can alter metabolic function, membrane structure, etc. Therefore, development and validation of suitable post-thaw assays are critical. Post-thaw function is most commonly assessed using physical integrity (e.g., membrane integrity), metabolic activity, mechanical activity (attachment or contraction), mitotic activity, or engraftment potential. The selection of assay depends strongly on the desired post-thaw function of the cell. Membrane integrity is used most often. Today, dyes like trypan blue are used less often to measure post-thaw physical integrity because the dye is difficult to validate on frozen and thawed cells. The method to test viability needs to be carefully selected and qualified for the particular cell type being measured with a protocol that specifies diluents and time. Fluorescent dyes are used with increasing frequency to determine cells' post-thaw physical integrity. Rigorous methods of post-thaw assessment typically involve multiple measures of cell viability and, in particular, at least two independent assays to measure post-thaw viability. For example, post-thaw attachment and proliferation commonly are used to evaluate viability. As the complexity of the desired cell function after thawing increases, so do the demands on post-thaw assessment. For example, post-thaw assessment of stem cells may require assay of membrane integrity as well as proliferation and the ability of the cells to differentiate into different lineages post-thaw. Post-thaw assays should be carefully developed and validated to avoid measurement bias. A certain fraction of cells will lyse during freezing, and methods of measuring cell recovery should include a complete assessment of cell losses (cells that have lysed as well as cells that are intact but not viable). Stability of cryopreserved cells can be assured by periodically thawing and testing a vial of the cells (also see ICH Q5D).

Testing for adventitious agents after the preparation of MCBs and WCBs should be routine, and *USP* general chapters *Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin* ⟨1050⟩ and *Virology Test Methods* ⟨1237⟩ provide additional testing guidance (see also the FDA 2010 guidance cited in the *Appendix*). Testing for mycobacterial contamination, which may not be isolated in standard sterility testing, also can be considered for some cell substrates. Representative vials should be retrieved and tested for contamination (bacteria, fungi, *Mycoplasma,* and viruses). Numerous well-established methods are available for detecting *Mycoplasma* in cell cultures (see *USP* general chapter *Mycoplasma Tests* ⟨63⟩).

The batch record should be detailed, including the history of the cells and all activities starting from their receipt to release of the cell banks or products for use. The record should include detailed information about the cryopreservation process, including the procedure, the equipment used (with unique identifier), and a printed record of the freeze profile. The viabilities of the cryopreserved cells should be monitored over time to ascertain the effectiveness of the freezing process and the storage conditions. The information captured in each batch file of a cell line must be traceable to the original source, and all documents should be maintained and updated according to the quality management system in place.

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# CRYOPRESERVATION OF HUMAN CELL THERAPY PRODUCTS

Cell preservation methods are used to ensure product stability during hold, storage, and transport steps for a wide range of human cell-based products (additional information is found in ▲*Cell-based Advanced Therapies and Tissue-based Products* ⟨1046⟩▲ (CN 1-May-2020)). Cells can be preserved in liquid suspension for up to a few days, but quantitative and qualitative changes in the cellular product invariably occur over time. Although preservation in the frozen state also affects the cellular product, it allows more predictable preservation of specific product characteristics over much longer time intervals.

For any given cell product, the decision to use cryopreservation depends primarily on the timing of final product administration in relationship to cell source collection and product manufacturing steps. Many patient-specific autologous and allogeneic products are maintained in liquid suspension, without cryopreservation, from starting cell source through final formulation and are released as fresh products after a relatively short time. However, many clinical applications require cryopreservation of the cell source, intermediate products, or final product. In these cases, cryopreservation can permit optimization of workflow during manufacturing, completion of lot-release testing, maintenance and management of a product inventory, transport of the product to the clinical site, and coordination of product administration with the patient's medical or surgical regimen. For example, umbilical cord blood is cryopreserved and stored in public banks for subsequent transport to, and temporary storage at, clinical transplant centers, where it is thawed immediately before infusion into a patient.

Development of a cryopreservation process for a clinical cell therapy must consider the consequences for the product, the patient, and the overall feasibility of the therapy. For the cell product, the manufacturer must ensure that expected cell losses because of cryopreservation and thaw occur in a manner that is reasonably predictable and must ensure that the final product administered to the patient will meet specifications for cell number, viability, and functional characteristics.

For the patient, cryopreservation may affect the efficacy and safety of the final product. For example, the cryoprotectant DMSO is associated with risk of predictable dose-dependent gastrointestinal, cardiovascular, and neurologic toxicities that typically are ascribed to histamine release. Residual DMSO in the final product should be estimated or measured. DMSO is categorized by ICH as a Class 3 (relatively low risk) solvent or excipient in pharmaceutical products, and amounts of up to 50 mg/day, or less, are considered acceptable without justification (see ICH Q3C). Cryopreserved cellular products frequently contain 10–20 times this amount unless they are washed after thawing. Even higher amounts of DMSO may occur with administration of multiple cryopreserved products, and this occurs commonly with autologous peripheral blood stem cell transplantation. A DMSO limit of 1 g/kg recipient weight/day is commonly used in clinical cell therapy practice. Procedures to prevent DMSO toxicity also should be considered. It is common clinical practice to premedicate patients with diphenhydramine or other antihistamine agents to prevent DMSO toxicity. Product washing methods by either manual (centrifugation) or automated methods also can be considered but must be validated to ensure adequate postwash recovery and cell function.

Use of cryopreserved cell therapy products may require the clinical site to receive, store, thaw, and perform other final preparation steps on the cryopreserved product. Feasibility assessment requires consideration of the site's capabilities with regard to specialized personnel, training, equipment, and facilities to execute those tasks.

If cryopreservation is planned as part of the manufacturing process, development teams must consider the effect of cryopreservation on cell number and characteristics and should require reliable methods for cell enumeration and assessment of cell viability and function. During development runs, more assays often are performed than will be required eventually for in-process and final product testing. This is done in order to evaluate the effects of each manipulation of the product. Because some cells may be more susceptible than others to freeze–thaw damage, these studies should include assessment of selective losses of important cell subpopulations within the product.

As described in the *Introduction,* several critical processes influence the outcome of a cryopreservation protocol. Following is a brief discussion of issues unique to cell therapies.

## Prefreeze Processing

If cells are harvested with plasma present, samples should be properly processed with an anticoagulant to prevent clotting. In addition, some cells are prone to clumping or aggregation when centrifuged, and some cell products may exhibit excessive damage or loss of one or more populations within the product. Cells harvested from adherent or nonadherent culture may include substantial numbers of dead or fragile cells. Therefore, centrifugation and wash steps should be optimized and specific for the product's cellular contents, suspension volume, suspension medium, and container, along with appropriate evaluation of the cellular product before and after these manipulations. Prefreeze processing should not result in cells that are stressed (e.g., cells that demonstrate elevated early apoptotic markers or temperature-shock responses) before the start of the freezing process, or cell losses will be higher than expected.

## Reagents and Containers

Clinical-grade reagents and containers should be used whenever possible (see *Ancillary Materials for Cell, Gene, and Tissue-Engineered Products* ⟨1043⟩). It is customary to use sterile, single-use, disposable plastic bags or cryovials that have been qualified for the specific cryopreservation process and subsequent storage conditions. Cryopreservation media for cell therapy products usually consist of isotonic saline-based solutions with one or more CPA, typically the intracellular cryoprotectant DMSO at 5%–10% final concentration with or without an extracellular cryoprotectant such as hydroxyethyl starch. The use of human-derived protein additives such as human serum albumin, serum, or plasma is common, but they may need extensive qualification, so they should be avoided if possible and alternatives should be evaluated. Additives such as animal-derived heparin, citrate-based anticoagulants, and DNase sometimes are used. Many centers formulate their own cryopreservation media, but commercial cryopreservation media, which typically include 5%–10% DMSO and other proprietary components,

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increasingly are used by cell therapy manufacturers to eliminate variability and the need for additional qualification activities associated with local formulation.

## Addition of Cryoprotectant Solution and Cooling

Procedures for introduction or removal of a cryopreservation solution should be assessed before freezing to ensure that cell losses resulting from this step are minimized. The cryopreservation medium usually is added to the cell suspension in steps or gradually (e.g., using a syringe pump) to prevent cell losses resulting from osmotic stress. It is common to prechill the cryopreservation medium and keep the cell suspension and the admixture chilled using cold packs, a frozen blanket, or a chilled work surface to prevent heat-related cell damage during addition of DMSO. Once the cryopreservation medium is added, the cell suspension typically is transferred to the precooled chamber of a controlled-rate freezer. During the freeze process, a record of chamber and product temperature over time, or freeze curve, is generated for inclusion in the production record. Product temperatures can be recorded from a probe placed on the product bag's outer surface or from the inside of a comparable product in a dummy bag or vial that undergoes concurrent freezing.

## Storage and Transport

Cryopreserved cell therapy products typically are stored and transported at temperatures of –150° or colder. FDA requires screening and testing for evidence of transmissible disease only for allogeneic donors of cell therapy products and not for autologous donations. However, many centers test autologous donors as well and segregate products from autologous donors who are known to have transmissible diseases when their products must be stored. A report of hepatitis B cross-contamination of cellular products within a liquid-nitrogen storage tank led to the currently common practices of storage in the vapor phase of liquid nitrogen and the use of overwrap bags to reinforce product containment. Liquid-nitrogen vapor phase storage may be associated with vertical temperature gradients: products at the top of the storage tank may have a warmer temperature than those stored at the bottom of the tank. Temperature gradients should be monitored, and vertical temperature gradients should be minimized, e.g., by use of metal heat shunts. Overwrap bags may reduce the warming rate of the sample if used during the subsequent thaw process, and their use should be qualified as part of the overall cryopreservation process validation.

Transport of cryopreserved cell therapy products usually is accomplished by using dry shippers containing absorbent material that can be charged with liquid nitrogen to maintain vapor-phase temperatures for up to 2 weeks if properly charged. Data loggers are used to document the temperature history during transport. These shipping containers and procedures must be validated before cell therapy products for clinical use are shipped in the containers.

## Warming (Thawing)

Although bedside thawing of cell therapy products before infusion has been a common clinical practice, the use of trained personnel in a controlled laboratory environment is now recognized as the preferred method for thawing because it allows a more standardized process and a higher degree of control when staff must respond to a container failure, which may require product salvage in a more sterile environment. Product thawing typically is done by immersion in 37° water baths and using overwrap bags to minimize product loss and contamination in case of primary container failure. Bags may be gently kneaded during thaw to reduce temperature gradients across the bag and to accelerate thawing. The product is removed from the water bath when some ice is still present in the product but the majority of the product is thawed.

## Post-Thaw Processing

DMSO is toxic to cells in liquid suspension. Toxicity may be reduced by diluting or washing the cell suspension before infusion or further manipulation. Because cryopreserved, thawed cells are more sensitive to volumetric expansion when the cells transition from a hypertonic solution to an isotonic solution, the dilution and washing solutions and methods must be carefully designed and validated. Cell washing using either a conventional centrifuge or an automated device can result in additional mechanical stress to the cells, so cell losses must be assessed by an appropriate method before a specific method is implemented in clinical practice.

## Quality Control Practices

Quality management of cryopreservation, storage, and thaw of clinical cell therapy products must incorporate quality system elements common to current Good Tissue Practices (cGTP) and current Good Manufacturing Practices (cGMP), including personnel qualification, facility controls, document control, control of equipment and materials, label control, and use of validated SOPs (21 CFR 1271, 210, and 211). Quality control practices specific to clinical cryopreservation typically include assessment and documentation of freeze curves for all products, retention of tubing segments and vials for subsequent testing, and regular monitoring of post-thaw product quality. Practices required by cGTPs and applicable to all human cell- and tissue-based products include measures to ensure accurate and complete labeling and records, to ensure that the correct product goes to the patient, and to allow tracking of the cell product from collection to infusion. The practical implications of these requirements are that labeling and records, including inventory systems, for cryopreserved products must be designed to prevent errors in identification of products. Identity verifications of products moving into and out of cryopreserved storage are performed routinely, e.g., with two people checking the product label against records. If necessary for additional verification of product identity, the contents of a tubing segment attached to the product bag can be thawed and tested before thaw of the entire product. ISBT 128, an internationally recognized system for labeling of blood and cell-therapy products, incorporates use of consistent product nomenclature and barcoding of the product from donor source through administration (see *Appendix*).

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## HEMATOPOIETIC STEM CELLS

Studies of the response of hematopoietic stem cells (HSCs) to freezing began in the 1950s, and cryopreserved HSCs have been widely used in clinical practice during the past 30 years. The most common method of cryopreserving HSCs for clinical applications involves the use of 10% DMSO and a controlled-rate freezer set at a cooling rate of 1°/min. Another method, less commonly used, involves the use of passive freezing of the HSC product in a –80° mechanical freezer and the use of 5% DMSO + 6% hydroxyethyl starch solution. Cells commonly are frozen at densities of 30–50 × $10^6$ cells/mL. Exceeding cytocrits of 20% (v/v) has been shown to reduce cell recovery. Post-thaw assessment of the sample consists of enumeration of nucleated cells, viable $CD34^+$ cells, and hematopoietic colony-forming units, along with subsequent calculation of recoveries from corresponding prefreeze values.

HSCs cryopreserved for clinical use may be obtained from bone marrow, mobilized peripheral blood, or umbilical cord blood (UCB). Each source has unique requirements for preservation. For example, peripheral blood progenitor cell products contain larger numbers of cells and may be frozen in multiple bags with relatively high cell concentrations. Protocols for UCB preservation may include the use of syringe pumps to introduce cryopreservation solutions while minimizing osmotic stresses. For UCB, specialized solutions often are used after thaw to dilute or remove DMSO while minimizing osmotic stress for the cells.

## MESENCHYMAL STEM CELLS

Research on the clinical use of mesenchymal stem cells (MSC) has grown rapidly since the mid-1990s. Reliable, safe, and efficient methods of cryopreservation and storage are critical, especially for allogeneic off-the-shelf MSC products manufactured in multiple product doses for treatment of a large number of patients with a range of clinical indications.

Because MSCs traditionally have been generated in cultures that contain fetal bovine serum (FBS), cryopreservation media for these cells often have incorporated FBS. More recently, alternatives to gamma-irradiated FBS are being explored for the culture expansion before cryopreservation, and cryopreservation has been successful in media that contain 5%–10% DMSO and other components without bovine sources of protein. Although there is no consistent method of post-thaw cell processing, some protocols include dilution or washing of the cells to mitigate the effects of DMSO. Emerging clinical applications for MSCs may require repeat dosing of the cellular product, a practice that requires attention to potential immunogenicity of components of the cryopreservation medium, e.g., FBS or other proteins.

Post-thaw assessment of MSCs typically has involved the use of membrane-integrity dyes such as trypan blue, surface antigen expression, and evidence that the cells are capable of multilineage differentiation. Because the mechanism of action of MSCs may involve the immunomodulatory or trophic properties of the cells, post-thaw assessment also should include relevant cell function.

## LYMPHOID CELLS

Lymphocytes are used for a variety of clinical applications including immunotherapy to treat cancer, viral infection, and autoimmune disease. Therapy based on lymphocytes may consist of mixed lymphocyte populations or lymphocyte subpopulations that have been selected or activated ex vivo, e.g., regulatory T cells, natural killer cells, and activated T cells. As with hematopoietic cells, lymphocytes typically are cryopreserved using a 10% DMSO solution and a controlled cooling rate of 1°/min.

Lymphocytes may undergo extensive post-thaw apoptosis, which can influence the clinical efficacy of the cells. Highly purified populations of lymphocytes may exhibit higher levels of post-thaw apoptosis than mixed lymphocyte populations. Strategies such as caspase inhibition and cytokine rescue have been used to diminish post-thaw apoptosis of lymphocytes.

## PRESERVATION OF HUMAN PLURIPOTENT STEM CELL LINES

Pluripotent stem cells (PSCs) are cells that appear to have the capacity to (1) undergo self-renewal and replicate indefinitely and (2) generate cells that are representative of the three germ-layer tissues required to create all cells of the human body as demonstrated by the capacity to generate teratomas in immune-deficient mice. The two predominant types of stem cell line used for in vitro laboratory research are human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs). hESCs are derived from donated surplus blastocysts by isolation and culture of the blastocyst inner cell mass (the tissue that would have progressed to form the embryo). hiPSCs are created by artificial reprogramming of somatic cells (by the delivery of reprogramming factors using a variety of methods) to yield cells that express the critical properties of PSCs listed above. hiPSCs can be derived from a range of somatic cell types using an increasing range of methods to secure expression of certain reprogramming factors. A range of tissue-derived cultures are known to harbor stem cell populations in vitro (e.g., subcutaneous fat, bone marrow, cord and blood of the fetal umbilicus, primordial germ cells from the fetal neural crest, and neural stem cell spheroid cultures) that have been shown to have a limited capacity for in vitro replication and have not been established as stable diploid PSC lines. Such cultures will not be covered in this chapter, and the following sections will deal specifically with hESC and hiPSC lines.

PSC lines are complex multicomponent cell cultures and may contain a variety of different cell populations with greater or lesser degrees of differentiation or lineage commitment. However, the key property of a stem cell culture, self-renewal, must be sustained. In addition, the cells must retain the capacity to undergo asymmetric division to yield two different daughter cells: one that is a stem cell identical to the parent cell and one with a reduced degree of potency (i.e., ability to generate different cell lineages).

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## Development of Current Methodologies for PSC

Currently, individual labs have their preferred methodology, no single approach appears to dominate for routine use, and it seems possible to obtain acceptable levels of post-thaw viability and recovery by either vitrification or controlled slow rate cryopreservation; however, a common method that results in better cell recovery is described here. Briefly, this technique involves placing colonies of hESCs in a vitrification solution composed of 20% DMSO + 20% ethylene glycol (EG) + 0.5 mol/L sucrose after equilibration with lower-concentration DMSO + EG solutions. The colonies are loaded into straws and plunged into liquid nitrogen. Another common method involves placing colonies in a solution consisting of 10% (v/v) DMSO and using either a passive freezing device or a controlled-rate freezer designed to achieve an average cooling rate of approximately 1°/min.

Vitrified IPS or hES samples have special storage and shipping requirements. All cryopreserved or vitrified samples must be stored at temperatures below the glass transition temperature of the sample, and for vitrified samples this is much lower (e.g., –150°) than for traditionally cryopreserved cell samples. In addition, fluctuations in temperatures during storage or shipment can lead to crystallization and therefore degradation of the sample. Because vitrified samples are sensitive to these temperature fluctuations, they should be shipped at liquid nitrogen temperatures and should not be shipped on dry ice.

Challenges for the reliable and reproducible preservation of PSC lines lie not just in the preservation process itself but also in the preparation, cryoprotection, and recovery procedures. Specifically, methods of harvesting and handling before cryopreservation may result in significant cell losses. Processing procedures should be made as reproducible as possible and with the use of an SOP to enhance the reproducibility of preservation outcomes. Furthermore, the effective banking of PSC lines is challenged by the methods by which the cells typically are passaged and harvested for preservation (i.e., individual colonies dissected and transferred as small colony fragments to fresh culture flasks for expansion or to preservation medium for freezing). First, in order to avoid extensive loss of viability in the earlier harvested cells, cultures used to make a bank actually may require preservation in small batches over a working day. Second, preparation of PSC banks comprising smaller pools of cell colony fragments is not only time consuming and laborious, but it also makes it impossible to homogenize the preparation of cells before aliquoting into vials, as would happen with more traditional methods of preserving cell lines. Thus, consistency between vials of PSC lines is compromised. Third, freezing PSCs as colony fragments preserves the gap junctions known to form between cells, potentially leading to intercellular propagation of ice crystals and extensive loss of viability.

To avoid some of these issues, analysts can use enzymatic disaggregation of colonies to simplify and accelerate cell harvesting and to enable cryopreservation of more homogenous, single-cell suspensions before preservation. To be prudent, some laboratories choose to perform enzymatic disaggregation solely before cryopreservation but not for routine culture. Whenever possible, analysts should use non–animal-sourced enzymatic agents for cell dissociation. Another important characteristic of PSC lines is the common but variable incidence of undirected differentiation that occurs within colonies and may vary considerably across the many colonies in a culture. Colonies with a high proportion of differentiated cells should be discarded because the differentiated cells are an undesirable component of a PSC culture and may affect the properties of the undifferentiated cells within the colony.

## Points to Consider in the Preservation of PSC Lines

Analysts should consider a number of important factors in the preservation of PSC lines that should be addressed at the levels of preparation, harvesting, banking, and testing of cryopreserved stocks.

### EVALUATION AND HARVESTING

Analysts should observe cell cultures on a regular basis. Cultures that exhibit high levels of differentiated cells should be discarded. An individual investigator or banking facility should have a quality control program that evaluates differentiation of cultures on a regular basis and develops threshold levels of acceptable differentiation in a culture. The protocol for harvesting followed by equilibration of the sample with CPAs should be carried out rapidly in order to minimize cell losses because of harvesting.

### THE BANKING PROCESS

Conventional cell banking procedures require the development of MCB and WCB. Cells in the MCB should be preserved at an early passage number (P10–P20) in order for experimental and cell line development work to be performed on cells at the lowest possible passage number. The first WCB should be established with the minimum passages to achieve the cell number required. To promote consistency, the WCBs subsequently can be replaced at the same passage level from the MCB as required.

### VIABILITY

Determination of post-thaw viability and function for PSCs can be complicated and is often done incorrectly. The most common method of post-thaw assessment is use of a membrane integrity dye. As a measure of viability, the fraction of cells with intact membranes is compared to those without. Another fairly common method is to quantitate PSC colony formations. The total number of colonies seeded into a plate is counted and then is counted again after a certain incubation time. This method also evaluates other functional characteristics of the cells, such as their ability to attach and proliferate. Note that procedures such as harvesting and cryopreservation may induce apoptosis. Staining for early (phosphatidylserine on the cell surface) or later (Annexin VI) apoptotic markers may provide insights into the general health of frozen and thawed cultures.

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### HOMOGENEITY

As already described, preservation of colony fragments by vitrification can exacerbate the lack of homogeneity between vials or straws of cells. When larger banks of PSCs are established, analysts should test vials from early, middle, and late positions in the filling sequence of the cell bank for viability, growth rate, and key markers as indicated in *Viability.*

### STEMNESS

In order to check that a PSC line has not lost any of its stem cell characteristics during preservation, analysts should check expression of a number of key stem cell-related markers. One extensive study analyzed 59 hESC lines and a panel of 94 genes and resolved five stem cell-related molecules for which mRNA was expressed consistently in hESCs. These genes now are included in commercially available microfluidics gene cards that are specifically designed for investigating stem cell populations. If it is crucial to demonstrate that the PSCs have retained their pluripotency, then a number of characterization tests can be performed, including teratoma formation in immune-deficient mice, formation of trilaminar embryoid bodies, and directed differentiation to demonstrate that the culture can produce representatives of each of the three germ layer tissues that are required to form all of the cells of the human body.

### GENETIC STABILITY

In both iPSC and hESC lines, it is not unusual for clones of abnormal karyotype to arise on extended passage and overgrow the culture. Thus analysts should monitor cultures for such abnormal cells. Traditionally, this has been performed by karyotypic studies of metaphase spreads of the cells using Giemsa staining. The occurrence of nondiploid cells, even at very low incidence, can be problematic. Guidance documents such as the 2009 International Stem Cell Banking Initiative are helpful to determine if such cultures should be discarded. However, newer procedures such as array comparative genome hybridization and single-nucleotide polymorphism arrays provide much more detailed analysis of genetic stability and can be used in parallel with Giemsa banding to give greater confidence in genetic stability.

### BEST PRACTICES

In addition to following good cell culture practices, analysts should note the availability of a specific guidance that contains principles and best practices in the procurement, banking, testing, and storage of hESCs for research purposes (see the ISCB 2009 reference in the *Appendix*). This guidance is useful for both iPSC and hESC lines.

## CELL SUBSTRATES USED IN PRODUCTION AND CHARACTERIZATION OF BIOTECHNOLOGY-DERIVED AND BIOLOGIC THERAPEUTIC PRODUCTS

A wide variety of both recombinant and nonrecombinant cells are cryopreserved and used in the production and characterization of human biologics and biotechnology-derived (B&B) products (more information can be found in ICH Q5D *Derivation and Characterisation of Cell Substrates Used for Biotechnological/Biological Products)*. The major groups include cell lines derived from mammals (including humans), insects (primarily moths), and selected strains of bacteria and yeast. The most common microbial substrates used for production of human biotechnology-derived products are recombinant strains of *Escherichia coli, Pichia pastoris,* or *Saccharomyces* spp. (yeasts). Despite the high degree of diversity among cell types used to manufacture B&B products, there is a surprising degree of uniformity across cryopreservation practices and the same principles apply to cells used for manufacture and those used in tests for adventitious agents and product potency.

Typically, a two- or three-tiered cell banking system is used for maintenance of manufacturing cell lines or microbial strains. For those that use a three-tier system, the first tier bank can be referred to as a research, seed, stock, accession, pre-MCB, or parent cell bank. The source of the pre-MCB can be a research or development laboratory, or cells can be purchased from a commercial repository. It is advisable to characterize the parental cell bank prior to its use in cloning. The second tier (or first tier in a two-bank system) MCB or Master Cell Stock (MCS) is prepared directly from this parent cell bank with minimal cell passages or generations. The MCB or MCS is extensively tested to confirm purity, phenotype, genotype, protein expression, or other important attributes. The WCB or Working Cell Stock (WCS) is derived from vials of the MCB after successive passages in culture. The WCB is the manufacturing cell substrate that is scaled up through repeated subcultures to seed the final production bioreactor, fermenter, or lot of culture vessels (e.g., roller bottles). At each tier in the cell banking system, proper cryopreservation is paramount to success in both product development and manufacturing. In some cases, the end of production cells also may be banked for testing purposes as part of cell bank qualification.

### Mammalian and Insect Cell Lines

Mammalian cell lines are the cellular substrates of choice for the production of complex protein molecules. Mammalian cells possess the intrinsic biological machinery required for posttranslational glycosylation of proteins that often is critical for stability and bioactivity in humans. Both diploid and heteroploid cell lines (including hybridomas) are used for production of biotechnology-derived therapeutics. Diploid cell lines are common vaccine substrates [e.g., WI-38 and MRC-5 (human fibroblast cell lines), BHK-21 (baby hamster kidney cell line), and MDCK (Madin-Darby canine kidney)]. Additionally, the African green monkey Vero cell line (a heteroploid cell line) is used for several US-licensed vaccines. Today, commonly used heteroploid cell lines include various recombinant Chinese Hamster Ovary and human embryonic kidney cell lines. Cryopreservation methods are fairly well standardized for these cell lines. However, investigators may find it useful to investigate the toxicity of different cryoprotectants and concentrations when they use a new cell substrate.

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Insect cell lines have proven their capability for production of various recombinant polypeptides. The most common cell lines are derived from the moths *Spodotera frugiperda* and *Trichoplusia ni,* and their established cell lines are called Sf9 and Tn5 (or High Five), respectively. Production of recombinant proteins employs recombinant baculovirus infection for transfer of heterologous genes. Although insect cell lines require different nutritional factors, lower incubation temperatures, and higher osmolarity than their mammalian counterparts, the same essential elements of cryopreservation apply to both groups.

In addition to the guidance given in the *Key Elements of Cryopreservation Practice* section, these additional points should be considered for animal cell line substrates:

### PREFREEZE PROCESSING

Analysts should ensure that cells are not contaminated and are still viable. For diploid cells, analysts should grow to a passage level to maintain diploidy and below the intended level for use. Analysts pool cell cultures and perform a cell count to determine the number of viable cells available for banking. Harvested cells are centrifuged at a relatively low speed for a short duration, e.g., 100–200 × *g* for 5–10 min, preferably using a refrigerated centrifuge.

### CPAS AND CRYOCONTAINERS

The membrane-permeable CPA of choice is 5%–10% (v/v) DMSO diluted in fresh growth medium. For certain sensitive cell lines, cell culture-conditioned medium can be added to supplement the cryopreservation medium. DMSO must be sterile and tissue-culture grade (>99% purity). The most appropriate cryocontainer is a presterilized polypropylene screw-cap vial designed for cryogenic storage in vapor phase liquid nitrogen.

### INTRODUCTION OF CRYOPRESERVATION MEDIUM

Immediately following centrifugation of cells, growth medium is removed from cell pellets, and cells are gently resuspended by slow addition of cryopreservation medium that is often precooled for many cell types. The cell suspension is immediately diluted with an appropriate volume of cryopreservation medium based on viable cell count and targeted cell density (typically, cell banks are produced at a viable cell density of approximately $1 \times 10^7$ cells/mL). As mentioned in the previous section, cryopreservation medium is highly hypertonic, and exposure time should be limited. The final cell suspension is transferred to a vessel in which the cells can be gently mixed during vial filling to facilitate uniformity of the cell bank.

### COOLING AND CONTROLLED FREEZING AND STORAGE

Vials can be filled manually by using a hand-held pipetting device or by using an automated vial-filling machine. In either case, vials are usually refrigerated as the filling progresses in order to minimize potential toxic effects of DMSO at higher temperatures. Time limits should be established for the entire filling process and recorded in the batch record. Immediately after the vials are filled, analysts should transfer them into a controlled-rate freezer or, alternatively, into an ultracold static freezer (e.g., –80°) using an insulated container designed for controlled cooling. Any controlled freezing system should be properly qualified to ensure expected cooling rates are delivered to all vials within the load. When using a controlled-rate freezer, analysts determine optimal cooling programs empirically, but a rate in the range of 1°–5°/min after transition through heat of fusion should be acceptable for most cell lines (colder than about –40°, the rate can be increased, e.g., 10°/min). After vials have been frozen to approximately –80° or below in the freezing system, they are transferred as quickly as possible to liquid-nitrogen vapor phase storage units.

### WARMING AND VIAL THAWING

In general, cells frozen at a slow cooling rate should be thawed as quickly as possible to maximize cell viability. In order to ensure uniformity of temperature, vials should be transferred directly from liquid-nitrogen vapor phase storage into a portable vapor phase (dry) shipper for transport to the laboratory. If a liquid-nitrogen Dewar is not available, then vials can sometimes be packed in dry ice, but this process should be demonstrated as suitable because it can result in detrimental pH changes. After transfer to the laboratory, vials are placed directly into a warm water bath (e.g., 37°, making sure caps are not immersed), a bead bath, or a thermoblock. Note that insect cell lines should be thawed at 27°–30°. Vials should be agitated to facilitate uniform thawing of cells. Immediately after thawing, vials should be transferred into the biological safety cabinet and sanitized before opening. Typically, growth medium is slowly added to thawed cells while agitating to dilute DMSO and to slowly reduce the osmolarity of the post-thaw milieu back to a physiological level.

### POST-THAW PROCESSING

Different methods can accomplish this step, but analysts should achieve sufficient dilution of the DMSO and return the cells to their normal isotonic growth environment. Manipulation of cells immediately post-thaw should be minimized because of the stresses induced by the freeze–thaw process. For example, pipetting and centrifugation should be minimized.

## Microbial Strains: *E. Coli,* Yeast, and Bacterial Vaccine Strains

Recombinant *E. coli* strains have a proven track record for production of a number of commercially viable biotechnology-derived products, including recombinant insulin analogs, human growth hormone, and parathyroid hormone. Compared to their mammalian and insect counterparts, recombinant *E. coli* strains are relatively simple to grow and scale up to large volumes, e.g., 40,000 L. However, bacteria lack the sophisticated cellular machinery for building more complex protein molecules that require post-translational modifications such as glycosylation. Yeasts are unicellular, eukaryotic cells that can be

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manipulated genetically to produce a wide range of recombinant proteins and peptides with limited complexity. Despite the evolutionary distances between the mammalian and microbial cell substrates, the same set of essential cryopreservation elements described above apply, with the following unique points:

### PREFREEZE PROCESSING

Cultures should be propagated in shake flasks to late logarithmic or early stationary phase using strain-specific growth conditions.

### CPAS AND CRYOCONTAINERS

The cell wall or cell membrane-permeable CPA of choice is glycerol (although DMSO is also sometimes used) at concentrations typically ranging from 5%–10% (v/v). Synthetic glycerol should be used for registration of commercial products along with other raw materials that are free of animal components.

### INTRODUCTION OF CRYOPRESERVATION MEDIUM

Because microbial cells possess cell walls for protection and support of cytoplasmic contents, physical manipulation and osmotic shifts do not have the same negative impact seen in animal cell lines. Immediately after centrifugation, cell pellets are vigorously resuspended in the cryopreservation medium (to a dilution based on requirements of the cell bank for number of vials and viable colony-forming units/mL). This dilution step can be based on active measurement of culture optical density or other cell enumeration assays. The final cell suspension is transferred to a vessel in which the cells can be mixed during filling of final containers to facilitate uniformity of the cell bank. Because of potential toxicity, analysts should limit the time of exposure to glycerol, despite the relative robustness of microbial cells. Vials can be filled manually by using a hand-held pipetting device or by using an automated vial-filling machine.

### COOLING AND CONTROLLED FREEZING AND STORAGE

Again, because of their inherent robustness, microbial cells do not require as strict control of cooling rate as do animal cell lines. Consequently, the choice of freezing system or method has a smaller effect on the viability of the cell bank. Filled cryocontainers simply can be transferred into an approximately –80° freezer overnight followed by transfer to vapor phase liquid nitrogen. Alternatively, microbial cell banks may not use liquid nitrogen storage but can be stored at approximately –80°. However, if liquid-nitrogen storage is available, it is preferred for long-term storage (e.g., years). If a controlled-rate freezer is employed, then it should be fully qualified to deliver a uniform cooling rate that is within an expected range, e.g., 1°–5°/min.

### WARMING AND CONTAINER THAWING

Controlling the rates of cooling and warming is less critical for microbial cells, but the cells still should be thawed as quickly as possible using a warm water bath (e.g., 30°–35°). Temperatures can be adjusted to the incubation temperature of the strain.

### POST-THAW PROCESSING

Growth medium is added to dilute the thawed cells to a desired level of colony-forming units/mL and to induce removal of intracellular glycerol.

All cells are not created equal and are divergent by nature. A broad span of evolutionary time separates the various cell substrates used for manufacture of human B&B products. Fortunately for the practitioner of industrial cell culture, cryopreservation strategies converge to a set of shared principles and methods that translate across evolutionary paths.

## APPENDIX

## Useful Guidances


- FDA. Guidance for industry: characterization and qualification of cell substrates and other biological materials used in the production of viral vaccines for infectious disease indications. 2010. http://www.fda.gov/downloads/biologicsbloodvaccines/guidancecomplianceregulatoryinformation/guidances/vaccines/ucm202439.pdf. Accessed 24 September 2012.
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# ⟨1046⟩ CELL-BASED ADVANCED THERAPIES AND TISSUE-BASED PRODUCTS

## INTRODUCTION

This general chapter provides a comprehensive overview of considerations for the development of cell-based advanced therapies and tissue-based products. A collection of terms commonly used in this field is provided in *Glossary*. Cell-based advanced therapies and tissue-based therapies are medical products that contain human or animal cells that will be administered to humans to repair, replace, regenerate, or augment a recipient's cells, tissues, or organs that are diseased, dysfunctional, or injured. The source cells or tissues can be harvested for use without manipulation or may be propagated, expanded, pharmacologically treated, or otherwise altered in biological characteristics ex vivo before administration. The diversity of clinical indications and types of cell-based advanced therapies are shown in *Table 1*. Examples of tissue-based products are included in *Table 2*.

**Table 1. Examples of Cell-Based Advanced Therapies**

| Disease/Condition | Example |
|---|---|
| Hematopoietic stem cell transplantation following ablative therapy | Hematopoietic stem and progenitor cells that have been harvested, propagated, selected, and/or treated for removal of contaminating cells by means of devices and/or reagents |
| Cancer | T cells, NK cells, dendritic cells, or macrophages targeted to cancer-specific peptides to elicit an anticancer response; autologous or allogeneic cancer cells, genetically or biochemically modified and irradiated to elicit an anticancer response |
| Diabetes | Encapsulated β-islet cells |
| Myocardial infarction | Autologous or allogeneic stem/progenitor cells; skeletal myocytes; cardiac-derived stem cells |
| Graft-versus-host disease | Allogeneic mesenchymal stem cells |
| Wound healing | Autologous keratinocytes or allogeneic dermal fibroblasts on a biocompatible scaffold |
| Focal defects in knee cartilage | Autologous or allogeneic chondrocytes with or without a biocompatible scaffold |
| Bone repair | Mesenchymal stem cells in a biocompatible scaffold |
| Neurodegenerative diseases | Neuronal progenitor cells derived from embryonic, fetal, or adult source tissues; cells genetically modified to secrete neurotrophic factors, with or without encapsulation |
| Infectious disease | Activated T cells |
| Autoimmune disease | Regulatory T cells ($T_{reg}$) |
| Spinal cord injury | Neuronal progenitor cells |
| Organ repair or regeneration | Autologous or allogeneic cells on biocompatible biomaterials (gels) or 3-dimensional scaffold structure |

Cell therapy products can be modified by treatment with integrating or non-integrating genetic materials (DNA, RNA, siRNA, etc.) so that the pattern of gene expression is changed. Typically, cells are taken from the patient or a healthy donor, and are modified outside of the body before they are given to the patient. Regulatory bodies consider the ex vivo gene-modified cellular product to be a gene therapy product. A great deal of information in this chapter is relevant to processing, characterization, manufacturing, and administration of genetically modified cells. However detailed information about the use of various gene transfer systems, patient monitoring considerations, genetic analysis, and other issues pertinent to gene therapy products are addressed in *Gene Therapy Products* ⟨1047⟩.

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**Table 2. Examples of Tissue-Based Products**

| Uses | Example Products |
|---|---|
| Musculoskeletal | Decellularized, freeze-dried demineralized human bone (cortical or cancellous) |
| | Decellularized, freeze-dried human bone (cortical or cancellous) |
| | Decellularized human dermis |
| | Decellularized bovine dermis |
| | Decellularized porcine dermis |
| | Decellularized porcine subintestinal submucosa (SIS) |
| | Human cancellous bone with adipose-derived stem cells |
| | Human demineralized cortical bone and cancellous bone with bone marrow-derived stem cells |
| Neurological | Decellularized human nerve tissue |
| Ophthalmology | Human amniotic tissue |
| | Human placental tissue |
| Wound care | Decellularized human dermis |
| | Decellularized porcine SIS |
| | Human amniotic tissue |
| | Human placental tissue |

For tissue-based grafts that contain non-autologous cells, the cells are derived from the same donor; they may be harvested with the tissue, or harvested from a different organ (e.g., bone marrow aspirate, adipose) and recombined with the tissue (e.g., bone).

This chapter describes issues related to the manufacturing, sourcing of components, and characterization of cell-based advanced therapies or tissue-based products to ensure their safety and efficacy. A list of relevant regulatory and guidance documents is presented in the *Appendix*. Manufacturers of cell-based advanced therapies or tissue-based products should consider and apply the controls and procedures outlined in this chapter to ensure the products' safe use in humans. New methodologies are continually being developed and validated and will be included in the United States Pharmacopeia (*USP*) as they become available. *USP* monographs for specific tissue and tissue-based products outline test specifications that should be met throughout a product's time in the market place. The term "cellular therapy product" refers to living human or animal cells or tissues that are subject to approval by the US FDA. This generally means clinical trials are required to demonstrate safety and efficacy prior to commercialization under a biologics license application (BLA), or for a medical device, premarket approval (PMA). Additionally, most cellular therapy products must comply with both good tissue practices (GTPs) and good manufacturing practices (GMPs). A subset of cell-based products, known as combination products, or tissue engineering products, refer to cells combined with a medical device, such as a natural or synthetic scaffold. These products must also meet similar standards for establishing safety and efficacy as well as comply with GTPs and GMPs, or medical device quality system regulations (QSR). Most cell-based advanced therapies and tissue engineering products are likely to fall within the legislation that defines regenerative medicine advanced therapies (RMAT). The majority of commercially available tissue-based products are human derived. Human tissue-based materials are often referred to as HCT/Ps, or human cells, tissues, cellular and tissue-based products. HCT/Ps that meet criteria identified in 21 Code of Federal Regulations (CFR) 1271.3(d)(1) and Section 361 of the Public Health Service (PHS) Act do not require premarket approval by FDA (i.e., BLA or PMA) and only need to comply with GTP regulations. These "361 products" meet criteria for "minimal manipulation", and are utilized in applications consistent with "homologous use". Human tissue products not meeting these criteria may require premarket clearance [510(k)] or PMA or BLA. While less common, some tissue-based materials are animal derived and generally require premarket clearance [510(k)].

## Considerations for Incorporating Quality System Concepts Early in Cell-Based Advanced Therapies and Tissue-Based Product Development

Current and future regulatory requirements will continue to challenge developers of cell-based advanced therapies and tissue-based products to incorporate robust quality attributes early in the design phase to ensure a focus on patient safety by means of a high degree of process understanding. Modern quality systems that harmonize current Good Manufacturing Practices (cGMPs) with other non-US pharmaceutical regulatory systems [such as the International Council for Harmonisation (ICH) and the ISO] and the FDA medical device quality system are being recognized for ensuring quality. These new standards include product development concepts such as Quality by Design (QbD) and Process Analytical Technology (PAT). Moreover, these quality systems integrate approaches to continual improvement and risk management that promote adoption of the latest scientific advances and innovative manufacturing technologies.

Employing the principles of Quality Risk Management (QRM) early in product development may identify areas of risk that can be mitigated before they are incorporated into the manufacturing process and affect the safety and efficacy of the product. Developers of cell-based advanced therapies and tissue-based products should employ risk management and assessment techniques as a key component of their quality systems. "Risk management" is defined as a systematic process for the identification, assessment, and control of risks to the quality of the cell-based advanced therapies or tissue-based product across

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the product lifecycle. Using QRM techniques can help achieve safe and efficacious products by assessing patient risks, determining design space boundaries, or ranking quality attributes. QRM can also establish and maintain a state of control by using risk management to drive process control. Finally, QRM can be used to facilitate continual improvement by prioritizing opportunities for improvement. The level of effort, formality, and documentation of the risk management process should be commensurate with the level of risk, should be based on scientific and medical knowledge, and ultimately should be linked to patient protection.

The elements of risk management have become an accepted paradigm; these are described in FDA and international regulatory guidance documents, especially ICH Q9. A number of tools have been developed to facilitate this assessment. These tools provide a quantifiable means of prioritizing risk so that higher-risk elements of a process can be identified and corrected.

Depending on the objective of the risk management program, risk analysis can be more or less formalized. A more formalized risk assessment system is necessary for process or product development. This is especially important when limited resources must be prioritized. Formalized systems are predicated on well-established tools that can quantify risk in every phase or step of manufacturing. These systems can also be used in evaluating raw material choices, validation prioritization, facility alterations, equipment changes, and utility deliberations.

Formal risk analysis tools include process mapping, preliminary hazard analysis, Hazard Analysis of Critical Control Points (HACCP), Hazard Operability Analysis (HAZOP), Fault Tree Analysis (FTA), Failure Mode Effects Analysis (FMEA), and Failure Mode Effects and Criticality Analysis (FMECA).

For cell-based advanced therapies and tissue-based products, FMEA has been commonly used to identify, quantify, and prioritize risk. FMEA can assign a numerical rating in one of three categories:

- "Severity", which is the consequence of a failure;
- "Occurrence", which is the likelihood of the failure happening based on past experience or nonconformance; and
- "Detection", based on the ability to detect the failure.

Each category is assigned a numerical rating (typically 1–5 or 1–10) consistent with the severity of the excursion from the operating parameter range, the probability of an excursion, and the likelihood of detecting an excursion before it has an effect on the product. Lower numbers refer to an unlikely probability of detection whereas higher numbers refer to the likelihood of a failure or hazardous effect. The product of the severity, occurrence, and detection values is a Risk Priority Number (RPN). In the risk-evaluation process RPNs are prioritized, and the most immediate remediation can be directed to areas of highest risk.

# COMPONENTS USED IN CELL-BASED ADVANCED THERAPIES AND TISSUE-BASED PRODUCT MANUFACTURING

## Introduction

Manufacturers of cell-based advanced therapies or tissue-based products must ensure that all components used in manufacturing are appropriately qualified. Examples of components used in the production of cell-based advanced therapies or tissue-based therapies include the source cells and tissues; natural or synthetic biomaterials; ancillary materials required during manufacturing but not intended to be present in the final therapeutic product; and excipients used in the formulation of cell-based advanced therapies or tissue-based therapies. For more information on ancillary materials, see *Ancillary Materials For Cell, Gene, And Tissue-Engineered Products* ⟨1043⟩.

Qualification is the process of acquiring and evaluating data to establish the source, identity, purity, biological safety, and overall suitability of a specific component to ensure quality. The diversity of cell-based advanced therapies and tissue-therapy products and the materials used to produce them makes it difficult to recommend specific tests or protocols for a qualification program. Therefore, rational and scientifically sound programs must be developed for each component.

Material qualification activities will change as products move from clinical trials to licensure and commercialization. A well-designed qualification program becomes more comprehensive as product development progresses. In the early stages of product development, safety concerns should be the primary focus of a material qualification plan. In the later stages, material qualification activities should be completely developed and should comply with cGMP.

## Qualification of Source Cells and Tissues

Various human- and animal-derived cells and tissues serve as source material for cell and tissue-based products. Three sources of donor cells for cell-therapy products include:

1. The patient's own cells (autologous cell products)
2. Cells from another human being (allogeneic cell products)
3. Cells derived from animals (xenogeneic cell products)

The source of cells used for a particular cell or tissue-based therapy largely depends on compatibility, purity, and availability. Use of autologous cells has the advantage of minimal concerns regarding immune rejection. However, an autologous source is not always available and appropriate if the cell type is dysfunctional, malignant, not readily obtainable, or contaminated.

The alternative is a compatible allogeneic cell source that may be more readily available. Of primary concern with the use of allogeneic cell sources is immune incompatibility, which could lead to rejection of the administered cell- or tissue-based therapy. In immunocompromised recipients, the donor cells may react to the patient's cells, leading to graft-versus-host disease, which can be life threatening. Despite the potential complications of using allogeneic donor cells or tissues, in the absence of other alternatives the risk-to-benefit ratio may be acceptable. A number of approaches successfully circumvent immune barriers for the use of allogeneic sources. Immunosuppressive drugs developed for solid organ transplantation and advances in inducing immune tolerance are increasingly applied to cell transplantation. Certain allogeneic cells elicit minimal immune reactions, even

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in human leukocyte antigen (HLA)-mismatched recipients. Examples include mesenchymal stem cells, certain dermal and epidermal cells, and fibroblasts. Cells may be obtained from living or deceased donors.

Despite advances in the derivation of new types of therapeutic cells, particularly stem cells (adult, fetal, embryonic and induced pluripotent cells), the ability to generate certain types of cells or tissues remains elusive. As a result, ongoing efforts use xenogeneic cells and tissues to treat a number of human diseases or conditions. Use of xenogeneic cells and tissues must address concerns about both immune rejection and transmission of animal viruses to humans (see *Animal Sources of Cells and Tissues* below). Both human- and animal-sourced cells and tissues can pose a risk for transmission of adventitious agents.

Some general principles in the sourcing of tissues include: 1) systems must allow the material to be traced back to the donor, while adhering to privacy legislation; 2) steps must be taken to prevent the transmission of infectious diseases from the donor to the recipient; and 3) while terminal sterilization is employed for some decellularized tissue-based materials, aseptic procurement and processing must ensure the safety of the final product because terminal sterilization of products containing living cells and tissues is not possible. The FDA has promulgated a specific set of regulations, referred to as GTPs, that specifically address the need to procure and process tissues in a manner that avoids transmission of a communicable disease. GTPs and/or GMPs must be followed for cell-based advanced therapies or tissue-based therapy products, depending on cell source.

## DONOR ELIGIBILITY

FDA has enacted a comprehensive set of regulations governing human tissues and human cells that are intended for implantation, transplantation, infusion, or transfer into a human recipient. These materials are referred to as HCT/Ps. Paramount for procurement of HCT/Ps for medical use is adherence to donor eligibility requirements. These dictate that a donor's relevant medical records must be reviewed to evaluate risk factors and clinical evidence of communicable disease agents. This includes obtaining a health history and could include performing a physical assessment on a donor to screen for communicable diseases. In addition, donors must also undergo appropriate laboratory testing using FDA-cleared or -approved test kits for specific relevant communicable disease agents and diseases (RCDADs). Required disease testing will expand as new RCDADs are identified and FDA-cleared or -approved tests or test kits become available. Sources for information about communicable disease testing are FDA's *Guidance on Eligibility Determination for Donors of Human Cells, Tissues, and Cellular and Tissue-Based Products (HCT/Ps)*, American Association of Tissue Banking (AATB) Standards, and subsequent recommendations for emerging RCDADs such as West Nile Virus and Zika Virus are available from the FDA website (https://www.fda.gov/regulatory-information/search-fda-guidance-documents/eligibility-determination-donors-human-cells-tissues-and-cellular-and-tissue-based-products).

## HUMAN CELLS, TISSUES, OR CELL-BASED ADVANCED THERAPIES OR TISSUE-BASED PRODUCTS

HCT/Ps may be sourced from normal healthy donors, cadaveric donors, or patients with diseases such as cancer. The suitability of tissue sourced from patients with cancer and other diseases should be assessed before collection to ensure adequate safety and function of the final cell therapy product. Additionally, the regulations in 45 CFR Part 46 apply to all federally supported human subject research. These regulations require that an Institutional Review Board review and approve the use of any tissue taken from a human donor. The regulations also include special considerations for research on prisoners, children, pregnant women, or gestational tissue. In all cases appropriate written consent must be obtained from the donor or the donor's next of kin describing the tissue that is being procured and its intended use.

The risk of disease transmission to the manufacturing operator should be minimized by appropriate training for handling potentially infectious materials and by the use of protective equipment and clothing. Tissues should be obtained under environmental conditions and controls that provide a high degree of assurance for aseptic recovery.

Hematopoietic progenitor cells (HPCs) are one of the most extensively used cell sources for human transplantation. These cells can be collected from the bone marrow, peripheral blood, or umbilical cord blood. The source of cells depends on the patient, the disease, and the clinical protocol. Regardless of the cell source, methods for processing the cells are similar. HPCs can be sourced from healthy donors or patients with hematological disorders. In addition to FDA's HCT/Ps regulations, applicable guidelines and standards for the collection and processing of these materials have been published by the American Association of Blood Banks (AABB), the Foundation for the Accreditation of Hematopoietic Cell Therapy, and the National Marrow Donor Program (NMDP).

For cell or tissue sources obtained from surgical specimens or cadaveric donors, standard hospital operating room practices are applicable. The air quality in a typical limited-access operating room is adequate for such procedures. Procurement personnel must be appropriately trained in all aspects of tissue recovery, such as surgical scrubbing, gowning, operating room behavior, anatomy, surgical site preparation, and aseptic technique. Special care is required when tissue or organ procurement requires extensive manipulation of the bowel, which may result in the inadvertent puncture of the bowel. Consideration should be given to sampling incoming tissues for microbial bioburden. Though it is recognized that results of such bioburden testing would likely not be available until after the tissue has been processed, in the event of a subsequently observed microbial contamination during downstream processing or positive sterility result, the bioburden results from the incoming tissue could aid in the investigation of the source of the contamination.

## ANIMAL SOURCES OF CELLS AND TISSUES

Ideally, cellular therapy products would consist of human cells manufactured with minimal exposure to animal-based materials. However, at present important unmet medical needs may potentially be addressed by cellular therapy products from animal cells or tissues. One example is pancreatic islets intended to treat diabetes. Currently human sources of pancreatic islets are available only from pancreas donated at the time of death. The quality of donor organ islets is variable, and the available supply is inadequate to meet potential demand. One approach is procurement of pancreatic islets from appropriately qualified animal sources for subsequent use in humans (xenotransplantation).

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Developers who intend to use viable animal cells or tissues in a cell-based advanced therapy or tissue-based product must adequately address public health concerns and must develop approaches to mitigate the potential risk of introduction and propagation of zoonotic infectious agents into the general human population. The *PHS Guideline on Infectious Disease Issues in Xenotransplantation* (January 2001) describes potential risks. The FDA guidance *Source Animal, Product, Preclinical, and Clinical Issues Concerning the Use of Xenotransplantation Products in Humans* (April 2016) reflects updated approaches and expectations to minimize risks of xenogeneic cellular products (https://www.fda.gov/regulatory-information/search-fda-guidance-documents/source-animal-product-preclinical-and-clinical-issues-concerning-use-xenotransplantation-products). In most cases, decellularized (i.e., nonviable) xenograft tissue-based products are not subject to the PHS guideline and FDA guidance mentioned above and are generally regulated in the US as medical devices and must follow the applicable regulatory pathway [e.g., 510(k)] and all related requirements.

The use of viable animal tissue in the manufacture of cell-based advanced therapies requires that the tissue be sourced in a controlled and documented manner from designated pathogen-free animals bred and raised in captivity in countries or geographic regions that have appropriate disease prevention and control systems. In addition, the care and use of animals should be approved by a certified institutional animal care and use committee. Donor animals must have documented lineage, be obtained from closed herds or colonies, and be under health maintenance and monitoring programs. The animal housing facility should be USDA certified (large vertebrate animals) or Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) certified (small vertebrate animals) and should meet the recommendations stated in the current version of the *Guide for the Care and Use of Laboratory Animals* (National Research Council), which can be obtained from AAALAC (www.aaalac.org). Such facilities should be staffed with veterinarians and other trained personnel who ensure animal health and disease prevention. The facility's procedures should be documented, and records should be kept. Health maintenance and monitoring programs should be based on standard veterinary care for the species, including physical examinations, monitoring, laboratory diagnostic tests, and vaccinations. A stepwise "batch" or "all-in–all-out" method of source animal movement through the facility can minimize the potential for transmission of infectious agents.

Feed components should be documented and should exclude recycled or rendered materials in order to reduce the risk of prion-associated diseases.

To provide a high degree of assurance of product safety, animal donors and tissues should be screened at several stages throughout the process to rule out the presence of microbial agents. These control tests should utilize assays that are sufficiently sensitive and specific to detect bacteria, mycoplasma, fungi, or viruses of interest. Donor animals should be screened for relevant diseases before tissue procurement. Post-tissue-retrieval necropsies, sentinel animal programs, and archival storage of donor organs, tissues, blood, and other specimens also ensure the safety of animal tissue for use in cellular therapeutic applications.

In general, similar aseptic procurement issues apply to animal and human tissues. The tissue should be obtained under environmental conditions and controls that provide a high degree of assurance of aseptic recovery. Specifically designed procurement facilities, usually closely associated with the animal holding facility, should be employed. Recommended design features and attributes of the animal tissue procurement facility should include the following: 1) staging of events such as shaving, sedation, and operating room preparation in separate rooms with appropriate environmental controls; 2) high-efficiency particulate air (HEPA) filtration; 3) adjacent but separate facilities for further tissue processing; and 4) dedicated areas for carcass removal. Issues relating to personnel training, bowel manipulation and puncture, and disinfection apply to the surgical procurement of both human and animal tissues (see *Human Cells, Tissues, or Cell-Based Advanced Therapies or Tissue-Based Products* above). When researchers establish animal cell lines for use in production, cell banks should be created, tested, and characterized as described in the next section.

## CELL BANK SYSTEM

A cell bank is a collection of cells obtained from pooled cells or derived from a single cell clone or donor tissue that is stored in bags or vials under defined conditions that maintain genotypic and phenotypic stability. The cell bank system usually consists of a master cell bank (MCB) and a working cell bank (WCB), although alternative approaches are possible. The MCB is produced in accordance with cGMP and preferably is obtained from a qualified source with known and documented history. Human cells and tissues should be obtained by means of a licensed tissue acquisition vendor with a donor qualification program in accordance to 21 CFR 1271. The WCB is produced or derived by expanding one or more vials of the MCB. The WCB or MCB becomes the source of cells for every batch produced for human use. Cell bank systems contribute greatly to production batch consistency because the starting cell material is always the same. However, it may not be possible or feasible to create a cell bank, so appropriately tested and qualified primary cells may be used in lieu of creation of cell banks. The MCB and WCB should at a minimum be tested for identity, sterility, purity, viability, and the presence of viruses and mycoplasma.

## CELL BANK QUALIFICATION

Cell bank safety testing and characterization are important steps toward obtaining a uniform final product with lot-to-lot consistency and freedom from adventitious agents. ICH Q5A, *Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin*, gives specific recommendations for testing cell banks for viral agents. While this guideline is not specifically intended to cover cell- or tissue-based products, the same tests are generally applicable. Additional virus testing may be needed depending on the prevalence of viral diseases endemic in the donor population. Testing to qualify the MCB is performed once and can be done on an aliquot of the banked material or on cell cultures derived from the cell bank. Specifications for qualification of the MCB should be prospectively established. It is important to document the MCB history, the methods and reagents used to produce the bank, and the storage conditions. All the ancillary materials required for production of the banks, such as media, sera, cytokines, growth factors, and enzymes, should also be qualified, documented, and appropriately tested.

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SAFETY TESTING OF MCB AND WCB

**Master cell bank:** Safety testing to qualify the MCB includes testing to demonstrate freedom from adventitious agents and endogenous viruses. The testing for adventitious agents should include tests for bacteria, fungi, mycoplasma, and viruses. Freedom from adventitious viruses should be demonstrated using both in vitro and/or in vivo test systems and appropriate species-specific tests, demonstrated using both in vitro and/or in vivo test systems, such as appropriate species-specific tests, massive parallel sequencing (MPS) methods, degenerate polymerase chain reaction (PCR), hybridization to oligonucleotide arrays, or mass spectrometry, as allowed by regulatory authorities.
**Working cell bank:** Safety testing of the WCB is less extensive and generally focuses on the potential for introduction of adventitious viruses or activation of latent virus during the additional culture required to create the WCB. End-of-production (EOP) safety testing should also be performed to ensure that the cells can be expanded a known maximum number of generations while still producing an acceptable product. For information about which types of adventitious virus testing should be performed on the MCB, WCB, and EOP cells, consult *Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin* ⟨1050⟩.

CHARACTERIZATION OF MCB AND WCB

Characterization of the MCB and WCB includes identity testing to establish species origin, e.g., isoenzyme analyses to confirm the human origin of the cells. However, cell bank characterization should encompass additional assessments such as the following:

- Growth kinetics and population doubling time
- Morphological assessment
- Percent confluence at passage
- Cell counts
- Viability (pre- and postcryopreservation)
- Phenotypic expression of desired and undesired cell types (pre- and postcryopreservation)
- Monitoring of unique biochemical markers (pre- and postcryopreservation)
- Assessments of functional activity (pre- and postcryopreservation)
- Gene and protein expression analysis (pre- and postcryopreservation)
- Expression of immune histocompatibility antigens (HLA/MHC)
- Molecular fingerprinting
- Chromosomal stability

## Biocompatible Scaffold Materials

As previously described, most natural or synthetic scaffold materials are regulated as medical devices, although scaffolds derived from human tissues that meet minimal manipulation and homologous use criteria (e.g., demineralized cortical bone particles, or cancellous bone matrix granules) are regulated as "361 tissue" HCT/Ps. When suitable, use scaffolds that have previously been approved for other clinical uses because these materials should have already undergone extensive safety and quality testing. For applications in cell or tissue-based products, the scaffold material should allow cells to attach, proliferate, and migrate, and high porosity is often desired to facilitate cell seeding within the material. The scaffold must provide adequate diffusion of nutrients for cell health and release of cell-excreted products. Depending on the intended use, the material should have adequate mechanical strength and be amenable to manipulation, chemical modification, and manufacture. The scaffold material should be biocompatible, relatively inert, and immunologically benign.

Scaffolds can generally be classified as hard or soft. Hard scaffolds are used in applications where a specific shape is required, such as forming a blood vessel or a bladder. Soft scaffolds are used in applications where the product needs to conform flexibly to an existing shape in the body.

Scaffold materials can be synthetic or natural polymers, biodegradable or permanent. Biodegradation allows the scaffold to be resorbed or removed from the body without manipulation and for the defect to be filled with viable tissue. The scaffold degradation rate must coincide with the rate of formation or regeneration of the tissue. The natural scaffold structure must replace the degrading scaffold in such a way that it maintains the structural integrity of the tissue or organ being regenerated. For example, a newly formed blood vessel must withstand both the internal blood pressure as well as external mechanical forces.

The most commonly used synthetic biodegradable polymer is polyglycolic acid (PGA). Polylactic acid (PLA) is also widely used, sometimes in combination with PGA. These polymers degrade within the body, are readily removed before degradation, and have a long history of use in suture materials. Polycaprolacton (PCL), which exhibits a slower rate of degradation than PLA or PGA, is used in applications that require a long presence in the body.

Extracellular matrix (ECM) and its derivatives are natural materials used for scaffolds in the manufacture of cell–biomaterial combination products. Example sources of ECM include dermis, subintestinal submucosa (SIS), pericardium, and other predominantly collagen containing organs. Proteins such as collagen or fibrin and polysaccharides such as chitosan or glycosaminoglycans (GAGs) have also been used in growing cells to make combination products. Collagen is by far the most popular substrate for cells and has been molded into scaffolds for a variety of products, mainly in tissue-engineered skin applications. Cross-linking agents such as glutaraldehyde and water-soluble carbodiimides have been used to enhance the strength of natural scaffolds. Depending on the source of the material, and the extent of decellularization, natural scaffolds can be immunogenic.

The safety and biocompatibility of the scaffold and product-contact materials must be established. A full battery of tests should be performed as recommended by *Biological Reactivity Tests, In Vitro* ⟨87⟩, *Biological Reactivity Tests, In Vivo* ⟨88⟩, ISO

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10993-1, or FDA Guidance: Use of International Standard ISO 10993-1. Process residuals and degradation products from the preparation of the scaffold should be quantified and limits should be established. The stability and storage conditions of scaffold materials should be established.

### Qualification of Ancillary Materials

Ancillary products include a wide variety of raw materials and components used in manufacturing, but are not intended to be present in the final therapeutic product. They include substances such as culture media, buffers, growth factors, cytokines, cultivation and processing components, monoclonal antibodies, and components of cell-separation systems.

Residual ancillary materials may be antigenic, so their removal from the final product should be assessed, and appropriate limits should be established when necessary.

Ancillary material quality can profoundly affect the safety, potency, and purity of the cellular or tissue-based product. Ideally, each ancillary material should be produced under conditions that are in compliance with cGMP. However, some complex or unique substances may not be available from cGMP–compliant suppliers. In these situations, the cellular or tissue-therapy product manufacturer should develop a scientifically sound strategy for qualifying the ancillary material. Such a qualification program should address each of the following areas: 1) identification and selection, 2) suitability for use in manufacturing, 3) characterization and acceptance criteria, 4) vendor qualification, and 5) quality assurance (QA). Lot history files should be constructed for each ancillary material.

Conformance to specifications should be compared to the certificate of analysis data. Traceability is essential, and lot numbers for each ancillary material used should be noted in the productions records of the cell-based product. Chapter ⟨1043⟩ contains specific information about implementing an appropriate qualification program for these materials. Other *USP* chapters provide considerations about the qualification of specific ancillary materials (e.g., *Bovine Serum* ⟨1024⟩, *Fetal Bovine Serum—Quality Attributes and Functionality Tests* ⟨90⟩, and *Growth Factors and Cytokines Used in Cell Therapy Manufacturing* ⟨92⟩).

### Qualification of Excipients

During the final steps in the manufacturing process, excipients or substances that increase stability and functionality of the therapeutic cells may be included. Examples of excipients include culture media, saline TS or other electrolyte solutions approved for injection, exogenous proteins such as human serum albumin, or cryoprotectants such as dimethyl sulfoxide (DMSO). Excipients are not intended to exert a direct therapeutic effect upon the patient; rather they are intended to contribute to the maintenance of the quality attributes of the final cellular product. Because excipients will be administered to the patient along with the cells, particular attention must be paid to their qualification. In general, excipients that are already FDA-approved for human use should be used whenever possible. If non-approved excipients are used, a complete safety assessment should be done. For novel excipients such as cryopreservation solutions, appropriately designed preclinical safety studies may be needed. In addition, preclinical studies should include product formulated the same way as intended for clinical use.

## MANUFACTURING OF CELL-BASED ADVANCED THERAPIES OR TISSUE-BASED PRODUCTS

### Introduction

The manufacturing of cell-based advanced therapies or tissue-based products requires a number of operations and manipulations by individuals who are well trained in aseptic processing techniques. The technical competence of the personnel is particularly crucial to product safety and efficacy.

### Cell Isolation and Selection

Regardless of the source, there exist several generally-accepted principles for processing human or animal cells and tissues. The source cell or tissue material should be packaged in sterile, leak-proof containers and transported from the procurement area to the processing area under controlled conditions optimized to maintain quality. The transport medium may contain antibiotics, but care should be taken during processing so that antibiotics are not present in the final cellular product. In the case of blood-derived materials, or tissues containing substantial amounts of blood, the transport medium should contain an anticoagulant.

#### ISOLATION

Solid organs or tissues are usually dissected to expose a desired region. If multicellular organoids (for instance, islets of Langerhans) or single-cell suspensions are desired, the tissue may be subjected to mechanical or enzymatic disaggregation. In order to prevent excessive cell clumping, enzymes such as deoxyribonuclease may be added to digest nucleic acids released from damaged cells.

Cell and tissue isolations involving open manipulation steps should be carried out in an ISO 5 (Class 100) biological safety cabinet; the environment surrounding the biological safety cabinet should maintain aseptic processing operations. For minimally manipulated HCT/Ps in closed systems, these environments may be controlled but unclassified. However, for cell and tissue-based therapies that are manipulated and manufactured under cGMPs, the environment surrounding the biological safety cabinet should be controlled and classified, usually as an ISO 7 (Class 10,000) clean room. In all cases, proper in-process labeling, line clearance, and lot segregation should be employed in order to avoid product cross-contamination.

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### SELECTION

Cell mixtures may require further processing in order to enrich a desired cell population or remove an undesirable cell type.

Cell populations can be enriched by varying the force and duration of centrifugation, often in conjunction with a variable density gradient medium. Continuous-flow elutriation centrifuges separate cell populations by subjecting them to opposing centrifugal and fluid stream forces within a special rotor chamber.

Cell separation can also be achieved by taking advantage of cytological or biochemical characteristics unique to different cell populations. Soybean agglutinin aggregates cells that bear a particular carbohydrate moiety expressed on mature blood cells, but not stem cells, allowing enrichment of the stem cells. Certain cell populations adhere to specific solid substrates such as tissue-culture plastic, collagen-coated materials, or polymeric scaffolds; this allows the surface-bound cell type to be selectively removed from the initial cell suspension.

Monoclonal antibodies directed against specific cell-surface proteins can be used for positive or negative cell selection. For example, a monoclonal antibody-bound cell population can be removed from the cell suspension after incubation with antibody-specific magnetic nanoparticles, followed by magnetic collection. Target cell populations can be isolated from unlabeled cell suspensions by incubating the suspension with antibody-coated plastic surfaces or microspheres. A fluorescence-activated cell sorter (FACS) can be used to separate different cell types bound to fluorescently-labeled antibodies.

Cell populations may also be enriched by destroying unwanted cells, e.g., via antibody binding and exogenous complement fixation, or by incubation with cytotoxic agents or mitotic inhibitors. Such destructive procedures require thorough processing to the remove dead cells, cell fragments, and cytotoxic agents from the final cell product.

## Cell Ex Vivo Expansion and Differentiation

### EX VIVO EXPANSION

A key issue for manufacturers of cell-based advanced therapies and tissue-based products is the ability to produce and deliver a therapeutically relevant dose of the required cell population to the patient. Depending on the application, the product may be a pure, homogeneous cell type, or it may be a mixture of different functional cell types. Many target cell populations are present at a low level or low purity in complex primary source tissues. In such cases, production of a therapeutic dose are typically achieved by specific enrichment and ex vivo expansion of the required cells.

Ex vivo expansion of cells may occur in suspension culture (e.g., T cells or hematopoietic stem and progenitor cells), adherent culture (e.g., mesenchymal stromal cells, embryonic stem cells, induced pluripotent stem cells, neuronal stem cells, or dermal fibroblasts), or a mixture of both (e.g., bone marrow stroma expansion). Numerous technologies exist for cell culture. Cells can be propagated in tissue-culture flasks (T flasks), in roller bottles, on polymeric scaffolds, or in nonrigid, gas-permeable bags, usually inside incubator units controlled for temperature, humidity, and gas composition. Multilayered, high-capacity cell culture systems composed of tissue culture plastic, multibag systems, and bioreactors using microcarriers enable expansion, harvesting, and formulation to be carried out in a closed system. Traditional small-scale fermenter units can be used for expansion of cells in suspension culture. It is also possible to expand adherent cells in such units either by providing a surface for attachment (microcarriers, coated beads, or disks) or by adapting the cells to propagate in suspension culture. Some culture systems are specifically designed for the propagation of cells for therapeutic applications. These tend to be closed systems that use disposable bioreactor cartridges in automated processing units with direct control of temperature, gas composition, and media perfusion rate. In some cases automated software allows patient–donor tracking and documentation of culture conditions and manipulations. These features are useful in the design and implementation of quality control (QC) product release testing programs and for the QA documentation of processing runs.

In adherent culture, the cells are usually harvested from the surface upon which they have expanded. Methods of release include physical agitation, enzymatic cleavage, and chelation of metal ions and competitive inhibition of adhesion or matrix molecules. As described above, consideration must be given to the source, safety, toxicology, and residual testing for any reagent used to release adherent cells during manufacturing. Some product-specific systems do not require the release of adherent cells. Cells are expanded on a biocompatible synthetic or natural scaffold that is then applied topically (for example, engineered skin substitutes), or the cells are grown inside or outside of fibers for ex vivo perfusion (for example, hepatocytes in hollow-fiber devices to treat liver disease).

In all cases standard cell culture parameters should be optimized for maximum process efficiency and consistency. Such parameters include composition of cellular source material, initial seeding density, media composition, rate of media exchange, temperature, gas composition, pH, and rate of delivery. Depending on the nature of the product, the potential effect of process parameters on the potency and function of the target cells should be defined.

**Bioreactors:** Specialized bioreactors and devices may be needed for manufacturing certain three-dimensional combination products. These bioreactors hold the biocompatible scaffold/matrices for the manufacture of the construct. Although the bioreactor can provide a closed system for construct manufacturing, it creates a challenge in providing access to the scaffold for seeding cells and sampling for product release testing while maintaining sterility. Bioreactors are often single-use devices that ensure that no cross-contamination occurs between products. Preferably the product will not be repackaged for transport and delivery. For example, bioreactors may also serve as the final container for product shipment.

Container–closure testing must be performed for all final container–closure systems. Compatibility for sterilization of the bioreactor and the scaffold should be verified, and the sterilization process must be validated for each product configuration. Leachables and extractables from product-contact materials such as bioreactors and packaging components should be quantified, and limits should be established.

In closed bioreactor systems it can be difficult to observe or sample cells. Measurement of metabolic parameters can provide a surrogate method that is amenable to validation with which to evaluate the rate of proliferation and predict when to harvest the cell product. The relationship of such parameters to the viability, potency, and function of the cell product should be well defined. Postexpansion purification and enrichment of target cells by using methods such as those described above may be required.

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### DIFFERENTIATION

Some cell therapies require lineage or functional differentiation of the source cells. For example, hematopoietic stem cell expansion processes normally result in products that contain a mixture of multipotent stem cells, lineage-committed progenitor cells, and lineage-differentiated cells. The composition of these products can be manipulated by different combinations of growth factors and cytokines during the expansion process. The inverse is true for processes in which mature cells are "de-differentiated" to enable them to then be recommitted to a lineage pathway (for example, chondrocytes in cartilage repair). Specific examples of ex vivo manipulation are the production of antigen-specific T cells to target various specific disease indications or derivation of therapeutic cell types from embryonic stem cells. Before release for clinical use, the resulting differentiated target cells should be appropriately characterized. Assessing the potential for de-differentiation of multipotent cells that have undergone differentiation may be necessary to ensure the safety of the product. Where the cells have been expanded and subsequently differentiated, karyotype analysis or in vitro transformation assays may be performed to demonstrate the cells are acceptable for clinical use.

### EX VIVO GENETIC MANIPULATION

Genetic modification of cells ex vivo is a common processing procedure that is used to alter the pattern of gene expression in a defined population. The introduction of integrating or nonintegrating genetic materials (DNA, RNA, siRNA, virus) is performed in order to induce the expression of new genes and products or to change endogenous gene expression. Ex vivo genetic modification in autologous transplantation settings involves the manipulation of a harvested or expanded cell population from a patient and subsequent re-administration of the cells to the donor. In a typical allogeneic transplant setting, a stable, genetically modified cell population that has been characterized and banked is administered to a broad patient population. In order to control graft-versus-host disease in allogeneic bone marrow transplants, selected donor T cells have been treated with lethal genes such as thymidine kinase that make the cells susceptible to gancyclovir treatment after transplant. Examples of autologous genetically modified cell therapy products include chimeric antigen receptor T-cells (CAR-T), the transduction of tumor cells with cytokine or other immunomodulatory genes, lymphocytes transduced with receptors for tumor antigens, and the introduction into harvested lymphocytes of an antiviral ribozyme vector as a strategy to treat human immunodeficiency virus infection. Allogeneic cell therapy product examples include CAR-T, genetically modified and irradiated tumor cell lines used as tumor vaccines, and encapsulated cells transfected with a gene to express a neurotrophic factor for localized therapeutic protein delivery in the central nervous system.

Ex vivo genetically modified cells are considered gene therapy. Issues associated with gene therapy products are addressed in detail in ⟨1047⟩, especially the production of the vector or genetic material used to accomplish gene transfer, analytical testing strategies, patient safety, and monitoring. The manufacturing, cell processing, and process control methodologies addressed above are applicable in the procedures used for genetic manipulation. Frequently cell populations that are genetically modified are isolated and/or expanded or selected before the introduction of the genetic material. Specialized equipment and processes for introduction of genetic material must be validated and monitored. Issues associated with cell banking and stability apply to cell lines used in cell therapy product manufacturing. Finally, issues associated with analysis and administration of the genetically modified cell population are discussed later in this chapter.

## Formulation of Cell and Tissue-Based Products

Approaches for formulating cell and tissue-based products depend largely on the planned storage time for the cells before delivery to the patient. For some cell-based products, the time between completion of manufacturing and delivery to the intended recipient can be measured on the order of hours to days. Other cell-based products may be cryopreserved in order to extend their shelf life. A different approach for formulating cell and tissue-based products may involve the addition of a natural or synthetic scaffold that can facilitate handling, protecting the cells from immunological responses, and creating a specific shape that contributes to the therapeutic effect. Considerations for formulating each of these types of cell and tissue-based products are discussed below.

## Decellularized Tissue-Based Products

The cells from decellularized tissue materials are killed, lysed, and removed from tissue using one or more of a variety of methods. Protocols may include physical methods (e.g., exposure to freezing temperature, force and pressure, or electrical disruption) and/or exposure to enzymes or chemicals (e.g., acids, bases, or detergents). These step(s) are typically followed by thorough rinsing and soaking in buffer to remove reagents and all cellular debris, as well as to neutralize reagents as needed. Tissues may then be further processed, such as demineralization (bone), before packaging, freeze-drying, and/or other final processing steps.

### NONCRYOPRESERVED CELL-BASED PRODUCTS

Products consisting of suspensions of cells for delivery to patients within hours after the completion of manufacturing frequently are formulated in sterile, buffered solutions suitable for direct administration. For other noncryopreserved cell-based products extension of shelf life from hours to days may be possible by use of solutions that contain appropriate nutrients and antioxidants. In most cases, these excipients are not intended for direct administration into patients. Consequently, the excipients may require removal before delivery to the patient (see *Clinical Site Preparation and Administration*). If an unapproved formulation buffer will be administered to patients, preclinical toxicology testing should be performed.

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### CRYOPRESERVED CELL-BASED PRODUCTS

Most cell cryopreservation medium formulations are supplemented with 5%–10% DMSO with or without hydroxyethyl starch (generally 6%) and a plasma protein such as 4%–10% human serum albumin in a balanced salt solution. DMSO prevents dehydration by altering the increased concentration of nonpenetrating extracellular solutions during ice formation. The high molecular weight polymeric hydroxyethyl solution protects the cells from dehydration as water is incorporated into extracellular ice crystals. The use of protein often results in maximum recovery and viability of cells after thawing. Some cryopreservation formulations are completely free of protein.

The optimal concentration of cells for cryopreservation depends on the cell type and should be determined empirically, but it generally ranges from $10^6$ to $10^7$ cells/mL. The homogeneity and viability of the cell population being cryopreserved can also differ after thawing and should be carefully assessed. In situations where the final cell-based product is intended to be thawed and administered immediately, the presence of DMSO in the formulation buffer does subject the patient to an increased level of infusion-related toxicity, although this is related to the volume administered and the final concentration of the cryopreservative. Refer to *Clinical Site Preparation and Administration* for additional considerations.

### CELLS COMBINED WITH BIOCOMPATIBLE SCAFFOLDS

Many cell and tissue-therapy products are administered in combination with a biocompatible scaffold (also referred to as tissue engineering). Some wound healing or skin substitute products contain cells seeded on a scaffold. The biochemical and physical structure of the scaffold and the method for combining cells with the scaffold are specific to the product. The biocompatible scaffold (e.g., demineralized cortical bone particles, cancellous granules) may also be human tissue derived from the same donor as the allogeneic cells.

Cells can be loaded into a semipermeable membrane device for delivery. Usually the pore size of the membrane is large enough to allow the cell-secreted therapeutic factors to pass, but it is small enough to stop immunoglobulins and host cells from making contact with, destroying, or mounting an immune response to the therapeutic cells. The device can be a single hollow-fiber or a semipermeable capsule with cells inside that secrete therapeutic compounds, or it can be part of a larger system of pumps and filters such as hollow-fiber modules with hepatocytes for the treatment of liver disease.

Cells can be seeded onto a three-dimensional scaffold and allowed to propagate and form a tissue-like structure. In the resulting product, the cells are oriented in a unique manner that is important for the intended use of the product (e.g., skin substitutes).

Cells can be encapsulated in a gel or cross-linkable polymer solution, and the resulting implantable structure can serve as a culture vessel, as a means to shield the cells from the host's immune system, or as a way to mold cells into a defined shape. Some of the polymers used include alginate, hyaluronic acid, collagen, chitin, or synthetic polymers. Encapsulated pancreatic β-islet cells have been implanted in patients to treat diabetes. To treat urinary incontinence, chondrocytes have been mixed with alginate to form a structure upon injection.

Cells can be adhered to scaffolds of defined shape that are then implanted. Some examples include osteogenic precursor cells on scaffolds of demineralized cadaveric human bone, ceramic hydroxyapatite, ceramic hydroxyapatite–tricalcium phosphate, or biodegradable glass, which can be used in the repair of bone defects.

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## ANALYTICAL METHODS

### General Considerations

The complexity and scope of cell-based therapies are reflected in the wide range of analytical methods that are used to establish in-process controls and final product release criteria. Quality specifications for cell-based advanced therapies and tissue products should be chosen to confirm the product's quality, safety, and potency. Selected tests should be product specific and should have appropriate acceptance criteria to ensure that the product exhibits consistent quality parameters within acceptable levels of biological variation, loss of activity, physicochemical changes, or degradation throughout the product's shelf life. The development and setting of specifications for cell and tissue products should follow the principles outlined in ICH Q6B, *Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products*.

Specifications are established on the basis of thorough characterization of the product during the development phase and an understanding of the process and its capability. Characterization should include measurements of the physicochemical properties, safety, purity, process and product-related impurities, potency, viability, sterility, and quantity. Manufacturers should develop specifications for each product developed from this information by application of appropriate statistical methods. The data should include lots used in preclinical and clinical studies, and should also include assay and process validation data that can be correlated to stability, safety, and efficacy assessments.

In-process controls and specifications for the product should ideally be anchored by use of an appropriate reference standard or reference material. An autologous product may rely on a reference standard generated from processing cells or tissue from a healthy donor or from a source that supplies cells and tissues to research institutions. The reference standard ensures that the process, as measured by the release assays, does not change significantly over time, and it verifies that a test produces acceptable results, i.e., system suitability requirements are met. The reference standard is made from a lot that is produced under controlled conditions and passes all in-process and final release testing. In addition, this reference standard is subjected to an additional level of characterization that includes tests not normally performed for product release. The reference standard need not be stored at the same dose, formulation, or temperature as the final product. However, the stability of this reference standard must be determined.

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Alternatively, a working standard can be used. If so, in the test it should behave like the reference standard. Changing to a new reference standard should include many tests, all of which are run side by side with the existing reference standard. The impact of any change in the properties of the new reference standard should be carefully evaluated before it is adopted. One option for a reference standard for a cell product with a short shelf life or for an autologous or patient-specific application can be a bank of normal donor cells of the appropriate cell type. This cell bank can be used to ensure that the manufacturing process is capable of making a consistent product.

## In-Process Controls

Manufacturing processes should have well-defined go–no go decision criteria that are established for key in-process manufacturing steps. In-process control tests ensure that the in-process material is of sufficient quality and quantity to ensure manufacture of an acceptable final product. Examples of in-process controls include:

- Cell enumeration and viability
- Microbiological (sterility, endotoxin, mycoplasma)
- Expression of phenotypic or genotypic markers
- Verification of morphology against visual reference standards
- Production of a desired bioactive substance
- Determination of population doublings, passage number, age of culture
- Assays for process impurities
- Monitoring of culture system parameters [% carbon dioxide ($CO_2$), % relative humidity, pH, glucose, lactate, etc.]
- Functional tests such as colony forming units (cfu) and expression of cell-specific proteins
- Content uniformity
- Quantitation of particulate matter
- Visual inspection for cellular aggregates and other impurities

A primary reason for in-process control tests is to reliably obtain adequate product quality and yield. A secondary reason is to gather process and product characterization data that can be useful in assessing the impact of process changes or excursions. Refer to ▲*Quality Systems*▲ (ERR 1-Nov-2020) for discussion on critical process parameters (CPP). In-process sampling must always be balanced against adequate final product yield. Therefore, only truly informative and timely in-process testing should be implemented. Intermediate process material that fails to satisfy in-process control criteria should not be used for further manufacturing. Non-conforming material may be reprocessed if there are procedures in place. The effect of reprocessing on other quality attributes such as stability must be defined before the material can undergo further manufacturing. The reprocessed material must satisfy the original in-process specifications. If several sub-lots (e.g., cells harvested from different culture vessels) will be pooled for further processing, non-conforming sub-lots should not be included even if the larger pool containing these failed sub-lots would pass the in-process assay criteria. For manufacturing processes that yield multiple containers of cell suspensions, in-process visual inspections are highly recommended. For example, fibers, polymer fragments and other visible particulate matter can accumulate and co-mingle with cells presenting a patient safety concern. In-process visual inspection after the final container filling, but prior to the final product labeling or cryopreservation (if frozen), allows the culling of nonconforming containers before final product sampling and testing. Timely in-process visual inspection may be challenging, particularly when attempting to limit cell exposure to cryopreservatives such as DMSO.[1]

During process development, assays for product quality and yield should be performed after most processing steps to determine which steps are quality-critical, stability-indicating, or sensitive to process deviations. Statistical process controls and critical product quality parameters, along with statistically valid sampling, should be used to establish limits for process validations and manufacturing investigations. In-process controls should be performed even for fully validated manufacturing processes to ensure proper process control. Assay results should be tracked and trended over time, and actions should be taken to correct problems as they arise.

## Final Product Release Specifications

Cell-based therapies regulated as biological products must comply with applicable sections of 21 CFR 211 and 21 CFR 610 to ensure they meet essential quality attributes such as identity, purity, potency, and microbiological safety.

Because terminal sterilization is not possible for a living cell-based product, essentially all cell-based products are required to meet acceptance criteria for product tests such as sterility, mycoplasma (not needed if cells are cultured less than 24 h, or if tissue has been decellularized), and endotoxin—typically, negative or no growth demonstrates sterility and the absence of mycoplasma, and products must demonstrate <5 endotoxin units (EU) per kilogram of patient body weight. In the case of intrathecal injection, the specified endotoxin limit is more stringent: ≤0.2 EU/kg of patient body weight. Adventitious virus testing is rarely performed on the final cell-based therapy product because the source cells or cell banks and ancillary materials of biological origin have been screened and tested for viral agents of concern before manufacturing.

For almost all other final product release criteria, such as those for identity, purity, and potency, the analytical procedures with methods and acceptance criteria are specific to the individual cell-based product. *Table 3* provides an overview of the expected final product release tests for cell-based therapies and lists examples of approaches that are used to satisfy the testing requirements.

[1] Clarke D, Jean S, Powers D, Karnieli O, Nahum S, Abraham E, et al. Managing particulates in cell therapy: guidance for best practice. *Cytotherapy*. 2016;18(9):1063–1076.

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**Table 3. Overview of Final Product Release Testing**

| Release Test | Examples | Criteria |
|---|---|---|
| Sterility | *Sterility Tests* ⟨71⟩ | Negative |
| Mycoplasma | Direct and indirect culture method (FDA *Points to Consider*) | Negative; not detected. Testing not usually required if cells are cultured less than 24 h. |
| Endotoxin | *Bacterial Endotoxins Test* ⟨85⟩ | <5 EU/kg (<0.2 EU/kg intrathecal) |
| Identity | • Surface marker determination<br>• Isoenzyme analysis<br>• Genetic fingerprint<br>• Morphology<br>• Bioassay<br>• Biochemical marker | Product specific |
| Purity | • Percentage of viable cells<br>• Percentage of cells expressing specific marker(s)<br>• Limits on undesired cell types<br>• Limits on process contaminants (e.g., serum)<br>• Scaffold composition | Product specific |
| Potency | • Viable cell number<br>• Colony-formation assay<br>• Change in expression of specific genes<br>• Secretion of desired macromolecule<br>• Expression of cell-surface molecules HLA<br>• Target specific cell killing<br>• Evidence of metabolic activity<br>• Evidence of cell function | Product specific |
| Dose | • Viable cell number<br>• Enumeration of specific cell population<br>• Total DNA<br>• Total protein | Product specific |
| Appearance | *Injections* ⟨1⟩, *Visible Particulates in Injections* ⟨790⟩ | Product specific. Cell suspensions should be free of unexpected visible particulate matter. |
| Others | • Morphology<br>• Size<br>• Tensile or burst strength | Product specific |

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## STERILITY

Cell and tissue-based products are required to comply with final product release testing requirements, including sterility. Sterility testing is also frequently performed in-process to establish microbial purity for cells that require extended culturing. A suitable sterility test includes the test described in *Sterility Tests* ⟨71⟩. These culture-based test methods require 14 days and thus are suitable only for cell-based therapy products that have extended shelf lives (e.g., following cryopreservation). Many cell-based therapies have short shelf lives and must be delivered to patients before the 14-day test results are available. In such situations, the FDA has identified an approach that will allow the administration of the cell-based product to patients in this setting [see *Guidance for FDA Reviewers and Sponsors: Content and Review of Chemistry, Manufacturing, and Control (CMC) Information for Human Somatic Cell Therapy Investigational New Drug Applications (INDs)*]:

- In-process sterility testing on a sample taken 48–72 h before final harvest or after the last refeeding of the cultures
- A rapid microbial detection test such as a Gram stain or other procedure on the final formulated product
- Sterility testing compliant with 21 CFR 610.12 on the final formulated product

Under this alternative approach, the release criteria for sterility would be based on a negative result of the Gram stain and a no-growth result from the 48- to 72-h in-process sterility test. In the event that the 14-day sterility test is determined to be positive after the product is administered to the subject, the manufacturer is required to report the sterility failure, results of investigation of the cause, and any corrective actions as an amendment to the IND within 30 calendar days after initial receipt of the positive culture test result.

Because of concerns regarding the sensitivity of a Gram stain and the inability to obtain full sterility results for 14 days after administration to the patient, there is widespread interest in the use of rapid microbiological methods as an alternative to the 14-day culture method. This is discussed in *Alternative Test Methodologies*.

## MYCOPLASMA

Testing for mycoplasma is recommended for all raw materials derived from a human or animal source; it is required as a lot-release assay for cell-and tissue-based products if they are cultured for longer than 24 h. The FDA has published a document (*Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals*) describing in detail the accepted methods for the cultivation and isolation of mycoplasma. Methods for mycoplasma detection are also described in *Mycoplasma Tests* ⟨63⟩.

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Because the classical assay takes 1 month to complete, alternative methods are being developed and validated for the rapid detection of mycoplasma; this is discussed in *Alternative Test Methodologies*.

## ENDOTOXINS

Endotoxins exert numerous biological effects: they can affect secretion and cytokine production, induce fever in recipients, or serve as powerful mitogens. Because of these wide-ranging impacts, endotoxin assessment must be included in raw material and manufacturing process qualifications for cell and tissue-based therapies. *Bacterial Endotoxins Test* ⟨85⟩ describes methods for measuring endotoxins, all based on the Limulus amoebocyte lysate assay. This assay can be validated for a wide range of biopharmaceutical products; one important feature is the ability to rapidly conduct the assay before the release of short-shelf-life products.

## IDENTITY

Lot-release testing for cell-based products must include an identity test to unequivocally identify the product. The complexity of the identity test depends on the nature of the specific product and the array of products being manufactured, e.g., in a single-product vs. a multi-product facility.

Identity tests for cell-based products must be relevant to the cell type and manipulations applied during processing. Differential surface markers are frequently used to ascertain product identity, as described in *Table 3*. Flow-cytometric immunoassay methods are the most common means of detecting and quantifying these markers. Isoenzyme analyses can confirm species of origin, which is especially useful for xenogeneic cell products. Cell morphology may be used to distinguish specific cell types. Genetic fingerprint technologies such as short tandem repeats can also establish cell line identity.

## PURITY

Purity methods specifically quantify the intended active product components, or unwanted impurities (product- or process-related residual contaminants), in the final product. *Table 3* describes examples of several impurity methods. The requirement to test for a particular impurity for product lot release depends on: 1) the capability of the manufacturing process to remove or inactivate the impurity, as demonstrated by process validation, and 2) the impact of the impurity, such as toxicity or altered product function.

Examples of process-related impurities associated with cell-based therapy products include residual production-medium components (e.g., serum, antibiotics, or exogenous cytokines), ancillary materials used in downstream processing (e.g., nucleases or proteases), and leachables (e.g., plasticizers from tubing or culture plastic). Impurities may be bioactive (e.g., cytokines or hormones) or immunogenic (e.g., aggregates, degradation products, or animal-derived proteins). Impurities may have other deleterious effects, depending on the dose of the product.

Product-related impurities are specific to each product type. Impurity examples include cell debris; cells that are dead, non-functional, altered, or otherwise unwanted. Analytical methods should quantitate both product and impurities, and should allow the manufacturer to assess the lot-to-lot consistency of the product-impurity profile. It may be possible to validate the manufacturing process to the extent that specific lot-release testing for impurities can be limited.

Testing for impurities is often extensive during product characterization and process validation, when the consistency of the manufacturing and purification process is being assessed. In-process testing for impurities can provide valuable information regarding the consistency of manufacturing and materials. *In-Process Controls* describes the role that in-process testing can play in monitoring and limiting impurities, both during process development and manufacturing. Lot-release impurity testing should reflect the safety risks associated with the impurity, and the ability of the process to consistently remove that impurity.

## POTENCY

Potency is defined under 21 CFR 600.3(s) as "the specific ability or capacity of the product, as indicated by appropriate laboratory tests or by adequately controlled clinical data obtained through the administration of the product in the manner intended, to effect a given result." Together with dose, potency defines the biological activity of each lot (see *Dose-Defining Assays*, below). The relationship between product potency measurements and clinical safety and efficacy is key to their use in batch release. Potency may be assessed by cell enumeration, in vitro or in vivo bioassays, or a combination of these. Because it is not uncommon for potency assays to have large coefficients of variation, these assays often require well-defined and fully characterized representative reference material. In part because of their inherent variability, patient-specific products such as autologous immunotherapies present a particular challenge in demonstrating potency. Potency assay development should focus on characterizing and controlling the sources of assay variability. Information about potency assay and product variability should inform the product stability study design, as well as the proposed statistical approach to expiration date assignment (see *Stability*, below).

Assays measuring the potency of cell-based therapy products vary widely, and depend on the products' unique characteristics and shelf life. For some cell-based products, product potency may correlate with clinical efficacy. For example, a colony-forming assay or enumeration of committed progenitor cells may correlate with clinical engraftment outcomes in clinical studies. Product potency may also correlate with responses measured in animal or cellular disease models. If the cell-based product releases a bioactive macromolecule, a potency assay could be based on the units of activity released. For example, the production of insulin in response to changes in glucose levels could be the basis of a potency assay for a cell-based therapy product intended to treat diabetes.

Approaches to measuring product potency should be discussed with regulatory authorities early in development. The FDA has issued guidance that discusses using a matrix of biological and nonbiological assays, including both qualitative and quantitative assays, to establish product potency. Information in this guidance is particularly relevant for cell-based products that have a short shelf life, a complex mechanism of action, or multiple biological activities (see the FDA's *Guidance for Industry: Potency Tests for Cellular and Gene Therapy Products*).

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A validated potency assay is typically required before regulatory approval, which means that a well-defined product potency assay (or assays) should be in place before the initiation of pivotal clinical trials. Therefore, implementation of one or more candidate potency assays early in clinical development is strongly encouraged. Data from these candidate potency assays can also be particularly useful for assessing proposed manufacturing changes, during technology transfer, and in determining product stability.

#### DOSE-DEFINING ASSAYS

An assay that precisely measures the amount of the product is referred to as a dose-defining assay, and it is selected on the basis of its accuracy and precision.

Cell-based therapy products may be dosed on the basis of enumeration of one or more cell populations. For products consisting of a homogeneous, single-cell suspension, viable cell number is the most frequently used assay. Such assays may include enumeration of all cells, total nucleated cells, or another subset of cells. Viability assays are usually based on a cell's ability to exclude a supravital dye, such as trypan blue. Results are expressed as the number of cells that exclude the dye and are therefore considered viable. Fluorescent compounds that bind to nuclear proteins and are excluded by viable cells may be incorporated into flow-cytometric methods for simultaneous determination of viability and cell-identity markers.

Cell counting may be performed rapidly by manual or automated methods. Manual cell counting by visual enumeration of cells in a hemacytometer chamber is a readily available technique with acceptable accuracy but a lower degree of precision than most automated methods. Typical instruments for automated blood cell counting provide reproducible enumeration of nonnucleated cells (e.g., erythrocytes and platelets) and nucleated cells and differential counting of the nucleated cells into mononuclear and polymorphonuclear leukocyte populations. Other automated cell counters ▲rely▲ (ERR 1-Nov-2020) on computer algorithms for visual field analysis of stained cell populations. Beyond cell counting, further identification and enumeration of specific cell populations usually requires cell-surface phenotype analysis by flow cytometric or other methods (see *Identity* above). The proportion of a specific subpopulation of cells may be determined by FACS analysis or by flow cytometry. An example of a specific cell enumeration assay that incorporates viability assessment is described in *Flow Cytometric Enumeration of CD34+ Cells* ⟨127⟩.

For products that contain cells in a nonhomogeneous suspension, such as cells that are combined with a biomaterial (e.g., a scaffold), alternative cell enumeration measures have been used, including total area of a cell sheet, wet weight, total protein, and total DNA. If such measures are used to determine product dose, then supplemental tests should be performed to establish relevance.

## Considerations for Release Testing of Cell–Biomaterial Constructs

For some cell-based therapy products such as cells combined with biomaterials to form combination products, it may not be feasible to directly test the cell–biomaterial construct. This is frequently the case when autologous cells are involved and the cell–biomaterial construct consists of a single unit and sampling of the construct is not feasible. In such cases, the individual components are tested before they are combined, and the final construct is not subjected to direct testing. Indirect measures such as sampling of the culture media can be employed to address regulatory requirements. The quality and stability of the formulated cell–biomaterial construct and relevance of indirect measures must be established by validation studies during product development.

## Sampling Issues

GMP samples from each lot must be retained after the completion of final product release testing, in case safety or quality issues arise later. Even if the product has a very short shelf life, retained samples can be used to detect impurities and other unwanted substances. If rapid-release strategies are employed, manufacturers may need to retain additional or alternatively stored (such as frozen or fixed) samples, so that product quality can be reassessed if necessary. In all cases, sample handling and storage must be validated against relevant testing methods and stability requirements. The FDA (in 21 CFR 820.250) requires product and material sampling plans to be written, specific, and justified on the basis of statistics, science, and risk. Sampling considerations are especially important for products dispensed into multiple containers. Sampling should be based on the appropriateness, stability, and expected distribution for each parameter tested. Proper sampling plan design should include considerations for samples (e.g., uniformity, size, handling, storage) and statistics (e.g., frequency, redundancy, confidence limits, replicates). Process development studies generally help determine the appropriate parameters and limits for the statistical sampling plan design; process validations must confirm or support those statistical sampling plan parameters and limits. See *Stability Protocol Development* (below) for additional considerations, and ⟨71⟩ or *Visible Particulates in Injections* ⟨790⟩ for examples of statistical sampling approaches.

## Alternative Test Methodologies

As described in *Final Product Release Specifications* (above), cell-based therapies must undergo testing for sterility, mycoplasma, and endotoxin. Additional acceptance criteria for tests relating to identity, purity, potency, dose, and other relevant characteristics must be met before clinical use. With the exception of sterility, mycoplasma, and endotoxin, most of the test procedures and their underlying methods used to ensure that the final product meets release acceptance criteria are unique to the product and can be adapted to meet the specific characteristics and applications of the cell-based therapy product. In general, test methods should be developed based on the best available science and should be suitable for use in a GMP manufacturing environment. The assays should be robust, reliable, and capable of being validated and should provide results before release for clinical use. *Validation of Compendial Procedures* ⟨1225⟩ provides basic considerations for methods validation.

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For some cell-based therapies, the sample size and volume of material required for testing or the length of time necessary to obtain test results can consume significant amounts of the final product, or the time required for obtaining results may exceed the product's shelf life—or both. This creates problems with the available supply of product to treat patients and in other situations precludes the possibility of obtaining results before administration to patients. This is a particular problem for the compendial sterility test as well as the FDA-recommended broth–agar culture method for mycoplasma. Consequently, both industry and regulatory authorities have shown considerable interest in facilitating the development of alternative test methods for both sterility and mycoplasma.

FDA regulations for biological products specifically address the use of equivalent methods provided they ensure that the safety, purity, potency, and effectiveness of the biological product is equal to or greater than the assurances provided by the specified method (21 CFR 610.9). Some of the available alternative test methods for sterility and mycoplasma are described below.

The range of available technologies is broad and continues to be developed by assay designers for use in the cell therapy industry. Attributes that should be included in any review of proposed technology include accuracy for the intended purpose, speed in productivity, cost, acceptability by the scientific community and regulatory agencies, simplicity of operation, training requirements and reagents, the reputation of the vendor, technical services provided by the vendor, and, finally, utility and space requirements.

Validation of these test methods and demonstration of equivalence as described in 21 CFR 610.9 are required at the time of BLA or a PMA submission.

### STERILITY

Detection platforms for alternative microbiological methods have been described in *Rapid Microbial Tests for Release of Sterile Short-Life Products: A Risk-Based Approach* ⟨1071⟩. The primary advantage of these systems is the automated nature of the test results and recovery of microorganisms for failure investigations and other microbial characterization methodologies. Principles of validation of alternative microbiological methods are also described in *Validation of Alternative Microbiological Methods* ⟨1223⟩.

### MYCOPLASMA

Compendial testing methodologies for mycoplasma are growth based in agar and broth cultures and require at least 28 days to monitor appropriately the presence of mycoplasma contamination. Because of this limitation, a number of rapid mycoplasma testing technologies have been developed based on nucleic acid amplification techniques such as PCR, as well as nonamplified nucleic acid hybridization assays, enzyme-linked immunosorbent assays (ELISA), and enzyme-based assays.

## QUALITY SYSTEMS

Quality systems weave together all aspects of manufacturing. As with biopharmaceutical and pharmaceutical products, the FDA requires manufacturers of cell-based therapies and tissue-based products to employ QC and QA programs to oversee manufacturing facilities, processes, validation efforts, document and change management systems, labeling, employees training, and the testing of all raw materials, in-process samples, bulk products, and final formulated products. Human cells and tissue must be handled in accordance with GTP as described in 21 CFR 1271. In addition, cGMP as outlined by the FDA in 21 CFR 210, 211, 600s (especially 21 CFR 610), and 820 apply to the manufacturing of cell-based therapies and tissue-based products that are subject to PMA. The extent of quality oversight increases as clinical development progresses, and full cGMP compliance is expected by the time Phase III clinical trial(s) begin.

Data obtained from in-process and final product release testing should be recorded and monitored. Results that are out of specification (OOS), or even those that are out of trend, must be investigated before disposition of the material. The FDA's *Guidance for Industry: Investigating Out-of-Specification (OOS) Test Results for Pharmaceutical Production* (October 2006) provides a systematic approach for conducting an investigation.

An effective risk-management approach at the earliest stages of cell-based product development helps ensure the highest product quality by proactively understanding, identifying, and mitigating potential quality issues. Risk management can also be used to establish meaningful specifications and CPPs to ensure that quality attributes are met.

Risk analysis and mitigation systems use experience and process knowledge to define risk categories. As an example, Risk Levels 1–4 are defined below and can be adopted as one means of conducting a preliminary risk assessment:

**Risk Level 1:** *Technicality*—Poses no risk to the patient and does not impact the safety and effectiveness of the product. Example: A missing signature on a batch record.

**Risk Level 2:** *Alert*—May pose a safety risk to the patient or may have a potential impact on the safety and efficacy of the product. Compliance must be re-established with appropriate justification to proceed after QA review and approval. Example: Digestion time for biopsy processing falls outside a defined range.

**Risk Level 3:** *Do not ship/reject lot*—May pose a safety risk to the patient or may impair the efficacy of the product even after corrective action. Shipment is not permitted. Example: Cultures fail to demonstrate adequate cell growth.

**Risk Level 4:** *Post-Distribution Event*—May pose a safety risk to the patient or may impair the safety, potency, or purity of the product. The defect is identified after product distribution. Example: Failed sterility test occurred after distribution of product.

## FACILITY DESIGN AND OPERATION CONSIDERATIONS

Like biopharmaceutical operations, cell-based advanced therapy and tissue-based product manufacturing facilities must be carefully designed to maintain cGMP-compliant aseptic processing operations while also accommodating unique product

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requirements. Cells or tissue starting materials may need to be received and processed in a segregated area under quarantine to avoid cross-contaminating the main facility. Specially designed equipment and spaces may be required for tissue or cell processing. The facility may need to store, process, and dispose of specialized chemicals or infectious waste. All of these issues may constrain the design of the facility, especially air-handling systems and clean room environments.

While protecting the cell-based advanced therapy or tissue-based product from inadvertent contamination is a primary concern, minimizing risk to the manufacturing operators must also be ensured through appropriate training, protective clothing (gowns, gloves, sleeves, surgical masks, eye protection, and head coverings) and engineering controls (biological safety cabinets, appropriate air quality, etc.).

The degree of control required for cell and tissue processing operations depends on a number of factors, including the complexity of the aseptic manufacturing process, the primary site of manufacturing, and the final product shelf life. ISO 7 (Class 10,000) clean rooms and ISO 5 (Class 100) biological safety cabinets are typical components for cell therapy manufacturing processes, especially those that involve open manipulations.

The differential pressures between classified manufacturing should comply with the FDA's September 2004 document, *Guidance for Industry: Sterile Drug Products Produced by Aseptic Processing—Current Good Manufacturing Practice*. The facility and processing areas should be monitored for air quality in a manner that provides a high level of aseptic assurances. For guidance in this area, see *Microbiological Control and Monitoring of Aseptic Processing Environments* ⟨1116⟩.

Facility cleaning, component and product segregation, sanitization procedures, and environmental monitoring systems must be in place to avoid microbial contamination and cross-contamination between production lots. Techniques such as bar-coding and radio frequency (RF) tags can be used for product tracking and segregation. For guidance in this area, see 21 CFR 211.42, 211.113, 1271, and the FDA's September 2004 *Guidance for Industry: Sterile Drug Products Produced by Aseptic Processing—Current Good Manufacturing Practice*.

Unlike facilities designed for allogeneic products which rely on volume scale-up, facilities for autologous products typically utilize multiple small-unit scale out, which must be considered in the design and operation of the facility. Automation can be used to effectively manage repetitive manual cell manipulations and multiple simultaneous production lots.

Process equipment and software that controls or monitors critical parameters must be qualified and validated; such equipment should also be fitted with alarm systems and emergency back-up power.

## CONSIDERATIONS FOR VALIDATION AND QUALIFICATION

The principles of validation recommended by ICH and FDA guidance documents and ⟨1225⟩ and *Validation of Microbial Recovery from Pharmacopeial Articles* ⟨1227⟩ apply to cell or tissue-based products. Analytical and manufacturing equipment and methods should be validated following the principles described in ⟨1225⟩, in addition to guidance documents issued by ICH [see Q2 (R1)].

Cell-based advanced therapies and tissue-based products present some unique validation challenges. First, the cellular or tissue starting materials can be quite variable, with a wide range in the quality and quantity. Second, manufacturing process steps (especially manual manipulations) can be complex and variable. Manufacturers should develop and validate raw material acceptance criteria, robust manufacturing processes, and analytical methods that consistently result in acceptable final products, even if the process relies on nonstandard or variable tissue materials.

Process validation should take this variability into consideration and should ensure that critical manufacturing and testing endpoints consistently meet specifications.

Aseptic process validations should be performed using microbial growth media in place of the usual process materials, in order to show that the manufacturing staff can execute the procedures and produce a product free of microbial contamination. Procedures intended to maintain segregation during manufacturing should be challenged to verify that there is minimal opportunity for cross-contamination or inadvertent exchange among different patient product lots. Personnel training plans should be established, and proficiency qualifications should be performed periodically. At facilities where raw materials are procured or products are administered, personnel may need to be trained and qualified in order to minimize variability.

Depending on the variability in the source cells or tissues and the complexity of the manufacturing process, it may be necessary to manufacture more than three qualification lots to verify the consistency and the robustness of the manufacturing process. Manufacturing data should be tracked and trended to enable manufacturers to discover and correct problems. Annual product reviews should summarize such factors as in-process and final product testing results, process failures, deviations, OOS, and effectiveness of corrective and preventive action (CAPA) over a 12-month period.

Equipment and facility cleaning validations should be performed to evaluate cell and DNA carry-over, and to demonstrate the efficacy of cleaning and sanitizing agents on both standard microbial and fungal contaminants and environmental isolates from the manufacturing facility. Measurement of residual cleaning agents should be addressed in equipment cleaning validations.

Shipping validations, including environmental challenges such as extremes of temperature and vibration, should be performed for the cellular or tissue starting materials, as well as the final product. Validations should also be performed on software that tracks and controls labeling, shipping, and inventory systems.

## CLINICAL SITE PREPARATION AND ADMINISTRATION

Before administration of some cell or tissue-based products, one or more product modifications or preparative steps may be required. These modifications or steps are frequently performed close to the time of administration, and, therefore, they may not be under the direct control of the original manufacturer. The nature of these modifications is largely dictated by characteristics of the product.

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At the clinical site, written procedures and process controls must be established for all product storage intervals, transport steps, and modifications, starting with a clear definition of critical control points. The unique and irreplaceable nature of many cell or tissue-based products heightens the need for well-established procedures for clinical site preparation and administration.

## Product Manipulations

Typical manipulations include the following:

- **Change in final container:** The manufactured product may have been stored or transported in one container and may require transfer to a different container for administration.
- **Change in physical state or temperature:** A product may require thawing or warming.
- **Change in solution or suspension:** A product may have to be dissolved, diluted, or suspended in a liquid.
- **Combination with a biomaterial:** Therapeutic cells may require combination with a scaffold material such as decellularized extracellular matrix sheets, gels, plugs, capsules, sponges, particles, or granules. In other cases, cells can be added to an existing medical device such as a hollow-fiber filtration unit before use.
- **Admixture or compounding:** For some cell products, mixing or compounding at the clinical site may be necessary.
- **Filtration or washing:** The presence of unwanted materials in the manufactured product, such as particulates, cellular debris, metabolites, or compounds remaining from previous manipulations, may require washing or filtration steps.
- **Sampling:** Sampling of the final product before administration may be required to test the final formulation.

## Thawing Cell-Based Products

Thawing is performed rapidly. If a small number of cells will be reinfused or transplanted, DMSO does not need to be removed from the suspension because most cell preparations can be concentrated adequately to keep the DMSO concentration within tolerable limits. DMSO use has two effects on cells after thawing: cells may clump if damaged, and DMSO reduces cell viability in minutes. If the DMSO must be removed or cells must be concentrated for administration, the thawed cell suspension is generally serially diluted (to avoid osmotic shock) and resuspended in a protein-containing medium. Cell viability and potency may be monitored after thawing, but the information is frequently intended only to gather information rather than as a specification that must be met for clinical use of the cellular product.

## Additional Release Testing of Clinical Site-Manipulated Cell Products

Cell-based therapy products that undergo preparative steps or manipulations at clinical sites must be subjected to appropriate checks or tests to ensure that all quality specifications are met before release for patient administration. The nature and extent of manipulations determines whether additional release requirements or critical specifications must be added to those required immediately after initial manufacture.

Prerelease steps usually include the following:

- Physical inspection of the product, including product appearance (color, turbidity, particulates, or foreign matter), container integrity, temperature, and accuracy and convenience of labeling
- Review of process records and/or certificate of analysis
- For patient-specific products, verification of product labeling and records related to identity of the intended recipient

## Administration to Patients

In all cases, adequate anesthesia and premedication must be carefully evaluated. For example, if the DMSO will remain in a thawed, cryopreserved cellular product, the patient may be given an antihistamine before administration. Pre-administration evaluation must also include assessment of concurrent therapies that may interact with the cell-based advanced therapy or tissue-based product to modify its effects. Some therapies may be adjunctive to the cell-based advanced therapy or tissue-based, such as cytokines that promote proliferation or differentiation of the infused or implanted tissue. Other commonly used drugs such as antibiotics, antineoplastics, anticoagulants, and anti-inflammatory agents must be evaluated for possible effects.

### DELIVERY OF CELL-BASED THERAPY TO PATIENTS

Some cell-based advanced therapy or tissue-based products are patient specific because they are manufactured from a selected autologous or allogeneic tissue source, cells, or tissue. Certain patient-specific products have a defined potential for benefit or adverse immunoreactivity. Systems must be in place to prevent administration of such a product to the wrong patient. Recommended systems include procedures similar to those used for administration of human blood products, and at least two people should verify the identity of the patient and patient-specific product immediately before administration.

A variety of delivery systems such as catheters, syringes, and intravenous (IV) lines are frequently used to administer cells to patients. Before clinical use manufacturers should ensure that these medical device components are compatible with the cells and formulation solutions. In all cases, standard operating procedures and a quality program must be in place to ensure that the product is administered in the intended manner.

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# STABILITY

## General Considerations

Stability studies for cell-based therapies and tissue-based products should be based on a comprehensive understanding of the final therapeutic product and its intended use. Manufacturers should assess the stability of the final product, in-process hold steps, cell banks, critical raw materials, and reference standards.

Where feasible, stability testing should be carried out in accordance with the principles described in ICH guideline Q5C, presented in *Quality of Biotechnological Products: Stability Testing of Biotechnological/Biological Products* ⟨1049⟩. Stability data should also be collected for bulk and other in-process materials that are stored before final processing and filling.

For some cell-based advanced therapies and tissue-based products, final lots may have small volumes and/or shelf lives. In such cases, stability protocols should be based on materials from multiple donors. Because it is difficult or unethical to obtain sufficient stability study cells or tissues from patients, cells or tissues from several sources such as normal donors, research tissue repositories, cadaveric sources, or well-characterized banked primary cells may be used in stability studies to validate storage, shipping, and expiration dating. These results must be interpreted with caution until data confirm that the actual product cells and "surrogate" source materials exhibit similar stability profiles.

For combination products that include cells and biomaterials, the stability of all components when combined must be considered. When biodegradable scaffold materials are present, scaffold degradation should be considered in determining the stability and shelf life of the combination product.

## Stability Protocol Development

Formal stability studies to support licensure and early-phase product stability information gathering should be detailed in a written plan that describes how stability data will be collected and analyzed to support the expiration date.

Stability studies must verify that the storage conditions maintain the quality attributes of the product so that it complies with stability specifications. These specifications may differ from release specifications, but they must address product potency.

Initial studies to establish a provisional expiration date must be conducted before administration to the first patient. Such initial studies are useful for determining which assays are stability indicating, that is, the best indicators of product degradation.

Shipping validations are a special type of stability study. Typically, the product is packaged and shipped (or subjected to simulated shipping) under normal and extreme conditions, and the material is tested before and after shipping to ensure that it still meets the product release requirements. As described in *Storage and Shipping* (below), special attention must be given to the specific thermal, mechanical, and radiological stresses that cell-based therapies and tissue-based products will likely encounter.

## Stability Challenge Conditions

Based on risk assessment, stability studies for cell-based advanced therapies and tissue-based products should include challenge conditions outside of the specified storage ranges, such as those encountered during periods of abnormal storage, shipping, or handling. Examples include brief incubator malfunctions, incubator or cold storage failure, periods of extreme temperature fluctuation caused by shipping to hot or cold climates, hypobaric conditions in the cargo hold of a commercial airliner, or temperatures likely to be encountered in the surgical suite.

A short exposure to an environmental condition well outside of an established limit and a long exposure to one just outside of an established limit may be equally detrimental. The effect of light on the stability-indicating profile should be investigated if it is scientifically warranted. Special attention should be given to products stored in fluids containing light-sensitive or light-reactive components that may give rise to cytotoxic by-products.

Accelerated aging studies are useful to characterize how the cell-based advanced therapy or tissue-based product degrades, and which assays are stability indicating. Such studies should be performed before formal stability studies begin so that the formal studies incorporate the validated stability-indicating assays into the protocol.

# STORAGE AND SHIPPING

## General Considerations

Storage conditions are chosen to preserve the quality of the cell-based advanced therapy or tissue-based product, so that product specifications are maintained throughout storage, shipping, and handling at the clinic. Before clinical trial use, initial studies must be conducted to determine acceptable storage, shipping, handling, and expiration dating. Once stability-indicating methods are developed and the final container–closure, storage, and shipping conditions are chosen, these conditions must be validated, as discussed in *Stability* (above).

The product in its final container closure should be placed in a lightproof, leakproof shipping container with adequate physical support to ensure stability and prevent leakage during shipment. Special consideration should be given to the ability of gas to permeate the shipping container, especially if the cell-based therapy product is stored or shipped on dry ice or liquid nitrogen.

## Storage

For each type of cell-based therapy product, the manufacturer should establish product storage specifications and acceptable storage conditions, including temperature range or liquid nitrogen level. Storage unit monitoring and alarm systems should

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immediately notify manufacturing of unacceptable storage conditions. The stability of the product during routine storage should be monitored and documented via a stability program (see *Stability*, above).

Cryopreservation is the main mode of long-term cell storage. Cell-based therapy products are most often cryopreserved using controlled-rate freezing, or equivalent procedures known to maintain viability. Freezer equipment should be validated and temperature mapped so that cells are stored at an appropriately low temperature. Product stability should be validated under the holding conditions at both the manufacturing facility and clinical site.

Many cell-based products cannot be cryopreserved. Because cells continue to metabolize during storage, their expiration period is short—on the order of hours or days—though the expiration date may be extended by increasing the volume of the storage medium, or by adjusting the storage temperature.

### Shipping

Shipping containers and procedures for cell-based therapies and tissue-based products must ensure acceptable temperatures are maintained under conditions of actual use. These conditions include extremes of temperature inside and outside the shipping container, and other shipping challenges such as X-rays or mechanical vibration. Shipping studies should be conducted during product development in order to identify which stresses affect the product. Bracing and insulating materials should then be chosen and validated to provide a packaging system that will protect the product against extreme temperatures and mechanical stresses and maintain the final container closure integrity. Shipping validations must ensure that the product meets quality specifications (including potency) once it reaches its final destination.

Most cell and tissue-based products are shipped by commercial shippers or courier systems. Commercial aircraft shipments must obtain special permission in order to bypass scanning by airport X-ray equipment. Special attention should be paid to shipping container labels because both biohazard and patient-specific information may be required in specific areas of the packaging. Shipping validations must be conducted under predefined protocols with predetermined acceptance criteria to ensure that the product meets quality specifications (including potency) once it reaches its final destination.

Cryopreserved cell-based therapy products are typically shipped to medical centers on dry ice or in liquid nitrogen dry shippers. Dry shippers may be preferable because temperature is more readily maintained and monitored. Dry shippers also allow continuous monitoring of the shipper's temperature, which can be collected and logged for up to 14 days. Dry ice and liquid nitrogen are both considered hazardous materials during shipping and must be labeled accordingly.

## LABELING

Labeling of cell therapy products is regulated by the FDA under 21 CFR 201, 601, 610, and 1271. For biologics, 21 CFR 610 Subpart G outlines the requirements for container and package labeling. When possible, a full label should be affixed to the product container. When partial labels are used, the container must be placed in a package that contains a label bearing all the items required for the package label. For containers that cannot accommodate any label, the container must be placed in a package that bears all the information required for a package label. When affixed to the container the label should not impede inspection of the contents. For products with very short shelf lives, expiration dating requires adjustment and correction for time zones to provide the user an accurate assessment of shelf life.

Regulations in 21 CFR 610.62 refer to the position and prominence of the proper or United States Adopted Names (USAN) name in relation to a trade name.

Additional labeling requirements in 21 CFR 1271.90 apply because some cell-based therapy products are also considered HCT/Ps. For autologous cell therapies, the manufacturer is exempt from the requirements of determining donor eligibility. However, if the recommended testing for pathogenic or microbial contaminants is not performed before release, the label must contain the statement "FOR AUTOLOGOUS USE ONLY" or "NOT EVALUATED FOR INFECTIOUS SUBSTANCES." The label must also contain the Biohazard legend shown in 21 CFR 1271.3(h) with the statement "WARNING: Advise patient of communicable disease risks." For patient-specific products, the patient's full name, initials, or a combination of these must appear on the labeling to ensure that the product will be administered to the appropriate patient. For materials containing human tissue, per AATB standards, transport package labels should also include prominent identification of contents as "DONATED HUMAN TISSUE" as well as prescribed storage conditions and transport expiration date (if applicable).

Regulations in 21 CFR 201.56 and 201.57 govern the content and format of the package insert.

Several groups have designed a standard for uniform labeling of cellular therapy products, ISBT 128, which defines the data structures and the placement of bar codes and their corresponding eye-readable text that appears beneath the bar code. In addition, ISBT 128 provides class names for different types of cellular products, and various other texts that must go on the labels. Although this voluntary ISBT 128 standard meets different organizations' requirements for labeling cellular products, it does not currently meet FDA regulatory requirements. Consequently, labels that comply with ISBT 128 must be supplemented with additional information required by FDA.

## CONSIDERATIONS FOR TECHNOLOGY TRANSFER

Transfer of the skills, knowledge, technologies, and methods of manufacturing necessary to create a cell or tissue-based product is essential to ensure that scientific and technological developments are accessible and transferable to others. Some general considerations for technology transfer activities are summarized below.

The process of developing a cell-based therapy or tissue-based product is complex and often involves several rounds of technology transfer throughout the product's life cycle. Some examples of technology transfer activities include: from bench research to translational research; transfer from research and development to GMP-compliant manufacturing; and change in manufacturing facility (for example, from in-house manufacturing to a contract manufacturer).

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Manufacturers should anticipate the need for technology transfer during the research and development stage of a cell or tissue-therapy process. This should result in good documentation practices for product research and development, including testing procedures. Data and results should be retained in the format of development reports or technical reports to provide historical information that can be referenced and used in regulatory filings. Critical raw materials, procedures, and equipment should also be identified during technology transfer. Product and process development progress should be monitored against milestones established as part of risk assessment and gap identification in the technology transfer plan. *Table 4* provides an overview of the steps involved in technology transfer.

**Table 4. Technology Transfer—Fundamental Steps**

| | |
|---|---|
| Preparation | • Define the scope, strategy, and risks associated with the project that will be transferred<br>• Identify overall gaps and process transferability<br>• Assess availability of documentation such as manufacturing and testing procedures, sampling plans, in-process and final product data and specifications, material specifications (including source, testing requirements, and quantities required for a manufacturing procedure or test procedure), equipment specifications, specialized training requirements, facility requirements, and infrastructure requirements<br>• Establish a governance body consisting of leads, experts, and mentors from both the sending and receiving sides; determine responsibilities for each group and individual<br>• Define communication and reporting channels<br>• Identify performance measurements, milestones, and timelines |
| Development and Implementation | • Establish a risk management plan<br>• Establish a technology transfer master plan<br>• Develop a training plan<br>• Establish documents at the receiving site (specifications, SOPs, batch records, and standard test methods)<br>• Train operations, quality, and support personnel for sustainability<br>• Qualify materials and vendors<br>• Establish and execute equipment comparability/suitability protocols<br>• Calibrate equipment at receiving site<br>• Qualify personnel, equipment, and facility at receiving site (includes execution of aseptic process validations, sterile media fills, and cleaning validations)<br>• Establish and execute methods/assay qualifications<br>• Establish a product stability program<br>• Perform engineering and consistency/qualification runs<br>• Assess need to establish comparability and prepare regulatory filings<br>• Establish and execute shipping qualifications |
| Maintenance | • Collect and trend process/product data<br>• Monitor product stability<br>• Manage change control<br>• Train and requalify personnel<br>• Recalibrate and requalify equipment<br>• Update regulatory filings |

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The ultimate goal of technology transfer is for the recipient to consistently reproduce a process in order to make a comparable product in compliance with regulations. It is not atypical for manufacturers to develop and implement process improvements during early stages of technology transfer to support scale-up and manufacturing for Phase I/II clinical trials. However, for technology transfer during Phase III studies, pivotal trials, or commercial manufacturing, changes to the process or product should be avoided because they could require additional clinical studies and adversely affect time to market.

***Change to read:***

## REGULATIONS AND STANDARDS

The Federal Food, Drug, and Cosmetic Act (FD&C Act) and the Public Health Service Act (PHS Act) provide the legal framework for FDA regulation of biological products, including cell-based therapy products. A list of frequently used terms in regulation of cellular-therapy products is presented in *Table 5*. In 1993 FDA provided notice that it intended to regulate cellular and gene-therapy products as biological products (*Federal Register* 1993;58:53248–53251). The FDA defined somatic cellular-therapy products as autologous (i.e., self), allogeneic (i.e., intraspecies), or xenogeneic (i.e., interspecies) cells that have been propagated, expanded, selected, pharmacologically treated, or otherwise altered ex vivo for administration to humans for the prevention, treatment, cure, diagnosis, or mitigation of disease or injuries. For other biological products and drugs, clinical trials involving somatic cellular therapy products must be initiated under an investigational new drug (IND) application. After a sponsor submits sufficient evidence of product safety and clinical effectiveness, FDA approval can be obtained for marketing in the form of a BLA or PMA.

As defined by the FDA, cellular therapy products are considered to be drugs, biological products but also HCT/Ps that are regulated under Section 351 and/or Section 361 of the PHS Act. This means that cell-based therapies are subject to cGMP (21 CFR 210 and 211), Biologics Product regulations (21 CFR 610), and HCT/P regulations (21 CFR 1271) including cGTP.

In recent years, the FDA has issued a number of regulations and guidance documents for human cell and tissue products (see *Appendix* and https://www.fda.gov/vaccines-blood-biologics/guidance-compliance-regulatory-information-biologics/biologics-guidances). Of particular importance are the regulations at 21 CFR 1271 that establish a tiered, risk-based approach for HCT/Ps. In this regulatory framework, many conventional human cells or tissues are not subject to premarket approval and have only to comply with GTPs, including donor eligibility. This lower tier of regulatory oversight is intended to prevent the

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introduction, transmission, or spread of communicable disease. When human cells or tissue are the starting material for the creation of a novel cell-based product, additional regulatory requirements are applicable. This higher tier of regulatory oversight includes compliance with GMPs, biological product standards, and premarket approval (see 21 CFR 1271.10). In almost all cases, the cell-based products described in this chapter should comply with the higher tier of regulatory oversight.

In addition to cellular therapy-specific regulations and guidance, many general guidelines such as those related to aseptic processing, GMP expectations during development, process validation, and others are relevant and applicable (see www.fda.gov). Additionally, ICH has issued guidance documents for qualifying cell-based therapy and tissue-based products (see *Appendix* and www.ich.org). Some of the guidelines and concepts in these documents are reproduced in *USP–NF*.

The regulatory pathway for cell-based therapy products parallels that of pharmaceuticals, and as the product moves from early research through pivotal trials and finally marketing approval, the degree of manufacturing control becomes increasingly stringent. This has implications for the manufacturing unit and may dictate that the site be moved. Standards-setting organizations encourage the use of a fully functional quality unit to oversee manufacturing progress. Information is available on the FDA website, along with references to groups charged with guiding the medical community and the manufacturing unit during development.

In addition to *USP* general chapters and monographs for cell-based therapies and tissue-based products, a number of professional standards-setting organizations (see *Table 6* and *Appendix*) have worked closely with regulatory authorities to develop standards and practices. These organizations ensure that standards are current and comply with governmental regulations. Such standards are a supplemental source of knowledge in identification of donors, donor screening and testing, product collections, processing of cellular products, administration, adverse event reporting, and follow-up after treatment. The AATB has developed guidelines for sourcing allogeneic tissue. Over the years various organizations have tried to harmonize standards, including the development of common information circulars that can be compared with package inserts. At present, however, compliance with one organization's standards does not ensure compliance with those of any other organization.

Many benefits accrue to manufacturing facilities that participate in voluntary standards programs. Professional standards-setting organizations participate in educational workshops and disseminate information about operational issues. They also maintain close surveillance of FDA activity and training of inspectors. Further, the FDA relies on accreditation by voluntary standards program, and the FDA's unannounced inspections have led to an increasingly high level of compliance in laboratory and clinical settings and has also undoubtedly increased patient safety. Third-party payors and hospital-ranking services have begun to use accreditation reports in their evaluation of quality programs.

**Table 5. Frequently Used Terms in Regulation of Cellular-Therapy Products**

| Term | Definition |
|---|---|
| 351 products | Regulated under Section 351 of the PHS Act |
| 361 products | Regulated under 21 CFR 1271, Human Cells, Tissues, and Cellular and Tissue-Based Products |
| BLA | Biologics License Application |
| CAR-T | Chimeric Antigen Receptor T-cells |
| CBER | Center for Biologics Evaluation and Research |
| CDRH | Center for Devices and Radiologic Health |
| cGMPs | current Good manufacturing practices |
| GTP | Good tissue practices, ▲21▲ (ERR 1-Nov-2020) CFR 1271, Human Cells, Tissues, and Cellular and Tissue-Based Products |
| HCT/Ps | Human cells, tissues, and cellular and tissue-based products |
| IDE | Investigational Device Exemption. An IDE allows the investigational device to be used in a clinical study in order to collect safety and effectiveness data required to support a Premarket Approval (PMA) application or a Premarket Notification [510(k)] submission to the FDA. |
| IND | Investigational New Drug. An IND is a request for FDA authorization to administer an investigational drug to humans. IND regulations are contained in 21 CFR 312. |
| MCB | Master cell bank |
| PMA | Premarket approval |
| WCB | Working cell bank |

**Table 6. Cellular Therapy Product Standards-Setting Organizations**

| | | |
|---|---|---|
| AABB | AABB, formerly known as the American Association of Blood Banks, is an international association representing individuals and institutions involved in activities related to transfusion and cellular therapies, including transplantation medicine. | www.aabb.org/ |

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**Table 6. Cellular Therapy Product Standards-Setting Organizations** *(continued)*

| | | |
|---|---|---|
| AATB | The American Association of Tissue Banks is an educational and scientific, tax-exempt organization that facilitates the provision of transplantable tissues of uniformly high quality to meet national needs. AATB publishes standards to ensure that the conduct of tissue banking meets acceptable norms of technical and ethical performance. AATB conducts an accreditation program for establishments that retrieve, process, store, or distribute human tissue for transplant. A certification program is administered for tissue-bank personnel to ensure that tissue-banking activities are performed in a professional manner consistent with the standards of the association. | www.aatb.org/ |
| ASTM | ASTM International (ASTM), originally known as the American Society for Testing and Materials, is one of the largest voluntary standards-development organizations in the world and provides technical standards for materials, products, systems, and services. ASTM International standards are used in the information infrastructure that guides design, manufacturing, and trade in the global economy. | www.astm.org/ |
| FACT | The Foundation for the Accreditation of Cellular Therapy is a nonprofit corporation co-founded by the International Society for Cellular Therapy (ISCT) and the American Society of Blood and Marrow Transplantation (ASBMT) for voluntary inspection and accreditation in the field of cellular therapy. | www.factwebsite.org/ |
| NMDP | The National Marrow Donor Program is a nonprofit organization that operates the federally funded registry of volunteer hematopoietic cell donors and umbilical cord blood units in the United States. | www.nmdp.org/ |
| ICCBBA | The International Council for Commonality in Blood Banking Automation was established and given the responsibility for implementation and management of the ISBT 128 standard, a system for identification, labeling, and processing of human blood, tissue, and cellular-therapy products using an internationally standardized system. | www.iccbba.org/ |

## GLOSSARY

**Adventitious agent:** A foreign material that is introduced inadvertently; not natural or hereditary (as in microbial, chemical, or biochemical contamination of a purified substance).

**Allogeneic:** From an unrelated member of the same species but with a different genotype.

**Ancillary materials:** Components used during manufacturing that should not be present in the final product. Examples: growth factors, cytokines, monoclonal antibodies, cell-separation devices, and media components.

**Apheresis:** Procedure of withdrawing blood from a donor, removing select components (e.g., platelets or leukocytes), and transfusing the remainder into the donor.

**Autologous:** From one's own body.

**Bioassay:** Measurement of the effectiveness of a compound by its effect on animals or cells in comparison with a standard preparation. (See also *Potency*.)

**Biological product:** Any virus, therapeutic serum, toxin, antitoxin, or analogous product applicable to the prevention, treatment, or cure of diseases or injuries in humans. (The term "analogous product" has been interpreted to include essentially all biotechnology-derived products and procedures including gene therapy, transgenics, and somatic cell therapy.)

**Biotechnology:** Any technique that uses living organisms (or parts of organisms) to make or modify products, to improve plants or animals, or to develop microorganisms for specific uses. The newer definition refers to the industrial and pharmaceutical use of rDNA, cell fusion, novel bioprocessing techniques, and gene therapy.

**B Lymphocytes** (B cells)**:** A class of lymphocytes that produce antibodies and are derived from bone marrow.

**Bone marrow cells:** A variety of undifferentiated cells (stem cells) and differentiated cells (lymphocytes, granulocytes, erythrocytes, and platelets) found in the internal cavities of bones or bone marrow.

**Bone marrow transplantation:** Transplantation of bone marrow cells that are capable of maintaining the hematological functions indefinitely. Technique used in the treatment of immunological disorders (severe combined immune deficiencies such as ADA deficiency), hematological disorders (anemia), metabolic disorders (Gaucher disease), and malignant diseases (leukemia, lymphoma, or solid tumor).

**CD34:** Cluster of differentiation cell-surface marker 34. CD34 is a protein that distinguishes stem and progenitor cells from more mature blood cells.

**Cell lines:** Cells that are derived from primary culture embryos, tissue, or organs. Such cell lines may have a finite life span or be immortalized (made to replicate indefinitely).

**Cellular therapy:** Therapy that uses whole cells to treat a disease, condition, or injury.

**cGMP:** Current good manufacturing practice.

**Chondrocytes:** Cells that produce the components of cartilage.

**Clonal:** Genes, cells, or entire organisms derived from and genetically identical to a single common ancestor gene, cell, or organism.

**Clonogenic assay:** Procedure based on the ability to give rise to a clone of cells.

**Combination products:** Therapeutic products that combine drugs, devices, and/or biological products.

**Culture medium:** The liquid that covers cells in a culture vessel and contains ingredients to nourish and support the cells. Culture medium may also include growth factors added to produce desired changes in the cells.

**Cytokine:** Any factor that acts on cells; usually a protein that promotes growth.

**Cytoplasm:** Cellular material that is within the cell membrane and surrounds the nucleus.

**Cytotoxic:** Able to cause cell death.

**Dendritic cells:** Cells that sensitize T cells to antigens.

**Differentiation:** A process of biochemical and structural changes by which cells become specialized in form and function.

**Enzyme-linked immunosorbent assay** (ELISA)**:** An immunoassay that uses an enzyme-labeled antigen or antibody to detect the binding of a molecule to a solid matrix.

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**Embryonic stem cell, human** (hESC)**:** Stem cell derived from the inner cell mass of the blastocyst.

**Endothelial cells:** Epithelial cells of mesodermal origin that line the internal cavities of the body, such as heart and blood and lymph vessels.

**Engraftment:** Process whereby cells, tissues, or organs are implanted or transplanted into another organism. Refers to both the mechanical and the biological processes necessary to have a fully functional graft.

**Epidermal:** Pertaining to the outermost and nonvascular layer of the skin derived from embryonic ectoderm.

**Epithelial cells:** Cells from the linings of various organs, e.g., respiratory, intestinal, or vascular epithelial cells.

**Ex vivo:** Outside of the living body. Refers to a medical procedure in which an organ, cells, or tissue are taken from a living body for a treatment or procedure, and then returned to the living body.

**Feeder cells:** Cells used in co-culture to maintain pluripotent stem cells. For hESC, typical feeder layers include mouse embryonic fibroblasts or human embryonic fibroblasts that have been treated to prevent them from dividing.

**Fibroblasts:** Connective tissue cells that have the capacity to produce collagen.

**Fluorescence-activated cell sorter** (FACS)**:** A machine that sorts cells based on fluorescent markers attached to them.

**Formulated:** Prepared in accordance with a prescribed method or conditions.

**Graft-versus-host disease:** Rejection of the transplanted tissue by the host. It is the leading cause of patient death when mismatched allogeneic tissue is used.

**Granulocyte:** One of three types of white blood cells. These cells digest bacteria and parasites.

**Granulocyte–macrophage colony-stimulating factor** (GM CSF)**:** A natural hormone that stimulates white blood cell production, particularly that of granulocytes and monocytes.

**Growth factors:** Factors responsible for regulatory cell proliferation, function, and differentiation.

**Hemacytometer:** A device used to manually count cells.

**Hematopoietic:** Pertaining to or affecting the formation of blood cells.

**Hematopoietic stem cells:** Stem cells that give rise to all red and white blood cells and platelets.

**Hepatocytes:** The predominant cell type in the liver that has an important role in metabolism and is a source of serum proteins. These cells are generally not dividing, but when injured they can divide and regenerate until the injured cells are replaced.

**Human leukocyte antigen** (HLA)**:** Proteins controlled by the major histocompatibility complex. These proteins play a key role in determining transplant compatibility.

**Immunoassay:** Technique for identifying substances based on the use of antibodies.

**Immunofluorescence:** Technique that combines an antibody detection strategy with a fluorescent label for visualization often used in combination with microscopy or fluorescence activated cell sorting.

**Immunogenic:** Substance capable of inducing an immune response; a form of antigen that induces an immune response, as opposed to a tolerogen that induces tolerance.

**In vitro:** In the laboratory (outside the body). The opposite of in vivo (in the body).

**In vivo:** Procedure performed in the living organism.

**Islet cells:** β-islet cells of the pancreas that secrete insulin.

**Keratinocytes:** Differentiated epidermal cells that constitute the top layer of cells in the skin.

**Lineage** (committed progenitor cells, differentiated cells)**:** Specific path of cell differentiation that can be traced to a single cell of origin.

**Macrophage:** Any of many forms of mononuclear phagocytes that are found in tissues and arise from hematopoietic stem cells in the bone marrow.

**Mesenchymal stem cells:** Multipotent stem cells that can differentiate into a variety of cell types.

**Monoclonal antibodies:** Antibodies that are derived from a single cell clone.

**Myocytes:** Fundamental cell units in the muscle. Target cells for insertion of genes that encode secretory proteins.

**Natural killer cells** (or NK cells)**:** Cytotoxic lymphocytes that constitute a major component of the innate immune system.

**Neuronal stem cells:** Stem cells found in neural tissue that can give rise to neurons and glial cells.

**Osteogenic cells:** Derived from or involved in the growth or repair of bone.

**Passage:** The process in which cells are disassociated, washed, and seeded into new cultures after a round of cell growth and proliferation. The number of passages is a good indication of the age of the cultures and expected stability.

**Process validation:** Means for providing documentation that the manufacturing process is controlled, reproducible, and capable of consistently producing a product that meets predetermined specifications.

**Polymerase chain reaction** (PCR)**:** Technique to amplify a target DNA or RNA sequence of nucleotides by cycles of polymerase-based copying, resulting in geometric increases in copy number.

**Potency:** A quantitative measure of biological activity based on the attribute of the product linked to the relevant biological properties.

**Progenitor cell:** Parent or ancestral cell, usually one that is already committed to differentiate into a specific type or lineage of cells.

**Regenerative medicine:** An emerging interdisciplinary field of research and clinical applications focused on the repair, replacement or regeneration of cells, tissues or organs to restore impaired function using a combination of approaches including, but not limited to, the use of soluble molecules, gene therapy, stem cell transplantation, tissue engineering, and the reprogramming of cell and tissue types.

**Serum-free:** Refers to cell growth medium that lacks a serum component.

**SIS:** Subintestinal submucosa.

**Somatic cells:** Cells other than the germ cells.

**Stem cell:** Immortal cell that is capable of proliferating and differentiating into different types of specialized cells. Each major tissue system is thought to have its own putative stem cell.

**Supravital dye:** A dye that stains only living cells.

**Suspension culture:** Growth, in suspension, of cells not requiring attachment to substrate in order to undergo cell division.

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**T cells:** Lymphocytes that acquire functional repertoires and the concept of self in the thymus and are responsible for cell-mediated immunity. There are several subsets of T cells (helper T cells, suppressor T cells, and cytotoxic T cells).

**Umbilical cord blood stem cells:** Stem cells derived from the blood that remains in the placenta and in the attached umbilical cord after childbirth.

**Undifferentiated cells:** Cells that have not yet developed into a specialized cell type or tissue.

**Working standard:** In-house or secondary standard that is qualified against and used in place of a reference standard.

**Xenogeneic:** From a different species.

**Xenotransplantation:** Transplantation of organs from one species to another (e.g., from pigs to humans).

## APPENDIX

### Lists of Relevant Regulatory References

Cellular therapies and cell-therapy components are regulated by FDA as biological products. The general requirements are listed in national laws and international guidance. In the US, national requirements are codified in different sections of 21 CFR, and additional recommendations are available in FDA guidance documents. International guidance documents are available from the ICH, the European Agency of Medicines (EMA), and the World Health Organization (WHO). Although guidance documents from the ICH are well referenced in this chapter, those from WHO and European Medicines Evaluation Agency (EMEA) are not, and manufacturers of cellular or tissue-based products intended for markets outside the United States are advised to refer to relevant guidances from relevant nations. Beyond *USP* chapters referenced in this chapter, the following lists include regulatory documents as well as best practices in product and process development, manufacturing, quality control, and quality assurance.

**Code of Federal Regulations (CFR)**

| Regulations | Scope |
|---|---|
| 21 CFR 201 | Labeling |
| 21 CFR 210 | cGMP for drugs |
| 21 CFR 211 | cGMPs for final products |
| 21 CFR 600.3 | Definitions of important terms for biological products |
| 21 CFR 601 | Requirements for marketing authorization of biologics under BLA |
| 21 CFR 610 | Identity, purity, potency, and microbial safety testing requirements for biological products |
| 21 CFR 610.12 | Sterility |
| 21 CFR 610.60–68 | Product and container labeling requirements for biological products |
| 21 CFR 820 | Quality systems for products regulated as medical devices |
| 21 CFR 1271 | Good tissue practices (GTPs) |
| 45 CFR Part 46 | Department of Health and Human Services, Part 46, Protection of Human Subjects |

**FDA Guidance Documents**

- *Guidance for FDA Reviewers and Sponsors: Content and Review of Chemistry, Manufacturing, and Control (CMC) Information for Human Somatic Cell Therapy Investigational New Drug Applications (INDs)*, April 2008. https://www.fda.gov/downloads/biologicsbloodvaccines/guidancecomplianceregulatoryinformation/guidances/xenotransplantation/ucm092705.pdf.
- *Guidance for Industry: Potency Tests for Cellular and Gene Therapy Products*, January 2011. https://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/CellularandGeneTherapy/UCM243392.pdf.
- *Guidance for Industry: Biologics License Applications for Minimally Manipulated, Unrelated Allogeneic Placental/Umbilical Cord Blood Intended for Hematopoietic and Immunologic Reconstitution in Patients with Disorders Affecting the Hematopoietic System*, March 2014. http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/CellularandGeneTherapy/UCM357135.pdf.
- *Guidance for Industry: Considerations for Allogeneic Pancreatic Islet Cell Products*, September 2009. https://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/CellularandGeneTherapy/UCM182441.pdf.
- *Guidance for Industry: Current Good Tissue Practice (cGTP) and Additional Requirements for Manufacturers of Human Cells, Tissues, and Cellular and Tissue-Based Products (HCT/Ps)*, December 2011. https://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Tissue/UCM285223.pdf.
- *Guidance for Industry: Eligibility Determination for Donors of Human Cells, Tissues, and Cellular and Tissue-Based Products (HCT/Ps)*, February 2007. https://www.fda.gov/downloads/biologicsbloodvaccines/guidancecomplianceregulatoryinformation/guidances/tissue/ucm091345.pdf.

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- *Guidance for Industry: Donor Screening Recommendations to Reduce the Risk of Transmission of Zika Virus by Human Cells, Tissues, and Cellular and Tissue-Based Products*, March 2016. https://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Tissue/UCM488582.pdf.
- *Guidance for Industry: Use of Nucleic Acid Tests to Reduce the Risk of Transmission of West Nile Virus from Living Donors of Human Cells, Tissues, and Cellular and Tissue-Based Products (HCT/Ps)*, May 2017. http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Tissue/UCM372084.pdf.
- *Guidance for Industry: Use of Nucleic Acid Tests to Reduce the Risk of Transmission of Hepatitis B Virus from Donors of Human Cells, Tissues, and Cellular and Tissue-Based Products*, August 2016. http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/UCM516650.pdf.
- *Guidance for Industry: Use of Donor Screening Tests to Test Donors of Human Cells, Tissues and Cellular and Tissue-Based Products for Infection with Treponema pallidum (Syphilis)*, September 2015. http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Tissue/UCM373311.pdf.
- *Guidance for Industry: Source Animal, Product, Preclinical, and Clinical Issues Concerning the Use of Xenotransplantation Products in Humans*, December 2016. https://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Xenotransplantation/UCM533036.pdf.
- *PHS Guideline on Infectious Disease Issues in Xenotransplantation*, January 2001. https://www.fda.gov/regulatory-information/search-fda-guidance-documents/phs-guideline-infectious-disease-issues-xenotransplantation.
- *Guidance for Industry: Investigating Out-of-Specification (OOS) Test Results for Pharmaceutical Production*, October 2006. www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070287.pdf.
- *Guidance for Industry: Sterile Drug Products Produced by Aseptic Processing—Current Good Manufacturing Practice*, September 2004. https://www.gmp-compliance.org/guidelines/gmp-guideline/fda-guidance-for-industry-sterile-drug-products-produced-by-aseptic-processing-current-good-manufacturing-practice-september-200.
- *Guidance for Industry and Food and Drug Administration Staff, Use of International Standard ISO 10993-1, "Biological evaluation of medical devices–Part 1: Evaluation and testing within a risk management process"*. https://www.fda.gov/downloads/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/UCM348890.pdf.

**National and International Regulatory Documents**

- The United States Consensus Standard for the Uniform Labeling of Cellular Therapy Products using ISBT 128. Available at: http://www.iccbba.org/.
- ISO 10993-1:2018, Biological evaluation of medical devices—Part 1: Evaluation and testing within a risk management process. Available at: http://www.iso.org.
- ICH Q2(R1): Validation of Analytical Procedures: Text and Methodology. Available at: http://www.ich.org.
- ICH Q5A(R1): Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin. Available at: http://www.ich.org.
- ICH Q5C: Quality of Biotechnological Products: Stability Testing of Biotechnological/Biological Products. Available at: http://www.ich.org.
- ICH Q6B: Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products. Available at: http://www.ich.org.
- ICH Q9: Quality Risk Management. Available at: http://www.ich.org.
- USAN Naming Guidelines for Cellular and Non-Cellular Therapies. Available at: http://www.ama-assn.org/.
- Guide for the Care and Use of Laboratory Animals (National Research Council, 1996). Available at: http://www.nap.edu/.

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# ⟨1047⟩ GENE THERAPY PRODUCTS

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## INTRODUCTION

Gene therapy products allow administration of nucleic acids to modify the genetic material of cells. Gene therapy products can be broadly classified based on the approach to delivery and include the following: 1) viral vectors [viruses that harbor the gene(s) of interest but usually without the mechanism to self-replicate in vivo]; 2) nucleic acids in a simple formulation (naked DNA); and 3) nucleic acids formulated with agents such as liposomes that enhance their ability to penetrate the cell. Where introduction of nucleic acid to cells takes place ex vivo, the cell population that is administered becomes the gene therapy product. Guidance specific to the manufacturing, processing, characterization, and administration of cell-based products is provided in ▲*Cell-based Advanced Therapies and Tissue-based Products* ⟨1046⟩.▲ (CN 1-May-2020)

Decisions regarding the choice of a gene vector can be complex (see *Design Considerations for Gene Vectors*). The viruses most commonly used include murine retroviruses, human adenoviruses, and human adeno-associated viruses (AAVs). It is inherent in the definition of gene therapy in this chapter that the administration of nucleic acid through transduction is expressed as RNA and then as protein. Examples of gene therapy products are shown in *Table 1*.

**Table 1. Examples of Gene Therapy Products**

| Categories or Strategies | Indication: Administered Product |
|---|---|
| Gene replacement<br>Short-term<br>Long-term | Cardiovascular disease: growth factor vector on a biocompatible scaffold[a]<br>Cystic fibrosis: transmembrane conductance regulatory vector<br>Hemophilia: factor VIII or IX vector |
| Direct cell killing | Cancer: recombinant oncolytic viruses |
| Immunotherapy | Cancer: autologous tumor cells transduced with cytokine or other immunomodulatory genes; lymphocytes transduced with receptors for tumor antigens<br>Arthritis: gene-modified autologous lymphocytes |
| Conditionally lethal genes[b] | Cancer (solid tumor): thymidine kinase (TK) or cytosine deaminase (CD) vector into tumor cells<br>Graft-versus-host disease (GVHD): TK or CD vector transduced into donor T cells |
| Gene disruption via antisense RNAs, ribozymes, and inhibitory RNAs expressed via a vector | Cancer: anti-oncogene vector<br>Cytomegalovirus retinitis: anti-viral vector<br>Human immunodeficiency virus (HIV): autologous lymphocytes transduced with antiviral ribozyme vector |
| Intrabodies | Cancer or HIV: vector encoding single-chain antibody to a tumor protein or a viral protein, respectively |

[a] This product promotes formation of new blood vessels.
[b] Cells with conditionally lethal genes as well as their neighboring cells are killed after the administration of a second drug in vivo. For TK, the drug is gancyclovir. For CD, the drug is 5-fluorocytosine.

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## CHAPTER PURPOSE AND ORGANIZATION

Clinical uses for gene therapy products, their manufacturing processes, and analytical schemes for determining identity, dose, potency, purity, and safety are rapidly evolving, and are as diverse as the products themselves. This chapter summarizes the issues and best current practices in the manufacturing, testing, and administration of gene therapy products. Usually *USP* chapters focus on materials that are commercially available. This chapter, however, not only discusses products for commercial applications, but it also addresses the production of clinical trial materials. When different approaches are options for clinical trial material compared to those used for commercial product, this is discussed.

Where appropriate, reference is made to applicable guidance including International Council for Harmonisation (ICH) quality guidelines because the principles apply even though gene therapy products may be outside the official scope. A list of regulatory and guidance documents applicable to gene therapy is presented in the *Appendix*, together with a list of terms commonly used in the gene therapy field. The traditional compendial perspective is to develop public standards that can be applied to a particular final product without providing production details. This chapter attempts to specify when traditional methodologies or standards can be adapted.

This chapter is extensive because of the diverse nature of the products and the special considerations that they require. Manufacturing has been divided into two sections: the first discusses general aspects of manufacturing and process development, and the second discusses vector design and class-specific topics. *On-Site Preparation and Administration* follows the manufacturing sections because the handling of these products at the clinic often requires facilities and expertise not found in a typical hospital. Other manufacturing-related sections include: *Analytical Methods*; *Stability*; *Storage and Shipping*; and *Labeling*. *Regulations and Standards* summarizes existing guidelines and highlights the need for the development and validation of new methodologies to assess product quality. The *Glossary* lists and defines the terms and abbreviations used in this chapter and those commonly employed in this field.

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# MANUFACTURING OVERVIEW

## Introduction

The manufacturing of gene therapy products has been divided into two sections. This section discusses five topics that apply to manufacturing of all gene therapy products: 1) raw materials, 2) characterization of banked materials, 3) in-process controls, 4) specifications, and 5) validation considerations. The second section, *Manufacturing of Gene Therapy Products*, addresses manufacturing of gene therapy vectors, both viral and nonviral, and discusses the design of gene vectors in detail.

All the general principles of current good manufacturing practice (cGMP) outlined by the FDA in 21 Code of Federal Regulations (CFR) 210, 211, 600s (especially 21 CFR 610), and 820, as well as other *USP* chapters apply to the manufacturing of gene therapy products. The manufacturing facility, equipment and process, raw materials, quality systems, and trained personnel are some of the key elements of cGMP. cGMPs are applied throughout clinical development. Typically, the extent of control increases as clinical development progresses, and full cGMP compliance is expected by initiation of manufacturing in support of Phase III clinical trial(s). The facility and equipment should be carefully designed, built, and validated to support the manufacturing process and to maintain the required product/facility segregation. Preventive maintenance and calibration should be performed routinely on critical equipment. Incubators, bioreactors, and freezers should be fitted with alarm systems that can remotely signal failure. Quality systems should be established to ensure manufacturing is consistent and in control. Systems include but are not limited to the following: change control, document control, environmental monitoring, training, validation master plans, raw material testing and release, vendor approval, product testing and release, stability testing, and corrective/preventive action (CAPA).

## Ancillary Materials

A wide variety of raw materials, including ancillary materials, may be used in manufacturing. Raw materials may include complex substances such as cells, tissues, biological fluids, growth factors, and monoclonal antibodies. Some of these materials may remain in the final therapeutic product as active substances, cryoprotectants, or excipients. An ancillary material exerts an effect on a therapeutic material (for example, a cytokine may activate a population of cells) but is not intended to be present in the final therapeutic product. The quality of raw materials used in the production of a gene therapy product can affect the safety, potency, and purity of the product. Therefore, qualification of this type of materials is necessary to ensure the consistency and quality of all gene therapy products. Activities involved with raw material qualification will change as products move through various stages of clinical development and on to licensure and commercialization. A well-designed qualification program becomes more comprehensive as product development progresses. A qualification program for raw materials used in the manufacturing of gene therapy products should address each of the following areas: 1) identification and selection, 2) suitability for use, 3) characterization, 4) animal-derived components, and 5) quality assurance. For all raw materials, it must be considered when and where each is used in the manufacturing process because this can help define selection criteria. *Ancillary Materials for Cell, Gene, and Tissue-Engineered Products* ⟨1043⟩ should be consulted for specific information about implementing an appropriate qualification program for these materials. Other *USP* chapters provide information about the qualification and standards of specific ancillary materials (e.g., *Bovine Serum* ⟨1024⟩, *Fetal Bovine Serum—Quality Attributes and Functionality Tests* ⟨90⟩, and *Growth Factors and Cytokines Used in Cell Therapy Manufacturing* ⟨92⟩).

## Characterization of Cell and Virus Banks

### CELL BANKS

A cell bank is a collection of vials containing cells stored under defined conditions, with uniform composition, and obtained from pooled cells derived from a single cell clone. The cell bank system usually consists of a master cell bank (MCB) and a working cell bank (WCB), although more tiers are possible. The MCB is manufactured in accordance with cGMP and preferably is obtained from a qualified repository source (source free from adventitious agents) with known and documented history. The WCB is produced or derived by expanding one or more vials of the MCB. The WCB, or MCB in early trials, becomes the source of cells for every batch produced for human use. Cell bank systems contribute greatly to consistency of production of clinical or licensed product batches because the starting cell material is always the same. Cell banks used for the preparation of virus banks or clinical product should be suitably characterized before use. Aspects of cell banking and validation are addressed in ⟨1046⟩, *Quality of Biotechnological Products: Analysis of the Expression Construct in Cells Used for Production of rDNA-Derived Protein Products* ⟨1048⟩, and *Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin* ⟨1050⟩.

### VIRUS BANKS

The master virus bank (MVB) is similar in concept to the MCB because it is derived from a single production run and is uniform in composition. The working virus bank (WVB) is derived directly from the MVB. As with the cell banks, the purpose of a virus bank is to have a consistent source of virus that is shown to be free of adventitious agents for use in production of clinical or product batches. In keeping with cGMP regulations, testing of the cell bank that will be used for production of the virus banks, including quality ▲control▲ (USP 1-May-2020) testing, should be completed before the use of this cell bank for production of virus banks.

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### QUALIFICATION

Cell and viral bank characterization is an important step toward obtaining a uniform final product with lot-to-lot consistency and freedom from adventitious agents. Testing to qualify the MCB or MVB is performed once, and can be done on an aliquot of the banked material or on cell cultures derived from the cell bank. Specifications for qualification of the MCB or MVB should be established. It is important to document the MCB and MVB history, the methods and reagents used to produce the bank, and the storage conditions. All the raw materials required for production of the banks—media, sera, trypsin, and similar substances—must also be tested for adventitious agents.

### QUALIFYING THE MASTER CELL BANK

The FDA *Guidance for Industry: Human Somatic Cell Therapy and Gene Therapy* (March 1998) provides specific recommendations for qualifying MCBs. Additional guidance is provided in ICH Q5D. A description and history of the cell line is required, along with a description of the freezing process, storage conditions, and the number of vials prepared. The identity of the cells should be analyzed by genotypic and/or phenotypic markers. For MCB containing vector sequences, the presence and integrity of the vector should be demonstrated using molecular assays (restriction endonuclease mapping and/or nucleic acid sequencing) and/or measurement of vector gene expression. Purity must be analyzed to exclude bacterial, mycoplasma, fungal, and viral contamination (other than vector sequences). Freedom from adventitious viruses should be demonstrated using both in vitro and in vivo virus tests and appropriate species-specific tests such as the mouse antibody production (MAP) test. Special attention should be given to the detection of replication-competent virus (RCV) arising from recombination of the vector and viral sequences. The MCB is further qualified by tests conducted on cells (from the MCB or WCB) expanded to the limit of in vitro cell age for production.

### QUALIFYING THE MASTER VIRUS BANK

Testing of the MVB is similar to that of the MCB and should include testing for freedom from adventitious agents in general (such as bacteria, fungi, mycoplasma, or viruses) and for organisms specific to the production cell line, including RCV. Identity testing of the MVB should establish the properties of the virus and the stability of these properties during manufacture.

### QUALIFYING THE WORKING CELL OR VIRUS BANK

Characterization of the WCB or WVB is generally less extensive and requires the following: 1) testing for freedom from adventitious agents that may have been introduced during generation of the WCB, 2) testing for RCV, if relevant, 3) routine identity tests to check for cell line cross-contamination, and 4) demonstration that aliquots can consistently be used for final product production. This assumes that the WCB and WVB were prepared in a controlled environment using media and equipment that were screened appropriately for adventitious agents. If not, additional release testing is required.

## In-Process Controls

Manufacturing processes should have well-defined go–no go decision criteria that are applied to key in-process intermediates and are used to pool material that has been processed through a step in several sublots. Quality must be built into the product as well as tested during batch release. In-process controls are the assays or tests that are performed to ensure that the in-process intermediate is of sufficient quality and quantity to ensure manufacture of a quality final product. Examples of in-process controls are listed in *Table 2*. The main reason for performing the in-process control is to ensure that the correct product with anticipated quality and yield is obtained. Intermediate in-process material that fails to satisfy the in-process control criteria should not be used for further manufacturing. This material may be reprocessed if there are procedures in place for such activities. The reprocessed material must satisfy the original in-process specifications before it can undergo further manufacturing. If several sublots will be pooled for further processing, sublots that fail to satisfy the criteria should not be included in the pool, even if the pool containing these failed sublots would pass the in-process assay criteria. During clinical development, assays for product quality and yield should be performed after most processing steps to determine which steps are critical and which assays are most sensitive to deviations in the process. The information from these runs is also used to set the criteria for the selected assays. In-process controls are performed for fully validated processes to ensure that the process continues to be under control. The results of these assays should be trended, and actions should be taken to correct problems as they arise.

**Table 2. Examples of In-Process Control Applications**

| Type of Product | Attribute to Control |
|---|---|
| Viral gene therapy | Quantity of virus after virus culture<br>Specific activity of virus in fractions after column chromatography<br>Quantity of host-cell DNA in fractions after column chromatography |
| Nonviral gene therapy | Optical density or change in oxygen consumption during culture<br>Amount and form of plasmid before culture harvesting<br>Amount and form of plasmid after extraction steps<br>Amount of pyrogen or endotoxin after extraction steps in plasmid pool |

## Specifications

The specification for a gene therapy product should be chosen to ensure the safety and efficacy of the product before use. Selected tests should be product-specific and should have appropriate acceptance criteria to ensure that the product exhibits

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consistent quality parameters within acceptable levels of biological variation, loss of activity, physicochemical changes, or degradation throughout the product's shelf life. The development and setting of specifications for cell and gene products should follow the principles outlined in ICH Q6B and should reflect the FDA's *Guidance for FDA Reviewers and Sponsors: Content and Review of Chemistry, Manufacturing, and Control (CMC) Information for Human Gene Therapy Investigational New Drug Applications (INDs)*.

Establishing specifications for a drug substance and drug product is part of an overall manufacturing control strategy that includes control of raw materials, excipients, and cell and virus banks; in-process testing; process evaluation and validation; stability testing; and testing for consistency of lots. When combined, these elements provide assurance that the process is in control and that the key quality attributes of the product are maintained. Appropriate specifications are established on the basis of thorough characterization of the product during the development phase and an understanding of the process and its capability. Characterization should include measurements of the physicochemical properties, safety, purity, process and product-related impurities, potency, viability, sterility, and quantity. Specifications for each product and its ingredients should be developed from this information by application of appropriate statistical methods. The data should include lots used in preclinical and clinical studies and should also include assay and process validation data that can be correlated to safety and efficacy assessments. Specifications should accommodate the inherent variabilities exhibited by the production process and by the assay. Some lot-release specifications typically applied to biologics may require re-examination for these product types.

The procedures in a specification for the product are anchored by appropriate reference standards. The reference standard for the product ensures that the product, as measured by the release assays, does not change significantly over time. The reference standard is manufactured using the same process as used for clinical production and is subject to all in-process and final release testing. In addition, the reference standard may be subjected to additional characterization not typically performed as part of lot release. The reference standard need not be stored at the same dose, formulation, or temperature as the product, but the stability of this reference standard should be determined. The reference standard verifies that a test produces acceptable results (passes its system suitability tests). A specific assay standard (working standard) can be used in the test, but it should be calibrated against and behave like the reference standard. Changing to a new reference standard (lot) should include many tests, all of which are run side by side with the existing reference standard. The impact of any change in the properties of the new reference standard should be carefully evaluated before it is adopted.

Additional specifications may be needed to produce a safe and effective gene therapy product. These might relate to some of the controls and action limits used to maintain standards and consistency for raw materials, excipients, and the manufacturing process (see *Ancillary Materials* and *In-Process Controls*). Specifications should be established to allow acceptance of raw materials and excipients used in the final formulation of the product. In addition, tests should be performed at critical decision steps during manufacture or at points where data serve to confirm consistency of the process. In-process release specifications should be established for each control step. Heterogeneity can result from the manufacturing process or storage of the product. Therefore, the manufacturer should define the pattern of heterogeneity within the product and should establish limits that will maintain the therapeutic efficacy and safety of the product.

In some cases, specifications may be established for lot release as well as for shelf life. As discussed in ICH Q5C and *Quality of Biotechnological Products: Stability Testing of Biotechnological/Biological Products* ⟨1049⟩, the use of different specifications should be supported by sufficient data to demonstrate that the clinical performance is not affected. Acceptance criteria should be established and justified on the basis of data obtained from lots used in preclinical and clinical studies and lots used for demonstration of manufacturing consistency and on the basis of relevant development data such as those arising from validated analytical procedures and stability studies. Acceptance criteria should also be correlated with safety and efficacy assessments.

Once specifications have been established, test results should be trended. Results that are out-of-specification (OOS)—or even those that are out of trend—should be investigated before the material is considered for further processing. The purpose of an investigation is to determine the cause of the discordant result. The FDA's *Guidance for Industry: Investigating Out-of-Specification (OOS) Test Results for Pharmaceutical Production* provides a systematic approach for conducting an investigation. An assay result can be rejected if it can be confirmed that an error, such as analyst error, calculation error, or equipment failure, has taken place. If the investigation concludes that the product is not within the specification, the lot should be rejected. In unique situations, a product that does not meet all specifications may have to be administered to a patient. However, procedures must be in place to govern the communication of the OOS results to the physician or to the person responsible for making the decision to use the product and to provide instruction for any follow-up testing, patient monitoring, and communication of those results.

## Considerations for Validation

The potential for wide biological variation in gene therapy products, particularly for patient-specific treatments, affects the validation effort. Nevertheless, the basic principles of process validation for any biological product, including recommendations by ICH, FDA guidance documents, *Validation of Compendial Procedures* ⟨1225⟩, and *Validation of Microbial Recovery from Pharmacopeial Articles* ⟨1227⟩, apply to the validation of most gene therapy products. Guidelines for validating viral vaccines can be relevant to gene therapy processes that produce viral vectors. The hold steps in a manufacturing process should be validated to ensure that in-process intermediates are within specification and that the quality attributes of the final product are maintained. Product-release assays should be validated before production of the materials for Phase III pivotal clinical trials.

Process validation demonstrates that the unit operations of the manufacturing process perform consistently and can generate a quality product that meets specifications. Because biological processes are prone to variability, the consistency and robustness of the manufacturing process should be determined by validating the process on at least three lots. Process validation issues pertinent to cell-based products are addressed in ⟨1046⟩.

If possible, the process should be validated for virus clearance according to principles discussed in ICH Q5A. If this is difficult because of the nature of the gene therapy vector (e.g., enveloped virus), additional characterization of cells and animal-derived components used in the production process should be considered. If the gene therapy product is manufactured in a multiproduct facility, validate cleaning of multiproduct equipment and rooms to demonstrate the effectiveness of cleaning agents to inactivate or remove virus.

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# MANUFACTURING OF GENE THERAPY PRODUCTS

## Introduction

Principles for the production of pharmaceutical or biological products are also relevant to the production of gene therapy vectors for use in humans. The same cGMP requirements are applied to ensure that a high-quality product is delivered to the patient. Because of the nature of gene therapy manufacturing systems, most manufacturers face development issues such as scalability, yield, cost efficiency, and product stability.

Most gene therapy vectors have been produced only in relatively small batches necessary to meet the needs of early clinical trials conducted in small numbers of patients. However, the promise of gene therapy in larger patient populations has led to progress in large-scale production and purification technology. This section focuses on designing vectors for gene therapy and choosing a suitable production technology.

## Design Considerations for Gene Vectors

### TYPES OF VECTORS

A typical gene therapy vector is composed of the following: 1) the vector backbone; 2) a promoter; 3) the therapeutic gene, either as cDNA or genomic sequence; and 4) a polyadenylation signal. A wide array of viruses—including murine and human retroviruses, adenoviruses, parvoviruses such as AAV, herpes viruses, poxviruses, toga viruses, and nonviral plasmid therapy systems—have been developed for gene therapy applications. These vectors (see *Table 3*) differ greatly in terms of their capacity to deliver genetic material and the duration of expression. Some viral vectors preferentially target dividing cells, but others are capable of transducing both dividing and nondividing cells. There are significant variations in transgene capacity (i.e., there are limitations on the size of the foreign DNA fragment that can be incorporated into the vector genome). The level, timing, and duration of gene expression required for a gene therapy product depends on the clinical indication. Low-level, long-term gene expression may be required for some diseases, including adenosine deaminase (ADA) deficiency or type A and type B hemophilia. High-level, short-term expression may be more appropriate for cancer when genes that induce apoptosis are used or for cardiovascular disease when preventing hyperproliferation of smooth-muscle cells that may impede restenosis of saphenous vein grafts.

**Table 3. Types of Gene Vectors**

| | Viral | | | | | | | Nonviral |
|---|---|---|---|---|---|---|---|---|
| Family | Retroviridae | | Adenoviridae | Parvoviridae | Herpesviridae | Togaviridae | Poxviridae | — |
| Examples species | Murine Leukemia Virus | HIV | Adenovirus | AAV | Herpes Simplex Virus | Sindbis | Poxvirus (Vaccinia) | Plasmid derived |
| **Vector Characteristics** | | | | | | | | |
| Insert size limit | 8 kb | 8 kb | 4.3–34 kb | 4–5 kb | 40–150 kb | 5 kb | 25–50 kb | 12 kb |
| Chromosome integration | Yes | Yes | No, episomal | Can be integrated or episomal | Can be integrated or episomal | No | No | Yes, but at very low frequency |
| Therapeutic expression | Stable | Stable | Stable or transient | Stable | Stable or transient | Transient | Transient | Stable or transient |
| Vector localization | Nucleus | Nucleus | Nucleus | Nucleus | Nucleus | Cytoplasm | Cytoplasm | Nucleus |
| Types of cells transduced | Dividing only | Dividing and quiescent | Dividing and quiescent | Dividing and quiescent | Dividing and quiescent | Dividing and quiescent | Dividing and quiescent | Dividing and quiescent |
| Efficiency of gene transfer | High | High | High | High | High | High | High | Low |
| Expression of viral proteins | No | No | Yes, unless viral genes deleted | No | Yes | Yes | Yes | No |
| Other | Tropism can be altered by pseudotyping | | — | — | — | Can be used as a plasmid therapy system | — | — |

### VECTOR DESIGN CRITERIA

Many types of gene therapy vectors are being developed, and the vector selected for a particular clinical application depends on the disease state, the target cell, and the intended route of administration. As shown in *Table 3*, capacity depends on vector type, so clinical applications that require a large amount of genetic material will limit the choice of vector system. The payload of a vector system becomes increasingly important when one designs vectors with genomic DNA or a vector that contains extensive regulatory sequences.

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Vectors are also selected based on the intended duration of expression and the target cell. For example, retroviral vectors integrate stably into target cells and are therefore well suited for stem cells or lymphocytes that are expected to undergo extensive cell division. In contrast, adenoviral and plasmid vectors are episomal and may be lost during cell division. However, adenoviral vectors are attractive for vaccine development and cancer applications where tumor cell elimination is the goal. Other vectors, such as AAV, do not integrate at high efficiency but can be expressed long-term in nondividing cells such as neurons or hepatocytes.

Target cell type can also play into the selection of an appropriate vector system (see *Targeting Transduction*). For example, the ▲▲ (USP 1-May-2020) Coxsackie virus B and adenovirus receptor (CAR) is expressed poorly on hematopoietic tissues, which limits the usefulness of the vector system for blood-derived cells. Vectors based on murine retroviruses require cell cycling and are not well suited to the transduction of nondividing cells such as neurons.

The immune system can target both the viral components of the vector and the expressed transgene. Pre-existing antibodies or cellular immunity to certain vector systems can exist and may limit their usefulness. Vectors can elicit an innate immune response that can decrease the efficiency of gene transfer and may also induce a severe adverse event. A large number of current gene therapy approaches seek to limit toxicity and immune response by administration of vector to cells ex vivo. Nevertheless, the majority of diseases suitable for gene therapy will require in vivo administration, and ongoing research seeks vectors with limited immune recognition.

The route of administration and manipulation of the total dose of vector are strategies that can be used to compensate for some limitations of specific vector systems. Additionally, there are advantages and disadvantages for the manufacture of each of the different vector systems that should be considered when planning a clinical application. Production consistency favors systems with well-defined fermentation or culture systems, such as plasmid, retroviral, or adenoviral vectors. For viral vector systems that require helper functions (see below), a rationally engineered cell line can overcome the scalability and consistency limitations of co-transfections. Use of a cell line that is adapted to suspension culture can affect scalability and cost efficiency.

## TARGETING TRANSDUCTION

To be effective, a vector must first find and transduce its target cell. Viruses have a natural host range that is strongly influenced by the expression of specific cell-surface receptors, the current phase of the cell cycle, and the route of administration. Integrins are a class of cell-adhesion receptors that interact with either the penton base or the fiber protein of adenoviruses. The fiber and penton base proteins of adenoviruses mediate binding to the CAR, CD46, and integrins. Adeno-associated viruses primarily interact with heparan sulfate proteoglycan and sialic acid receptors on the cell surface. However, interaction with secondary receptors such as integrins, laminin, and growth factor receptors is required for efficient cell entry and trafficking of virus particles to the nucleus. An amphotropic variant of the murine leukemia virus (MLV), commonly used for gene therapy applications, utilizes the sodium-dependent phosphate transporter RAM-1 to enter cellular targets. Expression levels of each of these receptors vary according to tissue type, which dictates the transduction efficiency of the vector.

The host and tissue range can be modified or targeted by biochemical and genetic manipulation of the vector. Alterations in the tissue and cell specificity of retroviruses—and lentiviruses in particular—occur largely through genetic pseudotyping. During this process, the envelope proteins that dictate virus binding and entry via a specific cellular receptor of one virus are replaced with the envelope protein of another retrovirus or with a protein from an entirely different virus such as the vesicular stomatitis virus glycoprotein. The relative complexity of adenovirus and adeno-associated virus capsids allows them to be genetically modified in several ways. Substitution of a single virus protein (e.g., adenovirus fiber or AAV VP1) with that of another serotype within the same family is very much like the pseudotyping process for retroviruses. However, mosaic virus particles created by interspersing individual capsid proteins from several different virus serotypes in one virion, and chimeric particles created by capsid proteins of two distinctly different serotypes on a particle of yet another serotype (e.g., adenovirus 35 knob on adenovirus 41 fiber on an adenovirus 5 particle) can also effectively change the types of cells and tissues that a vector can transduce. Although this approach may seem straightforward, certain modifications of virus capsid proteins at the genetic level do not facilitate virus particle formation. Although most modifications improve virus uptake in one specific cellular target, they may also increase uptake in several other tissues in which gene transfer would not be desirable. Thus, selection of proteins and ligands must be carefully considered and tested in preclinical models of disease before these vectors can be used extensively.

Viral coat proteins and nonviral vectors can be chemically modified for ligand-mediated receptor targeting. They can be conjugated to cell-targeting ligands by antibody–virus interactions with bi-specific antibodies. Molecular bridges like biotin–avidin complexes and chemical crosslinkers such as bifunctional polyethylene glycol (PEG) tethered to cell specific receptor-binding proteins are easily conjugated to virus particles and often are incorporated in targeting strategies. These approaches can easily be combined and/or exchanged with genetic modifications and with each other to create vectors that effectively target several receptors. Although the biochemical approach avoids the functional complications of introducing foreign domains into viral proteins, each lot of vector must be modified because progeny virus will default to their original genetically encoded tropism. Biochemical processes also require the use of multiple reagents, which may complicate transfer to the clinic.

Another common and effective strategy to target viral vectors to tumor cells takes advantage of the virus replication cycle. Deletion of genes critical for taking over functional cellular checkpoints to support normal virus replication allows the virus to replicate only in cancerous cells where those checkpoints are either defective or inactive. This effect can be further enhanced creating small mutations in virus replication genes driven by tumor- and tissue-specific promoters. With respect to cell cycling, adenoviruses and adeno-associated viruses easily infect both quiescent and dividing cells, but MLV-based retroviral vectors are efficient only when transducing rapidly dividing cells. Lentiviral vectors can infect quiescent cells, including cells of neuronal origin. In general, nonviral vectors can enter both dividing and nondividing cells but have lower transduction efficiencies than viral vectors. Transduction efficiencies of nonviral vectors can be enhanced by the formulation and direct injection in the tissue of interest.

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## INFLUENCE OF HUMORAL IMMUNE SYSTEM AND COMPLEMENT

One of the most significant barriers to effective gene transfer is the humoral immune response to the vector. Regardless of the route of administration, the intended target cell, and the dose, the vector is likely to encounter some component of the immune system. For viral vectors, the humoral immune system cannot readily distinguish between wild-type viral infections and recombinant viral vectors because the humoral response is directed against proteins in the viral envelope or capsid. Protein-containing formulations of nonviral vectors can also elicit a humoral immune response. Specific and cross-reacting humoral responses may pre-exist because of natural exposure to wild-type versions of viral vectors, or they may be elicited during dosing, and the antibody response may vary in its capacity to diminish gene transduction in individual patients. Because neutralizing capacity is frequently enhanced upon multiple dosing, repeated administration can also be problematic. Some of these issues may be remedied by the use of nonviral systems. However, the level of transduction efficiency of these vectors is not currently sufficient for many gene-transfer applications. Transduction efficiency of all vectors is also importantly compromised by the complement system. Many complement proteins have a natural affinity for virus capsid proteins. This interaction initiates release of cytokines and chemokines that facilitate rapid removal of vector from the systemic circulation. This affinity is often heightened (especially in the case of retroviruses) when nonhuman cellular proteins and culture components (e.g., fetal bovine serum) are incorporated into and/or coat the virus particle during large-scale production. Use of producer cell lines of human origin and serum-free culture conditions has decreased inactivation of vectors by complement.

Several strategies for mitigating and avoiding the humoral immune response and complement inactivation have been developed. Increasing the vector dose to compensate for the neutralizing activity of the antibodies and altering dosing regimens to coincide with periods of low antibody titer are logical choices, but possible toxicity associated with high doses of virus and the individual variability of the immune response are potential negative consequences of this approach. Alternatively, viral vectors can be engineered to evade the immune system. One approach involves increasing expression of specific viral genes that allow the virus to evade the host's humoral response. Recombinant viruses constructed from serotypes with limited exposure rates such as simian adenovirus 7 can avoid neutralization in those previously exposed to more common serotypes. Mosaic and chimeric viruses can also avoid neutralization. Both approaches effectively address the issue of efficient gene transfer in those with pre-existing immunity, but both approaches require further investigation in response to concerns regarding safety and large-scale production as well as induction of immune responses in naive patient populations. Covalent attachment of polyethylene glycol (PEGylation) to the virus capsid can both protect the virus from neutralization and blunt the immune response. Pharmaceutical methods such as embedding viral vectors in polymer matrices and administration of vectors to the mucosa (oral and nasal) can also protect viral vectors from the humoral immune response.

## INFLUENCE OF CELLULAR IMMUNE RESPONSES

Once transgene expression is initiated, cellular immune responses rapidly remove cells transduced by both viral and nonviral vectors. This decreases the overall therapeutic effectiveness of gene transfer of low-to-moderate vector doses and can be highly toxic when higher doses are administered. Active protein synthesis is not required for cellular immune responses to viral capsid proteins. For example, the capsid proteins of recombinant AAV vectors have been shown to be long-lived, leading to a delayed immune response and elimination of vector-transduced cells. De novo synthesis of viral gene products can also exacerbate host-cellular responses. Viral vectors have been designed with specific backbone deletions to eliminate the expression of viral structural genes and reduce this effect. Examples of such vectors include gutless adenoviruses, herpes viruses, and adeno-associated viruses in which all viral genes have been deleted, making them dependent on another helper virus for subsequent replication. Certain plasmid sequences, especially those with unmethylated CpG dinucleotides, can elicit a strong cellular immune response and have been used as adjuvants in some DNA-based vaccines. Amplifying plasmids in bacteria that express the CpG Methylase (M.SssI), removal of CpG sequences by site-directed mutagenesis, and removal of unnecessary prokaryotic sequences to create minimal plasmids have reduced the incidence of unwanted cellular responses. One nonmolecular approach to minimize the cellular response against the vector involves the use of immunosuppressants (cyclosporine, sirolimus, dacluzumab) at the time of initial vector administration as well as methods described for reducing the humoral response (use of chimeric vectors, PEGylation) outlined above.

## ANTIGENICITY OF THE GENE THERAPY PRODUCT

In many cases, the gene therapy product and associated promoter and enhancer elements are antigenic in certain cellular targets. When proteins that are retained in the target cell are used, cellular responses may eliminate the target cell. If sustained protein expression is required, the cellular immune response may decrease the effectiveness of the therapy or eliminate it entirely. In terms of treating genetic diseases, patients with a null mutation who have never seen the transgene product may be at a higher risk for immune response than patients who produce a defective protein. Also, truncation of a gene such as the cystic fibrosis transmembrane conductance regulator (CFTR) so that it fits within a chosen vector may result in creation of a distinct antigen.

In other applications, the transgene product is a foreign protein, e.g., thymidine kinase derived from the herpes simplex virus (HSV), and thus may elicit an immune response. In some cases this is the desired therapeutic effect, particularly in antigen-based immunotherapy for cancer or a viral disease. Efforts to minimize the immune response against elements associated with the transgene cassette include designing vectors with the ability to carry full-length humanized sequences for the transgene of interest and administration of immunosuppressants at initial dosing and other intervals throughout the treatment protocol.

## VECTOR LOCALIZATION WITHIN THE TARGET CELL

Once the vector reaches the target cell, several factors can affect the level and duration of therapeutic gene expression, and these factors dictate the choice of an appropriate vector system for a specific clinical indication. The localization of the vector genome within the cell, the strength of the gene expression control elements, the stability of the message, and the stability of

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the translated protein all affect therapeutic impact. Alphavirus-based vectors, such as those derived from Sindbis or Semliki Forest virus, reside in the cytoplasm and typically exhibit a very high level of gene expression. Retroviral, adenoviral, and other viral vectors have advantages in gene delivery with their natural mechanisms for nuclear delivery of the therapeutic gene and reasonable levels of gene expression from viral or other promoters. Nonviral plasmid vectors are episomal and are often susceptible to DNA degradation when they are shunted into cell endosomes. However, some nonviral systems incorporate nuclear targeting signals as a means of increasing therapeutic gene transcription efficiency.

### TISSUE-SPECIFIC EXPRESSION

Another means of controlling gene expression is the incorporation of tissue-specific promoters to stimulate or to restrict expression of the therapeutic gene. Unfortunately, many tissue-specific promoters do not provide high levels of gene expression, and incorporating these sequences into viral vectors may result in loss of specificity or low-level expression in cells that do not normally express the promoter. Tagging vector with sequences recognized by the microRNA system has also permitted tissue-specific expression and may offer tighter control than typically seen with tissue-specific promoter systems.

Drug-responsive promoters are being used to control gene expression. Rapamycin, mifepristone, and tetracycline (tet-on) systems have been used to repress gene expression. This type of regulation is particularly useful when constitutive expression of the vector transgene is toxic.

### IMPACT OF REPLICATION STATUS OF VECTOR

Replication status is another important consideration for vector design and selection. Viral vectors are most frequently constructed to be incompetent or replication-defective in order to limit uncontrolled vector spread and pathogenicity. However, the ability to replicate and spread within a specific cell population, e.g., within a tumor or to metastatic sites, may provide a significant therapeutic advantage over cell type-specific targeting of replication-incompetent vectors. Replication can be engineered to be conditional when, for example, specific viral gene interactions are matched with intracellular pathway targets by means of targeted deletions and/or changes in transcriptional or translational control. When these targets are defective or missing, as in cancer cells, the virus can replicate, but when the target cell is functioning normally, viral replication is repressed. Viruses that have been genetically engineered for selective oncolytic replication include: adenovirus, HSV, vaccinia, measles virus, picornaviruses, influenza virus, Coxsackie virus, and Sendai virus. Some nongenetically-modified viruses are inherently oncolytic in human cells, e.g., reovirus and Newcastle disease virus.

One of the risks inherent in the use of conditionally replicating viral vectors is that such systems are leaky, i.e., the growth of the virus is not absolutely restricted to a single cell type. Also, subsequent rounds of viremia become considerations in the evaluation of tissue distribution/exposure and shedding. The therapeutic promise of these approaches depends on the reliability with which conditionality of replication can be selected or engineered. This therapeutic potential will be realized only if balanced with steps to control the potential risks to patients (associated with replication competence of the viruses/viral vectors) and to address associated shedding-related issues of third party exposure and environmental concerns.

As a proactive contribution to the safety profile and to take advantage of scientific and clinical information already available, virus strains that have been used for human vaccination are often used as the vector backbone. Nevertheless, because the product is replication competent, it presents specific technical challenges for adventitious agent testing and product characterization.

Nonviral vectors are normally designed as nonreplicating systems, but some groups are experimenting with replicating nonviral plasmids to increase gene expression levels (because of the low transduction efficiency of most nonviral systems) and to increase the duration of gene expression. Additional preclinical studies are needed to establish the safety of these systems. Artificial chromosomes have also been designed to take advantage of normal mechanisms for retaining gene expression in rapidly dividing target cells.

### VECTOR INTEGRATION

The duration of gene expression is also a function of the persistence of the vector genome in target cells. Retroviral vectors can stably integrate into the host-cell genome, providing long-term expression. Adenoviruses and nonviral plasmid vectors, e.g., those not administered using electroporation, do not integrate, and expression generally decreases over time. Recombinant AAV vectors generally do not integrate, and when they do, it is not site-specific. However, stable episomes have been observed in certain cell types such as muscle cells.

Site-specific integration can be a desirable feature for vectors that are intended to correct genetic disorders. Although it is not currently efficient enough to be useful, the control of the site of integration is desirable in order to prevent insertional mutagenesis. Insertional mutagenesis has the potential to kill a cell if a critically functioning gene is inactivated or to predispose a cell to malignant transformation if a tumor-suppressor gene is inactivated. Of clinical relevance, promoter or enhancer elements within vectors can lead to activation of cellular oncogenes and have been associated with malignant transformation in children undergoing retroviral gene transfer for X-linked severe combined immunodeficiency.

The success of any gene therapy product depends on the relationship between the vector-delivery system and the requirements of the disease in terms of the site, level, and duration of therapeutic gene expression. A universal vector now appears unlikely, and the challenge lies in fitting one of several possible vectors to the disease and to the gene to be delivered.

## Manufacturing and Purification Strategies

### VECTOR CONSTRUCTION

Viral and nonviral gene-transfer vectors are constructed by using standard molecular biology protocols. For viral vectors, the vector backbone consists of viral RNA or DNA sequences from which the regions encoding viral structural genes or the regions

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required for replication have been deleted. The deleted region of the vector is usually modified with specific restriction endonuclease sites used to allow insertion of the gene of interest. For nonviral vectors, the plasmid DNA backbone contains multiple restriction sites for cloning and the bacterial elements necessary for plasmid production. Vector backbones can accommodate single or multiple gene insertions depending on the maximum amount of sequence they can carry. The promoter that facilitates transcription of the gene insert can be a related viral promoter, such as the murine leukemia virus long terminal repeat (MuLV LTR), or a heterologous promoter that is either tissue-specific, such as the alpha crystalline promoter (of the eye), or constitutive, such as the cytomegalovirus (CMV) late gene promoter. For example, in a retroviral vector construct containing two gene inserts, transcription of one is regulated from the 5′-LTR-promoter sequence, and a second gene insert can be linked to an internal heterologous promoter from Simian virus 40 (SV40).

The complementary DNA (cDNA) containing the therapeutic gene of interest, including its introns, is excised from its source using restriction enzymes and is inserted at the multiple cloning site of the gene-transfer vector. The polyadenylation signal can be derived from multiple sources such as the SV40 virus or human growth hormone gene. Characterization and testing of gene therapy vectors are described under *Analytical Methods*.

## HELPER FUNCTION SYSTEMS

Recombinant viral vectors are most often modified to be replication defective, a condition created by deletion or modification of the viral genes needed for replication and production of infectious virus. Because the vectors are stripped of some or all of the viral genes, a system must be developed to supply viral proteins and to encapsulate the vector into a viral particle. Generally, this is accomplished by two methods: transient transfection or stable packaging cell lines.

In the transient transfection method, a series of different plasmids are generated, including a plasmid containing the vector and another vector containing the viral genes. For example, retroviral vectors can be generated using a three-plasmid system: 1) the transgene-containing vector plasmid; 2) a plasmid containing the *gag/pol* viral gene region; and 3) a plasmid containing the viral envelope. All three plasmids are transfected into cells, e.g., HEK293 or HT1080, and vector-containing virions are harvested after 2–3 days. The separation of vector and viral genes on different plasmids, along with vector designs that minimize the homology between vector and viral sequences, decrease the chance for recombination and generation of replication-competent virus. Similar approaches can be taken with most viral vector systems.

The transient transfection method has the advantage of a rapid production time and flexibility when changing components of the vector or viral constructs. Nevertheless, it can be cumbersome when scaling up for manufacturing, and special care must be taken to provide consistent production yields. An alternative method has been the use of vector packaging cell lines. In this scenario, the viral genes are introduced stably into an immortalized cell line that yields persistent expression of viral genes. As with transient transfection, the viral genes generally are expressed from different plasmids to decrease the risk of recombination. Since plasmids integrate infrequently, considerable time and effort are required to isolate a high-titer packaging cell line and to generate an MCB. Vector constructs can be introduced into cells from the MCB, and researchers, by screening for a high-titer clone may allow isolation of a stable cell line that generates the vector of interest. These cell lines generally can be expanded to great numbers and often produce vector for up to a week at a time, facilitating vector scale-up and product consistency.

Typical helper function systems are as follows:

**Retroviral vector systems:** Initial packaging cells were based on the murine fibroblast cell line NIH 3T3. The PG13 cell line (expressing the Gibbon Ape Leukemia Virus envelope) has been used extensively with a low incidence of recombination events leading to RCV. More recently, the human HEK293 and HT1080 cell lines have been modified to serve as packaging cell lines for retroviruses. The use of a human cell line decreases elimination of vector particles by the human complement system (although this is generally not a concern for vectors used in ex vivo protocols).

**Adenoviral vector systems:** HEK293 cells are widely used to supply the E1 function necessary for efficient adenoviral replication that is deleted from first-generation adenoviral vectors. Other complementing cell lines, such as E1-modified A549 cells (human lung carcinoma) and the PER.C6 cell line (human embryonic retinoblast), have also been created to supply E1 or other missing functions. PER.C6 contains the E1 region under the control of a phosphoglycerate kinase (PGK) promoter and has no flanking adenoviral sequences in order to eliminate production of replication-competent adenovirus (RCA).

**AAV vector systems:** These systems classically use adenovirus-infected HEK293 cell lines transiently transfected with AAV helper plasmid containing the *rep* and *cap* genes, which are required for AAV replication and capsid formation, respectively, and are deleted from the AAV vector. In some AAV production systems, wild-type adenovirus has been removed from the process by using triple transfection of plasmids expressing *Ad* early genes, *rep* and *cap*, and the vector transgene. The HeLa cell line (from human uterine cervical carcinoma) has also been used as a transient production system. More recently, both of these cell lines have been used to establish stably transfected packaging cell lines that express *rep* and *cap* genes and in some cases express the adenoviral functions needed for AAV replication when *rep* and *cap* are present (E1a, E1b, E2a, E4, and VA RNA). AAV production systems using recombinant HSV and Baculovirus have also been developed.

**Gutless adenoviruses:** Early manufacturing systems for the adenovirus vector known as gutless adenovirus were similar to classical AAV vector manufacturing systems because HEK293 cells were transiently transfected with helper plasmid containing required adenoviral functions. Development of helper viruses housing a packaging signal flanked by loxP sites and complementing HEK293 cell lines that express the bacteriophage P1 site-specific Cre recombinase has greatly improved the yield of the gutless virus. This technology notably reduces the amount of helper virus contamination by preventing packaging of the helper virus genome while permitting it to replicate and support replication and encapsidation of the gutless vector.

## VIRAL GENE THERAPY VECTORS

Retrovirus and adenovirus vectors typically have been produced at laboratory, non-GMP scale by use of traditional cultivation methods for anchorage- and serum-dependent cell lines employing flasks, trays, and roller bottles. Initially, gene therapy vectors were produced by these methods because large volumes of product were not required for early clinical studies. Cell-bank systems are used as the source of cells, and virus banks are the source of virus for clinical production. In many cases, supernatant

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is collected, clarified, and stored frozen in bags at –70°. In many early clinical trials, unpurified supernatant has been used for ex vivo gene transfer.

Larger-scale upstream production methods have been reported and are commonly used. They include suspension, bioreactor, and fixed-bed or microcarrier culture methods. Some groups have reported adapting their process cells to serum-free culture conditions. Cells are harvested and lysed or supernatant is collected. The harvest is clarified and purified to remove host-cell debris, host-cell DNA, and other process-derived contaminants.

Traditionally, viruses are purified by gradient ultracentrifugation, but this is time-consuming and unsuitable for larger-scale production purposes. The selection of downstream process steps and their sequence is determined by the nature of the virus itself and the upstream process used for manufacturing the virus. As processes are being developed for the manufacture of gene therapy vectors, many different purification steps have been reported. These include ion-exchange and sulfonated-cellulose chromatography, zinc ion affinity chromatography, and size-exclusion chromatography. Typically, DNase or other nuclease treatments are used in the process in order to reduce host-cell or plasmid DNA. AAV production and lentiviral production are complicated by a need for transient transfection or co-transfection of plasmid or helper virus. These processes have generally required anchorage-dependent cell lines that are difficult to scale up. The development of stably transfected cell lines would allow large-scale production.

## PURIFICATION METHODS: VIRAL VECTORS

**Retroviruses:** To date, purification of retrovirus preparations for Phase I clinical trials has often been minimal at best, i.e., the simple concentration of culture supernatants is insufficient to meet the stringent quality standards required for in vivo therapy. Centrifugation and microfiltration techniques are very useful for clarification of culture supernatants and removal of cellular debris. Ion-exchange, size-exclusion, and affinity chromatography techniques have also been employed to remove excess salt, serum, and low molecular weight contaminants also concentrated with the virus.

**Adenoviruses:** Recombinant adenoviral vectors were often purified by cesium chloride density gradient ultracentrifugation. This is still used for research-scale preparations, but the procedure is neither scalable nor efficient for large quantities of clinical-grade virus. The most recent scalable purification methods use anion-exchange chromatography because of the strong affinity of intact virus particles for the resin with respect to that of cellular and individual capsid proteins. Published loading estimates for anion-exchange resins range from $0.5 \times 10^{12}$ to $5 \times 10^{12}$ virus particles/mL of resin or 0.14 mg of virus to 1.4 mg of virus/mL of resin. Gel filtration and immobilized metal affinity chromatography are often used in polishing steps following anion-exchange purification of recombinant adenovirus-based products.

**Adeno-associated viruses:** The toxicity of cesium chloride, the aggregation of AAV particles, and the fact that adenovirus is not completely removed after extensive centrifugation complicate AAV purification by cesium chloride density gradient ultracentrifugation. Another density separation medium, iodixanol, which is less toxic than cesium chloride and prevents AAV aggregation, has been employed in a single centrifugation step. Passage of the AAV fraction over an affinity column consisting of either a heparinized support matrix or monoclonal antibodies produced against AAV2 strongly increased purity and infectivity of final preparations. These methods are appropriate only for specific AAV serotypes. Ion-exchange chromatography is the most powerful and versatile method for AAV purification, although buffer pH, detergent concentration, and column medium must be tailored for each AAV serotype. Infectivity and purity of preparations obtained from these purification strategies are comparable to those obtained from affinity chromatographic methods and are complete within 3 h.

## PLASMID OR NONVIRAL VECTORS

Plasmids are double-stranded, circular DNA molecules that exist in bacteria as extrachromosomal, self-replicating molecules. They have been modified to serve as cloning systems, to contain multiple restriction endonuclease recognition sites for insertion of the cloned transgene, and to contain selectable genetic markers for identification of cells that carry the recombinant vector. Plasmid-based, nonviral vectors are frequently used as gene delivery systems for both in vivo and ex vivo gene therapies. These vectors are in the form of naked DNA or are complexed with lipids or other agents that facilitate transfer across the cell membrane and delivery to the cell nucleus without degradation. An advantage of a plasmid-vector system is the efficient production of large quantities of the vector that is easily characterized and avoids the risk of RCV associated with many viral systems.

Nonviral vectors are typically produced by using an *Escherichia coli* bacterial system. Plasmids are transfected into *E. coli*, and an appropriate single bacterial colony is selected and expanded to create an MCB. After reselection of a colony from a bacterial plate inoculated from the MCB, plasmid DNA is isolated from cultures that can range in size from 1 L on a laboratory scale to hundreds of liters in bacterial fermenters. Plasmid DNA can be purified by several methods including affinity or ion-exchange chromatography and cesium chloride–ethidium bromide density gradients. Cesium chloride–ethidium bromide density gradients are not recommended for production of clinical-grade material.

## PRODUCTION AND PROCESSING OF NONVIRAL VECTORS

One benefit of nonviral vectors for gene transfer is that the production process is rather generic and can be applied to any plasmid preparation regardless of composition or application. Because the current average human dose of plasmid DNA for gene transfer and vaccination is approximately 1 mg, the primary challenge associated with large-scale production of plasmids is to develop a process that is both scalable and economical. Thus, process development for plasmid-based vectors remains an active area of research and development. A standard process for large-scale production of recombinant DNA plasmids consists of the following five unit operations.

**Fermentation:** Fermentation processes must support growth of transformed bacteria and maximize the amount of plasmid produced by each cell. *E. coli* is the most common strain used for plasmid production. Amino acids, nucleosides, and the ratio of nitrogen to carbon-containing compounds present in a rich media formulation greatly improve plasmid yield.

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**Harvest:** Bacterial cells are harvested either by centrifugation or microfiltration. Centrifugation under GMP conditions can be costly, which makes microfiltration the accepted method of cell harvest. This also allows spent media, metabolic byproducts, extracellular debris, and impurities to be washed away before purification.
**Lysis:** Bacterial cells must be lysed to release the recombinant plasmids. This is one of the most critical steps in the production process because it can significantly affect the amount of usable [covalently closed circular (ccc)] and unusable [sheared, partially denatured, and open circular (oc)] forms of DNA in a preparation. The most widely used method of lysis for clinical-scale manufacturing is treatment with alkaline detergent and precipitation of cellular debris with acetate. This removes a large fraction of cellular impurities from the lysate, but it also increases the sensitivity of plasmids to mixing and localized concentrations of detergent, which are hard to manipulate on a large scale. Lysis of cells by heat exposure addresses this issue and effectively denatures cellular proteins and bacterial DNA.
**Isolation/purification:** Some processes include additional steps for removal of cellular debris and other contaminants from crude bacterial lysates by precipitation with detergents, polyethylene glycol, or salt. These reagents affect plasmid stability and are removed by column chromatography. Size-exclusion chromatography can effectively separate plasmid DNA from RNA, proteins, and other small molecules present in the cleared lysate. The degree of separation of plasmid DNA from contaminants is highly dependent on the type and concentration of salt in the running buffer. Resins used in anion-exchange chromatography have a high affinity for plasmid DNA and provide maximal sample concentration. Hydrophobic interaction and thiophilic aromatic chromatography are the methods of choice for selective separation of the different plasmid DNA isoforms and endotoxin reduction.
**Bulk preparation:** After purification, the bulk plasmid is placed in a suitable buffer and formulation by ultrafiltration using a membrane with a pore size of 50–100 kDa.

Plasmids for clinical use must be highly characterized. Impurities from production and processing steps are well known. Tests necessary to confirm the identity, purity, and potency of a plasmid-based product are well established and routine. These tests and the current specifications set by the FDA and the World Health Organization are summarized in *Table 4*.

**Table 4**

| Assay Type | Issue | Determined By | Acceptable Level in Final Product |
|---|---|---|---|
| Identity | Cross-contamination with other products | Restriction digest/gel electrophoresis | N/A |
| Purity | Residual bacterial chromosomal DNA | Real-time polymerase chain reaction (PCR) | <2 µg/mg DNA |
| | Residual RNA | Analytical HPLC | <0.2 µg/mg DNA |
| | Residual bacterial protein | Bicinchoninic acid (BCA) protein assay | <3 µg/mg DNA |
| | Endotoxin | Limulus amebocyte lysate (LAL) assay | ▲Suitable criteria based on the final manufacturing process▲ (USP 1-May-2020) |
| | Sterility (bacterial and fungal) | Method outlined in 21 CFR 610.12 | No growth |
| | Appearance | Visual inspection | Clear solution free of particulates |
| | pH | pH meter | Physiologic (7.0–7.4) but may be product-specific |
| | Plasmid confirmation (ccc vs oc) | HPLC or capillary gel electrophoresis (CGE) | >97% ccc |
| Potency | Labeled dose | In vitro<br>Enzyme-linked immunosorbent assay (ELISA)<br>Fluorescence-activated cell sorter (FACS)<br>Reverse transcription (RT)-PCR<br>Light absorbance ($A_{260}$) | Transgene/plasmid specific |

## Introduction of Genetic Material into Cells—Gene-Modified Cells

A common extension of cell therapy involves the introduction of genetic material, usually DNA, into cells to alter their pattern of gene expression. While discussion focuses on DNA, similar scenarios can be applied to RNA or a derivative of DNA, except that the stability and solubility of the particular nucleic acid may dictate modifications of certain steps. The general process is often referred to as ex vivo gene therapy because the cells are removed from the patient or donor and the genetic material is introduced while the cells are outside of the body. The genetically modified cells are then administered to the patient. The genetic material introduced can either cause expression of new genes and products or inhibit the expression of already expressed genes and products. The latter represents a type of antisense therapy. The genetic material can be introduced by the same range of reagents that are involved with gene therapy: viral vectors, nucleic acids in a simple formulation (naked DNA), or nucleic acids formulated with agents such as liposomes that enhance their ability to penetrate the cell. Most of the steps and considerations discussed above also apply to the ex vivo introduction of genetic material into cells. The main goal of ex vivo therapy is to develop robust processes that will work with the majority of the patient's or donor's cells. This takes considerably more effort than processes for cell lines.

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The method for introducing new genetic material into cells depends on the biology of the system and the desired stability of gene expression. If a simple retroviral vector such as ▲Moloney▲ (USP 1-May-2020) murine leukemia virus is used for transduction, the cells must be actively dividing because vector DNA is integrated into the cellular DNA only during replication. This usually leads to long-lasting expression of the desired gene product. Adenoviral vectors, naked DNA, or formulated DNA can be introduced into nondividing cells. However, gene expression will be transient because the introduced DNA will usually be extrachromosomal.

The main challenge in ex vivo gene therapy is to achieve efficient transduction or transfection, introducing sufficient DNA into the cell before the DNA degrades. In the case of transduction by simple retroviral vectors, cells are stimulated with reagents that cycle them into the S phase (replication) at the time that the vector is applied. Most retroviral vectors are stable in cell culture for a period up to a few hours. Because diffusion is minimal, only a small fraction of viral particles will come into contact with cells during this period. The following techniques can be used to increase the number of viral particles that contact the cell in a given time period:

1. Maximization of viral particle concentration and minimization of the media volume during the transduction step.
2. Multiple applications of the virus.
3. Centrifugation of virus particles onto the cells.
4. Placing cells on a filter and slow pulling viral media through the filter.
5. Addition of binding-enhancing polymers to the media. [NOTE—Co-culturing the target cells with the viral producer cells is not recommended. This technique increases the chance of a recombinant event and production of RCV. Furthermore, any product for which co-culturing is used to transduce the human cells would be considered a xenotransplant if the producer cells were not human. The second cell type, whether human or not, may cause inflammation.]

Each of these techniques has its own set of issues that must be addressed in order to develop a robust process. In technique 1, reduction of the volume during transduction results in rapid exhaustion of the medium, so supplemental medium should be added within a few hours. In technique 2, the cells may no longer be in the correct cell cycle phase during later applications, or cells may have become refractory because of unproductive transformation during the previous application. Techniques 3 and 4 can work well on a very small scale, but the number of cells that can be transduced may be insufficient to obtain an efficacious dose. In technique 5, polymers may fail to provide a benefit because virus binding may involve specific receptors for which surface density may prove to be the limiting factor.

Similar issues and techniques can apply with other viruses or DNA preparations. The issue of slow diffusion is even more marked for the use of DNA preparations. Factors such as the cell type in which the viral vector was produced, the media used for vector production, and the purity of the vector can have a pronounced effect on the efficiency of transduction. Although certain methods may not require cells to be actively cycling, in practice, most processes require that cells be capable of replication because of the following considerations:

1. Safety considerations may dictate that only cells that express the new DNA are returned to the patient, which requires that these cells be selected. As described below, the most common selection method uses an antibiotic-resistant gene that is co-introduced with the new genetic material.
2. Further propagation may be required to achieve the therapeutic dose of cells.
3. Economic, biological, or technical reasons may dictate that the DNA introduction step be carried out at a low cell number and that the desired cell population then be expanded to the required dose.

Therefore, conditions that enable the cell or maintain its ability to proliferate must be developed in almost all cases. The biology of the cells, the available technology, and process economics will determine whether cells are propagated before, after, or during the introduction of new genetic material. Most processes do in fact expand the population after the introduction of the new gene.

Whether cells that do not productively express the gene can be administered to patients depends on the biology of the application, the dose required versus the handling capability of the manufacturing system, and, most importantly, the toxicity of the nonproductive cell population. Selection of the genetically modified cell population is commonly carried out using an antibiotic-resistance marker gene, such as neomycin, which is co-introduced into the cell with the new genetic material. For neomycin selection, cells in culture are treated with the antibiotic G418 at a concentration and for a period that has been shown to kill cells with nonproductive expression while allowing the productively expressing cells to proliferate. In this manner cells that are resistant to the antibiotic are presumed also to express the DNA of interest. The expression should be tested as a lot-release requirement or verified in a series of mock runs. Because most antibiotics decrease cellular proliferation, optimization of the culture media composition may be necessary for efficient selection and propagation of the gene-modified cells.

Following the antibiotic selection step, a second phase of antibiotic-free cell propagation may be required in order to achieve the desired dose and to rinse residual G418 out of the system. The selected medium and the total time that the cells are in culture can be critical to maintaining the desired expression of the original differentiated functions. An additional issue associated with the use of selection markers is that they generally are nonhuman genes. The expression of these genes usually elicits an immune response. Process development is often carried out with cells from healthy donors. Consideration should be given to the fact that for very sick patients, healthy cells that can be stimulated to undergo efficient, sustained replication are difficult to obtain.

Manufacturing, cell processing, and analytical testing issues pertinent to cell-based products are addressed in ⟨1046⟩.

## Formulation of Gene Therapy Products

Final formulations for gene therapy products are still in early development, and currently most gene transfer vectors are stored in solution at ultra-low temperatures. Successful formulation of candidates for gene transfer relies on a thorough understanding of the physicochemical and biological characteristics unique to each vector system. Factors like solution pH, ionic strength, and osmolarity influence the thermal stability of viral and nonviral vectors. Organic carbohydrates such as mannitol, sorbitol, sucrose, and trehalose have been incorporated into preparations to prevent disruption of the native

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conformation of the vector in solution, during the freeze-thaw process, and during lyophilization. Amino acids such as arginine and leucine have been incorporated into formulations for their buffering effects and to prevent aggregation. Surfactants such as the Tweens, Spans, and Pluronics have been effective at preventing aggregation, but this effect is somewhat vector-specific because some vector products are easily disrupted by these reagents. Lipids, polymer, and extraneous proteins (human albumin and gelatin) have also been incorporated in many vector preparations because of their ability to prevent loss of vector from direct interaction with pharmaceutical surfaces and during freeze-thaw cycles.

Before initiating a program for formal screening of formulations for a vector, the following factors should be considered. The required dose and/or storage concentration of the final product, as well as the specifics of the container–closure and/or delivery system should be established. Analytical methods to assess potency and identify degradants should be in place. The expectations of the formulation must also be defined. Some pragmatic criteria for the design and selection of vaccine formulations for use worldwide are: 1) the final product should be in a formulation that affords an 18- to 36-month shelf life when stored at 2°–8° or above, 2) the formulation should have an acceptable stability profile at ambient temperatures to cover short-term storage and transportation in the field, 3) it should adequately protect the vector from damage during freeze-thaw cycling, and 4) it should consist of reagents that are pharmaceutically acceptable and within physiologically acceptable concentrations. Formulation changes during clinical development must be supported with preclinical studies and stability data. Sufficient time should be built into plans to account for this.

To date, little work in the area of formulation development of retroviral vectors with additives approved for human use has been described. The most significant effort to develop stable formulations for gene transfer has been with recombinant adenoviruses. Recently, identification of the mechanisms by which recombinant adenoviruses degrade in solution led to the development of several liquid formulations that stabilize the virus for up to 18 months at 4°. Adeno-associated viruses are regarded as one of the most stable viral vectors. This virus has been documented to be stable for approximately 4 months in phosphate buffered saline at 4°. Addition of cryoprotectants and surfactants prevents aggregation of virus particles and extends the shelf life to 1 year. Lyophilized formulations of both adenovirus and AAV with shelf lives of several years at room temperature have also been described. Although nonviral vectors have been found to be generally robust in standard buffers at 4°, their stability may be extensively influenced by extraneous components included to promote gene targeting. Consider the nature of those components when developing stability protocols and strategies. ▲▲ (USP 1-May-2020)

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## ON-SITE PREPARATION AND ADMINISTRATION

One or more product modifications or preparative steps may be required before administration of the gene therapy product to the patient. These modifications or steps are frequently performed close to the time of administration, and, therefore, they are performed under conditions not under control of the original manufacturing facility. The nature of these modifications is dictated largely by characteristics of the product in relationship to the particular application. These include thawing, washing, or filtration to remove unwanted product manufacture-related materials and also include defined physical space with appropriate environmental controls, trained personnel, detailed standard operating procedures, and a comprehensive quality program.

The unique and irreplaceable nature of many gene therapy products, e.g., gene-modified cells, many of which have originated from an autologous or a selected allogeneic tissue source, creates special considerations for product manufacture, release, and administration. Issues pertinent to the administration of cell-based products are addressed in detail in ⟨1046⟩.

### On-Site Preparation

#### PRODUCT MANIPULATIONS

Before administration, on-site preparation of the gene therapy product may involve one or more manipulations, including the following:

- **Change in final container:** The manufactured product may have been stored or transported in one container but may require transfer to a different container for administration.
- **Change in physical state or temperature:** A product may require thawing from the frozen state or warming from the refrigerated state.
- **Change in solution or suspension:** A product may have to be dissolved, diluted, or suspended in a liquid.
- **Addition to biocompatible structural material:** A gene therapy product may need to be combined with living, natural, or synthetic structural tissue or matrix. Examples of matrix material include hollow fibers, fibrous sheets, gels, plugs, capsules, sponges, or granules.
- **Admixture or compounding with other nonstructural materials:** A product may require mixing or compounding with drugs, cytokines, biologics, or other nonstructural materials.
- **Filtration or washing:** Unwanted materials in the manufactured product, such as particulates, cellular debris, metabolites, or compounds remaining from previous manipulations may require washing or filtration steps.
- **Sampling:** Sampling of the final product immediately before administration may be required for certain clinical protocols.

#### FACILITY REQUIREMENTS

Facility requirements for performing on-site preparative steps or administration of gene therapy products depend on the nature of the products, their applications, and the manipulations required. ▲▲ (USP 1-May-2020)

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### RELEASE OF FINAL PRODUCT

Gene therapy products that undergo on-site preparative steps or manipulations must be subjected to appropriate checks or tests to ensure that all quality specifications are met before release for patient administration. The nature and extent of manipulations will determine whether release requirements or critical specifications must be added to those required immediately after initial manufacture. Prerelease requirements usually include the following:

1. Physical inspection of the product, which typically includes measures to ensure appropriate product appearance with regard to color, turbidity, particulates or foreign matter, container integrity; product temperature; and accuracy and convenience of labeling
2. Review of process records
3. For patient-specific products, clerical checking of product labeling or records related to the identity of the intended recipient.

▲▲ (USP 1-May-2020)

## Administration to Patients

### PRE-ADMINISTRATION REQUIREMENTS

Depending on the specific gene therapy application, trained patient-care staff must take steps to prepare the patient for product administration. These steps are aimed at ensuring that the product will provide the intended therapeutic outcome and at minimizing the risk of adverse effects. Issues pertinent to administration of cell-based products are addressed in ⟨1046⟩. Generally, a thorough re-evaluation of the patient's general condition and suitability for therapy must be performed close to the time of product administration. This evaluation usually includes a patient history, physical examination, and laboratory studies such as blood counts and chemistries. In addition, staff may obtain baseline physical or functional measurements, laboratory tests, or imaging studies relevant to the specific application. Examples include pulmonary function tests for a therapy aimed at improving lung function, measurement of blood levels of an enzyme that is the gene product in a gene therapy application, and nuclear imaging of organs before anticancer therapies.

A variety of patient interventions related to route of administration may be required before product administration. For therapies that require intravenous administration, patients with poor peripheral venous access may require placement of a central venous catheter. In applications where gene-modified cells or matrices combined with cells are implanted into the patient, the site of implantation may require preparation in the operating room. This may involve surgically opening the site, removing the degenerated or damaged tissue, trimming of the adjacent tissue to accommodate the implant, and excising the tissue from a second site to be used as an anchor or support for the implant. For instance, in the case of products for wound healing, it is critical that the site for grafting be free from infection and that it demonstrates a well-prepared wound bed. Where gene-modified cells are intended to repair cartilage defects, the site of damage needs to be prepared so that the cells can be applied to a water-tight compartment. For applications involving direct administration of the product into an organ system (for example, bronchioalveolar system) or vascular network (for example, coronary arteries), the patient may require endoscopic or surgical access to these sites.

In all cases, the need for adequate anesthesia and premedication must be carefully evaluated in conjunction with these steps before product administration. Pre-administration patient evaluation must also include assessment of concurrent therapies that may interact with the gene therapy product to modify its effects. Some therapies may be considered adjunctive to the gene therapy, such as cytokines that promote proliferation or differentiation of the infused or implanted tissue. Other commonly used drugs such as antibiotics, antineoplastics, anticoagulants, and anti-inflammatory agents must be evaluated for possible effects on the efficacy of the gene therapy product.

### PATIENT TREATMENT

Some gene therapy products are patient-specific because they are manufactured from a selected tissue source, such as autologous, selected allogeneic, or xenogeneic tissue. Certain patient-specific products have a defined potential for benefit or adverse immunoreactivity. Systems must be in place to prevent administration of such a product to the wrong patient. Recommended systems include procedures similar to those used for administration of human blood products, including special attention to the correct identification of the patient and patient-specific product by at least two people immediately before administration. These issues are addressed in greater detail in ⟨1046⟩. Gene therapy products can be administered by a variety of routes. These include parenteral injection, inhalation, and gastrointestinal routes. Other possibilities include direct application of gene therapy products into regional vasculature, organs, tissues, or body cavities by means of needles or catheters or following surgical exposure of the tissue. Although parenteral administration can be accomplished in routine outpatient or inpatient facilities, the other means of administration may require specialized facilities such as an aseptic operating theater or endoscopic suite. In all cases, standard operating procedures and a quality program must be in place to ensure that the product is administered in the intended manner.

### POST-ADMINISTRATION MONITORING OF PATIENT

There should be written policies and procedures for monitoring patient outcomes and managing reports of adverse events. Patient outcome assessment should include indicators that are likely to detect errors or problems related to the entire manufacturing process, with special attention to manipulations, storage, or transportation after the initial manufacture of the product. Management of adverse reactions should include procedures for ensuring prompt medical evaluation and treatment of patients with suspected adverse effects and a system for reporting and evaluating adverse effects that may point to a potential

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defect in the administered product. Reporting procedures include providing details required for federal, state, or USP adverse-event reporting programs.

Follow-up and monitoring procedures should be implemented for patients who have received gene therapy vectors or ex vivo gene therapies. To the extent that it is relevant and that it can be assessed, vector or gene-modified cell biodistribution and persistence in vivo should be monitored. With direct administration of vectors, localization to the germ line may be an issue. Although preclinical studies can address this issue, useful information may be gained by patient monitoring. When a retroviral vector has been administered, patients should be monitored for replication-competent retrovirus (RCR) according to the FDA's *Guidance for Industry: Supplemental Guidance on Testing for Replication Competent Retrovirus in Retroviral Vector Based Gene Therapy Products and During Follow-up of Patients in Clinical Trials Using Retroviral Vectors* (October 2000). This involves active monitoring during the first year and archiving of patient samples thereafter if RCR is not detected initially.

Database systems to collate and track patient-monitoring results are essential to management of this information. National registries or publication of data should be considered for establishing the collective safety of gene therapy.

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## ANALYTICAL METHODS

The complexity and scope of gene therapy products are reflected in the wide range of analytical procedures and their methods that are used to assess product quality. Approved gene therapy products must comply with applicable sections of 21 CFR 211 and 610 to ensure their identity, dose, potency, purity, and safety. Specific guidance for the identification, development, and validation of analytical methodologies to support cell and virus bank characterization, final-product release, and stability studies is currently provided in FDA guidelines for gene therapy manufacturing and testing (see *Appendix*); in ⟨1225⟩; and in the ICH guidelines Q2(R1) and Q6B. Most product-specific analytical methods for gene therapy products have not been standardized. Even well-defined tests such as those described under *Sterility Tests* ⟨71⟩ may not be directly applicable to certain gene therapy products. For some gene therapy products, large quantities of clinical material may not be available during early clinical development. Some required tests (e.g., sterility) may require modification. Consultation with regulatory authorities is advised.

*Table 5* provides an overview of product-specific testing parameters for the biological component and general methods or approaches used to satisfy the testing requirements for nonviral, viral, and gene-modified cellular gene therapy products. The analysis of gene therapy products relies heavily on biological assays, but it also uses methodologies developed for biotechnology-derived products. The intent of this section is to outline the types of methods and their specific applications with regard to product characterization, stability, and release testing. Process validation may alleviate the need for certain specific lot-release tests. Development of appropriate reference materials and standards for viral, nonviral, and gene-modified cellular gene therapy products should be a part of product development. Reference materials should be fully characterized in order to provide continuity between standards over time. In the case of gene-modified cellular gene therapy products, the reference material may be a surrogate tissue or simulated product. Reference materials are briefly addressed in the FDA's *Guidance for Industry: Potency Tests for Cellular and Gene Therapy Products*.

Gene-modified cellular gene therapy products may require a rapid-release approach if they have a limited shelf life (see ⟨1046⟩). The rapid-release approach is not usually applied to viral and nonviral gene therapy products because these products are sufficiently stable for completion of testing before release. Some formulated nonviral gene therapy products also have limited shelf lives. In such cases, the individual components are tested before release and the formulated complex is not tested. The formation and stability of the formulated nonviral gene therapy complex is established via validation studies during product development.

As specified in CFR, product samples must be retained after product-release testing is completed. Retain additional samples if rapid-release strategies are employed so that product quality can be reassessed by alternative or traditional test methodologies, if necessary.

**Table 5. Analytical Tests for Cell and Gene Therapy Biological Products**

| Test | Gene-Modified Cellular Gene Therapy Product | Gene Therapy Products | |
|---|---|---|---|
| | | Viral | Nonviral |
| Identity of Biological Substance | • Surface marker determination<br>• Species<br>• Morphology<br>• Bioassay<br>• Biochemical marker | • Restriction enzyme map<br>• PCR<br>• Immunoassay for expressed gene<br>• Sequencing | • Restriction enzyme map<br>• PCR<br>• Immunoassay for expressed gene<br>• Sequencing |
| Dose | • Viable cell number<br>• Enumeration of specific cell population<br>• Total DNA<br>• Total protein | • Particle number<br>• Transducing units (DNA hybridization assay)<br>• Total protein<br>• HPLC assay using authenticated reference standard | • Plasmid–DNA weight<br>• Formulated-complex weight HPLC or capillary electrophoresis assay using authenticated reference standard |

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**Table 5. Analytical Tests for Cell and Gene Therapy Biological Products** *(continued)*

| Test | Gene-Modified Cellular Gene Therapy Product | Gene Therapy Products | |
|---|---|---|---|
| | | Viral | Nonviral |
| Potency | • Viable cell number (cells intended for structural repair)<br>• Bioassays<br>○ Colony-formation assay<br>○ Function of expressed gene<br>○ Induction of secondary effect [e.g., human leukocyte antigen (HLA) induction, secretion of cytokines, and up-regulation of surface marker] | • Function of expressed gene (induction of secondary effect and other bioassays) | • Function of expressed gene (induction of secondary effect and other bioassays) |
| Purity | • Percentage of viable cells<br>• Percentage of transduced cells<br>• Percentage of cells with specific surface marker<br>• Process contaminants (e.g., serum) | • Residual host-cell DNA<br>• Process contaminants (e.g., serum and cesium chloride)<br>• Residual helper virus<br>• Optical density ratio<br>• Residual host-cell proteins<br>• Viral protein profile (HPLC assay for defective or immature particles)<br>• Residual RNA | • Percentage of specific physical form (e.g., percentage supercoiled)<br>• Residual host-cell DNA<br>• Residual RNA<br>• Residual host-cell proteins<br>• Residual solvents<br>• Optical density ratio<br>• Process contaminants (e.g., cesium chloride) |
| Safety | • Mycoplasma<br>• Sterility<br>• Pyrogen and endotoxins<br>• Adventitious viruses<br>• Residual virus<br>• Replication-competent vector | • ▲ (USP 1-May-2020)<br>• Sterility<br>• Pyrogen and endotoxins<br>• Adventitious viruses<br>• RCV | • Mycoplasma<br>• Sterility<br>• Pyrogen and endotoxins |

## Sampling Issues

Sampling for lot-release testing should be based on the potential distribution for the parameter tested. See *Stability-Protocol Development* for additional considerations. Samples from each lot should be retained in case of a safety or quality issue with the lot. Even if the product has a very short shelf life, these retained samples can be used to detect impurities and other substances. The need for proper design of the sampling scheme is highlighted in safety testing for adventitious agents or in assessment of RCV for gene-modified cell or viral gene therapy products. In such cases, process validation assists in determining the appropriate statistically based sampling design.

## Safety

Safety testing for gene therapy products focuses on three issues: 1) detecting contamination from adventitious sources during product processing, 2) preventing the use of packaging cell lines and plasmids that potentially permit genetic recombination between vectors and the packaging cell lines or plasmids—or among the vectors themselves, and 3) testing the final product to ensure a safe level of undesired genetic and/or structural variants or other viruses used in processing.

The primary means of assessing safety are the performance of biological assays to measure adventitious agents directly. Molecular biology-based assays that measure adventitious agent DNA or RNA or detect undesired genetic variants are also used. Although live genetically engineered vectors officially fall outside its scope, the detailed information available in the ICH Q5A guideline, presented in ⟨1050⟩, should be consulted because the principles apply.

### VIRAL GENE THERAPY PRODUCTS

One of the primary safety concerns associated with viral vectors used for gene therapy is the occurrence of undesired genetic variants. Among them the most critical type, and probably the best studied, is RCV. RCV is more clearly defined for replication-incompetent viral vectors, but for conditionally replication-competent viruses it refers to undesired genetic variants that have lost selectivity toward the target cells and thus might raise safety concerns. Regardless of the virus, these concerns are based on the potential lack of predictability for the pathogenicity of a contaminating virus for a specific route of administration, particularly if it is not the normal route of infection or if humans are not a natural host for the virus. The pathogenesis of a wild-type adenovirus infection is known but may not be predictive for the routes of administration employed with recombinant adenoviral vectors. For replication-incompetent adenoviral vectors, a limit of 1 RCA per $3 \times 10^{10}$ viral particles is currently considered acceptable (see the FDA's *Guidance for FDA Reviewers and Sponsors: Content and Review of Chemistry, Manufacturing, and Control (CMC) Information for Human Gene Therapy Investigational New Drug Applications (INDs)*].

Typically, RCA levels are determined by a cell-based assay that allows amplification of the RCA while preventing replication of the product. The cell line most often used for amplification and detection of RCA is the A549 cell line. However, some recombinant adenoviral vectors express therapeutic genes that interfere with analysis on A549 cells. In such cases, ▲another cell line is used. The▲ (USP 1-May-2020) cell line is chosen on the basis of resistance to the effects of expression of the transgene and with subsequent passage of cell lysate or supernatant onto A549 cells for amplification and detection of the RCA. RCA is most often detected by visual observation of the cytopathic effect, but it can also be detected in the A549 cell culture by immuno- or polymerase chain reaction (PCR)-based methods.

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Quantitation of the RCA level is based on the quantity of sample tested and the detection limit of the assay. Typically, RCA bioassays are validated as being able to detect 1 plaque-forming unit or infectious unit of RCA in the test sample over a wide range of test-sample sizes. Test-sample sizes can range, but they are typically based on the FDA RCA acceptance limit. To verify detection limits, include spike controls as part of the test, even with validated assays. For recombinant adenoviruses produced using HEK293 cells, RCA detection by PCR on the final products or the progeny virus amplified in HEK293 cells can be confounded by detection of residual HEK293 host-cell DNA (detection of the E1 region). PCR assays, however, can be designed to specifically quantitate host cell DNA contamination and can be made specific to particular forms of slow-growing RCA. Quantitative PCR assays can be used in conjunction with a cell-based method for precise quantitation of RCA levels. When a tested sample is found to be positive, the identity of the RCA is usually confirmed by conducting PCR analysis. This rules out the possibility that contamination of the assay by exogenous wild-type adenovirus or other adventitious agents is responsible for the positive result.

For conditionally replication-competent adenoviruses or other replication-competent viral vectors, testing for RCV or undesired genetic variants is usually more complicated and vector specific. Usually one or two nonpermissive cell lines that are not target cells are infected with the replication-competent virus in attempts to produce progeny virus. In order to generate a sufficient quantity of progeny population for analysis, analysts subject the infection to multiple passages and extended culture time. Two normal fibroblast cell lines that are easy to culture, WI-38 and MRC-5, have been used as the model nonpermissive cell lines for detecting RCA in replication-competent adenovirus products. Even after multiple passages on the nonpermissive cell lines, it may be necessary to amplify the progeny (which tend to appear only in minute quantities) in permissive or packaging cell lines to a sufficient quantity for subsequent testing. The resulting progeny should be tested for changes in biological selectivity and genetic composition. Usually the genetic characterization of the progeny population includes restriction enzyme mapping followed by Southern blotting, PCR, or nucleotide sequencing. After the genetic elements unique to RCV or undesired genetic variants are identified, quantitative PCR assays can be designed to monitor the level of RCV after amplification in nonpermissive cell lines or sometimes, if the sensitivity is adequate, directly in the final product without biological amplification. Using a spike control in the biological assay for detecting RCV is encouraged but may not be applicable to all cases. Currently there is no specified acceptable limit of RCA for conditionally replication-competent adenovirus, although clinical safety has been reported for an oncology application with several thousands of RCA per dose.

For retroviral vectors, testing for RCR is required for cell banks, viral vector production lots, and any resulting ex vivo product lots (see FDA's *Guidance for Industry: Supplemental Guidance on Testing for Replication Competent Retrovirus in Retroviral Vector Based Gene Therapy Products and During Follow-up of Patients in Clinical Trials Using Retroviral Vectors*). Standard assays have been designed to detect replication-competent MLV. The pathogenesis and potential long-term toxicity of low-level amphotropic MLV in human beings is not known. Methods commonly used to detect RCR include an amplification of virus titer by application of product to a replication-permissive cell line such as *Mus dunni*. Because infection is limited by the ability of a virus to reach the cells by means of Brownian motion, procedures (e.g., centrifugation and filtration) that physically bring the virus into contact with the cells can be used to enhance detection. However, high-titer recombinant vector can interfere with the detection of low-level RCR, and this interference may be enhanced by such methods. Infected cells are passaged several times to allow viral replication. Culture medium is harvested at the end of the culture period, and RCR is detected by using an indicator cell line. If the product is an amphotropic MLV, RCR can be detected by using a feline cell-based PG4 S+L– assay, a mink cell-based MiCl S+L– assay, or a marker rescue assay. In S+L– assays, the RCR expresses proteins that lead to transformation and subsequent plaque formation on the monolayer. In a marker rescue assay, RCR infects a cell line that expresses a retroviral vector encoding a marker gene such as β-galactosidase, drug resistance, or a fluorescent protein. The vector is packaged by the proteins supplied to it in trans by the RCR. The potentially vector-laden supernatant is transferred to naive target cells that are then screened for expression of the marker vector.

Testing for RCR is performed by co-cultivation of the cell line or amplification of vector supernatant with an RCR replication-permissive cell line, typically *M. dunni*, for several passages. Culture medium is harvested at the end of this co-cultivation process and applied to an appropriate indicator cell line as described above. Note that artifacts may be generated during the co-cultivation assay by expression of an endogenous virus in the permissive cell line or by fusion if the vector-producing cell line is cultured directly with a marker rescue cell line. In addition, co-cultivation may not be possible for ex vivo cell products that have specific culture requirements or limited culture life spans.

Methodologies for testing the presence of RCR in crude, purified bulk or final vector products are not specified. The FDA has deposited a reference standard of an amphotropic hybrid MLV with the American Type Culture Collection (ATCC). This viral stock has been assigned a label titer and should be used in assay validation. Method validation should demonstrate the ability to reproducibly detect a single RCR particle in individual product types because the product and its related impurities can interfere with the detection of RCR. Currently, there are no acceptable limits for RCR contamination in products. Any product lot found to contain RCR cannot be used for human use. Reference standards for assessing RCV in other viral vectors including ecotropic, xenotropic, or pseudotyped MLV, adenovirus, and lentivirus have not been developed. The adenovirus reference material, which consists of wild-type human adenovirus type 5, has been used as a spike control and during validation of RCA assays, but this practice may not be applicable to all RCA assays. Amplification and detection of replication-competent human immunodeficiency virus (HIV), especially its pseudotyped variants, may warrant special containment and handling procedures.

Additional safety testing usually focuses on methods similar to those described in *Biological Reactivity Tests, In Vivo* ⟨88⟩, *Safety Tests—Biologicals* and ⟨71⟩. For viral gene therapy vectors produced using a human cell line, performance of the in vitro adventitious agent bioassays using three cell lines is recommended. For adenoviral vectors, specific tests for adeno-associated virus are also recommended. For adeno-associated virus, specific tests for adenovirus and herpes virus are recommended. Material for testing should be derived from the stage of manufacture that provides the greatest chance of detection, which could be prebulk (e.g., late-stage fermentation), the bulk, or the final product.

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Safety testing usually focuses on methods similar to those described in ⟨88⟩ and ⟨71⟩. Safety testing should be performed on nonviral formulated material. If the shelf life of the formulated nonviral product is very short, then the components should be tested individually.

Safety testing for undesired genetic variants that might emerge during the manufacturing process in nonviral gene therapy products is similar to that for RCV testing for viral vectors but with more vector-specific considerations. Typically, molecular biology-based methods are applied to the final product to test for variants. When genetic stability is established by process validation, the assays for monitoring the levels of undesired genetic variants may be limited to restriction enzyme mapping followed by further confirmation of critical genetic elements (such as transgenes or regulatory elements) by PCR or Southern blotting.

## Dose-Defining Assays

An assay that precisely measures the amount of the product is referred to as a dose-defining assay, and it is selected on the basis of its accuracy and precision. An assay that measures therapeutic activity of the product is referred to as a potency assay, and it is designed to measure product function. The design of the assay depends on the type of product. In the case of chemical and protein drugs, the assays measuring the amount of active ingredient (dose) are referred to as strength assays. Product dose can be defined as the concentration or amount of the drug product administered to the patient, and it is typically measured as product mass.

Particle concentration is a commonly used measure for viral vector product dose. Particle concentration may be measured by physical, biophysical, or in vitro cell-based assays. For example, quantitation of purified adenoviral particles may be determined by using the optical density of a solution of virus in 0.1% (w/v) sodium dodecyl sulfate (SDS) solution at 260 nm, because a relationship between absorption and particle concentration has been published for adenovirus. The particle number concentration is equivalent to the product of the absorbance at 260 nm in a 1-cm cell, the dilution factor, and $1.1 \times 10^{12}$ particles. A method that has become standard in determining particle concentration is integration of viral peak area of 260 nm and/or 280 nm absorbance against an authenticated reference standard in an anion-exchange resin-based HPLC assay. Compared to the optical density method, the HPLC method has the advantage of eliminating the interference of free DNA and/or capsid proteins on quantitation of viral particles. An adenoviral reference material (ARM) from ATCC has an HPLC-determined particle concentration established from a large-scale collaboration that involved many laboratories. Whenever possible, the ARM should be used to calibrate the internal HPLC method and reference material.

Virus concentration can also be assessed by the measurement of selected structural proteins with known molecular masses and known copy numbers within the virion. For this method, the virus must be lysed, and the structural proteins must be separated by using an appropriate, high-recovery chromatographic procedure (e.g., reversed-phase HPLC). The chromatographic separation and the identity and the purity of the selected structural protein must be verified during assay validation by methods such as SDS polyacrylamide gel electrophoresis (SDS-PAGE), peptide sequencing, and mass spectroscopy. The selected structural proteins can be quantified, for example, by integrating chromatographic peaks at 214 nm and comparing the area to that of an authenticated reference standard. The virus concentration can then be calculated based on the molecular mass, the copy number, and the measured mass of the protein. Very importantly, the virus concentration can be estimated simultaneously for multiple structural proteins, which allows the use of this assay in relatively impure virus preparations. This method has been applied to adenovirus and should be applicable to other viral vector types.

Biophysical methods of determining particle number include direct quantitation of vector nucleic acid by radiolabeled-probe hybridization and indirect quantitation by amplification of template nucleic acid [e.g., PCR and reverse transcription (RT)-PCR] or by signal amplification (e.g., branched-chain DNA using multiple-probe hybridization).

In cases where biophysical methods are not available, bioassays that measure gene-vector titer have been used. These involve infection, transfection, or transduction of a susceptible cell line in vitro, followed by some measure of the product uptake. Methods for quantitation or estimation of the number of infection, transfection, or transduction events include plaque-forming unit assays, tissue culture infectious dose assays based on cytopathic effect of 50% tissue culture infectious dose ($TCID_{50}$) or immunofluorescent detection of an expressed vector protein, or a quantitative DNA-hybridization assay. Examples follow: For replication-competent adenoviral gene therapy products, the ARM available from ATCC has a defined range of $TCID_{50}$ titer determined via a collaborative effort. Whenever applicable, it should be used in validation of an internal reference standard or assay control of infectious titer assays. However, because of the likelihood of genetic differences between the ARM, which is wild-type human adenovirus type 5, and the replication-competent adenoviral gene therapy product, it may not be reasonable to normalize the titer of the vector of interest to that of the ARM.

For retroviral or lentiviral gene therapy products or AAVs that carry a selectable marker (e.g., that for neomycin resistance) or a reporter gene (e.g., β-galactosidase) in addition to the therapeutic gene, the infectious titer is commonly determined by measuring the number of transduced or infected cells expressing these nontherapeutic proteins. Vector titer is typically reported as the number of colony-forming units (cfu) per mL for cells transduced with viral vectors that contain drug-resistance markers and are selected for growth in drug-containing medium. Titer based on β-galactosidase can be expressed in terms of blue cfu/mL after staining and counting the cells that convert the β-galactosidase substrate X-Gal into a blue chromophore. For vectors without a marker gene, quantitation of transduction has been measured precisely by using quantitative PCR or has been estimated by hybridization methods.

Most nonviral gene therapy products contain plasmid DNA, and their usual measure of dose is the DNA mass. The DNA mass may be determined in the formulated state, and, if recombinant protein is included in the formulation, the total combined mass of all formulation components based on a specific ratio can be used. DNA concentrations greater than 500 ng/mL are most simply determined by using optical density measurement at 260 nm. This method is not generally applicable to lipid-formulated DNA. Because RNA and proteins also have significant absorbance at 260 nm, other analyses must be performed to demonstrate minimal contamination with RNA, protein, or residual host-cell chromosomal DNA. Dyes that specifically bind to double-stranded DNA allow accurate measurement of DNA concentrations of less than 500 ng/mL when calculated against

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an authenticated DNA standard curve. PicoGreen is one such fluorescent dye, and it is minimally affected by single-stranded DNA, RNA, proteins, salts, and detergents. The fluorescent dye Hoechst 33258 also binds to both double-stranded and single-stranded DNA and it can be used to determine DNA concentrations as low as 0.3 ng/mL. The Hoechst 33258 does not bind to protein or RNA, and it can accurately determine the DNA concentrations in crude samples.

Methods such as capillary electrophoresis and HPLC employing an authenticated reference material can also be used to determine the strength of nonviral products.

## Potency

Potency is defined as the therapeutic activity of the drug product. Together with dose, potency defines the biological activity of each lot (see *Dose-Defining Assays*). Potency can be assessed by in vitro or in vivo bioassays. It is not uncommon for these assays to have coefficients of variation between 30% and 50%, although stringent assay design with good statistical consideration could help reduce assay variation. These assays require a well-defined, representative reference material that can be used as a positive control for the assay and/or in calculation of the relative potency of the test article. The general consideration for bioassays in current *USP* chapters on design and development of biological assays should be applied to the potency assay design for gene therapy products. The positive control qualifies the performance of an individual assay. Potency assay development should focus on characterizing and controlling variability. High-precision assays are more effective tools in monitoring product quality. Information about potency assay variability should be incorporated into the stability study design and the proposed statistical approach to assignment of expiration date (see *Stability*).

Bioassays employed to measure the potency of viral and nonviral gene therapy products generally involve infection, transfection, or transduction of a susceptible cell line in vitro, followed by some functional measure of the expressed gene of interest. Functional assays for the therapeutic gene (e.g., those measuring enzyme activity and cell growth stimulation or inhibition) should generally be used instead of analytical methods such as enzyme-linked immunosorbent assay (ELISA). When the biological function of the expressed transgene exhibits a broad range of activities or only generates semiquantitative results, the ELISA or other immunological or biochemical readouts can be used as a surrogate potency assay with a tight specification range if extensive characterization data is available to demonstrate that all expressed protein is biologically active. For example, in the case of a gene therapy product expressing a cytokine, cytokine expression is usually quantified by ELISA first, and the result is used to adjust the sample dilution for the functional assay. The potency of such vectors may be better controlled by the ELISA quantitation results, but the biological activity of expressed cytokine could be used to verify that the measured mass is biologically active without the requirement to meet a narrow specification range for the biological activity itself.

HPLC or flow cytometry, which provide information about the level of expression but only infer function, have also been used in a context like that described for immunoassays. In addition, for viral vectors, infectious titer measurements by themselves are generally not considered an adequate measure of product potency. For example, the $TCID_{50}$ titer or plaque-forming-unit assays for adenoviral vectors on HEK293 cells can indicate that the infectivity of adenovirus is preserved but do not confirm that the adenoviral product has maintained full biological function(s), especially transgene biological activity. The design and ultimate suitability of an assay system for determining product potency depends on the relationship between the intended human target cell in vivo and the following: 1) the transduction or transfection efficiency of the cell line used in vitro; 2) the protein expression levels; and, 3) the duration of expression required for the therapeutic effect.

In vivo tests can also be used to measure vector-product potency. Readouts can be based on a response per animal (e.g., blood levels of therapeutic protein 24 h after treatment) or a group response rate (e.g., percentage of animals that elicited an immune response or survived virus challenge). The availability of an appropriate in vivo test system depends on the vector-host range (for viral vectors), the pharmacokinetics and biodistribution of the vector and the resulting gene product relative to its human counterpart, and the time frame required to observe the therapeutic effect or surrogate. Issues of cost, facilities, validation, and ethics determine the practicality of an in vivo potency test.

## Purity

Analytical methods that separate, isolate, and specifically quantify the intended active product components determine product purity. Impurities are either product- or process-related components that can be carried through to the final product. The manufacturing and purification process should be optimized to consistently remove impurities while retaining product activity. The requirement to test for a particular impurity for product lot release depends on the following: 1) the demonstrated capability of the manufacture and purification process to remove or inactivate the impurity through process validation, and 2) the potential toxicity associated with the impurity.

Examples of process-related impurities associated with gene therapy products include residual medium components for the production [e.g., fetal bovine serum (FBS), antibiotics, cytokines, and *E. coli* chromosomal DNA in a plasmid product], ancillary products used in downstream processing (e.g., nucleases such as DNase I), and residual moisture for lyophilized vector products. Impurities may be bioactive (e.g., cytokines and hormones) or immunogenic (e.g., product aggregates, degradation products, plasmid-selection markers, and nonhuman-derived proteins), or they may have other deleterious effects (e.g., they may compete with the product) if administered at a dose equal to that of the product. Product-related impurities are specific to each product type. Examples include nicked plasmid forms in nonviral products and defective or immature virus particles in retroviral or adenoviral vector products. Analytical methodologies to assess purity require quantitation or physical separation of the intended product from its impurities. Common sense should drive the need to quantify specific impurities. Suitable validation of the manufacturing process may limit the need for specific lot-release testing for impurities. Manufacturers may place an emphasis on demonstrating the consistency of the product–impurity profile.

Testing for impurities is often extensive during product characterization and process validation when the consistency of the manufacturing and purification process is being demonstrated. Testing for impurities as part of lot-release testing should reflect the safety risks associated with the impurity and the ability of the process to consistently remove that impurity.

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## VIRAL GENE THERAPY PRODUCTS

Product-related impurities for viral vectors include aggregates and defective and immature particles that may be produced during the manufacture or purification of the recombinant vector. Aggregates of vector may form if the product is highly concentrated, stored under certain conditions (e.g., under a certain pH or temperature), or reconstituted after lyophilization. Assays to detect aggregates include particle size analysis by laser light-scattering and the use of nonreducing, nondenaturing PAGE, followed by staining of the gel or transfer and detection of viral proteins by Western blot analysis. Sedimentation rate analysis also allows separation of aggregates from monomers based on size. Optical density analyses of light scattering are also used to assess vector aggregation.

Defective particles are viral particles that do not contain the appropriate recombinant genome—that is, they contain some other nucleic acid or contain no genome at all, or the vector has some missing, defective, or otherwise altered structural component that impairs its ability to transduce a cell. For viral vector systems that have capsomeric symmetry that requires the appropriate nucleic acid incorporation for configuration, empty particles may be readily distinguished from those carrying genomes. For enveloped viruses, empty particles may not be as readily separated from those with encapsidated nucleic acid.

For some viral vector products, active viral particles can be separated from defective particles by using analytical HPLC. Anion-exchange resins have been used to separate active adenovirus from defective virus particles. However, this method might not be useful for an adenoviral vector purified by anion-exchange chromatography unless the resin for the assay is different from that used during manufacture. Depending on the nature of the viral vector and its nonactive or defective forms, other methods of separation, such as equilibrium centrifugation in a cesium chloride density gradient, may need to precede the quantitation of the active particle. Ideally, the method of separation will allow quantitation.

Defective particles that carry a non-cell-derived oncogene or other undesirable genes may pose a special concern. For example, in murine-based retroviral packaging cell lines, small viral elements called VL30 sequences can be packaged in about one-third of all particles. Assays may be needed to quantify specific defective particles if they are known to be present in quantities sufficient to pose a safety concern.

Virus quality and the comparability of preparations can also be assessed by measuring selected structural proteins with known molecular masses and known copy numbers within the virion. For this method, the virus is lysed, and the structural proteins are separated by using reverse-phase HPLC or some other high-recovery chromatographic procedure. The chromatographic separation should be validated, and the identity of the selected structural proteins should be verified by methods such as SDS-PAGE, peptide sequencing, or mass spectroscopy. Fingerprinting of the batch can be conducted based on quantification of the selected structural proteins and comparison to a reference standard. When the method incorporates mass spectroscopy, impurities such as structural variants can also be identified. For adenovirus preparations, some precursor and most mature virion proteins can be detected and distinguished, thus allowing monitoring of the product and of the immature virion forms.

Host cell-derived proteins may be considered impurities for some viral vector products and may be separated and quantified by PAGE or HPLC or detected by amino acid analysis, Western blot, or immunoassay-based methods. However, for enveloped viruses such as retroviruses, host cell-derived membrane proteins are an integral part of the product. In those vector systems, it may be difficult to determine the presence of contaminating exogenous host-derived proteins.

Presence of specific process-related impurities depends on the manufacture and purification process of each vector or product type. However, most products need to be tested for residual endotoxins (see *Bacterial Endotoxins Test* ⟨85⟩). Acceptable limits of endotoxins have been determined and can be directly applied to viral vector products. Although genomic DNA derived from continuous cell substrates used to manufacture biological products historically has been considered potentially tumorigenic, recent studies suggest that the risks are very low. However, every attempt should be made during process development to reduce levels of contaminating DNA. The need to test for residual DNA as part of product lot release should be evaluated on a case-by-case basis and may depend on the size distribution of the DNA, its association with the product or its formulation components, and the product's route of administration. Quantitative PCR assays can analyze the amount of residual host-cell DNA by using primers designed to amplify evolutionarily conserved and abundant target sequences such as 18S for HEK293 cells.

Quantitation of residual serum components such as bovine serum albumin (BSA) can be achieved by using ELISA and a BSA reference standard. Researchers may need to develop specific functional or immunological methods for other ancillary products, including other culture media or purification process components such as cytokines or enzymes (e.g., deoxyribonuclease 1 or benzon nuclease).

## NONVIRAL GENE THERAPY PRODUCTS

A plasmid used as a drug substance is considered a well-characterized biologic, and key impurities from the manufacturing process are well known. Testing is usually performed on each individual component: the plasmid DNA, lipid or lipoplex reagents, and protein components if any are present in the formulation. Plasmid DNA is characterized for a variety of impurities, including residual host-cell DNA, residual RNA, and residual protein. Residual protein testing is frequently included in lot-release testing. Optical density ratios (usually the measurement at 260 nm ▲is compared to the measurement▲ (USP 1-May-2020) at 280 nm) are frequently used in purity specifications for plasmid DNA.

In addition, the plasmid DNA should also be characterized with regard to its conformation in solution. Plasmid DNA exists as monomeric supercoiled, relaxed monomer, and linear forms. Because all forms can be generated during large-scale fermentation, and data about their relative in vivo potency is scarce, the relative quantity of each form is monitored to verify batch-to-batch consistency in the relative amounts of each conformation. Agarose gel electrophoresis can resolve each of these forms but is not highly quantitative for each individual species. Analytical anion-exchange HPLC serves as a quantitative assay for monomeric supercoil and other forms, including concatamers. Other analytical methods that have been valuable for characterization of plasmid constructs during process development and validation such as capillary gel electrophoresis (CGE), linear-flow dichroism, and atomic-force microscopy are also viable methods to assess the purity of a given plasmid preparation. The most appropriate method for lot release depends on how each plasmid conformation affects product potency. Specific details for each of these methods are outlined in *Nucleic Acid-Based Techniques—General* ⟨1125⟩.

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Tests should be conducted for process-related impurities such as residual organic solvents (phenol, alcohol), salts, and certain antibiotics such as kanamycin used during the fermentation process. Lipid and lipoplex formulation components must also be tested for their chemical purity. Testing for specific chemical impurities is commonly performed by using GC–MS, HPLC, or TLC methods. If protein is part of the formulated complex, then the protein must also be tested for purity. HPLC is capable of detecting trace amounts of residual antibiotics and can therefore be used during process validation or lot-release testing to confirm that they have been effectively removed. The specifics of these methods are outlined in *Biotechnology-Derived Articles—Peptide Mapping* ⟨1055⟩ or in *Biotechnology-Derived Articles—Total Protein Assay* ⟨1057⟩.

Bacterial protein, DNA, RNA, and endotoxins are the major types of host-derived process contaminants. Standard protein assays (e.g., Lowry, Bradford, or Coomassie), PAGE followed by silver staining or Western blot analysis, or ELISA can be used to detect residual host protein in the nanogram range. Host chromosomal DNA can be detected by slot blot hybridization (detection in picogram range) or by real-time PCR (detection sensitivity < 1 pg) using highly conserved target sequences (e.g., 18S for *E. coli*). PCR assays for this purpose must use recombinant polymerases that are highly purified to minimize residual bacterial DNA for which the presence can create background signals. PAGE or agarose gel electrophoresis followed by fluorescent dye staining can be used to detect residual RNA. Quantitation may not be required because of the labile nature of RNA and the low-level toxicity associated with it. The *Limulus* amobocyte lysate (LAL) test is the most sensitive and widely used method for endotoxins determination. Colorimetric assays offer sensitivities of 0.005 EU/mL. Details of the methods described here are outlined in ⟨1057⟩, *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* ⟨1056⟩, *Nucleic Acid-Based Techniques—Approaches for Detecting Trace Nucleic Acids (Residual DNA Testing)* ⟨1130⟩, ⟨1125⟩, and ⟨85⟩.

### LYOPHILIZED VIRAL AND NONVIRAL VECTOR PRODUCTS

Residual moisture can affect the stability of a lyophilized vector product. FDA's *Guideline for the Determination of Residual Moisture in Dry Biological Products* recommends a 1% residual moisture level, although data indicating no adverse effects on product stability at higher levels is considered acceptable. Residual moisture levels can be determined by using a standard method (see *Water Determination* ⟨921⟩) that is compatible with the formulated product.

## Identity

Lot-release testing for gene therapy products must include an identity test. This test clearly identifies the product and confirms that inadvertent substitution with another product has not occurred. The complexity of the identity test depends on the nature of the specific product and the array of products being manufactured. For example, more extensive and rigorous testing may be performed for an autologous gene-modified cell therapy product at a facility where multiple patient products are manufactured than for a viral vector product produced at a site that manufactures a single vector product.

### VIRAL GENE THERAPY PRODUCTS

For characterization purposes, restriction enzyme mapping and sequencing of the transcription unit DNA are the most commonly used approaches. PCR-based methods, restriction enzyme mapping, and transgene-expression-based immunoassays are commonly used to confirm the identity during lot-release testing.

### NONVIRAL GENE THERAPY PRODUCTS

Restriction enzyme mapping is the most common identity method for plasmid-based products. The number of enzymes used to create the vector fingerprint will vary according to the complexity of the DNA and the degree of similarity between multiple products. If lipids, lipoplex agents, or proteins are used to formulate the DNA, then their identity must also be tested and confirmed. Lipids and lipoplex components may be identified by procedures used for traditional pharmaceuticals, such as GC–MS and TLC. Protein components of the formulation may be identified by peptide mapping or other means outlined under ⟨1057⟩.

## STABILITY

The shelf lives of gene therapy products vary widely depending on the nature of the product, its intended clinical use, its specific attributes, and the recommended storage, packaging, and shipping conditions. Therefore, it is difficult to draft uniform guidelines regarding stability-study duration and testing frequency for all products. In all cases, the study should be designed on the basis of scientifically sound principles and approaches and a comprehensive understanding of the final therapeutic product and its intended use. The product's stability during in-process hold steps, cell and virus banks, critical raw materials, and reference standards also must be assessed. A well-designed and executed stability program will provide a high degree of assurance that the product is stable within the specified shelf life.

For viral and nonviral vector gene therapy products and gene-modified cellular gene therapy products that are not patient-specific, the selection of batches to support license application and final-product labeling should be carried out in accordance with the principles of stability testing, such as those described in ICH guideline Q5C and ⟨1049⟩. Stability data should also be collected for bulk material and at other in-process points if material is stored before final processing and filling. Issues related to the stability of cell-based products are addressed in ⟨1046⟩.

Nonviral DNA plasmid vectors are often formulated with specific mixtures of lipids, proteins, or lipoconjugates to form liposomes or encapsulated complexes. Depending on the formulation, a shelf life of hours to years can be attained. Where a product has a short shelf life, the final formulation may require preparation at the clinic just before administration. Instability is frequently observed as aggregation and precipitation. Formation and stability of the formulated complex should be

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characterized and established by validation studies during product development. Stability data should also be collected for major components of the formulated complex, such as the lipids, the liposomes, and the DNA itself.

## Stability-Protocol Development

Stability studies verify that the storage conditions maintain the purity and potency of the product for a defined period so that product administered to the patient is still capable of meeting the stability specifications. These specifications may differ from the manufacturing release specifications, but they must be verified with clinical data. Formal stability studies to support licensure as well as plans for gathering early-phase product stability information should be detailed in a written plan that describes how data will be collected and analyzed to support the product's expiration period. Protocols should follow the format recommended in existing guidelines and should include the scope, storage conditions, number of lots that will be tested, test schedule, assays that will be used, data analysis, and product specifications. Any assay used in a formal stability study for licensure must be validated before the study begins. The specific study design should take into account the problems the product may encounter during manufacturing, shipping, and processing at the clinical site (see *Accelerated and Most Appropriate Challenge Conditions* below). The study design should also incorporate the latest knowledge in the biological sciences and should address existing regulatory requirements. For instance, if the product's final formulation is performed at the clinical site, stability studies on this final formulation should be performed to establish the time and conditions under which the product can be held.

Stability assessment should include an evaluation of product functionality (potency). The potency assay often has a high degree of inherent variability. Measuring and calculating the decay of product activity by employing the standard statistical methodologies may require multiple, frequent sampling intervals over an extended period of time and may require analysis of more than three production lots to compensate for assay variability. Initial studies to establish a provisional expiration date must be conducted before administration to the first patient. Initial studies are also useful for determining which assays are stability indicating, that is, the best indicators of product degradation. Because existing compendial methods do not address the unique characteristics of gene therapy products, the development of assays that would address these unique characteristics is encouraged.

## Accelerated and Most Appropriate Challenge Conditions

The stability-indicating profile of a gene therapy product varies over time under the influence of a wide variety of environmental conditions, including temperature, extremes in physiological storage conditions, and light. Multifactorial degradation pathways must be considered when researchers investigate the effects of these parameters on the stability of the product. Studies should include conditions that are outside the specified storage ranges, that is, challenge conditions such as those encountered during periods of abnormal storage, shipping, or handling. Examples include brief incubator malfunctions, incubator or cold storage failure, periods of extreme temperature fluctuation due to shipping to hot or cold climates, hypobaric conditions experienced in the cargo hold of a commercial airliner, or temperatures likely to be encountered in the surgical suite. A short exposure to an environmental condition well outside of an established limit and a long exposure to an environmental condition just outside of an established acceptable range may be equally detrimental to the overall stability profile. The slow and constant rate of product degradation at a specified temperature may increase if a different set of storage conditions is applied. The effect of light on the stability-indicating profile should be investigated if it is scientifically warranted. Give special attention to products stored in fluids that contain light-sensitive or reactive components that may give rise to cytotoxic by-products.

Studies analogous to accelerated aging studies typically used in pharmaceutical stability-monitoring programs are also useful to determine how the product degrades and which assays are stability indicating. These studies can be the same as some of those mentioned in the preceding paragraph. Other studies include placing a product at 37°, or at 18° when its normal storage temperature is 25 ± 2°, or placing a lyophilized product in a high-humidity environment. Such studies should be performed before formal stability studies begin so that the latter can incorporate the validated stability-indicating assays.

# STORAGE AND SHIPPING

Appropriate conditions are chosen to preserve the purity and potency of the product so that its specification and those of its ingredients are maintained throughout storage, shipping, and handling at the clinic. Initial studies must be conducted before patient administration to determine acceptable storage, shipping, and handling conditions. The initial storage and shipping conditions need not be those envisioned for the commercial product but should ensure that the product specifications are maintained beyond the initial expiration date. For products with short shelf lives, storage and shipping conditions, even within a medical center, must be considered at the same time because shipping constitutes the bulk of storage time after manufacturing. Give special consideration to the ability of gas to permeate the shipping container, especially if the gene therapy product is stored or shipped on dry ice. Once stability-indicating methods are developed and the final storage and shipping conditions are chosen, these must be validated as discussed under *Stability*.

Most products with limited shelf lives are shipped by reliable overnight courier systems. In some cases, highly fragile products are hand-carried onto commercial aircraft. Special permission must be obtained by commercial carriers if scanning by airport X-ray equipment must be avoided. Cargo shipping studies should be designed during the development of packaging systems to identify stresses to which the product may be subjected. Bracing and insulating materials should then be chosen and validated to provide a packaging system that will tolerate, and protect the product against, the extreme conditions of shipping.

Most gene therapy products can be either lyophilized or formulated by means similar to those employed for many recombinant proteins or cell therapy products. These storage formulations typically have expiration periods longer than 1 year and no unusual shipping requirements. Nonviral gene therapy products, which may be unstable in their final formulation, can have similar expiration periods if they are stored in a multiple-vial kit with the nucleic acid in 1 vial and a carrier, such as lipids, in the other. The final formulation is performed at the medical center just before administration.

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## LABELING

Product labeling is regulated by the FDA, and compliance with existing policies is required. Because gene therapy products are regulated biologics, their labeling is subject to these rules. Biologics and devices must meet labeling requirements specific to the container and the package (21 CFR 610 and 801, respectively). Both the container label and the package label must include the expiration date. If the container is packaged, then the recommended storage conditions should be included on the outer package label. If the container is not packaged, the recommended storage conditions and all other requirements of a package label must appear on the container. Labeling must also comply with relevant national and international requirements.

If a product must be applied to the patient in a particular physical orientation or in a specifically defined area, labeling that indicates the correct orientation and/or area should be apparent even after the package is opened. Unless the product has been screened for pathogenic or microbial contaminants before release, appropriate biohazard labeling may be required. For products with very short shelf lives, expiration dating requires adjustment and correction for time zones to provide the user an accurate assessment of shelf life. Clinical procedures must be scheduled around these crucial time frames. For patient-specific products, the patient's full name, initials, or a combination of these will need to appear on the labeling, in addition to lot designation, to ensure that the product is administered to the appropriate patient.

## REGULATIONS AND STANDARDS

The technologies involved in manufacturing gene therapy products have been widely documented in the literature and continue to evolve. These products can be regulated as drugs or biologics, or uncommonly as devices, depending on how they are manufactured and used. The novel approaches permitted by these technologies may make it difficult to determine which FDA centers will be involved in their regulation, and the FDA has advised manufacturers to seek clarification in the early stages of development. Currently, the Center for Biologics Evaluation and Research (CBER) regulates most human gene therapy products. CBER relies on both the Public Health Service Act and the Federal Food, Drug, and Cosmetic Act. Regulation is the same as that for biotechnology-derived products. The general requirements are described primarily in 21 CFR. The federal government has issued many guidance documents as *Points to Consider* or *Guidelines* (see www.fda.gov). ICH guidance documents for many of the quality-related areas are relevant in varying degrees to qualifying gene therapy products (although some products are nominally outside the scope of the guidance documents, the principles still apply; see www.ifpma.org or www.ich.org). Some of these documents are reproduced in *USP–NF* as general chapters. ICH has also held a number of meetings about gene therapy products and has a Gene Therapy Discussion Group (GTDG) that addresses current issues in gene therapy product development and research and has released several ICH Considerations that reflect harmonized principles. The National Institutes of Health (NIH) has published *Guidelines for Research Involving Recombinant DNA Molecules* that require NIH review of research, including clinical research or trials conducted or sponsored by institutions that receive NIH funding. These guidelines apply to many gene therapy products.

Biological and biochemical standards for quality assurance (QA) of the production and analysis of gene therapy products are highly desirable. The diversity of gene therapy products, in particular viral vectors, has so far limited the development of standards that have wide applicability. A MuLV RCR preparation (VR-1450) with an assigned infectivity titer is available from ATCC for testing murine retroviral vectors for the presence of RCR. A wild-type adenovirus type 5 reference standard with assigned particle number and infectivity titer for characterization of adenoviral vectors is also available from ATCC. A working group has been set up to oversee the development of an AAV reference standard. However, several obstacles to choosing, developing, establishing, and circulating suitable standards are apparent. These include decisions about which virus serotype will be most commonly and successfully used for gene therapy, availability of GMP prepared materials, safety, long-term stability, transportation, and initiation and completion of collaborative studies to evaluate candidate standards. Thus, development of standards for other viral vectors, including lentiviral-, herpes viral-, and poxviral-vectors, remains challenging.

New methodologies, including proteomics, novel nucleic acid technologies (NATs), protein modification methods, and stem cell isolation and culture, are now available and, in many cases, are applicable to the development, characterization, and analysis of gene therapy products. In addition, the use of synthetic polymers both for the modification of existing viral vectors and for the development of chemically dynamic synthetic vectors provides advantages, e.g., improved systemic circulation, better targeting and delivery, and lower levels of immunostimulation and inflammation. The availability of defined stem cell populations and improved engrafting methods should lead to greater effectiveness of ex vivo transduced cells used in gene therapy protocols. The introduction of new methodologies will require the continual review and regulatory oversight to ensure the quality and safety of gene therapy products of the future.

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## GLOSSARY

**Adenovirus:** Virus belonging to the family Adenoviridae of DNA viruses having a nonenveloped virion with 252 capsomeres and a diameter between 70 and 90 nm; a single linear molecule of double-stranded DNA (36–38 kb); at least 10 structural ether-resistant and acid-stable proteins; virions are released by cell destruction.

**Adenovirus-associated virus** (AAV)**:** Human parvovirus contains a single-stranded DNA genome and depends on helper viruses (adenovirus, herpes virus, or vaccinia virus) for replication. Without co-infection, the wild-type virions integrate at a specific site on chromosome 19 and remain latent.

**Adventitious agent:** A foreign agent that is introduced accidentally or inadvertently; not natural or hereditary (as in microbial, chemical, or biochemical contamination of a purified substance).

**Amphotropic virus:** A virus that infects and replicates in cells from multiple species.

**Ancillary materials:** Components used during manufacturing that are not intended to be present in the final product. Examples: growth factors, monoclonal antibodies, cell-separation devices, and media components.

**Autologous:** From one's own body.

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**Base pair:** Two nucleotide bases on different strands of the nucleic acid molecule that bond together.

**Bioassay:** Measurement of the effectiveness of a compound by its effect on animals or cells in comparison with a standard preparation. (See also *Potency*▲in this *Glossary*.)▲ (USP 1-May-2020)

**Biological product:** Any virus, therapeutic serum, toxin, antitoxin, or "analogous product" applicable to the prevention, treatment, or cure of diseases or injuries in humans. (In this US FDA-derived definition, the term "analogous product" has been interpreted to include essentially all biotechnology-derived products and procedures including gene therapy, transgenics, and somatic cell therapy.)

**Biotechnology:** Any technique that uses living organisms (or parts of organisms) to make or modify products, to improve plants or animals, or to develop microorganisms for specific uses. The newer definition refers to the industrial and pharmaceutical use of rDNA, cell fusion, novel bioprocessing techniques, and gene therapy.

**Cell lines:** Cells that are derived from primary culture embryos, tissue, or organs. Such cell lines may have a finite life span or may be immortalized (modified to be able to replicate indefinitely).

**Cell therapy:** Therapy that uses whole cells to treat a disease, condition, or injury. Distinct from tissue and organ transplantation.

**CFTR:** Cystic fibrosis transmembrane conductance regulator.

**cGMP:** Current good manufacturing practices. The FDA outlined cGMPs in the 21 CFR, in the *Federal Register*, and its *Points to Consider.*

**Complementary DNA** (cDNA)**:** DNA synthesized from an mRNA rather than a DNA template. It is used for cloning or as a DNA probe for locating specific genes.

**Cytokine:** Any factor that acts on cells; usually a protein that promotes growth.

**Cytoplasm:** Cellular material that is within the cell membrane and surrounds the nucleus.

**Cytotoxic:** Able to cause cell death.

**Differentiation:** A process of biochemical and structural changes by which cells become specialized in form and function.

**Ecotropic virus:** A virus that infects and replicates in cells from only the original host species.

**Electroporation:** Method for enabling transfer of material into cells that involves use of a brief electrical field to create temporary pores in the cell membrane.

**Enzyme-linked immunosorbent assay (ELISA):** An immunoassay that utilizes an enzyme-labeled antigen or antibody to detect the binding of a molecule to a solid matrix.

**Enveloped viruses:** Viruses containing a lipoprotein bilayer surrounding the capsid and acquired by budding through the cell membrane of the host cells.

**Episomal:** Pertaining to any accessory extra-chromosomal genetic material.

**Ex vivo:** Procedure performed outside of the living organism.

**Fluorescence-activated cell sorter** (FACS)**:** A machine that sorts cells based on fluorescent markers attached to them.

**Formulated:** Prepared in accordance with a prescribed method or conditions.

**Fusion:** Joining of the membrane of two cells, creating a daughter that contains some of the same properties from each parent cell. It is used in making hybridoma cells in which antibody-producing cells are fused to mouse myeloma cells.

**Gene construct:** Expression vector that contains the coding sequence of the protein and the necessary elements for its expression.

**Gene therapy:** Therapy that uses nucleic acid that is subsequently expressed as RNA or protein to treat a disease or condition. The US FDA defines gene therapy products as products containing genetic material administered to modify or manipulate the expression of genetic material to alter the biological properties of living cells.

**Genome:** Total hereditary material of a cell.

**Germ cell:** Reproductive cell (sperm or egg), gamete, or sex cell.

**Graft-versus-host disease** (GVHD)**:** Rejection of the transplanted tissue by the host. It is the leading cause of patient death when mismatched allogeneic tissue is used.

**Growth factors:** Factors responsible for regulatory cell proliferation, function, and differentiation.

**Helper virus:** Aids the development of a defective virus by supplying or restoring the activity of a viral gene or by enabling the defective virus to form a functional envelope.

**Hematopoietic:** Pertaining to or affecting the formation of blood cells.

**Hepatocyte:** The predominant cell type in the liver that has an important role in metabolism and is a source of serum proteins. These cells generally do not divide, but when injured they can divide and regenerate until the injured cells are replaced.

**Herpes simplex virus** (HSV)**:** A DNA virus that is a member of the family Herpesviridae. It can infect both warm- and cold-blooded vertebrates by contact between moist mucosal surfaces.

**Human leukocyte antigen** (HLA)**:** Proteins controlled by the major histocompatibility complex. These proteins play a key role in determining transplant compatibility.

**Humoral:** Pertaining to elements found in body fluids (for example, humoral immunity and neutralizing antibodies).

**Hybridization dot blot** (DNA or RNA)**:** A technique for detecting, analyzing, and identifying protein; similar to the Western blot but without electrophoretic separation of proteins.

**Immunoassay:** Technique for identifying substances based on the use of antibodies.

**Insertional mutagenesis:** A type of mutation that is caused by the insertion of nucleic acid into a host-cell chromosome. There are multiple possible negative consequences of such an event, including death of a cell if an essential gene is inactivated or predisposition to cancer if a tumor suppressor gene is inactivated.

**Integration:** Assimilation (insertion via covalent binding) of genetic material (DNA) into the chromosome of a recipient cell.

**Intrabodies:** Intracellular antibodies that are not secreted and that are designed to bind and inactivate target molecules inside cells.

**In vivo:** Procedure performed in the living organism.

**In vitro:** Procedure performed outside of the living organism. It may involve cells or tissues derived from the organisms.

**Leukemia:** Malignant neoplasm of the blood-forming tissues.

**Lipoplex:** A formulation of lipids and polymers and/or proteins.

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**Liposome:** A spherical lipid bilayer ▲or multiple lipid bilayers▲ (USP 1-May-2020) enclosing ▲one or more▲ (USP 1-May-2020) aqueous ▲compartment(s). See *Injections and Implanted Drug Products* ⟨1⟩.▲ (USP 1-May-2020)

**Mock run:** A test run that deliberately omits some critical reagents.

**Monoclonal antibodies:** Antibodies that are derived from a single cell clone.

**Naked DNA:** Isolated, purified, and uncomplexed DNA (no protein or lipid).

▲▲ (USP 1-May-2020)

**Oncogenes:** Genes associated with neoplastic proliferation (cancer) following a mutation or perturbation in their expression.

**Oncogenic:** Cancer-causing.

**Packaging cell line:** Cell line that produces proteins required for packaging and production of viral vectors in an active form but does not produce replication-competent virus. It complements at the protein level what the vector is lacking genetically.

**Parvovirus:** DNA viruses of the family Parvoviridae. Host range includes many vertebrate species. Small, linear chain, single-stranded DNA with terminal hairpin loops.

**Plasmid:** A small circular form of DNA that carries certain genes and is capable of replicating independently in a host cell.

**Polymerase chain reaction** (PCR)**:** Technique to amplify a target DNA or RNA sequence of nucleotides by repeated cycles of polymerase-based copying, resulting in geometric increases in copy number.

**Potency:** A quantitative measure of biological activity based on the attribute of the product linked to the relevant biological properties.

**Process validation:** Means for providing documentation that the manufacturing process is controlled, reproducible, and capable of consistently producing a product that meets predetermined specifications.

**Producer cell line:** An established cell line used to produce virus vectors, often on a large scale.

**Promoter:** DNA sequence that is located at the front of a gene and controls gene expression. It is required for binding of RNA polymerase to initiate transcription.

**Recombinant DNA:** DNA produced by joining fragments of DNA from different sources by in vitro manipulations.

**Replication-competent virus:** A virus that can complete an entire replication cycle without a need for a helper virus; an autonomously replicating virus.

**Restriction endonuclease:** An endonuclease that recognizes a specific sequence of bases within double-stranded DNA.

**Retrovirus:** A virus that contains reverse transcriptase, which converts viral RNA into DNA that then integrates into the host cell in a form called a provirus.

**Serum-free:** Refers to cell growth medium that lacks a serum component.

▲**Southern blot:** Southern blotting refers to the transfer of DNA from an agarose or polyacrylamide gel to a nitrocellulose or nylon membrane.▲ (USP 1-May-2020)

**S phase:** Synthesis phase. Part of the cell cycle during which DNA replication occurs.

**Stem cell:** Immortal cell that is capable of proliferating and differentiating into different types of specialized cells. Each major tissue system is thought to have its own putative stem cell.

**Suspension culture:** Cells capable of growth in suspension, not requiring substrate (attachment) on which to grow.

**Transduction:** Transfer and expression of genetic material into a cell by means of a virus or phage vector.

**Transfection:** Transfer of DNA into cells by physical means such as by calcium phosphate coprecipitation.

**Transgene:** Refers to the foreign genetic material delivered as part of a vector construct.

**Vector:** The agent (plasmid, virus, or liposome–protein or DNA–protein complex) used to introduce nucleic acid into a cell.

**Viability:** State of being alive and functional.

**Virion:** An elementary viral particle consisting of genetic material (nucleocapsid) and a protein covering.

**Virus:** Submicroscopic infectious agent that contains genetic information necessary for reproduction. It is an obligate intracellular parasite.

**Western blot:** An electroblotting method in which proteins are transferred from a gel to a thin, rigid support (e.g., nitrocellulose membrane) and detected by binding radioactively labeled antibody or antibody coupled to an enzyme, allowing use of a precipitating chromogenic or chemiluminescent substrate.

**Xenogeneic:** From a different species.

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# APPENDIX

## Lists of Relevant Regulatory References

Gene therapy products are regulated by the FDA as biologics, and therefore their manufacturing, testing, labeling, and other factors are subject to the requirements codified in CFR and FDA guidance documents (www.fda.gov). Additional guidance is provided in ICH guidelines (www.ich.org). Manufacturers of gene therapy products that seek markets outside the US should refer to regulatory documents from relevant countries. Beyond *USP* chapters, the following lists include regulatory documents, as well as best practices for the development, manufacturing, quality control, and quality assurance of gene therapy products.

▲**Code of Federal Regulations (CFR)**

| Regulations | Title |
|---|---|
| 21 CFR 210 | Current Good Manufacturing Practice in Manufacturing, Processing, Packing, or Holding of Drugs; General |
| 21 CFR 211 | Current Good Manufacturing Practice for Finished Pharmaceuticals |

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| Regulations | Title |
|---|---|
| 21 CFR 600s | Biological Products: General |
| 21 CFR 610 Subpart G | General Biological Products Standards—Labeling Standards |
| 21 CFR 801 | Labeling |
| 21 CFR 820 | Quality System Regulation▲ (USP 1-May-2020) |

**FDA Guidance Documents**

- *Guideline for the Determination of Residual Moisture in Dried Biological Products*, January 1990.
- *Guidance for Industry: Human Somatic Cell Therapy and Gene Therapy*, March 1998. ▲https://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/CellularandGeneTherapy/ucm081670.pdf.▲ (USP 1-May-2020)
- *Guidance for Industry: Supplemental Guidance on Testing for Replication Competent Retrovirus in Retroviral Vector Based Gene Therapy Products and During Follow-up of Patients in Clinical Trials Using Retroviral Vectors*, ▲November 2006. https://www.fda.gov/downloads/biologicsbloodvaccines/guidancecomplianceregulatoryinformation/guidances/cellularandgenetherapy/ucm078723.pdf.▲ (USP 1-May-2020)
- *Guidance for Industry: Investigating Out-of-Specification (OOS) Test Results for Pharmaceutical Production*, October 2006. ▲https://www.fda.gov/downloads/drugs/guidances/ucm070287.pdf.▲ (USP 1-May-2020)
- *Guidance for FDA Reviewers and Sponsors: Content and Review of Chemistry, Manufacturing, and Control (CMC) Information for Human Gene Therapy Investigational New Drug Applications (INDs)*, April 2008. ▲https://www.fda.gov/downloads/biologicsbloodvaccines/guidancecomplianceregulatoryinformation/guidances/cellularandgenetherapy/ucm078694.pdf.▲ (USP 1-May-2020)
- ▲▲ (USP 1-May-2020)*Guidance for Industry: Potency Tests for Cellular and Gene Therapy Products*, ▲January 2011. https://www.fda.gov/downloads/biologicsbloodvaccines/guidancecomplianceregulatoryinformation/guidances/cellularandgenetherapy/ucm243392.pdf▲ (USP 1-May-2020)

**National and International Regulatory Documents**

- ICH Q5A(R1): Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin. ▲Available at: http://www.ich.org.▲ (USP 1-May-2020)
- ICH Q5C: Quality of Biotechnological Products: Stability Testing of Biotechnological/Biological Products. ▲Available at: http://www.ich.org.▲ (USP 1-May-2020)
- ICH Q5D: Quality of Biotechnological Products: Derivation and Characterisation of Cell Substrates Used for Production of Biotechnological/Biological Products. ▲Available at: http://www.ich.org.▲ (USP 1-May-2020)
- ICH Q6B Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products. ▲Available at: http://www.ich.org.▲ (USP 1-May-2020)
- ICH Q2(R1): Validation of Analytical Procedures: Text and Methodology. ▲Available at: http://www.ich.org.▲ (USP 1-May-2020)
- NIH Guidelines for Research Involving Recombinant ▲or Synthetic Nucleic Acid▲ (USP 1-May-2020) Molecules. ▲Available at: https://osp.od.nih.gov/biotechnology/nih-guidelines/.▲ (USP 1-May-2020)

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# ⟨1048⟩ QUALITY OF BIOTECHNOLOGICAL PRODUCTS: ANALYSIS OF THE EXPRESSION CONSTRUCT IN CELLS USED FOR PRODUCTION OF r-DNA DERIVED PROTEIN PRODUCTS[1]

## I. INTRODUCTION

This document presents guidance regarding the characterization of the expression construct for the production of recombinant DNA (r-DNA) protein products in eukaryotic and prokaryotic cells. The document is intended to describe the types of information that are considered valuable in assessing the structure of the expression construct used to produce r-DNA derived proteins. The document is not intended to cover the entire quality aspect of r-DNA derived medicinal products.

The expression construct is defined as the expression vector containing the coding sequence of the recombinant protein. Segments of the expression construct should be analyzed using nucleic acid techniques in conjunction with other tests performed on the purified recombinant protein for assuring the quality and consistency of the final product. Analysis of the expression construct at the nucleic acid level should be considered as part of the overall evaluation of quality, taking into account that this testing only evaluates the coding sequence of a recombinant gene and not the translational fidelity nor other characteristics of the recombinant protein, such as secondary structure, tertiary structure, and posttranslational modifications.

## II. RATIONALE FOR THE ANALYSIS OF THE EXPRESSION CONSTRUCT

The purpose of analyzing the expression construct is to establish that the correct coding sequence of the product has been incorporated into the host cell and is maintained during culture to the end of production. The genetic sequence of recombinant proteins produced in living cells can undergo mutations that could alter the properties of the protein with potential adverse consequences to patients. No single experimental approach can be expected to detect all possible modifications to a protein. Protein analytical techniques can be used to assess the amino acid sequence of the protein and structural features of the expressed protein due to posttranslational modifications such as proteolytic processing, glycosylation, phosphorylation, and acetylation. Data from nucleic acid analysis may be useful because protein analytical methods may not detect all changes in protein structure resulting from mutations in the sequence coding for the recombinant protein. The relative importance of nucleic acid analysis and protein analysis will vary from product to product.

Nucleic acid analysis can be used to verify the coding sequence and the physical state of the expression construct. The nucleic acid analysis is performed to ensure that the expressed protein will have the correct amino acid sequence, but is not intended to detect low levels of variant sequences. Where the production cells have multiple integrated copies of the expression construct, not all of which may be transcriptionally active, examination of the transcription product itself by analysis of m-RNA or c-DNA may be more appropriate than analysis of genomic DNA. Analytical approaches that examine a bulk population of nucleic acids, such as those performed on pooled clones or material amplified by the polymerase chain reaction, may be considered as an alternative to approaches that depend on selection of individual DNA clones. Other techniques could be considered that allow for rapid and sensitive confirmation of the sequence coding for the recombinant protein in the expression construct.

The following sections describe information that should be supplied regarding the characterization of the expression construct during the development and validation of the production system. Analytical methodologies should be validated for the intended purpose of confirmation of sequence. The validation documentation should, at a minimum, include estimates of the limits of detection for variant sequences. This should be performed for either nucleic acid or protein sequencing methods. The philosophy and recommendations for analysis expressed in this document should be reviewed periodically to take advantage of new advances in technology and scientific information.

## III. CHARACTERIZATION OF THE EXPRESSION SYSTEM

### A. Expression Construct and Cell Clone Used to Develop the Master Cell Bank (MCB)

The manufacturer should describe the origin of the nucleotide sequence coding for the protein. This should include identification and source of the cell from which the nucleotide sequence was originally obtained. Methods used to prepare the DNA coding for the protein should be described.

The steps in the assembly of the expression construct should be described in detail. This description should include the source and function of the component parts of the expression construct, e.g., origins of replication, antibiotic resistance genes,

[1] This guideline was developed within the Expert Working Group (Quality) of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) and has been subject to consultation by the regulatory parties, in accordance with the ICH process. This document has been endorsed by the ICH Steering Committee at *Step 4* of the ICH process, November 29, 1995. At *Step 4* of the process, the final draft is recommended for adoption to the regulatory bodies of the European Union, Japan and the USA. This guideline was published in the Federal Register on February 23, 1996 (61 FR 7006) and is applicable to drug and biological products. Although this guideline does not create or confer any rights for or on any person and does not operate to bind FDA or the industry, it does represent the agency's current thinking on the production of r-DNA derived protein products. For additional copies of this guideline, contact the Drug Information Branch, HFD-210, CDER, FDA, 5600 Fishers Lane, Rockville, MD 20857 (Phone: 301-827-4573) or the Manufacturers Assistance and Communication Staff (HFM-42), CBER, FDA, 1401 Rockville Pike, Rockville, MD 20852-1448. Send one self-addressed adhesive label to assist the offices in processing your request. An electronic version of this guidance is also available via Internet using the World Wide Web (WWW) (connect to the CDER Home Page at http://www.fda.gov/cder and go to the "Regulatory Guidance" section).

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promoters, enhancers, and whether or not the protein is being synthesized as a fusion protein. A detailed component map and a complete annotated sequence of the plasmid should be given, indicating those regions that have been sequenced during the construction and those taken from the literature. Other expressed proteins encoded by the plasmid should be indicated. The nucleotide sequence of the coding region of the gene of interest and associated flanking regions that are inserted into the vector, up to and including the junctions of insertion, should be determined by DNA sequencing of the construct.

A description of the method of transfer of the expression construct into the host cell should be provided. In addition, methods used to amplify the expression construct and criteria used to select the cell clone for production should be described in detail.

### B. Cell Bank System

Production of the recombinant protein should be based on well-defined MCB and Working Cell Banks (WCB). A cell bank is a collection of ampules of uniform composition stored under defined conditions, each containing an aliquot of a single pool of cells. The MCB is generally derived from the selected cell clone containing the expression construct. The WCB is derived by expansion of one or more ampules of the MCB. The cell line history and production of the cell banks should be described in detail, including methods and reagents used during culture, in vitro cell age, and storage conditions. All cell banks should be characterized for relevant phenotypic and genotypic markers, which could include the expression of the recombinant protein or presence of the expression construct.

The expression construct in the MCB should be analyzed as described below. If the testing cannot be carried out on the MCB, it should be carried out on each WCB.

Restriction endonuclease mapping or other suitable techniques should be used to analyze the expression construct for copy number, for insertions or deletions, and for the number of integration sites. For extrachromosomal expression systems, the percent of host cells retaining the expression construct should be determined.

The protein coding sequence for the recombinant protein product of the expression construct should be verified. For extrachromosomal expression systems, the expression construct should be isolated and the nucleotide sequence encoding the product should be verified without further cloning. For cells with chromosomal copies of the expression construct, the nucleotide sequence encoding the product could be verified by recloning and sequencing of chromosomal copies. Alternatively, the nucleic acid sequence encoding the product could be verified by techniques such as sequencing of pooled c-DNA clones or material amplified by the polymerase chain reaction. The nucleic acid sequence should be identical, within the limits of detection of the methodology, to that determined for the expression construct as described in section III.A., and should correspond to that expected for the protein sequence.

### C. Limit for In Vitro Cell Age for Production

The limit for in vitro cell age for production should be based on data derived from production cells expanded under pilot plant-scale or full-scale conditions to the proposed in vitro cell age or beyond. Generally, the production cells are obtained by expansion of the WCB; the MCB could be used to prepare the production cells with appropriate justification.

The expression construct of the production cells should be analyzed once for the MCB as described in section III.B., except that the protein coding sequence of the expression construct in the production cells could be verified by either nucleic acid testing or analysis of the final protein product. Increases in the defined limit for in vitro cell age for production should be supported by data from cells that have been expanded to an in vitro cell age that is equal to or greater than the new limit for in vitro cell age.

## IV. CONCLUSION

The characterization of the expression construct and the final purified protein are both important to ensure the consistent production of a r-DNA derived product. As described above, analytical data derived from both nucleic acid analysis and evaluation of the final purified protein should be evaluated to ensure the quality of a recombinant protein product.

## GLOSSARY

**Expression Construct:** The expression vector that contains the coding sequence of the recombinant protein and the elements necessary for its expression.

**Flanking Control Regions:** Noncoding nucleotide sequences that are adjacent to the 5′ and 3′ end of the coding sequence of the product that contain important elements that affect the transcription, translation, or stability of the coding sequence. These regions include, e.g., promoter, enhancer, and splicing sequences, and do not include origins of replication and antibiotic resistance genes.

**Integration Site:** The site where one or more copies of the expression construct is integrated into the host cell genome.

**In Vitro Cell Age:** Measure of time between thaw of the MCB vial(s) to harvest of the production vessel measured by elapsed chronological time in culture, by population doubling level of the cells, or by passage level of the cells when subcultivated by a defined procedure for dilution of the culture.

**Master Cell Bank (MCB):** An aliquot of a single pool of cells that generally has been prepared from the selected cell clone under defined conditions, dispensed into multiple containers, and stored under defined conditions. The MCB is used to derive all working cell banks. The testing performed on a new MCB (from a previous initial cell clone, MCB, or WCB) should be the same as for the MCB unless justified.

**Pilot Plant Scale:** The production of a recombinant protein by a procedure fully representative of and simulating that to be applied on a full commercial manufacturing scale. The methods of cell expansion, harvest, and product purification should be identical except for the scale of production.

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**Relevant Genotypic and Phenotypic Markers:** Those markers permitting the identification of the strain of the cell line that should include the expression of the recombinant protein or presence of the expression construct.

**Working Cell Bank (WCB):** The WCB is prepared from aliquots of a homogeneous suspension of cells obtained from culturing the MCB under defined culture conditions.

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# ⟨1049⟩ QUALITY OF BIOTECHNOLOGICAL PRODUCTS: STABILITY TESTING OF BIOTECHNOLOGICAL/BIOLOGICAL PRODUCTS[1]

## INTRODUCTION (1)

The guidance stated in the ICH harmonized tripartite guideline entitled "Stability Testing of New Drug Substances and Products" (issued by ICH on October 27, 1993) applies in general to biotechnological/biological products. However, biotechnological/biological products have distinguishing characteristics to which consideration should be given in any well-defined testing program designed to confirm their stability during the intended storage period. For such products in which the active components are typically proteins and/or polypeptides, maintenance of molecular conformation and, hence, of biological activity, is dependent on noncovalent as well as covalent forces. The products are particularly sensitive to environmental factors such as temperature changes, oxidation, light, ionic content, and shear. To ensure maintenance of biological activity and to avoid degradation, stringent conditions for their storage are usually necessary.

The evaluation of stability may necessitate complex analytical methodologies. Assays for biological activity, where applicable, should be part of the pivotal stability studies. Appropriate physicochemical, biochemical, and immunochemical methods for the analysis of the molecular entity and the quantitative detection of degradation products should also be part of the stability program whenever purity and molecular characteristics of the product permit use of these methodologies.

With these concerns in mind, the applicant should develop the proper supporting stability data for a biotechnological/biological product and consider many external conditions that can affect the product's potency, purity, and quality. Primary data to support a requested storage period for either drug substance or drug product should be based on long-term, real-time, real-condition stability studies. Thus, the development of a proper long-term stability program becomes critical to the successful development of a commercial product. The purpose of this document is to give guidance to applicants regarding the type of stability studies that should be provided in support of marketing applications. It is understood that during the review and evaluation process, continuing updates of initial stability data may occur.

## SCOPE OF THE ANNEX (2)

The guidance stated in this annex to "Stability Testing of New Drug Substances and Products" applies to well-characterized proteins and polypeptides, their derivatives and products of which they are components, and which are isolated from tissues, body fluids, cell cultures, or produced using recombinant deoxyribonucleic acid (r-DNA) technology. Thus, the document covers the generation and submission of stability data for products such as cytokines (interferons, interleukins, colony-stimulating factors, tumor necrosis factors), erythropoietins, plasminogen activators, blood plasma factors, growth hormones and growth factors, insulins, monoclonal antibodies, and vaccines consisting of well-characterized proteins or polypeptides. In addition, the guidance outlined in the following sections may apply to other types of products, such as conventional vaccines, after consultation with the appropriate regulatory authorities. The document does not cover antibiotics, allergenic extracts, heparins, vitamins, whole blood, or cellular blood components.

## TERMINOLOGY (3)

For the basic terms used in this annex, the reader is referred to the "Glossary" in "Stability Testing of New Drug Substances and Products." However, because manufacturers of biotechnological/biological products sometimes use traditional terminology, traditional terms are specified in parentheses to assist the reader. A supplemental glossary is also included that explains certain terms used in the production of biotechnological/biological products.

## SELECTION OF BATCHES (4)

### Drug Substance (Bulk Material) (4.1)

Where bulk material is to be stored after manufacture, but before formulation and final manufacturing, stability data should be provided on at least three batches for which manufacture and storage are representative of the manufacturing scale of production. A minimum of 6 months stability data at the time of submission should be submitted in cases where storage periods greater than 6 months are requested. For drug substances with storage periods of less than 6 months, the minimum amount of stability data in the initial submission should be determined on a case-by-case basis. Data from pilot-plant scale batches of

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drug substance produced at a reduced scale of fermentation and purification may be provided at the time the dossier is submitted to the regulatory agencies with acommitment to place the first three manufacturing scale batches into the long-term stability program after approval.

The quality of the batches of drug substance placed into the stability program should be representative of the quality of the material used in preclinical and clinical studies and of the quality of the material to be made at manufacturing scale. In addition, the drug substance (bulk material) made at pilot-plant scale should be produced by a process and stored under conditions representative of that used for the manufacturing scale. The drug substance entered into the stability program should be stored in containers that properly represent the actual holding containers used during manufacture. Containers of reduced size may be acceptable for drug substance stability testing provided that they are constructed of the same material and use the same type of container/closure system that is intended to be used during manufacture.

### Intermediates (4.2)

During manufacture of biotechnological/biological products, the quality and control of certain intermediates may be critical to the production of the final product. In general, the manufacturer should identify intermediates and generate in-house data and process limits that assure their stability within the bounds of the developed process. Although the use of pilot-plant scale data is permissible, the manufacturer should establish the suitability of such data using the manufacturing scale process.

### Drug Product (Final Container Product) (4.3)

Stability information should be provided on at least three batches of final container product representative of that which will be used at manufacturing scale. Where possible, batches of final container product included in stability testing should be derived from different batches of bulk material. A minimum of 6 months data at the time of submission should be submitted in cases where storage periods greater than 6 months are requested. For drug products with storage periods of less than 6 months, the minimum amount of stability data in the initial submission should be determined on a case-by-case basis. Product expiration dating should be based upon the actual data submitted in support of the application. Because dating is based upon the real-time/real-temperature data submitted for review, continuing updates of initial stability data should occur during the review and evaluation process. The quality of the final container product placed on stability studies should be representative of the quality of the material used in the preclinical and clinical studies. Data from pilot-plant scale batches of drug product may be provided at the time the dossier is submitted to the regulatory agencies with a commitment to place the first three manufacturing scale batches into the long-term stability program after approval. Where pilot-plant scale batches were submitted to establish the dating for a product and, in the event that the product produced at manufacturing scale does not meet those long-term stability specifications throughout the dating period or is not representative of the material used in preclinical and clinical studies, the applicant should notify the appropriate regulatory authorities to determine a suitable course of action.

### Sample Selection (4.4)

Where one product is distributed in batches differing in fill volume (e.g., 1 milliliter (mL), 2 mL, or 10 mL), unitage (e.g., 10 units, 20 units, or 50 units), or mass (e.g., 1 milligram (mg), 2 mg, or 5 mg), samples to be entered into the stability program may be selected on the basis of a matrix system and/or by bracketing.

Matrixing, i.e., the statistical design of a stability study in which different fractions of samples are tested at different sampling points, should only be applied when appropriate documentation is provided that confirms that the stability of the samples tested represents the stability of all samples. The differences in the samples for the same drug product should be identified as, for example, covering different batches, different strengths, different sizes of the same closure, and, possibly, in some cases, different container/closure systems. Matrixing should not be applied to samples with differences that may affect stability, such as different strengths and different containers/closures, where it cannot be confirmed that the products respond similarly under storage conditions.

Where the same strength and exact container/closure system is used for three or more fill contents, the manufacturer may elect to place only the smallest and largest container size into the stability program, i.e., bracketing. The design of a protocol that incorporates bracketing assumes that the stability of the intermediate condition samples are represented by those at the extremes. In certain cases, data may be needed to demonstrate that all samples are properly represented by data collected for the extremes.

## STABILITY-INDICATING PROFILE (5)

On the whole, there is no single stability-indicating assay or parameter that profiles the stability characteristics of a biotechnological/biological product. Consequently, the manufacturer should propose a stability-indicating profile that provides assurance that changes in the identity, purity, and potency of the product will be detected.

At the time of submission, applicants should have validated the methods that comprise the stability-indicating profile, and the data should be available for review. The determination of which tests should be included will be product-specific. The items emphasized in the following subsections are not intended to be all-inclusive, but represent product characteristics that should typically be documented to demonstrate product stability adequately.

### Protocol (5.1)

The dossier accompanying the application for marketing authorization should include a detailed protocol for the assessment of the stability of both drug substance and drug product in support of the proposed storage conditions and expiration dating

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periods. The protocol should include all necessary information that demonstrates the stability of the biotechnological/biological product throughout the proposed expiration dating period including, for example, well-defined specifications and test intervals. The statistical methods that should be used are described in the tripartite guideline on stability.

## Potency (5.2)

When the intended use of a product is linked to a definable and measurable biological activity, testing for potency should be part of the stability studies. For the purpose of stability testing of the products described in this guideline, potency is the specific ability or capacity of a product to achieve its intended effect. It is based on the measurement of some attribute of the product and is determined by a suitable in vivo or in vitro quantitative method. In general, potencies of biotechnological/biological products tested by different laboratories can be compared in a meaningful way only if expressed in relation to that of an appropriate reference material. For that purpose, a reference material calibrated directly or indirectly against the corresponding national or international reference material should be included in the assay.

Potency studies should be performed at appropriate intervals as defined in the stability protocol and the results should be reported in units of biological activity calibrated, whenever possible, against nationally or internationally recognized standards. Where no national or international reference standards exist, the assay results may be reported in in-house derived units using a characterized reference material.

In some biotechnological/biological products, potency is dependent upon the conjugation of the active ingredient(s) to a second moiety or binding to an adjuvant. Dissociation of the active ingredient(s) from the carrier used in conjugates or adjuvants should be examined in real-time/real-temperature studies (including conditions encountered during shipment). The assessment of the stability of such products may be difficult because, in some cases, in vitro tests for biological activity and physicochemical characterization are impractical or provide inaccurate results. Appropriate strategies (e.g., testing the product before conjugation/binding, assessing the release of the active compound from the second moiety, in vivo assays) or the use of an appropriate surrogate test should be considered to overcome the inadequacies of in vitro testing.

## Purity and Molecular Characterization (5.3)

For the purpose of stability testing of the products described in this guideline, purity is a relative term. Because of the effect of glycosylation, deamidation, or other heterogeneities, the absolute purity of a biotechnological/biological product is extremely difficult to determine. Thus, the purity of a biotechnological/biological product should be typically assessed by more than one method and the purity value derived is method-dependent. For the purpose of stability testing, tests for purity should focus on methods for determination of degradation products.

The degree of purity, as well as the individual and total amounts of degradation products of the biotechnological/biological product entered into the stability studies, should be reported and documented whenever possible. Limits of acceptable degradation should be derived from the analytical profiles of batches of the drug substance and drug product used in the preclinical and clinical studies.

The use of relevant physicochemical, biochemical, and immunochemical analytical methodologies should permit a comprehensive characterization of the drug substance and/or drug product (e.g., molecular size, charge, hydrophobicity) and the accurate detection of degradation changes that may result from deamidation, oxidation, sulfoxidation, aggregation, or fragmentation during storage. As examples, methods that may contribute to this include electrophoresis (SDS-PAGE, immunoelectrophoresis, Western blot, isoelectrofocusing), high-resolution chromatography (e.g., reversed-phase chromatography, gel filtration, ion exchange, affinity chromatography), and peptide mapping.

Wherever significant qualitative or quantitative changes indicative of degradation product formation are detected during long-term, accelerated, and/or stress stability studies, consideration should be given to potential hazards and to the need for characterization and quantification of degradation products within the long-term stability program. Acceptable limits should be proposed and justified, taking into account the levels observed in material used in preclinical and clinical studies.

For substances that cannot be properly characterized or products for which an exact analysis of the purity cannot be determined through routine analytical methods, the applicant should propose and justify alternative testing procedures.

## Other Product Characteristics (5.4)

The following product characteristics, though not specifically relating to biotechnological/biological products, should be monitored and reported for the drug product in its final container:

Visual appearance of the product (color and opacity for solutions/suspensions; color, texture, and dissolution time for powders), visible particulates in solutions or after the reconstitution of powders or lyophilized cakes, pH, and moisture level of powders and lyophilized products.

Sterility testing or alternatives (e.g., container/closure integrity testing) should be performed at a minimum initially and at the end of the proposed shelf life.

Additives (e.g., stabilizers, preservatives) or excipients may degrade during the dating period of the drug product. If there is any indication during preliminary stability studies that reaction or degradation of such materials adversely affect the quality of the drug product, these items may need to be monitored during the stability program.

The container/closure has the potential to affect the product adversely and should be carefully evaluated (see below).

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## STORAGE CONDITIONS (6)

### Temperature (6.1)

Because most finished biotechnological/biological products need precisely defined storage temperatures, the storage conditions for the real-time/real-temperature stability studies may be confined to the proposed storage temperature.

### Humidity (6.2)

Biotechnological/biological products are generally distributed in containers protecting them against humidity. Therefore, where it can be demonstrated that the proposed containers (and conditions of storage) afford sufficient protection against high and low humidity, stability tests at different relative humidities can usually be omitted. Where humidity-protecting containers are not used, appropriate stability data should be provided.

### Accelerated and Stress Conditions (6.3)

As previously noted, the expiration dating should be based on real-time/real-temperature data. However, it is strongly suggested that studies be conducted on the drug substance and drug product under accelerated and stress conditions. Studies under accelerated conditions may provide useful support data for establishing the expiration date, provide product stability information or future product development (e.g., preliminary assessment of proposed manufacturing changes such as change in formulation, scale-up), assist in validation of analytical methods for the stability program, or generate information that may help elucidate the degradation profile of the drug substance or drug product. Studies under stress conditions may be useful in determining whether accidental exposures to conditions other than those proposed (e.g., during transportation) are deleterious to the product and also for evaluating which specific test parameters may be the best indicators of product stability. Studies of the exposure of the drug substance or drug product to extreme conditions may help to reveal patterns of degradation; if so, such changes should be monitored under proposed storage conditions. Although the tripartite guideline on stability describes the conditions of the accelerated and stress study, the applicant should note that those conditions may not be appropriate for biotechnological/biological products. Conditions should be carefully selected on a case-by-case basis.

### Light (6.4)

Applicants should consult the appropriate regulatory authorities on a case-by-case basis to determine guidance for testing.

### Container/Closure (6.5)

Changes in the quality of the product may occur due to the interactions between the formulated biotechnological/biological product and container/closure. Where the lack of interactions cannot be excluded in liquid products (other than sealed ampules), stability studies should include samples maintained in the inverted or horizontal position (i.e., in contact with the closure), as well as in the upright position, to determine the effects of the closure on product quality. Data should be supplied for all different container/closure combinations that will be marketed.

In addition to the standard data necessary for a conventional single-use vial, the applicant should demonstrate that the closure used with a multiple-dose vial is capable of withstanding the conditions of repeated insertions and withdrawals so that the product retains its full potency, purity, and quality for the maximum period specified in the instructions-for-use on containers, packages, and/or package inserts. Such labeling should be in accordance with relevant national/regional requirements.

### Stability after Reconstitution of Freeze-Dried Product (6.6)

The stability of freeze-dried products after their reconstitution should be demonstrated for the conditions and the maximum storage period specified on containers, packages, and/or package inserts. Such labeling should be in accordance with relevant national/regional requirements.

## TESTING FREQUENCY (7)

The shelf lives of biotechnological/biological products may vary from days to several years. Thus, it is difficult to draft uniform guidelines regarding the stability study duration and testing frequency that would be applicable to all types of biotechnological/biological products. With only a few exceptions, however, the shelf lives for existing products and potential future products will be within the range of 0.5 to 5 years. Therefore, the guidance is based upon expected shelf lives in that range. This takes into account the fact that degradation of biotechnological/biological products may not be governed by the same factors during different intervals of a long storage period.

When shelf lives of 1 year or less are proposed, the real-time stability studies should be conducted monthly for the first 3 months and at 3 month intervals thereafter. For products with proposed shelf lives of greater than 1 year, the studies should be conducted every 3 months during the first year of storage, every 6 months during the second year, and annually thereafter.

While the testing intervals listed above may be appropriate in the preapproval or prelicense stage, reduced testing may be appropriate after approval or licensure where data are available that demonstrate adequate stability. Where data exist that

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indicate the stability of a product is not compromised, the applicant is encouraged to submit a protocol that supports elimination of specific test intervals (e.g., 9-month testing) for postapproval/postlicensure, long-term studies.

## SPECIFICATIONS (8)

Although biotechnological/biological products may be subject to significant losses of activity, physicochemical changes, or degradation during storage, international and national regulations have provided little guidance with respect to distinct release and end of shelf life specifications. Recommendations for maximum acceptable losses of activity, limits for physicochemical changes, or degradation during the proposed shelf life have not been developed for individual types or groups of biotechnological/biological products but are considered on a case-by-case basis. Each product should retain its specifications within established limits for safety, purity, and potency throughout its proposed shelf life. These specifications and limits should be derived from all available information using the appropriate statistical methods. The use of different specifications for release and expiration should be supported by sufficient data to demonstrate that the clinical performance is not affected, as discussed in the tripartite guideline on stability.

## LABELING (9)

For most biotechnological/biological drug substances and drug products, precisely defined storage temperatures are recommended. Specific recommendations should be stated, particularly for drug substances and drug products that cannot tolerate freezing. These conditions, and where appropriate, recommendations for protection against light and/or humidity, should appear on containers, packages, and/or package inserts. Such labeling should be in accordance with relevant national and regional requirements.

## GLOSSARY (10)

**Conjugated Product:** A conjugated product is made up of an active ingredient (e.g., peptide, carbohydrate) bound covalently or noncovalently to a carrier (e.g., protein, peptide, inorganic mineral) with the objective of improving the efficacy or stability of the product.

**Degradation Product:** A molecule resulting from a change in the drug substance (bulk material) brought about over time. For the purpose of stability testing of the products described in this guideline, such changes could occur as a result of processing or storage (e.g., by deamidation, oxidation, aggregation, proteolysis). For biotechnological/biological products, some degradation products may be active.

**Impurity:** Any component of the drug substance (bulk material) or drug product (final container product) that is not the chemical entity defined as the drug substance, an excipient, or other additives to the drug product.

**Intermediate:** For biotechnological/biological products, a material produced during a manufacturing process that is not the drug substance or the drug product but for which manufacture is critical to the successful production of the drug substance or the drug product. Generally, an intermediate will be quantifiable and specifications will be established to determine the successful completion of the manufacturing step before continuation of the manufacturing process. This includes material that may undergo further molecular modification or be held for an extended period before further processing.

**Manufacturing Scale Production:** Manufacture at the scale typically encountered in a facility intended for product production for marketing.

**Pilot-Plant Scale:** The production of the drug substance or drug product by a procedure fully representative of and simulating that to be applied at manufacturing scale. The methods of cell expansion, harvest, and product purification should be identical except for the scale of production.

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# ⟨1050.1⟩ DESIGN, EVALUATION, AND CHARACTERIZATION OF VIRAL CLEARANCE PROCEDURES

## INTRODUCTION

This chapter is a companion document to *Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin* ⟨1050⟩, which was adapted, essentially unchanged, from the International Conference on Harmonization Q5A. This chapter provides users with practical guidance regarding the design, evaluation, and characterization of viral clearance procedures. The chapter scope is the same as that described in ⟨1050⟩ and covers biotechnology products for human use that are derived from cell lines of human or animal origin. Viral clearance studies performed according to the principles outlined in this chapter will provide meaningful data about the ability of the overall production and purification processes to remove or inactivate a broad spectrum of viral types that may affect the safety of biotechnology-derived products. [NOTE—The *Glossary* contains definitions of terms used in this chapter that are not already defined in ⟨1050⟩.]

The regulations for licensing biotechnology products stipulate that cell banks, biologically derived raw materials, and bulk harvest must be controlled and tested for viral safety; however, many of the viral detection approaches that can be used have inherent limitations. In addition, certain detection methods may be so specific for a particular virus that they may fail to detect viral variants (e.g., noncytopathic strains, or non-laboratory-adapted wild-type strains, or mutated variants). Some newly emerging viruses may be missed. Finally, detection methods typically are limited by their sensitivities, and infectious viruses at a low titer may go undetected. Viral contamination of manufacturing processes can come from many sources, including cell substrates and raw materials of biological origin (such as cell culture supplements and other production raw materials), and contamination during production. In addition to the required viral safety testing of these materials, manufacturers must evaluate the effectiveness of downstream purification process steps, which together must remove or inactivate any viruses potentially present (see ⟨1050⟩). USP encourages readers to maintain and keep current best practices in the field by reviewing current peer-reviewed publications, regulatory guidances, and key opinion leaders and organizations.

As suggested in the U.S. Food and Drug Administration "Points to Consider" documents (*Points to Consider in the Manufacture and Testing of Monoclonal Antibody Products for Human Use* and *Draft Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals*), as well as ⟨1050⟩, a multi-step approach is typically used to ensure viral safety. Clearance studies can generally be considered generic or modular. As described in the FDA's *Points to Consider in the Manufacture and Testing of Monoclonal Antibody Products for Human Use*, a generic clearance study is one in which virus removal and inactivation is demonstrated for several steps in the purification process of a model monoclonal antibody (mAb) in which the data may be extrapolated to other mAbs following the same purification and virus removal/inactivation scheme as the model mAb. A modular clearance study demonstrates virus removal or inactivation of individual steps during the purification process (column chromatography, filtration, pasteurization, solvent/detergent, low pH, and others) so that each module in the purification scheme may be studied independently of the other modules. If necessary, an alternative model mAb can be used to demonstrate viral clearance in different modules. Identical clearance modules can be extrapolated to the product mAb. This chapter complements these documents by providing users strategies to perform and assess viral clearance.

## GOALS AND PRINCIPLES OF VIRAL CLEARANCE STUDIES

A viral clearance study should evaluate the ability of the overall purification process to remove or inactivate a broad spectrum of virus types, including viruses that are known to contaminate or have the potential to contaminate the raw materials, and those that can be introduced during manufacturing. Viral clearance studies typically involve substudies performed on specific and suitable individual steps of the manufacturing and purification process. The studies should be performed in a manner that generates quantitative data, allowing for identification of effective clearance steps and estimation of viral reduction factors (VRFs, also known as log reduction factors, LRF, or log reduction values, LRV). The VRFs of an individual clearance step represent the ratio of the viral load in the pretreatment material (used to challenge the clearance step) to the viral load in the post-treatment material. The VRFs derived from specific process steps are used to evaluate the overall capacity of the entire production process to remove or inactivate process-specific or nonspecific viruses.

One of the key goals of a manufacturing purification process is to achieve maximal viral clearance without compromising product quality. Critical attributes of strategic viral clearance steps in the manufacturing process must be characterized. In the context of viral clearance, robustness has two main components: 1) the ability of process steps to remove and/or inactivate viruses under worst-case conditions or over a wide operational range for parameters (e.g., temperature, protein concentration, pressure, flow rate, conductivity, and pH), and 2) the ability of process steps to consistently remove or inactivate nonspecific viruses that possess a broad spectrum of physical and chemical resistance characteristics (e.g., pH, heat, solvent/detergent treatment, or filtration). A validated process that provides robust viral clearance should establish the VRF achievable for a panel of "relevant" or "model" viruses (see next section for definitions). The validated process also should demonstrate robustness of dedicated inactivation or removal steps for which critical operational parameters that could affect viral clearance are well established. Therefore, a demonstration of viral clearance robustness provides confidence that the manufacturing process can remove or inactivate potential viral contaminants.

Viral clearance study protocols begin with an overall action plan and design of experiments. Chapter ⟨1050⟩ (see *Section V* and *Table 4*) describes five major action plans (*Cases A–E*) and can serve as a guide. Cell substrates categorized in *Cases C, D,* and *E* represent special cases, and manufacturers who use these substrates should discuss study design with the applicable regulatory authorities. Unique issues for each production process must be considered on a case-by-case basis. These issues may relate to the starting materials, production process, product, and intended use of the product. The overall action plan dictates the choice of viruses used in the viral clearance study, i.e., the study must include relevant, specific, and nonspecific model

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viruses. Further, the viral clearance study protocol may include the following elements: descriptions of the study facility and staff responsible for executing the study, the scaled-down purification models, justification of the appropriateness of the scaled-down model, and the study design. The study report may include these elements, as well as a stepwise analysis of calculated VRFs and the overall viral clearance capacity of the purification process. Viral clearance studies should be conducted in a well-documented, controlled manner that complies with current regulatory requirements.

## CONSIDERATIONS FOR PERFORMANCE OF VIRAL CLEARANCE STUDIES

### Selection of Viruses

The choice and number of viruses that may be used in a viral clearance study are dictated by the nature and origin of the production cell line, as well as the nature and origin of the animal-derived materials used in production and purification. In general, at least two viruses, one enveloped (typically a retrovirus, e.g., MuLV) and one nonenveloped (preferably a parvovirus, e.g., MVM), are used in the early clinical phases of product development, but three or more viruses may be used to generate data for registration-enabling studies. At least two orthogonal virus removal/inactivation steps (steps with different mechanisms of clearance) should be evaluated per virus. The reproducibility of an effective step should be assessed by performing at least two independent experiments, or reproducibility should be supported by the process development history (or experiences). Model viruses for process evaluation and process characterization studies should be similar to the virus that may contaminate the product; however, other viruses with a wide range of physical and chemical characteristics should also be examined. The latter is important for showing that the purification process is capable of inactivating or removing a wide variety of viruses, including newly emerging viruses (e.g., vesivirus 2117, circoviruses, or newly discovered animal and human parvoviruses) or unexpected contaminants (e.g., BVDV, MVM, epizootic hemorrhagic disease virus, and Cache valley virus). The manufacturer should justify the choice of viruses in accordance with the aims of the process evaluation and process characterization studies and the guidance provided in this document, as well as relevant regulatory guidelines.

Viruses used in clearance studies fall into three categories: relevant viruses, specific model viruses, and nonspecific model viruses. “Relevant viruses” are viruses used in process evaluation studies, which are either the identified virus, or of the same species as the virus that is known, or likely to contaminate the cell substrate or any other reagents or materials used in the production process. When a relevant virus is not available or when it is not well adapted for this purpose (e.g., it cannot be grown in vitro to a sufficiently high titer or cannot be detected using cell-based viral titration endpoints required for assessing inactivation), a “specific model virus” can be used as a substitute. An appropriate specific model virus may be a virus that is closely related to the known or suspected virus (same genus or family), having similar physical and chemical properties to those of the observed or suspected virus. For example, cell lines derived from rodents usually contain endogenous retroviral particles or retroviral-like particles that can be infectious or noninfectious. The capacity of the manufacturing process to remove and/or inactivate rodent retroviruses present in products obtained from such cells should be determined. In the case of rodent-derived cells, one can use a murine leukemia virus as a model virus. Manufacturers using human-derived B-lymphocyte cells immortalized by infection with Epstein–Barr virus should demonstrate the ability of the manufacturing process to clear a herpesvirus. In this instance, pseudorabies virus or another herpesvirus can be used as a specific model virus.

Finally, when the goal is to characterize the capacity of the manufacturing process to remove and/or inactivate viruses in general (i.e., to characterize the robustness of the clearance process), viral clearance studies can be performed with a panel of “nonspecific model viruses” that have a wide range of physical and chemical characteristics. Data obtained from studies with relevant or specific model viruses also can contribute to this assessment. It is not necessary to test all types of viruses; preference should be given to viruses with diverse physical and chemical properties that would be representative of a wide range of viral families. The results obtained for such viruses provide useful information about the ability of the production process to remove or inactivate viruses in general. *Table 1* gives examples of model viruses that represent a range of physicochemical properties for use in viral clearance studies, including those examples already described in ⟨1050⟩ (see *Table A-1*). Additional points to consider during viral selection are: 1) ability to grow the virus of interest to a sufficiently high titer, 2) ability to create stocks with minimal aggregates, 3) availability of a qualified/validated assay system for detection of the selected virus, 4) the selected viruses are not likely to pose a health hazard to personnel performing the study or the environment, and 5) potential to address new and emerging viruses.

**Table 1. Examples of Viruses Used in Viral Clearance Studies for Biotechnology Products Derived from Cell Cultures**

| Virus | Family | Genus | Natural Host | Genome | Enveloped | Size (nm) | Shape | Resistance[a] |
|---|---|---|---|---|---|---|---|---|
| Adenovirus 5 | Adeno | Mastadeno-virus | Human | DNA | No | 70–90 | Spherical | High |
| BVDV | Flavi | Pestivirus | Bovine | RNA | Yes | 50–70 | Pleomorphic/spherical | Low |
| Cache valley virus | Bunya | Bunyavirus | Bovine, ovine | RNA | Yes | 80–120 | Spherical | Low |
| Encephalomyocarditis virus (EMC) | Picorna | Cardiovirus | Mouse | RNA | No | 25–30 | Icosahedral | Medium |
| Feline calicivirus | Calici | Vesivirus | Feline | RNA | No | 35–40 | Icosahedral | High |
| Herpes simplex 1 | Herpes | Alpha-herpesvirus | Human | DNA | Yes | 120–200 | Spherical | Medium |
| MuLV | Retro | Type C oncovirus | Mouse | RNA | Yes | 80–110 | Spherical | Low |

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**Table 1. Examples of Viruses Used in Viral Clearance Studies for Biotechnology Products Derived from Cell Cultures** *(continued)*

| Virus | Family | Genus | Natural Host | Genome | Enveloped | Size (nm) | Shape | Resistance[a] |
|---|---|---|---|---|---|---|---|---|
| Parainfluenza virus | Paramyxo | Paramyxovirus | Various | RNA | Yes | 100–200 | Pleomorphic/ spherical | Low |
| Parvoviruses (MVM, PPV, CPV, BPV) | Parvo | Parvovirus | Murine, porcine, canine, bovine | DNA | No | 18–26 | Icosahedral | Very High |
| Poliovirus sabin type 1 | Picorna | Enterovirus | Human | RNA | No | 25–30 | Icosahedral | Medium |
| Porcine circovirus (PCV) | Circo | Circovirus | Porcine | DNA | No | 15–20 | Icosahedral | High |
| Pseudorabies virus | Herpes | — | Swine | DNA | Yes | 120–200 | Spherical | Medium |
| Reovirus 3 | Reo | Orthoreovirus | Various | RNA | No | 60–80 | Spherical | Medium |
| Simian virus 5 | Paramyxo | Rubulavirus | Simian | RNA | Yes | 150–300 | Pleomorphic/ spherical | Low |
| Sindbis virus | Toga | Alphavirus | Human | RNA | Yes | 60–70 | Spherical | Low |
| SV40 | Papova | Polyomavirus | Monkey | DNA | No | 40–50 | Icosahedral | Very high |
| Vesicular stomatitis virus | Rhabdo | Vesiculovirus | Equine, bovine | RNA | Yes | 70 × 150 | Bullet | Low |
| West Nile virus | Flavi | Flavivirus | Avian | RNA | Yes | 40–70 | Pleomorphic/ spherical | Medium |

[a] Resistance to physicochemical treatments based on studies of purification processes. Resistance is relative to the specific treatment, and it is used in the context of understanding the biology of the virus and the nature of the manufacturing process. Actual results will vary according to the treatment. These viruses are examples only, their use is not mandatory, and the table is not exhaustive.

## Process Clearance Capability

The viral clearance capability of a process is typically expressed as the logarithmic value of the virus reduction factor evaluated by the entire process. Although there are no specific requirements for the clearance capability that a process must achieve, the nature and origin of the cell type, raw materials used in production, as well as other factors may influence the need for greater or lower levels of viral clearance. For example, the target retroviral clearance capability for a CHO and NS0 cell manufacturing process could be 4–6 LRF of total-process retroviral clearance above the level found in the maximum therapeutic dose, based on the retroviral-like particle titer in the production bioreactor material. Minimum clearance capability for other types of viruses might include >4 LRF of clearance for additional enveloped viruses and >4 LRF of clearance for nonenveloped viruses. Specific clearance targets must be established and justified on a case-by-case basis. In general, for a single virus clearance step to be considered effective, 4 LRF or more of clearance must be demonstrated. In contrast, a clearance step demonstrating 1–3 LRF is considered a supportive step. The titer of the virus input and output for any given process step evaluated should include 95% confidence ($\alpha = 0.05$). A step can be supportive for some viruses and effective for others. When possible and as process knowledge allows, the analyst should perform viral clearance studies under expected worst-case conditions for the parameters defined as critical for that step (e.g., pH, temperature, and flow rate). Sufficient numbers of downstream purification process steps should be identified and evaluated so that the required overall clearance target can be reached.

## Downstream Processing Steps for Viral Clearance

Downstream processing steps should employ different (orthogonal) mechanisms of viral clearance because consideration of multiple steps of the same or similar clearance mechanisms may lead to overestimation of the overall viral reduction capability. In general, a purification process should include at least one robust viral inactivation step and/or one robust viral removal step. For every purification step assessed, the mechanism of viral clearance should be described as inactivation, removal, or a combination of both; if both, the primary clearance mechanism should be identified. For inactivation steps, clearance studies should be planned in such a way that they can determine the kinetics of viral inactivation. Ideally, a manufacturing process includes steps that are dedicated to viral clearance; that is, they are present in the production or purification process specifically for the removal or inactivation of viruses. Examples of dedicated steps include solvent and detergent treatment, low-pH inactivation, and virus-removal filtration.

## Qualification and Scale-Down of Purification Steps

Viral clearance studies using infectious virus spikes are not conducted at production scale in the manufacturing suite because this could contaminate the suite with viruses. Such studies should be performed in a segregated facility equipped for virological work and staffed with personnel who have virology expertise and familiarity with the operation of a scaled-down purification process. Each scaled-down step of the purification process must be qualified, i.e., found to be comparable to the full-scale production process by all relevant, measurable criteria. Comparability should be demonstrated using representative raw

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materials and intermediates from production, and equipment with process parameters through appropriate scale-down principles. The outputs should be measured with the appropriate analytical methods and statistical analyses.

In many instances, the monitored parameters are the same as those analyzed during performance of the actual manufacturing steps. For chromatography, the parameters should be representative of the respective clinical or commercial-scale manufacturing: [e.g., the column bed height, residence time, linear flow rate, chromatographic matrix, buffer composition (including pH, conductivity, and operating temperature), and product pool concentrations and composition]. Scaled-down and manufacturing-scale chromatography systems should produce similar elution profiles and step yields, and final product analytical profile (e.g., SEC-HPLC and/or SDS-PAGE analyses). Similar considerations apply for other types of procedures. Any unavoidable differences between scaled-down and manufacturing-scale procedures should be investigated to determine their potential influence on the viral clearance results.

## Selection of Sampling Points

Sampling points for assessing viral inactivation should include the starting materials (virus-spiked process solutions and appropriate controls) as well as samples taken at several time points during the inactivation process. This approach allows the analyst to monitor the kinetics of viral inactivation. Sampling points for virus-removal steps, such as chromatography and filtration, should include the feed-stream process solution that will be applied to the step, as well as the resulting process pool that is then processed in the subsequent steps. Chromatographic fractions (e.g., flow-through, washes, pre-peak, peak, post-peak, strip) of the mainstream, as well as pre- and post-mainstream samples, typically are evaluated in studies that support registration-enabling studies. Analyzing such side fractions might also be helpful in cases where the mechanism of partitioning is not understood. Sampling points are discussed further in the section *Performance of Viral Spiking Studies*.

## Selection of Assays for Viral Quantitation

Various types of viral quantitation assays can be used. Examples include infectivity assays such as tissue-culture infectious-dose assays and plaque quantitation assays. In addition, quantitative polymerase chain reaction (qPCR) assays detect and quantify the nucleic acids of both infectious and noninfectious viruses. These assays are described in *Virology Test Methods* ⟨1237⟩, *Nucleic Acid-Based Techniques—Extraction, Detection, and Sequencing* ⟨1126⟩, and *Nucleic Acid Based Techniques—Amplification* ⟨1127⟩. When using nucleic acid-based techniques (NAT), it is important to assure that the virus spike and the spiked samples to be analyzed do not contain significant amounts of free viral nucleic acids. The amount of free nucleic acids can be reduced by nuclease pretreatment of samples. It is important to characterize the impact of nuclease pretreatment on the virus spike since it could have a negative impact on the virus spike. Quantitative/quantal infectivity assays should have adequate sensitivity and reproducibility, and should be performed with a sufficient number of replicates to ensure adequate statistical validity of the results. All assays should include appropriate system suitability controls. Virus titration assays used in support of viral clearance studies must be shown to be suitable for this purpose by means of qualification or validation.

## Sample Matrix Effects on Viral Quantitation Assays

When a virus is cleared by inactivation or removal (e.g., filtration or column chromatography) methods, the analysts must perform preliminary cytotoxicity and viral interference analyses to determine if their viral quantitation assays are sensitive to matrix components present in their samples. These studies should be performed before the spiking studies to confirm that the results of the viral clearance spiking studies are actually due to viral clearance and not to other intrinsic or extrinsic factors yielding a false-negative result. For example, some process intermediates may be cytotoxic to the detector (indicator) cells or may interfere with virus detection by a particular assay system. The process solutions used in these studies should be representative of those from a full-scale good manufacturing practice (GMP) manufacturing run. The dilution of the process sample solution that does not cause cytotoxicity or interference is determined and the process solution is diluted accordingly before storage or testing samples from that manufacturing step in the virus-spiking studies.

In a cytotoxicity evaluation, the cells used in the viral quantitation assay are exposed to a series of dilutions of the sample matrix solution for that process step. Then the cells are assessed for viability and changes in morphology that could interfere with evaluation of the indicator cells for viral cytopathic effects or plaques and hence could have an impact on assay performance. The dilution of the process sample solution that does not cause cytotoxicity is determined, and the process solution is diluted accordingly before storage or testing samples from that manufacturing step in the virus-spiking studies.

The study involves the following steps:

1. Perform serial dilutions (e.g., 10-fold, 5-fold, or 2-fold) of the virus inactivation or filtration load using appropriate cell culture media as the diluent
2. Inoculate the different dilutions of virus inactivation or filtration load alone onto the indicator cells that will be used for each virus titration
3. Include appropriate buffer or media and cell controls
4. Identify the dilution of the virus inactivation load that does not exhibit measurable detector cell toxicity attributable to the load matrix

Process solutions also can interfere with virus detection by the in vitro cell-based viral infectivity assay system, either by inactivating the virus itself or by altering the indicator cells in a way that delays or prevents occurrence of a productive viral infection. To assess a process solution for viral interference, analysts prepare dilutions of the process solution and spike them with a known quantity of virus. Each dilution is subsequently titrated in the quantitative/quantal viral infectivity assay. If the difference between the known titer and the titer determined for the spiked solution in the assay (the viral interference titer) differ by more than the predetermined assay variability (typically ±0.5 log), then viral interference is suspected.

To assess the inactivation step load for viral interference, analysts should follow these steps:

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1. Perform serial dilutions (e.g., 10-fold, 5-fold, or 2-fold) of the virus stock using appropriate cell culture media as the diluent. This will serve as the positive control for the virus titer
2. Perform serial dilutions (e.g., 10-fold, 5-fold, or 2-fold) of the virus stock using the virus inactivation load as the diluent [NOTE—Information from the cytotoxicity study will determine the design of this step and the next one.]
3. Inoculate the dilution series onto the indicator cells
4. Include assay suitability controls (positive and negative) in the test
5. Identify the dilution of the virus inactivation load that does not result in significant changes in virus stock titer compared to the positive control virus titer

If qPCR assays are used to quantitate the virus, each process solution should be tested for interference and recovery with the nucleic acid extraction method used, as well as for interference with the qPCR reaction itself. Each process solution must be diluted to a level that does not cause interference. Although diluting a process solution to achieve acceptable assay performance is a commonly used strategy, this reduces the maximum demonstrable VRF for the clearance step under evaluation.

## Effects of Storing and Freezing Viral Clearance Samples

If process solutions are used within their established hold times, stability studies should be performed to better understand the effect on the clearance step of holding or storing the solutions, or on the virus (e.g., aggregation). For example, bacterial growth or precipitation of proteins in the solutions may occur, particularly after prolonged storage.

If the samples from the spiking study are stored frozen before virus quantitation, analysts should perform stability studies using the viral infectivity result as an endpoint. Ideally, this should be done before the viral spiking study by spiking dilutions of each process solution with a known amount of virus and then freezing each sample. After specified times in frozen storage, the samples are removed, and the titer of each sample dilution is determined. If the difference between the known titer and the titer determined from the spiked frozen solution is greater than the assay variability (typically ±0.5 log), this indicates that viral infectivity has been compromised by frozen storage.

## Qualification of Virus Stocks and Effects on Processing Steps

The virus stocks used in viral clearance studies are critical reagents. Each virus stock should have a traceable, certified source with full documentation of the controlled production procedures. Virus stocks should meet predetermined criteria for identity, purity (e.g., sterility, mycoplasma, and adventitious virus testing), infectious titer, stability at freezing temperatures, and minimal viral aggregates. Analysts should minimize the number of passages from the master or working viral bank in order to reduce the chance of mutation. Purity of virus preparations should be taken into account in cases when virus preparation impurities may influence the performance of a certain unit operation (e.g., virus filtration).

During qualification, analysts should obtain an initial virus titer for each virus stock. Ideally, analysts should independently assay viral stocks for titer on separate days using different passage numbers of the indicator culture cells that were used in the viral quantification assay. The resulting titers should agree within the predetermined variability of the assay (typically the expectation is ±0.5 log), and if so, the analyst can average the titers to determine the certified titer of the stock.

It may be advisable to perform a mock spiking study before the true spiking study. This mock spiking study evaluates the effects of the spiking virus matrix on the process step that is under evaluation. Mock spiking studies are important for all steps that will be tested for viral clearance, but they are particularly important for those steps that will be evaluated for the clearance of virus from a stock that contains impurities or additives (e.g., stabilizing protein). Mock spiking studies are conducted by adding the viral suspension matrix (including all components except the virus) to the process solution at a virus-spike to feed-stream-solution (load) ratio of NMT 10% (v/v) and then executing the processing step. The mock-spiked step is monitored for expected performance, and if results are different than expected, then reducing the virus:load spiking ratio may be helpful. This adjusted virus-spike to feed-stream-solution (load) ratio would be used in performing the actual clearance study for the processing step under study.

## Performance of Viral Spiking Studies

Viral clearance studies are usually conducted either at a site where both the spiking and viral quantitation assays are performed (e.g., a biosafety testing laboratory) or at two different sites: one where the spiking studies are done and a second where the samples are quantitated. Sponsors and analysts must gain knowledge and control factors that can influence each manipulation that is not part of the manufacturing process, for example, freezing and thawing (see *Effects of Storing and Freezing Viral Clearance Samples*) as well as shipping conditions.

Before initiating a clearance study, analysts should prepare a well-documented study design that clearly defines the following: the steps that will be tested, the sampling plan for each step, sample identification, sample handling and storage, sample shipment if required, critical operating parameters, process scale, rationale for worst-case conditions (if they have been established), and the appropriate controls for each step. At a minimum, the samples tested should include those used to determine: 1) the spiking virus titer, 2) the spiking virus titer after freeze–thaw (if applicable), 3) the virus titer in the production solution before processing, and 4) the virus titer after processing. The analyst may need to collect and assay additional samples, depending on the step under test and the phase of product development.

In a typical biomanufacturing process, viral clearance can be accomplished by virus inactivation (e.g., pH treatment, heat treatment, or solvent and detergent treatment) or by virus removal (e.g., filtration or column chromatography). *Figures 1A* and *1B* show examples of experimental designs for virus inactivation, *Figure 2* shows virus removal by filtration, and *Figure 3* shows virus removal by column chromatography. These examples are intended for general reference and do not represent every specific condition. In developing their study designs, sponsors should consider their own process conditions in order to plan their own specific steps.

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The overall concept of viral clearance procedures can be expressed as follows:

Viral Clearance = Viral Inactivation + Viral Removal, where

Viral Removal = Removal by Filtration + Removal by Column Chromatography

## VIRAL CLEARANCE BY VIRUS INACTIVATION

**General considerations:** Analysts should recognize that virus inactivation is not always a simple, first-order reaction. Typically, virus inactivation is complex and involves a fast initial phase followed by a slower phase. Thus, analysts should plan the inactivation process in such a way that samples are taken at different times in order to construct an inactivation time curve. Samples collected for inactivation studies should include the planned process time, and at a minimum, time zero; a time point or a suitable number of greater than zero but less than the minimum inactivating-agent exposure time; a time point equal to the minimum inactivating-agent exposure time; and in addition a time point beyond the minimum exposure time might be helpful in cases where there is a slow inactivation curve. Additional time points may be particularly important when there is no prior experience with virus inactivation kinetics.

When viral clearance by inactivation occurs, two scenarios generally are possible: 1) the inactivating agent is not present in the load material and inactivation is initiated by addition of the inactivating agent after virus spiking (*Figure 1A*); and 2) the load material already contains the inactivating agent before the virus spike, and inactivation is initiated by addition of the virus spike (*Figure 1B*). An example of the latter is a Protein A column eluate at low pH. Whenever possible, analysts should determine the initial virus load from the virus-spiked load material before they add an inactivating agent. However, this is not possible when the inactivating agent is already present in the load material at the start of the experiment. In these situations, the initial virus load can be calculated using the certified titer value of the spiking virus preparation and the virus:load spiking ratio. When viral inactivation occurs too rapidly to allow for plotting an inactivation time curve under normal process conditions, analysts should include appropriate controls to show that activity is indeed lost due to the inactivating agent present.

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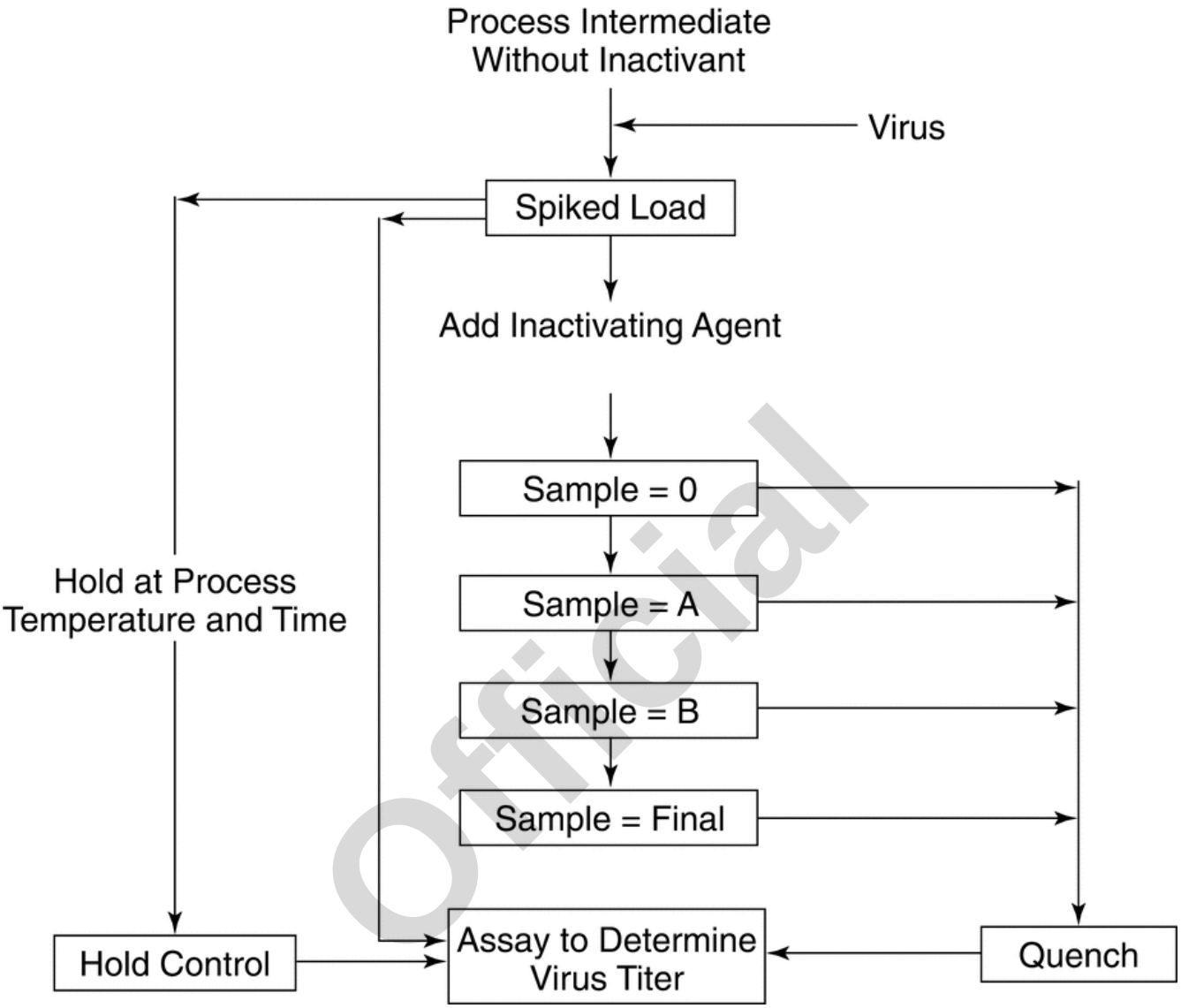


Figure 1A. Virus inactivation when load material does not contain an inactivating agent; the inactivating agent is added after viral spiking.

**Study design:** The general approach to the study design for assessing viral clearance by inactivation is outlined in *Figures 1A* and *1B*. In *Figure 1A*, the virus spike is added to the load material (process intermediate) before initiation of inactivation. The virus and load material are mixed well, and the spiked load and hold control samples are pulled. The spiked load should be tested immediately to determine virus titer. The actual titer of the spiked load (obtained experimentally) should be compared to the theoretical titer of the spiked load (obtained from the certified titer of the lot of virus used and the spiking ratio) to verify performance of virus detection assay and that the appropriate amount of virus was added to the load material. The hold control should be treated the same as the spiked load (i.e., prior to neutralization of the hold control, the sample should be pH adjusted in the same manner as the spiked load and then neutralized and spiked with virus). In addition, the hold control should remain at process temperature until the final sample in the experiment is pulled. The viral titer of the hold control then is assessed and compared to the actual titer of the spiked load to determine if any virus was inactivated in the presence of the load material over time and at the temperature of the inactivation study. This may be considered when determining the final VRF.

Inactivation should be initiated in a way that mimics the manufacturing process (e.g., with constant mixing) so that the inactivating agent (e.g., detergent, or acid utilized to lower pH) is mixed homogeneously into the solution as soon as possible. This minimizes the presence of localized high concentrations of the inactivating agent. If heat is used for inactivation, the heat should be applied in a way that mimics manufacturing (e.g., the ramp to target temperature should be identical) and controls should be kept unheated. However, the exact reproduction of addition (mixing) of inactivating agents or reproduction of a heating ramp can be very difficult at down-scale. In such cases it is advised to spike the virus directly into the inactivation agent-containing (or heated) material and to follow inactivation kinetics as described in *Figure 1B*. For control of the added virus, virus is spiked into process material without the inactivating agent or into unheated material.

Worst-case process conditions (e.g., pH, time, and temperature) should be applied if they have been established. Time points (samples) are pulled and the reaction is immediately quenched (e.g., by neutralization, dilution, or immediate cooling of the

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sample) so that further viral inactivation cannot occur. This allows the analyst to determine the kinetics of virus inactivation and then construct a virus inactivation versus time curve. Large-volume sampling can be applied to maximize clearance values.

In *Figure 1B*, the load material contains the inactivating agent and thus the addition of the virus spike starts the inactivation. In this scenario, it may not be possible to neutralize the process intermediate solution before the virus-spiking study because large fluctuations in pH may cause the protein product to fall out of solution. Also, the spiked load and hold control samples cannot be pulled from the virus-spiked load material, thus the control samples are pulled from a media or buffer control that does not contain the inactivating agent.

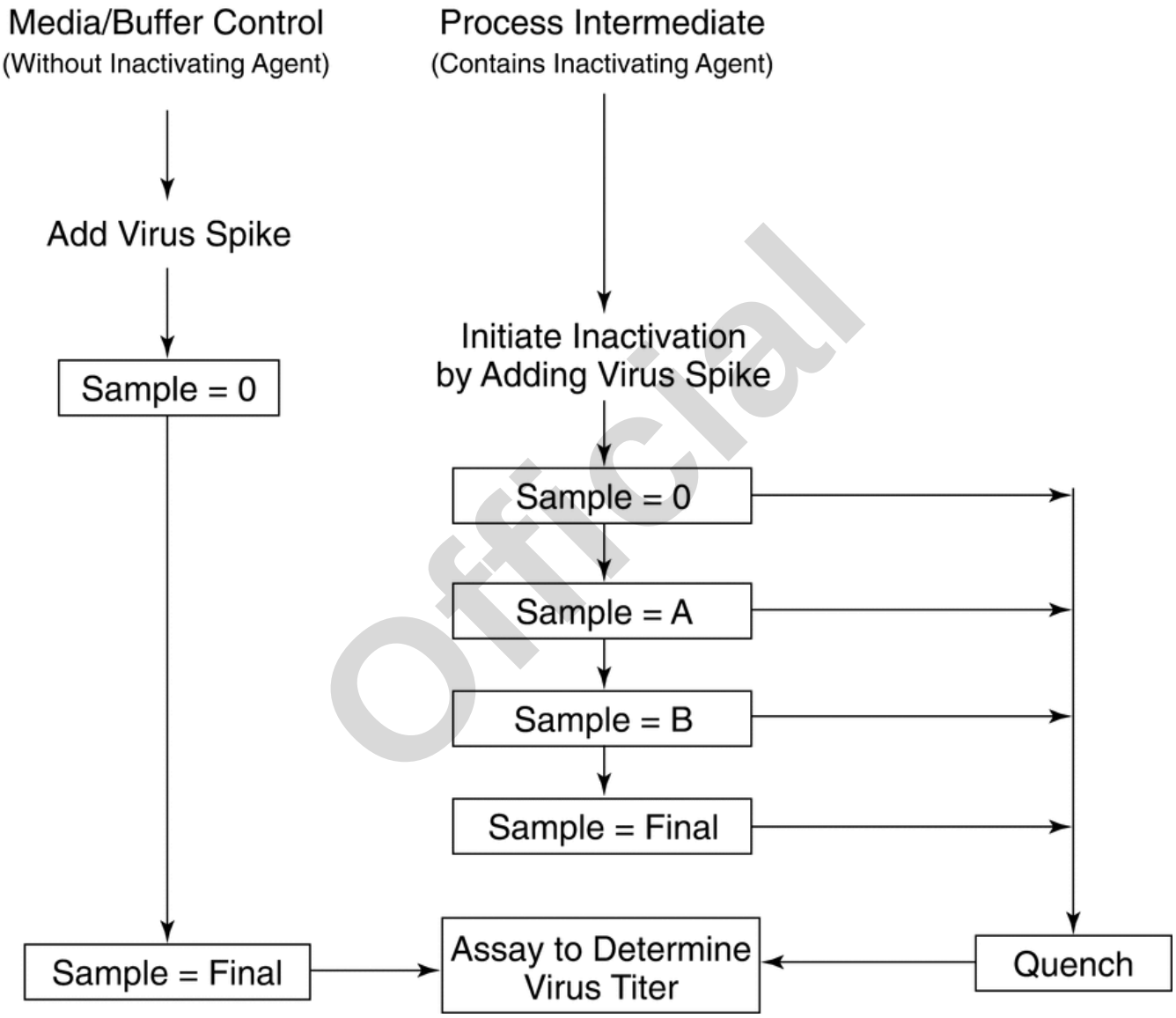


Figure 1B. Virus inactivation when the load material contains the inactivating agent before the virus spike; the addition of virus spike starts inactivation.

## VIRAL CLEARANCE BY FILTRATION

Analysts must perform preliminary cytotoxicity and viral interference analyses when preparing to perform viral clearance by filtration (see *Sample Matrix Effects on Viral Quantitation Assays*). The viral filter load and pool dilution that will be used in the clearance study is that dilution shown not to cause cytotoxicity or interference.

**Study design:** The general approach to the study design for assessing viral clearance by filtration is outlined in *Figure 2*. Once the process intermediate is spiked with virus, the spiked load sample should be assayed immediately for virus titer. Virus spiking can be associated with aggregation effects. The aggregation status of a contaminating virus in the respective product intermediate is difficult to predict and therefore, as a worst-case approach, a mono-disperse virus preparation is advised for spiking. Filtration of virus before spiking (e.g., 0.45 µm or 0.22 µm) reduces larger virus aggregates from frozen virus stocks. A pure high-titer virus preparation can be helpful to reduce virus spike-induced effects influencing performance of virus filtration. The hold control sample should be maintained at processing conditions (temperature and time) and then assayed to evaluate

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the impact of these conditions, as well as the process intermediate components, on virus titer. Worst-case process conditions should be applied, if they have been established. The virus titers obtained from the spiked load and hold control should be within the experimental variability of the virus titration assay. If there is a negative impact on virus titer because of the processing conditions (temperature, time, and process intermediate composition), then an investigation should begin to understand the process. If warranted, the final VRF may need to be adjusted to account for the impact of hold conditions on viral preparations. However, this approach might underestimate the removal capacity from virus filtration. Alternatively, use of another detection method (e.g., NAT) or another, more stable, model virus can be discussed.

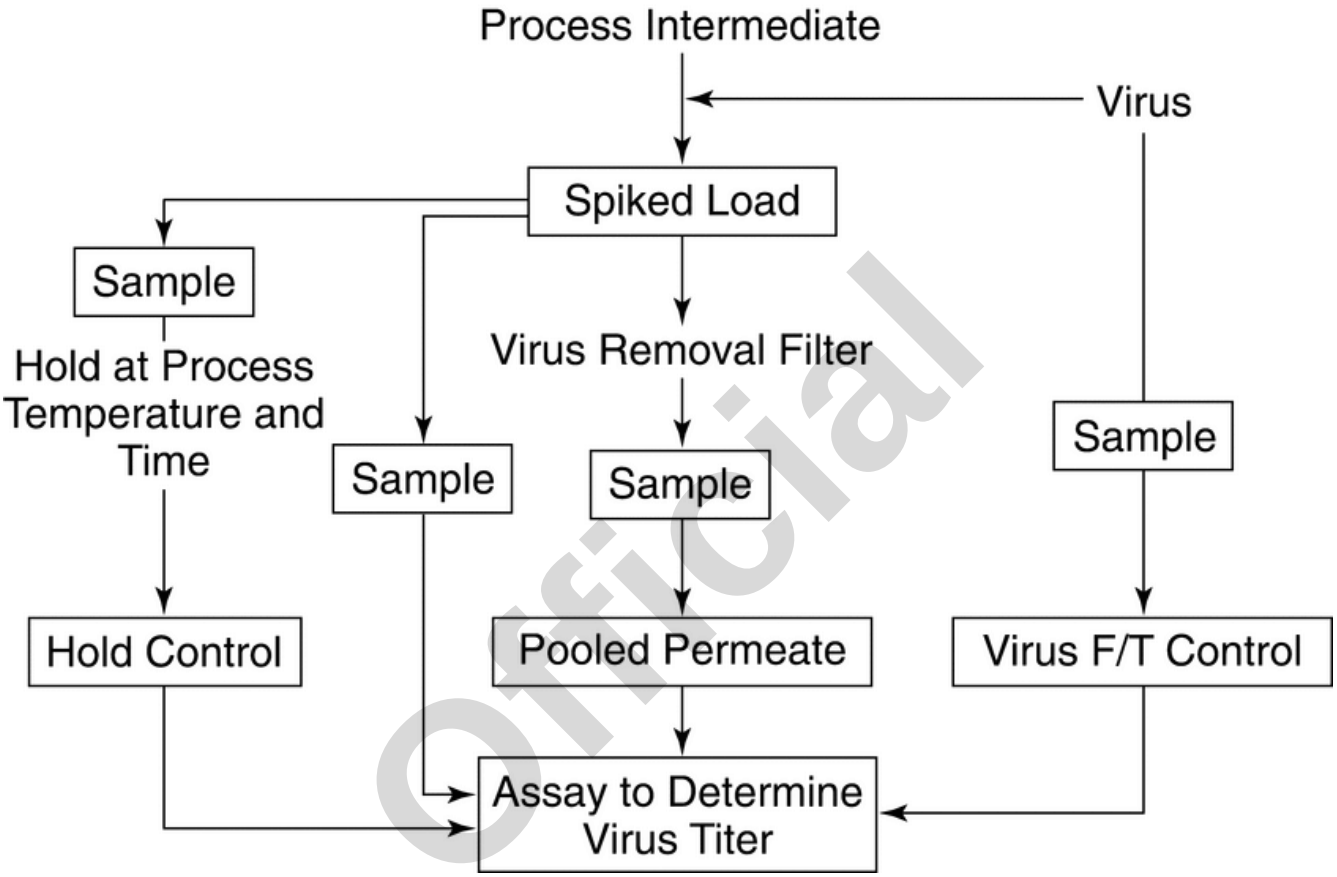


Figure 2. Clearance of virus by filtration methods.

Appropriate pre- and post-filtration controls should be used to assess removal of the virus spike by the prefiltration step. Samples are evaluated using virus titration assays. Test samples may require neutralization to appropriate pH levels for cell-culture-based titration assays. If a 0.22-µm or 0.45-µm prefilter is used during production in line with the virus-reduction filter, spiking could be performed on the intermediate before prefiltration. However, a sample should be taken from the stage after prefiltration in order to determine the VRF. Alternatively, the virus spike can be added to the intermediate after this prefiltration (using a similarly filtered virus spike) in order to determine virus reduction caused by the virus filter. To gain additional understanding of the virus clearance process, analysts can take samples at various time points throughout the run. In the event that samples are stored frozen prior to virus quantitation, a virus freeze/thaw (F/T) control is suggested to determine if there is any impact of the F/T cycle on virus titer (see also *Effects of Storing and Freezing Viral Clearance Samples*).

## VIRAL CLEARANCE BY COLUMN CHROMATOGRAPHY

**General considerations:** For registration-enabling studies, analysts should investigate the distribution of the virus load among different chromatography fractions. Generally, analysts add virus to the starting intermediate (feed stream or load) for each step tested and determine the virus titer of the spiked load material and product pool spiked load material before and after the step. Fractions, in addition to the main product pool, may require testing at the later stages of product development (phase 3 trials and beyond). As is usual for viral clearance, these studies should be performed in duplicate. With column chromatography, the ability of the columns as well as other devices to clear virus may increase or decrease after repeated use. Evidence of consistent viral clearance after multiple uses may provide support for repeated use of such columns. Typically, this evidence is obtained when analysts compare clearance on new resin versus resin that has been cycled to or slightly beyond the targeted column lifetime, i.e., the number of times the column will be used in commercial manufacturing. For registration-enabling studies, data should also be provided to show that any virus potentially retained by the resin (carry-over) will be adequately inactivated or removed by cleaning procedures before reuse of the column.

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For each manufacturing step assessed for viral clearance, the probable mechanism of reduction in viral infectivity should be known and described as virus inactivation or virus removal, or both. In some situations, it may be necessary to distinguish between removal and inactivation. For example, a column chromatography step that physically separates virus from product also may use a buffer capable of inactivating virus. In such situations, it may be possible to combine the use of viral infectivity assays with NAT assays to measure the individual contributions of the inactivation and removal mechanisms. Dissection of each step to determine the relative contribution of each mechanism of clearance allows for a thorough understanding of how viral clearance is achieved. This understanding may help to identify critical variables in each clearance step that should be controlled to support reproducible clearance. The testing of each critical variable (when it is well understood) at worst-case conditions helps to evaluate the process robustness of the step.

To measure virus clearance by a column chromatography system, analysts must perform preliminary cytotoxicity and viral interference analyses (see *Sample Matrix Effects on Viral Quantitation Assays* for details). The column load dilution that will be used in the clearance study is that dilution shown not to cause cytotoxicity or interference. [NOTE—Interference and cytotoxicity studies also can be performed on collected fractions, if warranted.]

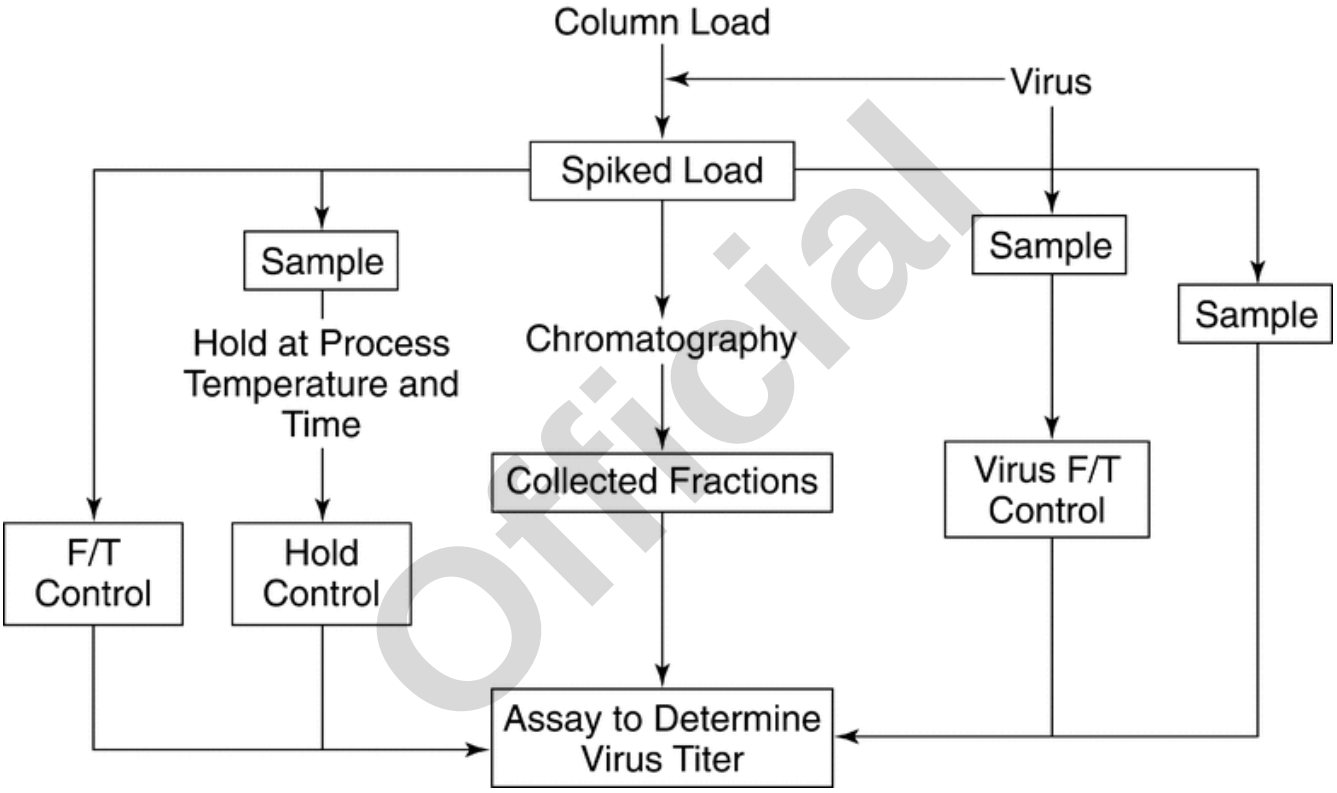


Figure 3. Clearance of virus by a column chromatography system.

*Study design:* *Figure 3* shows the general approach to study design for assessing viral clearance by chromatography. The column load is the material applied to the chromatography column. The load material in small-scale viral clearance studies should be comparable to material from clinical or commercial manufacturing in terms of composition and physicochemical attributes. The virus stock is a freshly thawed virus material of predetermined infectivity titer that is used to perform virus spiking of the column load material and also is used in the interference study. The virus stock can be sampled to determine the actual virus titer immediately after thaw. The spiked load control and additional samples should be tested immediately or immediately frozen. The titer of the frozen sample and other samples will be determined at the end of the chromatography step run. In certain cases, filtration of the spiked load with a 0.22-µm or 0.45-µm prefilter may be necessary to remove any viral aggregates formed when the process intermediate was spiked with the model virus.

The chromatography step experiment for viral clearance should closely mimic the actual manufacturing process step parameters. It should be performed under worst-case conditions (e.g., flow, protein concentration, and elution conditions) if they have been established. The product pool or multiple fractions can be collected, and the virus titer can be determined in each collected fraction (such as flow-through, washes, pre-peak, peak, post-peak, strip, and others). The titers of virus in all the fractions that comprise the product pool are used to calculate the clearance factor of the step. Interference and cytotoxicity studies also can be performed on collected fractions, if warranted. For the duration of the step, the hold control (the load plus virus) should be kept at the temperature at which the chromatography is performed. At the end of the chromatography run, the hold control should be titrated along with other collected samples/fractions (these can be titrated immediately, or if not feasible, can be frozen and tested at a later time). Samples are evaluated using virus titration assays. Test samples may require neutralization to appropriate pH levels for cell-culture-based titration assays.

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## GLOSSARY

(See also *Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin* ⟨1050⟩, *Glossary*.)[1]

**Process Robustness of Viral Clearance:** Ability of the downstream process or a single-unit operation to exhibit the characteristic performance and effectiveness in clearing viruses after minor or moderate changes of standard operating parameters or conditions.

**Process Solutions:** Production intermediates that are obtained for viral clearance studies. They should be obtained from a full-scale production facility or from a scaled-down process that has been shown to be representative of full-scale production.

**Production Process:** The manufacturing processes for biotechnology products are often varied and complex, and they are typically divided into upstream and downstream processing. Upstream activities produce the protein of interest, usually by cell culture or fermentation, and considerations include integrity and quality of the process, cell banks, expression systems, cultivation, media, process/product purity, impurities, and contaminants. Downstream processing involves separation and purification of the bulk bioproduct to make it suitable for its end use, e.g., purification, sterilization, and final formulation. Downstream activities include filtration, centrifugation, precipitation, numerous chromatographic separations, sterilization by terminal filtration, or lyophilization.

**Purification Process:** Separating and isolating the product of interest, in its desired form, from the fermentation supernatant or cell homogenate.

**Viral Clearance Effectiveness:** The efficacy of the entire purification process or an individual process step within a purification process to clear viruses, as determined by the viral reduction factor. In general, for a virus clearance step to be considered effective 4 logs or more of clearance must be demonstrated with 95% confidence ($\alpha = 0.05$).

**Viral Load:** The amount of virus added to the load material of a purification step and then subjected to an inactivation/removal treatment. The experimental determination of viral load before and after treatment enables calculation of a reduction factor that is specific to the virus and the treatment used.

**Viral Reduction Factor (VRF):** The VRF of an individual clearance step represents the $\log_{10}$ of the ratio of the virus load in the pretreatment material (used to challenge the clearance step) to the virus load in the post-treatment material. The overall VRF is calculated by adding all the individual steps with a $\log_{10}$ reduction factor >1. The $\log_{10}$ viral reduction factor is determined by the following equation:

$$Log_{10}\text{initial virus load} - log_{10}\text{final virus load} = Log_{10}\text{Viral Reduction Factor}$$

The $\log_{10}$ viral reduction value expresses levels of decreased viral contamination by factors of 10 that could be easily converted to percent reduction. For instance, a 1-log reduction is equivalent to a 90% reduction, a 2-log reduction is a 99% reduction, a 3-log reduction is a 99.9% reduction, and a 4-log reduction is a 99.99% reduction.

**Virus Removal/Inactivation Validation:** Virus removal/inactivation validation studies evaluate the capacity of the process to eliminate and/or to inactivate the viruses. Typically, this involves spiking the product with a known virus and then subjecting the product to inactivation/removal processes.

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[1] Cf. glossary in ⟨1050⟩.

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# ⟨1050⟩ VIRAL SAFETY EVALUATION OF BIOTECHNOLOGY PRODUCTS DERIVED FROM CELL LINES OF HUMAN OR ANIMAL ORIGIN

## I. INTRODUCTION

This document is concerned with testing and evaluation of the viral safety of biotechnology products derived from characterized cell lines of human or animal origin (i.e., mammalian, avian, insect), and outlines data that should be submitted in the marketing application/registration package. For the purposes of this document, the term virus excludes nonconventional transmissible agents like those associated with Bovine Spongiform Encephalopathy (BSE) and scrapie. Applicants are encouraged to discuss issues associated with BSE with the regulatory authorities.

The scope of the document covers products derived from cell cultures initiated from characterized cell banks. It covers products derived from in vitro cell culture, such as interferons, monoclonal antibodies, and recombinant deoxyribonucleic acid (DNA)-derived products including recombinant subunit vaccines, and also includes products derived from hybridoma cells grown in vivo as ascites. In this latter case, special considerations apply and additional information on testing cells propagated in vivo is contained in *Appendix 1*. Inactivated vaccines, all live vaccines containing self-replicating agents, and genetically engineered live vectors are excluded from the scope of this document.

The risk of viral contamination is a feature common to all biotechnology products derived from cell lines. Such contamination could have serious clinical consequences and can arise from the contamination of the source cell lines themselves (cell substrates) or from adventitious introduction of virus during production. To date, however, biotechnology products derived from cell lines have not been implicated in the transmission of viruses. Nevertheless, it is expected that the safety of these products with regard to viral contamination can be reasonably assured only by the application of a virus testing program and assessment of virus removal and inactivation achieved by the manufacturing process, as outlined below.

Three principal, complementary approaches have evolved to control the potential viral contamination of biotechnology products:

(1) Selecting and testing cell lines and other raw materials, including media components, for the absence of undesirable viruses which may be infectious and/or pathogenic for humans;

(2) Assessing the capacity of the production processes to clear infectious viruses;

(3) Testing the product at appropriate steps of production for absence of contaminating infectious viruses.

All testing suffers from the inherent limitation of quantitative virus assays, i.e., that the ability to detect low viral concentrations depends for statistical reasons on the size of the sample. Therefore, no single approach will necessarily establish the safety of a product. Confidence that infectious virus is absent from the final product will in many instances not be derived solely from direct testing for their presence, but also from a demonstration that the purification regimen is capable of removing and/or inactivating the viruses.

The type and extent of viral tests and viral clearance studies needed at different steps of production will depend on various factors and should be considered on a case-by-case and step-by-step basis. The factors that should be taken into account include the extent of cell bank characterization and qualification, the nature of any viruses detected, culture medium constituents, culture methods, facility and equipment design, the results of viral tests after cell culture, the ability of the process to clear viruses, and the type of product and its intended clinical use.

The purpose of this document is to describe a general framework for virus testing, experiments for the assessment of viral clearance, and a recommended approach for the design of viral tests and viral clearance studies. Related information is described in the appendices and selected definitions are provided in the glossary.

Manufacturers should adjust the recommendations presented here to their specific product and its production process. The approach used by manufacturers in their overall strategy for ensuring viral safety should be explained and justified. In addition to the detailed data that is provided, an overall summary of the viral safety assessment would be useful in facilitating the review by regulatory authorities. This summary should contain a brief description of all aspects of the viral safety studies and strategies used to prevent virus contamination as they pertain to this document.

## II. POTENTIAL SOURCES OF VIRUS CONTAMINATION

Viral contamination of biotechnology products may arise from the original source of the cell lines or from adventitious introduction of virus during production processes.

### A. Viruses That Could Occur in the Master Cell Bank (MCB)

Cells may have latent or persistent virus infection (e.g., herpesvirus) or endogenous retrovirus which may be transmitted vertically from one cell generation to the next, since the viral genome persists within the cell. Such viruses may be constitutively expressed or may unexpectedly become expressed as an infectious virus. Viruses can be introduced into the MCB by several routes such as: (1) Derivation of cell lines from infected animals; (2) use of virus to establish the cell line; (3) use of contaminated biological reagents such as animal serum components; (4) contamination during cell handling.

### B. Adventitious Viruses That Could Be Introduced During Production

Adventitious viruses can be introduced into the final product by several routes including, but not limited to, the following: (1) Use of contaminated biological reagents such as animal serum components; (2) use of a virus for the induction of expression

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of specific genes encoding a desired protein; (3) use of a contaminated reagent, such as a monoclonal antibody affinity column; (4) use of a contaminated excipient during formulation; and (5) contamination during cell and medium handling. Monitoring of cell culture parameters can be helpful in the early detection of potential adventitious viral contamination.

## III. CELL LINE QUALIFICATION: TESTING FOR VIRUSES

An important part of qualifying a cell line for use in the production of a biotechnology product is the appropriate testing for the presence of virus.

### A. Suggested Virus Tests for MCB, Working Cell Bank (WCB) and Cells at the Limit of In Vitro Cell Age Used for Production

*Table 1* shows examples of virus tests to be performed once only at various cell levels, including MCB, WCB, and cells at the limit of in vitro cell age used for production.

**Table 1. Examples of Virus Tests to be Performed Once At Various Cell Levels**

| | MCB | WCB[1] | Cells at the Limit[2] |
|---|---|---|---|
| *Tests for Retroviruses and Other Endogenous Viruses* | | | |
| Infectivity | + | – | + |
| Electron microscopy[3] | +[3] | – | +[3] |
| Reverse transcriptase[4] | +[4] | – | +[4] |
| Other virus-specific tests[5] | as appropriate[5] | – | as appropriate[5] |
| *Tests for Nonendogenous or Adventitious Viruses* | | | |
| In vitro Assays | + | ?[6] | + |
| In vivo Assays | + | –[6] | + |
| Antibody production tests[7] | +[7] | – | – |
| Other virus-specific tests[8] | +[8] | – | – |

[1] See text—section III.A.2.
[2] Cells at the limit: Cells at the limit of in vitro cell age used for production (See text—section III.A.3.).
[3] May also detect other agents.
[4] Not necessary if positive by retrovirus infectivity test.
[5] As appropriate for cell lines which are known to have been infected by such agents.
[6] For the first WCB, this test should be performed on cells at the limit of in vitro cell age, generated from that WCB; for WCB's subsequent to the first WCB, a single in vitro and in vivo test can be done either directly on the WCB or on cells at the limit of in vitro cell age.
[7] e.g., MAP, RAP, HAP—usually applicable for rodent cell lines.
[8] e.g., tests for cell lines derived from human, nonhuman primate, or other cell lines as appropriate.

#### 1. MASTER CELL BANK

Extensive screening for both endogenous and nonendogenous viral contamination should be performed on the MCB. For heterohybrid cell lines in which one or more partners are human or nonhuman primate in origin, tests should be performed in order to detect viruses of human or nonhuman primate origin because viral contamination arising from these cells may pose a particular hazard.

Testing for nonendogenous viruses should include in vitro and in vivo inoculation tests and any other specific tests, including species-specific tests such as the mouse antibody production (MAP) test, that are appropriate, based on the passage history of the cell line, to detect possible contaminating viruses.

#### 2. WORKING CELL BANK

Each WCB as a starting cell substrate for drug production should be tested for adventitious virus either by direct testing or by analysis of cells at the limit of in vitro cell age, initiated from the WCB. When appropriate nonendogenous virus tests have been performed on the MCB and cells cultured up to or beyond the limit of in vitro cell age have been derived from the WCB and used for testing for the presence of adventitious viruses, similar tests need not be performed on the initial WCB. Antibody production tests are usually not necessary for the WCB. An alternative approach in which full tests are carried out on the WCB rather than on the MCB would also be considered acceptable.

#### 3. CELLS AT THE LIMIT OF IN VITRO CELL AGE USED FOR PRODUCTION

The limit of in vitro cell age used for production should be based on data derived from production cells expanded under pilot-plant scale or commercial-scale conditions to the proposed in vitro cell age or beyond. Generally, the production cells are obtained by expansion of the WCB; the MCB could also be used to prepare the production cells. Cells at the limit of in vitro cell age should be evaluated once for those endogenous viruses that may have been undetected in the MCB and WCB. The

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performance of suitable tests (e.g., in vitro and in vivo) at least once on cells at the limit of in vitro cell age used for production would provide further assurance that the production process is not prone to contamination by adventitious virus. If any adventitious viruses are detected at this level, the process should be carefully checked in order to determine the cause of the contamination, and should be completely redesigned if necessary.

## B. Recommended Viral Detection and Identification Assays

Numerous assays can be used for the detection of endogenous and adventitious viruses. *Table 2* outlines examples for these assays. They should be regarded as assay protocols recommended for the present, but the list is not all-inclusive or definitive. Since the most appropriate techniques may change with scientific progress, proposals for alternative techniques, when accompanied by adequate supporting data, may be acceptable. Manufacturers are encouraged to discuss these alternatives with the regulatory authorities. Other tests may be necessary depending on the individual case. Assays should include appropriate controls to ensure adequate sensitivity and specificity. Wherever a relatively high possibility of the presence of a specific virus can be predicted from the species of origin of the cell substrate, specific tests and/or approaches may be necessary. If the cell line used for production is of human or nonhuman primate origin, additional tests for human viruses, such as those causing immunodeficiency diseases and hepatitis, should be performed unless otherwise justified. The polymerase chain reaction (PCR) may be appropriate for detection of sequences of other human viruses as well as for other specific viruses. The following is a brief description of a general framework and philosophical background within which the manufacturer should justify what was done.

**Table 2. Examples of the Use and Limitations of Assays Which May be Used to Test for Virus**

| Test | Test Article | Detection Capability | Detection Limitation |
|---|---|---|---|
| Antibody production | Lysate of cells and their culture medium | Specific viral antigens | Antigens not infectious for animal test system |
| In vivo virus screen | Lysate of cells and their culture medium | Broad range of viruses pathogenic for humans | Agents failing to replicate or produce diseases in the test system |
| In vitro virus screen for: | | Broad range of viruses pathogenic for humans | Agents failing to replicate or product diseases in the test system |
| 1. Cell bank characterization | 1. Lysate of cells and their culture medium (for co-cultivation, intact cells should be in the test article) | | |
| 2. Production screen | 2. Unprocessed bulk harvest or lysate of cells and their cell culture medium from the production reactor | | |
| TEM on: | | Virus and virus-like particles | Qualitative assay with assessment of identity |
| 1. Cell substrate | 1. Viable cells | | |
| 2. Cell culture supernatant | 2. Cell-free culture supernatant | | |
| Reverse transcriptase (RT) | Cell-free culture supernatant | Retroviruses and expressed retroviral RT | Only detects enzymes with optimal activity under preferred conditions. Interpretation may be difficult due to presence of cellular enzymes; background with some concentrated samples |
| Retrovirus (RV) infectivity | Cell-free culture supernatant | Infectious retroviruses | RV failing to replicate or form discrete foci or plaques in the chosen test system |
| Cocultivation | Viable cells | Infectious retroviruses | RV failing to replicate |
| 1. Infectivity endpoint | | | 1. See above under RV infectivity |
| 2. TEM endpoint | | | 2. See above under TEM[1] |
| 3. RT endpoint | | | 3. See above under RT |
| PCR (Polymerase chain reaction) | Cells, culture fluid and other materials | Specific virus sequences | Primer sequences must be present. Does not indicate whether virus is infectious. |

[1] In addition, difficult to distinguish test article from indicator cells.

### 1. TESTS FOR RETROVIRUSES

For the MCB and for cells cultured up to or beyond the limit of in vitro cell age used for production, tests for retroviruses, including infectivity assays in sensitive cell cultures and electron microscopy (EM) studies, should be carried out. If infectivity is not detected and no retrovirus or retrovirus-like particles have been observed by EM, reverse transcriptase (RT) or other appropriate assays should be performed to detect retroviruses that may be noninfectious. Induction studies have not been found to be useful.

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### 2. IN VITRO ASSAYS

In vitro tests are carried out by the inoculation of a test article (see *Table 2*) into various susceptible indicator cell cultures capable of detecting a wide range of human and relevant animal viruses. The choice of cells used in the test is governed by the species of origin of the cell bank to be tested, but should include a human and/or a nonhuman primate cell line susceptible to human viruses. The nature of the assay and the sample to be tested are governed by the type of virus which may possibly be present based on the origin or handling of the cells. Both cytopathic and hemadsorbing viruses should be sought.

### 3. IN VIVO ASSAYS

A test article (see *Table 2*) should be inoculated into animals, including suckling and adult mice, and in embryonated eggs to reveal viruses that cannot grow in cell cultures. Additional animal species may be used, depending on the nature and source of the cell lines being tested. The health of the animals should be monitored and any abnormality should be investigated to establish the cause of the illness.

### 4. ANTIBODY PRODUCTION TESTS

Species-specific viruses present in rodent cell lines may be detected by inoculating test article (see *Table 2*) into virus-free animals and examining the serum antibody level or enzyme activity after a specified period. Examples of such tests are the mouse antibody production (MAP) test, rat antibody production (RAP) test, and hamster antibody production (HAP) test. The viruses currently screened for in the antibody production assays are discussed in *Table 3*.

**Table 3. Virus Detected in Antibody Production Tests**

| MAP | HAP | RAP |
|---|---|---|
| Ectromelia Virus[2, 3] | Lymphocytic Choriomeningitis Virus (LCM)[1, 3] | Hantaan Virus[1, 3] |
| Hantaan Virus[1, 3] | Pneumonia Virus of Mice (PVM)[2, 3] | Kilham Rat Virus (KRV)[2, 3] |
| K Virus[2] | Reovirus Type 3 (Reo3)[1, 3] | Mouse Encephalomyelitis Virus (Theilers, GDVII)[2] |
| Lactic Dehydrogenase Virus (LDM)[1, 3] | Sendai Virus[1, 3] | Pneumonia Virus of Mice (PVM)[2, 3] |
| Lymphocytic Choriomeningitis Virus (LCM)[1, 3] | SV5 | Rat Coronavirus (RCV)[2] |
| Minute Virus of Mice[2, 3] | | Reovirus Type 3 (Reo3)[1, 3] |
| Mouse Adenovirus (MAV)[2, 3] | | Sendai Virus[1, 3] |
| Mouse Cytomegalovirus (MCMV)[2, 3] | | Sialoacryoadenitis Virus (SDAV)[2] |
| Mouse Encephalomyelitis Virus (Theilers, GDVII)[2] | | Toolan Virus (HI)[2, 3] |
| Mouse Hepatitis Virus (MHV)[2] | | |
| Mouse Rotavirus (EDIM)[2, 3] | | |
| Pneumonia Virus of Mice (PVM)[2, 3] | | |
| Polyoma Virus[2] | | |
| Reovirus Type 3 (Reo3)[1, 3] | | |
| Sendai Virus[1, 3] | | |
| Thymic Virus[2] | | |

[1] Viruses for which there is evidence of capacity for infecting humans or primates.
[2] Viruses for which there is no evidence of capacity for infecting humans.
[3] Virus capable of replicating in vitro in cells of human or primate origin.

## C. Acceptability of Cell Lines

It is recognized that some cell lines used for the manufacture of product will contain endogenous retroviruses, other viruses, or viral sequences. In such circumstances, the action plan recommended for manufacture is described in section V. of this document. The acceptability of cell lines containing viruses other than endogenous retroviruses will be considered on an individual basis by the regulatory authorities, by taking into account a risk/benefit analysis based on the benefit of the product and its intended clinical use, the nature of the contaminating viruses, their potential for infecting humans or for causing disease in humans, the purification process for the product (e.g., viral clearance evaluation data), and the extent of the virus tests conducted on the purified bulk.

# IV. TESTING FOR VIRUSES IN UNPROCESSED BULK

The unprocessed bulk constitutes one or multiple pooled harvests of cells and culture media. When cells are not readily accessible (e.g., hollow fiber or similar systems), the unprocessed bulk would constitute fluids harvested from the fermenter. A representative sample of the unprocessed bulk, removed from the production reactor prior to further processing, represents

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one of the most suitable levels at which the possibility of adventitious virus contamination can be determined with a high probability of detection. Appropriate testing for viruses should be performed at the unprocessed bulk level unless virus testing is made more sensitive by initial partial processing (e.g., unprocessed bulk may be toxic in test cell cultures, whereas partially processed bulk may not be toxic).

In certain instances, it may be more appropriate to test a mixture consisting of both intact and disrupted cells and their cell culture supernatants removed from the production reactor prior to further processing. Data from at least three lots of unprocessed bulk at pilot-plant scale or commercial scale should be submitted as part of the marketing application/registration package.

It is recommended that manufacturers develop programs for the ongoing assessment of adventitious viruses in production batches. The scope, extent, and frequency of virus testing on the unprocessed bulk should be determined by taking several points into consideration, including the nature of the cell lines used to produce the desired products, the results and extent of virus tests performed during the qualification of the cell lines, the cultivation method, raw material sources, and results of viral clearance studies. In vitro screening tests, using one or several cell lines, are generally employed to test unprocessed bulk. If appropriate, a PCR test or other suitable methods may be used.

Generally, harvest material in which adventitious virus has been detected should not be used to manufacture the product. If any adventitious viruses are detected at this level, the process should be carefully checked to determine the cause of the contamination, and appropriate actions taken.

## V. RATIONALE AND ACTION PLAN FOR VIRAL CLEARANCE STUDIES AND VIRUS TESTS ON PURIFIED BULK

It is important to design the most relevant and rational protocol for virus tests from the MCB level, through the various steps of drug production, to the final product including evaluation and characterization of viral clearance from unprocessed bulk. The evaluation and characterization of viral clearance plays a critical role in this scheme. The goal should be to obtain the best reasonable assurance that the product is free of virus contamination.

In selecting viruses to use for a clearance study, it is useful to distinguish between the need to evaluate processes for their ability to clear viruses that are known to be present and the desire to estimate the robustness of the process by characterizing the clearance of nonspecific "model" viruses (described later). Definitions of "relevant," specific, and nonspecific "model" viruses are given in the glossary. Process evaluation requires knowledge of how much virus may be present in the process, such as the unprocessed bulk, and how much can be cleared in order to assess product safety. Knowledge of the time dependence for inactivation procedures is helpful in assuring the effectiveness of the inactivation process. When evaluating clearance of known contaminants, in-depth, time-dependent inactivation studies, demonstration of reproducibility of inactivation/removal, and evaluation of process parameters should be provided. When a manufacturing process is characterized for robustness of clearance using nonspecific "model" viruses, particular attention should be paid to nonenveloped viruses in the study design. The extent of viral clearance characterization studies may be influenced by the results of tests on cell lines and unprocessed bulk. These studies should be performed as described in section VI. below.

*Table 4* presents an example of an action plan in terms of process evaluation and characterization of viral clearance as well as virus tests on purified bulk, in response to the results of virus tests on cells and/or the unprocessed bulk. Various cases are considered. In all cases, characterization of clearance using nonspecific "model" viruses should be performed. The most common situations are Cases A and B. Production systems contaminated with a virus other than a rodent retrovirus are normally not used. Where there are convincing and well justified reasons for drug production using a cell line from Cases C, D, or E, these should be discussed with the regulatory authorities. With Cases C, D, and E, it is important to have validated effective steps to inactivate/remove the virus in question from the manufacturing process.

**Table 4. Action Plan for Process Assessment of Viral Clearance and Virus Tests on Purified Bulk**

| | Case A | Case B | Case C[2] | Case D[2] | Case E[2] |
|---|---|---|---|---|---|
| *Status* | | | | | |
| Presence of virus[1] | – | – | – | – | (+)[3] |
| Virus-like particles[1] | – | – | – | – | (+)[3] |
| Retrovirus-like particles[1] | – | + | – | – | (+)[3] |
| Virus identified | not applicable | + | + | + | – |
| Virus pathogenic for humans | not applicable | –[4] | –[4] | + | unknown |
| *Action* | | | | | |
| Process characterization of viral clearance using non-specific "model" viruses | yes[5] | yes[5] | yes[5] | yes[5] | yes[7] |
| Process evaluation of viral clearance using "relevant" or specific "model" viruses | no | yes[6] | yes[6] | yes[6] | yes[7] |
| Test for virus in purified bulk | not applicable | yes[8] | yes[8] | yes[8] | yes[8] |

[1] Results of virus tests for the cell substrate and/or at the unprocessed bulk level. Cell cultures used for production which are contaminated with viruses will generally not be acceptable. Endogenous viruses (such as retroviruses) or viruses that are an integral part of the MCB may be acceptable if appropriate viral clearance evaluation procedures are followed.

[2] The use of source material which is contaminated with viruses, whether or not they are known to be infectious and/or pathogenic in humans, will only be acceptable under very exceptional circumstances.

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[3] Virus has been observed by either direct or indirect methods.
[4] Believed to be nonpathogenic.
[5] Characterization of clearance using nonspecific "model" viruses should be performed.
[6] Process evaluation for "relevant" viruses or specific "model" viruses should be performed.
[7] See text under Case E.
[8] The absence of detectable virus should be confirmed for purified bulk by means of suitable methods having high specificity and sensitivity for the detection of the virus in question. For the purpose of marketing authorization, data from at least 3 lots of purified bulk manufactured at pilot-plant or commercial scale should be provided. However for cell lines such as CHO cells for which the endogenous particles have been extensively characterized and adequate clearance has been demonstrated, it is not usually necessary to assay for the presence of the noninfectious particles in purified bulk.

### Case A

Where no virus, virus-like particle, or retrovirus-like particle has been demonstrated in the cells or in the unprocessed bulk, virus removal and inactivation studies should be performed with nonspecific "model" viruses as previously stated.

### Case B

Where only a rodent retrovirus (or a retrovirus-like particle that is believed to be nonpathogenic, such as rodent A- and R-type particles) is present, process evaluation using a specific "model" virus, such as a murine leukemia virus, should be performed. Purified bulk should be tested using suitable methods having high specificity and sensitivity for the detection of the virus in question. For marketing authorization, data from at least three lots of purified bulk at pilot-plant scale or commercial scale should be provided. Cell lines such as Chinese hamster ovary (CHO), C127, baby hamster kidney (BHK), and murine hybridoma cell lines have frequently been used as substrates for drug production with no reported safety problems related to viral contamination of the products. For these cell lines in which the endogenous particles have been extensively characterized and clearance has been demonstrated, it is not usually necessary to assay for the presence of the noninfectious particles in purified bulk. Studies with nonspecific "model" viruses, as in Case A, are appropriate.

### Case C

When the cells or unprocessed bulk are known to contain a virus, other than a rodent retrovirus, for which there is no evidence of capacity for infecting humans (such as those identified by footnote 2 in *Table 3*, except rodent retroviruses (Case B)), virus removal and inactivation evaluation studies should use the identified virus. If it is not possible to use the identified virus, "relevant" or specific "model" viruses should be used to demonstrate acceptable clearance. Time-dependent inactivation for identified (or "relevant" or specific "model") viruses at the critical inactivation step(s) should be obtained as part of process evaluation for these viruses. Purified bulk should be tested using suitable methods having high specificity and sensitivity for the detection of the virus in question. For the purpose of marketing authorization, data from at least three lots of purified bulk manufactured at pilot-plant scale or commercial scale should be provided.

### Case D

Where a known human pathogen, such as those indicated by footnote 1 in *Table 3*, is identified, the product may be acceptable only under exceptional circumstances. In this instance, it is recommended that the identified virus be used for virus removal and inactivation evaluation studies and specific methods with high specificity and sensitivity for the detection of the virus in question be employed. If it is not possible to use the identified virus, "relevant" and/or specific "model" viruses (described later) should be used. The process should be shown to achieve the removal and inactivation of the selected viruses during the purification and inactivation processes. Time-dependent inactivation data for the critical inactivation step(s) should be obtained as part of process evaluation. Purified bulk should be tested using suitable methods having high specificity and sensitivity for the detection of the virus in question. For the purpose of marketing authorization, data from at least three lots of purified bulk manufactured at pilot-plant scale or commercial scale should be provided.

### Case E

When a virus that cannot be classified by currently available methodologies is detected in the cells or unprocessed bulk, the product is usually considered unacceptable since the virus may prove to be pathogenic. In the very rare case where there are convincing and well justified reasons for drug production using such a cell line, this should be discussed with the regulatory authorities before proceeding further.

## VI. EVALUATION AND CHARACTERIZATION OF VIRAL CLEARANCE PROCEDURES

Evaluation and characterization of due virus removal and/or inactivation procedures play an important role in establishing the safety of biotechnology products. Many instances of contamination in the past have occurred with agents whose presence was not known or even suspected, and though this happened to biological products derived from various source materials other than fully characterized cell lines, assessment of viral clearance will provide a measure of confidence that any unknown, unsuspected, and harmful viruses may be removed. Studies should be carried out in a manner that is well documented and controlled.

The objective of viral clearance studies is to assess process step(s) that can be considered to be effective in inactivating/removing viruses and to estimate quantitatively the overall level of virus reduction obtained by the process. This should be achieved by the deliberate addition ("spiking") of significant amounts of a virus to the crude material and/or to different fractions

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obtained during the various process steps and demonstrating its removal or inactivation during the subsequent steps. It is not considered necessary to evaluate or characterize every step of a manufacturing process if adequate clearance is demonstrated by the use of fewer steps. It should be borne in mind that other steps in the process may have an indirect effect on the viral inactivation/removal achieved. Manufacturers should explain and justify the approach used in studies for evaluating virus clearance.

The reduction of virus infectivity may be achieved by removal of virus particles or by inactivation of viral infectivity. For each production step assessed, the possible mechanism of loss of viral infectivity should be described with regard to whether it is due to inactivation or removal. For inactivation steps, the study should be planned in such a way that samples are taken at different times and an inactivation curve constructed (see section VI.B.5.).

Viral clearance evaluation studies are performed to demonstrate the clearance of a virus known to be present in the MCB and/or to provide some level of assurance that adventitious viruses which could not be detected, or might gain access to the production process, would be cleared. Reduction factors are normally expressed on a logarithmic scale, which implies that, while residual virus infectivity will never be reduced to zero, it may be greatly reduced mathematically.

In addition to clearance studies for viruses known to be present, studies to characterize the ability to remove and/or inactivate other viruses should be conducted. The purpose of studies with viruses exhibiting a range of biochemical and biophysical properties that are not known or expected to be present is to characterize the robustness of the procedure rather than to achieve a specific inactivation or removal goal. A demonstration of the capacity of the production process to inactivate or remove viruses is desirable (see section VI.C.). Such studies are not performed to evaluate a specific safety risk. Therefore, a specific clearance value need not be achieved.

## A. The Choice of Viruses for the Evaluation and Characterization of Viral Clearance

Viruses for clearance evaluation and process characterization studies should be chosen to resemble viruses which may contaminate the product and to represent a wide range of physico-chemical properties in order to test the ability of the system to eliminate viruses in general. The manufacturer should justify the choice of viruses in accordance with the aims of the evaluation and characterization study and the guidance provided in this document.

### 1. "RELEVANT" VIRUSES AND "MODEL" VIRUSES

A major issue in performing a viral clearance study is to determine which viruses should be used. Such viruses fall into three categories: "Relevant" viruses, specific "model" viruses, and nonspecific "model" viruses.

"Relevant" viruses are viruses used in process evaluation of viral clearance studies which are either the identified viruses, or of the same species as the viruses that are known, or likely to contaminate the cell substrate or any other reagents or materials used in the production process. The purification and/or inactivation process should demonstrate the capability to remove and/or inactivate such viruses. When a "relevant" virus is not available or when it is not well adapted to process evaluation of viral clearance studies (e.g., it cannot be grown in vitro to sufficiently high titers), a specific "model" virus should be used as a substitute. An appropriate specific "model" virus may be a virus which is closely related to the known or suspected virus (same genus or family), having similar physical and chemical properties to the observed or suspected virus.

Cell lines derived from rodents usually contain endogenous retrovirus particles or retrovirus-like particles, which may be infectious (C-type particles) or noninfectious (cytoplasmic A- and R-type particles). The capacity of the manufacturing process to remove and/or inactivate rodent retroviruses from products obtained from such cells should be determined. This may be accomplished by using a murine leukemia virus, a specific "model" virus in the case of cells of murine origin. When human cell lines secreting monoclonal antibodies have been obtained by the immortalization of B lymphocytes by Epstein-Barr Virus (EBV), the ability of the manufacturing process to remove and/or inactivate a herpes virus should be determined. Pseudorabies virus may also be used as a specific "model" virus.

When the purpose is to characterize the capacity of the manufacturing process to remove and/or inactivate viruses in general, i.e., to characterize the robustness of the clearance process, viral clearance characterization studies should be performed with nonspecific "model" viruses with differing properties. Data obtained from studies with "relevant" and/or specific "model" viruses may also contribute to this assessment. It is not necessary to test all types of viruses. Preference should be given to viruses that display a significant resistance to physical and/or chemical treatments. The results obtained for such viruses provide useful information about the ability of the production process to remove and/or inactivate viruses in general. The choice and number of viruses used will be influenced by the quality and characterization of the cell lines and the production process.

Examples of useful "model" viruses representing a range of physico-chemical structures and examples of viruses which have been used in viral clearance studies are given in *Appendix 2* and *Table A-1*.

### 2. OTHER CONSIDERATIONS

Additional points to be considered are as follows:

(a) Viruses which can be grown to high titer are desirable, although this may not always be possible.

(b) There should be an efficient and reliable assay for the detection of each virus used, for every stage of manufacturing that is tested.

(c) Consideration should be given to the health hazard which certain viruses may pose to the personnel performing the clearance studies.

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## B. Design and Implications of Viral Clearance Evaluation and Characterization Studies

### 1. FACILITY AND STAFF

It is inappropriate to introduce any virus into a production facility because of good manufacturing practice (GMP) constraints. Therefore, viral clearance studies should be conducted in a separate laboratory equipped for virological work and performed by staff with virological expertise in conjunction with production personnel involved in designing and preparing a scaled-down version of the purification process.

### 2. SCALED-DOWN PRODUCTION SYSTEM

The validity of the scaling down should be demonstrated. The level of purification of the scaled-down version should represent as closely as possible the production procedure. For chromatographic equipment, column bed-height, linear flow-rate, flow-rate-to-bed-volume ratio (i.e., contact time), buffer and gel types, pH, temperature, and concentration of protein, salt, and product should all be shown to be representative of commercial-scale manufacturing. A similar elution profile should result. For other procedures, similar considerations apply. Deviations that cannot be avoided should be discussed with regard to their influence on the results.

### 3. ANALYSIS OF STEP-WISE ELIMINATION OF VIRUS

When viral clearance studies are being performed, it is desirable to assess the contribution of more than one production step to virus elimination. Steps which are likely to clear virus should be individually assessed for their ability to remove and inactivate virus and careful consideration should be given to the exact definition of an individual step. Sufficient virus should be present in the material of each step to be tested so that an adequate assessment of the effectiveness of each step is obtained. Generally, virus should be added to in-process material of each step to be tested. In some cases, simply adding high titer virus to unpurified bulk and testing its concentration between steps will be sufficient. Where virus removal results from separation procedures, it is recommended that, if appropriate and if possible, the distribution of the virus load in the different fractions be investigated. When virucidal buffers are used in multiple steps within the manufacturing process, alternative strategies such as parallel spiking in less virucidal buffers may be carried out as part of the overall process assessment. The virus titer before and after each step being tested should be determined. Quantitative infectivity assays should have adequate sensitivity and reproducibility and should be performed with sufficient replicates to ensure adequate statistical validity of the result. Quantitative assays not associated with infectivity may be used if justified. Appropriate virus controls should be included in all infectivity assays to ensure the sensitivity of the method. Also, the statistics of sampling virus when at low concentrations should be considered (*Appendix 4*).

### 4. DETERMINING PHYSICAL REMOVAL VERSUS INACTIVATION

Reduction in virus infectivity may be achieved by the removal or inactivation of virus. For each production step assessed, the possible mechanism of loss of viral infectivity should be described with regard to whether it is due to inactivation or removal. If little clearance of infectivity is achieved by the production process, and the clearance of virus is considered to be a major factor in the safety of the product, specific or additional inactivation/removal steps should be introduced. It may be necessary to distinguish between removal and inactivation for a particular step, for example, when there is a possibility that a buffer used in more than one clearance step may contribute to inactivation during each step, i.e., the contribution to inactivation by a buffer shared by several chromatographic steps and the removal achieved by each of these chromatographic steps should be distinguished.

### 5. INACTIVATION ASSESSMENT

For assessment of viral inactivation, unprocessed crude material or intermediate material should be spiked with infectious virus and the reduction factor calculated. It should be recognized that virus inactivation is not a simple, first order reaction and is usually more complex, with a fast “phase 1” and a slow “phase 2.” The study should, therefore, be planned in such a way that samples are taken at different times and an inactivation curve constructed. It is recommended that studies for inactivation include at least one time point less than the minimum exposure time and greater than zero, in addition to the minimum exposure time. Additional data are particularly important where the virus is a “relevant” virus known to be a human pathogen and an effective inactivation process is being designed. However, for inactivation studies in which nonspecific “model” viruses are used or when specific “model” viruses are used as surrogates for virus particles, such as the CHO intracytoplasmic retrovirus-like particles, reproducible clearance should be demonstrated in at least two independent studies. Whenever possible, the initial virus load should be determined from the virus that can be detected in the spiked starting material. If this is not possible, the initial virus load may be calculated from the titer of the spiking virus preparation. Where inactivation is too rapid to plot an inactivation curve using process conditions, appropriate controls should be performed to demonstrate that infectivity is indeed lost by inactivation.

### 6. FUNCTION AND REGENERATION OF COLUMNS

Over time and after repeated use, the ability of chromatography columns and other devices used in the purification scheme to clear virus may vary. Some estimate of the stability of the viral clearance after several uses may provide support for repeated use of such columns. Assurance should be provided that any virus potentially retained by the production system would be adequately destroyed or removed prior to reuse of the system. For example, such evidence may be provided by demonstrating that the cleaning and regeneration procedures do inactivate or remove virus.

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7. SPECIFIC PRECAUTIONS

(a) Care should be taken in preparing the high-titer virus to avoid aggregation which may enhance physical removal and decrease inactivation, thus distorting the correlation with actual production.

(b) Consideration should be given to the minimum quantity of virus which can be reliably assayed.

(c) The study should include parallel control assays to assess the loss of infectivity of the virus due to such reasons as the dilution, concentration, filtration or storage of samples before titration.

(d) The virus "spike" should be added to the product in a small volume so as not to dilute or change the characteristics of the product. Diluted, test-protein sample is no longer identical to the product obtained at commercial scale.

(e) Small differences in, for example, buffers, media, or reagents can substantially affect viral clearance.

(f) Virus inactivation is time-dependent; therefore, the amount of time a spiked product remains in a particular buffer solution or on a particular chromatography column should reflect the conditions of the commercial-scale process.

(g) Buffers and product should be evaluated independently for toxicity or interference in assays used to determine the virus titer, as these components may adversely affect the indicator cells. If the solutions are toxic to the indicator cells, dilution, adjustment of the pH, or dialysis of the buffer containing spiked virus might be necessary. If the product itself has anti-viral activity, the clearance study may need to be performed without the product in a "mock" run, although omitting the product or substituting a similar protein that does not have anti-viral activity could affect the behavior of the virus in some production steps. Sufficient controls to demonstrate the effect of procedures used solely to prepare the sample for assay (e.g., dialysis, storage) on the removal/inactivation of the spiking virus should be included.

(h) Many purification schemes use the same or similar buffers or columns repetitively. The effects of this approach should be taken into account when analyzing the data. The effectiveness of virus elimination by a particular process may vary with the manufacturing stage at which it is used.

(i) Overall reduction factors may be underestimated where production conditions or buffers are too cytotoxic or virucidal and should be discussed on a case-by-case basis. Overall reduction factors may also be overestimated due to inherent limitations or inadequate design of viral clearance studies.

## C. Interpretation of Viral Clearance Studies; Acceptability

The object of assessing virus inactivation/removal is to evaluate and characterize process steps that can be considered to be effective in inactivating/removing viruses and to estimate quantitatively the overall level of virus reduction obtained by the manufacturing process. For virus contaminants, as in Cases B through E, it is important to show that not only is the virus eliminated or inactivated, but that there is excess capacity for viral clearance built into the purification process to assure an appropriate level of safety for the final product. The amount of virus eliminated or inactivated by the production process should be compared to the amount of virus which may be present in unprocessed bulk.

To carry out this comparison, it is important to estimate the amount of virus in the unprocessed bulk. This estimate should be obtained using assays for infectivity or other methods such as transmission electron microscopy (TEM). The entire purification process should be able to eliminate substantially more virus than is estimated to be present in a single-dose-equivalent of unprocessed bulk. See *Appendix 5* for calculation of virus reduction factors and *Appendix 6* for calculation of estimated particles per dose.

Manufacturers should recognize that clearance mechanisms may differ between virus classes. A combination of factors should be considered when judging the data supporting the effectiveness of virus inactivation/removal procedures. These include:

(i) The appropriateness of the test viruses used;
(ii) The design of the clearance studies;
(iii) The log reduction achieved;
(iv) The time dependence of inactivation;
(v) The potential effects of variation in process parameters on virus inactivation/removal;
(vi) The limits of assay sensitivities;
(vii) The possible selectivity of inactivation/removal procedure(s) for certain classes of viruses.

Effective clearance may be achieved by any of the following: Multiple inactivation steps, multiple complementary separation steps, or combinations of inactivation and separation steps. Since separation methods may be dependent on the extremely specific physico-chemical properties of a virus which influence its interaction with gel matrices and precipitation properties, "model" viruses may be separated in a different manner than a target virus. Manufacturing parameters influencing separation should be properly defined and controlled. Differences may originate from changes in surface properties such as glycosylation. However, despite these potential variables, effective removal can be obtained by a combination of complementary separation steps or combinations of inactivation and separation steps. Therefore, well-designed separation steps, such as chromatographic procedures, filtration steps, and extractions, can be effective virus removal steps provided that they are performed under appropriately controlled conditions. An effective virus removal step should give reproducible reduction of virus load shown by at least two independent studies.

An overall reduction factor is generally expressed as the sum of the individual factors. However, reduction in virus titer of the order of 1 $\log_{10}$ or less would be considered negligible and would be ignored unless justified.

If little reduction of infectivity is achieved by the production process, and the removal of virus is considered to be a major factor in the safety of the product, a specific, additional inactivation/removal step or steps should be introduced. For all viruses, manufacturers should justify the acceptability of the reduction factors obtained. Results would be evaluated on the basis of the factors listed above.

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## D. Limitations of Viral Clearance Studies

Viral clearance studies are useful for contributing to the assurance that an acceptable level of safety in the final product is achieved but do not by themselves establish safety. However, a number of factors in the design and execution of viral clearance studies may lead to an incorrect estimate of the ability of the process to remove virus infectivity. These factors include the following:

1. Virus preparations used in clearance studies for a production process are likely to be produced in tissue culture. The behavior of a tissue culture virus in a production step may be different from that of the native virus, for example, if native and cultured viruses differ in purity or degree of aggregation.

2. Inactivation of virus infectivity frequently follows a biphasic curve in which a rapid initial phase is followed by a slower phase. It is possible that virus escaping a first inactivation step may be more resistant to subsequent steps. For example, if the resistant fraction takes the form of virus aggregates, infectivity may be resistant to a range of different chemical treatments and to heating.

3. The ability of the overall process to remove infectivity is expressed as the sum of the logarithm of the reductions at each step. The summation of the reduction factors of multiple steps, particularly of steps with little reduction (e.g., below 1 $\log_{10}$), may overestimate the true potential for virus elimination. Furthermore, reduction values achieved by repetition of identical or near identical procedures should not be included unless justified.

4. The expression of reduction factors as logarithmic reductions in titer implies that, while residual virus infectivity may be greatly reduced, it will never be reduced to zero. For example, a reduction in the infectivity of a preparation containing 8 $\log_{10}$ infectious units per milliliter (mL) by a factor of 8 $\log_{10}$ leaves zero $\log_{10}$ per mL or one infectious unit per mL, taking into consideration the limit of detection of the assay.

5. Pilot-plant scale processing may differ from commercial-scale processing despite care taken to design the scaled-down process.

6. Addition of individual virus reduction factors resulting from similar inactivation mechanisms along the manufacturing process may overestimate overall viral clearance.

## E. Statistics

The viral clearance studies should include the use of statistical analysis of the data to evaluate the results. The study results should be statistically valid to support the conclusions reached (see *Appendix 3*).

## F. Reevaluation of Viral Clearance

Whenever significant changes in the production or purification process are made, the effect of that change, both direct and indirect, on viral clearance should be considered and the system re-evaluated as needed. For example, changes in production processes may cause significant changes in the amount of virus produced by the cell line; changes in process steps may change the extent of viral clearance.

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# VII. SUMMARY

This document suggests approaches for the evaluation of the risk of viral contamination and for the removal of virus from product, thus contributing to the production of safe biotechnology products derived from animal or human cell lines, and emphasizes the value of many strategies, including:

A. Thorough characterization/screening of cell substrate starting material in order to identify which, if any, viral contaminants are present;

B. Assessment of risk by determination of the human tropism of the contaminants;

C. Establishment of an appropriate program of testing for adventitious viruses in unprocessed bulk;

D. Careful design of viral clearance studies using different methods of virus inactivation or removal in the same production process in order to achieve maximum viral clearance; and

E. Performance of studies which assess virus inactivation and removal.

# GLOSSARY

**Adventitious Virus:** See *Virus*.

**Cell Substrate:** Cells used to manufacture product.

**Endogenous Virus:** See *Virus*.

**Inactivation:** Reduction of virus infectivity caused by chemical or physical modification.

**In Vitro Cell Age:** A measure of the period between thawing of the MCB vial(s) and harvest of the production vessel measured by elapsed chronological time in culture, population doubling level of the cells, or passage level of the cells when subcultivated by a defined procedure for dilution of the culture.

**Master Cell Bank (MCB):** An aliquot of a single pool of cells which generally has been prepared from the selected cell clone under defined conditions, dispensed into multiple containers, and stored under defined conditions. The MCB is used to derive all working cell banks. The testing performed on a new MCB (from a previous initial cell clone, MCB, or WCB) should be the same as for the original MCB, unless justified.

**Minimum Exposure Time:** The shortest period for which a treatment step will be maintained.

**Nonendogenous Virus:** See *Virus*.

**Process Characterization of Viral Clearance:** Viral clearance studies in which nonspecific “model” viruses are used to assess the robustness of the manufacturing process to remove and/or inactivate viruses.

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**Process Evaluation Studies of Viral Clearance:** Viral clearance studies in which "relevant" and/or specific "model" viruses are used to determine the ability of the manufacturing process to remove and/or inactivate these viruses.

**Production Cells:** Cell substrate used to manufacture product.

**Unprocessed Bulk:** One or multiple pooled harvests of cells and culture media. When cells are not readily accessible, the unprocessed bulk would constitute fluid harvested from the fermenter.

**Virus:** Intracellularly replicating infectious agents that are potentially pathogenic, possess only a single type of nucleic acid (either ribonucleic acid (RNA) or DNA), are unable to grow and undergo binary fission, and multiply in the form of their genetic material.

**Adventitious Virus:** Unintentionally introduced contaminant virus.

**Endogenous Virus:** Viral entity whose genome is part of the germ line of the species of origin of the cell line and is covalently integrated into the genome of animal from which the parental cell line was derived. For the purposes of this document, intentionally introduced, nonintegrated viruses such as EBV used to immortalize cell substrates or Bovine Papilloma Virus fit in this category.

**Nonendogenous Virus:** Virus from external sources present in the MCB.

**Nonspecific Model Virus:** A virus used for characterization of viral clearance of the process when the purpose is to characterize the capacity of the manufacturing process to remove and/or inactivate viruses in general, i.e., to characterize the robustness of the purification process.

**Relevant Virus:** Virus used in process evaluation studies which is either the identified virus, or of the same species as the virus that is known, or likely to contaminate the cell substrate or any other reagents or materials used in the production process.

**Specific Model Virus:** Virus which is closely related to the known or suspected virus (same genus or family), having similar physical and chemical properties to those of the observed or suspected virus.

**Viral Clearance:** Elimination of target virus by removal of viral particles or inactivation of viral infectivity.

**Virus-like Particles:** Structures visible by electron microscopy which morphologically appear to be related to known viruses.

**Virus Removal:** Physical separation of virus particles from the intended product.

**Working Cell Bank (WCB):** The WCB is prepared from aliquots of a homogeneous suspension of cells obtained from culturing the MCB under defined culture conditions.

# APPENDICES

## Appendix 1: Products Derived from Characterized Cell Banks Which Were Subsequently Grown In Vivo

For products manufactured from fluids harvested from animals inoculated with cells from characterized banks, additional information regarding the animals should be provided.

Whenever possible, animals used in the manufacture of biotechnological/biological products should be obtained from well defined, specific pathogen-free colonies. Adequate testing for appropriate viruses, such as those listed in *Table 3*, should be performed. Quarantine procedures for newly arrived as well as diseased animals should be described, and assurance provided that all containment, cleaning, and decontamination methodologies employed within the facility are adequate to contain the spread of adventitious agents. This may be accomplished through the use of a sentinel program. A listing of agents for which testing is performed should also be included. Veterinary support services should be available on-site or within easy access. The degree to which the vivarium is segregated from other areas of the manufacturing facility should be described. Personnel practices should be adequate to ensure safety.

Procedures for the maintenance of the animals should be fully described. These would include diet, cleaning and feeding schedules, provisions for periodic veterinary care if applicable, and details of special handling that the animals may require once inoculated. A description of the priming regimen(s) for the animals, the preparation of the inoculum, and the site and route of inoculation should also be included.

The primary harvest material from animals may be considered an equivalent stage of manufacture to unprocessed bulk harvest from a bioreactor. Therefore, all testing considerations previously outlined in section IV. of this document should apply. In addition, the manufacturer should assess the bioburden of the unprocessed bulk, determine whether the material is free of mycoplasma, and perform species-specific assay(s) as well as in vivo testing in adult and suckling mice.

## Appendix 2: The Choice of Viruses for Viral Clearance Studies

### A. EXAMPLES OF USEFUL "*Model*" *Viruses:*

1. Nonspecific "model" viruses representing a range of physico-chemical structures:

- SV40 (Polyomavirus maccacae 1), human polio virus 1 (Sabin), animal parvovirus or some other small, nonenveloped viruses;
- a parainfluenza virus or influenza virus, Sindbis virus or some other medium-to-large, enveloped, RNA viruses;
- a herpes virus (e.g., HSV-1 or a pseudorabies virus), or some other medium-to-large, DNA viruses.

These viruses are examples only and their use is not mandatory.

2. For rodent cell substrates murine retroviruses are commonly used as specific "model" viruses.

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### B. EXAMPLES OF VIRUSES THAT HAVE BEEN USED IN VIRAL CLEARANCE STUDIES

Several viruses that have been used in viral clearance studies are listed in *Table A-1*. However, since these are merely examples, the use of any of the viruses in the table is not considered mandatory and manufacturers are invited to consider other viruses, especially those that may be more appropriate for their individual production processes. Generally, the process should be assessed for its ability to clear at least three different viruses with differing characteristics.

**Table A-1. Examples of Viruses Which Have Been Used in Viral Clearance Studies**

| Virus | Family | Genus | Natural Host | Genome | Env | Size (nm) | Shape | Resistance[1] |
|---|---|---|---|---|---|---|---|---|
| Vesicular Stomatitis Virus | Rhabdo | Vesiculovirus | Equine Bovine | RNA | yes | 70 × 150 | Bullet | Low |
| Parainfluenza Virus | Paramyxo | Paramyxovirus | Various | RNA | yes | 100–200 | Pleo/Spher | Low |
| MuL V | Retro | Type C oncovirus | Mouse | RNA | yes | 80–110 | Spherical | Low |
| Sindbis Virus | Toga | Alphavirus | Human | RNA | yes | 60–70 | Spherical | Low |
| BVDV | Flavi | Pestivirus | Bovine | RNA | yes | 50–70 | Pleo/Spher | Low |
| Pseudo-rabies Virus | Herpes | | Swine | DNA | yes | 120–200 | Spherical | Med |
| Poliovirus Sabin Type 1 | Picorna | Enterovirus | Human | RNA | no | 25–30 | Icosahedral | Med |
| Encephalomyocarditis Virus (EMC) | Picorna | Cardiovirus | Mouse | RNA | no | 25–30 | Icosahedral | Med |
| Reovirus 3 | Reo | Orthoreovirus | Various | RNA | no | 60–80 | Spherical | Med |
| SV 40 | Papova | Polyomavirus | Monkey | DNA | no | 40–50 | Icosahedral | Very high |
| Parvoviruses (canine, porcine) | Parvo | Parvovirus | Canine Porcine | DNA | no | 18–24 | Icosahedral | Very high |

[1] Resistance to physico-chemical treatments based on studies of production processes. Resistance is relative to the specific treatment and it is used in the context of the understanding of the biology of the virus and the nature of the manufacturing process. Actual results will vary according to the treatment. These viruses are examples only and their use is not considered mandatory.

## Appendix 3: Statistical Considerations for Assessing Virus Assays

Virus titrations suffer the problems of variation common to all biological assay systems. Assessment of the accuracy of the virus titrations and reduction factors derived from them and the validity of the assays should be performed to define the reliability of a study. The objective of statistical evaluation is to establish that the study has been carried out to an acceptable level of virological competence.

1. Assay methods may be either quantal or quantitative. Quantal methods include infectivity assays in animals or in tissue-culture-infectious-dose (TCID) assays, in which the animal or cell culture is scored as either infected or not. Infectivity titers are then measured by the proportion of animals or culture infected. In quantitative methods, the infectivity measured varies continuously with the virus input. Quantitative methods include plaque assays where each plaque counted corresponds to a single infectious unit. Both quantal and quantitative assays are amenable to statistical evaluation.

2. Variation can arise within an assay as a result of dilution errors, statistical effects, and differences within the assay system which are either unknown or difficult to control. These effects are likely to be greater when different assay runs are compared (between-assay variation) than when results within a single assay run are compared (within-assay variation).

3. The 95 percent confidence limits for results of within-assay variation normally should be on the order of $\pm 0.5 \log_{10}$ of the mean. Within-assay variation can be assessed by standard textbook methods. Between-assay variation can be monitored by the inclusion of a reference preparation, the estimate of whose potency should be within approximately $0.5 \log_{10}$ of the mean estimate established in the laboratory for the assay to be acceptable. Assays with lower precision may be acceptable with appropriate justification.

4. The 95 percent confidence limits for the reduction factor observed should be calculated wherever possible in studies of clearance of "relevant" and specific "model" viruses. If the 95 percent confidence limits for the viral assays of the starting material are +s, and for the viral assays of the material after the step are +a, the 95 percent confidence limits for the reduction factor are

$$\pm\sqrt{S^2 + a^2}$$

## Appendix 4: Probability of Detection of Viruses at Low Concentrations

At low virus concentrations (e.g., in the range of 10 to 1,000 infectious particles per L) it is evident that a sample of a few milliliters may or may not contain infectious particles. The probability, *p*, that this sample does not contain infectious viruses is:

$$p = ((V\text{-}v)/V)^n$$

where V (L) is the overall volume of the material to be tested; v (L) is the volume of the sample; and n is the absolute number of infectious particles statistically distributed in V.

If V >> v, this equation can be approximated by the Poisson distribution:

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$$p = e^{-cv}$$

where c is the concentration of infectious particles per L.

$$\text{or, } c = \ln p/-v$$

As an example, if a sample volume of 1 mL is tested, the probabilities p at virus concentrations ranging from 10 to 1,000 infectious particles per L are:

| | | | |
|---|---|---|---|
| c | 10 | 10 | 1,000 |
| p | 0.99 | 0.90 | 0.37 |

This indicates that for a concentration of 1,000 viruses per L, in 37 percent of sampling, 1 mL will not contain a virus particle.

If only a portion of a sample is tested for virus and the test is negative, the amount of virus which would have to be present in the total sample in order to achieve a positive result should be calculated and this value taken into account when calculating a reduction factor. Confidence limits at 95 percent are desirable. However, in some instances, this may not be practical due to material limitations.

## Appendix 5: Calculation of Reduction Factors in Studies to Determine Viral Clearance

The virus reduction factor of an individual purification or inactivation step is defined as the $\log_{10}$ of the ratio of the virus load in the pre-purification material and the virus load in the post-purification material which is ready for use in the next step of the process. If the following abbreviations are used:

Starting material: vol v′; titer $10^{a'}$;
virus load: $(v')(10^{a})$,
Final material: vol v″; titer $10^{a''}$;
virus load: $(v'')(10^{a''})$,
the individual reduction factors Ri are calculated according to
$10^{Ri} = (v')(10^{a'}) / (v'')(10^{a''})$

This formula takes into account both the titers and volumes of the materials before and after the purification step.

Because of the inherent imprecision of some virus titrations, an individual reduction factor used for the calculation of an overall reduction factor should be greater than 1.

The overall reduction factor for a complete production process is the sum logarithm of the reduction factors of the individual steps. It represents the logarithm of the ratio of the virus load at the beginning of the first process clearance step and at the end of the last process clearance step. Reduction factors are normally expressed on a logarithmic scale which implies that, while residual virus infectivity will never be reduced to zero, it may be greatly reduced mathematically.

## Appendix 6: Calculation of Estimated Particles per Dose

This is applicable to those viruses for which an estimate of starting numbers can be made, such as endogenous retroviruses.
Example:
I. Assumptions
Measured or estimated concentration of virus in cell culture harvest = $10^6$/mL
Calculated viral clearance factor = > $10^{15}$
Volume of culture harvest needed to make a dose of product = 1 L ($10^3$ mL)
II. Calculation of Estimated Particles/Dose

$$\frac{\left(10^{6}\text{virus units/mL}\right)\times\left(10^{3}\text{mL/dose}\right)}{\text{Clearance factor} > 10^{15}} = \frac{10^{9}\text{particles/dose}}{\text{Clearance factor} > 10^{15}} = < 10^{-6}\text{particles/dose}$$

Therefore, less than one particle per million doses would be expected.

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# ⟨1051⟩ CLEANING GLASS APPARATUS

## INTRODUCTION

Success in conducting Pharmacopeial assays and tests depends on the cleanliness of the glassware apparatus used. For this reason, commercial detergents or inorganic reagents for cleaning should be used when necessary.

In all cases, it is important to verify that the cleaning procedure is appropriate for the particular test or assay used. This can be accomplished in a number of ways, including use of experimental controls or verification of cleaning by utilization of residue/residual testing to ensure removal of any potential contaminants. The cleaning protocol should include a statement describing how the success of the cleaning procedure will be assessed.

Examples of particular tests where clean glassware is critical for success include but are not limited to: pyrogen and total organic carbon tests and assays of heparin sodium and vitamin $B_{12}$ activity. For optical measurements, special care is required for cleaning containers; however, the use of chromic acid or highly alkaline solutions should be avoided.

Additional references on cleaning glass apparatus are listed in the *Appendix*. USP does not endorse these citations, and they do not represent an exhaustive list. Further information about the effectiveness of the glassware apparatus cleaning procedures mentioned in this chapter may also be found in most quantitative chemical analytical textbooks.

***Change to read:***

## CLEANING VALIDATION BEST PRACTICES

The validation of a cleaning process may involve both a qualitative and quantitative assessment of cleaning performance and may be followed by specific assessments of cleanliness appropriate for the intended use. An established cleaning procedure may require revalidation when significant changes to the procedure are made or when the intended use of the glassware changes. The following outline illustrates this strategy:

General Cleaning Validation Procedure for General Laboratory Use

1. Qualitative assessment of cleaning process
    A. Take a soiled worst-case load according to intended use or prepare a load by applying formulation with fluorescent indicator (see *Table 1*)
    B. Inspect glassware with a UV lamp or black light to verify suitable coverage with fluorescent indicator
    C. Perform cleaning with process to be validated
    D. Inspect glassware with a UV lamp or black light to qualitatively evaluate cleanliness
2. Quantitative assessment of cleaning process
    A. Perform rinse of glassware with *Purified Water*
    B. Collect rinse water
    C. Test per *Total Organic Carbon* ⟨643⟩ and *Water Conductivity* ⟨645⟩
3. Perform additional (chemical) validation evaluations as appropriate for the intended use (see *Table 2*)

### Qualitative Assessment of Cleaning Process

A fluorescent dye/indicator is useful for qualitatively determining if a cleaning process or procedure is able to apply cleaning solution where required and remove the solution completely. Two fluorescent indicators are commonly used for this type of qualitative assessment of a cleaning process: riboflavin and fluorescein. The fluorescent indicator solution in *Table 1* may be prepared with little or no viscosity-increasing agent (e.g., hydroxyethyl cellulose [HEC]) if the solution will completely fill the equipment to be cleaned. A viscosity-increasing agent (e.g., 5 g/L of HEC) is recommended if the solution will be sprayed into or onto the equipment. The concentration of the fluorescent indicator may be increased if more sensitivity is required. The presence or absence of the fluorescent indicator can be assessed using a black light or UV lamp with excitation (output) of about 365 nm (UVA range).

**Table 1. Recommended Recipe for Fluorescent Indicator Solutions Used in Cleaning Validation Experiments**

| Ingredient | CAS | Function | Concentration (g/L) |
|---|---|---|---|
| *Riboflavin* or *Disodium Fluorescein* | [83-88-5] [518-47-8] | Fluorescent indicator | 0.2 |
| *Hydroxyethyl Cellulose*[a] | [9004-62-0] | Viscosity-increasing agent | 0–5 |
| *Purified Water* | [7732-18-5] | Solvent | *Quantum satis* |

[a] Natrosol 250 HHR or equivalent.

### Quantitative Assessment of Cleaning Process

For quantitative assessment of cleaning, the recommended approach is to follow the cleaning process by rinsing the glassware (or other laboratory equipment) with water of appropriate quality (e.g. *Purified Water*) and collecting the rinse water. The

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properties of this rinse water are evaluated to assess whether any contaminants are present at sufficient levels to significantly affect the properties of the rinse water and the involved analytical use of glassware. The amount of rinse water utilized should be commensurate to the quantities described in the methods in ⟨643⟩ and ⟨645⟩, which have been found to be good for assessing the cleanliness of the rinsed glassware. The results from these general chapter tests should show that the properties of the rinse water are not significantly changed after rinsing the glassware or remain compliant with the requested cleanliness level for the glassware. Determination of necessary cleanliness level should be risk-based and consider the intended use and the analytical test(s) involved.

### ADDITIONAL CLEANING VALIDATION ANALYSES

Depending on the intended use of the glassware (or other laboratory equipment), it may be advisable to perform additional experiments to verify suitable cleanliness after the proposed cleaning process. For example, glassware used to contain samples for gas chromatography (GC) analysis should also be shown to be free from residual volatiles that may interfere with the GC method. This may be assessed by sealing the top of the glassware and performing a headspace GC analysis to confirm that there are no residual volatiles that will interfere with planned analyses. See *Table 2* for additional validation experiments that are recommended for intended uses.

**Table 2. Additional Evaluation Recommended for Specific Use in Sensitive Analyses**

| Intended Use | Recommended Additional Cleaning Validation Experiment |
|---|---|
| General laboratory use | Default evaluation according to 1 and 2 of the outline in *Cleaning Validation Best Practices* |
| GC samples | Headspace GC analysis of residual volatile compounds |
| Liquid chromatography samples | Rinse with organic solvent (e.g., as the mobile phase) and assess for unknown peaks with similar retention times. |
| Elemental impurities samples | Evaluate Class 1 metals according to *Elemental Impurities—Procedures* ⟨233⟩ and verify levels are below ▲limit of detection.▲ (ERR 1-Sep-2021) |
| Particulate analysis samples | Rinse with particle-free water and assess for extrinsic particles in size range of interest. |
| Surface tension/surface energy analysis | Evaluate surface tension of rinse water. |
| Protein binding/activity | Evaluate rinse water per *Protein Determination Procedures* ⟨507⟩. |

### VALIDATION OF AUTOMATED GLASSWARE CLEANING

In laboratories that use automatic washers to clean glassware, it is important to conduct a validation procedure to demonstrate the effectiveness of the cleaning cycles by conducting a suitable validation procedure. One way this can be done is by first selecting a suitable molecule that is likely to be difficult to clean from soiled glassware. A suitable molecule is most relevant to the risks involved for each laboratory. It is recommended that laboratories choose a molecule that represents a worst-case scenario of the materials the glassware may be exposed to (e.g., poorly soluble molecules in common solvents). Next, items of glassware from a selection of different shapes and sizes (e.g., beakers, conical flasks, volumetric flasks) are spiked with known quantities of this analyte (i.e., analyte is dissolved into a good solvent and applied uniformly) and then allowed to dry. The spiked glassware is then distributed to selected positions in the glassware washer, which is then fully loaded with unspiked glassware so that all locations in the washer are populated. The wash cycle is then run, and all spiked items are extracted separately with a suitable solvent. Finally, the sample preparations are analyzed quantitatively to determine the residual contaminants in each item, where application of the quantitative method is based on the chosen soil test case.

## FAILURES OF CLEANING VALIDATION

If the cleaning procedures are not suitable to clean glassware to meet the desired validation criteria, it will be necessary to consider alternative cleaning procedures, additional rinsing procedures, or evaluate the use of disposable equipment.

### Alternative Cleaning Procedures

Typical cleaning solutions contain surfactants in either an acidic or alkaline buffer solution. The selection of either an acidic or alkaline cleanser is typically based on whether the compound to be removed has better solubility at low or high pH or is easily degraded at low or high pH. However, if the glassware becomes unduly clouded or dirty, or contains coagulated organic matter, it may be cleansed with chromic acid cleaning solution. The dichromate should be handled with extreme care because it is a powerful corrosive and carcinogen.

When these more aggressive cleaning solutions are used, the item may be rinsed with the cleaning solution or it may be filled and allowed to stand. The length of time it is allowed to stand depends on the amount of contamination on the glassware. Relatively clean glassware may require only a few minutes of exposure; however, if debris is present, such as blood clots, it may be necessary to allow the glassware to stand overnight. Due to the intense corrosive action of the chromic acid solution, it is good practice to place the stock bottle and the glassware being treated in flat glass pans made from lead or coated with lead or in plastic polymer pans compatible with the concentration of chromic acid used. These more aggressive cleaning solutions should be disposed of properly.

Special types of precipitates may require removal with nitric acid, aqua regia, or fuming sulfuric acid. These are very corrosive substances and should be used only when required. Grease is best removed by boiling in a weak solution of sodium carbonate.

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Acetone or any other lipophilic solvent may be used. Strong alkalis should not be used. Silicone grease is most easily removed by soaking the stopcock plug or barrel for 2 h in warm decahydronaphthalene.

## Alternative Rinsing Procedures

Generally, it is best to rinse glassware with purified or distilled water, and allow it to dry with or without blowing air into it to force it to dry. In some applications, it is important to avoid any nonvolatile residues on the glassware after cleaning (e.g., surface energy measurements). In these cases, it is recommended to use a procedure of alternatively rinsing with distilled water and a suitable volatile organic solvent (e.g., acetone). It is best to perform this final rinsing procedure just prior to use of the clean glassware. For example, a recommended rinse procedure would be as follows: water, acetone, water, acetone, water, acetone. This provides three rinses with each solvent and finishes with the last rinse being the volatile organic solvent, which will evaporate relatively quickly and leave the glassware clean, dry, and ready for immediate use.

## Sterilization of Laboratory Equipment and Glassware

When sterile or depyrogenated laboratory equipment is required, it is best to first clean and rinse the glassware thoroughly and then sterilize. Dry heat sterilization (e.g., >160° for 2 h) is generally recommended as it will provide both sterilization and depyrogenation. Autoclaving (e.g., >121° for 30 min) is recommended if the laboratory equipment is incompatible with the temperature of dry heat sterilization. Chemical sterilants (e.g., peracetic acid and hydrogen peroxide solutions) should be avoided, if possible, because they require extensive rinsing procedures to avoid residual oxidants that may lead to undesirable chemical reactions in subsequent use.

## Use of Disposable Laboratory Equipment

It is recommended that cost and environmental impact are assessed for the decision of whether to clean and reuse laboratory glassware or to use disposable laboratory plasticware. The environmental impact of cleaning and reusing laboratory glassware includes the cost of the production of the cleaning chemicals as well as the cost of proper disposal of any hazardous solvents and chemicals used or produced in the cleaning procedure. The environmental impact of the disposable plasticware includes the environmental impact of the production and disposal of the plasticware; however, it will reduce the production and disposal of any hazardous chemicals required for the cleaning process. In some cases, the use of disposable equipment may result in less of an environmental impact.

# APPENDIX

## Additional Sources of Information

Additional information and guidance can be found in the references listed below or in many quantitative chemical analytical textbooks:


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# ⟨1052⟩ BIOTECHNOLOGY-DERIVED ARTICLES—AMINO ACID ANALYSIS

This chapter provides guidance and procedures used for characterization of biotechnology-derived articles by amino acid analysis. This chapter is harmonized with the corresponding chapter in *JP* and *EP*. Portions of the chapter that are not harmonized with the other two pharmacopeias are marked by the symbol ♦. The footnote below is in the *USP* but is not in the *EP* or *JP*. Other characterization tests, also harmonized, are shown in *Capillary Electrophoresis* ⟨1053⟩, *Biotechnology-Derived Articles—Isoelectric Focusing* ⟨1054⟩, *Biotechnology-Derived Articles—Peptide Mapping* ⟨1055⟩, *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* ⟨1056⟩, and *Biotechnology-Derived Articles—Total Protein Assay* ⟨1057⟩.

## INTRODUCTION

Amino acid analysis refers to the methodology used to determine the amino acid composition or content of proteins, peptides, and other pharmaceutical preparations. Proteins and peptides are macromolecules consisting of covalently bonded amino acid residues organized as a linear polymer. The sequence of the amino acids in a protein or peptide determines the properties of the molecule. Proteins are considered large molecules that commonly exist as folded structures with a specific conformation, while peptides are smaller and may consist of only a few amino acids. Amino acid analysis can be used to quantify protein and peptides, to determine the identity of proteins or peptides based on their amino acid composition, to support protein and peptide structure analysis, to evaluate fragmentation strategies for peptide mapping, and to detect atypical amino acids that might be present in a protein or peptide. It is necessary to hydrolyze a protein/peptide to its individual amino acid constituents before amino acid analysis. Following protein/peptide hydrolysis, the amino acid analysis procedure can be the same as that practiced for free amino acids in other pharmaceutical preparations. The amino acid constituents of the test sample are typically derivatized for analysis.

## APPARATUS

Methods used for amino acid analysis are usually based on a chromatographic separation of the amino acids present in the test sample. Current techniques take advantage of the automated chromatographic instrumentation designed for analytical methodologies. An amino acid analysis instrument will typically be a low-pressure or high-pressure liquid chromatograph capable of generating mobile phase gradients that separate the amino acid analytes on a chromatographic column. The instrument must have postcolumn derivatization capability, unless the sample is analyzed using precolumn derivatization. The detector is usually a UV-visible or fluorescence detector depending on the derivatization method used. A recording device (e.g., integrator) is used for transforming the analog signal from the detector and for quantitation. It is preferred that instrumentation be dedicated particularly for amino acid analysis.

## GENERAL PRECAUTIONS

Background contamination is always a concern for the analyst in performing amino acid analysis. High-purity reagents are necessary (e.g., low-purity hydrochloric acid can contribute to glycine contamination). Analytical reagents are changed routinely every few weeks using only high-pressure liquid chromatography (HPLC) grade solvents. Potential microbial contamination and foreign material that might be present in the solvents are reduced by filtering solvents before use, keeping solvent reservoirs covered, and not placing amino acid analysis instrumentation in direct sunlight.

Laboratory practices can determine the quality of the amino acid analysis. Place the instrumentation in a low traffic area of the laboratory. Keep the laboratory clean. Clean and calibrate pipets according to a maintenance schedule. Keep pipet tips in a covered box; the analysts may not handle pipet tips with their hands. The analysts may wear powder-free latex or equivalent gloves. Limit the number of times a test sample vial is opened and closed because dust can contribute to elevated levels of glycine, serine, and alanine.

A well-maintained instrument is necessary for acceptable amino acid analysis results. If the instrument is used on a routine basis, it is to be checked daily for leaks, detector and lamp stability, and the ability of the column to maintain resolution of the individual amino acids. Clean or replace all instrument filters and other maintenance items on a routine schedule.

## REFERENCE STANDARD MATERIAL

Acceptable amino acid standards are commercially available* for amino acid analysis and typically consist of an aqueous mixture of amino acids. When determining amino acid composition, protein or peptide standards are analyzed with the test material as a control to demonstrate the integrity of the entire procedure. Highly purified bovine serum albumin has been used as a protein standard for this purpose.

## CALIBRATION OF INSTRUMENTATION

Calibration of amino acid analysis instrumentation typically involves analyzing the amino acid standard, which consists of a mixture of amino acids at a number of concentrations, to determine the response factor and range of analysis for each amino acid. The concentration of each amino acid in the standard is known. In the calibration procedure, the analyst dilutes the amino acid standard to several different analyte levels within the expected linear range of the amino acid analysis technique. Then, replicates at each of the different analyte levels can be analyzed. Peak areas obtained for each amino acid are plotted versus the known concentration for each of the amino acids in the standard dilution. These results will allow the analyst to determine the range of amino acid concentrations where the peak area of a given amino acid is an approximately linear function of the amino acid concentration. It is important that the analyst prepare the samples for amino acid analysis so that they are within the analytical limits (e.g., linear working range) of the technique employed in order to obtain accurate and repeatable results.

Four to six amino acid standard levels are analyzed to determine a response factor for each amino acid. The response factor is calculated as the average peak area or peak height per nmol of amino acid present in the standard. A calibration file consisting of the response factor for each amino acid is prepared and is used to calculate the concentration of each amino acid present in the test sample. This calculation involves dividing the peak area corresponding to a given amino acid by the response

* ♦Suitable standards may be obtained from NIST (Gaithersburg, MD), Beckman Instruments (Fullerton, CA), Sigma Chemical (St. Louis, MO), Pierce (Rockford, IL), or Agilent (Palo Alto, CA).♦

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factor for that amino acid to give the nmol of the amino acid. For routine analysis, a single-point calibration may be sufficient; however, the calibration file is updated frequently and tested by the analysis of analytical controls to ensure its integrity.

## REPEATABILITY

Consistent high quality amino acid analysis results from an analytical laboratory require attention to the repeatability of the assay. During analysis of the chromatographic separation of the amino acids or their derivatives, numerous peaks can be observed on the chromatogram that corresponds to the amino acids. The large number of peaks makes it necessary to have an amino acid analysis system that can repeatedly identify the peaks based on retention time and integrate the peak areas for quantitation. A typical repeatability evaluation involves preparing a standard amino acid solution and analyzing many replicates (i.e., six analyses or more) of the same standard solution. The relative standard deviation (RSD) is determined for the retention time and integrated peak area of each amino acid. An evaluation of the repeatability is expanded to include multiple assays conducted over several days by different analysts. Multiple assays include the preparation of standard dilutions from starting materials to determine the variation due to sample handling. Often, the amino acid composition of a standard protein (e.g., bovine serum albumin) is analyzed as part of the repeatability evaluation. By evaluating the replicate variation (i.e., RSD), the laboratory can establish analytical limits to ensure that the analyses from the laboratory are under control. It is desirable to establish the lowest practical variation limits to ensure the best results. Areas to focus on to lower the variability of the amino acid analysis include sample preparation, high background spectral interference due to the quality of reagents and/or to laboratory practices, instrument performance and maintenance, data analysis and interpretation, and analyst performance and habits. All parameters involved are fully investigated in the scope of the validation work.

## SAMPLE PREPARATION

Accurate results from amino acid analysis require purified protein and peptide samples. Buffer components (e.g., salts, urea, detergents) can interfere with the amino acid analysis and are removed from the sample before analysis. Methods that utilize postcolumn derivatization of the amino acids are generally not affected by buffer components to the extent seen with precolumn derivatization methods. It is desirable to limit the number of sample manipulations to reduce potential background contamination, to improve analyte recovery, and to reduce labor. Common techniques used to remove buffer components from protein samples include the following methods: (1) injecting the protein sample onto a reverse-phase HPLC system, removing the protein with a volatile solvent containing a sufficient organic component, and drying the sample in a vacuum centrifuge;(2) dialysis against a volatile buffer or water; (3) centrifugal ultrafiltration for buffer replacement with a volatile buffer or water; (4) precipitating the protein from the buffer using an organic solvent (e.g., acetone); and (5) gel filtration.

## INTERNAL STANDARDS

It is recommended that an internal standard be used to monitor physical and chemical losses and variations during amino acid analysis. An accurately known amount of internal standard can be added to a protein solution prior to hydrolysis. The recovery of the internal standard gives the general recovery of the amino acids from the protein solution. Free amino acids, however, do not behave in the same way as protein-bound amino acids during hydrolysis because their rates of release or destruction are variable. Therefore, the use of an internal standard to correct for losses during hydrolysis may give unreliable results. It will be necessary to take this particular point into consideration when interpreting the results. Internal standards can also be added to the mixture of amino acids after hydrolysis to correct for differences in sample application and changes in reagent stability and flow rates. Ideally, an internal standard is an unnaturally occurring primary amino acid that is commercially available and inexpensive. It should also be stable during hydrolysis, its response factor should be linear with concentration, and it needs to elute with a unique retention time without overlapping other amino acids. Commonly used amino acid standards include norleucine, nitrotyrosine, and α-aminobutyric acid.

## PROTEIN HYDROLYSIS

Hydrolysis of protein and peptide samples is necessary for amino acid analysis of these molecules. The glassware used for hydrolysis must be very clean to avoid erroneous results. Glove powders and fingerprints on hydrolysis tubes may cause contamination. To clean glass hydrolysis tubes, boil tubes for 1 hour in 1 N hydrochloric acid or soak tubes in concentrated nitric acid or in a mixture of concentrated hydrochloric acid and concentrated nitric acid (1:1). Clean hydrolysis tubes are rinsed with high-purity water followed by a rinse with HPLC-grade methanol, dried overnight in an oven, and stored covered until use. Alternatively, pyrolysis of clean glassware at 500° for 4 hours may be used to eliminate contamination from hydrolysis tubes. Adequate disposable laboratory material can also be used.

Acid hydrolysis is the most common method for hydrolyzing a protein sample before amino acid analysis. The acid hydrolysis technique can contribute to the variation of the analysis due to complete or partial destruction of several amino acids. Tryptophan is destroyed; serine and threonine are partially destroyed; methionine might undergo oxidation; and cysteine is typically recovered as cystine (but cystine recovery is usually poor because of partial destruction or reduction to cysteine). Application of adequate vacuum (less than 200 µm of mercury or 26.7 Pa) or introduction of an inert gas (argon) in the headspace of the reaction vessel can reduce the level of oxidative destruction. In peptide bonds involving isoleucine and valine, the amido bonds of Ile-Ile, Val-Val, Ile-Val, and Val-Ile are partially cleaved; and asparagine and glutamine are deamidated, resulting in aspartic acid and glutamic acid, respectively. The loss of tryptophan, asparagine, and glutamine during an acid hydrolysis limits quantitation to 17 amino acids. Some of the hydrolysis techniques described are used to address these concerns. Some of the hydrolysis techniques described (i.e., *Methods 4–11*) may cause modifications to other amino acids. Therefore, the benefits of using a given hydrolysis technique are weighed against the concerns with the technique and are tested adequately before employing a method other than acid hydrolysis.

A time-course study (i.e., amino acid analysis at acid hydrolysis times of 24, 48, and 72 hours) is often employed to analyze the starting concentration of amino acids that are partially destroyed or slow to cleave. By plotting the observed concentration of labile amino acids (i.e., serine and threonine) versus hydrolysis time, the line can be extrapolated to the origin to determine the starting concentration of these amino acids. Time-course hydrolysis studies are also used with amino acids that are slow to cleave (e.g., isoleucine and valine). During the hydrolysis time course, the analyst will observe a plateau in these residues. The level of this plateau is taken as the residue concentration. If the hydrolysis time is too long, the residue concentration of the sample will begin to decrease, indicating destruction by the hydrolysis conditions.

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An acceptable alternative to the time-course study is to subject an amino acid calibration standard to the same hydrolysis conditions as the test sample. The amino acid in free form may not completely represent the rate of destruction of labile amino acids within a peptide or protein during the hydrolysis. This is especially true for peptide bonds that are slow to cleave (e.g., Ile-Val bonds). However, this technique will allow the analyst to account for some residue destruction. Microwave acid hydrolysis has been used and is rapid but it requires special equipment as well as special precautions. The optimal conditions for microwave hydrolysis must be investigated for each individual protein/peptide sample. The microwave hydrolysis technique typically requires only a few minutes, but even a deviation of 1 minute may give inadequate results (e.g., incomplete hydrolysis or destruction of labile amino acids). Complete proteolysis, using a mixture of proteases, has been used but can be complicated, requires the proper controls, and is typically more applicable to peptides than proteins. [NOTE—During initial analyses of an unknown protein, experiments with various hydrolysis time and temperature conditions are conducted to determine the optimal conditions.]

- **METHOD 1**

  Acid hydrolysis using hydrochloric acid containing phenol is the most common procedure used for protein/peptide hydrolysis preceding amino acid analysis. The addition of phenol to the reaction prevents the halogenation of tyrosine.

  **Hydrolysis Solution:** *6* N hydrochloric acid containing 0.1% to 1.0% of phenol.

  **Procedure**

  **Liquid Phase Hydrolysis:** Place the protein or peptide sample in a hydrolysis tube, and dry. [NOTE—The sample is dried so that water in the sample will not dilute the acid used for the hydrolysis.] Add 200 µL of *Hydrolysis Solution* per 500 µg of lyophilized protein. Freeze the sample tube in a dry ice–acetone bath, and flame seal in vacuum. Samples are typically hydrolyzed at 110° for 24 hours in vacuum or inert atmosphere to prevent oxidation. Longer hydrolysis times (e.g., 48 and 72 hours) are investigated if there is a concern that the protein is not completely hydrolyzed.

  **Vapor Phase Hydrolysis:** This is one of the most common acid hydrolysis procedures, and it is preferred for microanalysis when only small amounts of the sample are available. Contamination of the sample from the acid reagent is also minimized by using vapor phase hydrolysis. Place vials containing the dried samples in a vessel that contains an appropriate amount of *Hydrolysis Solution*. The *Hydrolysis Solution* does not come in contact with the test sample. Apply an inert atmosphere or vacuum (less than 200 µm of mercury or 26.7 Pa) to the headspace of the vessel, and heat to about 110° for a 24-hour hydrolysis time. Acid vapor hydrolyzes the dried sample. Any condensation of the acid in the sample vials is minimized. After hydrolysis, dry the test sample in vacuum to remove any residual acid.

- **METHOD 2**

  Tryptophan oxidation during hydrolysis is decreased by using mercaptoethanesulfonic acid (MESA) as the reducing acid.

  **Hydrolysis Solution:** 2.5 M MESA solution.

  **Vapor Phase Hydrolysis:** About 1 to 100 µg of the protein/peptide under test is dried in a hydrolysis tube. The hydrolysis tube is placed in a larger tube with about 200 µL of the *Hydrolysis Solution*. The larger tube is sealed in vacuum (about 50 µm of mercury or 6.7 Pa) to vaporize the *Hydrolysis Solution*. The hydrolysis tube is heated to between 170° to 185° for about 12.5 minutes. After hydrolysis, the hydrolysis tube is dried in vacuum for 15 minutes to remove the residual acid.

- **METHOD 3**

  Tryptophan oxidation during hydrolysis is prevented by using thioglycolic acid (TGA) as the reducing acid.

  **Hydrolysis Solution:** a solution containing 7 M hydrochloric acid, 10% of trifluoroacetic acid, 20% of thioglycolic acid, and 1% of phenol.

  **Vapor Phase Hydrolysis:** About 10 to 50 µg of the protein/peptide under test is dried in a sample tube. The sample tube is placed in a larger tube with about 200 µL of the *Hydrolysis Solution*. The larger tube is sealed in vacuum (about 50 µm of mercury or 6.7 Pa) to vaporize the TGA. The sample tube is heated to 166° for about 15 to 30 minutes. After hydrolysis, the sample tube is dried in vacuum for 5 minutes to remove the residual acid. Recovery of tryptophan by this method may be dependent on the amount of sample present.

- **METHOD 4**

  Cysteine-cystine and methionine oxidation is performed with performic acid before the protein hydrolysis.

  **Oxidation Solution:** The performic acid is prepared fresh by mixing formic acid and 30 percent hydrogen peroxide (9:1), and incubating at room temperature for 1 hour.

  **Procedure:** The protein/peptide sample is dissolved in 20 µL of formic acid, and heated at 50° for 5 minutes; then 100 µL of the *Oxidation Solution* is added. In this reaction, cysteine is converted to cysteic acid and methionine is converted to methionine sulfone. The oxidation is allowed to proceed for 10 to 30 minutes. The excess reagent is removed from the sample in a vacuum centrifuge. This technique may cause modifications to tyrosine residues in the presence of halides. The oxidized protein can then be acid hydrolyzed using *Method 1* or *Method 2*.

- **METHOD 5**

  Cysteine-cystine oxidation is accomplished during the liquid phase hydrolysis with sodium azide.

  **Hydrolysis Solution:** 6 N hydrochloric acid containing 0.2% of phenol, to which sodium azide is added to obtain a final concentration of 0.2% (w/v). The added phenol prevents halogenation of tyrosine.

  **Liquid Phase Hydrolysis:** The protein/peptide hydrolysis is conducted at about 110° for 24 hours. During the hydrolysis, the cysteine-cystine present in the sample is converted to cysteic acid by the sodium azide present in the *Hydrolysis Solution*. This technique allows better tyrosine recovery than *Method 4*, but it is not quantitative for methionine. Methionine is converted to a mixture of the parent methionine and its two oxidative products, methionine sulfoxide and methionine sulfone.

- **METHOD *6***

  Cysteine-cystine oxidation is accomplished with dimethyl sulfoxide (DMSO).

  **Hydrolysis Solution:** *6* N hydrochloric acid containing 0.1% to 1.0% of phenol, to which DMSO is added to obtain a final concentration of 2% (v/v).

  **Vapor Phase Hydrolysis:** The protein/peptide hydrolysis is conducted at about 110° for 24 hours. During the hydrolysis, the cysteine-cystine present in the sample is converted to cysteic acid by the DMSO present in the *Hydrolysis Solution*. As an approach to limit variability and to compensate for partial destruction, it is recommended to evaluate the cysteic acid

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recovery from oxidative hydrolyses of standard proteins containing 1 to 8 mol of cysteine per mol protein. The response factors from protein/peptide hydrolysates are typically about 30% lower than those for nonhydrolyzed cysteic acid standards. Because histidine, methionine, tyrosine, and tryptophan are also modified, a complete compositional analysis is not obtained with this technique.

- **METHOD 7**

Cysteine-cystine reduction and alkylation is accomplished by a vapor phase pyridylethylation reaction.

**Reducing Solution:** Transfer 83.3 µL of pyridine, 16.7 µL of 4-vinylpyridine, 16.7 µL of tributylphosphine, and 83.3 µL of water to a suitable container, and mix.

**Procedure:** Add the protein/peptide (between 1 and 100 µg) to a hydrolysis tube, and place in a larger tube. Transfer the *Reducing Solution* to the large tube, seal in vacuum (about 50 µm of mercury or 6.7 Pa), and incubate at about 100° for 5 minutes. Then remove the inner hydrolysis tube, and dry it in a vacuum desiccator for 15 minutes to remove residual reagents. The pyridylethylated protein/peptide can then be acid hydrolyzed using previously described procedures. The pyridylethylation reaction is performed simultaneously with a protein standard sample containing 1 to 8 mol of cysteine per mol protein to improve accuracy in the pyridylethyl-cysteine recovery. Longer incubation times for the pyridylethylation reaction can cause modifications to the α-amino terminal group and the ε-amino group of lysine in the protein.

- **METHOD 8**

Cysteine-cystine reduction and alkylation is accomplished by a liquid phase pyridylethylation reaction.

**Stock Solutions:** Prepare and filter three solutions: 1 M Tris hydrochloride (pH 8.5) containing 4 mM edetate disodium (*Stock Solution 1*), 8 M guanidine hydrochloride (*Stock Solution 2*), and 10% of 2-mercaptoethanol in water (*Stock Solution 3*).

**Reducing Solution:** Prepare a mixture of *Stock Solution 2* and *Stock Solution 1* (3:1) to obtain a buffered solution of 6 M guanidine hydrochloride in 0.25 M Tris hydrochloride.

**Procedure:** Dissolve about 10 µg of the test sample in 50 µL of the *Reducing Solution*, and add about 2.5 µL of *Stock Solution 3*. Store under nitrogen or argon for 2 hours at room temperature in the dark. To achieve the pyridylethylation reaction, add about 2 µL of 4-vinylpyridine to the protein solution, and incubate for an additional 2 hours at room temperature in the dark. The protein/peptide is desalted by collecting the protein/peptide fraction from a reverse-phase HPLC separation. The collected sample can be dried in a vacuum centrifuge before acid hydrolysis.

- **METHOD 9**

Cysteine-cystine reduction and alkylation is accomplished by a liquid phase carboxymethylation reaction.

**Stock Solutions:** Prepare as directed for *Method 8*.

**Carboxymethylation Solution:** Prepare a solution containing 100 mg of iodoacetamide per mL of alcohol.

**Buffer Solution:** Use the *Reducing Solution*, prepared as directed for *Method 8*.

**Procedure:** Dissolve the test sample in 50 µL of the *Buffer Solution*, and add about 2.5 µL of *Stock Solution 3*. Store under nitrogen or argon for 2 hours at room temperature in the dark. Add the *Carboxymethylation Solution* in a 1.5 fold ratio per total theoretical content of thiols, and incubate for an additional 30 minutes at room temperature in the dark. [NOTE—If the thiol content of the protein is unknown, then add 5 µL of 100 mM iodoacetamide for every 20 nmol of protein present.] The reaction is stopped by adding excess of 2-mercaptoethanol. The protein/peptide is desalted by collecting the protein/peptide fraction from a reverse-phase HPLC separation. The collected sample can be dried in a vacuum centrifuge before acid hydrolysis. The *S*-carboxyamidomethylcysteine formed will be converted to *S*-carboxymethyl-cysteine during acid hydrolysis.

- **METHOD 10**

Cysteine-cystine is reacted with dithiodiglycolic acid or dithiodipropionic acid to produce a mixed disulfide. [NOTE—The choice of dithiodiglycolic acid or dithiodipropionic acid depends on the required resolution of the amino acid analysis method.]

**Reducing Solution:** a solution containing 10 mg of dithiodiglycolic acid (or dithiodipropionic acid) per mL of 0.2 M sodium hydroxide.

**Procedure:** Transfer about 20 µg of the test sample to a hydrolysis tube, and add 5 µL of the *Reducing Solution*. Add 10 µL of isopropyl alcohol, and then remove all of the sample liquid by vacuum centrifugation. The sample is then hydrolyzed using *Method 1*. This method has the advantage that other amino acid residues are not derivatized by side reactions, and the sample does not need to be desalted prior to hydrolysis.

- **METHOD 11**

Asparagine and glutamine are converted to aspartic acid and glutamic acid, respectively, during acid hydrolysis. Asparagine and aspartic acid residues are added and represented by Asx, while glutamine and glutamic acid residues are added and represented by Glx. Proteins/peptides can be reacted with bis(1,1-trifluoroacetoxy)iodobenzene (BTI) to convert the asparagine and glutamine residues to diaminopropionic acid and diaminobutyric acid residues, respectively, upon acid hydrolysis. These conversions allow the analyst to determine the asparagine and glutamine content of a protein/peptide in the presence of aspartic acid and glutamic acid residues.

**Reducing Solutions:** Prepare and filter three solutions: a solution of 10 mM trifluoroacetic acid (*Solution 1*), a solution of 5 M guanidine hydrochloride and 10 mM trifluoroacetic acid (*Solution 2*), and a freshly prepared solution of dimethylformamide containing 36 mg of BTI per mL (*Solution 3*).

**Procedure:** In a clean hydrolysis tube, transfer about 200 µg of the test sample, and add 2 mL of *Solution 1* or *Solution 2* and 2 mL of *Solution 3*. Seal the hydrolysis tube in vacuum. Heat the sample at 60° for 4 hours in the dark. The sample is then dialyzed with water to remove the excess reagents. Extract the dialyzed sample three times with equal volumes of *n*-butyl acetate, and then lyophilize. The protein can then be acid hydrolyzed using previously described procedures. The α-, β-diaminopropionic and α-, γ-diaminobutyric acid residues do not typically resolve from the lysine residues upon ion-exchange chromatography based on amino acid analysis. Therefore, when using ion-exchange as the mode of amino acid separation, the asparagine and glutamine contents are the quantitative difference in the aspartic acid and glutamic acid assayed contents with underivatized and BTI-derivatized acid hydrolysis. [NOTE—The threonine, methionine, cysteine,

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tyrosine, and histidine assayed content can be altered by BTI derivatization; a hydrolysis without BTI will have to be performed if the analyst is interested in the composition of these other amino acid residues.]

## METHODOLOGIES OF AMINO ACID ANALYSIS GENERAL PRINCIPLES

Many amino acid analysis techniques exist, and the choice of any one technique often depends on the sensitivity required from the assay. In general, about one-half of the amino acid analysis techniques employed rely on the separation of the free amino acids by ion-exchange chromatography followed by postcolumn derivatization (e.g., with ninhydrin or *o*-phthalaldehyde). Postcolumn detection techniques can be used with samples that contain small amounts of buffer components, such as salts and urea, and generally require between 5 and 10 µg of protein sample per analysis. The remaining amino acid techniques typically involve precolumn derivatization of the free amino acids (e.g., phenyl isothiocyanate; 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate or *o*-phthalaldehyde; (dimethylamino)azobenzenesulfonyl chloride; 9-fluorenyl-methylchloroformate; and 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole) followed by reverse-phase HPLC. Precolumn derivatization techniques are very sensitive and usually require between 0.5 and 1.0 µg of protein sample per analysis but may be influenced by buffer salts in the samples. Precolumn derivatization techniques may also result in multiple derivatives of a given amino acid, which complicates the result interpretation. Postcolumn derivatization techniques are generally influenced less by performance variation of the assay than precolumn derivatization techniques.

The following *Methods* may be used for quantitative amino acid analysis. Instruments and reagents for these procedures are available commercially. Furthermore, many modifications of these methodologies exist with different reagent preparations, reaction procedures, and chromatographic systems. Specific parameters may vary according to the exact equipment and procedure used. Many laboratories will utilize more than one amino acid analysis technique to exploit the advantages offered by each. In each of these *Methods*, the analog signal is visualized by means of a data acquisition system, and the peak areas are integrated for quantification purposes.

- **Method 1—Postcolumn Ninhydrin Detection General Principle**

  Ion-exchange chromatography with postcolumn ninhydrin detection is one of the most common methods employed for quantitative amino acid analysis. As a rule, a Li-based cation-exchange system is employed for the analysis of the more complex physiological samples, and the faster Na-based cation-exchange system is used for the more simplistic amino acid mixtures obtained with protein hydrolysates (typically containing 17 amino acid components). Separation of the amino acids on an ion-exchange column is accomplished through a combination of changes in pH and cation strength. A temperature gradient is often employed to enhance separation.

  When the amino acid reacts with ninhydrin, the reactant has characteristic purple or yellow color. Amino acids, except imino acids, give a purple color, and show maximum absorption at 570 nm. The imino acids, such as proline, give a yellow color, and show maximum absorption at 440 nm. The postcolumn reaction between ninhydrin and amino acid eluted from the column is monitored at 440 nm and 570 nm, and the chromatogram obtained is used for the determination of amino acid composition.

  Detection limit is considered to be 10 pmol for most of the amino acid derivatives, but 50 pmol for proline. Response linearity is obtained in the range of 20 to 500 pmol with correlation coefficients exceeding 0.999. To obtain good compositional data, samples larger than 1 µg before hydrolysis are best suited for this amino acid analysis of protein/peptide.

- **Method 2—Postcolumn OPA Fluorometric Detection General Principle**

  *o*-Phthalaldehyde (OPA) reacts with primary amines in the presence of thiol compound to form highly fluorescent isoindole products. This reaction is utilized for the postcolumn derivatization in analysis of amino acids by ion-exchange chromatography. The rule of the separation is the same as *Method I.* Instruments and reagents for this form of amino acid analysis are available commercially. Many modifications of this method exist.

  Although OPA does not react with secondary amines (imino acids, such as proline) to form fluorescent substances, the oxidation with sodium hypochlorite allows secondary amines to react with OPA. The procedure employs a strongly acidic cation-exchange column for separation of free amino acids followed by postcolumn oxidation with sodium hypochlorite and postcolumn derivatization using OPA and thiol compound, such as *N*-acetyl-L-cysteine and 2-mercaptoethanol. The derivatization of primary amino acids are not noticeably affected by the continuous supply of sodium hypochlorite.

  Separation of the amino acids on an ion-exchange column is accomplished through a combination of changes of pH and cation strength. After postcolumn derivatization of eluted amino acids with OPA, the reactant passes through the fluorometric detector. Fluorescence intensity of OPA-derivatized amino acids are monitored with an excitation wavelength of 348 nm and an emission wavelength of 450 nm.

  Detection limit is considered to be a few tens of pmol level for most of the amino acid derivatives. Response linearity is obtained in the range of a few pmol level to a few tens of nmol level. To obtain good compositional data, a sample greater than 500 ng before hydrolysis is best suited for the amino acid analysis of protein/peptide.

- **Method 3—Precolumn PITC Derivatization General Principle**

  Phenylisothiocyanate (PITC) reacts with amino acids to form phenylthiocarbamyl (PTC) derivatives, which can be detected with high sensitivity at 254 nm. Therefore, precolumn derivatization of amino acids with PITC followed by a reverse-phase HPLC separation with UV detection is used to analyze the amino acid composition.

  After the reagent is removed under vacuum, the derivatized amino acids can be stored dry and frozen for several weeks with no significant degradation. If the solution for injection is kept cold, no noticeable loss in chromatographic response occurs after three days.

  Separation of the PTC-amino acids on a reverse-phase HPLC with ODS column is accomplished through a combination of changes in concentrations of acetonitrile and buffer ionic strength. PTC-amino acids eluted from the column are monitored at 254 nm.

  Detection limit is considered to be 1 pmol for most of the amino acid derivatives. Response linearity is obtained in the range of 20 to 500 pmol with correlation coefficients exceeding 0.999. To obtain good compositional data, a sample larger than 500 ng of protein/peptide before hydrolysis is best suited for this amino analysis of proteins/peptides.

- **Method 4—Precolumn AQC Derivatization General Principle**

  Precolumn derivatization of amino acids with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) followed by reverse-phase HPLC separation with fluorometric detection is used.

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AQC reacts with amino acids to form stable, fluorescent unsymmetric urea derivatives (AQC-amino acids) which are readily amenable to analysis by reverse-phase HPLC. Therefore, precolumn derivatization of amino acids with AQC followed by reverse-phase HPLC separation is used to analyze the amino acid composition.

Separation of the AQC-amino acids on an ODS column is accomplished through a combination of changes in the concentrations of acetonitrile and salt. Selective fluorescence detection of the derivatives with an excitation wavelength at 250 nm and an emission wavelength at 395 nm allows for the direct injection of the reaction mixture with no significant interference from the only major fluorescent reagent byproduct, 6-aminoquinoline. Excess reagent is rapidly hydrolyzed ($t_{1/2}$< 15 seconds) to yield 6-aminoquinoline, *N*-hydroxysuccinimide and carbon dioxide, and after 1 minute no further derivatization can take place.

Peak areas for AQC-amino acids are essentially unchanged for at least 1 week at room temperature, and the derivatives have more than sufficient stability to allow for overnight automated chromatographic analysis.

The detection limit is considered to be ranging from about 40 fmol to 320 fmol for each amino acid, except for Cys. The detection limit for Cys is approximately 800 fmol. Response linearity is obtained in the range of 2.5 µM to 200 µM with correlation coefficients exceeding 0.999. Good compositional data can be obtained from the analysis of derivatized protein hydrolysates containing as little as 30 ng of protein/peptide.

- **Method 5—Precolumn OPA Derivatization General Principle**

Precolumn derivatization of amino acids with *o*-phthalaldehyde (OPA) followed by reverse-phase HPLC separation with fluorometric detection is used. This technique does not detect amino acids that exist as secondary amines (e.g., proline).

OPA in conjunction with a thiol reagent reacts with primary amine groups to form highly fluorescent isoindole products. 2-Mercaptoethanol and 3-mercaptopropionic acid can be used as thiol. OPA itself does not fluoresce and consequently produces no interfering peaks. In addition, its solubility and stability in aqueous solution, along with the rapid kinetics for the reactions, make it amenable to automated derivatization and analysis using an autosampler to mix the sample with the reagent. However, lack of reactivity with secondary amino acids has been a predominant drawback. This method does not detect amino acids that exist as secondary amines (e.g., proline). To compensate for this drawback, this technique may be combined with another technique described in *Method 7* or *Method 8*.

Precolumn derivatization of amino acids with OPA is followed by reverse-phase HPLC separation. Because of the instability of the OPA-amino acid derivative, HPLC separation and analysis are performed immediately following derivatization. The liquid chromatograph is equipped with a fluorometric detector for the detection of derivatized amino acids. Fluorescence intensity of the OPA-derivatized amino acids are monitored with an excitation wavelength of 348 nm and an emission wavelength of 450 nm.

The detection limits as low as 50 fmol via fluorescence have been reported, although the practical limit of analysis remains at 1 pmol.

- **Method 6—Precolumn DABS-Cl Derivatization General Principle**

Precolumn derivatization of amino acids with (dimethylamino)azobenzenesulfonyl chloride (DABS-Cl) followed by reverse-phase HPLC separation with visible light detection is used.

DABS-Cl is a chromophoric reagent employed for the labeling of amino acids. Amino acids labeled with DABS-Cl (DABS-amino acids) are highly stable and show the maximum absorption at 436 nm.

DABS-amino acids, all 19 naturally occurring amino acid derivatives, can be separated on an ODS column of a reverse-phase HPLC by employing gradient systems consisting of acetonitrile and aqueous buffer mixture. Separated DABS-amino acids eluted from the column are detected at 436 nm in the visible region.

This method can analyze the imino acids, such as proline, together with the amino acids, at the same degree of sensitivity. DABS-Cl derivatization method permits the simultaneous quantification of tryptophan residues by previous hydrolysis of the protein/peptide with sulfonic acids, such as mercaptoethanesulfonic acid, *p*-toluenesulfonic acid, or methanesulfonic acid, described for *Method 2* in *Protein Hydrolysis*. The other acid-labile residues, asparagine and glutamine, can also be analyzed by previous conversion into diaminopropionic acid and diaminobutyric acid, respectively, by treatment of protein/peptide with BTI, described for *Method 11* in *Protein Hydrolysis*.

The nonproteinogenic amino acid, norleucine, cannot be used as an internal standard in this method as this compound is eluted in a chromatographic region crowded with peaks of primary amino acids. Nitrotyrosine can be used as an internal standard because it is eluted in a clean region.

The detection limit of DABS-amino acid is about 1 pmol. As little as 2 to 5 pmol of an individual DABS-amino acid can be quantitatively analyzed with reliability, and only 10 ng to 30 ng of the dabsylated protein hydrolysate is required for each analysis.

- **Method 7—Precolumn FMOC-Cl Derivatization General Principle**

Precolumn derivatization of amino acids with 9-fluorenylmethyl chloroformate (FMOC-Cl) followed by reverse-phase HPLC separation with fluorometric detection is used.

FMOC-Cl reacts with both primary and secondary amino acids to form highly fluorescent products. The reaction of FMOC-Cl with amino acid proceeds under mild conditions, in aqueous solution, and is completed in 30 seconds. The derivatives are stable, with only the histidine derivative showing any breakdown. Although FMOC-Cl is fluorescent itself, the reagent excess and fluorescent side-products can be eliminated without loss of FMOC-amino acids.

FMOC-amino acids are separated by reverse-phase HPLC using an ODS column. The separation is carried out by gradient elution varied linearly from a mixture of acetic acid buffer, methanol, and acetonitrile (50:40:10) to a mixture of acetonitrile and acetic acid buffer (50:50), and 20 amino acid derivatives that are separated in 20 minutes. Each derivative eluted from the column is monitored by a fluorometric detector set at an excitation wavelength of 260 nm and an emission wavelength of 313 nm.

The detection limit is in the low fmol range. A linearity range of 0.1 µM to 50 µM is obtained for most amino acids.

- **Method 8—Precolumn NBD-F Derivatization General Principle**

Precolumn derivatization of amino acids with 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F) followed by reverse-phase HPLC separation with fluorometric detection is used.

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7-Fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F) reacts with both primary and secondary amino acids to form highly fluorescent products. Amino acids are derivatized with NBD-F by heating to 60° for 5 minutes.

NBD-amino acid derivatives are separated on an ODS column of reverse-phase HPLC by employing a gradient elution system consisting of acetonitrile and aqueous buffer mixture, and 17 amino acid derivatives that are separated in 35 minutes. *E*-aminocaproic acid can be used as an internal standard because it is eluted in a clean chromatographic region. Each derivative eluted from the column is monitored by a fluorometric detector set at an excitation wavelength of 480 nm and an emission wavelength of 530 nm.

The sensitivity of this method is almost the same as that for the precolumn OPA derivatization method (*Method 5*), excluding proline to which OPA is not reactive and might be advantageous for NBD-F against OPA.

The detection limit for each amino acid is about 10 fmol. Profile analysis was achieved for about 1.5 mg of protein hydrolysates in the final precolumn labeling reaction mixture for HPLC.

## DATA CALCULATION AND ANALYSIS

When determining the amino acid content of a protein/peptide hydrolysate, it should be noted that the acid hydrolysis step destroys tryptophan and cysteine. Serine and threonine are partially destroyed by acid hydrolysis, while isoleucine and valine residues may be only partially cleaved. Methionine can undergo oxidation during acid hydrolysis, and some amino acids (e.g., glycine and serine) are common contaminants. Application of adequate vacuum (less than 200 µm of mercury or 26.7 Pa) or introduction of inert gas (argon) in the headspace of the reaction vessel during vapor phase hydrolysis can reduce the level of oxidative destruction. Therefore, the quantitative results obtained for cysteine, tryptophan, threonine, isoleucine, valine, methionine, glycine, and serine from a protein/peptide hydrolysate may be variable and may warrant further investigation and consideration.

### Calculations

**Amino Acid Mole Percent:** This is the number of specific amino acid residues per 100 residues in a protein. This result may be useful for evaluating amino acid analysis data when the molecular weight of the protein/peptide under investigation is unknown. This information can be used to corroborate the identity of a protein and has other applications. Carefully identify and integrate the peaks obtained as directed for each *Procedure*. Calculate the mole percent for each amino acid present in the test sample by the formula:

$$100r_U/r$$

in which $r_U$ is the peak response, in nmol, of the amino acid under test; and r is the sum of peak responses, in nmol, for all amino acids present in the test sample. Comparison of the mole percent of the amino acids under test to data from known proteins can help establish or corroborate the identity of the sample protein.

**Unknown Protein Samples:** This data analysis technique can be used to estimate the protein concentration of an unknown protein sample using the amino acid analysis data. Calculate the mass, in µg, of each recovered amino acid by the formula:

$$mM_W/1000$$

in which m is the recovered quantity, in nmol, of the amino acid under test; and $M_W$ is the molecular weight, for that amino acid, corrected for the weight of the water molecule that was eliminated during peptide bond formation. The sum of the masses of the recovered amino acids will give an estimate of the total mass of the protein analyzed after appropriate correction for partially and completely destroyed amino acids. If the molecular weight of the unknown protein is available (i.e., by SDS-PAGE analysis or mass spectroscopy), the amino acid composition of the unknown protein can be predicted. Calculate the number of residues of each amino acid by the formula:

$$m/(1000M/M_{WT})$$

in which m is the recovered quantity, in nmol, of the amino acid under test; M is the total mass, in µg, of the protein; and $M_{WT}$ is the molecular weight of the unknown protein.

**Known Protein Samples:** This data analysis technique can be used to investigate the amino acid composition and protein concentration of a protein sample of known molecular weight and amino acid composition using the amino acid analysis data. When the composition of the protein being analyzed is known, one can exploit the fact that some amino acids are recovered well, while other amino acid recoveries may be compromised because of complete or partial destruction (e.g., tryptophan, cysteine, threonine, serine, methionine), incomplete bond cleavage (i.e., for isoleucine and valine), and free amino acid contamination (i.e., by glycine and serine).

Because those amino acids that are recovered best represent the protein, these amino acids are chosen to quantify the amount of protein. Well-recovered amino acids are, typically, aspartate-asparagine, glutamate-glutamine, alanine, leucine, phenylalanine, lysine, and arginine. This list can be modified based on experience with one's own analysis system. Divide the quantity, in nmol, of each of the well-recovered amino acids by the expected number of residues for that amino acid to obtain the protein content based on each well-recovered amino acid. Average the protein content results calculated. The protein content determined for each of the well-recovered amino acids should be evenly distributed about the mean. Discard protein content values for those amino acids that have an unacceptable deviation from the mean. Typically, a greater than 5% variation from the mean is considered unacceptable. Recalculate the mean protein content from the remaining values to obtain the protein content of the sample. Divide the content of each amino acid by the calculated mean protein content to determine the amino acid composition of the sample by analysis.

Calculate the relative compositional error, in percentage, by the formula:

$$100m/m_S$$

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in which m is the experimentally determined quantity, in nmol per amino acid residue, of the amino acid under test; and $m_S$ is the known residue value for that amino acid. The average relative compositional error is the average of the absolute values of the relative compositional errors of the individual amino acids, typically excluding tryptophan and cysteine from this calculation. The average relative compositional error can provide important information on the stability of analysis run over time. The agreement in the amino acid composition between the protein sample and the known composition can be used to corroborate the identity and purity of the protein in the sample.

## ⋆APPENDIX

### Amino Acid Analysis Procedures

The examples of the specific procedures for each *Method* described in *Methodologies of Amino Acid Analysis* are shown.

- **Method 1—Postcolumn Ninhydrin Detection**

One method for postcolumn ninhydrin detection is shown below. Many other methods are also available, with instruments and reagents available commercially.

**Mobile Phase Preparation**

**Solution A:** Transfer about 1.7 g of anhydrous sodium citrate and 1.5 mL of hydrochloric acid to a 100-mL volumetric flask, dissolve in and dilute with water to volume, and mix. Adjust, if necessary, with hydrochloric acid to a pH of 3.0.

**Solution B:** Transfer about 1.7 g of anhydrous sodium citrate and 0.7 mL of hydrochloric acid to a 100-mL volumetric flask, dissolve in and dilute with water to volume, and mix. Adjust, if necessary, with hydrochloric acid to a pH of 4.3.

**Solution C:** Prepare a solution containing 5% of sodium chloride, 1.9% of anhydrous sodium citrate, and 0.1% of phenol in water, and adjust to a pH of 6.

**Column Regeneration Solution:** Prepare a solution containing 0.8% of sodium hydroxide in water, and adjust to a pH of 13.

**Mobile Phase:** Use variable mixtures of *Solution A*, *Solution B*, and *Solution C* as directed for *Chromatographic System*.

**Postcolumn Reagent:** Transfer about 18 g of ninhydrin and 0.7 g of hydrindantin to 900 mL of a solution containing 76.7% of dimethyl sulfoxide, 0.7% of dihydrate lithium acetate, and 0.1% of acetic acid, and mix for at least 3 hours under inert gas, such as nitrogen. [Note—This reagent is stable for 30 days if kept between 2° and 8° under inert gas.]

**Buffer Solution:** Prepare a solution containing 2% of anhydrous sodium citrate, 1% of hydrochloric acid, 0.5% of thiodiglycol, and 0.1% of benzoic acid in water, and adjust to a pH of 2.

**Chromatographic System:** The liquid chromatograph is equipped with a detector with appropriate interference filters at 440, 570, or 690 nm and a 4.0-mm × 120-mm column that contains 7.5-µm sulfonated styrene-divinylbenzene copolymer packing. The flow rate is about 14 mL per hour. The system is programmed as follows. Initially equilibrate the column with *Solution A*; at 25 minutes, the composition of the *Mobile Phase* is changed to 100% *Solution B*; and at 37 minutes, the composition is changed to 100% *Solution C*. At 75 minutes into the run, the last amino acid has been eluted from the column, and the column is regenerated with *Column Regeneration Solution* for 1 minute. The column is then equilibrated with *Solution A* for 11 minutes before the next injection. The column temperature is programmed as follows. The initial temperature is 48°; after 11.5 minutes, the temperature is increased to 65° at a rate of 3° per minute; at about 35 minutes, the temperature is increased to 77° at a rate of 3° per minute; and finally at about 52 minutes, the temperature is decreased to 48° at a rate of 3° per minute.

**Procedure and Postcolumn Reaction:** Reconstitute the lyophilized protein/peptide hydrolysate in the *Buffer Solution*, inject an appropriate amount into the chromatograph, and proceed as directed for *Chromatographic System*. As the amino acids are eluted from the column, they are mixed with the *Postcolumn Reagent*, which is delivered at a flow rate of 7 mL per hour, through a tee. After mixing, the column effluent and the *Postcolumn Reagent* pass through a tubular reactor at a temperature of 135°, where a characteristic purple or yellow color is developed. From the reactor, the liquid passes through a colorimeter with a 12-mm flow-through cuvette. The light emerging from the cuvette is split into three beams for analysis by the detector with interference filters at 440, 570, or 690 nm. The 690-nm signal may be electronically subtracted from the other signals for improved signal-to-noise ratios. The 440-nm (imino acids) and the 570-nm (amino acids) signals may be added in order to simplify data handling.

- **Method 2—Postcolumn OPA Fluorometric Detection**

One method of postcolumn OPA fluorometric detection is shown below.

**Mobile Phase Preparation**

**Solution A:** Prepare a solution of sodium hydroxide, citric acid, and alcohol in HPLC-grade water having a 0.2 N sodium concentration and containing 7% of alcohol (w/v), adjusted to a pH of 3.2.

**Solution B:** Prepare a solution of sodium hydroxide and citric acid in HPLC-grade water having a 0.6 N sodium concentration, adjusted to a pH of 10.0.

**Solution C:** 0.2 N sodium hydroxide.

**Mobile Phase:** Use variable mixtures of *Solution A*, *Solution B*, and *Solution C* as directed for *Chromatographic System*.

**Postcolumn Reagent Preparation**

**Alkaline Buffer:** Prepare a solution containing 384 mM sodium carbonate, 216 mM boric acid, and 108 mM potassium sulfate, and adjust to a pH of 10.0.

**Hypochlorite Reagent:** To 1 L of *Alkaline Buffer*, add 0.4 mL of sodium hypochlorite solution (10% chlorine concentration). [Note—The hypochlorite solution is stable for 2 weeks.]

**OPA Reagent:** Transfer 2 g of *N*-acetyl-L-cysteine and 1.6 g of OPA to a 15-mL volumetric flask, dissolve in and dilute with alcohol to volume, and mix. Transfer this solution and 4 mL of 10% aqueous polyethylene (23) lauryl ether to a 1-L volumetric flask, dilute with 980 mL of *Alkaline Buffer*, and mix.

**Chromatographic System:** The liquid chromatograph is equipped with a fluorometric detector set to an excitation wavelength of 348 nm and an emission wavelength of 450 nm and a 4.0-mm × 150-mm column that contains 7.5-µm packing L17. The flow rate is about 0.3 mL per minute, and the column temperature is set at 50°. The system is programmed as follows. The column is equilibrated with *Solution A*; over the next 20 minutes, the composition of the *Mobile Phase* is changed linearly to 85% *Solution A* and 15% *Solution B*; then there is a step change to 40% *Solution A* and 60% *Solution B*; over the next 18 minutes, the composition is changed linearly to 100% *Solution B* and held for 7 minutes;

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then there is a step change to 100% *Solution C*, and this is held for 6 minutes; then there is a step change to *Solution A*, and this composition is maintained for the next 8 minutes.

**Procedure and Postcolumn Reaction:** Inject about 1.0 nmol of each amino acid under test into the chromatograph, and proceed as directed for *Chromatographic System*. As the effluent leaves the column, it is mixed with the *Hypochlorite Reagent*. The mixture passes through the first postcolumn reactor which consists of stainless steel 0.5-mm × 2-m tubing. A second postcolumn reactor of similar design is placed immediately downstream from the first postcolumn reactor and is used for the OPA postcolumn reaction. The flow rates for both the *Hypochlorite Reagent* and the *OPA Reagent* are 0.2 mL per minute, resulting in a total flow rate (i.e., *Hypochlorite Reagent*, *OPA Reagent*, and column effluent) of 0.7 mL per minute exiting from the postcolumn reactors. Postcolumn reactions are conducted at 55°. This results in a residence time of about 33 seconds in the OPA postcolumn reactor. After postcolumn derivatization, the column effluent passes through the fluorometric detector.

- **Method 3—Precolumn PITC Derivatization**

One method of precolumn PITC derivatization is described below.

**Mobile Phase Preparation**

**Solution A:** 0.05 M ammonium acetate, adjusted with phosphoric acid to a pH of 6.8.

**Solution B:** Prepare 0.1 M ammonium acetate, adjust with phosphoric acid to a pH of 6.8, and then prepare a mixture of this solution and acetonitrile (1:1).

**Solution C:** a mixture of acetonitrile and water (70:30).

**Mobile Phase:** Use variable mixtures of *Solution A*, *Solution B*, and *Solution C* as directed for *Chromatographic System*.

**Derivatization Reagent Preparation**

**Coupling Buffer:** a mixture of acetonitrile, pyridine, triethylamine, and water (10:5:2:3).

**Sample Solvent:** a mixture of water and acetonitrile (7:2).

**Sample Derivatization Procedure:** Dissolve the lyophilized test sample in 100 µL of the *Coupling Buffer*, and then dry in a vacuum centrifuge to remove any hydrochloride if a protein hydrolysis step was used. Dissolve the test sample in 100 µL of *Coupling Buffer*, add 5 µL of PITC, and incubate at room temperature for 5 minutes. The test sample is again dried in a vacuum centrifuge, and is dissolved in 250 µL of *Sample Solvent*.

**Chromatographic System:** The liquid chromatograph is equipped with a 254-nm detector and a 4.6-mm × 250-mm column that contains 5-µm packing L1. The flow rate is about 1 mL per minute, and the column temperature is maintained at 52°. The system is programmed as follows. The column is equilibrated with *Solution A*; over the next 15 minutes, the composition of the *Mobile Phase* is changed linearly to 85% *Solution A* and 15% *Solution B*; over the next 15 minutes, the composition is changed linearly to 50% *Solution A* and 50% *Solution B*; then there is a step change to 100% *Solution C*, and this is held for 10 minutes; then there is a step change to 100% *Solution A*, and the column is allowed to equilibrate before the next injection.

**Procedure:** Inject about 1.0 nmol of each PITC-amino acid under test (10-µL sample in *Sample Solvent*) into the chromatograph, and proceed as directed for *Chromatographic System*.

- **Method 4—Precolumn AQC Derivatization**

One method of precolumn AQC derivatization is shown below.

**Mobile Phase Preparation**

**Solution A:** Prepare a solution having a composition of 140 mM sodium acetate and 17 mM triethylamine, and adjust with phosphoric acid to a pH of 5.02.

**Solution B:** a mixture of acetonitrile and water (60:40).

**Mobile Phase:** Use variable mixtures of *Solution A* and *Solution B* as directed for *Chromatographic System*.

**Sample Derivatization Procedure:** Dissolve about 2 µg of the test sample in 20 µL of 15 mM hydrochloric acid, and dilute with 0.2 M borate buffer (pH 8.8) to 80 µL. The derivatization is initiated by the addition of 20 µL of 10 mM AQC in acetonitrile, and allowed to proceed for 10 minutes at room temperature.

**Chromatographic System:** The liquid chromatograph is equipped with a fluorometric detector set at an excitation wavelength of 250 nm and an emission wavelength of 395 nm and a 3.9-mm × 150-mm column that contains 4-µm packing L1. The flow rate is about 1 mL per minute, and the column temperature is maintained at 37°. The system is programmed as follows. The column is equilibrated with *Solution A*; over the next 0.5 minute, the composition of the *Mobile Phase* is changed linearly to 98% *Solution A* and 2% *Solution B*; then over the next 14.5 minutes to 93% *Solution A* and 7% *Solution B*; then over the next 4 minutes to 87% *Solution A* and 13% *Solution B*; over the next 14 minutes to 68% *Solution A* and 32% *Solution B*; then there is a step change to 100% *Solution B* for a 5-minute wash; over the next 10 minutes, there is a step change to 100% *Solution A*; and the column is allowed to equilibrate before the next injection.

**Procedure:** Inject about 0.05 nmol of each AQC-amino acid under test into the chromatograph, and proceed as directed for *Chromatographic System*.

- **Method 5—Precolumn OPA Derivatization**

One method of precolumn OPA derivatization is shown below.

**Mobile Phase Preparation**

**Solution A:** a mixture of 100 mM sodium acetate (pH 7.2), methanol, and tetrahydrofuran (900:95:5).

**Solution B:** methanol.

**Mobile Phase:** Use variable mixtures of *Solution A* and *Solution B* as directed for *Chromatographic System*.

**Derivatization Reagent:** Dissolve 50 mg of OPA in 1.25 mL of methanol (protein sequencing grade). Add 50 µL of 2-mercaptoethanol and 11.2 mL of 0.4 M sodium borate (pH 9.5), and mix. [Note—This reagent is stable for 1 week.]

**Sample Derivatization Procedure:** Transfer about 5 µL of the test sample to an appropriate container, add 5 µL of the *Derivatization Reagent*, and mix. After 1 minute, add not less than 20 µL of 0.1 M sodium acetate (pH 7.0). Use 20 µL of this solution for analysis. [Note—Use of an internal standard (e.g., norleucine) is recommended for quantitative analysis because of potential reagent volume variations in the sample derivatization. The sample derivatization is performed in an automated on-line fashion. Because of the instability of the OPA-amino acid derivative, HPLC separation and analysis are performed immediately following derivatization.]

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**Chromatographic System:** The liquid chromatograph is equipped with a fluorometric detector set at an excitation wavelength of 348 nm and an emission wavelength of 450 nm and a 4.6-mm × 75-mm column that contains 3-µm packing L3. The flow rate is about 1.7 mL per minute, and the column temperature is maintained at 37°. The system is programmed as follows. The column is equilibrated with 92% *Solution A* and 8% *Solution B*; over the next 2 minutes, the composition of the *Mobile Phase* is changed to 83% *Solution A* and 17% *Solution B*, and held for an additional 3 minutes; then changed to 54% *Solution A* and 46% *Solution B* over the next 5 minutes, and held for an additional 2 minutes; then changed to 34% *Solution A* and 66% *Solution B* over the next 2 minutes, and held for 1 minute; then over the next 0.3 minute changed to 20% *Solution A* and 80% *Solution B*, and held for an additional 2.6 minutes; and then finally over 0.6 minute changed to 92% *Solution A* and 8% *Solution B*, and held for an additional 0.6 minute.

**Procedure:** Inject about 0.02 nmol of each OPA-amino acid under test into the chromatograph, and proceed as directed for *Chromatographic System*.

- **Method 6—Precolumn DABS-Cl Derivatization**

One method for precolumn DABS-Cl derivatization is shown below.

**Mobile Phase Preparation**

**Solution A:** 25 mM sodium acetate (pH 6.5) containing 4% of dimethylformamide.

**Solution B:** acetonitrile.

**Mobile Phase:** Use variable mixtures of *Solution A* and *Solution B* as directed for *Chromatographic System*.

**Derivatization Reagent Preparation**

**Sample Buffer:** 50 mM sodium bicarbonate, adjusted to a pH of 8.1.

**Derivatization Reagent:** Dissolve 1.3 mg of DABS-Cl in 1 mL of acetonitrile. [Note—This reagent is prepared fresh shortly before the derivatization step.]

**Sample Dilution Buffer:** Prepare a mixture of 50 mM sodium phosphate (pH 7.0) and alcohol (1:1).

**Sample Derivatization Procedure:** Dissolve the test sample in 20 µL of *Sample Buffer*, add 40 µL of *Derivatization Reagent*, and mix. The sample container is sealed with a silicon-rubber stopper, and heated to 70° for 10 minutes. During the sample heating, the mixture will become completely soluble. After the derivatization, dilute the test sample with an appropriate quantity of the *Sample Dilution Buffer*.

**Chromatographic System:** The liquid chromatograph is equipped with a 436-nm detector and a 4.6-mm × 250-mm column that contains packing L1. The flow rate is about 1 mL per minute, and the column temperature is maintained at 40°. The system is programmed as follows. The column is equilibrated with 85% *Solution A* and 15% *Solution B*; over the next 20 minutes, the composition of the *Mobile Phase* is changed to 60% *Solution A* and 40% *Solution B*; over the next 12 minutes, the composition is changed to 30% *Solution A* and 70% *Solution B*, and held for an additional 2 minutes.

**Procedure:** Inject about 0.05 nmol of the DABS-amino acids into the chromatograph, and proceed as directed for *Chromatographic System*.

- **Method 7—Precolumn FMOC-Cl Derivatization**

One method for precolumn FMOC-Cl derivatization is shown below.

**Mobile Phase Preparation**

**Acetic Acid Buffer:** Transfer 3 mL of glacial acetic acid and 1 mL of triethylamine to a 1-L volumetric flask, and dilute with HPLC-grade water to volume. Adjust with sodium hydroxide to a pH of 4.20.

**Solution A:** a mixture of *Acetic Acid Buffer*, methanol, and acetonitrile (50:40:10).

**Solution B:** a mixture of acetonitrile and *Acetic Acid Buffer* (50:50).

**Mobile Phase:** Use variable mixtures of *Solution A* and *Solution B* as directed for *Chromatographic System*.

**Derivatization Reagent Preparation**

**Borate Buffer:** Prepare a 1 M boric acid solution, and adjust with sodium hydroxide to a pH of 6.2.

**FMOC-Cl Reagent:** Dissolve 155 mg of 9-fluorenylmethyl chloroformate in 40 mL of acetone, and mix.

**Sample Derivatization Procedure:** To 0.4 mL of the test sample add 0.1 mL of *Borate Buffer* and 0.5 mL of *FMOC-Cl Reagent*. After about 40 seconds, extract the mixture with 2 mL of pentane, and then extract again with fresh pentane. The aqueous solution with amino acid derivatives is then ready for injection.

**Chromatographic System:** The liquid chromatograph is equipped with a fluorometric detector set at an excitation wavelength of 260 nm and an emission wavelength of 313 nm and a 4.6-mm × 125-mm column that contains 3-µm packing L1. The flow rate is about 1.3 mL per minute. The system is programmed as follows. The column is equilibrated with *Solution A*, and this composition is maintained for 3 minutes; over the next 9 minutes, it is changed to 100% *Solution B*; then over the next 0.5 minute, the flow rate is increased to 2 mL per minute, and held until the final FMOC-amino acid is eluted from the column. The total run time is about 20 minutes.

**Procedure:** Inject not less than 0.01 nmol of each FMOC-amino acid under test into the chromatograph, and proceed as directed for *Chromatographic System*. The FMOC-histidine derivative will generally give a lower response than the other derivatives.

- **Method 8—Precolumn NBD-F Derivatization**

One method for precolumn NBD-F derivatization is shown below.

**Mobile Phase Preparation**

**Solution A:** a solution of 10 mM sodium citrate containing 75 mM sodium perchlorate, adjusted with hydrochloric acid to a pH of 6.2.

**Solution B:** a mixture of acetonitrile and water (50:50).

**Derivatization Reagent Preparation**

**Sample Buffer:** a 0.1 M boric acid solution, adjusted with sodium hydroxide to a pH of 9.2.

**Derivatization Reagent:** Dissolve 5 mg of NBD-F in 1.0 mL of alcohol, and mix.

**Sample Derivatization Procedure:** Dissolve the test sample in 20 µL of *Sample buffer*, add 10 µL of *Derivatization Reagent*, and mix. The sample container is heated at 60° for 5 minutes. After the derivatization, dilute the test sample with 300 µL of *Solution A*.

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**Chromatographic System:** The liquid chromatograph is equipped with a fluorometric detector set at an excitation wavelength of 480 nm and an emission wavelength of 530 nm and a 4.6-mm × 150-mm column that contains 5-µm particle size ODS silica packing. The flow rate is about 1.0 mL per minute, and the column temperature is maintained at 40°. The system is programmed as follows. The column is equilibrated with 94% *Solution A* and 6% *Solution B*; over the next 16 minutes, the composition is changed linearly to 63% *Solution A* and 37% *Solution B*; over the next 5 minutes, the composition is changed linearly to 62% *Solution A* and 38% *Solution B*; over the next 9 minutes, the composition is changed linearly to 100% *Solution B*, and held for an additional 5 minutes; then finally over 2 minutes, the composition is changed linearly to 94% *Solution A* and 6% *Solution B*; and then the column is allowed to equilibrate before the next injection.

**Procedure:** Inject about 15 pmol of each NBD-amino acid under test into the chromatograph, and proceed as directed for *Chromatographic System*.

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# ⟨1053⟩ CAPILLARY ELECTROPHORESIS

This chapter provides guidance and procedures used for characterization of biotechnology-derived articles by capillary electrophoresis. This chapter is harmonized with the corresponding chapter in *JP* and *EP*. Other characterization tests, also harmonized, are shown in *Biotechnology-Derived Articles—Amino Acid Analysis* ⟨1052⟩, *Biotechnology-Derived Articles—Isoelectric Focusing* ⟨1054⟩, *Biotechnology-Derived Articles—Peptide Mapping* ⟨1055⟩, *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* ⟨1056⟩, and *Biotechnology-Derived Articles—Total Protein Assay* ⟨1057⟩.

## INTRODUCTION

Capillary electrophoresis is a physical method of analysis based on the migration, inside a capillary, of charged analytes dissolved in an electrolyte solution under the influence of a direct-current electric field. In this section we are describing four capillary electrophoresis methods: *Capillary Zone Electrophoresis*, *Capillary Gel Electrophoresis, Capillary Isoelectric Focusing*, and *Micellar Electrokinetic Chromatography*.

## GENERAL PRINCIPLES

The migration velocity of the analyte under an electric field of intensity $E$ is determined by the electrophoretic mobility of the analyte and the electroosmotic mobility of the buffer inside the capillary. The electrophoretic mobility of a solute ($\mu_{ep}$) depends on the characteristics of the solute (electrical charge, molecular size, and shape) and the characteristics of the buffer in which the migration takes place (type and ionic strength of the electrolyte, pH, viscosity, and additives). The electrophoretic velocity ($v_{ep}$) of a solute, assuming a spherical shape, is as follows:

$$v_{ep} = \mu_{ep}E = \left(\frac{q}{6\pi\eta r}\right)\left(\frac{V}{L}\right)$$

in which $q$ is the effective charge of the solute; $\eta$ is the viscosity of the electrolyte solution; $r$ is the Stoke's radius of the solute; $V$ is the applied voltage; and $L$ is the total length of the capillary.

When an electric field is applied through the capillary filled with buffer, a flow of solvent, called electroosmotic flow, is generated inside the capillary. Its velocity depends on the electroosmotic mobility ($\mu_{eo}$), which in turn depends on the charge density on the capillary internal wall and the buffer characteristics. The electroosmotic velocity ($v_{eo}$) is given by the equation:

$$v_{eo} = \mu_{eo}E = \left(\frac{\varepsilon\zeta}{\eta}\right)\left(\frac{V}{L}\right)$$

in which $\varepsilon$ is the dielectric constant of the buffer; $\zeta$ is the zeta potential of the capillary surface; and the other terms are as defined above.

The velocity of the solute ($v$) is given by the equation:

$$v = v_{ep} + v_{eo}$$

The electrophoretic mobility of the analyte and the electroosmotic mobility may act in the same direction or in opposite directions, depending on the charge of the solute. In normal capillary electrophoresis, anions will migrate in the opposite direction to the electroosmotic flow and their velocities will be smaller than the electroosmotic velocity. Cations will migrate in the same direction as the electroosmotic flow and their velocities will be greater than the electroosmotic velocity. Under conditions in which there is a fast electroosmotic velocity with respect to the electrophoretic velocity of the solutes, both cations and anions can be separated in the same run. The time ($t$) taken by the solute to migrate the distance ($l$) from the injection end of the capillary to the detection point (capillary effective length) is as follows:

$$t = \frac{l}{v_{ep} + v_{eo}} = \frac{l(L)}{V\left(\mu_{ep} + \mu_{eo}\right)}$$

in which the other terms are as defined above.

In general, uncoated fused-silica capillaries above pH 3 have negative charge due to ionized silanol groups in the inner wall. Consequently, the electroosmotic flow is from anode to cathode. The electroosmotic flow must remain constant from run to run to obtain good reproducibility in the migration velocity of the solutes. For some applications, it might be necessary to reduce or suppress the electroosmotic flow by modifying the inner wall of the capillary or by changing the concentration, composition, and/or the pH of the buffer solution.

After the introduction of the sample into the capillary, each analyte ion of the sample migrates within the background electrolyte as an independent zone according to its electrophoretic mobility. Zone dispersion, that is the spreading of each solute band, results from different phenomena. Under ideal conditions, the sole contribution to the solute-zone broadening is molecular diffusion of the solute along the capillary (longitudinal diffusion). In this ideal case, the efficiency of the zone, expressed as the number of theoretical plates ($N$), is given by:

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$$N = \frac{\left(\mu_{ep} + \mu_{eo}\right)(VI)}{2DL}$$

in which $D$ is the molecular diffusion coefficient of the solute in the buffer.

In practice, other phenomena, such as heat dissipation, sample adsorption onto the capillary wall, mismatched conductivity between sample and buffer, length of the injection plug, detector cell size, and unleveled buffer reservoirs, can also significantly contribute to band dispersion. Separation between two bands (expressed by the resolution, $R_S$) can be obtained by modification of the electrophoretic mobility of the analytes, by the electroosmotic mobility induced in the capillary, and by increasing the efficiency for the band of each analyte as follows:

$$R_S = \frac{\sqrt{N}\left(\mu_{epb} - \mu_{epa}\right)}{4\left(\overline{\mu}_{ep} + \mu_{eo}\right)}$$

in which $\mu_{epa}$ and $\mu_{epb}$ are the electrophoretic mobilities of the two analytes to be separated; $\overline{\mu}_{ep}$ is the average electrophoretic mobility of the two analytes calculated as:

$$\overline{\mu}_{ep} = \frac{1}{2}(\mu_{epb} + \mu_{epa})$$

## APPARATUS

An apparatus for capillary electrophoresis is composed of a high voltage controllable direct current power supply; two buffer reservoirs held at the same level and containing specified anodic and cathodic solutions; two electrode assemblies (cathode and anode) immersed in the buffer reservoirs and connected to the power supply; a separation capillary usually made of fused-silica, sometimes with an optical viewing window aligned with the detector, depending on the detector type, with the ends of the capillary placed in the buffer reservoirs and the capillary being filled with a solution specified in a given monograph; a suitable injection system; a detector capable of monitoring the amount of substance of interest passing through a segment of the separation capillary at a given time, generally based on absorption spectrophotometry (UV and visible), fluorimetry, conductimetric, amperometric, or mass spectrometric detection, depending on the specific applications, or even indirect detection to detect non-UV-absorbing and nonfluorescent compounds; a thermostatic system capable of maintaining a constant temperature inside the capillary, recommended to obtain good separation reproducibility; a recorder; and a suitable integrator or a computer.

The definition of the injection process and its automation are critical for precise quantitative analysis. Modes of injection include gravity, pressure or vacuum, or electrokinetic injection. The amount of each sample component introduced electrokinetically depends on its electrophoretic mobility, leading to possible discrimination using this injection mode.

It is expected that the capillary, the buffer solutions, the preconditioning method, the sample solution, and the migration conditions will be specified in the individual monograph. The electrolytic solution employed is filtered to remove particles and degassed to avoid bubble formation that could interfere with the detection system or interrupt the electrical contact in the capillary during the separation run. To achieve reproducible migration time of the solutes, it would be necessary to develop, for each analytical method, a rigorous rinsing routine.

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## CAPILLARY ZONE ELECTROPHORESIS

### Principle

In capillary zone electrophoresis, analytes are separated in a capillary containing only buffer without any anticonvective medium. In this technique, separation takes place because the different components of the sample migrate as discrete bands with different velocities. The velocity of each band depends on the electrophoretic mobility of the solute and the electroosmotic flow on the capillary (see *General Principles*). Coated capillaries can be used to increase the separation capacity of those substances adsorbing on fused-silica surfaces.

This mode of capillary electrophoresis is appropriate for the analysis of small (MW < 2000) and large (2000 < MW < 100,000) molecules. Due to the high efficiency achieved in capillary zone electrophoresis, separation of molecules having only minute differences in their charge-to-mass ratio can be effected. This separation mode also allows the separation of chiral compounds by addition of chiral selectors to the separation buffer.

### Optimization

Optimization of the separation is a complex process where several separation parameters can play a major role. The main factors to be considered in the development of the separations are instrumental and electrolytic solution parameters.

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## Instrumental Parameters

### VOLTAGE

A Joule heating plot is useful in optimizing the applied voltage and column temperature. The separation time is inversely proportional to applied voltage. However, an increase in the voltage used can cause excessive heat production, giving rise to temperature and, as a result, viscosity gradients in the buffer inside the capillary, which causes band broadening and decreases resolution.

### POLARITY

Electrode polarity can be normal (anode at the inlet and cathode at the outlet) and the electroosmotic flow will move toward the cathode. If the electrode polarity is reversed, the electroosmotic flow is away from the outlet and only charged analytes with electroosmotic mobilities greater than the electroosmotic flow will pass to the outlet.

### TEMPERATURE

The main effect of temperature is observed on buffer viscosity and electrical conductivity, thus affecting migration velocity. In some cases, an increase in capillary temperature can cause a conformational change of some proteins, modifying their migration time and the efficiency of the separation.

### CAPILLARY

The length and internal diameter of the capillary affects the analysis time, the efficiency of separations, and the load capacity. Increasing both effective length and total length can decrease the electric fields, at a constant voltage, which increases migration time. For a given buffer and electric field, heat dissipation (thus, sample band broadening) depends on the internal diameter of the capillary. The latter also affects the detection limit, depending on the sample volume injected into the capillary and the detection system used.

The adsorption of sample components on the capillary wall limits efficiency; therefore, methods to avoid these interactions should be considered in the development of a separation method. In the specific case of proteins, several strategies have been devised to avoid adsorption on the capillary wall. Some of these strategies (use of extreme pH and adsorption of positively charged buffer additives) only require modification of the buffer composition to prevent protein adsorption. Other strategies include the coating of the internal wall of the capillary with a polymer covalently bonded to the silica that prevents interaction between the proteins and the negatively charged silica surface. For this purpose, ready-to-use capillaries with coatings consisting of neutral-hydrophilic, cationic, and anionic polymers are commercially available.

## Electrolytic Solution Parameters

### BUFFER TYPE AND CONCENTRATIONS

Suitable buffers for capillary electrophoresis have an appropriate buffer capacity in the pH range of choice and low mobility to minimize current generation.

To minimize band distortion, it is important to match buffer–ion mobility to solute mobility whenever possible. The type of sample solvent used is important to achieve on-column sample focusing, which increases separation efficiency and improves detection. Also, an increase in buffer concentration at a given pH decreases electroosmotic flow and solute velocity.

### BUFFER pH

The pH of the buffer can affect separation by modifying the charge of the analyte or additives and by changing the electroosmotic flow. For protein and peptide separation, a change in the pH of the buffer from above the isoelectric point to below the isoelectric point changes the net charge of the solute from negative to positive. An increase in the buffer pH generally increases the electroosmotic flow.

### ORGANIC SOLVENTS

Organic modifiers, such as methanol, acetonitrile, and others, may be added to the aqueous buffer to increase the solubility of the solute or other additives and/or to affect the ionization degree of the sample components. The addition of these organic modifiers to the buffer generally causes a decrease in the electroosmotic flow.

### ADDITIVES FOR CHIRAL SEPARATIONS

To separate ▲enantiomers,▲ (USP 1-Aug-2020) a chiral selector is added to the separation buffer. The most commonly used chiral selectors are cyclodextrins, although in some cases crown ethers, certain polysaccharides, or even proteins can be used. Because chiral recognition is governed by the different interactions between the chiral selector and each of the enantiomers, the resolution achieved for the chiral compounds depends largely on the type of chiral selector used. While developing a given separation it may be useful to test cyclodextrins having a different cavity size (α-, β-, or γ-cyclodextrin) or modified cyclodextrins with neutral (methyl, ethyl, hydroxyalkyl, etc.) or ionizable (aminomethyl, carboxymethyl, sulfobutylether, etc.) moities. When using modified cyclodextrins, batch-to-batch variations in the degree of substitution of the cyclodextrins must be taken into account because it will influence the selectivity. The resolution of chiral separations is also controlled by the concentration of

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the chiral selector, the composition and pH of the buffer, and the separation temperature. Organic additives, such as methanol or urea, can also affect the resolution of separation.

## CAPILLARY GEL ELECTROPHORESIS

In capillary gel electrophoresis, separation takes place inside a capillary filled with a gel that acts as a molecular sieve. Molecules with similar charge-to-mass ratios are separated according to molecular size because smaller molecules move more freely through the network of the gel and therefore migrate faster than larger molecules. Different biological macromolecules (for example, proteins and DNA fragments), which often have similar charge-to-mass ratios, can thus be separated according to their molecular mass by capillary gel electrophoresis.

### Characteristics of Gels

Two types of gels are used in capillary electrophoresis: permanently coated gels and dynamically coated gels. Permanently coated gels are prepared inside the capillary by polymerization of monomers. One example of such a gel is a cross-linked polyacrylamide. This type of gel is usually bonded to the fused-silica wall and cannot be removed without destroying the capillary. For protein analysis under reducing conditions, the separation buffer usually contains sodium dodecyl sulfate, and the sample is denatured by heating in a mixture of sodium dodecyl sulfate and 2-mercaptoethanol or dithiothreitol before injection. When nonreducing conditions are used (for example, analysis of an intact antibody), 2-mercaptoethanol and dithiothreitol are not used. Optimization of separation in a cross-linked gel is obtained by modifying the separation buffer (see *Capillary Zone Electrophoresis*) and by controlling the gel porosity during the gel preparation. For cross-linked polyacrylamide gels, the porosity can be modified by changing the concentration of acrylamide and/or the ratio of the cross-linker. As a rule, a decrease in the porosity of the gel leads to a decrease in the mobility of the solutes. Due to the rigidity of this type of gel, only electrokinetic injection can be used.

Dynamically coated gels are hydrophilic polymers (i.e., linear polyacrylamide, cellulose derivatives, dextran, etc.) which can be dissolved in aqueous separation buffers, giving rise to a separation medium that also acts as a molecular sieve. These polymeric separation media are easier to prepare than cross-linked polymers. They can be prepared in a vial and filled by pressure in a wall-coated capillary with no electroosmotic flow. Replacing the gel before every injection generally improves the separation reproducibility. The porosity of the dynamically coated gels can be increased by using polymers of higher molecular mass (at a given polymer concentration) or by decreasing the polymer concentration (for a given polymer molecular mass). A decrease in gel porosity leads to a decrease in the mobility of the solute for the same buffer. Both hydrodynamic and electrokinetic injection techniques can be used because the dissolution of these polymers in the buffer gives low viscosity solutions.

## CAPILLARY ISOELECTRIC FOCUSING

### Principle

In isoelectric focusing the molecules migrate under the influence of the electric field, so long as they are charged, in a pH gradient generated by ampholytes having pI values in a wide range (polyaminocarboxylic acids), dissolved in the separation buffer.

The three basic steps in capillary isoelectric focusing are loading, focusing, and mobilization.

#### LOADING

Two methods may be employed.

**Loading in One Step**—The sample is mixed with ampholytes and introduced into the capillary by pressure or vacuum.

**Sequential Loading**—A leading buffer, then the ampholytes, then the sample mixed with ampholytes, again ampholytes alone, and finally the terminating buffer are introduced into the capillary. The volume of the sample must be small enough so as not to modify the pH gradient.

#### FOCUSING

When the voltage is applied, ampholytes migrate toward the cathode or the anode according to their net charge, creating the pH gradient from anode (lower pH) to cathode (higher pH). During this step the components to be separated migrate until they reach a pH corresponding to their isoelectric point, and the current drops to very low values.

#### MOBILIZATION

If mobilization is required for detection, use one of the following methods. Three methods are available.

**Method 1**—Mobilization is accomplished during *Focusing*, under the influence of the electroosmotic flow when this flow is small enough to allow the focusing of the components.

**Method 2**—Mobilization is accomplished by application of positive pressure after *Focusing*.

**Method 3**—Mobilization is achieved after *Focusing*, by adding salts to the cathode reservoir or the anode reservoir, depending on the direction chosen for mobilization, in order to alter the pH in the capillary when the voltage is applied. As the pH is changed, the proteins and ampholytes are mobilized in the direction of the reservoir, which contains added salts, and pass the detector.

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The separation achieved is expressed as ΔpI and depends on the pH gradient ($d$pH/$dx$), the number of ampholytes having different pI values, the molecular diffusion coefficient ($D$), the intensity of the electric field ($E$), and the variation of the electrophoretic mobility of the analyte with the pH ($-d\mu/d$pH):

$$\Delta pI = 3\sqrt{\frac{D(d\text{pH}/dx)}{E(-d\mu/d\text{pH})}}$$

## Optimization

The major parameters that need to be considered in the development of separations are the following:

### VOLTAGE

The use of high fields from 300 V/cm to 1,000 V/cm during *Focusing*.

### CAPILLARY

Depending on the *Mobilization* strategy selected (see above), the electroosmotic flow must be reduced or suppressed. Coated capillaries tend to reduce the electroosmotic flow.

### SOLUTIONS

The anode buffer reservoir is filled with a solution of a lower pH than the pI of the most acidic ampholyte, and the cathode reservoir is filled with a solution with a higher pH than the pI of the most basic ampholyte. Phosphoric acid for the anode and sodium hydroxide for the cathode are frequently used.

Addition of a polymer, like methylcellulose, in the ampholyte solution tends to suppress convective forces (if any) and electroosmotic flow by increasing the viscosity. Commercial ampholytes covering many pH ranges are available and may also be mixed to obtain an expanded pH range. Broad pH ranges are used to estimate the isoelectric point (pI), whereas narrower ranges are employed to improve accuracy. Calibration can be made by correlating migration time with the isoelectric point of a series of standard protein markers. During *Focusing*, precipitation of proteins at their isoelectric point can be prevented, if necessary, using buffer additives such as glycerol, surfactants, urea, or zwitterionic buffers. However, depending on the concentration, urea can denature proteins.

# MICELLAR ELECTROKINETIC CHROMATOGRAPHY (MEKC)

## Principle

Separation takes place in an electrolytic solution that contains a surfactant at a concentration above the critical micellar concentration (cmc). The solute molecules are distributed between the aqueous buffer and the pseudo-stationary phase composed by the micelles according to the solute's partition coefficient. The technique can be considered as a hybrid of electrophoresis and chromatography. It is a technique that can be used for the separation of both neutral and charged solutes maintaining the efficiency, speed, and instrumental suitability of capillary electrophoresis. One of the most widely used surfactants in MEKC is the anionic surfactant, sodium dodecyl sulfate, although other surfactants, such as cationic surfactant cetyl trimethyl ammonium salts, have also been used.

The separation mechanism is as follows. At neutral and alkaline pH, a strong electroosmotic flow is generated and moves the separation buffer ions in the direction of the cathode. If sodium dodecyl sulfate is used as surfactant, the electrophoretic migration of the anionic micelle is in the opposite direction, towards the anode. As a result, the overall micelle migration velocity is slowed compared to the bulk flow of the electrolytic solution. In the case of neutral solutes, because the analyte can partition between the micelle and the aqueous buffer and has no electrophoretic mobility, the analyte migration velocity will depend only on the partition coefficient between the micelle and the aqueous buffer. In the electropherogram, the peaks corresponding to each uncharged solute are always between that of the electroosmotic flow marker and that of the micelle; and the time elapsed between these two peaks is called the separation window. For electrically charged solutes, the migration velocity depends on both the partition coefficient of the solute between the micelle and the aqueous buffer and on the electrophoretic mobility of the solute in the absence of micelles.

Because the mechanism in MEKC of neutral and weakly ionized solutes is essentially chromatographic, migration of the solute and resolution can be rationalized in terms of the retention factor of the solute (k′), also referred to as mass distribution ratio ($D_m$), which is the ratio between the number of moles of solute in the micelle to those in the mobile phase. For a neutral compound, k′ is as follows:

$$k' = \frac{t_r - t_0}{t_0\left(1 - t_r/t_{mc}\right)} = K\left(\frac{V_S}{V_M}\right)$$

in which $t_r$ is the migration time of the solute; $t_0$ is the analysis time of the unretained solute obtained by injecting an electroosmotic flow marker that does not enter the micelle (e.g., methanol); $t_{mc}$ is the micelle migration time measured by injecting a micelle marker, such as Sudan III, which migrates continuously associated in the micelle; K is the partition coefficient of the solute; $V_S$ is the volume of the micellar phase; and $V_M$ is the volume of the mobile phase.

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The resolution between two closely-migrating solutes ($R_S$) is as follows:

$$R_S = \frac{\sqrt{N}}{4} \times \frac{\alpha - 1}{\alpha} \times \frac{k_b'}{k_b' + 1} \times \frac{1 - \left(\frac{t_0}{t_{mc}}\right)}{1 + k_a' \times \left(\frac{t_0}{t_{mc}}\right)}$$

in which $N$ is the number of theoretical plates for one of the solutes; $\alpha$ is the selectivity; and $k_a'$ and $k_b'$ are retention factors for both solutes, respectively ($k_b' > k_a'$).

Similar, but not identical, equations give k′ and $R_S$ values for electrically charged solutes.

## Optimization

The main parameters to be considered in the development of separations by MEKC are instrumental and electrolytic solution parameters.

### INSTRUMENTAL PARAMETERS

**Voltage**—Separation time is inversely proportional to applied voltage. However, an increase in voltage can cause excessive heat production that gives rise to temperature gradients and viscosity gradients of the buffer in the cross section of the capillary. This effect can be significant with high conductivity buffers, such as those containing micelles. Poor heat dissipation causes band broadening and decreases resolution.

**Temperature**—Variations in capillary temperature affect the partition coefficient of the solute between the buffer and the micelles, the critical micellar concentration, and the viscosity of the buffer. These parameters contribute to the migration time of the solutes. The use of a good cooling system improves the reproducibility of the migration time for the solutes.

**Capillary**—As in *Capillary Zone Electrophoresis*, length and internal diameter of the capillary contribute to analysis time and efficiency of separations. Increasing both effective length and total length can decrease the electrical fields, working at constant voltage, and will increase migration time and improve the separation efficiency. The internal diameter controls heat dissipation, for a given buffer and electrical field, and consequently broadening of the sample band.

### ELECTROLYTIC SOLUTION PARAMETERS

**Surfactant Type and Concentration**—The type of surfactant, as the stationary phase in chromatography, affects the resolution because it modifies separation selectively. The log k′ of a neutral compound increases linearly with the concentration of surfactant in the mobile phase. When k′ approaches the value of

$$\sqrt{t_{mc}/t_0}$$

resolution in MEKC reaches a maximum. Modifying the concentration of surfactant in the mobile phase changes the resolution.

**Buffer pH**—pH does not modify the partition coefficient of nonionized solutes, but it can modify the electroosmotic flow in uncoated capillaries. A decrease in the buffer pH decreases the electroosmotic flow and, therefore, increases the resolution of the neutral solutes in MEKC, resulting in a longer analysis time.

**Organic Solvents**—To improve MEKC separation of hydrophobic compounds, organic modifiers (methanol, propanol, acetonitrile, etc.) can be added to the electrolytic solution. The addition of these modifiers generally decreases migration time and selectivity of the separation. The addition of organic modifiers affects critical micellar concentration, thus, a given surfactant concentration can be used only with a certain percentage of organic modifier before the micellization is inhibited or adversely affected, resulting in the absence of micelles and, therefore, the absence of the partition. The dissociation of micelles in the presence of a high content of organic solvent does not always mean that the separation will no longer be possible, because in some cases, the hydrophobic interaction between the ionic surfactant monomer and the neutral solutes forms solvophobic complexes that can be separated electrophoretically.

**Additives for Chiral Separations**—For the separation of enantiomers using MEKC, a chiral selector is included in the micellar system, either covalently bound to the surfactant or added to the micellar separation electrolyte. Micelles that have a moiety with chiral discrimination properties include salts, *N*-dodecanoyl-L-amino acids, bile salts, etc. Chiral resolution can also be achieved using chiral discriminators, such as cyclodextrins, added to the electrolytic solutions that contain micellized achiral surfactants.

**Other Additives**—Selectivity can be modified by adding chemicals to the buffer. Addition of several types of cyclodextrins to the buffer is also used to reduce the interaction of hydrophobic solutes with the micelle, increasing the selectivity for this type of compound. The addition of substances able to modify solute-micelle interactions by adsorption on the latter has been used to improve the selectivity of the separations in MEKC. These additives may consist of a second surfactant (ionic or nonionic), which gives rise to mixed micelles or metallic cations that dissolve in the micelle and form coordination complexes with the solutes.

## Quantification

Peak areas must be divided by the corresponding migration time to give the corrected area in order to compensate for the shift in migration time from run to run, thus reducing the variation of the response. Dividing the peak areas by migration time

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will also compensate for the different responses of sample constituents with different migration times. Where an internal standard is used, check that no peak of the substance to be examined is masked by that of the internal standard.

CALCULATIONS

From the values obtained, calculate the content of a component or components being determined. When indicated, the percentage of one (or more) components of the sample to be examined is calculated by determining the corrected area(s) of the peak(s) as a percentage of the total of the corrected areas of all the peaks, excluding those due to solvents or any added reagents (normalization procedure). The use of an automatic integration system (integrator or data acquisition and processing system) is recommended.

## SYSTEM SUITABILITY

In order to check the behavior of the capillary electrophoresis system, system suitability parameters are used. The choice of these parameters depends on the mode of capillary electrophoresis used. The parameters include the following: retention factor k′ used only for *Micellar Electrokinetic Chromatography*, apparent number of theoretical plates ($N$), the symmetry factor ($A_S$), and the resolution ($R_S$). Note that in previous sections, the theoretical expressions for $N$ and $R_S$ have been described, but more practical equations that allow for the determination of these suitability parameters using the electropherograms are described below.

### Apparent Number of Theoretical Plates

The apparent number of theoretical plates ($N$) may be calculated from the formula:

$$N = 5.54\ (t_R/w_h)^2$$

in which $t_R$ is the migration time or distance along the baseline between the point of injection and the perpendicular dropped from the maximum of the peak corresponding to the component; and $w_h$ is the peak width at half-height.

### Resolution

The resolution ($R_S$) between peaks of similar heights of two components may be calculated from the formula:

$$R_S = 1.18\ (t_{R2} - t_{R1}\ )/(w_{h1} + w_{h2})$$
$$t_{R2} > t_{R1}$$

in which $t_{R1}$ and $t_{R2}$ are the migration times or distances along the baseline between the point of injection and the perpendiculars dropped from the maxima of two adjacent peaks; and $w_{h1}$ and $w_{h2}$ are the peak widths at half-height.

When appropriate, the resolution ($R_S$) may also be calculated by measuring the height of the valley ($H_v$) between two partly resolved peaks in a standard preparation, the height of the smaller peak ($H_p$), and calculating the peak-to-valley ratio:

$$p/v = H_p/H_v$$

### Symmetry Factor

The symmetry factor of a peak ($A_S$) may be calculated using the formula:

$$A_s = w_{0.05}/2d$$

in which $w_{0.05}$ is the width of the peak at one-twentieth of the peak height; and $d$ is the distance between the perpendicular dropped from the peak maximum and the leading edge of the peak at one-twentieth of the peak height.

Other suitability parameters include tests for area repeatability (standard deviation of areas or of area/migration time) and tests for migration time repeatability (standard deviation of migration time). Migration time repeatability provides a test for the suitability of the capillary washing procedures. An alternative practice to avoid the lack of repeatability of the migration time is to use a migration time relative to an internal standard.

### Signal-to-Noise Ratio

A test for the verification of the signal-to-noise ratio for a standard preparation or the determination of the limit of quantification may also be useful for the determination of related substances. The detection limit and quantification limit correspond to a signal-to-noise ratio of 3 and 10, respectively. The signal-to-noise ratio ($S/N$) is calculated as follows:

$$S/N = 2H/h$$

in which $H$ is the height of the peak corresponding to the component concerned in the electropherogram obtained with the specified reference solution, measured from the maximum of the peak to the extrapolated baseline of the signal observed over a

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distance equal to twenty times the width at half-height; and *h* is the range of the background in an electropherogram obtained after injection of a blank, observed over a distance equal to twenty times the width at the half-height of the peak in the electropherogram obtained with the prescribed reference solution and, if possible, situated equally around the place where this peak would be found.

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# ⟨1054⟩ BIOTECHNOLOGY-DERIVED ARTICLES—ISOELECTRIC FOCUSING

This chapter provides guidance and procedures used for the characterization of biotechnology-derived articles by isoelectric focusing. This chapter is harmonized with the corresponding chapters in *JP* and *EP*. Other characterization tests, also harmonized, are shown in the USP general information chapters *Biotechnology-Derived Articles—Amino Acid Analysis* ⟨1052⟩, *Biotechnology-Derived Articles—Capillary Electrophoresis* ⟨1053⟩, *Biotechnology-Derived Articles—Peptide Mapping* ⟨1055⟩, *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* ⟨1056⟩, and *Biotechnology-Derived Articles—Total Protein Assay* ⟨1057⟩.

## GENERAL PRINCIPLES

Isoelectric focusing (IEF) is a method of electrophoresis that separates proteins according to their isoelectric points. Separation is carried out in a slab of polyacrylamide or agarose gel that contains a mixture of amphoteric electrolytes (ampholytes). When subjected to an electrical field, the ampholytes migrate in the gel to create a pH gradient. In some cases, gels containing an immobilized pH gradient, prepared by incorporating weak acids and bases to specific regions of the gel network during the preparation of the gel, are used. When the applied proteins reach the gel fraction that has a pH that is the same as their isoelectric point (pI), their charge is neutralized and migration ceases. Gradients can be made over various ranges of pH, according to the mixture of ampholytes chosen.

## THEORETICAL ASPECTS

When a protein is at the position of its isoelectric point, it has no net charge and cannot be moved in a gel matrix by the electric field. It may, however, move from that position by diffusion. The pH gradient forces a protein to remain in its isoelectric point position, thus concentrating it; this concentration effect is called “focusing”. Increasing the applied voltage or reducing the sample load results in improved separation of bands. The applied voltage is limited by the heat generated because the heat must be dissipated. The use of thin gels and an efficient cooling plate controlled by a thermostatic circulator prevents the burning of the gel while allowing sharp focusing. The separation is estimated by determining the minimum pI difference (ΔpI), which is necessary to separate two neighboring bands, as follows:

$$\Delta pI = 3\sqrt{\frac{D(dpH/dx)}{E(-d\mu/dpH)}}$$

in which D is the diffusion coefficient of the protein; dpH/dx is the pH gradient; E is the intensity of the electric field, in volts per centimeter; and –dμ/dpH is the variation of the solute mobility with the pH in the region close to the pI. Because D and –dμ/dpH for a given protein cannot be altered, the separation can be improved by using a narrower pH range and by increasing the intensity of the electric field.

Resolution between protein bands on an IEF gel prepared with carrier ampholytes can be quite good. Better resolution may be achieved by using immobilized pH gradients where the buffering species, which are analogous to carrier ampholytes, are copolymerized within the gel matrix. Proteins exhibiting pI values differing by as little as 0.02 pH units may be resolved using a gel prepared with carrier ampholytes, whereas immobilized pH gradients can resolve protein differing by approximately 0.001 pH units.

## PRACTICAL ASPECTS

From an operational point, special attention must be paid to sample characteristics and/or preparation. Salt in a sample can be problematic, and it is best to prepare the sample, if possible, in deionized water or 2% ampholytes using dialysis or gel filtration if necessary. The time required for completion of focusing in thin-layer polyacrylamide gels is determined by placing a colored protein (e.g., hemoglobin) at different positions on the gel surface and by applying the electric field: the steady state is reached when all applications give an identical band pattern. In some procedures the completion of the focusing is indicated by the time elapsed after the sample application.

The IEF gel can be used as an identity test when the migration pattern on the gel is compared to a suitable standard preparation and IEF calibration proteins; the IEF gel can be used as a limit test when the density of a band on IEF is compared subjectively with the density of bands appearing in a standard preparation, or it can be used as a quantitative test when the density is measured using a densitometer or similar instrumentation to determine the relative concentration of protein in the bands subject to validation.

## APPARATUS

An apparatus for isoelectric focusing consists of a controllable generator for constant potential, current, and power. Potentials of 2500 V have been used and are considered optimal under a given set of operating conditions. Supply of up to 30 W of constant power is recommended. The apparatus also includes a rigid plastic isoelectric focusing chamber that contains a cooled

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plate of suitable material to support the gel; and a plastic cover with platinum electrodes that are connected to the gel by means of paper wicks of suitable width, length, and thickness, impregnated with solutions of anodic and cathodic electrolytes.

## ISOELECTRIC FOCUSING IN POLYACRYLAMIDE GELS: DETAILED PROCEDURE

The following method is a detailed description of an IEF procedure in thick polyacrylamide slab gels, which is used unless otherwise stated in the monograph.

### Preparation of the Gels

#### MOLD

The mold is composed of a glass plate (A) on which a polyester film (B) is placed to facilitate handling of the gel, one or more spacers (C), a second glass plate (D), and clamps to hold the structure together (see *Figure 1*).

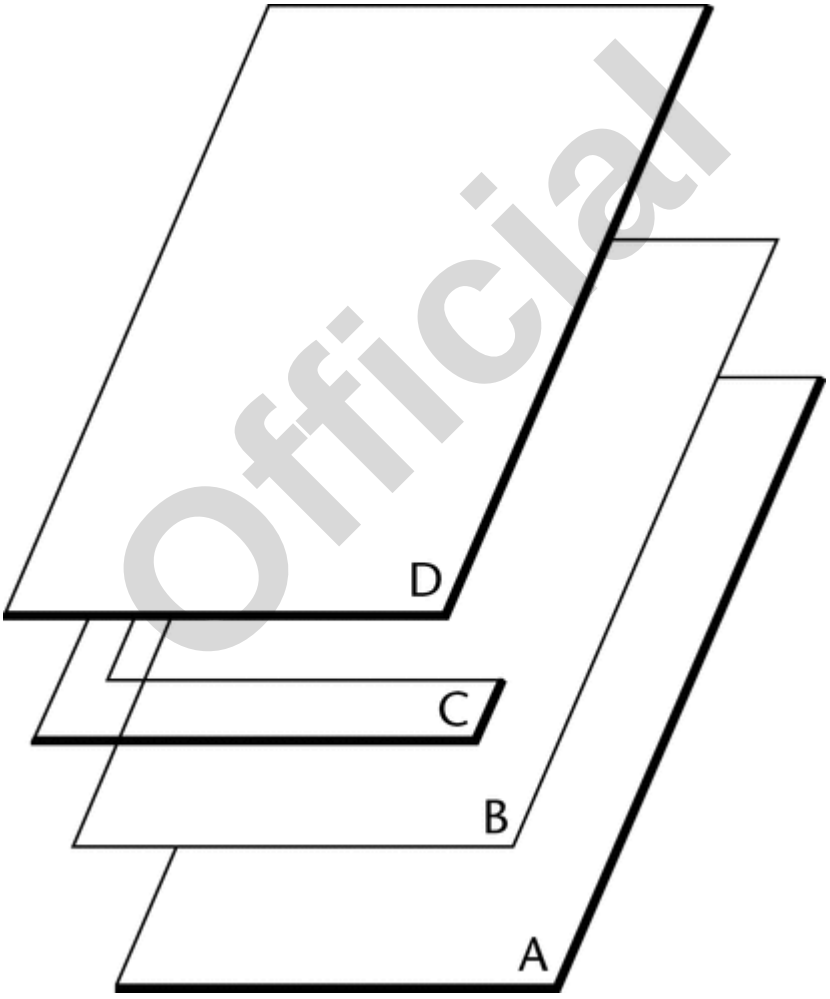


Figure 1. Mold

#### 7.5% POLYACRYLAMIDE GEL

Dissolve 29.1 g of acrylamide and 0.9 g of methylenebisacrylamide in 100 mL of water. To 2.5 volumes of this solution, add the mixture of ampholytes specified in the individual monograph, and dilute to 10 volumes with water. Mix carefully, and degas the solution.

#### PREPARATION OF THE MOLD

Place the polyester film on the lower glass plate, apply the spacer, place the second glass plate, and fit the clamps. Before use, place the mixture on a magnetic stirrer, and add 0.25 volumes of a 100 g/L solution of ammonium persulfate and 0.25 volumes of tetramethylenediamine. Immediately fill the space between the glass plates of the mold with the solution.

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FIXING SOLUTION FOR ISOELECTRIC FOCUSING POLYACRYLAMIDE GEL

Mix 35 g of sulfosalicylic acid and 100 g of trichloroacetic acid in 1000 mL of water.

COOMASSIE STAINING SOLUTION AND DESTAINING SOLUTION

Use the same solutions indicated in general information chapter *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* ⟨1056⟩.

## Procedure

Dismantle the *Mold*, and using the polyester film, transfer the gel onto the cooled support wetted with a few mL of a suitable liquid, taking care to avoid forming air bubbles. Prepare the test solutions and reference solutions as specified in the individual monograph. Place strips of paper for sample application, about 10 mm × 5 mm in size, on the gel, and impregnate each with the prescribed amount of the test and reference solutions. Also apply the prescribed quantity of a solution of proteins with known isoelectric points as pH markers to calibrate the gel. In some procedures, the gel has precast slots where a solution of the sample is applied instead of using impregnated paper strips. Cut two strips of paper to the length of the gel, and impregnate them with the electrolyte solutions: acid for the anode and alkaline for the cathode. The compositions of the anode and cathode solutions are given in the individual monograph. Apply these paper wicks to each side of the gel several mm from the edge. Fit the cover so that the electrodes are in contact with the wicks (with respect to the anodic and cathodic poles). Proceed with the isoelectric focusing by applying the electrical parameters described in the individual monograph. Switch off the current when the migration of the mixture of standard proteins has stabilized. Using forceps, remove the sample application strips and the two electrode wicks. Immerse the gel in *Fixing Solution for Isoelectric Focusing Polyacrylamide Gel*. Incubate with gentle shaking at room temperature for 30 minutes. Drain off the solution, and add 200 mL of *Destaining Solution*. Incubate with shaking for 1 hour. Drain the gel, and add *Coomassie Staining Solution*. Incubate for 30 minutes. Destain the gel by passive diffusion with *Destaining Solution* until the bands are well visualized against a clear background. Locate the position and intensity of the bands in the electropherogram, as prescribed in the individual monograph.

VARIATIONS TO THE DETAILED PROCEDURE (SUBJECT TO VALIDATION)

Where reference to the general method on isoelectric focusing is made, variations in methodology or procedure may be made subject to validation. These variations include the use of commercially available precast gels and of commercial staining and destaining kits; the use of immobilized pH gradients; the use of rod gels, and the use of cassettes of different dimensions, including ultra-thin (0.2 mm) gels; variations in the sample application procedure, including different sample volumes or the use of sample application masks or wicks other than paper; the use of alternate running conditions, including variations in the electric field depending on gel dimensions and equipment, and the use of fixed migration times rather than subjective interpretation of band stability; the inclusion of a prefocusing step; the use of automated instrumentation; and the use of agarose gels.

## VALIDATION OF ISOELECTRIC FOCUSING PROCEDURES

Where alternative methods to the detailed procedure are employed, they must be validated. The following criteria may be used to validate the separation: formation of a stable pH gradient of desired characteristics, assessed for example using colored pH markers of known isoelectric points; comparison with the electropherogram provided with the chemical reference substance for the preparation to be examined; and any other validation criteria as prescribed in the individual monograph.

## SPECIFIED VARIATIONS TO THE GENERAL METHOD

Variations to the general method required for the analysis of specific substances may be specified in detail in individual monographs. Variations may include the addition of urea in the gel (a 3 M concentration is often satisfactory to keep the protein in solution, but up to 8 M can be used). Some proteins precipitate at their isoelectric point. In this case, urea is included in the gel formulation to keep the protein in solution. If urea is used, only fresh solutions should be used to prevent carbamylation of the protein. Other variations include the use of alternative staining methods and the use of gel additives such as nonionic detergents (e.g., octylglucoside) or zwitterionic detergents (e.g., CHAPS or CHAPSO) and the addition of ampholyte to the sample to prevent proteins from aggregating or precipitating.

## POINTS TO CONSIDER

1. Samples can be applied to any area on the gel, but to protect the proteins from extreme pH environments, samples should not be applied close to either electrode. During method development, the analyst can try applying the protein in three positions on the gel (e.g., middle and both ends); the pattern of a protein applied at opposite ends of the gel may not be identical.
2. A phenomenon known as cathodic drift, where the pH gradient decays over time, may occur if a gel is focused too long. Although not well understood, electroendoosmosis and absorption of carbon dioxide may be factors that lead to cathodic drift. Cathodic drift is observed as focused protein migrating off the cathode end of the gel. Immobilized pH gradients may be used to address this problem.

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3. Efficient cooling (approximately 4°) of the bed that the gel lies on during focusing is important. High field strengths used during isoelectric focusing can lead to overheating and affect the quality of the focused gel.

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# ⟨1055⟩ BIOTECHNOLOGY-DERIVED ARTICLES—PEPTIDE MAPPING

This chapter provides guidance and procedures used for characterization of biotechnology-derived articles by peptide mapping. This chapter is harmonized with the corresponding chapter in *JP* and *EP*. Portions of the chapter that are not harmonized with the other two pharmacopeias are marked by the symbol ♦. Other characterization tests, also harmonized, are shown in *Biotechnology-Derived Articles—Amino Acid Analysis* ⟨1052⟩, *Capillary Electrophoresis* ⟨1053⟩, *Biotechnology-Derived Articles—Isoelectric Focusing* ⟨1054⟩, *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* ⟨1056⟩, and *Biotechnology-Derived Articles—Total Protein Assay* ⟨1057⟩.

## INTRODUCTION

Peptide mapping is an identity test for proteins, especially those obtained by rDNA technology. It involves the chemical or enzymatic treatment of a protein, resulting in the formation of peptide fragments, followed by separation and identification of the resultant fragments in a reproducible manner. It is a powerful test that is capable of identifying single amino acid changes resulting from events such as errors in the reading of complementary DNA (cDNA) sequences or point mutations. Peptide mapping is a comparative procedure because the information obtained, compared to a Reference Standard or Reference Material similarly treated, confirms the primary structure of the protein, is capable of detecting whether alterations in structure have occurred, and demonstrates process consistency and genetic stability. Each protein presents unique characteristics that must be well understood so that the scientific and analytical approaches permit validated development of a peptide map that provides sufficient specificity.

This chapter provides detailed assistance in the application of peptide mapping and its validation to characterize the desired protein product, to evaluate the stability of the expression construct of cells used for recombinant DNA products, to evaluate the consistency of the overall process, and to assess product stability, as well as to ensure the identity of the protein product or to detect the presence of protein variant. ♦The validation scheme presented differentiates between qualification of the method at an early stage in the regulatory process, the Investigational New Drug (IND) level, and full validation in support of New Drug Application (NDA), Product License Application (PLA), or Marketing Authorization Application (MAA). The validation concepts described are consistent with the general information chapter *Validation of Compendial Procedures* ⟨1225⟩ and with the International Conference on Harmonization (ICH) document *Analytical Methods Validation*.♦

## THE PEPTIDE MAP

Peptide mapping is not a general method, but involves developing specific maps for each unique protein. Although the technology is evolving rapidly, there are certain methods that are generally accepted. Variations of these methods will be indicated, when appropriate, in specific monographs.

A peptide map may be viewed as a fingerprint of a protein and is the end product of several chemical processes that provide a comprehensive understanding of the protein being analyzed. Four major steps are necessary for the development of the procedure: isolation and purification of the protein, if the protein is part of a formulation; selective cleavage of the peptide bonds; chromatographic separation of the peptides; and analysis and identification of the peptides. A test sample is digested and assayed in parallel with a Reference Standard or Reference Material. Complete cleavage of peptide bonds is more likely to occur when enzymes such as endoproteases (e.g., trypsin) are used instead of chemical cleavage reagents. A map should contain enough peptides to be meaningful. On the other hand, if there are too many fragments, the map might lose its specificity because many proteins will then have the same profiles.

### Isolation and Purification

Isolation and purification are necessary for analysis of bulk drugs or dosage forms containing interfering excipients and carrier proteins and, when required, will be specified in the monograph. Quantitative recovery of protein from the dosage form should be validated.

### Selective Cleavage of Peptide Bonds

The selection of the approach used for the cleavage of peptide bonds will depend on the protein under test. This selection process involves determination of the type of cleavage to be employed—enzymatic or chemical—and the type of cleavage agent within the chosen category. Several cleavage agents and their specificity are shown in *Table 1*. This list is not all-inclusive and will be expanded as other cleavage agents are identified.

**Table 1. Examples of Cleavage Agents**

| Type | Agent | Specificity |
|---|---|---|
| Enzymatic | Trypsin, EC 3.4.21.4 | C-terminal side of Arg and Lys |
| | Chymotrypsin, EC 3.4.21.1 | C-terminal side of hydrophobic residues (e.g., Leu, Met, Ala, aromatics) |
| | Pepsin, EC 3.4.23.1 and EC 3.4.23.2 | Nonspecific digest |
| | Lysyl endopeptidase (Lys-C endopeptidase), EC 3.4.21.50 | C-terminal side of Lys |

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**Table 1. Examples of Cleavage Agents** *(continued)*

| Type | Agent | Specificity |
|---|---|---|
| | Glutamyl endopeptidase; (from *S. aureus* strain V8), EC 3.4.21.19 | *C*-terminal side of Glu and Asp |
| | Peptidyl-Asp metalloendopeptidase (Asp-N endoproteinase), EC 3.4.24.33 | *N*-terminal side of Asp |
| | Clostripain, EC 3.4.22.8 | *C*-terminal side of Arg |
| Chemical | Cyanogen bromide | *C*-terminal side of Met |
| | 2-Nitro-5-thiocyanobenzoic acid | *N*-terminal side of Cys |
| | *o*-Iodosobenzoic acid | *C*-terminal side of Trp and Tyr |
| | Dilute acid | Asp and Pro |
| | BNPS-skatole | Trp |

## Pretreatment of Sample

Depending on the size or the configuration of the protein, different approaches in the pretreatment of samples can be used. For monoclonal antibodies, the heavy and light chains will need to be separated before mapping. If trypsin is used as a cleavage agent for proteins with a molecular mass greater than 100,000 Da, lysine residues must be protected by citraconylation or maleylation; otherwise, too many peptides will be generated.

## Pretreatment of the Cleavage Agent

Pretreatment of cleavage agents, especially enzymatic agents, might be necessary for purification purposes to ensure reproducibility of the map. For example, trypsin used as a cleavage agent will have to be treated with tosyl-L-phenylalanine chloromethyl ketone to inactivate chymotrypsin. Other methods, such as purification of trypsin by HPLC or immobilization of enzyme on a gel support, have been successfully used when only a small amount of protein is available.

## Pretreatment of the Protein

Under certain conditions, it might be necessary to concentrate the sample, or to separate the protein from added substances and stabilizers used in the formulation of the product if these interfere with the mapping procedure. Physical procedures used for pretreatment can include ultrafiltration, column chromatography, and lyophilization.

Other pretreatments, such as the addition of chaotropic agents (e.g., urea) can be used to unfold the protein prior to mapping. To allow the enzyme to have full access to cleavage sites and permit some unfolding of the protein, it is often necessary to reduce and alkylate the disulfide bonds prior to digestion.

Digestion with trypsin can introduce ambiguities in the tryptic map as a result of side reactions occurring during the digestion reaction, such as nonspecific cleavage, deamidation, disulfide isomerization, oxidation of methionine residues, or formation of pyroglutamic groups created from the deamidation of glutamine at the *N*-terminal side of a peptide. Furthermore, peaks may be produced by autohydrolysis of trypsin. Their intensities depend on the ratio of trypsin to protein. To avoid autohydrolysis, solutions of proteases may be prepared at a pH that is not optimal (e.g., at pH 5 for trypsin), which would mean that the enzyme would not become active until diluted with the digest buffer.

## Establishment of Optimal Digestion Conditions

Factors that affect the completeness and effectiveness of digestion of proteins are those that could affect any chemical or enzymatic reactions.

**pH**—The digestion mixture pH is empirically determined to ensure the optimal performance of the given cleavage agent. For example, when using cyanogen bromide as a cleavage agent, a highly acidic environment (e.g., pH 2, formic acid) is necessary; however, when using trypsin as a cleavage agent, a slightly alkaline environment (pH 8) is optimal. As a general rule, the pH of the reaction milieu should not alter the chemical integrity of the protein during the digestion and should not change during the course of the fragmentation reaction.

**Temperature**—A temperature between 25° and 37° is adequate for most digestions. The temperature used is intended to minimize chemical side reactions. The type of protein under test will dictate the temperature of the reaction milieu because some proteins are more susceptible to denaturation as the temperature of the reaction increases. For example, digestion of recombinant bovine somatropin is conducted at 4° because at higher temperatures it will precipitate during digestion.

**Time**—If a sufficient amount of sample is available, a time course study is considered in order to determine the optimum time to obtain a reproducible map and avoid incomplete digestion. Time of digestion varies from 2 to 30 hours. The reaction is stopped by the addition of an acid that does not interfere with the tryptic map, or by freezing.

**Amount of Cleavage Agent**—Although excessive amounts of cleavage agent are used to accomplish a reasonably rapid digestion time (i.e., 6 to 20 hours), the amount of cleavage agent is minimized to avoid its contribution to the chromatographic map pattern. A protein-to-protease ratio between 20:1 and 200:1 is generally used. It is recommended that the cleavage agent be added in two or more stages to optimize cleavage. Nonetheless, the final reaction volume remains small enough to facilitate the next step in peptide mapping—the separation step. To sort out digestion artifacts that might be interfering with the subsequent analysis, a blank determination is performed using a digestion control with all the reagents except the test protein.

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## Chromatographic Separation

Many techniques are used to separate peptides for mapping. The selection of a technique depends on the protein being mapped. Techniques that have been successfully used for the separation of peptides are shown in *Table 2*.

**Table 2. Techniques Used for the Separation of Peptides**

| |
|---|
| Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC) |
| Ion-Exchange Chromatography (IEC) |
| Hydrophobic Interaction Chromatography (HIC) |
| Polyacrylamide Gel Electrophoresis (PAGE), nondenaturating |
| Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) |
| Capillary Electrophoresis (CE) |
| Paper Chromatography—High Voltage (PCHV) |
| High-Voltage Paper Electrophoresis (HVPE) |

In this section, a most widely used reverse-phase HPLC (RP-HPLC) method is described as one of the procedures of chromatographic separation.

The purity of solvents and mobile phases is a critical factor in HPLC separation. HPLC-grade solvents and water that are commercially available are recommended for RP-HPLC. Dissolved gases present a problem in gradient systems where the solubility of the gas in a solvent may be less in a mixture than in a single solvent. Vacuum degassing and agitation by sonication are often used as useful degassing procedures. When solid particles in the solvents are drawn into the HPLC system, they can damage the sealing of pump valves or clog the top of the chromatographic column. Both pre- and post-pump filtration are also recommended.

### Chromatographic Column

The selection of a chromatographic column is empirically determined for each protein. Columns with 100 Å or 300 Å pore size and silica support can give optimal separation. For smaller peptides, octylsilane chemically bonded to totally porous silica particles 3 to 10 µm in diameter (L7) and of octadecylsilane chemically bonded to porous silica or ceramic microparticles 3 to 10 µm in diameter (L1) column packings are more efficient than the butyl silane chemically bonded to totally porous silica particles 5 to 10 µm in diameter (L26) packing.

### Solvent

The most commonly used solvent is water with acetonitrile as the organic modifier to which less than 0.1% of trifluoroacetic acid is added. If necessary, add isopropyl alcohol or *n*-propyl alcohol to solubilize the digest components, provided that the addition does not unduly increase the viscosity of the components.

### Mobile Phase

Buffered mobile phases containing phosphate are used to provide some flexibility in the selection of pH conditions, because shifts of pH in the 3.0 to 5.0 range enhance the separation of peptides containing acidic residues (e.g., glutamic and aspartic acids). Sodium or potassium phosphates, ammonium acetate, phosphoric acid, and a pH between 2 and 7 (or higher for polymer-based supports) have also been used with acetonitrile gradients. Acetonitrile-containing trifluoroacetic acid is also used quite often.

### Gradient Selection

Gradients can be linear, nonlinear, or include step functions. A shallow gradient is recommended in order to separate complex mixtures. Gradients are optimized to provide clear resolution of one or two peaks that will become “marker” peaks for the test.

### Isocratic Selection

Isocratic HPLC systems using a single mobile phase are used on the basis of their convenience of use and improved detector responses. Optimal composition of a mobile phase to obtain clear resolution of each peak is sometimes difficult to establish. Mobile phases for which slight changes in component ratios or in pH significantly affect retention times of peaks in peptide maps should not be used in isocratic HPLC systems.

### Other Parameters

Temperature control of the column is usually necessary to achieve good reproducibility. The flow rates for the mobile phases range from 0.1 to 2.0 mL per minute, and the detection of peptides is performed with a UV detector at 200 to 230 nm. Other methods of detection have been used (e.g., postcolumn derivatization), but they are not as robust or as versatile as UV detection.

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## System Suitability

The section *System Suitability* under *Chromatography* ⟨621⟩ provides an experimental means for measuring the overall performance of the test method. The acceptance criteria for system suitability depend on the identification of critical test parameters that affect data interpretation and acceptance. These critical parameters are also criteria that monitor peptide digestion and peptide analysis. An indicator that the desired digestion endpoint was achieved is the comparison with a Reference Standard or Reference Material, which is treated exactly as the article under test. The use of a USP Reference Standard in parallel with the protein under test is critical in the development and establishment of system suitability limits. In addition, a specimen chromatogram should be included with the USP Reference Standard or Reference Material for additional comparison purposes. Other indicators may include visual inspection of protein or peptide solubility, the absence of intact protein, or measurement of responses of a digestion-dependent peptide. The critical system suitability parameters for peptide analysis will depend on the particular mode of peptide separation and detection, and on the data analysis requirements.

When peptide mapping is used as an identification test, the system suitability requirements for the identified peptides cover selectivity and precision. In this case, as well as when identification of variant proteins is done, the identification of the primary structure of the peptide fragments in the peptide map provides both a verification of the known primary structure and the identification of protein variants by comparison with the peptide map of the USP Reference Standard or Reference Material for the specified protein. The use of a digested USP Reference Standard or Reference Material for a given protein in the determination of peptide resolution is the method of choice. For an analysis of a variant protein, a characterized mixture of a variant and a Reference Standard can be used, especially if the variant peptide is located in a less-resolved region of the map. The index of pattern consistency can be simply the number of major peptides detected. Peptide pattern consistency can be best defined by the resolution of peptide peaks. Chromatographic parameters—such as peak-to-peak resolution, maximum peak width, peak area, peak tailing factors, and column efficiency—may be used to define peptide resolution. Depending on the protein under test and the method of separation used, single peptide or multiple peptide resolution requirements may be necessary.

The replicate analysis of the digest of the USP Reference Standard or Reference Material for the protein under test yields measures of precision and quantitative recovery. Recovery of the identified peptides is generally ascertained by the use of internal or external peptide standards. The precision is expressed as the relative standard deviation (RSD). Differences in the recovery and precision of the identified peptides are expected; therefore, the system suitability limits will have to be established for both the recovery and the precision of the identified peptides. These limits are unique for a given protein and will be specified in the individual monograph.

Visual comparison of the relative retention times, the peak responses (the peak area or the peak height), the number of peaks, and the overall elution pattern is completed initially. It is then complemented and supported by mathematical analysis of the peak response ratios and by the chromatographic profile of a 1:1 (v/v) mixture of sample and USP Reference Standard or Reference Material digest. If all peaks in the sample digest and in the USP Reference Standard or Reference Material digest have the same relative retention times and peak response ratios, then the identity of the sample under test is confirmed.

If peaks that initially eluted with significantly different relative retention times are then observed as single peaks in the 1:1 mixture, the initial difference would be an indication of system variability. However, if separate peaks are observed in the 1:1 mixture, this would be evidence of the nonequivalence of the peptides in each peak. If a peak in the 1:1 mixture is significantly broader than the corresponding peak in the sample and USP Reference Standard or Reference Material digest, it may indicate the presence of different peptides. The use of computer-aided pattern recognition software for the analysis of peptide mapping data has been proposed and applied, but issues related to the validation of the computer software preclude its use in a compendial test in the near future. Other automated approaches have been used that employ mathematical formulas, models, and pattern recognition. Such approaches, for example the automated identification of compounds by IR spectroscopy and the application of diode-array UV spectral analysis for identification of peptides, have been proposed. These methods have limitations due to inadequate resolutions, co-elution of fragments, or absolute peak response differences between USP Reference Standard or Reference Material and sample fragments.

The numerical comparison of the retention times and peak areas or peak heights can be done for a selected group of relevant peaks that have been correctly identified in the peptide maps. Peak areas can be calculated using one peak showing relatively small variation as an internal reference, keeping in mind that peak area integration is sensitive to baseline variation and is likely to introduce error into the analysis. Alternatively, the percentage of each peptide peak height relative to the sum of all peak heights can be calculated for the sample under test. The percentage is then compared to that of the corresponding peak of the USP Reference Standard or Reference Material. The possibility of autohydrolysis of trypsin is monitored by producing a blank peptide map, that is, the peptide map obtained when a blank solution is treated with trypsin.

The minimum requirement for the qualification of peptide mapping is an approved test procedure that includes system suitability as a test control. In general, early in the regulatory process, qualification of peptide mapping for a protein is sufficient. As the regulatory approval process for the protein progresses, additional qualifications of the test can include a partial validation of the analytical procedure to provide assurance that the method will perform as intended in the development of a peptide map for the specified protein.

## Analysis and Identification of Peptides

This section gives guidance on the use of peptide mapping during development in support of regulatory applications.

The use of a peptide map as a qualitative tool does not require the complete characterization of the individual peptide peaks. However, validation of peptide mapping in support of regulatory applications requires rigorous characterization of each of the individual peaks in the peptide map. Methods to characterize peaks range from *N*-terminal sequencing of each peak followed by amino acid analysis to the use of mass spectroscopy (MS).

For characterization purposes, when *N*-terminal sequencing and amino acid analysis are used, the analytical separation is scaled up. Because scale-up might affect the resolution of peptide peaks, it is necessary, using empirical data, to ensure that there is no loss of resolution due to scale-up. Eluates corresponding to specific peptide peaks are collected,

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vacuum-concentrated, and chromatographed again, if necessary. Amino acid analysis of fragments may be limited by the peptide size. If the *N*-terminus is blocked, it may need to be cleared before sequencing. *C*-terminal sequencing of proteins in combination with carboxypeptidase digestion and matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) MS can also be used for characterization purposes.

The use of MS for characterization of peptide fragments is by direct infusion of isolated peptides or by the use of on-line LC-MS for structure analysis. In general, it includes electrospray and MALDI-TOF analyzers as well as fast atom bombardment (FAB). Tandem MS has also been used to sequence a modified protein and to determine the type of amino acid modification that has occurred. The comparison of mass spectra of the digests before and after reduction provides a method to assign the disulfide bonds to the various sulfhydryl-containing peptides.

If regions of the primary structure are not clearly demonstrated by the peptide map, it might be necessary to develop a secondary peptide map. The goal of a validated method of characterization of a protein through peptide mapping is to reconcile and account for at least 95% of the theoretical composition of the protein structure.

## ‣THE USE OF PEPTIDE MAPPING FOR GENETIC STABILITY EVALUATION

A validated peptide map can be used to assess the integrity of the predicted primary sequence of a protein product (i.e., its genetic stability). It can also be used to determine lot-to-lot consistency of the biotechnology-derived product process. Furthermore, the performance of the protein expression of the production system is best assessed by peptide mapping of the expressed protein. Peptide maps of protein produced at various times of the protein expression process, including a point well beyond the normal protein expression time, compared with those of a USP Reference Standard or Reference Material, will evaluate the genetic stability of the expression system as a function of time.

Variant protein sequences can arise from a genetic variation at the DNA level (point mutation) or as an error in the translation process. A validated peptide map is the best approach to the detection of protein variants. However, the limitations of the peptide mapping itself must be taken into consideration. The detection of a structured variant is possible only if the corresponding peptide variant is easily isolated and characterized. To establish genetic stability will require the use of a battery of biochemical methods, provided that the variants have properties different from those of the “normal” protein.‣

## ‣VALIDATION

### Critical Factors

Validation of peptide mapping requires that a protocol be designed, outlining in detail the experiment to be conducted and the criteria for acceptance of the map. Criteria for acceptance of mapping include detection limit, specificity, linearity, range, accuracy, precision, and reagent stability. Reproducibility of the peptide map is a critical element in the utilization of such a map as an identity test and for confirming genetic stability. Those technical aspects of peptide mapping that influence the reproducibility of the map will be discussed.

The setting of limits, with respect to quantification (peak area or height) and identification (retention times) for the selected group of relevant peaks is based on empirical observations. These limits detect significant differences between the sample and USP Reference Standard or Reference Material within a series of analyses.

Another critical issue is the recovery of peptides and its impact on peak area determination and reproducibility and on the establishment of acceptance criteria. The recovery criteria address all aspects of test methodology, from digestion to chromatographic conditions. Determination of peptide recovery includes quantitative amino acid analysis, spike addition, radiolabeling, and UV summation. An overall recovery of about 80% is considered satisfactory. Recovery of individual peptides is more problematic and is handled on a case-by-case basis. The critical factors considered in the validation of a peptide map are as follows.

#### Written Test Procedures

These procedures include a detailed description of the analytical method in which reagents, equipment, sample preparation, method of analysis, and analysis of the data are defined.

#### Validation Protocol

A protocol is prepared that contains a procedure for test validation.

#### Acceptance Criteria

The criteria can be minimal at the early stages, but need to be better defined as validation studies progress.

#### Reporting of Results

Results from the validation study are documented with respect to the analytical parameters listed in the validation protocol.

#### Revalidation of the Test Procedure

If the method used requires alteration that could affect the analytical parameter previously assessed in the validation of the procedure, the test procedure must be revalidated. Significant changes in the processing of the article, in laboratories

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performing the analysis, in formulation of the bulk or the finished products, and in any other significant parameter will require revalidation of the methods.

## Requirements

### Precision

**Intratest Precision**—This is a measure of the reproducibility of peptide mapping. The two critical steps in peptide mapping are fragmentation (i.e., digestion) and separation of peptides. An acceptable precision occurs where the absolute retention times and the relative peak areas are constant from run to run, and the average variation in retention time is small relative to that of a selected internal reference peak. The reproducibility of the map can be enhanced if a temperature-controlled column oven is used, if an extensive equilibration of the system is performed prior to the start of the test, if a blank (control digest mixture without protein) is run first to minimize "first run effects," and if a USP Reference Standard or a Reference Material digest is interspersed periodically with test samples to evaluate chromatographic drift.

The criteria for validation of the fragmentation step are similar to those described below for separation of peptides, but they are met for consecutive tests of a series of separately prepared digests of the protein under test.

The criteria for validation of the separation of peptides step include the following:

1. The average standard deviation of the absolute retention times of all major peaks for a set of consecutive tests of the same digest does not exceed a specified acceptance criterion.
2. The average standard deviation of absolute peak area for all fully resolved major peaks does not exceed a specified percentage.

**Intertest Precision**—This is a measure of the reproducibility of the peptide mapping when the test is performed on different days, by different analysts, in different laboratories, with reagents or enzymes from different suppliers or different lots from the same supplier, with different instruments, on columns of different makes or columns of the same make from different lots, and on individual columns of the same make from the same lot. Although it would be desirable, from a scientific perspective, to validate all of these variables in terms of their impacts on precision, a practical approach is to validate the test using those variables most likely to be encountered under operational conditions. Additional variables can be included when needed.

The experimental design allows the analyst to make comparisons using peak retention times and areas that are expressed relative to a highly reproducible internal reference peak within the same chromatogram. The relative peak area is expressed as the ratio of the peak area to that of the internal reference peak. The relative retention time can be expressed as the difference between the absolute retention time and that of the reference peak. The use of relative values eliminates the need to make separate corrections for differences due to injector-to-injector volumes, units of measure for peak areas, column dimensions, and instrument dead volumes. The variability in the retention times and peak areas for the *Intertest Precision* experiments is expected to be slightly higher than the variability observed for *Intratest Precision*.

### Robustness

Factors such as composition of the *Mobile Phase*, protease quality or chemical reagent purity, column variation and age, and digest stability are likely to affect the overall performance of the test and its reproducibility. Tolerances for each of the key parameters are evaluated and baseline limits established in case the test is used for routine lot release purposes.

**Mobile Phase**—The composition of the *Mobile Phase* is optimized to obtain the maximum resolution of peptides throughout the elution profile. A balance between optimal resolution and overall reproducibility is desired. A lower pH might improve peak separation but might shorten the life of the column, resulting in lack of reproducibility. Peptide maps at a pH above and below the pH of the procedure are compared to the peptide map obtained at the pH of the procedure and checked for significant differences; they are also reviewed with respect to the acceptance criteria established in the validation protocol.

**Protease Quality or Chemical Reagent Purity**—A sample of the USP Reference Standard or Reference Material for the protein under test is prepared and digested with different lots of cleavage agent. The chromatograms for each digest are compared in terms of peak areas, shape, and number. The same procedure can be applied to other critical chemicals or pretreatment procedures used during sample preparation, such as reducing and carboxymethylation reagents.

**Column Considerations**—Column-to-column variability, even within a single lot, can affect the performance of the column in the development of peptide maps. Column size may also lead to significant differences. A USP Reference Standard or Reference Material of the protein under test is digested and the digest is chromatographed on different lots of column from a single manufacturer. The maps are then evaluated in terms of the overall elution profile, retention times, selectivity resolution, and recovery. To evaluate the overall lifetime of the column in terms of robustness, perform a peptide mapping test on different columns and vary significantly the number of injections (e.g., from 10 injections to 250 injections). The resulting maps can then be compared for significant differences in peak broadening, peak area, and overall resolution. As a column ages, an increase in back pressure might be observed that might affect the peptide maps. A sensible precaution in the use of peptide mapping columns is to select alternative columns in case the original columns become unavailable or are discontinued. Perform a peptide mapping test using equivalent columns from different manufacturers, and examine the maps. Differences in particle shape and size, pore size and volume, carbon load, and end-capping can lead to significant differences in retention times, elution profile selectivity, resolution, and recovery. Slight modifications in the gradient profile may be required to achieve equivalency of mapping when using columns from different manufacturers.

[Note—The equivalency between instrumentation used for the validation of the test and for routine quality control testing should be considered. It might be preferable to use the same HPLC system for all applications. Otherwise, equivalency of the systems is determined, which may require some changes in the chromatographic test conditions.]

**Digest Stability**—The length of time a digest can be kept before it is chromatographed, as well as the conditions under which the digest is stored before chromatography, is assessed. Several aliquots from a single digest are stored at different storage conditions and chromatographed. These maps are then evaluated for significant differences.

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## REPRODUCIBILITY

Determination of various parameters indicated above is repeated using the same USP Reference Standard or Reference Material and test sample in at least two different laboratories by two analysts equipped with similar HPLC systems. The generated peptide maps are evaluated for significant differences.

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# ⟨1056⟩ BIOTECHNOLOGY-DERIVED ARTICLES—POLYACRYLAMIDE GEL ELECTROPHORESIS

## INTRODUCTION

### Scope

Polyacrylamide gel electrophoresis is used for the qualitative characterization of proteins in biological preparations, for control of purity, and for quantitative determinations.

### Purpose

Analytical gel electrophoresis is an appropriate method with which to identify and to assess the homogeneity of proteins in pharmaceutical preparations. The method is routinely used for the estimation of protein subunit molecular masses and for determination of the subunit compositions of purified proteins. Ready-to-use gels and reagents are commercially available and can be used instead of those described in this text, provided that they give equivalent results and that they meet the validity requirements given in *Validation of the Test* (below).

## CHARACTERISTICS OF POLYACRYLAMIDE GELS

The sieving properties of polyacrylamide gels are established by the three-dimensional network of fibers and pores that is formed as the bifunctional bisacrylamide cross-links adjacent polyacrylamide chains. Polymerization usually is catalyzed by a free radical–generating system composed of ammonium persulfate and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED).

As the acrylamide concentration of a gel increases, its effective pore size decreases. The effective pore size of a gel is operationally defined by its sieving properties, that is, by the resistance it imparts to the migration of macromolecules. There are limits on the acrylamide concentrations that can be used. At high acrylamide concentrations, gels break much more easily and are difficult to handle. As the pore size of a gel decreases, the migration rate of a protein through the gel decreases. By adjusting the pore size of a gel through manipulating the acrylamide concentration, analysts can optimize the resolution of the method for a given protein product. Thus, a given gel is physically characterized by its respective composition of acrylamide and bisacrylamide.

In addition to the composition of the gel, the state of the protein is an important component of electrophoretic mobility. In the case of proteins, the electrophoretic mobility depends on the pK value of the charged groups and the size of the molecule. It is influenced by the type, the concentration, and the pH of the buffer; by the temperature and the field strength; and by the nature of the support material.

## DENATURING POLYACRYLAMIDE GEL ELECTROPHORESIS

The method cited as an example is limited to the analysis of monomeric polypeptides with a mass range of 14,000–100,000 Da. It is possible to extend this mass range by various techniques (e.g., gradient gels and particular buffer system). For instance, tricine–sodium dodecyl sulfate (SDS) gels, using tricine instead of glycine (in the method described here) as the trailing ion in the electrophoresis running buffer, can separate very small proteins and peptides under 10,000–15,000 Da.

Denaturing polyacrylamide gel electrophoresis using glycine SDS (SDS-PAGE) is the most common mode of electrophoresis used in assessing the pharmaceutical quality of protein products and is the focus of the example method. Typically, analytical electrophoresis of proteins is carried out in polyacrylamide gels under conditions that ensure dissociation of the proteins into their individual polypeptide subunits and that minimize aggregation. Most commonly, the strongly anionic detergent SDS is used in combination with heat to dissociate the proteins before they are loaded on the gel. The denatured polypeptides bind to SDS, become negatively charged, and exhibit a consistent charge-to-mass ratio regardless of protein type. Because the amount of SDS bound is almost always proportional to the molecular mass of the polypeptide and is independent of its sequence, SDS–polypeptide complexes migrate through polyacrylamide gels with mobilities dependent on the size of the polypeptide.

The electrophoretic mobilities of the resultant detergent–polypeptide complexes all assume the same functional relationship to their molecular masses. SDS complexes migrate toward the anode in a predictable manner; low-molecular-mass complexes migrate faster than larger ones. The molecular mass of a protein therefore can be estimated from its relative mobility in calibrated SDS-PAGE, and the intensity of a single band relative to other undesired bands in such a gel can be a measure of purity.

Modifications to the polypeptide backbone, such as *N*- or *O*-linked glycosylation, can change the apparent molecular mass of a protein, because SDS does not bind to a carbohydrate moiety in a manner similar to that of a polypeptide; therefore, a consistent charge-to-mass ratio is not maintained.

Depending on the extent of glycosylation and other posttranslational modifications, the apparent molecular mass of proteins may not be a true reflection of the mass of the polypeptide chain.

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### Reducing Conditions

Polypeptide subunits and three-dimensional structure often are maintained in proteins by the presence of disulfide bonds. A goal of SDS-PAGE analysis under reducing conditions is to disrupt this structure by reducing disulfide bonds. Complete denaturation and dissociation of proteins by treatment with 2-mercaptoethanol (2-ME) or dithiothreitol (DTT) results in unfolding of the polypeptide backbone and subsequent complexation with SDS. Using these conditions, analysts can reasonably calculate the molecular mass of the polypeptide by linear regression (or, more closely, by nonlinear regression) in the presence of suitable molecular mass standards.

### Nonreducing Conditions

For some analyses, complete dissociation of the protein into subunit peptides is not desirable. In the absence of treatment with reducing agents such as 2-ME or DTT, disulfide covalent bonds remain intact, preserving the oligomeric form of the protein. Oligomeric SDS–protein complexes migrate more slowly than their SDS–polypeptide subunits. In addition, nonreduced proteins may not be completely saturated with SDS and hence may not bind the detergent in a constant mass ratio. Moreover, intrachain disulfide bonds constrain the molecular shape, usually in such a way that reduces the Stokes radius of the molecule, thereby reducing the apparent molecular mass, $M_r$. This makes molecular mass determinations of these molecules by SDS-PAGE less straightforward than analyses of fully denatured polypeptides because it is necessary that both standards and unknown proteins be in similar configurations for valid comparisons.

## CHARACTERISTICS OF DISCONTINUOUS BUFFER SYSTEM GEL ELECTROPHORESIS

The most popular electrophoretic method for the characterization of complex mixtures of proteins uses a discontinuous buffer system involving two contiguous but distinct gels: a resolving or separating (lower) gel and a stacking (upper) gel. The two gels are cast with different porosities, pH, and ionic strengths. In addition, different mobile ions are used in the gel and electrode buffers. The buffer discontinuity acts to concentrate large-volume samples in the stacking gel, resulting in improved resolution. When power is applied, a voltage drop develops across the sample solution and drives the proteins into the stacking gel. Glycinate ions from the electrode buffer follow the proteins into the stacking gel. A moving boundary region is rapidly formed, with the highly mobile chloride ions in the front and the relatively slow glycinate ions in the rear. A localized high-voltage gradient forms between the leading and trailing ion fronts, causing the SDS–protein complexes to form into a thin zone (stack) and to migrate between the chloride and glycinate phases. Within broad limits, regardless of the height of the applied sample, all SDS–proteins condense into a very narrow region and enter the resolving gel as a well-defined, thin zone of high protein density. The large-pore stacking gel does not retard the migration of most proteins and serves mainly as an anticonvective medium. At the interface of the stacking and resolving gels, the proteins undergo a sharp increase in retardation due to the restrictive pore size of the resolving gel and the buffer discontinuity, which also contributes to focusing of the proteins. Once in the resolving gel, proteins continue to be slowed by the sieving of the matrix. The glycinate ions overtake the proteins, which then move in a space of uniform pH formed by the tris(hydroxymethyl)aminomethane (Tris) and glycine. Molecular sieving causes the SDS–polypeptide complexes to separate on the basis of their molecular masses.

## PREPARING VERTICAL DISCONTINUOUS BUFFER SDS POLYACRYLAMIDE GELS

This section describes the preparation of gels using particular instrumentation. This does not apply to precast gels. For precast gels or any other commercially available equipment, the manufacturer's instructions must be used for guidance.

The use of commercial reagents that have been purified in solution is recommended. When this is not the case and when the purity of the reagents used is not sufficient, a pretreatment is applied. For instance, any solution sufficiently impure to require filtration must also be deionized with a mixed-bed (anion–cation exchange) resin to remove acrylic acid and other charged degradation products. When stored according to recommendations, acrylamide/bisacrylamide solutions and solid persulfate are stable for long periods.

### Gel Stock Solutions

#### 30% ACRYLAMIDE–BISACRYLAMIDE SOLUTION

Prepare a solution containing 290 g of acrylamide and 10 g of methylenebisacrylamide per liter of water. Filter.

#### AMMONIUM PERSULFATE SOLUTION

Prepare a small quantity of solution having a concentration of 100 g/L of ammonium persulfate. [NOTE—Ammonium persulfate provides the free radicals that drive polymerization of acrylamide and bisacrylamide. Because ammonium persulfate decomposes rapidly, fresh solutions must be prepared daily.]

#### TEMED

Use an electrophoresis-grade reagent.

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SDS SOLUTION

This is a 100 g/L solution of electrophoresis-grade SDS.

1.5 M BUFFER SOLUTION

Dissolve 90.8 g of Tris in 400 mL of water. Adjust the pH to 8.8 with hydrochloric acid, and dilute to 500.0 mL with water.

1 M BUFFER SOLUTION

Dissolve 60.6 g of Tris in 400 mL of water. Adjust the pH to 6.8 with hydrochloric acid, and dilute to 500.0 mL with water.

## Assembling the Gel-Molding Cassette

Clean the two glass plates (size, e.g., 10 cm × 8 cm), the polytetrafluoroethylene comb, the two spacers, and the silicone rubber tubing (e.g., 0.6 mm diameter × 35 cm length) with mild detergent; rinse extensively with water, followed by dehydrated alcohol; and allow the plates to dry at room temperature. Lubricate the spacers and the tubing with nonsilicone grease. Apply the spacers along each of the two short sides of the glass plate 2 mm away from the edges and 2 mm away from the long side corresponding to the bottom of the gel. Begin to lay the tubing on the glass plate by using one spacer as a guide. Carefully twist the tubing at the bottom of the spacer, and follow the long side of the glass plate. While holding the tubing with one finger along the long side, twist the tubing again and lay it on the second short side of the glass plate, using the spacer as a guide. Place the second glass plate in perfect alignment, and hold the mold together by hand pressure.

Apply two clamps on each of the two short sides of the mold. Carefully apply four clamps on the longer side of the gel mold, thus forming the bottom of the gel mold. Verify that the tubing is running along the edge of the glass plates and has not been extruded while the clamps were placed. The gel mold is now ready for pouring the gel.

## Preparation of the Gel

In a discontinuous buffer SDS polyacrylamide gel, it is recommended to pour the resolving gel, let the gel set, and then pour the stacking gel, because the composition of the two gels in acrylamide–bisacrylamide, buffer, and pH are different.

PREPARATION OF THE RESOLVING GEL

In a conical flask, prepare the appropriate volume of solution containing the desired concentration of acrylamide for the resolving gel, using the values given in *Table 1*. Mix the components in the order shown. Where appropriate, before adding the *Ammonium Persulfate Solution* and the *TEMED*, filter the solution if necessary under vacuum through a cellulose acetate membrane (pore diameter: 0.45 µm). Keep the solution under vacuum, while swirling the filtration unit, until no more bubbles are formed in the solution. Add appropriate amounts of *Ammonium Persulfate Solution* and *TEMED*, as indicated in *Table 1*, swirl, and pour immediately into the gap between the two glass plates of the mold. Leave sufficient space for the stacking gel (the length of the teeth of the comb plus 1 cm). Using a tapered glass pipet, carefully overlay the solution with water-saturated isobutanol. Leave the gel in a vertical position at room temperature to allow polymerization.

**Table 1. Preparation of the Resolving Gel**

| | Component Volume (mL) per Gel Mold Volume Below | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Solution component** | **5 mL** | **10 mL** | **15 mL** | **20 mL** | **25 mL** | **30 mL** | **40 mL** | **50 mL** |
| *6% Acrylamide* | | | | | | | | |
| Water | 2.6 | 5.3 | 7.9 | 10.6 | 13.2 | 15.9 | 21.2 | 26.5 |
| *30% Acrylamide–Bisacrylamide Solution* | 1.0 | 2.0 | 3.0 | 4.0 | 5.0 | 6.0 | 8.0 | 10.0 |
| *1.5 M Buffer Solution* | 1.3 | 2.5 | 3.8 | 5.0 | 6.3 | 7.5 | 10.0 | 12.5 |
| *SDS Solution* | 0.05 | 0.1 | 0.15 | 0.2 | 0.25 | 0.3 | 0.4 | 0.5 |
| *Ammonium Persulfate Solution* | 0.05 | 0.1 | 0.15 | 0.2 | 0.25 | 0.3 | 0.4 | 0.5 |
| *TEMED* | 0.004 | 0.008 | 0.012 | 0.016 | 0.02 | 0.024 | 0.032 | 0.04 |
| | | | | | | | | |
| 8% Acrylamide | | | | | | | | |
| Water | 2.3 | 4.6 | 6.9 | 9.3 | 11.5 | 13.9 | 18.5 | 23.2 |
| *30% Acrylamide–Bisacrylamide Solution* | 1.3 | 2.7 | 4.0 | 5.3 | 6.7 | 8.0 | 10.7 | 13.3 |
| *1.5 M Buffer Solution* | 1.3 | 2.5 | 3.8 | 5.0 | 6.3 | 7.5 | 10.0 | 12.5 |
| *SDS Solution* | 0.05 | 0.1 | 0.15 | 0.2 | 0.25 | 0.3 | 0.4 | 0.5 |
| *Ammonium Persulfate Solution* | 0.05 | 0.1 | 0.15 | 0.2 | 0.25 | 0.3 | 0.4 | 0.5 |

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**Table 1. Preparation of the Resolving Gel** *(continued)*

| | Component Volume (mL) per Gel Mold Volume Below | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Solution component** | **5 mL** | **10 mL** | **15 mL** | **20 mL** | **25 mL** | **30 mL** | **40 mL** | **50 mL** |
| *TEMED* | 0.003 | 0.006 | 0.009 | 0.012 | 0.015 | 0.018 | 0.024 | 0.03 |
| | | | | | | | | |
| 10% Acrylamide | | | | | | | | |
| Water | 1.9 | 4.0 | 5.9 | 7.9 | 9.9 | 11.9 | 15.9 | 19.8 |
| *30% Acrylamide–Bisacrylamide Solution* | 1.7 | 3.3 | 5.0 | 6.7 | 8.3 | 10.0 | 13.3 | 16.7 |
| *1.5 M Buffer Solution* | 1.3 | 2.5 | 3.8 | 5.0 | 6.3 | 7.5 | 10.0 | 12.5 |
| *SDS Solution* | 0.05 | 0.1 | 0.15 | 0.2 | 0.25 | 0.3 | 0.4 | 0.5 |
| *Ammonium Persulfate Solution* | 0.05 | 0.1 | 0.15 | 0.2 | 0.25 | 0.3 | 0.4 | 0.5 |
| *TEMED* | 0.002 | 0.004 | 0.006 | 0.008 | 0.01 | 0.012 | 0.016 | 0.02 |
| | | | | | | | | |
| 12% Acrylamide | | | | | | | | |
| Water | 1.6 | 3.3 | 4.9 | 6.6 | 8.2 | 9.9 | 13.2 | 16.5 |
| *30% Acrylamide–Bisacrylamide Solution* | 2.0 | 4.0 | 6.0 | 8.0 | 10.0 | 12.0 | 16.0 | 20.0 |
| *1.5 M Buffer Solution* | 1.3 | 2.5 | 3.8 | 5.0 | 6.3 | 7.5 | 10.0 | 12.5 |
| *SDS Solution* | 0.05 | 0.1 | 0.15 | 0.2 | 0.25 | 0.3 | 0.4 | 0.5 |
| *Ammonium Persulfate Solution* | 0.05 | 0.1 | 0.15 | 0.2 | 0.25 | 0.3 | 0.4 | 0.5 |
| *TEMED* | 0.002 | 0.004 | 0.006 | 0.008 | 0.01 | 0.012 | 0.016 | 0.02 |
| | | | | | | | | |
| 14% Acrylamide | | | | | | | | |
| Water | 1.4 | 2.7 | 3.9 | 5.3 | 6.6 | 8.0 | 10.6 | 13.8 |
| *30% Acrylamide–Bisacrylamide Solution* | 2.3 | 4.6 | 7.0 | 9.3 | 11.6 | 13.9 | 18.6 | 23.2 |
| *1.5 M Buffer Solution* | 1.2 | 2.5 | 3.6 | 5.0 | 6.3 | 7.5 | 10.0 | 12.5 |
| *SDS Solution* | 0.05 | 0.1 | 0.15 | 0.2 | 0.25 | 0.3 | 0.4 | 0.5 |
| *Ammonium Persulfate Solution* | 0.05 | 0.1 | 0.15 | 0.2 | 0.25 | 0.3 | 0.4 | 0.5 |
| *TEMED* | 0.002 | 0.004 | 0.006 | 0.008 | 0.01 | 0.012 | 0.016 | 0.02 |
| | | | | | | | | |
| 15% Acrylamide | | | | | | | | |
| Water | 1.1 | 2.3 | 3.4 | 4.6 | 5.7 | 6.9 | 9.2 | 11.5 |
| *30% Acrylamide–Bisacrylamide Solution* | 2.5 | 5.0 | 7.5 | 10.0 | 12.5 | 15.0 | 20.0 | 25.0 |
| *1.5 M Buffer Solution* | 1.3 | 2.5 | 3.8 | 5.0 | 6.3 | 7.5 | 10.0 | 12.5 |
| *SDS Solution* | 0.05 | 0.1 | 0.15 | 0.2 | 0.25 | 0.3 | 0.4 | 0.5 |
| *Ammonium Persulfate Solution* | 0.05 | 0.1 | 0.15 | 0.2 | 0.25 | 0.3 | 0.4 | 0.5 |
| *TEMED* | 0.002 | 0.004 | 0.006 | 0.008 | 0.01 | 0.012 | 0.016 | 0.02 |

## PREPARATION OF THE STACKING GEL

After polymerization is complete (about 30 min), pour off the isobutanol, and wash the top of the gel several times with water to remove the isobutanol overlay and any unpolymerized acrylamide. Drain as much fluid as possible from the top of the gel, and then remove any remaining water with the edge of a paper towel.

In a conical flask, prepare the appropriate volume of solution containing the desired concentration of acrylamide, using the values given in *Table 2*. Mix the components in the order shown. Where appropriate, before adding the *Ammonium Persulfate Solution* and the *TEMED*, filter the solution if necessary under vacuum through a cellulose acetate membrane (pore diameter: 0.45 µm). Keep the solution under vacuum, while swirling the filtration unit, until no more bubbles are formed in the solution. Add appropriate amounts of *Ammonium Persulfate Solution* and *TEMED*, as indicated in *Table 2*. Swirl, and pour immediately into the gap between the two glass plates of the mold directly onto the surface of the polymerized resolving gel. Immediately insert a clean polytetrafluoroethylene comb into the stacking gel solution, being careful to avoid trapping air bubbles. Add

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more stacking gel solution to fill the spaces of the comb completely. Leave the gel in a vertical position, and allow it to polymerize at room temperature.

**Table 2. Preparation of the Stacking Gel**

| | Component Volume (mL) per Gel Mold Volume Below | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Solution component** | **1 mL** | **2 mL** | **3 mL** | **4 mL** | **5 mL** | **6 mL** | **8 mL** | **10 mL** |
| Water | 0.68 | 1.4 | 2.1 | 2.7 | 3.4 | 4.1 | 5.5 | 6.8 |
| *30% Acrylamide–Bisacrylamide Solution* | 0.17 | 0.33 | 0.5 | 0.67 | 0.83 | 1.0 | 1.3 | 1.7 |
| *1.0 M Buffer Solution* | 0.13 | 0.25 | 0.38 | 0.5 | 0.63 | 0.75 | 1.0 | 1.25 |
| *SDS Solution* | 0.01 | 0.02 | 0.03 | 0.04 | 0.05 | 0.06 | 0.08 | 0.1 |
| *Ammonium Persulfate Solution* | 0.01 | 0.02 | 0.03 | 0.04 | 0.05 | 0.06 | 0.08 | 0.1 |
| *TEMED* | 0.001 | 0.002 | 0.003 | 0.004 | 0.005 | 0.006 | 0.008 | 0.01 |

## Preparation of the Sample

Unless otherwise specified in the specific monograph, the samples can be prepared as follows:

### SDS-PAGE SAMPLE BUFFER (CONCENTRATED)

Dissolve 1.89 g of Tris, 5.0 g of sodium lauryl sulfate, and 50 mg of bromophenol blue in water. Add 25.0 mL of glycerol, and dilute to 100 mL with water. Adjust the pH to 6.8 with hydrochloric acid, and dilute to 125 mL with water.

### SDS-PAGE SAMPLE BUFFER FOR REDUCING CONDITIONS (CONCENTRATED)

Dissolve 3.78 g of Tris, 10.0 g of SDS, and 100 mg of bromophenol blue in water. Add 50.0 mL of glycerol, and dilute to 200 mL with water. Add 25.0 mL of 2-ME. Adjust to pH 6.8 with hydrochloric acid, and dilute to 250.0 mL with water. Alternatively, DTT can be used as reducing agent instead of 2-ME. In this case prepare the sample buffer as follows: Dissolve 3.78 g of Tris, 10.0 g of SDS, and 100 mg of bromophenol blue in water. Add 50.0 mL of glycerol, and dilute to 200 mL with water. Adjust to pH 6.8 with hydrochloric acid, and dilute to 250.0 mL with water. Immediately before use, add DTT to a final concentration of 100 mM.

### SDS-PAGE RUNNING BUFFER

Dissolve 151.4 g of Tris, 721.0 g of glycine, and 50.0 g of sodium lauryl sulfate in water, and dilute to 5000 mL with the same solvent. Immediately before use, dilute to 10 times its volume with water, and mix. Measure the pH of the diluted solution. The pH is between 8.1 and 8.8.

### SAMPLE SOLUTION (NONREDUCING CONDITIONS)

Mix equal volumes of: a mixture comprising water plus the preparation or the reference solutions, and *SDS-PAGE Sample Buffer (Concentrated)*.

### SAMPLE SOLUTION (REDUCING CONDITIONS)

Mix equal volumes of: a mixture comprising water plus the preparation or the reference solutions, and *SDS-PAGE Sample Buffer for Reducing Conditions (Concentrated)* containing 2-ME (or DTT) as the reducing agent.

The concentration prescribed in the monograph can vary depending on the protein and staining method.

Sample treatment: Keep for 5 min in a boiling water bath or in a block heater set at 100°, and then chill. (Note that temperature and time may vary in the monograph because protein cleavage may occur during the heat treatment.)

### MOUNTING THE GEL IN THE ELECTROPHORESIS APPARATUS AND ELECTROPHORETIC SEPARATION

After polymerization is complete (about 30 min), remove the polytetrafluoroethylene comb carefully. Rinse the wells immediately with water or with the *SDS-PAGE Running Buffer* to remove any unpolymerized acrylamide. If necessary, straighten the teeth of the stacking gel with a blunt hypodermic needle attached to a syringe. Remove the clamps on one short side, carefully pull out the tubing, and replace the clamps. Proceed similarly on the other short side. Remove the tubing from the bottom part of the gel. Mount the gel in the electrophoresis apparatus. Add the electrophoresis buffers to the top and bottom reservoirs. Remove any bubbles that become trapped at the bottom of the gel between the glass plates. This is best done with a bent hypodermic needle attached to a syringe. Never prerun the gel before loading the samples, because this will destroy the discontinuity of the buffer systems. Before loading the sample, carefully rinse each well with *SDS-PAGE Running Buffer*. Prepare the test and reference solutions in the recommended sample buffer, and treat as specified in the individual monograph. Apply the appropriate volume of each solution to the stacking gel wells.

Start the electrophoresis using the conditions recommended by the equipment manufacturer. Manufacturers of SDS-PAGE equipment may provide gels of different surface area and thickness, and electrophoresis running time and current or voltage may vary in order to achieve optimal separation. Check that the dye front is moving into the resolving gel. When the dye is

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near the bottom of the gel stop the electrophoresis. Remove the gel assembly from the apparatus, and carefully separate the glass plates. Remove the spacers, cut off and discard the stacking gel, and immediately proceed with staining.

## SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS—GRADIENT CONCENTRATION GELS

Gradient gels (resolving gels) are prepared with an increasing concentration of acrylamide from the top to the bottom. Preparation of gradient gels requires a gradient-forming apparatus. Ready-to-use gradient gels are commercially available with specific recommended protocols.

Gradient gels offer some advantages over fixed-concentration gels. Some proteins that co-migrate on fixed-concentration gels can be resolved within gradient gels. During electrophoresis the proteins migrate until the pore size stops further progress, and therefore a stacking effect occurs, resulting in sharper bands. According to *Table 3*, gradient gels also allow separation of a wider range of protein molecular masses than do single, fixed-concentration gels.

*Table 3* gives suggested compositions of the linear gradient, relating the range of acrylamide concentrations to the appropriate protein molecular ranges. Note that other gradient shapes (e.g., concave) can be prepared for specific applications.

**Table 3. Acrylamide Gradient Percentages Recommended for Expected Protein Molecular Weights**

| Acrylamide (%) | Protein Range (kDa) |
|---|---|
| 5–15 | 20–250 |
| 5–20 | 10–200 |
| 10–20 | 10–150 |
| 8–20 | 8–150 |

Gradient gels also are used for molecular mass determination and protein purity determination.

## DETECTION OF PROTEINS IN GELS

Coomassie and silver staining are the most common protein staining methods and are described in more detail below. Several other commercial stains, detection methods, and commercial kits are available. For example, fluorescent stains are visualized using a fluorescent imager and often provide a linear response over a wide range of protein concentrations—often several orders of magnitude, depending on the protein.

Coomassie staining has a protein detection level of approximately 1–10 µg of protein per band. Silver staining is the most sensitive method for staining proteins in gels, and a band containing 10–100 ng can be detected. These figures are considered robust in the context of these gels. Improved sensitivity of one or two orders of magnitude has been reported in the literature.

Coomassie staining responds in a more linear manner than silver staining, but the response and range depend on the protein and development time. Both Coomassie and silver staining can be less reproducible if staining is stopped in a subjective manner, i.e., when the analyst deems the staining satisfactory. Wide dynamic ranges of reference proteins are important to use because they help assess the intra-experimental sensitivity and linearity. All gel-staining steps are done while wearing gloves, at room temperature, with gentle shaking (e.g., on an orbital shaker platform), and using any convenient container.

### Staining Reagents

#### DESTAINING SOLUTION

Prepare a mixture of 1 volume of glacial acetic acid, 4 volumes of methanol, and 5 volumes of water.

#### COOMASSIE STAINING SOLUTION

Prepare a 1.25 g/L solution of acid blue 83 in *Destaining Solution*. Filter.

#### FIXING SOLUTION

To 250 mL of methanol, add 0.27 mL of formaldehyde, and dilute to 500.0 mL with water.

#### SILVER NITRATE REAGENT

To a mixture of 3 mL of concentrated ammonia and 40 mL of 1 M sodium hydroxide, add 8 mL of a 200 g/L solution of silver nitrate, dropwise, with stirring. Dilute to 200 mL with water.

#### DEVELOPER SOLUTION

Dilute 2.5 mL of a 20 g/L solution of citric acid and 0.27 mL of formaldehyde to 500.0 mL with water.

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BLOCKING SOLUTION

A 10% (v/v) solution of acetic acid.

### Coomassie Staining

Immerse the gel in a large excess of *Coomassie Staining Solution*, and allow to stand for at least 1 h. Remove the staining solution.

Destain the gel with a large excess of *Destaining Solution*. Change the *Destaining Solution* several times until the stained protein bands are clearly distinguishable on a clear background. The more thoroughly the gel is destained, the smaller is the amount of protein that can be detected by the method. More rapid destaining can be achieved by including a few grams of anion-exchange resin or a small sponge in the *Destaining Solution*. [NOTE—The acid–alcohol solutions used in this procedure do not completely fix proteins in the gel. This can lead to losses of some low-molecular-mass proteins during the staining and destaining of thin gels. Permanent fixation is obtainable by allowing the gel to stand in a mixture of 1 volume of trichloroacetic acid, 4 volumes of methanol, and 5 volumes of water for 1 h before it is immersed in the *Coomassie Staining Solution*.]

### Silver Staining

Immerse the gel in a large excess of *Fixing Solution*, and allow it to stand for 1 h. Remove the *Fixing Solution*, add fresh *Fixing Solution*, and incubate for at least 1 h or overnight, if convenient. Discard the *Fixing Solution*, and wash the gel in a large excess of water for 1 h. Soak the gel for 15 min in a 1% (v/v) solution of glutaraldehyde. Wash the gel twice for 15 min in a large excess of water. Soak the gel in fresh *Silver Nitrate Reagent* for 15 min, in darkness. Wash the gel three times for 5 min in a large excess of water. Immerse the gel for about 1 min in *Developer Solution* until satisfactory staining has been obtained. Stop the development by incubation in the *Blocking Solution* for 15 min. Rinse the gel with water.

## RECORDING THE RESULTS

Gels are photographed or scanned while they are still wet or after an appropriate drying procedure. Currently, gel-scanning systems with data analysis software are commercially available to photograph and analyze the wet gel immediately.

Depending on the staining method used, gels are treated in a slightly different way. For Coomassie staining, after the destaining step, allow the gel to stand in a 100 g/L solution of glycerol for at least 2 h (overnight incubation is possible). For silver staining, add to the final rinsing a step of 5 min in a 20 g/L solution of glycerol.

Drying of stained SDS polyacrylamide gels is one of the methods to have permanent documentation. This method frequently results in gel cracking during drying between cellulose films.

Immerse two sheets of porous cellulose film in water, and incubate for 5–10 min.

Place one of the sheets on a drying frame. Carefully lift the gel, and place it on the cellulose film. Remove any trapped air bubbles, and pour a few mL of water around the edges of the gel. Place the second sheet on top, and remove any trapped air bubbles. Complete the assembly of the drying frame. Place in an oven or leave at room temperature until dry.

## MOLECULAR MASS DETERMINATION

Molecular masses of proteins are determined by comparison of their mobilities with those of several marker proteins of known molecular weight. Mixtures of prestained and unstained proteins with precisely known molecular masses blended for uniform staining are available for calibrating gels. They are available in various molecular mass ranges. Concentrated stock solutions of proteins of known molecular mass are diluted in the appropriate sample buffer and are loaded on the same gel as the protein sample to be studied.

Immediately after the gel has been run, mark the position of the bromophenol blue tracking dye to identify the leading edge of the electrophoretic ion front. This can be done by cutting notches in the edges of the gel or by inserting a needle soaked in India ink into the gel at the dye front. After staining, measure the migration distances of each protein band (markers and unknowns) from the top of the resolving gel. Divide the migration distance of each protein by the distance traveled by the tracking dye. The normalized migration distances are referred to as the relative mobilities of the proteins (relative to the dye front), or $R_F$. Construct a plot of the logarithm of the relative molecular masses ($M_r$) of the protein standards as a function of the $R_F$ values. Unknown molecular masses can be estimated by linear regression analysis (more accurately, by nonlinear regression analysis) or interpolation from the curves of log $M_r$ against $R_F$ if the values obtained for the unknown samples are positioned along the approximately linear part of the graph.

## VALIDATION OF THE TEST

The test is not valid unless the target resolution range of the gel has been demonstrated by the distribution of appropriate molecular mass markers, e.g., across 80% of the length of the gel. The separation obtained for the expected proteins must show a linear relationship between the logarithm of the molecular mass and the $R_F$. If the plot has a sigmoidal shape, then only data from the linear region of the curve can be used in the calculations. Additional validation requirements with respect to the test sample may be specified in individual monographs.

Sensitivity also must be validated. A reference protein control corresponding to the desired concentration limit that is run in parallel with the test samples can serve as a system suitability check of the experiment.

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## QUANTITATION OF IMPURITIES

SDS-PAGE is often used as a limit test for impurities. When impurities are quantitated by normalization to the main band using an integrating densitometer or image analysis, the responses must be validated for linearity. Note that depending on the detection method and protein, as described in the introduction of the section *Detection of Proteins in Gels*, the linear range can vary but can be assessed within each run by using one or more control samples containing an appropriate range of protein concentrations.

When the impurity limit is specified in the individual monograph, analysts should prepare a reference solution corresponding to that level of impurity by diluting the test solution. For example, when the limit is 5%, a reference solution would be a 1:20 dilution of the test solution. No impurity (any band other than the main band) in the electropherogram obtained with the test solution may be more intense than the main band obtained with the reference solution.

Under validated conditions, impurities can be quantified by normalization to the main band, using an integrating densitometer, or by image analysis.

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# ⟨1057⟩ BIOTECHNOLOGY-DERIVED ARTICLES—TOTAL PROTEIN ASSAY

***Delete the following:***

▲This chapter provides guidance and procedures used for characterization of biotechnology-derived articles. This chapter is harmonized with the corresponding chapter in *JP* and *EP*. Other characterization tests, also harmonized, are provided in *Biotechnology-Derived Articles—Amino Acid Analysis* ⟨1052⟩, *Capillary Electrophoresis* ⟨1053⟩, *Biotechnology-Derived Articles—Isoelectric Focusing* ⟨1054⟩, *Biotechnology-Derived Articles—Peptide Mapping* ⟨1055⟩, and *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* ⟨1056⟩.▲ (USP 1-Aug-2019)

***Change to read:***

## INTRODUCTION

▲A number of factors needs to be considered when choosing a procedure for the measurement of total protein content in a pharmacopeial preparation. Those factors include the complexity of the sample, constraints that impact the procedure, the required accuracy/variability of the procedure, and the desired analyst contact or automation associated with the procedure (e.g., sample availability, analysis time). The choice of a total protein measurement procedure balances these and other factors to meet the application need.

An analytical separation technique (e.g., HPLC, capillary electrophoresis) may be the best option as a total protein measurement procedure when analyzing a complex sample. Separating the sample into various components and integrating relevant peaks on a chromatogram or electropherogram can be used to manage 1) sample components that interfere with spectrophotometric protein measurements; 2) a mixture of proteins or peptides in the sample, including excipients (e.g., human serum albumin), where the individual protein components need to be quantitated; 3) other sample attributes that are being assessed (e.g., identity, impurity quantitation) during the analysis; 4) a limited sample quantity available for analysis; or 5) the balance between analysis time, instrument complexity, and other factors that favor a separation procedure over a spectrophotometric procedure.

Amino acid quantitation may also be suitable for quantitation of total protein and is often used as a primary method of calibration for other total protein measurement procedures (e.g., determination of an extinction coefficient used with protein absorbance at 280 nm).

Spectrophotometric procedures for total protein measurement are often employed for the analysis of pharmacopeial preparations due to their simplicity, high sample throughput for a given analysis time, and low cost.▲ (USP 1-Aug-2019)

Many of the total protein assay methods described below can be performed successfully using kits from commercial sources. ▲▲ (USP 1-Aug-2019)

***Add the following:***

## ▲SAMPLE EXTRACTION METHODS FOR TOTAL PROTEIN ASSAYS

Accurate measurement of total protein cannot always be accomplished if there are interfering substances in the sample. This interference can be observed with spectrophotometric methods of total protein measurement. In order to accurately measure the total protein content in samples with these interfering substances, a method to remove these substances needs to be part of the procedure and the total procedure (extraction in combination with a measurement method) should be validated as suitable for its intended purpose.

It is possible that an interfering substance exhibits a known reproducible effect on the assay that can be eliminated by using an appropriate blank containing the interfering substance to calibrate the spectrophotometer to zero. As an alternative, a spike control added to the *Test solution* or sample matrix can be used to adjust the test result based on the spike recovery.

Sample extraction techniques often involve precipitating protein from the sample, leaving the interfering substances in the liquid phase that is subsequently removed, and then solubilizing the precipitated protein in the assay buffer for analysis. Precipitating protein from the sample can also have the advantage of concentrating proteins from a dilute solution, thereby allowing a sample that was too dilute to use with a particular spectrophotometric procedure to now be within the useful range of the method. Gel filtration procedures to remove an interfering substance can result in sample dilution which should also be considered when developing the method of analysis. With all techniques, volumes of solutions used must be measured quantitatively to allow accurate calculation of concentration and dilution factors that are used in the final calculation of protein concentration in the starting sample.

The use of an extractive procedure may introduce a sample preparation error and affect the precision of the protein concentration result. When an extraction procedure is performed on *Test solutions*, it should also be performed for *Standard solutions*, system suitability solutions, and the *Blank*. Samples should be homogeneous and free of particles. Examples of several possible sample extraction procedures, depending upon the nature of the interfering substance, are described below but others may be demonstrated as suitable for use too.

### Procedure 1—Protein Precipitation with Acetone

1. Add acetone to the sample to bring the concentration of acetone to 85%–90%.
2. Let the sample stand for 1 h. If necessary for appropriate recovery, hold the sample at 4° overnight.
3. Centrifuge the sample at 14,000 × g or greater for 10 min. Discard the supernatant.

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4. Dissolve the pellet in the appropriate buffer for analysis.

## Procedure 2—Protein Precipitation with Trichloroacetic Acid

SODIUM DEOXYCHOLATE REAGENT

Prepare a solution of 1.5 g/L sodium deoxycholate in water.

TCA REAGENT

Prepare a solution of 720 g/L trichloroacetic acid (TCA) in water.

PROCEDURE

1. Add 0.1 mL of *Sodium deoxycholate reagent* to 1.0 mL of *Test solution*.
2. Mix using a vortex mixer, and then incubate for 10 min.
3. Add 0.1 mL of *TCA reagent*, and mix on a vortex mixer.
4. Centrifuge at a minimum of 6700 × g for 30 min.
5. Discard the supernatant.
6. Dissolve the protein pellet in 1.0 mL of the appropriate buffer for analysis.

## Procedure 3—Solid Phase Device Extraction

BUFFER PREPARATION

Prepare the appropriate buffer or solvent mixture depending upon the solid phase extraction (SPE) device being used for fractionation.

PROCEDURE

1. Load the sample onto the SPE device.
2. Wash with a suitable buffer or solvent mixture.
3. Elute the protein with an appropriate buffer or solvent mixture.

## Procedure 4—Column Gel Filtration

BUFFER PREPARATION

Prepare the appropriate buffer for the test method.

PROCEDURE

The protein is purified using either gravity or a centrifugal procedure per the manufacturer's instructions.[1] ▲ (USP 1-Aug-2019)

***Change to read:***

# ▲TOTAL PROTEIN MEASUREMENT PROCEDURES

Ideally, the protein in the *Standard solution* that is used to create the calibration curve should be the same protein as in the *Test solution*. This may not be practical and the use of a generic protein in the *Standard solution* may be substituted. Bovine serum albumin is often used because it is readily available and can be purchased at high purity. However, using a generic protein to create the standard curve may not be the ideal choice for all assays depending on the principle of the assay and the protein structure responsible for the response. Due to the potential variation in responses between different proteins that may be used to create the standard curve, the protein used for the standard curve should always be reported. In addition (unless otherwise specified in the individual monograph) prepare the Reference Standard or reference material for the protein under test in the same buffer used to prepare the *Test solution*.▲ (USP 1-Aug-2019)

## Method 1

Protein in solution absorbs UV light at a wavelength of 280 nm due to the presence of aromatic amino acids, mainly tyrosine and tryptophan. This property is the basis of *Method 1*. ▲▲ (USP 1-Aug-2019)If the buffer used to ▲solubilize▲ (USP 1-Aug-2019) the protein has a high absorbance relative to ▲▲ (USP 1-Aug-2019) water, there is an interfering substance in the buffer. This interference can be compensated for when the spectrophotometer is adjusted to zero buffer absorbance. The results may be compromised if the

[1] Suitable commercial columns are available. For example, PD-10 columns (available from GE Healthcare) for samples ranging from 1.0–2.5 mL, NAP-10 columns for samples up to 1.0 mL, NAP-5 columns for samples up to 0.5 mL, or another suitable alternative.

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interference results in a large absorbance that challenges the limit of sensitivity of the spectrophotometer. Furthermore, at low concentrations protein can be absorbed onto the cuvette, thereby reducing the content in solution. This can be prevented by preparing samples at higher concentrations or by using a nonionic detergent in the preparation.

▲To determine whether the protein sample is contaminated with nucleic acid, a 260/280 ratio is recommended if no other methods are used to quantitate the nucleic acids. *Table 1* can be used as a guide for protein purity.

**Table 1. 260/280 Ratio for Estimating Nucleic Acid Contamination and Protein Purity of the Sample**

| Protein (%) | Nucleic Acid (%) | 260/280 Ratio |
|---|---|---|
| 100 | 0 | 0.57 |
| 95 | 5 | 1.06 |
| 90 | 10 | 1.32 |
| 70 | 30 | 1.73▲ (USP 1-Aug-2019) |

### TEST SOLUTION

Dissolve a suitable quantity of the protein under test in the appropriate buffer to obtain a solution having a concentration of 0.2–2 mg/mL.

### STANDARD SOLUTION

Unless otherwise specified in the individual monograph, prepare a solution of Reference Standard or reference material for the protein under test in the same buffer and at the same concentration as the *Test solution*.

### PROCEDURE

Concomitantly determine the ▲absorbance values▲ (USP 1-Aug-2019) of the *Standard solution* and *Test solution* in quartz cells at a wavelength of 280 nm with a suitable spectrophotometer (see *Ultraviolet-Visible Spectroscopy* ⟨857⟩), using the buffer as the *Blank*. ▲ [NOTE—Keep the *Test solution*, *Standard solution*, and buffer at the same temperature during testing.]▲ (USP 1-Aug-2019) To obtain accurate results, the response should be linear in the range of protein concentrations to be assayed.

### LIGHT SCATTERING

The accuracy of the UV spectroscopic determination of protein can be decreased by the scattering of light by the ▲*Test solution*.▲ (USP 1-Aug-2019)If the proteins in solution exist as particles comparable in size to the wavelength of the measuring light (250–300 nm), scattering of the light beam results in an apparent increase in absorbance of the test specimen. To calculate the absorbance at 280 nm due to light scattering, determine the ▲absorbance values▲ (USP 1-Aug-2019) of the *Test solution* at wavelengths of 320, 325, 330, 335, 340, 345, and 350 nm. Using the linear regression method, plot the log of the observed absorbance versus the log of the wavelength, and determine the standard curve best fitting the plotted points. From the graph so obtained, extrapolate the absorbance value due to light scattering at 280 nm. Subtract the absorbance due to light scattering from the total absorbance at 280 nm to obtain the absorbance value of the protein in solution. Filtration with a filter having a 0.2-µm porosity or clarification by centrifugation may be performed to reduce the effect of light scattering, especially if the solution is noticeably turbid.

### CALCULATIONS

Calculate the concentration, $C_U$, of protein in the test specimen by the formula:

$$\text{Result} = C_S(A_U/A_S)$$

in which $C_S$ is the concentration of the *Standard solution*; and $A_U$ and $A_S$ are the corrected ▲absorbance values▲ (USP 1-Aug-2019) of the *Test solution* and the *Standard solution*, respectively (see ⟨857⟩).

## Method 2

This method, commonly referred to as the Lowry assay, is based on the reduction by protein of the phosphomolybdic–tungstic mixed acid chromogen in the Folin-Ciocalteu's phenol reagent, resulting in an absorbance maximum at 750 nm. The Folin-Ciocalteu's phenol reagent reacts primarily with tyrosine residues in the protein, which can lead to variation in the response of the assay to different proteins. Because the method is sensitive to interfering substances, a procedure for precipitation of the protein from the test specimen may be used. ▲Most interfering substances cause a lower color yield; however, some detergents cause a slight increase in color. A high salt concentration may cause a precipitate to form. Because different protein species may give different color response intensities, the standard protein and test protein should be the same.▲ (USP 1-Aug-2019)

The effect of interfering substances can be minimized by dilution, provided the concentration of the protein under test remains sufficient for accurate measurement. ▲Alternatively, procedures described in *Sample Extraction Methods for Total Protein Assays* could be used to remove interfering substances from the test sample.

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The Lowry assay can be used for protein concentrations of 5–100 µg/mL. Wider ranges may be acceptable if the range of standard concentrations selected results in a linear curve.▲ (USP 1-Aug-2019)

### STANDARD SOLUTIONS

Unless otherwise specified in the individual monograph, ▲solubilize▲ (USP 1-Aug-2019) the Reference Standard or reference material for the protein under test in the buffer used to prepare the *Test solution*. Dilute portions of this solution with the same buffer to obtain NLT 5 *Standard solutions* having concentrations between 5 and 100 µg of protein per mL, the concentrations being evenly spaced.

### TEST SOLUTION

Dissolve a suitable quantity of the protein under test in the appropriate buffer to obtain a solution having a concentration within the range of the concentrations of the *Standard solutions*. An appropriate buffer will produce a pH in the range of 10.0–10.5.

### BLANK

Use the buffer used to prepare the *Test solution* and the *Standard solutions*.

### REAGENTS AND SOLUTIONS

**Copper sulfate reagent:** Dissolve 100 mg of cupric sulfate and 200 mg of sodium tartrate in water, dilute with water to 50 mL, and mix. Dissolve 10 g of sodium carbonate in water to a final volume of 50 mL, and mix. Slowly pour the sodium carbonate solution into the copper sulfate solution with mixing. Prepare this solution fresh daily.
**SDS solution:** Dissolve 5 g of sodium dodecyl sulfate in water, and dilute with water to 100 mL.
**Sodium hydroxide solution:** Dissolve 3.2 g of sodium hydroxide in water, dilute with water to 100 mL, and mix.
**Alkaline copper reagent:** Prepare a mixture of *Copper sulfate reagent, SDS solution,* and *Sodium hydroxide solution* (1:2:1). This reagent may be stored at room temperature for up to 2 weeks.
**Diluted Folin-Ciocalteu's phenol reagent:** Mix 10 mL of Folin-Ciocalteu's phenol TS with 50 mL of water. Store in an amber bottle at room temperature.

### PROCEDURE

To 1 mL of each *Standard solution*, the *Test solution*, and the *Blank*, add 1 mL of *Alkaline copper reagent*, and mix. Allow to stand at room temperature for 10 min. Add 0.5 mL of the *Diluted Folin-Ciocalteu's phenol reagent* to each solution, mix each tube immediately, ▲and hold at room temperature for about 30 min.

[NOTE—Color development reaches a maximum in 20–30 min during incubation at room temperature, after which there is a gradual loss of color.]

Determine the absorbance values of the *Standard solutions* and *Test solution* at 750 nm with a suitable spectrophotometer, using the solution from the *Blank* to set the instrument to zero (see ⟨857⟩).▲ (USP 1-Aug-2019)

### CALCULATIONS

[NOTE—The relationship of absorbance to protein concentration is nonlinear; however, if the standard curve concentration range is sufficiently small, it will approach linearity.] Using the linear regression method, plot the ▲absorbance values▲ (USP 1-Aug-2019) of the solutions from the *Standard solutions* versus the protein concentrations, and determine the standard curve best fitting the plotted points. From the standard curve so obtained and the absorbance of the *Test solution*, determine the concentration of protein in the *Test solution*.

▲▲ (USP 1-Aug-2019)

## Method 3

This method, commonly referred to as the Bradford assay, is based on the absorption shift from 470 nm to 595 nm observed when the Brilliant Blue G dye binds to protein. The Brilliant Blue G dye binds most readily to arginyl and lysyl residues in the protein, which can lead to variation in the response of the assay to different proteins. ▲There are relatively few interfering substances, but detergents and ampholytes in the test specimen should be avoided. Highly alkaline specimens may interfere with the acidic reagent.▲ (USP 1-Aug-2019)

### STANDARD SOLUTIONS

Unless otherwise specified in the individual monograph, ▲solubilize▲ (USP 1-Aug-2019) the Reference Standard or reference material for the protein under test in the buffer used to prepare the *Test solution*. Dilute portions of this solution with the same buffer to obtain NLT 5 *Standard solutions* having concentrations of 100 µg–1 mg/mL of protein, the concentrations being evenly spaced.

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### TEST SOLUTION

▲Solubilize▲ (USP 1-Aug-2019) a suitable quantity of the protein under test in the appropriate buffer to obtain a solution having a concentration within the range of the concentrations of the *Standard solutions*.

### BLANK

Use the buffer used to prepare the *Test solution* and the *Standard solutions*.

### COOMASSIE REAGENT

Dissolve 100 mg of Brilliant Blue G[2] in 50 mL of alcohol. [NOTE—Not all dyes have the same Brilliant Blue G content, and different products may give different results.] Add 100 mL of phosphoric acid, dilute with water to 1 L, and mix. Pass the solution through filter paper (Whatman #1 or equivalent), and store the filtered reagent in an amber bottle at room temperature. [NOTE—Slow precipitation of the dye will occur during storage of the reagent. Filter the reagent before use.]

### PROCEDURE

Add 5 mL of the *Coomassie reagent* to 100 µL of each *Standard solution*, the *Test solution*, and the *Blank*, and mix by inversion. Avoid foaming, which will lead to poor reproducibility. ▲Incubate at room temperature for a suitable period of time, and then determine the absorbance values▲ (USP 1-Aug-2019) of the solutions from the *Standard solutions* and *Test solution* at 595 nm with a suitable spectrophotometer (see ⟨857⟩), using the *Blank* to set the instrument to zero. [NOTE—Do not use quartz (silica) spectrophotometer cells: the dye binds to this material. Because different protein species may give different color response intensities, the standard protein and test protein should be the same. ▲For consistent and accurate results, the absorbance values for all *Standard solutions*, *Blank*, and *Test solutions* should be determined within a suitable period of time to assure reproducible results of adding the *Coomassie reagent*.▲ (USP 1-Aug-2019)]

### CALCULATIONS

[NOTE—The relationship of absorbance to protein concentration is nonlinear; however, if the standard curve concentration range is sufficiently small, it will approach linearity.] ▲Plot the absorbance values of the *Standard solutions* versus the protein concentrations, and use linear regression to establish the standard curve. Determine the concentration of protein in the *Test solution* from the standard curve and the absorbance of the *Test solution*.▲ (USP 1-Aug-2019)

## Method 4

This method, commonly referred to as the bicinchoninic acid (BCA) assay, is based on reduction of the cupric ($Cu^{2+}$) ion to cuprous ($Cu^{1+}$) ion by protein. The bicinchoninic acid reagent is used to detect the cuprous ion. The method has few interfering substances. When interfering substances are present, their effect may be minimized by dilution, provided that the concentration of the protein under test remains sufficient for accurate measurement. ▲If substances that will cause interference in the test are present, proceed as directed in *Sample Extraction Methods for Total Protein Assays*. Because different protein species may give different color response intensities, the standard protein and test protein should be the same.▲ (USP 1-Aug-2019)

### STANDARD SOLUTIONS

Unless otherwise specified in the individual monograph, ▲solubilize▲ (USP 1-Aug-2019) the Reference Standard or reference material for the protein under test in the buffer used to prepare the *Test solution*. Dilute portions of this solution with the same buffer to obtain NLT 5 *Standard solutions* having concentrations of 10–1200 µg/mL of protein, the concentrations being evenly spaced.

### TEST SOLUTION

Dissolve a suitable quantity of the protein under test in the appropriate buffer to obtain a solution having a concentration within the range of the concentrations of the *Standard solutions*.

### BLANK

Use the buffer used to prepare the *Test solution* and the *Standard solutions*.

### REAGENTS

**BCA reagent:** Dissolve about 10 g of bicinchoninic acid, 20 g of sodium carbonate monohydrate, 1.6 g of sodium tartrate, 4 g of sodium hydroxide, and 9.5 g of sodium bicarbonate in water. Adjust, if necessary, with sodium hydroxide or sodium bicarbonate to a pH of 11.25. Dilute with water to 1 L, and mix.
**Copper sulfate reagent:** Dissolve about 2 g of cupric sulfate in water to a final volume of 50 mL.
**Copper–BCA reagent:** Mix 1 mL of *Copper sulfate reagent* and 50 mL of *BCA reagent*.

[2] Dye purity is important in the reagent preparation. Serva Blue G (Crescent Chemical Company, ▲Islandia,▲ (USP 1-Aug-2019) NY) is an acceptable grade.

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PROCEDURE

Mix 0.1 mL of each *Standard solution*, the *Test solution*, and the *Blank* with 2 mL of the *Copper–BCA reagent*. Incubate the solutions at 37° for 30 min, note the time, and allow ▲the mixtures▲ (USP 1-Aug-2019) to ▲cool▲ (USP 1-Aug-2019) to room temperature. Within 60 min following the incubation time, determine the ▲absorbance values for▲ (USP 1-Aug-2019) the *Standard solutions* and the *Test solution* ▲▲ (USP 1-Aug-2019) at 562 nm with a suitable spectrophotometer (see ⟨857⟩), using the *Blank* to set the instrument ▲calibration▲ (USP 1-Aug-2019) to zero. The color intensity continues to increase gradually after the solutions are cooled to room temperature. ▲▲ (USP 1-Aug-2019)

CALCULATIONS

[NOTE—The relationship of absorbance to protein concentration is nonlinear; however, if the standard curve concentration range is sufficiently small, it will approach linearity.] ▲Plot the absorbance values of the solutions from the *Standard solutions* versus the protein concentrations and determine the standard curve best fitting the plotted points. Determine the concentration of protein in the *Test solution* from the standard curve and the absorbance of the *Test solution*.▲ (USP 1-Aug-2019)

## Method 5

This method, commonly referred to as the Biuret assay, is based on the interaction of cupric ($Cu^{2+}$) ion with protein in an alkaline solution and the ▲▲ (USP 1-Aug-2019) development of absorbance at 545 nm. ▲This test shows minimal difference between IgG and albumin samples at the same concentration. Addition of the sodium hydroxide and the *Biuret reagent* as a combined reagent, insufficient mixing after the addition of the sodium hydroxide, or an extended time between the addition of the sodium hydroxide solution and the addition of the *Biuret reagent* will give IgG samples a higher response than albumin samples. The trichloroacetic acid method (described in *Procedure 2* of *Sample Extraction Methods for Total Protein Assays*) used to minimize the effects of interfering substances can also be used to determine the protein content in test specimens at concentrations below 500 µg/mL.▲ (USP 1-Aug-2019)

STANDARD SOLUTIONS

▲Prepare the▲ (USP 1-Aug-2019) Reference Standard or reference material for the protein under test in ▲0.9%▲ (USP 1-Aug-2019) sodium chloride solution. ▲▲ (USP 1-Aug-2019) Dilute portions of this solution with ▲0.9%▲ (USP 1-Aug-2019) sodium chloride solution ▲▲ (USP 1-Aug-2019) to obtain NLT 3 *Standard solutions* having concentrations of 0.5–10 mg/mL, the concentrations being evenly spaced. ▲▲ (USP 1-Aug-2019)

TEST SOLUTION

Prepare a solution of the test protein in ▲0.9%▲ (USP 1-Aug-2019) sodium chloride solution ▲▲ (USP 1-Aug-2019) having a concentration within the range of the concentrations of the *Standard solutions.*

BLANK

Use ▲0.9%▲ (USP 1-Aug-2019) sodium chloride solution. ▲▲ (USP 1-Aug-2019)

BIURET REAGENT

Dissolve about 3.46 g of cupric sulfate in 10 mL of hot water, and allow to cool (Solution 1). Dissolve about 34.6 g of sodium citrate dihydrate and 20.0 g of sodium carbonate in 80 mL of hot water, and allow to cool (Solution 2). Mix Solution 1 and Solution 2, and dilute with water to 200 mL. The *Biuret reagent* is stable at room temperature for 6 months. Do not use the reagent if it develops turbidity or contains any precipitate.

PROCEDURE

To 1 volume of ▲▲ (USP 1-Aug-2019) the *Test solution* add an equal volume of ▲6%▲ (USP 1-Aug-2019) sodium hydroxide solution ▲▲ (USP 1-Aug-2019) and mix. Immediately add a volume of *Biuret reagent* equivalent to 0.4 of the volume of the *Test solution*, and mix. Allow to stand at a temperature between 15°–25° for NLT 15 min. Within 90 min after the addition of the *Biuret reagent*, determine the ▲absorbance values▲ (USP 1-Aug-2019) of the *Standard solutions* and the solution from the *Test solution* at 545 nm with a suitable spectrophotometer (see ⟨857⟩), using the *Blank* to ▲calibrate▲ (USP 1-Aug-2019) the instrument to zero. [NOTE—Any solution that develops turbidity or a precipitate is not acceptable for calculation of protein concentration.]

CALCULATIONS

▲Within the given range of the standards, the relationship of absorbance to protein concentration is approximately linear. Plot the absorbance values of the *Standard solutions* versus the protein concentrations and use linear regression to establish the standard curve. Calculate the correlation coefficient for the line.▲ (USP 1-Aug-2019) A suitable system is one that yields a line having a correlation coefficient of NLT 0.99. ▲Determine the concentration of protein in the *Test solution* from the standard curve and the absorbance of the *Test solution*.▲ (USP 1-Aug-2019)

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## Method 6

This fluorometric method is based on the derivatization of the protein with *o*-phthalaldehyde (OPA), which reacts with the primary amines of the protein (i.e., $NH_2$-terminal amino acid and the ε-amino group of the lysine residues). The sensitivity of the test can be increased by hydrolyzing the protein before testing. Hydrolysis makes the α-amino group of the constituent amino acids of the protein available for reaction with the OPA reagent. The method requires very small quantities of the protein.

Primary amines, such as tris(hydroxymethyl)aminomethane and amino acid buffers, react with OPA and must be avoided or removed. Ammonia at high concentrations will react with OPA as well. The fluorescence obtained when amine reacts with OPA can be unstable. The use of automated procedures to standardize ▲the analysis▲ (USP 1-Aug-2019) may improve the accuracy and precision of the test.

### STANDARD SOLUTIONS

Unless otherwise specified in the individual monograph, ▲prepare▲ (USP 1-Aug-2019) the Reference Standard or reference material for the protein under test in the buffer used to prepare the *Test solution*. Dilute portions of this solution with the same buffer to obtain NLT 5 *Standard solutions* having concentrations of 10–200 µg/mL of protein, the concentrations being evenly spaced.

### TEST SOLUTION

▲Solubilize▲ (USP 1-Aug-2019) a suitable quantity of the protein under test in the appropriate buffer to obtain a solution having a concentration within the range of the concentrations of the *Standard solutions*.

### BLANK

Use the buffer used to prepare the *Test solution* and the *Standard solutions*.

### REAGENTS

**Borate buffer:** Dissolve about 61.83 g of boric acid in water, and adjust with potassium hydroxide to a pH of 10.4. Dilute with water to 1 L, and mix.
**Stock OPA reagent:** Dissolve about 120 mg of OPA in 1.5 mL of methanol, add 100 mL of *Borate buffer*, and mix. Add 0.6 mL of polyoxyethylene (23) lauryl ether, and mix. This solution is stable at room temperature for at least 3 weeks.
**OPA reagent:** To 5 mL of *Stock OPA reagent* add 15 µL of 2-mercaptoethanol. Prepare at least 30 min prior to use. This reagent is stable for 1 day.

### PROCEDURE

Adjust each of the *Standard solutions* and the *Test solution* to a pH between 8▲.0▲ (USP 1-Aug-2019) and 10.5. Mix 10 µL of the *Test solution* and each of the *Standard solutions* with 100 µL of *OPA reagent*, and allow to stand at room temperature for 15 min. Add 3 mL of 0.5 N sodium hydroxide, and mix. Using a suitable fluorometer (see *Fluorescence Spectroscopy* ⟨853⟩), determine the fluorescent intensities of solutions from the *Standard solutions* and the *Test solution* at an excitation wavelength of 340 nm and an emission wavelength between 440–455 nm. [NOTE—The fluorescence of an individual specimen is read only once because irradiation decreases the fluorescent intensity.]

### CALCULATIONS

The relationship of fluorescence to protein concentration is linear. Using the linear regression method, plot the fluorescent intensities of the solutions from the *Standard solutions* versus the protein concentrations, and determine the standard curve best fitting the plotted points. ▲Determine the concentration of protein in the *Test solution* from the standard curve and the fluorescence of the *Test solution*.▲ (USP 1-Aug-2019)

## Method 7

This method is based on nitrogen analysis as a means of protein determination. Interference caused by the presence of other nitrogen-containing substances in the test specimen can affect the determination of protein by this method ▲since non-proteinaceous nitrogen will also be detected.▲ (USP 1-Aug-2019) Nitrogen analysis techniques destroy the protein under test and are not ▲stand-alone methods since they quantitate all nitrogen present. If either *Procedure 1* or *Procedure 2* below is used to determine protein content, then a suitable orthogonal method must also be used to verify that no other nitrogen-containing sources are present and contributing to the apparent total protein content.▲ (USP 1-Aug-2019)

### PROCEDURE 1

Determine the nitrogen content of the protein under test as directed under *Nitrogen Determination* ⟨461⟩. Commercial instrumentation is available for the Kjeldahl nitrogen assay.

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## PROCEDURE 2

Commercial instrumentation is available for nitrogen ▲(elemental)▲ (USP 1-Aug-2019) analysis. Most nitrogen analysis instruments use pyrolysis (i.e., combustion of the sample in oxygen at temperatures approaching 1000°), which produces nitric oxide (NO) and similar oxides of nitrogen ($NO_x$) from the nitrogen present in the test protein. Some instruments convert the nitric oxides to nitrogen gas, which is quantified with a thermal conductivity detector. Other instruments mix nitric oxide (NO) with ozone ($O_3$) to produce excited nitrogen dioxide ($NO_2$), which emits light when it decays and can be quantified with a chemiluminescence detector. A protein reference material or reference standard that is relatively pure and is similar in composition to the test proteins is used to optimize the injection and pyrolysis parameters and to evaluate consistency in the analysis.

## CALCULATIONS

The protein concentration is calculated by dividing the nitrogen content of the sample by the known nitrogen content of the protein. The known nitrogen content of the protein can be determined from the chemical composition of the protein or by comparison with the nitrogen content of the Reference Standard or reference material.

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# ⟨1058⟩ ANALYTICAL INSTRUMENT QUALIFICATION

## INTRODUCTION

A large variety of analytical instruments, ranging from a simple apparatus to complex computerized systems, is used in the pharmaceutical industry to acquire data that will help ensure that products meet their specifications. Many of these instruments combine a metrological function with software control. There are many ways of demonstrating that an instrument is qualified and under control, and these can include qualification, calibration, validation, and maintenance. In order to ensure "fitness for purpose", an integrated approach, based upon a risk assessment, is recommended. For the purposes of this chapter, the term "instrument" includes any apparatus, equipment, instrument, or instrument system used in pharmacopeial analyses.

This informational chapter provides general guidance in a scientific, risk-based approach for carrying out an analytical instrument qualification (AIQ). Detailed instrument operating parameters to be qualified are found in the respective general chapters for specific instrument types. It is left to each laboratory to justify and document its specific approaches. The instrument owners/users and their management are responsible for assuring their instruments are suitably qualified.

This chapter uses various terms, acronyms, and activities common to analytical laboratories and validation disciplines. These terms and activities may not be identical to their usages in all laboratories. The reader is encouraged to be flexible in interpreting the application of these terms and activities in the context and intent of this chapter.

The risk assessment for an AIQ enables the classification of the instrument to determine the extent of qualification and actions needed to demonstrate fitness for purpose. Generally, the more complex the instrument, or the higher the criticality of the measurement, the greater the amount of work that is required to ensure that quality data will be generated. In addition, attention must be paid to ensuring that data integrity and security are maintained.

Instruments can generally be classified as belonging to Groups A, B, or C. It should be noted that the same type of instrument can fit into one or more categories, depending on its intended use.

Group A includes the least complex, standard instruments that are used without measurement capability or user requirement for calibration, such as a magnetic stirrer or vortex mixer. Proper function is ensured by observation, and no further qualification activities are needed for this group.

Group B includes instruments that may provide a measurement or an experimental condition that can affect a measurement. Examples include a pH meter or an oven. Proper function of instruments in this group may require only routine calibration, maintenance, or performance checks. The extent of activities may depend on the criticality of the application. Generally, these instruments may have firmware but not software that is updated by the user.

Group C comprises analytical instruments with a significant degree of computerization and complexity, such as high-pressure liquid chromatographs and mass spectrometers. All elements of qualification, including software validation, must be considered to ensure proper functioning of instruments in this group.

## COMPONENTS OF DATA QUALITY

There are four critical components involved in the generation of reliable and consistent data (quality data). *Figure 1* shows these components as layered activities within a quality triangle. Each layer adds to the overall quality. AIQ forms the base for generating quality data. The other components essential for generating quality data are analytical method validation, system suitability tests, and quality control check samples. These quality components are described below.

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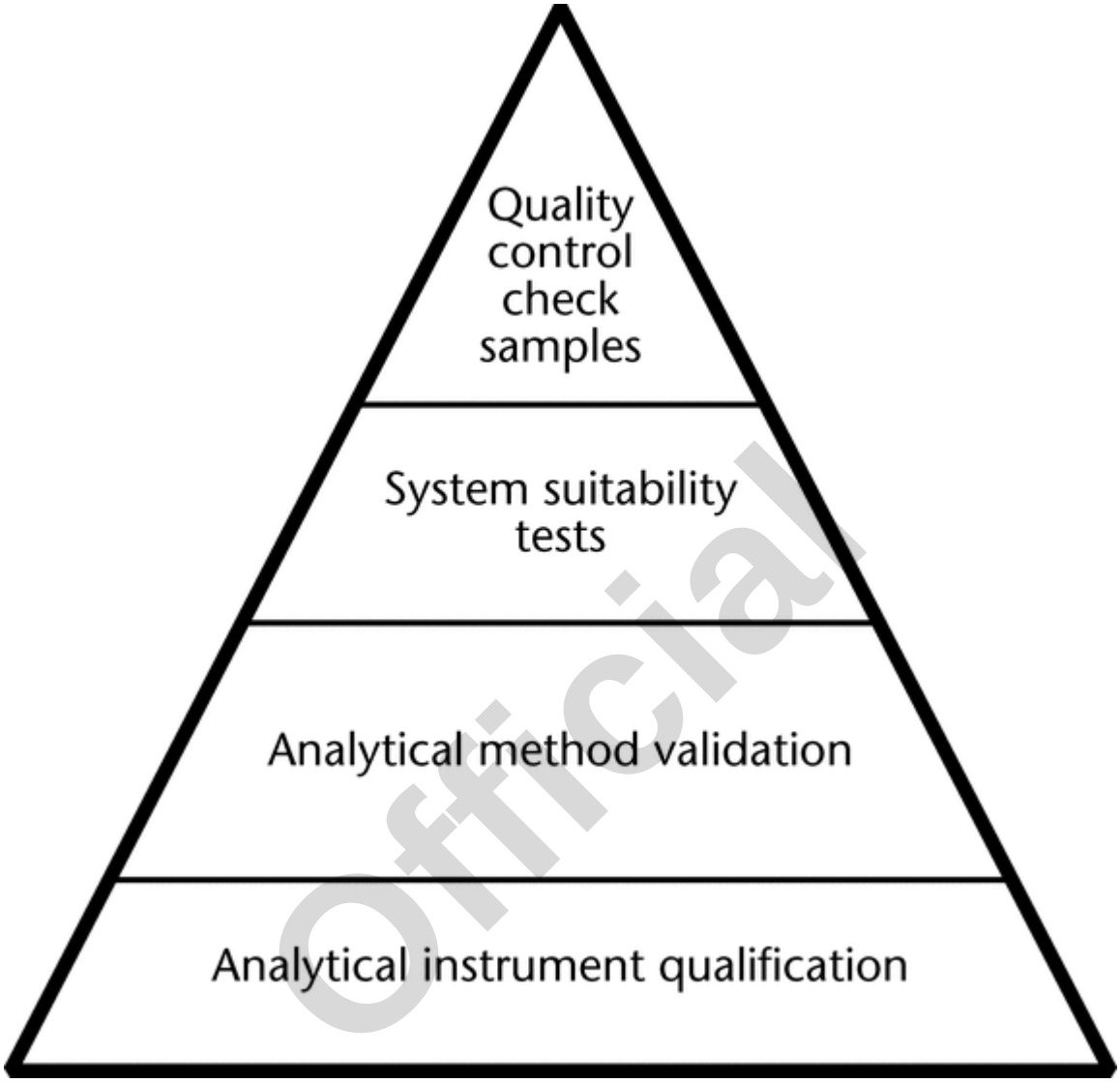


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Figure 1. Components of data quality.

## Analytical Instrument Qualification

AIQ is the collection of documented evidence that an instrument performs suitably for its intended purpose. Use of a qualified instrument in analyses contributes to confidence in the validity of generated data.

## Analytical Method Validation

Analytical method validation is the collection of documented evidence that an analytical procedure is suitable for its intended use. Use of a validated procedure with qualified analytical instruments provides confidence that the procedure will generate test data of acceptable quality. Additional guidance on validation of compendial procedures may be found in *Validation of Compendial Procedures* ⟨1225⟩.

## System Suitability Tests

System suitability tests verify that the system will perform in accordance with the criteria set forth in the procedure. These tests are performed along with the sample analyses to ensure that the system's performance is acceptable at the time of the test. *Chromatography* ⟨621⟩ presents a more detailed discussion of system suitability tests related to chromatographic systems.

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## Quality Control Check Samples

Many analysts carry out their tests on instruments that have been standardized by using reference materials and/or calibration standards. Some analyses also require the inclusion of quality control check samples to provide an in-process or ongoing assurance of the test's suitable performance. In this manner, AIQ and analytical method validation contribute to the quality of analysis before analysts conduct the tests. System suitability tests and quality control checks help ensure the quality of analytical results immediately before or during sample analysis.

# ANALYTICAL INSTRUMENT QUALIFICATION PROCESS

The following sections address the AIQ process in detail. The other three components of building quality into analytical data —analytical method validation, system suitability tests, and quality control check samples—are not within the scope of this chapter.

## Qualification Phases

AIQ is not a single, continuous process but instead results from interconnected activities over the lifetime of the instrument. The first activity is the generation of a user requirements specification (URS), which defines the laboratory's particular needs and technical and operational requirements that are to be met. The subsequent qualification activities necessary to establish fitness for purpose may be grouped into four phases: design qualification (DQ), installation qualification (IQ), operational qualification (OQ), and performance qualification (PQ). PQ is also sometimes called user acceptance testing (UAT). This framework may be extended for complex systems to include functional specifications (FS) and factory acceptance testing (FAT), if appropriate. Some activities normally undertaken in IQ, OQ, or PQ may be satisfied during the instrument installation and start-up. This is sometimes referred to as site acceptance testing (SAT). It is more important that all required activities be performed in a logical order and scientifically sound manner than the exact allocation within the IQ/OQ/PQ framework. The activities may also be performed as an integrated framework.

Some AIQ activities cover more than one qualification phase, and analysts could potentially perform them during more than one of the phases. However, there is a need for a logical, specific order to the AIQ activities; for example, IQ activities such as configuration must occur before OQ can start. However, where appropriate, qualification activities and associated documentation may be combined together (e.g., IQ and OQ). All AIQ activities should be predefined and contemporaneously documented.

OQ tests are specifically designed to verify the instrument's correct functionality and operation according to specifications in the user's environment, as documented in the URS. Repeating all the testing at regular intervals may not be required. However, following preventative maintenance, critical operational parameters should be confirmed. Routine analytical tests do not constitute OQ testing.

When the instrument undergoes major repairs or modifications, this should be evaluated using change control. Relevant IQ, OQ, and/or PQ tests should be repeated to verify that the instrument continues to operate satisfactorily. If an instrument is moved to another location, an assessment should be made of what, if any, qualification stage should be repeated.

### DESIGN QUALIFICATION

DQ is the documented collection of activities that define the functional and operational specifications and intended purpose of the instrument. DQ states what the laboratory wants the instrument to do and shows that the selected instrument is suitable. DQ may be performed by the instrument manufacturer or the user. It is expected that DQ requirements will be minimal for commercial, off-the-shelf instruments. Verification that the instrument specifications meet the desired functional requirements may suffice.

The supplier is generally responsible for robust design and maintaining documentation describing how the analytical instrument and any associated controlling software are manufactured (e.g., design specifications, functional requirements, and others) and tested, sometimes called factory acceptance tests. Nonetheless, the user should ensure that instruments are suitable for their intended application and may evaluate whether the supplier has adopted a quality system that provides for reliable instrumentation, software, and network connectivity. Users should also determine the supplier's capability to support installation, services, and training. This determination might be aided by the user's previous interaction with the supplier.

When use of an instrument changes or it is subject to a major upgrade, it may be necessary to review and/or update the user's DQ documentation.

### INSTALLATION QUALIFICATION

IQ is the documented collection of activities necessary to establish that an instrument is delivered as designed and specified, is properly installed in the selected environment, and that this environment is suitable for the instrument. IQ applies to an instrument that is new or was pre-owned. For any instrument that exists on site but has not been previously qualified, or not qualified to current industry standards, existing documents should be collated and a risk assessment should be undertaken to determine the best course of action.

Relevant parts of IQ would also apply to a qualified instrument that has been transported to another location or is being reinstalled for other reasons, such as prolonged storage.

The activities and documentation typically associated with IQ are as follows.

**Instrument delivery:** Ensure that the instrument, software, manuals, supplies, and any other instrument accessories arrive as specified by the user and that they are undamaged. For a pre-owned or existing instrument, manuals and documentation should be obtained.

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**Description:** Document information about the instrument and all components, including supplier(s), model(s), serial number(s), software version(s), and location.
**Utilities/Facility/Environment:** Verify that the installation site satisfactorily meets supplier-specified environmental requirements.
**Assembly and installation:** Assemble and install the instrument, and perform any preliminary diagnostics and testing. Assembly and installation may be done by the supplier, service agents, specialized engineers, or qualified in-house personnel. Supplier-established installation tests provide a valuable baseline reference for determining instrument acceptance. Any abnormal event observed during assembly and installation merits documenting. IQ documentation packages purchased from a supplier should be reviewed to ensure that they are acceptable to the user before and after execution.
**Software installation, network, and data storage:** Some analytical systems require the installation of software onto a qualified computer and to be connected to a network for communications and data storage at the installation site. Information technology involvement is often required with computerized laboratory systems.
**Installation verification:** Perform the initial diagnostics and testing of the instrument after installation. When required, connect the instrument to the network, and check its functionality.

## OPERATIONAL QUALIFICATION

OQ is the documented collection of activities necessary to demonstrate that an instrument will function according to its operational specification testing in the selected environment. OQ demonstrates fitness for the selected use, and should reflect URS. Testing activities in the OQ phase may consist of the following test parameters.
**Fixed parameters:** These tests measure the instrument's non-changing parameters such as length, height, weight, voltage inputs, acceptable pressures, and loads. If the manufacturer-supplied specifications for these parameters satisfy the user, the test requirements may be waived. However, if the user wants to confirm the parameters, testing can be performed at the user's site. Fixed parameters do not change over the life of the instrument, and therefore never need to be retested. [NOTE—These tests could also be performed during the IQ phase; if so, fixed parameters need not be retested as part of OQ testing.]
**Software functions:** Where applicable, OQ testing should include critical elements of the configured application software to show that the whole system works as intended. Functions to test would be those applicable to data capture, analysis of data, and reporting results under actual conditions of use as well as security, access control, and audit trail. The user can apply risk assessment methodologies and can leverage the supplier's software testing to focus the OQ testing effort.
**Secure data storage, backup, and archiving:** When applicable, test secure data handling, such as storage, backup, audit trails, and archiving at the user's site, according to written procedures.
**Instrument function tests:** Instrument functions required by the user should be tested to verify that the instrument operates as intended by the manufacturer. Supplier information is useful in identifying specifications for these parameters and in designing tests to verify that the instrument meets the supplier or user specifications in the user's environment.

The extent of OQ testing that an instrument undergoes depends on its intended applications, therefore no specific OQ tests for any instrument or application are offered in this chapter. Parameters to qualify are described in the general chapters pertaining to a specific analytical technique.

OQ tests can be modular or holistic. Modular testing of individual components of a system may facilitate interchanging of such components without requalification but requires a risk assessment to justify it. Holistic tests, which involve the entire system, demonstrate that the whole system complies with URS.

For OQ test packages purchased from a service provider or supplier, the user must review the material to assure themselves of the scientific soundness of the tests and compliance with applicable regulations. The user should review the documents before execution and approve the tests after execution to ensure completeness and accuracy of the completed document and the test data generated.
**Software configuration and/or customization:** Any configuration or customization of instrument software should occur before the OQ and be documented. Unless changes are needed for specific component tests, the OQ should be performed using the software configuration that will be used for routine analysis.

## PERFORMANCE QUALIFICATION

PQ is the documented collection of activities necessary to demonstrate that an instrument consistently performs according to the specifications defined by the user, and is appropriate for the intended use. The PQ verifies the fitness for purpose of the instrument under actual conditions of use. After IQ and OQ have been performed, the instrument's continued suitability for its intended use is demonstrated through continued PQ.

The user must define the PQ plans, including test procedures, acceptance criteria, and frequency. Preventive maintenance plans and documentation of repairs and other changes are also a necessary part of the overall instrument qualification.

PQ may include the following activities.
**Performance checks:** A test or series of tests to verify the acceptable performance of the instrument for its intended use. PQ tests are usually based on the instrument's typical on-site applications and may consist of analyzing known components or standards. The tests should be based on good science and reflect the general intended use of the instrument. Some system suitability tests or quality control checks that are performed concurrently with the test samples can be used to demonstrate that the instrument is performing suitably. PQ tests may resemble those performed during OQ, but the specifications for PQ results may be set differently if required. Nevertheless, user specifications for PQ tests should demonstrate trouble-free instrument operation for the intended applications. As is the case with OQ testing, PQ tests may be modular or holistic.

Testing frequency depends on the ruggedness of the instrument and the criticality of the analytic method. Testing may be unscheduled; for example, each time the instrument is used. It may also be scheduled for regular intervals. Experience with the instrument can influence this decision, which should be documented. It may be useful to repeat the same PQ tests each time the instrument is used so that a history of the instrument's performance can be compiled. Alternatively, the instrument may be

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incorporated into an integrated support system to ensure that it remains continually qualified. System suitability tests that are performed concurrently with the test preparations may also ensure that the instrument is performing suitably.

**Preventive maintenance and repairs:** Periodic preventive maintenance activities are required for many instruments. This may include calibration. Document the preventive maintenance plans, including procedures and frequency as part of the AIQ package. When an instrument fails to meet PQ criteria or otherwise malfunctions, the cause of the failure must be investigated and documented. The instrument may require maintenance or repair. The relevant OQ or PQ test(s) should be repeated after the needed maintenance or repair to ensure that the instrument remains qualified.

**Practices for PQ, change control, and periodic review:** Each PQ, maintenance, and calibration activity should be documented. Change control should be established to control changes to the instrument configuration, including firmware and software. Critical instruments should have a periodic review to ensure that the system is still under control. Typical areas for review can include qualification/validation status, currency of user procedures, change control records, correctness and completeness of records produced by the system, backup and recovery of electronic records, and review and sign-off of test results.

The instrument owner/user and their management are responsible for this work, although portions can be carried out on his/her behalf by internal staff or external suppliers or service providers.

## ROLES AND RESPONSIBILITIES

### Users

Users are ultimately responsible for specifying their needs and ensuring that a selected instrument meets them, and that data quality and integrity are maintained. The user's group encompasses analysts, their supervisors, instrument specialists, and organization management. Users should be adequately trained in the instrument's use, and their training records should be maintained as required by the regulations.

Users should also be responsible for qualifying their instruments, because their training and expertise in the use of instruments make them the best-qualified group to design the instrument test(s) and specification(s) necessary for a successful AIQ. Consultants, instrument manufacturers or suppliers, validation specialists, and quality assurance personnel can advise and assist as needed, but the final responsibility for qualifying instruments and validating systems lies with the users, who must ensure that the instrument is maintained in a qualified state through routine performance of PQ.

### Quality Unit

The role of the quality unit in AIQ remains the same as for any other regulated activity. Quality personnel are responsible for ensuring that the AIQ process meets compliance requirements, that processes are being followed, and that the intended use of the instrument is supported by complete, valid, and documented data.

### Manufacturers, Suppliers, Service Agents, and Consultants

Manufacturers are responsible for designing and manufacturing the instrument, and ensuring the quality of relevant processes used in manufacturing and assembly of the instrument. Manufacturers should test the assembled instruments before shipping them to users. To aid the user, suppliers are responsible for developing meaningful specifications for the users to compare with their needs and aid selection.

Where used, software should be developed and tested using a defined life cycle and should have evidence of work performed to support major and minor revisions. Release notes should accompany each version of software released.

Finally, it is desirable that suppliers should notify all known users about hardware or software defects discovered after a product's release; offer user training, service, repair, and installation support; and invite user audits as necessary.

There should be a quality or technical agreement between the user organization and manufacturers, suppliers, service agents, or consultants who supply calibration, maintenance, qualification, or validation services; the agreement should define the scope of work and the responsibilities of the two parties.

## SOFTWARE VALIDATION

There is an increasing inability to separate the hardware and software parts of modern analytical instruments. In many instances, the software is needed to qualify the instrument, and the instrument operation is essential when validating the software. Therefore, to avoid overlapping and potential duplication, software validation and instrument qualification can be integrated into a single activity.

Software used for analytical instruments can be classified into four groups: firmware, instrument control software, data acquisition software, and processing software. Although software validation is not the primary focus of this chapter, the following sections describe in which cases this activity is within the scope of the analytical instrument and system qualification.

One source of the validation of software is the guide *GAMP: A Risk-Based Approach to Compliant GxP Computerized Systems.*

### Firmware

Computerized analytical instruments contain integrated chips with low-level software (firmware). Such instruments will not function without properly operating firmware, and in most cases users generally cannot alter firmware function. Firmware is

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therefore considered a component of the instrument itself. Indeed, the qualification of hardware is not possible without operating it via its firmware.

Thus, when the hardware (that is, the analytical instrument) is qualified at the user's site, the firmware is also essentially qualified. No separate on-site qualification of the firmware is needed. Whenever possible, the firmware version should be recorded as part of the IQ activities. Any changes made to firmware versions should be tracked through the change control of the instrument (see *Change Control*, below).

In some instruments, firmware can also be capable of fixed calculations on the acquired data. These calculations need to be verified by the user. Some instruments have firmware that enables users to define programs for the instrument's operation; similarly, these user-defined programs need to be defined and verified to demonstrate that they are fit for the intended purpose. Any user-defined programs should be placed under change control and, if possible, access should be restricted to authorized personnel.

### Instrument Control, Data Acquisition, and Processing Software

Software for instrument control, data acquisition, and processing for many of today's computerized instruments is loaded on a computer connected to the instrument. Operation of the instrument is then controlled via the software, leaving fewer operating controls on the instrument. Also, the software is needed for data acquisition and post-acquisition calculations. Thus, both hardware and software, their functions inextricably intertwined, are critical to providing analytical results.

The software in this group can be classified into three types: 1) non-configurable software that cannot be modified to change the business process; 2) configurable software that includes tools from the supplier to modify the business process; and 3) configurable software with custom additions (i.e., custom software or macros to automate the business process).

The supplier of the system should develop and test the software according to a defined life cycle and provide users with a summary of the tests that were carried out. Ideally, this software development should be carried out under a quality management system.

At the user site, integrated qualification of the instrument, in conjunction with validation of the software, involves the entire system. This is more efficient than separating instrument qualification from validation of the software.

## CHANGE CONTROL

Changes to qualified instruments, including software, become inevitable as suppliers add new features and correct known defects. However, implementing all such changes may not always benefit users. Users should therefore adopt changes they deem useful or necessary. Changes also occur due to repair, maintenance, or relocation of the instrument. A change control process should be in place to guide the assessment, execution, documentation, and approval of any changes to instrumentation.

Change control applies to all elements of qualification and may follow the general qualification process. Users should assess the effects of changes to determine what, if any, requalification activities are required. If implementation of the change is needed, install the changes to the system. Consider if the change will affect the ability of the instrument to meet the user requirements or if the user requirements have changed. Evaluate which of the existing OQ and PQ tests need revision, deletion, or addition as a result of the installed change.

After implementation, perform any required testing to evaluate the effects of the change. Document all details of the change. Include a description of the change and a rationale, and list appropriate identification (e.g., part and serial numbers of new components and versions of new software or firmware).

## ANALYTICAL INSTRUMENT QUALIFICATION DOCUMENTATION

Documents obtained during qualification activities should be retained in an accessible manner. Where multiple instruments of one kind exist, documents common to all instruments and documents specific to an instrument may be stored separately. During change control, additional documents may supplement those obtained during the qualification process, and both sets of documents should be retained and maintained in a suitable manner that allows for appropriate protection and access.

## GLOSSARY

[NOTE—The definitions of these terms may be different than in other *USP* general chapters.]

**Calibration:** An operation that, under specified conditions, in a first step, establishes a relation between the quantity values, with measurement uncertainties provided by measurement standards, and corresponding indications with associated measurement uncertainties and, in a second step, uses this information to establish a relation for obtaining a measurement result from an indication. Note that:

1. A calibration may be expressed by a statement, calibration function, calibration diagram, calibration curve, or calibration table. In some cases, it may consist of an additive or multiplicative correction of the indication with associated measurement uncertainty.
2. Calibration should not be confused with adjustment of a measuring system, often mistakenly called "self-calibration", or with verification of calibration.
3. Often, the first step alone in the above definition is perceived as being calibration.

**Maintenance:** Actions performed to keep an analytical instrument in a state of proper function so that it continues to operate within the boundaries set during qualification or validation.

**Qualification:** Action of proving that any instrument works correctly and delivers the expected results; demonstration of fitness for purpose.

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**Software configuration:** Adaptation of software functions to a business process using tools provided within the application by the supplier of the software.

**Software customization:** Changing the way software automates a business process by the addition of externally custom-coded software modules using a recognized programming language or the development of macros within the application software.

**Software validation:** Confirmation by examination and provision of objective evidence that software conforms to user needs and intended uses, and that the particular requirements implemented through software can be consistently fulfilled.

**Supplier:** This term is used generically and can mean the manufacturer, a vendor, a service agent, or a consultant, depending on the circumstances.

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# ⟨1059⟩ EXCIPIENT PERFORMANCE

## INTRODUCTION

Excipients are used in virtually all drug products and are essential for product manufacturing and performance. Thus, the successful manufacture of a robust product requires the use of well-defined excipients and manufacturing processes that consistently yield a quality product. Excipients used in drug products typically are manufactured and supplied in compliance with compendial standards. However, the effects of excipient properties on the critical quality attributes (CQAs) of a drug product are unique for each formulation and process and may depend on properties of excipients that are not evaluated in *USP* or *NF* monographs. The effects of variations in excipient material attributes depend on the role of an excipient in a formulation and the CQAs of the drug product. This general chapter provides a framework for applying Quality by Design (QBD) principles to excipient quality and performance.

An excipient may be used in different ways or for different purposes in a formulation and may therefore require different material attributes to achieve the desired performance. Excipient functional categories are broad, qualitative, and descriptive terms for the purpose an excipient serves in a formulation. A list of excipients grouped by functional category is included in *NF* under *Front Matter, Excipients,* which summarizes some of the more common purposes that excipients serve in drug products. Also important are the material attributes of the ingredients that must be identified and controlled to ensure the excipient performs its intended function in a drug product. A critical material attribute (CMA) is a physical, chemical, biological, or microbiological property of a material that must be within an appropriate limit, range, or distribution to ensure that drug product CQAs are maintained throughout the product life cycle. Most, but not all, CMAs become tests in a compendial monograph. In some applications, excipient suppliers and users will need to identify and control material attributes in addition to monograph specifications. Identification of CMAs requires a thorough understanding of drug product CQAs; the manufacturing process(es); and the physical, chemical, biological, or microbiological properties of each ingredient. Manufacturers should anticipate lot-to-lot and supplier-to-supplier variability in excipient properties and should have in place appropriate control measures to ensure that CMAs are maintained within the required limits. Prior knowledge, experimental designs, and risk-assessment tools can be used to prioritize and identify CMAs of excipients as well as critical process parameters. A CMA of an excipient may not be related to the major component of the excipient because, for example, the presence of minor components (e.g., peroxides, elemental impurities, or microbiological content) may affect product stability or quality. Good product development practices, which at times are termed QBD principles, require understanding excipient CMAs that contribute to consistent performance and are the foundation of a control strategy that accommodates excipient variability, consistently achieving final product CQAs.

This informational general chapter provides an overview of the key functional categories of excipients and tests or procedures that can be used to monitor and control CMAs.[1]

In this chapter, the functional categories have been organized by their most typical use in common pharmaceutical dosage forms. However, functional categories can apply to multiple dosage forms. The association of a functional category with a particular dosage form does not limit the use of an excipient to a single type of dosage form or delivery system. Each functional category includes a general description; the mechanisms by which excipients achieve their function; physical properties common to these excipients; chemical properties; and a list of *USP* general chapters that can be useful in the development of specific tests, procedures, and acceptance criteria to ensure that CMAs are adequately monitored and controlled. Because of the complex nature and interplay of formulation ingredients, processing, and dosage form performance requirements, the information provided in this chapter should not be viewed as either restrictive or completely comprehensive.

***Change to read:***

## TABLETS AND CAPSULES

### Functional Category: Diluent

#### DESCRIPTION

Diluents are components that are incorporated into tablet or capsule dosage forms to increase dosage form volume or weight. Sometimes referred to as fillers, diluents often compose a large portion of the dosage form, and the quantity and type of diluent selected often depend on its physical and chemical properties. Thus, successful and robust manufacturing and dosage form performance depend on the measurement and control of the CMAs.

#### FUNCTIONAL MECHANISM

Among the most important functional roles diluents play is their ability to impart desirable manufacturing properties (e.g., powder flow, tablet compaction strength, wet or dry granule formation, or homogeneity) and performance (e.g., content uniformity, disintegration, dissolution, tablet integrity, friability, or physical and chemical stability). Some diluents (e.g., microcrystalline cellulose) occasionally are referred to as “dry binders” because of the high degree of tablet strength they impart to the final compressed tablet.

[1] This general information chapter provides nonmandatory information that does not create compendial requirements. For additional information about nonmandatory general chapters and alternative methods and procedures, see *General Notices, 6.30 Alternative and Harmonized Methods and Procedures.*

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### PHYSICAL PROPERTIES

The primary physical properties relevant to tablet/capsule diluents are those that can have a direct effect on diluent and formulation performance. These include: 1) particle size and size distribution, 2) particle shape, 3) bulk/tapped/true density, 4) specific surface area, 5) crystallinity, 6) moisture content, 7) powder flow, 8) solubility, 9) crystal form, and 10) compaction properties for tablet dosage forms.

### CHEMICAL PROPERTIES

Tablet diluents comprise a large and diverse group of materials that include inorganics (e.g., dibasic calcium phosphate or calcium carbonate), single-component organic materials (e.g., lactose monohydrate or mannitol), and multicomponent (e.g., silicified microcrystalline cellulose or sugar spheres), or complex organics (e.g., microcrystalline cellulose or starch). They may be soluble or insoluble in water, and they may be neutral, acidic, or alkaline in nature. These chemical properties can have a positive or negative effect on the drug substance physical or chemical stability and on performance. Appropriate selection of excipients with desirable physical and chemical properties can enhance the physical and chemical stability as well as the performance of the drug substance and dosage form. The detailed composition of an excipient may be important because excipient function could be influenced by the presence of minor concomitant components that are essential for proper performance. Pharmaceutical scientists may find it necessary to control the presence of undesirable components (e.g., elemental impurities or peroxides) to ensure adequate dosage form stability and performance.

### GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in diluent functions: *Light Diffraction Measurement of Particle Size* ⟨429⟩, *Bulk Density and Tapped Density of Powders* ⟨616⟩, *Crystallinity* ⟨695⟩, *Characterization of Crystalline Solids by Microcalorimetry and Solution Calorimetry* ⟨696⟩, *Density of Solids* ⟨699⟩, *Loss on Drying* ⟨731⟩, *Optical Microscopy* ⟨776⟩, *Particle Size Distribution Estimation by Analytical Sieving* ⟨786⟩, *Powder Fineness* ⟨811⟩, *Specific Surface Area* ⟨846⟩, *Water Determination* ⟨921⟩, and *Powder Flow* ⟨1174⟩.

## Functional Category: Wet Binder

### DESCRIPTION

Tablet and capsule binders are incorporated into formulations to facilitate the agglomeration of powder into granules during mixing with a granulating fluid such as water, hydroalcoholic mixtures, or other solvents. The binder may be either dissolved or dispersed in the granulation liquid or blended in a dry state, and other components and the granulation liquid may be added separately during agitation. Following evaporation of the granulation liquid, binders typically produce dry granules that achieve the desired properties such as granule size, size distribution, shape, content, mass, and active content. Wet granulation facilitates the further processing of the granules by improving one or more of the granule properties such as flow, handling, strength, resistance to segregation, dustiness, appearance, solubility, compaction, or drug release.

### FUNCTIONAL MECHANISM

Binders are soluble or partially soluble in the granulating solvent or, as in the case of native starches, can be made soluble. Concentrated binder solutions also have adhesive properties. Upon addition of liquid, binders typically facilitate the production of moist granules (agglomerates) by altering interparticle adhesion. They also may modify interfacial properties, viscosity, or other properties. During drying they may produce solid bridges that yield improved residual dry granule strength.

### PHYSICAL PROPERTIES

Dispersion or dissolution of a binder in the granulation liquid depends on its physical properties: depending on the application, then surface tension, particle size, size distribution, solubility, and viscosity are among the important properties. Homogeneous incorporation of a binder into a dry blend also depends on its physical properties such as particle size, shape, and size distribution. Viscosity often is an important property to consider for binders: for polymers, viscosity is influenced by the nature of the polymer structure, molecular weight, and molecular weight distribution. Polymeric binders may form gels.

### CHEMICAL PROPERTIES

Tablet and capsule binders can be categorized as: 1) natural polymers, 2) synthetic polymers, or 3) sugars. The chemical nature of polymers—including polymeric structure, monomer properties and sequence, functional groups, degree of substitution, and cross-linking—influences the complex interactions that can occur during granulation. Natural polymers in particular may exhibit greater variation in their properties because of variations in their sources and therefore their composition.

### GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in binder functions: *Bulk Density and Tapped Density of Powders* ⟨616⟩, *Chromatography* ⟨621⟩, *Crystallinity* ⟨695⟩, *Density of Solids* ⟨699⟩, *Loss on Drying* ⟨731⟩, *Particle Size Distribution Estimation by Analytical Sieving* ⟨786⟩, *Specific Surface Area* ⟨846⟩, *Viscosity—Capillary Methods* ⟨911⟩, and *Powder Flow* ⟨1174⟩.

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## Functional Category: Disintegrant

### DESCRIPTION

Disintegrants are functional components that are added to formulations to promote rapid disintegration into smaller units and to allow a drug substance to dissolve more rapidly. Disintegrants are natural, synthetic, or chemically modified natural polymeric substances. When disintegrants come in contact with water or stomach or intestinal fluid, they function by absorbing liquid and start to swell, dissolve, or form gels. This causes the tablet structure to rupture and disintegrate, producing increased surfaces for enhanced dissolution of the drug substance.

### FUNCTIONAL MECHANISM(S)

The ability to interact strongly with water is essential to the disintegrant function. Three major mechanisms describe the function of the various disintegrants: volume increase by swelling, deformation, and capillary action (wicking). In tablet formulations, the function of disintegrants is best described as a combination of two or more of these effects. The onset and degree of the locally achieved actions depend on various parameters of a disintegrant, such as its chemical nature and its particle size distribution and particle shape, as well as some important tablet parameters such as hardness and porosity.

### PHYSICAL PROPERTIES

The primary physical properties relevant to a disintegrant are those that describe the product's particle structure as a dry powder or its structure when in contact with water. These properties may include: 1) particle size distribution; 2) water absorption rate; 3) swelling ratio or swelling index; and 4) the characterization of the resulting product, whether it is still a particulate or a gel is formed.

### CHEMICAL PROPERTIES

Polymers used as disintegrants are either nonionic or anionic with counterions such as sodium, calcium, or potassium. Nonionic polymers are natural or physically modified polysaccharides such as starches, celluloses, pullulan, or cross-linked polyvinylpyrrolidone. The anionic polymers mainly are chemically modified starches, cellulose products, or low-cross-linked polyacrylates. These chemical properties should be considered in the case of ionic polymers. Disintegration performance is affected by pH changes in the gastrointestinal tract or by complex formation with ionic drug substances.

### GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in disintegrant functions: *Light Diffraction Measurement of Particle Size* ⟨429⟩, *Optical Microscopy* ⟨776⟩, *Particle Size Distribution Estimation by Analytical Sieving* ⟨786⟩, and *Powder Flow* ⟨1174⟩.

## Functional Category: Lubricant

### DESCRIPTION

Lubricants typically are used to reduce the frictional forces between particles and between particles and metal-contact surfaces of manufacturing equipment such as tablet punches and dies used in the manufacture of solid dosage forms. Liquid lubricants may be absorbed into the granule matrix before compaction. Liquid lubricants also can be used to reduce metal–metal friction on manufacturing equipment.

### FUNCTIONAL MECHANISM

Boundary lubricants function by adhering to solid surfaces (granules and machine parts) and by reducing the particle–particle friction or the particle–metal friction. The orientation of the adherent lubricant particles is influenced by the properties of the substrate surface. For optimal performance, the boundary lubricant particles should be composed of small, plate-like crystals or stacks of plate-like crystals. Fluid film lubricants melt under pressure and thereby create a thin fluid film around particles and on the surface of punches and dies in tablet presses, which helps to reduce friction. Fluid film lubricants resolidify after the pressure is removed. Liquid lubricants are released from the granules under pressure and create a fluid film. They do not resolidify when the pressure is removed but are reabsorbed or redistributed through the tablet matrix over the course of time.

### PHYSICAL PROPERTIES

The physical properties that are important for the function of boundary lubricants include particle size, surface area, hydration state, and polymorphic form. Purity (e.g., stearate:palmitate ratio) and moisture content also may be important. The physical properties of possible importance for fluid film lubricants are particle size and solid state/thermal behavior. Purity also may be important.

### CHEMICAL PROPERTIES

Lubricants can be classified as boundary lubricants, fluid film lubricants, or liquid lubricants. Boundary lubricants are salts of long-chain fatty acids (e.g., magnesium stearate) or fatty acid esters (e.g., sodium stearyl fumarate) with a polar head and fatty

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acid tail. Fluid film lubricants are solid fats (e.g., hydrogenated vegetable oil, type 1), glycerides (glyceryl behenate and distearate), or fatty acids (e.g., stearic acid) that melt when subjected to pressure. Liquid lubricants are liquid materials that are released from granules under pressure.

### GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in lubricant functions: *Light Diffraction Measurement of Particle Size* ⟨429⟩, *Crystallinity* ⟨695⟩, *Characterization of Crystalline Solids by Microcalorimetry and Solution Calorimetry* ⟨696⟩, *Loss on Drying* ⟨731⟩, *Optical Microscopy* ⟨776⟩, *Particle Size Distribution Estimation by Analytical Sieving* ⟨786⟩, *Specific Surface Area* ⟨846⟩, *Thermal Analysis* ⟨891⟩, *Water Determination* ⟨921⟩, and *Characterization of Crystalline and Partially Crystalline Solids by X-Ray Powder Diffraction (XRPD)* ⟨941⟩.

### ADDITIONAL INFORMATION

Certain lubricants, particularly those used in effervescent dosage forms, do not fall into the chemical categories defined above. These materials are used in special situations, and they are not suitable for universal application. Talc is an inorganic material that may have some lubricant properties. It is generally used in combination with fluid film lubricants to reduce sticking to punches and dies.

## Functional Category: Glidant and/or Anticaking Agent

### DESCRIPTION

Glidants and anticaking agents are used to promote powder flow and to reduce the caking or clumping that can occur when powders are stored in bulk. In addition, glidants and anticaking agents reduce the incidence of bridging during the emptying of powder hoppers and during powder processing.

### FUNCTIONAL MECHANISM

Glidants are thought to work by a combination of adsorption onto the surface of larger particles and reduction of particle–particle adhesive and cohesive forces, thus allowing particles to move more easily relative to one another. In addition, glidants may be dispersed among larger particles and thus may reduce friction between these particles. Anticaking agents may absorb free moisture that otherwise would allow the development of particle–particle bridges that are implicated in caking phenomena.

### PHYSICAL PROPERTIES

Primary physical properties of potential importance for glidants and anticaking agents are particle size, particle size distribution, and surface area. They may be slightly hygroscopic.

### CHEMICAL PROPERTIES

Glidants and anticaking agents typically are finely divided inorganic materials. Typically they are insoluble in water. Some of these materials are complex.

### GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in glidant or anticaking agent functions: *Light Diffraction Measurement of Particle Size* ⟨429⟩, *Loss on Drying* ⟨731⟩, *Particle Size Distribution Estimation by Analytical Sieving* ⟨786⟩, *Specific Surface Area* ⟨846⟩, and *Water Determination* ⟨921⟩.

## Functional Category: Coloring Agent

### DESCRIPTION

Coloring agents are incorporated into dosage forms to produce a distinctive appearance that may serve to differentiate a product from others that have a similar physical appearance or, in some instances, to protect photolabile components of the dosage form. These substances are subdivided into dyes (water-soluble substances), lakes (insoluble forms of a dye that result from its irreversible adsorption onto a hydrous metal oxide), inorganic pigments (substances such as titanium dioxide or iron oxides), and natural colorants (colored compounds not considered dyes per se, such as riboflavin). Coloring agents are subject to federal regulations, and consequently the current regulatory status of a given substance must be determined before its use.

The Federal Food, Drug, and Cosmetic Act defines three categories of coloring agents:

- FD&C colors: those certifiable for use in coloring foods, drugs, and cosmetics
- D&C colors: dyes and pigments considered safe in drugs and cosmetics when in contact with mucous membranes or when ingested
- Ext. D&C colors: colorants that, because of their oral toxicity, are not certifiable for use in ingestible products but are considered safe for use in externally applied products.

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### FUNCTIONAL MECHANISM

Water-soluble dyes usually are dissolved in a granulating fluid for use, although they also may be adsorbed onto carriers such as starch, lactose, or sugar from aqueous or alcoholic solutions. These latter products often are dried and used as formulation ingredients. Because of their insoluble character, lakes almost always are blended with other dry excipients during formulation. For this reason, direct-compression tablets often are colored with lakes.

### PHYSICAL PROPERTIES

Particle size and size distribution of dyes and lakes can influence product processing times (blending and dissolution), color intensity, and uniformity of appearance. A coloring agent should be physically nonreactive with other excipients and the drug substances.

### CHEMICAL PROPERTIES

The most important properties of a coloring agent are its depth of color and resistance to fading over time. Substances can be graded on their efficiency in reflecting desired colors of visible light, as well as on their molar absorptivities at characteristic wavelengths. A coloring agent should be chemically nonreactive with other excipients and the drug substances. The quality of a coloring agent ordinarily is measured by a determination of its strength, performance, or assay. The impurity profile is established by measurements of insoluble matter, inorganic salt content, metal content, and organic impurities.

### GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected coloring agent functions: *Light Diffraction Measurement of Particle Size* ⟨429⟩ and *Color—Instrumental Measurement* ⟨1061⟩. Instrumental methods should be used to determine the absolute color of a coloring agent.

### ADDITIONAL INFORMATION

Coloring agents are subject to federal regulations, and consequently the current regulatory status of a given substance must be determined before it is used.

## Functional Category: Capsule Shell

### DESCRIPTION

The word “capsule” is derived from the Latin *capsula,* which means a small container. Among other benefits, capsules enable pharmaceutical powders and liquids to be formulated for dosing accuracy, as well as ease of transportation. The capsule material should be compatible with all other ingredients in the drug product. Hard capsules typically consist of two parts: both are cylindrical, and one part is slightly longer than the other and is called the body. The cap fits closely on the body to enclose the capsule. In contrast, the soft capsule is a one-piece unit that may be seamed along an axis or may be seamless. The capsule material may be derived from hydrolysis of collagen that originates from porcine, bovine, or fish sources, or it can be of nonanimal origin, e.g., cellulosic or polysaccharide chemical entities. The capsule shell also contains other additives such as plasticizers, colorants, and preservatives. In some cases, capsule shells are sterilized to prevent microbial growth. The capsule shell is an integral part of the formulation, and therefore robust manufacturing and formulation performance depends on the measurement and control of CMAs. Capsules can be used to administer drugs by oral, rectal, vaginal, or inhalation routes.

### FUNCTIONAL MECHANISM

Capsules can enclose solid, semisolid, or liquid formulations. Capsules have a variety of benefits: masking unpleasant taste, facilitating blinding in clinical studies, promoting ease of swallowing, and presenting a unique appearance. Conventional capsule shells should dissolve rapidly at 37° in biological fluids such as gastric and intestinal media. However, the solubility properties of the shell can be modified (e.g., with enteric and controlled-release polymers) to control the release of the capsule contents.

### PHYSICAL PROPERTIES

The primary physical properties relevant to the capsule shell are those that can have a direct effect on product performance: 1) moisture content, 2) gas permeability, 3) stability on storage, 4) disintegration, 5) compactness, and 6) brittleness. The moisture content varies with the type of capsule. Hard gelatin capsules typically contain 13%–16% water compared to hypromellose (hydroxypropyl methylcellulose or HPMC) capsules that typically contain 4%–7% water content. Moisture content has an important effect on capsule brittleness. Soft gelatin capsules contain 5%–15% water. Equilibrium water content also may be crucial to dosage form stability because water migration can take place between the shell and capsule contents. Gas permeability may be important and generally is greater for HPMC capsules than for gelatin capsules because of the presence of open structures in the former. Unlike HPMC capsules, which do not cross-link, gelatin capsules have the potential to cross-link due to environmental and chemical exposure. Gelatin capsules may undergo cross-linking upon storage at elevated temperature and humidity [e.g., 40°(75% RH)]. Gelatin shell material is also well known to cross-link due to exposure to aldehydes, ketones, and certain dyes in shell formulations. Thus, presence of these materials in excipients should be considered for gelatin encapsulated products. Cross-linking slows in vitro dissolution and often necessitates introduction of enzymes in the test

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medium. Gelatin capsules should disintegrate within 15 min when exposed to 0.5% hydrochloric acid at 36°–38° but not below 30°. HPMC capsules can disintegrate below 30°.

### CHEMICAL PROPERTIES

Gelatin is a commercial protein derived from the native protein, collagen. The product is obtained by partial hydrolysis of collagen derived from skin, white connective tissue, and bones of animals. Type A gelatin is derived by acid treatment, and Type B gelatin is derived from base treatment. The common sources of commercial gelatin are pigskin, cattle hide, cattle bone, cod skin, and tilapia skin. The gelatin capsule shell also typically contains coloring agents, plasticizers such as polyhydric alcohols, natural gums and sugars, and preservatives such as sodium metabisulfite and esters of *p*-hydroxybenzoic acid. The more commonly used nongelatin capsules today are made from HPMC. Different capsule types contain different moisture levels and may thus influence drug product stability. The detailed composition of an excipient may be important because the shell function can be influenced by small amounts of impurities in the excipients (e.g., peroxides in oils or aldehydes in lactose and starches) that can cause capsule cross-linking. The presence in capsule shells of undesirable materials, such as metals, odorants, water-insoluble substances, and sulfur dioxide, should be evaluated to ensure stability and performance.

### GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected capsule shell functions: *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* ⟨61⟩, *Microbiological Examination of Nonsterile Products: Tests for Specified Microorganisms* ⟨62⟩, *Arsenic* ⟨211⟩, *Elemental Impurities—Limits* ⟨232⟩ and *Elemental Impurities—Procedures* ⟨233⟩, *Residue on Ignition* ⟨281⟩, *Disintegration* ⟨701⟩, *Dissolution* ⟨711⟩, *Water Determination* ⟨921⟩, and *Color—Instrumental Measurement* ⟨1061⟩.

### ADDITIONAL INFORMATION

In addition to the general chapters listed above, useful information for ensuring consistency in selected capsule shell functions may be found in *Gelatin, Gel Strength (Bloom Value)*.

## Functional Category: Coating Agent

### DESCRIPTION

Oral tablets may be coated using compression coating, sugar coating, or film coating. Compression coating (effectively making a tablet within a tablet) typically uses the same ingredients as a conventional tablet and thus is outside the scope of this section. The term “sugar coating” refers to a process and does not require that sucrose be part of the formulation. Oral capsules can be coated using film-coating procedures. Reasons for coating pharmaceutical dosage forms include masking unpleasant tastes or odors, improving ingestion and appearance, protecting active ingredients from the environment, and modifying the release of the active ingredient (e.g., controlled-release or gastrointestinal targeting). Materials used as coating agents differ depending on the coating process used. Sugar coating was the original coating process. However, today for technical and economic reasons, sugar coating largely has been replaced by film coating. Sugar coating is a complex process that typically involves the application of several different coats including a seal coat, key coat, subcoat, smoothing coat, color coat, and polishing coat. The coating solutions or suspensions are slowly poured or otherwise applied in aliquots onto a bed of tablets in a slowly rotating pan. The coating process typically takes an extended period (potentially several days) and results in a substantial increase in tablet weight. In contrast, film coating generally is a simpler process in which coating solution or suspension is sprayed onto tablets either in a rotating pan or in a fluid-bed apparatus and results in only a modest increase in capsule or tablet weight. The materials used in both sugar coating and film coating include natural, semisynthetic, and synthetic materials. These may be solutions, suspensions, or colloidal dispersions (latexes or pseudolatexes) that can be applied as either aqueous or nonaqueous systems. In addition, waxes and lipids can be applied as coatings in the molten state without the use of solvents. They also can be applied in the solid state as a polishing coat on top of sugar coating or film coating.

### FUNCTIONAL MECHANISM—SUGAR COATING

The seal coat is used to seal the surface of the tablet cores to prevent water in the coating solutions or suspensions from causing the tablet cores to disintegrate during coating. The seal coat typically is a polymer (e.g., shellac) that is insoluble in water and is applied as a thin coat from a solution in a nonaqueous solvent. The key component of the majority of sugar-coating solutions or suspensions is a solute, typically sucrose, that is highly soluble in water and forms a sticky, viscous solution (a syrup) at very high concentration. Other materials can be dissolved or suspended in the solution, depending on the stage during the coating process. As the coating dries, the dissolved coating material adheres to the surface of the tablets. The coating solution or suspension typically is applied in incremental steps, followed by drying, until the requisite coating has been achieved. The key coat is composed of another thin coat that is designed to adhere to the seal-coated cores to provide a base for the subcoat so the latter can adhere to the tablet surface. The subcoat typically contains a high concentration of suspended solids and is designed to increase the weight of the tablets comparatively quickly. The smoothing coat is designed to provide a smooth surface, and the color coat provides the final color if required. Finally, the tablets may be transferred to a polishing pan and polished using a mixture of waxes to provide a high-gloss finish.

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### FUNCTIONAL MECHANISM—FILM COATING

Film-coating agents are composed of film-forming materials (see *Functional Category: Film-Forming Agent*) that impart desirable pharmaceutical properties such as appearance and patient acceptance (e.g., taste masking and ease of swallowing). Film-coating agents also can serve other functional purposes such as providing a barrier against undesirable chemical reactions or untimely release of a drug from its components. After ingestion, the film coating may dissolve by processes such as hydration, solubilization, or disintegration, depending on the nature of the material used. Enteric coatings are insoluble in acidic (low pH) media but dissolve readily in neutral pH conditions. However, most common film-coating polymers do not have pH-specific solubility. The thickness of the film may vary by application and the nature of the coating agents. In the coating process, the polymer chains spread out on the core surface and coalesce into a continuous film as the solvent evaporates. Polymer solutions or dispersions with a low viscosity and high pigment-binding capacity reduce the coating time and facilitate relatively simple and cost-effective manufacturing. Plastic polymers, waxes, and lipid-based coatings can be applied without solvents by melting and atomization. When molten fluid droplets strike the surface of the fluidized drug particles, they spread and resolidify to form film layers. Therefore, film coating materials generally have the ability to form a complete and stable film around the substrate. The film coating typically is applied uniformly and is carefully dried to ensure that a consistent product is produced. Suitable plasticizers may be required to lower the minimum film-forming temperature of the polymer, and formulators should consider their potential effect on drug release.

### PHYSICAL PROPERTIES

Sugar coating is a lengthy, complex process. The physical properties of the seal-coating polymer and solution are important. The physical properties of the syrup component in the subsequent layers and any dissolved or suspended solids also are important, and coating agents must be sufficiently stable during use.

Film coating is a complex process, and the characteristics of the film-forming polymer are important. The particle size of colloidal dispersions varies with their composition and manufacture (latex, pseudolatex, or redispersed powder) and can have an effect on film formation. The surface tension of coating preparations can influence the spray pattern in the manufacturing process. The film should possess sufficient elasticity and mechanical strength to withstand the stresses during coating and packaging operations. For coatings that are applied in a molten state without solvents (plastic polymers, waxes, and lipid-based coatings), melting range and melt viscosity are the primary properties that formulators must consider.

### CHEMICAL PROPERTIES

Coating components can be of natural, semisynthetic, or synthetic origin and also can be available in different chemical grades. They comprise a diverse variety of different chemical materials. Formulators must consider the nature of the material and its intended use when they identify and quantitate chemical CMAs to ensure consistent performance.

### GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected coating agent functions: *Fats and Fixed Oils* ⟨401⟩, *Light Diffraction Measurement of Particle Size* ⟨429⟩, *Dissolution* ⟨711⟩, *Tensile Strength* ⟨881⟩, *Thermal Analysis* ⟨891⟩, *Viscosity—Capillary Methods* ⟨911⟩, *Viscosity—Rotational Methods* ⟨912⟩, and *Viscosity—Rolling Ball Method* ⟨913⟩. In addition, the general chapters listed under *Functional Category: Film-Forming Agent* (below) also may be appropriate for the evaluation of film-coating polymers.

### ADDITIONAL INFORMATION

Additives often are included in a coating formulation. Fillers (e.g., sugar alcohols, microcrystalline cellulose, calcium carbonate, and kaolin) may be added to increase the solids content of the coating agent without increasing viscosity. Stearic acid can be used to improve the protective function/moisture barrier of a coating. Coloring agents (e.g., titanium dioxide and iron oxides) may be added to modify appearance.

## Functional Category: Plasticizer

### DESCRIPTION

A plasticizer is a low molecular weight substance that, when added to another material—usually a polymer—makes the latter flexible, resilient, and easier to handle. Plasticizers are key components that determine the physical properties of polymeric pharmaceutical systems such as tablet film coatings and capsule shells.

### FUNCTIONAL MECHANISM

Plasticizers function by increasing the intermolecular and intramolecular mobility of the macromolecules that comprise polymeric materials. They achieve this by interfering with the normal intermolecular and intramolecular bonding mechanisms in such systems. The most effective plasticizers exert their effect at low concentrations, typically less than 5% w/w. Plasticizers commonly are added to film coatings (aqueous and nonaqueous systems) and capsule shells (hard and soft varieties) to improve their workability and mechanical ruggedness. Without the addition of plasticizers, such materials can split or fracture prematurely. Plasticizers also are added to semisolid pharmaceutical preparations, such as creams and ointments, to enhance their rheological properties.

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### PHYSICAL PROPERTIES

The most common plasticizers are low molecular weight (<500 Da) solids or liquids. They typically have low melting points (<100°) and can be volatile (i.e., exert an appreciable vapor pressure) at ambient temperature. Plasticizers can reduce the glass transition temperature ($T_g$) of the system to which they are added.

### CHEMICAL PROPERTIES

Many modern plasticizers are synthetic esters such as citrates and phthalates. Traditional pharmaceutical plasticizers include oils, sugars, and their derivatives.

### GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected excipient functions: *Residual Solvents* ⟨467⟩, *Melting Range or Temperature* ⟨741⟩, *Refractive Index* ⟨831⟩, *Specific Gravity* ⟨841⟩, *Thermal Analysis* ⟨891⟩, and *Water Determination* ⟨921⟩.

### ADDITIONAL INFORMATION

The choice of an appropriate plasticizer often is guided by reference to its solubility parameter, which is related to its cohesive energy density. Solubility parameter values for many common materials are tabulated in standard reference texts. To ensure maximum effectiveness, the solubility parameter of the plasticizer and the polymeric system being plasticized should be matched as closely as possible.

## Functional Category: Film-Forming Agent

### DESCRIPTION

Film-forming agents typically are polymers that can be used to prepare polymer films to coat tablets or capsules for oral administration, to modify appearance, to modify drug release, or to serve other purposes such as melt-in-the-mouth formulations. Polymeric materials used as film-forming agents can be derived from natural, semisynthetic, or synthetic sources, and they can be supplied as powders, granules, pre-prepared solutions, or colloidal dispersions. Colloidal dispersions may contain other components such as plasticizers, surface-active agents, preservatives, or stabilizers. Film-forming agents can be applied as colloidal dispersions (latexes or pseudolatexes) or as aqueous, hydroalcoholic, or nonaqueous polymeric solutions.

### FUNCTIONAL MECHANISM

Film-forming agents typically are composed of polymeric materials that possess the ability to form films after solvent evaporation from a solution of the polymer or from the continuous phase of a colloidal dispersion. Thus, the polymer alone must be a solid at ambient temperature and humidity. Some polymers can form films without the inclusion of added components, but other polymers may require the use of additional components such as plasticizers.

### PHYSICAL PROPERTIES

Many polymeric film-forming agents are available in a variety of physical grades that typically are based on the nominal viscosity of the particular grade. The physical properties of the polymer usually are those of a solid, and many polymers are available as powders and granules. In addition to the normal properties of bulk powders and granules, other important physical properties of a polymeric film-forming agent are the molecular weight distribution, which is linked to the nominal viscosity of the grade, and the glass transition temperature ($T_g$). If the film-forming agent is provided as a pre-prepared solution or dispersion, the viscosity of the solution or dispersion can affect performance and should be monitored.

### CHEMICAL PROPERTIES

Film-forming agents comprise a diverse group of materials, including natural, semisynthetic, and synthetic materials as discussed above. They may have ionizable functional groups that impart pH-dependent properties and also can be available in different chemical grades (e.g., with different degrees of chemical substitution). Pharmacopeial monographs often describe classes of polymeric materials that allow a considerable range of composition. Formulators should consider these factors when they identify critical material attributes and establish specifications to ensure consistent performance.

### GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected film-forming agent functions: *Fats and Fixed Oils* ⟨401⟩, *Light Diffraction Measurement of Particle Size* ⟨429⟩, *Bulk Density and Tapped Density of Powders* ⟨616⟩, *Chromatography* ⟨621⟩, *Density of Solids* ⟨699⟩, *Dissolution* ⟨711⟩, *Optical Microscopy* ⟨776⟩, *pH* ⟨791⟩, *Tensile Strength* ⟨881⟩, *Thermal Analysis* ⟨891⟩, *Viscosity—Capillary Methods* ⟨911⟩, *Viscosity—Rotational Methods* ⟨912⟩, *Viscosity—Rolling Ball Method* ⟨913⟩, *Bulk Powder Sampling Procedures* ⟨1097⟩, *Near-Infrared Spectroscopy—Theory and Practice* ⟨1856⟩, ▲*Raman Spectroscopy—Theory and Practice* ⟨1858⟩▲ (CN 1-Aug-2020), *Pharmaceutical Dosage Forms* ⟨1151⟩ *Powder Flow* ⟨1174⟩, and *Scanning Electron Microscopy* ⟨1181⟩.

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## Functional Category: Flavor and Fragrance

### DESCRIPTION

A flavor is a single chemical entity or a blend of chemicals of natural or synthetic origin that has the ability to elicit a taste or aroma (i.e., fragrance) response when orally consumed or smelled. The primary purpose of flavor that is added to a pharmaceutical preparation is to provide all or part of the taste and aroma of the product taken into the mouth. Flavors commonly are used in pharmaceutical oral disintegrating tablets, oral solutions, and oral suspensions to mask objectionable drug taste and to make the formulation more palatable, thus promoting patient compliance.

### FUNCTIONAL MECHANISM

Chemicals dissolved in saliva excite chemoreceptors on taste buds that reside primarily on the tongue and thus arouse taste perception. Dissolution also releases volatile chemicals that reach the olfactory receptors, triggering aroma perception. The total of taste and odor responses constitutes flavor. Humans can distinguish among five components of taste: sourness, saltiness, sweetness, bitterness, umami (savory), and a wide range of specific odors. Flavor enhancers and taste modifiers can be used to modify the sweetness profile of a sweetening agent or to mask off-flavors. For example, organic acids, such as aspartic and glutamic acids, are known to reduce bitterness.

### PHYSICAL PROPERTIES

Taste perception depends on physicochemical, physiological, and psychological factors. Physical properties such as particle size, solubility, humectancy, texture, and color all influence the senses. In addition to flavor, the sensory attributes of sight (e.g., appealing color), sound (e.g., crunch of a chewable tablet), and mouth feel (e.g., viscous, slimy, chalky, cloying, or watery) also contribute to and influence the overall sensory experience.

### CHEMICAL PROPERTIES

Chemicals that provide one of the five basic tastes possess a wide variety of structures, functional groups, and molecular weights. Chemicals used to flavor pharmaceuticals by providing both odor and taste tend to have low molecular weights (<250 Da) and polar functional groups such as esters, ketones, aldehydes, amines, or alcohols. To increase the stability of the flavor(s) in a solid dosage form and to minimize flavor–drug interactions, formulators can add flavors in an encapsulated or spray-dried form.

### GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in flavor functions: *Light Diffraction Measurement of Particle Size* ⟨429⟩, *Chromatography* ⟨621⟩, *Congealing Temperature* ⟨651⟩, *Loss on Drying* ⟨731⟩, *Melting Range or Temperature* ⟨741⟩, *Optical Rotation* ⟨781⟩, *Particle Size Distribution Estimation by Analytical Sieving* ⟨786⟩, *Refractive Index* ⟨831⟩, and *Specific Gravity* ⟨841⟩.

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## Functional Category: Release-Modifying Agents

Release-modifying agents are used to control drug release in extended-release formulations (also referred to as prolonged-release or controlled-release formulations). Sustained-release and enteric coating agents are included under *Functional Category: Coating Agent.*

### DESCRIPTION

Release-modifying agents change a medicinal product's drug-release pattern to achieve the desired drug plasma profile for a given time. The majority of release-modifying agents are polymers that differ in solubility, ease of erosion, rate of swelling, or sensitivity to the biological environment in which they are placed. These polymers have been used to fabricate matrix- or membrane-based drug delivery systems for oral, parenteral, transdermal, and other routes of administration. Matrix controlled-release drug delivery systems can be classified as hydrophilic eroding matrices, hydrophilic noneroding matrices, or hydrophobic matrices. In membrane controlled-release drug delivery systems, the drug reservoir is coated by a rate-controlling polymeric membrane that may consist of a blend of polymers to control release. Such devices may take the form of tablets, capsules, microspheres, vesicles, fibers, patches, and others. In addition to polymers, certain lipid-based excipients also can be used as release-modifying agents in hydrophobic matrix devices and other types of modified-release systems. Typically, these lipid-based materials are fats and waxes or related materials with melting ranges above 45°.

### FUNCTIONAL MECHANISM

Upon contact with a biological fluid, release-controlling polymers may undergo a variety of physical changes such as swelling, gelling, dissolution, or erosion, each of which can be triggered by simple aqueous exposure or can be modulated by pH, osmotic stress, or interactions with bile or other intestinal contents. In addition to physical changes, polymers may undergo chemical degradation by acids, bases, enzymes, water, heat, and others. Any or all of these mechanisms may act in concert to control the rate at which the drug is released from the delivery system.

For hydrophilic matrices in which drug diffusion dominates release rate, the rate of drug release depends on the properties of the polymer gel and the nature of the continuous phase in the interstices of the gel influences the dissolution and diffusion

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rates of the drug. In the case of eroding matrices, the gel erodes because of the mechanical action of the gastrointestinal tract as the water uptake increases, and the gel becomes more dilute, thus reducing the diffusion distance or releasing drug particles that subsequently dissolve. Hydrophobic matrix-forming materials are not soluble. Drug release from such systems is governed by drug diffusion through the tortuous pores that remain as soluble components dissolve.

Membrane-based drug delivery systems include polymer-coated tablets, capsules, and microspheres. Drug-release mechanisms from such systems are complex and depend on physicochemical characteristics of the drug and polymers or lipids used as well as biological factors in the case of biocompatible and biodegradable systems. Most commonly, drug release from such systems is governed by drug diffusion through the hydrated rate-controlling membrane.

Other modified-release systems for parenteral use include solid lipid nanoparticles and liposomes. The release mechanisms for these systems often involve a complicated interplay with biological processes such as potential clearance through the reticulo-endothelial system, targeted delivery, and cellular uptake.

Osmotic pump devices are a special case of membrane delivery systems. The rate-controlling polymer is insoluble and semipermeable—i.e., it will allow water but not drug molecules—to diffuse through the membrane. Release is controlled by the osmotic pressure of the core components and the viscosity of the resulting solution or suspension. The drug, either in solution or as a suspension, is forced out of a hole in the membrane, which is typically drilled by a laser during product manufacture.

### PHYSICAL PROPERTIES

The physical properties of the release-controlling excipient depend on the chemical type: hydrophilic polymer, hydrophobic polymer, semipermeable polymer blends, or lipid, wax, or biodegradable polymer (which can be hydrophilic or hydrophobic).

Hydrophilic polymers gel in contact with water or aqueous media. Because they should provide resistance to the mechanical action of the gastrointestinal tract during passage, they typically are higher molecular weight grades of the polymers. At the concentrations typically used during in vivo release, these high molecular weight polymers often do not exhibit Newtonian properties except in very dilute solution (if they are soluble). Formulators should monitor the kinetic and viscoelastic properties of the gels formed in the release medium.

Hydrophobic polymers are insoluble in water, and their solution properties are determined in nonaqueous solutions. The polymers used in the preparation of semipermeable membranes in osmotic pump devices also are insoluble in water, and similarly their solution properties are determined in nonaqueous solutions. Similarly, hydrophobic lipid-based materials are insoluble in water.

### CHEMICAL PROPERTIES

Release-controlling agents have many different types and origins and are available in a range of grades that reflect the considerable variation in their chemical structures and properties. Formulators must consider these variables during any chemical investigation and when they consider the effects of chemical structure on excipient performance. Properties of interest may include chemical composition for copolymers and cellulosic derivatives, degree of ionization, molecular weight, degree of cross-linking, or, for lipids, fatty acid composition. Residual impurities from the manufacturing process, e.g., monomers, initiators, quenching agents, peroxides, and aldehydes, may affect drug substance stability and should be monitored.

### GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected functions of release-modifying agents: *Fats and Fixed Oils* ⟨401⟩, *Light Diffraction Measurement of Particle Size* ⟨429⟩, *Crystallinity* ⟨695⟩, *Characterization of Crystalline Solids by Microcalorimetry and Solution Calorimetry* ⟨696⟩, *Dissolution* ⟨711⟩, *Loss on Drying* ⟨731⟩, *Melting Range or Temperature* ⟨741⟩, *Nuclear Magnetic Resonance Spectroscopy* ⟨761⟩, *Optical Microscopy* ⟨776⟩, *Particle Size Distribution Estimation by Analytical Sieving* ⟨786⟩, *Specific Surface Area* ⟨846⟩, *Mid-Infrared Spectroscopy* ⟨854⟩ and *Ultraviolet-Visible Spectroscopy* ⟨857⟩, *Tensile Strength* ⟨881⟩, *Thermal Analysis* ⟨891⟩, *Viscosity—Capillary Methods* ⟨911⟩, *Viscosity—Rotational Methods* ⟨912⟩, *Viscosity—Rolling Ball Method* ⟨913⟩, *Water Determination* ⟨921⟩, *Characterization of Crystalline and Partially Crystalline Solids by X-Ray Powder Diffraction (XRPD)* ⟨941⟩, *Powder Flow* ⟨1174⟩, and *Scanning Electron Microscopy* ⟨1181⟩.

### ADDITIONAL INFORMATION

Some release-modifying agents may include additives such as an antioxidant or an anticaking agent.

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## ORAL LIQUIDS

## Functional Category: pH Modifier (Acidifying/Alkalizing/Buffering Agent)

### DESCRIPTION

The hydrogen ion concentration, $[H^+]$, in an aqueous solution is expressed as $pH = -\log(H^+)$. The pH of pure water is 7 at 25°. An aqueous solution is acidic at pH <7 and alkaline at pH >7. An acid can be added to acidify a solution. Similarly, a base can be used to alkalize a solution. A buffer is a weak acid (or base) and its salt. When a buffer is present in a solution, the addition of small quantities of strong acid or base leads to only a small change in solution pH. Buffer capacity is influenced by salt/acid (or base/salt) ratio and total concentration of acid (or base) and salt. The pH of pharmaceutical solutions typically is controlled using acidifying/alkalizing and buffering agents to: 1) maintain a pH close to that of relevant body fluid to avoid irritation, 2)

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improve drug stability where it is found to be pH dependent, 3) control equilibrium solubility of weak acids or bases, and 4) maintain a consistent ionization state of molecules during chemical analysis, e.g., high-performance liquid chromatography.

### FUNCTIONAL MECHANISM

The ionization equilibria of weak bases, weak acids, and water are the key to the functions of acidifying, alkalizing, and buffering agents. The autoprotolytic reaction of water can be expressed as:

$$H_2O + H_2O \leftrightarrow H_3O^+ + OH^-$$

The autoprotolysis constant (or ion product) of water is $K_w = 1 \times 10^{-14}$ at 25° and varies significantly with temperature. Because the concentrations of hydrogen and hydroxyl ions in pure water are equal, each has the value of approximately $1 \times 10^{-7}$ mol/L, leading to the neutral pH of 7 at 25°. When an acid, base, or salt of a weak acid (or base) is added, the ionization equilibrium of water is shifted so that $[H^+][OH^-]$ remains constant, thus resulting in a solution pH that is different from 7.

### PHYSICAL PROPERTIES

pH modifiers typically are dissolved in liquid dosage forms. Physical properties may be important and should be considered because they may influence processing requirements (e.g., particle size may influence mixing time required to dissolve a pH modifier).

### CHEMICAL PROPERTIES

Buffers and pH modifiers influence solution pH, buffer capacity, osmolality, osmolarity, and water conductivity. When used in chemical analysis, buffers must be chemically compatible with the reagents and test substance. Buffers used in physiological systems should not interfere with the pharmacological activity of the medicament or the normal function of the organism.

### GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected pH-modifier or buffering-agent functions: *Water Conductivity* ⟨645⟩, *Osmolality and Osmolarity* ⟨785⟩, and *pH* ⟨791⟩.

## Functional Category: Wetting and/or Solubilizing Agent

### DESCRIPTION

Solubilizers can be used to dissolve otherwise insoluble molecules. They function by facilitating spontaneous phase transfer to yield a thermodynamically stable solution. A number of solubilizers are available commercially. Acceptable solubilizers for pharmaceutical applications have been fully evaluated in animals for safety and toxicology. Wetting agents increase the spreading and penetrating properties of a liquid by lowering its surface tension.

### FUNCTIONAL MECHANISM

Wetting and solubilizing agents comprise a variety of different chemical structures or classes. Some solubilizers have unique chemical structures. For example, a hydrophilic moiety may be tethered with a hydrophobic moiety to yield distinct micelle shapes and morphologies in water, thus facilitating solubilization. The mechanism of solubilization often is associated with a favorable interaction of the insoluble agent and the interior core of the solubilizer assembly (e.g., micelles). In other cases, unique hydrophobic sites that are capable of forming inclusion complexes are present. Other types of solubilizers use a range of polymeric chains that interact with hydrophobic molecules to increase solubility by dissolving the insoluble agent into the polymeric chains.

### PHYSICAL PROPERTIES

Wetting and solubilizing agents are typically solid, liquid, or waxy materials. Their physical properties depend on their chemical structures. The physical properties and performance of the wetting agents and solubilizers, however, depend on the surface-active properties of the solubilizers and on the hydrophilic–lipophilic balance (HLB). Materials with lower HLB values behave as emulsifiers, whereas those with higher HLB values typically behave as solubilizing agents. For example, the commonly used wetting and solubilizing agent sodium lauryl sulfate (HLB 40) is hydrophilic and highly water soluble and, upon dispersion in water, spontaneously reduces surface tension and forms micelles that function to solubilize lipophilic materials.

The unique hydrophilicity and hydrophobicity properties of solubilizers can be characterized by their chemical structures, aggregate numbers, or critical micelle concentrations (CMCs). The CMC value is unique to an individual solubilizer that bears hydrophilic, lipophilic, and/or hydrophobic chains. CMC is a measure of the concentration at which the surface-active molecules aggregate. These molecular aggregates can solubilize the solute by incorporating part into the hydrophobic interior. Such interactions with the insoluble molecule further stabilize the molecules in the entire assemblies without precipitation.

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### CHEMICAL PROPERTIES

The chemical and surface-active properties depend on the structures of the solubilizers. Because of the complex nature of solute–solvent–solubilizer interactions, pharmaceutical scientists must carefully consider, identify, and control the CMAs of solubilizers.

### GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected solubilizing agent functions: *Fats and Fixed Oils* ⟨401⟩, *Light Diffraction Measurement of Particle Size* ⟨429⟩, *pH* ⟨791⟩, *Specific Gravity* ⟨841⟩, *Specific Surface Area* ⟨846⟩, *Mid-Infrared Spectroscopy* ⟨854⟩ and *Ultraviolet-Visible Spectroscopy* ⟨857⟩, *Thermal Analysis* ⟨891⟩, *Viscosity—Capillary Methods* ⟨911⟩, *Viscosity—Rotational Methods* ⟨912⟩, *Viscosity—Rolling Ball Method* ⟨913⟩, and *Scanning Electron Microscopy* ⟨1181⟩.

## Functional Category: Antimicrobial Preservative

### DESCRIPTION

Antimicrobial preservatives are used to kill or prevent growth of bacteria, yeast, and mold in the dosage form.

### FUNCTIONAL MECHANISM

Preservatives work by a variety of mechanisms to control microbes. Most work at the cell membrane, causing membrane damage and cell leakage. Other modes of action include transport inhibition, protein precipitation, and proton-conducting uncoupling. Some preservatives are bactericidal (kill bacteria or yeast and mold); some are bacteriostatic (inhibit growth of microorganisms); and others are sporicidal (kill spores). Several of the preservatives can act synergistically (e.g., combinations of parabens).

### PHYSICAL PROPERTIES

Antimicrobials generally are soluble in water at concentration ranges at which they are effective. The vapor pressure of these agents is important, especially if the dosage form is intended for lyophilization or spray drying. Several of these agents are flammable. Understanding an excipient's partition coefficient is important because partitioning of a preservative into an oil phase can diminish the preservative's concentration in the aqueous phase, which in turn can reduce its value as a preservative.

### CHEMICAL PROPERTIES

Phenolic preservatives can undergo oxidation and color formation. Incompatibilities of preservatives (cationic and anionic mixtures, adsorption to tubes or filters, or binding to surfactants and proteins) should be taken into account during product development.

### GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected antimicrobial functions: *Injections and Implanted Drug Products* ⟨1⟩, *Antimicrobial Effectiveness Testing* ⟨51⟩, *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* ⟨61⟩, *Microbiological Examination of Nonsterile Products: Tests for Specified Microorganisms* ⟨62⟩, and *Antimicrobial Agents—Content* ⟨341⟩.

### ADDITIONAL INFORMATION

Safety and labeling requirements regarding the use of antimicrobial preservatives should be considered.

## Functional Category: Chelating and/or Complexing Agents

### DESCRIPTION

Chelating agents form soluble complex molecules with certain metal ions (e.g., copper, iron, manganese, lead, and calcium) and essentially remove the ions from solution to minimize or eliminate their ability to react with other elements and/or to precipitate. The agents are used in pharmaceuticals (oral, parenteral, and topical formulations), cosmetics, and foods to sequester ions from solution and to form stable complexes. Chelating agents also are referred to as chelants, chelators, or sequestering agents.

Complexing agents form soluble complex molecules (e.g., inclusion complexes) with other solutes (e.g., drug substances) and can influence physical and chemical properties such as solubility and stability.

### FUNCTIONAL MECHANISM

Chelating agents are used to sequester undesirable metal ions from solution. The chemical structure of chelating agents allows them to act as a "claw" to associate with the metal atom by forming a heterocyclic ring structure. Complexing agents function similarly but mechanistically and do not (by definition) require a two-point claw structure because they can associate via one or more binding sites. All chelating agents are complexing agents, but not all complexing agents are chelating agents.

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Chelating agents are used as antioxidant synergists, antimicrobial synergists, and water softeners. By sequestering metal ions from solution, chelating agents reduce the propensity for oxidative reactions. Chelating agents also have the ability to enhance antimicrobial effectiveness by forming a metal-ion–deficient environment that otherwise could feed microbial growth.

Complexing agents generally form soluble complex molecules with solutes (e.g., drug molecules) that can influence physical, chemical, and drug delivery properties. Complexing agents that form inclusion complexes typically contain a hydrophobic cavity into which a drug substance can enter and an outer, more hydrophilic region.

### PHYSICAL PROPERTIES

Chelating and complexing agents generally are soluble in water and typically are dissolved in liquid dosage forms. Physical properties may be important and should be considered because they may influence processing requirements (e.g., particle size may influence mixing time required to dissolve). Chelating and complexing agents exhibit different degrees of hygroscopicity. Because chelating agents are used in low levels, particle size distribution can be important to enable acceptable dosage form content uniformity.

### CHEMICAL PROPERTIES

Chelating agents complex with metal ions via any combination of ionic and covalent bonds. Dilute aqueous solutions may be neutral, acidic, or alkaline. Edetic acid and its salts are incompatible with strong oxidizers, strong bases, and polyvalent metal ions (e.g., copper and nickel). Specific agents are selected for a formulation based on their solubility, affinity for the target metal ion, and stability. Edetate salts are more soluble than the free acid. Unlike other edetate salts and the free acid, edetate calcium disodium does not sequester calcium and therefore is preferred to prevent hypocalcemia. It is also preferred to chelate metals with the release of calcium ions. Alternatively, disodium edetate can be used to treat hypercalcemia. Edetic acid will decarboxylate if heated above 150°.

Complexing agents generally form molecular complexes with drug substances that are dependent on complexing agent physical and chemical properties. The ability of a solute to form an inclusion complex is dependent on complexing agent molecular weight, chemical structure, and the dimensions of the hydrophobic cavity.

### GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected chelating and complexing functions: *Antimicrobial Effectiveness Testing* ⟨51⟩, *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* ⟨61⟩, *Elemental Impurities—Limits* ⟨232⟩ and *Elemental Impurities—Procedures* ⟨233⟩, *Iron* ⟨241⟩, *Lead* ⟨251⟩, *Antimicrobial Agents—Content* ⟨341⟩, *Light Diffraction Measurement of Particle Size* ⟨429⟩, *Loss on Drying* ⟨731⟩, *pH* ⟨791⟩, *Water Determination* ⟨921⟩, and *Cell-based Advanced Therapies and Tissue-based Products* ⟨1046⟩.

## Functional Category: Antioxidant

### DESCRIPTION

This category applies to antioxidants used as in vitro stabilizers of pharmaceutical preparations to mitigate oxidative processes. Antioxidants used for their biological activity in vivo may be regarded as active ingredients with therapeutic effects and are not discussed. Antioxidants delay the onset of and/or significantly reduce the rate of complex oxidative reactions that could otherwise have a detrimental effect on the drug substance. Antioxidants also can be considered for protecting nonactive components such as unsaturated oils, pegylated lipids, flavors, and essential oils. Thus, antioxidants preserve the overall integrity of the dosage form against oxidative stress. Antioxidants are most effective when incorporated in the formula to prevent or delay the onset of chain reactions and to inhibit free radicals and hydroperoxides from engaging in the cascading processes described above. Effective application of antioxidants and evaluation of their efficacy necessitate an understanding of oxidative mechanisms and the nature of the byproducts they generate. Autoxidation is initiated when oxygen reacts with a substrate to form highly reactive species known as free radicals ($RH \rightarrow R\cdot$). After "initiation" the free radicals in the presence of oxygen can trigger chain reactions ($R\cdot + O_2 \rightarrow ROO\cdot$ and $ROO\cdot + RH \rightarrow R\cdot + ROOH$) to form peroxy radicals, hydroperoxides, and new alkyl radicals that can initiate and then propagate their own chain reactions. The cascading reactions during the propagation phase can be accelerated by heat, light, and metal catalysts. In the presence of trace amounts of metal catalysts ($Cu^{+}$, $Cu^{2+}$, $Fe^{2+}$, and $Fe^{3+}$), hydroperoxides (ROOH) readily decompose to $RO\cdot$ and $ROO\cdot$ and subsequently can trigger reactions with the API and/or the excipients (e.g., hydrocarbons) to form hydroxyl acids, keto acids, and aldehydes that can have further undesirable effects. Note that hydroperoxides are not solely the reaction products of oxidative mechanisms within a formulation. Residual amounts of hydroperoxides also can be found in commonly used excipients like polyethylene glycols, polyvinylpyrrolidone, and polysorbates. The initiation phase generally is slow and has a limited effect on the quality of the finished product. The propagation phase, in contrast, involves rapid, irreversible degradation of chemical species.

### FUNCTIONAL MECHANISM

Antioxidants can be grouped by their mode of action. Phenolic antioxidants that block free radical chain reactions also are known as true or primary antioxidants. This group consists of monohydroxy or polyhydroxy phenol compounds with ring substitutions. They have very low activation energy to donate hydrogen atom(s) in exchange for the radical electrons that are rapidly delocalized by free radicals. By accepting the radical electrons, they stabilize free radicals. The reaction yields antioxidant free radicals that also can react with lipid free radicals to form other stable compounds. Thus, they can block oxidative chain reactions both in the initiation and propagation stages. Because of their solubility behavior, phenolic antioxidants are most effective in protecting oils and oil-soluble actives against oxidative stress. Reducing agents generally are water-soluble

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antioxidants (e.g., L-ascorbic acid) with lower redox potential than the drug or the excipient they are protecting. They delay the onset and the rate of oxidative reactions by sacrificially reacting with oxygen and other reactive species. The oxygen-scavenging potential of the reducing agents may be sensitive to pH and also can be negatively affected in the presence of trace elements. Chelating agents bind with free metals ($Cu^{+}$, $Cu^{2+}$, $Fe^{2+}$, and $Fe^{3+}$) that may be present in trace amounts in the formulation. The newly formed complex ions are nonreactive. Chelating agents therefore remove the capacity of the metal catalysts to participate in oxidative reactions that occur during the propagation stage.

The utility of antioxidants can be maximized by synergistic use of one or two primary antioxidants along with reducing and chelating agents. The combined effect often is greater than the sum of the individual effects of each antioxidant (synergistic effect).

#### PHYSICAL PROPERTIES

Solubility of the antioxidant should be greatest in the formulation phase (oily, aqueous, or emulsion interface), where the drug substance is most soluble. The temperature at which the antioxidant decomposes is critical for autoclaved preparations, where loss of antioxidant activity may occur. Stability of the antioxidant also must be considered and may be a function of pH and processing conditions. Metal ions may react with propyl gallate to form colored complexes. At alkaline pH, certain proteins and sodium salts may bring about discoloration of *tert*-butylhydroquinone.

#### CHEMICAL PROPERTIES

Activation energy, oxidation–reduction potential, and stability at different formulation (e.g., pH) and processing (e.g., heat) conditions are important chemical properties. If the dosage form's expected shelf life depends on the antioxidant's function, the concentration must be factored in and periodically assessed to ensure that a sufficient amount of antioxidant remains throughout the product shelf life.

#### GENERAL CHAPTERS

The following general chapters may be useful for assessing selected excipient antioxidant functions: *Iron* ⟨241⟩, *Chromatography* ⟨621⟩, *Crystallinity* ⟨695⟩, *Melting Range or Temperature* ⟨741⟩, *Specific Surface Area* ⟨846⟩, and *Water Determination* ⟨921⟩.

## Functional Category: Sweetening Agent

#### DESCRIPTION

Sweetening agents are used to sweeten oral dosage forms and to mask unpleasant flavors. See *Functional Category: Flavor and Fragrance* for more details.

#### FUNCTIONAL METABOLISM

Sweetening agents bind to receptors on the tongue that are responsible for the sensation of sweetness. The longer the sweetener molecule remains attached to the receptor, the sweeter the substance is perceived to be. The standard for sweetness is sucrose.

#### PHYSICAL PROPERTIES

The primary physical properties relevant to sweeteners relate to their compatibility with the other ingredients in the formulation (e.g., acidic ingredients), processing conditions (e.g., heating), particle size and distribution, moisture content, isomerism, sweetness, and taste-masking capability. These properties may be formulation dependent.

#### CHEMICAL PROPERTIES

Sweeteners can be divided into three main groups: sugars (which have a ring structure), sugar alcohols (sugars that do not have a ring structure), and artificial sweeteners. All sweeteners are water soluble. The stability of many sweeteners is affected by pH and other ingredients in the formulation. Some sweeteners may catalyze the degradation of some active ingredients, especially in liquids and in cases in which the manufacturing processes involve heating.

#### GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected sweetening functions: *Light Diffraction Measurement of Particle Size* ⟨429⟩, *Loss on Drying* ⟨731⟩, *Melting Range or Temperature* ⟨741⟩, *Optical Rotation* ⟨781⟩, and *Water Determination* ⟨921⟩.

#### ADDITIONAL INFORMATION

Products that contain aspartame must include a warning on the label stating that the product contains phenylalanine. Sugar alcohols have a glycemic index well below that of glucose. However, sorbitol is slowly metabolized to fructose and glucose, which raises blood sugar levels. Sugar alcohols in quantities generally greater than 20 g/day act as an osmotic laxative, especially

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when they are contained in a liquid formulation. Preservative systems should be carefully chosen to avoid incompatibility with the sweetener, and some sweeteners are incompatible with certain preservatives.

# SEMISOLIDS, TOPICALS, AND SUPPOSITORIES

## Functional Category: Suppository Base

### DESCRIPTION

Suppository bases are used in the manufacture of suppositories (for rectal administration) and pessaries (for vaginal administration). They can be hydrophobic or hydrophilic.

### FUNCTIONAL MECHANISM

Suppositories should melt at just below body temperature (37°), thereby allowing the drug to be released either by erosion and partition if the drug is dissolved in the base or by erosion and dissolution if the drug is suspended in the base. Hard fat suppository bases melt at approximately body temperature. Hydrophilic suppository bases also melt at body temperature and typically also dissolve or disperse in aqueous media. Thus, release takes place via a combination of erosion and dissolution.

### PHYSICAL PROPERTIES

The important physical characteristic of suppository bases is melting range. In general, suppository bases melt between 27° and 45°. However, individual bases usually have a much narrower melting range within these temperature boundaries, typically 2°–3°. The choice of a particular melting range is dictated by the influence of the other formulation components on the melting range of the final product.

### CHEMICAL PROPERTIES

Hard fat suppository bases are mixtures of semisynthetic triglyceride esters of longer-chain fatty acids. They may contain varying proportions of mono- and diglycerides and also may contain ethoxylated fatty acids. They are available in many different grades that are differentiated by melting range, hydroxyl number, acid value, iodine value, solidification range, and saponification number.

Hydrophilic suppository bases are mixtures of hydrophilic semisolid materials that in combination are solid at room temperature and yet release the drug by melting, erosion, and dissolution when administered to the patient. Hydrophilic suppository bases have much higher levels of hydroxyl groups or other hydrophilic groups than do hard fat suppository bases. Polyethylene glycols that show appropriate melting behavior are examples of hydrophilic suppository bases.

### GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected suppository base functions: *Fats and Fixed Oils* ⟨401⟩, *Congealing Temperature* ⟨651⟩, *Melting Range or Temperature* ⟨741⟩, and *Pharmaceutical Dosage Forms* ⟨1151⟩.

### ADDITIONAL INFORMATION

Some materials included in suppositories based on hard fats have much higher melting ranges. These materials typically are microcrystalline waxes that help stabilize molten suspension formulations. Suppositories also can be manufactured from glycerinated gelatin.

## Functional Category: Suspending and/or Viscosity-Increasing Agents

### DESCRIPTION

Suspending and viscosity-increasing agents are used in pharmaceutical formulations to stabilize dispersal systems (e.g., suspensions or emulsions), to reduce the rate of solute or particulate transport, or to decrease the fluidity of liquid formulations.

### FUNCTIONAL MECHANISMS

A number of mechanisms contribute to the dispersion stabilization or viscosity-increasing effect of these agents. The most common is the increase in viscosity—because of the entrapment of solvent by macromolecular chains or clay platelets—and the disruption of laminar flow. Other mechanisms include gel formation via a three-dimensional network of excipient molecules or particles throughout the solvent continuum and steric stabilization wherein the macromolecular or mineral component in the dispersion medium adsorbs to the surfaces of particles or droplets of the dispersed phase. The latter two mechanisms increase formulation stability by immobilizing the dispersed phase.

### PHYSICAL PROPERTIES

Each of the mechanisms—increased viscosity, gel formation, or steric stabilization—is a manifestation of the rheological character of the excipient. Because of the molecular weights and sizes of these excipients, the rheological profiles of their

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dispersions are non-Newtonian. Dispersions of these excipients display viscoelastic properties. The molecular weight distribution and polydispersity of the macromolecular excipients in this category are important criteria for their characterization.

### CHEMICAL PROPERTIES

The majority of the suspending and viscosity-increasing agents are: 1) hydrophilic carbohydrate macromolecules (acacia, agar, alginic acid, carboxymethylcellulose, carrageenans, dextrin, gellan gum, guar gum, hydroxyethyl cellulose, hydroxypropyl cellulose, hypromellose, maltodextrin, methylcellulose, pectin, propylene glycol alginate, sodium alginate, starch, tragacanth, and xanthan gum); and 2) noncarbohydrate hydrophilic macromolecules, including gelatin, povidone carbomers, polyethylene oxide, and polyvinyl alcohol. Minerals (e.g., attapulgite, bentonite, magnesium aluminum silicate, and silicon dioxide) comprise the second-largest group of suspending and viscosity-increasing agents. Aluminum monostearate is the one non-macromolecular, nonmineral excipient in this functional category. It consists chiefly of variable proportions of aluminum monostearate and aluminum monopalmitate.

### GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected viscosity-increasing functions: *Viscosity—Capillary Methods* ⟨911⟩, *Viscosity—Rotational Methods* ⟨912⟩, and *Viscosity—Rolling Ball Method* ⟨913⟩.

## Functional Category: Ointment Base

### DESCRIPTION

An ointment is a viscous semisolid preparation used topically on a variety of body surfaces. An ointment base is the major component of an ointment and controls its physical properties.

### FUNCTIONAL MECHANISM

Ointment bases serve as vehicles for topical application of medicinal substances and also as emollients and protective agents for skin.

### PHYSICAL PROPERTIES

Ointment bases are liquids with a relatively high viscosity so that solids can be suspended as a stable mixture.

Ointment bases are classified as: 1) oleaginous ointment bases that are anhydrous, do not absorb water readily, are insoluble in water, and are not removable by water (e.g., petrolatum); 2) absorption ointment bases that are anhydrous and absorb some water but are insoluble in water and are not water removable (e.g., lanolin); 3) emulsion ointment bases that are water-in-oil or oil-in-water emulsions and are hydrous, absorb water, and are insoluble in water (e.g., creams of water, oils, waxes, or paraffins); and 4) water-soluble ointment bases that are anhydrous and absorb water and are soluble in water and are water removable (e.g., polyethylene glycol).

### CHEMICAL PROPERTIES

Ointment bases are selected to be inert and chemically stable.

### GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected ointment base functions: *Congealing Temperature* ⟨651⟩, *Viscosity—Capillary Methods* ⟨911⟩, *Viscosity—Rotational Methods* ⟨912⟩, and *Viscosity—Rolling Ball Method* ⟨913⟩.

## Functional Category: Stiffening Agent

### DESCRIPTION

A stiffening agent is an agent or a mixture of agents that increases the viscosity or hardness of a preparation, especially in ointments and creams.

### FUNCTIONAL MECHANISM

In general, stiffening agents are high melting point solids that increase the melting point of ointments or increase the consistency or body of creams. Stiffening agents can be either hydrophobic (e.g., hard fat or paraffin) or hydrophilic (e.g., polyethylene glycol, high molecular weight).

### PHYSICAL PROPERTIES

The primary physical property relevant to stiffening agents is their high melting point or melting range. Typical melting ranges for stiffening agents range from 43° to 47° (cetyl esters wax), 53° to 57° (glyceryl distearate), 69° to 74° (glyceryl behenate), and 85° to 88° (castor oil, hydrogenated).

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CHEMICAL PROPERTIES

Stiffening agents comprise a diverse group of materials that include glycerides of saturated fatty acids, solid aliphatic alcohols, esters of saturated fatty alcohols and saturated fatty acids, saturated hydrocarbons, blends of fatty alcohols and a polyoxyethylene derivative of a fatty acid ester of sorbitan, and higher ethylene glycol polymers.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected stiffening-agent functions: *Congealing Temperature* ⟨651⟩, *Melting Range or Temperature* ⟨741⟩, *Viscosity—Capillary Methods* ⟨911⟩, *Viscosity—Rotational Methods* ⟨912⟩, and *Viscosity—Rolling Ball Method* ⟨913⟩.

ADDITIONAL INFORMATION

Some of the materials included as stiffening agents increase the water-holding capacity of ointments (e.g., petrolatum) or function as co-emulsifiers in creams. Examples include stearyl alcohol and cetyl alcohol.

## Functional Category: Emollient

DESCRIPTION

Emollients are excipients used in topical preparations to impart lubrication, spreading ease, texture, and softening of the skin and to counter the potentially drying/irritating effect of surfactants on the skin.

FUNCTIONAL MECHANISM

Emollients help form a protective film and maintain the barrier function of the epidermis. Their efficacy may be described by three mechanisms of action: protection against the delipidizing and drying effects of surfactants, humectancy due to occlusion (by providing a layer of oil on the surface of the skin, emollients slow water loss and thus increase the moisture-retention capacity of the stratum corneum), and lubricity, adding slip or glide to the preparation.

PHYSICAL PROPERTIES

Emollients impart one or more of the following attributes to a pharmaceutical preparation: spreading capacity, pleasant feel to the touch, softness of the skin, and indirect moisturization of the skin by preventing transepidermal water loss.

CHEMICAL PROPERTIES

Emollients are either oils or are derived from components of oils as esters of fatty acids. Depending on the nature of its fatty acid ester, an emollient may be liquid, semisolid, or solid at room temperature. Generally, the higher the molecular weight of the fatty acid moiety (carbon chain length) the richer the feel and softness of the touch. Fluidity generally is imparted by shorter chain length and higher degree of unsaturation in the fatty acid moiety. The degree of branching of ester bonds also influences the emollient properties.

GENERAL CHAPTER

The following general chapter may be useful in ensuring consistency in selected emollient functions: *Fats and Fixed Oils* ⟨401⟩.

# PARENTERALS

## Functional Category: Pharmaceutical Water

DESCRIPTION

Water is used as a solvent, vehicle, diluent, or filler for many drug products, especially those supplied in liquid form. These may include injectable drugs, ophthalmic drugs, inhalation solutions, and others. Water also is a vehicle for buffers and antimicrobial agents and is a volume expander for infusion solutions.

*USP* includes monographs for eight grades of pharmaceutical waters. Water for Injection is intended for use in the preparation of parenteral solutions. Where used for the preparation of parenteral solutions subject to final sterilization, use suitable means to minimize microbial growth, or first render the Sterile Water for Injection and, thereafter, protect it from microbial contamination. For parenteral solutions that are prepared under aseptic conditions and are not sterilized by appropriate filtration or in the final container, first render the Sterile Water for Injection and, thereafter, protect it from microbial contamination. Do not use Purified Water in preparations intended for parenteral administration. Where used for sterile dosage forms other than for parenteral administration, process the article to meet the requirements under *Sterility Tests* ⟨71⟩, or first render the Sterile Purified Water and, thereafter, protect it from microbial contamination. *USP* also contains references to other types of water, such as distilled water, deionized water, and others according to specific use as summarized in general information chapter *Water for Pharmaceutical Purposes* ⟨1231⟩.

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### FUNCTIONAL MECHANISM

A solvent is able to dissolve materials because it is able to disrupt the intermolecular attractive forces and to allow the individual molecules to become dispersed throughout the bulk solvent. Water is a favored solvent and vehicle in the majority of applications because it is easy to handle, safe, and inexpensive.

### PHYSICAL PROPERTIES

Water is liquid at normal temperature and pressure. It forms ice at the freezing temperatures of 0° or lower, and it vaporizes at a normal boiling temperature of 100°, depending on atmospheric pressure. Vaporized water in the form of steam is used for sterilization purposes because the latent heat of steam is significantly higher than that of boiling water.

### CHEMICAL PROPERTIES

Water in its pure form is neutral in pH and has very low conductivity and total organic carbon (TOC). However, pH, conductivity, and TOC are affected by storage conditions and exposure to gases in the air. Exposure to atmospheric carbon dioxide lowers water's pH. Storage in plastic containers may increase the TOC content of water over time. Water stored in glass containers may result in an increase in pH and conductivity over time.

### GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected pharmaceutical water functions: *Injections and Implanted Drug Products* ⟨1⟩, *Bacterial Endotoxins Test* ⟨85⟩, *Total Organic Carbon* ⟨643⟩, *Water Conductivity* ⟨645⟩, *Water for Hemodialysis Applications* ⟨1230⟩, and *Water for Pharmaceutical Purposes* ⟨1231⟩.

## Functional Category: Bulking Agent

### DESCRIPTION

Bulking agents used in lyophilized pharmaceuticals, also referred to as freeze-dried products, include various saccharides, sugar alcohols, amino acids, and polymers. The primary function of bulking agents is to provide a pharmaceutically elegant freeze-dried cake with noncollapsible structural integrity that will reconstitute rapidly before administration. In addition, bulking agents are selected to prevent product loss caused by blow-out during freeze drying, to facilitate efficient drying, and to provide a physically and chemically stable formulation matrix. Complementary combinations of bulking agents, e.g., mannitol and a polymer, frequently are used to improve performance.

### FUNCTIONAL MECHANISM

A bulking agent that readily crystallizes during lyophilization helps maintain the structural integrity of the cake formed during primary drying, thereby preventing macroscopic collapse and maintaining pharmaceutical elegance. Microscopic collapse of amorphous components in the formulation can still occur (with some potentially undesirable results) but does not result in macroscopic collapse or "meltback" if the bulking agent's properties and concentration are adequate. The bulking agent also should possess a high eutectic melting temperature with ice to permit relatively high primary drying temperatures with commensurate rapid and efficient drying and subsequent rapid reconstitution upon usage. Functional cake-forming excipients, such as mannitol, frequently are used because they crystallize during freezing, thereby allowing efficient drying and the formation of a structurally robust and stable cake. Amino acids and cosolvents also have been used to achieve this effect. Most biopolymer active ingredients remain amorphous upon freeze-drying, and bulking agents such as disaccharides can function as lyoprotectants by helping to maintain a stable amorphous phase during freezing and drying to prevent denaturation. Solubility enhancement of an insoluble crystalline active ingredient sometimes is achieved with the use of a biopolymer that enhances solubility or prevents crystallization during lyophilization or subsequent reconstitution. Bulking agents also are selected on the basis of biocompatibility, buffering capability, and tonicity-modifying properties.

Lyoprotectant properties of bulking agents (i.e., those that protect the drug substance during lyophilization) typically are achieved by the formation of a highly viscous glassy phase that includes the biopolymer drug substance in combination with low molecular weight amorphous saccharides such as sucrose, trehalose, or certain amino acids. A typical approach for protein pharmaceutical formulation is to combine a sugar alcohol that readily crystallizes and an amorphous diluent. This mixture acts as a lyoprotectant.

### PHYSICAL PROPERTIES

Bulking agents are dissolved in aqueous solution before lyophilization. Therefore, chemical purity and the absence of bioburden and pyrogenic materials are essential properties of the excipient. However, the physical form and particle properties of the excipient generally are not relevant to the final properties of the lyophilized formulation. The solubilization process and the drying process can be facilitated by the use of volatile cosolvents such as ethanol or tertiary butyl alcohol.

The physical properties that are essential to product performance during and after lyophilization include the glass transition temperature ($T_g$) of the amorphous frozen concentrate before drying, the glass transition temperature of the final dried formulation cake, and the eutectic melting temperature of the crystalline bulking agent with ice. The glass transition temperature ($T_g$) of the formulation depends on the glass transition temperatures of the individual components, concentrations, and interactions. Although approximations can be made based on reported transition temperatures for individual components,

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current practice includes the measurement of formulation glass transition temperatures by thermal analysis or freeze-drying microscopy.

The physical states of the bulking agent during and after lyophilization are important physical properties. Both formulation composition and processing parameters play roles in determining whether the bulking agent is amorphous or takes a specific crystalline form. Rate of freezing, drying temperatures, and annealing are among the important process parameters used to control the physical state of the formulation and its components. Moisture retention and adsorption after lyophilization also can contribute to formulation instability and poor reconstitution.

### CHEMICAL PROPERTIES

Reactivity of the bulking agent with other formulation components, especially the active ingredient, may be critical. Reducing sugars are well known to react with aromatic and aliphatic amines. Glycols may contain trace peroxide levels that can initiate oxidative degradation. The ability of saccharides and polyhydric alcohols to form hydrogen bonds to biopolymers may play a role in their lyoprotection effects.

### GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selecting bulking agent functions: *Injections and Implanted Drug Products* ⟨1⟩, *Crystallinity* ⟨695⟩, *Characterization of Crystalline Solids by Microcalorimetry and Solution Calorimetry* ⟨696⟩, *Thermal Analysis* ⟨891⟩, *Pharmaceutical Dosage Forms* ⟨1151⟩, and *Water–Solid Interactions in Pharmaceutical Systems* ⟨1241⟩.

## Functional Category: Tonicity Agent

### DESCRIPTION

To avoid crenation or hemolysis of red blood cells and to mitigate pain and discomfort if solutions are injected or introduced into the eyes and nose, solutions should be made isotonic. This requires that the effective osmotic pressure of solutions for injection must be approximately the same as that of blood. When drug products are prepared for administration to membranes, such as eyes or nasal or vaginal tissues, solutions should be made isotonic with respect to these tissues.

### FUNCTIONAL MECHANISM

Tonicity is equal to the sum of the concentrations of the solutes that have the capacity to exert an osmotic force across a membrane and thus reflects overall osmolality. Tonicity applies to the impermeant solutes within a solvent—in contrast to osmolarity, which takes into account both permeant and impermeant solutes. For example, urea is a permeant solute, meaning that it can pass through the cell membrane freely and is not factored when determining the tonicity of a solution. In contrast, sodium chloride is impermeant and cannot pass through a membrane without the help of a concentration gradient and, therefore, contributes to a solution's tonicity.

### PHYSICAL PROPERTIES

Solutions of sodium chloride, dextrose, and Lactated Ringer's are common examples of pharmaceutical preparations that contain tonicity agents. Not all solutes contribute to the tonicity, which in general depends only on the number of solute particles present in a solution, not the kinds of solute particles. For example, mole for mole, sodium chloride solutions display a higher osmotic pressure than glucose solutions of the same molar concentration. This is because when glucose dissolves, it remains one particle, but when sodium chloride dissolves, it becomes two particles: $Na^+$ and $Cl^-$. Several methods are available to calculate tonicity.

### CHEMICAL PROPERTIES

Tonicity agents may be present as ionic or nonionic types. Examples of ionic tonicity agents are alkali metal or earth metal halides such as calcium chloride ($CaCl_2$), potassium bromide (KBr), potassium chloride (KCl), lithium chloride (LiCl), sodium iodide (NaI), sodium bromide (NaBr) or sodium chloride (NaCl), sodium sulfate ($Na_2SO_4$), or boric acid. Nonionic tonicity agents include glycerol, sorbitol, mannitol, propylene glycol, or dextrose. Sodium or potassium chloride and dextrose commonly are added to adjust tonicity.

### GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected tonicity agent functions: *Injections and Implanted Drug Products* ⟨1⟩, *Osmolality and Osmolarity* ⟨785⟩, and *Pharmaceutical Calculations in Pharmacy Practice* ⟨1160⟩.

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# AEROSOLS

## Functional Category: Propellant

### DESCRIPTION

Propellants are compounds that are gaseous under ambient conditions. They are used in pharmaceuticals (nasal sprays and respiratory and topical formulations), cosmetics, and foods to provide force to expel contents from a container.

### FUNCTIONAL MECHANISM

Propellant substances are low boiling point liquids or compressed gases that are relatively inert toward active ingredients and excipients. They can be characterized by three properties: whether they form a liquid phase at ambient temperatures and useful pressures, their solubility and/or miscibility in the rest of the formulation, and their flammability. Their performance is judged by their ability to provide adequate and predictable pressure throughout the usage life of the product.

Propellants that have both a liquid and gas phase in the product provide consistent pressures as long as there is a liquid phase present—the pressure in the headspace is maintained by the equilibrium between the two phases. In contrast, the pressure provided by propellants that have no liquid phase may change relatively rapidly as the contents of the container are expelled. As the headspace becomes larger, the pressure within the container falls proportionately. Propellants that have no liquid phase but have significant pressure-dependent solubility in the rest of the formulation have performance characteristics between those of the other two systems. In such cases, as the headspace increases the propellant comes out of solution to help to maintain the pressure of the system.

In metered-dose inhalers, the propellant has a liquid phase that is an integral part of the dispensed pharmaceutical product. Actuating the metering valve dispenses a defined volume of the liquid contents. The propellant spontaneously boils and provides atomizing and propulsive force. A predictable change in active concentration occurs from the beginning to the end of the container life cycle as the liquid phase of the propellant vaporizes to reestablish the equilibrium pressure of the system as the headspace increases.

### PHYSICAL PROPERTIES

Propellants have boiling points well below ambient temperatures. A propellant's density for disperse systems and its solubility properties may be significant considerations when one selects a propellant. Apaflurane and norflurane have liquid-phase densities that are greater than that of water. Hydrocarbon propellants (butane, isobutane, and propane) and dimethyl ether have liquid-phase densities that are less than that of water.

### CHEMICAL PROPERTIES

Propellants typically are stable materials. The hydrocarbon propellants (butane, isobutene, and propane) and dimethyl ether are all flammable materials. Apaflurane, carbon dioxide, nitrogen, and norflurane are nonflammable. Nitrous oxide is not flammable but supports combustion. Chlorofluorocarbon propellants are considered to be ozone-depleting substances. Their use in foods, drugs, devices, or cosmetics is regulated by 21 CFR 2.125. Albuterol metered-dose inhalers formulated with chlorofluorocarbon propellants have not been available in the United States since January 1, 2009.

### GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected propellant functions: *Inhalation and Nasal Drug Products: Aerosols, Sprays, and Powders—Performance Quality Tests* ⟨601⟩, *Chromatography* ⟨621⟩, and *Water Determination* ⟨921⟩.

# DRY POWDER INHALERS

Dry powder inhalers (DPIs) commonly contain few functional excipients. For example, DPIs may contain a carrier and may use a capsule shell. Other useful excipients include glidants to improve flow during manufacture of the active carrier mix. A discussion of the use of a lubricant can be found in the tablet or capsule sections above in addition to the carrier properties discussed below.

## Functional Category: Carrier

### DESCRIPTION

Carriers are used to help deposit the active ingredient in the lung and may have a secondary role in diluting the active to ensure that dosages can be properly metered.

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FUNCTIONAL MECHANISM

The carriers are used to promote drug deposition into the lungs for better penetration or absorption in the appropriate lung location. In addition, the carrier is used to decrease the concentration of the active so the latter is adequately dosed in a uniform manner.

PHYSICAL PROPERTIES

The physical properties of carriers include appropriate morphology, hydration state, flowability, surface energy, and particle size distribution.

CHEMICAL PROPERTIES

Carriers must have suitable purity, including low microbial content and no extraneous proteins or impurities, to avoid interactions with the patient's immune system.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected carrier functions: *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* ⟨61⟩, *Microbiological Examination of Nonsterile Products: Tests for Specified Microorganisms* ⟨62⟩, *Elemental Impurities—Limits* ⟨232⟩ and *Elemental Impurities—Procedures* ⟨233⟩, *Light Diffraction Measurement of Particle Size* ⟨429⟩, *Nitrogen Determination* ⟨461⟩, *Inhalation and Nasal Drug Products: Aerosols, Sprays, and Powders—Performance Quality Tests* ⟨601⟩, *Bulk Density and Tapped Density of Powders* ⟨616⟩, *Crystallinity* ⟨695⟩, *Characterization of Crystalline Solids by Microcalorimetry and Solution Calorimetry* ⟨696⟩, *Density of Solids* ⟨699⟩, *Loss on Drying* ⟨731⟩, *Optical Microscopy* ⟨776⟩, *Particle Size Distribution Estimation by Analytical Sieving* ⟨786⟩, *Powder Fineness* ⟨811⟩, *Mid-Infrared Spectroscopy* ⟨854⟩ and *Ultraviolet-Visible Spectroscopy* ⟨857⟩, *Water Determination* ⟨921⟩, *Characterization of Crystalline and Partially Crystalline Solids by X-Ray Powder Diffraction (XRPD)* ⟨941⟩, and *Powder Flow* ⟨1174⟩.

## Functional Category: DPI Capsule Shells

DESCRIPTION

Capsule shells sometimes are used in DPIs. The capsule shell is used to contain the dosage amount and safeguard the inhalable powder in a DPI.

FUNCTIONAL MECHANISM

The use of capsule shell may speed pharmaceutical development because it does not require a complex device and can use premeasured drug substance or formulation. A capsule shell must not fragment into inhalable portions and should remain intact after the shell breaks to expose the powder for inhalation.

PHYSICAL PROPERTIES

Capsule shell composition generally is dictated by the drug substance's moisture content, brittleness, and electrostatic interactions with the inhalable powder.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected DPI capsule shell functions: *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* ⟨61⟩, *Microbiological Examination of Nonsterile Products: Tests for Specified Microorganisms* ⟨62⟩, *Arsenic* ⟨211⟩, *Elemental Impurities—Limits* ⟨232⟩ and *Elemental Impurities—Procedures* ⟨233⟩, *Residue on Ignition* ⟨281⟩, *Inhalation and Nasal Drug Products: Aerosols, Sprays, and Powders—Performance Quality Tests* ⟨601⟩, *Disintegration* ⟨701⟩, *Dissolution* ⟨711⟩, *Loss on Drying* ⟨731⟩, *Optical Microscopy* ⟨776⟩, *Particle Size Distribution Estimation by Analytical Sieving* ⟨786⟩, *Uniformity of Dosage Units* ⟨905⟩, *Water Determination* ⟨921⟩, *Color—Instrumental Measurement* ⟨1061⟩, and *Water–Solid Interactions in Pharmaceutical Systems* ⟨1241⟩.

**additional information**

In addition to the general chapters listed above, useful information for ensuring consistency in selected capsule shell functions may be found in *Gelatin, Gel Strength (Bloom Value)*.

# OPHTHALMIC PREPARATIONS

## Functional Category: Antimicrobial Preservatives

DESCRIPTION

The preservative system acts as a safeguard to kill or inhibit the growth of microorganisms that may be inadvertently introduced in the product after the manufacturing process either during storage or use.

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### FUNCTIONAL MECHANISM

Antimicrobial preservatives work by a number of mechanisms. Quaternary ammonium compounds affect microbial cell membranes via charge interactions with phospholipids, leading to disruption of the cell membrane. Parabens also disrupt cell membrane integrity. Alcohols such as chlorbutanol and benzyl alcohol work via lipid (membrane) solvation and protein denaturation. *N*-[3-(Dimethylamino)propyl]tetradecanamide has greater antimicrobial effectiveness toward fungi and protozoa than do quaternary ammonium compounds. Similar to quaternary ammonium compounds, it disrupts plasma membrane integrity. Sorbic acid works by reduction of the sulfhydryl groups of proteins. Hypochlorite is a strong oxidizing agent. Reactions of chloramines with the amine groups of proteins can cause changes in conformation and thus loss of protein activity. Chlorine released by these reactions can react with cellular constituents, such as proteins and lipid. Polyaminopropyl biguanide accumulates in the cell membrane, blocking the entry of nutrients.

### PHYSICAL PROPERTIES

To serve as an ophthalmic antimicrobial preservative, a compound should be at least sparingly soluble in water, thus providing an appreciable range of usable concentrations.

### CHEMICAL PROPERTIES

A preservative must be compatible with the active and inactive ingredients of the finished product. For example, quaternary ammonium compounds are incompatible with anionic surfactants. Benzyl alcohol is incompatible with oxidizing agents. Chlorbutanol is incompatible with some nonionic surfactants. Compatibility between compounds varies with the pH of the formula. The preservative should be stable in solution at the formulation pH, usually from 5 to 8. Formulation pH can affect preservative activity by influencing how the preservative partitions between the formulation and microbes and how the preservative interacts with the target sites of the microbial cell. For example, preservatives that must pass through cell membranes before exerting activity should be formulated at a pH at which the preservative is mainly in its un-ionized state.

### GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistent functions of selected antimicrobial preservatives: *Antimicrobial Effectiveness Testing* ⟨51⟩, *Sterility Tests* ⟨71⟩, *Bulk Density and Tapped Density of Powders* ⟨616⟩, *Chromatography* ⟨621⟩, *Density of Solids* ⟨699⟩, *Loss on Drying* ⟨731⟩, *Pharmaceutical Dosage Forms* ⟨1151⟩, *Powder Flow* ⟨1174⟩, *Sterility Assurance* ⟨1211⟩, and *Validation of Microbial Recovery from Pharmacopeial Articles* ⟨1227⟩.

## Functional Category: Polymers for Ophthalmic Use

### DESCRIPTION

Polymers are used in ophthalmic preparations to enhance the retention of active ingredients by reducing the amount of product that is lost from the eye when the patient blinks. In addition, polymers also can be components of artificial tears. Most water-soluble polymers commonly used as film-forming agents in ophthalmic preparations can be categorized as follows: 1) cellulose-based substances, 2) biologically produced gums, and 3) synthetically produced substances.

### FUNCTIONAL MECHANISM

Film-forming agents for ophthalmic preparations can enhance the retention of active ingredients in the eye by a number of mechanisms. They can be used as simple viscosity-modifying agents to reduce the flow of the product, thereby slowing the rate of product loss after administration. They also can be used to form films on the surface of the eye so the drug remains deposited on the eye after the liquid portion of the product has been expelled or has evaporated. These agents can be formulated to produce a film or a gel when the product warms to body temperature (upon contacting the surface of the eye), mixing with the tear film, and/or evaporating. Some polymers have shown bio-adhesive properties on the cornea and can increase drug retention.

### PHYSICAL PROPERTIES

To serve as an ophthalmic film-forming agent, a polymer typically must be at least slightly soluble in water, thus providing an appreciable range of usable concentrations. Such polymers often increase viscosity or exhibit film- or gel-forming properties when warmed to body temperature, when exposed to the pH or solute composition and ionic strength of the tear film, or when the product evaporates.

### CHEMICAL PROPERTIES

The finished product viscosity range that can be obtained with a film-forming agent is related to its chemical structure and molecular weight. Functional groups such as the pyruvate and acetate groups of xanthan gum can affect the relationship between viscosity and solution pH and ionic strength and also can determine film- and gel-forming properties. Polymer charge can influence interactions with the mucous layer of the eye. Molecular conformation, chain mobility, and degree of cross-linking also can affect the degree of swelling and thus performance.

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GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistent functions of polymers for ophthalmic use: *Bulk Density and Tapped Density of Powders* ⟨616⟩, *Chromatography* ⟨621⟩, *Density of Solids* ⟨699⟩, *Loss on Drying* ⟨731⟩, *Particulate Matter in Ophthalmic Solutions* ⟨789⟩, *Viscosity—Capillary Methods* ⟨911⟩, *Viscosity—Rotational Methods* ⟨912⟩, *Viscosity—Rolling Ball Method* ⟨913⟩, *Pharmaceutical Dosage Forms* ⟨1151⟩, and *Powder Flow* ⟨1174⟩. In addition, the general chapters listed under *Functional Category: Film-Forming Agents* also may be appropriate for the evaluation of polymers for ophthalmic use.

# TRANSDERMALS AND PATCHES

## Functional Category: Adhesive

DESCRIPTION

Topical drug delivery systems (e.g., transdermals or skin patches) require the use of adhesives to maintain contact between the applied drug delivery system and the skin. Adhesives can be intercalated as a separate layer between the formulation matrix and the skin surface, incorporated as a part of the formulation matrix itself, or applied to the periphery of the topical delivery system.

FUNCTIONAL MECHANISM

Adhesion is the tendency of dissimilar surfaces to adhere to one another as a result of one or more types of interactions. For topical drug delivery systems, these adhesive interactions generally are chemical (primarily electrostatic) or dispersive (van der Waals and/or hydrogen bonding) in nature, although there is the possibility of mechanical interaction via the interlocking of microscopic asperities.

PHYSICAL PROPERTIES

In general, the adhesives used in transdermals or skin patches are pressure-sensitive materials whose performance is best characterized by physical test methods for tackiness and viscoelasticity of the adhesive per se and viscosity of a solution of the adhesive.

CHEMICAL PROPERTIES

In transdermals, the most widely used pressure-sensitive adhesives are acrylic, rubber, and silicone polymers. Acrylic polymer adhesives include various esters of acrylic or methacrylic acid, acrylamide, methacrylamide, *N*-alkoxyalkyl, or *N*-alkyl-acrylamides. Polyisobutylenes and polysiloxanes are among the most common rubber-based and silicone-based adhesives, respectively. The molecular weight and compositional distribution of the polymers are critical to the replication of the adhesive's efficacy from batch to batch.

GENERAL CHAPTERS

The following general chapters may be useful in evaluating the suitability of adhesives used in transdermals: *Tensile Strength* ⟨881⟩ and *Viscosity—Capillary Methods* ⟨911⟩.

## Functional Category: Film-Forming Agent

DESCRIPTION

Film-forming agents used as the formulation matrix of topical drug delivery systems (e.g., transdermals or skin patches) or in conjunction with such systems comprise a flexible, nontacky but adherent film, in whole or in part, applied to the skin surface.

FUNCTIONAL MECHANISM

Film formation results from the progressive loss of solvent (or dispersion medium) from a solution (or dispersion) of a film-forming agent, whether in particulate or molecularly dispersed form. Solvent (or dispersion medium) loss leads to closer molecular or particulate packing and increased interaction among the film-forming agent molecules or particles. Ultimately, a continuous film is formed as a result of increased molecular entanglement or particulate sintering.

PHYSICAL PROPERTIES

Properties critical to successful film formation include the film-forming agent's glass transition temperature ($T_g$), the viscosity of the solution or dispersion, and the surface characteristics of the substrate. Viscoelastic properties such as elastic modulus, viscous modulus, and intrinsic or complex viscosity describe functional characteristics, such as adhesion, for a pressure-sensitive adhesive component. Adhesion to a substrate and tack and shear tests can be used for batch release.

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CHEMICAL PROPERTIES

Typical film-forming agents are thermoplastic or thermosetting high molecular weight polymers or copolymers, often in the form of aqueous dispersions or latex compositions. Cellulosic polymers, vinyl polymers and copolymers, and acrylic and methacrylic acid polymers and copolymers frequently are used in topical delivery systems as film-forming agents.

GENERAL CHAPTERS

The following general chapters may be useful in evaluating the suitability of film-forming agents used in transdermals and patches: *Thermal Analysis* ⟨891⟩, *Viscosity—Capillary Methods* ⟨911⟩, *Viscosity—Rotational Methods* ⟨912⟩, and *Viscosity—Rolling Ball Method* ⟨913⟩.

## RADIOPHARMACEUTICALS

Radiopharmaceuticals commonly contain categories of excipients that also are used in conventional drugs. For example, radiopharmaceutical capsules may contain diluents and necessarily use a capsule shell, and parenteral radiopharmaceuticals may contain pharmaceutical water, diluents, tonicity agents, pH modifiers, antimicrobial preservatives, chelating and/or complexing agents, and antioxidants. Many radiopharmaceuticals differ from conventional drugs, however, because their preparation (reconstitution) involves one or more chemical reactions that require unusual excipients. Furthermore, the self-absorption of emitted radiation may result in the radiolytic decomposition of many radiopharmaceuticals. Hence, several excipients are used predominately in radiopharmaceutical formulations, although they occasionally may be used for other drugs.

### Functional Category: Reducing Agent

DESCRIPTION

Reducing agents generally are required for technetium Tc 99m radiopharmaceuticals. Technetium Tc 99m, in the chemical form of sodium pertechnetate (+7 oxidation state), must be reduced to a lower oxidation state so that it can be chelated or otherwise complexed by the intended ligand to form the final Tc 99m radiopharmaceutical. The reducing agent, typically a stannous salt, generally is formulated in the kit for the preparation of the technetium Tc 99m radiopharmaceutical.

FUNCTIONAL MECHANISM

The reducing agent (e.g., stannous ion) must be present in sufficient quantity to reduce all of the technetium atoms to the intended oxidation state but must not produce undesired reduction products or other impurities (e.g., stannous hydroxide precipitates).

PHYSICAL PROPERTIES

Reducing agents (e.g., stannous salts) must be readily soluble in water.

CHEMICAL PROPERTIES

Reducing agents (e.g., stannous salts) are sensitive to oxidation by atmospheric oxygen and oxidizing species in solution. Hence, lyophilized contents of kit vials must be filled with a nonoxidative gas such as nitrogen or argon. The reducing agent also must be stable at the intended pH of the formulated product.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected reducing agent functions: *Chromatography* ⟨621⟩ and *Radioactivity* ⟨821⟩.

### Functional Category: Transfer Ligand

DESCRIPTION

In the preparation of certain radiopharmaceuticals, the radiometal (e.g., stannous-reduced technetium Tc 99m) is first chelated by a relatively weak chelating ligand and then is transferred to the principal chelating ligand or complexing moiety. Examples of such transfer ligands include citrate, gluconate, and tartrate.

FUNCTIONAL MECHANISM

Transfer ligands typically undergo rapid reactions with reduced technetium to form weak chelates, thus keeping the reduced technetium in a soluble form until it is transferred to the principal ligand. This procedure is especially useful when the kinetics of complexation with the principal ligand is slow or when a heating step is necessary to expose chelating groups on the principal ligand.

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PHYSICAL PROPERTIES

Transfer ligands must be readily soluble in water.

CHEMICAL PROPERTIES

Transfer ligands must have rapid complexation kinetics and must form relatively weak chelates compared to complexation with the principal ligand.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected transfer ligand functions: *Chromatography* ⟨621⟩ and *Radioactivity* ⟨821⟩.

## Functional Category: Colloid Stabilizing Agent

DESCRIPTION

Lyophobic colloids tend to clump together and form large aggregates to minimize their surface-area-to-volume ratio. Colloid stabilizing agents are relatively large lyophilic molecules that coat the surface of each individual colloid particle and prevent or inhibit clumping. Examples of colloid stabilizing agents include gelatin and dextran.

FUNCTIONAL MECHANISM

The colloid stabilizing agent coats the surface of the lyophobic colloid particles, making them appear lyophilic. Additionally, the colloid stabilizing agent may be charged, thus causing the coated colloid particles to repel one another.

PHYSICAL PROPERTIES

Colloid stabilizing agents must be readily soluble in water.

CHEMICAL PROPERTIES

Colloid stabilizing agents must be capable of coating the lyophobic colloid particles, e.g., by electrostatic attraction of an opposite charge.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected colloid stabilizing agent functions: *Chromatography* ⟨621⟩ and *Radioactivity* ⟨821⟩.

## Functional Category: Free Radical Scavenger

DESCRIPTION

Radiation interactions with water and other molecules frequently produce free radicals. Free radical scavengers preferentially interact with oxidative or reductive free radicals that otherwise would result in degradation of formulation components. In the case of radiopharmaceuticals, free radical scavengers can be used to enhance radiochemical purity. Examples of free radical scavengers include methylene blue and aminobenzoic acid.

FUNCTIONAL MECHANISM

Free radical scavengers preferentially interact with radiolytically produced free radicals before these free radicals can interact with the radiopharmaceutical and produce radiochemical impurities.

PHYSICAL PROPERTIES

Free radical scavengers must be readily soluble in water.

CHEMICAL PROPERTIES

Free radical scavengers must be capable of preferentially interacting with free radicals without causing other effects.

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# ⟨1061⟩ COLOR—INSTRUMENTAL MEASUREMENT

The observed color (see ⟨631⟩ *Color and Achromicity*) of an object depends on the spectral energy of the illumination, the absorbing characteristics of the object, and the visual sensitivity of the observer over the visible range. Similarly, it is essential that any instrumental method that is widely applicable take these same factors into account.

Instrumental methods for measurement of color provide more objective data than the subjective viewing of colors by a small number of individuals. With adequate maintenance and calibration, instrumental methods can provide accurate and precise measurements of color and color differences that do not drift with time. The basis of any instrumental measurement of color is that the human eye has been shown to detect color via three "receptors." Hence, all colors can be broken down into a mixture of three radiant stimuli that are suitably chosen to excite all three receptors in the eye. Although no single set of real light sources can be used to match all colors (i.e., for any three lights chosen, some colors require a negative amount of one or more of the lights), three arbitrary stimuli have been defined, with which it is possible to define all real colors. Through extensive color-matching experiments with human subjects having normal color vision, distributing coefficients have been measured for each visible wavelength (400 nm to 700 nm) giving the relative amount of stimulation of each receptor caused by light of that wavelength. These distribution coefficients $\bar{x}$, $\bar{y}$, $\bar{z}$, are shown below. Similarly, for any color the amount of stimulation of each receptor in the eye is defined by the set of *Tristimulus values* (X, Y, and Z) for that color.

The relationships between the distribution coefficient (see *accompanying figure*) and the tristimulus values are given in the equations

$$X = \int_0^\infty f_\lambda \bar{x}_\lambda P_\lambda d\lambda / Y',$$
$$Y = \int_0^\infty f_\lambda \bar{y}_\lambda P_\lambda d\lambda / Y', \text{ and}$$
$$Z = \int_0^\infty f_\lambda \bar{z}_\lambda P_\lambda d\lambda / Y',$$

in which

$$Y' = \int_0^\infty \bar{y}_\lambda P_\lambda d\lambda, P_\lambda$$

is the spectral power of the illuminant, and $f_\lambda$ is either the spectral reflectance ($\rho_\lambda$) or spectral transmittance ($\tau_\lambda$) of the material.

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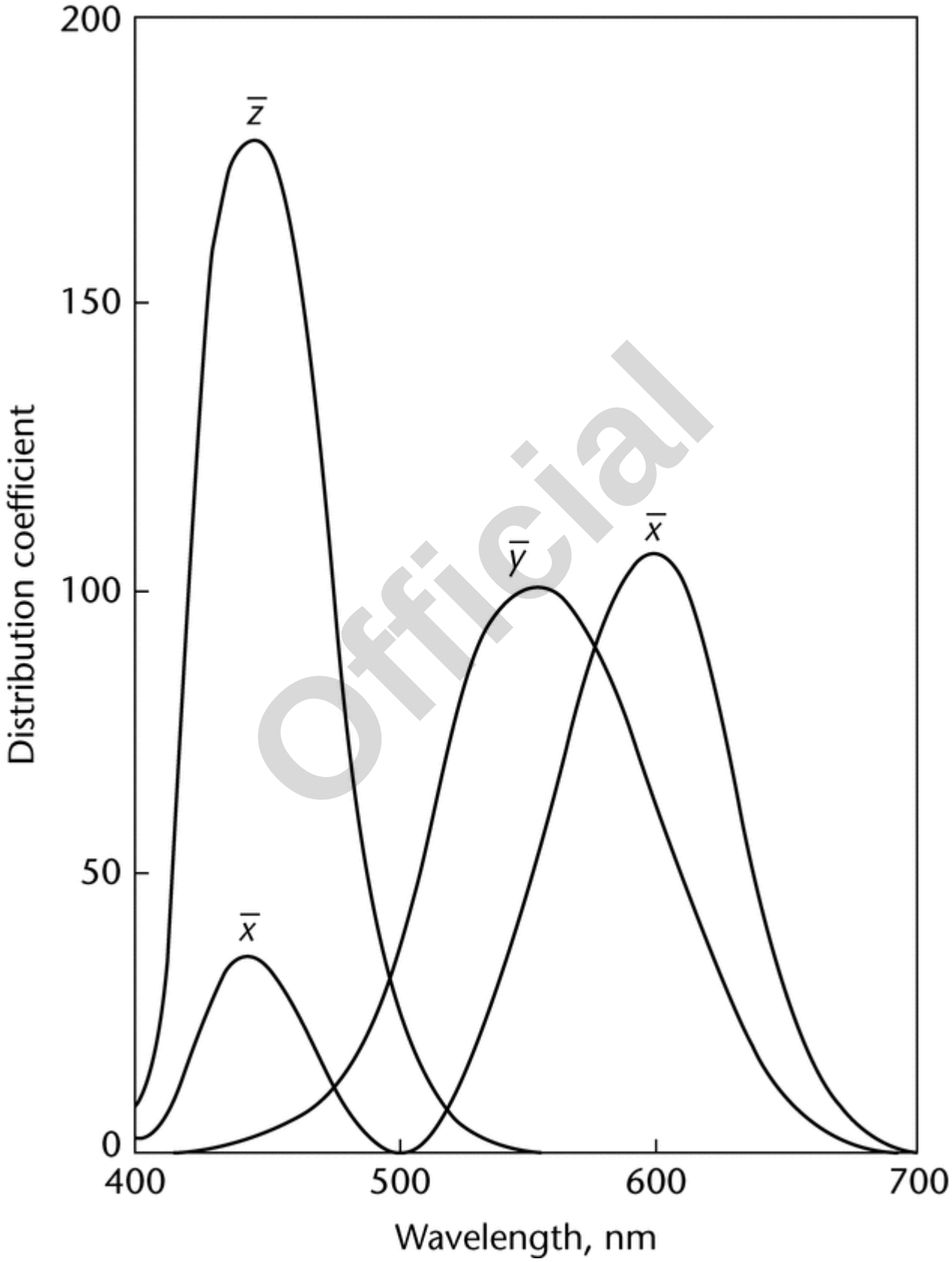


Distribution Coefficients from 400 to 700 nm

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Once the tristimulus values of a color have been determined, they may be used to calculate the coordinates of the color in an idealized three-dimensional color space referred to as a *visually uniform color space.* Many sets of color equations have been developed in an attempt to define such a space. The equations given in this chapter represent a compromise between simplicity of calculation and conformance with ideality.

The coordinates of a color in a visually uniform color space may be used to calculate the deviation of a color from a chosen reference point. Where the instrumental method is used to determine the result of a test requiring color comparison of a test preparation with that of a standard or matching fluid, the parameter to be compared is the difference, in visually uniform color space, between the color of the blank and the color of the test specimen or standard.

## PROCEDURE

The considerations discussed under *Ultraviolet-Visible Spectroscopy* ⟨857⟩ apply to instrumental color measurement as well. In the spectrophotometric method, reflectance or transmittance values are obtained at discrete wavelengths throughout the visible spectrum, a band width of 10 nm or less being used. These values are then used to calculate the tristimulus values through the use of weighting factors.[1] In the colorimetric method, the weighting is performed through the use of filters.

In the measurement of the spectral reflectance of opaque solids, the angle of viewing is separated from the angle of illumination in such a manner that only rays reflected diffusely from the test specimen enter the receptor. Specular reflection and stray light are excluded.

For the measurement of the spectral transmittance of clear liquids, the specimen is irradiated from within 5 degrees of the normal to its surface, and the transmitted energy measured is that confined within 5 degrees from the normal. The color of solutions changes with the thickness of the layer measured. Unless special considerations dictate otherwise, a layer 1 cm thick should be used.

The methods described here are not applicable to hazy liquids or translucent solids.

### Calibration

For purposes of calibration, one of the following reference materials may be used, as required by instrument geometry. For transmittance measurements, purified water may be used as a white standard and assigned a transmittance of 1.000 at all wavelengths. Then the tristimulus values X, Y, and Z for CIE source C are 98.0, 100.0, and 118.1, respectively. For reflectance measurements, opaque porcelain plaques, whose calibration base is the perfect diffuse reflector and whose reflectance characteristics have been determined for the appropriate instrumental geometry, may be used.[2] If the geometry of sample presentation precludes the use of such plaques, pressed barium sulfate, white reflectance standard grade, may be used.[3]

After calibration with the above-mentioned materials, it is desirable whenever possible to measure a reference material as close to the color of the sample as possible. If a sample of the material being tested is not suitable for use as a long-term standard, color chips are available[4] which span the entire visually uniform color space in small increments. The use of such a reference standard is encouraged as a means of monitoring instrument performance even for absolute color determinations.

### Spectrophotometric Method

Determine the reflectance or transmittance from 380 to 770 nm at intervals of 10 nm. Express the result as a percentage, the maximum being 100.0. Calculate the tristimulus values X, Y, and Z as follows.

REFLECTING MATERIALS

For reflecting materials the quantities X, Y, and Z are

$$X = \sum_{380}^{770} \rho_\lambda \bar{x}_\lambda P_\lambda \Delta\lambda / Y',$$

$$Y = \sum_{380}^{770} \rho_\lambda \bar{y}_\lambda P_\lambda \Delta\lambda / Y', \text{ and}$$

$$Z = \sum_{380}^{770} \rho_\lambda \bar{z}_\lambda P_\lambda \Delta\lambda / Y',$$

in which

$$Y' = \sum_{380}^{770} \bar{y}_\lambda P_\lambda \Delta\lambda \rho_\lambda$$

is the spectral reflectance of the material, $\bar{x}_\lambda P_\lambda$, $\bar{y}_\lambda P_\lambda$, and $\bar{z}_\lambda P_\lambda$ are known values associated with each Standard Source,[1,2] and $\Delta\lambda$ is expressed in nm.

[1] Typical weighting factors are given by ASTM Z58.7.1-1951 as reported in the *Journal of the Optical Society of America*, Vol. 41, 1951, pages 431-439.
[2] Suitable items are available from BYK-Gardner USA, 2431 Linden Lane, Silver Spring, MD 20910, or from Hunter Associates Laboratory, Inc., 11491 Sunset Hills Road, Reston, VA 22090.
[3] Suitable material is available from Eastman Kodak Company, Rochester, NY 14650, as "White Reflectance Standard."
[4] Centroid Color Charts may be obtained from suppliers of instruments for measurement of color.

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TRANSMITTING MATERIALS

For transmitting materials, the quantities X, Y, and Z are calculated as above, $\tau_\lambda$ (spectral transmittance) being substituted for $\rho_\lambda$.

## Colorimetric Method

Operate a suitable colorimeter[5] to obtain values equivalent to the tristimulus values, X, Y, and Z. The accuracy with which the results obtained from the filter colorimeter match the tristimulus values may be indicated by determining the tristimulus values of plaques of strongly saturated colors and comparing these values with those computed from spectral measurements on a spectrophotometer.

# INTERPRETATION

## Color Coordinates

The Color Coordinates, L*, a*, and b* are defined by

$$L^* = 116(Y/Y_0)^{1/3} - 16,$$

$$a^* = 500[(X/X_0)^{1/3} - (Y/Y_0)^{1/3}], \text{ and}$$

$$b^* = 200[(Y/Y_0)^{1/3} - (Z/Z_0)^{1/3}]$$

in which $X_0$, $Y_0$, and $Z_0$ are the tristimulus values of the nominally white or colorless standard, and $Y/Y_0 > 0.01$. Usually they are equal to the tristimulus values of the standard illuminant, with $Y_0$ set equal to 100.0. In this case $X_0 = 98.0$ and $Z_0 = 118.1$.

## Color Difference

The total Color Difference $\Delta E^*$ is

$$\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$$

in which $\Delta L^*$, $\Delta a^*$, and $\Delta b^*$ are the differences in color coordinates of the specimens being compared.

Instrumental variables can influence results. Although reliable comparisons can be made between similar colors measured concomitantly, results obtained on different instruments or under different operating conditions should be compared with caution. If it is necessary to compare data obtained from different instruments or taken at different times, etc., it is very helpful to have concomitant data obtained on a standard reference material such as color chips for opaque materials. Comparison of the readings on the reference material helps to identify variations caused by instrument performance.

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[5] A suitable tristimulus colorimeter is available from BYK-Gardner USA, 2431 Linden Lane, Silver Spring, MD 20910, or from Hunter Associates Laboratory, Inc., 11491 Sunset Hills Road, Reston, VA 22090.

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# ⟨1062⟩ TABLET COMPRESSION CHARACTERIZATION

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2. COMPRESSION PHASES
3. TABLET COMPRESSION CHARACTERIZATION EQUIPMENT
    3.1 Hydraulic Press
    3.2 Instrumented Research Tablet Press
    3.3 Tablet Press Emulator
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    3.5 Instrumented Production Tablet Press
4. TOOLING
5. PUNCH DISPLACEMENT–TIME PROFILES
6. TABLET MECHANICAL STRENGTH
7. TABLET POROSITY AND SOLID FRACTION
8. MANUFACTURABILITY PROFILE
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## 1. BACKGROUND

The tablet is currently the most widely used dosage form for oral drug delivery (see *Pharmaceutical Dosage Forms* ⟨1151⟩). The advantages of the tablet include economy of manufacture, patient convenience, and compliance. Tablets may also offer additional advantages over other dosage forms, such as superior physical or chemical stability.

Powder compression is a critical process in manufacturing the tablet dosage form. Although this process has been used routinely for over a century, problems related to powder compression in pharmaceutical formulation development and manufacturing persist. Common problems include tablet failures, such as capping and lamination (*1*–*3*), high friability, powder sticking to punch surfaces or the die wall, and insufficient mechanical strength to withstand stress in downstream processing. Some formulations may exhibit acceptable compression characteristics during early development where production volumes are usually low but become problematic during scale-up.

The properties of compressed tablets are sensitive to both material characteristics and process parameters. The characteristics of the equipment used and the ambient conditions of temperature and humidity are also key factors that influence tablet compression. Physical properties such as particle size (*4*), particle shape (*5*,*6*), surface texture (*7*), crystallinity, and moisture content (*8*) influence powder tableting performance by affecting the bonding strength and/or the bonding area (*9*,*10*). The powdered material mechanical properties, such as particle hardness, elastic properties, viscoelastic properties, plasticity, and particle brittleness, also affect tablet strength (*11*–*13*). The extent of lubrication is yet another important factor that affects powder compression. A lubricant is typically added to the drug product blend formulation as a final step prior to compression to reduce frictional forces during compression and tablet ejection. This added lubricant, however, may adversely impact tablet mechanical strength and dissolution release rate.

This chapter describes the current understanding of this specialized area and outlines experimental methodologies for characterization of tablet compression to provide guidance for standardized compression test procedures and use of terminology. Although the fundamental concepts described here are also applicable to other processes, such as plug formation during encapsulation and roller compaction, the focus of this article is on tableting.

[NOTE—The *Glossary* defines terms with the International System of Units (SI), commonly used in pharmaceutical tablet compression.]

## 2. COMPRESSION PHASES

Powder compression behavior is governed by the physical and mechanical properties of the material as well as aspects of the compression process such as pressure (i.e., stress), degree of deformation (i.e., strain), and rate of deformation (i.e., strain rate). Therefore, knowledge of stress, strain, and strain rate is important for understanding powder behavior during the compression process. The majority of pharmaceutical tablets are manufactured by “uniaxial powder compression”. That is, each tablet is formed by the densification of a loosely packed powder sample confined within a rigid die using two rigid punches that approach from above and below (in a vertical plane). This tablet formation process is often described as occurring in four stages, as shown in *Figure 1*:

1. Particle rearrangement
2. Compression

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3. Decompression
4. Ejection

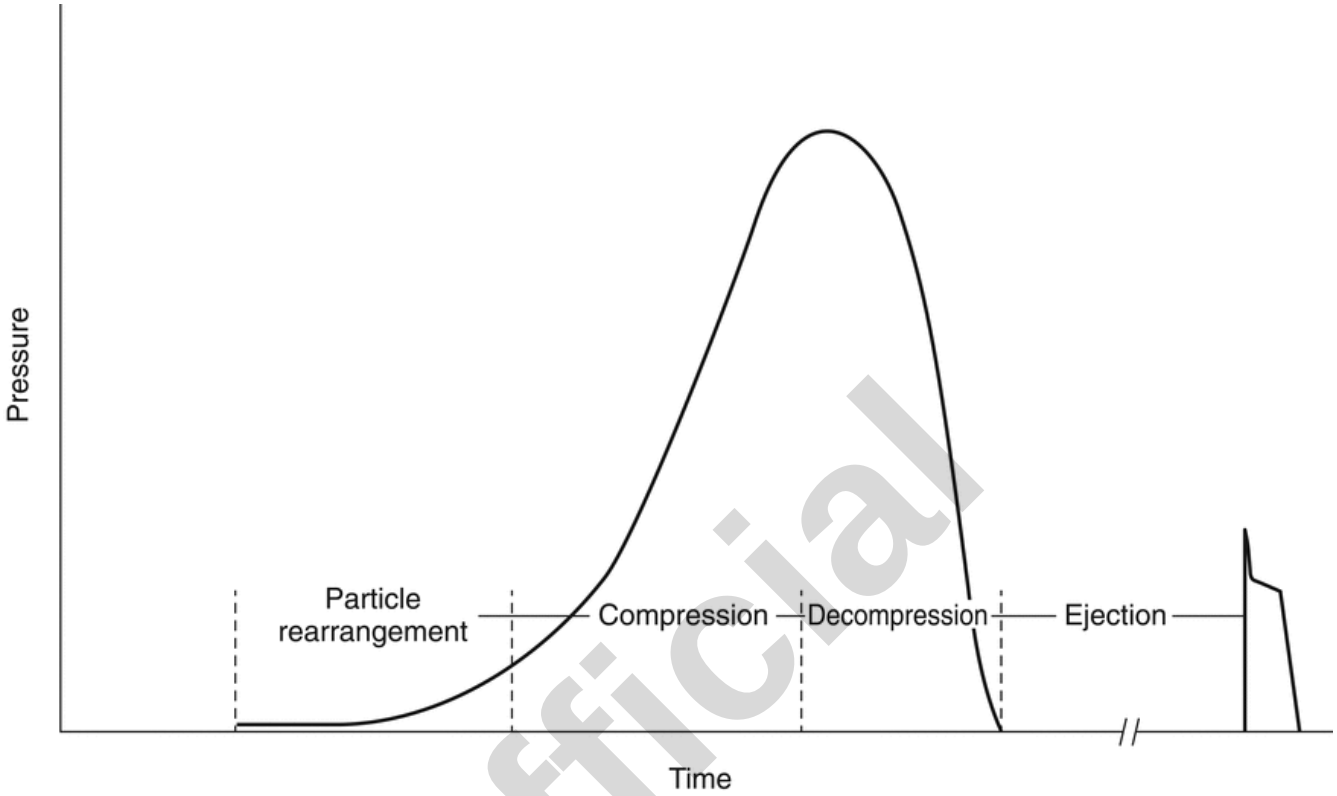


Figure 1. Stages of powder compression (lower punch pressure).

In the first stage, *Particle rearrangement*, particles typically change positions through slippage, rotation, or translational movement, thereby reducing or eliminating pores in the powder without significant irreversible deformation. At the end of the first stage, where the particles have reached their fullest extent of rearrangement, the powder is usually significantly denser than the starting powder because of reduced pore volume. Any further reduction in compact volume would require particle deformation.

During the second stage, *Compression*, particles are deformed at points of contact with other particles, the die wall, or the punch surfaces. During this stage, compression pressure often increases rapidly, causing volume reduction as the powder density increases. Under pressure, particles initially undergo elastic deformation. Depending on mechanical properties and stress at points of contact, particles can subsequently undergo varying degrees of fragmentation and/or plastic deformation. If fragmentation takes place early in the second stage, some of the fragments may undergo further rearrangement. However, relative particle movement is limited when the powder is highly consolidated. For most pharmaceutical materials, plastic deformation is an important part of the compression process that leads to an increase in the area of contact between particles, contributing to higher compact strength. Clean particle surfaces generated from fragmentation also contribute to higher compact strength. The deformation behavior and resultant tablet mechanical properties of many pharmaceutical powders are also sensitive to the compression speed (punch velocity) and the length of time at which the powder is held under pressure at a constant volume (dwell time). Tablet density and tensile strength of speed-sensitive powders (such as starch) depend on tableting speed, with higher speeds (i.e., shorter dwell times) generally producing less-dense tablets with lower strength. The end of the second stage is usually the time of highest compression pressure.

During the third stage, *Decompression*, the punches retract, resulting in a decreasing axial punch pressure. As axial pressure is reduced to zero during decompression, residual die wall pressure typically exists in the radial direction. During this phase, particles primarily undergo elastic recovery, depending on both the pressure and the mechanical properties of the particles. Elastic recovery may provide insight into the elastic deformation that the powder experienced during compression. Excessive elastic recovery may reduce the interparticle bonding and can result in a significant decrease in tablet mechanical strength. These same three stages apply to a pre-compression step in tablet manufacturing, which often involves a lower compression pressure and may be added as a precursor to the main compression step.

During the fourth stage, *Ejection*, the tablet is typically pushed out of the die by the lower punch. As the tablet emerges from the die, the ejected portion of the tablet is free to expand radially due to elastic recovery (i.e., release of residual die wall pressure). Significant shear stress may develop within the tablet and at the edges of the tablet–die interface because the lower portion of the tablet remains constrained by the die wall. In severe cases, this shear stress can result in tablet lamination or capping. The formation of a dense and defect-free compact depends on the ability of the particles to form interparticulate bonds during compression, and the ability of these bonds to withstand elastic expansion during the decompression and ejection phases. Tooling shape and size may also affect the properties of the compressed tablet because they affect density and stress distribution

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during compression. The thermodynamics of the compression process, e.g., via compression calorimetry (*14*,*15*), is another important aspect of powder compression, and it can be studied using instrumented presses (see *3. Tablet Compression Characterization Equipment*).

## 3. TABLET COMPRESSION CHARACTERIZATION EQUIPMENT

Various compression methods have been used to characterize the compression properties of pharmaceutical powders. Each method has benefits and limitations. To characterize powder-compression properties, a compression pressure (stress) is applied to a powder to produce a coherent, compacted specimen or tablet. The loading system used to apply the compression pressure may have several designs. Typical compression instruments used in the pharmaceutical industry include hydraulic presses, instrumented research tablet presses, tablet press emulators, compaction simulators, and instrumented production tablet presses.

### 3.1 Hydraulic Press

When a hydraulic press is used, typically only the peak hydraulic pressure value is easily accessible. Generally, compression and decompression speeds are not precisely controlled, and these processes occur over a period of seconds or more. Tablets produced on such devices may then be tested for strength and density, as well as other performance properties such as friability, disintegration, or dissolution. Such devices provide compression data for comparing materials and formulations but are not necessarily predictive of tableting performance during high-speed tableting.

### 3.2 Instrumented Research Tablet Press

Research tablet presses have two basic designs: eccentric single-station and rotary multi-station presses. Eccentric single-station presses typically are mechanically driven and compress the tablet using the upper punch with a sinusoidal position profile; the lower punch is typically fixed during the compression cycle. Research rotary multi-station tablet presses are scaled-down versions of production-scale presses, and they compress tablets via the movement of both the upper and lower punch as they pass under a pair of compression rolls. The geometry of the punch head (i.e., curvature and flat area) also influences the shape of the punch position profile on a rotary tablet press.

### 3.3 Tablet Press Emulator

Tablet press emulators are similar to rotary tablet presses in that they use compression rolls to move the punches together to form the tablet, and therefore punch displacement–time profiles (see *5. Punch Displacement–Time Profiles*) are highly representative of rotary machines. However, the tablet press cycle time is longer than that of rotary presses because the compression track is linear.

### 3.4 Compaction Simulator

Compaction simulators are single-station presses that utilize hydraulic or mechanical power to move the punches and are designed to match the displacement profile of a given high-speed press by means of a computer. Compaction simulators can also be programmed to simulate any punch position profile, which is useful for fundamental material characterization.

### 3.5 Instrumented Production Tablet Press

Instrumented production tablet presses also use a compression roll design to form a tablet. A large number of compression stations enables greater production throughput and/or multi-layer tablet or tablet-in-tablet capability.

## 4. TOOLING

Tablets can be manufactured with a variety of tooling sizes and shapes. Commonly used tooling shapes for basic material characterization are flat-faced, flat-faced with beveled edge, and standard round concave tooling that produces convex-shaped tablets. A wide variety of tooling shapes may be used in the production of pharmaceutical products, based on technical and commercial considerations (*16*). Tooling may be embossed (i.e., raised markings on punch surface), thus introducing debossed markings on the compressed tablets. Depending on the powder compression properties and manufacturing process parameters, the tablet tooling design may affect the tablet mechanical integrity.

## 5. PUNCH DISPLACEMENT–TIME PROFILES

Tablet compression equipment can be used to produce a variety of punch displacement–time profiles. *Figure 2* is an illustration of three of the simplest profiles used for fundamental material characterization.

The properties of a compressed powder are dependent upon several factors, including compression pressure, compression speed, and the compression profile. Compression experiments may be performed with one moving punch and one stationary punch; one moving upper punch and a fixed base (i.e., no lower punch); or two independently moving upper and lower

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punches (double-sided compression). Because the compression set-up and parameters can affect measured compact properties, it is important to specify the experimental details when reporting results. Typical compression profiles include:

- Linear compression and decompression phases that yield saw-tooth punch displacement–time profiles (*Figure 2A*)
- Square punch displacement–time profiles (*Figure 2B*)
- Modified sinusoidal punch displacement–time profiles typical of a rotary tablet press (*Figure 2C*)

Either one punch (single-sided compression) or both punches (double-sided compression) may follow these profiles. Accurate measurements of the displacement–time profiles, as well as the resultant forces applied, require high-accuracy instrumentation, and in some cases correction for system deformation (i.e., elastic deformation of punches and other machine components). Punch displacement–time profiles for most production-scale tableting machines are a combination of the sinusoidal and square profiles, where the compression and decompression phases follow the sinusoidal profile and between them there is a flat portion representing the dwell time.

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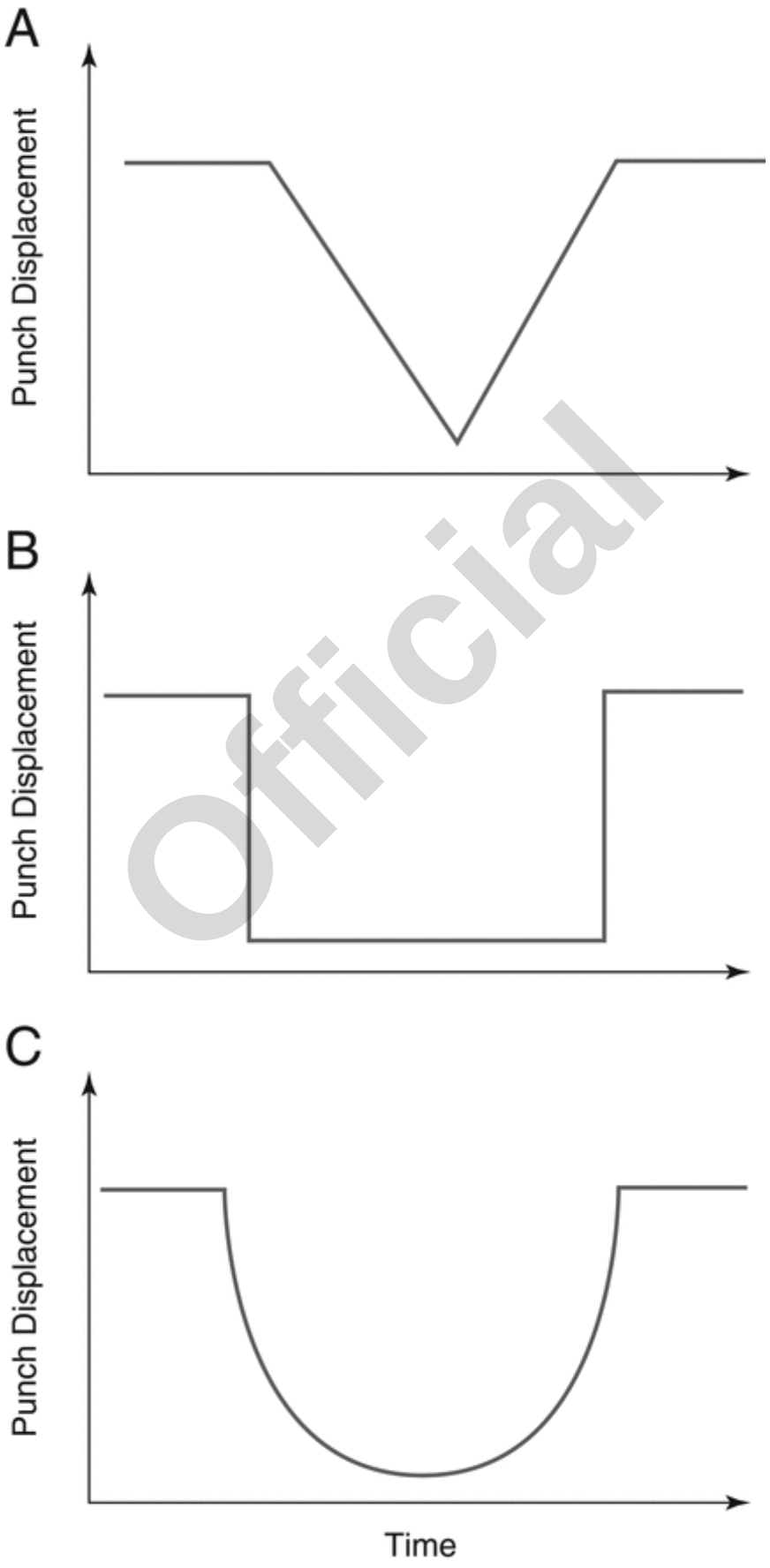


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Figure 2. Punch displacement–time profiles: A) saw-tooth profile, B) square profile, and C) modified sinusoidal profile. Only

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one curve for punch displacement over time is represented (e.g., upper-punch movement).

## 6. TABLET MECHANICAL STRENGTH

Tablet strength is primarily influenced by the particle–particle bond strength and true areas of contact (e.g., surface area over which attractive force between particles is significant) (*17*). When particle surfaces are brought into close proximity, interparticle interactions (e.g., van der Waals forces) are maximized and typically lead to strong interparticle bonding. Tablet mechanical strength can be quantified by measuring the maximum stress, either compressive or tensile, that a tablet can sustain prior to failure (breaking). A commonly used test is to place tablets between two platens and measure the force necessary to fracture the tablets; this test is described in *Tablet Breaking Force* ⟨1217⟩. For conventional round tablets with a circular cross-section, loading occurs across the diameter of the tablet and is sometimes referred to as "diametral loading". Other methods, such as three-point or four-point bending tests, are also available but are less frequently used in production settings because they can require more complex equipment and analysis. Tablet strength, as determined by these tests, is often referred to in the pharmaceutical industry as "hardness", although a more exact term is "breaking force". In material science, hardness refers to the resistance of a surface to penetration or indentation (e.g., Mohs hardness, indentation hardness, or permanent deformation pressure). The tablet breaking force value serves as both a criterion by which to guide product development and a quality control specification.

Tablet strength can be affected by several factors including:

- Tablet size and shape: Because breaking force is affected by tablet size and shape, a more reliable parameter for quantifying mechanical strength of a tablet is tensile strength. For cylindrical or convex tablets with simple shapes, tensile strength may be calculated from the diametral test, described in ⟨1217⟩.
- Relative density: Tablet strength increases as powders are compressed to a higher relative density.
- Time and storage conditions: Tablets can relax or be influenced by environmental conditions (e.g., relative humidity), therefore tablet storage conditions and length of storage time before testing should be specified for reproducible strength determinations.
- Formulation composition and manufacturing process: Each component has unique mechanical properties, and in some cases these properties can impact the mechanical properties of other components. For example, incorporation of a lubricant, which is meant to reduce adherence to manufacturing equipment, can reduce interparticulate bonds. Problems in manufacturing processes, such as over-mixing or over-granulation, also can influence tablet strength.

Tablet performance attributes, such as disintegration, dissolution, and friability, may be affected by tablet compression and may also be reflected in the tablet mechanical strength. Typically, a tablet of lower strength will have faster disintegration and dissolution as well as higher friability (see *Tablet Friability* ⟨1216⟩).

## 7. TABLET POROSITY AND SOLID FRACTION

Virtually all pharmaceutical compacts contain porous regions (pores). Tablet porosity is a measure of the volume of the tablet that consists of pores, or "void space". It is critically important to consider tablet porosity when quantitatively characterizing tableting properties, because it has a substantial effect on measured compact properties. Tablet solid fraction, also referred to as relative density, is a measure of the volume of solid material in a compact and may be calculated using *Equation 1*. Tablet solid fraction and porosity are related, as shown in *Equation 2*. The true density of a material is the average mass/unit volume (e.g., g/cm$^3$) exclusive of all voids (see *Density of Solids* ⟨699⟩). Typical true densities of organic powders of pharmaceutical interest are in the range of 1.0–1.7 g/cm$^3$, whereas inorganic ingredients may be in the range of 2.0–3.0 g/cm$^3$ (*18*,*19*).

$$Solid\ Fraction = \frac{(density\ of\ the\ tablet)}{(true\ density\ of\ material)} = \frac{\frac{(mass\ of\ tablet)}{(volume\ of\ tablet)}}{(true\ density\ of\ material)} \quad (1)$$

$$Porosity = 1 - Solid\ fraction \quad (2)$$

Simple tablet geometries (e.g., round flat-faced) are often used for research purposes, to simplify the determination of tablet volume by using the measurement of tablet dimensions. For more complex tablet shapes, alternative methods of determining tablet volume, such as the use of instruments that quantify envelope volume, may be used. Typical pharmaceutical tablets have porosities between 0.1 and 0.4, depending on the material properties and the conditions used to produce the tablet (*20*). A porosity of 0 would correspond to a theoretical tablet mass in which all pores had been eliminated, resulting in a compact consisting entirely of solid material (i.e., solid fraction = 1). With increasing compression pressure, pores are eliminated through particle rearrangement and deformation, and the tablet porosity decreases unless extensive elastic recovery of the tablet after decompression causes cracks or other defects in the tablet.

## 8. MANUFACTURABILITY PROFILE

Tablet breaking force is often measured as a function of compression force. A plot of breaking force versus compression force is useful for monitoring changes in the tableting behavior of a powder with a fixed tablet size, shape, and weight produced under similar compression conditions (i.e., production speed and force), such as those obtained using a specific rotary tablet press. In this case, the relationship between tablet breaking force and compression force may be termed "manufacturability", because it is often the criterion used in a production setting to monitor tablet compression. See *Figure 3* for an example of a manufacturability profile.

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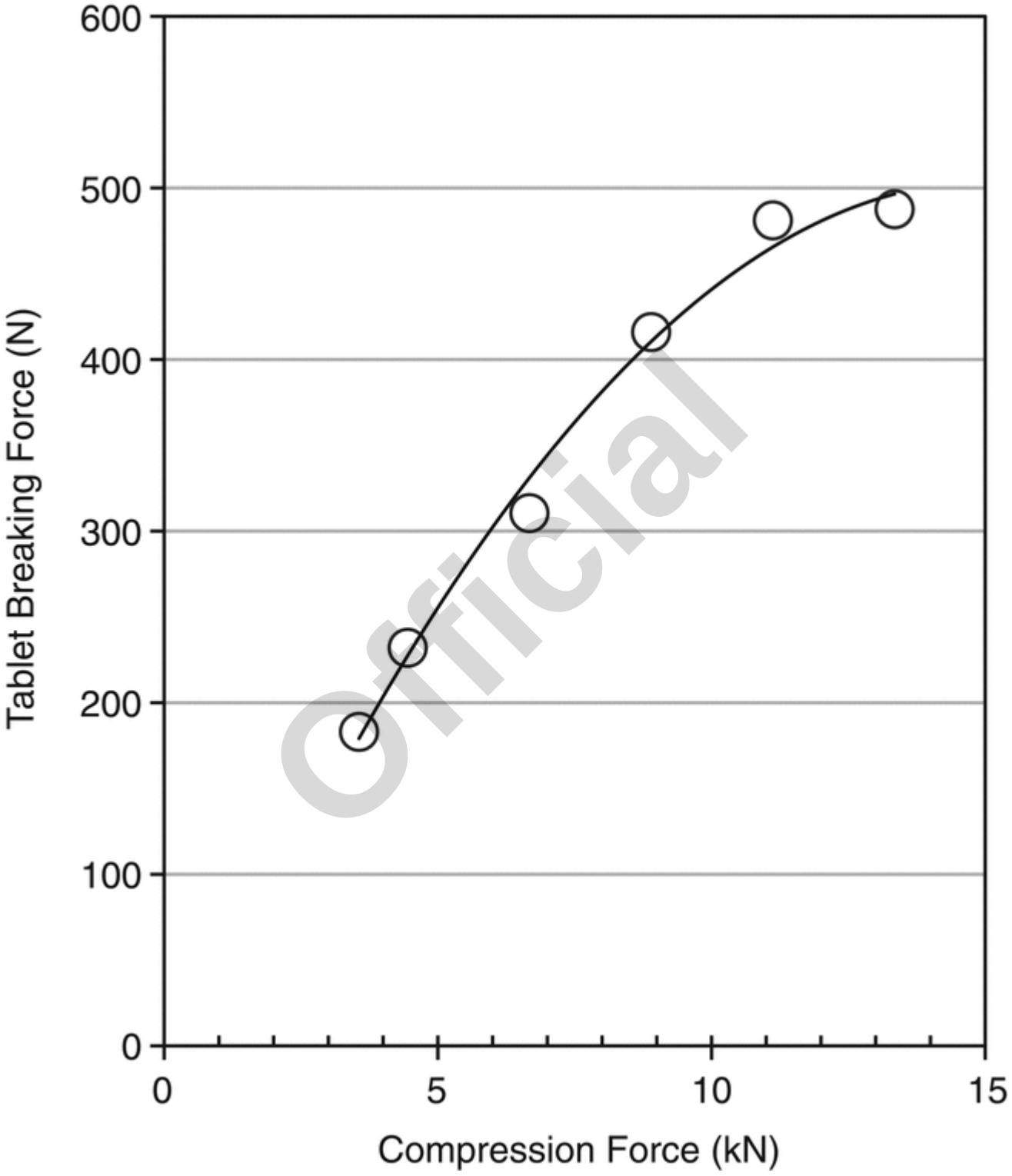


Figure 3. Example of a manufacturability profile.

## 9. TABLETABILITY PROFILE

Although valuable in a manufacturing setting, tablet breaking force should be replaced with tablet tensile strength for quantifying tablet mechanical strength (see ⟨1217⟩), and compression force should be replaced with compression pressure (compression force per unit area of the punch tip cross-section). These changes will minimize the impact of tablet size, thickness, and weight on compression data analysis. The relationship between tablet tensile strength and compression pressure is termed

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"tabletability". Tablet tensile strength usually increases initially with increasing compression pressure. Depending on tablet composition, tensile strength can either continue to increase or gradually level off at higher pressures. It is also possible that the tablet tensile strength may decrease with increasing pressure, a phenomenon known as overcompression. This decrease in tablet strength with increasing pressure is most often the result of tablet defects of some materials that occur at higher compression pressures. Because of the diversity of powder tabletability behaviors, it is beneficial to determine the tensile strength of tablets prepared under a range of compression pressures, instead of a single pressure, if possible. This will help obtain an accurate characterization of powder tabletability. When the available resources or materials are limited, the compression pressure required to make a compact at a specified tensile strength (e.g., 1 MPa) can be used to compare compression properties of different powders. The tabletability of pharmaceutical materials can often be described by *Equation 3* for a typical range of compression pressures, where *K* and *B* are empirical constants:

$$\log(\textit{tensile strength}) = K \log(\textit{compression pressure}) + B \quad (3)$$

See *Figure 4* for an example of a tabletability profile.

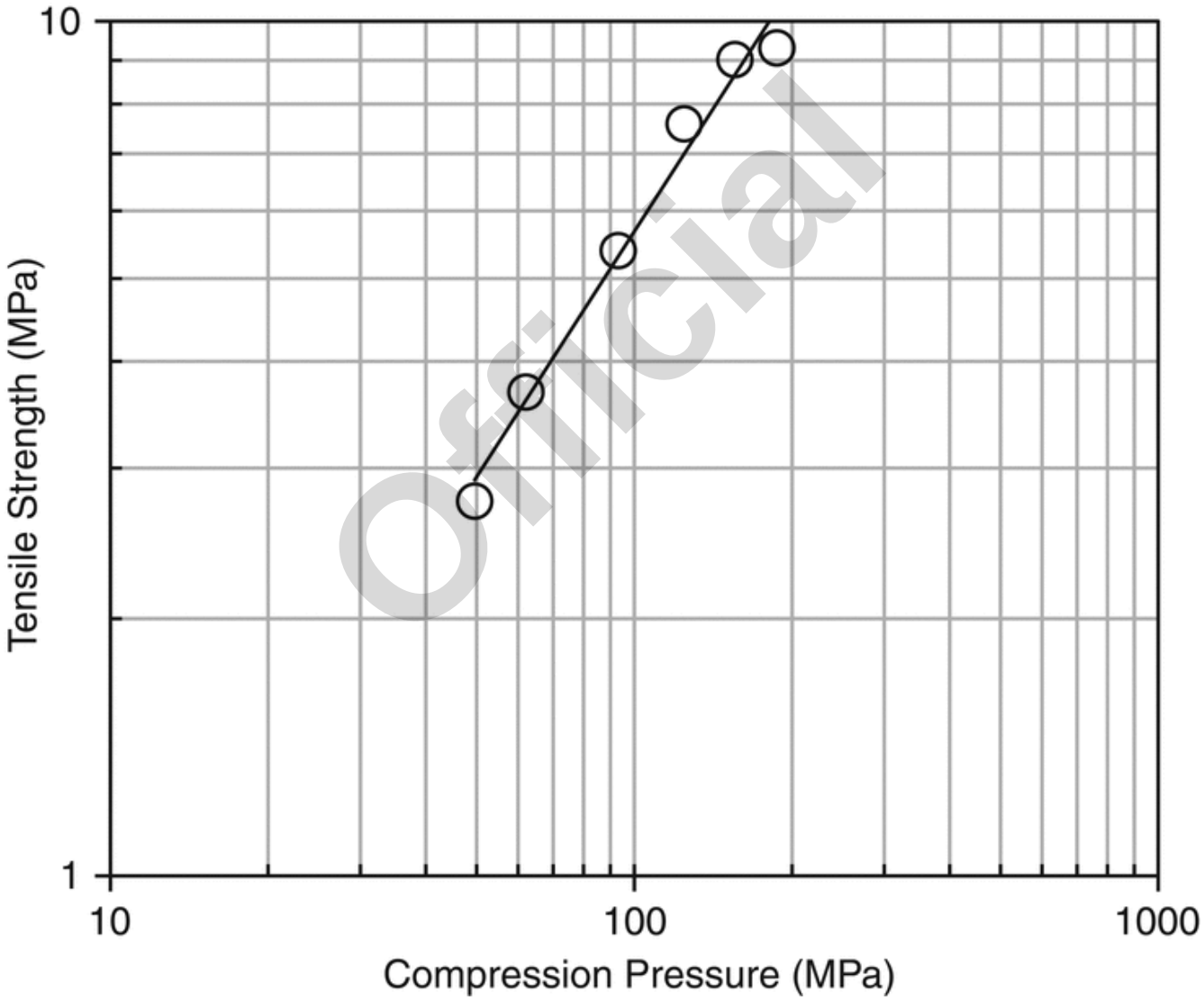


Figure 4. Example of a tabletability profile.

## 10. COMPRESSIBILITY PROFILE

Compressibility is the dependence of tablet solid fraction (or porosity) on compression pressure. The compressibility curve can be obtained by plotting tablet solid fraction as a function of compression pressure. See *Figure 5* for an example of a compressibility profile.

For many pharmaceutical materials, *Equation 4* can be used to describe compressibility over a typical range of tablet solid fractions, where *a* and *b* are empirical constants:

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$$\log(\textit{compression pressure}) = a \times (\textit{solid fraction}) + b \quad (4)$$

The compression pressure necessary to form a compact with a specified solid fraction (e.g., 0.85) may be used to compare diverse pharmaceutical materials. Use of 0.85 as a reference solid fraction is convenient because many, although not all, pharmaceutical powders can be compressed to this solid fraction, and a reference solid fraction enables comparative assessments of tablet property measurements. Alternative values for the reference solid fraction may be used as needed.

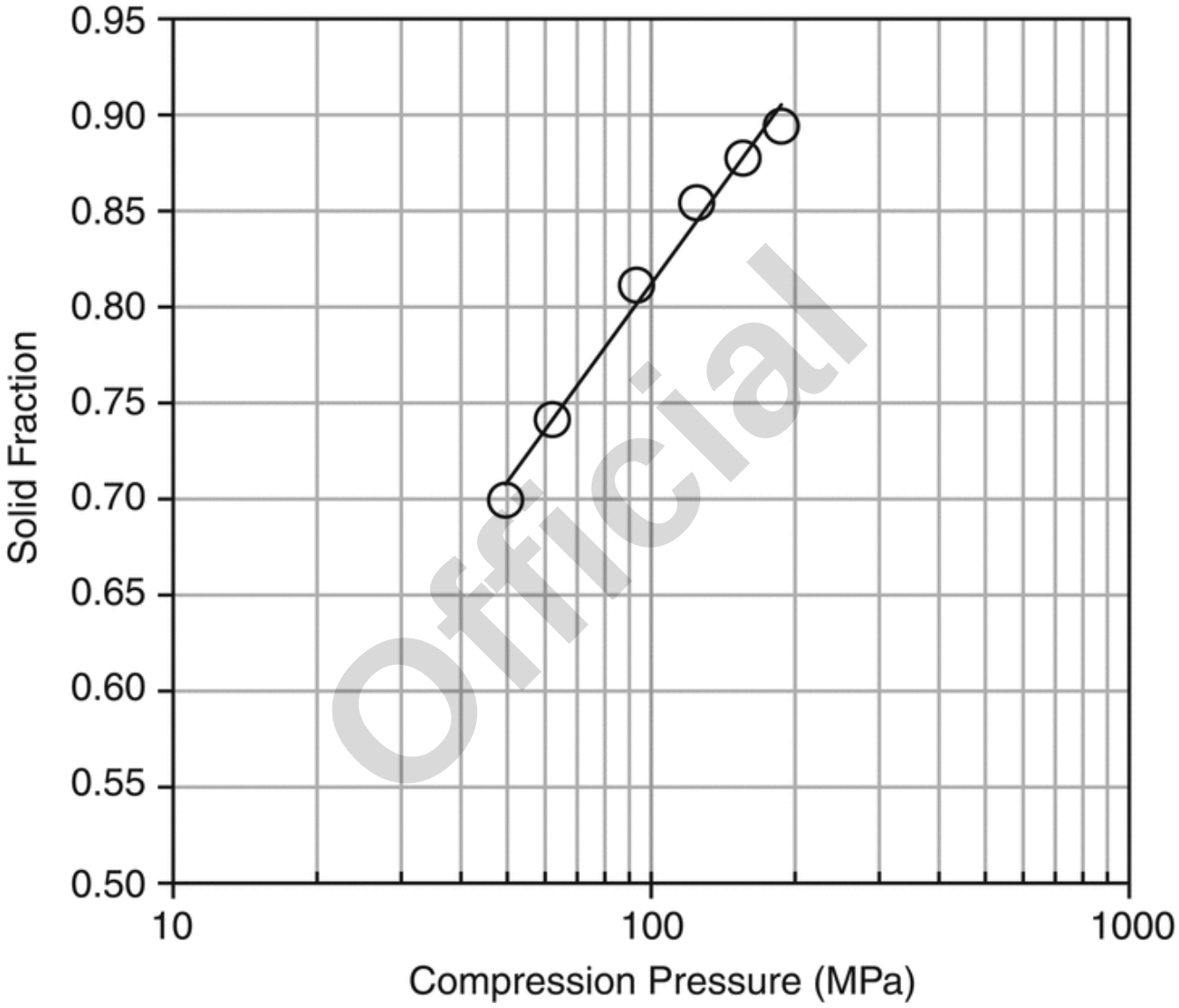


Figure 5. Example of a compressibility profile.

Compressibility has also been described using the Heckel equation (*Equation 5*), which predicts a linear dependence of the logarithm of tablet porosity versus compression pressure. In *Equation 5*, *K* and *B* are empirical constants.

$$-\ln(\textit{porosity}) = K \times (\textit{compression pressure}) + B \quad (5)$$

However, the Heckel equation is overly simplistic in that it does not adequately describe powder compressibility in the low-pressure region, where ln(*porosity*) versus pressure data are not linear. More sophisticated models, such as the modified Heckel equation (*21*) and the Drucker-Prager Cap Model (*22*), have proven to be more reliable in describing powder compressibility to account for the transition between the state of a powder and the state of a tablet.

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## 11. COMPACTIBILITY PROFILE

The relationship between tensile strength and solid fraction (or porosity) is termed “compactibility”. Generally, tablet tensile strength increases exponentially with increasing solid fraction, and powder compactibility is often well described by the Ryshkewitch-Duckworth equation (*Equation 6*) where *k* and *A* are empirical constants (*23*):

$$\log(\textit{tensile strength}) = k \times (\textit{solid fraction}) + A \quad (6)$$

This relationship is qualitatively reasonable, as the presence of more or larger pores in a compact weakens it. Moreover, this relationship highlights the importance of determining tablet porosity to gain a better understanding of powder tableting performance. For example, when a low tablet tensile strength is associated with high porosity (solid fraction), an effective strategy for overcoming the tableting problem is to add a more highly deformable excipient, which increases the plasticity of the powder and decreases tablet porosity. See *Figure 6* for an example of a compactibility profile.

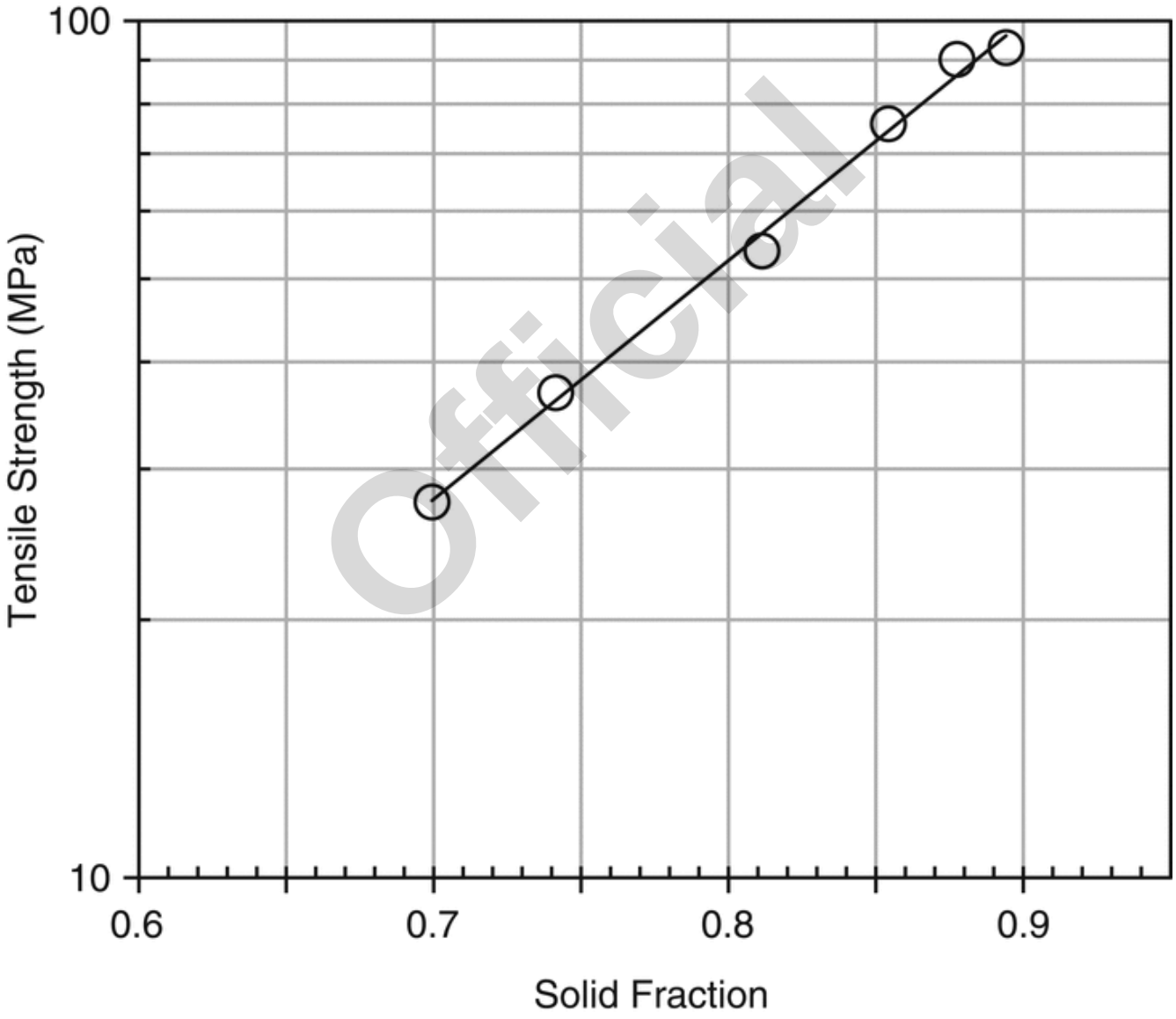


Figure 6. Example of a compactibility profile.

## 12. TABLET COMPRESSION PROFILE

*Figure 7* illustrates the relationships among tensile strength, compression pressure, and solid fraction (or porosity) and related tableting parameters. The relationships among tensile strength, compression pressure, and solid fraction (or porosity) can be presented in three dimensions as shown in *Figure 8*, where the three faces of the three-dimensional plot represent the tabletability, compressibility, and compactibility (*24*).

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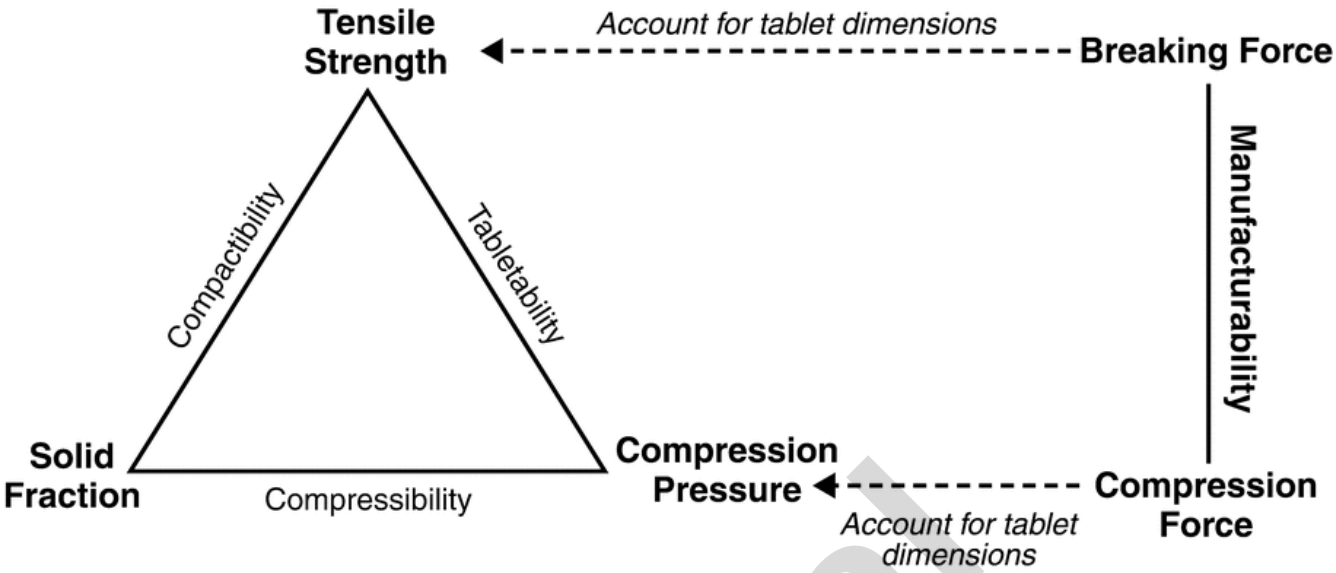


Figure 7. Relationships among tableting parameters for compression data analysis.

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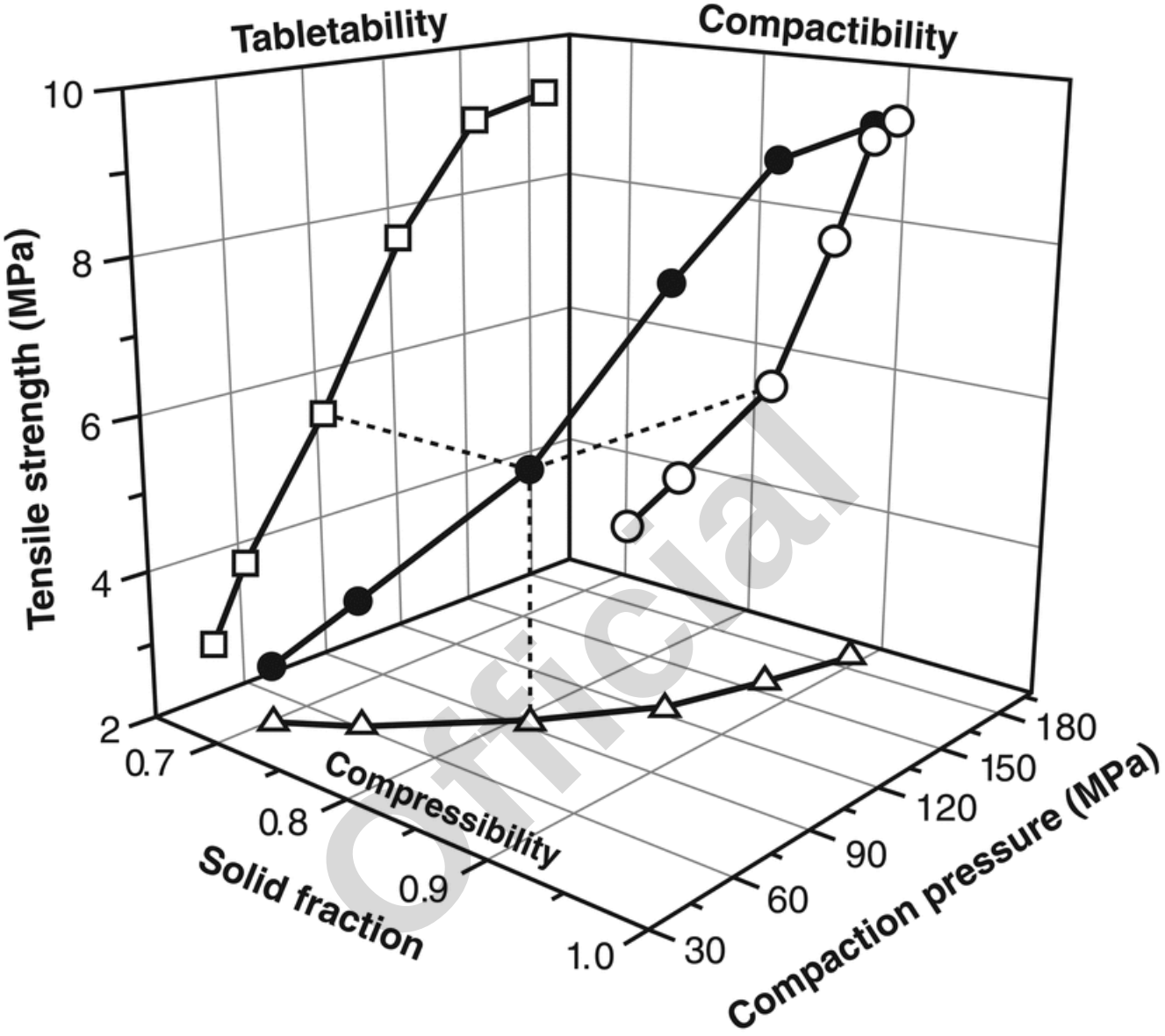


Figure 8. Three-dimensional compression profile.

## 13. MACHINE SPEED SENSITIVITY

The effect of compression speed may be assessed by comparing the properties of compacts that were prepared at different compression speeds. A range of compression speeds can be obtained by using a hydraulic press, a rotary tablet press, or a compaction simulator. For fundamental material assessments, tablet properties such as tensile strength, hardness, and porosity can be used to quantify strain rate sensitivity (*SRS*) of a powder using *Equation 7*.

$$SRS = \frac{\left|property_{(SR2)} - property_{(SR1)}\right|}{property_{(SR1)}} \quad (7)$$

*SR1* = low strain rates (e.g., low compression speed)
*SR2* = high strain rates (e.g., high compression speed)

## 14. CONCLUSIONS AND RECOMMENDATIONS

Tablet compression is a complex process dependent on the drug product composition, material properties, manufacturing process parameters, environment conditions, equipment, and tooling design. To gain an in-depth understanding of the

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compression behavior of a powder, it is beneficial to have knowledge of the particle size and shape, solid form, and water content of the powder. Without appropriate control and descriptions of these factors, the data obtained are not meaningful for accurately characterizing tablet compression properties of a powder. Therefore, these properties should always be considered during the analysis of the tablet compression results. *Table 1* shows examples of parameters that are commonly used in the characterization of the compression properties of pharmaceutical solids.

**Table 1. Important Parameters to Specify When Characterizing Powder Compression Properties**

| Experimental Parameter | Parameter Value(s) Used | Example Parameters in Common Use[a] |
|---|---|---|
| Tooling type | Specify | Round flat-faced; standard round concave |
| Tooling size | Specify | 8 mm, 10 mm, 13 mm |
| Compression speed | Specify | Tablets/minute, 0.03 mm/s, 300 mm/s |
| Punch displacement–time profile | Specify | Saw tooth, square, sinusoidal; single-sided, double-sided |
| Compression pressure range | Specify | 25–300 MPa |
| Solid fraction range | Specify | 0.6–0.95 |
| Tablet properties (weight, dimensions) | Specify | Tablet thickness, tablet diameter |
| Powder equilibration | Specify | 20°, 40% relative humidity (RH) |
| Lubrication | Specify | Type; external die; internal, % |
| Tablet storage (time, temperature, and RH) | Specify | None; 24 h at 20°, 40% RH |
| Tablet press configuration | Specify | With or without pre-compression setting |
| **Data Analysis** | | |
| Compressibility | Specify [NOTE—If possible, the entire curve is preferred.] | Compression pressure ($\sigma_C$) at specified solid fraction (SF) |
| Compactibility | | Tensile strength ($\sigma_X$) at specified SF |
| Tabletability | | $\sigma_X$ at specified $\sigma_C$, or $\sigma_C$ at specified $\sigma_X$ |
| Ejection force | | Ejection force at specified $\sigma_C$ |
| *SRS* | Specify | *SRS* = (P2 – P1)/P2, P2 = mean yield pressure at 300 mm/s; P1 = mean yield pressure at 0.033 mm/s, saw-tooth punch displacement–time profile |

[a] These examples are not required and they may not be suitable for all materials. They should not be viewed as prescribed values for characterizing powders.

## GLOSSARY

**Breaking force:** The force [in Newtons (N)] required to cause tablet mechanical failure. Often referred to as tablet hardness (see ⟨1217⟩).

**Brittleness:** The property that leads to particle or compact fracture, typically very rapidly.

**Capping:** Laminar splitting along the edge of the crown or band of a compressed tablet.

**Compactibility:** The ability of a powder to form an intact compact with measurable strength.

**Compactibility profile:** Change in tensile strength of a compressed body with solid fraction (or porosity).

**Compaction:** The transformation of a powder into an intact compact with measurable strength and defined shape by the application of compression pressure. Usually used synonymously with consolidation.

**Compaction emulator:** A device that physically approximates tablet press configurations where compression parameters may be applied; parameters may include pre-compression and compression roll dimensions, tableting speed, ejection angle, and punch design.

**Compaction simulator:** A device that permits powder compression, typically using a hydraulic source to control displacement of punches that may be designed to match the force displacement profile of a high-speed press by means of a computer.

**Compressibility:** The ability of a powder to be compressed (reduced in volume) by the application of stress.

**Compressibility profile:** Change in solid fraction (or porosity) of a compressed body with applied pressure.

**Compression:** The reduction in volume of a powder bed due to the application of a stress, e.g., loading.

**Compression force:** Force applied to compress a powder bed. The unit kN is commonly used in tableting.

**Compression pressure:** Pressure (force/area, in MPa) applied to specimen material; sometimes used interchangeably with the terms "compaction pressure" or "compression stress". See *Compaction* and *Compression*.

**Compression profile:** The relationship between *Compression pressure*, *Solid fraction* (or porosity), and tensile strength.

**Dwell time:** Duration of time (in ms) that the compression roll is in contact with the flat portion of the punch head. Often used to describe rotary compression processes with a modified sinusoidal punch displacement–time profile.

**Elastic deformation:** The change in shape of a stressed body that is completely recovered when stress is released. This is time-independent, recoverable deformation.

**Elastic limit:** The amount of stress at which a material deviates from linear elastic behavior, i.e., the smallest stress that leaves a detectable permanent deformation when unloaded.

**Failure:** The permanent collapse, breaking, or deforming of the material.

**Force:** A push or pull (in N) resulting from the interactions between two objects.

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**Hardness:** See *Breaking force*.

**Hydraulic press:** A device that uses liquid pressure to enable the application of force to a specimen.

**Indention hardness:** The resistance of a surface to permanent deformation (indentation) when subjected to pressure by a hard object.

**Lamination:** Condition in which a tablet splits or separates into layers.

**Manufacturability profile:** Change in breaking strength of a compressed body with applied force.

**Mechanical properties:** The characteristics of a material upon application of a stress. Examples include tensile strength, yield strength, plasticity, brittleness, hardness, elastic modulus, and bendability.

**Plastic deformation:** The permanent change in shape of a solid body, without fracture, resulting from the application of sustained stress beyond the elastic limit. Deformation occurs without a change in particle volume.

**Plasticity:** See *Plastic deformation*.

**Pressure:** Force applied to a unit area (in MPa), used interchangeably in this chapter with stress.

**Porosity or void fraction:** A measure of the empty spaces in a material. This is the fraction of the voids divided by the total volume. Porosity ranges between 0 and 1, or as a percentage between 0% and 100%.

**Shear stress:** The force per unit area (in MPa) acting along a plane through a body.

**Solid fraction:** The apparent density divided by the absolute density of the solid, sometimes referred to as relative density. Solid fraction = (1 − porosity).

**Sticking:** The adherence of material to the faces of tablet press punches or dies after compression.

**Stress:** Normal stress. Used interchangeably in this chapter with pressure. See *Pressure*.

**Tabletability profile:** Change in tensile strength of a compressed body with applied pressure.

**Tablet press:** A mechanical device that compresses powder into tablets of desired size and weight.

**True density:** The average mass per unit volume, exclusive of all voids that are not a fundamental part of the molecular packing arrangement.

**Viscoelastic deformation:** Time-dependent partially recoverable deformation.

**Yield strength:** The stress needed to produce a specified amount of plastic deformation (usually a 0.2% change in length).

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# ⟨1063⟩ SHEAR CELL METHODOLOGY FOR POWDER FLOW TESTING

1. INTRODUCTION
    1.1 Scope
2. THEORY AND PRINCIPLES
3. DESCRIPTION OF SHEAR CELL COMPONENTS AND DESIGNS
4. SHEAR CELL MEASUREMENTS
    4.1 Sample Preparation
    4.2 Instrument Preparation
5. SELECTION OF TEST CONDITIONS
6. TEST PROCEDURE
7. DATA ANALYSIS AND CALCULATIONS
8. REFERENCE MATERIALS AND REPRODUCIBILITY

## 1. INTRODUCTION

A large number of pharmaceutical processes involve powder transfer and feeding, and the ability of powders to flow in a controlled manner during these operations is critical to product quality. For example, drug product attributes such as weight and content uniformity depend on powder flow characteristics. Shear cells are among the most important methods for measuring powder flow properties, and the data from shear cell testing can be used to predict a wide variety of powder flow behaviors during pharmaceutical manufacturing. Shear cells have many advantages over simpler methods of measuring powder flow (see *Powder Flow* ⟨1174⟩), but their operation is more complex and the procedures for their use must be carefully controlled to produce accurate and reproducible data. This chapter describes best practices for obtaining reliable and accurate powder flow data using a shear cell.

### 1.1 Scope

This chapter focuses on the three most popular shear cell types used for measuring powder flow properties:

1. Translational (Jenike type)
2. Annular (Schulze type)
3. Rotational (Peschl type)

These three shear cell types are categorized as direct shear tests in which a region of the powder is sheared under a series of controlled normal stresses. From these data, a wide variety of parameters can be obtained, including the yield locus representing the shear-stress to normal-stress relationship at incipient flow, the angle of internal friction, the unconfined yield strength, powder cohesion, and a variety of related parameters such as the flow function. In addition, these three shear cells can be set up with wall coupons (see *Appendix*) to measure the powder wall friction. When the shear cell data are combined, they can be used for bin and hopper evaluation and design. Other testing approaches, such as triaxial testers and indirect or hybrid testers (e.g., the Johanson indicizer), are outside the scope of this chapter.

## 2. THEORY AND PRINCIPLES

The flow behavior of a powder is fundamentally different from the flow of a fluid. First, powder flow properties and shear behaviors are strongly dependent on the consolidation stresses applied to the powder and are minimally dependent on the strain or flow rate (under the assumption of quasi-static conditions such as flow in a bin). Fluid flow, in contrast, is strongly dependent on the strain rate (where viscosity describes the relationship between shear stresses and strain rates) and is minimally dependent on absolute pressure. Second, when shear stresses are applied to powders, they may not immediately fail (i.e., they can avoid flow under a sustained shear stress), whereas Newtonian and viscoelastic fluids do not behave this way and always flow under an applied shear stress. Thus, powders have the potential for arching and rat-holing, depending on the flow pattern (see *Appendix*).

However, powders can sustain a shear stress without flowing only up to a certain point. The yield locus for a given powder is a function of many variables, including its composition, particle size and shape, moisture content, temperature, time stored at rest, and the state of consolidation. Once a powder is subjected to stresses (whether by gravity or some mechanical means) that reach or exceed the yield locus, the powder flows. Hence, determining the yield locus for a given powder under conditions representative of its manufacturing process is an essential step in evaluating the flow behaviors for that process. In some circumstances, this may involve testing under controlled environmental conditions, as well as holding the powder under load for an extended period before shearing (a “time test”).

Because powder properties are highly dependent upon the degree of consolidation, the preparation of a uniform powder bed (consistent bulk density throughout the powder bed) is the first critical step of shear cell testing. The next stage of testing is the application of a normal stress ($\sigma$) and shear stress ($\tau$) to the powder bed to achieve steady-state shear, resulting in a known state of consolidation. The shear stress then is removed, and a reduced normal stress is applied. A shear stress then is applied and is progressively increased until the powder bed yields and begins to flow. This procedure is repeated at several different normal stress conditions to create a “yield locus” plot. To complete a full flow function analysis, the operator must determine several yield loci, which requires that the unconfined yield strength be determined under several different levels of consolidation.

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Although this chapter focuses on the powder (particle–particle) properties, the wall friction (particle–wall) properties and bulk density are also important. Such properties are used for bin design and also are essential when one compares different wall materials (e.g., different grades and finishes of stainless steel, or the effect of plastic coatings on powder flow behaviors). The most fundamental property of a wall material in this regard is $\Phi'$, the angle of friction between the bulk powder and the wall material, or, correspondingly, the coefficient of wall friction ($\mu_w$):

$$\mu_w = \tan(\Phi') = \tau_w/\sigma_w$$

$\tau_w$ = wall shear stress
$\sigma_w$ = wall normal stress

Note that $\Phi'$, and hence $\mu_w$, often are a function of the applied normal stress ($\sigma_w$).

## 3. DESCRIPTION OF SHEAR CELL COMPONENTS AND DESIGNS

*Figures 1*, *2*, and *3* provide schematics of the different types of shear cells considered in this general chapter. All have the same general operating principle of being able to measure the force required to shear a powder bed to which a normal load has been applied. The applied load or measured force can be expressed as a stress by dividing by the cross-sectional area of the shear plane under consideration.

The translational shear cell (*Figure 1*) has a fixed base with a movable ring above it, and both hold the bulk powder. A cover that fits within the ring is used to contain the powder and to provide a uniform application of the normal load (*N*). The ring and cover are pushed as a unit while a load cell records the shear forces (*F*) that are generated. The shear plane forms between the powder that is contained in the base and the powder contained in the ring.

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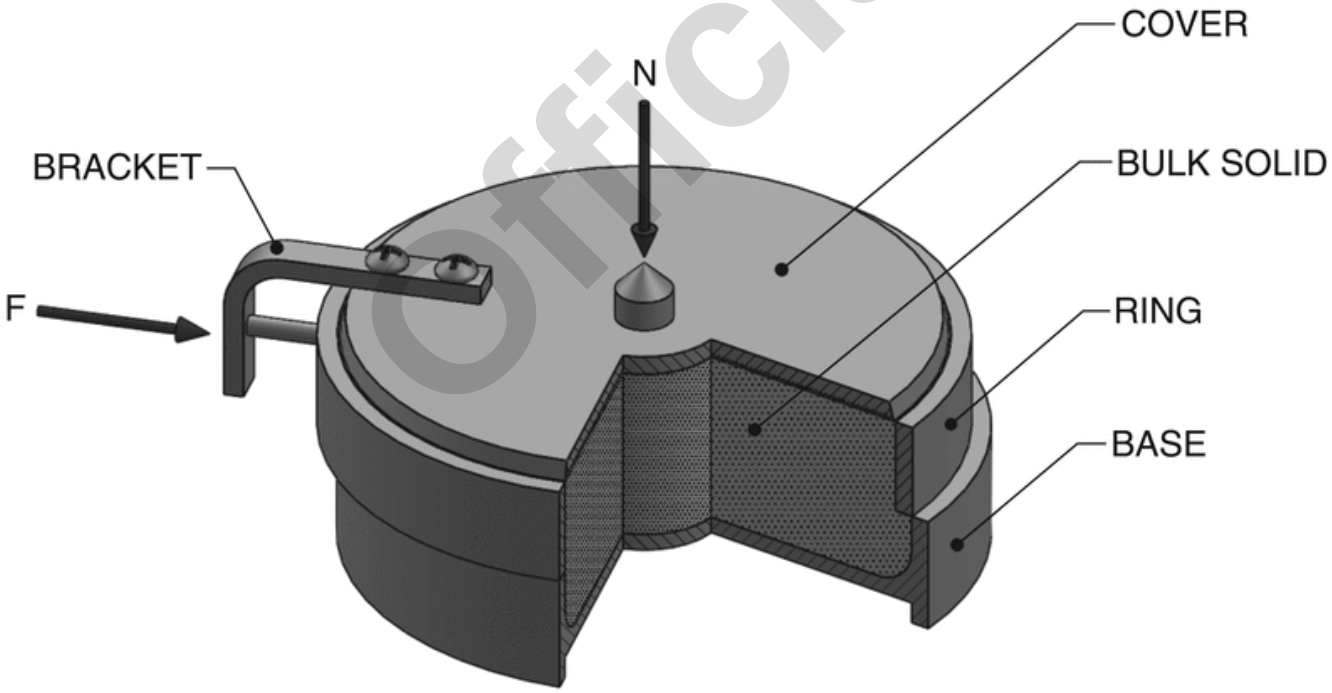


Figure 1. Descriptive schematic of the translational shear cell.

The annular shear cell (*Figure 2*) consists of a shear cell or base that holds the powder. A cover that fits within the cell is used to contain the powder and to provide a uniform application of the normal load (*N*). The cover is free to move up and down, but otherwise remains fixed in place by a load cell that measures the shear forces (*F*) that are generated. The shear cell is rotated at a constant angular velocity ($\omega$) to create a shear plane that forms in the powder bed somewhere between the cell bottom and the cover. The cover and shear cell usually have baffles or other surface features that prevent the powder from sliding or shearing at the interface between the powder and the cover or shear cell base.

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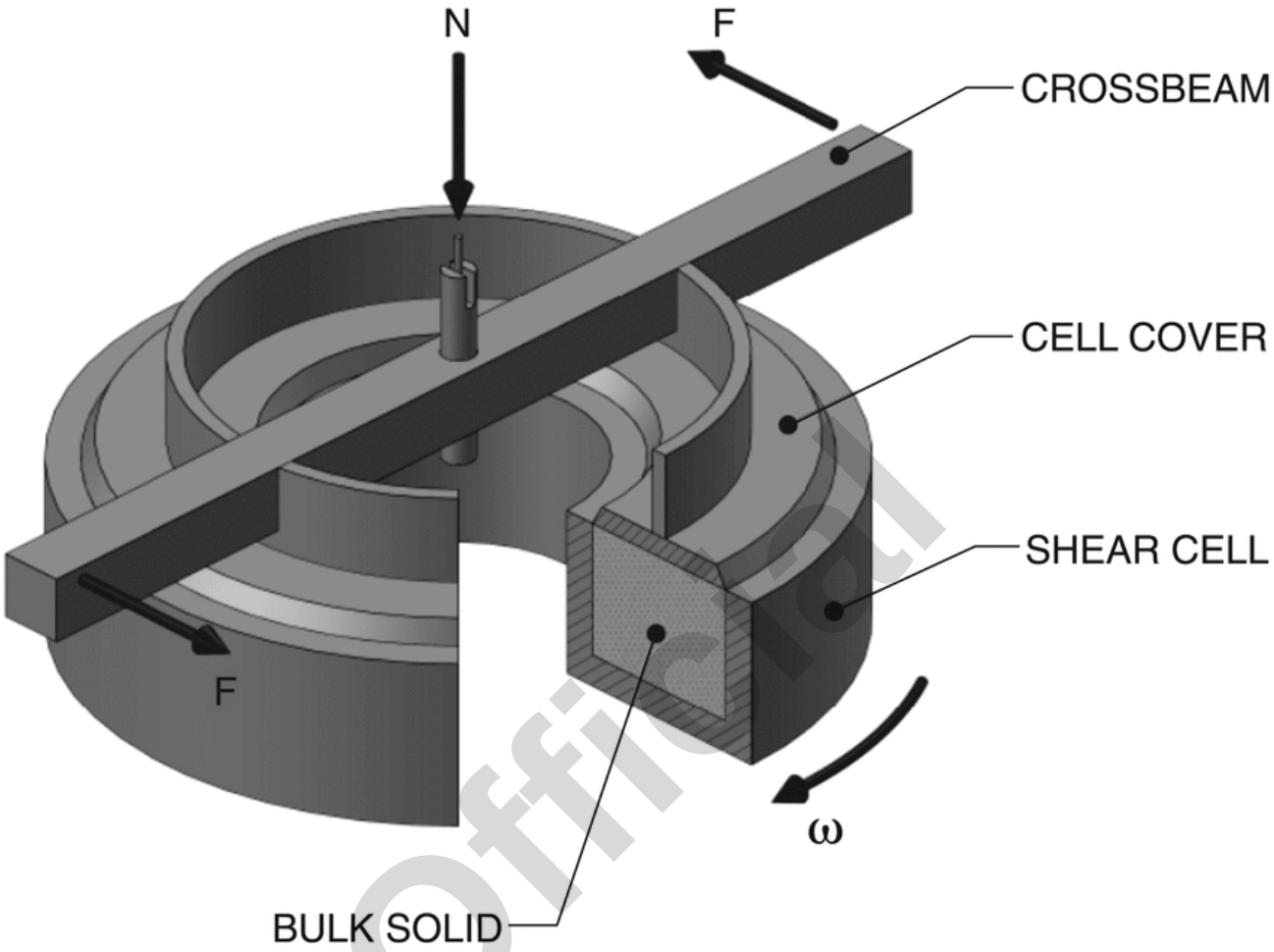


Figure 2. Descriptive schematic of the annular shear cell.

The rotational shear cell (*Figure 3*) has a base and ring that hold the powder. A loading lid that fits within the ring is used to contain the material and to provide a uniform application of the normal load (*N*). The loading lid is free to move up and down, but otherwise remains fixed in place, connected to a load cell that measures the shear forces (*F*) that are generated. The shear cell base then is rotated at a constant angular velocity ($\omega$) to create a shear plane that forms in the powder bed somewhere between the ring and the base. Alternatively, the base can be fixed, and the lid can be rotated to create the shear plane in the powder specimen. The ring and base usually have surface features that prevent the powder from sliding at the powder-surface interface.

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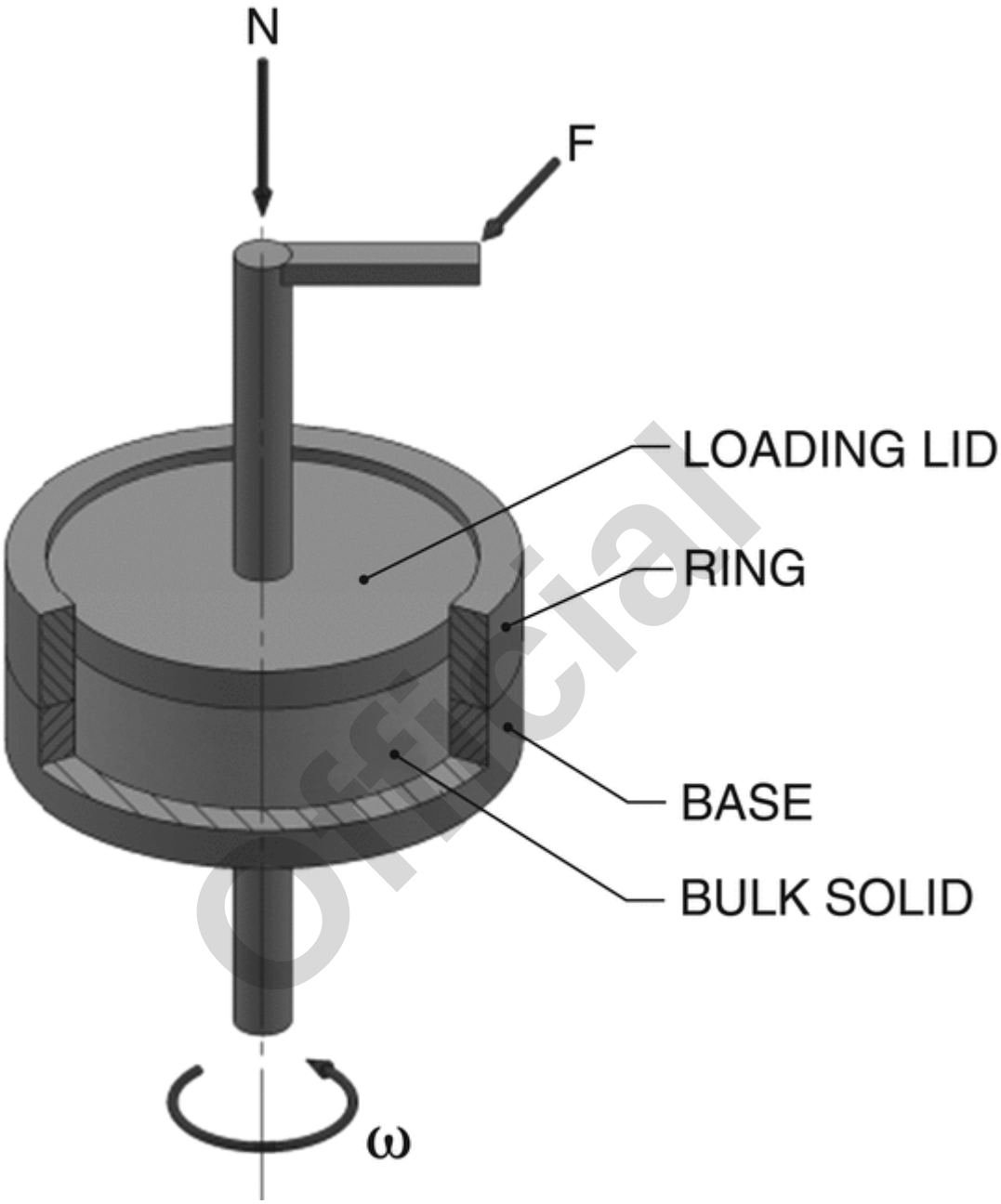


Figure 3. Descriptive schematic of the rotational shear cell.

The choice of one shear cell type over another depends on factors such as the availability of equipment and ease of use for a particular technician. Because of the infinite travel permitted with the annular and rotational shear cell geometry, these types of cells are more suited for powders for which reaching steady state requires large strains. Another advantage of the infinite travel of rotational testers is that a preconsolidation step may not always be required.

Wall friction tests can be carried out using all of the shear cells referenced in this chapter with only minor modifications, namely by creating a shear plane in which the powder slides over a suitable wall coupon rather than having the shear plane within the powder bed. This usually is achieved by replacing the base of the shear cell with a coupon of the wall material and adjusting the amount of powder used to create a shear plane at the powder–coupon interface.

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# 4. SHEAR CELL MEASUREMENTS

## 4.1 Sample Preparation

The applicability of shear cell data depends strongly on the use of a representative powder sample. Detailed discussion of appropriate sampling procedures can be found in *Bulk Powder Sampling Procedures* ⟨1097⟩.

The cell size (volume and diameter) usually is selected based on the maximum particle size of the powder sample, and the proportion of large particles in the sample. The particle size limit is usually reported by the supplier of commercially available testing equipment. For a translational cell, the maximum particle size is approximately 5% of the diameter of the cell, and for an annular cell the maximum particle size is approximately 10% of the width of the annulus (i.e., the difference between the outer and inner radius of the shear cell trough). Fresh powder is desired for each test, if possible, so the use of a smaller cell may be preferable to reusing the powder. Under some circumstances, a smaller cell may be needed to reach higher consolidation stresses. Smaller cell sizes may have reduced precision and increased bias, depending on the specific tester and the cell size under consideration, because of the larger contribution of non-idealities caused by wall effects.

The shear strength of some powders (e.g., fibrous or flaky solids) is often caused by interlocking of particles; therefore, such materials may prove to be unsuitable for testing with a conventional shear cell. Likewise, powders with high springback (rubber-like or highly elastic properties) sometimes can fall outside of the practical limits of a shear tester. Poor reproducibility and unexpected results may indicate a problem with testing these types of powders.

Powder samples must be handled and tested under conditions that are relevant from a practical standpoint. For example, many pharmaceutical powders will sorb/desorb moisture from their surroundings according to the ambient relative humidity and temperature. This sorbed moisture can significantly affect the measured powder flow properties. Controlling the environmental conditions during sample handling and testing so that sample behavior is representative of the processing conditions of interest is essential for most powders.

Some pharmaceutical powders “age” quickly after they are produced, and such powders may need to be tested immediately after they are manufactured. The testing of some powders may result in caking or particle attrition that can render a powder unsuitable for retesting. In these circumstances, fresh powder samples should be used for each test.

## 4.2 Instrument Preparation

The shear cell should be situated in an area that is free of vibrations. Vibrations can affect the instrument readings and also can densify or dilate the powder while it is being tested.

Because shear testers measure forces, their load cells must be calibrated for the forces being measured. Some testers also measure the displacement (linear or angular) of the cell (indicating travel) and/or the vertical displacement of the cover or lid (used to calculate the volume of the cell and hence an average bulk density). In these cases, the displacement transducers must also be calibrated. The rate of any changes in force and displacement will need to be confirmed as part of this calibration. In addition, the shear cell is operated under the assumption that all of the parts are precisely aligned and there is a minimal degree of wobble when the cell rotates. For example, if the cover and shear cell trough are not parallel during testing, it will be difficult to get reproducible results. Thus, the alignment and mechanical operation of the shear cell must be evaluated at regular intervals. No other adjustments with respect to calibration are performed, although the correct performance of the apparatus may be confirmed by the measurement of the yield locus of a reference powder under standardized test conditions.

Wall coupons used for testing must be representative of the surfaces upon which the powder will slide. Directional surfaces can be oriented in the direction of flow in the application (e.g., grain oriented down the length of a hopper), oriented in the worst-case condition (often, grain oriented perpendicular to the direction of flow in the hopper), or evaluated in both directions.

All shear cell components (including wall coupons) should be carefully cleaned before use with a method that does not abrade the surface or leave any chemical residue behind. In addition, the cleaning method should be capable of removing all of the components of the test sample, including lubricants and other additives.

# 5. SELECTION OF TEST CONDITIONS

Preshear normal stresses usually are selected based on the powder’s density values. Standards, such as ASTM Standard 6128, provide tables of specific initial preshear normal stresses as a function of the sample bulk density.[1] Subsequent preshear normal stresses are given in multiples of the initial preshear value (e.g., 2, 4, and 8 times the initial level). The number of preshear normal stress levels should be at least 4. It is often valuable to match preshear stresses in the test to the stresses expected in the processing situation of interest. Hold times for time tests are selected based on matching the hold time in the practical application. If long hold times are unrealistic, operators may consider several intermediate time points and extrapolation of the data.

Normal stress levels for shear are selected to provide a range of data points on the yield locus. Typically, a range between 25% and 80% of the preshear normal stress is valid, although powders with high unconfined yield strength or internal friction may require a narrower range. The range of normal shear stress levels should be sufficient to allow meaningful fitting and extrapolation of the data to determine the unconfined yield stress and other related parameters.

[1] ASTM. D6128-06. Standard test method for shear testing of bulk solids using the Jenike shear cell. West Conshohocken, PA: ASTM; 2006.

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## 6. TEST PROCEDURE

Shear cell testing involves a sequence of steps that consolidates the powder to a known extent and then shears it under carefully controlled conditions while recording the applied normal stresses and measuring the shear stresses. In most cases, the essential steps are as follows:

1. Fill the test cell with an appropriate and representative sample of the powder in a manner that provides a uniform bulk density and composition. This sample in the test cell is referred to as the "specimen". Detailed procedures vary according to the type of tester being used, but in all cases it is essential to evenly distribute the powder and avoid pockets of air that can be difficult to remove later.
2. For translational shear cells, perform a preconsolidation step to create the desired density in the cell. This is accomplished by twisting the cover while under a compressive normal load. This step reduces the travel needed to achieve a steady-state preshear value and can limit the total vertical displacement of the cover during the test. A preconsolidation step may be conducted in other cell types, but this is often not conducted, because the unlimited rotation of the annular and rotational cells can generally provide a sufficient state of consolidation. The appropriate level of preconsolidation is critical, and care must be taken not to over- or under-consolidate the specimen.
3. Consolidate the specimen by applying a known normal stress to the powder specimen via the cell cover/lid.
4. Preshear the specimen until a steady-state shear value is reached. Care should be taken to avoid over-consolidating the specimen. The applied shear stress is then reduced to zero.
5. An instantaneous shear test is run by shearing the specimen under a reduced normal stress (with respect to the applied preshear stress) until the shear stress goes through a maximum value and then begins to decrease.

Steps 1–5 are repeated at a series of different reduced normal stress conditions to create a complete set of data (yield locus) and then at a series of different preshear normal stress conditions to create a "flow function". The test cell is preferably emptied and refilled before generating each point on the yield locus. For annular or rotational shear cell types, it is common that steps 3–5 are repeated a number of times for the same specimen without emptying and refilling the cell. If the same specimen is used for multiple test points, caution must be taken to ensure that the specimen has not changed from test to test.

A wall friction test is run in an analogous manner by sliding the specimen over a coupon of wall material and measuring the shear stress as a function of the applied normal stress. In a time test, a normal stress is applied to the specimen for a predetermined period of time before shearing. Both wall-friction and time-consolidation tests can be conducted with the three types of shear cells described in this chapter, as long as directionality (if any) of the wall surface, relative to the cell movement, is taken into account.

## 7. DATA ANALYSIS AND CALCULATIONS

Illustrative results from the shear cell test procedure are presented in *Figure 4*. For analysis, the applied preshear normal stress ($\sigma_p$) and all valid applied normal stress points ($\sigma_{si}$) for $I$ = 1, 2, 3,…, $n$ and their corresponding measured shear stress points[2] ($\tau_p$, $\tau_{s1}$, $\tau_{s2}$,…, $\tau_{sn}$) are plotted in ($\sigma$, $\tau$)-coordinates. These data are then used to generate a smooth line through all the valid shear points to obtain the yield locus. Typically, the yield locus passes above or through the preshear point. If not, the test results should be analyzed in more detail. In some circumstances, the yield locus can be forced to pass through the preshear point and can be fitted to all yield points.

The "unconfined yield strength" of the powder is obtained by drawing a Mohr circle through the origin and tangential to the yield locus. The higher point of intersection of this Mohr circle and the $\sigma$-axis is the unconfined yield strength $f_c$. The "major consolidation stress", $\sigma_1$, is found by drawing a second Mohr circle through the preshear point and tangential to the yield locus. The higher point of intersection of this Mohr circle (consolidation locus) and the $\sigma$-axis is the major consolidation stress.

[2] For a given normal stress applied, the measured steady-state shear stress values should be consistent from test to test. To account for normal experimental variations, a given measured shear stress value may be prorated in an appropriate fashion, relative to the steady-state value associated with that point.

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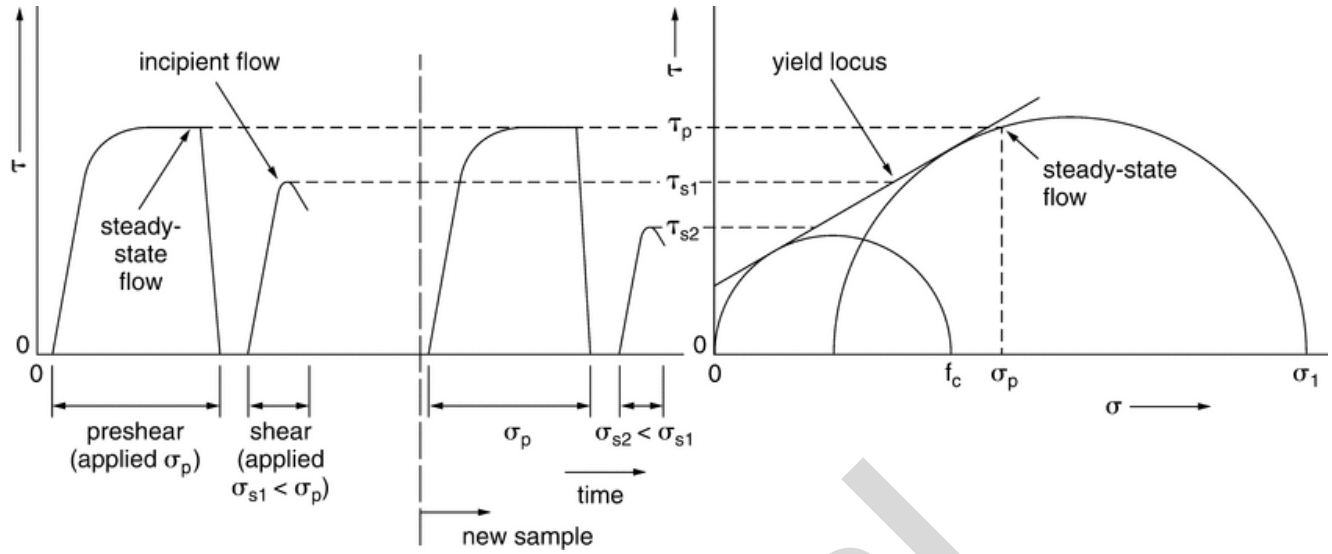


Figure 4. Shear cell testing and data analysis: shear cell raw data (*left*) and yield locus calculated from raw data (*right*).

The "angle of internal friction", $\Phi$, is defined as the slope of the yield locus, as shown in *Figure 5*. A line drawn through the origin and tangential to the steady-state Mohr circle has an angle, $\delta$, that is defined as the "effective angle of friction". The "cohesion," $C$, is the intersection of the yield locus and the $\tau$-axis.

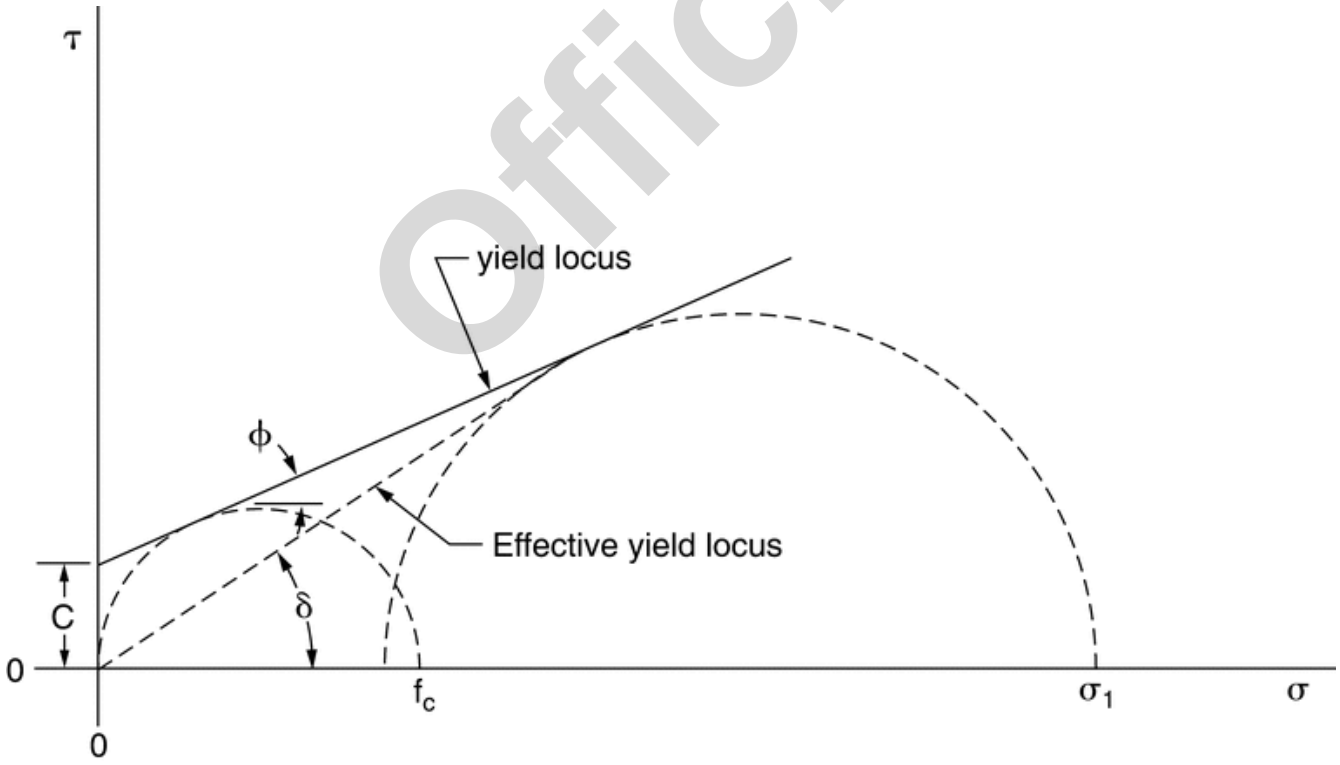


Figure 5. Graphical representation of the angle of internal friction ($\Phi$), effective angle of friction ($\delta$), and cohesion ($C$).

From a family of yield loci generated at different preshear normal stresses, it is possible to plot the unconfined yield strength, $f_c$, as a function of the major consolidation stress, $\sigma_1$, as shown in *Figure 6*. The best-fit curve to these points is called the "flow function" and can be used to calculate the potential for arching and rat-holing in a storage bin.

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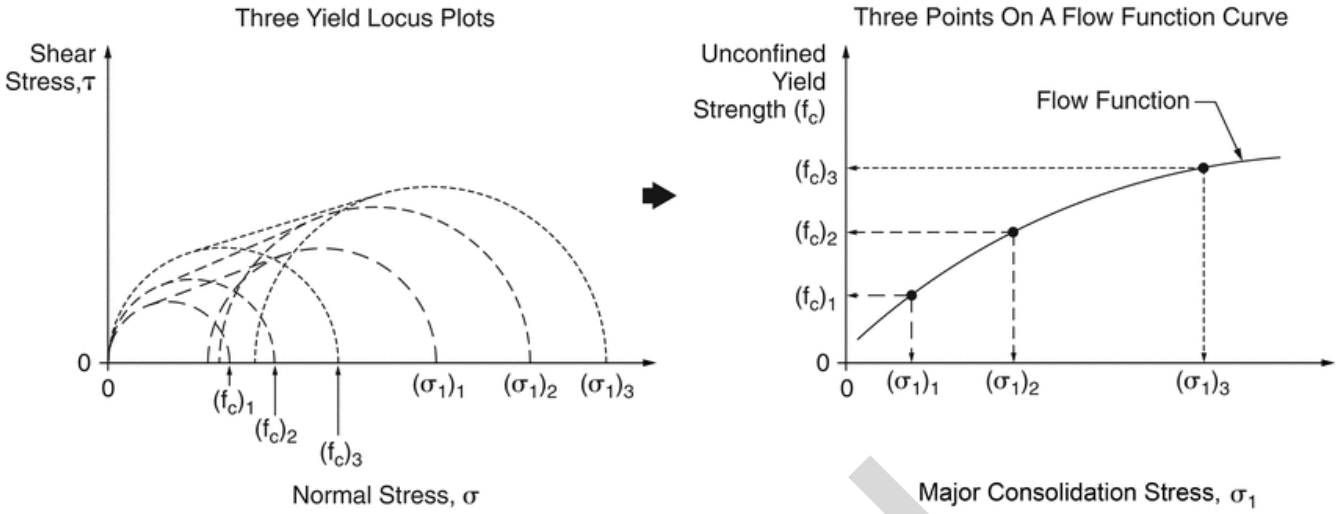


Figure 6. Calculation of the flow function from multiple yield locus plots.

The "kinematic wall yield locus" is developed in a manner similar to the calculation of the yield locus. This is depicted in *Figure 7*. The "wall friction angle", $\Phi'$, is defined as the inclination of a line from the origin to a point on the wall yield locus. In general, low wall friction angles are desirable.

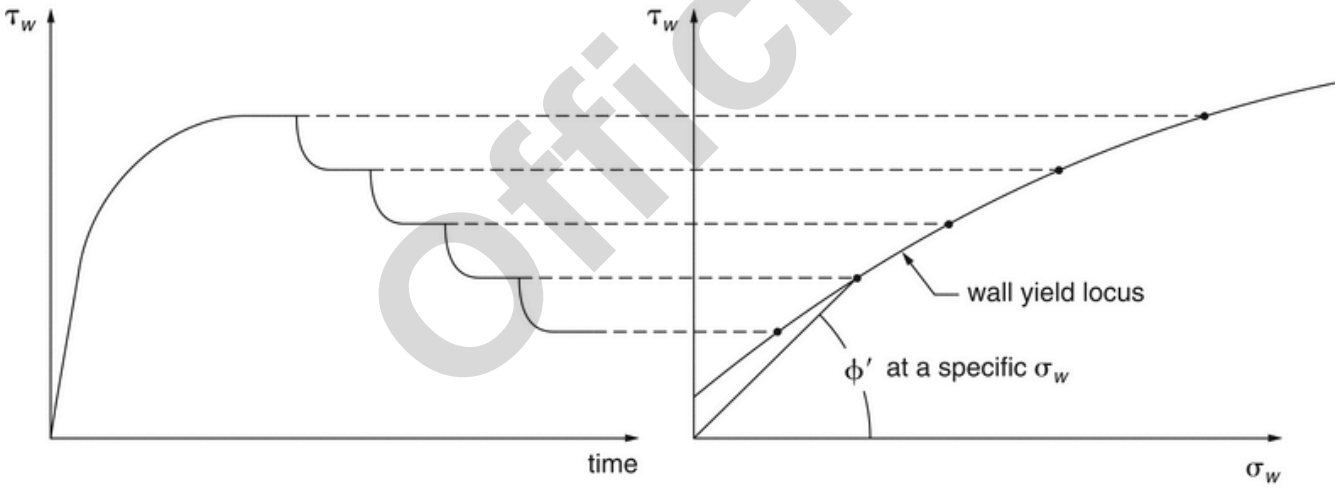


Figure 7. Calculation of the wall yield locus.

Test conditions should be carefully documented for every shear cell analysis to allow for accurate data interpretation and meaningful data comparisons. When analysts compare multiple powders, matching the tester type, specific tester model, test cell size, sample preparation, test procedures, applied normal stresses, and environmental conditions provides increased confidence that any differences in the results are attributable to the powder and not to the test. Changes in material sourcing or manufacturing may require the repetition of the initial studies.

Shear cell tests allow the analyst to characterize consolidated powders under quasi-static conditions. The tests are unable to directly measure powder flow properties at very low stress conditions or high shear rates, which may occur in some powder-handling situations. Thus, no single parameter can describe powder flowability. Instead, the parameters described in this chapter should be interpreted together and in the context of the conditions under which the powder will be stored and handled (e.g., equipment dimensions, environmental conditions, and others).

The ratio of the major consolidation stress, $\sigma_1$, to the unconfined yield strength, $f_c$, at a particular value of the major consolidation stress provides a general, simplified way of assessing powder flowability. As a general case, the flow of powdered materials is classified in *Table 1*.

**Table 1. General Classification of Flow Character[a]**

| $\sigma_1/f_c$ | Flow Character |
|---|---|
| <2 | Very cohesive, nonflowing |
| 2–4 | Cohesive |

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**Table 1. General Classification of Flow Character[a]** *(continued)*

| $\sigma_1/f_c$ | Flow Character |
|---|---|
| 4–10 | Easy-flowing |
| >10 | Free-flowing |

[a] Jenike A. Storage and flow of solids: bulletin no. 123 of the Utah Engineering Experiment Station. Salt Lake City, UT: University of Utah; 1964.

This approach can be useful for rank ordering the flow character of pharmaceutical powders as part of formulation and process development activities. However, it should be noted that this simplified view may lead to significant errors in interpretation because this approach does not take into account the flow pattern or the size or geometry of the manufacturing equipment being used.

## 8. REFERENCE MATERIALS AND REPRODUCIBILITY

For a tester that is installed and operated correctly, the performance of the instrument and operator can be assessed by testing a standard material. There is currently no specific pharmaceutical reference material for shear cell test method verification. Limestone powder has been used as a certified reference material.[3]

*Figures 8*, *9*, *10*, *11*, *12*, and *13* show representative yield loci and flow function plots for several common pharmaceutical excipient powders determined at a range of conditions. The plots are provided to illustrate the type of data that can be generated for pharmaceutical powders using a shear cell and the typical reproducibility of the data. The data in *Figures 8*, *9*, *10*, and *11* were collected using a procedure consistent with that described for *Figure 4*. These plots are not intended for use as reference data, and they should not be assumed to be representative of all test conditions and material types.

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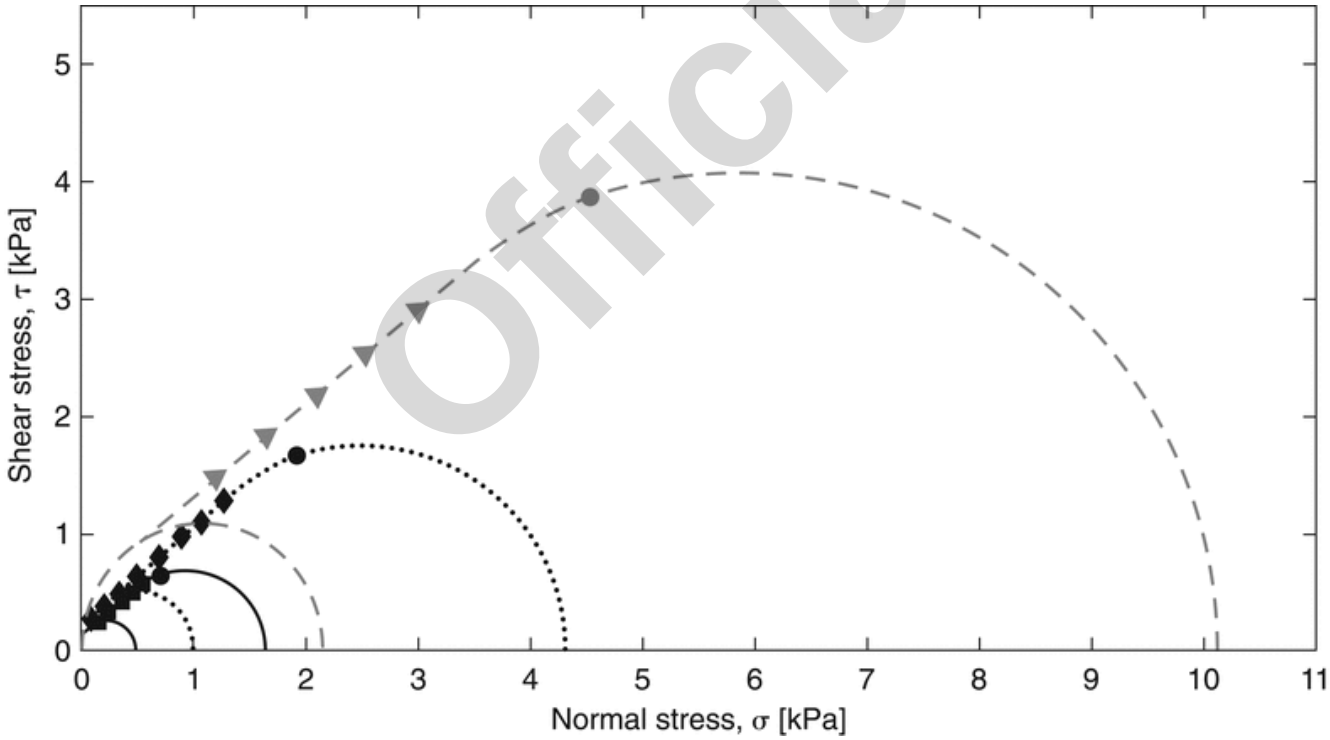


Figure 8. Representative yield loci for microcrystalline cellulose NF (Avicel PH-101) at preshear stresses of 730 Pa (*solid*), 1.9 kPa (*dot*), and 4.6 kPa (*dash*).

[3] Limestone powder certified reference material (BCR-116) is available from the Institute for Reference Materials and Measurements of the European Commission and instrument suppliers.

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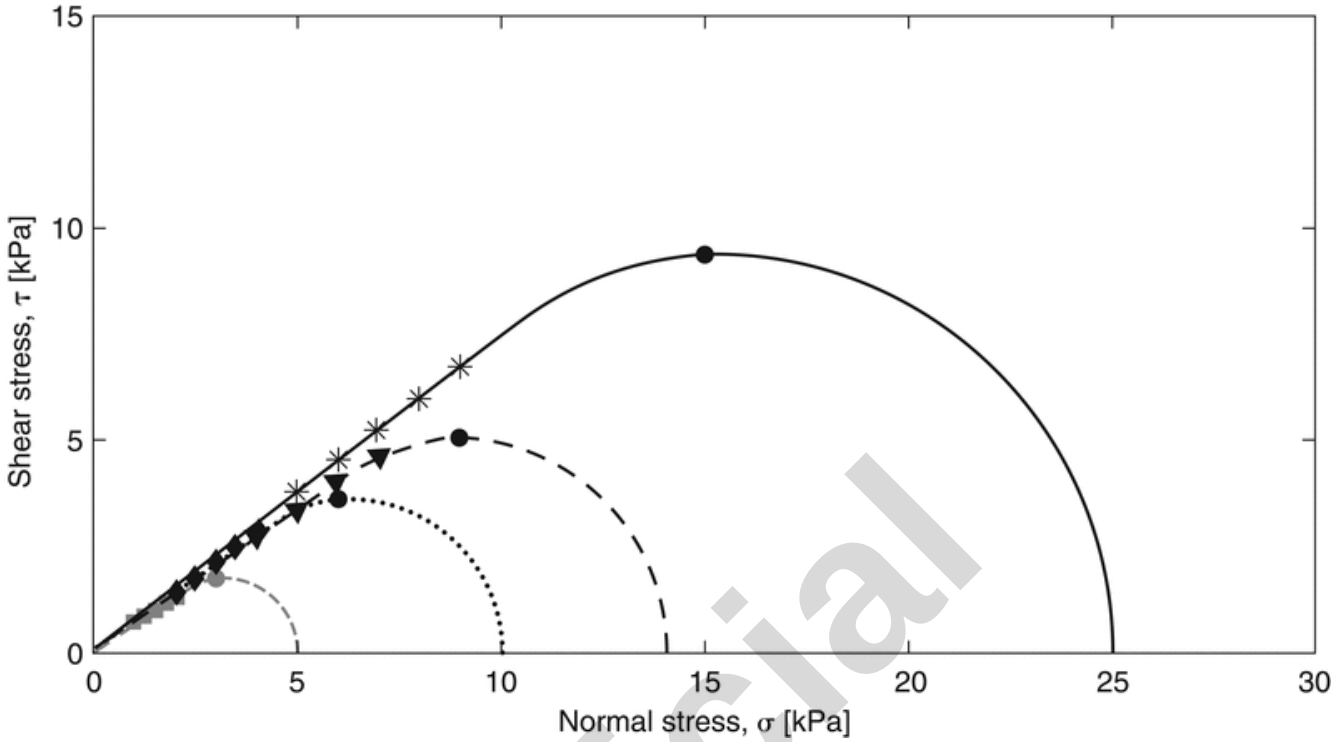


Figure 9. Representative yield loci for sorbitol at preshear stresses of 3 kPa (*gray, dash*), 6 kPa (*dot*), 9 kPa (*black, dash*), and 15 kPa (*solid*).

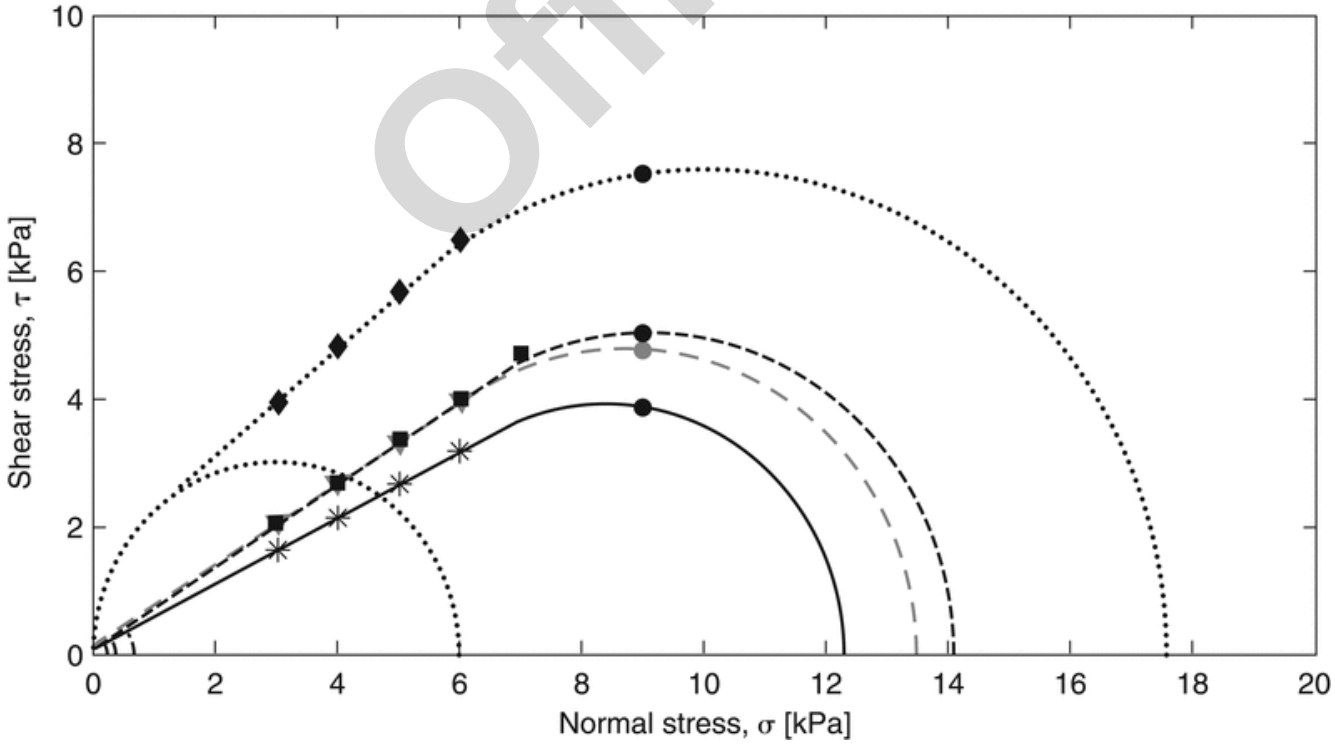


Figure 10. Representative yield loci for mannitol (*dot*), sorbitol (*black, dash*), sieved lactose (*gray, dash*), and spray-dried lactose (*solid*) at a preshear stress of 9 kPa.

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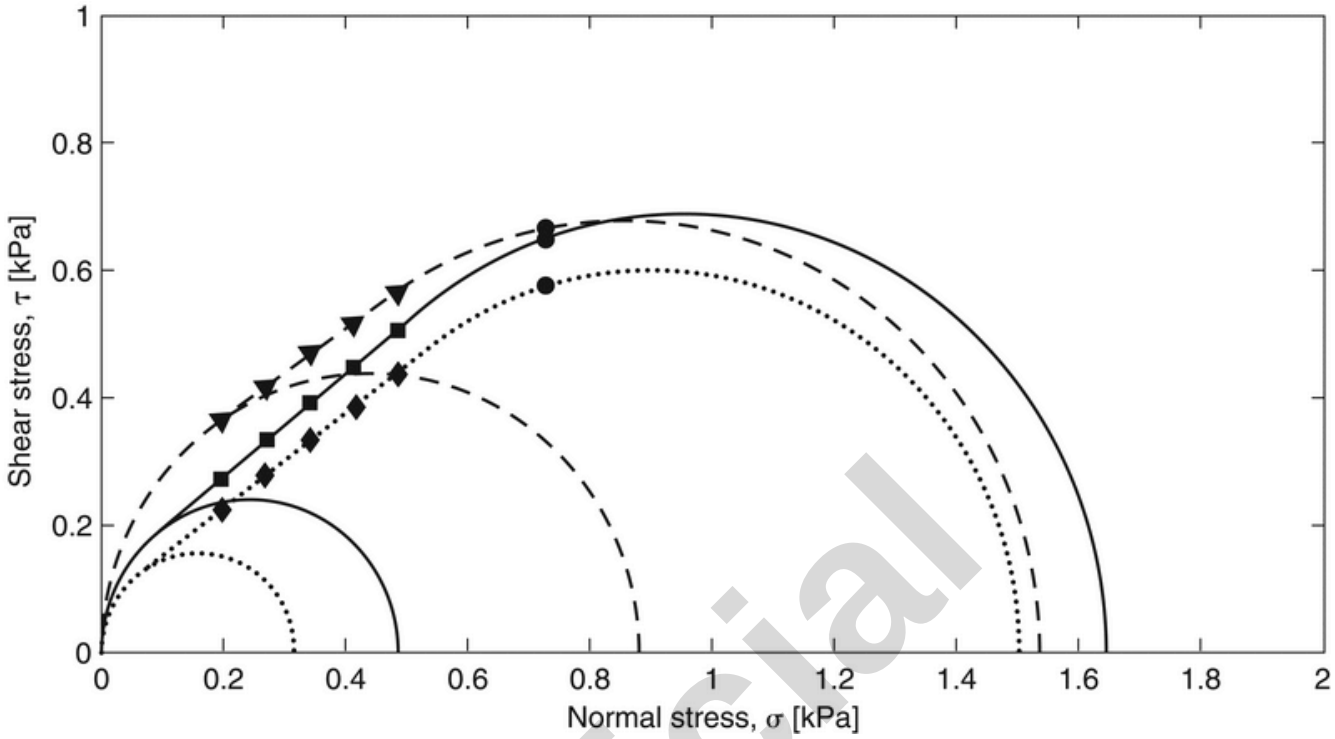


Figure 11. Representative yield loci for several different grades of microcrystalline cellulose NF. Avicel PH-101 (*solid*), Avicel PH-102 (*dot*), and Avicel PH-105 (*dash*) at a preshear stress of 730 Pa.

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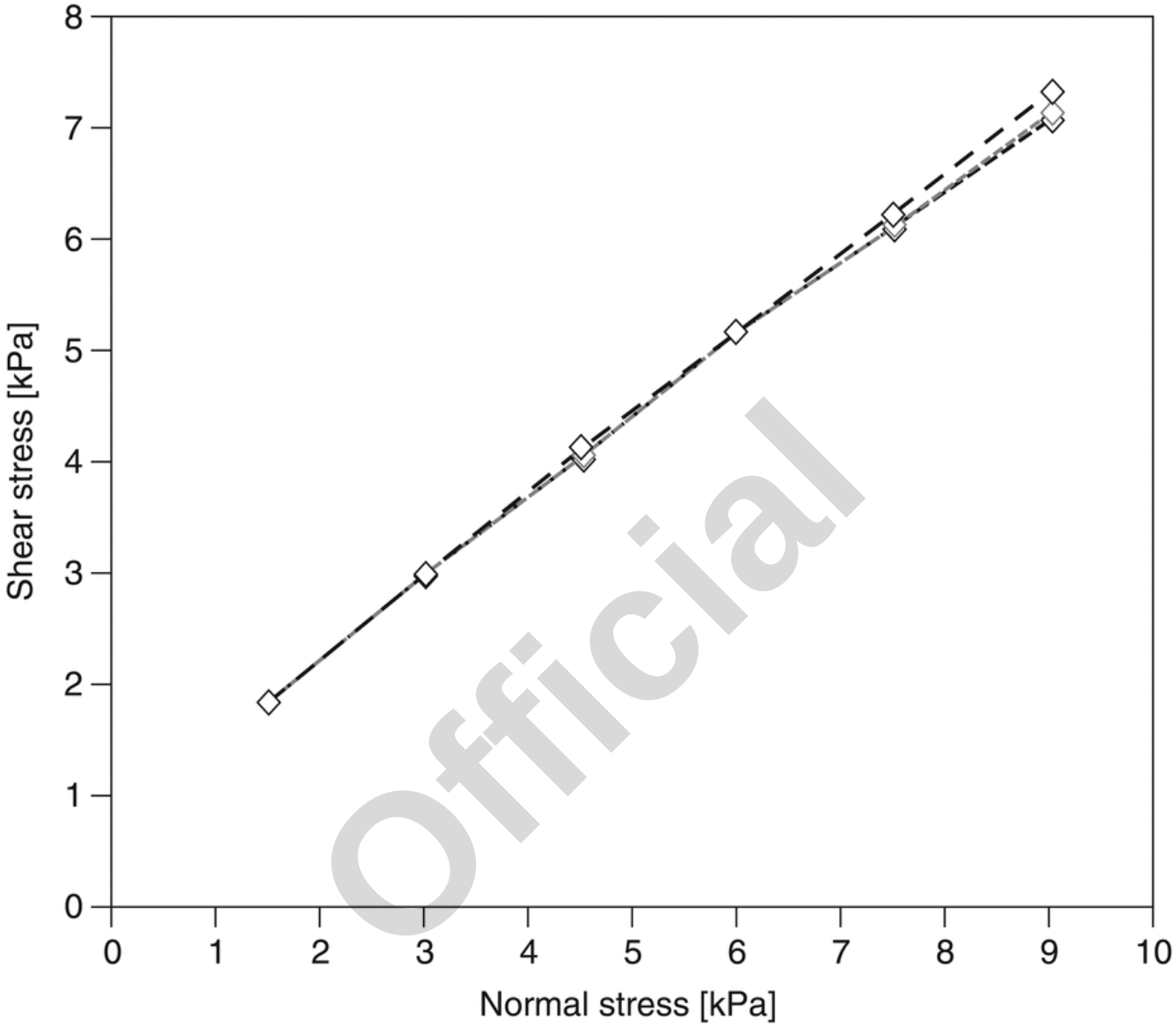


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Figure 12. Representative yield loci for microcrystalline cellulose (Avicel PH102) from three independent determinations conducted at a preshear stress of 9 kPa. [Figure adapted from Sun CC. Setting the bar for powder flow properties in successful high speed tableting. *Powder Technol.* 2010;201(1):106–108.]

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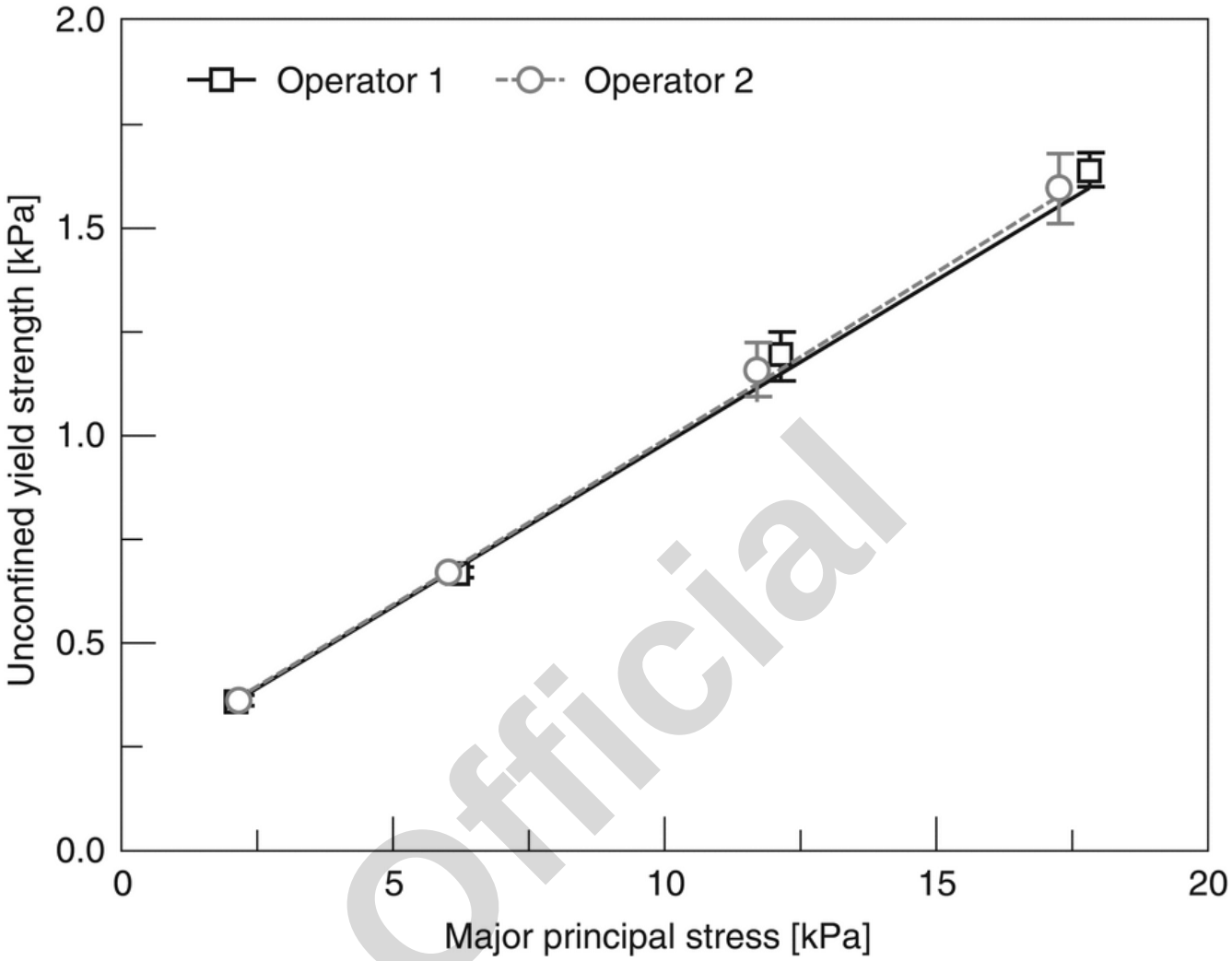


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Figure 13. Representative flow function plots for microcrystalline cellulose (Avicel PH102) from two independent operators. [Figure adapted from Shi L, Chattoraj S, Sun CC. Reproducibility of flow properties of microcrystalline cellulose—Avicel PH102. *Powder Technol.* 2011;212(1):253–257.]

## APPENDIX

**Notation**

| Term | Symbol and SI Units | Definition | Comments |
|---|---|---|---|
| Angle of internal friction | $\Phi$, degrees | The inclination of the yield locus (*Figure 5*). | |
| Angular velocity | $\omega$, degrees per second | The angular velocity at which the shear cell base rotates. | Is usually held constant. |
| Annular shear cell | N/A | A shear cell based on a rotating annulus design (*Figure 2*). | The Schulze Ring Shear Tester is a common annular shear cell. |
| Arching | N/A | The formation of a bridge of powder across an opening caused by, for example, attractive interactions between particles. | Also known as “bridging”. |
| Bulk density | $\rho$, kg/m$^3$ | The mass of a quantity of a powder divided by its total volume. | Varies with the applied normal stress and history of a sample. |
| Cohesion | *C*, *Pa* | The failure shear stress at zero normal stress, normally obtained by extrapolation of the yield locus (*Figure 5*). | An indication of the intrinsic strength of an unconfined powder. |

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**Notation** *(continued)*

| Term | Symbol and SI Units | Definition | Comments |
|---|---|---|---|
| Consolidation | N/A | The process of increasing the density of a powder, which usually results in increasing its unconfined yield strength. Achieved in a shear cell by applying a preshear normal load and shear load to the specimen. | |
| Coupon | N/A | Flat surface that is in contact with powder specimen during wall friction testing. | The coupon surface finish must be representative of the wall surface of interest. |
| Effective angle of friction | $\delta$, degrees | The inclination of the effective yield locus (*Figure 5*). | Sometimes used as a measure of relative flowability. |
| Effective yield locus | N/A | The straight line passing through the origin of the $\sigma - \tau$ plot and tangential to the steady-state Mohr's circle, corresponding to steady-state flow conditions of a powder of given bulk density (*Figure 5*). | |
| Flowability | N/A | A qualitative estimate of the relative flow properties of a powder. | Often based on measured values of the flow function or effective angle of friction. |
| Flow function | N/A | The plot of unconfined yield strength versus major consolidation stress for one specific powder (*Figure 6*). Quantified as the ratio of the major consolidation stress to the unconfined yield strength at a particular value of major consolidation stress. | |
| Funnel flow | N/A | A flow pattern where an active flow channel forms through stagnant material. | |
| Lid | N/A | Cover of the cell containing the specimen within a shear cell. | The lid position can be measured to determine displacement, bulk density, and powder dilation. |
| Major consolidation stress | $\sigma_1$, *Pa* | The major principal stress given by the steady-state Mohr circle. This circle is tangential to the yield locus and effective yield locus and passes through the preshear point (*Figure 4*). | |
| Mass flow | N/A | A flow pattern in a converging hopper where all material is in motion, including material sliding along the hopper walls. | |
| Mohr's circle | N/A | A graphical representation of a state of stress in coordinates of normal and shear stresses (*Figure 4*). | |
| Normal stress | $\sigma$, *Pa* | The stress acting normally (perpendicularly) to the considered plane. | $\sigma_p$ is the preshear normal stress; $\sigma_{si}$ for $i = 1, 2, 3, \ldots$ the test normal stresses. |
| Preconsolidation | N/A | Normal stress applied to consolidate a powder before testing in some shear cells (*Figure 4*). | |
| Preshear | N/A | Application of normal stress while shearing to attain steady-state shear conditions (*Figure 4*). | |
| Rat-holing | N/A | Tendency of powder to flow only in the region above an opening, thus forming a narrow rat hole in the powder. | Also known as "core flow". |
| Rotational shear cell | N/A | A shear cell operating under rotational shear conditions (i.e., rotational shearing perpendicular to the applied normal stress) (*Figure 3*). | The Peschl and Freeman shear cells are common rotational shear cells. |
| Shear stress | $\tau$, *Pa* | The stress required to shear the powder in a direction perpendicular to the normal stress. | |
| Time consolidated strength | $f_{ct}$, *Pa* | The unconfined yield strength of a powder after being held at fixed consolidation conditions for a certain time. | |
| Time yield locus | N/A | The yield locus of a powder that has remained at rest under a given normal stress for a certain time. | |
| Translational shear cell | N/A | A shear cell operating under translational shear conditions (i.e., linear shearing perpendicular to the applied normal stress) (*Figure 1*). | The Jenike shear cell is a common translational shear cell. |
| Unconfined yield strength | $f_c$, *Pa* | The major principal stress of the Mohr stress circle that is tangential to the yield locus when the minor principal stress is zero (*Figure 4*). | |
| Wall friction angle | $\Phi'$, degrees | The arctan of the wall friction coefficient (*Figure 7*). | |
| Wall friction coefficient | $\mu_w$ | The ratio of the wall shear stress to the wall normal stress at a given normal stress condition. | |
| Wall yield locus | N/A | A plot of the wall shear stress versus wall normal stress. Used to determine the wall friction angle for a powder-wall combination (*Figure 7*). | |

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**Notation** *(continued)*

| Term | Symbol and SI Units | Definition | Comments |
|---|---|---|---|
| Yield locus | N/A | A plot of shear stress versus normal stress at failure. The yield locus sometimes is called the instantaneous yield locus to differentiate it from the time yield locus (*Figure 4*). | A powder exhibits a series of yield loci for each different level of consolidation (density). |

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# ⟨1064⟩ IDENTIFICATION OF ARTICLES OF BOTANICAL ORIGIN BY HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY PROCEDURE

## INTRODUCTION

Identification of botanical articles can be achieved by the application of multiple techniques, including macroscopic and microscopic descriptions, DNA analysis, and chemical means. *Identification of Articles of Botanical Origin* ⟨563⟩ provides a detailed discussion of these approaches. Chemical identification typically employs chromatographic or spectroscopic procedures to achieve the identification by fingerprint comparison against that of a Reference Standard, monograph description, or a reference chromatogram. Thin-layer chromatography (TLC) is one of the chromatographic techniques used in *USP* monographs for botanical articles in this way. High-performance thin-layer chromatography (HPTLC) is the most advanced version of TLC (see *Chromatography* ⟨621⟩), and a general analytical procedure for the application of HPTLC to the identification of botanicals is described in *High-Performance Thin-Layer Chromatography Procedure for Identification of Articles of Botanical Origin* ⟨203⟩. In HPTLC, the stationary phase consists of a uniform, typically 200-µm layer of porous (pore size 60 Å), irregular particles of silica gel with a size between 2 and 10 µm and an average particle size of 5 µm, plus a polymeric binder and a fluorescence indicator ($F_{254}$) coated onto a support, which is typically a glass plate or aluminum foil. Other stationary phases, such as chemically bonded phases (C8, C18, CN, NH2; DIOL) or microcrystalline cellulose, are also available with and without a fluorescence indicator. Because of the greater separation efficiency of the fine particles in HPTLC, the chromatographic system is miniaturized, using smaller developing chambers and shorter developing distances of 6–8 cm, in comparison to classical TLC, where 12–15 cm are required for best separation. As a consequence, less mobile phase and less time are required for chromatogram development. Another effect of miniaturization is the use of smaller sample volumes and the resulting possibility of analyzing more samples per plate than in classical TLC.

In addition, improved layer quality due to smaller particle size positively affects the signal-to-noise ratio, and therefore affects the detectability of separated sample components. A system suitability test is used to qualify the results. This feature is of great importance for the identification of botanical ingredients, which have an intrinsic natural variability in chemical composition. HPTLC features electronic images of chromatograms that allow convenient visual comparison of results obtained for multiple samples against images of chromatograms generated with reference materials. For purposes of identification, the complexity of botanical ingredients is best represented by fingerprints obtained in multiple detection modes from the same chromatogram, e.g., UV 254 nm and UV 366 nm light without derivatization, and white and UV 366 nm light after derivatization. Because compendial methods are used to determine compliance, variability in the results caused by the analytical method selected should be avoided. Because HPTLC can control the variables within narrow ranges using a rigorously standardized methodology and appropriate equipment, HPTLC is able to increase significantly the reproducibility of a chromatographic result from plate to plate compared to traditional TLC. Following here is a discussion of the variables to be controlled in order to achieve reproducible results in the identification of articles of botanical origin using HPTLC.

## VARIABLES OF HPTLC

The plate is an open system affected by environmental factors, which must be controlled carefully. Unlike column chromatography, a closed system where samples are analyzed sequentially, in HPTLC multiple samples can be analyzed in parallel on the same plate. All steps of the planar chromatographic process are independent in time and location (an off-line process). For each of the steps—sample application, chromatogram development, visualization, detection, documentation, and evaluation—numerous parameters can be selected freely. This unique feature adds immense flexibility to the method and makes the planar chromatographic approach complementary to column-based techniques.

During method development, the many choices available for the various parameters can be overwhelming, and depending on the combination selected, there may be multiple results that all meet the respective analytical goal and yet are quite different. In this scenario, the labels “better” or “worse” are often assigned to the parameters according to personal preferences. In a cGMP (current Good Manufacturing Practices)-compliant environment, the reproducibility and integrity of results are of great importance. This is why the individual parameters of the HPTLC technique should be selected and combined thoughtfully in a standardized methodology so that the final result is optimized and can be compared to results obtained by different analysts. It may be important to avoid cumbersome and time-consuming steps, or to avoid the use of hazardous or toxic chemicals. Simplicity and clarity are desirable aspects of practical methods.

### Handling of Plates

HPTLC plates are delicate and must be handled with care to avoid damaging the layer. When moving the plate, it should be touched only in the upper part above the region of chromatography. Each plate should be labeled with a soft pencil in the upper right (or left) corner. The expected developing distance can be marked on the right (or left) edge with a short pencil mark that goes down to the glass support. Marking the developing distance across the entire plate should be avoided because it is difficult to judge when the rather-diffuse mobile phase front reaches that line. Aligning the front with a short mark on the edge is easier.

Plates should be stored in a place that is free of fumes and dust with the layer facing down to the stack. Shrink wrap of the package should not be in contact with the layer in order to avoid contamination with volatiles from the foil. Generally, HPTLC plates are ready for use without any pretreatment. Older or improperly stored plates may have accumulated impurities and therefore require precleaning. This is the case when after development, the solvent front (or additional secondary fronts) can be seen under UV 254 nm as an intense, broad dark band across the plate. Precleaning can be achieved by developing the plate

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in methanol to the upper edge. Subsequently, the plate is dried in a clean oven at 120° for 20 min. For cooling down to room temperature and for storage prior to further use, plates can be kept in an empty desiccator or wrapped in aluminum foil. Precleaning may slightly change the selectivity of a plate. Therefore, it is important to define in a method whether or not precleaned plates are to be used. Precleaning with the mobile phase is usually not a good option because it may be difficult to completely remove all components during the drying step.

## Plate Layout and Sample Application

The standard format of the HPTLC plate is 20 × 10 cm (width × height). Other sizes (e.g., 10 × 10 cm) could be used as well, but one should keep in mind that for obtaining reproducible results, each size of plate requires a different chamber to maintain the same geometrical aspects as, for example, a twin-trough chamber would have for the standard plate (see *Chromatogram Development*). The optimum developing distance in HPTLC is 6 cm. Cutting plates to less than 10 cm in height provides no additional advantages.

Exact positioning is essential for proper identification of separated zones. For the application of samples with respect to plate layout (*Figure 1*), the following parameters must be considered.

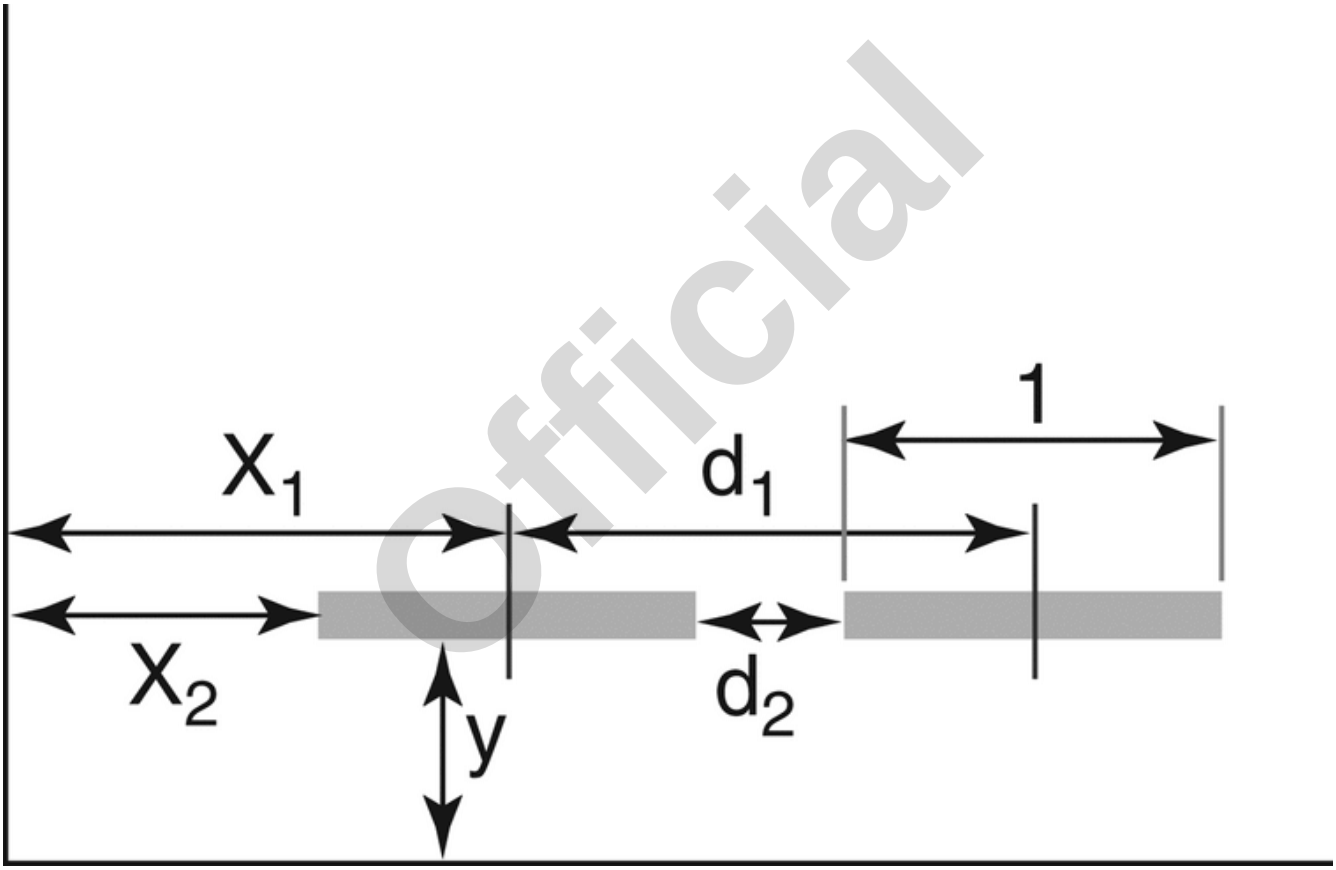


Figure 1. Plate layout.

For proper identification (migration distance/$R_F$ values), all samples must be applied on a (virtual) line parallel to the lower edge of the plate. The distance ($y$) must be large enough to avoid the sample being immersed in the developing solvent. During development, the velocity of the mobile phase decreases with increasing developing distance. Consequently, the final result depends on the application position relative to the level of developing solvent (*Figure 2*).

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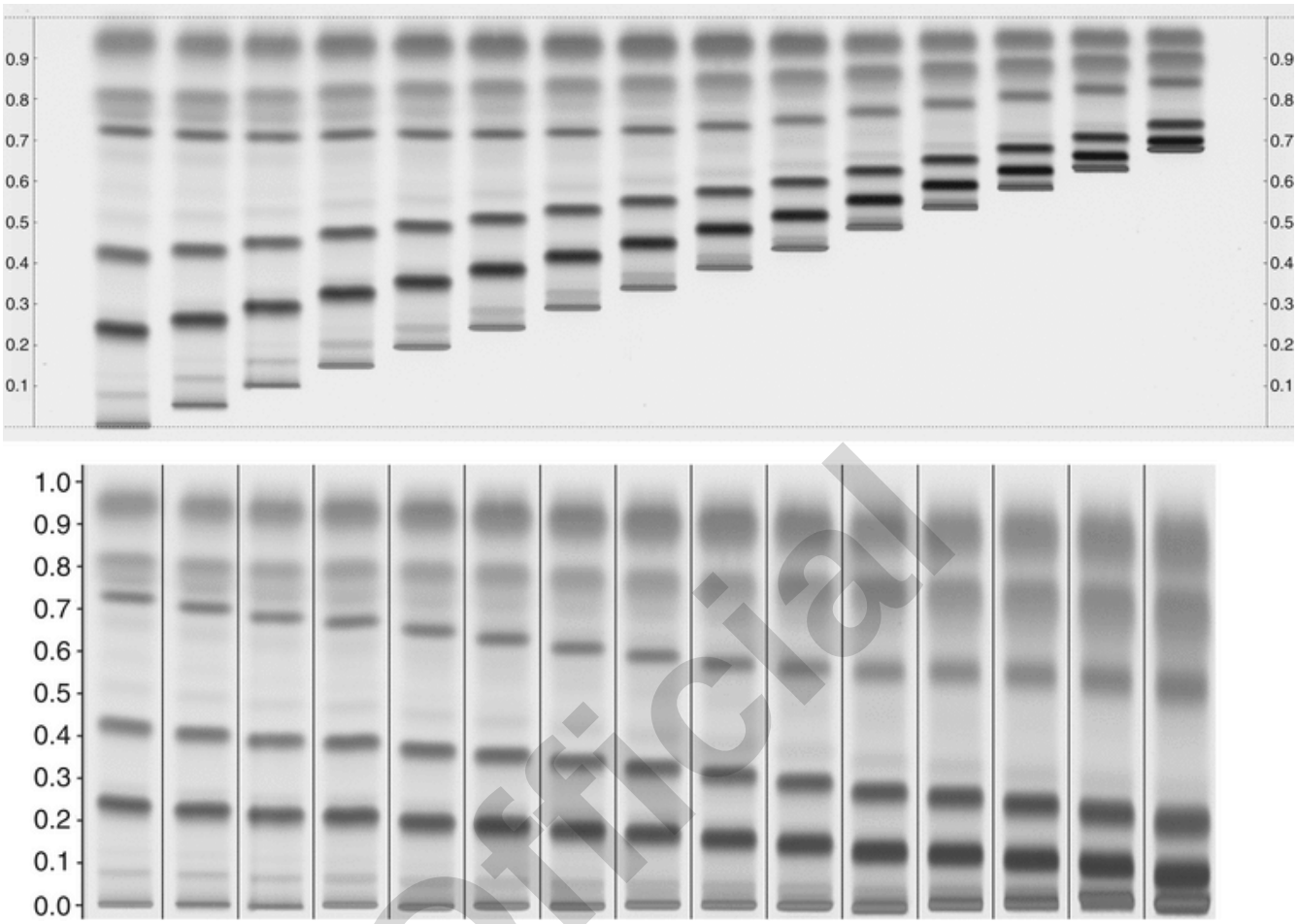


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Figure 2. Upper part: Effect of the application position relative to the level of developing solvent (5 mm). Distance from lower edge ranges from 8–50 mm in increments of 3 mm. Lower part: $R_F$ values calculated for the different effective developing distances. Sample is Houttuyniae herba; developing solvent is a combination of ethyl acetate, formic acid, and water (15:1:1, v/v/v); derivatization is Natural Products reagent (NP); detection is UV 366 nm.

For reproducible results, the distance from the lower edge ($y$) must be kept constant, and the amount of developing solvent placed into the chamber must also be fixed. To minimize solvent consumption, twin-trough chambers are used. The level of developing solvent in such chambers is typically 5 mm. A meniscus is formed on the plate at the immersion line. A sample applied at $y$ = 8 mm will be well above this meniscus.

The distance of the application position from the left and right edges of the plate ($x$) must be sufficient to avoid the so-called "edge effect" (*Figure 3*). During production, most precoated plates develop a small rim on their edges where the layer is slightly thicker. This causes the mobile phase to advance faster, and samples that are applied directly onto or too close to the rim will migrate unevenly.

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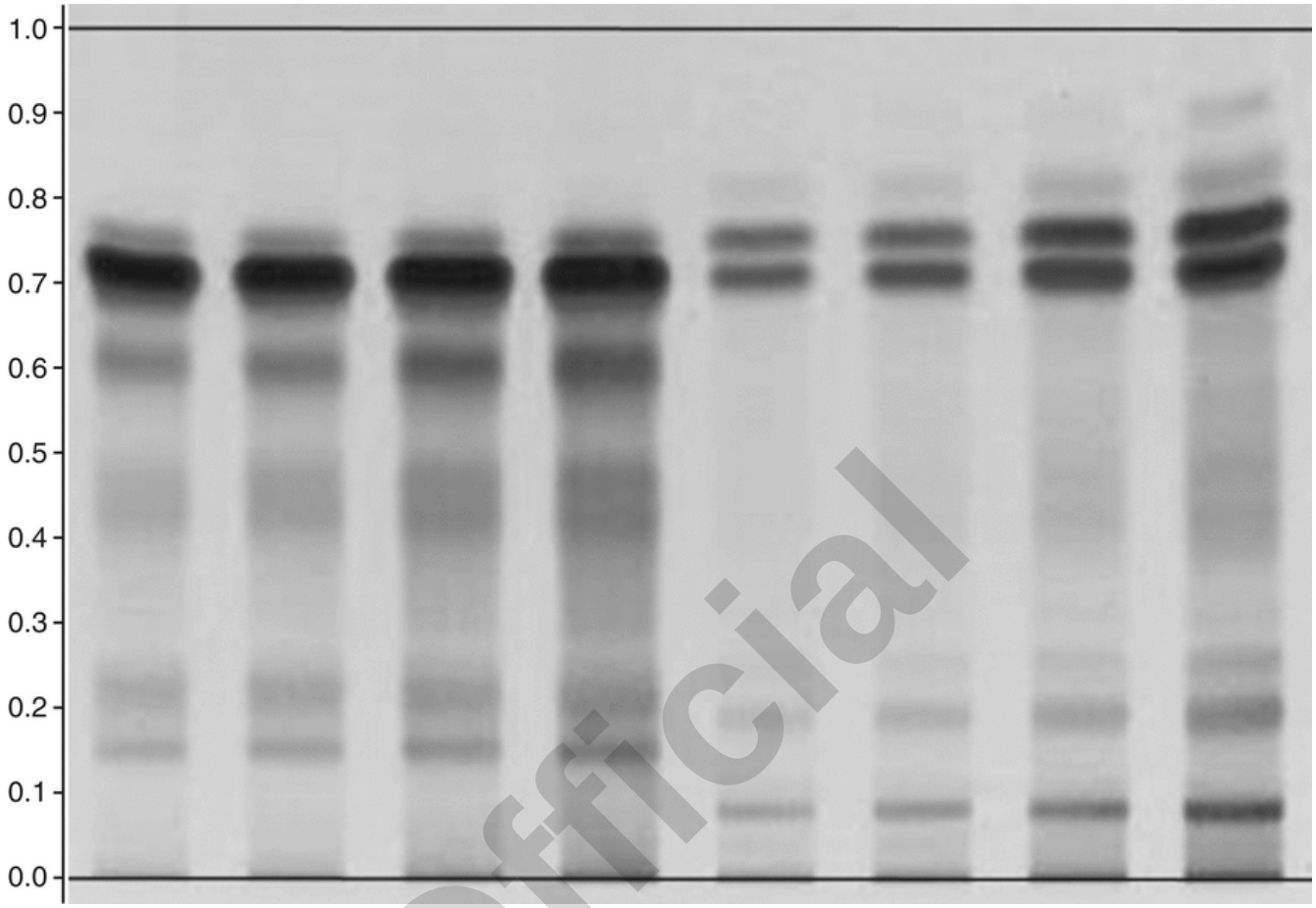


Figure 3. The “edge effect”.

The position $x_1$ is typically called “application position”. In order to avoid the edge effect, the length of the applied band ($l$) has to be taken into account when defining $x_2$ as the distance from the edge of the plate. A minimum of 15 mm is necessary for $x_2$. The distance between two samples ($d_1$) also considers the band length ($l$). For $d_2$, a minimum of 3 mm is recommended so that samples do not interfere with each other if larger volumes are applied. The band length ($l$) must be fixed for each method because it affects the concentration of samples per band if a defined volume of sample solution is applied. The applied quantity (across a defined band length) has an effect on the intensity of the separated zones and may affect whether or not a particular zone of a fingerprint is visible. An argument for selecting shorter bands would be that more samples can be applied on the plate. However, because chromatogram evaluation is primarily performed visually, it has to be noted that a band seems to look “sharper” (more like a band and less oblong) when it is >5 mm (*Figure 4*). A band length of 8 mm is a good compromise also from the perspective that ⟨621⟩ specifies bands of 5–10 mm for HPTLC plates.

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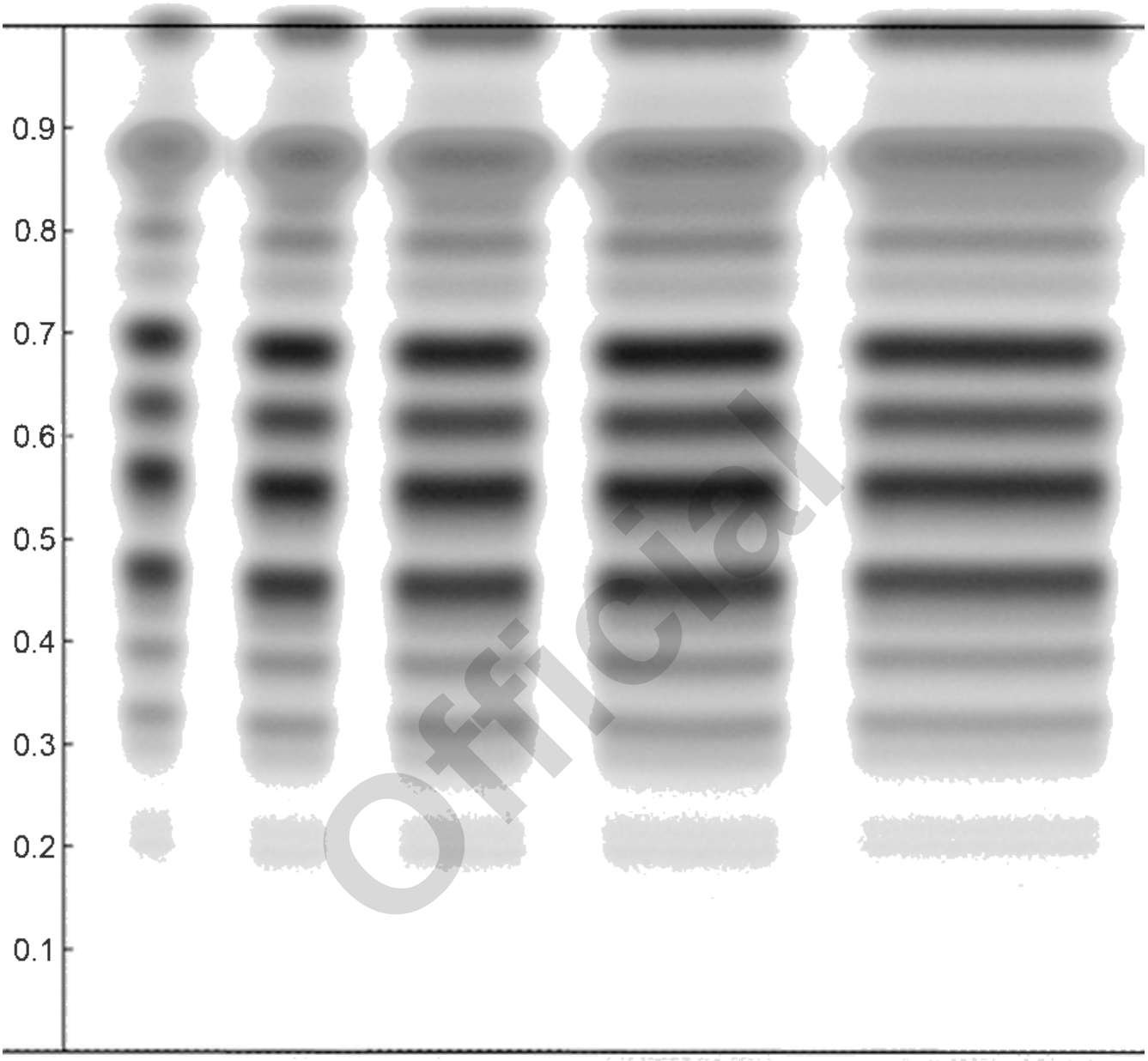


Figure 4. Effect of band length on the visual impression of separation. From left to right, 3 µL/3 mm, 5 µL/5 mm, 8 µL/8 mm, 11 µL/11 mm, and 15 µL/15 mm. Sample is Tiliae flos; developing solvent consists of ethyl acetate, formic acid, water, and methyl ethyl ketone (50:10:10:30, v/v/v/v); derivatization is NP/Polyethylene glycol reagent (PEG); detection is at UV 366 nm.

With the parameters just discussed, the standard plate layout will feature 15 bands of 8-mm length. If fewer samples are to be analyzed, it is recommended to either apply replicates or leave some tracks on the plate blank.

During application, samples are precisely deposited onto the HPTLC plate at defined positions and in defined quantities. Sample solutions can be applied by contact using a capillary or a syringe, or by spraying the sample solution without touching the plate (spray-on technique). During contact application, the sample's solvent can perform circular chromatography (*Figure 5*). Therefore, only small volumes should be applied in one stroke, and if possible, only nonpolar solvents should be used. During spray-on application, the sample's solvent is nebulized and evaporated, creating sharp zones regardless of the polarity of the solvent.

If samples are applied manually, a spotting guide can be used for proper positioning. Marking the application position with a pencil is not a good option, because the layer can be damaged easily.

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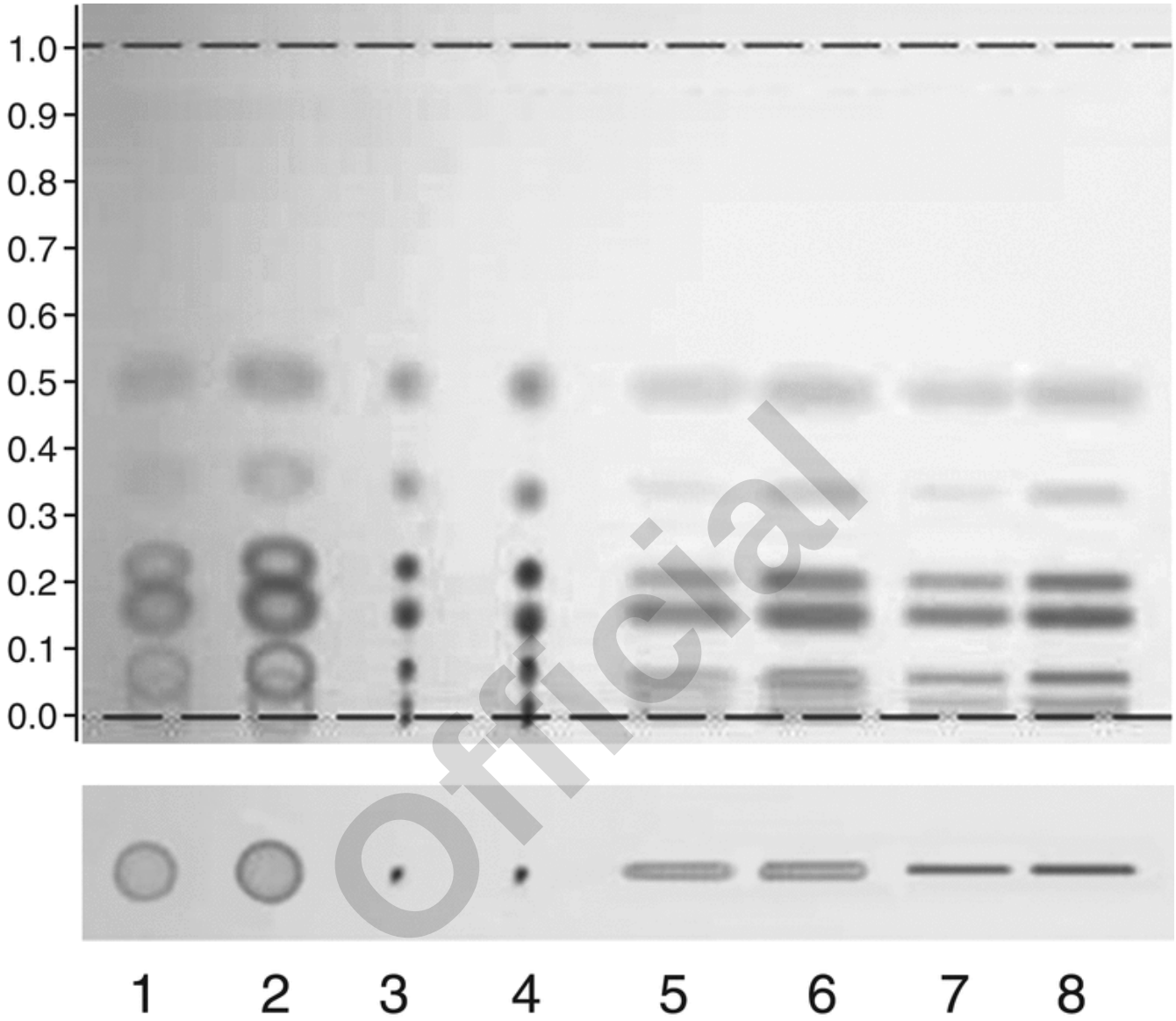


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Figure 5. Lower part: Sample application as spot (tracks 1–4) and band (tracks 5–8) using contact application (tracks 1, 2, 5, 6) or spray-on technique (tracks 3, 4, 7, 8); application volumes were 2 µL (tracks 1, 3, 5, 7) or 5 µL (tracks 2, 4, 6, 8); test dye mixture in methanol. Upper part: Chromatography with toluene as mobile phase.

## Chromatogram Development

The processes in a chromatographic chamber are highly complex and difficult to describe. Unlike column chromatography, which is assumed to take place in equilibrated chromatographic systems, planar chromatography always begins in a state of nonequilibrium and never reaches equilibrium. The sample has been applied onto the plate, which becomes the stationary phase only when it comes in contact with a liquid in the chromatographic chamber. The advancing mobile phase must be able to dissolve the sample for chromatography to begin. Driven by capillary action, the mobile phase velocity decreases with increasing migration distance because the resistance of the stationary phase against the flow is also increasing.

It can be shown experimentally that the optimum migration distance for mobile phases on HPTLC plates is about 6 cm. Depending on the viscosity of the mobile phase, development takes place between 10 and 20 min. To further move the solvent front just 10 mm will extend the developing time by 5–15 min, while moving an additional 20 mm will increase the developing time by 15–40 min. The contribution of that extra developing distance to improved separation is usually not justifiable, because the diffusion that occurs at decreased velocity of the mobile phase often offsets any gain in distance of separated zones.

Four distinct processes occur in a chromatographic chamber. These processes always take place simultaneously and affect each other (*Figure 6*).

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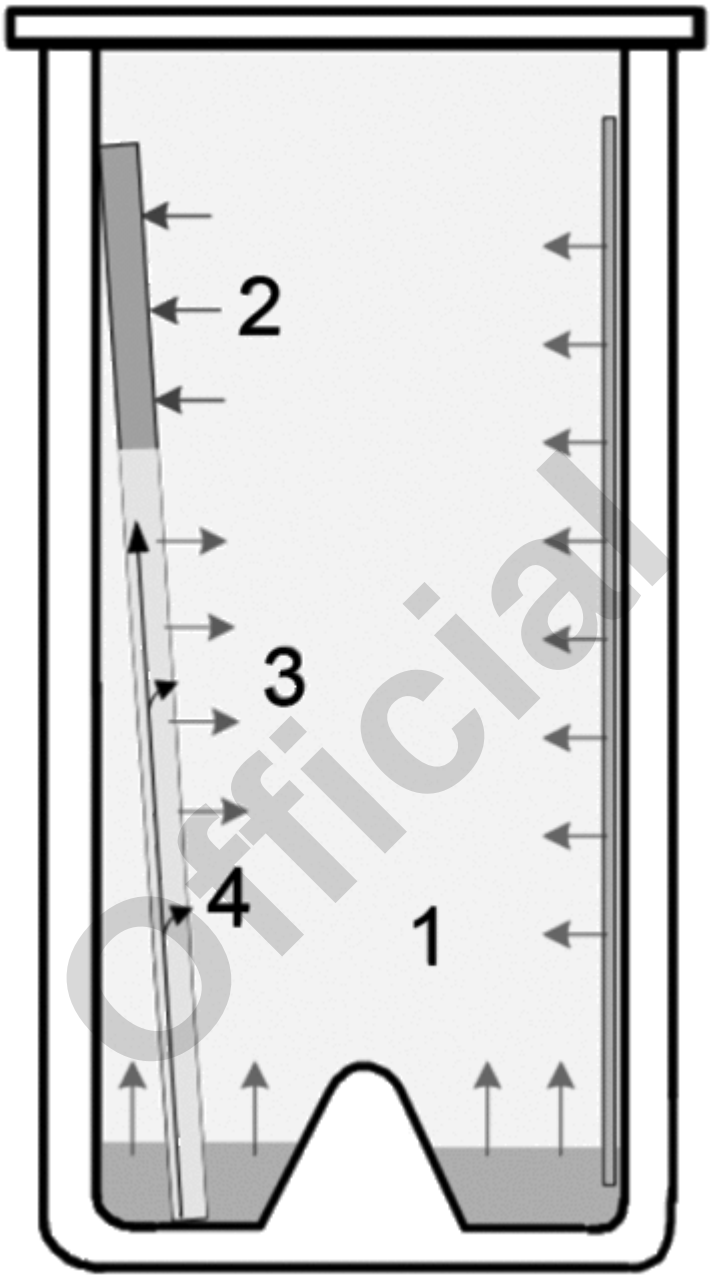


Figure 6. Processes occurring in the chromatographic chamber (twin-trough chamber): (1) chamber saturation; (2) plate preconditioning; (3) evaporation; (4) formation of secondary fronts.

Chamber saturation (*Figure 6*, process 1) is usually established before placement of the plate into the chamber. The developing solvent, which has a known composition, is placed into both troughs of the chamber, and the filter paper in the rear trough is wetted thoroughly. Chamber saturation occurs once the liquid in the tank is in equilibrium with its own vapor, and this is a function of time. After 20 min, the chamber will generally achieve a reproducible degree of saturation.

The plate should be placed into the chamber so that it rests in a vertical position against the front wall while the layer faces the rear wall. The dry portion of the layer absorbs solvent vapor from the gas phase (process 2) in order to achieve adsorptive saturation, which occurs when the surface is covered by a layer of solvent molecules. Generally, this process can lower $R_F$ values and also affect the selectivity of the separation. In the lower portion of the plate, the liquid in the chamber forms the mobile phase. Depending on the degree of saturation, a part of the mobile phase may evaporate off the plate (process 3). Multicomponent mobile phases may be separated by the stationary phase because of different interactions of the constituents. This process is affected by the degree of preconditioning.

In an unsaturated chamber, the situation is quite different. The rear trough does not contain liquid or filter paper. The plate is introduced immediately after the solvent, which does not allow enough time to achieve gas–liquid saturation equilibrium. Preconditioning has little effect, while evaporation and possibly formation of secondary fronts (process 4) dominate the

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chromatographic process. Both of these processes typically cause alterations in the chromatograms, thereby affecting the separation.

In conclusion, each chamber gives a somewhat different result, depending on the chamber's geometry, the presence or absence of liquid and filter paper in one of the troughs, and the waiting time before introducing the plate. The chromatograms shown in *Figure 7* were obtained with an automatic developing chamber with humidity control. In this example, a saturated chamber (A) gives better separation than an unsaturated chamber (C). Chromatograms are reproducible if all conditions are the same (A) and (B). If the filter paper for saturation is not exchanged but is reused without drying, a completely different result is obtained (D).

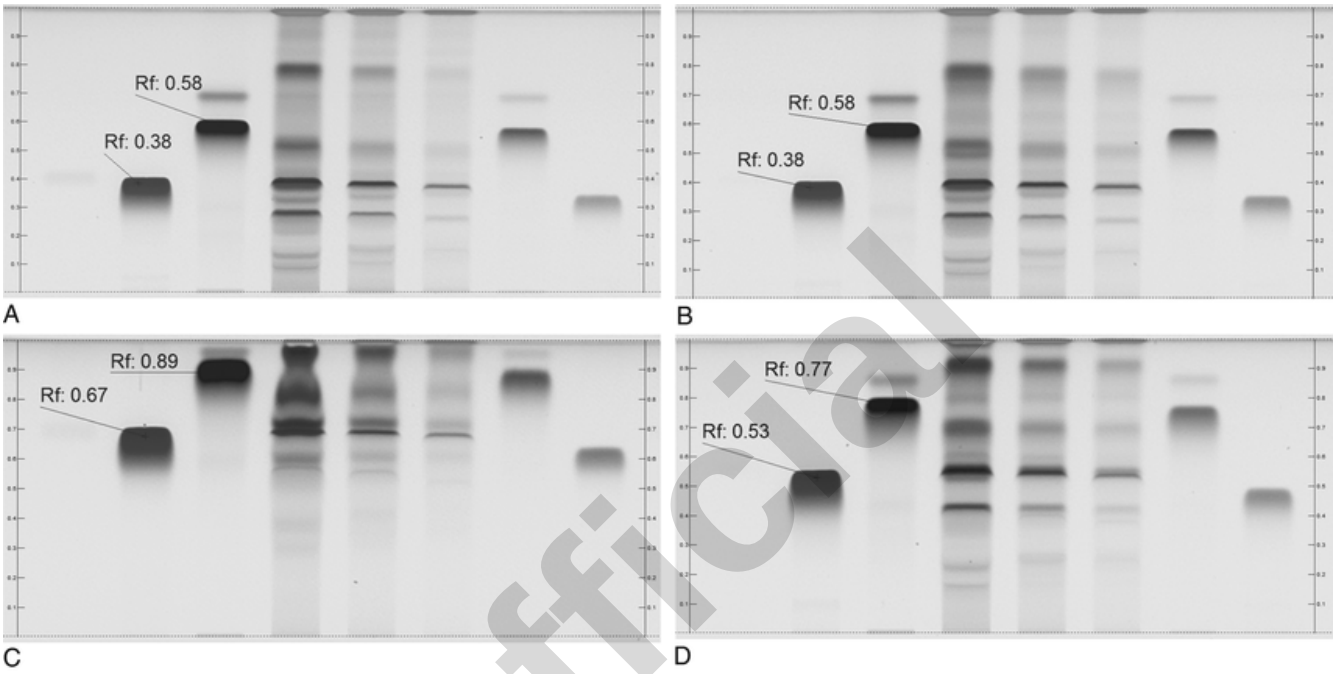


Figure 7. Effects of different chamber configurations at relative humidity 33%: (A) and (B) are saturated, (C) is unsaturated, and (D) is saturated with reused filter paper. Sample is *Lapsana communis*, references are chlorogenic acid ($R_F$ 0.38, Lane 1, Panel A), quercitrine ($R_F$ 0.58, Lane 2, Panel A), developing solvent: water, methanol, glacial acetic acid, and methylene chloride (2:3:8:15, v/v/v/v); derivatization NP/PEG; detection UV 366 nm.

For reproducible results, it is necessary to carefully standardize the development step. Chamber saturation is easy to achieve, as is proper timing. Unsaturated chambers are extremely sensitive to small changes and therefore, from a practical point of view, they should be avoided, even if they can give "better" separation. It is a good practice to keep developing distances constant (at optimum) for all methods, because other parameters, such as selectivity and condition of the gas phase, affect separation to a much greater degree.

## Drying

After the mobile phase has advanced the desired distance, the plate must be removed from the chamber and reproducibly dried. This is best achieved by exposing the plate in the vertical position to a flow of cold air. Elevated temperature during drying could cause volatile sample components to diffuse or evaporate from the plate. During the evaporation of the mobile phase, sample components move from deeper areas of the layer to the surface. Uneven drying across the plate may result in different intensities for zones with equal amounts of substances.

## Effects of Humidity

Silica gel has an extremely high affinity for water and is an effective drying agent. An HPTLC plate is always at equilibrium with the water vapor in the laboratory atmosphere (relative humidity, RH). Chromatographic results are affected by variation in the amount of water adsorbed onto the silica gel surface. The higher the RH, the more water is adsorbed. In a first approximation, this reduces the "activity" of the stationary phase and causes higher $R_F$ values. Also, because the adsorbed water can become part of the stationary phase, the selectivity of the chromatographic system often changes in response to variation in RH.

Unfortunately, it is nearly impossible to predict what effect a change in RH may have for a given separation (*Figure 8*). Some separations are more affected than others, and sometimes only parts of the chromatogram will change. In addition, there is no ideal activity of the plate that can solve all separation problems, and it is difficult to use defined activity changes of the stationary phase to influence separation in a desired way. The best option is to keep the RH constant at a moderate level (e.g., 33%) and then, try to adjust the selectivity of the mobile phase. One practical exemption is effective "drying off" of the plate using a molecular sieve. Activating a plate by heat (120° for 20 min) is possible, because under these conditions water adsorbed onto

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the silica gel is effectively removed, but when the plate is cooled down and stored, or during sample application it re-equilibrates to ambient relative humidity.

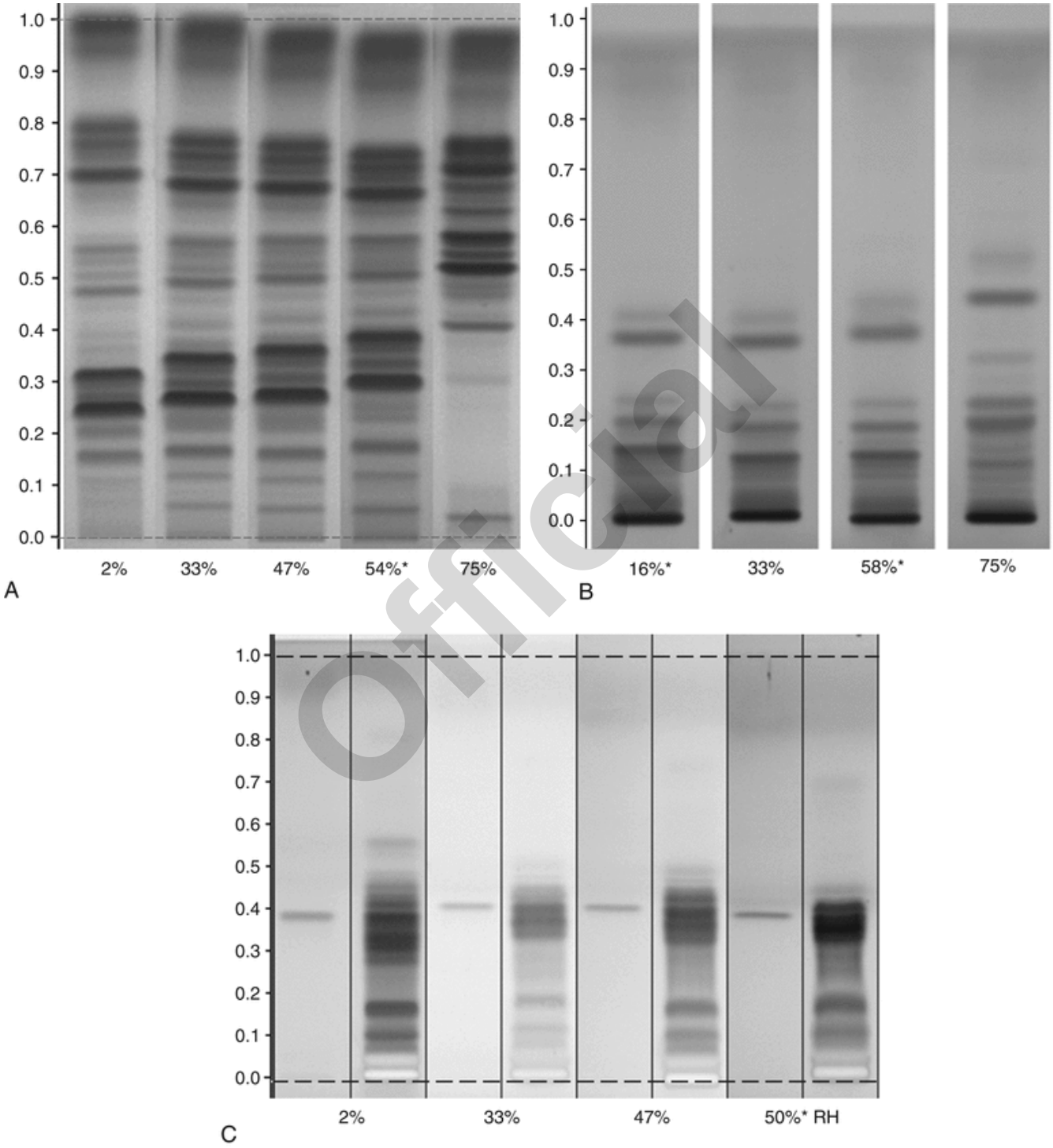


Figure 8. Effects of relative humidity on the separation of different samples. (A) *Hoodia gordonii*, developing solvent: chloroform, methanol, and water (70:30:3, v/v/v); derivatization: anisaldehyde reagent, detection white light. (B) Licorice root, developing solvent: ethyl acetate, formic acid, acetic acid, and water (15:1:1:2, v/v/v/v); derivatization: sulfuric acid reagent, detection white light. (C) Actein/Black cohosh rhizome, developing solvent: toluene, ethyl formate, and formic acid (5:3:2, v/v/v); derivatization: sulfuric acid reagent, detection UV 366 nm. Humidity: *ambient; 2%: molecular sieve; 33%: saturated solution of magnesium chloride ($MgCl_2$); 47%: saturated solution of potassium thiocyanate (KSCN); 75%: saturated solution of sodium chloride (NaCl).

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To achieve reproducible results independent of seasonal or regional changes in the RH in various laboratories, it is important to expose the plate to a defined RH before development and with the samples already applied. This will keep the activity and the selectivity of the stationary phase constant. Defined RH can be created in closed containers such as a desiccator over saturated salt solutions. Between 20° and 25°, saturated solutions of magnesium chloride establish 33% RH; of potassium thiocyanate (KSCN), 47% RH; and of sodium chloride (NaCl), 75% RH.

## Derivatization, Detection, and Evaluation

The possibility of convenient chemical derivatization of substances already separated on a plate is an outstanding advantage of planar chromatography. Solutions of derivatizing reagents can be applied onto the plate, either by spraying or immersion. For reproducible results, the amount of reagent of a given concentration applied needs to be defined either by volume (spraying) or by volume, and dwell time (immersion). Most derivatization reactions require a heating step performed either on a plate heater or in a drying oven. The plate should be dried in air before heating to avoid any disruption of the layer resulting from evaporation of residual solvent. Before the plate is heated, the heating device should reach the required temperature. For reproducible results, it is important that the entire derivatization process is strictly timed. This includes any waiting time (e.g., for cooling) before the detection step.

Detection is performed under white light, typically by using a light source above the plate (reflectance) in combination with light from below the plate (transmission). Additional information (typically before any chemical derivatization) can be obtained under UV 254 nm. The $F_{254}$ indicator of the HPTLC plate will emit a green light (blue for $F_{254s}$). Any substance that absorbs UV 254 nm will be visible as a dark "quenching" zone where the degree of quenching corresponds to the amount of that substance (and its extinction coefficient). Some substances can be excited to fluoresce by UV 366 nm, particularly after chemical derivatization. Such substances are seen as zones of specific colors on the dark background.

HPTLC benefits from digital images taken of the plate in different detection/illumination modes. Such images may sometimes look different from what the analyst's eye is seeing. This is due to some physical limitations, but it is possible to standardize the imaging process so that "true" data are generated. These parameters should always be the same for a given plate regardless of where and by whom the image is taken. The use of image editing software to change certain aspects is not permissible.

In the evaluation step, the analytical data obtained for a sample are compared against specifications of the method. HPTLC for identification of botanical ingredients typically produces fingerprints, i.e., sequences of zones that have specific positions, colors, and intensity. Those fingerprints are compared against data obtained with reference materials. Ideally, comparison is based on images rather than descriptions. Because of the natural variability of botanical materials, there will always be a range associated with the acceptance criteria for a fingerprint to pass the identity test. Fingerprints of different species (adulterants) should have distinguishing features not present in samples that pass the evaluation (*Figure 9*). It is necessary to include a range of botanical reference materials covering different accessions when defining the acceptance criteria for identification. Multiple detection modes are helpful in making decisions.

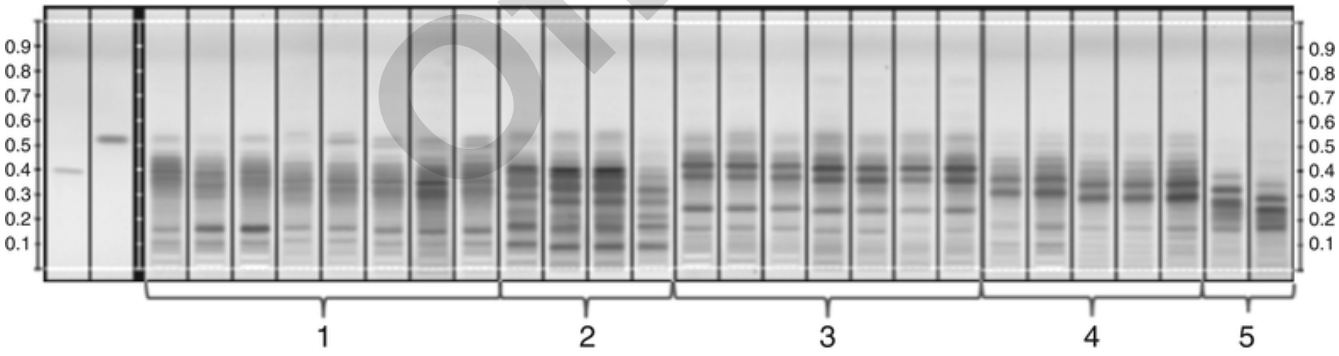


Figure 9. Similarities and differences among multiple samples of related species: (1) *Cimicifuga racemosa*; (2) *Cimicifuga foetida*; (3) *Cimicifuga heracleifolia/dahurica*; (4) *Cimicifuga pachypoda*; (5) *Cimicifuga americana*.

## System Suitability

Before evaluating and/or comparing data that originate from different HPTLC plates, each developed plate must be qualified. This is possible with a system suitability test, which is part of the analytical method. On each plate, the analyst selects two or more reference substances that have similar but just separable $R_F$ values under the chromatographic conditions to be used [e.g., chlorogenic acid (blue) and hyperoside (yellow-orange) in chromatographic systems used for flavonoids]. The substances designated to check the system suitability for resolution, position, and colors of the bands may be included in reference standard extracts or another matrix. Description of the resolution position and colors for the key bands of the reference material fingerprint should match the description in the reference within a specified tolerance range. Only when the system suitability requirements have been satisfied can the results obtained with the samples on the same plate be evaluated further to determine compliance. For reference to a standardized procedure for identification of articles of botanical origin controlling the variables described in this chapter, see ⟨203⟩.

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## CONCLUSIONS

As HPTLC is widely used for botanical identification to monitor the quality of articles of botanical origin on an increasingly globalized level, it seems important to thoroughly standardize the involved analytical procedures. All parameters discussed above must always be selected for each HPTLC analysis. Currently laboratories in different countries can make this selection widely within the framework of the *USP* general chapters; however, guidance about how to achieve specific HPTLC results in a reproducible manner is not available. A standardized procedure as proposed in ⟨203⟩ could fill this gap and serve as basis for harmonization of results obtained in different laboratories.

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# ⟨1065⟩ ION CHROMATOGRAPHY

## INTRODUCTION

Ion chromatography (IC) is a high-performance liquid chromatography (HPLC) instrumental technique used in USP test procedures such as identification tests, assays, and determination of impurities, including limit test and quantitative tests. IC is used to measure anions and cations derived from organic or inorganic molecules, from small molecules to larger biomolecules. This may include, but is not limited to, organic acids, carbohydrates, sugar alcohols, aminoglycosides, amino acids, amines, phosphonates, peptides, aminoglycosides, oligosaccharides, proteins, and glycoproteins.

IC has been applied to all aspects of the manufacturing and disposition of pharmaceutical products, including characterization of active ingredients, excipients, degradation products, impurities, and process streams. Raw materials, intermediates (including media and culture broths), bulk active ingredients, diluents, formulated products, production equipment cleaning solutions, process water, and waste streams may be analyzed using IC.

The majority of IC methods use either anion- or cation-exchange chromatography coupled with suppressed conductivity detection. IC is especially valuable for ionic or ionizable (in the mobile phase) analytes that have little or no native UV absorbance. In addition to suppressed conductivity detection, the ion-exchange separation can be coupled to other detection strategies, including pulsed amperometric detection (PAD), UV/Vis absorbance detection, inductively-coupled plasma mass spectrometric detection (ICPMS), and mass spectrometric detection, providing a wide range of analyte sensitivity and specificity. Ion-exclusion separations expand the range of IC applications to some nonionic analytes (e.g., alcohols) and provide a different selectivity for some analytes that can also be separated by ion exchange. The wide dynamic range of the majority of the IC detection methods makes IC applicable to the quantification of trace contaminants as well as major product components in the same run. IC typically uses dilute acids, alkalis, buffers, or salt solutions as the mobile phase, reducing solvent cost and simplifying disposal logistics. In most cases, the effluent can be disposed of after appropriate neutralization and, when necessary, after dilution with water.

IC is typically performed at or near ambient temperature. As with other forms of HPLC, IC separations are based on varying capacity factors and typically follow the Knox equation. IC is a technique often complementary to reversed-phase and normal-phase HPLC as well as atomic absorption and inductively-coupled plasma techniques in pharmaceutical analysis.

## APPARATUS

IC instruments can be either conventional HPLC instruments or instruments specially designed to only perform IC. What type of instrument is chosen depends on the application. Typical components of an IC instrument may include an autosampler, a high pressure pump, a sample loop of suitable size, a guard column, an analytical column, an optional suppressor if conductivity detection is used, a flow-through detector, and a data system (*Figure 1*).

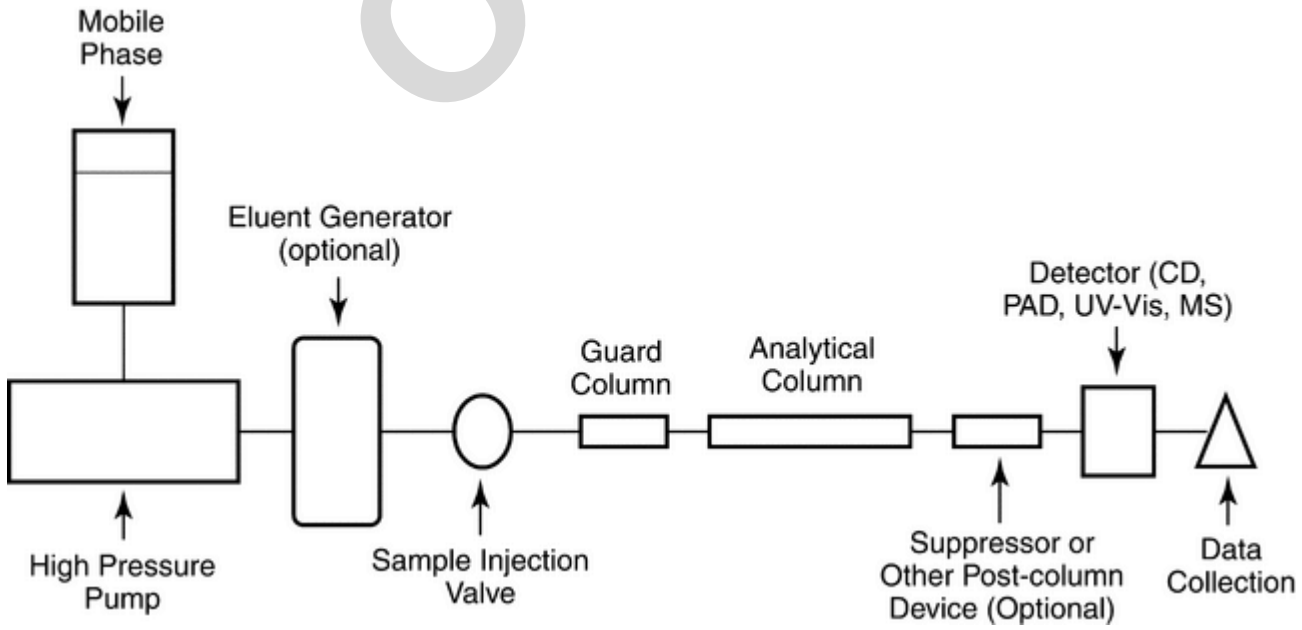


Figure 1. Components of a typical IC system illustrated schematically; CD = conductivity detector, PAD = pulsed amperometric detector, and MS = mass spectrometric detector. [NOTE—Mobile phase is water when the optional *Eluent Generator* is used.]

Because mobile phases generally consist of dilute acids, alkalis, or salt solutions, the components in contact with the mobile phase and the sample are typically made from inert materials, such as polyetheretherketone (PEEK). A conventional HPLC system

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can be used provided that its components are compatible with the mobile phase and injected sample solutions, though a metal-free system should be used for trace metal analysis. Following suitable preparation, if needed, the sample is introduced via the injection valve. Because IC uses a predominantly ionic mobile phase, a suppressor is typically necessary prior to conductometric detection when high sensitivity is needed, although nonsuppressed conductometric detection has been successfully used in pharmaceutical analysis.

## Mobile Phases

Nearly all IC separations require acids or bases diluted in high-purity water, generally with resistivity greater than 18 megohms-cm to prepare the mobile phases. As in every chromatographic method the sensitivity dictates the detection method, and the selectivity dictates the mobile phase and column selection.

For suppressed conductivity detection, the bases and acids used are suppressed to water or weakly dissociated species. If the analytes are anions, the mobile phase bases used as counterions by suppressed conductivity detection are sodium or potassium hydroxide, sodium carbonate, sodium bicarbonate, and less often sodium tetraborate. If the analytes are cations, the mobile phase acids used as counterions by suppressed conductivity detection are methanesulfonic acid, sulfuric acid, and less often hydrochloric acid. When determining low ion concentrations, appropriate trapping technology should be used to purify the mobile phase.

When the detection is based on UV or visible absorbance, then a wide variety of salt solutions may be used to prepare the mobile phase, including phthalic acid and *p*-hydroxybenzoic acid for the determination of anions, and methanesulfonic acid for the determination of cations.

Amperometric detection uses either a strong acid or base solution as the mobile phase, although some methods can use a salt solution near neutral pH or an acid or base solution containing a salt. When using alkaline mobile phases, carbon dioxide/carbonate contamination can be minimized through the use of high purity reagents and storage under nitrogen or helium headspace. Most IC-MS methods use the same mobile phases used for suppressed conductivity, though volatile amines and volatile salt solutions have also been used. IC methods that use an ion-exclusion column use solutions of strong acids such as sulfuric acid as mobile phases. Organic solvents commonly used for HPLC are sometimes used to prepare the mobile phase, typically at NMT 20% concentration. This addition is usually made to either modify selectivity or to enhance solubility of sample components that might otherwise contaminate the stationary phase.

Also, protein molecules may get a different surface charge distribution according to their structure and the pH of the mobile phase. In those cases, the pH, gradient of pH, ionic strength, or a combination of those parameters proved to be useful in the separation.

## Stationary Phases

IC separations rely on ion exchange, and therefore stationary phases for IC are anion or cation exchangers, and less commonly phases containing both functionalities. Stationary phases may be silica-based or polymer-based materials supporting ionic functional groups. However, due to the solubility of silica gel in water, particularly at alkaline pHs, the use of silica-based support is limited when the mobile phase is alkaline. In those cases, a polymeric support for IC is useful over an extended pH range. Most of the phases used today are constructed with highly cross-linked polymers, increasing their compatibility with the organic solvents that sometimes are needed to prepare the mobile phase. Polymeric anion-exchange phases for IC are typically constructed from polystyrene/divinylbenzene, ethylvinylbenzene/divinylbenzene, or polyvinyl alcohol polymeric substrate and polymethacrylate supports, with particles sizes ranging from 4 to 15 µm in diameter either nonporous or with pores up to 2000 Å. To provide functionality to the anion exchanger, the anion-exchange groups are attached to the substrate typically in one of two manners, electrostatically or covalently by condensation polymerization (a.k.a. a step-growth polymerization). Cation-exchange phases for IC use the same polymeric substrates as the anion-exchange phases, but because the mobile phases for cation IC are acidic, silica can also be used. Ion-exclusion chromatography uses a porous strong cation-exchange stationary phase, while ion-pair chromatography typically uses a polymer reversed-phase stationary phase.

## Detection

Conductivity detection is the most commonly employed mode of detection in IC, especially for suppressed conductivity detection.

In suppressed conductivity detection, the background conductance of the ionic mobile phase is significantly reduced as it flows through the suppression device (suppressor). For example, when diluted sodium hydroxide (10–50 mM) used as the mobile phase in IC of anions flows through the suppressor, it is converted to water ($H_2O$), which shows very poor conductivity. Analogous reactions occur using a suppressor for cations, where the acidic mobile phase is converted to water, and the analyte cations are converted to highly conducting hydroxide forms (due to higher equivalent conductance of hydroxide ions compared to other anions).

The reduced background conductance and the enhanced signal due to the ionic species result in a significantly enhanced signal-to-noise ratio for the conductometric detection of ions in suppressed IC. This results in reduced baseline noise while increasing the sensitivity and reproducibility of the analysis. Commonly used suppressors can be classified into two categories. In the first type, the reactions occur across an ion-exchange membrane with the regenerant ions furnished by either a chemical or as products of electrolysis of water. In the second type, the suppression reactions occur in a packed bed of high-exchange capacity resin or monolith material, with regeneration either by a chemical or by electrolysis of water.

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### PULSED/INTEGRATED AMPEROMETRIC DETECTION (PAD/IPAD)

PAD and IPAD are modes of amperometric detection that apply more than one potential to the working electrode. These detection modes are commonly used for the detection of electroactive species, e.g., organic compounds such as carbohydrates, sugar alcohols, amino acids, amines, and organic sulfur species that can be easily oxidized. Analytes are detected by an oxidative desorption process at the surface of an electrode located in the column effluent stream. PAD uses one potential for detection while IPAD uses multiple potentials. The current generated during the fixed time periods these detection potentials are applied is integrated to yield charge. Following detection, a series of potentials are applied for fixed time periods to clean the electrode surface. Unlike conventional direct current amperometry that suffers from electrode surface fouling, this rapidly repeating sequence of potentials for detection and electrode cleaning, referred to as a waveform, allows detection and removal of the products of redox reactions from the working electrode surface.

### UV DETECTION

Direct UV detection is used for inorganic and organic ions that absorb UV light, typically at low wavelengths. These include organic acids, bromide, iodide, nitrate, nitrite, thiosulfate, and cyano-metal complexes. Indirect UV detection uses eluents that strongly absorb in the visible or ultraviolet spectral region. A wavelength is selected where the eluent absorbs but the sample ions do not, and then negative peaks proportional to the analyte concentration are detected. In spectral detection after post-column reaction, some analytes are detected after the column effluent is combined with a reagent resulting in the formation of a compound that absorbs light at either a UV or visible wavelength. A classic example is the determination of metal ions, where the metal ions in the column effluent are chelated with 4-(2-pyridylazo)-resorcinol followed by detection at 510 and 530 nm.

### MASS SPECTROMETRY (MS)

Typically, analytes are detected after they have first passed through a suppressor to make the resulting effluent compatible with the mass spectrometer. Negative mode electrospray ionization is used for anions while the positive mode is used for cations. The suppressor effluent is sometimes augmented with an organic solvent to improve ionization for increased sensitivity. Certain metal ions can be determined by an ion-exchange separation followed by ICPMS.

## SAMPLE PREPARATION

Sample preparation may range from simple sample dissolution or dilution to the proper concentration, often followed by filtration, and in other cases more complex preparations need solid-phase extraction (SPE). If the solution is cloudy and/or contains particulates, then filtration through a syringe filter of 0.45-µm pore size that is suitable for IC is needed. Samples containing a high concentration of ions of the same charge as the target analyte may require a sample pretreatment to selectively remove the high concentration ion.

## PROCEDURE

The choice of mobile phase is typically dictated by the choice of column, which in turn is chosen based on the selectivity for the analyte(s) compared to other ions of the same charge known or likely to be present. In situations where the other ions are in high concentration, a column with higher capacity is chosen to prevent column overload. This is especially important for many limit tests, where low concentration of a target analyte is in the presence of a large concentration of another ion of the same charge state. For anion IC, some mobile phases can be prepared from the solid or from commercial concentrates or ready-to-use solutions, e.g., sodium bicarbonate/carbonate. Other mobile phases should be prepared and handled with care, e.g., sodium hydroxide solutions, minimizing air exposure, and prepared from 50% sodium hydroxide solutions. Sodium hydroxide pellets and commercial dilute solutions contain large amounts of carbonate, thus altering the desired composition of the mobile phase. The acid solutions for cation IC are prepared by diluting high-purity concentrated acids. Alternately, carbonate/bicarbonate, hydroxide, and methanesulfonic acid mobile phases can be produced by an eluent generator. Most analyses will require the injection of 5–50 µL of sample solution, but larger volumes may be required for the analysis of low concentration analytes. As in other LC techniques, quantification is made by either internal or external standardization procedures, where the concentration is calculated by interpolation of the sample response into a calibration curve. IC methods are validated according to the recommendations described in *Validation of Compendial Procedures* ⟨1225⟩.

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# ⟨1066⟩ PHYSICAL ENVIRONMENTS THAT PROMOTE SAFE MEDICATION USE

## INTRODUCTION

The physical work environment in healthcare settings is one of the most important potential contributors to medication errors. Data on medication errors reported to the United States Pharmacopeia (USP) Medication Error Reporting Program (2008) show that various physical attributes of the workplace affect human performance on the job. These attributes may help or hinder healthcare providers as they strive to deliver safe, high-quality care to every patient. As noted by the Institute of Medicine (IOM) in its landmark 2001 report, *Quality Chasm*, "Health care today harms too frequently and routinely fails to deliver its potential benefits."

This general chapter describes evidence-based standards for creating and maintaining a physical environment that supports and promotes accurate, safe medication use. The process of safe medication use involves multiple aspects, or stages: procurement, prescribing, transcribing, order entry, preparation, dispensing, and administration, as well as monitoring the medication's effects on the patient. A better understanding of these processes can form a solid foundation for improvement, allowing healthcare providers to reach optimal performance within the medication use system in various practice settings. This general chapter focuses on the characteristics of the physical environment that can promote accurate medication use. Standards are provided when justified by evidence and expert opinion, and a glossary of terms used in this general chapter is provided.

## ORGANIZATIONAL SUPPORT

The leadership of a healthcare organization typically determines the operations, policies, and procedures that influence safety. The leaders also establish the workplace design and cultural environment, and they have a powerful influence on the espoused safety culture versus the enacted safety culture. For example, the organizational hierarchy can either enhance safety by encouraging the free flow of information and reinforcing safety behaviors, or can discourage and downplay any safety initiatives. Two reports are available that document issues related to organizational actions and culture as they affect patient safety and care: The IOM's 1999 report, *To Err is Human*, and the IOM's 2006 report, *Preventing Medication Errors*.

Accreditation agencies such as The Joint Commission (TJC), the National Integrated Accreditation for Healthcare Organizations (NIAHO), and the International Organization for Standardization (ISO) emphasize the roles of the organization and leadership in determining safety standards. TJC defines the critical function of leadership and holds the leaders accountable for their systems and processes, whereas NIAHO and ISO call for management review of both quality and safety by the governing body, with the goal of promoting high-quality, safe healthcare.

Organizational influence can be understood as falling somewhere on a continuum ranging from prescriptive controls, in the form of rules and procedures, to discretionary controls, which may rely solely on the individual's experience, according to James Reason (*Human Error*, 1990). Organizational controls that foster a safe environment should include, at a minimum, the reporting, analysis, and intervention plans necessary to shore up defenses against adverse medication events. Systems analysis of errors should include the organizational influences that ultimately affect the workforce at the unit level.

Leadership that is supportive and empowering, at all levels of the organization, should result in optimal physical work environments that help to prevent medication errors and promote accurate medication use. This approach can improve the performance of all persons involved in the medication use process. An organization that aligns leadership goals and resources to a strategic safety focus based on evidence will create safer systems for medication delivery. Although hospitals have been "designed" for patient care, there is a growing body of research that points to evidence-based design (EBD) for safe delivery of care. Other high-risk industries with a strategic safety emphasis share common characteristics, including the use of robust processes for improvement throughout organizations; this can lead to interventions based on scientific data and evidence.

## MEDICATION SAFETY ZONES

In the medication use system, the degree of accuracy and safety accomplished is the end result of many interactions between humans, their physical work environments, and the equipment and technology they use. It is important to focus on areas of the work environment where (1) medications are prescribed; (2) orders are entered into a computer or transcribed onto paper; and (3) medications are prepared/compounded, dispensed, and administered. These work areas are commonly referred to as medication safety zones (MSZ). Examples include the work areas around a medication cart on a nursing unit; any location where prescribing decisions are made; the work space of an automated medication dispensing device; a pharmacy where prescriptions are prepared, inspected, and dispensed; and areas in patients' homes where medications are prepared, consumed, or administered. The patient's bedside in the healthcare facility or home is another important MSZ that presents unique challenges.

The physical environment of any procedure room deserves special attention because of the increased noise levels, variable illumination, and other distractions. In this challenging setting, providers are making critical medication decisions that can influence whether a patient lives or dies. Also, medication safety zones should include the provision for areas that allocate space to incorporate equipment for the safe disposal of sharps (needles and syringes). Overall, in any MSZ, it is important to ensure that appropriate quantities of supplies are maintained; product expiration dates are checked routinely; and temperature-sensitive products are kept in the correct storage conditions.

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## Principles From Human-Factors Research

The research literature describes several principles that may be useful in planning the physical environment of the MSZ.

### IMPORTANCE PRINCIPLE

Within the MSZ, important components should be placed in convenient locations. For example, information systems should be accessible within or near the MSZ so that drug information and patient data (e.g., lab results and vital signs) are readily available. Instructions about equipment function and troubleshooting should be located directly on or near the equipment to provide a quick answer to questions that arise.

### FREQUENCY OF USE PRINCIPLE

Supplies and equipment that are used frequently should be easy to find and accessible. This reduces the likelihood that staff will create workarounds, in which suboptimal equipment/supplies are substituted for the recommended items.

### FUNCTION PRINCIPLE

Displays, controls, or supplies related to a specific function should be grouped together. For example, syringes, needles, and alcohol swabs could be stored together in one drawer, and IV tubing and connectors that are used to prepare infusions could be placed together on the same shelf.

### SEQUENCE OF USE PRINCIPLE

Items should be placed in an order that reflects the sequence of steps needed to perform the task correctly. For example, sterile gloves are in or with sterile dressing kits, and the items are arranged in sequence. This allows personnel to complete tasks quickly and correctly.

## Bedside Workstations

Methods for performing workplace analysis are available. One important aspect is the medication administration areas at the bedside (in healthcare facilities or the patient's home), which should follow the same design as the centralized MSZ. Distractions are an even greater challenge at the bedside, and measures should be taken to minimize them whenever possible. Information and supplies should be kept accessible (according to the human-factor principles already discussed) and placed in an uncluttered area with adequate lighting. Sharps containers should be placed within easy reach for the provider, but out of high-traffic areas. Each bedside work station should be standardized in design, so that information and supplies do not need to be brought along when moving from one patient bed to another.

## Lean Production

Maintaining an efficient, effective workplace reduces the likelihood of errors. One efficient approach to workspace design uses lean-production techniques to enhance desirable, value-added activities while eliminating undesirable activities that lead to waste during work processes.

Three lean-production techniques are as follows: (1) *Visual Controls*, i.e., keeping work processes and indicators in view so that personnel can see the status of tasks at a glance; (2) *Streamlined Layout*, i.e., optimizing the sequence of work processes through facility design; and (3) *Point-of-Use Storage*, i.e., storing supplies at the location where they will be used whenever possible so that personnel can perform tasks more efficiently.[1]

## Simplification and Standardization

Making changes that simplify and standardize the patient-care environment decreases the cognitive load, reducing the likelihood of slips and lapses during routine tasks by minimizing decision and manipulation time. Standardization can be used for facility and room design, medical equipment, and medication-related functions (e.g., medication delivery, storage of patient-specific medications). Ensuring ready access to clinical information that is specific to the patient (or the drug) is essential for all areas in which personnel implement steps in the medication-use process.

## Innovative Solutions

Another approach to optimizing the design of the MSZ is to involve personnel who perform the tasks on a routine basis. Workers may suggest innovative solutions to work station problems. To support innovation, it is advisable to incorporate flexibility into the MSZ design.

[1] Sanders MS, McCormick EJ. *Human Factors in Engineering and Design*. New York: McGraw-Hill; 1993.

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## Forcing Functions

Constraint and forcing functions are an effective means of preventing errors, particularly for high-risk medications and situations. Forcing functions do not always refer to device design. The simplest of these do not require technology. One of the first forcing functions identified in healthcare was the removal of concentrated potassium from hospital units to eliminate the risk of inadvertent preparation of intravenous solutions with concentrated potassium, an error that has caused a small but stable number of deaths over the years.

Other examples would include sealing neuromuscular blockers in an intubation kit which lowers the chance of a paralyzing agent being administered to a patient without having access to ventilation support. Additionally, packaging an enteral product so it is physically unable to connect to an intravenous tubing luer lock connector would avert a wrong route error, even if the nurse was working in low-light conditions and initially misidentified the intended route for the tubing. The availability of medication safety technology is never a substitute for safe medication practices within an MSZ. Reports have warned of errors that result from ignoring or overriding safety checks, such as smart infusion pump drug libraries and alarms.

## Work System Elements

### CHARACTERISTICS OF INDIVIDUALS

Characteristics of the individual performing the work include his or her experience level, age, visual and hearing acuity, distractibility, and level of attention. Humans vary in their responses to the physical environment. In an ideal scenario, the physical environment could be modified on an individual basis. In that way, the environment could be adapted to match the needs of the current user, thereby optimizing the accuracy of his/her performance. Alternatively, the environment should be as supportive as possible to as wide a range of capabilities as possible.

## SOUND AND NOISE

The Environmental Protection Agency (EPA) recommends peak sound levels of 45 dB during the day and 35 dB at night in hospitals. The World Health Organization (WHO) guidelines state that background sound levels in a patient room should not exceed 35 dB. The International Noise Council recommends maximums of 45 dB during the day and 20 dB at night for acute care areas. Ear protection is required when workers are exposed to sound levels averaging 90 dB.

The standard for sound levels in MSZs is set at 45 dB. This is intended to ensure that critical verbal information can be heard accurately. Healthcare providers should be sensitive to their individual need for quiet, depending on the task being performed, and they should have a quiet area available to promote accurate performance. The total elimination of noise in patient-care settings is not feasible or desirable. Patient counseling areas in pharmacies should include sound-reduction methods to enhance audibility and learning, for example, the use of a closed room.

Noise is recognized as a serious health hazard to hospitalized patients, and as a source of interference with effective work performance. Most studies of the effects of noise in the work environment have been conducted in non-healthcare settings. However, noise levels as a factor contributing to stress for nurses is increasingly being documented. In healthcare facilities, sources of noise can range from overhead paging systems, equipment alarms, heating, ventilation, and air-conditioning (HVAC) systems, plumbing, televisions, and radios to ice machines. Noise has been cited as one obstacle to the effective performance of nurses. An in-depth study developed a noise map of a hospital, and found sound levels of 55 dB, which is 10–20 dB above EPA recommendations, depending on the time of day. Average sound levels in other hospitals have been measured between 45 and 68 dB, with peaks between 85 and 90 dB. A study of sound levels during shift changes measured 113 dB.

The following sound-related features may affect accuracy when dispensing medication: predictability of the sound; controllability of the sound; type of task (simple vs. complex); multitasking; and distraction due to noise (which may mask environmental cues and the worker's internal voice, used to rehearse and recall important tasks). Out of 58 studies, 7 showed that noise improved performance, while 29 showed that it impaired performance. Unpredictable but controllable sounds and noise were found in one study to improve prescription filling accuracy, contrary to previous research. This may indicate that some environmental stimuli are needed to maintain proper alertness and attention of workers. Researchers are attempting to identify optimal levels of arousal due to sound and noise for people performing different kinds of tasks (e.g., Yerkes–Dodson law).

Noise and other sensory interference can be reduced by employing activities, tools, and principles developed by human factors and engineering experts; many of these principles are already being used by some healthcare organizations. The effects of these and other design improvements for nursing workspaces on patient outcomes and facility performance are being studied as part of a research project (http://www.healthdesign.org/pebble) sponsored by the Center for Health Design, a nonprofit research and advocacy organization. The project has found decreases in medical errors, as well as reductions in patient transfers, nosocomial infections, patient falls, and medication usage. When permitted by infection control guidelines, reducing noise by installing materials that absorb sound (e.g., ceiling and wall materials, and carpeting) can be accomplished at modest cost. Acoustical engineers can provide additional methods for noise reduction. Workers who don't have to respond to any audible signals such as telephone calls or alarms may be able to wear noise-canceling headphones and listen to music, provided that performance is not adversely affected.

## ACTIVITIES AND TASKS PERFORMED

When designing an optimal physical environment that promotes accurate medication use, one should consider the activities and tasks that need to be performed. A poorly designed environment or other adverse conditions in the work area can lead to an unsafe adaptation of the procedures. For example, practitioners who have to listen to the constant barrage of equipment alarms that are not set within appropriate parameters (and are producing too many false alarms) may silence the alarms to

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reduce the noise. However, this limits the practitioner's ability to monitor the safety of the system. Similar situations occur with visual alarms and computer messages that are often ignored or overridden. In general, activities and tasks should be structured so they can be performed with the least amount of difficulty. If workarounds are detected, they should be investigated as an indication that the procedure or workflow is not suited for the task.

### Multi-Tasking

The term "multi-tasking" originated in the computer engineering industry; it is the ability of a person to perform more than one task simultaneously. The human brain is wired differently in every individual; therefore, not everyone can multi-task successfully. The need to multi-task is common in all areas of the healthcare environment, whether on a nursing unit, in a pharmacy, in an operating room, or in a special procedure area. Some people can focus on performing more than one task at a time, whereas others cannot. In the human brain, multi-tasking involves linearly switching back and forth among different activities while actually completing one task at a time.

This can be problematic in healthcare settings, particularly if something unexpected happens in relation to one task while the individual is focused on the other task. These interruptions and distractions can lead to medication errors and other forms of patient harm. Another type of multi-tasking error is related to "mental stacking," that is, when several items are under consideration at the same time. An excessive cognitive workload can lead to unintended consequences, and it is important to note that not all practitioners are able to handle established systems and processes in a particular physical environment.

Pharmacists and nurses are practitioners who typically multi-task. Although most prescriptions are prepared or administered one at a time, this does not mean that multi-tasking cannot occur. The work area should be designed to keep individual prescriptions separate while accommodating multi-tasking. Individuals vary in their abilities to perform sequential tasks versus multi-tasking. Currently there is not enough evidence to evaluate an individual's fitness to perform multi-tasking in healthcare settings, but human factors research in controlled laboratory settings suggests that in the future, it may be possible to assess and augment this skill in healthcare providers.

## TOOLS AND TECHNOLOGIES IN THE PHYSICAL ENVIRONMENT

In the healthcare setting, tools and technologies that are used to perform the tasks can also interact with the physical environment to affect the likelihood of medication errors. Therefore, the design of the environment should account for the specific tools and technologies that individuals will use to complete their work. For instance, the physical layout of an operating room should accommodate the size, maneuverability, and required spacing of the many devices used during surgery. Tools and technologies should also meet ergonomic design standards.

Another example is that labels on medications are sometimes too small to display instructions for preparation, dispensing, or administration; when this occurs, alternatives should be considered. One alternative is to include Quick Response (QR) codes on the labels, which allow healthcare personnel with a web-enabled device (such as an iPad, iPhone, or Android-powered device) to access prescriptive information and handle the product correctly. QR codes are two-dimensional bar codes that, when read by an imaging device, bring the user directly to the website containing the needed information.

Additional factors that should be considered include equipment malfunction, downtime, and vibration.

**Equipment malfunction:** Any equipment that malfunctions, is damaged, or is lost should be reported to the supervisor immediately. In the case of malfunction, a decision should be made either to repair or to replace the item as soon as possible. In many cases, the item can be replaced rapidly, but some pieces of equipment take longer to disassemble and reinstall. Personnel should confirm that the equipment is in good working order before and after each use. A routine program of confirming equipment function should be in place.

**Equipment downtime:** The term "downtime" refers to periods of time when a system or item of equipment is unavailable. The outage, or downtime duration, is the length of time that the equipment fails to perform its primary function. The concept of downtime is commonly applied to computer networks and servers but can also be used in other environments when discussing equipment failures. These failures can occur for various reasons, including damage; design flaws; procedural error, i.e., improper use by humans; engineering issues, i.e., how the equipment is used and deployed; overload occurring when system resources are stressed beyond capacity; environmental problems with support systems such as heating, ventilation, and air conditioning (HVAC) and power; and scheduled downtime for maintenance, upgrades, or expansion. Service agreements are a common strategy for minimizing downtime and outages. Procedures should be in place to address downtime.

**Equipment vibration:** Vibration is a mechanical phenomenon in which oscillations occur around an equilibrium point. Although vibration may be desirable, usually it is undesirable, wasting energy and creating unwanted noise. As examples, the vibrational motions of engines, electric motors, or any mechanical device are typically unwanted and may result from imbalances in the rotating parts, uneven friction, imperfect meshing of gear teeth, and other factors. Careful design and proper installation can usually minimize unwanted vibrations.

## DISTRACTIONS AND DISTRACTIBILITY INDEX

The healthcare work environment is replete with distractions and interruptions that can adversely affect work performance and lead to medication errors. Architects, engineers, and other professionals who play a role in workplace design have a responsibility to be educated about, and keenly aware of, this issue so they can design safe, ergonomically ideal workspaces. This approach should have a significant beneficial impact by counteracting the negative effects of interruptions and distractions on patient care. In general, MSZs should be located in areas where the potential for distraction and interruption is minimized. Nurses frequently cite distractions and interruptions as contributing to the incidence of medication errors. Distraction from competing tasks is likely to impair performance in several ways, such as sensory/perceptual interference (e.g., the nurse doesn't hear the alarm because a coworker interrupts with a question), cognitive cost of switching tasks (the nurse responds to an alarm

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more slowly because it takes time to reorient to the alarmed task after a coworker's question), or prospective memory failure (when returning to the task after the interruption, the nurse omits a step because of forgetting where he/she left off).

In addition, interruptions and distractions have been linked to higher rates of prescription dispensing errors in an ambulatory pharmacy. According to the 2008 USP MEDMARX Data Report, distractions continue to play a major role in medication errors, identified as a contributing factor in 45% of all medication errors in hospitals and health systems. However, interruptions and distractions can be prevented or reduced by giving staff the ability to control and manage their exposure to these disturbances. Personnel can be allowed to adjust features of the MSZ to maximize their concentration and attention levels and to optimize their performance. Adjustable features include a work station that is protected from interruptions and distractions, such as a separate medication room, versus a mobile cart with workspace for those that are not adversely affected by distractions. Individuals have different levels of distractibility, and should be sensitive to their own need for a distraction-free work area. Heightened self-awareness of the adverse impact of interruptions and distractions can help minimize problems. Also, workers can be trained in how to avoid interrupting coworkers for non-urgent requests, particularly while their coworkers are performing medication-related tasks. In a pharmacy study, the most frequent source of interruptions was coworkers asking for assistance.

Techniques that may be effective for decreasing interruptions and distractions include visual cues (such as wearing orange safety vests) and physical barriers, such as preparing doses in a medication room. Personnel can also use checklists to focus or refocus their own attention on the task. Scientific research about distractions and interruptions in the physical environment is limited; therefore, additional studies are needed to identify evidence-based corrective actions that will promote safe medication use.

## PHYSICAL ENVIRONMENT ELEMENTS

Factors in the physical environment, such as lighting, noise, temperature, layout, and workstation design, interact with human and task-related factors to influence the accuracy of medication use. The remainder of this general chapter focuses on providing guidance on the optimal environment to improve the performance of persons involved in the medication use process.

## WORK SYSTEM DESIGN

According to the Systems Engineering Initiative for Patient Safety (SEIPS) model, five work systems—humans, tasks, technology and tools, organization, and environment—interact to affect employee, organization, and patient outcomes. Because these five work systems are closely interrelated, designers should consider all of the systems. Whenever one work element changes, there will be implications for the other elements. The entire work system needs to be well designed to optimize performance and ensure positive outcomes. Although this general chapter describes all the work systems, it focuses on recommendations for the physical environment.

### Evidence-Based Design

Evidence-based design (EBD) is a field of study that draws upon the most reliable research data in planning the design of the workplace environment and equipment. EBD is closely related to the systematic hierarchy of evidence-based practice, and uses a systematic process of evaluating scientific research as the basis for design decisions that enhance human performance and reduce stress in the complex environment of healthcare. EBD intersects and combines the domains of ergonomics, human factors, usability, and cognitive psychology to arrive at the best possible work environment.

EBD principles are available to inform environmental design choices. In 2008, Ulrich and associates published a review of the research literature on evidence-based healthcare design, concluding that "The evidence indicates that well-designed physical settings play an important role in making hospitals safer and more healing for patients and better places for staff to work." For example, major advances such as reduced infection rates have been linked to the design and location of sinks for hand washing, proper ventilation, and suitable materials for surfaces.

Application of EBD principles to equipment design is just as essential as applying these principles to the work environment. The use of EBD for intravenous pumps, feeding tubes, and patient-controlled analgesic devices has been described in recent years. Research continues to support the use of standardized fonts and labels for drug identification, as well as EBD for medication storage areas. In each decision regarding the complex healthcare work environment, stakeholders should draw upon the most substantial research evidence available and use it as a major guiding factor. Objects such as furniture, cabinetry, and fixtures should be selected after careful consideration of EBD goals and research. For example, it is ideal if furniture can be configured to create a sense of privacy and minimize visual and auditory distractions during medication transcription, preparation, dispensing, and administration. Furniture should be adjustable to meet a worker's ergonomic needs. In addition, surface contamination can be reduced by selecting nonporous surface materials with no joints or seams, allowing for ease of cleaning.

## CHALLENGES IN THE PHYSICAL ENVIRONMENT

### Ordering and Transcribing

Errors in ordering medications or transcribing medication orders are well documented in the research literature. Because errors made during these tasks can directly endanger patient safety, ordering and transcribing should be treated as high-risk activities within the healthcare environment. Manual methods of ordering and transcribing are dependent on human performance. Errors may result when the reader misinterprets handwriting flaws, abbreviations, or decimal points, or when

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there is frank misreading of handwriting. Faxed orders involve additional concerns, because legibility may be poor after transmission and printing.

Computerized systems for ordering, known as computerized physician order entry (CPOE), and transcription of orders by electronic means are not completely protected from the possibility of error. For example, electronic healthcare records have been cited for failures in usability and visual display, and may lack the customization necessary to order for specific populations, such as pediatric patients. Although the electronic transmission of data is considered more secure from errors of interpretation, the addition of decision support in CPOE has not been established as an evidence-based intervention that assures safety. At all times, applications of technology should undergo the same degree of scrutiny as other methods to prevent medication errors.

## Preparation and Compounding

The preparation/compounding work area should be designed, arranged, and maintained to facilitate high-quality compounding that meets the standards of *Pharmaceutical Compounding—Nonsterile Preparations* ⟨795⟩ and *Pharmaceutical Compounding—Sterile Preparations* ⟨797⟩. The area for sterile drug preparation should be separate from the area for nonsterile drug preparation (see ⟨795⟩ and ⟨797⟩). Traffic in the preparation/compounding area should be kept to a minimum, with only authorized individuals allowed access. Lighting, temperature, and ventilation should be optimal to prevent decomposition of active pharmaceutical ingredients (APIs), excipients, and drug products. These measures can also ensure a workplace where personnel are not distracted by physical discomfort.

Humidity should be monitored and controlled, because drugs tend to degrade in the presence of moisture. Because of its effect on drug stability and integrity, the humidity in compounding and storage areas is second in importance only to temperature (see ⟨795⟩ and ⟨797⟩). A hygrometer should be placed on an interior wall, but not near an air-handling return, hot plate, or door entrance/exit, so that it will provide a representative reading of the relative humidity in the compounding area or storage facility.

The materials used for the floor, walls, shelving, cabinets, and ceiling should not retain dust, odors, or residues from the compounding activities. Also, the area should be free of dust-collecting overhangs (e.g., ceiling pipes, hanging light fixtures, and ledges). The actual work area should be level, smooth, impervious (free of cracks and crevices), and non-shedding, and the shelving and cabinets should be easy to clean.

Hazardous drugs should be prepared, stored, and handled by appropriately trained personnel under conditions that protect the healthcare workers and others who may come in contact with these drugs. Disposal of all hazardous drug wastes should comply with the applicable federal and state regulations. Any workers who perform routine custodial waste removal and cleaning activities in the storage and preparation areas for hazardous drugs should be trained in appropriate procedures to protect themselves and prevent contamination of the physical environment. This training should include procedures to be followed in the event of a spill.

For sterile compounding, the primary engineering control (PEC) unit should be placed in a location that will minimize noise and avoid conditions that could adversely affect its operation. For example, strong air currents from opened doors, personnel traffic, or air streams from the HVAC systems can disrupt the unidirectional airflow in open-faced workbenches. The PEC should be placed out of the traffic flow and in a position that will avoid disruption from the HVAC system and room cross-drafts. In general, all equipment should be selected and installed to create optimal working conditions, with minimal noise and sufficient lighting, so that personnel can perform accurate, high-quality work (see ⟨797⟩).

## Dispensing

There are many potential distractions in dispensing areas of pharmacies and other areas where medications are dispensed. These distractions may include ringing telephones and interruptions from patients, other staff, visitors, and others. Distractions should be minimized or eliminated to the greatest extent possible. Although dispensing functions often require multi-tasking, it is optimal that only one prescription or medication order be prepared, processed, and checked at a time. The final check should be done by a pharmacist or an authorized, licensed professional in an area that is free of distractions. Additional points include the following:

- Proper illumination is necessary because the labels on some containers have very small or lightly colored print.
- Noise levels should be kept to a minimum (see *Table 2*), which may require moving noisy equipment to a different room, or at least using a partition for separation.
- Odors in the pharmacy setting should be controlled by using appropriate exhaust systems, which should be maintained properly and monitored periodically. Pharmacies contain a combination of odors resulting from the storage, preparation, and dispensing of the drugs themselves; odors also emanate from other items such as disinfecting agents.
- Temperature should be maintained at appropriate levels for the comfort of pharmacy personnel and for the stability of the pharmaceuticals.
- The pharmacy workspace should be designed to enhance workflow efficiency and to maintain safety throughout the entire dispensing process.

## Administration

Some critically important MSZs for nurses include the medication preparation and administration areas. Information should be readily available in a user-friendly format that will facilitate the practitioner's synthesis of facts and data. Access to medication-related information should be efficient, with materials and records available at the proper sites. For example, a nurse may need both drug information and patient-specific data to make a decision about drug administration; therefore, these two sources should be near each other to support fact finding and/or decision making. The various information components within the space should be arranged according to specific principles that decrease distractions when seeking information and making decisions. Nurses also need to be aware of additional factors when administering medications, e.g., illumination, clutter, odor, noise levels, and temperature.

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### THE HOME ENVIRONMENT

The home healthcare environment differs from hospitals and other institutional environments in some important ways. For example, clinicians recognize that the homecare setting is the private domain of the patient. Thus the care they provide to each patient should be unique and individualized, based largely on that home setting. There may be situational variables in the patient's home that present risks to the patient and are difficult or impossible for the clinician to eliminate. For instance, hospitals have environmental safety departments to monitor air quality, as well as designers/engineers to ensure that the heights of stair risers are safe, but home healthcare providers are not likely to have the training or resources needed to assess and ameliorate such risks in the patient's home.

Yet the patient and caregiver still can promote a safe home environment by establishing proper storage, organization, and accessibility of medications, as well as adequate lighting in areas where drugs are administered. Several other physical elements also should be assessed to facilitate safe handling and administration of medications. There should be reliable telephone service to the home, an adequate area to prepare and administer medications (e.g., parenteral infusions), and intact, safe electrical outlets for any required equipment. All medicines should be stored in ways that facilitate both medication safety and compliance. The storage locations should be dry and cool. Medications needing refrigeration should be stored in areas where they will not freeze. All medications, whether prescription or over-the-counter, should be kept out of reach of children, pets, or mentally altered/impaired adults.

## PHYSICAL ENVIRONMENT FACTORS

Sensory interference resulting from extreme temperatures, noise, poor lighting, glare-producing surfaces, interruptions, or clutter can adversely affect the working memory and job performance of healthcare practitioners. The guidelines described here for the physical environment should be applied to MSZs.

### Illumination

Proper illumination levels can improve both accuracy and efficiency of performance. Prescription-filling accuracy improved significantly, from 96.2% to 97.4%, when lighting levels in a busy outpatient pharmacy were increased from 450 to 1460 lux (45–146 fc). One study found that pharmacists who rated lighting levels as at least adequate detected 38% more errors when filling prescriptions. In addition, as visual fatigue increases over a shift, increased light is needed. Pharmacists using task lights to increase illumination had a 10.7% reduction in product verification errors. A study of luminance in homes, offices, and public places found lower levels than recommended for reading, and also found that performance was related to age. Efforts should be made to prevent medication errors caused directly or indirectly by low lighting. For example, one incident report showed that poor lighting contributed to improperly connected patient-controlled analgesia (PCA) administration tubing, causing medication to run onto the floor, resulting in uncontrolled patient pain. Low lighting contributed to difficulty in seeing that the tubing was not connected properly. A study of lighting in a retail pharmacy revealed an error in strength and dosage form as dicyclomine 10-mg capsules were used to fill a prescription for 20-mg tablets. The light level at the shelf where the medications were stored was 220 lux (22 fc).

The recommendations described here take into consideration the need to work quickly and accurately during medication handling, the level of task visibility, and the comfort of personnel. Architects and lighting engineers can consult the Illuminating Engineering Society of North America (IESNA) reference "Lighting for Hospitals and Healthcare Facilities" for details about lighting medication areas. It is important to note that the illuminance levels recommended in the IESNA reference are below those listed in this guidance; the latter are higher because of evidence that lighting levels are inversely related to medication errors. Fluorescent cool white deluxe lamps or compact fluorescent lamps are recommended, because they have a color rendering index of 80 or more and are highly efficient compared to incandescent lamps. Providing the recommended color rendering index can help avoid misidentification of medications.

If illumination levels are below recommendations, task lighting is required in areas where critical visual tasks are performed. If task lighting is not available, then workers can cast shadows on the workspace, further reducing lighting levels. Critical tasks include reading handwritten prescriptions and small print on labels and inspecting medication dosage forms. Because individuals perceive lighting levels differently, adjustable 50-watt high-intensity task lights are recommended for situations where personnel encounter difficult-to-read prescriptions and product labels such as unit-dose package labels. Lighting levels are important for key healthcare providers involved in computer order entry (e.g., physicians or pharmacists), prescription filling, inspection, and patient counseling. Illumination levels for computer order entry areas should be at least 750 lux (75 fc). Higher levels [1000 lux (100 fc)] are recommended when handwritten orders are read.

Lighting should be positioned so there is no glare on the computer monitor that may make it difficult to read the screen accurately. Prescription preparation areas, medication inspection stations (for double-checking), and counseling areas should have illumination levels between 900 and 1500 lux (90–150 fc). These standards are all above the minimum of 200 lux (20 fc) for accurate reading of medication labels set by the American Society for Testing and Materials International (ASTM International). An ASTM International standard includes a legibility test requiring that the name and amount of the drug on the label be legible in 20 fc of light at a distance of about 20 inches (500 mm) by a person with 20/20 unaided or corrected vision. Lighting levels should be increased where the work force has an average age beyond 45 years to optimize legibility (general recommendation for treatment of presbyopia). Healthcare providers should also consider having a magnifying glass available to assist in the careful reading of labels with very small print and in situations where low lighting levels are unavoidable. Pharmacists using a magnification lens along with a task light reduced errors in product verification by 22% compared to a control group.

For nurses, key medication-related tasks that require good lighting include medication order review and medication selection, preparation, and administration. These tasks may take place in one or more locations on the nursing unit, such as the nursing station where patient charts are stored, the medication room, or a patient's room. Transitional lighting is recommended for

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medication areas on nursing stations and other patient care units to avoid dark and bright spots located next to dimly lit areas. Luminance should enable good color rendering (i.e., color rendering index of 80 or more) to assist with proper medication checking. Task lighting can help achieve appropriate levels of lighting and should be included on mobile medication carts (including those used with bar code medication verification systems). Glare on computer monitors should be controlled by ensuring that there are no light reflections that can wash out the screen and make it difficult to read.

Illumination levels for medication rooms located on nursing units should be at least 1000 lux (100 fc) based on the complexity of the task (e.g., reading small type on medication packages) and the need for accuracy and speed. The higher range of the lighting level should be used when the task requires reading small print. Lighting level recommendations are summarized in *Table 1*. Over time, lighting levels can decrease (e.g., by 25% over a 2-year period), so it is important to clean lighting fixtures routinely in order to maintain recommended luminance levels. Lighting levels should be measured on a quarterly basis. Burned out or flickering bulbs should be replaced promptly.

**Table 1. Lighting Level Recommendations for Healthcare Settings**

| | Illumination Level | |
|---|---|---|
| Work Area | Lux | Foot-Candle (fc) |
| Computer order entry | 1000 | 100 |
| Handwritten order processing | 1000 | 100 |
| Medication filling and checking (pharmacy) | 900–1500 | 90–150 |
| Patient counseling (pharmacy) | 900–1500 | 90–150 |
| Sterile compounding and preparation | 1000–1500 | 100–150 |
| Pharmacy medication storeroom | 500 | 50 |
| Medication preparation area (e.g., nursing station) | 1000 | 100 |
| Medication administration work area (e.g., cart surface, patient room) | 1000 | 100 |

Proper lighting is also essential at the point of care. Attempting to be patient- and family-friendly may run contrary to the necessary lighting conditions for safe medication administration. If practitioners administer medication at night under low luminance to avoid disturbing the patient or family, this an unsafe practice. Task or spot lighting should be available so practitioners can visually confirm that they have the correct patient (by reading armband or other identification technology), and so the medication and administration site is not compromised. Compact fluorescent lamps take time to come to the correct lighting level. Therefore, no critical tasks should be performed until the light is working at its rated level.

## Sound and Noise Recommendations

For hospitals, the Environmental Protection Agency (EPA) recommends peak sound levels of 45 decibels (dB) during the day and 35 dB at night in hospitals. The World Health Organization (WHO) guidelines state that background sound levels in a patient room should not exceed 35 dB. The International Noise Council recommends limits of 45 dB during the day and 20 dB at night for acute care areas. Ear protection is required when workers are exposed to sound levels averaging 90 dB. Sound level recommendations are shown in *Table 2*.

**Table 2. Peak Sound Level Recommendations**

| Work Area | Source[a] | Sound Level (dB) |
|---|---|---|
| Hospitals (daytime) | EPA | 45 |
| Hospitals (nighttime) | EPA | 35 |
| Patient room | WHO | 35 |
| Acute care areas (daytime) | INC | 45 |
| Acute care areas (nighttime) | INC | 20 |
| Ear protection required | INC | 90 (average) |
| Medication safety zones | — | 50 |

[a] EPA, Environmental Protection Agency; WHO, World Health Organization; INC, International Noise Council.

The standard for sound levels in MSZs is set at 45 dB, which is intended to ensure that critical verbal information can be heard accurately. However, this will be exceeded in many typical pharmacy dispensing and compounding areas. Healthcare providers should be sensitive to their individual needs for quiet, depending on the task being performed, and they should have a quiet area available to promote accurate performance. The total elimination of noise in patient-care settings is not feasible or desirable. Patient counseling areas in pharmacies should include sound-reduction methods (e.g., use of a closed room) to enhance audibility, learning, and privacy.

Excessive noise is recognized as a serious health hazard. Its effect on hospitalized patients and its possible interference with effective work performance by clinicians should be considered. Most studies of the effects of noise in the work environment have been conducted in non-healthcare settings. However, noise levels are being documented more often as a contributor to

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stress for nurses. In healthcare facilities, sources of noise can include overhead paging systems, equipment alarms, heating, ventilation, air-conditioning (HVAC) systems, plumbing, televisions, and radios to ice machines. Noise has been cited as one obstacle to the effective performance of nurses. An in-depth study developed a noise map of a hospital, and found sound levels of 55 dB, which is 10–20 dB above EPA recommendations, depending on the time of day. Average sound levels in other hospitals have been measured between 45 and 68 dB, with peaks between 85 and 90 dB. A study of sound levels during shift changes measured 113 dB.

Accuracy when dispensing medication can be affected by specific characteristics of the sound/noise, e.g., whether it is predictable and whether it can be controlled. These factors may mask environmental cues and the worker's internal voice, used to rehearse and recall important tasks. Out of 58 studies, 7 showed that noise improved performance, while 29 showed that it impaired performance. Unpredictable but controllable sounds and noise were found in one study to improve prescription filling accuracy, contrary to previous research. This may indicate that some environmental stimuli are needed to maintain proper alertness and attention of workers. Researchers are attempting to identify optimal levels of arousal due to sound and noise for people performing different kinds of tasks (e.g., Yerkes–Dodson law).

The adverse effects of noise and other sensory interference can be reduced by using activities, tools, and principles developed by human factors and engineering experts. Many of these principles and tools are already being used by some healthcare organizations. Researchers are studying these and other design characteristics of nursing workspaces to assess their effects on patient outcomes and facility performance; one study is sponsored by the Center for Health Design, a nonprofit research and advocacy organization, and a network of 11 healthcare providers (http://www.healthdesign.org/pebble). The data have shown reductions in medical errors, patient transfers, nosocomial infections, patient falls, and medication usage. Reducing noise by installing materials that absorb sound (e.g., ceiling and wall materials, and carpeting), when permitted by infection control guidelines, can be accomplished at modest cost. Acoustical engineers can provide additional methods for noise reduction. In addition, workers who do not have to respond to any audible signals such as telephone calls or alarms may be able to wear noise-canceling headphones and listen to music, provided that performance is not adversely affected.

## Odor

Areas where pharmaceuticals are stored or manipulated tend to have an odor that is characteristic of the respective drugs. These odors often can be minimized by using appropriate air handling/exhaust systems. Indoor environmental quality (IEQ) can be related to chemicals and other sources. The odors found in almost any working environment may result from caulks, sealants, coatings, adhesives, paints, varnishes, stains, wall coverings, cleaning agents, fuels and combustion products, carpeting, vinyl flooring, fabric materials, air fresheners, and other scented products, as well as personal products of employees (e.g., perfumes, shampoos, and others). If these odors are not controlled, IEQ problems can arise, especially if the building's ventilation system is improperly designed and/or poorly maintained. IEQ can be improved by installing appropriate exhaust systems to remove the odors emanating from drug products. This may also include "snorkel exhaust" facilities close to where the drug products are manipulated, as well as cabinet-type exhaust hoods. Other measures that may be helpful include establishing perfume-free zones, prohibiting smoking, and not allowing food storage or eating in the immediate area. The practice of introducing pleasant odors in the workplace may or may not be beneficial, but managing the response to odors and irritants is critical to maintaining the health and well-being of workers.

## Temperature Controls and Humidity

Temperature control is important for drug stability and for the comfort of personnel. In a room where equipment is present (e.g., laminar air flow hoods), heat tends to be generated, and therefore, appropriate temperature control measures should be designed and implemented.

In some situations such as compounding, it is important to control humidity and thereby minimize static electricity in powders, packaging, and other dosage forms (see ⟨*795*⟩ and ⟨*797*⟩). Humidity control is also important for related technologies, such as computers, tablets, and automated dispensing machines. The core temperature of the central processing unit in computers should be between 10° and 32° C, and the ideal humidity index is between 30% and 50%.

## Physical Design and Organization of Workspace Assessment

The design of the work environment influences the ability of providers to effectively use information and accurately perform tasks. The height of counters, height of shelving for storage of supplies, and lighting changes in lower drawers and cabinets that decrease visibility of products can contribute to errors if improperly adjusted. The effective integration of adjustable fixtures and appropriate counter heights, with the use of mobile carts, can improve efficiency as well as safety. Work counter clutter or lack of sufficient space to perform key tasks is typically an indicator of disorganization and poor planning. One study found that more dispensing errors occurred when medication storage containers were placed on shelves in a cluttered fashion (<1 inch between distinct drugs). When choosing appropriate furniture, an evidence-based-design furniture checklist may be used to make informed investment decisions and improve healthcare outcomes (see *Table 3* for a complete checklist).

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**Table 3. Evidence-Based Design Checklist**

| Findings | Evidence-Based Design Goals and Features[a] |
|---|---|
| | 1. Reduce surface contamination linked to healthcare associated infections<br>A. Surfaces are easily cleaned, with no surface joints or seams.<br>B. Materials for upholstery are impervious (nonporous).<br>C. Surfaces are nonporous and smooth.<br>2. Reduce patient falls and associated injuries<br>A. Chair seat height is adjustable.<br>B. Chair has armrests.<br>C. Space beneath the chair supports foot position changes.<br>D. Chair seat posterior tilt angle and seat back recline facilitate patient egress.<br>E. Chairs are sturdy, stable, and cannot be easily tipped over.<br>F. Rolling furniture includes locking rollers or casters.<br>G. Chairs have no sharp or hard edges that can injure patients who fall or trip.<br>3. Decrease medication errors<br>A. Lighting fixtures should provide 90–150 foot-candle illumination and an adjustable 50-watt high intensity task lamp for furniture with built-in lighting that is used in a medication safety zone.<br>B. Furniture is configurable to create a sense of privacy to minimize visual distractions and interruptions from sound and noise during medication transcription, preparation, dispensing, and administration activities.<br>4. Improve communication and social support for patients and family members<br>A. Furniture can be configured into small flexible groupings that are easily adjusted to accommodate varying numbers of individuals in a variety of healthcare settings.<br>B. Wide-sized and age variations are supported.<br>C. Acoustic and visual patient privacy are supported.<br>5. Decrease patient, family member, and staff stress and fatigue<br>A. Materials suggest a link to nature.<br>B. Appearance is attractive and noninstitutional.<br>C. Furniture is tested for safe and comfortable use by all, including morbidly-obese individuals.<br>6. Improve staff effectiveness, efficiency, and communication<br>A. Furniture is easily adjustable to individual worker's ergonomic needs.<br>B. Design enables care coordination and information sharing.<br>C. Materials are sound absorbing.<br>7. Improve environmental safety<br>A. Materials do not contain volatile organic compounds (VOCs), such as formaldehyde and benzene.<br>8. Represent the best investment<br>A. Reflect and reinforce the organizational mission, strategic goals, and brand.<br>B. Integrate new with existing furniture and objects for facility renovations projects.<br>C. Pieces can be flexibly reconfigured and moved to support changing and emerging missions.<br>D. Provide casters or glides to reduce floor damage.<br>E. Check that there are no protuberances that may damage walls; check chair rail heights.<br>F. Manufacturer provides results of safety and durability testing.<br>G. Manufacturer describes the specific evidence that has been used to design the product.<br>H. Manufacturer includes a warranty appropriate to use, such as furniture used all day, every day.<br>I. Replacement parts are available.<br>J. Repair can be done in the healthcare facility.<br>K. Manufacturer or local dealer can assist with furniture repair and refurbishing.<br>L. Environmental services/housekeeping staff can easily maintain furniture.<br>M. A Group Purchasing Organization (GPO) can be used when purchasing furniture. |
| **Findings Scale:** Present (+), Absent (–), More Information Needed (?), Not Applicable (N/A). | |

[a] Malone EB, Dellinger BA. *Furniture Design Features and Healthcare Outcomes*. The Center for Health Design; 2011 (see https://www.healthdesign.org/chd/research/furniture-design-features-and-healthcare-outcomes).

## METHODS FOR ASSESSING THE PHYSICAL ENVIRONMENT

### Illumination

An illuminance meter, also referred to as a light level meter or photometer, is an instrument that consists of a photodetector (with a digital or analog display) that measures illuminance in lux or foot-candles (fc). Lighting levels should be evaluated in all MSZs using point illuminance measurements. To do this, the photodetector should be placed in the area where the critical medication task is performed (e.g., medication inspection at a work counter), with the worker standing in a normal working position when the measurement is taken. In medication storage areas, measurements should include light levels at the top, middle, and bottom shelves, because light levels depend on the distance from the lighting source. Photometers are commercially available, or management engineers may be able to provide them, and they should be recalibrated annually.

Illumination levels also can be measured using a smart phone "Light Meter" application (app). These apps are easy to use and inexpensive. They are simply downloaded to a Smartphone, and after clicking on the app, the camera function is pointed to a lighted area/surface and the on/off button is pressed. The device takes measurements and also provides a description of

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the activities for which those light levels are appropriate. The apps can be calibrated and the sensitivity adjusted, with readouts in either lux or fc.

### Sound Levels

Sound level meters capable of reading from 30 to 130 decibels on the A scale (dBA) should be used to measure sound levels. The A scale is commonly used when measuring decibels because it most closely represents what the human ear hears in terms of loudness. The meters should be calibrated prior to each use. Measurements are taken while standing in a working position, using the instructions provided in the manual for the specific sound meter. Type 1 or Type 2 meters have acceptable levels of accuracy. Sound levels also can be measured easily and inexpensively by using a Smartphone app. The app is downloaded to a Smartphone, and the readout options are selected. The measurement is activated, and it continues to provide a complete readout of frequency (*x*-axis) and decibel sound pressure level (*y*-axis) in real time.

### Odor Detection

Odors can be detected by individuals, but this approach is very subjective. Some odors indicate the presence of a specific chemical entity or moiety. However, appropriate measuring devices for use in a work environment may not be available or practical. Consequently, it may be necessary to select a team of individuals to categorize what is acceptable and what is not acceptable in terms of odors in the work environment. Appropriate HVAC systems may play a role in reducing odors.

### Temperature/Humidity Measurement

Temperature is typically measured with digital thermometers, and humidity is measured with hygrometers. In both cases, a recording instrument (e.g., recording thermometer) offers the advantage of documentation.

## SUMMARY

As early as the 19th century, Florence Nightingale identified the importance of physical environments for patient healing, but only recently was it established that the physical environment can support the performance of critical tasks by healthcare personnel to protect the patient from iatrogenic harm. Currently, medication errors are recognized as complex, multifactorial, and system dependent, and full scientific understanding of error prevention has proven elusive. However, Nightingale's counsel, "above all, do no harm," compels us to use the most up-to-date evidence for safe practices in healthcare to prevent errors and protect the patient. Aspects and considerations of the physical work environment discussed in this general chapter are not exhaustive, but can serve as a stimulus for the assessment of existing work spaces and for consideration of the latest evidence from human factors science in the design of new facilities. The remaining constituent not discussed in this general chapter is the healthcare provider working in arduous conditions who may, with the best intent, contribute to patient harm through inadvertent actions in substandard conditions. The physical work environment should be structured to support and promote safe delivery of care and prevent harm to patients while also bolstering the practice of healthcare by clinicians.

## GLOSSARY OF TERMS

**Administering:** The preparation, directly prior to use, of a pharmacologic or other therapeutic agent for ingestion, injection, insertion, or application.

**Color rendering index (CRI):** An expression of how a light source affects the color appearance of objects or humans, compared to how they would appear under a reference light source.

**Compounding:** The preparation, mixing, assembling, altering, packaging, and labeling of a drug, drug-delivery device, or device in accordance with a licensed practitioner's prescription, medication order, or initiative based on the practitioner/patient/pharmacist/compounder relationship in the course of professional practice.

**Constraint:** A rule stating under what conditions an action is allowed or prohibited. Constraints are used in designing procedures or tools to prevent unsafe practices.

**Crowding:** A condition that occurs when multiple workers use the same workspace, adversely affecting the amount of space available for each person and also increasing the negative factors of distractions, interruptions, and noise.

**Decibel:** A unit used to measure the intensity of a sound by comparing it with a standard level on a logarithmic scale, thereby indicating the degree of loudness. The A scale is commonly used when measuring decibels, because it most closely represents what the human ear perceives in terms of loudness.

**Dispensing:** The act of providing a medication or prescription order; to fill a prescription.

**Distraction:** An external stimulus that occurs when a person is engaged in a task or activity that causes a cognitive or emotional disturbance, but does not result in the discontinuation of the activity, such as a telephone ringing or question from a coworker.

**Ergonomic design:** Arrangement of a workspace to accommodate each individual's capacities and limitations, allowing them to work safely and efficiently. This includes an optimum ambient environment and adjustable furniture.

**Forcing function:** An aspect of a design that prevents a target action from being performed, or that allows its performance only if another specific action is performed first.

**Human factors:** The scientific discipline concerned with interactions among humans and other elements of a system, and the profession that applies the theory, principles, data, and methods to design systems that optimize human well-being and overall system performance.

**Illumination level:** The quantity of light energy reaching an area as measured (in lux or foot-candles) by a photometer with an illuminance sensor; this indicates brightness. A lux is a unit of illuminance, measured in lumens per square meter. A

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foot-candle (fc) is lumens/square foot, and is also commonly measured by light meters. The term candela replaced fc as the International System (SI) measure of luminous intensity and represents 1 lumen/steradium (lm/st).

**Interruption:** The cessation of productive activity before a task is completed, caused by an externally imposed stimulus.

**Lean production:** High-quality work output achieved while eliminating waste and decreasing resources used, time spent, and errors.

**Medication safety zone:** A critical area where medications are prescribed; orders are entered into a computer or transcribed onto paper documents; or medications are prepared/compounded or administered. The characteristics of an optimal physical environment for accurate medication use will apply to MSZs.

**Multi-tasking:** The ability of human beings to perform multiple tasks simultaneously.

**Noise and sound:** Noise is defined as an auditory stimulus that bears no informational relationship to the task at hand. Sound is a change in volume that has some informational relationship to the task at hand. A quiet work environment is defined as an area where noise is absent and the worker is free from disturbance.

**Override:** To neutralize or counteract the action of an automatic control.

**Photometer:** An instrument for measuring photometric quantities such as illuminance.

**Physical environment:** The surroundings that can affect one or more human senses.

**Workaround:** A plan or method used to circumvent a problem (as in computer software) without eliminating it.

**Working conditions:** A set of factors that include the physical environment, workforce staffing, workflow design, personal/social factors, and organizational characteristics.

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# ▲⟨1071⟩ RAPID MICROBIAL TESTS FOR RELEASE OF STERILE SHORT-LIFE PRODUCTS: A RISK-BASED APPROACH

INTRODUCTION
USER REQUIREMENT SPECIFICATIONS FOR A RAPID MICROBIAL TEST FOR THE RELEASE OF STERILE SHORT-LIFE PRODUCTS
THE CONCEPT OF RISK-BASED MICROBIOLOGICAL MONITORING AND RELEASE TESTING
CRITICAL OPERATING PARAMETERS TO BE USED IN DETERMINING A RISK-BASED RAPID MICROBIAL TEST FOR THE RELEASE OF STERILE SHORT-LIFE PRODUCTS
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## INTRODUCTION

It is widely recognized that the current growth-based sterility tests with an incubation period of at least 14 days (see *Sterility Tests* ⟨71⟩) are not suitable for products with a short shelf-life or for products prepared for immediate use, which are usually infused into patients before the completion of the test (*1*). These short-life products include compounded sterile preparations (CSPs), positron emission tomographic (PET) products, and cell and gene therapies, which require a new generation of risk-based approaches that include rapid microbial tests. For a general discussion of the factors other than sterility testing that contribute to sterility assurance, see *Sterility Assurance* ⟨1211⟩. It should be noted that as with alternate test methods, the referee test in the event of a dispute is ⟨71⟩.

If a microbial test is conducted, patient safety is best served through the completion of a test that detects microbial contamination prior to product use.

The rapid microbial tests (RMTs) should be risk-based so the stakeholder can select the preferred technology for their intended use and balance user requirement specifications (URS) including time to result, specificity, limit of detection (LOD), sample size, and product attributes. For example, many radiopharmaceuticals, due to the short half-life of radiotracers, would benefit most from a real-time microbial test, while CSPs and cell therapy products, due to their short beyond-use dating, would benefit from an overnight test or at least one that is completed within 48 h.

This general informational chapter discusses the needs of those who manufacture/prepare and test products with a short shelf life and their URS, and includes a brief discussion of some suitable methods for risk-based rapid microbial testing for the release of short shelf-life sterile products (hereafter referred to in this chapter as "short-life products").

## USER REQUIREMENT SPECIFICATIONS FOR A RAPID MICROBIAL TEST FOR THE RELEASE OF STERILE SHORT-LIFE PRODUCTS

Selection of an appropriate technology for the rapid microbial testing for the release of a short-life product should be a risk-based decision. The URS (*2*) of different technologies include:

- As short as possible time to result, ideally in real time or less than 24 hours, preferably prior to the administration of the product
- Ability to detect, preferably less than 100 colony-forming units (cfu) in the test sample
- Ability to detect a wide range of viable microorganisms in a product
- Sample quantity, i.e., minimum number of articles tested and quantity per container tested that does not consume a large proportion of the available product; whenever feasible, manufacturers should consider assay requirements during process design
- Aseptic test material handling, i.e., closed systems to reduce inadvertent contamination during testing
- Availability of instruments and reagents from multiple vendors
- Availability of reference standards, negative and positive controls, appropriate for technologies that use signals other than the colony-forming unit
- Ease of use/simplicity of test and data interpretation
- Low rates of false positive and false negative results
- A method suitability testing strategy for each specific product
- Improved patient safety arising from:

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  - Completion of the test prior to administration
  - Tests that provide progressive monitoring and reports of a detection of sterility test failure
- Ability to identify the detected microorganisms, which may be useful to the clinician administering the products and investigating to determine its source
- Robustness and reliability of equipment and reagents used in the testing
- Sample preparation suitable for both manual and automated methods

## THE CONCEPT OF RISK-BASED MICROBIOLOGICAL MONITORING AND RELEASE TESTING

A review of the URS for an RMT shows that some risk-based decisions would need to be made especially in terms of time to results, LOD, sample size, and range of microbes detected to allow the use of such tests prior to administration. The stakeholders should carry out a risk assessment for choosing an RMT in cases where the current compendial sterility test is unsuitable.

Benefits may include, for instance:

- Use of RMT in cases where a substantial risk to patient survival exists when the product is administered too late. One striking example from the clinical literature is that of bloodstream infections that are rapidly progressive infections with mortality rates of up to 40% and in which each day of delay in administrating antibiotics leads to a 10% increase in mortality (*3*). In these cases, patient safety is clearly promoted by the completion of a microbial test prior to the administration.
- Use of growth-based RMTs with continuous reading applied for “negative to date” on risk release because fast growing microorganisms can be detected earlier, and if a failure is detected this would enable the clinician to intervene sooner with the patient. When a contaminated product is detected, the laboratory supervisor could inform the clinician treating the patient and intravascular fluid resuscitation and antibiotic treatment may be initiated to avoid septic shock. A sterility test that progressively monitors and automatically reports any failure will therefore have additional advantages as compared to a single reading at the end of the incubation period.
- Use of non-growth based RMTs with LOD above 1 cfu that is amplified by growth in microbiological media but very fast time to results. The ability to detect contamination, in real time, prior to the administration of the short-life product may be considered more important than detection of a single colony-forming unit in the product. When considering risk to the patient, choice of the RMT should factor in the sensitivity of the assay versus the time to detection. Assays should be reasonably sensitive to detect the presence of a low-level contaminant and should do so in a time frame that allows results to be available before product administration.
- Other advantages of nongrowth-based RMT methods also may include the inability to be affected by antibiotics in the test sample and the detection of culture-negative infectious agents. Some DNA–targeted antibiotics (e.g., polymyxin B and bacitracin) have been shown to inhibit PCR amplification while antibiotics such as penicillin G, chloramphenicol, amphotericin and nalidixic acid do not affect the resolving potential of the reaction. This would be most important to sterile compounding pharmacies producing injectable antibiotic solutions. Method suitability should determine whether any antibiotics in the test sample could affect the assay.

In addition, RMTs or other rapid microbiological methods (RMMs) may be used as in-process controls prior to the final product release sterility test to provide faster information on the effectiveness of microbial controls and the early detection of gross contamination (enabling to investigate and restart production sooner) or probability that a product may fail sterility.

For the risk assessment, one consideration that may be overlooked is the relative risk to the patient based on the volume of the product injected or infused and the site of administration. The greater the volume and the more invasive, the greater the risk of blood stream infection for the patient. The risk ranges from a small volume of an intradermal injection to a large volume of an intravenous infusion (see *Table 1*).

**Table 1. Typical Volumes by Route of Administration and the Relative Risk Level**

| Route of Administration | Typical Volume Administered (mL) | Risk Level |
|---|---|---|
| Intradermal | ≤0.1 | Very Low |
| Subcutaneous | ≤1 | Low |
| Intramuscular | ≤3 | Moderate |
| Intrathecal | 1–10 | High |
| Intravenous push | 1–60 | Moderate–High |
| Intravenous piggyback | 25–250 | High |
| Intravenous | >250 | High |

## CRITICAL OPERATING PARAMETERS TO BE USED IN DETERMINING A RISK-BASED RAPID MICROBIAL TEST FOR THE RELEASE OF STERILE SHORT-LIFE PRODUCTS

The estimated operating parameters, i.e., LOD, time to results, and sample size, for the candidate technologies suitable for an RMT are found in *Table 2*.

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**Table 2. Operational Parameters of the Candidate Rapid Microbial Technologies**

| Candidate Technology | LOD (cfu) | Time to Result | Sample Size Range (mL) |
|---|---|---|---|
| Gram stain (for comparative purposes only) | $10^4$–$10^5$ | 30 min | 0.1 |
| ⟨71⟩ | Theoretical LOD of 1–3 cfu based on a Poisson distribution | 14 days | 40–500 |
| Adenosine triphosphate (ATP) bioluminescence | 1–10 | 2–7 days (including pre-enrichment) | 1–1000 |
| ATP bioluminescence | $10^3$ | 30 min | 1–1000 |
| Flow cytometry | 10–100 | 6–8 h (pre-enrichment) | 0.1–2 |
| Isothermal micro-calorimetry | $10^4$ | 2–7 days | 1 |
| Nucleic acid-methods[a] | 10–100 | 2–4 h | 0.2–2 |
| Respiration | 1–10 | Overnight to 7 days | Up to 10 |
| Solid phase cytometry | 1–10 | 2–3 h | 1–1000 |

[a] For these methods, the signal would be in genomic units.

## SITUATIONS WHEN ⟨71⟩ IS UNSUITABLE FOR PRODUCT RELEASE TESTING

### Sample Size Consideration

The sample size tested may need to be reduced based on either the sample processing capability of the technology or the need to conserve the much-needed product.

The minimum quantity of product used for each medium and the minimum of units to be tested relative to batch size is found in ⟨71⟩, *Table 2* and *Table 3*. It is widely understood that for large pharmaceutical product batch sizes, the amount of units tested are not statistically based and has a reduced capability of detecting low contamination levels in individual product lots. However, with the smaller batch sizes of many short-lived products, this limitation will be reduced because the proportion of products tested relative to the batch size will increase.

This traditional sampling plan is not appropriate for a cell therapy product. For example, if 10 individual 60-mL IV bags of cells were prepared, then 4 bags would be sampled with 20 mL taken from each bag. With this sampling scheme 40% of the batch would be consumed for sterility testing representing not only a large economic loss, but also more importantly, a huge loss of therapeutic product that may prevent administration of a dose adequate to treat the patient. In contrast, if 40 individual 1-mL vials were consumed for the sterility testing of a 40,000-vial batch of an injectable drug product, this would represent only 0.1% of the batch.

Reducing the sample size and the number of units tested will reduce the sensitivity of the sterility test. *Table 3* and *Table 4* illustrate the relative insensitivity of the test for a pharmaceutical product and a CSP.

**Table 3. The Probability That a 20-Unit Sterility Test Passes Given an Increasing Contamination Rate for a Drug Product**

| | Contaminated Items in the Batch (%) | | | | | |
|---|---|---|---|---|---|---|
| | **0.1** | **1** | **5** | **10** | **20** | **50** |
| *p* | 0.001 | 0.01 | 0.05 | 0.1 | 0.2 | 0.5 |
| *q* | 0.999 | 0.99 | 0.95 | 0.9 | 0.8 | 0.5 |
| Probability (*p*) of drawing 20 consecutive sterile items | 0.98 | 0.82 | 0.36 | 0.12 | 0.012 | <0.00001 |

Calculated from the following (*4*):

$$p = (1 - p)^{20} = q^{20}$$

*p* = proportion of contaminated containers in the batch
*q* = proportion of non-contaminated containers in the batch

**Table 4. The Probability That a 6-Unit Sterility Test Passes Given an Increasing Contamination Rate for a CSP**

| | Contaminated Items in the Batch (%) | | | |
|---|---|---|---|---|
| | **1** | **5** | **10** | **20** |
| *p* | 0.001 | 0.01 | 0.05 | 0.1 |

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**Table 4. The Probability That a 6-Unit Sterility Test Passes Given an Increasing Contamination Rate for a CSP** *(continued)*

| | Contaminated Items in the Batch (%) | | | |
|---|---|---|---|---|
| | 1 | 5 | 10 | 20 |
| *q* | 0.999 | 0.99 | 0.95 | 0.9 |
| Probability (*p*) of drawing 6 consecutive sterile items | 0.995 | 0.94 | 0.73 | 0.53 |

Alternative sampling plans have been proposed in other compendia. One recommended approach to sterility testing of cell therapy products for a batch size of less than 40 units is found in *European Pharmacopoeia 9.0* chapter *2.6.27 Microbiological Examination of Cell-Based Preparations*.

The contamination test sample size for a cell preparation with a volume between 10 and 1000 mL would be 1% of the total volume; for a cell preparation with a volume between 1 and 10 mL, it would be a sample size of 0.1 mL; for a cell preparation less than 1 mL, the preparation would not be tested. As suggested in 21 CFR 610.12 (*Sterility*), in-process testing using rapid methods may be used in lieu of final product testing when fully justified.

In a similar fashion as cell-therapy preparations, the sample quantity and sampling plan for PET radiopharmaceuticals must also accommodate the limited number of vials (usually one) and the volume of product produced in a batch (usually less than 15 mL). If the batch is comprised of a single container, the sterility test sample size is at least 1% of the total batch volume. For example, if a batch is comprised of 1 vial containing 15 mL, use at least 0.15 mL for purposes of the sterility test. If the batch is comprised of more than one container, use a volume from a single container that represents at least 1% of the total batch volume. If a batch is comprised of 3 vials each containing 25 mL, use at least 0.75 mL from 1 vial for purposes of the sterility test.

### Limit of Detection

Within the limitations of preparing inocula from a cell suspension with one or more colony-forming units, growth-based sterility tests can be shown to have at least a theoretical LOD of 1–3 cfu based on a Poisson distribution. Setting an LOD of a single viable cell with all technologies is an unrealistic barrier of entry for any sterility test, especially when the signal is not the colony-forming unit that is amplified by cultural enrichment.

### Ability to Detect a Wide Range of Microorganisms

Although all the analytical platforms should have the ability to detect a wide range of bacteria, yeasts, and molds, it is of practical importance to demonstrate that technology chosen for an RMT is capable of detecting microorganisms implicated in sterility test failures, infection outbreaks, and product recalls associated with either CSPs, radiopharmaceuticals, cell therapies, or manufactured pharmaceuticals. This is especially true if the technology, after risk analysis, is shown to improve patient safety with the administration of the products unique to that stakeholder group.

## RAPID MICROBIAL TEST METHODS FOR THE RELEASE OF STERILE SHORT-LIFE PRODUCTS

Technologies recommended based on their match to the URS discussed above (*2*) suitable for an RMT are listed alphabetically as follows:

- Adenosine triphosphate (ATP) bioluminescence
- Flow cytometry
- Isothermal microcalorimetry
- Nucleic acid amplification
- Respiration
- Solid phase cytometry

### Brief Descriptions of the Technologies

Each of these candidate advanced analytical platforms is briefly discussed separately and key references are provided. For an overview, see Moldenhauer (*5*).

#### ADENOSINE TRIPHOSPHATE BIOLUMINESCENCE

This is a well-established technology with luminometers and reagents available from multiple instrument manufacturers. The energy from living cells is stored as ATP and can be measured as light when exposed to luciferase from the American firefly. Each ATP molecule consumed by luciferase produces 1 photon of light. The result detected by a luminometer is typically expressed in relative light units (RLU) and is instrument, reagent, and organism dependent. The ATP content of different microorganisms ranges from 2 to 4 × $10^{-18}$ mole/cfu for gram-negative bacteria, 5 to 8 × $10^{-18}$ mole/cfu for gram-positive bacteria, and 300 to 800 × $10^{-18}$ mole/cfu for fungi (*6*). Given the high signal-to-noise ratio of the measurement and the background ATP in microbiological culture media, the microbiologically relevant instrument detection limit in broth is in the order of 5000 RLU equivalent to approximately $10^3$ cfu.

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This LOD will detect the presence of microorganisms at levels which are 3–4 logs less within an aliquot of the media than that is required for visual detection of growth in the media. For a rapid microbial test for the release of sterile short-life products, an enrichment culture either in liquid media to reach a threshold ATP level or on a membrane filter on solid media for the formation of microbial colonies could be used with an incubation time of 2–7 days.

## FLOW CYTOMETRY

Flow cytometry may be used to detect fluorescently labeled viable microbial cells after an enrichment culture step of 24–48 h (*7*). A labeling reagent consisting of either a fluorogenic substrate or vital stain is used to differentiate viable cells from dead cells and cellular debris. While bacteria are very small and may be hard to distinguish from cell debris, they can be differentiated by size, shape, and fluorescent intensity. Cell viability is indicated by the ability of the intact cell membrane to retain a fluorochrome generated by nonspecific cellular esterase, or by labeling the cell with nucleic acid-specific vital stains. A laser illuminates each cell in the flow stream and the emitted light is detected by a dual photomultiplier array. The signal is digitized and interpreted by discrimination software. The LOD for this technology may be >1 cfu and an enrichment step may be necessary.

## ISOTHERMAL MICROCALORIMETRY

Isothermal microcalorimeters monitor enthalpy changes in closed vials (systems) related to microbial metabolic activity and growth. With current instruments, $10^4$ active microbial cells can release enough heat to be detected and enrichment is needed for detection (2–7 days to result). Recently its application in pharmaceutical microbiology has been evaluated, although its specific application to release testing of sterile products has not been established yet (*8*).

## NUCLEIC ACID AMPLIFICATION

Real-time quantitative polymerase chain reaction (PCR) has the potential to monitor the exponential phase of PCR through 36–48 cycles of amplification using universal primers and probes (termed "pan-bacterial" and "pan-fungal" methods) to estimate the initial quantity of the target DNA, which is in turn proportional to the number of microbial cells in the test sample. Unlike DNA, cellular RNA is turned-over rapidly metabolically and would be a better indicator of viable microorganisms. For example, *Escherichia coli* contains 2 molecules of DNA and $2 \times 10^4$ molecules of 16S rRNA per cell (*9*). This process is achieved by the conversion of RNA into a complementary copy of DNA (cDNA) by the enzyme reverse transcriptase and the cDNA can be analyzed in real time in either a quantitative (enumeration test) or qualitative assay (sterility test). Alternatively, for DNA-based PCRs, a sample pretreatment with ethidium monoazide or propidium monoazide may also provide the capability to differentiate live from dead microbial cells (*10*,*11*), or free microbial DNA may be removed from a test sample by a centrifugation/washing step and the bacterial pellet used for analysis.

Realistically, an LOD of a single viable cell is probably an insurmountable challenge, especially for a test relying on a DNA/RNA target and universal primers. Another challenge is the differing amounts of genomic material in different microorganisms.

Generally, the LOD ranges from 10 to 1000 viable cells/mL in a sample and, in some reported cases, from 10 to 100 viable cells/mL. Recently it was shown that PCR may actually achieve detection of microorganisms with a limit of $10^2$ to $10^3$ cfu/mL in a sample containing a high concentration of up to $10^6$ mammalian cells/ mL without the need for pre-incubation in microbial growth media (*12*). Adding a growth-based enrichment step for at least 24–48 h and comparing the PCR results before and after cultural enrichment may provide a practical solution for sterility testing. Alternatively, concentration methods could be applied to enrich the sample and reduce the sample volume. As noted by the authors of a recent study of the use of 16S rRNA PCR sterility test for stem cells, with the demonstrated bacterial sensitivity of 10–100 cfu/mL, a test method with a sensitivity of 100 cfu/mL would be suitable to detect clinically significant bacterial contamination of blood and cell products (*13*).

Direct comparison of growth-based and nucleic acid amplification-based assays is complicated by the fact that nucleic acid amplification-based assays also detect non-viable organisms and are a measure of microbial genome copy number, not colony-forming units. For that reason, the LOD for a nucleic acid amplification-based assay should be defined in terms of genome equivalents per milliliter.

Non-growth based RMTs for the release of sterile short-life products like nucleic acid amplification may have additional advantages due to the following:

- With close to real-time testing, the test will be completed before the short-life products are infused into a patient
- Detection of culture-negative infectious agents
- The test is minimally affected by antibiotics in the test sample, as indicated earlier in the chapter
- The test is less sensitive to background resulting from animal cell lysis (e.g., particles, ATP) as compared to other technologies since specific microbial genes are targeted

## RESPIRATION

This broad category ranges from classical respirometers, to gaseous headspace analyzers to automated blood culture systems. Aerobic and anaerobic broth formulations allow for the recovery of most microorganisms responsible for blood stream infections within a 5-day incubation. With some instruments, this includes incubation at both 20°–25° and 30°–35° as in ⟨71⟩. This technology has been successfully extended to sterility testing of cell therapy products cells using a 7-day incubation as an alternative to the compendial sterility test for lot release (*14*).

Other instruments are available to detect and enumerate respiring microorganisms. For example, tunable diode laser absorption spectroscopy (TDLAS) can measure oxygen ($O_2$) depletion or carbon dioxide ($CO_2$) increase in closed units containing growing microorganisms in culture medium. The technology was originally developed to monitor gas headspace composition in closed units and also could be used for automatic media fill inspection (*8*,*15*). The system has gaseous calibration

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standards and minor adaptations are needed if the system is to be used for sterility testing (e.g., calibrating for higher-volume containers). [NOTE—All the systems of the respiration platform require microbial growth and metabolic activity for detection, i.e., the usual time of 2–7 days to obtain results is required. However, when the results may be progressively monitored to detect a sterility test failure earlier in the incubation period, that is a huge advantage with short-life products as explained in the risk assessment section above.]

### SOLID PHASE CYTOMETRY

There are instrument systems based on solid phase cytometry combining fluorescent labeling and solid phase laser scanning to rapidly enumerate viable microorganisms in filterable liquids (*16*). Microorganisms are collected by filtration on 0.45-micron polyester membranes and treated with background and viability stains. The filters are scanned in a cytometer by a high-speed, 488 nM argon laser. Multiple photomultiplier tubes, processed to differentiate between labeled microorganisms and background noise, based on size, shape, and fluorescence intensity, detect fluorescence. The scan is displayed as a map that identifies the position of the fluorescent events that are verified using an epifluorescence microscope with an automated motorized stage to locate the individual events. The system is claimed to detect individual viable microorganisms in 2–3 h. It should be noted that most cell therapy products are non-filterable, so this technology may not be compatible with these types of products.

## METHOD SUITABILITY TESTING

The method suitability requirements for a growth-based sterility test are given in ⟨71⟩. The suitability of the test must be demonstrated for each product to be tested. The recovery of the USP challenge organisms at a level less than 100 cfu, in the presence of residual product, is demonstrated as clearly visible growth in the microbiological growth media for either the direct inoculation or membrane filtration method. With signals other than the colony-forming unit derived from laboratory culture (e.g., nucleic acid, ATP, and fluorescent labeling of viable microbial cells), the results from testing actual samples may give results that are not equivalent to that using other technologies. However, method suitability testing will confirm that product residues in the analyte will not inhibit the enzymatic steps associated with the signal generation by the rapid method.

## GLOSSARY

**Colony-forming unit (cfu):** Viable microorganisms capable of growth on solid microbiological culture media forming discrete, visible colonies.

**Limit of detection (LOD):** The lowest signal representing a viable microorganism that can be routinely detected.

**Patient safety:** Reduction of morbidity and mortality of the recipients of a contaminated product that is promoted by the completion of a rapid microbial test prior to the administration.

**Positron emission tomography (PET):** A nuclear medicine functional imaging technique that is used to observe metabolic processes in the body as an aid to the diagnosis of disease. The system detects pairs of gamma rays emitted indirectly by a positron-emitting radionuclide (tracer), which is introduced into the body.

**Risk-based rapid microbial test:** A rapid microbial test selected by the stakeholder after a consideration of the LOD, sample size, specificity, and time to result that promotes patient safety by completing the test prior to the administration of a short-lived product to detect contamination, if any.

**Specificity:** Ability to detect a wide range of different bacteria, yeasts, and molds.

**Time to result:** Time to complete the microbial test and reach a conclusion about the lack of contamination of the sample test.

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# ⟨1072⟩ DISINFECTANTS AND ANTISEPTICS

## INTRODUCTION

A sound cleaning and sanitization program is needed for controlled environments used in the manufacture of Pharmacopeial articles to prevent the microbial contamination of these articles. Sterile drug products may be contaminated via their pharmaceutical ingredients, process water, packaging components, manufacturing environment, processing equipment, and manufacturing operators. Current Good Manufacturing Practices (cGMPs) emphasize the size, design, construction, and location of buildings and construction materials, and the appropriate material flow to facilitate cleaning, maintenance, and proper operations for the manufacture of drug products. When disinfectants are used in a manufacturing environment, care should be taken to prevent the drug product from becoming contaminated with chemical disinfectants as a result of the inherent toxicity of the disinfectants. The requirements for aseptic processing include readily cleanable floors, walls, and ceilings that have smooth and nonporous surfaces; particulate, temperature, and humidity controls; and cleaning and disinfecting procedures to produce and maintain aseptic conditions. The cleaning and sanitization program should achieve specified cleanliness standards, control microbial contamination of products, and be designed to prevent the chemical contamination of pharmaceutical ingredients, product-contact surfaces and/or equipment, packaging materials, and ultimately the drug products. These principles also apply to nonsterile dosage forms where the microbial contamination is controlled by the selection of appropriate pharmaceutical ingredients, utilities, manufacturing environments, sound equipment cleaning procedures, products especially formulated to control water activity, inclusion of suitable preservatives, and product packaging design.

In addition to disinfectants, antiseptics are used to decontaminate human skin and exposed tissue and may be used by personnel prior to entering the manufacturing area. Chemical sterilants may be used to decontaminate surfaces in manufacturing and sterility testing areas. Furthermore, sterilants may be used for the sterilization of Pharmacopeial articles, and UV irradiation may be used as a surface sanitizer.

This general information chapter will discuss the selection of suitable chemical disinfectants and antiseptics; the demonstration of their bactericidal, fungicidal, and sporicidal efficacy; the application of disinfectants in the sterile pharmaceutical manufacturing area; and regulation and safety considerations. Biofilm formation and its relationship to disinfectants are outside the scope of this chapter. Additional information not covered in the chapter may be obtained from standard texts on disinfectants and antiseptics.[1]

## DEFINITIONS

**Antiseptic**—An agent that inhibits or destroys microorganisms on living tissue including skin, oral cavities, and open wounds.

**Chemical Disinfectant**—A chemical agent used on inanimate surfaces and objects to destroy infectious fungi, viruses, and bacteria, but not necessarily their spores. Sporicidal and antiviral agents may be considered a special class of disinfectants. Disinfectants are often categorized as high-level, intermediate-level, and low-level by medically oriented groups based upon their efficacy against various microorganisms.

**Cleaning Agent**—An agent for the removal from facility and equipment surfaces of product residues that may inactivate sanitizing agents or harbor microorganisms.

**Decontamination**—The removal of microorganisms by disinfection or sterilization.

**Disinfectant**—A chemical or physical agent that destroys or removes vegetative forms of harmful microorganisms when applied to a surface.

**Sanitizing Agent**—An agent for reducing, on inanimate surfaces, the number of all forms of microbial life including fungi, viruses, and bacteria.

**Sporicidal Agent**—An agent that destroys bacterial and fungal spores when used in sufficient concentration for a specified contact time. It is expected to kill all vegetative microorganisms.

**Sterilant**—An agent that destroys all forms of microbial life including fungi, viruses, and all forms of bacteria and their spores. Sterilants are liquid or vapor-phase agents.

Microorganisms differ greatly in their resistance to disinfection agents. The order of resistance of clinically significant microorganisms to chemical disinfectants from most to least resistant is listed in *Table 1*.

**Table 1. The Resistance of Some Clinically Important Microorganisms to Chemical Disinfectants (Listed in Order of Decreasing Resistance)**

| Type of Microorganisms | Examples |
|---|---|
| Bacterial spores | *Bacillus subtilis* and *Clostridium sporogenes* |
| Mycobacteria | *Mycobacterium tuberculosis* |
| Nonlipid-coated viruses | Poliovirus and rhinovirus |
| Fungal spores and vegetative molds and yeast | *Trichophyton, Cryptococcus,* and *Candida* spp. |
| Vegetative bacteria | *Pseudomonas aeruginosa, Staphylococcus aureus,* and *Salmonella* spp. |

[1] Ascenzi, J.M., Ed. *Handbook of Disinfectants and Antiseptics*, 5th ed.; Marcel Dekker: New York, 1995; Block, S.S., Ed. *Disinfection, Sterilization, and Preservation,* 5th ed.; Lippincott Williams & Wilkins Publishers: Philadelphia, 2000. Russell, A.D.; Hugo, W.B.; Ayliffe, G.A.J., Eds. *Principles and Practices of Disinfection, Preservation and Sterilization,* 3rd ed.; Blackwell Science Inc.: London, 1999.

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**Table 1. The Resistance of Some Clinically Important Microorganisms to Chemical Disinfectants (Listed in Order of Decreasing Resistance)** *(continued)*

| Type of Microorganisms | Examples |
|---|---|
| Lipid-coated viruses | Herpes simplex virus, hepatitis B virus, and human immunodeficiency virus |

## CLASSIFICATION OF DISINFECTANTS

Chemical disinfectants are classified by their chemical type. This includes aldehydes, alcohols, halogens, peroxides, quaternary ammonium compounds, and phenolic compounds (see *Table 2*).

**Table 2. General Classification of Antiseptics, Disinfectants, and Sporicidal Agents**

| Chemical Entity | Classification | Example |
|---|---|---|
| Aldehydes | Sporicidal agent | 2% Glutaraldehyde |
| Alcohols | General purpose disinfectant, antiseptic, antiviral agent | 70% Isopropyl alcohol, 70% alcohol |
| Chlorine and sodium hypochlorite | Sporicidal agent | 0.5% Sodium hypochlorite |
| Phenolics | General purpose disinfectant | 500 µg per g Chlorocresol, 500 µg per g chloroxylenol |
| Ozone | Sporicidal agent | 8% Gas by weight |
| Hydrogen peroxide | Vapor phase sterilant, liquid sporicidal agent, antiseptic | 4 µg per g $H_2O_2$ vapor, 10%–25% solution, 3% solution |
| Substituted diguanides | Antiseptic agent | 0.5% Chlorhexidine gluconate |
| Peracetic acid | Liquid sterilant, vapor phase sterilant | 0.2% Peracetic acid, 1 µg per g peracetic acid |
| Ethylene oxide | Vapor-phase sterilant | 600 µg per g Ethylene oxide |
| Quaternary ammonium compounds | General purpose disinfectant, antiseptic | Concentration dependent on application, Benzalkonium chloride |
| β-Propiolactone | Sporicidal agent | 100 µg per g β-Propiolactone |

The effectiveness of a disinfectant depends on its intrinsic biocidal activity, the concentration of the disinfectant, the contact time, the nature of the surface disinfected, the hardness of water used to dilute the disinfectant, the amount of organic materials present on the surface, and the type and the number of microorganisms present. Under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), the Environmental Protection Agency (EPA) registers chemical disinfectants marketed in the United States and requires manufacturers to supply product information on the use dilution, type of microorganisms killed, and the necessary contact time. Certain liquid chemical sterilizers intended for use on critical or semicritical medical devices are defined and regulated by the U.S. Food and Drug Administration (FDA).

## SELECTION OF AN ANTISEPTIC FOR HAND AND SURGICAL SITE DISINFECTION

Hands and surgical sites are disinfected in a hospital setting to reduce the resident flora and to remove transient flora (e.g., *Streptococcus pyogenes*) and methicillin-resistant *S. aureus* and *P. aeruginosa* that have been implicated in hospital-associated infection. Use of antiseptics to disinfect hands has been shown to be more effective than soap and water in reducing the counts of bacteria on the skin; repeated antiseptic use further reduces these counts. These principles may be applied to clean-room operators in the pharmaceutical industry.

Common antiseptics include 4% chlorhexidine, 10% povidone–iodine, 3% hexachlorophene, 70% isopropyl alcohol, and 0.5% chlorhexidine in 95% alcohol.

## SELECTION OF A DISINFECTANT FOR USE IN A PHARMACEUTICAL MANUFACTURING ENVIRONMENT

When selecting a disinfectant for use in a pharmaceutical manufacturing area, the following points should be considered: the number and types of microorganisms to be controlled; the spectrum of activity of commercially available disinfectants; the claims as a sterilant; the disinfectant or sanitizer supported by the EPA registrations; the concentration, application method, and contact time of the disinfectant; the nature of the surface material being disinfected and its compatibility with the disinfectant; the amount of organic compounds on the surface that may inactivate a disinfectant; the possible need to maintain a residual bactericidal activity of the disinfectant on the surface; the corrosiveness of the disinfectant to equipment with repeated application; the safety considerations for operators applying the disinfectant; the compatibility of the disinfectant with cleaning

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agents and other disinfectants; the planned disinfectant rotation; and the steps that need to be taken to avoid the contamination of pharmaceutical products by a disinfectant.[2]

## THEORETICAL DISCUSSION OF DISINFECTANT ACTIVITY

Plots of the log of the number of microorganisms per mL surviving in a disinfectant solution indicate that first-order kinetics can be applied as a gross approximation to the reduction in microbial count with respect to time. In practice, the plots show a more sigmoid curve with a slower initial reduction in numbers followed by an increasing rate with respect to time.

The rate constant, K, for the disinfection process can be calculated by the formula:

$$(1/t)(\log N_0/N)$$

in which t is the time, in minutes, for the microbial count to be reduced from $N_0$ to N; $N_0$ is the initial number of organisms, in cfu per mL; and N is the final number, in cfu per mL, of organisms.

As with a first-order chemical reaction, the same concentration of disinfectant reduces the number of organisms more rapidly at elevated temperatures. This can be expressed as a temperature, T, coefficient per 10° rise in temperature, $Q_{10}$, calculated by the formula:

$$\text{Time to decontamination at T°/Time to decontamination at T}$$

in which T is T° – 10.

Further evidence that a first-order reaction is an inadequate description of disinfection is that the $Q_{10}$ values for chemical and enzyme reactions are 2 to 3, while the common disinfectants phenol and alcohol have a $Q_{10}$ of 4 and 45, respectively.

Critical to the successful employment of disinfectants is an understanding of the effect of disinfectant concentration on microbial reduction. A plot of the log of the time to reduce the microbial population in a standard inoculum to zero against the log of the disinfectant concentration is a straight line with the slope of the line termed the concentration exponent, n. The relationship can be expressed as follows:

$$n = (\text{log of the kill time at concentration } C_2) - (\text{log of the kill time at concentration } C_1)/(\log C_1 - \log C_2)$$

in which $C_1$ and $C_2$ are the higher and lower disinfectant concentrations, respectively.

The wide differences in concentration exponents, n, have practical consequences in picking the use dilution of different disinfectants and in using dilution to neutralize a disinfectant in disinfectant-effectiveness testing and routine microbial monitoring of the manufacturing environment. For example, mercuric chloride has a concentration exponent of 1, so a 3-fold dilution will reduce the disinfectant activity by $3^1$ (or by one-third), while phenol with a concentration exponent of 6 will have a $3^6$ (or a 729-fold) reduction in disinfectant activity. Disinfectants with a larger concentration exponent or dilution coefficient rapidly lose activity when diluted. The concentration exponents for some disinfectants are listed in *Table 3*.

**Table 3. Concentration Exponents of Common Antiseptics, Disinfectants, and Sterilants**

| Disinfectant | Concentration Exponents |
|---|---|
| Hydrogen peroxide | 0.5 |
| Sodium hypochlorite | 0.5 |
| Mercuric chloride | 1 |
| Chlorhexidine | 2 |
| Formaldehyde | 1 |
| Alcohol | 9 |
| Phenol | 6 |
| Quaternary ammonium compounds | 0.8 to 2.5 |
| Aliphatic alcohols | 6.0 to 12.7 |
| Phenolic compounds | 4 to 9.9 |

Another important consideration may be the pH of the disinfectant. Many disinfectants are more active in the ionized form, while others are more active in the nonionized form. The degree of ionization will depend on the p$K_a$ of the agent and the pH of the disinfection environment. For example, phenol, with a p$K_a$ of 10, will be more effective at a pH below 7 where it is nonionized.

## MECHANISM OF DISINFECTANT ACTIVITY

*Table 4* lists the sites and modes of action of some representative disinfectants.

[2] Denny, V.F.; Marsik, F.J. Current Practices in the Use of Disinfectants within the Pharmaceutical Industry. *PDA J. of Pharmaceutical Sci. and Tech.*, **1997**, *51*, (6), 227–228.

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**Table 4. Mechanism of Disinfectant Activity Against Microbial Cells**

| Target | Disinfectant |
|---|---|
| Cell wall | Formaldehyde, hypochlorite, and glutaraldehyde |
| Cytoplasmic membrane, action on membrane potential | Anilides and hexachlorophene |
| Membrane enzymes, action on electron-transport chain | Hexachlorophene |
| Action on ATP | Chlorhexidine and ethylene oxide |
| Action on enzymes with –SH groups | Ethylene oxide, glutaraldehyde, hydrogen peroxide, hypochlorite, and iodine |
| Action on general membrane permeability | Alcohols, chlorhexidine, and quaternary ammonium compounds |
| Cell contents, general coagulation | Chlorhexidine, aldehydes, and quaternary ammonium compounds |
| Ribosomes | Hydrogen peroxide |
| Nucleic acids | Hypochlorites |
| Thiol groups | Ethylene oxide, glutaraldehyde, hydrogen peroxide, and hypochlorite |
| Amino groups | Ethylene oxide, glutaraldehyde, and hypochlorite |
| General oxidation | Hypochlorite |

## MICROBIAL RESISTANCE TO DISINFECTANTS

The development of microbial resistance to antibiotics is a well-described phenomenon. The development of microbial resistance to disinfectants is less likely to occur at significant levels, as disinfectants are more powerful biocidal agents than antibiotics. In addition, they are normally applied in high concentrations against low populations of microorganisms usually not growing actively, so the selective pressure for the development of resistance is less profound. However, the most frequently isolated microorganisms from an environmental monitoring program may be periodically subjected to use-dilution testing with the agents used in the disinfection program to confirm their susceptibility, as there are real differences among different species in resistance to the lethal effects of different sanitizers.

## DISINFECTANT CHALLENGE TESTING

Under FIFRA, the EPA requires companies that register public health antimicrobial pesticide products including disinfectants, sanitization agents, sporicidal agents, and sterilants to ensure the safety and effectiveness of their products before they are sold or distributed. Companies registering these products must address the chemical composition of their product, include toxicology data to document that their product is safe if used as directed on the label, include efficacy data to document their claims of effectiveness against specific organisms and to support the directions for use provided in the labeling, and provide labeling that reflects the required elements for safe and effective use. While these directions provide valuable information, they may not be helpful in terms of the products' use as disinfectants in a manufacturing environment.

In the United States, the official disinfectant testing methods are published by AOAC International[3] and include the Phenol-Coefficient Test, Use-Dilution Method Test, Hard Surface Carrier Method, and Sporicidal Carrier Test. A scientific study submitted for EPA review in support of disinfectant registration must be conducted at a laboratory facility that follows the Good Laboratory Practices (GLP) regulations (21 CFR 58). To demonstrate the efficacy of a disinfectant within a pharmaceutical manufacturing environment, it may be deemed necessary to conduct the following tests: (1) use-dilution tests (screening disinfectants for their efficacy at various concentrations and contact times against a wide range of standard test organisms and environmental isolates); (2) surface challenge tests (using standard test microorganisms and microorganisms that are typical environmental isolates, applying disinfectants to surfaces at the selected use concentration with a specified contact time, and determining the log reduction of the challenge microorganisms); and (3) a statistical comparison of the frequency of isolation and numbers of microorganisms isolated prior to and after the implementation of a new disinfectant. This is considered necessary because critical process steps like disinfection of aseptic processing areas, as required by GMP regulations, need to be validated, and the EPA registration requirements do not address how disinfectants are used in the pharmaceutical, biotechnological, and medical device industries. For the surface challenge tests, the test organisms are enumerated using swabs, surface rinse, or contact plate methods. Neutralizers that inactivate the disinfectants should be included in either the diluent or microbiological media used for microbial enumeration or both. Information on disinfectant neutralization may be found in *Validation of Microbial Recovery from Pharmacopeial Articles* ⟨1227⟩.

The disinfectant efficacy test must have realistic acceptance criteria. In practice, sufficient organisms need to be inoculated on a 2-inch × 2-inch square of the surface being decontaminated, i.e., a coupon, to demonstrate at least a 2 (for bacterial spores) to 3 (for vegetative bacteria) log reduction during a predetermined contact time (i.e., 10 minutes over and above the recovery observed with a control disinfectant application). The efficacy of the neutralizers and their ability to recover inoculated microorganisms from the material should be demonstrated during the use-dilution or surface-challenge studies. Points to remember are that disinfectants are less effective against the higher numbers of microorganisms used in laboratory challenge tests than they are against the numbers that are found in clean rooms (see *Microbiological Control and Monitoring of Aseptic Processing Environments* ⟨1116⟩); that inocula from the log growth phase that are typically employed in laboratory tests are more

[3] AOAC International Official Methods of Analysis, 15th, 16th, and 17th editions. Arlington, VA.

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resistant, with the exception of spores formed during the static phase, than those from a static or dying culture or stressed organisms in the environment; and that microorganisms may be physically removed during actual disinfectant application in the manufacturing area.

Although not all inclusive, typical challenge organisms that may be employed are listed in *Table 5*.

**Table 5. Typical Challenge Organisms**

| AOAC Challenge Organisms | Typical Environmental Isolates |
|---|---|
| Bactericide: *E. coli,* ATCC 11229; *S. aureus,* ATCC 6538; *P. aeruginosa,* ATCC 15442 | Bactericide: *M. luteus*, *S. epidermidis*, *Coynebacterium jeikeium*, *P. vesicularis* |
| Fungicide: *C. albicans,* ATCC 10231 or 2091; *Penicillium chrysogenum,* ATCC 11709; *A. brasiliensis*, ATCC 16404 | Fungicide: *P. chrysogenum, A. brasiliensis* |
| Sporicide: *B. subtilis,* ATCC 19659 | Sporicide: *B. sphaericus*, *B. thuringiensis* |

Because a wide range of different materials of construction are used in clean rooms and other controlled areas, each material needs to be evaluated separately to validate the efficacy of a given disinfectant. *Table 6* contains a list of common materials used in clean room construction.

**Table 6. Typical Surfaces to be Decontaminated by Disinfectants in a Pharmaceutical Manufacturing Area**

| Material | Application |
|---|---|
| Stainless steel 304L and 316L grades | Work surfaces, filling equipment, and tanks |
| Glass | Windows and vessels |
| Plastic, vinyl | Curtains |
| Plastic, polycarbonate | Insulation coating |
| Lexan® (plexiglass) | Shields |
| Epoxyl-coated gypsum | Walls and ceilings |
| Fiberglass-reinforced plastic | Wall paneling |
| Tyvek® | Equipment wraps |
| Terrazzo tiles | Floors |

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## DISINFECTANTS IN A CLEANING AND SANITIZATION PROGRAM

The selection of suitable disinfectants and the verification of their effectiveness in surface challenge testing is critical in the development of a cleaning and sanitization program.

Issues associated with the successful implementation of such a program are the development of written procedures, staff training, decisions on disinfectant rotation, institution of application methods and contact times, environmental monitoring to demonstrate efficacy, and personnel safety.

The cGMP 21 CFR 211.67, *Equipment Cleaning and Maintenance,* details the requirements for written procedures for cleaning, maintenance, and sanitization of pharmaceutical manufacturing equipment. These procedures should address the assignment of responsibility, establishment of schedules, details of cleaning operations, protection of clean equipment prior to use, inspection for cleanliness immediately prior to use, and maintenance of cleaning and sanitization records.

Staff involved in disinfection require training in microbiology, industry practices for cleaning and sanitization, safe handling of concentrated disinfectants, the preparation and disposal of disinfectants, and appropriate application methods. It should be emphasized that the preparation of the correct dilutions is critical because many disinfectant failures can be attributed to use of disinfectant solutions that are too dilute. Typically disinfectants used in aseptic processing and filling areas are diluted with Sterile Purified Water, and are prepared aseptically. Alternately, the disinfectant may be diluted with Purified Water, and then sterile filtered to eliminate microorganisms that may potentially persist in a disinfectant. Diluted disinfectants must have an assigned expiration dating justified by effectiveness studies.

The rotation of an effective disinfectant with a sporicide is encouraged. It is prudent to augment the daily use of a bactericidal disinfectant with weekly (or monthly) use of a sporicidal agent. The daily application of sporicidal agents is not generally favored because of their tendency to corrode equipment and because of the potential safety issues with chronic operator exposure. Other disinfection rotation schemes may be supported on the basis of a review of the historical environmental monitoring data. Disinfectants applied on potential product contact surfaces are typically removed with 70% alcohol wipes. The removal of residual disinfectants should be monitored for effectiveness as a precaution against the possibility of product contamination.

The greatest safety concerns are in the handling of concentrated disinfectants and the mixing of incompatible disinfectants. For example, concentrated sodium hypochlorite solutions (at a concentration of more than 5%) are strong oxidants and will decompose on heating, on contact with acids, and under the influence of light, producing toxic and corrosive gases including chlorine. In contrast, dilute solutions (at a concentration of less than 0.5%) are not considered as hazardous. Under no circumstances should disinfectants of different concentrations be mixed. Material Safety Data Sheets for all the disinfectants used in a manufacturing area should be available to personnel handling these agents. Appropriate safety equipment such as face shields, safety glasses, gloves, and uniforms must be issued to personnel handling the disinfectant preparation, and

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personnel must be trained in the proper use of this equipment. Safety showers and eye wash stations must be situated in the work area where disinfectant solutions are prepared.

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# ⟨1074⟩ EXCIPIENT BIOLOGICAL SAFETY EVALUATION GUIDELINES

## INTRODUCTION

This informational chapter presents a scientifically-based approach for the safety assessment of new pharmaceutical excipients (i.e., those excipients that have not been previously used or permitted for use in a pharmaceutical preparation). The guidelines presented herein provide a protocol for developing an adequate database upon which to establish conditions for the safe use of a new excipient intended for use in products administered by various dosage routes. [NOTE—The final section of this chapter, *Definition of Terms*, lists some terms referred to in this chapter.]

An excipient may perform a variety of functionality roles in a pharmaceutical product; but, unlike pharmacologically active drug entities, the excipient displays either no pharmacological activity or very limited and directed activity. Because of these differences between excipients and active drug substances in terms of risk and benefit relationships and expected biological activities, the approaches for safety assessments of excipients and active drug substances will differ. Therefore, it is important to note that the guidelines presented in this informational chapter apply only to the safety assessment of excipients, not to the safety assessment of active drug substances.

These testing guidelines are informational in nature and are intended to be used by professionals having a knowledge of toxicology and associated sciences. It is also intended that the applicable safety test method requirements of the receiving regulatory authority would be used in a proposal for market entry. For example, if a proposal is to be submitted to the U.S. Food and Drug Administration, that agency's safety test requirements would have to be met. These guidelines do not provide specific details regarding test methodology and data interpretation. Test procedures that are generally recognized by experts and by the regulatory agencies should be used. Alternatives to the use of living animals are encouraged wherever these alternative procedures have been validated for the intended purpose and where it is known that the alternative procedure will provide sufficient data upon which to base a safety judgment. It is recommended that the *Guiding Principles on the Use of Animals in Toxicology* of the Society of Toxicology (1996) and, in other countries, the appropriate legal and professional codes, be adhered to in the conduct of all test procedures. All studies must meet the requirements of the appropriate national good laboratory practice guidelines in effect in the country where the studies are being conducted.

In cases of extensive human experience based upon food use, there may be sufficient information to fulfill the requirements of the guidelines for orally-ingested excipients only. In addition, there may be animal-based data, which was developed for other purposes, that may be used to fulfill the testing guidelines requirements. If the data requirements have been met through prior human use experience and pertinent human data have been collected in a scientifically sound manner, there is no need to provide animal data for those endpoints evaluated by prior clinical experience.

Some dosage routes offer unique toxicological challenges, and the guidelines include provisions for these routes (e.g., inhalation). Also, further explanation is provided regarding numbers of species and other basic information (e.g., two species, one rodent and one nonrodent).

The extent of information required to define a set of baseline data, which constitute a toxicological and chemical database, is dependent upon the intended use of, and duration of, dosing of the candidate excipient material. It is critical that a thorough review of background information be conducted before embarking on a testing regimen. In addition to literature database reviews, information should be obtained regarding the physical and chemical properties of the compound; its manufacturing process (or processes); and product specifications including limits of impurities, potential for pharmacological activity, exposure conditions (i.e., dose, duration, frequency of use, dosage formulation, and route of administration), and potential user population. Also, base toxicity information covering the topics is fundamental. Particular attention should be addressed to the absorption/distribution/metabolism/excretion/pharmacokinetics (ADME/PK) studies because much of the later decision process will be dependent upon these data.

These guidelines provide a mechanism for obtaining sets of baseline data for all candidate excipient materials. The background information and baseline toxicity information alone may support the use of the candidate excipient either in a short half-life product that is not administered in a frequency that results in a residual excipient build-up in body tissue or in a product used only once or twice in a lifetime, such as a diagnostic agent. Additional tests, listed under *Step 4* of the *Safety Assessment Guidelines*, are necessary for candidate excipient material that is to be used in a manner that will result in short- or intermediate-term repeated exposure in humans—that is, a pharmaceutical product that will be administered for less than 10 days or for 30 to 90 consecutive days, respectively. For a candidate excipient material that is intended for use in a pharmaceutical product intended for either intermittent or chronic administration over a long time period, such as a treatment for psoriasis or an insulin preparation, further tests are required. These tests are listed under *Step 7* of the guidelines and in the appropriate section under *Additional Requirements for Specific Exposure Routes*. While providing guidance for consumer safety, some of the required tests are intended to provide information to address occupational safety (e.g., skin and eye irritation).

The guidelines are summarized in *Table 1*. Tests that are required (R) by the guidelines are distinct from those that are recommended conditionally (C). Whether conditional tests are conducted is dependent upon the conditions of use and available biological data. Consideration must also be given to the requirements of the regulatory authorities when making the decision to test.

**Table 1. Summary of Excipient Guidelines**

| | Routes of Exposure for Humans | | | | | |
|---|---|---|---|---|---|---|
| **Tests** | **Oral** | **Mucosal** | **Dermal/ Topical/ Trans-dermal** | **Injectable*** | **Inhalation/ Intranasal** | **Ocular** |
| *Baseline Toxicity Data* | | | | | | |

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**Table 1. Summary of Excipient Guidelines** *(continued)*

| | Routes of Exposure for Humans | | | | | |
|---|---|---|---|---|---|---|
| **Tests** | **Oral** | **Mucosal** | **Dermal/ Topical/ Trans-dermal** | **Injectable*** | **Inhalation/ Intranasal** | **Ocular** |
| Acute Oral Toxicity | R | R | R | R | R | R |
| Acute Dermal Toxicity | R | R | R | R | R | R |
| Acute Inhalation Toxicity | C | C | C | C | R | C |
| Eye Irritation | R | R | R | R | R | R |
| Skin Irritation | R | R | R | R | R | R |
| Skin Sensitization | R | R | R | R | R | R |
| Acute Injectable Toxicity | — | — | — | R | — | — |
| Application Site Evaluation | — | — | R | R | — | — |
| Pulmonary Sensitization | — | — | — | — | C | — |
| Phototoxicity/Photoallergy | R | — | R | R | R | — |
| Genotoxicity Assays | R | R | R | R | R | R |
| ADME/PK-Intended Route | R | R | R | R | R | R |
| 28-Day Toxicity (2 Species)-Intended Route | R | R | R | R | R | R |
| | | | | | | |
| *Additional Data: Short- or Intermediate-term Repeated Use* | | | | | | |
| 90-Day Toxicity (Most Appropriate Species) | R | R | R | R | R | R |
| Embryo-Fetal Toxicol. | R | R | R | R | R | R |
| Additional Assays | C | C | C | C | C | C |
| Genotoxicity Assays | R | R | R | R | R | R |
| Immunosupression Assays | R | C | C | R | C | C |
| | | | | | | |
| *Additional Data: Intermittent Long-term or Chronic Use* | | | | | | |
| Chronic Toxicity (Rodent, Nonrodent) | C | C | C | C | C | C |
| Reproductive Toxicity | R | R | R | R | R | R |
| Photocarcinogenicity | C | — | C | C | C | — |
| Carcinogenicity | C | C | C | C | C | C |
| R = Required<br>C = Conditional | | | | | | |

* Intravenous, intramuscular, subcutaneous, intrathecal, etc.

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## SAFETY ASSESSMENT GUIDELINES

### Background Information

Before proceeding to the steps under *Data Requirements and Checkpoints*, the following points should be reviewed and defined:

- Review literature information using all appropriate databases
- Define chemical and physical properties
- Define manufacturing process
- Define product specifications, including impurities and residual solvents (see applicable ICH guidelines)
- Estimate exposure conditions (dose, duration, frequency route)
- Define user population
- Assess potential for pharmacologic activity.

At this point evaluate what is known, and develop the initial approach to testing.

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## Data Requirements and Checkpoints

STEP 1

Toxicity Data (see *Baseline Toxicity Data*)
The toxicity data should take into account the following information:

- Effects of acute exposure by oral and intended routes
- Effects of repeated exposures by intended routes
- Effects of in vitro genotoxicity assays
- ADME/PK by oral or appropriate routes; single or multiple doses.

STEP 2

Depending on results of above, evaluate effects of a single dose in humans.

STEP 3

Checkpoint: Evaluate results of above and proposed exposure conditions and exposed population. The above data might allow use in a single product with a short half-life (e.g., a diagnostic agent).

STEP 4

Gather the following additional data:

- Effects of subchronic exposure in appropriate species and routes
- Embryo-fetal development studies via appropriate route of exposure
- Additional in vitro and in vivo genotoxicity tests.

STEP 5

Depending on results of above, consideration should be given to testing in humans as part of the clinical trials of an active ingredient or as a stand-alone procedure.

STEP 6

Checkpoint: Evaluate all of above information. Data might allow use in a variety of products intended for short-term, repeated intake (e.g., an antibiotic). If the ADME/PK studies for a noninjectable excipient show no absorption, data may permit using a product for 30 to 90 consecutive days.

STEP 7

Additional data should be obtained for use in a product taken chronically, either daily or intermittently, over a long time period depending on:

- Results of subchronic studies and long-term toxicity in appropriate mammalian nonrodents
- Reproductive toxicity studies
- Other test results and human exposure data and long-term toxicity or carcinogenicity in rodents.

## Baseline Toxicity Data

The following data should be taken into account:

- Appropriate acute toxicity by intended dose routes: skin sensitization, approximate lethal dose method, limit test, etc.
- Other appropriate acute toxicity studies: oral toxicity by limit test or approximate lethal dose method, skin irritation, etc.
- ADME/PK: single or multiple doses.
- Genotoxicity: for example, Ames Test, in vitro chromosome aberration test, mammalian cell mutation assay.
- 28-day repeated dosing studies in two species by appropriate routes (one rodent, one mammalian nonrodent): evaluation of injection site or similar considerations might be necessary depending on route of administration.

[NOTE—1. In those cases where intended route restrictions (e.g., volume, concentration) preclude an adequate assessment of the toxicity of the excipient, development of a toxicity profile by other relevant routes may be needed.

2. The comparison of toxicity and ADME/PK data between oral and intended routes is critical at this point because that knowledge may set the direction for future toxicity testing (e.g., reproductive toxicity testing conducted by oral route rather than intended route). In addition, relevant studies using the intended route and anticipated duration of exposure may preclude performance of additional studies.]

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## Additional Requirements for Specific Exposure Routes

### FOR ORAL EXPOSURE

No additional requirements beyond those presented for *Baseline Toxicity Data*.

### FOR MUCOSAL EXPOSURE

No additional requirements beyond those presented for *Baseline Toxicity Data*.

### FOR DERMAL, TOPICAL, OR TRANSDERMAL EXPOSURE

**Baseline Toxicity Data**

- *Effects of Acute Exposure by Transdermal Dose Route:* dermal sensitization study for repeat applications
- *Effects of Repeated Exposures by Transdermal Route*
    1. Photoallergy/phototoxicity study
    2. Studies in two species (one rodent, one mammalian nonrodent) by transdermal route.
- *Effects of Subchronic Exposure*, *Reproductive Toxicity Effects*—Initial toxicity studies may be performed by the IV route to adequately profile the toxicity of the excipient. This will provide an assessment of potential target organs if an adequate amount of the compound cannot be delivered via a transdermal dosage form. This is dependent upon the results from the ADME/PK studies.

Reproductive studies may also be conducted via oral or IV route with demonstration of absorption (oral) and pharmacokinetic comparisons of the chosen route versus transdermal.

Photocarcinogenicity studies may be required and should be considered if data and the proposed use indicate when evaluating materials to be placed on the skin for prolonged periods of time and exposure to UV light is a factor (e.g., sun block). This also applies to oral, parenteral, and inhalation products where skin drug concentrations exceed plasma drug concentrations for a substantial period of time, or where the candidate material would appear to have the potential for photo-activity or has demonstrated photo-activity.

### FOR INJECTABLE DOSAGE FORMS

**Background Information**

1. Define compatibility of the dosage form with blood, if appropriate, based on route of exposure.
2. Define the pH and tonicity of injectable dose form, if appropriate, based on the route of exposure.

**Baseline Toxicity Data**

- *Effects of Acute Exposure by Intended Injectable Dose Routes*
    1. Include evaluation of injection site irritation in rabbit or dog
    2. Include evaluation of rate of administration.

### FOR INHALATION OR INTRANASAL EXPOSURE[1]

**Baseline Toxicity Data**

- *Acute Inhalation Toxicity*—A limit test that would, for example, use the highest achievable concentration in a 4-hour exposure to vapor, aerosol, or solid particulate. Pulmonary sensitization may be performed along with other appropriate studies. If exposure is to be to an aerosol or solid particulate, particulates of appropriate mass median diameter should be generated.
- *Single and Repeated Dose ADME/PK by Inhalation or Intranasal and Oral Routes*
- *28-Day Repeated Dose Inhalation Study in Two Mammalian Species Using Vapor or Particulates of Appropriate Mass Median Diameter:* compare to similar oral toxicity data.

### FOR OPHTHALMIC EXPOSURE

**Background Information:** define pH and osmolarity of topical ocular dose form.

**Baseline Toxicity Data**

- *Effects of Acute Exposure by Ophthalmic Routes:* cytotoxicity tests (e.g., agar overlay)
- *Effects of Repeated Exposures by Ophthalmic Routes*
    1. Studies in two species (one rodent, one mammalian nonrodent)
    2. Examination of anterior and posterior segments of the eye
    3. Studies on allergenicity potential.

**Other Data**—Comparison of pharmacokinetic parameters of the route chosen for reproductive studies and the ophthalmic exposure are essential for extrapolation of potential toxicity via the ophthalmic route.

[1] When designing studies to evaluate use in products intended for use by the inhalation or intranasal route, consideration should be given to the dosing regimen that will be used by humans. The appropriate study protocol for a product intended for inhalation therapy that will result in prolonged exposures (e.g., several hours per day) may differ from that used to evaluate a product that would result in exposure to several metered doses per day.

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## GLOSSARY

**Acute:** exposure to a test agent within a single, 24-hour period. Doses may be single, multiple or continuous during a 24-hour period.

**Subacute:** repeated dosing of a test agent for up to 29 days. Daily doses may be single, multiple or continuous during a 24-hour period.

**Subchronic:** repeated dosing of a test agent for 30 days to 10% of the lifespan of the test species (90 days in rodents). Daily doses may be single, multiple or continuous during a 24-hour period.

**Chronic:** repeated dosing of a test agent for more than 10% of the lifespan of the test species (more than 90 days in rodents). Daily doses may be single, multiple or continuous during a 24-hour period.

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# ⟨1078⟩ GOOD MANUFACTURING PRACTICES FOR BULK PHARMACEUTICAL EXCIPIENTS

## BACKGROUND

This general information chapter provides guidelines for methods, facilities, and manufacturing controls to be used in the production of pharmaceutical excipients in order to ensure that excipients possess the quality, purity, safety, and suitability for use that they purport to possess. The principles and information in this chapter can be applied to the manufacture of all pharmaceutical excipients (referred to throughout this document as *excipient[s]*) intended for use in human drugs, veterinary drugs, and biologics. It covers the quality management system and the extent of good manufacturing practices (GMP) necessary throughout manufacturing for both batch and continuous processes. It is intended to assist manufacturers as well as auditors in establishing whether the facilities and controls used for the manufacture of excipients are adequate and whether the excipients possess the quality and purity that they purport to possess and are suitable for their intended use. The manufacture of certain excipients for specialist applications presents additional challenges that are outside the scope of this chapter. Examples include excipients for parenteral, ocular, inhalation, and open wound use and those that are sterile and/or pyrogen-free. It does not provide information for all national legal requirements nor does it cover in detail the particular characteristics of every excipient. The quality system standard used as a framework for this chapter is ISO 9001, which is appropriate to manufacturing. Because of the diversity of excipients, some principles in this information chapter may not be applicable to certain products and processes.

This chapter combines the concepts of existing GMP principles from the following sources:

- World Health Organization (WHO) GMP Guidelines for Excipients,
- International Pharmaceutical Excipients Council (IPEC) Good Manufacturing Practices Guide for Bulk Pharmaceutical Excipients 2001,
- Institute of Quality Assurance (IQA) Pharmaceutical Quality Group (PQG) PS 9100:2002, Pharmaceutical Excipients,
- International quality management system requirements as developed by the International Organization for Standardization (ISO).

In view of the increasing globalization of the pharmaceutical industry and the harmonization of pharmaceutical registration requirements, deference to all schemes is becoming necessary. Therefore, relevant portions of the manufacturing concepts are employed throughout this chapter.

The *General Guidance* section provides an overview of the appropriate manufacturing practice criteria applicable to excipient manufacture and the points of application of excipient good manufacturing practices and quality systems. The section also recommends measures to limit contamination of an excipient. Finally, it discusses the relationship of excipients to finished dosage forms. No attempt has been made to include details specific to particular excipients.

The information in *Appendix: Auditing Considerations* sets forth key criteria to aid in the audit of an excipient manufacturing facility.

For a list of terms used in this chapter and their definitions, see *Glossary.*

## INTRODUCTION

### Purpose and Scope

This chapter defines the extent and point of application of appropriate GMP principles for excipient manufacture and is applicable to the manufacture of excipients intended for use in drug products. It covers the quality management system and the extent of GMP necessary throughout manufacturing for both batch and continuous processes. It is intended to aid both auditors and manufacturers in establishing whether the facilities and controls used for the manufacture of excipients are adequate and whether the excipients possess the quality, purity, and safety that they purport to possess and are suitable for their intended use.

The manufacture of certain excipients for specialist applications presents additional challenges that are outside the scope of this chapter. Examples include excipients

- for parenteral, ocular, inhalation, and open wound use; and
- those that are purported to be sterile and/or pyrogen-free.

In these cases, detailed information pertaining to the intended use of an excipient as provided by the end user can be useful in determining appropriate GMP. This chapter does not address the specific GMP relating to good trade and distribution practices (GTDP).

### Principles Adopted

#### THE CHAPTER AND ITS USE

Pharmaceutical excipients are diverse and often have uses other than pharmaceutical applications. Each manufacturer should consider how the chapter might apply to its products and processes (for example, batch versus continuous processes). Because excipients are so diverse, some principles of this chapter may not be applicable to certain products and manufacturing processes.

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### APPLICATION

The text provides information necessary for the manufacture of excipients but does not provide all the details. It cannot specify national legal requirements or cover particular characteristics of every excipient.

### QUALITY SYSTEM STANDARD

The quality management system standard chosen as a framework for this chapter is ISO 9001, which is appropriate for manufacturing facilities. A manufacturer may apply the ISO standard with or without certification; but this possibility, as a business decision, is not discussed in this chapter. However, ISO certification has the benefit of providing assurance to customers that the excipient manufacturer's quality management system has been independently verified.

The headings in this chapter have been aligned with the ISO 9001 clause numbers, because many excipient manufacturers already use that standard as a basis for their quality management system. Additional headings are included as needed to introduce additional guidance on GMP when not covered by current ISO 9001 clauses.

### DOCUMENT STRUCTURE

The chapter combines the concepts of existing GMP principles from the following:

- World Health Organization (WHO), GMP Guidelines for Excipients,
- International Pharmaceutical Excipients Council (IPEC), Good Manufacturing Practices Guide for Bulk Pharmaceutical Excipients 2001,
- Institute of Quality Assurance (IQA) Pharmaceutical Quality Group (PQG) PS 9100:2002, Pharmaceutical Excipients, International quality management system requirements as developed by the International Organization for Standardization (ISO).

In view of the increasing globalization of the pharmaceutical industry and the harmonization of pharmaceutical registration requirements, relevant portions of the manufacturing concepts detailed in these schemes are employed throughout this chapter.

The *General Guidance* section provides an overview of the GMP criteria applicable to excipient manufacture and the point of application of excipient GMP.

The remaining sections provide guidance on GMP principles and implementation of a quality management system suitable for excipient manufacture. For example, these sections suggest measures to limit excipient contamination. No attempt has been made to include details specific to particular excipients, and individual manufacturers should address these as they apply to their own products and processes.

The Appendixes provide supporting guidance for excipient GMP. *Appendix: Auditing Considerations* describes key criteria to be considered in the audit of an excipient manufacturing facility. *Glossary* provides definitions of terms used in this chapter.

## GENERAL GUIDANCE

### Pharmaceutical Excipients

Pharmaceutical excipients are substances other than the active pharmaceutical ingredient (API) that have been appropriately evaluated for safety and are intentionally included in a drug delivery system. For example, excipients can do the following:

- aid in the processing of the drug delivery system during its manufacture,
- protect, support, or enhance stability, bioavailability, or patient acceptability,
- assist in product identification, and
- enhance any other attribute of the overall safety, effectiveness, or delivery of the drug during storage or use.

A more complete classification of excipients according to their functions can be found in *USP and NF Excipients, Listed by Category* in the *USP–NF.*

### Excipient GMP Implementation

The application of GMP is relevant once it has been determined that a chemical is intended for use as a component of a drug product. Excipient manufacture should be carried out in accordance with the GMP concepts consistent with this chapter. The objective of excipient GMP is to ensure that the manufacture of an excipient results in a consistent material with the desired quality characteristics. The emphasis of GMP for excipients is to ensure product integrity, avoid product contamination, and ensure that records are maintained.

As the excipient manufacturing process progresses, the degree of assurance concerning the quality of the product should increase. Manufacturing processes should be controlled and documented. However, at some logical processing step, as determined by the manufacturer, the GMP as described in this chapter should be applied and maintained.

Judgment based on risk analysis and a thorough knowledge of the process is required in order to determine from which processing step GMP should be implemented. This is usually well before the final finishing operation and, for example, may be identified using methods such as hazard analysis and critical control point (HACCP), failure mode and effects analysis (FMEA), or a detailed process flow diagram. Consideration should also be given to other factors such as batch versus continuous processing, dedicated versus multipurpose equipment, and open versus closed processes.

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# QUALITY MANAGEMENT SYSTEM: EXCIPIENT QUALITY SYSTEMS

## General Recommendations

The principles outlined in this chapter provide a comprehensive basis for the quality management system used in the manufacture of pharmaceutical excipients. Excipient manufacturers should identify the quality management processes required to ensure excipient quality. Where manufacturing, testing, or other operations that could affect excipient quality are outsourced, the responsibility for quality remains with the excipient manufacturer, and control measures should be defined (see also the subsection *Purchasing Information* in the *Product Realization* section).

## Documentation Recommendations

### GENERAL

The excipient manufacturer should have a system in place to control documents and data that relate to the requirements of the quality management system.

### QUALITY MANUAL

The excipient manufacturer should prepare a quality manual describing the quality management system, the quality policy, and the commitment of the excipient manufacturer to applying the appropriate GMP and quality management standards contained in this chapter. This manual should include the scope of the quality management system, reference to supporting procedures, and a description of the interaction between quality management processes.

### CONTROL OF DOCUMENTS

The excipient manufacturer should establish and maintain procedures for the identification, collection, indexing, filing, storage, maintenance, and disposition of controlled documents, including documents of external origin that are part of the quality management system.

Procedures used in the manufacture of excipients should be documented, implemented, and maintained. In addition, there should be formal controls relating to procedure approval, revision, and distribution. These controls should provide assurance that the current version of a procedure is being used throughout the operational areas and that previous revisions of documents have been removed.

Documents and subsequent changes to documents should be reviewed and approved by designated qualified personnel before issuance to the appropriate areas, as identified in the documents. Documents that affect product quality should be reviewed and approved by the quality unit (see also *Responsibility and Authority* in the section *Responsibility, Authority, and Communication* under *Management Responsibility*).

Controlled documents may include a unique identifier, the date of issue, and a revision number to facilitate identification of the most recent document. The department with the responsibility for issuing the documents should be identified. When it is practical, changes and the reasons for the changes should be documented.

Electronic documentation should meet the requirements for the document control system stated above. If electronic signatures are used on documents, they should be controlled to provide security equivalent to that provided by a handwritten signature. Electronic documents and signatures may also have to satisfy local regulatory requirements.

### CONTROL OF RECORDS

The excipient manufacturer should establish and maintain procedures for the identification, collection, indexing, filing, storage, maintenance, and disposition of records.

Records should be maintained to demonstrate achievement of the required quality and the effective operation of the quality management system. Records should be legible and identifiable with the product involved. Pertinent subcontractor quality data should be an element of these records.

Entries in records should be clear, indelible, made directly after performing the activity (in the order performed), and signed and dated by the person making the entry. Corrections to entries should be signed and dated, leaving the original entry legible.

Records should be kept for a defined period. This period should be appropriate to the excipient and to its expiry date or reevaluation interval. Records should be stored and maintained in such a manner that they are readily retrievable, in facilities that provide an environment suitable for minimizing deterioration or damage.

### CHANGE CONTROL

The excipient manufacturer should establish and maintain procedures to evaluate and approve changes that may affect the quality of the excipient. For example, this may include changes to the following:

- raw materials or packaging and their sources,
- material specifications,
- test methods,
- manufacturing and analytical equipment,
- production processes,
- manufacturing or packaging sites.

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A unit with a function that is independent from production (such as regulatory affairs or quality assurance) should have the responsibility and authority for the final approval of changes.

Customers should be notified, and, where applicable, excipient regulatory submissions (for example, for Drug Master Files [DMFs] or Certificates of Suitability to the *European Pharmacopoeia* [CEPs]) should be amended to reflect significant changes from established production and process control procedures that may affect excipient quality (see also *Customer Communication* in the section *Customer-Related Processes* under *Product Realization*).

# MANAGEMENT RESPONSIBILITY

## Management Commitment

Top management should demonstrate to the organization the importance it places on customer satisfaction and compliance with the appropriate regulations and standards. This should be accomplished through the development of a quality policy and establishment of quality objectives. Progress toward the documented quality objectives should be reviewed at planned intervals.

## Customer Focus

It is the responsibility of top management to ensure that customer requirements are determined and met. The excipient manufacturer should permit the customer or its representative to conduct audits of the manufacturer's quality management system, manufacturing processes, buildings, and facilities.

## Quality Policy

Top management should demonstrate its commitment to the corporate quality policy and ensure that it is implemented within the operational unit. The quality policy should support continual improvement of the quality management system. Management should participate in the development of the company's quality policy and provide the resources necessary for its development, maintenance, and deployment.

## Planning

### QUALITY OBJECTIVES

Top management should set objectives for adherence to GMP to ensure that the excipient manufacturer maintains and improves its performance. Objectives should be deployed throughout the organization and should be measurable and consistent with the quality policy.

### QUALITY MANAGEMENT SYSTEM PLANNING

Top management should provide adequate resources to ensure conformity to the provisions of this chapter. There should be a process for the identification of resources needed for adherence to GMP. A gap analysis based on audits by internal personnel, customers, regulatory agencies, or outside contractors, or based on the use of this chapter, could be created to identify resource requirements. Top management should ensure that the integrity of the quality management system is maintained when changes are planned and implemented.

## Responsibility, Authority, and Communication

### RESPONSIBILITY AND AUTHORITY

Responsibility and authority should be clearly defined by top management and communicated within the organization. It should be the responsibility of a unit that is independent of production, such as the quality unit, to do the following:

- ensure that quality-critical activities are undertaken as defined,
- approve suppliers of quality-critical materials and services,
- approve or reject raw materials, packaging components, intermediates, and finished excipients,
- ensure that there is a review of production records to confirm that no errors have occurred or, if errors have occurred, that they are fully investigated,
- participate in reviewing and authorizing changes to processes, specifications, procedures, and test methods that potentially affect quality (also see above, *Change Control* in the section *Documentation Recommendations* under *Quality Management System: Excipient Quality Systems*) and participate also in investigating failures and complaints,
- retain responsibility for approval or rejection of the excipient if it is produced, processed, packaged, or held under contract by another company,
- develop and implement a self-inspection program of the quality management system.

The excipient manufacturer may delegate some of the quality unit's activities to other personnel if appropriate controls (for example, periodic audits, training, and documentation) are in place.

An organization chart by function should show interdepartmental relationships as well as relationships to top management of the company. Personnel whose positions affect excipient quality should have job descriptions.

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#### MANAGEMENT REPRESENTATIVE

The excipient manufacturer should appoint a management representative with sufficient authority to ensure that the provisions of this chapter are properly implemented. The representative should periodically report to top management on conformity to the quality management system, including changing customer and regulatory requirements.

#### INTERNAL COMMUNICATION

The excipient manufacturer should ensure that appropriate systems are established to communicate GMP and regulatory requirements, quality policies, quality objectives, and procedures throughout the organization. The communication should also provide information about the effectiveness of the quality management system. Top management should be notified promptly of quality-critical situations, such as product retrievals, in accordance with a documented procedure.

### Management Review

#### GENERAL

The top management of the company should hold periodic reviews of the quality management system to confirm the organization's continued conformity to this chapter. The review should be recorded and should include assessing opportunities for improvement and the need for changes to the quality management system.

#### REVIEW INPUT

Management review inputs should include, for example, the following:

- results of internal and external audits,
- customer feedback of the company performance,
- product conformity and process performance,
- action items from the previous management review,
- customer complaints,
- status of corrective or preventive actions,
- changes that could affect the quality management system.

#### REVIEW OUTPUT

The management review should identify the resources needed and the opportunities presented for improving the quality management system and improving product conformity to customer and regulatory requirements. A record should be made of actions recommended and taken.

## RESOURCE MANAGEMENT

### Provision of Resources

There should be sufficient qualified personnel and resources (e.g., equipment, materials, buildings, and facilities) to implement, maintain, and improve the quality management system and to produce, package, test, store, and release each excipient in a manner consistent with this chapter.

### Human Resources

#### GENERAL

Personnel performing work affecting the quality of excipients should have the appropriate combination of education, training, and experience for their assigned tasks. Consultants advising on the design, production, packaging, testing, or storage of excipients should have sufficient education, training, and experience or any combination thereof to advise on the subject for which they are retained. Records should be maintained listing the name, address, and qualifications of consultants and the type of service they provide.

#### COMPETENCE, AWARENESS, AND TRAINING

The excipient manufacturer should establish and maintain procedures for identifying training needs and for providing the necessary training to personnel performing activities affecting excipient quality. Appropriate records of training should be maintained. Training should address the particular operations that the employee performs and GMP as they relate to the employee's functions. Qualified individuals should conduct GMP training frequently enough to ensure that employees remain familiar with applicable GMP principles. Management should establish adequate and continued personal-hygiene training for personnel who handle materials so that they understand the precautions necessary for preventing contamination of excipients. The training program should ensure that personnel understand that deviations from procedures may affect the customer's product quality.

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## PERSONNEL HYGIENE

To protect excipients from contamination, protective apparel such as head, face, hand, and arm coverings should be worn as appropriate to the duties performed. Jewelry and other loose items, including those in pockets, should be removed or covered. Only authorized personnel should enter the areas of the buildings and facilities designated as limited-access areas.

Personnel should practice good sanitation and health habits. Any person shown by either medical examination or supervisory observation to have an apparent illness or open lesions that may adversely affect the safety or quality of the excipient should be excluded from direct contact with raw materials, packaging components, intermediates, and finished excipients until the condition is corrected or until competent personnel determine that it will not jeopardize the safety or quality of the excipient. Personnel should be instructed to report to supervisory personnel any health conditions that may have an adverse effect on excipients. The storage and use of food, drink, personal medication, tobacco products, or similar items should be restricted to designated locations separate from manufacturing areas.

## INFRASTRUCTURE

The infrastructure should be managed, operated, cleaned, and maintained in accordance with GMP principles to ensure excipient quality and to avoid contamination (including, where critical to excipient quality, control of particulate matter, microbiological control, and control of water quality).

## BUILDINGS AND FACILITIES

The prevention of contamination should be considered in the design of the manufacturing processes and facilities, particularly when the excipient is exposed. Buildings and facilities used in the production, processing, packaging, testing, or storage of an excipient should be maintained in a good state of repair and should be of suitable size, construction, and location to facilitate cleaning, maintenance, and correct operation appropriate to the type of processing.

Manufacturing processes associated with the production of highly sensitizing or toxic products (for example, herbicides and pesticides) should be located in dedicated facilities or should use equipment separate from that used for excipient manufacture. If this is not possible, appropriate measures (for example, cleaning, inactivation) should be implemented to avoid cross-contamination. The effectiveness of these measures should be demonstrated. There should be adequate facilities for the testing of raw materials, packaging components, intermediates, and finished excipients.

## EQUIPMENT

Equipment used in the production, processing, packaging, testing, or storage of an excipient should be maintained in a good state of repair and should be of suitable size, construction, and location to facilitate cleaning, maintenance, and correct operation, depending on the type of processing (for example, batch versus continuous). Equipment should be commissioned before use to ensure that it is functioning as intended. Where equipment is located outdoors, there should be suitable controls to minimize the risk to excipient quality from the environment (for example, processing within a closed system).

## EQUIPMENT CONSTRUCTION

Process equipment should be constructed so that contact surfaces will not be reactive, additive, or absorptive and thus will not alter the quality of the excipient. Substances required for operation, such as lubricants or coolants, should preferably not come into contact with raw materials, packaging materials, intermediates, or finished excipients. Where contact is possible, substances suitable for use in food applications should be employed.

Equipment should be designed to minimize the possibility of contamination caused by direct operator contact in activities such as the unloading of centrifuge bags, the use of transfer hoses (particularly those used to transfer powders), and the operation of drying equipment and pumps. The sanitary design of transfer and processing equipment should be evaluated. To control the risk of contamination, equipment with moving parts should be assessed with regard to the integrity of seals and packing materials.

## EQUIPMENT MAINTENANCE

Documented procedures should be established and followed for maintenance of critical equipment used in the production, processing, packaging, testing, or holding of the excipient. There should be records of the use and maintenance of quality-critical equipment. These records can be in the form of a log, computer database, or other appropriate documentation.

## COMPUTER SYSTEMS

Computer systems that may affect excipient quality should have sufficient controls for operation and maintenance and for prevention of unauthorized access or changes to computer software, hardware, or data, including the following:

- systems and procedures that show that the equipment and software are performing as intended,
- procedures for checking the equipment at appropriate intervals,
- retention of suitable back-up or archival systems such as copies of the program and files,
- assurance that changes are verified and documented and are made only by authorized personnel.

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## UTILITIES

Utilities (for example, nitrogen, compressed air, and steam) used in the production, storage, or transfer of materials that could affect excipient quality should be assessed and appropriate action taken to control the risk of contamination and cross-contamination.

## WATER

Water used in the manufacture of excipients should be demonstrated to be of appropriate quality in consideration of purity requirements and the intended use of the excipient. Unless otherwise justified, process water should, at a minimum, meet regulatory requirements for drinking (potable) water. If drinking (potable) water is insufficient to ensure quality, or if tighter chemical and/or microbiological water quality specifications are required, appropriate controls and specifications should be set: for example, physical and chemical attributes, total microbial counts, and limits on objectionable organisms and/or endotoxins.

Where water used in the process is treated by the manufacturer to achieve a defined quality, the treatment process should be specified and monitored with appropriate action limits. Water that comes into contact with the excipient should be supplied under continuous positive pressure (or other means of preventing back flow) in a system free of defects to control the risk of contamination to the excipient.

## WORK ENVIRONMENT

Where the excipient is exposed during manufacture, it should be in an environment appropriate for minimizing contamination. The manufacturer should apply suitable controls to maintain that environment.

## AIR HANDLING

Where an air-handling system is installed to provide protection to the excipient, the excipient manufacturer should demonstrate its effectiveness. Excipient production unit air-handling systems should be designed to prevent cross-contamination. For dedicated areas processing the same excipient, it is permissible to recycle a portion of the exhaust air back into the same area. The adequacy of such a system for multiuse areas, especially if several products are processed simultaneously, should be assessed for potential cross-contamination.

## CONTROLLED ENVIRONMENT

A controlled environment may be necessary in order to avoid contamination or degradation caused by exposure to heat, air, or light. The degree of protection required may vary depending on the stage of the process. Special environments required by some processes should be monitored to ensure product quality (for example, inert atmosphere or protection from light). Where an inert atmosphere is required, the gas should be treated as a raw material. If interruptions in the special environment occur, adequate evidence and an appropriate rationale should be documented to show that such interruptions have not compromised the quality of the excipient. Such environmental concerns become increasingly important following purification of the excipient.

## CLEANING AND SANITARY CONDITIONS

Adequate cleanliness is an important consideration in the design of excipient manufacturing facilities. Buildings used in the production, processing, packaging, or holding of an excipient should be maintained in an appropriately clean and sanitary condition according to the type of processing conducted (for example, open/closed systems). Where maintenance of clean and sanitary conditions is critical to excipient quality, documented procedures should assign responsibility for cleaning and sanitation, describing in sufficient detail the cleaning schedules, methods, equipment, and materials to be used in cleaning the buildings and facilities. These procedures should be followed, and cleaning should be documented. Waste should be segregated and disposed of in a timely and appropriate manner. If waste is not disposed of immediately, it should be suitably identified.

## PEST CONTROL

Buildings should be free from infestation by rodents, birds, insects, and other vermin. Some raw materials, particularly botanicals, may contain some unavoidable contamination, such as rodent or other animal filth or infestation. The manufacturer should have sufficient control methods to prevent the increase of such contamination or infestation in holding areas and its spread to other areas of the plant.

## LIGHTING

Adequate lighting should be provided to facilitate cleaning, maintenance, and proper operations.

## DRAINAGE

In areas where the excipient is open to the environment, drains should be of adequate size and, where connected directly to a sewer, should be provided with an air break or other mechanical device to prevent back-siphoning.

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#### WASHING AND TOILET FACILITIES

Adequate personal washing facilities should be provided, including hot and cold water, soap or detergent, air dryers or single-service towels, and clean toilet facilities easily accessible to working areas. Adequate facilities for showering and/or changing clothes should be provided, where appropriate.

## PRODUCT REALIZATION

### Planning of Product Realization

The excipient manufacturer should plan and develop the processes and controls needed for product manufacture. These plans and controls should be appropriate to the production process, excipient specification, equipment, and facilities used in the manufacture of the product. Key aspects of the planning of a suitable process and its controls should include the following, as appropriate:

- documented testing programs, for quality-critical materials including excipients, that include appropriate specifications, sampling plans, and test and release procedures,
- generation and maintenance of records (also see above, *Control of Records* in the section *Documentation Recommendations* under *Quality Management System: Excipient Quality Systems*) that provide evidence that these plans have been realized as intended and that enable traceability to be demonstrated (also see below in this section, *Traceability* under *Identification and Traceability*),
- provision of resources to implement these plans,
- environmental and hygiene control programs to minimize contamination.

### Customer-Related Processes

#### DETERMINATION OF REQUIREMENTS RELATED TO THE PRODUCT

The excipient manufacturer should determine the excipient quality, labeling, and delivery requirements of the customer. Additional requirements, whether customer-specific, legal, or regulatory (for example, pharmacopeia material and general monographs), should be agreed on by both parties. Requirements not stated by the customer but necessary for specified or intended use, where known, should be considered.

#### REVIEW OF REQUIREMENTS RELATED TO THE PRODUCT

The excipient manufacturer and customer should mutually agree upon the requirements identified in the section above, *Determination of Requirements Related to the Product*, before supply commences. The manufacturer should have the facility and process capability to consistently meet the mutually agreed-upon specifications. Where the requirements determined in the section *Determination of Requirements Related to the Product* are changed, this review should be repeated before supply recommences.

#### CUSTOMER COMMUNICATION

There should be provision for providing accurate and pertinent communication to the customer. Master copies of documents such as specifications and technical reports should be controlled documents. Provision should be made for replying to customer inquiries, contracts, and order-handling requirements. Customer feedback and complaints should be documented. Customers should be notified of significant changes (also see above, *Change Control* in the section *Documentation Recommendations* under *Quality Management System: Excipient Quality Systems*).

#### DESIGN AND DEVELOPMENT

ISO 9001 includes requirements for ensuring control over design and development activities. It is recommended that companies involved in such activities follow the requirements of ISO 9001. Full GMP are not always applicable during the design and development of new excipients and/or manufacturing processes. However, development batches of excipients that are intended for use in drug products should be manufactured in accordance with the applicable provisions of this chapter.

### Purchasing

#### PURCHASING PROCESS

Excipient manufacturers should have a system for selecting and approving suppliers of quality-critical materials and services (for example, subcontract manufacturers and laboratories). Supplier approval by the quality unit should require an evaluation of the supplier's quality management system, including adequate evidence that they can consistently meet agreed-upon specifications and maintain traceability. This may require periodic audits of the supplier's manufacturing facility. Records of these activities should be maintained. Materials should be purchased against an agreed specification from approved suppliers.

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### PURCHASING INFORMATION

Purchasing agreements should describe the material or service ordered, including, where critical to excipient quality, the following:

- the name, type, class, style, grade, item code number or other precise identification traceable to the raw material and packaging specifications,
- drawings, process requirements, inspection instructions and other relevant technical data, including requirements for approval or qualification of product, procedures, process equipment, and personnel,
- adherence to the appropriate sections of this chapter for relevant contract manufacturers or laboratories, and
- a statement to notify the excipient manufacturer of significant changes in quality-critical raw materials.

### VERIFICATION OF PURCHASED PRODUCT

There should be procedures for the approval and release of quality-critical material. Upon receipt, quality-critical materials should be placed in quarantine and should not be used prior to acceptance. Effective quarantine can be established with suitable identifying labels, signs, and/or other manual documentation systems. When quarantine and stock control are managed with computer systems in lieu of a physical stock control, system controls should prevent the use of unreleased material. Quarantine may not be feasible for materials supplied via pipelines. In these cases the excipient manufacturer should establish an agreement with the supplier so that the manufacturer is notified of material that does not meet specification. Sampling activities should be conducted under defined conditions, in accordance with a defined sampling method and using procedures designed to prevent contamination and cross-contamination.

Quality-critical materials used in the manufacture of an excipient should be tested or otherwise verified prior to use. Verification should include availability and a check of the supplier certificate of analysis and, wherever feasible, at least an identification test. Testing schedules should be organized to separate routine tests from those that are performed infrequently or only for new suppliers. Bulk deliveries should have additional controls to ensure material purity and freedom from contamination (for example, dedicated tankers, tamper-evident seals, a certificate of cleaning, analytical testing, or audit of the supplier). These procedures, activities, and results should be documented.

## Production and Service Provision

### CONTROL OF PRODUCTION AND SERVICE PROVISION

Production activities should be carried out under controlled conditions (also see above, *Planning of Product Realization* under *Product Realization*). Specific examples of important controls, some of which may not be applicable to all excipient manufacturers, are illustrated in the following sections.

### PRODUCTION INSTRUCTIONS AND RECORDS

Production instructions and records are required but may differ for the type of operation: for example, batch versus continuous processes. There should be a controlled document that describes how the excipient is produced (for example, master production instructions, master production and control records, or process definitions). For batch processes, an accurate reproduction of the appropriate master production instructions should be issued to the production area. For continuous processes, a current processing log should be available. Records should be available for each batch of excipient produced and should include complete information relating to the production and control of each batch. For continuous processes, the batch and its records should be defined (for example, based on time or defined quantity). Records may be in different locations but should be readily retrievable. Records for both batch and continuous processing, where critical to excipient quality, should include the following:

- date and time each step was completed or date and time log of key parameters,
- identification of persons performing and directly supervising or checking each significant step, operation or control parameter,
- identification of major equipment and lines used,
- material inputs to enable traceability: for example, batch number and quantities of raw material/intermediate and time it was added,
- in-process and laboratory control results,
- the quantity produced for the defined batch and a statement of the percentage of theoretical yield, unless not quantifiable (for example, as in some continuous processes),
- inspection of the packaging and labeling area before and after use,
- labeling control records,
- description of excipient product containers and closures,
- description of sampling performed,
- failures, deviations and their investigations,
- results of final product inspection.

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## EQUIPMENT CLEANING

The manufacturer should design and justify cleaning and sanitization procedures and provide evidence of their effectiveness. In multipurpose plants the use of the *model product* approach (groups of product of similar type) may be used in justifying a suitable procedure. Cleaning and sanitization procedures should be documented. They should contain sufficient detail to allow operators to clean each type of equipment in a reproducible and effective manner. There should be a record confirming that these procedures have been followed. Equipment and utensils should be cleaned and sanitized where critical to excipient quality and at appropriate intervals to prevent contamination and cross-contamination of the excipient. The cleaning status of equipment should be recorded appropriately.

Where multipurpose equipment is in use, it is important to be able to determine previous usage when investigating cross-contamination or the possibility of such contamination (also see below in this section, *Records of Equipment Use*). During a production campaign, incidental carryover frequently occurs, and it is usually acceptable because cleanup between successive batches of the same excipient is not normally required in order to maintain quality levels. Products that leave residues that cannot be effectively removed should be produced in dedicated equipment. For continuous processing, the frequency of equipment cleaning should be determined by the manufacturer and justified.

## RECOVERY OF SOLVENTS, MOTHER LIQUORS, AND SECOND CROP CRYSTALLIZATIONS

Where solvents are recovered and reused in the same process or different processes, they should meet appropriate standards prior to reuse or mixing with other approved material. Mother liquors or filtrates containing recoverable amounts of excipient, reactants, or intermediates are frequently reused. Such processes should be documented in the production records or logs to enable traceability.

## IN-PROCESS BLENDING OR MIXING

In-process blending or mixing to ensure batch uniformity or to facilitate processing should be controlled and documented. If the intent of the operation is to ensure batch uniformity, it should be performed so as to ensure homogeneous mixing of materials to the extent feasible and should be reproducible from batch to batch.

## IN-PROCESS CONTROL

In-process inspection and testing, based on monitoring the process or actual sample analysis at defined locations and times, should be performed. Sampling methods should be documented to ensure that the sample is representative and clearly labeled. In-process samples should not be returned to production for incorporation into the final batch.

The results of in-process tests should be recorded and should conform to established process parameters or acceptable tolerances. Work instructions should define the procedure to follow and should indicate how to use the inspection and test data to control the process. There should be defined actions to be taken when the results are outside specified limits. Where approval to continue with the process is issued within the production department, the specified tests should be performed by trained personnel and the results recorded.

## PACKAGING AND LABELING

Procedures should be employed to protect the quality and purity of the excipient when it is packaged and to ensure that the correct label is applied to all containers. Packaging and labeling operations should be designed to prevent mix-ups. Procedures should be implemented to ensure that the correct labels are printed and issued and that the labels contain the correct information. The procedure should also specify that excess labels are immediately destroyed or returned to controlled storage. Excess labels bearing batch numbers should be destroyed. Packaging and labeling facilities should be inspected immediately before use to ensure that materials that are not required for the next packaging operation have been removed. When excipients are labeled on the packaging line, packaged in preprinted bags, or bulk-shipped in tank cars, there should be documentation of the system used to satisfy the intent of the above procedures.

## RECORDS OF EQUIPMENT USE

Records of quality-critical equipment use should be retained. These records should allow the sequence of cleaning, maintenance, and production activities to be determined.

## VALIDATION OF PROCESSES FOR PRODUCTION AND SERVICE PROVISION

An important factor in the assurance of product quality includes the adequate design and control of the manufacturing process, because product testing alone is not sufficient to reveal variations that may have occurred. Each step of the manufacturing process should be controlled to the extent necessary for ensuring that the excipient meets established specifications. The concept of process validation is a key element in ensuring that these quality assurance goals are met. The process reactions, operating parameters, purification steps, impurities, and key tests needed for process control should be documented, thus providing the basis for validation.

The full validation program that is typically performed in the pharmaceutical industry may not always be carried out by the excipient manufacturer. However, the excipient manufacturer should demonstrate the consistent operation of each manufacturing process: for example, through process capability studies, development, and scale-up reports.

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## Identification and Traceability

### TRACEABILITY

Quality-critical items (for example, raw materials, packaging materials, intermediates, and finished excipients) should be clearly identified and traceable through records. These records should allow traceability of the excipient both upstream and downstream. Identification of raw materials used in batch production processes should be traceable through the batch numbering system or other appropriate system. Identification of raw materials used in excipients produced by continuous processing should indicate the time frame during which a particular batch of raw material was processed through the plant. Excipient manufacturers should also have adequate knowledge about the origin of any raw materials derived from plant or animal matter.

Raw materials, including solvents, are sometimes stored in bulk tanks or other large containers, making precise separation of batches difficult. Nevertheless, the use of such materials and containers should be documented in production records.

### INSPECTION AND TEST STATUS

There should be a system for identifying the inspection status of quality-critical items, including raw materials, packaging materials, intermediates, and finished excipients. Although storing materials in identified locations is preferred, any means that clearly identifies the test status is satisfactory. Continuously fed materials may need special consideration in order to satisfy these requirements.

### LABELING

Labeling for excipient packages is subject to national and international regulatory requirements, which may include transportation and safety measures. At a minimum, labels should include the following:

- the name of the excipient and grade, if applicable,
- the excipient manufacturer's and/or distributor's name,
- the batch number from which the complete batch history can be determined,
- special storage conditions, if applicable.

### CUSTOMER PROPERTY

The excipient manufacturer should establish and maintain procedures for verification, storage, and maintenance of customer-supplied materials intended for incorporation into the customer's excipient. Verification by the manufacturer does not relieve the customer of the responsibility of providing an acceptable material. Material that is lost or that is damaged or otherwise unsuitable for use should be recorded and reported to the customer. In this case, procedures should be in place for acceptable disposition and replacement of the material. The manufacturer should also make provisions for protecting other real and intellectual property that is provided by the customer (for example, test equipment, test methods, and specifications).

## Preservation of Product

### HANDLING, STORAGE, AND PRESERVATION

Excipients, intermediates, and raw materials should be handled and stored under appropriate conditions of temperature, humidity, and light so that their identity, quality, and purity are not affected. Outdoor storage of raw materials (for example, acids, other corrosive substances, explosive materials) or excipients is acceptable, provided that the containers give suitable protection against deterioration or contamination of their contents, identifying labels remain legible, and containers are adequately cleaned prior to opening and use. Records of storage conditions should be maintained if they are critical for the continuing conformity of the material to specifications.

### PACKAGING SYSTEMS

An excipient packaging system should include the following features:

- documented specifications and examination or testing methods,
- cleaning procedures, where containers are reused,
- tamper-evident seals,
- containers that provide adequate protection against deterioration or contamination of the excipient during transportation and recommended storage,
- containers that do not interact with or contaminate the excipient,
- storage and handling procedures that protect containers and closures and minimize the risk of contamination, damage, or deterioration and that will avoid mix-ups (for example, between containers that have different specifications but are similar in appearance).

If returnable excipient containers are reused, previous labeling should be removed or defaced. If the containers are reused solely for the same excipient, previous batch numbers or the entire label should be removed or completely obliterated.

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### DELIVERY AND DISTRIBUTION

Identification and traceability of quality-critical aspects are required of excipient manufacturers. Distribution records of excipient shipments should be kept. These records should identify, by excipient batch, where and to whom the excipient was shipped, the amount shipped, and the date of shipment so as to facilitate retrieval if necessary. Where excipients are handled by a series of different distributors, it should be possible to trace them back to the original manufacturer, and not only to the previous supplier. The manufacturer should maintain the integrity and the quality of the product after final inspection and test. Where contractually specified, this protection should be extended to include delivery to the final destination. Excipients should be supplied only within their expiry and/or retest period.

### CONTROL OF MEASURING AND MONITORING DEVICES

Measuring and test equipment, including computerized systems, identified as being quality-critical should be calibrated and maintained. This includes in-process instruments as well as test equipment used in the laboratory. The control program should include the standardization or calibration of instruments and equipment at suitable intervals in accordance with an established, documented program. This program should contain specific directions, schedules, limits for accuracy and precision, and provisions for remedial action in the event that accuracy and/or precision limits are not met. Calibration standards should be traceable to recognized national or compendial standards as appropriate.

Instruments and equipment not meeting established specifications should not be used, and an investigation should be conducted to determine the validity of the previous results since the last successful calibration. The current calibration status of quality-critical equipment should be known and verifiable to users.

## MEASUREMENT, ANALYSIS, AND IMPROVEMENT

The organization should plan and implement the monitoring, measurement, and improvement activities that are required in order to demonstrate conformity of the excipient to customer requirements and to ensure conformity of the quality management system to this chapter. The organization should evaluate opportunities for improvements through the measurement and analysis of product and process trends.

## Monitoring and Measurement

### CUSTOMER SATISFACTION

The excipient manufacturer should establish measurement activities to assess customer satisfaction. Such measurements can include customer complaints, return of excipients, and customer feedback. This information should drive activities that strive to continuously improve customer satisfaction.

### INTERNAL AUDIT

The excipient manufacturer should carry out a comprehensive system of planned and documented internal quality audits. These should determine whether quality activities comply with planned arrangements and should also determine the effectiveness of the quality management system. Audits should be scheduled on the basis of the status and importance of the activity. Audits and follow-up actions should be carried out in accordance with documented procedures. Audit results should be documented and discussed with management personnel having responsibility in the area audited. Management personnel responsible for the area audited should take corrective action on the nonconformities found. *Appendix: Auditing Considerations* will be of assistance in establishing an internal audit program.

### MONITORING AND MEASUREMENT OF PROCESSES

The excipient manufacturer should identify the tests and measurements necessary for adequately controlling manufacturing and quality management system processes. When critical to excipient quality, techniques used to verify that the processes are under control should be established. When deviations from planned results occur, corrective action should be taken to ensure that the excipient meets requirements. Periodic reviews of key indicators such as process quality attributes and process failures should be conducted to assess the need for improvements.

### MONITORING AND MEASUREMENT OF PRODUCT

The excipient manufacturer should establish the test methods and procedures to ensure that the product consistently meets specifications. Analytical methods should be suited to their purposes. The analytical methods may be those included in the current edition of the appropriate pharmacopeia or another accepted standard. However, the methods may also be noncompendial. If the excipient manufacturer claims that its product is in compliance with a pharmacopeia or an official compendium, then

- noncompendial analytical tests should be demonstrated to be equivalent to those in the compendia;
- the product should comply with applicable *USP* general chapters and notices.

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## LABORATORY CONTROLS

Laboratory controls should include complete data derived from tests necessary for ensuring conformity with specifications and standards, including the following:

- a description of the sample received for testing, together with the material name, a batch number or other distinctive code, and the date the sample was taken,
- a statement referencing each test method used,
- a record of raw data secured during each test, including graphs, chromatograms, charts, and spectra from laboratory instrumentation, identified to show the specific material and batch tested,
- a record of calculations performed in connection with the test,
- test results and how they compare with established specifications,
- a record of the person who performed each test and the date(s) the tests were performed.

There should be a documented procedure for the preparation of laboratory reagents and solutions. Purchased reagents and solutions should be labeled with the proper name, concentration, and expiry date. Records should be maintained for the preparation of solutions and should include the name of the solution, the date of preparation, and the quantities of material used. Volumetric solutions should be standardized according to an internal method or by using a recognized standard. Records of the standardization should be maintained.

Where used, primary reference reagents and standards should be appropriately stored and need not be tested upon receipt, provided that a certificate of analysis from the supplier is available. Secondary reference standards should be appropriately prepared, identified, tested, approved, and stored. There should be a documented procedure for the qualification of secondary reference standards against primary reference standards. The reevaluation period should be defined for secondary reference standards, and each batch should be periodically requalified in accordance with a documented protocol or procedure.

## FINISHED EXCIPIENT TESTING AND RELEASE

Finished excipient testing should be performed on each batch to ensure that the excipient conforms to documented specifications. There should be a procedure to ensure that appropriate manufacturing documentation, in addition to the test results, is evaluated prior to release of the finished excipient. The quality unit should be responsible for the release of the finished excipient. For excipients produced by continuous processes, assurance that the excipient conforms to documented specifications may be achieved through the results of in-process testing or other process control records.

## OUT-OF-SPECIFICATION TEST RESULTS

Out-of-specification (OOS) test results should be investigated and documented according to a documented procedure. Retest sample results may be used to replace the original test result only if it is demonstrated on the basis of a documented investigation that the original result is erroneous. When statistical analysis is used, both the original and retest data must be included. The OOS procedure should define which statistical techniques are to be used and under what circumstances. These same principles apply when the sample is suspected of not being representative of the material from which it was taken.

## RETAINED SAMPLES

When practical, a representative sample of each batch of the excipient should be retained. The retention period should be appropriate to the expiry or reevaluation date. The retained samples should be stored and maintained in such a manner that they are readily retrievable in facilities that provide a suitable environment. The sample size should be at least twice the amount required to perform complete specification testing.

## CERTIFICATES OF ANALYSIS

The organization should provide certificates of analysis to the required specification for each batch of excipient.

## IMPURITIES

When possible, excipient manufacturers should identify and set appropriate limits for impurities. The limits should be based on appropriate safety data, limits as described in official compendia or other requirements, and sound GMP considerations. Manufacturing processes should be adequately controlled so that the impurities do not exceed such established limits. Many excipients are extracted from or purified using organic solvents. These solvents are normally removed by drying. It is important that excipient specifications include tests and limits for solvent residues.

## STABILITY

Although many excipient products are stable and may not require extensive testing to ensure stability, the stability of excipients is an important factor in the overall quality of the drug product. For excipients that have been on the market for a long time, historical data may be used to indicate stability. Where historical data do not exist, a documented testing and/or evaluation program designed to assess the stability characteristics of the excipient should be undertaken. The results of such stability testing and/or evaluation should be used in determining appropriate storage conditions and retest or expiry dates. The testing program should include the following:

- the number of batches, sample sizes and test intervals,
- storage conditions for samples retained for testing,

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- suitable stability-indicating test methods,
- storage of the excipient in containers that simulate the market container, where possible.

The stability of excipients may be affected by undetected changes in raw materials or subtle changes in manufacturing procedures or storage conditions. Excipients may also be shipped in a variety of packaging types that can affect their stability (for example, plastic or glass bottles, metal or plastic drums, bags, tank cars, or other bulk containers).

Some excipients may be available in different grades (for example, various molecular weights of a polymer or different monomer ratios, different particle sizes, bulk densities) or may be mixtures of other excipients. These excipients may be very similar to others within a product group. Minor quantitative differences of some of the components may be the only significant variation from one product to another. For these types of excipients, a model product approach may be appropriate for assessment of the stability of similar excipients. Stability studies of this type should involve selection of several model products that would be expected to simulate the stability of the product group being assessed. This selection should be scientifically sound and documented. Data from stability studies of these model products can be used to determine theoretical stability for similar products.

## EXPIRY/RETEST PERIODS

An expiry or retest period should be assigned to each excipient and communicated to the customer. Common practice is to use a retest period rather than an expiry period.

## CONTROL OF NONCONFORMING PRODUCT

Raw material, intermediate, or finished excipient found not to meet its specifications should be clearly identified and controlled to prevent inadvertent use or release for sale. A record of nonconforming product should be maintained. Incidences of nonconformity should be investigated to identify the cause. The investigation should be documented and action taken to prevent recurrence. There should be a documented procedure defining how the retrieval of an excipient from distribution should be conducted and recorded. Procedures should exist for the evaluation and subsequent disposition of nonconforming products. Nonconforming product should be reviewed in accordance with documented procedures to determine if it can be

- reprocessed or reworked to meet the specified requirements,
- accepted by the customer with customer agreement,
- regraded for other applications,
- destroyed.

## REPROCESSING

Repetition of an activity that is a normal part of the manufacturing process (reprocessing) should occur only when it has already been documented that the excipient may be made in that manner. In all other cases, the guidance for reworking should be followed.

## REWORKING

An activity that is not a normal part of the manufacturing process (reworking) should be conducted only following a documented review of risk to excipient quality and approval by the quality unit. As appropriate, when performing the risk assessment, consideration should be given to the following:

- new impurities that may be introduced as a result of reworking,
- additional testing to control the reworking,
- records and traceability to the original batches,
- suitable acceptance criteria for the reworked excipient,
- impact on stability or the validity of the reevaluation interval,
- performance of the excipient.

When the need to rework an excipient is identified, an investigation and evaluation of the cause are required. The equivalence of the quality of reworked material to original material should also be evaluated and documented to ensure that the batch will conform to established specifications and characteristics. Batches of excipients that do not conform to specifications individually must not be blended with other batches that do conform in an attempt to hide adulterated or substandard material.

## RETURNED EXCIPIENTS

Returned excipients should be identified and quarantined until the quality unit has completed an evaluation of their quality. There should be procedures for holding, testing, reprocessing, and reworking of the returned excipient. Records for returned products should be maintained and should include the name and the batch number of the excipient, the reason for the return, the quantity returned, and the ultimate disposition of the returned excipient.

## ANALYSIS OF DATA

The excipient manufacturer should develop methods for evaluating the effectiveness of its quality management system and use those data to identify opportunities for improvement. Such data can be derived from customer complaints, product reviews, process capability studies, internal audits, and customer audits. The analysis of such data may be used as part of the management review (also see above, *Management Review* under the *Management Responsibility* section). A periodic review of key indicators

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such as product quality attributes, customer complaints, and product nonconformities may be conducted to assess the need for improvements.

## Improvement

### CONTINUAL IMPROVEMENT

The excipient manufacturer should take proactive measures to continuously improve manufacturing and quality management system processes. To identify opportunities for continual improvement, analysis of the following performance indicators may be considered:

- causes of nonconforming product,
- results of internal and external audits,
- customer returns and complaints,
- process and operational failures.

**Corrective Action:** The excipient manufacturer should establish, document, and maintain procedures for the following:

- determining the root causes of nonconformities,
- ensuring that corrective actions are implemented and effective,
- implementing and recording changes in procedures resulting from corrective action.

**Preventive Action:** The excipient manufacturer should establish, document, and maintain procedures for the following:

- initiating preventive actions to deal with problems at a level corresponding to the risks,
- implementing and recording changes in procedures resulting from preventive action.

## GLOSSARY

The terms below are defined as used in this chapter. Wherever possible, definitions used by the International Conference on Harmonization have been used as the basis for the glossary.

**Acceptance Criteria:** numerical limits, ranges, or other suitable measures of acceptance for test results.

**Active Pharmaceutical Ingredient (API):** any substance or mixture of substances that is intended to be used in the manufacture of a drug product and that, when used in the production of a drug, becomes an active ingredient of the drug product. Such substances are intended to furnish pharmacological activity or other direct effect in the diagnosis, cure, mitigation, treatment, or prevention of disease, or to affect the structure or any function of the body of humans or animals.

**Adulterated Material:** a material that either has been contaminated with a foreign material or has not been manufactured using GMP. This does not pertain to a material that simply does not meet physical or chemical specifications.

**Batch (Lot):** a specific quantity of material produced in a process or series of processes so that it can be expected to be homogeneous. In the case of continuous processes, a batch may correspond to a defined fraction of the production. The batch size can be defined either by a fixed quantity or by the amount produced in a fixed time interval.

**Batch Number (Lot Number):** a unique combination of numbers, letters, and/or symbols that identifies a batch and from which the production and distribution history can be determined.

**Batch Process:** a process that produces the excipient from a discrete supply of raw materials that are present before the completion of the reaction.

**Batch Record:** documentation that provides a history of the manufacture of a batch of excipient.

**Blending (Mixing):** intermingling different conforming grades into a homogeneous lot.

**Calibration:** the demonstration that a particular instrument or measuring device produces results within specified limits by comparison with those produced by a reference or traceable standard, over an appropriate range of measurements.

**CEP (Certificate of Suitability to the *European Pharmacopoeia*):** certification granted to individual manufacturers by the European Directorate for the Quality of Medicines (EDQM) when a specific excipient or active ingredient is judged to be in conformity with a *European Pharmacopoeia* monograph.

**Certificate of Analysis:** a document listing the test methods, specification, and results of testing a representative sample from the batch to be delivered.

**Commissioning:** the introduction of equipment for use in a controlled manner.

**Contamination:** the undesired introduction of impurities of a chemical or microbiological nature or foreign matter into or onto a raw material, intermediate, or excipient during production, sampling, packaging or repackaging, storage, or transport.

**Continuous Process:** a process that continuously produces material from a continuing supply of raw material.

**Critical:** a process step, process condition, test requirement, or other relevant parameter or item that must be controlled within predetermined criteria to ensure that the excipient meets its specification.

**Cross-Contamination:** contamination of a material or product with another material or product.

**Customer:** the organization receiving the excipient once it has left the control of the excipient manufacturer; includes brokers, agents, and users.

**Deviation:** departure from an approved instruction or established standard.

**Drug Master File (DMF):** detailed information about the manufacture of an excipient that is submitted to the U.S. Food and Drug Administration (FDA).

**Drug (Medicinal) Product:** the dosage form in the final immediate packaging intended for marketing.

**Excipient:** substances other than the API that have been appropriately evaluated for safety and are intentionally included in a drug delivery system.

**Expiry (Expiration) Date:** the date designating the time during which the excipient is expected to remain within specifications and after which it should not be used.

**Impurity:** a component of an excipient that is not intended to be present but arises as a consequence of the manufacturing process.

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**In-Process Control/Testing:** checks performed during production to monitor and, if appropriate, to adjust the process and/or to ensure that the intermediate or excipient conforms to its specification.

**Intermediate:** material that must undergo further manufacturing steps before it becomes an excipient.

**Lot:** See *Batch.*

**Manufacturer/Manufacturing Process:** all operations of receipt of materials, production, packaging, repackaging, labeling, relabeling, quality control, release, and storage of excipients and related controls.

**Master Production Instruction (Master Production and Control Record):** documentation that describes the manufacture of the excipient from raw material to completion.

**Material:** a general term used to denote raw materials (starting materials, reagents, and solvents), process aids, intermediates, excipients, packaging, and labeling materials.

**Model Product:** a product that represents a group of similar products with respect to composition, functionality, or specification.

**Mother Liquor:** the residual liquid that remains after crystallization or isolation processes.

**Packaging Material:** a material intended to protect an intermediate or excipient during storage and transport.

**Production:** operations involved in the preparation of an excipient from receipt of materials through processing and packaging of the excipient.

**Quality Assurance:** the sum total of the organized arrangements made with the object of ensuring that all excipients are of the quality required for their intended use and that quality systems are maintained.

**Quality Control:** checking or testing that specifications are met.

**Quality-Critical:** describes a material, process step or process condition, test requirement, or any other relevant parameter that directly influences the quality attributes of the excipient and that must be controlled within predetermined criteria.

**Quarantine:** the status of materials isolated physically or by other effective means pending a decision on their subsequent approval or rejection.

**Raw Material:** a general term used to denote starting materials, reagents, and solvents intended for use in the production of intermediates or excipients.

**Record:** a document stating results achieved and/or providing evidence of activities performed. The medium may be paper, magnetic, electronic or optical, photographic, or another medium, or a combination thereof.

**Reevaluation Date (Retest Date):** the date when the material should be reexamined to ensure that it is still in conformity with the specification.

**Reprocessing:** repetition of an activity that is a normal part of the manufacturing process and that has been documented previously.

**Retrieval:** process for the removal of an excipient from the distribution chain.

**Reworking:** subjecting previously processed material that did not conform to standards or specifications to processing steps that differ from the normal process.

**Specifications:** list of tests, references to analytical procedures, and appropriate acceptance criteria that are numerical limits, ranges, or other criteria for the tests described for a material.

**Stability:** continued conformity of the excipient to its specifications.

**Top Management:** person or group of people who direct and control an organization at the highest level. The highest level can be at either the site level or the corporate level and will depend on the way in which the quality management system is organized.

**Traceability:** ability to determine the history, application, or location that is under consideration: for example, origin of materials and parts, processing history, or distribution of the product after delivery.

**Validation:** a documented program that provides a high degree of assurance that a specific process, method, or system will consistently produce a result meeting predetermined acceptance criteria.

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# APPENDIX

## Auditing Considerations

### INTRODUCTION

Many excipients are used in food, cosmetic, and industrial products as well as in pharmaceuticals. Thus, environmental conditions, equipment, and operational techniques employed in excipient manufacture are often those of the chemical industry as opposed to the pharmaceutical industry. Chemical processes can produce impurities from side reactions. Careful process control is therefore essential to minimize levels of impurities and contamination.

Excipients are often manufactured on a large scale, using continuous processing and automated process controls. Production equipment and processes vary depending on the type of excipient being produced, the scale of production, and the type of operation (for example, batch versus continuous process).

This appendix is intended as an aid in preparing for an audit of an excipient manufacturer. Both external and internal auditors (see also *Internal Audit* in *Monitoring and Measurement* under the *Measurement, Analysis, and Improvement* section) will find this appendix useful in identifying the significant issues with respect to GMP and quality that require examination. This section will assist excipient manufacturers in identifying key deliverables when adopting the GMP standards listed in the other sections of this chapter; in planning an audit, it will also help to verify the quality of the excipient manufacturing process and the manufacturer's quality management system.

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## GMP PRINCIPLES

**Control of Impurities and Contamination:** In general, the pharmaceutical customer does not perform further chemistry or purification steps on the excipient; it is used as purchased. Consequently, impurities present in the excipient are likely to be present in the drug product. Although dosage form manufacturers have some control over excipient quality through specifications, excipient manufacturers have greater control over the physical characteristics, quality, and presence of impurities in the excipients they produce.

External contamination of the excipient can arise from the manufacturing environment. However, chemical processes used to manufacture excipients are often performed in closed systems that afford protection against such contamination, even when the reaction vessels are not located in buildings. The external environment may require suitable controls to avoid potential contamination wherever the excipient or in-process material is exposed.

**Excipient Properties and Functionality:** Excipients are frequently used in those types of drug products for which physical characteristics, such as particle size, may be important. Although the manufacturer of the finished dosage form is primarily responsible for identifying the particular physical characteristics needed, it is also the responsibility of the excipient manufacturer to control excipient manufacturing processes to ensure consistent conformity to excipient specifications. Wherever possible, consideration should be given to the end use of the excipient. This is particularly important if the excipient is a direct component of a sterile drug product or one that is claimed to be pyrogen-free.

**Consistency of Manufacture and Change Control:** A thorough understanding of the manufacturing process and effective control of change can best ensure consistency of excipient quality from batch to batch. Implementation of changes may also have consequences for registration filings with regulatory agencies.

Changes in excipient manufacturing processes may result in changed physical or chemical properties of the excipient that are evident only during subsequent processing or in the finished dosage form. This is particularly important in the context of the pharmaceutical product approval process where bioequivalence comparisons are made between pivotal, clinical trial batch (*bio batch*) production and commercial scale-up batches. Changes made to the excipient supplied for the commercial product from the excipient supplied for the bio batch should not affect the quality and performance of the commercial drug product. Scale-up of excipients to commercial production may involve several stages, and data may be required to demonstrate consistency between batches through the scale-up process.

**Traceability:** Traceability of batch-related records to facilitate investigations and retrieval of product is also a key requirement of GMP.

## APPLICATION OF GMP PRINCIPLES

It is the responsibility of the excipient manufacturer to designate and document the rationale for the point in the manufacturing process at which appropriate GMP are to be applied. From this point on, appropriate GMP should be applied. The manufacturer should apply a level of GMP to each manufacturing stage commensurate with the importance of that step in ensuring product integrity. This may be demonstrated by means of the use of a risk assessment procedure (for example, HACCP, FMEA).

The stringency of GMP in excipient production should increase as the process proceeds from early manufacturing to final stages, purification, and packaging. Physical processing (for example, granulation, coating, or physical manipulation of particle size such as milling or micronizing) as well as chemical processing of excipients should be conducted at least to the standards suggested by this chapter.

It should be recognized that not all intermediates may require testing. An excipient manufacturer should, however, be able to identify critical or key points in the manufacturing process where selective intermediate sampling and testing are necessary in order to monitor process performance.

## GENERAL AUDITING CONSIDERATIONS

Audits of an excipient operation will be influenced by the purpose of the audit and the intended use of the excipient. The key stages of a production process should be examined to determine whether the manufacturer controls these steps so that the process performs consistently. Overall, an audit should assess the excipient manufacturer's capability to deliver a product that consistently meets established specifications.

The audit team may consist of engineers, laboratory analysts, purchasing agents, computer experts, maintenance staff, and other personnel as appropriate to the scope and purpose of the audit. External auditors must respect the confidentiality of the manufacturer's processes and other disclosures.

An audit should focus on the quality-critical processing steps that are necessary for producing an excipient that meets established physical and chemical criteria. These steps should be identified and controlled by the excipient manufacturer. Quality-critical processing steps can involve a number of unit operations or unit processes. Quality-critical steps can include, but are not limited to, the following:

- phase changes involving the desired molecule, solvent, inert carrier or vehicle (for example, dissolution, crystallization, evaporation, drying, sublimation, distillation, or absorption),
- phase separation (for example, filtration or centrifugation),
- chemical changes involving the desired molecule (for example, removal or addition of water of hydration, acetylation or formation of a salt),
- adjustments of the solution containing the molecule (for example, pH adjustment),
- precise measurement of added excipient components, in-process solutions, and recycled materials (for example, weighing or volumetric measurements),
- mixing of multiple components,
- changes that occur in surface area, particle size, or batch uniformity (for example, milling, agglomeration, or blending).

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## AUDIT CHECK POINTS

A good approach for an excipient plant audit is a review of the following areas:

- nonconformities—such as the rejection of a batch that did not meet specifications, customer complaints, return of a product by a customer, or retrieval of a product. The manufacturer should have determined the cause of the nonconformity, prepared a report of the investigation, and initiated and documented subsequent corrective action. Records and documents should be reviewed to ensure that nonconformities are not the result of a poorly developed or inconsistent process;
- customer complaint files—such as reports that some aspect of the product is not entirely suitable for use, because such problems may be caused by impurities or inconsistencies in the excipient manufacturing process;
- change control logs—to ascertain whether the company evaluates its significant changes to decide whether the customer and/or regulatory authority should be notified;
- nonconforming products meeting or Material Review Board documents and/or equivalent records that demonstrate that the disposition of nonconforming product is handled in an appropriate manner by responsible individuals;
- master formula and production records for frequent revisions that may reveal problems in the excipient production process;
- evidence for the presence of unreacted intermediates and solvent residues in the finished excipient;
- materials management systems, to ensure adequate control over nonconforming materials so that they cannot be sold to customers or used in manufacturing without authorization;
- review of a process flow diagram, to aid understanding of the various processing stages. The critical stages and sampling points should be identified as part of the review of the processing records;
- review of contamination control measures.

In evaluating the adequacy of measures taken to prevent contamination and cross-contamination of materials in the process, it is appropriate to consider the following risk factors:

- the type of system (for example, open or closed). Enclosed systems in chemical plants often are not closed when they are being charged and/or when the final product is being emptied. In addition, the same reaction vessels are sometimes used for different reactions;
- the form of the material (for example, wet or dry);
- the stage of processing and use of the equipment and/or area (for example, multipurpose or dedicated);
- continuous versus batch production.

## DOCUMENTATION AND RECORD REVIEW

Documentation required for the early steps in the process need not be as comprehensive as in the latter stages of the process. It is important that a chain of documentation exist and that it be complete when the following is the case:

- the excipient can be identified and quantified for processes where the molecule is produced during the course of the process. For batch production, a theoretical mass balance may also be established with appropriate limits, because deviations from tolerance are a good indicator of a loss of control;
- an impurity or other substance likely to adversely affect the impurity profile or form of the molecule is identified, and subsequent attempts are made to remove it.

As chemical processing proceeds, a chain of documentation should be established that includes the following:

- a documented process,
- the identification of critical processing steps,
- appropriate production records,
- records of initial and subsequent batch numbers,
- records of raw materials used,
- comparison of test results against meaningful standards.

If significant deviations from the normal manufacturing process are recorded, there should be evidence of suitable investigations and a review of the quality of the excipient. Complete documentation should be continued throughout the remainder of the process for quality-critical processing steps until the excipient is packaged and delivered to the end user. The batch should be homogeneous within the manufacturer's specifications. This does not necessitate the final blending of continuous-process material if process controls can demonstrate compliance with specifications throughout the batch.

In order to promote uniformity in excipient GMP inspections, the following basic requirements should be established:

- assignment of a unique batch number to the excipient, enabling it to be traced through manufacture to release and certification,
- suitable controls for the preparation of a batch record for batch processing and/or a production record, log sheet, or other appropriate documentation for continuous processing,
- demonstration that the batch has been prepared using GMP guidelines from the processing point at which excipient GMP have been determined to apply,
- confirmation that the batch is not combined with material from other batches for the purpose of either hiding or diluting an adulterated batch,
- records showing that the batch has been sampled in accordance with a sampling plan that ensures a representative sample of the batch,
- records showing that the batch has been analyzed using scientifically established test methods designed to ensure that the product meets established standards, specifications, and characteristics,

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- stability data adequate to support the intended period of use of the excipient; these data can be obtained from historical data, from actual studies on the specific excipient, or from applicable model product studies that can reasonably be expected to simulate the performance of the specific excipient.

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# ⟨1079.1⟩ STORAGE AND TRANSPORTATION OF INVESTIGATIONAL DRUG PRODUCTS

## INTRODUCTION

Clinical trials are drug studies that are performed to determine if an investigational medicine meets the effectiveness and safety criteria as has been outlined in the protocol for the clinical trial. Investigational drug products (IDPs) are products not commercially available for the indication or dosage being tested; there may be situations when a commercially available product may be used in a clinical trial as a positive control (comparator), a new indication, or a rescue medication. The term IDP is used throughout this document. Where IDP is used, others may be more familiar with the European Union term investigational medicinal product (IMP). The definition of an IMP is provided in European Commission Directive 2001/20/EC, Article 2 (d), as "a pharmaceutical form of an active substance or placebo being tested or used as a reference in a clinical trial, including products already with a marketing authorization but used or assembled (formulated or packaged) in a way different from the authorized form, or when used for an unauthorized indication, or when used to gain further information about the authorized form". For the purposes of this chapter, we will consider the two terms as equivalent. The pre-commercial nature of IDPs means the manufacturing ingredients, including active pharmaceutical ingredients (APIs), excipients, the clinical trial dosage, and any associated stability and packaging components may not be as defined as the final approved finished product. The Food and Drug Administration (FDA) current Good Manufacturing Practice regulations for finished drug products apply equally to commercial products and IDPs.

IDP distribution differs from commercial distribution in that the quantities for IDPs are often small (e.g., as little as one or two bottles or unit dose packages) and there are numerous final destinations, such as clinics and remote clinical settings. Another difference from a commercial drug product is the known stability of the IDP that is often a new chemical/molecular entity in the early stages of clinical trials and has not been through the robust stability program of commercial products. The IDP also creates a unique challenge when compared to a commercial product, which is ensuring the proper distribution of every packaging system, because a temperature excursion may jeopardize the entire clinical trial outcome.

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## SCOPE

Due to global security issues, regulations may vary and/or change for the distribution of drug products and for IDPs as well. Sponsors, clinical trial site personnel, or their designees are responsible for control of IDPs and should maintain an understanding of the current country distribution requirements. This chapter will address the aspects of storage and distribution that are unique to IDPs (e.g., unblinding, comparators, and academic studies); other chapters of interest may be *Monitoring Devices—Time, Temperature, and Humidity* ▲⟨1079.3⟩▲ (CN 1-Dec-2023), *Risks and Mitigation Strategies for the Storage and Transportation of Finished Drug Products* ⟨1079⟩, and *Good Distribution Practices for Bulk Pharmaceutical Excipients* ⟨1197⟩. This guidance chapter applies to all IDPs, including drug device combinations as well as non-commercial clinical IDPs, but does not apply to medical devices. The non-commercial clinical trial may have limited financial commercial return and is often performed in an academic setting or by a compounding pharmacy with limited industry participation (see *Figure 1*).

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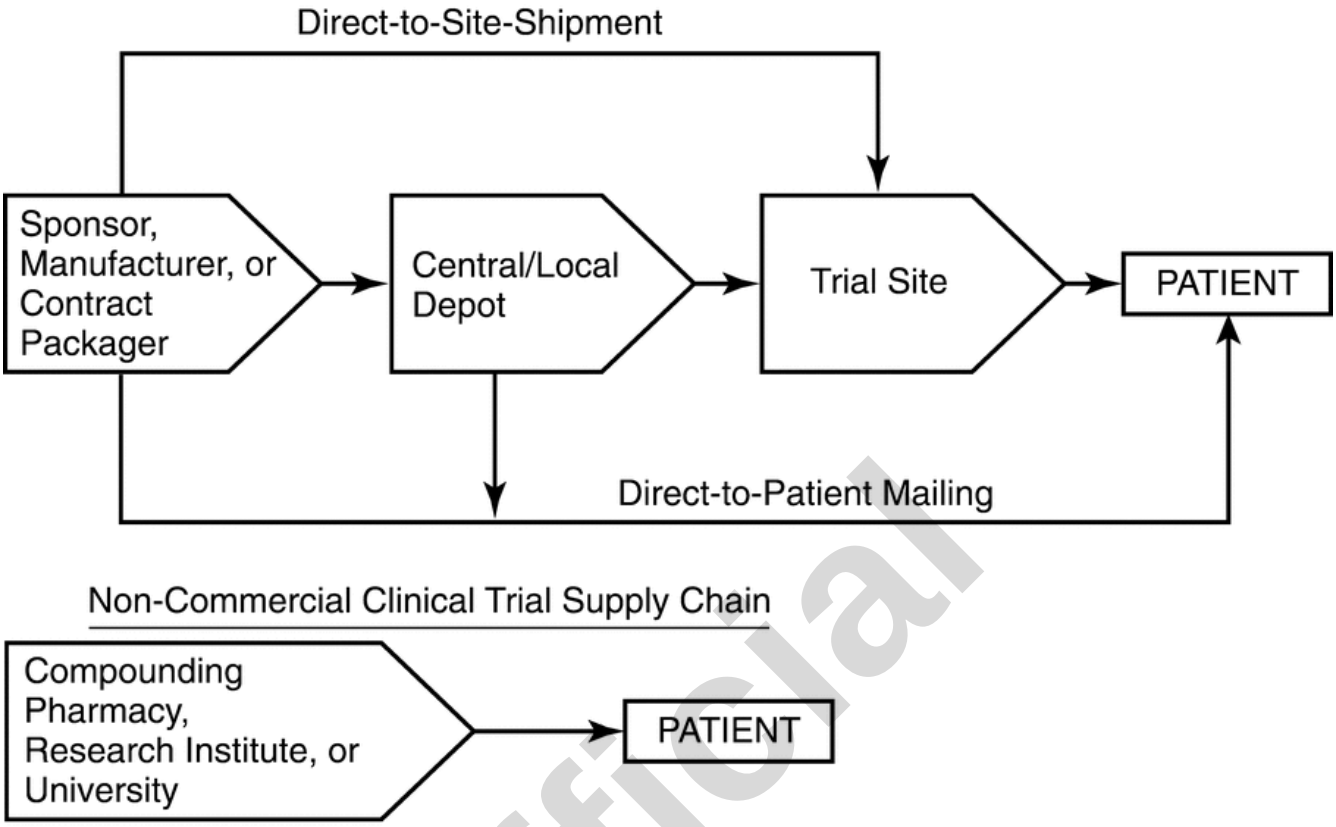


Figure 1. IDP supply chain scheme for commercial and non-commercial clinical trial studies.

## KEY FACTORS FOR IDP DISTRIBUTION

### Number of Clinical Trial Sites

The larger the number of clinical trial sites, the greater the risk involved, as this may mean more countries, shipments, and customs challenges. The IDP sponsor company and distributor should be aware of customs requirements in all countries where the IDP is to be distributed. IDPs may be transported from one clinical trial site to another, and the distribution of these IDPs must undergo the same distribution requirements as any other drug products being transported.

### Audits

#### CLINICAL TRIAL SITES

Clinical trial sites must store IDPs in an area that is safe and secure with limited access, and ensure products are maintained at their labeled storage conditions. A site qualification audit will ensure that the sites have adequate space and segregation for both IDPs that have not been dispensed to patients, as well as IDPs that are awaiting return to a sponsor or contractor for final reconciliation and disposition. Depending on the regulatory requirements of the countries where the studies are being conducted, calibrated thermometers or an automated temperature tracking and alarm system should be in place to document site compliance.

#### DEPOTS AND SUBCONTRACTORS

These places and individuals should follow both the sponsor requirements and associated country regulations in which the study is being conducted.

### Available Product Quantities

In early stage development (i.e., Phase 1 and early Phase 2), there are typically fewer APIs and IDPs available. Disruptions in the supply chain will have greater impact and consequences to the timely completion of clinical trials, and distribution delays (especially for biologics) may impact enrollment and study completion.

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## Expiration/Retest Dates

As the stability profile is being developed during the early phases of development, expiry or re-test date extensions should be anticipated. IDP expiry or re-test dates should follow local regulatory requirements throughout the life of the study. Expiry or re-test dates can be tracked through a validated electronic system, though IDP expiry or re-test dates at global sites or depots may need to be manually updated.

## Qualification of Packaging for IDP and Track and Trace

There is no difference in the packaging qualifications for IDPs and commercially available drug products.

Determining what the required environmental conditions of IDP shipments will be during the early stages of a clinical research protocol is of particular importance, as this is the best time to define the robust stability profile of the study drug. Important distribution risk factors to consider include the distance, time, temperature, and the number and type of handoffs that may occur. Packaging that is appropriate to the clinical phase should be used to protect the contents. When that is not possible, the intent is to use qualified components (i.e., components that have been tested to show positive outcomes for maintaining temperatures for a wide variety of products in a wide variety of environmental conditions) to ensure the IDPs inside the shipping containers are controlled and remain at the correct temperature during the journey from warehouse (or depot) to clinical trial site. There are, however, sophisticated track and trace technologies that permit the trial sponsor, packaging contractor, clinical research organization, and the clinical trial site to monitor (track) the IDP along the supply chain.

Performance qualification (PQ) of packaging systems for shipping IDPs should be carried out reflecting the actual load configuration, duration of travel, and expected environmental conditions. There should be no difference in the PQ rational for investigational and commercial drug products because the risks of storage and transport are not related to the marketing authorization. Details of PQ as a strategy for mitigating risks are provided in ⟨1079⟩.

## IDP Supply Chain Challenges

One of the larger IDP supply chain challenges is with the number of clinical trial supply shipments that are made in support of clinical trials. In IDP distribution circles, these shipments are referred to as “outbound” shipments. The faster the trial enrolls, the larger the number of shipments. With each shipment we now increase the risk of damage as well as temperature excursions. Each of these would mean shipping additional IDPs to support the trial and added cost to the sponsor company.

Among the challenges for the IDP supply chain are the frequency and size of shipments. These are influenced by such factors as number of patients, frequency of IDP administration to patients, the number and size of patient kits in each shipment, and the expense associated with each shipment.

## Timing

Clinical trial studies that are longer in duration than the original plan may mean more IDP shipments and the need to ensure these extensions continue being in compliance with current good distribution practices. There are instances when IDP inventory remains in the investigational site when enrollment is complete, even though enrolled patients continue to take their remaining treatment. In other instances, IDP may remain in the institution while awaiting return/destruction instructions from the sponsor. The guidelines in this chapter apply to both instances.

## Key Global Challenges for IDPs

Global challenges include security, language, culture, tariff codes/taxes, hiring, training, customs, holidays, country-specific import and export requirements, time differences, airline schedules, and documentation. Guidance for such issues can be found in regulatory guidance documents [e.g., FDA Drug Supply Chain Security Act (DSCSA) or International Council for Harmonisation (ICH) Q Series].

# IDP ENVIRONMENTAL CONDITIONS

The environmental controls for the distribution of IDPs may be provided for on container labeling, product certificate of analysis, safety data sheet, or other records available for shipping purposes; however, these environmental conditions are not always known at the time of transportation to the trial sites, such as the clinical setting or the patient’s home (for home administration). There must be confirmation that the proper steps are taken to ensure IDP supply chain integrity throughout the distribution process. At the point that the IDP is dispensed to the research participant (subject or patient), and is in their custody, the proper handling and storage of the IDP should be explained (in written form or verbally) at the clinical site, in the hospital, or in the participant’s home. IDPs that have been dispensed to a patient or subject may not be returned to research stock at the investigator site to be dispensed again. In the case where the IDP is provided to a member of the research team to be dispensed to the participant, but it is not dispensed to the subject or patient, and the chain of custody can be verified to have remained within the study team, the IDP may be received back to the investigational drug service to be dispensed again only if there are standard operating procedures (SOPs) and documentation in place to ensure control of the IDP. Please note that this redispensing guidance may be overruled if the specific protocol or sponsor SOPs or instructions forbids redispensing in any case.

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## Unblinding

Blinding of IDPs is performed to reduce or eliminate bias in clinical trials and assists in providing randomization across patient populations. In certain circumstances, in order to maintain the safety of research participants, emergency unblinding is necessary. This may be done for an individual dose or a batch/lot. In either case, the unblinded IDP would be separated from the supply of the IDP waiting to be dispensed. Depending upon the reason for the unblinding, the sponsor shall provide instruction regarding return of the IDP or destruction. The impact of unblinding a product can create a need for distribution of a new IDP, either from the manufacturer, depot, or from another clinical trial site. The supply chain partners should have a written procedure establishing responsibilities, procedures, precautions, records, and final destination regarding the unblinding of IDPs to ensure proper handling of this material and avoid the redistribution of IDPs with a broken randomized code.

## Comparators

Most IDPs in late Phase 2 and all of Phase 3 clinical trials will be compared to the current standard of care (i.e., commercially available product). This process is generally done using a comparator product that may or may not be currently in the market place in countries where the clinical trial is being conducted. The distribution process for the comparator should follow the shipping requirements on the IDP labeling for that product or from the manufacturer of the product. The IDP provider using comparators should understand and follow the distribution requirements of the comparator to ensure a safe and efficacious product for the clinical trial patient.

## Returned IDPs

There should be the same concern for environmental monitoring and supply chain integrity for returned IDPs, as some sponsors may choose to redistribute any returned IDPs that are unopened and undispensed to other clinical trial sites. Returned IDPs intended for redistribution must meet all requirements (i.e., initial release criteria) of the product and associated local regulations. The decision to reuse returned IDPs should be supported by a risk-based assessment.

# ASSESSING RISK EARLY IN THE DISTRIBUTION PROCESS

When shipping IDPs to depots and clinical trial sites, using common shipping lanes (routes between the point of origin and the destination point) and within appropriate temperature zones, a risk assessment can identify the probability and the impact missteps in the supply chain. ICH Q9 presents some risk assessment tools that can be used to help organizations to identify their risks and how to address them. Factors such as product category (e.g., narcotics, biologics, radiopharmaceuticals), storage conditions specification (e.g., temperature, light, humidity, vibration), environmental conditions that products will face according to the transportation route (e.g., origin, destiny, and lanes), duration of journey, shipping container (e.g., if temperature-controlled, if active or passive), and type of vehicle should be taken into consideration for risk assessment. Organizations should also evaluate if delays along the transportation route or due to custom holds can increase risk rates and should propose strategies to mitigate these risks.

# GLOSSARY

**Blinding:** The process through which one or more parties to a clinical trial are unaware of the treatment assignments. Blinded studies are conducted to prevent the unintentional biases that can affect subject data when treatment assignments are known.

**Comparator:** An investigational or marketed product (i.e., active control), or placebo, used as a reference in a clinical trial.

**Distribution:** Refers to elements such as shipping and transportation activities that are associated with the movement and supply of drug products.

**Investigational drug product (IDP):** Any pharmaceutical product (new product or reference product) or placebo being tested or used as a reference in a clinical trial.

**Protocol:** The study plan on which a clinical trial is based.

**Rescue medication:** Medicines identified in the protocol as those that may be administered to the patients when the efficacy of the investigational drug product is not satisfactory, or the effect of the IDP is too great and is likely to cause a hazard to the patient, or to manage an emergency situation.

**Standard operating procedure (SOP):** Detailed written instructions to achieve uniformity of performance of a specific function. ICH/ISO 9001 required for repeated application of processes that require standardization. All SOPs require quality assurance approval.

**Trial site:** The location where trial-related activities are actually conducted.

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# ⟨1079.2⟩ MEAN KINETIC TEMPERATURE IN THE EVALUATION OF TEMPERATURE EXCURSIONS DURING STORAGE AND TRANSPORTATION OF DRUG PRODUCTS

## 1. INTRODUCTION

The extent of physicochemical degradation of drug products depends on factors such as product stability, how a product is stored and shipped, and how it is packaged. Temperature at which a product is stored or shipped is likely to vary during the life of the product, which can impact its stability and degradation pathways and kinetics, thereby leading to the product failing to maintain its critical quality attributes.

Mean kinetic temperature (MKT) is a way to summarize the time history of a product's temperature exposure with a single "effective" or "virtual" temperature. It is defined as the single calculated temperature at which the total amount of degradation over a particular period is equal to the sum of the individual degradations that would occur at various temperatures. MKT integrates the time–temperature history by making assumptions about the kinetics of a product's chemical degradation. Therefore, MKT takes into account the fact that long temperature excursions at slightly elevated temperatures can be just as, or more, impactful than short temperature excursions at elevated temperatures.

## 2. SCOPE

This chapter applies to every link in the supply chain from the manufacturer through any entity that transports or stores a finished drug product, with the sole exception of the patient. Examples:

- Manufacturers of drug products, radiopharmaceuticals, biological products, and biotechnological products
- Repackaging operations in which the product may be owned by a company other than the primary manufacturer
- Healthcare providers and institutions such as hospitals; outpatient, ambulatory, and urgent care centers; home health providers; vaccine clinics; emergency departments; and medical, dental, and veterinary offices
- Pharmacies including but not limited to retail, infusion/compounding (sterile and nonsterile), specialty, mail order, hospital, nursing home, and hospice
- Importers and exporters
- Wholesale distributors
- Third-party logistics providers, brokers, freight forwarders, consolidators, and other organizations involved in storage; road, rail, sea, and/or air transport services; or mail distributors that offer expedited or controlled-temperature shipping services

Manufacturers of active pharmaceutical ingredients, excipients, medical devices (with the exception of drug combination devices), and dietary supplements are not within the scope of this chapter. However, the principles and mitigation strategies presented in this chapter may be useful for materials other than finished drug products.

## 3. MEAN KINETIC TEMPERATURE

MKT is the single calculated temperature at which the total amount of degradation over a particular period is equal to the sum of the individual degradations that would occur at various temperatures. MKT may be considered as an isothermal storage temperature that simulates the nonisothermal effects of storage temperature variation.[1] Seevers et al. demonstrated that the choice of activation energy doesn't change the MKT significantly. It is not a simple arithmetic mean. The use of MKT is considered an acceptable practice for storage and can be considered for excursions during transit.

The temperatures used for calculating MKT can be conveniently collected using electronic devices that measure temperatures at frequent intervals (e.g., every 15 min). MKT can be calculated directly or the data can be downloaded to a computer for processing. Software to compute the MKT is available commercially.

The arithmetic mean of the high and low temperatures of the observation period is then used in the calculation of MKT. MKT is calculated by the following equation (derived from the Arrhenius equation):

$$T_k = \frac{\Delta H/R}{-\ln\left(\frac{e^{-\Delta H/RT_1} + e^{-\Delta H/RT_2} + \ldots + e^{-\Delta H/RT_n}}{n}\right)}$$

$T_k$ = mean kinetic temperature
$\Delta H$ = heat of activation, 83.144 kilojoules · mole$^{-1}$ (unless more accurate information is available from experimental studies)
$R$ = universal gas constant, $8.3144 \times 10^{-3}$ kj · mole$^{-1}$ · K$^{-1}$
$n$ = total number of storage temperatures recorded during the observation period
$T_1$ = value for the temperature recorded during the first time period

[1] Seevers RH, Hofer J, Harber P, Ulrich D, Bishara R. The Use of Mean Kinetic Temperature (MKT) in the Handling, Storage, and Distribution of Temperature Sensitive Pharmaceuticals in *Pharmaceutical Outsourcing*; May–June 2009, 10(3); 30–35.

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$T_2$ = value for the temperature recorded during the second time period
$T_n$ = value for the temperature recorded during the *n*th time period

[NOTE—All temperatures, *T*, are absolute temperatures in degrees Kelvin (K).]

Drug products in the distribution supply chain may be held at temperatures outside of their labeled storage requirements as determined by the appropriate stability data. Drug products stored either in warehouse conditions or during transportation may experience excursions from their acceptable temperature ranges. Each product excursion outside of USP excursion limits must be evaluated and its impact on the final product determined. The means of evaluation must be scientifically sound with documented justification that the integrity and quality of the drug product have not been affected. One method of analysis for examining temperature excursion is the use of MKT.

Because MKT expresses the cumulative thermal stress that a drug product experiences, it is considered an acceptable practice for storage and can be considered for excursions during transit. The calculation must be justified for use with distribution excursions by confirming that the stability-limiting characteristic of the product follows zero-order or first-order kinetics over the temperature range encountered.

***Change to read:***

## 4. APPLICATION OF MKT

Mean kinetic temperature has been misunderstood and/or misused. The most significant misuse has been utilizing 52 weeks of temperature data to calculate MKT during a temperature excursion. Drug products typically do not spend 52 weeks in a single storage location. Thus, the 52 weeks of data used in the MKT calculation would not be a true reflection of the storage time. This approach skews results and could lead a company to overlook the impact of an excursion on the drug product.[1,2] A closely related concern is the idea that a temperature excursion above a product's storage temperature can be "fixed" just by lowering the temperature of a warehouse for an appropriate period of time so that the resulting MKT calculation would provide an acceptable value. This ignores the fact that any degradation due to the higher temperature is not reversible.[3]

Mean kinetic temperature is referenced in the controlled room temperature (CRT) and controlled cold temperature (CCT) definitions in *Packaging and Storage Requirements* ⟨659⟩. Within the definitions, the temperature range for an excursion and the maximum excursion time are defined, but the time frame used for calculating MKT is not mentioned. For CRT excursions, USP recommends using 30 days for calculating MKT or the average number of days that a product remains in the holder's possession. This recommendation is based on temperature data presented by Anderson et al.[3] and the fact that products, on average, spend 30 days in warehouse storage in the United States.[4]

For CCT excursions, USP recommends using 24 h for calculating MKT. While specific products may have stability data to support excursions within wider temperature and time frames, operations involved with storage and distribution (shipping) throughout the supply chain typically do not have access to information beyond the product labeling. This includes dispensing and healthcare providers. Excursions occur and these guidelines are intended to manage these excursions while being conscious of the impact of temperature and time excursions. It is important to note that an excursion is a nonconforming event (except as previously noted when provided by the manufacturer). MKT should not be used to justify a system out of control (e.g., repeated temperature and time excursions).

**Table 1. Use of MKT for CRT and CCT Excursions[a]**

| | Storage Range | Acceptable Excursion Range | Maximum Temperature | Maximum Excursion Time (NMT) | MKT (NMT) | Time Period of Calculation |
|---|---|---|---|---|---|---|
| CRT[b] | 20°–25° | 15°–30° | 40° | 24 h | 25° | 30 days |
| CCT[c] | 2°–8° | 8°–15° | 15° | 24 h | 8° | 24 h |

[a] Exposure to higher and/or lower temperatures beyond what is recommended in this chapter should be evaluated using monographs, product labels, or stability data provided by the manufacturer.
[b] Some CRT- labeled products may have stability issues with the higher and/or lower excursion limits. These products must be evaluated and shipped within the stability limits provided by the manufacturer.
[c] An article for which storage at CCT is directed may, alternatively, be stored and shipped in a cool place or refrigerated unless otherwise specified in the monograph or on the label (see ⟨659⟩).

In CRT storage environments, temperature monitoring (see *Monitoring Devices—Time, Temperature, and Humidity* ▲⟨1079.3⟩▲ (CN 1-Dec-2023)) captures the temperatures throughout the day (e.g., every 15 min). MKT can be calculated on an ongoing basis or anytime that there has been a temperature excursion using data going back 30 days from (and including) the high excursion temperature. 30 days must be from, and including, the high excursion temperature for the entire period of the excursion.

[2] Anderson C, Seevers R, Hunt D. The use of mean kinetic temperature to aid evaluation of temperature excursions: Proper and improper application. *Pharm. Forum* 2018;44(4).
[3] Anderson C, Seevers R, Hunt D. The use of mean kinetic temperature to aid evaluation of temperature excursions for controlled cold temperature drugs: Proper and improper application. *Pharm. Forum* 2019;45(5).
[4] Center for Healthcare Supply Chain Research. *Factbook: The Facts, Figures and Trends in Healthcare (2015–2016)*. 86th ed. Arlington, VA: Center for Healthcare Supply Chain Research; 2016:15.

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## Use of MKT for Controlled Cold Temperature Excursions

In CCT storage environments, temperature monitoring (see ▲⟨1079.3⟩▲ (CN 1-Dec-2023)) captures the temperatures throughout the day (e.g., every 15 min). MKT can be calculated on an ongoing basis or anytime that there has been a temperature excursion using data going back 24 h from the (and including) high excursion temperature. Excursion ranges, time, and MKT within allowances should be documented and can be reviewed for compliance and trending. The excursion temperature and time allowances for CCT products are much more restrictive than for most CRT products due to the more sensitive nature of these products to temperature excursions. Each excursion should be considered a separate event. A storage or transportation system that has repeated excursions should be considered a system out of control that needs to be corrected.

Excursion ranges, time, and MKT within allowances should be documented and can be reviewed for compliance and trending. Transportation systems that are monitored may also use MKT as outlined in this chapter. For transportation systems that are not monitored (a risk-based decision), lane temperature profile mapping may include the use of MKT when evaluating risks (see *Risks and Mitigation Strategies for the Storage and Transportation of Finished Drug Products* ⟨1079⟩).

## 5. CONCLUSION

While every effort should be made to store and distribute drugs within their labeled temperature range, excursions may occur. The use of MKT in evaluating a short-term excursion (as defined in ⟨659⟩) and the time period used for calculating MKT as recommended by USP will allow responsible management of excursions. It is important to note that excursions outside of USP allowable limits must be evaluated by the manufacturer, where conveying the excursion temperatures and time is critical to determining product disposition. MKT may not be used to justify a storage or transportation system that has repeated excursions; such a system is not in control and needs to be corrected.

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# ▲⟨1079.3⟩ MONITORING DEVICES—TIME, TEMPERATURE, AND HUMIDITY

## INTRODUCTION

A key aspect of protecting drugs during storage and transportation is monitoring the environment to which they are exposed. This chapter provides background information about the science and technology of temperature and humidity monitoring over time. It describes the available technologies and performance characteristics and provides recommendations for qualifying their performance. The shelf life of a drug product is a function of the temperature and humidity conditions during storage and transportation, as well as the drug product's chemical and physical properties. This chapter focuses strictly on supply chain temperature- and humidity-monitoring devices, both electronic and chemical.

Storage and distribution temperatures may be different if justified by appropriate stability studies and as indicated in the approved labeling. The effects of humidity are typically observed over much longer time periods of exposure (e.g., months to years versus hours to days) than effects of temperature, due to barriers to moisture ingress presented by primary and secondary drug product packaging.

The devices described in this chapter are those most commonly used to monitor the controlled storage and established distribution of drug products following good distribution practices (GDPs) (*1*–*2*). This chapter does not address measurement of temperature at extremes, which are temperatures above those that drugs are reasonably expected to experience in the supply chain. The types of devices described are already used in the worldwide distribution of pharmaceuticals and by other similar industries that require temperature control in distribution (e.g., the perishable food, blood component, and medical device industries). Devices also may be attached to individual items for the end user, e.g., vaccine vials in the World Health Organization/United Nations Children's Fund (WHO/UNICEF) global immunization program (*3*). Appropriate recycling practices should be followed for all devices as required by local regulations.

## TEMPERATURE-MEASUREMENT DEVICES

### Alcohol or Mercury Thermometers

These devices are based on the change in volume of a liquid as a function of temperature. Both types of thermometers can be designed to indicate the maximum and minimum temperatures (see *General Notices, 6.80.30. Temperature Reading Devices* for more information). Historically, these types of thermometers are used in a laboratory setting or in a pharmacy, rather than during supply chain monitoring. Alcohol thermometers can have a precision as good as 0.01°, but they must be quite large to measure temperatures in ranges of more than a few degrees.

Mercury thermometers are typically used in the ranges from 0° to 50° with a precision of about 0.1°. Some local regulations apply to mercury-based thermometers, and many states and local agencies have legislated or developed collection or exchange programs for mercury-containing devices. The US Environmental Protection Agency (EPA) also issues regulations requiring industries to reduce mercury releases to air and water, and to properly treat and dispose of mercury wastes to avoid potential health hazards (*4*). Globally, Health Care Without Harm (HCWH) and the WHO are co-leading a Health Care Initiative Products Partnership to reduce demand for mercury-containing devices by at least 70% and to shift production to accurate, affordable, and safer nonmercury alternatives (*5*).

Both alcohol and mercury thermometers are more fragile than other temperature-measuring devices described in this chapter.

### Infrared Devices

This device is used for measuring the infrared (IR) radiant heat from the article whose temperature is being determined, and the IR reading varies as a function of the object's temperature. The advantage of this type of device is that the article may be at some distance from the IR sensor. IR devices may give inaccurate higher or lower temperature readings because of the surface characteristics of packages (e.g., black versus white surfaces), and they also have the potential for operator error because of incorrect use of the IR reader (improper angle).

### Resistance Temperature Detectors

The resistance temperature detector (RTD) is based on the change in electrical resistance of a material as a function of temperature. The precision and accuracy of an RTD depend on the quality of the electronics used to measure the resistance. Although RTDs are among the most stable and accurate temperature sensors, their accuracy may change with the age and temperature of the device because its electronic components are affected by age and use. Although all temperature measurement devices should be placed on an appropriate calibration schedule as recommended by the manufacturer or user of the device, this calibration is particularly important for RTDs.

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### Solid-State Devices

Solid-state devices are based on the effect of temperature on either an integrated circuit (see *Thermistors*) or a micromechanical or microelectrical system. These devices are commonly referred to as data loggers and can attain high precision, and have the advantage of producing a digital output.

### Thermistors

A thermistor is a semiconductor device whose resistance varies with temperature. Thermistors are able to detect very small changes in temperature and are accurate over a broad range of temperatures.

### Thermocouples

Thermocouples are based on the change in the junction potential of two dissimilar metals as a function of temperature. Many metal pairs can be used, and each pair provides a unique range, accuracy, and precision. Precision and accuracy depend on the quality of the electronics used to measure the voltage across both metals and the type of temperature reference used.

### Thermomechanical Devices

Thermomechanical devices are based on the change in length of a solid material as a function of temperature. An example of such a device is a mechanical spring, which expands or contracts as a function of temperature, thus opening and closing an electrical circuit or moving a chart pen. Typical examples are chart recorders used in cold rooms.

## ELECTRONIC TIME-TEMPERATURE HISTORY RECORDERS

These recorders use one of the electronic temperature measurement technologies described above and create a record of the temperature history experienced.

### Electronic Time-Temperature Indicators

Electronic time-temperature indicators (TTIs) can be designed to issue an alert after a cold excursion, heat excursion, or after multiple temperature excursions and can provide a visual alarm via a colored light or liquid crystal display (LCD). The alarm(s) are generally programmable and can display conditions such as date, time, temperature, and duration of the alarm. A certificate of calibration is issued for individual units or lots. Multiple-use devices should have a calibration schedule, but single-use devices can rely on manufacturers' certificates of calibration.

### Electronic Temperature-Data Loggers

Electronic temperature-data loggers are recorders that monitor the temperature at programmable intervals and save the temperature history to a peripheral system, such as a personal computer. In addition, data loggers can record humidity using sensors described below. Electronic recorders monitor and save temperature values representative of the cumulative temperature history over a period of time and therefore have the advantage of being able to calculate the mean kinetic temperature (MKT) (*6*) based on the measurements. Data loggers equipped with transmitting devices (hardwired or radio transmission) can be used to monitor the temperature and humidity of a product while in transit and can download the recorded data when the data loggers arrive at a destination. Data loggers are increasingly required by worldwide ministries of health as part of a standard quality system for GDPs. Based on their communication capabilities, data loggers can be grouped into several different categories.

### Radio Frequency Data Loggers

In addition to data loggers that require a hardwired connection to a base unit or a computer, in recent years companies have adapted wireless (radio frequency or RF-enabled) temperature and humidity recorders. The effects of radio frequency identification (RFID) use on biologics have been studied on a variety of blood, blood components, monoclonal antibodies, and vaccines, and have demonstrated no effect (*7*). These loggers are integrated with chips capable of wireless RF communications that constitute the RFID sensor tags. The RFID chip inside the tag can be either active, which requires battery power for operation, or passive, which requires a nonzero RF field created by the RFID interrogator host unit (commonly called the reader) in the vicinity of the tag. RFID-enabled sensor tags (temperature and/or humidity) have the added capability of conveying recorded temperature history wirelessly to a host computer or database for seamless downstream processing. Multiple passive and active standards exist to control the communication between the tag and the host unit, including ISO-18000-6C (*8*), ISO-18000-7 (*9*), ZigBee (*10*), IEEE802.11 (*11*), and many proprietary standards.

When choosing between active and passive technologies, one needs to know that active technologies typically have extended communication range and memory capabilities at the expense of a higher price. Currently, reading ranges extend to 100 m, and with repeaters, longer distances can be achieved. Whether the communications circuitry is passive or active, these RF loggers still are electronic temperature recorders, which means their sensor circuitry uses external power from batteries or other sources.

A completely passive wireless RFID tag with an antenna capable of functioning as a sensor has been developed. The tag uses resonant antenna structures of RFID tags that are coated with specific sensor films. The passive wireless RFID tags act like analog

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sensors that, when interrogated by a wireless reader, show the instantaneous temperature. The film changes the antenna's reflection characteristics based on the monitored environmental variable (such as temperature and/or humidity), which then is decoded by the reader. The sensor film can be designed to work with different variables such as temperature, humidity, and various gas and chemical vapors. Although they lack some of the memory functionality included in electronic recorders, passive wireless sensors are relatively cost-effective compared to data loggers, and can be considered for item-level applications.

## CHEMICAL TEMPERATURE INDICATORS

Chemical temperature indicators are relatively cost-effective compared to electronic data loggers and can be considered for item-level applications. There are two basic types of chemical temperature indicators: 1) a threshold indicator that responds at a specific temperature and 2) a TTI that responds to cumulative heat exposure.

### Chemical Temperature Threshold Indicators

These indicators, sometimes referred to as critical temperature indicators, are based on a phase change or chemical reaction that occurs as a function of temperature. Examples include liquid crystals, waxes, polymers, and lacquers that change phase, and thereby their appearance, as a function of temperature. Chemical temperature threshold indicators are reversible or irreversible and are suitable for high or low temperatures. Temperature threshold indicators do not include any specified time delay to show a response and typically are single-use devices. These indicators provide a signal only when exposed to temperatures higher than (ascending indicator) or lower than (descending indicator) a predetermined threshold temperature.

**Ascending-Temperature Threshold Indicators**: Ascending-temperature threshold indicators are supplied as self-adhesive labels or cards and are normally composed of a heat-fusible compound that melts at the critical temperature. Melting of the compound gives rise to a color change or color development. Other types are provided as inks, lacquers, pellets, or crayons. Ascending-temperature threshold indicators are available from 0° to more than 200°, with as many as 10 temperatures on a single unit. Some ascending-temperature threshold indicators used to monitor frozen or refrigerated temperatures require an activation step (such as a pull tab or a reservoir that is ruptured by pressure).

**Descending-Temperature Threshold Indicators**: Descending-temperature threshold indicators show a response when exposed to temperatures below a threshold. These indicators do not include a specific time delay to show a response, and the response is typically caused by the time required for solidification of a liquid at the threshold temperature. Solidification of a liquid causes a visual change in the indicator. Examples include: 1) the expansion of the liquid to crack an ampule and release a colorant, 2) contraction of the liquid to mix components to develop color, or 3) aggregation of colloidal particles to change color.

### Chemical Time-Temperature Indicators

These TTIs include systems in which a reaction rate or diffusion process is used to estimate a temperature equivalent integrated over time. Thus, chemical TTIs provide a measure of accumulated heat rather than instantaneous temperature such as a spike or critical threshold (discussed above). The reactions generally are irreversible—once a color change, color development, or diffusion process has taken place, exposure to low temperatures will not restore the indicator to its original state, but lower temperatures (refrigeration) will slow the color change. The accuracy and precision of these indicators depend, to some extent, on human interpretation. Some versions of chemical indicators have been prepared in a bar code format and can be read with bar code readers. Other developments include reading a chemical indicator with an imaging device, such as a camera in a smart phone.

Chemical TTIs do not directly reflect the status of the drugs to which they are attached. In actual practice, the characteristics for degradation of a particular drug are known from accelerated and real-time stability studies that follow internationally accepted guidelines such as Parenteral Drug Association (PDA) Technical Report No. 53 (*12*) to guide selection of a suitable TTI (*13*). The activation energy of the TTI is not required to exactly match the activation energy of the degradation of the drug being monitored, and the latter, in fact, may not be known precisely. Therefore, the TTI should be chosen to provide an early warning if the drug is exposed to an excessive heat load before the expiration date.

An important characteristic of chemical TTIs is the precision with which the end point can be determined. For TTIs that change color, a reference color normally brackets the active portion of the TTI to show the end point color, which simplifies TTI interpretation. Accuracy can vary widely with the control and quality of the manufacturing process. Some TTIs are manufactured by procedures that comply with Quality System Regulations for Medical Devices. As discussed below in *Calibration of Temperature- and Humidity-Monitoring Devices*, it is not possible to calibrate any individual single-use device because the test is, by the nature of the TTI, necessarily destructive. This is analogous to any pharmaceutical product because each dose cannot be calibrated or validated, but validated processes should be used in the manufacturing process.

The two types of TTIs are partial-history indicators and full-history indicators. Partial-history TTIs provide a time- and temperature-dependent response when the temperature exceeds a predetermined value. A partial-history indicator normally is composed of a dyed, heat-fusible compound that diffuses along a porous strip or wick when the temperature exceeds the melting point of the compound. The diffusion process of the compound down the wick is temperature dependent, and therefore the partial-history TTI provides a time and temperature response above the melting point of the compound. Migration of the compound down the wick stops when the indicator is moved to a lower temperature at which point the compound solidifies. These TTIs normally have one or more viewing windows to monitor the length the dyed compound has traveled along the wick. Some indicators are activated by removing a barrier film that separates the dyed compound from the wick or rupturing a reservoir that contains the dyed compound. Other indicators do not require activation and must be stored below the melting point of the compound before use. These partial-history TTIs can have durations (service life) anywhere from several hours to several years. Full-history TTIs provide a continuous response to temperature. They change color or physical appearance as a

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result of exposure to time, and the rate of change increases with temperature so they are sensitive to cumulative heat exposure. Full-history TTIs are responsive to MKT (see *Risks and Mitigation Strategies for the Storage and Transportation of Finished Drug Products* ⟨1079⟩) and typically are single use, irreversible, and disposable because once the color changes it will not revert to the original color.

### Chemical-Physical Time-Temperature Indicators

This type of TTI is based on a temperature-dependent diffusion or chemical reaction process. It consists of a pressure-sensitive tape device that is composed of an indicator tape and an activator tape. In one example, the indicator tape contains a dye precursor dispersed in a polymer carrier. The activator is incorporated into an adhesive on the activator tape. Laminating the activator tape over the indicator tape causes activation. A color change or readable message occurs as the activator migrates into the indicator as a function of temperature and time. Other approaches to develop color changes include the use of a pH indicator or the etching of aluminum by the activator tape.

### Chemical Polymerization-Based Time-Temperature Indicators

This type of TTI uses a solid-state polymerization process in which a color develops intensity as a function of time and temperature. The color evolution is caused by the polymerization of a colorless monomer to a highly colored polymer. These TTIs can be applied by a print process that permits direct integration into a product label or stand-alone label. Because this type of TTI does not require activation, it must be shipped from the manufacturer on dry ice or under frozen conditions and stored at temperatures according to the manufacturer's instructions, normally below –24° before use. Chemical polymerization-based TTIs can be designed to reach the end point in as little as a few weeks at refrigerated temperature, or as long as a few years at controlled room temperature.

### Chemical Enzyme-Based Time-Temperature Indicators

This type of TTI uses an enzyme-catalyzed color-generating reaction that occurs as a function of time and temperature. The color change is caused by an enzyme reacting with a substrate, accompanied by a change in pH. The enzyme and substrate are in separate solutions in adjacent compartments. Breaking the barrier between the two compartments and mixing the two solutions activates the TTI.

### Chemical Organic Pigment-Based Time-Temperature Indicators

This type of TTI uses an organic pigment that is activated by exposure to ultraviolet light to develop a dark blue starting color. A filter is then placed over the label to protect it from deliberate or accidental reactivation. The colored pigment fades over time as a function of temperature.

## RELATIVE HUMIDITY MEASUREMENT TECHNOLOGIES

Relative humidity is the ratio of the partial pressure of water vapor in air to the vapor pressure of saturated air at a given temperature. In other words, the relative humidity is the amount of water vapor present, divided by the theoretical amount of moisture that could be held by that volume of air at a given temperature. Extensive tables of relative humidity data are available. Devices for measuring relative humidity are called hygrometers. Several different technologies exist for measuring relative humidity.

### Sling Psychrometer

The simplest type of hygrometer is based on the temperature difference observed between two identical thermometers, one ordinary and one with a wet cloth wick over its bulb. The two thermometers are whirled at the end of a chain, and the evaporation of water from the wick cools (based on evaporative cooling) the wet-bulb thermometer. The temperature difference between the wet and dry thermometers then is compared to a table specific to that psychrometer based on dry-bulb temperature, and the relative humidity is determined.

### Hair Hygrometer

This type of device is based on the fact that the length of a synthetic or human hair increases as a function of the relative humidity. This change is used to move an indicator or affect a strain gauge. A hair hygrometer can be accurate to ±3%, but it is unable to respond to rapid changes in humidity and loses accuracy at very high or very low levels of relative humidity.

### Infrared Hygrometer

This type of hygrometer determines relative humidity by comparing the absorption of two different wavelengths of IR radiation through air. One wavelength is absorbed by water vapor and the other is not. This type of hygrometer can accurately measure relative humidity in large or small volumes of air. It is sensitive to rapid changes of humidity and can be integrated with an electronic data-handling system.

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### Dew Point Hygrometer

This type of device uses a chilled mirror to determine the dew point of an air sample. The dew point is the temperature at which water vapor in the air begins to condense; that is, the temperature at which the relative humidity is 100%. The relative humidity can be calculated from this measurement and an accurate measurement of the ambient temperature. The dew point hygrometer is the standard against which most commercially available instruments are calibrated.

### Capacitive Thin-Film Hygrometer

The principle of this type of hygrometer is that the dielectric of a nonconductive polymer changes in direct proportion to the relative humidity. This change is measured as a change in capacitance. This type of hygrometer is accurate to ±3%.

### Resistive Thin-Film Hygrometer

This type of hygrometer is similar to the capacitive thin-film type because it uses the effect of changing relative humidity on an electric circuit. In the resistive thin-film hygrometer, the sensor is an organic polymer whose electrical resistance changes in logarithmic proportion to the relative humidity. This type of hygrometer is accurate to ±5%.

## CALIBRATION OF TEMPERATURE- AND HUMIDITY-MONITORING DEVICES

Thermometers and hygrometers that are used to provide data about the temperature and humidity exposure of a product must be suitable for their intended use. Specifically, they must be appropriately calibrated. A calibration program assures the user of the monitoring device that the device has been tested before use, either by the manufacturer or the user, to assess its suitability for the intended use. Calibrations should be performed with appropriate frequencies to support ongoing use. Monitors used in manufacturing, storage, and transport of drug products should be properly qualified by their users to ensure that the monitors have been received and maintained in proper working order. It is acceptable to use the calibration performed by the device's manufacturer based on the certificate of calibration and expiration date.

For temperature- and humidity-monitoring devices, measurement accuracy refers to the closeness of the value obtained with a particular device and the true value of the object or environment under measurement. In practice, this is determined by comparison with a device that has been calibrated against a standard that is obtained from or is traceable to the National Institute of Standards and Technology (NIST) or a comparable national metrology organization.

Any monitor takes time to respond to a change in the temperature or humidity. Measurement responsiveness typically is defined in a device's specifications for its operating range. Different data recording intervals are appropriate for different monitoring applications, and should be based on supply chain length (for example, transportation via ocean may require 30-min intervals, but 15-min intervals may be suitable for air transport). Most commonly, time accuracy is expressed as a ± percentage of total duration of the recording period. For pharmaceutical applications, a ±0.5% time accuracy is adequate.

Single-use electronic and chemical indicators should follow Good Manufacturing Practices (GMPs) with appropriate testing controls. Electronic indicators require proper calibration. Single-use indicator performance can be qualified by the supply chain user by sampling and testing of multiple production lots. For TTIs that calculate MKT, the performance of a batch can be assessed statistically by subjecting an appropriately sized sample to elevated temperature conditions for a set period of time and observing the results. Manufacturers should adopt appropriate acceptance criteria. It is acceptable to use the release test performed by the manufacturer of the indicator (based on the certificate of calibration or the certificate of analysis and the expiration date) in lieu of calibration or qualification.

## THE USE OF HISTORICAL TEMPERATURE DATA

Although historical geographic and seasonal trends may be used as a planning tool in selecting from among the various types of temperature- and humidity-monitoring devices, outside ambient temperatures are not necessarily reliable indicators of the temperatures experienced by different items in the distribution chain. For example, studies have reported important departures from ambient temperatures on summer days for mailboxes, trucks, and warehouses (*14*). Therefore, using lane-specific temperature monitoring is beneficial when manufacturers and shippers develop an ambient profile, and can be a valuable consideration for a risk-based approach to maintain product quality (*15*).

A drug product's quality (identity, strength, and purity) may be notably influenced by variations in temperature and humidity over the course of its shelf life, so manufacturers should appropriately monitor those environmental conditions. Pharmaceutical manufacturers perform stability testing to carefully evaluate the effects of temperature and humidity on their products. The packaging, shelf life, storage, and transportation conditions recommended for a product are chosen based on the results of these stability studies. Temperature effects can happen rapidly; therefore, temperature monitoring should be implemented on a risk-based approach taking into account the product's stability, distribution route, mode of transportation, and potential risks that may compromise the quality of the product. Relative humidity effects occur over a much longer time frame; humidity monitoring can be omitted when the drug product is sufficiently protected by the primary container proven by sound stability studies. Humidity monitoring is recommended when special environmental restrictions concerning the humidity are defined for the drug product.

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## STANDARDS FOR TEMPERATURE AND HUMIDITY DATA REPORTING

It is important to consider the records derived from temperature and humidity measurement. The same expectation of legibility, permanence, and traceability to the individual(s) who prepared the record applies to such records as would apply to any record prepared under GMPs.

For some types of devices, e.g., mercury or alcohol thermometers, chemical TTIs, or sling psychrometers, a handwritten log is typical. These logs should be kept in ink with the initials of the person who recorded the measurement included. For other devices, a paper graph is the standard output (e.g., temperature or humidity chart recorders). The paper graph thus creates a permanent record.

Many of the electronic devices described above for measurement of temperature of humidity have the capability of producing an electronic record. The requirements of 21 CFR §11, Electronic Records; Electronic Signatures—Scope and Application (*16*) apply to these records with respect to record permanence, audit trails, limiting system access to only authorized individuals, etc. One key concern is that many devices produce their reports in the form of portable document files (pdfs) that have different levels of security available. At the lowest level of security, a pdf can be edited with readily available software (e.g., Adobe Acrobat Pro or Microsoft Word). This creates a risk that what should be a permanent record of temperature or humidity can be altered. Therefore, it is recommended that the highest level of pdf security with appropriate encryption be employed with devices that use the pdf format for output.

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# ⟨1079⟩ RISKS AND MITIGATION STRATEGIES FOR THE STORAGE AND TRANSPORTATION OF FINISHED DRUG PRODUCTS

## 1. INTRODUCTION

Proper storage and transportation of finished drug products are critical activities in an integrated supply chain. These finished drug products include but are not limited to temperature-sensitive small molecules, vaccines, biologics, biotechnological products, radiopharmaceuticals, and combination products. With the globalization of the pharmaceutical industry, various individuals and organizations from locations around the world can come into contact with the finished drug product. The storage and transportation processes for a drug product may involve complex movements with differences in documentation, handling requirements, and communication between the various entities throughout the supply chain.

Environmental controls play a key role in maintaining drug safety, quality, and efficacy. Temperature is one of the most important parameters to control. Drugs must be stored and transported according to predetermined conditions (e.g., temperature) as supported by stability data. Temperature excursions outside of their respective labeled storage conditions, for brief periods, may be acceptable provided that stability data and scientific/technical justification exist, demonstrating that product safety, quality, and efficacy is not affected.

To maintain the original quality, every party involved in the storage and transportation of a finished product should have an in-depth understanding of the storage and transportation risks and have the appropriate mitigation strategies in place to control these risks. The intent of this chapter is to identify common risks in the storage and transportation of drug products and to recommend mitigation strategies. The chapter is not meant to prescribe specific approaches or discuss regulatory frameworks currently in place, but rather to focus on risks and mitigation strategies for quality processes to maintain product and supply chain integrity. The principles of this chapter can be used to facilitate the storage and transportation of drug products throughout a supply chain that is controlled, measured, and analyzed for continuous improvements while also maintaining the integrity of the drug product in its packaging during distribution.

## 2. SCOPE

This chapter applies to organizations and individuals involved in the storage and transportation of drug products, including but not limited to the following:

- Manufacturers of drug products, radiopharmaceuticals, biological products, and biotechnological products
- Repackaging operations in which the product may be owned by a company other than the primary manufacturer
- Healthcare providers and institutions such as hospitals; outpatient, ambulatory, and urgent care centers; home health providers; vaccine clinics; emergency departments; and medical, dental, and veterinary offices
- Pharmacies, including but not limited to retail, compounding (sterile and nonsterile), specialty, mail order, hospital, nursing home, and hospice
- Importers and exporters
- Wholesale distributors
- Third-party logistics providers, brokers, freight forwarders, consolidators, and other organizations involved in storage; road, rail, sea, and/or air transport services, or mail distributors that offer expedited or controlled-temperature shipping services

Manufacturers of active pharmaceutical ingredients, excipients, packaging materials, medical devices, and dietary supplements are not within the scope of this chapter. However, the concepts, risks, and mitigation strategies discussed in this chapter may be useful and can be applied in these cases, if desired.

## 3. RISK-BASED APPROACH TO THE STORAGE AND TRANSPORTATION OF FINISHED DRUG PRODUCTS

*Figure 1* illustrates the risk-based approach of a quality management system (QMS). It represents how product knowledge and process knowledge facilitate the identification of risk. The figure also illustrates how mitigation strategies that are planned to reduce the identified risks, categorized in clusters, form the pillars of a QMS.

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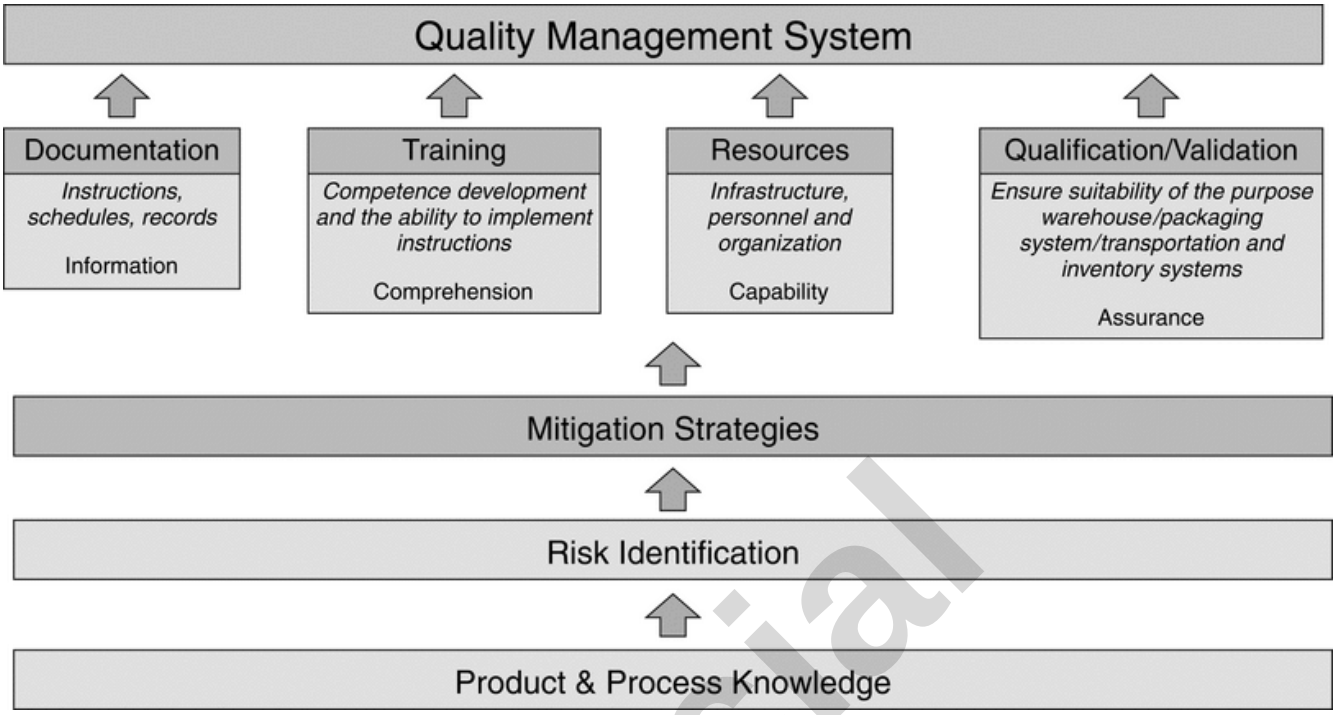


Figure 1. Risk-based approach for a QMS.

Product and process knowledge is the starting point in identifying risks related to the storage and transportation of drug products. Product knowledge includes but is not limited to the following: intended use; storage conditions; potential hazards to environment and personnel (e.g., hormones, cytotoxic drug products, and radiopharmaceuticals); and inherent vulnerability (e.g., high potential for abuse, high-value drugs, attractiveness of freight to criminals, counterfeiting, and diversion). Process knowledge includes but is not limited to the following: knowledge of supply chain partners; physical modes of transportation (air, sea, rail, road, or a combination of modes); transportation routes; and national and international regulation. Understanding these factors helps an organization identify their associated risks. Process mapping is a useful tool for organizations to gain further understanding of a particular process and/or operation (e.g., transport lane selection or loading/unloading patterns of warehouses and vehicles).

Risk identification is the systematic use of information to identify potential sources of harm (hazards). Information can include historical data, theoretical analysis, informed opinions, product and process knowledge, and the concerns of stakeholders. Risk identification addresses the question: "What might go wrong?" Mitigation strategies are part of the risk control process, specifically risk reduction. Risk reduction addresses the question: "What can be done to reduce or eliminate risks?" In this way, risk reduction can include actions taken to mitigate the severity or probability of harm. Processes that improve the detectability of hazards and quality risks can also be used as part of a risk control strategy.

Different stakeholders perceive risks differently; for example, they may assign different levels of risk based on their experience and knowledge or they may estimate the probability of risk to product differently. Regardless of where your organization fits into the supply chain, consideration of risks and mitigation actions taken should consider potential impact throughout the supply chain. The mitigation strategies can be divided into four categories that are fundamentals (or pillars) of a QMS (see *Figure 1*). These strategies, when implemented, give an organization the autonomy to plan, implement, measure, and improve their processes according to current regulations and associated risks. Generally, mitigation strategies fall within four categories related to: 1) documentation, i.e., providing instructions for a specific operation or process to standardize it and establish consistency; 2) training, i.e., ensuring competence; 3) resources, i.e., providing capability through infrastructure and human resources; and 4) qualification and validation, i.e., assurance that the resources and processes are reliable, reproducible, and robust.

Several informal and formal tools can be used to conduct risk assessments and control risk. Examples of tools used to perform risk identification include (but are not limited to): flow charts, process mapping, trends, historical data records (such as temperature records over a particular route), and observations. Other tools—such as failure mode effects analysis (FMEA), fault tree analysis (FTA), hazard analysis and critical control points (HACCP), hazard operability analysis (HAZOP), and preliminary hazard analysis (PHA)—can also be used for conducting risk management. [NOTE—See International Council for Harmonisation (ICH) in *Additional Sources of Information*] .

*Table 1* contains illustrative examples of the risks related to drug product storage and transportation, along with their mitigation strategies. The list presented below is not exhaustive and is meant to stimulate discussion and provide examples.

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**Table 1. Storage and Transportation Risks and Mitigation Strategies**

| Hazard | Effect | Mitigation Strategy | Mitigation Category |
|---|---|---|---|
| **General Risk** | | | |
| Human error due to excessive duties or lack of training or competence | Mishandling along the supply chain, which can affect product quality and integrity and patient safety | • Evaluate training effectiveness [are trainees competent in key aspects of the standard operating procedures (SOPs)?]<br>• Assign appropriate number of personnel to avoid excessive duties placed on one individual<br>• Assign employees to duties that they are qualified for based on education, experience, and competence | Training and Resources |
| **Procurement and Sales** | | | |
| Buy from or sell to unlicensed trading partners | Legal sanctions; patient safety | • Supplier qualification<br>• Customer qualification<br>• Checks to ensure license is current and appropriate<br>• Quality agreements between supplier and trading partners | Documentation and Resources |
| **Receiving and Shipping** | | | |
| Receive adulterated, falsified, or recalled product | Patient safety; introduction into legitimate supply chain of a product that is potentially substandard, illegal, or counterfeited | • Quarantine<br>• Quality control test<br>• Packaging identification fingerprints<br>• Recall awareness<br>• Notify regulatory authorities or trading partners<br>• Qualification of supply chain partners and on-going performance qualifications | Documentation and Training |
| Receive product that was not ordered | Unmatched transaction (e.g., wrong paperwork or transaction data sent); introduction into legitimate supply chain of a product that is potentially substandard, illegal, or counterfeited | • Adhere to receiving SOP | Documentation and Training |
| Mix products with different status (rejected, recalled, or returned) | Patient safety; shipping or selling of inappropriate product | • Product segregation, physical in the location and/or system<br>• Warehouse layout (logical flow and holding areas in order to avoid mix-ups)<br>• Adhere to receiving SOP | Storage, Documentation, and Resources |
| Shipping and receiving delays due to inclement weather, natural disasters, traffic disruption | Patient safety; arrival delays; temperature out of specification (temperature excursions, e.g., product freezes accidentally) | • Reschedule the delivery<br>• Temporary parking (waiting for opportunity to unload) or off-loading to a temperature-controlled facility or vehicle<br>• Recondition materials to ensure temperature maintenance during delay<br>• Rescue services<br>• Monitoring to demonstrate that the product integrity was not compromised; in cases when monitoring was not implemented due to qualifications and risk assessments, these delays might be out of scope due to the qualification parameters | Documentation, Training, and Resources |
| **Storage** | | | |
| Improper entry into a materials management system: wrong batch number, wrong expiration date, wrong status (e.g., product was approved but should be quarantined), or wrong amount | Inaccurate stock; picking and/or shipping product that should have been quarantined but was marked approved | • Adhere to stocking SOP<br>• Software validation | Documentation and Validation |

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**Table 1. Storage and Transportation Risks and Mitigation Strategies** *(continued)*

| Hazard | Effect | Mitigation Strategy | Mitigation Category |
|---|---|---|---|
| **General Risk** | | | |
| Product stored in wrong physical location | Patient safety because of picking error (software shows location, but staff can pick the wrong product if there is no check of the physical location) | • Adhere to stocking SOP<br>• Automated checking system | Documentation, Training, and Validation |
| | Product exposed to temperature excursions | • Refer to SOP showing list of products and their temperature specifications | Documentation |
| | Legal sanctions for controlled substance; risk of diversion for controlled substance | • Refer to SOP showing list of products and their license category (e.g., controlled, radiopharmaceutical) | Documentation |
| Environmental conditions out of specification | Affects product quality, product integrity, and patient safety (e.g., freezing of vaccine or biologic product); product loss causing financial loss | • Warehouse, packaging, and transportation qualification (temperature mapping)<br>• Product storage identification | Qualification and Validation, Training, and Documentation |
| | Out-of-range cold or hot areas; product storage temperature excursion product loss; financial loss; patient product availability | • Qualification (temperature mapping)<br>• Storage temperature monitoring program<br>• Homogenous airflow<br>• Monitoring and alarms<br>• Adhere to excursion-handling SOP | Documentation, Resources, and Qualification and Validation |
| Temperature monitoring device failure | Out-of-range cold or hot areas; product storage temperature excursion; product loss | • Backup monitoring devices with independent power source<br>• Adhere to excursion-handling SOP | Documentation and Resources |
| Storage or temperature system failure due to:<br>• Loss of electrical power<br>• Failure of temperature control or air circulation systems<br>• Unusual weather event | Out-of-range cold, warm, or hot areas; product storage temperature excursion; product loss | • Temperature and power alarms<br>• Backup power and coolant systems (redundant) and/or contingency storage<br>• Adhere to excursion-handling SOP | Documentation and Resources |
| Fear of reporting nonconformance and exception conditions | Affects product integrity and patient safety due to serious conditions not communicated | • Independent quality reporting structure<br>• Education on product integrity and impact on patients and the supply chain | Training and Resources |
| **Picking** | | | |
| Picking (human) error | Patient safety; wrong item shipped (product availability); product returns | • Automated checking system or second-person verification | Documentation, Training, and Qualification and Validation |

[NOTE—The effect of a hazard, if not mitigated, could impact product integrity and ultimately patient safety.]

***Change to read:***

## 4. RISK MITIGATION CATEGORIES AS QMS ELEMENTS

A QMS is necessary to implement, monitor, and maintain a robust storage and transportation process. Key elements of a QMS are management responsibility, documentation, training, resource management, complaint handling, deviation/excursion handling, returns and recalls management, qualification/validation, monitoring, audit, corrective action, preventive action, and continuous improvement. This chapter does not intend to provide a QMS framework; rather this chapter highlights how risk mitigation strategies are pillars of a QMS and how QMS elements ensure the quality and security of drug products throughout storage and transport.

The four categories of risk mitigation strategies are discussed in *Table 2* , with a matrix that links the mitigation strategy to the role that an organization plays within the supply chain.

**Table 2. Mitigation Strategies Used by Organizations within Supply Chain**

| | Role of Organization within Supply Chain | | | | | |
|---|---|---|---|---|---|---|
| **Applicable Mitigation Strategies** | **Manufacturer** | **Wholesaler and Distributor** | **Pharmacy or Compounding Pharmacy** | **Hospital and Healthcare Provider** | **Broker** | **Logistics Service Provider (LSP)** |
| **Documentation (Manuals, Procedures, Protocols, Records)** | | | | | | |
| Quality manual | Yes | Yes | Yes | Yes | Yes | Yes |
| Labeling | Yes | Yes | Yes | Yes | No | Yes |

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**Table 2. Mitigation Strategies Used by Organizations within Supply Chain** *(continued)*

| Role of Organization within Supply Chain | | | | | | |
|---|---|---|---|---|---|---|
| **Applicable Mitigation Strategies** | **Manufacturer** | **Wholesaler and Distributor** | **Pharmacy or Compounding Pharmacy** | **Hospital and Healthcare Provider** | **Broker** | **Logistics Service Provider (LSP)** |
| **Documentation (Manuals, Procedures, Protocols, Records)** | | | | | | |
| Procurement | Yes | Yes | Yes | Yes | Yes | Yes |
| Receiving | Yes | Yes | Yes | Yes | No | Yes |
| Picking | Yes | Yes | Yes | Yes | No | Yes |
| Packing | Yes | Yes | Yes | Yes | No | No |
| Sales | Yes | Yes | Yes | Yes | Yes | No |
| Storage | Yes | Yes | Yes | Yes | No | Yes |
| Transportation | Yes | Yes | Yes | Yes[a] | No | Yes |
| Supplier qualification | Yes | Yes | Yes | Yes | Yes | Yes |
| Customer qualification | Yes | Yes | No | No | Yes | No |
| Quality agreements | Yes | Yes | Yes | Yes | Yes | Yes |
| Licenses and authorizations | Yes | Yes | Yes | Yes | Yes | Yes |
| Recall | Yes | Yes | Yes | Yes | Yes | Yes |
| Return | Yes | Yes | Yes | Yes | No | No |
| Temporary parking | Yes | No[b] | Yes | No | No | Yes |
| Excursion handling | Yes | Yes | Yes | Yes | No | Yes |
| Disposal of expired and nonconforming drug products (e.g., suspect, expired, recalled, quarantined) | Yes | Yes | Yes | Yes | Yes | No |
| Pest control and pallet conservation | Yes | Yes | Yes | Yes | No | Yes |
| **Training** | | | | | | |
| Training | Yes | Yes | Yes | Yes | Yes | Yes |
| **Resources** | | | | | | |
| Product segregation | Yes | Yes | Yes | Yes | No | Yes |
| Storage area (layout/logical flow) | Yes | Yes | Yes | Yes | No | Yes[c] |
| Maintenance | Yes | Yes | Yes | Yes | No | Yes |
| Calibration | Yes | Yes | Yes | Yes | No | Yes |
| Monitoring systems and alarms | Yes | Yes | Yes | Yes | No | Yes |
| Appropriate number of personnel | Yes | Yes | Yes | Yes | Yes | Yes |
| Organizational chart and job descriptions | Yes | Yes | Yes | Yes | Yes | Yes |
| **Qualification and Validation** | | | | | | |
| Temperature mapping | Yes | Yes | Yes | Yes | No | Yes |
| Shipping packaging qualification | Yes | Yes | Yes | No[a] | No | No |
| Software validation (automated checking systems, inventory management system) | Yes | Yes | Yes | Yes | Yes | Yes |

[a] Unless the healthcare entity ships between owned facilities or to the patient.
[b] If the ownership of the product has already transferred to the distributors, then temporary parking could be the responsibility of the distributors/wholesaler.

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[c] Applied only if the logistics service provider has a temporary storage area.

## 4.1 Documentation and Procedures

Documentation provides written information that allows for consistency and traceability of actions. For this reason, documentation is a fundamental part of any quality system. In a risk-based approach, documentation is a category of risk mitigation. Some examples of documentation include quality manuals; standard operating procedures (SOPs); labeling; records pertaining to procurement, receiving, storage, and transportation; supplier qualification records; quality agreements; recalls; and excursion-handling records.

The sections that follow describe key types of documents that are fundamental for a QMS supply chain system. These documents can be used in a range of different organizations (see *Table 2*).

### 4.1.1 QUALITY MANUAL

A quality manual is a top-level quality document for all areas of the business affected by the quality system. The quality manual contains the quality policy, quality objectives, quality system structure, and information related to the management of a specific organization. The content may also include information on inspection management, customer complaints, recalls, withdrawals and holds, corrective and preventive actions, nonconformance and change control, information about regulatory issues, and performance evaluation through quality indicators.

### 4.1.2 STANDARD OPERATING PROCEDURES

SOPs are controlled documents, with document owners and approvers, effective dates, revision management, and scheduled reviews. Procedures should cover areas governed by the quality manual and should cover all aspects of the operation that may affect product quality, including handling, distribution, and all regulated activities in relation to the specific business (e.g., national and international laws). SOPs should also address actions that are performed to identify and mitigate risks.

Some key components are, but not limited to, the following:

- Corrective action/preventive action (CAPA)
- Documentation
- Record keeping
- Inventory management
- Licensing
- Management reviews
- Nonconforming product (for example, but not limited to, damaged or adulterated, expired, recall, suspect or illegitimate product, and temperature deviation)
- Order processing
- Purchasing
- Picking
- Packing and shipping
- Receiving
- Returns
- Storage
- Training

**Procurement:** One of the risks in procurement is buying and selling for transport with an unlicensed trading partner, leading to legal sanctions. A procurement procedure ensures that a product is purchased according to product specifications and that purchases are made from qualified partners that are licensed as appropriate.

**Receiving:** One potential risk when moving a product forward and backward along the supply chain is the introduction of substandard, illegal, or counterfeit products into the legitimate supply chain. Receiving is the operation related to the entrance of cargo into the operation's facility, starting with unloading the cargo from vehicles and then receiving, checking, and stocking the operation's facility. Each organization should have a receiving procedure that determines the appropriate checks for this operation. A checklist can be used as a reminder of what to inspect and what to record. Where appropriate, the transport vehicle can be inspected before unloading to verify that adequate protection from contamination was maintained during transit. To avoid the risk of receiving a product that was not ordered, all deliveries should be verified at the time of receipt in order to check that containers were not damaged and that the consignment corresponds to the order.

All incoming products should be segregated from salable inventory and identified. Products should be transferred to their respective storage areas according to their classification and storage specification. When products arrive at warehouse loading docks and other arrival areas, they should be transferred as quickly as possible to a designated storage area within a time period that is consistent with the organization's receiving SOP.

Inclement weather, natural disasters, and traffic disruption can cause receiving delays and potential temperature excursions. Rescheduling the delivery, temporarily parking while waiting for an opportunity to unload, rescue services, or even reconditioning materials to ensure temperature maintenance during delays are all mitigation strategies that can be implemented. Procedures to follow for these contingencies should be written, and personnel should be trained on them.

**Storage and transportation:** During storage and transportation, two approaches can be used to keep the product within its required labeled specifications:

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1. Controlling the environmental conditions within equipment, storage rooms, and transportation vehicles; and when applicable, using thermostatically controlled devices such as a heating, ventilation, and air conditioning (HVAC) system or refrigerators
2. Using packaging materials that allow for the control of environmental conditions (e.g., passive/thermal packaging, thermal blankets, temperature stabilizers, desiccants, and light-resistant material)

The organization should have written procedures for qualification of storage, shipping containers, and transportation (in-transit storage) of drug products, taking into account at a minimum:

- Product category (e.g., narcotics, medical devices, temperature-sensitive or hazardous products)
- Layout of the area [e.g., floor-standing pallets, pallet racking, and boxes inside refrigerators where practical and applicable (not feasible for air freight)]
- Volume of stored product (including peaks of storage)
- Air circulation and environmental conditions (e.g., temperature, relative humidity, pressure, shock, and vibration)
- Contingency plan for outages and employee breaks

The procedure should be written based on a risk assessment of factors that can impact product quality during storage and transportation. This procedure will be a measure to mitigate this risk.

If there are problems with vehicles during the transportation process (e.g., breakdowns, accidents, and loss of fuel), a product should be protected against environmental factors, theft, and diversion. The risk assessment and written procedures should take these situations into consideration. Depending on the probability of occurrence and the level of risk, an organization may consider backup systems or access to backup systems in the event of logistics disruption (e.g., severe weather).

**Recalls:** All supply chain partners are responsible for maintaining the quality and integrity of products under their control. Anytime a deviation is found that likely affects the safety or efficacy of a marketed product, an appropriate action should be taken, including a potential recall. A challenge involved in recalling a product is ensuring that the amount of product that was initially distributed is returned. Thus, sharing information on product recall increases the efficacy of the recall procedure, provides transparency to supply chain partners, and mitigates the risk of reintroducing a recalled product into the supply chain. The organization should have a written procedure that establishes the steps for recalling products and the control of recalled products, such as product identification and segregation. The extent of the recall needs to correspond to the level of risk. The extent of the recall may change if the risk is re-evaluated.

**Returns:** Accepting a returned product for restocking poses the risk that the product may not be authentic or its quality may have decreased. A risk-based evaluation should be performed to determine if the product will be acceptable for restocking and resale or if it needs to be destroyed. During the evaluations, returned products should be kept in a segregated area designated specifically for returns until final disposition. A written procedure for handling returns should be in place, taking into account:

- Reasons for return
- Appearance and integrity of the original packaging
- Evidence of conditions in which the cargo was transported and stored throughout the entire time span
- Duration of time between the original shipment and its return
- Authenticity of the product, to include product identifier verification covered by applicable traceability and serialization laws and evidence of proper storage while in possession of the entity returning the product (e.g., ongoing assurance)
- Representative sampling for quality control analysis (if applicable and following regulation)
- Expiry date and batch number
- Batch trace history of excursion
- Information from any track-and-trace system in place

**Supplier qualification (logistics service provider, third party logistics provider, material suppliers, maintenance providers, etc.):** Supplier qualification is a process in which the organization assesses its suppliers regarding their licenses, authorizations, and compliance with regulatory requirements for the distribution of drug products. The organization should establish a written procedure for how suppliers are selected and evaluated, including the criteria for qualification and the period for requalification on a risk-based approach.

### 4.1.3 LABELS

Labels are fundamental to material identification. For this reason, any label change should be communicated to downstream supply chain partners. Labels applied, even to small containers, should be clear, indelible, unambiguous, and permanently fixed in the format established by the manufacturer, packager, or repackager. The shipping label should include wording or icons to emphasize storage and transportation conditions, handling requirements, and hazards. The use of symbols that are recognized by international organizations is strongly recommended. ▲▲ (USP 1-Dec-2023)

### 4.1.4 QUALITY AGREEMENTS

Written agreements (e.g., quality agreement, technical agreement, service level agreement) should be in place between applicable organizations involved in the supply chain. Each supply chain partner should ensure that its respective service level agreements and supporting documents cover delivery and receiving responsibilities. The use of written agreements ensures clarity and transparency, while delineating the responsibilities of each organization in the supply chain.

### 4.1.5 EXCURSION HANDLING

Short-term temperature excursions can occur during distribution, storage, and transportation (see *Packaging and Storage Requirements* ⟨659⟩). Each excursion should be documented and handled with a deviation or appropriate risk assessment. Product disposition should be established on the basis of an assessment of the excursion (i.e., the temperature to which the

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material or product was exposed, and for how long), the stability data obtained from traditional stability studies (under accelerated, intermediate, if appropriate, and long-term conditions and performed in accordance with ICH guidelines), and distribution stability studies (e.g., extremes of temperature, thermal cycling, and freeze–thaw studies, as appropriate). Combining stability data from long-term and accelerated studies with mean kinetic temperature (MKT), temperature-excursion, and thermal-cycling studies should provide the information necessary to evaluate the effects of excursions. Excursions out of temperature range defined by thermostability data or ⟨659⟩ should be addressed/corrected in order to prevent recurrence using a risk-based approach. Downstream handlers of finished drug products may rely on the manufacturer's product disposition instructions. See ⟨659⟩ for excursion allowances and MKT limits and *Mean Kinetic Temperature in the Evaluation of Temperature Excursions During Storage and Transportation of Drug Products* ⟨1079.2⟩ for MKT. MKT should be calculated for the period of time that a drug is in residence at a warehouse and/or in transit on a truck to avoid the problem of diluting the impact of excursions by calculating annual MKT values.[1, 2]

## 4.2 Training

Training is a teaching-learning process used in the workplace to provide knowledge and develop skills and behaviors. The objective of training is to reduce the gap between the existing competencies and those required for performing the work. Training comprises knowledge of the topic to be taught and the types of training practices such as class-like, on-the-job training, web-based, blind sample analysis, mocks, and simulations. The type of training can influence the effectiveness of that training.

A written training procedure is necessary to establish who can be the trainer, how training needs are identified, and how training effectiveness will be evaluated. Selection of trainers must adhere to applicable laws as appropriate. Training needs could be identified and linked to job descriptions, complexity of duties, types of product handled, management reviews, and any kind of human resources program for competence development.

SOPs are the foundation of a quality system, and training must be provided for each SOP to the appropriate job roles responsible for executing the processes outlined in each SOP. Frequency of training and when training or retraining should occur should also be outlined in the overarching training SOP. Training records should be maintained either as hard copies or electronically.

The effectiveness of training should be considered and evaluated to determine its impact on task execution and quality. Evaluation of training effectiveness is not necessarily a separate document, but it may include a review of written or performance tests, observation, error rates, non-conformance or CAPAs, customer complaints, and internal and external audits. Any identified training effectiveness gaps should be corrected and evaluated. This may include retraining or evaluation and modification of SOPs, training materials, training method, or the instructor.

As a risk mitigation strategy, training should be performed before the SOP becomes effective. All employees whose actions have an impact on product quality and security should have initial and ongoing training based on an approved schedule. Basic training should be provided regarding risks and mitigation for storage and transportation of drug products so that employees will better understand how individual and collective actions impact product quality, and so they will develop awareness and a risk-based mindset.

## 4.3 Resources for Storage, Transportation, and Personnel

In the context of this chapter, resources are warehouses, vehicles, and organizational personnel. Facilities, equipment, and transportation vehicles are emphasized as systems that function to control environmental conditions in accordance with product specifications. Evaluation of facilities, equipment, packaging, and transportation systems should be part of the quality system of the organization. Sampling family or type of equipment/vehicles (e.g., shipping containers or trailers) should be part of the risk assessment, when evaluation of individual systems is not feasible. Additionally, trading partner facilities/vehicles can be evaluated by risk by family or type, which may include sample lane mapping to evaluate the exposure of product through the transportation system.

### 4.3.1 STORAGE

The facility should be designed to maintain the quality and integrity of the stored drug product. Buildings should be constructed in such a way that they are appropriate for the intended operations, taking into account:

- Security
- Product characteristics (e.g., narcotics, radiopharmaceuticals, fire/explosion risk)
- Product status (e.g., approved, recalled, returned, rejected, quarantined, falsified)
- Product required storage temperature
- Ease of cleaning and maintenance
- Logical flow of personnel and materials
- Means of preventing mix-ups and cross-contamination
- Ergonomic measures
- Product demand in order to prevent capacity constraints
- Any local, national, or international requirements
- Necessary environmental controls

[1] Seevers RH, Hofer J, Harber P, Ulrich DA, Bishara R. The use of mean kinetic temperature (MKT) in the handling, storage and distribution of temperature sensitive pharmaceuticals. *Pharmaceutical Outsourcing*. May/June 2009;12–17.

[2] Anderson C, Seevers R, Hunt D. The use of mean kinetic temperature to aid evaluation of temperature excursions: proper and improper application. *Pharm Forum*. 2018;44(4).

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Product segregation and proper identification can reduce the risk of mixing up products with different statuses, such as quarantined (rejected, expired, recalled, returned, or falsified) and released/salable. Products with special handling authorization, such as narcotics, should be segregated and locked in a secure area per applicable regulations. Radiopharmaceuticals should be contained in dedicated, locked storage areas. Hazardous products should be managed per applicable regulations for each supply chain partner. Other products that require special storage conditions such as hazardous products (see *Hazardous Drugs—Handling in Healthcare Settings* ⟨800⟩) should also be segregated per applicable safety standards. Any system used to replace physical segregation should offer the same level of security and protection.

Buildings and facilities used for the warehousing, storage, and/or holding of drug products should be of adequate size for their intended use to prevent product overcrowding. The building and facility should be designed to control environmental conditions, where necessary, and should be made of materials that are readily or easily cleaned. Sanitation and pest control procedures should be written, indicating the frequency of cleaning and the materials and methods to be used. The pest control program should prevent contamination and ensure the safe use of pesticides. Records of all cleaning and pest control activities should be maintained.

Storage facilities themselves, unless thermostatically controlled, cannot be validated. However, they can be qualified via a mapping process, with appropriate attention to geography and seasons. The generator backup power supply should be qualified.

Drug product storage areas are required to maintain the product temperature between the limits defined on the product label (see ⟨659⟩). Product storage areas/units should utilize recording systems to log and track temperatures. Alarm systems should be an integral part of the monitoring system for product temperatures. Although automated systems monitor units continuously, manual checks should be performed as appropriate to ensure functionality. When automated systems are not available, manual systems may be used. A risk-based approach should be applied when using a manual system.

Controlled access to warehouses and vehicles is a measure used to prevent unauthorized personnel from coming into contact with the product. Access control can be accomplished with automated systems or by procedure. Adequate precautions should be taken to prevent theft and diversion of products.

#### 4.3.2 TRANSPORTATION

All vehicles used in supply chain activities—such as trucks, vans, trains, airplanes, sea vessels, mail delivery vehicles, and emergency medical services vehicles—should be suitable for the intended purpose because they are providing in-transit storage and should prevent exposure to conditions that affect stability and package integrity. Thus, all of the precautions needed to maintain product quality, integrity, and security should be taken. Risk identification and mitigation strategies should be applied to determine whether the transportation method adequately protects the product from environmental exposures such as temperature and vibration without the need for additional packaging, or if additional packaging is necessary to mitigate the risk.

#### 4.3.3 PERSONNEL

Organizations should hire personnel and contractors who meet the requirements for handling drugs safely and securely per applicable laws and regulations. Job descriptions and individual profiles should be reviewed to ensure that all experience and training requirements are met and maintained.

### 4.4 Qualification and Validation

Qualification and validation for storage and transportation of drugs focus on the assurance that the storage and transportation methods meet predetermined criteria and that processes and procedures produce the desired outcome. Calibration is necessary for instruments used in qualification studies. Organizations need to include appropriate qualification, validation, and calibration activities in their master plan and SOPs, as well as schedules and the use of approved protocols to conduct these activities and produce final reports. The sections below provide brief explanations of calibration, qualification, and validation, focusing on storage and transportation. The scope for each stakeholder within the supply chain is described in *Table 3*.

#### 4.4.1 CALIBRATION OF INSTRUMENT OR DEVICE

Calibration ensures that measurements such as temperature and humidity meet recognized standards. Calibration frequency may be determined by the device manufacturer, device workload, operational demands, and/or damage that require repairs. Instruments or devices used for qualification or monitoring of set criteria must be calibrated to recognized standards such as those from the National Institute of Standards and Technology (NIST) and those found in *Monitoring Devices—Time, Temperature, and Humidity* ▲⟨1079.3⟩▲ (CN 1-Dec-2023).

**Table 3. Calibration, Qualification, and Validation Activities of Organizations**

| Calibration, Qualification, and Validation Activities | Role of Organization within Supply Chain | | | | | |
|---|---|---|---|---|---|---|
| | Manufacturer | Wholesaler and Distributor | Pharmacy and Compounding Pharmacy | Hospital and Healthcare Provider | Broker | Logistics Service Provider (LSP) |
| **Calibration** | | | | | | |
| Temperature recording devices used for temperature mapping or monitoring during storage and transportation | Yes | Yes | Yes | Yes | No | Yes |
| **Qualification** | | | | | | |

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**Table 3. Calibration, Qualification, and Validation Activities of Organizations** *(continued)*

| Calibration, Qualification, and Validation Activities | Role of Organization within Supply Chain | | | | | |
|---|---|---|---|---|---|---|
| | Manufacturer | Wholesaler and Distributor | Pharmacy and Compounding Pharmacy | Hospital and Healthcare Provider | Broker | Logistics Service Provider (LSP) |
| **Calibration** | | | | | | |
| Temperature mapping of warehouse and equipment (freezers, refrigerators) | Yes | Yes | Yes | Yes | No | Yes |
| Shipping packaging performance qualification | Yes | Yes | Yes | Yes | No | No |
| Transportation vehicle performance qualification | Yes | Yes | Yes | Yes | Yes | Yes |
| **Validation** | | | | | | |
| Software validation for systems that make quality decisions | Yes | Yes | Yes | Yes | Yes | Yes |

### 4.4.2 QUALIFICATION

For the purposes of this chapter, performance qualification is defined as all tests designed and executed to evaluate whether the storage rooms and areas, warehouse facilities, utilities, equipment, transport vehicles, and shipping containers are suitable for their intended purpose. Qualification studies should reflect actual load configurations and environmental conditions. Testing should be performed on both active and passive thermal packaging systems.

During the qualification process, temperature mapping is performed to provide information relative to temperature consistency throughout a product area. This information may confirm a uniform temperature over the mapped area or it may identify areas that need mitigation. Such mitigation may include the movement or installation of insulation, HVAC units, or fans, or the need to modify the relevant SOPs. The identification, documentation, and rationale for the mapping procedure are the foundation of temperature mapping of any temperature-controlled space (e.g., facility, vehicle, shipping containers, refrigerator, and freezer). Temperature variability in mapped locations and the level of thermal risk to the product should both be defined, unless another process to ensure environmental control has been put in place (see ▲ ⟨1079.3⟩▲ (CN 1-Dec-2023)).

**Temperature mapping in facilities and equipment:** The following factors, which may contribute to temperature variability in a facility, should be considered during the process of temperature mapping for storage locations: 1) size of the space; 2) location of HVAC equipment, space heaters, and air conditioners; 3) sun-facing walls; 4) low ceilings or roofs; 5) geographic location of the area being mapped; 6) airflow inside the storage location; 7) temperature variability outside the storage location; 8) workflow variation and movement of equipment (weekday vs. weekend); 9) loading or storage patterns of product; 10) equipment capabilities (e.g., defrost mode, cycle mode); and 11) SOPs. The duration of temperature recordings during the thermal mapping of a warehouse or cold room should capture workflow variation that may impact airflow and the resulting temperature fluctuation; for example, this process could last from 1 day to 1 week, depending on the workflow cycle.

**Temperature mapping for shipping packaging and vehicles:** Pharmaceutical manufacturers should consider primary, secondary, and tertiary packaging that best protects the drug product during storage and distribution. Shipping package performance testing should be documented as part of a QMS. Several standard test procedures are available to evaluate package performance for factors such as shock, vibration, pressure, compression, and other transit events. (See *Additional Sources of Information* for standards for test methods.)

Packaging at the tertiary level (e.g., outer, external, or shipping package) or thereafter for the distribution of the drug product should be selected and tested to ensure that product quality is maintained and to protect the contents from the rigors of distribution, including environmental or physical damage. Active, passive, or semi-active shippers and transport systems are typically subjected to operation performance qualification by the manufacturers or suppliers of such equipment.

Thermal packaging and vehicles used for transporting the drug product for pharmaceutical manufacturers, wholesalers, and pharmacies should be evaluated based on the labeled storage or transport conditions of the product as well as anticipated environmental conditions. Special consideration should be made for seasonal temperature differences, transportation between hemispheres, and the routes and modes of transport.

Identification of risks and mitigation strategies to protect the product should be based on documented studies of specific distribution environments, including domestic and international lanes (as appropriate), mode(s) of transport, duration, temperature, and other potential environmental exposures or sensitivities that may impact product quality. If risks to product integrity have been identified by using historical data, observation of current practices, or operational changes, mitigation strategies may be employed to address the identified risks. Strategies include temperature-controlled vehicles, active or passive thermal containers or packaging, or ambient conditions based upon identified risks (product storage labeling, time, temperature, and geography).

Temperature monitors/indicators may include calibrated monitoring or recording devices, real-time monitors such as GPS, and chemical indicators of temperature. Monitoring devices may include an alert mechanism if the preset ranges are breached (see ▲ ⟨1079.3⟩▲ (CN 1-Dec-2023)).

Thermal packaging and vehicles may be qualified based on historical data that are relevant to the process. If qualified thermal packaging is used without a temperature verification method (monitors or indicators), a plan should be in place to schedule transport within qualifying times and to mitigate and respond to exceptions. However, it may be acceptable to use product stability data from manufacturers and supply chain risk assessment to justify shipping without either continuous monitoring or qualification of the shipping system.

Operational and performance shipping studies should be part of a formal qualification protocol that may use controlled environments or actual field testing, depending on the projected transport channel. These studies should reflect actual load

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configuration conditions and expected environmental extremes. Testing should be performed on both active and passive thermal packaging systems.

When developing a thermal package qualification protocol, the following factors and actions should be considered:

- Transportation temperature profile for the shipping lane(s)
- Delivery cycle time, accounting for reasonable delays due to weather, traffic, or customs
- Testing beyond qualification time to obtain worst-case data for excursion dispositioning
- Seasonal changes in the environment
- Use of seasonal (warm/summer and cool/winter) configurations versus universal configuration
- Testing with actual payload, worst case configuration, or a representative sample
- Variable order sizes, making it difficult to select a representative sample; testing for the minimum and maximum payload possible in each package may be necessary
- Probe placement should be inside or directly attached to the product (or representative samples) or in the most vulnerable temperature locations within the package; scientific justification should be given for the differences between monitoring for qualification and operation
- Perform at least 3 replicate tests on a representative package containing a representative thermal payload per season; tests may be performed at the same time in an environmental chamber. ▲If a qualified environmental chamber is used for the test, it is only necessary to perform one test on a representative package containing a representative thermal payload per season.▲ (USP 1-Dec-2023)
- Allow the product payload as well as coolant to condition before beginning testing per protocol
- Utilize a recognized standard to conduct thermal qualification (see *Additional Sources of Information*) or develop a written rationale for a qualification

### 4.4.3 VALIDATION OF INFORMATION SYSTEMS

In the context of this chapter, before an information system that can impact the quality of the product in storage or in transportation is brought into use, it should be demonstrated through appropriate validation or verification studies that the system is capable of achieving the desired results accurately, consistently, and reproducibly. (See Pharmaceutical Inspection Convention in *Additional Sources of Information*). The following are considerations for using validation as a mitigation strategy. These considerations do not suggest replacing or enforcing any existing regulatory standards in place for various members of the supply chain [e.g., current good manufacturing practices (cGMP) for manufacturers, wholesale distributors, or pharmacies].

Prior to their use, information systems should be tested according to approved protocols. The extent of the study depends on the risk or impact the software can have on product or service quality. Validation or verification is not applied to systems that have no impact on quality. An inventory of information systems should be done periodically and include at least:

- Information system identification (name, version)
- System supplier
- Processes where system is used
- Process owner
- Risk assessment
- Status (validated, not validated, validation in progress, verified, not verified, verification in progress, not applicable)

A multidisciplinary team with representatives from information technology, quality, and operations should be responsible for protocols and report approvals. Responsibilities for the tests should be assigned in the protocols. Validation or verification tests should cover the following:

- Security (e.g., access levels, profiles, responsibilities inclusion, exclusion, or changing profiles)
- Data validity (e.g., challenge the software with entries above and below specification and with entry value errors)
- Documentation (e.g., system design in accordance with user requirements and other documents)
- Functionality (e.g., calculations, operations) [NOTE— Most of the functionality tests for embedded software are covered during equipment qualification (installation, operation, and performance qualifications]
- Data integrity (e.g., changes, traceability, backup, recovery, and protection)

After validation or verification studies, any modification to the system should be done according to change control procedures, and records of these changes should be kept.

## GLOSSARY

**Calibration:** A process that typically focuses on instruments or devices to provide assurance that they produce results within specified limits. Organizations need to include the appropriate qualification, validation, and calibration activities in their SOPs and master schedules and should follow protocols to conduct these activities and final reports.

**Qualification:** Qualification is the assurance that systems or equipment meet predetermined acceptance criteria. This process typically focuses on equipment and utilities such as refrigerators and HVAC systems, as well as packaging. There are several different types of qualification, and an organization should determine which to use and when. Some of these include design qualification, installation qualification, operational qualification, and performance qualification.

**Service level agreement** (SLA)**:** An SLA or contract is a negotiated agreement between the customer and service provider that defines the common understanding about materials or service quality specifications, responsibilities, guarantees, and communication mechanisms. It can either be a legally binding document or an information agreement. The SLA may also specify the target and minimum-level performance, operation, or other service attributes.

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**Temperature excursion:** An event in which a pharmaceutical product is exposed to temperatures outside of the range(s) prescribed for storage and/or transport. Temperature ranges for storage and transport may be the same or different; they are determined by the product manufacturer, based on stability data.

**Validation:** Validation typically focuses on processes and procedures, to provide assurance that the processes or equipment produce the desired outcome.

## ADDITIONAL SOURCES OF INFORMATION

American Society for Testing and Materials International. ASTM D3103-14. Standard Test Method for Thermal Insulation Performance of Distribution Packages.

American Society for Testing and Materials International. ASTM D4169-16. Standard Practice for Performance Testing of Shipping Containers and Systems.

American Society for Testing and Materials International. ASTM D4332-14. Standard Practice for Conditioning Containers, Packages, or Packaging Components for Testing.

Health Canada. National Guidelines for Vaccine Storage and Transportation. In: *Canada Communicable Disease Report*. June 15, 1995; Vol 21-11:93–97.

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World Health Organization. Supplement 8—Temperature mapping of storage areas (Technical supplement to WHO Technical Report Series, No. 961, 2011); 2015.

World Health Organization. Supplement 11—Qualification of refrigerated road vehicles (Technical supplement to WHO Technical Report Series, No. 961, 2011); 2015.

World Health Organization. Supplement 12—Temperature-controlled transport operations by road and by air (Technical supplement to WHO Technical Report Series, No. 961, 2011); 2015.

World Health Organization. Supplement 13—Qualification of shipping containers (Technical supplement to WHO Technical Report Series, No. 961, 2011); 2015.

World Health Organization. Supplement 14—Transport route profiling qualification (Technical supplement to WHO Technical Report Series, No. 961, 2011); 2015.

World Health Organization. WHO good distribution practices for pharmaceutical products (WHO Technical Report Series, No. 957, Annex 5); 2010.

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# ⟨1080⟩ BULK PHARMACEUTICAL EXCIPIENTS—CERTIFICATE OF ANALYSIS

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## 1. INTRODUCTION

### 1.1 Purpose

This general information chapter is derived from the *Certificate of Analysis Guide for Bulk Pharmaceutical Excipients*, prepared by the International Pharmaceutical Excipients Council of the Americas (IPEC-Americas) and is meant to serve as a guide for the preparation and appropriate use of a Certificate of Analysis (COA) for pharmaceutical excipients. The goals are to standardize the content and suggest a format for COAs for excipients, and to clearly define the roles and responsibilities for the excipient manufacturer and distributor. The detailed definitions and discussions are intended to establish a uniform approach. By providing this foundation for mutual understanding, the COA will serve as an important element of the overall supply chain controls needed to provide the user with assurance of excipient conformance to specification and its suitability for use in pharmaceuticals.

The chapter is divided into several parts. The first part provides background discussion necessary for the design and required elements of a COA. The section *4. COA Content* is provided to show the format and placement of information in the COA. This is followed by a detailed discussion to ensure that the purpose and meaning of the specific information contained in the COA is understood. For a list of terms used in this informational chapter and their definitions, see the *Glossary*.

### 1.2 Scope

This chapter is applicable to excipients used in the manufacture of pharmaceutical products.

### 1.3 Principles Adopted

As an international guide, this informational chapter cannot specify all national legal requirements or cover in detail the particular characteristics of every excipient. When considering the use of this chapter, manufacturers, distributors, and users should consider how it may apply to that specific organization's product. The diversity of excipients means that some principles

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of the chapter may not be applicable to certain products and processes. The terminology “should” and “it is recommended” do not necessarily mean “must” in the application of this chapter.

## 2. GENERAL GUIDANCE

The COA is a legal document that certifies the quality of the excipient and demonstrates that the batch conforms to the defined specifications, has been manufactured under recognized principles of good manufacturing practices (GMPs), and is suitable for use in pharmaceuticals. It should not be used in lieu of appropriate qualification of the supplier (*1*).

A COA for excipients should be prepared and issued by the company responsible for the material, following the general guidelines discussed below. It is expected that a complete and accurate COA is provided to the user for each batch and/or delivery of excipient. When analysis is performed by a distributor, the distributor should issue a COA to the user for any analysis that was performed by or on behalf of the distributor. In such cases, industry best practice is for the distributor to provide the user with the original manufacturer’s COA and the distributor’s COA.

Identification testing by the excipient manufacturer is not a regulatory requirement. The excipient manufacturer is not required to perform identity tests if they have process controls in place that together with testing ensure the identity of the excipient.

## 3. DESIGN AND REQUIRED ELEMENTS OF A COA

The elements of a COA listed below are included in *4. COA Content*. The excipient supplier may organize the elements on the COA at their discretion; however, the sections in this chapter have been designed to present the required and optional information in a logical manner.

The original manufacturer and manufacturing site should be identified if different from the supplier and supplier location. The intent is to enable the user to ensure that a change in manufacturing location has not occurred without their knowledge. It is essential that the manufacturer be known to the user. To protect confidentiality through the supply chain, the use of codes for manufacturers and manufacturing sites on the COA is acceptable as long as the user can link the code to the manufacturer and site of manufacture. The identity of the excipient should be definitively established by stating the compendial name and trade name (if any), the grade of the material, and the applicable compendial designations on the COA.

A batch number or other means of uniquely identifying the material quantity covered by the COA, and information relating specifically to it, are typically included in a body section (see *4.2 Body*). Unique identification of the excipient links the COA to the relevant specification and is traceable to a specified batch. The date of manufacture and, if applicable, the expiration date, recommended retest date, or other relevant statement regarding the stability of the excipient is typically included in this section (a detailed discussion of dates on the COA is contained in *6. Establishing Dates on a COA*). User-required information could also be included here.

The actual test results applicable to the quantity of material covered by the COA are included in *Appendix 1: Model COA*. Preferably, the acceptance criteria and test results are included for each characteristic listed. Test method designation and acceptance criteria may be communicated to the customer by reference to other controlled documents, e.g., sales specifications. Reporting of actual data and observations is recommended rather than nonspecific “passes” or “conforms” statements, unless the test is qualitative or this is the acceptance criteria as listed in a compendium or other specification.

If the reported results are not derived from sampling the finished excipient batch, it should be noted on the analysis section of the COA (see *7.2 Data versus Conformance* for a detailed discussion of such considerations). In such cases, alternative options for the origin of test results other than quality control laboratory testing include, for example (*2*):

- In-process testing
- Continuous monitoring of an attribute or variable and application of appropriate statistical process control (SPC) methods

It may be acceptable not to perform a test when the test attribute cannot be present or cannot fail to meet acceptance criteria, e.g., limited by upstream controls that involve measurement of an impurity to ensure it does not enter or form in the process. Not performing a specified test should be supported by a suitable documented rationale based on a documented risk assessment.

The section *4.3 Certification and Compliance Statements* is used to list various statements that may be required, depending on the excipient and agreed to user requirements. Any declaration by the supplier as to compliance with compendial and/or other regulatory requirements is typically included in this section.

The basis for COA approval should appear on the COA (see *8. Use of Electronically Generated COAs*).

## 4. COA CONTENT

The following information should appear on the COA or by reference. It is important that all pages of the COA are numbered and include the total number of pages for document control, to assure the customer that all pages of the COA are present. See *Appendix 1: Model COA* for an example.

### 4.1 Identifying Information

- Title “Certificate of Analysis”
- Identity and address of original manufacturing site: name or other suitable identifier that is unique to the manufacturer and site (e.g., code)

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- Responsible organization that issues the COA, address, and contact information (if different from the original manufacturer)
- Name (compendial or chemical) and compendial designation, as applicable
- Grade
- Trade name
- Batch number

### 4.2 Body

- Date of manufacture
- Unique identifier to the excipient specification
- Expiration or retest date (as applicable) or stability statement
- Specification
  - Test name
  - Reference to the test method
  - Acceptance criteria
- Analysis
  - Test results based on the finished excipient sample, or
  - Alternative test results, as appropriate (see *7.3 Alternatives to Excipient Testing*)
  - Date retested (if appropriate)

### 4.3 Certification and Compliance Statements

- Standard of GMP applied (e.g., IPEC-PQG Excipient, ICH Q7, NSF/IPEC/ANSI 363, EXCiPACT™)
- Additional compliance statements and applicable references to standards
- Potential to meet additional compendial standards
- Content listing and grade of ingredients (if a mixture)
- Customer specified information

### 4.4 Authorization

- Identity of authorized individual for approval or electronic signature statement
- Date of approval or suitable alternative
- Page number (i.e., 1 of X pages)

## 5. REQUIREMENTS FOR COMPENDIAL DESIGNATION

For a supplier to claim a compendial grade on the COA for an excipient, there are two requirements to be met. The first requirement is that the excipient is manufactured according to recognized principles of GMPs (see *General Notices, 3.10 Applicability of Standards*). The second requirement is that the excipient meets all of the acceptance criteria contained in the appropriate compendial monograph, unless its difference is stated on its label, as defined under *General Notices, 3.20 Indicating Conformance*. These expectations remain in effect until its expiration or recommended retest date, when stored according to the manufacturer's recommendations in the manufacturer's original unopened container.

Every compendial article must be so constituted that when examined in accordance with these assay and test procedures, it meets all the requirements in the monograph defining it, as well as meeting any provisions of the *General Notices*, general chapters, or rules, as applicable. However, it is not to be inferred that application of every analytical procedure in the monograph to samples from every production batch is necessarily a prerequisite for assuring compliance with compendial standards before the batch is released for distribution. Data derived from in-process testing or continuous monitoring of an attribute with SPC may be used. With appropriate scientific justification, analytical methods that are equivalent or better (i.e., more accurate, more precise, etc.) to that which appears in the monograph may be substituted by the supplier when judging compliance of the batch with the compendial standards (see *7. Reporting of Data*).

## 6. ESTABLISHING DATES ON A COA

### 6.1 General Guidance

In reporting dates on COAs for excipients, it is important that a clear and unambiguous format be used to prevent possible misinterpretation. To accomplish this, it is recommended that the name of the month be used to designate the month (it may be abbreviated), rather than a numerical representation. It is also recommended that the year include all four digits (i.e., Jan. 1, 2010, or 1 Jan. 2010, etc.).

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## 6.2 Date of Manufacture

The date of manufacture should be clearly defined by the original manufacturer and consistently applied for the particular excipient and process based on established policies and procedures.

It is important to note that while repackaging operations are to conform to GMP requirements, repackaging alone is not considered a processing step that can be used in determining the date of manufacture. To provide traceability for a specific excipient batch, other dates may be required in addition to the date of manufacture, to reflect additional steps such as repackaging.

## 6.3 Expiration Date and Recommended Retest Date

The stability of excipients may be an important factor in the stability of the finished pharmaceutical dosage forms that contain them. Therefore, it is important that the COA indicates stability of the excipient either by reporting the expiration date and/or the recommended retest date. This provides users with key information concerning the usability of the excipient in the period between the date of manufacture and the use of the excipient by the user.

Appropriate expiration and/or recommended retest dates for excipients should be established from the results of a documented stability-testing program or from documented historical data. Expiration and recommended retest dates should not be reported by a supplier without sufficient stability data or product history to support the assigned dates. Where the excipient is repackaged, the effect of this operation and the new packaging materials on the expiry or retest date should be evaluated to determine if such dates need to be changed.

The expiration date of an excipient cannot be extended. The retest date for an excipient is the date indicated by the supplier after which the excipient should be reevaluated to ensure continued compliance with appropriate specifications. An excipient retest date may be extended based upon appropriate testing. The reevaluation of the excipient may include physical inspection and/or appropriate chemical, physical, or microbiological testing.

It is acceptable to report both an expiration date and a recommended retest date on the COA for excipients, if applicable.

If stability data (*3*) are not available for an excipient, then an appropriate statement should be included on or with the COA to indicate what is known about the stability of the material, and/or whether stability studies are in progress.

## 6.4 Date Retested

If retesting is performed by an excipient supplier (as noted in *6.3 Expiration Date and Recommended Retest Date*), and the results are used by the supplier to extend the length of time that the material may be used, then the date retested should also be reported, preferably on the COA, but alternative communication means are acceptable. The specific tests that were subject to retesting should be clearly identified, and the results obtained upon retesting should be reported. After retesting, a new recommended retest date should be reported on the COA.

## 6.5 Additional Dates

Other dates may appear on a COA, if desired by the excipient supplier or requested by the user. Examples include the release date, shipping date, date of testing, and date the COA was printed or approved. Any additional dates that appear on a COA for excipients should include a clear indication of what the date represents.

***Change to read:***

# 7. REPORTING OF DATA

## 7.1 General Guidance

For the excipients listed in *USP–NF*, the product specifications are set by the supplier to include all attributes listed in the monograph. It is not required that analysis of all specification attributes be made on each lot. However, sufficient analysis and evidence of process stability should exist to ensure that the batch meets all specifications before it is released (see *7.3 Alternatives to Excipient Testing*). Periodic testing of all attributes should be performed to confirm continuing compliance. All the attributes should be verified at an appropriate frequency.

*General Notices, 6.30. Alternative and Harmonized Methods and Procedures* allows the use of alternative methods and/or procedures if they provide advantages in terms of accuracy, sensitivity, precision, selectivity, and in some other aspects of the procedure performance and handling, and give equivalent or better results.

For excipients that are not included in *USP–NF*, specifications should be set by the supplier to ensure that the quality of the material is maintained on a continuing basis and reflects both the inherent properties of the excipient and its manufacturing process. Specification methods should be demonstrated to provide accurate, reproducible, and repeatable results for the characteristic(s) being tested.

## 7.2 Data versus Conformance

Finished excipient tests are often performed on bulk excipient after all manufacturing processes are complete but prior to packaging. “Where an in-process or bulk excipient test result is traceable to the finished excipient material, such a test result can be reported on the COA” (*2*). When a compendial or specification test is not performed on the excipient batch, in-process,

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bulk, or package, this should be indicated on the COA. Typical statements or information in lieu of data may include the following:

- "Conforms"
- "If tested will meet compendial requirements"
- A footnote to indicate the last measurement or other suitable practice

Measurements reported on a COA can be derived from:

1. Testing a representative sample from the finished excipient batch
2. In-process testing of a representative sample where the attribute remains unaffected by further routine processing
3. Continuous monitoring of an attribute in combination with SPCs

Where number 2 or number 3 apply, the technique for how the test result was obtained should be described.

Some attributes [e.g., bovine spongiform encephalopathy (BSE)/transmissible spongiform encephalopathy (TSE)], *Residual Solvents* ⟨467⟩, *Elemental Impurities—Limits* ⟨232⟩, and/or genetically modified organism (GMO) statements may not be reported on the COA and may be provided separately.

## 7.3 Alternatives to Excipient Testing

For excipients used in drugs sold in the United States, if an excipient attribute "has required criteria, there must be some measurement or test of the material in each lot to ensure that the criteria are met. This may be a measurement from a surrogate test, from in-process control data, or from testing or measurement of the finished material in each lot. Conversely, FDA representatives believe that an approach, which allows for skip testing based on a satisfactory product quality history alone is not acceptable from a cGMP standpoint because such an approach does not adequately verify that each lot meets all of its specifications" (*2*).

It is noted that ICH Q6A allows for periodic/skip lot testing of the drug product and drug substance (*4*).

Results from in-process testing can also be used to replace testing on the finished excipient. "To ensure that a lot of excipient material complies with its required properties, it is acceptable to rely on tests or measurements conducted on samples of material taken at an in-process stage of production, provided that the in-process material will not be affected by subsequent processing or holding with respect to the attributes being verified. There should be justification that test results or measurements, or product performance characteristics, do not change from the in-process stage to the finished product" (*2*).

### 7.3.1 DOCUMENTATION

The supplier of an excipient should develop and maintain documentation that outlines the process control systems and validation data that justify the use of alternatives to finished excipient testing. This documentation should also include procedures for handling the impact of significant changes on the testing program (see ▲*Significant Change for Bulk Pharmaceutical Excipients* ⟨1195⟩▲ (CN 1-May-2021)).

### 7.3.2 EXAMPLES

See *Appendix 2: Alternatives to Excipient Testing—Examples*.

## 8. USE OF ELECTRONICALLY GENERATED COAs

COAs issued from computer systems without a handwritten signature are commonplace and are acceptable, provided the appropriate controls are in place. The following considerations should be met:

- Access to the computer system for COA management; entering and editing of data should be limited to authorized personnel
- Authentication by username and password as well as the change of each individual password at an appropriate frequency should be required
- Confirmation of the integrity and accuracy of the information stored in the system and transferred to the printed record should be completed during implementation and then periodically verified thereafter
- Data entered into a computer system from which information is extracted for a COA and changes made thereafter should be accompanied by time- and date-stamped audit trails

With these criteria met, the issuance of electronically generated COAs is acceptable, provided the COA includes contact information.

## 9. DISTRIBUTOR INFORMATION

Distributors provide excipients and associated services such as:

- Make excipient available in the manufacturer's unopened original package (pass through)
- Repackage excipients from bulk quantities
- Purchase excipients for repackaging under a different label

A distributor should not change the original title and data of the COA or other quality documents. Whenever possible, the original manufacturer's documentation should be used, or transcription of data should be verified.

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It is expected that the distributor will have the appropriate level of GMP in place (see *Good Manufacturing Practices for Bulk Pharmaceutical Excipients* ⟨1078⟩).

## GLOSSARY

**Acceptance criteria:** Numerical limits, ranges, or other suitable measures of acceptance for test results.

**Batch (lot):** A specific quantity of material produced in a process or series of processes so that batch can be expected to have uniform character and quality within specified limits. In the case of continuous processes, a batch may correspond to a defined fraction of the production. The batch size can be defined either by a fixed quantity or by the amount produced in a fixed time interval.

**Batch (lot) number:** A unique combination of numbers, letters, and/or symbols that identifies a batch and from which the production and distribution history can be determined.

**Batch process:** A manufacturing process or processing step that produces the excipient from a discrete supply of raw material that is present before the completion of the reaction.

**Certificate of analysis (COA):** A document listing the test methods, specification, and results of testing a representative sample from the batch to be delivered.

**Chemical property:** A quality attribute that is measured by chemical or physicochemical test methods.

**Continuous process or processing:** A process that continually produces the excipient from a continuing supply of raw material.

**Contract facility:** An internal or external facility that provides services to the manufacturer or distributor of an excipient. These can include, but are not limited to, the following: manufacturing facilities, laboratories, repackaging facilities (including labeling), and warehouses.

**Date of manufacture:** A date indicating the completion of the final manufacturing process (as defined by the supplier for the particular excipient and process).

**Date retested:** The date when retesting is performed by an excipient supplier to extend the length of time that the material may be used.

**Distributor:** All parties in the distribution/supply chain starting from the point at which an excipient is transferred outside the control of the original manufacturer's material management system including parties involved in trade and distribution, (re)processors, (re)packagers, transport and warehousing companies, forwarding agents, brokers, traders, and suppliers other than the original manufacturer.

**Excipient:** Substances other than the drug substance that have been appropriately evaluated for safety and are intentionally included in a drug delivery system.

**Expiry (expiration) date:** The date designating the time during which the excipient is expected to remain within specifications and after which it should not be used.

**Impurity:** Any component of an excipient that is not the intended chemical entity but is present as a consequence of the raw materials, excipient manufacturing process, or excipient degradation.

**Original manufacturer or manufacturer:** Person or company manufacturing a material to the stage at which it is designated as a pharmaceutical starting material.

**Packaging:** The container and its components that hold the excipient for storage and transport to the customer.

**Physical property:** A quality attribute that can be measured solely by physical means.

**Process:** The combination of operating steps (including synthesis, isolation, purification, packaging, etc.) that produce the finished excipient.

**Process capability index (*Cp*):** A statistical measurement that can be used to assess whether or not the process is adequate to meet specifications. A state of statistical control can be said to exist if the random variation in test results for a process attribute is such that the calculated process capability is >1.33 (see *Appendix 2: Alternatives to Excipient Testing—Examples* for further definition).

**Process step:** A documented instruction to the pharmaceutical excipient manufacturing personnel directing that an operation be done.

**Processing:** Operations to change product characteristics by mainly physical treatment through, e.g., milling, sieving, distilling, filtration, blending.

**Repackaging:** The action of changing the packaging of the material.

**Retest date:** The date when a specific batch of material must be reexamined to ensure that it is still suitable for use.

**Skip lot (periodic) testing:** The performance of specified tests at release on preselected batches and/or at predetermined intervals, rather than on a batch-to-batch basis, with the understanding that those batches not tested still must meet all the acceptance criteria established for that product. This represents a less than full schedule of testing and therefore should be justified, presented to, and approved by the regulatory authority before implementation. When tested, any failure of the starting material to meet the acceptance criteria established for the skip lot (periodic) test should be handled by proper notification of the appropriate regulatory authority or authorities. If these data demonstrate a need to restore routine testing, then batch-by-batch release testing should be reinstated.

**Significant change:** Any change that has the potential to alter an excipient's physical, chemical, or microbiological property from the norm, and/or that may alter the excipient's performance in the dosage form.

**Site:** A defined location of the equipment in which the excipient is manufactured. It may be within a larger facility. A change in site may be to a different part of the existing facility, but in a different operational area, or to a remote facility including a contract manufacturer.

**Specification:** A list of tests; references to analytical procedures; and pre-established numerical limits, ranges, or other criteria for the tests described for a material, that the material is required to meet.

**Stable process:** A process is considered stable when the output of the process, regardless of the nature of the processing (batch or continuous), can be demonstrated by appropriate means to show a level of variability that consistently meets all aspects of the stated specification (both USP specific and customer specific) and is thus acceptable for its intended use.

**Stability:** Continued conformance of the excipient to its specifications.

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**Supplier:** Person or company providing pharmaceutical starting materials on request. Suppliers may be distributors, manufacturers, traders, etc.

**Supply chain:** All steps in the entire chain of distribution starting from the point at which an excipient is transferred outside the control of the original manufacturer's material, to a management system downstream, to the final user of the excipient.

**User:** A party who utilizes an excipient in the manufacture of a drug product or another excipient.

# APPENDICES

## Appendix 1: Model COA

The following sample COA is provided to illustrate the principles discussed in the guide and is not meant to be prescriptive.

**Certificate of Analysis** (sample tests, limits, and statements are for demonstration purposes)

| | | | |
|---|---|---|---|
| Supplier Company Name | | | |
| Supplier Company Address | | | |
| Manufacturing Location | | | |
| Name of Manufacturer (if different from Supplier) | | Phone: **xxx-xxx-xxxx** | |
| Manufacturing Site Address | | Fax: **xxx-xxx-xxxx** | |
| Product: **Trade Name and Descriptor or Common Name** | | | |
| Grade: **Grade Designation** | | Customer Code: **xxxxxx** (if applicable) | |
| Batch Number: **xxxxxx** | | Date of Manufacture: **dd/mmm/yyyy** | |
| Recommended Retest Date: *<time from date of manufacture>* | | | |
| **Compendial Name and Listing**: *USP–NF*, *Ph.Eur.*, *JP*, or *JPE*<br>(List multiple names and designations if nomenclature is different in each compendium) | | | |
| Test Results (sample tests & limits for demonstration purposes) | | | |
| **Test** | **Test Method** | **Specification** | **Results** |
| Appearance | Visual examination | White granular powder | Complies |
| Foreign matter | Visual examination | Free from visible contamination | Complies |
| Identification—JPE | Tests A–C | Pass | Complies |
| Clarity and color | *JPE* | Clear and colorless | Complies |
| pH (x% solution) | *USP* | 5.0–7.0 | #.# |
| Residue on ignition | *JPE* | NMT 1.0% (450°–550°C) | #.# % |
| Viscosity (x% solution) | *Ph.Eur.* | 4.0–7.0 mPa-s (@20°C) | #.# mPa-s |
| Water insoluble sub. | *USP* | NMT 0.1% | #.# % |
| Loss on drying (110°C) | *USP* | NMT 5.0% | #.# % |
| Loss on drying (105°C) | *JPE* | NMT 6.0% | #.# % |
| Particle size | Supplier method # | 99.5% <150 µm | #### |
| Additional Information (sample tests & limits for demonstration purposes) | | | |
| Heavy metals | *JPE* | NMT 10 ppm (as Pb) | NMT 10 ppm[a] |
| Arsenic | *JPE* | NMT 2 ppm | NMT 2 ppm[b] |

[a] This test is performed in-process on each batch and the material has been shown not to change in the finished excipient sample.
[b] This test is performed quarterly based on process validation.

The following sample COA is provided to illustrate the principles discussed in the guide and is not meant to be prescriptive.

| |
|---|
| Supplier Company Name |
| Supplier Company Address |
| Product: **Trade Name and Descriptor or Common Name** |
| Grade: **Grade Designation** |
| Batch Number: **xxxxxxx** |
| **Certification and Compliance Statements** |
| **GMP compliance:** This batch of *<**Trade Name**>* has been manufactured using excipient GMP. |

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| |
|---|
| **Compendial standards:** This batch of <***Trade Name***> complies with all of the current requirements listed in the *United States Pharmacopeia* (*USP*), the *European Pharmacopoeia* (*Ph.Eur.*), and the *Japanese Pharmaceutical Excipients* (*JPE*). |
| **Other certification statements:** Any other type of certification e.g., Residual Solvents, GMO derived, or customer-specific information, should be listed here. These may vary depending on regional regulatory requirements, specific GMP issues, and customer desired information, based on their use of the excipient. |
| **Identity of authorized individual for approval:** xxxxxxxxxxxxxxxxxxxxxxxx |
| Title |
| **Date of approval:** dd/mmm/yyyy |
| This COA was released from a controlled electronic document management system. |

## Appendix 2: Alternatives to Excipient Testing—Examples

The following are examples of situations where alternatives to finished excipient testing might be justified. These are not the only situations where a sound technical basis can be demonstrated, neither are they examples of situations where alternatives to finished excipient testing will always be appropriate.

- An impurity, byproduct, or unreacted raw material could not be present in the product because the raw materials and chemical reactions used could not contain or generate it above the specified limits.
- The process capability index (*Cp*) for the relevant attribute is high and thus indicates a stable process. Statistical analysis of the reduced frequency data should show that the property remains stable and within specifications. A process is considered stable when the output of the process, regardless of the nature of the processing (batch or continuous), can be demonstrated, by appropriate means, to show a level of variability that consistently meets all aspects of the stated specification (both pharmacopeia and user specific) and is thus acceptable for its intended use. For continuous processing, it is also important to demonstrate that the material has been produced under conditions where the process has achieved a form of "steady state", i.e., minimal operator intervention and the in-process attributes have been stabilized.
- For a continuous process, the in-process analyses show that the property that is determined at a reduced frequency is stable and within specification. Repeating the test on each batch would be redundant.
- An analysis of an attribute that is determined on every batch in process has been shown to provide assurance that the final test requirement can be met. Such data can be used to support testing the finished excipient at reduced frequency.

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***Add the following:***

# ▲⟨1083⟩ SUPPLIER QUALIFICATION

## 1. INTRODUCTION

The manufacturers of pharmaceuticals primarily source their required ingredients, materials, components, and services through external local and global suppliers. External suppliers also provide services such as research and development, manufacturing, assembly, analysis, packaging, warehousing, transportation, and distribution, etc. The expectations on the part of regulatory authorities, healthcare professionals (including medical practitioners, nurses, dentists, and pharmacists), and consumers are that pharmaceutical products will be fitted for their intended use and be of the required quality. This means that active ingredients, excipients, components, other raw materials, and packaging components used in the end product have been manufactured to the appropriate standards and comply with regulations, pharmacopeial monographs, and/or all approved specifications. It also means that the processes in the supply chain for a material or service will also be compliant and do not increase risks to the product.

Reliable suppliers provide benefits through established risk management processes. Supplier qualification is a process to systematically evaluate suppliers based on i) the risk to the quality of the product or service supplied; ii) compliance of their quality systems to applicable regulations and requirements of the supply contract or quality agreement, and iii) the reliability of the supplier to avoid quality deviations and shortages of components and/or products.

This chapter provides a quality risk-based approach on how to select, assess, approve, and monitor suppliers of ingredients, packaging materials, and other components and services.

## 2. SCOPE

This chapter applies to organizations involved in identifying, selecting, assessing, approving, and monitoring suppliers of:

- Materials (e.g., active pharmaceutical ingredients, excipients, other materials, and components)
- Packaging materials (e.g., primary, secondary, and tertiary packaging)
- Service providers (e.g., contract manufacturing, packaging, and repackaging; logistic providers for warehousing and transportation; software; calibration and qualification services; and analytical services)

The principles discussed in this chapter apply to the different types of suppliers for pharmaceutical manufacturing companies and compounding pharmacies, to establish supplier reliability in providing appropriate materials and services. In this context, the fundamental principles of supplier qualification are the same for all such organizations. However, the details depend on the type of material or service, the stage of development, and the intended use of the product. This general chapter will focus on the high-level elements of supplier qualification that apply to all organizations.

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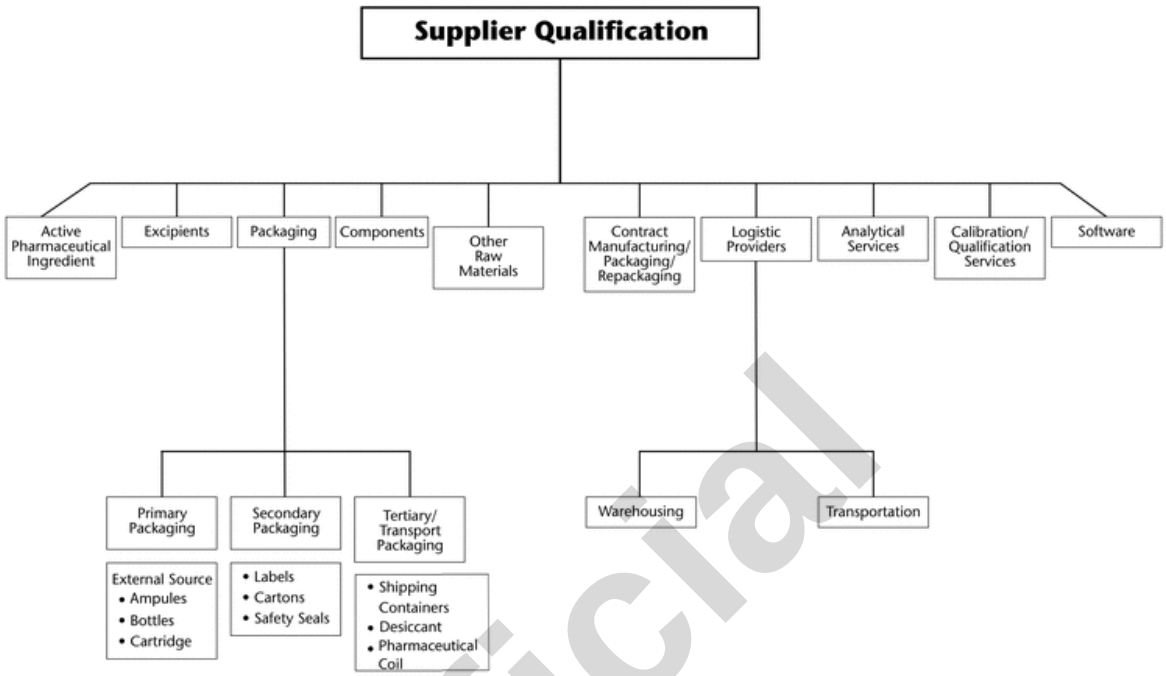


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Figure 1. Examples of some suppliers that should be qualified.

*Figure 1* shows types of suppliers for which the general concepts of this chapter may be applicable.

## 3. SUPPLIER QUALIFICATION LIFE CYCLE: STEPS FOR A SUPPLIER QUALIFICATION

The following sections provide expectations and the rationale of tasks for each step in the supplier qualification life cycle. Each activity in the supplier qualification life cycle should be documented and justified. The level of effort, formality, and documentation of each step should be commensurate with the risk level of material or service to be supplied, risk effect, and the availability of mitigation strategies. The supplier qualification life cycle is described in *Table 1*.

**Table 1. Supplier Qualification Life Cycle: Steps and Tasks**

| Step | Tasks |
|---|---|
| 1. Preparation | • Nominate a supplier qualification process owner<br>• Build a cross-functional team<br>• Identify material and/or service requirements<br>• Establish material and service criticality |
| 2. Identification and selection | • Establish supplier requirements<br>• Identify potential suppliers<br>• Sign confidentiality and nondisclosure agreements<br>• Make a risk assessment<br>• Select suppliers for evaluation |

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**Table 1. Supplier Qualification Life Cycle: Steps and Tasks** *(continued)*

| Step | Tasks |
|---|---|
| 3. Evaluation and acceptance | • Perform evaluation based on risk and criticality of the material and supplier and may include the following: i) documentation review; ii) audit; and iii) sample request for analysis<br>• Establish central location(s) for referencing supplier qualification (e.g., qualification database, list, or spreadsheet)<br>• Update supplier qualification status in the central list of suppliers, central location, or spreadsheet |
| 4. Performance monitoring | • Establish and share key performance indicators (KPIs) as necessary<br>• Monitor the supplier performance against the contract and/or quality agreement<br>• Evaluate the supplier performance at regular intervals<br>• Make decisions based on the evaluation, e.g., ask for corrective action and preventive action (CAPA), quality agreement review, contract termination |
| 5. Disqualification | • Document the circumstances<br>• Update the supplier database |
| 6. Conditional approval of an existing supplier | • Explanation and measures to mitigate the risk |

## 3.1 Preparation

### TEAM BUILDING

The involvement of subject matter experts (SMEs) with relevant experience is necessary to balance departmental objectives with goals of the overall organization. For a successful supplier qualification program, a cross-functional collaboration is required and a process owner should be nominated.

The objective of building cross-functional teams is to provide a forum to address supplier identification and selection, uncover risks and opportunities, qualify and if necessary, quantify those risks, and establish potential mitigation strategies, allowing all functional areas to align on critical requirements and assumptions to make appropriate decisions.

Functional areas to be considered for membership of a supplier qualification cross-functional team include (as needed):

- Technology development (e.g., research, development, concept, design, engineering)
- Technology launch and commercial support (e.g., pharmaceutical technology, engineering, industrial engineering, technical services, technology operations, process technology)
- Quality (e.g., quality compliance, quality assurance, quality control)
- Regulatory affairs and compliance
    - The regulatory framework will vary from region to region and country to country
- Environment, health, and safety
- Sales, marketing, business development, and customer service
- Commercial manufacturing, production, operations, packaging
- Purchasing, procurement, supply management, strategic sourcing, commodity management, category management
- Management of external contractors
    - Supply chain
    - Supplier management
    - Vendor relationship management
    - Strategic sourcing management
- Information technology, including customer and supplier data exchange
- Logistics, including warehouse

### MATERIAL AND/OR SERVICE REQUIREMENTS AND CRITICALITY

The identification and characterization of material and/or service requirements are important first steps in the product development process. Development teams identify materials and/or services needed for the finished product and begin studies to understand what requirements are necessary to support consistent product performance. These requirements can be further defined using a cross-functional development team to best identify supply chain partners.

Once the final product attributes and desired material performance are defined, the company teams should align on the material criticality needed to meet the final product specification. For this, the cross-functional development team agrees on material criticality in alignment with International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines Q8 (Pharmaceutical Development), Q9 (Quality Risk Management), Q10 (Pharmaceutical Quality System), and Q12 (Technical and Regulatory Considerations for Pharmaceutical Product Lifecycle Management).

Similar to the identification of material characteristics, service requirements should also be described by the organization, specifying what is required from the service suppliers. Storage conditions, category of product, licensing needed for handling a specific product, transportation modes, duration of trips, and types of shipping containers are some examples of topics that should be evaluated first to build service requirements for storage and transportation providers. For other services, such as contract manufacturing, analytical, calibration, or qualifications services, and service requirement, specification is also needed and is written according to the scope and risks of service that should be provided.

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## 3.2 Identification and Selection of Supplier for Materials and Services

Once the material and/or service requirements are identified, the cross-functional team can then establish supplier requirements. After determining requirements, the team can identify potential suppliers that should be considered for each material or service while assuring the procurement process is manageable and cost effective.

### SUPPLIER REQUIREMENTS

Appropriate requirements for material or service suppliers may include, but are not limited to:

- Ability to meet market demands, including regular lead times and responses to changes in demand
- Operational and financial viability, such as future manufacturing capacity, global presence, alternate sourcing options, and ability to manage supply chain risks
- Willingness and ability to communicate
- Quality systems and regulatory compliance are appropriate
- Controls are in place for material traceability and control of their own supply chain
- Possession of technical capability and expertise for the characterization of both the material or service and the manufacturing process
- The supplier has appropriate systems in place to prevent the introduction of falsified, diverted, or counterfeited products into the supply chain during the procurement, manufacturing, storage, transportation, disposal of waste, and destruction activities

### POTENTIAL SUPPLIER IDENTIFICATION

Once the material, service and supplier requirements are identified and aligned, the company can identify potential suppliers that meet the defined requirements.

First, the company defines a list of potential suppliers, and then engages with them to share subject matter expertise and verify expectations. Engagement with potential suppliers provides the best opportunity for improving understanding of the material or service in question such that it meets the criteria for the intended use, e.g., performance, variability, and impact on the quality attributes of the finished product.

The company should be able to provide the supplier with the critical information (such as intended use of the material or service, critical quality attributes of the product, storage conditions, technical capabilities needed from the supplier, and a provisional forecast) that will enable both the supplier and the company to support the finished product successfully.

Before exchanging information with suppliers, confidentiality and nondisclosure agreements should be signed.

### CONFIDENTIALITY AND NONDISCLOSURE AGREEMENTS

If needed, appropriate confidentiality agreements should be executed to allow for the exchange of confidential information needed for supplier qualification. When the information exchange moves beyond public knowledge, interacting with suppliers will necessitate a confidentiality or nondisclosure agreement. Suppliers operate in a competitive environment where compromised confidential knowledge of manufacturing processes, equipment, capacity, raw materials, process testing, facility layout, and other site-specific information could result in the loss of an economic advantage for the supplier or the gain in an economic advantage for their competitor. For suppliers where the portion of products sold into markets is relatively small, the risk of losing sensitive trade secrets may outweigh the benefit of selling to customers who require such information. Likewise, for a product manufacturer, compromised confidential information regarding a new product, existing formulations, material specifications, or production processes could have an adverse economic impact on their company. For these reasons, confidential information should only be shared and requested when necessary for ensuring the manufacturing, safety, and quality of the finished product.

### RISK MANAGEMENT

Risk assessment and control should be integrated into supplier qualification taking into account quality and patient safety. The supply chain's risk assessment process involves identifying the internal and external risks and evaluating their potential impact on the finished product, third party materials and services, and internal systems. This risk assessment effort is not a one-time event but rather a periodic recurring process to communicate and review risks.

It is not the aim of this chapter to provide a list of risk and mitigation strategies because risks are perceived differently and depend on experience and the services and materials that could be supplied. Instead, this section is meant to stimulate discussion and highlight points to be considered when assessing and controlling risks to select potential suppliers.

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**Table 2. Points to Be Considered When Assessing and Controlling Risks**

| Risk Category | Points to Consider |
|---|---|
| Material-related risks | • Use in patient and/or business critical products (irrespective of material cost)<br>• Degree of use across product lines (irrespective of material cost)<br>• Available inventory (internally and/or externally) irrespective of material cost<br>• Consider supplier's reject and discard rate<br>• Consider variability in process capability impacting the final product<br>• Material performance based on information supplied by supply chain intelligence<br>• Financial impact: cost of material and/or service, cost and time of the alternate source<br>• Product sensitivity<br>  ○ Sterility, temperature sensitivity, light sensitivity, etc.<br>• Impact of primary and secondary packaging on material integrity |
| Capability | • Manufacturing<br>  ○ Pharmaceutical: biologic, vaccine, sterile, nonsterile, etc.<br>  ○ Device: Class III, Class II, Class I, etc.<br>• Service<br>  ○ Familiarity with changing regulations in all countries of service<br>  ○ Upgrade capabilities<br>  ○ Package, delivery, and route qualification and traceability |
| Stock-out risk to patients | • New product<br>• Orphan product<br>• Availability of alternates |
| Financial risk to the business | • High cost of discards, loss of market share, impact to shareholders, etc. |
| Location and supply chain complexity | • Physical distance<br>• Political boundaries and regulations |

Each company needs to develop priorities based on the impact on all stakeholders: patient, public, employees, and shareholders. When measuring impact, it is important to focus on the effects on safety, efficacy, continuity of supply, and business and financial performance, and not only on the cost or volume.

For more information on risk and mitigation strategies related to storage and transportation of finished drug products, please see *Risks and Mitigation Strategies for the Storage and Transportation of Finished Drug Products* ⟨1079⟩. For further information on quality risk management, please see ICH Q9.

### SUPPLIER SELECTION

This selection process is intended to identify a short list of suppliers that will be evaluated further. The selection may include, but is not limited to, consideration of the following key points.

- **Supplier product and process technical and operational capability:** The supplier has the specific technical expertise, acceptable process capabilities, extensive experience with the material, service in question, and a defined process for improvements to afford future economies of scale.
- **Quality and regulatory compliance system:** The supplier has controls in place for material traceability, acceptable quality control, and control over their supply chain.
- **Relationship alignment and business capability:** The supplier is socially responsible, shares market intelligence, and meets the strategic needs of the company culture (e.g., commitment, trust, and reputation and promotes financial stability).
- **Supplier operating systems and business continuity:** The supplier supports speed to market, meets lead time demands, and responds to any changes in a timely manner.

There is no ranking implied and there may be different emphasis of importance depending on each company's needs. The organization could submit a questionnaire to the supplier in order to obtain initial feedback on these key points.

## 3.3 Evaluation and Acceptance

Based on the information gathered during the supplier selection stage, the selected supplier(s) are then further evaluated. The evaluation criteria are based on the risk assessment of the materials and services to be supplied. For example, the tolerable risk from a supplier and/or reagent used in an early process step of an active drug substance synthesis is usually higher than a supplier and/or an excipient used in the finished drug product. Similarly, the tolerable risk for an early development analytical service is usually higher than that for a registration stability program. In general, the closer you get to the finished product, the higher the risk from goods and services obtained from suppliers.

With an established risk assessment, the suppliers could be evaluated for their technical capability and quality system compliance. The evaluation may include, but is not limited to:

1. **Documentation review:**

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A. Authorizations, permits, or licenses for the intended service (e.g., manufacturing license, good practice regulations (GxP) certificates)
B. Site master file or drug master file references (where applicable)
C. A recent audit report, if possible
D. Recent supplier qualification report (between both organizations involved in the audit)
E. Recent regulatory inspection reports, if available
F. Quality management system (QMS) review (e.g., deviation handling, change control system, customer complaints handling, recall, product rejection and return handling)
G. Trend data for potential critical material attributes and compendial tests (if appropriate)
H. For service providers, examples may include design, operational, and performance qualification protocols and reports. Additional documentation for lane studies and applicable performance as appropriate

2. **Audit:** An audit could be necessary or required to verify and qualify the information obtained during the documentation review. It also provides the opportunity to engage with the supplier in a more detailed discussion to gain firsthand knowledge of the QMS, as well as the technical capabilities, such as personnel, equipment, facility, etc. When an on-site audit is not feasible, the use of remote or virtual audits should be taken into consideration and a third party's certification may be acceptable for the assessment of the supplier's QMS and conformance to GxP.
3. **Sample request:** For each material supplier, tests of at least three batches of materials should be performed to evaluate if the supplier can consistently provide the material with the quality suitable for the intended use. Information of data trending for potential critical material attributes or compendial test can also be provided. For labeling verification, one proof is acceptable.

With a satisfactory evaluation outcome, the contract and quality agreement negotiation will be initiated with the supplier. The following elements (not all-inclusive) should be included in the contract or quality agreement:

- Mode of transport and delivery
- Roles and responsibilities
- Good manufacturing practices (GMP) requirements
- Technical requirements
- Product and service quality monitoring
- Rejection, deviation, complaint, etc.

The organization should have a central location for referencing a supplier's qualification (e.g., qualification database, list, or spreadsheet) that shows those who were qualified, with their status, and important dates (initial qualification, requalification, monitoring, disqualification, etc.), which is continuously updated. Additionally, the date a supplier is first approved should be included in this location.

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## 3.4 Performance Monitoring

The supplier's performance should be appropriately monitored and recorded at receipt and/or use against the contract and/or quality agreement, key performance indicators (KPIs), and relevant external standards (e.g., International Organization for Standardization (ISO) standards, industry standards, GxP). The KPIs should reflect the most critical quality parameters for the supplied product or service. The following are examples of KPIs. [NOTE—Some of these may not apply in all cases and other KPIs may be considered.]

- The quality of the supplied product or service
- The timeliness of the supply and service
- Flexibility in response to unexpected and urgent requests and response to urgent issues and matters
- Response time of resolving audit observations, deviations, or customer complaints
- Effectiveness and timeliness of communication
- The ability to provide supplies and services without any interruption

EVALUATION OF SUPPLIER PERFORMANCE AND PERFORMANCE MONITORING

At regular intervals, the supplier performance should be evaluated and documented based on the results of the continuous monitoring detailed above. The frequency of the evaluation is based on the criticality of the supplied product or service; it is recommended to be at least once a year. Root cause analysis, severity, and frequency of unfulfilled KPI(s) should be included in the evaluation. Furthermore, the validity of the supplier's required certificates and authorizations, as well as the financial viability of the supplier, should be checked. Any trends in the supplier's performance should be identified and investigated. In addition, the quality agreement should be reviewed and updated as necessary on a regular basis.

Depending on the nature of any contract breach, unfulfilled KPI(s), or deviating trend(s), the supplier may be contacted with the request for improvement, corrective and preventive actions, or even termination of the contract in certain cases. The organization should consider how the deficiencies can be mitigated either at source or in-house.

## 3.5 Supplier Disqualification

There are many reasons to remove or disqualify a supplier. The removal of a supplier must be thoroughly evaluated to minimize the impact on product quality and disruption of supply. The circumstances surrounding the disqualification of a supplier must be documented through a change control program.

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When a supplier can no longer meet the criteria that were determined during the approval process, the supplier should be disqualified and removed from the supplier list.

A supplier is removed based on documented actions as follows:

1. Undertake an investigation to determine the root cause
2. Determine a timeline for supplier removal
3. Establish agreement of completion of tasks prior to being disqualified
4. Establish a procedure to audit the current status of the project
5. Establish a process to retrieve outstanding work and samples
6. Establish a risk mitigation strategy and determine the impact of the supplier disqualification on product quality
7. Implement the changes necessary
8. Remove the supplier's name from the approved supplier's list

## 3.6 Conditional Approval of an Existing Supplier

When an existing supplier's performance is not considered satisfactory, but their disqualification would adversely impact the patient, the organization should implement measures to mitigate the risk. This can either be by working with the supplier to improve their quality systems, etc., or by establishing and implementing further controls needed to assure the required quality is achieved and maintained. If the supplier agrees to work to improve their quality systems, there should be an agreed-upon action plan with criteria and timelines to be met.

## Glossary

**Material:** Any active pharmaceutical ingredient, excipient, component, packaging, or other raw material that is intended to be included as part of the product or used in its manufacture.

**Organization:** In the context of this chapter, an organization is the company that needs to purchase a material or outsource a contract service. It may also be referred to as company or supplier's customer. It is the entity that will perform the steps to qualify a supplier.

**Other raw materials:** In the context of biological products, other raw materials are defined as any other substances different from starting materials that are used for manufacturing or extracting the active substance(s) but from which this active substance is not directly derived (starting materials). Other raw materials could be reagents, culture media, fetal calf serum, additives, and buffers used in the production of a product.

**Product:** Any tangible output from the organization or service provider.

**Quality agreement:** A negotiated and comprehensive written agreement between the organization and the material supplier or service provider that defines and establishes each party's activities, the common understanding about materials or services, quality specifications, responsibilities, guarantees, and communication mechanism, providing key contacts for both parties.

**Service:** Activity related to manufacturing, quality, storage, or distribution of a product or material.

**Specification:** A list of tests, references to analytical procedures, and appropriate acceptance criteria, which are numerical limits, ranges, or other criteria for the tests described. It establishes the set of criteria to which a material should conform to be considered acceptable for its intended use. "Conformance to specifications" means that the purchased material, when tested according to the listed analytical procedures, will meet the listed acceptance criteria.

**Supplier:** In the context of this chapter, a supplier is the entity that will provide a service or material to the organization. It may also be called a vendor.

**Supplier qualification:** Supplier qualification is a process to systematically evaluate suppliers based on i) the risk to the quality of the product or service supplied, ii) compliance of their quality systems to applicable regulations and requirements of the supply contract or quality agreement, and iii) the reliability of the supplier to avoid quality deviations and shortages of components and/or products. ▲ (USP 1-Aug-2023)

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# ⟨1084⟩ GLYCOPROTEIN AND GLYCAN ANALYSIS—GENERAL CONSIDERATIONS

## OVERVIEW

A number of glycoprotein drugs have been developed as a result of advances in biotechnology, and many naturally derived protein drugs possess complex glycan structures. Glycosylation, a posttranslational modification of these proteins, can play an important role in determining the function, pharmacokinetics, pharmacodynamics, stability, and immunogenicity of these agents. The two main types of protein glycosylation are *N*-glycosylation and *O*-glycosylation. Unlike transcription and translation, glycosylation is not a template-driven process; therefore variability in the glycosylation pattern of a protein can arise, caused by different sources or different manufacturing processes. Differences in this pattern are known to affect biological activity. Glycosylation patterns may therefore be an important set of attributes that arise in characterizing a candidate glycoprotein intended for therapeutic use and in ensuring its stability and quality.

The first part of this chapter provides a brief introduction to glycobiology and describes the complexity of glycan structures. The subsequent parts provide flow charts and a series of general analytical strategies that can be used to characterize glycoprotein glycans by means of the following:

1. Direct analysis of glycoproteins; and
2. Analysis of released nonderivatized or derivatized glycans by various methods of chromatographic and electrophoretic separation and mass spectrometry (MS).

Different approaches to analyzing monosaccharides are described at the end of the chapter.

For selected analytical methods, this chapter cross-references other USP chapters, particularly those relating to biotechnology-derived articles (see chapters *Capillary Electrophoresis* ⟨1053⟩, *Biotechnology-Derived Articles—Isoelectric Focusing* ⟨1054⟩, *Biotechnology-Derived Articles—Peptide Mapping* ⟨1055⟩, and *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* ⟨1056⟩).

## PROTEIN GLYCOSYLATION

Most proteins in eukaryotic cells undergo glycosylation and other posttranslational modifications before being trafficked to lysosomes, becoming membrane bound at the cell surface, or being secreted. Glycosylation varies significantly from cell to cell, tissue to tissue, and species to species because of the varying expression of hundreds of glycosyltransferases and glycosidases located throughout the Golgi apparatus and endoplasmic reticulum (ER). Four main types of enzymatic glycosylation are found in proteins:

1. *N*-Glycosylation, which involves the initial transfer of oligosaccharides to the nitrogen on the terminal amide group of asparagine and their subsequent processing and modification to a series of glycan chains;
2. *O*-Glycosylation, which in general involves the initial transfer of monosaccharides to the hydroxyl groups of serine and threonine and subsequent elongation and branching of the saccharide chain by the addition of monosaccharides;
3. Glycosylphosphatidylinositol (GPI) anchor, which is a glycolipid linked to the *C*-terminus of a protein; and
4. *C*-Glycosylation, which involves the formation of a carbon–carbon bond between the C2 carbon of the indole ring of tryptophan and the C1 carbon of an α-mannopyranosyl residue.

Any given protein may contain multiple *N*-, *O*-, or *C*-glycosylations, but not more than one GPI anchor. A nonenzymatic addition of saccharides, called glycation, can occur when proteins are mixed with reducing sugars via a complex series of reactions. The two protein glycosylation types that are generally of concern and that are analyzed in glycoprotein drug substances are *N*- and *O*-glycosylation. Each of these is discussed below.

### *N*-Glycosylation

The biosynthesis of *N*-glycans in glycoproteins can be described as a four-step process:

1. Lipid-linked glycan chain initiation and elongation;
2. Transfer of oligosaccharide to the protein or nascent polypeptide chain;
3. Processing of the *N*-glycan chain by removal of specific glucose and mannose residues; and
4. Modification of the *N*-glycan chain by the addition of residues to the nonreducing ends of the glycan chain.

The consensus amino acid sequence for *N*-glycosylation is Asn–Xaa–Thr/Ser (where Xaa is any amino acid other than proline). Overall, only about two-thirds of all potential sequences, termed sequons, are glycosylated, and currently there is no method to predict which sequon will be glycosylated. The role of protein *N*-glycosylation is usually protein trafficking and secretion.

*N*-glycans can be categorized as high-mannose, hybrid, or complex, depending on the extent of processing *(Figure 1)*. High-mannose structures (*Appendix 1*) lack galactose or *N*-acetylglucosamine (GlcNAc) residues in the antennae, branches at the distal end of the chain. In hybrid structures, both substituted GlcNAc residues and terminal mannose residues are present in the antennae, whereas complex structures have both α1,6- and α1,3-mannose residues substituted with GlcNAc moieties. Hybrid and complex glycans can exist with two or more branches, frequently termed antennae; such glycans are therefore often termed, for example, biantennary, triantennary, or tetraantennary. Both monoantennary and pentaantennary *N*-glycans are also known to exist. In complex glycans, antennae frequently carry terminal sialic acid (neuraminic acid) residues. Sialylation has been shown to have a great effect on both the pharmacokinetics and the pharmacodynamics of many therapeutic glycoproteins.

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Common type of asparagine (Asn) linked glycans (*N*-glycans).

Fuc, fucose; Gal, galactose; GlcNAc, *N*-acetylglucosamine; Man, mannose; Neu5Ac, *N*-acetylneuraminic acid

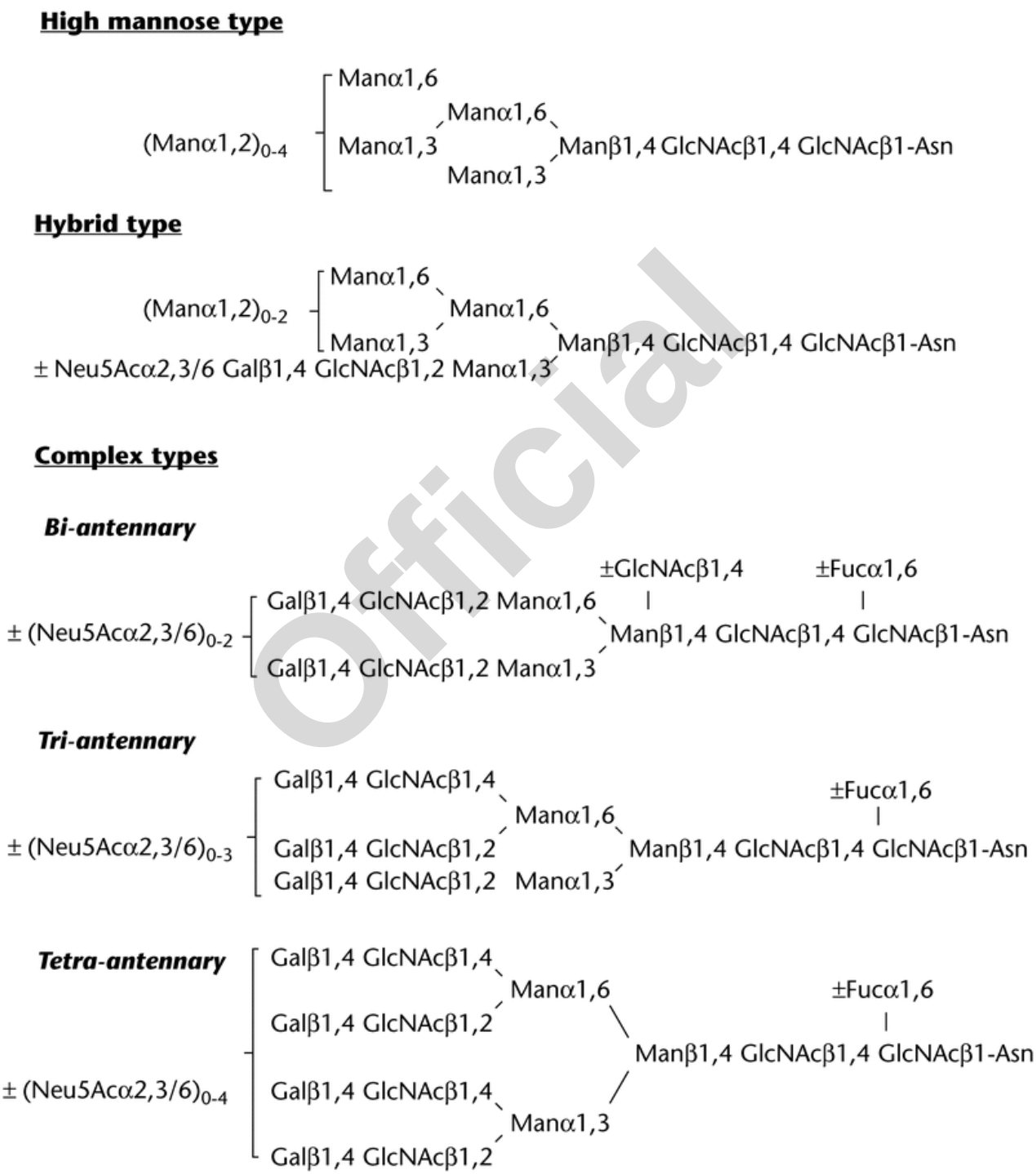


Figure 1. Common types of *N*-glycans. For abbreviations, see *Appendix 1*.

## *O*-Glycosylation

*O*-Glycan chains are built up sequentially via an initial GalNAc residue linked to serine, threonine, and tyrosine, as well as to the less common amino acids hydroxyproline and hydroxylysine. Multiple glycan core structures are known. The sequence and

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isomeric linkage of monosaccharides show greater variety than that in *N*-glycans, and at least eight different types have been identified *(Figure 2)*. Although no consensus amino acid sequence for *O*-glycosylation has been determined, glycosylation is usually favored by the presence of proline one residue before or three residues after the glycosylation site and the absence of charged amino acid residues proximal to serine or threonine. The disaccharide unit *N*-acetyllactosamine, Galβ1,4GlcNAc, is the most common chain extension. Additional modifications, including terminal capping of Gal with sialic acid and fucosylation along the chain, are also frequent.

*O*-Glycosylation can occur in cluster form, the mucin type, which usually forms part of the cell surface extracellular matrix or secreted glycoproteins. Other *O*-glycosylation, such as *O*-GlcNAc, is found on many nucleocytoplasmic proteins; and *O*-Man–linked glycosylation is found in some muscular and neural glycoproteins and in yeast. *O*-Fuc– and *O*-Glc–linked glycosylation types are found on many epidermal growth factor-like proteins that are associated with the Notch signaling pathway.

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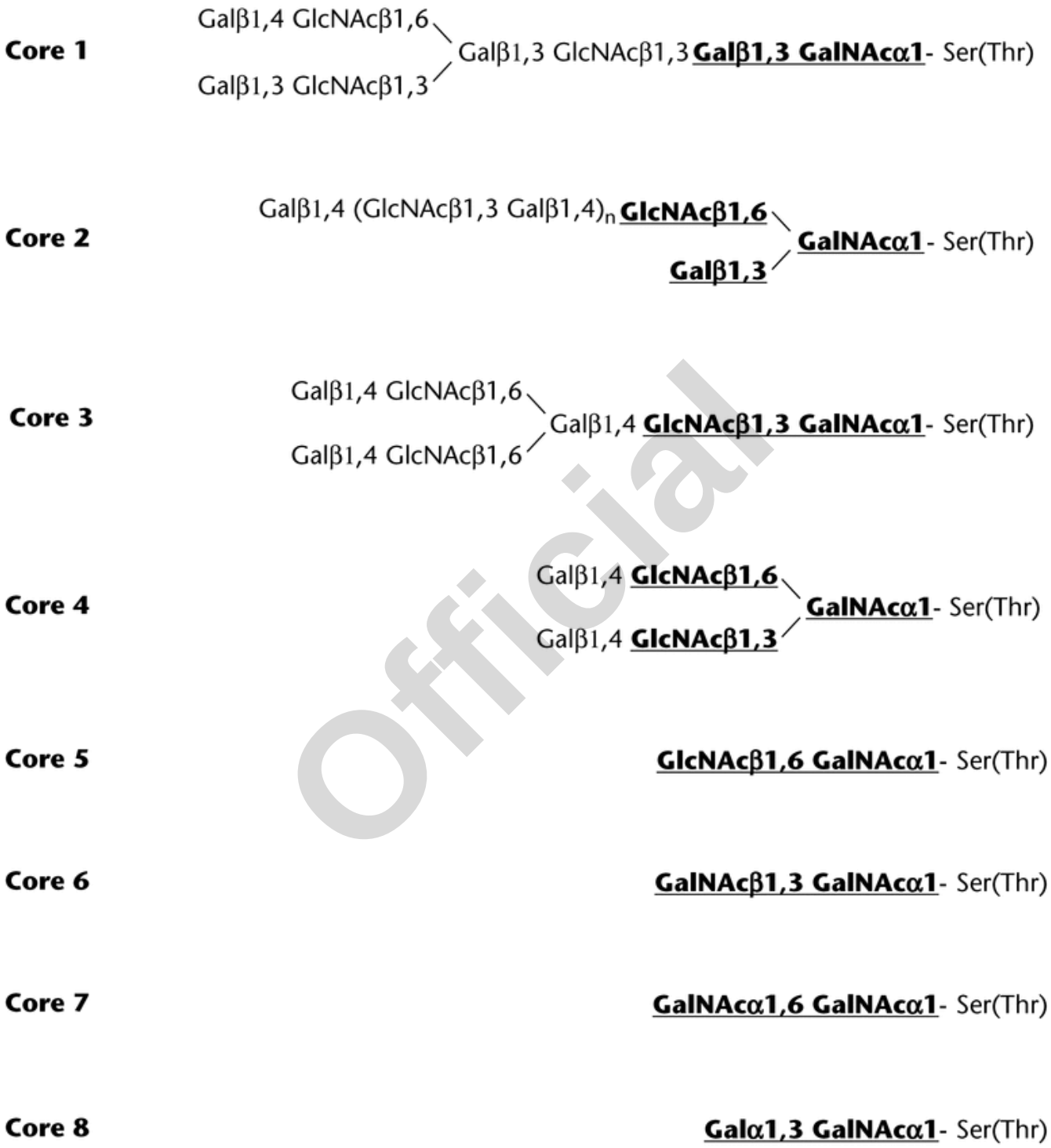


Figure 2. Common core structures of *O*-glycans (bold and underlined). Sugar abbreviations as in *Appendix 1*.

## Glycan Heterogeneity

Not only the type of glycosylation (*N*- or *O*-linked), site occupancy, and the site of glycosylation can vary from glycoprotein to glycoprotein, but also the actual oligosaccharide structures (branching and linkages) can differ, even on the same site. This structural variation arises because glycosylation is a process that is not driven by a template. The glycosylation pattern at a given site depends on many factors, including cell-specific and growth-dependent availability of glycosyltransferases and *exo*-glycosidases found in the Golgi bodies and ER. Heterogeneity leads to different physical and biochemical properties and, therefore, also to functional diversity.

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## Host-Cell Expression Systems and Glycosylation

### BACTERIA

Although both *O*- and *N*-glycosylation have been shown to occur in a variety of prokaryotes, *Escherichia coli*, the bacterium of choice for many therapeutic products, does not produce glycosylated proteins.

### YEAST

Yeast produces both *N*-glycosylated and *O*-glycosylated proteins. In yeast hypermannosylation with the *N*-glycan chain that contains more than 100 mannose residues can occur, but sialylation does not occur unless the organism is genetically modified. The development of recombinant strains of *Pichia pastoris* that contain inserted heterologous genes for various glycosylation enzymes has allowed the humanization of *N*-glycosylation pathways in this yeast. *O*-Glycosylation in yeast is also significantly different from that in mammalian cells. In contrast to mammalian cells, serine or threonine *O*-glycosylation is linked via mannose and often consists of linear chains of as many as six mannose residues.

### INSECT CELLS

*N*-glycan chains of insect cells usually are of the high-mannose, trimannose or paucimannose, and truncated complex types (see *Appendix 1* for definitions). Insect cells also produce glycoproteins bearing the Fucα1,3 residue linked to the proximal GlcNAc residue in the core chitobiose. This core fucose residue is a potent immunogen and allergen. *O*-Glycosylation in insect cells has not been well-studied, and although *O*-linked GalNAc–Ser(Thr) residues have been found, very few are processed further beyond the Galβ1,3GalNAc–Ser(Thr) sequence. Sialic acid residues have not been found on proteins produced in insect cells.

### PLANTS AND PLANT CELLS

Plant *N*-glycans contain mainly oligosaccharides of the oligomannose type, but also present are hybrid and truncated complex types of structures, with or without Xylβ1,2 attached to the β-linked mannose residue of the trimannosyl core and Fucα1,3 attached to the proximal GlcNAc residue of the core chitobiose. Both the Fuc and Xyl residues are immunogenic and have been shown to be part of the glyco-epitopes of several plant allergens. *O*-Glycosylation in plants has not been well studied but is known to consist predominantly of the addition of arabinogalactan chains attached to hydroxyproline, threonine, and serine residues that are located in the plant cell wall or on the outer surface of the plasma cell membrane. These glycans are immunogenic.

### ANIMAL CELLS

The majority of glycosylated therapeutic proteins are produced in continuous animal cell lines. Chinese hamster ovary (CHO), baby hamster kidney (BHK), human embryonic kidney (HEK), and mouse myeloma (SP2/0 or NS0) cells have all been employed. These animal cells generally produce proteins with humanlike glycosylation. Although there are several differences in glycosylation between rodent and human cells, such as the presence of *N*-glycolylneuraminic acid not found in humans, CHO cells have become a workhorse of the biotechnology industry.

## GLYCAN ANALYSIS FOR GLYCOSYLATED BIOLOGICAL DRUGS

Glycosylation of proteins may affect biological activity, either directly or indirectly, and variability in glycosylation arises not only from cellular diversity but also from the manufacturing process. The glycosylation pattern thus may be important as a part of characterization studies in assuring process consistency and may be also be important in ensuring the consistent quality of a biological drug product after market access. Appropriately characterized reference materials are needed in order to support biological and physicochemical testing of production batches to ensure batch-to-batch consistency. Glycosylation analysis may be appropriate for the following:

1. Characterizing the structure and stability of novel products and their stability to processing steps and storage;
2. Batch release testing and in process control testing; and
3. Assessing comparability between products (e.g., when one or more process changes have been made).

An understanding of the relationship between glycan structure and biological function underpins decisions about the information required at each development stage. For biological/biotechnological drug substances, the characterization criteria and specifications for batch release are generally set forth in the guidelines ICH Q6B, *Test Procedures and Acceptance Criteria for Biotechnological/Biological Products*;[1] and ICH Q5E, *Comparability of Biotechnological/Biological Products Subject to Changes in Their Manufacturing Process*.[2] Numerous approaches and methodologies are applied for glycan mapping. This variety is a consequence of the diversity and complexity of glycan structures and the available technology and detection systems.

Because of the diversity and complexity of glycan structures and the increasing availability and improvement of various detection systems and technology, analytical methods are wide ranging. Different methods that support step-by-step procedures depend on the glycoproteins, the availability of equipment, the expertise of individual scientists and groups, and the information required. The two most studied types of protein glycosylation that affect bioactivity are *N*- and *O*-glycosylation.

[1] http://www.ich.org/products/guidelines/quality/article/quality-guidelines.html; accessed 12/15/2011.
[2] http://www.ich.org/products/guidelines/quality/article/quality-guidelines.html; accessed 12/15/2011.

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Glycan analysis can serve in different applications; the most important are general product characterization, process validation, comparability evaluation, stability testing, monitoring manufacturing process consistency, and release testing. The selection of the analytical techniques and their applications in product development and routine manufacturing depend on many factors, such as the complexity of the glycoprotein, the understanding of the relationships between glycosylation and safety and efficacy, and the overall design of the strategy for manufacturing process control. For example, even when the biological relevance of glycosylation is not certain, control of glycosylation could be considered as a measure of manufacturing consistency.

The *Figure 3A* flow chart assists in the choice of applications for glycan analysis, and *Figure 3B* provides an overview of available analytical techniques and equipment employed.

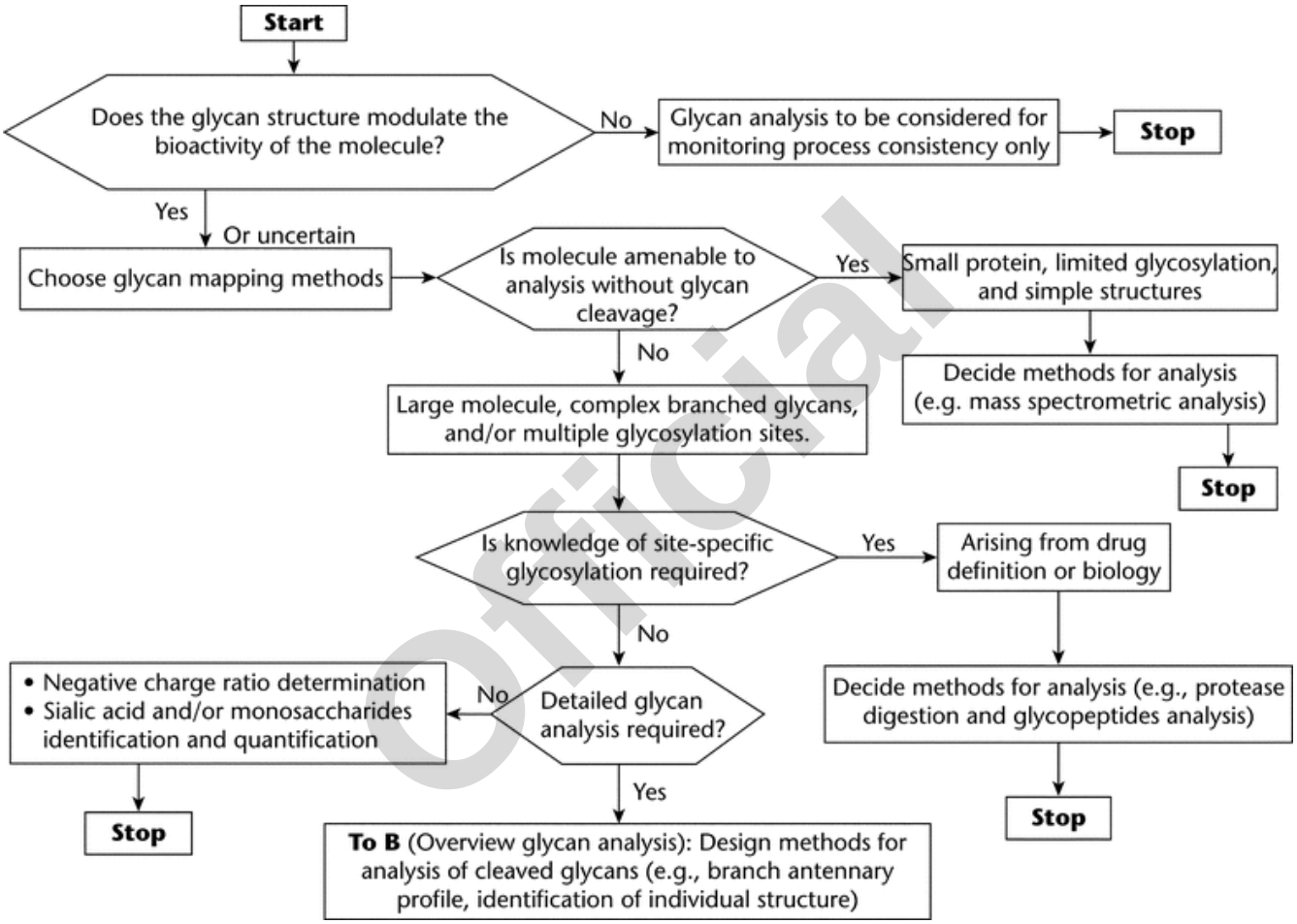


Figure 3A. Flow diagram assisting in the choice of options for glycan analysis.

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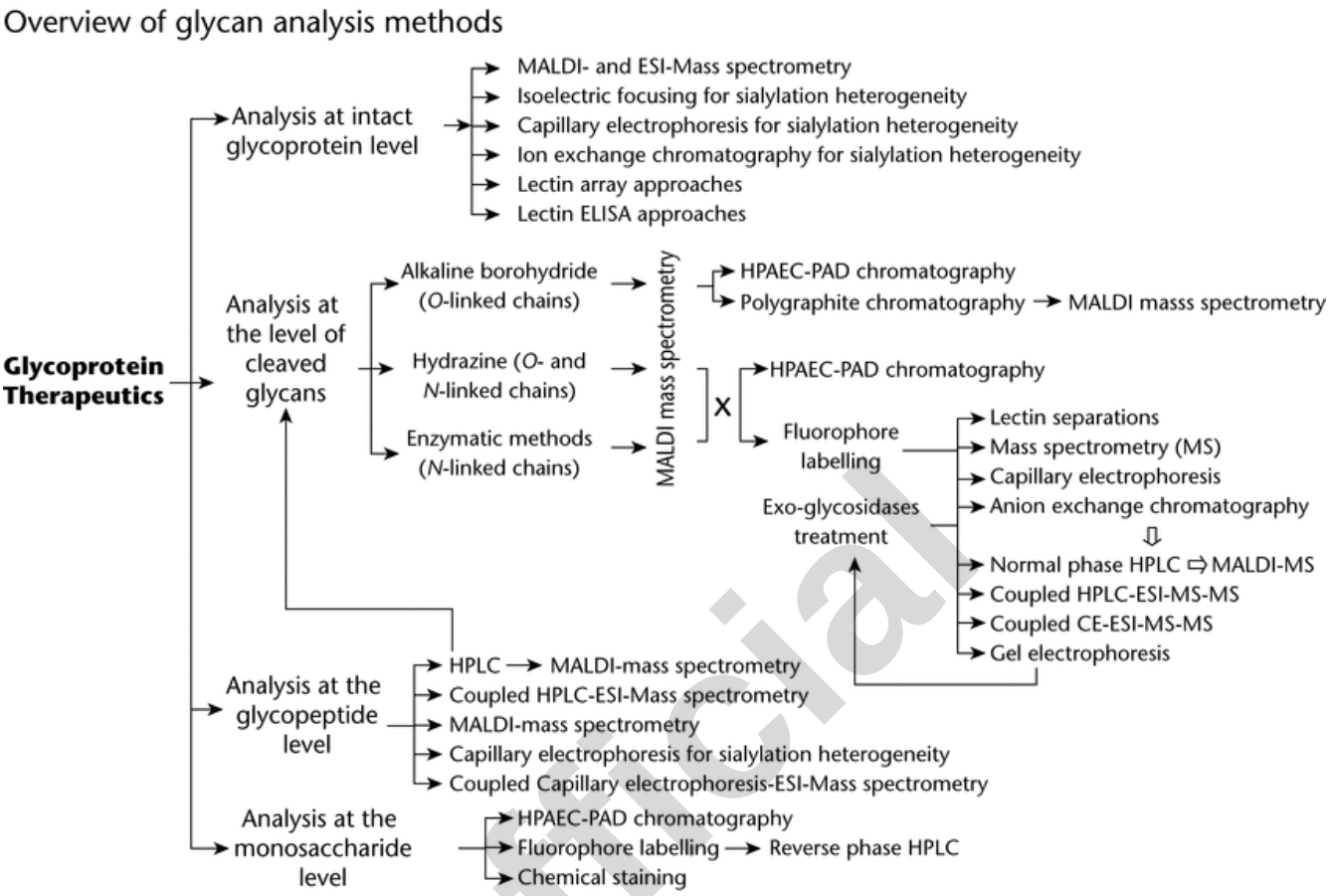


Figure 3B. Overview of glycan analysis techniques and equipment employed.

## CHOICE OF GLYCAN ANALYSIS FOR CHARACTERIZATION AND SPECIFICATION OF GLYCOSYLATED BIOLOGICAL DRUGS

### Analysis of Intact Glycoprotein

The most direct mode of analysis is direct study of the intact molecule. This mode provides information about the glycosylation profile of the glycoprotein. However, this approach provides limited information when the molecule is large and contains multiple glycosylation sites. One of the most important glycosylation factors defining biological activity is the degree of sialylation, which often determines the half-life of glycoproteins in circulation. This makes ionic-charge–based electrophoresis and ion-exchange chromatography obvious choices of technique. Nearly all types of gel electrophoresis have been used to probe protein glycosylation, including polyacrylamide gel electrophoresis (PAGE), (see *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* ⟨1056⟩) and isoelectric focusing (IEF), (see *Biotechnology-Derived Articles—Isoelectric Focusing* ⟨1054⟩). Similarly, capillary electrophoresis (CE), (see *Capillary Electrophoresis* ⟨1053⟩) has also been found suitable. Strong anion-exchange chromatography has been used for the same purpose, but the resolution is often inferior to that of IEF and CE. Direct mass spectrometry (MS) is another option for the analysis of posttranslational modification. Along with ongoing improvements in the resolution of MS, more and more complex glycoproteins become accessible for direct characterization by this method.

### Analysis of Glycopeptides

Analysis of glycopeptides provides information about site-specific glycosylation properties, the degree of occupancy, and oligosaccharide structures. Site-specific glycosylation can be affected by cell culture process conditions. Therefore, if a known glycosylation site is critical, manufacturers must monitor site-specific glycan structures. The typical approach is first to generate glycopeptides by protease digestion and to separate them by, for example, RP (reversed-phase) HPLC (see *Biotechnology-Derived Articles—Peptide Mapping* ⟨1055⟩). Subsequently the separated glycopeptides can be further characterized individually by, e.g., direct analysis using MS, or deglycosylation and subsequent glycan profiling, as described below in the section *Profiling of Cleaved Oligosaccharides*.

Direct identification of the mixture of glycopeptides and nonglycosylated peptides by MS is limited by masking effects (ion suppression) of peptide signals on glycopeptide signals. One approach to overcoming this effect is to separate peptides and

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glycopeptides before analysis by MS, e.g., by offline coupling (matrix-assisted laser desorption ionization [MALDI]) or online coupling (electrospray ionization [ESI]). MS analysis of glycopeptides plays an important role in the characterization of *O*-glycans because these glycans are not always released quantitatively and because, as a result of their smaller size, they are more amenable to characterization by MS as glycopeptides.

The use of CE for high-resolution separation may also be appropriate, especially for the analysis of sialylation.

## Profiling of Cleaved Oligosaccharides

Profiling of total glycans cleaved from glycoprotein is the most common approach for the characterization of glycoproteins. It provides a way to obtain information about the various populations of glycans present on the protein. The degree of sialylation can also be addressed at this stage. Depending on the chosen method, prior derivatization/labeling may be needed to allow the detection of the glycans. Many protocols are available, and most of the steps in the analysis are well established. The possible drawback with such flexibility is the lack of consensus about which methods to choose under which circumstances; because of the variety of analytical techniques, comparison of results obtained by different platforms may not always be possible. So far, the majority of the work has been done on *N*-glycosylation, because of the following factors:

1. *N*-glycans usually are more clinically relevant in biologicals than *O*-glycans; or
2. The release of *N*-glycans, either by chemical means (hydrazine) or by enzymes (endoglycosidases and peptide *N*-glycosidase [PNGase] F), is more straightforward than is the release of *O*-glycans.

### DEGLYCOSYLATION

The approach used for the release of glycans depends on the glycoprotein under test. The cleavage agent is chosen according to the type of cleavage needed and the level of information required. Enzymatic or chemical cleavage may be used. *Table 1* gives a nonexhaustive list of enzymatic cleavage agents and their specificity. Digestion efficiency generally depends on the accessibility of the glycans on the protein, and hence the protein should be denatured to maximize glycosylation site exposure unless analysts want to distinguish between surface and buried glycans. Chemical cleavage agents can also be used, e.g., hydrazine or alkaline borohydride for β-elimination of *O*-linked glycans.

**Table 1. Examples of Enzymatic Cleavage Agents**

| Agent | Specificity |
|---|---|
| ***N*-linked glycan release** | |
| Peptide-$N^4$-(*N*-acetyl-β-glucosaminyl) asparagine amidase (EC 3.5.1.52) | Hydrolysis of peptide-$N^4$-(*N*-acetyl-β-glucosaminyl) asparagine residue in which the glucosamine residue may be further glycosylated, to yield a (substituted) *N*-acetyl-β-D-glucosaminylamine and a peptide containing an aspartate residue |
| Peptide *N*-glycosidase F (PNGase F) | Release of *N*-glycan chain but no release of *N*-glycan chain containing (α1,3)-linked core fucose |
| Peptide *N*-glycosidase A (PNGase A) | Release of *N*-glycan chain containing (α1,3)-linked core fucose |
| Mannosyl-glycoprotein endo-β-*N*-acetylglucosaminidase (EC 3.2.1.96) | Endohydrolysis of the *N,N′*-diacetylchitobiosyl unit in high-mannose glycopeptides/glycoproteins containing the –[Man(GlcNAc)$_2$]Asn structure |
| Endo-β-*N*-acetylglucosaminidase F (endo F) | Release of high-mannose, hybrid, and complex oligosaccharides |
| Endo-β-*N*-acetylglucosaminidase H (endo H) | Release of high-mannose and hybrid oligosaccharides |
| ***O*-linked glycan release** | |
| Glycopeptide α-*N*-acetylgalactosaminidase (EC 3.2.1.97)* | Hydrolysis of terminal D-galactosyl-*N*-acetyl-α-D-galactosaminidic residues |

* This enzyme has limited usage because of its high substrate specificity.

**Chemical or Enzymatic Release of *N*-Glycans**—PNGase F (*Flavobacterium meningosepticum*) is the enzyme of choice for the release of *N*-glycans for most glycoproteins except for some insect cell and plant glycoproteins that may contain a Fucα1,3 linked to the chitobiosyl core. *N*-Glycan chains having this structure can be cleaved from the glycopeptide only by the almond enzyme, PNGase A. Chemical release by anhydrous hydrazine is much less common, mainly because of the limited availability of the reagent, which is considered a hazardous chemical. In addition, hydrazinolysis produces de–*N*-acetylated *N*-glycans.

**Chemical or Enzymatic Release of *O*-Glycans**—Currently only one enzyme, *O*-glycanase from *Diplococcus pneumoniae*, is available to release *O*-glycans, and this enzyme has a limited usage because of its high substrate specificity: it cleaves only Galβ1,3GalNAcα1-Ser/Thr. In addition, no ideal chemical procedure is available; but Ser- and Thr-linked *O*-linked glycan can usually be released by the reductive alkali-catalyzed β-elimination reaction (alkaline borohydride reaction), in which the released glycans are reduced as soon as they are cleaved in order to prevent formation of degradation products due to peeling. However, this reaction is not specific, and in the reaction, approximately 10%–20% of *N*-glycans are generally known to be released as well. The released glycans lack a reducing group used for the attachment of fluorescent labels by reductive amination. Fortunately, with advances in sensitive MS, direct identification of reduced glycans is possible. Relatively good quality reducing *O*-glycans can be obtained by alkali-catalyzed β-elimination using primary amines such as ethylamine and hydrazine. However, both reagents have the potential to produce peeled degradation products. Furthermore, *O*-glycan release by ethylamine is not quantitative. Hydrazine, although it may be better for use than ethylamine, requires strict control of reaction conditions and handling and does not have a commercial source in Europe.

**Separation of Cleaved Glycans Without Fluorescent Labeling**—*N*-Glycans can also be resolved by HPAEC high-pH anion-exchange chromatography with pulsed amperometric detection (PAD; see *Chromatography* ⟨621⟩), which shows high sensitivity, can also separate some isomers, and affords the ability to directly detect native glycans without labels or tags.

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However, LC/MS for this separation approach is challenging because this HPAEC system uses high-pH and high-salt mobile phases that interfere with ionization of glycans. In addition, absolute quantification of the glycan is only possible if the individual PAD response factors for the different glycan structures are known, e.g., if an appropriate oligosaccharide reference library is available. Porous graphitic carbon (PGC) chromatography can also be used to separate glycans, and this method adds an orthogonal selectivity compared to other columns. A PGC-electrospray-ionization-MS approach also can be applied for direct glycan analysis.

MALDI/ESI-MS is a powerful method for the analysis of glycan mixtures either in the native or derivatized form. Permethylation of released glycans is a common method for direct analysis using MALDI/ESI-MS especially for sialylated glycans.

## LABELING OF GLYCANS TO INCREASE DETECTION SENSITIVITY AND/OR TO MODIFY THEIR PHYSICOCHEMICAL PROPERTIES

Chemical derivatization is the most commonly used method for labeling glycans at their reducing end by reductive amination. One fluorescent label can be attached to each mono- and oligosaccharide, which facilitates determination of molar quantities. *Table 2* illustrates the most common examples of fluorescent labels and their most common uses.

**Table 2. Examples of Fluorescent Labels**

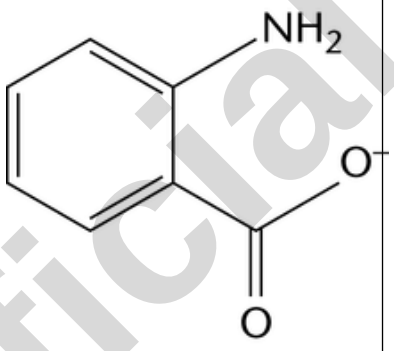
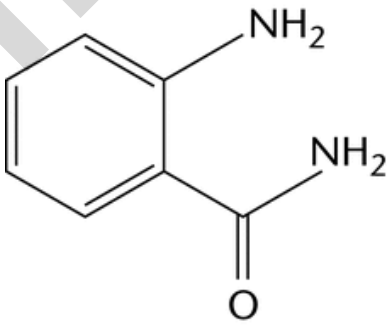
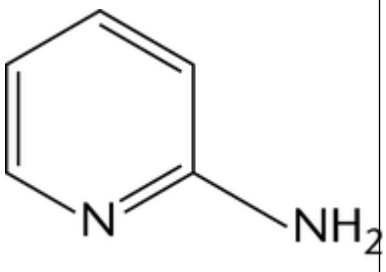
| Name | Acronym | Structure | Analytical Technique |
|---|---|---|---|
| 2-Aminobenzoic acid | 2-AA |  | HPLC |
| 2-Aminobenzamide | 2-AB |  | HPLC MS |
| 2-Aminopyridine | 2-AP |  | HPLC |

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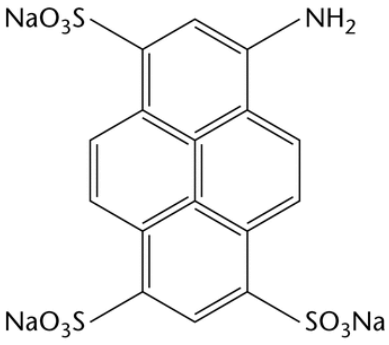
**Table 2. Examples of Fluorescent Labels** *(continued)*

| Name | Acronym | Structure | Analytical Technique |
|---|---|---|---|
| Trisodium 8-aminopyrene-1,3, 6-trisulfonic salt | APTS |  | CE |



### *N*-GLYCAN PROFILING

Released glycans can be analyzed or profiled by chromatographic, electrophoretic, or MS procedures and, in general, by a combination of these. The choice of method can be grouped according to the nature of the glycans and level of information required. Analysis of glycans provides information about the various populations of glycans present on the protein (high-mannose, hybrid, complex).

**Profiling of Glycans by HPLC and/or by Electrophoresis and MS**—Profiling of fluorescent-tag–labeled glycans by HPLC has become the most common approach. One label can be attached to every single mono- and oligosaccharide by reductive amination at their reducing end, which facilitates determination of molar quantities. With the appropriate label, glycans can be profiled with high sensitivity using reversed-phase, normal-phase, and anion-exchange HPLC (see *Chromatography* ⟨621⟩). Routinely, analysts use a combination of these methods in order to increase separation resolution and to better differentiate glycan structures. The accuracy of the glycan identification can be validated by means of glycan standards and/or by coupling the HPLC system with MS. Thus, anion-exchange, normal-phase, and reversed-phase HPLC–ESI–MS–MS form powerful combinations; and in-line analysis, if possible, may provide both relative quantitative profiling and information on glycan structure in a single run. Peak identification through retention time is acceptable if their identities have been previously validated by complementary methods and peak homogeneity can be assured.

The degree of sialylation of glycan chains can be a crucial factor for clinical efficacy, because sialylation often defines the half-life of the molecule in vivo. Anion-exchange HPLC is the simplest method for its determination, and glycan structures based on charge can then be identified by MS. Desalting of each fraction is required before MS if the ionization interface is designed for low-salt-containing sample flows only.

High-resolution separation systems such as CE have been used to identify glycan structures without MS when well characterized standards are used for comparison. The development of an online CE–MS system has further increased the power of glycan analysis using this approach.

**Structural Identification by Micro-Enzyme Sequencing and Mass Spectrometry**—Traditionally, when detailed structural information is required, the analysis is usually performed using micro-enzyme sequencing. This procedure is highly dependent on the specificity and quality of the enzymes used. Recently, tandem MS has been used more regularly to confirm, determine, and sequence known and novel glycan structures; this method is feasible especially when glycans are released from well-known glycoproteins and production sources.

## Monosaccharide Analysis

Different quantitative monosaccharide assays are carried out for a number of purposes. In the glycoprotein field, they provide information about the relative amounts of saccharide in a glycoprotein and about the degree of sialylation of a glycoprotein; and by the measurement of monosaccharide composition, they provide some information about the structure of the glycan chains present.

The simplest assays used are colorimetric tests to demonstrate that the product is glycosylated and to quantify the total amount of saccharide present in the product. These have poor specificity between different types of sugar residues.

Assays of monosaccharide composition are generally simpler to perform than is oligosaccharide profiling, but they provide less information. The most widely used assay is quantification of sialic acid content, because loss of sialylation and exposure of terminal Gal residues may lead to faster clearance of the glycoprotein from the circulation.

The assays can be divided into two types: (1) those that provide compositional information about the intact sample without prior degradation; and (2) others, principally chromatographic, that require hydrolysis of the saccharide chains before analysis and generate quantitative information about several different monosaccharide species simultaneously. In general, the former are colorimetric and the latter are chromatographic. The hydrolysis step is a significant source of assay variability and may require careful optimization for specific samples.

The presence of certain monosaccharides is diagnostic of specific glycan structures. For example, observation of GalNAc is usually a marker for the presence of *O*-linked glycan chains, and fucose denotes the presence of specific types of chains. As a consequence of the limited diversity of monosaccharide residues present in glycoprotein glycans, accurate quantification of Man, Gal, or GlcNAc residues is required in order to distinguish between large numbers of structurally diverse glycans. The

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monosaccharide *N*-glycolylneuraminic acid (Neu5Gc) is not produced in humans and is generally regarded as an unwelcome and potentially immunogenic component of biopharmaceutical products.

## SAMPLE PREPARATION

Glycoprotein samples for monosaccharide analysis should be free of salts, excipients, and other carrier materials (low molecular weight sugars are often used as excipients for biopharmaceuticals). This can be achieved by a number of methods, including the following:

1. Dialysis against water or a volatile buffer, using an appropriate membrane, and lyophilization;
2. HPLC on an appropriate gel-permeation column eluted with water or a volatile buffer, monitored by UV absorbance or refractive index, and followed by lyophilization of the sample; or
3. Sample trapping on a conventional RP-SPE cartridge such as a C18 or C8 SPE system, followed by washing away of salts and excipients and elution of the required sample.

## QUANTIFICATION

The common method for quantification of neutral sugars in glycoproteins depends on the color generated by heating glycans or glycoproteins in the presence of aqueous phenol in concentrated sulfuric acid. In many cases, the heat required for this reaction is generated by addition of concentrated sulfuric acid to the glycoprotein–phenol mixture in water. Rapid and efficient mixing of the solutions is critical for consistent results. Quantitative results are obtained by the simultaneous analysis of standards to generate a standard curve of absorbance against amount of saccharide and/or against a reference sample of the product under analysis.

## HYDROLYSIS PROCEDURES FOR POLYSACCHARIDES AND GLYCOPROTEIN GLYCAN CHAINS

Chromatographic methods for the identification and quantification of monosaccharide components require hydrolysis of the sample before analysis. Appropriate sample preparation is required because excipients or process-related impurities may be saccharides, and residual salts may interfere with the hydrolysis or the subsequent chromatographic separation or with fluorophore labeling. Sialic acid residues can be released either by mild acid hydrolysis or by enzymatic treatment, which leaves other sugar residues attached to the peptide backbone. Quantification of the amount of saccharide present is based on addition of an internal standard before or after hydrolysis. The most commonly used standard for sialic acid analysis by HPAEC is 3-deoxy-D-glycero-D-galacto-2-nonulosonic acid (KDN), and 2-deoxyglucose is widely used for neutral sugars. Both of these sugars are acid labile and should be added after the hydrolysis step. Accurate quantification depends both on stoichiometric hydrolysis and a lack of degradation of the monosaccharide products during hydrolysis.

## DETERMINATION OF TOTAL SIALIC ACIDS

Sialic acids occur in bacterial polysaccharides and glycoproteins generally as *N*-acetyl and *N*-glycolyl derivatives of neuraminic acid (Neu5Ac and Neu5Gc). The sialic acids can be determined together with other monosaccharides by a procedure that includes acid hydrolysis to liberate constituent monosaccharides, followed by HPLC using an appropriate standard mixture. Alternatively, total sialic acid content can be determined by colorimetric procedures without the need for hydrolysis. One method, commonly referred to as the Warren method, is based on the reaction of thiobarbituric acid with the product of periodate oxidation of neuraminic acid released in situ from the glycoprotein. Alternatively, the color can be generated by the reaction of resorcinol with neuraminic acid. For accurate quantification, include a reference standard sample is included in each measurement.

**Selective Release of Sialic Acids**—Mild acid hydrolysis or enzymatic digestion can be used to selectively release sialic acid from glycoprotein glycan chains for quantification by chromatographic methods and for quantification of unwelcome forms such as Neu5Gc. More aggressive acid conditions are required in order to release neutral and amino sugars before chromatographic analysis. The protocol must be optimized with respect to yield and saccharide degradation for each protein to be analyzed.

**Neuraminidase Digestion for the Release of Sialic Acid from Intact Glycoproteins**—Several types of neuraminidases have been isolated and studied; the enzyme derived from *Clostridium perfringens* is the one most commonly used for the enzymatic release of sialic acids from glycoproteins. Recombinant enzyme is available from commercial suppliers. Other enzymes with different specificities are available and can be used to distinguish different types of linkages. Hydrolysis conditions should be optimized for each product, because kinetic parameters for different linkages and for Neu5Ac and Neu5Gc may differ. Selective removal of Neu5Acα2→,3-linked and Neu5Acα2→,6- from cleaved glycans is a convenient means of defining linkages. For quantitative analyses, a known quantity of a suitable internal standard, often 2-deoxyglucose, is added after hydrolysis and removal of the acid.

## SEPARATION AND QUANTITATION OF UNLABELED MONOSACCHARIDES

Essentially the only method used for the simultaneous identification and quantification of unlabeled monosaccharide in hydrolysates is HPAEC-PAD. HPAEC-PAD methods are also applicable to oligosaccharide separations, and a single instrumental approach can be used for both applications.

For the general principles and components of chromatography, see *Chromatography* ⟨621⟩. HPAEC-PAD facilitates analysis of monosaccharides and all classes of oligosaccharides without derivatization. Carbohydrates, because they are polyhydric compounds, are weak acids that have pKa values of 12–14, and at high pH even neutral carbohydrates are ionized and can be separated as oxyanions by ion-exchange chromatography. Although separations can be performed on alkali-stable porous polystyrene–divinylbenzene anion exchangers, carbohydrates tend to exhibit broad peaks as a result of mass transfer problems. In microbead pellicular anion-exchange column packings, small functionalized latex beads (<0.1-µm diameter) are attached to

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larger (<10-µm diameter) uniform nonporous beads. The carbohydrate analyte interacts with the functional groups at the surface of the latex microbeads, eliminating diffusion into and out of pores and the associated peak broadening.

PAD is the method of choice for the detection of carbohydrates in HPAEC because it relies on the high-pH solutions that HPAEC provides by default. Amperometric detection measures the current, or charge, resulting from the oxidation or reduction of analyte molecules at the surface of a working electrode. Electrons are transferred from the electroactive analyte to the electrode during oxidation reactions and in the opposite direction during reduction reactions. This process allows sensitive and highly selective detection of analytes that can be oxidized or reduced, but interfering species that are not electroactive remain undetected. Carbohydrates are easily oxidized at gold and platinum electrodes at high pH, and the current generated is proportional to the carbohydrate concentration.

A typical amperometric detection system contains a working electrode and a reference electrode. Gold electrodes are most common for carbohydrate analysis, but oxidation products poison the electrode surface and inhibit further oxidation. Maintaining a stable, active electrode surface is accomplished by cyclical pulsing between high positive and negative potentials. This timed series of different potentials is referred to as a waveform, and repeated application of a waveform is the basis of pulsed amperometry. Different waveforms are used for different HPAEC-PAD applications and for different working electrodes: disposable gold electrodes require the use of fast, quadruple waveforms, but other gold electrodes allow a wider range of waveforms to be used without damaging the electrode surface. Disposable electrodes and fast waveforms were introduced to minimize the influence of electrode recess on the sensitivity and precision of quantitative monosaccharide applications.

### FLUOROPHORE LABELING OF MONOSACCHARIDES BEFORE SEPARATION AND QUANTIFICATION

An alternative approach to the identification and quantification of monosaccharides present in a hydrolysate is to modify the monosaccharides by reductive amination with an easily detected fluorophore label that allows high-sensitivity detection and improves the chromatographic separation of monosaccharides. Essentially standard HPLC equipment can be used and, because the same labeling approaches are applicable to cleaved oligosaccharides, a consistent analytical approach can be applied. Fluorophore labeling has been much less widely used than HPAEC-PAD for monosaccharide identification and quantification. Labeling of sialic acid derivatives is usually undertaken with 1,2-phenylenediamine (or DMB, the 4,5-methylenedioxy derivatives), and the resulting products are separated on a C-18 column and using fluorescence detection.

## CONCLUSION

Because of the complexity of glycoprotein glycan structures and their inherent variation during production processes, manufacturers are generally required by means of characterization studies to develop criteria for the control of the glycosylation pattern of a biological drug substance when glycosylation occurs, as well as to develop the level of information required at each stage of production and at batch release. Then analytical procedures can be derived in a manner that provides information relevant to fulfilling quality requirements. In general, a combination of approaches and techniques is needed, and more detailed glycan structural analysis at early drug development stages is required. Validation considerations are central as method development and product knowledge progress.

## APPENDIX 1

### Abbreviations

| | |
|---|---|
| Fuc | L-Fucose |
| Gal | D-Galactose |
| GalNAc | *N*-Acetyl-D-galactosamine |
| Glc | D-Glucose |
| GlcNAc | *N*-Acetyl-D-glucosamine |
| Man | D-Mannose |
| Neu5Ac | *N*-Acetylneuraminic acid |
| Neu5Gc | *N*-Glycolylneuraminic acid |
| Xyl | D-Xylose |

## Additional Definitions

**High mannose**—Glycan chains containing two core GlcNAc residues and between five and nine Man residues, and lacking Gal, GlcNAc, or Neu5Ac residues in the antennae. Such chains are typically found in mammalian glycans.

**Hypermannosylation**—(i) Addition of Man residues to high mannose chains creating chains with large numbers of Man residues, and (ii) *O*-Man linked glycan chains with multiple Man residues synthesized by yeast.

**Paucimannose**—Glycan chains containing two core GlcNAc residues between two and four Man residues. Core-linked Fucα1,3 and/or Fucα1,6 residues may be present.

**Oligomannose**—Used here as a generic term to include high mannose, paucimannose, and *N*-linked hyper-mannosylated chains.

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# ⟨1085⟩ GUIDELINES ON THE ENDOTOXINS TEST

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## INTRODUCTION

The first version of *Bacterial Endotoxins Test* ⟨85⟩ appeared in *USP 20–NF 15* (1980). The chapter was subsequently harmonized with the *Japanese* and *European Pharmacopeias*, and the first harmonized chapter appeared in *USP 25–NF 20* (2002). Since its first publication, the content has changed very little, but years of experience, increasing knowledge, and more complex parenteral formulations suggest that the basic methodologies described herein could benefit from additional supporting information. The purpose of this information chapter is to provide additional background information and guidance for the performance and proper application of the compendial bacterial endotoxins tests.

## BACKGROUND: PYROGENS AND ENDOTOXINS

Pyrogens, or fever-causing agents, are possible contaminants in parenteral products. Many substances can cause fevers when injected, infused, implanted, or when they come in contact with the bloodstream or cerebrospinal fluid of mammals. Although some biologics, vaccines, and cell and gene therapies may, by their nature, elicit pyrogenic responses in patients, the predominant and most potent pyrogenic contaminants in the manufacturing of parenteral drugs and medical devices are bacterial endotoxins, which are components of the cell walls of Gram-negative bacteria (GNB).

Endotoxins are integral with the outer cell membrane of GNB. If GNB cannot grow, endotoxins cannot be generated. However, endotoxins may remain active in cell wall fragments after cells die, so a material may be sterile but may still contain quantifiable levels of endotoxins activity. Bacterial endotoxins, when present in parenteral products (including biological products) or medical devices, indicate that the growth of GNB occurred at some point during manufacture of the product. Endotoxins can be introduced into the process stream by pharmaceutical ingredients including water, raw materials (particularly from natural sources), the active pharmaceutical ingredients (API), drug product formulation excipients, primary (product contact) packaging components, improperly cleaned or stored manufacturing equipment, and/or ineffective microbial contamination control practices.

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# ENDOTOXINS

The terms "bacterial endotoxins" and "endotoxins" refer to a complex component of the outer cell membrane of GNB. Although still an active area of research, natural contaminants in sterile parenteral drug products or medical devices are thought to include outer membrane vesicles (OMV), which are mini-spheres of outer membrane material that are released from actively proliferating cells as part of the normal bacterial growth cycle (*1–6*) as well as outer membrane fragments that come from disrupted or dead GNB cells. The biologically active lipopolysaccharide (LPS) is embedded in or associated with other Gram-negative outer membrane components, including various proteins, phospholipids, and lipoproteins (*1,3,6*). The hydrophobic lipid A portion of the LPS molecule is embedded in the membrane and the hydrophilic polysaccharide portion is exposed to the external environment surrounding the cells. Some current research suggests that it is doubtful that pure LPS is released as part of the normal growth cycle of GNB (*3*).

The current USP Endotoxin Reference Standard (RSE) and commercially prepared control standard endotoxins (CSE) are extracted from the GNB cell membrane, generally by the Westphal hot phenol method, and are purified to remove any surrounding cell membrane components (*7,8*). Additionally, these purified preparations are often further formulated with stabilizing agents (*8*). As such, LPS prepared using Westphal, or other extraction methods, cannot contaminate pharmaceutical products because they do not exist in this form in nature. Endotoxin standards, attributed to differences in source and manner of preparation, could "react differently from native sources of endotoxins" (*9*). It is possible that standards extracted by the Westphal method or other denaturing methods and then formulated may not always be representative surrogates for modeling the behavior of natural endotoxins in some pharmaceutical, biopharma, or medical device extraction experiments (*10,11*).

Although general test methods in the compendial chapters numbered below ⟨1000⟩ are considered to be validated, all laboratories, including contract testing laboratories, must demonstrate that the chosen BET methodology is suitable for a specific product or material tested according to *Bacterial Endotoxins Test* ⟨85⟩*, Gel-Clot Technique, Preparatory Testing, Test for Interfering Factors* or *Photometric Quantitative Techniques, Preparatory Testing, Test for Interfering Factors* (also known as inhibition/enhancement testing). This suitability testing will require the following prerequisites:

- Calculation or assignment of an endotoxins specification or limit for the material under test, and calculation of the maximum valid dilution (MVD). See the following sections: *Method Suitability, Calculating Endotoxin Limits for Drug and Biological Products*, *Calculating Endotoxin Limits for Medical Devices*, *Calculating Endotoxin Limits for Combination Products*, and *Calculating Endotoxin Limits for Active Substances and Excipients* below.
- Demonstration that the positive product control (PPC) can be recovered at a dilution of material that does not exceed the MVD. See *Bacterial Endotoxins Test* ⟨85⟩*, Gel-Clot Technique, Preparatory Testing, Test for Interfering Factors* or *Photometric Quantitative Techniques, Preparatory Testing, Test for Interfering Factors*.

Once assay suitability has been demonstrated, the laboratory may perform routine testing according to the calculations and sample preparation conditions that were described in the suitability study. Changes to those conditions (e.g., the lysate and/or endotoxins source, product component(s), formulation, or changes in manufacturing) are subject to change control and may require that the laboratory repeat the suitability study.

## PREPARATORY REQUIREMENTS

### RSE: CSE Calibration

It is well known that purified LPS from different genera/species/strains of GNB, when experimentally administered to rabbits on a uniform weight or mass basis, can produce significantly different pyrogenic reactions (*12,13*). However, by relating these effects to the administered activity—meaning their ability to elicit a fever in the rabbit (*Pyrogen Test* ⟨151⟩) or initiate an LAL reaction ( ⟨85⟩)—rather than weight or mass, the structural variability of LPS molecules can be normalized to a defined unit of activity called the endotoxin unit (EU). Two notes apply here:

- One USP EU is equivalent to one International Unit (IU) as indicated in ⟨85⟩.
- Lysate reagents currently described in ⟨85⟩ are licensed in the United States by the FDA and have a sensitivity associated with them; however, the FDA does not license CSE preparations.

The RSE is the purified LPS primary standard that is formulated from a common bulk preparation of *E. coli* 0113:H10:K LPS that is shared among the World Health Organization (WHO), *European Pharmacopoeia* (*Ph.Eur.*), *Japanese Pharmacopoeia* (*JP*), and *U.S. Pharmacopeia* (*USP*). The RSE was originally developed by the FDA in the 1970s to calibrate lysate reagents from multiple manufacturers, and it remains the primary standard for lysate calibration, calibration of secondary standards (e.g., CSE), and the generation of assay parameters such as standard curves and PPC assay controls.

Although RSE is available from USP for routine use, most BET assays are performed using a CSE, which is a secondary calibration analyte that may be included in LAL test kits purchased from reagent manufacturers. Many CSEs are provided as lyophilized preparations of a purified LPS that was filled and packaged by weight, not by activity. The purpose of the standardization study is to determine the specific activity, or potency, of the CSE in EU/unit of weight of the material against the RSE primary standard.

All the reagents used in BET assays are biological in nature and, therefore, can exhibit some variability in sensitivity and potency, making calibration against the RSE an important task. Calibration against RSE is necessary for each unique combination of lysate lot and CSE lot. If a kit is purchased from a reagent vendor, the vendor will conduct the lot-specific standardization and provide a lot-specific certificate of analysis (CoA) in the kit, which should be retained for reference in the laboratory. However, there may be circumstances when a laboratory might choose to purchase CSE from a third party or to calibrate a liquid endotoxin preparation, which requires that the laboratory conduct its own calibration study.

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## CSE Calibration/Potency Determination Using the Gel-Clot Method

CSE potency determination in the gel-clot test is accomplished by comparing the geometric mean (GM) endpoints of separate dilution series of RSE and CSE made in Water for BET [see *Bacterial Endotoxins Test* ⟨85⟩, *Reagents and Test Solutions, Water for Bacterial Endotoxins Test (BET)*] and tested in quadruplicate. The endpoint is defined as the last tube in a two-fold series of RSE or CSE dilutions showing a positive reaction (gel). To have an endpoint, the last positive tube must be followed in the series by at least one negative dilution. The GM is calculated as follows:

$$\text{Geometric mean} = \text{antilog}\left\{\frac{\Sigma\log_{10}\text{endpoints}}{\text{number of replicates}}\right\}$$

The RSE is labeled in units of activity (EU/vial), and the units for the GM endpoint for the RSE are expressed as EU/mL. Because the CSE is filled by weight, the units for the GM endpoint for the CSE are expressed as ng/mL. The potency of the CSE in EU/ng is calculated as follows:

$$\text{Potency of the CSE (EU/ng)} = \frac{\text{GM endpoint of the RSE series in EU/mL}}{\text{GM endpoint of the CSE series in ng/mL}}$$

For example, to calibrate a new lot of CSE for use with a gel-clot lysate lot with a labeled sensitivity of 0.125 EU/mL, dilute the RSE in EU/mL to bracket the label claim of the lysate reagent. For this example, the GM of the RSE confirms the label claim of 0.125 EU/mL. Dilute the CSE in ng/mL so that an endpoint will be reached. For this example, the endpoint of the CSE series is 0.00625 ng/mL. The potency of the CSE for the particular lysate lot is calculated as follows:

$$\frac{\text{GM of the RSE}}{\text{GM of the CSE}} = \frac{0.125\ \text{EU/mL}}{0.00625\ \text{ng/mL}} = 20\ \text{EU/ng of CSE}$$

For this lot of lysate, the potency of the new CSE lot is 20 EU/ng. If the lab wants to use this CSE lot with a different lysate lot, the calibration study must be repeated because potency determination references only a specific combination of lysate lot and CSE lot.

## CSE Calibration/Potency Determination Using Quantitative Kinetic and Endpoint Assays

Determining the potency of CSE in a quantitative assay is performed much like a standard assay for an unknown. Onset/reaction times from dilutions of the CSE diluted in ng/mL are interpolated from an RSE standard curve to yield the activity of the CSE dilution in EU/mL. Since the concentrations in ng/mL of these dilutions are known, calculation of EU/ng is a simple mathematical function. For any given concentration of CSE, calculate as follows:

$$\text{EU/ng} = (\text{EU/mL}) \div (\text{ng/mL})$$

For example, to calibrate a new CSE lot for use with a chromogenic assay, the first step is to prepare a standard curve using RSE in Water for BET. The standard curve range is left to the discretion of the analyst, but appropriate choices would be either the same range as is routinely used in the laboratory or the maximum range suggested by the reagent manufacturer. Next, the analyst dilutes the CSE to a known concentration in ng/mL and tests the dilutions as unknowns (see *Table 1*). Points to consider:

1. Because of linearity requirements for a standard curve, data used in the calculation of potency may not be extrapolated beyond the range of the curve, but rather must fall within the range of the referenced standard curve
2. Standard curves should not exceed the maximum ranges that are suggested by the reagent manufacturer
3. For CSE calibration, it is recommended that replicate determinations for any given dilution be considered individually (not averaged) when calculating the correlation coefficient to assess variability when calibrating the CSE

**Table 1. Example of Calibration of a CSE Analyte Against RSE in a Quantitative Assay**

| Example concentration (ng/mL) | Result (EU/mL) | Result (EU/ng) |
|---|---|---|
| 1 | 15 | 15 |
| 0.5 | 6 | 12 |
| 0.25 | 3 | 12 |
| 0.125 | 1.8 | 14 |
| — | — | Average: 13.25 |

In this case, the potency of the new CSE lot with kinetic chromogenic reagent is the mean of the four separate determinations. That calculation is 13.25 EU/ng, which is properly rounded down to 13 EU/ng. If the lab wants to use this CSE lot with a different lot of lysate—be it kinetic, endpoint, or gel clot—the calibration must be repeated because potency determination references a specific combination of lysate lot and CSE lot.

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## Activity Determination for a Liquid CSE

A liquid CSE such as a native endotoxin preparation does not have potency unless the actual weight of the source material is known (*Endotoxin Indicators for Depyrogenation* ⟨1228.5⟩). Typically, liquid CSE are described in terms of a concentration of activity expressed in EU/mL. The activity of a liquid CSE in EU/mL is determined by GM endpoint determination for replicate tubes in gel clot or by treating the solution as an unknown against an RSE standard curve for quantitative assays.

## Screening and Qualification of Consumables: Compendial Requirement

The harmonized compendial chapter on the BET requires that the laboratory screen consumable plastics for test interferences. From ⟨85⟩, *Apparatus*:

> If employing plastic apparatus, such as microplates and pipet tips for automatic pipettes, use apparatus that is shown to be free of detectable endotoxins and does not interfere in the test. [NOTE—In this chapter, the term "tube" includes any other receptacle such as a microtiter well.]

Plastics used in the performance of the test are molded, meaning that plastic pellets have been heated to a very high temperature to produce molten plastic in preparation for the molding process. This high temperature will destroy bacterial endotoxins, so molded plastics are free of detectable endotoxins when they are released from the molds. However, depending on the subsequent handling, recontamination is possible. Control measures should be in place to ensure appropriate storage conditions and avoid contact with potentially contaminating substances. However, unless depyrogenated plastics are stored under damp conditions or encounter substances during handling in which GNB could proliferate, the probability of recontamination is remote.

If a laboratory accepts a vendor CoA for the endotoxin content of a disposable component, there should be an understanding of the methods used to determine the reported test result. Two examples of CoAs are provided below:

- If a shipment of dilution tubes is received from a vendor and is labeled "non-pyrogenic", what does this really mean? Was the lot tested for pyrogens using a rabbit pyrogen test or a validated monocyte activation test? Was it tested using a standard ⟨85⟩, and if so, at what level of endotoxin does the vendor consider the material to be "non-pyrogenic"? It is important to ask the vendor how the material was prepared and tested.
- If a shipment of 10-mL tubes is received from a vendor with the label of "<0.5 EU/mL", what does that mean? With what volume was the tube extracted (1, 5, or 10 mL)? A 1-mL extraction would mean <0.5 EU/tube, whereas a 5-mL extraction would mean <2.5 EU/tube and a 10-mL extraction would mean <5 EU/tube. The laboratory should ask the vendor about the method of endotoxin extraction from the tube as well as the BET test conditions that were used to generate the result.

A default method to confirm the CoA result is to treat the plastic disposable as if it were a medical device and proceed according to the methodology provided in *Medical Devices—Bacterial Endotoxin and Pyrogen Tests* ⟨161⟩. However, while ⟨161⟩ defines the endotoxin limit for medical devices as <20 EU/device, a laboratory may want to consider redefining the limits for disposables used in the test to be less than the value of the most sensitive test used in the laboratory. For example, if the laboratory is using sterile polystyrene tubes for sample dilution, and the most sensitive test is a kinetic chromogenic test with a $\lambda = 0.05$ EU/mL, then consider setting a limit low enough to prevent interference across all test methods.

Likewise, some materials could contain extractable or leachable substances that could inhibit the LAL-bacterial endotoxins assay. The PPC will indicate whether the normal, routine use of the plastic results in any leached inhibitory substance. If a laboratory is considering use of disposable plastic containers such as sterile polystyrene for long-term storage of materials that ultimately will be tested, it is suggested that they do testing to confirm that there are no inhibitory or enhancing factors that could affect the accuracy of the test.

## Analyst Qualification

General laboratory training for analysts is good laboratory practice, as well as a current good manufacturing practice (cGMP) requirements (*Microbiological Best Laboratory Practices* ⟨1117⟩). Typically, training for performing any BET involves demonstration of acceptable proficiency for both sample preparation and assay method(s). It is suggested that training be divided into two parts:

1. Classroom training delivered by a subject matter expert (SME) develops an understanding of the principles and limitations of the test methods as well as the effects of the analyst's technique on the test result. Differences between analysts in the accuracy and precision of executing basic laboratory tasks such as pipetting, weighing raw materials, and making dilutions may introduce bias. Due to the inherent variability of the reagents (lysate and CSE) routinely used in the laboratory, poor or inconsistent techniques may significantly affect the accuracy of test results. Analysts must be retrained if a new test method is introduced into the laboratory (e.g., a change in laboratory procedures from gel clot to a kinetic assay).
2. Training effectiveness should be confirmed by the demonstration of analyst competency in performing the test. Competency or proficiency training may be divided into two parts:
    A. For compendial assays where confirmation of assay sensitivity is required, it is recommended one part of performance training follow the provision in *Bacterial Endotoxins Test* ⟨85⟩, *Gel-Clot Technique, Preparatory Testing* or *Photometric Quantitative Techniques, Preparatory Testing*. For the gel-clot method, this is the test for confirmation of labeled lysate sensitivity, and for quantitative tests, this requires the construction of a linear standard curve. For training on quantitative tests, it is recommended that replicate determinations for any given dilution be considered individually (not averaged) when calculating the correlation coefficient to assess the variability in the proficiency study. Both of these proficiency assessments require analysts to prepare and dilute RSE or CSE standards. If the laboratory uses a cartridge system with an on-board archived standard curve, it is recommended that the laboratory devise another method of ensuring that the analyst's technique in sample preparation and dilution is sound. This proficiency training should be described, justified, and included in the firm's standard operating

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procedures to ensure consistency across all analysts. Although there are many ways to determine proficiency, one possibility is to ask the analyst to prepare a sample with a known and confirmed level of endotoxin activity and ultimately compare the result to the known value. If the calculated values are incorrect, this may raise a concern about the analyst's technique.

B. The second part of the proficiency training should be an "on the job" training where analysts are required to demonstrate their ability to calculate endotoxin limits, prepare samples according to the results of suitability testing, and appropriately execute positive and negative controls in accordance with product-specific instructions and ⟨85⟩.

The following should be stressed during training:

- Appropriate laboratory aseptic technique is important so the analyst does not contaminate samples, diluents, or accessories used to perform the test.
- Use of a vortex mixer or another validated method (e.g., sonication for sample preparation) is important to optimize the distribution of endotoxins in samples and the aggregation state of the purified standards in the standard series. Because the formulations of the CSEs provided in LAL test kits are proprietary, it is highly recommended that analysts follow the manufacturers' instructions for vortexing time, both for reconstitution of the vial of LPS and in between dilutions. However, vortexing of lysate is not recommended as it may result in bubbles in the reagent.
- If reagents are saved, ensure that the "open" date and "expiration" date are clearly marked on the primary containers and that any holding of unused reagents follows manufacturers' instructions.
- Do not store RSE or CSE dilutions without a validation study that includes vessel type and materials of manufacture, concentrations of RSE or CSE that are to be held, hold temperature, and volume of the dilutions to be held.
- When reading gel-clot results, pick tubes up one at a time and invert 180°. Picking up more than one tube could jostle the contents. Once a gel is broken, it will not re-form, and the result may be a false-negative.
- When inoculating a microplate, tube, or cartridge care should be taken to avoid the formation of bubbles, because they will impact the accuracy of the test result.
- When using heating equipment (e.g., bead baths, water baths, plate readers), be certain that the equipment is qualified.
- If using a water bath for gel-clot incubation, change the water frequently. The recommended frequency is at least once a week.
- Ensure that all mechanical pipettors are calibrated, and use them only within the calibrated range.
- When possible, use larger volumes (milliliters) for dilution rather than smaller volumes (microliters), as smaller volumes increase variability.
- Standard curves for photometric tests are constructed based on the $\log_{10}$ of the measured onset or reaction times as a function of $\log_{10}$ of the endotoxin concentration. Pay attention to the onset times of the standards to ensure that they are consistent from run to run, analyst to analyst, and day to day for any given combination of CSE lot and lysate lot. See *Routine Testing, Standard Curve Control*.
- If using a monocyte activation test, be sure to include at least one non-endotoxin control.

An analyst requires additional training if any of the following is noted by a supervisor:

- Failure to meet the requirements of the initial performance training.
- Frequent inability to meet system suitability parameters, yielding invalid test results (e.g., confirmation of label claim for gel clot, demonstration of linearity for quantitative assays, inability to ensure that negative controls are nonreactive). Note that the inability to recover the PPC within the required range may signal an issue with analyst technique or a change in the product's manufacturing or formulation that changes the product's interference profile.
- Erratic results for slope and *y*-intercept for quantitative assays. See *Routine Testing, Standard Curve Control*.
- Adverse trends for out-of-specification (OOS) or out-of-trend (OOT) test results.

## Equipment and Instrumentation Calibration and Qualification

All instrumentation and equipment used in the performance of an LAL test, including (but not limited to) mechanical pipettors, water baths, heat blocks, and incubating plate readers, should be qualified using proper scientific standards and according to approved protocols and maintenance schedules. Where appropriate, user requirement specifications (URS), as well as IQ/OQ/PQ protocols and reports, should be written and ultimately approved by the quality unit. See *Analytical Instrument Qualification* ⟨1058⟩ for additional guidance. All equipment used in performing the BET should be properly calibrated and maintained at frequencies that are in accordance with the equipment manufacturer's recommendations. Because BET incubation temperatures are critical, equipment such as incubating plate readers, heat blocks, and water baths should be evaluated for uniformity of heat distribution.

Incubating plate or tube readers should reference a URS, an installation qualification (IQ), an operational qualification (OQ), and a performance qualification (PQ). Very often the vendors of these instruments will provide the laboratory with IQ/OQ/PQ templates and hands-on assistance that are very useful for the execution of these studies.

If a laboratory or production dry heat oven is used to depyrogenate glassware or other heat-stable items used in the performance of any of the BET assays, it must be validated to ensure appropriate time/temperature exposure and load pattern (see *Dry Heat Depyrogenation* ⟨1228.1⟩ for additional guidance).

Computer software must be compliant with all federal regulations and standards (21 CFR part 11 in the United States). It must allow for individual user passwords and audit trails. Quality control should understand how the vendors of the BET software have programmed their calculations. For example, if the instrument reports out an averaged result for the assay of three individual samples tested in duplicate, did the software program average the onset times to get the result or did it average the replicate results?

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## Laboratory Environmental Conditions

The BET can be performed in most modern laboratories under controlled conditions. Appropriate aseptic technique is important when preparing and diluting standards and handling samples. Gowning practice outside of normal laboratory personal protective equipment (PPE) requirements is not a concern unless the product under test demands specific analyst safety considerations due to toxicity or infectiousness. Gloves should be talc-free, as the talc may contain significant levels of endotoxins. Plate readers, water baths, and dry heat blocks used for sample incubation should be on a laboratory bench away from heating, ventilation, and air conditioning (HVAC) ducts, significant vibration, and laboratory traffic that could affect the test results. Sample hold times and conditions should be determined and subsequently documented, if necessary, to ensure that accurate test results can be generated in the qualified time. For example, if the laboratory receives a *Water for Injection* (WFI) or in-process sample, must it be refrigerated or can it remain at room temperature, and for how long? Prior to testing, it is recommended that the primary sample container(s) be adequately mixed before removing the test aliquot(s) for either direct testing or subsequent dilution.

***Change to read:***

# METHOD SUITABILITY

## Calculating Endotoxin Limits for Drug and Biological Products

An endotoxin limit specification for a compendial article is the allowable amount of endotoxin activity that can be safely contained in a parenteral product, according to current understanding and experimental evidence (*5*). The endotoxin limit calculation for any drug product is dependent on three variables: 1) the route of administration, which largely defines *K*, the numerator in the endotoxin limit formula, 2) the dose of the product per kilogram of body weight, and 3) the duration (time) of administration. This information can be found in the package insert for an approved drug product or can be obtained from the product development group for a product that is either still in development or in early clinical trials.

An endotoxin limit specification is calculated for each drug product formulation and set of administration conditions as follows:

$$\frac{K}{M}$$

*K* = threshold pyrogenic dose of 5 EU/kg for most routes of administration or 0.2 EU/kg for intrathecally administered drugs (see *Table 2*)

*M* = maximum recommended dose of product per kilogram of body weight of the patient. This dose relates to the concentration of active ingredient (potency of the active ingredient) in the finished product formulation. If a product is infused or injected into a patient at frequent intervals over an extended time, then *M* is based on the maximum total dose administered in a 1-hour period. If the pediatric dose per kilogram per hour is higher than the adult dose, the pediatric dose must be used for the calculation.

When calculating the endotoxin limit specification, body weight is defined according to the intended patient population, which can differ in terms of geographical regions or patient populations. For example, the average adult in the United States is assumed to weigh 70 kg, whereas the average adult in Japan is assumed to weigh 60 kg (*14*). Pediatric patients could be 30 kg or below. The average weights for children can be found on the Centers for Disease Control and Prevention clinical growth charts page (see www.cdc.gov/growthcharts/clinical_charts.htm). The body weight factor selected for pediatric and other special category patients should consider the worst case, i.e., lowest body weight in targeted patient populations that can receive the greatest recommended dose. There is also a special consideration with respect to body weights for veterinary products. Veterinary drug products may be administered to a variety of different species or subspecies. Generally, the smallest animal will have the greatest dose per kilogram. Reference to the product package insert is highly recommended when establishing veterinary endotoxin limit specifications.

Different routes of administration or types of product, e.g., radiopharmaceuticals or oncology products administered per square meter of body surface, have defined values for *K* in the endotoxin limit calculation as described in ⟨85⟩ and above. Where the product package insert describes multiple patient populations, indications, and routes of administration, it is suggested that the laboratory calculates the limit specification for each administration and chooses the most conservative one as the endotoxin limit specification for the product. A summary is shown in *Table 2*.

**Table 2. Defined Values for *K* in Terms of Route of Administration**

| Route of Administration | *K* | *M* |
|---|---|---|
| Intravenous (IV) for parenteral products | 5 EU/kg of body weight | Maximum dose per kilogram administered in 1 h |
| IV for radiopharmaceuticals | 175 EU | Volume of the maximum recommended dose |
| Intrathecal (IT) for parenteral products | 0.2 EU/kg of body weight | Maximum dose per kilogram administered in 1 h |
| IT for radiopharmaceuticals | 14 EU | Volume of the maximum recommended dose |
| Parenterals administered per square meter of body surface | 100 EU/$m^2$ | Maximum dose per square meter per hour |
| Injections other than IV (intramuscular, subcutaneous, etc.) | 5 EU/kg of body weight | Maximum dose per kilogram administered in 1 h |

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**Table 2. Defined Values for *K* in Terms of Route of Administration** *(continued)*

| Route of Administration | *K* | *M* |
|---|---|---|
| Intraocular fluids | 0.2 EU/mL (*15*) | — |
| Anterior segment solid devices | 0.2 EU/device (*15*) | — |
| Ophthalmic irrigation products | 0.5 EU/mL (⟨771⟩) | — |
| Injected or implanted ophthalmic drug product | 2 EU/dose (⟨771⟩) | — |

Some *USP* product monographs have endotoxin specifications defined at a targeted concentration for administered product. However, endotoxin limit specifications should be calculated for all indications in the product's package insert because indications and administrations for the product may be different from the data used to calculate the original *USP* monograph limit. If a firm's most stringent limit is lower than the USP limit, the firm should use its lower calculated endotoxin limit.

It is important that the endotoxin limit specification, as a critical quality attribute for a new product, be calculated early in development and monitored throughout development and early-stage clinical trials. If a dose has not been established, the limit should be calculated based on the worst case (highest) dose that is anticipated for the product with respect to the target patient population and the route of administration. Early phase endotoxin limits may change based on dosing and/or formulation changes prior to commercialization.

## Relevance of Limits for Compounded Sterile Preparations

When sterile compounding pharmacies prepare therapies for injection or infusion, care must be taken to avoid the addition of endotoxins to the preparations. The compounding pharmacy should use only product contact materials that they have depyrogenated in house or that they have received as sterile and free of detectable endotoxins (see *Screening and Qualification of Consumables: Compendial Requirement* earlier in the chapter). When diluents or intravenous (IV) solutions are used for preparing a product intended for IV, IM, intraocular, or intrathecal (IT) administration, the diluents should be commercially obtained and should meet the compendial limits, which most commonly are 0.5 EU/mL. If the required diluent is not a *USP* monograph article, it should be manufactured to meet the compendial limit of 0.5 EU/mL. If the required diluent is to be used for IT administration, it is essential that the laboratory ensure that the diluent plus drug product does not exceed the more stringent IT endotoxin limit.

## Calculating Endotoxin Limits for Active Substances and Excipients

The control of the levels of endotoxins in excipients and active substances can minimize the risk of finished drug product contamination. Suppliers and drug manufacturers should perform risk assessments of these substances based on raw material origins, production methods, representative sampling and testing, and storage conditions. For example, materials of natural origin such as sugars, heparins, and enzymes may contain significant levels of endotoxins and/or glucans. Suppliers of these materials should be audited or closely evaluated to ensure control of bioburden and endotoxins in their manufacturing operations. If these suppliers provide a CoA regarding the endotoxins or glucan content for individual lots of material, the evaluation should also include an assessment of the validity of test methods and the accuracy of test results and any testing history.

Consideration should be given to using a glucan blocker for products or materials that may contain glucans in addition to endotoxins. Glucan blockers are used to ensure that all tests are specific to endotoxins, preventing a false-positive reaction of the test to glucans. Because products do not have glucan specifications, glucan-blocking reagents are specific to assay methods and lysate formulations and are generally offered by lysate reagent manufacturers. Because glucan-blocking reagents are used in different ways, it is recommended that laboratories follow the reagent manufacturers' instructions for the use of these reagents.

If noncompendial materials or articles are tested and released in-house, endotoxin limits should be assigned after a thorough understanding of their potential contribution to the formulation. Working backwards from the calculated drug product specification for endotoxins, limits can be assigned to each individual component in the formulation with the assurance that the drug product limit would not be exceeded if each component were at its limit. If the same component is used in multiple formulations, the lowest limit should be used for the testing and release of that component.

## Calculating Endotoxin Limits for Combination Products

A combination product is defined as a product comprised of two or more regulated components (i.e. drug/device, biologic/device, drug/biologic, or drug/device/biologic) that are physically, chemically, or otherwise combined or mixed and produced as a single entity. It also is defined as two or more separate products packaged together in a single package or as a unit and comprised of drug and device products, device and biological products, or biological and drug products (*16,17*). Typically, these products can be two or more regulated components presented as a single-entity product (e.g., prefilled syringe), or a kit where two or more regulated products are packaged for use together as a single-entity product (e.g., a lyophilized drug packaged with a diluent and a syringe). Although a manufacturer can propose and justify unique limits for review in regulatory submissions, there are some general points to consider:

- If the combination product is a drug/device combination such as a prefilled syringe, the prefilled syringe may be tested as a filled unit and the endotoxin limit for the drug product prevails. Any endotoxins contributed by the container (device) are assumed to be eluted with the drug product during sample preparation and, therefore, are accounted for in the product's assayable endotoxins.

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- If the combination product is two drugs to be administered simultaneously [IV or intramuscular (IM)], then the endotoxin content of the combined dose may not exceed the endotoxin limit for drugs of 5 EU/kg/h for IV or IM administrations or 0.2 EU/kg/h for IT administration.
- If the combination product is a kit containing multiple components that are administered as a single entity (e.g., a lyophilized product/diluent/syringe) the endotoxin content of the combined dose may not exceed the endotoxin limit for drugs of 5 EU/kg/h for IV or IM administrations or 0.2 EU/kg/h for IT administration.

## Calculating Endotoxin Limits for Medical Devices

Chapter ⟨161⟩ assigns the endotoxin limit for medical devices as 20 EU/device except for those medical devices that come in contact with the cerebrospinal fluid, which has an assigned limit of 2.15 EU/device. Devices that contact the anterior segment of the eye should not exceed a limit of 0.2 EU/mL or 0.2 EU/device, as appropriate. Endotoxins in or on solid matrix medical devices are not measured directly, but rather the device is rinsed, soaked, or extracted in an appropriate volume of solvent (generally *WFI*) and the extracts are tested; in some cases extracts from several devices are pooled for testing. As a result, the endotoxin limit for a device extract is expressed in EU/mL, which can later be converted mathematically to EU/device. The endotoxin limit for an extract is inversely proportional to the volume of solvent used for the extraction. The relationship is:

$$\text{Endotoxin Limit} = \frac{K \times N}{V}$$

*K* = where endotoxin limit per device (20 EU unless otherwise defined and justified; 2.15 EU/device for IT devices)
*N* = number of devices represented in the pool
*V* = total volume of solvent used to extract the devices

For example, if the laboratory tests 10 IT devices, each with an extraction volume of 50 mL, the endotoxin limit specification for the pooled extract is:

$$\text{Endotoxin Limit} = \frac{K \times N}{V} = \frac{(2.15\ \text{EU/device}) \times (10\ \text{devices})}{500\ \text{mL}} = 0.04\ \text{EU/mL}$$

[NOTE—Although primary packaging components such as vials or stoppers may be tested using the techniques described in ⟨161⟩, they are not considered to be medical devices. The laboratory should assign endotoxin limits to these components that are much lower than the limits for standard medical device, but appropriate for the drug formulation and presentation. Also some medical devices are liquid (e.g., dialysis fluid) or a solid (e.g., an enzyme). For these products, the endotoxin limit is calculated or assigned and tested as if the device were a drug.]

## Maximum Valid Dilution

As product-associated interferences are diluted, so will any endotoxins in the sample be diluted. Therefore a calculation called the MVD is included in the compendial chapter to define the upper bound of allowable product dilution. The MVD is dependent on the endotoxin limit for the product, the starting concentration of the product (generally the concentration of the active ingredient), and the sensitivity of the test method. The MVD is defined as:

$$\text{MVD} = \frac{(\text{Endotoxin Limit}) \times (\text{Product Concentration})}{\lambda}$$

where

- The endotoxin limit is the calculated limit for the product or device.
- The product concentration equals the concentration of the active ingredient in units per milliliter. For those products administered on a milliliter per kilogram basis or for medical device extracts, the product concentration equals 1.
- $\lambda$ = the confirmed label claim sensitivity for gel-clot method or the lowest point on the referenced standard curve for the quantitative tests

Additional information on the MVD includes the following:

- The endotoxin limit is constant for any given formulation/dose/administration.
- The MVD is directly related to the starting concentration of active ingredient, and the higher the starting concentration, the higher the MVD.
- The MVD is inversely related to the numerical value given to the test method sensitivity. The more sensitive the test ($\lambda$ as the denominator in the MVD formula gets lower), the higher the MVD. Changing the test sensitivity to a lower number (more sensitive test) will assist in providing additional dilution room for interfering products.
- The units in the formula cancel out. The resulting calculated value is a dilution factor with no units. For example, an MVD of 100 means that maximum dilution for the product concentration can be diluted no further than 1:100 to have a valid test.
- The MVD does not limit the necessary product dilution when determining the true amount of product contamination in a sample that fails to meet the endotoxin limit. When a product fails at the MVD, it does not meet the endotoxin limit requirement. In this case, it is recommended that the laboratory determine the total bacterial endotoxin content in that product by dilution to extinction (a negative test for gel clot and a valid reading on the quantitative standard curve). The actual level of endotoxin contamination in the product may prove very helpful for trending purposes and to determine the root cause of the contamination during the OOS investigation.

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# Method Suitability Testing

## INTERFERENCE SCREENING FOR DRUG PRODUCTS, INCLUDING BIOLOGIC DRUGS

The principles and practice for performing inhibition/enhancement testing are provided in ⟨85⟩, *Gel-Clot Technique, Preparatory Testing, Test for Interfering Factors* or ⟨85⟩, *Photometric Quantitative Techniques, Preparatory Testing, Test for Interfering Factors*. Although historically suitability tests utilizing three consecutive lots of drug product were considered sufficient to assess suitability, it is recommended that an appropriate number of lots of product be determined prospectively for suitability testing to enable a valid assessment for the potential of lot-to-lot variability in endotoxins content. This is especially important for biological products or products where product development and process validation has indicated significant lot-to-lot variability. Products with greater variability in their starting material, API, and manufacturing process will typically require more than three lots for suitability testing, whereas for products with little or no process or product variability, three lots may suffice. The number of lots tested for suitability should be supported by a risk assessment including information on the life cycle stage of the product (clinical/commercial), known sources of variability, and material testing history that could support an increased or decreased number of lots chosen for suitability testing. All materials that are being tested using methods described in ⟨85⟩ including excipients and raw materials, should have a suitability study to assure that any interferences and variability are identified, mitigated (if necessary) and taken into account in the testing procedures.

## COMMON TEST INTERFERENCES

Most pharmaceutical products have been found to interfere to some extent with BET performance (*18*). Because of the high assay sensitivity, these product-specific interferences can usually be overcome by dilution in Water for BET [see *Bacterial Endotoxins Test* ⟨85⟩, *Reagents and Test Solutions, Water for Bacterial Endotoxins Test (BET)*], not to exceed the product-specific MVD. Interferences may affect either the enzyme cascade of the LAL reaction itself or the analyte used as the PPC (e.g., purified LPS), standard, or both. *Table 3* is a listing of common interferences and mitigations that may be considered and implemented beyond mere dilution (*19*). Where buffers or solvents other than Water for BET are used for dilution they should be free of detectable endotoxins.

**Table 3. Common Interferences and Mitigations**

| Interference | Interferes With | Mitigation |
|---|---|---|
| pH | LAL cascade | Dilution in Water for BET<br>Adjust pH of the product with ▲hydrochloric acid▲ (ERR 1-Dec-2020), sodium hydroxide, or dilution in tris buffer so that lysate + sample is within the optimal range specified by the lysate manufacturer. |
| Osmolarity | LAL cascade | Dilution in Water for BET is usually sufficient. |
| Chelating agents | LAL cascade<br>LPS aggregation | Dilution in Water for BET<br>Add a magnesium ion-containing buffer. |
| Glucan | LAL cascade | Use a glucan blocker supplied by the reagent manufacturer. |
| Nonspecific protein interference (e.g., serine proteases) | LAL cascade | Dilution in Water for BET<br>Dilution in *Water for BET* combined with heat |
| Heavy metals | LAL cascade<br>LPS aggregation | Dilution in Water for BET or diluents containing 1 mM chelating agent ethylenediaminetetraacetic acid (EDTA) |
| Proteins | LAL cascade<br>LPS aggregation | Dilution in Water for BET or normal saline<br>Dilution in Water for BET combined with heat |
| High detergent | LAL cascade<br>LPS aggregation | Dilution in Water for BET is usually sufficient. |
| Calcium cation | LAL cascade | Dilution in Water for BET or diluents containing 1 mM chelating agent EDTA |

A majority of test interferences are overcome by simple dilution. Dilution can be in Water for BET, buffers (e.g., tris or HEPES), buffers containing glucan-blocking agents, and buffers containing divalent cations or chelating agents as required. Dispersing agents may be added to diluents to further mitigate interference, and their value as an additive is typically considered during method development. It is noteworthy that glucan interference is a common interference in some biological products. Where glucan interference is likely, the use of a glucan blocker should be considered during assay suitability test method development. Diluents other than Water for BET and common buffers described above should be checked to assure that they do not interfere with the BET assay. For example, some neutralizing agents (e.g., proteinase K) may not be manufactured with appropriate controls against their contamination with GNB. If these reagents are used, they should be checked to assure that they do not contain endotoxins at levels that may contribute to the levels ultimately reported for the product. All BET assays must be conducted at neutral pH. Neutral pH for BET assays is generally defined as 6.0–8.0, but because all lysates are different in their formulations, the laboratory should confirm the proper range as described in the lysate manufacturer's package insert. However, the reagent itself may provide buffering capacity so the mixture of lysate and product dilution should be tested for pH during the suitability study. pH may be adjusted to neutrality by the use of buffers as diluents or endotoxin-free ▲hydrochloric

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acid▲ (ERR 1-Dec-2020) (HCl) or sodium hydroxide (NaOH). If adjustment of pH is required using acid or base, it is suggested that the pH be measured during the suitability study to assess lot-to-lot variation in the product.

Lysates from different manufacturers have different proprietary formulations, and all are calibrated for sensitivity against the RSE to ensure that they all detect LPS activity equally well in water. However, LPS activity may not be recovered equally in different products, or by using different compendial test methods. If there is significant product-specific interference that cannot be overcome by dilution to within the MVD or by standard mitigation methodologies (*Table 3*), the laboratory should consider a different lysate formulation or test method before concluding that the LAL-bacterial endotoxins test is either invalid or unacceptable (*20*).

### Qualifying Test Preparation Methods Other than Dilution

For sample preparation that requires manipulation other than simple dilution, e.g., heating or ultrafiltration, ⟨85⟩ instructs the analyst to proceed as follows:

> Interference may be overcome by suitable treatment such as filtration, neutralization, dialysis, or heating. To establish that the chosen treatment effectively eliminates interference without loss of endotoxins, perform the assay described above using the preparation to be examined to which Standard Endotoxin has been added and which has then been submitted to the chosen treatment.

## ROUTINE TESTING

### Sampling

All materials used to sample materials for endotoxin content (scoops, pipettes, bottles) must be inert with respect to the material(s) being sampled, and they must be sterile and free of detectable endotoxins. Even though samples are often taken in the field without the aid of a laminar flow hood, samplers must take precautions that a) they do not contaminate the sample itself, and b) in taking the sample, they do not contaminate the rest of the material in its original container.

Historically, the sampling scheme for finished drug products is to take at least 3 units representing the beginning, middle, and end of the batch. However, for a standard run of a small-volume parenteral, biological therapy, or large-volume parenteral, 3 units may not be a representative sample and may identify only those lots that are uniformly and highly contaminated. Sampling schemes should be justified and should be based on the known variability of the process, the unit operations of the process, historical knowledge of the process, and materials used in manufacture.

Bacteria and endotoxins are generally not homogeneously distributed in any product or material, liquid or powder. Care must be taken to flush ports in a circulating water loop before sampling and use the same equipment for sampling that is used for manufacture to assure a representative sample. For example, if a hose is attached to a port for the purpose of transferring water from a circulating loop to another formulation vessel, the water sample must be taken from the same hose with the same flush times as used by manufacturing. So, while the water within a properly constructed and controlled loop may theoretically be homogeneous, differences in the ports along the distribution loop may add endotoxins to samples.

For highly viscous materials, a suitable inert rod free of detectable endotoxins may be used to mix the material prior to sampling. Sterile, pyrogen-free spatulas and scoops are used for powdered and granular solids. Because of the lack of assurance of homogeneity, the number of samples from viscous materials and powders should be determined relative to the number of units initially received using appropriate statistical procedures.

Particularly for samples of viscous and powdered materials, samplers should have training on how to spot signs of non-uniformity. Such indicators may include differences in shape, size, or color or evidence of moisture in powdered materials. For viscous materials, samplers should be trained to look for stratification of the material, differences in viscosity or colors, and particulate contamination. Samples of raw materials that could exhibit variability in endotoxin content should not be pooled (see *Pooling*).

In the case of a new vendor of a raw material, the methodology and calculations used in the determination of the endotoxin content of the material should be reviewed. If the material is designated as critical for manufacturing, a site audit of the vendor may be in order. In addition, it is highly suggested that an in-house confirmation of the accuracy of the endotoxin level stated on the CoA be performed. For critical materials, the quality agreement should require that manufacturers of these materials inform the pharmaceutical manufacturer of changes in processes, controls, or raw materials so that the changes can be discussed at change control and the need for re-confirmation of materials can be determined. Once the CoAs have been successfully confirmed for a predetermined number of shipments justified in the sampling plan by history, and depending on the variability of the manufacturing process, the laboratory can consider accepting the CoA with periodic testing of the incoming materials, as long as the manufacture of the materials has not changed.

### Pooling

The term "pooling" refers to creating a composite sample preparation that includes the total contents of several individual units or equal aliquots from the units taken from the same lot or batch. Pooling is often done for batch-release testing. Pooling is an acceptable option for laboratories performing any of the BET assays on drug products (*9*). However, pooling has a number of drawbacks; the most obvious is that it will dilute endotoxins that may be in any one of the units, potentially concealing the variability in endotoxin content and distribution among the units that were pooled. Sampling plans, including instructions to pool samples, should be scientifically justified.

If units are pooled, the MVD must be adjusted to account for the possibility of endotoxins in just one of the samples (*9*). The adjusted MVD is calculated by dividing the originally calculated MVD by the number of units contributing to the pool. For example, if the MVD for a small volume parenteral is 240 and a manufacturer chooses to pool three vials for testing, the adjusted

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MVD is 240/3, which is 80. This reduction in MVD effectively reduces the endotoxin limit for the product by a factor of three to compensate for pooling. While pooling may result in an incremental savings in reagent usage and therefore save some money, there are a number of points to consider when pooling:

- Pooling may obscure any non-uniformity in endotoxin content between the individual sample units. Information on variability may be valuable in troubleshooting or investigations. For example, random contamination in one of multiple filling needles may cause some vials to contain endotoxins and others not.
- Taking aliquots of samples for pooling should always be performed using aseptic technique and with individual units vigorously mixed prior to removing the aliquots. The original containers with remaining product should be retained for investigation in the event of an OOS test result. Removing the aliquot through the disinfected rubber stopper using a pyrogen-free syringe is advisable to maintain the integrity of the unit container during subsequent storage for investigative testing.
- The concept of adjusted MVD does not apply to medical devices as they are, by convention, commonly pooled for testing.
- Any sampling scheme for drug products must represent the beginning, middle, and end of the batch. Additional samples may be taken if interventions in manufacturing raise concerns about possible endotoxin contamination.
- If testing at the adjusted MVD causes an unacceptable increase in product-specific interference, samples should be tested individually.
- Products with low calculated MVDs, or suspensions where there is no assurance of homogeneity in the removed aliquots, may not be good candidates for pooling.
- Pooling is not appropriate for in-process samples, particularly those representing different stages of manufacturing.

## Calculation of Endotoxin Content

All BET results are provided in EU/mL. If the product being tested has a limit expressed in EU per unit of weight or activity (e.g., EU/mg), a calculation must be made to convert EU/mL to EU/mg. For example, the endotoxin content for a product with a starting concentration of 10 mg/mL, after adjustment for the dilution factor, is determined by gel clot to be 5 EU/mL. The endotoxin content expressed in EU/mg is:

$$(5\ \text{EU/mL}) \div (10\ \text{mg/mL}) = 0.5\ \text{EU/mg}$$

## Out-of-Specification Results and Retesting Considerations

An endotoxins test result that does not meet product specifications necessitates an investigation (*21*). The suspect result is considered valid unless a comprehensive investigation clearly demonstrates otherwise.

Invalid tests, however, are not OOS results. Invalid tests are those where system suitability parameters such as negative controls, PPCs, confirmation of label claim in gel clot, or generation of a linear standard curve do not function as expected and therefore may affect the accuracy of the test results. Although invalid tests should be tracked and trended to look for patterns and trends that might require a corrective action, a true OOS test failure only exists when a valid assay has been performed and generates results that exceed the specification.

A finding of a nonconforming test result requires a laboratory assessment to assure the accuracy of the data. The FDA OOS guidance for industry terms this a Phase I investigation (*21*). Despite the fact that a failed BET assay is rare, the investigation should be done in a timely manner to ascertain if there was anything atypical about this sample, its preparation, calculations, or performance of the test that may affect the accuracy of the test result. Phase I should include a critical and in-depth review of sampling methods and techniques, sample hold conditions, sample preparation, test method parameters, and any documentation, including real-time notations of testing errors by analysts and a review to find any errors in calculation. An organizing tool, such as a fault tree analysis (FTA) or Ishikawa (fishbone) diagram, may help the team members to organize their thoughts and complete the investigation in a timely and compliant manner.

The use of a checklist approach will promote completeness and consistency across all assessments.

The purpose of the Phase I laboratory investigation is to confirm the accuracy of the original test result. A Phase I checklist should include the following, at a minimum:

- The testing history of the material under test: Has a specific assay problem or repeated failures for the same product been previously observed?
- Sampling procedures, sample container integrity, and sample preparation: Were samples taken, transported, and stored correctly, following approved methods?
- Reagent preparation, storage, and use.
- Calibration and maintenance status of dispensing, incubation, and reading instrumentation.
- Appropriateness of sample contact equipment and disposables.
- The test procedure: Is it consistent with the suitability data, and was it precisely followed?
- Were all controls and other system suitability indicators within normal limits? If not, then the test is not OOS and is invalid.
- Were all calculations (product endotoxin limits, MVD, and endotoxin contamination levels) performed correctly? Were all transcriptions performed accurately?
- Was the analyst adequately trained? What is the analyst's OOS history?

An approved BET standard operating procedure (SOP) should include two important directions to analysts:

1. If an analyst realizes and documents that a mistake has been made during the testing, including an error in calculation, sample preparation, dilution, or performance of the assay that could affect the accuracy of the test results, the test should be stopped and the reason for the termination of the test documented at the time that the error was identified.

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2. Analysts should not discard the original sample, any of the sample preparation dilution tubes, or the reagents used until the results of the assay are known and evaluated against the product specification. The tubes and reagents from an OOS assay may be an important part of the laboratory investigation.

If there is no analytical failure or documentation error found, the result is considered valid and the product is OOS. On the other hand, if the analysis was conducted incorrectly and documented error(s) are the root cause of the failure, the initial assay is invalid and the product may be tested again with appropriate controls.

Investigation of production practices, otherwise known as Phase II investigations, require assessment of all manufacturing activities that could have impacted the endotoxin content of the failed product sample. Phase II may be more easily conducted by a cross-functional team including operations, facilities, and engineering, with assistance from a microbiologist to identify potential sources of contamination relating to contamination control requirements during manufacturing.

The Phase II investigation is actually a reassessment of validated process control, particularly if the failure was not the first failure for the product. A product manufactured under a state of control should not fail endotoxins testing, so the Phase II investigation should focus on anything that may have been different about the lot in question.

At any point in the investigation, testing can be performed to challenge hypotheses regarding the reason for the failure of the material to meet its limit. However, it is important to note that these investigational tests are not considered to be re-tests and should not be used to release material. Rather, they are used to identify conditions that might have contributed to an inaccuracy in the testing of the product. A laboratory SOP or policy should also be written that precisely describes the procedure(s) that must be followed for the purposes of investigational testing. Such a plan eliminates situational decisions regarding the appropriateness of investigational testing, retesting, or resampling; the plan should be approved by the quality unit.

Investigational testing should be well-defined and justified, pre-approved, and well-documented. If an investigational second test of the original sample preparation finds that the product meets the specifications for the test, it is possible that an error was made in some aspect of the execution of the original assay or just as easily in the execution of the second test. If this is the case, any hypotheses should be proposed and testing executed to confirm that an error was made. The intended purpose of the investigational tests on the sample is not to reverse the original OOS result but rather to gain information regarding a potential failure so that a more effective effort can be made to pinpoint the problem and implement any necessary corrective and preventive actions.

The statement in ⟨85⟩ that reads “In the event of doubt or dispute, the final decision is made based upon the gel-clot limit test unless otherwise indicated in the monograph for the product being tested” has been widely misinterpreted and is relevant *only* to the specific case of a conflicting result between a regulatory agency using the gel-clot method for lot release testing and a testing laboratory using a different LAL-bacterial endotoxins test platform. It is not intended to allow a laboratory to use a gel-clot result that provides passing results when the laboratory’s customary assay method for the product shows an OOS test result.

In the event of a product failure, the total level of endotoxins activity in the failed sample(s) should be determined. For gel-clot assays, this may involve testing of dilutions beyond the MVD until an endpoint is reached for gel clot or a valid test result is obtained for quantitative tests. These values may provide a clue as to the source of potential or actual product contamination, or a case of mishandling of a positive control, or a tube mix-up.

## Standard Curve Control

Standard curves are created for endpoint chromogenic assay by evaluating the direct relationship between color intensity and standard concentration. The resulting curve has a positive slope, and ⟨85⟩ indicates that the correlation coefficient of the curve must be ≥0.980.

The standard curve is created by plotting the $\log_{10}$ of the onset or reaction time required for each standard to reach a predetermined optical density for kinetic turbidimetric assays or color intensity for kinetic chromogenic assays, as a function of the $\log_{10}$ of the standard concentration. This transformation of the data results in a standard curve with a negative slope, and the correlation coefficient must be $\leq -0.980$. Therefore, it can be stated that the linearity requirement for all compendial quantitative BET assays is $|r| \geq 0.980$. The requirement is expressed to three significant figures because the last “0” is important; it is inappropriate to have a standard curve with $|r| = 0.979$ and round up. However, in a well-controlled laboratory, correlation coefficients should routinely be greater than 0.980.

All standard curves have a corresponding linear equation $y = mx + b$ where $m$ is the slope of the standard curve regression line and $b$ is the $y$-intercept. Because the $y$-intercept is the point where $x = 0$, and because in a kinetic assay the $\log_{10}$ of 1 is 0, the $y$-intercept is really at the 1 EU standard. Because of the transformation of data to $\log_{10}$, standard curves for quantitative assays can be very sensitive to small changes in onset times.

Accuracy of test results depends on the accuracy of the standard curve. Therefore, the onset times for standards representing unique combinations of lysate lot and CSE lot from run to run, instrument to instrument and analyst to analyst should all be monitored. For example, an analyst can make a twofold or tenfold dilution error in the dilution of the standards, yet still produce a linear standard curve that meets the requirement $|r| \geq 0.980$. The dilution error would not be noticed by looking at the correlation coefficient alone, but would be evident in the onset times of the standards. An overdiluted set of standards would run more slowly (longer onset/reaction times) than a properly diluted standard series, and an underdiluted set of standards would run more quickly (shorter onset/reaction times) than a properly diluted standard series. The dilution error would also be reflected in the values generated for the $y$-intercept. Changes in slope can also affect the accuracy of the test data. It is suggested that part of an analyst’s training in quantitative assay performance is to understand the impact of variability on the accuracy of the test result (*22*,*23*).

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## ALTERNATE TEST METHODS

The methods listed in ⟨85⟩ for the detection of bacterial endotoxins (gel-clot limits test, gel-clot assay, kinetic chromogenic, endpoint chromogenic, kinetic turbidimetric) are considered to be validated. However, a laboratory may choose to use an assay methodology that is not listed in ⟨85⟩. If such a choice is made, the alternate test for the detection of bacterial endotoxins must be fully validated to ensure that decisions made using the alternate methodology are equivalent to or better than decisions made using the validated USP methods and ultimately approved by the appropriate regulatory authority. Although endotoxin testing is not specifically cited, guidance on how to think about the validation of alternate methods can be found in *Validation of Alternative Microbiological Methods* ⟨1223⟩ and *Validation of Compendial Procedures* ⟨1225⟩.

## GLOSSARY

**Bacterial endotoxins:** A GNB outer membrane macromolecular complex of polysaccharide, lipid, and protein. Extracted, purified, and highly concentrated (protein free) endotoxin is generally referred to as lipopolysaccharide or LPS to distinguish it from the more natural complexed cell membrane associated form (*24*).

**Bacterial endotoxins test (BET):** Compendial bacterial endotoxins test.

**Control standard endotoxin (CSE):** A preparation with a stable endotoxin concentration calibrated against RSE. CSEs are generally prepared by vendors or by laboratories, and although called "standards," are not certified for either purity or activity by a third party. CSEs are best described as calibration analytes. CSEs may be purified or they may be a preparation of native endotoxins. If a CSE is a preparation not already adequately characterized, its evaluation should include characterizing parameters such as activity, uniformity, and stability, which would demonstrate the suitability of the material to serve in the calibration of a BET. Detailed procedures for its preparation and use to ensure consistency in performance should also be included.

**Intrathecal:** A parenteral injection that results in the product coming in contact with the cerebrospinal fluid.

**Lysate or limulus amebocyte lysate (LAL):** The reagent used in the performance of the BET

**Parenteral:** Drugs or medical devices that are injected, infused, or implanted or that may otherwise come in contact with the bloodstream or cerebrospinal fluid.

**USP Endotoxin Reference Standard (RSE):** Primary endotoxin standard. RSE is an extracted, purified, and formulated preparation of *E. coli* 0113:H10:K LPS.

**Suitability:** Demonstration that the chosen assay is appropriate for its defined purpose in testing the material.

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# ⟨1086⟩ IMPURITIES IN DRUG SUBSTANCES AND DRUG PRODUCTS

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## ▲INTRODUCTION

Impurities are critical quality attributes of drug substances and drug products because they have the potential to affect the safety and efficacy of the product. This general information chapter provides guidance on the control of impurities in drug substances (process-related impurities and degradation products) and drug products (degradation products). (Definitions of key terms used in this chapter can be found in the *Glossary*).

This chapter covers drug substances and drug products described in the *USP–NF*. This chapter addresses impurities arising during the manufacturing process and/or storage of the drug substance. Also the chapter covers those impurities in drug products classified as degradation products of the drug substance or reaction products of the drug substance with an excipient and/or immediate container–closure system (collectively referred to as "degradation products").

This chapter does not cover veterinary products, biological/biotechnological products, peptides, oligonucleotides, fermentation products and semisynthetic products derived from them, polymorphic forms, radiopharmaceuticals, herbal products, or crude products of animal or plant origin. In addition, impurities in excipients and leachables from the container–closure system are out of the scope of this chapter.

The regulatory and compendial standards for the control of impurities continue to evolve due to advancements in analytical science, technology, and toxicology. Therefore, communications about impurities and degradation products in drug substances and drug products can be improved by including in this Pharmacopeia the definitions for terms and the contexts in which these terms are used (see the *Glossary*). See *General Notices, 5.60. Impurities and Foreign Substances* for additional information about impurities. Some other general chapters added over the years have also addressed topics of purity or impurity as these have come into focus or as analytical methodology has become available. Analytical aspects are enlarged upon in *Chromatography* ⟨621⟩, *Validation of Compendial Procedures* ⟨1225⟩, and *Verification of Compendial Procedures* ⟨1226⟩.

Impurity measurements for drug products using chromatographic methods could present a challenge to the Pharmacopeial standards setting due to low concentration of the impurity and complexity of matrix. As a consequence, many monographs for Pharmacopeial preparations rely on chromatographic assays. Where more significant impurities are known, some monographs set forth specific tests for these impurities. In general, however, this Pharmacopeia does not quantify in drug products impurities that are evaluated in drug substances and that are not expected to increase in the drug product during manufacturing or storage (i.e., impurities that are not degradation products). It is assumed that adequate retention specimens are in storage for the exact batch of drug substances used in any specific lot of a drug product. Whenever analysis of a drug product raises a question of the quality attributes of any of the drug substances used, subsequent analysis of retention specimens is in order.

## DRUG SUBSTANCE

Impurities in drug substances can be classified into the following categories:

1. Organic impurities
2. Inorganic impurities
3. Residual solvents

Organic impurities can arise during the manufacturing process and/or storage of the drug substance. They can be identified or unidentified, volatile or nonvolatile, and include the following:

1. Starting materials
2. Reaction byproducts
3. Intermediates
4. Degradation products
5. Reagents and ligands

Inorganic impurities can result from the manufacturing process. They are normally known and identified and include the following:

1. Reagents and catalysts
2. Elemental impurities
3. Inorganic salts
4. Other materials (e.g., filter aids)

Elemental impurities can include catalysts and environmental contaminants that may be present in drug substances. These impurities may occur naturally, be added intentionally, or be introduced inadvertently (e.g., by interactions with processing equipment and the container–closure system). When elemental impurities are known to be present, have been added, or have the potential for introduction, assessment of the drug substance in the context of its use in the finished product is required (see *Elemental Impurities—Limits* ⟨232⟩).

Residual solvents originate from organic liquids used as vehicles in the synthesis of a drug substance, as starting material, or as the intermediates involved in the synthesis. Because these are generally of known toxicity, the selection of appropriate controls is easily accomplished (see *Residual Solvents* ⟨467⟩).

Concepts for setting impurity limits in drug substances are based on chemistry and safety concerns. As such, limits for organic and inorganic impurities and residual solvents should be established according to the applicable guidances. Elemental impurity

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limits for a drug substance may be required depending on the outcome of the risk assessment for the finished product. The basic tenet for setting limits is that levels of impurities in a drug substance must be controlled throughout its development to ensure its safety and quality for use in a drug product.

Documented evidence that the analytical procedure used to evaluate impurities is validated and suitable for the detection and quantitation of impurities should be established.

## DRUG PRODUCT

The specification for organic impurities in a drug product should include a list of degradation products expected to occur during the manufacture of the commercial product and under the recommended storage conditions. Stability studies, forced degradation studies, knowledge of degradation pathways, product development studies, compatibility studies, and laboratory studies should be used to characterize the degradation profile. The selection of degradation products in the drug product specification should be based on the degradation products found in batches manufactured by the proposed commercial process.

The rationale for specifications should include a discussion of the degradation profiles observed in the safety and clinical development batches and in stability studies, together with a consideration of the degradation profile of batches manufactured by the proposed commercial process. For degradation products known to be unusually potent or to produce toxic or unexpected pharmacological effects, the quantitation/detection limit of the analytical procedures should be commensurate with the level at which the degradation products should be controlled.

For drug products, the concept for setting degradation product limits is based on sound scientific assessment as applied to available data on the safety and stability of the drug product, data that may include the degradation pathways of the drug substance, the manufacturing process, known excipient interactions, any safety assessment studies (e.g., predictive toxicology programs, mutagenicity assessments, and in vitro and in vivo toxicology studies), stability studies conducted under the recommended storage conditions, and ancillary studies that may provide additional information on the stability profile of the drug product. Impurities that are not degradation products (i.e., process-related impurities from the drug substance) are often not controlled in the drug product, as they are typically controlled in the drug substance and these impurities are not expected to increase over time. In cases where the studies show that impurities from the drug substance can increase over time (i.e., they are also drug product degradation products), they should be controlled in the drug substance and also in the drug product. Additional guidance for setting limits can be found in various International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines, such as ICH Q3A, Q3B, and M7; FDA guidance documents; and *USP* monograph submission guidelines.

Documented evidence that the analytical procedure used to evaluate impurities is validated and suitable for the detection and quantification of impurities should be established.

General chapter ⟨232⟩ specifies limits for the amounts of elemental impurities in drug products.

Drug products should contain no higher levels of residual solvents than can be supported by safety data (see ⟨467⟩).

## ORGANIC IMPURITIES IN DRUG SUBSTANCES AND DRUG PRODUCTS

All drug substances and drug products are subject to control of organic impurities. A threshold-based approach described in the ICH Q3A and Q3B guidelines may be used for the control of organic impurities in drug substances or drug products generated during the manufacturing process or storage (see *Control of Organic Impurities in Drug Substances and Drug Products* ⟨476⟩ for additional information). In this chapter, "impurity" can refer to process-related impurities and degradation products for a drug substance, and "degradation products" can refer to process-related impurities and degradation products for a drug product (see *Glossary* for additional information).

The organic impurities to be controlled in drug substances are the process-related impurities and degradation products. The organic impurities to be controlled in the drug product are those resulting from the degradation of the drug substance or from the interaction of the drug substance with excipients and/or the primary container–closure system. Drug substance process-related impurities need not be controlled in the drug product unless they are also degradation products. However, in some cases drug substance process-related impurities may be included in the drug product specifications, if appropriate, and limited by appropriate acceptance criteria.

As described in ⟨476⟩, unusually toxic impurities (e.g., mutagenic impurities) in drug substances and drug products require more stringent control compared to non-mutagenic impurities.

Manufacturers are responsible for controlling organic impurities in accordance with current regulatory standards. Manufacturers should consider the chemical characteristics and safety aspects of impurities when they identify and classify impurities in a drug substance or drug product. Analytical procedures for the detection and quantitation of impurities should be verified or validated. (For additional information, see ⟨1226⟩ and ⟨1225⟩). For impurities that are known or suspected to be highly toxic (e.g., mutagenic) or that produce undesired pharmacological effects, the quantitation/detection limit of the analytical procedures should be commensurate with the acceptance criteria for the suspected highly toxic impurity and current applicable regulatory guidance to ensure patient safety. Impurities or degradation products that are also significant metabolites and present in animal and/or human studies are generally considered qualified.

If an individual monograph does not include a procedure for quantifying an impurity or acceptance criterion for an observed impurity, the manufacturer is responsible for developing and validating analytical procedures and establishing appropriate acceptance criterion. USP requests submission of the alternative/new procedure to evaluate for potential inclusion in the appropriate monograph(s).

In cases of complex impurity profiles, it may not be feasible to resolve each of the impurities individually or to detect them and quantify them using a single analytical procedure. In such cases, manufacturers should consider alternate approaches such as the use of multiple analytical procedures to test for impurities.

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The acceptance criteria for impurities shall be based on applicable guidances or other acceptable scientific means, with safety as the primary consideration, and not solely based on process capability. In cases of complex impurity profiles, acceptance limits may be established based on grouping of impurities, as appropriate.

Similar principles may be applied to set thresholds and acceptance criteria for degradation products in FDA over-the-counter (OTC) monograph drug products, which are not discussed in ICH guidelines or FDA guidances. Degradation products in these drugs should be reported, identified, and/or qualified.

Measurement of degradation products can be challenging for products containing multiple drug substances and complex formulations. The use of placebo products as controls in stability studies may aid in the deconvolution of chemical changes that could be related to excipients rather than the drug substance.

(Definitions for key terms used in this chapter can be found in the *Glossary*. Additional sources of guidance on impurities in drug substances and drug products may be found in *Appendix 1: Additional Sources of Information and Guidance*.)

## ORGANIC IMPURITIES DECISION TREE

The decision tree shown in *Figure 1* provides guidance for the control of organic impurities in drug substances and drug products.

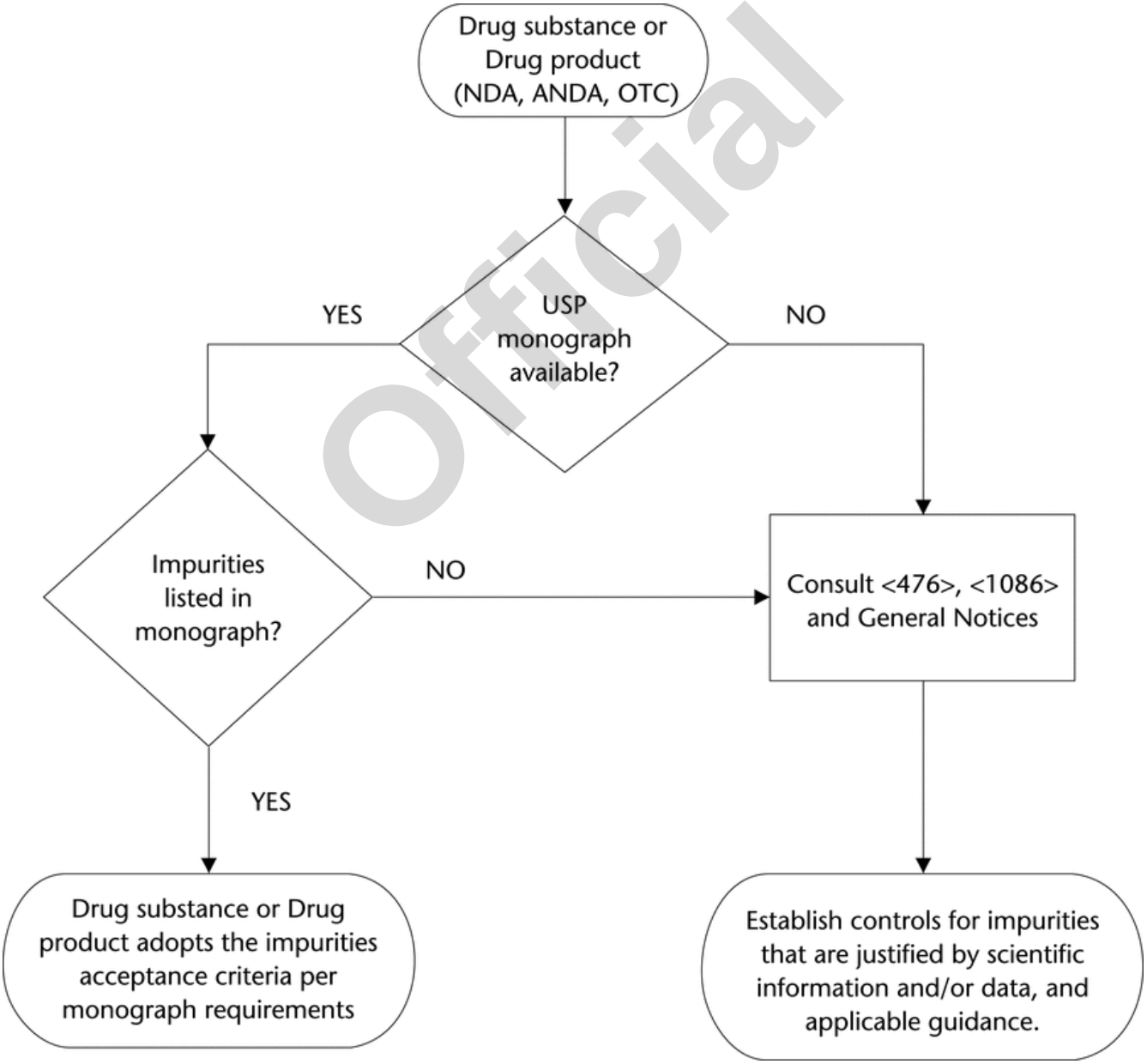


Figure 1. Decision tree for the control of organic impurities in drug substances and drug products. NDA = New Drug Application; ANDA = Abbreviated New Drug Application.

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Note that all impurities specific to a given drug product formulation may or may not be included in the *USP* monograph. If the impurity is listed in the monograph, follow monograph limits. If the impurity is not listed, consult this chapter and ⟨476⟩ for guidance. Nonmonograph tests and acceptance criteria suitable for detecting and controlling impurities that may result from a change in the processing methods or that may be introduced from external sources should be employed in addition to the tests provided in the individual monograph, where the presence of the impurity is inconsistent with applicable good manufacturing practices or good pharmaceutical practices.

## TERMINOLOGY ASSOCIATED WITH ORGANIC IMPURITIES USED IN DRUG SUBSTANCE AND DRUG PRODUCT MONOGRAPHS

The *Organic Impurities* section in a monograph generally includes impurities/degradation products that are known to be detected by the tests prescribed in the monograph. There are several scenarios for citing specified organic impurities in *USP–NF* monographs.

For identified impurities/degradation products (i.e., the ones that have known and characterized chemical structure), the chemical name is usually provided (e.g., in an impurities table or as a table footnote or in the Reference Standard section). They can have reference material available or not, depending on the substance.

Unidentified impurities/degradation products (i.e., the ones for which a structural characterization has not been achieved) are named with a general designation (e.g., impurity 1, impurity 2) or by an analytical property (e.g., chromatographic retention time). The reference material is not available.

Specified impurities/degradation products can be either identified or unidentified. They are listed and limited with a specific acceptance criterion in monographs.

If an impurity/degradation product is not listed and is NMT the identification threshold, it is controlled as an individual unspecified impurity. In all cases the terms “any individual impurity” or “any other individual impurity” correspond to unspecified impurities.

A *USP* monograph of a drug substance may contain acceptance criteria for: specified identified and unidentified organic impurities; “any individual impurity”, "any unspecified impurity", or “any other individual impurity”; and total impurities. Total impurities in the drug substance monographs are the sum of all specified and unspecified impurities above the reporting threshold.

A *USP* monograph of a drug product may contain acceptance criteria for: specified identified and unidentified degradation products; “any individual degradation product”, “any other individual degradation product”, or "any unspecified degradation product"; and total degradation products. Total degradation products in the drug product monographs are the sum of all specified and unspecified degradation products above the reporting threshold.

The preferred terms applicable to organic impurities procedures are “impurity” and “total impurities” in the drug substance monographs and “degradation product” and “total degradation products” in the drug product monographs. Only if the drug substance process-related impurities are included in the drug product organic impurities procedure should the term “impurity” be used and the total be expressed as “total impurities”. Drug substance process-related impurities also detected in the drug product and included in its specification may include a note that certain drug substance process-related impurities are listed for information only and should not be included in the “total degradation products”. When this note is included, the total degradation products should only include all specified and unspecified impurities/degradation products above the reporting threshold, with the exception of these designated process-related impurities.

Some monographs may state “disregard any peak below [… certain value]” in chromatographic tests. Typically, the disregard limit for substances covered by a monograph is set in accordance with the reporting threshold given in ⟨476⟩. In this context, the disregard limit is the decision criterion (the numerical value) for the user whether a peak response of an impurity is to be included or excluded in the total impurities.

In chromatographic tests, during quantitation, peaks caused by solvents and reagents or arising from the mobile phase or the sample matrix shall be disregarded, as well as other peaks that the monograph explicitly states are to be disregarded. When the impurity test prescribes the total of impurities or there is a quantitative determination of an impurity, choice of an appropriate threshold setting and appropriate conditions for the integration of the peak areas is important. Principles from ⟨621⟩, ⟨1225⟩, and ⟨1226⟩, such as signal-to-noise ratio, quantification limit, and quantitation methods, should be observed. The quantitation limit for the analytical procedure should be NMT (≤) the reporting threshold.

## GLOSSARY

**Degradation product:** An impurity resulting from a chemical change in the drug substance brought about during manufacturing and/or storage of the drug substance or drug product by the effect of, for example, light, temperature, pH, water, or by reaction with an excipient and/or the immediate container–closure system.

**Disregard limit:** See *Reporting threshold*. [NOTE—It may appear in a monograph as “disregard any peak below [.. certain value]”.]

**Drug substance process-related impurity:** An impurity generated during drug substance manufacturing. Process-related impurities may include starting materials, byproducts, intermediates, reagents, ligands, and catalysts. In this chapter, it is also called “process impurity”.

**Identification threshold:** A limit above which an impurity should be identified (i.e., its structural characterization).

**Identified impurity/degradation product:** An impurity or degradation product for which the structural characterization has been achieved.

**Impurity:** For a drug substance, any component of the drug substance that is not the chemical entity that is defined as the drug substance; for a drug product, any component of a drug product that is not the drug substance or an excipient in the drug product.

**Qualification:** The process of acquiring and evaluating data that establish the biological safety of an individual impurity or a given impurity profile at the level(s) specified.

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**Qualification threshold:** A limit above which an impurity should be qualified.

**Reporting threshold:** In chromatographic tests, the limit above which an impurity should be reported and should be taken into account for calculating total impurities/total degradation products. Synonyms include "reporting level" and "reporting limit". The older term "disregard limit" still appears in some *USP–NF* monographs; however, the ICH Q3 term "reporting threshold" is preferred. Peak responses must be corrected by the relative response factor when the information is provided in the individual monograph.

**Specified impurity/degradation product:** An impurity or degradation product that is individually listed and limited with an acceptance criterion in the drug substance or drug product monograph. A specified impurity or specified degradation product can be either identified or unidentified.

**Total impurities/total degradation products:** In a drug substance monograph, "total impurities" are the sum of all specified and unspecified impurities above the reporting threshold. Unless otherwise indicated, "total degradation products" in a drug product monograph are the sum of all specified and unspecified degradation products above the reporting threshold. Drug substance process-related impurities also detected in the drug product and included in its specification may include a note that certain drug substance process-related impurities are listed only for information and should not be included in the total degradation products. When this note is included, the total degradation products should only include all specified and unspecified impurities/degradation products above the reporting threshold, with the exception of these designated process-related impurities. For drug product monographs, the term "total degradation products" is considered a synonym to "total impurities", with "total degradation products" being a preferred term.

**Unidentified impurity/unidentified degradation product:** An impurity or degradation product for which structural characterizations has not been achieved and that is identified solely by qualitative analytical properties (e.g., chromatographic retention times).

**Unspecified impurity/degradation product:** An impurity or degradation product that is not individually listed with its own specific acceptance criterion in the drug substance or drug product monograph. In Pharmacopeial monographs, any impurity/degradation product that is not individually listed is considered "unspecified" and is limited by a general acceptance criterion. Synonyms for "unspecified impurity/degradation product" include "other impurity/degradation product" or "other individual impurity/degradation product", with "unspecified impurity/degradation product" being preferred terms.

# APPENDIX

## Appendix 1: Additional Sources of Information and Guidance

1. International Council for Harmonisation. Q3A(R2) Impurities in new drug substances. 2006. https://database.ich.org/sites/default/files/Q3A%28R2%29%20Guideline.pdf. Accessed 6 September 2017.
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# ⟨1087⟩ ▲▲ (USP 1-Dec-2020) INTRINSIC DISSOLUTION—DISSOLUTION TESTING PROCEDURES FOR ROTATING DISK AND STATIONARY DISK

This general information chapter discusses the determination of dissolution rates from nondisintegrating compacts exposing a fixed surface area to a given solvent medium. Compact, as used here, is a nondisintegrating mass resulting from compression of the material under test using appropriate pressure conditions. A single surface having specified physical dimensions is presented for dissolution. Determination of the rate of dissolution can be important during the course of development of new chemical entities because it sometimes permits prediction of potential bioavailability problems and may also be useful for characterizing compendial articles such as excipients or drug substances. Intrinsic dissolution studies are characterization studies and are not referenced in individual monographs. Information provided in this general information chapter is intended to be adapted via a specific protocol appropriate to a specified material.

Dissolution rate generally is expressed as the mass of solute appearing in the dissolution medium per unit time (e.g., mass $s^{-1}$), but dissolution flux is expressed as the rate per unit area (e.g., mass $cm^{-2}$ $s^{-1}$). Reporting dissolution flux is preferred because it is normalized for surface area and, for a pure drug substance, is commonly called intrinsic dissolution rate. Dissolution rate is influenced by intrinsic solid state properties such as crystalline state, including polymorphs and solvates, as well as degree of noncrystallinity. Numerous investigational approaches are available for modifying the physicochemical properties of chemical entities so that their solubility and dissolution properties are enhanced. Among these approaches are the use of co-precipitates and the use of amorphous solid dispersions. The effect of impurities associated with a material can also significantly alter its dissolution properties. Dissolution properties are also influenced by extrinsic factors such as surface area, hydrodynamics, and dissolution medium properties, including solvent (typically water), presence of surfactants, temperature, fluid viscosity, pH, buffer type, and buffer strength.

Rotating disk and stationary disk dissolution procedures are sufficiently versatile to allow study of the characteristics of compounds of pharmaceutical interest under a variety of test conditions. Characteristics common to both apparatuses include the following:

1. They are adaptable to use with standard dissolution testing stations, and both use a tablet die to hold the nondisintegrating compact during the dissolution test.
2. They rely on compression of the test compound into a compact that does not flake or fall free during the dissolution test.
3. A single surface of known geometry and physical dimension is presented for dissolution.
4. The die is located at a fixed position in the vessel, which decreases the variation of hydrodynamic conditions.

A difference between the two procedures is the source of fluid flow over the dissolving surface. In the case of the rotating disk procedure, fluid flow is generated by the rotation of the die in a quiescent fluid, but fluid flow is generated by a paddle or other stirring device for the stationary disk procedure.

## EXPERIMENTAL PROCEDURE

The procedure for carrying out dissolution studies with the two types of apparatus consists of preparing a nondisintegrating compact of material using a suitable compaction device, placing the compact and surrounding die assembly in a suitable dissolution medium, subjecting the compact to the desired hydrodynamics near the compact surface, and measuring the amount of dissolved solute as a function of time.

Compacts are typically prepared using an apparatus that consists of a die, an upper punch, and a lower surface plate fabricated out of hardened steel or other material that allows the compression of material into a nondisintegrating compact. An alternative compaction apparatus consists of a die and two punches. Other configurations that achieve a nondisintegrating compact of constant surface area also may be used. The nondisintegrating compact typically has a diameter of 0.2–1.5 cm.

### Compact Preparation

Attach the smooth lower surface plate to the underside of the die, or alternatively, insert the lower punch using an appropriate clamping system. Accurately weigh a quantity of material necessary to achieve an acceptable compact, and transfer to the die cavity. Place the upper punch into the die cavity, and compress the powder on a hydraulic press at a compression pressure required to form a nondisintegrating compact that will remain in the die assembly for the length of the test. Compression for 1 min at 15 MPa usually is sufficient for many organic crystalline compounds, but alternative compression conditions that avoid the formation of capillaries should be evaluated. For a given substance, the compact preparation, once optimized, is standardized to facilitate comparison of different samples of the substance.

Changes in crystalline form may occur during compression; therefore, confirmation of a solid state form should be performed by powder X-ray diffraction or other techniques. Remove the surface plate or lower punch. Remove loose powder from the surface of the compact and die by blowing compressed air or nitrogen over the surface.

### Dissolution Medium

The choice of dissolution medium is an important consideration. Whenever possible, testing should be performed under sink conditions to avoid artificially retarding the dissolution rate due to approach of solute saturation of the medium. Dissolution measurements are typically made in aqueous media. To approximate in vivo conditions, measurements may be run in the physiological pH range at 37°. The procedure when possible is carried out under the same conditions that are used to determine

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the intrinsic solubility of the solid state form being tested. Dissolution media should be deaerated immediately before use to avoid air bubbles forming on the compact or die surface.[1]

The medium temperature and pH must be controlled, especially when dealing with ionizable compounds and salts. In the latter cases, the dissolution rate may depend strongly on the pH, buffer species, and buffer concentration. A simplifying assumption in constant surface area dissolution testing is that the pH at the surface of the dissolving compact is the same as the pH of the bulk dissolution medium. For non-ionizable compounds, this is relatively simple because no significant pH dependence on dissolution rate is expected. For acids and bases, the solute can alter the pH at and near the surface of the compact as it dissolves. Under these conditions, the pH at the surface of the compact may be quite different from the bulk pH due to the self-buffering capacity of the solute. To assess intrinsic solubility, experimental conditions should be chosen to eliminate the effect of solute buffering, alteration of solution pH, and precipitation of other solid state forms at the surface of the compact. For weak acids, the pH of the dissolution medium should be 1–2 pH units below the p$K_a$ of the dissolving species. For weak bases, the pH of the dissolution medium should be 1–2 pH units above the p$K_a$ of the dissolving species.

## Apparatus

### ROTATING DISK

A typical apparatus (*Figure 1*) consists of a punch and die fabricated out of hardened steel. The base of the die has three threaded holes for the attachment of a surface plate made of polished steel, providing a mirror-smooth base for the compacted pellet. The die has a cavity into which is placed a measured amount of the material whose intrinsic dissolution rate is to be determined. The punch is then inserted in the die cavity, and the test material is compressed with a hydraulic press. [NOTE—A hole through the head of the punch allows insertion of a metal rod to facilitate removal from the die after the test.] A compacted pellet of the material is formed in the cavity with a single face of defined area exposed on the bottom of the die.

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[1] One method of deaeration is the following: Heat the medium, while stirring gently, to about 41°. Immediately filter under vacuum using a filter of 0.45-µm or less pore size, with vigorous stirring, and continue stirring under vacuum for about 5 min. Other deaeration techniques for removal of dissolved gases may be used.

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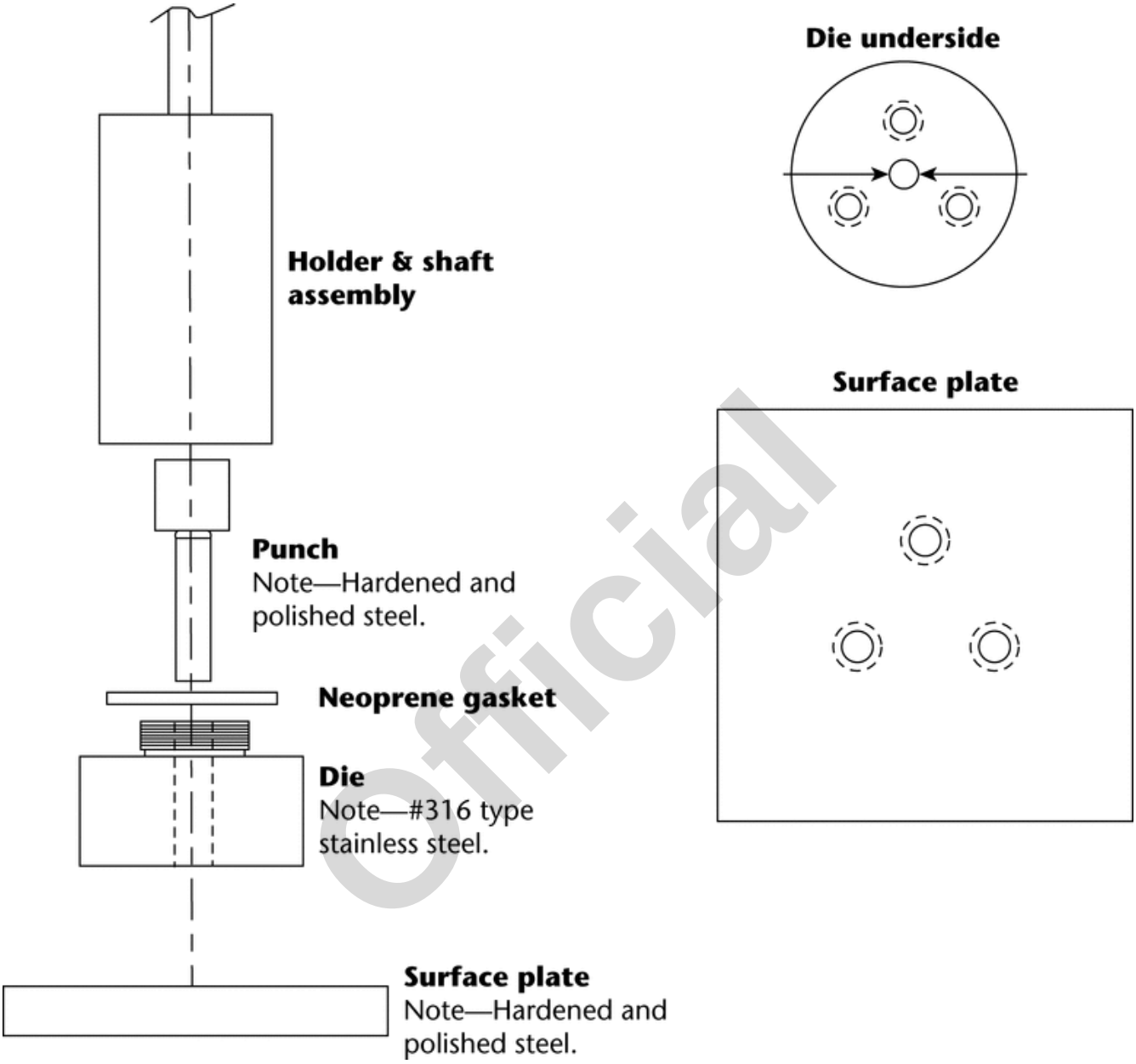


Figure 1.

The die assembly is then attached to a shaft constructed of an appropriate material (typically steel). The shaft holding the die assembly is positioned so that when the die assembly is lowered into the dissolution medium (*Figure 2*), the exposed surface of the compact will be NLT 1.0 cm from the bottom of the vessel and nominally in a horizontal position. The die assembly should be aligned to minimize wobble, and air bubbles should not be allowed to form on the compact or die surface.

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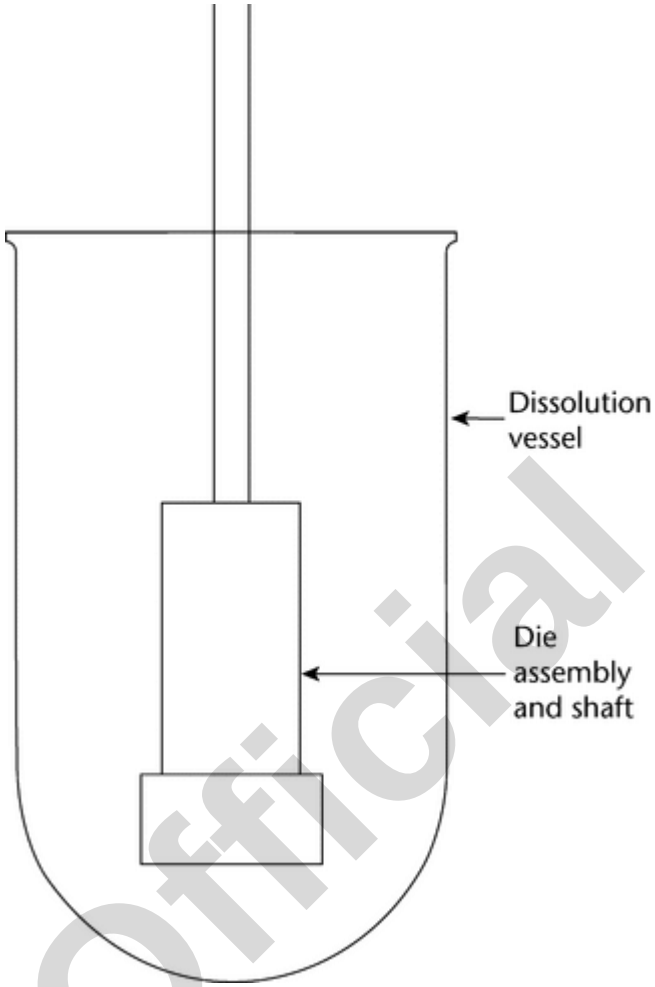


Figure 2.

A rotating disk speed of 300 rpm is recommended. Typical rotation speeds may range from 60 to 500 rpm. The dissolution rate depends on the rotation speed used. This parameter should be selected in order to admit at least 5 sample points during the test, but excessive stirring speeds may create shear patterns on the surface of the dissolving material that could cause aberrant results (i.e., nonlinearity). Typically, the concentration of the test specimen is measured as a function of time, and the amount dissolved is then calculated. The sampling interval will be determined by the speed of the dissolution process. If samples are removed from the dissolution medium, the cumulative amount dissolved at each time point should be corrected for losses due to sampling.

## STATIONARY DISK

The apparatus (*Figure 3*) consists of a steel punch, die, and a base plate. The die base has three holes for the attachment of the base plate. The three fixed screws on the base plate are inserted through the three holes on the die and then fastened with three washers and nuts. The test material is placed into the die cavity. The punch is then inserted into the cavity and compressed, with the aid of a bench top press. The base plate is then disconnected from the die to expose a smooth compact pellet surface. A gasket is placed around the threaded shoulder of the die, and a polypropylene cap is then screwed onto the threaded shoulder of the die.

The die assembly is then positioned at the bottom of a specially designed dissolution vessel with a flat bottom (*Figure 4*). The stirring unit (e.g., paddle) is positioned at an appropriate distance (typically 2.54 cm) from the compact surface. The die assembly and stirring unit should be aligned to ensure consistent hydrodynamics, and air bubbles should not be present on the compact surface during testing. Alternative configurations may be utilized if adequate characterization and control of the hydrodynamics can be established.

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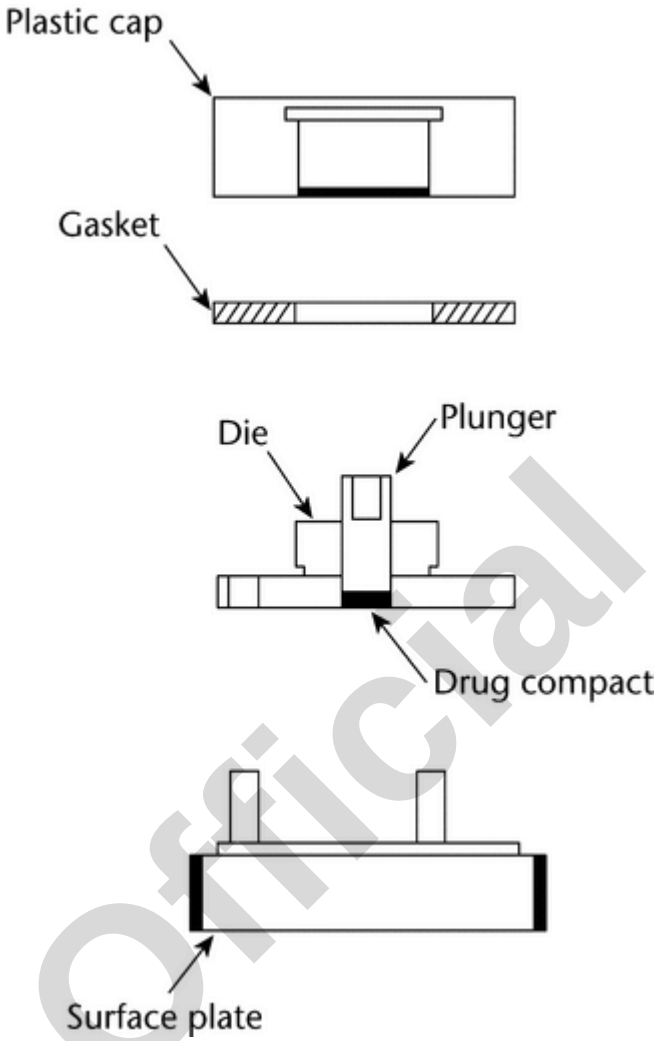


Figure 3.

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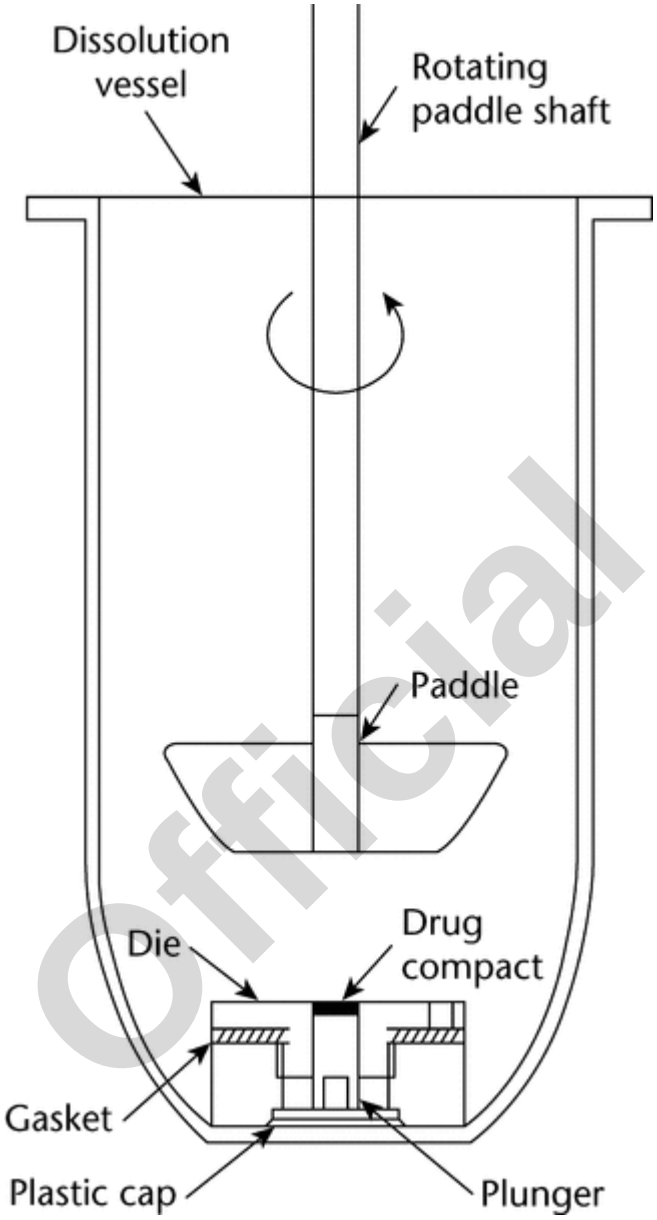


Figure 4.

The dissolution rate depends on the rotation speed and precise hydrodynamics that exist. Typically, the concentration of the test specimen is measured as a function of time, and the amount dissolved is then calculated. The sampling interval will be determined by the speed of the dissolution process (see *Rotating Disk*). If samples are removed from the dissolution medium, the cumulative amount dissolved at each time point should be corrected for losses due to sampling.

## DATA ANALYSIS AND INTERPRETATION

The dissolution rate is determined by plotting the cumulative amount of solute dissolved against time. Linear regression analysis is performed on data points in the initial linear region of the dissolution curve. The slope corresponds to the dissolution rate (mass $s^{-1}$). (More precise estimates of slope can be obtained using a generalized linear model that takes into account correlations among the measurements of the cumulative amounts dissolved at the various sampling times.)

The amount versus time profiles may show curvature. When this occurs, only the initial linear portion of the profile is used to determine the dissolution rate. Upward curvature (positive second derivative) of the concentration versus time data is typically indicative of a systematic experimental problem. Possible problems include physical degradation of the compact by cracking, delaminating, or disintegration. Downward (negative second derivative) curvature of the dissolution profile is often indicative of a transformation of the solid form of the compact at the surface or when saturation of the dissolution medium is inadvertently being approached. This often occurs when a less thermodynamically stable crystalline form converts to a more stable form. Examples include conversion from an amorphous form to a crystalline form or from an anhydrous form to a hydrate form, or

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the formation of a salt or a salt converting to the corresponding free acid or free base. If such curvature is observed, the crystalline form of the compact may be assessed by removing it from the medium and examining it by powder X-ray diffraction or another similar technique to determine if the exposed surface area is changing.

The constant surface area dissolution rate is reported in units of mass $s^{-1}$, and the dissolution flux is reported in units of mass $cm^{-2}$ $s^{-1}$. The dissolution flux is calculated by dividing the dissolution rate by the surface area of the compact. Test conditions, typically a description of the apparatus, rotation speed, temperature, buffer species and strength, pH, and ionic strength should also be reported with the analyses.

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# ⟨1088⟩ IN VITRO AND IN VIVO EVALUATION OF ▲ORAL▲ (USP 1-May-2021) DOSAGE FORMS

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## PURPOSE

This chapter ▲focuses on the in vitro and in vivo performance of oral dosage forms and▲ (USP 1-May-2021) provides an overview of the methodology for characterizing the ▲▲ (USP 1-May-2021) properties of a drug substance as well as its associated ▲oral dosage form that are critical to establish the relationship to the pharmacokinetic▲ (USP 1-May-2021) properties of the drug product. Results of in vitro methods are linked with information from in vivo evaluations through an in vitro–in vivo correlation (IVIVC), ▲or, if an IVIVC is not possible, an in vitro–in vivo relationship (IVIVR). Establishing an IVIVC may enable a waiver of in vivo bioequivalence study requirements as well as support scale-up and post-approval changes.▲ (USP 1-May-2021)

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## SCOPE

The ultimate goal of these characterization studies is an understanding of the relationship between the physicochemical ▲▲ (USP 1-May-2021) properties of the drug substance ▲and the in vitro performance of the drug product to the pharmacokinetic profile of the drug product.▲ (USP 1-May-2021) This chapter outlines the in vitro and in vivo testing that goes into the development of the body of data ▲required for▲ (USP 1-May-2021) decision making relating to the formulation, manufacturing, ▲post-approval changes (see FDA guidance *Extended Release Oral Dosage Forms: Development, Evaluation, and Application of In Vitro/In Vivo Correlations*; www.fda.gov/media/70939/download),▲ (USP 1-May-2021) and related regulatory activities ▲of an oral dosage form. An IVIVC is linked to the in vitro test conditions and the product critical quality attributes (CQAs) controlling the in vitro and in vivo release characteristics. If either is changed, the relationship may be compromised. An IVIVC includes the verification of relevant in vivo differences predicted by a discriminating in vitro method.▲ (USP 1-May-2021) This chapter complements the information in general chapters, *Assessment of Solid Oral Drug Product Performance and Interchangeability, Bioavailability, Bioequivalence, and Dissolution* ⟨1090⟩ and *The Dissolution Procedure: Development and Validation* ⟨1092⟩ by detailing the essential in vitro and in vivo data elements underlying an understanding of bioequivalence and bioavailability. This chapter's text recognizes that regulatory guidances and a wealth of text books are available to elaborate on the content provided, and it is not the purpose of this chapter to provide an exhaustive ▲discussion▲ (USP 1-May-2021) on the subjects presented but rather to provide a guide and listing of the issues of interest.

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## BACKGROUND INFORMATION

Establishing a meaningful relationship between dissolution behavior and in vivo drug performance (i.e., IVIVC) has long been sought from the perspectives of both bioavailability and bioequivalence and quality control considerations. ▲In order to ascertain lot-to-lot equivalence to the biobatch, clinically relevant specifications should be established preferably with an IVIVC. If an IVIVC is not possible, the use of an IVIVR may be acceptable.▲ (USP 1-May-2021)

The earliest achievable in vitro characteristic thought to predict an acceptable in vivo performance was tablet and capsule disintegration. A test for disintegration was adopted in *USP XIV* (1950). At that time, no quantitative work was done to attempt to demonstrate ▲a relationship▲ (USP 1-May-2021) to in vivo product performance. Advances in instrumental methods and analytical precision ultimately opened up prospects for this work. The *USP–NF* Joint Panel on Physiologic Availability recognized that the disintegration test was insufficiently sensitive and in 1968 directed the identification of candidate articles for the first 12 official dissolution tests that used *Apparatus 1*.

▲▲ (USP 1-May-2021) The special sensitivity of the dissolution test to changes in composition or method of manufacturing that do not result in significant changes in performance in vivo is well recognized. An understanding of the full complement of information given by in vitro and in vivo evaluation of the drug substance and product is the starting point in the development of a meaningful in vitro performance test.

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## IN VITRO ▲CHARACTERIZATION

For the purpose of an IVIVC, the goal of the in vitro characterization is to understand the performance of the formulation under different conditions and to understand the CQAs that influence the in vivo performance of the dosage form. In order to best characterize the performance of the dosage form, an understanding of the drug substance physicochemical properties is needed.

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## Drug Substance Properties

With the goal of achieving an IVIVC, the required intrinsic physicochemical information will include the pH-solubility profile, stability in the gastrointestinal environment, and other dissolution-controlling variables of the drug substance that are related to the extremes of the physiological environment experienced by the dosage form. Permeability, another drug substance property, can be determined in vitro by cellular permeability models such as Caco–2 cells.

## Drug Product Properties

The purpose of in vitro performance testing is to challenge the dosage forms under different conditions to predict the in vivo performance. The primary characterization is related to the rate-determining mechanism with different considerations for each release pattern. Immediate-release dissolution is a stochastic process but extended-release and delayed-release have intentional modifications that enable the performance characteristics. The variables of importance for immediate-release would include but not be limited to wetting, disintegration, disaggregation, erosion, and dissolution of particles in suspension. In addition to factors critical to the in vivo performance of immediate-release formulations, the key variables of importance for delayed-release are onset of the dissolution and pH dependency. The rate-controlling mechanisms of importance for extended-release include but are not limited to erosion, diffusion, swelling, and osmotic pressure. Identification of the rate-limiting steps in the dissolution process that can be linked to in vivo performance is of great importance.

## Design of In Vitro Characterization

The goals of the in vitro characterization studies are topographical characterization of the in vitro performance of the dosage and evaluation of dosage form robustness. A robustness evaluation would include such aspects as effect of the pH of the gastrointestinal environment, effect of mechanical factors (e.g., agitation), and effects of food components and alcohol.

## Experimental Variables

Experimental variables of the dissolution method parameters to consider in characterizing the dosage form include the media pH value, volume and composition of the dissolution medium, and agitation (hydrodynamic). Also use of different dissolution apparatus should be considered as a potential experimental variable.

The media composition may contain surfactants, lipids, or enzymes. Organic solvents may be added to the dissolution medium with appropriate justification. There should be consideration of the osmotic pressure of the dissolution medium used (e.g., compendial buffers are usually hypoosmotic compared to physiological values). See *Osmolality and Osmolarity* ⟨785⟩ for additional information.

## Experimental Design

Experimental design should be reflective of the physiological conditions prevailing within the gastrointestinal tract. The variables may be constant throughout each experiment or change along the time course of the experiment. The experimental setup could stipulate parallel or sequential design. One should consider the combinations of experimental variables in the gastrointestinal tract such as pH and compositions as they would occur in human (or veterinary) gastrointestinal physiology. The addition of surfactants and enzymes may also be considered. Conversely the setup may be simply run at a typical intestinal pH value. Other combinations of experimental parameters may be defined according to a factorial design or other statistical methods.

## Technical Requirements

One major criterion for selecting the dissolution method should be its discriminatory power to show changes in the critical attributes of the release mechanism of the chosen formulation.

The in vitro characterization should include a sufficient number of data points to support pivotal phases of dissolution kinetics and data points that provide a full profile with nearly if not complete dissolution (100% release). Analytical validation of the method is required. Results should be based on an adequate number of samples for regulatory acceptance. For example, a sample of 12 is needed.

The selected dosage form then will be varied—such as by composition, by critical material attributes (e.g., drug substance particle size), and by manufacturing process—so there are different formulations for in vitro and in vivo testing that represent different levels of drug release in vitro and in vivo. These different levels should show rank order at a minimum, that is rank order of the fastest in vitro result corresponds to the dosage form that shows the highest rate of absorption, and so forth with the slowest in vitro results to the slowest absorption rate (see *Figure 1*). The IVIVC is for a particular formulation and cannot be applied to other formulations. Ideally, the dissolution method used to establish the IVIVC should be identical to the regulatory dissolution method used for routine quality control testing. In general, a quality control method that is not based on an IVIVC is sensitive to differences of in vitro performance, such as changes in manufacturing, which may not have in vivo significance. An IVIVC method is based on differences in in vivo performance. To prove the discriminatory power and to set a meaningful specification, the IVIVC needs to be valid for critical formulation batches.

As the method development evolves, the guidance in ⟨1092⟩ is essential. In early drug product development, the dissolution method may be complex attempting to explain the in vivo performance. As the understanding of the product evolves, the method should be robust and eventually suitable for the regulatory dissolution test.

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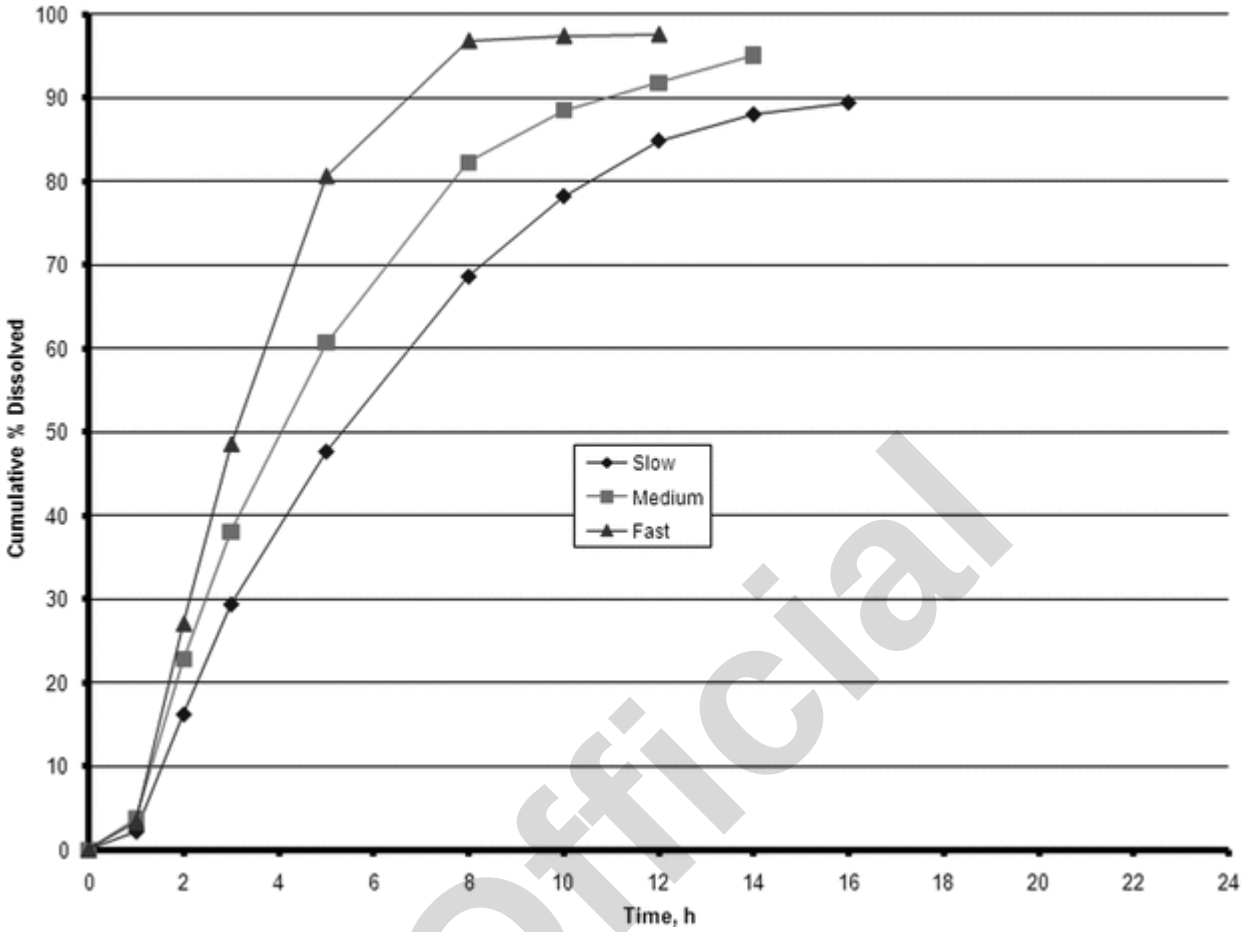


▲ (USP 1-May-2021)

Figure 1. Dissolution profiles of three modifications of a modified-release formulation (USP Apparatus 2, 50 rpm, 900 mL of water, 37°).

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## IN VIVO EVALUATION OF DOSAGE FORMS

In evaluating a drug product's performance, analysts fundamentally must ask what type of study should be performed to give reasonable assurance of bioequivalence ▲▲ (USP 1-May-2021) to the clinical trial product that demonstrated safety and efficacy.[1] ▲ (USP 1-May-2021) Although they provide important information concerning the release characteristics of the drug from the dosage form, in vitro dissolution studies at present are used primarily for setting or supporting specifications for drug products (e.g., shelf life) and manufacturing process control (e.g., scale-up or postapproval changes).

▲▲ (USP 1-May-2021)

The following discussions are intended to provide guidance for drug substance evaluation and the design, conduct, and evaluation of studies involving dosage forms. Although these guidelines focus on oral drug delivery systems, the principles may be applicable to other routes of drug administration (e.g., transdermal, subcutaneous, intramuscular).

▲Animal data can be used only for veterinary products and not for establishing an IVIVC for human drug products based on animal models.

### The Biopharmaceutics Classification System (BCS)

FDA has issued a guidance document titled *Waiver of In Vivo Bioavailability and Bioequivalence Studies for Immediate-Release Solid Oral Dosage Forms Based on a Biopharmaceutics Classification System* (see www.fda.gov/regulatory-information/search-fda-guidance-documents/waiver-vivo-bioavailability-and-bioequivalence-studies-immediate-release-solid-oral-dosage-forms). An additional guidance document from FDA is *M9 Biopharmaceutics Classification System-Based Biowaivers* (see www.fda.gov/media/117974/download).

[1] 21 CFR 320.22 Criteria for waiver of evidence of in vivo bioavailability or bioequivalence.

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## Pharmacokinetic Profiling

Analysts should thoroughly characterize the input absorption profile of the drug substance from a formulation that shows a rapid absorption rate (an oral solution, or a well-characterized immediate-release drug product). An intravenous solution provides instantaneous and complete absorption and is required by the Loo-Riegelman model for level A correlations. In turn, this formulation serves as a reference to evaluate the input profile of the modified-release dosage form. This information, together with the pharmacokinetics of the drug, can characterize drug absorption and can predict changes in drug absorption rate when input is modified as in modified-release dosage forms. For example, if the drug exhibits saturable first-pass hepatic metabolism, a change in systemic availability could result after oral administration if the input rate is altered.

The information required may include the following.

1. Pharmacokinetic parameters–clearance, area under the time–plasma concentration curve (AUC), maximum plasma concentration ($C_{max}$), time to maximum plasma concentration ($T_{max}$), volume of distribution, half-life, mean residence time, or model-dependent parameters
2. Linearity or characterization of nonlinearity over the dose or concentration range that could be encountered
3. Drug/active metabolite accumulation
4. Metabolic profile and excretory pathway, with special attention to the active metabolites and active enantiomers of racemic mixtures
5. Enterohepatic circulation
6. Protein-binding parameters and effect of dialysis
7. The effects of age, gender, race, and relevant disease states
8. Plasma/blood ratios
9. Information on chronopharmacokinetics▲ (USP 1-May-2021)

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## ▲CHARACTERIZATION OF DRUG SUBSTANCE

## The Biopharmaceutics Classification System (BCS)

FDA has issued a guidance titled "Waiver of In Vivo Bioavailability and Bioequivalence Studies for Immediate-release Solid Oral Dosage Forms Based on a Biopharmaceutics Classification System" (www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070246.pdf). A key assumption in the approach is that drug release and dissolution is sufficiently rapid so that an in vitro–in vivo correlation is not possible and/or useful. When applicable, the BCS allows dissolution rate data in lieu of BA or BE studies for product approval.

## Pharmacokinetic Properties

Analysts should thoroughly characterize the input absorption profile of the active drug entity from a formulation that shows rapid BA (an intravenous solution, oral solution, or a well-characterized immediate-release drug product). In turn, this formulation serves as a reference to evaluate the input profile of the modified-release dosage form. This information, together with the pharmacokinetics of the active drug entity, can characterize drug absorption and can predict changes in drug BA when input is modified as in modified-release dosage forms. For example, if the active drug entity exhibits saturable first-pass hepatic metabolism, a reduction in systemic availability could result after oral administration if the input rate is decreased.

In designing an oral modified-release dosage form, analysts may find it useful to determine the absorption of the active drug entity in various segments of the gastrointestinal tract, particularly in the lower gastrointestinal tract (colon) for delayed-release dosage forms that release drug in this region. Food effects also may be important and should be investigated.

## Drug Disposition

The information required to characterize drug disposition may include the following.

1. Disposition parameters—clearance, area under the time—plasma concentration curve (AUC), maximum plasma concentration ($C_{max}$), time to maximum plasma concentration ($T_{max}$), volume of distribution, half-life, mean residence time, or model-dependent parameters.
2. Linearity or characterization of nonlinearity over the dose or concentration range that could be encountered.
3. Drug/metabolite accumulation.
4. Metabolic profile and excretory pathway, with special attention to the active metabolites and active enantiomers of racemic mixtures.
5. Enterohepatic circulation.
6. Protein-binding parameters and effect of dialysis.
7. The effects of age, gender, race, and relevant disease states.
8. Plasma: blood ratios.
9. A narrow therapeutic index or a clinical response that varies significantly as a function of the time of day (chronopharmacokinetics).

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## Pharmacodynamic Properties

Before developing a dosage form, analysts should obtain concentration–response relationships over a dose range sufficiently wide to encompass important therapeutic and adverse responses. In addition, the equilibration-time[2] characteristics between plasma concentration and effect should be evaluated. For modified-release products that typically have larger drug doses in the dosage form, these concentration–response relationships should be sufficiently characterized so that a reasonable prediction of the safety margin can be made if dose dumping should occur. If there is a well-defined relationship between the plasma concentration of the active drug substance or active metabolites and the clinical response (therapeutic and adverse), the clinical performance of a new modified-release dosage form could be characterized by plasma concentration–time data. If such data are not available, clinical trials of the modified-release dosage form should be carried out with concurrent pharmacokinetic and pharmacodynamic measurements.▲ (USP 1-May-2021)

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# CHARACTERIZATION OF THE ▲ORAL▲ (USP 1-MAY-2021) DOSAGE FORM

## Pharmacokinetic Properties: Immediate-Release Products

The types of pharmacokinetic studies that should be conducted are based on how much is known about the ▲drug,▲ (USP 1-May-2021) its clinical pharmacokinetics, and its BCS class ▲▲ (USP 1-May-2021) when an FDA-approved drug product undergoes changes in the manufacturing of the product after the product has been approved. Such changes are common and can be caused by expansion in the size of the lots manufactured, new manufacturing locations, or the introduction of new technology. Necessary in vitro dissolution tests and/or in vivo bioequivalence tests are described in the FDA guidance *SUPAC-IR: Immediate-Release Solid Oral Dosage Forms: Scale-Up and Post-Approval Changes: Chemistry, Manufacturing and Controls, In Vitro Dissolution Testing, and In Vivo Bioequivalence Documentation* (see ▲www.fda.gov/regulatory-information/search-fda-guidance-documents/supac-ir-immediate-release-solid-oral-dosage-forms-scale-and-post-approval-changes-chemistry).▲ (USP 1-May-2021)

## Pharmacokinetic Properties: Modified-Release Products

▲Similar to immediate-release products,▲ (USP 1-May-2021) the types of pharmacokinetic studies that should be conducted for modified-release products are based on how much is known about the drug, ▲▲ (USP 1-May-2021) its pharmacokinetics, and biopharmaceutics, and whether pharmacokinetic studies are intended to be the sole basis for product approval. At a minimum, two studies are required to characterize the product when no reference modified-release product exists: (1) a single-dose crossover study for each strength of a modified-release dosage form and (2) a multiple-dose, steady-state study using the highest strength of a modified-release dosage form. A ▲▲ (USP 1-May-2021) study to evaluate the potential ▲for food effects including robustness of the dosage form (including dose dumping) from extended-release dosage forms also is required as a separate study or is included as an arm of a crossover study. Oral solutions, or well-characterized immediate-release drug products are possible reference products to evaluate a modified-release formulation. If the drug exhibits saturable first-pass hepatic metabolism from the small intestine, a reduction in systemic availability could result after oral administration if the input rate is decreased. An increase in systemic availability could be observed if a drug is absorbed from the colon from a delayed-release dosage form that targets the colon, thus avoiding a first-pass effect.

PK studies are required for IVIVC of modified-release dosage forms. The pharmacokinetic studies may serve as the basis for characterization of the dosage form. Techniques to develop IVIVC assume linear pharmacokinetics in the dosing range. Manufacturers seeking regulatory approval without conducting clinical trials should consult the authorities to ensure that an adequate database of safety and efficacy information exists. These studies also apply to an FDA-approved product that has undergone scale-up and postapproval changes (SUPAC). Necessary in vitro dissolution tests and/or in vivo bioequivalence tests are described in the FDA guidance *SUPAC-MR: Modified Release Solid Oral Dosage Forms: Scale-Up and Postapproval Changes: Chemistry, Manufacturing, and Controls; In Vitro Dissolution Testing and In Vivo Bioequivalence Documentation* (www.fda.gov/regulatory-information/search-fda-guidance-documents/supac-mr-modified-release-solid-oral-dosage-forms-scale-and-postapproval-changes-chemistry).▲ (USP 1-May-2021)

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# IN VITRO–IN VIVO CORRELATIONS

The term "IVIVC" first appeared in the pharmaceutical literature as a result of the ▲importance of identifying the relationship between bioavailability parameters and in vitro dissolution of a dosage form.▲ (USP 1-May-2021) IVIVC refers to the establishment of a ▲mathematical▲ (USP 1-May-2021) relationship between ▲▲ (USP 1-May-2021) a parameter derived from drug plasma concentrations produced by a dosage form, and a ▲dissolution▲ (USP 1-May-2021) property or characteristic of the same dosage form. The ▲bioavailability parameters▲ (USP 1-May-2021) most commonly used are one or more pharmacokinetic parameters such as $C_{max}$ or

[2] Equilibration time is a measure of the time-dependent discontinuity between measured plasma concentrations and measured effects. The discontinuity is more often characterized by the degree of hysteresis observed when the effect-concentration plot for increasing concentrations is compared with that for decreasing concentrations. Where the equilibration time is very short (i.e., rapid equilibration with no active metabolites generated), there will be little or no hysteresis. That is, the same effect will be observed for a given concentration independent of the interval between the time of dosing and the time that measurements are made.

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AUC, obtained following the administration of the dosage form. The ▲in vitro▲ (USP 1-May-2021) property most commonly used is a dosage form's in vitro dissolution ▲profile▲ (USP 1-May-2021) (e.g., percent of drug released under a given set of conditions ▲versus time).

**In vitro–in vivo correlation (IVIVC):** A predictive mathematical model describing the relationship between an in vitro property of an extended-release dosage form (usually the rate or extent of drug dissolution or release) and a relevant in vivo response (e.g., plasma drug concentration or amount of drug absorbed).▲ (USP 1-May-2021)

In many cases the actual drug plasma concentration profile can be predicted from in vitro dissolution data.

▲IVIVC analysis is most successful for oral drug products, where compared to in vivo absorption, in vivo dissolution is the rate-limiting step for bioavailability. For extended-release products the dissolution rate is intentionally designed so that in vivo dissolution is the rate-limiting step for bioavailability. Some correlations with immediate-release products can be expected where in vivo dissolution is the rate-limiting step. As described in the BCS, solubility and permeability classification are helpful in assessing feasibility of IVIVC with immediate-release products. Taking into account the changes in the absorption rate along the gastrointestinal transit, non-linear regression analysis may be useful.▲ (USP 1-May-2021)

## General Considerations

▲In an ideal case, correlations should be generated by using the entire profiles of both in vitro dissolution testing and plasma concentrations from bioavailability studies. A point-to-point data analysis allows establishment of a functional relationship, which does not imply a full correlation. One formulation of the product under investigation suffices for that determination. Data analysis performed with in vitro and in vivo parameters require at least three formulations to establish a correlation. Three correlation levels are available. Each correlation level displays important differences in the quality of the correlation. This chapter provides a discussion of each type of correlation as to when each may be the most appropriate type of relationship to establish and then to describe its potential utility within the framework of product development, the development of release specifications, and its regulatory utility in supporting biowaivers. Typically, one target formulation and one or two alternative formulations with expected higher and/or lower drug release are included in the IVIVC study protocol. If feasible an oral solution is administered to a selected number of subjects in order to compute the relative oral bioavailability and to perform a model-free numerical deconvolution procedure. The target formulation will be used for the correlation development, and it should be near a commercial form, although further modifications may be planned. This allows the task of developing an IVIVC to occur early enough in product development so that it helps in predicting the in vivo linkage and minimizes bio studies, and it also has a role in specification selection. If the IVIVC is established too early in the drug product development process, the formulation used in the study may not be representative of the final dosage form.▲ (USP 1-May-2021)

## Correlation Levels

Three correlation levels have been defined and categorized in descending order of quality. The concept of correlation level is based on the ability of the correlation to reflect the entire plasma drug concentration–time curve that results from administration of the given dosage form. The relationship of the entire in vitro dissolution curve to the entire plasma concentration–time profile defines the strength of the correlation and, therefore, the predictability. ▲If permeability rather than dissolution is rate limiting, or if there is solubility-limited absorption, the development of an IVIVC/R should not be attempted.▲ (USP 1-May-2021)

### LEVEL A

This level is the highest category of correlation. It represents a point-to-point relationship between in vitro dissolution and the in vivo input rate (e.g., absorption rate of the drug from the dosage form). For a *Level A* correlation, a product's in vitro dissolution curve is compared to its in vivo input curve (i.e., the curve produced by deconvolution of the plasma profile). Deconvolution can be accomplished using mass balance model-dependent methods, such as the Wagner-Nelson or Loo-Riegelman methods, or by model-independent, mathematical deconvolution ▲(linear system analysis). The model-dependent mass balance approaches do not allow for prediction of oral bioavailability, whereas the numerical deconvolution requires linear pharmacokinetics and is based on the assumption of absorption and elimination processes being invariant over time.

In an ideal correlation, the in vitro dissolution and in vivo absorption rate curves are superimposable or can be made superimposed by the use of a constant offset value or a multiplier of the time scale. The equations describing each curve are the same or stand in a linear relationship. Superimposition is not an absolute requirement for a *Level A* correlation. If the dissolution and absorption curves are different and a mathematical relationship can be developed to relate the two, the plasma level profile is predictable from the in vitro dissolution data. This is called an internal validation. If the same correlation is used to predict the plasma profile of a lot not used to establish the IVIVIC; this is an external validation. This relationship must be true not only at that single input rate but also over the entire quality control dissolution range for the product, provided that the in vitro dissolution profiles are similar in shape (i.e., they can be made superimposable by multiplication).▲ (USP 1-May-2021)

The advantages of a *Level A* correlation are as follows.

1. It develops a point-to-point correlation. This is not found with any other correlation level. It is developed using every plasma level and dissolution point collected at different time intervals, so it reflects the complete plasma level curve. ▲A *Level A* correlation is reversible and allows computation of in vivo profiles from in vitro profiles and vice versa.▲ (USP 1-May-2021) As a result, in the case of a *Level A* correlation, an in vitro dissolution ▲test▲ (USP 1-May-2021) can serve as a surrogate for in vivo performance ▲studies.▲ (USP 1-May-2021) A change in manufacturing site, method of manufacture, raw material supplies,

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minor formulation modifications, and even product strength using the same formulation can be justified without the need for additional bioavailability–bioequivalence (BA–BE) studies.[2, 3]

2. A truly meaningful quality control procedure that indicates in vivo performance and is predictive of a dosage form's performance is defined for the dosage form ▲because specifications may be set in a way to assure bioequivalence.▲ (USP 1-May-2021)
3. The extremes of the in vitro quality control standards can be justified ▲▲ (USP 1-May-2021) by deconvolution (using the upper and lower confidence interval limits). ▲Interpolation of specifications from the so-called side batches is a commonly accepted way, whereas extrapolation requires further efforts.▲ (USP 1-May-2021)

LEVEL B

This correlation uses the principles of statistical moment analysis. The mean in vitro dissolution time is compared to either the mean residence time or the mean in vivo dissolution time. As with a *Level A* correlation, *Level B* uses all of the in vitro and in vivo data but is not considered a point-to-point correlation. It does not correlate the actual in vivo plasma profiles but rather ▲parameters that result▲ (USP 1-May-2021) from statistical moment analysis of a plasma profile, ▲▲ (USP 1-May-2021) such as mean residence time. Because a number of different plasma profiles can produce similar mean residence time values, one cannot rely on a *Level B* correlation alone to predict a plasma profile from in vitro dissolution data. ▲The advantage of a *Level B* correlation is its applicability for non-linear pharmacokinetics.▲ (USP 1-May-2021) In addition, in vitro data from such a correlation ▲can▲ (USP 1-May-2021) be used to justify values at the extremes of quality control standards, ▲if all in vitro profiles are similar in shape and the in vivo performance of the side batches are fully included in the confidence intervals defining bioequivalence.▲ (USP 1-May-2021)

LEVEL C

This category relates one dissolution time point (e.g., $t_{50\%}$, $t_{90\%}$) to one pharmacokinetic parameter such as AUC, $C_{max}$, or $T_{max}$. It represents a single-point correlation and does not reflect the complete shape of the plasma profile. ▲*Level C* correlations do not reflect the shape of the profiles; therefore, *Level C* correlations cannot be used to predict plasma profiles.▲ (USP 1-May-2021) Because this type of correlation is not predictive of ▲the full rate and extent of in vivo product dissolution characteristics, it generally serves primarily as a guide in formulation development or as a production quality control procedure.▲ (USP 1-May-2021) Because of its obvious limitations, a *Level C* correlation has limited usefulness in predicting in vivo drug performance and is subject to the same caveats as a *Level B* correlation in its ability to support product and site changes as well as justification of the extreme values in quality control standards. ▲The advantage of a *Level C* correlation is that it selects parameters from the in vivo profiles, which are of a therapeutic relevance.▲ (USP 1-May-2021) The FDA guidance *Extended Release Solid Oral Dosage Forms: Development, Evaluation, and Application of In Vitro/In Vivo Correlations* (▲www.fda.gov/regulatory-information/search-fda-guidance-documents/extended-release-oral-dosage-forms-development-evaluation-and-application-vitroin-vivo-correlations▲ (USP 1-May-2021)) states that manufacturers can obtain biowaivers based on multiple *Level C* correlations. The guidance shows how manufacturers can achieve this correlation. ▲▲ (USP 1-May-2021)

## Developing a Correlation

This chapter does not define the only procedures for developing an IVIVC, and any well-designed and scientifically valid approach is acceptable. To assist the pharmaceutical scientist, one possible procedure for developing a *Level A* correlation follows:

1. In order to perform deconvolution properly, analysts should be familiar with the pharmacokinetics of the drug ▲substance.▲ (USP 1-May-2021)
2. ▲The formulation should be modified to produce different dissolution profiles so that the formulation has the same excipients in all the lots that will be tested. The products under investigation should be pharmaceutically equivalent. The formulation modifications used should be based on factors that would be expected to influence the product's modified-release rate and could occur during normal product manufacture or represent stability effects. In vitro drug release is performed on the formulation that will be used in the bioavailability study. *Figure 2* illustrates an example of dissolution profiles in which the formulations were tested in an acid buffer.▲ (USP 1-May-2021)

[2] FDA guidance *SUPAC-MR: Modified Release Solid Oral Dosage Forms: Scale-Up and Postapproval Changes: Chemistry, Manufacturing, and Controls; In Vitro Dissolution Testing, and In Vivo Bioequivalence Documentation* (1997).
[3] FDA guidance *Extended Release Solid Oral Dosage Forms: Development, Evaluation, and Application of In Vitro/In Vivo Correlations*: "If an IVIVC is developed with the highest strength, waivers for changes made on the highest strength and any lower strengths may be granted if these strengths are compositionally proportional or qualitatively the same, the in vitro dissolution profiles of all the strengths are similar, and all strengths have the same release mechanism."

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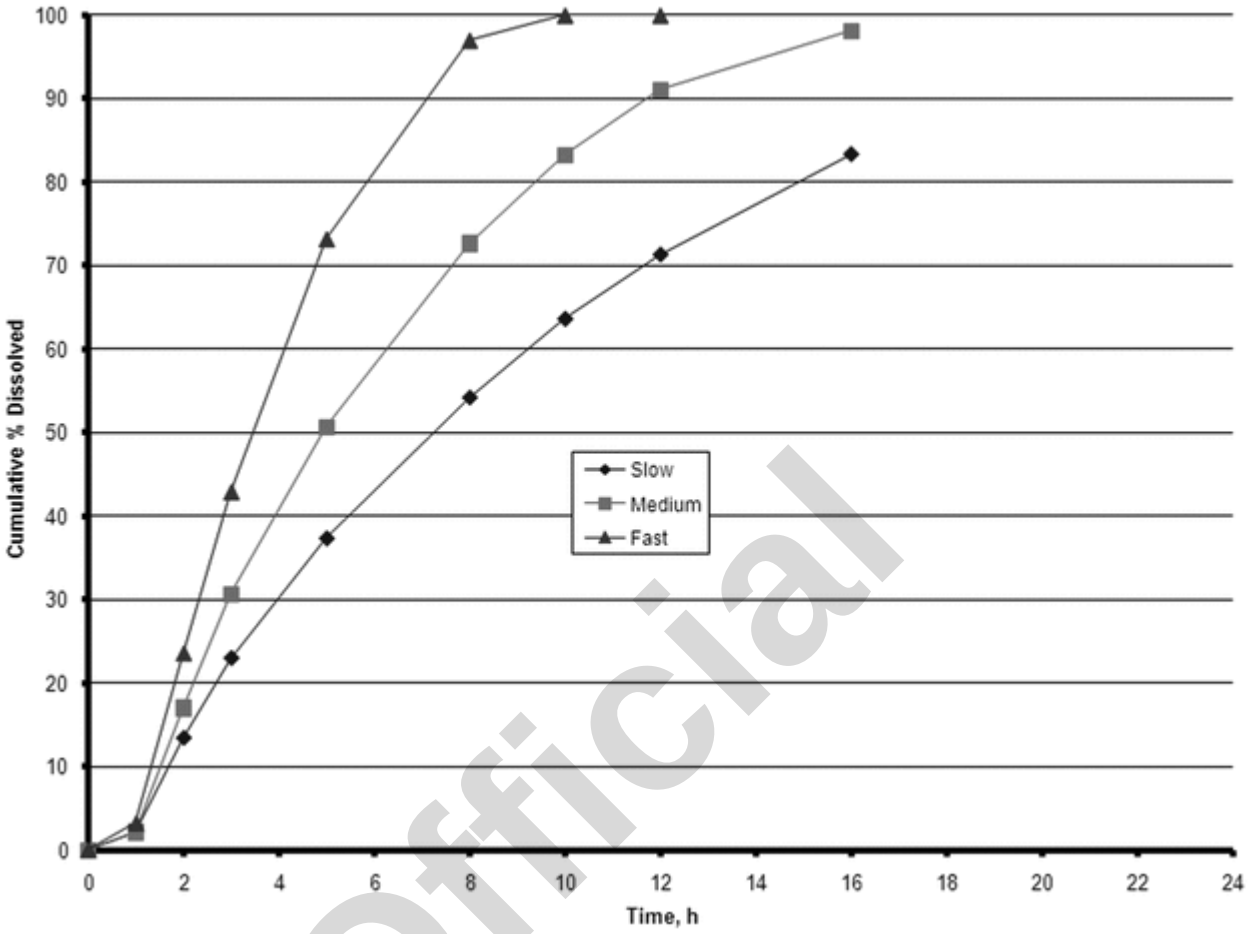


Figure 2. Mean dissolution profiles of ▲three modifications of a▲ (USP 1-May-2021) modified-release formulation (USP Apparatus 2, 50 rpm, ▲900 mL,▲ (USP 1-May-2021) pH 4.5 buffer, 37°).

3. The plasma ▲levels▲ (USP 1-May-2021) obtained in the definitive bioavailability study of the modified-release dosage form are treated by a deconvolution procedure. The resulting data may represent the ▲apparent▲ (USP 1-May-2021) drug input rate of the dosage form. They also represent in vivo dissolution when the rate-controlling step of the dosage form is its dissolution rate (i.e., drug absorption after dissolution is considered to be instantaneous). Any deconvolution procedure (e.g., mass balance or mathematical deconvolution) will produce acceptable results. ▲However, the results of mass balance algorithms versus numerical deconvolution are not comparable.▲ (USP 1-May-2021) *Figure 3* illustrates the results of numerical deconvolution of the plasma ▲profiles▲ (USP 1-May-2021) obtained for the ▲formulation in▲ (USP 1-May-2021) *Figure 2*.▲The plasma levels obtained in the definitive bioavailability study of the modified-release dosage form are analyzed by using a deconvolution procedure. The resulting data may represent the apparent drug input rate of the dosage form. The deconvolution represents in vivo dissolution when the rate-controlling step for oral drug absorption is its dissolution rate (i.e., drug absorption after dissolution is considered to be instantaneous). Any deconvolution procedure (e.g., mass balance or mathematical deconvolution) will produce acceptable results if properly validated. However, the results of mass balance algorithms versus numerical deconvolution are not comparable. *Figure 3* illustrates the results of numerical deconvolution of the plasma profiles obtained for the formulation in *Figure 2*.▲ (USP 1-May-2021)

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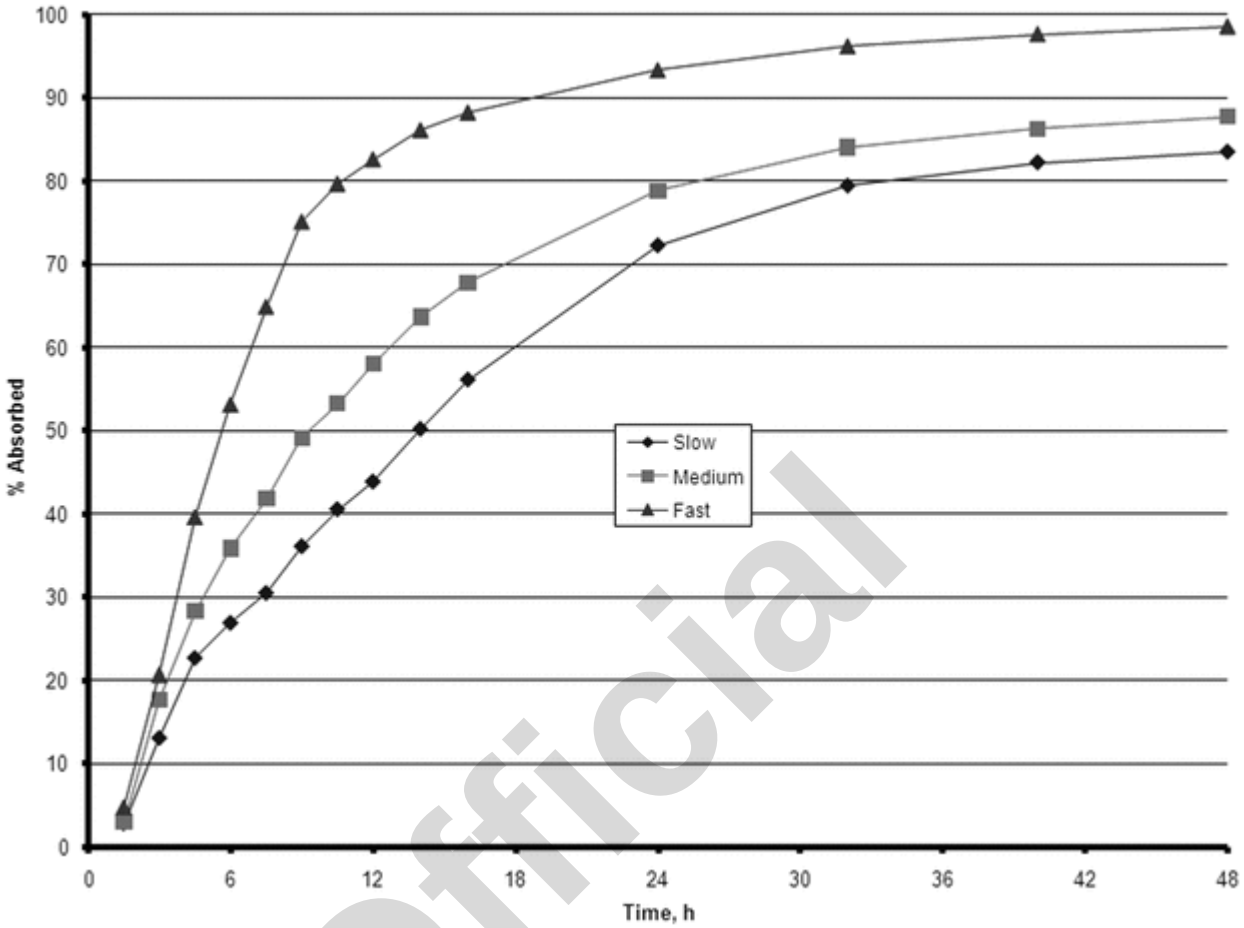


Figure 3. Mean absorption profiles from numerical deconvolution of plasma concentration–time plots ▲of the three examples from *Figure 2*.

The in vitro dissolution curve is then compared to the drug absorption curve. This can be performed by various methods. Simply positioning one curve on the other often can indicate the existence of a correlation, which then may be quantified by defining the equation for each curve and comparing the corresponding constants. The simplest way to demonstrate a correlation is to plot the fraction absorbed in vivo versus the fraction released in vitro, as illustrated in *Figure 4*. With a *Level A* correlation, this relationship is often linear, as illustrated in *Figure 4*. Alternatively, as illustrated in *Figure 5*, a correlation may be curvilinear. The intercept may or may not be zero depending on whether there is a lag time before the system begins to release the drug in vivo, or the absorption rate is not instantaneous, resulting in the presence of some finite quantity of dissolved but unabsorbed drug. In either case, it is a point-to-point or a *Level A* correlation when the least-squares fit of the line approaches a coefficient of determination, $r^2$, of 1.

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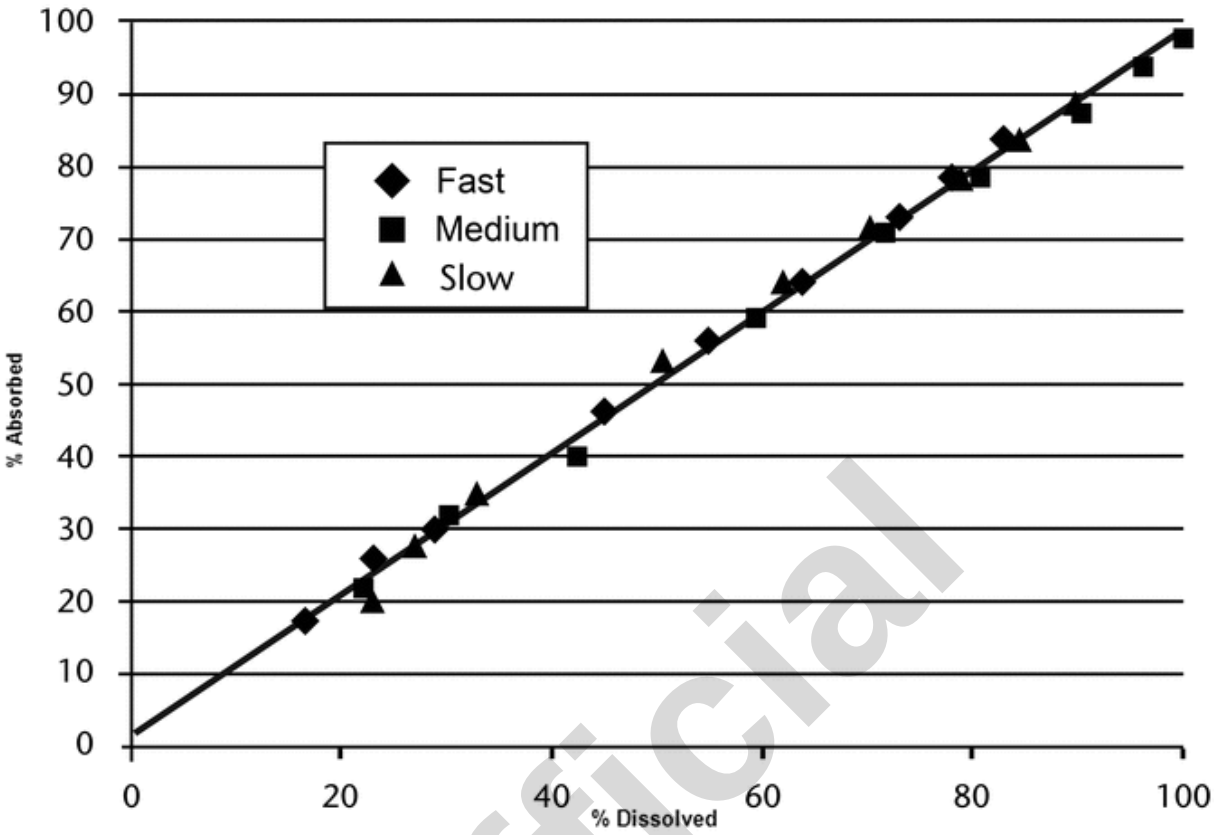


▲ (USP 1-May-2021)

Figure 4. Linear correlation plot for three formulations.

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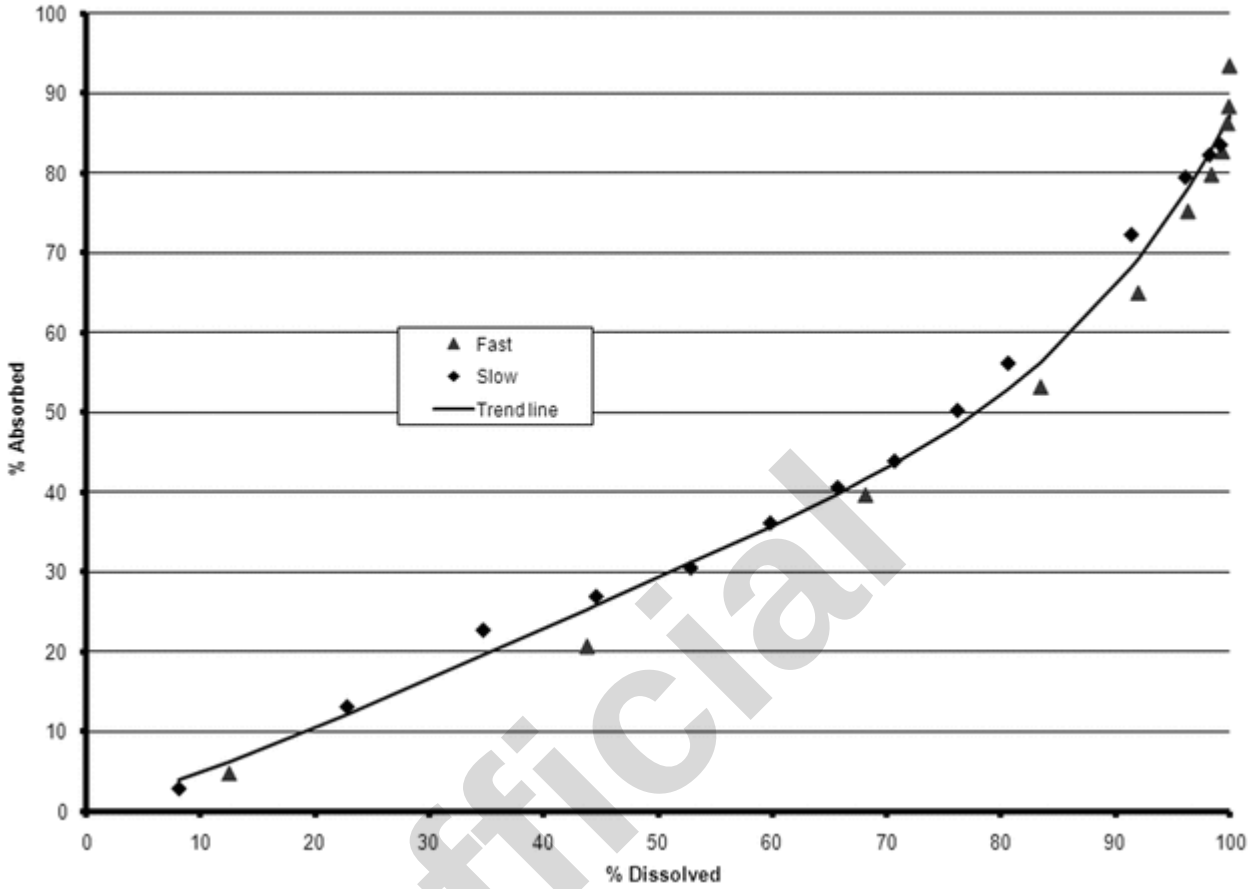


Figure 5. IVIVC attempt: pH 4.5 buffer.

4. ▲A discriminative in vitro method establishes that in vitro is predictive of in vivo absorption differences. This is achieved by preparing at least two formulations that have significantly different in vitro behavior. One should demonstrate a more rapid release while the other should be a slower release than the clinical bioavailability batch (or biobatch). A pilot BA–BE study should be performed with these formulations, and the previously established correlation should be demonstrated for both. The modifications of these formulations should be based on factors that are expected to influence the product's drug release rate.▲ (USP 1-May-2021)
5. ▲In vivo performance of the two biobatch formulations can be computed based on the correlation developed with the formulations that were used in the BA–BE study. Analysts can then compare the predicted and experimentally determined values (e.g., the prediction error). *Figure 6* and *Figure 7* illustrate an exercise that serves as an internal validation of the *Level A* correlation. An external validation would involve computing the drug plasma profile and comparing it with the observed plasma profiles for a formulation not included in the *Level A* correlation calculations. *Figure 8* illustrates the results of such a validation using the in vivo data from the formulation of the lot ultimately selected.▲ (USP 1-May-2021)
6. ▲Once a *Level A* correlation is established, in vitro testing can be used to justify dissolution specifications and biowaivers.▲ (USP 1-May-2021)

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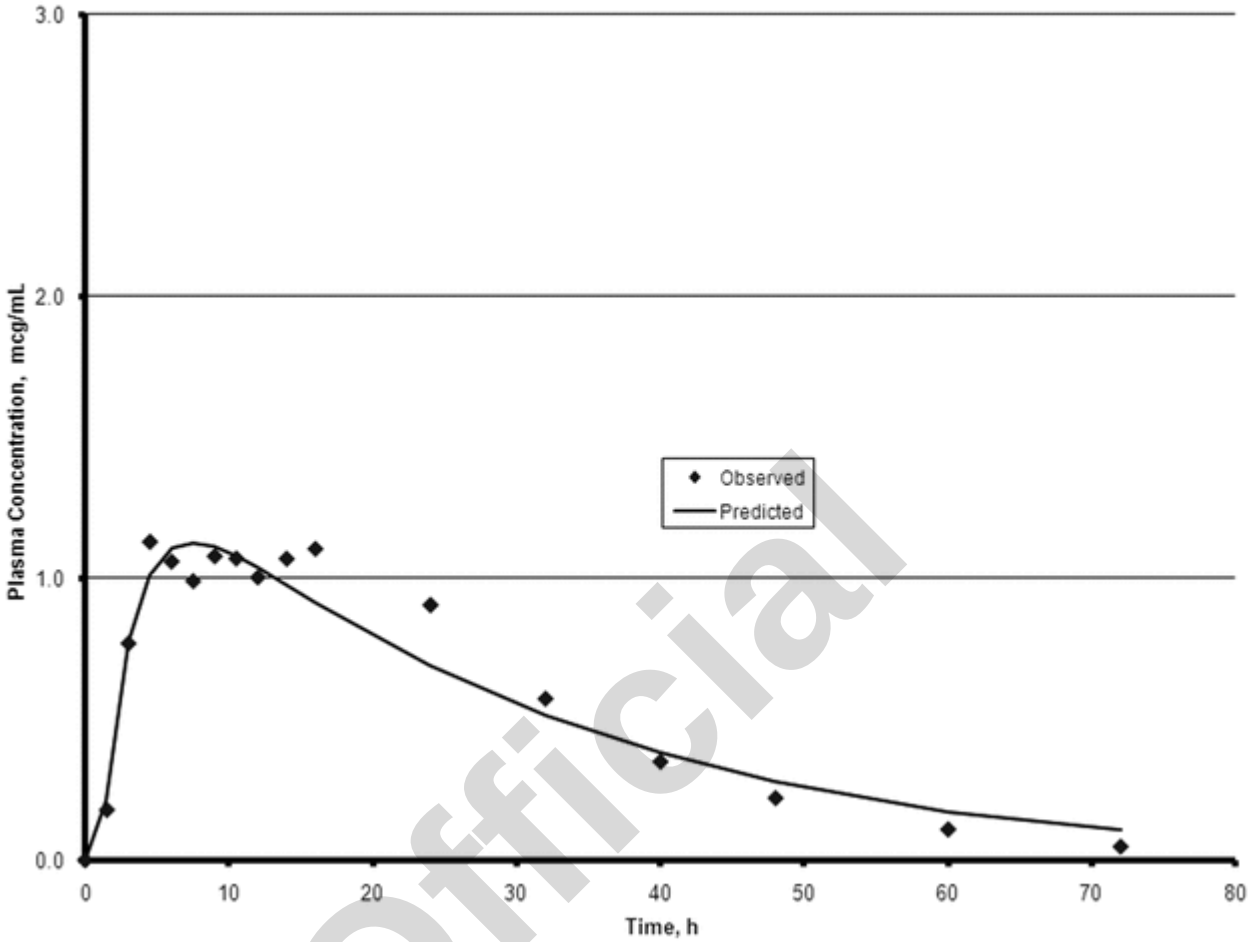


Figure 6. Observed and predicted mean plasma profiles: slow formulation.

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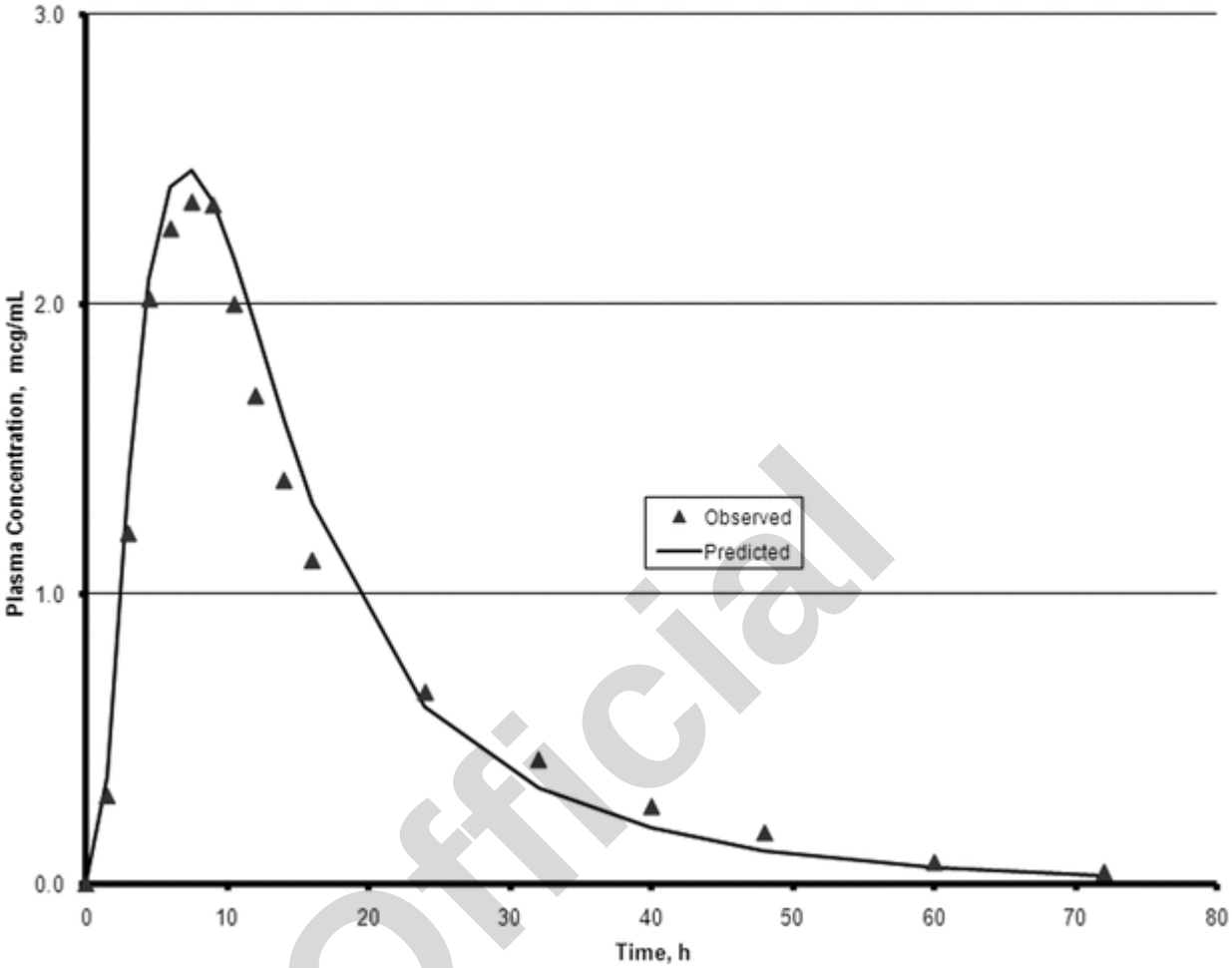


Figure 7. Observed and predicted mean plasma profiles: fast formulation.

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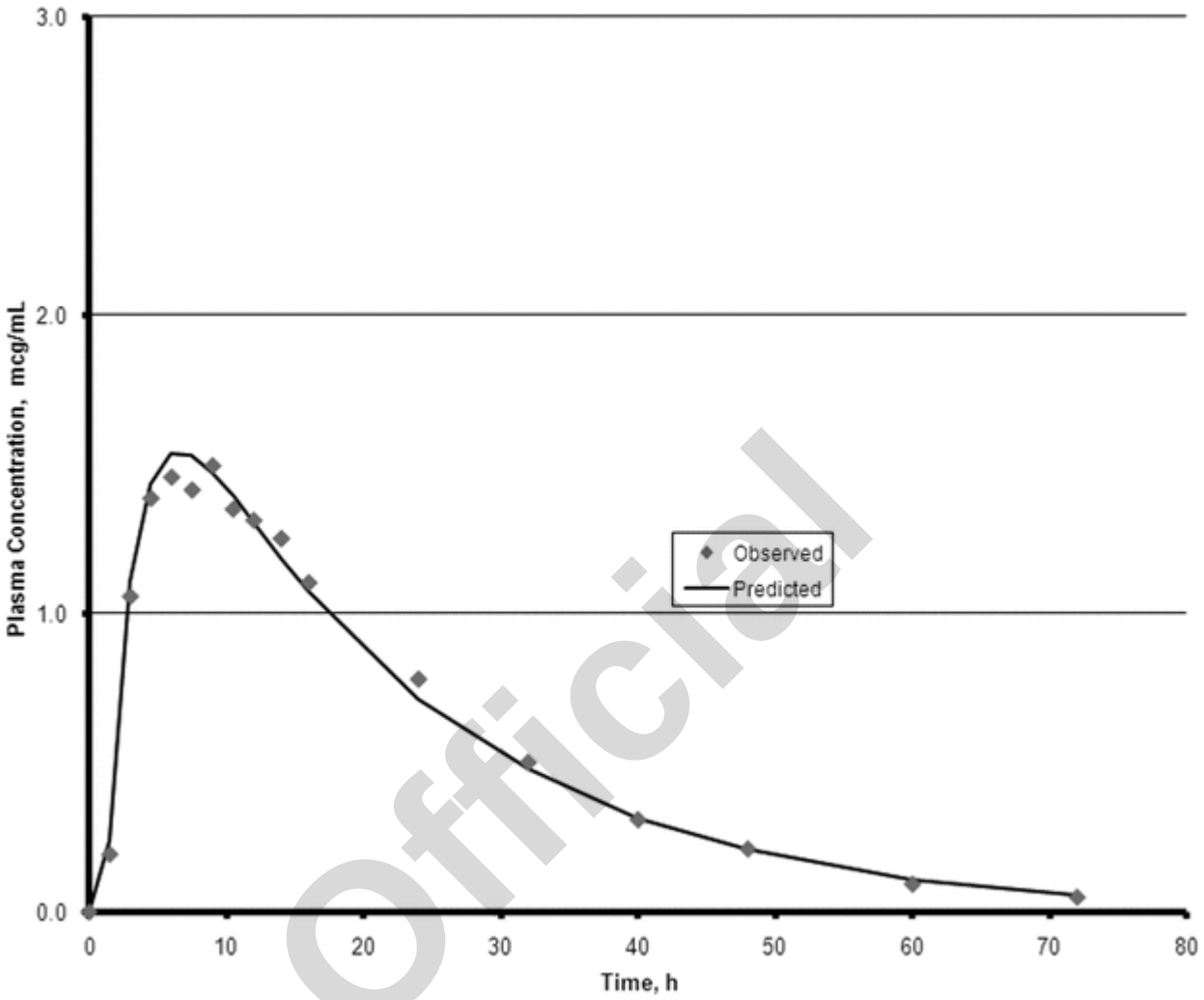


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Figure 8. Observed and predicted mean plasma profiles: medium formulation.

▲▲ (USP 1-May-2021)

## Establishment of Dissolution Specification Ranges

▲When establishing▲ (USP 1-May-2021) a multipoint dissolution specification for a modified-release dosage form, the dissolution behavior of the biobatch can be used to define the amount that will be released at each time point. The difficulty arises in the variation that will be allowed around each time point. In the case of a *Level A* correlation, this can be done in two ways, both of which use IVIVC: convolution and deconvolution.

### CONVOLUTION

Reasonable upper and lower dissolution values are selected for each time point established from the biobatch. ▲▲ (USP 1-May-2021) The dissolution curves defined by the upper and lower ▲limits▲ (USP 1-May-2021) are convoluted to project the anticipated plasma level curves that would result from administration of these formulations to the same patients to whom the biobatch was administered. If the resulting plasma level data fall within the ▲90%▲ (USP 1-May-2021) confidence intervals obtained in the definitive BA–BE study, these ranges can be considered acceptable. ▲▲ (USP 1-May-2021)

### DECONVOLUTION

An acceptable set of plasma-level data is established both for a ▲formulation▲ (USP 1-May-2021) demonstrating a more rapid release and for one demonstrating a slower release than that of the biobatch. These can be selected by using the extremes of the ▲90%▲ (USP 1-May-2021) confidence intervals or ±1 standard deviation of the mean plasma level. These curves are then deconvoluted, and the resulting input rate curve is used to establish the upper and lower dissolution specifications at each time point. In the case of *Level B* and *C* correlations, ▲formulations▲ (USP 1-May-2021) must be made at the proposed upper and lower

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limits of the dissolution range, and it must be demonstrated that these ▲formulations▲ (USP 1-May-2021) are acceptable by a BA–BE study.

## ▲Considerations for▲ (USP 1-May-2021) Immediate-Release Dosage Forms

Because the mechanisms for drug release from modified-release dosage forms are more complex and variable than those associated with immediate-release dosage forms, one would anticipate that an IVIVC would be easier to develop with the latter formulations. Unfortunately, most of the correlation efforts to date with immediate-release dosage forms have been based on the correlation *Level C* approach, although there also have been efforts employing statistical moment theory (*Level B*). Although it is conceivable that the same *Level A* correlation approach can be used with immediate-release dosage forms, until data have been gathered to support this concept, *Level B* and *Level C* are the best approaches that can be recommended with these dosage forms.

***Add the following:***

## ▲GLOSSARY

**Biobatch:**
The lot of drug product formulated for purposes of pharmacokinetic evaluation in a bioavailability/bioequivalency study. For modified-release solid oral products, this batch should be 10% or greater than the proposed commercial production batch or at least 100,000 units, whichever is greater.

**Biopredictive dissolution method:**
A set of testing conditions for which in vitro dissolution profiles are capable of predicting pharmacokinetic profiles. These are typically based on classical or mechanistic IVIVC.

**Biorelevant dissolution method:**
A set of testing conditions for monitoring in vitro dissolution designed to closely mimic a relevant biological fluid and physiological environment.

**Clinically relevant dissolution specification:**
A set of in vitro dissolution testing conditions and acceptance criterion(ia) that can identify and reject drug product batches that are not expected to be bioequivalent to clinical pivotal product batches.

**Discriminating dissolution specifications:**
A set of in vitro dissolution testing conditions that, along with the acceptance criterion(ia), are able to differentiate drug products manufactured under target conditions versus drug products that are intentionally manufactured with meaningful variations (i.e., formulation and manufacturing variants) for the relevant manufacturing variables (e.g., drug substance particle size, compression force, tablet hardness).

**In vitro–in vivo correlation (IVIVC):**
A predictive mathematical model describing the relationship between an in vitro property of an extended release dosage form (usually the rate or extent of drug dissolution or release) and a relevant in vivo response (e.g., plasma drug concentration or amount of drug absorbed).

**In vitro–in vivo relationship (IVIVR):**
An IVIVR is a relationship which simply states that an in vitro change will result in an in vivo change (in rank order), but the amount of change is not mathematically predictable.

**Modified release:**
A dosage form release pattern when the rate and/or time of release of the drug substance is altered as compared to what would be observed or anticipated for an immediate-release product. Two modified-release profiles, delayed-release and extended-release, are recognized.

**Pharmaceutically equivalent:**
Drug products containing the same active ingredient(s), having the same route of administration, and with identical strength or concentration of the same dosage form.

**Percent prediction error:** Calculated as [(observed value – predicted value)/observed value] × 100.

**Predictability:**
Verification of the model's ability to describe in vivo bioavailability results from a test set of in vitro data (external predictability) as well as from the data used to develop the correlation (internal predictability).

**Prediction error:**
A measure of the prediction by the IVIVC model of in vivo performance from in vitro dissolution data.▲ (USP 1-May-2021)

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# ⟨1090⟩ ASSESSMENT OF SOLID ORAL DRUG PRODUCT PERFORMANCE AND INTERCHANGEABILITY, BIOAVAILABILITY, BIOEQUIVALENCE, AND DISSOLUTION

BACKGROUND
BIOAVAILABILITY, BIOEQUIVALENCE, AND DISSOLUTION
BIOEQUIVALENCE
- Immediate-Release Drug Products
- Modified-Release Drug Products
- Orally Administered Drug Products, Not for Systemic Effect
- Bioequivalence Studies
- Statistical Analysis

DISSOLUTION AND IN VITRO PRODUCT PERFORMANCE
- Dissolution and In Vitro BA
- Dissolution and In Vitro Equivalence
- Dissolution Profile Comparisons

INTERCHANGEABILITY OF DRUG PRODUCTS
SOLID FORM AND PARTICLE SIZE
DIFFERENCES IN EXCIPIENTS
MANUFACTURING PROCESS
BIOWAIVER
- Biowaiver Based on Dosage Form Proportionality
- Biowaiver Based on the BCS

APPENDIX

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## BACKGROUND

This chapter provides recommendations for the in vivo and in vitro assessment of solid oral drug product performance. The chapter is intended as a guide for scientists and clinicians seeking to evaluate drug product performance by surrogate procedures correlative and/or antecedent to clinical trials in humans. *USP–NF* provides quality standards for drug substances, excipients, and finished preparations. A *USP–NF* monograph for an official substance or preparation includes the article's definition; packaging, storage, and other requirements; and a specification. The specification consists of a series of universal tests (description, identification, impurities, and assay) and specific tests—one or more analytical procedures for each test—and acceptance criteria. Quality standards are important attributes that must be built into the drug product. Meeting *USP–NF* standards is accepted globally as assurance of high quality and is part of the requirements necessary for approval of a bioequivalent , interchangeable drug product. Drug products must meet certain in vivo and/or in vitro performance standards to be considered therapeutically equivalent (TE) and interchangeable. Drug product performance may be defined as the release of the drug substance from the drug product dosage form, normally leading to systemic exposure or less often to local activity of the drug substance necessary for achieving a desired therapeutic response. Bioavailability (BA) is a measure of systemic exposure. This chapter discusses in vivo and in vitro approaches to determining drug product performance. The focus of the chapter is primarily on the performance of solid oral drug products.

This chapter references two Food and Drug Administration (FDA) guidances: *Guidance for Industry: Bioavailability and Bioequivalence Studies Submitted in NDAs or INDs—General Considerations* (draft guidance March 2014) and *Guidance for Industry: Bioequivalence Studies with Pharmacokinetic Endpoints for Drugs Submitted Under an ANDA* (draft guidance December 2013) (search by document title; http://www.fda.gov/); a European Medicines Agency (EMA) guidance: *Guideline on the Investigation of Bioequivalence* (2010) (search by document title; http://www.ema.europa.eu/ema/), and a World Health Organization (WHO) document, *Annex 7: Multisource (Generic) Pharmaceutical Products: Guidelines on Registration Requirements to Establish Interchangeability*, WHO Expert Committee on Specifications for Pharmaceutical Products: Fortieth Report. World Health Organization: Geneva; 2006: Annex 7 (WHO Technical Report Series, No. 937). TRS 992 (2015) (search by document title; http://who.int/en/). FDA guidances are used in the United States, and WHO, FDA, EMA, and national/regional guidelines may be used by national/regional drug regulatory authorities outside the U.S. Following approval, control of the quality of a drug product can be achieved in part by using the private and/or public specification, which can include a performance test. USP provides the general chapters *Disintegration* ⟨701⟩, *Dissolution* ⟨711⟩, *Drug Release* ⟨724⟩, *In Vitro and In Vivo Evaluation of Dosage Forms* ⟨1088⟩, *The Dissolution Procedure: Development and Validation* ⟨1092⟩, and *Capsules—Dissolution Testing and Related Quality Attributes* ⟨1094⟩, which describe these tests and procedures.

This chapter provides general information about the conduct of bioequivalence (BE) studies as a surrogate measure of in vivo drug product performance and dissolution profile comparisons as a measure of in vitro drug product performance as stated in the guidelines cited in this chapter. The chapter also discusses conditions when an in vivo BE requirement may be waived (biowaiver) for certain drug products and shows how the Biopharmaceutics Classification System (BCS) can be used as a predictor of a drug product's performance. An *Appendix* to this chapter defines key scientific terminology and provides a comparison between FDA, EMA, and WHO in terms of drug product performance assessment. Other BE guidance may exist outside of that given in this chapter.

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## BIOAVAILABILITY, BIOEQUIVALENCE, AND DISSOLUTION

BA studies focus on determining the process and time frame by which a drug substance is released from the oral dosage form and moves to the site of action [see *Guidance for Industry: Bioavailability and Bioequivalence Studies Submitted in NDAs or INDs—General Considerations* (FDA draft guidance March 2014) and *Guidance for Industry: Bioequivalence Studies with Pharmacokinetic Endpoints for Drugs Submitted Under an ANDA* (FDA draft guidance December 2013) (search by document title; http://www.fda.gov/]. BA is an indirect or surrogate measure of the rate and extent to which the drug substance or active moiety is absorbed from a drug product and becomes available at its target sites of action. BA data provide an estimate of systemic drug exposure, including fraction of drug absorbed. The determination of availability of drug substance from drug products that are not intended to be absorbed into the bloodstream is outside the scope of this chapter. Drug products are considered bioequivalent if a test (*T*) drug product does not show a significant difference in rate and extent of absorption by comparison with a designated reference (*R*) drug product when administered at the same dose under similar experimental conditions in either a single dose or in multiple doses.

BA and BE generally can be obtained by serially measuring drug substance and/or metabolite concentrations in the systemic circulation over a prescribed period. BE studies can use other approaches when systemic drug concentrations cannot be measured or are not appropriate. For these cases, more indirect approaches to BE determination include acute pharmacodynamic endpoints, clinical endpoints, and in vitro studies that typically involve comparisons of the dissolution profiles of (*T*) and (*R*) drug products. In the absence of an in vitro–in vivo correlation (IVIVC) or considerations based on the BCS, in vitro performance studies are not used in place of BA studies.

BA and BE information are important in regulatory submissions. BA information broadly addresses the absorption, distribution, metabolism, and excretion of the drug substance. For an innovator product, BE studies establish the performance of the product intended for marketing by comparing the BA of the product as developed for marketing approval to the clinical trial material, the drug product used in safety/efficacy trials. For the development and regulatory approval of a generic drug product, the *T* must be bioequivalent to the *R* (usually the brand or innovator drug product that is designated by the applicable regulatory authority).

The ICH guideline document titled *Specifications: Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Chemical Substances Q6A* (2000) (search by document title; http://www.fda.gov/) provides approaches for setting acceptance criteria for drug product performance. These approaches rely on dissolution or disintegration based on clinically acceptable batches, as does FDA's approach. BE studies focus on the performance of the drug product and usually involve comparisons of two drug products: *T* and *R*. The required studies and the determination of BE are the province of regulatory agencies. In the United States, *R* is termed the RLD and is so noted in FDA's *Approved Drug Products with Therapeutic Equivalence Evaluations,* 37th Edition [*Orange Book* (2017) (http://www.accessdata.fda.gov/scripts/cder/ob/)]. To assist countries and regions where the *R* product may not always be readily identifiable, WHO has prepared a document titled *Annex 11: Guidance on the Selection of Comparator Pharmaceutical Products for Equivalence Assessment of Interchangeable Multisource (Generic) Products* (2002) (search by document title; http://www.who.int/en/). In the WHO document, *R* is termed the "comparator pharmaceutical product" (CPP). When a country or region has a clearly defined set of CPPs, the task becomes one of requiring that a manufacturer demonstrate, to the satisfaction of its regulatory authority, that its product is pharmaceutically equivalent (PE) and bioequivalent to the corresponding CPP.

## BIOEQUIVALENCE

An interchangeable drug product must be PE. The EMA and WHO documents allow pharmaceutical alternatives to be considered TE and interchangeable if they are bioequivalent. Further, generic products must be shown to be bioequivalent to be considered TE to the *R* product . For the product to be considered PE, it must have the same drug substance, same strength, same dosage form, same route of administration, and same labeling as the *R* product. Several methods exist to assess and document BE. These include the following:

1. *Comparative pharmacokinetic studies in humans.* In these studies, the drug substance and/or its metabolite(s) are measured as a function of time in accessible biological fluid such as blood, plasma, serum, or urine to obtain pharmacokinetic measures such as area under the plasma drug concentration versus time curve (AUC) and maximum concentration ($C_{max}$) that are reflective of systemic exposure. Other pharmacokinetic parameters such as time to peak concentration ($T_{max}$) may be informative for safety and efficacy assessment.
   BE studies are designed to compare the in vivo performance of 1) a generic product with an *R* product or 2) a new formulation of an *R* product with the originally approved product. Generally the design is a two-period, two-sequence, single-dose, crossover randomized study. The number of subjects should be statistically justified and NLT 12. The necessary statistical power of the study will determine the number of subjects >12. During the study, blood samples are collected at sufficient intervals for assessing $C_{max}$, AUC, and other parameters. Blood samples are analyzed using appropriately validated bioanalytical methodology with standard pharmacokinetic measures and statistical approaches. The statistical method for testing pharmacokinetic BE is based on the determination of the 90% confidence interval around the geometric mean ratio of the log-transformed population means (*T*/*R*) for AUC and $C_{max}$ by carrying out two one-sided tests at the 5% level of significance.
2. *Other options.* In addition, comparative pharmacodynamic studies in humans and comparative clinical trials can be used to document or supplement BE assessment. In vivo documentation of equivalence is especially important for the following: drugs with a narrow therapeutic range; documented evidence of BE problems; modified-release pharmaceutical products designed to act by systemic absorption; and fixed-dose combination products with systemic action when at least one of the drug substances requires an in vivo study.

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## Immediate-Release Drug Products

Single-dose, crossover BE studies are carried out at the highest dose comparing *T* and *R* products under fasting conditions. A parallel study design can be used for drugs that have a very long elimination half-life ($t_{½}$). Sampling truncation at 72 h may be allowable by regulatory agencies. Lower strength(s) of the dosage form can be given a biowaiver based on linear pharmacokinetics, dosage form proportionality, and dissolution profile similarity. Food-effect studies are required as described within scale-up and postapproval changes-immediate release (SUPAC-IR). Food effect studies are required for all *T* immediate-release oral products except where: 1) the *T* and *R* products are both rapidly dissolving with similar dissolution profiles and containing highly soluble and highly permeable drug substances; or 2) where the labeling of the *R* product states that the product must be taken on an empty stomach; or 3) where the *R* product labeling does not make a statement about food effect on absorption or administration [see FDA *Guidance for Industry: Food-Effect Bioavailability and Fed Bioequivalence Studies* (2002); (search by document title; http://www.fda.gov/)].

## Modified-Release Drug Products

BE studies for modified-release dosage forms are carried out as single-dose, crossover studies under fasting and fed conditions at the highest dose to compare *T* and *R* products. A single-dose study is more sensitive than multiple-dose, steady-state studies in assessing in vivo drug product performance, particularly with regard to the phenomenon of dose dumping, i.e., the rapid and unintended premature release of the drug substance from an extended-release product into the gastrointestinal tract. Lower strengths of an extended-release dosage form may not require an in vivo study based on use of the same drug-releasing mechanism, dosage form proportionality, and similar dissolution profile. The FDA *Guidance for Industry: SUPAC-MR: Modified Release Solid Oral Dosage Forms Scale-Up and Postapproval Changes: Chemistry, Manufacturing, and Controls; In Vitro Dissolution Testing and In Vivo Bioequivalence Documentation* (search by document title; http://www.fda.gov/) provides guidance on the need for BE studies when changes are made to the formulation. BE studies under fed conditions are required for all generic orally administered modified-release products [see FDA *Guidance for Industry: Food-Effect Bioavailability and Fed Bioequivalence Studies* (2002)].

## Orally Administered Drug Products, Not for Systemic Effect

Some oral drug products are intended for local activity. Mesalamine and cholestyramine are examples of drugs that are intended for local activity. For these types of drugs, systemic absorption from the gastrointestinal tract is minimal; thus a comparative clinical trial is required while a systemic drug exposure profile also may be required. In some cases, in vitro studies may be appropriate, such as studies including comparison of cholestyramine binding to bile salts.

## Bioequivalence Studies

**Objective:** The objective of a BE study is to measure and compare formulation performance between two or more drug products. Drug availability from *T* and *R* products should not be statistically different when the drug is administered to patients or subjects at the same molar dose under similar experimental conditions.

**Design:** The design of a BE study depends on the objectives of the study, the ability to analyze the drug substance (and/or metabolites where appropriate) in biological fluids, the pharmacodynamics of the drug substance, the route of drug administration, and the nature of the drug and drug product. Pharmacokinetic parameters, pharmacodynamic parameters, clinical observations, and/or in vitro studies may be used to determine drug BA from a drug product.

Some possible BE study designs include the following:

1. Single-dose, two-way crossover study under fasted conditions
2. Single-dose, two-way crossover study under fed conditions
3. Single-dose, parallel study under fasted conditions
4. Single-dose, replicate design
5. Single-dose, partial replicate design
6. Multiple-dose, two-way crossover study, fasted conditions
7. Pharmacodynamic or clinical endpoint study
8. In vitro dissolution profile comparisons

The standard BE study is a crossover design (e.g., Latin square crossover design) in which each subject receives the *T* drug product and the *R* drug product on separate occasions. Studies are usually evaluated by a single-dose, two-period, two-treatment, two-sequence, open-label, randomized crossover design comparing equal doses of the *T* and *R* products in fasted or fed adult healthy subjects. A multiple-dose study may be required for some extended-release drug products. A washout is scheduled between the two periods to allow the subjects to completely eliminate the drug absorbed from the first dose before administration of the second dose of drug product. If the predose concentration is ≤5% of the $C_{max}$ value in that subject, the subject's data without any adjustments can be included in all pharmacokinetic measurements and calculations. Samples of an accessible biologic fluid such as blood are used to characterize the drug concentration versus time profile. During the fasting study, subjects are fasted at least 10 h. A predose (0 time) blood sample is taken. The drug product is given with 240 mL (8 fluid ounces) of water. No food is allowed for at least 4 h post dose. The *T* and *R* drug products are administered at the same time of day to avoid diurnal effects. Blood sampling is performed periodically after administration of drug product according to protocol. A food intervention or food effect study is conducted with standard meal conditions that are expected to provide the greatest effects on gastrointestinal physiology so that systemic drug availability is maximally affected. In addition, the high lipid content of the meal may affect the rate of drug release from

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the product, in situ. A high-fat (approximately 50% of total caloric content of the meal) and high-calorie (approximately 800–1000 calories) meal is recommended as a test meal for food-effect BA and fed BE studies. This test meal should derive approximately 150, 250, and 500–600 calories from protein, carbohydrate, and fat, respectively. The drug product is given with 240 mL (8 fluid ounces) of water after ingestion of the standard meal. Subjects should consume identical meals within the same interval before administration of the *T* or *R* drug products.

**Analysis of samples:** Samples, usually plasma, are analyzed for the drug substance and, on occasion, active metabolite concentrations by a validated bioanalytical method.

**Pharmacokinetic parameters:** Pharmacokinetic parameters are obtained from the resulting concentration–time curves. Two major pharmacokinetic parameters are used to assess the rate and extent of systemic drug absorption. AUC reflects the extent of drug absorption, and the $C_{max}$ reflects the rate of drug absorption. Other pharmacokinetic parameters may include the $T_{max}$, the elimination rate constant ($k$), elimination half-life ($t_{½}$), lag time ($T_{lag}$), and others.

## Statistical Analysis

Pharmacokinetic parameters are analyzed statistically to determine whether the *T* and *R* products yield comparable values. Because BE studies may use small sample sizes, log transformation of the data allows the frequency distribution of the data to be more normalized so that parametric statistical analyses may be performed (see FDA's *Guidance for Industry: Statistical Approaches to Establishing Bioequivalence* (2001); search by document title; http://www.fda.gov/).

Parametric (normal-theory) general linear model procedures are recommended for the analysis of pharmacokinetic data derived from in vivo BE studies. An analysis of variance (ANOVA) should be performed on the pharmacokinetic parameters AUC and $C_{max}$ using appropriate statistical programs and models. For example, for a conventional two-treatment, two-period, two-sequence (2 × 2) randomized crossover study design, the statistical model often includes factors accounting for the following sources of variation:

- Sequence (sometimes called Group or Order)
- Subjects, nested in sequences
- Period (or Phase)
- Treatment (sometimes called Drug or Formulation)

The sequence effect should be tested using the [subject (sequence)] mean square from the ANOVA as an error term. All other main effects should be tested against the residual error (error mean square) from the ANOVA. The least-squares means (LSMEANS) statement should be used to calculate LSMEANS for treatments. Estimates should be obtained for the adjusted differences between treatment means and the standard error associated with these differences.

The statistical assumptions underlying the ANOVA are as follows:

- Randomization of samples
- Homogeneity of variances
- Additivity (linearity) of the statistical model
- Independence and normality of residuals

In BE studies, these assumptions can be interpreted as follows:

- The subjects chosen for the study should be randomly assigned to the sequences of the study.
- The variances associated with the two treatments, as well as between the sequence groups, should be equal or at least comparable.
- The main effects of the statistical model, such as subject, sequence, period, and treatment effect for a standard 2 × 2 crossover study, should be additive. There should be no interactions between these effects.
- The residuals of the model should be independently and normally distributed.

If these assumptions are not met, additional steps should be taken prior to the ANOVA, including data transformation to improve the fit of the assumptions or use of a nonparametric statistical test in place of ANOVA. However, the normality and constant variance assumptions in the ANOVA model are known to be relatively robust (i.e., a small or moderate departure from one or both of these assumptions will not have a significant effect on the final result). The rationale for log transformation is provided in FDA's *Guidance for Industry: Statistical Approaches to Establishing Bioequivalence* (2001) [search by document title; see FDA http://www.fda.gov/].

**The two one-sided tests procedure:** A testing procedure termed the two one-sided tests procedure is used to determine the comparability of geometric mean values for pharmacokinetic parameters measured after administration of the *T* and *R* products.[1] The two one-sided tests procedure determines whether *T* is not importantly less than *R* and whether *R* is not importantly less than *T*. Other statistical approaches to the determination of a clinically meaningful difference may be used. Most often, 20% defines a clinically meaningful difference. The statistical procedure involves the calculation of a confidence interval for the ratio (or difference) between *T* and *R* pharmacokinetic variable averages. The limits of the observed confidence interval must fall within a predetermined range for the ratio (or difference) of the product averages. Point estimate mean ratios ($T/R$) derived from the log-transformed AUC and $C_{max}$ data must be between 80% and 125%. Because data are log transformed, $T/R = 80/100 = 80\%$ and $R/T = 100/80 = 125\%$. In addition, the 90% confidence intervals for the geometric mean ratios ($T/R$) for AUC and $C_{max}$ must be between 80.00% and 125.00%. The regulatory requirements for the range of 90% confidence intervals for $C_{max}$ are different in countries outside of the United States.

**Bio-inequivalence:** The failure to demonstrate BE may be due to a performance failure of the *T* product or to an inadequate study design. The failure to demonstrate BE because of an inadequate study design can be due to improper sampling in which: 1) the sampling time for $C_{max}$ was not properly obtained, or 2) the number of samples taken did not adequately describe the

[1] Schuirmann DJ. A comparison of the two one-sided tests procedure and the power approach for assessing the equivalence of average bioavailability. *J Pharmacokinet Biopharm.* 1987;15(6):657–680.

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plasma drug concentration versus time profile. Often, with highly variable drugs (e.g., % coefficient of variation [CV] of $C_{max}$ or AUC > 30%), where failure to demonstrate BE was observed, the study was not powered adequately due to too few subjects used.

**Presentation of data:** The drug concentration in biological fluid at each sampling time point should be furnished untransformed for all the subjects who participated in the study. The derived pharmacokinetic parameters also should be furnished untransformed. The mean, the standard deviation, and the CV for each variable should be computed and tabulated in the final report.

To facilitate BE comparisons, pharmacokinetic parameters for each individual should be displayed in parallel for the formulations tested. In particular, for AUC and $C_{max}$, the difference ($T - R$), the ratio ($T/R$), and the log of ratio (log $T/R$ or ln $T/R$) between the $T$ and $R$ values should be tabulated side-by-side for all the subjects. For each subject, the summary tables should indicate in which sequence ($T$ then $R$, or $R$ then $T$) the subject received the product. Histograms showing the frequency distribution of the difference and ln ratio (or log ratio) for the major pharmacokinetic parameters (AUC and $C_{max}$) are useful in the submission. NMT 20% of the total AUC (zero to infinity) should be obtained by an extrapolated terminal elimination.

In addition to the arithmetic mean for the $T$ and $R$ products, the geometric means (antilog of the means of the logs), means of the logs, and standard deviations of the logs should be calculated for AUC and $C_{max}$. All means, including arithmetic mean, geometric mean, and means of the logs, as well as standard deviations and CVs, should be included in the report.

## DISSOLUTION AND IN VITRO PRODUCT PERFORMANCE

As noted for an official preparation, *USP* monographs provide a public specification that includes a list of tests, references to analytical procedures, and acceptance criteria. Most solid oral dosage forms, including oral suspensions, require a dissolution or drug release test. Drug dissolution and drug release testing are described in ⟨*711*⟩ and ⟨*724*⟩. These public specifications are used for quality control tests and for market approval. The *USP* dissolution test in the monograph is related to BA and BE only when closely allied with a sound regulatory determination. Without this association, the *USP* dissolution test should be regarded solely as a quality control test for batch release. FDA guidances are: 1) *Guidance for Industry: Dissolution Testing of Immediate Release Solid Oral Dosage Forms* (1997) (http://www.fda.gov/; search by document title); 2) *Guidance for Industry: Extended Release Oral Dosage Forms: Development, Evaluation, and Application of In Vitro/In Vivo Correlations* (1997); and 3) *Guidance for Industry: Dissolution Testing and Specification Criteria for Immediate-Release Solid Oral Dosage Forms Containing Biopharmaceutics Classification System Class 1 and 3 Drugs* (draft July 2015) (search by document title; http://www.fda.gov/).

### Dissolution and In Vitro BA

Drug dissolution and release tests are very useful during drug product development for identifying critical manufacturing attributes such as the impact of ingredient properties and the impact of the manufacturing process on drug product performance. During product development, optimum dissolution conditions need to be developed to discriminate among drug product formulations and changes in manufacturing processes. After the finished dosage form is approved for marketing, drug dissolution and release tests may be useful in predicting possible changes in performance due to scale-up and postapproval changes. See the following FDA guidances:

*Guidance for Industry: Immediate Release Solid Oral Dosage Forms, Scale-Up and Postapproval Changes: Chemistry, Manufacturing, and Controls, In Vitro Dissolution Testing, and In Vivo Bioequivalence Documentation* (1995) (search by document title; http://www.fda.gov/) and

*Guidance for Industry: SUPAC-MR: Modified Release Solid Oral Dosage Forms, Scale-Up and Postapproval Changes: Chemistry, Manufacturing, and Controls; In Vitro Dissolution Testing and In Vivo Bioequivalence Documentation* (1997) (search by document title; http://www.fda.gov/).

For some oral drug products, in vitro drug dissolution can be related to in vivo performance, such as BA and/or systemic drug exposure. Chapter ⟨*1088*⟩ describes various approaches to IVIVC.

### Dissolution and In Vitro Equivalence

An appropriate dissolution test is a powerful in vitro physiochemical test that measures drug product quality and performance for a variety of dosage forms, such as solid oral dosage forms, transdermal dosage forms, suspensions, and certain semisolid dosage forms. The *USP* tests for finished dosage forms can be divided into two types: 1) drug product quality tests, and 2) drug product performance tests. Product quality tests are intended to assess attributes such as assay and content uniformity; product performance tests are designed to assess product performance, and in many cases relate to dissolution. For details regarding the performance of a dissolution/drug release test, see ⟨*711*⟩, ⟨*724*⟩, ⟨*1088*⟩, ⟨*1092*⟩,and *Semisolid Drug Products—Performance Tests* ⟨*1724*⟩.

The in vitro dissolution test was initially developed as a quality control tool to ensure drug product quality and batch-to-batch consistency. The test procedures for conducting dissolution/drug release tests are described in ⟨*711*⟩ and ⟨*724*⟩. The development of the BCS brings new understanding and power to the dissolution test. The BCS classifies the drug substance according to its aqueous solubility, as well as its permeability through a biomembrane, such as the intestinal mucosal cells. The dissolution rate of the drug substance from the dosage form is important in substantiating biowaivers based on the BCS.

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## Dissolution Profile Comparisons

In vitro drug dissolution and release testing can be related to in vivo drug performance, such as BA. The comparisons of dissolution profiles are gaining importance as a means of documenting comparative BA studies—that is, BE. A biowaiver is the replacement or waiver of in vivo BE studies by an in vitro test.

A model independent mathematical approach is used to compare the dissolution profiles of two products: 1) to compare the dissolution profile between the *T* product and *R* product in biowaiver considerations; 2) to compare the dissolution profile between the two strengths of products from a given manufacturer; and 3) for SUPAC after the product is approved. For comparing the dissolution profile, the similarity factor $f_2$ should be computed using the equation:

$$f_2 = 50 \cdot \log\left\{\left[1 + (1/n)\sum_{t=1}^{n}\left(R_t - T_t\right)^2\right]^{-0.5} \cdot 100\right\}$$

where $R_t$ and $T_t$ are the cumulative percentage of the drug dissolved at each of the selected *n* time points of the *R* and *T* product, respectively. An $f_2$ value of 50 or greater (50–100) ensures dissolution profile similarity and thus equivalence of the performance of the two products. FDA guidance recommends that, at a minimum three points, NMT 1 point exceeding 85% of the label claim dissolved, should be used for similarity profile comparison. For products that dissolve very rapidly (≥85% dissolution in 15 min), a profile comparison is not necessary. Different guidances have slightly different considerations in the application of dissolution profile comparisons that are given in *Table A-1*.

## INTERCHANGEABILITY OF DRUG PRODUCTS

The interchangeability of drug products is a major concern for physicians, pharmacists, and others who prescribe, dispense, or purchase them. Interchangeability of drug products is a regulatory decision requiring a determination of therapeutic equivalence. The FDA determines therapeutic equivalence of products in the U.S. Because the formulation and method of manufacture of the drug product can affect its BA and stability, the manufacturer must demonstrate that the *T* drug product is bioequivalent and *TE* to the *R*. The factors that can affect the drug product performance and therefore interchangeability of the finished drug product include:

- Solid form and particle size of the drug substance
- Differences in excipients in the formulation
- Differences in the manufacturing process

## SOLID FORM AND PARTICLE SIZE

As a result of the synthetic route and method of purification, the drug substance may be present with different particle size or solid form. Different solid forms, crystalline or amorphous, can dissolve at different rates, and therefore the extent of absorption and BA of these substances from the same dosage form, e.g., oral tablet, may be different. For example, crystalline structures are more thermodynamically stable than amorphous forms and as a consequence may dissolve more slowly. Different polymorph, hydrate, or salts of the drug substance may also have different physical chemical properties. These differing forms of the drug may have different aqueous solubilities or may dissolve at different rates. Particle size or particle size distribution of the drug may also affect the rate of dissolution. A drug substance having a particle size distribution with a large number of very fine particles may dissolve faster and lead to systemic absorption that is more rapid than for a particle size distribution with larger particles.

## DIFFERENCES IN EXCIPIENTS

A pharmaceutical dosage form typically consists of excipients as well as the drug substance(s). An excipient is any component, other than the drug substance(s), intentionally added to the formulation of a dosage form. Excipients, which may have no pharmacodynamic activity, play a critical role in manufacturing, stability, and performance. Excipients are manufactured to comply with compendial standards that address quality. However, the physical and chemical properties of the excipients affect the performance of a finished dosage form at least as much as the physical and chemical properties of the drug substance.

## MANUFACTURING PROCESS

Variation in the manufacturing process can have a dramatic effect on drug product performance. Blending, sieving, compression force, precompression or granulation, and coating are all manufacturing operations that can impact product performance. Interchangeability can be affected by the manufacturing operation that is as important a consideration as the control of the drug substance form and the properties of the excipients used in the formulation.

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# BIOWAIVER

The term "biowaiver" is applied to a regulatory approval process when the application (dossier) is approved on the basis of evidence of equivalence other than an in vivo BE test. For solid oral dosage forms, the evidence of equivalence is determined on the basis of an in vitro dissolution profile comparison between the *T* and the *R* product.

## Biowaiver Based on Dosage Form Proportionality

When a single-dose fasting BE study is conducted on the designated (usually highest) strength of the drug product, the requirement for the conduct of additional in vivo BE studies on the lower strengths of the same product can be waived, provided that the lower strength: 1) is in the same dosage form; 2) is proportionally similar in its active and inactive ingredients; 3) has the same drug release mechanism (for extended-release products); 4) meets an appropriate in vitro dissolution profile comparison criterion (e.g., $f_2 \geq 50$); and 5) both lower and higher strengths are within the linear pharmacokinetic range.

## Biowaiver Based on the BCS

BCS provides a regulatory platform for replacing certain BE studies with in vitro dissolution tests. The BCS takes into account three major factors that govern the rate and extent of drug absorption from immediate-release dosage forms. These are aqueous solubility and intestinal permeability of the drug substance in conjunction with the dissolution of the pharmaceutical dosage form. These properties are assessed and categorized as being low or high for the purpose of classification. On the basis of the solubility and permeability of the dosage form, the drug substance is categorized in different classes:

- Class 1: high solubility, high permeability
- Class 2: low solubility, high permeability
- Class 3: high solubility, low permeability
- Class 4: low solubility, low permeability

Solubility classification of a drug substance as low or high depends on the dose of the drug product. FDA defines a drug substance as being highly soluble "when the highest dose strength is soluble in 250 mL or less of aqueous media over the pH range of 1.0–6.8" (See *FDA Guidance for Industry: Waiver of In Vivo Bioavailability and Bioequivalence Studies for Immediate-Release Solid Oral Dosage Forms Based on a Biopharmaceutics Classification System* (December 2017) (search by document title; http://www.fda.gov/). However, EMA uses the highest single dose administered, and WHO uses the highest therapeutic dose approved by a competent authority, typically defined by the labeling for the innovator product.

The classification of drug substance permeability as high or low differs among the FDA, WHO, and EMA. FDA states that a drug substance is highly permeable where the extent of absorption is 90% or more of an administered dose in the absence of evidence of instability in the gastrointestinal tract. WHO considers a drug substance (API) highly permeable when the extent of absorption is 85% or more of an administered dose. EMA finds complete absorption to be established where the measured extent of absorption is ≥85% and goes on to say that complete absorption is generally related to high permeability. FDA, WHO, and EMA all use mass balance or comparison to an intravenous reference dose (EMA: absolute BA) in support of the claim of extent of absorption.

The FDA requirements for BCS-based biowaiver apply to immediate-release drug products when the drug substance is BCS Class 1 and the *R* and *T* products are rapidly dissolving (NLT 85% in NMT 30 min) or BCS Class 3 where *R* and *T* are very rapidly dissolving (NLT 85% in NMT 15 min) and contain the same excipients. *T* and *R* products should be PE and exhibit similar dissolution profiles. The FDA biowaiver excludes immediate-release products that have a narrow therapeutic index or are designed to be absorbed in the oral cavity. The EMA requirements for BCS-based biowaiver are applied to immediate-release solid oral drug products considered not to have a narrow therapeutic index. Both BCS Class 1 and 3 compounds can be considered. The *T* and *R* must have the identical drug substance, but different salts are acceptable as long as both belong within BCS Class 1. For BCS Class 1 drug substances, in vitro dissolution is either very rapid (i.e., >85% in 15 min) or similarly rapid (i.e., 85% in <30 min and similar dissolution profiles). For BCS Class 3 drug substances, a biowaiver would require very rapid in vitro dissolution for both *T* and *R*. Excipients that might affect BA are the same and other excipients are similar qualitatively and in similar amounts.

The WHO requirements for BCS-based biowaiver apply to BCS Class 1 and 3 drug substances. For BCS Class 1 substances, both *T* and *R* products should be rapidly dissolving and demonstrate similar dissolution profiles. For BCS Class 3 drug substances, dissolution should be very rapid in vitro. The same excipient composition for both *T* and *R* drug products is required.

Use of the BCS has become a means of documenting BE without the conduct of an in vivo study; see the FDA guidance *Guidance for Industry: Waiver of In Vivo Bioavailability and Bioequivalence Studies for Immediate-Release Solid Oral Dosage Forms Based on a Biopharmaceutics Classification System* (December 2017) (search by document title; http://www.fda.gov/).

The in vitro dissolution studies are generally carried out by basket method at 100 rpm or by paddle method at 50 rpm [FDA guidance cited immediately above and EMA guidance, *Guideline on the Investigation of Bioequivalence* (2010)] or 75 rpm [WHO guidance, *Annex 7: Multisource (Generic) Pharmaceutical Products: Guidelines on Registration Requirements to Establish Interchangeability*, WHO Expert Committee on Specifications for Pharmaceutical Products: Fortieth Report. WHO: Geneva; 2006: Annex 7 WHO Technical Report Series, No. 937). TRS 992] (search by document title; http://www.who.int/en/) in 900 mL of medium at pH 1.2, 4.5, and 6.8. On the basis of dissolution rate, the pharmaceutical dosage forms are classified as: 1) very rapidly (FDA, EMA and WHO) dissolving, if 85% or more of the labeled content dissolves in 15 min or less, or 2) rapidly dissolving, if 85% or more of the labeled content dissolves in 30 min.

For biowaiver, the dissolution tests should be carried out for both *T* and *R* product under the same test conditions. For the generic product to be eligible for biowaiver, the *R* product should belong to the same BCS class and should meet dissolution

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profile comparison criteria. On the basis of the BCS classification and dissolution profile comparison, a biowaiver can be considered by regulatory authorities, provided the dissolution profile similarity criteria are met *Table A-1*).

## DISSOLUTION AS A QUALITY CONTROL TEST AND VERSUS AS AN IN VITRO EQUIVALENCE TEST

There is a clear difference between dissolution as a quality control test and dissolution as an in vitro equivalence test. For immediate-release dosage forms, the quality control test involves a single-point dissolution test in only one medium (generally a compendial test). On the other hand, the in vitro equivalence test involves dissolution profile comparison in pH 1.2, 4.5, and 6.8 between the *T* product and the *R* product.

# APPENDIX

## Comparisons Among FDA, EMA, and WHO

**Table A-1. Comparison of Approaches to BCS-Based Biowaiver**

| | FDA | EMA | WHO |
|---|---|---|---|
| Conditions for $f_2$ calculation | Only one measurement should be considered after 85% dissolution of both the products | A minimum of 3 time points (0 excluded) and NMT 1 mean value of >85% dissolved for any of the formulations | A maximum of 1 time point should be considered after 85% dissolution of the reference (comparator product) has been reached. In cases where 85% dissolution cannot be reached, the dissolution should be conducted until an asymptote (plateau) has been reached. |
| Requirements | $f_2 \geq 50$ | $50 \leq f_2 \leq 100$ | $50 \leq f_2 \leq 100$ |
| Very rapidly dissolving | NLT 85% in 15 min (using the method according to the guideline) | >85% of the labeled amount dissolves in 15 min (using the method according to the guideline) | NLT 85% of the labeled amount dissolves in 15 min (using the method according to the guideline) |
| Rapidly dissolving | ≥85% of the labeled amount dissolves in 30 min (using the method according to the guideline) | ≥85% of the labeled amount dissolves within 30 min (similarly rapid) (using the method according to the guideline) | NLT 85% of the labeled amount dissolves in 30 min (using the method according to the guideline) |
| Biowaiver | BCS Class 1 drug product (*T* and *R*) is rapidly dissolving, and *T* does not contain any excipients that would affect rate and extent of absorption of the drug. BCS Class 3: drug product (*T* and *R*) is very rapidly dissolving, and *T* is qualitatively very similar in composition to *R*. | BCS Class 1 drugs and either very rapid dissolution or similarly rapid. Excipients that might affect BA are qualitatively and quantitatively the same; BCS Class 3 drugs and very rapid dissolution and excipients that might affect BA are qualitatively and quantitatively the same; and other excipients are qualitatively the same and quantitatively very similar. | BCS Class 1: *T* and *R* are very rapidly dissolving or similarly rapidly dissolving drugs;<br>BCS Class 3: *T* and *R* are very rapidly dissolving. |
| Apparatus | USP Apparatus 1<br>USP Apparatus 2 | Basket<br>Paddle | Basket<br>Paddle |
| Dissolution media | 0.1 N hydrochloride or simulated gastric fluid (without enzymes)<br>Buffer pH 4.5<br>Buffer pH 6.8 or simulated intestinal fluid (without enzymes) | pH 1.2 (0.1 N hydrochloride or SGF)<br>Buffer pH 4.5<br>Buffer pH 6.8 or SIF | Buffer pH 1.2<br>Buffer pH 4.5 (acetate buffer)<br>Buffer pH 6.8 |
| | For capsules and tablets with gelatin coating, simulated gastric and intestinal fluids USP (with enzymes) can be used. | Absolutely no addition of surfactants. Use of enzymes is allowed for gelatin capsules. | International pharmacopoeia buffers are preferred |
| Volume | 500 mL or less | 900 mL or less | 900 mL or less |
| Temperature | 37 ± 0.5° | 37 ± 1° | 37° |
| Agitation | Apparatus 1: 100 rpm<br>Apparatus 2: 50 rpm (or 75 rpm when appropriately justified) | Basket: 100 rpm<br>Paddle: 50 rpm | Basket: 100 rpm<br>Paddle: 75 rpm |
| Sample number | 12 | 12 | 12 |
| Sampling time | Sufficient number of intervals, e.g., 10, 15, 20, 30 min | 10, 15, 20, 30, 45 min | 10, 15, 20, 30, 45, 60 min |

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**Table A-2. Comparison of FDA, EMA, and WHO Definitions**

| Term | FDA | EMA | WHO |
|---|---|---|---|
| Pharmaceutical equivalents | Drug products are considered pharmaceutical equivalents if they contain the same active ingredient(s), are of the same dosage form, have the same route of administration, and are identical in strength or concentration. Pharmaceutically equivalent drug products are formulated to contain the same amount of active ingredient in the same dosage form and to meet the same or compendial or other applicable standards (strength, quality, purity, and identity); but they may differ in characteristics such as shape, scoring configuration, release mechanisms, packaging, excipients, expiration time, and, within certain limits, labeling. | Medicinal products are pharmaceutically equivalent if they contain the same amount of the same active substance(s) in the same dosage forms that meet the same or comparable standards. Pharmaceutical equivalence does not necessarily imply bioequivalence as differences in the excipients and/or the manufacturing process can lead to faster or slower dissolution and/or absorption. | Products are pharmaceutical equivalents if they contain the same molar amount of the same API(s) in the same dosage form, if they meet comparable standards, and if they are intended to be administered by the same route. Pharmaceutical equivalence does not necessarily imply TE as differences in the API solid state properties, the excipients and/or the manufacturing process and other variables can lead to differences in product performance. |
| Pharmaceutical alternatives | Drug products are considered pharmaceutical alternatives if they contain the same therapeutic moiety but are different salts, esters, or complexes of that moiety or are different dosage forms or strengths. | Medicinal products that contain different salts, esters, ethers, isomers, mixtures of isomers, complexes or derivatives of the active moiety, or which differ in dosage form or strength | Products are pharmaceutical alternative(s) if they contain the same active pharmaceutical moiety or moieties but differ in dosage form (e.g., tablets vs. capsules), strength, and/or chemical form (e.g., different salts, different esters). Pharmaceutical alternatives deliver the same active moiety by the same route of administration but are otherwise not pharmaceutically equivalent. They may or may not be bioequivalent or TE to the comparator product. |
| Therapeutic equivalents | Drug products are considered to be therapeutic equivalents only if they are pharmaceutical equivalents and if they can be expected to have the same clinical effect and safety profile when administered to patients under the conditions specified in the labeling. | A medicinal product is TE to another product if it contains the same active substance or therapeutic moiety and, clinically, shows the same efficacy and safety as that product, whose efficacy and safety have been established. In practice, demonstration of BE is generally the most appropriate method of substantiating therapeutic equivalence between medicinal products, which are pharmaceutically equivalent or pharmaceutical alternatives, provided they contain excipients generally recognized as not having an influence on safety and efficacy and comply with labeling requirements with respect to excipients. However, in some cases where similar extent of absorption but different rates of absorption are observed, the products can still be judged TE if those differences are not of therapeutic relevance. A clinical study to prove that differences in absorption rate are not therapeutically relevant may be necessary. | Two pharmaceutical products are considered to be TE if they are pharmaceutically equivalent or pharmaceutical alternatives and after administration in the same molar dose, their effects, with respect to both efficacy and safety, are essentially the same when administered to patients by the same route under the conditions specified in the labeling. This can be demonstrated by appropriate equivalence studies, such as pharmacokinetic, pharmacodynamic, clinical, or in vitro studies. |
| BA (bioavailability) | This term means the rate and extent to which the active ingredient or active moiety is absorbed from a drug product and becomes available at the site of action. | The rate and extent to which a substance or its active moiety is delivered from a pharmaceutical form and becomes available in the general circulation (taking into consideration that the substance in the general circulation is in exchange with the substance at the site of action). | The rate and extent to which the active moiety is absorbed from a pharmaceutical dosage form and becomes available at the site(s) of action. Reliable measurements of active pharmaceutical ingredient concentrations at the site of action are not usually possible. The substance in systemic circulation, however, is considered to be in equilibrium with the substance at the site(s) of action. Based on pharmacokinetic and clinical considerations, it is generally accepted that in the same subject an essentially similar plasma concentration time-course will result in an essentially similar concentration time-course at the site(s) of action. |

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**Table A-2. Comparison of FDA, EMA, and WHO Definitions** *(continued)*

| Term | FDA | EMA | WHO |
|---|---|---|---|
| Bioequivalent drug products | This term describes pharmaceutical equivalent or pharmaceutical alternative products that display comparable BA when studied under similar experimental conditions. | Two medicinal products are bioequivalent if they are pharmaceutically equivalent or pharmaceutical alternatives and if their bioavailabilities after administration in the same molar dose are similar to such degree that their effects, with respect to both efficacy and safety, will be essentially the same. | Two pharmaceutical products are bioequivalent if they are pharmaceutically equivalent or pharmaceutical alternatives and their BA, in terms of rate $C_{max}$ and $t_{max}$ and the extent of absorption (area under the curve [AUC]) after administration of the same molar dose under the same conditions, are similar to such a degree that their effects can be expected to be essentially the same. |
| *R* drug product | An RLD [21 CFR 314.94(a)(3)] means the listed drug identified by FDA as the drug product upon which an applicant relies in seeking approval of its ANDA. | A reference medicinal product is a medicinal product authorized under Article 6, in accordance with the provisions of Article 8 (EC Directive 2001/83/EC) | The comparator pharmaceutical product (CPP) is a pharmaceutical product with which the multisource product is intended to be interchangeable in clinical practice. The comparator product normally will be the innovator product for which efficacy, safety, and quality have been established. The selection of the comparator product usually is made at the national level by the drug regulatory authority. |
| *T* drug product | A generic product is a product that is therapeutically equivalent to the RLD and is intended to be interchangeable with the innovator product. | A generic medicinal product which has the same qualitative and quantitative composition in active substances and the same pharmaceutical form as the reference medicinal product, and whose BE with the reference medicinal product has been demonstrated by appropriate BA studies. The different salts, esters, ethers, isomers, mixtures of isomers, complexes or derivatives of an active substance shall be considered to be the same active substance, unless they differ significantly in properties with regard to safety and/or efficacy. In such cases, additional information providing proof of the safety and/or efficacy of the various salts, esters, or derivatives of an authorized active substance must be supplied by the applicant. The various immediate-release oral pharmaceutical forms shall be considered to be one and the same pharmaceutical form. | See *Multisource pharmaceutical products*. |
| Multisource pharmaceutical products | | | Pharmaceutically equivalent or pharmaceutically alternative products that may or may not be therapeutically equivalent. Multisource pharmaceutical products that are therapeutically equivalent are interchangeable. |
| Interchangeable pharmaceutical product | | | An interchangeable pharmaceutical product is one which is therapeutically equivalent to a comparator product and can be interchanged with the comparator in clinical practice. |

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# ⟨1091⟩ LABELING OF INACTIVE INGREDIENTS

This informational chapter provides guidelines for labeling of inactive ingredients present in dosage forms.

Within the past few years a number of trade associations representing pharmaceutical manufacturers have adopted voluntary guidelines for the disclosure and labeling of inactive ingredients. This is helpful to individuals who are sensitive to particular substances and who wish to identify the presence or confirm the absence of such substances in drug products. Because of the actions of these associations, the labeling of therapeutically inactive ingredients currently is deemed to constitute good pharmaceutical practice.

Although the manufacturers represented by these associations produce most of the products sold in this country, not all manufacturers, repackagers, or labelers here or abroad are members of these associations. Further, there are some differences in association guidelines. The guidelines presented here are designed to help promote consistency in labeling.

In accordance with good pharmaceutical practice, all dosage forms [NOTE—for requirements on parenteral and topical preparations, see the *General Notices, 5.20.20 Added Substances (Excipients and Ingredients) in Official Products*] should be labeled to state the identity of all added substances (therapeutically inactive ingredients) present therein, including colors, except that flavors and fragrances may be listed by the general term "flavor" or "fragrance." Such listing should be in alphabetical order by name and be distinguished from the identification statement of the active ingredient(s).

The name of an inactive ingredient should be taken from the current edition of one of the following reference works (in the following order of precedence): (1) the *United States Pharmacopeia* or the *National Formulary;* (2) *USAN and the USP Dictionary of Drug Names;* (3) CTFA *Cosmetic Ingredient Dictionary;* (4) *Food Chemicals Codex*. An ingredient not listed in any of the aforementioned reference works should be identified by its common or usual name (the name generally recognized by consumers or health-care professionals) or, if no common or usual name is available, by its chemical or other technical name.

An ingredient that may be, but not always is, present in a product should be qualified by words such as "or" or "may also contain."

The name of an ingredient whose identity is a trade secret may be omitted from the list if the list states "and other ingredients." For the purposes of this guideline, an ingredient is considered to be a trade secret only if its presence confers a significant competitive advantage upon its manufacturer and if its identity cannot be ascertained by the use of modern analytical technology.

An incidental trace ingredient having no functional or technical effect on the product need not be listed unless it has been demonstrated to cause sensitivity reactions or allergic responses.

Inactive ingredients should be listed on the label of a container of a product intended for sale without prescription, except that in the case of a container that is too small, such information may be contained in other labeling on or within the package.

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# ⟨1092⟩ THE DISSOLUTION PROCEDURE: DEVELOPMENT AND VALIDATION

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## INTRODUCTION

### Purpose

This chapter provides a comprehensive approach covering items to consider for developing and validating dissolution procedures and the accompanying analytical procedures. It addresses the use of automation throughout the test and provides guidance and criteria for validation. It also addresses the treatment of the data generated and the interpretation of acceptance criteria for immediate- and modified-release solid oral dosage forms.

### Scope

This chapter addresses the development and validation of dissolution procedures, with a focus on solid oral dosage forms. Many of the concepts presented, however, may be applicable to other dosage forms and routes of administration. ▲For products containing more than a single active ingredient, develop and validate the method(s) for each active ingredient.▲ (USP 1-Dec-2020) General recommendations are given with the understanding that modifications of the apparatus and procedures as given in *USP* general chapters need to be justified.

The organization of this chapter follows the sequence of actions often performed in the development and validation of a dissolution test. The sections appear in the following sequence.

1. PRELIMINARY ASSESSMENT (FOR EARLY STAGES OF PRODUCT DEVELOPMENT/DISSOLUTION METHOD DEVELOPMENT)
    - 1.1 Performing Filter Compatibility
    - 1.2 Determining Solubility and Stability of Drug Substance in Various Media
        - ▲1.2.1 Solubility
        - 1.2.2 Stability▲ (USP 1-Dec-2020)
    - 1.3 Choosing a Medium and Volume
    - 1.4 Choosing an Apparatus
2. METHOD DEVELOPMENT
    - 2.1 Deaeration
    - 2.2 Sinkers
    - 2.3 Agitation
    - 2.4 Study Design
        - 2.4.1 Time Points
        - 2.4.2 Observations
        - 2.4.3 Sampling
        - 2.4.4 Cleaning
    - 2.5 Data Handling
    - 2.6 Dissolution Procedure Assessment
3. ANALYTICAL FINISH
    - 3.1 Sample Processing
    - 3.2 Filters
    - 3.3 Centrifugation
    - 3.4 Analytical Procedure
    - 3.5 Spectrophotometric Analysis
    - 3.6 ▲Chromatography▲ (USP 1-Dec-2020)
4. AUTOMATION
    - 4.1 Medium Preparation
    - 4.2 Sample Introduction and Timing
    - 4.3 Sampling and Filtration
    - 4.4 Cleaning
    - 4.5 Operating Software and Computation of Results
    - 4.6 Common Deviations from the Compendial Procedures that May Require Validation
5. VALIDATION
    - 5.1 Specificity/Placebo Interference
    - 5.2 Linearity and Range
    - 5.3 Accuracy/Recovery

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5.4 Precision
5.4.1 Repeatability of Analysis
5.4.2 Intermediate Precision/Ruggedness
5.4.3 Reproducibility
5.5 Robustness
5.6 Stability of Standard and Sample Solutions
5.7 Considerations for Automation
6. ACCEPTANCE CRITERIA
6.1 Immediate-Release Dosage Forms
6.2 Delayed-Release Dosage Forms
6.3 Extended-Release Dosage Forms
6.4 Multiple Dissolution Tests
6.5 Interpretation of Dissolution Results
6.5.1 Immediate-Release Dosage Forms
6.5.2 Delayed-Release Dosage Forms
6.5.3 Extended-Release Dosage Forms
REFERENCES

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# 1. PRELIMINARY ASSESSMENT (FOR EARLY STAGES OF PRODUCT DEVELOPMENT/ DISSOLUTION METHOD DEVELOPMENT)

Before method development can begin, it is important to characterize the ▲drug substance▲ (USP 1-Dec-2020) so that the filter, ▲dissolution▲ (USP 1-Dec-2020) medium, volume of medium, and apparatus can be chosen properly in order to evaluate the performance of the dosage form. ▲Throughout the chapter, it is assumed that the drug substance is the analyte, unless otherwise stated. The analyte is the compound whose concentration is being measured. In some cases, however, the analyte may be a degradation product or a derivatized product whose concentration is reflective of the dissolution rate of the drug substance.▲ (USP 1-Dec-2020)

## 1.1 Performing Filter Compatibility

Filtration is a key sample preparation step in achieving accurate test results. The purpose of filtration is to remove undissolved drug and excipients from the withdrawn solution. If not removed from the sample solution, particles of the drug ▲substance may▲ (USP 1-Dec-2020) continue to dissolve and can bias the results. Therefore, filtering the dissolution samples is usually necessary and should be done immediately if the filter is not positioned on the cannula.

Filtration also removes insoluble excipients that may otherwise interfere with the analytical finish. Selection of the proper filter material is important and should be accomplished, and experimentally justified, early in the development of the dissolution procedure. Important characteristics to consider when choosing a filter ▲▲ (USP 1-Dec-2020) are ▲material,▲ (USP 1-Dec-2020) type, filter size, and pore size. The filter that is selected based on evaluation during the early stages of dissolution procedure development may need to be reconsidered at a later time point. Requalification ▲may need▲ (USP 1-Dec-2020) to be considered after a change in composition of the drug product ▲or dissolution medium▲ (USP 1-Dec-2020) or ▲after▲ (USP 1-Dec-2020) changes in the quality of the ingredients ▲either of the drug product or the dissolution medium.▲ (USP 1-Dec-2020)

Examples of filters used in dissolution testing can be cannula filters, filter disks or frits, filter tips, or syringe filters. The filter material ▲should▲ (USP 1-Dec-2020) be compatible with the media and the drug ▲substance.▲ (USP 1-Dec-2020) Common pore sizes range from 0.20 to 70 µm, however, filters of other pore sizes ▲(such as 0.02 µm for nanomaterials)▲ (USP 1-Dec-2020) can be used as needed. ▲In addition to pore size, filter design may affect effective particle size exclusion.▲ (USP 1-Dec-2020) If the drug substance particle size is very small (e.g., micronized or nanoparticles), it can be challenging to find a filter pore size that excludes these small particles. ▲Consider ways to verify that drug particles have not passed through the filter.▲ (USP 1-Dec-2020)

Adsorption of the ▲drug substance(s)▲ (USP 1-Dec-2020) by the filter may occur and needs to be evaluated. Filter materials will interact with dissolution media to affect the recovery of the individual solutes and must be considered on a case-by-case basis. Different filter materials exhibit different drug-binding properties. Percentage of drug loss from the filtrate due to binding may be dependent on the drug concentration. Therefore the adsorptive interference should be evaluated on sample solutions at different concentrations bracketing the expected concentration range. Where the drug adsorption is saturable, discarding an initial volume of filtrate may allow the collection of a subsequent solution that approaches the original solution concentration. Alternative filter materials that minimize adsorptive interference can usually be found. Prewetting of the filter with the medium may be necessary. In addition, it is important that leachables from the filter do not interfere with the ▲analyte.▲ (USP 1-Dec-2020) This ▲condition▲ (USP 1-Dec-2020) can be evaluated by analyzing the filtered dissolution medium and comparing it with the unfiltered medium.

The filter size should be based on the volume to be withdrawn and the amount of particles to be separated. Use of the ▲appropriate▲ (USP 1-Dec-2020) filter dimensions will improve throughput and recovery, and also reduce clogging. Use of a large filter for small-volume filtration can lead to loss of sample through hold-up volume, whereas filtration through small filter sizes needs higher pressures and longer times, and the filters can clog quickly.

Filters used for USP Apparatus 4 need special attention because they are integrated in the flow-through process. Undissolved particles may deposit on the filters, creating resistance to the flow.

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In the case of automated systems, selection of the filter with regard to material and pore size can be done in a similar manner to manual filtration. Flow rate through the filter and clogging may be critical for filters used in automated systems. Experimental verification that a filter is appropriate may be accomplished by comparing the responses for filtered and unfiltered standard and sample solutions. ▲The acceptable recovery range for a filtered standard or sample solution when compared with the unfiltered solutions needs to be assessed.▲ (USP 1-Dec-2020) This is done by first preparing a suitable standard solution and a sample solution. For example, prepare a typical dissolution sample in a beaker and stir vigorously with a magnetic stirrer to dissolve the drug load completely. For standard solutions, compare the results for filtered solutions (after discarding the appropriate volume) to those for the unfiltered solutions. For sample solutions, compare the results for filtered solutions (after discarding the appropriate volume) to those for centrifuged, unfiltered solutions. ▲The qualified filter (types, pore size, filter size, etc.) should be recorded in detail as part of standard and sample preparation as the method is finalized.▲ (USP 1-Dec-2020)

## 1.2 Determining Solubility and Stability of Drug Substance in Various Media

### ▲1.2.1 SOLUBILITY▲ (USP 1-Dec-2020)

Physical and chemical characteristics of the drug substance need to be determined as part of the process of selecting the proper dissolution medium. When deciding the composition of the medium for dissolution testing, it is important to evaluate the influence of buffers, pH, and if needed, different surfactants on the solubility and stability of the drug substance. Solubility of the drug substance is usually evaluated by determining the saturation concentration of the drug in different media at 37° using the shake-flask solubility method (equilibrium solubility, ▲see *Solubility Measurements* ⟨1236⟩).▲ (USP 1-Dec-2020) To level out potential ion effects between the drug substance and the buffers used in the media, mixtures of hydrochloric acid and sodium hydroxide are used to perform solubility investigations; this is in addition to the typical buffer solutions. In certain cases, it may be necessary to evaluate the solubility of the drug substance at temperatures other than 37° (i.e., 25°). The pH of the clear supernatant should be checked to determine whether the pH changes during the solubility test. Alternative approaches for solubility determination may also be used ▲(such as dynamic solubility, potentiometric titration, or turbidity measurement methods).▲ (USP 1-Dec-2020)

Typical media for dissolution may include the following (not listed in order of preference): diluted hydrochloric acid, buffers (phosphate or acetate) in the pH range of 1.2–▲7.2,▲ (USP 1-Dec-2020) simulated gastric or intestinal fluid (with or without enzymes), and water. For some ▲drug substances,▲ (USP 1-Dec-2020) incompatibility of the drug ▲substance▲ (USP 1-Dec-2020) with certain buffers or salts may influence the choice of buffer. The ▲concentration▲ (USP 1-Dec-2020) of the buffers and acids used can influence the solubilizing effect, and this factor may be evaluated.

Aqueous solutions (acidic or buffer solutions) may contain a ▲▲ (USP 1-Dec-2020) surfactant ▲▲ (USP 1-Dec-2020) to enhance the solubility of the drug substance. The surfactants selected for the solubility investigations should cover all common surfactant types, i.e., anionic, nonionic, and cationic. When a suitable surfactant has been identified, different concentrations of that surfactant should be investigated to identify the lowest concentration needed to achieve sink conditions. Typically, the surfactant concentration is above its critical micellar concentration (CMC). *Table 1* shows a list of some of the surfactants used in dissolution media. Approximate CMC values are provided with references when available. ▲CMC values are dependent on medium and temperature.▲ (USP 1-Dec-2020) The list is not comprehensive and is not intended to exclude surfactants that are not listed. Other substances, such as hydroxypropyl β-cyclodextrin, have been used as dissolution media additives to enhance dissolution of ▲compounds with low solubility.▲ (USP 1-Dec-2020) The US FDA ▲and USP▲ (USP 1-Dec-2020) maintain databases of dissolution methods, including information on dissolution media that have been used (*1*▲*,2*). Ideally,▲ (USP 1-Dec-2020) the amount of surfactant added is sufficient to achieve sink conditions in the desired volume of dissolution medium ▲(see *1.3 Choosing a Medium and Volume* for discussion of sink conditions). With some formulations, the use of surfactant concentrations that produce less than sink conditions may be more suitable. Appropriate surfactant concentration is formulation dependent and should be verified by testing the formulation.▲ (USP 1-Dec-2020)

It is important to control the grade and purity of surfactants because use of different grades could affect the solubility of the drug. For example, sodium dodecyl sulfate (SDS) is available in both a technical grade and a high-purity grade. Obtaining polysorbate 80 from different sources can affect its suitability when performing HPLC analysis.

There may be effects of counter-ions or pH on the solubility or solution stability of the surfactant solutions. For example, a precipitate forms when the potassium salt of the phosphate buffer is used at a concentration of 0.5 M in combination with SDS. This can be avoided by using the sodium phosphate salt when preparing media with SDS.

**Table 1. Commonly Used Surfactants with Critical Micelle Concentrations**

| Surfactant | | CMC (% wt/volume) | Reference |
|---|---|---|---|
| Anionic | SDS; sodium lauryl sulfate (SLS) | 0.18–0.23 | (*3–5*) |
| | Taurocholic acid sodium salt | 0.2 | (*4*) |
| | Cholic acid sodium salt | 0.16 | (*4*) |
| | Desoxycholic acid sodium salt | 0.12 | (*4*) |
| Cationic | Cetyltrimethyl ammonium bromide (CTAB, Hexadecyltrimethylammonium bromide) | 0.033–0.036 (0.92–1.0 mM) | (*6.7*) |
| | Benzethonium chloride (Hyamine 1622) | 0.18 (4 mM) | (*3*) |

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**Table 1. Commonly Used Surfactants with Critical Micelle Concentrations** *(continued)*

| Surfactant | | CMC (% wt/volume) | Reference |
|---|---|---|---|
| | Polysorbate 20 (Polyoxyethylene (20) sorbitan monolaurate, Tween 20) | 0.07–0.09 | (*4,8*) |
| | Polysorbate 80 (Polyoxyethylene (20) sorbitan monooleate, Tween 80) | 0.02–0.08 | (*4,8*) |
| | Caprylocaproyl polyoxyl-8 glycerides (Labrasol) | 0.01 | (*5*) |
| | Polyoxyl 35 castor oil (Cremophor EL) | 0.02 | (*9*) |
| | Polyoxyethylene 23 lauryl ether (Brij 35) | 0.013 | (*10*) |
| Nonionic | Octoxinol (Triton X-100) | 0.01–0.03 | (*4,11*) |
| Zwitterion | Lauryldimethylamine *N*-oxide (LDAO) | 0.023 | (*12*) |

Routinely, the dissolution medium is buffered; however, the use of purified water as the dissolution medium is suitable for products with a dissolution behavior independent of the pH of the medium. There are several reasons why purified water may not be preferred. The water quality can vary depending on its source, and the pH of the water is not as strictly controlled as the pH of buffer solutions. Additionally, the pH can vary from day to day and can also change during the run, depending on the drug substance and excipients. Use of an aqueous–organic solvent mixture as a dissolution medium is discouraged; however, with proper justification this type of medium may be acceptable.

### ▲1.2.2 STABILITY

Investigations of the stability of the drug substance should be carried out in the selected dissolution medium alone and with excipients present, at 37°. This elevated temperature has the potential to increase degradation. Stability should allow for sufficient time to complete or repeat the analytical procedure. In some cases, antioxidants may be used in the dissolution medium to improve the chemical stability of the drug substance in the dissolution medium.

The solution containing the drug substance is stored under conditions that ensure stability. The stability of this solution is analyzed over a specified period of time (for at least the time of the entire dissolution procedure), using a freshly prepared solution at each time interval for comparison. The acceptable range for solution stability is influenced by the drug concentration and is typically between 98% and 102% of the expected final concentration.

The solution containing the drug substance in the presence of excipients is typically stored at room temperature. This solution is analyzed over a specified period of time, using the original solution response for comparison. The typical acceptable range for solution stability may be between 98% and 102%, compared with the initial analysis of the solutions. If the solution is not stable, aspects to consider include temperature (refrigeration may be needed), light protection, and container material (plastic or glass). If degradation still occurs, refer to *3.4 Analytical Procedure* for guidance on quantification of the drug substance and degradation products.

The procedure may state that the solutions need to be analyzed within a time period demonstrating acceptable solution stability.

Physical stability of the sample solution also may be of concern; any modifications should be justified. Precipitation may occur because of lower solubility at room temperature than at the temperature of the dissolution test.▲ (USP 1-Dec-2020)

## 1.3 Choosing a Medium and Volume

When developing a dissolution procedure, one goal is to have sink conditions, which are defined as having a volume of medium ▲($V$) at least three times the volume required to form a saturated solution ($V_{sat}$) of the drug substance; that is, $V/V_{sat} \geq 3$. On the other hand, if the $V/V_{sat}$ ratio is too large, the dissolution test may not detect critical changes in the formulation and manufacturing process. When the medium and volume chosen provide proper sink conditions, it is more likely that dissolution results will reflect the properties of the dosage form and will be able to discriminate between an acceptable and an unacceptable batch. A medium that fails to provide sink conditions ($V/V_{sat} < 3$) may be acceptable if it is appropriately justified (see *1.2.1 Solubility*). The composition and volume of dissolution medium are guided by the solubility investigations. For example, the choice and concentration of a surfactant needs to be justified from the solubility data and the dissolution profiles of the product candidates. Appropriate surfactant concentration in the dissolution medium is formulation dependent and should be verified by testing the formulation. When the solid form of the drug substance is modified, typically to enhance solubility (e.g., amorphous solid dispersion or modified crystal form), re-evaluate the solubility of the processed material in the proposed dissolution medium.▲ (USP 1-Dec-2020)

The use of enzymes in the dissolution medium is permitted, in accordance with *Dissolution* ⟨711⟩, when dissolution failures occur as a result of cross-linking with gelatin capsules or gelatin-coated products. A discussion of the phenomenon of cross-linking and method development using enzymes can be found in *Capsules—Dissolution Testing and Related Quality Attributes* ⟨1094⟩. Validation should be performed with the method using enzymes according to *5. Validation*.

Another option is to use media that follow more closely the composition of fluids in the stomach and intestinal tract. These media may contain physiological surface-active ingredients, such as taurocholates. The media also may contain emulsifiers

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(lecithin) and components such as saline solution that increase osmolality. Also, the ionic strength or molarity of the buffer solutions may be ▲adjusted.▲ (USP 1-Dec-2020) The media are designed to represent the fed and fasted state in the stomach and small intestine. These media may be very useful in modeling in vivo dissolution behavior of immediate-release (IR) dosage forms, in particular those containing lipophilic drug substances, and may help in understanding the dissolution kinetics of the product related to the physiological make-up of the digestive fluids. Results of successful modeling of dissolution kinetics have been published, mainly for IR products. In the case of extended-release dosage forms with reduced effect of the drug substance on dissolution behavior, the use of such media needs to be evaluated differently. In vitro performance testing does not necessarily require media modeling the fasted and postprandial states (*13,14*).

An acid stage is part of the testing of delayed-release products by *Dissolution* ⟨711⟩*, Procedure, Apparatus 1 and Apparatus 2, Delayed-Released Dosage Forms, Method A* or *Delayed-Released Dosage Forms, Method B*. For drug ▲substances▲ (USP 1-Dec-2020) with ▲solubility in acidic media▲ (USP 1-Dec-2020) less than 10% of the label claim or ▲those that degrade in acidic media,▲ (USP 1-Dec-2020) the usefulness of the acid stage in detecting a coating failure is compromised. This would be handled on a case-by-case basis. Possible resolutions include the addition of surfactant to the acid stage, or adjustment of the specifications ▲(see *6.5.2 Delayed-Release Dosage Forms*).▲ (USP 1-Dec-2020)

During selection of the dissolution medium, care should be taken to ensure that the drug substance is suitably stable throughout the analysis. ▲For compounds that rapidly degrade to form a stable degradation product, monitoring the degradation product alone or in combination with a drug substance may be more suitable than analyzing only the drug substance. Refer to *3.4 Analytical Procedure*.▲ (USP 1-Dec-2020)

For compendial Apparatus 1 (basket) and Apparatus 2 (paddle), the volume of the dissolution medium can vary from 500 to 1000 mL. Usually, the volume needed for the dissolution test can be determined in order to maintain sink conditions. In some cases, the volume can be increased to between 2 and 4 L, using larger vessels and depending on the concentration and sink conditions of the drug; justification for this approach is expected. In practice, the volume of the dissolution medium is usually maintained within the compendial range given above. Alternatively, it may be preferable to switch to other compendial apparatus, such as a reciprocating cylinder (Apparatus 3), reciprocating holder (Apparatus 7), or flow-through cell (Apparatus 4). Certain applications may require low volumes of dissolution media (e.g., 100–200 mL) when the use of a paddle or basket is preferred. In these cases, an alternative, noncompendial apparatus (e.g., small-volume apparatus) may be used.

## 1.4 Choosing an Apparatus

The choice of apparatus is based on knowledge of the formulation design and the practical aspects of dosage form performance in the in vitro test system. In general, a compendial apparatus should be selected.

For solid oral dosage forms, Apparatus 1 and Apparatus 2 are used most frequently. When Apparatus 1 or Apparatus 2 is not appropriate, another official apparatus may be used. Apparatus 3 (reciprocating cylinder) has been found especially useful for chewable tablets, soft gelatin capsules, delayed-release dosage forms, and nondisintegrating-type products, such as coated beads. Apparatus 4 (flow-through cell) may offer advantages for modified-release dosage forms and immediate-release dosage forms that contain active ingredients with limited solubility. In addition, Apparatus 4 may have utility for multiple dosage form types such as soft gelatin capsules, beaded products, suppositories, or depot dosage forms, as well as suspension-type extended-release dosage forms. Apparatus 5 (paddle over disk) and Apparatus 6 (rotating cylinder) are useful for evaluating and testing transdermal dosage forms. Apparatus 7 (reciprocating holder) has application to non-disintegrating, oral modified-release dosage forms, stents, and implants, as well as transdermal dosage forms. For semisolid dosage forms, the generally used apparatus include the vertical diffusion cell, immersion cell, and flow-through cell apparatus with the insert for topical dosage forms (see *Semisolid Drug Products—Performance Tests* ⟨1724⟩).

Some changes can be made to the compendial apparatus; for example, a basket mesh size other than the typical 40-mesh basket (e.g., 10-, 20-, or 80-mesh) may be used when the need is clearly documented by supporting data. Care must be taken that baskets are uniform and meet the dimensional requirements specified in ⟨711⟩.

A noncompendial apparatus may have some utility with proper justification, qualification, and documentation of superiority over the standard equipment. For example, a small-volume apparatus with mini paddles and baskets may be considered for low-dosage strength products. A rotating bottle or dialysis tubes may have utility for microspheres and implants, ▲apex▲ (ERR 1-Dec-2020) vessels ▲may be useful to eliminate coning (mounding of material at the bottom of the vessel),▲ (USP 1-Dec-2020) and modified flow-through cells ▲may be useful▲ (USP 1-Dec-2020) for special dosage forms including powders and stents.

***Change to read:***

## 2. METHOD DEVELOPMENT

A properly designed test should yield data that are not highly variable, and ▲ideally▲ (USP 1-Dec-2020) should be free of significant stability problems. High variability in the results can make it difficult to identify trends or effects of formulation changes. ▲The number of dosage units tested▲ (USP 1-Dec-2020) can affect the observed variability. One guidance defines dissolution results as highly variable if the relative standard deviation (RSD) is more than 20% at time points of 10 min or less and more than 10% at later time points for ▲12 dosage units tested (*15*).▲ (USP 1-Dec-2020) Most dissolution results, however, exhibit less variability. In the development of a dissolution procedure the source of the variability should be investigated, and attempts should be made to reduce variability whenever possible. The two most likely causes are the formulation itself (e.g., drug substance, excipients, or manufacturing process) or artifacts associated with the test procedure (e.g., coning, tablets sticking to the vessel wall or basket screen). Visual observations are often helpful for understanding the source of the variability and whether the dissolution test itself is contributing to the variability. Any time the dosage contents do not disperse freely throughout the vessel in a uniform fashion, aberrant results can occur. Depending on the problem, the usual remedies include changing any of the following factors: the apparatus type, speed of agitation, level of deaeration, sinker type, or composition of the medium.

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Many causes of variability can be found in the formulation and manufacturing process. For example, poor content uniformity, process inconsistencies, excipient interactions or interference, ▲▲ (USP 1-Dec-2020) coating, capsule shell aging, and hardening or softening of the dosage form ▲during storage▲ (USP 1-Dec-2020) may be sources of variability and interferences.

## 2.1 Deaeration

The significance of deaeration of the dissolution medium should be determined because air bubbles can act as a barrier to the dissolution process if present on the dosage unit or basket mesh and can adversely affect the reliability of the test results. Furthermore, bubbles can cause particles to cling to the apparatus and vessel walls. Bubbles on the dosage unit may increase buoyancy, leading to an increase in the dissolution rate, or may decrease the available surface area, leading to a decrease in the dissolution rate. ▲Low-solubility drug substances▲ (USP 1-Dec-2020) are most sensitive to interference from air bubbles; therefore, deaeration may be needed when testing these types of products. A deaeration method is described in ▲footnote 5▲ (USP 1-Dec-2020) in *Dissolution* ⟨711⟩*, Procedure*. Typical steps include heating the medium, filtering, and drawing a vacuum for a short period of time. Other methods of deaeration are available and are in routine use throughout the industry. Once a suitable deaeration process is identified, it should be documented as part of the dissolution procedure. The extent of deaeration can be evaluated by measuring the total dissolved gas pressure or by measuring the concentration of dissolved oxygen in water. For example, an oxygen concentration below 6 mg/L has been found effective as a marker for adequate deaeration of water for *Dissolution* ⟨711⟩*, Apparatus, Apparatus Suitability, Performance verification test* with USP Prednisone Tablets RS.

▲Typically,▲ (USP 1-Dec-2020) media containing surfactants usually are not deaerated because the process results in ▲foaming, and because▲ (USP 1-Dec-2020) the effect of dissolved air on the dissolution process is ▲usually▲ (USP 1-Dec-2020) mitigated by the reduced surface tension of the medium. Sometimes, deaerating the medium before adding surfactants can be effective.

To determine whether deaeration of the medium is necessary, compare results from dissolution samples run in non-deaerated medium and medium deaerated using a compendial technique, as described above ▲(see footnote 5 in ⟨711⟩).▲ (USP 1-Dec-2020) If no effect of deaeration is detected, this experiment could serve as justification that deaeration is not required in the future. If there is an effect, however, then it is necessary to carefully control this parameter, and ▲validate the deaeration process as described in footnote 5 in ⟨711⟩ and *5.5 Robustness*.▲ (USP 1-Dec-2020) The dissolved gas content of deaerated media under atmospheric pressure is unstable and will tend toward saturation. Manipulation of the deaerated medium such as stirring or pouring can increase the rate at which atmospheric gases are redissolved.

## 2.2 Sinkers

Sinkers are often used to adjust the buoyancy of dosage forms that would otherwise float during testing with Apparatus 2. When sinkers are used, a detailed description of the sinker must be provided in the written procedure. It may be useful to evaluate different sinker types, recognizing that sinkers can significantly influence the dissolution ▲behavior▲ (USP 1-Dec-2020) of a dosage unit. When transferring the procedure, the same sinkers should be used, or if a different design is used, it should be shown to produce equivalent results. There are several types of commercially available sinkers. A harmonized sinker is described in *Dissolution* ⟨711⟩, *Figure 2a*.

A standard sinker can be made by using the appropriate length of wire and coiling it around a cylinder. For materials, use 316 stainless steel wire, typically 0.032 inch/20 gauge, or other inert material, and wind the wire around cylinders of appropriate diameter (e.g., cork borers) for an appropriate number of turns to fit the ▲tablet or▲ (USP 1-Dec-2020) capsule shell type. Sizes are shown in *Table 2*. The ends of the coil can be curved to retain the ▲tablet or▲ (USP 1-Dec-2020) capsule within the sinker when they are immersed. Because the ends of the wire may be rough, they may need to be filed. If the sinker is handmade, the sinker material and construction procedure instructions should be documented (e.g., dimension, design, number of coils); if a commercial sinker is used, the vendor part number should be reported if available.

**Table 2. Wire Sinkers Used With Common Capsule Shell Sizes**

| Capsule Shell Size | Length of Wire (cm) | Diameter Size (cm) | Cork Bore Number |
|---|---|---|---|
| #0, elongated | 12 | 0.8 | 4 |
| #1 and #2 | 10 | 0.7 | 3 |
| #3 and #4 | 8 | 0.55 | 2 |

Although sinkers are typically used to keep the dosage form at the bottom of the vessel, they can also be used to keep dosage forms from sticking to the vessel (e.g., film-coated tablets). The sinker should be appropriate to the dosage form; therefore, the same sinker size may not be suitable for all dosage-form sizes. The sinker should not be too tight around the dosage form because this may restrict interaction with the medium. Conversely, if wrapped too loosely, the dosage form may escape soon after the test begins. The sinker should be small enough that the capsule does not change its orientation within the sinker. Care should be taken when testing capsules that have some cross-linking present, to keep the sticky shell from attaching to the vessel bottom. In this case, the harmonized sinker design provided in *Dissolution* ⟨711⟩, *Figure 2a* will be advantageous.

## 2.3 Agitation

For immediate-release capsule or tablet formulations, Apparatus 1 (baskets) at 50–100 rpm or Apparatus 2 (paddles) at 50 or 75 rpm are commonly used. Other agitation speeds are acceptable with appropriate justification. Rates outside 25–150 rpm for both the paddle and the basket are usually not appropriate because of mixing inconsistencies that can be generated by stirring too slow or too fast. Agitation rates between 25 and 50 rpm are generally acceptable for suspensions.

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For dosage forms that exhibit coning (mounding) under the paddle at 50 rpm, the coning can be reduced by increasing the paddle speed to 75 rpm, thus reducing the artifact and ▲reducing variability in▲ (USP 1-Dec-2020) the data. If justified, 100 rpm may be used with Apparatus 2, especially for extended-release products. Decreasing or increasing the apparatus rotation speed may be justified if to achieve an in vitro–in vivo correlation (IVIVC) the resulting profiles better reflect in vivo performance, or if the method results in better discrimination without adversely affecting method variability.

Apparatus 3 (reciprocating cylinder) can be used at dip rates ranging from 5 to 30 dips/min. The hydrodynamics are influenced by the cylinder's reciprocating motion and the resulting movement of the sample in the medium. The reciprocating motion of the cylinder and screen may cause foaming if the medium contains surfactants. Addition of an anti-foaming agent such as simethicone or *n*-octanol may be useful for avoiding foaming from surfactants.

Apparatus 4 (flow-through cell) is described in ⟨711⟩ with standard flow rates of 4, 8, and 16 mL/min. Other flow rates for Apparatus 4 can be used if justified and if within the capacity of the pump to conform with the requirements in ⟨711⟩. Agitation in Apparatus 4 is not only related to the pump speed but can also be affected by cell diameter. At a set flow rate, as measured by volume, the 12-mm cell will develop a greater linear fluid velocity than is achieved in the 22.6-mm cell. Apparatus 4 can be configured with the addition of glass beads in the entry cone of the flow-through cell (packed column) or without glass beads (open column).

The flow characteristics of the flow-through cell are discussed in the scientific literature (*16*). The placement of the sample in the flow-through cell will influence the flow patterns that occur and thus should be a consideration in the attempt to reduce variability of the results.

## 2.4 Study Design

Selection of the agitation rate and other study design elements for the dosage form, whether immediate release or modified release, should conform to the requirements and specifications (i.e., apparatus, procedures, and interpretation) given in ⟨711⟩.

### 2.4.1 TIME POINTS

For immediate-release dosage forms, the duration of the dissolution procedure is typically ▲15▲ (USP 1-Dec-2020)–60 min; in most cases, a single time point specification is adequate for pharmacopeial purposes. For method development, however, a sufficient number of time points should be selected to adequately characterize the ascending and plateau phases of the dissolution curve. Industrial and regulatory concepts of product comparability and performance may require additional time points, which may also be required for product registration or approval. ▲▲ (USP 1-Dec-2020) Very rapidly dissolving products need not be subjected to a profile comparison if they can be shown to release 85% or more of the drug substance within 15 min ▲(*15*).▲ (USP 1-Dec-2020) For these types of products, a one-point test ▲▲ (USP 1-Dec-2020) will suffice. However, most products do not fall into this category. Dissolution profiles of immediate-release products typically show a gradual increase reaching 85%–100% at about 30–45 min. Thus, ▲based on the dissolution profile,▲ (USP 1-Dec-2020) sufficient dissolution time points are chosen to characterize the performance for most immediate-release products ▲(see *6.1 Immediate-Release Dosage Forms*).▲ (USP 1-Dec-2020) For some products, including suspensions, useful information may be obtained from earlier points, e.g., 5–10 min. For slower-dissolving products, time points later than 60 min may be useful. Dissolution test times for compendial tests are usually established on the basis of an evaluation of the dissolution profile data.

The $f_2$ similarity factor ▲is not▲ (USP 1-Dec-2020) useful when more than 85% is dissolved at 15 min. If the $f_2$ similarity factor is to be used, ▲for the dissolution test for profile comparison, at least three▲ (USP 1-Dec-2020) time points ▲▲ (USP 1-Dec-2020) are required. ▲Specifically, the mean percent dissolved for 12 dosage units tested must be less than or equal to 85% dissolved for at least two time points and no more than▲ (USP 1-Dec-2020) one point above 85% for both products (*17*). Therefore, the addition of early time points may be useful. ▲The use of the $f_2$ similarity factor in the comparison of dissolution profiles is discussed in ▲*Assessment of Solid Oral Drug Product Performance and Interchangeability,▲* (ERR 1-Dec-2020) *Bioavailability, Bioequivalence, and Dissolution* ⟨1090⟩.▲ (USP 1-Dec-2020)

For testing an extended-release dosage form, at least three time points are chosen, to guard against dose dumping, to define the in vitro release profile, and to show that essentially complete release (>80%) of the drug is achieved. Additional sampling times may be useful. Certain IVIVC criteria, such as Level B correlation (according to *In Vitro and In Vivo Evaluation of Dosage Forms* ⟨1088⟩), require the experimental determination of the time to dissolve 100% of the label claim. Selection of the final time points is reflective of the drug release profile data that are generated during development. ▲▲ (USP 1-Dec-2020)

Delayed-release dosage forms usually require specifications for at least two time points; therefore, it is important during development to evaluate the entire dissolution profile. In the case of enteric-coated dosage forms, the functionality of the coating is usually proven by challenge in an acid medium, followed by a demonstration of dissolution in a higher-pH medium. Chapter ⟨711⟩ gives a standard buffer medium for that stage of testing but other media may be used if justified. The timing of the acid stage is typically 2 h, and release in the buffer is similar to the timing for immediate-release forms. For delayed-release dosage forms that are not enteric-coated, the setting of specifications is different. ▲▲ (USP 1-Dec-2020) The onset of release is not determined by the experimental design, which is the pH change; multivariate specifications, therefore, may be needed to define time ranges and corresponding percentage ranges.

▲Determining the amount of drug dissolved after vigorous agitation rate and/or long-time interval, also called▲ (USP 1-Dec-2020) infinity points, can be useful during development studies. To obtain ▲this measurement,▲ (USP 1-Dec-2020) the paddle or basket speed is increased at the end of the run (after the last time point) for a sustained period (typically, 15–60 min), after which time an additional sample is taken. ▲To verify that dissolution is complete, additional points may be needed.▲ (USP 1-Dec-2020) Although there is no requirement for 100% dissolution in the profile, the infinity point can be compared to content uniformity data and may provide useful information about formulation characteristics during initial development or about method bias.

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### 2.4.2 OBSERVATIONS

Visual observations and recordings of product dissolution and disintegration behavior are useful because dissolution and disintegration patterns can be indicative of variables in the formulation or manufacturing process. For visual observation, proper lighting (with appropriate consideration of photo-degradation) of the vessel contents and clear visibility ▲into the vessel▲ (USP 1-Dec-2020) are essential. Documenting observations by ▲taking photographs or videos and by drawing sketches▲ (USP 1-Dec-2020) can be instructive and helpful for those who are not able to observe the real-time dissolution test. Observations are especially useful during method development and formulation optimization. It is important to record observations of all ▲▲ (USP 1-Dec-2020) vessels to determine if the observation is seen in all ▲▲ (USP 1-Dec-2020) vessels, or just a few. ▲▲ (USP 1-Dec-2020) Provide any unique observations to the formulator. Examples of typical observations include, but are not limited to, the following:

1. Uneven distribution of particles throughout the vessel. This can occur when particles cling to the sides of the vessel, when there is coning or mounding directly under the apparatus (e.g., below the basket or paddle), when particles float at the surface of the medium, when film-coated tablets stick to the vessel, and/or when off-center mounds are formed.
2. Air bubbles on the inside of the vessel or on the apparatus or dosage unit. Sheen on the apparatus is also a sign of air bubbles. This observation would typically be made when assessing the need to deaerate the medium.
3. Dancing or spinning of the dosage unit, or the dosage unit being hit by the paddle.
4. Adhesion of particles to the paddle or the inside of the basket, which may be observed upon removal of the stirring device at the end of the run.
5. Pellicles or analogous formations, such as transparent sacs or rubbery, swollen masses surrounding the capsule contents.
6. Presence of large floating particles or chunks of the dosage unit, especially at the surface of the media.
7. Observation of the disintegration rate (e.g., percentage reduction in size of the dosage unit within a certain time frame).
8. Complex disintegration of the coating of modified or enteric-coated products [e.g., the partial opening and splitting apart (similar to a clamshell) or incomplete opening of the shell], accompanied by the release of air bubbles and excipients.
9. Whether the dosage form lands in the vessel center or off-center, and if off-center, whether it sticks there.
10. Time required for the complete dissolution of the capsule shell or for tablet disintegration.

Observations also help to document that the proper procedure has been followed, or more importantly, that a deviation has occurred. Examples include the confirmation that a dosage ▲unit▲ (USP 1-Dec-2020) is actually in the vessel during the test or that more than one dosage ▲unit▲ (USP 1-Dec-2020) are inadvertently in the same vessel, or that a filter from the autosampler has dropped into the vessel.

### 2.4.3 SAMPLING

**Manual:** For manual sampling, use chemically inert devices (e.g., polymeric or glass syringes, and polymeric or stainless steel cannula), a filter, and/or a filter holder. The sampling site must conform to specifications in ⟨711⟩. When the agitation conditions are very slow, e.g., a 50-rpm basket, care should be taken to sample consistently in the same location in the vessel because there may be a concentration gradient; avoid sampling very close to the shaft or vessel wall. During method development, a decision should be made regarding whether to replace the media after each time point. Replacement is not preferred because the dosage unit may be disturbed during delivery of the media. However, replacement may be necessary if maintaining sink conditions is a challenge. With replacement, the volume used in the calculations remains the same throughout the time points, but there is some drug substance withdrawn with each sample that will need to be accounted for in the calculations.

Metal surfaces may interact with the sample. For example, adsorption onto metal surfaces may occur, or the metal surfaces may release metal ions into aqueous media. The ions can then catalyze degradation reactions, leading to artifacts during the analytical procedures. The surfaces of stirring elements and metal locks of syringes may be sources of interference to accurate sampling.

**Autosampling:** Autosampling is discussed in *4. Automation*.

### 2.4.4 CLEANING

Importance is placed on evaluation of the cleaning process between tests. Changes of dissolution medium and/or product necessitate the need for cleaning. Residues on the vessels can affect the results (e.g., adsorbed residues may dissolve and alter subsequent media properties or interfere with the sample analysis), and effective cleaning will return them to a suitable state. Automated systems are discussed in *4.4 Cleaning*.

## 2.5 Data Handling

Dissolution rates are calculated from the change in drug concentration in the dissolution medium. For procedures in which the volume of medium is fixed, such as for Apparatus 1 and Apparatus 2 testing of immediate-release dosage forms with only one sampling time, the concentration of the sample is multiplied by the medium volume to arrive at the mass of drug dissolved usually expressed as percentage of label claim. When multiple time points are taken, the total amount of drug removed at earlier time points should be assessed and may be part of the calculation of the amount dissolved, if considered important. Similarly, if the medium volume is not fixed, for example, when the sample volume is not replaced in testing extended-release products, the change in medium volume must be part of the calculation for successive sampling points. Dissolution tests performed with Apparatus 4 in the closed-loop configuration with in situ detection provide a convenient control of the medium volume. For testing with Apparatus 4 in the open configuration, the test time and flow rate will determine the volume of medium used in the dissolution calculations.

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Dissolution results can be evaluated as either cumulative rates or fractional rates. Cumulative rates represent the sum of all drug dissolution that occurs during an interval (*Figure 1*). Fractional rates are assessed at a specific time point or during a portion of the total test time (*Figure 2*). Typically, the rate of release will be expressed as either mass or percentage of label claim per unit time. For most compendial dissolution testing, the dissolution rate is expressed as a percentage of the label claim dissolved at the indicated test time.

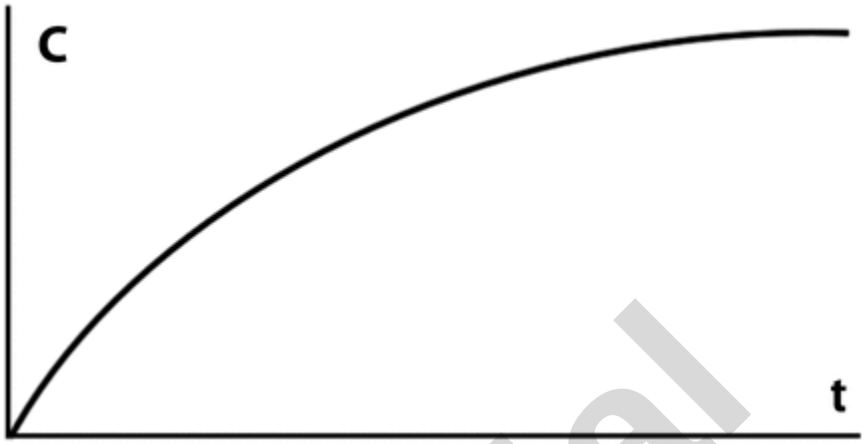


Figure 1. An example of a plot of dissolution as a cumulative process. Concentration, *C*, is the amount of drug released per volume of medium, and *t* represents time. This type of plot is readily observed in constant-volume dissolution systems, such as Apparatus 1 or Apparatus 2, or Apparatus 4 in closed-loop configuration.

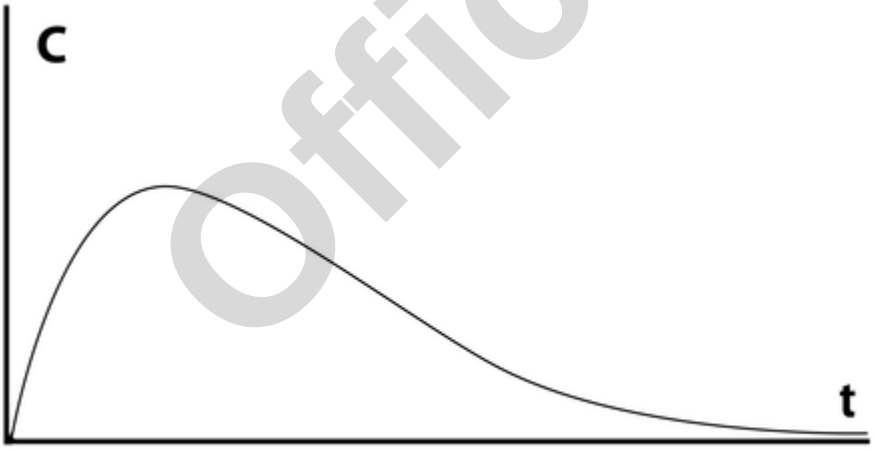


Figure 2. An example of a plot of the observed concentration of the sample taken for an interval that is negligibly small in relation to the time of the overall dissolution process. This concentration is ▲proportional▲ (USP 1-Dec-2020) to the instantaneous or fractional dissolution rate (*dc*/*dt*). This type of plot is readily observed in continuous-flow dissolution systems, such as Apparatus 4 in ▲open▲ (USP 1-Dec-2020) configuration.

Cumulative dissolution profiles represent the total amount of drug dissolved from the formulation over time. When cumulative dissolution is measured in a constant-volume system, no correction for the amount lost in sampling needs to be made. If sample is removed from the system, the amount consumed in analysis must be accounted for in the calculation. Recirculated sampling with Apparatus 1 or Apparatus 2, or with Apparatus 4 in the closed-loop configuration (*Figure 3*), are all examples of systems that will produce cumulative dissolution rates. With Apparatus 4 in the open configuration (*Figure 4*), cumulative rates accounting for the total amount of drug dissolved across the testing interval are obtained by collecting and analyzing the entire outflow from each individual flow-through cell. With Apparatus 3 (*Figure 5*), the medium in each tube is sampled at the end of the programmed interval, and the analyzed concentration represents the cumulative dissolution rate during that interval.

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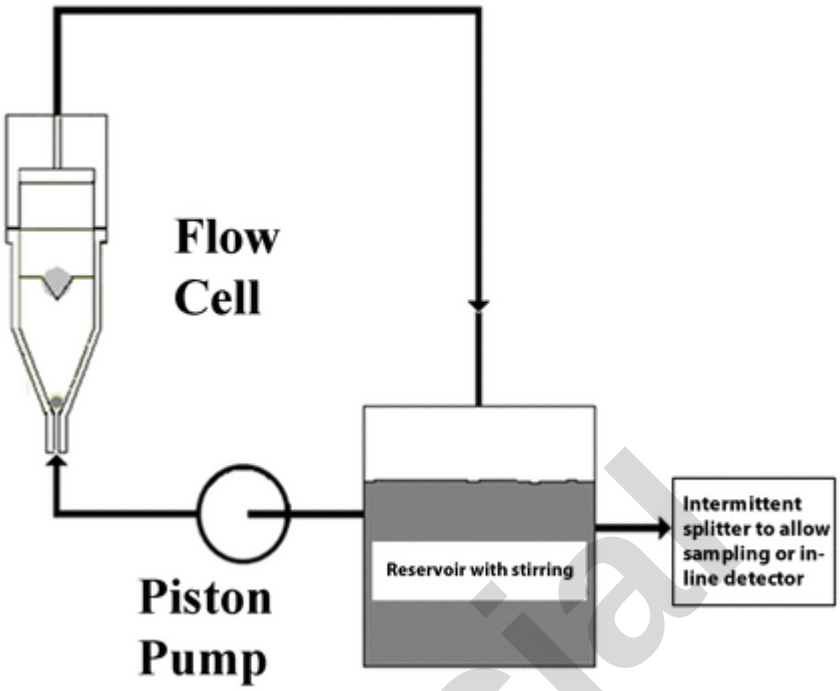


Figure 3. Apparatus 4 in the closed-loop configuration.

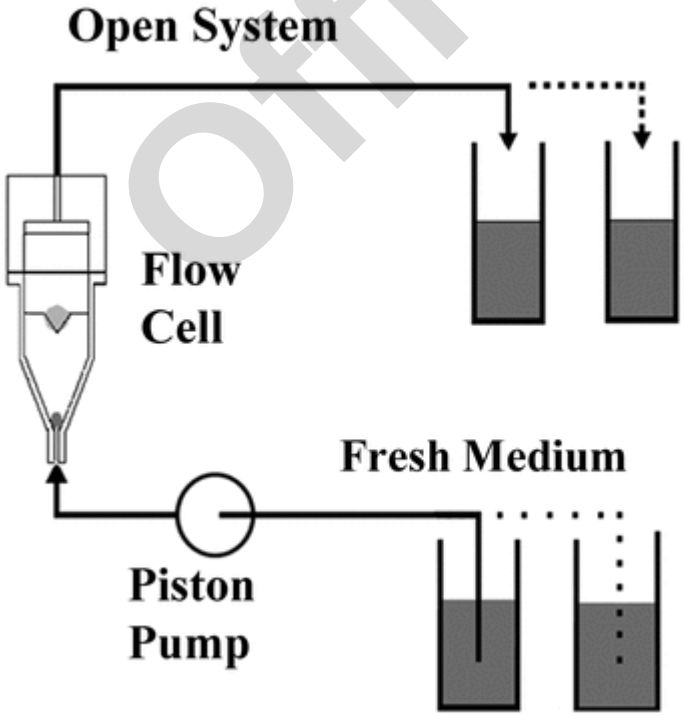


Figure 4. Apparatus 4 in the ▲open▲ (USP 1-Dec-2020) configuration. The sample can be collected in fractions, as shown at the top. The medium can be changed by using successive reservoirs.

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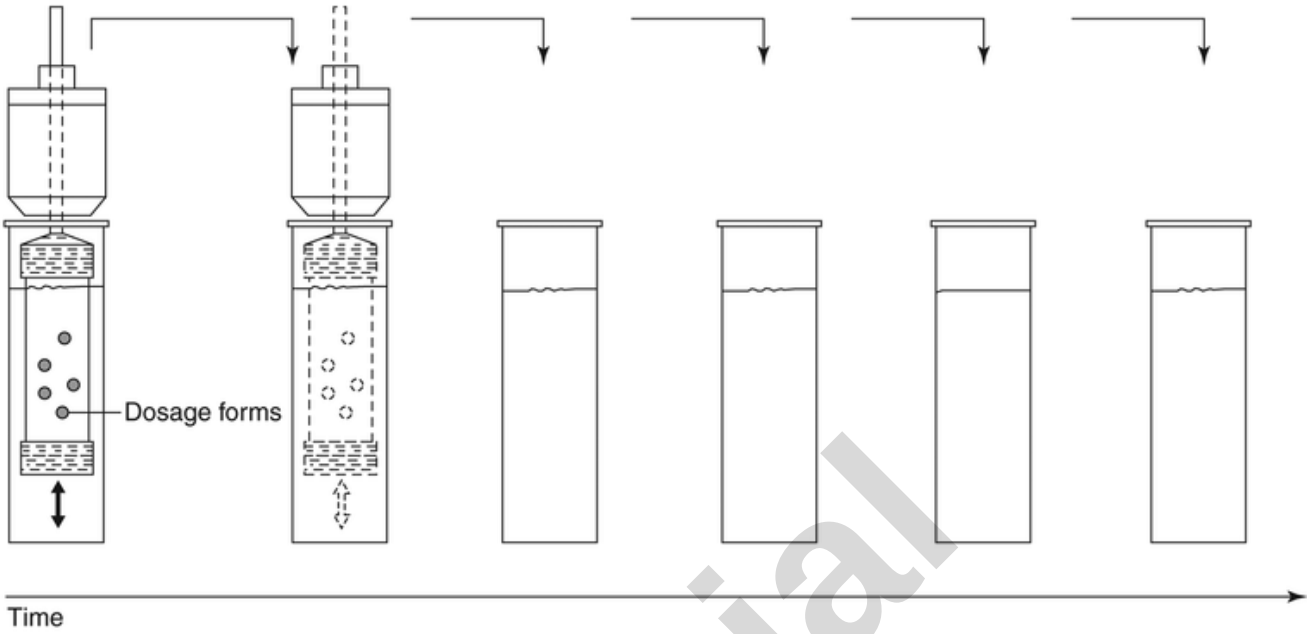


Figure 5. The progression that is possible for one reciprocating cylinder from Apparatus 3. The reciprocating cylinder can move from vessel to vessel. This feature facilitates changing the dissolution medium and testing for different intervals in successive tubes.

Fractional dissolution rates are typically measured for a discrete interval. A series of such rates will produce a step function as the dissolution profile. At any time, the cumulative dissolution rate from this type of profile is the sum of the preceding intervals. This type of profile is represented by Apparatus 3 using multiple tubes or Apparatus 4 in the ▲open▲ (USP 1-Dec-2020) configuration where the total outflow is collected and analyzed for successive intervals.

A number of algebraic and numerical methods exist for transforming cumulative and fractional dissolution results. The difference in amount released for successive time points can be calculated, and the average release rate is determined by the formula:

$$\text{Result} = (M_2 - M_1)/(t_2 - t_1)$$

Where:

$M$ = mass or percentage of label claim
$t$ = time

As the difference of $t_2$ from $t_1$ is reduced, the average rate can be considered to approach an instantaneous rate. Sampling considerations and physical constraints on measurement of the mass transfer at the medium interface of the dosage form make the measurement of true instantaneous dissolution impractical for routine determination in the laboratory. Fractional dissolution is measured for intervals where the difference between $t_2$ and $t_1$ is small, relative to the total test time. The design of Apparatus 4 in the open configuration permits a direct measurement of the fractional dissolution over small time intervals. For example, if a 4-mL fraction of outflow for Apparatus 4 running 16 mL/min is sampled, either by in situ detection or offline, the amount of drug detected represents the dissolution occurring in a 15-s interval.

Pooled dissolution has been used in a number of monographs. The pooled dissolution procedure produces an average release rate for the units tested by combining equal volumes from each vessel or cell and performing analysis of only the one resulting solution. Because this approach uses only the average release rate for comparison with the acceptance table, the pooled dissolution procedure has been viewed as reducing the amount of data available from the dissolution test and, thus, reducing its value. However, it should be noted that the pooling of equal sample volumes is equivalent, from a calculation standpoint, to determining the arithmetic mean of the individual sample results.

▲▲ (USP 1-Dec-2020)

## 2.6 Dissolution Procedure Assessment

The dissolution procedure requires an apparatus, a dissolution medium, and test conditions that together provide a method that is sensitive to changes in critical quality attributes ▲of a drug product, yet it is▲ (USP 1-Dec-2020) sufficiently rugged and reproducible for day-to-day operation. The method should be ▲transferrable▲ (USP 1-Dec-2020) between laboratories.

The ideal dissolution procedure will not contribute an unacceptable degree of variability and will provide a profile with adequate points below 85% dissolved. ▲▲ (USP 1-Dec-2020)

There are many ways to challenge the sensitivity of the method. One option is to compare dissolution profiles of formulations that are intentionally manufactured with meaningful variations for the most relevant critical manufacturing variables, for

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example, ±10%–20% change to the ranges of these variables. Similarly, samples that have been stressed may be used to demonstrate sensitivity to changes ▲during storage.▲ (USP 1-Dec-2020) This concept may be used to establish the factors that are most significant in their influence on the dissolution rate. These studies can focus on either the dissolution parameters (e.g., media concentration, agitation rate, and deaeration) or the product attributes (e.g., excipient ratios, particle size, compression). The ultimate goal is to understand the release mechanisms and determine whether the dissolution procedure ▲discriminates deviations in the process and formulation parameters▲ (USP 1-Dec-2020) of a drug product.

***Change to read:***

## 3. ANALYTICAL FINISH

The dissolution step has been described as an involved sample preparation. The sample handling and analytical procedure that are used to determine the amount of drug substance dissolved during the dissolution procedure are termed the "analytical finish". Although spectrophotometric determinations and HPLC are used most commonly and are discussed in this chapter, any suitable analytical technology may be used. Section *5. Validation* describes criteria for the methods.

### 3.1 Sample Processing

After the samples are withdrawn from the dissolution medium, they may require additional processing to make them suitable for the analytical methodology used to determine the amount released. For example, filtration ▲is often▲ (USP 1-Dec-2020) used to remove undissolved particulate matter, or samples may need to be protected from exposure to light or may need refrigerated storage. In addition, samples may have to be diluted to a level that is within the linear range of the method. With analysis by HPLC, dilution of the sample with mobile phase may be necessary to reduce the effect on the separation of injecting the dissolution medium. Other types of treatment may be necessary depending on the product formulation, such as the inactivation or elimination of interference caused by components of the formulation by the addition of appropriate reagents. However, separation may not be possible or needed in all cases. In some cases, in situ measurements obtained with methods such as fiber optics or electrochemical determination may be useful.

### 3.2 Filters

The topic of filtration is discussed in *1.1 Performing Filter Compatibility*.

### 3.3 Centrifugation

Centrifugation of samples is not preferred, for several reasons: dissolution can continue to occur until the solids are removed, a concentration gradient may form in the supernatant, and energy imparted may lead to increased dissolution of the drug substance particles. Possible exceptions, when centrifugation could be preferred, might include the use with compounds that adsorb onto all common filters, or situations when the potential filter leachables and extractables might interfere in the quantitative step of the dissolution test (e.g., when fluorescence procedures are used in quantitation). Centrifugation may prove useful during method development for evaluating the suitability of the filter material ▲and the filtration step.▲ (USP 1-Dec-2020)

### 3.4 Analytical Procedure

The usual assay for a dissolution sample employs either a spectrophotometric procedure or a liquid chromatographic procedure. Spectrophotometric determination may be direct or may provide the detection for HPLC. Spectrophotometric determination is used often because results can be obtained faster, the analysis is simpler, it is easier to automate, and fewer solvents are needed. The use of direct spectrophotometric determination typically requires confirmation of specificity. HPLC is preferred for a number of reasons such as providing a wide dynamic range that reduces the need to dilute some samples while also providing sensitivity in the analysis of dilute samples, and greater selectivity when excipients or multiple drugs in the formulation present a significant interference. Modern ▲chromatographic▲ (USP 1-Dec-2020) systems employ autosamplers that provide speed and simplicity advantages comparable to spectrophotometric analysis.

▲Ideally, the response of the drug substance is measured (see *Figure 6*, Case 1).

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| | CASE 1 (Typical) Stable Standard and Sample | CASE 2A Complete Degradation of Standard and Sample | CASE 2B Derivatized Standard and Sample | CASE 3A Stable Standard and Complete Degradation of Sample | CASE 3B Stable Standard and Partial Sample Degradation |
|---|---|---|---|---|---|
| Chromatogram of Standard Solution A | | | | | |
| Chromatogram of Sample | | | | | |
| Analytical Finish: Chromatography | Acceptable | Acceptable (no RRF correction is necessary) | Acceptable (no RRF correction is necessary) | Acceptable with RRF correction unless scientifically justified | Acceptable with RRF correction unless scientifically justified |
| Analytical Finish: Spectophotometry | Acceptable | Acceptable | Acceptable | Acceptable if wavelength of detection is at the isosbestic point | Acceptable if wavelength of detection is at the isosbestic point |
| | Degradation during dissolution procedure or during analytical finish $A \rightarrow Z$ | | Derivatization of standard and sample during analytical finish $A \rightarrow D$ | | |

Figure 6. Cases representing the analysis when degradation or derivatization occurs. RRF = relative response factor.

When degradation of the drug substance occurs, it is necessary to adjust the analysis in recognition of that fact. When degradation of the drug substance is quantitative in the standard and sample solutions (Case 2A) or when the drug substance is quantitatively derivatized in the standard and sample solutions (Case 2B), measurement of the degraded or derivatized material is acceptable, and no correction for relative response factor (RRF) is needed. In cases in which the drug substance is not stable in the sample solution and is stable in the standard solution (Cases 3A and 3B), chromatography is the preferred analytical finish and response factors of the degradation product and drug substance must be used in the calculations. If spectrophotometry is chosen as the analytical finish, the analytical wavelength must be at the isosbestic point of the drug substance and degradation product.▲ (USP 1-Dec-2020)

## 3.5 Spectrophotometric Analysis

Direct spectrophotometric analysis may be performed on samples that are manually introduced to the cuvette. Alternatively, samples may be automatically introduced into the spectrophotometer using autosippers and flow cells. Routine performance checks, cleaning, and maintenance, as described in the standard operating procedures (SOPs) or metrology documents, help to ensure reliable operation of these instruments. Cells with path lengths ranging from 0.02 to 1 cm are typically used, and longer path-length cuvettes can be used to increase the range for quantification of dilute samples. Cell alignment and air bubbles could be sources of error. The shorter path-length cells are used to avoid diluting the sample; in all cases, however, acceptable linearity and standard error need to be demonstrated. ▲Fiber optics, as a sampling and determinative method, with proper validation, is an option.▲ (USP 1-Dec-2020)

The choice of wavelength for the determination should be based on the spectrum of the drug in solution. In some cases, where the drug substance can degrade in the dissolution medium (e.g., dosage forms containing aspirin) ▲the measurements must be made▲ (USP 1-Dec-2020) at the isosbestic point. Excipients can also ▲interfere with the analytical finish,▲ (USP 1-Dec-2020) but performing analysis at multiple wavelengths can minimize their effects. The contribution of the absorbance from an excipient at the analytical wavelength can sometimes be determined by ratio from its absorbance at a wavelength where the absorbance of the drug substance is minimal.

Using a validated analytical finish, standard solutions are typically prepared in dissolution ▲medium▲ (USP 1-Dec-2020) and analyzed at just one concentration, either at 100% of the dosage strength or the selected *Q* value because linearity of the

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analytical finish has been established ▲over the analytical range.▲ (USP 1-Dec-2020) Prior to validation, dissolution profile analysis, or analysis of products of various strengths, requires using multiple standard solutions covering the expected range of concentration, ▲unless linearity has been established (linear across the required range, no significant intercept). In a typical sequence, the medium blank and standard bracket the sample solutions, especially at the beginning and end of the analysis. If the sample solution is analyzed in sequence with the blank and standard solutions, it may provide evidence of consistent analytical conditions or may signal changes over the time course of the analysis that could affect quantification.▲ (USP 1-Dec-2020) The standard and sample solutions should both be prepared in the dissolution medium in the linear concentration range and measured at the same wavelength. However, small amounts of an organic solvent may be used in the preparation of the standard, provided that the accuracy criteria can be met during validation.

The absorptivity is calculated by dividing the mean standard absorbance by the concentration, in mg/mL, ▲and▲ (USP 1-Dec-2020) by the cell path length in cm. A rearrangement of the Beer–Lambert expression gives the absorptivity, *a*, as:

$$a = A/bc$$

*A* = absorbance
*b* = path length (cm)
*c* = concentration (mg/mL)

Typical units for absorptivity that are used for dissolution testing are in terms of AU ▲(cm × mg/mL)▲ (USP 1-Dec-2020) where AU is absorbance unit. Historical data may be used to provide an acceptable absorptivity range for the analyte (using the appropriate ▲cell path length).▲ (USP 1-Dec-2020) This value may be useful in troubleshooting aberrant data.

▲▲ (USP 1-Dec-2020)

### 3.6 ▲Chromatography▲ (USP 1-Dec-2020)

For ▲chromatographic▲ (USP 1-Dec-2020) analysis, the effect on the chromatogram of peaks resulting from ▲▲ (USP 1-Dec-2020) dissolution ▲medium and excipients▲ (USP 1-Dec-2020) requires enumeration. A large solvent disturbance may affect accuracy and precision of response if it is poorly resolved from the peak of interest. This is even more important if large injection volumes (>100 µL) are needed. System suitability tests may evaluate peak shape; separation of the main peak from solvent disturbance and from closely eluting peaks; and injection precision. At a minimum, the precision is critical.

Ideally, the standard solutions should be diluted with the dissolution ▲medium▲ (USP 1-Dec-2020) at a concentration within the linear range of the method, e.g., 100%, or the selected *Q* value of the dosage strength. However, organic solvent may be used in the preparation of the standard, provided that the accuracy criteria can be met during validation. In some cases, the sample may be diluted with mobile phase to improve the peak shape. The standard and sample solutions should both be prepared in the linear concentration range and measured at the same wavelength. ▲A similar sequencing of standard, sample, and blank solutions described for spectrophotometric analysis in *3.5 Spectrophotometric Analysis* can be used.▲ (USP 1-Dec-2020)

***Change to read:***

## 4. AUTOMATION

▲Depending on the design of the instrument, different parts of the dissolution process may be automated, e.g., the▲ (USP 1-Dec-2020) elements of test preparation, initiation, sampling and timing, ▲media preparation,▲ (USP 1-Dec-2020) and cleaning. ▲▲ (USP 1-Dec-2020) Fully automated systems are available.▲▲ (USP 1-Dec-2020) This section will discuss operational steps that can be automated. The level of complexity for automation depends on whether the instrument configuration is open or closed loop and also whether the analytical device is coupled online or offline. Online analysis returns the sample aliquot to the test system, as in the case of spectrophotometry with flow-through cuvettes. Offline analysis removes the sample aliquot from the dissolution medium for subsequent analysis, typically by HPLC, where the analysis consumes the sample. The decision on the configuration usually depends on the number of samples to be processed and the time required for their analysis.

Automation may require deviations from the pharmacopeial specifications of the instruments, such as incorporation of an integrated outlet on the bottom of the vessel for cleaning and replacement of medium.

Operational steps that are not part of the compendial procedure should be validated. Deviations from the standard procedure described in ⟨711⟩, such as use of sampling probes or fiber-optic probes, should be validated against the standard procedure.

### 4.1 Medium Preparation

Automated media preparation generally is accomplished by diluting concentrates. Automated media preparation systems typically dispense the volume of medium into the vessel by monitoring either the weight or volume. Chemical and physical stability of the concentrates as well as homogeneity of the dilutions over the intended period of use are important issues and should be understood. Concentrates of buffer solutions and surfactants may have stability issues, such as chemical degradation and pH change. Physical instability may manifest as precipitation, re-crystallization, or phase separation and should be prevented.

If deaeration of the medium is required, the level of deaeration should be specified.

The concentration of the dissolved oxygen can be used to evaluate the efficiency of deaeration procedures discussed in *2.1 Deaeration*.

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## 4.2 Sample Introduction and Timing

Samples should be inserted in the vessel in a reproducible way. Automated sample introduction and aliquot withdrawal provide an advantage over manual sampling because the automated techniques can reduce the variability in the vessel-to-vessel timing of the test intervals. However, ▲in some automated systems,▲ (USP 1-Dec-2020) sample handling may impose timing limitations that need to be considered. The pharmacopeial tolerance of ±2% of the specified dissolution test time may be difficult to meet for early time points.

## 4.3 Sampling and Filtration

Autosampling is a useful alternative to manual sampling, especially if the test includes several time points. The transfer and filtration of sample solutions from the dissolution instrument to the analytical unit may be undertaken via tube connections or via robotic devices operated in a stepwise procedure. Sample volumes may be removed from the dissolution medium and not returned (consumptive sampling), or the sample volume may be returned to the dissolution medium (recirculated sampling).

There are many brands of autosamplers, including semi-automated and fully automated systems. Routine performance checks, cleaning, and maintenance, as described in the pertinent SOPs or metrology documents, help to ensure reliable operation of these devices.

Sampling probes may or may not remain in the vessel throughout the entire run. Sampling probes or fiber-optic probes can disturb the hydrodynamics of the vessel; therefore, adequate validation should be performed to ensure that the probes are not causing a significant change in the dissolution rate. If filters are used that are different from those used for manual sampling, then these different filters should also be evaluated separately. The position of the pharmacopeial sampling zone for Apparatus 1 and Apparatus 2 is midway from the top of the stirring element to the medium surface and depends on the medium volume. Sampling probes should pull the sample from the sampling zone. Instruments for which the sampling occurs through the hollow shaft should be designed with a means to adjust the depth of the inlet aperture to allow conformance with this requirement. The programmed sampling volume depends on the dead volume of the tubing, cuvettes, and other devices and has to be adjusted accordingly.

A recirculated sampling alignment can be operated either by discharging the tubing contents into the vessel after each sampling or by allowing the tubing to remain filled with solution in the intervals between sampling points. In the latter case, the dead volume and carryover effects are important considerations.

The need for sample volume replacement should be considered. In consumptive sampling with multiple sampling time points, the withdrawn volume may be replaced with an equal volume of fresh medium. The sampling volume may be critical if, in total, it exceeds 1% of the stated volume of dissolution medium required by the procedure. If it can be shown that replacement of the medium is not necessary, the volume change must be part of the calculation of results. See *2.5 Data Handling*.

Carryover may occur when subsequent samples are affected by residues or conditions of previous samples; the effect of the first sample or condition “carries over” to the second. In liquid handling, residues of liquids previously in the sample solution may contaminate subsequent sample solutions. Dissolution media containing surfactants or lipids may present problems. Carryover may occur for successive samples taken over a multiple time-point test, as well as at the beginning of a new test due to the cleaning solution. This topic is discussed in *4.4 Cleaning*.

Interaction of dissolved drug substance with the sampling and transfer devices is an important consideration. When adsorption of the dissolved drug substance occurs, it most often involves surfaces of the dissolution apparatus or sampling filters and tubing. Adsorption may be pH dependent in the case of charged, dissolved drug substance. Adsorption of the dissolved drug to the parts of the sampling device should be assessed using a typical sample solution (dissolution sample from the product or drug substance with formulation matrix) with known concentration. The typical design is a cross-validation with aliquots of the same sample solution passing and bypassing the sampling device (including the sampling probe, filter, tubing, valves, and pump). There is no general recommendation that may give preference to any kind of material or equipment construction (e.g., glass or specific polymers). See *5.7 Considerations for Automation* for more information.

In addition to the information in *2.4.3 Sampling*, connections of pumps and tubing may be sources of contamination in automated systems. Interferences with the spectroscopic analytical procedures, which are commonly used for dissolution testing, are less of a concern. However, interferences must be evaluated if the product under investigation contains low-dose metal salts, as do some dietary supplements.

Liquid transfer usually is undertaken via polymeric tubing. Inert materials such as polytetrafluoroethylene (PTFE) sometimes cannot be used because of their mechanical properties. Where flexible tubes are required, for example, in peristaltic pumps or for coiling in a small radius, polypropylene (PP) or high-density polyethylene (HDPE) may be the preferred materials. Depending on the type of polymer and its crystallinity and density, leaching of constituents, mainly plasticizers, may occur. Leachables can interfere with the analytical procedure. The concentration leached to the sample solution usually depends on the surface, the temperature, the exposure time, the hydrodynamic conditions, and the composition of the media.

## 4.4 Cleaning

In addition to the information in *2.4.4 Cleaning*, automated systems have specific cleaning issues. For example, evaluation of the effectiveness of purging and rinsing between sampling times and within-run condition of the tubing is recommended. Also it is important to evaluate the cleaning process between tests.

## 4.5 Operating Software and Computation of Results

The software systems for data evaluation and instrument operation must be validated as per 21 Code of Federal Regulations (CFR) 11 (*18*).

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## 4.6 Common Deviations from the Compendial Procedures that May Require Validation

Some common areas of deviation from compendial procedures include the following:

- Sample introduction relative to start of spindle rotation
- Residence time and positioning of sampling probes
- Recirculated versus consumptive sampling
- Sample volume replacement in consumptive sampling

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# 5. VALIDATION

The validation topics described in this section are typical but not all-inclusive and can be viewed in the context of *Validation of Compendial Procedures* ⟨1225⟩ as well as the International Council for Harmonisation (ICH) document, *Validation of Analytical Procedures: Text and Methodology* (*19*). Validation for both parts of the dissolution procedure, the analytical finish and the dissolution step, will be discussed in this section. The dissolution step is the release of the drug ▲substance▲ (USP 1-Dec-2020) in the dissolution medium and sampling. The analytical finish ▲is the quantification of the analyte and▲ (USP 1-Dec-2020) is defined in *3. Analytical Finish*. Validation of the ▲suitability of the▲ (USP 1-Dec-2020) analytical finish will evaluate the attributes, linearity and range, precision, specificity, accuracy/recovery, robustness, and stability of the sample and standard solutions. Validation of the dissolution step will include evaluation of precision and robustness of the dissolution sample preparation. Validation of the analytical finish is performed either using a standard solution or spiked placebo or by the method of standard addition (▲▲ (USP 1-Dec-2020) as described in *Validation of Compendial Procedures* ⟨1225⟩*, Validation, Analytical Performance Characteristics, Accuracy*), as specified in the sections below. Validation of the dissolution step requires the use of a well-characterized dosage form (e.g., having tight content uniformity and uniform performance). ▲See *Table 3* for an overview guide.▲ (USP 1-Dec-2020) Depending on the parameter of interest, validation of the sample handling and analytical procedure can be performed in situ, e.g., within the dissolution vessel. The validation parameters addressed and the extent of the validation may vary, depending on the phase of development or the intended use for the data.

The acceptance criteria are presented as guidelines only, and may differ for some products. Manufacturers should document the appropriate acceptance criteria for their products in pertinent SOPs or in validation protocols. Other considerations may be important for special dosage forms. Validation studies should be performed across the range of profile time points. For products containing more than a single active ingredient, the dissolution procedure needs to be validated for each active ingredient. It is expected that investigations into filter suitability and the potential for glass adsorption will have been undertaken already (see *1.1 Performing Filter Compatibility*). Validation of these assessments may occur during spiked recovery experiments.

**▲Table 3. Samples Used in the Assessment of Validation Attributes**

| Attribute | Comment | Analytical Finish | Dissolution Procedure |
|---|---|---|---|
| Linearity | — | **STD**; [a] SP*[b] | — |
| Range (linear, accuracy, precision) | — | **STD**; SP* | — |
| Accuracy/recovery | — | SP* | — |
| Repeatability | Individual preparations | **STD**; SP* | WCDF[c] |
| Intermediate precision | At least, two analysts/one laboratory | SP* | WCDF |
| Reproducibility | At least, two analysts/two laboratories | SP* | WCDF |
| Robustness | Deliberate changes to parameters | SP* | WCDF |

[a] **STD**: Standard solution (preferred material).
[b] SP*: Spiked placebo (standard addition method is acceptable).
[c] WCDF: Well-characterized dosage form.
▲ (USP 1-Dec-2020)

## 5.1 Specificity/Placebo Interference

It is necessary to demonstrate that the results are not unduly affected by ▲dissolution medium blank,▲ (USP 1-Dec-2020) placebo constituents, other active ▲drug substances,▲ (USP 1-Dec-2020) or ▲potential degradation products from the dissolved drug substance in the dissolution medium.▲ (USP 1-Dec-2020) The placebo consists of all the excipients and coatings, with inks and capsule shells included if appropriate, without the ▲drug substance.▲ (USP 1-Dec-2020) Placebo interference can be evaluated by using a spiked placebo that is prepared by weighing samples of the placebo blend, dissolving or dispersing them in dissolution medium at concentrations that would be encountered during testing, and adding a known amount of the drug in solution. It may be preferable to perform this experiment at 37°, comparing the solution to a standard solution at the concentration expected to be encountered during testing, by using the formula:

$$\text{Result} = (A_P/A_S) \times C_S \times (V/L) \times 100$$

Where:

$A_P$ = absorbance ▲or response▲ (USP 1-Dec-2020) of the placebo

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$A_S$ = absorbance ▲or response▲ (USP 1-Dec-2020) of the standard
$C_S$ = concentration of the standard (mg/mL)
$V$ = volume of the medium (mL)
$L$ = label claim (mg)

▲The blank is the dissolution medium without dissolved sample, and it is treated in the same manner as the sample. The effect of the absorbance of the blank at the analytical wavelength should be evaluated. In most cases, the absorbance of the dissolution medium blank may not exceed 1% of the standard solution at the concentration used for analysis. Values >1% should be evaluated on a case-by-case basis.▲ (USP 1-Dec-2020)

The interference should not exceed 2%. Note that for extended-release products, a placebo version of the finished dosage form may be more appropriate than blends because this placebo formulation will release the various excipients in a manner more nearly reflecting the product than will a simple blend of the excipients. In this case, it may be appropriate to evaluate potential interference at multiple sampling points in the release profile, with worst-case interference expected at the later sampling points.

▲▲ (USP 1-Dec-2020)

If the placebo interference exceeds 2%, modification of the method may be necessary. Possible modifications include choosing another wavelength, subtracting baseline using a longer wavelength, transforming absorbance values (e.g., first derivative), and using an alternative analytical technique such as HPLC. Other means for minimizing the placebo interference would be acceptable with appropriate justification. When other ▲▲ (USP 1-Dec-2020) drug substances or ▲degradation products▲ (USP 1-Dec-2020) are present, it is necessary to show that these do not ▲interfere with the measurement.▲ (USP 1-Dec-2020) One procedure for doing this is to measure the matrix in the presence and absence of the other active drug substance or ▲degradation product:▲ (USP 1-Dec-2020) any interference should not exceed 2%. Similar approaches may be used if other techniques are used for the analytical finish.

## 5.2 Linearity and Range

Linearity is typically established by preparing solutions of the drug substance, ranging in concentration from less than the lowest expected concentration to more than the highest concentration during release. The solutions may be prepared either using 1) a standard solution or spiked solution or 2) by the method of standard addition. A minimum of five concentrations is normally used (see ⟨1225⟩). Typically, solutions are made from a common stock if possible. The concentration range may not exceed the linearity limits of the method, including the instrumentation. Organic solvents may be used to enhance drug solubility for the preparation of the linearity standard solutions. However, NMT 5% (v/v) of organic solvent should be present in the final solution unless validated. Linearity is typically calculated by using an appropriate least-squares regression program. Typically, a square of the correlation coefficient ($r^2 \geq 0.98$) demonstrates linearity. In addition, the *y*-intercept must not be importantly different from zero.

The range of the procedure is the interval between the upper and lower concentrations of the drug substance (including these levels) that has been demonstrated to have a suitable level of precision, accuracy, and linearity using the procedure as written.

## 5.3 Accuracy/Recovery

Accuracy/recovery is typically established by preparing multiple ▲sample solutions▲ (USP 1-Dec-2020) containing the drug substance and any other constituents present in the dosage form (e.g., excipients, coating materials, capsule shell) ranging in concentration from less than the lowest expected concentration to more than the highest concentration during release. Accuracy/recovery may be done in conjunction with linearity determination. The method of standard addition can also be used. Before this activity, it is expected that filter assessment will already have been performed, and adsorption of drug onto the glass has also been investigated and ruled out.

Individual solutions may be directly prepared in the dissolution medium. Alternatively, to enhance drug solubility it may be appropriate to prepare a stock solution by dissolving the drug substance ▲typically▲ (USP 1-Dec-2020) in a small amount of organic solvent ▲▲ (USP 1-Dec-2020) and diluting to the final concentration with dissolution medium. ▲The amount of organic solvent should not exceed 5% in the sample solution and should not interfere with the analysis.▲ (USP 1-Dec-2020) An amount of stock solution equivalent to the targeted label claim may be used instead of the drug substance powder. Similarly, for very low strengths, it may be more appropriate to prepare a stock solution than to attempt to weigh very small amounts. The measured recovery is typically 95%–105% of the amount added. Bracketing or matrixing of multiple strengths may be useful.

A special case for validation ▲are the *Acid stage* criteria described in *Dissolution* ⟨711⟩, *Acceptance Table 3*.▲ (USP 1-Dec-2020)The limits of NMT 10% ▲and of NMT 25%▲ (USP 1-Dec-2020) need to be validated. Recovery experiments for drug ▲substances▲ (USP 1-Dec-2020) that have low solubility in acidic media may be challenging or impossible to perform and may need to be addressed on a case-by-case basis. ▲If the drug substance degrades in acidic media, the degradation product(s) should be used to quantify the dissolved drug and the validation experiment must address this fact. Two examples of dissolution of a delayed-release dosage form are provided to illustrate this case. In these examples, the *Q* value for the buffer phase is 80% at 15 min; the $B_1$ criterion is 85% label claim (LC). Please refer to *6.5.2 Delayed-Release Dosage Forms* for further information on acceptance criteria.

Example 1: The analytical results from the acid stage of dissolution experiment of an individual unit indicated that 1% LC of drug substance ($DS_{acid}$) and 5% LC of a degradant ($DEG_{acid}$) were present after 2 h. The total amount of drug substance dissolved in the acid stage is calculated:

$$DS_{acid} + DEG_{acid} = 6\%\ \text{LC}$$

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which meets the $A_1$ criterion of ≤10% LC that is specified in ⟨711⟩. This calculation is repeated for each of the remaining 5 individual units.

The unit was transferred to the buffer stage and the measured amount of drug substance ($DS_{buffer}$) is 82% LC after 15 min. The total amount of drug substance dissolved in both stages is calculated:

$$DS_{acid} + DEG_{acid} + DS_{buffer} = 88\%\ LC$$

which meets the $B_1$ criterion of 85% LC. This calculation is repeated for each of the remaining 5 individual units.

Example 2: The analytical results from the acid stage of the dissolution experiment indicated that $DS_{acid}$ and $DEG_{acid}$ are 8% and 20% LC, respectively, for an individual unit. The total amount of drug substance dissolved in the acid stage is:

$$DS_{acid} + DEG_{acid} = 28\%\ LC,$$

This result does not meet the $A_1$ criterion of NMT 10% LC. Furthermore, neither the $A_2$ nor the $A_3$ criterion is met because the calculated value exceeds the NMT 25% LC limit.▲ (USP 1-Dec-2020)

## 5.4 Precision

### 5.4.1 REPEATABILITY OF ANALYSIS

For the analytical finish, repeatability is evaluated by obtaining replicate measurements of standard and/or spiked placebo/standard addition solutions. It can be determined by calculating the RSD of the multiple injections or spectrophotometric readings for each standard solution, or by using the accuracy or linearity data. The ICH guidance, ▲Q2(R1) *Validation of Analytical Procedures: Text and Methodology*,▲ (USP 1-Dec-2020)recommends that repeatability should be assessed using a minimum of nine determinations covering the specified range for the procedure (i.e., 3 concentrations and 3 replicates of each concentration) or using a minimum of 6 determinations at 100% of the test concentration ▲(*19*).▲ (USP 1-Dec-2020) A typical acceptance criterion is an RSD of <2%. The demonstration of the repeatability for the dissolution step is conducted by performing the dissolution step on separate units of a well-characterized dosage form or equivalent composite.

### 5.4.2 INTERMEDIATE PRECISION/RUGGEDNESS

Assuming that the major contributor to the variance is from the dissolution step, intermediate precision may be evaluated to determine the effects of random events on the precision of the dissolution procedure. This evaluation is typically done later in the development of the drug product and is required for full method validation. For many analytical procedures intermediate precision is typically assessed by determination of contributions to variance and, possibly, by a comparison of means. The use of an experimental matrix design is encouraged for evaluation of intermediate precision because interaction effects may be observed more clearly relative to a single variable experiment. In dissolution testing, a ruggedness approach that compares means alone is often taken to investigate the factors that contribute to intermediate precision. The ruggedness can be evaluated across the range of product strengths. Typical variations to be studied include different days, analysts, and equipment. If possible, ruggedness can be evaluated using a ▲well-characterized dosage form, but if this is not available, a premeasured placebo with drug substance(s)▲ (USP 1-Dec-2020) may be used to investigate the intermediate precision. The use of such a spiked placebo would additionally support the assessment of the contribution of the analytical finish to the observed variability of results.

The dissolution procedure on the same lot of well-characterized dosage form may be run by at least two different analysts from the same laboratory, with each analyst preparing the standard solutions and the medium and following the defined extraction/quantification procedure. Typically, the analysts use different dissolution baths, spectrophotometers or HPLC equipment (including columns), and autosamplers, and they perform the test on different days. Full profiles are assessed where relevant to the product. This procedure may not be necessary at each ▲dosage▲ (USP 1-Dec-2020) strength; instead, bracketing with high and low strengths may be acceptable.

Acceptance criteria for intermediate precision or for ruggedness are predetermined. A typical acceptance criterion for ruggedness is that the difference in the mean value for dissolution results between any two conditions, using the same ▲dosage▲ (USP 1-Dec-2020) strength, does not exceed an absolute 10% at time points with <85% dissolved and does not exceed 5% for time points ▲NLT▲ (USP 1-Dec-2020) 85%. Acceptance criteria may be product specific, and other statistical tests and limits may be used.

### 5.4.3 REPRODUCIBILITY

Reproducibility follows the general concepts of intermediate precision, but is performed by, ▲at least,▲ (USP 1-Dec-2020) two different analysts at different labs.

## 5.5 Robustness

Evaluation of robustness, which assesses the effect of making small, deliberate changes to the dissolution conditions, typically is done later in development of the drug product and is a requirement for full method validation. It is performed using a well-characterized lot of drug product. ▲▲ (USP 1-Dec-2020) The number of replicates (typically 3 or 6) is dependent on the intermediate precision. All profile points should be evaluated.

Selection of parameters to be varied depends on the dissolution procedure and analysis type. The parameters may include medium composition (e.g., buffer or surfactant concentration, pH, deaeration), volume, agitation rate, sampling time, and

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temperature. Statistical analysis of the data generated will help determine the extent to which the parameters must be controlled in the method. The robustness assessment is well suited to Design of Experiments (DoE) methodologies to efficiently investigate the impact of the individual parameters and/or their interaction.

Robustness of analytical finish is referenced in ⟨1225⟩. HPLC analysis parameters may include mobile phase composition (percentage organic, buffer concentration, pH), flow rate, wavelength, column temperature, and multiple columns (of the same type). For spectrophotometric analysis, the wavelength may be varied.

▲Robustness follows the general concepts of intermediate precision and uses similar acceptance criteria.▲ (USP 1-Dec-2020)

## 5.6 Stability of Standard and Sample Solutions

▲As part of method validation, the stability of the standard and sample solutions should be demonstrated (see *1.2.2 Stability*).▲ (USP 1-Dec-2020)

## 5.7 Considerations for Automation

Automated methods offer opportunities for increased precision and reproducibility; however, bias may be introduced. In particular, the sampling probe and the sample lines warrant attention as places where inaccuracies may occur. Deviations from the procedure described in ⟨711⟩, such as resident sampling probes, sampling through the stirring element shaft (hollow-shaft sampling), or fiber-optic probes, should be validated. Other aspects of automation validation may include carryover of residual drug, effect of an in-residence probe, adsorption of drug, and cleaning and/or rinse cycles. Validation is performed using the automated dissolution system including materials. Therefore, any change in materials will require demonstration of suitability based on the validation attributes that are impacted by the change.

Manual and automated procedures should be compared to evaluate the interchangeability of the procedures. This is done by performing two automated runs at each dosage concentration, ▲(12 dosage units in total),▲ (USP 1-Dec-2020) using all sampling points, compared to ▲two▲ (USP 1-Dec-2020) manually sampled runs ▲(12 dosage units in total)▲ (USP 1-Dec-2020) of the same samples. The effect of the in-resident probe cannot be determined by sampling both ways from the same vessel. Results should be consistent with the requirements for intermediate precision if the procedures are to be considered interchangeable. The difference in the mean value for dissolution results between any two conditions using the same ▲dosage▲ (USP 1-Dec-2020) strength should not exceed an absolute 10% at time points with <85% dissolved nor exceed 5% for time points >85%. Acceptance criteria may be product specific, and other statistical tests and limits may be used.

Revalidation may be necessary when the automated system is used with different formulations because of the interaction with excipients. Dissolution media containing surfactants or lipids may require additional validation efforts.

***Change to read:***

# 6. ACCEPTANCE CRITERIA

▲The selection of dissolution acceptance criterion/criteria should be consistent with the dissolution data generated from bio/pivotal or clinical batches at the point of release as well as from stability batches. Generally,▲ (USP 1-Dec-2020) there is an expectation that acceptable batches will have results that fall within the acceptance ▲criterion/▲ (USP 1-Dec-2020) criteria and that all manufactured batches should have similar dissolution behavior, thus highlighting the importance of having a ▲dissolution specification (method and acceptance criterion/criteria) which is optimal and discriminating for the drug product. The selected acceptance criterion/criteria should, therefore,▲ (USP 1-Dec-2020) discriminate between an acceptable and an unacceptable batch. In addition, the dissolution test results are viewed as a link to the pivotal clinical trial batches. When changes in dissolution rate have been shown to ▲significantly affect bioavailability, the dissolution acceptance criterion/criteria▲ (USP 1-Dec-2020) should distinguish batches with unacceptable bioavailability (*20*). Likewise, when changes in the formulation and manufacturing process significantly affect dissolution and such changes are not controlled by another aspect of the ▲drug product's specification, the selected dissolution test should distinguish these changes. Additionally, if the dissolution characteristics of the drug product change upon storage, whether or not the specifications should be revised will depend on demonstrating bioequivalence of the changed drug product to the original biobatch or pivotal batch.▲ (USP 1-Dec-2020)

## 6.1 Immediate-Release Dosage Forms

▲For immediate-release dosage forms, the dissolution acceptance criterion and test time are established by evaluating the dissolution profile data. The acceptance criterion for a dissolution test is a function of *Q*. The term *Q* is used to describe the cumulative percent of drug dissolved with respect to label claim at the selected time point of the dissolution test. A *Q* value of 80% is generally recommended, except when a justification with adequate data are provided to support a lower *Q* value (e.g., clinical or bioavailability/bioequivalence data) (*15*). *Q* values above 80% are not generally used.

During product development, the collection of complete dissolution profile data is recommended from biobatches and stability batches at specified intervals, such as 10, 15, 20, 30, 40, 50, and 60 min or 15, 20, 30, 45, and 60 min, because these data are used to establish the dissolution acceptance criterion/criteria. From the dissolution profile data, a *Q* value of 80% should be set at the first time point where the average dissolution is at least 85%. However, this time point should not be less than 15 min.

After registration, for routine batch release and on stability, the dissolution testing of immediate-release drug products usually becomes a single time-point test because dissolution data are collected at only the sampling time point(s) listed in the approved regulatory dissolution test of the product. The collection of dissolution profile data is, in general, not required.▲ (USP 1-Dec-2020)

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## 6.2 Delayed-Release Dosage Forms

The discussion about dissolution of delayed-release dosage forms in ⟨*711*⟩ focuses on enteric-coated dosage forms, which is ▲a pH-dependent release and▲ (USP 1-Dec-2020) the most common delayed-release dosage form. ▲Other types of delayed-release dosage forms are discussed at the end of this section.▲ (USP 1-Dec-2020) A dissolution test for a delayed-release tablet or capsule is a two-part test, and each part has acceptance ▲criterion/criteria. First, the dosage unit is▲ (USP 1-Dec-2020) exposed to an acid medium, followed by exposure to a buffer medium. To ensure that the enteric coating performs properly, a "NMT" acceptance criterion is indicated in *Dissolution* ⟨*711*⟩, ▲*Acceptance Table 3*▲ (USP 1-Dec-2020) for the acid stage. The medium used for an acid stage is usually 0.1 N hydrochloric acid (HCl), and the duration of this stage is typically 2 h. The dosage ▲unit is▲ (USP 1-Dec-2020) then exposed to a buffer medium, usually 0.05 M phosphate buffer at pH 6.8, but other buffers and pH targets may be used if justified. The duration of the buffer stage ▲will depend on whether the in vivo release of the drug substance in the intestinal tract is intended to be immediate or extended. When the release in the intestinal tract is immediate, the sampling in the buffer stage is usually 60 min for compendial tests. This duration may vary, however, depending on the drug product. As with immediate-release dosage forms, the *Q* value and time point are determined by evaluating the entire dissolution profile in the buffer stage (see *6.1 Immediate-Release Dosage Forms*).

When the release in the intestinal tract is extended, the buffer stage time will depend on the extended release characteristics. In general, sampling should be done at 30 min, 1 h, 2 h, and after that every 2 h until complete drug release. As with extended-release dosage forms, the acceptance criteria limits and time points are determined by evaluating the entire dissolution profile in the buffer stage (see *6.3 Extended-Release Dosage Forms*).

There are other delayed release dosage forms with a multistage pH-dependent mechanism, in order to ascertain drug release in the distal region of the gastrointestinal (GI)-tract. For those, in vitro performance testing may need to be adapted to multimedia at different pH values and a sequential test design with more than one switch at predefined time points. The specifications are adapted accordingly.

Beside these, delayed release dosage forms exist with release mechanisms, that are non-pH dependent. For those, the general testing procedure as outlined in *USP* for enteric-coated dosage forms does not apply. Instead, product-based testing procedures in one dissolution medium with a defined pH are in place. As a consequence, the time to release may be part of the specifications (i.e., time, $t \pm$ min combined with NLT *Q*%).▲ (USP 1-Dec-2020)

## 6.3 Extended-Release Dosage Forms

A dissolution test for an extended-release dosage form is generally similar to that used for an immediate- or delayed-release drug product, except that the duration of the test is longer, and ▲the acceptance criteria include at least three time points (*21*). Additional time points may be required for drug product▲ (USP 1-Dec-2020) approval purposes. An early time point, usually 1–2 h, is chosen to show that dose dumping is not probable. An intermediate time point is chosen to define the in vitro release profile of the dosage form, and a final time point is chosen to show essentially complete release of the drug (*21*). ▲The acceptance criteria time points for the test should be determined by evaluating the complete dissolution profile across the desired test duration.

During product development, it is recommended that sampling be conducted at 30 min, 1 h, 2 h, and every 2 h until complete drug release, to aid with selection of the appropriate time points for the product's dissolution acceptance criteria. For routine batch release and testing of stability samples after registration, the dissolution testing of extended-release drug products usually becomes a test with at least 3 time points because dissolution data are collected at only the sampling time points listed in the approved regulatory dissolution test of the product. The collection of dissolution data at additional time points is, in general, not required.▲ (USP 1-Dec-2020)

As with an immediate- or delayed-release drug product, the acceptance criteria and time points for an extended-release drug product should discriminate between an acceptable and an unacceptable batch. The acceptance criteria for the first stage of testing ($L_1$) should be established on the basis of available batch data (*20,21*). If human bioavailability data are available for formulations exhibiting different release rates, then, ▲if possible, it is encouraged that an in vitro–in vivo relationship (see ⟨1088⟩ and ⟨1090⟩) be used to establish acceptance criteria (*20,21*).▲ (USP 1-Dec-2020) Acceptance criteria for the second ($L_2$) and third ($L_3$) stages are derived from the $L_1$ criteria using *Dissolution* ⟨*711*⟩, *Acceptance Table 2*.

## 6.4 Multiple Dissolution Tests

Typically, monographs for extended-release dosage forms contain multiple dissolution tests representing specific products. In accordance with *General Notices, 4.10.10 Applicability of Test Procedures*, the appropriate test, if not *Test 1*, is indicated on the product labeling. For example, the *USP* monograph for *Oxycodone Hydrochloride Extended-Release Tablets* (*22*) lists 2 dissolution tests, each of which has either three or four time points. If the Tablets are analyzed using *Test 2* and the dissolution results comply with the criteria provided ▲for *Test 2*, the labeling for the drug product▲ (USP 1-Dec-2020) can indicate that the Tablets meet USP *Dissolution Test 2*. Multiple dissolution tests also can be found in monographs for immediate- and delayed-release dosage forms. For example, the *USP* monographs for *Levothyroxine Sodium Tablets* and *Pantoprazole Sodium Delayed-Release Tablets* provide 4 dissolution tests (*23,24*).

## 6.5 Interpretation of Dissolution Results

*Dissolution* ⟨*711*⟩*, Interpretation* discusses immediate-, delayed-, and extended-release dosage forms. The discussion for each of these release patterns is expanded here with examples to assist with applying the criteria during the various stages of testing. Understanding how these criteria are applied will assist in setting appropriate acceptance criteria.

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▲For the purpose of correlation with in vivo data, parameters of mathematical models are obtained by fitting to dissolution data to establish a continuous functional relationship called in vivo/in vitro correlation (IVIVC) (see ⟨1088⟩). In the case of low-solubility drug substances, an oral solution for demonstration of bioavailability may not be available. Therefore, plasma drug concentration time profiles from human permeability predictions and the first in-human trial can be used to synthesize pharmacokinetic profiles for a solution and provide an initial estimate of the in vivo dissolution profile that can be used as a target for the in vitro dissolution profile (*25*).▲ (USP 1-Dec-2020)

### 6.5.1 IMMEDIATE-RELEASE DOSAGE FORMS

Once the *Q* value ▲and the associated time point are▲ (USP 1-Dec-2020) established, the dissolution test is a staged test of 3 levels. In the first level of testing called $S_1$, 6 dosage ▲units are tested. Each unit must be NLT▲ (USP 1-Dec-2020) *Q* + 5% (absolute percentage points) dissolved at a specified time. For example, the time and tolerances in a monograph would be:

**Time:** 30 min

**Tolerances:** NLT 80% (*Q*) of the labeled amount of "drug substance" is dissolved.

If the *Q* value for a 200-mg label claim (LC) immediate-release tablet is specified as 80% and the time point is 30 min, then ▲for level $S_1$▲ (USP 1-Dec-2020) NLT 85% LC (170 mg) of the drug substance in each tablet must be dissolved at 30 min.

If this criterion is not met, then 6 additional tablets are tested at level 2 ($S_2$). To pass the $S_2$ acceptance criteria, the average of all 12 tablets must be equal to or greater than *Q* (80% LC; 160 mg in the above example), and no tablet has less than *Q* – 15% (65% LC; 130 mg in the above example).

If these criteria are not met, then level 3 or $S_3$ testing must be performed by testing 12 additional tablets. To pass $S_3$, the average of all 24 tablets must be equal to or greater than *Q* (80% LC; 160 mg in the above example). Two additional criteria must be met as well: 1) NMT 2 tablets are less than *Q* – 15% (65% LC; 130 mg in the above example), and 2) no tablet is less than *Q* – 25% dissolved (55% LC; 110 mg in the above example.)

### 6.5.2 DELAYED-RELEASE DOSAGE FORMS

An aliquot of the ▲sample solution (drug substance or drug substance plus degradation product)▲ (USP 1-Dec-2020) from each vessel is analyzed at the end of the acid stage. For the acid stage, the acceptance criteria have 3 levels. Level 1 ($A_1$) testing is passed if no individual value exceeds 10% dissolved. If the $A_1$ criteria are not met, then the dissolution test is performed on 6 additional ▲units▲ (USP 1-Dec-2020) for level 2 ($A_2$) testing. Level $A_2$ criteria are passed if the average of all 12 ▲units▲ (USP 1-Dec-2020) in the acid stage is NMT 10% dissolved and if no individual ▲unit▲ (USP 1-Dec-2020) is more than 25% dissolved. Level 3 testing is performed if the $A_2$ criteria are not met. The $A_3$ criteria are passed if the average of all 24 ▲units▲ (USP 1-Dec-2020) in the acid stage is NMT 10% dissolved and if no individual ▲unit▲ (USP 1-Dec-2020) is more than 25% dissolved. For the special case in which the solubility of the drug in an acidic medium ▲is too low (because of conversion to the free acid)▲ (USP 1-Dec-2020) to support an acceptance criterion of NMT 10%, the drug product should be exposed to the acid stage for the defined duration and then exposed to the buffered medium. Alternate acceptance criteria for the acid stage based on drug solubility may be justified.

For delayed-release dosage forms, the total percentage dissolved is determined by adding the measured amounts ▲of drug substance or of drug substance and degradation product in the acid stage to the drug substance dissolved in the buffer stage for each individual unit (see Example 1 in *5.3 Accuracy/Recovery*). If the dosage form is designed for immediate release of the drug in the intestinal tract,▲ (USP 1-Dec-2020) these calculated values are then compared to staged acceptance criteria ($B_1$, $B_2$, and $B_3$) that are based on a *Q* value. The $B_1$, $B_2$, and $B_3$ criteria are ▲analogous▲ (USP 1-Dec-2020) to those for the immediate release $S_1$, $S_2$, and $S_3$ criteria.

▲For example, the time and tolerances in a monograph would be:

**Acid stage**

**Time:** 120 min

**Tolerances:** NMT 10% of the labeled amount of "drug substance" is dissolved.

**Buffer stage**

**Time:** 45 min

**Tolerances:** NLT 80% (*Q*) of the labeled amount of "drug substance" is dissolved.

If the dosage form is designed for extended release of the drug in the intestinal tract, the sum of the measured amounts in the acid and buffer stages for each individual unit are then compared to criteria described in the next section.▲ (USP 1-Dec-2020)

### 6.5.3 EXTENDED-RELEASE DOSAGE FORMS

In the following hypothetical example, which is used to describe the criteria for an extended-release dosage form, the time points are 1, 4, and 8 h. ▲▲ (USP 1-Dec-2020)

Acceptance ranges are often expressed in tabular form in the *USP–NF* (see *Table 4*).

**Table 4. *L*, Criteria**

| Time (h) | Amount Dissolved (%) |
|---|---|
| 1 | ▲14–34▲ (USP 1-Dec-2020) |
| 4 | ▲46–66▲ (USP 1-Dec-2020) |
| 8 | ▲NLT 80▲ (USP 1-Dec-2020) |

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Six ▲units▲ (USP 1-Dec-2020) are analyzed at level 1 ($L_1$); acceptance criteria are met if no individual value lies outside each of the stated ranges, and no individual value is less than the percentage specified for the final time point. If the $L_1$ criteria are not met, then 6 additional ▲units▲ (USP 1-Dec-2020) are analyzed at level 2 ($L_2$). The $L_2$ criteria are met if these 3 conditions are met:

1. The average value of the 12 ▲units▲ (USP 1-Dec-2020) lies within each of the stated ranges and is NLT the stated range of the final time point.
2. None of the 12 ▲units▲ (USP 1-Dec-2020) is >10% of the labeled content outside each of the stated ranges.
3. None of the 12 ▲units▲ (USP 1-Dec-2020) is >10% of the labeled content below the stated amount at the final test time.

For the above example, the $L_2$ acceptance criteria for the 12 ▲units▲ (USP 1-Dec-2020) (see *Table 5*) are as follows:

**Table 5. $L_2$ Criteria**

| | ▲1 h | 4 h | 8 h▲ (ERR 1-Dec-2020) |
|---|---|---|---|
| Average | ▲14%–34% | 46%–66% | NLT 80%▲ (USP 1-Dec-2020) |
| Individual ▲units | 4%–44% | 36%–76% | NLT 70%▲ (USP 1-Dec-2020) |

If the $L_2$ criteria are not met, then 12 additional ▲units▲ (USP 1-Dec-2020) are tested at level 3 ($L_3$). The $L_3$ criteria are met if these 5 conditions are met:

1. The average value of the 24 ▲units▲ (USP 1-Dec-2020) lies within each of the stated ranges and is NLT the stated range of the final time point.
2. NMT 2 of the 24 ▲units▲ (USP 1-Dec-2020) are >10% of labeled content outside each of the stated ranges.
3. NMT 2 of the 24 ▲units▲ (USP 1-Dec-2020) are >10% of the labeled content below the stated amount at the final test time.
4. None of the 24 ▲units▲ (USP 1-Dec-2020) is >20% of the labeled content outside each of the stated ranges.
5. None of the 24 ▲units▲ (USP 1-Dec-2020) is >20% of the labeled content below the stated amount at the final test time.

The $L_3$ acceptance criteria for the 24 ▲units▲ (USP 1-Dec-2020) in the above example are summarized in *Table 6*.

**Table 6. $L_3$ Criteria**

| | ▲1 h | 4 h | 8 h▲ (ERR 1-Dec-2020) |
|---|---|---|---|
| Average | ▲14%–34% | 46%–66% | NLT 80%▲ (USP 1-Dec-2020) |
| Individual ▲units▲ (USP 1-Dec-2020) | NMT 2 ▲units▲ (USP 1-Dec-2020) are outside the range of ▲4%–44%,▲ (USP 1-Dec-2020) and no individual ▲unit▲ (USP 1-Dec-2020) is outside the range of ▲0%–54%▲ (USP 1-Dec-2020) | NMT 2 ▲units▲ (USP 1-Dec-2020) are outside the range of ▲36%–76%,▲ (USP 1-Dec-2020) and no individual ▲unit▲ (USP 1-Dec-2020) is outside the range of ▲26%–86%▲ (USP 1-Dec-2020) | NMT 2 ▲units▲ (USP 1-Dec-2020) release ▲<70%▲ (USP 1-Dec-2020) and no individual ▲unit▲ (USP 1-Dec-2020) releases ▲<60%▲ (USP 1-Dec-2020) |

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# ⟨1094⟩ CAPSULES—DISSOLUTION TESTING AND RELATED QUALITY ATTRIBUTES

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## 1. INTRODUCTION

This general information chapter provides approaches for the development of dissolution test procedures for capsules, which are not provided by ▲*Disintegration* ⟨701⟩,▲ (USP 1-Dec-2023)*Dissolution* ⟨711⟩, *Drug Release* ⟨724⟩, *The Dissolution Procedure: Development and Validation* ⟨1092⟩, ▲*Oral Dosage Forms—Performance Tests* ⟨1711⟩,▲ (USP 1-Dec-2023)and *Disintegration and Dissolution of Dietary Supplements* ⟨2040⟩. The chapter also discusses quality attributes associated with capsules that may affect the outcome of dissolution testing.

### 1.1 Types of Capsules

Capsules can be classified as two main types based on the physical characteristics of the shell: soft capsules and hard capsules. For the purpose of this chapter, soft-shell capsules and hard-shell capsules are referred to as softgels and hardgels, respectively.

▲Softgel capsule is one piece where the manufacture of capsule shell, capsule filling, and final capsule sealing occur in one operation, whereas a hardgel capsule is two pieces (body and cap) where the manufacture of the two pieces and capsule filling occur in two separate operations, very often at different sites.▲ (USP 1-Dec-2023)

Softgels have a thicker shell and typically exhibit a higher degree of elasticity because of the added plasticizer. They also have a slightly longer rupture time when compared with hardgels. By comparison, hardgel capsules have a thinner and more rigid shell than softgel capsules. Both softgels and hardgels are composed of a polymer, e.g., gelatin, starch, ▲pullulan,▲ (USP 1-Dec-2023) or a cellulose derivative such as hypromellose (HPMC), or other polymers, as well as a plasticizer, and water. For hardgels, water acts as the plasticizer, whereas softgels contain high-boiling-point polyols such as glycerol or sorbitol as a plasticizer, and also contain water. Although many parameters affect the physical and chemical properties of the shell, the ratio of polymer to plasticizer primarily determines the rigidity, brittleness, and dissolution performance of the shell.

Capsules can also be characterized by the chemical properties of the fill material (hydrophobic-based versus hydrophilic-based) or by the physical properties of the fill material (solution versus dispersion versus solid). Hydrophobic solutions include neat oils, combinations of miscible oils, or active ingredients dissolved in oil vehicles. Hydrophobic dispersions include active ingredients dispersed or suspended in oil or in oil-wax mixtures. The latter often are termed semisolids. Hydrophilic solutions can be neat liquids, combinations of water-miscible liquids, or active ingredients dissolved in water-miscible vehicles. Hydrophilic dispersions or suspensions include active ingredients dispersed or suspended in hydrophilic vehicles such as polyethylene glycol. Solid fill materials consist of mixtures of excipients and active ingredients whose properties like hydrophilicity/hydrophobicity, polymorphism, particle size, etc., drive the dissolution behavior.

▲Capsules are used for the oral administration of formulations and can be swallowed as a whole unit. Capsules can also be used as a means of measuring a dose of a formulation. One example is in the case of granules, pellets or powders administered with food or beverages, where the capsule is opened and its contents sprinkled over the food or beverage. These capsules can be used when they may be difficult to swallow (e.g., pediatric populations and patients with difficulty swallowing). Another example is capsules that are used in inhalation powder drug products, where the device punctures the capsule to release its content for inhalation by the user. Yet another example is softgels containing liquids or semisolid formulations that have a special design, allowing the user to twist the top of the softgel, then pressing it in order to release its contents for application.▲ (USP 1-Dec-2023)

### 1.2 Manufacturing and Packaging Issues That Can Affect Dissolution Testing

A number of issues affect the development of a dissolution procedure and the dissolution behavior for capsules, including:

- Properties of capsule shell material
- Properties of the fill material
- Interaction between capsule shell material and fill material

Gelatin is a hygroscopic material, and its moisture content affects the properties of hard and soft gelatin capsules. Since certain excipients are known hygroscopic agents, it is particularly important to monitor the mechanical properties of gelatin capsules stored under various conditions of temperature and relative humidity. The factors that can affect the capsule properties include: moisture exchange between the shell and the fill material, which potentially can create brittleness in the gelatin shell, and chemical interactions between the fill material and gelatin, which can result in gelatin cross-linking.

The potential for aldehydic impurities and formation of degradants and degradation of the active ingredients or excipients in the formulation should be investigated during product development by means of stability studies (aldehydes contribute to cross-linking; see *2. Cross-Linking in Gelatin Capsules*). Understanding the possible routes of aldehyde or ketone formation, and possible sources of aldehyde, helps to predict capsule behavior. The rate of cooling and drying may modify the characteristics of the active ingredient release from the matrix. The possibility of migration of the active ingredient into the capsule shell, particularly when the softgel is made of gelatin, should be investigated for its impact on dissolution testing.

Capsule shells can become reactive depending on the storage conditions. Product packaging and storage conditions are chosen to prevent adverse effects on capsule quality. The ingress of moisture and/or oxygen into the packaging and through the capsule shell, as well as the rate of ingress, can affect the final shelf life for the product. In some cases, an increase in water

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content of the capsule shell may produce a measurable reduction in the dissolution time because a moistened product may facilitate polymer hydration.

Due to the water content of gelatin shell (usually between 13% and 16%), this type of capsule behaves as a moisture reservoir which may affect the stability of humidity-sensitive active ingredients.

When a capsule is immersed in an aqueous medium, first the water permeates the walls, then the polymer becomes hydrated, and swells. When fully hydrated, the shell starts to dissolve. The amount of time it takes for water to penetrate capsule shells varies, depending on the nature of the capsule shell and other factors. This time has been reported to be approximately 40 s for gelatin ▲and pullulan▲ (USP 1-Dec-2023) capsules, and about 3 min for HPMC capsules. This delay may be significant only for dissolution testing of immediate-release dosage forms, while it should have minor or no impact on dissolution testing of modified-release formulations.

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## 2. CROSS-LINKING IN GELATIN CAPSULES

Cross-linking involves the formation of chemical links stronger than the simple hydrogen and ionic bonding between gelatin chains, and affects the thermal reversibility of the sol-gel transition of gelatin in the shell. Cross-linking can be caused by agents present in the capsule fill that react with gelatin molecules, resulting in the formation of a pellicle on the internal surface of the shell. Less often, a pellicle may form on the external surface of the shell arising from reactive agents present in, or derived from the intermediate or final packaging components.

A pellicle is a thin, water-insoluble, clear membrane of cross-linked protein on the inner or outer surface of the capsule that prevents the capsule fill from being released. Cross-linking is evidenced by the observation of a thin membrane or a gelatinous mass during dissolution testing because the pellicle itself may be difficult to observe.

Cross-linking can also be caused by agents or impurities present in the shell, thereby rendering the entire shell matrix insoluble under conditions that normally would dissolve the gelatin shell. One of the strongest and most common types of cross-linking involves the covalent bonding of the amine group of a lysine side chain of one gelatin molecule to a similar amine group on another molecule. This reaction is typically caused by trace amounts of reactive aldehydes. Formaldehyde, glutaraldehyde, glyoxal, and reducing sugars are the most common cross-linking agents. The covalent bonding produced with this type of cross-linking is, for all practical purposes, irreversible, and ▲in vitro▲ (USP 1-Dec-2023) dissolution of the shell must involve the breaking of other bonds, such as the enzyme-mediated breaking of peptide bonds in protein chains.

Gelatin that is chemically modified, e.g., by the addition of succinic acid groups to the lysine side chains, can prevent or at least hinder aldehyde-mediated cross-linking. A weaker type of cross-linking involves complexation of free carboxylic acid groups from two different gelatin molecules with trivalent metal ions such as $Fe^{3+}$ and $Al^{3+}$. These cations can be found in some of the dyes used as colorants or as low-level contaminants of excipients. For high bloom gelatin (see *6.1.1 Gelatin*), which typically is considered higher quality, cross-linking occurs more readily because fewer links are needed to join greater lengths of gelatin chains.

It is extremely important to know and understand the product formulation to identify possible sources of cross-linking agents and take measures to eliminate or minimize their role in promoting cross-linking. This knowledge can help in the case of post-approval changes in the formulation, and/or packaging material.

Common causes of cross-linking include the following:

- Aldehydes that are present in the active ingredient, excipients, or packaging materials (e.g., in residual solvents), or that may be formed in-situ during storage
- High humidity▲/temperature▲ (USP 1-Dec-2023) (leading to higher oxygen permeability)
- ▲▲ (USP 1-Dec-2023)
- Substances that ▲may decompose to form aldehydes (e.g.,▲ (USP 1-Dec-2023) hexamethylenetetramine ▲in corn starch▲ (USP 1-Dec-2023)), resulting in the formation of ammonia and formaldehyde▲▲ (USP 1-Dec-2023)
- Rayon coilers that contain an aldehyde functional group (furfural)
- Polyethylene glycol may contain peroxides and aldehydes
- UV light, especially with high heat and humidity
- Aldehyde formation promoted by elevated temperatures

Dissolution testing of cross-linked capsules can result in slower release of the drug or no release at all. On rare occasions, if there are defects in the liquid-filled capsule seam, the capsule can rupture at the seam even in the presence of cross-linking in the gelatin, resulting in an early release of the capsule fill in the dissolution medium. The degree of cross-linking is not uniform within one capsule or among different capsules. As consequence, there is a higher variability in the dissolution results if the gelatin capsules are cross-linked. Enzymes can be added to the dissolution medium to overcome this problem (see ⟨711⟩ ▲and *3.2.3 Use of Enzymes*▲ (USP 1-Dec-2023)). Enzymes should not be used in the absence of such evidence.

▲The evidence of cross-linking can be confirmed visually or experimentally in the following ways:

- Visual observation of a thin membrane (pellicle) or gelatinous mass around the capsule during the dissolution test. Pellicle formation indicates that cross-linking has occurred to an advanced degree. Lower levels of cross-linking which would not result in a pellicle, but perhaps a gelatinous mass, could cause a reduction in the dissolution rate.
- Capsule switching test: the content of the cross-linked capsule is transferred into a fresh shell; subsequently, the emptied cross-linked capsule shell is filled with a fresh blend of the same formulation. The switched capsules are then subjected to dissolution testing. A comparison of the in vitro dissolution results of the cross-linked capsule shell filled with fresh blend versus that of fresh capsules containing the aged formulation blend can confirm whether the dissolution slow down of the aged gelatin capsules is due to cross-linking.
- Dissolution results that show, randomly, a very slow or reduced dissolution rate when compared to the previous data.

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Some instrumental analytical techniques have been used to investigate the capsule shell to determine the nature and extent of cross-linking. Examples of such techniques are:

- Ultraviolet/Visible (UV-Vis) spectroscopy—This technique involves making an aqueous solution of the samples, treating with a reagent, and measuring the change in the UV-Vis spectra. This method has failed to be reproducible due to an aqueous solution not being representative of the intact capsule.
- Fourier Transform Infrared (FTIR)—This technique is not reproducible due to the fact that changes in the IR spectrum of gelatin due to cross-linking are very small, which are difficult to reproducibly quantitate.
- Differential Scanning Calorimetry (DSC)—In theory the glass transition temperature of gelatin should increase with increasing degree of cross-linking. The gelatin glass transitions are very weak thermal transitions, which are often masked by thermal transitions of many additives commonly found in gelatin capsules.
- X-ray diffraction—This may be a promising technique for measuring cross-linking because a higher degree of diffraction may indicate a higher level of cross-linking.

[NOTE—Precise quantitation of cross-linking is difficult and unlikely to be achieved using these methods; however, a relative comparison may be possible.▲ (USP 1-Dec-2023)]

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## 3. DISSOLUTION PROCEDURE DEVELOPMENT

Design of a procedure for dissolution testing of capsules depends on the product formulation. The composition of the fill, the solubility of the active ingredient(s) in the fill, and the dispersion of the fill into the dissolution media all have an effect on the dissolution behavior of a given fill formula. For example, a formulation that contains a lipid-soluble active ingredient with hydrophobic excipients and a melting point in excess of 37° will likely not release the active ingredient into solution in aqueous media in a timeframe that is consistent with the expectations for immediate-release dosage forms. By contrast, a freely water-miscible active ingredient dissolved or dispersed in a water-soluble or water-dispersible fill formula at room temperature will be released into solution very soon after the shell ruptures and the dosage form releases the fill material into the media.

The specific design of the formulation and the target release profile should be known when developing a dissolution method for any given product. For lipid-based formulations that often are less dense than aqueous media, the release of the fill from the capsule may be driven by buoyancy. If the formulation has been designed to be self-emulsifying or self-microemulsifying, the formulation will efficiently disperse into most aqueous media. Formulations such as those that consist simply of a triglyceride with no additional co-solvent or emulsifier will rapidly float to the dissolution medium surface in the vessel. In this situation, the quantitative results from a dissolution test provide the rate of drug partition from this floating layer. The apparatus selection is perhaps the most critical step for these types of capsules because the efficiency with which the capsule contents mix with dissolution media is highly influenced by the hydrodynamics of the agitation. Once the apparatus has been selected, other variables to consider in developing a dissolution test are rotation speed, dip rate or flow rate, surfactant/solubility enhancer type and concentration, medium volume▲, mesh size of the basket or screens, media deaeration, sinkers,▲ (USP 1-Dec-2023) and pH (see ⟨711⟩, ⟨1092⟩, and ⟨2040⟩).

To better guide dosage form design, it may be useful to consider other characteristics of the product such as solubility of the active ingredient(s) in different media ▲(see *Solubility Measurements* ⟨1236⟩)▲ (USP 1-Dec-2023), intrinsic dissolution of the active ingredient(s) (see *Intrinsic* ▲*Dissolution*—▲ (USP 1-Dec-2023)*Dissolution Testing Procedures for Rotating Disk and Stationary Disk* ⟨1087⟩), dispersibility, globule size for emulsions, micelle formation, digestion, precipitation on dilution in media, phase behavior studies, and burst tests to detect gelatin cross-linking.

The rupture test may be a useful tool in the early steps of the development and evaluation of the formulation.▲Rupture occurs when the capsule shell is breached, exposing the fill contents. Typically, the rupture test is used only in the early stages of development. Later, it is replaced by a dissolution or a disintegration test, with appropriate justification.

The use of enzymes to address cross-linking is considered a second method. Special considerations in method development with the addition of enzymes is addressed in *3.2.3. Use of Enzymes*.▲ (USP 1-Dec-2023)

### 3.1 Apparatus

As is the case with other oral solid dosage forms, USP *Apparatus 1* (basket), USP *Apparatus 2* (paddles), USP *Apparatus 3* (reciprocating cylinder), and USP *Apparatus 4* (flow-through cell) are most often chosen as the dissolution apparatus for capsules (see ⟨711⟩ and ⟨2040⟩). However, several specific ▲physical and chemical properties and characteristics▲ (USP 1-Dec-2023) must be considered in selecting and using a dissolution apparatus for capsules:

- Capsules may be filled with a material that has a specific gravity less than that of water. Therefore, the capsules may float in an aqueous dissolution medium ▲and consequently must be submerged via the basket or with the aid of a sinker when using the paddle apparatus.▲ (USP 1-Dec-2023)
- Instead of dissolving completely during the dissolution test, the capsule shell may soften and disintegrate into a sticky or waxy mass that can adhere to any point in the dissolution vessel ▲, basket, or the underside of the basket shaft holding the basket, which may generate▲ (USP 1-Dec-2023) high variability in the results.
- ▲During the course of the test,▲ (USP 1-Dec-2023) the capsule fill material may form ▲an oily layer on the surface of the dissolution medium. This may require the use of surfactants to solubilize the fill material in the aqueous dissolution media.▲ (USP 1-Dec-2023)

The capsule dissolution process involves three stages: 1) rupture of the capsule shell, 2) release and dispersion of the capsule fill material, and 3) dissolution of the active ingredient(s) in the medium. Each dissolution apparatus referred to previously can achieve these three stages, but they may cause different hydrodynamic effects upon capsules as on any other dosage form as well.

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#### 3.1.1 USP APPARATUS 1 (BASKET)

This apparatus has the advantage of enclosing the capsules, preventing them from floating freely in the medium. For certain capsules, however, baskets may not be suitable. As the capsule ruptures, the material from the capsule shell may clog the basket's mesh, and for hydrophobic fill materials the oil phase released from the capsule may not disperse into fine enough droplets in the basket to efficiently pass through the mesh. A larger-size mesh may be needed to overcome these limitations.

#### 3.1.2 USP APPARATUS 2 (PADDLES)

This apparatus does not ▲▲ (USP 1-Dec-2023) prevent the capsules ▲ or contents ▲ (USP 1-Dec-2023) from floating. In these instances, wire coils can be wound around the capsules, or commercially available sinkers can be used to encase the capsules and hold them on the bottom of the vessel, allowing the fill to become exposed to more of the medium (see, ⟨1092⟩, *1.4 Choosing an Apparatus*, *2.2 Sinkers*, *2.3 Agitation*). Sinkers can also be used to prevent the capsule from sticking to the vessel walls and to provide better contact with the dissolution medium, even if the capsule does not float and remains at the bottom of the vessel. The shape and size of the sinker can play an important role in the dissolution profile and should be selected carefully. The swelling that occurs when the capsule is placed in contact with the dissolution medium should be considered when defining the size of the sinker to be used.

#### 3.1.3 USP APPARATUS 3 (RECIPROCATING CYLINDER)

This apparatus, like *Apparatus 1*, encloses the capsules. The mesh at the bottom of the cylinder, however, may become clogged with undissolved shell. Changing the mesh size may alleviate this problem. The different mechanism of agitation of *Apparatus 3* provides very different hydrodynamics compared with *Apparatus 1* and *2*. This characteristic may assist in dispersing hydrophobic droplets, avoiding the formation of layers, and some problems caused by the buoyancy of the capsule. In the case of *Apparatus 3*, a change to different media during the course of the dissolution experiment is possible. This allows dissolution profiles to be determined at different pH values, which is useful for targeted-action or modified-release dosage forms. For cases where the active ingredient has been dispersed but not dissolved, this apparatus may not be a good choice since significant sample loss will occur when moving the cylinder from one ▲ row ▲ (USP 1-Dec-2023) to the other. However, USP *Apparatus 3* has a tendency to generate foam when surfactants are added to the media. In this situation, an appropriate antifoaming agent may be added to the medium.

An alternative to *Apparatus 3* is the disintegration apparatus described in ⟨701⟩. The use of disks should be avoided because of clogging. If disks are not used, the capsule will float and may not be uniformly wetted. This apparatus offers more turbulent conditions that could be useful in the dispersion of hydrophobic filling, avoiding the formation of layers and counteracting the buoyancy of the capsule.

#### 3.1.4 USP APPARATUS 4 (FLOW-THROUGH CELL)

This apparatus also encloses the capsules and has a filter. Several types of cells are available depending upon the application. The use of the flow-through cell designed for lipid-filled softgels may be useful for certain types of formulations. ▲▲ (USP 1-Dec-2023) Use of *Apparatus 4* also makes it possible to change to different media and to alter the flow during the course of the dissolution experiment. In addition, this equipment can be set up for low or high volumes of media that can be used with low-strength products and with poorly soluble active ingredient(s), respectively.

Other apparatus may be considered with proper justification, with each candidate apparatus evaluated for variations in media composition, media volume, agitation or flow rate, and other test parameters to determine what effect each has on the dissolution performance of the product.

### 3.2 Medium

General recommendations for the selection of appropriate dissolution media can be found in chapters ⟨1092⟩ and ⟨2040⟩, FDA Guidance for Industry *Dissolution Testing of Immediate-Release Solid Oral Dosage Forms*, ▲ FDA Guidance for Industry *Dissolution Testing and Acceptance Criteria for Immediate-Release Solid Oral Dosage Form Drug Products Containing High Solubility Drug Substances*, ▲ (USP 1-Dec-2023) and FDA Guidance for Industry *Extended Release Oral Dosage Forms: Development, Evaluation, and Application of In vitro/In vivo Correlations*.

Characteristics of the dissolution medium that may affect the opening or rupture of the capsule shell, the release and dispersion of the capsule fill material, and the dissolution of the active ingredient(s) are discussed in the following sections.

#### 3.2.1 ACHIEVING SINK CONDITIONS

As in the development of any effective dissolution procedure, the medium preferably, but not necessarily, should provide sink conditions for the active ingredient(s) in an environment that ensures suitable stability and, preferably, is physiologically relevant for the product (see ⟨1092⟩).

If the fill is water-soluble or at least readily dispersible in an aqueous medium, and if the active ingredient itself is also soluble, sink conditions may be achieved in a manner comparable to that for any other solid oral dosage form. Liquid-filled capsules, however, may contain either a matrix or an active ingredient (or both) that are hydrophobic or water-insoluble. In this case, a medium with surfactants may be needed (see below). The use of organic co-solvents to improve sink conditions is discouraged and should be employed only as a last resort and at a minimum amount with appropriate justification. In practice, the presence of organic solvents in the medium may inhibit the dissolution of the shell.

▲▲ (USP 1-Dec-2023)

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To establish a suitable medium, several different dissolution media should be evaluated to identify the one that achieves appropriate sink conditions with the lowest quantity of solubilizing/dispersing agent. The effects of pH, ionic strength, buffer counter-ion, and/or co-solvent on active ingredient solubility and enzyme activity, particularly for hydrophobic active ingredients, in addition to the relative partitioning of the active ingredient between the matrix and medium, must also be evaluated.

### 3.2.2 USE OF SURFACTANTS/DISPERSING AGENTS/SOLUBILITY ENHANCERS

Surfactants, dispersing agents, or solubility enhancers may be used in the dissolution medium when the capsule fill and/or the active ingredient are hydrophobic or water-insoluble. They may also be used if the media described in ⟨1092⟩ and in the FDA guidances are ineffective in dispersing the capsule fill or in achieving proper sink conditions for the active ingredient.

Surfactants, dispersing agents, and solubility enhancers include:

- Anionic:
  - Sodium dodecyl sulfate (SDS; or sodium lauryl sulfate, SLS)
  - Bile salts (sodium deoxycholate, sodium cholate)
- Cationic:
  - Cetyltrimethylammonium bromide (CTAB)
  - Hexadecyltrimethylammonium bromide (HTAB)
  - Methylbenzethonium chloride (Hyamine)
- Nonionic:
  - Polysorbates (Tween)
  - Polyoxyethylene sorbitan esters
  - Octoxynol (Triton X100)
  - *N,N*-dimethyldodecylamine-*N*-oxide
  - Brij 721
  - Polyoxyl castor oil (Cremophor)
  - Nonylphenol ethoxylate (Tergitol)
  - Cyclodextrins
  - Polyoxyl 10 lauryl ether
- Zwitterionic:
  - Lauryl dimethyl amine oxide (dodecyldimethylamine oxide, DDAO)
  - Lecithin

Although useful as solubilizing agents, surfactants should be used cautiously because they can interact with the gelatin in the capsule shell and can hinder disintegration or dissolution. They can also inhibit enzymes that may be used to hydrolyze the gelatin shell and/or the fill.

One of the most common anionic surfactants used for dissolution testing of tablets, SLS, exhibits both of these adverse effects, particularly at lower pH (e.g., in simulated gastric fluid). In addition, SLS forms insoluble precipitates in the presence of potassium ions. SLS is one of the surfactants less compatible with enzymes. Only a very high quality grade of SLS should be employed in dissolution media because of the potential interference of its impurities in the quantitation step in dissolution testing. Therefore, the use of SLS should be considered only in the absence of other alternatives.

Other anionic or cationic surfactants have also been shown to affect gelatin solubility, and the extent of these interactions should be considered in the selection of the dissolution medium. Cationic surfactants should be avoided in formulations that contain fatty acids, because the combination potentially forms insoluble precipitates.

▲In addition to dispersing/dissolving the matrix and active ingredient(s), the medium must neither interfere with the activity of any enzyme used nor negatively interact with the capsule shell or formulation. Developing a suitable medium for hydrophobic systems therefore may require considerable experimentation.▲ (USP 1-Dec-2023)

### 3.2.3 USE OF ENZYMES

Proteolytic enzymes ▲▲ (USP 1-Dec-2023) may be used when ▲there is evidence of the presence of cross-linking in▲ (USP 1-Dec-2023) the gelatin capsule shell ▲(see *2. Cross-linking in Gelatin Capsules* and ⟨711⟩).▲ (USP 1-Dec-2023) Pancreatin▲, an example of a proteolytic enzyme,▲ (USP 1-Dec-2023) has the advantage of also possessing lipase activity, which makes it useful when the capsule fill material is a triglyceride.

Tests should be carried out to ensure that the enzymes used in the dissolution testing do not adversely interact with the formulation. ▲Enzymes have been reported to increase the risk of hydrolysis of substances which have an amide bond.▲ (USP 1-Dec-2023)The dissolution medium should also be optimized (e.g., by adjusting the pH, ionic strength, etc.) so that it preserves enzymatic activity while maintaining sink conditions for the active ingredient.

▲**Enzyme Activity:** The actual enzyme activity value is critical to the efficiency of the function of digesting the gelatin in the cross-linked capsule shells.

Enzyme activity is heavily dependent on the substrate being used to measure it. The protease activity may be different depending on the type of substrate being used. The protease activity must be determined by the procedure stated under the respective reagent specification in the Reagent Specifications section of USP for pepsin and bromelain or the appropriate *USP* monograph for *Pancreatin* and *Papain*.

**Dissolution Testing:** The dissolution testing of the capsules should be conducted following the instructions in ⟨711⟩. In the presence of cross-linking in the gelatin capsules, the test should be repeated. New dissolution medium should be prepared with

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the appropriate enzyme in the recommended amount, and the dissolution testing should be done with new capsules following the instructions in ⟨711⟩.

**Dissolution Testing During Stability Studies:** If the capsules were stored according to the conditions stated in the *USP* monographs for capsule shells and the formulation development was done considering the possible sources of cross-linking, typically, cross-linking in gelatin capsules is going to be found during stability studies. The dissolution testing during stability studies should start with the dissolution medium stated in the original method without the enzyme. When evidence of cross-linking is found, the test should be done with the addition of the appropriate enzyme to the dissolution medium. As cross-linking does not stop even if the agent causing it is removed, from this time point forward, the dissolution testing of stability samples can be carried out directly with the addition of the appropriate enzyme to the dissolution medium.

**Surfactants or Other Medium Components That May Denature Enzymes:** Surfactants and other medium components may denature the enzyme. If such ingredients are part of the dissolution medium and if enzymes are going to be used to digest the cross-linked gelatin, a special step should be included in the final dissolution method to preserve the protease activity of the enzyme. This procedure is commonly known as Tier 2, with Tier 1 being the original dissolution procedure without the enzyme.

**Tier 2 Dissolution Testing:** Typically, the Tier 2 dissolution testing will have two dissolution media: **Medium A** and **Medium B**.

**Medium A** contains the appropriate enzyme in the solvent of the original dissolution medium (e.g., water, acid, or buffer solution) in the amount stated in ⟨711⟩, in a smaller volume of dissolution medium than the one stated in the original method. The enzyme is going to digest the cross-linked gelatin, allowing the release of the capsule filling to the medium. Typically, the duration of the pre-treatment step is not more than 15 min. All the other conditions of the test (apparatus, rotation, or flow rate) should remain as described in the method or monograph.

**Medium B** is a solution in the appropriate solvent (e.g., water, acid, or buffer solution) with the surfactant or the component that may denature the enzyme in a concentration that when mixed with *Medium A* is going to reach the final concentration stated in the original dissolution procedure. The dissolution test is going to be run for additional time, with all the other test conditions (apparatus, rotation, or flow rate) as described in the method or monograph, to reach the total time specified in the method or monograph. As an example, if the total time of the dissolution test is 45 min, the samples will be in contact with *Medium A* for 15 min and, after the addition of *Medium B* to the apparatus, for 30 more min. The volume of *Medium A* and *Medium B* should be selected in a case-by-case approach, with *Medium A* volume being smaller than the volume stated in the original method or monograph. The volume of *Medium B* should be such that it will reach the volume stated in the original method or monograph after its addition to *Medium A*. Any deviations from these recommendations above need to be scientifically justified.

The first example of Tier 2 testing is located within the *USP* monograph for *Acitretin Capsules, Performance Tests, Dissolution Test 2*, where in *Tier 2* the volume of *Medium A* and *Medium B* is 450 mL each, resulting in the original medium volume of 900 mL. The second example of Tier 2 testing is located within the *USP* monograph for *Ziprasidone Capsules, Performance Tests, Dissolution Test 2*, where in *Tier 2* the volume of *Medium A* is 700 mL, and *Medium B* is 200 mL, resulting in the original volume of 900 mL.

**Forced Cross-Linking:** During product and dissolution method development, forced formation of cross-linking in the gelatin capsule may be useful to establish the type and amount of enzyme that will be used in the test, and to better understand the behavior of the formulation in the dissolution medium. This can be achieved by spiking excipients with known amounts of formaldehyde or other cross-linking agents, or exposing the capsules to formaldehyde or high humidity and high temperature for an extended period of time. One possible procedure to generate cross-linking in the gelatin capsules is the one described in Gold TB et al.[1] Other procedures can be found in the literature.▲ (USP 1-Dec-2023)

### 3.2.4 pH

In addition to establishing a drug pH-solubility profile during the dissolution development, the following specific issues should also be evaluated:

- The effect of media pH on the swelling or dissolution of the capsule based on the type of gelatin used in the product: either Type A (for which the medium pH typically is ▲7.0–9.0▲ (USP 1-Dec-2023)) or Type B (for which the medium pH typically is 4.7–5.4)
- The need for enzymes may influence the selection of a specific pH range in order to be adequate for the active ingredient solubility and stability, and to minimize effects on enzyme activity

▲▲ (USP 1-Dec-2023)

## 3.3 Sampling

Establishing the proper sampling technique and location for capsules follows the procedures described in chapters ⟨711⟩ and ⟨1092⟩. In the case of hydrophobic liquid filled capsules, where the fill material typically forms a film on the surface of the dissolution medium during the course of the test, sampling must be performed in such a way that the cannula penetrates the oily layer without becoming clogged. In addition to standard validation and compatibility studies, care must be taken when a filter is used to ensure that it does not become clogged with oil or undissolved capsule shell material when the sample is taken. Similar considerations apply to automated sampling equipment because filters and transfer lines may become obstructed during sampling. To address this issue, the use of surfactants and/or enzymes in dissolution media may be needed to better solubilize the capsule shell and fill. Another point to be considered in the case of automated sampling equipment is the probe. If it remains inside the medium during the test, it could perturb the oily layer and possibly influence the hydrodynamics, thus changing the dissolution profile. An alternative approach is to remove the probe and to introduce it just at the time of the sampling. The sampling method should be validated for each product.

[1] Gold TB, Buice RG, Lodder RA, Digenis GA. Determination of extent of formaldehyde-induced cross-linking in hard gelatin capsules by near-infrared spectrophotometry. *Pharm. Res.* 1997, 14(8): 1046-50. doi: 10.1023/a:1012105412735.

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## 3.4 Quantitation

Like dissolution samples from other oral solid dosage forms, dissolution samples from capsules can be quantitated using chromatographic, spectrophotometric, tandem chromatography-mass spectrometry, and other techniques after adequate method development and validation (see ⟨1092⟩ and where special considerations are described under *4. Method Validation*).

***Change to read:***

# 4. METHOD VALIDATION

In addition to the general method validation parameters discussed in ⟨1092⟩, the following performance characteristics may be evaluated:

- Effect of pH and ionic strength on drug solubility and on enzyme activity, if enzymes are used.
- For hydrophobic active ingredient(s), the relative partitioning of the active ingredient(s) between the matrix and the medium, as well as the potential for an adverse effect on release into the aqueous medium.
- For chromatographic procedures, the potential adverse effect of the surfactant, if used, on the chromatographic separation; as part of the robustness study, evaluation of different concentrations of surfactant, different surfactant types, interaction with buffer salts, etc.
- For spectrophotometric procedures, absorbance from the potential contribution of the capsule shell should be evaluated.
- Potential adverse effects of the surfactant or enzyme, if present, on the lifetime of the chromatographic column.
- Qualification of the sampling procedure to prevent clogging of the cannula, transfer tubing, or filters.
- Validation of the ▲dissolution method step with enzyme (Tier 2) and of the pre-treatment step when the dissolution medium contains components that may denature the enzyme.
- Verification that the conditions of the Tier 2 step do not affect the dissolution profile when compared to the Tier 1 test. This may be accomplished by performing parallel analysis with the drug formulation filled in non-cross-linked capsules run in the required dissolution media (Tier 1) and the same media with the enzyme added (Tier 2). The resulting dissolution profiles should be the same, showing that the enzyme did not adversely affect the drug release profile.
- Verification that the quantitative procedure in the Tier 2 step is specific, accurate, precise, linear, and has solution stability.

## 4.1 Cleaning Considerations

Cleaning methods for dissolution apparatus components (e.g., vessels and stirring elements) and for automated systems, if employed, should be developed and qualified for their effectiveness in removing traces of residue from product or media from previous tests. Interference from residual active ingredient(s), excipients, and media (including surfactants) should be mitigated to ensure the integrity of each subsequent dissolution test. While established glassware cleaning procedures may document best practices for cleaning laboratory glassware, more stringent procedures may be needed for dissolution equipment, especially automated equipment, to ensure that run-to-run cleaning procedures are effective and that in-run procedures, which adequately prime and purge sampling probes, inline filters, tubing, pumps, and dispensing systems, were effective in avoiding carryover from previous tests or dilution from rinse solutions from each successive sample collected during an automated sampling sequence for the dissolution test.▲ (USP 1-Dec-2023)

***Change to read:***

# 5. SUGGESTIONS FOR STARTING POINTS

Based on the considerations discussed above, possible starting points for establishing a dissolution test for capsules based on the solubility characteristics of the fill and the active ingredient(s) are the following:

Possible formulations:

1. Hydrophilic fill/active ingredient is soluble in aqueous media
2. Hydrophobic fill/active ingredient is poorly soluble in aqueous media
3. Hydrophilic fill/active ingredient is poorly soluble in aqueous media
4. Hydrophobic fill/active ingredient is soluble in aqueous media

## 5.1 Medium

Evaluate the solubility of the active ingredient(s) in aqueous media within a pH range of 1.0 to 7.2–7.5.

## 5.2 Medium Additives

Enzymes ▲▲ (USP 1-Dec-2023) may be used if the product shows evidence of cross-linking in the gelatin capsule. ▲See *3.2.3 Use of Enzymes*.▲ (USP 1-Dec-2023)If the product is a formulation such as *2* or *3* above, evaluate the use of surfactants and possible interaction with enzymes.

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### 5.3 Apparatus

USP *Apparatus 1* or *2*. If the capsule is filled with low specific gravity liquid and has a tendency to float, sinkers should be used. If the product is a formulation such as *2* or *4* above, consider *Apparatus 3* as an option.

### 5.4 Agitation Speed

Agitation speed typically is 50–100 rpm for baskets and 50–75 rpm for paddles. Higher speeds should be justified.

### 5.5 Time Points

A dissolution profile should be established during the development phase to identify when the rate of active ingredient release has leveled off.

### ▲5.6 In Vitro/In Vivo Correlations

An in vitro/in vivo correlation (IVIVC) is possible with capsules, as with any solid oral dosage form. See *In Vitro and In Vivo Evaluation of Oral Dosage Forms* ⟨1088⟩. However, if cross-linking becomes an issue with an IVIVC, the Tier 2 procedure with enzyme would have to be validated to show that the correlation still presents with the new media.▲ (USP 1-Dec-2023)

***Change to read:***

## 6. CRITICAL QUALITY ATTRIBUTES

### 6.1 Shell Composition

Knowledge of the composition of the shell (polymer, plasticizers, water content, etc.), as discussed at the beginning of this chapter, is an important aspect and helps determine critical quality attributes. Other important aspects of understanding the critical quality attributes are the composition and properties of the gel mass and finished shell. In its simplest form, the shell of a capsule is prepared from a molten gel mass, which in the case of hardgels, is comprised of gelatin or other polymers and water, and in the case of softgels, the shell is comprised of gelatin and a plasticizer dissolved in an aqueous vehicle. The ratio of polymer to plasticizer varies depending on the desired performance traits of the shell, the size of the shell, and the composition of the fill material. Other minor components added to the gel mass may include colorants, flavors, stabilizers, buffers, and opacifiers. The physical characteristics and quality of these minor components are central to the design of high-quality and robust formulations. The composition of hardgels usually does not vary with capsule size.

#### 6.1.1 GELATIN

**Gelatin capsule manufacturing process:** For hardgels, there are two separate manufacturing steps: 1) manufacturing the empty shells, and 2) the filling process. Capsule shells are produced, packaged, and shipped to the dosage form manufacturers. Capsules are then filled and sealed. Most modern capsule-filling machines are designed to allow accurate filling of powders, granules, pellets, tablets, and combinations of these can be modified to allow hot or cold liquid to be filled into hardgels. An essential part of a liquid-filling operation is the ability to effectively seal the capsule. This sealing process is a critical process parameter, and detailed knowledge of all the aspects of this process is important.

For softgels, the formation of the capsule, the filling process (i.e., encapsulation process), and sealing of the capsules occur simultaneously. During the encapsulation process, it is important to monitor the shell thickness, seam quality, capsule weight, and the fill weight using statistical process controls.

**Chemistry of gelatin:** Understanding the chemical nature of gelatin is another important component of the critical attributes of the formulation. Gelatin is graded primarily on the strength of the gel. Depending on the process and the tissue source, noticeable differences in ▲bloom▲ (USP 1-Dec-2023) strength are apparent among suppliers and even between lots from the same supplier. Consequently, controlling the strength of the gelatin from batch to batch, measured as bloom strength, is key to obtaining a consistently performing product. Bloom strength is a measure of the strength of the gel prepared at a set concentration of gelatin in water under controlled conditions, and is a function of the molecular weight of the gelatin, the concentration of the gelatin in the gel, and the pH of the gel. It is a measure of the resultant gel's resistance to compression and is reported in bloom-grams or grams. Bloom strength increases when the gelatin concentration in the gel increases, when the average molecular weight of the gelatin increases, and when the pH of the gel approaches neutrality (from either direction). In addition, as bloom strength increases, the cost of gelatin increases and the rate of gel dissolution decreases. Bloom strength also has an effect on the clarity and color of liquid-filled capsules. For gelatin with higher bloom strength, less gelatin is needed to produce a suitable shell, which results in a clearer shell and a reduced need for colorants and dyes in order to produce the desired hue. Although gelatin can be purchased with bloom strengths ranging from 50 to 300, most gelatins used in the manufacture of liquid-filled capsules have a bloom strength of about 150–200 for softgels and 220–280 for hardgels. Gelatin manufacturers commonly blend different sublots of gelatin to meet bloom requirements. These important characteristics of gelatin must be taken into account when characterizing the product under development.

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#### 6.1.2 CHEMISTRY OF OTHER POLYMERS

Suitable consideration should be given to the other polymers used to manufacture capsule shells. Iota and kappa carrageenans, modified corn, potato, and pea starches, and modified celluloses, together with plasticizers are also used for the preparation of capsule shells. Carrageenans are polysaccharides extracted from sea weeds. They possess the required gelling characteristics similar to gelatin. However, kappa carrageenan produces a brittle gel, and iota carrageenan a soft and elastic gel. Carrageenans are usually combined with modified starches and plasticizers to form the capsule shell. Modified celluloses, such as HPMC, ▲and other polymers such as pullulan▲ (USP 1-Dec-2023) are also used for the manufacturing of hardgels. Compared to gelatin shell, the shell prepared using the polymers mentioned above will have some advantages, such as non-crosslinking, being able to handle wider pH ranges, and tolerance of high fill temperatures.

#### 6.1.3 PROCESSING AIDS

Processing aids include gelatin ribbon lubricants in the softgel manufacturing process. Commonly used ribbon lubricants include mineral oil and medium chain triglyceride. Since ribbon lubricants are applied to both the inner and outer surfaces of the capsule shell, the evaluation of their potential adverse effects is focused on the capsule itself and primarily on gelatin cross-linkage. ▲SLS is used as a processing aid for hard gelatin capsules to improve glide properties of capsules during the filling process at the dosage form manufacturers.▲ (USP 1-Dec-2023)

### 6.2 Stability and Storage Conditions

Knowledge of the stability and reactivity of gelatin or other polymers is essential to anticipate their influence on final product quality. At room temperature, the capsule shells as supplied are relatively effective in protecting the fill from oxygen and its effects, and when an opacifier such as titanium dioxide is added to the shell, it can prevent photodegradation of the fill. In addition, the low water activity of the shell does not promote microbial growth. For bacteria, yeasts, and molds to grow, a higher water activity of at least ▲0.80▲ (USP 1-Dec-2023) $a_w$ is required; the water activity of capsule shells typically is less than 0.40 $a_w$.

Storage conditions of the final product range from refrigeration to standard room temperature. Products should be stored according to the label directions. Brief excursions outside of these conditions should be evaluated to determine their influence on the final product quality. Storage conditions and duration for the empty capsule shells should also be considered. ▲See *USP* monographs for Hard Gelatin Capsule Shells, Hard Hypromellose Capsule Shells, and Hard Pullulan Capsule Shells.▲ (USP 1-Dec-2023)

### 6.3 ▲Brittleness

The strength and durability of hydrophilic polymer films can be affected by their moisture content which, in turn, will be affected by changes in the relative humidity of the immediate environment. This applies to hardgel capsule shells since they are, in essence, hydrophilic polymer films. Hardgel capsule shells are designed with tight tolerances, and a change in moisture content of the capsule shell can impact capsule filling. If the relative humidity around the capsules increases, the capsule shells will absorb moisture and can swell, thereby changing their dimensions. If the relative humidity drops, then the capsule shells will lose moisture and become brittle, which may also impact capsule fill operations. This phenomenon is more problematic with hard gelatin and hard pullulan capsules than with hypromellose capsules. Thus, it is important to store empty capsule shells in moisture-tight packaging and under controlled room temperature. Embrittlement can also occur in storage with hard gelatin capsules containing hygroscopic fills, since such materials will draw water out of the shell. In general, if the fill has a water activity of <0.2 $a_w$, water will be lost from gelatin hard capsule shells and embrittlement may occur.

For capsules used in inhalation devices, both embrittlement and softening due to moisture absorption can impact the safety and efficacy of the drug product. Brittle capsules may break rather than be punctured and release gelatin particles on piercing. Softer capsules may not puncture properly, thus interfering with the release of its contents.

There are several methods for the assessment of capsule brittleness; however, there is no universally accepted test. Users of hardgel capsules should confirm with their suppliers as to the testing details if capsule brittleness is a concern.

### 6.4 Overall Potential Capsule Defect Assessment

During the capsule manufacturing process, the following defects may occur:

- Critical Defect—Affects the ability of the capsule to function as a dosage form delivery system, or contributes to major processing issues during filling operations (i.e., holes, split, mashed, etc.)
- Major Defect—Has the potential to cause a processing problem during capsule filling operations (i.e., dents, short cap, long body, etc.)
- Minor Defect—Has no effect on a capsule's performance as a whole or on the capsule filling operation, but detracts from the capsule's cosmetic appearance (i.e., bubbles, strings, crimps, etc.)▲ (USP 1-Dec-2023)

### 6.5 Formulation Development and Manufacturing for Liquid-Filled Capsules

It is important to understand the properties of the active ingredient(s) during formulation development.

Formulations developed using liquid-filling technology may be applicable when the active ingredients display 1) poor solubility in aqueous systems, 2) short half-life requiring frequent dosing, 3) low melting point, 4) low dose/high potency where containment is important, 5) requirement for taste or odor masking, and 6) critical chemical or physical stability as in the case of highly hygroscopic active ingredients. Other factors to consider during formulation development include:

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- Compatibility and stability of excipients and active ingredient(s) over time.
- Temperature-dependent solubility of the active ingredient(s) in the lipid.
- Aging/polymorphic characteristics of the lipid.
- Adequate characterization of the saturation/supersaturation status of the active ingredient(s) in the lipid formulation in order to avoid precipitation of the active ingredient(s). Optimally, saturation status should be determined when the formulation is at equilibrium with the shell.
- Stability in solution under stress conditions.
- Aldehyde formation and degradation of active ingredient(s).
- Influence, if any, of the rate of cooling on the structure of certain excipients, which may modify the release characteristics of the active ingredient(s).

Important factors to consider during a liquid-filling operation are temperature and viscosity of the fill material and, in the case of a dispersion or suspension, the particle size of the dispersed active ingredient(s). In principle, any excipient found to be compatible with the shell can be used, but in a manufacturing environment the viscosity of the fill material is important. ▲For hardgel capsule filling,▲ (USP 1-Dec-2023) excipients that are solid at room temperature but melt at temperatures up to 70° may be suitable (depending on the shell polymer) for formulating active ingredient(s), provided those excipients yield the desired in vivo performance.

Both shell and fill excipients should be controlled for levels of known cross-linking agents such as formaldehyde and reducing sugars.

Imperfections in the shell and/or in the seam may affect dissolution. They may also give rise to leaking of the capsules contents.

The appropriate in-process controls should be in place to monitor and reduce the lot-to-lot variability.

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# ⟨1097⟩ BULK POWDER SAMPLING PROCEDURES

## INTRODUCTION

The goals of this chapter are to provide guidance on bulk powder sampling procedures, identify important bulk powder sampling concepts, and collect a knowledge base of useful practices and considerations that can lead to the ideal physical sampling of bulk powder materials. The terminology used here is well established in the field of material sampling (see *Appendix 3*, for instance reference *7*). Sampling is undertaken as part of an estimation process. The parameter of primary interest here is the mean level of some analyte in the bulk powder as a whole.

The purpose of a sampling plan is to obtain a representative sample of a population so that reliable inferences about the population sampled can be drawn to a certain level or degree of confidence. Acquiring a representative sample from a lot is critical because without a representative sample all further analyses and data interpretations about the lot are in doubt. An ideal sampling process is a process in which every particle or at least every equal-size portion of the population has an equal probability of being chosen in the sample. In addition, sampling procedures should be reproducible, i.e., if the sampling protocol were repeated, a high probability should exist of obtaining similar results. Also, the integrity of the sample should be preserved during and after sampling. The details of how to sample depend on a variety of factors. For example, criteria for sampling to evaluate particle segregation may differ from criteria for evaluating moisture content or identification.

Because of the propensity of a powder to segregate, heterogeneous powder systems can make it difficult to obtain an ideal sample. Thus, to extract representative samples requires careful development of a sampling plan that accounts for and mitigates the segregation tendencies of a particular powder system. Developing a general guidance for bulk powder sampling is challenging because every situation is different, and therefore different approaches must be used to deal with each situation. Thus, the goal of this general information chapter is to outline recommended steps for developing a sampling scheme or plan for a particular system that is consistent with good sampling practices.

The primary difficulty in acquiring a representative sample is that the size of the sample for measurement, typically a few milligrams to grams, must be withdrawn from a large population on the order of hundreds to thousands of kilograms. The few milligrams analyzed in a laboratory must be taken from a large population of particles in a warehouse in such a manner that the measurement sample is representative of all the particles in the lot. Any bias or error in the sampling process will cause all future inferences to be in error. Over the years methods have been developed and refined to attempt to ensure that the measurement sample is representative of the whole population. A typical strategy is shown in *Figure 1*. The strategy is to sample in stages, starting with the initial gross or primary sample withdrawn directly from the received containers. In the laboratory, the gross sample must be reduced in size until it is the appropriate size for measurement. This should be done in a manner that minimizes the introduction of sampling errors. The key to reducing the sampling error is to ensure that every particle of the population has an equal probability of being included in the sample. However, because of segregation or the nonrandom nature of powders, many obstacles can cause bias and contribute to sampling errors. Following the flow chart in *Figure 1* and the steps outlined in subsequent discussions will help to minimize sampling errors.

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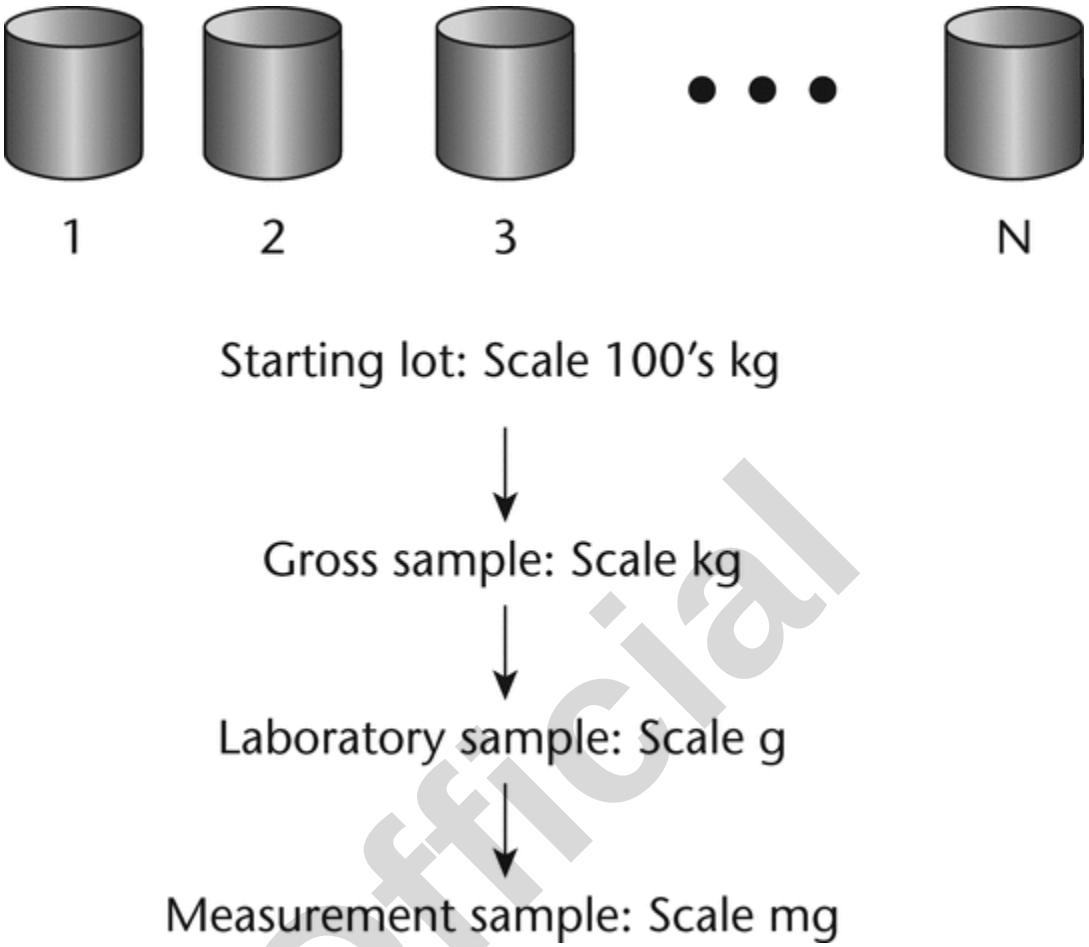


Figure 1. Overall sampling strategy for reducing the sample size from the hundreds of kg scale to the mg scale.

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To acquire a representative sample, a suitable sampling plan must be developed and implemented. A good sampling plan includes: (1) population determination and sample size selection, (2) a sample collection procedure and a method for sample size reduction, and (3) summary calculations that demonstrate that the sampling plan will yield samples that accurately characterize the population to within a stated level of acceptance. In addition, an infrastructure is needed to maintain the integrity of the samples and sampled materials.

This chapter begins with a brief introduction to sampling theory and terminology. The technical content of the chapter requires a basic scientific understanding of physical particle characteristics (e.g., mass, density, shape, and size) and statistics (e.g., acceptance sampling and binomial distribution).

## SAMPLING THEORY AND TERMINOLOGY

### Fundamental Sample Size (Sample Mass)

Sample size is considered from two perspectives: (1) the mass of the sample intended to represent the entire population, sometimes termed the composite sample, and (2) the number of samples taken with a mass sufficient to independently evaluate, compare, or provide confidence to ensure the reproducibility of the composite or the uniformity of the population. The key to obtaining an ideal sample is to understand and account for the degree of heterogeneity of the characteristic being evaluated in the system under study. For example, heterogeneity of a particle system arises from two sources: the intrinsic, constitutive, or compositional heterogeneity and the spatial distribution heterogeneity. The intrinsic heterogeneity of the powder system reflects the fundamental differences in the individual particles. Statistical heterogeneity (differences between individuals), or variance, is expected to maintain assumed properties. For a normal population the general expression for a statistical sample size suggests that the number of independent samples is proportional to the square of the normal quantile at the desired confidence level ($Z$) and the population variance ($\sigma^2$) and is inversely proportional to the square of the minimum detectable difference required ($\delta$), as shown in equation 1:

$$n \propto \frac{Z^2\sigma^2}{\delta^2} \quad (1)$$

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In order to apply the normal theory sample size equation to sample mass with a discrete number of particles, consideration for material characteristics is needed. For a heterogeneous bulk material, such as a bulk powder, the sample mass required to ensure adequate representation of the intrinsic or fundamental population heterogeneity or variation is determined by the size, shape, and density of the particles. The total sampling error (TSE) measures the difference between the analyte concentration estimated in the sample ($a_{sample}$) and the mean analyte concentration in the lot ($a_{lot}$) relative to the mean analyte concentration in the lot ($a_{lot}$), as shown in equation 2:

$$TSE = \frac{a_{sample} - a_{lot}}{a_{lot}} \quad (2)$$

When ideal sampling is employed, the TSE is reduced to a fundamental sampling error, limited only by the intrinsic heterogeneity of the material. The relative variance of the fundamental sampling error ($S_{fse}^2$) has been empirically estimated in particle size applications by characterizing the critical particle mass, heterogeneity, size (diameter), shape, density, and weights of the material. Empirical estimates require a thorough and complete knowledge of the material and process. Established material characterization and methods are critical aspects of avoiding unacceptable estimates. As shown in equation 3:

$$S_{fse2} \propto f_{shape}\, g_{CF}\, c_{max}\, id_{max3} \left( \frac{1}{m_{sample}} - \frac{1}{m_{lot}} \right) \quad (3)$$

where $f_{shape}$ is a measure of cubicity or shape factor of the analyte particles; $g_{CF}$, the granulometric factor, is an empirical correction factor of differences in particle size; $c_{max}$ is the compositional maximum heterogeneity and is calculated as if the material consists of the analyte particles and everything else; l, the liberation factor, is an empirical factor representing the proportion of critical content particles separated from the non-analyte containing particles of the lot; $d_{max}$ is the particle diameter [e.g., the maximum diameter or the diameter (cm) of the size of the opening of a screen retaining 5% by weight of the lot to be sampled]; $m_{sample}$ is the mass of the sample; and $m_{lot}$ is the mass of the lot being sampled. [NOTE—A liberation factor is needed when the analyte does not appear as separate particles. A high liberation value (1.0) suggests heterogeneity of particles. A low liberation value (0.05) suggests very homogeneous particles. See *Appendix 1* for examples of potential applications of equation 3 in the estimation of the fundamental sample mass needed to account for constitutional heterogeneity of the powder mixture.] Use of equation 3 requires prior estimates of $f_{shape}$, $g_{CF}$, $c_{max}$, l, and $d_{max}$.

## Segregation Error

Distribution heterogeneity is the difference between samples or groups of particles spatially or temporally. For example, small particles are located preferentially in the lower portion of a powder bed. This type of situation can arise as a result of powder bed segregation and is common in some particle systems with a broad particle size distribution. In other words, smaller particles may not be randomly distributed throughout the lot. This spatial heterogeneity introduces variation in the sample and is a source of variation that contributes to the total variation. Together, fundamental and segregation error give rise to sampling error, which dictates how variable the samples will be, how large the sample size and numbers of samples should be (e.g., 10 containers, sampled at top and bottom, with sample sizes of 50 g each), and how hard it will be to obtain a representative sample.

Minimizing the effects of segregation error during lot material characterization while still ensuring a representative sample mass requires collecting many small samples that average out the variation of the segregation error. This assumes one is interested in estimating the overall average, not characterizing lot heterogeneity. Segregation error is difficult to control because segregation may be the result of changes in particle size, shape, and density, as well as inputs into the determination of sample mass. Minimizing the effects of segregation error when reducing the primary sample size requires adequate physical mixing or randomization of the primary samples before analysis, thus providing equal selection probability.

## Total Sampling Method Error

Intrinsic or compositional heterogeneity is a function of the powder system and represents the true characteristics of the material (e.g., equation 3). Thus, intrinsic heterogeneity is often the minimal variance a system can have. The difference between the true state of the system and what is actually measured when ideal sampling is employed is called the fundamental error (equation 2). The relative variance of TSE ($S^2_{Total}$) is represented in equation 4 as the sum of the relative variances of all error components:

$$S^2_{total} = S^2_{fundamental} + S^2_{segregation} + S^2_{extraction} + S^2_{delimitation} + S^2_{preparation} + S^2_{trends,\,shifts} + S^2_{cycles} + S^2_{analytical\,method} \quad (4)$$

The $S^2_{Total}$ can be reduced by employing ideal sampling. Ideal sampling will limit or adjust for the effects of error contributed by particle segregation, extraction error created by the sampling device, delimitation error created by not considering the three-dimensional nature of the bulk material, and sample handling errors such as product degradation. The total variation is the sum of these sources of error, illustrated in equation 4 as independent, additive components. To the end of reducing these errors, an important goal of material characterization by sampling is the determination of the relevant errors within the bulk sample. Knowing the source of the error helps determine how to best minimize these errors.

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Fundamental error arises from the intrinsic heterogeneity of particles within a sample of the material population. Reducing fundamental error requires changing the intrinsic characteristics of the material, such as reducing the particle size by milling or grinding. Segregation error is the spatial distributional difference of particles across the population. This type of error can be minimized by mixing or randomization of the particles being selected. Segregation error is affected by the characteristics of fundamental error. Additionally, for the determination of both fundamental and segregation error, it is assumed that mechanical sampling is carried out correctly and is not invasive, i.e., that mechanical sampling does not alter the characteristics being measured and provides a true representation. In instances where sampling of the bulk material does not provide unbiased representation or is so invasive that it alters material characteristics, then, in order to obtain noninvasive, unbiased samples, operators may need to change sampling from a bulk form to a stream form of processing, either upstream or downstream from the sample point (see *Appendix 2*). The mechanical sampler may need to mix the sample sufficiently to facilitate random sampling with equal probability of selection in order to obtain an adequate representation of the entire bulk lot. The process may also require mixing or sampling from a location in the process that will provide a random sample from material that is susceptible to segregation.

Extraction, delimitation, and handling errors occur as a result of the mechanical sampler and sample handling prior to analysis, which also are affected by fundamental error. Trends, shifts, and cycles are temporal sources of error that affect total error. The analytical error of the method of analysis contributes to the overall error of the reported result. In addition to obtaining representative subsamples from the bulk material, the method must also obtain a representative subsample from the particulate laboratory sample before analysis.

## Sampling Strategy

A typical sampling strategy consists of two basic steps: (1) the primary or gross sample, followed by (2) the secondary sample, which reduces the primary sample to a size that is suitable for laboratory measurement. In short, the goal is to select from the lot a quantity of material suitable for measurement without significantly changing the attribute for which one is sampling. In parallel with the sample size reduction, sample size calculations must be done in such a way that the sampling strategy has sufficient statistical power to determine whether the attributes of interest lie within the specification ranges with a reasonable degree of certainty. Each step must be done correctly, or the sampling strategy as a whole will not provide a sample that is representative of the original population.

To successfully withdraw a sample from a bulk container that is representative of the population, one needs to have an idea of the population's heterogeneity, i.e., how segregated or stratified the system is. Knowing what factors can accentuate segregation and knowing the patterns of segregation that are likely will help one to account for segregation in a powder bed and to take better samples. Many factors can affect the degree of powder bed segregation. For segregation to occur, sufficient energy needs to be put into the powder bed to induce motion between particles. When a sufficient amount of energy is supplied, segregation can occur via three modes: percolation (in the powder bed), rolling (on the free surfaces of a powder bed), and free flight (when the powder bed is fluidized). These modes are illustrated in *Figure 2*.

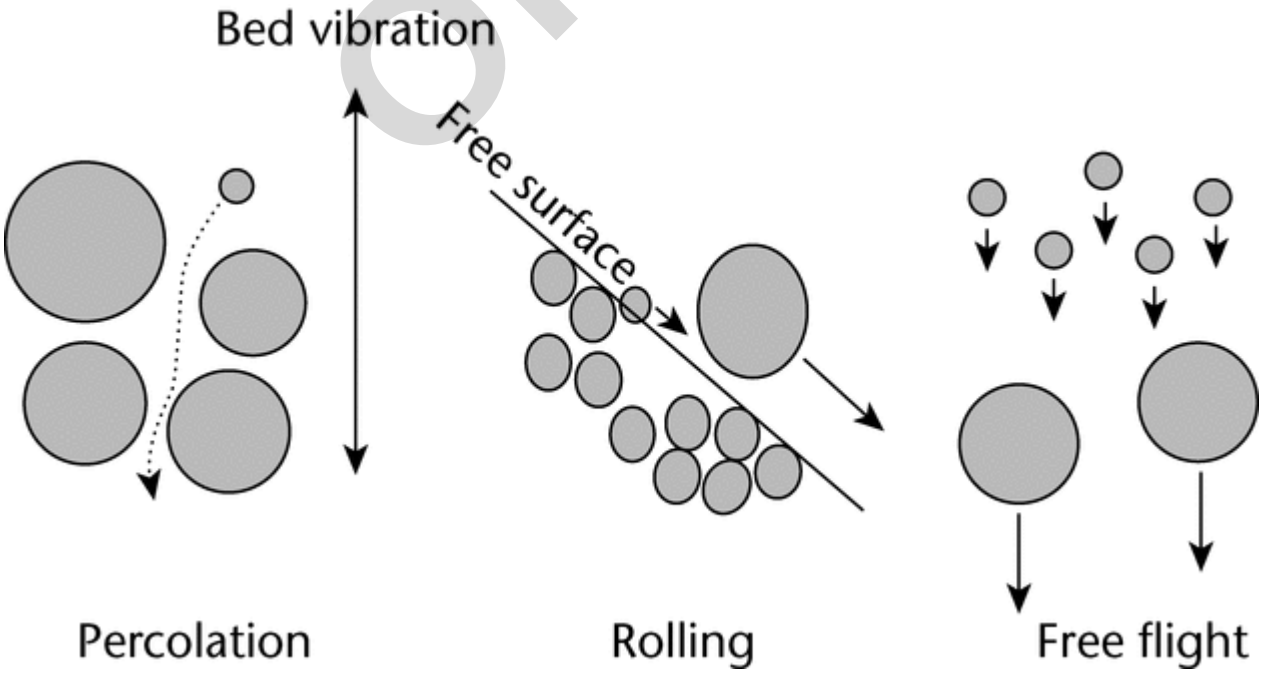


Figure 2. Illustration of the three modes of particle segregation: percolation, rolling, and free flight.

Within the powder bed, segregation can occur by means of percolation, also called sifting segregation, as well as through the movement of coarse particles to the top via vibration. During sifting segregation, smaller particles acting under the influence of gravity can more easily migrate downward into the void spaces between larger particles when the particle bed is perturbed. The net effect of these movements is that the smaller particles percolate down into the powder bed, resulting in the top of the

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powder bed having a higher proportion of larger particles. A common example of sifting segregation is unpopped corn kernels that are found at the bottom of a bag of popped popcorn.

For free surfaces, rolling segregation can occur any time that particles can roll down a free surface. In other words, segregation can occur on any non-level surface that allows the relative movement of particles. When particles roll down these free surfaces, larger particles tend to tumble farther down the surface than the smaller particles (see *Figure 3*). For example, if a conical heap or pile is formed in the middle of a hopper during loading, larger particles are more likely to roll farther down the heap, toward the outer edge of the hopper. This creates a situation in which the smaller particles tend to be in the center of the hopper, and the larger particles accumulate toward the outer wall of the hopper. The formation of these free surfaces can be a major factor in segregation.

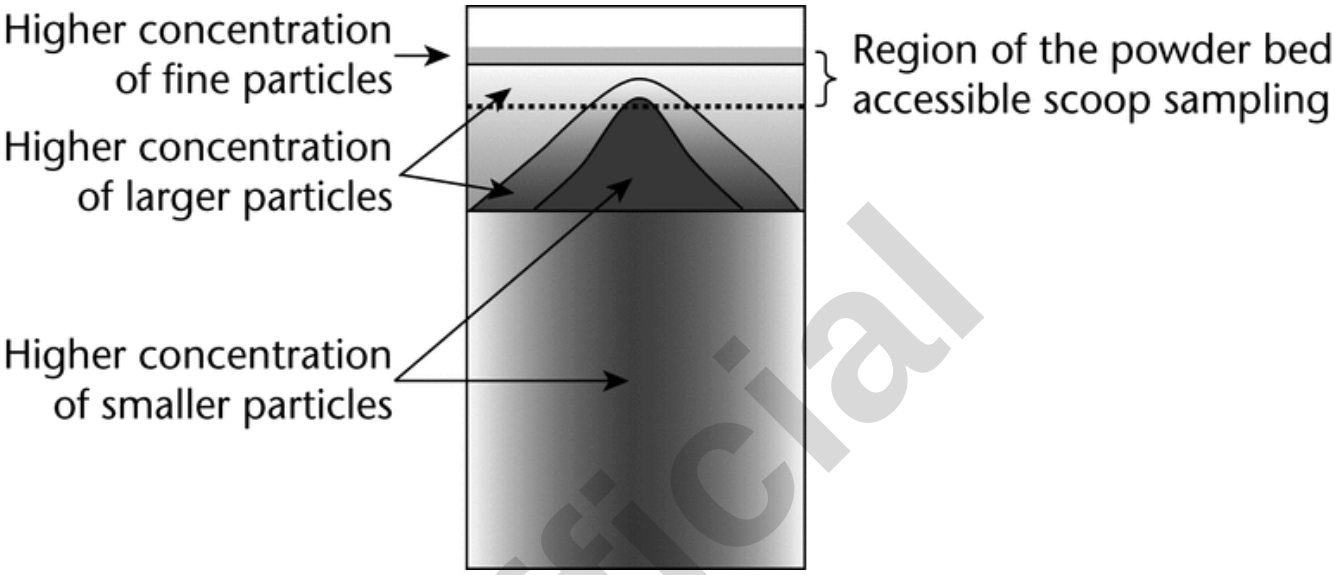


Figure 3. Example of extensive powder segregation within a drum.

When powder beds are fluidized, a large amount of air is incorporated into the powder bed and, when this air is moving, the air velocity may exceed the terminal velocity of the smaller particles. When this happens, the fine particles are suspended in the air stream while the coarse particles settle out. The fine particles eventually settle on top of the powder bed, forming a top layer that has a higher concentration of fine particles. This type of segregation, sometimes called elutriation segregation, can occur when a powder is discharged from a hopper, or is poured into the top of a hopper, and a large volume of air is displaced.

In summary, for a highly segregating system, the powder bed could have a particle distribution similar to that shown in *Figure 3*, where, as a result of elutriation segregation, a layer of fine particles on the top overlies larger particles deposited by percolation segregation, and a radial distribution of larger particles appears toward the outer wall as a result of rolling segregation.

In general, the primary factors that affect segregation are particle size and size distribution, density, and shape and shape distribution. Of secondary importance are surface roughness, surface coefficient of friction, moisture content, and container shape and design. Particle size is the most important single factor, and subtle differences in particle size can cause measurable segregation. If the attribute of interest is associated with particle size, then this attribute will segregate along with the different particle sizes. For example, if a manufacturer makes a granulation in which the larger particles contain more drug than the smaller particles, then drug content can be very prone to segregation—i.e., drug content will show segregation patterns similar to those associated with particle size segregation.

Segregation can notably increase sampling error because it decreases the probability that certain particle types will be in the sample. In addition, the powder bed may already be segregated when material is received, and poor sample handling can also cause segregation. To avoid further segregation during sample handling, the operator should avoid situations that promote segregation, such as the following: pouring where the powder forms a sloping surface, pouring into the core of a hopper, vibrations, shaking, and stirring (unless done to promote mixing). In addition, the use of mass flow hoppers reduces segregation.

Two basic strategies help promote ideal sampling: (1) use of a sampling thief and (2) sampling from a moving powder stream.

A sampling thief is a long spearlike probe that can be inserted into the powder bed and, once inserted, can collect powder samples from points adjacent to the spear. With a sampling thief, particles from almost any point in the powder bed can be included in the sample. The second method relies on fundamental principles of sampling, namely that (1) a powder should always be sampled when in motion, and (2) the whole stream of powder should be sampled for many short periods rather than sampling a part of the stream for a longer period.

For example, if the container to be sampled is emptied onto a conveyer belt, all the material will pass by a single point that can be sampled. Thus, no matter how segregated the system is, the collection of the powder at random time points ensures that every particle has an equal probability of being included in the sample. The second fundamental principle accounts for material segregation on the conveyer belt: by collecting the entire stream, one gets a cross section of all the particles, no matter how much segregation occurs on the conveyer belt.

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Many methods are available for obtaining a sample from a powder system. Unfortunately, many of these methods involve setting the powder bed in motion or performing in-process sampling. Because of concerns about cross-contamination and containment of potentially toxic materials, most of these methods are impractical for the bulk sampling required for compliance with current Good Manufacturing Practices (cGMPs). Hence, most of the sampling done in the pharmaceutical industry is static sampling, done by either (1) scoop or grab sampling or (2) stratified sampling, typically employing a sampling thief. The choice of method is dictated by the distribution of the attribute being sampled in the container, as discussed below.

## GENERAL SAMPLE COLLECTION: CONSIDERATIONS AND TOOLS

### Types of Systems and General Considerations

#### HOMOGENEOUS SYSTEMS

For powder systems where the attribute of interest is uniformly distributed throughout the container—so that any sample is an unbiased representation of the entire container, lot, or population of interest—scoop sampling is adequate. Scoop sampling is a straightforward procedure in which the operator, after selecting representative containers for sampling, opens a container, scoops out a sufficient amount of material from the top of the powder bed, and then seals the container. If a thin layer of material on top of the powder bed is suspected of being different from the bulk, samples should be taken from a point below this top layer. For example, in cases of elutriation segregation, a thin layer of fine particles may lie on top of the powder bed, and the operator should dig down into the powder bed to avoid sampling from this layer. The scoop should be large enough that no material is lost during handling, because lost material may result in sample bias. In other words, one should avoid the use of a heaping scoop from which material can roll off the sides. The advantages of scoop sampling are convenience and cost, and, for highly potent materials, low-cost disposable scoops that can be used to minimize cross-contamination.

#### HETEROGENEOUS SYSTEMS

If the attribute of interest is spatially distributed in a heterogeneous manner throughout the sample, then scoop sampling is prone to potentially significant errors. Scoop sampling is a non-probabilistic method because only the most accessible fraction of the container is sampled. Obviously, only the material in the top layer can be reached with a scoop. For example, a sample from the top outer edge of the drum shown in *Figure 3* could be biased because, in this example, the larger particles are preferentially distributed toward the top and outer edges of the drum. Hence the smaller particles have a lower probability of appearing in the sample. As a result, the smaller particles will be underrepresented in the sample, and any analysis of particle size will not reflect the true particle size distribution of the original population.

For heterogeneous systems, the initial primary sample is the most difficult to obtain. Use of a sampling thief, sometimes called a grain probe or sampling spear, is needed. The advantage of a sampling thief is that much more of the powder bed is accessible because the sampling thief can sample from different points in the powder bed, thus helping to reduce sampling bias. Many types of sampling thieves are available, including: (1) the concentric sleeve with slotted compartments, (2) the concentric sleeve with grooves, sometimes called the open-handled probe, (3) the end sampler, and (4) the core sampler. Each type has its own unique operating procedures, as described below.

The concentric sleeve with slotted compartments is probably the most popular type of sampling thief used in the pharmaceutical industry. This type consists of two concentric tubes or cylinders in which the inner tube is divided into compartments. This design makes it possible to detect differences in the attribute of interest across the depth of the container. To collect a sample, the operator closes the compartments and inserts the sampling thief into the powder bed with the collection zone openings facing upward. The handle is turned to open the sample zones, then the handle is moved up and down with two quick short strokes to help fill the compartments. The sampling thief is then closed and removed from the powder bed. The operator should visually inspect the powder bed through its depth before emptying the sampling thief. The powder from the individual compartments can be combined on a clean surface or in a collection container. In certain situations the material from each compartment may be analyzed separately, that is, without mixing.

In the concentric sleeve with grooves (open-handled probe), the inner tube is not divided into compartments. The probe is first inserted into the powder bed with the groove open, the outer sleeve is rotated to close, and the sampling thief is then withdrawn from the powder bed. The probe's contents are emptied from the handle end by holding the probe upright and letting the sample slide out from the handle, a method more convenient than the one using the thief with slotted compartments. However, this type of thief makes it more difficult to perform visual inspection to examine for material inconsistencies according to depth.

An end sampler probe, often used to sample slurries, has a single entry zone at the bottom of the sampling thief. Frequently the end sampling zone is larger than the rest of the sampling thief. This feature is a disadvantage because the larger the probe, the more it perturbs the powder bed, possibly resulting in the introduction of sampling bias.

Core samplers have a hollow outer cylinder with a tapered outer wall on the open end. This probe is inserted into the powder bed, and the intrinsic cohesion of the particles keeps them from flowing out when the probe is withdrawn. The contents of the cylinder are then emptied into a clear container.

#### GENERAL CONSIDERATIONS

The most reliable and reproducible results in powder size measurements are obtained when the particle size ranges from 2 to 10 µm; otherwise, the powder is too cohesive and does not flow properly into the sampling thief. In addition, particles larger than about one-third the width of the slot give poor results. Samples should be taken from several sites throughout the container. The probe should be long enough to penetrate at least three-quarters of the depth of the powder bed, ensuring that material from all depths can be captured in the sample. The choice of sites should be dictated by an understanding (often

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subjective) of the powder bed's degree of heterogeneity, which may have been caused by handling or movement during transport. Sampling plans can call for the insertion of the probe either at random locations and random angles or at predetermined locations and angles. For example, the plan may call for the probe to be inserted at the center and at two locations near the edges. Also, many operators recommend that probes always be inserted at a 10° angle from vertical, which increases the range of locations sampled.

Some of the disadvantages of sampling thieves include the labor-intensive nature of the procedure. The probe must be physically inserted into the powder bed, often multiple times; the contents of the probe must be emptied; and then the probe must be thoroughly cleaned. For settled powder beds, the sampling probe can be difficult to insert. In addition, the sampling probe can introduce errors as a result of the following: fine particles can lodge between the inner and outer tubes; particles can fracture; fine particles can compact and not flow well into the sampling compartments; segregation can occur during flow into the sampling zone; and the act of inserting the probe can disrupt the powder bed by dragging powder from the top layers of the bed down through the bed.

## Representative Lot Sampling

Statistically-based sampling plans are based on statistical principles and depend on the population's spatial heterogeneity and intrinsic variability. Statistically-based plans are efficient and allow the collection of a sufficient number of samples to yield the desired degree of certainty without collecting too many or too few samples for the test method, scale, product variation, risk requirements, and tolerance for a stated product's quality level or specification. The commonly used $\sqrt{N+1}$ sampling plan given in *Table 1* is not a statistically based sampling plan and may result in collection of too few samples for small populations and too many samples for large populations. The use of statistically-based sampling plans is advantageous because it facilitates risk management. However, in cases where prior knowledge of the population to be sampled is insufficient, a nonstatistical sampling plan such as that given in *Table 1* can be considered.

*Figure 4* illustrates the sample size selection scheme paths. The first choice is whether to use a statistical or nonstatistical sampling plan. Statistical plans are preferred when a variable attribute like particle size or drug content is being determined. General sampling approaches are outlined in *USP* general information chapter *Analytical Data—Interpretation and Treatment* ⟨1010⟩. Statistically-based lot acceptance sampling plans require a valid rationale with known quality levels for the determination of product lot characteristics. As noted, the application of statistical sampling plans, including lot acceptance sampling plans, requires specific and thorough knowledge of the material being sampled. Reference statistical sampling plans state the rationale for sampling as part of the sampling scheme. Manufacturers who use a statistically-based lot acceptance sampling method should refer to an appropriate standard such as ANSI/ASQ Z1.9-2003 for bulk materials or ANSI/ASQ Z1.4-2003 for multiunit or discrete populations. These standards are readily available via sources such as the American Society for Quality (http://www.asq.org/) or the American National Standards Institute (http://www.ansi.org/).

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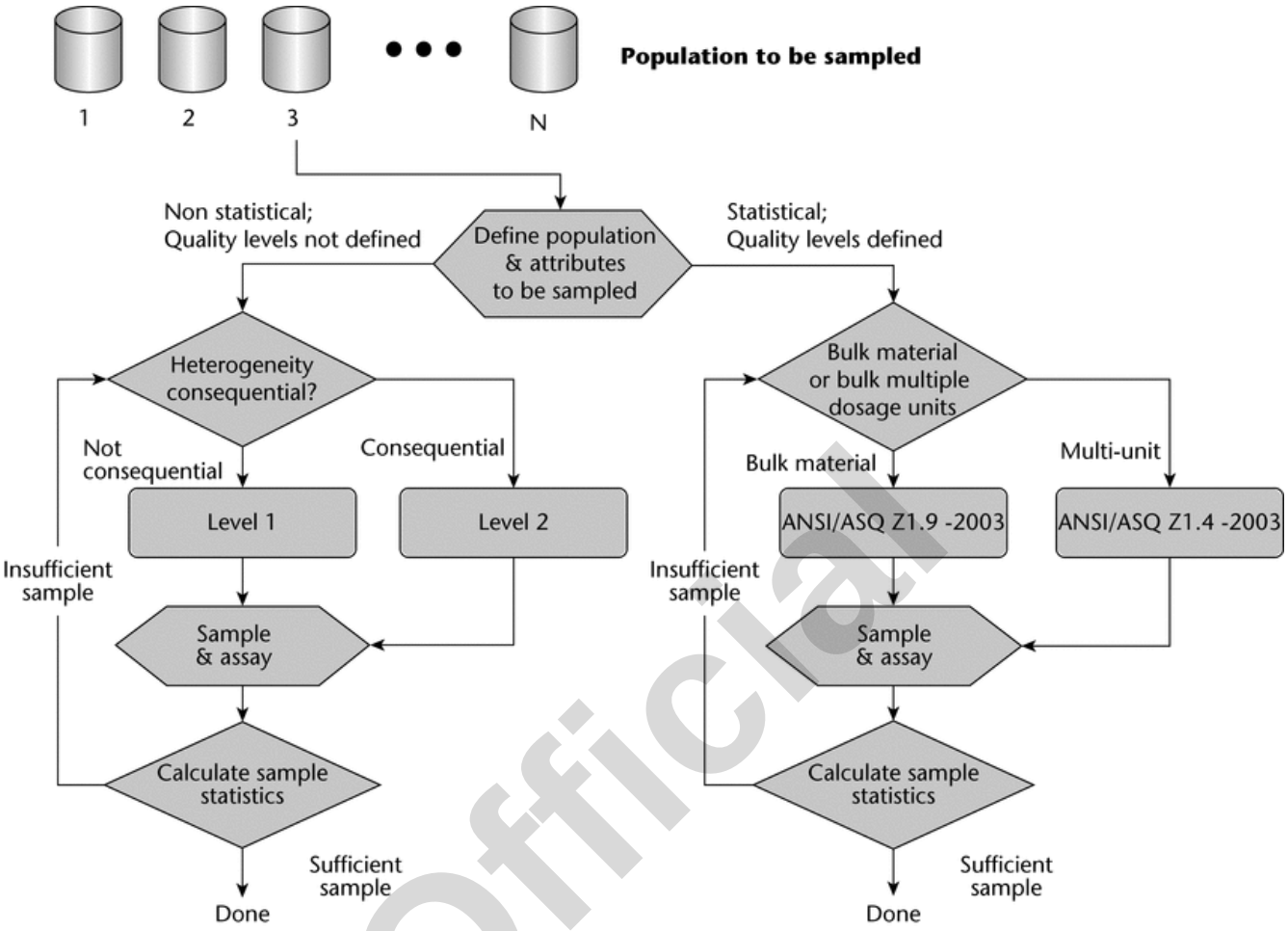


Figure 4. Sample size selection scheme.

If one is developing a nonstatistical sampling plan for which the quality level is not known, *Table 1* gives suggested sample sizes for the number of containers in the lot that should be sampled.

The *Level 1* sampling plan is relevant to materials when heterogeneity does not affect the analysis and the customer seeks to sample more than one container, when the sampling plan can be proportional to the square root of the number of containers received, and when the material comes from a known and trusted source. In such cases, the sample can be withdrawn from any point in the container. For adequately homogenous systems, scoop sampling from the top of the container is suitable.

The *Level 2* sampling plan involves a 50% increase in sample size when compared with *Level 1* and is used when a larger proportion of the number of containers is needed, for example, when a material's heterogeneity is suspected of being consequential and acceptance sampling quality levels are not defined, or when the material comes from a less trusted source. Depending on the material's degree of heterogeneity, a sampling thief may be used. However, if the degree of heterogeneity will not significantly affect the results for the attribute being sampled, then scoop sampling from the top of the drum may still be suitable.

*Table 1* shows the number of containers, n, to be sampled for a lot segregated into N containers. Note that the value of n from the formula is rounded at 0.5 up to the next higher integer. For example, if N = 6: for *Level 1*, $n = \sqrt{6} + 1 = 3.45$ rounds to n = 3; for *Level 2*, $n = 1.5 \times \sqrt{6} = 3.67$, which rounds to n = 4.

**Table 1**

| N (Number of Containers Comprising Lot) | n (Sample Size) | |
|---|---|---|
| | Level 1 | Level 2 |
| $N \leq 3$ | All | All |
| $N \geq 4$ | $\sqrt{N} + 1$ | $1.5 \times \sqrt{N}$ |

These initial decisions, as illustrated in *Figure 4*, are often difficult and sometimes must be made without sufficient information. If there is uncertainty about which method or level is appropriate, sometimes a quick, small-scale informal test of the system may help determine the best way to proceed. In addition, for some systems and attributes, the *Level 1* and *Level 2* sampling plans may result in oversampling. For example, when one is sampling for identification from the same lot, the suggested levels

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may result in collecting more samples than are statistically needed; in such cases, the statistically-based sampling plans referenced in *Figure 4* can be used.

## Sample Collection

Acquiring a representative sample from a lot of bulk powder is a difficult procedure that requires special consideration, and the basic procedures for acquiring a representative sample are discussed below. Note that every situation requires techniques that are appropriate for the given population to be sampled. The methods presented here are applicable to the sampling of static powders stored in midsize bulk containers such as 1-ton super sacks, 50-kg drums, or 50-lb bags. These methods are not necessarily applicable to the sampling of liquids, large storage containers such as train cars or silos, or in-process systems such as blenders or moving conveyer belts. In addition, the procedures described here are most applicable to particles in the size range from approximately ~1 µm to approximately ~1000 µm. Significantly smaller or larger particles require special procedures that are not covered here.

# PRIMARY SAMPLE COLLECTION

Lot acceptance samples are generally transferred or delivered in containers. To collect a representative primary or gross sample (see *Figure 1*), the appropriate container or containers must first be selected from the population of N containers; second, a representative sample must be withdrawn from each of the selected containers.

## Container Selection

To avoid bias and other sampling errors, the containers to be sampled must be randomly selected. To make a random selection, first number all containers in the lot, then use a random number table (or computer-generated random numbers) to choose from which container or containers to withdraw the samples.

For systems in which containers are grouped together in such a manner that many of the individual containers are not practically accessible (e.g., 50-lb bags stacked and bound in shrink wrap on a pallet), the sampling plan may need to take into account the larger container, in addition to the smaller container, as a sampling unit, in order to ensure a representative sample.

## Withdrawing Sample from a Container

### CONTAINER TYPES

The three most popular container types are the bag, drum, and super sack. Because bags are generally closed and not resealable, special sampling thieves, sometimes called bag triers, have been designed to puncture the bag. If the system to be sampled is heterogeneous, the samples should be obtained from the bottom, center, and top of the bag; and, depending on how the bags are stacked on the pallet, they should also be sampled from the front and the back. When sampling from bags, particular attention should be paid to the corners, because they can disproportionately trap fine particles. If no bag trier is available, use a knife to cut open the bag for sampling. When sampling from a bag, be sure to clean the external surface sufficiently that the sample is not contaminated and foreign material is not introduced into the bulk material. Once the sample has been taken, place a compatible material over the hole in the bag, then fix this patch with an appropriate adhesive tape. Depending on the heterogeneity of the drum, a scoop or a sampling thief is used. Super sacks are large sack containers that usually have a fill spout on the top and a discharge spout on the bottom. For adequately homogeneous material, scoop sampling is appropriate; but if there is any concern about the heterogeneity of the material, a thief should be used. The large size of super sacks makes the use of a thief more important for representative sampling than in the case of a drum or bag, in order to limit potential delimitation error.

## Sample Handling

The samples collected can be either assayed individually or combined; then a subset of the gross sample can be assayed, as depicted in *Figure 1* and described below. Sample increments should be combined on a clean, dry surface or in a suitable container or bag. All containers with which the sample comes into contact should be inert and should not chemically or physically react with the sample. In addition, samples should be accurately labeled and good records kept. A portion should be kept for possible future analysis.

# PRIMARY SAMPLE SIZE REDUCTION

As mentioned above, the primary sample typically consists of multiple samples taken from containers and mixed together. To obtain an analysis or measurement sample (*Figure 1*), the gross or primary sample must be reduced to a size appropriate for the analytical method. Gross or primary sample size reduction is an often overlooked aspect of a sampling plan, but it is an important step. The factors that cause segregation in a container can also cause segregation in the primary sample, and any bias in the size reduction method for the primary sample will lead to erroneous results. The advantage of secondary samples is that the mass has been reduced to a point at which it is much easer to obtain a representative sample because every element in the powder bed is readily accessible. Such accessibility makes it easier to adhere to sampling best practices. Generally speaking, sample measurement takes place under either wet or dry conditions; the choice is dictated by the requirements of

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the analytical method. For example, the Coulter counter requires that samples be uniformly suspended in an electrolyte, but other methods, like sieving, are typically performed with dry powders.

Before dividing an agglomerated sample, the agglomerates should be broken apart by a suitable technique such as sieving.

## Dry Analysis Methods

Many laboratory devices are available for the reduction of the primary sample to an analytical sample. The three most important methods used in the pharmaceutical industry are: (1) scoop sampling, (2) cone and quartering, and (3) the spinning riffler or rotary sample divider (manual method of fractional shoveling); see *Figure 5*.

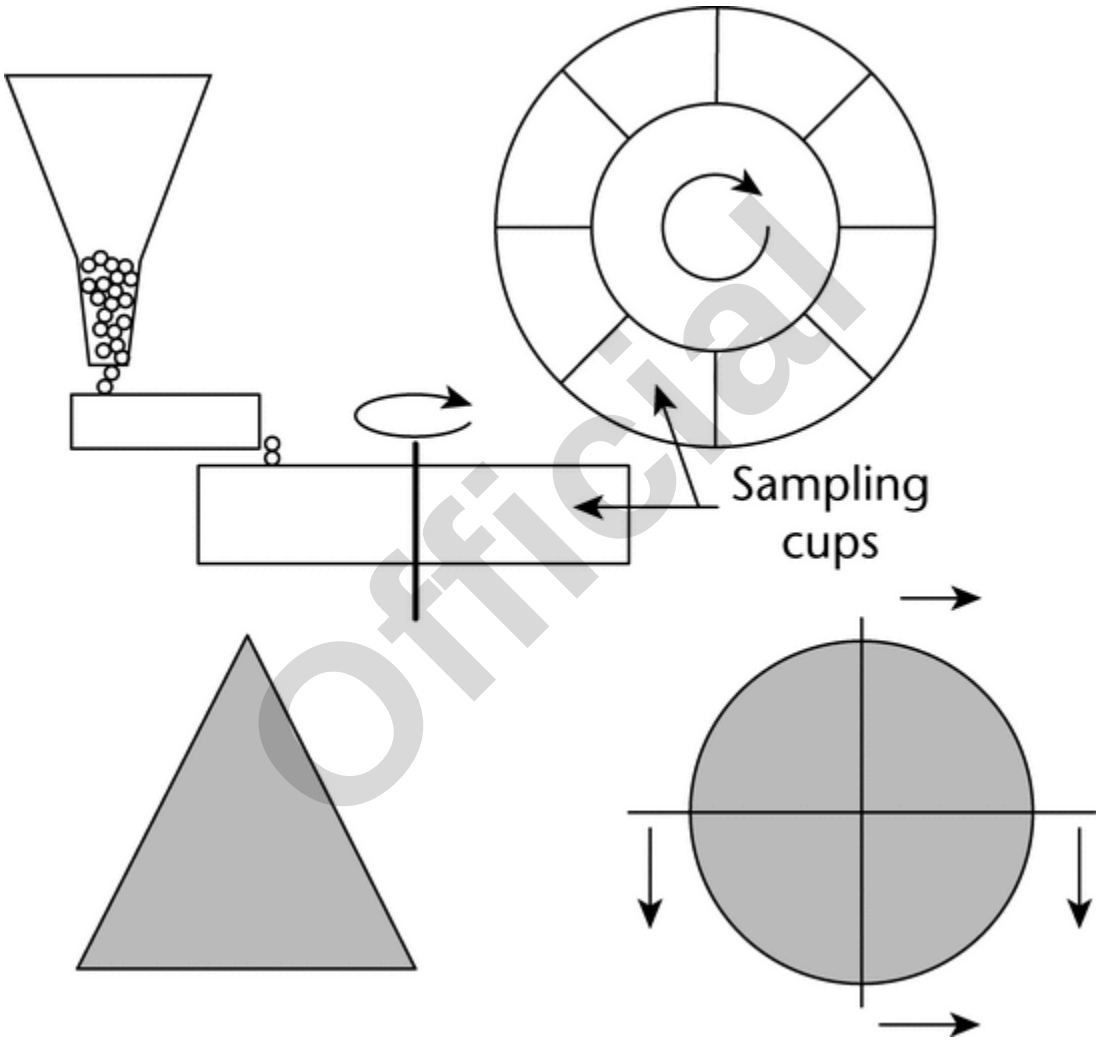


Figure 5. Two procedures for dividing samples. Top: spinning riffler, in which a circular holder rotates at a constant speed, and the sample is loaded at a constant rate into the containers via a vibratory chute, which is fed by a mass-flow hopper. Bottom: cone and quartering. (Cone, left, is flattened and quartered; quarters can be formed into cones and further subdivided.)

### SCOOP SAMPLING

Scoop sampling is done as previously described, but generally with a smaller scoop or spatula. Great care must be taken when removing material from the primary sample, because this material could be highly segregated as a result of handling. Scoop sampling is appropriate for homogeneous or cohesive powders. However, if the powder is prone to segregation, scoop sampling can introduce significant errors. Moreover, scoop sampling has several serious disadvantages. First, the method depends on the operator's deciding from which part of the primary sample to scoop the material and what quantity of the sample to extract, which are features that can introduce operator bias. Second, in scoop sampling, operators have a natural tendency to withdraw the sample from the free surface, which is highly prone to segregation and is not representative of the bulk. Third, operators need to avoid creating a heap where rolling segregation can occur, because material could fall off the edges of the spatula or scoop and bias the sample. Ideally, the operator should make some attempt to mix the primary sample before using the scoop, but this too can exacerbate segregation problems and should be done only with great caution.

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### CONE AND QUARTERING

Cone and quartering is done by pouring the primary sample into a symmetric cone on a flat surface. The cone is then flattened by a flat surface such as a spatula, and is divided into four identical quarters (*Figure 5*). One quarter is taken as the sample. This procedure can be repeated (e.g., quarter-samples can be subdivided into quarters) until the desired sample size is obtained. The theory of this method is that when a symmetric cone is created, all the segregation processes also occur symmetrically around the cone, and hence symmetry is used to mitigate the effect of segregation. In practice, it is very difficult to actually make a symmetric powder cone, and the method becomes very operator-dependent and often unreliable. Differences in how operators form the heap and subdivide it can lead to a lack of precision and significant errors. In addition, if the method is done more than once, errors can propagate each time the cone and quartering is performed. Some experts do not recommend this method.

### SPINNING RIFFLER

A spinning riffler (*Figure 5*) includes a series of containers mounted on a circular holder. The circular holder rotates at a constant speed, and the sample is loaded at a constant rate into the containers via a vibratory chute, which is fed by a mass-flow hopper. Once the material has been divided among the different holders, an individual holder can be removed for testing or further sample division. The angular velocity of the circular holders and the amplitude of the vibratory feeder can be controlled to accommodate powders with different flow properties. The holder velocity and feed rate should be adjusted so that the containers fill uniformly and so that a heap does not form on the vibratory feeder. Spinning rifflers are available in different sizes, making possible subdivisions of powders ranging from a few milligrams to hundreds of grams. The only drawbacks of the spinning riffler are the time required to process the sample and clean the device, and the capital expense. Despite these minor disadvantages, the spinning riffler is by far the best method for subdivision of free-flowing powders.

Fractional shoveling is the manual version of the spinning riffler. In this method, scoop samples are taken from the original sample and placed into a sufficient number of aliquots, and then subsequent scoops are taken from the original sample and placed into one of the aliquots in sequential order. This process is repeated until the original samples are gone. Then one of the aliquots is randomly taken as the reduced sample. As is the case with all manual methods, operator error and variability can be significant factors.

## Wet Analysis Methods

Wet analysis methods require dispersing the sample in a liquid suitable for analysis, and then withdrawing an aliquot using a syringe or pipet. Effective secondary sampling requires making a stable homogenous suspension (i.e., the sample must be stable from the time of formation of a suspension to the time when the analysis is complete). Some important factors in wet analysis are sample solubility in the dispersion vehicle, aggregation of sample, the use of suspending agents, and deaggregation of primary particles in the dispersion vehicle. Even though a uniform suspension is created, the sample should be homogenized, typically by shaking, immediately before withdrawing the sample with a syringe or pipet. The diameter of the syringe or pipet should be large enough so that particles are not excluded and clogging does not occur. The diameters of the largest particles should not exceed 40% of the syringe or pipet tip diameter. If for practical reasons the amount of material from the primary sample is too large, the sample size should be reduced before a suspension is made. To reduce the sample size, use the methods described above in the *Dry Analysis Methods* section. As a precaution, collect and retain enough sample to repeat all tests a minimum of five times.

# APPENDICES

## Appendix 1: Subsampling Examples

The examples provided below describe the importance of material particle characterization during the selection of an appropriate sample mass. Four examples are presented. In the first example, similarity in the fundamental or intrinsic material characteristics is assumed. In the second example, the density of the heavy metal analyte being measured is changed. In the third example, the effect of changing the particle size is evaluated. In the fourth example, the adequacy of the fundamental particle characteristics in a formulation needed for a given unit dose or mass is evaluated.

### EXAMPLE 1. SAMPLE MASS DETERMINATION

Assuming the lot size is 1 kg, the maximum particle diameter is 1000 µm, and the concentration of the analyte is expected to be 1%, what sample mass of round, equal-sized and -shaped 1000-µm particles with a density of 1 g/cm$^3$ would be needed to estimate the average concentration of the analyte with a percent relative standard deviation (%RSD) of 5%?

Rearranging equation 3, one can estimate the sample mass as shown in equation 5:

$$m_{sample} \approx \frac{1}{\frac{s_{fse}^2}{f_{shape}\, g_{CF}\, C_{max}\, Id_{max}^3} + \frac{1}{m_{lot}}} \quad (5)$$

The compositional maximum heterogeneity ($c_{max}$) can be estimated by considering the analyte and matrix density ($\lambda_a$ and $\lambda_m$, respectively, and their average $\lambda$) and analyte concentration ($a_L$) (equation 6):

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$$C_{max} = \frac{(1 - a_L)^2 \lambda_a \lambda_m}{a_L \bar{\lambda}} \quad (6)$$

For low analyte concentrations, the compositional maximum heterogeneity is simplified to equation 7:

$$C_{max} \approx \lambda_a / a_L \quad (7)$$

For high analyte concentrations, the compositional maximum heterogeneity is simplified to equation 8:

$$C_{max} \approx \lambda_m (1 - a_L) \quad (8)$$

The shape factor is approximated by equation 9:

$$f_{shape} \approx \text{Volume}/d^3 \quad (9)$$

Where d is the nominal particle diameter for a sphere, and the shape factor is [(4/3)π/8], or approximately 0.5.

The granulometric factor can be approximated by the typical minimum diameter noted as the 5th percentile size, divided by the typical maximum diameter noted as the 95th percentile size, as shown in equation 10:

$$g_{CF} \approx d_{5\%}/d_{95\%} \quad (10)$$

Because all particles are the same size, the granulometric factor, $g_{CF}$, is 1.0. Because the analyte exists in a state liberated from the matrix particles, the liberation factor is also 1.0. The sample mass for a 5% RSD (using equation 5) is then:

$$m_{sample} = \frac{1}{\frac{0.05^2}{0.5 \times 1 \times 100 \times 0.1^3} + \frac{1}{1000}} = 19.6 \text{ g}$$

A sample mass of 19.6 g will provide a sampling error of approximately 5% RSD. Note that in this example the particle characteristics are simplified to demonstrate that a lot mass of 1000 g contains $2 \times 10^6$ particles of 0.5 mg mass. The sample mass of 19.6 g contains approximately 39,216 particles, yielding a 5% RSD, using the binomial distribution where p is the concentration of the analyte ($a_L$) and n is the number of particles sampled, as shown in equation 11.

$$\text{Binomial RSD} = \sqrt{(1 - p)/np} = \sqrt{(1 - 0.01)/39,216 \times 0.01 = 0.0498 \approx 0.05} \quad (11)$$

(See *Table 2* for a summary of calculations.)

**Table 2. Summary of Calculations for *Example 1*, Equal-Sized and -Shaped Particles**

| $m_{Lot}$ (g) | d (cm) | $f_{shape}$ | $g_{CF}$ | $c_{max}$ | $a_L$ | $\lambda_a$(g/cm³) | l | $m_s$ (g) |
|---|---|---|---|---|---|---|---|---|
| 1000 | 0.1 | 0.5 | 1 | 100.0 | 0.01 | 1 | 1 | 19.6 |
| Mass per Particle $P_s$<br>$d^3 f_{shape} \lambda_a$ | | | Particles in 19.6 g<br>$m_s/(P_s/g_{CF})$ | | | | Binomial RSD<br>p = a(L) = 0.01<br>n = 39,216<br>(Eq. 11) | |
| 0.005 | | | 39,216 | | | | 0.05 | |

In determining the required sample mass, it is assumed that the sample is representative of the population. Moreover, when using a single representative sample, it is assumed that the uniformity of the sample mass is consistent with the remaining population. Note that the granulometric and liberation factors allow proportional adjustment of the sample size, depending on the nature of the particles. The inclusion of a liberation factor in the equation allows for particles to exist with a proportion of the analyte residing within every particle or a proportion thereof. The granulometric factor permits adjustment of the sample mass by accounting for the relationship in size between the smallest and largest particles represented in the lot.

This approximation also can be applied to liquid suspensions in which each particle is considered discrete and the sample can be characterized with respect to size, density, mass, and volume.

## EXAMPLE 2. HEAVY METAL

In this example, it is assumed that the analyte is the heavy metal lead, with a density of 11.34 g/cm³, with a limit of not more than 5 ppm, where the shapes of the particles are cubes ($f_{shape}$ = 1.0), the particles are approximately 50 µm, and a 5-g sample is taken from screened material ($g_{CF}$ = 0.55). On the basis of equation 3, the %RSD is 17.7%. Using equation 5, one finds that a sample mass of approximately 60 g is needed to achieve a 5% RSD, assuming that $a_L$ is equal to the limit allowed and that the analyte cannot be assumed to be liberated from the material (l = 1.0). (See *Table 3* for a summary of calculations.)

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**Table 3. Summary of Calculations for *Example 2*, Heavy Metal**

| $m_{Lot}$ (g) | d (cm) | $f_{shape}$ | $g_{CF}$ | $c_{max}$ | $a_L$ | $\lambda_a$ (g/cm³) | I | $m_S$ (g) |
|---|---|---|---|---|---|---|---|---|
| 1000 | 0.005 | 1.0 | 0.55 | $2.3 \times 10^{6}$ | $5 \times 10^{-6}$ | 11.34 | 1.0 | 58.71 |

If the sample were assumed to be homogeneous (I = 0.1) with respect to presence of the analyte with all particles, then a sample mass of 6.2 g would be required. Moreover, if the shape of the particles were between round and cubic ($f_{shape}$ = 0.8), then a sample mass of 5 g would be required to complete the analysis.

### EXAMPLE 3. SUBSAMPLING

Ideal sampling, as noted earlier, is fundamental to understanding the important role of subsampling. In many instances it is desirable to reduce the sample size in a manner that results in a representative sample and lessens the need to test a large sample mass. In some cases the particle size and compositional heterogeneity can result in an unwieldy sample mass. This may occur with larger-sized particles or when a composite sample of many containers is required. Samples with larger-sized particles may need to be physically reduced.

For example, using *Example 2* above, if the maximum particle size were 1000 µm or 1 mm, then a 997-g sample would be suggested by equation 3. Reducing the particle size by grinding and subsampling to achieve a predetermined sampling %RSD may require subsampling more than once to achieve the desired particle size. For example, the entire sample may be reduced to 100 µm to reduce the %RSD to approximately 3%; then, with ideal sampling, a subsample could be selected and entirely reduced to 50 µm to achieve a 5% RSD. Finally, a 5-g subsample could be correctly taken and tested. If certain particles have a large size with high concentration of the analyte, then samples should be selected to ensure that at least 1, but preferably at least 5–6, particles would be selected with 95% probability or chance of selection.

### EXAMPLE 4. MINIMUM UNIT DOSAGE MASS

A formulator would like to know the minimum mass required for a dosage form to ensure with 95% confidence a unit dosage of 1% active drug powder. The drug and the excipient have a similar round shape ($f_{shape}$ = 0.5) and a density of 0.33 g/cm³. The active drug is milled to 1 µm, but the size of excipients can be as large as 200 µm. The value for $g_{CF}$ is taken from equation 10 using the expected range of the excipient that accounts for 95% of the formulation, as 10 µm/200 µm, or $g_{CF}$ = 0.05. The quantity $c_{max}$ from equation 7 is taken as 0.33/0.01. The drug particles are completely liberated from the excipient. The batch size is 100 kg.

A minimum sample mass of approximately 3 mg is needed to ensure with 95% confidence (2 RSDs) that the average drug content is 0.9%–1.1%. The proposed dosage form has an active concentration of 100 µm/10 mg total unit mass. The unit dosage form mass is adequate, but the formulation requires that the mixing process, unit dosage production, bulk sampling device, and lab sample preparation or subsampling from bulk samples result in equal probability of selection of drug particles. Only if these conditions for mixing, production, sampling, and testing are met can it be reliably demonstrated that the unit dosage and test determination acceptance criteria of 1% (0.01 µg/mg) are met. Acceptable outcomes of such testing also indicate that the particle size, shape, and density must be controlled. For example, an increase in the sizes of particles to 500 µm results in a need for a 42-mg sample mass and dose. Assuming a cubic, as opposed to a rounded, particle increases the sample mass to 5 mg, which for a fixed dosage form mass may result in less room for the variation contributed by other characteristics, or in lesser confidence. If the acceptance criteria were changed to 0.95%–1.05%, requiring a 1% RSD, then the minimum sample mass would increase to approximately 70 mg. (See *Table 4* for a summary of calculations.)

**Table 4. Summary of Calculations for *Example 4*, Minimum Unit Dose Mass**

| $m_{Lot}$ (g) | d (cm) | $f_{shape}$ | $g_{CF}$ | $c_{max}$ | $a_L$ | $\lambda_a$ (g/cm³) | I | $m_S$ (g) |
|---|---|---|---|---|---|---|---|---|
| $10^{5}$ | 0.02 | 0.5 | 0.05 | 33 | 0.01 | 0.33 | 1.0 | 0.00264 |

## Appendix 2: Material Characterization and Sampling

Specific and thorough knowledge of the material's synthesis, composition, and usage is critical to developing a bulk material sampling plan. Material characterization is important because bulk material can exist in many forms throughout the material process flow. As illustrated in *Figure 6*, the type of sampling can vary by process step and ultimately affects the use of the material in the drug product. Appropriate material characterization considers the material process step, the type of sampling, the objective of the process step, and ultimately the drug product.

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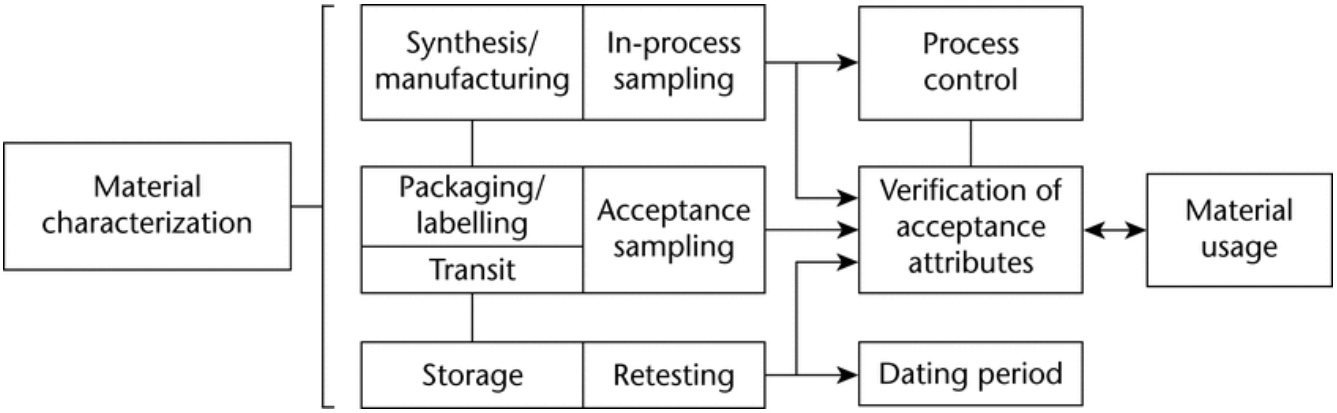


Figure 6. Material process flow.

For example, material can be synthesized or mixed in a large container where sampling may be limited or ideal for the characteristic needed. If the characteristic is important but the sampling conditions are not ideal, perhaps because of the heterogeneity of a powder mixture, then sampling for that characteristic may be more appropriately performed at a different stage upstream or downstream to assess the heterogeneity of the contents and ensure ideal sampling. This is sometimes performed to reduce the sampling dimension. The sampling dimension is reduced when the 3 dimensional bulk container space is sampled in a 1 or 2 dimensional stream sampled over time. Reducing the spatial sampling dimension may result in conditions that will allow for more accurate measurement of the heterogeneity of material while limiting sampling error through ideal sampling.

Acceptance attributes (see *Table 5*) depend on material characterization and process. Acceptance attributes may be applicable throughout the life of the bulk material. Both the number and size of samples require an understanding of the material's variation.

**Table 5. Examples of Acceptance Attributes**

| Acceptance Attributes | | | |
|---|---|---|---|
| **Physical** | **Chemical** | **Microbiological** | **Packaging** |
| Particle size<br>Viscosity<br>Density | Purity<br>pH<br>Identity<br>Strength | Sterility<br>Pyrogens<br>Microbial load | Label accuracy<br>Integrity |

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## Appendix 3: Additional Sources of Information

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# ▲⟨1099⟩ LIMIT ON NUMBER OF LARGE DEVIATIONS WHEN ASSESSING CONTENT UNIFORMITY IN LARGE SAMPLES

## INTRODUCTION

The uncertainty around the application of the zero tolerance criterion (ZTC) to sample sizes larger than 30 may inhibit the collection of uniformity data from large samples. This chapter provides a process for limiting the number of observed results that fall outside of the ZTC ($c_2$), as described in *Uniformity of Dosage Units* ⟨905⟩, when samples larger than 30 are collected. It should be noted that the criterion described in this chapter is not intended as a batch release test, nor as a replacement of or alternative to ⟨905⟩. It also is not intended as an extension to go beyond second tier testing in ⟨905⟩. The use is solely to help judging if a large data set is consistent with the ZTC element of ⟨905⟩; whether the large data set complies with the complete set of requirements in ⟨905⟩ must be decided by other means.

The ZTC in ⟨905⟩ states that no individual content of any dosage unit can be less than $[1 - (0.01)(L2)]M$ nor more than $[1 + (0.01)(L2)]M$, where $L2$ is 25.0% unless otherwise specified in the applicable monograph, and $M$, the "reference value", depends on the sample mean, $\overline{X}$ (expressed as a percentage of the label claim), as follows:

$M$ = 98.5% if $\overline{X}$ <98.5, $M$ = 101.5% if $\overline{X}$ >101.5, and $M = \overline{X}$ otherwise, with a sample size ($N$) of 30

## PROCEDURE

When a sample that includes the contents of more than 30 units has been collected, the following procedure can be used to confirm that the results in that sample are consistent with the ZTC of ⟨905⟩. The criterion is applicable both when the content is determined directly by assaying a number of units and when the content is determined indirectly by weighing the units in the situations allowing this as described in ⟨905⟩. The procedure is as follows:

1. Express individual results $x_1$, $x_2$, ..., $x_N$ as a percentage of the label claim
2. Calculate the mean ($\overline{X}$) of the contents of the $N$ units in the sample
3. Calculate the reference value $M$: $M$ = 98.5% if $\overline{X}$ <98.5, $M$ = 101.5% if $\overline{X}$ >101.5, and $M = \overline{X}$ otherwise
4. Determine $S_N$, the number of sample results less than $[1 - (0.01)(L2)]M$ or more than $[1 + (0.01)(L2)]M$, where $L2$ = 25.0% unless otherwise specified in the applicable monograph
5. The sample is consistent with the ZTC of ⟨905⟩ if $S_N \leq c_2$, where $c_2$ depends on the sample size as detailed in *Table 1*

**Table 1. Limit on Number of Observed Results Falling Outside of the ZTC Based on Sample Size**

| $N$ | $c_2$ |
|---|---|
| 31–100 | 0 |
| 101–181 | 1 |
| 182–265 | 2 |
| 266–353 | 3 |
| 354–442 | 4 |
| 443–533 | 5 |
| 534–624 | 6 |
| 625–717 | 7 |
| 718–810 | 8 |
| 811–903 | 9 |
| 904–998 | 10 |
| 999–1092 | 11 |
| 1093–1187 | 12 |
| 1188–1283 | 13 |
| 1284–1379 | 14 |
| 1380–1475 | 15 |
| 1476–1571 | 16 |
| 1572–1667 | 17 |
| 1668–1764 | 18 |
| 1765–1861 | 19 |

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Values of $c_2$ for other sample sizes ($N$) are determined as

$$c_2 = \max\left\{c: \sum_{i=0}^{c} \binom{N}{i} f'(1-f)^{N-i} \leq 0.75\right\},$$

where $f = 1 - 0.75^{1/30} = 0.00954357$. The $c_2$ value can be calculated in a spreadsheet with a cumulative binomial function.▲ (Postponed on 1-Mar-2019)

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# ⟨1102⟩ IMMUNOLOGICAL TEST METHODS—GENERAL CONSIDERATIONS

***Change to read:***

## INTRODUCTION

This general information chapter provides a high-level description of principles for immunological test methods (ITMs) that can be used in specified monograph tests, along with information and approaches to analytical development and validation for ITMs. The scope of this chapter is to provide general information that is applicable to all ITMs. The chapter provides a foundation for specific chapters about different types of ITMs, e.g., *Immunological Test Methods—Enzyme-Linked Immunosorbent Assay (ELISA)* ⟨1103⟩, *Immunological Test Methods—Immunoblot Analysis* ⟨1104⟩▲▲ (USP 1-May-2021), and *Immunological Test Methods—Surface Plasmon Resonance* ⟨1105⟩. This suite of general information chapters is related to the bioassay general information chapters. Use of ITMs for process monitoring, diagnosis, and evaluation of clinical response, assessment of pharmacokinetics/pharmacodynamics/absorption, distribution, metabolism, and excretion (PK/PD/ADME), and other product characterization (nonrelease testing) is outside the scope of this chapter.

The basis of all ITMs used to measure a quality attribute of a biologic drug substance or drug product is the highly specific noncovalent binding interaction between an antibody and antigen. The antigen typically is an analyte of interest (e.g., protein, carbohydrate, virus, or cell), and the binder is usually an antibody (e.g., monoclonal antibody or polyclonal antiserum). ITMs are applicable to molecules that are either directly antigenic (immunogens) or can be rendered indirectly antigenic (haptens). The measurand in ITM is directly related to a quality attribute of the product under test.

ITMs are valuable because they exhibit high sensitivity and specificity for an analyte in complex matrices. They typically are used for qualitative and quantitative assessment of both an antibody and antigen, but their application also extends to the measurement of hapten, complement, antigen–antibody complexes, and other protein–protein interactions. These properties of ITMs allow their use for assessing identity, potency (strength), purity, impurities, stability, and other quality attributes of biological drug substances and drug products.

ITMs are useful for many applications because they can measure molecules over a wide range of sizes and binding types. In general, antibodies are stable during various chemical modifications that do not have a significant adverse influence on interactions with an antigen. Antibody molecules tend to withstand moderate acidic and alkaline pH changes better than other proteins do. Because of this characteristic, a variety of ITMs with high degrees of sensitivity and specificity are possible. The ability to accelerate contact between an antigen and antibody enables ITM formats that provide rapid or real-time results.

Generally, ITMs have higher precision and shorter turnaround time than do traditional biologically-based (i.e., cell-based and animal) assays. Although in some cases these advantages can support the replacement of a biological assay with an immunoassay, such changes should be approached systematically and with caution. Often it is challenging to prove the equivalence, or comparability, of results from bioassays and immunoassays because the interaction between antigen and antibody may not reflect the functional attributes observed in bioassays.

One major limitation of ITMs compared to physicochemical methods (such as liquid or gas chromatography) is that the latter generally are more precise and can simultaneously identify a set of impurities or unexpected substance(s). Another major limitation is that generally ITMs operate at high molar dilutions at which they are sensitive to disturbances caused by environmental factors in the sample matrix (i.e., matrix effects). Matrix effects can depend on ITM format and are not fully understood. Their specificity, a hallmark of ITMs, is sometimes compromised by structural or sequence similarities between the analyte and a closely related molecular impurity (cross-reactivity).

Most ITMs reflect physical interaction (binding) between an antigen and antibody and not the analyte's functional properties. Therefore, analysts must pay attention in the selection and execution of ITM format. Cell-based ITMs that can provide functional information about the analyte are beyond the scope of this chapter.

## GENERAL CHARACTERISTICS OF ITMS

ITMs are based on the principle of specific, noncovalent, and reversible interactions between an antigen and antibody. In general, the primary antigen–antibody reaction is brought about by complementarity, which creates macromolecular specificity. This noncovalent interaction determines the degree of intrinsic affinity. Intrinsic affinity contributes to functional and/or relative affinity that depends on factors like reaction phase and valency, which in turn determines the degree of reversibility of an interaction. A better understanding of factors that affect antigen–antibody interactions provides the rationale for the development of a suitable ITM format (e.g., solid or liquid phase, competitive or noncompetitive binding, etc.).

A defining characteristic of ITMs is that they employ an antigen (or hapten) and antibody. In addition, ITMs may contain companion molecules such as complement components. The components of ITMs are defined as follows:

- **Antigens**—Comprise a wide range of molecules that are capable of binding to the antibody in a specific interaction. Generally, part(s) of an antigen [the immunogenic epitope(s)] is/are capable of eliciting antibody response.
- **Haptens**—Small molecules that, by themselves, are not capable of eliciting an antibody response but are capable of eliciting an immune response when attached to a large carrier such as a protein. Antibodies produced to a hapten–carrier adduct also may bind to the small-molecule hapten in a specific interaction.
- **Complements**—Companion molecules that, under certain conditions, aid in the functionality of antigen–antibody complexes but are not required for antigen–antibody or hapten–antibody interaction.
- **Antibodies**—Proteins with regions that impart a high degree of specific binding to antigens (and haptens). The structural elements of an immunoglobulin G (IgG) antibody are shown in *Figure 1*.

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In addition to these components, ITMs require some means to detect or monitor the binding reaction between the antigen and antibody.

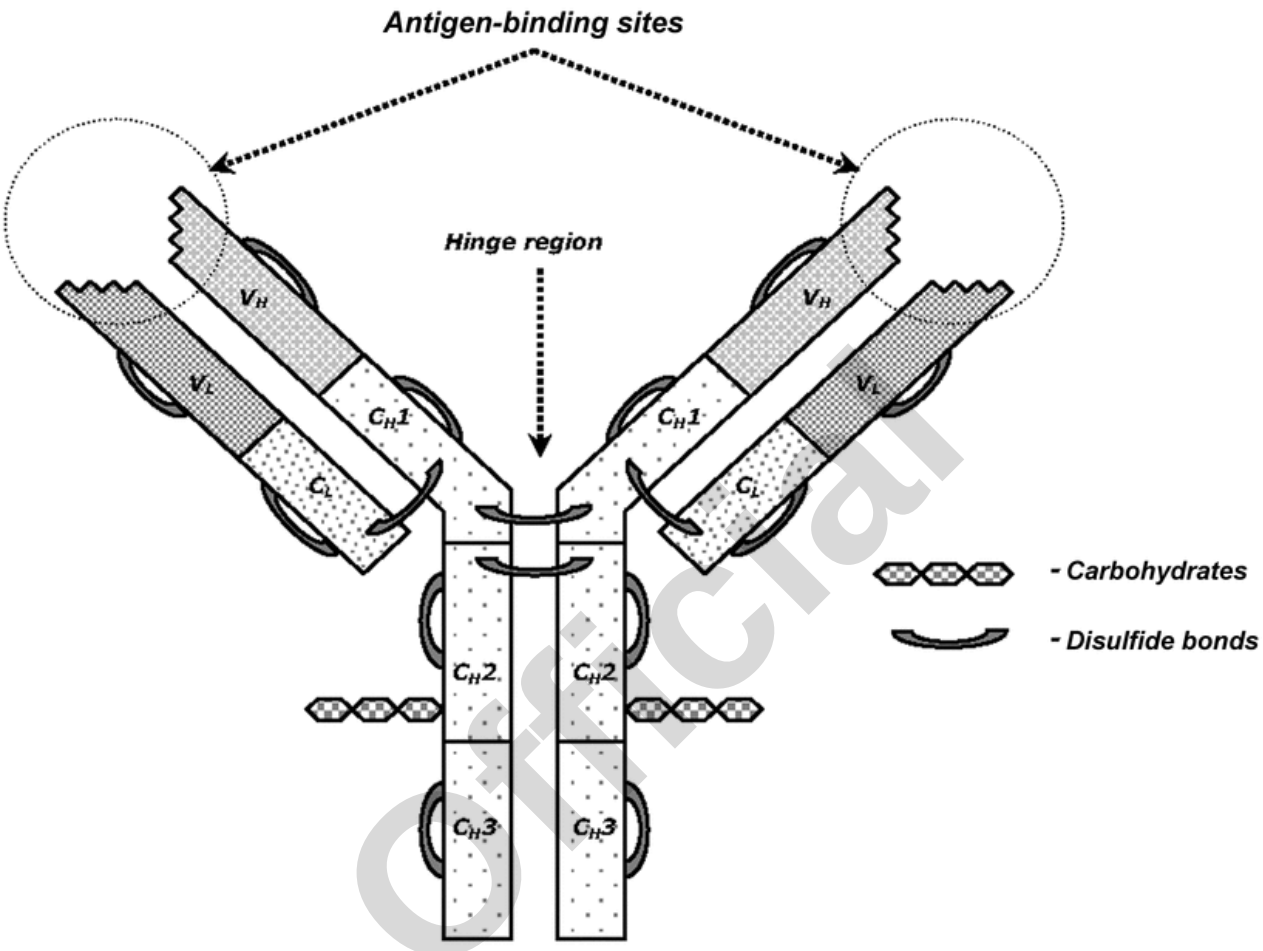


Figure 1. The structure of IgG. The IgG molecule is characterized by a distinctive domain structure of heavy (*H*) and light (*L*) chains, both of which are divided into variable and constant regions (*V* and *C*, respectively). Light chains consist of $V_L$ and $C_L$ domains, and heavy chains consist of a variable domain ($V_H$) and three constant domains ($C_H1$, $C_H2$, and $C_H3$). All domains are stabilized by disulfide bonds, and $C_H2$ domains contain carbohydrates. The flexible hinge region between the $C_H1$ and $C_H2$ domains allows the independent behavior of two antigen-binding sites formed by variable domains.

## TYPES OF ITMS

Measurement of antigen–antibody binding can be performed in a variety of assay types and formats: solid or liquid phase, manual or automated, labeled or nonlabeled, competitive or noncompetitive, qualitative or quantitative, homogeneous or heterogeneous, or combinations of some of these. The distinguishing characteristic of all these assays is the binding of an antibody or antigen to the analyte (which can be an antigen or antibody as well), followed by detection of the antigen–antibody complex. Although many different formats can be used for the binding reaction, along with different methods for detection, quantification of the analyte in the test article is always performed by comparison of the measurement to a reference standard. Thus a number of ITM technologies support investigations of product quality. Commonly used assay designs include enzyme-linked immunosorbent assay (ELISA), Western blotting, flow cytometry, competitive enzyme-linked immunosorbent assay, surface plasmon resonance (SPR), rate nephelometry, radioimmunoassay (RIA), radial immunodiffusion, precipitation, and agglutination. These methods are described below.

### Enzyme-Linked Immunosorbent Assay

An ELISA is a quantitative, solid-phase immunological method for the measurement of an analyte following binding to an immunosorbent and its subsequent detection using enzymatic hydrolysis of a reporter substrate either directly (analyte has enzymatic properties) or indirectly (e.g., horseradish peroxidase– or alkaline phosphatase–linked antibody subsequently bound

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to the immunosorbed analyte). The analyte usually is quantitated by interpolation against a standard curve of a reference material. Chapter ⟨1103⟩ discusses ELISA in greater detail, including ELISA development for quantitative analysis.

## Western Blotting

A Western blot is a semiquantitative or qualitative method for the measurement of a protein analyte that has been resolved by polyacrylamide gel electrophoresis and subsequently transferred to a solid membrane (e.g., nitrocellulose, nylon, or polyvinylidene difluoride). Detection can be achieved directly by reacting with a labeled primary antibody (antibody specific to the analyte of interest) or indirectly by reacting labeled secondary antibody (antibody against the primary antibody) to the primary antibody bound to the membrane-immobilized antigen. The label can be a radioisotope or an enzyme that uses the substrate to produce color, fluorescence, or luminescence. This method is semiquantitative, especially when proteins are present in low concentration and in very complex mixtures. It is commonly used in early process development (e.g., antibody screening, protein expression, protein purification, etc.). Western blotting is a powerful method for analyzing and identifying proteins in complex mixtures, particularly after separation using 2-dimensional gel electrophoresis, which separates proteins based on size and charge (pI).

## Flow Cytometry

Flow cytometry is a laser-based semiquantitative technology that permits the measurement of fluorophore-conjugated probes as they interact with their respective ligands on cells or particles. More details for flow cytometry can be found in *Flow Cytometry* ⟨1027⟩.

## Surface Plasmon Resonance

SPR is a quantitative method for measurement of an analyte in a sample where the antibody–antigen complex formation can be measured in real time at the interface of a liquid and solid (e.g., gold surfaces or particles). The measurement taken is the real-time change in refraction of a polarized light and occurs during the formation of the antibody–antigen complex, resulting in changes to the plasmon resonance minima (i.e., the sensorgram). The quantity of analyte is determined by comparison to the measurement of a reference standard curve determined in the same assay. More details for SPR can be found in ⟨1105⟩.

## Rate Nephelometry

Rate nephelometry is a quantitative method for measurement of an analyte in a sample in solution by measuring the light scatter introduced by small aggregates formed by the antigen–antibody complex. The quantity of analyte is determined by comparison to the measurement of a reference standard curve determined in the same assay.

## Radioimmunoassay

RIA, a sensitive ITM first developed in the 1950s, is a quantitative method for measurement of an analyte in a sample. RIA usually uses a competitive antibody–antigen binding reaction, but it also can be used in sandwich immunoassay format, including immunoprecipitation. In competitive RIAs the analyte competes for binding with a radiolabeled (e.g., using $^{125}I$ or $^{3}H$) reference antigen that is identical to the analyte; therefore, the analyte and the antigen both compete for binding to a fixed and limiting dilution of a specific (often polyclonal) antibody. The radiolabeled antigen is present in excess. The same unlabeled antigen in the test sample competes in binding to the same site on the antibody, which is present in a fixed quantity. Binding of the unlabeled antigen to the antibody leads to the displacement of the labeled antigen, resulting in a decrease in the radioactivity of the antigen–antibody complex fraction. To separate the antigen–antibody complex from the excess unbound antigen, the complex generally is either precipitated with a secondary antibody (or Protein G) immobilized on a solid matrix (e.g., glass or resin beads) or with an already immobilized primary antibody. The quantity of analyte usually is determined by interpolation against a standard curve of a reference material, where a fixed amount of antibody and radiolabeled antigen is mixed with an increasing amount of unlabeled antigen. Hence, even a small quantity of unlabeled antigen will result in a relative quantitative decrease in total bound radioactivity.

## Single Radial Immunodiffusion

Single radial immunodiffusion (SRID or SRD) is a quantitative method for measurement of an analyte in a sample by measuring the diameter of the ring of precipitin formed by the antigen–antibody complex. Antigen is applied to a well in a gel infused with a constant level of antibody. Solutions with higher concentrations of antigen diffuse farther before being saturated with antibody and then precipitated. The quantity of analyte is determined by comparison to a reference standard curve measured by the same assay.

## Precipitation

The underlying principle for this method is that the interaction of a multivalent antibody and antigen leads to the formation of a complex. In some cases a visible precipitate is formed. Other immunoprecipitation techniques involve the use of Protein A or Protein G beads to capture the antigen–antibody complex and facilitate the separation of the antigen–antibody complexes

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from the other antigens in the solution. Precipitation is not commonly used for quantitative analytical purposes because of the time required (days to complete), lack of sensitivity, and requirement for large quantities of antigen and antibodies.

### Agglutination

Agglutination and inhibition of agglutination, respectively, provide qualitative and quantitative measures of certain antigens and antibodies. Inhibition of agglutination is a modification of the agglutination reaction that provides higher sensitivity to detect small quantities of proteins, chemicals, viruses, and other analytes. The principle of agglutination is similar to that for precipitation except that the interaction takes place between an antibody and a particulate antigen and leads to a visible clump or agglutination. The most common example of this application is for blood typing (i.e., A, B, or O antigen).

## CHOICE OF ITM

When choosing an ITM, analysts should consider sensitivity and specificity as well as the complexity of the sample. *Table 1* provides an assay developer with a comparative view of the advantages and disadvantages of a variety of ITM formats. The intended application of the ITM should govern the choice of the most suitable format.

**Table 1. ITMs Used in Biopharmaceutical Laboratories**

| Method | Advantages | Disadvantages | Typical Industry Uses |
|---|---|---|---|
| ELISA | • High sensitivity<br>• Often wide dynamic range<br>• High throughput<br>• Low cost | • Multistage process highly dependent on proper execution of each stage<br>• Wash steps add time and often biohazardous waste<br>• Reagent labeling required | • Potency assessment<br>• Specific protein concentration analysis in complex samples<br>• Protein identification<br>• Purity assessment<br>• Immunogenicity assessment |
| Western blot | • Gives information about antigen size and/or charge<br>• Allows separation of various antigens (or degradation/aggregation products) bearing same epitope<br>• Can tolerate complex mixtures | • Typically works only with linear epitopes<br>• Labor intensive<br>• Low throughput, output<br>• Subject to interpretation<br>• Immobilization can alter binding<br>• Limited to proteins | • Protein purity assessment<br>• Protein stability assessment<br>• Protein identity test |
| Flow cytometry | • High throughput<br>• Highly automated | • Use limited to cells, particles, and samples bound to beads<br>• Sensitive to aggregates and sample matrix | • Potency assessment<br>• Cell identity in cell-therapy products |
| SPR | • Direct detection of binding<br>• Can measure affinity precisely, including on and off rates | • Immobilization can alter binding<br>• Regeneration can alter binding<br>• Low throughput, output | • Immunogenicity assessment<br>• Potency assessment<br>• Specific protein concentration analysis in complex samples |
| Rate nephelometry | • Easily automated<br>• Rapid | • Small detection range<br>• High background for turbid samples | • Assay for individual vaccine components for check of stability and purity |
| RIA | • Binding occurs in native conformation<br>• Low-concentration samples can be analyzed<br>• High sensitivity antibody used at limiting dilution that conserves reagent<br>• Can be plate-based for higher throughput (e.g., scintillation proximity assays) | • Requires radioactive labeling for detection<br>• Shorter half-life of some radioisotopes requires periodic preparation of the tracer<br>• Hazardous waste | • Protein identification (e.g., hormones)<br>• Specific protein concentration analysis in complex samples |
| SRD | • Precise<br>• Simple setup | • Semiquantitative<br>• Low precision<br>• Low sensitivity | • Vaccine release test |
| Precipitation | • Low equipment cost | • Subject to interpretation<br>• Slow<br>• Poor sensitivity (µg range) | • Vaccine identification |
| Agglutination | • Rapid<br>• Low equipment cost | • Subject to interpretation<br>• Slow<br>• Low specificity because of interfering substances | • Vaccine identification |

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# KEY CONSIDERATIONS IN ITM DEVELOPMENT

The goal during method development is to produce an accurate assay that is practically feasible and possesses an acceptable degree of intra- and inter-assay precision. To minimize the overall imprecision, the sources of variability should be identified and minimized.

## Reagent Selection

Immunoassays are subject to several sources of interference such as cross-reactivity, endogenous interfering substances, buffer matrices, sample components, exposed versus masked epitopes, conformation changes in the antigen of interest, and other factors. Hence, during method development, analysts must identify possible sources of interference both to develop a robust method and to aid future troubleshooting.

Cross-reactivity is a major obstacle during immunoassay development. This arises when the specificity of an antigen–antibody reaction is compromised by the cross-reactivity binding of structurally similar molecules with the reaction binder. Some common examples are protein isoforms, degraded analyte entities, molecules of the same class, precursor proteins, metabolites, etc. Cross-reactivity can be minimized by rigorous reagent characterization and selection.

Reagents used in ITM applications generally fall into one of two categories: critical reagents and noncritical reagents. Critical reagents are specific and unique to the particular ITM or reagents that are intolerant of very small changes in composition or stability. Examples of critical reagents generally include assay-specific antibodies and reference or method calibration standards. Equivalence in the assay format must be established before replacement with a new lot. Noncritical reagents are those that can vary to some degree in composition without adversely affecting ITM performance. Reagents are often assumed to be noncritical (e.g., buffers, water quality, blocking buffer, or substrate) but later may be identified as critical components if assay ruggedness fails and troubleshooting of ITM reagents begins. ITM-specific reagents, including vendor and catalog number, should be defined in test procedure documents.

Antibody selection is critical for development of a successful immunoassay because it defines the assay's specificity and sensitivity. Furthermore, during antibody generation, analysts should ensure that the immunization protocols support the end use of the antibodies. For some applications a more specific antibody can be generated by the selection of a small and specific immunogen and affinity purification of the antibody, resulting in highly defined epitope coverage. In other applications it may be critical to ensure broad coverage of the different available epitopes on the molecules of interest, and a polyclonal antibody (pAb) pool may be the best choice. Currently, monoclonal antibodies (mAb) are preferred for some applications for the detection of single analytes because of their high specificity, lot-to-lot consistency, and indefinite supply. Compared to polyclonal antibodies, mAb have a higher initial cost to produce, but for these applications, the advantages generally outweigh the initial cost. Other applications may require more comprehensive epitope selection to ensure that subtle changes in the molecule(s) do not prevent recognition of the entire antigen, and thus a pool of monoclonal antibodies, or a pAb pool, would be the preferred choice. The latter are widely used for detection in a complex mixture of analytes (e.g., host-cell proteins). ▲However, the characteristics of a polyclonal antiserum are not solely determined by the immunogen. Different animals of the same species will not necessarily produce identical antisera to the same immunogen and they may even vary during the immunization protocol. This warrants performing qualification of each lot of reagent which should not be assumed to transfer between antisera produced in the same way.▲ (USP 1-May-2021) Similarly, immunoassays may use two distinct epitopes on an antigen—one for capture and the other for detection—which greatly reduces cross-reactivity. Another approach to minimize cross-reactivity is to purify the antigen before immunoanalysis. Variations in incubation temperature and time can affect the reaction kinetics of antibody interactions with similar yet different antigens. Thus this property should be optimized to increase the specificity of antigen–antibody interactions.

## Development of Immunoassays

Development is an important stage in the establishment of a suitable ITM. During development of an ITM, analysts explore various settings of assay parameters and interactions between parameters to identify conditions under which the assay will consistently produce reliable results using minimal reagents, effort, and time. In Quality by Design terminology, the "possible operating space" is the collection of settings of assay parameters explored, and the "design space" refers to the conditions under which the assay performs well. The necessary performance properties of the ITM (precision, accuracy, specificity, etc.) required depend on the intended use(s). During ITM development, analysts should consider the following:

- Antigen–antibody ratio;
- In sandwich immunoassays, the ratio of capture antibody to detector antibody;
- Antigen–antibody reaction kinetics in the sample matrix (antigen–antibody binding generally is not linear);
- Selection of the standard (full-length antigen for the standard or just a small portion of the antigen containing the antibody-binding epitope, among other considerations); and
- Matrix effects.

The use of design of experiments (DOE) is strongly recommended, and different DOE methods may be appropriate in each stage of development. Early in development, screening designs are particularly useful (generally two-level geometric fractional factorial designs). After screening (with a modest number of factors to study), full factorials or response surface designs are often appropriate. As development activities shift to qualification (ideally, if not typically, as the focus shifts to robustness), robust response surface designs often are a good choice. During qualification or validation, analysts may find it practical to simultaneously study robustness to assay operating conditions (using a small geometric fractional factorial) and validation

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parameters such as precision (via nested or crossed designs for random factors associated with repeatability, intermediate precision, and reproducibility).

Experiments that assess dilutional linearity and components of specificity, including matrix effects, usually involve construction of spiked samples. Although spiking often is performed in a dilution matrix, spiking a collection of actual samples or mixing actual samples is an important component of demonstrating robustness of dilutional linearity and components of specificity to the sample and matrix components.

▲Quantitative ITMs generate test sample data with an estimated concentration based on a calibration curve fit to reference (or standard) samples using an appropriate mathematical model. For data reporting, depending on the units of the reference standard, nature of the measured analyte, the degree of measurement standardization, the geographic region, and the history of the method, analysts may express concentration in terms of weight per volume, mole per volume, or weight of analyte per weight of product.

ITMs employ calibration curves prepared with reference standards of known (nominal) concentrations which helps control variation associated with repeatability, intermediate precision, and reproducibility and permits the estimation of results for unknown test samples. Common simple statistical analyses assume that the (possibly transformed) data are normally distributed, have constant variance, are independent, and that an appropriate model has been used. Analysts should assess these assumptions using a substantial body of data (typically tens of assays).▲ (USP 1-May-2021)

## Reagent Considerations

A procedure for qualifying reagent sources and vendors (including audits), ordering, receiving, and disposing of commercial reagents and consumables should be outlined in a standard operating procedure (SOP). The preparation of internal reagents must be documented in a manner that allows reconstruction. Commercial and internally prepared reagents must be labeled with identity, concentration, lot number, expiration, and storage conditions. The stability and assignment of expiration dates for internally prepared reagents often are based on available literature and scientific experience, but analysts may need to confirm these empirically. An SOP for extending expiration dating of critical reagents is recommended. In addition, analysts should implement a mechanism for reagent tracking and linking lot numbers to analytical run numbers. Unacceptable reagent performance is detected by tracking quality control (QC) samples. Shifts in QC samples should prompt a review of analytical runs and changes in reagent lot numbers or review of possible deterioration of critical reagents. To avoid such shifts, analysts can cross-validate critical reagent lot changes.

The impact of collection and storage containers on analytical performance often is overlooked. When defining the stability and expiration of in-house reagents, analysts should record information about the storage container vendor, catalog, and lot number. The importance of a suitable reference standard and its characterization cannot be overemphasized for ITMs for biological products. Because of their inherent complexity, reference and calibration standards of macromolecular biologics often are less well characterized than are conventional small-molecule drug reference standards. If the calibration standard represents a mixture of different antigens (e.g., host-cell proteins), it should be shown to be representative of the antigen profile in the samples being tested. Consistency in ITM results depends on the availability of a suitable representative reference standard material.

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# VALIDATION

Analytical validation involves the systematic execution of a defined protocol and prespecified analysis that includes prespecified acceptance criteria. A validation demonstrates that an analytical method is suitable for one or more intended uses [see *Validation of Compendial Procedures* ⟨1225⟩, *Biological Assay Validation* ⟨1033⟩, and International Council for Harmonisation (ICH) Q2(R1)]. Qualification may involve similar or identical experiments and procedures as validation, but qualification does not require prespecified protocols, analyses, or acceptance criteria. In certain situations (e.g., use of a commercial kit), assay development may not be required before qualification. Chapter ⟨1225⟩ discusses which assay performance characteristics must be examined during validation for four primary categories of intended uses. For example, analytical procedures that quantitate major bulk drug substances or active ingredients may not require validation of the detection and quantitation limits but do require validation of accuracy, precision, specificity, linearity, and range.

## System Suitability or Assay Acceptance Criteria

The purpose of system suitability or assay acceptance criteria is to ensure that the complete system—including the instrumentation, software, reagents, and analyst—is qualified to perform the intended action for the intended purpose.

▲System suitability criteria should be selected based on method type and purpose. Identity methods should include positive and negative control samples. Methods intended for the measurement of sample activity should include appropriate criteria regarding performance of standards as well as response levels of blanks. If possible, a control of known activity can be included to help demonstrate suitable assay performance. Samples should be replicated and criteria for precision should be included in suitability criteria. Procedures for repeat sample analysis and identification and treatment of outliers, when applicable, should be included [see the FDA *Guidance for Industry: Analytical Procedures and Methods Validation for Drugs and Biologics* (2015)].▲ (USP 1-May-2021)

All processes should be controlled by well-defined SOPs that ensure consistency, reduce errors, and promote reproducibility of laboratory processes. Training files for all personnel should be contemporaneous and should include some demonstration that analysts are qualified to perform the method and the specific ITM.

Instrument and software qualification begins with a definition of the design qualifications, including a risk assessment and gap analysis that identify potential threats to the collection, integrity, and permanent capture of ITM data. Qualification also

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includes installation qualifications (IQ) and operational qualifications (OQ)▲ [see *Analytical Instrument Qualification* ⟨1058⟩ and *Good Documentation Guidelines* ⟨1029⟩].

## Data▲ (USP 1-May-2021) Tracking

Regulatory agencies have strict requirements about maintaining the identity and integrity of both samples and data. A quality process driven by SOPs must be implemented to ensure the correct identity and integrity of test and reserve samples. Ideally, a bar code system should be used to track the collection, identity, location, chain of custody, number of sample freeze/thaw cycles, storage temperature, and length of time that a sample is stored. This information should be captured and should be auditable from the time of collection to disposal (or sample depletion). ▲▲ (USP 1-May-2021)

Quality systems must be in place to minimize data errors ▲which might occur during the generation of data reports from the bioanalytical laboratories.▲ (USP 1-May-2021) Errors may also be introduced by test sample misplacement or identification, incorrect data reduction, miscalculations, transcription errors, omissions, and other factors. Ideally, validated software and laboratory information management systems are used when possible to generate, transfer, and archive data ▲[see the FDA *Guidance for Industry: Data Integrity and Compliance with cGMP* (2016)].▲ (USP 1-May-2021)

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# ⟨1103⟩ IMMUNOLOGICAL TEST METHODS—ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

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## INTRODUCTION

Immunological test methods (ITMs) utilize bindings between an antigen (Ag) and antibody (Ab). (See *Appendix 1: Abbreviations* for a complete list of acronyms used in this chapter.) Enzyme-linked immunosorbent assay (ELISA) is one of the most widely used ITMs for characterization, release, and stability testing of biotechnology products to help ensure the quality of biological drug substances and drug products. The term "ELISA" is used here in a broader sense and includes enzyme immunoassays (EIAs), as well as alternative detection methods, e.g., chemiluminescence and fluorescence.

This chapter provides analysts with general information about principles, procedures, experimental configurations, assay development, and validation for solid-phase ITMs like ELISA and can be used for the other immunoassay variations mentioned above. The chapter also covers reference standards and controls used for immunoassays. The information can be adapted to the specific procedures of a monograph. This chapter does not cover immunoassays for the measurement of immune responses to product in animals or humans (e.g., serological or cellular assays), non-immunoassays (e.g., receptor-ligand interactions), or other related approaches.

The chapter is part of a group of general information chapters for ITMs (*Immunological Test Methods—General Considerations* ⟨1102⟩, *Immunological Test Methods—Immunoblot Analysis* ⟨1104⟩, and *Immunological Test Methods—Surface Plasmon Resonance* ⟨1105⟩), and also is related to the general information chapters for bioassays (*Design and Development of Biological Assays* ⟨1032⟩, *Biological Assay Validation* ⟨1033⟩, and *Analysis of Biological Assays* ⟨1034⟩).

### Definition

ELISA can be defined as a qualitative or quantitative solid-phase immunological method to measure an analyte following its binding to an immunosorbent surface and its subsequent detection by the use of enzymatic hydrolysis of a reporter substrate, either directly (as with an analyte that has enzymatic properties or is directly labeled with an enzyme) or indirectly (by means of an enzyme-linked antibody that binds to the immunosorbed analyte). Qualitative results provide a simple positive or negative result for a sample. Converting quantitative to qualitative results based on a cutoff value that separates positive and negative results is common practice. Because the performance properties of the assay depend heavily on the cutoff value, the process used to determine the cutoff should be evidence-based and well documented. Quantitative assays determine the quantity of the analyte based on the interpolation of a standard calibration curve with known analyte concentration, run simultaneously in the same assay. This standard should be an appropriate, preferably homologous, reference or calibration material that is representative of the analyte(s) of interest. The power of immunoassays has been demonstrated by the variety of procedures that have evolved, including alternative solid surfaces such as beads of different sorts, various plastics in plates of different configurations, and alternative detection methods, e.g., chemiluminescence and fluorescence. ELISA assays are widely used in the biopharmaceutical industry for various applications such as identity, purity, potency, detection or quantitation of antibody or antigen, and other purposes.

### Basic Principles

The essential steps of an ELISA can be broken down as follows (see *Figure 1*):

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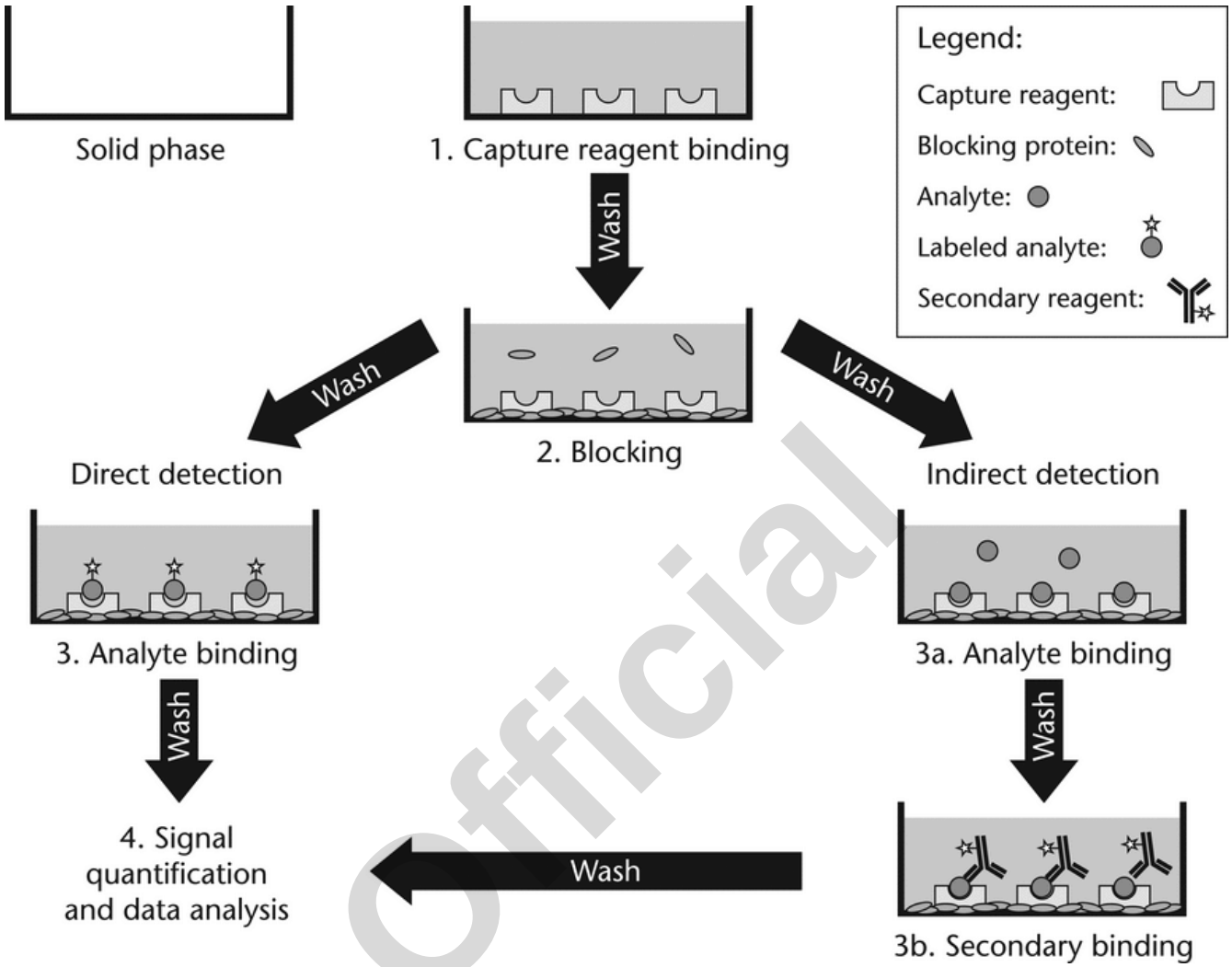


Figure 1. Essential steps for performing an ELISA.[1]

1. Binding of the capture reagent (generally an antibody or antigen), which functions as an immunosorbent for capture of the analyte, to a solid surface;

2. Removal of excess, unbound capture reagent followed by blocking of unoccupied binding sites with a blocking protein such as albumin, gelatin, casein, or other suitable material;

▲3 or▲ (USP 1-Dec-2019) 3a. Incubation of the analyte (in the test sample or reference standard) with the capture reagent to bind the analyte onto the solid surface, followed by the washing away of unbound material in the test sample and detection of the analyte.

▲3.▲ (USP 1-Dec-2019) Direct detection occurs when the analyte has enzymatic activity or has been linked to a detector molecule (e.g., enzyme); or

3b. Incubation of the analyte (in the test sample or reference standard) with the capture reagent to bind the analyte onto the solid surface, followed by the washing away of unbound material in the test sample and subsequent detection of the analyte (*Figure 1*, step 3a). Indirect detection occurs when the analyte is detected by the addition of a secondary enzyme-labeled reagent (*Figure 1*, step 3b); and

4. Quantification of the analyte by addition of a substrate suitable for the detector used [e.g., 3,3′, 5,5′-tetramethylbenzidine (TMB)], followed by comparison of the test sample to the reference standard.

[1] Capture reagent binding, blocking, analyte binding, detector antibody binding, and analysis are the five basic steps in an ELISA. Capture reagent binding, blocking, and analyte binding steps are each followed by a washing step to remove unbound reagents before the addition of the next reagent. Before analysis an appropriate substrate is added, followed by measurement of the substrate by appropriate equipment for detection. Quantitation of unknowns takes place by comparison with a standard curve.

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## ASSAY DESIGN

▲Four▲ (USP 1-Dec-2019) general categories of ELISA are described in *Table 1* and in the sections that follow. The assay designs are flexible and, depending on specific needs, can be modified from these procedures. The choice of format depends primarily on the amounts and purity of reagents and equipment available. On some occasions the analyte being characterized actually is an antibody, as in the case of a monoclonal antibody that is being developed as a drug. In this case, anti-idiotypic or other antibodies specific for the antibody are used to develop the assays.

**Table 1. Representative ELISA Types**

| ELISA Type | Required Reagents | Attributes | Disadvantages |
|---|---|---|---|
| Direct detection | • Capture analyte[a]<br>• Labeled primary antibody specific for antigen | • Rapid because only one antibody is used<br>• Uses less reagent<br>• Analyte is immobilized | • May modify the conformation of the analyte<br>• Sensitive to matrix and adjuvant components<br>• Not commonly used<br>• Poor sensitivity |
| Indirect detection | • Capture analyte[a]<br>• Primary antibody specific for antigen<br>• Labeled secondary detector antibody that binds to the primary antibody | • Versatile because a variety of primary antibodies can be used with the same secondary detector<br>• Improved sensitivity because of signal amplification<br>• Analyte is immobilized | • Longer because of more incubation and washing steps |
| Competitive | • Analyte[a] can be used as a capture reagent or can be labeled with a detection label<br>• Antibody specific for analyte can be used for capture or labeled for detection<br>• Labeled secondary antibodies to bind to primary antibody if an indirect format is used | • Good for assessing antigenic cross-reactivity<br>• Appropriate for smaller proteins with single epitopes<br>• Requires only a single antibody<br>• Analyte in solution competes for binding to primary antibody | • Format difficult to troubleshoot<br>• Limited dynamic range |
| Sandwich | • Primary capture antibody specific for analyte<br>• Sample solution containing analyte[a]<br>• A different primary enzyme-antibody conjugate specific for analyte | • Improved sensitivity<br>• Good for quantitative assays for larger multi-epitope molecules<br>• Analyte measured in solution | • Requires relatively large amounts of pure or semipure specific antibody<br>• Not suited for smaller proteins that may have only a single epitope or a few closely spaced epitopes |

[a] This reagent can be either purified or partially purified. The terms “analyte” and “antigen” are used interchangeably when describing ELISAs.

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### Direct ELISA

#### DIRECTLY LABELED ANTIBODY

In this assay an antigen is coated onto a solid surface and the remaining unbound reactive sites are blocked [*Figure 2* (A)]. Then a solution containing a specific antibody labeled with a detector is added. After incubation, the unbound antibody is washed away, followed by the addition of an appropriate substrate for the detector used.

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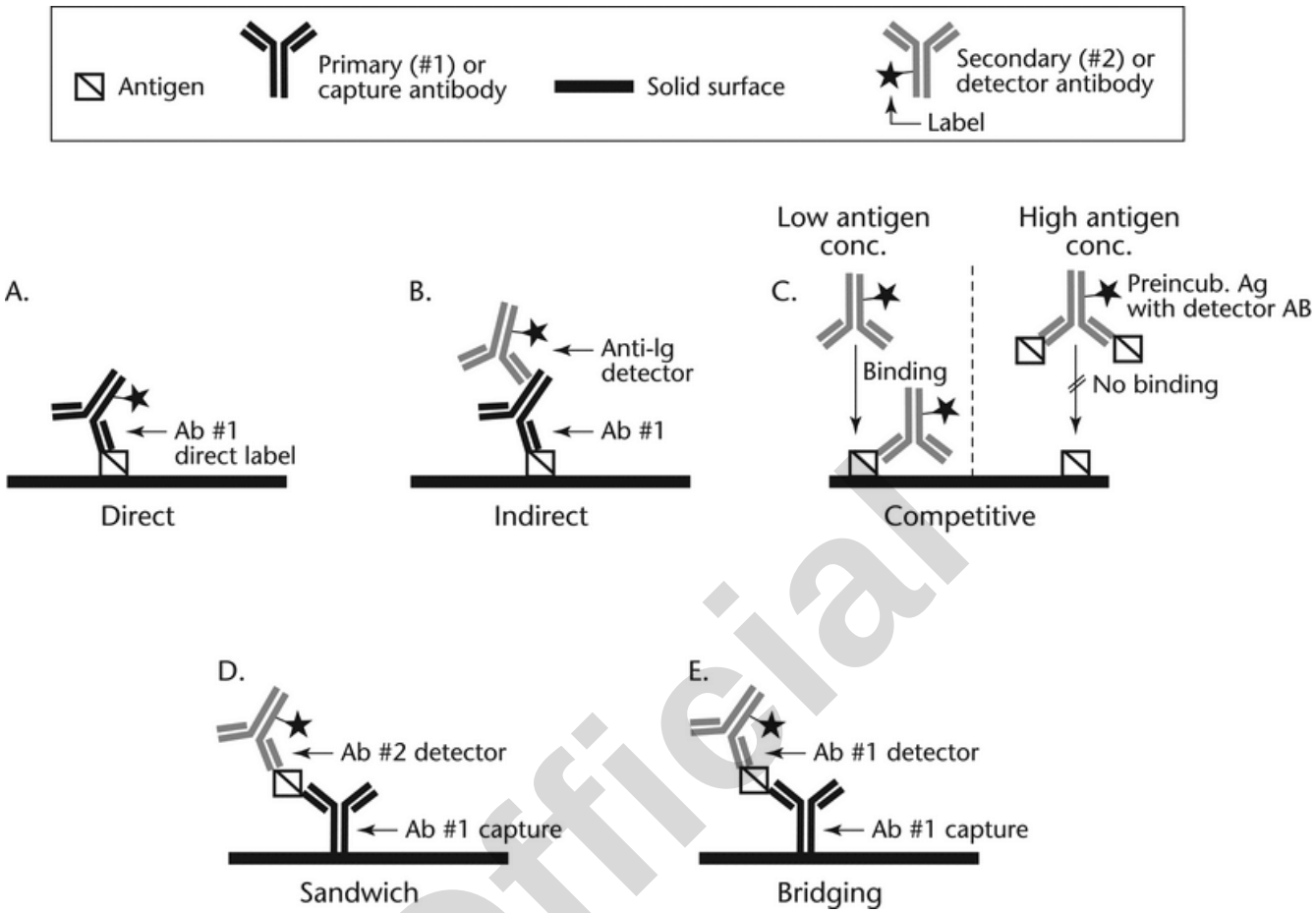


Figure 2. Schematic representations of direct, indirect, competitive, sandwich, and bridging ELISAs.[2] [Ab = antibody; Anti-Ig = anti-immunoglobulin; Ag = antigen (or analyte); Conc = concentration; Preincub = preincubation]

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#### DIRECTLY LABELED ANTIGEN

This assay is similar to that using a directly labeled antibody, except that the antibody is coated onto the solid surface and a labeled antigen is used as the detector.

## Indirect ELISA

In this assay an antigen is coated onto a solid surface and then, after blocking, a solution containing a specific antibody is added [*Figure 2* (B)]. After incubation, the unbound antibody is washed away, followed by the addition of an anti-immunoglobulin (anti-Ig) detector antibody. Anti-Ig detectors are available commercially for specific Ig classes and subclasses from a variety of species, which makes this assay format useful for isotyping of antibodies. In addition, the use of a labeled anti-Ig detector amplifies the signal compared to a *Direct ELISA*, thereby increasing assay sensitivity.

## Competitive ELISA

#### DIRECT ANTIBODY COMPETITIVE ELISA

This assay is used to detect or quantitate soluble antigens [*Figure 2* (C)]. It requires an antigen-specific antibody that has been conjugated to an appropriate detector, e.g., horseradish peroxidase (HRP), alkaline phosphatase (AP), ruthenium, or fluorescein. It also requires a purified or partially purified antigen for coating. The antigen is coated onto a solid surface, followed by a blocking step. The antibody–conjugate is incubated with the test solution containing soluble antigen. The mixture is then added to the immobilized antigen, incubated, and unbound antigen–antibody complex is washed away. Substrate is added, and the inhibition of the reaction (e.g., colorimetric, electrochemiluminescence, fluorescence, or chemiluminescence) is

[2] The type of ELISA format depends on the availability of reagents, the intended purpose of the assay, and the physicochemical characteristics of the analyte of interest. For a *Bridging ELISA*, the capture and detector antibodies recognize the same epitope, and therefore the target antigen must have at least two epitopes available for binding.

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measured relative to the reaction when no competitor antigen is added. The amount of inhibition is inversely proportional to the amount of antigen in the test sample. Competitive assays can also measure small molecules by coating an antibody to the plate that is specific to the small molecule. The small molecule is often biotinylated with a long linker that does not interfere with binding between the capture antibody on the plate and the small molecule. Antigen (the small molecule) in the sample then competes with the labeled small molecule for binding to the capture antibody. After washing, a detection reagent (e.g., streptavidin labeled with HRP) is added to detect the binding complex.

#### DIRECT ANTIGEN COMPETITIVE ELISA

This assay is similar to the *Direct Antibody Competitive ELISA*, except that it is used to detect soluble antibodies. The antigen is conjugated to the detector and the antibody is coated onto the solid surface.

#### INDIRECT ANTIBODY COMPETITIVE ELISA

This assay is similar to the *Direct Antibody Competitive ELISA*, except that instead of directly labeling the antibody, the test uses a labeled anti-Ig reagent for detection.

#### INDIRECT ANTIGEN COMPETITIVE ELISA

This assay is similar to the *Direct Antigen Competitive ELISA*, except that instead of directly labeling the antigen, the test uses a labeled secondary antibody for detection.

### Sandwich ELISA

#### DIRECT SANDWICH ELISA

In this assay an antibody is immobilized onto a solid surface and blocked, and then a solution containing a specific antigen is added [*Figure 2* (D)]. After an incubation step, the unbound material is washed away, and a labeled detector antibody is added. This assay format requires two antibodies, each of which binds to different epitopes on the surface of the large and complex molecule. The two antibodies are specific for the antigen, and the antigen should be sufficiently large and complex to accommodate the binding of two antibodies.

#### INDIRECT SANDWICH ELISA

Alternatively, instead of directly labeling the detector antibody, an anti-Ig antibody detector can be used. Indirect sandwich immunoassay formats can be considered only if each binding reagent is from a unique species (e.g., a sandwich assay using two mouse monoclonal antibodies for capture and detection could not be detected indirectly because the resulting signal may become independent of the antigen concentration).

#### BRIDGING ELISA

This subset of *Sandwich ELISA* assays often uses a single antibody for both capture and detection [*Figure 2* (E)]. If a monoclonal antibody is used, it requires that the target antigen have at least two identical epitopes that are adequately spaced to prevent steric hindrance so that one epitope binds to the capture antibody and the other epitope binds to the detector antibody. Alternatively, a polyclonal antibody can be used but still requires that the target antigen be large enough to accommodate the binding of two antibody molecules. With respect to specificity and sensitivity, bridging assays usually are suitable for most large molecules.

## CHOICE OF ASSAY

Deciding which ELISA procedure or format to use often depends on individual choice and availability of reagents, instruments, and other equipment. For example, sometimes a laboratory repeatedly engineers a particular epitope into multiple fusion proteins. In this case, the laboratory can use certain common qualified reagents (e.g., an antibody to a glutathione S-transferase region in multiple fusion proteins), facilitating rapid sandwich immunoassay development. Small antigens with a limited number of epitopes available for antibody binding restrict ELISA format choices. If there is only one binding epitope, then ELISA methods that use the sandwich/two-site binding or other bridging formats cannot be used because they require at least two available epitopes for antibody binding. In addition, small molecules are not usually used as a capture reagent on a plate because the process may interfere with binding to the detection reagent. Examples of such small molecules are some peptides, oligosaccharides, nucleotides, and antibacterials. Analysts usually adopt a competitive assay format for such small analytes.

Different assays and formats may demonstrate different properties and characteristics, e.g., specificity, precision, accuracy, sensitivity, dynamic range, dose-response ratio, sample throughput, sensitivity to interference, and simplicity or efficiency for automation. Ease of validation also may vary between different assay protocols and formats. Assay designs with replicates in adjacent wells could be biased if there are location effects; hence, in this case, replicates should not be in adjacent wells. Assay designs that are convenient to perform on 96-well plates, using relatively few single-channel pipet actions and more multi-channel pipet actions, are usually easier to adapt to automation. Assays with steep dose-response curves are generally better able to deliver high precision estimates; however, some assays with steep dose-response curves are imprecise in the $EC_{50}$ and require a wider dose range.

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# PROCEDURES

## Solid Phase

Solid phases are available in a variety of forms (e.g., membrane, plate, or bead) and chemistries [e.g., nylon, nitrocellulose, polyvinylidene fluoride (PVDF), polyvinyl, polystyrene, or a chemically derivatized surface]. The selection of the solid phase determines the most likely binding mechanism, i.e., hydrophobic, hydrophilic, or covalent interactions. In general, compared to plates, beads offer higher capacity and are more commonly used in clinical assays whereas plates are more commonly used to test biotechnology products. Additional information on plates is provided below.

### COATING THE SOLID PHASE—IMMOBILIZATION OF CAPTURE REAGENT

Capture reagents are coated onto a solid phase by adding a solution containing the capture reagent to the surface. The most commonly used solid-phase materials for capture reagent immobilization are plastic 96-well microtiter plates. Those with flat-bottom wells are recommended for spectrophotometric readings, and round-bottom well plates are useful for visual assessment of a dye's color development. The degree of coating is influenced by the concentration of capture reagent, temperature during coating, duration of capture reagent adsorption, the surface properties of the solid-phase material, and the nature of the buffer of the capture reagent solution. Although the optimum coating concentration must be determined for each capture reagent, concentrations of 1–10 µg/mL are most commonly used. The volume of capture reagent added to each well usually corresponds to the sample volume that will be analyzed, i.e., 50–100 µL. Coating duration, temperature, and buffers are discussed separately below. During the coating procedure analysts should avoid introducing bubbles. Proteins that bind to plastic can be denatured, which alters antigenicity. In such cases, a capture antibody or an intermediary protein such as Protein A or Protein G can be used. In addition, streptavidin can be used if the reagent is biotinylated. The pH of the coating buffer should be optimized based on the isoelectric point of the capture reagent and the surface properties of the assay plate chosen.

### MICROTITER PLATES

The composition and commercial source of the microtiter plate can influence binding of the capture reagent during coating. Several microtiter plates from different suppliers should be compared using a single coating procedure to select those that provide high specificity for the capture reagent of interest and low nonspecific background. Comparisons of different grades of plates from a single supplier also may be needed. Clear plates typically are used for colorimetric ELISA, and opaque plates often are used for chemiluminescent and fluorometric ELISA. Acidic capture reagents may require a lower pH solution to neutralize repulsive forces between the protein and solid phase. Peptides often require optimization of buffer pH based on their charge for optimal coating conditions during assay development. Polysaccharides, lipopolysaccharides, or glycoproteins may be difficult to coat directly to the plate and may require a capture antibody or a buffer that contains lysine or glutaraldehyde. Coating with an antibody can be enhanced by precoating the microtiter plate with Protein A or Protein G or a combination of the two, which allows binding to the Fc region so that the Fab portion can bind to the analyte of interest. However, care must be taken to ensure that subsequent secondary antibodies do not react with the Protein A- or Protein G-coated wells. In this case, for example, chicken IgY or another appropriate antibody class could be used. Microtiter plate formats other than the 96-well variety, such as half-volume 96-well or 384-well plates, can be used to increase throughput and/or conserve reagents.

### COATING TIME

Coating time depends on binding kinetics, stability, concentration of capture reagent, and incubation temperature. Although different combinations of coating times and temperatures often result in the same coating efficiency, the stability of the capture reagent (which should be determined during method development) influences which conditions to select. Analysts must assess the impact of varying the coating time in order to determine the robustness of the assay procedure.

### COATING TEMPERATURE

Coating temperature and time are closely related assay parameters. The coating temperature depends on the binding kinetics and stability of the antigen. Higher temperatures can increase the rate of adsorption and may shorten the coating time, but they are likely to affect interaction sites and to reduce antigen-antibody affinity. Typical combinations of time and temperature are 1–4 h at ambient temperature, 15 min to 2 h at 37°, or overnight at 4°. Analysts should determine the effects of variations in temperature in order to assess the robustness of the assay procedure.

### BUFFERS

Buffers used for diluents, coating, blocking, and washing plates can affect overall assay performance. Buffer components can interact with the test sample and inhibit binding. They also can cause low antigen sensitivity or high nonspecific background activity.

**Diluent:** Buffers [e.g., phosphate-buffered saline (PBS) or imidazole-buffered saline] with polysorbate 20 (0.01%–0.1%) are used commonly for different ELISA steps as a diluent and washing buffer.

**Coating buffers:** Coating buffers should maximize assay consistency and promote binding of the capture reagent to the solid phase. Commonly used coating buffers include 50 mM carbonate, pH 9.6; 20 mM tris-hydrochloride (tris-HCl), pH 8.5; and 10 mM PBS, pH 7.2. The choice of coating buffer depends on the nature of the individual antigens and should be determined empirically.

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**Blocking agents and buffers:** A blocking agent is a compound (e.g., protein or detergent) that should saturate the remaining immunosorbent binding sites following capture reagent (antibody or antigen) binding. This reduces nonspecific binding of analyte and nonanalyte components to the immunosorbent matrix and/or the absorbed reagent. Nonspecific binding occurs when protein in the test sample binds to the plastic of the microtiter plate or absorbed reagent instead of specifically binding to the capture reagent of interest. Nonspecific binding can be reduced by adding blocking reagent to the wells and by the addition of another protein such as bovine serum albumin (BSA) to the dilution buffer. The choice of blocking agent should be governed by the nature of the capture reagent, plate, coating buffer, test sample diluent, and related factors. If any of these parameters changes, a change in blocking agent may be needed. Commonly used blocking agents include BSA, nonfat milk, gelatin, casein, normal horse serum, fetal bovine serum, polysorbate 20, and others. Several grades of BSA are available commercially, and the optimal grade should be empirically determined for each assay. In addition, many commercial blocking and assay diluent reagents are available for ITM.

## Adding Samples and Reagents

Samples and reagents generally are pipetted into the ELISA plate wells. Care should be taken to avoid cross-contamination, frothing, or bubbles. ▲A sample loading pattern should be included in the test method procedure. For reproducibility and accuracy of results, consistency between the wells of the ELISA plate is very important. This can be achieved by using replicates; however, as mentioned above, care should be taken to avoid replicates in adjacent wells. A common way of avoiding the edge effect is not to use the edge wells at all. Additionally, plate edge effects can also be avoided by reducing assay time, using a low evaporation lid, or sealing the plate with a clear or breathable sterile tape.▲ (USP 1-Dec-2019) Labor-saving equipment such as electronic pipets, automated liquid handlers, plate washers, and robotic pipets also can be used to improve precision, reduce analyst-to-analyst variability, and increase throughput.

### PIPETS

Single, multi-channel, and robotic pipets with set or fixed volumes are available. The type and accuracy of pipets should be evaluated for each application. Regular maintenance and professional calibration of pipets should be performed and documented.

### PIPET TIPS

A variety of pipet tips are available, some of which are specific to the type of pipet. The type and accuracy of the pipet tip, particularly related to the viscosity and nonspecific binding of the materials, should be evaluated for each application.

## Washing

Wash steps are included throughout the ELISA procedure to remove the unbound coating antigen, sample, and detection reagents. Washing is critical for assay performance, can be a source of assay failure, and is important to evaluate during method development. Multiple approaches can be used for washing. Manual procedures include using a squeeze bottle, dipping the microtiter plate in wash buffer, and adding wash buffer with a multi-channel pipet or hand-held multi-channel (8- or 12-pin) manifolds. Analysts should wash carefully to avoid cross-well contamination. Automatic microplate washers generally provide more washing consistency. Strip-well and multi-well washers are available. Most automatic washers can be programmed for different dispensing volumes and speeds, number of washes, speed of buffer aspiration, and amount of residual buffer left in the well. Incorrectly programmed or maintained, as well as incompletely cleaned, automatic washers can cause assay variation and elevated assay background.

## Incubation

ELISAs are incubated following the addition of samples and reagents. The optimal time, conditions, and temperature of each incubation step should be determined during method development. Incubation times vary from minutes to overnight. Commonly used incubation temperatures are ambient temperature, 4°, and 37°. ELISA plates commonly are sealed or placed in a secondary container to avoid evaporation or contamination during incubation. Atmospheric conditions such as dry or humidified incubation should be evaluated during method development. Rocking, shaking, or rotating the microtiter plates may be necessary or desirable depending on the kinetics of binding.

## Blocking Conditions and Nonspecific Reactions

After immobilization and removal of the unbound antigen or antibody, unoccupied binding sites are blocked to ensure that the measured analyte in the test article or subsequent (detection) reagents does not bind nonspecifically to the solid surface or to the coated antigen or antibody. If nonspecific binding occurs, any reported signal could bias the measurement and may reduce the sensitivity and dynamic range of the assay. Blocking is critical to ensure the sensitivity and/or specificity of the assay. Sources of nonspecific binding fall into two general categories:

1. Ionic or hydrophobic interactions occur when binding is mediated by nonspecific ionic or hydrophobic interactions between assay reagents and the solid surface or another assay reagent.
2. Immunological interactions occur when binding is mediated by unintended antigen–antibody interactions. This occurs when antibody preparations used in the assay interact with other assay reagents. For example, if an ELISA was designed to test a serum-derived analyte using murine capture and detection antibodies, antibodies in the test article with reactivity to murine Ig (also known as heterophilic antibodies) could be nonspecifically detected in the assay.

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The choice of blocking agent (examples are found in *Blocking agents and buffers*) is determined empirically, and the balance between the reduction in nonspecific binding and the impact on assay sensitivity should be assessed during method development. Cross-reactivity with other assay reagents should be considered; for example, endogenous biotin is found in milk and serum, and serum may contain antibody to viral or bacterial proteins. Therefore, screening of serum lots may be necessary. The volume of blocking solution added to the well should be greater than the maximum reaction volume used for later steps so that all of the potential surface area that may interfere with the binding reaction is blocked.

In addition, Ig in the test materials can be removed by using buffers that inhibit antibody conformation or aggregate the heterophilic antibodies, by blocking with nonimmune serum, or by removing Fc regions in critical antibody reagents, thereby reducing or eliminating undesired immunological interactions that cannot be addressed by the blocking reagents described above. Negative control wells can be included to monitor nonspecific reactions. The nature of the negative control wells depends on the assay but can include blocked wells without coating antigen, eliminating the primary or secondary antibody, or using buffer in place of sample. Control wells also can be useful as part of system suitability testing.

## Pretreatment of Samples

Although ELISA methods are designed to measure an analyte in complex mixtures, the presence of other materials can prove problematic if they interfere with analyte detection. In order to ensure assay specificity, the specific procedure to treat samples to remove nonspecific interfering substances (e.g., reducing agents or precipitates) can be determined empirically during method development and then can be incorporated into the validated assay. Any sample-processing step should be evaluated against the potential that the treatment will alter the test article's properties and/or introduce further variability that results in biased measurements. Samples, standards, and controls should be prepared and handled in processes as similar to each other as possible. Analysts should verify that sample pretreatments have not damaged the sample so much that it can no longer be measured (e.g., by spiking experiments).

## Detector Antibodies

Depending on the ELISA format, detector antibodies labeled with enzyme or other labels can be used as primary or secondary reagents to enable detection of the immobilized analyte. In a direct or competitive ELISA [*Figure 2* (A and C)], after the analyte is bound to the immunosorbent surface, excess analyte is washed away and the immobilized analyte is detected using a detector antibody that is considered to be the primary antibody. In other ELISA formats [*Figure 2* (B, D, and E)], the analyte-specific Ig (nonconjugated primary antibody) is allowed to bind to the immobilized analyte, and any excess antibody is washed away before the addition of a detector antibody, which is termed the secondary antibody.

To facilitate detection, in all ELISA formats that use enzyme-conjugated antibodies, a substrate specific for the conjugated enzyme is introduced into the assay system. An enzymatic reaction ensues, converting a substrate into a soluble product that can be measured using appropriate wavelengths and a suitable reader.

ELISA sensitivity depends on the quality of the reagents and the detection system, including the label and substrate. If multiple differently conjugated antibodies are available, analysts should select one appropriate for the assay. During this evaluation, the dilution of each conjugate that yields desirable sensitivity and specificity should be determined using appropriate controls.

The most commonly used labeling enzymes for conjugating to antibodies include AP, HRP, and galactosidase. These enzymes are highly specific, sensitive, and stable in catalyzing chromogenic, luminescent, or fluorescent reactions. *para*-Nitrophenyl phosphate (pNPP) is a commonly used substrate for AP. Commonly used substrates for HRP include TMB, *o*-phenylenediamine dihydrochloride (OPD), and [2,2′-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt (ABTS)] (see *Table 2*). The substrates for AP and HRP are chromogenic and result in the formation of a colorimetric product that can be measured using a spectrophotometer. Chemiluminescent and fluorescent substrates for AP and HRP also are available, and in many cases they are available as commercial kits. Disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1$^{3,7}$]decan}-4-yl) phenyl phosphate (CSPD) is a known chemiluminescent substrate for AP (see *Table 2*). Well-known fluorescent substrates for galactosidase include 4-methylumbelliferyl galactoside (MG) and nitrophenyl galactoside (NG). If a chemiluminescent substrate is used, then a luminometer is required to quantitate the formed product. A fluorometer is needed if a fluorescent substrate is used in the ELISA.

*Table 2* also provides a summary of the advantages and disadvantages of different types of ELISA substrates. Colorimetric substrates have been prevalent since the origin of ELISAs and may yield robust assays that generally are more cost efficient than assays that use chemiluminescent and fluorescent substrates. Nevertheless, chemiluminescent and fluorescent ELISA methods may yield more rapid and sensitive assays with a wider dynamic range than assays that use a colorimetric readout. The final choice of readout should be governed by the assay's purpose and the requirements of the assay.

**Table 2. Enzyme Conjugates and Substrates**

| Readout | Principle of the Enzymatic Reaction | Enzyme | Substrate | Reader | Advantages | Disadvantages |
|---|---|---|---|---|---|---|
| Colorimetric | Produces a colored product that yields absorbance values directly proportional to analyte concentration | AP, HRP | pNPP<br>TMB<br>OPD<br>ABTS | Spectrophotometer | • Robust<br>• Economical<br>• Reagent availability | • Less sensitive |

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**Table 2. Enzyme Conjugates and Substrates** *(continued)*

| | | | | | | |
|---|---|---|---|---|---|---|
| Chemilu - minescent | Produces a light emission that is directly proportional to analyte concentration | AP | CSPD | Luminometer | • Wide assay dynamic range<br>• Lower coating concentrations<br>• More sensitive<br>• Rapid signal generation | • Requires special plates<br>• Costly |
| Fluorescent | Produces excitation-induced light emission that is directly proportional to analyte concentration | Galactosidase | MG<br>NG | Fluorometer | • Rapid<br>• Sensitive | • Requires special plates<br>• Costly<br>• Interference by excipients |

# ASSAY DEVELOPMENT AND VALIDATION PLAN

## Critical Reagent Development

Key considerations for critical reagents are source, purity, specificity, and stability. For quality measurements, ITMs use reference standards along with critical reagents for analyte capture and detection. Any changes of critical biological reagents should be evaluated (see, for example, guidance contained in ⟨1032⟩).

### SOURCE

The availability and quality of the starting material should be controlled so that manufacturing of the (purified) reagent can be reproducibly and consistently performed, potentially over several decades. Because critical reagents are biological molecules, sources can range from chemical synthesis (e.g., peptides) to complex biological matrices (e.g., antibodies prepared from serum, monoclonal antibody from ascites/cell culture, or fermentation/cell culture products). When appropriate for the intended use of the assay, a single lot of a critical reagent can be manufactured to establish a substantial supply and to prevent lot-to-lot variability. In other instances, it may be appropriate to include in the validation multiple lots or multiple suppliers in order to demonstrate that the assay is sufficiently robust for its intended use.

### PURITY

In general, the purity of critical reagents should be assessed to ensure the removal of impurities and manufacturing process residuals that can influence reagent performance and/or stability.

### SPECIFICITY

The specificity of a critical reagent refers to its ability to capture or detect only the analyte of interest. The reagent must be specific to the analyte and should show little nonspecific binding or no cross-binding to off-target molecules in complex test materials.

### STABILITY

The stability of critical reagents should be empirically determined to ensure assay performance over time (issues include accuracy, precision, reproducibility, and assay drift). Long-term (months to years) stability of critical reagents under required storage conditions (e.g., with defined temperatures and containers) should be determined so that appropriate expiry dating can be assigned. Short-term (minutes to days) stability (and freeze/thaw and room temperature stability for frozen critical reagents) also is required to ensure day-to-day assay accuracy, precision, and reproducibility.

## Feasibility/Pilot Studies

The steps of the process by which an ELISA method is developed, validated, and used in routine sample analysis are described below:

1. Generate or purchase critical reagents to measure the analyte. Determine storage conditions and stability.
2. Understand the performance goals for the assay system.
3. Develop the assay to the point that there is a detectable concentration response curve.
4. Perform method development/robustness testing.
5. Prepare the reference/calibration standard and control and assess stability.
6. Establish assay procedures, appropriate controls and limits, assay and sample acceptance criteria, and instrumentation.
7. Determine method performance, and qualify method for accuracy, specificity, precision, and robustness, including qualification of all applicable sample types to be analyzed.
8. Validate the assay.
9. Implement the method (technology transfer) in the testing laboratory, including training and qualification of analysts.
10. Monitor assay performance.

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During assay development, the critical parameters and reagents that are required for the assay should be assessed and set at levels that yield desired assay performance. In many instances several parameters may be evaluated, and well-designed experiments can accelerate assay development, particularly for assessing the potential interaction of several inputs.

Many ELISA procedures are product specific, and external reference/calibration standards may not be available. The preparation and stability of reference/calibration standards should be considered early in assay development.

### Assay Validation

Assay validation is executed according to guidances from appropriate regulatory bodies [e.g., International Council for Harmonisation (ICH) Q2] to demonstrate that the particular test used for an analyte is appropriate for its intended use. More information about assay validation can be found in *Validation of Compendial Procedures* ⟨1225⟩ or ⟨1033⟩ if the ELISA is used as a surrogate potency assay. See *Appendix 2: Additional Sources of Information about Specific Topics in Validation and Data Analysis*.

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## DATA ANALYSIS

The analysis of ELISA data can be simple (e.g., a linear calibration with inverse regression) or complex (e.g., a nonlinear calibration curve with inverse regression). The type and rigor of data analysis depend largely on the assay system and the intended uses of the assay. For example, data reduction may estimate a concentration (e.g., ng/mL) of an unknown sample using a calibration curve. Other approaches include estimation of the half-maximal inhibitory concentration ($IC_{50}$) or effective concentration ($EC_{50}$), estimation of the amount of a sample that yields the same response as the $EC_{50}$ (or $IC_{50}$) on a standard curve, and an estimate of the relative activity of a test sample compared to a reference/calibration standard. More extensive guidance about statistical methods for potency analysis is given in ⟨1032⟩ and ⟨1034⟩.

In general, ELISA curves are characterized by a nonlinear relationship between the concentration of the analyte of interest and the calculated mean response. Typically, this response curve is defined by a sigmoidal relationship of response to concentration. A wide range of mathematical models can fit standard/calibration curves, and analysts should take care in the selection of an appropriate curve-fitting algorithm. In other cases, ELISA assays are used for qualitative purposes to determine whether a sample is positive or negative based on a sensitivity threshold.

### Basic Statistical Analysis

Basic statistical methods are not detailed here. *Analytical Data—Interpretation and Treatment* ⟨1010⟩ addresses important fundamentals, including data handling; computation of means, standard deviations, and standard errors; detection of and methods to address nonconstant or nonnormal variation; detection of and management of outliers; and procedures for and interpretation of statistical tests and confidence intervals. The concepts behind validation, goals, designs, analysis, and practical methods for validation are described in ⟨1010⟩, ⟨1225⟩, and ⟨1033⟩. *Design and Analysis of Biological Assays* ⟨111⟩ contains guidance on combining results from independent assays, ▲outliers, and confidence intervals.▲ (USP 1-Dec-2019)

### Nonlinear Statistical Analysis

Nonlinear calibration for immunoassays draws on many sources for statistical design and analysis. These include methods for assessing and addressing nonconstant variance, designs and analysis methods for experiments with complex structures, and validation. The concepts behind linear calibration design, analysis, and inverse regression apply in nonlinear calibration, and professional statisticians can help apply these appropriately.

### Reporting Results

Reported estimates of concentration should be understood as having an associated confidence interval based on the results of the validation. The reported value or estimate used to describe a sample can be based on a combined result from multiple assays.

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## APPENDICES

### Appendix 1: Abbreviations

**Ab:** Antibody
**ABTS:** 2,2′-Azino-bis[3-ethyl-benzothiazoline-6-sulfonic acid]diammonium salt
**Ag:** Antigen
**Anti-Ig:** Anti-immunoglobulin
**AP:** Alkaline phosphatase
**BSA:** Bovine serum albumin
**CSPD:** Disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1$^{3,7}$]decan}-4-yl)phenyl phosphate

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**EIA:** Enzyme immunoassay
**ELISA:** Enzyme-linked immunosorbent assay
**HRP:** Horseradish peroxidase
**Ig:** Immunoglobulin
**ITM:** Immunological test method
**MG:** 4-Methylumbelliferyl galactoside
**NG:** Nitrophenyl galactoside
**OPD:** *o*-Phenylenediamine dihydrochloride
**PBS:** Phosphate-buffered saline
**pNPP:** *para*-Nitrophenyl phosphate
**PVDF:** Polyvinylidene fluoride
**TMB:** 3,3′,5,5′-Tetramethylbenzidine

## Appendix 2: Additional Sources of Information about Specific Topics in Validation and Data Analysis

| | **Analytical Data—Interpretation and Treatment** ⟨1010⟩ | **Design and Analysis of Biological Assays** ⟨111⟩ | **Validation of Compendial Procedures** ⟨1225⟩ | **Biological Assay Chapters** ⟨1032⟩, ⟨1033⟩, and ⟨1034⟩ |
|---|---|---|---|---|
| Means | X | — | — | — |
| Standard deviations | X | — | — | — |
| Standard errors | X | — | — | — |
| Non-normality | X | — | — | X |
| Nonconstant variance | X | — | — | X |
| Outliers | X | ▲X▲ (USP 1-Dec-2019) | — | X |
| Tests | X | — | — | — |
| Confidence intervals | X | ▲X▲ (USP 1-Dec-2019) | — | X |
| Validation | — | — | X | X |
| Combining results from multiple assays | — | X | — | X |

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# ⟨1104⟩ IMMUNOLOGICAL TEST METHODS—IMMUNOBLOT ANALYSIS

## INTRODUCTION

### Definition and Scope

The chapter is part of a group of general information chapters for immunological test methods (*Immunological Test Methods—General Considerations* ⟨1102⟩, *Immunological Test Methods—Enyzme-Linked Immunosorbent Assay (ELISA)* ⟨1103⟩, and *Immunological Test Methods—Surface Plasmon Resonance* ⟨1105⟩) that provides analysts with general information about principles, method development, method validation, and data evaluation for immunoblot analysis. Immunoblot analysis is defined as any method in which an antibody is used for detection of one or more analytes (e.g., proteins, polysaccharides) that has been transferred to a test membrane surface. Immunoblot methods are typically classified by whether electrophoretic separation occurs as a part of the immunoblot procedure. Electrophoretic separation is based on molecular weights and charge differences of a population of molecules. See *Capillary Electrophoresis* ⟨1053⟩, *Biotechnology-Derived Articles—Isoelectric Focusing* ⟨1054⟩, and *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* ⟨1056⟩ for a detailed description of electrophoretic separation methods. An example of immunoblot analysis involving electrophoretic separation is the Western blot, which was first described in the scientific literature in the late 1970s. Another approach for immunoblot analysis is to perform molecule detection using an antibody without prior electrophoretic separation. Examples of this nonelectrophoretic type of approach are the slot or dot blot (slot/dot).

The scope of this chapter includes only those methods in which an antibody is used for the detection of a molecule bound to a membrane. Therefore this chapter does not discuss procedures that use nonimmunological means of detection.

## ASSAY SELECTION

### Nonelectrophoretic Assay (Slot/Dot Blot)

The slot/dot blot method is a simplified, nonelectrophoretic method in which a mixture containing the analyte(s) for detection is first applied directly to the membrane using a vacuum manifold machined to contain regularly spaced rectangular slots. The slot/dot blot method is faster and simpler because there is no electrophoretic separation of the multiple, individual analytes that may be present in the mixture. For these reasons, it can be readily adapted for automated analysis of multiple samples, for which a number of systems are commercially available, but it offers no information about the molecular weight and only limited information regarding the quantity of sample. Although it can be set up in a quantitative format, the method usually is used to produce a qualitative result, e.g., confirming identity by demonstrating the presence or absence of specific antigens by means of an immunocomplex detection system. After the analytes are bound to the membrane and unbound sites are blocked, analysts use a detector antibody to determine the presence or absence of the analyte or analytes of interest. The uniform shape of the slot blot and its greater surface area for analyte binding make it better suited than the dot blot for quantitative applications and analysis by densitometry.

### Electrophoretic Assay

Electrophoretic blotting methods (commonly called Western blots) are widely used for analyzing mixtures of proteins. The Western blot is a powerful tool to study the identification, relative concentration, relative molecular weight, and posttranslational modifications of specific proteins. In Western blots, the proteins of the sample are separated using gel electrophoresis. Protein separation may be based on molecular weight alone or on isoelectric point (pI) and molecular weight. Proteins migrate either in one dimension (1D) or in two dimensions (2D) through a gel. When proteins are separated by their molecular weights, the smaller proteins migrate faster and separate according to molecular weight. When analysts use a 2D gel, proteins are separated according to pI in the first dimension, and then according to their molecular weights in the second dimension. After separation, the proteins are transferred to a membrane, the membrane is blocked to avoid nonspecific binding of subsequent assay reagents, and the protein of interest is detected using specific antibodies.

A bound antibody can be detected by different methods, including colorimetric detection, fluorescent detection, chemiluminescent detection, and radioactive detection. Upon detection of the protein(s) of interest, immunoblot quantitation can be indirectly performed by densitometry.

#### 1D ELECTROPHORESIS

In 1D electrophoresis, individual proteins or groups of proteins are separated by molecular weight for further analysis by Western blot. Using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), proteins migrate in response to differences in electrical charge through a 3D network of fibers and pores. The network is formed as the bifunctional bisacrylamide, or other cross-linker, cross-links adjacent polyacrylamide chains to form a gel (see also *USP* general chapter ⟨1056⟩). The combination of gel pore size and protein characteristics determines the migration of proteins. Separated proteins are detected subsequently by Western blot analysis using antibodies specific to the target proteins. By means of Western blot analysis, a test sample can be compared to a standard, and the appearance of degradants and impurities specifically related to the target proteins can be monitored if the detection antibody can still recognize the altered forms of the protein. Although a high level of sensitivity can be achieved by this approach, separation of individual proteins at similar molecular weights may not be achieved. If analysts must probe individual proteins at similar molecular weights, 2D separations may be required.

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### 2D ELECTROPHORESIS

In 2D electrophoresis, individual proteins or groups of proteins are separated in the first dimension by isoelectric focusing (IEF; charge) and in the second dimension by electrophoresis in the presence of SDS (molecular weight). Separating proteins this way allows information to be obtained not only about molecular weight, as in 1D gels, but also about the charge of a protein. Two-dimensional gels are a useful choice for resolving complex mixtures and for assessing protein antibody specificity (e.g., evaluation of host cell proteins).

## Membrane, Reagent, and Detection Options

### MEMBRANES

Generally, both nitrocellulose and polyvinylidine fluoride (PVDF) membranes are used for immunoblot methods. For cost considerations, nitrocellulose membranes are often preferred over PVDF membranes for slot/dot blots (or vacuum blotting), but due to their greater mechanical strength, PVDF membranes should be considered if stripping and reprobing are required.

### BLOCKING REAGENTS

Following transfer or binding of protein to membranes, the unoccupied binding sites on the membranes must be blocked to prevent nonspecific binding of subsequent reagents. Most detection probes are proteins that also can bind to the membrane. Failure to appropriately block the membrane sites can result in nonspecific binding and high background. A number of blocking reagents are available, including gelatin, nonfat milk, and bovine serum albumin (BSA). Proteins should be unrelated to the antigens used in the study. Because these reagents often have lot-to-lot variability, they may require qualification. They must be evaluated with the detection system selected and optimized using that detection system for minimal background with no loss of signal. If the blocking reagent is derived from a biological source, it must not contain trace levels of the protein under measurement, because the latter can increase the background.

### METHODS OF DETECTION

Immunological detection of analytes in any type of immunoblot can be direct or indirect. The choice of format depends on a combination of the level of sensitivity required and the quality of the antisera available. For identity or product detection, sensitivity usually is not critical, and direct detection via a conjugated antibody is commonly used, which often simplifies and shortens the time required to execute the method. Alternatively, indirect detection, usually by the use of a conjugated anti-species reagent, can be used to improve sensitivity. On some occasions, the analyte being detected is actually an antibody, as in the case of a monoclonal antibody that is being developed as a drug. In this case, antibodies specific for the antibody (e.g., anti-idiotypic antibodies) can be used.

**Primary antibody:** The primary antibody is selected based on its specificity for the analyte or protein. Although polyclonal anti-sera can offer a broad range of detection against a potentially large set of epitopes, an unwanted cross-reaction resulting in decreased specificity may occur. If this cannot be overcome by assay optimization, a monoclonal antibody or groups of selected monoclonal antibodies can be used. Monoclonal antibodies are often advantageous for long-term studies, because they yield a consistent supply of antibody against a specific epitope. The use of monoclonal antibodies directly limits the number of epitopes involved in the detection of the target. This must be evaluated for each application. The primary antibody may be directly conjugated or used in conjunction with a secondary antibody and an appropriate detection system. The optimal antibody concentration usually is considered to be the greatest dilution of antibody that results in a strong positive signal with minimal background. This must be optimized in conjunction with the block and detection system selected. The primary antibody should be qualified before assay use.

**Secondary antibody:** The secondary antibody typically is directed against the species of the primary antibody immunoglobulin (which is specific for the analyte, e.g., goat anti-mouse IgG). Enzymes such as horseradish peroxidase (HRP) or alkaline phosphatase (AP) typically are linked to the secondary antibody, but other labels such as fluorophores or gold particles can be used for detection. If the secondary antibody is biotinylated, biotin–avidin–HRP or –AP complexes can be used for detection.

**Detection enzyme and substrate:** Once an immunocomplex containing the enzyme–conjugate reagent has formed, analysts add a suitable substrate to the assay. This reaction results in production of a colored precipitate or a fluorescent or chemiluminescent product that can be recorded, measured, and analyzed further. A broad range of detection options is available to best fit individual applications and intended uses. A number of these are described in ⟨1103⟩ and ⟨1102⟩, as well as in *Table 1*.

**Table 1. Detection Reagents and Methods**

| Readout | Principle of the Enzymatic Reaction | Enzyme | Substrate | Detection | Advantages | Disadvantages |
|---|---|---|---|---|---|---|
| Colorimetric | Produces a colored product that yields absorbance values directly proportional to analyte concentration | AP[a] HRP[c] | pNPP[b] TMB[d] OPD[e] ABTS[f] | Spectro - photometer | — Robust — Economical — Reagent availability | — Time-consuming — Less sensitive than other methods |
| Chemiluminescent | Produces a light emission that is directly proportional to analyte concentration | AP, HRP | CSPD[g] | Luminometer, photographic film (CCD[h] camera) | —Wide assay dynamic range — Very sensitive — Rapid signal generation | — Reproducibility can be challenging |

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**Table 1. Detection Reagents and Methods** *(continued)*

| Readout | Principle of the Enzymatic Reaction | Enzyme | Substrate | Detection | Advantages | Disadvantages |
|---|---|---|---|---|---|---|
| Fluorescent | Produces excitation-induced light emission that is directly proportional to analyte concentration | Galactosidase, fluorescently labeled antibody | MG[i] NG[j] | Fluorometer (CCD camera with filters) | — Rapid — Sensitive | — Interference by excipients |
| Radioactive | Antigen is labeled with a radioactive isotope. Radiation is proportional to analyte concentration. | — | — | Scintillation counter | — Easy to quantitate — Rapid | — Safety risk with exposure — Radioactive waste |

[a] Alkaline phosphatase.
[b] *para*-Nitrophenyl phosphate.
[c] Horseradish peroxidase.
[d] 3,3′,5,5′-Tetramethylbenzidine.
[e] *o*-Phenylenediamine dihydrochloride.
[f] 2,2′-Azino-bis[3-ethyl-benzothiazoline-6-sulfonic acid]diammonium salt.
[g] Disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1$^{3,7}$]decan}-4-yl)phenyl phosphate.
[h] Charge-coupled device.
[i] 4-Methylumbelliferyl galactoside.
[j] Nitrophenyl galactoside.

## METHOD DEVELOPMENT

Method development can proceed on the basis of the background information just provided. The scope of method development, and eventually method validation, are dictated by the purpose of the method. The purpose determines the format for the assay and other requirements for the test, and therefore, the purpose should be determined first. The following sections explore considerations for each method's purpose.

### Intended Purpose of the Method

#### IDENTITY TESTING

In the case of identity tests, analysts want to detect the presence of a protein; therefore, demonstration of specificity is essential and required. For this purpose, analysts also control the quantity of protein in the sample. Thus, the limits of detection (LOD), limits of quantitation (LOQ), and other measures of quantity are not required attributes of the method. Examples include material identity assays that demonstrate the isotype of an IgG and, in some cases, demonstrate the specificity of an antibody in a method validation. If there is no interference from the matrix or potential cross-reaction with other materials present in the sample, then a simple slot/dot blot may suffice. If multiple proteins in the sample display immunoreactivity and must be distinguished from each other, another separation procedure must be used before blotting and immunostaining. The complexity of the proteins in the sample and the usefulness of the additional information gained using an electrophoretic separation help determine if a slot/dot blot can meet the needs of the test.

#### LIMIT TESTING

In other applications, analysts may want to show that an impurity has been removed to a level below toxicological concern. In many cases, a limit test is used when it is possible to say *yes* or *no* about the presence or absence of a protein below a predetermined level. This simplifies the development and validation of the method. With densitometry (scanning or imaging) equipment, the intensity of spots or bands can be determined relative to a standard curve, resulting in an estimate of concentration. An LOD should be determined to establish the appropriate limit threshold for the method. A dot blot may be suitable for either circumstance if the specificity of the antibody in the sample matrix can be demonstrated.

Another common purpose for an immunoblot is to show the presence or absence of a protein expressed from a culture. In this situation, analysts want to establish the identity of the protein by immunostaining, as well as verify that the protein has the expected molecular weight. This provides further assurance of no nonspecific interactions with other proteins in a complex matrix that generates the signal in the blot.

#### SPECIFICITY TESTING

Characterizing the specificity of the reagents for an ELISA impurity test or an immunoaffinity column also is a common immunoblot purpose. This is another form of an identity test in which the desired endpoint is demonstration of the specificity of binding between the antigen and the antibody. The result of the measurement is a demonstration of binding to a select group of the total protein population in the sample or binding to the whole population of proteins in the sample, as required for host-cell protein assays. In order to demonstrate the specificity of an antibody relative to a population of proteins, analysts typically must carry out electrophoretic or other separations. Showing, by means of immunostaining, that a protein of the right molecular weight or pI can be recognized by the antibody is a powerful demonstration both of specificity toward a given protein and the absence of binding to other proteins. In addition, having, within the same experiment, the appropriate positive control samples that are known to contain the protein, and the appropriate negative samples that are known not to contain the protein,

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makes a convincing argument for the specificity and selectivity of the antibody when analysts validate an ELISA method for protein impurities. Electrophoretic separations can be done in one dimension using either SDS–PAGE (for molecular weight) or isoelectric focusing (IEF; for isoelectric point) for a limited number of proteins with known molecular weights. Electrophoretic separations also can be done in two dimensions (e.g., IEF followed by SDS–PAGE) to show selectivity and specificity toward a more heterogeneous population of proteins. A 2D Western blot commonly is used to demonstrate the specificity of a polyclonal antibody candidate directed against a host-cell protein (HCP) antigen preparation before development of a quantitative ELISA for that purpose.

## Assay Mode and Sample Introduction

After considering the critical elements required for each purpose of the method, the analyst can use this information to select the most appropriate assay mode. The main points to consider when developing an immunoblot method are shown in *Figure 1*. If appropriate, spotting samples on a membrane or applying by vacuum is the easiest and most convenient way to introduce a sample to an immunoblot membrane. However, low levels of nonspecific binding from multiple proteins can create additive nonspecific interference in dot blots or slot blots, resulting in background levels that appear to be the desired analyte.

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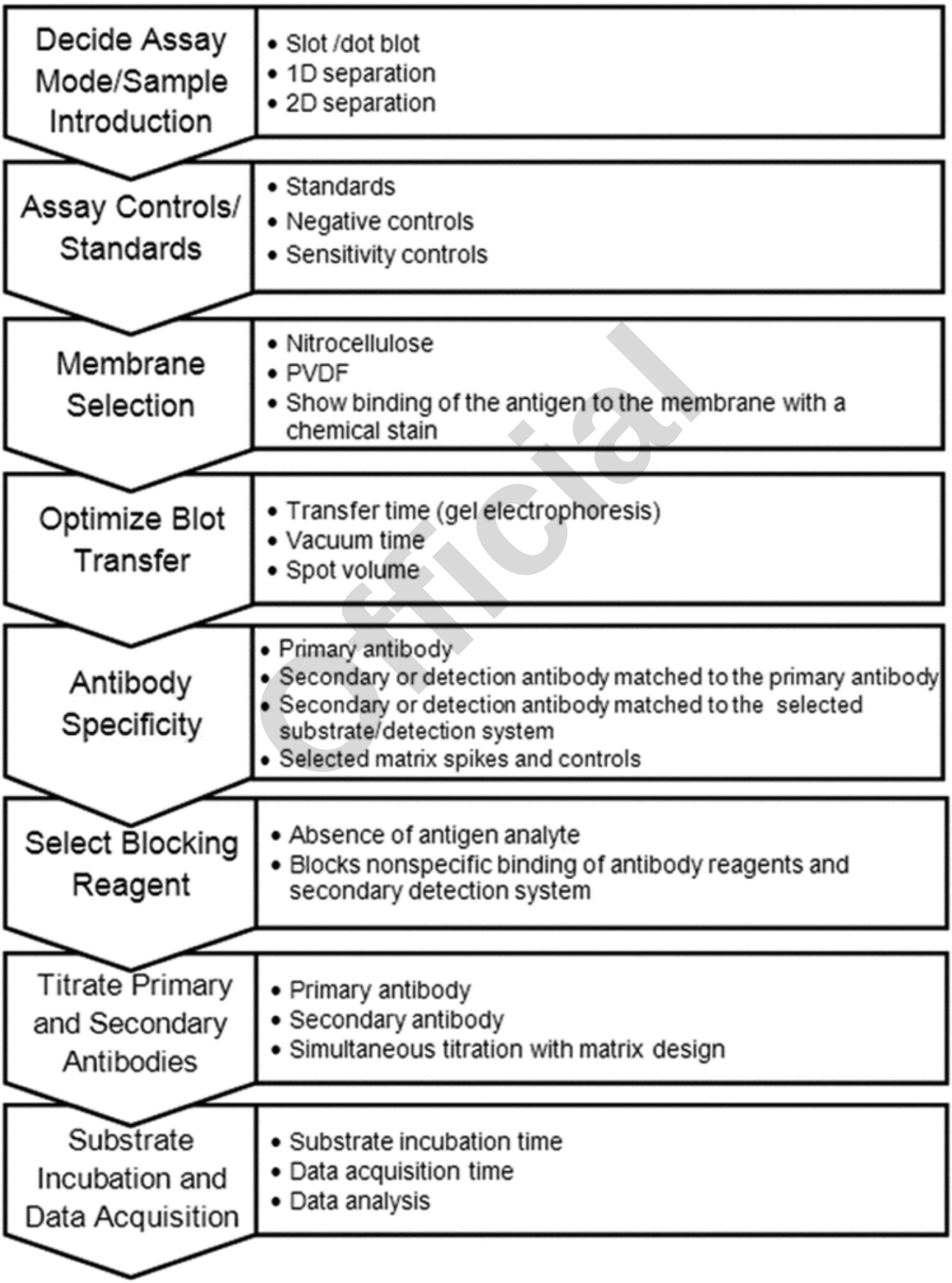


Figure 1. Method Development Flow Chart.

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Electrophoretic separations, although time consuming, can be useful for separating and further distinguishing specific and nonspecific binding. Analysts must trade sensitivity for selectivity in going from a single dimension to two dimensions because of the further separation of immunoreactive species from a single band into multiple spots, as is the case with the heterogeneity seen in sialylated proteins or deamidated species.

## Assay Controls and Standards

Controls and standards are selected based on the purpose of the assay and the information needed during development. Protein molecular weight markers can be used to obtain an accurate estimate of the molecular weight of immunoreactive species. The use of positive and negative controls is helpful for troubleshooting throughout the experimental design process. Standards or positive and negative controls can be used to assess system suitability and to establish method performance. A positive control can confirm appropriate protein migration and can confirm that membrane transfer has reached completion. A negative control is useful for assessing nonspecific interactions. A method sensitivity control near the LOQ can be used to measure the consistency of the method near the LOQ to evaluate changes in assay performance.

## Membrane Selection

A membrane is selected based on the application and the protein being measured. Membranes with various pore sizes should be available and should be suitable for the molecular weight of the protein of interest to aid in appropriate transfer of different sizes of proteins. If a chemical staining method is known to work on a specific membrane with a specific protein, then it is advantageous to show that the protein binds to the membrane and is stained before analysts work on the immunostaining steps for the assay. Electrophoretic separations followed by transfer to a membrane should be optimized with chemical staining, e.g., with sensitive fluorescent stains or silver stains and at potentially higher loads before analysts work on lower load levels required for blot optimization. Stains such as Coomassie or colloidal Coomassie may not have sufficient sensitivity to detect a low level of impurities required for certain applications.

## Optimize Blot Transfer

Analysts should optimize transfer times from the gel to the membrane. Larger proteins require more time for transfer than smaller proteins. Small proteins may be lost during long transfer times and can transfer from the gel all the way through the membrane and be lost on the other side. The density of the gel and gradient gels can result in nonuniformity of transfer from the top to the bottom of the gel. During transfer optimization, many method developers use multiple membranes in order to capture proteins that transfer through the first membrane. Chemical staining of both the gel and the membranes can provide useful information about the location of the proteins transferred from the gel to support, either extending or reducing the transfer time.

After they select the assay mode, analysts next investigate spotting of the antigen or transfer from a gel to the appropriate membrane at various relevant concentration levels. Levels of analyte above the concentration needed for a Western blot may be required at first to determine if transfer and recognition by the antibodies is possible. If the analyte is present in low concentrations, spiking may be necessary to show its location during transfer optimization. Because of the potential variability of immunostaining and transfer, a sensitivity control or several levels of controls should be incorporated into the method based on the analyte titration above the background level. This can be adjusted as method development progresses.

## Antibody Specificity

Analysts should demonstrate antibody specificity early in immunoblot method development. If possible, they should test samples of the matrix without the analyte and should show an absence of response. In contrast, samples that contain analyte spiked into the matrix should show a positive response, demonstrating the specificity of the antibodies.

Analysts also should demonstrate the specificity of the secondary antibody conjugate or label. Control immunoblots with lanes or spots of primary antibody and control matrix samples containing the analyte as a negative control can show that the secondary antibody is binding to the primary antibody and not to proteins found in the matrix. Commercial sources for enzyme conjugates or fluorescent-labeled anti-species antibodies are readily available and normally are screened or affinity purified against the species of antibody being detected, which eliminates some of the early work needed to achieve the desired specificity. The secondary antibody or detection system must be matched with the detection equipment and the desired sensitivity of the assay, e.g., fluorescence, colorimetric precipitating substrates, or chemiluminescence.

## Select Blocking Reagent

Replicate membranes can be blocked with previously described blocking agents as analysts select the most appropriate blocking reagent and the amount of time required to minimize background by means of subsequent primary and secondary antibody incubations. Analytes titrated at multiple concentrations on the membrane allow analysts to assess the amount of signal to the amount of noise (background) with various blocking reagents followed by immunostaining with the primary antibody, labeled secondary antibody, and substrate, if needed, for visualization. This titration also serves as the starting point for examining LOD and LOQ for limit tests and quantitative measurements. The LOD for immunoblots is determined by the level of nonspecific background relative to the specific signal from the analyte. As is the case with any other analytical method, if the background and signal are equal, there is no distinction between the signal and the noise.

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## Titrate Primary and Secondary Antibodies

Titrating the level of primary and secondary antibody from low to high dilutions can also, as with a blocking reagent, be used to select an antibody concentration that reduces background binding in the blank regions surrounding protein spots or bands, and can optimize the signal from the analyte. A matrix grid that varies the level of primary signal against secondary signal can be useful for optimizing the background, improving analyte signal, and reducing the consumption requirements for expensive antibody reagents.

Immunoaffinity chromatography against a highly purified antigen can be used to reduce the level of nonspecific interference for all of the immunological reagents used in an immunoblot. The method developer must be cautious that the selectivity, specificity, and affinity of the primary antibody are not lost in affinity purification because of high-affinity antibodies that remain on the antigen column or because of the destruction of antibody binding caused by elution conditions. For the secondary antibody, immunoaffinity-purified anti-species antibodies are available commercially with a variety of possible labels conjugated to the antibody.

## Substrate Incubation and Data Acquisition

Analysts can optimize substrate development time for enzymes in order to minimize background and improve the LOD and LOQ. Excessive substrate development times for precipitating substrates can result in an intensification of the background level relative to the specific signal from the desired analyte. If the blot is agitated during substrate incubation, undesired swirling patterns of product from precipitating substrates can form. Too short an incubation time results in a less-specific signal, but too long a time can result in high background and poor resolution. Most enzyme conjugates have an optimum development time. Fluorescent labels and chemiluminescent labels have the advantage of acquisition by scanning instrumentation that can store data electronically and perhaps acquire image signals in an additive manner. Fluorescent labels have the added advantages that the signal is stable with time, numerous experiments for development time can be obtained with a single blot, and optimization of signal acquisition can be performed on a single blot.

# PROCEDURES

## Slot/Dot Blots

Using an appropriate slot/dot apparatus, analysts can make antigens of interest adhere to a suitable membrane (e.g., nitrocellulose) by gravity or vacuum filtration, followed by addition and incubation of antigen-specific antibodies that bind to epitopes on the antigens. Remaining binding sites on the membrane are blocked by the addition of nonspecific antigen (e.g., BSA), followed by probing of the antigen-specific antibodies with a detection system [e.g., protein A/G conjugated to HRP binds to the antibodies that then are visualized using a 4-chloro-naphthol (4-CN) peroxidase substrate]. Positive identification is the development of dots or bands on the membrane. A negative result remains white or exhibits faint bands that are considerably lighter than positive bands.

## 1D Immunoblotting

### PREPARATION OF SDS–PAGE GELS

Analysts should choose an SDS–PAGE gel with a content of acrylamide–bisacrylamide suitable for the molecular weight(s) of the protein(s) of interest; i.e., the smaller the molecular weight of the protein, the higher the percentage of mono- or bisacrylamide, and conversely, the larger the molecular weight of the protein, the lower the percentage of mono- or bisacrylamide.

Uniform-concentration gels have separation ranges as shown in *Table 2*, and gradient gels have a separation range as shown in *Table 3*. Gels can be purchased ready-made or can be produced in the laboratory according to procedures in ⟨1056⟩.

**Table 2. Linear Range of Separation (kD) for Uniform-Concentration Gels**

| Acrylamide Concentration (%) | Linear Range of Separation (kD) |
|---|---|
| 5 | 57–212 |
| 7.5 | 36–94 |
| 10 | 20–80 |
| 12 | 12–60 |
| 15 | 10–43 |

**Table 3. Linear Range of Separation (kD) for Gradient Gels**

| Acrylamide (%) | Protein Range (kD) |
|---|---|
| 5–15 | 20–250 |
| 5–20 | 10–200 |

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**Table 3. Linear Range of Separation (kD) for Gradient Gels** *(continued)*

| Acrylamide (%) | Protein Range (kD) |
|---|---|
| 10–20 | 10–150 |
| 8–20 | 8–150 |

## SAMPLES AND STANDARD

To prepare samples, analysts typically must lyse cells and tissues in order to release the proteins of interest. The main consideration when choosing a lysis buffer is whether the antibody chosen for detection of the protein(s) of interest can recognize denatured samples. When this is not the case, analysts use buffers without detergent or with relatively mild, nonionic detergent.

Samples should be treated (e.g., reduced, nonreduced, or denatured) according to general chapter ⟨1056⟩, and when a sample of unknown protein content is used, a series of dilutions should be loaded onto the gel. Standards (molecular weight markers) should be treated according to the manufacturer's instructions.

## ELECTROPHORESIS

Before applying samples to the stacking gel wells according to ⟨1056⟩, analysts denature samples (e.g., heat at 95°–100° for 5 min). An appropriate volume of sample is loaded onto the gel, and a voltage of 8 V/cm applied until the dye has moved into the resolving gel. Afterward, the voltage is increased to 15 V/cm, and the separation is run until the bromophenol blue reaches the bottom. If a commercially available gel is used, the manufacturer's recommendations are followed. *Table 4* shows common sample-loading volumes for particular gels.

**Table 4. Common Sample-Loading Volumes**

| Wells | Gel Thickness (mm) | Maximum Sample Load Volume (µL) |
|---|---|---|
| 10 | 1.0 | 25 |
| 10 | 1.5 | 37 |
| 12 | 1.0 | 20 |
| 15 | 1.0 | 15 |
| 15 | 1.5 | 25 |

## TRANSFER

After electrophoresis, the proteins of interest can be blotted to a membrane such as nitrocellulose or PVDF with a pore size that is appropriate for the molecular weight of the proteins of interest. Both nitrocellulose and PVDF have a protein-binding capacity of about 100–200 µg/cm$^2$. PVDF is more chemically resistant than nitrocellulose and is easier to handle. Detailed instructions for the transfer process can be found on the websites of the manufacturers of transfer apparatus and vary depending on the system.

Transfer can be done in wet or semi-dry conditions. Semi-dry transfer generally is faster, but wet transfer is especially recommended for large proteins >100 kD. For both kinds of transfer, the membrane is placed next to the gel. The two are sandwiched between absorbent materials, and the sandwich is clamped between solid supports to maintain tight contact between the gel and membrane.

A standard buffer for transfer is the same as the buffer used for the migration or running buffer without SDS, but with the addition of methanol to a final concentration of 20%. For proteins larger than 80 kD, SDS should be included at a final concentration of 0.1%. Lowering methanol in the transfer buffer also promotes swelling of the gel, allowing large proteins to transfer more easily. *Table 5* contains common buffers used for Western blot methods.

**Table 5. Common Western Blot Buffer Formulations**

| Buffer | Content |
|---|---|
| Sample buffer 2× (nonreducing) 1D electrophoresis | 1.89 g of Tris<br>5.0 g of SDS<br>50 mg of bromophenol blue<br>25.0 mL of glycerol<br>100 mL of water<br>Adjust with HCl to a pH of 6.8.<br>Add water to 125 mL. |
| Sample buffer 2× (reducing) 1D electrophoresis | To nonreducing sample buffer:<br>Add 12.5 mL of 2-mercaptoethanol before adjusting the pH.<br>Alternatively, use 1.93 g of Tris, and add a suitable quantity of DTT[a] to obtain a final concentration of 100 mM DTT. |

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**Table 5. Common Western Blot Buffer Formulations** *(continued)*

| Buffer | Content |
|---|---|
| Running buffer 10×<br>1D electrophoresis | 151.4 g of Tris<br>721.0 g of glycine<br>50.0 g of SDS<br>Add water to 5000 mL.<br>Adjust to a pH of 8.1–8.8. |
| Transfer buffer 10× | 151.4 g of Tris<br>721.0 g of glycine<br>Add water to 5000 mL.<br>Adjust to a pH of 8.1–8.8. |
| Transfer buffer 1× | 100 mL of 10× stock<br>500 mL of water<br>200 mL of methanol<br>Add water to 1000 mL. |
| TBS 10× | 24.23 g of Tris base<br>80.06 g of NaCl<br>Mix in 800 mL of ultrapure water.<br>Adjust with pure HCl to a pH of 7.6.<br>Add water to 1000 mL. |
| TBS-T | 100 mL of TBS 10×<br>900 mL of water<br>1 mL of polysorbate 20 |
| 8.5 M urea stock | 510 g of urea<br>Add water to 1000 mL. |
| Sample buffer<br>2D electrophoresis | 47 mL of 8.5 M urea stock<br>385 mg of tributyl phosphine (TBP)<br>2 g of CHAPS[b]<br>25 mg of bromophenol blue<br>1% carrier ampholytes of choice |

[a] Dithiothreitol.
[b] 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate.

Methanol is necessary only if analysts use nitrocellulose. If they use PVDF, they can remove methanol from the transfer buffer and need only to activate the PVDF before they assemble the gel and membrane sandwich.

In semidry transfer, a sandwich of paper/gel/membrane/paper wetted in transfer buffer is placed directly between the cathode and anode. During wet transfer the membrane should be closest to the positive electrode, and the gel should be closest to the negative electrode. The composition of the transfer buffer is not necessarily the same as the migration or running buffer. Analysts should consult the apparatus manufacturer's protocol, and it is common to add both SDS and methanol. The balance of SDS and methanol in the transfer buffer, the proteins' molecular weights, and the gel percentage can affect transfer efficiency for both wet and semidry transfers.

## BLOCKING

Blocking the membrane prevents nonspecific background binding of the primary and secondary antibodies to the membrane. Traditionally, one of two blocking solutions is used: nonfat milk or BSA (Cohn fraction V). Milk is cheaper but is not recommended for studies of phosphoproteins. To prepare a 5% milk or BSA solution, weigh 5 g/100 mL of Tris-buffered saline containing polysorbate 20 buffer (TBS-T; see *Table 5*). Mix well, and filter. Failure to filter can lead to spotting in which tiny dark grains contaminate the blot during development. Incubate at 4° for 1 h with gentle shaking. Rinse in TBS-T after the incubation.

## PRIMARY ANTIBODY AND INCUBATION BUFFER

Dilute the antibody with blocking buffer at a proper dilution (1:100–1:3000, depending on antibody titer), and optimize the dilution according to the results. Too much antibody can result in nonspecific bands.

## INCUBATION TIME

Incubation time can vary between a few hours and overnight, and depends on the binding affinity of the antibody for the protein and the abundance of protein. A more dilute antibody with a prolonged incubation may improve specific binding.

## INCUBATION TEMPERATURE

It is best to incubate under cold temperatures. When analysts incubate in blocking buffer overnight, they should incubate at 4° to prevent contamination from bacterial growth, and should gently agitate the antibody solution to enable adequate homogeneous covering of the membrane.

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### SECONDARY ANTIBODY AND INCUBATION BUFFER

Handle the secondary antibody and incubation buffer as follows. Wash the membrane several times in TBS-T while agitating to remove residual primary antibody. Dilute the secondary antibody with TBS-T at the suggested dilution. Too much secondary antibody can result in nonspecific bands. Incubate the blot at room temperature for 1–2 h with gentle agitation. *Table 1* shows multiple options for secondary detection reagents and methods. More details are available in the *Immunoblot Data Analysis* section below.

## Slot/Dot Blot

The procedure is similar to the procedure for 1D immunoblotting, but differs because protein samples are not separated electrophoretically but are spotted directly onto the membrane either manually or by use of a blotting unit (dot or slot blot format).

### PROCEDURE USING MANUAL SPOTTING

Handle manual spotting as follows. Place a dry filter paper on a stack of dry paper towels. Place filter paper that is pre-wet with transfer buffer on top of the dry filter paper. Place a pre-wet membrane on top of the pre-wet filter paper. Samples are spotted onto the pre-wet membrane and are allowed to absorb into the membrane. After the sample is absorbed, place the membrane on a clean, dry filter paper to dry.

### PROCEDURE USING A VACUUM-BLOTTING UNIT

Analysts typically use a vacuum-blotting unit as follows. Prepare a membrane, and place it in the blotting unit according to the manufacturer's instructions. Apply vacuum to the blotting unit to remove excess buffer. To improve solubility, dissolve the sample in a buffer, and if it is not clear, remove precipitates by centrifugation. If the sample is too viscous to pipet, then dilute it further with buffer. With the vacuum off, carefully pipet samples into the wells, and apply vacuum to the blotting unit. After all the samples have filtered through the membrane, turn off the vacuum, add buffer to each well to wash down the sides, and apply vacuum again. Remove the membrane, and proceed with immunoblotting.

## 2D Immunoblotting

### SAMPLE PREPARATION

The compounds used to solubilize proteins must not increase the ionic strength of the solution. For example, a common sample solubilization solution is the following: 8 M urea, 50 mM dithiothreitol (DTT) or 2 mM tributyl phosphine (TBP), 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 0.2% carrier ampholytes, and 0.0002% bromophenol blue. The addition of carrier ampholytes enhances the solubility of proteins as they approach their isoelectric points. The use of ampholytes produces an approximately uniform conductivity across the pH gradient without affecting its shape, meaning that the concentration of carrier ampholytes should be optimized.

### CHARGE SEPARATION

Several vendors produce and sell immobilized pH gradient (IPG) strips, or they can be made in-house according to ⟨1054⟩. The choice of IPG strips depends on the pI of the proteins of interest. The size of the IPG should match the size of the second-dimension gel. The amount of protein in each sample should be determined, and the amounts loaded on the IPG strips should be in the range of 10–300 µg, depending on the size of the IPG. The sample and standard should be loaded according to the manufacturer's instructions or according to ⟨1054⟩. Analysts then proceed with the isoelectric focusing by applying the electrical parameters described in ⟨1054⟩ or by the manufacturer.

### MOLECULAR WEIGHT SEPARATION

After charge separation, analysts must equilibrate the strip in SDS-containing buffer before separation in the second dimension to determine molecular weight (as described previously in the *1D Immunoblotting* section). Analysts should position the strip directly on top of the gel, then secure the strip by overlaying it with 0.5%–1.0% agarose prepared in SDS–PAGE running buffer. To track the ion front in the second dimension, analysts can add bromophenol blue to the agarose.

# IMMUNOBLOT DATA ANALYSIS

The presence or absence of bands usually is determined by comparison to a control (highly characterized antigens known or qualified to give a precise or expected response) of a type that is similar to the antigen being processed. Although analysts usually perform a qualitative comparison, bands or dots can be quantitated using a detection system (e.g., after incubating in a solution containing 4-CN peroxidase substrate; also see *Table 1*), and are compared to the control bands run in parallel (e.g., in the same gel).

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## Detection Options

### ENHANCED CHEMILUMINESCENCE

Enhanced chemiluminescence is a popular method for detection in immunoblot analysis because it is highly sensitive (detection to pg or lower levels), and can be used to quantitate the relative concentration of the protein of interest. The method depends on incubation of the blot with a substrate that luminesces when exposed to the reporter on the secondary antibody. The light is detected using either photographic film or a charge-coupled device (CCD) camera. The image then is analyzed by densitometry to evaluate the relative amount of protein staining in terms of optical density. By using an appropriate set of molecular weight standards as markers, analysts can estimate molecular weight.

### FLUORESCENCE DETECTION

Direct fluorescence can be used to detect proteins on blots. Direct fluorescence is simple, rapid, sensitive, and has a greater linear range than enhanced chemiluminescent detection. The advantage of direct fluorescence is the ability to detect many different fluorescent signals. This analysis avoids the need to reprobe the blot. Compared to enhanced chemiluminescence, fluorescence methods are easier to visualize and quantitate on CCD or laser-scanning imaging systems. Some data-acquisition systems permit extending the time of data acquisition to optimize signal-to-noise levels. Fluorescence-labeled blots that can be re-examined are useful for this purpose.

Enhanced chemifluorescence (ECF) is another common fluorescence method. ECF uses secondary antibodies conjugated to either HRP or AP. The enzyme-conjugated antibodies react with specific substrates that produce fluorescence after enzymatic cleavage. Analysts visualize the resulting signals using UV epi-illumination and capture digital images. An ECF signal has a greater linear range than traditional enhanced chemiluminescence. For example, direct fluorescence has a limit of detection in the pg range, and also has about 2 logs of linear dynamic range.

Quantum dots also are an alternative to detect proteins in immunoblot analysis. Quantum dots are a type of probe that can be conjugated to antibodies simultaneously or sequentially to detect multiply labeled antigens, without the need for blot stripping. Similarly, near-infrared (NIR) fluorophore–linked antibody is a method for antibody detection whereby light produced from the excitation of a fluorescent dye is measured in a static state. Light measured in a static state allows more precise and accurate detection than light measured in a dynamic state (e.g., chemiluminescence).

### RADIOACTIVE DETECTION

Proteins also can be detected by labeling an antigen with a radioactive isotope (e.g., iodine). On the one hand, this method has the advantage that the radioactivity in a band is easy to quantitate by means of time exposure to film and densitometry, or by directly excising the band from the membrane and counting using a scintillation counter. On the other hand, radioactivity also introduces the disadvantage of safety because analysts must manage radioactive material, and analytical laboratories must have a program to control and monitor waste management and individual exposure.

## Immunoblot Quantitation

### NONELECTROPHORETIC QUANTITATION

The quantitation of a specific protein is achieved when the blot procedure is properly optimized and generates a linear response range over a particular time frame. Immunoblot quantitation includes several elements: adequate antigen and antibody concentrations and purity, antibody specificity, blocking conditions, sufficient washes, and the duration and intensity of the signals. Once the exposures are captured on a film or electronically under optimized conditions, analysts use densitometric methods to quantitate results by comparing a specific protein on the blot and on the standard. Analysts can correct results for background by including a negative control.

The intensity of the bands depends on the amount of protein. Different commercial software packages are available for image analysis of bands on a film. Alternatively, digital imaging systems containing CCD cameras usually include software designed to perform data analysis.

### ELECTROPHORETIC QUANTITATION

Proteins of various molecular weights are identified by the extrapolation of plots of relative mobilities of prestained proteins of known molecular weight and can be compared to the positive control. Positive controls are trended to determine the limit range of the densitometry results compared to the nominal concentration results. Independent of the detection method, the following criteria must be met for a valid Western blot result.

- Ensure adequate development by minimizing membrane overexposure and visualizing staining controls.
- The prestained molecular weight markers must be visible and must cover the anticipated range.
- The band(s) should have the appropriate location and intensity for the standard, the control, and the protein of interest.
- There should be no blot or staining artifacts that obscure the visualization and interpretation of bands.

# METHOD VALIDATION

As outlined by ICH Guideline Q2(R1) Validation of Analytical Procedures: Text and Methodology (effective November 2005) and *USP* general chapter *Validation of Compendial Procedures* ⟨1225⟩, a qualitative assay such as the slot/dot blot requires

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validation of specificity. Specificity is the ability to detect the analyte in the presence of other components. For validation, it should be shown that the particular steps of the slot/dot blot method can detect the antigen when present and do not report false positive results when the antigen is absent. In addition, demonstration of the specificity of the antigen-specific antibodies is part of the specificity evaluation.

*USP* general chapter ⟨1225⟩ provides guidelines for the validation of analytical procedures, and analysts should consider this resource when they validate immunoblot methods. All methods require a demonstration of the specificity of the antibody to the antigen and the lack of recognition of other proteins and reagents in the matrix. Identity tests require only specificity. Limit tests require specificity and LOD. A sensitivity control incorporated into each test can show that the LOQ is met on each determination to account for potential changes in the sensitivity of the method. A quantitative test requires all ICH validation parameters, including robustness testing.

Demonstration of electrophoretic immunoblot specificity should include the following: stained gels to show protein separation, stained blots to show adequate protein transfer to the membrane, blots with control samples to show the specificity of the conjugate to the primary antibody, and blots that show the binding of the antibody to the appropriate antigen. Method validation also can identify the need for control membranes for each assay, as well as protein sensitivity controls as measures of system suitability.

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# ⟨1105⟩ IMMUNOLOGICAL TEST METHODS—SURFACE PLASMON RESONANCE

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## INTRODUCTION

Surface plasmon resonance (SPR) optical detection is a useful method for the label-free assays (procedures) that study biomolecular interactions. Commercially available SPR biosensors that incorporate these assays can collect real-time, information-rich data from binding events. These data can be used widely from basic research to drug discovery and development to manufacturing and quality control (QC). SPR can characterize binding events with samples ranging from proteins, nucleic acids, and small molecules to complex mixtures, lipid vesicles, viruses, bacteria, and eukaryotic cells. Typical quality and safety attributes addressed with SPR analysis include:

- Interaction specificity
- Interaction affinity
- Kinetic binding parameters
- Thermodynamic parameters
- Biologically active concentration of an analyte

This chapter provides an overview of the physics underlying ▲the principle of▲ (USP 1-Dec-2020) SPR and common instrument configurations, as well as the range of molecules that can be studied and general considerations for experimental design as determined by the assay objective. ▲Biolayer interferometry (BLI), which is another optical technique that is useful for label-free assays that study biomolecular interactions, is not in the scope of this chapter. The general principles of SPR discussed in this chapter may apply to the use of BLI.▲ (USP 1-Dec-2020)

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## OVERVIEW

### HISTORY

The physical principles of SPR were first explained in the early 1900s, starting with a description of the uneven distribution of light in a diffraction grating spectrum caused by the excitation of surface plasmon waves. A landmark series of experiments showed the optical excitation of surface plasmons under conditions of total internal reflection and fostered detailed studies of the application of SPR for chemical and biological sensing. Since then, SPR's potential for characterizing thin films and monitoring interactions at metal interfaces has been recognized, and significant research and development have yielded instruments that can quantitatively evaluate the binding interactions of small and large molecules.

### PHYSICS

SPR is an optical phenomenon that occurs when a thin conducting film is placed between two media that have different refractive indices. In many commercially available instruments, the two media are glass and the sample solution, and the conducting film is preferentially a gold layer applied to the glass, although other conducting metals such as silver have been used. The glass–metal component comprises a solid support that is often referred to as a *sensor*.

Light applied to the glass under conditions of total internal reflection produces an electromagnetic component that is called an *evanescent wave*. The evanescent wave penetrates the medium of lower refractive index (typically the sample solution) without losing net energy. The amplitude of the evanescent wave decays exponentially with distance from the surface, roughly one-half of the wavelength of the incident light (e.g., for a light source of 760 nm the evanescent wave penetrates approximately 300 nm).

For a specific combination of wavelength and angle of incident light, electron charge density waves called *plasmons* are excited in the gold film. As energy is absorbed via the evanescent wave, a decrease in the intensity of the reflected light at a specific angle (the SPR angle) is observed. Analysts can conduct an SPR experiment by fixing the wavelength and varying the angle of incident light.

An increase in mass at the sensor surface caused by a binding interaction between two or more molecules causes a change in the local refractive index (RI) that gives rise to an SPR response, which is observed as a shift in the SPR angle. By monitoring the shift in the SPR angle as a function of time, an analyst can generate a *sensorgram* (*Figure 1*). The change in RI is very similar for different proteins, so the SPR measurement depends primarily on the mass change at the sensor surface and is relatively independent of the nature of the molecules being measured.

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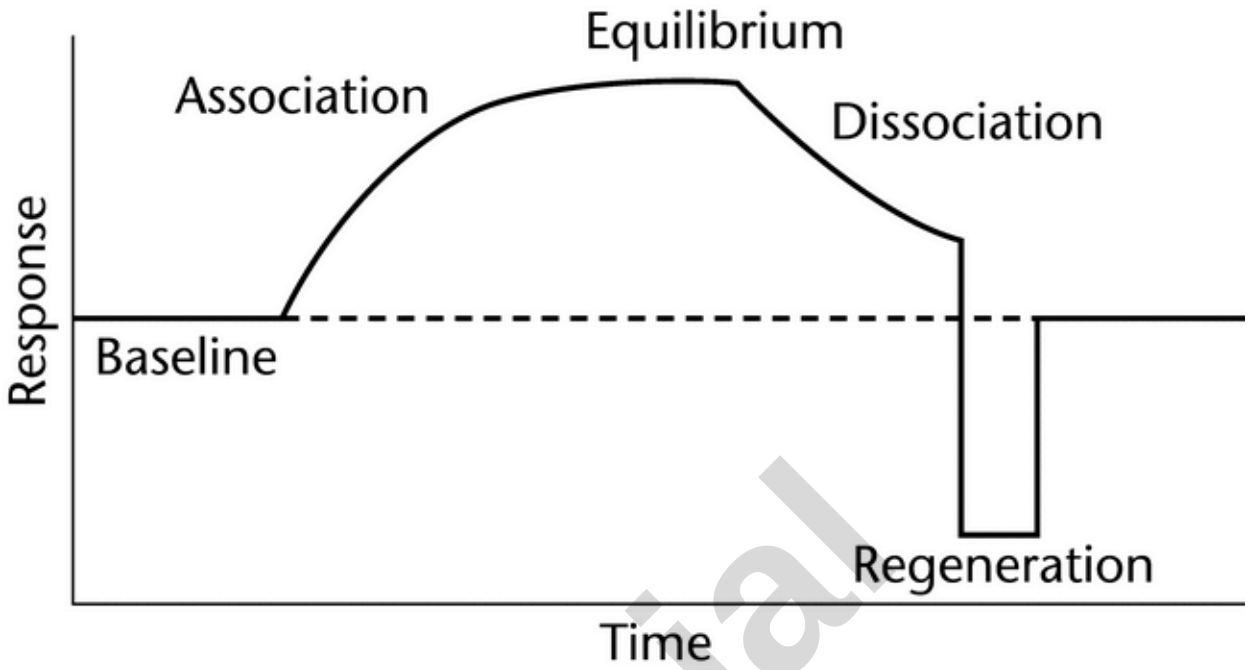


Figure 1. Representative sensorgram.

**INSTRUMENTS**

The main components of commercially available SPR instruments are (1) a light source, typically a high-efficiency light-emitting diode, (2) an optical detector such as a diode-array or charge-coupled device camera, (3) a solid support containing the conducting film and some means for attaching molecules, (4) a sample delivery system, frequently a microfluidic device capable of delivering samples using single serial or parallel injections via single or multiple needles, and (5) a computer with appropriate software for instrument control, data collection, and analysis.

Prism-based and diffraction-grating instrument systems are commercially available. Most prism-based systems follow the Kretschmann configuration (*Figure 2*). The light is focused onto the sensor surface (away from the samples) via a prism with a refractive index matching that of the surface. In this configuration the incident light does not penetrate the sample solution, which permits SPR measurements for heterogeneous, turbid, or opaque samples. In systems that utilize a diffraction grating (*Figure 3*) the analyte solution is placed over a plastic surface on which a metal has been deposited. The plastic acts as an attenuated total internal reflection prism in which light reflected from the grating is reflected many times back to the grating surface. In this configuration light passes through the analyte sample solution, and thus turbid or opaque samples are not suitable for measurement. The diffraction grating does permit sampling of a larger surface area and is applicable for SPR measurements of arrays.

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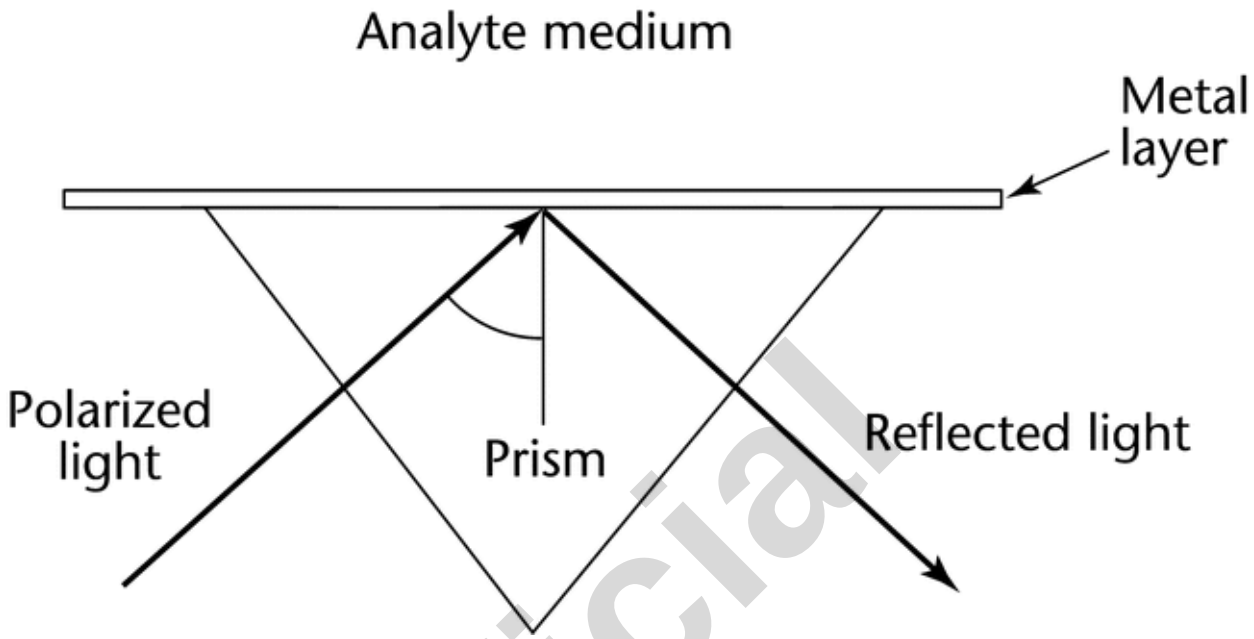


Figure 2. Kretschmann SPR configuration.

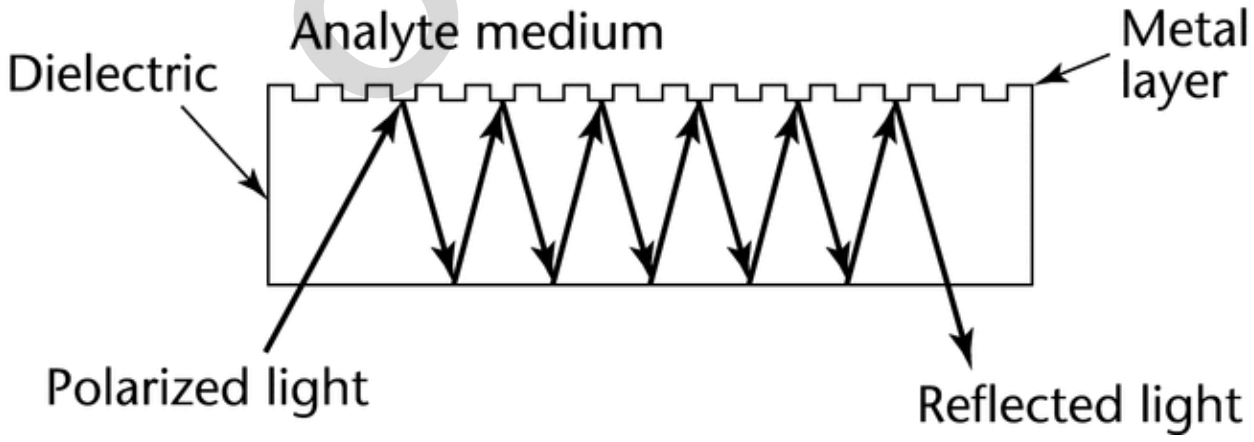


Figure 3. Diffraction grating SPR configuration.

The instruments are compatible with a wide range of biological samples and buffers as well as some organic solvents.

**BIOMOLECULAR INTERACTIONS THAT CAN BE STUDIED BY ASSAYS USING SPR**

A diverse range of biological entities can be studied using SPR, including small molecules (<100 Da), proteins, nucleic acids, lipids, bacteria, viruses, and whole cells. Most published SPR research involves protein–protein interactions, of which antibody–antigen interactions represent a dominant subset. Improvements in instrument sensitivity and experimental protocols have helped analysts make studies of small molecules, lipids, and nucleic acids. Protein interactions with larger entities such as whole cells and some bacteria and viruses are limited by the exponential decay of the evanescent wave as described above. In practice these large molecules can be studied effectively, but the information obtained may be limited to qualitative or semiquantitative (e.g., relative ranking) data.

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#### ASSAY TYPES

Several types of SPR assays are useful, including binding specificity, concentration analysis, kinetics and affinity analysis, and thermodynamics. Each assay type generates unique information that is helpful for profiling biomolecules.

SPR is also suitable for use in qualitative studies to confirm the specificity of interactions. Analysts can monitor ▲▲ (USP 1-Dec-2020) sequential binding events because each individual event yields a mass increase on the sensor chip surface, and all stages in the binding process are monitored. Examples include epitope mapping, antibody isotyping, and immunogenicity measurements.

Most chemical and spectroscopic methods used to quantify proteins (1) measure total protein content, (2) do not distinguish active from inactive molecules, and (3) cannot be used in conjunction with unpurified samples. Because SPR is a noninvasive method (no light penetrates the sample), it can measure small amounts of analyte molecules from complex matrices such as food products, serum or plasma, and cell extracts. Direct or indirect (inhibition or competitive) formats for measuring concentration are possible. SPR biosensors are uniquely suited for measurement of kinetic association and dissociation rate constants from real-time measurement of binding interactions. Affinity can be derived either from interactions that have reached equilibrium or from the ratio of the dissociation and association rate constants. The typical working range for affinity measurements is pM to high µM concentrations. Association rate constants that can be measured typically range from $10^3$ to $10^7$ $M^{-1}s^{-1}$ and dissociation rate constants from $10^{-5}$ to 0.5 $s^{-1}$. By studying temperature dependence of rate and affinity constants, analysts can determine thermodynamic parameters for a binding interaction. Not only can the equilibrium values for changes in enthalpy ($\Delta H$) and entropy ($\Delta S$) associated with complex formation be determined, but transition state energetics can also be evaluated. Subsequent sections of this chapter address the specific details for these different assay types.

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## SPR ASSAY

The typical SPR assay involves five steps:

1. Sample and buffer preparation
2. Surface preparation
3. Analyte binding
4. Surface regeneration
5. Data analysis and interpretation

Careful attention to experimental design leads to high-quality data and results. In SPR experiments, mass transport is essential for binding interactions to take place in instruments that use thin-layer flow-cell systems. Analyte molecules are transferred from the bulk solution to the binding surface via mass transport. When a limitation for binding occurs as a result of fast binding kinetics combined with high surface density, the binding interaction is considered mass-transport limited. In this case, the binding kinetics and complex formation are influenced by the availability of analyte molecules. The advantages and disadvantages of mass-transport–limited binding are discussed later in the application examples.

#### SAMPLE AND BUFFER PREPARATION

Both purified and crude samples can be analyzed in a variety of matrices including serum, plasma, cell supernatants, and lysates. Crude samples containing particulates (e.g., cell debris or precipitates) may require clarification ▲▲ (USP 1-Dec-2020) to help minimize unwanted binding ▲and avoid clogging of microfluidic channels.▲ (USP 1-Dec-2020) A short spin (30–60 s) in a benchtop centrifuge or filtration (0.22–1.0 µm) using low–protein-binding filters is recommended. The concentration range for evaluation depends on the experimental objective (yes/no binding, concentration, or kinetic/affinity analysis) as well as the binding affinity of the interacting molecules. In general, sample concentrations an order of magnitude below the equilibrium dissociation constant ($K_D$), can be detected by SPR, but determination of an exact concentration is influenced by the analyte size (large vs. small molecules), binding specificity, and overall biological activity of the samples.

Most biological buffers and several organic solvents can be used in SPR experiments. The addition of salts and detergents to buffer solutions frequently can stabilize biomolecules. High-quality grade (e.g., molecular biology grade or higher) buffer components should be used. To simplify experiments, analysts should add only components that are absolutely required for biological activity or function. Buffers should be filtered and degassed. ▲▲ (USP 1-Dec-2020)

#### SURFACE PREPARATION

Surface preparation involves the attachment of one of the binding partners to a solid support (surface). This process is frequently referred to as *immobilization*, and the resulting surface with the attached biomolecule is the sensor for the experiment. The choices of binding partner, solid support, and immobilization method are influenced by (1) the nature and demands of the application or experimental objective; (2) the availability of surfaces with different properties (e.g., charge density, hydrophobicity, or hydrophilicity); (3) the characteristics and supply of biomolecule to be used for immobilization; and, most importantly, that (4) biological activity be maintained and binding sites be available to interacting partners. Depending on the experimental objective, ▲either heterogeneous (random orientation) or homogeneous (orientation-specific) attachment to the solid support can be performed.▲ (USP 1-Dec-2020) The two main categories of immobilization methods are (1) direct immobilization, in which the molecule is covalently attached to the surface, and (2) indirect or capture immobilization, which takes advantage of tags or native groups on the protein or biomolecules (*Table 1*).

**Table 1. Surface Preparation Techniques**

| Chemistry | Immobilization Method | Biomolecules | Comments |
|---|---|---|---|
| Amine | Direct | Proteins, peptides | Amino terminus, Lys residues |
| Thiol—native | Direct | Proteins, peptides | Native Cys residue |

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**Table 1. Surface Preparation Techniques** *(continued)*

| Chemistry | Immobilization Method | Biomolecules | Comments |
|---|---|---|---|
| Thiol—added | Direct | Proteins, peptides | Carboxyl groups derivatized |
| Aldehyde | Direct | Glycoproteins | *Cis–diol* required |
| Biotin capture | Indirect | Biotinylated peptides, nucleic acids, proteins | Stable, irreversible capture |
| Affinity tags | Indirect | Proteins, peptides | His, Glutathione *S*-transferase (GST), etc. |
| Protein A, Protein G | Indirect | Antibodies, IgG-tagged molecules | IgG species–dependent |
| Protein A, Protein G | Indirect | Biomolecules specific to the capturing antibody | Mono- or polyclonal antibodies may be suitable—testing recommended |
| Hydrophobic adsorption, membrane capture | Indirect | Lipids, membranes, membrane-associated proteins | Monolayer or bilayer attachment possible |

**Direct immobilization:** For direct immobilization, several chemistries are available for attaching proteins or other biomolecules to the surface. The properties of the surface determine the specific sequence of steps and length of time required to prepare the surface. Many commercially available surfaces have a biologically compatible layer (e.g., a hydrogel) that contains functional groups such as carboxyl that can be used for immobilization. To ensure binding specificity, the purity of the biomolecule that is attached to the surface should be ▲of sufficient purity (e.g., ▲ (USP 1-Dec-2020) 95% or greater) and the required concentration should range from 1 to 1000 µg/mL. Direct immobilization chemistries frequently result in heterogeneous surfaces because of random orientation of biomolecules on the surface.

Immobilization via free primary amine groups such as lysine residues in proteins or the amino terminus of proteins or peptides is one of the most generally applicable covalent chemistries for attaching proteins to a surface. Carboxyl groups on the surface are converted to reactive esters using a mixture of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) or sulfo-NHS (sNHS). The protein or biomolecule is applied in high concentrations (mg/mL) to maximize the efficiency of amine coupling. Finally, free esters are blocked with ethanolamine. The contact time with the surface, the protein concentration, or the EDC/NHS concentration can be varied to adjust the immobilization level.

When amine coupling interferes with the binding site, the biomolecule can be attached using alternative coupling chemistries or a high-affinity capture approach. For example, for biomolecules with free thiol groups (typically cysteine residues), a disulfide group is introduced by treating the surface with NHS and EDC to attach 2-(2-pyridinyldithio)ethaneamine (PDEA). Adding the biomolecule to the surface results in thiol–disulfide exchange, and excess PDEA groups are inactivated with cysteine–HCl. If the biomolecule lacks a free thiol group, a reactive disulfide (PDEA) can be linked to carboxyl groups. Subsequently the pyridyldisulfide groups can be attached to thiol groups on the surface that have been derivatized via injection of NHS and EDC, followed by cystamine, then reduction with dithioerythritol (DTE) or dithiothreitol (DTT). Attachment of maleimide groups to the surface makes possible an alternative form of immobilization via thiol groups in which a stable thioether bond is formed. Surfaces prepared using this method have the capacity to withstand basic pH (> 9.5) and reducing agents such as β-mercaptoethanol and dithiothreitol. Several heterobifunctional reagents are available commercially for introduction of reactive maleimido groups to the surface, including sulfo-MBS (*m*-maleimidobenzoyl-*N*-hydroxysulfosuccinimide ester), sulfo-SMCC (sulfosuccinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate), GMBS [*N*-(γ-maleimidobutyrloxy)sulfosuccinimide ester], EMCH [*N*-(ε-maleimidocaproic acid)-hydrazide] or BMPH [*N*-(β-maleimidopropionic acid)-hydrazide]. For biomolecules containing either native aldhehyde groups or *cis–diols*, which may be converted into aldehydes by mild oxidation, surface attachment via a hydrazone bond is an option. Hydrazide groups on the sensor surface react with aldehyde groups on the biomolecule to form a stable bond. Immobilization via aldehyde groups is most useful for glycoconjugates, glycoproteins, and polysaccharides.

**Indirect (high-affinity capture) immobilization:** Indirect or high-affinity capture immobilization approaches use tags commonly used for protein purification. This technique exploits the high-affinity capture of the biomolecule by a capturing molecule that has been immobilized covalently using one of the techniques described above. The requirement for biomolecular purity is less stringent for indirect vs. direct immobilization because the capturing step for the biomolecule can also provide purification. Indirect immobilization frequently yields a homogenous surface because all biomolecules are oriented similarly via the tag. The affinity between the biomolecule and its capturing agent should be sufficiently high to ensure little or no dissociation from the surface for the duration of an analysis cycle. Monoclonal antibodies are frequently used as capture molecules. For example anti-GST antibodies can be attached to the sensor chip surface via amine chemistry ▲▲ (USP 1-Dec-2020) to capture GST-tagged molecules. Protein A, Protein G, and anti-IgG antibodies are useful capturing molecules for use with antibodies.

The high-affinity interaction between streptavidin or related molecules and biotin ($K_D \approx 10^{-15}$ M) makes it a useful system for the capture of biotinylated molecules (e.g., proteins, peptides, nucleic acids, membranes, and liposomes). Frequently, the biotin-binding protein is attached to the surface using primary amine groups. Because of the high affinity of the interaction, biotinylated molecules are considered permanently immobilized, and in contrast to most other capture approaches biotinylated molecules cannot be removed without damaging the surface. Histidine (His)-tagged recombinant proteins can be captured via nickel–NTA chemistry or covalently immobilized anti-His antibodies.

Lipids and membrane-associated proteins can be captured to the surface as either a lipid monolayer or bilayer. Lipids from micelles or liposomes adsorb to a hydrophobic surface, creating a lipid monolayer with the hydrophobic lipid

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tails oriented toward the solid support and the hydrophilic heads towards the aqueous sample. This approach provides a stable environment for proteins associated with a membrane surface or partially inserted into the membrane, but it is not ideal for transmembrane proteins because the resulting surface presents only half the membrane structure for binding interactions. Intact membrane structures (lipid bilayers) with associated or incorporated proteins can be captured by preparing liposomes with a specific antigenic component or with biotinylated lipids, allowing capture of the liposomes with immobilized antibody or streptavidin, respectively.

**Additional considerations:** Once the biomolecule has been attached to the sensor using either a direct or indirect immobilization approach, analysts should assess the baseline stability of the newly created surface. If the baseline is decreasing (downward drift), the most likely cause is the presence of unattached biomolecules, possibly because of self-association or aggregation. If the baseline is increasing (upward drift) refolding or re-orientation may be causing the change. In either case, the newly created surface should be conditioned before use by one or more of the following: (1) multiple injections of biologically compatible buffer; (2) washing the surface with buffer at a fast flow rate; (3) multiple injections of either high ionic strength (e.g., 1 M NaCl) or detergent (e.g., 20 mM CHAPS or 0.05% Polysorbate 20 (P20)) solutions; or (4) repeated analyte binding and regeneration injections. [NOTE—Recommendations (3) and (4) should be used only if the activity of the biomolecule in the presence of these reagents has been evaluated previously.] Large baseline drifts caused by low-affinity capture may be overcome by using EDC/NHS as a cross-linking step, but this may compromise biomolecule activity if active sites of the biomolecule are involved in the cross-linkages. The effect of cross-linking on biomolecule activity must be tested empirically for each biomolecule–analyte system. In general, cross-linking should be as brief as possible: 15 s is often sufficient to achieve acceptable baseline stability without compromising biomolecule activity.

**How much to immobilize:** The amount of biomolecule to immobilize depends on the experimental objective. Equations 1 and 2 are useful for calculating the appropriate surface density:

$$R_{max} = (MW_A/MW_L) \times R_L \times S_m \quad \text{[Equation 1]}$$
$$R_L = R_{max} \times (1/S_m) \times (MW_L/MW_A) \quad \text{[Equation 2]}$$

$R_{max}$ = theoretical maximum binding response (assuming a surface that is 100% active and 100% bound with analyte)
$R_L$ = response of the immobilized molecule
$MW_A$ = molecular weight of the analyte
$MW_L$ = molecular weight of the immobilized molecule
$S_m$ = molar binding stoichiometry

For kinetic experiments, a low density of immobilized molecule is preferred ▲▲ (USP 1-Dec-2020) to avoid steric hindrance, aggregation, and/or mass-transport–limited binding. Low density is defined as $R_L$ that limits $R_{max}$ to 5–50 response units. For other applications, e.g., concentration analysis where mass-transport–limited binding is desired, $R_{max}$ can be 100–200 times higher than for kinetic experiments provided that steric hindrance or aggregation are not induced. Specific recommendations for immobilization density are included in the application examples for this chapter.

### ANALYTE BINDING

Samples that will be evaluated for binding using SPR do not require the same purity as biomolecules intended for direct immobilization onto the surface. Because the light source does not penetrate the sample, turbid or opaque samples can be analyzed by SPR. Whenever practical, samples should be clarified according to the recommendations given under *Sample and Buffer Preparation*, and buffer additives should be minimized, including only the amount required for biological activity.

Differences between the refractive index of the bulk and sample buffers give rise to a response. The use of control surfaces and samples aids in demonstrating binding specificity for the molecules in SPR. For direct immobilization methods, suitable control surfaces can be (1) the sensor surface without any modification or biomolecule attached, (2) a surface that has been chemically treated in the same manner as the surface containing the biomolecule, or (3) a related but known nonbinding biomolecule. For surfaces prepared using indirect (capture) immobilization the capturing molecule in the absence of the tagged binding partner should be used as the control surface. The difference in response between the control and active surfaces gives an initial indication of the binding specificity. Concentration-dependent responses and inhibition of binding by incubating the sample with the biomolecule on the surface can further establish the binding specificity.

If nonspecific or unwanted binding is observed, analysts should determine the source. Frequently changes in pH or ionic strength of the buffers used in the experiment will reduce or eliminate the unwanted binding. Additional suggestions for reducing nonspecific binding are summarized in *Table 2*.

**Table 2. Suggested Actions for Reducing Nonspecific Binding**

| Category | Action |
|---|---|
| Experimental Design | 1. Optimize running buffers:<br>A. Increase salt (150–500 mM)<br>B. Add detergent (0.001%–0.05%)<br>C. Match composition of sample and running buffers<br>2. Change ligand immobilization method<br>3. Evaluate ligand quality<br>4. Increase or decrease temperature in detection chamber |

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**Table 2. Suggested Actions for Reducing Nonspecific Binding** *(continued)*

| Category | Action |
|---|---|
| Choice of Surface | 1. Change properties of sensor surface:<br>A. Reduce electrostatic interactions<br>B. Evaluate hydrophobic vs. hydrophilic character of surface<br>C. Consider alternative ligand to use for control surface<br>2. Pre-immobilize amino—PEG<br>3. Change blocking molecule (e.g., ethylenediamine) |
| Additions to Sample | 1. Add nonspecific binding reducer to sample:<br>A. Increase ionic strength of running and sample buffers (e.g., 150–500 mM NaCl)<br>B. Add detergent to running and sample buffers (e.g., 0.001%–0.05% surfactant P20)<br>C. Add soluble carboxymethyl dextran (1–10 mg/mL, for dextran-based surfaces only)<br>2. Simplify sample buffer—include only components required for biological activity<br>3. Evaluate analyte quality |

Equations 1 and 2 are also useful for assessing surface activity. The higher the binding response, the more active the surface is unless the observed binding response exceeds the calculated $R_{max}$ value. In this case, the molar binding stoichiometry is incorrect, the analyte molecule is aggregated, or the analyte is binding nonspecifically to the surface. Binding responses that are low (<10% of $R_{max}$) suggest that the analyte concentration selected for the experiment is too low or that the surface activity of the immobilized molecule is low. In the former case, increasing the analyte concentration should increase the binding response, and in the latter situation using a different immobilization method may be helpful.

**SURFACE REGENERATION**

Surface regeneration refers to the process of removing bound analyte from the surface ▲▲ (USP 1-Dec-2020) to reuse the surface for subsequent binding interactions. In some instances, complex dissociation is fast and bound analyte is simply washed away with buffer, so regeneration is not needed. Alternatively, the instrument configuration may allow multiple samples to be injected either sequentially or in parallel across several immobilized surfaces simultaneously, thereby limiting the need for regeneration. Inadequate surface regeneration may affect the reproducibility of an assay and negatively affect the overall quality of the resulting data. To identify the correct conditions, analysts should consider the nature of the specific interaction and the experimental objective. For example, a slight baseline drift will not affect the results when a simple yes/no answer is sought, but in concentration determination or kinetic studies, optimization of the regeneration step is critical.

Most biochemical interactions involve non-covalent bonds such as hydrogen, electrostatic, van der Waals, and hydrophobic bonds. Because the combination of physical forces responsible for binding and the regeneration conditions critical for not causing irreversible conformational changes are unknown for most interactions, the final conditions must be evaluated empirically.

The ideal condition for regeneration dissociates all the bound material without affecting the biological properties of the immobilized biomolecule. An incomplete regeneration or too stringent conditions may result in decreased analyte binding capacity in subsequent cycles because of either blocking of binding sites by nondissociated analyte or partial denaturation of the biomolecule. Regeneration buffers and solutions can be divided into different classes by the effect they have on the interaction. Any combination of buffers can be used.

The major classes of regeneration buffers are: acidic, basic, ionic/chaotropic, detergent, hydrophobic/nonpolar, and chelating (see *Table 3* for examples of each class). Analysts should start with mild conditions, moving progressively to more harsh conditions. In many cases, especially when one is working with antibodies, change in pH is the most effective method of regenerating the surface. Contact time with the surface is important for efficient regeneration. When analysts use pH change, the contact times should be short, one-half to 2 min. When analysts use high ionic strength or chaotropes, longer contact times of 2–4 min are usually effective.

**Table 3. Examples of Regeneration Solutions**

| Acid | Base | Ionic/ Chaotropic | Detergent | Hydrophobic/ Nonpolar | Chelating |
|---|---|---|---|---|---|
| 1–100 mM HCl | 1–100 mM NaOH | 0.5–5 M NaCl | 0.02%–0.5% SDS | 25%–100% ethylene glycol | 10–20 mM EDTA or EGTA |
| 10–100 mM glycine, pH 1.3–3.0 | 10–100 mM glycine, pH 9.0–10.0 | 1–4 M $MgCl_2$ | 40 mM octyleneglycol + 20 mM CHAPS | 5%–50% DMSO | 10–200 mM imidazole |
| 10–100 mM phosphoric acid | 1 M ethanolamine HCl, pH 9.0 or above | 1 M KSCN | 40 mM octylglucoside | 1%–10% acetonitrile | |
| 0.1% TFA | 100 mM sodium carbonate + 1 M NaCl, pH 9–11 | 2–6 M guanidine HCl | | | |
| 100 mM formic acid | 20–100 mM NaOH containing 0.5% surfactant P20 or 0.05% SDS | | | | |

The purpose of optimizing the regeneration conditions is to find the mildest possible regeneration solution that completely dissociates the complex. Analysts should maintain a constant level of activity over the binding–regeneration cycles even if the baseline changes a little. Repeated cycles of analyte binding followed by regeneration of the surface will provide

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insight into the overall performance of the surface. Ideally the surface performance should be evaluated for the same number of cycles that will be used during the SPR experiment.

The surface must be monitored for signs of accumulation ▲(*Figure 4A*) ▲ (USP 1-Dec-2020)and also degradation of the immobilized ligand (*Figure 4*▲*B*▲ (USP 1-Dec-2020)). This can be accomplished by monitoring both the baseline at the beginning of each injection cycle and binding signal (slope or bound response) of a quality control sample. Appropriately defined acceptance criteria for system suitability such as baseline drift and quality control performance help to monitor the integrity of the immobilized ligand on the surface.

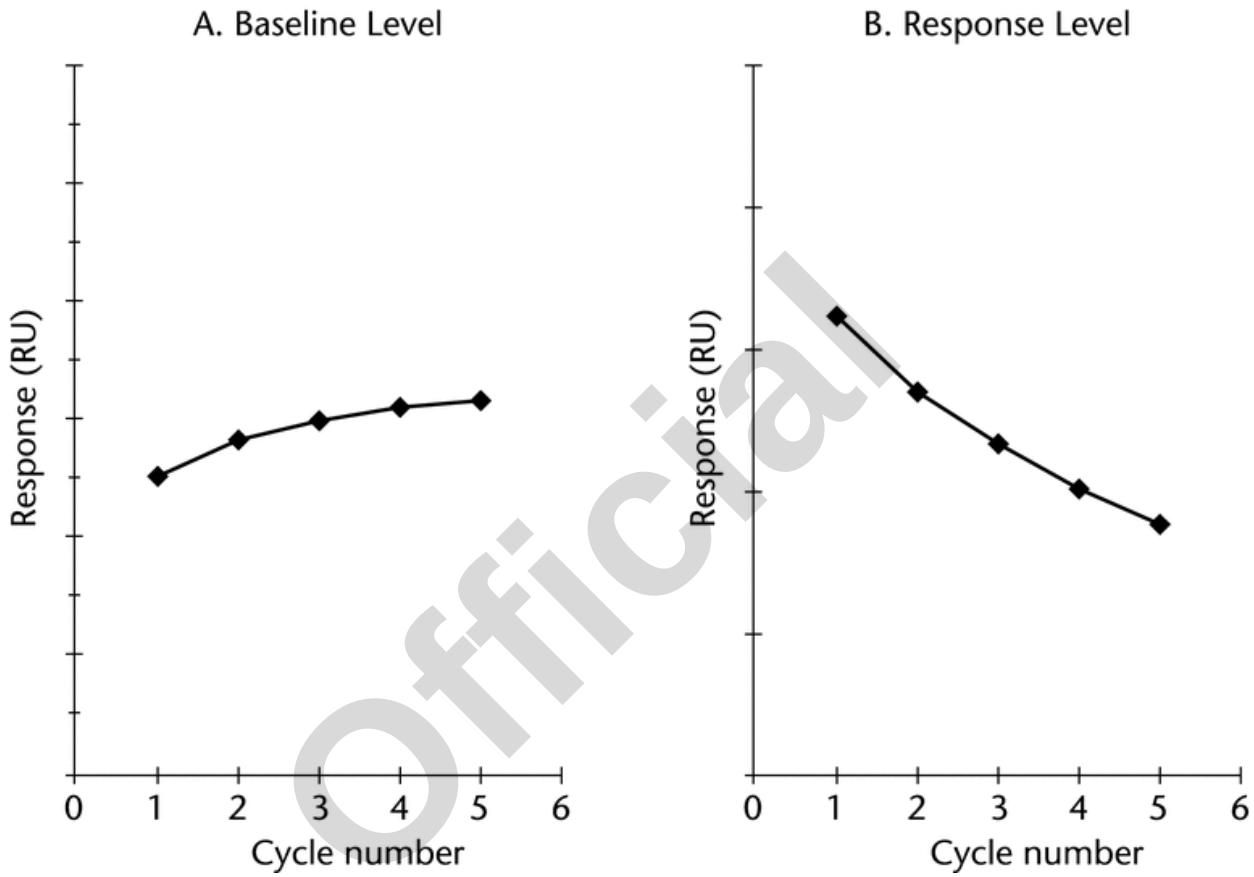


Figure 4. Evaluating surface performance (A) accumulation on surface and (B) degradation of immobilized ligand.

If the binding response is slowly decreasing, there are two possible explanations:

1. If the baseline increases, the accumulation of analyte ▲results in a decrease of binding capacity▲ (USP 1-Dec-2020) on the surface, which in turn decreases the amount of analyte that can be bound to the surface. Analysts can increase the strength of the regeneration solution slightly or can change to a regeneration solution of equal strength within the same class. There may be a difference between regeneration solutions in their ability to solubilize the analyte.
2. If the baseline of the raw data sensorgram remains constant but the binding response still decreases, the regeneration conditions cause an irreversible change to the biomolecule that decreases the binding capacity of the surface, which in turn decreases the amount of analyte that can be bound on the surface. Analysts can decrease the strength of the regeneration solution slightly or can change to another regeneration solution of equal strength within the same class.

Once a suitable regeneration solution has been determined, it should be tested in a series of analyte binding and regeneration cycles. Because the binding activity of the surface typically decreases with time and/or use, analysts must empirically determine the binding response threshold and consequent number of cycles for surface use. Examples for determining binding threshold and number of cycles are presented below (see *Applications 1–3*).

In some cases, the baseline will drop or rise, and/or the binding capacity will decrease somewhat in the first few injections before it stabilizes. This is caused by either the dissociation of electrostatically bound biomolecules from the surface (depending on surface characteristics) or to binding to a high-affinity non-regenerable fraction of the surface. For this reason, each newly immobilized surface should be conditioned with repeated analyte binding and regeneration cycles before collection of quantitative data. Alternative immobilization methods, such as a different chemistry or indirect capture, should be evaluated when the immobilized biomolecule is difficult to regenerate.

## DATA ANALYSIS AND INTERPRETATION

Analysis and interpretation of the data are specific to the experimental objective. Several data analysis programs exist to aid in the calculation of kinetics and affinity constants from SPR data. The validity and quality of the results are linked

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directly to experimental design. The fitting process is purely mathematical, without regard to the biological significance of the values obtained.

**Data analysis algorithm:** Global analysis seeks a single set of kinetic rate constants for all of the analyte concentrations used in the experiment. Using a data-fitting algorithm such as Marquardt-Levenberg the data analysis software begins an iterative process starting with an initial approximation to find the best set of parameters that produces agreement between the experimental data (sensorgram) and the calculated fit to the data. The iterative process continues until the difference between the experimental and calculated (theoretical) curves is minimized as measured by the sum of the squared residuals.

**Preparing the data for analysis:** Before conducting kinetic analysis, analysts should inspect the experimental data visually for anomalies or artifacts such as baseline disturbances or out-of-range data (often due to air bubbles) lasting for a predefined time period (e.g., 4–8 s). Outliers should be removed from the data set according to pre-established criteria. Nonessential data, such as capture or regeneration injections, should be removed from the sensorgram, and the data at each analyte concentration should be adjusted using the double-referencing procedure described below.

Before analysis the raw data should be processed in the following manner:

- Align the injection start to zero seconds for all concentrations and buffer injections for both the reference and active surfaces.
- Align the baseline to zero response for all sensorgrams.
- Subtract the reference surface sensorgram from the active surface sensorgram in order to create a corrected data set.
- Subtract the corrected buffer sensorgram from the sensorgrams at different concentrations in order to create a double-referenced data set.

The double-referencing procedure removes systematic errors (e.g., instrument noise) and low levels (less than 5% of total binding response) of nonspecific binding. It should not be used to correct for significant nonspecific binding events because this can lead to erroneous measurements.

When analyzing the data for kinetic information, analysts use the association (injection) and dissociation (buffer flow) phases for all of the concentrations in the series. For steady-state affinity analysis, the response at equilibrium $R_{eq}$ (data plateau or no change in response vs. time) is measured for each sensorgram to create a binding isotherm with $R_{eq}$ vs. concentration. This isotherm is analyzed using the equations described below.

**Kinetics and steady-state affinity models:** The Langmuir kinetic model assumes a 1:1 interaction between the binding partners so that

$$A + B \underset{k_d}{\overset{k_a}{\rightleftharpoons}} AB$$

The association and dissociation rate constants are defined below:

$$\frac{d[AB]}{dt} = k_a \times \left[A\right] \times \left[B\right]$$

$$-\frac{d[AB]}{dt} = k_d \times \left[AB\right]$$

Combining these two equations and defining $[B_{free}] = [B_{tot} - AB]$, the net rate expression is

$$d[AB]/dt = k_a \times [A_{free}]\,[B_{tot} - AB] - k_d \times [AB]$$

which can be translated into terms from the SPR experiment as follows:

$$dR/dt = k_a \times C \times (R_{max} - R) - k_d \times R$$

where $R$ is the binding response at any point along the sensorgram and $C$ is the known ▲molar▲ (USP 1-Dec-2020) analyte concentration. Using global analysis as described above, $k_a$, $k_d$, and $R_{max}$ are calculated from the experimental data using the rate equations shown below:

Association: $dR/dt = k_a \times C \times (R_{max} - R) - k_d \times R$

Dissociation: $dR/dt = -k_d \times R$

Because the concentration of analyte is zero during dissociation, the rate equation for dissociation depends only on the response, $R$, and the dissociation rate constant, $k_d$.

Application of the equilibrium condition where the complex formation (association) equals complex decay (dissociation)

$$k_a \times [A] \times [B] = k_d \times [AB]$$

yields the following equation for the equilibrium dissociation constant

$$K_D = \frac{\left[A\right] \times \left[B\right]}{[AB]} = \frac{C\left(R_{max} - R_{eq}\right)}{R_{eq}}$$

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where $R_{eq}$ is the binding response at equilibrium that is measured in the experiment for a given analyte concentration ▲(*C*) assumed not to change during the course of association,▲ (USP 1-Dec-2020) and $K_D$ and $R_{max}$ are calculated using global analysis.

Kinetics binding models can be used to describe non-1:1 interactions, e.g., bivalent interactions that occur if an antibody is used as the analyte, heterogeneity in binding partners, conformational change, or more complex interactions such as cooperative binding. SPR analysts are cautioned against using more complex models to assess data unless experimental design has been confirmed.

**Assessing the fit:** The quality and validity of the fit to the kinetic data can be assessed by ▲(1) $k_a$ and $k_d$ based on the operating range of the instrument,▲ (USP 1-Dec-2020) (2) visual inspection of the agreement between the experimental and calculated curves, (3) the size and the shape of residual plots, (4) the biological relevance of the results, and (5) statistical parameters such as $\chi^2$ ▲(*Chi*$^2$,▲ (USP 1-Dec-2020)average of squared residuals), and standard error (SE), *T*-value or *U* (uniqueness) factor. The best parameter fit to the experimental data should be superimposed on the curve for each concentration in the experiment. The residual plot visualizes the difference between the calculated and experimental data. The ▲distribution▲ (USP 1-Dec-2020) of the residuals should be random without trending (waviness or curving up or down). The height of the residual plot should reflect the instrument noise. Further, $\chi^2$ should be minimized for a good fit with values that depend on the instrument noise, number of data points, and overall binding response. The parameter values should be considered for biological and experimental relevance. For example, is the calculated $k_a$ value slow when the interaction is known to be fast, or is the calculated $R_{max}$ value higher than the value that was calculated using Equations 1 and 2. Parameter significance is evaluated based on standard error, *T*-values, and *U* factor. Parameters that are significant cannot be changed without affecting the quality of the fit. All ▲▲ (USP 1-Dec-2020) the criteria should be within acceptable limits.

A similar set of criteria can be used for assessing the fit to steady-state affinity data, but because there are fewer data points (6–12 total, depending on the number of concentrations used), the statistical parameters and residual plots are less predictive of fit quality. Visual inspection of the agreement between the experimental and calculated binding isotherms and the parameter relevance are good tools to use for assessing the fit. Additionally, according to the relationship between concentration, $K_D$, and $R_{max}$, when concentration equals to $K_D$ , ▲$R_{eq}$ will be▲ (USP 1-Dec-2020) 50% of $R_{max}$. Confirming that the analyzed data follow this relationship provides another way to check the validity of the calculated result. When it is practical to calculate the $K_D$ using both kinetic and steady-state analysis approaches, the $K_D$ values should agree within experimental error.

**Addressing a lack of fit:** When the data do not fit or the parameter values do not make sense, often the problem can be resolved by a systematic approach that considers potential sources for deviations and tests each hypothesis. Items to consider include reagent purity, immobilization chemistry or surface density, analyte concentration errors, nonspecific binding, loss of ligand activity, or mass-transport–limited binding. Reviewing the raw (uncorrected) data helps determine the source of nonspecific binding or concentration errors. *Table 4* lists potential sources for deviations from 1:1 binding and recommended actions.

**Table 4. Common Sources for Deviation from 1:1 Binding Kinetics**

| Source of Deviation | Recommended Action |
|---|---|
| Nonzero baseline before injection | • Normalize response to zero and reanalyze |
| Incorrect injection start and stop times or poorly defined injection start/stop | • Adjust injection start/stop<br>• Remove sensorgram artifacts (e.g., injection or air bubble spikes) |
| Concentration input errors | • Verify concentration values and reanalyze |
| Bulk refractive index contribution too high | • Use double-referencing approach before analysis<br>• Set RI = 0 |
| Mass-transport–limited binding | • Vary flow rate (slow to fast) for a single concentration and overlay sensorgrams (should be identical for same association and dissociation time)<br>• Include mass transport term, $k_m$, in fitting model<br>• Reduce surface density |
| Nonspecific binding | • Change immobilization chemistry<br>• Change sensor surface properties<br>• Buffer additives—add or minimize<br>• Reagent purity—repurify samples |
| Loss in binding partner activity | • Change immobilization chemistry<br>• Change regeneration solution<br>• Re-analyze data using local instead of global parameter fitting for $R_{max}$ |
| Multi-valent binding interaction | • Immobilize multivalent binding partner<br>• ▲Select sensor surface (e.g., planar surface) that minimizes potential for multi-valent binding▲ (USP 1-Dec-2020) |

Recommendations for data analysis will be introduced in the subsequent sections of this chapter.

**APPLICATION 1—IMMUNOGENICITY ASSESSMENT**

SPR has emerged as a powerful technique for assessing immunogenicity of protein therapeutics. An advantage of this platform for detecting antibodies in serum (or plasma) samples is that it allows label-free detection based on mass accumulation in real time, which potentially allows detection of low-affinity antibodies of all classes and subclasses. This technology is useful both for screening assays (first-tier immunoassays that are used to detect the presence of antibodies

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capable of binding to a protein therapeutic) and ▲▲ (USP 1-Dec-2020) characterization assays. Characterization assays are useful for defining generated antibodies that bind to the protein and can include analysis of antibody concentration, isotype(s) represented, relative binding affinity, and binding specificity. A limitation is that SPR it is not appropriate for determination of the neutralizing capability of antibodies, which is best determined using cell-based biological assays. When designing and validating SPR assays for immunogenicity assessment, analysts should consider critical parameters including protein immobilization to ensure immunological reactivity, immobilized protein stability, and surface regeneration conditions.

**Protein immobilization:** The first step in the development of immunogenicity assessment assays is to identify the optimum mechanism for immobilization of the target protein. When considering the target density for immobilization, analysts often recommend that a high-density surface be used. The advantage of a high-density surface is that it maximizes the opportunity that anti-therapeutic antibodies will ▲encounter▲ (USP 1-Dec-2020) an immobilized ligand. A high-density surface also provides excellent assay sensitivity. An important aspect of these assays is that the chemistry chosen for immobilization should provide random orientation rather than a site-directed orientation so that all potential epitopes on the therapeutic protein are available for binding by the anti-therapeutic antibodies. The effectiveness of immobilization is determined by evaluating the ability of positive control antibodies to bind to the immobilized protein. When evaluating the effectiveness of immobilization, analysts should test multiple antibodies with different epitope specificities. When panels of antibodies that cover a range of affinities and bind to different epitopes on the target protein are all capable of binding, this provides confidence that antibodies contained in clinical specimens also will be detected. If any of the positive control antibodies do not demonstrate binding, this suggests that the immobilization is not optimal and should be modified. Although SPR is demonstrably efficient at detecting low-affinity antibodies, analysts should confirm that the immobilization protocol chosen is effective for detection of low- and high-affinity antibodies.

**Protein stability upon immobilization:** The positive control antibody must be able to bind to the immobilized protein ▲▲ (USP 1-Dec-2020) for an assay result to be acceptable. This confirmation of binding provides confidence that if antibodies against a protein are present in a sample, they will bind to the immobilized protein on the surface of the sensor. Because SPR relies on re-using the immobilized protein surface for multiple analyses, a regeneration protocol is required to effectively remove any bound material from the immobilized protein. This regeneration procedure is based on the ability to remove bound material without damaging or removing the immobilized protein ▲(see *Surface Regeneration*).▲ (USP 1-Dec-2020)

The stability following regeneration should be established during assay development and should be confirmed during assay validation. ▲▲ (USP 1-Dec-2020) To monitor the performance of the sensor during an assay, analysts should periodically test a positive control sample during an assay run. If the performance of the positive control samples indicates the immobilized protein has been compromised, analysts should re-analyze test samples obtained after the performance of the assay dropped below acceptable limits. Acceptance parameters for immobilization may vary by compound and should be established for each assay.

**Availability of epitopes after immobilization:** Once the protein is immobilized, the availability of multiple epitopes should be confirmed. Ideally this is done by testing for binding of positive control antibodies with different epitope specificity. One method for testing epitope availability is to use a panel of monoclonal antibodies that are known to recognize different regions of the protein. If the protein has been randomly immobilized, all the different positive control antibodies should be able to bind. The reason for evaluating epitope availability is to prevent false-negative results when serum samples are evaluated. If the immobilization is not random, it would be possible to consistently immobilize the protein via a specific epitope, thus making that epitope unavailable for binding by an antibody. Another possibility is that chemical modification of the protein to facilitate immobilization altered the protein's conformation.

**Surface regeneration and subsequent protein stability:** Using the previous guidelines, analysts should monitor the surface for signs of accumulation and degradation of the immobilized ligand and discontinue use when necessary. For example, when the binding capacity of a positive control antibody (diluted in test serum) drops below 80% of initial capacity the surface should not be used.

**Assay cut-point determination:** When performing assays to determine if a ▲complex sample (e.g., serum)▲ (USP 1-Dec-2020) contains antibodies against a protein, analysts sometimes observe a background level of binding. That background binding can vary depending on the nature of the immobilized protein and also the patient population being tested. ▲▲ (USP 1-Dec-2020) To determine if a test sample contains antibodies ▲of interest,▲ (USP 1-Dec-2020) analysts compare binding to control samples that do not contain antibodies against the protein. ▲In doing so,▲ (USP 1-Dec-2020) a cut-point ▲can be▲ (USP 1-Dec-2020) established, ▲which defines the response level of the assay where the sample can be regarded as positive for containing the desired antibodies.▲ (USP 1-Dec-2020) Analysts determine the assay cut-point by analyzing a series of serum samples that do not contain antibodies against the immobilized protein and then performing statistical analysis to determine the level of binding consistent with a sample that does not contain antibodies. The cut-point should be established using the same conditions that will be used for sample analysis. Although different approaches are used for determining a cut-point, a common approach is to establish the mean from the binding of 50–100 serum samples from healthy volunteers and set the cut-point at 95% (equivalent to the mean plus 1.645 times the standard deviation for a normal distribution). Analysts should remove statistical outliers from the calculations because their inclusion can cause a high bias and raise the cut-point. This higher cut-point will result in identification of fewer samples with antibodies against the immobilized protein. The statistically evaluated cut-point is the response unit value that serum samples must exceed to be considered positive for the presence of antibodies against the therapeutic protein. An important feature of cut-point determination is that it may be different in different patient populations. For example, patients with inflammatory disease, may show a higher level of nonspecific reactivity compared to a normal population. This higher level of nonspecific binding would result in samples being identified as positive when they did not contain any antibodies specific for the protein. When this situation arises, pre-dose serum samples ▲from those patients with higher levels of nonspecific binding▲ (USP 1-Dec-2020) can be used to establish a new patient population-specific mean and assay cut-point.

**Analytical procedure development and validation:** Once the stability of the immobilized protein is confirmed, a regeneration procedure has been defined, and the cut-point established, the antibody testing method can be developed and validated. The conditions used for analyzing samples should be identical to those used to establish the assay cut-point. An important parameter to consider is the optimal dilution of the serum sample. Increasing the dilution factor reduces nonspecific binding by serum proteins but also reduces overall sensitivity. Most antibody assessment procedures use between 5% and 50% serum. As the percentage of serum that is tested decreases, the percentage of the binding signal that is due to nonspecific interaction also decreases, and subsequently the percentage of the signal mediated by antibodies binding to the immobilized protein increases. Besides dilution, other means to reduce nonspecific interaction include adding surfactants, increasing salt concentration, adding BSA or HSA, or adding soluble sensor surface support material such as carboxymethyl dextran or alginate to the dilution and running buffer.

Other important variables to optimize include flow rate and sample volume. The combination of flow rate and sample volume defines the contact time, the length of time during which a given sample is in contact with the immobilized protein. The longer a sample is in contact with the immobilized protein, the greater the chances for antibody binding. The next important aspect to consider is verification that initial binding is a result of an antibody and not some other serum component. This can be accomplished by adding an anti-human immunoglobulin reagent and monitoring subsequent binding. If the initial binding observed was due to an anti-protein antibody, this reagent will bind to that antibody (the anti-protein antibody remains bound to the immobilized protein). When a therapeutic monoclonal antibody is the immobilized protein, the confirmatory reagent must be screened and verified not to bind directly to the immobilized therapeutic monoclonal antibody. One option here is the immobilization of the Fab' fragment rather than the intact therapeutic monoclonal antibody. The confirmatory reagent must be verified for specificity. Once all of the parameters are optimized, the assay can be validated. Validation parameters include those typically associated with immunogenicity assays (precision, specificity, sensitivity, and robustness) as well as parameters specific to SPR assays (protein immobilization, stability of immobilized surface, and number of regeneration cycles).

**Interference by serum components:** Depending on the immobilized protein, serum components other than antibodies specifically directed against the immobilized protein possibly could bind to the immobilized surface. It is also possible that serum components that block the ability of antibodies to bind to the immobilized protein could be present. Both ▲▲ (USP 1-Dec-2020) can be evaluated by testing the binding of serum samples from the target subject population that are known not to contain antibodies against the immobilized protein and then monitoring to determine if any binding does occur. If nonspecific binding is identified, steps can be taken to reduce or eliminate it. These steps can include pretreatment of samples to remove the nonspecific reactant, addition of surfactant, or alteration of salt concentration in sample buffers to reduce nonspecific binding.

Analysts should verify that serum samples do not contain agents that are capable of inhibiting antibody binding to the immobilized protein (these could include soluble forms of the immobilized protein or soluble receptors that could bind to the immobilized protein and block binding of the antibodies to the immobilized protein). Analysts can add the positive control antibody to target serum samples and can evaluate binding. If binding is inhibited by the target serum samples compared with binding to normal human serum samples, steps can be taken to remove the inhibiting agent. Failure to identify target serum interference can result in either false-positive or false-negative results.

**Implementation of multiplex assays:** When a therapeutic protein is a second-generation product that has been modified from an original therapeutic protein (e.g., via pegylation or increased glycosylation), the presence of antibodies against both the original and the second-generation product should be evaluated simultaneously. This can be accomplished by immobilizing each protein on separate channels in the microfluidic device and allowing serum samples to bind in series or in parallel to both immobilized proteins. The rationale for testing for binding to both the original and the modified therapeutic protein is that antibodies generated against the modified protein could have binding specificity to the original protein as well. As part of the characterization of the immune response, analysts must understand the specificity of antibodies for both first- and second-generation products. When possible, binding to an endogenous counterpart might also be tested by immobilizing the endogenous protein on a separate flow cell or channel.

**Characterization of anti-therapeutic protein antibodies:** Once antibodies against a therapeutic protein have been captured by binding to the immobilized therapeutic protein, those antibodies can be characterized. The important features of anti-therapeutic antibodies that can be studied include the relative binding affinity, the amount of antibodies present in the serum sample, the isotype(s) of antibodies present in the sample, and binding specificity.

By monitoring the rate at which the response units decrease after the conclusion of sample addition to the sensor, analysts can determine the relative affinity of the antibodies. A high rate of dissociation is characteristic of a low-affinity antibody, and a slow rate of dissociation suggests the presence of high-affinity antibodies. It is useful to compare the dissociation rates with both the positive control antibody (typically a high-affinity antibody preparation) as well as a panel of monoclonal antibodies of known binding affinities.

The relative active concentration of antibodies present in a sample can be estimated by comparing the binding signal with the signal produced from a dilution series of the positive control. Analysts can generate a standard curve from the standard and can compare the active concentration of antibodies in the sample with that standard curve. Because the positive control does not exactly mimic the mixture of antibodies contained in the sample—in fact, the positive control is often obtained from hyperimmunized animals such as rabbits—the concentration value obtained is relative to the standard. This value only approximates the actual concentration of human antibodies. Because the same positive control can be used throughout clinical development, analysts can compare the amount of antibodies obtained from different subjects using this strategy. Because the instrument's signal is proportional to the mass that is binding to the sensor, this type of analysis provides value. Analysts should consider that IgM antibodies have five times the mass of IgG antibodies. Another approach for determining the concentration of antibodies is described in the concentration analysis section of this chapter (see *Application 2* below).

The isotype of captured antibodies can be readily determined by monitoring binding associated with sequential addition of isotyping reagents. For example, if IgM antibodies are present and have bound to the immobilized protein, the

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addition of an anti-human IgM reagent will produce an additional signal. Isotyping reagents can be found with specificity towards IgM, IgG, IgE, IgA, IgG1, IgG2, IgG3, and IgG4. Because of steric hindrance, analysts may be required to repeat isotyping analyses in different sequences to be certain the presence of previously bound isotyping reagents has not hindered subsequent analyses. For example, assume a sample contains both IgG1 and IgG4 antibodies against a protein and both species have bound to the immobilized protein. Because the anti-IgG1 isotyping reagent has bound to the IgG1 antibodies, the isotyping reagent bound to them may prevent subsequent additions of an anti IgG4 reagent from binding to the IgG4, and the presence of the IgG4 would be undiscovered. Analysts will conclude that only IgG1 antibodies are present, but if the order of isotyping reagent addition were reversed the IgG4 antibodies would be discovered. This example underscores the importance of careful interpretation of isotyping results. This is a problem for subsequent analysis only if there is observed binding by a previous cycle of isotype reagent addition. The specificity of isotyping reagents should be confirmed before use. Analysts should, for example, verify that an anti-human IgG reagent binds only to human IgG and does not cross-react with human IgM.

The region of the therapeutic protein recognized by the antibodies can sometimes be determined by immobilizing versions of the protein that have been truncated, have point mutations, or contain only a fragment of the protein. If the antibodies fail to bind to the changed version of the protein, it suggests that the epitope toward which the antibody is directed was influenced by the change. It should be kept in mind that point mutations and truncations not only influence the primary sequence of a protein, but can also influence the tertiary structure (▲e.g., folding and▲ (USP 1-Dec-2020) conformation ▲▲ (USP 1-Dec-2020)) of a protein. Also, a subject is likely to generate a population of antibodies with different specificities for a variety of epitopes. Despite this concern, the strategy just described can prove useful for identifying the region on the protein where the antibodies are binding.

**APPLICATION 2—CONCENTRATION ANALYSIS**

SPR can be used to determine the concentration of biologics in defined buffer systems, e.g., eluates from purification columns, formulation buffers, and complex mixtures such as serum, fermentation broths, crude cell extracts, and cell suspensions. The concentration of an analyte is measured by its binding to the specific ligand or other molecules that can interact with any portion of the analyte. The analyte concentration is determined on a surface where the analyte-specific ligand or an analyte-specific capture reagent is immobilized. The binding rate or the mass of analyte bound is determined, and the analyte concentration is calculated using either a standard curve obtained from a concentration series of a purified and well characterized reference material or by a calibration-free analysis that is based on the relationship between the diffusion properties of the analyte and the absolute analyte concentration.

**Immobilization of ligand:** To determine concentration the ligand is immobilized covalently or non-covalently on the surface. Analysts select an appropriate coupling mechanism and chemistry to ensure the ligand's functional integrity. ▲▲ (USP 1-Dec-2020) To provide conditions that favor partial or full independence of kinetic parameters, a high surface density of the ligand is desired.

A high-density surface allows the analyte to bind to the ligand under conditions that limit mass transport. The interaction between the analyte and the ligand can be described by the following two-step process:

$$A \xrightarrow{k_m} A_{surface} + B \underset{k_d}{\overset{k_a}{\rightleftarrows}} AB$$

where $A$ is the concentration of analyte in the sample, $A_{surface}$ is the concentration of analyte at the sensor surface, $k_m$ is the mass transport coefficient, $B$ is the immobilized ligand, $AB$ is the analyte–ligand complex, and $k_a$ and $k_d$ are the rate constants for the reaction between $A$ and $B$. The mass transport coefficient $k_m$ depends on the flow rate, the dimensions of the flow cell, and the diffusion coefficient of the analyte. The analyte first must be transported from the bulk to the sensor surface to react with the immobilized ligand molecules. If this mass transport of the analyte is much faster than the association step between ligand and analyte, the overall observed binding will be driven by the kinetic rate constants of $A$ and $B$, a prerequisite for accurately determining the kinetic parameters. If the mass transport of analyte is much slower than the association step, the binding will be limited by the mass transport process, and kinetic parameters for the specific interaction between $A$ and $B$ will be difficult to obtain. However, these conditions are desired for determining the active concentration of an analyte. A high density of ligand on the sensor surface and slower flow rates favor limited mass transport. Between these extremes, the overall binding is determined by contributions from both mass transport and interaction kinetics. It may not be possible to achieve limited conditions of mass transport for concentration analyses of interactions with relatively slow association rate constants (e.g., $k_a = 10^4\ M^{-1}s^{-1}$).

The ligand and the analyte reference material should be of sufficient purity with special attention to the presence of aggregated material. Aggregates of the analyte can interfere with the regeneration of the ligand surface because they can bind with multiple binding sites.

The reference material must be comparable (e.g., molecular weight and kinetic parameters) to the test samples. Under certain conditions the active concentration in unknown samples can be determined using a calibration-free procedure that is based on the relationship between the diffusion properties of the analyte and the absolute analyte concentration. These two methodologies are described separately below.

**Concentration determination with a reference standard curve:** In typical concentration-determination assays the analyte concentration is calculated from a standard curve that is obtained with a reference material injected at select concentrations. Three different approaches can be used to measure concentration with a reference standard calibration curve.

**Direct binding assay:** Determine the quantity of analyte bound after an arbitrarily fixed sample injection time. A sandwich method can be performed as an extension of the single-step direct binding approach ▲▲ (USP 1-Dec-2020) to increase assay sensitivity.

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**Binding rate determination:** Determine the initial binding rate for a sample rather than the amount bound. Under conditions of mass-transport limited binding, the binding rate is directly proportional to analyte concentration, and is independent of binding kinetics. This allows one to measure the concentration of related molecules that might have different binding characteristics.

**Inhibition or competition assays:** When the mechanism of action for an analyte is binding to a soluble ligand and thereby disrupting a ligand–receptor interaction, an inhibition assay can be used. In an inhibition assay, a receptor is attached to the sensor surface by a covalent linkage. The interaction between the analyte and the soluble ligand is indirectly measured by mixing a fixed concentration of ligand with varying concentrations of the analyte and injecting the ligand–analyte mixture across the immobilized receptor surface. Competitive methods in solution can also be used for large molecules and particles such as viruses, as well as for small analytes that give low direct responses. In parallel systems the assay can be designed so that the standard samples and the unknown sample are injected in parallel. This method can be useful for ligands that are difficult to regenerate.

Analysts can plot the signal (amount bound or rate of binding) of the reference material standards against concentrations and then can generate a standard curve using an appropriate mathematical model such as a linear or a logistic four-parameter curve fit. Samples can be injected at one or more dilutions. Fewer dilutions can be employed if a linear relationship between sample and reference standard has been demonstrated. Concentrations of unknown samples are either obtained by back-calculation from the standard curve or, if they are analyzed at the same target concentrations as those of the reference standard curve, by comparison of curve-fit parameters.

Parameters that can influence assay performance and results include but are not limited to flow rate, ligand density on the surface, sample purity, sample matrix, and reproducibility of surface regeneration. These parameters must be evaluated during assay qualification or validation. Interference with binding of analyte to the immobilized ligand can be minimized by salts, detergents, or sensor-surface support material. A commonly used sensor surface consists of carboxymethylated dextran, so the addition of dextran to the sample dilution buffer can minimize nonspecific interactions. Injections over a negative control surface can also be used to mathematically subtract the nonspecific binding data from the data obtained on the positive surface. A qualified or validated concentration determination SPR assay should include QC samples that can serve as measure to determine the accuracy of the standard curve that has been prepared to analyze samples with unknown analyte concentration. They can be conveniently prepared in larger batches, qualified for use with a Certificate of Analysis for the target concentration, and stored in small aliquots under appropriate storage conditions.

After each analyte injection the ligand surface is regenerated and all bound analyte is removed. This regeneration must be strong enough to remove all bound analyte, but the conditions also must leave the immobilized ligand intact so that injections can be compared to each other.

**Concentration determination without calibration:** Calibration-free concentration assays are based on the relationship between the diffusion properties of the analyte and the absolute analyte concentration. By measuring the initial binding rate analysts can derive the analyte concentration if specific properties of analyte and the analytical environment are known. This approach can be useful when no satisfactory reference standard is available.

To determine the analyte concentration in a sample, analysts use the relationship between initial binding rate and analyte concentration. On a sensor surface with a high immobilization level, the initial binding rate (slope) can be described as a function of the molecular weight, the mass transport coefficient $k_m$, and the concentration of the analyte. Before a sample is analyzed analysts must determine the mass transport coefficient. It depends on the diffusion coefficient ($D$), flow rate, and flow cell dimensions and is described by the following formula:

$$k_m = 0.98 \times \sqrt[3]{\frac{D^2 \times f}{0.3 \times h^2 \times w \times l}}$$

where $D$ is the diffusion coefficient, $f$ is the flow rate, and $h$, $w$, and $l$ are the flow cell height, width, and length, respectively. Flow rate and flow cell dimensions typically are known for a given instrument, and the diffusion coefficient is determined by the size and shape of the molecule ▲using▲ (USP 1-Dec-2020) instrument-specific tools, literature references, or experiments, e.g., by analytical ultracentrifugation or light scattering.

In a typical experimental setup the evaluation requires two flow rates. By using measurements at two widely separated flow rates, analysts can assess the influence of flow rate on binding rate. The robustness of the assay is also improved by fitting the data obtained at two different flow rates, which give correspondingly two different values for $k_m$ (because $k_m$ depends on the flow rate), to a model with a global variable for analyte concentration (so that the model is constrained to find a single concentration value that best fits both curves simultaneously).

Calibration-free concentration analysis is suitable only for proteins with MW ≥ 5000 Da. It requires fast analyte–ligand association ($k_a > 5 \times 10^4$ $M^{-1}s^{-1}$) and it cannot handle mixtures of analytes with different diffusion properties. The dynamic range of the method is approximately 0.05–5 µg/mL.

### APPLICATION 3—KINETIC AND AFFINITY ANALYSIS

Because of its ability to detect binding interactions in real time, SPR provides valuable information about the kinetics of complex formation and dissociation. SPR instruments can be used to determine the association rate constant and dissociation rate constant for a ▲▲ (USP 1-Dec-2020) binding interaction, and these values can be used subsequently to calculate the dissociation equilibrium constant ($K_D = k_d/k_a$). Obtaining $K_D$ from a ratio of $k_a$ and $k_d$ is useful when the binding interaction does not reach equilibrium in a timeframe that is suitable for an SPR binding experiment. For binding interactions that reach equilibrium (rate of complex formation equals the rate of complex decay) in min (vs. h), $K_D$ can be determined directly from a steady-state binding response. The length of time required to reach equilibrium is influenced by the dissociation rate, so quickly dissociating complexes (e.g. $k_d = 10^{-2}$ $s^{-1}$) will reach equilibrium faster than those that dissociate slowly (e.g., $k_d = 10^{-5}$ $s^{-1}$). Software programs capable of simulating 1:1 binding kinetics are

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useful for predicting the length of time required to reach equilibrium. The typical working range for affinity measurements with commercially available SPR instruments is from $10^{-12}$ M (pM) to $10^{-4}$ M (μM).

Proper experimental design is required to accurately measure $k_a$, $k_d$, and $K_D$. Several questions must be considered when designing kinetic analysis or steady-state affinity experiments, including:

- Which binding partner should be immobilized?
- How will the analyst immobilize one of the binding partners?
- What type of reference surface should be used?
- How much binding partner should be immobilized?
- Does the binding partner maintain activity after immobilization?
- Is binding to the immobilized binding partner specific?
- What regeneration conditions, if necessary, should be used?

When selecting which binding partner to immobilize for most protein–protein interactions, analysts must consider several factors: (1) the purity and availability of the proteins, (2) the presence of a tag or functional group to aid in immobilization, (3) maintaining biological activity, and (4) the binding valency (e.g., monovalent vs. multivalent binding).

A reference surface is required for all detailed kinetic and affinity analysis experiments. If direct immobilization is used, then a reference surface is created using the same immobilization protocol, omitting the protein during the coupling step. Alternatively, a mutant form of the protein with a modified binding site can be used. The reference surface for high-affinity capture typically consists of either the capture molecule and no binding partner or uses an unrelated molecule for a mock capture surface. For the specific case of antibody–antigen interactions, an unrelated monoclonal antibody often serves as the capture reagent on the reference surface.

After deciding on the immobilization approach, analysts must decide how much binding partner to immobilize. For kinetic analysis, the primary consideration is to minimize the surface density to avoid mass-transport–limited binding of the analyte molecule to the immobilized binding partner. Analysts also must consider the immobilization level when conducting steady-state affinity analysis because high immobilization levels can cause steric hindrance or can induce secondary effects such as nonspecific binding or aggregation.

Before performing a kinetic experiment or steady-state affinity analysis, analysts must assess the activity of the surface by injecting the analyte molecule at a single concentration. The concentration should be high enough that the equilibrium binding response provides a close approximation of the experimental maximum response ($R_{max}$). This condition is typically met when the target molecule concentration is at least 10-fold higher than the $K_D$ of the binding interaction. For a protein–protein interaction having a $K_D$ value of 100 pM this means that the target molecule should be injected at a concentration of at least 1 nM. Using the $R_{max}$ equation (*Equations 1* and *2*), analysts can calculate the theoretical $R_{max}$ based on the amount of binding partner immobilized or captured. If the experimental $R_{max}$ exceeds the theoretical $R_{max}$, then the analyte molecule is larger than expected or the analyte exists in a higher-order structure than expected (e.g., following aggregation or as a multimer). If the experimental $R_{max}$ is significantly lower (<50%) than the theoretical $R_{max}$, this suggests that the immobilization procedure has compromised the binding site, and an alternative immobilization procedure should be investigated. An advantage of using high-affinity capture instead of direct coupling is that the surface activity typically remains close to 100%, provided that the specific activity of the immobilized binding partner is 100% before capture.

Injecting the target molecule across the reference surface also provides a quick assessment of the amount of nonspecific binding that exists on the sensor surface. Nonspecific binding that is electrostatic in nature can be eliminated or reduced by addition of salt (e.g., 0.5–1.0 M NaCl) to the sample diluent buffer and the running buffer or by using sensor surfaces with a low charge density. Nonspecific binding that arises from hydrophobic interactions can be minimized or eliminated by the addition of detergents such as 0.05% Polysorbate 20 or 10 mM CHAPS to the sample diluent buffer and the running buffer. Before using buffer additives, analysts should test whether the specific binding interaction or binding activity is affected. Nonspecific binding to a capture molecule can be resolved by switching to a different capture molecule. Reducing surface density also may eliminate nonspecific binding.

Many protein–protein interactions dissociate slowly ($k_d = 10^{-3}$ to $10^{-6}$ $s^{-1}$), with complex half-lives ($t_{1/2}$) of more than 2 h. A regeneration step is important for these types of binding interactions.

In some protein–protein interactions, surface regeneration may not be possible because of a high-affinity binding interaction between the molecules. If this situation occurs, analysts can consider a titration kinetic experiment or the use of instruments that ▲can perform▲ (USP 1-Dec-2020) parallel analyte injections. In a kinetic titration, increasing concentrations of target molecule are injected consecutively across the immobilized surface, and the resulting data are analyzed using a titration kinetics model. In parallel instruments the analyte concentration series is injected in one step, thus eliminating the need for regeneration.

Surface regeneration typically is not performed for steady-state affinity experiments because the binding interactions in this type of experiment have $k_d$ values in the range of $10^{-2}$ to 0.5 $s^{-1}$, and therefore have $t_{1/2}$ values of <2 min. The bound analyte dissociates from the surface as buffer flows over the surface, and regeneration is not required.

Analysts should have an accurate analyte concentration for kinetic analysis because the calculation of $k_a$ depends on the analyte ▲molar▲ (USP 1-Dec-2020) concentration. Typically, an absorbance reading at a wavelength of 280 nm ($A_{280}$) is used for this purpose, but analysts should remember that the $A_{280}$ value reflects the total bulk protein in solution and does not reflect the actual concentration of protein that is capable of binding (i.e., the active concentration).

Sample diluent injections should be included in replicate for kinetics and steady-state affinity experiments so that nonspecific responses due to instrumentation or sample diluent can be removed during the data-evaluation process.

The recommended analyte concentration range is 10-fold below and above the $K_D$ for the interaction. By keeping the surface density low ($R_{max}$ = 5–50 RU) and extending the association time, analysts should be able to collect data with

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enough curvature to accurately define $k_a$ and $k_d$ values. When the affinity of the interaction is high ($K_D$ = low nM to pM), higher analyte concentrations may be required to build a kinetic profile with sufficient curvature to complete the kinetic analysis. Increasing the analyte concentration should not be a substitute for changing other experimental design parameters (e.g., surface density and contact time). Although it is desirable to use analyte concentrations that approach $R_{max}$, unusually high concentrations may induce aggregation of the analyte in solution or nonspecific binding to the surface.

Within an analyte concentration series, replicate samples should be used to assess surface activity. Additionally, each concentration series should be tested 3–5 times using different surfaces in order to establish confidence intervals for the resulting kinetics and affinity constants.

The amount of data that is collected during the kinetic or steady-state affinity experiment affects the accuracy of the results. For kinetic experiments involving binding interactions with slow $k_d$ values, analysts should collect enough dissociation data so that a measurable dissociation response (vs. instrument noise) is acquired. For example, a binding interaction between a therapeutic monoclonal antibody and its target molecule that has a $k_d$ of $10^{-5}$ $s^{-1}$ ▲may▲ (USP 1-Dec-2020) require the collection of at least 4 h of dissociation data. Rather than collecting this much dissociation data for all analyte concentrations, analysts can use a long dissociation time for the highest concentration of analyte that will be injected, and they can use a short dissociation time (2–5 min) for all other analyte concentrations. With parallel-injection instruments one can collect the long dissociation data for all of the concentrations. For data evaluation analysts should collect both long and short dissociation time data for sample diluent injections. For steady-state affinity experiments, the analyte injection time should be long enough to allow for a steady-state binding response to occur at all analyte concentrations that are tested. Dissociation data do not have to be collected for a steady-state affinity experiment because dissociation data are not used in the evaluation of this type of experiment, but the complete dissociation of analyte is required before beginning the next injection.

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**USE OF SPR IN A REGULATED ENVIRONMENT**

When SPR assays are used for lot release and stability testing, the assay must exist within a controlled setting so that decisions can be made about the use of product within the clinic or marketplace. SPR instrumentation, including software, should be 21 CFR Part 11 compliant and should be amenable to validation. For example, an installation and operation qualification should be conducted prior to the use of the instrument in any regulated application. These requirements are important because they help ensure the integrity of both data acquisition and data evaluation as well as critical instrument hardware performance.

Besides using SPR instrumentation that meets regulatory requirements, analysts should establish system suitability criteria for an SPR assay. Including these criteria in an SPR assay ensures that the results obtained for the test sample are generated by an assay that is performing within its operating parameters. A discussion of assessing system suitability parameters is not within the scope of this chapter, but the reader is referred to USP general chapters *Design and Analysis of Biological Assays* ⟨111⟩ and *Analysis of Biological Assays* ⟨1034⟩ for more detailed discussions. Some examples of system suitability parameters for an SPR assay can include:

- Ligand immobilization density
- Parameters from a curve fitting model (e.g. four parameter logistic curve fit)
  - $EC_{50}$ values for reference standard curve and positive QC curve
  - Effective asymptotes (response range) for reference standard curve and positive QC curve
  - $R^2$ values for reference standard curve and positive QC curve
  - Parallelism between reference standard curve and positive QC curve
- Relative bioactivity for positive QC ($EC_{50}$ ratio of reference standard to positive control)
- Calculated concentration for positive QC (single-point positive QC measurement)
- Binding response for negative QC (nonspecific analyte or diluent)

Multiple lots of ligand, analyte, coupling reagents, and sensor surfaces should be used to establish assay system suitability criteria that reflect normal assay conditions. Assay results for reference standard and QC samples should be tracked over time. Regular trending analyses should be done on the data to show whether the SPR assay remains in control over its required lifecycle. If trending in the data is observed, remedies can be performed proactively, preventing assay failure.

For SPR assays that are used in lot-release testing, it is also important to establish sample acceptance criteria. These criteria are used to accept or reject sample data and can include:

- Relative bioactivity for test sample
- Coefficient of variation (CV) for sample replicates
- Parallelism between reference standard curve and positive QC curve

▲Above criteria described for system and sample suitability are mainly applicable to SPR concentration-based or classical potency assays. In the case of SPR affinity and kinetic assay formats, other criteria should be used to accept or reject the assay or sample data. The following additional parameters are suggested for SPR affinity and kinetic assays:

- $R_{max}$
- Parameters for a 1:1 fitting model
  - $Chi^2 \leq 10\%$ $R_{max}$
  - $K_D$ affinity constant within defined range
  - $k_a$ and $k_d$ rate constants within defined range▲ (USP 1-Dec-2020)

Another point to consider for SPR assays in regulated environments is the identification of noncritical and critical reagents. Noncritical reagents typically include coupling buffer, regeneration buffer, and continuous flow (running) buffer. Critical

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reagents typically include the ligand and analyte for direct binding assays, and ligand, analyte, and competitor molecule in inhibition binding assays. Critical reagents should be qualified/requalified on a routine basis to ensure that they are suitable for use in the SPR assay. Best practices for the characterization of critical reagents are not within the scope of this chapter, so the reader is referred to current regulatory documents for such discussion. Analysts should ▲refer to current▲ (USP 1-Dec-2020) regulatory documents such as ICH Guideline Q2(R1) *Validation of Analytical Procedures*, ▲2015 FDA Guidance for Industry—Analytical Procedures and Methods Validation for Drugs and Biologics, USP *Biological Assay Validation* ⟨1033⟩,▲ (USP 1-Dec-2020) and USP *Validation of Compendial Procedures* ⟨1225⟩ when they validate SPR assays in a regulated laboratory.

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# ⟨1106.1⟩ IMMUNOGENICITY ASSAYS—DESIGN AND VALIDATION OF ASSAYS TO DETECT ANTI-DRUG NEUTRALIZING ANTIBODY

## INTRODUCTION AND SCOPE

Administration of natural source or recombinant biologic medicines may elicit some degree of immune response leading to development of anti-drug antibodies (ADAs) in treated subjects. Neutralizing antibodies (NAbs) are a subset of ADAs that affect the biological activity of the biologic drug product. For the purposes of this chapter, NAbs are defined by their ability to neutralize the biological activity of a therapeutic in an in vitro system. This chapter does not address antibodies that may impact drug clearance. [NOTE—Two helpful references on this topic can be found in the *Appendix*.] Further clinical studies would be needed to assess changes in biologic activity on therapeutic outcomes.

NAbs can alter the biological activity of the therapeutic molecule by binding to one or more epitopes that lie within its active site(s). In addition, NAbs can interfere with active sites through steric hindrance (i.e., binding to areas of the protein that are near the active site), or by allosteric interactions (i.e., binding to a site on the drug and inducing a change in conformation that can interfere with the drug's activity). Therefore, it is important to monitor the immunogenicity of biological therapeutics throughout the drug product development cycle by using sensitive and reliable methods that not only determine the presence or absence of ADAs but also characterize whether they have neutralizing capability. The objective of this general information chapter is to provide practical recommendations on best practices that may be used for risk-based design and validation of anti-drug NAb assays.

As described in *Immunogenicity Assays—Design and Validation of Immunoassays to Detect Anti-Drug Antibodies* ⟨1106⟩, immunoassays (or ligand binding assays) are typically used to screen for the presence of ADAs. Several assay formats can be used to determine whether a detected ADA is also a NAb. The first assay format, defined here as a functional NAb assay (thus having an actual biological readout), is most commonly used and can take the form of either a cell-based functional assay or non-cell-based functional assay (e.g., a biochemical assay for an enzyme therapeutic). Another major group of assay formats is based on NAb-mediated inhibition of ligand binding between therapeutics and their targets. These assays can take the form of either cell-based binding assays (e.g., where a cell-surface target exists) or non-cell-based binding assays (e.g., where a purified soluble receptor or drug target is utilized).

The decision to use a cell-based assay versus a non-cell-based assay is dependent on factors that include the drug's mechanism of action (MoA) and immunogenicity risk assessment, as well as the availability of relevant and sensitive NAb assays (*Figure 1*). Another important consideration in selecting the assay format is the degree of risk to patient safety that NAb formation would pose; thus, for therapeutics where antibodies pose a high risk to patients, the assay format should be sufficiently sensitive for detecting clinically relevant NAbs. The speed at which results are required can have an influence on the type of assay selected if the cell-based assay requires extended time to execute. Some practical considerations for making this decision are presented in the section *Risk-Based Approach to Assessing Neutralizing Antibodies and Their Consequences*.

Detection of drug-neutralizing activity in such in vitro assays facilitates improved understanding of any observed clinical effects resulting from altered pharmacological activity. A thorough investigation of the presence of NAbs and their characterization in relation to clinical parameters such as pharmacokinetics (PK), pharmacodynamics (PD), efficacy, and safety is therefore strongly recommended. NAbs would have to be produced at a concentration that is biologically relevant such that the effect of the antibodies could be detected in an in vitro assay, and these antibodies could bind with sufficient affinity to remain tightly bound to the therapeutic. In contrast, a low-affinity-binding antibody that recognizes an active region of a therapeutic protein might not remain bound to the therapeutic, and thus would not readily mediate a biological effect. However, one cannot rule out the possibility of low-affinity antibodies having an effect in vivo, and thus NAb assays should be designed to detect the widest range of antibody affinities that is practical (e.g., limit the number of wash steps).

Although the principles described in this chapter are generally applicable to most commonly used cell-based and non-cell-based NAb assays, modified approaches to assay design and validation may be required for certain products (e.g., enzymes), clinical uses (e.g., different dosing regimens or routes of administration), or patient populations. [NOTE—A list of helpful regulatory guidances and white papers is contained in the *Appendix*.]

## FACTORS THAT INFLUENCE THE DEVELOPMENT OF NEUTRALIZING ANTIBODIES

Although it is not entirely clear why biological therapeutics induce NAbs only in some individuals, or why certain therapeutics induce more NAbs than others, research has shown that certain factors are typically associated with the generation of NAbs. The initial low-affinity response to a therapeutic protein (primarily composed of IgM antibodies) is rarely able to effectively neutralize the biological effect of the therapeutic protein. However, antibody response to a therapeutic protein can mature, usually upon repeated exposure. As the immune response matures, more epitopes of the therapeutic protein may be recognized by ADAs leading to an increased possibility of NAbs, as epitopes within the active region of the therapeutic protein are now recognized.

Ultimately, those factors that trigger development of a robust immune response require the trigger of T-cell mediated immune responses. Some of these factors include the presence of multiple T-cell epitopes (linear, 7–9 amino acid sequence recognized in the context of the major histocompatability complex class II or MHC II); specific product attributes, such as aggregation, that would encourage uptake into antigen-presenting cells; genetic sequence differences between the therapeutic protein and endogenous counterparts; degree of immunologic tolerance; and the amount, route, and schedule of the administration of the protein therapeutic. There is also a potential for residual host cell proteins to act as adjuvants. Therapeutic proteins that are very close in amino acid sequence to endogenous proteins are less likely to trigger a robust immune response. When the sequence is identical and the endogenous counterpart is fully exposed to the immune system, a high level of tolerance

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to the therapeutic protein is anticipated. In subjects with autoimmune disorders, this may not be the case, as tolerance to self-proteins has already been compromised.

NAbs are always defined within the context of the assay that identifies them. Thus, it is possible that not all subjects with NAbs are identified in any program. Also, it is important to recognize that if the sensitivity of a NAb assay improves, then the number of subjects with NAbs that are detected may increase. If the incidence of NAb-positive subjects increases, it is important to determine whether the change is related to assay performance or to an alteration in the product quality attributes of the therapeutic protein. NAb specificity is also important. For assays with relatively poor specificity, baseline (pretreatment) samples may show positive responses; however, such a response may not be due to antibodies directed specifically to the therapeutic under study.

## DETERMINATION OF PRECLINICAL AND CLINICAL IMMUNOGENICITY

### Nonclinical: Relevance and Scope of Preclinical Immunogenicity

Preclinical toxicology studies are generally used to suggest dosimetry and to define safety margins by evaluating idiosyncratic toxicity and specific target-organ toxicity caused by therapeutics. The primary requirement for these studies is to demonstrate that the animals are exposed to the therapeutic at the intended dosage levels throughout the study. As stated in the ICH S6 Guidance, this can be accomplished by analyzing the circulating drug concentration (PK data) in combination with data on PD markers, and/or by using immunogenicity assessments to facilitate interpretation of the exposure-to-toxicity correlation. As discussed in ⟨1106⟩, animal test systems have significant limitations in their ability to support specific conclusions on the potential for clinical immunogenicity, especially as it relates to MHC restriction, T-dependent B-cell responses, and the potential to neutralize the pharmacological effect of the therapeutic. However, evaluation of immunogenicity in nonclinical studies may be useful when there is substantial homology of the therapeutic to an endogenous counterpart in animals [e.g., erythropoietin (EPO) and thrombopoietin (TPO)]. Severe thrombocytopenia was reported in several animal species after dosing with autologous TPO molecules, due to development of circulating anti-TPO NAbs. In these studies, NAbs directly affected the pharmacological activity of both the endogenous protein and the exogenously administered therapeutic, making it difficult to interpret the toxicology study findings.

NAbs are usually directly detected and characterized by in vitro assays. However, a decrease in the expression or activity level of a PD marker may be indicative of NAbs. If confirmation that the PD marker is due to the presence of NAb is desired then this can be confirmed with a NAb assay as a surrogate to infer the presence of NAbs. It is important to confirm that the change in the reduced PD marker is not merely due to clearance of drug from the circulation, an effect that can be caused by ADA, rather than due to “true” neutralization. As part of the immunogenicity evaluation, it also may be useful to characterize the immune-dominant epitopes for such high-risk proteins. Nonclinical sample collection and related considerations are discussed in ⟨1106⟩.

Although the measured incidence and titer of NAbs may not be the same in different animal species, induction of NAbs in multiple species may suggest a higher probability of NAb development in humans where significant homology exists within the biologically active site. Immunogenicity studies in transgenic animals expressing the gene encoding the protein of interest may be useful to help predict the relative potential of various forms of drug product to induce NAbs.

### Clinical: Relevance and Scope of Immunogenicity Assessments

Evaluation of immunogenicity, including development of NAbs, remains an important part of the safety assessment in clinical drug development.

The scope of the immunogenicity assessment for therapeutics is based on the type of therapeutic, selected indication, pharmacology, route of administration, duration of treatment, and immune competence of the patient. The frequency of sample collection and analysis should be based on the timing and incidence of antibody responses as well as the occurrence and severity of clinical sequelae (see ⟨1106⟩ for more information). Because NAbs can trigger a range of possible clinical effects, specific and sensitive analytical methods are needed to (1) investigate the characteristics of antibodies induced over time (non-neutralizing and neutralizing), (2) reveal the duration of the ADA response (transient versus persistent), and (3) clarify the implications of the potential immunogenicity for clinical outcomes.

Adequate patient follow-up for measuring neutralizing ADAs should be implemented, because persistent NAbs generally develop later in the course of treatment and it is important to fully characterize the temporal nature of the antibody response. For example, careful follow-up could reveal a transient response in which antibodies appear, yet then disappear after a period of time such as 2–3 months. In addition to the scheduled, routine repetitive sampling, patients should be evaluated clinically in a symptom-driven manner whenever antibody development is suspected. Pre-approval clinical trials may fail to detect rare immunogenic events due to insufficient patient numbers for adequate statistical evaluation and/or an inadequate duration of drug exposure. Therefore, a postapproval immunogenicity surveillance program may be necessary for any therapeutic protein that carries a risk of generating clinically meaningful NAbs.

## RISK-BASED APPROACH TO ASSESSING NEUTRALIZING ANTIBODIES AND THEIR CONSEQUENCES

Chapter ⟨1106⟩ describes in detail the principles of risk-based approaches to detection and characterization of antibodies directed against biologic drug products. The two primary concerns related to NAbs are (1) the therapeutic can lose pharmacological activity, which in turn can affect its efficacy, and (2) NAbs can cross-react with an endogenous, nonredundant protein. In addition, as with all ADAs, NAbs can contribute to other adverse effects such as the formation of immune complexes

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and potential deposition of these complexes in tissues and the vascular system. *Table 1* in ⟨1106⟩ summarizes important risk factors that may influence the severity of the clinical consequences from an ADA or NAb response. The basic risk assessment criteria outlined in *Table 1* in ⟨1106⟩ provide a useful orientation to this type of risk assessment. The various combinations of factors from the three risk categories (lower, medium, and higher risk), as well as the actual antibody properties, could result in a continuum of intermediate risks. For example, NAbs directed against recombinant human growth hormone can neutralize endogenous growth hormone, which is a vital, nonredundant factor. Yet decades of therapy with a number of different recombinant human growth hormone preparations have shown that attenuation of growth was not observed, even in the presence of the cross-reactive NAbs detected with an in vitro assay. Therefore, the actual risk associated with the recombinant human growth hormone could be viewed as intermediate between the high- and medium-risk categories, despite the existence of the endogenous counterpart.

Potential assay formats are selected based on MoA on a case-by-case basis with proper consultation with regulatory agencies when needed, while the analytical (or immunogenicity assessment) strategy is driven by the risk of immunogenicity for the specific therapeutic. *Figure 1* presents a flow chart with some useful options for NAb assay format selection.

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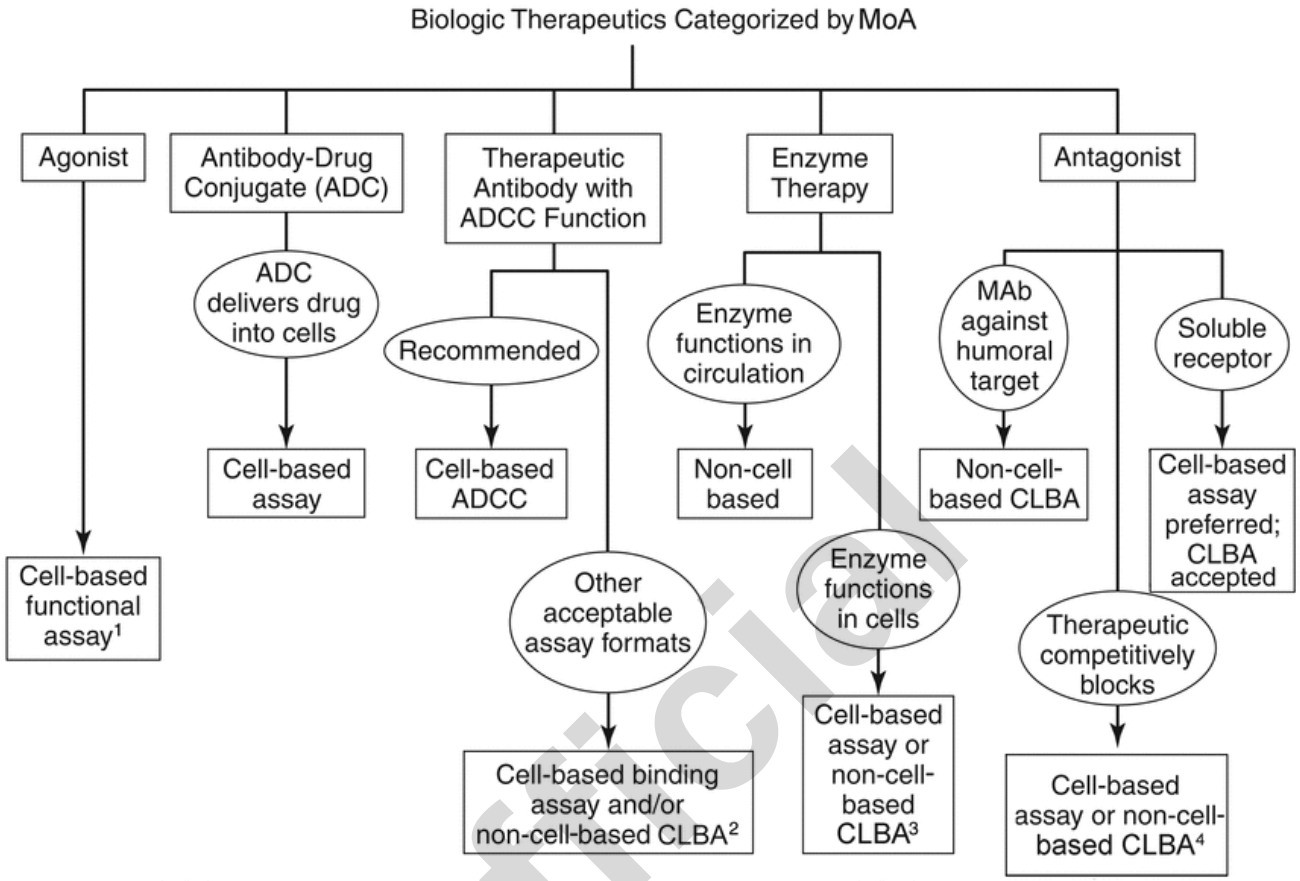


[NOTE—ADCC is "antibody-dependent cell-mediated cytotoxicity;" CLBA is "competitive ligand binding assay;" MAb is "monoclonal antibody."]

[1] A cell-based functional NAb assay is recommended for therapeutic agonists targeting cellular receptor. This includes therapeutics with a nonredundant or redundant endogenous counterpart.

[2] For therapeutics with ADCC function, a cell-based ADCC assay potentially has technical challenges. In addition, ADAs specific to the Fc region of a humanized antibody therapeutic often bind to other nonspecific IgGs in serum matrix and are difficult to detect in a binding ADA assay format. Under this situation, a ligand binding assay using a bridging format or an assay method based on antigen–drug interaction could be a viable platform for the NAb assay.

[3] Non-cell-based assays should be considered, in consultation with a regulatory agency, if the NAb-induced inhibition of lysomal enzyme therapeutics that function at low pH is not feasible under cell-based assay conditions.

[4] For therapeutics that function by binding to more than one receptor, a cell-based assay is recommended.

Figure 1. NAb assay format selection examples.

For agonistic therapeutics that interact directly with cellular receptors, such as growth factors and hormones, development of cell-based NAb assays is appropriate to reflect the MoA of the drug. This category includes therapeutics with a nonredundant endogenous counterpart that may generate clinical sequelae with a high level of risk to patients. For these therapeutics, assays with the capability to determine NAb levels with high specificity and sensitivity are required; the results are generally expressed in titers or equivalents of neutralized drug. These recommendations stem from the concern that NAbs induced by therapeutic proteins can cross-react with and neutralize vital endogenous homologs and cause autoimmune-type deficiency syndromes [e.g., that seen with megakaryocyte growth and development factor (MGDF) and EPO]. Early detection and continued testing for NAbs directed against high-risk molecules are also recommended because NAb data can influence therapeutic decisions.

For antagonistic therapeutics (e.g., anti-IgE or anti-coagulation factors), some regulatory agencies have accepted non-cell-based competitive ligand binding assays for detection of NAbs that are directed against therapeutics with humoral targets. However, for antagonistic therapeutics that interact with a receptor and a coreceptor to exert a pharmaceutical effect on target cells, cell-based assays are generally recommended as this type of MoA cannot be reflected accurately in a

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non-cell-based ligand binding assay because an ADA that does not block drug–target interactions may interfere with target coreceptor interactions, causing functional neutralization.

In terms of other therapeutics, such as antibody-drug conjugate (ADC), antibody therapeutics with effector functions for clinical efficacy, and enzyme therapeutics that function in target cells, cell-based assays are generally recommended to reflect the drug's MoA. However, for lysosomal enzyme therapeutics that function at pH 4–5, it may be challenging to generate any NAb controls that can inhibit the enzyme at low pH with sufficient sensitivity. In this situation, the development of a non-cell-based assay (e.g., inhibition of binding between an enzyme drug and its cognate receptor) is warranted in consultation with a regulatory agency. In addition, a non-cell-based enzyme activity assay could be appropriate for enzyme therapeutics, even those that exert their activities within cells. However, results of such an assay will be conclusive only if the conditions required for the intracellular activity of an enzyme drug also allow for the effective binding of a putative NAb to the drug.

Generally, more frequent NAb testing (e.g., monthly or bimonthly) is recommended for high-risk situations. Sampling should be extended post-study for patients who test positive until they test negative in two consecutive tests at least 2 months apart, or based on the half-life of the ADA (e.g., 3–5 half-lives). This may include post-marketing testing. For medium- and lower-risk situations, testing should be conducted on case-by-case basis in consultation with regulatory agencies.

To fully assess the potential risk associated with NAbs, it is also important to understand the exposure–response relationship of a given biotherapeutic, because this relationship could suggest the extent to which NAbs can affect drug activity in vivo. For example, a high dose of drug would be more difficult to neutralize because more NAb would be necessary; thus, there is a lower risk of efficacy loss. In contrast, when a low concentration of drug is sufficient to achieve therapeutic effect, low levels of NAbs could neutralize drug activity, hence increasing the risk level. Similar situations can occur when the concentration of an endogenous drug counterpart is low; even low levels of NAb could neutralize its physiological function and thereby increase the antibody-related risk.

## DESIGN OF NAB TEST METHODS

### General Considerations

The selection of a NAb assay type is heavily driven by the MoA of the therapeutic, as well as the nature of its target. An example is a monoclonal antibody therapeutic that binds to a receptor on the cell surface, which in turn affects interactions with other cell surface receptors. For this situation, the analyst should use a cell-based NAb assay because it is unlikely that such interactions could be mimicked on a microtiter plate surface. *Figure 1* shows various selections of NAb assays, categorized by their dependence on the MoA of the therapeutic. NAb assay results are generally reported as quasi-quantitative values using a titer, or are qualitative, with just a positive or negative result. Certain technology platforms report quantitative values, such as a surface plasmon resonance (SPR) assay (see ⟨1106⟩ and *Immunological Test Methods—Surface Plasmon Resonance* ⟨1105⟩) or in special cases, neutralization of specific amounts of drug. It could be argued that any numerical value of sample neutralizing capacity is quasi-quantitative when using an antibody preparation as a reference standard, because the affinities and nature of the antibodies within the standard are unlikely to mimic exactly what is contained in a test sample. In addition, comparing sample values between different laboratories that use different standard preparations will likely yield different results for the same sample. Therefore, although a NAb sample value in mass units is helpful for making relative comparisons, all the caveats associated with that value should be considered. A titer value is recommended as the primary readout for NAb assays and is created by testing a sample using serial dilutions. The reciprocal of the test sample dilution that produces a positive result, as defined by the assay cut-point, represents the assay titer. This assay titer should be multiplied by the initial minimum required dilution (MRD; see also ⟨1106⟩), if not accounted for, to report the titer of NAbs present in the neat sample matrix.

### Cell-Based Methods for NAb Assessment

As stated previously, cell-based NAb assays can have two general types of readouts: one based on binding events and the other requiring a series of intracellular events that lead to a functional readout. Drug binding to a receptor or surface ligand on the cell can result in its internalization, phosphorylation, or changes in cyclic adenosine monophosphate (cAMP). Functional assays have more complex cellular readouts, such as proliferation, reporter gene expression, or protein synthesis and secretion.

As shown in *Figure 1*, if a cell-based NAb assay is appropriate, it is important to study the performance parameters of the critical components in the assay system, namely the cell line, drug product, positive control (PC) NAb, and test species matrix. Cell-based NAb assays can be technically challenging because of the need to optimize the cell line, culture conditions, and sample matrix components. Lack of optimization can compromise assay precision, robustness, and sensitivity. Initially, the cells used for the drug's biological potency assay could be evaluated for use in the NAb assay. Use of the same cell line provides several advantages in that cell line maintenance and culture conditions have been optimized. Often, this system can be adapted for a biological matrix. Alternatively, commercial sources of cell lines are available, or a drug-responsive cell line could be genetically engineered. Cell lines transfected with target receptors can offer the advantage of more controlled receptor density, specificity, and enhanced signaling. Reporter gene expression cell lines can provide sensitive assay endpoints.

The cells' responsiveness to the drug should be characterized, particularly before and after freezing, as well as during continuous culture (see also general information chapter *Cryopreservation of Cells* ⟨1044⟩). Drug dose-response experiments in the appropriate biological matrix (e.g., serum) can identify early interference issues. Ideally, several matrix samples should be evaluated. Specificity should be studied to allow use with serum from the species of interest that developed NAb after exposure to the drug. PC antibodies are often isolated from antisera of this species, but because this is not always available during assay development, a surrogate control antibody reagent is produced to use during assay development and validation (see the section *Development of Positive and Negative Controls in NAb Assays*). Cell-based assay formats that have been used successfully in drug product development are shown in *Table 1*. The type of assay format chosen is influenced by the mechanism by which the drug interacts with cells. These interactions can either be direct or indirect. A direct interaction is one in which the drug product exerts its effect by acting directly on cells, such as with cytokines, peptides, or monoclonal antibodies to cell surface

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determinants. Indirect interactions are those where the drug product blocks the interaction of a ligand with its cell surface receptor and consequently interferes with the biologic activity of the ligand. Examples of such drugs include therapeutic monoclonal antibodies to soluble factors and soluble receptors.

**Table 1. Frequently Used Cell-Based NAb Assay Formats**

| Assay Endpoint | Assay Platform Examples | NAb Action | Advantages | Disadvantages |
|---|---|---|---|---|
| Cell surface interactions | Fluorescence cytometry, plate-based cell surface binding | Interferes with binding of drug to target site on the cell, or affects internalization of drug product into the cell. | Drug, target, and NAb interaction occurs only on the surface of cells, which may result in simple and robust assays. | Does not apply to drugs with a MoA involving signaling pathways within cells; inadequate receptor expression on cell may be a limitation. |
| Phosphorylation of intracellular substrates | Kinase receptor activation, enzyme-linked immunosorbent assays (ELISAs), intracellular staining | Affects drug-induced phosphorylation of target receptor/substrate, or affects ligand-induced phosphorylation by blocking drug. | Utilizes early event in cell signaling, which may result in a shorter assay. | Needs phosphorylation site-specific antibodies; multiple assay steps are needed if using ELISA to detect phosphorylation. |
| Cell proliferation | $^{3}$H incorporation, Alamar Blue | Affects cell proliferation that is induced or inhibited by drug, or affects ligand-induced cell proliferation by blocking drug. | Well-established assay endpoint for growth factor-type drugs reflects what happens to the cells. | Assay endpoint is the outcome of multiple intracellular pathways and steps, which may result in a long assay; prone to interference by serum factors; assay needs to show specificity. |
| Reporter gene expression | Luciferase, β-galactosidase, chloramphenicol transacetylase | Affects reporter gene expression by drug product, or affects ligand-induced reporter gene expression by blocking drug. | Assay can be quick, sensitive, and robust. | Need to invest time to construct reporter gene cell line that can respond robustly in presence of test species serum; need to demonstrate specificity. |
| Protein expression or secretion | ELISA, enzyme immunoassay, radioimmunoassay, electrochemiluminescent assay (ECL), scintillation proximity assays | Influences protein expression by drug, or influences ligand-induced protein expression by blocking drug. | The protein can be measured in the cell supernatant or in cell lysate by using an ELISA. | Assay endpoint is the outcome of multiple intracellular pathways and steps, which may result in a long assay; assay involves cell culture followed by ligand binding assay to detect synthesized protein, thus two assays are required. |
| Effector functions | Complement-dependent cytotoxicity (CDC), ADCC, Fcγ receptor binding | Influences target cell fate mediated by effector functions of therapeutic antibodies. | Required for antibody therapeutics where both Fab and Fc domains are involved in function. | Multiple effector functions (ADCC, CDC, phagocytosis, etc.) may be involved, which can make assay choice, assay development, and interpretation of results challenging. |
| Cytotoxicity/apoptosis | Luminescent cell viability assays, terminal deoxynucleotidyl transferase dUTP nick end labeling assay | Affects cell cytotoxicity/apoptosis induced by drug, or affects ligand-induced cell cytotoxicity/apoptosis by blocking drug. | Directly reflects MoA of cell killing mediated by therapeutics. | Needs to demonstrate specificity. |

Consideration is given to the assay endpoint, which can reflect either early or late cellular responses. Early responses include the initial binding to the cell surface, internalization, an early event in cell signaling, or gene expression. Late responses reflect the outcome of multiple intracellular pathways that result in a biological outcome such as proliferation, induction of measurable products, apoptosis, or effector functions such as ADCC or CDC.

Care should be taken to optimize the concentrations of critical assay components that influence its sensitivity for detecting NAbs. In the case of a direct NAb assay, the dose response at several concentrations of the drug product should be evaluated. The amount of drug product used in the assay should be within the linear portion of the dose-response curve that produces a reproducible response (e.g., often a 30%–80% maximal assay response). The sensitivity of an indirect NAb assay depends on both the ligand concentration and the drug product concentration.

In all cases, the appropriate controls should be incorporated. For example, in a direct assay format in which the drug acts directly on the cells to induce a biological response, the assay controls should include cells alone, cells with drug product, and cells with drug product and PC NAb. An indirect assay format should include cells alone, cells with ligand, cells with ligand and drug product, and cells with ligand, drug product, and PC NAb. The assay can be set up as a single dilution to provide qualitative information (positive or negative), as is generally the case for preclinical studies, or can use multiple dilutions to give a quasi-quantitative readout, such as the titer. Designing well-controlled assays facilitates monitoring of assay performance over time and identification of factors to consider as root causes of assay failure.

## Non-Cell-Based Methods for NAb Assessment

Cell-based functional assay formats have traditionally been recommended by the regulatory agencies for NAb assessment. However, in comparison to the technical challenges of cell-based assays described above, non-cell-based immunoassays are capable of overcoming some of the technical limitations inherent to the cell-based bioassays, and have therefore become another useful technology platform for NAb evaluation. Non-cell-based immunoassay platforms, especially competitive ligand

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binding assays (CLBAs) when relevant to the MoA of the drug, can be used for NAb assessment if proven to specifically detect NAbs (*Figure 2A* and *Figure 2B*).

For a range of biological therapeutics (e.g., antagonistic MAb therapeutics against a soluble ligand), the drug exerts its pharmaceutical effect by binding to its target and blocking the interaction of the ligand with its cell surface receptor; consequently, the drug interferes with the biological activity of the ligand. In this instance, a non-cell-based assay method is appropriate for NAb assessment because the assay method reflects the MoA of the drug by measuring drug binding to its target and inhibition of such binding activity by NAbs.

The CLBA-based NAb assay design is based on the competition between the ligand and NAbs for a limited number of binding sites on the ligand binding therapeutic. As the level of NAb increases, less ligand binds to the drug and the measured assay response decreases when compared to a control sample that does not contain any neutralizing activity. Therefore, within the linear range of the assay, the NAb concentration is proportional to the percentage change in assay response. In theory, any ligand binding assays, such as solid- or liquid-phase immunoassays (e.g., radioligand, ELISAs, chemiluminescence, and ECL assays), radioimmunoprecipitation assays (RIPA), and SPR, may be adapted to the CLBA format for the detection of NAb in test samples (see *Appendix* and *Immunological Test Methods—General Considerations* ⟨1102⟩ and *Immunological Test Methods—Enzyme-Linked Immunosorbent Assay (ELISA)* ⟨1103⟩, and ⟨1105⟩ for more information).

Two assay formats are available for non-cell-based CLBA (*Figure 2A* for direct format and *Figure 2B* for indirect format).

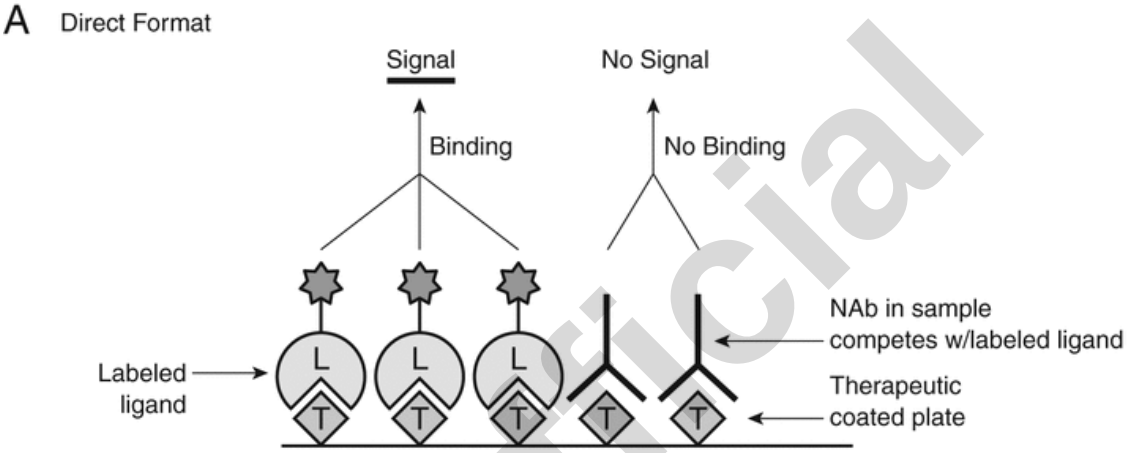


Figure 2A. Direct CLBA format: In this assay format example, the therapeutic product is coated on a plate and serves as a capture molecule to bind to the ligand labeled with a detection molecule (e.g., an enzyme, a fluorescent label, or an ECL label). Binding between the therapeutic and the ligand is inhibited when NAb is present in test samples, resulting in a lower signal. "T" represents "therapeutic product" and "L" represents "ligand."

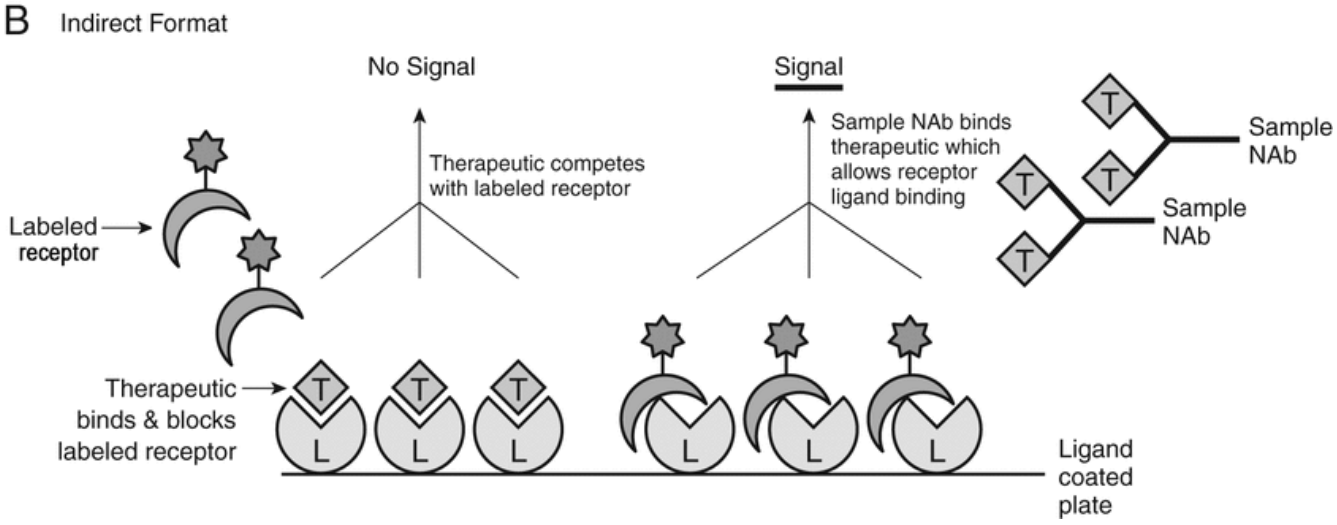


Figure 2B. Indirect CLBA format: In this assay format example, the ligand is coated on the plate, and the therapeutic competes with the labeled receptor for binding to the ligand. When NAb is present in test samples, it binds to the therapeutic and the neutralized therapeutic is unable to bind to the ligand; therefore, the signal will increase because the labeled receptor is now able to access and bind to the ligand.

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The direct CLBA NAb assay format, based on measuring the binding of a drug to its target, is a simple approach (*Figure 2A* and *Table 2*), whereas the indirect CLBA NAb assay format monitors drug-mediated inhibition of ligand–receptor binding (*Figure 2B* and *Table 2*). The two assay formats both utilize the MoA of the drug (inhibition of drug–ligand or ligand–receptor binding) but measure the neutralizing activity using different assay endpoints.

**Table 2. CLBA NAb Assay Format and Critical Assay Components**

| Assay Format | Assay Measurement | Assay Design for Solid-Phase Immunoassays | Assay Components | Advantage | Disadvantage |
|---|---|---|---|---|---|
| Direct CLBA | Binding of a drug to its ligand (target) | The drug as capture molecule and the labeled ligand as detection molecule (the reverse format is more prone to drug interference) | Unlabeled or labeled drug, conjugated target, test species samples, positive and negative controls | Simple assay design | If the binding prevents signaling or attachment of another ligand to its receptor, only the very early binding step is reflected in this assay. |
| Indirect CLBA | Drug-mediated inhibition of ligand–receptor binding | The ligand as capture molecule and the labeled receptor as detection molecule (the reverse format is feasible) | Unlabeled or conjugated ligand, conjugated receptor, unconjugated drug, test species samples, positive and negative controls | Reflects a consequence of NAb activity that is further downstream. | The complexity of receptor purification makes it challenging to maintain proper protein folding and consistent assay performance. |

For the direct CLBA NAb assay format applied in a solid-phase immunoassay, the immobilized drug usually serves as the capture molecule, while the labeled target serves as the detection molecule, generating an assay signal after it binds to the drug. The neutralizing activity can be assessed as the level of inhibition of drug-target binding when NAbs are present in test samples. The assay sensitivity of the direct CLBA NAb assay format is largely determined by the drug concentration selected. A lower drug concentration typically leads to a more sensitive NAb assay, but a low dynamic range may limit the detection of a broad range of NAbs. Therefore, it is important to optimize the drug concentration during assay development. To further optimize the assay, implementation of design of experiment (DoE) can be considered to systematically assess the interactions among key assay-operation parameters and identify the most optimal assay conditions.

In certain cases, such as when the ligand or receptor is a highly charged molecule and tends to bind nonspecifically to surfaces, the assay format also can be reversed by using the ligand or receptor protein as the capture molecule and the drug as the detection molecule. However, because of the use of the ligand as a coating agent, this reverse format can be much more prone to drug interference even if comparable assay sensitivity is achieved. Therefore, the direct CLBA NAb assay format using the drug as the capture molecule and the conjugated ligand as the detection molecule is usually preferred.

An indirect CLBA NAb assay format, which is based on drug-mediated inhibition of ligand–receptor binding, also can be used for the detection of NAb to antagonistic therapeutics that neutralize soluble ligands. One applicable format for an indirect CLBA NAb assay uses the ligand as the capture molecule and the conjugated receptor protein as the detection molecule in a solid-phase immunoassay. The reverse format also may be feasible. The immobilized ligand binds to the conjugated receptor, generating an assay signal. Ligand–receptor binding is inhibited in the presence of drug, but occurs when the drug is neutralized by NAb. The neutralizing activity in the test sample is thereby estimated by the level of restoration of ligand–receptor binding when drug function is blocked by NAb.

The sensitivity of the indirect CLBA NAb assay is largely dependent on the concentrations of both the ligand and drug. A lower ligand concentration will result in a lower drug concentration selected for the assay, leading to a higher NAb assay sensitivity. Other operational parameters also can be effectively evaluated through the DoE approach to determine the optimal assay conditions.

When an oligomeric receptor protein is included in the non-cell-based NAb assay, specific consideration should be given to the challenges presented by this type of receptor, which may be less likely to retain the original conformation necessary to bind to the ligand when not associated with the cellular membrane. In addition, cellular receptors typically have one or multiple transmembrane domains that need to be truncated to facilitate receptor purification. Therefore, concerns arise regarding proper protein folding of these truncated ectodomains and retention of the structure necessary for ligand–receptor binding. The complexity of receptor purification further requires that lot-to-lot variation and stability of the protein products be effectively managed in order to maintain consistent assay performance.

Positive and negative controls are critical for monitoring NAb assay performance. Unlike cell-based NAb assays, the design of assay controls for non-cell-based CLBA assays is usually more straightforward, without the need to include background controls. The selection of positive and negative controls is described in detail in the section *Development of Positive and Negative Controls in NAb Assays*.

## VALIDATION OF NAB ASSAYS

As described above, there are two main formats for NAb assays: cell-based functional assays and binding-based assays. The various components of validation that should be carried out prior to study initiation are described in the sections that follow. The principles of each aspect of validation apply to both assay formats unless noted otherwise.

### Minimum Required Dilution

Determination of the appropriate dilution of assay matrix is an important part of NAb assay optimization because this will affect the minimal test sample dilution and therefore the assay sensitivity. The considerations for defining the minimum-required

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dilution (MRD) used for the ADA screening immunoassay described in ⟨1106⟩ also may be applied to NAb assays. The effect of sample matrix on assay capability should be evaluated at multiple dilutions, preferably by using different pools of the test species matrix. The dilution of sample matrix that has a minimal effect on the assay response should be selected and further evaluated using the PC antibody spiked into sample matrix. A NAb assay should be able to detect antibodies in the presence of assay matrix components that may be expected to be present. These might include complement, coagulation factors, soluble targets, lipids, concomitant medications, and the endogenous homologous counterpart, as well as the administered drug product. As described in ⟨1106⟩, the MRD can be objectively determined and defined as a dilution level that achieves an optimal signal-to-background ratio with acceptable variability.

Factors such as soluble targets that either bind the drug product or act directly on the cells can interfere in the assay, leading to false positive results. Alternatively, certain factors may alter the assay response in a manner that masks the presence of a NAb in the sample (e.g., as noted with the presence of interfering levels of biotherapeutics or growth factors). Confirmation of assay specificity can help to determine the presence of such interfering substances (see the section *Assay Specificity*). Assessment of matrix effects during assay development can be accomplished using pooled matrix. Ideally, the assay matrix should be defined using multiple individual matrix samples because of heterogeneity of the various possible interfering substances. Diseased-state (treatment-naive) matrix may contain additional factors that interfere in the assay and should be evaluated whenever possible.

If lipemic, hemolyzed, incompletely clotted, and, preferably, disease-state sera from naive subjects are available, they should be screened in the assay both with and without the addition of the PC NAb. If the unspiked sera give a response in the assay, and/or the sera interfere in the detection of the control, the analytical procedure should state that samples compromised in such a manner may not yield reliable results and may have to be excluded from testing.

## Development of Positive and Negative Controls in NAb Assays

NAb assays, including cell-based functional assays and non-cell-based immunoassays, are designed to detect heterogeneous and often polyclonal anti-drug immunoglobulins. Because of the diverse nature of immune responses to the drug, it is not possible to generate a true NAb reference standard. Assay performance is therefore monitored by utilizing surrogate positive and negative controls (PCs and NCs). Typically, an NC sample is generated by pooling relevant matrix that is negative for ADA, such as matrix collected from subjects with no previous exposure to the drug. One should consider whether the NC pool can appropriately represent the target study population, such as by comparing assay response produced by the NC to the mean response produced by the individual target population matrix samples (at least 20 individuals). In some cases, it can be difficult to obtain a sufficient number of study population-relevant matrix samples or to ensure that samples used in assay validation accurately represent matrix characteristics of the study population samples. The incurred study baseline samples should be assessed for any evidence of predose reactivity to the compound.

NAb assay PCs typically consist of a hyperimmunized animal serum, a monoclonal Ab, or material that otherwise has specific, and generally high, affinity neutralizing ADA reactivity. The antibody preparation used to generate a NAb-assay PC must be able to neutralize the biological activity of the drug compound in vitro. Ideally, the PC may be prepared by spiking immunoaffinity-purified ADA (polyclonal or monoclonal), or a protein-A/G purified preparation spiked into an appropriate neat matrix may be used. Alternatively, for preclinical studies only, whole hyperimmunized animal serum spiked into appropriate neat matrix can be used. For MAb biotherapeutics, the PC generally has anti-idiotypic antibody reactivity. The PC antibody is considered a critical reagent and should be documented and characterized for use in the assay. Pertinent documentation and evaluations that should be considered include immunization scheme, purification procedure and yield, protein concentration, relative affinity, cross-reactivity, isotype, and NAb titer. Thorough characterization will promote reagent consistency over time and minimize lot-to-lot variation. Refer to ⟨1106⟩ for stability monitoring of assay controls.

Although PCs are used to develop, validate, and monitor performance of NAb assays, these controls are not assumed to represent actual incurred study samples. A great diversity of immune responses to drug molecule, with a broad range of binding affinity and specificity characteristics, should be expected. Therefore, PC and NC performance is used primarily to ensure that the assay performs as expected (i.e., to confirm system suitability).

## Assay Cut-Points

The cut-point of a NAb assay is the assay response used to determine whether a sample is positive for neutralizing activity. All individual drug-naive subject samples and NC samples used for the cut-point evaluation should be spiked with a fixed concentration of drug determined prior to validation; however, this would not apply to the direct format for non-cell-based NAb assays. The assay response can be expressed as assay signal or as the ratio of assay signal from a test sample to that derived from the NC. The NAb-positive samples would have a response above the cut-point if NAb increases the assay response and below the cut-point if NAb reduces the assay response. Alternatively, the assay response may be normalized and computed as percentage change from the NC.

Because ⟨1106⟩ includes an outline of the cut-point evaluation process, this chapter only provides a summary of the evaluation process, with emphasis on and clarification of some key steps for NAb assays (additional helpful resources can be found in the *Appendix*). Alternative statistical methods can be applied in some steps of the cut-point evaluation process (e.g., outlier evaluation) with appropriate justification.

Based on statistical and practical considerations, at least 30 individual subject sera from the target disease population (if available) or healthy donors should be used for cut-point evaluation. Samples from these subjects should be tested over at least three independent runs (e.g., days, analysts) by at least two analysts using a balanced-design framework. At least three reportable results for the negative control should be available from each plate, where a reportable result may be the average of duplicate sample results if the study samples are also tested in duplicate. Also, these three results should come from different locations in the plate, such as the first column, middle column, and last column of the plate.

If the cut-point is estimated from only healthy donor sera during this validation phase, data from the target disease population should be evaluated during the in-study phase to determine statistically whether the distributions of assay response are similar to those of the healthy population. If the variances are significantly different, the cut-point should be re-evaluated using the

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target disease population. If only the means are significantly different, the same cut-point can be used after redefining the NC based on the target population. Other population differences relevant to the clinical study with respect to gender, age, and other factors also should be considered in the sample selection process.

The distributions of original data and log-transformed data (or other transformations), averaged across assay runs, can be evaluated in terms of the skewness coefficient and normal probability plot. Data from the scale (e.g., original, log) that provides the most symmetric or close-to-normal distribution should be used in all subsequent analyses, such as outlier evaluation, cut-point calculations, and comparisons of means and variances across assay runs. Analytical and biological outliers should be identified using appropriate statistical methods such as using the conditional residuals and subject mean estimates from a mixed-effects model that includes the relevant sources of variation in the cut-point experiment (e.g., this model may include Subjects nested within Subject Groups, Run Number nested within Analyst, and Plate ID as random-effects, as well as Subject Groups, Analyst, Plate Testing Order, and the interaction of Analyst and Plate Testing Order as fixed-effects).

The analytical outliers should be identified and removed before identifying the biological outliers, and the distribution of assay response should be evaluated after removing the analytical and biological outliers. If the distribution is adequately normal (i.e., Shapiro–Wilk test is not significant), the parametric approach [mean +2.33 × standard deviation (SD), if NAb increases the assay response, and mean –2.33 × SD, if NAb reduces the assay response] can be used for determining the cut-point from these validation data. This calculation will yield a 1% untreated false positive rate from a normal distribution. The cut-point may be defined in terms of other false positive rates, such as 0.1%–5% on a case-by-case basis, with appropriate justification and discussions with regulatory agencies. If the distribution is not adequately normal (i.e., Shapiro–Wilk test is significant) but is still symmetric enough (skewness coefficient <1), and the departure from normality is mostly due to the heavy tails or some extreme values that may not have been identified as statistical outliers, robust alternatives to the mean and SD, such as the median and 1.4826 times the median absolute deviation, respectively, can be used in the above cut-point calculation formula. If the distribution is highly nonsymmetric (skewness coefficient >1), the nonparametric 99th percentile is recommended, although this should be a last resort as it requires a much larger number of subjects (>100) to obtain a reliable estimate.

Another important consideration is that the SD estimate used in the cut-point estimation should include all the different sources of variation that are relevant to the context where study samples will be tested during the in-study phase. Because study samples are typically tested by multiple analysts over several plates and assay runs, the SD used in the cut-point evaluation in such cases should include, at the minimum, interanalyst, interplate/run, intraplate/run, and intersubject variability.

In order to understand the nature of variability in the assay, and to determine whether the same cut-point evaluated during the validation phase can be used for identifying samples with neutralizing activity during the in-study phase, the means and variances of the distribution of the assay signal from approximately six runs should be compared using a mixed effects model and Levene's test, respectively (see ⟨1106⟩). If these are not significantly different, then the same cut-point (fixed cut-point) can be used during the in-study phase. Otherwise, the cut-point evaluated as above using these validation data should be divided by (or subtracted from) the NC. This is called a multiplicative (or additive) normalization factor. This factor may be multiplied (or added) to the NC used during each run of the bioanalysis phase to define the run- or plate-specific cut-point. Such a cut-point is called the floating cut-point. If the original data are found to be approximately symmetric or normal, then an additive correction factor may be used. If a log transformation is necessary to ensure approximate symmetry or normality of the distribution or if the distribution of the ratio of original sample results (assay signal) to NC has been shown to be adequately symmetric or normal, then a multiplicative correction factor may be used. In such cases, all of the analyses for cut-point evaluation also may be done in terms of the ratio of assay signal from the individual subject sample to the NC from the corresponding plate.

In practice, regardless of whether the means and variances are significantly different between assay plates or runs, the use of a floating cut-point is recommended as it is more accommodating to minor drifts between assay runs during the in-study sample testing phase.

If a fixed cut-point is justified from the above evaluations and therefore is implemented during the in-study phase, a higher level of attention is required to monitor and validate changes in reagents and other assay conditions. Because the cut-point has been fixed in relation to that assay as it existed at the time of the experiments that determined the cut-point, it is essential to ensure that the assay signal results from the controls and test samples are consistent and stable in the event that any changes are made to the assay (e.g., new reagents, analysts, or machinery).

## System Suitability Criteria

NAb assays typically have an intricate design with multistep operations. These assays use complex reagents and equipment and also require extensive data collection. It is therefore important to conduct system suitability assessments in order to evaluate and verify overall method validity and utility.

Specifically, negative and positive assay controls and additional background controls (e.g., cells alone and/or cells with drug product) should be included as part of each analytical run during assay validation and during the sample testing phase. The exact nature of background controls will depend on the type of NAb assay developed (see the section *Design of NAb Test Methods*). Data obtained during assay validation are used to develop assay acceptance criteria.

Generally, monitoring performance of the PC at low level (LPC) and high level (HPC) is most critical. Inclusion of the LPC helps monitor the established assay sensitivity. Exclusive use of a mid-level PC (MPC) should be avoided because the middle range of the assay response versus PC concentration may not be affected as significantly by changes in assay conditions (e.g., reagent, changes) as are LPC and HPC. Alternatively, performing an analysis of a PC tested in a dilution series provides coverage of multiple PC levels, including LPC. In some circumstances, use of an NC that includes a non-neutralizing antibody may be helpful for identifying run failure and preventing reports of false positive results. Typical acceptance criteria for the assay control samples include (a) precision of the raw assay signal for PC and NC samples, (b) performance of the LPC sample or reportable titer value for the PC, and (c) upper and/or low limit for the assay response generated by the assay NC. Other criteria may be applied.

When the floating cut-point approach is used for the NAb assay cut-point evaluation, the system suitability criteria or limits can be defined for the ratio of the LPC to the NC and for the ratio of the HPC to the LPC, instead of defining limits separately

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for each PC. For the in-study phase, it is also useful to apply acceptance criteria for intra-assay precision (variability of response of replicates in an assay). As discussed in ⟨1106⟩, setting criteria for passing or failing assays in pre-study validation experiments should be avoided and all assays performed during pre-study validation should be included except for those rejected for an assignable cause. The appropriate choice of LPC concentration and the acceptable range of performance are important for ensuring the ability to monitor sustained assay sensitivity. The failure rate for LPC is expected to be 1% based on the PC performance. Similarly, an assay failure rate can be set at 1% based on the performance of the NC. If applicable, higher rates of failure (e.g., 5%) may be used. To set a specific failure rate based on the NC and LPC sample performance, appropriate assay response limits should be calculated based on control performance during assay pre-study validation. Alternatively, limits may be established for the PC titer value. These should be defined based on the PC performance observed during the pre-study assay validation phase. Importantly, depending on the specifics of the NAb assay format, the signal in the assay is expected to increase (*Figure 3A*) or decrease (*Figure 3B*) in response to the increasing PC concentration. Hence, an appropriate NC limit should be set. For example, in the first case, where the assay signal is increased, an upper limit for the NC should be established. In the second case, a lower limit for the NC will be most important.

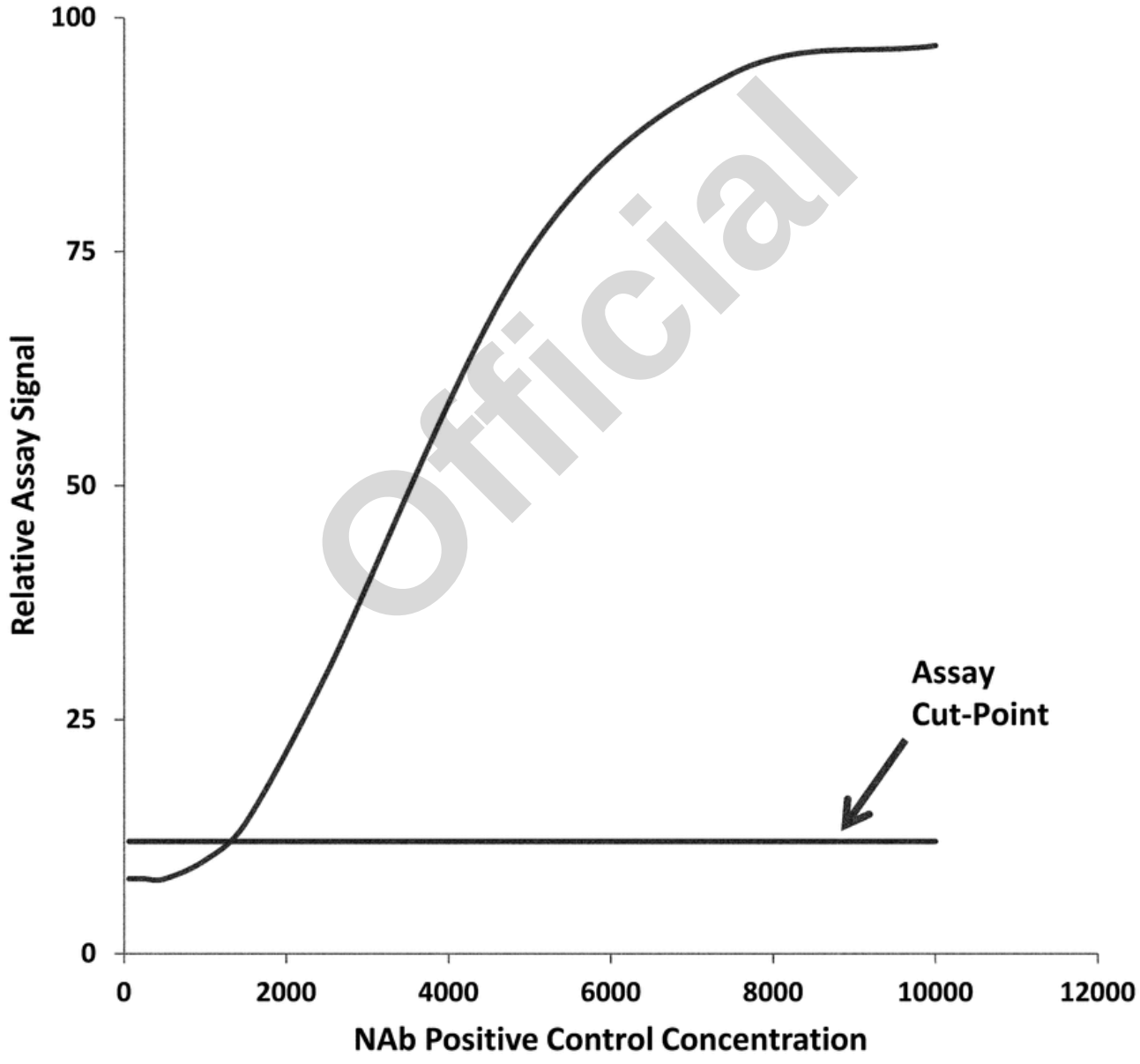


Figure 3A. NAb assay response as a function of PC concentration. Assay signal increases with increasing PC concentrations.

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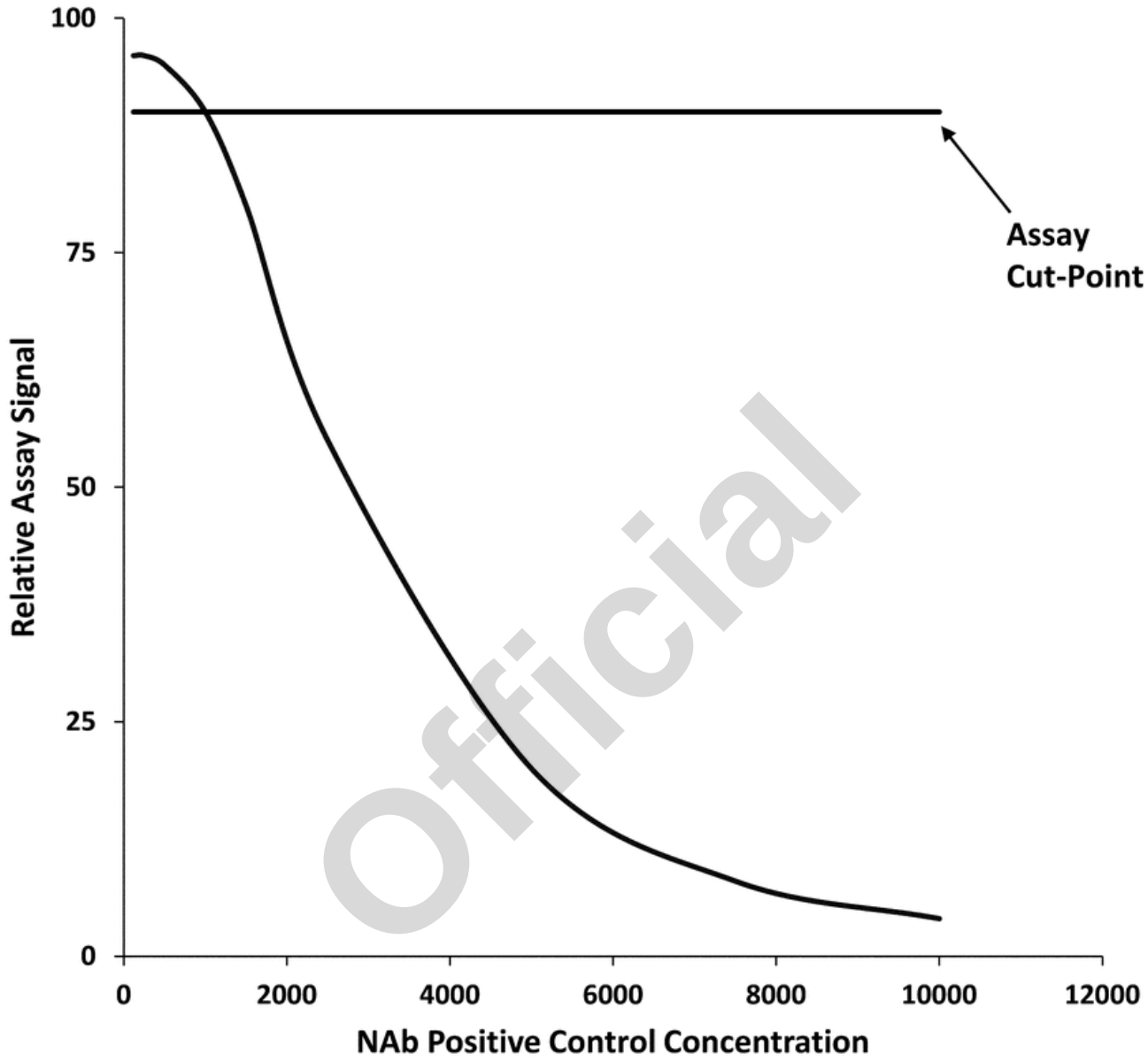


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Figure 3B. NAb assay response as a function of PC concentration. Assay signal decreases with increasing PC concentrations.

Initial assay control acceptance criteria may be based on the existing information about assay performance obtained during the assay development and qualification phase. Alternatively, standard criteria that are based on the specific, existing NAb assay validation standard operating procedure may apply. Important technical details such as instrument signal readout capabilities should be considered when setting expectations for the assay control performance. Actual criteria for the assay controls can only be determined after a holistic evaluation of the assay validation information in its entirety.

Because of the complexity of the NAb assays, specifically the cell-based NAb protocols, an effort should be made to identify particular steps and conditions with the highest potential to affect assay performance. Generally, assay control performance should be monitored during the study support phase to ensure consistency of reported data. Assay control performance data should be monitored for any short- or long-term trends and variations relative to the predefined range. If a suspicious trend in the control sample performance is identified or the signal falls close to a limit of the acceptable range, an investigation should be considered.

## Relative Sensitivity

NAb assay sensitivity is defined as the lowest concentration of an assay PC antibody that can be detected reliably by the method. The result obtained for this validation parameter is highly dependent on the characteristics of the PC used to conduct the experiments, including its neutralizing capacity and binding affinity to the drug. The relative sensitivity of NAb assays is also inversely dependent on the concentration of drug used in the assay.

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To conduct the experiment, PC is spiked into assay-relevant pooled matrix. These spiked samples are tested in multiple runs, commonly in at least three independent runs by two operators for a total of six runs. It is recommended that more than one antibody curve be performed per run per operator.

Generally, linear interpolation between values just above and below the assay cut-point is conducted to calculate the assay sensitivity parameter. In some cases, a four-parameter model is used for which at least six values generated by various concentrations of PC should be available to appropriately analyze the resulting data set. One of the values should fall below the assay cut-point. When using linear interpolation or a four-parameter-fit approach, the PC concentration that would generate an assay response equal to the cut-point value is calculated. Values generated in multiple tests over multiple days are averaged, and the result is reported as the relative sensitivity of the assay. Once established, the assay sensitivity value can be used to guide selection of a LPC concentration to be used when monitoring assay performance during assay validation and when testing incurred study samples. Generally, the LPC concentration is chosen so that the rate of assay failure due to performance of the PC is NMT 5%.

Because the assay sensitivity depends greatly on the characteristics of the assay PC, assay sensitivity will vary between different NAbs. The assay sensitivity value determined during assay validation cannot be used to predict the actual NAb concentration that could be detected in study samples. The NAb assay sensitivity parameter is useful when evaluating various analytical platforms, during assay development and optimization, and for selecting appropriate PC concentrations for assay validation and system suitability testing. Typical targets for the NAb assay sensitivity are often 0.5–2 µg/mL; however, because NAb assays are complex, a case-by-case, fit-for-purpose approach to selecting an appropriate assay sensitivity is used with the goal of detecting clinically relevant NAbs.

## Assay Specificity

Assay specificity is defined as the ability to unambiguously detect the analyte of interest. For example, in the case of cell-based assays, an initial assessment should be done to investigate the ability of the chosen cell line to respond to biological components expected to be present in the assay matrix that may structurally or functionally resemble the drug molecule or its molecular target; the goal is to ensure the specificity of the NAb format. Such an assessment may aid in determining the assay format, the analytical platform, the type of cell line to be used in the assay, any sample pretreatment, and other assay conditions. Careful attention should be paid to potential differences between normal and disease-state samples. Assessment of assay specificity may include the testing of irrelevant ADA (e.g., antibodies to other similar molecules) and should include, where possible, specific binding ADA that are known to lack neutralizing activity. The comparison may be made either by evaluating an antibody dilution profile or by spiking the antibody in excess into a pooled negative matrix sample. The irrelevant antibodies are not expected to score positive in a well-designed, specific NAb assay. Other matrix components (e.g., soluble forms of receptors or other drug binding partners) may exhibit inhibitory effects on the drug activity. It is important to understand whether true confirmatory testing should be included as part of an incurred sample analysis routine. Such confirmatory testing should demonstrate that the inhibition of the drug activity is specific to NAbs and not other factors found in the assay matrix. NAb confirmatory testing, also referred to as matrix interference assays, generally employ a cut-point-based approach. If a decision is made to include a matrix interference NAb confirmatory assay during routine sample testing, the validation should follow the general principles described above for the assay cut-point determination.

## Selectivity and Interference

Selectivity evaluates the ability of a NAb assay to detect a NAb PC in a matrix sample containing potential interfering factors. These matrix factors may bind to NAb through specific or nonspecific interactions, interfering with NAb detection. General matrix interference can be investigated by evaluating the recovery of the NAb assay response generated by the HPC and LPC prepared in 10–20 individual relevant matrix samples.

One of the major interfering agents in a NAb assay is the drug itself, when present in test samples from dosed subjects. The drug interferes with the ability of the assay to detect NAbs, causing false negative or false positive results, depending on assay design. The magnitude of drug interference is dependent on multiple factors such as the circulating drug concentration, the concentration and other characteristics (affinity, avidity) of the PC antibody, the half-life of the drug, and the assay design. Therefore, it is not possible to establish a universal “drug tolerance level” for all NAb assays. Nevertheless, drug interference in the NAb assay should be addressed in the assay design and testing strategies. During assay development or optimization, drug interference may be assessed by adding titrated concentrations of drug into undiluted matrix containing fixed concentrations of a positive NAb control, based on assay sensitivity. The limit of drug tolerance is reported as the highest concentration of drug at which PC NAb remains detectable, where detectability should be defined (e.g., a certain signal-to-noise ratio, a selected level above background). To ensure that the assay method is sensitive enough to detect NAbs in the presence of circulating drug, the positive NAb control could be titrated in undiluted pooled matrix sample (e.g., at 250 ng/mL or 500 ng/mL for clinical studies or 1000 ng/mL for nonclinical studies) to assess drug tolerance level for the assay method. Thus, based on the sensitivity of the NAb assay, the PC should be diluted to that level when trying to detect NAb in the presence of circulating drug. The drug tolerance level can vary considerably when different concentrations of the positive NAb control are added to the assay matrix. NAbs may also differ in affinity and/or avidity. Therefore, the drug tolerance level assessed using a PC may not predict the actual levels of drug interference in study samples.

Because NAb assays tend to be highly susceptible to drug interference, it is generally not recommended to test for NAbs in samples from time points when drug levels are expected to be high. However, it may be necessary to analyze NAb activity in study samples containing circulating drug to investigate when the onset of the NAb response occurs and its impact on drug exposure. Under these circumstances, methods need to be applied to overcome drug interference and enable NAb detection in the presence of high levels of circulating drug. Strategies commonly used to improve drug tolerance level include acid dissociation and removal of excess drug through physical separation or solid-phase absorption. An alternate approach is a drug quantitation-based approach in which samples are tested for the bioactivity of circulating or exogenously added drug. Each method has its own caveat. For example, acid pretreatment may affect the activity of NAbs. In cell-based NAb assays, excess

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acid contained in samples may negatively affect the cellular response and decrease the assay signal, thereby compromising the advantage offered by using the acid dissociation procedure.

In addition to drug interference, soluble drug ligands may also interfere with NAb assays, potentially generating false positive results. Target interference also can be introduced when the drug target is released from drug–target complexes during acid dissociation. These interfering factors may be addressed by optimizing sample pretreatment methods. One feasible approach is to pretreat the samples with beads conjugated to an anti-target antibody. After removal of beads, the target-depleted samples can be used in the NAb assay.

## Precision

Precision—intra-assay and interassay—is the quantitative expression of variability, and it provides a measure of the amount of random error that occurs during the execution of an analytical procedure. Precision estimates are useful indicators of assay performance in the specified assay matrix.

### QUALITATIVE NAb ASSAYS

Per general information chapter *Validation of Compendial Procedures* ⟨1225⟩ and ICH Q2(R1), intra-assay precision (repeatability) is the degree of agreement between results generated by consecutive analysis (replicate testing) of the same assay controls or samples under the same operating conditions, by the same operator using the same equipment in a laboratory, within a short period of time. Four to six independent preparations of LPC, HPC, and/or NC samples in a single lot of pooled donor serum (normal or disease state) are evaluated in duplicate or triplicate, in multiple positions on the same plate in a randomized manner, to determine the relevant sources contributing to response variability. The imprecision of the assay response (optical density, fluorescence unit, luminescence unit, or percentage change in assay signal after normalization or interpolation), is calculated and reported as percent coefficient of variation (%CV), which equals (SD/mean) × 100. The %CV values of the mean assay response obtained with the various assay controls should suffice for assessment of intra-assay precision. The %CV may vary depending on the technology used for detection, assay methodology, and procedural complexity. The expected target CV or pooled %CV for intra-assay precision should therefore be defined based on assay capability, as well as on intended use. It is not possible to generalize acceptable precision as it depends on the use of the assay and drug type, risk to patient, and other factors, but the need to rely on the assay result should drive acceptable limits.

Interassay precision (also called intermediate or total precision) encompasses within-laboratory variation among assay runs, and therefore represents the overall precision of the assay. The experiment described above should be executed over multiple days with at least two operators, especially if the sample testing will be executed by more than one operator in the study phase. The pooled intraplate SD and the SD of the mean for each sample tested on multiple plates over multiple days can be used to calculate the intermediate precision, assuming that most of the variability is attributable to plate variability and that the sample size is the same on every plate. Intermediate precision is highly dependent on the assay methodology and procedural complexity. The target intermediate precision, therefore, should be defined based on assay capability, as well as on intended use (fit-for-purpose).

Using an alternate approach, interassay precision may be assessed by deriving the mean, SD, and %CV of the NCs and PCs from all of the experiments conducted during assay development (provided that the plate location effects are negligible), with a few exclusions. The runs to be excluded are those with an assignable operator or equipment error, or with method variations introduced intentionally for robustness testing.

### QUASI-QUANTITATIVE NAb ASSAYS

To assess the precision of the reported titers, operators generally use the LPC and one to two concentrations of the HPC. The HPCs (minimum of three independent preparations) should be diluted in a two- or three-fold titration series using undiluted pooled assay matrix as the diluent and then should be tested in the assay. The HPC can also be diluted in MRD matrix pool, as long as future samples are diluted in the same manner. To measure intra-assay precision, it is generally recommended that three titration curves of the low and high PC be analyzed by one operator on the same day. To measure interassay precision, three titration curves each of the low and high PC should be analyzed on a minimum of 2–3 different days by two operators. Titers are determined as a reciprocal value of the highest dilution of the PC that tests positive. Target titers can be determined and assigned to each low and high PC or can be calculated as mean values by averaging the titer values obtained for the low and high PCs in the precision assessment. Intra-assay and interassay precisions of titers are then evaluated by comparing titers obtained for individually prepared curves to the target titer assigned for the low PC and high PC, respectively. As described in ⟨1106⟩, a recommended but more rigorous approach is to use these data to define a minimum significant ratio (MSR). The calculated MSR reflects the smallest fold-change in the titer values that can be considered statistically significant ($P < 0.05$); for instance, if MSR = 5, then titers that are different by more than five-fold can be considered significant.

In general, the acceptance criterion for titer precision is that the assigned titer value should be within one dilution of the target titer in independent titration series. This, however, will depend on the method capability, the dilution level (e.g., this criterion may be suitable for a two- or three-fold serial dilution assay format but not a 10-fold serial dilution format), and the intended use of the reported titer data in the clinical setting. If using the calculated mean titer approach, occasionally the mean titer may fall in between the dilutions because it is derived from observed values from multiple analyses. In this scenario, the ±1 dilution rule needs to be modified, and titers observed for a defined PC are rounded to the nearest dilution to yield the target titer.

## Robustness and Reproducibility

Robustness and reproducibility of immunogenicity assays are discussed in ⟨1106⟩, which is in harmony with ⟨1225⟩ and ICH Q2(R1).

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Robustness testing should be done as part of assay optimization during assay development, if at all possible. This is because it is unlikely that during validation an analyst would specifically attempt to make changes that might routinely occur (e.g., switching lots of materials or varying incubation times within certain limits). It is therefore necessary to understand which parameters of the method require strict adherence (e.g., concentration of coating antibody), with precise limits for the parameter delineated in the method, versus parameters that need less control and can have "approximate" descriptions in the method.

Robustness experiments can be performed in a simple fashion by varying one or two variables at a time or by DoE approaches, depending on how many factors will be tested at any one time and the extent to which interactions among parameters will be examined. The breadth of robustness testing depends on the intended use of the assay (e.g., a small study looking for gross changes in a toxicology experiment might need less robustness testing than some other studies). However, any assay used for clinical studies involving the registration of a drug should be examined thoroughly to ensure that it performs as expected on a routine basis over an extended period. Plate edge effect (or uniformity) should be examined during assay development. Other common factors that should be evaluated for impact include incubation times and temperatures, reagent concentrations, and cell densities.

If an assay needs to be transferred to a different laboratory, analysts should assess the reproducibility of the method in the new laboratory. This can be done as part of the assay transfer qualification and/or the validation of the method at the new facility. It is important to note that a properly designed and optimized assay, where critical parameters have been understood and controlled, should be robust enough to transfer without any issues. However, to ensure that the assay will perform in the same manner in each laboratory, several useful indicators can be assessed. Shared samples can be tested at both sites, and estimates of assay variability and quality control performance can be evaluated (see also the section *Transfers to Other Laboratories* in *Life Cycle Management*).

### Stability

It is important to understand the optimal storage and handling conditions for assay samples, controls, materials, and reagents (see ⟨1106⟩ for additional guidance). For example, it may be important to provide advice to clinics on aliquoting procedures, time to freeze samples, storage temperature, and shipping conditions.

A key consideration for cell-based NAb assays is the stability of the cell line itself. Cell lines, as living entities, exhibit inherent variability and the potential to change over time or react to the environment in a way that can influence their response in the assay. For example, variations in levels of cell surface receptors may be affected by the number of cell doublings, time in culture, presence of certain media components (e.g., serum), and/or cell density. Therefore, during NAb assay development, cell line performance should be thoroughly characterized. Appropriate controls should be established for parameters including passage number, reagents, and media changes. An example is that the use of frozen cell aliquots of the same passage number for each assay can sometimes reduce variation because of changes in continuous culture.

### Documentation of Pre-Study and In-Study Validation

The recommended documentation is described in chapter ⟨1106⟩ for both pre-study and in-study validation.

#### IN-STUDY VALIDATION ASSAY MONITORING

The ability of a NAb assay to perform in a reliable manner over time is important for conducting historical comparisons of NAb incidence for a single biotherapeutic as it advances through the clinical development life cycle. Also, verification that a validated assay continues to perform as expected is an ongoing process, and once the NAb assay is implemented, it is good scientific practice to monitor its performance by trending the results obtained with assay controls over time. It is recommended to use a statistical approach (e.g., Westgard rules) for assigning the threshold for assay failure and investigation based on behavior of the assay control (positive or negative). This may be easier to accomplish for highly precise assays than for assays that tend to be more variable. Regardless, it is recommended to tabulate the assay control values obtained from a minimum of 10 runs over an appropriate time period to establish a threshold and an approach for identifying assays that seem to be trending toward the assay limits. If the assay performs outside pre-established expectations, an investigation should be performed to identify the cause of the observation; implementation of appropriate step(s) may be necessary. If corrective step(s) are required, assay performance verification may be necessary to demonstrate that performance has returned to its original level. It is also important to identify variables that are likely to contribute to assay drift, such as the use of a new working cell bank, changes in lots of critical reagents, and different assay operators.

## LIFE CYCLE MANAGEMENT

### Changing the NAb Assay Format

During the drug development phase, it may be necessary to change the NAb assay format. For example, the assay format might be changed from cell-based to non-cell-based or vice versa to obtain an assay with better sensitivity or specificity, or other desirable characteristics. If NAb-related adverse events tend to be serious or life threatening (e.g., in the case of growth factor or cytokine drug products that have a nonredundant function), it is recommended to seek regulatory agency advice when considering a different assay format to ensure its suitability for detection of clinically relevant NAbs.

For all NAb assay format changes, an assessment of assay sensitivity and specificity is important. If the switch is being made for greater ease of performance without a significant improvement in assay characteristics, the ability of the previous and new formats to detect NAbs should be compared. This comparison should be made by using donor serum samples spiked with the

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PC antibody, as well as incurred study samples that have previously tested positive for NAbs. For the latter, a rate of concordance for sample results should be predetermined and met.

## Transfers to Other Laboratories

### NON-CLIA LABORATORIES

If the receiving laboratory is not certified under the Clinical Laboratory Improvement Amendments (CLIA) program, the capability of this lab to follow good laboratory practices (GLP) should be assessed before initiating any assay transfer activities. CLIA-certified laboratories are regulated by the Centers for Medicare and Medicaid Services of the U.S. Department of Health and Human Services. These regulations are defined in 24 CFR Part 493 and apply to laboratories testing human specimens for the purpose of disease diagnosis, prevention, monitoring, or treatment. For non-CLIA labs, the capability assessment may include evaluation of existing infrastructure for conducting cell-based or non-cell-based NAb assays, review of staff training records, and other activities. An informal feasibility study may be useful for assessing assay performance in the receiving lab. The transferring lab can simply provide the assay and reagents to the receiving lab, which can attempt a few runs and evaluate the assay controls. This exercise helps evaluate both the clarity of the written method and the receiving lab's ability to conduct the assay. The feasibility study results should be useful when determining the level of formal training that the transferring lab should provide to the receiving lab. Formal training, if needed, should adhere to a documented training plan. Assay controls and training samples prepared by spiking matrix samples with the PC NAb may be run by both the trainer and trainee following the same detailed method. The acceptance criteria for successful training should be clearly described in the training plan.

Formal assay transfer activities may commence after the receiving lab has been trained successfully and its equipment has been qualified. An assay transfer protocol should be written to describe the experiments that will be conducted during the assay transfer phase. The acceptance criteria for the experiments should be clearly defined. Some typical experiments that should be conducted by the receiving lab during assay transfer may include (a) running PC antibody curves, (b) testing matrix samples that have been left unspiked or have been spiked with PC NAb, and (c) testing different lots of critical reagents, performed by more than one analyst. For the experiments designated (b) and (c), it may be useful for both the transferring lab and receiving lab to conduct the experiments using the same training samples. Statistical analyses then should be performed on the data generated, to assess the degree of concordance between the two labs. The extent of concordance required for a successful assay transfer should be detailed in the protocol. In certain situations, it may be necessary to derive a new assay cut-point or a change in assay sensitivity because of a change in reagents or PC NAb. All changes to the method should be captured in a revised method supplemented by an assay transfer report that details all the experiments that support the changes. After a successful assay transfer, the receiving lab may implement the method for analyzing study samples. Appropriate assay trending approaches should be implemented to monitor assay performance and thereby ensure that it remains within recommended specifications.

### CLIA LABORATORIES

For NAb assay transfers to a CLIA lab, one should follow the relevant guidelines for laboratory staff qualifications and for review and approval of the assay training and transfer documents by personnel who provide oversight to CLIA tests. The approach for NAb assay transfer to GLP (non-CLIA) labs, described above, may be used for CLIA lab transfers as well. Annual competency assessments, quality assurance monitoring and review of assay controls, reporting of individual patient test results to treating physicians, and proficiency testing are required of the CLIA-compliant laboratory. Proficiency testing (PT) should be performed twice annually. The testing may include preparation of pooled matrix samples that are tested to establish a baseline. Aliquots are then provided to the testing group in a blinded manner (at least 5 PT samples/PT event), and the results are compared to baseline. Overall, 4 of 5 samples must pass PT, and any discrepant results are investigated.

## Cross-Validation and Bridging

Cross-validation may be necessary if the same assay needs to be run or maintained at multiple labs simultaneously. The first step is training of the personnel who run the assay at the new location; this is often provided by the originator lab. Several components of the assay transfer activities mentioned above may be applied during a cross-validation effort. Assay performance may be monitored using assay controls and matrix samples, which are spiked or left unspiked at both locations. If the NAb assay is altered within a study, bridging experiments should be performed. This may be accomplished by running incurred samples previously tested by the old method in the new method and assessing concordance. For a certain period of time, study samples may be run in both the old and new methods at the same time to ensure that the new method is acceptable.

## Method Improvements or Changes

The laboratory should implement a process for introducing any known change in the assay that might impact assay performance. Appropriate qualification experiments should be conducted before introducing the change to ensure that it will not interfere with the historical performance of the assay. If the change requires an adjustment of the assay acceptance criteria, appropriate documentation may be required to show the impact of the adjustment on the intended use of the assay. A statistician should be consulted if needed.

For cell-based NAb assays, a change in the method might involve use of a different cell line or selection of a different assay endpoint with the same cell line to achieve improved assay characteristics. A change in the NAb assay readout platform is sometimes made (e.g., ELISA yielding absorbance values versus ECL values). In cases of method improvement, both assay development and assay validation will be needed.

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### CROSS-VALIDATION TO OTHER SPECIES

During the drug development cycle, there may be a need to change the assay matrix from one species to another (e.g., cynomolgus or chimp to human) or across patient populations. The change of assay matrix may require an investment in both assay development and assay validation efforts to ensure that the assay shows acceptable sensitivity, specificity, and other characteristics.

### REAGENT REPLACEMENT

Reagent qualification is required if a critical reagent needs replacement using previously established qualification criteria. Ideally, the reference lot and the new lot should be compared by preparing assay controls. However, in many cases a reference lot may not be available for conducting a comparison. In this case, the new reagent should be tested and if the assay behaves as expected, it may be considered acceptable.

## Assay Standardization

There is an emerging clinical need and apparent value in assay standardization (i.e., use of reference standards, platform, and reference method) to facilitate harmonization of the approach used for the detection of NAbs directed toward drugs in the same class of therapeutic [e.g., interferons, anti-tumor necrosis factor (TNF) MAbs, or erythropoiesis-stimulating agent therapies]. If several companies are involved, a consensus approach to standardizing the method should be undertaken. This requires agreement on a universal method and protocol with a common set of reagents in all laboratories concerned. For cell-based NAb assays, establishment and implementation of a common, master cell bank is critical. The reporting units for NAb-positive samples also must be unified, such as, expressing results as positive or negative versus specific values with units and/or using a standard protocol and common reagents for calculation of NAb activity. To confirm that standardization has been implemented successfully, the universal method should be used by all labs involved in the standardization effort to analyze a set of matrix samples spiked or unspiked with NAb PC in a blinded manner. Analysis of known positive and negative study samples also should be performed.

# APPENDIX: ADDITIONAL SOURCES OF INFORMATION

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# ⟨1106⟩ IMMUNOGENICITY ASSAYS—DESIGN AND VALIDATION OF IMMUNOASSAYS TO DETECT ANTI-DRUG ANTIBODIES

## INTRODUCTION AND SCOPE

Anti-drug antibodies (ADA) can be induced when animal or human immune systems recognize a protein drug product as foreign. The administration of biopharmaceuticals can elicit product-specific ADA, and various types of ADA responses can develop in either nonclinical or clinical studies. [NOTE—A list of regulatory documents, white papers, and other relevant references is contained in the *Appendix*.] Although the main focus of this chapter is ADA immunoassay design and validation, the chapter also includes discussion of an overall risk-based immunogenicity assay testing strategy that includes preclinical and clinical studies.

ADA assay results are directly influenced by assay design, assay reagents, how the assay is run, what samples are run in the assay (timing of sample collection, etc.), and how assay data are analyzed. In fact, it essentially is impossible to compare the results of studies that use different ADA assays. Guidance, such as this general information chapter, recommending best practices and considerations for ADA assay development helps ensure that the assays produce results that are meaningful for patient safety and product efficacy.

The primary concern with unintended or unwanted immunogenicity of biological products is whether antibodies produced by patients receiving the product lead to some form of clinical response (e.g., an effect on safety or efficacy). The utility and interpretation of preclinical toxicology studies also can be influenced by the presence of ADA.

The pharmacokinetics (PK) or the pharmacologic activity of the drug can be altered by ADA that either enhance or reduce the clearance of the drug, alter bioavailability, or inhibit or exacerbate the pharmacological action of the drug. If an endogenous counterpart of a drug exists, ADA that inhibit the activity of the product also can bind to and cross-react with an endogenous protein counterpart of the product, potentially leading to a deficiency syndrome. Under some circumstances, ADA can form immune complexes that can induce serum sickness-type clinical responses. Moreover, IgE isotype ADA responses can result in anaphylaxis.

Immunogenicity assessments are playing an increasing role in biopharmaceutical development as part of Product Quality Risk Assessments (PQRAs) and the assessment of the criticality of quality attributes (as described in ICH Q8 and Q9). They also play a role in the demonstration of process and product comparability after manufacturing process changes. Often the manufacturing process for a biological therapeutic will be refined during clinical development, and often changes occur after the sponsor obtains marketing authorization. Such changes, however minor, potentially could affect the bioactivity, efficacy, or safety of a biotherapeutic, and immunogenicity is a key consideration. Changes in the levels and types of degradation products (oxidized, deamidated, aggregates, or others), isoforms of the protein, and process-related impurities such as host cell protein and DNA could affect immunogenicity and warrant closer evaluation.

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## FACTORS THAT AFFECT THE IMMUNOGENIC POTENTIAL OF A THERAPEUTIC PROTEIN

Many factors can influence whether administration of a biological product will induce an immune response in the recipient, including the structure of the protein product itself, product variants, product formulations, the immune status and genetic makeup of the patient, and the dosing route and regimen used in the clinic.

### Protein Structure

The primary amino acid structure of the product and its variants can determine if there are immunogenic epitopes that the patient's immune system recognizes as foreign, leading to an immune response. Amino acid sequences that are not found in human proteins and thus could be recognized as foreign by the human immune system (e.g., those derived from a nonhuman cell line or created by protein engineering, e.g., fusion proteins) not surprisingly can induce an immune response in humans. In addition, chemical modification of amino acids (e.g., oxidation or deamidation) may result in a sequence that can stimulate an immune response, although few data to date have confirmed such occurrences following administration of therapeutic proteins. Truncation of the protein could expose amino acid motifs (neoepitopes) not normally exposed in the native protein, stimulating an immune response.

In general, glycosylation does not appear to play a major role in the induction of immune responses to biological products, although nonhuman (e.g., murine) or nonmammalian (e.g., products derived from plants) glycosylation can induce immune responses. An example is a human monoclonal antibody that contained a terminal galactose-α-1,3-galactose because of posttranslational modification by its murine production cell line. This antibody was antigenic and caused severe hypersensitivity reactions in presensitized individuals bearing cross-reactive IgE. There was no evidence that this glycan induced primary immune reactions in naïve (i.e., not presensitized) individuals. Such examples are rare. In fact, in many cases complex carbohydrates may prevent or reduce the antigenicity of immunogenic proteins by shielding epitopes from binding antibodies.

Aggregated protein has been shown to induce immunogenicity in animal models. Proposed mechanisms of action include either presentation of high molecular weight, repeating subunits (multimers) directly to B-cells thereby inducing a T-cell–independent response or by inducing a T-cell–dependent response via enhanced antigen presentation. Addition of polyethylene glycol (PEG) molecules (PEGylation) to recombinant therapeutics has been attempted in order to attenuate immunogenicity and antigenicity of recombinant proteins by limiting exposure of epitopes. Although no clear evidence establishes that the immunogenicity of proteins is diminished by PEGylation, some clinical data suggest that PEGylation can limit antibody binding (i.e., antigenicity) to the protein backbone. PEG itself can be immunogenic and anti-PEG antibodies should be monitored, especially in subjects with known PEG hypersensitivity. In fact, a background level of anti-PEG antibodies is present in the general

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population, leading to the requirement to develop an anti-PEG antibody assay as well as an ADA assay during the clinical development of PEGylated products.

### Process-Related Impurities

Process-related impurities such as endotoxins, host cell DNA, or proteins can act as adjuvants and can provoke an immune response by evoking danger signals via activation of immune cellular receptors such as Toll-like receptors. Some postulate that leachables from primary packaging components also can act as immune stimulators or affect the higher-order structure of the protein product and induce an immunogenic response, although firm data are not yet available.

### Immune Status of the Patient

The ability of the patient's immune system to recognize and respond to a protein product can dictate the level of immune response. Patients who are taking immunosuppressive drugs such as glucocorticoids, cyclosporine, or methotrexate may have a lower likelihood of immune response to a protein product despite its immunogenic potential. Conversely, autoimmune diseases and inflammation may involve the overactivation of an immune system so that a product's level of immunogenicity may be much greater than one would anticipate. The pharmacologic activity of the protein therapeutic itself should also be taken into account. Some protein therapeutics directed against B-cell antigens can deplete peripheral B-cells. Conversely, other protein therapeutics may have immune-modulatory activities, e.g., altering patterns of T-cell trafficking. These activities may affect an individual patient's or a patient population's ability to mount an immune response to a protein product.

A patient's immune status should also be considered when the patient exhibits specific pre-existing ADA, cross-reacting ADA (for example, in the case of murine- or plant-derived products), or antibodies against production cell line-related impurities that might induce a clinical response.

### Genetic Background of the Patient

Molecules that recognize and present protein-derived peptides to the adaptive immune system—the human leukocyte antigen or major histocompatability antigen molecules—show considerable genetic diversity between individuals and between populations in different parts of the world. This diversity is one reason why different patients may have different immune responses to the same product. Consequently, if clinical studies include only a population of limited genetic diversity, then the immunogenicity profile of a protein therapeutic in that population may not reflect its immunogenicity profile in the larger, more diverse population that would be exposed to product after approval.

### Dose and Route of Administration

The way a therapeutic protein product is used can influence its potential for immunogenicity. Different routes of administration appear to have different effects on immunogenicity, and subcutaneous injection generally is perceived to be a more immunogenic route of exposure than is intravenous administration. The dosing regimen also can influence immunogenicity. A protein therapeutic that typically is administered one time as a single dose (e.g., a thrombolytic protein) is less likely to induce an immune response than is a protein therapeutic that is used in a multidose regimen because the immune system usually requires a prime followed by a boost to ensure a robust response. Patients may have pre-existing sensitization even without any known exposure to a therapeutic protein product, and they may exhibit adverse clinical responses on first exposure. However, products with long half-lives or those that are particularly immunogenic (e.g., those with multiple T-cell epitopes) have been shown to induce ADA after a single injection. A chronic dosing regimen has a greater chance of inducing an immune response because the immune system receives multiple exposures to the product and this can lead to a strong memory T-cell response.

Another regimen that has caused ADA with clinical sequelae is one whereby a product is given for a short period of time, stopped, and then introduced again only after a long lag period. This has the effect of priming the immune system, and the reintroduction can cause immune-related events such as allergic responses. The use of chronic dosing on a regular basis, although it repeatedly provides the drug product, appears to avoid such hyper-responsiveness by inducing some form of tolerance.

## DETERMINATION OF PRECLINICAL AND CLINICAL IMMUNOGENICITY

### Preclinical: Relevance and Scope of Preclinical Immunogenicity

The immunogenicity of many biotherapeutics is greater in preclinical studies and has low predictive value for humans because an immune response to human or humanized proteins tends to be greater in animals than in humans because of the perceived foreignness of the protein in animals and the absence of an endogenous biological counterpart. Even though detection of ADA in animals may not be clinically relevant, researchers must assess ADA for the interpretation of the required toxicity data necessary for regulatory submissions (see ICH S6R1 in the *Appendix*).

Generally ADA assessments, PK data, and pharmacodynamic (PD) data aid in the interpretation of the results and validity of animal toxicology studies. In some instances preclinical immunogenicity data also can be used to compare the relative immunogenicity of products before and after manufacturing changes, although only with the grossest of changes in relative immunogenicity does this appear to be meaningful. Preclinical studies, particularly in higher animal species, are typically not statistically powered to draw conclusions on relative immunogenicity but, when they can, it should be recognized that differences in MHC restriction and natural immune tolerance of animals versus humans may not permit translation of such

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information to effects in humans. Furthermore, it should also be acknowledged that strains of preclinical species are limited in genetic diversity compared to the human population.

ADA may alter exposure to an active drug by blocking the therapeutic agent from binding to the target or by accelerating the clearance of the therapeutic agent from circulation, resulting in reduced exposure. ADA may increase the half life of biologic drugs and with this the exposure if the drug–ADA complex is still bioactive. Typically, samples should be collected for possible ADA analysis from all preclinical safety studies in which animals are exposed to the drug for more than 7 days (see references in the *Appendix* for more information). Because the key consideration for including immunogenicity analysis in nonclinical studies is to demonstrate the exposure to the drug for the duration of the study, this also can be achieved by demonstrating drug-mediated modulation of a PD marker. In addition, an absence of an effect on the serum concentration or PK profile of the drug also can indirectly demonstrate the absence of detectable effects of an ADA on drug exposure. However, in some situations the development of an ADA may not significantly affect the clearance of the drug, but instead the ADA may affect the drug's binding to and activity at the target (e.g., anti-idiotype antibodies). Therefore, lack of an effect on a PD marker and/or on PK also should be considered to ensure activity is not affected by the ADA.

For most nonclinical studies, samples from various phases of the study should be collected, banked, and analyzed with regard to any observed pharmacological or toxicological changes. In fact, most nonclinical toxicology studies do not evaluate the kinetics of ADA development, and samples for the assessment of ADA usually are taken at baseline, end of treatment, and end of recovery periods.

Analyses of samples taken during the dosing phase also can be performed if unusual PK data or toxicological findings are observed at the end of the study. In this case it needs to be taken into consideration that analysis of samples taken during the dosing phase may be complicated by drug interference. Therefore, it is important to take samples at appropriate time-points (e.g., before the next dose) and to have suitable assays with high drug tolerance in place. In some instances when a soluble target may be present in the circulation, understanding of ADA level (titers or relative mass units) can facilitate the interpretation of toxicological findings.

A risk-based approach should be used to determine if further characterization (e.g., demonstrating neutralizing activity) is necessary for preclinical studies. Factors such as the presence of endogenous counterparts, utility of a PD marker, or the PK assay may affect this decision. If data from a neutralizing antibody assay (NAb) are deemed necessary, then this assay could be either a target-binding inhibition immunoassay or cell-based assay, irrespective of the risk level. Immunogenicity evaluation and study data interpretation typically require serial sampling and analysis of serum samples for PK, PD, and ADA. Such repeated and frequent sampling may not be feasible when researchers conduct studies using rodents, particularly mice. In such cases, the study can be designed to allow discrete analyses of toxicity, PK, PD, and ADA endpoints from similarly treated cohorts of mice with sample collection at similar study time points to allow inferential analysis of effects observed across treatment groups.

## Clinical: Relevance and Scope of Immunogenicity Assessments

In clinical studies, ADA detection and characterization is important to understand the safety and exposure and efficacy profile of the therapeutic. Typically, the ADA analysis strategy in clinical studies involves a screening assay for binding antibodies then a confirmatory assay, followed by further characterization in NAb. Immunogenicity data from clinical studies generally are analyzed in the context of their relevance to the PK and PD of the therapeutic and on adverse events. For replacement therapies (e.g., enzyme-replacement therapies, blood clotting factors, and erythropoietin), a comprehensive monitoring program should be designed based on ADA detection combined with multiple safety parameters to monitor the potential for serious adverse events.

Researchers should consider the kinetics of the appearance of ADA during clinical studies because this can affect not only ADA detection but also the potential clinical sequelae. Some products induce antibodies rapidly, but other biotherapeutics can take years before an immune response is detected or can be correlated to any clinical sequelae. Investigators also should consider whether an antibody response is transient or persistent. Therefore, understanding the kinetics of ADA appearance is important. This goal can be achieved by carefully selecting ADA sample collection times and taking care in developing the ADA assay for clinical studies. Samples should be collected during each phase of the study (pretreatment, during treatment, and during any washout phases). The sensitivity and drug tolerance of the ADA assay also must be appropriate for the intended use of the assay. For example, when products have long terminal half-lives (e.g., monoclonal antibodies) scientists should develop ADA assays that are capable of detecting ADA in the presence of product levels that are expected to be present in patient test samples. In addition, during the design of ADA sampling plans, researchers should consider the appropriateness of obtaining samples after a washout period.

The number of patients assessed for ADA in clinical trials and the duration and timing of ADA sample collection are critical factors to understand the incidence and clinical impact of ADA. Other factors may confound ADA analysis, including the nature of the therapeutic itself, the presence of pre-existing cross-reactive antibodies, rheumatoid factors, heterophilic antibodies, soluble targets, or ligands. Samples should be taken to assess the levels of these interfering factors in serum (and other PD markers as applicable) at the same time as ADA samples.

Besides determining the presence of binding antibodies, clinical immunogenicity assessments typically include further characterization of positive samples in titer assays as well as in NAb to determine the potential effect of ADA levels to neutralize the drug's effect or to mediate safety events. In addition, understanding the kinetics of ADA and NAb development by detection of ADA in sequential samples taken throughout the study phases and elucidation of the ADA immunoglobulin class(es) and subclass(es) may aid in better understanding of patient- and treatment-related factors and the mechanisms by which the therapeutic induces ADA development.

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# RISK-BASED APPROACH TO ASSESSING IMMUNOGENICITY AND ITS CONSEQUENCES

## Assessing the Potential Risk of Product-Specific Immunogenicity

The concept of risk is defined in ICH Q9 as the combination of the probability of occurrence of *harm* and the *severity* of that harm. In relation to pharmaceuticals, the protection of the patient by managing the risk to safety should be considered of prime importance and thus risk assessments of product-induced immune responses should focus on the potential severity of clinical consequences from ADA responses rather than the probability of occurrence of ADA responses. A few patients with severe or life-threatening ADA-related side effects are of more concern than many ADA-positive patients who have no clinical consequences. Risk mitigation should also be factored into the overall risk-assessment process (e.g., elimination of clinical impact by co-medication).

Although ADA testing strategies can be based on immunogenicity risk assessment, this may not always be feasible during early drug development when reliable assays may not be available.

ADA-induced safety events can range from mild side effects to life-threatening conditions. The potential severity of the consequences of an ADA response should be considered as early as possible. *Table 1* summarizes some but not all of the risk factors that may influence the severity of clinical consequences from an ADA response.

**Table 1. Factors That May Influence the Severity of ADA-Related Clinical Sequelae**

| | Lower Risk[1] | Medium Risk | Higher Risk |
|---|---|---|---|
| Presence of an identical endogenous counterpart (includes families of proteins that share domains) | No endogenous counterpart (e.g., Botulinum toxin) | Redundant counterpart (e.g., interferons or growth hormones) | Nonredundant counterpart [e.g., erythropoietin or megakaryocyte growth and development factor (MGDF)] |
| Presence of a structurally related endogenous molecule or domain | No structurally related endogenous molecule or domain | Medium structurally related endogenous molecule or domain exists | Highly structurally related endogenous molecule or domain exists |
| Patient's disease state | Life-threatening | Moderate-to-severe | Mild |
| Potential clinical consequences of immunogenicity | No clinically meaningful impact on safety or efficacy | Manageable impact to safety and/or efficacy | Extensive clinical influence on safety or loss (or dramatic increase) of efficacy |

[1] Risk is a consequence of both severity and frequency.

A number of factors may contribute to the incidence of an ADA response, and some but not all of these factors are shown in *Table 2*. During an immunogenicity risk assessment the factors shown in *Table 2* should be considered in conjunction with those in *Table 1*. The clinical consequences of immunogenicity are unpredictable, even with the risk assessments outlined above.

An immunogenicity risk assessment is of real value only when all the factors that influence the likelihood and severity of a potential immune response are carefully considered. Risk assessments should be done in a cross-functional manner, including input from clinicians, safety assessment, PK, bioanalytical scientists, as well as process scientists. Consultations with regulators and clinical safety monitoring boards also may be helpful and should be carried out iteratively during the product development process as clinical data are obtained.

ADA-mediated clinical sequelae and ADA incidence rate are separate entities, but the two factors are interrelated because the number of patients with ADA-mediated serious adverse events may rise with a higher ADA incidence rate.

**Table 2. Factors That May Influence ADA Incidence**

| | Lower Incidence | Medium Incidence | Higher Incidence |
|---|---|---|---|
| Circulating level of endogenous counterpart | Abundant | Scarce | None |
| Patient's immune status | Suppressed | Normal | Activated |
| Exposure: dosing regimen or frequency | Single dose | Chronic (maintenance) | Episodic dosing |
| | | Multiple dosing | |
| Route of administration | Intravenous or oral | Subcutaneous, intramuscular, mucosal (non-inhalational) | Intradermal or inhalational |
| Product Characteristics | Lowest levels (or absence) of product or process-related impurities (e.g., aggregates or denaturation, fragmentation) | Intermediate levels of product- or process-related impurities, and potential epitope content | Higher levels of product- or process-related impurities (e.g., aggregates; denaturation, fragmentation) |
| | Molecular integrity of active substance maintained | | Higher level of antigenic epitopes (derived from murine cell lines, contains novel mutated sequences, etc.) |
| | No or low content of potential T-cell epitopes | | Mechanism of action: immune activation |
| | Mechanism of action: e.g., immunosuppressant | | |

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## Risk-Based Approach to ADA Testing Strategy

The ADA testing strategy should be based on an immunogenicity risk assessment. Appropriately designed, validated, and executed immunogenicity assays and testing schemes provide the data that make risk assessments possible and predict the eventual outcome for patients. The frequency of sampling, neutralizing activity assessments, and qualitative or quasi-quantitative measurements may all depend on perceived risk.

In clinical studies, patient safety is of primary concern, and the extent of ADA characterization necessary depends on the potential risk of ADA-related sequelae. The type of drug should also be taken into account. For example, for a multicomponent fusion protein that contains at least one component with a potentially high risk of adverse events, a domain-mapping method (i.e., reactivity of ADA with individual components) is recommended.

Generally, more extensive ADA testing and characterization should be applied if the risk of clinical adverse effects is high. Samples should be analyzed and characterized based on the timing and incidence of the ADA response as well as the occurrence and severity of clinical side effects. A higher risk of ADA incidence normally does not warrant extensive characterization of ADA, and usually the risk of clinical consequences drives the bioanalytical strategy. Nevertheless, some investigations may be driven by the need to understand the cause of a high ADA incidence (e.g., the reactivity of ADA with aggregated versus nonaggregated drug).

The following step-wise, risk-based testing strategy can be refined depending on the product's level of risk and during the design of the clinical studies to ensure that the maximum amount of data can be gained, including correlations to PK, PD, etc.

STEP 1

Develop ADA methods that are fit for purpose and are consistent with current industry best practices and regulatory guidance. Incorporate baseline and postdose ADA testing into the clinical study design, together with concurrent testing for PK plus any relevant PD, safety, or efficacy markers that will facilitate interpretation of ADA data. The analysis of results from ADA testing should be built into study analysis plans.

STEP 2

Test all pre- and post-dosing samples for ADA. Two important tests that should be carried out in all cases are the ADA screening assay and the drug inhibition or immunoglobulin depletion confirmation assay. Report as negative any ADA results below the assay cut-point and with drug levels below the interference levels, as well as those that test negative in the confirmatory assay. Test methods that are capable of sensitive ADA detection despite the presence of trough levels of drug are desirable. In their absence, samples containing drugs above the interference levels should be reported as inconclusive with a statement of possible drug interference. In such cases, ADA analysis could be performed later following a drug washout period to reach a conclusive result (refer to the section *Relative Sensitivity* of this chapter for further information). For the confirmed positive samples, ADA levels should be estimated, preferably by titration, but they can be reported in terms of relative mass units. Certain mass-based technology platforms may necessitate the use of relative mass units.

STEP 3

Samples deemed positive in *Step 2* should be tested for neutralizing ability and potentially other characteristics, depending on the risk assessment. In high-risk situations, NAb activity should be measured, typically using a cell-based assay. Depending on the drug's mechanism of action, sometimes a ligand-binding NAb assay format can be used if it is adequately proven to specifically detect NAb. Concurrently generated PK/PD/safety or biomarker data should be used to help interpret the clinical relevance of neutralizing antibody activity. In addition, determination of ADA isotypes and affinity may be helpful in the overall assessment of the immune response. Allergic reactions associated with drug administration may necessitate measurement of drug-specific IgE, although detection may depend on the sampling scheme and method design.

# DESIGN OF IMMUNOASSAY-BASED TEST METHODS

Immunoassay methods for ADA detection generally are complex and require a broad understanding of multiple technical challenges. Screening assays that serve as the key first step in the immunogenicity testing scheme are designed to have a certain false positive (rather than false negative) rate in order to maximize the sensitivity for detecting ADA. In addition, using a risk-based approach, it is more appropriate to have 5% false positives rather than false negatives during this initial screening phase. Typically, positive screening samples are confirmed to contain drug-specific ADA in confirmation assays before the determination of the level of ADA (titers) or any additional characterization.

## Screening Assays

In their simplest form, screening ADA assays immobilize the therapeutic protein on a microtiter plate or onto beads to capture ADA (solid phase) or co-incubate a labeled therapeutic protein at a predetermined concentration with the sample containing ADA (solution assay). The bound polyclonal ADA is then detected using a labeled secondary reagent or labeled drug. Limiting the number of wash steps or reducing wash fluid dispensing rates may increase the detection of antibodies with fast off-rates on assay platforms that use wash steps. Generally, screening assays are designed to detect classes of antibodies that may be most relevant to the product's route of administration, e.g., IgA for mucosal routes of administration. Although the most common ADA raised against protein therapeutics are of the IgM and IgG isotypes, other isotypes of ADA including IgE and IgA may require detection based on the clinical response and the route of product administration. In addition, depending on how

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rapidly ADA responses develop and the half-life of the therapeutic, it may be feasible to detect the development of ADA initially of the IgM isotype that later affinity matures to an IgG isotype following repeat administration of product.

Because screening assays serve as the first step in the immunogenicity testing program, these assays generally are configured to have moderate throughput and often are automated. The various technology platforms used to develop screening immunogenicity assays have inherent strengths and weaknesses as outlined in *Table 3*. Development of a bioanalytical strategy to use a certain technology platform for assay development should take into consideration the nature of the product (e.g., a therapeutic protein or a monoclonal antibody), potential sources of interference in the assay (e.g., therapeutic concentration anticipated in patient samples, soluble target based on co-medications, and biology), and disease-specific interfering or cross-reacting factors (e.g., rheumatoid factor).

**Table 3. Advantages and Disadvantages of Various Assay Types Currently Used to Assess ADA**

| Assay Type | Advantages | Disadvantages |
|---|---|---|
| Direct/Indirect ELISA | High throughput | Potential for high background |
| | Readily available | May not be specific |
| | Easy to automate | Utility depends on capability to detect different Ig subtypes |
| | Inexpensive | Drug tolerance lower for solid-phase ELISAs |
| | Readouts can increase sensitivity (e.g., electrochemiluminescence) | Excessive washes can decrease detection of low-affinity ADA |
| | Drug tolerance higher for solution-phase ELISAs | |
| Bridging Format | Low background | Difficult to confirm presence of IgM |
| | Highly specific | Need to label product |
| | Readily available | Reduced ability to detect low-affinity antibodies and $IgG_4$ because of single-arm binding between bound ligand and detector |
| | Easy to automate | |
| | Inexpensive | |
| | Format can be used across species and detects all isotypes | |
| | Can be used across detection platforms (colorimetric, electrochemiluminescence, etc.) | |
| Surface Plasmon Resonance/Biolayer Interferometry Assays | Flexibility to characterize immune response (concentration and relative affinity) | Expensive technology |
| | Higher drug tolerance and can detect low-affinity antibodies | Limited suppliers |
| | | Moderate throughput |
| Radioimmunoprecipitation Assays | Inexpensive | Radioactive waste |
| | Highly sensitive | Need to frequently requalify radiolabeled reagents because of short half-life |
| | Better for high-affinity antibodies | Utility depends on capability to precipitate all relevant antibody present |
| | | Can be less useful for low-affinity antibodies |

In addition, analysts should consider whether the method does not result in an underestimation of ADA-positive samples because the ADA binding epitopes on the capture reagent are blocked with a tag or by coating on plates or beads. Another point for consideration in the design and development of an ADA assay is the adaptability of the test for both nonclinical and clinical matrices. Although most assay formats can transfer readily from nonclinical to clinical use, there are exceptions. Further, clinical ADA assays should be qualified or validated for use with samples collected from a similar patient population. Here again, although most ADA screening assays may not show unique matrix interferences between one disease matrix and another, there may be exceptions. Immunoassays used for ADA detection generally are quasi-quantitative methods because standardized, species-specific (especially human) polyclonal ADA calibrators generally are not available. The positive controls, typically developed in-house as hyperimmune serum in animals or by phage display serve as surrogates for the drug-induced ADA in treated patients.

As depicted in *Table 3*, some of the more common assay formats currently in use for development of screening assays include plate-based or bead-based enzyme-linked immunosorbent assays (ELISA; see also USP general chapter *Immunological Test Methods—Enzyme Linked Immunosorbent Assays (ELISA)* ⟨1103⟩) with colorimetric, fluorometric, or luminescent read-outs, plate-based or solution-phase electrochemiluminescent (ECL) or ELISA assays, surface plasmon resonance assays (SPR; see also USP general chapter *Immunological Test Methods—Surface Plasmon Resonance* ⟨1105⟩), or biolayer interferometry assays, and radioimmunoprecipitation assays (RIPA). In order to differentiate positive from negative responses, assay cut-points are statistically determined using samples collected from the target population. The assay cut-point also helps to determine the assay sensitivity. An incorrectly established cut-point may result in false negatives or too many false positive responses that should be ruled out as drug-specific responses in the confirmation assay. Assay performance typically is optimized during development by evaluation of the following parameters: sensitivity (lowest amount of detectable antibody in a sample demonstrated using surrogate controls); specificity (likely detection of a true positive rather than a nonspecific interaction);

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precision (reproducibility of results from multiple analyses); interference (interfering substances in sample matrix, including the administered drug, that affects assay sensitivity); and stability and robustness (likelihood of optimal assay performance over time). After optimizing these parameters, analysts typically validate the method for its intended use. If the initial assay cannot meet the performance goals (e.g., because of poor sensitivity or high backgrounds), then analysts should either improve and validate the first assay format again or develop and validate more than one assay format.

### Confirmatory Assays

Samples that are positive in the screening assay usually are confirmed in a second assay that includes adding a certain fold excess of the therapeutic. This is intended to demonstrate that a positive signal seen in the ADA screening assay is caused by the presence of drug-specific antibodies. Because the cut-point for the screening assay is set to result in the detection of approximately 5% false positives, the confirmatory assay is used to rule out the false positive samples from further analysis. Multiple options are available for performing confirmatory assays. Usually a soluble drug is added to the sample and should compete with the plate-bound immobilized drug for binding to sample ADA. A specific interaction to a soluble drug results in a decrease in the assay signal. As with the screening assay, the cut-point for the confirmation assay is established statistically. Verification of the presence of drug-specific antibody can also be performed using an orthogonal method on a different assay platform that may have different nonspecific binding profiles. Analysts should take care while adopting this approach to ensure an adequately sensitive assay in order to avoid a false negative reaction. Finally, the specificity of ADA to a drug can also be confirmed by depleting all immunoglobulin from a sample (e.g., using a Protein A or G column) followed by reanalysis of the depleted sample. In the latter approach, the depleted sample scores negative in the assay if an antibody caused the original signal. Validation of the confirmatory assays helps ensure that the results from the confirmatory assay are appropriately interpreted.

### Characterization Assays

Following screening and confirmation, the relative level of ADA in a positive sample is assessed by titration. The most common approach is to serially dilute the sample and report the reciprocal of the highest dilution factor at which the sample tests positive, or the titer of the sample. The higher the sample dilution (and therefore the ADA titer value), the higher is the concentration of circulating ADA in that sample. This approach has been used historically to report serological data from vaccine studies. Another less frequently used approach is to express the amount of ADA in the sample in mass units relative to a surrogate standard. Although this approach has the advantage of relating the amount of antibody in the sample to assay sensitivity, analysts should recognize that the calibrator may not be representative of the polyclonal ADA responses under measurement.

Traditional approaches to dilutional linearity testing do not apply to ADA assays. However, when analysts express ADA data in terms of titer values, they also should demonstrate that the positive control(s) displays reasonable (relative) linearity of dilution.

In addition to performing titration, analysts routinely characterize positive ADA samples in neutralization assays to determine the in vitro effect of ADA that might reflect the in vivo biological or pharmacological activity of the therapeutic product. Additionally, the isotype(s) of the ADAs also can be analyzed. Isotype(s) identification is sometimes performed in a multiplexed manner. Using one fluorescent multiplex platform, analysts mix each sample with multiple secondary reagents that are specific to different immunoglobulin isotypes and are labeled with unique fluorochrome labels.

An SPR-based platform also can be used for this purpose (also see *Immunological Test Methods—Surface Plasmon Resonance* ⟨1105⟩). The isotype of an ADA can be determined by observing binding of isotype-specific reagents (such as an anti-human $IgG_1$ Ab) to the ADA that has been captured by immobilized drug. Analysts should take care when identifying and validating the isotype-specific reagents because unexpected cross-reactivity often is observed. Isotyping can help understand the maturation of the immune response. For example, an ADA response that is comprised solely of IgM antibodies is an immature immune response without T-cell involvement and may or may not progress further. In contrast, an immune response comprised of $IgG_1$ and $IgG_4$ antibodies represents a more mature response that has already engaged more components of the immune system. ADA titration and characterization assays are validated routinely using many of the same parameters as screening and confirmation assays to ensure consistent assay performance.

## VALIDATION OF IMMUNOASSAYS

Method validation is a process of demonstrating, by the use of specific laboratory investigations, that the performance characteristics of an analytical method are suitable for its intended use (see also USP general chapter *Validation of Compendial Procedures* ⟨1225⟩). The level of method validation depends on the stage of product development and the risks associated with the product. A partial validation involving assessments of method sensitivity, specificity, and precision requirements with less emphasis on robustness, reproducibility, and stability may be adequate for the earlier stages of clinical development (Phase 1–Phase 2 studies), whereas fully validated methods are required for pivotal and postmarketing studies.

Validation of an assay before use of the method for sample bioanalysis is called *pre-study validation*, and amendments to this process may be made between studies. This process maps out the performance characteristics of the assay and should demonstrate that the method is suitable for its intended purpose when it is subsequently applied to study samples. In contrast, *in-study validation* refers to the monitoring of assay performance during study-phase applications of the assay in order to ensure that the assay remains valid and that the resulting bioanalytical data are reliable.

Reliable performance of the assay also depends on all the elements spanning bioanalytical testing and data manipulation, such as assay reagents, analysts, equipment, and computer programs. In essence, the assay is a system comprising several elements other than assay steps and reagents alone. Pre-study validation therefore establishes system suitability (establishment of criteria for control samples that are used to accept or reject runs and imprecision limits for individual samples), and then

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in-study validation continues to verify it. Critical changes to methods often require additional validation (partial or full), sometimes leading to the revision of the system suitability criteria.

## Minimum Required Dilution

During assay development the minimum required dilution (MRD) can be defined as the dilution level of the ADA negative sample that results in the highest signal-to-variability ratio (or *Z′* factor).

The ability to dilute such samples also should be assessed to ensure that the chosen MRD is adequately distal to any prozone (hook) effects that may have been observed. Although they are rare, some unusual prozone effects may require the test method to include more than one dilution of a test sample to minimize false negative data.

The MRD should be evaluated during the assay development/design phase, i.e., before analysts initiate the validation experiments, so analysts do not need to repeat the evaluation during validation. It can be established using 10 individual drug-naïve ADA negative samples, each tested in 2-fold serial dilutions (e.g., a range of 1:5 to 1:80). The MRD is sometimes defined as the dilution level that results in the highest signal-to-background ratio when typically the background is the dilution matrix. However, this definition ignores the variability in the signal. Therefore it is preferable to define the signal-to-background value and include variability.

One way of doing this is to use a *Z′* factor that includes both the intensity of the assay readout and its variability at different dilutions (see the *Appendix* for more information). The *Z′* factor for each dilution level is obtained from the formula [(mean (*S*) – 3 SD (*S*)] – [mean (*B*) + 3 SD (*B*)]/[mean (*S*) – mean (*B*)], where *S* is the assay signal of the diluted sample and *B* is the background signal. Thus, the MRD is the dilution that results in a desired value for the *Z′* factor and signal-to-background ratio. This metric is widely used for high-throughput screening assays to ensure adequate confidence in the ability to differentiate between truly active versus inactive compounds. An inappropriately large MRD can compromise the sensitivity of an assay.

## Pre-Study Validation

Pre-study validation establishes the following:

1. Assay cut-points
2. System suitability criteria
3. Relative sensitivity
4. Specificity
5. Selectivity/interference
6. Precision
7. Robustness
8. Reproducibility
9. Stability

## Assay Cut-Points

Because of the quasi-quantitative nature of ADA detection methods, the use of a decision threshold or cut-point becomes necessary to discriminate between ADA-positive and -negative samples. Because the screening assay and specificity confirmation assay produce different types of results, separate cut-points are necessary. In some instances, a different cut-point also may be necessary for evaluating titers of confirmed positive samples from the titration assay. Some key points in the evaluation process are summarized briefly below (more information is available in the *Appendix*).

### SAMPLES FOR CUT-POINT EVALUATION

Samples from an appropriate population should be used for the development of assay cut-points. In some cases it may not be practical or feasible to obtain matrix samples from a population that has a target disease before initiating pre-study validation experiments. Consequently, samples from healthy drug-naïve subjects are used commonly to establish the initial cut-points. This approach is preferred for a conventional Phase 1 study with normal volunteers. When the clinical program progresses beyond Phase 1 and samples from the target disease population become available, it is more appropriate to re-evaluate cut-point data for the target population. If the distribution of assay responses with respect to both the mean and variability are comparable between the target population and the normal volunteers, then the same cut-point can be used. If not, target disease-specific cut-points are more appropriate, and fixed or floating cut-points computed from the data obtained from the baseline samples from a clinical trial can be considered.

### SCREENING CUT-POINTS

The screening assay cut-point is a signal in the screening assay that identifies a sample that is likely to contain ADA (termed a *screen positive* or *potential positive* sample) versus an ADA-negative sample. A screening assay cut-point is established during pre-study validation based on a systematic and statistically rigorous analysis of assay responses from a panel of individual samples that are considered to be representative of a drug-naïve target patient population.

To determine the screening method cut-points for clinical assays, analysts should use samples from at least 50 drug-naïve individuals for a robust evaluation. If additional indications are targeted, analysts should test at least 20 drug-naïve individuals per indication. If the variability is significantly different from the original indication, then additional drug-naïve individuals may need to be tested. If not, then the original cut-point can be applied to the additional indication. For nonclinical assays a total of at least 25 drug-naïve individuals should suffice. To ensure this cut-point is robust, at least two analysts should perform this

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experiment over three days in at least two different plate layouts. A balanced experimental design and plate layouts will help avoid potential confounding between analysts, subject samples, run dates, etc. For clinical ADA assays, if multiple disease-state populations are being tested they should be distributed evenly across the plates to ensure they are properly balanced across plates and runs. Statistical outliers of the sample results should be examined and eliminated, e.g., using outlier box plots defined in terms of quartiles and interquartile range. In addition, confirmed reactive samples (e.g., via immunodepletion) can be excluded as well.

Three types of screening cut-points can be calculated for application during the study phase—fixed, floating, and dynamic—and one of these should be appropriately chosen for study phase bioanalysis.

**Fixed cut-point**—A fixed cut-point is a cut-point that is determined in pre-study validation and subsequently is used for the in-study phase. The fixed cut-point is used for analyses of test samples until there is a need to revalidate or change the cut-point (e.g., because of a critical change in the assay, assay transfer to another laboratory, upgraded instruments, etc.). The cut-point value can be fixed within a given study, for a target population, or across studies and multiple target populations. In order to use this approach, one should statistically demonstrate similar means and variances across runs during pre-study validation. A fixed cut-point can be determined based on the mean + 1.645 SD (standard deviation), which represents the 95$^{th}$ percentile of the population under normal distribution (and therefore is expected to identify approximately 5% of the samples as false positives). The standard deviation should include different sources of variation such as the intra-run, inter-run, inter-analyst, and inter-subject variability. If the data are not normally distributed, appropriate transformations (typically log transformations) can be used. If transformation doesn't help, usually it is acceptable to determine the nonparametric 95$^{th}$ percentile. However, in preclinical trials it may be considered adequate to use a cut-point at the 99$^{th}$ or 99.9$^{th}$ percentile because immunogenicity of a protein normally results in high antibody titers. Alternatively, even if the validation data suggest similar means and variances across runs, to account for possible deviations between assay runs during the in-study bioanalysis phase, it would be safer to apply a floating cut-point.

**Floating cut-point**—A floating cut-point is a cut-point calculated by applying an additive or multiplicative normalization factor, determined from the pre-study validation data, to the biological background obtained during the in-study phase (see Appendix G of Shanker et al., 2008, in the *Appendix* for details). The biological background may be represented by the negative control (pool of matrix from subjects that are negative for ADA), the assay diluent, or the predose subject sample (subject-specific cut-point). The method for determining floating cut-point uses the variation estimate from the pre-study validation that includes different sources of variation such as the intra-run, inter-run, inter-analyst, and inter-subject variability. Such a cut-point is recommended when the means of drug-naïve samples are not similar but the variances are similar across runs. When a negative control is used for normalization, analysts also should ensure that the negative control results represent the drug-naïve matrix sample results of the target population. This is accomplished by demonstrating that the signal of the negative control trends directionally with the signal of the individual samples. Alternatively, the use of assay diluent for normalization or pretreatment subject ("baseline") sample results may be more appropriate. However, a pre- versus post-dose ratio might be a better solution.

**Dynamic cut-point**—A dynamic cut-point is a cut-point that changes between the plates in a run, between runs in a study, or between studies, and it does not apply the variation estimates from pre-study validation. The latter characteristic differentiates it from a floating cut-point. This approach is necessary only where means and variances significantly differ between runs. Because this approach entails testing of several individual drug-naïve samples for the evaluation of a run-specific cut-point, it consumes a large portion of the plates from each in-study run and therefore is not practically feasible, especially when analysts use 96-well plates instead of 384-well plates. Differences in variability between assay runs sometimes can be resolved by further optimization of some key steps in the assay protocol or by resolving some analytical issues. In some cases, the differences in variability can be attributed to different analysts or instruments, and use of an analyst-specific or instrument-specific floating cut-point may resolve this issue. If further optimization does not resolve the situation or if the causes are not clear, another practical alternative may be to pool the variability across all runs and use this pooled variance for floating cut-point evaluation during the in-study phase.

## SPECIFICITY CONFIRMATION METHOD CUT-POINT

Because of the conservative approach of incorporating a 5% false-positive rate into the computation of the screening cut-point, the elimination of false-positive samples via confirmation of drug-specific binding is an important component of ADA bioanalysis. It is also important to understand the level, if any, of the drug itself within the sample.

The amount of change in assay signal that differentiates drug-specific binding from nonspecific binding is referred to as the *specificity cut-point*. The specificity cut-point should be determined by an objective approach in the context of assay variability near the low positive range of the assay. To determine the specificity cut-point, drug-naïve negative samples from at least 25 individuals should be evaluated (however, more are commonly used when available), with and without drug preincubation. Ideally these samples should be the same as those tested during the screening cut-point evaluation, and the unspiked and spiked counterparts of the individual subject samples should be tested together in the same plates.

The mean percent change from the unspiked sample (inhibition) and SD are calculated. The mean inhibition plus 3.09 SD (if a 0.1% false positive rate is desired) represents the specificity cut-point. As in determination of the screening cut-point, specifically reactive samples after preincubation with drug (i.e., those that contain pre-existing antibodies) and statistical outliers should be eliminated in order to make the specificity cut-point more conservative. The analytical process outlined above for the screening cut-point applies for the evaluation of the specificity cut-point as well.

Alternative approaches such as the use of *mock low positive* samples in which the individual drug-naïve samples are spiked with a low concentration of a positive control sometimes is considered for this cut-point evaluation. However this method is subjective and is not recommended because it depends on the concentration of the positive control and the unique affinity/avidity of the positive control that may or may not represent true positive patient samples. Additional sources of information regarding the relative statistical merits of these approaches and methods for verifying the assumptions are listed in the *Appendix*.

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### TITRATION METHOD CUT-POINT

The titration method cut-point is a test result value below which further serial dilution of an ADA-positive sample produces negative assay results. Typically, the screening assay cut-point is used as the titration cut-point, but the validation of a separate titration method cut-point can become necessary when the signal from the assay diluent or matrix causes higher results than the screening assay cut-point (because of a blocking effect of serum) or if samples at a dilution higher than the MRD do not generate consistently negative results, i.e., when the screening cut-point falls on the lower plateau of the positive-control dilution curve. In such instances, the same data generated during a screening cut-point experiment can be used to define the titration cut-point using a 0.1% false positive rate threshold criterion (i.e., Mean + 3.09 SD). During bioanalysis, confirmed positive patient samples that fall between the screening cut-point and titration cut-point can be assigned a titer value equal to that of the MRD.

### CROSS-REACTIVITY METHOD CUT-POINT

If applicable, ADA-positive samples that are confirmed to be specific to the drug can be further characterized for cross-reactivity to other related antigens. Like the specificity confirmation assay, a cross-reactivity test method may require a preincubation step with and without the related antigen. Cross-reactivity to the antigen is confirmed when the percent inhibition of signal in the presence of the antigen is greater than or equal to the cross-reactivity method cut-point. The methods for determining the cross-reactivity method cut-point are similar to those for the specificity method cut-point, although it also may be acceptable to apply the drug specificity confirmation cut-point.

## Defining System Suitability

### ASSAY CONTROLS

ADA-positive controls can comprise polyclonal or monoclonal anti-idiotypic antibodies. They should be affinity purified and quantitated to enable assay validation. Each run (or plate) should include at least a low level of positive control (*low positive control*) and a negative control, but the inclusion of a higher level control (*high positive control*) also can be useful in monitoring method performance. Tracking all of these controls over time can help ensure that the method is performing suitably. A low positive control helps ensure that the assay remains as sensitive during study phase bioanalysis as during the pre-study validation.

On the one hand, the low positive control should produce a response that can be seen reproducibly above the cut-point, but sometimes it may result in a signal that is below the cut-point (thereby failing or invalidating the assay). On the other hand, choosing an unreasonably high concentration for a low positive control may produce an assay signal that is substantially above the cut-point, which is inappropriate. To provide objectivity to the selection of a low positive control concentration, it is useful to think in terms of assay rejection rates, i.e., the percentage of assays (plates) that fail because the low positive control produces a result below the cut-point. As an example and in order to understand if the low positive control is sufficiently low, a 1% rejection rate may be a reasonable target for a low positive control. This is calculated as mean + $t_{0.01,df}$ × SD, where mean and SD are determined using the data from the sensitivity experiment or related assay development data, and $t_{0.01,df}$ is the critical value determined from the *t*-distribution corresponding to a 1% false positive rate and *df* is the degrees of freedom that depends on the number of samples and runs used in the calculation. This theoretically implies that about 99% of the data from the low positive controls will be at or above the cut-point.

An optional high positive control can be useful for methods that are prone to hook effects, tracking assay performance, reagent qualifications, and troubleshooting. The concentration of the high positive control should be chosen from the upper end of the linear range of the dilution curve, usually just below the upper plateau of the curve.

### SYSTEM SUITABILITY CRITERIA

System suitability criteria using assay controls help ensure that an analytical procedure remains valid for use. Acceptance ranges (system suitability criteria) for quality controls should be established by statistical evaluation of the experimental data acquired during assay validation.

When the floating cut-point approach is deemed necessary and is used for the screening cut-point evaluation, the system suitability criteria or limits can be defined for the ratio of the low positive control to the negative control and for the ratio of the high positive control to the low positive control or a negative control instead of defining limits separately for each positive control. It is also useful to apply acceptance criteria for intra-assay precision (variability of signals of replicates in an assay) for the in-study phase. Although data from assays that fail acceptance criteria during the in-study phase should be rejected, setting criteria for passing or failing assays in pre-study validation experiments should be avoided because these potentially can lead to the exclusion of some validation data, resulting in an inaccurate estimate of analytical error. All assays during pre-study validation should be included, and the only exceptions should be those rejected for an assignable cause (e.g., technical error).

## Relative Sensitivity

No ADA-positive control can be expected to represent the spectrum of humoral immune responses observed in individuals treated with study compounds. The sensitivity of ADA assays is highly dependent on the nature of the positive control reagent(s) so that high-affinity positive controls often produce better sensitivity values than lower affinity positive controls in the same assay. Analysts should consider this when they choose controls, as well as when they estimate assay sensitivity. Moreover, because the drug itself can interfere with ADA detection, the sensitivity of ADA detection becomes progressively worse in the presence of increasing concentrations of drug within the sample. Despite these caveats, the determination of assay sensitivity is valuable when analysts choose an optimal ADA detection method or platform, a low positive control for validation, or assess

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the suitability of an assay. The assessment of assay sensitivity in the presence of an interfering drug (drug tolerance) is critical for understanding the suitability of the method for detecting ADA in dosed patients. ADA assay sensitivity should be defined not as a single value, but as a set of at least two values: (1) the concentration of positive-control ADA detected within undiluted matrix in the absence of any drug and (2) the concentration of positive-control ADA detected within undiluted matrix in the presence of drug levels expected at the time points when samples for ADA analysis are taken. Assays should, in general, demonstrate a sensitivity of at least 500 ng/mL for methods applied to clinical studies (or 1000 ng/mL for nonclinical studies) to show suitability for intended purpose—that is, for the detection of clinically meaningful ADA, although assay sensitivity should be justified on a case-by-case basis. It is not useful to express sensitivity in terms of antiserum titers, and thus sensitivity should be assessed using monoclonal antibody or affinity-purified polyclonal preparations. Analysts can evaluate sensitivity by means of two assay runs performed by two independent operators (when feasible) for a total of at least three runs.

To assess sensitivity in the absence of a drug, analysts should prepare mock samples with known concentrations of ADA that are serially diluted (usually 2- to 3-fold serial dilutions) in matrix pooled from drug-naïve individuals and evaluated according to the screening method until the assay results of the dilutions in matrix are below the screening assay cut-point. The lowest concentration of ADA that is consistently found (for example, using a 95% upper confidence limit based on the number of runs or operators) above the screening assay cut-point is determined to be the sensitivity of the assay. Alternatively, it can be the lowest concentration of ADA that is found to be above the screening assay cut-point in all runs by all operators or in 19 of 20 runs (see the *Appendix* for more information).

To assess sensitivity in the presence of a drug, two alternative experimental approaches could be considered: (1) titrate the drug into undiluted matrix containing set concentrations of a positive-control ADA (e.g., 250, 500, or 750 ng/mL). Report the highest concentration of the drug at which ADA remains detectable. (2) Alternatively, because immunogenicity samples often are taken at drug trough time points, prior knowledge of the anticipated trough drug concentration range could be used to determine the assay sensitivity in the presence of the expected concentrations of the drug.

## Specificity

Specificity refers to the ability of a method to detect ADA that specifically binds the drug molecule, its domains, or components. The assay is developed and optimized based on the ability of the positive-control ADA to specifically bind the drug. During validation, results of the specificity confirmation assay support assay specificity.

## Selectivity and Interference

The selectivity of an ADA assay is its ability to identify a positive control in biological matrix samples that may contain potential interfering substances and is an important concern for ADA detection assays. Such matrix effects typically arise from nonspecific binding interactions between a matrix-based factor and the ADA or from specific binding of unknown factors. During validation, analysts assess the selectivity of the ADA assay by looking at the recovery of analyte (represented by a positive control sample) from matrix samples that contain the potential interferent(s). One caveat here is that the selectivity of an ADA assay, as assessed using the positive control, may not reflect the selectivity of the assay when it is used with actual nonclinical or clinical samples.

Interference is the property of a factor (most commonly the drug itself and its target, if soluble) to affect assay results positively or negatively. It should be evaluated using a low positive ADA test sample that is spiked into a sample matrix from drug-naïve patients. Each potential interfering factor should be tested at a physiologically or pharmacologically relevant range of concentrations. The highest concentration of the interfering factor that does not alter the classification of the test sample (e.g., an ADA sample that remains positive relative to the screening assay cut-point) is defined as the tolerance of the assay to that interfering factor. For therapeutics that have a long terminal half-life, the main interferent in an ADA assay is the drug itself. As discussed previously, the drug tolerance of an assay should be interpreted as the sensitivity of the method in the presence of interfering drug.

Other endogenous interferents include oligomeric drug targets, or the target's soluble receptor may interfere with ADA detection. In addition, certain sample pretreatments performed to reduce drug interference can release drug target from drug-target complexes, leading to subsequent interference problems. Hence, analysts should carefully evaluate pretreatment steps such as acid dissociation during assay development to mitigate the risk of inaccurate data.

## Precision

Precision is a measure of the variability in a series of measurements for the same material run in a method. The acceptance criteria for the precision of ADA assays should be within the range commonly expected for immunoassays. These criteria also should be appropriate for the assay platform and should be fit for purpose. During assay validation, precision should be determined in experiments that are run at the level of intended use during the study phase (e.g., number of plates, samples per plate, etc.).

The acceptance criteria for precision should be within the range commonly expected for immunoassays. These criteria also should be appropriate for the platform used, guided by assay development data and experience with the technology platform and assay method. Additional information is found in the *Appendix*.

### SCREENING AND CONFIRMATORY METHOD PRECISION

For ADA screening assays with numeric readouts (as opposed to categorical yes/no readouts), assay precision can be determined using data from at least six independent assay runs of the assay positive controls (low positive and high positive controls). Typically, estimates of intra-assay precision (interreplicate variability, also called intra-assay repeatability) and interassay precision (also called interassay repeatability, or intermediate, total, or overall precision) are reported as percent coefficient of variation (%CV).

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Intra-assay precision (repeatability) is the variability of assay results when the same material is tested multiple times within the same run. Interassay precision (also termed intermediate or total precision) is the variability of assay results when the same sample is tested in separate runs, over separate days, and by multiple operators (or only one operator if the study phase bioanalysis is intended to be performed by only one operator). These are expressed as %CV of ADA signals. Data from the replicates of negative and positive controls from each of all the runs tested during the pre-study validation phase are pooled and analyzed within the framework of random-effects ANOVA, resulting in estimates of intra-assay %CV and interassay %CV. Analysts should consider positional effects by varying sample position on microtiter plates because these effects (e.g., edge effects) can influence the assay precision. One should use the same number of test and control sample replicates during validation as are used in the assay during routine use.

Similarly, intra- and inter-assay precision estimates for the confirmatory assay can be derived using the percent inhibition data of the spiked versus unspiked low positive control samples from multiple assay runs (at least six) in the pre-study validation.

### TITRATION ASSAY PRECISION

In order to determine the precision of a titer, two or more analysts should assay serial two-fold dilutions of five or more mock high positive control samples in at least six runs. Mock high positive control samples can be obtained by spiking individual negative sera from the target population with a high positive sample. The titer then is determined by interpolation of each of the dilution curves, and the overall mean and SD are calculated. Then intra- and inter-assay precision (%CV) can be determined.

A recommended but more rigorous approach is to use these data to define a minimum significant ratio (MSR): $MSR = 10^{t \cdot \mathrm{sqrt}(2) \cdot SD}$, where SD is the overall standard deviation (intra-run plus the interrun variation) of the titers in common (base 10) log scale, and $t$ is the threshold from Student's $t$-distribution with $n - 1$ degrees of freedom ($n$ = number of runs). The calculated MSR reflects the smallest fold-change in the titer values that can be considered as statistically significant ($P < 0.05$)—i.e., if MSR = 5, then titers that are different by more than five-fold can be considered significant. In addition to serving as an indicator of the level of variability in the titers of the positive control, this MSR evaluation also can be an approximate criterion for comparing samples with confirmed pre-existing antibodies in baseline versus posttreatment samples in order to assess treatment-induced immunogenicity. The MSR applies only if the titer is interpolated and does not apply to endpoint titers.

## Robustness

Robustness is an indication of the reliability of an assay. It is assessed by the capacity of the assay to remain unaffected by small but deliberate variations in method performance that would be expected under relevant, real-life changes in standard laboratory situations. The choice of robustness variables to test during validation should be based on the knowledge of the assay and its associated risks. Some common variables are microtiter plate lots, incubation times, temperature, and reagent lot and concentrations. Study samples or positive control samples can be used to test assay robustness. The use of acceptance criteria for system suitability controls during robustness validation (computed from the assay development and optimization data or validated system suitability control acceptance criteria) is recommended. Continuous monitoring of an assay during validation and beyond with strict records of key assay parameters (e.g., incubation times, pipetted volumes of critical reagents, operators, etc.) may help identify some of the robustness factor interactions if sufficient data are accumulated.

## Reproducibility

Assay reproducibility according to USP general chapter *Validation of Compendial Procedures* ⟨1225⟩ and ICH Q2(R1) *Validation of Analytical Procedures: Text and Methodology* is the reliability of a method when performed in two or more laboratories. In the context of method transfers and interlaboratory method validity demonstrations, assay reproducibility is the same as a cross-validation.

Reproducibility is applicable only if an assay will be run by two or more independent laboratories during in-study phase bioanalysis. Reproducibility is an assessment of the transferability of an assay, i.e., the validity of testing samples in two or more laboratories and the comparability of data produced by them. Reproducibility assessments do not consider routine changes in an assay such as interequipment or interanalyst imprecision. Such contributors to variability (often referred to as intermediate precision factors) are part of the reproducibility variability.

Study phase acceptance criteria for system suitability controls are established in the originator laboratory (see below) during the original assay validation process. The performance of these controls can be compared across multiple laboratories. When only a single laboratory performs the ADA assay, however, reproducibility need not be validated until the method will be transferred to another laboratory.

## Stability

It is useful to understand the optimal storage conditions for assay samples, controls, materials, and reagents, and they should be investigated as part of assay optimization before validation. Later, during assay validation, stability studies should evaluate assay performance following intended storage conditions. Ideally, stability testing conditions should mimic the expected sample, material, and reagent handling conditions, storage temperature(s), and varying lengths of storage time.

### MATERIAL AND REAGENT STABILITY

ADA assays are stability indicating with respect to the applicable materials and reagents, and thus separate tests for reagent stability usually are not required for assay validation. During study phase bioanalysis, assay materials and reagents are presumed to be stable if the system suitability controls meet validated acceptance criteria. However, analysts should validate the stability of plates that have been prepared in advance (e.g., coated with capture antibody and blocked) and stored.

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SAMPLE HANDLING AND STABILITY

ADA samples typically are collected in a serum or plasma matrix. For samples stored at or below –20°C, the stability of ADA are universally accepted, so this sample storage condition may not require validation. It is generally accepted that an ADA sample in serum or plasma will be stable after three freeze–thaw cycles and up to 2 years when stored at –70°C.

## Documentation of Pre-Study Validation

Typically three types of assay-specific documents are created during pre-study validation: an assay validation plan or protocol, an assay method description, and an assay validation report.

An assay validation plan or protocol is recommended before analysts initiate pre-study validation experiments. This document should state the intended purpose of the method, a detailed description of the immunoassay and reagents or materials, a summary of the performance characteristics that will be validated, and a priori acceptance criteria for precision, robustness, stability, and, when appropriate, reproducibility. Some experimental detail and data-handling procedures should be presented in the validation plan because these details provide a clear guidance to the validation analysts and ensure better control over the resulting data.

A method description typically is established after pre-study validation but before the study. This provides a detailed description of the reagents, controls, and equipment needed to run the assay, together with a step-by-step operating procedure and information about processes for data reduction and interpretation. The point at which such a description becomes a Standard Operating Procedure (SOP) is specific to each manufacturer's quality system.

When validation is completed, manufacturers generally conduct a technical peer review of validation data, followed by a validation data audit. An assay validation report is created after the validation work is completed. This documents all of the study validation data, together with information about the methods and batches of reagents that were used. An audited report is approved by management and then is archived.

## In-Study Validation

In-study validation (monitoring the maintenance of system suitability) and revalidation are critical components of any bioanalytical method. Hence, the validation of a method actually does not end until the method is retired from analytical use.

For in-study performance of quantitative bioanalytical methods, acceptance criteria for precision and accuracy generally are required. Because accuracy is *not* applicable for ADA methods, monitoring the performance of quality control samples reassures analysts that the assay system is suitable for its intended use, i.e., that the assay remains valid and is performing as well as it did during pre-study validation.

The use of a low positive control ensures the assay remains sensitive. Generally during study sample analysis the intra-assay (interreplicate) precision of the results of positive controls, as well as test samples (with assay signal at or higher than the screening cut-point), are controlled using system suitability acceptance criteria to ensure that meaningful data are consistently obtained. Results below the cut-point, however, may not be required to meet CV limit criteria.

# LIFE CYCLE MANAGEMENT

Management of the performance of immunogenicity assays from initial clinical development through subsequent product life cycle requires a comprehensive understanding of the strengths, weaknesses, and capabilities of the method format, as well as of the critical assay reagents and assay performance characteristics. In addition, a well-defined plan for critical reagent production, characterization and qualification, qualification of suppliers of critical reagents, and characterization and qualification parameters for reagents produced in-house (aggregate level and labeling efficiency) help manage the risk of maintaining the assay and transferring the method to other laboratories.

When there are changes in critical method components, equipment, or the population that is studied with a particular ADA assay, an assay revalidation may be required. The revalidation may cover some or all validation characteristics (i.e., it may be a partial or whole assay revalidation). Use of lots or batches of assay critical reagents that are different from those used in pre-study validation do not require assay revalidation, but they must be supported by appropriate experimental qualification data for the new reagent to ensure maintenance of system suitability.

Another critical aspect of life cycle management is the development of a strategy to bridge clinical data between an existing and an improved assay format. Such changes typically occur in a product's life cycle because of postmarketing commitments or other needs. To facilitate comparison and cross-validation of the existing method to the revised versions, analysts should retain sufficient aliquots of the original lots of critical assay reagents. In addition, archiving of analyte-spiked samples as well as blinded patient samples is useful to bridge between reagent lots and methods in order to minimize drift in assay performance. Analysts should develop a written plan outlining the sort of changes to the existing assay or critical reagents that will warrant an assay qualification versus a cross-validation or full validation. A quality management document should include details such as the number of assays that must be performed, the number of analysts that will be used, required training for analysts, acceptance criteria to demonstrate equivalence between existing and revised methods, data analysis, and reporting method. This information demonstrates the robustness and consistency of the assay following changes. Quality controls that ensure assay equivalence include %CV, tolerance limits, $EC_{50}$ values of slope, titer level, and signal-to-noise ratio. One approach commonly used to demonstrate equivalence of two immunogenicity methods is the demonstration of ≥90% concordance in archived sample results between the existing and revised methods.

Analysts should use archived samples with a range of positive values as well as an appropriate number of negatives to verify that a new assay segregates samples into positive and negative categories in the same manner as an existing one.

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Another important consideration for life cycle management of critical assay reagents is the monitoring of long-term reagent stability under different storage conditions. A detailed stability testing plan includes storage temperatures (4°C, –20°C, and –70°C), aliquot volume, freeze–thaw cycles, and acceptable performance characteristics for assay qualification, and results should be documented. In this context, it may be prudent to archive patient samples to demonstrate the long-term stability of the polyclonal ADA response in actual patient samples.

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***Add the following:***

# ▲⟨1108⟩ ASSAYS TO EVALUATE FRAGMENT CRYSTALLIZABLE (FC)-MEDIATED EFFECTOR FUNCTION

INTRODUCTION
BACKGROUND
CELL-BASED FUNCTIONAL ASSAYS
- Antibody-Dependent Cell-Mediated Cytotoxicity
- Complement-Dependent Cytotoxicity
- Antibody-Dependent Cellular Phagocytosis
- Fc-Dependent Apoptosis

BINDING ASSAY FORMATS
- Non-Cell-Based Binding Assays
- Cell-Based Binding Assays

CRITICAL REAGENTS
- Complement
- Recombinant Fc Receptors
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- Qualification of Reagents

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## INTRODUCTION

Therapeutic modalities such as antibodies and fragment crystallizable (Fc)-fusion molecules are often engineered with a specific immunoglobulin G (IgG) isotype and subtype in order to impart particular functionalities, such as half-life extension or induction of cytotoxic activity. Alternatively, a specific isotype and subtype may be selected to mitigate, or lessen, potential induction of unwanted biological activities. These modalities have defined structure/function domains that impart the therapeutic benefit.

In vitro assays that assess complex Fc domain interactions are critical to the biological characterization of Fc-containing therapeutics. This chapter provides information and points to consider about the more commonly used techniques available in both cell-based and non-cell-based formats for measuring these interactions. Finally, the chapter also discusses critical reagents, the foundation for these various assays, as well as assay validation, data analysis, and reporting strategies, but it is not intended to cover all potentially applicable techniques.

## BACKGROUND

An example of an IgG1 monoclonal antibody (mAb) is shown in *Figure 1*. The interactions mediated by each structural domain, including antigen binding via the fragment antigen binding (Fab) domain, Fc gamma receptor (FcγR) binding, complement component 1q (C1q) binding, and neonatal Fc receptor (FcRn) binding, are highlighted. The different glycans that may be present in the Fc domain are also noted.

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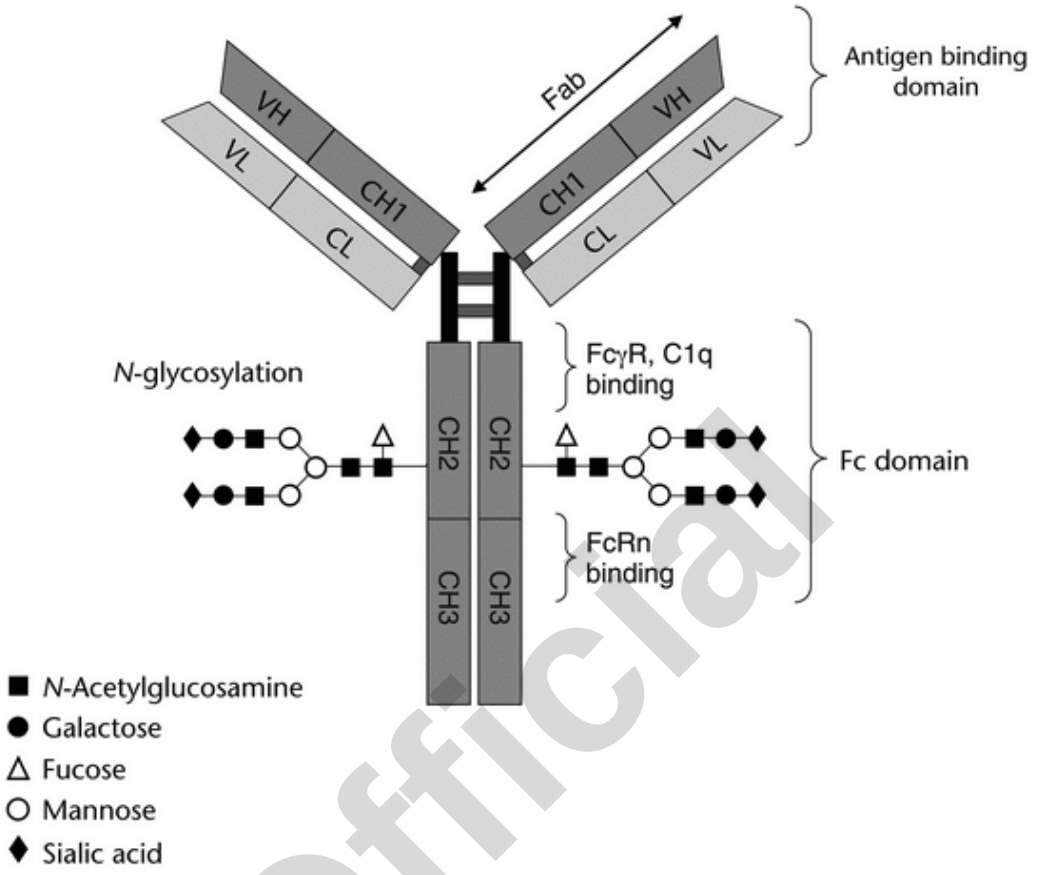


Figure 1. Basic structure of an IgG1 mAb.

The different Fc-mediated effector function activities may include one or more of the following:

- Antibody-dependent cell-mediated cytotoxicity (ADCC)
- Complement-dependent cytotoxicity (CDC)
- Antibody-dependent cellular phagocytosis (ADCP)
- Fc-dependent apoptosis

Fc effector function activities are mediated via interaction of the Fab domain with the target antigen, and the interaction of the Fc domain with complement or various FcγR expressed on cells of hematopoietic origin (e.g., natural killer, macrophage, dendritic, monocyte, and neutrophil). In some cases, the targeted killing or destruction of cells is part of the mechanism of action (MOA) of the therapeutic and has been linked to its clinical efficacy. In other cases, the unintentional killing of normal cells via these mechanisms can be an undesirable consequence of the therapeutic with potential impact on safety. In addition, the interactions of the Fc-domain with FcRn have implications for the circulating half-life.

Fc receptors (FcRs) represent a large class of glycoproteins in the immunoglobulin superfamily. The FcγR subclass is most involved in interactions with Fc-containing therapeutics. The Fc–FcγR interaction is complex due to the fact that there are multiple subtypes of FcγR with different binding affinities toward the Fc of the various human IgG subtypes: IgG1, IgG2, IgG3, and IgG4. *Table 1* shows FcγRs and their relative affinity to each IgG subtype.

**Table 1. FcR Class and Affinity[a, b]**

| Receptor Type | IgG1 | IgG2 | IgG3 | IgG4 |
|---|---|---|---|---|
| FcRn | +++ | +++ | ++ | +++ |
| FcγRI | +++ | – | +++ | ++ |
| FcγRIIa 131H | ++ | + | ++ | + |
| FcγRIIa 131R | ++ | + | ++ | + |
| FcγRIIb | + | +/– | + | + |
| FcγRIIc | + | +/– | + | + |

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**Table 1. FcR Class and Affinity[a, b]** *(continued)*

| Receptor Type | IgG1 | IgG2 | IgG3 | IgG4 |
|---|---|---|---|---|
| FcγRIIIa 158V | ++ | +/– | ++ | + |
| FcγRIIIa 158F | + | +/– | ++ | + |
| FcγRIIIb | + | – | ++ | – |

[a] Key: "–" is no measurable affinity; "+/–" is low affinity, barely detectable; "+" to "+++" is increasing affinity.
[b] Stewart R, Hammond SA, Oberst M, Wilkinson, RW. The role of Fc gamma receptors in the activity of immunomodulatory antibodies for cancer. *J ImmunoTherapy of Cancer*, 2014;2(1):1.

It should be noted that affinity measurements are highly dependent on assay format and assay parameters. Consequently, the relative affinities in *Table 1* are a compilation of general trends reported from numerous studies and are presented as such for the purpose of making comparisons. Although for most IgG subtypes monomeric IgG interaction with FcγRI is of relatively high affinity while that for other FcγRs is of relatively low affinity, higher-avidity interactions resulting from cross-linking-induced interactions of FcγRs with immune complexes may occur. This is important for the biological function of FcγRs and for the Fc-mediated effector function of IgGs co-bound to target antigen as part of immune complexes, underscoring the importance of cell-based Fc effector function assays in addition to binding assays when potential for known Fc effector function exists. One or more FcγRs on FcγR-expressing hematopoietic cells may be involved in mediating ADCC, ADCP, or Fc-dependent apoptosis. Note also that antigen density and Fc *N*-glycan composition can impact FcγR-binding interactions and associated effector functions.

The classical pathway of the complement system involved in CDC is a cascade of complement protein complex formation comprising several different complement proteins, which ultimately form a membrane attack complex that can kill complement-bound target cells. The formation of this complement complex is dependent on the first step in which C1q, a component of the complement, binds to the Fc region of the Fc-containing therapeutic, which in turn is bound to the antigen or other binding partner through the Fab or the binding domain of an alternative mAb. IgG1 and IgG3 subtypes have higher CDC activity than IgG2 and IgG4. A comparison of all four together, in general, reveals that the relative C1q binding activity is determined to be IgG1≈IgG3 > IgG2≈IgG4. (*1*) It is also important to take into consideration the fact that epitope presentation, antigen density, and Fc *N*-glycan composition can impact C1q binding and CDC activity.

Recycling of antibodies after binding through the Fc region to FcRn is known to prolong their persistence in the bloodstream. Fc fusion proteins are engineered with the Fc for this purpose. The interaction of the Fc domain with FcRn occurs in a strict pH-dependent manner in which maximum binding is observed at a slightly acidic pH (6.0) and dissociation at neutral pH. Although it is always best practice to maintain assay temperature and pH control in any protein–protein interaction measurement, particular attention should be paid to pH level when assessing FcRn binding to Fc.

Specifically, the Fc-containing protein is pinocytosed at the cell surface and associates with FcRn in the acidic environment of the endosome. It is then recycled back to the cell surface where it is released from the FcRn at the more neutral pH of the blood. Human IgG1, IgG2, and IgG4 bind well to FcRn, whereas most human IgG3 binds poorly. Serum half-life is considerably shortened with lower-affinity binding, and this is one reason why IgG3s and the IgG3 Fc domain are generally not used as protein therapeutics. During studies performed at pH 5.8 and 25° that compared all four IgG subtypes together, the rank order of binding affinity to FcRn was IgG1≈IgG2≈IgG4 > IgG3. In contrast to FcγRs, differential glycosylation patterns in the Fc do not appear to alter FcRn binding based on affinity measurements from assays performed at pH 5.8 and 25°.

## CELL-BASED FUNCTIONAL ASSAYS

This section describes the most commonly used cell-based assays. It is important to note that the intent is to describe these assays from a chemistry, manufacturing, and control perspective, as opposed to a discovery research perspective. As such, the differentiation between the use of relevant reagents and materials, such as primary cells, versus engineered cell lines that overexpress certain proteins, will be highlighted, where appropriate.

For the purpose of drug release, stability, and comparability studies, it is important to have robust, accurate, precise, and stability-indicating analytical assays that have been appropriately developed and qualified. This process should include an evaluation of the method sensitivity to the post-translational modifications, including glycosylation, which is known to impact Fc function. However, an in-depth discussion of each glycan and its respective impact is complex and beyond the scope of this chapter.

Three common components of cell-based assays where technical considerations are important are: 1) the choice of the target cell, 2) concentrations of the therapeutic antibody, and 3) endpoint detection for cell killing or surrogate marker.

Assessment of lack of effector function for a modality is challenging. Points to consider include:

- IgG1 positive control match for IgG2 and IgG4 modalities
- Physiologically relevant versus engineered cell lines that over-express targets
- Soluble targets bound to cell surface receptors or membrane expressed versions of soluble targets

The most effective option may be to carefully consider assay design to address lack of effector function, potentially including proven assay formats for modalities with demonstrated effector function.

*Table 2* summarizes the commonly used cell-based assay formats for each Fc-mediated effector function activity. Each assay is then described in more detail. Because fundamental assay optimization approaches such as design of experiment (DOE), as well as assay qualification or validation strategies, are generally applicable to most bioassays and are covered in *Design and Development of Biological Assays* ⟨1032⟩ and *Biological Assay Validation* ⟨1033⟩, these topics will not be covered extensively in this chapter.

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**Table 2. Commonly Used Cell-Based Effector Function Assay Formats**

| Activity | Assay Format | Points to Consider |
|---|---|---|
| Antibody-dependent cell-mediated cytotoxicity (ADCC) | Cytotoxicity: Peripheral blood mononuclear cells (PBMCs), NK cell-based assays using chromium-51 ($^{51}Cr$) radioactivity or fluorescence-labeled target cells, or release of intracellular enzymes or adenosine triphosphate (ATP) as readouts | • Primary vs. engineered cell lines<br>• Primary cells are expensive and can be unreliable; donor-to-donor effector cell variability and FcγR polymorphisms exist<br>• Stability of engineered NK or target cells needs to be established<br>• Format of cells (e.g., continuously cultured, thaw-and-use, or prelabeled thaw-and-use target cells) |
| | | • Chromium-51 ($^{51}Cr$) is nontoxic to cells; no background from effector cells, but radioactivity is associated with safety; waste concerns<br>• Fluorescent labels may be toxic to cells; extra time is required for labeling/washing cells; prelabeled thaw-and-use target cell banks could be used |
| | | • For assays using release of intracellular enzymes or ATP, signals from spontaneous death of effector cells or target cells increase the background, and assay controls need to be included to calculate ADCC-specific cell death |
| | Reporter gene assays: Readout quantifying ADCC signaling pathway activation in engineered effector cells | • Faster and more consistent, are quality control (QC) friendly, and often give higher signal-to-background ratios, but licensing fees can be expensive<br>• Consider selection of ADCC assay format for best suitability for intended use (e.g., QC friendly vs. demonstration of target cell killing); consider bridging studies |
| Complement-dependent cytotoxicity (CDC) | Cytotoxicity: Single target cell-based assays using viability indicators, fluorescence-labeled target cells, or release of intracellular enzymes or ATP as readouts | • Single cell involved in assay; easy-to-detect activity if using cell viability reagent because no labeling required<br>• Cell viability readout may take longer than assessment of label released |
| | | • Fluorescent labels may be toxic to cells, extra time required for labeling/washing cells; prelabeled thaw-and-use target cell banks could be used |
| | | • Source of complement and choice of target cell line are critical; some cells are more susceptible to CDC than others |
| | Reporter gene assays: Using target cells constitutively expressing luciferase | • Faster and more consistent and often give higher signal-to-background ratios, but licensing fees can be expensive; consider bridging studies |
| Antibody-dependent cell-mediated phagocytosis (ADCP) | Phagocytosis: Primary macrophages or monocyte cell lines with flow cytometry assay, live cell digital imaging, use of pH-sensitive dye to confirm actual phagocytosis | • Complex assays using primary macrophages or monocyte cell lines; difficult to transfer; variable; not QC friendly<br>• Primary vs. engineered cell lines<br>• Primary cells are expensive and can be unreliable; donor-to-donor effector cell variability and FcγR polymorphisms exist; need for in vitro differentiation to macrophages can add to interassay variability<br>• Stability of engineered cell lines needs to be established<br>• Format of cells (e.g., continuously cultured, thaw-and-use, prelabeled thaw-and-use target cells)<br>• *Assay* accuracy and precision; ability to validate the assay for QC use<br>• Confirmation of actual phagocytosis: primary macrophages or monocytic cell lines used together with pH-sensitive dyes or live cell imaging |
| | Reporter gene assays: Readout quantifying ADCP signaling pathway activation in engineered effector cells | • Faster, more consistent and precise; QC friendly; addresses the FcγRIIa most often predominant in ADCP<br>• Consider selection of ADCP assay format for best suitability for intended use (e.g., QC friendly vs. demonstration of actual phagocytosis); consider bridging studies |

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**Table 2. Commonly Used Cell-Based Effector Function Assay Formats** *(continued)*

| Activity | Assay Format | Points to Consider |
|---|---|---|
| Fc-dependent apoptosis | Apoptosis: Cell-based assay with cross-linking reagents; detection modes include caspase activators, DNA strand breaks via nucleic acid stains, and mitochondrial membrane potential changes by fluorescent probes; fluorescent detection of membrane phospholipid phosphatidylserine | • Primary vs. engineered cell lines<br>• Stability of engineered cell lines needs to be established<br>• Homogeneous formats available<br>• Antigen density on target cell may be difficult to achieve<br>• Optimization and quality of cross-linking reagents important |

## Antibody-Dependent Cell-Mediated Cytotoxicity

ADCC is a cell-mediated innate immune mechanism whereby an effector cell of the immune system (e.g., NK cell, monocyte, macrophage, or eosinophil) actively lyses a target cell that has been recognized by target-specific antibodies. ADCC can be important for the efficacy and MOA of some mAb therapeutics. However, for other molecules, ADCC activity may be a potential liability that needs to be assessed; for example, if the target is located on normal cells. As *Figure 2* shows, to induce ADCC, the Fab domain of the antibody has to bind antigen on the surface of the target cell while its Fc domain binds to the FcγR on the surface of the effector cell.

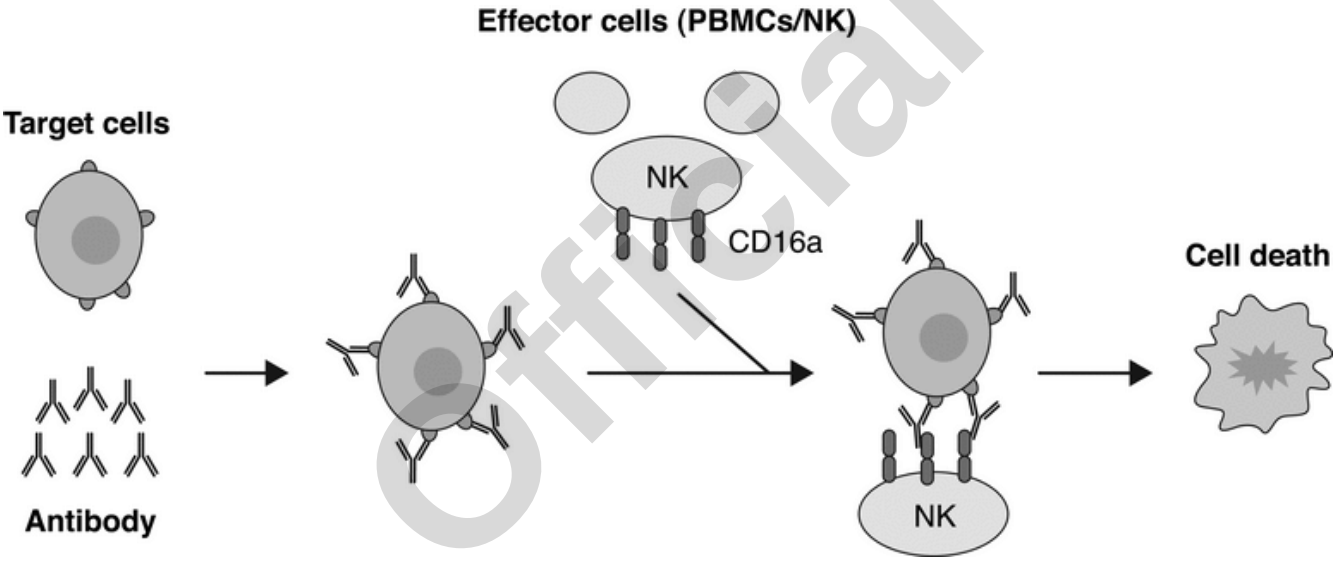


Figure 2. ADCC assay.

The development of ADCC assays is complicated because two different cell types are involved and many parameters need to be optimized, such as target and effector cell numbers; target-to-effector cell ratio; plating procedure, media, and extracellular matrix; drug concentrations and dilutions; volumes; incubation times; signal reading; and data processing and analysis. In addition, the type of FcγR and genetic polymorphism should be considered as these components can affect the affinity of Fc binding.

Traditional ADCC assays use PBMCs or isolated NK cells from human donors to induce chromium-51 ($^{51}Cr$) release from preloaded target cells after lysis. Although such effector cells are the most physiologically relevant, they also represent an expensive, variable, and unreliable material source. Importantly, donor-to-donor differences due primarily to FcγR polymorphism can significantly affect the precision and accuracy of the results. Consequently, cells are generally pooled from multiple donors.

The use of a radioactive readout in traditional ADCC methods also has been shown to have both advantages and disadvantages. Chromium is non-toxic to cells, and because only target cells are loaded with it, there is no background from effector cells. However, the use of radioactivity is associated with safety and waste concerns. In general, the traditional ADCC assays are challenging to perform and transfer.

To avoid radioactivity, target cells can be preloaded with fluorescent dyes such as calcein *O,O′*-diacetate tetrakis(acetoxymethyl) ester (calcein AM), carboxyfluorescein succinimidyl ester (CFSE), 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF), or europium. Note that these reagents may be toxic to cells and the sensitivity to these dyes may vary significantly between different cell types. The cell penetration of dyes and the ability to retain the dyes intracellularly also may be cell type-dependent.

The advantage of prelabeling cells is that only target cell death is measured with no background from effector cells, which usually provides higher signal-to-background ratios, especially for fluorescent dyes with amplification. The general drawback is the extra time and manipulation required for the loading and washing steps. However, it can be mitigated by implementing thaw-and-use cell banks prelabeled with the fluorescent dye.

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Alternatively, the release of intracellular enzymes such as lactate dehydrogenase (LDH) or other markers such as ATP with luminescent, fluorescent, or colorimetric readouts can be used to assess ADCC. However, signals from effector cells or spontaneous death of target cells increases the assay background. Use of a target cell line constitutively expressing a marker, such as luciferase, is an attractive option as well, because it permits a homogeneous assay format.

Engineered effector cell lines can be employed instead of primary cells collected from human donors. The use of engineered cells significantly decreases assay variability and provides a reliable and unlimited source of a critical reagent, which enables high throughput; this makes these assays more amenable to execution for product release and stability testing. Although these types of cells have been accepted for release and stability purposes, it is important to understand how the data generated using engineered cell lines compare to data from the more biologically relevant primary cells.

The final results from an ADCC assay can be presented in terms of potency relative to a reference standard. However, the calculation of percentage cytotoxicity should also be performed during assay development and qualification in order to understand what percentage of the cell population is actually being killed. The calculation is done according to the formula:

$$\% \text{ Cytotoxicity} = \frac{\text{Experimental lysis} - \text{Spontaneous lysis}}{\text{Maximal lysis} - \text{Spontaneous lysis}} \times 100$$

"Spontaneous lysis" in this case is the lysis that occurs in the absence of antibody but in the presence of effector cells.

The use of reporter gene assays as a format is also an option; for example, those assays that use engineered effector cells expressing FcγRIIIa and a reporter gene with ADCC-specific, MOA-relevant, response elements such as nuclear factor of activated T cells (NFAT). The reporter gene assay reflects the early pathway activation of ADCC as a surrogate for cell death. Pathway activation in these assays can only occur when antibody is bound to both target and effector cells. The assays are usually faster and more precise and have higher signal-to-background ratio, making them very suitable for demonstrating consistency of manufacturing and for batch release of the therapeutic. The assays also allow for an assessment of relative potency, but not cytotoxicity. The reporter gene assays are useful for characterization and comparability needs where ADCC is a presumed MOA, especially because accuracy and precision are better than with ADCC assays using primary effector cells.

## Complement-Dependent Cytotoxicity

CDC represents another mechanism by which a therapeutic monoclonal antibody can destroy target cells. As *Figure 3* illustrates, CDC is initiated by the binding of the Fab domain of the antibody to the cell-associated target, and binding of the Fc portion to a plasma C1q protein, which triggers the complement cascade, leading to the formation of the membrane attack complex (MAC) (C5b to C9) at the surface of the target cell, resulting in the killing of the target cell. Given the importance of this activity, a robust and quantitative CDC assay can be an important analytical tool to guide drug process and product development as part of a complete analytical control strategy. Different IgG isotypes have different potential to elicit CDC, based on a number of factors including the inherent affinity to C1q protein. In general, the level of CDC is high for human IgG1 and IgG3 and low for IgG2 and IgG4. The ability of an antibody to mediate CDC is also dependent on the location of the epitope on the target receptor. Binding to a specific epitope can impact the conformational display of the antibody, thereby impacting its interaction with C1q protein. In addition to specific epitopes, the density of the target antigen can influence the level and extent of the CDC activity. In general, cell lines with higher target antigen densities tend to be more sensitive to CDC activity than cell lines with lower target antigen densities.

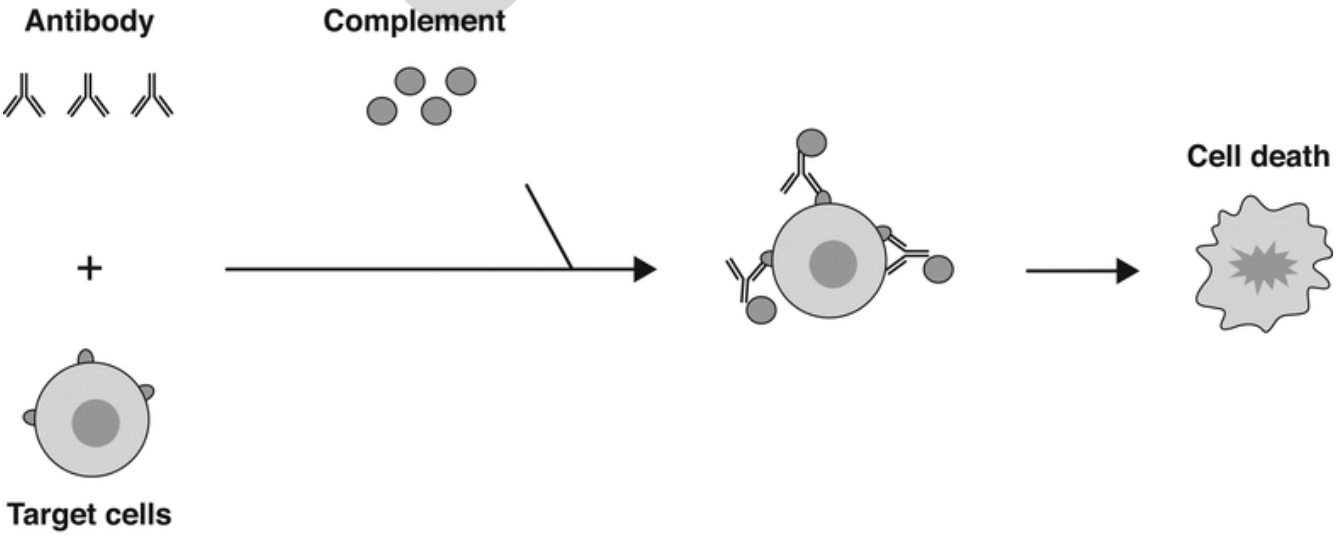


Figure 3. CDC assay.

The selected target cell may be a cell that is physiologically representative for the disease indication, or alternatively, a cell line that recombinantly expresses target antigen. However, it might be beneficial to ascertain that target levels in an engineered cell line are representative of target levels in physiologically relevant cells, which can be achieved with techniques such as flow cytometry.

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The choice of complement protein is also an important consideration for the development of the CDC assay. Different sources of complement such as rabbit, primate, or human have been used. It is important to note that some cancer cell lines express receptors that inhibit complement activation, thereby masking the potential activity of the therapeutic. This again underscores the importance of the choice of the target cell line as well as the choice of the complement.

The choices of cell killing readout for the development of the CDC assay are numerous and can affect the sensitivity, precision, and robustness of the assay. The choices are similar to those mentioned in the ADCC assay in *Antibody-Dependent Cell-Mediated Cytotoxicity*.

Cell killing also can be assessed by measuring the activity of released enzymes upon cell death, such as LDH or adenylate kinase. This method has the advantage of not preloading the cells and is based on loss of membrane integrity. However, the assay also can be variable because of added steps and manipulations of performing the assay. An alternative assay format is to measure ATP in an assay with luminescent, fluorescent, or colorimetric readouts. Luminescence-based assays measuring ATP levels in particular have become more popular because cell killing can be measured by adding the detection reagent without the need for further manipulation, and the assays are sensitive. In addition, like the ADCC assay in *Antibody-Dependent Cell-Mediated Cytotoxicity*, a cell line constitutively expressing a marker, such as luciferase, as a surrogate measure of viable cell number also may be employed.

## Antibody-Dependent Cellular Phagocytosis

ADCP is an Fc-dependent, cell-mediated, and innate immunity mechanism in which antibody-opsonized target cells, or in some cases, specific fibrillar proteins, engage the FcγR on the surface of immune effector cells to induce phagocytosis. This causes the internalization and degradation of the target (see *Figure 4*).

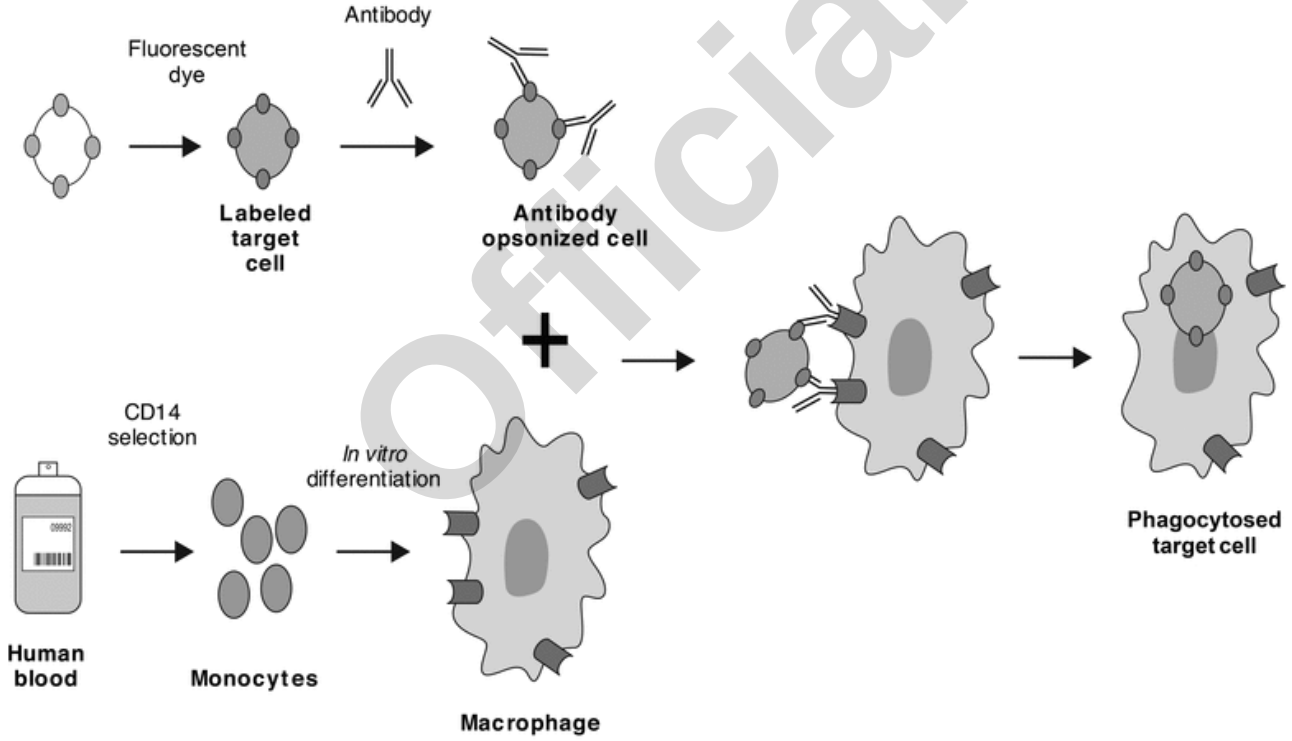


Figure 4. ADCP assay.

Effector cells capable of mediating ADCP include macrophages, monocytes, neutrophils, and microglia. ADCP assays are generally more complex than ADCC or CDC assays. Part of this complexity is driven by the immune cell types capable of eliciting ADCP. In contrast to ADCC mediated by NK cells expressing FcγRIIIa, the macrophage, monocyte, or neutrophil effector cells involved in ADCP express more than one type of FcγR. The FcγRs that are present and the ratio of their cell surface expression can vary with both cell type and cytokine induction, although FcγRIIa often appears to be the predominant FcγR expressed. In addition, distinct IgG subtypes have different potential to elicit ADCP through the various FcγRs. Fcγ receptor polymorphisms can also affect ADCP activity, notably for IgG2 interactions with FcγRIIa, although also for IgG1 interactions with FcγRIIIa on immune cells capable of ADCP. Furthermore, Fc engineering that enhances effector function may alter the potential contribution in vivo of particular effector cells and their FcγRs on ADCP activity (also true for ADCC). Consideration of these complexities should be used to drive decisions on in vitro ADCP assays to use during antibody drug development.

Traditionally, ADCP assays use primary macrophages as physiologically relevant effector cells. Because macrophages are not a circulating immune cell type, human primary macrophages for ADCP assays must be prepared from blood donor monocytes and differentiated in culture for a week. It is preferable to use differentiation treatments that lead to macrophages representing the desired subtype (i.e., the subtype that most closely represents the specific in vivo situation).

Monocytes also may be used as effector cells to evaluate ADCP activity, but they are less effective at ADCP.

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The complexity of primary effector cell preparation, particularly for macrophages, has potential for introducing assay variability. Rigorous QC and development of a robust process of cell preparation, including cell differentiation, are essential. Flow cytometry can be used to evaluate specific markers that represent the desired cells so that their representation in the population is understood and can be used for "go/no go" decisions on use. Expression of specific FcγRs can be confirmed using flow cytometry and quantified using commercially available kits. *Flow Cytometry* ⟨1027⟩ provides a thorough review of best practices for flow cytometry and includes references to functional methods for measuring apoptosis, cell viability, and intracellular cytokine expression. Pooling of ADCP effector cells from different donors is not possible because of major histocompatibility (MHC) restrictions, resulting in greater overall potential for assay run-to-run variability.

Several established cell lines, such as monocytic THP-1, pro-monocytic U937, and BV-2 microglia, are used as effector cells for ADCP assay development. Additionally, cell lines such as Chinese hamster ovary (CHO) and Jurkat cells expressing FcγRIIa or other FcR have been used successfully as surrogates for traditional ADCP assays.

An appropriate target cell selection is equally important. The stability of antigen expression level across cell passage can be monitored. Target cell size and shape can also influence ADCP activity. Different physiologically relevant target cells should be analyzed for suitability or bridging to data using engineered cells line(s), as part of assay development. When the target is extracellular fibrils, the preparation of fibrils needs thorough development as well.

ADCP assays typically involve the tracking of fluorescently labeled target cells by either flow cytometry or confocal microscopy. As such, ADCP is more complex than the ADCC assay. For flow cytometry, effector and target cells used in the ADCP assay are made distinguishable through differential fluorescent dye labeling of cell markers specific for each cell type. Two or more fluorochromes can be used simultaneously in flow cytometry if their peak emission wavelengths are not close to each other and are thus detected by different detectors. The amount of fluorescent signal is proportional to the number of fluorochrome-containing molecules associated with specific cells. The ADCP assay involves reaction of effector and target cells incubated with a dose range of antibody. Different conditions can be evaluated including effector: target cell ratio, assay duration, and antibody dose range. An antibody isotype control should be included as a negative control.

A subsequent wash step is performed and then flow cytometry is used to evaluate percentage of target cells phagocytosed. A uniform population of effector cells, such as an established cell line, simplifies the assay significantly. For example, percentage of labeled cells that are doubly stained compared with total target cells that are singly stained could represent the percentage of target cells phagocytosed. Analysis involves identification and quantification of the different cells as well as gating, which is setting a numerical or graphical boundary to analyze a subset of data. The subset could be target cells alone or cells positive for specific markers of both effector cells and target cells. Forward and side scatter of cells is often used as an initial gating process to remove debris from the analysis before focusing on gating based on specific, fluorescently labeled markers.

As an alternative to using target cells in ADCP assays, some investigators have used fluorescent beads coated with antigen or fluorescently labeled fibrils. These beads or fibrils are incubated with antibodies while the immune complexes are incubated with effector cells. Effector cells then can be analyzed for fluorescent bead or fibril uptake by using flow cytometry, and ADCP activity also can be evaluated; however, antigen-coated beads may not accurately reflect target cells. It is important to understand how the data generated from this approach compare to data from physiologically relevant target cells.

Because flow cytometry using specific labeling of external markers of target and effector cells actually represents evidence of cell surface association rather than proof of phagocytosis, it is advisable to confirm phagocytosis. This can be done by using a pH-sensitive fluorescent dye, available commercially, to label target cells and thereby detect their presence in the acidic environment of the phagocyte lysosomes. Alternatively, target cell engulfment can be confirmed with confocal fluorescence microscopy or microscopy-based live-cell imaging, including time lapse, to evaluate ADCP by live cells. Effector cells also can be treated with trypsin for removal of bound but non-internalized fluorescence. Live-cell digital imaging equipment and associated software have advanced to a level capable of quantification of ADCP dose responses; this is in addition to qualitative assessment using fluorescent, pH-sensitive or other dyes, and provides a viable, high-quality alternative to flow cytometry analysis. Some additional options of these systems include capability for environmental control for cells in assay plates in the chambers where the cells are digitally imaged. This process also allows cell responses to be evaluated at different times during the assay reaction. For this type of analysis, appropriate balancing of imaging and cell needs is important.

Reporter gene assay formats are also an option (e.g., assays that use engineered effector cells expressing a relevant FcγR and a reporter gene with ADCP-specific, MOA-relevant, response elements such as NFAT). The reporter gene assay reflects the early pathway activation of ADCP as a surrogate for cellular phagocytosis. Pathway activation in these assays can only occur when antibodies are bound to both target and effector cells. Different engineered effector cells expressing FcγRIIa, FcγRIIIa, or FcγRI allow evaluation of the potential impact of factors such as manufacturing changes on different FcγRs involved in ADCP. The assays are usually faster, more precise and accurate, and have higher signal-to-background ratio, making them very suitable for demonstrating consistency of manufacturing and for characterization, for example. They allow for an assessment of relative potency, but not phagocytosis. Comparison to data generated from assays measuring actual cellular phagocytosis is recommended, and because the correlation may differ between particular therapeutics, the correlation should be investigated for each one.

## Fc-Dependent Apoptosis

Apoptosis, or programmed cell death, is the genetically controlled destruction of cells that occurs as a normal part of an organism's growth or development. Apoptosis is characterized by nuclear condensation, cell shrinkage, membrane blebbing, and chromosomal DNA fragmentation. Fc-dependent apoptosis is one of the Fc effector functions in which ligation of certain cell surface molecules by antibodies in the presence of cross-linking antibodies or FcγR-expressing cells initiates signal transduction events that lead to apoptosis of the cells.

Fc-dependent apoptosis has been implicated as a potential MOA for several therapeutic antibodies. As *Figure 5* illustrates, the induction of Fc-dependent apoptosis in vivo depends on specific target molecules capable of activating apoptosis pathways and requires cross-linking of the Fc regions by FcγRs on NK cells and other effector cells such as neutrophils and monocytes. Depending on the effector cells involved, Fc-dependent apoptosis may be induced via cross-linking by any of the subclasses of FcγRs (i.e., I, IIa, IIb, IIIa, IIIb). Factors affecting FcγR binding activity, such as antibody isotypes and glycoforms, would also

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impact Fc-dependent apoptosis. Additionally, the degree of Fc-dependent apoptosis is positively influenced by the expression level of the antigen.

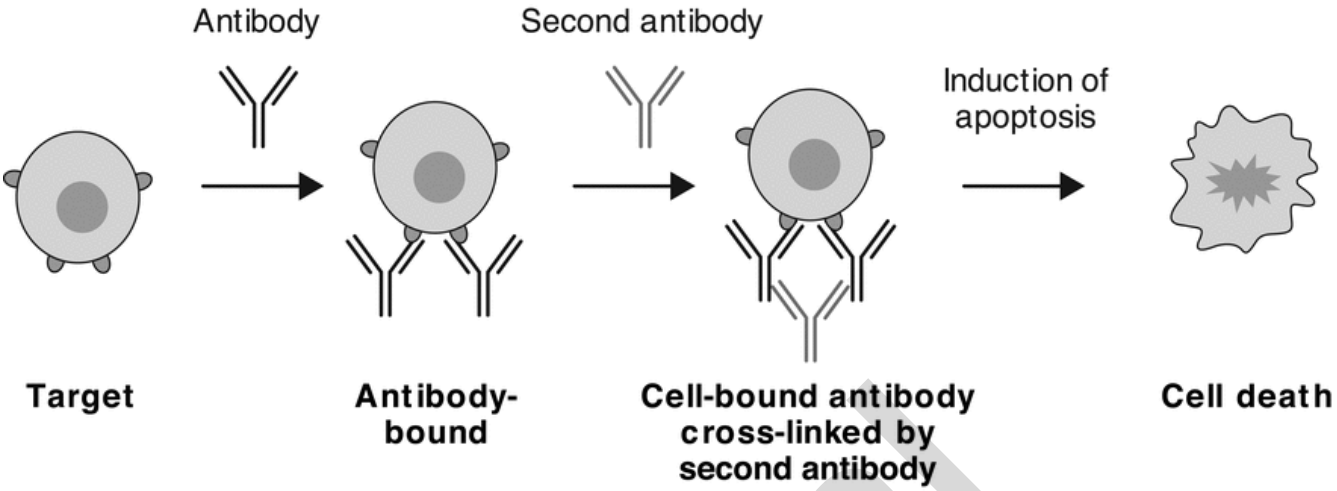


Figure 5. Fc-dependent apoptosis.

Design of in vitro Fc-dependent apoptosis assays involves selection of relevant target cells and cross-linking agents. Considerations for target cells are similar to those for ADCC and ADCP. Common cross-linking agents include anti-Fc antibodies and protein A. Note that the optimum composition of antibody and cross-linking agents in the assay should be determined empirically. Apoptosis of target cells can be assessed by detection of chromatin condensation, DNA strand breaks or fragmentation with various nucleic acid stains, changes in the permeability of cell membranes or mitochondrial membrane potential by fluorescent probes, translocation of anionic phospholipid phosphatidylserine with fluorescent Annexin V conjugates, and/or activation of caspase enzymes by caspase activity assays. As some of these tools measure events that may not always lead to apoptosis, their selection should be justified based on knowledge of the apoptosis pathways in the specific target cell.

## BINDING ASSAY FORMATS

*Cell-Based Functional Assays* provided information on the biological pathways and associated proteins that are critical to measure these mechanisms of action. In contrast, this section is structured to describe the most commonly used binding assay formats and link the various tools used to the biology described in *Cell-Based Functional Assays*. Cell-based binding assay formats are not used as commonly as non-cell-based assays. A synopsis of the more commonly used formats for cell-based binding assays follows the more detailed descriptions of the non-cell-based binding assays.

*Table 3* summarizes the commonly used non-cell-based binding assay formats and cell-based binding formats. Each of these assays is then described in more detail.

**Table 3. Commonly Used Binding Assay Formats**

| Assay Format | Merits/Limitations | Points to Consider |
|---|---|---|
| Enzyme-linked immunosorbent assay (ELISA) | • High throughput, automatable, standard equipment, economical, easily transferred<br>• Multiple wash steps, lengthy, requires plate precoating | • Presence of aggregates may complicate the interpretation of results, particularly for IgG2, IgG4, and FcRn binding<br>• Consider cross-linking to improve sensitivity, particularly when the binding interactions are weak (e.g., with FcγRII and FcγRIII)<br>• Critical reagent quality and concentration impact the affinity of the interaction being measured |

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**Table 3. Commonly Used Binding Assay Formats** *(continued)*

| Assay Format | Merits/Limitations | Points to Consider |
|---|---|---|
| Surface plasmon resonance (SPR), biolayer interferometry (BLI) | • Label-free formats; more precise kinetic data; can measure a relatively wide range of binding affinities; sample preparation and data analysis can be automated; can incorporate the use of a reference material to express relative equilibrium dissociation constant ($K_D$)<br>• Medium throughput; requires specialized equipment and technical expertise; harder to transfer; requires more technical skills than other non-cell-based methods | • Generation of valid and useful binding data is generally dependent on the thorough characterization and consistently high quality of critical reagents such as FcγR, FcRn, and Fc-containing reagents, and the experimental design format (e.g., FcRn binding pH, data analysis)<br>• For experimental setup, consider the type of biosensor chip and alternate approaches to immobilization of ligand, including ligand density, on the sensor chip surface; minimize mass transport, steric hindrance, and non-specific interactions; avoid avidity artifacts due to overloading ligand on the chip; direct or indirect ligand immobilization is possible, although indirect immobilization is preferred; consider flowing a higher concentration of analyte over the chip surface to mitigate low-affinity interactions (e.g., FcγRII, FcγRIII)<br>• Preferably, FcRn protein should comprise the extracellular domain of the α-chain of FcRn and beta-2 microglobulin (β2M) for efficient, pH-dependent binding to IgG. The α-chain of FcRn also has been used alone in comparative SPR binding studies of Fc-containing molecules; however, IgG binding is decreased relative to that observed using the native FcRn heterodimer. Whether produced commercially or in-house, mammalian cell expression is recommended to maximize proper protein folding and post-translational modification<br>• BLI is less sensitive than SPR but generally has higher throughput |
| Kinetic exclusion | • Label-free format measures high-affinity interactions; can measure binding beyond limits of popular surface-based methods (e.g., very slow dissociation); useful across a broad range of affinities; measurements can be made using unpurified mixtures; can measure concentrations<br>• Low to medium throughput; possible non-specific binding to beads; less useful for weaker binding interactions such as some FcγR interactions with antibodies; may not reflect physiological binding if physiological binding is not in solution in vivo; specialized equipment needed | • Confirm that binding partners normally bind in solution; consider which binding partner is captured in the flow cell; determine availability of purified binding partners |
| Bead-based proximity assay format | • High throughput, homogeneous, suitable for lower-affinity interaction, high signal-to-noise ratio, easily automatable, transferable<br>• Expensive reagents require labeling and relatively expensive plate reader; sensitive to the presence of aggregates in the samples due to avidity effects | • Concentrations of the FcR and Fc-containing molecules should be varied to produce the most desirable dose-response curve<br>• Bead-coupling ratios should be optimized based on the amount of each binding partner used in the assay |
| Fluorescence resonance energy transfer | • High throughput, homogeneous, fast, wide assay response window, easily automatable, transferable<br>• Does not work well for lower-affinity interactions; requires labeling and specialized plate reader | • Distance between binding partners should be smaller than that for bead-based proximity assays |
| Cell-based binding assays | • More physiologically relevant as receptors are presented in cell membrane environment; uses standard equipment; medium to high throughput<br>• Need to maintain cell lines in culture; consider stability of expression for relevant receptors over time in culture; more challenging to automate and transfer than non-cell-based assays; levels of receptors may lead to avidity artifacts for high-molecular-weight aggregates | • Cell licensing fees<br>• Cell detachment can be a challenge; potentially stronger plate effects; multiple wash steps; lengthy; requires precoating of plates with cells<br>• In FcRn cell-based binding assays, FcRn expressing human embryonic kidney (HEK) and CHO cells have been used to study Fc-FcRn. To achieve full functionality of the expressed FcRn, the entire complex should be transfected and co-expressed, including the large subunit p51 (FCGRT) and p14 (β2M) |

For all of these assay formats, the reagents used in the assay are critical to the assay performance. In some cases, specific recombinant proteins will need to be obtained, whereas in other instances, vendor-supplied secondary antibodies, detection reagents, and specialized plates or binding surfaces are required.

Because of the large number of assay formats discussed in the following sections, each one is structured in a manner that introduces the technique and then discusses the advantages, disadvantages, and points to consider.

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# Non-Cell-Based Binding Assays

## ELISA

The enzyme-linked immunosorbent assay (ELISA) is the most common type of binding assay. This non-cell-based assay format is flexible, and many different formats exist. *Immunological Test Methods—Enzyme-Linked Immunosorbent Assay (ELISA)* ⟨1103⟩ provides information on the various formats. Different ELISA assays have been developed and used to evaluate Fc-containing protein binding to C1q and to various FcγRs, and to evaluate pH-dependent binding to FcRn.

**Advantages:** High throughput, automatable, standard equipment, economical, easily transferred.

**Disadvantages:** Multiple wash steps, lengthy, requires plate precoating, may be sensitive to the presence of aggregates in the samples depending on the assay format. The presence of aggregates may complicate the interpretation of results, especially for IgG2, IgG4, and FcRn binding. In addition, the ELISA format may have binding limitations, particularly when the binding interactions are weak (e.g., FcγRII and FcγRIII). In these cases, consider cross-linking to improve sensitivity (refer to ⟨1103⟩).

**Points to consider:** Critical reagent quality, inventory and concentration, affinity of interaction being measured.

## SURFACE PLASMON RESONANCE

Surface plasmon resonance (SPR) technology can be used for label-free, real-time detection and characterization of ligand binding and dissociation in FcR and Fc-containing protein interactions. Association rate ($k_a$) and dissociation rate ($k_d$) constants, and the equilibrium dissociation constant or affinity constant ($K_D = k_d/k_a$) can be determined. Typical systems use microfluidics flow cells. One of the interacting proteins is immobilized, either directly or indirectly via capture, as the ligand on a biosensor chip surface within the microfluidics system. Typically, the other protein partner, as an analyte, is then flowed in a solution over the surface of the biosensor chip with immobilized ligand to a desired association level to measure association; then a subsequent dissociation step follows with buffer alone. Association and dissociation of FcR with Fc-containing protein can be measured through change in SPR or SPR response units (RUs), which, in turn, reflect a change in mass at the sensor chip surface reflective of binding.

The technique is sensitive, and different formats and conditions can be used to obtain data under specific conditions. For interactions where a steady state equilibrium between association and dissociation cannot be reached in a reasonable time, but kinetic information exists, a kinetic analysis to obtain $k_a$ and $k_d$ allows the affinity constant $K_D$ to be determined. When the kinetics of association and dissociation are very fast and a steady state can be reached in a reasonable time, a steady state equilibrium analysis can be used to determine $K_D$. Different fit models of the binding data are used to determine further information about the binding interaction (e.g., best fit to a 1:1 binding model), as well as to obtain values for the desired binding parameters. A more extensive discussion of SPR as an immunological test method is provided in *Immunological Test Methods—Surface Plasmon Resonance* ⟨1105⟩, which provides guidance on practical aspects of methods, controls, and analyses, including those pertinent to obtaining accurate information on $k_a$, $k_d$ and $K_D$, as well as how to avoid pitfalls.

**Advantages:** No labeling required; provides more precise kinetic data; can measure a relatively wide range of affinities; can automate sample preparation; data analysis is straightforward; and can use with a reference material to express relative $K_D$.

**Disadvantages:** Lower throughput, specialized equipment needed, harder to transfer and validate, and requires greater technical expertise than some other non-cell-based methods.

**Points to consider:** Technology requires special expertise and careful assessment of the quality of the critical reagent quality.

The generation of a valid and useful SPR binding data set for an FcR and Fc-containing protein interaction can be generally described as being dependent on three major factors: the quality of the FcγR or FcRn and Fc-containing reagents, the experimental design format, and data analysis.

More specifically, for FcRn, binding occurs at somewhat acidic pH (approximately pH 6) in the endosome after the Fc-containing therapeutic, such as a monoclonal antibody, is pinocytosed at the cell surface and associates with FcRn when released into the endosome. Typically, in SPR-based FcRn assays, binding and dissociation at pH 6 are analyzed to obtain the equilibrium dissociation constant or affinity, $K_D$, at this pH. Additionally, binding at pH 6 and dissociation at a neutral pH, such as pH 7.2–7.4, can be performed to obtain an understanding of dissociation at the neutral pH; dissociation at neutral pH is essential for recycling of the antibody in vivo through release again at the cell surface. Antibody engineering to improve binding to FcRn, as a component potentially contributing to improved pharmacokinetics (PK), may alter the ability of FcRn to release antibody at neutral pH, so evaluating dissociation under neutral pH buffer flow can provide important information in such work. For Fc-containing proteins directed at soluble targets, evaluating FcRn binding of the protein in the presence of its soluble target also should be considered.

FcRn protein should preferably comprise the soluble extracellular domain of the heavy chain (α-chain) of FcRn and associated beta-2 microglobulin (β2M) because the β2M is important for efficient pH-dependent IgG binding and reflects physiological binding more closely. Recombinant FcRn heterodimer is available commercially or can be produced to obtain better control over quality, consistency, and supply. Mammalian cell expression is best to maximize proper protein folding and post-translational modifications. The soluble extracellular domain of FcRn with associated, species-specific β2M that is copurified can be used, or a fusion protein of the FcRn extracellular domain with β2M is also possible. Alternatively, the heavy chain (α-chain) of FcRn can be, and often is, used alone in comparative SPR binding studies of Fc-containing molecules, although IgG binding is decreased compared with that of native FcRn heterodimer.

For FcγRs (other than to FcγRI) binding of Fc-containing proteins such as antibodies is a low-affinity interaction. To obtain suitable results, a higher concentration of analyte than usual may need to be flowed over the chip surface to which the binding partner is bound.

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For all FcRs, thorough characterization and high and consistent functional quality of protein is important for an accurate and reproducible assay. For example, it is important in obtaining $k_a$ in kinetic analysis because concentration is a factor of $k_a$ and the concentration should reflect that of functionally active protein.

Consideration should be given to whether to immobilize the FcR or the Fc-containing protein to the surface of the SPR chip since both formats are possible. Different biosensor-prepared surfaces are available commercially. Some chip surfaces can slow association of the specific binding partners so that mass-transport limitations or steric hindrance are avoided in order to obtain accurate results. A low density of ligand on the chip surface is desirable for the same reasons. Direct or indirect ligand immobilization is possible. Overall, surface preparation that avoids avidity artifacts and achieves relatively low-level ligand binding under gentle immobilization to avoid mass-transport-limited conditions and to direct simple analyte binding may best reflect conditions on the cell.

Indirect immobilization of ligand is preferred because it is gentler. Examples include amine-immobilized neutravidin to capture biotinylated IgG ligand or immobilized antigen to capture IgG ligand. Direct immobilization formats, such as amine coupling of the ligand directly to the chip, can be considered, but with caution, because this type of direct immobilization through multiple sites throughout the protein has the potential to alter protein structure and binding characteristics. Outputs of SPR analyses that signify achievement of appropriate binding conditions include good fitting to simple binding models and low and randomly scattered residuals around the fitted data. Prior determination of the concentration of active analyte being tested against a specific immobilized partner allows more accurate input of values toward binding parameter determination.

## BIOLAYER INTERFEROMETRY

Biolayer interferometry (BLI) is another label-free, real-time detection technology used to detect intermolecular interactions. The technology is suitable for measuring FcR and Fc-containing protein interactions. It is an optical analytical method that measures interference pattern changes of light wavelengths (optical interferometry) and is dependent on a fiber-optic biosensor. When target molecules bind to or dissociate from the surface of the biosensor to which the binding partner is bound, a spectral shift occurs to a degree proportional to the number of in-solution binding partner molecules bound to the biosensor surface. The change is monitored in real time and can provide kinetic data or relative $K_D$ data on molecular interactions between the two binding partners. BLI also can be used to obtain concentrations of molecules.

**Advantages:** No labeling; high throughput; can provide kinetic and quantitation data; semi-automatable.

**Disadvantages:** Specialized equipment and biosensors needed; more difficult to transfer.

**Points to consider:** Selection of the appropriate biosensor and assay orientation (i.e., which molecule to immobilize) is important. The affinity of FcγR–IgG interactions is often relatively low, requiring that high concentrations of analyte be used. An excess of ligand molecules on the biosensors can lead to data artifacts due to crowding, steric hindrance, avidity, mass-transport effects, and non-specific binding, while a low loading density could result in poor signal-to-noise ratio. The buffer conditions should account for optimal binding yet reduce as much as possible the non-specific binding. Length of the association step should be considered because shorter times may prevent secondary interactions.

## KINETIC EXCLUSION ASSAY

The kinetic exclusion assay is a solution equilibrium/exclusion-based method. It is particularly sensitive in determining $K_D$ in the pM to low nM range, but low-affinity interactions are more of a challenge because of sample volume and/or concentration needs. The kinetic exclusion assay can also determine concentrations of interacting binding partners. In the assay, one binding partner is held constant in concentration, the other is titrated into the solution, and the binding is allowed to come to equilibrium. For antibody–antigen interactions, the antibody is normally titrated in. The solution is then rapidly passed into a flow cell. The flow cell contains antigen-coated beads that bind any free antibody, but antigen-bound antibodies in the solution do not have time to dissociate; they are kinetically excluded from binding to the bead. The free antibody bound to the beads is subsequently detected with fluorescently labeled secondary antibodies of appropriate specificity; they are then quantified, giving a measure of free antibody remaining at equilibrium. This value then can be used to determine binding parameters.

**Advantages:** Can measure high-affinity interactions and a broad range of affinities; measurements can be made with unpurified mixtures and unmodified proteins; can measure concentrations and binding beyond the limits of popular surface-based methods (e.g., very slow dissociation).

**Disadvantages:** Low to medium throughput; possible non-specific binding to beads; less useful for weaker binding interactions such as some FcγR interactions with antibodies; may not reflect physiological binding if physiological binding is not in solution in vivo; specialized equipment needed.

**Points to consider:** Whether the binding partners normally bind in solution; orientation of the assay (i.e., which binding partner is captured in the flow cell); whether a binding partner is available only in an unpurified state.

## BEAD-BASED PROXIMITY ASSAY

Bead-based assay technologies are now widely used to study biomolecular interactions in a microplate format. These technologies are non-radioactive, homogeneous proximity assays. Binding of molecules captured on the beads leads to an energy transfer from one bead to another, ultimately producing a luminescent/fluorescent signal. The technology allows the detection of molecules in a wide range of sample matrices. Almost any sandwich assay can be developed to detect an analyte of interest. An example FcR assay may have the following competitive assay format. An Fc or Fc-containing therapeutic can be biotinylated and coupled to streptavidin donor beads, while a recombinant histidine-tagged FcR (FcR-His) molecule is conjugated to acceptor beads. In the absence of an unlabeled Fc-containing sample, the beads come into close proximity and a signal (e.g., luminescence) is generated. However, when a different Fc-containing moiety (i.e., test article) is added into the reaction, the Fc region competes with the Fc-biotin reagent in solution at pH 6.0 for binding to FcR-His. The assay would measure the dose-dependent decrease in signal when an Fc-containing test article is added to a reaction containing FcR-His

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and Fc-biotin. Test sample activity would be determined by comparing the test sample response to the response obtained for the reference standard. Results would be reported as percent relative binding values.

**Advantages:** High throughput; homogeneous; suitable for lower-affinity interaction; high signal-to-noise ratio; easily automatable; transferable.

**Disadvantages:** Expensive reagents; requires labeling and relatively expensive plate reader; more sensitive to the presence of aggregates in the samples due to avidity effects.

**Points to consider:** A number of variables should be considered during the development and optimization of the assay. The concentration of the FcR and Fc-containing molecules should be varied to produce the most desirable dose-response curve. Bead-coupling ratios should be optimized based on the amount of each binding partner used in the assay.

### FLUORESCENCE RESONANCE ENERGY TRANSFER

Fluorescence resonance energy transfer (FRET) is based on the transfer of energy between two fluorophores, a donor and an acceptor, when in close proximity. Molecular interactions between biomolecules can be assessed by coupling each partner with a fluorescent label and by detecting the level of energy transfer. A donor–acceptor complex can be detected without the need for physical separation from the unbound partners, allowing for fully homogeneous assays that do not require separation steps such as centrifuging, washing, or filtration.

However, traditional FRET is affected by fluorescence from sample components such as buffers, proteins, chemical compounds, and cell lysate. This type of background fluorescence is extremely transient and therefore can be eliminated using time-resolved methodologies. Homogeneous time-resolved fluorescence (HTRF) combines standard FRET technology with time-resolved measurement of fluorescence, eliminating short-lived background fluorescence. Introducing a time delay of approximately 50–150 µs between the system excitation and fluorescence measurement allows the signal to be cleared of all non-specific, short-lived emissions. In contrast, HTRF acceptors emit long-lived fluorescence when engaged in a FRET process. Therefore, long-lived emissions signify energy transfer due to the proximity of the labeled biomolecules. Also, FRET is governed by the physics of molecular proximity, which only allows this phenomenon to occur when the distance between the donor and the acceptor is short enough. In practice, FRET systems are characterized by the Förster radius ($R_0$) distance at which FRET efficiency is 50%. For HTRF, $R_0$ lies between 50 and 90 Å, depending on the acceptor used.

**Advantages:** High throughput, homogeneous, fast, wide assay response window, easily automatable, transferable

**Disadvantages:** Does not work well for lower-affinity interaction, requires labeling and specialized plate reader

**Points to consider:** Distance between binding partners is smaller than that for bead-based proximity assays

## Cell-Based Binding Assays

This format can be a suitable choice when a recombinant reagent such as a receptor extracellular domain (ECD) is not commercially available or when it is challenging to produce the recombinant reagent in sufficient amounts. Two types of cell-based binding assays can be established, as shown in *Table 4*. A cell-based binding assay is often preferred for measurement of FcRn binding because of the structure of the FcRn. As such, this assay format is discussed in the context of an FcRn binding assay.

**Table 4. Cell-Based Binding Assay Formats**

| Type | | Platform |
|---|---|---|
| Direct assay | Conventional | Plate-based or flow cytometry |
| Competitive assay | Nonhomogeneous | Plate-based |
| | Homogeneous | Flow cytometry, HTRF |

**Advantages:** Arguably more physiologically relevant because receptors are presented in a membrane environment; no need to purify receptors; standard equipment; medium-to-high throughput.

**Disadvantages:** Need to express receptors and maintain cell lines for a longer time. In general more difficult to automate and transfer than non-cell-based methods. Levels of receptors may lead to avidity artifacts for high-molecular-weight aggregates. Cell detachment can be a challenge; wash steps must be gentle to reduce cell detachment from well bottoms, which may be partially mitigated by use of round-bottom well plates and centrifugation. Potentially stronger plate effects; multiple wash steps; lengthy; requires precoating of plates with cells. In addition, cell licensing fees may be a consideration.

**Points to consider:** The following points apply for FcRn cell-based assays. A cell-based assay in which the Fc region of IgG binds to the cell surface expressed FcRn complex may be more indicative of physiology where the FcRn is present as a membrane-bound form and the IgG floats freely as a soluble form. Therefore, this model system may be more appropriate to measure Fc-FcRn interactions. Typically, binding of Fc to FcRn takes place under mildly acidic conditions (pH 6–6.5) and releases the IgG at neutral to basic pH (7–7.4).

# CRITICAL REAGENTS

The validity of testing results for Fc functionality is highly dependent on the quality of the critical reagents used in these assays. Just as the purity and glycan composition can impact the effector function activity of an Fc-containing therapeutic, the composition of the reagent with which they interact is equally important. As highlighted in *Cell-Based Functional Assays*, it is important that purified FcRs utilized in binding based assays represent the appropriate genetic polymorphisms in that receptor. For cell-based assays, the cells themselves are considered critical reagents. Specifically, it is important to characterize receptor

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densities and changes to receptor expression on cells used in ADCC, ADCP, and CDC assays because these changes can affect assay sensitivity. Implementation of critical reagent standards is a compelling idea that would permit standardization of the evaluation of the Fc domain across a multitude of therapeutics.

As with most potency assays, Fc-mediated effector function assays require product reference standards that are well-characterized and controlled. Reference standards are generally derived from a representative product batch. It is important to note that glycosylation and glycan patterns need to be well-characterized in reference standards because these attributes affect Fc functionality. It is not uncommon to observe statistically significant differences in the levels of glycan structures when comparing different batches. As such, the choice of a batch as a reference standard becomes important. One approach that can be considered is to select a batch that represents the midpoint of process variability as the reference standard batch.

### Complement

Serum, complement, and cells are important critical reagents for CDC assays. Assays use commercially available, non-heat-treated serum complement fractions. Assays also incorporate sources of complement that include human, rabbit, guinea pig, and mouse. It is important to note that the lot-to-lot variability of complement batches can complicate results. This can be mitigated by the purchase and qualification of a large lot size that can last a few years based on the testing requirements of the lab.

### Recombinant Fc Receptors

Many FcRs are commercially available or can be manufactured in-house or by an external contract manufacturing organization (CMO). The FcR heterodimerizes with the invariant gamma chain for functional activity, or in the case of FcγRII, the gamma chain is contiguous with the FcR region. However, because the gamma chain is not involved in any direct interactions with the Fc region, the FcR component of the heterodimer is sufficient for direct interaction studies. If FcRs are purchased as a catalog item from an external vendor, it is important to ascertain lot-to-lot variability of the materials purchased. This can be mitigated by the purchase of a large lot size that can last several months to years based on the testing requirements of the lab. The host cell line is an important consideration because glycosylation of some FcRs, such as FcγRIIIa, can affect binding to Fc regions.

### Quality of Reagents

The quality and consistency of FcR critical reagents used in Fc function assays should be ensured by using biochemical analytical techniques to assess purity, homogeneity, stability, lot-to-lot variability, and desired functional properties such as pH specificity of binding for FcRn. These analyses help ensure proper protein folding necessary for FcR binding to Fc-containing protein. If the reagents were purchased from vendors, this information is still important to collect and to supplement what is provided by the vendor. It is important to understand the best design for engineered proteins such as FcRn alpha chain and co-expressed β2M. For the Fc-containing therapeutic protein, aggregation and other modifications (e.g., specific methionine oxidations in the Fc region known to affect FcRn binding or levels of other post-translational modifications such as *N*-glycans known to affect FcγR binding) also should be assessed prior to assay so that this information can be used to help interpret results.

The quality of critical cell reagents should be ensured through assessment of stability, lot-to-lot variability, and consistent, desired functional properties. Stability of the cell line should be established during several passages to ensure a functional assay and the ability to create cell banks. Stability can be evaluated using flow cytometry to evaluate, for example, receptor expression, and also by evaluating consistency of assay response. If cells require a pretreatment before an assay, then the consistency of response to this pretreatment also needs to be established.

### Qualification of Reagents

For the qualification of reagents, appropriate criteria need to be developed that are based not only on passing the method acceptance criteria but also on consistency and linkage to data generated from previous batches. Careful qualification is essential to preventing drift in the assay response when changing batches of critical reagent. The stability of FcR and FcRn proteins needs to be adequately monitored during long-term storage.

## ASSAY VALIDATION

The fundamentals of potency assay validation, including key method parameters to be evaluated, are outlined in ⟨1033⟩. Use of a validated assay is a requirement for assays that support product lot release and stability testing. Characterization assays are not subject to current good manufacturing practices (cGMPs) or good laboratory practices (GLPs). However, potency assay validation principles should be considered to demonstrate the method performance characteristics and the fitness for use of the assay results.

## DATA ANALYSIS AND REPORTING

Data analysis and the reporting of results are important outputs from each assay. Fundamental aspects of these are described in ⟨1032⟩, *Analysis of Biological Assays* ⟨1034⟩, and *Design and Analysis of Biological Assays* ⟨111⟩. For lot release and stability testing, the convention is to use “relative potency” or “percent relative potency” for the reporting of results. With this approach, a test sample response is compared to the response obtained with a reference standard. As described in ⟨1032⟩ and

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⟨1034⟩, the central tenet of this approach is the assumption that the same active ingredient is present in both materials. Only after parallelism, or similarity, has been established for the sample response relative to the reference standard response is it permissible to compute the relative potency result. For non-cell-based binding assay formats such as SPR, BLI, and kinetic exclusion, a number of strategies can be used for determining activity, such as reporting the absolute affinity constants, $K_D$ values, kinetic measurements, or relative binding.

A key consideration for the biological characterization of Fc effector function is post-translational modifications and their impact on activity. Following the modeling of the response, consideration should be given to the comparison of the dose-response curves for the reference standard and the test sample(s) as well as the impact of certain attributes, such as glycans, on those responses. In some cases, the use of relative potency as a result output may not be warranted.

## REFERENCES

1. Noubia Abdiche Y, et al. The neonatal Fc receptor (FcRn) binds independently to both sites of the IgG homodimer with identical affinity. *MAbs* 2015;7(2):331–343.

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# ⟨1111⟩ MICROBIOLOGICAL EXAMINATION OF NONSTERILE PRODUCTS: ACCEPTANCE CRITERIA FOR PHARMACEUTICAL PREPARATIONS AND SUBSTANCES FOR PHARMACEUTICAL USE

The presence of certain microorganisms in nonsterile preparations may have the potential to reduce or even inactivate the therapeutic activity of the product and has a potential to adversely affect the health of the patient. Manufacturers have therefore to ensure a low bioburden of finished dosage forms by implementing current guidelines on Good Manufacturing Practice during the manufacture, storage, and distribution of pharmaceutical preparations.

Microbial examination of nonsterile products is performed according to the methods given in the texts on *Microbial Enumeration Tests* ⟨61⟩ and *Tests for Specified Microorganisms* ⟨62⟩. Acceptance criteria for nonsterile pharmaceutical products based upon the total aerobic microbial count (TAMC) and the total combined yeasts and molds count (TYMC) are given in *Tables 1* and *2*. Acceptance criteria are based on individual results or on the average of replicate counts when replicate counts are performed (e.g., direct plating methods).

When an acceptance criterion for microbiological quality is prescribed, it is interpreted as follows:

- $10^1$ cfu: maximum acceptable count = 20;
- $10^2$ cfu: maximum acceptable count = 200;
- $10^3$ cfu: maximum acceptable count = 2000; and so forth.

**Table 1. Acceptance Criteria for Microbiological Quality of Nonsterile Dosage Forms**

| Route of Administration | Total Aerobic Microbial Count (cfu/g or cfu/mL) | Total Combined Yeasts/Molds Count (cfu/g or cfu/mL) | Specified Microorganism(s) |
|---|---|---|---|
| Nonaqueous preparations for oral use | $10^3$ | $10^2$ | Absence of *Escherichia coli* (1 g or 1 mL) |
| Aqueous preparations for oral use | $10^2$ | $10^1$ | Absence of *Escherichia coli* (1 g or 1 mL) |
| Rectal use | $10^3$ | $10^2$ | — |
| Oromucosal use | $10^2$ | $10^1$ | Absence of *Staphylococcus aureus* (1 g or 1 mL) |
| | | | Absence of *Pseudomonas aeruginosa* (1 g or1 mL) |
| Gingival use | $10^2$ | $10^1$ | Absence of *Staphylococcus aureus* (1 g or 1 mL) |
| | | | Absence of *Pseudomonas aeruginosa* (1 g or 1 mL) |
| Cutaneous use | $10^2$ | $10^1$ | Absence of *Staphylococcus aureus* (1 g or 1 mL) |
| | | | Absence of *Pseudomonas aeruginosa* (1 g or 1 mL) |
| Nasal use | $10^2$ | $10^1$ | Absence of *Staphylococcus aureus* (1 g or 1 mL) |
| | | | Absence of *Pseudomonas aeruginosa* (1 g or 1 mL) |
| Auricular use | $10^2$ | $10^1$ | Absence of *Staphylococcus aureus* (1 g or 1 mL) |
| | | | Absence of *Pseudomonas aeruginosa* (1 g or 1 mL) |
| Vaginal use | $10^2$ | $10^1$ | Absence of *Pseudomonas aeruginosa* (1 g or 1 mL) |
| | | | Absence of *Staphylococcus aureus* (1 g or 1 mL) |
| | | | Absence of *Candida albicans* (1 g or 1 mL) |
| Transdermal patches (limits for one patch including adhesive layer and backing) | $10^2$ | $10^1$ | Absence of *Staphylococcus aureus* (1 patch) |
| | | | Absence of *Pseudomonas aeruginosa* (1 patch) |
| Inhalation use (special requirements apply to liquid preparations for nebulization) | $10^2$ | $10^1$ | Absence of *Staphylococcus aureus* (1 g or 1 mL) |
| | | | Absence of *Pseudomonas aeruginosa* (1 g or 1 mL) |
| | | | Absence of bile-tolerant Gram-negative bacteria (1 g or 1 mL) |

**Table 2. Acceptance Criteria for Microbiological Quality of Nonsterile Substances for Pharmaceutical Use**

| | Total Aerobic Microbial Count (cfu/g or cfu/mL) | Total Combined Yeasts/Molds Count (cfu/g or cfu/mL) |
|---|---|---|
| Substances for pharmaceutical use | $10^3$ | $10^2$ |

*Table 1* includes a list of specified microorganisms for which acceptance criteria are set. The list is not necessarily exhaustive, and for a given preparation it may be necessary to test for other microorganisms depending on the nature of the starting materials and the manufacturing process.

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If it has been shown that none of the prescribed tests will allow valid enumeration of microorganisms at the level prescribed, a validated method with a limit of detection as close as possible to the indicated acceptance criterion is used.

In addition to the microorganisms listed in *Table 1,* the significance of other microorganisms recovered should be evaluated in terms of the following:

- The use of the product: hazard varies according to the route of administration (eye, nose, respiratory tract).
- The nature of the product: does the product support growth? does it have adequate antimicrobial preservation?
- The method of application.
- The intended recipient: risk may differ for neonates, infants, the debilitated.
- Use of immunosuppressive agents, corticosteroids.
- The presence of disease, wounds, organ damage.

Where warranted, a risk-based assessment of the relevant factors is conducted by personnel with specialized training in microbiology and in the interpretation of microbiological data. For raw materials, the assessment takes account of the processing to which the product is subjected, the current technology of testing, and the availability of materials of the desired quality.

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# ⟨1112⟩ APPLICATION OF WATER ACTIVITY DETERMINATION TO NONSTERILE PHARMACEUTICAL PRODUCTS

The determination of the water activity of nonsterile pharmaceutical dosage forms aids in the decisions relating to the following:

1. optimizing product formulations to improve antimicrobial effectiveness of preservative systems,
2. reducing the degradation of active pharmaceutical ingredients within product formulations susceptible to chemical hydrolysis,
3. reducing the susceptibility of formulations (especially liquids, ointments, lotions, and creams) to microbial contamination, and
4. providing a tool for the rationale for reducing the frequency of microbial limit testing and screening for objectionable microorganisms for product release and stability testing using methods contained in the general test chapter *Microbial Enumeration Tests* ⟨61⟩ and *Tests for Specified Microorganisms* ⟨62⟩.

Reduced water activity ($a_W$) will greatly assist in the prevention of microbial proliferation in pharmaceutical products; and the formulation, manufacturing steps, and testing of nonsterile dosage forms should reflect this parameter.

Low water activity has traditionally been used to control microbial deterioration of foodstuffs. Examples where the available moisture is reduced are dried fruit, syrups, and pickled meats and vegetables. Low water activities make these materials self-preserved. Low water activity will also prevent microbial growth within pharmaceutical drug products. Other product attributes, for example, low or high pH, absence of nutrients, presence of surfactants, and addition of antimicrobial agents, as well as low water activity, help to prevent microbial growth. However, it should be noted that more resistant microorganisms, including spore-forming *Clostridium* spp., *Bacillus* spp., *Salmonella* spp. and filamentous fungi, although they may not proliferate in a drug product with a low water activity, may persist within the product.

When formulating an aqueous oral or topical dosage form, candidate formulations should be evaluated for water activity so that the drug product may be self-preserving, if possible. For example, small changes in the concentration of sodium chloride, sucrose, alcohol, propylene glycol, or glycerin in a formulation may result in the creation of a drug product with a lower water activity that can discourage the proliferation of microorganisms in the product. This is particularly valuable with a multiple-use product that may be contaminated by the user. Packaging studies should be conducted to test product stability and to determine that the container–closure system protects the product from moisture gains that would increase the water activity during storage.

Reduced microbial limits testing may be justified through risk assessment. This reduction in testing, when justified, may entail forgoing full microbial limits testing, implementing skip-lot testing, or eliminating routine testing.

Nonaqueous liquids or dry solid dosage forms will not support spore germination or microbial growth due to their low water activity. The frequency of their microbial monitoring can be determined by a review of the historic testing database of the product and the demonstrated effectiveness of microbial contamination control of the raw materials, ingredient water, manufacturing process, formulation, and packaging system. The testing history would include microbial monitoring during product development, scale-up, process validation, and routine testing of sufficient marketed product lots (e.g., up to 20 lots) to ensure that the product has little or no potential for microbial contamination. Because the water activity requirements for different Gram-reactive bacteria, bacterial spores, yeasts, and molds are well described in the literature,[1] the appropriate microbial limit testing program for products of differing water activities can be established. For example, Gram-negative bacteria including the specific objectionable microorganisms, *Pseudomonas aeruginosa, Escherichia coli,* and *Salmonella* species will not proliferate or survive in preserved products with water activities below 0.91, while Gram-positive bacteria such as *Staphylococcus aureus* will not proliferate below 0.86, and *Aspergillus niger* will not proliferate below 0.77. Furthermore, even the most osmophilic yeast and xerophilic fungi will not proliferate below 0.60, and they cannot be isolated on compendial microbiological media.[1] The water activity requirements measured at 25° for the growth of a range of representative microorganisms are presented in *Table 1*.

**Table 1. Water Activities ($a_W$) Required to Support the Growth of Representative Microorganisms**

| Bacteria | Water Activity ($a_W$) | Molds and Yeast | Water Activity ($a_W$) |
|---|---|---|---|
| *Pseudomonas aeruginosa* | 0.97 | *Rhyzopus nigricans* | 0.93 |
| *Bacillus cereus* | 0.95 | *Mucor plumbeus* | 0.92 |
| *Clostridium botulinum,* Type A | 0.95 | *Rhodotorula mucilaginosa* | 0.92 |
| *Escherichia coli* | 0.95 | *Saccharomyces cerevisiae* | 0.90 |
| *Clostridium perfringens* | 0.95 | *Paecilomyces variotti* | 0.84 |
| *Lactobacillus viridescens* | 0.95 | *Penicillium chrysogenum* | 0.83 |
| *Salmonella* spp. | 0.95 | *Aspergillus fumigatus* | 0.82 |
| *Enterobacter aerogenes* | 0.94 | *Penicillium glabrum* | 0.81 |
| *Bacillus subtilis* | 0.90 | *Aspergillus flavus* | 0.78 |

[1] J. A. Troller, D. T. Bernard, and V. W. Scott. Measurement of Water Activity. In: *Compendium of Methods for the Microbiological Examination of Foods*. American Public Health Association, Washington, DC, 1984 pp.124–134.

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**Table 1. Water Activities ($a_W$) Required to Support the Growth of Representative Microorganisms** *(continued)*

| Bacteria | Water Activity ($a_W$) | Molds and Yeast | Water Activity ($a_W$) |
|---|---|---|---|
| *Micrococcus lysodekticus* | 0.93 | *Aspergillus niger* | 0.77 |
| *Staphylococcus aureus* | 0.86 | *Zygosachharomyces rouxii* (osmophilic yeast) | 0.62 |
| *Halobacterium halobium* (halophilic bacterium) | 0.75 | *Xeromyces bisporus* (xerophilic fungi) | 0.61 |

Pharmaceutical drug products with water activities well below 0.75 (e.g., direct compression tablets, powder and liquid-filled capsules, nonaqueous liquid products, ointments, and rectal suppositories) would be excellent candidates for reduced microbial limit testing for product release and stability evaluation. This is especially true when pharmaceutical products are made from ingredients of good microbial quality, when manufacturing environments do not foster microbial contamination, when there are processes that inherently reduce the microbial content, when the formulation of the drug product has antimicrobial activity, and when manufacturing sites have an established testing history of low bioburden associated with their products. *Table 2* contains suggested microbial limit testing strategies for typical pharmaceutical and over-the-counter (OTC) drug products based on water activity. Other considerations, as listed above, would be applied when setting up the microbial limits testing program for individual drug products because water activity measurements cannot solely be used to justify the elimination of microbial content testing for product release.

Similar arguments could be made for the microbial limits testing of pharmaceutical ingredients. However, this would require pharmaceutical manufacturers to have a comprehensive knowledge of the pharmaceutical ingredient manufacturer's manufacturing processes, quality programs, and testing record. This could be obtained through a supplier audit program.

**Table 2. Microbial Limit Testing Strategy for Representative Pharmaceutical and OTC Drug Products Based on Water Activity**

| Products | Water Activity ($a_W$) | Greatest Potential Contaminants | Testing Recommended |
|---|---|---|---|
| Nasal inhalant | 0.99 | Gram-negative bacteria | TAMC,* TCYMC, absence of *S. aureus* and *P. aeruginosa* |
| Hair shampoo | 0.99 | Gram-negative bacteria | TAMC, TCYMC, absence of *S. aureus* and *P. aeruginosa* |
| Antacid | 0.99 | Gram-negative bacteria | TAMC, TCYMC, absence of *E. coli* and *Salmonella* spp. |
| Topical cream | 0.97 | Gram-positive bacteria | TAMC, TCYMC, absence of *S. aureus* and *P. aeruginosa* |
| Oral liquid | 0.90 | Gram-positive bacteria and fungi | TAMC and TCYMC |
| Oral suspension | 0.87 | Fungi | TAMC and TCYMC |
| Topical ointment | 0.55 | None | Reduced testing |
| Lip balm | 0.36 | None | Reduced testing |
| Vaginal and rectal suppositories | 0.30 | None | Reduced testing |
| Compressed tablets | 0.36 | None | Reduced testing |
| Liquid-filled capsule | 0.30 | None | Reduced testing |

* TAMC = Total aerobic microbial count; TCYMC = Total combined yeast and mold count.

[NOTE—The water activities cited in *Table 2* for the different dosage forms are representative, and companies are urged to test their individual products before developing a testing strategy.]

Water activity, $a_W$, is the ratio of vapor pressure of $H_2O$ in product (P) to vapor pressure of pure $H_2O$ (Po) at the same temperature. It is numerically equal to 1/100 of the relative humidity (RH) generated by the product in a closed system. RH can be calculated from direct measurements of partial vapor pressure or dew point or indirect measurement by sensors whose physical or electric characteristics are altered by the RH to which they are exposed.

The relationship between $a_W$ and equilibrium relative humidity (ERH) is represented by the following equations:

$$a_W = P/Po \text{ and } ERH(\%) = a_W \times 100$$

The $a_W$ measurement may be conducted using the dew point/chilled mirror method.[2] A polished, chilled mirror is used as the condensing surface. The cooling system is electronically linked to a photoelectric cell into which light is reflected from the condensing mirror. An air stream, in equilibrium with the test sample, is directed at the mirror which cools until condensation occurs on the mirror. The temperature at which this condensation begins is the dew point from which the ERH is determined. Sample preparation should be considered as it may affect the water activity level of the material tested. Commercially available instruments using the dew point/chilled mirror method or other technologies need to be evaluated for suitability, validated, and calibrated when used to make water activity determinations. These instruments are typically calibrated using saturated salt solutions at 25°, as listed in *Table 3*.

[2] AOAC International Official Method 978.18. In: *Official Methods of Analysis of AOAC International,* 17th edition, AOAC International, Gaithersburg, Maryland.

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**Table 3. Standard Saturated Salt Solutions Used to Calibrate Water Activity Determination Instruments**

| Saturated Salt Solutions | ERH (%) | $a_w$ |
|---|---|---|
| Potassium sulfate ($K_2SO_4$) | 97.3 | 0.973 |
| Barium chloride ($BaCl_2$) | 90.2 | 0.902 |
| Sodium chloride (NaCl) | 75.3 | 0.753 |
| Magnesium nitrate [$Mg(NO_3)_2$] | 52.9 | 0.529 |
| Magnesium chloride ($MgCl_2$) | 32.8 | 0.328 |

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# ⟨1113⟩ MICROBIAL CHARACTERIZATION, IDENTIFICATION, AND STRAIN TYPING

## INTRODUCTION

Microorganisms, if detected in drug substances, excipients, water for pharmaceutical use, the manufacturing environment, intermediates, and finished drug products, typically undergo characterization. This may include identification and strain typing, as appropriate. [NOTE—A *Glossary of Terms* is provided at the end of this chapter.] Routine characterization of microorganisms may include the determination of colony morphology, cellular morphology (rods, cocci, cell groupings, modes of sporulation, etc.), Gram reaction or other differential staining techniques, and certain key biochemical reactions (e.g., oxidase, catalase, and coagulase activity) that can be diagnostic. Microbial characterization to this level is sufficient for many risk-assessment purposes in nonsterile pharmaceutical manufacturing operations and in some sterile product manufacturing environments.

In some cases a more definitive identification of the microorganisms yields genus- and species-level identification. Beyond this, available methodologies can perform strain-level identification, which can be useful in an investigation to determine the source of the microorganism. Identification is especially common when organisms are recovered at atypically high rates or in numbers that exceed recommended levels for specific categories of products. Additionally, microbial identification is useful in aseptic processing and is necessary where sterility test positives have occurred and in the assessment of contamination recovered from failed aseptic process simulations, i.e., media fills.

Microbiological identification systems are based on different analytical methodologies, and limitations may be inherent to the method and/or arise from database limitations. Identification is accomplished by matching characteristics (genotypic and/or phenotypic) to an established standard (reference) organism such as a type strain. If a microorganism is not included in the database it will not be identified, so manufacturers should review the breadth of the database of the identification system they plan to use and its applicability to their needs. Users should consider which microbiological identification system(s) is (are) most applicable to their requirements. Bearing in mind both these limitations and the level of identification required (genus, species, strain), users also must select the appropriate technology to use in routine microbiological identification testing.

The need for microbial identification is specifically cited in USP general test chapter *Microbiological Examination of Nonsterile Products: Tests for Specified Microorganisms* ⟨62⟩. This chapter indicates a requirement for confirmatory identification tests for organisms that grow on selective or diagnostic media and demonstrate defined morphological characteristics. Also, USP general test chapter *Sterility Tests* ⟨71⟩ allows for invalidation of the test, if after identification of the microorganisms isolated from the test, the growth of this (or these) species may be unequivocally ascribed to faults with respect to the material and/or the technique used in conducting the sterility test procedure. USP general information chapter *Microbiological Control and Monitoring of Aseptic Processing Environments* ⟨1116⟩ recommends that microbial isolates be identified at a rate sufficient to support the environmental monitoring program.

## ISOLATION OF PURE CULTURES

The first step in identification is to isolate a pure culture for analysis. This is typically accomplished by successive streaking of the colony of interest in a quadrant pattern on appropriate general microbiological solid media with the objective of obtaining discreet colonies that usually yield pure cultures. This technique also allows phenotypic expression and growth of sufficient inoculum for succeeding identification procedures. Analysts should recognize that expression of the microbial phenotype (i.e., cell size and shape, sporulation, cellular composition, antigenicity, biochemical activity, and sensitivity to antimicrobial agents) may be affected by isolate origins, media selection, and growth conditions (see *Table 1*). Therefore, the preparatory media for identification and the number of subcultures may affect the results of phenotype identification methods.

**Table 1. Phenotypic Characteristics Used in Microbial Taxonomy**

| Categories | Characteristics |
|---|---|
| Culture | Colony morphology, colony color, shape and size, pigment production |
| Morphological | Cellular morphology, cell size, cell shape, flagella type, reserve material, Gram reaction, spore and acid-fast staining, mode of sporulation |
| Physiological | Oxygen tolerance, pH range, temperature optimum and range, salinity tolerance |
| Biochemical | Carbon utilization, carbohydrate oxidation or fermentation, enzyme patterns |
| Inhibition | Bile salt-tolerance, antibiotic susceptibility, dye tolerance |
| Serological | Agglutination, fluorescent antibody |
| Chemo-taxonomic | Fatty acid profile, microbial toxins, whole cell composition |
| Ecological | Origin of the organism |

In contrast, the microbial genotype generally is well conserved and unaffected by cultural conditions. Therefore, once the isolation of a pure, monoclonal colony is assured, the microorganism may be analyzed without concern over the most recent growth media or the viability of the isolate. *Table 2* lists genotypic characteristics that can be determined.

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**Table 2. Genotypic/Phylogenetic Characteristics That Can Be Used in Microbial Taxonomy**

| Categories | Characteristics |
|---|---|
| Genotypic | DNA base ratio (G + C content), restriction fragment patterns, and DNA probes |
| Phylogenetic | DNA–DNA hybridization, and 16S and 23S rRNA sequences |

Bacterial taxonomy as described in *Bergey's Manual of Systematic Bacteriology* (*Bergey's Manual*)[1] is at present accomplished by comparative analysis of genetic material. When the DNA from an unknown organism is compared to the DNA from a known organism, the degree of relatedness can be determined. Genotypic identification (*Table 2*) is accomplished through the use of DNA hybridization, restriction fragment pattern comparisons, and/or DNA probes. For example, greater than 70% relatedness with DNA–DNA hybridization indicates the organisms are the same species. Phylogenetic analysis (*Table 2*) is typically performed by comparing the base sequence of a portion of the 16S ribosomal RNA gene for bacteria, or the 23S ribosomal RNA gene for fungi. Polymerase chain reaction (PCR) is used to amplify these genes, and the amplified region is then isolated and base sequenced using an electrophoretic or dideoxy chain termination method. Comparisons can be made using validated proprietary databases or those that are publicly available. [**CAUTION**—Publicly available databases may not be validated.]

## PRIMARY SCREENING AND CHARACTERIZATION

Microorganisms isolated on compendial media from samples of pharmaceutical ingredients, water for pharmaceutical use, the manufacturing environment, intermediates, and finished products may be physiologically stressed. The microorganisms will pass from a metabolic state suitable for survival under adverse ambient conditions to culture conditions that are far richer nutritionally and are at an optimal incubation temperature. This transition can be managed by careful handling of the isolates. In preparation for identification, individual representative colonies from the primary isolation media are streaked for monoclonal colonies onto solid media as described above. The first step is to determine the Gram reaction, cellular morphology, and in some cases diagnostic biochemical reactions of the bacteria isolates. This is a critical step for many phenotypic identification schemes. If the wrong characteristics are assigned to an isolate, subsequent testing may be conducted using the wrong microbial identification kit, resulting in an incorrect result. Several common preliminary screening tests are described below.

### Gram Staining

Gram staining methods include the four-step method: crystal violet (primary stain), iodine (mordant), alcohol or alcohol–acetone (decolorizer), and safranin (counterstain). In the three-step method the decolorization and counterstaining steps are combined. Under optimal conditions, Gram-positive organisms retain the crystal violet stain and appear blue violet. Gram-negative organisms lose the crystal violet stain, so they contain only the counterstain safranin and appear red. Some bacteria may be Gram-variable. Common pitfalls in this method are that heat fixation may cause Gram-positive cells to stain Gram-negative, and older cultures may give Gram-variable reactions. Using too much decolorizer could result in a false Gram-negative result, and not using enough decolorizer may yield a false Gram-positive result. One variation that has advantages in some situations is to perform a methanol, rather than heat, fixation of the bacterial smear. In some cases alcohol fixation may give more consistent Gram stain results. In either method a Gram-positive and a Gram-negative control should be included to allow identification of errors in staining. Because the Gram-staining reaction must be read under a microscope, cellular morphology can be simultaneously ascertained.

### Spore Staining

Spore staining can be accomplished using a malachite green stain for bacterial spores. A positive control should be included to allow identification of errors in spore staining.

### Biochemical Screening

Key biochemical screening tests include (1) the oxidase test to separate Gram-negative, rod-shaped bacteria into nonfermenters (oxidase positive) and enteric (oxidase negative) bacteria, (2) the catalase test to separate *Staphylococci* (catalase positive) from *Streptococci* (catalase negative), and (3) the coagulase test to separate *Staphylococci* into coagulase negative (presumptively nonpathogenic) and coagulase positive (more likely pathogenic) *Staphylococci*.

For many types of investigations and routine surveying of manufacturing environmental bioburden, these few tests can provide sufficient information for ongoing evaluation. However, when circumstances dictate greater in-depth assessment, identification to the genus, species, or strain level can yield valuable insights about the nature and source of environmental bioburden. Also, microbial identification to the species and even strain level can be critical in assessing and mitigating risk from microbial contamination.

## MICROBIAL IDENTIFICATION BY PHENOTYPIC METHODS

Phenotypic methods use expressed gene products to distinguish among different microorganisms. Generally, these require a relatively large number of cells in pure, monoclonal culture. Recovery and growth methods for microbial enumeration and

[1] *Bergey's Manual of Systematic Bacteriology, 2nd Edition,* 2003.

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identification are limited by the length of incubation and the fact that many organisms present in the environment are not recovered by general microbiological growth media. Additionally, freshly isolated, stressed microorganisms by subculture from primary recovery may not result in a full expression of phenotypic properties. However, methods based on carbon utilization and biochemical reaction, as well as fatty acid profiles by gas–liquid chromatography and whole-cell composition by MALDI–TOF mass spectrometry, are always based on inocula development for a specific identification system. These systems rely on specified culture media and incubation conditions to achieve consistent identification. Phenotypic microbial identification methods are successfully used in food, water, clinical, and pharmaceutical microbiological testing laboratories.[2] Phenotypic microbial identification methods provide information that enables microbiologists to make informed decisions regarding product risk and to recognize changes in environmental microflora. In many quality control investigations, phenotypic identification alone is sufficient and will enable scientists to conduct a thorough investigation and to recommend appropriate corrective actions as needed.

## MICROBIAL IDENTIFICATION BY GENOTYPIC METHODS

Genotypic microbial identification methods are theoretically more reliable because nucleic acid sequences are highly conserved in most microbial species. Applicable genotypic methods include DNA–DNA hybridization, PCR, 16S and 23S rRNA sequencing, multilocus sequence typing (MLST), pyrosequencing, DNA probes, and analytical ribotyping. These methods can be technically challenging for microbiologists. They also require more expensive analytical equipment and supplies. Often these analyses are conducted by contract laboratories, government laboratories, universities, research institutes, or specialized laboratories within industrial firms. Therefore, the use of genotypic identification methods is typically limited to critical microbiological investigations such as product failure investigations. Further, if strain-level identification is done in the course of an investigation, analysts must ensure that the method is appropriate.

DNA sequencing of the first 500 base pairs of the 16S rRNA sequence is useful for identification to the species level but may not provide sufficient power to resolve among closely related species or strains of the same species. In contrast, Southern hybridization of restriction endonuclease digests is powerful and can be effective in demonstrating differences between two strains. If the banding patterns appear identical, this shows only that restriction endonuclease has similar cleavage sites in that region of the two organisms. Demonstration that the two organisms are the same should include two or more different restriction endonuclease digests, each of which yields bands in the area of interest. All bands from the two organisms must be identical.

In contrast to microbial identification, nucleic acid-based methods can be used to screen for specific microorganisms. The steps associated with this activity are sample collection, nucleic acid extraction, target amplification, hybridization, and detection. The problem of amplifying DNA from nonviable bacterial cells can be overcome by using reverse transcription to convert rRNA that is transitional, hence related to viability, to DNA for PCR amplification. Issues include the detection of microbial variants, limits of detection, matrix effects, positive cutoff verification, instrument and system carry-over, diagnostic accuracy, and reproducibility.

## VERIFICATION OF MICROBIAL IDENTIFICATION METHODS

Microbial identification tests include serological tests, chemical reagents, reference organisms, and instrumentation. The verification of an identification test system can include one of the following: (1) using an existing system for parallel testing of microbial isolates obtained from routine testing (the number of isolates tested may be as high as 50, and any discrepancies in identification can be arbitrated using a referee method); (2) testing 12–15 known representative stock cultures of different commonly isolated species for a total of 50 tests; or (3) confirming that 20–50 organism identifications, including 15–20 different species, agree with the results of a reference laboratory testing of split sample.[3] In each case the appropriate quality control organisms, as recommended by the supplier and the compendia, should be included in the verification process.

With identification systems, verification of the identity of the species should be evaluated and the level of agreement should be considered. Typically greater than 90% agreement can be achieved with samples of microorganisms that are appropriate for the identification system. Groups of organisms that are challenging to identify (e.g., nonfermenting bacteria, corynebacteria, and coagulase-negative *Staphylococci*) may be included, when appropriate, in the verification process but may yield lower levels of agreement.

The hierarchy of microbial identification errors in descending order of impact is (1) misidentification to genera, (2) misidentification to species, and (3) no identification. Misidentification could lead to inappropriate corrective and preventive actions and product disposition.

A microbial identification system may not be able to identify an isolate because the organism is not included in the database, the system parameters are not sufficiently comprehensive to identify the organism, the isolate may be nonreactive in the system, or the species may not have been taxonomically described. Such isolates can be sent to the supplier of the microbial identification system for additional study and, if appropriate, added to the database. Alternatively, genotypic identification tests can be conducted, and the species can be added to an in-house database. Misidentification is more difficult to determine, but any microbial identification should be reviewed for reasonableness in terms of the microorganism's morphology, physiological requirements, and source of isolation. Organisms identified only to genus may be common for the numerous nonpathogenic species of *Staphylococcus, Corynebacterium* (and other small pleomorphic Gram-positive rods), and *Micrococcus*.

The most important verification tests are accuracy and reproducibility. These measurements can be defined as follows:

$$\text{Accuracy \%} = (\text{Number of correct results}/\text{Total number of results}) \times 100$$

[2] O'Hara, C.M., M.P. Weinstein, and J.M. Miller. Manual and automated systems for detection and identification of microorganisms. *ASM Manual of Clinical Microbiology, 8th Edition,* 2003.

[3] Cumitech 31. *Verification and Validation of Procedures in the Clinical Microbiology Laboratory.* Elder, B.L., S.A. Hansen, J.A. Kellogg, F.J. Marsik, and R.J. Zabransky, ASM, February 1997.

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Reproducibility % = (Number of correct results in agreement/Total number of results) × 100

The user should establish suitable acceptance criteria for accuracy and reproducibility, taking into account method capability.

Other measurements are sensitivity, specificity, and positive and negative predictive value. These measurements are best illustrated by an example. A clinical microbiology laboratory compared the frequency of isolation of a DNA hybridization probe to a culture method for the sexually transmitted bacterium *Neisseria gonorrhaeae.*[3] The frequency of isolation from clinical specimens was historically 10%. The lab ran 100 split samples, and the results are presented in *Table 3.*

**Table 3. Comparison of the Distribution of Negative and Positive Results for the DNA Probe and Culture Methods**

| | Culture Results | |
|---|---|---|
| DNA Probe Results | Positive | Negative |
| Positive | 9 | 2 |
| Negative | 1 | 88 |

Sensitivity = [9/(9 + 1)] × 100 = 90%
Specificity = [88/(88 + 2)] × 100 = 97.7%
Positive Predictive Value = [9/(9 + 2)] × 100 = 81.8%
Negative Predictive Value = [88/(88 + 1)] × 100 = 98.9%

Note that the positive predictive value (PPV) is not intrinsic to the test; it also depends on the prevalence of the microorganism in clinical samples. PPV is directly proportional to the prevalence of the disease or condition. In this example, if the group of people tested had included a higher proportion of people with infection, then the PPV would probably be higher and the negative predictive value (NPV) lower. If all persons in the group had infection, the PPV would be 100% and the NPV 0%. The mathematical derivation of these functions is outlined in *Table 4.*

**Table 4. A Two-Row by Two-Column Contingency Table with Respect to the Reference Culture Method and the Alternate PCR Method (After ISO 5725-1 and 5725-2 2004)***

| | PCR | | |
|---|---|---|---|
| **Culture** | **Positive** | **Negative** | **Sum** |
| Positive | a<br>True Positive | b<br>False Negative | a + b |
| Negative | c<br>False Positive | d<br>True Negative | c + d |
| Sum | a + c | b + d | |

* ISO 5725-1:1994 Accuracy (trueness and precision) of measurement methods and results—Part 1: General principles and definitions and ISO 5725-2:1994 Accuracy (trueness and precision) of measurement methods and results—Part 2: Basic methods for the determination of repeatability and reproducibility of standard measurement methods.

Inclusivity (%) = [a/(a + b)] × 100
Exclusivity (%) = [d/(c + d)] × 100
Positive Predictivity (%) = [a/(a + c)] × 100
Negative Predictivity (%) = [d/(b + d)] × 100
Analytical Accuracy (%) = [(a + d)/(a + b + c + d)] × 100
Kappa Index = 2(ad − bc)/[(a + c) × (c + d) + (a + b) × (b + d)]

## Phylogenetic Considerations

The second edition of *Bergey's Manual* represented a major departure from the first edition, and also from the eighth and ninth editions of the *Manual of Determinative Bacteriology*. The organization of content in *Bergey's Manual* follows a phylogenetic framework, based on analysis of the nucleotide sequence of the ribosomal small subunit 16S RNA, rather than a phenotypic structure.

Phylogenetic trees or dendrograms show the closest genetically related organisms. The application of this technology has resulted in taxonomic revisions and the renaming of some well-known microorganisms; e.g., the fungus *A. niger* ATCC 16404 was renamed *A. brasiliensis*. In general, organisms with relatedness less than or equal to 97% are considered different genera and those with relatedness less than or equal to 99% are considered different species,[4] but there are many exceptions to this generalization.

Differences in genotype and phenotype are relatively uncommon, e.g., same or very similar genotype shared by microorganisms with different phenotypes, similar phenotypes but different genotypes, and microorganisms that are genotypically too distant to be the same species or genus. The concept of polyphasic taxonomy[5] that refers to assembly and use of many levels of information, e.g., microbial characterization, phenotypic and genotypic data, and origin of the

[4] J.E. Clarridge III. The Impact of 16S rRNA Gene Sequencing Analysis for Identification of Bacteria on Clinical Microbiology and Infectious Diseases, *Clin. Microbiol. Rev. 17 (2)* 840–862, 2004.
[5] Gillis, M., P. Vandamme, P. De Vos, J. Swings and K. Kersters. Polyphasic Taxonomy. *Bergey's Manual of Systematic Bacteriology, 2nd Edition,* 2003.

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microorganisms, can be successfully applied to microbial identification. This avoids decisions made solely using genotypic data that make no sense when the microbial characteristics, testing history, and source of isolation are considered.

## GLOSSARY

**Microbial classification:** The arrangement of microorganisms into taxonomic groups based on their similarities and relationships.

**Microbial identification:** The determination of which broad group (e.g., bacteria, yeast, or mold) or narrow group (e.g., genus and/or species) to which a laboratory isolate belongs.

**Microbial characterization:** The use of colony growth, cellular morphology, differential staining, and key diagnostic features to characterize a laboratory isolate for trending and investigative purposes without identification, e.g,. nonpathogenic Staphylococci.

**Mol %GC:** The molecular percentage of guanine–cytosine range within the chromosomal DNA. [NOTE—The %GC + %AT = 100%.]

**Phylogenetic species:** A species consisting of many strains including the type of strain that shares at least 70% total genome DNA–DNA hybridization and less than 5° ΔTm (difference in melting point of the hybrid).

**Polyphasic taxonomy:** Taxonomy that assembles and assimilates many levels of information from molecular, physiological, morphological, serological, or ecological sources to classify a microorganism.

**Relatedness:** The extent of relationship or similarity of two (or more) organisms on a Phylogenetic Tree or a Dendrogram.

**rRNA Sequence:** The DNA sequences that encode rRNA used in protein synthesis are highly conserved among microorganisms of a common ancestry. They are used to determine the phylogenetic distance between organisms and are useful in microbial taxonomy and identification.

**Strain:** A specific isolate of a species that is maintained in pure culture and is characterized. The type strain is representative of the species that provides a reference for the species based on its historic isolation, characterization, and deposition in recognized culture collections.

**Strain typing:** Strain typing is an integral part of epidemiological investigations in clinical and public health microbiology. Methods including pulsed-field gel electrophoresis, riboprinting, arbitrarily primed polymerized chain reaction, and whole genome ordered restriction or optical mapping can be used to demonstrate that microbial species are the same strain and most likely are from a common source.

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# ⟨1115⟩ BIOBURDEN CONTROL OF NONSTERILE DRUG SUBSTANCES AND PRODUCTS

## INTRODUCTION

In terms of microbiological contamination risk control, there are two broad categories of drug products: (a) sterile products, in which the bioburden is essentially eliminated using validated methodologies, and (b) nonsterile products for which the final product bioburden is controlled to appropriate levels based on product attributes, route of administration, and target patient population.

Microbial content in nonsterile products is controlled to a level consistent with patient safety. Use of excessive controls that would add complexity or cost without a commensurate safety benefit is not advantageous in terms of added value to either the patient or the manufacturer. Therefore, a pragmatic scientific approach to management of the microbial bioburden in nonsterile products requires consideration of patient risk and contamination control objectives to achieve a practical and appropriate level of risk management.

A critical consideration in ensuring product quality is to prevent conditions within the manufacturing facility or manufacturing process that favor the proliferation or ingress of microorganisms. Microbial growth in excipients, components, and drug substances is a concern because it creates the possibility that viable microbial content could reach unacceptable levels. Bioburden levels in the ranges of those recommended in *Microbiological Examination of Nonsterile Products: Acceptance Criteria for Pharmaceutical Preparations and Substances for Pharmaceutical Use* ⟨1111⟩ are recognized as safe and do not pose risk of infection or microbial toxins. Manufacturers should have a clear understanding of situations that could favor microbial growth within their facilities and materials and should implement practical countermeasures.

This chapter outlines a risk-based approach to the control of contamination in nonsterile product manufacturing. The manufacture of nonsterile products and the management of their microbiological content are distinctly different from those required for sterile products. Sterile products are administered parenterally by means of injection or are applied topically to sensitive tissues where the risk of infection is comparatively high.

In contrast, nonsterile products are administered to regions of the human body that are rich in microbial flora and have physical or immunological barriers to infection. Examples include the oral cavity, skin, nasopharynx, vagina, and rectum, which harbor a high and diverse viable microbial population. Recent findings from the human microbiome project (*1*) underscore the enormous size and diversity of bacterial populations associated with humans. A healthy adult has a bacterial population that averages approximately $10^{14}$ bacteria, a number that exceeds the individual's own cells by a factor of 10. All humans carry on or in their bodies microorganisms that under certain conditions may cause infection in other humans. Because these microorganisms are present on humans means they may be present transiently in the nonsterile manufacturing environment. Their ubiquity in nature and infrequent association with infection confirms that the risk associated with them is very low. However, good hygienic controls and selection of suitable gowning systems are important considerations for products that are intended for patients who may be immunocompromised.

The following list provides a hierarchy of broad categories of nonsterile pharmaceutical products with respect to potential risk of microbiological contamination (from high to low) (*2*):

- metered-dose and dry powder inhalants
- nasal sprays
- otics
- vaginal suppositories
- topicals
- rectal suppositories
- oral liquids (aqueous)
- liquid-filled capsules
- oral tablets and powder-filled capsules

When formulators evaluate the susceptibility of nonsterile pharmaceutical products to microbial hazards, considerations include whether the active ingredient has inherent antimicrobial activity, the microbiological content of excipients, inclusion of antimicrobial preservatives in the formulation, and water activity. In addition, manufacturers should consider whether processing steps and hold periods could result in changes in the bioburden.

Nonsterile products are expected to have some bioburden, which should be controlled within a suitable range (see ⟨1111⟩). The risk of infection resulting from a nonsterile drug product generally is low, regardless of the route of administration, provided appropriate precautions and procedures are followed. General chapters *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* ⟨61⟩ and *Microbiological Examination of Nonsterile Products: Tests for Specified Microorganisms* ⟨62⟩ provide methods, and ⟨1111⟩ provides information for the evaluation of microorganisms isolated during nonsterile drug product manufacturing. It is not possible to provide a comprehensive product-by-product list of objectionable microorganisms. The degree to which any organism may be objectionable depends on the product attributes, route of administration, and patient population. Manufacturers are responsible for determining whether microorganisms recovered from drug products are objectionable. In general, objectionable microorganisms are those that are known to be truly pathogenic, considering the product's route of administration. Guidance for risk factors to be considered is also provided as a bulleted list in ⟨1111⟩. Risk may also arise where microorganisms are able to proliferate in sufficient numbers that could result in an unacceptable level of patient risk to a level above the ranges recommended in ⟨1111⟩. Microbiological risk should be assessed on a case-by-case basis during the development of a new product and should be evaluated during the validation of the manufacturing process.

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The proliferation of microbial contamination in a production facility, in products, or in product ingredients is an objectionable condition. Microbial proliferation within a facility creates conditions that favor the spread of contaminants, potentially in dangerous numbers, into ingredients, primary packaging materials, and even into product itself. Proliferation of microorganisms in ingredients or production intermediates must be prevented because it creates a risk due to microbial toxin and could result in damage to the chemical and pharmacological properties of the drug.

## U.S. REGULATORY GUIDANCE DOCUMENTS

The U.S. Code of Federal Regulations includes the Food and Drug Administration's Good Manufacturing Practice (GMP) requirements, which are found in Part 211 (*3*). These regulations contain general requirements for the manufacture of pharmaceutical products. Pertinent sections of 21 CFR 211 include: *211.42 Design and construction*—requires that buildings used for pharmaceutical manufacturing and associated activities are of suitable size, construction, and location; *211.46 Ventilation, air filtration, air heating and cooling*—requires adequate equipment to control microorganisms, dust, humidity, and temperature and differential air pressures be provided when appropriate for drug product manufacturing; *211.56 Sanitation*—requires written procedures for use of suitable rodenticides, insecticides, fungicides, fumigating agents, and cleaning and sanitizing agents, and written procedures shall be designed to prevent the contamination of equipment, components, drug product containers, closures, packaging, and labeling materials; *211.82 Receipt and storage of untested components, drug product containers, and closures*—requires an acceptance, quarantine, testing, and release procedure for components and containers; *211.110 Sampling and testing of in-process materials and drug products*—requires testing of in-process manufacturing controls that will include bioburden determination. Objectionable organism considerations: *21 CFR 211.84(d)(6)*—requires microbiological testing of incoming containers and components with potential for microbiological contamination that is objectionable in view of their intended use; *21 CFR 211.113(a)*—requires written procedures directed to control of objectionable microorganisms in drug products not required to be sterile; *21 CFR 211.165(b)*— requires appropriate laboratory testing, as necessary, of each batch of drug product required to be free of objectionable microorganisms.

## MICROBIAL CONTROL CONSIDERATIONS DURING PRODUCT DEVELOPMENT

A formal risk assessment program that identifies risk modalities and assigns critical control points for manufacture of nonsterile product is useful. Hazard Analysis and Critical Control Point programs (*4*) are widely used to assess and mitigate microbial risk in food manufacturing and may also be useful for manufacturers of nonsterile drug products. Points to be considered by pharmaceutical microbiologists when they assess the potential risk associated with nonsterile drug product manufacture are listed below:

- synthesis, isolation, and final purification of the drug substance
- microbiological attributes of the drug substance
- microbiological attributes of excipients and intermediates
- formulation and chemical and physical attributes of the drug product
- antimicrobial properties of the material, e.g., water activity or others
- manufacturing process
- delivery system
- packaging
- storage conditions for intermediates and the finished dosage form
- route of administration
- expected treatment procedure and dosage regimen
- population to which the product is delivered (e.g., neonates, immunocompromised patients, etc.)

Thorough consideration of these factors is valuable in defining appropriate manufacturing facility requirements and process control point measures that limit conditions that favor microbial proliferation and/or ingress.

## MICROBIAL CONTROL CONSIDERATIONS DURING MANUFACTURING

Although many factors (*Figure 1*) can result in the introduction of microorganisms, some of these are more likely to result in microbial contamination. These manufacturing factors are, in descending order (*5*): (1) ingredient water, (2) pharmaceutical ingredients, (3) process equipment, (4) manufacturing personnel, and (5) manufacturing environment.

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Figure 1. Factors that contribute to nonsterile product bioburden.

## Water Systems and Use

Water used in active ingredient manufacturing, formulation, cleaning, and housekeeping is the single most important risk element contributing to the contamination of nonsterile products. The quality or type of water used for nonsterile product formulation and final rinse of clean equipment should be chosen based on product risk. Purified waters used in pharmaceutical manufacturing are deionized and thus do not contain chlorine to control microbial growth. Substantial populations of Gram-negative rod-shaped bacteria and some molds are able to grow in such purified dechlorinated water, particularly in holding tanks at or around ambient temperatures. Standing water should be drained or physically removed quickly and efficiently from production vessels and equipment, as well as work surfaces and floors. Chlorinated potable water (city water) may be appropriate for some cleaning, housekeeping, and sanitization activities. Water quality for processing application uses should be determined on a case-by-case basis. Additional guidance for water system design and operation can be found in *Water for Pharmaceutical Purposes* ⟨1231⟩.

Process waters used for manufacturing of excipients, and, in some cases, active ingredients for nonsterile products present a substantial risk for microbial colonization and proliferation, particularly for ingredients of natural origin that have received minimal processing to reduce bioburden or to control microbial proliferation. Formulating and manufacturing equipment can be a source of contamination, and risks are higher when water and ingredients that are susceptible to microbial survival or growth are used. Therefore, cleaning, drying, and, where appropriate, sanitization of manufacturing equipment can be beneficial, but disinfectant residues should be limited in the operating environment and should be removed from product-contact surfaces. The isolation of waterborne organisms, particularly Gram-negative rods, is a likely indicator of failure to remove standing water on equipment and environmental surfaces.

## Active Pharmaceutical Ingredients, In-Process Materials, and Excipients

Ingredients and excipients used in nonsterile product manufacturing processes are important sources of microbiological contamination. Vendor evaluation, specifications, testing, package selection, shipping, storage conditions, expiry dates, and assessment for likely contamination or proliferation risk are all critically important in the reduction of microbial risk associated with these materials. Of particular concern are unprocessed materials of natural origin, those that have high water activity (see *Application of Water Activity Determination to Nonsterile Pharmaceutical Products* ⟨1112⟩ for additional information), synthetic

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processes with aqueous isolation steps or open processing, and biological processes with limited downstream purification or lacking defined microbial removal step(s). In-process bioburden monitoring points should be established at points in the process that are either immediately before or immediately follow a potential bioburden reduction process (such as an organic solvent extraction, heating, large change in pH) and/or immediately prior to final fill as determined by the risk analysis performed as suggested in the section *Microbial Control Considerations during Product Development*.

Manufacturers should sample incoming materials and should ensure proper contamination-control conditions for weighing and material addition to process equipment. All sampling and weighing equipment should be properly cleaned, sanitized, stored, and identified. The activities and the associated controls implemented to prevent microbial colonization and proliferation should be based on a documented, prospective risk-assessment and contamination-control strategy.

Pharmaceutical ingredients of consistently suitable microbial quality are an important element of the microbiological control program for nonsterile products. Procurement of ingredients of appropriate microbial quality requires the identification of vendors with the demonstrated capacity to produce drug substances or excipients of suitable quality. Supplier survey assessments may be conducted periodically to establish that the supplier has a well-designed and maintained microbiological control program for its manufacturing and primary packaging facilities. Materials that have low water activity, possess high or low pH, are not of natural origin, are inherently antimicrobial, or contain an antimicrobial preservative are generally not at risk for microbial proliferation. Risk assessments should consider ingredient characteristics regarding microbial survival, support of microbial growth, or frank antagonism to microbial survival.

The introduction of moisture into stored materials notably increases the risk of microbial contamination. Condensation in storage tank headspace or impermeable storage containers can result in contamination of materials with waterborne organisms or fungi even when the product under storage is expected to preclude microbial colonization or proliferation.

Microbiological examination of pharmaceutical ingredients (see chapters ⟨61⟩, ⟨62⟩, and ⟨1111⟩) can provide important insights into the microbiological quality of drug substances and excipients.

Primary packaging and intermediate containers (e.g., drum liners, plastic bags, and so on) can be a source of microbial contamination, and manufacturers should consider their initial quality, storage conditions, preparation, and handling procedures.

## MICROBIAL CONTROL OF DRUG SUBSTANCE MANUFACTURING

The approaches to microbial control described in this chapter can be applied with minimal change to drug substances manufactured either by biological processes or organic synthesis. The processes in organic synthesis often use extremes of temperature, pressure, pH, and other conditions that inhibit or actively destroy microorganisms. When the chemical process for manufacturing a drug substance is complex, the final steps often have the greatest potential effect on the bioburden. As is the case for drug product manufacturing, the water used in the process presents the greatest challenge to microbial control. The types of water used in these processes vary. Potable water typically is used in early-stage processing and cleaning, and Purified Water and Water for Injection often are used for downstream processing and cleaning. Use of Water for Injection is typically restricted to the final steps of bulk chemical or biological drug substance manufacturing that will ultimately be used in the formulation of sterile products. These processes are frequently performed almost entirely within closed vessels, which minimizes external facility considerations. Some process equipment is suitable for clean-in-place operations that use agents that are not only effective cleansers but also in some cases inhibit microbial growth/proliferation. The materials used in drug substance manufacturing include natural substances, organic compounds of various types, inorganic salts, acids, bases, and organic solvents, and the potential for microbial contamination from these materials clearly is variable. Personnel interventions in these processes are limited by the nature of the equipment and consideration for worker safety, and in biologics processing and certain late stages of organic synthesis the manufacturing environments may be classified (e.g., ISO 7 and ISO 8). Limiting the involvement of personnel and use of controlled environments both reduce opportunities for microbiological contamination.

### Equipment Design and Use

When possible, specifications for the selection of equipment that will be used in the manufacture of nonsterile products should include sanitary design. Equipment and utensils should be cleanable so contaminants and residual products can be reliably removed. Equipment should use sanitary fittings and should be designed for easy use of cleaning and sanitizing agents and complete rinse water drainage. Residual water in tanks, piping, or on equipment surfaces introduces the risk of colonization by waterborne organisms. Manufacturing equipment that cannot be cleaned in place should be readily accessible for manual cleaning, and parts that must be cleaned out of place should be not only easily accessible but also readily or easily removable. A further consideration is the compatibility of equipment with the typical range of disinfectants, including sporicides, used in cleaning procedures to sanitize equipment.

The preferred material of construction for equipment and utensils that will be in contact with product is austenitic stainless steel. Manufacturers should consider the surface finish on product-contact materials, for which a roughness average (RA) of 15–20 µm is a reasonable rule of thumb. Surfaces for general equipment and machine surfaces need not be polished beyond these RA values. Other materials of construction should be nonporous, smooth, and compatible with the products and cleaning materials. Process piping systems also should follow sanitary design principles and should be sloped to facilitate drainage, and the equipment should contain flush gaskets to prevent material build-up and to facilitate cleaning. The 3A Sanitary Standards (see www.3-A.org) provide generally useful guidance for process layout and design and machinery selection. Equipment cleaning procedures should be detailed and should ensure that the equipment is completely dry after cleaning and is stored in a manner that prevents microbial proliferation. Manufacturers should implement procedures for the protection of cleaned equipment and utensils before their next use. Cleaning and sanitization process validation should include the evaluation of microbial content both after sanitization and before use. Properly designed storage protection should mitigate the possibility of microbial growth before use, so after proper storage conditions are validated ongoing monitoring of equipment and utensils

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should not be required. Surface microbial sampling either immediately after cleaning or immediately before use must be done with caution; media residues and residual moisture must be carefully eliminated if sampling is performed.

Manufacturers should evaluate whether products that are manufactured using a piece of processing equipment may, under some processing circumstances, promote the growth of microorganisms. This evaluation is necessary to properly establish processing hold times and to define equipment use conditions following cleaning. The use of sanitizing agents on product-contact surfaces is not required when the cleaning procedures that remove chemical residues also remove microorganisms. In addition, there is a risk of product contamination with sanitization agents. With a validated cleaning process, the absence of chemical residues and visible standing water may provide assurance of the cleanliness of the equipment without routine chemical disinfection and also may obviate the need for microbiological monitoring of the equipment.

### Personnel

In addition to maintaining personal hygiene, operators should be trained and dressed appropriately for the function they are performing (*Table 1*).

**Table 1. Gowning in Manufacturing Areas**

| Protective Clothing | Operators in Formulation and Primary Packaging Areas |
|---|---|
| Plant uniform or plant uniform with overall for high-risk product and environment | Yes |
| Hair/beard covering | Yes |
| Safety glasses | Yes |
| Dedicated shoes or shoe coverings | Yes |
| Gloves | Yes (if in direct product contact) |
| Face masks | Yes (if in direct product contact) |

### The Manufacturing Environment

As noted, the environmental risks and controls for nonsterile products are different from those for aseptically manufactured sterile products. Unlike aseptic processing for which facility requirements are generally uniform in specification and performance, nonsterile product manufacturing environments typically involve diverse products and microbial contamination control requirements. In general, liquid, cream, or ointment products require a greater level of contamination risk mitigation than do solid dosage forms.

Common design elements to control microbial contamination may include the following:

- Walls, ceilings, and floors are constructed of nonporous materials that are readily cleanable and are resistant to cleaning agents and disinfectants.
- Floor drains are permitted in nonsterile product manufacturing areas provided that they can be closed during processing or fitted with a suitable air break if they are open during area and equipment cleaning.
- Access should be limited to essential personnel.
- Material, equipment, and personnel flows should avoid contamination.
- Ventilation and air filtration should be adequate to maintain the specified cleanliness, space pressurization (if required), temperature, and relative humidity.
- Good housekeeping and good general hygiene should be applied at all times.
- Cleaning and use status of all tools and implements used in production and all process equipment should be known at all times.
- Product-contact or water-supply tubing, valves, and fittings should be cleaned and sanitized according to a defined schedule, should be stored dry, and should be labeled with respect to status.
- Manufacturers should implement a formal housekeeping and sanitization program for operating areas, corridors, equipment storage, material staging, and other common areas.

Classified environments are not required for nonsterile product manufacturing, e.g., those specified in ISO 14644-1 (*6*). ISPE Baseline Guide No. 2, Oral Solid Dosage Forms (*7*) provides minimal acceptable design features for facilities for manufacturing nonsterile products.

## MICROBIAL ASSESSMENT OF NONSTERILE PRODUCT MANUFACTURING ENVIRONMENTS

As described above, microbiological monitoring of the manufacturing environment can serve as an adjunct to control and generally is a qualitative assessment tool in a properly implemented formal risk-based microbiological control program. A monitoring program commensurate with the product bioburden risk can help confirm the effectiveness of microbiological controls and may facilitate early detection of potential problems. Microbial methods and practices for aseptic facilities may be used, but the contamination recovery rates defined in *Microbiological Control and Monitoring of Aseptic Processing Environments* ⟨1116⟩ are not intended for nonsterile environments. Classified environments of the same class do not necessarily have similar microbiological control capabilities. Because the majority of organisms recovered in environmental monitoring are of human origin, the levels of transient contamination recovered depend largely on the level of human activity and gowning requirements.

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Gowning requirements are not as extensive in nonsterile product manufacturing as they are in aseptic processing, and thus in nonsterile environments manufacturers can monitor bioburden with reduced frequency and with expectations of higher bioburden recovery. Periodic assessments of production plant hygiene can offer useful insights into the effectiveness of the facility's cleaning and environmental controls.

During nonsterile product manufacturing, microbiological monitoring of the environment need not be as rigorous as that required during aseptic processing. Similarly, during nonsterile product manufacturing microbial risk mitigation is different from that for sterile products. In nonsterile products, manufacturers can expect intrinsic microbial bioburden that, properly controlled, does not result in risk to the end user. Manufacturers should establish acceptable levels of microorganisms within each product (*3*) and should sufficiently identify microorganisms to gain an appropriate understanding of bioburden patterns and seasonal variability.

## Microbial Sampling

During nonsterile product manufacturing, the air sampling methods used for environmental monitoring are active or passive. Active devices sample appropriate air volumes and deposit viable organisms on solid media plates or strips. Results typically are expressed as colony-forming units per unit volume although alternate methods may be used in lieu of recovery and growth on media. Passive sampling in the form of settling plates can be used in lieu of active air samplers.

Personnel monitoring typically is not required in nonsterile product manufacturing except when near-aseptic gowning materials are employed.

Sampling locations should be selected based on risk evaluation followed by a general hygienic survey of the environment. High-traffic areas where materials are transferred into and out of the area may be particularly prone to transient microbial contamination. Microbiological monitoring is not required in areas beyond the point where product has been placed into primary packaging containers.

Alternate nongrowth-based microbiological methods can be substituted for growth-based methods at the option of the user. Because there are no standardized environmental sampling methods and because monitoring is intended as a qualitative evaluation of general facility hygiene, there is no need for comparative studies between growth- and nongrowth-based methods. A small number of parallel samples is sufficient to establish a comparative baseline for an environment.

The frequency of monitoring should reflect the potential risk associated with the dosage form (see *Introduction*). Additionally, some products may have innate antimicrobial activity because of their attributes such as low water activity or inclusion of an antimicrobial preservative or an active ingredient that is itself an antimicrobial agent, e.g., an antibiotic or antitumor agent. Products that are resistant to microbial colonization or have microbiocidal or microbiostatic characteristics require little or no microbiological monitoring.

In general, environments for tablet and powder- and liquid-filled capsule manufacturing should require no monitoring or infrequent monitoring. Monitoring programs should be risk based, and the frequency and number of sampling sites should reflect the risk level. Manufacturing areas for higher-risk dosage forms such as inhalant products require more frequent monitoring and typically are manufactured in rooms classified to a particulate air quality level, e.g., ISO 8.

For most nonsterile product manufacturing environments, because of their limited environmental controls and comparatively low product risk, the establishment of alert and action levels may not be required. Environmental monitoring is considered an informational survey of the general hygienic conditions of the environment and should not be used in product-release decisions. Monitoring of unclassified environments is not required.

General information chapter *Microbial Characterization, Identification, and Strain Typing* ⟨1113⟩ contains general information about microbial characterization. In most nonsterile hygienic assessments, characterization of the microorganisms, cellular morphology, Gram reaction, and simple diagnostic testing are sufficient.

## Active Measures for Microbial Control

In addition to facility and process design considerations and equipment cleaning and storage controls, there are instances in which active means for addressing the contamination risk are required. The microbiological control of nonsterile products can be enhanced by the adoption of direct contamination control processes such as the following:

- decontamination of product contact surfaces, materials, and containers, typically by means of heat treatment (in the most critical applications sterilization can be used)
- chemical or physical (e.g., dry or moist heat) bioburden reduction treatments for raw materials and active ingredients
- use of closed, cleaned or decontaminated systems for handling and transfer of materials
- use of disposable components or utensils
- improved gowning materials for operational personnel
- use of classified environments in high-risk operations

These measures can be applied as required to improve the microbial contamination control during the manufacture of nonsterile products.

# OVERALL MANAGEMENT OF A MICROBIOLOGICAL CONTROL PROGRAM

The management of a successful microbiological control program includes the following: identification of suitable suppliers of pharmaceutical ingredients and excipients that have good microbiological quality; conducting a microbial risk assessment of the manufacturing process and packaging system; and the establishment of an appropriate monitoring and control system.

Although environmental contamination is by no means the most significant cause of nonsterile product recalls or contamination events, environmental monitoring may be a program adjunct to the microbiological control program. Microbial monitoring is an assessment and is not by itself a contamination control activity. There have been no scientifically controlled

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studies demonstrating what linkage, if any, exists between airborne or surface monitoring results and microbiological safety of the final product.

The microbiological contamination control program should be developed for identifying and controlling product risk, based on a formal assessment of risk modalities. The risk analysis should result in the identification of critical control points and should facilitate proper equipment selection, process layout and design, and facility design requirements.

Critical factors for the prevention of microbiological contamination during nonsterile product manufacturing are the control of the microbiological quality of ingredients and water, along with the development of proper cleaning and sanitization procedures. Microbiological monitoring does not mitigate risk, but it may serve as a sentinel.

No monitoring program can provide the assurance of contamination control as effectively as sound, proactive and preventive measures. Consistent control of contamination can be achieved mainly by an overall process evaluation assessing each of the control elements described above via risk assessment. Risk assessment may be coupled with active evaluation studies to ensure that appropriate measures are in place to prevent conditions conducive to contamination.

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# ⟨1116⟩ MICROBIOLOGICAL CONTROL AND MONITORING OF ASEPTIC PROCESSING ENVIRONMENTS

Microbiologically controlled environments are used for a variety of purposes within the healthcare industry. This general information chapter provides information and recommendations for environments where the risk of microbial contamination is controlled through aseptic processing. Products manufactured in such environments include pharmaceutical sterile products, bulk sterile drug substances, sterile intermediates, excipients, and, in certain cases, medical devices. Aseptic processing environments are far more critical in terms of patient risk than controlled environments used for other manufacturing operations —for example, equipment and component preparation, limited bioburden control of nonsterile products, and processing of terminally sterilized products. In this chapter, the type of aseptic processing is differentiated by the presence or absence of human operators. An advanced aseptic process is one in which direct intervention with open product containers or exposed product contact surfaces by operators wearing conventional cleanroom garments is not required and never permitted. [NOTE—A description of terms used in this chapter can be found in the *Glossary* at the end of the chapter.]

The guidance provided in this chapter and the monitoring parameters given for microbiological evaluation should be applied only to clean rooms, restricted-access barrier systems (RABS), and isolators used for aseptic processing. ISO-classified environments used for other purposes are not required to meet the levels of contamination control required for aseptically produced sterile products. The environments used for nonsterile applications require different microbial control strategies.

A large proportion of products labeled as sterile are manufactured by aseptic processing rather than terminal sterilization. Because aseptic processing relies on the exclusion of microorganisms from the process stream and the prevention of microorganisms from entering open containers during processing, product bioburden as well as the bioburden of the manufacturing environment are important factors governing the risk of unacceptable microbial contamination. The terms *aseptic* and *sterile* are not synonymous. *Sterile* means having a complete absence of viable microorganisms or organisms that have the potential to reproduce. In the purest microbiological sense, an *aseptic* process is one that prevents contamination by the exclusion of microorganisms. In contemporary aseptic healthcare-product manufacturing, *aseptic* describes the process for handling sterilized materials in a controlled environment designed to maintain microbial contamination at levels known to present minimal risk.

In any environment where human operators are present, microbial contamination at some level is inevitable. Even the most cautious clean-room environment design and operation will not eliminate the shedding of microorganisms if human operators are present. Thus, an expectation of zero contamination at all locations during every aseptic processing operation is technically not possible and thus is unrealistic. There are no means to demonstrate that an aseptic processing environment and the product-contact surfaces within that environment are sterile. Monitoring locations should be determined based upon a assessment of risk. Although manufacturers should review environmental monitoring results frequently to ensure that the facility operates in a validated state of control, monitoring results can neither prove nor disprove sterility. Because of the limitations of monitoring, manufacturers cannot rely directly on monitoring, statistics, or periodic aseptic-processing simulations to ensure a sterility assurance level.

Environmental monitoring is usually performed by personnel and thus requires operator intervention. As a result, environmental monitoring can both increase the risk of contamination and also give false-positive results. Thus, intensive monitoring is unwarranted, particularly in the ISO 5 environments that are used in the most critical zones of aseptic processing.

A number of sampling methods can be used to assess and control the microbiological status of controlled environments for aseptic processing. At present, nearly all of these methods rely on the growth and recovery of microorganisms, many of which can be in a damaged state caused by environmental stress and therefore may be difficult to recover. The numerical values for air, surface, and personnel monitoring included in this chapter are not intended to represent limits or specifications but are strictly informational. Because of the variety of microbiological sampling equipment and methods, it is not scientifically reasonable to suggest that the attainment of these values guarantees microbial control or that excursions beyond values in this chapter indicate a loss of control. The assessment of risks associated with manufacturing environments must be made over a significant period; and in each case, the contamination recovery rate metric should be established on the basis of a review of actual findings within the facility. The objective of each user should be to use contamination recovery rates to track ongoing performance and to refine the microbiological control program to foster improvements. When optimum operational conditions are achieved within a facility, contamination recovery rate levels typically become relatively stable within a normal range of variability.

There are no standard methods for air sampling, and available literature indicates that air-sampling methods are highly variable. It should not be assumed that similar sample volumes taken by different methods will produce similar rates of recovery. Many factors can affect microbial recovery and survival, and different air sampler suppliers may have designed their systems to meet different requirements. Also, sample-to-sample variation in microbial sampling can be extensive. Limited data are available regarding the accuracy, precision, sensitivity, and limits of detection of monitoring methods used in the aseptic processing of healthcare products.

Surface sampling methods are also not standardized. Different media are employed, and in the case of swabs, different results have been reported for wet and dry swab methods and contact plates. Replicate sample contact plates should be expected to give similar results under identical conditions, but rates of recovery have been reported to be both lower than expected and highly variable. In general, surface monitoring has been found to recover <50%, even when used with relatively high inoculum levels on standardized coupons. In actual production environments where organisms are stressed to varying degrees, recovery rates may be lower.

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## ADVANCED ASEPTIC TECHNOLOGIES

Advanced aseptic technologies can be defined as those that do not rely on the direct intervention of human operators during processing. At present, technologies such as isolators, blow/fill/seal, and closed RABS (designs that are never opened during setup or operation) may be considered advanced aseptic technologies, provided that direct intervention by gowned personnel is disallowed during processing. In recent years, isolator technology has found a broad acceptance in healthcare manufacturing. Isolators and closed RABS effectively separate the operator from the critical aseptic processing environment. Because these systems substantially reduce contamination risk, their microbiological control levels are higher than those of conventional clean rooms that have comparable particulate air classification level, for example, ISO 5.

## CLEAN ROOM CLASSIFICATION FOR ASEPTIC PROCESSING ENVIRONMENTS

The design and construction of clean rooms and controlled environments are covered in ISO 14644 series. This standard defines the performance of a clean environment with respect to the concentration of total particulates per unit volume. ISO 14644-1 stipulates the total particulate counts allowed for a clean environment to meet the defined air quality classifications. The reader is referred to this standard regarding the design characteristics and certification of clean environments.

Pharmaceutical manufacturers are concerned with nonviable particulate contamination in injectable products (see *Particulate Matter in Injections* ⟨788⟩). Unlike microbial contamination in which experimental data suggest that humans are the only significant source, nonviable particulates can arise both from humans and from processing equipment. Studies indicate that gowned humans slough particulate and microbial contamination at a rather consistent rate. However, the relationship between microbial (viable) and nonviable contamination does not hold for particulates shed by processing equipment. Where equipment is the primary source of particulate matter, the resulting particulates are essentially all nonviable.

The argument that if fewer total particulates are present in a clean room, it is less likely that airborne microorganisms will be present is true only if human operators are the source of particulate matter. It is not possible to clearly distinguish between background total particulate contamination generated largely by mechanical operations and the total particulates contributed by personnel. Thus, it is both commonplace and proper for clean-room environmental monitoring programs to consist of both a total particulate component and a microbiological component. *Table 1* describes the clean room classifications commonly used in the pharmaceutical industry. In aseptic processing, clean environments of ISO 14644-1 Classes 5–8 are typically used.

**Table 1. Airborne Total Particulate Cleanliness Classes[a]**

| ISO Class[b] | Particles ≥0.5 µm/m³ |
|---|---|
| ISO 5 | 3520 |
| ISO 6 | 35,200 |
| ISO 7 | 352,000 |
| ISO 8 | 3,520,000 |

[a] Taken from ISO International Standard 14644 Part 1, published by the International Organization for Standardization, May 1999.
[b] The four ISO 14644-1 classes correspond closely to former U.S. Federal Standard 209E classifications. The relationships are ISO 5/Class 100, ISO 6/Class 1000, ISO 7/Class 10,000, and ISO 8/Class 100,000.

Isolators and closed RABS present a different picture, because personnel are excluded from the aseptic processing environment and manipulations are made using glove-and-sleeve assemblies and half-suits made of thick, flexible plastic (such as polyvinyl chloride or synthetic rubber). Personnel have far less effect on the microbial quality of the environment within an isolator enclosure than in clean room environments. Some users have chosen to operate RABS in a manner that allows open, direct human intervention. In an open operational state, these systems are more similar in operation to conventional clean rooms and therefore cannot be considered advanced aseptic processing systems. In an open RABS, the ability of operators to adversely affect microbial contamination risk is higher than with closed RABS or isolators.

Specifications for air changes per hour and air velocities are not included in ISO 14644, nor were they included in Federal Standard 209E. Typically, ISO Class 8/Class 100,000 rooms are designed to provide a minimum of 20 air changes per hour; ISO Class 7/Class 10,000 rooms are designed to provide more than 50 air changes per hour; and ISO Class 5/Class 100 clean rooms provide more than 100 air changes per hour. The design of some facility criteria may differ. By diluting and removing contaminants, large volumes of air are likely to reduce airborne contamination in aseptic production. Optimum conditions vary considerably, depending on process characteristics, particularly the amount of contamination derived from personnel. These specifications should be used only as a guide in the design and operation of clean rooms, because the precise correlations among air changes per hour, air velocity, and microbial control have not been satisfactorily established experimentally.

Manufacturers should maintain a predominantly unidirectional flow of air (either vertical or horizontal) in a staffed Class 5 clean room environment, particularly when products, product containers, and closures are exposed. In the evaluation of air movement within a clean room, studying airflow visually by smoke studies or other suitable means is probably more useful than using absolute measures of airflow velocity and change rates. Risk assessment models are another useful way of reducing contamination risk and should be considered.

Air velocity and change rates are far less important in isolators or closed RABS than in clean rooms because personnel are more carefully separated from the product, product containers, and closures. Air velocities substantially lower than those used in human-scale clean rooms have proved adequate in isolator systems and may be appropriate in RABS as well. In zones within isolators where particulate matter poses a hazard to product quality, predominantly vertical or horizontal unidirectional airflow can be maintained. Experience has shown that well-controlled mixing or turbulent airflow is satisfactory for many aseptic processes and for sterility testing within isolators (see *Sterility Testing—Validation of Isolator Systems* ⟨1208⟩).

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# IMPORTANCE OF A MICROBIOLOGICAL EVALUATION PROGRAM FOR CONTROLLED ENVIRONMENTS

Monitoring of total particulate count in controlled environments, even with the use of electronic instrumentation on a continuous basis, does not provide information on the microbiological content of the environment. The basic limitation of particulate counters is that they measure particles of 0.5 µm or larger. While airborne microorganisms are not free-floating or single cells, they frequently associate with particles of 10–20 µm. Particulate counts as well as microbial counts within controlled environments vary with the sampling location and the activities being conducted during sampling. Monitoring the environment for nonviable particulates and microorganisms is an important control function because they both are important in achieving product compendial requirements for *Foreign and Particulate Matter* and *Sterility* in *Injections and Implanted Drug Products* ⟨1⟩.

Total particulate monitoring may provide a better means of evaluating the overall quality of the environment in isolators and closed RABS than in most conventional clean rooms. The superior exclusion of human-borne contamination provided by an isolator results in an increased proportion of nonviable particulates. Total particulate counting in an isolator is likely to provide an immediate indicator of changes in contamination level. Microbial monitoring programs should assess the effectiveness of cleaning and sanitization practices by and of personnel who could have an impact on the bioburden. Because isolators are typically decontaminated using an automatic vapor or gas generation system, microbial monitoring is much less important in establishing their efficiency in eliminating bioburden. These automatic decontamination systems are validated directly, using an appropriate biological indicator challenge, and are controlled to defined exposure parameters during routine use to ensure consistent decontamination.

Microbial monitoring cannot and need not identify and quantify all microbial contaminants in these controlled environments. Microbiological monitoring of a clean room is technically a semiquantitative exercise, because a truly quantitative evaluation of the environment is not possible, given the limitations in sampling equipment. Both the lack of precision of enumeration methods and the restricted sample volumes that can be effectively analyzed suggest that environmental monitoring is incapable of providing direct quantitative information about sterility assurance. Analysts should remember that no microbiological sampling plan can prove the absence of microbial contamination, even when no viable contamination is recovered. The absence of growth on a microbiological sample means only that growth was not discovered; it does not mean that the environment is free of contamination.

Routine microbial monitoring should provide sufficient information to demonstrate that the aseptic processing environment is operating in an adequate state of control. The real value of a microbiological monitoring program lies in its ability to confirm consistent, high-quality environmental conditions at all times. Monitoring programs can detect changes in the contamination recovery rate that may be indicative of changes in the state of control within the environment.

Environmental microbial monitoring and analysis of data by qualified personnel can assist in ensuring that a suitable state of control is maintained. The environment should be sampled during normal operations to allow the collection of meaningful, process-related data. Microbial sampling should occur when materials are in the area, processing activities are ongoing, and a full complement of personnel is working within the aseptic processing environment.

Microbial monitoring of manufacturing clean rooms, RABS, and isolators should include compressed gases, surfaces, room or enclosure air, and any other materials and equipment that might produce a risk of contamination. The analysis of contamination trends in an aseptic environment has long been a component of the environmental control program. In aseptic processing environments and particularly in ISO Class 5 environments, contamination is infrequently observed. In isolator enclosures, contamination is rarer still because of superior exclusion of human-borne contamination. Because of the criticality of these environments, even minor changes in the contamination incident rates may be significant, and manufacturers should frequently and carefully review monitoring data. In less critical environments, microbial contamination may be higher, but changes in recovery rates should be noted, investigated, and corrected. Isolated recoveries of microorganisms should be considered a normal phenomenon in conventional clean rooms, and these incidents generally do not require specific corrective action, because it is almost certain that investigations will fail to yield a scientifically verifiable cause. Because sampling itself requires an aseptic intervention in conventional clean rooms, any single uncorrelated contamination event could be a false positive.

When contamination recovery rates increase from an established norm, process and operational investigation should take place. Investigations will differ depending on the type and processing of the product manufactured in the clean room, RABS, or isolator. Investigation should include a review of area maintenance documentation; sanitization/decontamination documentation; the occurrence of nonroutine events; the inherent physical or operational parameters, such as changes in environmental temperature and relative humidity; and the training status of personnel.

In closed RABS and isolator systems, the loss of glove integrity or the accidental introduction of material that has not been decontaminated are among the most probable causes of detectable microbial contamination. Following the investigation, actions should be taken to correct or eliminate the most probable causes of contamination. Because of the relative rarity of contamination events in modern facilities, the investigation often proves inconclusive. When corrective actions are undertaken, they may include reinforcement of personnel training to emphasize acceptable gowning and aseptic techniques and microbial control of the environment. Some additional microbiological sampling at an increased frequency may be implemented, but this may not be appropriate during aseptic processing because intrusive or overly intensive sampling may entail an increased contamination risk. When additional monitoring is desirable, it may be more appropriate during process simulation studies. Other measures that can be considered to better control microbial contamination include additional sanitization, use of different sanitizing agents, and identification of the microbial contaminant and its possible source.

In any aseptic environment, conventional or advanced, the investigation and the rationale for the course of action chosen as a result of the investigation must be carefully and comprehensively documented.

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## PHYSICAL EVALUATION OF CONTAMINATION CONTROL EFFECTIVENESS

Clean environments should be certified as described in ISO 14644 series in order to meet their design classification requirements. The design, construction, and operation of clean rooms vary greatly, so it is difficult to generalize requirements for parameters such as filter integrity, air velocity, air patterns, air changes, and pressure differential. In particularly critical applications such as aseptic processing, a structured approach to physical risk assessment may be appropriate.

One such method has been developed by Ljundqvist and Reinmüller. This method, known as the L-R method, challenges the air ventilation system by evaluating both airflow and the ability of an environment to dilute and remove airborne particles. In the L-R method, a smoke generator allows analysts to visualize the air movements throughout a clean room or a controlled environment, including vortices or turbulent zones, and the airflow pattern can be fine-tuned to minimize these undesirable effects. Following visual optimization of airflow, particulate matter is generated close to the critical zone and sterile field. This evaluation is done under simulated production conditions but with equipment and personnel in place. This type of test can also be used to evaluate the ability of RABS and isolator systems, particularly around product exit ports in these systems, to resist the effects of contamination.

Visual evaluation of air movement within clean rooms is a subjective process. Complete elimination of turbulence or vortices is not possible in operationing clean rooms that contain personnel and equipment. Air visualization is simply one step in the effort to optimize clean room operations and is not a definitive pass/fail test, because acceptable or unacceptable conditions are not readily definable.

Proper testing and optimization of the physical characteristics of the clean room, RABS, or isolator are essential before implementation of the microbiological monitoring program. Assurance that the clean room, RABS, or isolator is in compliance with its predetermined engineering specifications provides confidence that the ability of the facility systems and operating practices to control the bioburden and nonviable particulate matter are appropriate for the intended use. These tests should be repeated during routine certification of the clean room or advanced aseptic processing systems, and whenever significant changes are made to the operation, such as personnel flow, equipment operation, material flow, air-handling systems, or equipment layout.

## TRAINING OF PERSONNEL

Good personnel performance plays an essential role in the control of contamination, proper training and supervision are central to contamination control. Aseptic processing is the most critical activity conducted in microbiological controlled environments, and manufacturers must pay close attention to details in all aspects of this endeavor. Rigorous discipline and strict supervision of personnel are essential in order to ensure a level of environmental quality appropriate for aseptic processing.

Training of all personnel working in controlled environments is critical. This training is equally important for personnel responsible for the microbial monitoring program, because contamination of the clean working area could inadvertently occur during microbial sampling. In highly automated operations, monitoring personnel may be the employees who have the most direct contact with the critical surfaces and zones within the processing area. Microbiological sampling has the potential to contribute to microbial contamination caused by inappropriate sampling techniques or by placing personnel in or near the critical zone. A formal training program is required to minimize this risk. This training should be documented for all personnel who enter controlled environments. Interventions should always be minimized, including those required for monitoring activities; but when interventions cannot be avoided, they must be conducted with aseptic technique that approaches perfection as closely as possible.

Management of the facility must ensure that personnel involved in operations in clean rooms and advanced aseptic processing environments are well versed in relevant microbiological principles. The training should include instruction about the basic principles of aseptic technique and should emphasize the relationship of manufacturing and handling procedures to potential sources of product contamination. Those supervising, auditing, or inspecting microbiological control and monitoring activities should be knowledgeable about the basic principles of microbiology, microbial physiology, disinfection and sanitation, media selection and preparation, taxonomy, and sterilization. The staff responsible for supervision and testing should have academic training in medical or environmental microbiology. Sampling personnel as well as individuals working in clean rooms should be knowledgeable about their responsibilities in minimizing the release of microbial contamination. Personnel involved in microbial identification require specialized training about required laboratory methods. Additional training about the management of collected data must be provided. Knowledge and understanding of applicable standard operating procedures are critical, especially those procedures relating to corrective measures taken when environmental conditions require. Understanding of contamination control principles and each individual's responsibilities with respect to good manufacturing practices (GMPs) should be an integral part of the training program, along with training in conducting investigations and in analyzing data.

The only significant sources of microbial contamination in aseptic environments are the personnel. Because operators disperse contamination and because the ultimate objective in aseptic processing is to reduce end-user risk, only healthy individuals should be permitted access to controlled environments. Individuals who are ill must not be allowed to enter an aseptic processing environment, even one that employs advanced aseptic technologies such as isolators, blow/fill/seal, or closed RABS.

The importance of good personal hygiene and a careful attention to detail in aseptic gowning cannot be overemphasized. Gowning requirements differ depending on the use of the controlled environment and the specifics of the gowning system itself. Aseptic processing environments require the use of sterilized gowns with the best available filtration properties. The fullest possible skin coverage is desirable, and sleeve covers or tape should be considered to minimize leaks at the critical glove–sleeve junction. Exposed skin should never be visible in conventional clean rooms under any conditions. The personnel and gowning considerations for RABS are essentially identical to those for conventional clean rooms.

Once employees are properly gowned, they must be careful to maintain the integrity of their gloves, masks, and other gown materials at all times. Operators who work with isolator systems are not required to wear sterilized clean-room gowns, but inadequate aseptic technique and employee-borne contamination are the principal hazards to safe aseptic operations in

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isolators, as well as RABS, and in conventional clean rooms. Glove-and-sleeve assemblies can develop leaks that can allow the mechanical transfer of microorganisms to the product. A second glove, worn either under or over the primary isolator/RABS glove, can provide an additional level of safety against glove leaks or can act as a hygienic measure. Also, operators must understand that aseptic technique is an absolute requirement for all manipulations performed with gloves within RABS and isolator systems.

The environmental monitoring program, by itself, cannot detect all events in aseptic processing that might compromise the microbiological quality of the environment. Therefore, periodic media-fill or process simulation studies are necessary, as is thorough ongoing supervision, to ensure that appropriate operating controls and training are effectively maintained.

## CRITICAL FACTORS IN THE DESIGN AND IMPLEMENTATION OF A MICROBIOLOGICAL ENVIRONMENTAL MONITORING PROGRAM

Since the advent of comprehensive environmental monitoring programs, their applications in capturing adverse trends or drifts has been emphasized. In a modern aseptic processing environment—whether an isolator, RABS, or conventional clean room—contamination has become increasingly rare. Nevertheless, a monitoring program should be able to detect a change from the validated state of control in a facility and to provide information for implementing appropriate countermeasures. An environmental monitoring program should be tailored to specific facilities and conditions. It is also helpful to take a broad perspective in the interpretation of data. A single uncorrelated result on a given day may not be significant in the context of the technical limitations associated with aseptic sampling methods.

### Selection of Growth Media

A general microbiological growth medium such as soybean–casein digest medium (SCDM) is suitable for environmental monitoring in most cases because it supports the growth of a wide range of bacteria, yeast, and molds. This medium can be supplemented with additives to overcome or to minimize the effects of sanitizing agents or of antibiotics. Manufacturers should consider the specific detection of yeasts and molds. If necessary, general mycological media such as Sabouraud's, modified Sabouraud's, or inhibitory mold agar can be used. In general, monitoring for strict anaerobes is not performed, because these organisms are unlikely to survive in ambient air. However, micro-aerophilic organisms may be observed in aseptic processing. Should anoxic conditions exist or if investigations warrant (e.g., identification of these organisms in sterility testing facilities or *Sterility Tests* ⟨71⟩ results), monitoring for micro-aerophiles and organisms that grow under low-oxygen conditions may be warranted. The ability of any media used in environmental monitoring, including those selected to recover specific types of organisms, must be evaluated for their ability to support growth, as indicated in ⟨71⟩.

### Selection of Culture Conditions

Time and incubation temperatures are set once the appropriate media have been selected. Typically, for general microbiological growth media such as SCDM, incubation temperatures in the ranges of approximately 20°–35° have been used with an incubation time of not less than 72 hours. Longer incubation times may be considered when contaminants are known to be slow growing. The temperature ranges given above are by no means absolute. Mesophilic bacteria and mold common to the typical facility environment are generally capable of growing over a wide range of temperatures. For many mesophilic organisms, recovery is possible over a range of approximately 20°. In the absence of confirmatory evidence, microbiologists may incubate a single plate at both a low and a higher temperature. Incubating at the lower temperature first may compromise the recovery of Gram-positive cocci that are important because they are often associated with humans.

Sterilization processes for preparing growth media should be validated. When selective media are used for monitoring, incubation conditions should reflect published technical requirements. Contamination should not be introduced into a manufacturing clean room as a result of using contaminated sampling media or equipment. Of particular concern is the use of aseptically prepared sampling media. Wherever possible, sampling media and their wrappings should be terminally sterilized by moist heat, radiation, or other suitable means. If aseptically prepared media must be used, analysts must carry out preincubation and visual inspection of all sampling media before introduction into the clean room. The reader is referred to *Microbiological Best Laboratory Practices* ⟨1117⟩ for further information regarding microbiology laboratory operations and control.

## ESTABLISHMENT OF SAMPLING PLAN AND SITES

During initial startup or commissioning of a clean room or other controlled environment, specific locations for air and surface sampling should be determined. Locations considered should include those in proximity of the exposed product, containers, closures, and product contact surfaces. In aseptic processing, the area in which containers, closures, and product are exposed to the environment is often called the *critical zone*—the critical zone is always ISO 5. For aseptic operations the entire critical zone should be treated as a sterile field. A nonsterile object, including the gloved hands of clean room personnel or an RABS/isolator glove, should never be brought into contact with a sterile product, container closure, filling station, or conveying equipment before or during aseptic processing operations. Operators and environmental monitoring personnel should never touch sterile parts of conveyors, filling needles, parts hoppers, or any other equipment that is in the product-delivery pathway. This means that surface monitoring on these surfaces is best done at the end of production operations.

The frequency of sampling depends on the manufacturing process conducted within an environment. Classified environments that are used only to provide a lower overall level of bioburden in nonsterile product manufacturing areas require relatively infrequent environmental monitoring. Classified environments in which closed manufacturing operations are

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conducted, including fermentation, sterile API processing, and chemical processes, require fewer monitoring sites and less frequent monitoring because the risk of microbial contamination from the surrounding environment is comparatively low. Microbiological monitoring of environments in which products are filled before terminal sterilization is generally less critical than the monitoring of aseptic processing areas. The amount of monitoring required in filling operations for terminal sterilization depends on the susceptibility of the product survival and the potential for proliferation of microbial contamination. The identification and estimated number of microorganisms that are resistant to the subsequent sterilization may be more critical than the microbiological monitoring of the surrounding manufacturing environments.

It is not possible to recommend microbial control levels for each type of manufacturing environment. The levels established for one ISO Class 7 environment, for example, may be inappropriate for another ISO Class 7 environment, depending on the production activities undertaken in each. The user should conduct a prospective risk analysis and develop a rationale for the sampling locations and frequencies for each controlled environment. The classification of a clean room helps establish control levels, but that does not imply that all rooms of the same classification should have the same control levels and the same frequency of monitoring. Monitoring should reflect the microbiological control requirements of manufacturing or processing activities. Formal risk assessment techniques can result in a scientifically valid contamination control program.

*Table 2* suggests frequencies of sampling in decreasing order of frequency and in relation to the criticality or product risk of the area being sampled. This table distinguishes between aseptic processing where personnel are aseptically gowned and those where a lesser gowning is appropriate. Environmental monitoring sampling plans should be flexible with respect to monitoring frequencies, and sample plan locations should be adjusted on the basis of the observed rate of contamination and ongoing risk analysis. On the basis of long-term observations, manufacturers may increase or decrease sampling at a given location or eliminate a sampling location altogether. Oversampling can be as deleterious to contamination control as undersampling, and careful consideration of risk and reduction of contamination sources can guide the sampling intensity.

**Table 2. Suggested Frequency of Sampling for Aseptic Processing Areas[a]**

| Sampling Area/Location | Frequency of Sampling |
|---|---|
| **Clean Room/RABS** | |
| *Critical zone (ISO 5 or better)* | |
| Active air sampling | Each operational shift |
| Surface monitoring | At the end of the operation |
| *Aseptic area adjacent critical zone* | |
| All sampling | Each operating shift |
| *Other nonadjacent aseptic areas* | |
| All sampling | Once per day |
| **Isolators** | |
| *Critical zone (ISO 5 or better)* | |
| Active air sampling | Once per day |
| Surface monitoring | At the end of the campaign |
| *Nonaseptic areas surrounding the isolator* | |
| All sampling | Once per month |

[a] All operators are aseptically gowned in these environments (with the exception of background environments for isolators). These recommendations do not apply to production areas for nonsterile products or other classified environments in which fully aseptic gowns are not donned.

## SELECTION OF SAMPLE SITES WITHIN CLEAN ROOMS AND ASEPTIC PROCESSING AREAS

ISO 14644 suggests a grid approach for the total particulate air classification of clean rooms. This approach is appropriate for certifying the total particulate air quality performance against its design objective. Grids may also have value in analyzing risk from microbial contamination, although in general, grids that have no personnel activity are likely to have low risk of contamination. Microbial contamination is strongly associated with personnel, so microbiological monitoring of unstaffed environments is of limited value.

Microbiological sampling sites are best selected with consideration of human activity during manufacturing operations. Careful observation and mapping of the clean room during the qualification phase can provide useful information concerning the movement and positioning of personnel. Such observation can also yield important information about the most frequently conducted manipulations and interventions.

The location and movement of personnel within the clean room correlate with contamination risk to the environment and to the processes conducted within that environment. Sample sites should be selected so that they evaluate the impact of personnel movement and work within the area, particularly interventions and manipulations within the critical zone.

The most likely route of contamination is airborne, so the samples most critical to risk assessment are those that relate to airborne contamination near exposed sterile materials. Other areas of concern are entry points where equipment and materials move from areas of lower classification to those of higher classification. Areas within and around doors and airlocks should be included in the monitoring scheme. It is customary to sample walls and floors, and indeed sampling at these locations can provide information about the effectiveness of the sanitization program. Sampling at these locations can take place relatively

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infrequently, because contamination there is unlikely to affect product. Operators should never touch floors and walls, so mechanical transmission of contamination from these surfaces to critical areas where product is exposed should not occur.

Manufacturers typically monitor surfaces within the critical zone, although this should be done only at the end of operations. Residues of media or diluent from wet swabs should be avoided on surfaces, because they could lead to microbial proliferation. Also, cleaning surfaces to remove diluent or media requires personnel intervention and movements that can result in release of microbial contamination into the critical zone and can disrupt airflow.

## MICROBIOLOGICAL CONTROL PARAMETERS IN CLEAN ROOMS, ISOLATORS, AND RABS

Since the early 1980s, manufacturers have established alert and action levels for environmental monitoring. In recent years the numerical difference between alert and action levels has become quite small, especially in ISO 5 environments. Growth and recovery in microbiological assays have normal variability in the range of ±0.5 $\log_{10}$. Studies on active microbiological air samplers indicate that variability of as high as tenfold is possible among commonly used sampling devices. As a result of this inherent variability and indeterminate sampling error, the supposed differences between, for example, an alert level of 1 cfu and an action level of 3 cfu are not analytically significant. Treating differences that are within expected, and therefore, normal ranges as numerically different is not scientifically valid and can result in unwarranted activities. In a practical sense, numerical values that vary by as much as five- to tenfold may not be significantly different.

Because of the limited accuracy and precision of microbial growth and recovery assays, analysts can consider the frequency with which contamination is detected rather than absolute numbers of cfu detected in any single sample. Also, a cfu is not a direct enumeration of microorganisms present but rather is a measure of contamination that may have originated from a clump of organisms.

Mean contamination recovery rates should be determined for each clean room environment, and changes in contamination recovery rate at a given site or within a given room may indicate the need for corrective action. Within the ISO 5 critical zone, airborne and surface contamination recovery rates of 1% or less should be attainable with current methods. Contamination recovery rates for closed RABS and isolator systems should be significantly lower still and can be expected to be <0.1%, on the basis of published monitoring results.

Contamination observed at multiple sites in an environment within a single sampling period may indicate increased risk to product and should be carefully evaluated. The appearance of contamination nearly simultaneously at multiple sites could also arise from poor sampling technique, so careful review is in order before drawing conclusions about potential loss of control. Resampling an environment several days after contamination is of little value, because the conditions during one sampling occasion may not be accurately duplicated during another.

Surface samples may also be taken from clean room garments. Personnel sampling should be emphasized during validation and is best done at the completion of production work in order to avoid adventitious contamination of the garments. In this case the average should be <1% for these sample sites as well. Gloves on closed RABS and isolators should meet the more rigorous expectation of <0.1% contamination recovery rates.

Because of the inherent variability of microbial sampling methods, contamination recovery rates are a more useful measure of trending results than is focusing on the number of colonies recovered from a given sample. *Table 3* provides recommended contamination recovery rates for aseptic processing environments. The incident rate is the rate at which environmental samples are found to contain microbial contamination. For example, an incident rate of 1% would mean that only 1% of the samples taken have any contamination regardless of colony number. In other words, 99% of the samples taken are completely free of contamination. Contamination recovery rates that are higher than those recommended in *Table 3* may be acceptable in rooms of similar classification that are used for lower-risk activities. Action should be required when the contamination recovery rate trends above these recommendations for a significant time.

**Table 3. Suggested Initial Contamination Recovery Rates in Aseptic Environments[a]**

| Room Classification | Active Air Sample (%) | Settle Plate (9 cm) 4 h Exposure (%) | Contact Plate or Swab (%) | Glove or Garment (%) |
|---|---|---|---|---|
| Isolator/Closed RABS (ISO 5 or better) | <0.1 | <0.1 | <0.1 | <0.1 |
| ISO 5 | <1 | <1 | <1 | <1 |
| ISO 6 | <3 | <3 | <3 | <3 |
| ISO 7 | <5 | <5 | <5 | <5 |
| ISO 8 | <10 | <10 | <10 | <10 |

[a] All operators are aseptically gowned in these environments (with the exception of background environments for isolators). These recommendations do not apply to production areas for nonsterile products or other classified environments in which fully aseptic gowns are not donned.

Detection frequency should be based on actual monitoring data and should be retabulated monthly. Action levels should be based on empirical process capability. If detection frequencies exceed the recommendations in *Table 3* or are greater than established process capability, then corrective actions should be taken. Corrective actions may include but are not limited to the following:

- Revision of the sanitization program, including selection of antimicrobial agents, application methods, and frequencies
- Increased surveillance of personnel practices, possibly including written critiques of aseptic methods and techniques
- Review of microbiological sampling methods and techniques

When higher-than-typical recovery levels for glove and garment contamination are observed, additional training for gowning practices may be indicated.

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## SIGNIFICANT EXCURSIONS

Excursions beyond approximately 15 cfu recovered from a single ISO 5 sample, whether from airborne, surface, or personnel sources, should happen very infrequently. When such ISO 5 excursions do occur, they may be indicative of a significant loss of control when they occur within the ISO 5 critical zone in close proximity to product and components. Thus, any ISO 5 excursion >15 cfu should prompt a careful and thorough investigation.

A key consideration for an abnormally high number of recovered colonies is whether this incident is isolated or can be correlated with other recoveries. Microbiologists should review recovery rates for at least two weeks before the incident of abnormally high recovery so that they can be aware of other recoveries that might indicate an unusual pattern. Microbiologists should carefully consider all recoveries, including those that are in the more typical range of 1–5 cfu. The identity of the organisms recovered is an important factor in the conduct of this investigation.

In the case of an isolated single excursion, establishing a definitive cause probably will not be possible, and only general corrective measures can be considered. It is never wise to suggest a root cause for which there is no solid scientific evidence. Also, there should be an awareness of the variability of microbial analysis. Realistically, there is no scientific reason to treat a recovery of 25 cfu as statistically different from a recovery of 15 cfu. A value of 15 cfu should not be considered significant in terms of process control, because realistically there is no difference between a recovery of 14 cfu and one of 15 cfu. Microbiologists should use practical scientific judgment in their approach to excursions.

## FURTHER CONSIDERATIONS ABOUT DATA INTERPRETATION

In the high-quality environments required for aseptic processing, detection frequency typically is low. As can be seen from the rates recommended in *Table 3*, the majority of samples taken in an aseptic processing area will yield a recovery of zero contamination. In the most critical areas within an aseptic processing operation, it is expected that less than 1% of the samples will yield any recoverable contamination. In the most advanced of modern aseptic operations that use separative technologies such as isolators or closed RABS, the recovery rate will approach zero at all times.

The microbiologist responsible for environmental control or sterility assurance should not take this to mean that the environmental quality approaches sterility. The sensitivity of any microbial sampling system in absolute terms is not known. In environmental monitoring, a result of zero means only that the result is below the limit of detection of the analytical system. A false sense of security should not be derived from the infrequency of contamination recovery in aseptic processing.

Sterility assurance is best accomplished by a focus on human-borne contamination and the facility design features that best mitigate risk from this contamination. Greatest risk mitigation can be attained by reducing or eliminating human interventions through proper equipment design and by providing sufficient air exchanges per hour for the intended personnel population of the facility. Other risk mitigation factors include effective personnel and material movement and the proper control of temperature and humidity. Secondary factors for risk mitigation include cleaning and sanitization. Risk analysis models that analyze processes prospectively to reduce human-borne contamination risk by minimizing operator interventions are more powerful tools for sterility assurance than monitoring. Environmental monitoring cannot prove or disprove in absolute terms the sterility of a lot of product. Environmental monitoring can only assure those responsible for a process that a production system is in a consistent, validated state of control. Care should be taken to avoid drawing inappropriate conclusions from monitoring results.

## SAMPLING AIRBORNE MICROORGANISMS

Among the most commonly used tools for monitoring aseptic environments are impaction and centrifugal samplers. A number of commercially available samplers are listed for informational purposes. The selection, appropriateness, and adequacy of using any particular sampler are the responsibility of the user.

### Slit-to-Agar Air Sampler (STA)

The unit is powered by an attached source of controllable vacuum. The air intake is obtained through a standardized slit below which is placed a slowly revolving Petri dish that contains a nutrient agar. Airborne particles that have sufficient mass impact the agar surface, and viable organisms are allowed to grow. A remote air intake is often used to minimize disturbance of unidirectional airflow.

### Sieve Impactor

This apparatus consists of a container designed to accommodate a Petri dish that contains a nutrient agar. The cover of the unit is perforated with openings of a predetermined size. A vacuum pump draws a known volume of air through the cover, and airborne particles that contain microorganisms impact the agar medium in the Petri dish. Some samplers feature a cascaded series of sieves that contain perforations of decreasing size. These units allow determination of the size range distribution of particulates that contain viable microorganisms based on the size of the perforations through which the particles landed on the agar plates.

### Centrifugal Sampler

The unit consists of a propeller or turbine that pulls a known volume of air into the unit and then propels the air outward to impact on a tangentially placed nutrient agar strip set on a flexible plastic base.

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### Sterilizable Microbiological Atrium

The unit is a variant of the single-stage sieve impactor. The unit's cover contains uniformly spaced orifices approximately 0.25 inch in size. The base of the unit accommodates one Petri dish containing a nutrient agar. A vacuum pump controls the movement of air through the unit, and a multiple-unit control center as well as a remote sampling probe are available.

### Surface Air System Sampler

This integrated unit consists of an entry section that accommodates an agar contact plate. Immediately behind the contact plate is a motor and turbine that pulls air through the unit's perforated cover over the agar contact plate and beyond the motor, where it is exhausted. Multiple mounted assemblies are also available.

### Gelatin Filter Sampler

The unit consists of a vacuum pump with an extension hose terminating in a filter holder that can be located remotely in the critical space. The filter consists of random fibers of gelatin capable of retaining airborne microorganisms. After a specified exposure time, the filter is aseptically removed and dissolved in an appropriate diluent and then plated on an appropriate agar medium to estimate its microbial content.

### Settling Plates

This method is still widely used as a simple and inexpensive way to qualitatively assess the environments over prolonged exposure times. Published data indicate that settling plates, when exposed for 4- to 5-hour periods, can provide a limit of detection for a suitable evaluation of the aseptic environment. Settling plates may be particularly useful in critical areas where active sampling could be intrusive and a hazard to the aseptic operation.

One of the major drawbacks of mechanical air samplers is the limited sample size of air being tested. When the microbial level in the air of a controlled environment is expected to contain extremely low levels of contamination per unit volume, at least 1 cubic meter of air should be tested in order to maximize sensitivity. Typically, slit-to-agar devices have an 80-L/min sampling capacity (the capacity of the surface air system is somewhat higher). If 1 cubic meter of air were tested, then it would require an exposure time of 15 min. It may be necessary to use sampling times in excess of 15 min to obtain a representative environmental sample. Although some samplers are reported to have high sampling volumes, consideration should be given to the potential for disruption of the airflow patterns in any critical area and to the creation of turbulence.

Technicians may wish to use remote sampling systems in order to minimize potential risks resulting from intervention by environmental samplers in critical zones. Regardless of the type of sampler used, analysts must determine that the extra tubing needed for a remote probe does not reduce the method's sensitivity to such an extent that detection of low levels of contamination becomes unlikely or even impossible.

## SURFACE SAMPLING

Another component of the microbial-control program in controlled environments is surface sampling of equipment, facilities, and personnel. The standardization of surface sampling methods and procedures has not been as widely addressed in the pharmaceutical industry as has the standardization of air-sampling procedures. Surface sampling can be accomplished by the use of contact plates or by the swabbing method.

Contact plates filled with nutrient agar are used for sampling regular or flat surfaces and are directly incubated for the appropriate time and temperature for recovery of viable organisms. Specialized agar can be used for the recovery of organisms that have specific growth requirements. Microbial estimates are reported per contact plate.

The swabbing method can be used to supplement contact plates for sampling of irregular surfaces, especially irregular surfaces of equipment. The area that will be swabbed is defined with a sterile template of appropriate size. In general, it is in the range of 24–30 $cm^2$. After sample collection the swab is placed in an appropriate diluent or transport medium and is plated onto the desired nutrient agar. The microbial estimates are reported per swab of defined sampling area.

Surface monitoring is used as an environmental assessment tool in all types of classified environments. In ISO 5 environments for aseptic processing, surface monitoring is generally performed beside critical areas and surfaces. Component hoppers and feed chutes that contact sterile surfaces on closures and filling needles can be tested for microbial contamination. Often in conventional staffed clean rooms, these product contact surfaces are steam sterilized and aseptically assembled. The ability of operators to perform these aseptic manipulations are evaluated during process stimulations or media fills, although true validation of operator technique in this manner is not possible. Surface monitoring on surfaces that directly contact sterile parts or product should be done only after production operations are completed. Surface sampling is not a sterility test and should not be a criterion for the release or rejection of product. Because these samples must be taken aseptically by personnel, it is difficult to establish with certainty that any contamination recovered is product related.

## CULTURE MEDIA AND DILUENTS

The type of medium, liquid or solid, used for sampling or plating microorganisms depends on the procedure and equipment used. Any medium used should be evaluated for suitability for the intended purpose. The most commonly used all-purpose solid microbiological growth medium is soybean–casein digest agar. As previously noted, this medium can be supplemented with chemicals that counteract the effect of various antimicrobials.

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## IDENTIFICATION OF MICROBIAL ISOLATES

A successful environmental control program includes an appropriate level of identification of the flora obtained by sampling. A knowledge of the flora in controlled environments aids in determining the usual microbial flora anticipated for the facility and in evaluating the effectiveness of the cleaning and sanitization procedures, methods, agents, and recovery methods. The information gathered by an identification program can be useful in the investigation of the source of contamination, especially when recommended detection frequencies are exceeded.

Identification of isolates from critical and immediately adjacent areas should take precedence over identification of microorganisms from noncritical areas. Identification methods should be verified, and ready-to-use kits should be qualified for their intended purpose.

## CONCLUSION

Environmental monitoring is one of several key elements required in order to ensure that an aseptic processing area is maintained in an adequate level of control. Monitoring is a qualitative exercise, and even in the most critical applications such as aseptic processing, conclusions regarding lot acceptability should not be made on the basis of environmental sampling results alone. Environments that are essentially free of human operators generally have low initial contamination rates and maintain low levels of microbial contamination. Human-scale clean rooms present a very different picture. Studies conclusively show that operators, even when carefully and correctly gowned, continuously slough microorganisms into the environment. Therefore, it is unreasonable to assume that samples producing no colonies, even in the critical zone or on critical surfaces, will always be observed. Periodic excursions are a fact of life in human-scale clean rooms, but the contamination recovery rate, particularly in ISO 5 environments used for aseptic processing, should be consistently low.

Clean-room operators, particularly those engaged in aseptic processing, must strive to maintain suitable environmental quality and must work toward continuous improvement of personnel operations and environmental control. In general, fewer personnel involved in aseptic processing and monitoring, along with reduction in interventions, reduces risk from microbial contamination.

## GLOSSARY

**Airborne Particulate Count (also referred to as *Total Particulate Count*):** The total number of particles of a given size per unit volume of air.

**Airborne Viable Particulate Count (also referred to as *Total Airborne Aerobic Microbial Count*):** The recovered number of colony-forming units (cfu) per unit volume of air.

**Air Changes:** The frequency per unit of time (minutes, hours, etc.) that the air within a controlled environment is replaced. The air can be recirculated partially or totally replaced.

**Air Sampler:** Devices or equipment used to sample a measured amount of air in a specified time to quantitate the particulate or microbiological status of air in the controlled environment.

**Aseptic:** Technically, the absence of microorganisms, but in aseptic processing this refers to methods and operations that minimize microbial contamination in environments where sterilized product and components are filled and/or assembled.

**Aseptic Processing:** An operation in which the product is assembled or filled into its primary package in an ISO 5 or better environment and under conditions that minimize the risk of microbial contamination. The ultimate goal is to produce products that are as free as possible of microbial contamination.

**Barrier System:** Physical barriers installed within an aseptic processing room to provide partial separation between aseptically gowned personnel and critical areas subject to considerable contamination risk. Personnel access to the critical zone is largely unrestricted. It is subject to a high level disinfection.

**Bioburden:** Total number and identity of the predominant microorganisms detected in or on an article.

**Clean Room:** A room in which the concentration of airborne particles is controlled to meet a specified airborne particulate cleanliness Class. In addition, the concentration of microorganisms in the environment is monitored; each cleanliness Class defined is also assigned a microbial level for air, surface, and personnel gear.

**Commissioning of a Controlled Environment:** Certification by engineering and quality control that the environment has been built according to the specifications of the desired cleanliness Class and that, under conditions likely to be encountered under normal operating conditions (or worst-case conditions), it is capable of delivering an aseptic process. Commissioning includes media-fill runs and results of the environmental monitoring program.

**Contamination Recovery Rate:** The contamination recovery rate is the rate at which environmental samples are found to contain any level of contamination. For example, an incident rate of 1% would mean that only 1% of the samples taken have any contamination regardless of colony number.

**Controlled Environment:** Any area in an aseptic process system for which airborne particulate and microorganism levels are controlled to specific levels, appropriate to the activities conducted within that environment.

**Corrective Action:** Actions to be performed that are according to standard operating procedures and that are triggered when certain conditions are exceeded.

**Critical Zone:** Typically the entire area where product and the containers and closures are exposed in aseptic processing.

**Detection Frequency:** The frequency with which contamination is observed in an environment. Typically expressed as a percentage of samples in which contamination is observed per unit of time.

**Environmental Isolates:** Microorganisms that have been isolated from the environmental monitoring program.

**Environmental Monitoring Program:** Documented program implemented via standard operating procedures that describes in detail the methods and acceptance criteria for monitoring particulates and microorganisms in controlled environments (air, surface, personnel gear). The program includes sampling sites, frequency of sampling, and investigative and corrective actions.

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**Equipment Layout:** Graphical representation of an aseptic processing system that denotes the relationship between and among equipment and personnel. This layout is used in the *Risk Assessment Analysis* to determine sampling site and frequency of sampling based on potential for microbiological contamination of the product/container/closure system. Changes must be assessed by responsible managers, since unauthorized changes in the layout for equipment or personnel stations could result in increase in the potential for contamination of the product/container/closure system.

**Isolator for Aseptic Processing:** An aseptic isolator is an enclosure that is over-pressurized with HEPA filtered air and is decontaminated using an automated system. When operated as a closed system, it uses only decontaminated interfaces or rapid transfer ports (RTPs) for materials transfer. After decontamination they can be operated in an open manner with the ingress and/or egress of materials through defined openings that have been designed and validated to preclude the transfer of contamination. It can be used for aseptic processing activities or for asepsis and containment simultaneously.

**Material Flow:** The flow of material and personnel entering controlled environments should follow a specified and documented pathway that has been chosen to reduce or minimize the potential for microbial contamination of the product/closure/container systems. Deviation from the prescribed flow could result in increase in the potential for microbial contamination. Material/personnel flow can be changed, but the consequences of the changes from a microbiological point of view should be assessed by responsible managers and must be authorized and documented.

**Media Fill:** Microbiological simulation of an aseptic process by the use of growth media processed in a manner similar to the processing of the product and with the same container/closure system being used.

**Media Growth Promotion:** Procedure that references *Growth Promotion Test of Aerobes, Anerobes, and Fungi* in *Sterility Tests* ⟨71⟩ to demonstrate that media used in the microbiological environmental monitoring program, or in *media-fill* runs, are capable of supporting growth of indicator microorganisms and of environmental isolates from samples obtained through the monitoring program or their corresponding ATCC strains.

**Product Contact Areas:** Areas and surfaces in a controlled environment that are in direct contact with either products, containers, or closures and the microbiological status of which can result in potential microbial contamination of the product/container/closure system.

**Restricted Access Barrier System (RABS):** An enclosure that relies on HEPA filtered air over-spill to maintain separation between aseptically gowned personnel and the operating environment. It is subject to a high level of disinfection prior to use in aseptic process. It uses decontaminated (where necessary) interfaces or RTPs for materials transfer. It allows for the ingress and/or egress of materials through defined openings that have been designed and validated to preclude the transfer of contamination. If opened subsequent to decontamination, its performance capability is adversely impacted.

**Risk Assessment Analysis:** Analysis of the identification of contamination potentials in controlled environments that establish priorities in terms of severity and frequency and that will develop methods and procedures that will eliminate, reduce, minimize, or mitigate their potential for microbial contamination of the product/container/closure system.

**Sampling Plan:** A documented plan that describes the procedures and methods for sampling a controlled environment; identifies the sampling sites, the sampling frequency, and number of samples; and describes the method of analysis and how to interpret the results.

**Sampling Sites:** Documented geographical location, within a controlled environment, where sampling for microbiological evaluation is taken. In general, sampling sites are selected because of their potential for product/container–closure contacts.

**Standard Operating Procedures:** Written procedures describing operations, testing, sampling, interpretation of results, and corrective actions that relate to the operations that are taking place in a controlled environment and auxiliary environments. Deviations from standard operating procedures should be noted and approved by responsible managers.

**Sterile or Aseptic Field:** In aseptic processing or in other controlled environments, it is the space at the level of or above open product containers, closures, or product itself, where the potential for microbial contamination is highest.

**Sterility:** Within the strictest definition of sterility, an article is deemed sterile when there is complete absence of viable microorganisms. *Viable*, for organisms, is defined as having the capacity to reproduce. Absolute sterility cannot be practically demonstrated because it is technically unfeasible to prove a negative absolute. Also, absolute sterility cannot be practically demonstrated without testing every article in a batch. Sterility is defined in probabilistic terms, where the likelihood of a contaminated article is acceptably remote.

**Swabs for Microbiological Sampling:** Devices used to remove microorganisms from irregular or regular surfaces for cultivation to identify the microbial population of the surface. A swab is generally composed of a stick with an absorbent tip that is moistened before sampling and is rubbed across a specified area of the sample surface. The swab is then rinsed in a sterile solution to suspend the microorganisms, and the solution is transferred to growth medium for cultivation of the microbial population.

**Trend Analysis:** Data from a routine microbial environmental monitoring program that can be related to time, shift, facility, etc. This information is periodically evaluated to establish the status or pattern of that program to ascertain whether it is under adequate control. A trend analysis is used to facilitate decision-making for requalification of a controlled environment or for maintenance and sanitization schedules.

## APPENDIX

### Additional Resources

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# ⟨1117⟩ MICROBIOLOGICAL BEST LABORATORY PRACTICES

***Add the following:***

▲INTRODUCTION
MEDIA PREPARATION AND QUALITY CONTROL
- Media Preparation
- Media Storage
- Quality Control Testing

MICROBIOLOGICAL MEDIA INCUBATION TIMES
MAINTENANCE OF MICROBIOLOGICAL CULTURES
LABORATORY EQUIPMENT
USING MODERN TECHNOLOGIES
LABORATORY LAYOUT AND OPERATIONS
SAMPLE HANDLING
INCUBATION TEMPERATURE EXCURSIONS
COMPETENCIES AND TRAINING OF PERSONNEL
QUALIFICATION OF ANALYSTS
CONSIDERATIONS FOR MICROBIOLOGICAL RISK ASSESSMENTS
LABORATORY RESOURCES
- Oversight of Contract Laboratories
- Oversight of Suppliers

METHOD TRANSFER
DOCUMENTATION
MAINTENANCE OF LABORATORY RECORDS
QUANTITATIVE MICROBIOLOGY
DATA INTEGRITY OF MICROBIOLOGICAL DATA
INTERPRETATION OF ASSAY RESULTS
- Use of Negative Controls
- Investigations

REFERENCES▲ (USP 1-Aug-2022)

***Change to read:***

## INTRODUCTION

Good ▲▲ (USP 1-Aug-2022) practices in a microbiology laboratory consist of activities that depend on several principles: aseptic technique, control of media, control of test strains, operation and control of equipment, diligent recording and evaluation of data, and training of the laboratory staff ▲in related competencies.▲ (USP 1-Aug-2022) Because of the inherent risk of variability in microbiology data, reliability and reproducibility are dependent on the use of accepted methods and adherence to good ▲practices in the laboratory. This chapter does not cover the unique and specific laboratory activities for virology and mycoplasma recovery and detection.▲ (USP 1-Aug-2022)

***Change to read:***

## MEDIA PREPARATION AND QUALITY CONTROL

### Media Preparation

Culture media are the basis for most microbiological tests. ▲Culture media may be either prepared in-house or obtained commercially. The term "prepared media" used in this chapter covers both these types of media.▲ (USP 1-Aug-2022) Safeguarding the quality of the media is therefore critical to the success of the microbiology laboratory. Media preparation, proper storage, and quality control testing can ensure a consistent supply of high-quality media.

It is important to choose the correct media or components in making media based on the use of accepted sources or references for formulas. The manufacturer's formula and instructions for preparation routinely accompany dehydrated and ready-made media. Because different media types may have different preparation requirements (e.g., heating, additives, and pH adjustment), it is important to follow these instructions to ensure preparation of acceptable media quality. A certificate of analysis describing expiration dating and recommended storage conditions accompanies ready-made media, as well as the quality control organisms used in growth-promotion ▲testing, inhibitory and indicative property testing, as appropriate, and the results of performance testing with acceptance criteria for that media. For in-house prepared media, similar quality control criteria should be established.▲ (USP 1-Aug-2022)

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Water is the universal diluent for microbiological media. *Purified Water* is most often used for media preparation, but in certain cases the use of deionized or distilled water may be appropriate. Water of lesser quality should not be used for microbiological media preparation. The volume of the water used should be recorded.

Consistent preparation of media requires accurate weighing of dehydrated media or media constituents. A calibrated balance with the appropriate weight range for the ingredients should be used (see *Weighing on an Analytical Balance* ⟨1251⟩). Clean weighing containers and tools (such as spatulas) should be used to prevent foreign substances from entering the formulation. The weight of the components should be recorded.

Dehydrated media should be thoroughly dissolved in water before dispensing and sterilization. If heating is necessary to help dissolve the media, care should be taken not to overheat the media because all culture media, to a greater or lesser extent, are heat-sensitive. Equipment used in the preparation of media should be appropriate to allow for controlled heating, constant agitation, and mixing of the media. Darkening of media (Maillard-type reaction or nonenzymatic browning) is a general indication of overheating. When adding required supplements to media, adequate mixing of the medium after adding the supplement should be performed.

Preparation of media in poorly cleaned glassware can allow inhibitory substances to enter the media. Inhibitory substances can come from detergent residue after cleaning glassware or from prior materials used in the glassware. Be sure that the cleaning process removes debris and foreign matter, and that the detergent is thoroughly rinsed out with *Purified Water*. See *Cleaning Glass Apparatus* ⟨1051⟩ for additional guidance.

Sterilization of media should be performed within the parameters provided by the manufacturer or validated by the user. Commercially prepared media should provide documentation of the sterilization method used. Autoclaving by moist heat is the preferred sterilization technique, except in instances when boiling is required in order to avoid deterioration of heat-labile components of the media. Sterilization by filtration may also be appropriate for some formulations.

The effects of the sterilization method and conditions on the media should be validated by sterility and growth-promotion testing of the media. In addition, if sterilized by moist heat, the autoclave cycle should be validated to ensure proper heat distribution for selected loads and volumes. Typically, manufacturers recommend an autoclave cycle of 121° for 15 min using a validated autoclave. These conditions apply to time at temperature of the media. As container size and the load configuration of the autoclave will influence the rate of heating, longer cycles may be required for larger loads.▲▲ (USP 1-Aug-2022) Sterilization time will be dependent on the media volume and autoclave load. Sterilization cycles in which the autoclave is slow to come up ▲or come down▲ (USP 1-Aug-2022) to temperature may result in overheating of the media. Therefore, care must be taken to validate a sterilization cycle, balancing the need for sterile media against the tendency of the media to degrade under excessive heating. ▲Holding▲ (USP 1-Aug-2022) of the media in the autoclave after the liquid ▲or slow exhaust cycle▲ (USP 1-Aug-2022) is completed is not recommended after cooling, as it may damage the media. Improper heating or sterilizing conditions—for commercially prepared or internally prepared media—may result in a difference in color,▲▲ (USP 1-Aug-2022) loss of clarity, altered gel strength, or pH drift from the manufacturer's recommended range, as well as reduced growth-promotion activity and/or selectivity. ▲All sterilization cycle parameters should be documented.

Unless it is specified to confirm the pH of the medium prior to the sterilization process,▲ (USP 1-Aug-2022) the pH of each batch of medium should be confirmed after it has cooled to room temperature (20°–25°) by aseptically withdrawing a sample for testing. Refrigerated purchased media should be allowed to warm up to ambient room temperature if it is to be checked for pH confirmation. A flat pH probe is recommended for agar surfaces, and an immersion probe is recommended for liquids. See *pH* ⟨791⟩ for guidance with pH measurement and instrument calibration. The pH of media should be in a range of ±0.2 of the value indicated by the manufacturer unless a wider range is acceptable by the validated method.

Prepared media should be checked by appropriate inspection of plates and tubes for the following ▲quality and integrity parameters:▲ (USP 1-Aug-2022)

- Cracked containers or lids
- Unequal filling of containers
- Dehydration resulting in cracks or dimpled surfaces on solid media
- Hemolysis
- Excessive darkening or color change
- Crystal formation from possible freezing
- Excessive number of bubbles
- ▲▲ (USP 1-Aug-2022)
- Status of redox indicators (if appropriate)
- Lot number and expiration date▲▲ (USP 1-Aug-2022)
- Sterility▲▲ (USP 1-Aug-2022)
- Cleanliness of plates (lid should not stick to dish)

## Media Storage

It is prudent to ▲understand▲ (USP 1-Aug-2022) how the manufacturer or supplier transports and stores media before distribution to the end user. Manufacturers of media should use transport and storage conditions that minimize the loss of moisture, control the temperature, prevent microbial contamination, and provide mechanical protection to the prepared media.

Media should be labeled properly with batch or lot numbers, preparation and expiration dates, and media identification. Media should be stored according to the manufacturer's instructions. Media prepared in-house should be stored under validated conditions. Do not store ▲media plates containing▲ (USP 1-Aug-2022) agar at or below 0°, as freezing could damage the gel structure. Protect stored media from exposure to light, excessive temperature, ▲and temperature changes, *as this may cause condensation*. Consideration should be given to storing agar plates in▲ (USP 1-Aug-2022) a sealed package or container to ▲prevent▲ (USP 1-Aug-2022) moisture loss ▲from the plates.▲ (USP 1-Aug-2022)

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Remelting of an original container of solid media, ▲if not containing heat-labile components (*1*),▲ (USP 1-Aug-2022) should be performed only once to avoid media whose quality is compromised by overheating or potential contamination. It is recommended that remelting be performed in a heated water bath or ▲by other suitable methods that will not compromise media quality.▲ (USP 1-Aug-2022) The use of microwave ovens and heating plates is common, but care should be taken to avoid damaging media by overheating and to avoid the potential injury to laboratory personnel from glass breakage and burns. ▲It is recommended that▲ (USP 1-Aug-2022) molten agar medium be held in a monitored water bath ▲until it has been tempered to▲ (USP 1-Aug-2022) 45°–50°, ▲and not further held▲ (USP 1-Aug-2022) for ▲▲ (USP 1-Aug-2022) more than 8 h, ▲or as determined appropriate for the medium composition.▲ (USP 1-Aug-2022) Caution should be taken when pouring the media from a container immersed in a water bath to prevent water from the bath commingling with the poured sterile media. ▲Careful wiping of▲ (USP 1-Aug-2022) the exterior of the container dry before pouring ▲is recommended.▲ (USP 1-Aug-2022)

Disposal of used cultured media (as well as expired media) should follow local biological hazard safety procedures.

## Quality Control Testing

Although growth media can be prepared in a laboratory from individual components, many laboratories, for ▲convenience,▲ (USP 1-Aug-2022) use dehydrated media or purchase commercially prepared media in plastic plates or glass containers. Manufacturers of media attempt to standardize raw materials from biological sources but must constantly deal with unavoidable differences in raw materials obtained from natural sources, and therefore lot-to-lot variability of media must be considered. In addition, the performance of media prepared in a laboratory or by a manufacturer is highly dependent on preparation and storage conditions. Improper media preparation can cause unsatisfactory conditions for microbial growth or recovery and unreliable results.

Therefore, quality control tests should be performed on all prepared media, including media associated with swabs or media in strips ▲▲ (USP 1-Aug-2022). Tests routinely performed on in-house prepared media should include pH, growth promotion, inhibition, and indicative properties (as appropriate). Periodic stability checks to confirm the expiration dating ▲are recommended only until media expiry has been validated.

When media has not been terminally sterilized in its final container and secondary packaging (e.g., irradiated plates of media), an inspection for absence of microbial contamination is recommended for plated or liquid media in tubes to prevent unintended use of contaminated media. The sterility inspection can be performed on a representative sample of the specific media-and-container combination(s) on the basis of the risk of impact on its use in microbiological control testing. The intent of the inspection should be to detect evidence of growth in the relevant medium, such as turbidity in liquid broth media or colonies forming on plated media. Similar to the practice of pre-incubation of non-irradiated, plated media before use in an aseptic manufacturing area, media incubation should be performed at the same temperature range and for at least the maximum time period for which the media is to be incubated in the test (e.g., if incubation time is 3–5 days in the test, the minimum incubation time should be 5 days).

Every in-house sterilized batch of media should be tested for growth promotion.▲ (USP 1-Aug-2022) Test organisms may be selected from the appropriate compendial test chapter. In addition, microorganisms used in growth-promotion testing may be based on the manufacturer's recommendation for a particular medium or may include representative environmental isolates (but these latter ▲isolates▲ (USP 1-Aug-2022) are not to be construed as compendial requirements).

▲It is commonly stated that a satisfactory growth-promotion outcome includes growth that is visibly comparable to that obtained with a previously tested and approved batch of medium. This is for a qualitative comparison; the comparison medium should be of the same formula, consistency, and volume. If quantitative comparison is desired, follow guidance in *Validation of Microbial Recovery from Pharmacopeial Articles* ⟨1227⟩; the medium should be of the same formula, consistency, and volume. For qualitative and quantitative comparison, direct physical comparison with a previously tested batch is not necessary. Numerical results comparison in quantitative testing should be appropriate for the intended purpose, with adequate knowledge of microbiological growth variability (i.e., factor of 2).▲ (USP 1-Aug-2022)

Expiration dates on media should have supporting growth-promotion testing to indicate that the performance of the media still meets acceptance criteria up to and including the expiration date. The length of shelf life of a batch of media will depend on the stability of the ingredients and formulation under specified conditions, as well as the type of container and closure.

When a batch of media does not meet the requirements of growth-promotion testing, an investigation should be initiated to identify the cause. This investigation should include a corrective action plan to prevent the recurrence of the problem. Any batch of media that fails growth-promotion testing is unsuitable for use. [NOTE—Failed growth-promotion test results may not be used to negate positive test results.]

Some reagents are used for diagnostic purposes to help support identification of ▲microorganisms,▲ (USP 1-Aug-2022) e.g., Gram stain, ▲▲ (USP 1-Aug-2022) oxidase, ▲and coagulase test▲ (USP 1-Aug-2022) reagents. These may have attributes that can be quality control tested similar to microbiological media. Select the correct quality control standard microorganisms, following the manufacturer's instructions, and perform the testing before unknown sample diagnostic testing. All relevant diagnostic reagents should be subjected to incoming quality confirmation before use.

Special care should be taken with media that are used in sterility tests (see *Sterility Tests* ⟨71⟩ for requirements) and in environmental monitoring studies. Media used for environmental monitoring of critical areas should preferably be double wrapped and terminally sterilized. If terminal sterilization is not performed, media should be subjected to 100% pre-incubation and inspection before use within a critical area. [NOTE—Growth-promotion testing for this media ▲should▲ (USP 1-Aug-2022) be performed ▲to qualify that▲ (USP 1-Aug-2022) pre-incubation ▲does not impact recovery.▲ (USP 1-Aug-2022)] This will prevent extraneous contamination from being carried into controlled environments and will prevent false-positive results. A raised agar level for surface contact plates should be verified.

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***Add the following:***

## ▲MICROBIOLOGICAL MEDIA INCUBATION TIMES

Incubation times for microbiological tests with a duration of less than 3 days should be expressed in hours: e.g., "Incubate at 30°–35° for 18–72 h". Tests with a duration longer than 72 h should be expressed in days: e.g., "Incubate at 30°–35° for 3–5 days". For tests with incubation times expressed in hours, incubate for the minimum specified time and exercise good microbiological judgment when exceeding that time. For tests with incubation times expressed in days, incubations started in the morning or afternoon should generally be concluded at that same time of day *in the morning or afternoon, respectively.*▲ (USP 1-Aug-2022)

***Change to read:***

## MAINTENANCE OF MICROBIOLOGICAL CULTURES

Biological specimens can be the most delicate standards to manage because their viability and characteristics are dependent on adequate handling and storage. Standardizing the handling and storage of cultures by the user laboratory should be done in a way that will minimize the opportunity for contamination or alteration of growth characteristics. The careful and consistent treatment of stock cultures is critically important to the consistency of microbiological test results. Cultures for use in compendial tests should be acquired from a national culture collection or a qualified secondary supplier ▲and have documented equivalency to relevant ATCC strains (*2*).▲ (USP 1-Aug-2022) They can be acquired frozen, freeze-dried, on slants, or in ready-to-use forms. Confirmation of the purity of the culture and the identity of the culture should be performed before its use in quality control testing. Ready-to-use cultures should be subjected to incoming testing for purity and identity before use. The confirmation of identity for commonly used laboratory strains ideally should be done at the level of genus and species.

Preparation and resuscitation of cultures should follow the instructions of the supplier or a validated, established method. The "seed-lot technique" is recommended for storage of stock cultures.

The original sample from the national culture collection or a qualified secondary supplier is resuscitated and grown in an appropriate medium. Aliquots of this stock culture (the first transfer or passage) are suspended in a cryoprotective medium, transferred to vials, and frozen at –30° or below until use. If stored at –70° ▲or below,▲ (USP 1-Aug-2022) or in lyophilized form, strains may be kept indefinitely. These frozen stocks can then be used to inoculate monthly or weekly working cultures. Once opened, do not refreeze unused cell suspensions after culturing a working suspension. The unused portion should be discarded to minimize the risk of loss of viability and contamination of the stock.

The number of transfers of working control cultures should be tracked to prevent excessive subculturing that increases the risk of phenotypic alteration or mutation. The number of transfers allowable for specific compendial tests may be specified in that test. One passage is defined as the transfer of organisms from a viable culture to a fresh medium with growth of the microorganisms. Any form of subculturing is considered to be a transfer/passage.

***Change to read:***

## LABORATORY EQUIPMENT

Most equipment (incubators, water baths, and autoclaves) are subject to standard validation practices of ▲installation▲ (USP 1-Aug-2022) qualification, operational qualification, and performance qualification. ▲Some useful guidance can be found in *Analytical Instrument Qualification* ⟨1058⟩.▲ (USP 1-Aug-2022)Additionally, periodic calibration (generally annually ▲or on a frequency based on a risk assessment▲ (USP 1-Aug-2022)) is commonly required. New equipment, critical to the operation of the laboratory, should be qualified according to a protocol approved by the quality assurance unit (QAU). In addition, regular cleaning and sanitization of equipment such as incubators, refrigerators, and water baths should be performed to minimize the potential for contamination in the laboratory. Door seals of incubators and refrigerators should be cleaned and checked for state of repair. ▲These activities should be incorporated into the equipment preventative maintenance program.▲ (USP 1-Aug-2022)

Instruments (pH meters and spectrophotometers) used in a microbiology laboratory should be calibrated on a regular schedule and tested to verify performance on a routine basis. The frequency of calibration and performance verification will vary based on the type of instrument and the importance of that equipment to the generation of data in the laboratory. ▲Laboratory equipment with software should be evaluated for maintaining data integrity (*Code of Federal Regulations Title 21* (21 CFR), Part 11).▲ (USP 1-Aug-2022)

Equipment that is difficult to sanitize (such as refrigerators and incubators) should be ▲segregated from▲ (USP 1-Aug-2022) aseptic operations (such as storage of media for testing and incubation of sterility test samples) and live culture operations to minimize the potential for inadvertent contamination of the tests.

Autoclaves are central to the operation of the laboratory and must have proper validation in place to demonstrate adequate sterilization for a variety of operations. Autoclave resources must be available (and validated) to sterilize waste media (if performed in that laboratory) as well as the media prepared in that laboratory. The choice of one or several autoclaves is not driven by a need to separate aseptic and live operations (everything in the properly maintained autoclave is sterile after the cycle), but rather driven by resource considerations (see below).

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***Add the following:***

## ▲USING MODERN TECHNOLOGIES

The use of new technologies in a microbiology laboratory requires new learning and training to ensure a proper implementation in the laboratory (for examples, see *Validation of Alternative Microbiological Methods* ⟨1223⟩, *Microbial Characterization, Identification, and Strain Typing* ⟨1113⟩), and *Rapid Microbial Tests for Release of Sterile Short-Life Products: A Risk-Based Approach* ⟨1071⟩. In addition to the laboratory specialists, the laboratory supervisors or quality assurance personnel evaluating qualification of systems or deviations must also have the skills to analyze and interpret the complex data that may be generated by new techniques. For each new technology, it is valuable to develop rules and principles that will build consistency and accuracy when laboratory personnel are using that technology to perform the relevant procedure. The rules and principles should be included in the written procedure.▲ (USP 1-Aug-2022)

***Change to read:***

## LABORATORY LAYOUT AND OPERATIONS

Laboratory layout and design should carefully consider the requirements of good ▲▲ (USP 1-Aug-2022) practices ▲in a microbiology laboratory▲ (USP 1-Aug-2022) and laboratory safety. It is essential that cross-contamination of microbial cultures ▲or DNA/RNA samples for PCR testing▲ (USP 1-Aug-2022) be minimized to the greatest extent possible, and it is also important that microbiological samples be handled in an environment that makes contamination highly unlikely.

In general, a laboratory should be divided into clean or aseptic areas and live culture areas. Areas in which environmental ▲and other microbiological test▲ (USP 1-Aug-2022) samples are handled and incubated should be maintained completely free of live cultures, if possible. If complete separation of live and clean culture zones cannot be accomplished, then other barriers and aseptic practices should be employed to reduce the likelihood of accidental contamination. These barriers include protective clothing, sanitization and disinfection procedures, and ▲containment by▲ (USP 1-Aug-2022) biological safety cabinets designated for clean or aseptic operations only. Procedures for handling spills or mishaps with live cultures ▲or DNA/RNA▲ (USP 1-Aug-2022) should be in place, and all relevant technical personnel should be trained regarding these methods.

Some samples will demonstrate microbial growth and require further laboratory analysis to identify the contaminants. When growth is detected, the sample should be taken from the clean section of the laboratory to the live culture section without undue delay. Subculturing, staining, microbial identification, or other investigational operations should be undertaken in the live culture section of the laboratory. If possible, any sample found to contain growing colonies should not be opened in the clean zone of the laboratory. Careful segregation of contaminated samples and materials will reduce false-positive results.

Staff engaged in sampling activities should not enter or work in the live culture handling section of a laboratory unless special precautions are taken, including wearing ▲dedicated▲ (USP 1-Aug-2022) protective clothing ▲that should not be worn outside the laboratory (e.g., coats)▲ (USP 1-Aug-2022) and gloves and carefully ▲washing and▲ (USP 1-Aug-2022) sanitizing hands upon exiting. Ideally, staff assigned to sampling activities, particularly those in support of aseptic processing, should not work in the vicinity of live culture laboratory operations.

It is important to consider that microbial contamination of samples, which leads to false-positive results, is always possible unless careful aseptic precautions are taken. Facilities should be designed so that raw material and excipient sampling can be done under controlled conditions, including proper gowning and the use of sterilized sampling equipment. It may not always be possible to sample utility systems, such as water systems, under full aseptic conditions; however, it should be noted that when samples are not taken aseptically, their reliability is inevitably compromised.

Environmental sampling methods should require minimal aseptic handling in loading and unloading sampling instruments. Whenever possible, sampling equipment should be loaded with its microbiological recovery media in the environment that is to be sampled.

All testing in laboratories used for critical testing procedures—such as sterility testing of final dosage forms, bulk product, seed cultures for biological production or ▲PCR testing of▲ (USP 1-Aug-2022) cell cultures used in biological production—should be performed under controlled conditions. ▲Sterility tests should preferably be carried out in an isolator with ISO 5 classification.▲ (USP 1-Aug-2022) Isolators have been shown to have lower levels of environmental contamination than manned clean rooms and, therefore, are generally less likely to produce false-positive results. Proper validation of isolators is critical both to ensure environmental integrity and to prevent the possibility of false-negative results due to chemical disinfection of materials brought into or used within isolators (see *Sterility Testing—Validation of Isolator Systems* ⟨1208⟩). ▲Adequate disinfection of materials prior to loading a sterility test isolator is another important step to reduce the possibility of false-positive results.

For less-critical microbiological testing, such as with nonsterile product or intermediate bioburden samples, testing under a unidirectional airflow (UDAF) cabinet, or as applicable is preferable. During qualification, the UDAF air must meet the air quality grade for which it is being qualified. In addition to the qualification, the cabinet's environment (air and surfaces) may be tested periodically. The testing frequency and levels defined would depend on the microbiological grade of the product tested, the quality of air that can be delivered by the UDAF design and filters, the fact that nonsterile material may be present in the UDAF and the cleaning/disinfection regime.▲ (USP 1-Aug-2022)

***Change to read:***

## SAMPLE HANDLING

Viable microorganisms in most microbiology samples—particularly water, environmental monitoring, and bioburden samples—are sensitive to handling and storage conditions. Critical parameters in these conditions include product (or sample)

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composition, container composition, time of storage, and temperature of storage. Therefore, it is important to minimize the amount of time between the sampling event and the initiation of testing and to control, as much as possible, the conditions of storage. ▲Microbiological samples should never be stored in a frozen state before testing because this practice leads to loss of cell viability.▲ (USP 1-Aug-2022) If the sample is to be transported to a distant location for testing, then the conditions of transport (time, temperature, etc.) should be qualified as suitable for that test and sample. ▲For example, when monitoring water or bioburden prior to bioburden-reducing steps or sterilizing steps, samples may be held at 2°–8° for up to 24 h from the time of sample collection until the start of the analysis (*3*). When testing within 24 h is not possible (e.g., when contract laboratories are used), the actual maximum hold time between collection and testing should be supported by experimental studies (e.g., challenge tests). For bacterial endotoxin testing, studies may be performed to demonstrate that endotoxins present in the product and stored in the original container for a certain time may be adequately recovered. Storage conditions (e.g., 2°–8°) in the laboratory should apply. Highly purified lipopolysaccharides (LPS) such as reference standard endotoxins (RSE) or control standard endotoxins (CSE) might not be the most relevant endotoxin indicators for such studies and use of laboratory-derived endotoxin indicators would provide a more realistic assessment as discussed in *Endotoxin Indicators for Depyrogenation* ⟨1228.5⟩.

If environmental monitoring samples cannot be incubated within a reasonable time frame (e.g., due to the use of an off-site testing laboratory), the time from sample collection to the start of incubation should be supported with experimental data.

All samples should be examined and the tests completed prior to release, whenever possible.▲ (USP 1-Aug-2022)

Product mixing before sampling may need to be evaluated and applied ▲▲ (USP 1-Aug-2022) to ensure ▲adequate dispersal▲ (USP 1-Aug-2022) and representation in the sample aliquot.

All microbiological samples should be taken using aseptic techniques, including those taken in support of nonsterile products. If possible, all microbiological samples should be taken under full aseptic conditions in specialized sampling areas. The areas ▲and methods of transport of samples should be designed▲ (USP 1-Aug-2022) to minimize contamination▲▲ (USP 1-Aug-2022).

Samples submitted to the microbiology laboratory should be accompanied by documentation detailing source of the sample, date the sample was taken, date of sample submission, person or department responsible for the submission, ▲storage conditions,▲ (USP 1-Aug-2022) and any potentially hazardous materials associated with the sample. The testing department should acknowledge receipt of the sample and reconcile the identity and number of samples as part of this sample documentation.

***Delete the following:***

## ▲MICROBIOLOGICAL MEDIA INCUBATION TIMES

Incubation times for microbiological tests of less than 3 days' duration should be expressed in hours: e.g., "Incubate at 30° to 35° for 18 to 72 hours". Tests longer than 72 hours' duration should be expressed in days: e.g., "Incubate at 30° to 35° for 3 to 5 days". For incubation times expressed in hours, incubate for the minimum specified time, and exercise good microbiological judgment when exceeding the incubation time. For incubation times expressed in days, incubations started in the morning or afternoon should generally be concluded at that same time of day.▲ (USP 1-Aug-2022)

***Add the following:***

## ▲INCUBATION TEMPERATURE EXCURSIONS

Incubation equipment qualification and ongoing calibration provide the acceptable range for temperature conditions relative to the expected procedural incubation temperatures or range. When an excursion occurs outside of the procedural incubation temperature(s), an assessment should be performed to determine the potential impact on the test samples and what, if any, action should be taken with the test samples. The effect of a higher or lower temperature on microbial recovery from the test samples for the recorded period of time should be evaluated. An investigation of the excursion should be documented along with the disposition of the test samples. For instance, a slightly lower temperature excursion (e.g., 1°–2°) for a brief time could be compensated with an increase in incubation time; or, for a higher temperature excursion (e.g., greater than 40° for a total aerobic microbial count or greater than 30° for a total yeasts and molds count) consider any negative impact on recovery.▲ (USP 1-Aug-2022)

***Change to read:***

## ▲COMPETENCIES AND▲ (USP 1-AUG-2022) TRAINING OF PERSONNEL

Each person engaged in each phase of pharmaceutical manufacture should have the education, training, and experience to do his or her job. The demands of microbiological testing require that the core educational background of the staff, supervisors, and managers be in microbiology or a closely related biological science. They should be assigned responsibilities in keeping with their level of skill and experience.

A coherent system of standard operating procedures (SOPs) is necessary to run the microbiology laboratory. These procedures serve two purposes in a training program. Firstly, these SOPs describe the methodology that the microbiologist will follow to obtain accurate and reproducible results, and so serve as the basis for training. Secondly, by tracking the procedures in which a particular microbiologist has demonstrated proficiency, the procedure number or title also serves to identify what training the microbiologist has received specific to his or her job function.

Training curricula should be established for each laboratory staff member specific to his or her job function. He or she should not independently conduct a microbial test until qualified to run the test. Training records should be current, documenting the microbiologist's training in the current revision to the particular SOP.

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▲Performing USP microbiology methods requires a quality control infrastructure that will support accuracy and consistency of method outcomes. While this chapter provides information about effective microbiology practices, there are other *USP* chapters that offer guidance in relevant areas where microbiology is integrated (see *Guide to General Chapters* and *Chapter Charts*).▲ (USP 1-Aug-2022)

Periodic performance assessment is a wise investment in data quality. This performance testing should provide evidence of competency in core activities of the microbiology laboratory such as hygiene, plating, aseptic technique, documentation, and others as suggested by the microbiologist's job function.

Microbiologists with supervisory or managerial responsibilities should have appropriate education and in-house training in supervisory skills, laboratory safety, scheduling, budgeting, investigational skills, technical report writing, relevant SOPs, and other critical aspects of the company's processes as suggested in their role of directing a laboratory function.

Competency may be demonstrated by specific course work, relevant experience, and routinely engaging in relevant continuing education. Achieving certification through an accredited body is also a desirable credential. Further, it is expected that laboratory supervisors and managers have a demonstrated level of competence in microbiology at least as high as those they supervise. Expertise in microbiology can be achieved in a variety of ▲ways▲ (USP 1-Aug-2022) in addition to academic course work and accreditation. Each company is expected to evaluate the credentials of those responsible for designing, implementing, and operating the microbiology program. Companies can thus ensure that those responsible for the program understand the basic principles of microbiology, can interpret guidelines and regulations based on good science, and have access to individuals with theoretical and practical knowledge in microbiology to provide assistance in areas in which the persons responsible for the program may not have adequate knowledge and understanding. It should be noted that microbiology is a scientifically based discipline that deals with biological principles substantially different from those of analytical chemistry and engineering disciplines. Many times it is difficult for individuals without specific microbiological training to make the transition.

***Add the following:***

## ▲QUALIFICATION OF ANALYSTS

The hiring of experienced personnel combined with periodic training and qualification of analysts, including regular vision checks, should limit to an acceptable level the variability of microbiological test results produced by each analyst's handling and reading of test results, as well as ensure that the defined good documentation practices are understood and followed.

These also apply to personnel who may be involved in visual inspection of media fill units.▲ (USP 1-Aug-2022)

***Add the following:***

## ▲CONSIDERATIONS FOR MICROBIOLOGICAL RISK ASSESSMENTS

A microbiological risk assessment can be beneficial and provide information to assist in decision-making about the impact of microbiological quality concerns. (See *Microbiological Examination of Nonsterile Products: Acceptance Criteria for Pharmaceutical Preparations and Substances for Pharmaceutical Use* ⟨1111⟩ for additional specific parameters for evaluating non-specified microorganisms that may be present in nonsterile products.)

Some examples of when a microbiological risk assessment might be used are: 1) elevated bioburden counts (but within specification); 2) growth of atypical colonies after enrichment on selective agar plates for compendium-specified microorganisms; 3) exceeded alert/action levels; and 4) identification of a recovered species of concern.

The nature and intent of a risk assessment can differ depending on the relevant situation. The following is a list of example topics that could be included as part of the risk assessment.

- **Scope of the Event**—Providing information on the number of product lots that are directly or indirectly impacted on the basis of shared raw materials, shared equipment, and similar manufacturing processes.
- **Microorganism Identification**—Identifying the microorganism(s) to the species level whenever possible.
- **Characterization and Source of the Microorganism(s)**—Researching the literature to determine where the natural habitat for the microorganism can be found; the potential physiological states for the microorganism (e.g., spore vs. vegetative, planktonic vs. biofilm); and the potential pathogenicity of the species, the disease potential of the microorganism for the intended route of administration, and the number of microorganisms recovered.
- **Manufacturing Process**—Evaluating the product/material manufacturing process (or for an API or raw material) to determine if it contains microbial ingress and/or growth-control points or growth-promoting, or microbial-reducing steps.
- **Target Patient Base and Intended Use of the Product(s)**—Identifying potential health effects to the probable user group(s) targeted by the product based on the product insert and label information.
- **Route of Administration of the Product(s)**—Identifying the route of administration of the product (e.g., oral non-aqueous/aqueous, topical, inhalant, etc.). If it is an API or raw material, identify the route of administration of the product that it will be used to manufacture.
- **Intrinsic Product(s) Characteristics**—Evaluating the intrinsic characteristics of the product and the effect they could have on the level of microorganism(s) over shelf life. This means identifying the nature of the product (such as its water activity or pH) and whether the product can support growth or has antimicrobial activity.
- **Assessment Conclusion**—Providing a sound, scientifically-based determination. For example, determination that a microorganism is objectionable or is not objectionable in the test article.

The risk assessment should include determination of the level of risk to the patient or product for an impacted material that is used to manufacture finished product(s) or for a finished product that will be released to the market. An investigation to determine the source of a recovered species of concern and its contamination risk may be required to be completed before a

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drug product is released to the market. Writing and approval of a microbiological risk assessment should be performed by personnel with specialized education and experience in microbiology. ▲ (USP 1-Aug-2022)

***Change to read:***

## LABORATORY RESOURCES

The laboratory management is responsible for ensuring that the laboratory has sufficient resources to meet the existing testing requirements ▲(*4*). ▲ (USP 1-Aug-2022) This requires some proficiency in budget management and in determining appropriate measures of laboratory performance. A measure of laboratory performance is the number of investigations performed on tests conducted by the laboratory, but this measure alone is not sufficient. In addition to tracking investigations, the period of time between sample submission and initiation of testing should be tracked, as well as the period of time between end of test and report release (or test closure). Significant delays in these measures are also indications of an under-resourced laboratory staff.

The laboratory management should have sufficient budget to meet testing requirements. Particular measures of budgetary requirements will be specific to the given laboratory, but budgetary considerations related directly to the need of the laboratory for sufficient resources must be addressed to ensure reliable testing results ▲(*4*).

### Oversight of Contract Laboratories

Some companies do not have the laboratory space, specialized equipment, or capacity to conduct high-volume microbiological analyses, so they may outsource this type of testing to contract laboratories. It is the company's responsibility to thoroughly investigate the reputation and quality control performance of any new or unfamiliar contract laboratory with which they engage in a quality agreement. Before signing an agreement, a manufacturer should confirm that the contract laboratory complies with current good manufacturing practice (GMP) regulations regarding laboratory qualifications, can perform the required USP compendial testing for microbial recovery and bacterial endotoxins (see *Tests for Burkholderia Cepacia Complex* ⟨60⟩, *Microbial Enumeration Tests* ⟨61⟩, *Tests for Specified Microorganisms* ⟨62⟩, ⟨71⟩, and *Bacterial Endotoxins Test* ⟨85⟩), and an onsite audit can be conducted or, perhaps, a sample batch submitted for testing to ensure the quality of testing and data integrity. The qualifications of the laboratory management staff to interpret microbiological sample results need to be documented, especially if these staff will serve as subject matter experts for data review or if a laboratory investigation of questionable sample results ever needs to be conducted. Reports written by the contract laboratory should include complete analytical worksheets from the analysis performed. The raw data should be available to and periodically reviewed by a qualified microbiologist of the client company in order to verify the reliability of finished work. All the data used for calculations, including all the positive (growth-promotion results) and negative controls used during analysis, should also be included in the final report. In contrast, a certificate of analysis with a summary of analytical results does not provide all the information needed to verify sample test data or satisfy a regulatory audit. Relevant method-suitability test results should also be available to support the subsequent testing.

### Oversight of Suppliers

The impact of the microbiological quality of materials from a supplier can be significant for the laboratory (e.g., media, reagents, cultures) or for manufacturing (e.g., excipients, components); thus, microbiology knowledge and experience is critical for adequate oversight of a supplier. Since the microbiology laboratory can perform specification tests for excipients and products, alignment of knowledge and understanding with a supplier is valuable. Based on evaluation of the appropriate microbiological quality for each material, adequate supplier oversight from a microbiological perspective can provide increased knowledge, with consistent quality of the materials, and improved trust in the relationship with the supplier. For the laboratory supplies, materials of good quality are an important input to systematically maintain laboratory capability for performing consistent tests. For the manufacturing operations, an excipient supply of good quality provides the foundation for a consistently produced product that is microbiologically safe for patients. The full understanding and evaluation of a supplier's microbiological testing strategy, along with sampling, test methodology, and training, provides a means for developing a relationship with transparency and integrity. An experienced microbiologist should initiate ongoing communications in this type of customer–supplier relationship and serve as a technical expert. The controls in place for testing and inspection of incoming lab supplies are key to supply control. ▲ (USP 1-Aug-2022)

***Add the following:***

## ▲METHOD TRANSFER

Performance of validated methods can change from one laboratory to another. Differences in media, reagents, equipment, procedures, and in levels of experience of personnel can all affect the outcome of a microbiological method. When transferring a method from one laboratory to another, control of the change should be evaluated and directed so that there is minimal negative impact. Each combination of method and test substance should be performed, according to the requisite procedure, by both the sending and receiving laboratories in an appropriately defined and documented activity (with a defined number of replicates) that will ensure that method testing capability and experience have been transferred effectively.

For method transfers, comparative testing of the same sample may not be relevant for microbiological methods due to the non-homogeneity of microbial cells in the sample. Since no contamination is frequently observed, such testing would result in comparing zero counts. Carrying out a method suitability verification of the analytical method of the product to be transferred

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would provide greater assurance that the transfer-receiving unit may adequately perform the tests under the conditions with the material used (e.g. nutrient media).▲ (USP 1-Aug-2022)

***Change to read:***

## DOCUMENTATION

Documentation ▲(either manually or by an electronic laboratory management system)▲ (USP 1-Aug-2022) should be sufficient to demonstrate that the testing was performed in a laboratory and by methods that were under control. This includes, but is not limited to, documentation of the following:

- Microbiologist training and verification of proficiency
- Equipment validation, calibration, and maintenance
- Equipment ▲monitoring▲ (USP 1-Aug-2022) during test (e.g., 24-h/7-day chart recorders ▲for incubators▲ (USP 1-Aug-2022))
- Media preparation, sterility checks, growth-promotion,▲indicative, and inhibitory▲ (USP 1-Aug-2022) capabilities
- Media inventory ▲quality▲ (USP 1-Aug-2022) control ▲and shelf-life▲ (USP 1-Aug-2022) testing
- Critical aspects of test conducted as specified by a procedure
- Data and calculations verification
- Reports reviewed by QAU or a qualified responsible manager
- Investigation of data deviations (when required)

▲Data integrity guidance will be addressed later in this chapter.▲ (USP 1-Aug-2022)

***Change to read:***

## MAINTENANCE OF LABORATORY RECORDS

Proper recording of data and studies is critical to the success of the microbiology laboratory. The overriding principle is that the test should be performed as written in the SOP, the SOP should be written to reflect how the test is actually performed, and the laboratory notebook should provide a record of all critical ▲information▲ (USP 1-Aug-2022) needed to reconstruct the details of the testing and confirm the integrity of the data. At a minimum, a laboratory write-up should include the following:

- Date
- Material tested
- Microbiologist's name
- Procedure number
- Documented test results
- Deviations (if any)
- Documented ▲significant method steps and▲ (USP 1-Aug-2022) parameters (equipment used, microbial stock cultures used, media lots used)
- Management/Second review signature

Every critical piece of equipment should be ▲documented▲ (USP 1-Aug-2022) in the write-up, and all should be on a calibration schedule documented by SOP and maintenance records. Where appropriate, logbooks or forms should be available and supportive of the laboratory notebook records. Equipment temperatures (water baths, incubators, autoclaves) should be recorded and traceable.

The governing SOP and revision should be clearly noted in the write-up. Changes in the data should be crossed off with a single line and initialed. Original data should not be erased or covered over.

Test results should include the original plate counts, allowing a reviewer to recreate the calculations used to derive the final test results. Methods for data analysis should be detailed in cited SOPs. If charts or graphs are incorporated into ▲paper▲ (USP 1-Aug-2022) laboratory notebooks, they should be secured with clear tape and should not be obstructing any data on the page. The chart or graph should be signed by the person adding the document, with the signature overlapping the chart and the notebook page. Lab notebooks should include page numbers, a table of contents for reference, and an intact timeline of use. ▲Similar requirements should be established for electronic laboratory notebooks that capture this same information.▲ (USP 1-Aug-2022)

All laboratory records should be archived and protected against catastrophic loss. A formal record-retention and -retrieval program should be in place.

***Add the following:***

## ▲QUANTITATIVE MICROBIOLOGY

Classical microbiology utilizes the colony forming unit (cfu) as the basis of quantitation for growth-based methods (*5*). The heterogeneous nature of microbial contamination can lead to inaccuracies in quantitative methods. While representative sampling is a high priority in all methods, calculating colony counts with averaging can lead to subjective and varied results depending on the plate reader and analyst experience. It is important to understand that although averaging colony counts from multiple plates can make the analysis simpler, a wide range of individual plate counts could lead to underestimation of actual contamination. Rounding of cfu, another quantitative principle, should be carefully evaluated for fit with the method to

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avoid reporting inaccurate results. [NOTE—Microbial colonies represent biological entities; thus, fractions or decimals in colony count results are meaningless.] There are methods, such as kinetic bacterial endotoxin testing, where decimal place calculations are expected. Be careful of placing too much significance on the decimal portion of a result when evaluating a result compared to an endotoxin limit specification.▲ (USP 1-Aug-2022)

***Add the following:***

## ▲DATA INTEGRITY OF MICROBIOLOGICAL DATA

Microbiological methods are classically performed manually and on the basis of visual evaluation by an analyst performing the test. Therefore, the interpretation of test results or the number of colonies tested may be prone to a certain subjectivity and variability. To further improve data integrity and reduce subjectivity, alternative methods for the reading of plates, such as the use of automated plate readers or high-resolution photographs of the plate, may be used. However, these systems can have inherent challenges such as difficulty with the following: counting colonies embedded in the agar gel from pour-plated dishes, counting satellite colonies, differentiating overlapping colonies, differentiating particles from colonies, and interpreting the photo consistently from one individual to another. Automated enumeration methods that stack images to capture colonies growing in time may overcome some of these challenges.

It should be noted that, in order to avoid jeopardizing the asepsis status required during testing, contemporaneous recording of actions during execution of the microbiological testing cannot be followed in all cases. It is acceptable for recording of actions to take place immediately after the working session upon exiting the UDAF hood instead of constantly having to go back and forth from aseptic to non-aseptic areas to write down test actions executed.

Counts from Petri plates are considered original data on the day that the method requires the plates to be read and recorded. After reading, if these same plates are subsequently stored at room temperature or under refrigeration, it is not possible to confirm the original results because the microbial counts may increase during storage. Many microbial colonies continue to grow during refrigeration but at a slower rate. The colony counts derived from conducting a microbe test using a compendial method depend on adherence to the incubation time and temperature stated in the prescribed method (i.e., ⟨61⟩).

It is common practice for the raw data sheet that contains the original data, as well as the data entry from the raw data sheet to the laboratory information management systems (LIMS), to be reviewed for accuracy and completion by a qualified analyst, an analyst who has not executed or evaluated the test, or the supervisor.

An important data integrity threat with microbiological testing resides with falsification of data and intentional omission of testing results. To control this risk, the company culture and ethical standards are essential as well as the application of a rigorous quality management system.

For the compendial sterility test that combines criticality of the test and higher risk of misinterpretation of results, it is now a standard practice to have a second analyst perform a contemporaneous evaluation of the sample (in test media) for microbial growth. Nonetheless, applying uncritically a contemporaneous reading by a second analyst (four-eyes principle) for all samples and microbiological tests is not recommended. Precision in counts may vary from one analyst to another (even if they are trained and qualified) as colonies may overlap, swarm over media, etc., allowing for misinterpretation. Microbiology is a "logarithmic science" (⟨1223⟩); sample size is statistically weak and testing procedures have inherent variability. By tolerating no differences in counts, a high number of non-critical deviations will be generated, thus consuming resources unreasonably. As an alternative to a contemporaneous enumeration, a contemporaneous verification by a second person that the testing activity is performed correctly may be executed for higher risk tests. A second person could verify, for instance, if the reading of results is correctly executed according to the procedure, if the result on the Petri plate is correctly transcribed onto the GMP recording sheet (i.e., if growth is observed this is captured in the GMP sheet), and if the description of the sample corresponds to the description on the GMP recording sheet. An assessment of the risk due to a misinterpreted result and its impact on patient safety is performed to determine the high risk test outlined above.▲ (USP 1-Aug-2022)

***Change to read:***

## INTERPRETATION OF ASSAY RESULTS

Analytical microbiological assay results can be difficult to interpret for several important reasons: 1) microorganisms are ubiquitous in nature, and common environmental contaminants—particularly organisms associated with humans—predominate in many types of microbiological analysis; 2) the analyst has the potential to introduce contaminating organisms during sample handling or processing in the laboratory; 3) microorganisms may not be homogeneously distributed within a sample or an environment; and 4) microbiological assays are subject to considerable variability of outcome. Therefore, apparent differences from an expected outcome may not be significant.

Because of these characteristics of microbiological analysis, laboratory studies should be conducted with the utmost care ▲(using aseptic techniques)▲ (USP 1-Aug-2022) to avoid exogenous contamination as previously discussed in this chapter. Equally important, results must be interpreted from a broad microbiological perspective, considering not only the nature of the putative contaminant but the likelihood of that organism(s) surviving in the pharmaceutical ingredient, excipient, or environment under test. In addition, the growth characteristics of the microorganism should be considered (especially in questions of the growth of filamentous fungi in liquid media).

▲Microbiological data should be scheduled to be reviewed and trended to assess the capability of the measures to control contamination and ensure the microbiological quality of products or raw materials remains within the acceptance criteria defined and is not worsening. Trending of microbiological data can be performed in several ways, but the end goal is to determine if an adverse trend is arising. An adverse microbiological trend is an early warning of a potential degradation or loss of control within the environment, the utility, raw material, or product tested. Multiple designations may be used to define adverse trends, and it is up to the user to define which is the most relevant definition in the context of the application. For

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instance, an adverse trend can be defined as repeating higher than usual counts or increasing amount and variety of microorganisms or contamination occurrences over a certain time period. An adverse trend may be systematically related to multiple events.

To reduce the role of subjectivity and to distinguish counts that are out of expectation or that differ from the norm, monitoring levels could be calculated using statistical methods. There is no absolute standard that can be used to calculate levels using historical data as data distribution may differ among the different microbiological tests. Microbiological data are generally not normally distributed, may contain many zero counts (for highly controlled or aseptic areas), and are highly variable. Therefore, classical process control tools based on normally distributed data may not apply. The percentile ranking method using the negative binomial or gamma fit distribution has been shown to be reliable and covers a large diversity of microbiological data (*6*). As an alternative, if enough values are available, a non-parametric fit may be used.

## Use of Negative Controls

Some microbiological methods specify the use of a negative control, but their use in other situations should be relevant and appropriate. For example, the testing of rinse fluid without product in a membrane filtration sterility test could provide useful information in a laboratory investigation of an out-of-specification result. Also, for bioburden testing, a negative control can provide ongoing trending of lab equipment sterilization and good aseptic techniques.

## Investigations ▲ (USP 1-Aug-2022)

When results are observed that do not conform to a compendial monograph or other established acceptance criteria, an investigation ▲▲ (USP 1-Aug-2022) is required. There are generally two distinct reasons for the observation of microbial contamination that does not comply with a target or requirement: A laboratory error or laboratory environmental conditions may have produced an invalid result, or the product contains a level of contamination or specific types of contaminants outside established levels or limits. ▲In such cases, conduct an investigation using an appropriate investigative tool (e.g., Ishikawa "fishbone" diagrams or the Kepner-Tregoe method).▲ (USP 1-Aug-2022) Laboratory management and, in most cases, the QAU should be notified immediately. ▲Immediate actions may be required if the product's quality is potentially impacted by the deviation. These may include, for instance, putting the batch in quarantine and assessing the potential impact for batches produced in the same campaign or already released on the market.▲ (USP 1-Aug-2022)

A full and comprehensive evaluation of the laboratory situation surrounding the result should be undertaken. All microbiological conditions or factors that could bring about the observed condition should be fully considered, including the magnitude of the excursion compared to established limits or levels. In addition, an estimate of the variability of the assay may be required to determine whether the finding is significant.

The laboratory environment, the protective conditions in place for sampling, historical findings concerning the material under test, and the nature of the material, particularly with regard to microbial survival or proliferation in contact with the material, should be considered in the investigation. In addition, interviews with the laboratory analyst(s) may provide information regarding the actual conduct of the assay that can be valuable in determining the reliability of the result and in determining an appropriate course of action. If laboratory operations are identified as the cause of the nonconforming test outcome, then a corrective action plan should be developed to address the problem(s). Following the approval and implementation of the corrective action plan, the situation should be carefully monitored and the adequacy of the corrective action determined.

If assay results are invalidated on the basis of the discovery of an attributable error, this action must be documented. Laboratories also should have approved procedures for ▲additional▲ (USP 1-Aug-2022) testing (retesting), and if necessary, resampling where specific regulatory or compendial guidance does not govern the conduct of an assay investigation. ▲Since microorganisms are not necessarily homogeneously distributed in a sample; microbial numbers evolve following storage; all volume of the sample is used during testing in some cases; and sampling is a snapshot of a short time; the classical retesting rules for chemical analysis should not be applied in microbiological testing. Additional testing of the original product, raw materials, intermediates, environment, etc., however, may serve to provide supportive information for the root cause investigation but not be used to invalidate the original results.▲ (USP 1-Aug-2022)

***Add the following:***

## ▲REFERENCES

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5. Sutton S. Accuracy of Plate Counts. *J Val Technol*. Summer, 2011; 42–46.
6. Gordon O, Goverde M, Pazda J, Staerk A, Roesti D. Comparison of different calculation approaches for defining microbiological control levels based on historical data, *PDA Journal of Pharmaceutical Science and Technology*. 2015; 69:383–398.▲ (USP 1-Aug-2022)

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# ⟨1121⟩ NOMENCLATURE

## INTRODUCTION

The *USP* (or *NF*) titles for monograph articles are legally recognized under the Federal Food, Drug, and Cosmetic Act as the designations for use in labeling the articles to which they apply.

The value of designating each drug substance by one and only one nonproprietary[1] name is important in terms of achieving simplicity and uniformity in drug nomenclature. In support of the U.S. Adopted Names program (see *Mission and Preface* in *USP–NF*), of which the U.S. Pharmacopeial Convention is a co-sponsor, the USP Council of Experts gives consideration to the adoption of the U.S. Adopted Name, if any, as the official title for any compound that attains compendial recognition.

A compilation of the U.S. Adopted Names (USAN) published from the start of the USAN program in 1961, as well as other names for drugs, both current and retrospective, is provided in the *USP Dictionary of USAN and International Drug Names.* This publication serves as a book of names useful for identifying and distinguishing all kinds of names for drug substances, whether public, proprietary, chemical, or code-designated names.[2]

A nonproprietary name of a drug serves numerous and varied purposes. Its principal function is to identify the substance to which it applies by means of a designation that may be used by the professional and lay public free from the restrictions associated with registered trademarks. Teaching in the health sciences requires a common designation, especially for a drug that is available from several sources or is incorporated into a combination drug product; nonproprietary names facilitate communication among healthcare providers; nonproprietary names must be used as the titles of the articles recognized by official drug compendia; a nonproprietary name is essential to the pharmaceutical manufacturer as a means of protecting trademark rights in the brand name for the article concerned; and, finally, the manufacturer is obligated by federal law to include the established nonproprietary name in advertising and labeling.

Under the terms of the Drug Amendments of 1962 to the Federal Food, Drug, and Cosmetic Act, which became law October 10, 1962, the Secretary of Health and Human Services is authorized to designate an official name for any drug wherever deemed "necessary or desirable in the interest of usefulness and simplicity."[3]

The Commissioner of Food and Drugs and the Secretary of Health and Human Services published in the *Federal Register* regulations effective November 26, 1984, which state, in part:

> "**Sec. 299.4 Established names of drugs.**"
> "(e) The Food and Drug Administration will not routinely designate official names under section 508 of the act. As a result, the established name under section 502(e) of the act will ordinarily be either the compendial name of the drug or, if there is no compendial name, the common and usual name of the drug. Interested persons, in the absence of the designation by the Food and Drug Administration of an official name, may rely on as the established name for any drug the current compendial name or the USAN adopted name listed in *USAN and the USP Dictionary of Drug Names*."[4]

It will be noted that the monographs on the biologics, which are produced under licenses issued by the Secretary of the U.S. Department of Health and Human Services, represent a special case. Although efforts continue toward achieving uniformity, there may be a difference between the respective title required by federal law and the *USP* title. Such differences are fewer than in past revisions of the Pharmacopeia. The *USP* title, where different from the FDA Center for Biologics Evaluation and Research title, does not necessarily constitute a synonym for labeling purposes; the conditions of licensing the biologic concerned require that each such article be designated by the name appearing in the product license issued to the manufacturer. Where a *USP* title differs from the title in the federal regulations, the former has been adopted with a view to usefulness, simplicity, and conformity with the principles governing the selection of monograph titles generally.

## MONOGRAPH NAMING POLICY FOR SALT DRUG SUBSTANCES IN DRUG PRODUCTS AND COMPOUNDED PREPARATIONS

The titles of *USP* monographs for drug products and compounded preparations formulated with a salt of an acid or base use the name of the active moiety, as defined below. The strength of the product or preparation is also expressed in terms of the active moiety.

An active moiety is the molecule or ion, excluding those appended portions of the molecule that cause the drug to be a salt (including a salt with hydrogen or coordination bonds), or other noncovalent derivative (such as a complex, chelate, or clathrate) of the molecule. The active moiety is responsible for the physiological or pharmacological action of the drug substance, without regard to the actual charged state of the molecule in vivo. For example, the active moiety of a hydrochloride salt of a base is the free base and not the protonated form of the base. The active moiety of a metal salt of an acid is the free acid.

This Policy is followed by USP in naming drug products and compounded preparations that are newly recognized in the *USP*. Revising existing monographs to conform to this Policy is not intended, except where the USP Council of Experts determines that, for reasons such as safety, a nomenclature change is warranted.

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[1] The term "generic" has been widely used in place of the more accurate and descriptive term "nonproprietary" with reference to drug nomenclature.
[2] *USP Dictionary of USAN and International Drug Names* is obtainable on order from U.S. Pharmacopeia, Customer Service Department, 12601 Twinbrook Parkway, Rockville, MD 20852.
[3] F.D.&C. Act, Sec. 508 [358].
[4] 53 Fed. Reg. 5369 (1988) amending 21 CFR § 299.4.

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## Labeling

The labeling clearly states the specific salt form of the active moiety that is present in the product or preparation because this information may be useful to practitioners and patients. The names and strengths of both the active moiety and specific salt form (when applicable) are provided in the labeling.

## Exceptions

In rare cases in which the use of the specific salt form of the active moiety in the title provides vital information from a clinical perspective, an exception to this policy may be considered. In such cases, when the monograph title contains the specific salt form of the active moiety, the strength of the product or preparation also is expressed in terms of the specific salt form.

# GENERAL NOMENCLATURE FORMS

List of the specific drug products with extensive examples can be found in the USP Nomenclature Guidelines (www.usp.org). USP also developed some general practices for drug product nomenclature:

- The [ROUTE OF ADMINISTRATION] is omitted for those dosage forms for which the route of administration is understood. The general form then becomes simply [DRUG] [DOSAGE FORM].
  - Thus, oral will not be included as the route of administration for orally administered capsules, tablets, and lozenges.
  - The route of administration is omitted for topically applied products—creams, ointments, lotions, and pastes. However, if some other route of administration is intended (e.g. ophthalmic), it will be included in the monograph title.
- In some instances, the drug is supplied in one dosage form for the preparation of the intended dosage form. In such cases, the dosage form provided in the container is named first and the word “for” appears, followed by the final dosage form that is suitable for administration. The general format becomes [DRUG] [DOSAGE FORM] for [ROUTE OF ADMINISTRATION] [DOSAGE FORM]., e.g. Aspirin Effervescent Tablets for Oral Solution.
- The term “for” is included in the names of solid preparations which must be dissolved or suspended in a suitable liquid to obtain a dosage form suitable for administration, and the general format becomes [DRUG] for [ROUTE OF ADMINISTRATION] [DOSAGE FORM]. e.g. Ampicillin for Oral Suspension, Cytarabine for Injection.
- The term “Vaginal Inserts”, rather than “Vaginal Tablets”, “Vaginal Capsules”, or “Vaginal Suppositories” is used in the title of this type of vaginal preparation to decrease the potential for misadministration of these products.
- The term “Suppositories” is used in the titles of solid preparations that are intended for rectal administration.
- Solutions administered by injection are officially titled Injections (see *Injections and Implanted Drug Products* ⟨1⟩). The route of administration is omitted for drugs that are injected, because the route (e.g. intravenous, intramuscular, subcutaneous, etc.) must appear on the labels and in the labeling. USP defines seven types of injections:
  1. [DRUG] Injection—Liquid preparations that are drug substances or solutions thereof.
  2. [DRUG] for Injection—Dry solids that, upon the addition of suitable vehicles, yield solutions conforming in all respects to the requirements for Injections.
  3. [DRUG] Injectable Emulsion—Liquid preparations of drug substances dissolved or dispersed in a suitable emulsion medium.
  4. [DRUG] Injectable Suspension—Liquid preparations of solids suspended in a suitable liquid medium.
  5. [DRUG] for Injectable Suspension—Dry solids that, upon the addition of suitable vehicles, yield preparations conforming in all respects to the requirements for Injectable Suspensions.
  6. [DRUG] Extended-Release Injectable Suspension—Liquid preparations of solids suspended in a suitable liquid medium and formulated in a manner that allows the contained API to be available over an extended period of time.
  7. [DRUG] for Extended-Release Injectable Suspension—Dry solids that, upon the addition of suitable vehicles, yield preparations conforming in all respects to the requirements for Extended-Release Injectable Suspensions.

Injections intended to be administered through an intravenous secondary line (“piggyback”) formulated in vehicles other than Water for Injection shall be named [DRUG] in [VEHICLE]. Examples of Vehicle formats which currently appear in *USP* monograph titles are:

1. [DRUG] in Dextrose Injection
2. [DRUG] in Dextrose and Sodium Chloride Injection
3. [DRUG] in Lactated Ringer’s and Dextrose Injection
4. [DRUG] in Sodium Chloride Injection

# POLICY FOR IMPLEMENTATION OF NOMENCLATURE REVISIONS

It is the practice of USP to set the official dates of nomenclature revisions (change of the established name or nomenclature aspects of the labeling sections in the monographs) to allow a reasonable time for product label changes to be made and to allow health practitioners and consumers time to become familiar with the new terminology. The assignment of an implementation schedule is handled by the USP Expert Committee. USP’s implementation schedule, shown below, is automatic, unless an exception is sought.

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## 18 Months

Implementation period of 18 months is usually applied when only one or a small number of products is affected.

## 30 Months

Implementation period of 30 months is usually applied when names or labeling of multisource products or multiproduct lines of a firm's preparations are being changed.

## 60 Months

Implementation period of 60 months is usually applied for title and labeling changes that affect excipients, because such changes would require relabeling of very large numbers of prescription-only and OTC preparations.

There may be exceptions to this schedule where a shorter time is needed in order to specify nomenclature and labeling changes in cases where public health and safety are a concern. Extensions to the implementation schedule are rarely made, and must have suitable justification as well as the approval of the USP Expert Committee. Any questions or concerns regarding this postponement schedule may be addressed to the USP staff liaison assigned to the Expert Committee.

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# ⟨1125⟩ NUCLEIC ACID-BASED TECHNIQUES—GENERAL

## SCOPE

Nucleic acid-based assays are used in a variety of settings, the most common of which include the detection of infectious agents (viruses, bacteria, etc.), and cellular materials, as well as disease profiling. More recently such assays have also been used for forensic purposes and for the detection of trace contamination in biological materials. The latter include pharmaceutical development applications, such as viral clearance and adventitious agent testing in vaccine seed lots and tissue culture cell banks. This chapter introduces a series of general information chapters that provide techniques that support procedures for the detection and analysis of nucleic acids (see *Figure 1*). The assays using these techniques may be presented in a USP general chapter or in a private specification.

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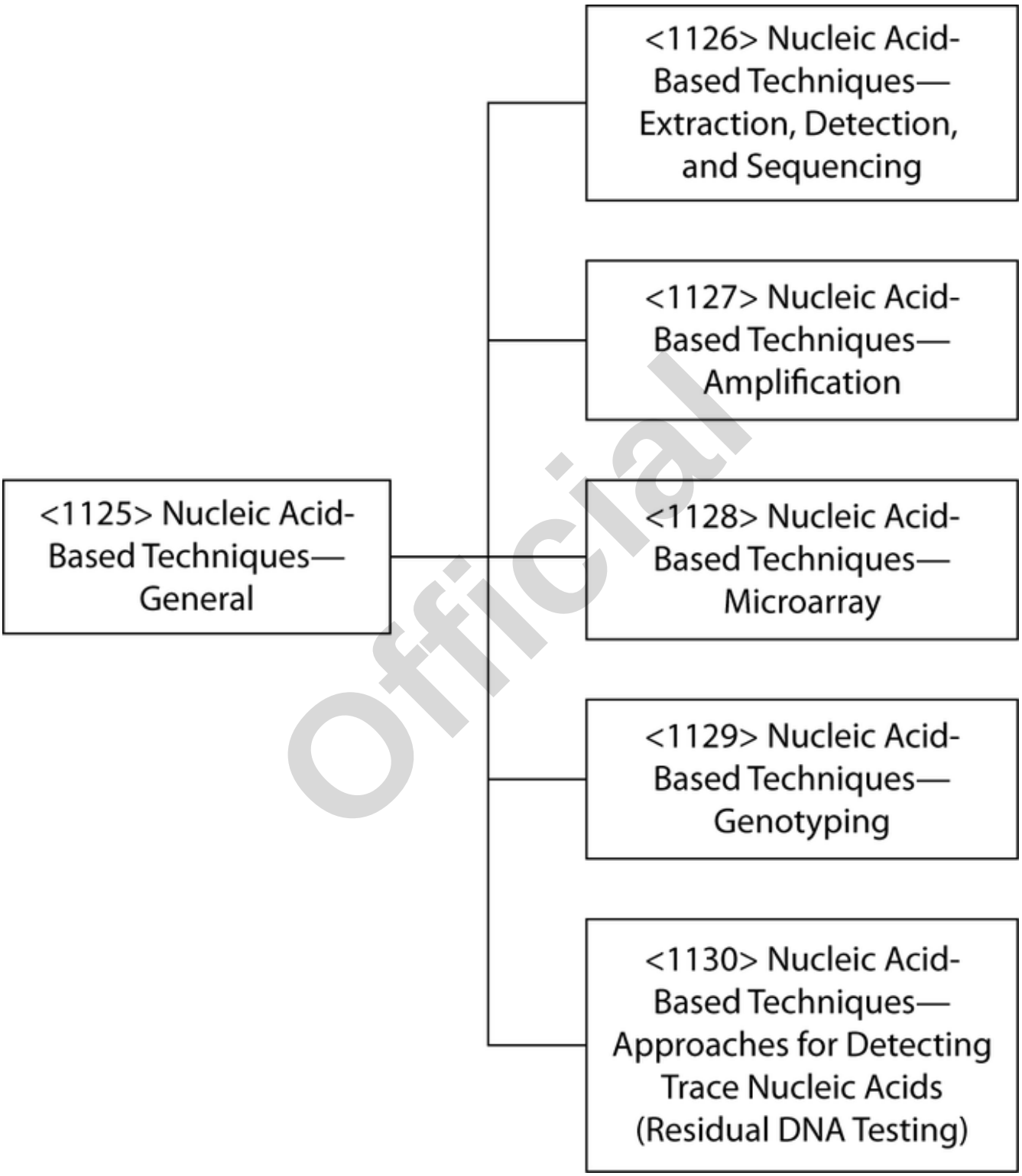


Figure 1

The major requirement for any nucleic acid analytical procedure is the availability of pure, intact nucleic acids for analysis. The information in *Nucleic Acid-Based Techniques—Extraction, Detection, and Sequencing* ⟨1126⟩ discusses procedures available for nucleic acid extraction and handling. Hybridization is the core mechanism underlying many molecular biology techniques, and in addition to the detection of nucleic acids by absorbance and fluorescence measurements and size measurement by gel electrophoresis, this chapter also covers blotting and identification of nucleic acid species by hybridization assays using labeled probes. Hybridization probes are oligonucleotides that have a sequence that is complementary to the target of interest. Probes

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contain radioactive, fluorescent, biotin, digoxigenin, or other tags that, upon binding of the probe to the target, allow visualization and identification of the target. Probes are capable of detecting target sequences that are present in concentrations too low to be detected by absorbance measurements or gel electrophoresis.

These analytical procedures require a minimum quantity of nucleic acid, typically in the nanogram to microgram range. However, in the vast majority of cases, e.g., in the detection of viruses or rare cellular RNA species, the nucleic acid under assay is present in minute quantities (in the picogram to femtogram range), and an amplification step must be performed before the nucleic acid can be detected and identified. The amplification step may be directed either at the signal used for detection (signal amplification), such as the branched DNA assay (bDNA assay), or at the target as in nucleic acid amplification technologies (NAT).

In 1983 a revolutionary yet simple process termed polymerase chain reaction (PCR) was developed for amplifying the number of specific nucleic acid fragments present in a sample, and in just a few years after its discovery PCR became the most frequently used procedure for amplifying nucleic acids, especially DNA. Since the inception of PCR, the number of applications has expanded rapidly, and the technique, which now includes quantitative and multiplex assays, is currently used in almost every field of research and development in biology and medicine. Numerous variations of assay procedures have been developed for specific analytes. The general information chapter, *Nucleic Acid-Based Techniques—Amplification* ⟨1127⟩, describes amplification procedures used for DNA and RNA analysis as well as qualitative and quantitative NAT assays. Signal amplification procedures in which the signals, typically fluorescent signals, are used to detect the nucleic acid of interest, are not very common. The major signal amplification procedure, the branched DNA or bDNA assay, is used predominantly for viral nucleic acid detection.

Quality assurance aspects of the methodology are also covered, together with a summary of current regulatory requirements for NAT assays. The need for globally comparable, accurate, and reliable results in the diagnostics field has driven the quest for, and development of, national and international standards within an increasingly sophisticated and metrologically sound, highly developed international regulatory environment devoted to the highest standards of regulatory science. Because NAT has become the most widely used of nucleic acid techniques, the majority of guidance documents and standards are related to NAT. The general information chapter, *Nucleic Acid-Based Techniques—Microarrays* ⟨1128⟩, addresses a still-emerging field that is of increasing relevance to molecular DNA analysis. Detailed treatment of various microarrays, including data analysis and validation, are excluded from ⟨1128⟩ at this time. The general information chapter, *Nucleic Acid-Based Techniques—Genotyping* ⟨1129⟩, focuses on the specific modifications of the techniques that are necessary to enable detection of single base differences and common genetic variations, e.g., single nucleotide polymorphisms (SNPs). The final general information chapter in the series, *Nucleic Acid-Based Techniques—Approaches for Detecting Trace Nucleic Acids (Residual DNA Testing)* ⟨1130⟩, describes residual DNA testing in the context of pharmaceutical manufacturing. Applications relevant to viral adventitious agents, however, are discussed in the general information chapter *Virology Test Methods* ⟨1237⟩.

Two major uses of nucleic acid testing are excluded from this family of NAT chapters: viral testing for blood and blood product safety and genetic testing. The traditional perspective of USP is to develop public standards that can be applied to a particular final product without expressively defining a product and/or its production details. This chapter aims to specify when traditional methodologies or existing standards can be adapted. Novel methodologies for amplification and detection by NAT are also highlighted. As these new methodologies become mature and properly validated, they will be included in subsequent revisions.

Due to rapid development in the field, compendial and regulatory affairs scientists are advised to consult the current edition of *USP* and its *Supplements* regularly.

## GLOSSARY

**3′–5′ Exonuclease activity:** Enzymatic activity to remove a mispaired nucleotide from the 3′ end of the growing strand. The reaction is a hydrolysis of a phosphoester bond. The presence of a 3′–5′ exonuclease, or proofreading, activity improves the fidelity of the polymerization.

**5′–3′ Exonuclease activity:** Enzymatic activity to remove a mispaired nucleotide from the 5′ end of a polynucleotide strand. This activity is actually that of a single-strand-dependent endonuclease and is needed to remove RNA primers of Okazaki fragments, the RNA strand in the intermediate DNA–RNA heteroduplex during reverse transcription, and during DNA repair.

**Absorbance:** [Symbol: A]The logarithm, to the base 10, of the reciprocal of the transmittance (T). [NOTE—Descriptive terms used formerly include optical density, absorbancy, and extinction.]

**Accuracy:** The accuracy of an analytical procedure is the closeness of test results obtained by that procedure to the true value.

**Allele:** One of two or more alternative forms of a gene at a given position (locus) on a chromosome, caused by a difference in the sequence of DNA.

**Amplicon:** A short segment of DNA generated by the PCR process whose sequence is defined by forward and reverse primers. Sometimes referred to as an amplimer.

**Amplification:** The enzymatic in vitro replication of a target nucleic acid.

**Annealing:** Hybridizing or binding of complementary nucleic acids, usually at an optimal temperature.

**Concatenation:** The process in which a DNA segment composed of repeated sequences is linked end-to-end.

**Complementary dna (cdna):** DNA synthesized from an RNA template in an enzymatic reaction catalyzed by the enzyme reverse transcriptase.

**Denaturation:** The process of separating double-stranded DNA into single strands by breaking the hydrogen bonds. This is typically accomplished by heating the DNA solution to temperatures greater than 90° or by treating it with strong alkali.

**Deoxyribonucleic acid (dna):** The genetic material that is passed from parent to daughter cells and propagates the characteristics of the species in the form of genes it contains and the proteins for which it codes. DNA contains the following four deoxyribonucleosides: dA, dC, dT, and dG.

**Deoxyribonucleotide triphosphate (dntp):** A base that is added to a primer during the PCR that comprises the newly synthesized strand. Examples of dNTPs are dATP, dUTP, dCTP, dGTP, and dTTP.

**Detection limit:** It is the lowest amount of analyte in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions.

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**DNA polymerase:** An enzyme that can synthesize new complementary DNA strands using a DNA template and primer. Several of these enzymes are commercially available, including *Taq* DNA polymerase and rTth DNA polymerase.

**Endonuclease:** An enzyme that cleaves phosphodiester bonds in a polynucleotide chain.

**Energy transfer:** This describes the process in which an excited state of one molecular entity (the donor) is deactivated to a lower-lying state by transferring energy to a second molecular entity (the acceptor), which is thereby raised to a higher energy state.

**Extension:** Refers to the elongation of the DNA chain that is being synthesized using the parent DNA strand as the template for synthesis of that daughter strand. This is a natural process that occurs during DNA replication. Extension occurs during the PCR process with DNA polymerases.

**Extinction coefficient:** [Symbol: ε]—The quotient of the absorbance (A) divided by the product of the concentration, expressed in moles/L, of the substance and the absorption path length, in cm. [NOTE—Terms formerly used include molar absorbancy index; molar absorptivity; and molar absorption coefficient.]

**Fidelity:** Fidelity is a measure of the accuracy of nucleic acid replication. The polymerase enzyme used is only one of the elements that influences fidelity. Other elements include buffer conditions, thermal cycling parameters, number of cycles, efficiency of amplification, and the sequence of the DNA being copied.

**Fluorophore:** A functional group in a molecule that makes the molecule fluorescent by absorbing energy of a specific wavelength and re-emits the energy at another wavelength.

**Fluorescence:** The emission of one or more photons by a molecule or atom activated by the absorption of a quantum of electromagnetic radiation. X-rays, UV, visible light, and IR radiations may all stimulate fluorescence. For details on the spectroscopic measurement of fluorescence, see *Fluorescence Spectroscopy* ⟨853⟩.

**Genome:** The complete genetic complement or the complete set of instructions for reproducing an organism and carrying out its biological function in life. The DNA in our cells comprises our genome. When our cells divide, the complete genome in our cells is duplicated for transmission to each of the remaining daughter cells.

**Genotype:** The genetic constitution of an organism as revealed by genetic or molecular analysis, i.e., the complete set of genes, both dominant and recessive, possessed by a particular cell or organism.

**Genotyping:** The process of assessing genetic variations present in an individual.

**Hairpin:** Antiparallel duplex structure that forms by pairing of inverted repeat sequences within a single-stranded nucleic acid. The helical section is called the stem and the unpaired base segment at the end of the structure is called the loop.

**Hot-start PCR:** Technique that is commonly used to improve the sensitivity and specificity of PCR amplification. A hot start is performed by withholding from the reaction mix a key component necessary for amplification until the reaction reaches a temperature above the optimal annealing temperature of the primers. The component withheld from the reaction mix can be primers, DNA polymerase, $MgCl_2$, or dNTPs.

**Hybridization:** The process of forming a double-stranded nucleic acid molecule, for example between a nucleotide sequence (probe) and a target.

**Ligation:** The process of joining two or more DNA fragments.

**Melting temperature ($T_m$):** The temperature at which 50% of the DNA becomes single-stranded.

**Microarray:** Sets of miniaturized chemical reaction areas that are used to test DNA fragments, antibodies, or proteins. Usually the probes are immobilized on a chip and hybridized with target.

**Mismatch:** Unconventional base pairing (other than C with G, and A with T or U). A mismatched base pair has lower bonding energy and decreases the stability of the DNA molecule.

**Nucleic acid:** Linear polymers of nucleotides, linked by 3′, 5′ phosphodiester linkages. In DNA, deoxyribonucleic acid, the sugar group is deoxyribose, and the bases consist of adenine, guanine, thymine, and cytosine. RNA, ribonucleic acid, has ribose as the sugar, and uracil replaces thymine.

**Oligonucleotide:** Linear sequence comprising as many as 25 nucleotides joined by phosphodiester bonds, generally used as a DNA synthesis primer.

**Photobleaching:** Photobleaching is the irreversible destruction of a fluorophore in the excited state. Different fluorophores have different rates of photobleaching. For example, fluorescein photobleaches very easily. Often the rate of decomposition is proportional to the intensity of illumination. A simple and practical way to overcome this is to reduce the incident radiation.

**Polymerase:** An enzyme that catalyzes the synthesis of nucleic acids on pre-existing nucleic acid templates, assembling RNA from ribonucleotides or DNA from deoxyribonucleotides.

**Polymerase chain reaction (PCR):** A laboratory technique that rapidly amplifies a specific region of double-stranded DNA, predetermined by the pair of primers used for amplification. Generally involves the use of a heat-stable DNA polymerase.

**Precision:** The degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of a homogenous sample.

**Primer:** Nucleic acid polymerases link a mononucleotide to a chain of nucleic acids, which is called the primer. RNA polymerases are able to use a single nucleotide as a primer, but DNA polymerases always require an oligonucleotide.

**Probe:** A specific DNA or RNA sequence that has been labeled by radioactive, fluorescent, or chemiluminescent tags and is used to detect complementary sequences by hybridization techniques such as blotting or colony hybridization. In addition, probes can also be used for quantitation of amplicons as described for quantitative PCR assays. A more detailed description of such probes is given in the general information chapter, *Nucleic Acid-Based Techniques—Amplification* ⟨1127⟩.

**Processivity:** The ability of an enzyme to repetitively continue its catalytic function without dissociating from its substrate.

**Proofreading activity:** Literally to read for the purpose of detecting errors for later correction. DNA polymerase has a 3′ to 5′ exonuclease activity that is used during polymerization to remove recently added nucleotides that are incorrectly paired.

**Quantitation limit:** It is the lowest amount of analyte in a sample that can be determined with an acceptable precision and accuracy under the stated experimental conditions.

**Quenching:** The process of extinguishing, removing, or diminishing a physical property such as heat or light. Fluorescence quenching can be either collisional or static.

**Reverse transcriptase:** An enzyme that requires a DNA primer and catalyzes the synthesis of a DNA strand from an RNA template. An enzyme that can use RNA as a template to synthesize DNA.

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**Reverse transcription (rt):** The process of making cDNA using an RNA template.

**Real-time PCR:** May often be referred to as Quantitative PCR or Real-Time Quantitative PCR but not RT-PCR and is a procedure for simultaneous DNA quantitation and amplification. The generation of amplicons monitored as they are generated by the use of a fluorescent reporter system and captured by sophisticated instrumentation.

**Real-time (RT-PCR):** The combination of real-time PCR and reverse transcription PCR.

**Reverse transcriptase polymerase chain reaction (RT-PCR):** A variation of the PCR technique in which cDNA is made from RNA via reverse transcription. The cDNA is then amplified using standard PCR protocols.

**Ribonucleic acid (RNA):** A type of nucleic acid composed of a specific sequence of ribonucleotides linked together. RNA contains the following four ribonucleosides: A, C, G, and U.

**Robustness:** The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in procedural parameters listed in the procedure documentation and provides an indication of its suitability during normal usage.

**rTth dna polymerase:** Recombinant thermostable DNA polymerase originally isolated from the bacterium *Thermus thermophilus*. rTth has optimal activity at 70°–80° and survives the denaturation steps of PCR. In addition to DNA polymerase activity, it has efficient reverse transcriptase activity in the presence of manganese.

**Specificity:** The ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation proeducts, and matrix components.

**Taq dna:** Thermostable DNA polymerase that is originally isolated from the bacterium *Thermus aquaticus*, *Taq* has optimal activity at 70°–80° and is not degraded during the high-heat denaturation steps of PCR.

**Template:** A master copy used to start the DNA or RNA replication process.

**Transcription:** The synthesis of RNA using a DNA template.

***Change to read:***

# APPENDICES

## Appendix 1: Regulations and Standards

Nucleic acid-based techniques have rapidly transformed almost every field of research, pharmaceutical development, and diagnostics. The need for globally comparable, accurate, and reliable results in the diagnostic field has driven the development of national and international standards as well as fostered a highly developed regulatory environment. Because NAT has become the most widely used of nucleic acid techniques, the majority of guidance documents and standards are related to NAT.[1] Virus-specific regulations and reference standards will be addressed in the Appendix to General Information chapter *Virology Test Methods* ⟨1237⟩. The following is a selective list of national guidance documents. For application-specific guidance the compendial user is referred back to the relevant regulatory agency for the most current guidance.

- FDA Center for Biologics Evaluation (CBER) "Nucleic Acid Based In Vitro Diagnostic Devices for Detection of Microbial Pathogens" (2005)
- FDA Center for Biologics Evaluation (CBER) "Guidance for Industry: Content and Format of Chemistry, Manufacturing and Controls Information and Establishment Description Information for a Biological In Vitro Diagnostic Product" (1999)
- FDA Center for Biologics Evaluation (CBER) "Guidance for Industry: In the Manufacture and Clinical Evaluation of In Vitro Tests to Detect Nucleic Acid Sequences of Human Immunodeficiency Viruses Types 1 and 2" (1999)
- FDA Center for Biologics Evaluation (CBER) "Guidance for Industry: Use of Nucleic Acid Tests on Pooled and Individual Samples from Donations of Whole Blood and Blood Components (including Source Plasma and Source Leukocytes) to Adequately and Appropriately Reduce the Risk of Transmission of HIV-1 and HCV" (2004)

## Appendix 2: Abbreviations

| | |
|---|---|
| AABB | American Association of Blood Banks |
| ACD | acid citrate dextrose |
| ASO | allele-specific oligonucleotides |
| bDNA | branched DNA assay |
| BMA | bone marrow aspirate |
| CE-LIF | capillary electrophoresis and laser-induced fluorescence |
| CCD | charge-coupled device |
| cDNA | complementary DNA |
| CPR | cyclic probe reaction |
| CsCl | cesium chloride |
| Ct | cycle threshold |
| DEPC | diethylpyrocarbonate |

[1] Reference materials for nucleic acid-based techniques are available from National Institute of Standards and Technology (NIST), ▲https://www.nist.gov/srm.▲ (ERR 1-Aug-2020)

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| | |
|---|---|
| DHPLC | denaturing high-performance liquid chromatography |
| DNA | deoxyribonucleic acid |
| DNase | deoxyribonuclease |
| dNTP | deoxyribonucleotide triphosphate |
| DMSO | dimethyl sulfoxide |
| DOP-PCR | degenerated oligonucleotide primed PCR |
| dsDNA | double-stranded DNA |
| ssDNA | single-stranded DNA |
| dUTP | 2′-deoxyuridine 5′-triphosphate |
| dTTP | 2′-deoxythymidine 5′-triphosphate |
| ESI | electrospray ionization |
| EDTA | ethylenediaminetetraacetic acid |
| FDA | Food and Drug Administration |
| FEN | flap endonuclease |
| FISH | fluorescent in situ hybridization |
| FFPE | formalin-fixed paraffin embedded |
| FRET | fluorescence resonance energy transfer |
| GLP | good laboratory practice |
| HCV | hepatitis C virus |
| HIV | human immunodeficiency virus |
| ICH | International Conference on Harmonization |
| LAPS | light-addressable potentiometric sensor |
| LCR | ligase chain reaction |
| LED | light-emitting diode |
| LNA | locked nucleic acid |
| MALDI | matrix-assisted laser desorption–ionization |
| MDA | multiple-displacement amplification |
| MOPS | 3-[*N*-morpholino]propanesulfonic acid |
| MS | mass spectrometry |
| mRNA | messenger RNA |
| NAT | nucleic acid amplification technologies |
| NASBA | nucleic acid sequence-based amplification |
| NTP | nucleotide triphosphate |
| OLA | oligonucleotide ligation assay |
| PAGE | polyacrylamide gel electrophoresis |
| PCR | polymerase chain reaction |
| PEP | primer-extension-preamplification |
| PPi | pyrophosphate |
| QA | quality assurance |
| QC | quality control |
| RCA | rolling circle amplification |
| RFLP | restriction fragment length polymorphism |
| RNA | ribonucleic acid |
| RNAse | ribonuclease |
| RT | reverse transcriptase |
| RT-PCR | reverse transcription-polymerase chain reaction |
| rTth | recombinant *Thermus thermophilus* |

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| | |
|---|---|
| SDS | sodium dodecyl sulfate |
| SNP | single nucleotide polymorphism |
| 3SR | self-sustained sequence replication |
| SSCP | single-strand conformation polymorphism |
| STR | short tandem repeat |
| Taq | *Thermus aquaticus* |
| Tm | melting temperature; the temperature at which 50% of the double-stranded nucleic acid molecule becomes single-stranded |
| TMA | transcription-mediated amplification |
| TOF | time-of-flight |
| UNG | uracil-*N*-glycosylase |
| WGA | whole-genome amplification |

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# ⟨1126⟩ NUCLEIC ACID-BASED TECHNIQUES—EXTRACTION, DETECTION, AND SEQUENCING

## NUCLEIC ACID EXTRACTION

### Introduction

The basic principles of nucleic acid amplification technology (NAT) and definitions of the various techniques are covered in *Nucleic Acid-Based Techniques—General* ⟨1125⟩. The current chapter covers general steps in the extraction and purification of nucleic acids from a variety of samples.

The expanding discipline of molecular biology in pharmaceutical and biomedical research and development is characterized by the rapid discovery of new markers for disease and technologies for their detection. Nucleic acid targets are isolated from a wide variety of specimens, and the quality and quantity of the extracted target are highly affected by specimen collection, handling, and choice of extraction procedure.

The analysis of complex organisms by molecular biological techniques requires the isolation of pure, high molecular weight genomic DNA and intact full-length RNA. The application of these techniques then allows the detection, identification, and characterization of the associated organism or adventitious agent. Recently developed tests employing purified human DNA enable genetic testing for the presence, predisposition, or carrier status of inherited diseases such as cystic fibrosis, hereditary hemochromatosis, or Tay–Sachs disease, to name a few examples, or the analysis of single nucleotide polymorphisms (SNPs).

DNase and RNase are the major sources of nucleic acid instability. Although both enzymes are ubiquitous and are easily released during nucleic acid extraction, RNases are far more stable and harder to inactivate than are DNases because they generally do not require co-factors in order to function. Minute amounts of RNase are sufficient to destroy RNA, so great care should be taken to avoid inadvertently introducing these enzymes into the sample during or after the isolation procedure. If RNA is collected for the specific application of gene expression analysis, researchers should keep in mind that the sample collection process itself can alter the resulting expression profile.

Because of the ubiquity of RNases, measurement of intracellular RNA targets has lagged behind that of DNA targets in contributing to patient management and characterization of targets for pharmaceutical purposes. However, RNA represents the current status of the organism and is an important tool for correlating a phenotype with its associated genetic activity. The unstable nature of RNA has made standardization of NAT tests difficult, and false negative results can easily arise from a poorly handled sample because of target degradation rather than from the absence of disease or regulation of gene activity. Nevertheless, commercially available isolation and detection systems provide a high level of standardization and robustness, resulting in the implementation of RNA-based assays in recent years. The following sections discuss general steps in the extraction and purification of nucleic acids from a variety of samples, focusing on (1) collection, handling and storage of samples; (2) disruption of samples; (3) subsequent extraction and purification of nucleic acids; and (4) storage of purified nucleic acids.

### Sample Source

The broad diversity of possible specimens requires different procedures for collection. For example, blood samples are collected in an appropriate anticoagulant- or additive-containing tube. Ethylenediaminetetraacetic acid (EDTA) and acid citrate dextrose (ACD) are the recommended anticoagulants for tests that require plasma or bone marrow aspirate (BMA) samples. When extraction from tissues is appropriate, the optimal amount of tissue is usually 1 to 2 g, depending on the type of tissue, because the amount of DNA and RNA per weight of tissue varies greatly from tissue to tissue. In general, more than 10 mg of tissue is required to obtain >10 µg of DNA or RNA. Because of the highly variable amounts and types of proteins and other contaminants present in different tissues, nucleic acid isolation protocols are tissue-specific, and a broad range of ready-to-use isolation systems are available from different manufacturers of kits for nucleic acid extraction. The tissue type also influences the stability of both DNA and RNA in specimens, and the two types of nucleic acid differ importantly with respect to sample preparation and downstream analysis. These issues are described later in the chapter.

### Pre-Analytical Steps and Sample Collection

Although the genetic makeup of the organism remains mostly unchanged over time, the mRNA population represents the current status of a cell under any given set of conditions, and thus is highly dynamic. To prevent degradation of mRNA and/or to preserve the original transcription pattern of the cellular mRNA, tissue should be placed immediately on ice or snap-frozen in liquid nitrogen. However, freezing disrupts the cellular structure and releases RNases. Hence, for RNA isolation in general (mRNA, ribosomal RNA, viral RNA, etc.), thawing in an RNase-inactivating buffer is essential. A more convenient procedure employs a stabilizing agent at ambient temperature. Several reagents for different types of sample material (e.g., tissue or bacteria) are commercially available. Vanadium salts were once used to inhibit RNase activity, but they have been superseded by the use of chaotropic agents for the inhibition of RNase and stabilization of RNA. The sample can easily be collected in such reagents and stored for several days to weeks prior to RNA isolation.

For reliable gene-expression analysis, the immediate stabilization of the RNA expression pattern and of the RNA itself is an absolute prerequisite. Directly after the biological sample is harvested or extracted, changes in the gene-expression pattern occur because of specific and nonspecific RNA degradation as well as transcriptional induction. Such changes in the gene-expression pattern should be avoided for all reliable quantitative gene-expression analyses, such as biochip and array analyses and quantitative reverse transcription-polymerase chain reaction (RT-PCR).

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The use of gloves while handling reagents and RNA samples is mandatory to prevent RNase contamination arising from contact with the surface of the skin or from laboratory equipment. In order to create and maintain an RNase-free environment, laboratory personnel should treat water or buffer solutions with diethylpyrocarbonate (DEPC), which inactivates RNases by covalent chemical modification. Care should be taken because DEPC is irritating to the eyes, skin, and mucous membranes and is also a suspected carcinogen. Alternatively, commercially available RNase-free solutions and reagents may be used. Commercially available RNase inhibitor proteins are also available for use in reactions but with different levels of effectiveness with respect to various RNase types. However, it should be noted that DEPC cannot be used with Tris-buffered solutions. Many scientists recommend the use of disposable vessels when working with RNA. Nondisposable glassware should be cleaned with a detergent, thoroughly rinsed, and oven baked at 240° for 4 or more hours before use (autoclaving alone will not fully inactivate many RNases). Alternatively, glassware can also be treated with DEPC. Nondisposable plasticware should be thoroughly rinsed with 0.1 M sodium hydroxide and 1 mM EDTA, followed by RNase-free water. Alternatively, chloroform-resistant plasticware can be rinsed with chloroform to inactivate RNases. The use of aerosol-resistant filter tips is also important for avoiding RNase contamination. These issues are not critical for DNA, and following the rules of Good Laboratory Practice (GLP) is generally sufficient for successful isolation of DNA.

As a general precaution, staff should follow all applicable safety precautions when handling tissue or body fluids (human or other). Some of these precautions (e.g., the use of disposable gloves) also prevent contamination of the sample. Applicable guidelines and standards for the collection and processing of human-derived materials have been published by the American Association of Blood Banks, the International Conference on Harmonization, and the FDA.

## Sample Disruption and Homogenization

Prior to extraction, source material is disrupted and homogenized. Disruption is the complete breakage of cell walls and plasma membranes of solid tissues and cells in order to release all DNA and RNA contained in the specimen. This is usually done using a lysis buffer that also inactivates endogenous nucleases. In addition to disrupting tissues, homogenization shears high molecular weight DNA and cellular components. During RNA isolation, scientists often must reduce the viscosity of cell lysates (caused by the presence of high molecular weight DNA molecules) prior to final isolation in order to make the subsequent extraction steps easier and more efficient. Incomplete homogenization may interfere with subsequent RNA purification steps (e.g., inefficient binding of RNA to silica membranes) and therefore result in significantly reduced yields. A typical procedure to shear high molecular weight DNA and homogenize the sample is to repeatedly pass the lysate through a small-gauge needle. However, this procedure is time-consuming and is not suitable for high throughput of samples. Better procedures to achieve complete disruption and homogenization of cells and tissue include rapid agitation in the presence of beads and lysis buffer (bead milling) or rotor–stator homogenization.

During the bead milling process, disruption and simultaneous homogenization occur by the shearing and crushing action of the beads as they collide with the cells. Disruption efficiency is influenced by the size and composition of the beads, the speed and configuration of the agitator, the ratio of buffer to beads, the disintegration time, and the amount of starting material. These parameters must be determined empirically for each application. For disruption with mortar and pestle, the samples should be frozen in liquid nitrogen and ground to a fine powder under liquid nitrogen. Standard safety precautions and the use of safety clothing to protect the skin and eyes should be employed when working with liquid nitrogen. Rotor–stator homogenizers are able to disrupt and homogenize animal and plant tissues within 5 to 90 seconds, depending on the sample. The rotor turns at very high speed, causing the sample to be disrupted by a combination of turbulence and mechanical shearing. Other alternatives are commercial spin-column homogenizers in combination with silica-membrane technology, which provide a fast and efficient way to homogenize cell and tissue lysates without cross-contamination of samples.

In order to achieve complete disruption, different sample types require different procedures. Cells from tissue culture grown as a monolayer or in suspension are easily disrupted by the addition of a lysis buffer that typically contains a mixture of an anionic detergent, a protease, and a chaotropic agent in a buffered salt solution. In contrast, nucleic acid isolation from fibrous tissues such as skeletal muscle, heart, and aorta can be difficult to disrupt because of the abundance of contractile proteins, connective tissue, and collagen. Fresh or frozen tissue samples should be cut into small pieces to aid lysis. Blood samples, including those treated to remove erythrocytes, can be efficiently lysed using a lysis buffer and a proteinase.

In general, the same procedures are applicable for extraction of DNA and RNA. For DNA isolation more gentle procedures are preferable, but during RNA isolation, cells and tissues can be disrupted using a mixer mill because there is no risk of shearing the RNA. Certain downstream applications require high molecular weight DNA, and care should be taken not to shear the DNA molecules and thus render the DNA unsuitable for further analysis.

## Extraction and Purification

Although several procedures are available for nucleic acid extraction, the suitability of a procedure depends on the starting material, the type and purity of nucleic acid isolated, and possibly the downstream application. The principal procedures are described below; several commercial kits are available to accommodate different sample types and applications.

### PHASE EXTRACTION

The original technique for extraction of DNA and RNA from lysed samples is phase extraction, which involves nucleic acid extraction using a mixture of phenol and chloroform. Depending on pH and salt concentration, either DNA or RNA partitions in the aqueous phase. At neutral/basic pH, the DNA remains in the aqueous phase, and RNA remains in the organic phase or in the interphase (with the proteins). However, at acidic pH, DNA in the sample is protonated, neutralizing the charge and causing it to partition into the organic phase. RNA, which remains charged, partitions in the aqueous phase. The two phases are separated by centrifugation, and the aqueous phase is re-extracted with a mixture of phenol and chloroform, followed by extraction with chloroform to remove any residual phenol. The nucleic acid is recovered from the aqueous phase by precipitation with alcohol. For RNA, this procedure is often combined with a protease digestion, alcohol or lithium chloride precipitation,

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and/or cesium chloride (CsCl) density gradients. A potential problem is contamination of the recovered DNA or RNA with organic solvents that may interfere with enzymatic downstream applications or spectrometry readouts.

### CESIUM CHLORIDE DENSITY GRADIENT CENTRIFUGATION

For the isolation of high molecular weight genomic DNA, CsCl density gradient centrifugation is the traditional procedure. Cells are lysed using a detergent, and the DNA is isolated from the lysate by alcohol precipitation. The DNA is then mixed with CsCl and ethidium bromide and centrifuged for several hours at a high g force (typically 100,000 × g). The DNA band, which can be visualized under UV light as a result of the intercalation of the ethidium bromide with the DNA, is collected from the centrifuge tube, extracted with isopropanol to remove the ethidium bromide, and then precipitated with ethanol to recover the DNA. This procedure allows the isolation of high-quality DNA, but it is time consuming and also a safety concern because of the high quantity of EtBr involved.

### ANION-EXCHANGE CHROMATOGRAPHY

An alternative procedure for the purification of high molecular weight genomic DNA is anion-exchange chromatography based on the interaction between the negatively charged phosphate groups of the nucleic acid and positively charged surface molecules on the anion-exchange resin. Binding occurs under low-salt conditions, and impurities such as RNA, cellular proteins, and metabolites are washed away using medium-salt buffers. Pure DNA is eluted with a high-salt buffer and is desalted and concentrated by alcohol precipitation. This procedure yields DNA of a purity and biological activity equivalent to two rounds of purification in CsCl gradients, but in much less time. The procedure also avoids the use of toxic substances, and it can be adapted for different scales of purification. DNA up to 150 kilobases (kb) in length may be isolated using this procedure. Several kits are available for the isolation of DNA based on anion-exchange technology, and procedures vary in processing times and the quality and size of the isolated DNA.

### SILICA TECHNOLOGY

The current procedure of choice for most applications is based on silica technology and can be used for isolation of full-length RNA or DNA with an average size of 20 to 50 kb. However, higher molecular weight DNA exceeding 100 kb is not efficiently extracted by this technology. The procedure relies on the selective adsorption of nucleic acids to silica in the presence of high concentrations of chaotropic salts. Although both types of nucleic acid adsorb to silica, the use of specific buffers in the lysis procedure ensures that only the desired nucleic acid is adsorbed while other nucleic acids, cellular proteins, and metabolites remain in solution. The contaminants are washed away, and high-quality RNA or DNA is eluted from the silica using a low-salt buffer. The silica matrix can be used as particles in suspension, in the form of magnetic beads, or as a membrane. This technique is suitable for high throughput, and several kits and automated systems are commercially available. However, these aqueous lysis buffers (in contrast to lysis buffers based on an organic solvent such as phenol) are not ideally suited for difficult-to-lyse samples (e.g., fatty tissues). Kits designed to facilitate lysis of fatty tissues and to inhibit RNases are available. Silica-based kits provide a fast and reliable procedure for both DNA and RNA purification and are commonly used for nucleic acid extraction.

Although these procedures yield pure nucleic acids, for some applications in which even trace contaminations with either RNA or DNA may interfere, pretreatment with DNase or RNase may be necessary. Alternatively, procedures that use specific probe capture may be used. Relevant applications requiring such ultra-pure nucleic acids are discussed in *Nucleic Acid-Based Techniques—Amplification* ⟨1127⟩.

## Specific Applications for Hard-to-Extract Materials

### EXTRACTION FROM FORMALIN-FIXED AND PARAFFIN-EMBEDDED BIOPSIES

The nucleic acids in formalin-fixed paraffin embedded (FFPE) biopsies are usually heavily fragmented and chemically modified by formaldehyde. Although formaldehyde modification cannot be detected in standard quality control assays such as gel electrophoresis, formaldehyde modification does interfere with enzymatic analyses. Sufficient extraction and demodification for DNA can be achieved by prolonged digestion with protease, but this will lead to heavy fragmentation and degradation of RNA. Some isolation systems have been optimized to reverse as much formaldehyde modification as possible without further RNA degradation. Nevertheless, RNA purified from FFPE samples should not be used in downstream applications that require full-length RNA. Some applications may require modifications to allow the use of fragmented RNA (e.g., designing small amplicons for RT-PCR).

### EXTRACTION FROM BACTERIA AND PATHOGENS

Although Gram-negative bacteria are relatively easy to lyse, Gram-positive bacteria or yeasts typically need an enzymatic pretreatment to remove the cell wall for efficient lysis. This methodology can be applied only to DNA isolation because the enzymatic treatment will influence the expression profile of the organism, and therefore RNA isolation requires a more rapid lysis procedure. Another factor to consider is that microorganisms normally occur against the background of a host or an environmental matrix (e.g., soil), which makes detection by polymerase chain reaction (PCR) often difficult because of inhibitory components. This means that the isolation procedure has to be carefully adapted and optimized for the specific organism and sample type. Commercial kits are available, and most are based on the use of lysozyme for the removal of cell walls.

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### SPECIAL CONSIDERATIONS FOR LIMITED SAMPLE AMOUNTS

Multiple genetic testing techniques, including SNP analysis, short tandem repeat analysis, sequencing or genotyping using arrays, real-time PCR, and other procedures depend on the availability of high-quality DNA. Because human genomic DNA or samples of individual genotypes are often limited, a process to immortalize nucleic acid samples can overcome this limitation. Procedures applicable to genotyping are discussed in *Nucleic Acid-Based Techniques—Genotyping* ⟨1129⟩. Whole-genome amplification (WGA) has recently been employed to amplify limited genomic DNA from already purified DNA or directly from clinical or casework samples without any DNA purification. Two basic technologies for WGA are available and are PCR-based or rely on isothermal multiple-displacement amplification. These applications are described in more detail in *Nucleic Acid-Based Techniques—Amplification* ⟨1127⟩.

## Sample Handling and Long-Term Storage

DNA is a relatively stable macromolecule, and once isolated it can be kept at 2° to 8° for at least 1 year. However, where DNA is present in very small quantities, such as in a test of residual DNA, it may be advisable to store the DNA at less than or equal to –20°. Generally, DNA is stored in solution. Distilled water can be used if DNA will be used for PCR and/or endonuclease digestion within a few days after its isolation. However, Tris–EDTA at pH 7.5–8.5 is the preferred buffer for DNA storage because DNA degradation can occur in water because of the limited buffering capacity of this medium. Purified nucleic acids retain recognizable characteristics during long-term storage, provided the samples are stored as frozen solutions. The DNA solution should be stored as a primary stock solution frozen at –80°. DNA can also be lyophilized and stored dry without the need for refrigeration. In some cases DNA can be stored for years on special filter papers that bind DNA and allow storage in a dried state at ambient temperature.

The ubiquity of RNases requires extra precautions when handling RNA. Isolated RNA should be kept on ice when aliquots are pipetted. Filter tips that prevent RNase carry-over from the pipette and sterile, disposable polypropylene tubes are recommended throughout the procedure because these tubes are generally RNase-free and do not require any pretreatment to inactivate RNases. Purified RNA can be stored at –20° or –80° in water. Under these conditions no degradation is normally detectable. Unlike DNA, RNA does not benefit from basic buffer solutions during long-term storage because of its sensitivity to alkaline conditions. Generally, if nucleic acid samples are required for multiple testing, RNA and DNA samples should be frozen in multiple aliquots at –80° for subsequent analysis in order to avoid repeated freeze–thaw cycles that can lead to degradation, and also to minimize the possibility of contamination, which could result in analytical inaccuracy.

# QUALITATIVE AND QUANTITATIVE EVALUATION OF NUCLEIC ACIDS

## Introduction

This section describes procedures that assess the purity, integrity, and quantity of purified nucleic acids, including spectroscopic procedures, electrophoresis of nucleic acid fragments, and probe-based techniques. Detection and quantitation by amplification are discussed in *Nucleic Acid-Based Techniques—Amplification* ⟨1127⟩.

### ABSORBANCE SPECTROSCOPY

The basic principles of spectroscopy are addressed in *Ultraviolet-Visible Spectroscopy* ⟨857⟩. For nucleic acids, absorbance is determined at 260 nm, but this procedure does not distinguish between DNA and RNA. Absorbance can also be used to estimate protein contamination in nucleic acids. Proteins maximally absorb at 280 nm, and nucleic acids maximally absorb at 260 nm. Thus the calculation of the A260/A280 ratio is used as an estimation of protein contamination in nucleic acid preparations. A ratio of 1.8 to 2.0 is considered desirable. As an example, double-stranded DNA has an extinction coefficient of 20 for 1 mg per mL of DNA at 260 nm and a coefficient of 10 at 280 nm. In contrast, for 1 mg per mL of protein, the extinction coefficients are on the order of 1 at 280 nm (depending on tyrosine and tryptophan content) and 0.57 at 260 nm. Thus a large protein contamination could exist at a 260/280 ratio of greater than 1.8 because of the lower sensitivity of protein absorbance. In addition, the change of absorbance of DNA with wavelength ($\Delta A/\Delta\lambda$) is steep at 280 nm, and this could lead to an incorrect determination if the spectrophotometer is out of calibration. The peak at 260 nm is broad, and thus readings are less sensitive to calibration issues.

Information on contamination by nonproteinaceous materials can be provided by a scan of DNA from 220 nm to 320 nm. Pure DNA has a mostly symmetric peak around 260 nm, zero absorbance at 320 nm, and a minimum at 230 nm. Absorbance rises again from 230 nm to 220 nm. Interfering substances can co-purify with DNA and absorb in the lower UV range (around 230 nm). These substances can interfere with and lead to an overestimation of DNA content, thus showing the utility of a scan —or at least a measurement of absorbance—at 230 nm in addition to 260 nm and 280 nm. Absorbance above 300 nm can arise from other contaminants and particulate matter. Common reagents used in the isolation of DNA, particularly solvents such as phenol and alcohols if they are not completely removed, can interfere with DNA absorbance measurements. Analysts should be aware of the limitations of this type of measurement. Finally, the absorbance of DNA and the 260/280 ratio is dependent on ionic strength—a difference as large as 30% can exist. Absorbance of genomic DNA is higher, and the 260/280 ratio is lower in pure water when compared with the same DNA in a buffer or a salt solution.

For the purposes of quantitation of nucleic acids, the respective extinction coefficients for DNA and RNA are used. An absorbance of 1 in a 1-cm cuvette corresponds to 50 µg per mL of double-stranded DNA [$E$ (specific absorption coefficient) = 0.02 (µg per mL)$^{-1}$ cm$^{-1}$]. The specific absorption coefficient for RNA at 260 nm is $E$ = 0.025 (µg per mL)$^{-1}$ cm$^{-1}$ (absorbance of 1.0 corresponds to 40 µg per mL), and for single-stranded DNA $E$ = 0.027 (absorbance of 1.0 corresponds to 37 µg per mL). A solution of DNA is read against a blank of the same buffer solution in which the DNA is dissolved. Ideally, readings should fall within a range of 0.1 to 1.0 absorbance for adequate linearity. Absorbance above 1.0 becomes increasingly nonlinear as

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the absorbance rises. The accuracy of readings below 0.1 (5 µg per mL DNA) depends on the quality and noise level of the spectrophotometer.

## Fluorescence Protocols for DNA and RNA Quantitation

Cyanine dye derivatives are used for the quantitation of nucleic acids because they specifically interact with nucleic acids (DNA, RNA, and oligonucleotides) and fluoresce only upon binding. The exact mechanism of interaction is not always fully understood but may involve intercalation in double-stranded DNA and surface binding.

Measurements can be performed using a fluorometer or a plate reader. The sensitivity of fluorescence with these dyes is much higher than that of absorbance, which gives these dyes great utility when DNA concentration is low (down to 25 pg per mL). The dye must be protected from light to avoid photobleaching. Linearity is maintained over three to four orders of magnitude. Calf thymus and Lambda phage DNA are often used as calibrants to construct a standard curve. Some of these dyes have been optimized to bind double-stranded DNA or single-stranded RNA and oligonucleotides. A DNA-binding dye will also bind to single-stranded DNA and RNA but at low ionic strength, and the signal is about 10% or less than that seen with double-stranded DNA for an equivalent mass of material. Thus, this methodology is preferred for DNA measurements when no effort has been made to remove RNA from the preparation. Another fluorescent dye is available and is optimized for RNA measurements. Using two different concentrations of this dye, analysts can detect RNA in amounts as low as 1 ng per mL and as high as 1 µg per mL. The dye also fluoresces with DNA but does not display an equivalent ability to minimize binding by the use of particular conditions (e.g., with DNA and the double-strand binding dye). Quantitation may be affected by contaminating nucleic acid (e.g., DNA in an RNA preparation and vice versa). Treatment with a DNase is needed if DNA is present in the RNA preparation. Proteins are unlikely to interfere with these dyes, but some detergents as well as phenol result in loss of fluorescence. Nucleic acid extraction reagents should thus be checked for effect on subsequent fluorescent assays.

Bisbenzimide fluorochrome dyes such as (2′-[4-hydroxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5′-bi-1*H*-benzimidazole) represent another option for measuring DNA. Researchers have studied the binding of these dyes to the minor groove of DNA and have found that sequences of adenine or thymine in the DNA sequence provide a minor groove dimension that binds the dyes best. Thus the fluorescent signal can show DNA sequence dependence, and the calibrant DNA should have a nucleotide composition that is similar to that of the DNA to be measured. These dyes are not as sensitive as cyanine dyes but are more sensitive than absorbance measurements. Low dye concentrations and high ionic strength are required in order for analysts to distinguish double-stranded DNA from RNA. Low ionic strength conditions are required in order to differentiate double-stranded DNA from single-stranded DNA.

## Detection by Size

### AGAROSE GEL ELECTROPHORESIS

Agarose gel electrophoresis provides a simple and accurate procedure for separating nucleic acids by fragment size. The technique can be adapted to separate fragments over a large range of sizes and can be used in a preparative or analytical fashion. For example, gel electrophoresis can be used to verify that a product of a PCR reaction is of the correct size. DNA fragments can be retrieved from a gel slice and provide a sufficiently pure PCR product for cloning or sequencing. The general integrity of an RNA preparation can be determined by gel electrophoresis as well. The stoichiometry of the nucleic acid fragment size (in base pairs) and negative charge from the phosphate provide the basis for the separation. With the exception of plasmids, electrophoresis is generally free of DNA conformation-induced effects. Supercoiled plasmid DNA will migrate ahead of linear or open-circle/nicked plasmid, which is useful for determining the conformation of a plasmid preparation. In contrast, denaturing gels are used for RNA because of RNA's tendency to form inter- and intramolecular secondary structures.

Agarose gel electrophoresis utilizes a horizontal setup wherein the gel is cast in a box and placed on a bridge between two buffer compartments that are filled with the buffer of choice. The gel is also covered with a thin layer (~1 mm) of buffer. Although the main electrical resistance resides in the gel itself, there is sufficient charge on the nucleic acids to move fragments through the gel toward the anode. The fragments move in proportion to size, the smallest moving the fastest. The parameters that most affect electrophoresis are gel pore size, buffer concentration, and the voltage gradient. The ability to separate the fragments of choice is largely a function of the gel pore size, which depends on agarose concentration. Generally the agarose concentration is in the range of 0.5% to 1.0% for DNA fragments of <100 to 25,000 base pairs, and the higher concentration is used when it is important to distinguish the smallest fragments. Lowering the agarose concentration in the gel results in the resolution of larger fragments but also in a loss of resolution of small fragments. For the largest fragments pulsed, (reversed)-field electrophoresis is utilized.

To achieve uniform electrophoresis, all of the agarose must be completely melted. Electrophoresis-grade agarose is dissolved in the same buffer that will be used for electrophoresis. The buffers most commonly used for DNA separations are TBE (tris-borate-EDTA) or TAE (tris-acetate-EDTA). TBE has a higher buffering capacity than TAE, but TAE should be used if the DNA is going to be retrieved from the gel. Denaturing RNA gels use MOPS buffer (40 mM MOPS, 10 mM sodium acetate, 1 mM EDTA, pH 7.0). Melting the agarose is conveniently achieved with the assistance of a microwave oven. The agarose will easily come to a boil, but this may not result in complete melting of the agarose, which may require bringing the solution to a boil several times, with intermittent mixing and holding periods, until the agarose is completely melted. Agarose particles transform from white to transparent before melting. Any partially melted agarose can be detected by swirling the flask while holding it up to the light. If the solution does not appear uniform, then it requires additional heating. The agarose is poured into the gel box after partial cooling but before setting up. Commercially available ready-to-use gels suitable for a particular application can also be used. For RNA-denaturing gels, formaldehyde is added under a fume hood to the melted agarose to a final concentration of 2.2 M or 6.7%. Before the agarose has hardened, the analyst places a comb in the gel to provide wells for the samples and size standards. Once solidified, the gel is placed in the electrophoresis box, and buffer is added until both sides are filled and there is a layer of buffer across the surface of the gel. Then 10X tracking buffer (40% sucrose with 0.25% bromphenol blue or

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0.25% xylene cyanol or both) is added to each DNA sample to increase the sample density and to provide a tracking dye that is used to assess when the electrophoresis is finished. The increased density allows the sample to be transferred into the well and to remain there until it migrates into the gel during electrophoresis.

One or more lanes should be used for a DNA size standard containing fragments in the range that is relevant to the samples and agarose concentration. Size standards in various ranges are readily available. Bracketing the samples in wells between standards is useful to determine whether the electrophoresis gradient has been uniform over the width of the gel. However, in the case of eukaryotic RNA preparations, the 18S and 28S ribosomal RNAs that are co-extracted from prominent bands (corresponding to 1900 and 4700 nucleotides) can also be used as size standards. In addition, the rRNA provides information on the RNA integrity because missing or fuzzy rRNA bands indicate problems with the quality of the RNA preparation. Once the wells are filled, the cover is placed over the gel box, and the box is connected to the power supply. The indicator dye in the tracking buffer added to the samples and size standard allows the easy determination of how far the electrophoresis has proceeded. Bromophenol blue will migrate with DNA fragments of <500 base pairs, and xylene cyanol will migrate with fragments of 5000 base pairs.

The power supply is frequently run under conditions of constant voltage (1 to 10 V per cm) of gel length. Elevated voltage can cause high current, resulting in the generation of damaging heat and exhaustion of the buffer.

## PULSED-FIELD ELECTROPHORESIS

This variation on agarose gel electrophoresis is used to separate a range of large DNA fragments and is most useful when resolution of 50,000 to 200,000 base-pair fragments is needed. The main difference is the addition of an alternating-field device that controls the power supply operating under constant voltage. Large fragments of DNA change conformation in order to move through the agarose pores, and the larger pieces take longer to readjust when the field is reversed and thus move more slowly than do smaller fragments. This allows resolution of fragments over the period of hours that the pulsed-field procedure operates. A commonly used ratio of forward to reverse is 3:1, and, in addition, the procedure typically calls for a stepwise increase in the unit time between reverses of the field. Electrophoresis may continue for 10 to 16 hours to avoid fluctuation in gel temperature, viscosity, and other properties that may cause artifacts.

## POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

The format for performing PAGE is quite different from that for agarose gel electrophoresis. The general procedure for PAGE is described in *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* ⟨1056⟩. For resolution of small fragments of DNA in the 10 to 500 base-pair range, nondenaturing polyacrylamide gel electrophoresis is more suitable than agarose gel electrophoresis because separation of fragments of this size requires much smaller pore size than is achievable in agarose gels. The gel is prepared by polymerization of acrylamide monomers. The percentage of acrylamide dictates the range of fragment sizes that can be best resolved. For example, 20% acrylamide is suitable for the 10–100 base-pair range, and 5% acrylamide is useful in the 100–500 base-pair range. Commercially available ready-to-use polyacrylamide gels suitable for the particular size discrimination can also be used. The separated nucleic acids are visualized by staining with, for example, silver nitrate solution rather than with ethidium bromide or cyanine dye. However, staining with silver nitrate is laborious and time-consuming and not suitable for preparations that contain a large amount of protein, because proteins will also stain with silver nitrate.

## CAPILLARY ELECTROPHORESIS AND LASER-INDUCED FLUORESCENCE (CE–LIF)

CEF has been used for many years to separate DNA fragments (for the general principles of CE, see *Capillary Electrophoresis* ⟨1053⟩). The procedure relies on a principle similar to that underlying agarose gel electrophoresis. CE can utilize the cross-linked buffer systems applied in gel electrophoresis, but the technique can also use polymer-containing solutions (e.g., polymethylcelluloses) that are designed to create pores that entangle proteins. These polymer solutions may be added to the capillary between injections, allowing a "fresh" gel prior to each run. In addition, capillaries can be used for more injections than are possible for polymerized gel-filled capillaries. The resolving power of the separation depends on the size of the pores, which is based on the composition of the gel. Kits are available to separate fragments into the desired size ranges. Fragment sizes outside the resolution window can possibly be separated, but the separation may not be reliable or reproducible when the gel capability is exceeded.

Fragments can be detected by a variety of mechanisms. Detection utilizing UV absorbance is possible, but the preferred and most common detection procedure is laser-induced fluorescence (LIF). Fluorescence offers improvements over UV detection in terms of selectivity and sensitivity. In addition, the detection limits for fluorescence are two to three orders of magnitude better than those for UV. Although DNA is intrinsically fluorescent, the background fluorescence and complex laser spectroscopy required preclude routine use. The most common way to label DNA is described in the section above on fluorescent protocols for RNA and DNA quantitation. This system is widely employed because of its simplicity (the dyes are added to the sample or into the reaction buffer) and effectiveness. The advantages of CE include speed of analysis, sensitivity using minimum sample volumes, and the potential for automation. These are achieved mainly by the inherent miniaturization of the gel. Automated systems allow robust analysis of the quality, quantity, and fragment size of both RNA and DNA. CE applications have been especially important for evaluating the integrity of RNA because of the instability and progressive degradation of RNA caused by ubiquitous RNases, and new technologies that compare the ratios of 28S and 18S are improving the capabilities of these procedures.

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# FILTER HYBRIDIZATION AND IN VITRO LABELING OF PROBES

## Introduction

Hybridization techniques were used early in molecular biology to identify individual nucleic acids and to estimate the degree of similarity between species. Hybridization is widely used in the procedures described in this and other chapters to visualize and identify nucleic acid sequences (see *Nucleic Acid-Based Techniques—Amplification* ⟨1127⟩, *Nucleic Acid-Based Techniques—Genotyping* ⟨1129⟩, and *Nucleic Acid-Based Techniques—Approaches for Detecting Trace Nucleic Acids (Residual DNA Testing)* ⟨1130⟩). With the advent of restriction endonuclease digestion of DNA and electrophoretic separation by molecular mass, hybridization using labeled probes provided a way to visualize the organization of genes within a specific genome.

The hybridization techniques described are dot and slot blotting, Northern blotting, Southern blotting, in situ hybridization, and fluorescent in situ hybridization (FISH). All these techniques rely on the use of nucleic acid probes. Probes are oligonucleotides with specific DNA or RNA sequences that have been labeled with radioactive, fluorescent, chemiluminescent, chemical tags or enzymes (reporter molecules). Hybridized probes bind to complementary sequences on the target nucleic acids and are used to visualize and characterize targets, as described below.

## Dot and Slot Blotting

Dot blotting is the simplest and quickest of the hybridization techniques. The nucleic acids are directly applied to a support membrane, which may be a nitrocellulose or nylon membrane, without prior separation of the nucleic acid species by agarose gel electrophoresis. The nucleic acids are spotted onto the filter using a micropipettor or an apparatus such as a dot blot or slot blot apparatus. This consists of a membrane frame with a membrane sandwiched in between the two pieces of the frame. The bottom frame plate is connected to a vacuum manifold, and the top piece of the frame has slots through which the nucleic acids are loaded. The samples are loaded under vacuum and pulled through the membrane by vacuum, with the nucleic acid binding to the membrane, and then the filter is air-dried. The nucleic acids are fixed to the filter either by heating to 80° for nitrocellulose membranes or by exposure to UV light for a predeterrmined time for nylon filters. Hybridization with a labeled probe provides confirmation of the identity of the nucleic acid but does not provide any information about the number or sizes of the species. The nucleic acid species of interest can be quantitated by spotting known concentrations of the purified nucleic acid on the filter and comparing the signal generated by the unknown samples with those of the standard preparations.

## Southern Blotting

Southern blotting refers to the transfer of DNA from an agarose or polyacrylamide gel to a nitrocellulose or nylon membrane. Small, single-stranded DNA probes can then be used to visualize and identify the DNA species of interest. Southern blot analysis is based on a transfer and immobilization methodology developed in 1975, coupled with the electrophoretic separation of fragmented DNA. More specifically, the procedure typically is used to identify specific nucleic acid sequences in the context of a defined genetic topography, such as a restriction endonuclease map. The position of genes within the viral genome can be accurately mapped using a variety of restriction endonucleases in combination with Southern blot analysis. The procedure requires that DNA be obtained in sufficient quantity for analysis. Fragmented DNA is separated according to size using agarose gel electrophoresis. Double-stranded DNA fragments must be denatured before they are transferred and immobilized on a membrane by capillary action. The immobilized DNA is then cross-linked to the filter, which may be composed of nitrocellulose or nylon, as described above. However, the use of positively charged nylon membranes eliminates the need to fix the DNA to the nylon membrane. Nitrocellulose membranes are more fragile and may be probed up to 3 times with separate probes. Nylon membranes are more robust and may be probed 10 to 12 times, but they may present more background noise, particularly when they are used with chromogenic probes.

## Northern Blotting

Northern blot analysis comprises a series of steps for the separation, transfer, and immobilization of RNA in a manner similar to the treatment of DNA using Southern blot analysis. Denaturation of the RNA is required to reduce secondary structure to ensure that the RNA separates in the agarose uniformly according to length. Denaturation of RNA is accomplished either prior to electrophoresis using glyoxal or dimethyl sulfoxide (DMSO) or during electrophoresis by means of gels that contain formaldehyde. Transfer is achieved in a manner identical to that used for Southern blotting. However, in the case of Northern blotting, it is unnecessary to denature the RNA prior to transfer because denaturation is accomplished before electrophoretic separation of the RNA species. The immobilized RNA is cross-linked to the membrane in a manner similar to the cross-linking of DNA.

## In Situ Hybridization and Fluorescent In Situ Hybridization (FISH)

Hybridization of a nucleic acid in situ classically refers to determining the location of that nucleic acid sequence in its natural state—in tissue, in individual cells, or on a chromosome. In situ hybridization probes are designed to bind to complementary nucleic acid sequences, whether they be DNA or RNA. The purpose of these hybridization procedures is to discover where in a tissue a gene is being expressed, in which case the target is RNA, or to map a specific DNA sequence to its location on a chromosome, in which case the target is DNA.

Chromosome mapping of DNA sequences is accomplished by chemically attaching silver grains to the probe sequences and then counting the density of the grains in a metaphase chromosome spread. Although, historically, these procedures worked well, sensitivity was always an issue. The solution was to use a reporter that was more sensitive and safer than the other reporters,

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namely, fluorescence used in the technique of fluorescent in situ hybridization (FISH). FISH has an additional benefit in that the different colors available in fluorescence afford the ability to observe multiple hybridization events simultaneously, a feature not available with other detection systems.

### Detection of DNA and RNA in Hybridization Assays Using Labeled Probes

Visualization and location of individual nucleic acid species of interest are achieved by the specific hybridization of DNA or RNA probes that are labeled for easy visualization. The filter or sample (fixed cells or tissues in the cases of in situ hybridization and FISH) is incubated with the labeled probe at an appropriate temperature and salt concentration that allows hybridization of desired stringency. This is followed by washing with buffers of varying detergent and salt concentrations and at varying temperatures in order to minimize background signal due to nonspecific hybridization. The labeling and types of probes are discussed below.

Probes can be RNA probes generated in vitro or DNA probes, either double-stranded fragments, plasmids, or single-stranded oligonucleotides containing moieties to facilitate detection of fragments that contain portions of the gene of interest. Probes can be labeled with radioactive tracers such as $^{32}P$ or $^{35}S$ by incorporation of a labeled nucleotide in the probe sequence or with a nonradioactive label such as biotin by incorporation of a modified base, such as adenine monophosphate linked to biotin. Radioactive probes are visualized with X-ray film placed over the blot. Biotin-labeled probes are detected with a conjugate of streptavidin–alkaline phosphatase. An enzymatic reaction is run with alkaline phosphatase and a substrate that yields an insoluble colored product at the site of the probe. Variations on nonradioactive probes utilize other modifications to the DNA and linked antibody–alkaline phosphatase, as well as chemiluminescent probes that are detected on film.

Nucleic acids can be synthesized and manipulated by either enzymatic or chemical means. These same systems can be used to modify nucleic acid structure and to introduce foreign moieties to create unique molecules that can provide an advantage to the detection of limiting viral nucleic acids against a background of host nucleic acids. The chemical synthesis of nucleic acids and their purification has become routine, and high-quality synthesis and purification are commonly achieved. Moreover, larger segments can be synthesized, and when even larger segments are required, the subsections can be designed for concatenation and ligation.

Custom synthesis of DNA oligonucleotides is readily achievable in the laboratory using commercially available reagents and equipment. Alternatively, probes can be custom ordered from numerous commercial providers. Size-exclusion procedures for purification generally are used to eliminate incomplete oligonucleotides. RNA oligonucleotides also may be chemically synthesized or generated in vitro using complementary cloned DNA fragments under the control of various prokaryotic RNA polymerase promoter sequences. The use of DNA probes is much more common, but there may be some applications in which the increased association of RNA–RNA or RNA–DNA hybrids is advantageous.

The principal procedures of labeling DNA are direct labeling using a kinase reaction to attach a labeled nucleotide to the end of each DNA strand, by incorporating labeled nucleotides into a nicked DNA by utilizing the DNA repair function of the Klenow fragment of *Eschericia coli* DNA polymerase I enzyme (nick translation), and by PCR. This last procedure generates a relatively higher yield of internally labeled probe because each round of thermal cycling doubles the amount of labeled probe, whereas the former procedures result in a ratio of less than one probe molecule per template molecule. The PCR procedure also is used to generate unique probes with a variety of moieties located at the termini.

## NUCLEIC ACID SEQUENCING

### Introduction

The first DNA sequencing procedure, described in 1977, utilized chemical cleavage to specifically introduce chain breaks in a DNA sequence (Maxam and Gilbert sequencing). The procedure proved to be of significant utility in the early years of molecular biology, but it has not been used to perform high-volume sequencing and therefore is not discussed in detail here. The majority of sequencing performed today is based on the dideoxysequencing procedure, also described in 1977 (Sanger sequencing). This procedure fundamentally changed sequencing by exploiting the enzymatic specificity of polymerases that introduce strand interruptions at specific bases. This is the most widely recognized sequencing procedure and is considered a routine assay in molecular biology laboratories. Innovations in instrumentation, sample preparation and collection, data management, data analysis, and sequence assembly have relied on this sequencing procedure as their fundamental sequence generator.

High-throughput sequencing takes all the elements of the sequencing procedures and applies them to a mass collection of sequence data, typically for larger genomes, but high-throughput sequencing certainly may be used for smaller projects as well. Obtaining the final sequence information includes all processes associated with sample preparation, sequencing, data assembly, and data finishing. The technology to achieve these individual objectives includes the instrumentation, disposables, protocols, and procedures.

### Sequencing Reaction

The dideoxysequencing procedure takes advantage of specificity of the Klenow enzyme to introduce chain-terminating nucleosides, called dideoxynucleotides, intermittently during the polymerase extension process. The sequencing of each sample requires four separate reactions (one for each base). The resulting mixture of various nucleotide chain lengths is then separated on the basis of individual molecular masses. The incorporation of radioactively labeled nucleotides during the sequencing reaction permits the detection of the nucleotide chains.

Improvements in biotechnology have led to the discovery of more robust enzymes with high fidelity, improved stability, and other attributes that have led to longer reads and improved sequence fidelity. These improvements have made possible the introduction of cycle sequencing, which is now commonly used. The principle of the cycle sequencing procedure is a

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combination of Sanger sequencing and aspects of PCR amplification, whereby dideoxynucleotides are incorporated into the amplified DNA. Cycle sequencing leads to a higher concentration of labeled fragments covering a wider range of sizes than does Sanger sequencing, leading in turn to a higher read length.

## Separation Procedures for DNA Sequencing Fragments

The previous sections of this chapter deal with the treatment of intact DNA and RNA molecules; the following sections address the challenges of separating the fragments that result from the sequencing reactions, notably slab gel sequencing and capillary electrophoresis. Subsequent sections address detection technologies and sequence integrity.

## Slab Gel Sequencing

Polyacrylamide gel electrophoresis, frequently referred to as slab gel electrophoresis, was the first separation mechanism employed for the separation of DNA sequencing fragments. As described above, the electrophoretic separation of DNA fragments is driven by the size of the fragments in the reaction mixture. However, for slab gel sequencing the pore sizes are chosen so that single-base resolution for many hundreds of bases is possible. In addition to the polyacrylamide in the gel, a denaturant such as urea is frequently included to ensure denaturation of the fragments. Until the implementation of multicapillary sequencing systems, the separation power and throughput of slab gel separation mechanisms were often considered state of the art.

## Capillary Electrophoresis Sequencing

As noted above, capillary electrophoresis offers significant advantages over gel-based separations. However, as with slab gel sequencing, the pore sizes are chosen so that single-base resolution for many hundreds of bases is possible. Multicapillary systems that utilize 8 to 384 capillaries are commercially available. These systems are the primary systems used for large-scale DNA sequencing, and, theoretically, they yield more than 1.1 billion base pairs of DNA sequences per year.

## Detection

### RADIOACTIVITY

The first detection strategies for DNA sequencing reactions utilized radioactive isotopes such as $^{32}P$ or $^{35}S$, primarily because these were practical for gel separations. The advantages are that detection is universal, low limits of detection are possible, mobility shifts are eliminated, and fidelity differences for the DNA polymerases do not occur. Disadvantages include the high disposal and safety costs, the inability to multiplex (ultimately limiting throughput), and the need for 24 to 36 hours of exposure time (i.e., no real-time detection).

### FLUORESCENCE

Fluorescence dyes have largely replaced radioactive isotopes as detection tools during DNA sequencing, mainly because they do not have the disadvantages of radioactive probes. Because the dyes can be discriminated by means of their emission maxima, multiplexing is possible, so four sequencing reactions per sample can be replaced by a single reaction using four different labels. Thus a single lane can be used rather than the four separate lanes that were necessary with radioactive probes. Additional advantages are higher throughput and automated data collection in real time.

### MASS SPECTROMETRY

Mass spectrometry (MS) has revolutionized the field of biochemistry and has significant potential in the area of nucleic acid sequencing. Soft-ionization techniques such as electrospray ionization and matrix-assisted laser desorption–ionization have expanded the potential application of MS to DNA sequencing. MS offers some advantages over other detection methodologies, including speed of fragment detection (signal acquisition is in the range of microseconds versus hours for conventional approaches) and accuracy (e.g., the molecular mass of each fragment can be determined with a high degree of accuracy). The Sanger procedure makes use of mass differences of the fragments generated as part of the polymerization reaction. MS is sufficiently precise to resolve fragment sizes that differ by only one base pair. Unfortunately, the sensitivity of MS detection suffers as fragment length increases, and the 100-base-pair barrier has yet to be crossed.

More recently, other sequencing technologies have emerged that are based on massively parallel sequencing techniques that attempt to achieve low-cost sequencing. These techniques are based, for example, on solid-phase sequencing or they make use of highly parallel and miniaturized pyrosequencing, which is described in *Nucleic Acid-Based Techniques—Genotyping* ⟨1129⟩.

## Sequence Integrity

A prerequisite for automated data collection and interpretation is that the data must be of good quality, which means minimizing human intervention and allowing the system to make base identifications following detection steps. It is a critical step to ensure accurate base identification by minimally sequencing both strands of the DNA several times. In addition, other tactics may be employed, such as using primers at different sequence positions, which can improve the accuracy of the developed consensus sequence. This task can be facilitated by the use of specialized software packages that are commercially available. More recent technology developments have produced alternative sequencing platforms that are more amenable to

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large-scale sequencing projects. These techniques include array-based platforms on which short stretches of target are sequenced on a chip that supplies raw data to sophisticated computational programs that reconstruct the sequence. Other sequencing approaches have been developed for the rapid sequencing of short nucleic acid sequences such as oligonucleotides of short PCR products. These technologies include MS-based and pyrosequencing platforms, the latter of which is described in *Nucleic Acid-Based Techniques—Genotyping* ⟨1129⟩.

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# ⟨1127⟩ NUCLEIC ACID-BASED TECHNIQUES—AMPLIFICATION

## INTRODUCTION

The basic principles of nucleic acid amplification technologies (NAT) and definitions of the various techniques are covered in *Nucleic Acid–Based Techniques—General* ⟨1125⟩. The current chapter covers major techniques that result in amplification of targeted nucleic acid sequences. The most common NAT assay is the polymerase chain reaction (PCR), which was first described by Kary Mullis. This procedure has been further refined to amplify a DNA fragment starting from RNA (reverse transcription-PCR, or RT-PCR). Initially, PCR was used in a qualitative manner to amplify and detect DNA molecules because its exquisite sensitivity paired with its high specificity made it a useful tool for the detection of nucleic acid targets. Since its inception, the number of PCR applications has expanded rapidly, and the technique, which now includes quantitative and multiplex assays, is currently used in almost every field of research and development in biology and medicine. In addition to the changes and improvements to the original design of the PCR procedure, alternatives to PCR are techniques used to amplify target nucleic acids to generate RNA instead of DNA amplicons. The most commonly used techniques are nucleic acid sequence–based amplification (NASBA) and the transcription-mediated amplification (TMA) which are described here in detail. In contrast to PCR, which relies on incubating the sample at three different temperatures, NASBA and TMA are based on isothermal conditions.

In addition to amplification of the target nucleic acid, the amplification step also can be directed at the signal used for detection (signal amplification). The most commonly employed technique is the branched DNA (bDNA) assay, in which the signal, typically a fluorescent probe that binds to the target sequence, is amplified. The bDNA assay is used predominantly for viral nucleic acid detection and quantitation.

This chapter describes the main assay components necessary for a PCR procedure and includes a discussion of the general optimization of PCR assays. The various PCR assay formats, including PCR, nested PCR, and RT-PCR are covered, and a discussion of the detection of the resulting amplicons follows. Although all these assays are essentially qualitative procedures, they can be modified for semiquantitation, and the various modifications are described. For accurate and reliable quantitation, real-time PCR has now replaced the methods listed above; real-time PCR and real-time RT-PCR are described in the *NAT Assays* section. The same section includes a discussion about probes and dyes that are an essential component of real-time PCR and the methods of quantitation using the generation of standard curves. The next PCR technique discussed is multiplex PCR, which is used for simultaneous detection of multiple targets or for normalization of assay results. Apart from PCR, the major alternative NAT tests that are used routinely, primarily in blood screening and clinical diagnostic screening are NASBA and TMA. The final technique described is whole-genome amplification, wherein the complexities of amplification require modifications to the PCR procedures. The chapter concludes with a discussion about the evolution of instrumentation used in NAT assays and the quality assurance and quality control issues associated with NAT because this is probably one of the most highly regulated biological techniques, especially when applied to blood screening.

## ASSAY COMPONENTS

### Enzymes

The essential components for NAT assays—polymerases, reaction buffers which include desoxynucleotides, ions, primers, probes, and fluorescent dyes—can be chosen from a broad selection of commercially available NAT reagent kits and vendors. Polymerases suitable for NAT applications can, in principle, be grouped into *Taq* DNA polymerases or DNA I polymerases from other *Thermus* species that are polymerases with features that are similar to those of *Taq* DNA polymerase. In addition, so-called proofreading polymerases are available (e.g., from *Pyrococcus* species) that display a 3′–5′ exonuclease activity capable of removing wrongly incorporated DNA bases from the growing DNA strand under amplification conditions. *Taq* DNA polymerase is the standard NAT enzyme and is the one most often used in NAT assays. Modifications of *Taq* DNA polymerase, such as deletions of the 5′–3′ exonuclease domain (Klenow fragment, Stoffel fragment) or point mutations for improved incorporation of dideoxynucleotides are also employed (e.g., for PCR-based sequencing reactions). Proofreading DNA polymerases or mixtures of *Taq* DNA polymerase with a proofreading polymerase are used if either fidelity of the NAT product is critical (e.g., for DNA cloning experiments) or longer NAT products are to be amplified. For RT–PCR, a reverse transcriptase is necessary to first convert the RNA target to copy DNA (cDNA) that can subsequently be amplified. For TMA reverse transcriptase with an RNAse H activity is needed to convert the RNA target to double-stranded template DNA, while for NASBA exogenous RHAse H is added to the reaction mixture. Depending on the reaction environment, two types of enzymes can be used to generate cDNA: a reverse transcriptase isolated from retroviral sources or a DNA polymerase that can function both as reverse transcriptase and DNA polymerase. Finally, chemical modification of the polymerase, resulting in an inactive enzyme at temperatures below 90°, is now typically used to prevent mispriming of templates at sub-optimal temperatures (see section on *Assay Optimization*).

### Reaction Buffers

Reaction buffers vary with respect to ion composition, pH, and additives and are sometimes specifically adopted for particular applications such as multiplex PCR, real-time PCR, RT–PCR, TMA and NASBA. An important component of the reaction mixture is $Mg^{2+}$ ions, or, in the case of polymerases with both reverse transcriptase and DNA polymerase functions, such as *Thermus thermophilus* (*Tth*), $Mn^{2+}$ ions. Other additives that enhance the sensitivity and specificity of the assay may be present. The concentration of the four deoxynucleotide triphosphates (dNTPs) must be optimized.

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## Primers

Primer sets are oligonucleotides with sequences that are designed specifically to prime the amplification of a portion of a target nucleic acid of interest. Synthetic oligonucleotide primers for both standard PCR and for real-time or quantitative PCR are designed for the specific recognition of and binding to a single DNA or RNA sequence. Such specificity is achieved through design that involves both the length and the sequence of the primers. Length and sequence specifications have separate criteria that must be simultaneously met in order for the primers to perform properly. The length of a primer is a statistical issue that relates to the issue of the minimum length of a specific sequence necessary to guarantee that the desired target sequence is unique, regardless of the size or complexity of the genome. As an example, in the case of the human genome, with its 3.2 billion DNA bases, that length is 17 bases. For this reason the vast majority of PCR primers are between 20 and 25 bases long. The specificity of a primer should be determined by comparison with sequences in all known databases. Tools available on the Web facilitate such comparisons.

In terms of primer sequence, the issues to consider are $T_m$ (the temperature at which 50% of the double-stranded nucleic acid molecule becomes single-stranded) and secondary structure. Every DNA has its own characteristic $T_m$, determined by length, sequence composition, and reaction environment. PCR primers are designed to bind to a perfectly complementary DNA sequence via guanine-cytosine (G-C) and adenine-thymine (A-T) base pairing. The $T_m$ of the two PCR primers used in a reaction should be as close as possible. In terms of secondary structure, the formation of secondary structures by intra- or intercomplementarity should be minimized. Interaction between different primers can result in primer–dimers that will compromise assay sensitivity and specificity. All of the design issues presented are accounted for in any one of the dozens of primer design software packages that are available and can be found on the Internet.

## Assay Optimization

NAT assay optimization is necessary for successful amplification that is sensitive and specific. Parameters that should be optimized include the thermocycling conditions, both temperatures and cycling times (that depend to a large extent on the target, primer, and probe sequences), concentrations of template, concentrations of NAT reagents, sample matrix and the number of amplification cycles. In the case of multiplex PCR, a compromise among elements of the reaction conditions is usually necessary because of the difficulties in optimizing the conditions for all the primer and probe sets. Recent changes have been made to improve sensitivity and specificity of NAT assays. One change is hot-start PCR, in which the addition of one of the essential components of the NAT assay, typically the DNA polymerase, is temporarily withheld. When this occurs during reaction setup, the initial nucleic acid denaturation step prevents nonspecific amplification due to mispriming at suboptimal temperatures. Early hot-start procedures made use of wax barriers that effectively separated essential components into two liquid phases that were mixed only when the wax melted. However, this procedure has been replaced by two important hot-start technologies that do not require physical separation of the components by inconvenient additional handling steps. In the first procedure, antibodies directed against the DNA polymerase are complexed with the enzyme and lose their binding avidity at elevated temperature at the start of the reaction. The second procedure uses chemical modification of the polymerase, resulting in an inactive enzyme. At temperatures above 90°, typically in the first denaturation step, the modifier dissociates from the enzyme, and the enzymatic activity is restored. The advantage of an antibody-mediated hot start is the immediate release of enzyme activity at the start of the reaction by a very short heat incubation step. However, antibody-mediated hot-start chemistries tend to be less stringent when compared with chemically activated enzymes if there is a large excess of active polymerase molecules.

# NAT ASSAYS

This section describes the basic techniques of PCR, nested PCR, and RT-PCR and procedural modifications that allow semiquantitation.

## Polymerase Chain Reaction

The PCR technique is based on a three-step process: denaturing double-stranded DNA (dsDNA) into single strands (ssDNA), annealing primers to the ssDNA, and enzymatic extension of primers that are complementary to the ssDNA templates. Each step is usually carried out at a different temperature. By cycling the temperature steps many times (usually 30 to 45 times), a billion-fold amplification of the target nucleic acid can be achieved, but the optimal number of cycles should be determined empirically. In some cases, especially where sensitivity is more important than false positive results due to excessive cycling, such as in blood screening, extra sensitivity can be gained by increasing the number of cycles to 60 to ensure that extremely low levels of target are detected. In a typical reaction, PCR product (amplicon) doubles at each cycle of amplification (exponential amplification). The increase in amplification in the early cycles follows a sigmoidal curve. In later cycles, the concentrations of the template strands and amplicons favor template strands re-annealing instead of PCR primer annealing to the template. At this point the concentration of the PCR product no longer doubles after each cycle, and the curve begins to plateau. A thermostable enzyme such as *Taq*-polymerase is a prerequisite because temperature cycling at 95° (the typical temperature step used to denature double-stranded templates) would inactivate a thermolabile polymerase.

### NESTED PCR

An early variation of the PCR assay was nested PCR, which was designed to increase the assay's sensitivity and specificity. In this procedure amplicons from the initial PCR reaction are subjected to a second round of amplification using a different set of primers. This set of primers is specific to the amplicon sequence but is within the first set of primers (nested primers). The

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advantage of amplification with two sets of target-specific primers is increased specificity (any nonspecific amplification during the first amplification round would be reduced) and increased sensitivity (due to initial amplification of the target in the first amplification round). In addition, amplification of a product of the expected size is taken as confirmation of the presence of the target. However, a major drawback of this procedure is the high likelihood of cross-contamination due to the increased manipulation of amplicons generated in the first round of amplification. The use of highly specific primers and probes and the optimization of reaction conditions have resulted in the diminished applications of this procedure for routine testing, but the procedure is sometimes used for samples that are difficult to amplify by conventional PCR.

### RT-PCR

In amplifying RNA targets, analysts prepare cDNA before the amplification step (RT-PCR). One-step and two-step RT-PCR procedures are available. In one-step RT-PCR the reverse transcription of RNA into cDNA and the subsequent amplification step are carried out in a single reaction without intermediate procedures. Therefore the reaction mixture for one-step RT-PCR includes the gene-specific amplification primers that are used for both reverse transcription and amplification. The advantage of this procedure is the overall reduction in handling time, increased throughput, and reduced contamination risk because reopening the reaction vessel is not necessary. In contrast, in two-step RT-PCR the reverse transcription and amplification are performed as two separate steps. In general, random primers or oligo-d(T) primer rather than gene-specific primers are used for the reverse transcription step. An aliquot of the cDNA synthesis reaction is then transferred into the NAT reaction for subsequent amplification. The advantage of this procedure is the standardization of the reverse transcription reaction, which can be used as a single source for the analysis of multiple transcripts in gene expression analysis.

## DETECTION OF AMPLICONS

Following amplification, analysts can employ a variety of procedures for detection of the amplicon as described in detail in the general information chapter, *Nucleic Acid–Based Techniques—Extraction, Detection, and Sequencing* ⟨1126⟩. These include agarose gel electrophoresis with ethidium bromide or other dyes, capillary electrophoresis, and laser-induced fluorescence and hybridization followed by chromogenic detection such as streptavidin horseradish peroxidase detection, chemiluminescence, or fluorescent detection using labeled probes.

**Quantitation—**The original PCR and RT-PCR assays were qualitative and detected amplicons at the end of the reaction. Such detection is not easy to quantitate because at this stage the amplification is in a plateau phase at the end of the assay, and the amount of amplicon is not necessarily directly related to the quantity of the starting template. Several approaches have been deployed to attempt to overcome the shortcoming of PCR to produce reliable, quantitative results. Initial attempts at quantitation relied on assessing the amount of amplified DNA during the early or exponential part of the assay, but this procedure was fraught with problems because the aliquots had to be taken from the reaction mixtures at regular intervals, thus greatly increasing the risk of cross-contamination. One of the earliest and most straightforward approaches to quantifying PCR products was to measure the amount of amplicons that were generated during the exponential phase of the reaction by comparing this to a serially diluted external control. Several aspects, including variability in sample preparation and variations in reaction conditions, however, hampered this approach. Because of the exponential amplification of NAT procedures, even small errors or variances can lead to distinct differences.

Compared with dilution procedures, competitive PCR proved to be a much more precise approach to achieving reliable estimates of the originally present target molecules. This procedure relies on the simultaneous co-amplification of a specific target sequence in the presence of increasing concentrations of an exogenous target molecule (control) which shares the primer binding sites with the target sequence but whose sequence is slightly modified or shortened in order to facilitate discrimination from wild-type amplicons. In addition, the concentration of the control is known. The close sequence homology and similar size of the control and target amplicons are designed to ensure that the template and internal control are amplified with comparable efficiency. The relative strength of the amplicon bands of template and control can be assayed, for example, on ethidium bromide–stained agarose gels, giving a relatively precise quantitation of the wild-type target. A drawback of this approach is that the internal control and the template should be present in the reaction in approximately the same quantity in order to yield correct results. The development of real-time, quantitative PCR has eliminated the variability associated with quantitative PCR, thus allowing the routine and reliable quantification of PCR products.

## REAL-TIME PCR AND REAL-TIME RT-PCR

Although gene quantitation by quantitative PCR was a widely used procedure, its applications were expanded by the advent of real-time PCR and real-time RT-PCR. Real-time PCR displays the same advantages as standard quantitative PCR—sensitivity, specificity, and a wide dynamic range—but the real-time procedure offers the additional advantage of requiring no post amplification processing because it combines amplification and detection in a single step. Real-time PCR collects data throughout the amplification process by measuring a fluorescence signal created as amplification progresses. A multitude of fluorescence chemistries allows the correlation of generated PCR product to fluorescence intensity. In principle, fluorescence intensity will increase with every cycle performed. Once the intensity is greater than background fluorescence, the so-called cycle threshold ($C_t$) value is achieved. This value, which represents the first cycle in which there is a detectable increase in fluorescence above the background level, is used to measure relative or absolute target quantities. The $C_t$ value is inversely proportional to the number of target molecules in the sample and thus provides a means to quantitate the amount of target in the starting material (i.e., the greater the number of target molecules present, the lower the $C_t$ value).

The reaction conditions for real-time PCR applications have to take into account the presence of the probe(s) and will require optimization. The most commonly used probes currently are hydrolysis probes, although hybridization probes are an alternative. In most cases, the amplification and detection steps can be combined into a two-step cycling reaction, but these conditions have to be optimized. In contrast, DNA-binding dyes which may also be used for amplicon detection require separation of the annealing and extension steps since the dye binding occurs during the extension step which is usually done at 72°.

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A fluorescent DNA intercalating dye is used for detection of the PCR product in real-time mode. This dye emits light when bound to double-stranded DNA and the subsequent increase in fluorescence can be detected by real-time PCR instruments. Dyes that bind to dsDNA bind not only to the specific PCR product but also to artifacts such as nonspecific PCR products and primer–dimers. Analysts have observed substantial differences in the specificity of dsDNA-binding dyes in use with real-time PCR kits. Therefore, some analysts recommend verifying the presence of a single PCR product by gel electrophoresis to determine the correct size of the PCR product. Also, a melting curve analysis is advisable to ensure the absence of artifacts that could contribute to the fluorescent signal and thereby lead to misinterpretation of quantitative data. Alternatively, sequence-specific labeled probes can be employed. A wide variety of fluorescence-labeled probes and primers exist for use in real-time PCR and are described in the next section.

**Real-Time PCR Probes**—The difference between conventional PCR and real-time PCR is the presence of a third chemically synthesized oligonucleotide, the probe, which, for the most basic hybridization probes, contains some type of reporter molecule, usually a fluorescent molecule or fluorophore. Non-nucleic acid materials can be added to chemically synthesized DNAs that are then incorporated into oligonucleotide probes for real-time PCR. Other applications include hybridization probes such as those used for fluorescence in situ hybridization (FISH) and microarrays and probes designed to capture other nucleic acids. A challenge arises in using fluorescent probes for real-time PCR because the unbound or free probe is not removed before detection, thus requiring a means to distinguish between signal obtained from bound and free probe. In contrast, FISH assays involve washing away free probe following hybridization.

All of the issues associated with primer design for conventional PCR apply to real-time PCR primers as well as to the probe sequence. As a general rule only two additional considerations apply to the probe sequence. One of these is thermodynamic, and the other specifically concerns the reporter moieties themselves. Thermodynamically, a good probe molecule that is designed to bind in the sequence somewhere between the two PCR primers will have a $T_m$ that is about 5° higher than that of the two primers. In the large majority of cases the amplicon will be between 100 and 500 DNA bases in length, although for real-time PCR a smaller amplicon between 100 and 150 bases long results in a more efficient reaction. Thus it is rarely a problem to find a sequence inside a PCR amplicon that meets the necessary criteria.

Current probe designs overcome the problems of background from unbound probe using simple hybridization probes. In the original design, two probes that hybridize to adjacent sequences on the target nucleic acid are labeled. The reporter moiety is a fluorescent molecule attached to the 3′ end of the upstream probe sequence, and a second fluorescent molecule is attached to the 5′ end of the second probe. Excitation of the 5′ fluorophore with light energy of the proper wavelength results in absorption of that energy, followed by emission of light energy of a slightly longer or less energetic wavelength (Stoke's Law). This emitted energy then excites the 3′ fluorophore if it is close enough to the emitter and compatible with it in the sense that the emitted energy from the 5′ fluorophore can excite the 3′ fluorophore. When this occurs, the observed fluorescent light wavelength will be that of the acceptor molecule and not that of the donor. Fluorescence absorption and emission spectra are readily available for all of the commonly used fluorophores, and the only applicable rules are that the two fluorescent molecules must be fewer than 40 DNA bases apart and that the emission spectrum of the donor must overlap the absorption spectrum of the acceptor. Thus hybridization of the two probes, also known as hybridization probes or FRET probes (Fluorescence Resonance Energy Transfer), results in the emission of a fluorescent signal by the acceptor, and the latter signal can be detected. In the absence of hybridization, the probes are sufficiently separated in solution so that energy transfer cannot occur, and only background fluorescence is emitted by the donor.

Issues of fluorophore compatibility have been resolved by the increased use of a special class of molecule called a quencher. Quenchers are fluorescent molecules that absorb fluorescence energy over a wide range of wavelengths. Instead of re-emitting that energy as light they simply dissipate it as heat. Thus, if a quencher molecule is placed at the 3′ end of a probe and a fluorophore at the 5′ end, the probe will remain dark even when excitation energy is present so long as the molecule remains intact (hydrolysis probes). These probes utilize the 5′ nuclease activity of the DNA polymerase to hydrolyze a probe bound to its target amplicon. Cleavage results in separation of the reporter and quencher and permits fluorescence of the reporter. This reduces much of the work of optimization of the assay conditions (since only a single probe is used) and background noise generated with two probes.

A variation on hydrolysis probes involves placing the reporter and quencher molecules on a single oligonucleotide that is constructed so that, in the unbound state, the quencher and reporter are in close proximity, resulting in efficient quenching of the reporter. When the probe hybridizes to its complementary sequence on the amplicon, the probe undergoes a conformational change that forces the quencher and reporter apart, permitting fluorescence of the reporter. A variation on these kinds of probes is a combined primer and probe in which, again, the quencher and reporter are in close proximity in the native probe, thus resulting in no signal. Priming and subsequent elongation of the primer–probe results in hybridization to the newly synthesized DNA strand, causing spatial separation of the quencher and reporter and resulting in the generation of a signal.

**Probe Labeling**—Modern synthetic oligonucleotide modification chemistries permit the manufacture of oligonucleotides with non-nucleic acid materials. Placement of modifications is carried out in one of two ways: during synthesis or after synthesis. For the former, modifications are constructed in such a way that they behave like the four DNA or RNA bases that are routinely placed in the sequence. The modification is then presented in the desired location during the synthesis as if it were just another base in the series. In the latter, usually employed when more than one modification occurs, the synthesis contains a linker, such as an amino group, to which the desired modification is then attached. This process is often called "hand-tagging."

Perhaps the best-known example of hand-tagging is the conventional dual-labeled probe used in real-time PCR. The quencher is placed at the 3′ end of the sequence during synthesis, and the fluorescent reporter molecule is hand-tagged to an amino modification at the 5′ end of the sequence after the synthesis is finished and has undergone purification. Some modifications, such as biotins, are designed so that multiple modifications can be carried out in a single synthesis. Thus, it is possible to modify a synthetic DNA or RNA sequence to contain a number of different non-nucleic acid molecules. A cost is associated with such modifications insofar as alterations often are achieved with a loss of mass due either to an inherently lower efficiency of modifications to bind to the oligonucleotide as compared with standard DNA or RNA bases or to the requirement that the synthesis must be purified before modification, after modification, or both.

Proof

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The benefits of modifying synthetic DNAs or RNAs usually outweigh the costs. The standard, quenched, dual-labeled, real-time PCR probe has permitted precise quantification of gene expression. Fluorescently labeled DNA oligonucleotides are also essential components of in situ hybridizations and microarrays. Some modifications confer increased thermal stability when synthetic DNAs or RNAs are hybridized to complementary DNAs or RNAs by comparison with unmodified DNA–DNA and DNA–RNA duplexes. These analogues include peptide nucleic acids, 2′-fluoro N3-P5′-phosphoramidates, and 1′, 5′-anhydrohexitol nucleic acids. Although such analogues succeed to varying degrees in achieving increased thermal stabilities, they fail to provide enhanced target recognition. Another approach is to use base analogues such as locked nucleic acid, which is an analogue that contains a 2′-O, 4′-C methylene bridge. This bridge restricts the flexibility of the ribofuranose ring and locks the structure into a rigid bicyclic formation, conferring enhanced hybridization performance and stability.

The modification of a probe typically is governed by its intended use. Generally, fluorescent reporters are used in real-time PCR and for in situ hybridization. The range of available fluorescent reporters covers the spectrum from 517 nm to 778 nm. For hybridization probes, base modifications are preferred because these primarily alter thermodynamic interactions between bases, leading to improved specificity. Amino attachment groups, both with and without C-spacers, are used to attach other modifications to DNA sequences and to attach DNA sequences to solid surfaces such as glass slides. An example is the attachment of biotin molecules to DNA sequences. Biotin forms a strong bond with streptavidin-coated materials such as magnetic beads, allowing capture of specific nucleic acids that may themselves be hybridized to other molecules.

**Quantitation**—PCR products may be quantified using a standard curve drawn from replicate serial dilutions of a reference reagent or standard for the nucleic acid sequence of interest. The concentration of the nucleic acid in the reference reagent is known. Real-time PCR quantitation based on a standard curve may utilize plasmid DNA or other forms of DNA. However, the efficiency of PCR must be the same for the standards and the target samples. Performing PCR from purified targets can in some cases be more efficient than performing PCR with complex nucleic acid mixtures. The cycle threshold (Ct) values and concentrations of the dilutions of the reference reagent can be used to construct a standard curve from which the concentration of the unknown sample can be estimated. When the assay run conditions have been well standardized and the standard curve for a particular target has been well calibrated, in subsequent assay runs it may be sufficient to co-amplify only two dilutions of a reference reagent (usually dilutions containing known amounts of nucleic acid at high and low concentrations). These dilutions, or calibrators, can then be used to quantitate any unknown samples by comparison of the $C_t$ values.

**Multiplex PCR**—Multiplex PCR describes the simultaneous amplification of several nucleic acid targets in a single assay reaction. This is a particularly demanding variation of PCR because it requires the use of a single set of reaction conditions for the amplification of multiple targets with different sequence characteristics. Additional complications can arise due to the increased chance of nonspecific amplification products arising from multiple primer interactions. In addition, the differing individual target amplification efficiencies can result in weaker reactions being out-competed by stronger, more efficient reactions.

Both qualitative and quantitative applications of multiplex PCR have been described in the literature, as have multiplex RT-PCR assays. Quantitative multiplex PCR relies on either the generation of multiple standard curves to enable quantitation of each target in the assay, or the inclusion of internal competitor sequences that can be used as calibrants.

Hybridization kinetics of primers and probes may be significantly different, even when designed using the same algorithm. This leaves the analyst with very limited room to optimize reaction conditions. However, optimization may include adjustment of DNA polymerase amount, $Mg^{2+}$ to increase hybridization efficiency, or primer concentration. Especially in real-time PCR, optimization of primer concentration is critical for quantitative co-amplification of target genes. These are contained in the sample at significantly different amounts. Increasing hybridization efficiency of the primer–probe system can be achieved by providing sufficient reagents, such as $Mg^{2+}$, as well as adding a “molecular crowding” reagent that increases the effective concentration of all reaction components in the mixture. Multiplex PCR is not only used for genotyping applications, but also for quantitative real-time PCR because it offers several advantages over standard single real-time PCR reactions. Some of these advantages are a minimized amount of sample used, increased precision through the use of an internal control (e.g., housekeeping gene) co-amplified with the target gene in the same reaction, no separate pipetting steps, and cost-effectiveness.

Most PCR assays, however, suffer from a common problem—that of minimizing differences in extractions or amplifications between different samples. Multiplex PCR is useful in cases where it is critical to ensure that variability in quantitation of different samples is not due to differences in nucleic extraction or amplification measurements (usually when one measures the production of an mRNA species). Certain precautions and techniques can be employed to minimize these challenges; they are discussed in the next section on normalization of assay results.

**Normalization of Assay Results**—To minimize the effects of assay variables, analysts sometimes use a relative quantitation procedure that normalizes the target transcript level to a control that can be employed and compared for all samples included in the gene expression study. Probably the most reliable and most frequently used relative quantitation procedure relies on the measurement of “housekeeping” or control genes to normalize the expression of the target gene in a multiplex PCR format. This procedure is preferred because the quantitation of both the housekeeping gene and the target gene are influenced by varying cDNA synthesis efficiencies or the presence of enzyme inhibitors contained in the sample. However, it should be noted that the efficiency of conversion of target RNA to cDNA is not necessarily consistent even within a single-tube reaction but is a function of primer design, target sequence, etc. which may differ between target and housekeeping genes. The selection of appropriate control genes can cause problems because they may not necessarily be equally expressed across all unknown samples and may vary under experimental conditions. Normalizing measurements to a set of housekeeping genes in order to avoid the problem of variability may circumvent this concern. Alternatively, analysts can establish a thorough evaluation of housekeeping genes that do not alter gene expression levels under the experimental conditions.

All the NAT techniques described thus far are variations on the PCR assay, which is the most widely used of the NAT techniques. However, isothermal assays that are based primarily on the amplification of RNA are used for routine purposes. This is known as the transcription-mediated amplification (TMA) assay, which is closely related to the nucleic acid sequence–based amplification (NASBA) assay. Both assays are described in more detail in the following section.

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### NUCLEIC ACID CEQUENCE-BASED AMPLIFICATION AND TRANSCRIPTION-MEDIATED AMPLIFICATION

Both NASBA and TMA rely on in vitro isothermal amplification for detection and amplification of nucleic acids, also referred to as self-sustained sequence replication or 3SR. The major difference between the assays is that NASBA uses three enzymes—reverse transcriptase (RT), RNA polymerase, and RNase H—whereas TMA uses only two enzymes: RT and RNA polymerase. The complete procedure generally is performed at 41° to 42° using two primers. Both NASBA and TMA are especially suited to amplifying RNA analytes, including rRNA, mRNA, pathogens that have RNA as their genetic material, as well as DNA targets.

One of the primers that has a promoter sequence for the RNA polymerase at the 5′ end binds to the RNA target and is extended via the DNA polymerase activity of the RT. The product of this reaction is an RNA–DNA hybrid. RNase H activity then specifically digests the RNA strand of the hybrid, leaving only the cDNA to which the second primer can bind. A complementary strand of DNA is then synthesized by the RT, resulting in a dsDNA molecule with a T7 promoter at the 5′ end. The T7 RNA polymerase then transcribes multiple copies of the RNA amplicon. The RNA copies may undergo the same cycle to create new duplex DNA molecules with a T7 promoter from which many molecules of RNA are transcribed. Thus, unlike the action of PCR, the amplicon amplified in this case is of an RNA species.

Some of the characteristics of this technology are that only relatively short target sequences can be amplified efficiently (around 100–250 nucleotides); it uses a single temperature, which eliminates the need for special thermocycling equipment; the fidelity of the technique is comparable to that of other amplification processes; and the RNA amplicons are exponentially amplified. Carryover contamination is minimized because of the labile nature of the RNA amplicon in the laboratory environment. Containment procedures built into the assay procedure further help to minimize contamination. Detection of amplicons is typically achieved by the use of labeled probes and, in TMA technology, a common method is detection of chemiluminescent signals from hybridized probes that remain intact during the subsequent alkaline hydrolysis step used to destroy free probe.

The NAT techniques described, both PCR and TMA, are optimized for amplifying specific, small fragments of a genome. In cases when whole genome amplification is desirable, such as for mutation analysis or identity testing, modifications of the PCR procedure are necessary in order to ensure adequate sequence representation of genetic loci, as described in the following section.

### WHOLE GENOME AMPLIFICATION

Historically, whole genome amplification (WGA) has been performed using modified PCR procedures. These procedures have relied on the nonspecific amplification of the genome using primers that bind under low-stringency conditions to the DNA template. PCR-based approaches differ mainly in terms of the type of primer employed in the reaction: in primer-extension-preamplification (PEP), short 15 base random primers are used in an initial cycling reaction at low stringency to make multiple random copies of segments of the genome. This product is then used as target for the specific PCR reaction. Amplification bias of favorable sequence contexts leading to uneven representation of the genome is the major drawback of this technique. The generation of increasingly shorter fragments during each round of amplification is a further drawback. Another procedure called degenerate oligonucleotide primed-PCR (DOP-PCR) uses tagged primers and low stringency amplification for the first few cycles of amplification followed by an increase in annealing stringency in later cycles. The tagged primers are characterized by defined sequence tags at the 3′ and 5′ ends and a random sequence in the centre of the primer. Under the later, more stringent conditions, the target DNA fragments generated during the first cycles containing the amplification tag sequences are amplified preferentially without any further shortening of the fragment length. PCR-based WGA typically employs *Taq*-like polymerases that possess the disadvantage of introducing variations into the amplified DNA due to their relatively low processivity and fidelity which become compounded by the very high number of amplification cycles used in these methods. This may cause problems in downstream applications such as genotyping analysis. These limitations as well as the relatively poor sequence representation of genomic loci inherent to PCR-based WGA can be overcome by an isothermal reaction called multiple displacement amplification (MDA).

The enzyme that is used for MDA comprises a high processivity polymerase with proofreading and strand-displacement activity. The isothermal reaction is performed at 30° without any change in reaction temperature. The reaction starts with the annealing of multiple random primers to the target DNA and elongation of the primers using a DNA polymerase from the *Bacillus subtilis* phage Phi29. Because the polymerase is able to displace DNA strands in a 5′–3′ direction, the polymerase reaction is not stopped when the elongating strands meet downstream DNA strands. The displaced DNA strand serves again as a target for multiple primed elongation reactions so that the DNA template is amplified exponentially in a branched-like manner, yielding high molecular weight DNA with a good representation of the genomic loci. Compared with PCR-based WGA, the error rate is very low. In particular, the mutation rate of repetitive sequence structures is low because of the limited strand-displacement activity of Phi29-polymerase. This permits reliable genotyping of genomic DNA (e.g., SNP analysis, mutation analysis, identity testing, or analysis of case work samples) on different platforms such as real-time PCR or array analysis.

## INSTRUMENTATION

The development of the numerous and varied NAT techniques described in this chapter has been facilitated by the evolution of instrumentation that has served to automate these complex procedures. A general description of the major changes in instrumentation is discussed in this section.

The continuous control of the temperature steps necessary to achieve exponential amplification for PCR assays is carried out by fully automated thermocyclers that consist of a heating block in which the temperature can be rapidly cycled. Temperature changes are induced by water, or more recently, by using the Peltier effect. These instruments may be coupled to a fluorometer apparatus if they are used for real-time PCR analysis. In the latter case certain instruments are equipped with a rotor device that is heated and cooled by air instead of a metal block that typically is used as a heating module. In the case of endpoint PCR,

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PCR products are usually analyzed according to size on agarose or polyacrylamide gels, or by capillary electrophoresis using fluorophore-labeled primers. They may also be analyzed by an array-based approach or other hybridization procedures.

Because no post-PCR processing or label-separation steps are required, real-time PCR assays are simple to perform, making them useful for high-throughput applications. Real-time PCR instruments combine the properties of a thermocycler and a fluorometer to allow determination of PCR products by fluorescence measurement. In each PCR cycle, either one or several fluorescence readouts are taken to monitor the PCR reaction for generation of amplicons, usually at the extension step of the PCR reaction.

Real-time PCR instruments vary with regard to simultaneous sample throughput (32–384 reaction vessels), sample volume (5–100 µL), excitation source, and detector used. These compositions define the suitable range of fluorescent dyes for multiplex real-time PCR as well as size and heating/cooling principle (see above). The excitation source of real-time thermocyclers is either a laser-based system, halogen bulbs, or light-emitting diodes (LED). Optical filters are used to select the wavelength of interest. In most instruments, the emitted light is detected by a charge-coupled device (CCD) that consists of an array of light-sensitive cells. Light projected onto the CCD is converted to an electric charge, resulting in a signal that is proportional to the light intensity.

The versatility of the PCR assay has resulted in the widespread and diverse use of this technique. With the advent of real-time PCR, it has been possible to design high-throughput instrumentation for automated testing. Similarly, the TMA assay has also been automated. Such technology is used by laboratories doing high-throughput, highly regulated testing, typically blood screening for hepatitis C virus (HCV) or human immunodeficiency virus-1 (HIV-1) because automated tests are ideal in a regulated environment where minimum human intervention is required. The use of NAT in a highly regulated environment has resulted in the development of guidances for managing the quality assurance (QA) and quality control (QC) aspects of testing, as well as the validation of systems and assays as described in the following section.

## QUALITY ASSURANCE AND QUALITY CONTROL FOR NAT

This section serves as a general guidance for the development of laboratory- and procedure-specific QC and QA procedures for NAT. Aspects such as waste management, management of radioactive material, or working with hazardous material are not covered. NAT is a technology that offers extreme sensitivity with its ability to generate millions of amplicons from as little as a single nucleic acid template, resulting in a detectable signal. The advantages of this technology can be offset by the necessity of establishing complex assay protocols and the requirement to follow carefully very stringent QC/QA protocols. Deviation from these protocols can cause major problems, such as false positive results due to the contamination of templates by amplicons generated in previous assay runs. Similarly, failure to control inhibitors could lead to suboptimal amplification and possible false negative results. Given the myriad factors that can greatly influence the outcome of a NAT assay, all aspects concerning NAT need to be covered by appropriate and stringent QC/QA procedures. This requires careful facility design, workflow, and selection of equipment suitable to the purpose. Data recording, record keeping, and data interpretation are other aspects that should be covered by QC/QA. Thus, QA for NAT assays includes assay validation, establishment of acceptance criteria and specifications, and adherence to good manufacturing/laboratory practices. These aspects are also described in this section. In addition, reference should be made to other published guidelines such as the ICH Guideline *Validation of Analytical Methods: Methodology* (Q2B) and the NCCLS Guidelines.

### Laboratory QC/QA

An NAT laboratory should be designed and operated in a manner that prevents contamination of reactions with products from previous amplifications (carry-over) as well as cross-contamination between samples. Historically, the application of PCR required strict separation of the various steps of the assay in order to prevent cross-contamination of PCR by amplicons. This was necessary because early procedures for analysis of PCR products involved the transfer of the product, which potentially could lead to contamination. Therefore, in an open system the best measure to prevent contamination has been the strict separation of working areas for individual process steps. This includes individual areas for template preparation, master mix setup, distribution of the master mix to individual reaction wells and addition of template, space for cycling the PCR assays and, optionally, a separate work space for PCR product analysis. These requirements are not necessary with closed systems. With both open and closed systems it is still necessary to take additional precautions. These safety measures include UV illumination of work spaces overnight to inactivate residual DNA by crosslinking. In case of contamination, laboratory benches and pipettes can be decontaminated by cleaning with a 10% solution of commercial bleach, which usually contains about 5% sodium hypochlorite, taking appropriate safety measures such as wearing gloves and eye protection. Afterwards, benches and pipettes should be rinsed with distilled water. A unidirectional workflow will reduce the opportunity for contamination to occur. Also, no materials, supplies, or equipment should be exchanged between designated working areas or rooms.

### Equipment QC/QA

Other good laboratory practices that are related to the prevention of carry-over contamination include the use of suitable and clean equipment. Generally, disposable consumables (tubes, pipette tips, etc.) are highly preferable to reusable equipment. The use of disposable tips containing hydrophobic filters is another very effective measure to minimize cross-contamination. All samples, primer, probes, etc. must be labeled with relevant information such as identity of the content, date of use or preparation, expiration date, concentration, and storage information. Dedicated laboratory coats or disposable lab coats should be available in each room (or section) of the NAT laboratory. Appropriate gloves should be used during all processing steps to prevent sample contamination. The gloves should be changed frequently. Because heat sterilization does not completely destroy DNA, PCR products may lead to detectable contamination of, for example, glass surfaces. Following unique sterilization procedures for different materials such as waste and glass laboratory equipment is advisable.

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## Carry-Over Prevention with Uracil-*N*-Glycosylase

Contamination by PCR product carry-over can be mitigated by using the commercially available uracil-*N*-glycosylase (UNG) procedure. The procedure involves substituting 2′-deoxyuridine 5′-triphosphate (dUTP) for 2′-deoxythymidine 5′-triphosphate (dTTP) in the PCR setup and treating all PCR mixtures with UNG prior to PCR amplification, which can be easily incorporated as a first step into PCR cycling programs. Incorporating dUTP into the amplicon makes the PCR products biochemically distinct from the native DNA template. The enzyme UNG cleaves the deoxyuridine-containing PCR products by opening the deoxyribose ring at the C1 position. When the deoxyuridine-containing DNA is heated during the first thermal cycle, the amplicon DNA chain breaks at the position of the deoxyuridine at the alkaline pH of the PCR reaction mixture and thereby renders the carried-over PCR product nonamplifiable. Thus, any previously generated U-containing amplicon that might have contaminated another sample will become nonamplifiable. As a consequence, false positive results can be avoided. However, it should be noted that UNG has concentration limits above which it does not fully remove PCR carry-over products.

### VALIDATION OF NAT SYSTEMS

Assay validation is achieved by

1. ensuring the quality and consistency of assay components, including primers, probes, and enzymes; (including shelf life and contamination control) and
2. establishing the performance characteristics of the NAT assay in terms of reproducibility, accuracy, ruggedness, robustness, specificity, precision, and analytical and clinical sensitivity.

The analytical sensitivity of an assay is defined as the minimum concentration of a reference reagent or standard detected by the test while the clinical sensitivity of a test is determined by testing clinical specimens and determining the 95% LOD. The clinical sensitivity of a test is not necessarily the same as the analytical sensitivity. The closer the reference or standard material is to the samples being tested the closer the correlation.

The principal steps of assay validation are

1. sample preparation;
2. consistent production of critical reagents;
3. use of controls, calibrators, and quantitation standards;
4. specimen and reagent stability;
5. functionality of instruments and software;
6. operator training; and
7. laboratory surveillance for proficiency.

Following assay validation, further QA is necessary to monitor specifications and functional characteristics that have been established by the use of well-characterized reagents of known potency.

## Quality Control of Reagents

### DNA TEMPLATES

The test specimens used are usually, but not limited to, whole blood, plasma, and serum. Specimen preparation is a key step in the NAT assay and has a major influence on the performance and variability of the assay. Specimen collection is the first step in sample preparation. QC/QA staff should carefully evaluate the effects on the integrity of DNA of collection tubes and temperatures during sample transport. To prevent cross-contamination during specimen collection, aseptic techniques should be used along with closed sampling systems in order to avoid specimen contamination. The use of appropriate sample handling techniques, temperature conditions, and anticoagulants or preservatives should help reduce the risk of contamination. Anticoagulants such as heparin or EDTA may interfere with the NAT assay.

### SAMPLE EXTRACTION

The buffers, reagents, and detergent or chaotropic agents used for nucleic acid extraction should be evaluated for inhibitory effects on the NAT assay. Extraction controls, including spiked materials, should be included to monitor the efficiency and reproducibility of the extraction method. Reproducibility of the sample preparation method should be determined under the specimen processing conditions, including sample handling, storage, and shipping conditions. DNA is generally stable, but personnel should take care to avoid storage at refrigerated temperatures for extended periods of time to avoid sample degradation. Repeated freeze–thaw cycles can sometimes cause DNA fragmentation. In the case that the target is RNA, it should be noted that RNA is very unstable and specimens should be frozen.

### PRIMERS

Primers and probes should be qualified in terms of purity, identity, and functional potency. Purity can be assessed by use of HPLC or mass spectrometry; identity can be established by sequencing; and functionality can be established by the use of reference reagents. However, in many cases, these methods may not be available for in-house testing. In these cases, it may be sufficient to compare lot-to-lot variation of purity and functional potency using relevant methods available in-house coupled with the use of reference reagents.

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DNA POLYMERASES

The functionality of enzymes should be determined using reference materials. Enzyme preparations should be tested for other enzymatic activities; for example, exonucleases and DNA- and RNA-dependent polymerase activities and specifications should be established. Lot-to-lot comparison, as well as comparison with the manufacturer's CoA should also be done. Storage conditions recommended by the manufacturer should be strictly followed, and appropriate controls should be used to monitor the stability of enzymes.

## Run Controls

The use of controls affords the operator assurance that the assay has performed within accepted specifications. In PCR testing, several steps in the testing process, as outlined above, should be monitored and verified. Multiple controls or controls that serve multiple purposes may be needed for a PCR assay. Controls should reflect the specific technology under development but should typically allow monitoring of ultracentrifugation, extraction, amplification, hybridization, quantitation, contamination, etc. Controls should be similar to the specimen type whenever feasible, although spiked controls may be acceptable.

A negative control is one that does not contain the target sequence or pathogen that is being tested. It should resemble as closely as possible the sample matrix under testing. Multiple negative controls should be examined, including nontarget sequences and nucleic acid-free controls to monitor for false positives resulting from contamination. Because of the high sensitivity of amplification assays, QC/QA personnel highly recommend that sponsors include control measures for the prevention of contamination events.

A positive control is one that contains the target sequence of interest. It should resemble as closely as possible the specimen matrix being tested and should contain an appropriate and defined amount of target sequences. (e.g., kit control).

Specifications for both positive and negative controls should be provided, as well as validation data supporting the proposed assay cut-off/reporting threshold value or the assay's limit of detection. The laboratory should define the source of the controls and calibrators and have a plan for their continued renewal. Controls can be infectious or non-infectious. In the latter case, validation of viral inactivation should be provided.

Reagent controls are often referred to as blanks and could include samples that have no target sequence, no enzyme, no primers, etc. These controls provide additional information about problems encountered in PCR assays.

An internal control is added to each specimen to ensure the overall validity of the individual test results. Internal controls are used to verify sample extraction, amplification, and detection.

## External Quality Assessment and Proficiency Testing

Quality assessment of the laboratory is achieved by participation in periodic competency assessment and laboratory proficiency programs. The latter should include the testing of reference reagents and well-characterized panels to measure the technical proficiency of operators. Therefore, care should be taken to prevent cross-contamination, to monitor workflow, and to ensure careful specimen and test sample handling. Evaluation of operator proficiency should include participation in competency and quality assessment programs. Each operator in a particular laboratory should participate in such programs and should demonstrate comparable results.

## Data Management

Complete and consistent documentation of all activities performed and all data generated is necessary. Such documentation does not only require the maintenance of records of the data generated through sample testing but also information about reagents and equipment calibration and maintenance. Moreover, any alteration in the assay procedure needs to be introduced through a planned change control process and documented in such a way that change can be assessed by an independent party.

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# ⟨1128⟩ NUCLEIC ACID-BASED TECHNIQUES—MICROARRAY

## INTRODUCTION

Microarrays are microscopic spots of DNA (measured in micrometers) arranged in an ordered manner (columns and rows) on a planar surface so that each DNA spot can be uniquely identified to facilitate an accurate analysis of the data. The DNA spots, also called array elements, are specific DNA molecules of known or unknown sequences and can be of similar or different nucleotide lengths. Samples of these mixtures are placed in fixed locations on the microarray.

Unlike conventional probes, which are a specific DNA or RNA sequence labeled with radioactive, fluorescent, or chemiluminescent tags (see *Nucleic Acid-Based Techniques—General* ⟨1125⟩*, Glossary*), the array elements are referred to as probes when the sequence information of the array elements is known, despite not being labeled. In this context, the target refers to labeled nucleic acids in solutions that are hybridized to the array elements or probes. The purpose of a microarray experiment is to identify the sequence of these labeled nucleic acids and/or determine their content. Compendial applications at this time are limited but may increase with wider use of microarrays in diagnostics and in drug discovery, development, registration, and control applications. When used for compendial purposes, standard assay development and validation approaches with availability of suitable reference materials are likely to apply.

Microarrays can range from hundreds to thousands of array elements (low density), tens to hundreds of thousands of array elements (high density), to millions of array elements (very high density). In addition to the use of planar surfaces for microarrays, the array elements can also be immobilized on individual support particles, such as beads. In these cases the array elements are identified by the particles themselves rather than specific locations on an array. The advantages of using microscopic spots on the array include high density, fast hybridization kinetics, and low sample volumes. Microarrays greatly speed up the acquisition of data, and in some cases increase the predictive power of results, by comparison with conventional nucleic acid-based assays. This is achieved by miniaturization, multiplexing, and parallel execution of nucleic acid-based tests that traditionally are performed in tubes, plates, or capillaries as described in general chapter ⟨1125⟩ (see also *Nucleic Acid-Based Techniques—Extraction, Detection, and Sequencing* ⟨1126⟩, *Nucleic Acid-Based Techniques—Amplification* ⟨1127⟩, *Nucleic Acid-Based Techniques—Genotyping* ⟨1129⟩, and *Nucleic Acid-Based Techniques—Approaches for Detecting Trace Nucleic Acids (Residual DNA Testing)* ⟨1130⟩).

The principle of microarray analysis is the specific binding of the target DNA molecules to the probes or array elements. The ordered array of rows and columns of spots allows highly automated detection and analysis. DNA microarrays are manufactured, processed, detected, and analyzed in a number of different ways and have many applications. With the aid of computers, laboratory automation, and high-resolution detection devices, microarrays produce large amounts of data and are the analytical tool of choice to unravel the molecular complexity of DNA or expressed RNA.

The basic principles of nucleic acid amplification technologies (NAT) and definitions of the various techniques are described in chapter ⟨1127⟩. The present chapter covers the general field of microarrays, but detailed treatment of various application-specific microarrays, including data analysis and validation, are excluded from this chapter at this time. The following sections address the major applications of microarrays, sample processing, labeling, workflow, detection, and analysis of data. Several of these sections, for example, sample preparation and labeling, overlap with chapters ⟨1126⟩ and ⟨1127⟩, and cross references are made accordingly. Finally, regulatory aspects of microarrays will be discussed.

## GENERAL PRINCIPLES OF MICROARRAY EXPERIMENTS

### Types and Applications

Microarrays are most widely used in three types of analysis: gene expression, microarray-based comparative genomic hybridization (or array comparative genome hybridization, aCGH), and single nucleotide polymorphism (SNP). In brief, gene expression microarrays generally measure messenger RNA in a cell; aCGH analyzes DNA copy number variations, chromosomal additions, and deletions in genomic DNA; and SNP microarrays are used in genotyping to analyze single nucleotide polymorphisms (see ⟨1129⟩). Within each type of microarray, various platforms, both manual and with various levels of automation, are available. *Table 1* summarizes the three major types and most common applications, as well as the target for each application, the probe, and the complementary nucleic acid techniques (see ⟨1126⟩, ⟨1127⟩, and ⟨1129⟩).

**Table 1. Major Types and Applications of Microarrays**

| Types | Application | Target | Probe | Complementary Technology |
|---|---|---|---|---|
| Gene Expression | Gene Expression | mRNA | Oligonucleotide/cDNA | qRT/PCR, Northern Blotting |
| aCGH | aCGH<br>CNV | DNA | Oligonucleotide/<br>cDNA/Pac,<br>Yac, Bac | Cytogenetic chromosome analysis |
| SNP | SNP Genotyping | DNA | Oligonucleotides | Sequencing |
| | SNP | Amplicons | Oligonucleotide | Sequencing |
| | SNP | Oligonucleotide | Amplicon | Sequencing |

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### GENE EXPRESSION MICROARRAYS

Gene expression microarrays are used to measure the relative level at which a certain gene is expressed. They are a powerful tool for target gene discovery, molecular tumor characterization, diagnosis, classification, treatment, and monitoring of diseases. Underlying molecular subgroups that are active in diseases have been identified by observing distinct and recurring gene expression subsets found within diseased tissues. Gene expression microarrays are also used to measure changes in gene expression over a given period of time, e.g., within various stages of a cell cycle or by identification of gene mutation(s) that lead to cancerous growth. Another application for gene expression microarrays is the development of new drugs, e.g. by measuring the down-regulation of a gene associated with a particular disease to monitor the effectiveness of a new drug. When the expression levels from a set of genes are measured, the term gene expression signature (biomarker or classifier) is used. Other examples of biomarkers or classifiers are drug activity classifiers that are used to diagnose the mechanism of action of a drug or toxicity classifiers that are used to diagnose and develop dosage parameters for a patient.

### aCGH MICROARRAYS

In contrast to gene expression microarrays, aCGH microarrays target segments of DNA rather than individual genes (this is, similar to chromosomal banding and traditional comparative genomic hybridization). In an aCGH microarray, the array elements, which are large pieces of genomic DNA or specially designed oligonucleotides, are used to identify a known chromosomal location or changes. The primary advantage of aCGH is the ability to detect DNA copy changes at multiple loci in a single assay and to do so at a much greater resolution compared to traditional CGH. Depending on their design, aCGH microarrays provide distinct advantages over conventional cytogenetic analysis such as karyotyping and fluorescence in situ hybridization (FISH) because they have the potential to detect the majority of microscopic and submicroscopic chromosomal abnormalities. Compared to aCGH, these conventional cytogenetic techniques have low throughput, are labor-intensive, and often require specially trained staff to perform tests in a consistent manner. aCGH microarrays are also useful for the detection of cancer by monitoring the loci of oncogenes and tumor suppressor genes.

### SNP MICROARRAYS

SNP microarrays identify the presence of known sequence polymorphisms by analysis of the pattern of hybridization to a series of probes that are specifically complementary either to wild-type or mutant sequences. If the SNP or set of SNPs associated with a particular disease are known, SNP microarrays can be used to identify a disease in an individual. SNP microarrays provide an efficient and inexpensive tool for simultaneously studying multiple genetic variations in multiple samples.

## Design of Microarrays

The following sections discuss the design of the three types of microarrays described above and the suitability of the materials used for the microarray probes for each of the three types.

### GENE EXPRESSION MICROARRAY

These microarrays are the most common type of microarray in use today. The array elements consist of either cDNA derived from mRNA of known genes but of unknown sequence, or oligonucleotides for which detailed sequence information is available. Oligonucleotides are preferred array elements because of the affordable cost of synthesis and the large amount of sequence information now available for specific genes or gene fragments. These can be arrayed in specific patterns to enable accurate analysis of related gene sequences and gene families in a single hybridization assay. The following general principles apply to oligonucleotide design for gene expression microarrays:

1. Oligonucleotides should be 25–70 mers.
2. Oligonucleotides should include appropriate controls (i.e., oligonucleotides corresponding to sequences from a different organism).
3. All oligonucleotides should map to within 1000 nucleotides of the 3′ end of cDNAs and should correspond to the coding strand.
4. Sequence repeats, stretches of polyA, G, C, and T and extremes of $T_m$s should be avoided.
5. Oligonucleotides should be compared to sequences in existing databases to avoid cross-reactivity (less than 70% sequence identity with nontarget sequences is preferable).

In addition to oligonucleotides, PCR amplicons and double-stranded DNA (dsDNA) are also used as probes. However, the PCR amplicons require purification to remove enzymes, salts, nucleotides, and other contaminants from the amplification process that could interfere with the binding of the probes and could also inhibit hybridization. In addition, the preparation of dsDNA probes for spotting is labor intensive and expensive. Moreover, dsDNA probes can have repetitive sequences that compromise hybridization specificity. When sequence information is unavailable, dsDNA remain the probes of choice because unknown dsDNA probes can still be used to study gene expression.

### aCGH MICROARRAYS

These microarrays traditionally use bacterial artificial chromosomes (BACs) of 100–200 kilo-base pairs per DNA segment as the array elements. However, the large-scale DNA isolations or PCR amplifications of such large-insert clones are elaborate and time consuming. As is the case in expression profiling applications, aCGH microarrays have transitioned from dsDNA targets to oligonucleotide targets. Oligonucleotide libraries or ready-made microarrays can now be purchased, saving considerable time and effort.

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Depending on the application, SNP microarrays can use both amplicons and oligonucleotides as probes. In one of the most common formats to detect mutations in a gene sequence, the probe is that of a single gene in which the sequence differs by a single nucleotide polymorphism from the sequence of the other probes for that gene in the same microarray. For the discrimination of only one mismatch, short oligonucleotide probes (15–30 bp) maximize the destabilization caused by mispairing and are therefore used for the detection of SNPs.

## Manufacturing of Microarrays

Microarray elements are deposited onto a solid support, the most widely used of which is glass. Microarray manufacturing can be divided into two main categories, direct synthesis of the probes on the microarray (in situ) or synthesis of the probes before spotting on the microarray (ex situ). In situ synthesis is generally used for higher density microarrays but is limited to nucleotides of approximately 25–100 bases. With increasing nucleotide length, the likelihood of truncated products increases because of the limited stability of building oligonucleotides in situ. In contrast, ex situ microarray manufacturing can put any premade material into a microarray format, including oligonucleotides, PCR products (amplicons), complementary DNA (cDNAs), and BACs.

The main techniques for in situ synthesis are photolithography, maskless lithography, and ink jetting. Microarrays are generally manufactured commercially, although for a small number of low-density microarrays, the end user can manufacture the microarrays using a low-throughput microarray manufacturing robotic instrument (a personal microarrayer). However, only maskless lithography and ink jetting are available for end user manufacturing. In photolithography, a glass substrate containing a photomask, which is chemically prepared so that particular nucleotides bind to specific positions, is used to synthesize the oligonucleotides on the substrate. The masks predetermine which of the nucleotides are activated when flooded with one of the four types of nucleotides. The process is repeated until the required number of bases is synthesized. The manufacture of these microarrays uses computer algorithms and multiple spots to cover the gene of interest. Maskless lithography uses a digital micromirror device that uses a solid-state array of miniature aluminum mirrors to create virtual masks that replace the physical photomasks. A computer controls the desired pattern of UV light via individual mirrors. Each digital micromirror in turn controls the pattern of UV light projected onto the glass in the reaction chamber, which is coupled to a DNA synthesizer. The UV light selectively cleaves a UV-labile protecting group at the precise location where the next nucleotide will be coupled. The patterns are coordinated with the DNA synthesis chemistry in a parallel, combinatorial manner so that hundreds of thousands of unique oligonucleotides can be synthesized in a single microarray. Ink jetting is accomplished by building up the nucleotides, base-by-base, in repetitive print layers using standard phosphoramidite chemistry. Inkjet heads similar to those used in commercial inkjet printers are connected to bottles that contain the four different phosphoramidite nucleotides that make up the building blocks of in situ nucleic acid synthesis. The advantages of inkjetting and maskless lithography are flexibility in design and the ability to make small batches of arrays quickly.

The two main types of ex situ manufacturing techniques are microspotting pins (contact printing) and piezoelectric printing (noncontact). The technology excels at printing multiple probes many times over numerous surfaces with one small-volume loading of probe. Spot size and delivery volume are controlled by the size of the end of the tip, and many tip sizes are available. A piezoelectric printing mechanism uses a small dielectric crystal in contact with a glass capillary that holds the sample fluid. Application of the voltage results in ejection of fluid from the tip, resulting in drop volumes from hundreds of picoliters to several microliters.

## General Experimental Considerations

Regardless of the type and application, all microarray experiments have a similar workflow: amplification step, labeling, hybridization, and wash steps, followed by scanning, quantitation, and reporting. The experimental design determines the type of microarray used, number of spots required, and the specific sets of nucleic acids on the microarray. The experimental design also influences the platform used, such as the number of spots, surface type, nucleic acid type, throughput, resolution, and number of colors that can be detected in a single assay. Platforms can be open (support is available from multiple vendors) or closed (support from a single vendor). In general, experimental designs that require a high density of spots and quantitation are more difficult and expensive to implement than qualitative assays.

## Microarray Sample Considerations

Sample extraction, isolation, and preparation should be carefully chosen in order not to alter the ability of the resulting target to hybridize to the microarray. In general, sample preparation issues are the same for microarrays as for other laboratory techniques such as qPCR (quantitative PCR) and sequencing (described in chapters ⟨1126⟩ and ⟨1127⟩). RNA, cDNA, genomic DNA, and PCR products are some of the sample types analyzed with microarrays. In some genotyping applications, specific alleles are used both as array elements and targets.

As with any nucleic acid technique, the quality of the nucleic acid is critical for the microarray experiment. The nucleic acid should be pure, intact, and accurately quantitated before use ⟨1126⟩. In particular, the presence of contaminating DNA in total RNA samples may cause problems in microarray analysis because some labeling methods label both RNA and DNA with equal efficiency. For some applications in which even trace contaminants with either RNA or DNA may interfere, pretreatment with DNase or RNase may be necessary. For example, contaminating, labeled DNA can hybridize with microarray targets leading to high-level hybridization signals that are not derived from RNA transcripts, thus resulting in an inaccurate estimation of the target RNA concentration because both nucleic acid species are quantitated at the same wavelength.

A major consideration in any microarray experiment is the availability of adequate amounts of sample nucleic acid for analysis. For example, sample from laser-capture microdissection, needle tissue biopsies, or other small clinical samples do not yield

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sufficient RNA (for expression microarrays) or DNA (for aCGH microarrays) and must be amplified before analysis. It is critical that the amplification procedures for amplification of mRNA be so designed that the final mixture of amplicons accurately reflect the distribution of mRNA species in the sample. Uniform amplification of genomic DNA for aCGH microarrays can be achieved by the use of multiple displacement amplification (MDA), which overcomes the nonuniform amplification of genomic DNA that occurs in PCR-based amplification methods that use degenerate oligonucleotide primed PCR (DOP-PCR). For SNP arrays where specific alleles are the target of interest, nonuniform amplification is not an issue, and samples can be amplified (and labeled) by PCR, multiplex PCR, and WGA (see ⟨1127⟩).

## Microarray Labeling

The targets for a microarray are a population of nucleic acids that are extracted from a sample and are appropriately labeled. Many methods can be used for labeling targets (see ⟨1127⟩), but fluorescent labeling is the most widely used because it offers high sensitivity and a superior dynamic range. An added advantage is the ability to detect two or more signals in a single experiment. The method of labeling depends on the microarray type. The two methods used to fluorescently label targets for gene expression microarrays, direct and indirect labeling, have been described in ⟨1127⟩. In general, the second method (indirect labeling), in which the label is added via a linker, requires less starting material and is less expensive. Published reports have shown that this method yields results similar to those obtained from directly labeled samples. In microarray aCGH, a patient's DNA and reference DNA (300–1000 ng) are typically fluorescently labeled with red and green fluorescent dyes, respectively, often using a random priming protocol. Random prime labeling uses a high concentration of Klenow enzyme whereby genomic DNA is digested with restriction enzymes and hybridized with random primers. The primers are extended by the 5′–3′ polymerase activity of Klenow, resulting in a strand displacement activity with the direct incorporation of labeled nucleotides. SNP microarrays using oligonucleotides as array elements are labeled using fluorescently labeled nucleotides in both single and multiplexed PCR reactions, followed by a purification step to remove unincorporated dyes. Where amplicons are used as array element, labeled oligonucleotide probes are synthesized using phosphoramidite chemistry.

## Hybridization and Wash

Hybridization should be carried out under conditions that minimize annealing of noncomplementary fragments. The wash steps following a hybridization reaction are optimized to provide the highest possible specificity, signal-to-noise ratio, and reproducibility (see ⟨1126⟩). Before hybridization, double-stranded probes and targets should be denatured, and nonspecific sites should be blocked. Microarray surface chemistries are designed to capture all nucleic acids with high efficiency, so the free-binding groups on the surface must be blocked or inactivated to prevent nonspecific binding of labeled material that could compromise the signal-to-noise ratio. Surfaces are blocked and washed with various aqueous-based buffers that typically include salts, detergents, and blocking agents such as low molecular weight, hydrolyzed proteins. The purpose of the posthybridization washes is to remove all unattached and nonspecifically bound label from the surface and probes. In general, both automated and manual washes are done in saline sodium citrate/sodium dodecyl sulfate (SSC/SDS) buffers of various concentrations and at different elevated temperatures depending on the stringency required. After the final wash step, microarrays using fluorescent targets are dried immediately by centrifugation or in a nitrogen stream. Hybridized microarrays must be stored in the dark and should be scanned as soon as possible. Some fluorescent dyes used in microarray analysis are subject to degradation by environmental ozone, and in these cases ozone levels in the experimental environment must be less than 5 parts per billion. Specialized ozone-free hoods are made to protect microarray dyes.

## Microarray Detection

Regardless of the microarray type, each spot on a microarray represents a unique probe sequence to which a single, labeled target is bound, and this specific binding allows detection and quantitation of the target. This is achieved by the emission of light (photons) at a particular wavelength by the fluorescently labeled duplexes when the microarray is exposed to light of specific wavelength from an excitation source. The emitted fluorescent light is converted to electrical energy by a detector. The detector is either a photomultiplier tube (PMT) or a charge-coupled device (CCD) with specially designed optical paths that collect the raw data from microarrays (scanning). The detector filters and optical paths are designed to detect specific fluorescent dyes at sufficient resolution while eliminating crosstalk when two or more dyes are used on a single microarray. The resulting signal is proportional to the number of photons emitted by the microarray. These signals are used to create a digitized image showing the presence and quantitation of specific targets.

Samples can be scanned from a single wavelength channel or can be sequentially scanned from two channels. For instance, for a single-channel microarray platform, a sample is typically labeled with a fluorophore that emits a signal in the red channel. For a dual-channel microarray format, a second sample can be labeled with a dye that emits in the green channel. Dual labeling is used in some experimental designs, such as expression microarrays, to measure the overexpression of a gene associated with a disease state. In such experiments, cDNAs derived from the mRNA of normal and diseased tissues are differentially labeled, mixed, and tested on the same slide in a competitive hybridization reaction. The resulting ratios of the two colors reflect the relative abundance of the labeled material within each sample. Similarly, calculating the fluorescent ratios from each target on an aCGH microarray allows the mapping of gains and losses for a chromosome of interest.

## Microarray Image Processing

Most microarray scanners detect and acquire one, two, or more colors (via one, two, or more channels). The optical path of the system minimizes overlap between the spectra (crosstalk) and allows acquisition of two spectrally separate images. In many cases, the images are represented as a red and a green image. When two colors are used, the ratio of the two fluorescence images eliminates artifacts caused by regional bias and irregular spot size. When one color is used, the fluorescence signals from

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two or more microarrays are normalized and can be compared with each other. Diameters of spots printed on the arrays range from 10 µm to just under 1000 µm, and the resolution of scanners ranges from 1 to 50 µm. Thus, depending on scanner resolution, variable amounts of pixel data can be collected per scan over an entire microarray.

## MICROARRAY IMAGE ANALYSIS

The analysis of scanned images usually involves three tasks: spot finding or gridding, image segmentation, and spot quantification.

Spots are initially assigned specific coordinates, and the process of spot finding or gridding can range from manual to fully automatic, depending on the image-processing software used. This takes into account the individual size and shape of each spot and adjusts for uneven rows and columns that may be produced by the printing process.

The process of segmentation partitions the entire image to foreground or background pixels and relies on the spatial and intensity properties of each pixel. There are four main types of signal segmentation that have been used for spotted arrays. The simplest method is spatial segmentation which places two circles (inner and outer circles) of fixed but different sizes over each spot to demarcate probe signal from the immediate background signal. On the one hand, because of the irregularity of spot sizes on some microarrays, the actual area inner circle may be larger than the diameter of a spot and thus will contain background pixels. On the other hand, artifacts and signal can be found in the area between the inner and outer circles and contribute to the background signal. The second method, intensity-based segmentation, distinguishes signal pixels from background pixels based on the spot intensities within a target region. In this case, a certain percentage of pixels within the top-ranked intensities may be classified as signal pixels. The advantages of this method are simplicity and speed, but the drawback is the inability to distinguish between artifacts and signal and the tendency to detect low signals that are close to background. The third method is a statistical approach known as Mann-Whitney segmentation that combines information from spatial and intensity-based analysis. Here, background pixels located outside the inner circle set are used to determine a threshold intensity level for a signal within the inner circle. The limitation of this method is that a large amount of spot irregularities and artifacts can reduce its accuracy. The fourth method, the trimmed measurement segmentation method, also combines spatial and intensity information and measures signal distributions inside and outside the inner circle. The method trims the upper and lower extremes of each distribution to allow removal of signal from artifacts and incorrectly located background or foreground signal pixels.

The main assumption of spot quantification is that the total fluorescent intensity from a spot is proportional to the expression level of the labeled transcript. This is highly dependent on a number of factors, including target preparation, hybridization conditions, and signal detection within the linear dynamic range. If the amount of probe deposited during the microarray manufacturing procedure varies from spot to spot and from array to array, thus resulting in different sized spots, the sum or total signal intensity can be variable and inaccurate. To correct for this variation, microarrays should be spotted via homogenous surface chemistry that has a fixed binding capacity. This ensures the same amount of probe at each spot location. Alternatively, spots can be quantified by taking the mean, median, or mode of intensities of all signal pixels determined to be foreground signal. The more robust methods that protect against outlier signals are the trimmed mean (where a certain percentage of top and bottom signals are trimmed before calculation of the mean) and median signal intensities. When two different fluorophores are used, the intensity ratio can be used to correct for variable probe amounts and can be calculated from mean, median, and mode intensities from each channel.

## MICROARRAY DATA ANALYSIS

Particularly dense formats of microarrays that contain tens of thousands to millions of probes per chip or slide generate a large volume of raw data per array, which requires the use of specialized data-analysis software. Microarray software programs are designed to extract primary data, normalize the data to remove the influence of experimental variation, and link probes to relevant gene and sequence-derived targets. Software programs are also available to apply statistical methods, analyze, visually display, and manage data in order to extract biologically meaningful information. The major parts of data analysis are normalization, background correction, and ratio calculation.

Normalization systematically adjusts microarray raw data in an effort to reduce the variability brought about by differences in the manufacture and processing of the microarrays and by technical variables so that true biological differences between samples can be detected. The wide range of normalization methods precludes a detailed discussion of the topic in this chapter, and currently there are no standards for normalization. Commonly used algorithms are selected based on the microarray type, the number of fluorophores used, and the samples being studied. Some methods are built into the manufacturer's software, but others are available from commercial sources or open-source software providers.

Background correction eliminates low levels of noise in microarrays stemming from both the inherent noise of the detection instruments and from the surface chemistry used in manufacturing. Several contaminants acquired from microarray processing can cause high levels of background that must be corrected before data analysis.

In two-color microarrays, the ratio of signal intensities of array elements of two co-hybridized samples is used as a relative measure of gene expression. In single-channel systems, the ratio can be calculated between signals taken from two different samples (one sample is a reference sample) hybridized on individual microarrays. Thus, the resulting data from microarrays does not represent an absolute quantification but rather a relative level of RNA or DNA against a reference sample or control.

### Quality Control and Quality Assurance

As with any diagnostic assay, quality control and quality assurance are critically important. Microarrays must demonstrate robustness and reproducibility. The general quality control and assurance steps outlined in chapter ⟨1127⟩ for nucleic acids and NAT also apply to microarrays. Unlike other diagnostic tests, no reference reagents are available at present for quality control

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of microarrays, and regulatory guidance is emerging. FDA has issued a draft guideline titled "In Vitro Diagnostic Multivariate Index Assays." This guidance addresses the definition and regulatory status of a class of in vitro diagnostic devices referred to as in vitro diagnostic multivariate index assays (IVDMIAs), and microarrays fall into this category. The guidance addresses premarket pathways and postmarket requirements with respect to IVDMIAs.

Several unintended sources of variability that are specific to microarrays can extensively affect signal intensities and the accurate derivation of a true signal that accurately reflects the labeled transcript. A major source of variability is spot quality. Measurements of spot quality at the processing stage permit removal of spots with poor or questionable quality. Other sources of variability are artifacts, for example, regional shifts (rise or fall) in an array's overall signal that can be visualized within single chips or in-composite data derived from multiple chips. These changes can be distinguished from actual variability because they are nonrandom, and patterns can be detected by visualizing signals over the entire area of the chip. When dyes of different spectral properties are used to label two different samples in a competitive hybridization reaction using a single array, differences may arise because of labeling bias rather than gene expression level. For instance, the green channel may appear consistently brighter than the red channel despite the fact that there are no real differences in expression. Hybridization with reverse dyes can ensure detection and elimination of dye bias effects. As with any quantitative assay for RNA, the integrity of the sample affects its measurement, and sample quality is an important determinant for accuracy. For instance, because labeling is directed from the 3′ to the 5′ end but RNA degrades from the 5′ end, degraded RNA leads to high 3′/5′ ratios, resulting in nonuniform labeling across the entire transcript. Finally, variability can be introduced during the processing of microarrays, which is a relatively complex procedure that involves multiple steps such as labeling, hybridization, washing, and staining (technical variables). Such variability can mask true differences in the samples tested.

When used as a diagnostic test, the microarray should demonstrate robustness, reproducibility, a high degree of correlation to the original format, and reliable prognosis prediction. The microarray ideally should contain at least 2–3 replicate spots for each reporter gene to ensure intra-assay reproducibility. With a two-channel microarray, a reference sample pool can be hybridized in the complimentary fluorescent channel so that data can be expressed as log ratios, which reduces the need for extensive normalization. Interassay reproducibility of test results and stability over time can be tracked by using a number of reference samples that, when labeled and hybridized, represent a spectrum of predictive endpoints (for instance, high risk, borderline risk, low risk) and that should fall within a predetermined range of results. Failure of these controls should result in rejection of results of samples in the same assay run. If the assay is performed at many sites, site-to-site reproducibility is imperative and must be assessed. The reproducibility of the assay with regard to tissue extraction also must be determined, and the quality of tissue specimens or RNA should be specified clearly (for instance, percentage of tumor cells within a specimen).

In conclusion, microarray experiments should be carefully designed and conducted in order to minimize variability and to yield data that accurately relate to the samples analyzed. In addition, biomarkers of interest should be analyzed and verified using an alternative platform such as qRT-PCR that should be shown to be reproducibly detected in the same and different samples. The development of reference standards, especially when microarrays are used as diagnostic tests, is the next step to ensuring the quality and validation of microarray results. With the shift from custom-built to commercial microarrays, issues with reproducibility, standardization, and quality control have been largely addressed by the stringent quality controls used in commercial manufacturing.

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# ⟨1129⟩ NUCLEIC ACID-BASED TECHNIQUES—GENOTYPING

## INTRODUCTION

This chapter outlines techniques for detecting single-base DNA differences and other types of polymorphic DNA sequences that occur in the three billion bases that make up the human genome. The most common genetic variation is a single nucleotide polymorphism (SNP), which is a simple change in one base of the gene sequence. SNPs occur on average every 1000 bases and account for a significant amount of inter-individual variability. SNPs can predispose individuals to disease or influence their response to a drug. Approximately 1.8 million human SNP loci have been identified, and more are likely to be discovered in the coming years.[1]

Common approaches for detecting SNPs and other types of polymorphic DNA sequences are described in the following sections. These approaches encompass a variety of techniques, such as nucleic acid amplification techniques (NAT), real-time NAT, and microarrays, the principles of which are covered in more detail in related chapters. This chapter focuses on the specific modifications of the techniques that are necessary to enable detection of single base differences.

## SNP GENOTYPING TECHNOLOGIES

Although the usefulness of studying SNPs for gene mapping and disease association studies is apparent, a single standardized procedure for SNP genotyping has not been adopted. Various approaches for performing SNP genotyping have been developed to meet a wide range of needs, including throughput capacity, ease of assay design, accuracy, and reliability. Available procedures can also be divided according to whether they are based on identifying known SNPs or whether they can be used to screen for unknown SNPs. To identify the most appropriate SNP genotyping procedure for a specific application, the throughput requirements in terms of the number of SNPs to be analyzed per sample (multiplexing level) and the sample throughput need to be determined because different approaches may work best depending on these requirements.

Most procedures used for genotyping SNPs depend on polymerase chain reaction (PCR) amplification of the genomic regions that span the SNPs followed by the actual genotyping reaction. PCR provides the required sensitivity and specificity for distinguishing between heterozygous and homozygous genotypes in large, complex genomes. The difficulty of designing and carrying out multiplex PCR reactions limits the throughput of many of the current SNP genotyping assays. The following sections outline several of the major approaches currently in use for SNP genotyping. In many cases the underlying technology can be modified to meet the specific application requirements in terms of sample throughput and number of SNPs detected. In general, real-time PCR-based procedures are better suited to higher sample numbers, and array-based procedures are better suited to the simultaneous detection of many SNPs. Newer technologies based on multiplexed array formats are also emerging and will be suitable for high sample numbers and many SNP applications.

### Sequencing

Sequencing is the definitive procedure for DNA analysis, and its use for SNP detection allows unambiguous identification of base changes (see *Nucleic Acid-Based Techniques—Extraction, Detection, and Sequencing* ⟨1126⟩ for nucleic acid sequencing). The standard technology is expensive, and the procedure is time consuming and labor intensive and suffers from low sample throughput. Sequencing is a useful confirmatory tool, and it has applications in situations when other technologies are not appropriate, but is not the most cost-effective solution for the majority of SNP genotyping applications that require the identification of only one or a few bases.

### Restriction Fragment Length Polymorphism Analysis

The first widely used procedure for the detection of polymorphisms exploited alterations in restriction enzyme sites caused by SNPs, leading to the gain or loss of cutting events. PCR–restriction fragment length polymorphism (RFLP) analysis comprises PCR amplification of a fragment of interest and subsequent digestion with a restriction enzyme. The fragments produced are typically analyzed by a size fractionation procedure, usually gel electrophoresis. Because of its simplicity, the procedure has been and still is extensively used, although it entails certain limitations: only a subset of polymorphisms that reside in an endonuclease restriction site can be studied with the conventional procedure; incomplete digestion due to suboptimal processing can produce misleading digestion patterns; and the procedure is less amenable to automation than are other SNP genotyping procedures.

### Probe Hybridization

The basis of many SNP genotyping procedures are DNA hybridizations that make use of the stronger binding of a DNA probe to a perfectly matched complementary target than to a target that contains a single base mismatch. The ability of hybridization with allele-specific oligonucleotides (ASO) to detect a single base mismatch was first shown in the late 1970s and subsequently was used to detect the sickle-cell mutation in the beta-globin gene by Southern blot hybridization. The invention of PCR facilitated the further development of probe-based assays for genotyping SNPs in complex genomes.

[1] Database of Single Nucleotide Polymorphism (db SNP) Build 128 is available from National Center for Biotechnology Information (NCBI), http://www.ncbi.nlm.nih.gov/projects/SNP/index.html.

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The thermal stability of a hybrid between an ASO probe and its SNP-containing target sequence is not only determined by the stringency of the reaction conditions but also by the secondary structure of the target sequence and the nucleotide sequence flanking the SNP. Therefore, prediction a priori of the reaction conditions or the sequence of the ASO probe that will allow optimal discrimination between two alleles using ASO hybridization is difficult. These parameters should be established empirically and separately for each SNP. Consequently, there is no single set of reaction conditions that would be optimal for genotyping all SNPs, which makes the design of multiplex assays based on hybridization with ASO probes an extremely difficult task.

One approach to counter the problem of assay design is to carry out multiplex ASO hybridization reactions on arrays that carry multiple probes for each SNP that will be analyzed. This involves using probe sets in which the SNP occurs at different positions along the probes. It becomes feasible to include large numbers of ASO probes per SNP when one uses high-density arrays that can carry as many as $10^6$ probes per $cm^2$.

Another approach is to use base analogues such as locked nucleic acid (LNA), which is described in detail in *Nucleic Acid-Based Techniques—Amplification* ⟨1127⟩. For applications that involve few SNPs but many samples, homogeneous real-time PCR approaches have been developed. These include the use of fluorescent probe chemistries such as hydrolysis probes, stem-loop probes, and FRET (fluorescence resonance energy transfer) hybridization probes. The principle of these assays is discussed in more detail in *Nucleic Acid-Based Techniques—Amplification* ⟨1127⟩. For SNP detection, the basis of many assays is the selective binding of the ASO probe to its perfectly matched target sequence, resulting in energy transfer and generation of a fluorescence signal. Probes designed with specific secondary structures tend to form a stem–loop structure that destabilizes mismatched hybrids, increasing their power of allele distinction as compared with that of linear ASO probes. Hydrolysis probes modified with minor groove-binder molecules that increase target affinity show improved powers of allele discrimination. The use of two probes, each labeled with a different reporter fluorophore, allows both SNP alleles to be detected in a single tube. Limited multiplexing can be achieved by using probes labeled with different fluorophores. In the fluorescent probe–based assays, the increase in fluorescence due to accumulating PCR product is usually monitored in real time in 96-well or 384-well microtiter plates. Alternatively, the fluorescence generated from the two alleles can be measured after completion of the PCR. In this case the results are expressed as a signal ratio that reflects the hybridization of the two oligonucleotides to the target sequence, and so differences in amplification efficiency between samples do not affect interpretation of the genotyping results.

A third approach involves heating the reaction after PCR has been completed in order to disassociate the probe from the target. Each duplex has its own specific $T_m$, which is defined as the temperature at which 50% of the DNA becomes single stranded. The $T_m$ depends on the stability of the probe–target duplex. Perfectly matched probe–target duplexes have a greater stability and hence a higher $T_m$ than does the same duplex containing a single base mismatch. By continuously monitoring the fluorescence during the heating phase, analysts generate a "melt curve" that measures the changes in fluorescence that result when the probe denatures, or "melts," away from the amplicon. This approach can be used only for systems that do not rely on hydrolysis of the probe to generate a signal and is therefore not suitable for hydrolysis probe assays.

Because no post-PCR processing or label-separation steps are required, homogeneous real-time PCR assays are simple to perform, making them useful for high-throughput genotyping applications. The optimal probes must be designed individually for each SNP, and the assays are therefore most efficient when a limited number of SNPs is analyzed. The cost of probes modified with fluorescent and quenching moieties may also be a limiting factor in the high-throughput application of the assays.

## Primer Extension

In this technique, an oligonucleotide is used to prime DNA synthesis by a polymerase enzyme, as performed in a standard PCR or sequencing reaction. Variations of the technique exist. Allele-specific PCR uses two primers, each fully complementary to one of the SNP alleles, with the SNP position being at the 3′ end of the primer, and with a common reverse PCR primer to selectively amplify the SNP alleles. Because only perfectly matched oligonucleotides will prime DNA polymerase extension, product will be detected only from the reaction containing the perfectly matched primer.

Agarose gel electrophoresis is used to detect the amplified products, although homogeneous, real-time, allele-specific PCR approaches have also been developed using primers labeled with different fluorophores or a fluorescent dye that intercalates with the double-stranded PCR products or by performing amplicon detection using probes such as hydrolysis and hairpin (stem–loop) probes. When using intercalating dyes or labeled allele-specific PCR primers without a consecutive target-specific detection reaction or size-separation step, one may find that the specificity of the procedure may be compromised owing to primer–dimers and other spurious amplification products that will not be distinguished from the actual PCR product. A limitation of all variants of allele-specific PCR is that the reaction conditions or primer design for selective allele amplification must be optimized empirically for each SNP. Like the hydrolysis and hairpin probe assays, the homogeneous allele-specific PCR procedures are best suited for the analysis of a limited number of SNPs in large sample collections. Array-based approaches for greater SNP multiplexing have also been developed.

In procedures based on single nucleotide primer extension (sometimes known as minisequencing), allele discrimination is based on the high accuracy of nucleotide incorporation by DNA polymerase. A primer is used, and its 3′ end is positioned on the base just preceding the SNP to be tested. The DNA polymerase is then used to incorporate labeled ddNTPs, each labeled with different fluorescent dyes. After the labeled oligonucleotides are separated from the nonincorporated ddNTPs, the results can be scored on a fluorescence plate reader. In addition to fluorescent tags, ddNTPs may be labeled with biotin or haptens and then detected indirectly through antibodies conjugated to alkaline phosphatase or peroxidase using colorimetric or chemiluminiscent markers in ELISA formats.

Multiplexing of this procedure has also been described to reduce costs and improve throughput. In these procedures, the different loci genotyped simultaneously are separated either by gel electrophoresis or by hybridization to arrayed tags. Primer extension directly on a solid support such as a microarray is also possible. The immobilization of the single-stranded primers on the solid support may be through biotin–avidin–streptavidin reaction or covalently via 5′ disulfide groups.

Mass spectrometry using techniques such as matrix-assisted laser desorption–ionization time-of-flight mass spectrometry (MALDI-TOF MS) can also be used to determine the identity of the ddNTP incorporated based on mass. A difficulty with MALDI-TOF MS is that the primer extension products must be rigorously purified before measurement to avoid background

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from biological material present in the sample. Such enzyme-assisted procedures have proven to be more robust and to provide more specific allele discrimination than does ASO hybridization at similar reaction conditions. These features are advantageous for high-throughput applications because the effort required for assay design and optimization is minimized.

## Ligation

In the oligonucleotide ligation assay (OLA), oligonucleotides are designed so that they meet at the position of the SNP to be tested. Enzymatic joining, using a DNA ligase, occurs only when the match is perfect. The test is usually performed by designing two oligonucleotides specific for each allele and labeled differently on one side of the SNP, and one common oligonucleotide on the other. Detection of the alleles can be performed directly in the microplate wells by colorimetric approaches. Multiplexing and the use of gel separation have also been described.

OLA has also been used in microarray formats with one of the ligation probes immobilized or with immobilized single stem–loop probes. Alternatively, ligation can be carried out in solution followed by capture of the ligation products on microarrays or on microparticles that carry a generic set of oligonucleotides that are complementary to a "tag" sequence on one of the ligation probes. In practice, thermostable ligases are frequently used for genotyping SNPs in combination with PCR before allele-specific ligase detection reactions. Because the reaction mechanisms for PCR and ligation are different, the reagents for both reactions can be combined. This feature is used in a homogeneous, real-time PCR assay with ligase-mediated genotyping and detection by FRET. Compared with DNA-polymerase-assisted primer extension procedures, a drawback of the OLAs is that detection of each SNP requires three oligonucleotides, which increases the costs of these assays.

Padlock probes are linear oligonucleotides, the ends of which are complementary to the target and have a central stretch of random sequence. When perfectly hybridized to their target sequence, padlock probes can be circularized by ligation, whereas a mismatch with the target sequence prevents ligation. Circularized oligonucleotides can act as templates for DNA-polymerase-assisted rolling circle amplification (RCA). RCA can be used to amplify the ligated circularized padlock probes to a level required for detecting single-copy sequences. A homogeneous, isothermal assay for genotyping individual SNPs in a microtiter plate format has been devised by combining exponential amplification of ligated padlock probes using a branched rolling circle amplification reaction with detection by energy-transfer-labeled hairpin primers.

## Displacement

The invader assay uses the property of flap endonucleases (FENs) for removing redundant portions (flap) from the 5′ end of a downstream DNA fragment overlapping an upstream (invader) DNA fragment. An invader oligonucleotide is designed with its 3′ end on the SNP to be tested. Two oligonucleotide signal probes are also designed, overlapping the polymorphic site and each corresponding to one of the alleles. After displacement of the signal probes by the invader probe, FEN-mediated cleavage occurs only for the perfectly matched allele-specific signal probe. Generation of the cleaved fragment is monitored by using it in a second reaction as an invader probe to cleave a FRET probe. This assay does not require PCR amplification of the locus to be tested, and scoring can be done using a simple fluorescence plate reader.

## Pyrosequencing

In the pyrosequencing procedure, primer extension is monitored by enzyme-mediated luminometric detection of pyrophosphate (PPi), which is released on incorporation of deoxynucleotide triphosphates. The genotype of an SNP is deduced by sequential addition and degradation of the four nucleotides using apyrase in a dedicated instrument that operates in a 96-well or 384-well microtiter plate format. Using pyrosequencing, the apparatus can determine short 30 to 50 bp sequences of DNA that flank an SNP. A limitation of the procedure is that the sequential identification of bases prevents genotyping of several SNPs per reaction in diploid genomes. An advantage of the procedure is that any new polymorphism will be detected. However, specific equipment is needed for the injection of the nucleotides.

## Single-Strand Conformation Polymorphism and Heteroduplex Analysis

Single-strand conformation polymorphism (SSCP) and heteroduplex analysis were among the first procedures established for the detection of SNPs. Conventional SSCP analysis involves denaturing PCR-amplified fragments and subsequent formation of sequence-specific secondary and tertiary structures of the single strands during nondenaturing gel electrophoresis. The electrophoretic mobility then depends on the 3-D shape of the single-stranded molecules. One single base difference in DNA fragments of up to 300 bp will usually change the conformation in a way that can be detected by nondenaturing PAGE.

The traditional polyacrylamide gels and $^{32}$P-labeled fragments are frequently being replaced by fluorescently labeled fragments and automated capillary electrophoresis. The simplicity of the procedure, combined with automation and short analysis time, contribute to high-throughput analysis at relatively low cost. If the denatured PCR products are allowed to slowly re-nature, they form DNA duplexes. The duplexes with the same sequence on both strands (homoduplexes) or with a single base pair mismatch on one strand (heteroduplexes) have different electrophoretic mobility in a native gel. In the case of a single base pair substitution, the heteroduplex can easily be separated from a homoduplex.

In other versions of the technique, denaturing high-performance liquid chromatography (DHPLC) is used for the separation of the heteroduplex and homoduplex strands. The mutation analysis with DHPLC can be almost totally automated with an autosampler on one end and a fraction collector on the other. Analysis is rapid (about 5 minutes per sample), and simple evaluation of data distinguishes between simple and multiple peaks in the elution profiles, allowing lengths as large as 1.5 kb of DNA to be analyzed. A disadvantage may be the recommended use of Pfu DNA polymerase, which, as a high-fidelity enzyme, allows sharper peaks but may be less successful in amplifying some regions.

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### Short Tandem Repeat Profiling

A short tandem repeat (STR) is a type of DNA polymorphism that occurs when a pattern of two or more nucleotides is repeated and the repeated sequences are directly adjacent to each other. The pattern can range in length from 2 to 10 bp (e.g., $CATG_n$ in a genomic region) and is typically in the noncoding intronic, or upstream/downstream regions. By examining several STR loci and counting how many repeats of a specific STR sequence there are at a given locus, one can create a unique genetic profile of an individual. Currently more than 10,000 STR sequences in the human genome have been published. STR analysis has become the prevalent analysis procedure for determining genetic profiles in forensic cases. STR analysis in the field of forensics came into popularity in the mid to late 1990s. The STRs in use for forensic analysis are tetra- or penta-nucleotide repeats (4 or 5 repeat units) because these give a high degree of error-free data while being robust enough to survive degradation in nonideal conditions. Shorter repeat sequences tend to suffer from artifacts such as stutter and preferential amplification; several genetic diseases are associated with tri-nucleotide repeats, including Huntington's disease. Longer repeat sequences suffer more highly from environmental degradation and do not amplify by PCR as well as do shorter sequences.

The analysis is performed by extracting nuclear DNA from the cells of a forensic sample of interest and then PCR amplifying specific polymorphic regions of the extracted sample. Once these sequences have been amplified, they are resolved either by gel electrophoresis or capillary electrophoresis, which allow the analyst to enumerate the repeats of the STR sequence in question. If the DNA is resolved by gel electrophoresis, the DNA can be visualized either by silver staining or an intercalating dye such as ethidium bromide or, as in most modern forensics labs, by fluorescent dyes. Instruments built to resolve STR fragments by capillary electrophoresis also use fluorescent dyes. In the United States, 13 core STR loci have been selected as the basis by which an individual genetic profile can be generated. These profiles are stored in local, state, and national DNA databanks such as the Combined DNA Index System (CODIS).

Forensic reference materials are available. The DNA Profiling Standard is composed of well-characterized human DNA in two forms: genomic DNA and DNA to be extracted from cells spotted onto filter paper.

## ASSAY VALIDATION CONSIDERATIONS

The difficulty in reproducing and validating existing and emerging SNP genotyping assays due to factors such as variation in performance of PCR thermal cyclers, efficiency of different enzymes, personnel, and the presence of PCR inhibitors in the sample matrix (discussed in more detail in *Nucleic Acid-Based Techniques—Amplification* ⟨1127⟩ for general NAT assays) can hamper appropriate implementation of the technologies. Also, in the clinical laboratory the use of in-house assay formats often makes comparisons between laboratories difficult. Incorrect diagnosis of a genetic mutation can have significant consequences, so accuracy of 99.99% or higher is essential for such assays. To determine the accuracy of a technology, the new procedure should be validated on multiple samples in which the genotype has been previously determined with a gold standard procedure, such as sequencing. Even with the most accurate procedure of analysis, sample preparation and amplification and detection procedures must be optimized to eliminate any potential inaccuracies.

Some genotyping errors can be minimized by careful planning of the laboratory procedures, the inclusion of well-defined controls, and increased automation. However, errors due to the processes used for genotyping are sometimes difficult to overcome and need to be taken into account. The types of errors and the frequency with which they occur differ between different approaches. Situations in which preferential amplification of one allele or nonspecific probe hybridization occur can all result in SNP miscalls. Additional unanticipated polymorphisms present within the primer/probe sequences can lead to amplification bias, highlighting the need for careful assay design and validation using alternative techniques. Limited and degraded samples can also result in preferential allelic amplification due to chance PCR priming events at low copy number.

It is preferable to have a no-call result, which would require the test to be repeated, than a miscall that provides incorrect results that are subsequently reported. Performance of replicate assays may also help to ensure accuracy. Data interpretation can also affect accuracy. Wild-type, heterozygous, and homozygous mutant results should be clearly distinguished from one another, and a well-defined measure of uncertainty should be attributed to them. Proficiency testing schemes and ring-trials go some way toward ensuring that individual assays are fit for the purpose for which they are intended for specific applications and that the staff performing them are competent. Sharing of technical information for assay design and sample preparation will also help. The availability of reference panels of well-characterized samples aids assay design and evaluation and allows sound interlaboratory comparisons to be made.

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# ⟨1130⟩ NUCLEIC ACID-BASED TECHNIQUES—APPROACHES FOR DETECTING TRACE NUCLEIC ACIDS (RESIDUAL DNA TESTING)

## INTRODUCTION

The basic principles of nucleic acid amplification technologies (NAT) and definitions of the various techniques are covered in *Nucleic Acid-Based Techniques—General* ⟨1125⟩. This chapter covers the analytical procedures used to quantify residual DNA in biopharmaceuticals.

Process characterization and the theoretical safety concerns associated with process-related impurities highlight the need for residual DNA testing in biopharmaceutical products. The ability of a manufacturing process to remove residual DNA from a biopharmaceutical product is an indicator of the quality and consistency of the process and that the process is under control. Additionally, the cells used to produce a biopharmaceutical can be sources of a range of complex, heterogeneous, and potentially unsafe impurities, with host cell DNA among these impurities. For continuous cell lines, the potential risk of residual DNA arises from both of its biological activities, namely infectivity and oncogenicity. Infectivity could be due to the presence of an infectious viral genome in the cellular DNA of the cell substrate. The oncogenicity activity of residual DNA could arise through its capacity to induce a normal cell to become transformed, which may lead to tumorigenicity. Although animal testing has shown that extraneous DNA can cause tumors or infections, no reports to date have demonstrated this risk in humans. Residual DNA content, up to 10 ng of residual DNA per parenteral dose, may be considered for DNA originating from mammalian cell cultures, but the acceptable residual DNA content may vary depending on the source of the residual DNA and the route of administration of the product. One can address residual DNA in biopharmaceutical processes in two ways: 1) by validating clearance during process validation; and/or 2) by monitoring residual DNA levels through routine testing of the drug substance. Generally, 10 ng per dose is the accepted limit, by health authorities, of host residual DNA derived from mammalian cell cultures. The level of concern regarding residual DNA can be tied to its source and the route of administration, so the residual DNA specification and procedure for monitoring DNA clearance for a given product should be developed in consultation with regulatory agencies. Regardless of whether routine testing of a drug substance is used to determine residual DNA content or whether DNA clearance is demonstrated by process validation, analytical procedures for the quantification of residual DNA are required. DNA amplification techniques, such as quantitative PCR (qPCR), are used most often for residual DNA testing because of their superior sensitivity and unique advantages (e.g., high specificity). The expectation is that the analytical procedure used to quantify residual DNA in biopharmaceuticals has a detection limit well below the DNA level allowed by regulators for biopharmaceuticals (often 10 ng/dose). Assays based on hybridization, DNA-binding protein, and qPCR are typically the techniques of choice because they can meet the sensitivity expectation.

## SAMPLE PRETREATMENT

Analysis of residual DNA requires accurate quantification of picogram levels of DNA in mg (or larger) quantities of product, which may be in a variety of matrices. In certain circumstances, the sample can be analyzed neat in the analytical procedure with acceptable recovery and precision. When the product or other sample components interfere with the assay sample, dilution may be all that is required to overcome the interference, so long as the specified DNA content of the sample remains within the useful range of the analytical procedure. When sample dilution is not effective in reducing assay interference, it may be necessary to use more extensive sample pretreatment procedures, such as proteolytic digestion, chemical dissociation, or extractions. It may be necessary to use a combination of different pretreatment steps to remove interference to an acceptable level. Extensive sample manipulation can lead to losses of DNA or introduction of environmental DNA, and should be a consideration when using one or more sample pretreatment steps. Contamination with environmental DNA may only be a concern when using a residual DNA procedure that is not sequence specific.

Protein samples may only require digestion with proteinase (e.g., Proteinase K, Pronase) to allow the analytical method to quantitatively recover the residual DNA. It may also be possible that the DNA is bound to the sample components, and chemical dissociation (e.g., detergents) may disrupt the binding, allowing sufficient recovery in the residual DNA assay. Residual DNA test procedures often use protein reagents and the use of a chemical dissociation reagent. These materials must be used at a sufficiently low level or removed so that the analysis is not compromised.

It may be necessary to extract the DNA from the sample to remove the inhibitory substances that are causing the reduced DNA recovery. Extraction procedures are typically based on precipitating the DNA from the sample or DNA-specific binding to a matrix (e.g., magnetic beads). Historically, extraction methods based on phenol and chloroform, followed by ethanol precipitation, have been applied to the purification of DNA in molecular biology research. The phenol/chloroform extraction technique may be a useful pretreatment for residual DNA samples before analysis, but the phenol/chloroform extraction technique might not be the best choice for the low levels of DNA typically found in biopharmaceutical samples. Because of these low levels, quantitative DNA recovery with ethanol precipitation may be difficult. For this reason, a carrier molecule (e.g., glycogen) may be necessary to aid in DNA recovery if the phenol/chloroform extraction technique is used. Commercial kits are available and have been used successfully for pretreatment of residual DNA samples for improved recovery in the residual DNA assay. For example, some kits use a chaotrope (sodium iodide) and a detergent (sodium *N*-lauroyl sarcosinate) to disrupt the association of the DNA with the sample. The DNA is then co-precipitated using glycogen as the carrier molecule in the presence of isopropanol. Extraction of DNA from the sample, based on binding to a solid matrix, can be found in various formats. One of the most popular formats uses magnetic beads, where the beads are added to a sample with a binding solution to capture the sample DNA on the beads. The beads are then captured and held in the sample tube using a magnetic stand while the supernatant containing the interfering substances is removed and discarded. The beads are washed repeatedly using a magnetic

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stand and a wash solution. Finally, the DNA is eluted from the beads for the assay using an elution buffer, with the beads being removed from the sample preparation using the magnetic stand.

The sample manipulation involved with pretreatment may reduce the recovery of the residual DNA or introduce environmental DNA into the sample. Great care must be taken during any sample manipulations to avoid DNA losses or contamination. The addition of target DNA-spiked samples in the residual DNA assay is a common practice. The target DNA-spiked sample should not be confused with the internal positive control (IPC), which is typically a nontarget DNA added after the sample pretreatment step to detect the presence of PCR inhibitors and to evaluate DNA amplification during the analysis. The IPC may also be introduced before the extraction to improve the control of this step. A recovery of 50%–150% of the spiked target DNA is often applied to residual DNA assays to ensure that the assay yields acceptable results. When sample characteristics (e.g., matrix effects or sample preparation method) make achieving a recovery acceptance criterion of 50%–150% impractical, then correcting the observed DNA concentration by using the load recovery percentage is also an acceptable approach.

## HYBRIDIZATION-BASED RESIDUAL DNA ASSAY

The first residual DNA assays were based on DNA hybridization, wherein a DNA probe created from host cell DNA detects and quantifies the amount of complementary DNA present in the product under assay. Double-stranded host cell DNA consists of two complementary strands of DNA that are held together by hydrogen bonding. The double-stranded DNA in the test sample is denatured to single strands and immobilized to a membrane, typically a nitrocellulose or nylon membrane. The sample is probed using host cell DNA that has been denatured and labeled. The host cell DNA probe is not a specific sequence but is prepared by a random labeling procedure during which a radioactive or fluorescent label is introduced into the host cell DNA to produce the probe. When the denatured, labeled DNA probe is brought into contact with the membrane-immobilized DNA, the probe will bind to complementary sequences of the host cell DNA. If the probe is radioactive, the membrane is placed against autoradiography film for a sufficient length of time, the film is developed, and a dark spot will be observed where the test DNA was immobilized. The level of hybridization can be measured using a phosphor-imaging system. If the probe has a fluorescent label, the intensity of the spots is determined using a fluorescence-imaging system. The intensity of the spot is proportional to the amount of probe that was hybridized to the test DNA and therefore is proportional to the amount of residual DNA in the sample. The intensity of the spot can be compared visually with the intensity of spots that correspond to a standard curve yielding semiquantitative results (i.e., visual quantitation), or the intensity can be determined using an instrument (e.g., densitometer) to create a quantitative value that is compared with the values obtained from the standard curve.

## DNA-BINDING PROTEIN-BASED RESIDUAL DNA ASSAY

Instrumentation is commercially available for the quantitation of residual DNA in biopharmaceuticals. The instrumentation requires reagents that use DNA-binding protein and antibodies targeted for DNA in a four-step analytical procedure.

1. The first step requires that the DNA be denatured into single-stranded DNA by sample heating. The denatured DNA is mixed with a single reagent that contains DNA-binding protein that is conjugated with streptavidin and a monoclonal anti-DNA antibody that is conjugated to urease. The DNA-binding protein and the monoclonal antibody are specific for single-stranded DNA but do not have any sequence specificity. This liquid phase facilitates the formation of reaction complexes that contain DNA, streptavidin, and urease.
2. During the second step, the sample is filtered through a biotinylated membrane that binds to the streptavidin and captures the complexes on the membrane, which is washed to remove any reagents that are not bound to the membrane.
3. During the third step, the membrane is inserted into a sensor on the instrument, where the urease in the DNA complex reacts with a urea solution in the sensor, producing ammonia and a change in pH that is detected using a light-addressable potentiometric sensor (LAPS). The change in pH directly correlates with the amount of DNA in the sample.
4. In the fourth step, the raw data from the instrument are analyzed using the appropriate software to determine the residual DNA content of the sample.

## POLYMERASE CHAIN REACTION TECHNIQUES

Real-time qPCR is a procedure that is well adapted to fast sample throughput and has applications in many areas of biopharmaceutical manufacture (e.g., copy number detection, virus detection). The technique can quantify the amount of a nucleic acid target sequence in DNA from a variety of samples. The DNA probe and primers used in the analysis are key to the procedure. The most common qPCR method for detection of this amplification is referred to as the 5′ nuclease assay. In this format, the probe has a reporter dye attached to one end and a quencher dye attached to the other end. A pair of DNA primers is also added to the reaction. During the amplification reaction, a thermostable DNA polymerase initiates DNA synthesis where the DNA primer binds to the single-stranded sample (template) DNA and moves along the sample DNA, synthesizing new complementary DNA. While following the template DNA, DNA polymerase I cleaves any complementary DNA in the path. If DNA polymerase I encounters the labeled DNA probe, DNA polymerase I will cleave the probe. The reporter dye is released into the solution and, in the absence of the quencher dye, the resulting fluorescence is measured. Repeating the reaction cycle results in an amplification of the fluorescence signal. The number of cycles required for the fluorescence measurement to exceed a threshold value correlates to the amount of starting residual DNA in the sample. By comparing the fluorescence obtained from a sample to a standard curve, analysts can quantify the residual DNA in the sample.

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### Alternative Detection Strategies

A number of innovative detection strategies have been developed and commercialized beyond that described above. A few of the most common are as follows:

1. Intercalating cyanine dyes fluoresce after binding to double-stranded DNA. The amount of dye incorporated is proportional to the amount of target amplicon generated. These dyes are inexpensive and easy to use. The disadvantage of this technique is the lack of a specific probe to confer sequence specificity beyond that afforded by the primers, and the dye will also bind somewhat to single-stranded DNA and RNA molecules. Consequently, primer dimers or nonspecific products may affect the quantification. However, it is possible to check for the specificity of the system by running a melting curve at the end of the PCR run, based on the principle that every product has a different dissociation temperature and depending on the size and base content.
2. Other sensitive probes exist that contain a stem-loop structure with a fluorophore and a quencher at their 5′ and 3′ ends, respectively. The stem is usually six bases long, mainly consisting of cytosines and guanines, and holds the probe in the hairpin configuration. The “stem” sequence keeps the fluorophore and the quencher in close proximity, but only in the absence of a sequence complementary to the “loop” sequence. In the presence of a complementary sequence, the probe unfolds and hybridizes to the target, leading to separation between the fluorophore and the quencher, and the probe fluoresces. The amount of signal is proportional to the amount of target amplicon sequence. The increase in fluorescence that occurs is reversible, because there is no cleavage of the probe. It is also possible to design the stem structure to add specificity to this type of probe. However, these probes are often expensive, and the signal can be weak due to the limited possible physical separation between the fluorophore and the quencher.
3. A variation on the second example described above uses a single-stranded nucleic acid sequence containing the specific PCR primers, the specific probe with a stem-loop tail separating a fluorophore and a quencher, and a blocking group. The stem-loop tail is separated from the PCR primer sequence by a “PCR blocker”, a chemical modification that prevents the polymerase from copying the stem-loop sequence of the primer. After extension of the primer during PCR amplification, the specific probe sequence is able to bind to its complement within the same strand of DNA. This hybridization event opens the hairpin loop so that fluorescence is no longer quenched, and an increase in signal is observed. Covalent attachment of the probe to the target amplicon ensures that each probe has a target in close proximity. Enzymatic cleavage is not required, nor a separate probe hybridization step, thereby reducing the time needed for signaling. This can result in stronger signals and lower background with faster cycling; however, these probes can be quite expensive and complicated to design.
4. Fluorogenic minor groove binder probes are short linear probes that have a minor groove binder with a nonfluorescent quencher on the 5′ end and a fluorophore on the 3′ end. The minor groove binder prevents the exonuclease activity of the DNA polymerase from cleaving the probe. Quenching occurs when the random coiling of the probe in the free form brings the quencher and the fluorophore close together. The probe is elongated when bound to its target and quenching is decreased, leading to an increase in fluorescent signal proportional to the amount of accumulated amplicon. These probes are also expensive and can produce a low signal-to-noise ratio.

### Quantitative Multiplex PCR-Based Residual DNA Assay

An evolution of qPCR is quantitative multiplex PCR, where several pairs of primers and the corresponding probes are introduced in the reaction medium to simultaneously detect multiple targets. Benefits include higher throughput and better control of false-negative results, whereas disadvantages come from amplification and detection interferences, as outlined in *Nucleic Acid-Based Techniques—Amplification* ⟨1127⟩. One of the applications of this technique is a duplex qPCR, where the introduction of an exogenous DNA, called IPC (internal positive control, see *Sample Pretreatment* above), enhances confidence in the accuracy of the analysis when appropriately amplified. Multiplex qPCR is not used as often as single-target qPCR to assay host cell residual DNA in biopharmaceuticals.

## RESIDUAL DNA TESTING POINTS TO CONSIDER

When developing a residual DNA assay, one should consider how the assay will be used, the structure of the DNA available (e.g., fragment length), and regulatory issues. The cost of analysis can be significant and should be considered when evaluating an assay format. In addition, environmental, health, and safety aspects should be considered. Traditionally, hybridization assays were performed using phosphorus ($^{32}$P)-labeled DNA and autoradiography. Because $^{32}$P decays quickly, probes prepared with $^{32}$P have a limited shelf life, and the precautions necessary for handling radioactive material can be cumbersome. These issues with $^{32}$P labeling may make fluorescence labeling of the hybridization probe a more desirable option. If the hybridization assay is assessed visually, this process represents a semiquantitative assay, but if the intensity of the spots is determined using a densitometer or other imaging system, the results may be quantitative. DNA-binding protein assays and qPCR give quantitative results. Quantitative assays are typically preferred instead of semiquantitative assays (e.g., older hybridization-based methods), because the results are considered more accurate and precise, which allows better process monitoring and control. Because of sample matrix interference, a sample pretreatment step is often required to obtain accurate and reproducible results. Pretreatment steps can influence the recovery of DNA, so it is usually necessary to design the assay with a spike-recovery control with an acceptance criterion to ensure assay performance.

In-house controls are usually prepared in the laboratory and qualified by ultraviolet spectroscopy, using standard techniques employed in molecular biology, to determine the DNA content and purity. The hybridization assay uses genomic and/or vector DNA, labeled randomly throughout the DNA, as the hybridization probe reagent. For this reason, the hybridization assay is specific for the source of DNA but is not specific for a given sequence. A synthesized probe, specific for a specific sequence, can be prepared and used in the hybridization assay if this level of specificity is desirable. The DNA-binding protein residual DNA

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assay is not sequence specific and hence not specific for the host DNA. Therefore, laboratory personnel should avoid contaminating samples for this assay with environmental DNA before denaturing the DNA; otherwise, the DNA result may be falsely elevated. The qPCR probe has the advantage of being sequence specific, but this creates some special challenges for development of a qPCR residual DNA assay. The qPCR-specific sequence must be a stable sequence within a suitable region of DNA. The recovery of the target sequence must consistently represent the recovery of all the residual DNA. Biopharmaceutical manufacturing processes may typically include operations that shear DNA into smaller fragments, and this must be taken into consideration when selecting an assay. Procedures exist to determine whether the DNA fragments in a sample are too small for adequate residual DNA recovery with a given assay. When bridging from one DNA assay technique to another, a thorough understanding of the DNA analyte is critical. Some assays can detect both single-stranded and double-stranded DNA, whereas some can only detect double-stranded DNA (e.g., some fluorescent dye-binding assays). There are assays that are not sequence specific, and those assays that are sequence specific can be influenced by the copy number of the target sequence present in the DNA. There are assays that require two or more antibody molecules to bind to the DNA fragment (e.g., DNA-binding protein-based residual DNA assay), and if the DNA fragments are too small and present in sufficient quantity, they can saturate the reagents and inhibit the assay (hook effect).

Although safety concerns regarding residual DNA impurities are not as prominent as they once were, the levels of residual DNA in any bioprocess remain a key quality attribute and provides valuable characterization of the manufacturing process.

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# ⟨1132⟩ RESIDUAL HOST CELL PROTEIN MEASUREMENT IN BIOPHARMACEUTICALS

## 1. INTRODUCTION AND SCOPE

Many medicinal products are produced through recombinant technology via a host cell (e.g., bacteria, yeast, or mammalian, insect, or plant cell lines). During the manufacture of such products, some amount of non-product, host cell-derived material will inevitably be introduced into the process stream. This process results in a mixture of the desired product and host cell-derived impurities, including host cell proteins (HCPs), and other process-related impurities that will be targeted for clearance through bioprocessing.

Residual HCPs have the potential to affect product quality, safety, and efficacy; therefore, the quantity of HCPs should be low. The product purification processes must be optimized to consistently remove as many HCPs as feasible, with the goal of making the product as pure as possible.

The primary concern with HCPs in biopharmaceutical products is their potential to induce anti-HCP antibodies that could induce a clinical effect in patients. In addition, HCPs may possibly act as adjuvants, which can induce anti-drug antibodies that can affect the safety or efficacy of the drug. A more extensive discussion of immunogenicity and its effect on preclinical and clinical studies is described in *USP* general chapter *Immunogenicity Assays—Design and Validation of Immunoassays to Detect Anti-Drug Antibodies* ⟨1106⟩. HCPs can also have a direct effect on the quality of the product itself. For example, proteolytic HCPs, even in minute quantities, can cleave the desired protein product over time, reducing or eliminating biological potency or altering stability.

This chapter focuses on HCP immunoassays for recombinant therapeutic products. It does not address products such as vaccines or gene-, cell-, or tissue-based therapies, although the general principles discussed may apply to the measurement of HCPs in these products. The design and validation of immunoassays for HCPs involve unique and significant challenges due to: 1) the wide variety of possible HCPs in medicinal products; 2) the general use of polyclonal antibody reagents to detect them; 3) the lack of exactly matched standards for quantitation; 4) in some cases, a considerable effect from sample dilution effects; and 5) inherent limitations to measure single HCP species.

The chapter includes assay development strategies throughout the product and process development lifecycle, and it describes approaches to demonstrate that the assay is fit for use (e.g., illustrates unit operation clearance of HCPs, lot release). Because of the complexity of HCP immunoassays, careful development and characterization of critical reagents are required, particularly for the immunogen used to elicit the anti-HCP antibodies, the antibody reagent(s), and the assay HCP standard. Because HCP testing is an essential part of process development and product quality control, HCP testing is also discussed in conjunction with regulatory requirements and other considerations for guidance on an overall control strategy for HCPs. A brief outline of the general chapter follows:

*1. Introduction and Scope*
*1.1 Considerations for Manufacturing, Characterization, and Consistency*
*2. Terminology*
*3. HCP Immunoassay Methods*
*3.1 The Assay Development Cycle*
*3.2 Development and Characterization of HCP Reagents*
*3.3 Immunoassay Method Development and Qualifying as Fit for Use*
*4. HCP Immunoassay Method Validation*
*4.1 Accuracy*
*4.2 Sensitivity and Assay Range*
*4.3 Sample Linearity*
*4.4 Specificity*
*5. Supporting Technologies for Residual HCP Detection, Identification, and Measurement*
*5.1 Considerations for Electrophoretic Methods*
*5.2 Considerations for Western Blot Methods*
*5.3 Considerations for Chromatographic and Proteomic Methods*
*5.4 Concluding Remarks on Supporting Technologies for HCPs*
*6. Use of HCP Immunoassays for Process Development, Characterization, and Validation*
*6.1 Assays for Individual HCPs*
*6.2. Control Strategy*
*7. Summary and Conclusions*
*8. Bibliography*

### 1.1 Considerations for Manufacturing, Characterization, and Consistency

Different cell-based expression systems are used to manufacture medicinal products, such as bacteria (*Escherichia coli*, *Pseudomonas fluorescens*), yeast (*Saccharomyces cerevisae*, *Pichia pastoris*), mammalian cells (e.g., Chinese hamster ovary (CHO), mouse myeloma cell line NSO, and others), insect cells (baculovirus-infected *Spodoptera frugiperda* cells), and plant cells (tobacco, *Arabidopsis*, rice). The particular HCP profile is unique and specific to the particular host cells under specific culture conditions and manufacturing processes. HCPs can vary in pI (~3–11) and hydrophobicity, and HCPs display a wide range of molecular weights (from ~5 kDa to at least ~250 kDa), depending on the host cell and manufacturing process used. The number of HCPs in upstream samples can run anywhere from several hundred to more than one thousand proteins, depending on the host cell and culture conditions. Although many cellular hosts have been used in biopharmaceutical manufacturing historically,

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the most experience has been gained using *E. coli* and the mammalian cells CHO, NSO, SP2/0, and human embryonic kidney cell line HEK293. The guidance in this chapter draws most heavily from the experience with these expression systems; however, general principles apply broadly to any host cell system.

In mammalian cells, the recombinant protein is typically secreted from the cells into the cell culture fluid (CCF), along with many of the HCPs. However, it has been observed that intracellular protein trafficking may not proceed in a normal fashion in production cultures. For example, proteins usually associated with intracellular organelles, such as lysosomes, may be found in the CCF of largely viable cell cultures, because the clones have been selected for maximum protein export. In addition, as some of the cells die, their soluble, intracellular proteins are released into the CCF. Some harvest operations also lyse cells; therefore, the resulting harvested CCF typically contains both secreted and intracellular HCPs. While this mixture of proteins incubates in the fermenter, additional changes in the HCP population may occur, for example, as the result of enzymatic activity (e.g., proteinases or sialidases).

HCP assays provide important information about the composition of the material entering the downstream recovery process and how each purification step affects HCP clearance. In some cases, HCPs can even bind to, and co-purify with, certain products. Process characterization and validation studies are needed to show which process steps remove HCPs and also to demonstrate the robustness of these steps for consistently removing HCPs. As such, HCP assays are an essential part of purification process development and help ensure manufacturing consistency. Lastly, reproducible and reliable HCP assays may be required to measure residual HCPs remaining in the drug substance (DS) used to make drug product (DP) that is delivered to the patient. HCP levels should be measured in: 1) preclinical lots used in toxicology assessment, 2) all lots during clinical development, and 3) process validation samples from the final manufacturing process. After approval, HCP monitoring may be required as an element of the control system. Subsequent sections of this chapter discuss in more detail the use of HCP assays in process validation and in a good manufacturing practices (GMP) control system.

## 2. TERMINOLOGY

To help establish a common nomenclature in the literature and with regulatory agencies, *Table 1* lists common terms with their definitions (indicating how they are used in this chapter) in addition to synonyms that have been used historically. Note that the term "platform" indicates that the same set of standards and reagents is used within a company to test a variety of products made from the same type of expression system (e.g., CHO cells) grown under similar upstream conditions. In the case of platform HCP assays, the antibodies to HCP are obtained from animals immunized with HCP antigens generated from a common upstream process that is applicable to many products, even if the downstream purifications are different. This approach allows the knowledge from prior products to be leveraged. Justification that an assay is suitable for a new product, using the same expression system and common upstream conditions, is therefore often relatively straightforward.

The HCP immunogen used to generate platform anti-HCP antibodies and used often as the assay calibration standard is, by design, comprised of a broad set of HCPs. In contrast, the qualifier "process-specific" indicates that the immunogen/standard has been prepared from a set of HCPs unique to a given process (either a unique upstream cell culture process or a unique downstream purification process). Process-specific assays are, therefore, limited in their utility, and each must be fully qualified for each process. Process-specific immunogens and calibration standards are, by intent, more narrow and specific to a given process. "Commercially available" assays produced by vendors are often derived from a combination of strains and harvest/purification procedures, and these assays are intended to have a broad application; but these commercially available assays are not specifically designed for a given manufacturer's proprietary cell line, and users do not have control over reagent availability and lot-to-lot consistency.

**Table 1. HCP-associated Terminology**

| Term | Definition | Historical Synonyms |
|---|---|---|
| Commercially available | Available to the public for commercial sale; typically a combination of upstream isolates and corresponding antibodies made by the vendor and sold as reagents or kits. | Generic |
| Platform | The same set of an HCP standard and antibodies is developed with a company's proprietary host cell strain and used broadly within a type (e.g., CHO) across several products when the upstream conditions are similar. | Custom, in-house, proprietary |
| Upstream process specific | An assay designed from material where the upstream culture process deviates significantly from the platform.<br>This is generally before any purification and may be applied to more than one product if these parameters are similar. | Custom, in-house, product-specific, proprietary |
| Downstream process specific | An assay designed from materials where the downstream unit operations are used to enrich the HCP population.<br>This may be applied to more than one product if these parameters are similar. This is rarely used today and is not recommended except for certain products with exceptional downstream processing. | Custom, in-house, product-specific, proprietary |
| Assay for an individual HCP | An assay using a standard composed of an identified, single, known HCP and its specific antibody/antibodies. | Single analyte assay or Custom, HCP-specific |
| Coverage | Describes the assessment of how completely a population of polyclonal antibodies recognize the population of HCPs.<br>The coverage assessment may be made on the HCP population used as the HCP antigen or from the product production culture. | |
| Qualification | Demonstration of suitability of analytical methods (including reagents used in these methods) for their intended application to a given process and in-process samples. | |

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**Table 1. HCP-associated Terminology** *(continued)*

| Term | Definition | Historical Synonyms |
|---|---|---|
| Protein A/G-affinity purification | Affinity purification of antibodies with immobilized Protein A or G[a] | Affinity chromatography |
| HCP-affinity purification | Affinity purification of antibodies using immobilized HCP (antigen)[a] | Affinity chromatography, immunoaffinity chromatography |
| Null cell | The cell strain used for production that does not contain the product-specific genetic elements; includes untransfected parental cells and cells transfected with the expression vector but without the product gene. | Parental, blank, or mock-transfected cell |
| ng/mg | The numerical quantity (ratio) of HCP per product, where ng represents HCP mass and mg represents the product mass. It is calculated by dividing the HCP concentration (ng/mL) by the product protein concentration (mg/mL). | ppm[b] |

[a] In some cases, both purifications are performed, typically the protein A/G first, then the HCP affinity.
[b] Although ppm has been used historically, it is not advised because this term is used to reflect mass per unit volume for other types of tests. It is recognized that ng is used conventionally as a value derived from interpolation from an HCP standard curve (in units of ng/mL), where the signal is reflective of antibody binding and, unlike the therapeutic protein concentration measurement, does not strictly reflect the mass of HCP that may be present.

## 3. HCP IMMUNOASSAY METHODS

Immunoassay methods rely on antibodies that recognize, as broadly as possible, the population of HCPs entering the downstream purification process; therefore, the sandwich immunoassay, designed with polyclonal antibodies, is the workhorse of HCP monitoring and quantitation. This assay format offers a combination of high sensitivity, specificity, throughput, automation potential, rapid turnaround, quantitative results, and low cost per assay that is unmatched by any other currently available assay technology. Other immunoassay formats (e.g., competitive immunoassays) may or may not be suitable, because they lack either the specificity or the sensitivity afforded by the sandwich format. Although these methods result in a single HCP value for a given lot, the number can give greater weight to HCPs for which high-affinity antibodies are present in the reagent(s)—and no or low weight to HCPs which are either not recognized or recognized by low-affinity antibodies in the assay. For these reasons, orthogonal measures of product purity are often needed. More details on these methods can be found in *5. Supporting Technologies for Residual HCP Detection, Identification, and Measurement*.

The basic principles and design of immunoassays are discussed in *USP* chapter *Immunological Test Methods—Enzyme-Linked Immunosorbent Assay (ELISA)* ⟨1103⟩. The format that is most commonly used for HCP testing is the sandwich immunoassay with detection systems such as colorimetric, electrochemiluminescent (ECL), chemiluminescent, radioactive, or others. Homogeneous immunoassays, including competitive assays, where all of the reagents are combined at once and the binding occurs in a single step without washing, may be problematic due to antigen excess leading to antibody insufficiency issues (discussed later in the chapter); therefore, these formats should be used with caution. The heterogeneous sandwich immunoassay format described in chapter ⟨1103⟩ is generally preferred, because the dynamic range and sensitivity may be reduced in the homogeneous format. The formats, with their advantages and disadvantages, are discussed further in chapter *Immunological Test Methods—General Considerations* ⟨1102⟩. Data analysis is typically performed with a nonlinear fit of the sigmoidal curve generated by a wide range of standard concentrations, although some analyses may focus on the low end of the curve for greater sensitivity.

### 3.1 The Assay Development Cycle

*Figure 1* illustrates common assay development plans, depending on the reagents available at various stages. Fewer bridging studies are required when: 1) platform reagents are available, and 2) upstream processes are historically consistent. *Figure 1A* illustrates a scenario where platform methods are not available, and a commercially available assay is used up to the stage of process validation with appropriate assay qualification. For phase III and beyond, either a platform or upstream, process-specific method is preferred. A bridging study should be performed to support assay replacement. If the commercial assay is intended for phase III and post-approval, care must be taken to fully demonstrate that the assay reagents are applicable to the process HCPs. An additional consideration is that the reagents are from an outside vendor over whom the biopharmaceutical manufacturer has less control.

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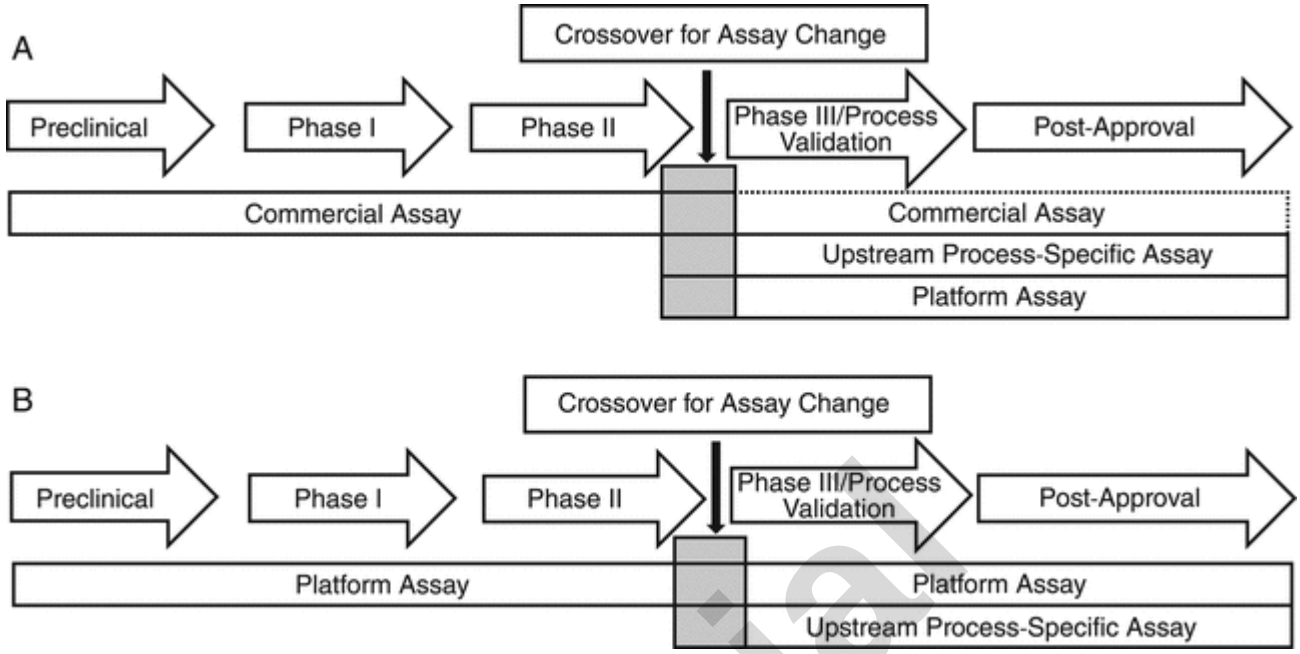


Figure 1A. If there is no platform assay before product development starts. Figure 1B. If there is a platform assay before product development starts.

*Figure 1B* suggests that a platform assay can be used through all stages of product development if already available when product development starts. The platform assay should be qualified for each new product. Switching to an upstream, process-specific assay may be necessary for phase III or process validation and beyond, if the cell culture process is significantly changed from the platform process, and may introduce significantly different HCP populations. A bridging study should be performed to support assay replacement.

The limitations of a downstream-specific HCP assay should be considered, because it may be tempting to think that HCP assays are improved by taking the HCPs from null expression through the first column(s) and immunizing animals with a downstream column pool. Historically, the logic was that the immunogen and standard would be enriched in those HCPs most likely to enter the recovery process and be in the final product. This strategy was based on the assumption that, rather than having thousands of irrelevant proteins in the immunogen, only those most likely to be in the process will be present in the immunogen; thus, the resulting assay will be focused on those HCPs of greatest interest. Potential concerns that may arise as a result of this approach are:

1. If only HCPs from a null cell run pool from the first column are used, then later changes during process development (e.g., changes in column run conditions) could invalidate the HCP assay. Thus, process development becomes very restricted and involves the risk of needing to develop multiple HCP assays for slight process changes (or the need to manage the uncertainty).
2. HCPs that co-purify with a product may do so because they bind directly or indirectly to the product protein. A null cell run of a column without the product protein would miss these HCPs.
3. Nonspecific adsorption to chromatography resins is not uncommon and, often, is not a reproducible phenomenon. Compared to the first passage, subsequent passage of the null cell run material over a column will likely produce a different set of HCPs after passage over a new column resin.

## 3.2 Development and Characterization of HCP Reagents

### 3.2.1 PREPARATION OF ANTIGEN/HCP STANDARD

As described in *1.1 Considerations for Manufacturing, Characterization, and Consistency*, the total HCP "antigen" is a complex population of proteins; therefore, when generating the HCP antigen/standard reagent, it is important to ensure that: 1) the calibration standard is representative of the cell line and manufacturing process, 2) its protein concentration is accurately quantified, and 3) the immunogen is administered in a way that generates polyclonal antibodies with reactivity to as many different HCPs as possible. The HCP antigen composition should also be comprehensive enough to tolerate normal process manufacturing changes during the life cycle of the product(s).

In addition to the uses above, the HCP antigen may also be needed to prepare the affinity column for purification of the antisera. If affinity purification is used, the quantity of HCP antigen needed should be carefully planned. The amount produced should be large enough to provide sufficient inventory for many years (often 10–20 years); ideally, for the whole life span of the product. However, the antigen preparation process should be performed and documented in a way that facilitates a potential resupply.

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Because the HCP assay is used to test DS samples that contain trace HCP impurities, any cross-reactivity of the anti-HCP antibodies with the product may compromise the test method and yield biased results. Therefore, any contamination of the HCP antigen with product must be avoided to prevent the generation of anti-product antibodies.

**3.2.1.1 Preparation of HCP antigen from mammalian cells:** The majority of biopharmaceutical products produced today are expressed in mammalian cells, e.g., CHO cells. A typical process for the preparation of CHO HCP antigen is outlined in *Figure 2*.

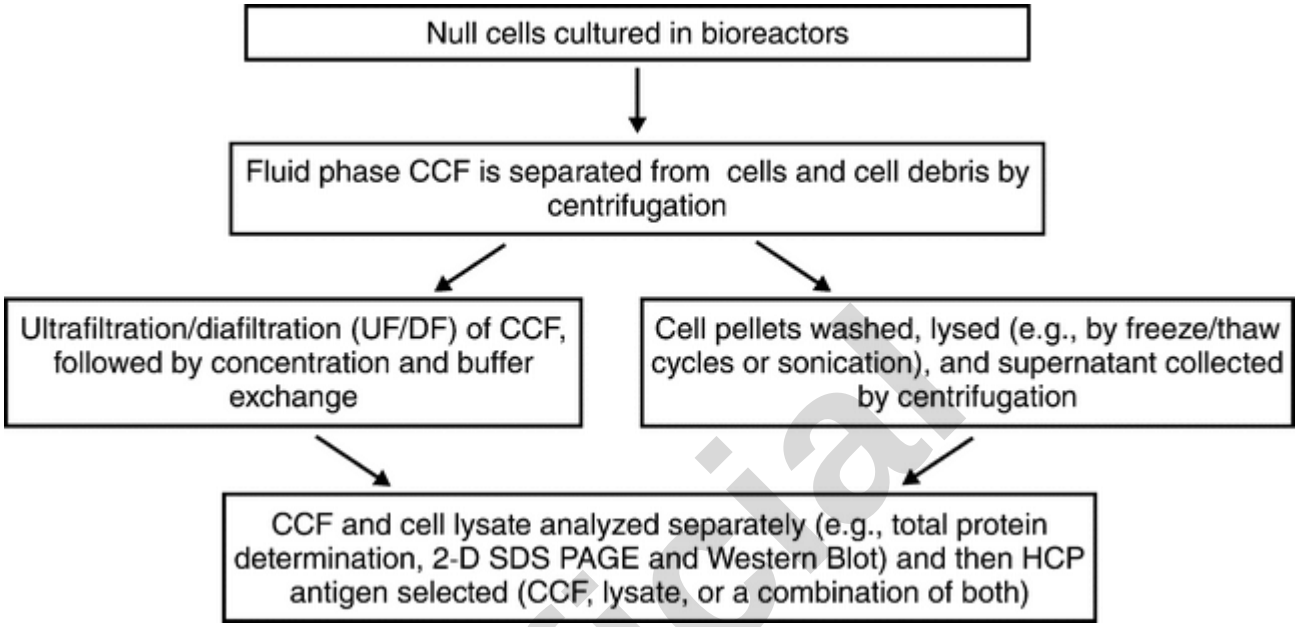


Figure 2. Example of mammalian HCP antigen preparation.

The first step is to establish a null cell that does not express the product gene, either by using non-transfected (i.e., parental) cells with a common origin to those used to make product alone, or to transfect them with the vector used to create the production cell line without the product coding gene (historically described as a "mock" transfection). The main advantage to the latter is that selective markers (e.g., dihydrofolate reductase or glutamine synthetase) are expressed. Alternatively, antibodies can be raised to selective markers independently. Another advantage is that mock-transfected null cells express selection markers and may be grown in cell culture conditions more closely resembling the manufacturing process. Using pools of mock transfected null lines which have not been cloned allows for the maximum potential genetic diversity and therefore gives the highest potential of generating a broad host cell protein population. However, these cultures, which are seeded with cell pools (not cloned), may demonstrate more variability from run to run. Both approaches [non-transfected (parental) and mock transfected] are commonly used. Both are also subject to the following issues: the cell culture process optimized for the production clone may not lead to similar viability and cell density of the null cells. In addition, in the absence of the stresses experienced by cells producing large quantities of product protein, the null cell lines may exhibit a different HCP profile. These changes in viability, cellular metabolism, and cell density may alter the HCP profile of the antigen, making it less representative of the manufacturing process. More about choosing appropriate HCP antigen preparation conditions is discussed below.

Once the null cells have been established, the HCP antigen is prepared in a bioreactor, cell culture flask, or cell culture bag reflective of the product or process cell culture conditions. A platform cell culture process may be applied, which represents a common manufacturing process for several products (e.g., CHO-derived monoclonal antibodies). Typically, the resulting HCP antigen is minimally processed to ensure a broad HCP spectrum, making the antigen suitable for HCP monitoring of multiple products generated from the platform process. A platform HCP antigen also may be produced by combining several null cell runs with slightly different cell culture conditions (e.g., allowing the culture to remain in the bioreactor for longer periods of time to vary the cell viability). This approach is used to obtain a broader HCP spectrum, and antisera raised against this immunogen may be suitable for HCP detection in products generated from a variety of slightly different processes. These reagents also may increase the robustness of the HCP immunoassay toward process changes.

Alternatively, the HCP antigen may be prepared using an upstream, process-specific approach in which the cell culture process is tailored to mimic the production process for a unique product or specific process. The advantage of this approach may be the high relevance of the resulting HCP antigen to a particular upstream process. As with platform reagents, because no additional processing purification steps are included, the antigen contains a broad range of HCPs, and the antigen will often remain suitable for HCP monitoring after downstream production-process changes. The disadvantage of this approach is that the use of the reagents typically will be limited to a single (or few) product(s) or process(es).

The HCP antigen can be produced from a representative small scale, pilot scale, or production scale run. There are pros and cons for each approach. Pilot or production scale may mimic the actual process best; however, normal variation between cell culture runs may not be reflected if only one run at large scale is applied. Conversely, several small-scale preparations can be pooled and may better reflect the run-to-run variability of the cell culture process. Regardless of the scale, the presence of product should be aggressively avoided, because its presence in the HCP immunogen will compromise the quality of the resulting immunoassay antisera.

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After performing the null cell run, the HCP antigen can be prepared from the cell lysate, the concentrated CCF, or as a mixture of both as shown in *Figure 2*. If the antigen is prepared from CCF, the cells may be harvested at a time when production line harvest is performed or some days later, to allow for some of the cells to lyse and release HCPs into CCF and thereby broaden the HCP spectrum.

To prepare immunogen from cell lysate, the cells are harvested by centrifugation, and the cell pellets are washed and lysed (e.g., by using repeated cycles of freeze/thaw, or high pressure homogenization). Conditions are typically selected to mimic the production process (i.e., to prepare immunogen from CCF, cells and debris are removed by centrifugation, and the CCF is then treated by ultrafiltration/diafiltration). The buffer is exchanged (e.g., into PBS or HEPES), and the CCF is concentrated. The cut-off for filtration should be chosen to minimize loss of HCP, e.g., 10 kDa or less.

**3.2.1.2 Preparation of HCP antigen from bacterial cells:** Many of the above-described principles used in the preparation of mammalian HCP antigens also apply to prokaryotic cell culture. However, some unique challenges should be considered. In particular, manufacturing processes for bacterial expression system come in more varieties, such as those yielding concentrated protein deposits known as inclusion bodies, which can represent up to 95% of the total cell protein. HCP impurities are also present in the inclusion bodies, including bacterial membrane proteins, ribosomal subunit proteins, and cytoplasmic proteins, such as small heat shock proteins or chaperones. The formation of inclusion bodies in the production strain makes it more difficult to generate a representative HCP preparation from a null cell fermentation process. The null cell may not have the same HCP profile, and it will not generate inclusion bodies with which specific HCPs may co-purify, as described above. In other cases, HCPs have been created from periplasmic secretion systems, if the production process can be replicated in the null cell.

Because there is no obvious approach for overcoming these limitations, in practice the bacterial HCP antigen is usually generated from the lysates of washed cells, using null cell fermentations, that are grown and induced under conditions representing the upstream manufacturing process as closely as possible. As for any HCP antigen, it is necessary to demonstrate that the HCP profile is representative of the manufacturing process before use in assay development. This approach yields the full complement of HCPs expressed and hence is very broad.

Because most animals have been exposed to bacterial antigens and may have pre-existing antibodies to many bacterial cell proteins, it is important to characterize the pre-existing antibody responses, and if appropriate, consider the use of specific pathogen-free (SPF) animals. The presence of significant levels of pre-existing anti-HCP antibodies may confound the analysis of the antibody responses; therefore, it is important to screen preimmune sera from animals for pre-existing antibodies prior to immunization.

Null bacterial cells transfected with the expression vector contain chaperone genes that may be expressed at high levels (e.g., 5% of the total HCPs). Such overexpression of particular HCP impurities might necessitate the development of single-analyte assays for these particular impurities (see *6.1 Assays for Individual HCPs*).

**3.2.1.3 Characterization of the HCP antigen:** Several analyses are recommended before immunization: 1) protein content (total protein assay); 2) absence of product (as shown by Western blotting, immunoassay, and/or MS analysis); and 3) characterization by 1-D or 2-D-polyacrylamide gel electrophoresis (PAGE). Protein concentration is measured to establish the standard concentration for future use and to determine the overall amount of the prepared antigen. The most commonly used methods are the bicinchoninic assay (BCA), the Bradford assay, and amino acid analysis (AAA), although the colorimetric methods require a standard (e.g., BSA) that is not well matched to the (HCP) analyte. Absorbance at 280 nm (A280) may also be used although it is less specific for protein (e.g., nucleic acids will also be measured). These methodologies often provide similar results, but some methods may be more significantly affected by the presence of interfering substances than others. One should consider using two orthogonal methods to exclude a gross over- or underestimation of the protein concentration by one particular method. For more information, see general chapter *Biotechnology-Derived Articles—Total Protein Assay* ⟨1057⟩, which discusses the advantages and disadvantages of various protein analysis methods. The HCP concentration must be assigned with a scientifically sound approach that is used consistently for its lifetime. The lack of product can be assessed with gels, anti-product Western blots, or other suitable methods. Lastly, the 1-D and 2-D gels help characterize the pattern of HCPs, show that a broad spectrum of proteins is present, and show that a reasonable match to production is present. However, this can be challenging because of the presence of large amounts of the product, which can obscure HCP detection. Comparisons may be made between the specific populations of host proteins produced in the null cultures and production cultures using the orthogonal methods discussed in *5. Supporting Technologies for Residual HCP Detection, Identification, and Measurement*.

**3.2.1.4 HCP standard reference reagents:** Frozen HCP material is usually stable for a very long time, but stability of the HCP standard reagents should be monitored over time because the assay lifetime can be very long. HCP standard stability can be confirmed by monitoring the HCP standard performance in the HCP immunoassay as well as in orthogonal methods.

Appropriate controls within the assay range may be established to monitor assay performance. Controls may be prepared from independent dilutions of the HCP standard, product samples or intermediate pools, or spiked product samples. However, there is a slight risk that control samples prepared as a dilution from the same HCP standard material can degrade at the same rate as undiluted HCP standard, and degradation of the standard will not be detected. To mitigate the risk, data from several standard curve parameters (e.g., signal, background, slope, coefficient of determination) are often assessed for each assay and used to support HCP standard stability. Using material different from the reference to prepare controls will help ensure that the degradation rate will be independent, and HCP standard degradation can be detected easily.

Assay control charts can be established and used to record reference curve parameters, control sample values, and lot-specific information for the critical reagents, such as labeled HCP antibodies. Acceptable ranges for the controls should be established on the basis of multiple runs (usually 20–30) and may be used as part of routine assay acceptance criteria. In addition, the values for % coefficient of variation (CV) of standard and control replicates are often a part of the assay acceptance criteria. Differences over time in HCP standard curve performance or HCP control levels, or an increase in assay variability, may indicate a lack of HCP standard stability.

### 3.2.2 PREPARATION AND CHARACTERIZATION OF ANTIBODIES

**3.2.2.1 Preparation of antibodies to HCPs:** The anti-HCP antibodies prepared are often used for many applications (Western blots, immunoassay) over a long period of time (e.g. the anticipated life of a product or platform). Therefore, material needs

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should not be underestimated. The recommended antisera quantity will depend on many factors including the intended use, the predicted quantity purified from a given volume of antisera, the amount coated on microtiter plates, and many other considerations. The choice of animal species used to generate anti-HCP antibodies is driven by the immunogen, the requirements for the particular method, and the preferences of the individual investigator. Rabbits, sheep, and goats are often chosen for immunization programs. In some cases, multiple animal species have been used. In such cases, using species more phylogenetically distant from mammals (e.g., chickens) have helped to generate anti-HCP antibodies to conserved mammalian HCP proteins. The choice of animal species may also be driven by a desire to either increase the amount of antisera available from one or several animals (goats or sheep), or to use a strategy to obtain a greater diversity of responses provided by the use of many more individual animals from a single species (e.g., rabbits). The number of animals chosen to generate the antiserum pool(s) depends on the species used and the duration of the immunization protocol. If smaller animals are used to generate the antiserum, more individual animals are generally needed (e.g., 10–20 rabbits). In contrast, if larger animals (goats or sheep) are used, investigators usually will immunize 3–10 animals per protocol. Typical immunization protocols provide approximately 100–150 mL of antiserum per rabbit or approximately 500 mL of antiserum per goat, and the total yield of Protein A or Protein G purified antibody is approximately 1 gram per rabbit or 5 grams per goat. Animals should be screened for pre-existing antibodies against drug substance prior to immunization. If positive results are found, then these animals should be excluded from immunization.

A portion of the prepared HCP antigen is mixed with an appropriate adjuvant (most commonly, Freund's adjuvant or in combination with incomplete adjuvant) and used for the immunization of animals. Each animal receives an initial priming immunization, followed by multiple (often 4 to 8) booster immunizations, to mature the immune response and generate high-titer, high-affinity antibody responses. Antiserum is collected from each animal before the initial priming immunization (time 0) and at appropriate intervals (usually 10–14 days) after booster doses. Four to six bleeds are usually collected from each animal. However, the duration of the immunization protocol may be extended by increasing the number of booster immunizations, making it possible to collect additional high-titer antisera. Other immunization strategies may also be helpful (e.g., cascade immunization or size fractionation of the HCP immunogen).

Investigators should perform titer or Western blot analyses of the individual test bleeds before antiserum pooling to screen for and remove sera that 1) have low titer, 2) display an immunodominant immune response to a small group of HCPs, or 3) have nonspecific binding characteristics or anti-product reactivity. This evaluation should also be performed on the final antibody pools to demonstrate broad coverage of HCPs and a lack of binding to the therapeutic product. In addition, some laboratories have found it useful to evaluate antibodies for aggregates using size exclusion chromatography as described below. Acid elution conditions may cause some antibodies to aggregate, and multimers of this type, particularly when labeled with either biotin or horseradish peroxidase, may lead to increased assay background. In addition, some laboratories include a final process-scale size-exclusion chromatography step to reduce aggregate levels to low levels (e.g., <5%).

Multiple approaches to the purification of antibodies from antiserum pools have successfully generated antibodies suitable for use in HCP immunoassays. Antibody purification is often performed by Protein A, Protein G, or HCP column affinity chromatography. The application of coated magnetic beads can also provide benefits in some scenarios. The choice of Protein A or Protein G is driven by which animal species was used to generate the antibody. Manufacturers provide standard protocols for these routine antibody purifications. When affinity purifying anti-HCP antibodies, materials such as chromatography resins, which may have been used previously for other products, should be avoided, and additional cleaning cycles should be in place to minimize carryover. Ideally, manufacturers should use a resin that has never been exposed to a DP. Before the affinity purification step, some laboratories also include an initial 50% ammonium sulfate precipitation of the crude antiserum to enrich and concentrate the antibody fraction and thereby extend the performance and duration of use of affinity columns. The use of Protein A or Protein G purification strategies generates reagents in which the anti-HCP-specific antibodies compose a smaller proportion of the total antibody. In contrast, HCP affinity purification strategies selectively purify anti-HCP-specific antibodies from the antisera or antibody-enriched preparations. In this case, the HCP antigens are coupled to an activated resin, and the antiserum is purified over the column following established protocols or the resin manufacturer's instructions. The purified antibodies may be further purified by size-exclusion chromatography to remove aggregates, then dialyzed, concentrated, divided into aliquots, and stored frozen (typically –70° or colder). Neat antisera should also be stored frozen (see ⟨1106⟩ for additional information regarding storage of serum samples).

Affinity purification of specific antibodies using HCP immobilized on a column requires careful management of the column preparation and use so that it can be reproduced reliably for future purifications. In addition, if reused, appropriate resin storage and regeneration procedures should be evaluated and controlled to avoid degradation during storage. Done properly, it is a reliable, reproducible process that has led to consistent anti-HCP antibodies and HCP immunoassays. After loading the column with antisera, an optimized wash procedure is critical. Because the preparation is a mixture of antibodies with differing affinities, extensive washing may remove low-affinity antibodies. Similarly, loading large amounts of anti-HCP antisera onto the column may result in high-affinity antibodies, displacing low-affinity antibodies if they recognize epitopes that are in close proximity. In either case, the low-affinity antibodies may be removed. In theory, higher affinity antibodies may also be more challenging to elute. Whatever load and wash conditions are initially selected for the antigen-specific affinity purification, these conditions need to be maintained in future production lots of the antibodies. A comparison between these two purification strategies is shown in *Table 2*. Both approaches are commonly used.

**Table 2. Antibody Purification Strategies**

| Type of Purification | Pros | Cons |
|---|---|---|
| Protein A or G Affinity Column Chromatography | Robust and reproducible for quickly separating IgG fraction from other serum proteins | Lower proportion of antibodies specific to HCPs and may have a higher amount of low affinity antibodies |
| HCP Affinity Column Chromatography | Enriches for specific high affinity anti-HCP antibodies; excludes nonspecific antibodies and serum proteins | More skill and documentation required to make and maintain resins consistently. Some high affinity anti-HCP antibodies may not be eluted in usable form and may not be recovered |

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**3.2.2.2 Characterization of antibodies to HCPs:** Antibodies prepared for capture and detection are assessed independently and as part of the sandwich immunoassay pair. The concentration of the unmodified antibody is determined most commonly by absorbance at 280 nm, AAA, or BCA. Each approach is acceptable, provided it is applied consistently and (if colorimetric) is standardized similarly. It is important to determine the concentration of the capture and detection antibodies in each batch because they will be diluted to a certain concentration in the optimized immunoassay method. The detection and capture antibodies are characterized by their performance in the immunoassay method(s). The best label to antibody conjugation ratio should also be empirically evaluated primarily based on the best performance in the assay. This optimal ratio should be targeted in the future when new labeled antibodies are made. Finally, although antibodies are generally very stable when stored frozen (e.g., –70°), investigators should ensure storage integrity and determine a defined period of use. This can be accomplished by continuous monitoring with trending charts or by requalification experiments at specified time intervals.

The quality of the antibody pair (capture and detector) is often evaluated in two ways:

1. Show that the antibody pairs are specific and sensitive in an immunoassay format for the HCPs present in a series of samples taken from the unit operations of a given purification scheme, including the DS. This topic is also addressed in more detail in *4. HCP Immunoassay Method Validation*. In concept, the ability to detect and measure HCPs (immunoreactivity) in a series of actual process samples from a given process for a product in development is demonstrated with data showing: 1) sequential clearance as the product is purified, 2) sample linearity throughout a dilution series, and 3) lack of cross-reactivity to product or matrix. The reduction of HCPs during the purification process is determined by clearance factors where the HCP content (in ng/mg) at each step in the process is divided by the amount in the prior step. Typically, starting samples from the cell culture may have HCP levels ranging from several hundred thousand to several million ng/mg, and final products may have HCP levels ranging from <1 to 100 ng/mg (showing many logs of clearance). When these trends are observed, they suggest that the process and the assay are functioning effectively, and the antibodies used in the immunoassay have suitable quality attributes. If these trends are not observed, it may not necessarily reflect a problem with the antibodies, because it is also possible that the purification process is not effective.
2. Show that a broad range of HCPs in the calibration standard is recognized (i.e., that the coverage of the HCP population is adequate). Coverage is evaluated for at least the capture antibodies using 2-D gel Western blots or immunoaffinity fractionation of the total HCP population by immobilizing the anti-HCP antibodies on a column. In addition it is recommended to test the coverage of the detection antibody if different coating and detection antibodies are used. The benefits and limitations of using either 2-D gels or immunoaffinity fractionation to demonstrate coverage is discussed more fully below. Whichever method is used, the extent of coverage must be addressed in qualifying the immunochemical reagents.

HCP coverage evaluations help assess the ability of the antibodies to recognize a wide range of HCPs in the calibration standard and those present in in-process and DS samples. Two methods (2-D gels followed by Western blot analysis and immunoaffinity purification followed by 2-D gel analysis) are in fairly common usage, and both have the limitation that they tend to underestimate the true antigen binding in immunoassay methods but may have value in comparing two preparations head-to-head (or a platform to a commercial reagent). Both procedures use reducing 2-D gel electrophoresis to separate HCPs in the HCP standard or in early process sample(s) (e.g., harvest). Numerical coverage comparisons should be used with caution because of the many method variables and the art required to reproduce results, even with the same reagents in the same laboratory. Because of this variability, the results are best evaluated qualitatively. Comparisons of batches (or sources) of antibodies should be done side by side as much as possible to determine if antibody lots are comparable or if one is superior to another. One approach, the SDS-PAGE/Western, is essentially a comparison of the number of spots present in the immunologically stained membrane versus the number in a duplicate gel (or blot) stained for total protein (e.g., by fluorescent dye or silver; see also *USP* chapter *Immunological Test Methods—Immunoblot Analysis* ⟨1104⟩). Differential staining of each antibody preparation may power the analysis, because it can be used to analyze the same 2-D gel. The second approach, immunoaffinity binding/SDS-PAGE, involves comparing the flow through and eluate to the load from the HCP calibration standard (or early process sample) passed over a resin to which the capture antibodies have been covalently immobilized. The resin is washed after loading and before elution to remove nonspecifically bound HCPs. For the purpose of sample comparison, difference gel electrophoresis (DIGE) technology could be helpful. *Table 3* lists the advantages and disadvantages of these two coverage methods.

**Table 3. Comparison of Coverage Methods**

| | Pros | Cons |
|---|---|---|
| 2-D SDS-PAGE/Western blot | Good separation of individual HCPs allows for individual spot counting. | HCP antigens are denatured, may not represent what is seen in immunological assays (e.g., ELISA). |
| | | High variability—numerical "percent coverage" values vary widely with the same material tested within a single lab and between different laboratories. |
| | | Transfer efficiency of a broad range of HCPs difficult to optimize, leading to underestimates due to over-transfer through the membrane or failure to transfer from the gel, dependent on molecular weight. |
| | | Matching spots between blots and gels are difficult and not standardized. |

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**Table 3. Comparison of Coverage Methods** *(continued)*

| | Pros | Cons |
|---|---|---|
| Immunoaffinity binding/ 1- or 2-D SDS-PAGE | HCPs bind to antibody resin in solution under native conditions similar to the sandwich immunoassay. | May underrepresent some HCPs if they are bound too tightly and do not elute, resulting in an underestimation of coverage. |
| | Analysis of spots in gels does not require immunoblotting and is simpler because the problems of transfer are avoided. | Preparation of the anti-HCP resin must be done carefully and may be difficult to reproduce. Results can be dependent on resin loading and elution conditions. |
| | | Proteins at low concentration that only show up in Westerns will not be detected. |

More information about the appropriate use of SDS-PAGE/Western blot methods and ways to minimize the disadvantages above can be found in the later section *5.2 Considerations for Western Blot Methods*.

An example of a suitable use of this approach is shown in *Figure 3*.

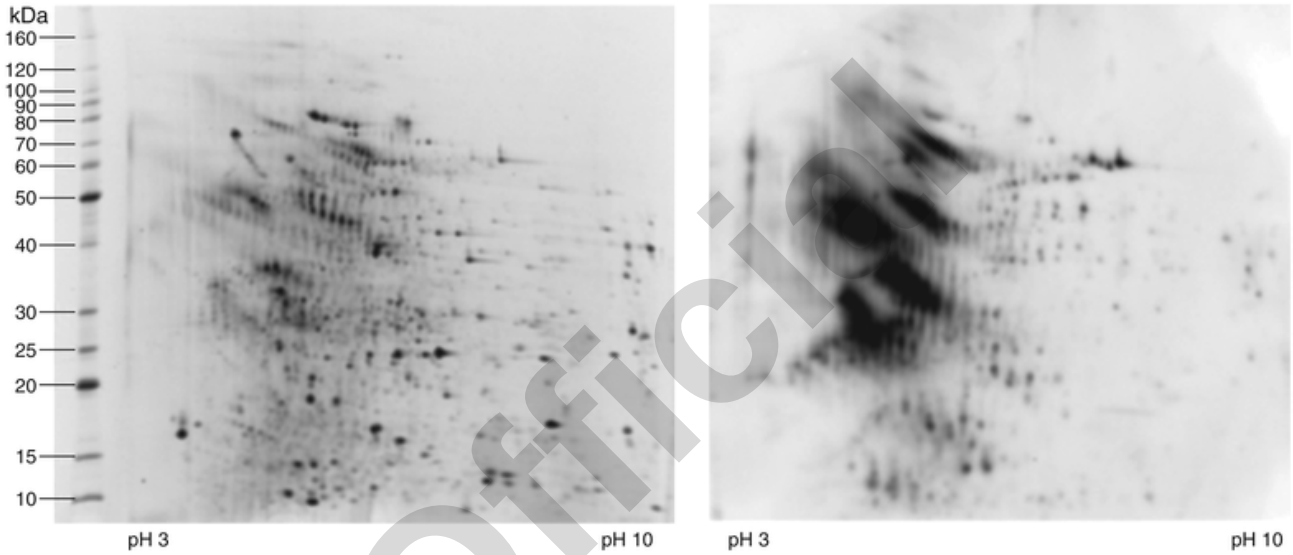


Figure 3. Left panel: 2-D IEF/SDS-PAGE analysis of representative CHO HCP calibration standard stained with a sensitive fluorescent stain. Right panel: Western blot analysis of the same gel shown in the left panel.

### 3.2.3 GENERATION AND QUALIFICATION OF REPLACEMENT REAGENTS

When the supply of HCP reagents are depleted or if their performance declines, new reagents may be needed. Often, new HCP antigens and new HCP antibodies are both generated at the same time. In theory, the new set of reagents should be created by following the same protocol that was used for the previous set of reagents to match the performance of the new and old assays as closely as possible and produce consistent data for a given program. In practice, however, it is not always possible (nor advisable) to generate the assay reagents exactly the same way that they were originally made. For example, if the manufacturing process has been optimized over time and the original platform assay reagents are no longer relevant, a careful assessment should be made. When such changes are required, the ideal way to confirm the quality of the replaced reagents is through characterization of antibodies (see below) and demonstrating that they can detect similar or greater HCP log reduction, from harvest to DS, using the same samples tested head to head with the original assay reagents.

When replacing an HCP antigen in which the current production process is essentially the same as the old process, the new HCP antigen should be prepared as similarly to the old as possible. It is important to compare the protein concentrations of the old and new HCP antigens side by side, using the same method to correctly assign the protein concentration for the new antigen and to ensure that the protein concentrations are comparable in the new and old HCP calibration standard. Characterization by 1-D and 2-D SDS-PAGE should also be performed as a side-by-side comparison of the old and new HCP antigens (see above) to be sure that gross changes in protein composition have not occurred.

For antibody replacement, the same animal species should be used, if possible. In cases where a better antibody response is desired, antibodies produced by different species may be evaluated. The selection of the species should take into account all the points discussed in this chapter. A side-by-side comparison of results from process samples using old and new antibodies in a sandwich immunoassay format should be included to demonstrate suitability of the replacement antibodies. Additional characterization by orthogonal methods (e.g., coverage using 1-D and 2-D SDS-PAGE) is recommended, as described above. Assay qualification or validation should also be considered after changing reagents.

To fully understand the effect of replacing HCP critical reagents on assay results requires testing of process samples in bridging studies using multiple samples from harvest, process intermediates from all appropriate purification steps, and the final DS. These studies can be very resource intensive, which underscores the importance of making sufficient quantities of HCP critical reagents at the outset (and minimizing replacement).

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## 3.3 Immunoassay Method Development and Qualifying as Fit for Use

As noted in *1. Introduction and Scope*, HCP immunoassays serve two important functions in the development and control of biopharmaceutical manufacturing processes. HCP immunoassays serve as a measure of product purity, and therefore are potentially related to patient safety. They also serve as a measure of manufacturing consistency, and therefore they are reflective of process control. To meet these requirements, HCP assays are often validated and incorporated into the cGMP control system, which includes specific reject limits (in the DS as a Certificate of Analysis (C of A) method or as an in-process control). If the final DS is a conjugated protein, the testing should be done on the protein intermediate (before conjugation). In addition, HCP assays are used to guide process development and are often used to demonstrate robust process performance in the context of formalized process validation activities.

HCP immunoassays are often formatted as a sandwich ELISA, and multiple antibody labeling options are available (see ⟨1103⟩). Depending on the label chosen, appropriate conjugates and substrates are selected; examples are described in ⟨1103⟩. Most proteins can be biotinylated because of their lysine content and, because biotin is small, it is less likely to affect the protein's binding activity. Because antibodies are large and have many lysine residues, the conjugation ratio of biotin to antibody can be higher than that of smaller proteins. However, it is important to not over- or under-label the antibody (e.g., with biotin or HRP), because this might lead to either less antigen binding or lower signal/sensitivity, respectively. Controlling and defining the labeling stoichiometry (e.g., mole of biotin to mole of IgG) is useful for making future batches consistently. Multiple ratios of any antibody label should be tested and optimized for performance in the immunoassay. Excess unconjugated reagents should be removed, either by dialysis, affinity purification, or by using a suitable desalting column.

Often, the standard is first screened to see whether a dose-response curve can be generated, and if so, in which concentration range of analyte. The results can also reveal a starting point for initial reagent concentrations that can be optimized further. If more than one antibody pair has been prepared, then each is tested to find the best performance (see *USP* chapter ⟨1103⟩ for more information). The signal (low calibration standard) to noise (background) ratios generated with each antibody candidate can be used to select the best pair. Typically, HCP immunoassays do not always possess a full dose-response curve with both asymptotes; therefore, for the purposes of residual HCP detection, the assay for DS release should focus on the low end of the curve.

### 3.3.1 QUALIFYING A NEW MANUFACTURING PROCESS WITH A PLATFORM HCP ASSAY

As noted earlier, if the upstream/isolation procedure is matched, then a new product produced in that system can benefit from a platform assay approach that can decrease qualification time and effort. However, in cases where there are variations that occur in optimizing the production cultures and the cell culture design space is not completely defined, it is important to understand whether these variations are significant enough to render the platform assay unsuitable. In these cases, the platform assay should be qualified experimentally to demonstrate that it is suitable for measuring HCPs from the new process. Assay accuracy, precision, sensitivity, linearity, range, and specificity must be evaluated (see *4. HCP Immunoassay Method Validation*). In addition, the range of HCPs in the platform HCP standard should be compared to those present in the new process. The antibodies should also be characterized using samples from the new process or product using approaches discussed previously.

The following approaches and concepts may be useful in determining if a platform assay is suitable for a product made with a new process:

1. Conduct a representative, small-scale null cell run grown under conditions used in the new culture process, and determine the protein concentration of the null cell run CCF. Assay the null cell run CCF at the same nominal concentration as the assay HCP standard using the HCP sandwich immunoassay, and compare the dilution curve from the null cell run CCF to the standard used in the assay. If the standard and new null cell run CCF curves are similar (e.g., in shape and amplitude, and the calculated HCP concentrations are within a factor of two), then the new process is considered not different from the platform, and a process-specific HCP assay is not usually needed.
2. With the CCF from the above null cell run from the new process, make a comparison to the HCP calibration standard using 2-D gel electrophoresis. This will compare the diversity of proteins produced by the two processes. However, be aware that new "spots" may appear in the 2-D gels that may not reflect immunochemical differences in the assay, for example, post-translational modifications or limited proteolysis. As such, the appearance of a few new spots or up-regulated or down-regulated spots in the process-specific null cell run is not a basis for invalidating the application of the platform assay. A qualitative assessment (e.g., versus a Western blot) is recommended.
3. Comparison of HCP levels at harvest in actual production runs (CCF samples) with the same product produced in the original versus the "new" process is also valuable. In general, immunoassay results within a factor of two are often considered similar, thereby confirming the platform HCP reagents.
4. HCPs in CCF from production cultures may also be estimated on the basis of total protein measurements. The total protein in the CCF is the sum of product (measured using a product-specific titer assay) and HCPs. Samples from harvests of the manufacturing run cell culture can be dialyzed, as long as they are sufficiently concentrated to make membrane losses negligible, to remove small peptides and amino acids, and then the total protein is determined (e.g., by amino acid analysis). Subtraction of the product concentration from the total protein provides an estimate of the non-product protein, HCP, in mg/mL. This value may be compared with the HCP concentration from the immunoassay. Comparable values (e.g., within 2×) indicate that the HCP platform is confirmed for the new process.

### 3.3.2 NEED FOR NEW REAGENTS AFTER PROCESS CHANGES

Major changes, such as substantial and unambiguous changes in cell culture conditions (e.g., moving from a serum-containing process to a serum-free production process), typically require production of new reagents and development and validation of a new HCP assay. In this example, the HCP reagents created using the old process recognize a number of serum proteins but may not recognize many new HCP proteins in the serum-free process. Because of both the process change

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and the assay change, different HCP levels will be observed when the same samples are measured for HCP in the old and new assays (i.e., the samples from the serum-containing process may have a high level of HCP measured by the old assay and low level of HCP measured by the new assay, whereas the samples from the serum-free process may have much lower HCP results by the old assay but more with the new assay).

If the assay was developed as a downstream, process-specific assay, changes in the purification steps can also require replacing the assay with a new one that is relevant to the new downstream process. This is the biggest problem with these very specialized assays and why platform-based assays are usually preferred, because they often do not require a new assay when the downstream steps are changed.

#### 3.3.3. SAMPLE STABILITY

It is important to demonstrate that the sample handling procedures do not affect the assay results. This is particularly true when comparing results for various intermediate process pools. Occasionally, HCPs can precipitate during freezing or when the sample pH is adjusted from acidic to neutral. In addition, storage at 2°–8° or multiple freeze-thaws may result in loss of reactivity. The analyst may need to add specific stabilizing components (e.g., divalent cations) to the samples to ensure stability.

## 4. HCP IMMUNOASSAY METHOD VALIDATION

HCP immunoassays for product purity assessment typically are part of the cGMP control system, either as an in-process test or on the C of A, and need to be validated appropriately. The validation approach depends in part on the types of samples that will be tested with the method. All validation parameters for a quantitative impurity test are needed when used for the final DS, whereas validation for in-process samples usually focuses on dilution linearity, interference, and precision. Regardless of whether the assay is an in-process or release assay (DS), action limits are valuable as a part of an overall control strategy. Readers are referred to ICH Q2(R1) guidelines and chapter *Validation of Compendial Procedures* ⟨1225⟩ for general expectations for assay validation, particularly those requirements for cGMP assay validation, and to chapter ⟨1103⟩ for sandwich immunoassays. Although the main focus of this section is on cGMP assay validation, many of the same principles apply to the assays used to demonstrate that process purification steps are suitable for their intended purpose.

This section will focus on those aspects of HCP immunoassays that are unique or require special attention and documentation. HCP immunoassays differ from more conventional immunoassays in that they are multi-analyte assays. There are potentially thousands of proteins in the immunogen and standard, and a correspondingly diverse set of antibodies is produced, yet only upstream CCF will contain a diversity of proteins that approximate the HCP calibration standard. Intermediate pools and final products typically contain progressively fewer of the HCP proteins that are present in the standard. As discussed previously, this is the aspect of accuracy that is compromised by HCP immunoassays, and why the mass unit ratios (ng of HCP per mg of DS) are not a literal "mass" relationship. However, below are common industry approaches that address these limitations as much as possible.

The validation summary below is for the final DS or a penultimate process step, whichever is chosen for the C of A method. It should be noted that for in-process testing, the focus is on method accuracy (spike recovery), dilution linearity, and precision. In the case of accuracy, some samples will have more potential interfering substances (other process impurities or additives) that may need to be confirmed as non-interfering (e.g., residual DNA, leached protein A, virus inactivators, chaotropes, and low pH buffers).

### 4.1 Accuracy

To assess HCP assay accuracy in the DS, at a minimum the analytical assay standard should be added to appropriate samples and accurate spike recovery demonstrated over the range of the assay. Matrix interference can come from buffer components and product, and the minimum dilution required for acceptable spike recovery (typically between 70% and 130%) needs to be determined for each sample type. Typically, spike recovery experiments are conducted with spikes using at least three and potentially as many as five different levels. Because samples are assayed at multiple dilutions, the investigator may spike and then dilute the sample, or dilute the sample and then spike the diluted samples. Spikes near the quantitation limit (QL) help to evaluate the accuracy and repeatability of the assay near the QL, which is where the measurement is often the most variable. A spike recovery of 50%–200% may be acceptable for a spike at or near the QL.

Although these are minimum requirements for assay validation, they should not be interpreted as demonstrating accuracy for any one specific HCP that may co-purify with the product. To accomplish that, comparison to a standard of that particular HCP species is needed; however, because this HCP is rarely known, this may not be possible. Tips for discerning these situations are provided below in the dilution linearity section and in *5. Supporting Technologies for Residual HCP Detection, Identification, and Measurement*.

### 4.2 Sensitivity and Assay Range

HCP sandwich immunoassays often achieve standard curve sensitivity in the low single digits of ng/mL. For sample protein concentrations where the test dilution is 10 mg/mL, this implies that a sensitivity of about 1 ng of HCP per mg of product is possible. However, this sensitivity may be difficult to achieve for products with lower protein concentrations. It is important to highlight that the reported HCP ratio (ng of residual HCP relative to mg of product) is not really the ratio of masses implied by the unit ng/mg but rather "immunological equivalents" per mg of product.

The first aspect of determining QL is typically determined experimentally for each product in a given sample buffer matrix in spike recovery studies. The minimum recommended dilution (MRD) for the DS should be established by spiking the HCP standard in a sample dilution series. For example, an HCP spike of 10 ng/mL added to each of a series of solutions containing undiluted (e.g., 10 mg/mL) protein and serially diluted DS. Those dilutions in which the spike recovery is 70%–130% (50%–

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150% is also commonly used for levels near the QL) are considered acceptable. Typically, a proposed DS MRD is tested further to ensure that spike recovery is consistently achieved.

For the second part of the QL determination, the DS is tested at the MRD (if the DS samples have high levels of detectable HCP, a formulation buffer may be used). The QL is generally determined by the analysis of spiked concentrations of HCP and by establishing the minimum level at which the HCP can be determined with acceptable accuracy and precision. This study is typically performed at least three times, preferably by different analysts or on different days. For example, a spike of 3 ng/mL of HCP in a product protein concentration of 5 mg/mL has a QL of 0.6 ng/mg or 3 ng/mL if spike recovery is achieved in, e.g., at least four of six tests or if mean spike recovery criteria are met.

Typically, assays are set up to measure the range from a few ng/mL to >100,000 ng/mL. This range allows the analyst to test a variety of samples at multiple dilutions (see discussion of *4.3 Sample Linearity*) and allows for a practical assay that can accommodate in-process pools with highly differing HCP levels. For example, upstream samples may contain >100,000 ng/mL of HCPs and require large dilutions to obtain results in range of the standard curve.

For routine commercial DS manufacture where the protein product concentration is known and the process impurities are well understood, testing at a single dilution may be used for release. The results are reported as the ratio of measured HCP (ng/mL) to the product concentration (mg/mL) resulting in units of ng/mg. When the DS has undetectable levels the results are reported as "less than" the assay QL (ng/mL) divided by the product concentration (mg/mL; e.g., <0.6 ng/mg in the example above). Before setting this target concentration for testing, however, the dilution linearity of the samples should be well understood and a robust manufacturing process established. In the event that the level or species of HCPs vary run-to-run it may be necessary to test each sample at multiple dilutions (see below).

## 4.3 Sample Linearity

HCP assay nonlinearity is not uncommon for some samples and should be assessed for multiple batches of a given sample type (e.g., from several clinical DS lots or process validation batches) to ensure consistency and proper reporting of results. Because a single HCP (or a few individual HCPs) may co-purify with product, it is possible that particular HCP(s) will be present in excess of the available antibodies in the HCP immunoassay. This is possible in multi-analyte assays where only a limited surface area is available on assay microtiter plates or beads, and because thousands of different anti-HCPs are needed, antibodies to any one HCP will, of necessity, be limited. Furthermore, the relative amount of antibody to each separate HCP is also not controlled, so the binding capacity for each separate HCP differs. For samples exhibiting this behavior, the analyst must dilute the sample into the range of the assay, i.e., to a point where nonlinear behavior is no longer observed because the HCP has been diluted to a concentration where it no longer exceeds the available antibody. In some cases, the sensitivity of the assay becomes limiting, and one never reaches a dilution where the assay result is independent of the sample dilution. In such cases, the highest HCP ratio value (corrected for sample dilution) within the validated assay range should be reported. A detailed example of how to handle nonlinear dilutions arising as a result of antibody insufficiency follows.

*Table 4* and *Figure 4* show data for three different samples tested in an ELISA that has a range of 1–100 ng/mL. In this example, all samples were diluted initially to 10 mg/mL (the MRD, where spike recovery had been previously confirmed), and then serially diluted using a twofold dilution series to below the assay QL of 1 ng/mL. Whereas *Sample 1* (diamonds) dilutes linearly over the full range tested, both *Sample 2* (squares) and *Sample 3* (triangles) HCP results plateau at higher concentrations of the sample. Saturation of the antibodies reflects antigen excess, and determination of the HCP value is made by diluting the sample into the range of linear dilution; visually estimated as <2 mg/mL for the squares and <1 mg/mL for the triangles. [NOTE—If large sample dilutions are required to get into the range of the HCP assay, consider making intermediate dilutions to limit dilution-related errors.]

**Table 4. Raw Data for the Graph in *Figure 4*[a]**

| Product | Sample 1 | | | Sample 2 | | | Sample 3 | | |
|---|---|---|---|---|---|---|---|---|---|
| Conc. (mg/mL) | HCP conc. (ng/mL) | HCP ratio (ng/mg) | % max ratio value | HCP conc. (ng/mL) | HCP ratio (ng/mg) | % max ratio value | HCP conc. (ng/mL) | HCP ratio (ng/mg) | % max ratio value |
| 10.00 (neat) | 49 (neat) | 4.90 | 83% | 20 (neat) | 2.00 | <20 | 3.2 (neat) | 0.32 | 22% |
| 5.00 | 28.5 | 5.70 | 96% | 16.5 | 3.30 | 54% | 2.5 | 0.50 | 35% |
| 2.50 | 12 | 4.80 | 81% | 10 | 4.00 | 66% | 1.5 | 0.60 | 42% |
| 1.25 | 7.4 | 5.92 | 100% | 7.4 | 5.92 | 97% | 1.1 | 0.88 | 61% |
| 0.625 | 3.1 | 4.96 | 84% | 3.3 | 5.28 | 87% | 0.9 | 1.44 | 100% |
| 0.3125 | 1.6 | 5.12 | 86% | 1.9 | 6.08 | 100% | <1 | <6 | NA |
| 0.15625 | <1 | <6 | NA | <1 | <6 | NA | <1 | <6 | NA |
| Reported HCP ratio value with guide and *n* | Guide 1 = 5.2 (*n* = 6); Guide 2 = 5.2 (*n* = 6) | | | Guide 1 = 5.8 (*n* = 3); Guide 2 = 5.3 (*n* = 4) | | | Guide 1 = 1.2 (*n* = 2); Guide 2 = 1.4 (*n* = 1) | | |

[a] Numerical data for three samples (also graphed in *Figure 4*) show differing degrees of saturation of the response at higher concentrations of product (and HCP impurity) tested. Two guides on how to interpret these data (guides 1 and 2) illustrate how the "linear range" of the assay is limited to the most dilute data points shown in *Table 4* and *Figure 4*. Note—Samples are always diluted until the HCP concentration is below the QL of the assay (in this example the QL is 1 ng/mL). Guide 1: All values within 20% of maximum value are averaged. Guide 2: Values are averaged as long as the CV of the values is <20%, removing less diluted samples first. A third guide might also be used that reports the highest value measured above the QL but the suitability of this approach should be very well demonstrated by method validation.

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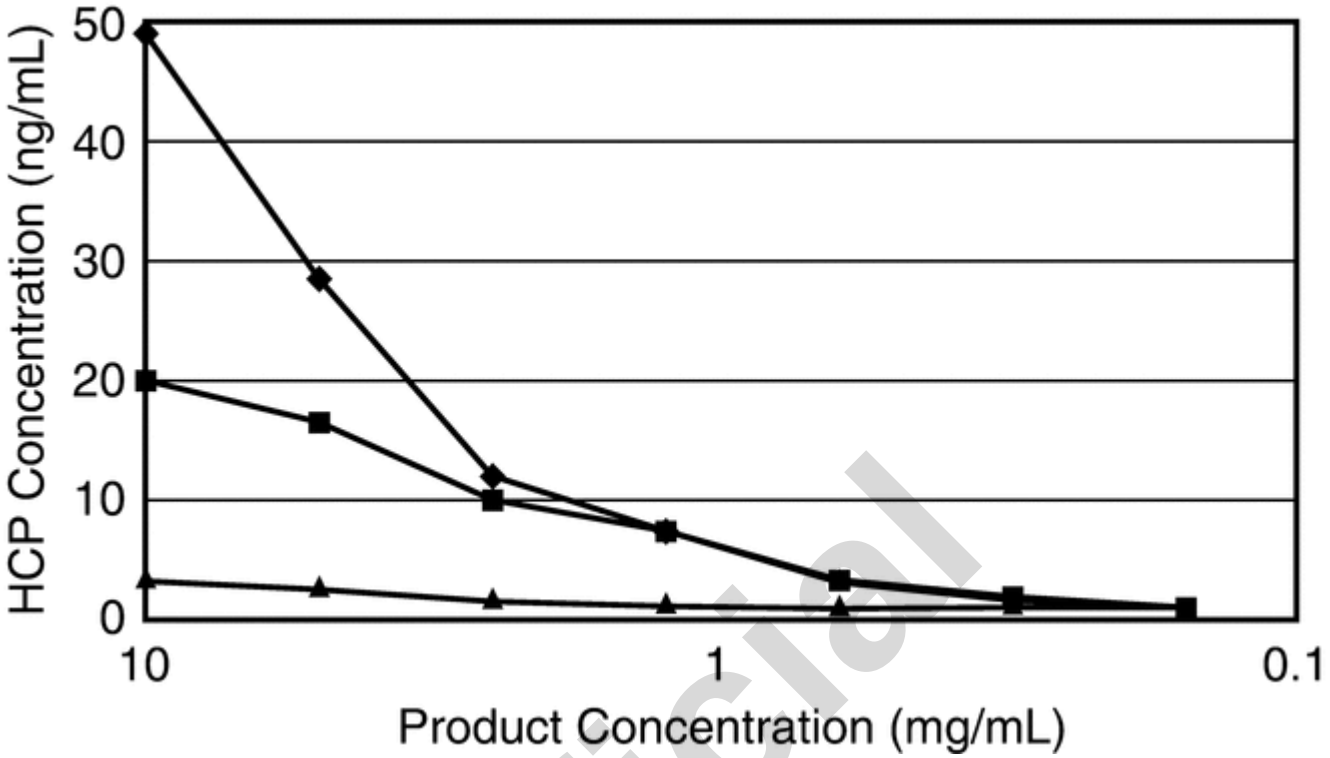


Figure 4. Twofold serial dilution of the three samples listed in *Table 4*. HCP concentration results are plotted against the product concentration in the samples, which serves as a measure of sample dilution.

Because measurements near the QL may have higher variability and less reproducibility than measurements farther from the assay limit, usually (if possible) values within the sample linear HCP concentration response range are averaged. The procedure for which data points may be averaged must be specified in advance of running the assay and is typically documented as part of the test procedure. In the example shown in *Table 4*, two different guidelines are illustrated. *Guide 1* averages all values within 20%–25% of the maximum HCP ratio value. *Guide 2* averages values only if the CV for the resulting averaged values is <20%–25%. Although their data sets are not identical, both rules are justified, based on the argument that variations of 20%–25% are within the variation seen in assay replicates and reflect the underlying reproducibility of immunoassay methods. In this example, both methods give very similar results. In contrast, the third potential guide, which reports the highest value measured above the QL, would yield results that are at least 10% higher. In addition, the validity of the third guide would depend highly on the quality of the method development.

Homogeneous (one-step incubations without washes between reagent additions) sandwich immunoassays are more prone to antigen excess and resulting dilution nonlinearity problems and may exhibit “high-dose hook effects” in which samples with large amounts of antigen give inaccurately low readings, because the antigen excess prevents formation of the complete sandwich. Suitable use of this format should be confirmed experimentally for each sample type and could be acceptable if the samples are diluted to assay QL and lack of hook effects is confirmed.

Historically, some companies have used spike-recovery data produced in the validation of accuracy to demonstrate linearity. Although this demonstrates the linear dilution of the standard, it does not demonstrate the linear dilution of the HCPs in the samples. For the reasons of antigen excess discussed above, the linear dilution of the standard is a necessary condition but not sufficient to demonstrate linearity of the assay for different sample types.

Finally, although antibody insufficiency is generally believed to be the primary cause of a lack of dilution linearity, other potential causes are: 1) reactivity of the antibodies with product protein, 2) the presence of non-specific antibodies (“sticky” antibodies), 3) reactivity of the antibodies to non-HCP proteins present in samples (such as peptones or BSA), 4) HCP aggregation, 5) HCP standard dilution profile that is not representative of the in-process or final product HCP, and 6) sample matrix interference. Cross-reactivity and matrix interference are discussed below.

## 4.4 Specificity

Assessment of potential matrix interference in samples is addressed in section *4.1 Accuracy* and is primarily used to establish the lack of formulation interference (in the DS) and other impurities that are normally present (for upstream process samples). Another specificity issue to evaluate is the potential cross-reactivity of the anti-HCP antibodies with the product itself. Although this is possible, antibodies to HCPs that cross-react with product epitopes are rarely observed.

In those cases where cross-reactivity is observed, more false-positive assay signals are risks that must be managed. If cross-reactivity is suspected, it should be carefully and scientifically established. In the vast majority of cases, signals in HCP immunoassays are real, indicating the presence of HCP impurities. In particular, instances where the HCP(s) are noncovalently associated with product, and they co-purify, may give the appearance of cross-reactivity. However, because of the tremendous

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variety of antibodies in a polyclonal anti-HCP pool, when measuring residual HCP in the presence of excessive levels of product, some antibodies may still bind product, yielding a false-positive signal. In this case, it may be necessary to modify the assay or the anti-HCP reagent. If a platform HCP assay is used to test multiple batches (for the same program), and different levels of HCP between batches are observed, this is strong evidence that the HCP-positive signal is real and not due to cross-reactivity.

Evidence for cross-reactivity is often seen in a product that has been purified in a sequence of steps and where the HCP levels are not reduced by typical purification processes. The HCP/product ratio (ng/mg) should be used so that changes in the HCP/product ratio during purification can be compared. First, Western blots of DS are usually probed with anti-HCP antibodies to determine if HCP(s) are noncovalently associated with product. If bands are detected that are unrelated to the product, this suggests that cross-reactivity is not occurring, and further process development may be required if lower HCP levels are sought.

Alternatively, if the anti-HCP is recognizing a product-associated band, this suggests cross-reactivity. Caution should be exercised, however, because the product is denatured (by SDS-PAGE), and binding may be to epitopes not accessible when in the native, solution phase (i.e., in the immunoassay). In this case, a positive Western blot could be an artifact (conversely, a negative Western blot could also miss cross-reactivity, but this is less probable). Regardless, if cross-reactivity is suspected, it can be confirmed and corrected by either: 1) addition of a high-purity product (or related structures, such as human IgG from a different source, for a monoclonal antibody) to block the cross-reacting antibodies in the assay, or 2) depletion of the anti-HCP in the reagents using a product-affinity column made from highly purified product. In either case, it is important to ensure that the antigen used to block or deplete the serum is itself free of residual HCPs. This information is available from the Western blots probed with anti-HCP. If HCP bands are present, this indicates that a higher-purity product may be needed and can be prepared with orthogonal, lab-scale purifications. Sometimes reversed-phase HPLC can be effective in making this material, although this is not useful during product production because of its denaturing properties.

## 5. SUPPORTING TECHNOLOGIES FOR RESIDUAL HCP DETECTION, IDENTIFICATION, AND MEASUREMENT

Biopharmaceutical manufacturers need an integrated control system that offers assurance of overall product purity and manufacturing consistency. Typically, product purity is demonstrated by using a combination of methods, and this is very much the case for HCPs. In addition to the most common sandwich immunoassay format, other assay types provide valuable complementary information. *Tables 5*, *6*, and *7* provide overviews for commonly used methods for HCP detection, quantitation, and characterization. *Table 5* discusses electrophoretic methods; *Table 6* discusses Western blot methods; and *Table 7* discusses chromatographic and proteomic methods. Gel electrophoresis and immunoblots are also discussed in *3.2.2.2 Characterization of antibodies to HCPs*.

Orthogonal assay methods used to provide additional assurance of purity and the lack of HCPs may be used in any of three modes: “detection”, “identification”, or “quantitation”. In the case of detection, orthogonal assays address the question, “Is there any protein other than a form of the product that the immunoassay may have missed?” If this is the case, as knowledge accumulates regarding the identity of the HCP(s), it becomes possible to perform a specific protein risk assessment and, if necessary, generate a standard and separate test for that particular HCP impurity. The orthogonal method may then be used in a “quantitative” mode by comparing the product sample with the appropriate HCP standard for the known HCP. For example, a quantitative HPLC-MS/MS method may be developed, based on monitoring peptides that ionize well from digests of the analytical standard protein and the sample.

### 5.1 Considerations for Electrophoretic Methods

Three electrophoretic methods that are useful in impurity analysis are 1-D and 2-D SDS-PAGE and SDS-capillary electrophoresis using non-gel sieving (CE-SDS). In all these methods, excess sample is loaded to ensure detection of trace protein impurities. The drawback is that excess product obscures impurities that migrate close to the product and cause interference. However, 1-D gels or CE-SDS may already be part of the GMP control system for monitoring product-related fragments; therefore, extending these methods for HCP monitoring does not involve additional testing. 2-D gels have been used to investigate the purity of the final product and to identify minor spots in the gels associated with either product variants or HCP impurities. The presence of a new band in the gel, or a new peak in the CE-SDS electropherogram, may represent either a new product-related substance/impurity or may reflect the presence of an HCP impurity (“process related”). In either case, an investigation is warranted. If not part of the routine control system, a 1-D gel is still an excellent method for extended characterization of important lots, such as registration lots, and for manufacturing investigations. Gels and CE-SDS are useful for confirming the absence of gross levels of HCPs (should they be present and missed in the immunoassay). Gels are typically stained with a high sensitivity stain, such as a fluorophore or silver. Ideally, the stain should be compatible with in-gel proteolytic digestion and subsequent analysis by mass spectrometry to identify the protein.

Proteins differ in their staining intensity with silver or fluorescent dyes; therefore, only semiquantitative estimates of the amount of protein in a band or spot are possible (e.g., glycosylated forms may not stain as intensely). Nonetheless, because these methods do not rely on anti-HCP antibodies, they provide an important orthogonal measure of purity.

Some proteins may appear in gels as multiple bands or spots that result from post-translational modifications or proteolytic clipping. To the extent that a single gene product is distributed to multiple locations in the gel, this further reduces the sensitivity of gels to detect impurities.

As discussed following *Table 7* on proteomic methods, it is often possible to excise bands or spots from the gels. These excised gel fragments may then be incubated with proteases to provide *in situ* digestion of the protein into peptides. These peptides may then be fractionated and sequenced using LC-MS/MS and compared with databases to identify the origin of the peptides as either product-related or HCPs.

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**Table 5. Analytical and Characterization Electrophoretic Methods for HCP Testing**

| Method | Use | Advantages | Disadvantages | Approximate Sensitivity for HCPs and Ability to Quantify |
|---|---|---|---|---|
| 1-D gel, either reduced or nonreduced, stained with silver or high-sensitivity fluorescent dye. | Screening a large number of samples for consistency and presence of nonproduct protein bands. May be used as part of GMP control system. | High resolution separation; provides approximate molecular weight (MW) information; relatively simple to run. Side-by-side analyses of samples provide powerful comparisons for manufacturing consistency. | Large excess of product protein may obscure HCP bands. Different proteins show differences in staining intensities so only semi-quantitative. Limited capacity of gel lanes for total protein load. | 100 ng/mg. Semiquantitative unless used with an analytical standard for a known HCP. |
| Large-format 2-D gel (e.g., 20 × 25 cm) stained with silver or a high-sensitivity fluorescent dye. | Screening a small number of samples for consistency and presence of unknown protein spots. Not suitable for routine use in lot release but may be used for characterization of specific lots or HCP standards. | High-resolution separation of trace HCP impurities from product. Provides approximate MW and pI information on protein spots. | Large excess of product protein may obscure HCP spots. Different proteins show differences in staining intensities so only semiquantitative. Highly technique-dependent results requiring skilled staff and sophisticated equipment. | 100 ng/mg of total protein load). Semiquantitative unless used with an analytical standard for a known HCP. |
| CE-SDS with laser-induced fluorescence (LIF) detection. Fluorescently labeled samples are separated as either reduced or nonreduced samples. | Screening a large number of samples for consistency and presence of unknown protein peaks. May be used as part of GMP control system. | Rapid, high-resolution method to separate fluorescently labeled proteins. | Sensitivity may not be as high as a 1-D gel with a highly sensitive stain. Fluorescent labels are not specific for HCP proteins, so there is no distinction between HCP and product-related peptides. Difficult to extend to other measures, such as Western blots, or to identify protein peaks. | 1000 ng/mg (0.1% of total protein load). Semiquantitative unless used with an analytical standard for a known HCP. |

## 5.2 Considerations for Western Blot Methods

The Western blot technique is complementary to the sandwich immunoassay for demonstrating product purity and for characterizing the immunochemical reagents used in the immunoassay (see *3.2.2.2 Characterization of antibodies to HCPs*). When the anti-HCP antibody is used as a probe, it may be useful to: 1) detect HCPs that are noncovalently associated with the product and (potentially) missed in immunoassay, or 2) monitor an individual HCP. In other cases, a protein that reacts well in the immunoassay may not be detected in Western blot because a conformational epitope was lost following denaturation. Immunoblots require only one antibody to a given protein, whereas sandwich immunoassays require two. Previous work has shown that high-affinity antibodies that react with a given HCP can be more sensitive than silver or fluorescent dye staining of gels.

1-D Western blots of final product provide a simple reliable orthogonal method for demonstrating product purity. Although the same antibody preparation may be used in both the immunoassay and Western blot, for the reasons noted above, the blot may provide additional information on trace proteins (and miss others), hence its orthogonal nature. *Table 6* presents the advantages and disadvantages of 1-D and 2-D Western blot methods for these purposes.

Below is a list of the major considerations for optimization of SDS-PAGE/Western methods:

- 2-D-gel format and size: The larger formats (18-cm length or longer) resolve proteins better and come in a variety of isoelectric focusing (IEF) application modes and molecular weight dimensions (e.g., gradients). Total protein staining of various formats can be studied to optimize the separation in both dimensions and thereby find the balance that separates most (or the maximum number) of HCPs.
- The load on 2-D gels should be optimized to balance sensitivity needed (higher load) versus resolution desired (lower load). The protein load, ionic content of the sample, and speed of separation need to be balanced to avoid over-heating and to optimize resolution. The stains or substrates to be used will influence this choice. Typically, more is loaded for the immunoblot (e.g., 200 µg) than for the total protein-stained gel or blot (e.g., 50 µg for silver stains). The difference in load may confound the separation to some extent; therefore, finding the right balance will require experimentation.
- Signal development for both the total protein (gel or blot) and the Western blot requires significant experimentation (e.g., dye types, exposure times, buffer conditions) to optimize. For example, because it is important to detect as many bands as possible, sometimes the signal is amplified so much that the most abundant species obscure the others, or the background increases and contrast is lost. A related issue is standardization and calibration of the densitometer or imager, which must be fine-tuned to achieve consistency under experimentally determined conditions. Both reagent and instrument optimization can require significant time to reproducibly establish the optimized conditions where signal is maximized and background is minimized (see also ⟨1104⟩).
- The transfer and blocking reagents require experimentation to identify the best conditions (such as buffers, instrument power/time, temperature, and blockers).

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**Table 6. Analytical and Characterization Western Blot Methods for HCP Testing**

| Method | Use | Advantages | Disadvantages | Approximate Sensitivity and Ability to Quantify HCPs |
|---|---|---|---|---|
| 1-D gel, Western blot | Screening a large number of samples for consistency and presence of unknown protein bands that react with anti-HCP antibodies. May be used as part of GMP control system. | High-resolution separation; provides approximate MW information; relatively simple to run. Side-by-side analyses of samples provide powerful comparisons of lots. | Requires high-quality anti-HCP antibody source. Large excess of product in the gel can lead to nonspecific staining of product bands. SDS-induced denaturation of proteins leads to loss of conformational epitopes. | Not quantitative for HCPs unless a specific protein standard is used. Sensitivity depends entirely on the quality of the antibody. |
| Large-format 2-D gel (e.g., 20 × 25 cm) Western Blot with chemiluminescence or similar high-sensitivity detection. | Screening a small number of samples for consistency and presence of unknown protein spots reacting with HCP antibodies. Not suitable for routine use but may be used for characterization of specific lots of product or characterization of antibody reagents. | High-resolution separation of trace HCP impurities from product. Provides approximate MW and pI information on protein spots. | Requires high-quality anti-HCP antibody source. Large excess of product protein may obscure HCP spots. SDS-denaturation of proteins leads to loss of conformational epitopes. Produces highly technique-dependent results requiring skilled staff and sophisticated equipment. | Not quantitative for HCPs unless a specific protein standard is used. Sensitivity depends entirely on the quality of the antibody, but 100 ng/mg is possible. |

## 5.3 Considerations for Chromatographic and Proteomic Methods

Mass spectrometric techniques for the detection, identification, and quantitation of individual HCPs are rapidly evolving and leverage much of the technology developed for proteomics. Furthermore, as new genomic databases become available (e.g., the CHO genome), mass spectrometric approaches are more useful. These methods (see also *Applications of Mass Spectrometry* ⟨1736⟩ for additional information) often combine sample preparation such as reduction, alkylation, and proteolytic digestion, followed by separation (e.g., with reversed-phase chromatography RP-HPLC) before introduction into a mass spectrometer that fragments all proteins thus providing an amino acid sequence for each peptide. The resulting sequence information is compared to the product sequence to identify product-related fragments and to a database related to the host (e.g., *E. coli*, CHO) to identify HCPs.

A challenge for MS analysis stems from the overwhelming number of product-derived peptides relative to impurity peptides. One approach to address this issue of competitive ionization of the peptides (also called ion suppression of the HCP peptides by the product peptides), is to apply LC-MS/MS analysis on partially resolved HCP preparations (e.g., HPLC fractions). This purification reduces the product contribution to total ions in the mass spectrometer. Other approaches are in development. Recent technological improvements such as 1) chromatography resins able to resolve effectively using MS-friendly mobile phases, 2) improved interfaces to front end LC and/or IEF separation systems, and 3) mass spectrometers with higher mass resolution, accuracy, and faster scan rates, now make it possible to identify and quantify specific HCPs in DS with a high degree of confidence. Major challenges in terms of sensitivity and quantitation of sufficiently large sets of heterogeneous HCPs, cost, and QC-related issues remain to be met before this technology can replace immunoassays for the control of HCPs in DS.

As a characterization method orthogonal to the HCP immunoassay, LC-MS/MS data may be used in two ways. First, if the MS-based method does not find HCPs in samples that were also below QL in the immunoassay, then these orthogonal techniques can increase confidence that the HCP ELISA did not miss an HCP that has co-purified. The detection limit for many LC-MS/MS methods is currently in the range of about 10 to 100 ng of HCP per mg of product. It is therefore sufficiently sensitive to rule out a single HCP being present at a high level and is more sensitive than gels. Second, if an impurity is identified at a high level or one that is a safety concern, then one can assess whether the purification process needs to be improved to remove it, whether the current control system is adequate, and whether development of a specific assay is required. Absence of signal does not prove absence of HCPs, but once an HCP is identified, the sensitivity of LC-MS/MS can be enhanced. LC-MS/MS is complementary to other HCP technologies and is increasingly valuable as an orthogonal method. Detailed discussion of all the proteomic approaches to the analysis of HCPs is beyond the scope of this chapter, in part because the field is evolving so rapidly that any review would become out of date quickly.

Intact HCPs also may be separated using HPLC with UV detection. The profile has been used for comparing different lots of null cell runs, for example. Readers are referred to *Chromatography* ⟨621⟩ for the core science behind HPLC chromatographic methods. Because HCPs differ in abundance, and the chromatography may not provide unique resolution of all proteins, an individual HCP must constitute about 1% (or less with good resolution) of the total protein population for possible detection (even then, a given HCP may be obscured by product, depending on its retention time). Nonetheless, investigation of unknown peaks as a general practice and comparisons of the chromatographic profiles of null cell run HCPs may be useful in determining the similarity of HCPs produced under different culture conditions.

As with the electrophoretic methods discussed in this chapter, reversed-phase separation of proteolytically cleaved product samples with UV detection may already be included in a GMP control system as part of product testing. The presence of new or unexpected peaks in the UV chromatogram may indicate the presence of an HCP impurity, although this method is not overly sensitive (~1% impurity if not obscured by other peaks). However, because no new testing or sample preparation is required, this opportunity to use an orthogonal method for determining purity should be considered.

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**Table 7. Analytical and Characterization Chromatographic and Proteomic Methods for HCP Testing**

| Method | Use | Advantages | Disadvantages | Approximate Sensitivity and Ability to Quantify |
|---|---|---|---|---|
| Mass Spectrometry – HPLC/electrospray ionization (ESI)-MS/MS or cIEF/ESI-MS/MS or MALDI-TOF of protein digests | Identification of individual HCPs. May be applied to the characterization of a purified product lot, or null cell run HCP standards. | Allows identification and monitoring of individual HCPs. May be combined with gels to identify the individual proteins seen in gels. | Low-throughput method requiring significant sample preparation, skilled staff, and sophisticated instrumentation. | 100 ng/mg and semi-quantitative if used in the discovery mode for unknown HCPs. 10 ng/mg (or better may be possible) and quantitative if used with an analytical standard for a known HCP. |
| RP-HPLC/UV or RP-UHPLC/UV. May be applied to intact proteins or proteolytic digests | Suitable for comparing null cell run standards if proteins are well separated. Larger proteins may need limited proteolysis or reduction to improve resolution. | High resolution, separates HCPs based on hydrophobicity and size; direct comparison of null strain and product strain is possible via overlays. | Difficult to find a stationary phase and mobile phase combination able to resolve and elute all HCPs. Co-elution may hinder quantitation of specific proteins. High concentration of product peak(s) may interfere with detection of trace HCPs. | 10,000 ng/mg (1%). Semi-quantitative unless used with an analytical standard for a known HCP. |

## 5.4 Concluding Remarks on Supporting Technologies for HCPs

A number of various hybrid analytical techniques have been attempted over the years. One example is fractionation of HCP standards by ion exchange, followed by RP chromatography, and then an immunoassay or MS analysis (2-D MS) on the proteins in the final fractions. Alternatively, "product subtraction" has been evaluated to remove the bulk of the total protein from final product samples before gel electrophoresis of LC-MS/MS analysis. These hybrid methods for coverage determination are very labor intensive, and they may introduce artifacts or biases of their own (e.g., antibodies to product do not typically remove all the myriad of heterogeneous forms). As such, they have not found widespread application, although in individual cases they may provide insight into the nature of product impurities.

Electrophoresis, Western blotting, and immunoassay remain the most widely used methods for HCP reagent characterization and for demonstration of HCP clearance by purification processes, because they are robust, sensitive, and can cover a broad range of protein impurities. Immunoassay and (increasingly) mass spectrometry are highly complementary and the most powerful methods for monitoring residual HCP levels in samples and confirming their absence in final DSs.

# 6. USE OF HCP IMMUNOASSAYS FOR PROCESS DEVELOPMENT, CHARACTERIZATION, AND VALIDATION

As described previously, although HCP testing has limitations as quantitative assays, if the immunoassay can rapidly and robustly detect most of the HCPs, then it is extremely valuable during process development, characterization, and validation. HCP clearance at each intermediate process step under a variety of conditions is valuable data. Application to process validation requires that the assay be demonstrated to be scientifically sound, suitable for its intended purpose, and appropriately documented. As stated earlier, process pools can differ widely in terms of buffer components, product concentration, and HCP composition. At a minimum, acceptable spike recovery of the HCP standard into in-process pool samples is necessary to qualify test dilutions of the various pools in the assay. In addition, testing each sample type at multiple dilutions is helpful during development to verify that: 1) results are within the range of the assay, and 2) nonlinearity in sample dilution is considered. In cases where the process is shown to clear HCPs robustly, process validation may also be used to justify not having a routine HCP test as part of the cGMP control system.

*Figure 5* illustrates data gathered during process development and validation and shows the dilution dependence for a number of in-process pools (*Pool 1* is after the first CCF purification step, *Pool 2* the next, and so on) through the process. *Figure 5* shows HCP ratio (in ng/mg) on the *y*-axis plotted against the product dilution in the test well on the *x*-axis. Each sample is serially diluted through three or four twofold dilutions. The starting dilution is determined by both the need to dilute samples with high levels of HCP into the range of the assay and the need to test only at dilutions where acceptable spike recovery has already been demonstrated. In *Figure 5*, the CCF has slightly more than 1 million ng of HCP per mg of product, meaning that a little less than one-half of the total protein in the CCF is product. The first column pool reduces the HCP level to just over 10,000 ng/mg. The second column has minimal HCP clearance; the third column reduces the HCP ratio to 1,000 ng/mg. The final column in the process reduces the HCP level by an additional 2 logs, resulting in a final HCP ratio of 10 ng/mg. All of the intermediate pools show HCP ratio values that are independent of the dilution of the sample.

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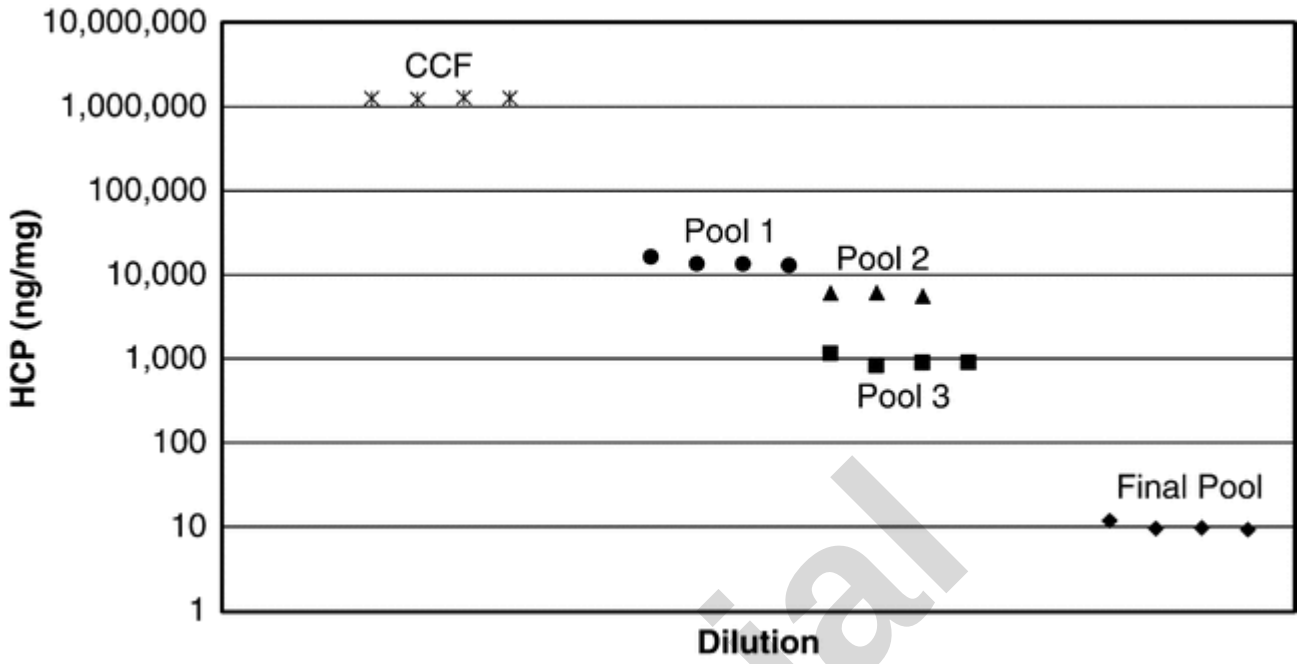


Figure 5. Example of HCP clearance by each purification step. Within a sample set, the first value is the most dilute in its dilution series.

*Figure 6* presents a set of data for a different recovery process. As in *Figure 5*, the HCP ratio data for a serial twofold dilution series of each in-process pool sample are plotted. For CCF and the column 1 and column 2 pools, the HCP ratio (ng/mg) is independent of sample dilution (dilution linearity is achieved as in *Figure 5*). But after processing over the third column, distinct dilution dependence is apparent in the assay. This nonlinear dilution is likely associated with antigen excess relative to available antibody for the particular set of HCP(s) in the sample (i.e., a single or limited number of HCP species co-purify with the product). With product purification, more product must be put in the assay well for residual HCPs to be detectable in immunoassay. The product concentration in the assay may be increased with each purification step, and thus there is a similar increase in the concentration of the co-purifying HCP impurity. By column 3, the amount of the impurity likely exceeds the capacity of the available antibodies. This phenomenon is repeated in the final pool, which involves no HCP clearance but only buffer exchange by UF/DF. The reported value for these pools is the one obtained by diluting the sample to near the QL of the assay (the most dilute sample in the series) using a method described in *Table 4*, which is about 300 ng/mg in this case. Additional discussion of assay nonlinearity is found in the *4.3 Sample Linearity* subsection of *4. HCP Immunoassay Method Validation*.

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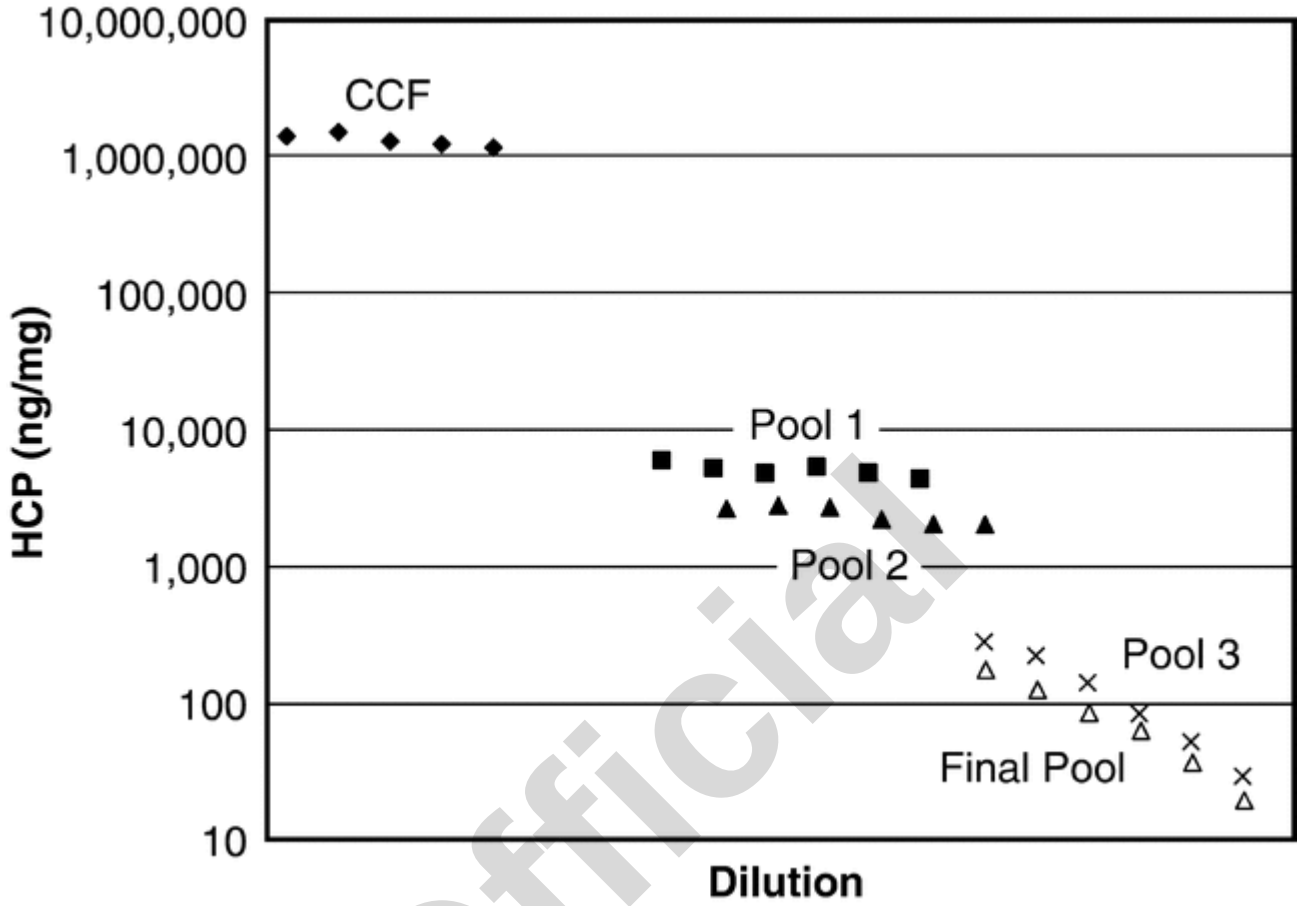


Figure 6. Example of HCP clearance with a purification process. Within a sample set, the first value is the most dilute in its dilution series.

## 6.1 Assays for Individual HCPs

In some cases, a manufacturing process might result in production of relatively high levels of a single HCP that can result in a bias in the reported results. As discussed above, in this situation the concentration of antibodies present in the total HCP assay may not be sufficient to capture all of that particular HCP present. In such cases, where a single, known HCP is present, another assay for that single HCP may be needed. For example, in the case of *E. coli* production cultures, co-expressing chaperones or other processing enzymes that aid in folding or disulfide formation can be produced in very high levels. In addition, these chaperones may be present in the immunogen used to develop the HCP assay reagents, in a conformation that is different from the way the protein is presented in samples. Thus, antibodies produced to chaperones not bound to product may not recognize, or may poorly recognize, product-bound HCPs. In this case, separate assays using appropriate antibodies may need to be developed. The existence of high levels of a single HCP is not unique to bacterial cell cultures and is possible with any cellular expression system. Another example is when a protein is intentionally added as part of the processing, then it is treated as a single HCP, and a specific assay will provide better data (than the total HCP immunoassay).

## 6.2 Control Strategy

### 6.2.1 TARGETS, ALERT LIMITS, AND REJECT LIMITS

In both United States and European Union regulations, and generally in other markets, HCPs are described as process-related impurities, and guidances state that their presence should be minimized by the use of appropriate, well-controlled manufacturing processes. U.S. 21 CFR 610.13 requires biological products to be "...free of extraneous material except that which is unavoidable...". ICH Q6B indicates that biologic product manufacturers should assess impurities that may be present and develop acceptance criteria for the impurities based on their preclinical and clinical experience. However, regulatory guidance also recognizes the inherent challenges involved in using assay-specific data and product- and process-specific reagents. Guidance states "The absolute purity of biotechnological and biological products is difficult to determine and the results are method-dependent" (ICH Q6B), and, "In the same manner, standardization of the analytical methods would be problematic since the reagents used are product and production system-related" [European Agency for the Evaluation of Medicinal Products (EMEA) 1997].

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Although FDA guidance describes the need to test for HCPs, it also allows, through validation, removal of the release testing requirement, once clearance has been shown to be consistent. An expectation exists that, whenever possible, impurities that were introduced by manufacturing processes and remain in products should be below detectable levels based on a highly sensitive analytical method. However, an appropriately conducted clearance study performed as part of process validation can be an acceptable substitute for lot-to-lot (C of A) testing (FDA 1997), even when low levels of HCPs are present in the DS. In addition, "If impurities are qualitatively and quantitatively (i.e., relative amounts and/or concentrations) the same as in the DS, testing of the DP is not necessary" (ICH Q6B). This is the case for HCPs, the concentration of which in the DP may be inferred from the DS, because the manufacturing process does not introduce, or clear, HCPs when the DS is converted to the DP. Thus, in these instances, HCP testing of the DP is generally not required.

The overarching goal is to develop processes that minimize the quantities of residual HCPs in DSs. When using HCP immunoassays as part of a manufacturing control system, limits for acceptable results need to be established. The "target" for HCP in final product gives the purification development group an objective for the process, which may or may not be achievable. Antibody processes with highly selective affinity chromatography steps (e.g., using Protein A) are sometimes able to achieve a sensitivity of 1 ng of HCP per mg of protein. Target HCP levels determine the design criteria that are then used to develop the process.

Preclinical toxicology lots are the first lots for which official HCP values should be determined, because at this point in product development, the investigator is building the data set that will be used to support the safety of subsequent clinical studies. As the molecule advances through clinical development, the preceding studies are used to support the projected safety (and risk) associated with subsequent studies. Ideally, changes in HCP level and thus clinical exposure should decrease as the product moves through clinical development.

As part of an overall control strategy, the "reject limits" are those levels which, if exceeded, render the product no longer fit for use. During product development, knowledge about the process and the product increases; therefore, it is expected that reject limits can be tightened as one moves from toxicology material to phase I/II material, to phase III, and finally to commercial material. Each drug candidate profile and its clinical program are unique; hence, no single numerical limit is applicable to all.

Reject limits are primarily concerned with patient safety. Typically, one does not have specific data documenting "unsafe" levels of HCPs. Rather, acceptable levels for early stage products should be determined through a risk assessment (including non-clinical data, available literature, previous experience with products manufactured using the same or a similar cell line, etc.). For commercial products, the acceptable levels are also based on the experience gathered during clinical trials. Because the HCP impurity profiles may be influenced by the purification process, the registration batches used in the pivotal clinical trials must be representative of the to-be-marketed process. Reject limits for commercial product should be set to exclude HCP values that are outside the range of clinical experience upon which the risk/benefit decision was made for the biopharmaceutical.

Alert limits, trend limits, or action limits (different companies use different terms) reflect considerations of manufacturing consistency. In a commercial process, there should be sufficient manufacturing history to be able to define out-of-trend results. However, early in product development, where there is little or no manufacturing history, action limits may be used to set upper limits on the expected HCPs, based on experience with similar products for which there is a history, or in comparison to the target value. Exceeding these limits should trigger an investigation to determine whether the lot is releasable. Orthogonal measures of product purity are valuable in such investigations.

With the emergence of orthogonal measures of purity, any non-product related atypical signals should be evaluated. Efforts should be made to revise the purification process to remove any unwanted HCPs present at higher than desirable levels (based on, for example, clinical and non-clinical data, available literature, etc.), or a specific justification of its acceptability must be provided. This is accomplished as part of a risk analysis that considers the clinical exposure similar to that described above for immunoassays.

In cases where HCP assays are improved or changed due to unforeseen events, the reject limits may need to be modified, even increased in some cases (if the new assay has broader coverage). However, it is essential that samples be tested in bridging studies to ensure that no changes in quality would be missed in the new, improved assay. In some cases, improved assays give higher values, so bridging studies (head-to-head with original versus improved assay) will be needed to justify the new specification limit.

### 6.2.2 SAFETY CONSIDERATIONS IN SETTING LIMITS

HCP specification limits in units of ng/mg are typically set using process capabilities and also on the basis of clinical experience (i.e., the levels used in trials where the clinical outcomes are known). Although there are several theoretical safety concerns about HCP impurities, the immunogenicity of the impurity is a primary consideration. An immune response may include formation of antibodies directed against the HCP or directed against the therapeutic protein, where the HCP is believed to have acted as an adjuvant. Readers are referred to ⟨1106⟩ for a more in-depth discussion of factors influencing immunogenicity. When patients receive more than one biotherapeutic protein with their associated impurities, immunogenicity is an even greater concern. Although high-dose products may contain a higher mass of residual HCPs per dose and this should be considered during risk assessments, the potential effect is not necessarily predictable of a clinical outcome. In some cases, very low levels of certain HCPs have been shown to have clinical effects, whereas high levels of other HCPs have had none.

Different limits may be set, depending on whether the product has only one or a very limited number of co-purifying HCPs or it has a multiplicity of HCPs, each present as a small fraction of the total HCP level in the product. This could lead to a separate assay and specification for individual HCP(s) that cannot be cleared from the process and are expected to be present in the DS and may have bioactivity.

The host cell origin can also be a consideration. It is possible that HCPs from human cell lines (e.g., HEK293 cells) may pose less immunogenicity risk than HCPs from more distantly related species because of sequence homology. However, if these proteins are normally intracellular, then they might still be immunogenic and seen as "foreign" by the patient's immune system. If such antibodies develop, then they could cross-react with endogenous human proteins and neutralize or render ineffective one or more necessary biological systems. As a consequence, arguments can be made on both sides; hence, the risks based on

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production cell line (microbial versus mammalian) are generally believed to be the same and cannot be practically informative *a priori*.

In addition to considering immunogenicity, the HCP may be bioactive and a risk. For example, a hamster homolog of a therapeutic protein expressed in CHO cells could be sufficiently similar to the product to co-purify. Alternatively, an anti-human cytokine antibody product expressed in CHO cells could bind to the homologous hamster cytokine, and the complex could co-purify with the antibody product. In both cases, the homologous hamster proteins could be biologically active when administered to patients.

## 7. SUMMARY AND CONCLUSIONS

Biopharmaceutical products should be as free of residual HCPs as reasonably possible. The industry standard for measuring HCPs is a sandwich immunoassay using polyclonal antibodies directed against the broadest possible spectrum of potential impurity proteins. Because the diversity and proportion of HCPs in the calibration standard is never matched exactly to the HCPs in any sample and due to the broad range of antibodies in the immunochemical reagents, different HCP assays will quantify individual HCPs to a differing extent. Thus, the single numerical value readout of the HCP immunoassay is "immunologically weighted". Highly immunogenic proteins will overquantify relative to the average population, and weakly immunogenic proteins will underquantify. Some proteins may not induce an immune response in the animal species used to generate antibodies, and the HCP immunoassay will be blind to those proteins. Because of this possibility, orthogonal methods for judging product purity also should be included in the control system. The best strategy is to use the HCP immunoassay in conjunction with other analytical tools.

Finally, one should use caution in extrapolating safe HCP values across products. No single specification limit will suffice. Differences in the biological properties of particular HCPs that co-purify with product, the route of administration, the patient population, and product/HCP dose all contribute to possible factors influencing patient risk. Over the history of recombinant biologic therapeutics, there have been examples where individual co-purifying proteins have been immunogenic, acted as adjuvants to increase the patient immune response to the therapeutic protein, or had biological activities themselves. Although these examples exist, they are rare, and overall there is an excellent safety record for biologics. In spite of the limitations and complexities of HCP assays, careful and diligent development and application of these assays have helped to ensure patient safety for millions of doses. HCP analysis continues to be a complex problem demanding time, resources, and careful thought by biopharmaceutical manufacturers with the goal of ensuring that products consistently meet the standard of having as low an HCP impurity level as can reasonably be achieved.

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***Add the following:***

# ▲⟨1149⟩ GUIDELINES FOR ASSESSING AND CONTROLLING THE PHYSICAL STABILITY OF CHEMICAL AND BIOLOGICAL PHARMACEUTICAL RAW MATERIALS, INTERMEDIATES, AND DOSAGE FORMS

## INTRODUCTION

The physical stability of pharmaceutical materials (e.g., excipients, active ingredients, intermediates) and drug products is an important property to consider when developing any new medicine, as the following considerations illustrate:

- Physical changes have been the cause of some high-profile product recalls and reductions in product shelf-life (*1*). Recalls have occurred for a drug product from the US marketplace due to changes in the unexpected appearance of a more stable and less soluble crystal form and for a transdermal system after crystals formed unexpectedly and compromised its efficacy.
- As recently as the 1980s, the screening of drug substances for polymorphism and for the ability to form salts/co-crystals and hydrates/solvates ("pseudopolymorphs") was not routine at many companies. Hence, there was limited awareness of the risks of physical transformations occurring during the manufacture, storage, and use of pharmaceutical dosage forms (*2*).
- Since the 2000s, there has been a marked increase in the use of amorphous drug substances and intermediates, increasing the probability of physical instability occurring because of recrystallization (*3*).
- There has been an increased understanding of the risks of developing salt forms of weak bases, which can disproportionate in the presence of excipients commonly used to manufacture immediate-release solid oral dosage forms (*4*).

This general information chapter is focused on changes in those physical attributes that can directly affect the safety, quality, and efficacy of pharmaceutical materials, systems, or products. Further, the scope of this chapter is intended to cover all materials and dosage forms described in the *USP–NF*, including drug substances, drug product intermediates, excipients, solid/liquid/semi-solid dosage forms, sterile and nonsterile preparations, and diagnostic materials.

Even small changes in the critical physical attributes of an excipient, drug substance, intermediate, or drug product over time can affect the shelf life of a material and any product manufactured from it. Several health authorities have discussed this topic in guidance documents and articles (see *Appendix 1*). In addition, several national pharmacopeias have mentioned physical stability testing and control topics in their monographs and chapters.

Clearly, certain health authorities and official compendia consider physical properties to be potentially critical quality attributes (CQAs) for excipients, drug substances, drug products, and related materials (e.g., intermediates) for some dosage forms. As a result, demonstration of 1) manufacturing processes capable of producing drug substances and drug products of consistent physical quality at release and 2) the physical stability of those materials during storage is required for successful regulatory filing and approval of marketing applications (e.g., new drug applications and marketing authorization applications).

## DEFINITION OF PHYSICAL STABILITY

Physical stability is the ability of a material to remain physically unchanged over time under stated or reasonably expected conditions for manufacturing, storage, and use. However, while this simple definition is quite useful, it does not cleanly distinguish between a purely physical change and a purely chemical change. For instance, if the color of a solution (a physical property) changes, this could be due either to a change in the ionization state of the compound containing the chromophore (a physical change) or to a change in the chemical structure of the compound containing the chromophore (a chemical change). Furthermore, ultraviolet-visible spectrophotometry (a physical test method) could be used to characterize either the change in ionization state or the change in chemical composition. Additionally, sometimes degradation pathways are synergistic: a chemical degradation event triggering a physical event, such as when oxidation is followed by aggregation in a protein biologic. Thus, while this chapter focuses on instabilities that arise from physical causes, its discussion unavoidably overlaps (in terms of cause, observation, and consequence) with instabilities arising from chemical changes.

According to fundamental physics, physical instability is often the result of the physical forces of interaction. The commonly accepted physical forces of interaction are van der Waals forces (such as the London dispersion force, Debye force, and Keesom force); hydrogen bonding; and electrostatic interactions. Hydrogen bonding is separate from the Keesom force because a hydrogen bond has too much electrostatic character to be considered a true Keesom force. An important characteristic of all of these forces is that they are reversible. In other words, a physical bond or interaction can be readily reversed to restore the original species, in contrast to covalent bond formation, wherein the original reactants cannot be easily restored. Thus, the physical forces of interaction are fundamentally different in both mechanism and consequence from the formation of a chemical (or covalent) bond.

Temperature, pH, light, pressure (including water vapor pressure), gravity, and shear force may all influence the aforementioned physical forces. They may also influence the interfaces across which the physical forces of interaction may be operating or the bulk phase through which those physical forces of interaction may be operating. The interplay among temperature, pressure, gravity, and the physical forces of interaction can lead to some seemingly inconsistent observations. For example, someone who has tried to resuspend a non-flocculated caked suspension will likely find it hard to accept the fact that physical interactions are reversible. In that case, the settling that occurred under the influence of gravity created significant areas of true contact between the particle surfaces. Those areas of true contact lead to an increased number of interparticulate

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interactions. Weak interactions, when present in great number, can be significant. However, in spite of that strength, the input of a sufficient amount of energy will regenerate the original particles. An exception to this example would be when the particles have been settled for sufficiently long periods of time and drug dissolution or precipitation have further strengthened the cake. Dissolution and precipitation are reversible processes. However, as a result of these processes, the original particles cannot be regenerated because they no longer exist. Interestingly, all of the individual processes resulting in such cake formation are reversible, but, in total, they may lead to an irreversible change in the system.

There also may be cases for which the system is reversible, but the packaging makes it functionally irreversible. An example would be the phase separation of an ointment. If the phase-separated ointment has not been packaged, it could be presumably returned to its original state by high-shear mixing. However, such mixing is not possible when the separation occurs after packaging (assuming that the ointment was packaged in a collapsible tube). Thus, the packaged product may have undergone an irreversible physical change.

In conclusion, when considering the physical stability of pharmaceutical materials and products, one should think carefully about the underlying mechanism involved and not solely consider the measurement technique or observation.

## COMMON TYPES OF PHYSICAL INSTABILITY

Several common types of physical instability may occur for excipients, drug substances, drug product intermediates, and drug products (see *Table 1*, *Table 2*, *Table 3*, *Table 4*, *Table 5*, and *Table 6*). Such instabilities can occur during storage of the raw materials, during manufacture of the drug product, or during the storage, distribution, and use of the drug product. With the proper controls in place, these potential instabilities can be often minimized or avoided. However, without such precautions, the integrity of the product can be seriously compromised.

The common instabilities or "failure modes" can be classified as "critical," "major," or "minor" depending on the defect's impact on patient safety or product efficacy. Critical failure modes are known to affect patient safety or product efficacy, whereas a major failure mode is likely to affect safety or efficacy. Finally, minor failure modes do not affect patient safety or product efficacy.

A major challenge when classifying any given failure mode is that the classification of a failure mode is often highly dependent on the type of product being considered. For example:

- For small molecule drug substances, if the crystal form of an oral Biopharmaceutics Classification System (BCS) Class I drug (i.e., drug with high solubility and high permeability) changes and the new crystal form still meets the solubility requirements for a BCS Class I drug, the failure mode could be considered minor. However, if the solubility of the new crystal form becomes substantially lower than the original crystal form, the failure mode is likely major or critical because the physical instability leads to a change in the dissolution rate of the drug product.
- For biologic substances and products (as opposed to small molecule pharmaceutical products), there are different types of physical instability, and thus different failure modes and risk factors. The unique structures of protein biologics are critical for their precise biochemical function; therefore, physical stability is dependent on the proper folding of their native state and appropriate structural maintenance. Aggregation is a primary mode of physical instability. Aggregation can occur in solution or on surfaces due to absorption, and it can be either reversible or essentially irreversible. Such changes can lead to a loss of activity as well as other critical problems, such as toxicity and immunogenicity.

**Table 1. Examples of Physical Instability Leading to Changes in Appearance**

| Material | Failure Mode | Example Cause(s) |
|---|---|---|
| Powders (such as excipients and drug substances) | Caking or agglomeration/aggregation | Temperature cycling during storage and distribution, water sorption, and presence of electrostatic charge |
| | Appearance change upon storage | Change of crystallinity, polymorph change, change in hydration state |
| Tablets | Appearance change upon storage | Spontaneous salt formation between a drug substance and an excipient |
| | Breakage or chipping | Water sorption or loss by an excipient |
| Solution | Appearance change upon storage | Chemical reaction or degradation |
| | Particulate formation | Insoluble salt formation between drug substance and preservative |
| | Haze formation/opalescence, which can lead to subvisible or visible particulate | Chemical degradation leading to the formation of an insoluble product, microbial growth, or product–package interaction |
| | Fibril or particulate formation | Aggregation (biologics) due to stressors such as shear forces |
| | Gelling | Protein unfolding, hydrogen bonding, liquid crystal formation, intermolecular interactions |
| Powder (prepared as a lyophilized cake) | Collapse of lyophilized cake | Crystallization of excipient or drug substance; storage temperature greater than glass transition temperature |
| | Increased reconstitution time | Crystallization of excipient or drug substance; storage temperature greater than glass transition temperature |
| Emulsion | Appearance change | Droplet coalescence over time |
| | Phase separation | "Cracking" of emulsion due to temperature cycling upon storage |

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**Table 1. Examples of Physical Instability Leading to Changes in Appearance** *(continued)*

| Material | Failure Mode | Example Cause(s) |
|---|---|---|
| Gel | Appearance change | Dehydration of gel due to inappropriate storage conditions |
| | Inhomogeneous polymer cross-linking of the gel or a change in composition of the liquid | Weakness of gel structure |

**Table 2. Examples of Physical Instability Leading to Changes in Dissolution and Disintegration**

| Material | Failure Mode | Example Cause(s) |
|---|---|---|
| Excipient | Failure (for a super-disintegrant) to promote disintegration | Prior water sorption under high humidity storage conditions |
| Drug substance | Slowed dissolution rate | Particle agglomeration upon storage due to surface disorder induced by milling; crystallization of amorphous drug; polymorphic transitions |
| Tablets/capsules | Change in dissolution rate | • Crystal form change<br>• Crystallization of amorphous drug<br>• Water migration within dosage form<br>• Disproportionation of drug substance salt<br>• Cross-linking of gelatin capsule |
| | Change in disintegration time | • Water migration within dosage form<br>• Cross-linking of gelatin capsule |
| Suspension | Change in dissolution (of the suspended particles) rate | Increase in particle size, agglomeration |

**Table 3. Examples of Physical Instability Leading to Changes in Tablet or Capsule Mechanical Properties**

| Material | Failure Mode | Example Cause(s) |
|---|---|---|
| Excipient | Loss of tabletability | Water sorption from environment, polymorph change |
| Drug substance | Loss of tabletability | Particle agglomeration upon storage due to surface disorder induced by milling |
| Tablets | Change in breaking force | • Crystal form change<br>• Water sorption or desorption<br>• Water migration within dosage form |
| | Change in disintegration time | • Water uptake<br>• Water migration within dosage form |
| Capsules (hard or soft shell) | Loss of mechanical strength or integrity | • Water sorption by gelatin capsule<br>• Water redistribution within dosage form<br>• Inadequate primary packaging |

**Table 4. Examples of Physical Instability Leading to Changes in Rheological Properties of Suspensions or Solutions**

| Material | Failure Mode | Example Cause(s) |
|---|---|---|
| Suspension | Change in viscosity leading to dosage non-uniformity via sedimentation | Molecular weight change of suspending agent caused by increased temperature |
| | | Increase in suspended particle size/change in morphology (e.g., Ostwald ripening) |
| Solution | Change in viscosity, precipitation of the solute | Molecular weight reduction of the solvent caused by increased temperature |
| | | • Increase in solute size/change in morphology (e.g., Ostwald ripening)<br>• Changes in solubility with temperature |

**Table 5. Examples of Physical Instability Leading to Changes in Rheological Properties of Semi-Solid Products**

| Material | Failure Mode | Example Cause(s) |
|---|---|---|
| Excipient | Change in viscosity | Molecular weight reduction caused by high-shear |
| Emulsion | Change in viscosity | Increase in globule size upon temperature cycling |
| Gel | Change in yield stress | Phase separation ("syneresis") caused by temperature cycling or other improper storage conditions |
| Ointment | Change in viscosity | Drug substance recrystallization |

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**Table 6. Examples of Physical Instability Leading to Changes in Performance of Inhaled and Nasal Drug Products**

| Material | Failure Mode | Example Cause(s) |
|---|---|---|
| Dry powder inhaler (drug product) | Variable dose delivery due to change in aerodynamic particle size distribution (APSD) | Change in interparticulate interactions due to uncontrolled storage humidity and temperature (e.g., capillary forces progress into solid bridges) |
| | | Crystallization of amorphous material (excipient and/or drug substance) |
| Metered dose inhaler (particles in liquid suspension, drug product) | Variable dose delivery | Agglomeration and then aggregation of drug substance primary particles due to temperature excursions during storage |
| Nasal spray (drug product) | Subpotent dose delivered | Precipitation of less soluble drug substance polymorph or solvate/hydrate |
| | | Precipitation of insoluble complex between drug substance and excipient |

## COMMON RISK FACTORS

Based on the observations of common physical instabilities (see *Common Types of Physical Instability*) a number of common risk factors can be identified. Examples are listed below.

For excipients and drug substances:

- Materials with a low melting point (typically less than 100°) may have a greater tendency to undergo phase changes during manufacturing, distribution, storage, or use
- Acids and bases will have a tendency to form salts when mixed together
- Hygroscopic materials will tend to absorb water from their surroundings, and this may accelerate moisture-mediated physical changes (such as the softening of tablets or the crystallization of amorphous components)
- Materials with a tendency to form hydrogen bonds may be susceptible to forming hydrates or co-crystals when mixed with polar materials
- Amphiphilic materials may form liquid crystalline phases and undergo gelling

For drug substance and drug product manufacturing processes:

- High-shear processes (such as milling, homogenization, or stirring) can induce physical changes in common pharmaceutical materials
- Processes that involve heating and cooling can trigger unwanted phase changes
- Processes utilizing solvents (including water) also can be risk factors for physical instabilities due to the possibility of dissolution and precipitation
- Changes in pH or ionic strength may induce aggregation of proteins and peptides, sometimes synergistically with specific processing conditions (such as high shear forces, changes in hydrophobic surface interactions)
- Terminal sterilization processes may induce physical changes (for example, processes involving high heat sterilization may affect viscosity and particle size distribution and osmolality of a suspension; high temperature and changes in hydrophobic surface interactions may induce aggregation/fibrillation of proteins and peptides)

For the storage and distribution of raw materials, intermediates, and drug products:

- Poorly controlled temperature and humidity conditions can trigger physical instabilities for many materials
- Primary packaging that does not provide adequate protection from environmental conditions (such as humidity) can be a risk factor for physical instability
- Physical changes often can be triggered by in-use conditions that expose the product to elevated temperature or humidity or excessive agitation

All of these material and process risk factors should be considered carefully during the design of the formulation (including excipient choice) and the selection of the manufacturing process and the packaging system. These risk factors may not be important for all types of dosage forms (e.g., simple solutions), but they need to be determined for each individual situation. Consideration of the various risk factors should also occur when deciding upon the best control strategy to ensure product quality and when determining whether or not detailed physical testing is needed during product development.

## ANALYTICAL TESTING CHALLENGES

Historically, the detection and quantitation of physical changes in a drug substance, excipient, drug product intermediate, or final dosage form have created significant analytical challenges. Only in recent years has instrumentation, with the necessary selectivity and sensitivity, become commonly available so that significant numbers of samples (needed for meaningful trend analysis) can be quickly and reproducibly tested.

Usually, the physical properties of greatest interest will be those related directly to product performance or CQAs. These properties may be directly or indirectly dependent on the physical stability of individual components or on the product as a whole. Such properties may be investigated as part of the pharmaceutical development process, may be monitored during manufacturing or at the time of product release, or may be tested throughout long-term stability studies.

Common analytical testing challenges include the following:

- Physical stability testing methods (see *Appendix 2*) may require specialized instrumentation and expertise. Examples include optical microscopy, scanning electron microscopy with energy dispersive spectroscopy (SEM/EDS), moisture sorption, laser diffraction (Mie or Fraunhoffer), thermal analyses, Fourier-transform infrared spectroscopy (FTIR), Raman spectroscopy, near-infrared (NIR) spectroscopy, rheology, mercury porosimetry, X-ray diffraction, and solid-state nuclear

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magnetic resonance (solid-state NMR). Additional techniques used for biologics include circular dichroism, size-based analysis (analytical ultracentrifuge, light scattering, size-exclusion chromatography), and techniques to study the three-dimensional structure such as hydrogen/deuterium exchange mass spectrometry, X-ray crystallography, and electron paramagnetic spin resonance spectroscopy. Some of these methods are more complex than other orthogonal techniques, and therefore are suitable for the product development phase and not for routine testing of commercial products. For example, solid-state NMR may be used as a development tool, but Raman spectroscopy may suffice for physical form monitoring of a commercial product. Similarly, a rheometer may be needed to fully characterize a liquid, ointment, or cream, but a viscometer may suffice to show the consistency of well-characterized commercial drug product.

- Physical test methods often require slow and very labor-intensive sample preparation that does not perturb the physical properties of the samples. Ideally, whole dosage forms should be analyzed without the need for pulverization, sub-sampling, dilution, or other alterations.
- The limits of detection for physical changes often can be much higher than for chemical test methods. For example, detecting a physical polymorphic change of less than 5% (by volume or mass/weight) is quite difficult in most cases and often requires exquisite control of the sample preparation as well as the test methodology. Likewise, viscosity changes (e.g., in semi-solids) are frequently logarithmic in nature, and in this situation 5% to 10% variability would be quite normal.
- Among the most important properties to understand for solid dosage forms is the moisture sorption behavior of the individual components. Water is one of the most influential factors affecting the physical stability of solids. Understanding the moisture content and how it may change during the testing process is also critical.
- Single-component materials represent a limiting case of "product". These generally include the drug substances, excipients, specific phase impurities, and perhaps packaging components (e.g., a desiccant). Formulated products present additional challenges, not only due to matrix effects but also for appropriate sample preparation.
- Quantitative measures are desired where possible. This depends first on the inherent characteristics of the technique; some are inherently qualitative or non-specific.
- Another challenge may be the availability of suitable, stable reference materials and the ability to present them in a context that is equivalent to the analyte. Spiking known amounts of a drug substance into a placebo formulation is one common approach, but accurate results only can be obtained with proper sample preparation, especially for low concentrations and/or low drug loads in solids, semi-solids, and high-viscous liquids.
- For best quantitation, an analyte must be stable during sample preparation and analysis. Ideally, each analyte would be prepared in an identically processed matrix; however, this is rarely possible. The best practice is to keep the matrices as similar as possible.
- It may be necessary to build empirical models to describe the concentration-response relationship using reference standards to estimate the amount of physical change that has occurred. Such models may not transfer well from instrument to instrument, or they may "drift" over time because of changes in instrument sensitivity.
- Standard chemical stability approaches may not work when applied to physical stability studies. For example, applying the Arrhenius extrapolation could be misleading because physical changes can occur abruptly with a shift in temperature. Additionally, elevated temperatures (e.g., 40°) may be above a phase transition point (e.g., the melting point of a semi-solid). Therefore the "accelerated" conditions commonly used for chemical stability tests may prove to be meaningless for physical stability studies.

As mentioned above, there often will be a need for enhancing the sensitivity of physical stability-indicating methods, for several reasons. In managing the product matrix effects, two factors should be considered: dilution and interference. For a single component in a drug product (such as the drug substance), detection and quantitation limits may be affected simply by the lower concentration of the component in the product matrix (e.g., for a product with a 10% drug load, a 5% detection limit for a phase impurity in a drug substance could result in an effective detection limit of only 50%). Product components other than the analyte could also give responses. If such responses overlap with those of the analyte, the sensitivity of the method would be diminished or the method could become unsuitable. Especially for early studies, improved sensitivity could allow for more accurate prediction of the expected shelf-life using shorter time frames. Common approaches for increasing sensitivity and managing matrix effects include: 1) using longer data collection times to provide better signal-to-noise ratios, 2) changing or limiting the spectral or scan range to avoid interferences, and 3) adjusting the concentration or separation of the analyte.

Some analytical challenges may require the use of orthogonal techniques to provide desired information; for example, where interferences or other matrix effects preclude the use of a desired technique. Even if qualitative, such methods could still be useful. Additionally, the use of multiple techniques may be required to profile the physical stability characteristics of the molecule or product to maximize the potential to detect issues or differences (useful in biological comparability exercises). These considerations may be important in the development of product understanding and ultimately an appropriate control strategy, as discussed in the next section.

## BUILDING AN APPROPRIATE CONTROL STRATEGY

A risk-based scientific approach is recommended to ensure product quality throughout development and commercialization. An enhanced product development approach, such as Quality-by-Design (QbD), should be applied to ensure a systematic evaluation and understanding of the critical physical attributes of materials/products and of functional relationships that link the material/product physical attributes and process parameters to product CQAs.

Attributes of QbD include:

- Identifying all possible influences that may affect product performance
- Using risk assessment, guided by scientific knowledge and experience to identify potentially high-risk attributes or parameters

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- Designing and executing experiments to determine whether the influence should be considered critical and at what levels (as such, edge of failure experiments at early development stages are encouraged)
- Developing a control strategy that focuses on defining acceptable ranges for the critical attributes/parameters
- Submission strategy and impact classification are determined by the innovator based upon regulatory expectations and sound scientific strategy, which may include design space experimentation but may not meet the expectations of a QbD submission

Perhaps the most important aspect of developing a physical stability control strategy is understanding the underlying mechanisms of physical instability. This usually requires understanding the properties and variability of individual components as well as the product as a whole. Some CQAs may be indirectly related to the causative physical changes. For example, the dissolution behavior of an oral dosage form could be affected by multiple underlying physical changes, such as changes in tablet breaking force or interactions between the drug substance and excipients, or drug substance polymorphic change.

The potential physical instability of a product should be considered throughout the product development process. Ideally, it should be addressed as early as the point at which the drug substance form is selected. If solubility, purity, crystallinity, and mechanical properties are not limiting the manufacture and bioavailability of a drug substance, the solid form that exhibits better physical stability should be selected. For example, the polymorph of a drug that is thermodynamically stable at ambient conditions is usually chosen to minimize the chance that a physical form change could occur during normal storage and use.

Formulation design and packaging selection may be used to build physical stability into the product. For biological pharmaceuticals, the formulation design may include choosing such excipients as buffers, amino acids, polyols/disaccharides/polysaccharides, or surfactants, to optimize physical stability (e.g., at a specific pH) or protect the protein. Platform formulations may be used early in development, followed by further characterization and screening of excipients/ranges to define the optimum combination to ensure physical stability throughout shelf-life. Reformulation may be required to maintain physical stability as target product profile changes occur during development or product life-cycle changes occur (e.g., moving to a subcutaneous formulation at a higher protein concentration).

The International Council for Harmonisation [ICH Q8(R2)] recommends drug-excipient compatibility studies to facilitate the early detection of, and understanding of, physical and chemical incompatibilities (*5*). Compatibility studies can enhance the understanding of drug-excipient interactions and can help with root cause analysis if stability problems occur.

In some cases, excipient hygroscopicity may facilitate phase changes such as disproportionation of a salt, hydration of an anhydrous drug, or crystallization of an amorphous form. Thus, if possible, hygroscopic excipients should be avoided. When hygroscopic excipients must be used for certain functionalities, appropriate strategies to control exposure to high humidity should be implemented.

Some excipients may react with drug substances to form a new solid phase, such as a salt or co-crystal. Ion exchange between some drugs and excipients may lead to changes in the dissolution rate of tablets. A drug substance may also form a less-soluble complex with an excipient and thus could precipitate out from a solution formulation.

Major changes in physical properties are often mediated by exposure to high temperature or humidity. To avoid unintended physical instability of a drug product, it is important to understand the temperature and humidity sensitivity of the components in the product. For example, an anhydrous drug or an excipient may undergo hydration when exposed to high relative humidity (RH). In that case, high RH conditions should be avoided during distribution, storage, and use.

When problems are recognized but a simple solution is not readily available, the formulation design, critical manufacturing process parameters, in-process controls, packaging selection, and controlled storage conditions should be used to minimize physical instability and help ensure adequate shelf-life of the drug product. The selection of protective packaging must align with the purpose of stabilizing the drug product. In cases where moisture-induced phase changes in a drug product are problematic, moisture-resistant packaging may be used to maintain physical stability of the drug product after it has been dispensed to patients. Blister packing of individual tablets is one example. Adding moisture-absorbing desiccant packs to a bottle is another example. Sometimes, tablets do not have sufficient strength to withstand stress during handling. For example, use of rayon/polyester coil or the use of a desiccant sachet instead of a desiccant canister should be considered to avoid excessive agitation/stress. In those cases, the package should provide protection against excessive stresses during storage and handling. If the drug product's sensitivity to temperature is confirmed, high-temperature situations should be avoided during transportation and use of the product by patients. In extreme cases, refrigerated storage may be required.

For products that do have potential physical instability problems, a modified testing plan may be required. For example, it may be necessary to demonstrate using additional studies that the physical changes do not have any impact on the bioperformance of the product.

Alternatively, a limit test may need to be developed to determine the extent of physical change that occurs over the shelf-life of the product or during normal use by patients. Additional tests also may be needed when a product has arrived at its intended destination, or even before it is dispensed to patients.

Finally, where a test method is implemented to assess the extent of a physical change in a raw material or dosage form, it is then necessary to ask how much change is acceptable. There is no universal answer to this question, and each case must be considered on its own merits. A color change that only occurs once the primary package is opened and has no impact on product performance or patient safety may have quite wide limits of acceptability, whereas a drug substance phase change that slows the dissolution rate of a solid dosage form may require quite stringent limits set over the entire lifetime of the product. To ensure that an appropriate control strategy is in place, extensive testing may be needed during product development. Such testing may then alleviate the need for validated methods for those products when manufactured commercially, either by showing that under standard conditions adverse physical changes do not occur, or that such occurrences may be alleviated by use of proper packaging.

In general, validated physical test methods are less prevalent than validated chemical test methods. The development of a validated solid-state analytical method may be required in some instances. In other cases, less complex approaches may be preferred provided they are supported by earlier research during the development phase of the product. See *Validation of Compendial Procedures* ⟨1225⟩ for more information on the validation of physical methods.

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## CONCLUSION

Physical instability of pharmaceutical materials, including active ingredients, excipients, drug product intermediates, and finished drug products, is a well-recognized phenomenon that is documented in the literature. Regulatory agencies around the world have provided guidance that highlight the importance of physical stability in pharmaceutical development and its role in the stability of drug products over their shelf-life. Physical instability can affect the quality, safety, and efficacy (bioperformance) of the drug product and its subsequent life-cycle. Irrespective of the level of changes in product quality or performance, a systematic investigation and selection of suitable forms of drug substance and formulation ingredients are recommended early in the drug development process to create a mechanistic understanding of the process involved in the instability. Particularly pivotal for the enhancement of this understanding will be the judicious selection of a sensitive analytical technique and the optimization of the data acquisition process.

The overarching understanding, such as that may be gained through a QbD approach, will assist in identifying risks and vulnerabilities associated with drug product manufacturing, storage, and use, which will ultimately facilitate implementation of a control strategy. The understanding will also contribute to informed decisions regarding the selection of storage conditions and appropriate packaging materials, as well as the possible need for validated test methods.

## APPENDIX 1

### Examples of Guidelines and Guidances Used by Health Authorities That Mention Physical Stability

| Health Authority | Guideline or Guidance |
|---|---|
| International Council for Harmonisation (ICH) | Q1A: Stability Testing of New Drug Substances and Products (*6*)<br>Q1B: Stability Testing: Photostability Testing of New Drug Substances and Products (*7*)<br>Q6A: Specifications: Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Chemical Substances (*8*)<br>Q8 (R2): Pharmaceutical Development (*5*)<br>Q11: Development and Manufacture of Drug Substances (Chemical Entities and Biotechnological/Biological Entities) (*9*) |
| US Food and Drug Administration (FDA) | Drug Stability Guidelines (*10*) (for animal drugs)<br>Metered Dose Inhaler (MDI) and Dry Powder Inhaler (DPI) Products—Quality Considerations (*11*)<br>Nasal Spray and Inhalation Solution, Suspension, and Spray Drug Products—Chemistry, Manufacturing, and Controls Documentation (*12*)<br>Liposome Drug Products—Chemistry, Manufacturing, and Controls; Human Pharmacokinetics and Bioavailability; and Labeling Documentation (*13*)<br>ANDAs:[a] Stability Testing of Drug Substances and Products (*14*)<br>ANDAs: Stability Testing of Drug Substances and Products—Questions and Answers (*15*)<br>ANDAs: Pharmaceutical Solid Polymorphism—Chemistry, Manufacturing, and Controls Information (*16*) |
| European Medicines Agency (EMA) | Note for Guidance on In-Use Stability Testing of Human Medicinal Products (*17*)<br>Guideline on Stability Testing for Applications for Variations to a Marketing Authorization (*18*)<br>Guideline on the Pharmaceutical Quality of Inhalation and Nasal Products (*19*)<br>Reflection Paper on the Data Requirements for Intravenous Liposomal Products Developed with Reference to an Innovator Liposomal Product (*20*) |
| World Health Organization (WHO) | Stability Testing of Active Pharmaceutical Ingredients and Finished Pharmaceutical Products (*21*) |

[a] Abbreviated new drug applications.

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# APPENDIX 2

## Examples of Physical Test Methods and Information in the *USP–NF*

| General Chapters |
|---|
| *Spectroscopic Identification Tests* ⟨197⟩ |
| *Light Diffraction Measurement of Particle Size* ⟨429⟩ |
| *Visual Comparison* ⟨630⟩ |
| *Color and Achromicity* ⟨631⟩ |
| *Completeness of Solution* ⟨641⟩ |
| *Globule Size Distribution in Lipid Injectable Emulsions* ⟨729⟩ |
| *Dissolution* ⟨711⟩ |
| *Optical Microscopy* ⟨776⟩ |
| *Optical Rotation* ⟨781⟩ |
| *Osmolality and Osmolarity* ⟨785⟩ |
| *Particle Size Distribution Estimation by Analytical Sieving* ⟨786⟩ |
| *Subvisible Particulate Matter in Therapeutic Protein Injections* ⟨787⟩ |
| *Particulate Matter in Injections* ⟨788⟩ |
| *Particulate Matter in Ophthalmic Solutions* ⟨789⟩ |
| *Visible Particulates in Injections* ⟨790⟩ |
| *Refractive Index* ⟨831⟩ |
| *Specific Gravity* ⟨841⟩ |
| *Specific Surface Area* ⟨846⟩ |
| *Mid-Infrared Spectroscopy* ⟨854⟩ |
| *Near-Infrared Spectroscopy* ⟨856⟩ |
| *Ultraviolet-Visible Spectroscopy* ⟨857⟩ |
| *Raman Spectroscopy* ⟨858⟩ |
| *Thermal Analysis* ⟨891⟩ |
| *Viscosity—Capillary Methods* ⟨911⟩ |
| *Viscosity—Rotational Methods* ⟨912⟩ |
| *Viscosity—Rolling Ball Method* ⟨913⟩ |
| *Viscosity—Pressure Driven Methods* ⟨914⟩ |
| *Characterization of Crystalline and Partially Crystalline Solids By X-Ray Powder Diffraction (XRPD)* ⟨941⟩ |
| *Tablet Friability* ⟨1216⟩ |
| *Tablet Breaking Force* ⟨1217⟩ |
| *Water–Solid Interaction in Pharmaceutical Systems* ⟨1241⟩ |
| *Applications of Nuclear Magnetic Resonance Spectroscopy* ⟨1761⟩ |
| *Measurement of Subvisible Particulate Matter in Therapeutic Protein Injections* ⟨1787⟩ |
| *Visual Inspection of Injections* ⟨1790⟩ |
| *Mid-Infrared Spectroscopy—Theory and Practice* ⟨1854⟩ |
| *Near-Infrared Spectroscopy—Theory and Practice* ⟨1856⟩ |
| *Ultraviolet-Visible Spectroscopy—Theory and Practice* ⟨1857⟩ |
| *Raman Spectroscopy—Theory and Practice* ⟨1858⟩ |
| *Rheometry* ⟨1911⟩ |

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# ⟨1151⟩ PHARMACEUTICAL DOSAGE FORMS

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## GENERAL CONSIDERATIONS

This chapter provides general descriptions of and definitions for drug products, or dosage forms, commonly used to administer the drug substance (active pharmaceutical ingredient; API). It discusses general principles involved in the manufacture or compounding of these dosage forms. A glossary is provided as a nomenclature resource and should be used in conjunction with the *Nomenclature Guidelines*.[1]

A dosage form is a pharmaceutical preparation consisting of drug substance(s) and/or excipient(s) to facilitate dosing, administration, and delivery of the content of the drug product or placebo to the patient. The design, materials, manufacturing, and testing of all dosage forms target drug product quality. A testing protocol must consider not only the physical, chemical, microbiological, and biological properties of the dosage form as appropriate, but also the administration route and desired dosing regimen. These considerations, organized by route of administration, are detailed in *Injections and Implanted Drug Products (Parenterals)—Product Quality Tests* ⟨1⟩, *Oral Drug Products—Product Quality Tests* ⟨2⟩, *Topical and Transdermal Drug Products—Product Quality Tests* ⟨3⟩, *Mucosal Drug Products—Product Quality Tests* ⟨4⟩, *Inhalation and Nasal Drug Products—General Information and Product Quality Tests* ⟨5⟩, and *Ophthalmic Products—Quality Tests* ⟨771⟩.[2] The organization of this general information chapter is by the quality attributes of each particular dosage form, generally without specific reference to the route of administration. The following list provides the preferred dosage ▲form terms▲ (USP 1-May-2021) used in official article titles. In addition to the preferred dosage ▲form terms▲ (USP 1-May-2021) the *Glossary* contains other ▲dosage form▲ (USP 1-May-2021) terms that have been used in current official article titles but are not preferred and should not be used for new drug product titles.

**Official Dosage Forms Used in Official Article Titles**

- Aerosols
- Capsules
- Creams
- Emulsions
- Films
- Foams
- Gases
- Gels
- Granules
- Gums
- Implants
- Injections
- Inserts
- Irrigations
- Liquids
- Lotions
- Lozenges
- Ointments
- Pastes
- Pellets
- Pills
- Powders
- Rinses
- Shampoos
- Soaps
- Solutions
- Sprays
- Strips
- Suppositories
- Suspensions
- Systems
- Tablets

Tests to ensure compliance with *USP* standards for dosage form performance fall into one of the following areas.

### Dose Uniformity

(See also *Uniformity of Dosage Units* ⟨905⟩.) Consistency in dosing for a patient or consumer requires that the variation in the drug substance content of each dosage unit be accurately controlled throughout the manufactured batch or compounded lot of drug product. Uniformity of dosage units typically is demonstrated by one of two procedures: content uniformity or weight variation. The procedure for content uniformity requires the appropriate assay of the drug substance content of individual units. The procedure for weight variation uses the weight of the individual units to estimate their content. Weight variation may be used where the underlying distribution of the drug substance in the blend is presumed to be uniform and well-controlled, as in solutions. In such cases, the content of the drug substance may be adequately estimated by the net weight. Content uniformity does not rely on the assumption of blend uniformity and can be applied in all cases. Successful development and manufacture of dosage forms requires careful evaluation of the drug substance particle or droplet size, incorporation techniques, and excipient properties.

### Stability

Drug product stability involves the evaluation of chemical stability, physical stability, and performance over time. The chemical stability of the drug substance in the dosage form matrix must support the expiration dating for the commercially prepared dosage forms and a beyond-use date for a compounded dosage form. Test procedures for potency must be stability indicating (see *Validation of Compendial Procedures* ⟨1225⟩). Degradation products should be quantified. In the case of dispersed or emulsified systems, consideration must be given to the potential for settling or separation of the formulation components. Any physical changes to the dosage form must be easily reversed (e.g., by shaking) prior to dosing or administration. For tablets, capsules, oral suspensions, and implants, in vitro release test procedures such as dissolution and disintegration provide a measure of continuing consistency in performance over time (see *Dissolution* ⟨711⟩, *Disintegration* ⟨701⟩, and *Drug Release* ⟨724⟩).

[1] *Nomenclature Guidelines*, http://www.usp.org/health-quality-safety/compendial-nomenclature.
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## Bioavailability

(See also *In Vitro and In Vivo Evaluation of Dosage Forms* ⟨1088⟩ and *Assessment of Solid Oral Drug Product Performance and Interchangeability, Bioavailability, Bioequivalence, and Dissolution* ⟨1090⟩.) Bioavailability is influenced by factors such as the method of manufacture or compounding, particle size, crystal form (polymorph) of the drug substance, the properties of the excipients used to formulate the dosage form, and physical changes as the drug product ages. Assurance of consistency in bioavailability over time (bioequivalence) requires close attention to all aspects of the production (or compounding) and testing of the dosage form. With proper justification, in vitro release testing (e.g., disintegration and dissolution) may be used as a surrogate to demonstrate consistent availability of the drug substance from the formulated dosage.

## Release Profile

Two principal categories of drug release are recognized: immediate-release and modified-release.

"Immediate-release" is observed when no deliberate effort has been made to modify the drug substance release profile. For example, capsules and tablets are considered immediate-release even if a disintegrating agent or a lubricant has been used.

"Modified-release" is a term used when the rate and/or time of release of the drug substance is altered as compared to what would be observed or anticipated for an immediate-release product. Two modified-release profiles, delayed-release and extended-release, are recognized. The term "modified-release" is not used for official article titles.

"Delayed-release" is used when deliberate formulation achieves a delay in the release of the drug substance for some period of time after initial administration. For oral products, expressions such as "enteric-coated" or "gastro-resistant" also have been used where release of the drug substance is prevented in the gastric environment but promoted in the intestinal environment. However, the term "delayed-release" is used for official article titles.

"Extended-release" is used when the deliberate formulation achieves prolongation of drug substance release compared to that observed or anticipated for an immediate-release dosage form. Expressions such as "prolonged-release", "repeat-action", "controlled-release", "long-acting", and "sustained-release" also have been used to describe such dosage forms. However, the term "extended-release" is used for official article titles.

The *Nomenclature Guidelines*[1] should be consulted for naming conventions for products with a single drug substance or for products with a combination of more than one drug substance displaying the combination of release profiles of immediate-release and extended-release, immediate-release and delayed-release, or extended-release and delayed-release.

## Manufacture

Although detailed instructions about the manufacture of any of these dosage forms are beyond the scope of this general information chapter, general manufacturing principles have been included.[3] Information relative to extemporaneous compounding of dosage forms can be found in *Pharmaceutical Compounding—Nonsterile Preparations* ⟨795⟩ and *Pharmaceutical Compounding—Sterile Preparations* ⟨797⟩.

## Route of Administration

The primary routes of administration for pharmaceutical dosage forms can be defined as parenteral (see ⟨1⟩), gastrointestinal (see ⟨2⟩), topical (see ⟨3⟩), mucosal (see ⟨4⟩), and inhalation (see ⟨5⟩). Each has subcategories as needed. Many tests used to ensure quality generally are applied across all of the administration routes, but some tests are specific for individual routes. For example, products intended for injection must be evaluated using *Sterility Tests* ⟨71⟩, *Bacterial Endotoxins Test* ⟨85⟩, or *Pyrogen Test* ⟨151⟩, and the manufacturing process (and sterilization technique) employed for parenterals (by injection) should ensure compliance with these tests. Tests for particulate matter may be required for certain dosage forms depending on the route of administration (e.g., by injection—*Particulate Matter in Injections* ⟨788⟩, mucosal—*Particulate Matter in Ophthalmic Solutions* ⟨789⟩, ▲or inhalation—⟨5⟩▲ (USP 1-May-2021)). Additionally, dosage forms intended for the inhalation route of administration must be monitored for particle size and spray pattern (for a metered-dose inhaler or dry powder inhaler) and droplet size (for nasal sprays). Further information regarding administration routes and suggested testing can be found in *Chapter Charts*, *Charts 4a*, *4b*, *5*, *6*, *7*, *8*, *10a*, *10b*, and *13*.

An appropriate manufacturing process and testing regimen helps ensure that a dosage form can meet the appropriate quality attributes for the intended route of administration.

## Packaging and Storage

Suitable packaging is determined for each product. For additional information about meeting packaging requirements listed in the individual labeling, refer to *Packaging and Storage Requirements* ⟨659⟩, *Containers—Performance Testing* ⟨671⟩, and *Good Repackaging Practices* ⟨1178⟩. Product labeling must specify storage requirements that describe environmental conditions, limitations, and restrictions. For instance, exposure to excessive temperature, humidity, and light can influence the ability of the packaging to protect the product.

## Labeling Statements

Some dosage forms or articles have mandatory labeling statements that are given in the *Code of Federal Regulations* (e.g., 21 CFR §201.320 and 21 CFR §369.21). The text of 21 CFR should be consulted to determine the current recommendations.

[3] The terms "manufacture" and "preparation" are used interchangeably in this general chapter.

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# PRODUCT QUALITY TESTS, GENERAL

International Council for Harmonisation (ICH) Guidance Q6A (available at www.ich.org) recommends specifications (list of tests, references to analytical procedures, and acceptance criteria) to ensure that drug products are safe and effective at the time of release and over their shelf life. Tests that are universally applied to ensure safety, efficacy, strength, quality, and purity include description, identification, assay, and impurities.

## Description

The *Definition* section (see *General Notices, 4.10 Monographs*) in a *USP* monograph describes the drug product and specifies the range of acceptable assayed content of the drug substance(s) present in the dosage form. For certain products, the *Definition* includes any relevant additional information, such as the presence or absence of other components, excipients, or adjuvants, and cautionary statements on toxicity and stability. While appearance information to aid in identification is used in a regulatory submission (e.g., a qualitative description of size, shape, color, etc.) it is typically not required as part of a *USP* monograph. This information is drug product specific.

## Identification

Identification tests are discussed in *General Notices, 5.40 Identification*. Identification tests should establish the identity of the drug substance(s) present in the drug product and should discriminate between compounds of closely related structure that are likely to be present. Identification tests should be specific for the drug substance(s). For example, the infrared absorption spectrum is often used (see *Mid-Infrared Spectroscopy* ⟨854⟩ and *Spectroscopic Identification Tests* ⟨197⟩). If no suitable infrared spectrum can be obtained, other analytical methods can be used. Near-infrared (NIR) or Raman spectrophotometric methods also could be acceptable as the sole identification method of the drug product formulation (see *Near-Infrared Spectroscopy—Theory and Practice* ⟨1856⟩ and *Raman Spectroscopy—Theory and Practice* ⟨1858⟩). Identification by a chromatographic retention time from a single procedure is not regarded as specific. The use of retention times from two chromatographic procedures for which the separation is based on different principles or a combination of tests in a single procedure can be acceptable (see *Chromatography* ⟨621⟩ and *Thin-Layer Chromatographic Identification Test* ⟨201⟩).

## Assay

A specific and stability-indicating test should be used to determine the strength (drug substance content) of the drug product. Some examples of these procedures are *Antibiotics—Microbial Assays* ⟨81⟩, ⟨621⟩, or *Assay for Steroids* ⟨351⟩. In cases when the use of a nonspecific assay is justified (e.g., *Titrimetry* ⟨541⟩), other supporting analytical procedures should be used to achieve specificity. When evidence of excipient interference with a nonspecific assay exists, a procedure with demonstrated specificity should be used.

## Impurities

Process impurities, synthetic byproducts, and other inorganic and organic impurities may be present in the drug substance and excipients used in the manufacture of the drug product. These impurities are evaluated by tests in the drug substance and excipients monographs. Impurities arising from degradation of the drug substance or from the drug-product manufacturing process should be monitored. *Residual Solvents* ⟨467⟩ is applied to all products where relevant.

▲*Elemental Impurities—Limits* ⟨232⟩ is applied to all products where relevant.▲ (USP 1-May-2021)

In addition to the universal tests listed, the following tests may be considered on a case-by-case basis.

## Physicochemical Properties

Examples include *pH* ⟨791⟩, *Viscosity—Capillary Methods* ⟨911⟩ or *Viscosity—Rotational Methods* ⟨912⟩, and *Specific Gravity* ⟨841⟩.

## Particle Size

For some dosage forms, particle size can have a significant effect on dissolution rates, bioavailability, therapeutic outcome, and stability. Procedures such as those found in *Inhalation and Nasal Drug Products: Aerosols, Sprays, and Powders—Performance Quality Tests* ⟨601⟩ and *Particle Size Distribution Estimation by Analytical Sieving* ⟨786⟩ could be used.

## Uniformity of Dosage Units

See the discussion of *Dose Uniformity* in the *General Considerations* section.

## Water Content

A test for water content is included when appropriate (see *Water Determination* ⟨921⟩).

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## Microbial Limits

The type of microbial test(s) and acceptance criteria are based on the nature of the nonsterile drug product, method of manufacture, and the route of administration (see *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* ⟨61⟩, *Microbiological Examination of Nonsterile Products: Tests for Specified Microorganisms* ⟨62⟩, and *Microbiological Examination of Nonsterile Products: Acceptance Criteria for Pharmaceutical Preparations and Substances for Pharmaceutical Use* ⟨1111⟩).

## Antimicrobial Preservative Content

Acceptance criteria for preservative content in multidose products should be established. They are based on the levels of antimicrobial preservative necessary to maintain the product's microbiological quality at all stages throughout its proposed usage and shelf life (see *Antimicrobial Effectiveness Testing* ⟨51⟩).

## Antioxidant Content

If antioxidants are present in the drug product, tests of their content should be performed to maintain the product's quality at all stages throughout its proposed usage and shelf life.

## Sterility

Depending on the route of administration (e.g., ophthalmic preparations, implants, aqueous-based preparations for oral inhalation, and injections) sterility of the product is demonstrated as appropriate (see ⟨71⟩).

## Dissolution

A test to measure the release of the drug substance(s) from the drug product normally is included for dosage forms such as tablets, capsules, suspensions, granules for suspensions, implants, transdermal delivery systems (TDS), and medicated chewing gums. Single-point measurements typically are used for immediate-release dosage forms. For modified-release dosage forms, appropriate test conditions and sampling procedures are established as needed (see ⟨711⟩ and ⟨724⟩). In some cases, dissolution testing may be replaced by disintegration testing (see ⟨701⟩).

## Breaking Force and Friability

These parameters are evaluated as in-process controls. Acceptance criteria depend on packaging, supply chain, and intended use (see *Tablet Friability* ⟨1216⟩ and *Tablet Breaking Force* ⟨1217⟩).

## Leachables

When evidence exists that leachables from the container–closure systems (e.g., rubber stopper, cap liner, or plastic bottle) have an impact on the safety or efficacy of the drug product, a test is included to evaluate the presence of leachables.

## Other Tests

Depending on the type and composition of the dosage form, other tests such as alcohol content, redispersibility, particle size distribution, rheological properties, reconstitution time, endotoxins/pyrogens, particulate matter, functionality testing of delivery systems, delivered dose uniformity, viscosity, and osmolarity may be necessary.

***Change to read:***

# DOSAGE FORMS

## Aerosols

Aerosols are dosage forms packaged under pressure and contain therapeutic agent(s) and ▲propellant(s)▲ (USP 1-May-2021) that are released upon actuation of an appropriate valve system. Upon actuation of the valve system, the drug substance is released as a plume of fine particles or droplets. Only 1 dose is released from the preparation upon actuation of a metered valve. In the case of topical products and depending on the nature of the drug substance and the conditions being treated, actuation of the valve may result in a metered release of a controlled amount of the formulation or the continuous release of the formulation as long as the valve is depressed.

The aerosol dosage form refers only to those products packaged under pressure that release a fine mist of particles or droplets when actuated (see *Glossary*). Other products that produce dispersions of fine droplets or particles will be covered in subsequent sections (e.g., ▲*Sprays* and *Powders*▲ (USP 1-May-2021)).

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### TYPICAL COMPONENTS

Typical ▲major▲ (USP 1-May-2021) components of aerosols are the formulation containing one or more drug substance(s) and ▲propellant(s),▲ (USP 1-May-2021) the container, the valve, and the actuator. Each component plays a role in determining various characteristics of the emitted plume, such as droplet or particle size distribution, uniformity of delivery of the therapeutic agent, delivery rate, and plume velocity and geometry. The metering valve and actuator act in tandem to generate the plume of droplets or particles. The metering valve delivers an accurate volume of the pressurized liquid formulation from the container. The actuator directs the metered volume to a small orifice that is open to the atmosphere. Upon actuation, the formulation is forced through the opening, forming the fine mist of ▲droplets/▲ (USP 1-May-2021)particles that are directed to the site of administration.

Aerosol preparations may consist of either a two-phase (gas and liquid) or a three-phase (gas, liquid, and solid or liquid) formulation. The two-phase formulation consists of drug substance(s) dissolved in liquefied propellant. Co-solvents such as alcohol may be added to enhance the solubility of the drug substance(s). Three-phase inhalation and nasal aerosol systems consist of suspended drug substance(s) in propellant(s), co-solvents, and potentially other suitable excipients. The suspension or emulsion of the finely divided drug substance is typically dispersed in the liquid propellant with the aid of suitable biocompatible surfactants or other excipients.

Propellants for aerosol formulations are typically low molecular weight hydrofluorocarbons or hydrocarbons that are liquid when constrained in the container, exhibit a suitable vapor pressure at room temperature, and are biocompatible and nonirritating. Compressed gases do not supply a constant pressure over use and typically are not used as propellants.

Metal containers can withstand the vapor pressure produced by the propellant. Excess formulation may be added to the container to ensure that the full number of labeled doses can be accurately administered. The container and closure must be able to withstand the pressures anticipated under normal use conditions as well as when the system is exposed to elevated temperatures.

### TYPES OF AEROSOL DOSAGE FORMS

Aerosol dosage forms can be delivered via various routes. The container, actuator, and metering valve, as well as the formulation, are designed to target the site of administration.

Inhalation aerosols, commonly known as metered-dose inhalers (MDIs), are intended to produce fine particles or droplets for inhalation through the mouth and deposition in the pulmonary tree. The design of the delivery system is intended to release measured mass and appropriate quality of the active substance with each actuation.

Nasal aerosols, commonly known as nasal MDIs, produce fine particles or droplets for delivery through the nasal vestibule and deposition in the nasal cavity. Each actuation of the valve releases a measured mass of the drug substance with appropriate quality characteristics.

Lingual aerosols are intended to produce fine particles or droplets for deposition on the surface of the tongue. The design of the delivery system releases 1 dose with each actuation.

Topical aerosols produce fine particles or droplets for application to the skin.

### LABELING FOR PROPER USE

Refer to 21 CFR §201.320 and 21 CFR §369.21.

## Capsules

Capsules are solid dosage forms in which the drug substance and/or excipients are enclosed within a soluble container or shell or coated on the capsule shell. The shells may be composed of two pieces (a body and a cap), or they may be composed of a single piece. Two-piece capsules are commonly referred to as hard-shell capsules, and one-piece capsules are often referred to as soft-shell capsules. This two-piece and one-piece capsule distinction, although imprecise, reflects differing levels of plasticizers in the two compositions and the fact that one-piece capsules typically are more pliable than two-piece capsules.

The shells of capsules are usually made from gelatin. However, they also may be made from cellulose polymers ▲(e.g., hypromellose)▲ (USP 1-May-2021) or other suitable material. Most capsules are designed for oral administration. When no deliberate effort has been made to modify the drug substance release rate, capsules are referred to as immediate-release.

### TWO-PIECE OR HARD-SHELL CAPSULES

Two-piece capsules consist of two telescoping cap and body pieces in a range of standard sizes.

### ONE-PIECE OR SOFT-SHELL CAPSULES

One-piece capsules typically are used to deliver a drug substance as a solution or suspension. Liquid formulations placed into one-piece capsules may offer advantages by comparison with dry-filled capsules and tablets in achieving content uniformity of potent drug substance(s) or acceptable dissolution of drug substance(s) with poor aqueous solubility. Because the contact between the shell wall and its liquid contents is more intimate than in dry-filled capsules, undesired interactions may be more likely to occur (including gelatin cross-linking and pellicle formation).

### MODIFIED-RELEASE CAPSULES

The release of drug substance(s) from capsules can be modified in several ways. Two categories of modified-release capsule formulations are recognized by USP.

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**Delayed-release capsules:** Capsules are sometimes formulated to include enteric-coated granules to protect acid-labile drug substances from the gastric environment or to prevent adverse events such as irritation. Enteric-coated multiparticulate capsule dosage forms may reduce variability in bioavailability associated with gastric emptying times for larger particles (i.e., tablets) and may minimize the likelihood of a therapeutic failure when coating defects occur during manufacturing. Alternatively, a coating may be applied to the capsule shell to achieve delayed release of the contents.

**Extended-release capsules:** Extended-release capsules are formulated in such a manner as to make the contained drug substance available over an extended period of time following ingestion. Requirements for dissolution (see ⟨711⟩) are typically specified in the individual monograph.

Methods for modifying drug substance release from capsules include coating the filled capsule shells or the contents, in the case of dry-filled capsules.

### PREPARATION

**Two-piece capsules:** Two-piece gelatin capsules are usually formed from blends of gelatins that have relatively high gel strength in order to optimize shell clarity and toughness or from hypromellose. They may also contain colorants such as Drug & Cosmetic (D&C) and Food, Drug, & Cosmetic (FD&C) dyes[4] or various pigments, opaquing agents such as titanium dioxide, dispersing agents, plasticizers, and preservatives. Gelatin capsule shells normally contain between 12% and 16% water.

The shells are manufactured in one set of operations and later filled in a separate manufacturing process. Two-piece shell capsules are made by a process that involves dipping shaped pins into gelatin or hypromellose solutions, followed by drying, cutting, and joining steps.

Powder formulations for two-piece gelatin capsules generally consist of the drug substance and at least one excipient. Both the formulation and the method of filling can affect release of the drug substance. In the filling operation, the body and cap of the shell are separated before filling. Following the filling operation, the machinery rejoins the body and cap and ensures satisfactory closure of the capsule by exerting appropriate force on the two pieces. The joined capsules can be sealed after filling by a band at the joint of the body and cap or by a designed locking joint between the cap and body. In compounding prescription practice, two-piece capsules may be hand-filled. This permits the prescriber the choice of selecting either a single drug substance or a combination of drug substances at the exact dose level considered best for an individual patient.

**One-piece capsules:** One-piece capsules are formed, filled, and sealed in a single process on the same machine and are available in a wide variety of sizes, shapes, and colors. The most common type of one-piece capsule is that produced by a rotary die process that results in a capsule with a seam. The soft gelatin shell is somewhat thicker than that of two-piece capsules and is plasticized by the addition of polyols such as glycerin, sorbitol, or other suitable materials. The ratio of the plasticizer to the gelatin can be varied to change the flexibility of the shell depending on the nature of the fill material, its intended usage, or environmental conditions.

In most cases, one-piece capsules are filled with liquids. Typically, drug substances are dissolved or suspended in a liquid vehicle. Classically, an oleaginous vehicle such as a vegetable oil was used. However, nonaqueous, water-miscible liquid vehicles such as the lower molecular weight polyethylene glycols are now more common. The physicochemical properties of the vehicle can be chosen to ensure stability of the drug substance as well as to influence the release profile from the capsule shell.

## Creams

(See *Emulsions*.)

## Emulsions

An emulsion is a dispersed colloidal system consisting of two immiscible liquid phases generally stabilized with one or more suitable agents.

Typical pharmaceutical emulsions are prepared from immiscible aqueous and organic (oil) liquids. Complex multiple-phase systems may exist in an emulsion. Whether the organic or the aqueous phase is the dispersed phase depends on the volumes of the two phases, the emulsifier chosen, and the method of preparation. When an oil phase is dispersed in an aqueous phase, the emulsion is termed an oil-in-water (O/W) emulsion and water is referred to as the continuous phase. When water is dispersed in oil, the emulsion is referred to as a water-in-oil (W/O) emulsion. Emulsions have dispersed phases typically ranging from 0.1 to 100 µm. Emulsions are opaque while microemulsions are usually transparent or translucent. Microemulsions have dispersed phases less than 0.1 µm.

Emulsions may exhibit three types of instability: flocculation, creaming, and coalescence. Flocculation describes the process by which the dispersed phase comes out of suspension in the form of flakes. Coalescence is another form of instability—small droplets within the media continuously combine to form progressively larger droplets. Emulsions can also undergo creaming, where one of the phases migrates to the top (or the bottom, depending on the relative densities of the two phases) of the emulsion. To prevent flocculation, creaming, and coalescence of the emulsions, manufacturers commonly add surfactants, pH-modifying agents, or emulsifying agents to increase the stability of emulsions so that the emulsion does not change significantly with time.

[4] In 1960 Congress enacted the Color Additive Amendments, requiring the US FDA to regulate dyes, pigments, or other coloring agents in foods, drugs, and cosmetics separately from food additives. Under the law, color additives are deemed unsafe unless they are used in compliance with FDA regulations. The law provides a framework for the listing and certification of color additives. See FDCA §721; see FDA regulations at 21 CFR Part 70. Colors also must be listed in pertinent FDA regulations for specific uses; the list of color additives for drugs that are exempt from certification is published at 21 CFR Part 73, Subpart B. FDA also conducts a certification program for batches of color additives that are required to be certified before sale; see 21 CFR Part 74 (Subpart B re: drugs). Regulations regarding certification procedures, general specifications, and the listing of certified provisionally listed colors are at 21 CFR Part 80. FDA maintains a color additives website with links to various legal and regulatory resources at: http://www.fda.gov; search by document title.

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Emulsions are widely used as pharmaceutical dosage forms. Oral emulsions have been prepared to improve taste, solubility, stability, or bioavailability. Emulsions for topical administration are referred to as creams, lotions, and sometimes ointments. Parenteral emulsions have been used for anesthetics and parenteral nutrition and to deliver poorly water-soluble drugs.

### CREAMS

Creams are semisolid emulsion dosage forms. They often contain more than 20% water and volatiles, and/or typically contain less than 50% hydrocarbons, waxes, or polyols as the vehicle for the drug substance. Creams are generally intended for external application to the skin or to the mucous membranes. Creams have a relatively soft, spreadable consistency and can be formulated as either a W/O emulsion (e.g., *Cold Cream* or *Fatty Cream* as in the *European Pharmacopoeia*) or as an O/W emulsion (e.g., *Betamethasone Valerate Cream*). Creams are generally described as either nonwashable or washable, reflecting the fact that an emulsion with an aqueous external continuous phase is more easily removed than one with a nonaqueous external phase (W/O emulsion).

### LOTIONS

Lotions are an emulsified liquid dosage form intended for external application to the skin. Historically, some topical suspensions such as calamine lotion have been called lotions but that nomenclature is not currently preferred. Lotions share many characteristics with creams. The distinguishing factor is that they are more fluid than semisolid and thus pourable. Due to their fluid character, lotions are more easily applied to large skin surfaces than semisolid preparations. Lotions may contain antimicrobial agents as preservatives.

### INJECTABLE EMULSIONS

Injectable emulsions are sterile liquid dosage forms of drug substances dissolved or dispersed in a suitable emulsion medium. Injectable emulsions are for parenteral administration of poorly water-soluble drugs.

### OINTMENTS

Ointments are sometimes semisolid emulsion dosage forms (see *Dosage Forms, Ointments*).

### PREPARATION

▲▲ (USP 1-May-2021)

**Creams:** Creams may be formulated from a variety of oils, both mineral and vegetable, and from fatty alcohols, fatty acids, and fatty esters. Emulsifying agents include nonionic surfactants, detergents, and soaps. Soaps are usually formed in situ during the preparation of creams from a fatty acid in the oil phase hydrolyzed by a base dissolved in the aqueous phase.

Preparation usually involves separating the formula components into two portions: lipid and aqueous. The lipid portion contains all water-insoluble components and the aqueous portion contains the water-soluble components. Both phases are heated to a temperature above the melting point of the highest melting component. The phases are then mixed and the mixture is stirred until reaching ambient temperature or until the mixture has congealed. Mixing is generally continued during the cooling process to promote uniformity. Traditionally, the aqueous phase is added to the lipid phase, but comparable results have been obtained with the reverse procedure. High-shear homogenization may be employed to reduce particle or droplet size and to improve the physical stability of the resultant dosage form.

The drug substance(s) can be added to the phase in which it is soluble at the beginning of the manufacturing process, or it can be added after the cream is prepared by a suitable dispersion process such as levigation or milling with a roller mill. Creams usually require the addition of a preservative(s) unless they are compounded immediately prior to use and intended to be consumed in a relatively short period of time.

**Lotions:** Lotions are usually prepared by dissolving or dispersing the drug substance into the more appropriate phase (oil or water), adding the appropriate emulsifying or suspending agents, and mixing the oil and water phases to form a uniform fluid emulsion.

**Injectable emulsions:** Chapter ⟨1⟩ provides guidance on sterile preparations. Emulsions intended for parenteral administration can be formulated using the same principles as creams and lotions. The formulation should be designed for ease of administration. The particle size of the dispersed phase can vary by route of administration. For example, emulsions intended for intravenous administration should comply with *Globule Size Distribution in Lipid Injectable Emulsions* ⟨729⟩. The procedure to assure sterility should be validated by media fills. Preservatives are generally not used in injectable emulsions.

**Ointments:** (See *Dosage Forms, Ointments*.)

## Films

Films are thin sheets that are placed in the oral cavity. They contain one or more layers. A layer may or may not contain the drug substance. Typically, these thin sheets are formed by casting or extrusion that results in a dispersion of the components through the film. Films are classified by the site of application. “Oral films” can be formulated to deliver medication to the mouth such as oral hygiene products or to deliver medication to the gastrointestinal tract for absorption. “Buccal films” and “sublingual films” are formulated to facilitate absorption through the proximal mucosal membranes avoiding first pass metabolism or degradation in the gastrointestinal tract and providing a quick onset of action.

Films can be formulated with edible polymers such as pullulan or with water-soluble polymers such as modified cellulose, edible gums, and copolymers. The dissolution rate of the film is controlled to facilitate incorporation of the medication into saliva or for absorption by the proximal mucosa. These films must be substantial enough to maintain their integrity during

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manufacture and packaging, and permit handling by the patient. Because of the rapid dissolution, taste and mouth feel are important considerations.

## Foams

Foams are dispersions of gas in a liquid or solid continuous phase wherein the liquid or solid contains the drug substance and suitable excipients. Typical excipients intended for foam dosage forms include surfactants to ensure distribution of the gas/propellant in the formulation, aqueous or nonaqueous vehicles, and propellants (for pressurized systems). Foams are produced by mechanical means or via interaction of propellant gas and the formulation under pressure. Foams dispensed from nonpressurized containers use mechanical force to mix the formulation and air resulting in foam generation. Foams dispensed from pressurized containers use the ▲propellant(s)▲ (USP 1-May-2021) present in the gas phase to increase pressure inside the container. When the nozzle of the actuator is opened, the liquid phase is pushed out through specific actuators resulting in foam generation.

▲Foams are primarily intended for application to the skin or mucous membranes. Foams can also be delivered by the injection route. Foams can be formulated to quickly break down into liquid or to remain as a foam to ensure prolonged contact.▲ (USP 1-May-2021)

Medicated foams intended to treat severely injured skin, ▲▲ (USP 1-May-2021) open wounds, ▲or administered by injection▲ (USP 1-May-2021) must be sterile.

### PREPARATION

A foam may contain one or more drug substances, surfactants, and aqueous or nonaqueous liquids, and is produced with or without the aid of propellants. When a propellant is not used, mechanical work is required to generate the foam. If the propellant is in the internal (discontinuous) phase, a stable foam is discharged. If the propellant is in the external (continuous) phase, a quick-breaking foam is discharged.

## Gases

Medical gases are products that are administered directly as a gas. A medical gas has a direct pharmacological action or acts as a diluent for another medical gas. Gases used as excipients for administration of aerosol products, as adjuvants in packaging, or produced by other dosage forms, are not included in this definition.

### COMPONENTS

Medical gases may be single components or defined mixtures of components. Mixtures also can be extemporaneously prepared at the point of use.

### ADMINISTRATION

Medical gases may be administered to the patient using several methods: nasal cannulas, face masks, atmospheric tents, and endotracheal tubes for the pulmonary route; hyperbaric chambers for the pulmonary and topical routes of administration; jetted tubes that are directed at dental tissue to promote drying in preparation for fillings and crowns; tubes for expanding the intestines to facilitate medical imaging during colonoscopy; tubes for expanding the pelvis via transuterine inflation in preparation for fallopian tubal ligation; and tubes for expanding angioplasty devices. The dose of medical gas is typically metered by a volume rate of flow under ambient temperature and pressure conditions. Administration of a highly compressed gas generally requires a regulator to decrease the pressure, a variable-volume flow controller, and suitable tubing to conduct the gas to the patient. For pulmonary administration, the gas flow will be directed to the nose or mouth by a suitable device or into the trachea through a mechanical ventilator. When medical gases are administered chronically, provision for humidification is common. Care should be exercised to avoid microbial contamination.

### SPECIAL CONSIDERATIONS

The container and system fittings should be appropriate for the medical gas. Adaptors should not be used to connect containers to patient-use supply system piping or equipment. Large quantities of gases such as oxygen or nitrogen can be stored in the liquid state in a cryogenic container and converted into a gas, as needed, by evaporation. Additional rules concerning the construction and use of cryogenic containers are promulgated by governmental agencies (e.g., U.S. Department of Commerce).

Containers, tubing, and administration masks employed for gases containing oxygen are free of any compound that would be sensitive to oxidation or that would be irritating to the respiratory tract.

A significant fraction of the dose of a medical gas may be released into the general vicinity of the patient due to incomplete absorption. Adequate ventilation may be necessary to protect health care workers and others from exposure to the gas (e.g., nitrous oxide).

## Gels

A gel is solid or semisolid. Gels can be classified in two groups, chemical and physical gels. Chemical gels are usually covalently cross-linked gels, while physical gels consist of small molecules or molecular chains that are physically cross-linked into networks, or solutions, or colloidal dispersions that are stiffened by a gelling agent. Typically, gels hold their form being self-supporting.

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Some gels may exhibit a range of behavior under mechanical forces. Gels may be thixotropic, forming semisolids on standing and becoming less viscous on agitation. Like emulsions, gels can be characterized as having a continuous phase as well as a dispersed phase. A variety of routes are available for gel administration such as topical, mucosal, or oral. In veterinary medicine, gels also can be administered via ▲the intramammary route.▲ (USP 1-May-2021)

Gels may consist of a network of small discrete particles (e.g., *Aluminum Hydroxide Gel*, *Bentonite Magma*, or *Psyllium Hemicellulose*). Because these gels may be thixotropic, forming semisolids on standing and becoming less viscous on agitation, they should be shaken before use to ensure homogeneity and should be so labeled.

Gels can consist of organic macromolecules uniformly distributed throughout a liquid in such a manner that no apparent boundaries exist between the dispersed macromolecules and the liquid continuous phase. These gels may be made from natural or synthetic macromolecules (e.g., carbomer, hypromellose, or starch) or natural gums (e.g., tragacanth). Although these gels are commonly aqueous based, alcohols and oils may be used as the continuous phase.

Chewable gels are used to deliver drug substances or dietary supplements via the oral route. In addition to drug substances(s) or dietary supplements, chewable gels can consist of all or some of the following components: gelling agent(s), sugars, water, sweeteners, and flavoring agents. The sweeteners and flavoring are intended to enhance patient acceptance and mask the taste of the delivered labeled drug substance or dietary supplement. Chewable gels maintain their molded shape, are elastic, and yield to mastication. They are intended to be chewed before swallowing. Chewable gels are also known as "gummies" in the confectionary and dietary supplement industries but that term is not used in official article titles.

### PREPARATION

Gels may be formed by dispersing the gelling agent in the continuous phase (e.g., by heating starch), by cross-linking the dispersed phase gelling agent, by changing the pH (as for *Carbomer Copolymer*), or by reducing the continuous phase by heat or vacuum (as for gels formed with sucrose).

Care should be taken to ensure uniformity of the drug substances by dispersing them by vigorous mixing or milling, or by shaking if the preparation is less viscous.

Chewable gels are formulated with one or more gelling agents (such as gelatin or starch), sugars (such as sucrose, fructose, or corn syrup), flavoring agents, sweeteners, colorants, and water. The ingredients are blended and heated to form a viscous solution that is poured into molds (e.g., corn starch molds). After cooling, the individual units are separated from the molds.

## Granules

Granules are solid dosage forms that are composed of agglomerations of smaller particles. These multicomponent compositions are prepared for oral administration and are used to facilitate flexible dosing regimens as granules or as suspensions, address stability challenges, allow taste masking, or facilitate flexibility in administration (for instance, to pediatric patients, geriatric patients, or animals). Granular dosage forms may be formulated for direct oral administration and may facilitate compounding of multiple drug substances by allowing compounding pharmacists to blend various granular compositions in the retail or hospital pharmacy. More commonly, granules are reconstituted to a suspension by the addition of water or a supplied liquid diluent immediately prior to delivery to the patient. Effervescent granules are formulated to liberate gas (carbon dioxide) upon addition of water. Common examples of effervescent granules include antacid and potassium supplementation preparations. Common therapeutic classes formulated as granule dosage forms include antibiotics, certain laxatives (such as senna extract products), electrolytes, and various cough and cold remedies that contain multiple drug substances.

### PREPARATION

Granules are often the precursors used in tablet compression or capsule filling. Although this application represents a pharmaceutical intermediate and not a final dosage form, numerous commercial products are based on granules. In the typical manufacture of granules, the drug substance(s) is blended with excipients (processing aids) and wetted with an appropriate pharmaceutical binding solution, solvent, or blend of solvents to promote agglomeration. This composition is dried and sized to yield the desired material properties.

Frequently, granules are used because the drug substance is unstable in aqueous environments and cannot be exposed to water for periods sufficient to accommodate manufacture, storage, and distribution in a suspension. Preparation of the liquid dosage form from the granules immediately prior to dispensing allows acceptable stability for the duration of use. Granules manufactured for this purpose are packaged in quantities sufficient for a limited time period—usually one course of therapy that typically does not exceed 2 weeks. In addition to the drug substances, other ingredients may be added to ensure acceptable stability (e.g., buffers, antioxidants, or chelating agents) or to provide color, sweetness, and flavor; and for suspensions, to provide acceptable viscosity to ensure adequate suspension of the particulate to enable uniform dosing.

Effervescent granules are typically formulated from sodium or potassium bicarbonate and an acid such as citric or tartaric acid. To prevent untimely generation of carbon dioxide, manufacturers should take special precautions to limit residual water in the product due to manufacture and to select packaging that protects the product from moisture. The manufacture of effervescent granules can require specialized facilities designed to maintain very low humidity (approximately 10% relative humidity). Effervescent powder mixtures are purposely formed into relatively ▲coarse▲ (USP 1-May-2021) granules to reduce the rate of dissolution and provide a more controlled effervescence.

Reconstitution of granules must ensure complete wetting of all ingredients and sufficient time and agitation to allow the soluble components to dissolve. Specific instructions for reconstitution provided by the manufacturer should be carefully followed.

Reconstituted suspensions should be thoroughly mixed or shaken before use to resuspend the dispersed particulates. This is especially true of suspension preparations dosed from multiple-dose containers. For particularly viscous suspensions prone to

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air entrapment, instructions may advise the user how to shake the preparation to resuspend settled particulates while minimizing air entrapment.

For granules reconstituted to form suspensions for oral administration, acceptable suspension of the particulate phase depends on the particle size of the dispersed phase as well as the viscosity of the vehicle. Temperature can influence the viscosity, which influences suspension properties and the ease of removal of the dose from the bottles. In addition, temperature cycling can lead to changes in the particle size of the dispersed phase via Ostwald ripening. Thus, clear instructions should be provided regarding the appropriate storage temperature for the product.

## Gums

Medicated gum is a pliable dosage form that is designed to be chewed rather than swallowed. Medicated gums release the drug substance(s) into the saliva. Medicated gums can deliver therapeutic agents for local action in the mouth or for systemic absorption via the buccal or gastrointestinal routes (e.g., nicotine or aspirin). Most gums are manufactured using the conventional melting process derived from the confectionary industry or alternatively may be directly compressed from gum powder. Medicated gums are formulated from insoluble synthetic gum bases such as polyisoprene, polyisobutylene, isobutyleneisoprene copolymer, styrene butadiene rubber, polyvinyl acetate, polyethylene, ester gums, or polyterpenes. Plasticizers and softeners such as propylene glycol, glycerin, oleic acid, or processed vegetable oils are added to keep the gum base pliable and to aid in the incorporation of the drug substance(s), sweeteners, and flavoring agents. Sugars as well as artificial sweeteners and flavorings are incorporated to improve taste, and dyes may be used to enhance appearance. Some medicated gums are coated with magnesium stearate to reduce tackiness and improve handling during packaging. A preservative may be added.

### PREPARATION

**Melted gum:** The gum base is melted at a temperature of about 115° until it has the viscosity of thick syrup and, at that point, is filtered through a fine-mesh screen. This molten gum base is transferred to mixing tanks where the sweeteners, plasticizers, and typically the drug substance are added and mixed. Colorings, flavorings, and preservatives are added and mixed while the melted gum is cooling. The cooled mixture is shaped by extrusion or rolling and cutting. Dosage units of the desired shape and potency are packaged individually. Additional coatings such as powder coatings to reduce tackiness or film or sugar coatings may be added to improve taste or facilitate bulk packaging.

**Directly compressed gum:** The gum base is supplied in a free-flowing granular powder form. The powder gum base is then dry blended with sweeteners, flavors, the drug substance, and lubricant. The blend is then processed through a conventional tablet press and tableted into desired shapes. The resulting medicated gum tablets can be further coated with sugar or sugar-free excipients. These tablets can be packaged in blisters or bottles as needed.

### SPECIAL CONSIDERATIONS

Medicated gums are typically dispensed in unit-dose packaging. The patient instructions also may include a caution to avoid excessive heat.

## Implants

Implants are long-acting dosage forms that provide continuous release of the drug substance often for periods of months to years. They are administered by the parenteral route and are sterile. Some implants approved as animal drugs to be administered subcutaneously to the ears are not required to be sterile. Typically for systemic delivery, they may be placed subcutaneously, or for local delivery they can be placed in a specific region in the body (e.g., in the sinus, in an artery, in the eye, in the brain). Implants are usually administered by means of a suitable injector or by surgical procedure.

Polymer implants can be formed as a single-shaped mass such as a cylinder. The polymer matrix must be biocompatible (see *The Biocompatibility of Materials Used in Drug Containers, Medical Devices, and Implants* ⟨1031⟩), but it can be either bioabsorbable or nonbioabsorbable. Shaped polymer implants are administered by means of a suitable special injector. Release kinetics are typically not zero-order, but zero-order kinetics are possible. Drug substance release can be controlled by the diffusion of the drug substance from the bulk polymer matrix or by the properties of a rate-limiting polymeric membrane coating. Polymer implants are used to deliver potent small molecules like steroids (e.g., estradiol for cattle) and large molecules like peptides [e.g., luteinizing hormone-releasing hormone (LHRH)]. Example durations of drug substance release are 2 and 3 months for bioabsorbable implants and up to 3 years for nonbioabsorbable implants. An advantage of bioabsorbable implants is that they do not require removal after the release of all drug substance content. Nonbioabsorbable polymer implants can be removed before or after a drug substance release is complete or may be left in situ. An implant can have a tab with a hole in it to facilitate suturing it in place (e.g., for an intravitreal implant for local ocular delivery). Such implants may provide therapeutic release for periods as long as 2.5 years.

Drug substance-eluting stents combine the mechanical effect of the stent to maintain arterial patency with the prolonged pharmacologic effect of the incorporated drug substance (to reduce restenosis, inhibit clot formation, or combat infection). As an example, a metal stent can be coated with a nonbioabsorbable or bioabsorbable polymer-containing drug substance. The resultant coating is a polymeric matrix that controls the extended release of the drug substance.

### PREPARATION

Cylindrical polymeric implants are commonly made by melt extrusion of a blend of drug substance and polymer, resulting in a rod that is cut into shorter lengths. Polymer implants also can be made by injection molding. Still other implants are assembled from metal tubes and injection-molded plastic components.

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Sterility can be achieved by terminal sterilization or by employing aseptic manufacturing procedures.

## Injections

(See *Emulsions*, ▲*Foams*,▲ (USP 1-May-2021)*Powders*, *Solutions*, and *Suspensions* for information on injectable dosage forms.)
Injections are not treated as a dosage form in this chapter. Chapter ⟨1⟩ provides quality and other information about injectable products. Information on specific dosage form terminology can be found in the *Glossary*. For appropriate injection nomenclature, see *Nomenclature* ⟨1121⟩.

### EXCESS VOLUME IN INJECTIONS

Each container of an injection is filled with a volume in slight excess of the labeled "size" or the volume that is to be withdrawn. The excess volumes recommended in *Table 1* are usually sufficient to permit withdrawal and administration of the labeled volumes.

**Table 1**

| Labeled Size (mL) | Recommended Excess Volume | |
|---|---|---|
| | For Mobile Liquids (mL) | For Viscous Liquids (mL) |
| 0.5 | 0.10 | 0.12 |
| 1.0 | 0.10 | 0.15 |
| 2.0 | 0.15 | 0.25 |
| 5.0 | 0.30 | 0.50 |
| 10.0 | 0.50 | 0.70 |
| 20.0 | 0.60 | 0.90 |
| 30.0 | 0.80 | 1.20 |
| 50.0 or more | 2% | 3% |

## Inserts

Inserts are solid dosage forms that are inserted into a naturally occurring (nonsurgical) body cavity other than the mouth or rectum (see *Suppositories*). The drug substance in inserts is delivered for local or systemic action. Vaginal inserts are usually globular or oviform and weigh about 5 g each. Inserts intended to dissolve in vaginal secretions are usually made from water-soluble or water-miscible vehicles such as polyethylene glycol or glycerinated gelatin.

### PREPARATION

▲▲ (USP 1-May-2021) Inserts vary considerably in their preparation. Inserts may be molded (using technology similar to that used to prepare lozenges, suppositories, or plastics), compressed from powders (as in tableting), or formulated as special applications of capsules (soft gelatin capsules and hard gelatin capsules have been employed for extemporaneously compounded preparations). Inserts may be formulated to melt at body temperature or disintegrate upon insertion. Design of the dosage form should take into consideration the fluid volume available at the insertion site and minimize the potential to cause local irritation. Most inserts are formulated to ensure retention at the site of administration.

## Irrigations

(See *Solutions*.)

## Liquids

As a dosage form, a liquid consists of a pure chemical in its liquid state. Examples include mineral oil, isoflurane, and ether. This dosage form term is not applied to solutions.

## Lotions

(See *Emulsions*.)

## Lozenges

Lozenges are solid oral dosage forms that are designed to dissolve or disintegrate slowly in the mouth. They contain one or more drug substances that are slowly liberated from the typically flavored and sweetened base. They are frequently intended to provide local action in the oral cavity or the throat but also include those intended for systemic absorption after dissolution.

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The typical therapeutic categories of drug substances delivered in lozenges are antiseptics, analgesics, decongestants, antitussives, and antibiotics. Molded lozenges are called cough drops or pastilles but these terms are not used in official article titles. Lozenges prepared by compression or by stamping or cutting from a uniform bed of paste are sometimes known as troches (a term not used in official article titles). Compressed or stamped lozenges are often produced in a circular shape.

Lozenges can be made using sugars such as sucrose and dextrose, or can provide the benefits of a sugar-free formulation that is usually based on sorbitol or mannitol. Polyethylene glycols and hypromellose are sometimes included to slow the rate of dissolution.

### PREPARATION

Excipients used in molded lozenge manufacture include gelatin, fused sucrose, sorbitol, or another carbohydrate base.

Molded lozenges can be prepared by mixing the ingredients with water and heating to reduce the water content. The viscous solution is then poured into molds (e.g., corn starch molds). The lozenges are quickly cooled in the molds to trap the base in the glassy state. Once formed, the lozenges are removed from the molds and packaged. Care is taken to avoid excessive moisture during storage to prevent crystallization of the sugar base.

Compressed lozenges are made using excipients that may include a filler, binder, sweetening agent, flavoring agent, and lubricant. Sugars such as sucrose, sorbitol, and mannitol are often included because they can act as a filler and binder as well as serve as sweetening agents. Approved FD&C and D&C dyes or lakes (dyes adsorbed onto insoluble aluminum hydroxide) also may be present.

The manufacturing of compressed lozenges is essentially the same as that for conventional tableting, with the exception that a tablet press capable of making larger tablets and exerting greater force to produce harder tablets may be required (see *Tablets*).

The paste used to produce lozenges manufactured by stamping or cutting contains a moistening agent, sucrose, and flavoring and sweetening agents. The homogenous paste is spread as a bed of uniform thickness, and the lozenges are cut or stamped from the bed and are allowed to dry. Some lozenges are prepared by forcing dampened powders under low pressure into mold cavities and then ejecting them onto suitable trays for drying at moderate temperatures.

## Ointments

Ointments are semisolid preparations generally intended for external application to the skin or mucous membranes. Drug substances delivered in ointments are intended for local action or for systemic absorption. Ointments usually contain less than 20% water and volatiles and more than 50% hydrocarbons, waxes, or polyols as the vehicle. Ointment bases recognized for use as vehicles fall into four general classes: hydrocarbon bases, absorption bases, water-removable bases, and water-soluble bases.

### HYDROCARBON BASES

Also known as oleaginous ointment bases, hydrocarbon bases allow the incorporation of only small amounts of an aqueous component. Ointments prepared from hydrocarbon bases act as occlusive dressings and provide prolonged contact of the drug substance with the skin. They are difficult to remove and do not change physical characteristics upon aging.

### ABSORPTION BASES

Absorption bases allow the incorporation of aqueous solutions. Such bases include only anhydrous components (e.g., *Hydrophilic Petrolatum*) or W/O emulsions (e.g., *Lanolin*). Absorption bases are also useful as emollients.

### WATER-REMOVABLE BASES

O/W emulsions (e.g., *Hydrophilic Ointment*) are sometimes referred to as creams (see *Emulsions*). Water-removable bases may be readily washed from the skin or clothing with water, making them acceptable for cosmetic reasons. Other advantages of the water-removable bases are that they can be diluted with water and that they favor the absorption of serous discharges in dermatological conditions.

### WATER-SOLUBLE BASES

Also known as greaseless ointment bases, they are formulated entirely from water-soluble constituents. *Polyethylene Glycol Ointment* is the only official preparation in this group. Water-soluble bases offer many of the advantages of the water-removable bases and, in addition, contain no water-insoluble substances such as petrolatum, anhydrous lanolin, or waxes. They are more correctly categorized as gels (see *Gels*).

The choice of an ointment base depends on the action desired, the characteristics of the incorporated drug substance, and the latter's bioavailability if systemic action is desired. The product's stability may require the use of a base that is less than ideal in meeting other quality attributes. Drug substances that hydrolyze rapidly, for example, are more stable in hydrocarbon bases than in bases that contain water.

### PREPARATION

Ointments are typically prepared by either direct incorporation into a previously prepared ointment base or by fusion (heating during the preparation of the ointment). A levigating agent is often added to facilitate the incorporation of the medicament into the ointment base by the direct incorporation procedure. In the fusion method, the ingredients are heated. Homogenization

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is often necessary. The rate of cooling is an important manufacturing detail because rapid cooling can impart increased structure to the product of the fusion method.

## Pastes

Pastes are semisolid preparations of stiff consistency and contain a high percentage (20%–50%) of finely dispersed solids. Pastes are intended for application to the skin, oral cavity, or mucous membranes. Pastes ordinarily do not flow at body temperature and thus can serve as occlusive, protective coatings. As a consequence, pastes are more often used for protective action than are ointments.

Fatty pastes that have a high proportion of hydrophilic solids appear less greasy and are more absorptive than ointments. They are used to absorb serous secretions and are often preferred for acute lesions that have a tendency toward crusting, vesiculation, or oozing.

Dental pastes are applied to the teeth. Other orally administered pastes may be indicated for adhesion to the mucous membrane for a local effect. ▲Although rare, pastes can be administered orally, for example to evaluate pharyngeal function.▲ (USP 1-May-2021)

In veterinary medicine, pastes are typically administered orally and are intended for systemic delivery of drug substances. The paste is squeezed into the mouth of the animal, generally at the back of the tongue, or is spread inside the mouth.

## Pellets

The use of the term "pellet" for implantable dosage forms is no longer preferred (see *Implants*). In veterinary medicine, medicated articles and feeds may be pelletized but are not considered dosage forms (see *Animal Drugs for Use in Animal Feeds* ⟨1152⟩).

Pellets are small solid dosage forms that can be designed as single or multiple entities. They can have a spherical or nearly spherical shape, although such a shape is not required. Spherical pellets are sometimes referred to as beads. Pellets used in veterinary medicine may instead be cylindrical in shape. Pellets can provide several advantages, including physical separation for chemically or physically incompatible materials and for control of the release of drug substance. Pellets may be designed with the drug substance dispersed in a matrix or the pellets may be coated with an appropriate polymer. Pellets may be administered by the oral (gastrointestinal) route. ▲Depending on the design, pellets▲ (USP 1-May-2021) for oral administration can:

1. Protect stomach tissues from irritation
2. Sometimes minimize variability associated with gastric retention of larger dosage forms
3. Solely extend the release of the drug substance
4. Solely delay the release
5. Both extend and delay the release of the drug substance

Some pellets can be sprinkled on food. In the case of delayed-release formulations, the coating polymer is chosen to resist dissolution at the lower pH of the gastric environment but to dissolve in the higher pH of the intestinal environment.

Pellets may be administered by injection. One or several pellets can be injected or surgically administered to provide continuous therapy for periods of months or years (see *Implants*).

In veterinary medicine, pellets may be used to improve palatability of the drug product and pellets for oral administration may be delivered on top of an individual animal's food or feed.

### PREPARATION

The desired performance characteristics determine the manufacturing method chosen. In general, pellet dosage forms are manufactured by compression, or by wet or dry extrusion processes sometimes followed by spheronization, or followed by wet or dry coating processes. Manufacture of pellets by wet coating usually involves the application of successive coatings upon nonpareil seeds. This manufacturing process is frequently conducted in fluid-bed processing equipment. Dry powder coating or layering processes are often performed in specialized rotor granulation equipment. The extent of particle growth achievable in wet coating processes is generally more limited than the growth that can be obtained with dry powder layering techniques, but either method allows the formulator to develop and apply multiple layers of coatings to achieve the desired release profile. The manufacture of pellets by compression is largely restricted to the production of material for subcutaneous implantation. This method of manufacture provides the necessary control to ensure dose uniformity and is generally better suited to aseptic processing requirements.

Alternatively, microencapsulation techniques can be used to manufacture pellets. Coacervation coating techniques typically produce coated particles that are much smaller than those made by other techniques.

## Pills

Pills are drug substance-containing small, spherical, solid bodies intended for oral administration. The pill dosage form has been largely replaced by compressed tablets and by capsules. Unlike tablets, pills are usually prepared by a wet massing, piping, and molding technique. This term is frequently incorrectly used as a general term to describe solid oral dosage forms, such as tablets and capsules.

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### PREPARATION

Excipients are selected on the basis of their ability to produce a mass that is firm and plastic. The drug substance is triturated with powdered excipients in serial dilutions to attain a uniform mixture. Liquid excipients that act to bind and provide plasticity to the mass are subsequently added to the dry materials. The mass is formed by kneading. The properties of firmness and plasticity are necessary to permit the mass to be worked and retain the shape produced. Cylindrical pill pipes are produced from portions of the mass. The pill pipe is cut into individual lengths corresponding to the intended pill size, and the pills are rolled to form the final shape. Pill-making machines can automate the preparation of the mass, production of pill piping, and the cutting and rolling of pills.

## Plasters

A plaster is a semisolid substance for external application that is supplied on a support material. Plasters are applied for prolonged periods to provide protection, support, or occlusion (maceration). This term is not preferred and should not be used for new drug product titles ▲(see *Systems*).▲ (USP 1-May-2021) Plasters consist of an adhesive layer that may contain active substances. This layer is spread uniformly on an appropriate support that is usually made of a rubber base or synthetic resin. Unmedicated plasters are designed to provide protection or mechanical support to the site of application. Plasters are available in a range of sizes or cut to size to effectively provide prolonged contact to the site of application. They adhere firmly to the skin but can be peeled off the skin without causing injury.

## Powders

Powders are defined as a single solid or a mixture of solids in a finely divided state. Powders used as pharmaceutical dosage forms may contain one or more drug substances and can be used as is or can be mixed with a suitable vehicle for administration (see *Solutions* or *Suspensions*). Powders can be intended for internal or external use. Powders for external use are typically dusted onto the skin or applied to bandages or clothing. Powders for internal use can be applied to accessible mucous membranes with suitable applicators or are entrained in air streams for application to the nose or lungs.

The performance of powder dosage forms can be affected by the physical characteristics of the powder. Selection of relevant and appropriate powder characteristics depends on the dosage form and its route of administration. For example, particle size can influence the dissolution rate of the particles and thus the bioavailability and/or effectiveness at the site of action. Externally applied powders should have a particle size of 150 µm or less (typically in the 50–100-µm range to prevent a gritty feel on the skin that could further irritate traumatized skin). The particle size of powders delivered to the lung or nose influences where the powder is deposited. Particle size may influence the mixing, segregation, and aggregation of the particles, which can affect the delivery and uniformity of the dosage form. For more information, see *Powder Fineness* ⟨811⟩ and ⟨5⟩.

▲In veterinary medicine, a powder that needs to be reconstituted prior to administration previously has been called a concentrate. The term "concentrate" is no longer preferred.▲ (USP 1-May-2021)

### INHALATION POWDERS AND NASAL POWDERS

Inhalation powders and nasal powders consist of an appropriately finely divided solid and a suitable container–closure delivery system. For additional information, see ⟨5⟩ and ⟨601⟩.

### PREPARATION

Powder dosage forms can be produced by the combination of multiple components into a uniform blend. This preparation can also involve particle size reduction, a process referred to as comminution. Milling, spray drying, supercritical fluid, high-pressure homogenization, precipitation technologies, and porous microparticle fabrication techniques may be used to reduce the particle size of powders. As the particle size is decreased, the number of particles and the surface area increase, which can increase the dissolution rate and bioavailability, and/or the rate and extent of local action, of the drug substance.

Blending of powders may be accomplished by different techniques. Industrial processes may employ sifting or tumbling the powders in a rotating container. One of the most common tumble blenders is a V-blender, which is available in a variety of sizes suitable for small-scale and large-scale compounding and industrial production. Depending on the particle size of the drug substance, a random mixture of powders may be employed. Blending techniques for powders include those used in compounding pharmacy such as spatulation and trituration. ▲▲ (USP 1-May-2021)

Powder flow can be influenced by both particle size and shape. Larger particles generally flow more freely than do fine particles. Powder flow is an important attribute that can affect the packaging or dispensing of a powder.

## Rinses

(See *Solutions*.)

## Soaps and Shampoos

Soaps and shampoos are solid or liquid preparations intended for topical application to the skin or scalp followed by subsequent rinsing with water. Soaps and shampoos are emulsions, suspensions, or surface-active compositions that readily form emulsions, micelles, or foams upon the addition of water followed by rubbing. Incorporation of drug substances in soaps and shampoos combines the cleansing/degreasing abilities of the vehicle and facilitates the topical application of the drug substance to affected areas, even large areas, of the body. The surface-active properties of the vehicle facilitate contact of the

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drug substance with the skin or scalp. Medicated soap and shampoo formulations frequently contain suitable antimicrobial agents to protect against bacteria, yeast, and mold contamination.

### PREPARATION

The preparation of medicated soaps and shampoos follows techniques frequently used for the preparation of emulsified systems. To ensure uniformity, the drug substance(s) must be added to the vehicle prior to congealing (in the case of soaps) followed by thorough mixing. If the medication is present as a suspension, the particle size must be controlled to promote uniform distribution of the drug substance and possibly optimize performance. Because soap manufacture frequently involves processing the ingredients at an elevated temperature, care must be exercised to avoid excessive degradation of the drug substance during processing.

## Solutions

A solution is a preparation that contains one or more dissolved chemical substances in a suitable solvent or mixture of mutually miscible solvents. Because molecules of a drug substance in solution are uniformly dispersed, the use of solutions as dosage forms generally provides assurance of uniform dosage upon administration and good accuracy when the solution is diluted or otherwise mixed.

Substances in solutions are more susceptible to chemical instability than they are in the solid state and, dose-for-dose, are generally heavier and more bulky than solid dosage forms. These factors increase the cost of packaging and shipping relative to that of solid dosage forms. Some solutions are prepared and ready for use, and others are prepared as powders or other solids intended for reconstitution with an appropriate vehicle just before use (see *Powders*). Solution dosage forms can be administered by injection, inhalation, and the mucosal, topical, and gastrointestinal routes. A solution administered by injection is officially titled "injection" (see ⟨1⟩).

Some solutions are designed to form a mass in situ. These solutions comprise polymer, drug substance, and solvent for the polymer. The polymer solvent can be water or an organic solvent. After administration of the solution to a patient by subcutaneous or intramuscular administration, it forms a gel or a solid polymeric matrix that traps the drug substance and extends the drug substance release for days or months.

Solutions intended for oral administration usually contain flavorings and colorants to make the medication more attractive and palatable for the patient or consumer. When needed, they also may contain stabilizers to maintain chemical and physical stability and preservatives to prevent microbial growth.

Solutions are sometimes placed on devices such as swabs, cloths, or sponges, that aid application.

▲▲ (USP 1-May-2021) A solution that needs to be diluted prior to administration ▲previously▲ (USP 1-May-2021) has been called a concentrate. ▲▲ (USP 1-May-2021) The term "concentrate" is no longer preferred.

## Sprays

Spray preparations may deliver either accurately metered or nonmetered amounts of formulation.

A spray drug product is a dosage form that contains a drug substance in the liquid state as a solution or suspension and is intended for administration as a mist. Sprays are distinguished from aerosols in that spray containers are not pressurized. Most of the sprays are generated by manually squeezing a flexible container or actuation of a pump that generates the mist by discharging the contents through a nozzle.

Depending on the design of the formulation and the valve system, the droplets generated may be intended for immediate inhalation through the mouth and deposition in the pulmonary tree, or for inhalation into the nose and deposition in the nasal cavity.

The mechanism for droplet generation and the intended use of the preparation distinguish various ▲types of spray drug products.▲ (USP 1-May-2021) A spray ▲drug product▲ (USP 1-May-2021) may be composed of a pump, container, actuator, valve, nozzle, or mouthpiece in addition to the formulation containing the drug(s), solvent(s), and any excipient(s). The design of each component plays a role for the appropriate performance of the drug product and in determining the critical characteristics of the droplet size distribution. Droplet and particle size distributions, delivered dose uniformity, plume geometry, and droplet velocity are critical parameters that influence the efficiency of drug delivery. When the preparation is supplied as a multidose container, the addition of a suitable antimicrobial preservative may be necessary. Spray formulations intended for local or systemic effect typically have an aqueous base and may contain excipients to control pH and viscosity. In addition, depending on the route of administration, the formulation may be isotonic. For additional information, see ⟨5⟩ and ⟨601⟩.

### LABELING AND USE

Refer to the Center for Drug Evaluation and Research (CDER) *Guidance for Industry: Nasal Spray and Inhalation Solution, Suspension, and Spray Drug Products—Chemistry, Manufacturing, and Controls Documentation*.

## Strips

A strip is a dosage form or device in the shape of a long, narrow, thin, absorbent, solid material such as filter paper. Typically it is sterile and it may be impregnated with a compound or be gauged to allow measurements for diagnostic purposes, such as in measuring tear production. The term "strip" should not be used when another term such as "film" is more appropriate.

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## Suppositories

Suppositories are dosage forms adapted for application into the rectum. They melt, soften, or dissolve at body temperature. A suppository may have a local protectant or palliative effect, or may deliver a drug substance for systemic or local action.

Suppository bases typically include cocoa butter, glycerinated gelatin, hydrogenated vegetable oils, mixtures of polyethylene glycols of various molecular weights, and fatty acid esters of polyethylene glycol. The suppository base can have a notable influence on the release of the drug substance(s). Although cocoa butter melts quickly at body temperature, it is immiscible with body fluids, and this inhibits the diffusion of fat-soluble drug substances to the affected sites. Polyethylene glycol is a suitable base for some antiseptics. In cases when systemic action is desired, incorporating the ionized rather than the nonionized form of the drug substance may help maximize bioavailability. Although nonionized drug substances partition more readily out of water-miscible bases such as glycerinated gelatin and polyethylene glycol, the bases themselves tend to dissolve very slowly, which slows drug substance release. Cocoa butter and its substitutes (e.g., *Hard Fat*) perform better than other bases for allaying irritation in preparations intended for treating internal hemorrhoids. Suppositories for adults are tapered at one or both ends and usually weigh about 2 g each.

### PREPARATION

Cocoa butter suppositories have cocoa butter as the base and can be made by incorporating the finely divided drug substance into the solid oil at room temperature and suitably shaping the resulting mass, or by working with the oil in the melted state and allowing the resulting suspension to cool in molds. A suitable quantity of hardening agents may be added to counteract the tendency of some drug substances (such as chloral hydrate and phenol) to soften the base. The finished suppository melts at body temperature.

A variety of vegetable oils, such as coconut or palm kernel, modified by esterification, hydrogenation, or fractionation, are used as cocoa butter substitutes to obtain products that display varying compositions and melting temperatures (e.g., *Hydrogenated Vegetable Oil* and *Hard Fat*). These products can be designed to reduce rancidity while incorporating desired characteristics such as narrow intervals between melting and solidification temperatures, and melting ranges to accommodate formulation and climatic conditions.

Drug substances can be incorporated into glycerinated gelatin bases by addition of the prescribed quantities to a vehicle consisting of about 70 parts of glycerin, 20 parts of gelatin, and 10 parts of water.

Several combinations of polyethylene glycols that have melting temperatures that are above body temperature are used as suppository bases. Because release from these bases depends on dissolution rather than on melting, there are significantly fewer problems in preparation and storage than is the case for melting-type vehicles. However, high concentrations of higher molecular weight polyethylene glycols may lengthen dissolution time, resulting in problems with retention.

Several nonionic surface-active agents closely related chemically to the polyethylene glycols can be used as suppository vehicles. Examples include polyoxyethylene sorbitan fatty acid esters and the polyoxyethylene stearates. These surfactants are used alone or in combination with other suppository vehicles to yield a wide range of melting temperatures and consistencies. A notable advantage of such vehicles is their water dispersibility. However, care must be taken with the use of surfactants because they may either increase the rate of drug substance absorption or interact with the drug substance to reduce therapeutic activity.

Compounding suppositories using a suppository base typically involves melting the suppository base and dissolution or dispersion of the drug substance in the molten base (see ⟨795⟩). When compounding suppositories, the compounding professional prepares an excess amount of total formulation to allow the prescribed quantity to be accurately dispensed. In compounding suppositories, avoid caustic or irritating ingredients, carefully select a base that will allow the drug substance to provide the intended effect, and in order to minimize abrasion of the rectal membranes, reduce solid ingredients to the smallest reasonable particle size.

## Suspensions

A suspension is a biphasic preparation consisting of solid particles dispersed throughout a liquid phase. Suspension dosage forms may be formulated for specific routes of administration such as oral, topical, inhalation, ophthalmic, otic, and injection. ▲In veterinary medicine, suspensions also can be administered via the intramammary route.▲ (USP 1-May-2021) Some suspensions are prepared and ready for use, and others are prepared as powders or other solids intended for reconstitution with an appropriate vehicle just before use (see *Powders*).

Inhalation suspensions (see ⟨5⟩), ophthalmic suspensions, injectable suspensions, and some otic suspensions are prepared in sterile form. Suspensions are generally not injected intravenously, epidurally, or intrathecally unless the product labeling clearly specifies these routes of administration.

Some liposomal drug products are referred to as suspensions because they can settle and require resuspension prior to administration (see ⟨1⟩).

Resorbable microparticles can provide extended release of a drug substance over periods varying from a few weeks to months. They can be administered subcutaneously or intramuscularly for systemic delivery, or they may be deposited in a desired location in the body for site-specific delivery. Resorbable microparticles (or microspheres) generally range from 20 to 100 µm in diameter. They are composed of a drug substance dispersed within a biocompatible, bioabsorbable polymeric excipient (matrix). Poly(lactide-co-glycolide) polymers have been used frequently. These excipients typically resorb by hydrolysis of ester linkages. The microparticles are typically administered by suspension in an aqueous vehicle followed by injection with a conventional syringe and needle. Release of the drug substance from the microparticles begins after physiological fluid enters the polymer matrix, dissolving some of the drug substance that is then released by a diffusion-controlled process. Drug release also can occur as the bioresorbable polymer molecular weight decreases and as the matrix erodes.

Some suspensions are designed to form a mass in situ. These suspensions comprise polymer, drug substance, and solvent for the polymer. The polymer solvent can be water or an organic solvent. After administration of the suspension to a patient

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by subcutaneous or intramuscular administration, it forms a gel or a solid polymeric matrix that traps the drug substance and extends the drug substance release for days or months.

Historically, the term "milk" was sometimes used for suspensions in aqueous vehicles intended for oral administration (e.g., *Milk of Magnesia*). The term "magma" is often used to describe suspensions of inorganic solids, such as clays in water, that display a tendency toward strong hydration and aggregation of the solid, giving rise to gel-like consistency and thixotropic rheological behavior (e.g., *Bentonite Magma*). In the past, the term "lotion" referred to both topical suspensions and topical emulsions. Now the term only refers to topical emulsions (see *Emulsions*).

Limited aqueous solubility of the drug substance(s) is the most common rationale for developing a suspension. Other potential advantages of an oral suspension include taste masking and improved patient compliance because of the more convenient dosage form. When compared to solutions, suspensions can have improved chemical stability. Ideally, a suspension should contain small uniform particles that are readily suspended and easily redispersed following settling. Unless the dispersed solid is colloidal, the particulate matter in a suspension will likely settle to the bottom of the container upon standing. Such sedimentation may lead to caking and solidification of the sediment and difficulty in redispersing the suspension upon agitation. To prevent such problems, manufacturers commonly add ingredients to increase viscosity and the gel state of the suspension or flocculation, including clays, surfactants, polyols, polymers, or sugars. Frequently, thixotropic vehicles are used to counter particle-settling tendencies, but these vehicles must not interfere with pouring or redispersal. Additionally, the density of the dispersed phase and continuous phase may be modified to further control settling rate. For topical suspensions, rapid drying upon application is desirable.

Temperature can influence the viscosity (and thus suspension properties and the ease of removing the dose from the bottle), and temperature cycling can lead to changes in the particle size of the dispersed phase via Ostwald ripening. When manufacturers conduct stability studies to establish product shelf life and storage conditions, they should cycle conditions (freeze/thaw) to investigate temperature effects.

Unless studies confirm that the formulation will not support microbial growth, suspension preparations packaged to provide multiple doses should contain suitable antimicrobial agents to protect against bacterial, yeast, and mold contamination (see ⟨51⟩) or other appropriate measures should be taken to avoid microbial contamination.

Suspensions for reconstitution are dry powder or granular mixtures that require the addition of water or a supplied formulated diluent before administration. This formulation approach is frequently used when the chemical or physical stability of the drug substance or suspension does not allow sufficient shelf life for a preformulated suspension. Typically, these suspensions are refrigerated after reconstitution to increase their shelf life. For this type of suspension, the powder blend is uniform and the powder readily disperses when reconstituted.

Injectable suspensions are generally intended for either subcutaneous or intramuscular routes of administration and should have a controlled particle size, typically in the range of 5 µm and smaller. The rationale for the development of injectable suspensions may include poor drug substance solubility, improved chemical stability, prolonged duration of action, and avoidance of first-pass metabolism. Care is needed in selecting the sterilization technique because it may affect product stability or alter the physical properties of the material.

▲▲ (USP 1-May-2021) A suspension that needs to be diluted prior to administration ▲previously▲ (USP 1-May-2021) has been called a concentrate. ▲▲ (USP 1-May-2021) The term "concentrate" is no longer preferred.

### PREPARATION

Suspensions are prepared by adding suspending agents or other excipients and purified water or oil to solid drug substances and mixing to achieve uniformity. In the preparation of a suspension, the characteristics of both the dispersed phase and the dispersion medium should be considered. During development, manufacturers should define an appropriate particle size distribution for the suspended material to achieve the desired effectiveness and to minimize the likelihood of particle size changes during storage.

In some instances, the dispersed phase has an affinity for the vehicle and is readily wetted upon its addition. For some materials, the displacement of air from the solid surface is difficult, and the solid particles may clump together or float on top of the vehicle. In the latter case, a wetting agent may be used for certain types of suspensions to facilitate displacement of air from the powder surface. Surfactants, alcohol, glycerin, and other hydrophilic liquids can be used as wetting agents when an aqueous vehicle will be used as the dispersion phase. These agents function by displacing the air in the crevices of the particles and dispersing the particles. In the large-scale preparation of suspensions, wetting of the dispersed phase may be aided by the use of high-energy mixing equipment such as colloid mills or other rotor–stator mixing devices.

After the powder has been wetted, the dispersion medium (containing the soluble formulation components such as colorants, flavorings, and preservatives) is added in portions to the powder, and the mixture is thoroughly blended before subsequent additions of the vehicle. A portion of the vehicle is used to wash the mixing equipment free of suspended material, and this portion is used to bring the suspension to final volume and ensure that the suspension contains the desired concentration of solid matter. The final product may be passed through a colloid mill or other blender or mixing device to ensure uniformity.

Suspensions are resuspended before the dose is dispensed. Because of the viscosity of many suspension vehicles, air entrainment may occur during dosing. The formulation process allows evaluation of this possibility; adjustments in vehicle viscosity or the incorporation of low levels of antifoaming agents are common approaches to minimize air entrainment. Alternatively, specific instructions for resuspending the formulation may be provided to minimize air incorporation and ensure accurate dosing.

## Systems

Systems are preparations of drug substance(s) in carrier devices, often containing adhesive backing, that are applied topically or inserted into body cavities. The drug substance is designed to be released in a controlled manner over a specified period of time or the drug substance is released based on its concentration in the formulation. Unless otherwise stated in the labeling,

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the carrier device is removed after use. The term “system” should not be used when another dosage form term is more appropriate (e.g., inserts and implants).

The notation of strength is either defined in terms of the amount of the drug substance released from the system over a specific period of time or as the drug concentration within the formulation (e.g., the percentage of the drug). Various routes of administration are possible, so the route must always be indicated in the compendial name when a specific location for application is essential for proper use (e.g., “intrauterine”, “ocular”, or “periodontal” as the route of administration). For example, systems applied to the eye are called ocular systems. The route is named “transdermal” when, for example, systemic absorption of the drug substance may take place through the dermis without specifying the region of the body to which the system is applied.

The term “patch” has sometimes been used but is not preferred for use in drug product monograph nomenclature when referring to a system.

Intrauterine systems are intended for placement in the uterus. Release of the drug substance can be up to 5 years.

Ocular systems are intended for placement in the lower conjunctival fornix from which the drug diffuses through a membrane at a constant rate.

Periodontal systems are intended for placement in the pocket between the tooth and the gum. In some cases, periodontal systems may be formed in situ in the periodontal pocket and release the drug substance(s) for several weeks.

TDSs are placed onto intact skin to deliver the drug to the systemic circulation. They are designed for prolonged release (up to 7 days). Specific quality tests for TDS are found in ⟨3⟩.

## Tablets

Tablets are solid dosage forms in which the drug substance is generally blended with excipients and compressed into the final dosage. Tablets are the most widely used dosage form in the United States. Tablet presses use steel punches and dies to prepare compacted tablets by the application of high pressures to powder blends or granulations. Tablets can be produced in a wide variety of sizes, shapes, and surface markings. Capsule-shaped tablets are commonly referred to as caplets, although the term is not used in official article titles. Specialized tablet presses may be used to produce tablets with multiple layers or with specially formulated core tablets placed in the interior of the final dosage form. These specialized tablet presentations can delay or extend the release of the drug substance(s) or physically separate incompatible drug substances. Tablets may be coated by a variety of techniques to provide taste masking, protection of photo-labile drug substance(s), extended or delayed release, or unique appearance (colors). When no deliberate effort has been made to modify the drug substance release rate, tablets are referred to as immediate-release.

### BUCCAL TABLETS

Intended to be inserted in the buccal pouch, where the drug substance is absorbed directly through the oral mucosa. Few drug substances are readily absorbed in this way (examples are nitroglycerin and certain steroid hormones).

### CHEWABLE TABLETS

Formulated and manufactured to produce a pleasant-tasting residue in the mouth and to facilitate swallowing. Hard chewable tablets are typically prepared by compaction, usually utilizing mannitol, sorbitol, or sucrose as binders and fillers, and contain colors and flavors to enhance their appearance and taste. Soft chewable tablets are typically made by a molding or extrusion process, frequently with more than 10% water to help maintain a pliable, soft product. Hard chewable tablets in veterinary medicine often have flavor enhancers like brewer’s yeast or meat/fish-based flavors.

Tablets for human use that include “chewable” in the title must be chewed or crushed prior to swallowing to ensure reliable release of the drug substance(s) or to facilitate swallowing. If tablets are designed so that they may be chewed (but chewing is not required for drug substance release or ease of swallowing), the title should not include a reference to “chewable”. In that case, the product may still be described as “chewable” in the ancillary labeling statement.

Tablets for veterinary use that are intended to be chewed will include “chewable” in the title. However, it is understood that for veterinary products it is not possible to ensure that tablets are chewed prior to ingestion. Chewable tablets may be broken into pieces and fed to animals that normally swallow treats whole.

### EFFERVESCENT TABLETS

Prepared by compaction and contain, in addition to the drug substance(s), mixtures of acids (e.g., citric acid or tartaric acid) and carbonates, and/or sodium bicarbonate. Upon contact with water, these formulations release carbon dioxide, producing the characteristic effervescent action.

### HYPODERMIC TABLETS

Molded tablets made from completely and readily water-soluble ingredients; formerly intended for use in making preparations for hypodermic injection. They may be administered orally or sublingually when rapid drug substance availability is required.

### MODIFIED-RELEASE TABLETS

Two categories of modified-release tablet formulations are recognized by USP.

**Delayed-release tablets:** Tablets are sometimes formulated with acid-resistant or enteric (also called “gastro-resistant”) coatings to protect acid-labile drug substances from the gastric environment or to prevent adverse events such as irritation.

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**Extended-release tablets:** Extended-release tablets are formulated in such a manner as to make the drug substance available over an extended period of time following ingestion. Requirements for dissolution (see ⟨711⟩) are typically specified in the individual monographs.

## ORALLY DISINTEGRATING TABLETS

Orally disintegrating tablets are intended to disintegrate rapidly within the mouth to provide a dispersion before the patient swallows the resulting slurry where the drug substance is intended for gastrointestinal delivery and/or absorption. Some of these dosage forms have been formulated to facilitate rapid disintegration and are manufactured by conventional means or by using lyophilization or molding processes. Further details may be found in the CDER *Guidance for Industry: Orally Disintegrating Tablets*.

## SUBLINGUAL TABLETS

Sublingual tablets are intended to be inserted beneath the tongue, where the drug substance is absorbed directly through the oral mucosa. As with buccal tablets, few drug substances are extensively absorbed in this way, and much of the drug substance is swallowed and is available for gastrointestinal absorption.

## TABLETS FOR ORAL SOLUTION

Before administration, tablets for oral solution are intended to be solubilized in a liquid diluent. In some cases, tablets for oral solution also may be chewed or swallowed.

## TABLETS FOR ORAL SUSPENSION

Tablets for oral suspension are intended to be dispersed in a liquid before administration as a suspension. The dosage form is tablets for oral suspension when either the drug substance or the excipients do not dissolve when dispersed in a liquid. In some cases, tablets for oral suspension also may be chewed or swallowed.

## TABLET TRITURATES

Small, usually cylindrical, molded or compacted tablets. Tablet triturates traditionally were used as dispensing tablets in order to provide a convenient, measured quantity of a potent drug substance for compounding purposes, but they are rarely used today.

## PREPARATION

Most compacted (compressed) tablets consist of the drug substance(s) and a number of excipients. These excipients may include fillers (diluents), binders, disintegrating agents, lubricants, and glidants. Approved FD&C and D&C dyes or lakes, flavors, and sweetening agents also may be present.

Fillers or diluents are added when the quantity of drug substance(s) is too small or the properties of the drug substance do not allow satisfactory compaction in the absence of other ingredients. Binders impart adhesiveness to the powder blend and promote tablet formation and maintenance of drug substance uniformity in the tableting mixture. Disintegrating agents facilitate reduction of the tablet into small particles upon contact with water or biological fluids. Lubricants reduce friction during the compaction and ejection cycles. Glidants improve powder fluidity, powder handling properties, and tablet weight control. Colorants are often added to tablet formulations for aesthetic value or for product identification.

Tablets are prepared from formulations that have been processed by one of three general methods: wet granulation, dry granulation (roll compaction or slugging), or direct compression.

**Wet granulation:** Involves the mixing of dry powders with a granulating liquid to form a moist granular mass that is dried and sized prior to compression. It is particularly useful in achieving uniform blends of low-dose drug substances and facilitating the wetting and dissolution of poorly soluble, hydrophobic drug substances.

**Dry granulation:** Can be produced by passing powders between rollers at elevated pressure (roll compaction). Alternatively, dry granulation also can be carried out by the compaction of powders at high pressures on tablet presses, a process also known as slugging. In either case, the compacts are sized before compression. Dry granulation improves the flow and handling properties of the powder formulation without involving moisture in the processing.

**Direct compression:** Tablet processing involves dry blending of the drug substance(s) and excipients followed by compression. The simplest manufacturing technique, direct compression, is acceptable only when the drug substance and excipients possess acceptable flow and compression properties without prior process steps.

Tablets may be coated to protect the ingredients from air, moisture, or light; to mask unpleasant tastes and odors; to improve tablet appearance; and to reduce dustiness. In addition, coating may be used to protect the drug substance from acidic pH values associated with gastric fluids or to control the rate of drug release in the gastrointestinal tract.

The most common coating in use today is a thin film coating composed of a polymer that is derived from cellulose. Sugar coating is an alternative, less common approach. Sugar-coated tablets have considerably thicker coatings that are primarily sucrose with a number of inorganic diluents. A variety of film-coating polymers are available and enable the development of specialized release profiles. These formulations are used to protect acid-labile drug substances from the acidic stomach environment as well as to prolong the release of the drug substance to reduce dosing frequency (see ⟨711⟩ or ⟨701⟩).

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## Tapes

A tape is a dosage form suitable for delivering drug substances to the skin. It consists of a drug substance(s) impregnated into a durable yet flexible woven fabric or extruded synthetic material that is coated with an adhesive agent. Typically the impregnated drug substance is present in the dry state. The adhesive layer is designed to hold the tape securely in place without the aid of additional bandaging. Unlike TDSs, tapes are not designed to control the release rate of the drug substance. The term "tape" is not preferred and should not be used for new official article titles ▲(see *Systems*).▲ (USP 1-May-2021)

The drug substance content of tapes is expressed as amount per surface area with respect to the tape surface exposed to the skin. The use of an occlusive dressing with the tape enhances the rate and extent of delivery of the drug substance to deeper layers of the skin and may result in greater systemic absorption of the drug substance.

***Change to read:***

## GLOSSARY

This glossary provides definitions for terms in use in medicine and serves as a source of official titles for official articles, except when the definition specifically states that the term is not to be used in drug product titles. Examples of general nomenclature forms for the more frequently encountered categories of dosage forms appear in ⟨1121⟩. In an attempt to be comprehensive, this glossary was compiled without the limits imposed by current preferred nomenclature conventions. To clearly identify/distinguish preferred from not preferred terms, entries indicate when a term is not preferred and generally direct the user to the current preferred term. ▲Terms are also used to modify dosage forms, and such terms can be used in the naming of dosage forms.▲ (USP 1-May-2021) For example, ▲a tablet can be called a chewable tablet. The term "chewable" designates that the tablet▲ (USP 1-May-2021) must be chewed prior to swallowing.

**Aerosol:** A dosage form consisting of a liquid or solid preparation packaged under pressure and intended for administration as a fine mist. When not used in naming, the term "aerosol" also refers to the fine mist of small droplets or solid particles that are emitted from the product.

**Aromatic water** (not used in official titles; see *Solution*)**:** A clear, saturated, aqueous solution of volatile oils or other aromatic or volatile substances.

**Aural (Auricular)** (not used in official titles; see *Otic*)**:** For administration into, or by way of, the ear.

**Bead** (not used in official titles; see *Pellet*)**:** A solid dosage form in the shape of a small sphere. In most products a unit dose consists of multiple beads.

**Bolus** (not preferred; see *Tablet*)**:** A large tablet intended for administration to large animals. Occasionally, the term "bolus" is used to describe a method of administration.

**Buccal:** Administration directed toward the cheek, generally from within the mouth.

**Caplet** (not used in official titles; see *Tablet*)**:** Tablet dosage form in the shape of a capsule.

**Capsule:** A solid dosage form in which the drug substance, with or without other ingredients, is filled into either a hard or soft shell or coated on the capsule shell. Most capsule shells are composed mainly of gelatin.

**Chewable:** A term for a solid dosage form that is intended to be chewed or crushed before swallowing.

**Chewable gel:** Formed or molded oral gel dosage forms that maintain their shape, are elastic, and yield to mastication. Chewable gels are also known as "gummies," but this term is not used for official article titles.

**Coating (Coated)** (not used in official titles)**:** A term for the outer solid covering applied to a solid dosage form. This outer deposit is also referred to as a film. Coatings are applied for functional or aesthetic purposes such as taste masking, stability, modifying release characteristics, product identification, and appearance.

**Collodion** (not preferred; see *Solution*)**:** A preparation that is a solution dosage form composed of pyroxilin dissolved in a solvent mixture of alcohol and ether, and applied externally.

**Colloidal dispersion:** A term for a preparation or formulation in which particles of colloidal dimension (i.e., typically between 1 nm and 1 µm) are distributed uniformly throughout a liquid.

**Concentrate** (not a preferred term for human or veterinary drug products)**:** The current use is for drug substances that are not intended for direct administration to humans or animals. The use in drug product nomenclature is being phased out (see ⟨1121⟩ and *Nomenclature Guidelines*[1] ).

**Conventional-release** (not used in official titles; see *Immediate-release*)**:** A term describing a dosage form in which no deliberate effort has been made to modify the release rate of the drug substance. In the case of capsules and tablets, the inclusion or exclusion of a disintegrating agent is not interpreted as a modification.

**Cough drop** (not used in official titles; see *Lozenge*)

**Cream:** A semisolid emulsion dosage form often containing more than 20% water and volatiles, and/or containing less than 50% hydrocarbons, waxes, or polyols as the vehicle for the drug substance. Creams are generally intended for external application to the skin or mucous membranes.

**Delayed-release:** A type of modified-release dosage form. When used in naming dosage forms, this term denotes a dosage form deliberately formulated to delay release of the drug substance for some period of time after initial administration. For oral products, expressions such as "enteric-coated" or "gastro-resistant" have been used where release of the drug substance is prevented in the gastric environment but promoted in the intestinal environment. However, the term "delayed-release" is used for official article titles.

**Dental:** When used in naming dosage forms, this term denotes a preparation that is applied to the teeth for localized action.

**Dip** (not preferred; see *Immersion*)

**Disintegrating tablet** (not used in official titles; see *Tablet*, *Tablet for oral suspension*, or *Tablet for oral solution*; see also *Orally disintegrating*)

**Dispersible tablet** (not used in official titles; see *Tablet*, *Tablet for oral suspension*, or *Tablet for oral solution*)

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**Dosage form** (not used in official titles)**:** A combination of drug substance(s) and/or excipient(s) in quantities and physical form designed to allow the accurate and efficient administration of the drug substance to the human or animal patient.

**Dry powder inhaler** (not used in official titles)**:** A device used to administer an inhalation powder in a finely divided state suitable for oral inhalation by the patient.

**Effervescent:** A term for an oral dosage form, frequently tablets or granules, containing ingredients that, when in contact with water, rapidly release carbon dioxide. The dosage form is dissolved or dispersed in water to initiate the effervescence prior to ingestion.

**Elixir** (not preferred; see *Solution*)**:** A preparation that typically is a clear, flavored, sweetened hydroalcoholic solution intended for oral use. The term should not be used for new drug products in *USP–NF* but is commonly encountered in compounding pharmacy practice.

**Emollient** (not used in official titles)**:** A term for a cream or ointment indicating an increase in the moisture content of the skin following application of bland, fatty, or oleaginous substances.

**Emulsion:** A dosage form consisting of a two-phase system composed of at least two immiscible liquids, one of which is dispersed as droplets (internal or dispersed phase) within the other liquid (external or continuous phase), generally stabilized with one or more emulsifying agents. "Emulsion" is not used as a dosage form term if a more specific term is applicable (e.g., *Cream*, *Lotion*, or *Ointment*).

**Enteric-coated** (not used in official titles; see *Delayed-release*)**:** A term for a solid dosage form in which a polymer coating has been applied to prevent the release of the drug substance in the gastric environment.

**Excipient** (not used in official titles)**:** An ingredient of a dosage form other than a drug substance. The term “excipient” is synonymous with inactive ingredient.

**Extended-release:** A term denoting a dosage form that is deliberately formulated to prolong the release of the drug substance compared to that observed for an immediate-release dosage form. Expressions such as “prolonged release”, “repeat action”, “controlled release”, “long acting”, and “sustained release” also have been used to describe such dosage forms. However, the term “extended-release” is used for official article titles.

**Extended-release injectable suspension:** Liquid preparations of solids suspended in a suitable vehicle and formulated to allow the drug substance to be available over an extended period of time. The term "for extended-release injectable suspension" indicates dry solids that, upon the addition of a suitable vehicle, yield a preparation that conforms in all respects to the requirements for extended-release injectable suspensions.

**Film:** A term used to describe a thin sheet of material, usually composed of a polymer. Films are used in various routes of administration including as a means of oral administration of material in a rapidly dissolving form.

**Foam:** A dosage form containing gas dispersed in a liquid or solid continuous phase. Foams are formed at the time of application by dispensing product from the canister or other appropriate container and can be formulated to quickly break down into a liquid or to remain as a foam to ensure prolonged contact.

**Gas** (not used in official titles)**:** One of the states of matter having no definite shape or volume and occupying the entire container when confined.

**Gastro-resistant** (not used in official titles; see *Delayed-release*)**:** A term for a solid dosage form in which a polymer coating has been applied to prevent release in the gastric environment.

**Gel:** A dosage form that is a semisolid dispersion of small particles or a solution of large molecules interpenetrated by a solution containing a gelling agent to provide stiffness.

**Gelcap** (not used in official titles)**:** A capsule that is coated is sometimes referred to as a gelcap.

**Geltab/Filmtab** (not used in official titles)**:** A tablet that is coated is sometimes referred to as a geltab or filmtab.

**Granules:** A dosage form composed of dry aggregates of powder particles that may contain one or more drug substances, with or without other ingredients. They may be swallowed as such, dispersed in food, or dissolved in water. Granules are frequently compacted into tablets or filled into capsules, with or without additional ingredients. More commonly, granules are reconstituted as suspensions.

**Gum:** A dosage form in which the base consists of a pliable material that, when chewed, releases the drug substance into the oral cavity.

**Gummies** (not used in official titles; see *Chewable gel*)

**Hard-shell capsule** (not preferred; see *Capsule*)**:** A type of capsule in which one or more drug substances, with or without other ingredients, are filled into a two-piece shell. Most hard-shell capsules are composed mainly of gelatin and are fabricated prior to the filling operation.

**Immediate-release** (not used in official titles)**:** A term for a dosage form in which no deliberate effort has been made to modify the drug substance release rate.

**Immersion:** A veterinary route of administration via partial or complete submersion in a specified environment such as liquid or air.

**Implant:** A dosage form that is a solid or semisolid material containing the drug substance that is placed into the body. The implantation process is invasive, and the material is intended to reside at the site for a period consistent with the design release kinetics or profile of the drug substance(s).

**Inhalation (By Inhalation):** A route of administration for aerosols characterized by dispersion of the drug substance into the airways during inspiration.

**Injection (By Injection):** A route of administration of a liquid or semisolid deposited into a body cavity, fluid, or tissue by use of a needle.

**Injection:** Liquid preparations that may contain drug substances and/or excipients or solutions thereof. The term “for injection” indicates dry solids that, upon the addition of a suitable vehicle, yield solutions conforming in all respects to the requirements for injections.

**Injectable emulsion:** Liquid preparations of drug substances dissolved or dispersed in a suitable emulsion medium.

**Injectable suspension:** Liquid preparations of solids suspended in a liquid medium. The term “for injectable suspension” indicates dry solids that, upon the addition of a suitable vehicle, yield preparations conforming in all respects to the requirements for injectable suspensions. For extended-release preparations, see *Extended-release injectable suspension*.

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**Insert:** A solid dosage form that is inserted into a naturally occurring (nonsurgical) body cavity other than the mouth or rectum. It should be noted that a suppository is intended for application into the rectum and is not classified as an insert (see *Suppository*).

**▲Intramammary:** Administration of a drug product into the mammary gland, including via the teat canal.▲ (USP 1-May-2021)

**Intraocular:** A route of administration to deliver a sterile preparation within the eye.

**Irrigation:** A sterile solution or liquid intended to bathe or flush open wounds or body cavities.

**Jelly** (not preferred; see *Gel*)**:** A semisolid dispersion of small particles or a solution of large organic molecules interpenetrated by a solution containing a gelling agent to promote stiffness.

**Liposomes:** A term for preparations of amphiphilic lipids that have low water solubility (see ⟨1⟩).

**Liquid** (not used in official titles)**:** A dosage form consisting of a pure chemical in its liquid state. This dosage form term should not be applied to solutions. When not used in dosage form naming, the term, "liquid" is used to indicate a material that is pourable and conforms to its container at room temperature.

**Lotion:** An emulsion liquid dosage form applied to the outer surface of the body. Historically, this term was applied to topical suspensions and topical emulsions. The current definition of a lotion is restricted to an emulsion.

**Lozenge:** A solid dosage form intended to disintegrate or dissolve slowly in the mouth.

**Modified-release:** A term for a dosage form with a drug substance release pattern that has been deliberately changed from that observed for the immediate-release dosage form of the same drug substance. The two types of modified-release are extended-release and delayed-release. The term "modified-release" is not used in official article titles.

**Molded tablet** (not used in official titles)**:** A tablet that has been formed by dampening the ingredients and pressing into a mold, then removing and drying the resulting solid mass.

**Mouthwash** (not used in official titles; see *Rinse*)**:** Term applied to a solution preparation used to rinse the oral cavity.

**Nasal:** Route of administration (mucosal) characterized by administration to the nose or by way of the nose for local or systemic effect.

**Ocular** (not preferred; see *Intraocular*)**:** Route of administration indicating deposition of the drug substance within the eye.

**Ointment:** A semisolid dosage form, usually containing less than 20% water and volatiles and more than 50% hydrocarbons, waxes, or polyols as the vehicle. This dosage form generally is for external application to the skin or mucous membranes.

**Ophthalmic:** A route of administration characterized by application of a sterile preparation to the external parts of the eye.

**Oral:** Route of administration characterized by application to the mouth or delivery to the gastrointestinal tract through the mouth.

**Orally disintegrating:** When used in naming a dosage form, this term denotes a solid oral dosage form that disintegrates rapidly in the mouth prior to swallowing. The drug substance is intended for gastrointestinal delivery and/or absorption. See also CDER *Guidance for Industry: Orally Disintegrating Tablets*.

**Orodispersible** (not used in official titles; see *Orally disintegrating*)

**Oro-pharyngeal:** A route of administration characterized by deposition of a preparation into the oral cavity and/or pharyngeal region to exert a local or systemic effect.

**Otic:** A route of administration characterized by deposition of a preparation into, or by way of, the ear. Sometimes referred to as *Aural* (*Aural* not preferred).

**Parenteral:** General route of administration which is characterized by injection through the skin or other external boundary tissue or implantation within the body. Specific parenteral routes include intravenous, intraventricular, intra-arterial, intra-articular, subcutaneous, intramuscular, intrathecal, intracisternal, and intraocular (see ⟨1⟩).

**Paste:** A semisolid dosage form containing a high percentage (20%–50%) of finely dispersed solids with a stiff consistency. This dosage form is intended for application to the skin, oral cavity, or mucous membranes.

**Pastille** (not used in official titles; see *Lozenge*)

**Patch** (not preferred; see *System*)**:** Frequently incorrectly used to describe a *System*.

**Pellet:** A small solid dosage form of uniform, sometimes spherical, shape intended for direct administration. Spherical pellets are sometimes referred to as *Beads*. Pellets used in veterinary medicine are typically cylindrical in shape. Pellets intended as implants must be sterile, except for some ear implants used in animal drugs. The use of the term "pellet" for implantable dosage forms is no longer preferred (see *Implant*).

**Periodontal:** A term for a preparation that is applied around a tooth for localized action.

**Pill:** A solid, spherical dosage form usually prepared by a wet massing, piping, and molding technique. This term is frequently incorrectly used as a general term to describe solid oral dosage forms such as tablets or capsules.

**Plaster** (not preferred; ▲see *System*)▲ (USP 1-May-2021)**:** A dosage form containing a semisolid composition supplied on a support material for external application. Plasters are applied for prolonged periods of time to provide protection, support, or occlusion (for macerating action).

**Powder:** A dosage form composed of a solid or mixture of solids reduced to a finely divided state and intended for internal or external use.

**Powder, inhalation:** A powder containing a drug substance for oral inhalation. The powder is used with a device that aerosolizes and delivers an accurately metered amount.

**Premix** (not preferred; see Animal Drugs for Use in Animal Feeds ⟨1152⟩, Scope, Type A Medicated Articles and Type B Medicated Feeds)

**Prolonged-release** (not used in official titles; see *Extended-release*)

**Rectal:** A route of administration characterized by deposition into the rectum to provide local or systemic effect.

**Rinse** (see *Solution*)**:** A liquid preparation used to cleanse by flushing. A rinse is used to swish in the mouth and then expectorated. The nonpreferred term "mouthwash" sometimes has been used for "rinse".

**Semisolid** (not used in official titles)**:** A term for a material that exhibits plastic flow behavior. A semisolid material is not pourable, does not readily conform to its container at room temperature, and does not flow at low shear stress.

**Shampoo:** A solution, emulsion, or suspension dosage form used to clean the hair and scalp. May contain a drug substance intended for topical application to the scalp.

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**Soap:** The alkali salt(s) of a fatty acid or mixture of fatty acids used to cleanse the skin. Soaps used as dosage forms may contain a drug substance intended for topical application to the skin. Soaps also have been used as liniments and enemas.

**Soft gel capsule** (not preferred; see *Capsule*)**:** A specific capsule type characterized by increased levels of plasticizers producing a more pliable and thicker-walled material than hard gelatin capsules. Soft gel capsules are further distinguished because they are single-piece sealed dosages. Frequently used for delivering liquid compositions.

**Soluble tablet** (not used in official titles; see *Tablet* and *Tablet for oral solution*)**:**

**Solution:** A clear, homogeneous liquid dosage form that contains one or more chemical substances dissolved in a solvent or mixture of mutually miscible solvents.

**Spirit** (not preferred; see *Solution*)**:** A liquid dosage form composed of an alcoholic or hydroalcoholic solution of volatile substances.

**Spot on (Pour On)** (not used in official titles)**:** A method of delivering liquid veterinary drug products by administering them onto the animal's skin, usually between the shoulder blades (spot on) or down the back (pour on).

**Spray:** A spray is a dosage form that contains drug substance(s) in the liquid state, either as a solution or as a suspension, and is intended for administration as a mist. Sprays are distinguished from aerosols in that spray containers are not pressurized. Most of the sprays are generated by manually squeezing a flexible container or actuation of a pump that generates the mist by discharging the contents through a nozzle.

When not used in the naming of a dosage form, the term "spray" describes the generation of droplets of a liquid or solution to facilitate application to the intended area.

**Stent, drug-eluting:** A specialized form of implant used for extended local delivery of the drug substance to the immediate location of stent placement.

**Strip** (only used for diagnostic products, otherwise not preferred; see *Film*)**:** A dosage form or device in the shape of a long, narrow, thin, absorbent, solid material such as filter paper.

**Sublingual:** A route of administration characterized by placement underneath the tongue and for release of the drug substance for absorption in that region.

**Suppository:** A solid dosage form in which one or more drug substances are dispersed in a suitable base and molded or otherwise formed into a suitable shape for insertion into the rectum to provide local or systemic effect.

**Suspension:** A liquid dosage form that consists of solid particles dispersed throughout a liquid phase.

**Syrup** (not preferred; see *Solution*)**:** A solution containing high concentrations of sucrose or other sugars. This term is commonly used in compounding pharmacy.

**System:** A preparation of drug substance(s) in a carrier device that is applied topically or inserted into a body cavity. The drug substance is designed to be released in a controlled manner over a specified period of time or the drug substance is released based on its concentration in the formulation. Unless otherwise stated in the labeling, the carrier device is removed after use.

**Tablet:** A solid dosage form prepared from powders or granules by compaction.

**Tablet for oral solution:** A tablet that is intended to be dispersed in a liquid before administration. When dispersed in the liquid, a solution results.

**Tablet for oral suspension:** A tablet that is intended to be dispersed in a liquid before administration. When dispersed in the liquid, a suspension results.

**Tape** (not preferred; ▲see *System*)▲ (USP 1-May-2021)**:** A dosage form or device composed of a woven fabric or synthetic material onto which a drug substance is placed, usually with an adhesive on one or both sides to facilitate topical application. The rate of release of the drug substance is not controlled.

**Tincture** (not preferred; see *Solution*)**:** An alcoholic or hydroalcoholic solution prepared from vegetable materials or from chemical substances.

**Topical:** A route of administration characterized by application to the external surface of the body.

**Transdermal:** A route of administration characterized by drug product application to the skin where the drug substance passes through the dermal layer with the intent to achieve a systemic effect.

**Troche** (not used in official titles; see *Lozenge*)**:** A solid dosage form intended to disintegrate or dissolve slowly in the mouth and usually prepared by compaction in a manner similar to that used for tablets.

**Urethral:** A route of administration characterized by deposition into the urethra.

**Vaginal:** A route of administration characterized by deposition into the vagina.

**Vehicle** (not used in official titles)**:** A term commonly encountered in compounding pharmacy that refers to a component for internal or external use that is used as a carrier or diluent in which liquids, semisolids, or solids are dissolved or suspended. Examples include water, syrups, elixirs, oleaginous liquids, solid and semisolid carriers, and proprietary products (see *Excipient*).

**Veterinary:** A term for dosage forms intended for nonhuman use.

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# ⟨1152⟩ ANIMAL DRUGS FOR USE IN ANIMAL FEEDS

## PURPOSE

This chapter provides general descriptions of, and definitions for, animal drugs and drug products delivered in animal feeds. It discusses general principles involved in the manufacture, packaging, and labeling of these drugs and drug products.

## SCOPE

Medicated articles and feeds that are used to deliver animal drug(s) via the food given to animals are discussed in this general information chapter. Drugs approved for further manufacture into medicated animal feeds are not dosage form drugs. Dosage form drugs administered with feeds are not medicated articles or feeds. Drug dosage forms are listed in *USP* general chapter *Pharmaceutical Dosage Forms* ⟨1151⟩.

Animal drugs approved for further manufacture into medicated animal feeds may be in either dry or liquid form. They are sometimes referred to as premixes. The term "premix" is no longer used for animal drugs for use in animal feeds but is still used in some older drug monographs. Animal drugs in feeds are regulated as Type A medicated articles and Type B and Type C medicated feeds.

### Type A Medicated Articles

Type A medicated articles [21 CFR 558.3(b)(2)] are concentrated forms of animal drugs intended solely for further manufacture of other approved Type A medicated articles or Type B or C medicated feeds. This means that Type A medicated articles cannot be fed directly to animals. They consist of one or more animal drug(s) with or without a carrier (e.g., calcium carbonate, rice hull, corn, gluten) and with or without other inactive ingredients. They can be prepared in dry or liquid form. They are sold to feed mills or livestock producers and are intended to be further diluted by mixing into feed before consumption by animals.

### Type B Medicated Feeds

Type B medicated feeds [21 CFR 558.3(b)(3)] are intermediate medicated feeds for animals. They are manufactured from either Type A medicated article(s) or other Type B medicated feed(s) by dilution with non-medicated feed ingredients. In addition to the animal drug(s), Type B medicated feeds contain a substantial quantity of nutrients that comprise NLT 25% of the total feed weight. They can be prepared in dry or liquid form. Similar to Type A medicated articles, Type B medicated feeds are intended only for further dilution by mixing into feed, and they are not approved for feeding to animals.

### Type C Medicated Feeds

Type C medicated feeds [21 CFR 558.3(b)(4)] are intended to be fed directly to animals. They are manufactured from Type A medicated articles, Type B medicated feeds, or other Type C medicated feeds diluted with non-medicated feed ingredients. Type C medicated feeds may be prepared in dry or liquid form. Type C medicated feeds can be either fed as the complete feed for the animals, top-dressed onto the animals' normal daily rations, or offered "free-choice" (21 CFR 510.455). Type C medicated feeds approved to be offered free-choice are not intended to be fully consumed in a single feeding or to constitute the entire diet of the animals.

## PREPARATION

Type A medicated articles in dry form are typically produced by blending the drug substance(s) with carriers and other excipients to promote uniform mixing when subsequently added to the animal feed. The drug substance(s) may first be mixed with an excipient (e.g., starch or sodium aluminosilicate) that has a similar particle size and can help distribute the drug substance(s) uniformly throughout the final mixture. This pre-blend may then be mixed with bulk excipients (e.g., calcium carbonate or soybean hulls). The product may be granulated and/or oil (e.g., mineral oil, soybean oil) may be added to aid uniform distribution, to prevent particle segregation during shipping, and/or to minimize formation of airborne drug substance particles during production of another Type A medicated article or Types B or C medicated feeds.

Type A medicated articles in liquid form are produced by mixing the drug substance(s) with a suitable solvent (e.g., water or propylene glycol). The drug substance(s) is usually dissolved to produce a solution, but suspension products could be produced also.

Types B or C medicated feeds are typically manufactured at feed mills or on-farm by livestock producers. To manufacture Types B or C medicated feeds in dry form, Type A medicated articles are added to the feeds during the mixing process. Liquid Type A medicated articles may be sprayed in at set rates, and dry Type A medicated articles are added using methods that facilitate uniform distribution in the feeds. Types B and C medicated feeds in dry form may be further processed by heating, steaming, and extruding into pellets. The pellets may be rolled or broken up to create crumbles. Types B and C medicated feeds may also be prepared in liquid form. Liquid feeds are typically molasses based and contain an animal drug(s) dissolved or suspended in the liquid matrix. The liquid feed may need to be recirculated or agitated on a routine basis to maintain a uniform distribution of the drug(s).

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A Type B medicated feed may also be prepared by diluting another Type B medicated feed. A Type C medicated feed may also be prepared by diluting a Type B medicated feed or another Type C medicated feed.

## LABELING AND PACKAGING

Labeling for Type A medicated articles and Types B and C medicated feeds provides all information necessary for their safe and effective use. The label for a Type A medicated article includes mixing directions for the manufacture of medicated feeds from the Type A medicated article and feeding directions for the Type C medicated feeds. The label for a Type B medicated feed provides mixing directions for the manufacture of medicated animal feeds. Labels for both Type A medicated articles and Type B medicated feeds indicate that they are not to be fed directly to animals. The label for Type C medicated feeds includes directions for feeding.

Type A medicated articles are packaged in bags (e.g., paper with polyethylene liners) for dry products or in appropriate containers (e.g., plastic) for liquids. Typical sizes are 50-lb bags or several-gallon containers. Dry Types B and C medicated feeds may be packaged in bags for storage and delivery, or they may be shipped in bulk form for storage or immediate use. Free-choice Type C medicated feeds may be packaged in bags (e.g., loose minerals), wrapped in film (e.g., compressed blocks), or packaged in tubs (e.g., molded blocks). Liquid Types B and C medicated feeds are stored and shipped in bulk in tanks.

## NOMENCLATURE

The drug product's established (non-proprietary) name consists of the drug substance (active moiety) and an appropriate type of medicated article or feed. The following nomenclature options are available to indicate types of medicated articles or feeds as part of the product established name:

[Drug] Type A medicated article
[Drug] Type A liquid medicated article
[Drug] Type B medicated feed
[Drug] Type B liquid medicated feed
[Drug] Type C medicated feed
[Drug] Type C liquid medicated feed
[Drug] Type C free-choice medicated feed
[Drug] Type C liquid free-choice medicated feed
[Drug] Type C top-dress medicated feed

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***Add the following:***

# ▲⟨1153⟩ DRUG PRODUCTS CONTAINING NANOMATERIALS

## 1. INTRODUCTION

The current interest in nanotechnology and its application to the medical field has historical precedent. Initial products employed general physical chemistry of colloids to produce stable drug suspensions and improve bioavailability. The later introduction of liposomes began the evolution to today's more sophisticated and potentially revolutionary new technologies, which have accompanied the rapid growth in biotechnology product opportunities, notably including targeted drug delivery products.

The term "nanomaterials" by recent convention describes materials that have features or structures that exist on the 1- to 100-nm scale in any of the three spatial dimensions. The existence of a large number of formulations containing nanomaterials that are currently under development or review, or that have already received approval from the United States Food and Drug Administration (US FDA), emphasizes the need to identify important properties of these formulations, as well as methods of measuring these properties to ensure quality and performance. The US FDA has authored guidance on nanomaterials (*1*). In summary, the guidance recognizes materials with one or more dimensions within and outside of the nanoscale range but with properties attributed to its dimensions (less than 1000 nm). These materials often require measurements with specialized instruments (see *Table 1*).

**Table 1. Names and Definitions**

| Name of the Nanomaterial | Subcategory | Definition, Structure, and Description |
|---|---|---|
| Dendrimers (*2*) | — | Dendrimers are highly branched star-shaped macromolecules consisting of polymers [e.g., polyamidoamine (PAMAM)]. These structures can be linked to drug substances or used by physical/steric association to encapsulate drug substances. |
| Drug nanoparticles/Nanocrystals (*3*) | — | Nanoparticles consist of crystalline or amorphous forms of pure drug substance and are used as a formulation strategy to enhance bioavailability by increasing drug solubility as described by the (*4*) Kelvin equation and dissolution rate as described by the Noyes-Whitney equation. Examples of nanocrystal products include those used for cancer (megestrol acetate), organ rejection (sirolimus), and emesis (aprepitant) and dyslipidemia (fenofibrate). Nanocrystal drug products may be solid dosage forms or prepared as aqueous or nonaqueous suspensions. The latter often require stabilization with surfactants or polymeric stabilizers. A common route of administration is the oral route. Nanocrystals are made using milling, homogenization, and precipitation methods, as well as a combination of these methods. |
| Liposomes (*5*) | — | Lipid vesicles are composed of one or more concentric lipid bilayers enclosing a single or multiple aqueous compartments. Liposomes have been employed to formulate both water-soluble and water-insoluble drug substances. Due to their hydrophilic nature, the former can be incorporated into the internal aqueous compartment(s) of liposomes and the latter into the hydrophobic core of the lipid bilayer. Examples of drug substances used in liposome drug products include anticancer agents (i.e., doxorubicin, daunorubicin, vincristine, irinotecan, cytarabine), antifungals (i.e., amphotericin B, itraconozole), analgesics (i.e., morphine, bupivacaine), and vaccines (i.e., influenza, hepatitis A). Liposomes can be manufactured by extrusion (filtration), homogenization, and micromixing processes. The drug substance can be incorporated during liposome formation or by loading into empty preformed liposomes. Routes of administration include topical; parenteral, with intravenous administration as the most common route; and inhalation. |

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**Table 1. Names and Definitions** *(continued)*

| Name of the Nanomaterial | Subcategory | Definition, Structure, and Description |
|---|---|---|
| Micelles | — | Nano-scaled colloidal structures are formed by self-assembly of amphiphilic molecules. The constituents of the micelles can be either surfactants or block copolymers (*6*). For block copolymers, there are constructs in which the polymers employ different hydrophobic blocks [e.g., poly(propylene oxide), poly(L-amino acids), or poly(esters). An example of such block copolymers is, poly(ethylene glycol)-block-polylactide (PEG-PLA) polymers]. Polymeric micelles are used to target biological sites and minimize toxicity. As an example, a PEG-PLA polymeric micelle product improves the solubility of paclitaxel. Its route of administration is typically intravenous.<br>Classical micelles (*7*) are thermodynamically stable self-associating surfactant structures used to increase the solubility of sparingly soluble drug substances. Micelles are frequently used for parenteral delivery of anticancer agents, e.g., docetaxel. |
| Nanobubbles (*8*) | — | Nanobubbles are created using oscillating vibration sources to disperse air in water, usually in the presence of surfactant. Nanobubbles can be employed as contrast agents and also to deliver drug substance by virtue of their incorporation into the stabilizing agent at the surface of the bubble. |
| Nanoemulsions | — | Submicron oil-in-water or water-in-oil emulsions are stabilized with surfactants and cosurfactants. By definition, they have droplet sizes below 100 nm, but typically ranging less than 1 µm. Nanoemulsions (o/w) are used primarily to administer water-insoluble drug substances. They are made by high-shear emulsification methods or low-energy emulsification methods (phase inversion). Nanoemulsions are used in ophthalmic and oral products, for example, to deliver cyclosporin for dry eyes and for prophylaxis of organ rejection and immune regulation (*9*). |
| Nanofibers (*10*) | — | Nanofibers are rarely seen in pharmaceutical preparations. Nanofibers are nanoscale in one dimension and are used frequently in electrospun polymer-containing drugs (*11*) or alone, for example, as a wound-healing matrix (*12*). |
| Nanotubes | Carbon (*13*) | Nanotubes are rarely seen in pharmaceutical preparations. May be manufactured from a variety of substances. Usually an allotrope of carbon that forms into cylindrical carbon structures. These nanotubes have novel properties that make them potentially useful for a variety of pharmaceutical applications (e.g., controlled drug release) and tissue-engineering applications (e.g., as a scaffold for cell growth). |
| Nanotubes | Self-Assembled | Another nanotube example is the peptide drug lanreotide, which self-assembles at high concentrations to form nanotubes (liquid crystals). The lanreotide nanotube structure extends the bioavailability of the peptide for 1 month following a single intramuscular injection (*14*). |
| Nanoparticles | Inorganic (*15*) | Inorganic nanoparticles are discrete nanomaterials consisting of inorganic materials (e.g., gold, silver, silica, etc). These inorganic nanoparticles are used as a core substrate to which active moieties, e.g., small molecules or biomolecules, can be attached, particularly for antimicrobial agents, immune therapeutics and vaccines. |
| | | Iron oxide nanoparticles (colloids) are used for diagnostic imaging and iron replacement therapies. |
| | | Titanium dioxide and zinc oxide nanoparticles are used in sunscreens for the prevention of erythema. |

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**Table 1. Names and Definitions** *(continued)*

| Name of the Nanomaterial | Subcategory | Definition, Structure, and Description |
|---|---|---|
| Nanoparticles | Polymeric (natural and synthetic) (*16*) | Solid particles prepared by one of several methods involving either dispersion of preformed polymer or in situ polymerization. Polymers used in preformed particles include various naturally occurring proteins, polysaccharides, and synthetic polymers, e.g., polylactide (PLA), poly(lactide-co-glycolide) (PLG), poly(ethylene glycol)-block-polylactide (PEG-PLA), and other polyesters. In situ polymerization of monomers requires cross-linking. The drug is often incorporated into polymeric nanoparticles by an emulsion evaporation technique (*17*). |
| Nanoparticles | Solid lipid (*18*) | Polymeric nanoparticles are drug substances embedded in a solid lipid core, which is usually stabilized by a monolayer of phospholipids. Lipid nanoparticles are proposed for use in the delivery of hydrophobic drug substances. This also includes drug complexes with other components such as protein, lipid, polymer, or metal. |

## General Quality Tests of Drug Products Containing Nanomaterials

The following list is not comprehensive but represents the most common properties characterized for nanomaterials used in drug products.

### AERODYNAMIC PARTICLE SIZE DISTRIBUTION (APSD)

Products for inhalation are subject to aerodynamic particle size assessment by multistage cascade impactor in accordance with *Inhalation and Nasal Drug Products: Aerosols, Sprays, and Powders—Performance Quality Tests* ⟨601⟩ if intended for administration by inhalation aerosol or powder or in accordance with *Products for Nebulization—Characterization Tests* ⟨1601⟩ if intended for administration by a nebulizing system. The proportion of therapeutic aerosol found in the cascade impactor size fraction is determined by differences in particle inertia. Furthermore, quantification of drug substance mass is possible through chemical assay of the collected subfractions. These differences are related directly to the aerodynamic size, which is indicative of the likely deposition location(s) in the human respiratory tract. Although the size range within which these apparatuses operate is larger than the size range associated with single nanoparticles, their delivery in the formulated drug product to the respiratory tract will likely occur as microaggregated powder particles or as a suspension in liquid droplets. A small proportion of the aerosol may be generated as nanosized particles, for which alternative methods, in particular, low-pressure cascade impactors, might be considered in conjunction with conventional cascade impactors to extend the range within which sizing is undertaken (*19*).

### COMPOSITION AND STRUCTURE

Methods of determining composition depend on the nature of the material under investigation. For example, for inorganic molecules, elemental analysis by atomic absorption spectroscopy and atomic emission spectroscopy with flame ionization or inductively coupled plasma mass spectrometry can be employed. For organic polymeric systems, gel permeation chromatography/size exclusion chromatography with refractive index detection may be supplemented with viscosity determinations and application of the Mark-Houwink equation to estimate the molecular weight (*20*). If a multi-angle light scattering detector (MALS) is added the weight average molecular weight can be determined. Photon correlation spectroscopy (dynamic light scattering) also may be used for molecular weight determination. At a fundamental level, surfactants and polymer-based systems can be subjected to elemental analysis or spectroscopy methods. Electron microscopy techniques such as cryogenic transmission electron microscopy may be used for liposome/lipid-based nanoparticle formulations to determine their size and morphology (e.g., lamellarity). Other scattering methods, e.g., small-angle X-ray scattering (SAXS) and small-angle neutron scattering (SANS), may also be used to generate information on nanomaterial size. These scattering techniques are not routine due to their technical complexity and instrument availability but can also provide detailed information on form (shape), surface, and material interactions.

### DISSOLUTION/IN VITRO RELEASE

Multiple methods exist for dissolution testing. Importantly, care should be taken to ensure that the nanomaterial does not interfere with or interact with the testing materials or otherwise confound results. For example, an undissolved nanomaterial may pass through filters, resulting in a false elevation of solubility. To address this problem, the use of membrane diffusion and microdialysis has been proposed to separate particles from dissolved material (*21*). Fiber optic probes may also be employed. Some caution may be required when using the fiber optic probe technique with ultraviolet-visible (UV-Vis) detection to accurately estimate the quantity of dissolved material (*22*). However, the use of a fiber optic probe with different detection systems, such as infrared (IR) (*23*) or Raman (*24*) spectroscopy, may be a viable approach. Nanomaterials may also influence the accuracy of results by reacting with the surfaces of the dissolution apparatus. Therefore, novel dissolution methods may need to be developed.

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## ENCAPSULATION EFFICIENCY

For nanomaterials in which the drug substance is encapsulated in an excipient, e.g., lipid or polymer, the loading and encapsulation efficiency is usually determined. Encapsulation efficiency can be achieved by separating the drug substance in the nanomaterial from the free drug substance, typically using centrifugal filter devices. Size-exclusion spin columns or solid phase extraction (SPE) columns are used to separate free from encapsulated or carrier-associated drugs, performing an appropriate quantitative assay of free drug versus encapsulated drug as a proportion of the total drug present.

## PARTICLE SIZE

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) can be valuable tools to measure the particle size of individual nanoparticles. Atomic force microscopy (AFM) also may be used to visualize individual particles and may aid in measuring their size, shape, and surface texture.

## PARTICLE SIZE DISTRIBUTION

Dynamic light scattering (DLS), also known as photon correlation spectroscopy (PCS), can be used to measure the particle size and distribution of nanomaterials. This method measures fluctuations in scattered light intensity from particles in Brownian motion to calculate the sample particle size and distribution. Laser light scattering can also be used to measure the particle size and distribution of nanoparticles, although this technique may be somewhat limited depending on the size of the nanomaterials to be measured.

## PURITY

In some cases, the purity of the materials used to generate nanostructures is characterized to assure homogeneity among different batches. For example, the chemical composition of the bilayer of liposomes or micelles or the chemical purity of inorganic particles can be characterized to demonstrate batch-to-batch consistency.

## SHAPE

AFM is a useful tool for three-dimensional analysis of nanoparticles. This technique will allow measurement of particle size, shape, and surface texture. SEM and TEM may also be used for morphology information. SAXS and SANS may also be used to generate information on nanomaterial shape. Multiangle light scattering (MALS) may be used for determining shape, as it can also give information on the conformation (e.g., sphere, coil, and rod) of the molecules when coupled with separation techniques.

## SOLUBILITY

Typical methods for assessing solubility (e.g., the shake-flask method) may not be suitable for use with nanomaterials because the undissolved particles cannot be readily separated from the solution. Methods to assess the solubility of nanomaterials (i.e., nanoparticles or nanocrystals) sometimes use characterization of the particle size distribution and changes in the particle size distribution as a function of time as a surrogate or complementary data to dissolution data. More generally, solubility assessments for nanomaterials often use highly selective separation techniques (e.g., size exclusion chromatography or ultracentrifugation) capable of differentiating the dissolved and undissolved nanomaterials. To address this problem, the use of membrane diffusion and microdialysis has been proposed to separate particles from dissolved material (*21*). Fiber optic probes may also be employed, although some caution may be required when using the fiber optic probe technique with UV-Vis detection to accurately estimate the quantity of dissolved material (*22*). However, the use of a fiber optic probe with different detection systems, such as IR (*23*) or Raman (*24*) spectroscopy, may be a viable approach.

## SURFACE AREA

Gas adsorption techniques (generally using nitrogen or krypton) allow measurement of the nanoparticle surface area. Samples are exposed to varying pressures at which the incrementally dosed analysis gas will adsorb onto the surface of the nanoparticles. The surface area is then calculated. This technique may also be utilized to measure the average particle size.

## SURFACE PROPERTIES

Chemical composition (for example, as detected by X-ray photoelectron spectroscopy/secondary-ion mass spectrometry), charge, and reactivity influence surface properties. Measurement of the zeta potential and isoelectric point via electrophoretic or electroacoustic methods are often used to help predict whether nanomaterials will tend to aggregate or agglomerate or whether they will remain as discrete particles in the liquid vehicle. A high (positive or negative) zeta potential is one indication of a system in which nanoparticles will repel each other and remain as discrete particles, whereas a system with a low zeta potential (near zero, the isoelectric point) may be more likely to aggregate or agglomerate. Freeze-fracture scanning electron microscopy may also be useful in studying the aggregate or agglomerate morphology.

## PHYSICAL STABILITY

Physical stability, in terms of propensity of nanomaterial to aggregate or agglomerate can be assessed using methods such as controlled centrifugation techniques, which apply a force to the system and assess the level of segregation/separation/sedimentation, combined with DLS to assess the apparent particle size (hydrodynamic diameter) distribution. These techniques

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may be used as a part of standard stability testing. The integrity of the nanosystem with regard to the retention of drug substance should also be evaluated.

## 2. GENERAL QUALITY TESTS

The methods shown in *Table 2* relate to the properties of a drug product that may alter due to the presence of nanomaterials.

**Table 2**

| Name | Recommended Methods |
|---|---|
| Carbon nanotubes | **Dissolution/In vitro release**—New or existing general chapter<br>**Scanning, transmission, and atomic force microscopy**—*Scanning Electron Microscopy* ⟨1181⟩. Transmission and atomic force microscopy are described elsewhere (*25*).<br>**Dynamic light scattering**—*Analytical Methodologies Based on Scattering Phenomena—Light Diffraction Measurements of Particle Size* ⟨1430.2⟩<br>**Solubility**—*Solubility Measurements* ⟨1236⟩<br>**Gas adsorption surface area measurement**—*Specific Surface Area* ⟨846⟩<br>**X-Ray photoelectron spectroscopy and secondary ion mass spectrometry**—Techniques are described elsewhere (*26*).<br>**Zeta potential**—*Determination of Zeta Potential by Electrophoretic Light Scattering* ⟨432⟩ and *Analytical Methodologies Based on Scattering Phenomena—Electrophoretic Light Scattering (Determination of Zeta Potential)* ⟨1430.4⟩<br>**Sedimentation methods/Centrifugation**—Techniques are described elsewhere (*27*). |
| Dendrimers | All general quality tests identified in *General Quality Tests of Drug Products Containing Nanomaterials* [a] |
| Nanocrystals and nanoparticles in the amorphous state | All general quality tests identified in *General Quality Tests of Drug Products Containing Nanomaterials*[a] |
| Drug nanoparticles | All general quality tests identified in *General Quality Tests of Drug Products Containing Nanomaterials*[a] |
| Inorganic nanoparticles | All general quality tests identified in *General Quality Tests of Drug Products Containing Nanomaterials*[a] |
| Liposomes | **Dissolution/In vitro release**[a]—*Dissolution* ⟨711⟩ and *Oral Dosage Forms—Performance Tests* ⟨1711⟩<br>**High resolution microscopy**—⟨1181⟩. Transmission and atomic force microscopy are described elsewhere (*25*).<br>**Dynamic light scattering**—*Analytical Methodologies Based on Scattering Phenomena—Light Diffraction Measurements of Particle Size* ⟨1430.2⟩<br>**Solubility**—*Solubility Measurements* ⟨1236⟩<br>**Zeta potential**—⟨432⟩ and ⟨1430.4⟩ |
| Micelles | **Dissolution/In vitro release**—⟨711⟩ and ⟨1711⟩<br>**High resolution microscopy**—⟨1181⟩. Transmission and atomic force microscopy are described elsewhere (*25*).<br>**Dynamic light scattering**—⟨1430.2⟩<br>**Solubility**—⟨1236⟩<br>**Zeta potential**—⟨432⟩ and ⟨1430.4⟩ |
| Nanobubbles | **Dissolution/In vitro release**—⟨711⟩ and ⟨1711⟩<br>**High resolution microscopy**—⟨1181⟩. Transmission and atomic force microscopy are described elsewhere (*25*)<br>**Dynamic light scattering**—⟨1430.2⟩<br>**Zeta potential**—⟨432⟩ and ⟨1430.4⟩ |
| Nanoemulsions | **Dissolution/In vitro release**—⟨711⟩ and ⟨1711⟩<br>**High resolution microscopy**—⟨1181⟩. Transmission and atomic force microscopy are described elsewhere (*25*)<br>**Dynamic light scattering**—⟨1430.2⟩<br>**Solubility**—⟨1236⟩<br>**Zeta potential**— ⟨432⟩ and ⟨1430.4⟩ |
| Nanofibers | All general quality tests identified in *General Quality Tests of Drug Products Containing Nanomaterials*[a] |
| Polymeric nanoparticles (natural and synthetic) | All general quality tests identified in *General Quality Tests of Drug Products Containing Nanomaterials*[a] |
| Solid lipid nanoparticles | All general quality tests identified in *General Quality Tests of Drug Products Containing Nanomaterials*[a] |

[a] Where included in an aerosol dosage form, ⟨601⟩ should be consulted.

## 3. DESCRIPTION OF PRODUCT QUALITY TESTS

- **Dissolution:** ⟨711⟩ and ⟨1711⟩
- **Dynamic light scattering:** ⟨1430.2⟩

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- **Crystallinity:** ⟨*696*⟩
- **Gas adsorption surface area measurement:** ⟨846⟩
- **High resolution microscopy (scanning, transmission, and atomic force microscopy):** ⟨1181⟩. Transmission and atomic force microscopy are described elsewhere (*25*).
- **Inertial impaction:** ⟨601⟩
- **Solubility:** ⟨1236⟩
- **Ultracentrifugation (sedimentation):** Techniques are described elsewhere (*27*).
- **X-Ray photoelectron spectroscopy and secondary ion mass spectrometry:** Techniques are described elsewhere (*26*).
- **Zeta potential:** ⟨432⟩ and ⟨1430.4⟩.

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# ⟨1160⟩ PHARMACEUTICAL CALCULATIONS IN PHARMACY PRACTICE

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## 1. INTRODUCTION

The purpose of this general chapter is to provide general information to assist pharmacists and support personnel in performing the necessary calculations for compounding and dispensing medications. This general chapter is not inclusive of all the information necessary for performing pharmaceutical calculations. For additional information regarding pharmaceutical calculations, consult a pharmaceutical calculations textbook. For additional information on pharmaceutical compounding and drug stability, see *Pharmaceutical Compounding—Nonsterile Preparations* ⟨795⟩, *Pharmaceutical Compounding—Sterile Preparations* ⟨797⟩, *Packaging and Storage Requirements* ⟨659⟩, *Quality Assurance in Pharmaceutical Compounding* ⟨1163⟩, and *Stability Considerations in Dispensing Practice* ⟨1191⟩.

Correct pharmaceutical calculations can be accomplished by using proper conversions from one measurement system to another and properly placing decimal points (or commas, in countries where it is customary to use these in the place of decimal points), by understanding the arithmetical concepts, and by paying close attention to the details of the calculations. Before proceeding with any calculation, pharmacists should do the following: (a) read the entire formula or prescription carefully; (b) determine the materials that are needed; and then (c) select the appropriate methods of preparation and the appropriate calculations.

Logical methods that require as few steps as possible should be selected to ensure that calculations are done accurately and correctly. A pharmacist should double-check each calculation or have someone else double-check, e.g., a technician, if another pharmacist is not available, before proceeding with compounding the preparation. One expedient method of double-checking is estimation, which consists of convenient rounding (e.g., 0.012 to 0.01, 0.44 to 0.5, 18.3 to 20, and 476 to 500) to approximate the magnitude of answers.

## 2. AMOUNTS OF ACTIVE INGREDIENTS

The pharmacist must be able to calculate the amount or concentration of drug substances in each unit or dosage portion of a compounded preparation at the time it is prepared and again at the time it is dispensed. Pharmacists must perform calculations and measurements to obtain, theoretically, 100% of the amount of each ingredient in compounded formulations. Calculations must account for the active ingredient, or active moiety, and water content of drug substances, which includes those in the chemical formulas of hydrates. Official drug substances and added substances must meet the requirements in general chapter *Loss on Drying* ⟨731⟩, which must be included in the calculations of amounts and concentrations of ingredients. The pharmacist should consider the effect of ambient humidity on the gain or loss of water from drugs and added substances in containers subjected to intermittent opening over prolonged storage. Each container should be opened for the shortest duration necessary and then closed tightly immediately after use.

The nature of the drug substance to be weighed and used in compounding a prescription must be known. If the substance is a hydrate, its anhydrous equivalent weight may need to be calculated. On the other hand, if there is adsorbed moisture present that is either specified on a Certificate of Analysis (CoA) or that is determined in the pharmacy immediately before the

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drug substance is used in the preparation (see ⟨731⟩), this information must be used when calculating the amount of drug substance that is to be weighed to determine the exact amount of anhydrous drug substance required.

There are cases in which the required amount of a dose is specified in terms of a cation (e.g., $Li^+$), an anion (e.g., $F^-$), or a molecule (e.g., theophylline in aminophylline). In these instances, the drug substance weighed is a salt or complex, a portion of which represents the pharmacologically active moiety. Thus, the exact amount of such substances weighed must be calculated on the basis of the required quantity of the pharmacological moiety.

The following formula may be used to calculate the theoretical weight of an ingredient in a compounded preparation:

$$W = AB/CD$$

$W$ = actual weighed amount
$A$ = prescribed or pharmacist-determined weight of the active or functional moiety of drug or added substance
$B$ = molecular weight (MW) of the ingredient, including waters of hydration for hydrous ingredients
$C$ = MW of the active or functional moiety of a drug or added substance that is provided in the MW of the weighed ingredient
$D$ = the fraction of dry weight when the percent by weight of adsorbed moisture content is known from the loss on drying procedure (see ⟨731⟩) or from the CoA. The CoA should be lot specific.

## 2.1 Active Ingredients

### 2.1.1 CALCULATING DRUGS DOSED AS SALT FORM AND HYDRATE

**Examples—Drugs dosed as salt form and hydrate**

1. Drugs dosed as salt form and hydrate

Triturate morphine sulfate and lactose to obtain 10 g in which there are 30 mg of morphine sulfate for each 200 mg of the morphine–lactose mixture. [NOTE—Morphine is dosed as the morphine sulfate, which is the pentahydrate.]

$W$ = weight of morphine sulfate (g)
$A$ = weight of morphine sulfate pentahydrate in the prescription, 1.5 g
$B$ = MW of morphine sulfate pentahydrate, 759 g/mol
$C$ = MW of morphine sulfate pentahydrate, 759 g/mol
$D$ = 1.0

To solve the equation:

$$W = (1.5\ \text{g} \times 759\ \text{g/mol})/(759\ \text{g/mol} \times 1) = 1.5\ \text{g of morphine sulfate pentahydrate}$$

2. Active drug moiety and correction for moisture

Accurately weigh an amount of aminophylline to obtain 250 mg of anhydrous theophylline. [NOTE—In this example, the powdered aminophylline dihydrate weighed contains 0.4% w/w absorbed moisture as stated in the CoA received by the pharmacy.]

$$W = AB/CD$$

$W$ = weight of aminophylline dihydrate (mg)
$A$ = weight of anhydrous theophylline, 250 mg
$B$ = MW of aminophylline dihydrate, 456 g/mol
$C$ = MW of anhydrous theophylline, 360 g/mol
$D$ = 0.996

[NOTE—One mol of aminophylline contains 2 mol of theophylline. Theophylline has a MW of 180.]

To solve the equation:

$$W = (250\ \text{mg} \times 456\ \text{g/mol})/(360\ \text{g/mol} \times 0.996) = 318\ \text{mg of aminophylline dihydrate}$$

## 2.2 Hydrates, Salts, and Esters

Frequently, for stability or other reasons such as taste or solubility, the base form of a drug is administered in another form such as a salt or an ester. This altered form of the drug usually has a different MW, and at times it may be useful to determine the amount of the base form of the drug in the altered form.

### 2.2.1 CALCULATING HYDRATES, SALTS, AND ESTERS

**Examples—Hydrates, salts, and esters**

1. Hydrates

If a prescription for 100 g of lidocaine hydrochloride 2% gel is to be made, 2 g of anhydrous lidocaine hydrochloride could be used, or the equivalent amount of lidocaine hydrochloride monohydrate could be calculated as follows:

$W$ = weight of lidocaine hydrochloride monohydrate (g)
$A$ = weight of anhydrous lidocaine hydrochloride in the prescription, 2 g

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*B* = MW of lidocaine hydrochloride monohydrate, 288.81 g/mol
*C* = MW of anhydrous lidocaine hydrochloride, 270.80 g/mol
*D* = 1.0

To solve the equation:

$$W = (2\ \text{g} \times 288.81\ \text{g/mol})/(270.80\ \text{g/mol} \times 1) = 2.133\ \text{g of lidocaine hydrochloride monohydrate}$$

2. Salts

A prescription calls for 10 mL of a fentanyl topical gel at a concentration 50 mcg fentanyl/0.1 mL prepared from fentanyl citrate. The amount of fentanyl citrate required for the preparation could be calculated as follows:

Amount of fentanyl needed for the preparation:

$$(50\ \text{mcg fentanyl}/0.1\ \text{mL}) \times 10\ \text{mL} = 5000\ \text{mcg of fentanyl}$$

*W* = weight of fentanyl citrate in the prescription (mcg)
*A* = weight of fentanyl in the prescription, 5000 mcg
*B* = MW of fentanyl citrate, 528.59 g/mol
*C* = MW of fentanyl, 336.47 g/mol
*D* = 1.0

To solve the equation:

$$W = (5000\ \text{mcg} \times 528.59\ \text{g/mol})/(336.47\ \text{g/mol} \times 1) = 7855\ \text{mcg of fentanyl citrate}$$

3. Esters

The amount of cefuroxime axetil contained in a single 250-mg cefuroxime tablet can be calculated as follows:

*W* = weight of cefuroxime axetil in tablet (mg)
*A* = weight of cefuroxime in the prescription, 250 mg
*B* = MW of cefuroxime axetil, 510.47 mg/mmol
*C* = MW of cefuroxime, 424.39 mg/mmol
*D* = 1.0

To solve the equation:

$$W = (250\ \text{mg} \times 510.47\ \text{g/mol})/(424.39\ \text{g/mol} \times 1) = 300\ \text{mg of cefuroxime axetil}$$

# 3. DOSAGES BY BODY WEIGHT AND SURFACE AREA

## 3.1 Dosing by Weight

Doses are frequently expressed as mg of drug per kg of body weight per a dosing interval.

### 3.1.1 CALCULATING DOSING BY WEIGHT

**Example—Dosing by weight**

A physician orders azithromycin for oral suspension at a dose of 15 mg/kg/day, divided every 12 h, for a child that weighs 36 lb. Calculate the volume of oral suspension, in mL, that should be administered for each dose of a 200-mg/5-mL suspension as follows:

1. Calculate the child's weight in kg:

$$36\ \text{lb} \times \text{kg}/2.2\ \text{lb} = 16.4\ \text{kg}$$

2. Multiply the weight, in kg, by the dosing rate:

$$16.4\ \text{kg} \times 15\ \text{mg/kg/day} = 246\ \text{mg/day}$$

3. Divide the total daily dose by the number of doses/day:

$$246\ \text{mg}/2\ \text{doses} = 123\ \text{mg/dose}$$

4. Calculate the volume of each dose using ratio and proportion:

$$(123\ \text{mg/dose})/(200\ \text{mg}/5\ \text{mL}) = 3.1\ \text{mL/dose}$$

Some calculations may also be completed using dimensional units analysis (DUA). The DUA should begin at the left end with a factor containing the numerator answer units. All units other than those in the answer should cancel. If using DUA, the preceding equation would be as follows:

$$36\text{-lb child} \times \frac{\text{kg}}{2.2\ \text{lb}} \times \frac{15\ \text{mg}}{\text{kg} \cdot \text{day}} \times \frac{5\ \text{mL}}{200\ \text{mg}} \times \frac{\text{day}}{2\ \text{doses}} = \frac{3.1\ \text{mL}}{\text{dose}}$$

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## 3.2 Dosing by Body Surface Area (Humans)

Some medications, including chemotherapeutic agents, require dosing by body surface area (BSA). The dose is expressed as amount of drug per meter squared ($m^2$). BSA may be calculated using the following formulas:

$$BSA\left(m^2\right) = \sqrt{[Height(in)] \times Weight(lb)]/3131}$$
$$BSA\left(m^2\right) = \sqrt{[Height(cm) \times Weight(kg)]/3600}$$

### 3.2.1 CALCULATING BY BODY SURFACE AREA (HUMAN)

**Example—Dosing by BSA (humans)**
A physician orders rituximab at a dose of 375 mg/$m^2$ every week for 6 weeks for a patient who is 6 ft 2 in tall and weighs 183 lb. Calculate the volume, in mL, of 10-mg/mL rituximab injection needed to make each IV infusion dose as follows:

1. Calculate the patient's BSA:

$$m^2 = \sqrt{[74in \times 183\,lb]/3131} = 2.08m^2$$

2. Multiply the BSA by the dosing rate:

$$2.08\ m^2 \times 375\ mg/m^2 = 780\ mg/dose$$

3. Calculate the volume of each dose using ratio and proportion:

$$(780\ mg/dose)/(10\ mg/mL) = 78\ mL/dose$$

The preceding calculation may also be completed using DUA as follows:

$$2.08\text{-}m^2\ patient \times \frac{375\ mg}{m^2 \cdot dose} \times \frac{mL}{10mg} = \frac{78\ mL}{dose}$$

## 3.3 Dosing By Body Surface Area (Animals)

BSA for cats and dogs may be calculated using the following formulas. For other animals, consult an appropriate veterinary medicine reference.

Body surface area for cats:

$$BSA\ (m^2) = \{10 \times [body\ weight\ (g)]^{0.667}\}/10{,}000$$

Body surface area for dogs:

$$BSA\ (m^2) = \{10.1 \times [body\ weight\ (g)]^{0.667}\}/10{,}000$$

### 3.3.1 CALCULATING DOSING BY BODY SURFACE AREA (ANIMALS)

**Example—Dosing by BSA (animals)**
A veterinarian orders oral cyclophosphamide therapy at a dose of 50 mg/$m^2$ for a cat who weighs 5.8 kg. Calculate the dose of cyclophosphamide as follows:

1. Calculate the cat's BSA:

$$BSA\ (m^2) = [10 \times (5800\ g)^{0.667}]/10{,}000 = 0.324\ m^2$$

2. Multiply the BSA by the dosing rate:

$$0.324\ m^2 \times 50\ mg/m^2 = 16.2\ mg$$

# 4. BIOLOGICAL POTENCY UNITS

Because some substances cannot be completely characterized by chemical and physical means, it may be necessary to express quantities of activity in biological units of potency [see the *USP General Notices 5.50.10, Units of Potency (Biological)*].

## 4.1 Calculating by Use of Potency Units

### EXAMPLES—USE OF POTENCY UNITS

1. Potency units-to-milligrams conversion
A dose of penicillin G benzathine for streptococcal infection is 1.2 million units administered intramuscularly. If a specific product contains 1180 units/mg, calculate the amount, in mg, of penicillin G benzathine in the dose as follows:

$$(1{,}200{,}000\ units)/(1180\ units/mg) = 1017\ mg\ of\ penicillin\ G\ benzathine$$

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2. Potency units-to-milligrams conversion

A prescription calls for 60 g of an ointment containing 150,000 units of nystatin per gram. Calculate the quantity of nystatin with a potency of 4400 units/mg that should be weighed for the prescription as follows:

60 g × (150,000 units of nystatin/g) = 9,000,000 units

9,000,000 units/(4400 units/mg) = 2045 mg of nystatin

## 5. VOLUME AND WEIGHT SUMS

Weights are additive in most mixtures of liquids, semisolids, and solids. Volumes in mixtures of miscible solutions and pure liquids may or may not be additive, based primarily on the effects of volume proportions and intermolecular hydrogen bonding. For example, mixtures containing equal or near-equal volumes of water and ethanol (and other miscible mono-hydroxy alcohols) will be exothermic and result in a volume contraction of <5%, e.g., 50 mL of water + 50 mL of ethanol yield 97–98 mL at 20°–25°. Negligible volume contraction occurs between water and polyhydroxy or polyhydric alcohols, e.g., glycerin and propylene glycol. Volumes are additive with usually negligible error in aqueous mixtures that contain <10% of mono-hydroxy alcohols, i.e., there is <0.5% volume contraction.

## 6. DENSITY AND SPECIFIC GRAVITY

Density is defined as the mass of a substance in air at a specific temperature (typically 25°) per unit volume of that substance at the same temperature. Density may be calculated with the following equation:

Density = (mass of substance/volume of substance) at a particular temperature and pressure

Specific gravity (SG) is the unitless ratio of the density of a substance to the density of water at 4°, or [(g of substance/mL)/1.00 g/mL]. Alternatively, SG can be calculated at a particular temperature in some common units of density from density of substance per density of water.

SG may be calculated with the following equation:

SG = (weight of the substance)/(weight of an equal volume of water)

### 6.1 Calculating Density and Specific Gravity

EXAMPLES—DENSITY AND SPECIFIC GRAVITY

1. Density calculation

2.3 g of activated charcoal powder occupies a bulk volume of 5.2 mL at 20° and 1 atm. The density of activated charcoal powder can be calculated as follows:

Density = 2.3 g/5.2 mL = 0.44 g/mL

2. SG calculation

125 g of glycerin occupies a volume of 99 mL at 25°. [NOTE—The density of water at 25° is 0.997 g/mL.] The SG of glycerin can be calculated as follows:

SG = (125 g/99 mL)/(0.997 g/mL) = 1.266

3. Concentrated acid calculation

Hydrochloric acid is approximately a 37% w/w solution of hydrochloric acid in water. Calculate the amount, in g, of hydrochloric acid contained in 75 mL of hydrochloric acid as follows. [NOTE—The SG of hydrochloric acid is 1.18.]

37% w/w × 1.18 = 43.7% w/v

(43.7 g/100 mL) × 75 mL = 32.8 g of hydrochloric acid

## 7. MILLIEQUIVALENTS AND MILLIMOLES

[NOTE—This section addresses milliequivalents (mEq) and millimoles (mmol) as they apply to electrolytes for dosage calculations. See also *8. Concentration Expressions*.]

The quantities of electrolytes administered to patients are usually expressed in terms of mEq. Weight units such as mg or g are not often used for electrolytes because the electrical properties of ions are best expressed as mEq. An equivalent (Eq) is the weight of a substance that supplies 1 unit of charge. An equivalent weight is the weight, in g, of an atom or radical, divided by the valence of the atom or radical. A mEq is $1/1000^{th}$ of an Eq. The equivalent weight of a compound may be determined by dividing its formula or MW in g by the valence of its largest valence ion.

A mole equals one gram-atomic weight or gram-molecular weight of a substance. A millimole equals $1/1000^{th}$ of a mole.

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## 7.1 Calculating Milliequivalents and Millimoles

EXAMPLES—MILLIEQUIVALENTS AND MILLIMOLES

1. Calculate the mEq weight of calcium. [NOTE—Calcium has a MW of 40.08, and the valence of calcium is $2^+$.]

Eq weight = 40.08 g/2 = 20.04 g

mEq weight = 20.04 g/1000 = 0.02004 g = 20.04 mg

2. Calculate the quantity, in mEq, of potassium in a 250-mg Penicillin V Potassium Tablet. [NOTE—Penicillin V potassium has a MW of 388.48 g, there is one potassium atom in the molecule, and the valence of potassium is $1^+$.]

Eq weight = 388.48 g/1 = 388.48 g

mEq weight = 388.48 g/1000 = 0.38848 g = 388.48 mg

(250 mg/tablet)/(388.48 mg/mEq) = 0.644 mEq of potassium/tablet

3. Calculate the mEq of magnesium and sulfate in a 2-mL dose of 50% Magnesium Sulfate Injection. [NOTE—Magnesium sulfate ($MgSO_4 \cdot 7H_2O$) has a MW of 246.47, and the highest valence ion is magnesium $2^+$ and sulfate $2^-$.]

(50 g/100 mL) × (2 mL/dose) = 1 g/dose

Eq weight = 246.47 g/2 = 123.24 g/Eq

(1g/dose)/(123.24 g/Eq) = 0.008114 Eq = 8.114 mEq of both magnesium and sulfate per dose

This problem may also be worked using DUA as follows:

$$\frac{50\ \text{g}}{100\ \text{mL}} \times \frac{2\ \text{mL}}{\text{dose}} \times \frac{2\ \text{Eq}}{246.47\ \text{g}} \times \frac{1000\ \text{mEq}}{\text{Eq}} = \frac{8.114\ \text{mEq}}{\text{dose}}$$

4. A vial of sodium chloride injection contains 3 mEq/mL of sodium chloride. Calculate the strength, in % w/v, of the injection. [NOTE—Sodium chloride has a MW of 58.44.]

$$\frac{3\ \text{mEq}}{\text{mL}} \times \frac{58.44\ \text{g}}{1\ \text{Eq}} \times \frac{\text{Eq}}{1000\ \text{mEq}} = \frac{0.1753\ \text{g}}{\text{mL}}$$

(0.1753 g/mL) × 100 mL = 17.53 g in 100 mL = 17.53% w/v

5. Calculate the weight of potassium in mmol. [NOTE—Potassium has a MW of 39.1.]
The weight of 1 mol is 39.1 g and the weight in mmol is:

39.1 g/1000 = 0.0391 g or 39.1 mg

6. Calculate the mmol of penicillin V potassium in a 250-mg Penicillin V Potassium Tablet. [NOTE—Penicillin V potassium has a MW of 388.48.]
The weight of 1 mol is 388.48 g, and the weight of 1 mmol is:

388.48 g/1000 = 0.38848 g or 388.48 mg

$$\frac{250\ \text{mg}}{\text{tablet}} \times \frac{\text{mmol}}{388.48\ \text{mg}} = \frac{0.644\ \text{mmol of Penicillin V potassium}}{\text{tablet}}$$

## 8. CONCENTRATION EXPRESSIONS

The concentration expressions in this section refer to homogeneous mixtures of the following states of matter at a temperature of 20°–30° and pressure of 1 atm (29.92 in Hg, 760 mm Hg, 101.3 kPa, 1013.3 mb): gas in gas, gas in liquid, liquid in liquid, liquid in semisolid, solid in liquid, solid in semisolid, and solid in solid. Concentration expressions used in pharmacy practice and pharmaceutical research include, but are not limited to, those listed in *Table 1*. Common metric drug strength and clinical concentrations include, for example, mcg/mL, mg/dL, g or mg per L, and ng/µL (see *General Notices 8.240, Weights and Measures*).

**Table 1**

| Title | Abbreviation | Definition |
|---|---|---|
| Mass in volume ratios | None is standard | Mass of a dispersed or dissolved ingredient per volume amount of mixtures containing that ingredient |
| mEq[a] per volume | mEq/volume unit | mEq of an electrolyte or salt per unit of volume of solutions containing that electrolyte or salt |

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**Table 1** *(continued)*

| Title | Abbreviation | Definition |
|---|---|---|
| Molality | m | mol[b] of a solute/kg of a solvent containing that solute[c] |
| Molarity | M | mol of a solute/L of a solvent containing that solute[d] |
| Normality[e] | N | Equivalents (Eq[f]) of a solute/L of a solvent containing that solute[g] |
| Parts per million | ppm | Parts of a gas, liquid, or solid per 1 million part of another gas, liquid, or solid containing the first gas, liquid, or solid |
| % Volume in volume | % v/v | mL of liquid per 100 mL of a solvent containing that liquid |
| % Weight in volume | % w/v | g of a solute per 100 mL of a solvent containing that solute |
| % Weight in weight | % w/w | g of a solute per 100 g of a mixture containing that solute |
| Ratio strength | 1:*R* | 1 part of an ingredient per $R^h$ parts of a mixture containing that ingredient |
| | 1 in *R* | 1 part of an ingredient in $R^h$ parts of a mixture containing that ingredient |
| | *X*:*Y* | $X^h$ parts of one ingredient per $Y^h$ parts of another ingredient in a mixture |

[a] 1 mEq = Eq/1000.
[b] The abbreviation for mole is mol.
[c] 1 mol of solute per 1 kg of solvent is a 1 molal (1 m) solution.
[d] 1 mol of solute per 1 L of solution of that solute is a 1 molar (1 M) solution.
[e] Normality = (Molarity × largest valence ion of a compound), e.g., (18 M $H_2SO_4$ × 2) = 36 N $H_2SO_4$, where 2 derives from the $2^-$ valence of $SO_4$.
[f] Eq of a compound = (1 mol × largest valence ion of a compound), e.g., 1 mol of lithium citrate = 3 Eq of lithium citrate; 1 mol of $Ca(gluconate)_2$ = 2 Eq of $Ca(gluconate)_2$; and 1 mol of KCl = 1 Eq of KCl.
[g] 1 Eq of solute per 1 L of solution of that solute is a 1 normal (1 N) solution.
[h] *R*, *X*, and *Y* are whole numbers.

## 8.1 Calculating Normality

EXAMPLE—NORMALITY

Calculate the amount of sodium bicarbonate powder needed to prepare 50 mL of a 0.07 N solution of sodium bicarbonate ($NaHCO_3$). [NOTE—Sodium bicarbonate has a MW of 84.01.] In an acid or base reaction, because $NaHCO_3$ may act as an acid by giving up one proton, or as a base by accepting one proton, one Eq of $NaHCO_3$ is contained in each mole of $NaHCO_3$.

$$0.050\ \text{L} \times \frac{0.07\ \text{Eq}}{\text{L}} \times \frac{1\ \text{mol}}{1\ \text{Eq}} \times \frac{84.01\ \text{g}}{1\ \text{mol}} = 0.294\ \text{g of sodium bicarbonate}$$

## 8.2 Calculating Percentage Concentrations

Percentage concentrations of solutions and other homogeneous mixtures are usually expressed in one of three common forms in which numerator and denominator quantities are in g and mL measurement units.

1. Volume percent (% v/v) = (volume of liquid solute/volume of solution or suspension) × 100
   or % v/v = mL of liquid solute in 100 mL of solution or suspension
2. Weight percent (% w/w) = (weight of solute/weight of mixture) × 100
   or % w/w = g of ingredient in 100 g of mixture
3. Weight in volume percent (% w/v) = (weight of solute/volume of solution or suspension) × 100
   or % w/v = g of solute in 100 mL of solution or suspension

The preceding three equations may be used to calculate any one of the three values (i.e., weights, volumes, or percentages) in a given equation if the other two values are known (see also *General Notices 8.140, Percentage Concentrations*).

EXAMPLES—PERCENTAGE CONCENTRATIONS

1. Weight percent
   A prescription order reads as follows (see *Table 2*):

**Table 2**

| | |
|---|---|
| Zinc oxide | 7.5 g |
| Calamine | 7.5 g |
| Starch | 15 g |
| White petrolatum | 30 g |

Calculate the percentage concentration for each of the four components using the preceding weight percent equation as follows:

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A. The total weight of ointment = 7.5 g + 7.5 g + 15 g + 30 g = 60.0 g
B. The weight percent of zinc oxide = (7.5 g of zinc oxide/60 g of ointment) × 100% = 12.5%
C. The weight percent of calamine = (7.5 g of calamine/60 g of ointment) × 100% = 12.5%
D. The weight percent of starch = (15 g of starch/60 g of ointment) × 100% = 25%
E. The weight percent of white petrolatum = (30 g of white petrolatum/60 g of ointment) × 100% = 50%

2. Volume percent
A prescription order reads as follows:
Rx: Eucalyptus Oil 3% v/v in Mineral Oil.
Dispense 30 mL.
Calculate the quantities of ingredients in this prescription using the volume percent equation as follows:
A. The amount of eucalyptus oil.

$$3\%\ v/v = (\text{volume of oil in mL}/30.0\ \text{mL}) \times 100\%$$

$$\text{volume in oil} = 0.9\ \text{mL of eucalyptus oil}$$

B. The amount of mineral oil.

$$30\ \text{mL} - 0.9\ \text{mL} = 29.1\ \text{mL of mineral oil}$$

## 8.3 Conversions of Concentration Expressions

### 8.3.1 SOLID-IN-LIQUID SOLUTION CONVERSIONS

The calculations used to convert from percent weight in volume, % w/v, to other concentrations and vice versa, using the same densities and formula or MWs, are illustrated as follows for calcium chloride, magnesium sulfate, and potassium chloride solutions in water.

**8.3.1.1 Calculating solid-in-liquid conversions**

*Examples—Solid-in-liquid conversions*

1. Convert 10% w/v calcium chloride ($CaCl_2 \cdot 2H_2O$) to molality (m). [NOTE—Calcium chloride has a MW of 147.01 g; 10% w/v solution has a density of 1.087 g/mL.]

$$10\%\ w/v = 10\ \text{g of calcium}/100\ \text{mL of solution}$$

Using the density of the solution:

$$100\ \text{mL of solution} \times 1.087\ \text{g/mL} = 108.7\ \text{g of solution}$$

$$108.7\ \text{g of solution} - 10\ \text{g of calcium chloride} = 98.7\ \text{g of water} = 0.0987\ \text{kg of water}$$

$$10\ \text{g of calcium chloride}/(147.01\ \text{g of calcium chloride/mol of calcium chloride}) = 0.068\ \text{mol of calcium chloride}$$

$$0.068\ \text{mol of calcium chloride}/0.0987\ \text{kg of water} = 0.689\ \text{m}$$

2. Convert 50% w/v magnesium sulfate ($MgSO_4 \cdot 7H_2O$) to molarity (M). [NOTE—Magnesium sulfate has a MW of 246.47 g.]

$$\frac{50\ \text{g}}{100\ \text{mL}} \times \frac{\text{mol}}{246.47\ \text{g}} \times \frac{1000\ \text{mL}}{1\ \text{L}} = 2.092\ \text{M}$$

3. Convert 10% w/v calcium chloride ($CaCl_2 \cdot 2H_2O$) to normality (N).

$$\frac{10\ \text{g}}{100\ \text{mL}} \times \frac{1\ \text{mol}}{147.01\ \text{g}} \times \frac{2\ \text{Eq}}{1\ \text{mol}} \times \frac{1000\ \text{mL}}{1\ \text{L}} = 1.36\ \text{N}$$

*2 Eq/mol derived from the $2^+$ valence of calcium

4. Convert 10% w/v calcium chloride ($CaCl_2 \cdot 2H_2O$) to mEq/mL.

$$\frac{10\ \text{g}}{100\ \text{ml}} \times \frac{1\ \text{mol}}{147.01\ \text{g}} \times \frac{2\ \text{Eq}}{1\ \text{mol}} \times \frac{1000\ \text{mEq}}{1\ \text{Eq}} = 1.36\ \text{mEq/mL}$$

5. Convert 0.1% w/v calcium chloride ($CaCl_2 \cdot 2H_2O$) to ppm.

$$(0.1\ \text{g}/100\ \text{mL}) \times (1 \times 10^6\ \text{ppm}) = 1000\ \text{ppm}$$

6. Convert 33% w/v potassium chloride (KCl) to 1:*R* ratio strength.

$$(1/R) = (33\ \text{g}/100\ \text{mL})$$

$$R = 3.03$$

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1:*R* = 1:3

### 8.3.2 LIQUID-IN-LIQUID SOLUTION CONVERSIONS

The calculations used to convert from percent weight in weight, % w/w, and volume in volume, % v/v, to other concentrations and vice versa using the same densities and formula or MWs, are illustrated for glycerin and isopropyl alcohol in water. Besides liquid-in-semisolid, solid-in-semisolid, and solid-in-solid mixtures, % w/w is used for viscous liquids, such as coal tar, glycerin, and concentrated acids.

**8.3.2.1 Converting liquid-in-liquid solutions**

*Examples—Liquid-in-liquid conversions*

1. Convert 50% w/w glycerin to % w/v. [NOTE—50% w/w glycerin has a density of 1.13 g/mL.]

(50 g/100 g) × (1.13 g/mL) = 0.565 g/mL

56.5 g/100 mL = 56.5% w/v

2. Convert 70% v/v isopropyl alcohol to % w/w. [NOTE—Isopropyl alcohol has a density of 0.79 g/mL, and 70% v/v isopropyl alcohol has a density of 0.85 g/mL.]

70 mL of isopropyl alcohol × (0.79 g/mL) = 55.3 g of isopropyl alcohol

100 mL of solution × (0.85 g/mL) = 85 g of solution

(55.3 g of isopropyl alcohol/85 g of solution) × 100 = 65.06% w/w

3. Convert 70% v/v isopropyl alcohol to % w/v. The following values are from example 2.

55.3 g of isopropyl alcohol/100 mL of solution = 55.3% w/v

4. Convert 50% w/w glycerin to molality (m). [NOTE—Glycerin has a MW of 92.1.]

50 g of glycerin/(92.1 g/mol) = 0.543 mol of glycerin

100 g of solution – 50 g of glycerin = 50 g of water = 0.05 kg of water

(0.543 mol of glycerin/0.05 kg of water) = 10.86 m

5. Convert 70% v/v isopropyl alcohol to molality (m). [NOTE—Isopropyl alcohol has a density of 0.79 g/mL and a MW of 60.1; 70% v/v isopropyl alcohol has a density of 0.85 g/mL.]

70 mL of isopropyl alcohol × (0.79 g/mL) = 55.3 g of isopropyl alcohol

100 mL of solution × (0.85 g/mL) = 85 g of solution

(85 g of solution – 55.3 g of isopropyl alcohol) = 29.7 g of water = 0.0297 kg of water

55.3 g of isopropyl alcohol/(60.1 g/mol) = 0.92 mol of isopropyl alcohol

(0.92 mol of isopropyl alcohol/0.0297 kg of water) = 30.98 m

6. Convert 50% w/w glycerin to molarity (M). [NOTE—Glycerin has a MW of 92.1 g.]

From example 1, 50% w/w glycerin = 56.5% w/v glycerin

(56.5 g/100 mL) × (mol/92.1 g) × (1000 mL/L) = 6.13 M

7. Convert 50% w/w glycerin to % v/v. [NOTE—50% w/w of glycerin has a density of 1.13 g/mL; 100% glycerin has a density of 1.26 g/mL.]

50 g of glycerin/(1.26 g/mL) = 39.7 mL of glycerin

100 g of solution/(1.13 g/mL) = 88.5 mL of solution

(39.7 mL of glycerin/88.5 mL of solution) × 100% = 44.8% v/v

9. Convert 50% w/w glycerin to 1 in *R* ratio strength.

1/*R* = (50 g of glycerin/100 g of solution)

*R* = 2

1 in *R* = 1 in 2

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8.3.3 SOLID AND SEMISOLID IN SOLID AND SEMISOLID MIXTURE CONVERSIONS

The calculations used to convert from percent weight in weight (% w/w) to ppm and ratio strengths are illustrated as follows for fluocinonide and tolnaftate in topical semisolids and powders.

**8.3.3.1 Calculating solid and semisolid in solid and semisolid mixture conversions**

*Examples—Solid and semisolid in solid and semisolid mixture conversions*

1. Convert 0.05% w/w fluocinonide ointment to ppm.

$$(0.05 \text{ g}/100 \text{ g}) \times (1 \times 10^6 \text{ ppm}) = 500 \text{ ppm}$$

2. Convert 1.5% w/w tolnaftate powder to 1:*R* ratio strength.

$$1/R = (1.5 \text{ g of tolnaftate}/100 \text{ g of powder})$$

$$R = 67$$

$$1{:}R = 1{:}67$$

3. Convert 1% w/w tolnaftate in talcum powder to *X*:*Y* ratio strength.

$$100 \text{ g of powder} - 1 \text{ g of tolnaftate} = 99 \text{ g of talcum}$$

$$X{:}Y = 1 \text{ g of tolnaftate}{:}99 \text{ g of talcum}$$

## 8.4 Dilution and Concentration

A more concentrated solution can be diluted to a lower concentration to obtain appropriate strength and precision when compounding preparations. Powders and semisolid mixtures can be triturated or mixed to achieve lower concentrations. The amount of an ingredient in the diluted mixture is the same as that in the portion of the more concentrated source used to make the dilution; thus, the following equation can be applied to dilution problems $(Q_1)(C_1) = (Q_2)(C_2)$, where $Q_1$ and $Q_2$ are the quantity of solutions 1 and 2, respectively, and $C_1$ and $C_2$ are concentrations of solutions 1 and 2, respectively. Any quantities and concentration terms may be used but the units of those terms must be the same on both sides of the equation.

8.4.1 CALCULATING DILUTION AND CONCENTRATION

**Examples—Dilutions and fortifications**

1. Semisolid dilution

Calculate the quantity $(Q_2)$, in g, of diluent that must be added to 60 g of a 10% w/w ointment to make a 5% w/w ointment.

$$(Q_1) = 60 \text{ g}, (C_1) = 10\% \text{ w/w, and } (C_2) = 5\% \text{ w/w}$$

$$60 \text{ g} \times 10\% \text{ w/w} = (Q_2) \times 5\% \text{ w/w}$$

$$(Q_2) = 120 \text{ g}$$

$$120 \text{ g} - 60 \text{ g} = 60 \text{ g of diluent to be added}$$

2. Solid dilution

Calculate the amount of diluent that should be added to 10 g of a trituration (1 in 100) to make a mixture that contains 1 mg of drug in each 10 g of the final mixture.

$$\text{Convert mg to g: 1 mg of drug} = 0.001 \text{ g of drug}$$

$$10 \text{ g of mixture should contain } 0.001 \text{ g of drug}$$

$$(Q_1) = 10 \text{ g}, (C_1) = (1 \text{ in } 100), \text{ and } (C_2) = (0.001 \text{ in } 10)$$

$$10 \text{ g} \times (1/100) = (Q_2) \times (0.001/10)$$

$$(Q_2) = 1000 \text{ g}$$

Because the final mixture of 1000 g contains 10 g of the trituration, 990 g (or 1000 g – 10 g) of diluent is required to prepare the mixture at a concentration of 0.001 g of drug in each 10 g.

3. Liquid dilution

Calculate the percentage strength $(C_2)$ of a solution obtained by diluting 400 mL of a 5.0% w/v solution to 800 mL.

$$(Q_1) = 400 \text{ mL}, (C_1) = 5.0\% \text{ w/v, and } (Q_2) = 800 \text{ mL}$$

$$400 \text{ mL} \times 5\% \text{ w/v} = 800 \text{ mL} \times (C_2)$$

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$(C_2)$ = 2.5% w/v

4. Liquid fortification

Calculate the additional amount, in g, of codeine phosphate that need to be added to 180 mL of a 12 mg/5 mL elixir of acetaminophen with codeine to have a final concentration of 30 mg/5 mL of codeine phosphate.

Amount to add = Total amount required – Amount present

Total amount required: (30 mg/5 mL) × 180 mL = 1080 mg of codeine phosphate

Amount present = (12 mg of codeine/5 mL) × 180 mL = 432 mg of codeine phosphate

Amount to add: 1080 mg – 432 mg = 648 mg of codeine phosphate

## 9. ALCOHOL AND ETHANOL

To achieve compliance with the statements in the *General Notices* about alcohol and the *USP* monograph for *Alcohol*, some conventions and special calculations are needed. See *General Notices 5.20.20.1 In Compounded Preparations*, *8.30 Alcohol Content*, and *Labeling ⟨7⟩, Labels and Labeling for Products in Other Categories, Alcohol* for information. The *USP* monograph for *Alcohol* states that it contains 92.3%–93.8% by weight corresponding to 94.9%–96.0% by volume of alcohol ($C_2H_5OH$) at 15.56°. The percent concentration for alcohol is generally taken to be 95% v/v of alcohol ($C_2H_5OH$) in water.

In summary:

- When the word alcohol is written on a prescription order or in a formula, as for example “alcohol 10 mL” or “dissolve in 5 mL of alcohol”, the compounder should use the Alcohol, USP [that is 95% alcohol ($C_2H_5OH$)].
- When the word alcohol is written with a percent, for example “alcohol 20%”, this means 20% v/v of alcohol ($C_2H_5OH$). If this percent is on a label of a commercial product, it means the product contains 20% v/v alcohol ($C_2H_5OH$). If this is part of a compounding formula, it means the compounder must add the equivalent of 20% v/v alcohol ($C_2H_5OH$), which may require special calculations.
- Labels of products and compounded preparations are to include the content of alcohol ($C_2H_5OH$) in % v/v. For compounded preparations, this value must often be calculated based on the volume(s) of alcohol-containing ingredients added.

For calculations when preparing compounded drug preparations using Alcohol, USP, the first step is to determine the quantity, in mL, of alcohol needed, and the second step is to determine the % v/v of alcohol ($C_2H_5OH$) in the final preparation so that it can be properly labeled.

### 9.1 Calculating Alcohol

EXAMPLES—ALCOHOL

1. Determine the quantity of alcohol needed for the prescription (see *Table 3*):

**Table 3**

| | |
|---|---|
| Clindamycin | 1% |
| Alcohol | 15% |
| Propylene glycol | 5% |
| Purified water, a sufficient quantity to make | 60 mL |

A. In this prescription order, the alcohol 15% means the preparation contains 15% v/v of alcohol ($C_2H_5OH$).

B. Calculate the quantity of alcohol ($C_2H_5OH$) needed for 60 mL of preparation:

$$\frac{\text{15 mL of alcohol}}{\text{100 mL of preparation}} = \frac{x\text{mL of alcohol}}{\text{60 mL of preparation}}$$

$$x = 9 \text{ mL of alcohol}$$

C. Because alcohol or ethanol content is required in drug labeling, the content in this preparation would be labeled as either alcohol 15%, or ethanol 14.3%, where 14.3% results from (15 mL × 0.9545) in which 0.9545 is the mean fraction by volume of ethanol in Alcohol, USP.

D. Determine the % v/v alcohol content for labeling. Because labeling of alcohol is in % v/v of alcohol ($C_2H_5OH$), the alcohol content of this preparation would be labeled: Alcohol 15%.

2. Determine the alcohol content, in % v/v, for the prescription (see *Table 4*):

**Table 4**

| | |
|---|---|
| Castor oil | 40 mL |

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**Table 4** *(continued)*

| | |
|---|---|
| Acacia | As needed |
| Alcohol | 15 mL |
| Cherry syrup | 20 mL |
| Purified water, a sufficient quantity to make | 100 mL |

A. In this formulation, alcohol 15 mL means the preparation contains 15 mL of Alcohol, USP.
B. In 100 mL of preparation, calculate the % v/v of ethanol: (15 mL × 0.9545) = 14.3 mL of ethanol, where 0.9545 is the mean fraction by volume of ethanol in Alcohol, USP.
C. Because alcohol or ethanol content is required in drug labeling, the content in this preparation would be labeled as either alcohol 15% or ethanol 14.3%.

## 10. ALLIGATION ALTERNATE AND ALGEBRA METHODS FOR COMBINING MULTIPLE STRENGTHS OF THE SAME ACTIVE PHARMACEUTICAL INGREDIENT

### 10.1 Alligation Alternate

Alligation is a method of determining the proportions in which substances of different strengths are mixed to yield a desired strength or concentration. Once the proportion is found, the calculation may be performed to find the exact amounts of substances required.

Set up the problem as follows.

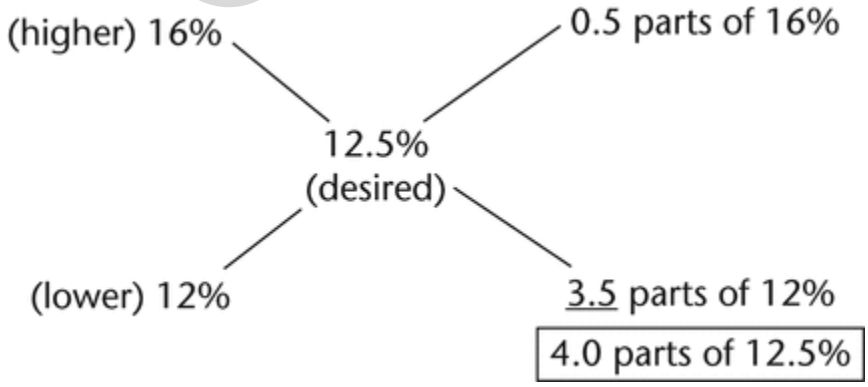
1. Place the desired percentage or concentration in the center.
2. Place the percentage of the substance with the lower strength on the lower left-hand side.
3. Place the percentage of the substance with the higher strength on the upper left-hand side.
4. Subtract the lower percentage from the desired percentage, and place the obtained difference on the upper right-hand side.
5. Subtract the desired percentage from the higher percentage, and place the obtained difference on the lower right-hand side.

The results on the right side determine how many parts of the two different percentage strengths should be mixed to produce the desired percentage strength of a drug mixture. The total parts will equal the final weight or volume of the preparation.

#### 10.1.1 CALCULATING BY USING THE ALLIGATION ALTERNATE

**Examples—Alligation alternate**

1. Determine the amount of ointment containing 12% drug concentration and the amount of ointment containing 16% drug concentration must be used to make 1 kg of a preparation containing a 12.5% drug concentration.




In a total of 4 parts of 12.5% preparation, 3.5 parts of 12% ointment and 0.5 parts of 16% ointment are needed.

4 parts correspond to 1 kg or 1000 g.

1 part corresponds to 250 g.

3.5 parts correspond to 3.5 × 250 g or 875 g of 12% ointment.

0.5 parts correspond to 0.5 × 250 g or 125 g of 16% ointment.

2. Determine the volume, in mL, of 20% dextrose in water and 50% dextrose in water needed to make 750 mL of 35% dextrose in water.

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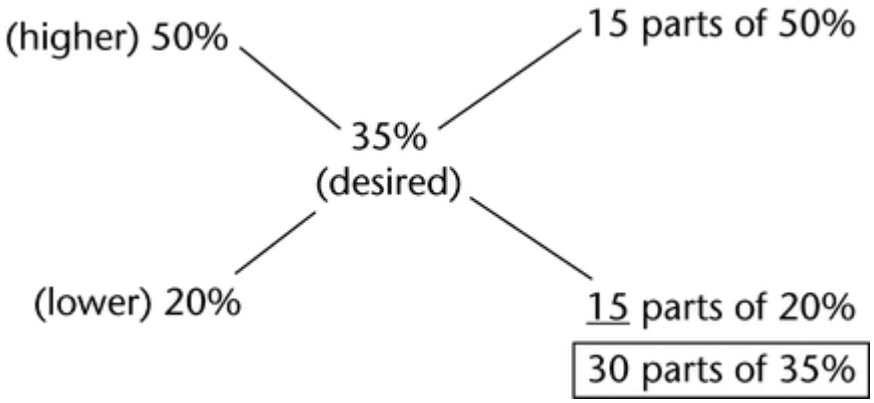


In a total of 30 parts of 35% dextrose in water, 15 parts of 50% dextrose in water and 15 parts of 20% dextrose in water are required.

30 parts correspond to 750 mL.

15 parts correspond to 375 mL.

Thus, use 375 mL of the 20% solution and 375 mL of the 50% solution to prepare the preparation.

## 10.2 Algebra Method

The following algebraic equation may be used instead of alligation to solve problems of mixing two different strengths of the same ingredient:

$(C_s \times Q_s) + (C_w \times Q_w) = (C_f \times Q_f)$, where $C$ is concentration or strength, $Q$ is the quantity; and the subscript $s$ identifies the strongest strength, $w$ identifies the weakest strength, $f$ represents the final mixture with a strength less than $s$ and greater than $w$, $(Q_s + Q_w) = Q_f$, $Q_s = (Q_f - Q_w)$, and $Q_w = (Q_f - Q_s)$.

### 10.2.1 CALCULATING BY USING THE ALGEBRA METHOD

**Examples—Algebra method**

1. Determine the amount, in g, of 16% w/w drug ointment and 12% w/w drug ointment required to prepare 1 kg of 12.5% w/w drug ointment.

$$(16\% \times Q_s) + [12\% \times (1000\ \text{g} - Q_s)] = 12.5\% \times 1000\ \text{g}$$

$$16\%\ Q_s + 120\ \text{g} - 12\%\ Q_s = 125\ \text{g}$$

$$4\%\ Q_s = 5\ \text{g}$$

$$Q_s = 5\ \text{g}/4\% = 125\ \text{g of 16\% ointment}$$

$$Q_w = 1000\ \text{g} - 125\ \text{g} = 875\ \text{g of 12\% ointment}$$

2. Determine the volume, in mL, of 10% dextrose injection and 50% dextrose injection needed to make 750 mL of 35% dextrose injection.

$$(50\% \times Q_s) + [10\% \times (750\ \text{mL} - Q_s)] = 35\% \times 750\ \text{mL}$$

$$50\%\ Q_s + 75\ \text{mL} - 10\%\ Q_s = 262.5\ \text{mL}$$

$$40\%\ Q_s = 187.5\ \text{mL}$$

$$Q_s = 187.5\ \text{mL}/40\% = 468.75\ \text{mL (470 mL practically)}$$

$$Q_w = 750\ \text{mL} - 468.75\ \text{mL} = 281.25\ \text{mL (280 mL practically)}$$

# 11. ALIQUOT DILUTIONS

When the quantity of drug desired requires a degree of precision in measurement that is beyond the capability of the available measuring devices, the pharmacist may use the aliquot method of measurement. It applies when potent drug substances are compounded, or when the total amount of the active drug in a single dose or individualized doses is less than the minimum accurately weighable quantity (MAWQ). Even if the amount of drug needed is greater than the MAWQ per unit, an aliquot will provide more material per unit, which will aid in handling and administration. Aliquot means "containing an exact number of

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times in something else;" the aliquot must be a proportional part of the total. Therefore, 5 is an aliquot part of 15, because 5 is contained exactly 3 times in 15. Both the total volume of solution or weight of powder triturate and the aliquot volume/weight should be easily and accurately measurable. If the solution or powder triturate is highly concentrated and a small error is made in measuring the aliquot, a large error can occur in the quantity of drug brought to the final formulation.

Aliquots can be: solid–solid, when the active drug and the diluents are solids; solid–liquids, when the active drug is solid and is to be incorporated into a liquid preparation, such as a solution, an emulsion, or a suspension; and liquid–liquid, when the active drug is liquid and the diluents are liquids. It can be a pure liquid or a concentrated solution of a drug. Aliquots of pure liquids are relatively uncommon because few drugs are liquid in their pure state. Aliquots involving concentrated solutions are more common.

There are two general methods to prepare aliquots:

1. Aliquot method 1 is applicable to drugs or substances that have to be within the degree of accuracy provided by the measuring device. It is the simplest method and can be applied to solid and liquid aliquots.
2. Aliquot method 2, also known as the dilution factor method, is useful when there is more flexibility in the amount of drug that may be measured.
   Aliquot Method 1
   A. The MAWQ amount of drug is measured.
   B. The drug is diluted with an arbitrary amount of diluent.
   C. The amount of dilution that will give the desired amount of drug is calculated, and the amount is measured.
   Aliquot Method 2
   A. The quantity of drug to be measured is determined by multiplying the amount of drug needed by an appropriately determined factor, called the dilution factor. The dilution factor must be a whole number more than or equal to the MAWQ divided by the amount of drug needed.
   B. An arbitrary amount of diluent is measured and added. The amount of diluent used can be determined by different methods, provided the amount of diluent chosen will give an aliquot greater than or equal to the MAWQ.
   C. The amount of aliquot needed is determined by multiplying the weight or volume of the dilution by the inverse of the dilution factor. Dilution factors are usually chosen to be whole numbers.

The general calculations can be shown as:

$$A/B = C/D$$

$A$ = amount of drug desired
$B$ = amount of drug measured
$C$ = amount of drug in aliquot
$D$ = aliquot total amount

## 11.1 Calculating Aliquots

EXAMPLES—ALIQUOTS

1. Solid-in-liquid dilution (Aliquot Method 1)
   Prepare 100 mL of a solution containing 0.2 mg/mL of clonidine using water as the diluent. To prepare this solution, 20 mg of clonidine is needed.
   A. Select the weight of drug desired ($A$) to be equal to or greater than the MAWQ. In this situation, the MAWQ of the balance is 120 mg.
   B. Select the aliquot volume ($D$) in which the desired amount of drug ($C$) will be contained. This establishes the concentration of the solution to be prepared. Clonidine solubility is 1 g/13 mL, so if 5 mL is selected as the aliquot volume, the concentration in that solution will be 20 mg/5 mL. Therefore, solubility will not be a problem in this aqueous solution.
   C. Using the preceding formula, calculate the volume of solution ($B$) to be prepared.

   $$120 \text{ mg of clonidine}/B = 20 \text{ mg of clonidine}/5 \text{ mL of aliquot}$$

   $$B = 30 \text{ mL}$$

   D. Prepare the solution containing 120 mg of clonidine in 30 mL of *Purified Water*. Transfer a 5-mL aliquot from this solution to a final container, and add sufficient *Purified Water* to bring the formulation to a final volume of 100 mL.
2. Solid-in-solid dilution (Aliquot Method 2)
   Prepare an individual dose of codeine phosphate 20 mg.
   A. Select a dilution factor that will yield a quantity that is greater than or equal to the MAWQ, and weigh this amount. In this case, the dilution factor may be greater than or equal to 6 because 6 × 20 mg = 120 mg. The smallest dilution factor that may be chosen is 6 if the MAWQ of the balance is 120 mg.
   B. Weigh an amount of diluent that will give an aliquot greater than or equal to the MAWQ. In this example, 600 mg of diluent is weighed.
   C. Mix the two powders thoroughly by geometric trituration in a mortar.
   D. Calculate the total weight of the dilution: 120 mg codeine phosphate + 600 mg diluent = 720 mg.
   E. Calculate the aliquot part of the dilution that contains 20 mg of codeine phosphate by multiplying the total weight of the dilution by the inverse of the dilution factor: 720 mg × (1/6) = 120 mg.

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F. Weigh this calculated amount of the dilution (120 mg) to get the desired 20 mg of codeine phosphate per dose.

# 12. POWDER VOLUME DISPLACEMENT IN LIQUIDS

## 12.1 Displacement in Suspension

### 12.1.1 CALCULATING POWDER VOLUME

**Examples—Powder volume**

1. Powder displacement in suspension
The directions to reconstitute a 150-mL bottle of an amoxicillin for oral suspension of 250 mg/5 mL require 111 mL of *Purified Water*. The physician has requested that the product be reconstituted at a concentration of 500 mg/5 mL. Calculate the amount of *Purified Water* required for the higher concentration.
   A. Calculate the volume of the suspension occupied by the amoxicillin powder:

$$150\ \text{mL} - 111\ \text{mL} = 39\ \text{mL}$$

   B. Calculate the quantity of amoxicillin present in the entire bottle:

$$150\ \text{mL} \times (250\ \text{mg}/5\ \text{mL}) = 7500\ \text{mg}$$

   C. Calculate the total volume of the suspension at the requested concentration (500 mg/5 mL):

$$7500\ \text{mg}/(500\ \text{mg}/5\ \text{mL}) = 75\ \text{mL}$$

   D. Calculate the volume of *Purified Water* needed to reconstitute the powder by subtracting the powder volume calculated in step a:

$$75\ \text{mL} - 39\ \text{mL} = 36\ \text{mL of } \textit{Purified Water}$$

[NOTE—Such formulations may be too viscous to flow freely.]

2. Powder volume in drugs for injection
If the powder volume of 250 mg of ceftriaxone for injection is 0.1 mL, calculate the amount of diluent that should be added to 500 mg of ceftriaxone for injection to make a suspension with a concentration of 250 mg/mL.
   A. Calculate the total volume of injection:

$$500\ \text{mg}/(250\ \text{mg/mL}) = 2\ \text{mL}$$

   B. Calculate the volume occupied by 500 mg of ceftriaxone for injection:

$$500\ \text{mg}/(250\ \text{mg}/0.1\ \text{mL}) = 0.2\ \text{mL}$$

   C. Calculate the volume of the diluent required:

$$(2\ \text{mL of suspension}) - (0.2\ \text{mL of ceftriaxone for injection}) = 1.8\ \text{mL of diluent}$$

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# 13. INTRAVENOUS FLOW OR INFUSION RATES

Intravenous (IV) solutions and emulsions may be administered by gravity flow and infusion or syringe pumps. Gravity-flow IV sets are regulated by an adjustable clamp on the tubing, and the approximate flow rate is determined by counting the number of drops per 10–15 seconds, then adjusting that to a per minute rate. Manufactured IV sets are typically calibrated to deliver from 15 to 60 drops/mL, depending on the particular set.

## 13.1 Solving by Multiple or Separate Steps

As in previous sections, the following examples may be solved by multiple separate steps, or a single-DUA procedure.

### 13.1.1 CALCULATING INTRAVENOUS FLOW OR INFUSION RATES

**Examples—IV or infusion rates**

1. An IV infusion of dextrose 5% in water with 20 mEq of potassium chloride is to be administered to a 6-year-old child at the rate of 12 mL/hour. An IV administration set that delivers 60 drops/mL is available. Calculate the flow rate in drops per minute:

$$\frac{12\ \text{mL}}{\text{hour}} \times \frac{60\ \text{drops}}{\text{mL}} \times \frac{\text{hour}}{60\ \text{minutes}} = \frac{12\ \text{drops}}{\text{minute}}$$

2. A 63.6-kg patient is admitted to the Emergency Department and requires a dopamine hydrochloride infusion to maintain an adequate blood pressure. The drug is ordered at an initial rate of 2 mcg/kg/minute. A 400-mg/250-mL dopamine hydrochloride injection is available. Calculate the flow rate in mL/hour to be administered by infusion pump:

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$$63.6\ \text{kg} \times \frac{2\ \mu\text{g}}{\text{kg} \cdot \text{minute}} \times \frac{250\ \text{mL}}{400\ \text{mg}} \times \frac{\text{mg}}{1000\ \mu\text{g}} \times \frac{60\ \text{minutes}}{\text{hour}} = \frac{4.8\ \text{mL}}{\text{hour}}$$

# 14. OSMOLARITY AND TONICITY OF SOLUTIONS

The following discussion and calculations have therapeutic implications in preparations of dosage forms intended for ophthalmic, subcutaneous, intravenous, and intrathecal administration as well as for neonatal use.

## 14.1 Tonicity

Cells of the body, such as erythrocytes, will neither swell nor shrink when placed in a solution that is isotonic with body fluids. The measurement of tonicity, however, which is a physiological property, is somewhat difficult. A 0.9% w/v sodium chloride injection, which has a freezing point (FP) of –0.52°, is both isotonic and isoosmotic with body fluids. In contrast to isotonicity, FP depression is a physical property. Some solutions that are isoosmotic with body fluids are not isotonic, because they contain solutes to which cells are freely permeable rather than semipermeable. Freely permeable solutes (e.g., boric acid and urea) can cause erythrocyte lysis, i.e., behave as if they were hypotonic in concentrations that are hyperosmotic relative to body fluids. Nevertheless, many pharmaceutical products are prepared using FP data or related sodium chloride data to prepare solutions that are isoosmotic with body fluids. A closely related topic is osmolarity (see *Osmolality and Osmolarity* ⟨785⟩).

FP data or sodium chloride equivalents of pharmaceuticals and excipients (see *Table 5*) may be used to prepare isoosmotic solutions, as shown in the following examples.

### 14.1.1 CALCULATING TONICITY

**Example—Tonicity**

Determine the amount of sodium chloride (NaCl) required to prepare 60 mL of an isoosmotic solution of atropine sulfate injection 0.5% using the E values and the FP depression values in *Table 5*.

**Table 5. Sodium Chloride Equivalents (E) and FP Depressions for a 1% Solution of the Drug or Excipient**

| Drug or Excipient | E | FP Depression |
|---|---|---|
| Atropine sulfate | 0.13 | 0.075 |
| Sodium chloride | 1.00 | 0.576 |

Using the E values:

1. The total amount of substances equivalent to a 0.9% sodium chloride injection = (0.9 g/100 mL) × 60 mL = 0.54 g.
2. The amount of atropine sulfate required = (0.5 g/100 mL) × 60 mL = 0.3 g.
3. 1 g of atropine sulfate is equivalent to 0.13 g of sodium chloride.
4. 0.3 g of atropine sulfate is equivalent to 0.3 × 0.13 g = 0.039 g of sodium chloride.
5. Thus, the required amount of sodium chloride is 0.54 g – 0.039 g = 0.501 g or 0.5 g.

Using FP depression values:

1. The FP depression required is 0.52°.
2. A 1% solution of atropine sulfate causes an FP depression of 0.075°.
3. A 0.5% solution of atropine sulfate causes an FP depression of 0.5 × 0.075° = 0.0375°.
4. The additional FP depression required is 0.52° – 0.0375° = 0.483°.
5. A 1% solution of sodium chloride causes an FP depression of 0.576°.
6. Therefore, an FP depression of 1° is caused by a 1%/0.576 = 1.736% solution of sodium chloride.
7. 1.736% × 0.483 = 0.838% solution of sodium chloride causes an FP depression of 0.482°.
8. The required amount of sodium chloride is (0.838%) × 60 mL = 0.502 g or 0.5 g.

# 15. pH AND pH BUFFERS

## 15.1 pH Calculations

See *Appendix 1* for logarithmic definitions and applications.

pH = –log [$H_3O^+$], and pKa = –log ([$H_3O^+$][$A^-$])/[HA], where [$H_3O^+$] is the hydrodium ion concentration in an aqueous solution, [$A^-$] is the ionic form of the relevant acid, and Ka is the ionization constant of either a monoprotic acid or a particular proton from a polyprotic acid in aqueous solution. The [$H^+$] = the antilogarithm of (–pH) or $10^{-pH}$; and Ka = the antilogarithm of (–pKa) or $10^{-pKa}$.

The pH of an aqueous solution containing a weak acid may be calculated using the Henderson–Hasselbalch equation:

$$\text{pH} = \text{pKa} + \log\ [(\text{base form})/(\text{acid form})]$$

The buffer equation symbol (↔) represents the equilibrium between conjugate base and acid forms or pairs of the same molecule. It is called the buffer equation, because small changes in the ratio of concentrations of the conjugate forms result

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in a logarithmically smaller change in pH. The salt form can be an acid or base, depending on structure; thus, its conjugate form is a base or acid, respectively.

Example 1:

B and $BH^+$ represent a nonionized or "free" base and cationic acid pair, $BH^+ \leftrightarrow B^+H^+$

Example 2:

HA and $A^-$ represent a nonionized or "free" acid and anionic base pair, $HA \leftrightarrow A^- + H^+$

Example 3:

$H_nA^-$ and $H_{n-1}A^{2-}$, such as $H_2PO_4^-$ and $HPO_4^{2-}$, represent an anionic acid and anionic base relative to each other; the pKa = 7.2 for $H_2PO_4^- \leftrightarrow HPO_4^{2-} + H^+$.

#### 15.1.1 CALCULATING pH

**Example—pH**

A solution contains 0.020 mol/L of sodium acetate and 0.010 mol/L of acetic acid, which has a pKa value of 4.76. Calculate the pH and the $[H^+]$ of the solution as follows:

$$pH = 4.76 + \log (0.020/0.010) = 5.06$$

$$[H^+] = \text{antilogarithm of } (-5.06) = 8.69 \times 10^{-6}$$

### 15.2 Buffer Solutions

#### 15.2.1 DEFINITION

A buffer solution is an aqueous solution that resists a change in pH when small quantities of acid or base are added, when diluted with the solvent, or when the temperature changes. Most buffer solutions are mixtures of a weak acid and one of its salts, or mixtures of a weak base and one of its salts. Water and solutions of a neutral salt, such as sodium chloride, have very little ability to resist the change of pH and are not capable of effective buffer action.

#### 15.2.2 PREPARATION, USE, AND STORAGE OF BUFFER SOLUTIONS

Buffer solutions for Pharmacopeial tests should be prepared using freshly boiled and cooled water (see *Reagents, Indicators and Solutions—Solutions, Buffer Solutions*). They should be stored in containers such as Type I glass bottles and used within 3 months of preparation.

Buffers used in physiological systems are carefully chosen so as to not interfere with the pharmacological activity of the medicament or the normal function of the organism. Commonly used buffers in parenteral products, for example, include: the nonionized acid and base salt pairs of acetic acid and sodium acetate, citric acid and sodium citrate, glutamic acid and sodium glutamate, and monopotassium or monosodium phosphate and dipotassium or disodium phosphate; and the acid salt and nonionized base pair tris(hydroxymethyl)aminomethane hydrochloride and tris(hydroxymethyl)aminomethane. Buffer solutions should be freshly prepared.

The Henderson–Hasselbalch equation, noted in *15.1 pH Calculations*, allows calculation of the pH and concentrations of conjugate pairs of weak acids and their salts and weak bases and their salts in buffer solutions when the pKa of the acid form of the buffer pair is known. Appropriately modified, this equation may be applied to buffer solutions composed of a weak base and its salt.

#### 15.2.3 BUFFER CAPACITY

The buffer capacity of a solution is the measurement of the ability of that solution to resist a change in pH upon addition of small quantities of a strong acid or base. An aqueous solution has a buffer capacity of 1 when 1 L of the buffer solution requires 1 g equivalent of strong acid or base to change the pH by 1 unit. Therefore, the smaller the pH change upon the addition of a specified amount of acid or base, the greater the buffer capacity of the buffer solution. Usually, in analysis, much smaller volumes of buffer are used to determine the buffer capacity. An approximate formula for calculating the buffer capacity is g equivalents of strong acid or base added per L of buffer solution per unit of pH change, i.e., (g equivalents/L)/(pH change).

#### 15.2.4 CALCULATING BUFFER CAPACITY

**Example—Buffer capacity**

The addition of 0.01 g equivalents of sodium hydroxide to 0.25 L of a buffer solution produced a pH change of 0.50. The buffer capacity of the buffer solution is calculated as follows:

$$(0.01 \text{ Eq}/0.25 \text{ L})/0.50 \text{ pH change} = 0.08(\text{Eq/L})/(\text{pH change})$$

## 16. TEMPERATURES

The relationship between Celsius or Centigrade (°C) and Fahrenheit (°F) temperature scale is expressed by the following equations:

$$°C = (°F - 32) \times (5/9)$$

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$$°F = (°C \times 1.8) + 32$$

## 16.1 USP Temperatures

According to the *General Notices 8.180 Temperatures*, temperatures are expressed in centigrade (Celsius) degrees, and all other measurements are made at 25° unless otherwise indicated. For instructional purposes, °F is shown in the examples.

16.1.1 CALCULATING TEMPERATURES

**Examples—Temperatures**

1. Convert 77°F to Celsius degrees.

$$°C = (77°F - 32) \times (5/9) = 25°C$$

2. Convert 30°C to Fahrenheit degrees.

$$°F = (30°C \times 1.8) + 32 = 86°F$$

The relationship between the Kelvin or absolute (K) and the Celsius (°C) scales is expressed by the equation:

$$K = °C + 273.15$$

# 17. ENDOTOXINS

An endotoxin is a lipopolysaccharide that comes from a particular source, where species and strain number are usually indicated.

## 17.1 Endotoxin Concentrations

For more information concerning endotoxins, see *Bacterial Endotoxins Test* ⟨85⟩.

17.1.1 CALCULATING ENDOTOXINS

**Example—Endotoxins**

A 71.8-kg patient is to receive an intrathecal infusion of morphine sulfate at a rate of 0.3 mg/hour. The solution will be prepared by diluting preservative-free morphine sulfate injection, which contains 10 mg/mL of morphine sulfate, with 0.9% sodium chloride injection to produce an infusion rate of 2 mL/hour.

1. Determine the volume, in mL, of morphine sulfate injection (10 mg/mL) and 0.9% sodium chloride injection needed to prepare a 24-hour infusion.

0.3 mg of morphine sulfate per hour × 24 hours = 7.2 mg of morphine sulfate

7.2 mg of morphine sulfate/(10 mg/mL) = 0.72 mL of morphine sulfate injection

2 mL of infusion per hour × 24 h = 48 mL of total volume

48 mL total volume − 0.72 mL morphine sulfate injection = 47.28 mL of 0.9% sodium chloride injection

2. Calculate the maximum potential endotoxin load per hour for this preparation. [NOTE—*USP* monographs specify upper limits of 14.29 USP Endotoxin Units (EU)/mg of morphine sulfate in injections for intrathecal use, and 0.5 EU/mL for injections containing 0.5%–0.9% sodium chloride.]

7.2 mg of morphine sulfate injection × 14.29 EU/mg of morphine sulfate = 102.89 EU from morphine sulfate

47.28 mL of sodium chloride injection × 0.5 EU/mL = 23.64 EU from 0.9% sodium chloride injection

Endotoxin load = 102.89 EU + 23.64 EU = 126.53 EU

126.53 EU/24 hour = 5.27 EU/hour

3. Determine if the endotoxin load in step 2 exceeds the allowable USP limit for this patient. [NOTE—The maximum endotoxin load by intrathecal administration is 0.2 EU/kg/hour (see ⟨85⟩).]

Maximum endotoxin load = (0.2 EU/kg/hour) × 71.8 kg-patient = 14.36 EU/hour

The endotoxin load of 5.27 EU/hour does not exceed the allowable limit of 14.36 EU/hour.

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# 18. STABILITY KINETICS AND EXPIRATION DATE PREDICTION

## 18.1 Stability Based on Rate Calculations

Calculation of a predetermined minimum percentage of initial drug strength or other quality parameter, e.g., in vitro dissolution of active pharmaceutical ingredients (APIs) or active drugs in solid oral dosage forms, is based on component-specific assays and other validated scientific testing. The expiration date or time elapsed until such minimum acceptable limits are reached for a specific manufactured product is exclusive to the specific formulation, packaging, and environmental conditions, e.g., temperature, humidity, and illumination, to which the item is subjected. See also ⟨659⟩, ⟨795⟩, ⟨797⟩, ⟨1163⟩,and ⟨1191⟩.

The degradation or concentration loss rates or kinetics of most APIs can be accurately represented or modeled by either zero order (constant) or first order (mono-exponential) rate equations. Zero order calculations are generally applicable to solids, semisolids, suspensions in which a majority of the drug strength is present as solid particles, and auto-oxidation in solutions. First order calculations are generally applicable for drug hydrolysis in solutions.

## 18.2 Zero Order Rate Calculations

The isothermal zero order or constant rate equation for a particular formulation is $C = C_0 - kt$, where $C$ is the concentration of API at any time, $C_0$ is the concentration at origination or time zero, $k$ is the reaction rate constant, and $t$ is any time after origination or zero. The values and units of the rate, $dC/dt$, and rate constant, $k$, are the same for zero order processes, i.e., the units are concentration/time, such as mg/mL/day.

### 18.2.1 ZERO ORDER RATE EQUATION DERIVED FROM ORIGINAL DATA

The following examples illustrate calculations of the zero order rate equation from original concentration assay and time data, and an expiration date using that equation.

**18.2.1.1 Calculating zero order rate**

*Examples—Zero order rate*

1. Calculate the zero order rate equation based on the assay results for a drug suspension at 25° (see *Table 6*):

**Table 6**

| *C* (mg/mL) | *t* (days) |
|---|---|
| 49 | 3 |
| 47.5 | 8 |
| 44.8 | 17 |
| 42.3 | 26 |

Linear regression of the $C$ (ordinate) versus $t$ (abscissa) values yields the equation, $C = 49.84 - 0.292t$ with a correlation coefficient of 0.9996.

2. Calculate the time when $C = 0.9 \times C_0$, i.e., the expiration date where the concentration will be 90% of the original concentration ($t_{90}$):

$$C = 49.84 - 0.292t$$

$$0.9 \times 49.84 = 49.84 - 0.292(t_{90})$$

$$t_{90} = (44.86 - 49.84)/{-0.292} = 17.05 \text{ days}$$

3. Using the previous linear regression equation, calculate the $C$ of the drug suspension at 25° when $t = 12$ days:

$$C = 49.84 - (0.292 \times 12) = 46.34 \text{ mg/mL}$$

4. Calculate $t$ when $C = 45$ mg/mL:

$$45 = 49.84 - 0.292t$$

$$t = (45 - 49.84)/{-0.292} = 16.6 \text{ days}$$

### 18.2.2 ZERO ORDER VALUES CALCULATED FROM A RATE EQUATION

The following are examples of expiration dates calculated from a rate equation derived from original concentration assay and time data.

**18.2.2.1 Calculating zero order values from a rate equation**

*Examples—Zero order from a rate equation*

1. Calculate the $t_{80}$ expiration date of a drug cream at 25° using the equation, $C = 0.05 - 0.0003t$, where the $C$ unit is % w/w and the $t$ unit is months. At $t_{80}$, $C = 0.8C_0$.

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$$0.8 \times 0.05 = 0.05 - 0.0003(t_{80})$$

$$t_{80} = 33.3 \text{ months}$$

2. Calculate the $t_{80}$ expiration date of the drug cream formulation in example 1, but for which $C_0$ is 0.1:

$$0.8 \times 0.1 = 0.1 - 0.0003(t_{80})$$

$$t_{80} = 66.7 \text{ months}$$

## 18.3 First Order Rate Calculations

The isothermal first order rate equation for a particular formulation in exponential form is $C = C_0e^{-kt}$, and in linear form is $\ln(C) = \ln(C_0) - kt$, where $C$ is the concentration of an API at any time, $C_0$ is the concentration at origination or time zero, $k$ is the reaction rate constant, and $t$ is any time after origination or zero. The constantly changing rate, $dC/dt$, and rate constant, $k$, are not the same for first order processes. The rate units are concentration/time, e.g., mg/mL/hour, but the rate constant unit is reciprocal time, time$^{-1}$, e.g., hour$^{-1}$.

### 18.3.1 FIRST ORDER LINEAR RATE EQUATION DERIVED FROM ORIGINAL DATA

The following examples illustrate calculation of the linear first order rate equation from original concentration assay and time data and calculation of an expiration date using that equation.

**18.3.1.1 Calculating first order linear rate equations**

*Example—First order linear rate*

1. Calculate the linear first order rate equation based on the assay results for a drug solution at 27° (see *Table 7*):

**Table 7**

| $C$ (mg/mL) | $t$ (h) |
|---|---|
| 12.3 | 2 |
| 11.9 | 6 |
| 11.5 | 14 |
| 10.6 | 24 |

Linear regression of the $\ln(C)$ (ordinate) versus $t$ (abscissa) values yields the equation, $\ln(C) = 2.522 - 0.0065t$ with a correlation coefficient of 0.992.

2. From the linear regression equation, calculate the time when 95% of the original concentration is reached, $t_{95}$, when $C = 0.95C_0$, which is the predetermined expiration date:

$$\ln(C_0) = 2.522; \text{ thus, } C_0 = e^{2.522} = 12.45 \text{ mg/mL}$$

$$\ln(0.95 \times 12.45) = 2.522 - 0.0065(t_{95})$$

$$t_{95} = (2.470 - 2.522)/-0.0065 = 8 \text{ hours}$$

### 18.3.2 FIRST ORDER VALUES CALCULATED FROM A LINEAR EQUATION

The following are examples of an expiration date, concentration, and time calculated for the same drug solution at 22° from the rate equation, $\ln(C) = 4.382 - 0.076t$, where the $C$ units are mcg/mL and the $t$ unit is days, derived from the original concentration assay and time data.

**18.3.2.1 Calculating first order values from a linear rate equation**

*Examples—First order from a linear rate*

1. Calculate the $t_{90}$ expiration date:

$$\ln(C_0) = 4.382; \text{ thus, } C_0 = e^{4.382} = 80$$

$$\ln(0.9 \times 80) = 4.382 - 0.076(t_{90})$$

$$t_{90} = (4.277 - 4.382)/-0.076 = 1.4 \text{ days}$$

2. Calculate the time at which $C = 75$ mcg/mL:

$$\ln(75) = 4.382 - 0.076t$$

$$t = (4.317 - 4.382)/-0.076 = 0.86 \text{ day}$$

3. Calculate whether $C = 70$ mcg/mL occurs before or after $t_{90}$:

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$$\ln(70) = 4.382 - 0.076t$$

$$t = (4.248 - 4.382)/-0.076 = 1.8 \text{ days}$$

$C$ = 70 mcg/mL occurs at 1.8 days, after a $t_{90}$ of 1.4 days

### 18.3.3 FIRST ORDER EXPIRATION DATE CALCULATED FROM TWO VALUES OF CONCENTRATION AND TIME

When degradation or other cause of concentration loss is known from experience or reference information to obey first order kinetics, the rate constant can be accurately estimated from accurate assays of only two concentrations at their respective times. In this case, the linear first order rate equation, $\ln(C) = \ln(C_0) - kt$, may be transformed or integrated as $\ln(C_2) = \ln(C_1) - k(t_2 - t_1)$, which when rearranged is $k = \ln(C_1/C_2)/(t_2 - t_1)$. The following examples apply these equations to calculate expiration dates, concentrations, and times.

**18.3.3.1 Calculating first order expiration date from two values**

*Examples—First order expiration date from two values*

1. At 25°, the concentration of an antibiotic in solution was 89 mg/mL after 3 hours and 74 mg/mL after 8 hours. Calculate the initial concentration at time zero:

$$k = \ln(89/74)/(8 - 3) = 0.037 \text{ hour}^{-1}$$

$$\ln(89) = \ln(C_0) - (0.037 \text{ hour}^{-1} \times 3 \text{ hour})$$

$$\ln(C_0) = 4.489 + 0.111 = 4.6$$

$$C_0 = e^{4.6} = 99.5 \text{ mg/mL}$$

2. Calculate the $t_{90}$ expiration date using the data in example 1. At $t_{90}$, $C = 0.9C_0$.

$$\ln(0.9 \times 99.5) = \ln(99.5) - 0.037(t_{90})$$

$$t_{90} = (4.495 - 4.600)/-0.037 \text{ hour}^{-1} = 2.8 \text{ hours}$$

3. Calculate the concentration at 6 hours using the data in example 1:

$$\ln(C) = \ln(99.5) - (0.037 \times 6)$$

$$\ln(C) = 4.378$$

$$C = e^{4.378} = 79.7 \text{ mg/mL}$$

### 18.3.4 FIRST ORDER TIMES, $t_n$, FOR 0.$n$ FRACTION OR $n$% OF REMAINING ORIGINAL CONCENTRATION

The two most common first order pharmaceutical $t_n$ values are the $t_{50}$, which is a primary parameter factor in clinical pharmacokinetics, and the $t_{90}$, which is the most common stability shelf life or expiration date. Values of any $t_n$, where $0 < n < 100$, are derived from the linear first order equation, $\ln(C) = \ln(C_0) - kt$. The equations for $t_{50}$ and $t_{90}$ in particular are derived in the following examples. The value of $k$ by definition is constant for a specific drug chemical in a specific formulation at a specific temperature; thus, $t_n$ values derived from such values of $k$ are also constant.

**18.3.4.1 Calculating first order times for remaining original concentrations**

*Examples—First order times for remaining original concentrations*

1. At $t_n$, $C = 0.n \times C_0$.

$$\ln(0.n \times C_0) = \ln(C_0) - kt_n$$

$$t_n = [\ln(0.n \times C_0) - \ln(C_0)]/-k = \ln[(0.n \times C_0)/C_0]/-k = \ln(0.n)/-k$$

$$t_n = \ln(0.n)/-k$$

2. At $t_{50}$, $C = 0.5(C_0)$.

$$\ln(0.5 \times C_0) = \ln(C_0) - kt_{50}$$

$$t_{50} = [\ln(0.5 \times C_0) - \ln(C_0)]/-k = \ln[(0.5 \times C_0)/C_0]/-k = \ln(0.5)/-k = -0.693/-k = 0.693/k$$

$$t_{50} = 0.693/k$$

3. At $t_{90}$, $C = 0.9(C_0)$.

$$\ln(0.9 \times C_0) = \ln(C_0) - kt_{90}$$

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$$t_{90} = [\ln(0.9 \times C_0) - \ln(C_0)]/-k = \ln[(0.9 \times C_0)/C_0]/-k = \ln(0.9)/-k = -0.105/-k = 0.105/k$$

$$t_{90} = 0.105/k$$

## 18.4 Stability Prediction Based on Arrhenius Theory

The basis of the Arrhenius theory is that reaction rates and rate constants change exponentially in the direction of arithmetic temperature change. The pharmaceutical application of the Arrhenius theory is based on scientifically accurate and statistically valid assay data obtained at three or more temperatures that are ≥10° warmer than the intended drug storage temperature and each other. The Arrhenius equation may be expressed in an exponential form, $k = Ae^{-(Ea/RT)}$, a linear form, $\ln(k) = \ln(A) - (E_a/RT)$, and an integrated form, $\ln(k_2/k_1) = E_a(T_2 - T_1)/[R(T_2 \times T_1)]$, where $k$, $k_1$, and $k_2$ are isothermal rate constants, $A$ is a thermodynamic factor, $E_a$ is energy of activation for the degradation reaction, $R$ is the gas constant ($1.987 \times 10^{-3}$ kcal mol$^{-1}$K$^{-1}$ or $8.314 \times 10^{-3}$ J K$^{-1}$ mol$^{-1}$), and $T$, $T_1$, and $T_2$ are absolute or Kelvin temperatures.

### 18.4.1 ARRHENIUS LINEAR EQUATION DERIVED FROM ORIGINAL DATA

The following examples illustrate derivation of a linear Arrhenius equation from original assay data and its application to predicting a drug stability expiration date at a cooler or lower storage temperature.

**18.4.1.1 Calculating Arrhenius equations**

*Examples—Arrhenius equations*

1. Calculate the linear Arrhenius equation based on the rate constants and temperatures for a beta-lactam antibiotic that decomposes in solution at a first order rate (see *Table 8*):

**Table 8**

| $T$ (°C) | $T$ (K) | $1/T$ ($K^{-1}$) | $k$ (hour$^{-1}$) | ln($k$) |
|---|---|---|---|---|
| 40 | 313 | $3.195 \times 10^{-3}$ | 0.0014 | –6.571 |
| 50 | 323 | $3.096 \times 10^{-3}$ | 0.005 | –5.298 |
| 60 | 333 | $3.003 \times 10^{-3}$ | 0.016 | –4.135 |

Linear regression of the ln($k$) (ordinate) *versus* $1/T$ (abscissa) values yields the equation, $\ln(k) = 33.977 - (12{,}689/T)$ with a correlation coefficient of 0.99997.

2. Calculate the $t_{90}$ shelf life expiration date, in days, at 25°C (298 K) using the equation in example 1:

$$\ln(k_{25}) = 33.977 - (12{,}689/298) = 33.977 - 42.581 = -8.604$$

$$k_{25} = e^{-8.604} = 1.834 \times 10^{-4} \text{ hour}^{-1} = 4.402 \times 10^{-3} \text{ day}^{-1}$$

$$t_{90} = 0.105/k = 0.105/4.402 \times 10^{-3} \text{ day}^{-1} = 23.85 \text{ days}$$

$$t_{90} \text{ at } 25°C = 23.85 \text{ days}$$

### 18.4.2 STABILITY PREDICTIONS USING THE INTEGRATED ARRHENIUS EQUATION

The following examples illustrate stability predictions based on one accurately determined isothermal rate constant and adherence to the same degradation rate order, e.g., first order, at temperatures at which stability is to be calculated from the equation, $\ln(k_2/k_1) = E_a(T_2 - T_1)/[R(T_2 \times T_1)]$.

**18.4.2.1 Calculating stability prediction using integrated Arrhenius equation**

*Example—Stability using integrated Arrhenius equations*

1. Calculate the $t_{85}$ stability expiration date at 4°C (277 K) for an ester hydrolysis with an $E_a$ = 15 kcal/mol and $k$ = 0.0045 hour$^{-1}$ at 23°C (296 K):

$$\ln(k_{277}/0.0045) = 15(277 - 296)/[1.987 \times 10^{-3}\ (277 \times 296)]$$

$$\ln(k_{277}) - \ln(0.0045) = -285/162.92$$

$$\ln(k_{277}) = -1.749 + \ln(0.0045) = -7.153$$

$$k_{277} = e^{-7.153} = 7.825 \times 10^{-4} \text{ hour}^{-1}$$

$$t_{85} = \ln(0.850)/k_{277} = -0.163/-7.825 \times 10^{-4} \text{ hour}^{-1} = 208.3 \text{ hours}$$

$$t_{85} \text{ at } 4°C = 208.3 \text{ hours } (t_{85} \text{ at } 23°C \text{ is } 36.2 \text{ hours})$$

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### 18.4.3 ARRHENIUS-BASED $Q_{10}$ STABILITY ESTIMATION

The temperature coefficient ($Q_{10}$) represents the multiplicative factor by which a chemical reaction rate constant changes in the same direction as the temperature for each 10°C change. For drug molecules, $Q_{10}$ ranges from 2 to 5, corresponding to an $E_a$ range of 10–25 kcal/mol or 42–105 kJ/mol. A $Q_{10}$ of 3 yields reasonable estimates of drug stability in the equation,

$$\left(t_n \text{ at } T_2\right) = \left(t_n \text{ at } T_1\right) / \left\{Q_{10}^{\left[\left(T_2 - T_1\right) / 10\right]}\right\}$$

$n$ = percentage of remaining $C_0$
$T_1$ = temperature at which $t_n$ is known
$T_2$ = temperature at which $t_n$ is to be estimated

Calculations using $Q_{10}$ values of both 2 and 4 may be used to obtain the shortest or most conservative stability estimate, but $Q_{10}$ = 3 is applied in the following two examples.

**18.4.3.1 Calculating Arrhenius-based $Q_{10}$ stability estimation**

*Example—Arrhenius-based $Q_{10}$ stability*

1. Estimate the $t_{90}$ expiration date in hours of an antibiotic suspension stored in a closed automobile at 57° for which the 8° refrigeration $t_{90}$ is 14 days.

$$t_{90} \text{ at } 57° = [14 \text{ days} \times (24 \text{ hours/day})]/\{3^{[(57-8)/10]}\} = 336 \text{ hours}/3^{4.9} = 336 \text{ hours}/217.7 = 1.54 \text{ hours}$$

$$t_{90} \text{ at } 57° = 1.54 \text{ hours}$$

***Change to read:***

## 19. MEAN KINETIC TEMPERATURE

### 19.1 Definition of Terms

Mean kinetic temperature (MKT) is the single calculated temperature at which the total amount of degradation over a particular period is equal to the sum of the individual degradations that would occur at various temperatures. MKT may be considered as an isothermal storage temperature that simulates the non-isothermal effects of storage temperature variation. It is not a simple arithmetic mean (see ▲*Mean Kinetic Temperature in the Evaluation of Temperature Excursions during Storage and Transportation of Drug Products* ⟨1079.2⟩▲ (CN 1-Dec-2020)).

Controlled room temperature (CRT) is the temperature thermostatically maintained that encompasses the usual and customary working environment of 20°–25° (68°–77°F). The following conditions apply to the application of MKT to CRT:

- MKT must not exceed 25°.
- Excursions between 15° and 30° (59° and 86°F) that are experienced in pharmacies, hospitals, and warehouses, and during shipping are allowed.
- Provided the MKT does not exceed 25°, transient spikes up to 40° are permitted as long as they do not exceed 24 h. Spikes above 40° may be permitted only if the manufacturer provides documentation that such spikes are permitted.
- Articles may be labeled for storage at “controlled room temperature” or at “up to 25°,” or other wording based on the same MKT (see ⟨659⟩).

### 19.2 MKT Equation

(See ▲ ⟨1079.2⟩▲ (CN 1-Dec-2020).)

$$\text{MKT } or\ T_k = \frac{-\Delta H/R}{\ln\left(\frac{\Sigma e^{-\Delta H/RT_1} + \ldots + e^{-\Delta H/RT_n}}{n}\right)}$$

| | |
|---|---|
| $\Delta H$ = | 83.144 kJ/mol[a] |
| $R$ = | $8.3144 \times 10^{-3}$ kJ/mol · K (universal gas constant) |
| $T_1$ = | value for the temperature recorded during the first time period, e.g., the first week |
| $T_n$ = | value for the temperature recorded during the $n$th time period, e.g., $n$th week |
| $n$ = | total number of storage temperatures recorded during the observation period |

[a] $\Delta H$ = 83.144 kJ/mol or 19.86 kcal/mol is a typical or approximately average Arrhenius energy of activation, Ea, for hydrolysis of amide, ester, lactam and peptide bonds from more than 100 drug stability reports.[1,2,3]

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[NOTE—All temperatures, *T*, are absolute temperatures in degrees Kelvin (K).]

## 19.3 MKT Equation Stepwise Solution

1. Calculate the inverse ln of the negative value of ($\Delta H/RT$) for each of the total *n* mean temperatures.
   For example, for 27°C or 300.15 K:

$$e^{-\Delta H/RT} = e^{-\left[\left(83.144\ \text{kJ/mol}\right)/\left[\left(8.3144\times10^{-3}\text{kJ/mol}\cdot\text{K}\right)\times 300.15\ \text{K}\right]} = e^{-(83.144/2.496)} = e^{-33.311} = 3.414\times10^{-15}$$

2. Sum, Σ, all the step 1 values.
3. Divide the step 2 sum by *n*.
4. Take ln of the step 3 result.
5. Divide $-\Delta H/R$ (equivalent to –10,000 K) by the step 4 answer.

$$-\Delta H/R = \left(83.144\ \text{kJ/mol}\right)/8.3144\times10^{-3}\text{kj/mol}\cdot\text{K}\right) = -10,000\ \text{K}$$

6. MKT = step 5 answer.
7. Compare step 6 answer with MKT ≤25°C for determination of whether the temperature falls within the range for CRT.

## 19.4 Example Calculations of MKT for CRT Storage Evaluation

### EXAMPLE 1—CALCULATION OF 1-MONTH MKT

1. Calculate the 1-month MKT in °C and determine whether it falls within the definition of CRT from the data in *Table 9* in which the far right-hand column comprises the step (1) calculation in *19.3 MKT Equation Stepwise Solution*.

**Table 9**

| Week | Temperatures, *T* | | | | $\Delta H/RT$ | $e^{-\Delta H/RT}$ |
|---|---|---|---|---|---|---|
| | Lowest (°C)[a] | Highest (°C)[a] | Mean (°C)[a] | Mean (K) | | |
| 1 | 24 | 28 | 26 | 299.15 | 33.428 | $3.037\times10^{-15}$ |
| 2 | 23 | 26 | 25 | 298.15 | 33.540 | $2.715\times10^{-15}$ |
| 3 | 19 | 24 | 22 | 295.15 | 33.881 | $1.930\times10^{-15}$ |
| 4 | 21 | 26 | 24 | 297.15 | 33.653 | $2.425\times10^{-15}$ |

[a] Rounded to nearest whole °C.

2. $\Sigma(3.037\times10^{-15} + 2.715\times10^{-15} + 1.930\times10^{-15} + 2.425\times10^{-15}) = 1.011\times10^{-14}$
3. $(1.011\times10^{-14})/4 = 2.528\times10^{-15}$
4. $\ln(2.528\times10^{-15}) = -33.612$
5. –10,000 K/–33.612 = 297.51 K
6. MKT (°C) = 297.51 K – 273.15 K = 24.36°C
7. MKT of 24.36°C meets the CRT between 20° and 25°C; thus, the MKT falls within the CRT range in ⟨659⟩.

### EXAMPLE 2—CALCULATION OF ANNUAL MKT

1. Calculate the annual MKT in °C and determine whether the MKT falls within the range of CRT from the data in *Table 10* in which the far right-hand column comprises the step (1) calculation in *19.3 MKT Equation Stepwise Solution*.

**Table 10**

| Month | Temperatures, *T* | | | | $\Delta H/RT$ | $e^{-\Delta H/RT}$ |
|---|---|---|---|---|---|---|
| | Lowest (°C)[a] | Highest (°C)[a] | Mean (°C)[a] | Mean (K) | | |
| April | 23 | 27 | 25 | 298.15 | 33.540 | $2.715\times10^{-15}$ |
| May | 23 | 28 | 26 | 299.15 | 33.428 | $3.037\times10^{-15}$ |
| June | 22 | 25 | 24 | 297.15 | 33.653 | $2.425\times10^{-15}$ |
| July | 23 | 27 | 25 | 298.15 | 33.540 | $2.715\times10^{-15}$ |
| August | 27 | 29 | 28 | 301.15 | 33.206 | $3.791\times10^{-15}$ |
| September | 24 | 26 | 25 | 298.15 | 33.540 | $2.715\times10^{-15}$ |

[1] Kennon L. Use of models in determining chemical pharmaceutical stability. *J Pharm Sci.* 1964;53(7):815–8.
[2] Bailey LC, Medwick T. Mean kinetic temperature—a concept for storage of pharmaceuticals. *Pharm Forum.* 1993;19:6163–6.
[3] Yoshioka S, Stella VJ. *Stability of Drugs and Dosage Forms*. New York, NY: Kluwer Academic Publishers; 2002:62–5.

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**Table 10** *(continued)*

| Month | Temperatures, *T* | | | | $\Delta H/RT$ | $e^{-\Delta H/RT}$ |
|---|---|---|---|---|---|---|
| | Lowest (°C)[a] | Highest (°C)[a] | Mean (°C)[a] | Mean (K) | | |
| October | 24 | 27 | 26 | 299.15 | 33.428 | $3.037 \times 10^{-15}$ |
| November | 23 | 26 | 25 | 298.15 | 33.540 | $2.715 \times 10^{-15}$ |
| December | 23 | 27 | 25 | 298.15 | 33.540 | $2.715 \times 10^{-15}$ |
| January | 24 | 28 | 26 | 299.15 | 33.428 | $3.037 \times 10^{-15}$ |
| February | 21 | 24 | 23 | 296.15 | 33.767 | $2.164 \times 10^{-15}$ |
| March | 23 | 26 | 25 | 298.15 | 33.540 | $2.715 \times 10^{-15}$ |

[a] Rounded to nearest whole °C.

2. $\Sigma(2.715 \times 10^{-15} + 3.037 \times 10^{-15} + 2.425 \times 10^{-15} + 2.715 \times 10^{-15} + 3.791 \times 10^{-15} + 2.715 \times 10^{-15} + 3.037 \times 10^{-15} + 2.715 \times 10^{-15} + 2.715 \times 10^{-15} + 3.037 \times 10^{-15} + 2.164 \times 10^{-15} + 2.715 \times 10^{-15}) = 3.378 \times 10^{-14}$
3. $3.378 \times 10^{-14}/12 = 2.815 \times 10^{-15}$
4. $\ln(2.815 \times 10^{-15}) = -33.504$
5. $-10{,}000\ \text{K}/-33.504 = 298.473\ \text{K}$
6. MKT (°C) = 298.473 K − 273.15 K = 25.32°C
7. 25.32°C exceeds the CRT limit of ≤ 25°C for MKT; thus, MKT does not fall within the CRT range in ⟨659⟩

EXAMPLE 3—CALCULATION OF ANNUAL MKT

1. Calculate the annual MKT in °C and determine whether the MKT falls within the range of CRT from the data in *Table 11* in which the far right hand column comprises step (1) calculation in *19.4 Example Calculations of MKT for CRT Storage Evaluation*.

**Table 11**

| Month | Temperature, *T* | | | | $\Delta H/RT$ | $e^{-\Delta H/RT}$ |
|---|---|---|---|---|---|---|
| | Lowest, °C[a] | Highest, °C[a] | Mean, °C[a] | Mean, K[b] | | |
| 1 | 23 | 27 | 25 | 298.15 | 33.540 | $2.715 \times 10^{-15}$ |
| 2 | 22 | 28 | 25 | 298.15 | 33.540 | $2.715 \times 10^{-15}$ |
| 3 | 18 | 22 | 20 | 293.15 | 34.112 | $1.532 \times 10^{-15}$ |
| 4 | 23 | 27 | 25 | 298.15 | 33.540 | $2.715 \times 10^{-15}$ |
| 5 | 23 | 26 | 25 | 298.15 | 33.540 | $2.715 \times 10^{-15}$ |
| 6 | 24 | 26 | 25 | 298.15 | 33.540 | $2.715 \times 10^{-15}$ |
| 7 | 24 | 26 | 25 | 298.15 | 33.540 | $2.715 \times 10^{-15}$ |
| 8 | 27 | 33 | 30 | 303.15 | 32.987 | $4.720 \times 10^{-15}$ |
| 9 | 23 | 27 | 25 | 298.15 | 33.540 | $2.715 \times 10^{-15}$ |
| 10 | 24 | 25 | 25 | 298.15 | 33.540 | $2.715 \times 10^{-15}$ |
| 11 | 21 | 29 | 25 | 298.15 | 33.540 | $2.715 \times 10^{-15}$ |
| 12 | 23 | 26 | 25 | 298.15 | 33.540 | $2.715 \times 10^{-15}$ |

[a] Rounded to nearest whole °C.
[b] K = °C + 273.15

2. $\Sigma(2.715 \times 10^{-15} + 2.715 \times 10^{-15} + 1.532 + \times 10^{-15} + 2.715 \times 10^{-15} + 2.715 \times 10^{-15} + 2.715 \times 10^{-15} + 2.715 \times 10^{-15} + 4.720 \times 10^{-15} + 2.715 \times 10^{-15} + 2.715 \times 10^{-15} + 2.715 \times 10^{-15} + 2.715 \times 10^{-15} + 2.715 \times 10^{-15}) = 3.340 \times 10^{-14}$
3. $3.340 \times 10^{-14}/12 = 2.783 \times 10^{-15}$
4. $\ln(2.783 \times 10^{-15}) = -33.515$
5. $-10{,}000\text{K}/-33.515 = 298.372\text{K}$
6. MKT(°C) = 298.372 K − 273.15 K = 25.22°C
7. 25.22°C exceeds the CRT limit of ≤25°C for MKT; thus, MKT does not fall within the CRT range in ⟨659⟩

The 2 months of mean temperatures 20°C and 30°C, which arithmetically yield 2 months of mean 25°C, resulted in an MKT of 25.22°C, exceeding the range for CRT. This ▲results in higher temperatures given greater weight in the calculation (see ⟨1079.2⟩).▲ (CN 1-Dec-2020) However, higher temperatures are not intentionally ▲given greater weight in the MKT calculation,▲ (CN 1-Dec-2020) but higher temperatures have a greater influence over the MKT result, because of the logarithmic or exponential increase in reaction rate with arithmetic or linear increase in temperature.

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# APPENDIX 1: COMMON AND NATURAL LOGARITHMS

The logarithm of a number is the exponent or power to which a given base number must be raised to equal that number. Thus, the logarithm of $Y$ to the base, $b$, equals $X$, or $\log_b(Y) = X$. The logarithm of 0 and all negative numbers is undefined or nonexistent. The logarithm of 1 is 0 and of numbers <1 is negative in all systems (see *Table A-1*).

**Table A-1 Common (or Briggsian) and Natural (or Napierian) Logarithms**

| Logarithmic System | Abbreviation or Symbol | Base Number | Format | Antilogarithm or Inverse Logarithm |
|---|---|---|---|---|
| Common | Log | 10 | $\log Y = X$ | $10^X = Y$ |
| Natural | Ln | $e$ or 2.7183[a] | $\ln Y = X$ | $e^X = Y$ |

[a] $e$ is an irrational number derived from an infinite series of reciprocal whole number factorials, $e = 1 + 1/1! + 1/2! + 1/3! + 1/4! \ldots + 1/n!$, where $n$ = infinity. $e$ rounds to 2.7183 when $n \geq 8$.

The relationships between common and natural logarithms are the following:

1. $\log Y = \ln Y/\ln 10 = \ln Y/2.303$
2. $\ln Y = \ln 10 \times \log Y = 2.303 \times \log Y$

Rules for some common calculations with logarithms are shown in *Table A-2*.

**Table A-2 Rules for Calculating with Logarithms**

| | Formula | Example |
|---|---|---|
| Additions and multiplications | $\ln(A) + \ln(B) = \ln(A \times B)$<br>$\log(A) + \log(B) = \log(A \times B)$ | $\ln(0.62) + \ln(1.73) = \ln(0.62 \times 1.73) = \ln(1.0726) = 0.070$<br>$\log(5.7) + \log(0.43) = \log(5.7 \times 0.43) = \log(2.451) = 0.389$ |
| Subtraction and quotients | $\ln(A) - \ln(B) = \ln(A/B)$<br>$\log(A) - \log(B) = \log(A/B)$ | $\ln(0.5) - \ln(4) = \ln(0.5/4) = \ln(0.125) = -2.079$<br>$\log(1.57) - \log(2.48) = \log(1.57/2.48) = \log(0.6330645) = -0.199$ |
| Simple non-base exponentials | $\ln(Y^Z) = Z \times \ln(Y)$<br>$\log(Y^Z) = Z \times \log(Y)$ | $13.6^{-Z} = 1.25$<br>$\ln(1.25) = -Z \times \ln(13.6)$<br>$Z = -\ln(1.25)/\ln(13.6) = -0.223/2.610 = -0.085$<br>$0.57^Z = 2.3$<br>$\log(2.3) = Z \times \log(0.57)$<br>$Z = \log(2.3)/\log(0.57) = 0.362/-0.244 = -1.484$ |
| Base exponentials | $\ln(a \times e^{\pm b}) = \ln(x) \leftrightarrow \ln(a) \pm b = \ln(x)$<br>$\log(a \times 10^{\pm b}) = \log(x) \leftrightarrow \log(a) \pm b = \log(x)$ | $67 \times 10^b = 15.1$<br>$\log(67) + b = \log(15.1)$<br>$1.826 + b = 1.179$<br>$b = 1.179 - 1.826 = -0.647$ |

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# ⟨1163⟩ QUALITY ASSURANCE IN PHARMACEUTICAL COMPOUNDING

## INTRODUCTION

The need for a quality assurance system is well documented in *United States Pharmacopeia (USP)* chapters for compounded preparations (see *Quality Control* under *Pharmaceutical Compounding—Nonsterile Preparations* ⟨795⟩ and *Quality Assurance (QA) Program* under *Pharmaceutical Compounding—Sterile Preparations* ⟨797⟩). A quality assurance program is guided by written procedures that define responsibilities and practices that ensure compounded preparations are produced with quality attributes appropriate to meet the needs of patients and health care professionals. The authority and responsibility for the Quality Assurance program should be clearly defined and implemented and should include at least the following nine separate but integrated components: (1) training; (2) standard operating procedures (SOPs); (3) documentation; (4) verification; (5) testing; (6) cleaning, disinfecting, and safety; (7) containers, packaging, repackaging, labeling, and storage; (8) outsourcing, if used; and (9) responsible personnel.

The definition of compounding for the purpose of this chapter is defined in general test chapter ⟨795⟩.

The safety, quality, and efficacy and/or benefit of compounded preparations depend on correct ingredients and calculations; accurate and precise measurements; appropriate formulation, facilities, equipment, and procedures; and prudent pharmaceutical judgment. As a final check, the compounder shall review each procedure in the compounding process. To ensure accuracy and completeness, the compounder shall observe the finished preparation to ensure that it appears as expected and shall investigate any discrepancies and take appropriate corrective action before the prescription is dispensed to the patient.

The water used in all aspects of compounding should meet the requirements of *Waters for Pharmaceutical Purposes* ⟨1231⟩.

Radiopharmaceuticals and radiolabeled materials have unique characteristics requiring additional quality assurances described in *Positron Emission Tomography Drugs for Compounding, Investigational, and Research Uses* ⟨823⟩ and the *Radiopharmaceuticals as CSPs* section under ⟨797⟩.

The responsibilities of the compounder and compounding personnel can be found in chapters ⟨795⟩ and ⟨797⟩.

## TRAINING

Personnel involved in nonsterile or sterile compounding require additional, specific training and periodic retraining beyond the training needed for routine dispensing duties. A thorough quality assurance program for compounded preparations requires documentation of both training and skill competency. In addition, the authority and responsibility for the QA program should be clearly defined as implemented. Training for nonsterile compounders should meet or exceed the standards set forth in ⟨795⟩, and personnel training for sterile preparation compounders should meet or exceed the standards set forth in ⟨797⟩.

## STANDARD OPERATING PROCEDURES

SOPs for pharmaceutical compounding are documents that describe how to perform routine and expected tasks in the compounding environment, including but not limited to procedures involving:

- Beyond-Use dating
- Chemical and physical stability
- Cleaning and disinfecting
- Component quality evaluation
- Compounding methods
- Dispensing
- Documentation
- Environmental quality and maintenance
- Equipment maintenance, calibration, and operation
- Formulation development
- Labeling
- Materials and final compounded preparation handling and storage
- Measuring and weighing
- Packaging and repackaging
- Patient monitoring, complaints, and adverse event reporting
- Patient or caregiver education and training
- Personnel cleanliness and garb
- Purchasing
- Quality Assurance and Continuous Quality Monitoring
- Safety
- Shipping
- Testing
- Training and retraining

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SOPs are itemized instructions that describe when a task will be performed, how a task will be performed, who will perform the task, why the task is necessary, any limitations in performing the task, and what action to take when unacceptable deviations or discrepancies occur.

SOPs must be reviewed regularly and updated as necessary. Auditing and verifying compliance with established SOPs should be performed periodically. The SOP should be specific to each device and process used in compounding. Properly maintained and implemented SOPs are vital to preparation quality.

## DOCUMENTATION

The purpose of documentation is to provide a record of all aspects of compounding operations and procedures that are described in this chapter, in ⟨795⟩, and in ⟨797⟩. Information on the compounding record should ideally be entered as the tasks are performed or as testing data is received. Compounding records should be reviewed for accuracy, completeness (as appropriate) and approved by QA personnel, prior to dispensing. Additionally, beyond-use dating and sterility studies, where appropriate, should be documented by reference to at least one of the following:

- Stability studies published in peer-reviewed literature,
- In-house or laboratory conducted stability and/or sterility studies,
- National compendia, or
- An extrapolation of above based on professional judgment.

## VERIFICATION

Verification involves authoritatively signed assurance and documentation that a process, procedure, or piece of equipment is functioning properly and producing the expected results. The act of verification of a compounding procedure involves checking to ensure the calculations, weighing and measuring, order of mixing, and compounding techniques and equipment were appropriate and accurately performed. The quality of ingredients should be verified upon receipt (e.g., Certificate of Analysis, manufacturer's label on commmercial products, etc.). Verification may require outside laboratory testing when in-house capabilities are not adequate. Equipment verification methods are sometimes available from manufacturers of the specific equipment or can be developed in-house. The responsibility for assuring that equipment performance is verified, including work completed by contractors, resides with the compounder.

See *Component Selection, Handling, and Storage* under ⟨795⟩.

## TESTING

A quality assurance program for compounded preparations should include testing during the compounding process and of the finished compounded preparation, when appropriate, as described in chapters ⟨795⟩ and ⟨797⟩. The compounder should have a basic understanding of pharmaceutical analysis to ensure that valid results are obtained when tests are being conducted, whether they are done in-house or outsourced. Acceptance criteria shall be determined prior to testing. Testing every compounded preparation is neither practical nor officially required, but compounders should conduct visual inspections and know: (1) the importance of testing in the overall quality program in the compounding facility, (2) when to test, (3) what to test, (4) what appropriate method(s) and equipment to use, (5) how to interpret the results, (6) the limits of the test, and (7) specific actions required when a preparation does not meet specifications. Investigative and corrective action should extend to other preparations that may have been associated with the specific failure or discrepancy. Testing may involve one or more quality attributes, and each test will have one or more acceptable procedures, usually with well-defined acceptance criteria.

The goal in testing is to determine accurately the adequacy of the compounding process and the quality of the preparation. Any testing procedure used should have accuracy, reproducibility, and specificity. No single testing procedure is suitable for all drugs or preparations because a number of factors determine the validity and reliability of results.

Compounding professionals have two options for the testing that is required for compounded preparations or their ingredients. Some testing methods can easily be performed at the compounding site, but some may need to be outsourced to a contract laboratory. Some testing methods can be conducted in-house by an individual who possesses a good understanding of pharmaceutical analysis and proper training. See *Table 1* for a list of compendial testing methods and *USP* chapters for reference.

**Table 1. U.S. Pharmacopeia Chapters for Selected Quality Testing Methods and Procedures**

| | Chapter Title | Chapter |
|---|---|---|
| **General Testing** | | |
| Boiling point | Distilling Range | ⟨721⟩ |
| Density | Density of Solids | ⟨699⟩ |
| Ion selective potentiometry | — | — |
| Loss on drying | Loss on Drying | ⟨731⟩ |
| | Pharmaceutical Calculations in Prescription Compounding | ⟨1160⟩ |
| Melting point | Melting Range or Temperature | ⟨741⟩ |

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**Table 1. U.S. Pharmacopeia Chapters for Selected Quality Testing Methods and Procedures** *(continued)*

| | Chapter Title | Chapter |
|---|---|---|
| Osmolality and osmolarity | Pharmaceutical Calculations in Prescription Compounding | ⟨1160⟩ |
| | Osmolality and Osmolarity | ⟨785⟩ |
| Particle size | Powder Fineness | ⟨811⟩ |
| Particulate matter in injections | Particulate Matter in Injections | ⟨788⟩ |
| pH | pH | ⟨791⟩ |
| Refractive index | Refractive Index | ⟨831⟩ |
| Viscosity change | Viscosity—Capillary Methods | ⟨911⟩ |
| Volumetric | Prescription Balances and Volumetric Apparatus | ⟨1176⟩ |
| Weight | Prescription Balances and Volumetric Apparatus | ⟨1176⟩ |
| **Spectroscopy** | | |
| Atomic absorption spectroscopy | Atomic Absorption Spectroscopy | ⟨852⟩ |
| Fluorescence spectroscopy | Fluorescence Spectroscopy | ⟨853⟩ |
| Mid-Infrared spectroscopy | Mid-Infrared Spectroscopy | ⟨854⟩ |
| Ultraviolet/visible spectroscopy | Ultraviolet-Visible Spectroscopy | ⟨857⟩ |
| **Chromatography** | | |
| Column chromatography (CC) | Chromatography | ⟨621⟩ |
| Gas chromatography (GC) | Chromatography | ⟨621⟩ |
| High-performance liquid chromatography (HPLC) | Chromatography | ⟨621⟩ |
| Paper chromatography (PC) | Chromatography | ⟨621⟩ |
| Thin-layer chromatography (TLC) | Chromatography | ⟨621⟩ |
| **Microbiology** | | |
| Endotoxin testing | Bacterial Endotoxins Test | ⟨85⟩ |
| Microbial limit testing | Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests | ⟨61⟩ |
| | Microbiological Examination of Nonsterile Products: Tests for Specified Microorganisms | ⟨62⟩ |
| Preservative effectiveness testing | Antimicrobial Effectiveness Testing | ⟨51⟩ |
| Sterility | Sterility Tests | ⟨71⟩ |

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If testing is done at the compounding site, appropriate equipment shall be obtained and qualified either by the manufacturer upon sale or by the compounding professional upon receipt and shall be maintained, calibrated, and used properly. If testing is outsourced, the compounding professional should determine what to outsource, how to select a laboratory, and should develop an ongoing relationship with the laboratories chosen. Contract laboratories shall follow standards set forth in *USP* general chapters, as appropriate, and preferably should be registered with the U.S. Food and Drug Administration (FDA).

## Selection of a Testing Method

One general consideration in testing procedure selection is the type of information needed, such as quantitative (strength, concentration), semiquantitative (where a tolerance level is involved, as in endotoxin levels), or qualitative (presence/absence testing, including substance identification, sterility). Another consideration involves the physical and chemical characteristics of the analyte, including solubility, partition coefficient, dissociation constant (pKa), volatility, binding, and the quantity present. The testing method selected also depends upon factors such as sample handling/preparation/purification requirements; type of data needed; and accuracy, reproducibility, and specificity required.

The degree of quantitative measurement and specificity must be considered in the verification process. The typical analytical characteristics used in method verification include accuracy, precision, specificity, detection limit, quantitation limit, linearity, range, and ruggedness. Generally, the greater the level of accuracy, precision, or specificity required, the more sophisticated and expensive the testing methods needed. The methods used are also governed by the types of instrumentation available and the standards available for comparison.

Pharmaceutical analysis decisions include procedure selection, obtaining a representative sample (the number of preparation units selected to adequately represent the entire formulation, e.g., 10 randomly selected capsules from a preparation of 100 capsules), storage/shipping of the sample, sample preparation for analysis, the actual analysis, data acquisition, data treatment, and interpretation.

The compounding professional is responsible for implementing a program using selected testing methods for the preparations compounded in the facility. *USP* chapters on spectroscopy and chromatography methods are referenced in *Table*

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*1*. Examples of general and microbiological testing methods are discussed later in this chapter. Examples of selected testing methods for bulk substances and various dosage forms (see *Pharmaceutical Dosage Forms* ⟨1151⟩) are shown in *Table 2*.

**Table 2. Selected Compendial Testing Methods for Bulk Substances and Various Dosage Forms**

| | Testing Method[a] | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Bulk Substances and Dosage Forms | Wt | Vol | pH | Osm | RI | Sp Gr | MP | UV/Vis | HPLC | GC | IR | Sterile | Endo-toxin | PM |
| Bulk substances | – | – | + | – | + | – | + | + | + | + | + | – | +[b] | – |
| Capsules | + | – | – | – | – | – | – | – | + | + | – | – | – | – |
| Emulsions | + | + | + | – | – | + | – | – | + | + | – | – | – | – |
| Gels | + | + | + | – | + | + | – | – | + | + | – | – | – | – |
| Inhalations | + | + | + | + | + | + | – | + | + | + | – | + | + | – |
| Injections | + | + | + | + | + | + | – | + | + | + | – | + | + | + |
| Inserts | + | – | – | – | – | + | + | – | + | + | – | – | – | – |
| Irrigations | + | + | + | + | + | + | – | + | + | + | – | + | + | – |
| Lozenges | + | – | – | – | – | – | – | – | + | + | – | – | – | – |
| Nasals | + | + | + | + | + | + | – | + | + | + | – | *[c] | – | – |
| Ophthalmics | + | + | + | + | + | + | – | + | + | + | – | + | – | +[d] |
| Otics | + | + | + | + | + | + | – | + | + | + | – | – | – | – |
| Powders | + | – | – | – | – | – | – | – | + | + | – | – | – | – |
| Semisolids | + | – | + | – | – | + | + | – | + | + | – | – | – | – |
| Solutions, nonsterile | + | + | + | + | + | + | – | + | + | + | – | – | – | – |
| Sterile implant gels | + | + | + | + | + | + | – | + | + | + | – | + | + | – |
| Sterile implant solids | + | + | – | – | – | – | + | + | + | + | – | + | + | – |
| Sticks | + | – | – | – | – | + | + | – | + | + | – | – | – | – |
| Suppositories | + | – | – | – | – | + | + | – | + | + | – | – | – | – |
| Suspensions, nonsterile | + | + | + | – | – | + | – | – | + | + | – | – | – | – |
| Tablets | + | – | – | – | – | – | – | – | + | + | – | – | – | – |

[a] Wt, weight; Vol, volume; Osm, osmolality/osmolarity; RI, refractive index; Sp Gr, specific gravity; MP, melting point; UV/Vis, ultraviolet/visible spectroscopy; HPLC, high-performance liquid chromatography; GC, gas chromatography; IR, infrared spectroscopy; PM, particulate matter; +, test applicable; –, test not applicable.
[b] Endotoxin testing may be needed for bulk substances used in compounding some sterile preparations.
[c] *, microbial limits (see *Microbiological Examination of Nonsterile Products: Acceptance Criteria for Pharmaceutical Preparations and Substances for Pharmaceutical Use* ⟨1111⟩ and *Pharmaceutical Compounding—Sterile Preparations* ⟨797⟩).
[d] Solutions only, not suspensions or ointments.

## Sampling Requirements

Before collecting samples for testing, compounding professionals should consider the following factors:

- Quantity of preparation being compounded, for a specific prescription versus in anticipation of prescriptions routinely received
- Number of samples needed
- Destructive or nondestructive testing
- Appropriate methods of obtaining representative samples
- Physical state of the samples (solid, liquid, or gas)
- Type of container required for collection and storage
- Any special handling and shipping requirements or restrictions (e.g., controlled drug substances, dangerous or hazardous chemicals, flammable or caustic substances, and refrigerated or frozen preparations)

## Storage Requirements

Storage requirements for samples must be specified, including type of container, temperature, humidity, and light protection (see *General Notices and Requirements* and the *Containers, Packaging, Repackaging, Labeling, and Storage* section in this chapter).

The effect(s) that any substance has on the compounded preparation that may interfere or alter the results must be known beforehand. When sending a preparation to a contract laboratory, the compounder should provide the complete written formulation so that the laboratory can quickly determine if there may be any interfering substances present.

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### Data Interpretation Requirements

The collection of raw data from the testing process must be completed accurately. One must ensure that appropriate and valid descriptive statistics (e.g., mean, standard deviation) are used to analyze the data and that the operating parameters of the analytical instruments are well-established. Reference values, if available, should be provided with the analytical results. A description of the analytical controls used by the laboratory is important for documentation, as is the source of reference standards used to establish standard curves.

### Personnel Requirements and Considerations

If testing is done in-house, personnel involved in this activity must be appropriately trained and evaluated with documentation of the training and evaluation. If testing is outsourced, the compounder must be assured of the credentials, proper training, and continuing competency activities of the personnel in the contract laboratory.

## PHYSICAL TESTING OF DOSAGE UNITS

[NOTE—In this section the terms "unit" and "dosage unit" are synonymous. To ensure the consistency of dosage units, each unit in a batch should have a uniform weight within a narrow range. Dosage units are defined as dosage forms containing a single dose or a part of a dose in each unit. If multiple dose units are compounded in a batch formulation, the total number of units should not deviate outside of ±10% of the theoretical number of units.]

## WEIGHT ASSESSMENT

First, zero or tare the balance. During the compounding process intermediate weighing may be necessary to ensure that all substances have been included and weighed accurately.

At the end of the compounding process, for the dosage form and quantity designated, take care to preserve the integrity of each dosage unit during the following assessment procedures. Assume the concentration (weight of drug substance per weight of dosage unit) is uniform. The following are examples of weight assessment.

### Hard Capsules

- Zero or tare balance with an empty capsule.
- Accurately weigh each individual filled capsule from a representative sample of the finished batch (for example, a minimum of 5% of total capsules or 10 individual capsules, whichever is less) and record the weight of each finished capsule on the compounding record.
- Calculate the theoretical weight of a finished capsule's contents.
- Compare the actual content weight of each finished capsule in the representative sample with the theoretical weight of a finished capsule's contents.
- Determine if there is a deviation outside ±10% with any weight of a finished capsule's contents and the theoretical weight of a finished capsule, and if so,
  - Review the compounding record to ensure no steps were omitted.
  - Repeat with a larger representative sample of the finished batch (10% of total capsules or 20 individual capsules, whichever is less). Do not mix with the first batch tested.
- If a deviation outside of ±10% is discovered in the second representative sample, then destroy the batch.

### Other Solids (Including Tablets, Suppositories, Inserts, and Lozenges)

- Accurately weigh each individual dosage unit from a representative sample of the finished batch (for example, a minimum of 5% of total tablets or 10 individual tablets, whichever is less) and record the weight of each dosage unit on the compounding record.
- Calculate the theoretical weight of the dosage unit.
- Compare the actual weight of each dosage unit in the representative sample with the theoretical weight of a dosage unit.
- Determine if there is a deviation outside ±10% with any weight of a finished dosage unit and the theoretical weight of a finished dosage unit, and if so,
  - Review the compounding record to ensure no steps were omitted.
  - Repeat with a larger representative sample of the finished batch (10% of total tablets or 20 individual tablets, whichever is less). Do not mix with the first batch tested.
- If a deviation outside of ±10% is discovered in the second representative sample, then destroy the batch.

### Semi-Solids (Including Creams, Gels, and Ointments)

- Accurately weigh an empty container and record the weight on the compounding record.
- Fill an empty container with the final compounded preparation.

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- Calculate the theoretical weight of the compounded preparation.
- Weigh the filled container.
- Determine if there is a deviation outside of ±10%, and if so, review the compounding record to ensure no steps were omitted. If the deviation cannot be explained, destroy the batch and prepare a new one.

*Additional Quality Assurance Checks Before Packaging Semi-Solids—*

- Visually inspect the preparation for foreign materials and expected appearance.
- Measure pH, when applicable.

## MICROBIOLOGICAL TESTING

Microbiological testing for pharmacy compounding includes sterility, endotoxin, preservative effectiveness testing, and microbial limit testing (see ⟨797⟩).

### Sterility Testing

Sterility tests may be conducted using commercial kits or by developing and verifying USP sterility testing protocols. Standards and procedures are explained in ⟨71⟩.

### Endotoxin Testing

Endotoxin tests may be conducted using commercial kits or by purchasing the components separately. Endotoxin testing may be performed in-house with appropriate training and experience. See ⟨85⟩.

### Preservative Effectiveness Testing

Preservative effectiveness testing may be conducted when preparing a frequently compounded formulation that contains a preservative. When such a test is performed, the results shall support the beyond-use-date (BUD) assigned to the compounded preparations. See ⟨51⟩.

### Microbial Limit Testing

Microbial limit testing may be conducted to provide an estimate of the number of viable aerobic microorganisms (see ⟨61⟩) or to demonstrate freedom from designated microbial species (see ⟨62⟩).

## CLEANING, DISINFECTING, AND SAFETY

This section applies to both equipment and facilities (see ⟨795⟩, ⟨797⟩, and *Disinfectants and Antiseptics* ⟨1072⟩).

***Change to read:***

## CONTAINERS, PACKAGING, REPACKAGING, LABELING, AND STORAGE

For storage, packaging, repackaging, and labeling of compounded preparations and repackaging of manufactured products (when defined as compounding in *USP*), refer to *USP General Notices and Requirements* and the following general chapters:

- *Containers—Glass* ⟨660⟩
- *Plastic Packaging Systems and their Materials of Construction* ⟨661⟩
- *Plastic Materials of Construction* ⟨661.1⟩
- *Plastic Packaging Systems for Pharmaceutical Use* ⟨661.2⟩
- ▲*Elastomeric Components Used in Injectable Pharmaceutical Packaging/Delivery Systems* ⟨381⟩▲ (CN 1-Dec-2020)
- *Good Repackaging Practices* ⟨1178⟩
- ▲*Risks and Mitigation Strategies for the Storage and Transportation of Finished Drug Products* ⟨1079⟩▲ (CN 1-Dec-2020)
- *Labeling* ⟨7⟩
- *Packaging and Storage Requirements* ⟨659⟩
- *Pharmaceutical Dosage Forms* ⟨1151⟩

## OUTSOURCING

[NOTE—This section addresses only the purchasing or selling of compounded preparations from pharmacy to pharmacy, not the outsourcing of analytical testing of compounded preparations.]

For pharmacies that prepare outsourced compounded preparations or repackaged commercial products, documentation of beyond-use dating, as defined previously in the *Documentation* section of this chapter, is required and shall be provided upon

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request. In addition, documentation of compliance with *USP* chapters ⟨795⟩ and ⟨797⟩ is required and shall be provided upon request.

For facilities that receive outsourced compounded preparations or repackaged commercial products, documentation shall be on file for all BUDs assigned to those preparations or products.

## RESPONSIBLE PERSONNEL

The responsibility and authority for a quality assurance program should be clearly defined and implemented. Personnel responsible for the quality assurance program should have the education, training, and experience necessary to perform the assigned functions. Quality assurance personnel should assure that documentation, verification, and testing are performed in accordance with written policies and procedures. If deviations from approved policies and procedures occur, it is the responsibility of the quality assurance personnel to investigate and to implement appropriate corrective action. Documentation of any investigations and corrective actions is the responsibility of the quality assurance personnel. Responsible personnel in the quality assurance program are essential in assuring the safety, identity, strength, quality, and purity of compounded drug preparations.

## SUMMARY

A quality assurance program is necessary to ensure the quality of compounded preparations. A sound quality assurance program includes detailed SOPs, documentation, verification, analytical and microbiological testing as appropriate to particular compounded preparations, and responsible quality assurance personnel. Compounding professionals must determine the types of testing and degree of testing that will be a part of their quality assurance program. They also must decide whether to perform testing in-house or outsource it to a contract laboratory.

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***Add the following:***

# ▲⟨1168⟩ COMPOUNDING FOR PHASE I INVESTIGATIONAL STUDIES

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    1.2 Background
    1.3 Applicable Regulatory Requirements
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## 1. INTRODUCTION

### 1.1 Scope

The objective of this chapter is to guide compounders in the compounding of investigational preparations and placebos that are used in investigational studies, specifically Phase I studies for humans. For the purpose of this chapter, the terms "study" or "studies" are used to refer to investigational studies, specifically Phase I investigational studies or trials. When compounding a preparation, the standards in *Pharmaceutical Compounding—Nonsterile Preparations* ⟨795⟩, *Pharmaceutical Compounding—Sterile Preparations* ⟨797⟩, and *Hazardous Drugs—Handling in Healthcare Settings* ⟨800⟩ apply. In addition to these standards, this chapter applies when a compounder is preparing an agent for use as an investigational agent in a Phase I clinical study.

### 1.2 Background

Investigational studies are biomedical or health-related research studies that follow a predefined protocol to ensure subject protection and data integrity. Before drugs are tested in humans, usually a battery of preclinical animal studies is conducted to provide information about the medication's safety profile and pharmacokinetic parameters. Investigational studies in humans can provide a critical understanding of a medication's safety profile (i.e., adverse drug reactions and drug interactions) and pharmacokinetic parameters, and can provide insight into early indications of therapeutic efficacy. Clinical studies generally are conducted in four phases, each with a different purpose to answer different questions.

1. **Phase I studies:** Researchers test an investigational drug or treatment in a small group of people (generally in the range of 20–80 patients or healthy volunteers) for the first time to evaluate the drug's safety, determine a safe dosage range, understand the pharmacokinetic profile, and identify side effects
2. **Phase II studies:** The investigational drug or treatment is administered to a larger group of people (usually NMT several hundred subjects with the specific disease state to be treated) to see if the drug is effective and to further evaluate its safety
3. **Phase III studies:** The investigational drug or treatment is administered to large groups of people (usually several hundred to several thousand subjects with the disease state of interest plus concomitant medical conditions) to confirm the drug's effectiveness, monitor side effects, compare it to commonly used treatments, and collect information that allows the investigational drug or treatment to be used safely

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4. **Phase IV studies:** Post-marketing studies delineate additional information about the drug's risks, benefits, and optimal use

Compounding of investigational preparations may be useful 1) when only a small number of doses are needed to support the study, 2) to evaluate various dosage forms or dosage regimens before choosing one for further study, or 3) to develop age-appropriate dosage forms for certain study populations. Compounding investigational preparations may provide flexibility in evaluating dosing ranges and alternative routes of administration, and may reduce the time and cost to do so while maintaining subject safety and high product quality.

Sponsors of studies involving investigational preparations being developed for commercial use usually provide the investigational agents to be used in Phase I studies. Sometimes, however, investigational agents require additional preparation before administration to patients. For example, a lyophilized powder may require reconstitution, or a vial of investigational agent may need to be combined with a solution for infusion. In some cases, a sponsor may use a compounder to prepare the investigational agent from a bulk drug substance or approved drug that the sponsor supplies. For investigator-initiated studies, the investigator may ask a compounder to prepare the investigational agent from either approved drugs or bulk drug substances that are available from commercial sources.

Virtually all dosage forms can be used in investigational studies (see *Pharmaceutical Dosage Forms* ⟨1151⟩). Investigational preparations may include tablets, capsules, powdered drug substance pre-weighed in unit doses, powdered drug substance in bulk for multiple subjects, solution or suspension in unit doses, solution or suspension in bulk for multiple subjects, sterile solutions for injection or infusion, sterile lyophilized powdered drug substance, powder for inhalation, nasal spray, radio-labeled bulk and unit doses, bulk drug substance and excipients for compounding, topical preparations such as creams or lotions, and/or placebos.

The formulation to be used in the study should be based on the questions to be answered by the study and should exclude any ingredient (i.e., excipient) that may adversely affect the patient's response to the investigational preparation.

When a third party (e.g., a compounder outside the sponsor's organization) is involved in an investigational study, the sponsor of the study will supply clinical study documentation that provides guidance to the compounder or contract research organization on how to conduct the study. Examples of clinical study documentation include but are not limited to the study protocol, study manual, compounding logs, investigator brochure, and consent forms. Clinical study documentation should be written to clearly specify the scope, roles, and responsibilities of each party involved, including the responsibility of the compounder to follow the clinical study documentation. If a sponsor initiates a contract with a compounder to perform part or all of the Phase I compounding of an investigational preparation, the sponsor and the compounder are both responsible for ensuring that the Phase I investigational preparation is prepared in compliance with applicable requirements. The sponsor should evaluate the compounder according to their auditing practices to ensure that effective quality control (QC) functions are in place.

## 1.3 Applicable Regulatory Requirements

Regulatory bodies in many countries specify requirements for the compounding of investigational preparations as part of an investigational drug application. Personnel engaged in compounding of preparations for investigational use must comply with these requirements, which vary according to applicable laws, regulations, and guidelines of the regulatory jurisdiction.

This chapter references ⟨795⟩, ⟨797⟩, ⟨800⟩, and *Quality Assurance in Pharmaceutical Compounding* ⟨1163⟩, which provide standards for compounding of sterile and nonsterile preparations. With respect to any provisions in these chapters that are inconsistent with applicable regulatory requirements, compounders must comply with the more stringent requirement(s).

## 1.4 Additional Considerations

A Phase I investigational agent is experimental by definition, and its safety profile in the study population may be unknown. Therefore, the following are examples of questions compounders should be able to answer before they begin compounding any investigational agent. Compounders should be aware of the potential implications of the answers.

1. Will this be a Phase I study?
2. Will there be an Investigational New Drug (IND) application in place for this investigational agent?
3. Will there be an Institutional Review Board (IRB) approval in place for this investigational agent/preparation?
4. Will there be an established triad relationship between the pharmacist, investigator, and patient?
5. Will all the materials supplied by the sponsor or being used to compound the investigational preparation be appropriate for human use as determined by the study sponsor?
6. Will the necessary checks and balances as described in this chapter be in place to ensure subject safety and the compounding of a quality preparation (e.g., independent verification of calculations)?
7. What is the dosage form and what type of testing criteria is specified for this dosage form?
8. Will the investigational preparation interact with any of the dosing devices that are being used in the study?
9. How will the investigational agent be dosed and is the investigational preparation appropriate for that route of administration?
10. What release testing (e.g., assay, pH) will be required for the investigational preparation?
11. Will this preparation be sent to other sites/facilities/locations participating in the clinical study?
12. Has the sponsor provided clear preparation instructions supported by stability and in-use data?
13. Are the available stability data adequate to support the beyond use date (BUD) of the preparation?
14. Is the investigational agent or any components of the final compounded preparation hazardous as defined by the National Institute for Occupational Safety and Health (NIOSH) criteria (see ⟨800⟩)?

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## 2. PERSONNEL TRAINING

In general, all personnel should have the education, experience, and training—or any combination thereof—to enable each individual to perform their assigned function. In particular, personnel should have the appropriate experience to prepare the Phase I compounded investigational preparation and be familiar with QC principles and acceptable methods for complying with applicable regulatory requirements. Additionally, personnel must be knowledgeable with regard to the standards in ⟨795⟩, ⟨797⟩, and ⟨800⟩, and should be knowledgeable about ⟨1163⟩. In addition to the training described in ⟨795⟩, ⟨797⟩, and ⟨800⟩, training for compounding for investigational studies should be described in standard operating procedures (SOPs), should be documented, and should include, but is not limited to, the following, as appropriate for specific protocols:

- Overview of new drug development
- Investigator obligations in FDA-regulated clinical research
- Managing investigational agents and preparations
- Detection, evaluation, and reporting of adverse events
- Audits and inspections in clinical studies
- Monitoring of clinical studies by industry sponsors
- Health Insurance Portability and Accountability Act (HIPAA) privacy rules for human subject protection
- IRB roles
- Recruitment for participation in research studies and informed consent
- Good Clinical Practice (GCP)
- Human Subjects Protection (HSP)

## 3. BUILDINGS AND FACILITIES

Facilities should be properly designed and constructed for compounding of the investigational preparations. The design should include special controls (e.g., separate storage of investigational agents) to ensure that the investigational agents/preparations are not commingled with approved drugs used for treatment. The areas used for labeling (see *8. Labeling*), storage, handling, packaging, and transport (see *11. Storage, Handling, Packaging, and Transport*) should be secure, with restricted access. Facility design and use considerations must comply with ⟨795⟩, ⟨797⟩, ⟨800⟩, and applicable regulatory requirements. Additionally, facilities should comply with *Physical Environments That Promote Safe Medication Use* ⟨1066⟩ and ⟨1163⟩. Sponsors should conduct an audit to ensure compliance. Facilities should consider the value of being accredited by a national accreditation agency or organization.

## 4. EQUIPMENT AND COMPONENTS

### 4.1 Equipment

Equipment must meet the standards in ⟨795⟩, ⟨797⟩, ⟨800⟩, and applicable regulatory requirements. Additionally, equipment should comply with applicable standards in ⟨1163⟩ and *Prescription Balances and Volumetric Apparatus Used in Compounding* ⟨1176⟩. A number of technologies and resources are available to facilitate and streamline compounding of investigational preparations. Some examples include:

- Disposable equipment and process aids to reduce cleaning burden and risk of cross contamination
- Commercial, prepackaged materials (e.g., *Sterile Water For Injection*, and presterilized containers and closures) to eliminate the need for sterilization of additional materials
- Closed processing equipment (e.g., robotic compounding systems)

The compounder should consider and understand the impact of drug delivery devices (e.g., infusion tubing, pumps, and syringes) used to deliver investigational preparations on the stability and systemic availability of the investigational agent. Information should be evaluated to ensure that the investigational preparation does not interact with or create stability issues when used with the drug delivery device such that it could impact the safety or effectiveness of the preparation (e.g., investigational agent, excipient, or vehicle binding to the IV tubing).

### 4.2 Components

For sponsor-initiated studies, all materials (e.g., drug substance, excipients, commercial product, packaging components, and in-process material) will likely be supplied by the sponsor. All materials must comply with the standards in ⟨795⟩, ⟨797⟩, ⟨800⟩, available monographs, and applicable regulatory requirements. Any materials not supplied by the sponsor should be appropriate for human use as determined by the study sponsor.

#### 4.2.1 BULK DRUG SUBSTANCES AND EXCIPIENTS

Compounders should have SOPs in place that describe the receipt, handling, review, acceptance, storage, and control of materials to be used to compound preparations for investigational studies. Receipt records should be maintained, and the active substances and excipients used should be traceable to the individual patient. Bulk drug substances and excipients preferably should be official compendial articles, but noncompendial ingredients and substances may be used if they are approved or provided by the sponsor, are evaluated for safety, and the evaluation is appropriately documented. A Certificate of Analysis

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(COA) or similar product/substance release document confirming the identity, strength, purity, and quality should accompany the bulk drug substance. For human- and animal-derived material, documentation should include information about sourcing and test results for adventitious agents. All bulk investigational agents and other ingredients should be visually inspected for any physical damage and should be quarantined until examined or tested, as appropriate, before they are released for use. Storage and handling conditions for investigational agents (bulk drug substances), other ingredients (excipients), and the final preparation should be described in SOPs and should be maintained to prevent degradation or contamination and to ensure preparation quality. Temperature, humidity, light protection, and other specifications should be provided by the sponsor or supplier. Investigational agent labeling and other ingredient-container labeling should be displayed prominently and understandably with respect to the requirements for proper storage and retest date. If required, in-package temperature-monitoring devices should be used during transport and their information recorded after the package is received at the study site.

#### 4.2.2 CONVENTIONALLY MANUFACTURED PRODUCTS AS DRUG SOURCE MATERIAL

Conventionally manufactured drug dosage forms (e.g., tablets, capsules, injectables, or liquids) may be used in investigational studies if they are approved or provided by the sponsor. Examples of compounding with conventionally manufactured dosage forms may include, but are not limited to, reconstitution of injectable preparations, over-encapsulation, and incorporating comminuted tablets into an oral liquid or capsule preparation. Generally, immediate-release tablets should be used. Controlled-release tablets can be used if the sponsor determines they are acceptable after careful consideration of the controlled-release mechanism.

#### 4.2.3 IN-PROCESS MATERIALS

In-process materials include any item or preparation that is prepared in advance and held for use during the compounding of the investigational preparation (e.g., premixes, triturations, stock solutions, primary emulsions, and gel components).

## 5. STANDARD OPERATING PROCEDURES

Appropriate SOPs should be in place to facilitate the compounding of the investigational preparation, which must follow ⟨795⟩, ⟨797⟩, ⟨800⟩, and any applicable regulatory requirements. The procedures should be written clearly and should contain sufficient detail to allow reproducibility of the compounding process and traceability of materials. In addition, the procedures should take into account the complexity of the process and any risks involved. For instance, weighing and compounding a nonsterile preparation starting with a nonsterile powder is less complex and has fewer associated risks compared to preparing and dispensing a sterile preparation compounded from nonsterile ingredients, which is highly complex and entails high associated risks.

The compounding of Phase I investigational preparations should follow written procedures that provide for the following:

- A record that details the materials, equipment, procedures used, and any problems encountered during compounding. Compounders should retain records sufficient to replicate the compounding process. Similarly, if the compounding of a Phase I investigational preparation is initiated but not completed, the record should include an explanation of why compounding was terminated
- Records for handling, compounding, packaging, storage, and transporting investigational agents and final preparations
- A document that identifies procedures for reviewing, approving, and monitoring investigational agents and final preparations
- A record of changes in procedures and processes used for subsequent batches along with the rationale for any changes

## 6. PREPARATION ACTIVITIES

Before compounding the investigational preparation, the sponsor-designated person should review the protocol and the intended use of the investigational preparation to ensure that adequate space, facilities, equipment, and trained personnel are available to compound the investigational preparation.

The compounder should do a trial run (compound the investigational preparation in accordance with the compounding record) and have the compounded preparation tested per the sponsor protocol. Based on the results of the trial run, the compounding record may need to be changed and additional trial runs conducted to confirm that a quality preparation can be compounded by the site using the compounding record.

In addition, based on these verification studies, appropriate tests (e.g., measurement of final pH of preparation, weight checking all over-encapsulated products) and acceptable limits should be selected, and all future batches evaluated using these agreed upon tests, which should be incorporated into the compounding record. The finished preparation includes the dosage form, package, labeling, and any other required items. Based on the nature of the final preparation (e.g., simple dilution as compared to a powder-filled capsule), the final preparation should be analyzed for conformance to the specifications provided by the primary investigator or sponsor. Additionally, sufficient quality assurance (QA) measures should be incorporated in the process to ensure that the actual yield matches the theoretical yield of finished preparation or that any deviation is accounted for and documented.

Compounded investigational preparations require: correct ingredients and calculations; accurate and precise measurements; and appropriate formulation, facilities, equipment, and procedures. As a final release check, and after obtaining the results of any release testing conducted in accordance with the compounding record and *7. Release of Investigational Agent/Preparation*, the compounder should review each step of the compounding process in the compounding record to ensure that it was completed appropriately and should examine the finished preparation to ensure that it appears as expected. The compounder

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should investigate any deviations and discrepancies identified during the release check and take appropriate corrective action. Based on information gathered during the investigation, a decision on the outcome of the final preparation should be made and documented. The decision could range from rejection and disposal of the compounded preparation to the release of the preparation for use.

### 6.1 Retention Samples

A representative sample from each lot of investigational agent and each lot of compounded investigational preparation (finished preparation in the container used in the investigational study) must be retained and properly stored according to the clinical study documentation following study termination or withdrawal of the IND application. If the clinical study documentation does not specify the retention time, the sample should be retained for the length of time determined by the sponsor. Sponsors should have access to signed compounding records and individual retained components of the compounded preparation. These individual components must be kept according to the clinical study documentation and retention policies. The sample should consist of a quantity adequate for the performance of additional testing or investigation if required at a later date.

### 6.2 Disposition of Unused Materials and Preparations

If permitted, unused investigational preparations can be reallocated from one subject to another or from one site to another in accordance with the sponsor's protocol. Unused investigational agents, excipients, or finished preparations must be accounted for and disposed of in accordance with SOPs and sponsor requirements. The disposition (i.e., dispensed, returned to the sponsor, or destroyed) of all investigational preparations should be documented. Any discrepancies should be noted (e.g., preparation of doses not dispensed or that were in broken or breached containers). At the completion of the study, the sponsor should visit the compounding facility to account for all used and unused supplies of the investigational agent. The sponsor should verify the accountability and note the quantity returned for reconciliation and destruction. The compounding facility should verify the quantity returned for destruction or destroyed on-site, and should complete and sign the necessary forms.

## 7. RELEASE OF INVESTIGATIONAL AGENT/PREPARATION

The final investigational preparation used for subject dosing should be released according to sponsor procedures, which are usually identified in the sponsor-provided clinical study documentation. Integral to release is the assurance that preparation activities have been conducted in accordance with the appropriate quality requirements and as defined by the sponsor, including receipt, handling, preparation, dispensing, labeling, blinding (when necessary), and storage. Any discrepancies should be documented and discussed with the sponsor to determine possible effects and appropriate steps that should be taken. The sponsor is responsible for approval of the final investigational preparation prior to subject dosing, or the responsibility may be delegated to the designated person according to the clinical study documentation.

Sponsors may require the evaluation of one or more quality attributes (e.g., physical, chemical, and microbiological testing) before the investigational preparation is released. Each release test should include one or more procedures, usually with well-defined acceptance criteria. Investigative and corrective actions associated with any specific failure or discrepancy should be documented. Regardless of the source, each investigational preparation and excipient should have predetermined acceptance criteria.

The sponsor or sponsor-investigator should identify a designated person, which may include the compounder, to be responsible for either implementing an in-house testing program or working with a contract laboratory to confirm performance of appropriate testing methods for the investigational preparations. Results of any testing that is undertaken on an investigational preparation should be shared with and discussed with the sponsor of the study. If testing will be done at the compounding facility, appropriate equipment should be obtained and qualified either by the manufacturer upon sale or by the compounder upon receipt, and should be properly maintained, calibrated, and used. All personnel conducting in-house testing should be trained, skilled, and proficient in the procedure(s) necessary for testing.

If a compounding facility has the necessary equipment, supplies, and personnel who are skilled and qualified, many QC tests can be conducted on-site. Appropriate SOPs should be developed and implemented to ensure that equipment and instruments are working satisfactorily and that preparations are tested properly. Compounders can perform physical QC tests to ensure the uniformity and accuracy of compounded preparations. These tests address individual dosage unit weights (including the average), total preparation weight, pH, and physical attributes such as appearance, taste, and smell.

If testing is outsourced, the sponsor and the designated person identified by the sponsor should determine what to outsource and how to select a laboratory, and should develop an ongoing relationship with the laboratory chosen. Contract laboratories must follow standards set forth in *USP* chapters, as appropriate, and preferably should be registered with the FDA.

Factors to coordinate and consider in testing requirements include:

- Quantity of preparation being compounded according to the clinical study documentation for a specific prescription
- Number of samples needed
- Destructive or nondestructive testing
- Appropriate methods for obtaining representative samples
- Physical state of the samples (solid, liquid, or gas)
- Type of container required for collection and storage
- Any special handling and shipping requirements or restrictions (e.g., controlled drug substances, dangerous or hazardous chemicals, hazardous drugs, flammable or caustic substances, and refrigerated or frozen preparations)

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- Sponsor-specified storage requirements for samples including type of container, temperature, humidity, and light resistance (see *Packaging and Storage Requirements* ⟨659⟩)

## 8. LABELING

The term "labeling" encompasses all the written, printed, and graphic material accompanying the preparation, including information on the immediate container received by the patient. Labeling also includes the instructions to the investigators involved in the study, package inserts, cartons, outer wrapping (if used), and any other materials accompanying the investigational preparation. Labeling control is important. Only the required quantity of labels should be printed and all labels should be accounted for. An example label should be retained with the compounding record and should be retrievable.

The label includes all written, printed, or graphic matter on the immediate container received by the patient. Appropriate labels should be selected after consideration of the font type and size as well as the adhesive to be used. The printed label should be legible and should adhere to the investigational preparation container during short-term storage and use. The label adhesive should not come in direct contact with the dosage form (e.g., tablet, capsule), leach into packaging materials, or contaminate the investigational preparation.

There should be complete agreement among all of the labeling materials in terms of the information provided. Information on the label should be verified for accuracy by a second person prior to application of the label to the final packaging of the investigational preparation.

Labeling of investigational agents and preparations should follow applicable requirements of the regulatory agency and sponsor.

The investigational protocol or the compounding facility manual should provide labeling instructions as well as label content. Labels may be provided by the sponsor or may be produced on-site at the compounding facility. If labels are provided by the sponsor, the site may be required by laws, regulations, or guidelines of the regulatory jurisdiction, or internal procedures, to provide additional separate and unique labeling. The compounded investigational preparation should be labeled with a unique identifier that allows traceability and recall, if necessary, and a BUD.

However, in the case of investigational preparations, there may be instances when including this information on the preparation labeling might have an adverse effect on blinding. Regulatory bodies may permit exclusion of control numbers and BUDs from the preparation labeling for blinding purposes, provided this information is made available separately (e.g., to the clinical investigator) in case the blinding or randomization code needs to be broken. An auxiliary label for supplying additional information may need to be affixed to individual compounded investigational preparations or a bag that holds a supply of vials or containers of the same drug strength or concentration. Such labels should supply information that is missing on the preparation label or information that is poorly visible on the label. A highlighter pen can be used to focus attention on key information on the label.

## 9. ESTABLISHING BEYOND USE DATES

A BUD should be established for compounded investigational preparations by the sponsor. Due to the lack of data on investigational agent stability (e.g., if it is a new chemical entity) or stability of the final compounded preparation, the sponsor may not rely on the default BUDs established in ⟨795⟩ and ⟨797⟩. A contract analytical laboratory can help establish an appropriate BUD by performing either real-time or accelerated stability testing. Stability studies may be ongoing simultaneously with the investigational study. In such situations, the sponsor is responsible for ensuring regular communication with the compounder regarding updated stability data and how those data affect a previously identified BUD. New stability data could lead to an increase or decrease in a previously identified BUD. Additionally, compounders should notify the sponsor of any stability issues which may lead to treatment failures in study subjects. The available data should support material storage for as long as intended during the investigational trial.

## 10. QUALITY ASSURANCE AND QUALITY CONTROL

A QA program for compounding investigational preparations is important for the integrity of a study. QA encompasses all of the processes and procedures undertaken to ensure that compounded preparations are of the quality required for their intended purposes, and also the proper documentation of all steps taken and data obtained. The effectiveness and suitability of the QA program should be assessed regularly per the facility's SOPs.

QA for compounding investigational preparations ensures the following:

- Compounded investigational preparations are designed and prepared according to the methods and procedures in the clinical study documentation, applicable regulatory requirements, and *USP–NF* standards
- Compounding and control operations are clearly specified and implemented according to the regulatory requirements, and *USP–NF* standards
- Compounded investigational preparations are dispensed only if they have been correctly prepared, verified, and stored in accordance with the procedures and parameters defined by the sponsor
- Adequate measures are in place to ensure that the compounded investigational preparations are released, stored, and handled in such a way that the required quality can be ensured until the BUD
- Required documentation is maintained

A QC program for investigational agents and preparations should address the following five components:

1. Bulk drug substances and other ingredients
2. In-process items

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3. Packaging materials (e.g., container and closures)
4. Labels
5. Finished preparations

A designated person should be assigned overall responsibility for the establishment and execution of the quality program. Responsible personnel are essential in ensuring the identity, strength, quality, and purity of investigational agents and their components (see ⟨1163⟩).

A QA program for compounded preparations may include testing during the compounding process and of the finished compounded preparation as determined by the study sponsor.

Sponsors, or their contract facilities including pharmacies or clinics, should conduct audits according to the study requirements. The audit should verify that the designated person identified by the sponsor is performing quality inspections.

## 11. STORAGE, HANDLING, PACKAGING, AND TRANSPORT

### 11.1 Storage

Storage conditions (see ⟨659⟩) in all storage areas for investigational agents and preparations should be carefully monitored and controlled, the data should be documented throughout the entire study process, and any deviations should be handled as specified by the clinical study documentation. Any temperature deviations that are outside the sponsor's indicated storage conditions should be investigated, recorded (with duration), and reported to the sponsor. Electronic monitoring and recording devices are recommended because they can provide a detailed record of storage conditions.

### 11.2 Handling

All materials used in compounding should be handled appropriately and in accordance with information from the Safety Data Sheets (SDS) when available. When compounding with hazardous drugs, compounders should pay careful attention to all aspects of handling these substances to protect personnel and the environment, and must also comply with ⟨800⟩ and all applicable laws, regulations, or guidelines of the regulatory jurisdiction.

### 11.3 Packaging

All packaging materials—including the immediate container and closure—should be supplied or specified by the sponsor. Packaging materials should meet *USP–NF* standards and should be sourced and selected based on physical and chemical characteristics and compatibility with the final preparation to avoid possible preparation–container interactions. They should also be accompanied by documentation of their composition and size specifications. The packaging may include, but is not limited to, glass or plastic bottles, metal or plastic caps, paper, cardboard, plastic parts and film, metal foil, drums, cans, tubes, vials, or jars.

The packaging should protect and ensure the stability of the preparation. Specifically, the investigational preparation should be packaged to protect it from alteration, contamination, and damage during storage, handling, and transport (see *Storage and Transportation of Investigational Drug Products* ⟨1079.1⟩). If the investigational preparation is sensitive to temperature fluctuations and will be transported to another facility, consideration should be given to using an in-package temperature monitoring device. If used, the information from this device should be recorded immediately after the package is received at the study site.

### 11.4 Transport

Distribution and dispensing are potentially the least controllable part of the overall scheme from compounding to administration. If distribution and dispensing occur within the same facility, the potential problems are reduced. However, if distribution and dispensing occur in different locations, and external carriers are used, the potential problems can be substantial, especially if overnight delivery is required or distribution needs to occur during weather extremes.

## 12. DOCUMENTATION

After study termination, records of investigational agents and preparations must be maintained by the compounding facility according to the sponsor's requirements. This includes records pertaining to the preparation, release, and disposition of each lot of material (e.g., drug substance and excipients) used, as well as source documentation and release testing, as appropriate, for bulk materials. Records pertaining to reference standards, if any, that are used to support investigational agents or preparations must be retained according to study requirements.

### 12.1 Safety Data Sheets

SDS, when available, should be on-site or should be readily retrievable electronically. They should be reviewed by all personnel who will be working with the compounding materials.

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## 12.2 Certificate of Analysis

A COA, or equivalent document if outside the U.S., should be obtained for active pharmaceutical ingredient(s) (API) and excipient(s) used in the compounding of an investigational preparation, and should be maintained throughout the study. It is recommended that the sponsor supply all clinical materials. If all materials are supplied by the sponsor, the sponsor is responsible for maintaining COAs for the ingredients and supplying these to the compounding facility if requested.

However, if some or all of the clinical materials are supplied by the compounding facility, a COA or equivalent document should be collected from the supplier of the material being used, and the COA should be maintained by the compounding facility and should be provided to the sponsor at the end of the study if requested.

## 12.3 Records Management

The required documentation for investigational preparations includes the original study specifications, the compounding records, test results, and the COAs. Sponsors should retain all records for at least 2 years after approval of an IND application, according to the clinical study documentation and applicable regulatory requirements, or, if an IND application is not submitted or approved, for 2 years after discontinuation of shipment and delivery of the investigational agent and FDA notification.

# APPENDIX

See *Table A-1* for the list of acronyms included in this chapter.

**Table A-1. Acronyms Included in ⟨1168⟩**

| Acronym | Description |
|---|---|
| API | Active pharmaceutical ingredient |
| BUD | Beyond use date |
| COA | Certificate of Analysis |
| FDA | Food and Drug Administration |
| GCP | Good Clinical Practice |
| HIPAA | Health Insurance Portability and Accountability Act |
| HSP | Human Subjects Protection |
| IND | Investigational New Drug |
| IRB | Institutional Review Board |
| NIOSH | National Institute for Occupational Safety and Health |
| QA | Quality assurance |
| QC | Quality control |
| SDS | Safety Data Sheets |
| SOPs | Standard operating procedures |

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# ⟨1174⟩ POWDER FLOW

The widespread use of powders in the pharmaceutical industry has generated a variety of methods for characterizing powder flow. Not surprisingly, scores of references appear in the pharmaceutical literature, attempting to correlate the various measures of powder flow to manufacturing properties. The development of such a variety of test methods was inevitable; powder behavior is multifaceted and thus complicates the effort to characterize powder flow. The purpose of this chapter is to review the methods for characterizing powder flow that have appeared most frequently in the pharmaceutical literature. In addition, while it is clear that no single and simple test method can adequately characterize the flow properties of pharmaceutical powders, this chapter proposes the standardization of test methods that may be valuable during pharmaceutical development.

Four commonly reported methods for testing powder flow are (1) angle of repose, (2) compressibility index or Hausner ratio, (3) flow rate through an orifice, and (4) shear cell. In addition, numerous variations of each of these basic methods are available. Given the number of test methods and variations, standardizing the test methodology, where possible, would be advantageous.

With this goal in mind, the most frequently used methods are discussed below. Important experimental considerations are identified and recommendations are made regarding standardization of the methods. In general, any method of measuring powder flow should be practical, useful, reproducible, sensitive, and yield meaningful results. It bears repeating that no one simple powder flow method will adequately or completely characterize the wide range of flow properties experienced in the pharmaceutical industry. An appropriate strategy may well be the use of multiple standardized test methods to characterize the various aspects of powder flow as needed by the pharmaceutical scientist.

## ANGLE OF REPOSE

The angle of repose has been used in several branches of science to characterize the flow properties of solids. Angle of repose is a characteristic related to interparticulate friction or resistance to movement between particles. Angle of repose test results are reported to be very dependent upon the method used. Experimental difficulties arise as a result of segregation of material and consolidation or aeration of the powder as the cone is formed. Despite its difficulties, the method continues to be used in the pharmaceutical industry, and a number of examples demonstrating its value in predicting manufacturing problems appear in the literature.

The angle of repose is the constant, three-dimensional angle (relative to the horizontal base) assumed by a cone-like pile of material formed by any of several different methods (described briefly below).

### Basic Methods for Angle of Repose

A variety of angle of repose test methods are described in the literature. The most common methods for determining the static angle of repose can be classified on the basis of the following two important experimental variables:

1. The height of the "funnel" through which the powder passes may be fixed relative to the base, or the height may be varied as the pile forms.
2. The base upon which the pile forms may be of fixed diameter or the diameter of the powder cone may be allowed to vary as the pile forms.

### Variations in Angle of Repose Methods

In addition to the above methods, the following variations have been used to some extent in the pharmaceutical literature:

- *Drained angle of repose* is determined by allowing an excess quantity of material positioned above a fixed diameter base to "drain" from the container. Formation of a cone of powder on the fixed diameter base allows determination of the drained angle of repose.
- *Dynamic angle of repose* is determined by filling a cylinder (with a clear, flat cover on one end) and rotating it at a specified speed. The dynamic angle of repose is the angle (relative to the horizontal) formed by the flowing powder. The internal angle of kinetic friction is defined by the plane separating those particles sliding down the top layer of the powder and those particles that are rotating with the drum (with roughened surface).

### Angle of Repose General Scale of Flowability

Although there is some variation in the qualitative description of powder flow using the angle of repose, much of the pharmaceutical literature appears to be consistent with the classification by Carr[*], which is shown in *Table 1*. There are examples in the literature of formulations with an angle of repose in the range of 40° to 50° that were manufactured satisfactorily. When the angle of repose exceeds 50°, the flow is rarely acceptable for manufacturing purposes.

**Table 1. Flow Properties and Corresponding Angles of Repose[*]**

| Flow Property | Angle of Repose (degrees) |
|---|---|
| Excellent | 25–30 |
| Good | 31–35 |

[*] Carr, R.L. Evaluating Flow Properties of Solids. *Chem. Eng.* **1965**, *72*, 163–168.

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**Table 1. Flow Properties and Corresponding Angles of Repose*** *(continued)*

| Flow Property | Angle of Repose (degrees) |
|---|---|
| Fair—aid not needed | 36–40 |
| Passable—may hang up | 41–45 |
| Poor—must agitate, vibrate | 46–55 |
| Very poor | 56–65 |
| Very, very poor | >66 |

## Experimental Considerations for Angle of Repose

Angle of repose is not an intrinsic property of the powder; i.e., it is very much dependent upon the method used to form the cone of powder. The following important considerations are raised in the existing literature:

- The peak of the cone of powder can be distorted by the impact of powder from above. By carefully building the powder cone, the distortion caused by impact can be minimized.
- The nature of the base upon which the powder cone is formed influences the angle of repose. It is recommended that the powder cone be formed on a "common base," which can be achieved by forming the cone of powder on a layer of powder. This can be done by using a base of fixed diameter with a protruding outer edge to retain a layer of powder upon which the cone is formed.

## Recommended Procedure for Angle of Repose

Form the angle of repose on a fixed base with a retaining lip to retain a layer of powder on the base. The base should be free of vibration. Vary the height of the funnel to carefully build up a symmetrical cone of powder. Care should be taken to prevent vibration as the funnel is moved. The funnel height should be maintained approximately 2–4 cm from the top of the powder pile as it is being formed in order to minimize the impact of falling powder on the tip of the cone. If a symmetrical cone of powder cannot be successfully or reproducibly prepared, this method is not appropriate. Determine the angle of repose by measuring the height of the cone of powder and calculating the angle of repose, $\alpha$, from the following equation:

$$\tan(\alpha) = \text{height}/0.5\ \text{base}$$

# COMPRESSIBILITY INDEX AND HAUSNER RATIO

In recent years the compressibility index and the closely related Hausner ratio have become the simple, fast, and popular methods of predicting powder flow characteristics. The compressibility index has been proposed as an indirect measure of bulk density, size and shape, surface area, moisture content, and cohesiveness of materials because all of these can influence the observed compressibility index. The compressibility index and the Hausner ratio are determined by measuring both the bulk volume and the tapped volume of a powder.

## Basic Methods for Compressibility Index and Hausner Ratio

Although there are some variations in the method of determining the compressibility index and Hausner ratio, the basic procedure is to measure (1) the unsettled apparent volume, $V_O$, and (2) the final tapped volume, $V_f$, of the powder after tapping the material until no further volume changes occur. The compressibility index and the Hausner ratio are calculated as follows:

$$\text{Compressibility Index} = 100 \times [(V_O - V_f)/V_O]$$

$$\text{Hausner Ratio} = (V_O/V_f)$$

Alternatively, the compressibility index and Hausner ratio may be calculated using measured values for bulk density ($\rho_{bulk}$) and tapped density ($\rho_{tapped}$) as follows:

$$\text{Compressibility Index} = 100 \times [(\rho_{tapped} - \rho_{bulk})/\rho_{tapped}]$$

$$\text{Hausner Ratio} = (\rho_{tapped}/\rho_{bulk})$$

In a variation of these methods, the rate of consolidation is sometimes measured rather than, or in addition to, the change in volume that occurs on tapping. For the compressibility index and the Hausner ratio, the generally accepted scale of flowability is given in *Table 2**.

**Table 2. Scale of Flowability***

| Compressibility Index (%) | Flow Character | Hausner Ratio |
|---|---|---|
| ≤10 | Excellent | 1.00–1.11 |

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**Table 2. Scale of Flowability*** *(continued)*

| Compressibility Index (%) | Flow Character | Hausner Ratio |
|---|---|---|
| 11–15 | Good | 1.12–1.18 |
| 16–20 | Fair | 1.19–1.25 |
| 21–25 | Passable | 1.26–1.34 |
| 26–31 | Poor | 1.35–1.45 |
| 32–37 | Very poor | 1.46–1.59 |
| >38 | Very, very poor | >1.60 |

### Experimental Considerations for the Compressibility Index and Hausner Ratio

Compressibility index and Hausner ratio are not intrinsic properties of the powder; i.e., they depend on the methodology used. In the existing literature, there are discussions of the following important considerations affecting the determination of (1) the unsettled apparent volume, $V_o$, (2) the final tapped volume, $V_f$, (3) the bulk density, $\rho_{bulk}$, and (4) the tapped density, $\rho_{tapped}$:

- The diameter of the cylinder used
- The number of times the powder is tapped to achieve the tapped density
- The mass of material used in the test
- Rotation of the sample during tapping

### Recommended Procedure for Compressibility Index and Hausner Ratio

Use a 250-mL volumetric cylinder with a test sample weight of 100 g. Smaller weights and volumes may be used, but variations in the method should be described with the results. An average of three determinations is recommended.

## FLOW THROUGH AN ORIFICE

The flow rate of a material depends upon many factors, some of which are particle-related and some related to the process. Monitoring the rate of flow of material through an orifice has been proposed as a better measure of powder flowability. Of particular significance is the utility of monitoring flow continuously because pulsating flow patterns have been observed even for free flowing materials. Changes in flow rate as the container empties can also be observed. Empirical equations relating flow rate to the diameter of the opening, particle size, and particle density have been determined. However, determining the flow rate through an orifice is useful only with free-flowing materials.

The flow rate through an orifice is generally measured as the mass per time flowing from any of a number of types of containers (cylinders, funnels, hoppers). Measurement of the flow rate can be in discrete increments or continuous.

### Basic Methods for Flow Through an Orifice

There are a variety of methods described in the literature. The most common method for determining the flow rate through an orifice can be classified on the basis of three important experimental variables:

1. The type of container used to contain the powder. Common containers are cylinders, funnels, and hoppers from production equipment.
2. The size and shape of the orifice used. The orifice diameter and shape are critical factors in determining powder flow rate.
3. The method of measuring powder flow rate. Flow rate can be measured continuously using an electronic balance with some sort of recording device (strip chart recorder, computer). It can also be measured in discrete samples (for example, the time it takes for 100 g of powder to pass through the orifice to the nearest tenth of a second or the amount of powder passing through the orifice in 10 seconds to the nearest tenth of a gram).

### Variations in Methods for Flow Through an Orifice

Either mass flow rate or volume flow rate can be determined. Mass flow rate is the easier of the methods, but it biases the results in favor of high-density materials. Because die fill is volumetric, determining volume flow rate may be preferable. A vibrator is occasionally attached to facilitate flow from the container; however, this appears to complicate interpretation of the results. A moving orifice device has been proposed to more closely simulate rotary press conditions. The minimum diameter orifice through which powder flows can also be identified.

### General Scale of Flowability for Flow Through an Orifice

No general scale is available because flow rate is critically dependent on the method used to measure it. Comparison between published results is difficult.

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### Experimental Considerations for Flow Through an Orifice

Flow rate through an orifice is not an intrinsic property of the powder. It very much depends on the methodology used. Several important considerations affecting these methods are discussed in the existing literature:

- The diameter and shape of the orifice
- The type of container material (metal, glass, plastic)
- The diameter and height of the powder bed.

### Recommended Procedure for Flow Through an Orifice

Flow rate through an orifice can be used only for materials that have some capacity to flow. It is not useful for cohesive materials. Provided that the height of the powder bed (the "head" of the powder) is much greater than the diameter of the orifice, the flow rate is virtually independent of the powder head. Use a cylinder as the container because the cylinder material should have little effect on flow. This configuration results in flow rate being determined by the movement of powder over powder rather than powder along the wall of the container. Powder flow rate often increases when the height of the powder column is less than two times the diameter of the column. The orifice should be circular and the cylinder should be free of vibration. General guidelines for dimensions of the cylinder are as follows:

- Diameter of opening > 6 times the diameter of the particles
- Diameter of the cylinder > 2 times the diameter of the opening

Use of a hopper as the container may be appropriate and representative of flow in a production situation. It is not advisable to use a funnel, particularly one with a stem, because flow rate will be determined by the size and length of the stem as well as the friction between the stem and the powder. A truncated cone may be appropriate, but flow will be influenced by the powder-wall friction coefficient, making selection of an appropriate construction material an important consideration.

For the opening in the cylinder, use a flat-faced bottom plate with the option to vary orifice diameter to provide maximum flexibility and to better ensure a powder-over-powder flow pattern. Rate measurement can be either discrete or continuous. Continuous measurement using an electronic balance can more effectively detect momentary flow rate variations.

## SHEAR CELL METHODS

In an effort to put powder flow studies and hopper design on a more fundamental basis, a variety of powder shear testers and methods that permit more thorough and precisely defined assessment of powder flow properties have been developed. Shear cell methodology has been used extensively in the study of pharmaceutical materials. From these methods, a wide variety of parameters can be obtained, including the yield loci representing the shear stress-shear strain relationship, the angle of internal friction, the unconfined yield strength, the tensile strength, and a variety of derived parameters such as the flow factor and other flowability indices. Because of the ability to more precisely control experimental parameters, flow properties can also be determined as a function of consolidation load, time, and other environmental conditions. The methods have been successfully used to determine critical hopper and bin parameters.

### Basic Methods for Shear Cell

One type of shear cell is the cylindrical shear cell that is split horizontally, forming a shear plane between the lower stationary base and the upper moveable portion of the shear cell ring. After powder bed consolidation in the shear cell (using a well-defined procedure), the force necessary to shear the powder bed by moving the upper ring is determined. Annular shear cell designs offer some advantages over the cylindrical shear cell design, including the need for less material. A disadvantage, however, is that because of its design, the powder bed is not sheared as uniformly; i.e., material on the outside of the annulus is sheared more than material in the inner region. A third type of shear cell (plate-type) consists of a thin sandwich of powder between a lower stationary rough surface and an upper rough surface that is moveable.

All of the shear cell methods have their advantages and disadvantages, but a detailed review is beyond the scope of this chapter. As with the other methods for characterizing powder flow, many variations are described in the literature. A significant advantage of shear cell methodology in general is a greater degree of experimental control. The methodology is rather time-consuming and requires significant amounts of material and a well-trained operator.

### Recommendations for Shear Cell

The many existing shear cell configurations and test methods provide a wealth of data and can be used very effectively to characterize powder flow. They are also helpful in the design of equipment such as hoppers and bins. Because of the diversity of available equipment and experimental procedures, no specific recommendations regarding methodology are presented in this chapter. It is recommended that the results of powder flow characterization using shear cell methodology include a complete description of equipment and methodology used.

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# ⟨1176⟩ PRESCRIPTION BALANCES AND VOLUMETRIC APPARATUS USED IN COMPOUNDING

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    1.2 Electronic Balances
    1.3 Minimum Accurately Weighable Quantity on the Balance
2. VOLUMETRIC APPARATUS
    2.1 Selection and Use of Graduates
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    2.3 Dispensing Bottles
    2.4 Syringes
    2.5 Pipets
    2.6 Volumetric Flasks
    2.7 Additional Considerations When Using Volumetric Apparatus
GLOSSARY

## 1. BALANCES

Pharmacies that perform materials measurements for compounding and dispensing should have access to a well-maintained National Institute of Standards and Technology (NIST) Class III torsion prescription balance or superior balance (preferably an electronic balance) to weigh masses accurately. The pharmacy should have a set of calibration weights, or the balance should have internal calibration capability to standardize the precision and accuracy of the balance.

For more information regarding standards for weights and balances, see *Balances* ⟨41⟩. The standards in NIST Handbook 44 called "Specifications, Tolerances, and Other Technical Requirements for Weighing and Measuring Devices" may also be useful to compounders.[1] Some balances offer digital or direct-reading features and printing capabilities, which may be desirable for ease of use. All balances should be calibrated or verified and tested frequently using appropriate test weights.

### 1.1 Mechanical Prescription Balances

A Class III (formerly Class A) prescription balance is a device designed to accurately weigh drugs and other substances and materials required in pharmaceutical compounding and dispensing practice.[1] It is constructed to support its full weighing capacity without developing undue stresses, and its adjustment is not altered by repeated weighing of the capacity load. The removable pans or weighing vessels should be of equal weight and shape. The balance should have leveling feet or screws and a built-in means to observe its level. The balance may feature dial-in weights and also a precision spring and dial instead of a weighbeam. A balance that has a graduated weighbeam must have a stop that halts the rider or poise at the zero reading, and the reading edge of the rider must be parallel to the graduations on the weighbeam. The distance from the face of the index plate to the indicator pointer or pointers should be NMT 1.0 mm; the points should be sharp; and when there are two pointers, the ends should be separated by NMT 1.0 mm when the scale is in balance. The indicating components and the lever system should be protected against drafts; the balance lid should permit free movement of the loaded weighing pans when the lid is closed; and the balance must have a mechanical arresting device.

#### BALANCE TESTING PROCEDURE

A Class III prescription balance meets the criteria in the following four tests using an ANSI/ASTM E617 set of Class 6 or better test weights. A typical apothecary weight set contains weights ranging from 10 mg to 50 g in sizes of 10 mg, 20 mg, 50 mg, 100 mg, 200 mg, 500 mg, 1 g, 2 g, 5 g, 10 g, 20 g, and 50 g.

Four tests are required to evaluate the suitability of a Class III prescription balance. A balance is acceptable if it passes all four tests. If the balance fails any test, it should not be used until it has been repaired to meet specifications and retested. The four tests are:

1. Sensitivity requirement
2. Arm ratio test
3. Shift test
4. Rider and graduated beam

**Sensitivity requirement test:** The following requirements are based on (1) the NIST standard that a 6-mg load must displace the pointer 2 mm,[1] and (2) the absence of a 6-mg weight in a typical apothecary weight set.

1. For balances with pointer scale division marks 2 mm apart, a 10-mg weight on one pan and no weight on the other pan, dial, or rider beam of a leveled balance should displace the pointer 1.5-scale division marks, or approximately 6.7 mg/mark, as observed visually. This same 1.5-scale division mark displacement shall be observed with a 10-g weight on one pan and 10-g plus 10-mg weights on the other pan of a level balance.

[1] NIST Handbook 44 (2015) Specifications, Tolerances, and Other Technical Requirements for Weighing and Measuring Devices. Section 2.2.0 Scales (http://www.nist.gov/pml/wmd/pubs/h44-11.cfm).

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2. For balances on which the pointer scale division marks are 1 mm apart, a 10-mg weight on one pan and no weight on the other pan, dial, or rider beam of a leveled balance should displace the pointer 3-scale division marks, or approximately 6.7 mg/two marks, as observed visually. This same 3-scale division marks displacement shall be observed with a 10-g weight on one pan and 10-g plus 10-mg weights on the other pan of a level balance.
3. NLT 0.12 g (120 mg) shall be weighed on a Class III prescription to reduce reading errors of less than one mark and two marks in cases (a) and (b), respectfully, to NMT 5% weighing error.

When a balance has been observed or suspected to have been moved, unleveled, contaminated, or otherwise damaged, misaligned, or misused, then its sensitivity must be tested and confirmed to be accurate.

**Arm ratio test:** The arm ratio test will determine whether both arms of the balance are of equal length.

1. Level the balance.
2. With the pans empty, adjust the balance until the pointer is in the middle of the marker plate.
3. Place a 30-g weight in the center of each pan.
4. When the balance comes to rest, record the rest point.
5. If the rest point has changed from the middle of the marker plate, place a 30-mg weight on the lighter side.
6. When the balance comes to rest, this new rest point should either return to or go farther than the middle of the marker plate.

**Shift test:** The shift test checks the mechanics of the arm and lever components of the balance.

1. Level the balance.
2. With the pans empty, adjust the balance until the pointer is in the middle of the marker plate.
3. Place a 10-g weight in the center of the left pan and place another 10-g weight successively toward the right, left, front, and back side of the right pan, noting the rest point in each case.
4. In any case where the rest point has changed from the center of the marker plate, add a 10-mg weight to the lighter side.
5. When the balance comes to rest, this new rest point either should return to or go farther than the middle of the marker plate.
6. Level the balance and adjust the balance until the pointer is in the middle of the marker plate.
7. Repeat the procedure with the 10-g weight in the center of the right pan, and vary the position of the 10-g weight on the left pan.
8. Level the balance and adjust the balance until the pointer is in the middle of the marker plate. Make several observations in which both 10-g weights are shifted simultaneously to off-center positions on their pans (i.e., both toward the inside, both toward the outside, one front and the other back). In any case where the rest point is shifted from the middle of the marker plate, the addition of a 10-mg weight on the lighter side should equalize or overcome the shift.

**Rider and graduated beam test:** The rider and graduated beam test checks the accuracy of the calibrated dial or rider on the balance.

1. Level the balance.
2. With the pans empty, adjust the balance until the pointer is in the middle of the index plate.
3. Place a 500-mg weight on the left pan and move the dial or rider to the 500-mg point.
4. When the balance comes to rest, record the rest point.
5. If the rest point has changed from the middle of the index plate, place a 10-mg weight on the lighter side.
6. When the balance comes to rest, this new rest point should either return to or go farther than the middle of the index plate.
7. Follow the same procedure using a 1-g weight on the left pan and the dial or rider on the 1-g point. If the new rest point is shifted from the middle of the index plate, a 10-mg weight to the lighter side should equalize or overcome the shift.

## 1.2 Electronic Balances

A typical electronic prescription balance is an instrument that provides essential readability for materials weighed within the range of capacities for the balance. The display should have prompts to guide users through the balance function, as well as an output port for printing if necessary. Most balances sold for prescription compounding meet or exceed Class I or II accuracy requirements according to NIST Handbook 44 and come with certificates issued under the National Type Evaluation Program (NTEP) of the National Conference on Weights and Measures.

Calibration/certification of the balance should be performed according to the standard operating procedures of the facility. Many electronic balances contain internal calibration programs that automatically calibrate the balance daily. If there is no internal calibration feature, external calibration may be conducted using a calibration weight, according to the procedure supplied by the manufacturer. Weights for use in calibrating an electronic balance should be kept in a special rigid and compartmentalized box and handled with plastic or plastic-tipped forceps, or gloves that are provided with the weights, to prevent scratching or soiling. These calibration weights should meet or exceed ASTM Class 1 criteria. In addition, there are companies that offer calibration services to certify that the balance is performing adequately. For more information regarding the use of electronic balances, see ⟨41⟩ and *Weighing on an Analytical Balance* ⟨1251⟩.

***Change to read:***

## 1.3 Minimum Accurately Weighable Quantity on the Balance

The minimum accurately weighable quantity (MAWQ) is the smallest weight or mass that will produce no greater than a predetermined fraction of error on a properly calibrated, situated, and operated balance. The predetermined weighing error is

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assigned based on either a professional standard (such as NMT 0.05 or 5% error in the weight of any prescription ingredient) or scientific rigor, for example, NMT 0.005 or 0.5% error in the weight of an ingredient that is in limited supply. The compounder should use professional judgment when assigning the acceptable error for each process.

The formula for determining MAWQ for a typical Class III torsion balance is:

$$\text{MAWQ} = \text{Sensitivity requirement/Acceptable error}$$

Example: Calculate the MAWQ for a Class III torsion balance with a sensitivity requirement of 6 mg and an acceptable error of 5% or 0.05.

$$\text{MAWQ} = 6\ \text{mg}/0.05 = 120\ \text{mg}$$

For electronic balances, the MAWQ is calculated using the linearity or the absolute error over the range of the balance. This value is provided by the balance manufacturer. Note that the balance linearity and the readability of the smallest mass unit may not be the same.

Example: Calculate the MAWQ for an electronic balance with a linearity of 0.002 g and an acceptable error of 5% or 0.05.

$$\text{MAWQ} = 0.002\ \text{g}/0.05 = 0.04\ \text{g or } 40\ \text{mg}$$

▲The MAWQ equation in this section should be used if the linearity of an electronic balance is known. If the linearity of an electronic balance is not known, the minimum weight of an analytical balance equation described in *Weighing on an Analytical Balance* ⟨1251⟩*, Qualification, Minimum Weight* should be used and verified at regular intervals as described in the facility's standard operating procedures (SOPs) or at least annually and after any repairs.▲ (USP 1-May-2019)

## 2. VOLUMETRIC APPARATUS

An assortment of appropriate volumetric devices should be available to accurately measure fluids and liquids of different volumes and densities. Pharmaceutical devices approved for measuring volumes of liquids, including burets, pipets, and cylindrical graduates marked in metric or metric and apothecary units, are to meet the standard specifications for glass volumetric apparatus described in "Specifications and Tolerances for Reference Standards and Field Standard Weights and Measures, 2. Specifications and Tolerances for Field Standard Measuring Flasks".[2] Conical graduates are to meet the standard specifications described in NIST Handbook 44.[1] There are ASTM standards (ASTM E542) for the calibration of laboratory volumetric apparatus that may be useful to compounders as well.[3]

### 2.1 Selection and Use of Graduates

#### CAPACITY

The capacity of a cylindrical graduate is the volume at the maximum graduation mark at the specified temperature. Volumes for prescription compounding and dispensing that are measured in cylindrical graduates should be adequate to not exceed 5% error.

#### CYLINDRICAL AND CONICAL GRADUATES

The error in a measured volume caused by a deviation of ±1 mm in reading the lower meniscus in a graduated cylinder remains constant along the height of the uniform column. The same deviation of ±1 mm causes a progressively larger error in a conical graduate, the extent of the error being further dependent upon the angle of the flared sides to the perpendicular of the upright graduate. A deviation of ±1 mm in the meniscus reading causes an error of approximately 0.5 mL in the measured volume at any mark on the uniform 100-mL cylinder graduate. The same deviation of ±1 mm can cause an error of 1.8 mL at the 100-mL mark on an acceptable conical graduate marked for 125 mL.

A general rule for selection of a graduate for use is to use the graduate with a capacity equal to or just exceeding the volume to be measured. Measurement of small volumes in large graduates tends to increase errors, because the larger diameter increases the volume error in a deviation of ±1 mm from the mark. The relation of the volume error to the internal diameters of graduated cylinders is based on the equation:

$$V = \pi r^2 h$$

$V$ = volume
$r$ = radius
$h$ = height

[2] Harris GL. Specifications and tolerances for reference standards and field standard weights and measures. 2. Specifications and tolerances for field standard measuring flasks. 1996. NTIS Order Number: PB96-178926. National Technical Information Service, Alexandria, Virginia 22312 (http://www.ntis.gov).

[3] ASTM E542-01. Standard practice for calibration of laboratory volumetric apparatus. West Conshohocken, PA: ASTM International; 2012. www.astm.org.

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An acceptable 10-mL cylinder having an internal diameter of 1.18 cm holds 109 µL in 1 mm of the column. Reading 4.5 mL in this graduate with a deviation of ±1 mm from the mark causes an error of about ±2.5%, and the same deviation in a volume of 2.2 mL in the same graduate causes an error of about ±5%. *Table 1* shows the accuracy limits for cylindrical graduates.

**Table 1. Tolerance or Accuracy Limits for Class A Cylindrical Graduates[a]**

| Capacity (mL) | Smallest Graduation Mark Interval (mL) | Tolerance (±mL)[b] | Minimum Volume for 5% Error (mL)[c] |
|---|---|---|---|
| 5 | 0.1 | 0.05 | 1.0 |
| 10 | 0.1 or 0.2 | 0.10 | 2.0 |
| 25 | 0.2 or 0.5 | 0.17 | 3.4 |
| 50 | 1 | 0.25 | 5.0 |
| 100 | 1 | 0.50 | 10.0 |
| 250 | 2 | 1.00 | 20.0 |
| 500 | 5 | 2.00 | 40.0 |
| 1000 | 10 | 3.00 | 60.0 |
| 2000 | 20 | 6.00 | 120.0 |
| 4000 | 50 | 14.50 | 290.0 |

[a] Adapted from ASTM E1272-02. Standard specification for laboratory glass graduated cylinders. West Conshohocken, PA: ASTM International; 2012. www.astm.org. Some brands exceed the ASTM limits; for example, one source of a Class A 10-mL graduate lists the tolerance as ±0.08 mL.
[b] The constant volume error in each measurement.
[c] The minimum volume for *N*% error = [Tolerance (mL) × 100]/*N* (%); e.g., for *N* = 5% for a 10-mL graduate, the minimum volume is 2.0 mL. [The minimum volume for 5% error = (0.1 mL × 100)/5 = 2.0.]

There is an inverse relationship between the temperature and density of liquids. For compounding and dispensing purposes, deviations will be negligible when volume measurements are performed at temperatures NMT 5° from that specified on the particular cylindrical graduate, which is usually 20°. For example, the densities in g/mL of water, ethanol, and glycerin vary, respectively, from 0.999 to 0.997, 0.791 to 0.785, and 1.265 to 1.262 over the temperature range of 15°–25°. The accuracy of each cylindrical graduate used in pharmacy practice is recommended to be verified as follows, at a measuring temperature of 20°–25° with the assumption that deviations follow a normal or Gaussian distribution:

1. Tare a clean, dry graduate on a properly calibrated balance of adequate capacity, linearity, and readability for the volumes to be measured.
2. Fill the tared, dry graduate identically five or more times on a level surface to the smallest, a mid-range, and the maximum capacity graduation marks with *Purified Water* or deionized water, precluding and wiping spills and splashes from the exterior and interior above the target fill line.
3. Record the weight of each filling.
4. Calculate the mean weight of each set of fillings.
5. Calculate the percent deviation of each weight from the theoretical weight as follows:

$$\text{Percent deviation weight} = \{[\text{Actual weight} - (\text{intended volume} \times 0.9975)]/(\text{Intended volume} \times 0.9975)\} \times 100\%$$

6. Calculate the percent mean deviation weight as follows:

$$\text{Percent mean deviation weight} = \{[\text{Mean actual weight} - (\text{intended volume} \times 0.9975)]/(\text{Intended volume} \times 0.9975)\} \times 100\%$$

Deviations for cylindrical graduates used in compounding and dispensing should not exceed 5.0% for individual weights or 2.5% for mean weights of the corresponding volumes of *Purified Water*. *Table 1* and *Table 2* show that a wider range of volumes can be measured in Class A cylindrical graduates compared to Class B. According to ASTM standards, Class A cylindrical graduates must be marked with the letter "A" to designate compliance with applicable construction and accuracy requirements. Class B cylindrical graduates are the same basic design as Class A cylindrical graduates and are considered to be for general purpose use. However, volumetric tolerances may be up to twice the allowable range for Class A cylindrical graduates.

**Table 2. Tolerance or Accuracy Limits for Class B Cylindrical Graduates[a]**

| Capacity (mL) | Smallest Graduation Mark Interval (mL) | Tolerance (±mL)[b] | Minimum Volume for 5% Error (mL)[c] |
|---|---|---|---|
| 5 | 0.1 | 0.10 | 2.0 |
| 10 | 0.1 or 0.2 | 0.2 | 4.0 |
| 25 | 0.2 or 0.5 | 0.34 | 6.8 |
| 50 | 1 | 0.50 | 10.0 |
| 100 | 1 | 1.00 | 20.0 |

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**Table 2. Tolerance or Accuracy Limits for Class B Cylindrical Graduates[a]** *(continued)*

| Capacity (mL) | Smallest Graduation Mark Interval (mL) | Tolerance (±mL)[b] | Minimum Volume for 5% Error (mL)[c] |
|---|---|---|---|
| 250 | 2 | 2.00 | 40.0 |
| 500 | 5 | 4.00 | 80.0 |
| 1000 | 10 | 6.00 | 120.0 |
| 2000 | 20 | 12.00 | 240.0 |
| 4000 | 50 | 29.00 | 580.0 |

[a] Adapted from ASTM E1272-02. Standard specification for laboratory glass graduated cylinders. West Conshohocken, PA: ASTM International; 2012. www.astm.org.
[b] The constant volume error in each measurement.
[c] The minimum volume for *N*% error = [Tolerance (mL) × 100]/*N* (%); e.g. for *N* = 5% for a 5-mL graduate, the minimum volume is 2.0 mL. [The minimum volume for 5% error = (0.1 mL × 100)/5 = 2.0.]

## 2.2 Medicine Droppers

Medicine droppers meet the specifications in *Packaging and Storage Requirements* ⟨659⟩. Medicine droppers should be used only for qualitative purposes, such as pH adjustment with an acid, alkali, or buffer, and visual identification testing with reagents. Medicine droppers are not approved for volumetric measurements for compounding. Calibrated medicine droppers have markings to guide delivery of the prescribed quantity of medication. Their purpose should be limited to the measurement of a dose for administration.

## 2.3 Dispensing Bottles

Some dispensing bottles may be supplied by the manufacturer as part of the packaging, whereas others may be selected and supplied by the pharmacist. These containers are not necessarily accurately calibrated and should not be used for measurement during compounding and dispensing unless they are appropriately calibrated by the compounder.

## 2.4 Syringes

Syringes are available in a variety of sizes with calibrated increments used for measuring and may be used to accurately measure and deliver a wide range of liquid volumes. For viscous liquids, measurements made with syringes are usually more accurate than those made with cylindrical graduates.

### ORAL SYRINGES

Oral syringes are available as a device for accurately providing a dose of liquid medication to a patient. The performance of the syringes may be user dependent. For this reason, it is important that suitable operating procedures are documented and followed, and that operators are specifically trained in the correct use of the instruments. Users should be cautious about relying on manufacturers' performance figures. It is more appropriate to perform calibration, taking into account the variation between different users. Oral syringes provide an improvement in dosing accuracy for viscous medications, compared with medication droppers.

### SYRINGE CALIBRATION

Syringe calibration is based on the gravimetric determination of the quantity of water either contained or delivered, and the conversion of this value to true volume at the standard temperature of 20°. At 80% of the nominal syringe volume, 5–10 measurements should be performed.

Calibration procedure:

1. Aspirate and dispense an exact volume of deionized water that is 80% of the nominal syringe volume.
2. Determine the mass of the dispensed water.
3. Calculate the volume of the dispensed water using mass and density.
4. Document the measurement values.
5. Calculate the accuracy (*R*).

Calibration formula:

$$\text{Accuracy } (R) = [(\text{Average value} - \text{target value})/\text{Target value}] \times 100$$

## 2.5 Pipets

Pipets are thin glass tubes used to deliver volumes <25 mL. The two types of pipets are the single-volume pipet and the calibrated pipet. The single-volume pipet is the most accurate and the simplest to use, but the single-volume pipet is limited to the measurement of a single fixed volume; it is not capable of partial volume measurements.

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The calibrated pipet has graduation marks from a point near the tip of the pipet to the capacity of the pipet. In addition to delivering its entire contents, the calibrated pipet can be used to deliver partial volumes with good volumetric precision.

Micropipets generally are used when very small volumes (<1 mL) are required; micropipets are available in a variety of sizes. Each micropipet can be adjusted, usually by turning a dial, to deliver a volume within a limited range. For example, one micropipet may deliver volumes of 0–20 µL, another delivers 0–100 µL, and yet another delivers 0–1000 µL. The pipet selected should provide the greatest accuracy for the volume to be measured. Pipets should be calibrated/certified periodically as specified in the facility's standard operating procedures. Calibrating micropipets is a very specialized process that requires adequate training and appropriate equipment. There are companies that offer contract calibration services to certify that pipets are performing correctly.

## 2.6 Volumetric Flasks

Volumetric flasks have a slender neck and wide, bulb-like base. They are single-volume glassware and come in a variety of sizes. Only one calibration mark is etched on the neck of the flask. When the flask is filled to that mark, the flask contains the volume indicated on the flask. Volumetric flasks are difficult to use if dissolving solids in liquid because of the narrowness of the neck. If solids are to be dissolved, the flask should be partially filled with liquid, the solid material added and completely dissolved, and then the flask should be filled to the calibration mark.

## 2.7 Additional Considerations When Using Volumetric Apparatus

The use of volumetric apparatus requires some working knowledge of viscosity, density, surface tension, and adhesion. Each of these properties may affect measurement accuracy. For example, higher-viscosity liquids will be drawn into the vessel at a slower rate, and the operator should allow time for complete filling. Delivery can also be slow, because higher-viscosity fluids travel through the orifice at a slower rate. Density can affect filling and emptying in a manner similar to viscosity. In addition, liquids with very low surface tension may tend to "crawl up" the vessel wall or leak from the tip of a syringe or pipet. Adhesion can affect accuracy by resulting in a slow rate of vessel emptying. The operator should confirm that all of the liquid has been drained from the vessel if performing a quantitative transfer.

Understanding the terms "To Contain (TC)" and "To Deliver (TD)" is important, because they apply to glassware. TC and TD glassware consists of vessels that range from 1 to about 100 mL and are individually marked to indicate whether they are TC or TD vessels. A TC vessel is designed to deliver the entire measured content of the operation, and it may require forced air to expel the final quantity. A TD vessel is designed to deliver the entire measured amount via gravity flow.

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# GLOSSARY

**Accuracy:** The closeness of the displayed weight, as measured by the balance, to the true weight, as known by the use of a calibration weight or weights.

**Balance indicator:** A combination of elements, one or both of which will oscillate with respect to the other, to indicate the equilibrium state of the balance during weighing.

**Capacity:** Maximum weight, including the weight of tares to be placed on one pan. The nominal capacity of a prescription scale (Class III prescription balance) is assumed to be one-half apothecary ounce (15.55 g), unless otherwise marked. [NOTE—This is applicable only to scales not marked with an accuracy class. Past and currently available Class III prescription balances typically have a capacity of either 60 or 120 g and bear a label stating such.]

**Linearity:** The ability to maintain the same sensitivity over the entire weighing range of the balance. This is the constant weighing error in every amount weighed within the capacity range of the balance. This term is used only with electronic balances. Analogous terms are "absolute error" or "linear accuracy". The linearity is determined by the balance manufacturer.

**Minimum accurately weighable quantity (MAWQ):** The smallest weight or mass that will produce no greater than a predetermined fraction of error on a properly calibrated, situated, and operated balance.

**Precision:** The reproducibility of the weighing measurement as expressed by a standard deviation. A similar term, "repeatability", is sometimes used in specifications for electronic balances.

**Readability:** The smallest division at which the balance increments.

**Repeatability:** An instrument's ability to consistently deliver the same weight reading for a given object, and to return to a zero reading after each weighing cycle. This is tested by repeatedly weighing the same test weight.

**Rest point:** The point on the index plate at which the indicator or pointer stops when the oscillations of the balance cease, or the index plate position of the indicator or pointer calculated from recorded consecutive oscillations in both directions past the zero of the index plate scale. If the balance has a two-pointer indicating mechanism, the position or the oscillations of only one of the pointers need to be recorded or used to determine the rest point.

**Sensitivity requirements (SR):** The maximum change in load that will cause a specified change, such as a particular quantity of pointer scale division marks, of the pointer or indicating component of the balance.

**Tare bar:** An auxiliary ungraduated weighbeam bar with a movable poise. It can be used to correct for variations in weighing glasses or papers.

**Weighbeam** or **Beam:** A graduated bar equipped with a movable poise or rider. Metric graduations are in 0.01-g increments up to a maximum of 1.0 g.

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# ⟨1178⟩ GOOD REPACKAGING PRACTICES

## INTRODUCTION

This chapter is intended to provide guidance to those engaged in repackaging of oral solid drug products; and the chapter provides information to any person who removes drugs from their original container–closure system (new primary package) and repackages them into a different container–closure system for sale and/or for distribution.

This chapter does not apply to pharmacists engaged in dispensing prescription drugs in accordance with state practice of pharmacy. The pharmacist needs to apply

1. the principal information provided in the USP general information chapters *Plastic Packaging Systems and Their Materials of Construction* ⟨661⟩, *Plastic Materials of Construction* ⟨661.1⟩, and *Plastic Packaging Systems for Pharmaceutical Use* ⟨661.2⟩ and
2. other beyond-use date references in *Labeling* ⟨*7*⟩*, Expiration Date and Beyond Use Date*.

## ESTABLISHING EXPIRATION DATE

In the absence of stability data, the following criteria should be considered by repackagers when assigning an expiration date.

### Unit-Dose Packaging

1. The original container–closure system of the drug product to be used for repackaging must be received un-opened and show no outward signs of having been previously opened.
2. The unit-dose container–closure system must meet the testing requirements under *Containers—Performance Testing* ⟨671⟩ for either *Class A* or *Class B* containers.
3. The contents of the original bulk drug product to be repackaged are repackaged at one time unless the repackager has data and/or other scientific information from literature sources demonstrating that the drug product is not sensitive to exposure to moisture, oxygen, or light.
4. The unit-dose container–closure system must meet or exceed the original container's specification for light resistance.
5. The conditions of storage must meet the storage specifications provided in *Packaging and Storage Requirements* ⟨659⟩. Where no specific storage conditions are specified, the product must be maintained at controlled room temperature and in a dry place during the repackaging process, including storage.
6. The expiration dating period used for the repackaged product does not exceed (1) 6 months from the date of repackaging; or (2) the manufacturer's expiration date; or (3) 25% of the time between the date of repackaging and the expiration date shown on the manufacturer's bulk article container of the drug being repackaged, whichever is earlier.
7. Nitroglycerin Sublingual Tablets or any other drug product known to have stability problems should not be repackaged. This would include any drug known to be oxygen-sensitive or one that exhibits extreme moisture or light sensitivity. In deciding whether a particular drug product is suitable for repackaging, the repackager should take into consideration any available information from the manufacturer, published literature, the USP, and the FDA.
8. Documentation must be maintained to demonstrate that the preceding criteria are met.
9. Documentation must be maintained that specifies the container–closure packaging material used in repackaging operations.

### Multiple-Unit Packaging

1. A repackager may use the manufacturer's original expiration date without additional stability testing if the drug product is repackaged into an equivalent container–closure system that is at least as protective as, or more protective than, the original system and complies with criteria established for equivalency.
2. The original container–closure system of the drug product to be used for repackaging must be received un-opened and shows no outward signs of having been previously opened.
3. The contents of the original bulk drug product to be repackaged are repackaged at one time unless the repackager has data and/or other scientific information from literature sources demonstrating that the drug product is not sensitive to exposure to moisture, oxygen, or light.
4. The conditions of storage meet the storage specifications in *Packaging and Storage Requirements* ⟨659⟩. When no specific storage conditions are specified, the product should be maintained at controlled room temperature and in a dry place during repackaging operations.
5. The type of container–closure system used for repackaging must be at least as protective or more protective than the original container–closure system in terms of moisture vapor transmission rate (MVTR), oxygen transmission, light transmission, and compatibility of the container–closure system with the drug product. System equivalency extends to any special protective materials, such as for light transmission, seals, or desiccants associated with the original container–closure system.
6. The container–closure system must meet or exceed the original container–closure system's results for light transmission.
7. Nitroglycerin Sublingual Tablets or any other drug product known to have stability problems should not be repackaged. This would include any drug known to be oxygen-sensitive or one that exhibits extreme moisture or light sensitivity. In

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deciding whether a particular drug product is suitable for repackaging, the repackager should take into consideration any available information from the manufacturer, published literature, the USP, and the FDA.

8. Documentation must be maintained to demonstrate that the preceding criteria are met.
9. Documentation must be maintained that specifies the container–closure packaging material used in repackaging operations.

***Change to read:***

## REFERENCES FOR REPACKAGING REGULATIONS AND GUIDANCES

The references listed below are not meant to be all inclusive: specific repackaging operations may have additional requirements.

- **Food, Drug, and Cosmetic Act**
- **Food and Drug Administration Regulations and Guidances**
  *Enforcement Policy: 21 CFR, Part 7*
  *General Labeling Provisions: 21 CFR, Part 201, Subpart A*
  *Drug Establishment Registration and Listing: 21 CFR, Part 207.20*
  *Current Good Manufacturing Regulations: 21 CFR, Parts 210–211*
  *Special Requirements for Specific Human Drugs: 21 CFR, Part 250*
  *Controlled Substances: 21 CFR, Part 1300*
  *Potable Water: 40 CFR, Part 141*
  FDA *Compliance Policy Guides,* including the following:
    Sub Chapter 430 Labeling and Repackaging
    Sub Chapter 460 Pharmacy Issues
    Sub Chapter 480 Stability/Expiration Dating
- **Applicable USP Chapters**
  ⟨660⟩ *Containers—Glass*
  ⟨661⟩ *Plastic Packaging Systems and Their Materials of Construction*
  ⟨661.1⟩ *Plastic Materials of Construction*
  ⟨661.2⟩ *Plastic Packaging Systems for Pharmaceutical Use*
  ⟨671⟩ *Containers—Performance Testing*
  ▲ ⟨1079⟩ *Risks and Mitigation Strategies for the Storage and Transportation of Finished Drug Products*▲ (CN 1-Dec-2020)

## GLOSSARY

⟨659⟩ *Packaging and Storage Requirements* provides definitions related to repackaging. For the purposes of this chapter, a repackager, a contract packager, and an equivalent container–closure system are defined as follows:

**Repackager:** A repackager is an establishment that repackages drugs and sends them to a second location in anticipation of a need. Repackaging firms repackage preparations for distribution (e.g., for resale to distributors, hospitals, or other pharmacies), a function that is beyond the regular practice of a pharmacy. Distribution is not patient-specific in that there are no prescriptions. Unlike dispensers, repackaging firms are required to register with the FDA and to comply with the Current Good Manufacturing Practice Regulations in 21 CFR 210 and 211.

**Contract packager:** A contract packager is an establishment that is contracted to package or repackage a drug product into a single- or multi-unit container. These containers should meet all of the applicable requirements in this chapter. A contract packager does not take ownership from the manufacturer and generally receives the assigned expiration date from the contractor.

**Equivalent container–closure system:** This term refers to a container–closure system that is at least as protective or more protective than the original container–closure system in terms of moisture vapor transmission rate (MVTR), oxygen transmission, light transmission, and compatibility of the container–closure system with the drug product. System equivalency extends to any special protective materials, such as for light transmission, seals, or desiccants associated with the original container–closure system. These values may be determined by the repackager, or they may be obtained from the container–closure vendor for the specific container–closure system under consideration.

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# ⟨1180⟩ HUMAN PLASMA

## SCOPE

This chapter provides a consolidated source of information regarding human plasma, with emphasis on plasma for fractionation. Specifically, the chapter addresses plasma classification and nomenclature; collection and processing procedures required for ensuring product safety; details of specific plasmas; and quality systems relating to plasma collection. The chapter also includes, at the end of the text sections, a glossary; a list of abbreviations used in the chapter; and appendices that provide plasma definitions, donor selection criteria, and testing requirements.

Plasma originating from U.S.-licensed collection facilities provides the major supply for the global plasma derivative market. The U.S. Food and Drug Administration (FDA) regulates the collection and processing of plasma used for further manufacture. Title 21 of the Code of Federal Regulations (CFR) details Good Manufacturing Practice (GMP) requirements and product standards to protect the health of the blood donor and to ensure the safety and efficacy of blood products. CFR regulations are updated periodically, but between revisions, existing regulations may not address the most current issues and scientific developments. Therefore, the FDA periodically publishes guidance documents.[1] (For further information, see *Quality Systems*, below).

This chapter emphasizes U.S. practices, but because plasma and its derivatives are shipped globally, it is important to recognize that there are regional differences in recommendations, requirements, and regulations. Therefore this chapter provides information regarding the European Union (EU), the United Kingdom (UK), and Australia.

## OVERVIEW

### Composition of Plasma

Plasma constitutes approximately 55% of the total blood volume. It is a clear, straw-colored, complex liquid that is 7% protein, 91% water, and 0.9% mineral salts. The majority (approximately 70%) of total plasma protein is albumin. Additional plasma proteins relevant to fractionation include immunoglobulins, coagulation factors, fibrinolytic proteins, proteases, and protease inhibitors. These constituent plasma proteins can be isolated on the basis of the different solubility characteristics of each protein when subjected to specific conditions of pH, temperature, ionic strength, and ethanol concentration. The major products derived from fractionation are listed in *Table 1*.

**Table 1. The Major Fractions and Products from the Cohn Process**

| Fraction | Product |
|---|---|
| Cryoprecipitate | Antihemophilic factor (FVIII) |
| Cryosupernatant | Antithrombin III, factor IX complex |
| Fraction I | Fibrinogen, factor XIII |
| Fraction II | Immune globulin G (IgG) |
| Fraction III | IgA, IgM, prothrombin, plasminogen |
| Fraction IV-1 | Factor IX complex, activated factor IX complex |
| Fraction IV-4 | Plasma protein fraction, alpha-1 proteinase inhibitor |
| Fraction V | Albumin |

### Plasma for Manufacture of Derivative Products

The two methods for collection of human plasma are automated apheresis (for definitions, see the *Glossary* preceding the appendices) and centrifugation of whole blood donations. Source Plasma collected by apheresis constitutes the majority of plasma used in the manufacture of plasma derivatives in the United States. Plasma collected for transfusion but not so used (i.e., recovered plasma) also may be used for manufacture. Flow charts delineating how apheresis and whole blood–derived plasma can be used in the manufacture of plasma derivatives in the United States and Europe are presented in *Figures 1* and *2*, respectively.

[1] FDA. Blood Guidances. www.fda.gov/cber/blood/bldguid.htm. FDA Memoranda to Blood Establishments available at www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/OtherRecommendationsforManufacturers/MemorandumtoBloodEstablishments/default.htm.

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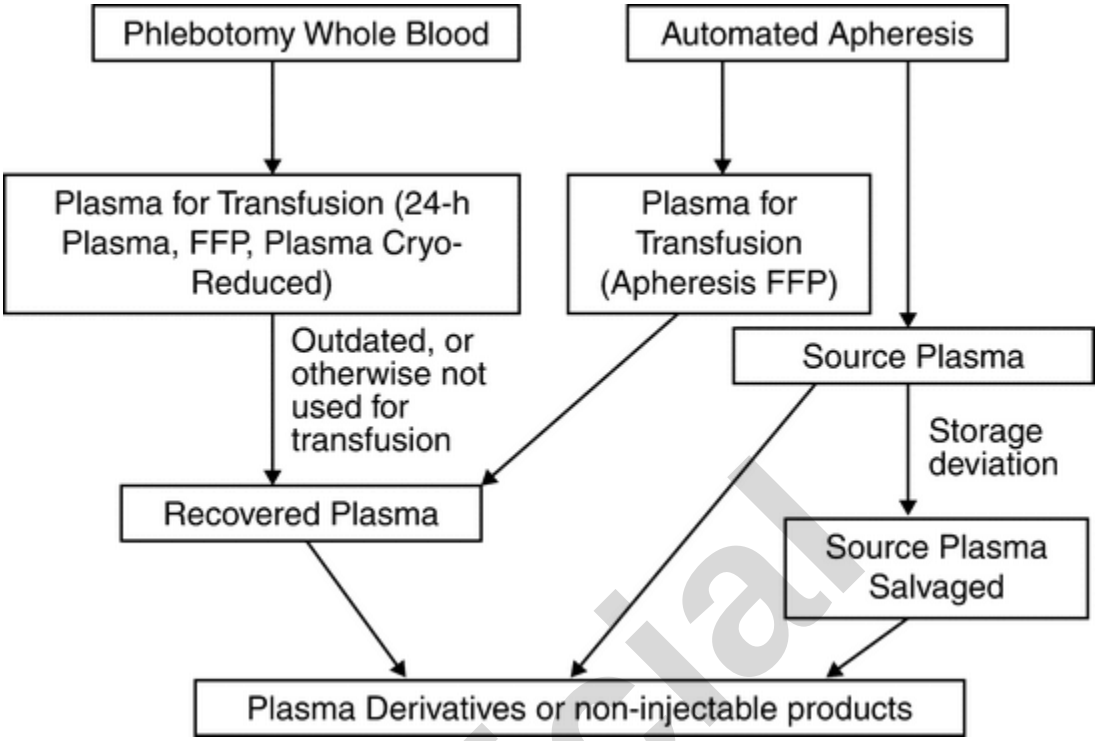


Figure 1. U.S. plasma derivative manufacture: FDA standards.

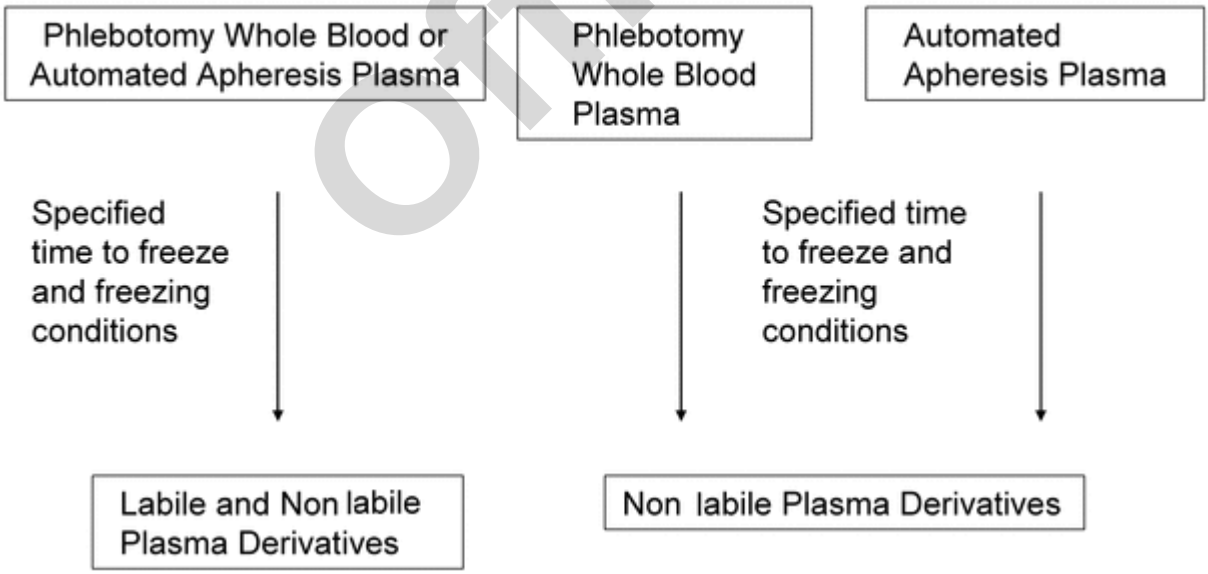


Figure 2. EU plasma derivative manufacture: EU standards.

Regardless of the collection method, plasma for fractionation should be a clear to slightly turbid liquid without visible sign of hemolysis; it may vary in color from light yellow to green; it should be ±10% of the stated volume; and it should show no sign of clots.

## Source Plasma

Licensed Source Plasma may be manufactured only in collection centers that are approved by the FDA for the collection and distribution of Source Plasma in interstate commerce. Currently, federal regulations governing the manufacture of Source Plasma, including minimal requirements for donors, are found in 21 CFR 640, Subpart G. By definition, Source Plasma is plasma intended for further manufacture. Source Plasma donors can donate as often as twice a week and may be compensated. In addition to FDA requirements, most plasma collectors and fractionators also comply with voluntary standards established by

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the Plasma Protein Therapeutics Association (PPTA), a trade and standards-setting organization.[2] PPTA voluntary standards address several areas of donor, plasma unit, plasma pool, and center management, and are designed to supplement existing regulatory requirements.

### Plasma for Transfusion

Plasma for transfusion is not intended for further manufacture but for direct transfusion to patients. It may be collected by either whole blood or apheresis donation. In the United States, plasma for transfusion comes from unpaid volunteer donors. Blood collecting facilities that collect plasma for transfusion typically comply with requirements of both the CFR and a voluntary trade organization, the American Association of Blood Banks (AABB). Currently, sections 640.3 and 640.31 of 21 CFR outline requirements for donors of whole blood and therefore govern most donors of plasma for transfusion, regardless of the collection method. AABB voluntary standards include information contained in FDA regulations and guidance pertaining to blood and plasma, as well as additional standards.[3] Plasma for transfusion may be converted to recovered plasma, an unlicensed product that may be used for further manufacture.

### Plasma for Ancillary Use in Biologics Manufacturing

Human plasma and its derivatives are used in the manufacture of other biologic products. In this role, the plasma or plasma derivative falls into the category of ancillary use. This is defined as use of a reagent or material as a processing or purification aid or a reagent that exerts an effect on the therapeutic substance but is not intended to be part of the final product formulation (see the *USP* general information chapter *Ancillary Materials for Cell, Gene, and Tissue-Engineered Products* ⟨1043⟩).

Human plasma is commonly used in manufacturing processes that involve primary human cells or cell lines intended for therapeutic applications. In these applications, plasma provides a source of protein and possibly other factors that enhance expansion and differentiation of cell populations. A variety of methods have been used to prepare human plasma and derivatives for ancillary use, but the practices are not standardized. Allogeneic plasma typically is obtained from either apheresis or whole blood, using citrate anticoagulation. Allogeneic plasma typically is collected from paid donors who, like Source Plasma donors, have been screened for the absence of transfusion-transmissible diseases. Preferred donors may be blood type group AB, because they lack anti-A and anti-B isohemagglutinins. Other preferred donors include untransfused males, because this group is unlikely to have human leukocyte antigen (HLA) antibodies that could react with cells in a given culture system. Serum is prepared either from nonanticoagulated whole blood that has been allowed to clot or by the addition of calcium to plasma obtained from citrated whole blood or apheresis. Heating plasma or serum to 56° inactivates heat-labile complement and other proteins.

Although there are no standardized specifications for plasma products used as ancillary materials, assays often include safety testing associated with general biologics (e.g., bacterial/fungal cultures, endotoxin, and mycoplasma). In addition, characterization of the products may include tests for irregular erythrocyte and HLA antibodies, osmolality, pH, total protein and immunoglobulin concentrations, hemoglobin concentration, and chemistries such as Na, K, Cl, Ca, and glucose.

In some instances, plasma from bovine sources has been used instead of human plasma. The differences between bovine and human plasma products include factors relevant to their efficacy as ancillary materials, as well as safety of the final manufactured product when administered to humans (see the *USP* general information chapter *Bovine Serum* ⟨1024⟩). Fetal bovine serum is preferred to human serum for some applications because it may be superior in promoting human cell growth in vitro. Safety considerations, especially the risk of transmissible spongiform encephalopathy and allergic reactions related to antibovine antibodies, have led to recommendations that human plasma sources be used whenever possible instead of bovine sources. However, substitution of human for bovine plasma does not completely eliminate the risk of infectious and immunologic sequelae, even when human plasma is used as an ancillary material.

## PLASMA COLLECTION AND PROCESSING

This section discusses the standard principles involved in plasma collection and the methods used to ensure the safety of the plasma and subsequently manufactured plasma derivatives. Principles for screening and testing of donors are presented in other parts of this chapter.

### Collection

Source Plasma is collected by apheresis. Recovered plasma can be obtained either by apheresis or as a by-product of whole blood collection. Collection should take place via an FDA-approved, closed, sterile, pyrogen-free collection system that contains an anticoagulant. No antibacterial or antifungal agent should be added to the plasma. Donations must be collected aseptically. The skin of the donor must be aseptically prepared. Source Plasma is collected using 4% sodium citrate as the anticoagulant. The composition of sodium citrate is given in *Table 2*.

Three anticoagulant solutions are licensed in the United States for collection of whole blood: citrate phosphate dextrose (CPD), citrate phosphate double dextrose (CP2D), and citrate phosphate dextrose adenine (CPDA-1). The composition of blood collection bags containing these anticoagulants is shown in *Table 3*. Plasma for transfusion or further manufacture can be made from a unit of whole blood collected in any of the three anticoagulant solutions. Regulations relating to plasma make no distinction among the three anticoagulant solutions. Consequently, collection, storage, and transport requirements are identical regardless of the anticoagulant solution used in the primary collection.

[2] PPTA. www.pptaglobal.org.
[3] AABB. *Standards for Blood Banks and Transfusion Services*. 25th ed. Bethesda, MD: AABB; 2008.

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**Table 2. Anticoagulant Solution for Collection of Source Plasma by Apheresis (4% Sodium Citrate)**

| Volume | Sodium Citrate Dihydrate | Citric Acid Anhydrous | pH (25°) | Ratio of Solution to Whole Blood |
|---|---|---|---|---|
| 250 mL or 500 mL | 40 g/L | As required for pH adjustment | 6.4–7.5 | 1:16 |

**Table 3. Anticoagulant Solutions Used during Whole Blood Collection for Recovery of Plasma (500-mL collection bags)**

| Anticoagulant | CPD[a] | CP2D[b] | CPDA-1[c] |
|---|---|---|---|
| Volume (mL) | 70 | 63 | 63 |
| Dextrose (mg) | 1780 | 3220 | 2010 |
| Sodium citrate dihydrate (mg) | 1840 | 1660 | 1660 |
| Citric acid anhydrous (mg) | 209 | 206 | 206 |
| Monobasic sodium phosphate (mg) | 155 | 140 | 140 |
| Adenine (mg) | — | — | 17.3 |
| pH (25°) | 5.3–5.9 | 5.3–5.9 | 5.3–5.9 |
| Ratio of solution to whole blood | 1.4:10 | 1.4:10 | 1.4:10 |

[a] Citrate phosphate dextrose.
[b] Citrate phosphate double dextrose.
[c] Citrate phosphate dextrose adenine.
Note: Collection of Source Plasma typically involves the use of sodium citrate as the anticoagulant. The specification for sodium citrate is given in *Table 2*. Plasma for transfusion is stored at 2° to 8° after collection. Plasma collected by apheresis should be frozen immediately at –18° or colder.

### Labeling

The labeling for Source Plasma should comply with 21 CFR 640.70 and 21 CFR 640.69(b). The labeling for whole blood should comply with 21 CFR 606.121 and 606.122, and with internal licenses.

A unique identification number is assigned so that the donation can be related to the individual donor records and test results. The origin of each donation in a plasma pool and the results of the corresponding donation and laboratory tests must be traceable while the required degree of confidentiality concerning the donor's identity is maintained. Whole blood must be labeled "This Product may transmit infectious agents" [21 CFR 121(c)(9)]. Source Plasma or recovered plasma must be labeled "Caution: For Manufacturing Use Only" if the product is intended for use in fractionation. For plasma to be used as a reagent or for in vitro use, the required labeling statement is "Caution: For Use in Manufacturing Noninjectable Products Only" [21 CFR 121(e)(5)(ii)].

### Storage

Plasma for fractionation should be stored at or below –20°. The plasma can still be used for fractionation if its temperature exceeds –20° on (at most) one occasion for not more than 72 hours and if the plasma has been maintained at a temperature of –5° or lower at all times. Storage temperatures must be maintained during transport.

## PLASMA SAFETY CONSIDERATIONS

Plasma is protected by five overlapping safeguards that the FDA has termed the "Five-Layer Safety Net": donor screening, blood testing, donor deferral, quarantine, and investigation. For guidance in this area, see FDA Publication No. FS 02-1 February 2002.

Voluntary measures that provide an additional margin of safety include recruitment and retention of suitable donors and inventory hold procedures. In the manufacture of plasma-derived products, steps taken for viral clearance are very important for ensuring safety.

None of these measures is sufficient by itself; the safety net is the overlapping combination of the activities.

### Donor Screening

The selection of a suitable site for blood and plasma donation activities is a first and very important step to ensure safe donations. Areas with low disease prevalence are preferred as locations for donation centers, thereby reducing the likelihood of collecting plasma from an infected donor.

One of the PPTA voluntary standards is the viral marker standard, which obliges plasma centers to report viral marker rates for human immunodeficiency virus (HIV), hepatitis B virus (HBV), and hepatitis C virus (HCV) in the donor populations. A center's rates are compared to the industry average. Alert limits are set to take into account the number of annual donations in the center. If a center exceeds the limit for any of these viruses or the aggregate of all three, the center must implement corrective actions that will bring the center into compliance with the standard.

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Appropriate donor selection helps provide a safe plasma supply. A detailed donor history questionnaire in conjunction with a careful medical examination allows center personnel to recognize unsuitable donors whose behavior puts them at risk for transfusion-transmitted disease or who have underlying medical conditions that preclude donation.

An additional measure put in place by centers that collect Source Plasma is the PPTA National Donor Deferral Registry (NDDR). It lists donors throughout the United States who have been previously deferred from donation (although it provides no information about the reason for deferral). Other countries have different systems depending on their national regulations concerning personal data gathering. Any individual who tests positive for HIV, HBV, or HCV is entered into the national database (the National Donor Deferral Registry) used by all U.S. plasma centers that are certified under the International Quality Plasma Program (IQPP). All individuals who present at a U.S. plasma center for the first time are checked against the NDDR. In this manner, donors who have previously been deferred for positive test results at any participating facility can be identified and rejected quickly. This standard ensures that donors deferred for positive test results do not donate in other facilities.

A voluntary safety initiative, the Qualified Donor Standard, implemented by the plasma fractionation industry, builds on the fact that many plasmapheresis donors contribute plasma frequently. A donor who enters a plasmapheresis center for the first time is called an Applicant Donor, and the first donation is used for further manufacturing only if the donor returns a second time. Potential donors must pass two separate medical screenings and testing for HIV, HBV, and HCV on two different occasions. Only after satisfactory screenings and negative test results does that person become a Qualified Donor. If a donor does not return within 6 months, that person loses his/her Qualified Donor status and must qualify again. This standard means that plasma from a one-time-only donor (even when all test results are negative) cannot be used for further manufacture. This standard results in committed donors and eliminates the risk that plasma centers will accept so-called test seekers. The interval between permitted donations of whole blood is too long to allow a similar screening program, although quite a number of donors in whole-blood donor centers are regular and repetitive donors.

Another PPTA voluntary standard addresses donor management criteria. The Community-Based Donor Standard allows only donors who permanently reside within its defined donor recruitment area to donate at a given center. In addition, a Donor Education Standard requires new donors to engage in an educational program and follow-up assessment regarding HIV/acquired immune deficiency syndrome (AIDS) and activities that place them at risk for HIV/AIDS.

In addition to donor management strategies and standards, PPTA has issued a plasma unit management standard called Inventory Hold. This standard states that collected plasma will be held in inventory for at least 60 days from the time of collection. This allows the retrieval of units as a result of post-donation information (information that was not known at the time of donation) that would have disqualified the donor. This information could include admitting high-risk behavior; becoming reactive for HIV, HBV, or HCV; or providing incorrect information about international travel.

## Blood Testing

Testing of donations is an important safety measure both for plasma intended for transfusion and plasma intended for further manufacturing. Both enzyme-linked immunosorbent assays (ELISA) and nucleic acid amplification technologies (NAT) are used to screen donations for the presence of infectious disease. Automation provides the necessary throughput to screen every donation for a variety of potential pathogens.

Testing strategies differ depending on whether the plasma donation is intended for transfusion or further manufacture. Plasma for transfusion requires more extensive infectious disease testing, because there are no pathogen inactivation/removal technologies licensed for this product in the United States. On the other hand, plasma for further manufacture is subjected to several pathogen inactivation/removal steps during manufacture, thereby obviating the need for some disease testing. *Table 4* outlines current infectious disease tests required by the FDA for plasma donations collected in the United States. *Appendix 3* compares EU and U.S. disease testing and donor deferral requirements.

**Table 4. FDA Disease Test Requirements for Plasma for Transfusion and Plasma for Further Manufacturing**

| Disease | Plasma for Transfusion | Plasma for Further Manufacturing[a] |
|---|---|---|
| Hepatitis B | Hepatitis B surface Antigen (HBsAg)<br>Hepatitis B core antibody | HBsAg |
| Hepatitis C | Anti-HCV<br>HCV RNA | Anti-HCV<br>HCV RNA |
| HIV | Anti-HIV I/II<br>HIV RNA | Anti-HIV I/II<br>HIV RNA |
| Human T-lympho - tropic virus (HTLV) I/II | Anti-HTLV I/II | Not required |
| Syphilis | Serologic test for syphilis, every donation | Serologic test for syphilis, every 4 months for donors only |
| West Nile virus (WNV)[b] | WNV RNA | Not required |

[a] The FDA also encourages in-process NAT testing for parvovirus B19 and hepatitis A. HBV NAT testing also is performed on most Source Plasma.
[b] Testing for WNV is recommended in an FDA draft guidance. The FDA is considering recommendations regarding testing for *Trypanosoma cruzi* (Chagas disease).

NAT, of which polymerase chain reaction (PCR) is the most widely used form, does not rely on the detection of antibodies produced by the infected host after exposure, but targets the nucleic acid of the infecting agent. By means of the selection of suitable priming molecules (the so-called primers), the assay is highly specific for the infecting virus (see the *USP* general

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information chapter *Nucleic Acid-Based Techniques—Amplification* ⟨1127⟩). Through several cycles of amplification, the polymerase enzyme can repetitively generate copies of the targeted fragment of the viral nucleic acid, providing an exponential amplification of a very short stretch of the viral deoxyribonucleic acid (DNA) [or ribonucleic acid (RNA)]. The exponential amplification leads to the generation of many copies of the target molecule and allows the subsequent detection of this virus-specific fragment, even if the original viral load was exceedingly low. This methodology has brought a new degree of safety.

NAT testing, because of its complexity and expense, is difficult to conduct on individual donations. Generally, aliquots from several donations are combined into a single pool, often called a minipool. Testing in pooled format remains more sensitive than serological ELISA screening of individual donations. In addition, the NAT principle circumvents several of the limitations in detecting pathogens by means of serological methods. Pooling can influence overall sensitivity, depending on the pool size and the analytical sensitivity of the NAT assay employed.

In many countries, the maximal load of a pathogen acceptable for a single donation defines the overall NAT sensitivity required. Assays of higher analytical sensitivity can use larger pools, but those of lower analytical sensitivity must test smaller pools in order to comply with regulations. The availability of commercial NAT test kits with defined analytical sensitivity has made minipools up to 512 very common, because these pool sizes, in combination with the analytical sensitivity of the assays used, comply with common regulations on overall sensitivity.

Effective NAT screening requires that the viral load of the plasma pool at the beginning of production be less than the inactivation and/or removal capacity of the process. Differences between the plasma transfusion and fractionation industries have led to different applications of NAT. For individual donations intended for transfusion, where there is no inactivation and/or removal process and where testing is the only method to interdict a contaminated donation, the safety of each individual donation must be ensured by testing with the most sensitive assays possible. Plasma intended for further manufacturing, in contrast, is pooled and serves as the starting material for a multistep process that has built-in pathogen inactivation methodologies. Therefore, NAT screening for plasma for further manufacture is focused on ensuring safe donations and limiting the viral load of the plasma pool to levels less than the known viral inactivation/removal capacity of the inactivation process.

To avoid the loss of large amounts of plasma from a reactive pool, the fractionation industry has implemented a prescreening strategy, the minipool screening concept mentioned earlier. Aliquots of plasma donations are combined to form minipools, and the minipools are tested by NAT. If a minipool is reactive for a virus tested, the individual donation that gave rise to this positive result can be identified and interdicted. The other donations demonstrated to be free of infection can be used for further manufacturing. The donations are then combined into a production pool, a sample of which is subjected to NAT testing as required by regulations.

As indicated, the NAT test portfolio is not uniform and depends on the intended use of the donation and the regulatory environment. Although screening for HCV RNA is done in most countries, screening for HIV is not universally required. NAT detection of HBV is used mainly for plasma for manufacturing. Screening for B19 virus or hepatitis A virus (HAV) is performed only on plasma for manufacturing. HBV screening using NAT detection is more widespread in European and Asian countries than in the United States.

## Donor Deferral

A donor may be deferred from further donation as a result of answers provided on the donor history questionnaire, counseling of donors for reasons for deferral, a medical examination performed at the time of donation, or positive tests for infectious diseases. These processes ensure both the eligibility of the donor and the suitability of the donation. In the event that either is not acceptable, standard processes permanently remove the donor and interdict unused units previously donated. The donor registries mentioned earlier are one means of ensuring that a donor deferred at one center cannot donate elsewhere.

## Quarantine and Inventory Hold

Each individual unit of plasma, whether for transfusion or further manufacture, is held in quarantine until all the required tests have been completed. If all required tests have been performed and found acceptable, the unit can be released; if not, the unit must be destroyed. The plasma industry has voluntarily implemented the inventory hold protocol (also discussed in the previous section *Donor Screening*) for plasma for further manufacture. According to inventory hold requirements, during a 60-day hold period an individual plasma donation cannot be used for further manufacture. The rationale for the hold is that donors who have been recently infected with a pathogen may not have developed levels of antibody at the time of donation, thereby donating an infectious unit despite negative disease tests. The hold provides sufficient time for an infectious donor to develop levels of antibody that will be detected during a subsequent donation if the plasma was intended for transfusion. The 60-day hold also reduces the chance of releasing an infectious unit into the manufacturing process.

The introduction of NAT may have decreased the need for inventory hold, because NAT targets the infecting virus directly and thus does not rely on the time-delayed production of antibodies. Because NAT cannot detect all viruses and because even NAT has a certain (although very low) limit of detection, inventory hold is still of value and thus remains in place in the plasma fractionation industry.

## Investigation

Each plasma donation must be traceable from donation to ultimate disposition in order to minimize the potential transmission of an infectious agent. Traceability encompasses all data concerning donation site, donor identifying information, test results, and data regarding transport, storage, and consignee(s).

Look-back is a process to identify and interdict (quarantine) previous donations from a donor who, at a subsequent donation event, has been found to be (1) infected with a transmissible agent or (2) unsuitable for donating plasma because of history,

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physical examination, or post-donation information. Although look-back strategies are similar in most countries, specific procedures may vary.

## QUALITY SYSTEMS

The intent of this section is to outline the general principles and regulations that are the basis of quality systems relating to plasma collection. U.S. collection centers must follow cGMPs that originate in CFR and are elaborated in FDA regulations, guidance documents, and industry standards.

The GMP regulations specifically governing plasma are found in 21 CFR 600, Biological Products: General; and 606, Current Good Manufacturing Practice (cGMP) for Blood and Blood Components. More general cGMP regulations are found in 21 CFR 210, Current Good Manufacturing Practice in Manufacturing, Processing, Packing, or General Holding of Drugs, General; and 21 CFR 211, Current Good Manufacturing Practice for Finished Pharmaceuticals. Although quality systems regulations are part of 21 CFR for medical devices, they have been extended to other manufacturing as part of the "c" in cGMP and FDA Guidance documents.[4]

The quality system is divided into four major parts: management responsibility, resources, manufacturing operations, and evaluation activities. These are the foundation of the five manufacturing systems: production, facilities and equipment, laboratory control, materials, and packaging and labeling. The procedures of each system are designed to allow operations that facilitate implementation of cGMP requirements. In many instances, these requirements relate to providing facilities and expertise to achieve the requirements for the Five-Layer Safety Net (donor screening, blood testing, donor deferral, quarantine, and investigation), discussed above.

Management responsibilities include providing leadership; building a quality system for the organization that meets requirements; establishing policies, objectives, and plans; and reviewing the quality systems with defined frequency. Resources include having sufficient resources for operational activities, personnel development plans, adequate facilities, and suitable equipment; and controlling outsourced operations. Manufacturing includes designing, developing, and documenting product and processes, performing and monitoring operations, and addressing nonconformities. Evaluation activities include analyzing data for trends, conducting internal audits, and initiating corrective and preventive actions.

A number of required routine activities related to collection and release of plasma or normal recovered plasma from whole blood are linked to both cGMP guidelines and quality systems. These include the requirement for having SOPs to cover all aspects of collection, testing, and release. It is also necessary to validate equipment and systems used by the collection center, including temperature-controlled areas, laboratory equipment, water systems, and computer systems. The design and operation of the facility must be adequate to perform the tasks at hand and prevent cross-contamination. Plasma collection facilities must have an adequate number of knowledgeable and trained staff as well as procedures for acceptance and release of raw materials. To the extent possible, collection facilities must adhere to the GMP requirements for a pharmaceutical manufacturing facility.

## GLOSSARY

**Apheresis:** A method of obtaining one or more blood components by machine processing of whole blood; the residual components of the blood are returned to the donor during or at the end of the process.

**Blood component:** A constituent of human blood: red cells, white cells, platelets, or plasma.

**Blood establishment:** Any structure or organization responsible for any aspect of the collection and testing of human blood or blood components, whatever their intended purpose, and their processing, storage, and distribution. Hospital transfusion services engaged only in compatibility testing and transfusion of blood and blood products are not included within the definition of blood establishment.

**Blood product:** Any therapeutic product derived from human blood or plasma.

**Center:** Collection site or location where blood or plasma is collected (and also may be processed and stored). *Center* is also applicable to a testing *Site* (see entry in this glossary).

**Cryoprecipitate:** A plasma component prepared from fresh-frozen plasma by freeze-thaw precipitation of proteins and subsequent concentration and resuspension of the precipitated proteins in a small volume of the plasma.

**Deferral:** Temporary or permanent suspension of the eligibility of an individual to donate blood or blood components.

**Distribution:** The act of delivery of blood and blood components to other blood establishments, hospital blood banks, and manufacturers of blood products.

**Manufacturing pool:** A combination of a specified number of plasma donations used as the first step in the manufacture of plasma derivatives.

**Donation minipool:** A combination of a small number of units or samples representative of donations used for pretesting prior to pooling units for manufacture.

**Quarantine:** The physical isolation of blood components or incoming materials/reagents over a variable period of time while awaiting acceptance, issuance, or rejection of the blood components or incoming material/reagents.

**Site:** Any location at which a blood establishment carries out blood collection, not including any location not owned or managed by the blood establishment at which blood is collected or any mobile blood collection unit.

**Validation:** The establishment of documented and objective evidence that the particular requirements for a specific intended use can be consistently fulfilled.

## ABBREVIATIONS

| | |
|---|---|
| AABB | American Association of Blood Banks |

[4] FDA. Guidance for Industry: Quality Systems Approach to Pharmaceutical cGMP Regulations. 2006. Available at www.fda.gov/downloads/Drugs/.../Guidances/UCM070337.pdf.

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| | |
|---|---|
| AIDS | Acquired immune deficiency syndrome |
| CFR | Code of Federal Regulations |
| CJD | Creutzfeldt-Jakob disease |
| CMV | Cytomegalovirus |
| CNS | Central nervous system |
| DNA | Deoxyribonucleic acid |
| EBV | Epstein-Barr virus |
| EIA | Enzyme immunoassay |
| EU | European Union |
| FBS | Fetal bovine serum |
| FDA | Food and Drug Administration |
| FFP | Fresh-frozen plasma |
| FP24 | Plasma frozen within 24 hours after phlebotomy |
| cGMP | Current Good Manufacturing Practice |
| HAV | Hepatitis A virus |
| HBsAg | Hepatitis B virus surface antigen |
| HBV | Hepatitis B virus |
| HCT | Hematocrit |
| HCV | Hepatitis C virus |
| HIV | Human immunodeficiency virus |
| HLA | Human leukocyte antigen |
| HTLV | Human T-lymphotropic virus |
| IgA, IgG, IgM | Immunoglobulins A, G, and M, respectively |
| IQPP | International Quality Plasma Program |
| IU | International Unit |
| NAT | Nucleic acid amplification technology |
| NDDR | National Donor Deferral Registry |
| PCR | Polymerase chain reaction |
| PPTA | Plasma Protein Therapeutics Association |
| PRP | Platelet-rich plasma |
| RNA | Ribonucleic acid |
| SARS | Severe acute respiratory syndrome |
| TEP | Therapeutic exchange plasma |
| WBDP | Whole blood-derived plasma |
| WNV | West Nile virus |

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# APPENDICES

## Appendix 1

[NOTE—The collection, processing, and uses of plasma have generated a large number of terms and definitions that reflect the diversity of operations. In addition to the FDA standards and terms, industrywide voluntary standards are discussed in the *Plasma Safety Considerations* section.

Advisory Note: These terms are not meant as regulatory definitions, because plasma term definitions can vary from region to region and among industry sectors. The reader is advised to consult with regulatory authorities responsible for the region and industry sector. Often specific process variables must be considered.]

**Appendix 1: Plasma Types and Specifications as Assigned by Regulatory Agencies in Selected Jurisdictions**

| Plasma Type and Agency or Agency Type | Specification |
|---|---|
| **Recovered plasma** | |

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**Appendix 1: Plasma Types and Specifications as Assigned by Regulatory Agencies in Selected Jurisdictions** *(continued)*

| Plasma Type and Agency or Agency Type | Specification |
|---|---|
| CFR | Plasma derived from single units of whole blood as a by-product in the preparation of blood components from whole blood collection and intended for further manufacturing. Compliance Policy Guides Manual (CPG 7134.12), Sec. 230.100. |
| AABB | Plasma for use in manufacturing and prepared from allogenic donations. Plasma selected for manufacture that has been collected from whole blood or apheresis plasma collected for transfusion that has expired. |
| Inter-region, Inter-sector | Plasma separated from whole blood most often by manual centrifugation or by apheresis. The priority for the blood collected is usually for the production of red blood cells. However, the plasma can be suitable for further manufacture of biotherapeutics and transfusion. The time from collection to freezing can vary depending on the distance of collection and processing sites. Volunteer donors typically are used. |
| **Source Plasma** | |
| CFR | Fluid portion of human blood collected by plasmapheresis and intended as source material for further manufacturing use (21 CFR 640.60). |
| Inter-region, Inter-sector | Plasma separated from whole blood by plasmapheresis where the cellular components can be returned to the donor. The priority for the plasma usually is for further manufacture of biotherapeutic products. However, the plasma can be suitable for transfusion. It is rapidly frozen after collection. |
| **Fresh-frozen plasma (FFP)** | |
| CFR | Fresh-frozen plasma shall be prepared from blood collected by a single uninterrupted venipuncture with minimal damage to and minimal manipulation of the donor's tissue. The plasma shall be separated from the red blood cells and placed in a freezer within 8 hours or within the timeframe specified in the directions for use for the blood collecting, processing, and storage system and stored at –18° or colder [21 CFR 640.34(b)]. |
| AABB | Plasma separated from the blood of an individual donor and placed at –18° or colder within 6 to 8 hours of collection from the donor or within the timeframe specified by the manufacturer's instructions. |
| Inter-region, Inter-sector | Plasma that is collected and frozen quickly after preparation. Transfusion is the primary intended use. However, FFP can be suitable for further manufacture of biotherapeutic products. |
| Council of Europe | A component for transfusion or for fractionation prepared either from whole blood or from plasma collected by apheresis, frozen within a period of time and to a temperature that will adequately maintain labile coagulation factors in a functional state (Chapter 21). |
| UK | Supernatant plasma separated from a whole blood donation or plasma collected by apheresis, frozen, and stored. |
| Australia | Plasma, Fresh Frozen is a component for transfusion or for fractionation prepared either from whole blood or from plasma collected by apheresis, frozen within a period of time and to a temperature that will adequately maintain the labile coagulation factors in a functional state. If prepared from whole blood, it should preferably be recovered within 6 hours, and not more than 18 hours after collection if the unit has been refrigerated. Plasma may also be collected up to 24 hours if the collected blood has been immediately cooled and maintained at 20°–24°. Separated plasma must be frozen to below –30° within one hour. Freezing of plasma collected by apheresis, as above, must commence within 6 hours of collection or within 24 hours if the collected blood has been immediately cooled and maintained at 20°–24°. |
| **Concurrent plasma** | |
| Inter-region, Inter-sector | Plasma collected concurrently with cellular components. Concurrent plasma may be suitable for transfusion or for further manufacture of biotherapeutics. |
| **Applicant Donor** | |
| Inter-region, Inter-sector | Source Plasma obtained during the first collection from a new donor. The plasma is reserved for testing, and any remainder or products derived from the remainder are not allowed for use in humans or are quarantined until the donor passes appropriate tests and returns for a second donation which also clears testing. At that time, both collections are reclassed as "Qualified". |
| **Platelet-rich plasma (PRP)** | |
| CFR | PRP shall be prepared from blood collected by a single uninterrupted venipuncture with minimal damage to and manipulation of the donor's tissue. The plasma shall be separated from the red blood cells by centrifugation within 4 hours after completion of the phlebotomy or within the timeframe specified in the directions for use for the blood collecting, processing, and storage system. The time and speed of the centrifugation shall have been shown to produce a product with at least 250,000 platelets per µL. The plasma shall be stored at a temperature between 20° and 24° immediately after filling the final container. A gentle and continuous agitation of the product shall be maintained throughout the storage period if stored at a temperature of 20° to 24° [21CFR 640.34(d)]. |
| Inter-region, Inter-sector | Plasma that is a product of the first centrifugation of blood where it is separated from red cells. Platelets are fractionated into the plasma layer. |
| **Platelet-poor plasma** | |
| Inter-region, Inter-sector | Plasma that is further purified from platelets by a second centrifugation of PRP. |
| **Cryo-poor plasma** | |
| CFR | Plasma that remains after both platelets and cryoprecipitated AHF have been removed may be labeled "Plasma, Cryoprecipitate Reduced" [21CFR 640.34(e)(2)]. |
| AABB | Plasma Cryoprecipitate Reduced; Fresh-frozen Plasma from which cryoprecipitate has been removed. |

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**Appendix 1: Plasma Types and Specifications as Assigned by Regulatory Agencies in Selected Jurisdictions** *(continued)*

| Plasma Type and Agency or Agency Type | Specification |
|---|---|
| Inter-region, Inter-sector | Plasma that has been thawed by maintaining the temperature just above freezing (usually 4°). A large portion of certain plasma proteins (e.g., FVIII, cryoprecipitate, fibrinogen, fibronectin, or FXIII) has been precipitated from the plasma. |
| Council of Europe | Plasma, Fresh-Frozen, Cryoprecipitate-Depleted (Chapter 23). A component prepared from plasma by the removal of cryoprecipitate. |
| UK | Plasma cryoprecipitate-depleted for transfusion means a plasma component prepared from a unit of plasma, fresh-frozen. It comprises the residual portion after the cryoprecipitate has been removed. |
| Australia | Plasma, Fresh Frozen, Cryoprecipitate-Depleted is a component prepared from Fresh Frozen Plasma by the removal of cryoprecipitate. The content of albumin, immunoglobulin, and most clotting factors is maintained, but the levels of Factors V and VIII and fibrinogen are reduced. It can be stored for up to 36 months at below −25°. |
| **Cryo-rich plasma** | |
| Inter-region, Inter-sector | Plasma that has been thawed by gentle heat input (e.g., in a 37° water bath) where the cryoprecipitate remains dissolved. |
| **Plasma for labile products** | |
| Inter-region, Inter-sector | Plasma that has been collected and best maintains the activity and integrity of labile plasma proteins as exemplified by clotting Factor VIII. Generally the time from collection through processing to freezing is rapid. |
| **Plasma for stable products** | |
| Inter-region, Inter-sector | Plasma that has been collected where conditions for preservation of labile products was not achieved, however conditions were sufficiently moderate so relatively stable products like IgG and albumin would not be impacted. |
| **Less than 6-hour plasma** | |
| Inter-region, Inter-sector | This is generally recovered plasma that has been collected, processed, and frozen prior to 6 hours after collection. This plasma generally is considered to be acceptable for the production of labile products. |
| **6- to 12-hour plasma** | |
| Inter-region, Inter-sector | Generally this is recovered plasma that has been collected, processed, and frozen more than 6 hours and less than 12 hours after collection. This plasma is generally considered to be acceptable for the production of labile products but is inferior to less than 6-hour plasma for this purpose. |
| **12- to 24-hour plasma** | |
| Inter-region, Inter-sector | Generally this is recovered plasma that has been collected, processed, and frozen more than 12 hours and less than 24 hours after collection. This plasma may be acceptable for the production of labile products but is inferior to less than 6-hour plasma and 6- to 12-hour plasma for this purpose. |
| **Less than 12-hour plasma** | |
| Inter-region, Inter-sector | Generally this is recovered plasma that has been collected, processed, and frozen less than 12 hours after collection. This plasma may be acceptable for the production of labile products but is inferior to less than 6-hour plasma and 6- to 12-hour plasma for this purpose. |
| **More than 24-hour plasma** | |
| Inter-region, Inter-sector | Generally this is recovered plasma that has been collected, processed, and frozen more than 24 hours after collection and usually less than 72 hours after collection. This plasma generally is not acceptable for the production of labile products. |
| **Pooled plasma** | |
| Inter-region, Inter-sector | Plasma that has been pooled for manufacturing from several donors. Some plasma pools for further manufacture of biotherapeutic products can be derived from several hundred to a few thousand donors. |
| **Single-donor plasma** | |
| Inter-region, Inter-sector | Plasma derived from a single donor. It can be a single unit or a pool of several units derived from multiple collections from the same donor. |
| **Hyperimmune plasma** | |
| Inter-region, Inter-sector | Plasma derived from donors with high titers to specific disease agents. Titers are elevated in these donors mostly as a result of immunization with a vaccine (e.g., hepatitis B, tetanus, or rabies) or exposure to disease agents (e.g., HCV or SARS). Hyperimmune plasma usually is intended for the preparation of IgG to provide passive immunity against target disease agents. |
| **S/D plasma** | |
| Inter-region, Inter-sector | Plasma that has been treated with solvent/detergent, an inactivation method effective against envelope virus disease agents (e.g., HIV, HBV, or HCV). Some plasma protein components are inactivated or damaged by the process (e.g., alpha-1 proteinase inhibitor, Protein S, anti-plasmin, or FVIII). |
| EU (PharmEuropa) | Human Plasma Pooled and Treated for Virus Inactivation is a frozen or freeze-dried, sterile, nonpyrogenic preparation obtained from human plasma derived from donors belonging to the same ABO blood group. The preparation is thawed or reconstituted before use to give a solution for infusion. The human plasma used complies with the monograph on Human Plasma for Fractionation. |
| **Therapeutic exchange plasma (TEP)** | |

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**Appendix 1: Plasma Types and Specifications as Assigned by Regulatory Agencies in Selected Jurisdictions** *(continued)*

| Plasma Type and Agency or Agency Type | Specification |
|---|---|
| Inter-region, Inter-sector | Similar to Source Plasma in its collection. However, the donors are patients who are having their plasma replaced with electrolytes, protein solutions, or plasma from another donor. The objective usually is to remove disease elements from the patient's plasma. Generally, TEP is not advisable for further manufacture of biotherapeutic products. However, there may be cases where a specialty product may propose a specific TEP as a source material. |
| **Quarantine plasma** | |
| Inter-region, Inter-sector | Plasma that has been collected and not had initial testing completed and/or stored as part of a controlled donor program. The donor is retested for disease agents (e.g., 6 months after collection). If the donor again is negative for the tested disease agents, then the plasma is released for use for further manufacture and/or use in humans. |
| **Quarantine residual plasma** | |
| Inter-region, Inter-sector | Plasma that has been collected and stored as part of a controlled donor program. The donor was not retested for disease agents (e.g., 6 months after collection). An example would be that the donor did not return to the collection facility to permit the later test. Quarantine Residual Plasma is not recommended for further manufacture and/or use in humans. |
| **Salvaged plasma** | |
| Inter-region, Inter-sector | Plasma that has experienced a storage or transport temperature deviation but may still be useful for the preparation of nonlabile products such as albumin or IgG. |
| **Plasma for fractionation (Processing requirements)** | |
| CFR | Placed in a freezer within 8 hours and stored at –18° or colder. |
| Australia | The liquid part of human blood remaining after separation of the cellular elements from blood collected in a receptacle containing an anticoagulant or separated by continuous filtration or centrifugation of anticoagulated blood in an apheresis procedure. It is intended for the manufacture of plasma-derived products. When the plasma is intended for the recovery of proteins that are labile in plasma, it is frozen rapidly to –25° or below within 24 hours of collection. For the recovery of nonlabile proteins, the plasma should be frozen to –20° or below as soon as possible and at the latest within 72 hours of collection. Frozen plasma is stored and transported in conditions designed to maintain the temperature at or below –20°. For accidental reasons, the storage temperature may rise to above –20° on one or more occasions during storage and transport, but the plasma is nevertheless considered suitable for fractionation if all the following conditions are fulfilled: the total period of time during which the temperature exceeds –20° does not exceed 72 hours; the temperature does not exceed –15° on morethan one occasion; the temperature at no time exceeds –5°. |
| EU (PharmEuropa) | Human plasma for fractionation is the liquid part of human blood remaining after separation of the cellular elements from blood collected in a receptacle containing an anticoagulant or separated by continuous filtration or centrifugation of anticoagulated blood in an apheresis procedure. It is intended for the manufacture of plasma-derived products.<br>When obtained by plasmapheresis or from whole blood (after separation from cellular elements), plasma intended for the recovery of proteins that are labile in plasma is frozen within 24 hours of collection by cooling rapidly in conditions validated to ensure that a temperature of –25° or below is attained at the core of each plasma unit within 12 hours after it is placed in the freezing apparatus.<br>When obtained by plasmapheresis, plasma intended solely for the recovery of proteins that are not labile in plasma is frozen by cooling rapidly in a chamber at –20° or below as soon as possible and at the latest within 24 hours of collection.<br>When obtained from whole blood, plasma intended solely for the recovery of proteins that are not labile in plasma is separated from cellular elements and is frozen in a chamber at –20° or below as soon as possible and at the latest within 72 hours of collection.<br>Frozen plasma is stored and transported in conditions designed to maintain the temperature at or below –20°. For accidental reasons, the storage temperature may rise to above –20° on one or more occasions during storage and transport, but the plasma is nevertheless considered suitable for fractionation if all the following conditions are fulfilled: the total period of time during which the temperature exceeds –20° does not exceed 72 hours; the temperature does not exceed –15° on more than one occasion; the temperature at no time exceeds –5°. |
| **Plasma frozen within 24 hours after phlebotomy** | |
| CFR | Plasma manufactured from whole blood should be frozen within 24 hours after phlebotomy. Blood component must be labeled "Plasma Frozen Within 24 Hours after Phlebotomy." |

**Appendix 2: Donor Criteria**

| | Region | | | |
|---|---|---|---|---|
| **Criterion** | **United States** | **United Kingdom** | **European Union** | **Australia** |
| General criteria for blood donation | | | | |
| Appearance | Donor should appear to be in good health (AABB) | Donor should be in good health | Only donors in good health accepted | Nothing specific noted |
| Underlying medical conditions | Most serious medical conditions are grounds for deferral under United States, EU, and UK guidelines. FDA guidelines require deferral only if a person has used bovine insulin manufactured from UK cattle. However, AABB guidelines require deferral for cancer, heart, liver, or lung disease, and bleeding tendency unless approved by a medical director. Cancer and cardiac disease and diabetes treated with insulin require permanent deferral under EU and UK guidelines | | | Most serious medical conditions are grounds for deferral.<br>Cancer: accepted 5 years of remission.<br>Cardiac disease: varies depending on clinical condition.<br>Diabetes: acceptable if controlled. |

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**Appendix 2: Donor Criteria** *(continued)*

| | Region | | | |
|---|---|---|---|---|
| **Criterion** | **United States** | **United Kingdom** | **European Union** | **Australia** |
| Age | ≥16 or conform to applicable state law (AABB, whole blood) | Between 18 and 65; donation at 17 permitted if in accord with national legislation; first-time donors >60 only if permitted by physician | Whole blood: between 17 and 65; no first-time donors >60<br>Apheresis: first-time donors between 18 and 60; may donate up to age 65 | Whole blood: can start at 16–17 with consent of parents and continue to 80, but medical review required at >70.<br>Apheresis: accept new donors 18–65, with medical evaluation required when >60.<br>Existing donors require annual medical review if >65. |
| Weight | None stated: no more than 10.5 mL/kg may be withdrawn (AABB)<br>FDA requires Source Plasma donors to weigh at least 110 lb | Whole blood: ≥50 kg<br>Apheresis: no specific weight requirement | Whole blood: ≥50 kg<br>Apheresis: ≥50 kg | <45 kg—defer.<br>Medical opinion required for unexplained weight loss |
| Blood pressure | Systolic ≤180 mm Hg<br>Diastolic ≤100 mm Hg (AABB) | Systolic ≤180 mm Hg<br>Diastolic ≤100 mm Hg | No specified blood pressure parameters; donors with high blood pressure may donate provided (1) they have not suffered any complications caused by high blood pressure, (2) they are taking only beta blockers and/or diuretics, and (3) their disease is stable, as determined by qualified medical personnel. | Acceptable ranges:<br>Systolic 90–180 mm Hg.<br>Diastolic 60–90 mm Hg.<br>Hypertension 180–100: defer.<br>Hypotension 90–60: defer. |
| Pulse | Between 50 and 100 beats per minute and regular; lower pulses acceptable at discretion of physician (AABB) | Between 50 and 100 beats per minute and regular | No specific pulse rate parameters stated | Regular pulse between 50 and 100: accept<br>Pulse between 40 and 49: accept if donor is physically fit and not on medication |
| Temperature | ≤37.5° taken orally at time of donation (AABB) | Donors who have had a temperature of ≥38° may not donate for 2 weeks | Donors who have had a temperature of ≥38° or flu-like symptoms may not donate for 2 weeks | No requirement |
| HgB/HCT | ≥125 g/L or HCT 38% | Males: ≥135 g/L<br>Females: ≥125 g/L | Males: ≥135 g/L<br>Females: ≥125 g/L | Whole blood: females, 120–165 g/L; males, 130–185 g/L.<br>Apheresis: females, 115–165 g/L; males, 125–185 g/L.<br>If high, defer. If low, defer for 6 months and test for ferritin. |
| Skin examination | Free of infectious skin disease at site of phlebotomy; no skin punctures or scars indicative of addiction to self-injected narcotics (CFR)<br>Free of infectious diseases (AABB) | Skin at venipuncture site should be clear of lesions, including eczema | There should be no skin disease at venipuncture site | Avoid venesection where there is evidence of inflammation or infection |
| Pregnancy | Defer for 6 weeks after delivery (AABB) | Defer 6 months after delivery | Defer for 1 week for every completed week of pregnancy | Current: defer 9 months from estimated date of confinement.<br>After third-trimester delivery: defer 9 months.<br>Miscarriage or termination: defer 3, 6, or 9 months, respectively for 1st, 2nd, and 3rd trimester. |
| Underlying medical conditions that require deferral and that do not pose a risk of transfusion-transmissible infection | | | | |
| Cancer | Permanent deferral unless deemed suitable by medical director (AABB) | Permanent deferral although physician may make exceptions. Permitted after cervical cancer or basal cell carcinoma if successfully treated | Malignant neoplasms, including leukemias and myeloproliferative disorders, are cause for permanent deferral; exceptions may be made for certain conditions after successful therapy | Permanent deferral for haematological malignancies.<br>Skin cancer–basal–cell carcinoma: accept.<br>Other cancers: defer 5 years after completion of treatment |

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**Appendix 2: Donor Criteria** *(continued)*

| | Region | | | |
|---|---|---|---|---|
| **Criterion** | **United States** | **United Kingdom** | **European Union** | **Australia** |
| Cardiac disease | Free of major organ disease (heart, liver, and lungs) unless deemed suitable by medical director (AABB) | Permanent deferral for persons with a history of heart disease, especially coronary disease, angina pectoris, severe cardiac arrhythmia, arterial thrombosis, or recurrent venous thrombosis. 2-year deferral for rheumatic heart disease with no evidence of chronic disease | Permanent deferral for persons with active or past serious cardiovascular disease, except congenital abnormalities with complete cure | Permanent deferral for arrhythmias, endocarditis, ischaemic heart disease, heart surgery, myocardial disease.<br>Accept: congenital heart disease if surgically corrected.<br>Heart murmurs: accept, subject to medical opinion.<br>Accept after full recovery: pericardial disease, rheumatic heart disease. |
| Cerebrovascular diseases | No specific guideline other than that the donor must be free of major organ disease (AABB) | Permanent deferral for history of cerebrovascular diseases | Permanent deferral for donors with a history of serious CNS disease | Permanent deferral |
| Epilepsy | No deferral | Must be free of epileptic attack for 3 years and have been taken off all medication | Permanent deferral unless at least 3 years have elapsed since the date that donor last took anticonvulsant medication and there has been no recurrence of symptoms | Defer for 2 years from last seizure |
| Gastrointestinal disease | No specific deferral; free of major organ disease unless deemed suitable by medical director (AABB) | No specific deferral | Deferral (not noted to be a permanent deferral) for disease that renders the individual liable to impaired iron absorption or blood loss | Ulcers: defer indefinitely |
| Genitourinary and renal disease | No specific deferral.<br>Free of major organ disease unless deemed suitable by medical director (AABB) | Five-year deferral after complete recovery from acute glomerulonephritis | Permanent deferral for donor with serious genitourinary or renal disease | Permanent deferral: chronic pyelonephritis, chronic kidney infection, chronic dialysis.<br>Accept if resolved: haematuria, acute kidney infection.<br>Urinary catheter present: plasma only for fractionation if underlying condition acceptable.<br>Acute dialysis: defer for 12 months.<br>Acute glomerulonephritis: defer 5 years after recovery. |
| Diabetes | No specific deferral except that receipt of bovine insulin manufactured in the UK requires permanent deferral (FDA).<br>Free of major organ disease unless deemed suitable by medical director | Permanent deferral if insulin therapy required | Permanent deferral for donors on insulin treatment | Permanent deferral if diabetes-associated complications present<br>Accept if disease is controlled, even if patient is taking insulin |
| Respiratory disease | Free of acute respiratory disease (CFR).<br>Free of major organ disease (lungs) unless deemed suitable by medical director (AABB) | Permanent deferral for chronic bronchitis; common cold acceptable | Permanent deferral for serious disease | Permanent deferral: chronic abscess, bronchiectasis, or emphysema with respiratory insufficiency.<br>Accept if mild and controlled: asthma, bronchiectasis without respiratory insufficiency<br>Plasma for fractionation only: chronic bronchitis.<br>Acute bronchitis: defer 2 weeks after recovery and being off antibiotics for 5 days.<br>Pleurisy, pneumonia: defer 4 weeks after recovery.<br>Acute pulmonary embolism: defer 12 months after recovery |

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**Appendix 2: Donor Criteria** *(continued)*

| | Region | | | |
|---|---|---|---|---|
| **Criterion** | **United States** | **United Kingdom** | **European Union** | **Australia** |
| Hematologic disorders | Free of abnormal bleeding tendency unless determined suitable by medical director (AABB)<br>Free of major organ disease (cancer) unless deemed suitable by medical director (AABB) | No specific deferral. Donors who are heterozygous for beta thalassemia eligible if HgB values are within normal limits | Permanent deferral for donors with serious hematologic diseases | Permanent deferral: donors with serious hematologic diseases (sickle cell disease, thalassaemia major).<br>Accept: thalassaemia minor.<br>Defer: anaemia<br>Plasma for fractionation only: elliptocytosis, glucose-6-phosphate dehydrogenase (G6PD) deficiency, spherocytosis.<br>Apheresis only permitted for patients with G6PD deficiency |
| Immunologic disorders | No specific deferral.<br>Free of major organ disease unless deemed suitable by medical director (AABB) | Permanent deferral for donors with autoimmune disease that affects more than one organ.<br>Defer: documented history of anaphylaxis | Permanent deferral for donors with serious immunologic diseases | Permanent deferral: autoimmune disorders.<br>Accept: If asymptomatic, only one organ system involved, not on immunosuppressive therapy.<br>Accept: Sjogren's syndrome |
| Metabolic disease | No specific deferral.<br>Free of major organ disease unless deemed suitable by medical director (AABB) | No specific deferral | Permanent deferral for serious metabolic disease | No specific reference |
| Bone disease | No specific deferral.<br>Free of major organ disease unless deemed suitable by medical director (AABB) | Two-year deferral after having been declared cured of osteomyelitis | No specific deferral | No specific reference |
| Surgery | No specific deferral unless blood was transfused; in which case, a 12-month deferral applies (CFR) | Major surgery requires evaluation of risk for transfusion-transmissible disease | Permanent deferral for persons with history of resection of the stomach. Major surgery requires a 6-month deferral; minor surgery requires a 1-week deferral | Minor (e.g., skin lesions, arthroscopy): defer until recovered.<br>Routine minor (e.g., appendectomy, laparoscopy): defer for 2 months.<br>Major surgery (if donor received autologous blood only): defer 6 months.<br>Neurosurgery; medical assessment required. |
| Medications that require deferral | | | | |
| Antibiotics | As defined by medical director (AABB) | Donors treated with any prescribed drug should be deferred for a period consistent with the pharmacokinetic properties of the drug | Defer for 2 weeks from full recovery or 1 week from cessation of antibiotic therapy, whichever is longer | Acute treatment: defer until recovered and off antibiotics for 5 days.<br>Prophylactic: accept plasma only for fractionation.<br>Topical: accept if skin is unbroken and not infected. |
| Drugs with teratogenic potential | Etretinate: permanent deferral (FDA).<br>Acitretin: 3-year deferral from last dose (FDA).<br>Dutasteride: 6-month deferral from last dose (FDA).<br>Isotretinoin: 1-month deferral from last dose (FDA).<br>Finasteride: 1-month deferral from last dose (FDA). | Donors treated with drugs with proven teratogenic effect should be deferred for a period consistent with the pharmacokinetic properties of the drug | Donors taking drugs that are proven or potential teratogens or who are taking drugs that accumulate in the tissues over long periods of time should not be used as blood donors | Raloxifene (Evista): defer for 6 months after completion of treatment.<br>Acitretin (Neotigason): defer for 3 years after completion of treatment.<br>Etretinate (Tigason): permanent deferral.<br>Finasteride (Proscar): 7 days after completion of treatment.<br>Isotretinoin: defer for 8 weeks after completion of treatment. |
| Growth hormone from human pituitary glands | Permanent deferral (FDA) | | | Permanent deferral |

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**Appendix 2: Donor Criteria** *(continued)*

| | Region | | | |
|---|---|---|---|---|
| **Criterion** | **United States** | **United Kingdom** | **European Union** | **Australia** |
| Other drugs | No other deferrals by FDA or AABB.<br>Any other deferral at discretion of medical director of blood center (AABB).<br>Warfarin: 7-day deferral for plasma donation (AABB). | Recommended that a list of commonly used drugs with rules for acceptability of donors, approved by the medical staff of the transfusion center, be available. | Other drugs acceptable as long as the underlying condition for which the drug is taken is acceptable. | Most medications that are taken by donors are not harmful to recipients; therefore people taking medications can be acceptable as blood donors. Eligibility is based on the assessment of the underlying condition and specific medication guidelines. |
| Insulin | Permanent deferral: bovine insulin made in UK (FDA). | Permanent deferral if treated with insulin | Permanent deferral if treated with insulin | Defer if diabetes is poorly controlled |
| Immunizations | | | | |
| Toxoids | No deferral | No deferral | No deferral | |
| Licensed killed bacterial vaccines | No deferral | No deferral | | No deferral |
| Licensed inactivated viral vaccines | No deferral | No deferral | No deferral except 1-week deferral after hepatitis B vaccination | No deferral except 1-week deferral after hepatitis B vaccination |
| Unlicensed killed vaccines | 1-year deferral (AABB) | | | Defer 3 months after vaccination |
| Inactivated rickettsial vaccines | No deferral | No deferral | | |
| Live attenuated bacterial and viral vaccines | 4 weeks for varicella and rubella<br>2 weeks for rubeola, yellow fever, mumps, polio (oral), typhoid (oral) (AABB) | 4 weeks | 8 weeks | Plasma only for fractionation for 4 weeks after vaccination |
| Transfusion-transmissible infections that require deferral | | | | |
| HIV infection/AIDS and sexual partners | Permanent deferral if present or past clinical or laboratory evidence of HIV infection/AIDS: positive EIA with positive or indeterminate confirmatory test; positive NAT test; clinical signs include unexplained weight loss, night sweats, blue or purple spots in mouth or on skin, white spots or unusual sores in the mouth, swollen lymph nodes for more than 1 month, persistent cough or shortness of breath, persistent diarrhea, fever for more than 10 days; sexual partners deferred for 1 year from time of last contact (FDA) | Permanent deferral for donors found to have a confirmed positive marker for HIV. Donors found to have a repeat positive marker for HIV that cannot be confirmed should be informed according to the nationally agreed algorithm. | Donors with HIV I or II must be permanently deferred | Infection: permanent deferral. Relevant symptoms within the last 6 months: defer for 12 months.<br>Sexual contact with HIV-positive partner: defer for 12 months after last sexual contact. |
| Hepatitis and sexual partners and household contacts | Permanent deferral for the following:<br>History of viral hepatitis after the 11th birthday.<br>Confirmed repeatedly reactive for HBsAg.<br>Positive test for anti-HBc on more than 1 occasion (testing is not required for Source Plasma donors).<br>Present or past laboratory or clinical evidence of infection with HCV.<br>Sexual partners of patients with hepatitis deferred for 1 year from last contact; household contacts of persons with hepatitis B deferred for 1 year from last contact (CFR). | Permanent deferral for donors whose blood gives a positive reaction for the presence of HBsAg and/or anti-HCV. Donors with a history of jaundice or hepatitis may, at the discretion of the appropriate competent medical authority, be accepted as blood donors, provided that an approved test for HBsAg and anti-HCV is negative. | Permanent deferral for hepatitis B and C: donors with history of hepatitis B may donate after 12 months after recovery, provided that all markers are negative or core antibody positive, HBsAg is negative, and anti-HbS ≥100 IU/L; donors with documented current or past infection with hepatitis C are permanently deferred; donors with hepatitis A are deferred for 12 months. | Hepatitis B acute or past infection: defer for 12 months after recovery, then perform hepatitis testing.<br>Hepatitis B chronic carrier: permanent deferral.<br>Hepatitis B contact, sexual, mucosal, household: defer for 12 months from last exposure unless immune.<br>Hepatitis B other contact: accept.<br>Hepatitis C positive past infection: permanent deferral.<br>Hepatitis C contact, sexual, mucosal, household: defer for 12 months from last exposure;<br>other contact: accept. |

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**Appendix 2: Donor Criteria** *(continued)*

| | Region | | | |
|---|---|---|---|---|
| **Criterion** | **United States** | **United Kingdom** | **European Union** | **Australia** |
| HTLV | Present or past clinical or laboratory evidence of infection with HTLV I/II (positive EIA on 2 occasions).<br>[HTLV not tested in Source Plasma donors] (FDA) | Permanent deferral for carriers of HTLV I/II | Permanent deferral for donors with HTLV I/II | Infection: permanent deferral.<br>Repeat reactive status: plasma only for fractionation.<br>Sexual contact: defer for 12 months after last contact.<br>Household contact: accept. |
| West Nile virus | Donors with symptoms suspicious of or actual diagnosis of WNV deferred for 120 days. Donor testing positive on WNV NAT deferred for 120 days. Donor who develops symptoms of WNV within 2 weeks of donation should be deferred for 120 days. Donors implicated in possible transfusion-transmitted WNV infection should be deferred for 120 days (FDA). | Defer for 28 days after donor leaves an area with ongoing transmission to humans. | Defer for 6 months if donor was in area endemic for WNV and was diagnosed with or had symptoms consistent with WNV.<br>Defer for 28 days after donor returns from endemic area, provided donor has no symptoms of WNV. | Infection: defer for 3 months after full recovery.<br>Area exposure: plasma only for fractionation for 8 weeks after leaving risk area. |
| Chagas disease and babesiosis | Permanent deferral for history of Chagas disease; current FDA regulations do not require testing for Chagas disease. However, although not required, most facilities collecting blood for transfusion perform EIA test for Chagas and permanently defer following positive test. (AABB)<br>FDA probably will not require antibody screening for fractionated or recovered plasma used for further manufacture. FDA has granted exemptions and permitted the collection and distribution of Source Plasma for further manufacture into noninjectable products from a donor known to have Chagas disease (CFR).<br>Permanent deferral for history of babesiosis (AABB) | Permanent deferral for individuals with Chagas disease or history of Chagas disease; blood of persons who were born or have been transfused in areas where the disease is endemic should be used only for plasma fractionation products unless a validated test for infection is negative | Permanent deferral for individuals with Chagas disease. Individuals in the following categories may donate 6 months after leaving an endemic area, provided that a validated test for Chagas disease is negative (if a validated test is positive or not performed, the donor is permanently deferred): born in South or Central America, mother born in South or Central America, transfused in South or Central America; lived or worked in a rural subsistence community in South or Central America for 4 weeks or more | Infection: permanent deferral.<br>Contact: accept.<br>Chagas disease area resident: plasma only for fractionation, permanently.<br>Chagas disease area visitor: plasma only for fractionation for 12 months after leaving endemic area |
| Creutzfeld-Jakob disease (CJD) and variant CJD | Permanent deferral if donor:<br>has diagnosis of CJD or nvCJD;<br>has a blood relative diagnosed with CJD;<br>received dura mater graft;<br>received human-derived pituitary growth hormone;<br>received bovine insulin made in UK;<br>spent a cumulative 3 months in UK between 1980 and 1996;<br>received a blood transfusion in the UK at any time since 1980;<br>spent 6 months between 1980 and 1990 on a US military base in Northern Europe;<br>spent 6 months between 1980 and 1996 on a US military base elsewhere in Europe;<br>spent a cumulative 5 years in Europe<br>(Source Plasma donors are not deferred for the latter) (FDA) | Permanent deferral if donor:<br>treated with extracts derived from human pituitary glands;<br>has been recipient of dura mater or corneal graft;<br>has a family risk of CJD or any other TSE.<br>For vCJD:<br>Member states should determine on the basis of the prevalence of BSE within individual countries, of the endogenous exposure of the population to bovine products imported from countries with a high BSE prevalence, and of the incidence of cases of vCJD, what precautionary measures they may need to take to minimize the risk of transmission of vCJD via blood transfusion | Permanent deferral if donor:<br>Diagnosed with CJD, vCJD, or any other prion-associated disease;<br>has family risk of CJD;<br>at increased risk from surgery, transfusion, or transplant of tissues or organs;<br>received a dura mater graft;<br>received a corneal, scleral, or ocular graft;<br>received human-derived pituitary extract;<br>received a blood transfusion in UK since 1980;<br>received intravenous immunoglobulin (IVIg) of UK origin;<br>donated unit of blood implicated in possible case of transfusion-related vCJD.<br>Additionally, all plasma from British donors cannot be used for fractionation | Permanent deferral if diagnosed with any prion-related disease.<br>Permanent deferral if donors have spent a cumulative time of 6 months in England, Wales, Scotland, Northern Ireland, the Channel Islands, or the Isle of Man between 1 January 1980 and 31 December 1996.<br>Permanent deferral if donors received a blood transfusion or blood products in England, Wales, Scotland, Northern Ireland, the Channel Islands, or the Isle of Man from 1 January 1980 onwards unless the blood products were processed plasma products and were given after 31 December 2001.<br>Permanent deferral if the donor had ear surgery performed between 1972 and 1989 and dura mater was used.<br>Permanent deferral for donors who received human-derived pituitary growth and gonadotrophic fertility hormones prior to 1986. |
| Visceral leishmaniasis (Whole Blood) | Donors who have been to Iraq are deferred for 1 year. Permanent deferral for signs and symptoms of visceral leishmaniasis (FDA) | No specific deferral | Permanent deferral for visceral leishmaniasis | Cutaneous: plasma only for fractionation, permanently<br>Visceral: permanent deferral<br>Contact: accept. |

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**Appendix 2: Donor Criteria** *(continued)*

| Region | | | | |
|---|---|---|---|---|
| **Criterion** | **United States** | **United Kingdom** | **European Union** | **Australia** |
| Medical conditions and behaviors that place an individual at risk for a transfusion-transmissible infection and require deferral. | | | | |
| Xenotransplant | No current deferral required for blood donation, but draft regulations for tissue and organs require permanent deferral. | Permanent deferral | Permanent deferral | Permanent deferral |
| Blood transfusion organ and tissue transplant; treatment with plasma-derived clotting factor concentrates | One-year deferral (dura mater graft is permanent deferral); permanent deferral if patient received clotting factor concentrates and sexual partner deferred for 1 year after last contact | Six-month deferral: if NAT test for hepatitis C is negative, may donate after 4 months | Permanent deferral if donor ever received clotting factor concentrate or was transfused after 1 January 1980; 1 year for tissue or organ transplant | Blood transfusion homologous: defer 12 months<br>Coagulation factor, blood derived, short term: defer 12 months from last treatment.<br>Coagulation factor, blood derived, continuous: permanent deferral.<br>Human tissue recipients:<br>Organ/haematological: permanent deferral.<br>Homologous, bone, tendon, skin: accept.<br>Collagen: accept.<br>Corneal: permanent deferral for iatrogenic cCJD risk. |
| Surgery or use of endoscope with biopsy | No specific deferral criteria; general health and transfusion criteria apply | Six-month deferral for major surgery; stomach resection requires permanent deferral; 1-week deferral for minor surgery; 6-month deferral for endoscope with biopsy; if NAT test for hepatitis C is negative, may donate after 4 months | Six-month deferral for major surgery or procedure using an endoscope; 1-week deferral for minor surgery | Defer 6 months |
| Nonsterile skin penetration or mucous membrane exposure to blood or body fluids not the donor's own | Twelve-month deferral | Six-month deferral; if NAT test for hepatitis C is negative, may donate after 4 months | Twelve-month deferral | Defer 12 months |
| Acupuncture, tattoo, body piercing, etc. | Twelve-month deferral unless performed by a state-regulated entity, using sterile needles and disposable dyes | Six-month deferral; if NAT test for hepatitis C is negative, may donate after 4 months; exception can be made according to national risk assessment | Twelve-month deferral; 6-month deferral if validated test for hepatitis B core antibody is negative; for acupuncture, no deferral if performed by state-regulated entity | If using single-use items: plasma only for fractionation for 12 months<br>If not single-use or unsure: defer for 12 months |

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**Appendix 2: Donor Criteria** *(continued)*

| Criterion | Region | | | |
|---|---|---|---|---|
| | United States | United Kingdom | European Union | Australia |
| Injection of medications or steroids not prescribed by a physician | Permanent deferral; sexual partner deferred for 1 year | All blood donors should be provided with accurate and updated information about HIV transmission and AIDS so that persons who have unsafe sex practices or other risk behavior exposing them to potential infection will refrain from donating. The information provided may vary among countries according to local epidemiological data. | Permanent deferral if donor ever injected or has been injected with drugs; sexual partner deferred for 1 year | Permanent deferral |
| Males who have sexual contact with another male | Permanent deferral for sexual contact, even once, since 1977; female sexual partner deferred for 1 year from last contact | | Permanent deferral for oral or anal sexual contact even if protection used; female sexual partner deferred for 1 year from last contact. | Defer for 12 months after last sexual contact |
| Accepted money or drugs or other payment in exchange for sex since 1977 | Permanent deferral; sexual partner deferred for 1 year from last contact | | Permanent deferral; sexual partner deferred for 1 year from last contact. | Defer for 12 months after last sexual contact |
| Incarceration for more than 72 hours in the past year | One-year deferral | | No deferral | Defer for 12 months after release |
| Born in or lived in Africa | Permanent deferral if born or lived in countries where HIV I subtype O is endemic (Cameroon, Central African Republic, Chad, Congo, Equatorial Guinea, Gabon, Niger, Nigeria); sexual partner deferred for 1 year from last contact unless tested with a test validated to detect Group O | | No specific deferral for Africa; malaria rules apply; however, sexual partners of persons who were sexually active in areas where HIV is endemic deferred for 1 year from last contact | No specific deferral: donors who have visited a malaria-endemic area are subject to a plasma-only restriction period of at least 12 months. The restriction period is extended to 3 years if residence has been for 6 continuous months or more within the past 3 years. With a negative malaria test, the restriction period can be reduced to 4 months. Donors who have traveled to an HIV risk area must be asked if they had sexual contact with a resident of that area. |

**Appendix 3: Disease Testing**

| Disease Test | United States | European Union |
|---|---|---|
| Hepatitis | | |
| HBsAg | Permanent deferral for repeatedly reactive test results (FDA) | ***European Pharmacopoeia:*** **Human Plasma for Fractionation** |
| Hepatitis B core antibody | Permanent deferral if reactive on 2 or more separate occasions; permanent deferral if core antibody positive results are coupled with prior or concurrent repeatedly reactive HBsAg test (FDA). Testing is not required for Source Plasma donors | Laboratory tests are carried out for each donation to detect the following viral markers:<br>(1) antibodies against HIV-1 (anti-HIV-1).<br>(2) antibodies against HIV-2 (anti-HIV-2).<br>(3) hepatitis B surface antigen (HBsAg).<br>(4) antibodies against hepatitis C virus (anti-HCV).<br>If a repeat-reactive result is found in any of these tests, the donation is not accepted. |
| Hepatitis C antibody EIA | Permanent deferral if repeatedly reactive for hepatitis C antibody; may re-enter donor after 6 months if confirmatory test is negative (FDA) | **Blood Directive 2002/98/EC (Annex IV): Basic Testing Requirements for Whole Blood and Plasma Donations** |
| Hepatitis C NAT testing | Permanent deferral if positive on single testing (FDA) | The following tests must be performed for whole blood and apheresis donations, including autologous predeposit donations:<br>ABO group (not required for plasma intended only for fractionation).<br>Rh D group (not required for plasma intended only for fractionation).<br>Testing for the following infections is required in donors:<br>Hepatitis B (HBsAg),<br>Hepatitis C (Anti-HCV),<br>HIV 1 and 2 (Anti-HIV 1 and 2).<br>Additional tests may be required for specific components or donors or epidemiological situations.<br>**Blood Directive 2004/33/EC (Annex III): Permanent Deferral Criteria (excerpt):**<br>Hepatitis B, except for HBsAg-negative persons who are demonstrated to be immune<br>Hepatitis C<br>HIV 1 or 2 |
| HIV | | |
| HIV NAT | Permanent deferral if positive on single testing (FDA) | |

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**Appendix 3: Disease Testing** *(continued)*

| Disease Test | United States | European Union |
|---|---|---|
| HIV I or II Antibody (EIA) | Permanent deferral for repeatedly reactive HIV EIA test; may reenter after 6 months if confirmatory test negative (FDA). Testing is not required for Source Plasma donors | ***European Pharmacopoeia:*** **Human Plasma for Fractionation**<br>Laboratory tests are carried out for each donation to detect the following viral markers:<br>(1) antibodies against HIV-1 (anti-HIV-1)<br>(2) antibodies against HIV-2 (anti-HIV-2)<br>(3) hepatitis B surface antigen (HBsAg)<br>(4) antibodies against hepatitis C virus (anti-HCV)<br>If a repeat-reactive result is found in any of these tests, the donation is not accepted<br>**Blood Directive 2002/98/EC (Annex IV): Basic Testing Requirements for Whole Blood and Plasma Donations**<br>The following tests must be performed for whole blood and apheresis donations, including autologous predeposit donations:<br>ABO group (not required for plasma intended only for fractionation)<br>Rh D group (not required for plasma intended only for fractionation)<br>Testing for the following infections are required in donors:<br>Hepatitis B (HBsAg)<br>Hepatitis C (Anti-HCV)<br>HIV 1 or 2 (Anti-HIV 1 and 2)<br>Additional tests may be required for specific components or donors or epidemiological situations.<br>**Blood Directive 2004/33/EC (Annex III), Permanent Deferral Criteria (excerpt)**<br>Hepatitis B, except for HBsAg-negative persons who are demonstrated to be immune<br>Hepatitis C<br>HIV 1 or 2 |

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# ⟨1181⟩ SCANNING ELECTRON MICROSCOPY

## INTRODUCTION

Over the last few decades, electron microscopy has become a reliable investigative tool for the study of solid and semi-solid materials. Since the invention of the electron microscope attributed to Max Knoll in 1935 and commercialization in the 1960's by Cambridge Instrument Co. and JEOL, recent improvements in resolution, stability, and specimen accommodation have resulted in a robust array of commercial instrumentation that spans a wide range of capabilities. This chapter provides a review of the common electron microscopy technologies and techniques.

## UTILITY OF THE TECHNIQUE

### General Description of the Technique

Scanning electron microscopy (SEM) is an electron optical imaging technique that yields both topographic images and elemental information and is used in conjunction with a variety of detection systems and elemental detectors. All electron microscopy systems utilize a high energy, focused electron beam as source illumination with concurrent increase in resolution compared to photon (light) microscopy. The resolution increase is realized by electron beam wavelength being three orders of magnitude smaller than that of visible light. The optics consist of condenser and objective lenses in conjunction with selected apertures. A focused e-beam is rastered over the surface of a specimen, generating a variety of signals from which an image is collected.

### Traditional High Vacuum SEM

Traditional SEM systems operate at high vacuum, so specimens must be clean, dry, and able to withstand imaging at high vacuum, up to $10^{-6}$ torr. Additionally, the specimens must be inherently conductive or coated to conduct the high surface charges. Four general principles of these systems are:

- e-beam generation
- image formation
- specimen preparation
- elemental composition.

Typically, SEM analysis requires only a small amount ($10^{-3}$ to $10^{-12}$ g, depending on the application) of a solid specimen. The specimen is presented to the collimated e-beam and scanned in a controlled raster pattern. Images, or micrographs, are generated using secondary electron (SE) and/or back-scattered electron (BSE) detection. Specimen detail may be resolved to 10 nm even in the most simple systems. The collimation of the e-beam also yields great depth of field that is evident in a wide dynamic range of magnification, practically spanning five orders of magnitude (5×–100,000×).

The size of the final aperture controls the beam diameter and, accordingly, the image resolution and total current at a specimen. Small apertures are required for high resolution and large apertures provide high current for optimal X-ray emission intensity. In many systems, the objective aperture can be adjusted during use with a sliding or rotating holder. Flexibility in trading resolution for specimen current is also important because specimen characteristics affect these two criteria differently.

Image magnification is controlled by altering the area of the electron beam raster; smaller areas yield higher magnification. An Everhart-Thornley (ET) detector is used for electron detection; the resultant images are most similar to those of reflected light microscopy. An ET detector consists of a Faraday cage and a scintillator disk connected by a light pipe to a photomultiplier tube. The Faraday cage serves three functions: (1) at positive bias it attracts SE; (2) at negative bias it repels SE to enable the ET detector to collect BSE signals alone; and (3) it shields the primary electron (PE) beam from the scintillator potential. Various scintillator coatings are used. For example, phosphorus-based coatings yield intense, high-contrast images. Aluminum-based coatings, although less sensitive, can withstand the high SE flux generated during elemental analyses. Additionally, solid-state detectors provide up to 10 times greater sensitivity for BSE collection. They can be placed at a variety of positions and distances with respect to a specimen and used in conjunction with ET detectors.

### Variable Pressure or Environmental Scanning Electron Microscopy (ESEM)

Robust and common variations of the traditional high vacuum system prevail in today's labs. So-called variable pressure, "environmental" or low vacuum systems (LVSEM) appeared in the mid 1980s. These are electron microscope systems operating at a much higher, near ambient pressure state (up to 50 torr) at the specimen site, plus gas over-blanketing (almost any may be used) and temperature control to 1500°C as well.

There are two key advantages for ESEM's: First, non-conducting samples do not require a conductive carbon or metal thin film to be applied. The gas in the sample chamber is ionized by the electrons generated from the beam/sample interaction. These ions will diffuse to the sample surface, thereby dissipating any negative charge buildup. Secondly, if the sample in question is prone to outgassing volatile substances such as solvents and water, the near-ambient pressures used in the sample chamber can greatly diminish the likelihood of this occurring.

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### Scanning Transmission Electron Microscopy

The combination of a traditional transmission electron microscope system with scanning control yields specimen analysis with high micrograph resolution, information at the atomic scale such as atomic structure, chemical information, and interfacial bonding. Image acquisition is conducted in the same manner as SEM; however, use of thin specimens allows collection of transmission images as well. Great increase in resolution is attained by controlling the e-beam probe volume, to atomic dimensions. *Figure 1* shows the lattice image of a ceramic that was obtained using a scanning transmission electron microscopy (STEM) instrument. As with any electron- or ion-beam method, the sample must be able to withstand the electron bombardment. This may not always be the case with pharmaceutical materials, as they can degrade in the e-beam during the analysis.

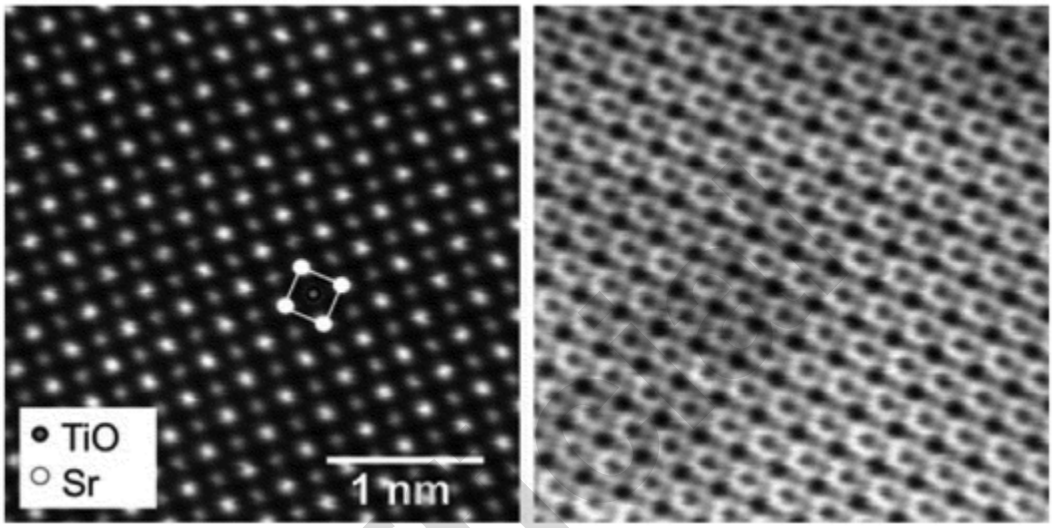


Figure 1. Scanning transmission electron micrograph of an oxide ceramic (perovskite), shown in both dark field (left) and bright field (right) imaging modes. Image courtesy of "... © Carl Hanser Verlag, Muenchen".

## ELECTRON BEAM GENERATION

The basis for the formation of an electron image is the generation of an energetic flux of electrons that impinge upon the sample surface. If the beam is too energetic, the sample will be burned as the beam is rastered across the sample's surface. This is an important consideration when analyzing pharmaceutical materials.

Electron guns can be classified into two types: thermionic emitters and field emitters. With the former, the filament is heated under high vacuum, and a large potential is applied. The field that is created, combined with the heated filament, "boils" off electrons that are subsequently focused in the microscope optics. To increase the flux of electrons from thermionic emitters, one must increase the potential that is applied across the filament, which also increases the kinetic energy of the electrons and the likelihood that the sample surface will be burned.

Another method used to produce electronic beams is to apply a large electric field across an electronic emitter. The filament can have a current passing through it or not. Where surface damage is a prime consideration, cold emitters are the best way to obtain a clear image at high magnification without burning the surface or damaging the material. Typically tungsten is used as the electron emitter, as it is strong enough to withstand the mechanical forces. When using a field emission gun, the cathode must be kept at very high vacuum, and no foreign contaminants must be allowed to deposit on the filament. For these reasons, the column must be differentially pumped. Field emission instruments are better suited for the analysis of pharmaceutical compounds, as they produce a very bright beam that is less damaging to the sample. Additionally, field emission electron SEMs have higher spatial resolution, making them better suited for the identification of foreign particulate matter.

### Electron Beam–Sample Interactions

Interaction of the electron beam (PE) with the specimen produces a variety of physical phenomena that, when detected, are used to form images and provide elemental information about the specimen. These phenomena include:

- emission of inelastic scattered secondary electrons (SE), proportional to specimen topography
- reflection of elastically scattered back-scattered electrons (BSE), proportional to the atomic number (*Z*) of the specimen
- component X-ray emission (XRE, X in *Figure 2*)
- emission of Auger electrons (AE)
- cathodoluminescence (CL)
- conduction of current (specimen current)
- charging from induced voltages (IV) or adsorbed electrons

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- electron transmission
- heat generation
- electromotive forces (see *Figure 2*).

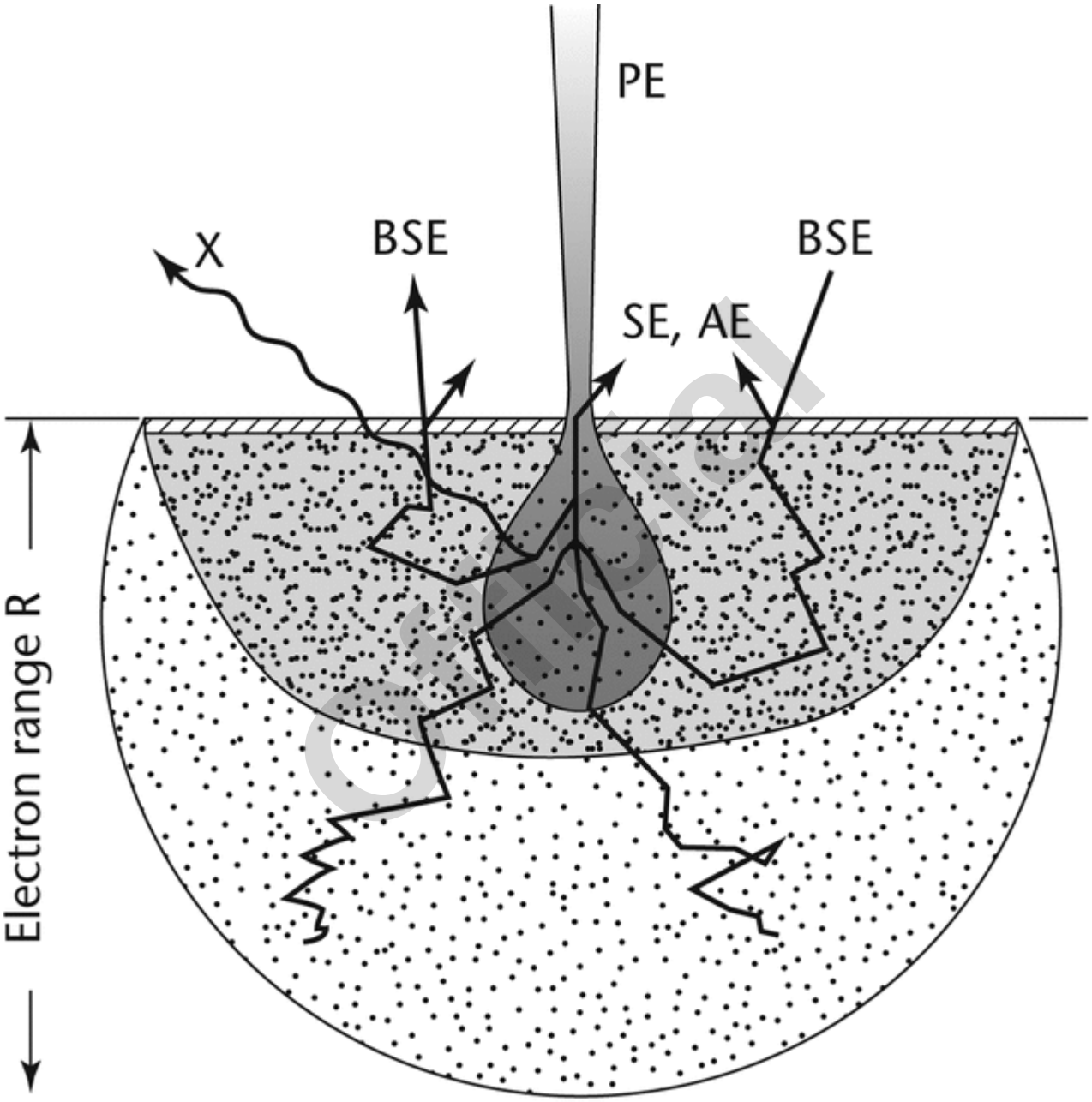


Figure 2. Interaction diagram.

SE are emitted from a specimen surface as the result of inelastic collisions between primary (incident) electrons and the specimen. When the energy imparted to a specimen electron exceeds the work function of a specimen, that electron is emitted as SE. Most secondary electrons have energies of 5–20 eV; electrons in this low-energy range can be efficiently collected, yielding high signal-to-noise images. Because such low-energy electrons can penetrate only short distances through the specimen, SE originate from within 2–30 nm of the surface and generate highly resolved images. The actual e-beam penetration depth is dependent on e-beam accelerating voltage, specimen elemental composition, specimen density, and specimen mounting angle. Excitation volumes of 0.5–5 µm in diameter (dark gray) are typical for a wide range of materials from metallic to biological.

Back-scattered electrons are primaries that have been reflected from the specimen. The primary electrons can undergo multiple collisions prior to exiting from the specimen; therefore, BSE have energies over a broad range and emerge from relatively deep specimen penetration (≈0.1–5 µm) (see *Figure 3*).

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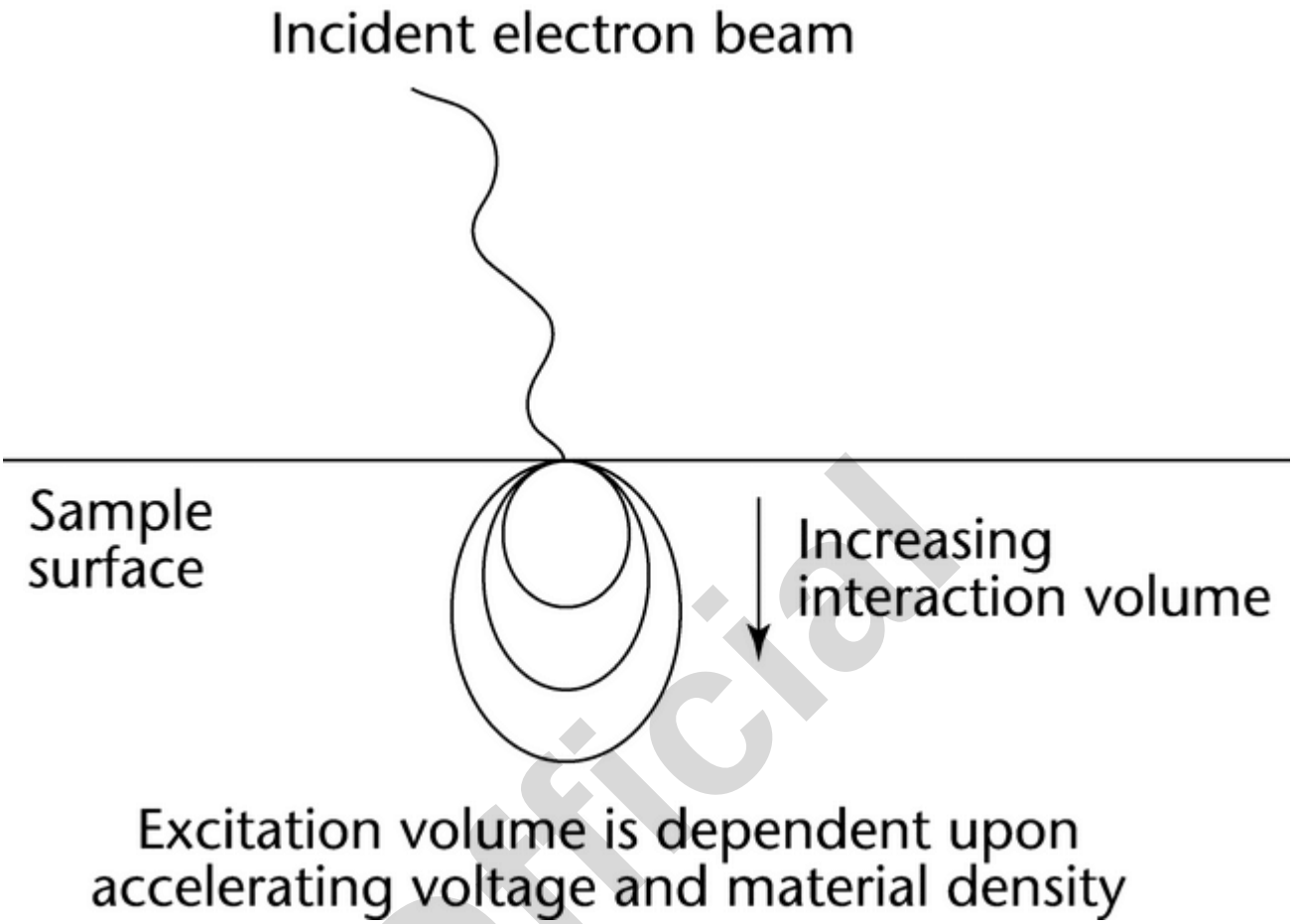


Figure 3. Bulk penetration.

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These high-energy (15–25 keV) BSE are collected less efficiently than SE, and they yield images with poorer resolution. The efficiency of BSE reflection is a function of the atomic number (*Z*) of the specimen atoms; thus, the contrast of BSE images depends on elemental composition. The penetration depth of all electrons is affected by elemental composition, specimen density, specimen tilt, and incident beam energy (accelerating voltage). There are a number of software algorithms to correct for these influences, when known such as ZAF, Bence-Albee, etc. For example, the SE images of sodium phosphate and zinc phosphate crystals are quite similar. However, the heavier nuclei of the zinc species produce more efficient BSE reflection, and BSE images will reveal higher contrast. BSE images of heavy- versus light-element phases, or mixtures of species, show dramatic contrast differences that are representative of elemental heterogeneity. Elemental mapping by energy dispersive X-ray spectroscopy (EDS), discussed later, can provide complementary information.

## SAMPLE PREPARATION

As scanning electron microscopy has advanced as an analytical technique, different options have become available for sample preparation. Ideally, the objective is to obtain the required image or chemical analysis of the material to be examined with as little intervention as possible. Additionally, one must consider the purpose of the analysis and the amount of material that is available. If the material to be examined is a foreign particulate in a parenteral product, sample preparation could be quite different than if one is intending to image a bulk drug substance material or excipients. The decision tree (see *Figure 4*) below represents a simplified version of the available sample preparation method and is one possible construct to determine the appropriate sample preparation method.

For example, in addition to metal (primarily aluminum) sample stubs there are also various metal meshes that can be used to support samples. If the particle in question is isolated from liquid, the filter itself may serve as a suitable substrate for the powder without having to transfer the sample to another sample holder. Additionally, adhesive carbon tabs are a common means to secure the sample to the stub. There are a variety of other adhesive materials such as double-sided copper, silver and aluminum tape, or carbon and silver paint for this purpose.

### Sample Coating

Historically, pharmaceutical materials, which are typically poor electrical conductors, had to be coated with a very thin layer of conductive material (carbon, gold, platinum, or other metals) to ensure that the electron beam used for imaging could be

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dissipated from the sample surface. Coating was also used to create a layer of “electron rich” material that would serve as a source for secondary electron emission.

With the advent of environmental scanning electron microscopy (ESEM) or variable pressure scanning electron microscopes, coating of non-conductive materials is no longer a prerequisite. The atmosphere in the sample chamber itself generates positively charged ions that will dissipate any charge that accumulates on the sample surface.

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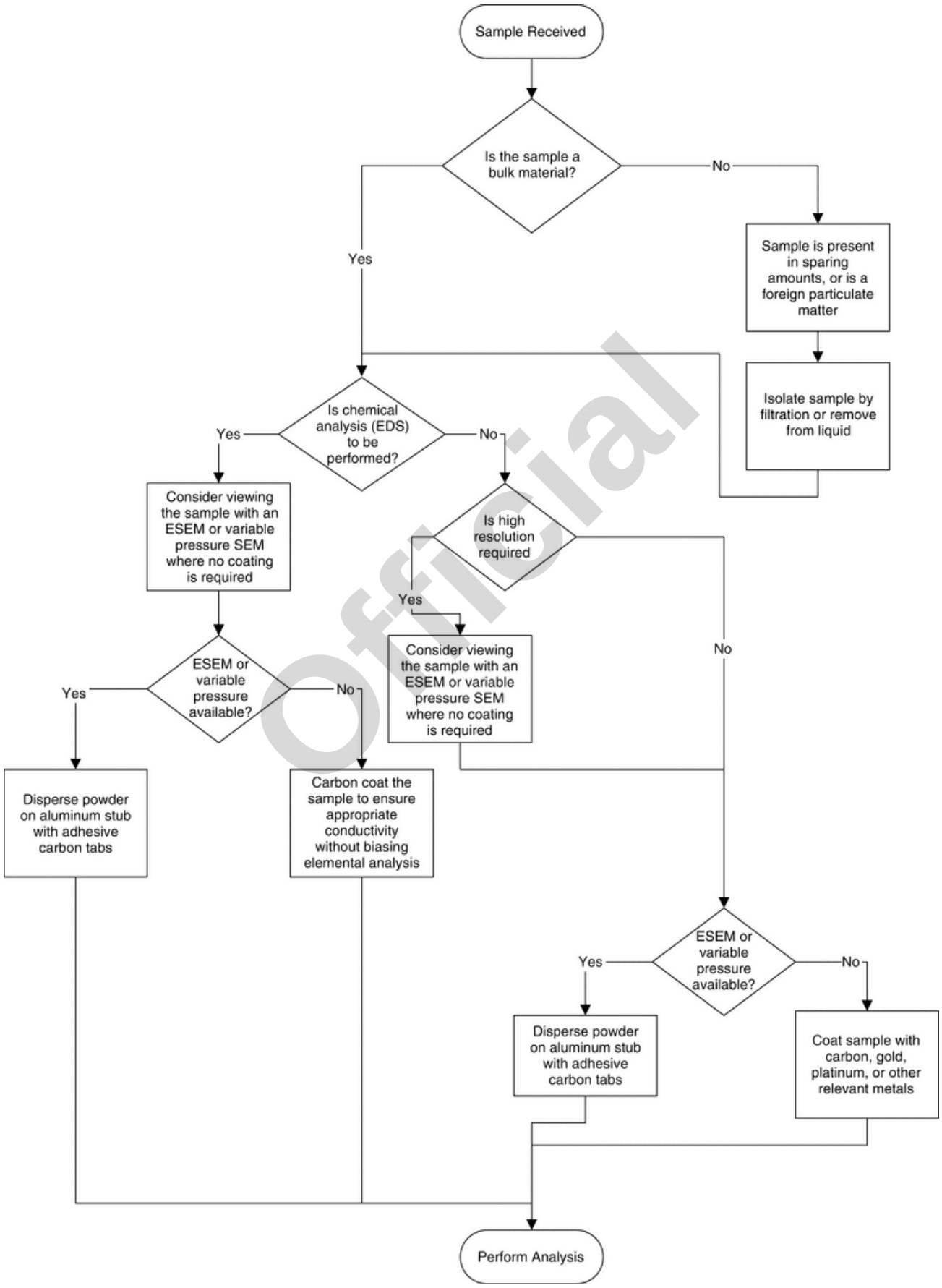


Figure 4. Decision tree for SEM sample preparation.

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## X-RAY GENERATION AND ELEMENTAL COMPOSITIONAL ANALYSIS

When a PE encounters an orbital electron in an atom, the resultant collision can either promote that orbital electron to a higher energy level or ionize the atom. Stabilization of an atom by relaxation of a higher energy electron to fill a vacancy results in the emission of an X-ray photon. These X-ray energies are discrete and element-specific; they equal the differences between the shell electron energies for the various shells of a given element. For instance, an ejected K-shell electron can be stabilized by a higher energy L-shell electron, yielding a net energy ($E_L - E_K$), which is specific for the X-ray photon energy of the elemental K line. X-ray emission lines are classified according to the electron shell in which the vacancy existed, e.g., K, L, M. The lines are further categorized according to the shell from which the relaxing electron originates. Thus, a Kα X-ray line will arise from a vacancy in a K-shell that is filled from an L-shell; a Kβ X-ray line will arise from a K-shell vacancy filled from an M-shell, and so on (see *Figure 5*).

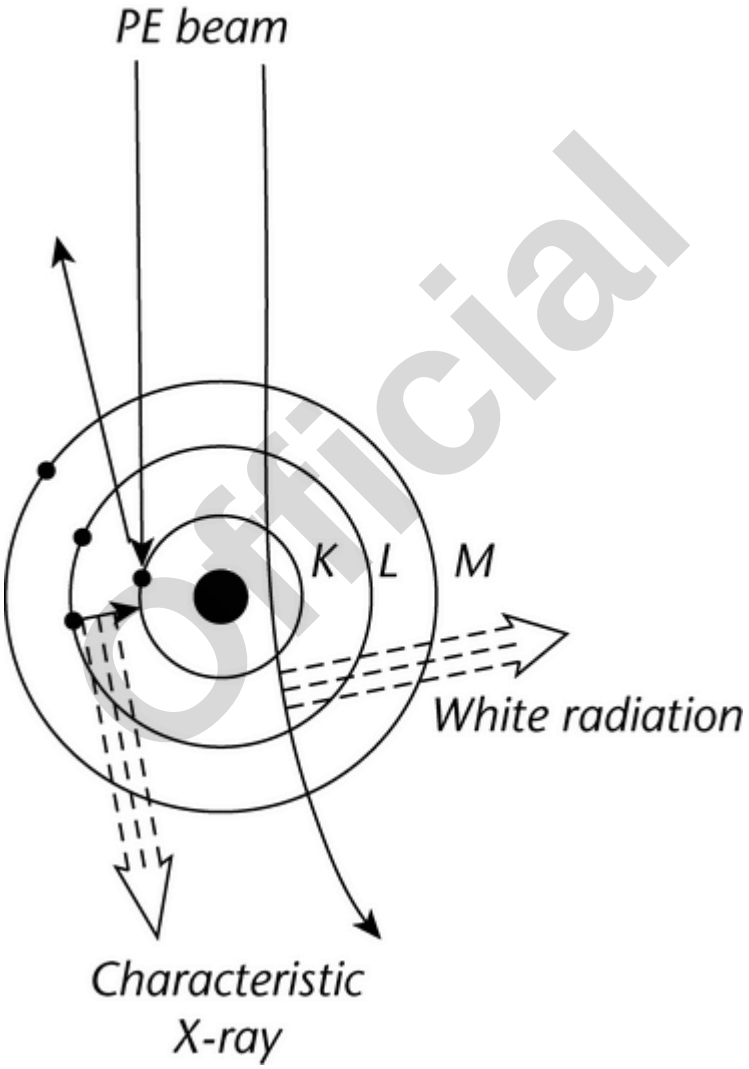


Figure 5. Atom model.

Detection of the generated X-rays and subsequent elemental analysis of the feature of interest can be accomplished by two different kinds of detectors: wavelength and energy dispersive X-ray spectrometers (WDS and EDS, respectively). Each has its individual benefits and challenges. Historically, wavelength dispersive detectors are much less susceptible to spectral artifacts that can create incorrect peak identification such as peak broadening and distortion, silicon escape peaks, absorption, etc. Additionally, most Si(Li) EDS detectors require liquid nitrogen cooling, which can cause ice build-up on the detector window in the sample chamber. The much larger EDS collection angle makes it more robust and less susceptible to detector misalignment than WDS. *Figure 6* illustrates the superior spectral resolution that is obtained with WDS. With WDS, it is much easier to detect characteristic X-rays that are energetically similar. The EDS exhibits peak broadening due to the close proximity of the peaks.

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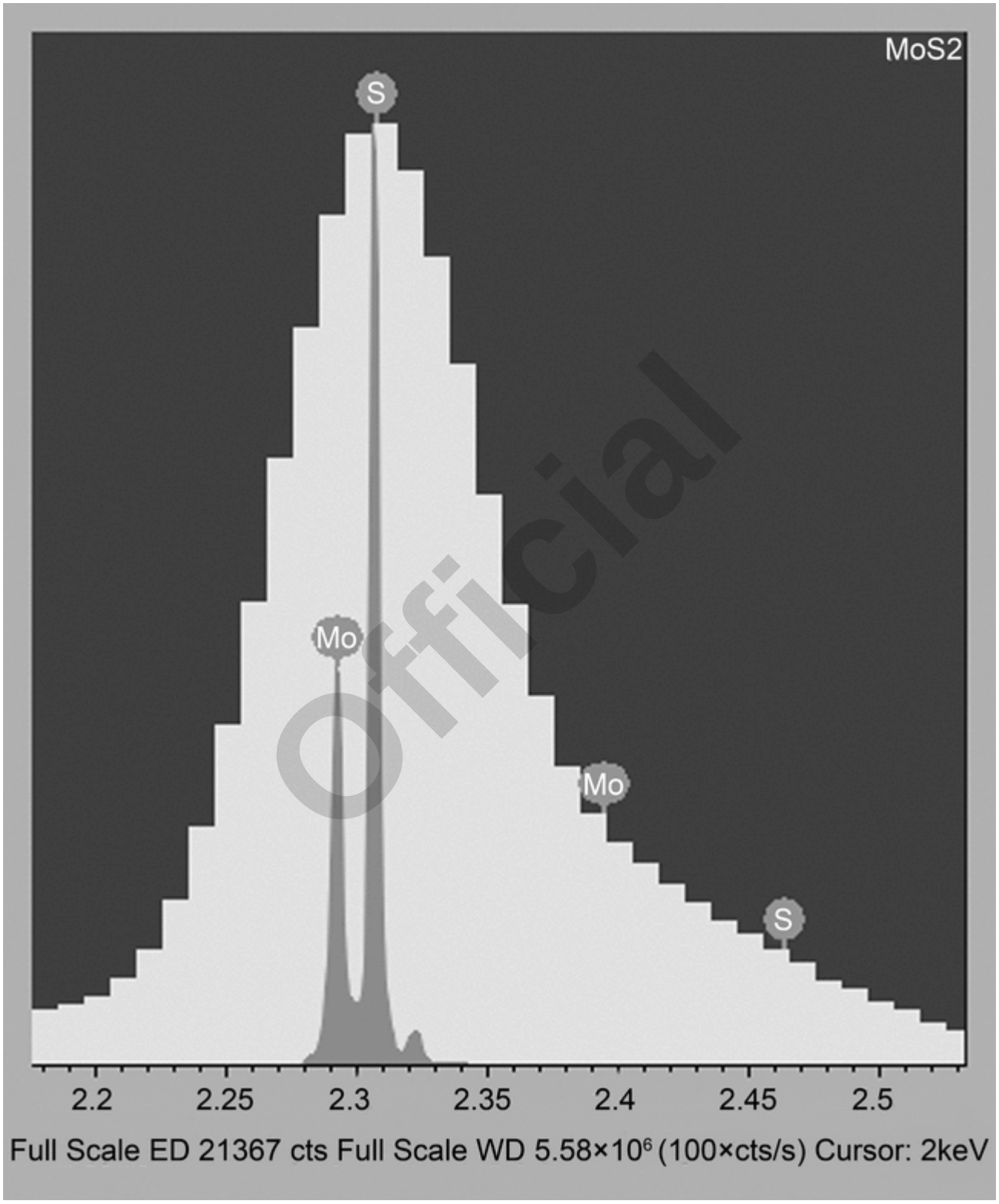


Figure 6. Example of spectral resolution obtained with wavelength-dispersive detectors where S Kα and Mo Lα can be clearly resolved by WDS (gray peaks) but are not resolved by EDS (broad yellow peak). "Used by Permission. Courtesy of Oxford Instruments."

Advantages of EDS reside in the ease of use and speed to collect the entire X-ray spectrum making it ideal for the routine identification of particles and features within a sample. The two types of detector technology most widely available for energy dispersive X-ray spectroscopy (EDS) analysis are based on lithium-drifted silicon [Si(Li)] and silicon drift technology. While Si(Li)

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detectors have been around for the last 40 years, silicon drift detectors (SDD) have recently emerged as the preferred type due to several key advantages including better detection of light elements ($Z \geq 4$), ability to handle high count rates, excellent energy resolution even at high count rates, and even elimination of the need for liquid nitrogen to cool the detector prior to use due to the use of Peltier cooling systems. While Si(Li) detectors were generally recommended to be run at 1,000–2,500 counts per second with an energy resolution of 129 eV at Mn-Kα, current SDD technology has superior energy resolution of 121 eV even at the high count rate of 600,000 cps. For the average user, this means that the SDD detector may drastically shorten analysis time provided that a high count rate may be obtained and with significantly better resolution for detection of light elements. In practicality, the achievable count rate is determined by the accelerating voltage and probe current, solid angle of the detector and working distance to the sample, and ability of the sample to survive the electron beam without charging or material change. Whether the user has an Si(Li) or SDD detector, it is a good rule of thumb to keep the dead time to 30% or below.

The elemental content of a specimen has a bearing on the selection of conditions for analysis. The most useful range of accelerating voltage is approximately 3–20 kV; most elements of interest can be ionized by electrons with energies in this range. The energy required in order to excite X-ray emission from a given line is termed its critical excitation potential. The critical excitation potential for a K line can be approximated by the sum of the primary line energies (Kα + Lα + Mα). Selection of an accelerating voltage equal to 1.5 times this sum is usually sufficient for semi-quantitative analysis. For example, copper has Kα at 8.05 keV + Lα at 0.93 keV = 8.98 keV: and 1.5 × 8.98 keV = 13.47 keV. Selection of 15-kV accelerating voltage yields sufficient energy to ionize the K-shell of copper atoms and generate a useful analytical signal. It is generally suggested that EDS practitioners should use the minimum accelerating voltage necessary to adequately excite the elements of interest (typically 1.5–10 times the critical excitation energy). The interaction volume increases with increasing accelerating voltage, thus better spatial resolution can be achieved by using lower accelerating voltage. Depending on the dimensions and density of the particles, a signal from the substrate may be detected. Additionally, larger interaction volume causes increased absorbance and fluorescence within the sample volume. Several accelerating voltages may be experimented with to determine the minimum accelerating voltage required. Routine use of a copper and a lighter metal standard such as aluminum allows quick verification of elemental primary line energies bracketing the 0- to 10-keV range. An elemental collection for the metal standard prior to or within the experimental protocol is conducted to verify the primary line is ±0.04 keV of reference (but may depend upon the detector manufacturer specification). This is a practical internal standard practice.

Inter-element interferences originate from many effects. High-energy X-rays emitted from heavy atoms can ionize lighter elements to produce secondary X-ray emission from the lighter species. Lower high-Z element fluorescence and higher low-Z element fluorescence can be observed, in contrast to that expected from the PE-induced signal of a pure element. Conversely, X-ray emission from a light element may be absorbed by a heavier matrix to yield a negative bias in the light-element signal. These effects always exist in heterogeneous specimens and must be corrected for during any quantitative analysis. A common algorithm, ZAF, may be used to correct for Z-dependent interferences due to absorption and secondary X-ray emission.

Point analysis and mapping are common analysis modes available for the identification of particles by EDS regardless of detector type. Most modern user interfaces allow the user to view a region of interest with secondary electron imaging and collect a spectrum for the entire region or a defined sub-region. Upon collecting an initial spectrum, it is important the user check that it appears satisfactory in terms of energy range for the elements present, optimum count rate, and is free from artifacts such as charging. Once optimized for collection, the user can choose their preferred analysis mode to identify their sample. Point analysis is generally useful if different particle morphologies can be readily observed by SEM. The user can place a cross-hair at the center of each particle to collect a spectrum for later comparison. Care should be taken for the analysis of particles less than 5 µm since the interaction volume may spread in the sample combined with the beam/sample interaction and can excite electrons from the surrounding environment. This will produce spectral peaks that are not native to the feature of interest. For this reason, chemical analysis of small objects in an SEM is difficult. Other more surface-sensitive techniques such as X-ray photoelectron spectroscopy (XPS), scanning auger spectroscopy, and others provide more accurate spectral information. Orthogonal analysis, both within and outside any given technique, is of great value.

When there is no obvious morphological difference for a collection of particles, EDS mapping may be the best mode of analysis. In this mode, the EDS detector collects information as the electron beam rasters across the sample, thus the elemental information is in registry with the SE/BSE image. The user can define the region of interest from the entire region observable in the SE/BSE image to a sub-region. Additionally, the user can define the scan speed, dwell time/pixel, and resolution of the map to suit their needs in terms of speed or resolution. A significant factor to consider for EDS mapping is that the X-ray signal will vary based on the surface texture and slope of the particle's surface. In the most severe case, a shadow may be observed if that surface is no longer within the line of sight of the detector. It may be possible to minimize these artifacts due to tilting the sample further towards the detector. It is often useful to map the elements of interest versus an element that does not exist in the sample. The X-ray counts for the non-existent element should provide a uniform background across the entire region if the surface is level.

Examples of elemental maps overlaid on the SE image are shown in *Figure 7*. The first image (*Figure 7*, left) shows maps for barium and potassium overlaid on the corresponding SE image, thus showing that the particles represent an inhomogeneous mixture of barium/potassium precipitates (left). For the second example, EDS mapping was used with Cryo-SEM to examine a cryo-fractured silicone/water emulsion where the Si map represents silicone and the O map represents water. The internal structure of a large silicone droplet within a continuous water phase can be observed in *Figure 7* (right) where it is apparent the silicone droplet contains several internal water droplets. Many current EDS interfaces allow the user to extract a spectrum from any location within the map.

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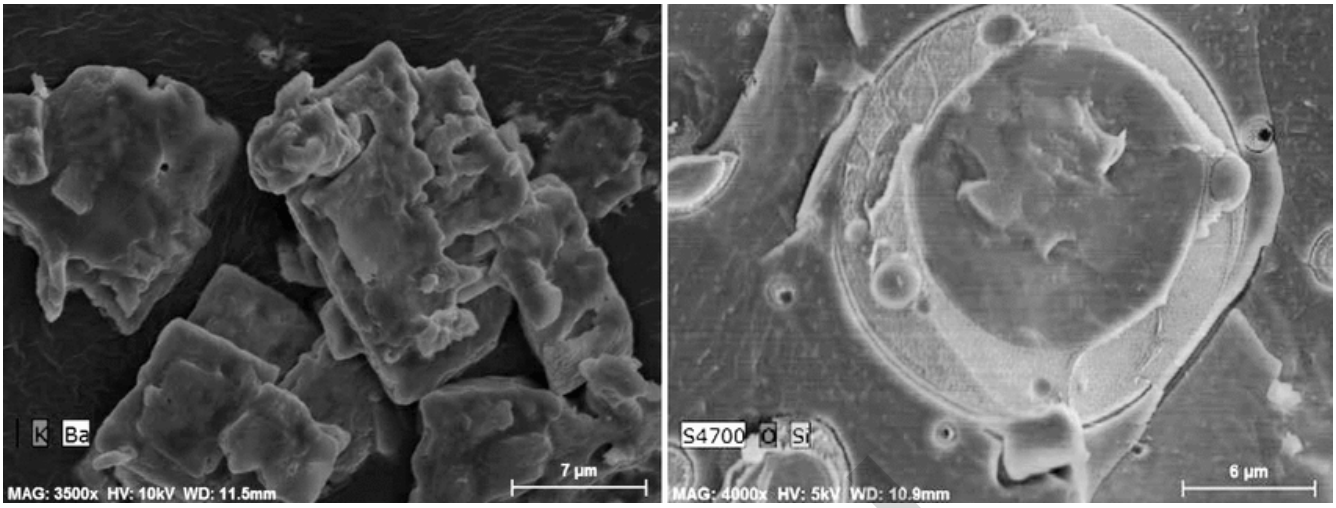


Figure 7. Left: Potassium and barium maps overlaid on the SE image of a mixed potassium/barium precipitate. Right: Oxygen and silicon maps overlaid on SE image of a vitrified silicone/water emulsion.

## IMAGE OPTIMIZATION

Instrumental parameters needed to acquire a quality image can be highly dependent on sample type and sample preparation. Some important aspects with regard to the sample include the form (powders, monoliths, fibers, vitrified liquids, etc.), overall dimensions, texture, atomic composition, and inherent conductivity. Since many instrumental parameters for a modern SEM can be readily modified with the PC-based user interface with only slight re-optimization, it is highly suggested that the operator experiment with operating conditions in order to choose the best imaging conditions for the features of interest. Parameters that affect image quality can be further categorized as instrumental settings and observation settings.

Common instrumental settings that may affect image quality include accelerating voltage, emission current, working distance, SE/BSE detectors (if multiple modes are used), condenser lens current setting, and objective lens aperture diameter. Of these, the accelerating voltage may be the most important instrumental parameter since it directly affects the penetration depth of incident electrons thereby determining the features that can be observed. In general, choosing the optimal accelerating voltage is a balance of achieving the best contrast for the features that the user is most interested in, while minimizing unwanted effects such as loss of topographic information or charging. The best approach to choosing an ideal accelerating voltage for an unknown sample is to experiment with up to 3 accelerating voltages (e.g., 1, 3, and 10 kV), then choose the best one to continue to further optimize the image. The working distance and choice of SE/BSE detector are normally chosen in combination depending on the desired resolution, depth of focus, and sample tilt. Generally, shorter working distances result in higher resolution but less depth of focus. Likewise, based on the position the SE detectors are placed in the column, a tradeoff exists for spatial resolution versus surface topography, where upper detectors generally detect a high resolution SE signal and lower detectors show more surface topography. Lastly, the condenser lens setting and objective lens aperture diameter can be optimized to provide the ideal specimen current, resolution, and depth of focus required for a specific sample type.

Upon choosing an initial set of instrumental conditions as mentioned above, basic image observation can be optimized in the following manner:

1. Select a low magnification (1000–3000×) initially
2. Course adjust image brightness
3. Course adjust focus
4. Select an easy-to-visualize area of the specimen
5. Check/adjust to optimize the electromagnetic alignment of the electron optics (beam, aperture, stigmation)
6. Select a feature of interest at higher magnification
7. Adjust focus and astigmatism
8. Adjust image brightness and contrast
9. Collect image.

Image collection can be accomplished using different resolutions (1280 × 960 is most common) and capture speeds using either slow scan collection or frame integration. In general, slow scan collection is used to collect high-resolution images whereas frame integration is used for specimen types that have charging or stability issues. A collection using a higher capture resolution, slower scan speed, or additional frames may improve the image resolution, however the user must take caution of the increased likelihood of specimen drift over this period which would negate any benefit.

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# FORENSIC ANALYSIS OF PARTICLES

## Shape Analysis

Because of the three-dimensional nature of images obtained via scanning electron microscopy, particle shape provides a great deal of information about the composition and source of the particle without obtaining a chemical analysis via EDS.

The most commonly identified particles found in pharmaceutical compounds generally fall into several distinct categories. Note that particles in parenteral products arise from three general sources: (a) extrinsic or outside of the product, as truly foreign solids; (b) intrinsic or associated with the product, its package or manufacture; (c) inherent particles expected to compose the formulation, such as proteins in biotherapeutic products (see *Subvisible Particulate Matter in Therapeutic Protein Injections* ⟨787⟩), and may be encountered when probing those formulae. Some examples of extrinsic particles are the following:

- Corrosion products from one or more equipment sources, and may occur from formulation interaction
- Materials from the manufacturing environment such as cellulose (from paper bags), extraneous fibers (clothing, filters, etc.)
- Inhomogeneities in the product, specifically incomplete mixing (for solid oral dosage products) that can create cosmetic or elegance issues with tablets
- Environmentally-sourced biological materials
- Material that has been exposed to extreme heat that might char. The causes for this can vary from paper pyrolized during depyrogenation to material sticking to components that are exposed to friction (powder milling, compaction, etc.). If residue unintentionally builds up in these areas, the exposure to friction can decompose the material, making it appear burnt.

*Figure 8* shows representative images of some of the particulate matter mentioned above that the user might encounter in a forensic investigation.

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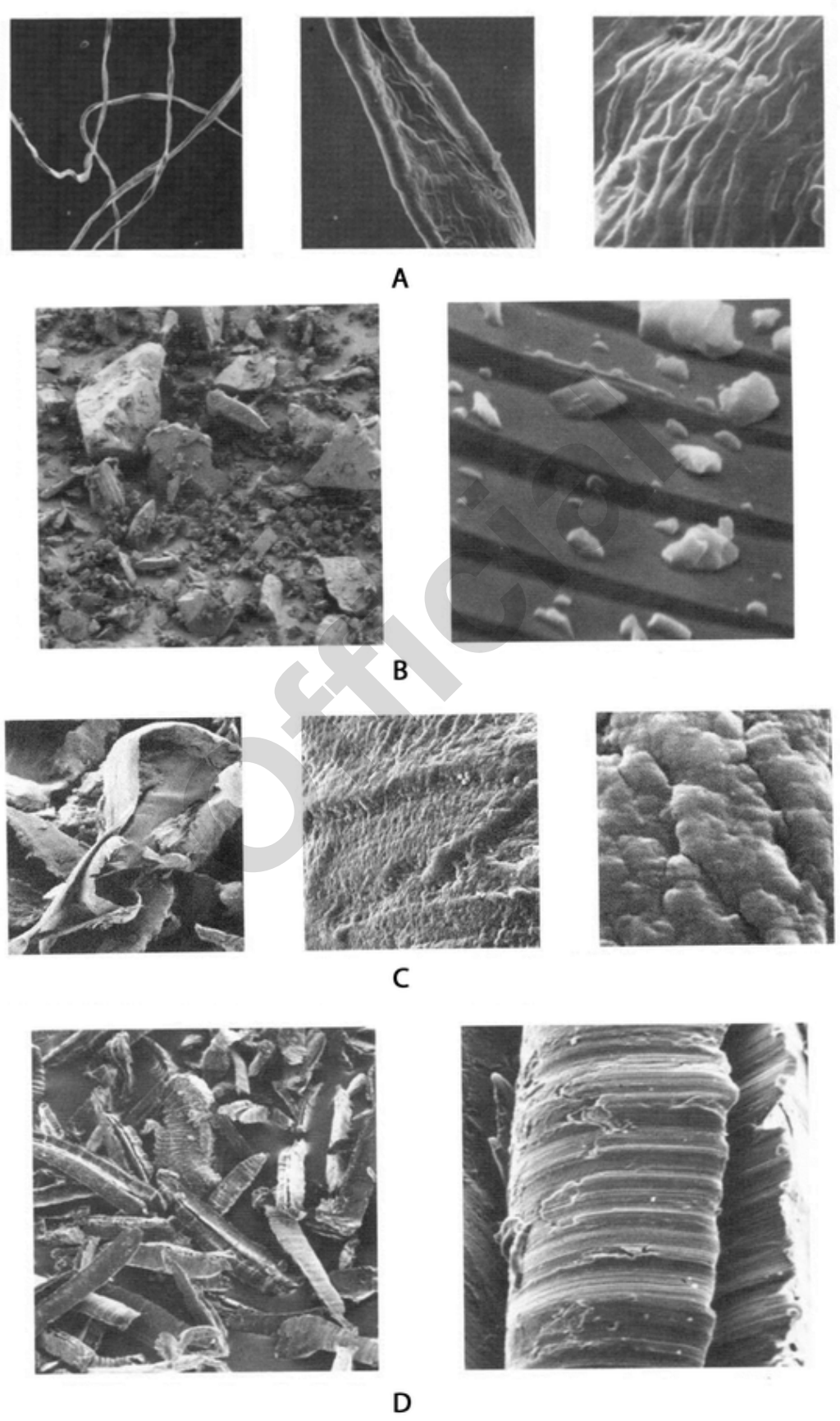


Figure 8. Scanning electron micrographs of commonly found particles in pharmaceutical manufacturing areas: A. cotton

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fibers, B. glass particles, C. Teflon shavings, D. aluminum shavings. (All of the images provided courtesy of McCrone Atlas (www.mccroneatlas.com.)

Interpretation of a particle's origin from characteristics observed by SEM analysis depends on the particle features; for instance, any particle with sharp corners and well-defined edges is made from a material that is not soluble in the product liquid. If it is transparent to the beam, then the user could conclude it consists of very low Z elements. Crystalline particles exhibit obvious symmetry and growth patterns. Slow growth is characterized by large crystals and fast growth by small, often aggregated crystal masses.

Particle morphology and surface characteristics may be helpful in sourcing unknowns. Aspect ratio, or the ratio of the length of a particle divided by its width, indicates fibrous materials. Analysis by optical microscopy often serves as a more effective tool to analyze fibers by determining diagnostic optical properties (refractive indices, birefringence, elongation, etc.) using plane and crossed polarized light that can distinguish among various possible sources. With a scanning electron microscope, however, elemental analysis can determine (a) if the fiber is inorganic or organic and (b) the presence of surface additives. Scanning electron microscopy also offers the added benefit of being able to image at much higher magnification to reveal morphological structures of biological origin or features indicative of commercial manufacture.

As mentioned previously, different kinds of electrons are emitted when an electron beam interacts with a solid surface. The two most useful types of electrons are secondary and back-scattered. By choosing the appropriate detector or instrument set up, the image can be biased towards one or the other. Back-scattered electrons can provide useful information regarding the chemical composition of the feature in question. If the instrument is set up to use BSE to form the image, elements possessing higher atomic mass will appear brighter than those having a lower atomic mass. By selecting this imaging mode, organic particles are easily distinguished from metallic debris, for example. For instance, at low kV, glass or plastic may appear as solid objects, whereas at higher kV they may appear somewhat transparent. Further, appearance at low kV on a tungsten filament SEM may mean something completely different than that at low kV on an FE SEM or environmental SEM. Different materials require different operating conditions to suitably image using SE, to prevent charging and to improve contrast. Typically, use low kV (3 kV or less using a semi-in-lens or in-lens FE SEM) for non-conductive materials such as glass and plastic. If the material is extremely thin (<100 nm), the material can be visualized in transmission mode (STEM) as well as SE mode.

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# ⟨1184⟩ SENSITIZATION TESTING

## INTRODUCTION

This chapter considers sensitization and hypersensitization in the context of medical devices and implants, and describes methodologies for testing such articles for their potential to cause sensitization.

There are four types of hypersensitization reactions according to the Gell and Coombs classification system. Type I reactions involve the fixation of IgE to mast cells that subsequently release pharmacologically active substances, such as histamine. Type II reactions are the result of IgG and/or IgM binding to target cells, followed by complement fixation and cell lysis. Type III reactions are caused by the presence of antigen-antibody complexes that cause physical damage such as kidney damage due to glomerular blockage. Type IV reactions are cell-mediated (involve the action of T cells and their interaction with the human lymphocyte antigens). Type IV reactions are also called delayed-type hypersensitivity reactions. *Table 1* below summarizes the types of reactions, the mediators of the reactions, and examples of representative diseases.

**Table 1. The Four Types of Hypersensitization Reactions[*], Mediators, and Disease Examples**

| Reaction Class | Mediators | Disease Examples |
|---|---|---|
| Type I | IgE molecules bound to mast cells interact with antigen to release pharmacologically active substances | Hay fever, bronchial asthma, other atopic reactions |
| Type II | IgM and/or IgM molecules interact with target cells, fix complement, cell lysis | Various drug allergies, erythroblastosis fetalis, hemolytic anemia, thrombocytopenia |
| Type III | Antigen-antibody complexes, complement | Arthus reaction, serum sickness, allergic glomerulonephritis |
| Type IV | T lymphocytes, antigen, monocytes, macrophages | Contact dermatitis |

[*] According to Gell and Coombs classification scheme

A multi-step process, delineated in chapter *The Biocompatibility of Materials Used in Drug Containers, Medical Devices, and Implants* ⟨1031⟩ is followed in determining which, if any, toxicological tests need to be performed on a given article. In some cases, sufficient evidence to satisfy toxicology requirements may be available from previously marketed articles (See *Figure 1* in chapter ⟨1031⟩). Important factors addressed in *Figure 1* (chapter ⟨1031⟩) include the type and extent of contact with the body, the chemical composition, the manufacturing process, the sterilization process, and, as mentioned above, similarity to previously marketed articles.

If further toxicological testing is necessary, the classification of medical devices provided in *Table 2* from general information chapter ⟨1031⟩ is important, because the degree and extent of toxicological testing that is required is strongly influenced by the nature and duration of the bodily contact with the article. The classification derived from *Table 2* in chapter ⟨1031⟩, coupled with the length of exposure to the article, is used in *Tables 3–5* of chapter ⟨1031⟩ to determine which toxicological tests need to be performed. *Table 2* below presents information extracted from *Tables 3–5* of chapter ⟨1031⟩ and indicates those circumstances for which sensitization testing should be considered.

**Table 2. Articles For Which Sensitization Testing Should Be Considered Based on Article Category and Length of Exposure**

| Device Category | Body Contact | Contact Duration |
|---|---|---|
| Surface devices | Skin | A[a], B[b], C[c] |
| | Mucosal membrane | A, B, C |
| | Breached or compromised surfaces | A, B, C |
| External communicating devices | Blood path, indirect | A, B, C |
| | Tissue, bone, or dentin communicating | A, B, C |
| | Circulating blood | A, B, C |
| Implant devices | Tissue or bone | A, B, C |
| | Blood | A, B, C |

[a] A: limited (less than 24 hours)
[b] B: prolonged (24 hours to 30 days)
[c] C: permanent (more than 30 days)

There are nine test methodologies reviewed in this chapter. *Table 3* lists the methods and the species with which they are performed.

**Table 3. Test Methodologies That May Be Used in Sensitization Testing, and Species Required for Test**

| Test | Species Used in Test |
|---|---|
| Magnusson & Kligman Maximization | Guinea pig |

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**Table 3. Test Methodologies That May Be Used in Sensitization Testing, and Species Required for Test** *(continued)*

| Test | Species Used in Test |
|---|---|
| Standard Buehler | Guinea pig |
| Open Epicutaneous | Guinea pig |
| Freund's Complete Adjuvant | Guinea pig |
| Optimization | Guinea pig |
| Split Adjuvant | Guinea pig |
| Local Lymph Node Assay | Mouse |
| Mouse Ear Swelling | Mouse |
| Vitamin A Enhancement | Mouse |

Given the preponderance of testing performed with either the *Magnusson & Kligman Guinea Pig Maximization Test* (GPMT) or *Buehler Tests* (BT), those tests will be reviewed in detail in this chapter. A brief summary of the remaining tests is provided as alternatives to the more frequently used procedures.

Each test should be periodically validated in the performing laboratory using positive controls such as hexyl cinnamic aldehyde, mercaptobenzothiazole, or benzocaine (positive controls recommended by the Organization for Economic Cooperation and Development [OECD]).

## MAGNUSSON & KLIGMAN GUINEA PIG MAXIMIZATION TEST (GPMT)

### Animals

Either male and female albino guinea pigs or both may be used. All animals should be in good health and weigh between 300 g and 500 g at the start of the experiment. The females should not be pregnant, nor should they have borne young previously. Prior to use, it is essential to acclimatize the animals to the laboratory conditions for at least 5 days. All animals should be handled in accordance with the guidelines in the appropriate regulatory requirements established for the humane treatment of animals. At least 10 test animals and 5 control animals should be used. To obtain sufficient analytical power (i.e., to detect weak sensitizers) it may be necessary to use 20 test animals and 10 control animals. Additional animals may be required to establish the proper doses to administer (see *Determination of Test Article Concentration*).

### Housing and Feeding

The animal room should be held at 20 ± 3°, at 30% to 70% relative humidity, with 12 hours of light and dark. Animals may be housed individually or in group housing. Standard laboratory diets may be used (those satisfactory for guinea pigs ensure an adequate amount of ascorbic acid). Drinking water should be available ad libitum.

### Animal Pretest Preparation

Animals should be randomized via a validated randomization method. For example, such methods may utilize random number tables or computer-generated random numbers. Sites on the animals intended for test article application (intrascapular region) should have the hair removed in a manner that does not abrade the skin. This may be accomplished via clipping, shaving, or with chemical depilatories. The chemical depilatory must not elicit irritation of its own. General observations of the animals prior to use in the test should be recorded, including any indication of ill health (do not use such animals in tests), and body weights.

### Test Article Preparation[1]

The use of this test requires that the test article can be injected intradermally. When the test article is not suitable for direct administration, extracts should be prepared according to the procedure provided in general chapter *Biological Reactivity Tests, In Vivo* ⟨88⟩.

### Determination of Test Article Concentration

The purpose of this preliminary study is to determine the concentrations of *Test Article Preparation* to be used during the initial induction phase and the second challenge phase of a GPMT study. Two or three animals may be used for the concentration determination.

A range of concentrations of the test article, or extracts of the article, should be injected intradermally (0.1 mL per site), using the solvent that will be employed in the *Test Procedure.* The concentration that causes only mild to moderate irritation (no

[1] For further information on sample preparation, see ANSI/AAMI/ISO/CEN Standard 10993–12—1996: Biological Evaluation of Medical Devices—Part 12: Sample Preparation and Reference Materials.

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extensive skin destruction, with no evidence of overt systemic toxicity to the animals) should be used in the *Intradermal Injection Induction Phase* of the *Test Procedure*.

Using two or more animals, apply via occlusive dressings and patches, a range of concentrations of test article or extracts of the article. Remove the dressings/patches after 24 hours, and examine the sites for erythema. Choose the concentration that causes only slight erythema for the *Topical Application Induction Phase* of the *Test Procedure*. Use the highest concentration of test article or extract that does not cause erythema for the *Challenge Phase* of the *Test Procedure*. If the irritation threshold is not reached, then select the highest possible concentration for the *Topical Application Induction Phase* and *Challenge Phase* of the *Test Procedure*.

## Test Procedure

### INTRADERMAL INJECTION INDUCTION PHASE

This phase requires three pairs of injections administered intradermally, with the test and control injection of each pair on opposite sides intrascapularly. Each injection should contain 0.1 mL, with injection pairs 1 and 2 administered nearer to the head, and injection pair 3 administered slightly farther towards the tail. The pairs are nominally within an area of 8 $cm^2$. The pairs of injections consist of the following:

| | |
|---|---|
| Injection pair 1: | A 1:1 (v/v) mixture of Freund's Complete Adjuvant (FCA), an oil–water emulsion containing mycobacteria, and the appropriate solvent/vehicle (see *Biological Reactivity Tests, In Vivo* ⟨88⟩). Control animals receive a mixture of FCA and physiological saline (1:1). |
| Injection pair 2: | The *Test Article Preparation* in the concentration as specified in *Determination of Test Article Concentration,* using the appropriate solvent/vehicle. Control animals receive only the solvent/vehicle. |
| Injection pair 3: | The *Test Article Preparation* in the concentration as specified in *Determination of Test Article Concentration* in a 1:1 (v/v) mixture with FCA. Control animals receive an injection of a 1:1 (v/v) mixture of FCA and solvent/vehicle. |

### TOPICAL APPLICATION INDUCTION PHASE

Seven days (±1 day) after completion of the *Intradermal Injection Induction Phase,* administer the test sample by topical application to the intrascapular region of each animal. For both test and control animals, if the *Test Article Preparation* does not cause skin irritation, apply 10% sodium lauryl sulfate in petrolatum approximately 24 hours before the start of the *Topical Application Induction Phase* to induce a local irritation.

Test animals should have 2- × 4-cm pieces of filter paper or absorbent gauze fully loaded with the *Test Article Preparation* (prepared within 24 hours of use) using the concentration selected in *Determination of Test Article Concentration* applied to each injection site. The filter paper or absorbent gauze should be secured to the animals using occlusive dressings. Control animals receive the same treatment, except that the appropriate solvent/vehicle is used instead of the test article.

Remove the dressings and patches approximately 48 hours after application.

### CHALLENGE PHASE

This phase should occur 14 ± 1 days after the *Topical Application Induction Phase.* Hair should be removed from the test application sites. Filter paper patches or chambers are soaked with a freshly prepared *Test Article Preparation* in the concentration specified in *Determination of Test Article Concentration.* This is done for all test and control animals. The patches or chambers are secured with an occlusive dressing and removed after 24 ± 2 hours.

## Observations

At approximately 24, 48, and 72 hours after removal of the challenge patches, the application sites should be examined for signs of reactions. Of particular importance are instances where the reaction of the test animals exceeds that of the control animals. All signs of reactivity should be recorded, with particular attention paid to signs of erythema and edema. A true edematous reaction will blanch under gentle pressure. The longer the period of blanching, the greater the severity of edema.

## Interpretation

There is more than one way of evaluating and grading the results from GPMT. *Tables 4*, *5*, and *6* list details for three such grading systems. Grades of 1 or higher in the test animals, with grades of less than 1 in control animals, are indicative of sensitization. If control animals display grade 1 reactivity, and if the test animals display reactivity above the greatest reactivity seen in the control animals, sensitization due to the test article is again suspected. The percentages in *Table 4* need to be revised if there are only 10 test animals (i.e., the categories would be 0, <10%, 10%–30%, 31%–60%, 61%–80%, and 81%–100%.) If there are 20 test animals, then multiples of 5% are appropriate.

**Table 4. Classification Based on Percent of Responsive Test Animals**

| % of Positives in Test Group | Assigned Grade Class | |
|---|---|---|
| 0 | — | Nonsensitizer |
| <8 | 1 | Weak |

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**Table 4. Classification Based on Percent of Responsive Test Animals** *(continued)*

| % of Positives in Test Group | Assigned Grade Class | |
|---|---|---|
| 8–28 | 2 | Mild |
| 29–64 | 3 | Moderate |
| 65–80 | 4 | Strong |
| 81–100 | 5 | Extreme |

**Table 5. Classification Based on Erythema and Edema Formation**

| Erythema and Eschar | Grade |
|---|---|
| No erythema | 0 |
| Slight or equivocal erythema | <1 |
| Well-defined erythema | 2 |
| Moderate erythema | 3 |
| Severe erythema to slight eschar formation | 4 |
| **Edema** | |
| No edema | 0 |
| Slight or equivocal edema | <1 |
| Well-defined edema | 2 |
| Moderate edema | 3 |
| Severe edema | 4 |

**Table 6. Classification Based on Erythema Formation Alone**

| Erythema formation | Grade |
|---|---|
| No erythema | 0 |
| Discrete or patchy erythema | 1 |
| Moderate and confluent erythema | 2 |
| Intense erythema and swelling | 3 |

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The results should be submitted for statistical analysis (e.g., chi-square contingency table) to determine if the differences in scores between treated and control animals are significant. The response of the test group versus the control group should be compared statistically. (The Mann-Whitney U test can be used for the comparison.)

## Rechallenge

The extent of any response in the negative control group, under experimental conditions, shows the irritation potential of the *Test Article Preparation*. In this case, test and control animals should be rechallenged 1 week later on the untreated side of the animal, with a reduced concentration of the *Test Article Preparation*. A sensitized guinea pig will react to some degree to both challenges. A weak reaction occurring at a single time point in only one challenge should cast strong doubt as to whether that guinea pig is truly sensitized.[2]

# STANDARD BUEHLER TESTS (SBT)

## Animals

See *Animals* in the *Magnusson & Kligman Guinea Pig Maximization Test (GPMT)*.

## Housing and Feeding

See *Housing and Feeding* in the *Magnusson & Kligman Guinea Pig Maximization Test (GPMT).*

[2] Basketter D.A. Guinea pig predictive tests for contact hypersensitivity. In Immunotoxicology and Immunopharmacology, 2nd ed.; Dean, J.H, Luster, M.I., Munson, A.E., Kimber, I., Eds; Raven Press, Ltd: New York, 1994; pp 693–702.

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## Animal Pretest Preparation

See *Animal Pretest Preparation* under *Magnusson & Kligman Guinea Pig Maximization Test (GPMT).* The fur of the guinea pig may be removed from one flank by clipping.

## Test Article Preparation

See *Test Article Preparation* in the *Magnusson & Kligman Guinea Pig Maximization Test (GPMT).*

## Determination of Test Article Concentration

The purpose of this preliminary study is to determine the concentrations of *Test Article Preparation* to be used during the initial induction phase and the second challenge phase of an SBT study. Two or three animals may be used for the concentration determination.

A range of concentrations of the test article, or extracts of the article, should be applied using patches (for example, four 4 $cm^2$ absorbent pads) or chambers. The patches should be held in place using tape (if necessary) and occlusive dressings. The patches should be removed after approximately 6 hours, and any residues of the test chemical are removed from the test site. Observations are made at that time, and at 24 and 48 hours.

The concentration that causes only mild to moderate irritation (slight erythema, with no evidence of overt toxicity to the animals) and can be applied repeatedly to the same site should be used in the *Induction Phase* of the *Test Procedure*. Use the highest concentration of test article or extract that does not cause erythema for the *Challenge Phase* of the *Test Procedure*.

## Test Procedure

### INDUCTION PHASE

Apply 0.4 mL of the *Test Article Preparation* in an appropriate solvent/vehicle at the dose identified in *Determination of Test Article Concentration.* Use patches similar to those used in *Determination of Test Article Concentration.* The patches should be applied to one flank (hair clipped off) and held in place occlusively for 6 hours. The animals may need to be restrained to ensure occlusion. Patches and any visible residues should be removed after 6 hours. Control animals also receive patches, but these contain only the appropriate solvent/vehicle. This process should be repeated three times a week for both test and control animals on the same site for three consecutive weeks (weekly intervals are used in the modified Buehler Test).

### CHALLENGE PHASE

This phase should be carried out 14 days after the last application of the *Induction Phase*. Clip the hair off the previously untested flank of each animal 24 hours before the challenge application. As in the *Induction Phase,* apply patches containing the test article (concentration specified in *Determination of Test Article Concentration*) or solvent/vehicle alone to the untested areas of the test and control animals. To obtain well-defined edges at the application sites, commercial chambers with a lipped edge are preferred. Secure the patches with occlusive dressings, and keep them in place for 6 hours. Remove all patches after 6 hours.

### OBSERVATIONS

At 22 ± 2 hours after removal of the patches, the application sites should have the animal's fur removed via clipping or depilation. After approximately 2 more hours, grade the sites (*Tables 4*, *5* or *6* may be employed). All signs of reactivity should be recorded, with particular attention paid to signs of erythema and edema. Repeat the grading once again after 24 to 48 hours more have elapsed. The response of the test group versus the control group can be compared statistically. (The Mann-Whitney U test can be used for the comparison.)

### INTERPRETATION

The results should be submitted for a statistical analysis (e.g., chi-square contingency table) to determine if the differences in scores between treated and control animals are significant.

See *Interpretation* in the *Magnusson & Kligman Guinea Pig Maximization Test (GPMT).*

### RECHALLENGE

See Rechallenge in the *Magnusson & Kligman Guinea Pig Maximization Test (GPMT).*

# OTHER SENSITIZATION TEST PROCEDURES

The *Magnusson & Kligman Guinea Pig Maximization Test* and the *Standard Buehler Tests* are the most frequently performed sensitization tests. However, there are a number of other methods that may be useful in the assessment of the potential for sensitization. Some may be applicable to both solid test articles and extracts, some only to extracts.

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Where the use of guinea pigs is called for in the following tests, the animals and their housing should meet the requirements as specified for *Animals* in the *Magnusson & Kligman Guinea Pig Maximization Test.* The fur of the guinea pig should be removed from test sites as indicated for *Animal Pretest Preparation* in the *Magnusson & Kligman Guinea Pig Maximization Test.*

## Draize Test

This was the first predictive test accepted by the regulatory agencies, and is still in use. The test uses guinea pigs and the test article is administered via intradermal injections.

### TEST ARTICLE PREPARATION

This test requires that the test article be in the form of a solution that may be directly applied to the animal's skin. Therefore, extracts of the material would need to be made. See *Biological Reactivity Tests, In Vivo* ⟨88⟩ for information on the preparation procedure.

### INDUCTION PHASE

One flank of each of 20 guinea pigs is shaved, then 0.05 mL of a 0.1% solution of test article is injected into the anterior flank. The next day, and then every other day thereafter up to day 20, 0.1 mL of the test article is injected into a new site on the same flank.

### CHALLENGE PHASE

This phase begins 2 weeks after the final injection of the *Induction Phase*. The untreated flank is shaved, then 0.05 mL of test article is injected into each of the 20 guinea pigs. Twenty previously untreated animals serve as the controls, and receive injections of the test article as well.

### OBSERVATIONS

The test sites of all control and test animals are evaluated for erythema at 24 and 48 hours after the challenge injections. The degree of reaction in test animals is compared to the reaction in control animals. A larger and/or more intense response by the test animals versus the control animals is indicative of sensitization.

## Open Epicutaneous Test

This test uses guinea pigs. The goal is to determine the dose required to induce sensitization by simulating human usage via topical application of the test article.

### TEST MATERIAL PREPARATION

This test requires that the test article be in the form of a solution that may be directly applied to the animal's skin. Therefore, extracts of the material need to be made. See *Biological Reactivity Tests, In Vivo* ⟨88⟩ for information on the preparation procedure.

### PRELIMINARY TESTING

A series of concentrations of test article is applied to 2 $cm^2$ areas of skin on the anterior flank of 6 to 8 guinea pigs (0.025 mL per application). The test sites should be examined for erythema 24 hours after test article administration. The highest concentration that does not cause irritation (maximum nonirritant concentration) and the lowest concentration causing erythema in approximately 25% of the animals (minimum irritant concentration) are determined.

### INDUCTION PHASE

The test article (or control vehicle) is applied to 8 $cm^2$ areas of the flank skin of 6 to 8 guinea pigs daily for 3 weeks, or five times a week for 4 weeks. The amount per application is 0.01 mL. A set of increasing concentrations is again employed, ranging from the minimum irritant concentration using a stepwise progression. The test article should be applied to the same sites each time, unless irritation develops, in which case a new site on the same flank should be used. Control animals receive the same series of treatments using the vehicle instead of the test article.

### CHALLENGE PHASE

Each animal is challenged on the untreated flank 24 to 72 hours after the last *Induction Phase* treatment using 0.025 mL applied to 2 $cm^2$ areas. A set of increasing concentrations is used, from minimum irritant concentration to the maximum nonirritant concentration, and five lower concentrations are also used.

### OBSERVATIONS

The test sites are evaluated at 24, 48, and 72 hours post-treatment. The maximum concentration that does not cause irritation in the control group is determined. Animals from the test groups that develop inflammatory responses at concentrations lower than the maximum nonirritating concentration in the controls should be considered to be sensitized.

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## Freund's Complete Adjuvant Test

This test is based upon the use of intradermal injections using the test article in a mixture of Freund's complete adjuvant and distilled water (50:50).

### TEST MATERIAL PREPARATION

Because this test uses intradermal injections, extracts of the test material need to be made in order to use this procedure. See *Biological Reactivity Tests, In Vivo* ⟨88⟩ for information on the extraction procedure.

### PRELIMINARY TESTING

The minimum irritating and the maximum nonirritating concentrations are determined in the same manner as for *Preliminary Testing* in the *Open Epicutaneous Test.*

### INDUCTION PHASE

The test area consists of six 2 $cm^2$ areas across the shoulders of the guinea pigs. Two groups of 10 to 20 guinea pigs each should be used. The test group animals are injected intradermally with 0.1 mL of a 5% solution of the test article extract in FCA/water. Control animals receive injections with FCA/water without the test article. These injections are repeated every 4 days until a total of three injections have been given.

### CHALLENGE PHASE

This phase should begin 2 weeks after the last injection of the *Induction Phase*. Topical applications of 0.025 mL of test article at the minimum irritating and the maximum nonirritating concentrations, plus two lower concentrations, are administered to 2 $cm^2$ areas of the shaved flank. The test sites should remain uncovered.

### OBSERVATIONS

The test sites are examined for the presence of erythema 24, 48 and 72 hours after the topical applications. The minimum nonirritating concentration in the control animals should be determined. Those test animals that display erythema at concentrations lower than the minimum nonirritating concentration in the control animals should be considered to be sensitized.

## Optimization Test

This test has some similarities to the older *Draize Test.* Unlike the *Draize Test,* however, this test uses both intradermal and topical treatments, and includes adjuvant for some induction injections.

### TEST MATERIAL PREPARATION

As with other test procedures that incorporate intradermal injections, the test article needs to be in a form suitable for injection. See *Biological Reactivity Tests, In Vivo* ⟨88⟩ for information on the extraction procedure.

### INDUCTION PHASE

Twenty test and 20 control guinea pigs are used. A total of 10 intradermal injections should be given to each animal. Test animals receive 0.1 mL of a mixture of 0.1% test article and 0.9% saline (50:50) on day 1, with one injection into a shaved flank, and another into a portion of shaved dorsal skin. Two and 4 days later, one intradermal injection of the test article in saline is given to eight new dorsal sites. Every other day during weeks 2 and 3, the test article is injected intradermally into 10 sites over the shoulders in a 50:50 mixture of saline and FCA. The same sequence of injections is given to the 20 control animals, except that no test article is included with the saline or saline/FCA injections.

### CHALLENGE PHASE

Thirty-five days after the first injection, the animals are challenged topically with 0.1 mL of the 0.1% solution of test article in saline (for test animals). The control animals receive saline injections only. At 45 days after the first injection, a second topical challenge is given. A nonirritating concentration of test article (0.05 mL) is applied topically to a 1 $cm^2$ area of untreated skin. This site should then be covered with a 2 $cm^2$ piece of filter paper, after which an occlusive dressing should be applied. The patch should be removed after 24 hours.

### OBSERVATIONS

Twenty-four hours after each injection during week 1, the thickness of a fold of skin over the injection sites for each animal should be measured using a caliper (mm), and the two largest cross-diameters of each erythematous reaction should be recorded (mm). The reaction volumes are calculated by multiplying the fold thickness by the products of the two cross-diameters (expressed as µL). The mean reaction (+1 SD) volume during week 1 should be calculated for each animal.

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Challenge reaction volumes are calculated for each animal following the injections at day 35. If an animal develops a challenge reaction volume greater than its mean reaction volume + 1 SD, it should be considered sensitized.

Following the patch testing challenge, the test sites are evaluated for erythema and edema. Evaluations should be made using *Table 5*.

The number of positive animals should be compared statistically with the pseudopositive control animals. This should be done for both intradermal injection results and patch testing results. The Fisher exact test may be used.

The results from the intradermal injections and the patch testing, following separate statistical analysis, may be combined and evaluated using *Table 7* in order to classify a test article as a strong, moderate, or weak sensitizer; or not a sensitizer.

**Table 7. Classification Scheme for Test Articles Based on the Optimization Test**

| Intradermal % of Positive Animals | Patch Test % of Positive Animals | Classification |
|---|---|---|
| S*, > 75 | and/or S, > 50 | Strong sensitizer |
| S, 50–75 | and/or S, 30–50 | Moderate sensitizer |
| S, 30–50 | N.S.*, 0–30 | Weak sensitizer |
| N.S., 0–30 | N.S., 0 | Not a sensitizer |

* S = significant; N.S. = not significant

## Split Adjuvant Test

This test makes use of both FCA and skin damage. The test article is applied topically.

### TEST MATERIAL PREPARATION

Because this test employs topical test article applications, the article can be either in solid or liquid form. If extracts are to be made, see chapter *Biological Reactivity Tests, In Vivo* ⟨88⟩ for extraction procedures.

### INDUCTION PHASE

Ten to 20 guinea pigs are used for both test and control groups. An area of back skin immediately behind the scapulas should be shaved to the extent that the skin becomes glistening. The shaved areas should then be treated with dry ice for 5 to 10 seconds. A dressing made of loose mesh gauze with stretch adhesive and a 2- × 2-cm opening should be placed over the treated area, then secured with adhesive tape. The test article (0.2 mL of viscous materials, 0.1 mL of liquids, or solid material) is placed within the opening in the dressing on top of the treated skin. Two layers of #2 filter paper should be placed over the test article, then backed by occlusive tape. Then the filter paper/occlusive backed material should be secured to the surrounding dressing with adhesive tape. After 2 days have passed, the filter paper should be lifted from the test sites, and the test article reapplied on the same site. The filter paper and backing should be secured once again. After 2 more days, the filter paper should be lifted and two injections of 0.075 mL of FCA should be administered into the edges of the test site. Then the test material is once again applied, and the filter paper/backing resecured. The test article should be reapplied once more on day 7 and the filter paper/backing resealed. On day 9, the filter paper and all associated dressing material should be removed.

### CHALLENGE PHASE

On day 22 following the induction treatment, 0.5 mL of test material (or the solid article) should be applied to a 2-× 2-cm area of shaved midback. The test sites should be covered by filter paper and backed by adhesive tape. This should be held in place with an elastic bandage secured with adhesive tape. Control animals receive the same challenge phase treatment. The preparation should be removed after 24 hours.

### OBSERVATIONS

Twenty-four, 48, and 72 hours after the removal of the challenge phase preparation, the test sites should be evaluated for erythema and edema. The grading scheme of *Table 5* could be employed.

## Mouse Ear Swelling Test

There are a number of potential advantages in using mice versus guinea pigs for sensitization methods. The classic guinea pig tests tend to be costly and require a long time to complete. Moreover, with the dependence upon relatively subjective scoring based on edema and erythema, methodological robustness, and ruggedness may be questionable. This test uses mice and employs both topical exposures and injections.

### ANIMALS

Female, 6- to 8-week old CF-1, Balb/c, or Swiss mice should be used. They may be group housed in direct bedding cages. Acclimatization should be for at least 5 to 7 days. Food (appropriate mouse feed) and water should be available ad libitum. No

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animals with damaged pinnae should be used in the study. The thickness of both ears of each animal should be measured and recorded at this time.

TEST MATERIAL PREPARATION

As with other test procedures that incorporate intradermal injections, the test article needs to be in a form suitable for injection. See *Biological Reactivity Tests, In Vivo* ⟨88⟩ for information on the extraction procedure.

PRELIMINARY TESTING

The minimally irritating and maximally nonirritating concentrations of test article for this procedure should be determined. This is done by using four groups of two mice and examining the effects of at least four concentrations of test article.

INDUCTION PHASE

The abdomens of the animals should be shaved, then tape-stripped using a surgical adhesive tape until the test area is glistening. A single injection of 0.05 mL of FCA is subdivided into two injection sites administered intradermally within the shaved/stripped area, but along the borders. After the adjuvant injections, 100 µL of test article (using the minimally irritating concentration) or vehicle (controls) is applied to the center of the shaved test areas. After the test areas dry, the mice should be returned to their cages. The tape stripping and application of test article (but not FCA) is repeated each day for the next 3 days.

CHALLENGE PHASE

This phase should occur 7 days after the final topical induction application. The test article (highest nonirritating concentration) should be applied topically (20 µL) to one ear, while the opposite ear receives 10 µL of vehicle alone. This should be done for both test and control animals.

OBSERVATIONS

The thickness of both ears of each animal should be recorded after 24 and 48 hours postchallenge. The measurements should be made with a caliper (a spring-loaded caliper is preferable). A sensitized animal is one in which the test article-treated ear is at least 20% thicker than its opposite ear. For the test to be valid, the test article-treated ears of control animals should not be more than 10% thicker than the opposite ears. If the control animal ears do not meet the requirements, the test should be repeated using lower concentrations.

## Local Lymph Node Test

This test is based on the observation that exposure of the mice to sensitizers can cause hyperplasia of T cells within the auricular lymph nodes of mice. The method combines both in vivo and in vitro phases, and requires the use of radioisotopes. An unusual aspect of this test is that no challenge phase is required.

ANIMALS

Four groups of four mice at least, male or female CBA/ca mice (only one sex in a given test) between the ages of 8 to 12 weeks should be used.

TEST MATERIAL PREPARATION

Although in theory one could apply a solid test article to the dorsal surface of the ear of a mouse, in practice an extract of such an article should be used. See *Biological Reactivity Tests, In Vivo* ⟨88⟩ for information on the extraction procedure.

PRELIMINARY TESTING

A nontoxic concentration of test article should be used. If not already established, a preliminary test for overt toxicity may be required to establish a suitable dose.

INDUCTION PHASE

Twenty-five µL of the appropriate test article concentration, or vehicle (controls), should be applied to the dorsal surface of each pinna for 3 consecutive days. Five days after the first treatment, the animals should be injected, via the tail vein, with 2.5 mL of phosphate buffered saline containing 20 µCi of $^{3}$H-methyl thymidine. Five hours after the isotopic injection, the animals should be euthanized. The draining auricular lymph nodes should be removed from each animal of each test and control group. The nodes from all animals within a given group should be combined, such that a single cell suspension can be made from each group of animals. The cell suspension can be made by passing the nodes through a 200-mesh stainless steel gauze using a syringe plunger. The cells should then be centrifuged at 190 × g for 10 minutes, resuspended in 3 mL of 5% trichloroacetic acid (TCA), and held overnight at 4°.

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The resulting precipitate should be recovered by centrifugation, and the pelleted precipitate should be resuspended in 1 mL of 5% TCA. The suspension should then be placed in scintillation vials with 10 mL of scintillation fluid, and the disintegrations/minute (dpm) counted with a β-counter.

### OBSERVATIONS

The ratio of dpm for each test group should be compared to the dpm for the control group. If the ratio equals or exceeds 3 for any test group, the concentration of test article used with that group may be considered to be sensitizing.

## Vitamin A Enhancement Test

This test is similar to the *Mouse Ear Swelling Test* in that test articles are applied topically to the abdomen, with a challenge application to the ears, followed by measurements of ear thickness. A principal difference is the use of mouse feed supplemented with vitamin A acetate. The purpose of the supplementation is to increase the reactivity of the immune system, thereby increasing the potential sensitization reaction.

### ANIMALS

Male, 3- to 4-week old Balb/c mice should be maintained on a diet supplemented with vitamin A acetate. The diet may be prepared by mixing each kg of feed with 0.477 g of gelatinized vitamin A acetate. The feed mixture should be used within 3 weeks of preparation. Mice intended for use in sensitization studies should have been on the supplemented diet for at least 4 weeks. The mice at the time of the sensitization study should therefore be between 7 and 10 weeks old. The thickness of both ears of each animal should be measured and recorded at this time.

### TEST MATERIAL PREPARATION

Although, in theory, one could apply a solid test article to the dorsal surface of the ear of a mouse, in practice an extract of such an article should be used. See *Biological Reactivity Tests, In Vivo* ⟨88⟩ for information on the extraction.

### PRELIMINARY TESTING

The maximally nonirritating dose and minimally irritating concentrations should be determined using separate groups of animals. This could be done as described for *Preliminary Testing* in the *Mouse Ear Swelling Test.*

### INDUCTION PHASE

The fur of the abdomen and thorax of 10 mice per group should be shaved. Then 100 µL of test article (at the minimally irritating concentration) should be applied to the test areas on days 0, 2, 4, 7, and 11. Control animals receive 100 µL of vehicle alone on the same schedule.

### CHALLENGE PHASE

This phase should occur 4 days after the final application of the *Induction Phase*. Twenty-five µL of test article (at the maximally nonirritating concentration) should be applied to each ear of each animal in the test and control groups.

### OBSERVATIONS

Ear thickness for both ears of each animal should be recorded after 24 and 48 hours postchallenge. The measurements should be made with a caliper (a spring-loaded caliper is preferable). The percent increase in ear thickness should be calculated for each ear by subtracting the pretreatment measurement from the post-treatment measurement, dividing the result by the pretreatment measurement, then multiplying by 100. The response of the test group versus the control group should be compared statistically. (The Mann-Whitney U test could be used for the comparison.)

The results of individual animals should also be calculated. If an increase in ear thickness for an animal from the test group is at least 50% greater than the largest increase of a control animal, that is indicative of sensitization. As an overall evaluation, should the results of the study provide a significant result of the statistical test at $p < 0.01$ for the control versus test group comparisons, or if at least two test animals have ear thickness increases in excess of 50% of the maximum control thickness changes and the group comparison showed a $p < 0.05$, sensitization is indicated for the test article.

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# ⟨1191⟩ STABILITY CONSIDERATIONS IN DISPENSING PRACTICE

[NOTE—Inasmuch as this chapter is for purposes of general information only, no statement in the chapter is intended to modify or supplant any of the specific requirements pertinent to Pharmacopeial articles, which are given elsewhere in this Pharmacopeia.]

Aspects of drug product stability that are of primary concern to the pharmacist in the dispensing of medications are discussed herein.

Pharmacists should avoid ingredients and conditions that could result in excessive physical deterioration or chemical decomposition of drug preparations, especially when compounding (see *Pharmaceutical Compounding—Nonsterile Preparations* ⟨795⟩). The stability and clinical effect of manufactured dosage forms can be greatly compromised by seemingly negligible alterations or inappropriate prescription compounding. Pharmacists should establish and maintain compounding conditions that include the ensuring of drug stability to help prevent therapeutic failure and adverse responses.

**Stability—**Stability is defined as the extent to which a product retains, within specified limits, and throughout its period of storage and use (i.e., its shelf-life), the same properties and characteristics that it possessed at the time of its manufacture. Five types of stability generally recognized are shown in the accompanying table.

**Criteria for Acceptable Levels of Stability**

| Type of Stability | Conditions Maintained Throughout the Shelf Life of the Drug Product |
|---|---|
| Chemical | Each active ingredient retains its chemical integrity and labeled potency, within the specified limits. |
| Physical | The original physical properties, including appearance, palatability, uniformity, dissolution, and suspendability, are retained. |
| Microbiological | Sterility or resistance to microbial growth is retained according to the specified requirements. Antimicrobial agents that are present retain effectiveness within the specified limits. |
| Therapeutic | The therapeutic effect remains unchanged. |
| Toxicological | No significant increase in toxicity occurs. |

## FACTORS AFFECTING PRODUCT STABILITY

Each ingredient, whether therapeutically active or pharmaceutically necessary, can affect the stability of drug substances and dosage forms. The primary environmental factors that can reduce stability include exposure to adverse temperatures, light, humidity, oxygen, and carbon dioxide. The major dosage form factors that influence drug stability include particle size (especially in emulsions and suspensions), pH, solvent system composition (i.e., percentage of "free" water and overall polarity), compatibility of anions and cations, solution ionic strength, primary container, specific chemical additives, and molecular binding and diffusion of drugs and excipients. In dosage forms, the following reactions usually cause loss of active drug content, and they usually do not provide obvious visual or olfactory evidence of their occurrence.

### Hydrolysis

Esters and β-lactams are the chemical bonds that are most likely to hydrolyze in the presence of water. For example, the acetyl ester in aspirin is hydrolyzed to acetic acid and salicylic acid in the presence of moisture, but in a dry environment the hydrolysis of aspirin is negligible. The aspirin hydrolysis rate increases in direct proportion to the water vapor pressure in an environment.

The amide bond also hydrolyzes, though generally at a slower rate than comparable esters. For example, procaine (an ester) will hydrolyze upon autoclaving, but procainamide will not. The amide or peptide bond in peptides and proteins varies in the lability to hydrolysis.

The lactam and azomethine (or imine) bonds in benzodiazepines are also labile to hydrolysis. The major chemical accelerators or catalysts of hydrolysis are adverse pH and specific chemicals (e.g., dextrose and copper in the case of ampicillin hydrolysis).

### Epimerization

Members of the tetracycline family are most likely to incur epimerization. This reaction occurs rapidly when the dissolved drug is exposed to a pH of an intermediate range (higher than 3), and it results in the steric rearrangement of the dimethylamino group. The epimer of tetracycline, epitetracycline, has little or no antibacterial activity.

### Decarboxylation

Some dissolved carboxylic acids, such as *p*-aminosalicylic acid, lose carbon dioxide from the carboxyl group when heated. The resulting product has reduced pharmacological potency.

β-Keto decarboxylation can occur in some solid antibiotics that have a carbonyl group on the β-carbon of a carboxylic acid or a carboxylate anion. Such decarboxylations will occur in the following antibiotics: carbenicillin sodium, carbenicillin free acid, ticarcillin sodium, and ticarcillin free acid.

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## Dehydration

Acid-catalyzed dehydration of tetracycline forms epianhydrotetracycline, a product that both lacks antibacterial activity and causes toxicity.

## Oxidation

The molecular structures most likely to oxidize are those with a hydroxyl group directly bonded to an aromatic ring (e.g., phenol derivatives such as catecholamines and morphine), conjugated dienes (e.g., vitamin A and unsaturated free fatty acids), heterocyclic aromatic rings, nitroso and nitrite derivatives, and aldehydes (e.g., flavorings). Products of oxidation usually lack therapeutic activity. Visual identification of oxidation, for example, the change from colorless epinephrine to its amber colored products, may not be visible in some dilutions or to some eyes.

Oxidation is catalyzed by pH values that are higher than optimum, polyvalent heavy metal ions (e.g., copper and iron), and exposure to oxygen and UV illumination. The latter two causes of oxidation justify the use of antioxidant chemicals, nitrogen atmospheres during ampul and vial filling, opaque external packaging, and transparent amber glass or plastic containers.

## Photochemical Decomposition

Exposure to, primarily, UV illumination may cause oxidation (photo-oxidation) and scission (photolysis) of covalent bonds. Nifedipine, nitroprusside, riboflavin, and phenothiazines are very labile to photo-oxidation. In susceptible compounds, photochemical energy creates free radical intermediates, which can perpetuate chain reactions.

## Ionic Strength

The effect of the total concentration of dissolved electrolytes on the rate of hydrolysis reactions results from the influence of ionic strength on interionic attraction. In general, the hydrolysis rate constant is inversely proportional to the ionic strength with oppositely charged ions (e.g., drug cation and excipient anions) and directly proportional to the ionic strength with ions of like charge. A reaction that produces an ion of opposite charge to the original drug ion because of the increasing ionic strength, can increase the drug hydrolysis rate as the reaction proceeds. High ionic strength of inorganic salts can also reduce the solubility of some other drugs.

## pH Effect

The degradation of many drugs in solution accelerates or decelerates exponentially as the pH is decreased or increased over a specific range of pH values. Improper pH ranks with exposure to elevated temperature as a factor most likely to cause a clinically significant loss of drug, resulting from hydrolysis and oxidation reactions. A drug solution or suspension, for example, may be stable for days, weeks, or even years in its original formulation, but when mixed with another liquid that changes the pH, it degrades in minutes or days. It is possible that a pH change of only 1 unit (e.g., from 4 to 3 or 8 to 9) could decrease drug stability by a factor of 10 or greater.

A pH buffer system, which is usually a weak acid or base and its salt, is a common excipient used in liquid preparations to maintain the pH in a range that minimizes the drug degradation rate. The pH of drug solutions may also be either buffered or adjusted to achieve drug solubility. For example, pH in relation to pKa controls the fractions of the usually more soluble ionized and less soluble nonionized species of weak organic electrolytes.

The influence of pH on the physical stability of two phase systems, especially emulsions, is also important. For example, intravenous fat emulsion is destabilized by acidic pH.

## Interionic ($Ion^{N+}$–$Ion^{N-}$) Compatibility

The compatibility or solubility of oppositely charged ions depends mainly on the number of charges per ion and the molecular size of the ions. In general, polyvalent ions of opposite charge are more likely to be incompatible. Thus, an incompatibility is likely to occur upon the addition of a large ion with a charge opposite to that of the drug.

## Solid State Stability

Solid state reactions are relatively slow; thus, stability of drugs in the solid state is rarely a dispensing concern. The degradation rate of dry solids is usually characterized by first-order kinetics or a sigmoid curve. Therefore, solid drugs with lower melting point temperatures should not be combined with other chemicals that would form a eutectic mixture.

When moisture is present, the solid drug decomposition may change to zero-order chemical kinetics because the rate is controlled by the relatively small fraction of the drug that exists in a saturated solution, which is located (usually imperceptibly) at the surface or in the bulk of the solid drug product.

## Temperature

In general, the rate of a chemical reaction increases exponentially for each 10° increase in temperature. This relationship has been observed for nearly all drug hydrolysis and some drug oxidation reactions. The actual factor of rate increase depends on the activation energy of the particular reaction. The activation energy is a function of the specific reactive bond and the drug

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formulation (e.g., solvent, pH, additives). As an example, consider a hydrolyzable drug that is exposed to a 20° increase in temperature, such as that from cold to controlled room temperature. The shelf life of the drug at controlled room temperature should be expected to decrease to one-fourth to one-twenty-fifth of its shelf life under refrigeration.

The pharmacist should also be aware that inappropriately cold temperatures may cause harm. For example, refrigeration may cause extreme viscosity in some liquid drugs and cause supersaturation in others. Freezing may either break or cause a large increase in the droplet size of emulsions; it can denature proteins; and in rare cases, it can cause less soluble polymorphic states of some drugs to form.

## STABILITY STUDIES IN MANUFACTURING

The scope and design of a stability study vary according to the product and the manufacturer concerned. Ordinarily the formulator of a product first determines the effects of temperature, light, air, pH, moisture, trace metals, and commonly used excipients or solvents on the active ingredient(s). From this information, one or more formulations of each dosage form are prepared, packaged in suitable containers, and stored under a variety of environmental conditions, both exaggerated and normal. At appropriate time intervals, samples of the product are assayed for potency by use of a stability-indicating method, observed for physical changes, and, where applicable, tested for sterility and or for resistance to microbial growth and for toxicity and bioavailability. Such a study, in combination with clinical and toxicological results, enables the manufacturer to select the optimum formulation and container and to assign recommended storage conditions and an expiration date for each dosage form in its package.

### Responsibility of Pharmacists

Pharmacists help to ensure that the products under their supervision meet acceptable criteria of stability by (1) dispensing oldest stock first and observing expiration dates, (2) storing products under the environmental conditions stated in the individual monographs, labeling, or both, (3) observing products for evidence of instability, (4) properly treating and labeling products that are repackaged, diluted, or mixed with other products, (5) dispensing in the proper container with the proper closure, and (6) informing and educating patients concerning the proper storage and use of the products, including the disposition of outdated or excessively aged prescriptions.

#### ROTATION OF STOCK AND OBSERVANCE OF EXPIRATION DATES

Proper rotation of stock is necessary to ensure the dispensing of suitable products. A product that is dispensed infrequently should be closely monitored so that old stocks are given special attention, particularly with regard to expiration dates. The manufacturer can guarantee the quality of a product up to the time designated as its expiration date only if the product has been stored in the original container under recommended storage conditions.

#### STORAGE UNDER RECOMMENDED ENVIRONMENTAL CONDITIONS

In most instances, the recommended storage conditions are stated on the label, in which case it is imperative to adhere to those conditions. They may include a specified temperature range or a designated storage place or condition (e.g., "refrigerator," or "controlled room temperature") as defined in the *Packaging and Storage Requirements* ⟨659⟩. Supplemental instructions, such as a direction to protect the product from light, also should be followed carefully. Where a product is required to be protected from light and is in a clear or translucent container enclosed in an opaque outer covering, such outer covering is not to be removed and discarded until the contents have been used. In the absence of specific instructions, the product should be stored at controlled room temperature (see *Packaging and Storage Requirements* ⟨659⟩). The product should be stored away from locations where excessive or variable heat, cold, or light prevails, such as those near heating pipes or fluorescent lighting.

#### OBSERVING PRODUCTS FOR EVIDENCE OF INSTABILITY

Loss of potency usually results from a chemical change, the most common reactions being hydrolysis, oxidation-reduction, and photolysis. Chemical changes may also occur through interaction between ingredients within a product, or rarely between product and container. An apparent loss of potency in the active ingredient(s) may result from diffusion of the drug into, or its combination with, the surface of the container-closure system. An apparent gain in potency usually is caused by solvent evaporation or by leaching of materials from the container–closure system.

The chemical potency of the active ingredient(s) is required to remain within the limits specified in the monograph definition. Potency is determined by means of an assay procedure that differentiates between the intact molecule and its degradation products. Chemical stability data should be available from the manufacturer. Although chemical degradation ordinarily cannot be detected by the pharmacist, excessive chemical degradation sometimes is accompanied by observable physical changes. In addition, some physical changes not necessarily related to chemical potency, such as change in color and odor, formation of a precipitate, or clouding of solution, may serve to alert the pharmacist to the possibility of a stability problem. It should be assumed that a product that has undergone a physical change not explained in the labeling may also have undergone a chemical change, and such a product is never to be dispensed. Excessive microbial growth, contamination, or both, may also appear as a physical change. A gross change in a physical characteristic such as color or odor is a sign of instability in any product. Other common physical signs of deterioration of dosage forms include the following.

**Solid dosage forms:** Many solid dosage forms are designed for storage under low-moisture conditions. They require protection from environmental water and therefore should be stored in tight containers (see *Packaging and Storage Requirements* ⟨659⟩) or in the container supplied by the manufacturer. The appearance of fog or liquid droplets, or clumping of the product, inside

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the container signifies improper conditions. The presence of a desiccant inside the manufacturer's container indicates that special care should be taken in dispensing. Some degradation products, for example, salicylic acid from aspirin, may sublime and be deposited as crystals on the outside of the dosage form or on the walls of the container.

*Hard and soft gelatin capsules:* Since the capsule formulation is encased in a gelatin shell, a change in gross physical appearance or consistency, including hardening or softening of the shell, is the primary evidence of instability. Evidence of release of gas, such as a distended paper seal, is another sign of instability.

*Uncoated tablets:* Evidence of physical instability in uncoated tablets may be shown by excessive powder and/or pieces (i.e., crumbling as distinct from breakage) of tablet at the bottom of the container (from abraded, crushed, or broken tablets); cracks or chips in tablet surfaces; swelling; mottling; discoloration; fusion between tablets; or the appearance of crystals that obviously are not part of the tablet itself on the container walls or on the tablets.

*Coated tablets:* Evidence of physical instability in coated tablets is shown by cracks, mottling, or tackiness in the coating and the clumping of tablets.

*Dry powders and granules:* Dry powders and granules that are not intended for constitution into a liquid form in the original container may cake into hard masses or change color, which may render them unacceptable.

*Powders and granules intended for constitution as suspensions:* Dry powders and granules intended for constitution into solutions or suspensions require special attention. Usually such forms are antibiotics or vitamins that are particularly sensitive to moisture. Since they are always dispensed in the original container, they generally are not subject to contamination by moisture. However, an unusual caked appearance necessitates careful evaluation, and the presence of a fog or liquid droplets inside the container generally renders the preparation unfit for use. Presence of an objectionable odor also may be evidence of instability.

*Effervescent tablets, granules, and powders:* Effervescent products are particularly sensitive to moisture. Swelling of the mass or development of gas pressure is a specific sign of instability, indicating that some of the effervescent action has occurred prematurely.

**Liquid dosage forms:** Of primary concern with respect to liquid dosage forms are homogeneity and freedom from excessive microbial contamination and growth. Instability may be indicated by cloudiness or precipitation in a solution, breaking of an emulsion, nonresuspendable caking of a suspension, or organoleptic changes. Microbial growth may be accompanied by discoloration, turbidity, or gas formation.

*Solutions, elixirs, and syrups:* Precipitation and evidence of microbial or chemical gas formation are the two major signs of instability.

*Emulsions:* The breaking of an emulsion (i.e., separation of an oil phase that is not easily dispersed) is a characteristic sign of instability; this is not to be confused with creaming, an easily redispersible separation of the oil phase that is a common occurrence with stable emulsions.

*Suspensions:* A caked solid phase that cannot be resuspended by a reasonable amount of shaking is a primary indication of instability in a suspension. The presence of relatively large particles may mean that excessive crystal growth has occurred.

*Tinctures and fluidextracts:* Tinctures, fluidextracts, and similar preparations usually are dark because they are concentrated, and thus they should be scrutinized carefully for evidence of precipitation.

*Sterile liquids:* Maintenance of sterility is of course critical for sterile liquids. The presence of microbial contamination in sterile liquids usually cannot be detected visually, but any haze, color change, cloudiness, surface film, particulate or flocculent matter, or gas formation is sufficient reason to suspect possible contamination. Clarity of sterile solutions intended for ophthalmic or parenteral use is of utmost importance. Evidence that the integrity of the seal has been violated on such products should make them suspect.

**Semisolids (creams, ointments, and suppositories):** For creams, ointments, and suppositories, the primary indication of instability is often either discoloration or a noticeable change in consistency or odor.

*Creams:* Unlike ointments, creams usually are emulsions containing water and oil. Indications of instability in creams are emulsion breakage, crystal growth, shrinking due to evaporation of water, and gross microbial contamination.

*Ointments:* Common signs of instability in ointments are a change in consistency and excessive "bleeding" (i.e., separation of excessive amounts of liquid) and formation of granules or grittiness.

*Suppositories:* Excessive softening is the major indication of instability in suppositories, although some suppositories may dry out and harden or shrivel. Evidence of oil stains on packaging material should warn the pharmacist to examine individual suppositories more closely by removing any foil covering. As a general rule (although there are exceptions), suppositories should be stored in a refrigerator (see *Packaging and Storage Requirements* ⟨659⟩).

## PROPER TREATMENT OF PRODUCTS SUBJECTED TO ADDITIONAL MANIPULATIONS

In repackaging, diluting a product or mixing it with another product, the pharmacist may become responsible for its stability.

**Repackaging:** In general, repackaging is inadvisable. However, if repackaging is necessary, the manufacturer should be consulted concerning potential problems. In the filling of prescriptions, it is essential that suitable containers be used. Appropriate storage conditions and, when appropriate, an expiration date and beyond use date should be indicated on the label of the prescription container. Single-unit packaging calls for care and judgment and for strict observance of the following guidelines: (1) use appropriate packaging materials, (2) if stability data on the new package are not available, repackage at any one time only sufficient stock for a limited time, (3) include on the unit-dose label a lot number and an appropriate beyond-use date, (4) if a sterile product is repackaged from a multiple-dose vial into unit-dose (disposable) syringes, discard the latter if not used within 24 hours, unless data are available to support longer storage, (5) if quantities are repackaged in advance of immediate need, maintain suitable repackaging records showing name of manufacturer, lot number, date, and designation of persons responsible for repackaging and for checking (see *General Notices*), (6) if safety closures are required, use container closure systems that ensure compliance with compendial and regulatory standards for storage.

**Dilution or mixing:** If a product is diluted, or if two products are mixed, the pharmacist should observe good professional and scientific procedures to guard against incompatibility and instability. For example, tinctures such as those of belladonna and digitalis contain high concentrations of alcohol to dissolve the active ingredient(s), and they may develop a precipitate if they

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are diluted or mixed with aqueous systems. Pertinent technical literature and labeling should be consulted routinely; it should be current literature, because at times formulas are changed by the manufacturer. If a particular combination is commonly used, consultation with the manufacturer(s) is advisable. Since the chemical stability of extemporaneously prepared mixtures is unknown, the use of such combinations should be discouraged; if such a mixture involves an incompatibility, the pharmacist might be responsible. Oral antibiotic preparations constituted from powder into liquid form should never be mixed with other products.

Combining parenteral products necessitates special care, particularly in the case of intravenous solutions, primarily because of the route of administration. This area of practice demands the utmost in care, aseptic technique, judgment, and diligence. Because of potential unobservable problems with respect to sterility and chemical stability, all extemporaneous parenteral preparations should be used within 24 hours unless data are available to support longer storage.

## INFORMING AND EDUCATING THE PATIENT

As a final step in meeting responsibility for the stability of drugs dispensed, the pharmacist is obligated to inform the patient about the proper storage conditions (for example, in a cool, dry place—not in the bathroom) for both prescription and nonprescription products, and to suggest a reasonable estimate of the time after which the medication should be discarded. When beyond-use dates are applied, the pharmacist should emphasize to the patient that the dates are applicable only when proper storage conditions are observed. Patients should be encouraged to clean out their drug storage cabinets periodically.

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***Change to read:***

# ⟨1195⟩ SIGNIFICANT CHANGE ▲▲ (USP 1-May-2021) FOR BULK PHARMACEUTICAL EXCIPIENTS

***Change to read:***

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- 1.1 Purpose
- 1.2 Scope
- 1.3 Principles Adopted
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2. SIGNIFICANT CHANGE
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## INTRODUCTION

### 1.1 Purpose

This informational general chapter is intended to establish a uniform approach to the evaluation of the significance of changes involving the manufacture and distribution of pharmaceutical excipients. The purpose of the evaluation is to consider the impact of the change on the excipient and to determine whether or not the excipient user and/or regulatory authority should be informed. It is recommended that users and excipient suppliers utilize this chapter as the basis for notification requirements in quality and/or supply agreements.

### 1.2 Scope

This chapter is applicable to all excipients used in the manufacture of pharmaceutical products. Although the principles of good manufacturing practices (GMP) as described in *Good Manufacturing Practices for Bulk Pharmaceutical Excipients* ⟨1078⟩ are the focus of this chapter, there are instances where the changes affect the principles and practices described in *Good Distribution Practices for Bulk Pharmaceutical Excipients* ⟨1197⟩.

This chapter is intended to guide the assessment of a change that affects the manufacture and/or supply of the excipient. All significant changes should be considered as requiring user notification. The level of change is determined by the type of change as well as the results of the evaluation. Conclusions regarding the level of change should be justified and documented.

The principles set forth in this chapter should be applied once the excipient manufacturer has determined that an excipient is intended for use as a component of a drug product. This chapter applies to excipients manufactured by either batch processing or continuous processing, and the use of the term "batch" or "lot" may refer to either type of processing.

### 1.3 Principles Adopted

This chapter is internationally applicable, reflecting the diverse nature of pharmaceutical excipients, which often have uses other than pharmaceutical applications. It provides minimum recommendations when considering the impact of a change on

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the excipient. This general chapter cannot specify all national legal requirements or cover in detail the particular characteristics of every excipient.

When considering how to use this chapter, each excipient manufacturer should consider how it may apply to their product's manufacturing processes. The diversity of excipients means that some principles of the chapter may not be applicable to certain excipients products and processes. The terminology "should" and "it is recommended" does not mean "must" and common sense should be used in the application of this chapter. However, the excipient manufacturer has a responsibility to follow the principles and guidance provided herein and to inform (notify) the drug product manufacturer(s) of any significant change (as outlined in this chapter), enabling the appropriate evaluation of the potential impact on the end drug product(s) due to the change.

The underlying principle of this chapter is that all changes should be regarded as significant (Level 2) and thus notifiable unless otherwise scientifically justified and documented.

Level 2 changes are discussed further in *2.2 Change Risk Levels*.

### 1.4 Layout

The first section of this chapter provides the background discussion necessary for evaluating a change and determining the need to inform the user and/or regulatory authorities. The second section defines the term "significant change" and is followed by guidance on determining the risk that a change will be significant. Notification processes to customers and possibly regulatory authorities follows, and the chapter concludes with a series of specific changes in which the classification possibilities are examined. The *Glossary* contains terms and definitions used in all sections of this chapter. *Appendix 1* includes case studies to show how the significance of change can be determined. *Appendix 2* provides decision trees useful in considering the potential impact of a change on excipient performance.

### 1.5 General Considerations

#### EXCIPIENT COMPOSITION

Excipients frequently function because of their heterogeneous composition. They may contain multiple components that are dependent on their source and manufacturing process and may be necessary for their established functions. Potential change in composition is an important consideration when assessing significance of change.

#### DIFFERENTIATION OF EXCIPIENT MANUFACTURE

Whereas drug substances are typically of high purity, well characterized, and used in a limited number of therapeutic applications, the pharmaceutical excipient is often a natural substance, mixture, or polymer whose chemical and physical properties are more difficult to quantify. Unlike drug substances, pharmaceutical excipients are often used with a broad range of active ingredients and in a variety of finished dosage forms. Evaluating the impact of a change in the manufacture of an excipient on its performance in the finished dosage form/medicinal product is complex. Compounding the complexity of such evaluations by the excipient manufacturer are the unlimited possibilities for excipient function relative to new combinations of excipients, drug substances, and or processing conditions that users may choose to apply.

#### EXCIPIENT GMP

At some logical processing step, as determined by the excipient manufacturer, GMP as described in ⟨1078⟩ and the NSF/IPEC/ANSI 363: *Good Manufacturing Practices (GMP) for Pharmaceutical Excipients* (*1*) or in the EXCiPACT cGMP standard (*2*) should be applied and maintained. Judgment, based on risk assessment and a thorough knowledge of the process and intended application, is required to determine from which processing step GMPs should be applied. Generally, this chapter should be applied from the point at which GMPs are applied; nevertheless, it may be important to consider changes that occur prior to this point (e.g., in raw materials) and evaluate them for significance.

## 2. SIGNIFICANT CHANGE

### 2.1 Definition of Significant Change

Significant change is defined as any change that alters an excipient's physical, chemical, or microbiological properties from the norm, and/or any change that has the potential to alter the excipient's performance in the dosage form.

### 2.2 Change Risk Levels

In the evaluation of the impact of changes to the excipient, it is recognized that even with objective criteria some judgment may be necessary. To facilitate the decision as to the significance of a change and the potential impact on the pharmaceutical dosage form, the types of changes are classified using two levels (examples of two case studies can be found in *Appendix 1*). The impact of the change should be assessed against the guiding principles listed in *3.2 Guiding Principles for Evaluating Change Significance*, which often reflect the potential impact of the change on the performance of the excipient. Evaluation according to the principles of this chapter, the types of changes, and, where appropriate, risk assessment principles will determine the classification. The risk assessment needs to take into consideration the complexity of the change and the ability to fully characterize the impact. The two levels of change are:

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- Level 1: Not Significant
- Level 2: Significant

The notification of Level 1 changes is not mandatory, and it is up to the excipient supplier to determine whether to notify the user. All Level 2 changes require user notification and, where appropriate, regulatory authority notification (see *4. Notification Requirements*).

Unless otherwise justified and documented, all changes should be regarded as Level 2.

Guidance on specific changes is given in *5. Specific Changes*, which includes examples that reinforce the position that certain changes are always notifiable (i.e., Level 2).

## 3. DETERMINATION OF SIGNIFICANCE/RISK ASSESSMENT

### 3.1 General

It is recommended that the evaluation of changes and the processes for the determination of significance are integrated into the documented procedure for change management. If the level of change is not specifically defined in *5. Specific Changes*, further assessment is needed utilizing the risk assessment principles described in this section of the document.

All changes should be assumed to be Level 2 unless otherwise scientifically justified and documented.

### 3.2 Guiding Principles for Evaluating Change Significance

The following principles should be considered in determining the significance of the change with or without the use of a formalized risk assessment approach:

1. Complexity of the change(s) (including possible cumulative effects)
2. Level of understanding of historical norms
3. The ability to fully characterize the impact of the change on the:
    - Excipient properties (i.e., chemical, physical, microbiological, composition profile, etc.)
    - Intended excipient performance
    - Equivalency of the composition profile comparing pre-change and post-change batches
        - No new component is present at or above 0.10%; neither has a component that was previously present at this level disappeared (*3*). Minor components, including residual solvents and elemental impurities, remain within historical norms for the batches produced before the change.
4. The ability to assess the change in trial batches and/or model products
5. Manufacturer's understanding of the users' application(s) and use(s) of the product or critical material attributes
6. The content and requirements of any quality or technical agreements that are in place
7. In the case of raw material changes—the level of knowledge, understanding, credibility, and reliability of the raw material manufacturer and the relationships that exist within the raw material supply chain
8. The content of regulatory documents used in the applications under the excipient manufacturer [drug master file (DMF), Certificate of Suitability to the monographs of the *European Pharmacopoeia* (CEP)] or submitted to customers for their own regulatory applications (technical dossier)

### 3.3 Change Management Documentation

The change management documentation should describe the nature of the change, the reason it may be significant, the testing to be performed to evaluate the change, the criteria for determining the significance, and the final decision on the level of the change.

The associated risk assessment and decisions made should be documented.

Evidence may be obtained after implementation or testing that requires the original decision on the level of change to be re-evaluated. Under such circumstances, the reasons for the re-evaluation and the decision based on the re-evaluation should be scientifically justified and fully documented.

### 3.4 Justification for Level 1 Change

Level 1 changes that are specifically given in this section or in the decision tree do not need further justification. However, a Level 1 change that is determined through risk assessment should be justified and documented. It is recommended that the justification includes a detailed rationale explaining the conclusion that the change does not pose a significant risk to the medicinal drug product quality.

### 3.5 Testing

The results from the testing of an appropriately determined number of pre- and post-change batches of excipient, or results from predefined operational time periods, should be compared for evaluation of the change prior to final implementation.

Where the manufacture of a predetermined number of multiple post-change batches for evaluation is not practical, concurrent evaluation of batches produced after the change has been implemented should be compared to historical data from a predetermined and sufficient number of batches manufactured before the change.

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One method to compare the new data with historical data is the two one-sided test (TOST) for demonstrating equivalence (see *Analytical Data–Interpretation and Treatment* ⟨1010⟩. As a further check on consistency, the new batch property specifications can be plotted on standard statistical quality control (SQC) charts, along with the batch results from the selected pre-change batches or operational time periods and in-process testing and controls. The choice of statistical test, if used, should be justified and documented.

Samples for comparison purposes must be suitable to evaluate the impact of the change. Consideration should be given to the stability of the samples since the batch was produced. The comparison should encompass, where appropriate, chemical and physical properties, microbiological properties, composition profile, stability, and performance. Sample types could include retention samples or other samples that have been stored under appropriate conditions.

Chemical and physical properties lend themselves to quantitative measurement. Often these properties are part of the specification for the excipient. As such, for those properties potentially affected, there should be a large body of historical data for comparison with the corresponding data for the excipient made after the change. However, there may be additional properties that should be assessed on the basis of the type of change being made.

Where appropriate, the process validation should be updated to reflect the changed process.

## 4. NOTIFICATION REQUIREMENTS

Level 2 changes always require user notification. The user should be given as much advance notice of impending changes as is reasonably possible. The timing of the notification will rely on the specifics of the particular change being made. The notification should include the date of implementation and the urgency of the change.

The user may require time to complete the evaluation of the impact of the change on drug products. During this period the user may request inventory of the excipient produced before the change was made. Where possible, the manufacturer should plan for the change with this eventuality in mind and collaborate with the user to develop an appropriate implementation plan.

It is recommended that a summary of the changes and any supporting data and information be provided to the user to aid in the evaluation. As further applicable data become available (e.g., stability studies), they should be communicated.

On occasion, there may be a need for emergency changes. In such cases, it should be understood that notice periods may be very short and supporting data and information, as detailed above, may not be available at the time of initial notification.

If a regulatory filing exists, such as an excipient DMF or CEP, the authorities may require notification of significant changes involving the manufacture of excipients. Holders of United States DMFs should consult the FDA *Guideline for Drug Master Files* (*4*) for more details.

## 5. SPECIFIC CHANGES

The types of change described in this section should drive decisions on the significance and determination as to whether a change is Level 1 or Level 2. The following information should be considered when assessing the types of change. If a decision cannot be made by using the guidance in this section, then the risk assessment approach in *3. Determination of Significance/ Risk Assessment* should be used to make a decision.

### 5.1 Changes to the Site, Infrastructure Used to Manufacture, and Distribution of the Excipient

#### SITE CHANGE

A change in manufacturing site could be for either the production or packaging of the excipient. A change in the manufacturing site is a Level 2 change.

If the change involves the site of the quality control laboratory, then the impact hinges on the test method. If the method remains the same, the change is Level 1 provided a formal method transfer or validation is conducted. If the new laboratory uses a different analytical technique, then the change is Level 2.

#### SCALE

Manufacturers may change the scale of their production. If the process is being scaled outside the historical norms, and the excipient as evaluated is also outside the historical norms, the change is significant and thus Level 2. If the existing equipment is optimized to increase capacity without altering the process, often found in continuous processing, the change is Level 1 provided that a comparison of pre- and post-change data is within historical norms. However, careful consideration should be given to changes that can clearly impact the properties and/or functionality of the excipient.

A change in batch size for a continuous process does not necessarily mean a change in scale. If the same process and equipment train is used and there is no change in the process and control parameters (i.e., simply a longer time of running to define a batch), then the change is Level 1. If the process equipment, process parameters, or control parameters are changed, then the change is Level 2.

#### PRODUCTION EQUIPMENT

The evaluation of equipment change is predicated on whether the new equipment is equivalent to the equipment it replaces. Generally, equipment that is a replacement in kind is a Level 1 change. If the new equipment is not a replacement in kind but was included in the most current equipment qualification, then the change is still Level 1. If the new equipment is not a replacement in kind and was not included in the most current equipment qualification, then change is Level 2. If an equipment

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change could potentially affect the excipient or manufacturing process, it should be evaluated using the risk assessment approach in *3. Determination of Significance/Risk Assessment*.

PRODUCTION PROCESS

A change in production process involves changes to the synthetic route or target levels for parameters such as temperature, pressure, flow rate, the processing aids to be used, the sequence of operating steps, and the operation to be performed. Each type of process change is further described in *Appendix 2*.

If there is a change in a process parameter within the intended range, such as operating at a new target within that range, then the change is Level 1.

If a process parameter or processing step is outside the intended range (i.e., validated range and/or design space) and the excipient as evaluated is also outside the historical norms, then the change is Level 2.

If a change in the production process is made that increases the level of process control within historical norms, then the change is Level 1.

Introduction of new products into production equipment that was, until that point, dedicated to one excipient is a Level 2 change.

PACKAGING, LABELING, AND DOCUMENTATION

Any change in the primary or barrier packaging that is a replacement in kind is Level 1. Replacement in kind applies to packaging constructed of the same materials and sealed in the similar manner and liners made of the same materials such that the protection provided to the excipient by the packaging system (container/closure system) is the same as before the change. Any change that is not a replacement in kind is Level 2.

Any change to seals that are intended to be tamper evident is Level 2.

Any change to labeling or documentation pertaining to the company name, product name, batch/lot numbering scheme, site of manufacture or testing, species origin, additives, or storage and handling conditions is a Level 2 change.

Changes in secondary packaging—packaging materials that do not have direct contact with the excipient and are not considered barrier packaging components—should be assessed using the risk assessment principles outlined in *3. Determination of Significance/Risk Assessment* to determine the appropriate level of change.

EXCIPIENT SPECIFICATIONS AND TEST METHODS

Changes to excipient specifications are Level 2 unless the specification is tightened within the existing range.

Any change to an excipient specification or test method made to comply with routine compendial changes is Level 1.

Replacement of an excipient test method with an equivalent validated alternative method is a Level 1 change.

SUPPLY CHAIN

Changes by the excipient manufacturer of their official distributor(s) are Level 2.

[NOTE—For distributors, any change to their supply source is Level 2. Changes due to excipient discontinuation are Level 2.]

Changes to distribution and warehousing locations should be evaluated using risk assessment principles (see *3. Determination of Significance/Risk Assessment*).

Changes to processes or locations related to repackaging or relabeling are Level 2.

For more information on good distribution practices, please refer to ⟨1197⟩.

## 5.2 Determination of Impact of Changes on Excipient Quality and Performance

INTRODUCTION

It is important to give careful consideration to any processing changes after the excipient has been synthesized or isolated but prior to packaging. However, it must be recognized that a change made earlier in the process can result in a change in the excipient performance and it is recommended that such changes also be considered.

When determining impact of change, as a minimum, the criteria below should be evaluated. For those identified as applicable, all conclusions or decisions should be scientifically justified and documented.

The following represents the minimum criteria that should be used for evaluating the impact of change:

1. Change in the physical properties of the excipient
2. Change in the chemical properties of the excipient
3. Change in the microbiological properties of the excipient
4. Change in the composition profile of the excipient
5. Change in the origin, type, or site of any raw materials
6. Change in the distribution of the excipient
7. Change in the origin and/or type of packaging and/or labeling
8. Change in excipient stability
9. Change in the regulatory status of the excipient
10. Change in the compliance with compendia or other regulation
11. Potential to change the intended performance of the excipient based on the excipient manufacturer's understanding

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Additional guidance on these points is provided in the following sections. Changes to any of these attributes may impact the excipient quality and/or performance in the dosage form, and it is important to identify objective criteria for evaluation. An assessment of the impact of such changes provides the excipient manufacturer with the rationale for determining the significance of the change to the user of the excipient and the justification for notifying the user and/or the regulatory authorities.

## PHYSICAL PROPERTIES

Evaluation of the physical properties of an excipient should include, at a minimum, all applicable specifications and other relevant parameters that define the physical properties of the excipient. Physical properties should be considered based upon the physical form of the excipient. The number of batches chosen for evaluation should be justified. A comparison of these test results for the excipient pre- and post-change should be carried out to determine if there has been a change from historical norms as well as to assess the likely impact of such change(s). For example, the following properties should be considered if relevant:

- Bulk density (loose and tapped)
- Surface area
- Particle shape and structure
- Particle size distribution
- Color
- pH
- Viscosity
- Molecular weight distribution

## CHEMICAL PROPERTIES

Evaluation of the chemical properties of an excipient should include, at a minimum, all applicable specifications and other relevant parameters that define the chemical attributes of the excipient. The number of batches chosen for evaluation should be justified. A comparison of these test results for the excipient pre- and post-change should be carried out to determine if there has been a change from historical norms and to assess the likely impact of such change(s).

## MICROBIOLOGICAL PROPERTIES

Change in processing steps, raw materials, water, or equipment, can impact control of microorganisms in the excipient. Therefore, the effect of the change on the microbiological properties should be evaluated, particularly for excipients susceptible to microbial growth. When the risk-based evaluation determines that testing is required, a comparison of the microbiological properties pre- and post-change should be carried out to determine if there has been a change from historical norms and to assess the likely impact of such change(s). The number of batches chosen for evaluation should be justified.

## POTENTIAL IMPACT ON THE INTENDED PERFORMANCE OF THE EXCIPIENT BASED ON THE EXCIPIENT MANUFACTURER'S UNDERSTANDING

Although performance/functionality is often defined by the previous parameters (physical, chemical, and compositional properties), objective criteria for evaluating other potential changes to excipient performance or functionality are desirable. However, the nature of this type of study can vary broadly based upon the excipient, its application in the dosage form, and the capabilities of the excipient manufacturer. It must also be recognized that the excipient manufacturer may not always be aware of all applications of the excipient. Therefore, this chapter cannot provide objective criteria for such studies but stresses the importance of such consideration by the excipient manufacturer. If there is a potential that the performance or functionality of the excipient may be impacted by the change, users should be notified. Material samples should be provided if requested so the user can determine the impact of the change on their finished pharmaceutical product(s). *Excipient Performance* ⟨1059⟩ can provide guidance in this area.

## COMPOSITION PROFILE

Objective criteria are also necessary when considering the impact on the composition profile for an excipient as a result of changes (*3*).

The composition profile may include, if relevant:

- Identified organic components
- Unidentified organic components
- Residual solvents
- Identified inorganic components
- Water content

The feasibility of developing a composition profile will vary with the nature and origin of the excipient. It is important to note that the presence of impurities and concomitant components in some excipients is extremely difficult to quantify. Thus, an excipient manufacturer may not have developed a complete quantitative composition profile. In such cases, it is important for the excipient manufacturer to document efforts to identify and quantify the concomitant components that may be present so as to justify the limited results and to justify other means by which changes may be evaluated.

A comparison of the composition profile of the excipient pre- and post-change should be carried out to determine if there has been a change from historical norms. The number of batches chosen for evaluation should be justified.

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Changes in the levels of residual solvents should be considered when determining the significance of a change. See *Residual Solvents* ⟨467⟩ for details.

Changes in the levels of elemental impurities should be considered when determining the significance of a change.

Water content can have an impact on excipient performance in the preparation of the pharmaceutical dosage form and the performance of the dosage form in vivo. Therefore, a change in the water content beyond the range typical for excipient production, even if within the compendial or specification limit, can impact the stability and performance of the drug product and/or end use of the excipient.

## CHANGE IN THE ORIGIN, TYPE, OR SITE OF RAW MATERIALS

It is recommended that excipient manufacturers and their raw material suppliers agree to a change notification process wherein they are notified of significant changes to the raw materials. Changes in the properties of the raw materials outside of historical norms should be evaluated using risk assessment principles (see *3. Determination of Significance/Risk Assessment*).

Changes to the specifications of the raw materials that may impact the regulatory status of the excipient are Level 2.

Changes in the type of raw material (natural, synthetic, or mineral) are Level 2.

Changes to the origin of plant materials (e.g., corn vs. potato) are Level 2.

Changes to the origin of animal materials (e.g., bovine vs. porcine) are Level 2.

Changes in the country of origin of raw materials, which may impact regulatory status of the excipient, are Level 2.

Changes in the manufacturing process of the raw material should be evaluated using risk assessment principles (see *3. Determination of Significance/Risk Assessment*).

Changes to the supplier or site of the raw material manufacturer should be evaluated using risk assessment principles (see *3. Determination of Significance/Risk Assessment*).

The origin of the raw material includes the country of origin, geological origin, and species (animal or plant) origin. The type of raw material includes whether the material is natural or synthetic, the physical form and/or preliminary extraction of the raw material, and/or processing prior to delivery to the excipient manufacturing site. The site of the raw materials includes the actual manufacturing site or distribution points.

Changes in the species of origin, from animal to animal or from plant to animal, may cause a change in the viral and microbiological safety profile of the excipient.

A change in the country of origin of a raw material can impact the status of the excipient as it relates to the potential presence of bovine spongiform encephalopathy (BSE) or transmissible spongiform encephalopathies (TSE) material or genetically modified organisms (GMO). The country of origin of animal-derived raw material, or components used in the manufacture of the raw material, can result in noncompliance with relevant TSE regulations (*5–7*). These aspects may have impact on the regulatory status (as discussed further in this section and in *4. Notification Requirements*.

Switching raw material from animal-derived to plant-derived, or from one plant species to another, raises the potential for the presence of plant-based allergenic material in the excipient. Changes to plant-derived raw materials can also affect the GMO status of the excipient.

A change in the geological origin of mineral-based excipients can alter the composition of the excipient. Geological formations containing the same mineral can still differ in their chemical composition (particularly relating to minor concomitant components), crystalline structure, density, inorganic components, etc. A change in geological origin of a raw material can impact the excipient chemical or physical properties, the composition profile, or excipient performance/functionality.

Changes to or additions of a further site of manufacture, even from the same supplier, can result in changes to the raw material that can impact the properties of the excipient. The equipment and processes between sites may differ. Changes in the distribution points of the raw material supply chain may impact its quality.

## CHANGE IN THE DISTRIBUTION OF THE EXCIPIENT

Assurance of the quality (purity, integrity, safety) of the excipient may be impacted by how the excipient is transported from the manufacturer to the end user, considering the key distribution points within the supply chain. Each partner in the supply chain has the potential to affect the quality of the excipient. For example, storage and transportation conditions may affect excipient quality, excipient stability, or the potential to become contaminated. Therefore, changes in the distribution or supply chain can be important. It is not anticipated that all changes in carriers must be a notifiable change; however, the excipient manufacturer must evaluate any known carrier changes to be assured that there will be no changes in storage and/or transportation conditions.

## CHANGE IN THE ORIGIN OR TYPE OF PACKAGING OR LABELING

A change in the primary or barrier packaging components can involve the manufacturer, country of origin, or materials of construction. The evaluation of the primary packaging should include the impact on the composition profile, excipient stability (see *Impact on Excipient Stability*), and interactions between the excipient and the packaging (leachables/extractables). The evaluation of barrier packaging, if separate from primary packaging, should include, as a minimum, the impact on excipient stability.

A change in the labeling may impact information that the user needs to properly identify or use the excipient. In some cases minor labeling changes that involve simple things such as graphic design may not be significant. However, if information on the label changes from what was previously provided, this must be carefully assessed to determine the level of notification necessary.

Such changes may necessitate notifying the regulatory authority, if the excipient company has filed information with regulators that would require notification (such as a DMF or CEP).

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IMPACT ON EXCIPIENT STABILITY

An assessment should be made for the potential of the change to impact the stability of the excipient. Where this potential is identified, stability studies should be initiated as part of the evaluation of change. If the risk assessment shows that stability implications are predictable, stability studies may be done concurrently with notification and implementation of the change (*8*).

CHANGE IN THE REGULATORY STATUS OF THE EXCIPIENT

Changes can occur in regulations, guidelines, and directives that may affect the regulatory status of the excipient. An evaluation of the change(s) should be carried out for the potential of the change to impact registration dossiers, such as drug master files, certificates of suitability, drug import/export licenses, and manufacturing authorization registrations (as applicable).

CHANGE IN COMPLIANCE WITH A COMPENDIUM OR OTHER REGULATION

When changes to compendial monographs or regulations occur, evaluation should be carried out to confirm continued compliance with these requirements.

Removal of an existing compendial claim is a significant change; however, the expansion of claims to include compliance with additional regulatory requirements is not necessarily a significant change.

## 5.3 Multiple Changes

Multiple changes involving more than one type of change, as discussed here, may occur simultaneously. Where Level 2 changes have been identified, user notification should proceed without delay. The other changes should be evaluated cumulatively using risk assessment principles (*3. Determination of Significance/Risk Assessment*) to determine the appropriate level for the totality of changes.

## 5.4 Discontinuation of an Excipient

If an excipient is to be discontinued, this will have a significant impact on the user since the user will have to qualify an alternative excipient and/or supplier. Depending on the particular use of the excipient, the new excipient source may well have a significant impact on performance and will require careful evaluation by the excipient user.

# GLOSSARY

**Bovine Spongiform Encephalopathy (BSE):** A slowly progressive, degenerative, fatal disease affecting the central nervous system of adult cattle. The exact cause of BSE is not known but it is generally accepted by the scientific community that the likely cause is an infectious form of a type of protein, called a prion, that is normally found in animals. In cattle with BSE, these abnormal prions initially occur in the small intestines and tonsils. They are found in central nervous tissues, such as those of the brain and spinal cord, and other tissues of animals experiencing later stages of the disease. A disease similar to BSE, called Creutzfeldt-Jakob Disease (CJD), is found in people. A variant form of CJD (vCJD) is believed to be caused by eating contaminated beef products from BSE-affected cattle.

**Bulk Pharmaceutical Excipient (BPE):** See *Excipient*.

**Chemical Property:** A quality parameter that is measured by chemical or physicochemical test methods.

**Certificate of Suitability to the monographs of the European Pharmacopoeia (CEP):** Certificate granted by the European Directorate for the Quality of Medicines (EDQM) to manufacturers of active ingredients or excipients confirming that the applicable *European Pharmacopoeia* (*Ph. Eur.*) monographs and general chapters are adequate to control the chemical purity of the material. A CEP also can be granted to confirm that material conforms to the *Ph. Eur.* general chapter 5.2.8 "Minimising the risk of transmitting animal spongiform encephalopathy agents via medicinal products" even if the material itself does not have a *Ph. Eur.* monograph.

**Concomitant Component:** A minor component of an excipient that accompanies the nominal component, which is identified either in the title or definition of a monograph. Concomitant components are characteristic of many excipients and are not considered to be impurities if there is no negative impact on drug products. Some, but not all, concomitant components are defined or specified in excipient monographs. Added substances are not considered concomitant components. (Any component that can be considered a toxic impurity because of significant undesirable biological effect is not considered to be a concomitant component.)

**Continuous Process:** A process that continually produces material from a continuing supply of raw material.

**Decision Tree:** A visual presentation of the sequence of events that can occur, including decision points.

**Drug Master File (DMF):** Detailed information concerning a specific facility, process, or product submitted to a drug regulatory authority (such as the US Food and Drug Administration, Health Canada, and Japan's Pharmaceuticals and Medical Devices Agency) and intended for incorporation by reference into a new drug application, supplemental new drug application, abbreviated new drug application, investigational new drug application, or biological license application.

**Drug Substance:** Any substance or mixture of substances intended for use in the manufacture of a drug product that, when used in the production of a drug, becomes an active ingredient of the drug product. Such substances are intended to furnish pharmacological activity or other direct effect in the diagnosis, cure, mitigation, treatment, or prevention of disease or to affect the structure or any function of the body of humans or animals.

**Excipient:** Substances other than the drug substances that have been appropriately evaluated for safety and are intentionally included in a drug delivery system.

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**Foreign Substance:** A component present in the bulk pharmaceutical excipient but NOT introduced into the excipient as a consequence of its synthesis or purification and not necessary to achieve the required functionality (formerly referred to as contaminant).

**Functionality:** A desirable property of an excipient that aids and/or improves the manufacture, quality, or performance of the drug product.

**Genetically Modified Organism (GMO):** An organism, with the exception of human beings, in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination.

**Impurity:** Any substance that detracts from the quality of the excipient (i.e., that is not the substance appearing in the official name, or concomitant component, or an added substance as defined above).

**Packaging:** The container and its components that hold the excipient for transport to the customer.

**Physical Property:** A quality parameter that can be measured solely by physical means.

**Process:** The combination of operating steps including synthesis, isolation, purification, packaging, etc., that produces the finished excipient.

**Process Parameter:** A measurable operating condition.

**Process Validation:** A documented program that provides a high degree of assurance that a specific process will consistently produce a result that will meet predetermined acceptance criteria.

**Raw Material:** A general term used to denote starting materials, reagents, and solvents intended for use in the production of intermediates or excipients. Raw material and starting material are not equivalent; starting material has a different meaning in a regulatory context.

**Replacement In Kind:** Manufacturing equipment that uses the same operating principles and is of similar construction; packaging components that are made with the same materials of construction and sealed in a similar manner.

**Residual Solvent:** Organic chemical solvents that are used or produced in the manufacture of active substances or excipients or in the preparation of medicinal products.

**Significant Change:** Any change that alters an excipient's physical, chemical, or microbiological properties from the norm, and/or any change that has the potential to alter the excipient's performance in the dosage form.

**Site:** A defined location of the equipment where the excipient is manufactured. It may be within a larger facility. A change in site may be to a different part of the existing facility, but in a different operational area, or to a remote facility including that of a contract manufacturer.

**Solvent:** An inorganic or organic liquid used as a vehicle for the preparation of solutions or suspensions in the manufacture of an excipient.

**Specification:** A list of tests, references to analytical procedures, and appropriate acceptance criteria, which are pre-established numerical limits, ranges, or other criteria for the tests described, that the material is required to meet.

**Starting Material:** A raw material or intermediate defined at the starting point for excipient GMPs and used in the production of an excipient that is incorporated as a significant structural fragment or that is purified to meet the quality requirement for an excipient.

**Statistical Quality Control (SQC):** The plotting of sequential test results to show their variation relative to the specification range and their normal variation.

**Transmissible Spongiform Encephalopathy (TSEs):** TSEs are rare forms of progressive neurodegenerative disorders that affect both humans and animals and are caused by similar uncharacterized agents that generally produce spongiform changes in the brain. Specific examples of TSE's include: scrapie, which affects sheep and goats; BSE, which affects cattle; transmissible mink encephalopathy; feline spongiform encephalopathy; chronic wasting disease (CWD) which affects mule deer, white-tailed deer, black-tailed deer, and elk; and Creutzfeldt-Jakob disease, variant Creutzfeldt-Jakob disease (vCJD), Gerstmann-Straussler syndrome, fatal familial insomnia, and kuru, which affect humans.

# APPENDICES

## Appendix 1: Case Studies

CASE STUDY EXAMPLES

The two examples below describe changes that are indeterminate and require risk assessment of the significance of change.

EXAMPLE 1

The excipient is a polymer. The processing involves taking the raw materials and polymerizing them, then applying a finishing process leading to the final product. The conditions for the polymerization are to be changed. No other aspects of processing are to be changed. The processing changes lead to an improved control of the polymerization process. However, the product still meets the existing compendial requirements, and none of the measured parameters are out of trend as a result of the change. Schematically the process is as follows:

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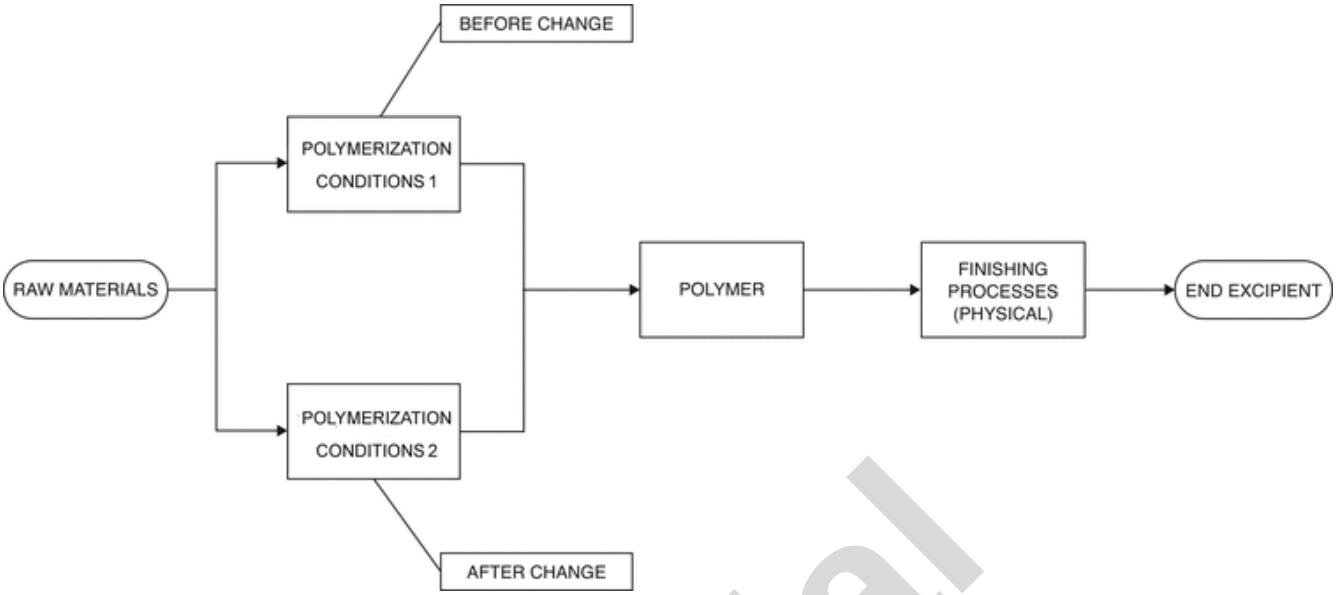


Applying the guidance in this general chapter, this is not clearly a Level 2 change to warrant user notification, neither is it a case where the change is automatically a Level 1 change.

Key to the assessment of this change is to determine if there are any other changes to the characteristics of the product resulting from the change. Changes in the method of polymerization can lead to different molecular weight distributions (which may not be a compendial test) and differences in the composition profile of the excipient. Therefore, these aspects of the excipient need to be assessed against the historic norms for the original process before a decision can be made. The difference in composition is especially relevant because minor components and even impurities resulting from the original process, which were contributing to the performance and functionality of the excipient, have been removed from the excipient after the change.

If there is no evidence to indicate that the excipient has changed in any specification parameter or within the other assessments that the manufacturer can define, then the change is classified as Level 1. The manufacturer will document this rationale in the management of change system.

If no justification can be determined for designating this a Level 1 change, then this is a Level 2 change requiring user notification.

EXAMPLE 2

An excipient that is a proprietary blend of ingredients is prepared by a continuous manufacturing process involving a high temperature step. The proposed change is to increase the flow rates within the originally defined equipment capability (i.e., within the overall process design space) although these flow rates are outside the current operating ranges. No other aspects of processing are to be changed—only this step is being altered—and all raw materials and final processing steps are the same.

The increased flow rate is desired for economic reasons. The product made after the implementation of this change still meets the existing selling specification. Minor degradation of one of the components is technically unavoidable in the manufacturing process at the temperatures required for processing. As a result of the increased flow rates, the residence time at high temperature is reduced and the levels of degradants, though within historical ranges, are consistently toward the low end of that range.

Applying the guidance of this chapter, this is not automatically a Level 2 change to warrant user notification nor is it a case where the change is automatically a Level 1 change. Given the circumstances above, a reasonable justification for this being a Level 1 change can be made.

However, the manufacturer has information that the degradants may have an impact on some user applications. Although the degradants remain within typical historical ranges, there is reason to believe they will now trend lower within the permitted ranges due to the changed process. With this additional information, the change becomes Level 2 and requires user notification.

## Appendix 2: Decision Tree

A decision tree has been developed to graphically aid and clarify the change levels in this chapter. The decision tree begins with the proposed change and guides the manufacturer to an indication of the likelihood the change will impact the excipient user. The decision tree classifies the types of change that occur in excipient manufacture as involving the site of manufacture, processing steps, packaging, or testing and quality control.

For convenience, the decision tree has been split into three parts (see *Figure 1*, *Figure 2*, and *Figure 3*):

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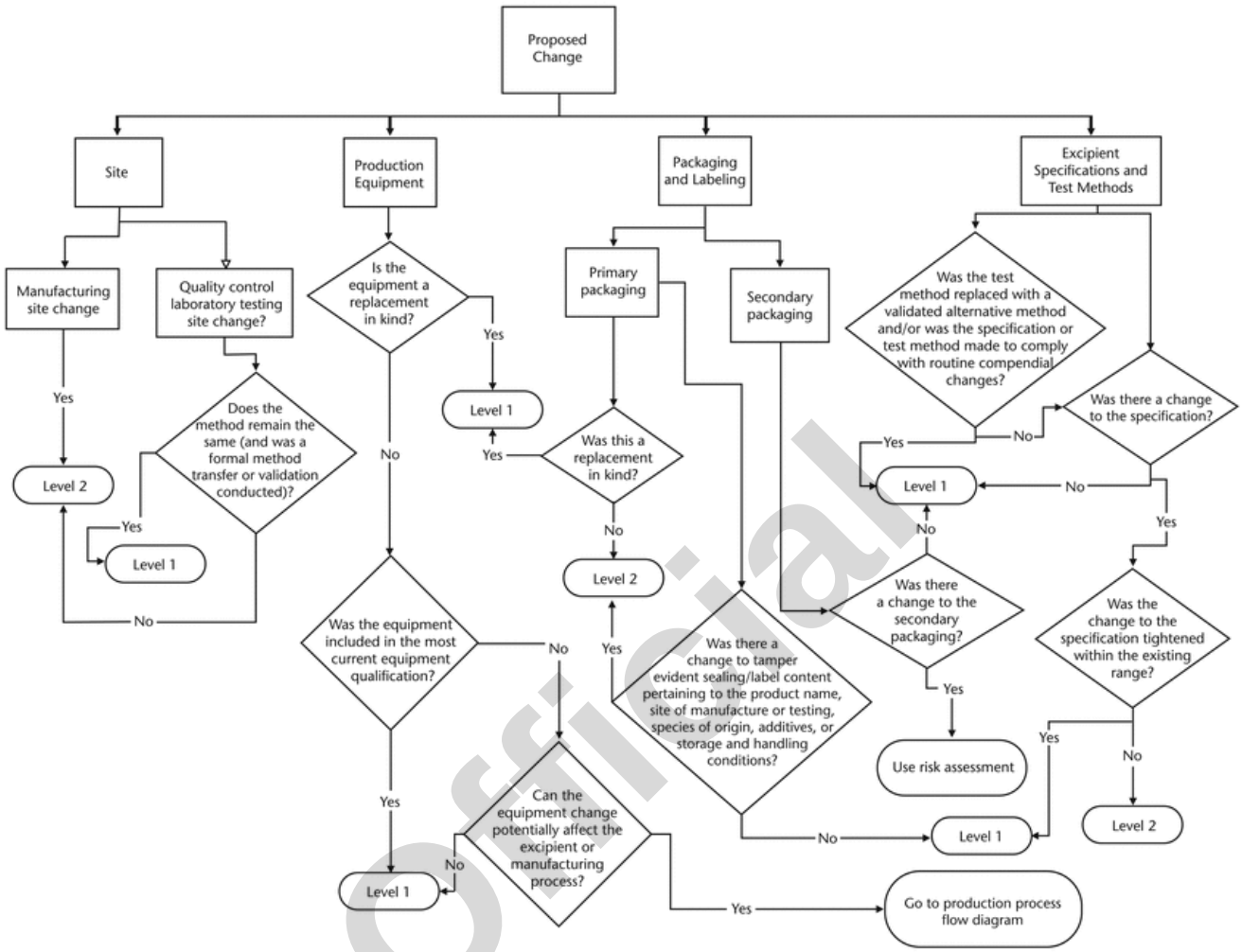


Figure 1. Decision tree covering site, production equipment, packaging and labeling, and excipient specifications and test methods.

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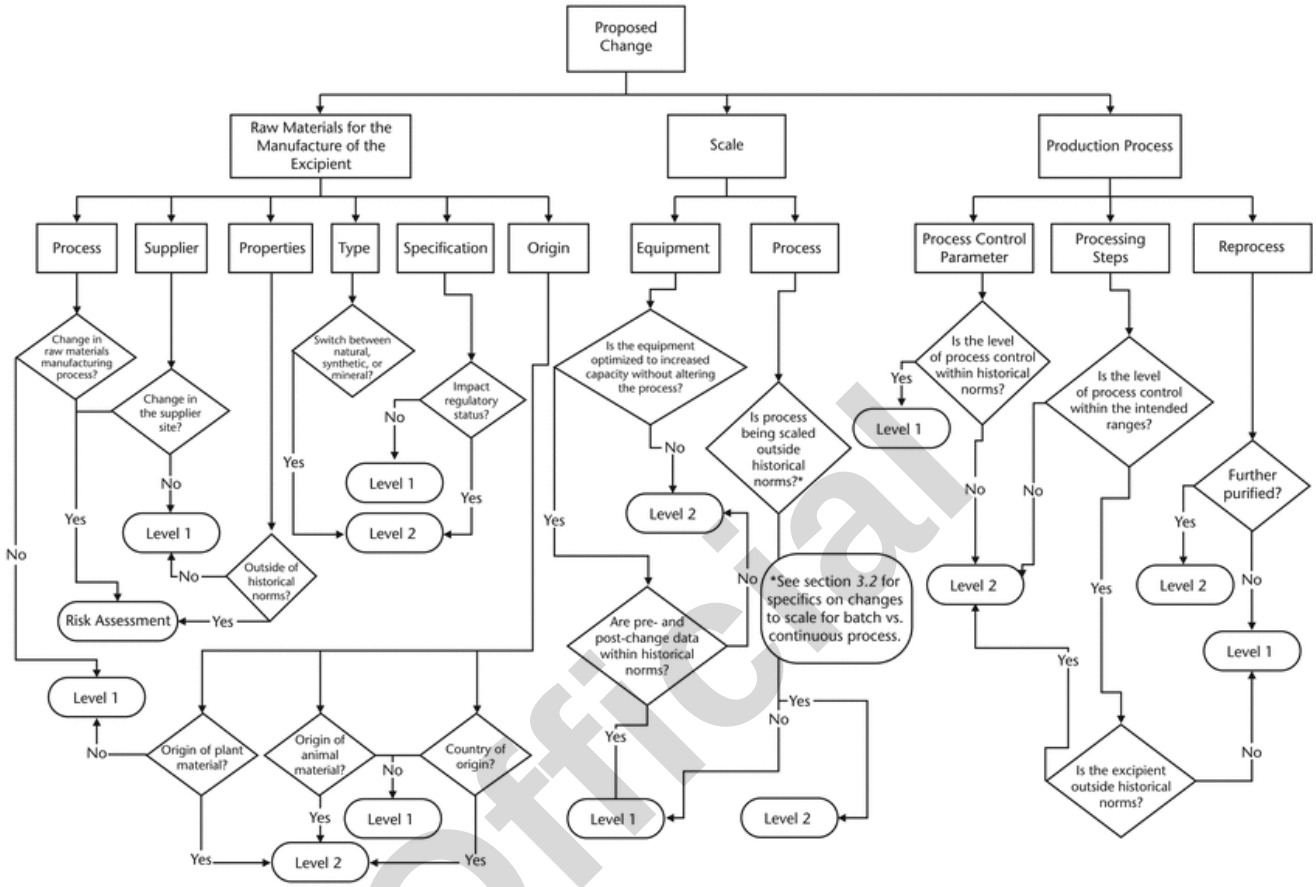


Figure 2. Decision tree covering raw materials for the manufacture of the excipient, scale, and production processes.

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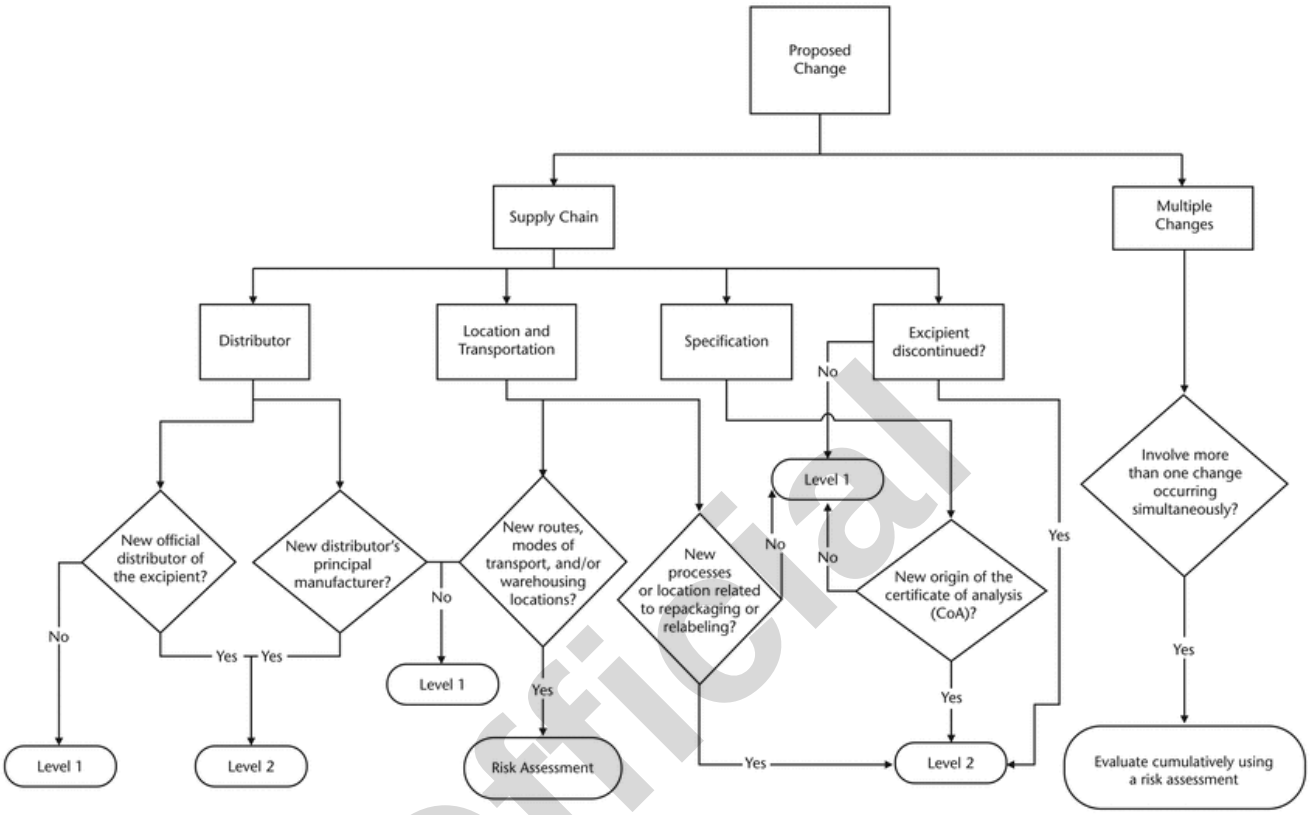


Figure 3. Decision tree covering supply chain and multiple changes.

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8. IPEC-Europe. The IPEC Excipient Stability Program Guide 2010. https://www.gmp-compliance.org/guidelines/gmp-guideline/the-ipec-excipient-stability-program-guide-2010.▲ (USP 1-May-2021)

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# ⟨1197⟩ GOOD DISTRIBUTION PRACTICES FOR BULK PHARMACEUTICAL EXCIPIENTS

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## SECTION 1. INTRODUCTION AND SCOPE

### 1.1 Introduction

Excipients are used in virtually all drug products and are essential to product performance and quality. Typically, excipients are manufactured and supplied so that they comply with compendial standards. The pharmaceutical excipient supply chain participants include manufacturers, distributors, brokers, suppliers, traders, transporters, forwarding agents, and repackagers. The quality of pharmaceutical excipients is affected by inadequate control of activities including distribution, packaging, repackaging, labeling, and storage. Improper or inadequately controlled trade and distribution practices can pose a significant risk to the quality of pharmaceutical excipients and can increase the risk of contamination, cross-contamination, adulteration, mix-ups, degradation, or changes in physical or chemical properties. To maintain the original and intended quality, all participants in the excipient supply chain should carry out their activities according to appropriate standards for good trade and distribution practices as discussed in this chapter.

[NOTE—The Appendix consists of definitions and acronyms.]

### 1.2 Scope

This general information chapter provides recommendations for those activities and practices that ensure good trade and distribution practices for pharmaceutical excipients in order to ensure their intended quality. These activities and practices include quality management, organization, documentation, premises, storage, equipment, stability, prevention of adulteration, importation, packaging, repackaging, labeling, dispatch, transport, returned goods, and compounding practices. In addition, personnel, authenticity of data, expiration dating, retesting, complaints and recalls, handling of nonconforming materials, internal/external/third-party audits, quality agreements, shelf life, traceability, economically motivated adulteration, and conformance to compendial monographs are included. The procedures outlined here are applicable to all persons and manufacturers involved in the handling of pharmaceutical excipients and apply to every step in the supply chain. This chapter covers all materials designated as, or intended for use as pharmaceutical excipients, beginning with the point in the manufacturing process at which the final excipient is designated for pharmaceutical use.

### 1.3 General Considerations

Manufacturers, distributors, users, regulators, and consumers expect pharmaceutical excipients to be manufactured, packed, stored, and transported in a manner that does not compromise their suitability for use in medicinal products for human or veterinary use. Because they are components of drug products, excipients are drugs within the meaning of the U.S. Federal Food, Drug, and Cosmetic Act (FD&C Act), and thus the U.S. Food and Drug Administration (FDA) definition of adulteration applies when an excipient is not fit for its intended use.

Excipients are a diverse group of materials. They can be of animal, mineral, synthetic, or vegetable origin, and they include materials that are solids, liquids, or gases. Excipients can be packed and transported in container sizes ranging from a few grams to a railroad tank car.

Because of their diverse nature and the number of ways in which excipients can be transported from the manufacturing site through the supply chain to the ultimate site of use, this general information chapter cannot provide exhaustive detail for specific materials and modes of transport. Rather, this chapter provides general guidance about what is expected of those people and organizations involved in the supply and distribution of pharmaceutical excipients intended for use in the manufacture of pharmaceutical finished products. Hence, there are instances when *USP–NF* chapters *Good Manufacturing Practices for Bulk Pharmaceutical Excipients* ⟨1078⟩, *Bulk Pharmaceutical Excipients—Certificate of Analysis* ⟨1080⟩, and ▲*Significant Change for Bulk Pharmaceutical Excipients* ⟨1195⟩ ▲ (CN 1-May-2021) provide a more detailed guide about what is expected in these specific areas.

Excipients also are used in a variety of industries. Although most drug substances typically are made exclusively for use in pharmaceutical finished products, the pharmaceutical use of an excipient may be only a small fraction of the total use of the material across all industries. This complicates the regulation of both the manufacture and the supply of pharmaceutical excipients. Excipients often are manufactured outside the United States, which further complicates the regulation of the manufacture and the supply of pharmaceutical excipients. Thus, all stages in the supply chain for the pharmaceutical excipient require transparency and proper flow of the necessary information regarding the excipient shipment. In addition, to ensure compliance with this chapter, suppliers of pharmaceutical excipients must follow all applicable national, regional, and local laws and regulations.

### 1.4 Pharmaceutical-Grade Excipients

Pharmaceutical excipients must be prepared according to the recognized principles of good manufacturing practices (GMPs) using ingredients that comply with specifications designed to ensure that the resulting substances meet the requirements of

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the compendial monograph (see *General Notices, 3.10. Applicability of Standards* and chapter *Good Manufacturing Practices for Bulk Pharmaceutical Excipients* ⟨1078⟩).

*USP* or *NF* standards apply to any excipient marketed in the United States that is recognized in the compendium and is intended or labeled for use as an ingredient in a pharmaceutical product. The applicable standard applies to such articles whether or not the added designation *"USP"* or *"NF"* is used (see *General Notices, 3.10.10. Applicability of Standards to Drug Products, Drug Substances, and Excipients*). An ingredient may include the designation *"USP"* or *"NF"* in conjunction with its official title or elsewhere on the label only when a monograph is provided in the compendium and the article complies with the monograph standards and other applicable standards in the compendium including, but not limited to, the principles of GMP manufacture (see *General Notices, 3.20. Indicating Conformance*).

When *USP*- or *NF*-grade excipients are unavailable, manufacturers should first explore the use of materials which claim to comply with other pharmacopeias (e.g. EP, JP). If unavailable, pharmaceutical manufacturers should then consider appropriate alternatives (e.g. food-grade ingredients), provided such materials are suitable for the intended use. If a pharmacopeial grade is not used, a written justification should be available. The pharmaceutical manufacturer/user is responsible for the development and confirmation of suitable quality tests, procedures, and attributes to ensure that the material is appropriate for its intended use and that manufacturing is carried out under GMPs or a quality management system that demonstrates the same level of assurance of quality as that provided in *USP* (see ⟨1078⟩). It is an unacceptable practice to upgrade technical- or industrial-grade material to pharmaceutical-grade quality based only on analytical results that show compliance with the requirements of a pharmacopeial monograph.

### 1.5 Authenticity of Data

In the United States, the responsibility for the quality of the components of a finished pharmaceutical product lies with the organization that guarantees the quality of the finished pharmaceutical product. Thus, an important consideration in the purchase and supply of a pharmaceutical excipient is confirmation that the material is what it purports to be, that it meets specifications, that it was manufactured under applicable GMPs, that it has not been tampered with in any way before arriving at the site of intended use, that the appearance of the containers and other attributes of the shipment are comparable to those of previously received shipments of the same excipient and grade from the same supplier, and that it is fit for its intended use. Certain paperwork should accompany all shipments of pharmaceutical excipients. This paperwork should include a bona fide and legible copy of a Certificate of Analysis (COA) (see *Bulk Pharmaceutical Excipients—Certificate of Analysis* ⟨1080⟩).

When they receive a COA, manufacturers should take appropriate steps to verify the authenticity of the COA and the data contained therein. This has become particularly important in recent years because of instances of adulteration of excipients intended for use in the manufacture of pharmaceutical products. Steps to verify the authenticity of the COA should be taken at all stages in the supply chain.

Data on the COA can be verified in a number of ways, but the excipient user is responsible for confirming that the data are authentic by means of periodic verification of compliance with established specifications as stated in 21 Code of Federal Regulations Part 211 (21 CFR 211; see *Current Good Manufacturing Practice For Finished Pharmaceuticals*, http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=211, http://www.fda.gov/downloads/AboutFDA/CentersOffices/CDER/UCM095852.txt). In addition, other documents such as dispatch notes from previous stages in the supply chain can provide further evidence of the pedigree of the excipient shipment. Such documents are termed "pedigree documents".[1]

This chapter may present additional challenges for certain excipient users, e.g., compounding pharmacies. However, those who compound still are obliged to take all reasonable steps to verify that excipients they receive are fit for their intended use. Part of this verification can include an examination of pedigree documents and a signed certificate of conformance (COC) from the suppliers. Information contained in the *USP–NF* monograph's labeling requirements, FDA's Inactive Ingredient Database, and the CFR provide specific information about the excipient's permitted use in FDA-regulated products. All purchasers of pharmaceutical excipients should establish written procedures for the verification of data and verification that the excipient is fit for its intended purpose.

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## SECTION 2: QUALITY, ORGANIZATION, AND DOCUMENTATION

### 2.1 Quality Management

A Quality Management System (QMS) is a tool by which all parties involved in the excipient supply chain maintain the quality of the excipient. A documented quality policy is the cornerstone of the QMS and formally describes the company's overall philosophy with regard to quality as authorized by top or senior management. Additionally, an appropriate QMS should include:

- An organizational structure capable of supporting the elements of the quality policy
- Documented procedures and relevant records that demonstrate that a product will meet established quality criteria. This is commonly known as quality assurance (QA)
- Established procedures for approving suppliers of starting materials and verifying that they continue to meet agreed-upon requirements
- A material-release testing procedure to confirm the quality of excipients for their intended purpose(s)

Excipient manufacturers and suppliers should prepare a Quality Manual. The Quality Manual describes the elements of the QMS and includes the quality organizational structure, written policies, procedures, and processes or references to them, and a

[1] IPEC. The IPEC Excipient Pedigree White Paper. Arlington, VA: IPEC; ND. Available at: http://ipecamericas.org/sites/default/files/Excipient_Pedigree.pdf (Accessed July 6, 2011).

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description of departmental functions as they relate to the policies, procedures, and processes (see *Section 2.3 Documentation Requirements*). In implementing the QMS, companies must ensure that adequate qualified personnel are available to carry out the actions called for in the QMS and must avoid giving any one individual such extensive responsibilities that quality could be at risk.

COC to quality systems such as applicable International Organization for Standardization (ISO) guides or hazard analysis and critical control point (HACCP) analyses are not mandatory but provide assurance that products are produced and handled appropriately. However, certification to these quality systems should not be viewed as a substitute for the information contained in this chapter. In addition, internal audits should be conducted at regular intervals to confirm compliance with GMP (as applicable) and good distribution practices (GDP), and manufacturers should seek opportunities for improvement (see *Section 2.7 Audits: Internal, External, and Third-Party*).

All parties involved in the excipient supply chain share responsibility for the quality and safety of pharmaceutical excipients. These responsibilities should be delineated in a quality agreement between parties in the supply chain (see *Section 2.9 Quality Agreements*). All parties and their activities in the supply chain should be documented, and records should be maintained according to written procedures that ensure the traceability of all products acquired and distributed. All members of the supply chain have an obligation to protect excipients in their custody from deliberate economically motivated adulteration or deliberate introduction of foreign materials that could compromise the quality or performance of the excipient or adversely affect human or animal health.

## 2.2 Organization and Personnel

The organizational structure should be adequate and sufficiently staffed, and workers should be appropriately authorized for the activities they conduct. An organizational chart should delineate the responsibilities and interrelationships of personnel. Management ultimately is responsible for implementation of GDPs and ongoing verification that the QMS is maintaining the intended excipient quality.

Individuals within the company should have clearly defined responsibilities that are documented in writing. All individuals should understand their responsibilities and should be suitably qualified to perform their assigned duties. Their qualifications should be assessed for adequacy for their responsibilities and should be documented. Qualifications can include a combination of formal education, training, and experience. This also extends to any contracted service providers. Procedures should be in place to ensure that permanent, temporary, and contract employees minimize the possibility that unauthorized individuals will handle products.

An employee at each supply chain site should be designated and given the authority and responsibility for the implementation and maintenance of the QMS. The designated employee should have sufficient authority, qualifications, and resources to perform this function, as well as to identify and correct deviations from the QMS. Management and other personnel must not be subject to conflicts of interest or other pressures that could have an adverse effect on their ability to perform their duties related to product quality.

Staff should be aware of the principles of GDP included in this chapter and should receive regular, on-going training relevant to their responsibilities and to general quality principles. All training should be conducted according to a written training plan, and records of this training should be maintained. Personnel who have special duties such as handling hazardous materials or supervising activities required by local legislation may require additional training, including specific hazard management. Effectiveness of training should be verified regularly.

Personnel working with open product must understand and maintain good hygiene, health, and sanitation practices. Staff should use appropriate, nonshedding, protective apparel that will protect the product from the sampler as well as the sampler from the hazards of the product. Established procedures should eliminate the potential for product contamination by personal items such as jewelry, food, drink, or tobacco products. Written procedures that address hygiene, health, sanitation, and protective apparel should be in place.

Each supply channel party should have in place disciplinary procedures to address situations when personnel involved in the handling of products are suspected of or are implicated in inappropriate or illegal activities.

Some quality-related duties may be contracted to third parties, persons, or entities outside of the direct employ of the supplier. The delegation of these activities should be documented in a quality agreement or contract with the third party, and the organization should confirm compliance with the principles of GDP by conducting periodic on-site audits of these third parties. Delegation to a third party does not remove the organization's overall responsibilities for these activities.

## 2.3 Documentation Requirements

### 2.3.1 GENERAL

Organizations should have in place a system to control documents and data that relate to the requirements of the QMS.

### 2.3.2 QUALITY MANUAL

Organizations also should maintain a quality manual that describes the QMS, the quality policy, and the company's commitment to applying the appropriate GDP and quality management standards contained in this chapter. This manual should include the scope of the QMS, reference(s) to supporting procedures, and a description of the interaction between quality management processes.

### 2.3.3 DOCUMENT CONTROL

Procedures for the identification, collection, indexing, filing, storage, withdrawal, archiving, maintenance, and disposition of controlled documents, including documents of external origin that are part of the QMS, should be established and

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maintained. Procedures used for the handling and distribution of excipients should be documented, implemented, and maintained. In addition, organizations should establish formal controls relating to procedure approval, revision, and distribution. These controls should provide assurance that the current version of a procedure is used throughout the operational areas and that previous revisions of documents have been removed or withdrawn.

Designated qualified personnel should review documents and subsequent changes to the documents before the latter are issued to the appropriate areas. Documents that influence product quality should be reviewed and approved by the quality unit. Controlled documents may include a unique identifier, date of issue, and revision number to facilitate identification of the most recent document. The department with the responsibility for issuing the documents should be identified. The reasons for changes and the implementation date should be documented.

Electronic documentation should meet the requirements stated above for the document control system. If electronic signatures are used, they should be controlled to provide security equivalent to that given by a hand-written signature. Electronic documents and signatures also may need to satisfy local regulatory requirements.

#### 2.3.4 CONTROL OF RECORDS AND DATA

Procedures for the identification, collection, indexing, filing, storage, maintenance, and disposition of records and data should be established and maintained. Records and data should be maintained to demonstrate achievement of the required quality and the effective operation of the QMS. Records and data should be legible and clearly linked with the product or process involved. Pertinent third-party quality data also should be an element of these records.

Entries in records and data should be clear and indelible and should be made directly after the person performs the activity and then should be signed and dated by the person who made the entry. Corrections to entries should be signed and dated, leaving the original entry legible and with an explanation for the change, especially if this may not be obvious to subsequent reviewers.

Records and data should be kept for a defined period that is appropriate for the excipient, its use, and its retest or re-evaluation date. Records and data should be stored and maintained in such a manner that they are readily retrievable and in facilities that provide a suitable environment to minimize deterioration or damage. Electronic records and automated data-capture systems should meet the requirements for controlled records and data as stated above.

#### 2.3.5 CHANGE CONTROL

Procedures to evaluate and approve all changes, including evaluating the impact of the change on the quality of the excipient, should be established and maintained, for example, changes to the following:

- Authorized excipient manufacturer or packaging material supplier
- Manufacturing or packaging sites
- Excipient or packaging material specifications
- Test methods and laboratory
- Repackaging, labeling, and storage equipment
- Analytical equipment
- Repackaging, labeling, and storage processes
- Process and equipment changes at the original excipient manufacturer's site (see ▲*Significant Change for Bulk Pharmaceutical Excipients* ⟨1195⟩▲ (CN 1-May-2021))

An independent QA group should have the responsibility and authority for the final approval of any changes. The QA group may be part of another operational unit such as regulatory affairs or research and development.

Customers and, if applicable, regulatory authorities (e.g., those responsible for drug master files or certificates of suitability to the *European Pharmacopoeia*) should be notified of significant changes to established production and process control procedures that could affect excipient quality. The original manufacturer and downstream intermediaries (distributors and traders) should have excipient change control agreements in place defining the extent of notification by the original manufacturer in case of a change as described above. Each of the handling parties within the supply chain should have change control agreements to ensure that changes from the original excipient manufacturer are communicated to the end user. This change control agreement is part of the overall contractual agreements between the parties.

### 2.4 Complaints and Deviations

Customer complaints and information about possible defects should be systematically documented and investigated based on a written procedure with assigned responsibilities that describes the action that will be taken and includes the criteria on which a decision to recall a product should be based. Investigations should be formally conducted and written up in a timely manner to establish if the complaint is justified, to identify the origin or reason for the complaint (e.g., the repackaging procedure, the original manufacturing process, etc.), to identify root cause(s), to define any initial and follow-up action(s), and the method of communication (e.g., to the customer, original excipient manufacturer, authorities, etc.). Complaint records should be retained and regularly evaluated for trends, frequency, and criticality in order to identify possibly needed corrective or preventive actions.

Investigations should identify whether the reported defect is limited to a single batch of material or if other batches must be investigated. If additional batches are implicated, they should be identified and labeled accordingly (e.g., "under quarantine"). As necessary, appropriate follow-up action, possibly including a recall (as outlined in *Section 2.5 Recalls*), should be taken after investigation and evaluation of the complaint. Confirmed serious problems related to product quality (e.g., faulty manufacturing, packaging, or product deterioration) should be communicated upstream to the manufacturer and downstream to customer(s) in case they received material with the same batch number. A similar process should be implemented for the handling of deviations and product defects not identified by a customer complaint.

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## 2.5 Recalls

Those involved in the excipient supply chain should have a system for recalling promptly and effectively any materials known or suspected to be defective. Entities involved in the supply chain should implement written procedures to manage excipient recall (retrieval) in a timely manner. The procedures should:

- Describe how the process of recall (retrieval) should be managed based on the risk involved
- Describe a decision-making process with defined responsibilities
- Define the functions involved in the process (e.g., QA, sales, logistics, senior management, competent authorities, etc.)
- Define the communication process and documentation to parties within the supply channel as well as to regulatory authorities
- Define the steps needed to retrieve the material

If the original excipient manufacturer does not initiate a recall, it should be informed of the recall. Entities in the supply chain should have written procedures for the organization of any recall activity, and these should be regularly checked and updated. All recalled materials should be stored in a secure, segregated (quarantined) area while their disposition is decided. In the event of serious or potentially life-threatening situations, all customers and competent authorities in all countries to which an excipient potentially was distributed should be promptly informed of any intention to recall the excipient. All records should be readily available to the designated person(s) responsible for recalls. These records should contain sufficient information about materials supplied to customers (including exported materials). At regular intervals, QA groups in supply-chain organizations should evaluate the effectiveness of recall arrangements.

## 2.6 Handling of Nonconforming Materials

Nonconforming materials should be handled in accordance with a procedure that will prevent their inadvertent introduction or reintroduction into the market. They should be stored separately, either physically separated or under electronic control, to prevent their inadvertent introduction into commerce. Firms that conduct recalls should maintain records covering all activities, including destruction, disposal, return, and reclassification, and should perform an investigation to establish whether any other batches also are affected. They should document the investigation and actions taken to prevent recurrence of the problem. As necessary, firms should take corrective measures. Procedures should exist for the evaluation and subsequent disposition of nonconforming products, and the disposition of the material, including downgrading to other suitable purposes, should be documented. Nonconforming materials should never be blended with materials that comply with specifications.

## 2.7 Audits: Internal, External, and Third-Party

To verify compliance with the principles of GDP for pharmaceutical excipients, firms in the excipient supply chain should perform regularly scheduled internal audits in accordance with approved procedures. Firms should document audit findings and corrective actions and ensure that they are brought to the attention of responsible management. Accepted corrective actions should be completed in a timely and effective manner and should be conducted by designated, qualified individuals. Qualified individuals may be employees of the company, but they must be sufficiently removed from the function under audit so that their independence is not compromised.

Firms should perform external audits in accordance with approved procedures and schedules to assess the capability of suppliers to meet requirements for a product or service, as specified. A response to a questionnaire may be considered in the auditing process but generally does not take the place of on-site inspections and should not be considered a substitute when an audit is required. Independent auditing organizations can perform third-party audits to determine the level of compliance or conformance to specified standards and regulations (e.g., GMP, GDP, and ISO).

## 2.8 Contract Activities

Any GDP-related activity that is delegated to another party should be agreed upon in writing in an approved contract with clearly defined responsibilities. The contract should clearly establish which party is responsible for each applicable quality activity. Before entering into an agreement, the contract giver should evaluate the proposed contract acceptor's compliance with GDP as described in this general chapter. The evaluation should include an initial on-site audit of the contract acceptor's premises and quality system, giving special consideration to the prevention of cross-contamination and maintaining traceability. The contract should also include the responsibilities of the contract giver for measures to avoid the entrance of counterfeit or adulterated materials into the distribution chain.

There should be no gaps or unexplained lapses in the application of GDP. The contract acceptor should conduct periodic on-site auditing of contracted distribution activities with regard to the application of GDP by the contract giver. Subcontracting may be permissible under certain conditions, subject to approval by the original contract giver, especially for activities such as sampling, analysis, repacking, and labeling. If subcontracting occurs, the subcontractor should conform to the same GDP standards as the primary contract giver. The subcontractor also should permit an on-site audit by the contract acceptor's quality unit or its designee.[2, 3]

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[2] WHO. *Good Trade and Distribution Practices for Pharmaceutical Starting Materials*. Geneva; WHO: Technical Report Series, No. 917, 2003, Annex 2. Available at: http://who.int/medicines/areas/quality_safety/quality_assurance/GoodtradeDistributionPracticesTRS917Annex2.pdf?ua=1 (Accessed March 2, 2015).

[3] WHO. *Finished Products: Good Distribution Practices for Pharmaceutical Products*. Geneva; WHO: Technical Report Series 957, 2010, Annex 5. Available at: http://whqlibdoc.who.int/trs/WHO_TRS_957_eng.pdf (Accessed July 7, 2011).

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## 2.9 Quality Agreements

Quality agreements are legally binding and are mutually negotiated between parties involved in the supply chain for pharmaceutical excipients. The quality agreement identifies who is responsible for certain quality activities and how quality issues will be resolved between the parties. Although they are intended to address the parties' quality commitments, quality agreements are not designed to take the place of an audit.

Suppliers should have in place quality agreements between themselves and the parties with whom they do business. Original excipient manufacturers should have quality agreements in place with their direct customers and authorized distributors of their products. Distributors should also have agreements with end users and other parties in the supply chain to whom they supply products. All entities in the supply chain should fully understand which entity is responsible for the GDP-related activities (as outlined in this chapter) at each step in the supply chain.

Quality agreements should address the quality systems requirements, but they are not intended to list every element of the quality system. It is not necessary to reiterate agreement on every point of the quality system when the parties state general agreement on the applicable quality standard. Quality responsibilities included in a quality agreement should be those that may require action by one or both parties to the agreement.

A key element that must be defined in the quality agreement is the communication pathways and timing for quality events. Parties must be clear about their responsibility for notifying the next party in the supply channel and for notifying the applicable regulatory authorities in the case of a significant quality event. Many times a decision about who should notify the regulatory authority is a collaborative effort between the parties. Depending on the issue's impact, the timing of these notifications relative to the time of the incident should be specified within the quality agreement.

Both parties to the agreement are responsible for ensuring that the quality agreement is maintained as an accurate document throughout the life of the business relationship. Revisions to this document may be needed as regulatory requirements change, new products are supplied, or a new material risk arises. The parties should maintain a history of the revisions to the quality agreement.

***Change to read:***

# SECTION 3: PREMISES, STORAGE, REPACKAGING, AND STABILITY

## 3.1 Buildings and Facilities

Organizations should establish operating procedures for the use of buildings and facilities, including the areas discussed below, and firms should consider protective measures to ensure the security of the grounds (e.g., fencing or perimeter walls).

The buildings and facilities used in the storage and handling of excipients should restrict access to allow entrance only by authorized persons to areas used for the manufacture, packaging, and holding. Organizations should take precautions to prevent unauthorized persons from entering limited-access areas. When the status of excipients requires protection from use (e.g., quarantine), organizations must have clearly marked limited-access controls in place, or they should use validated computerized systems to prevent material distribution before approved release.

Buildings should be of adequate size and capacity to allow the orderly flow of materials, proper storage and handling of materials, and appropriately controlled environmental conditions for the final dispatch of excipients into and out of the premises. Buildings should be maintained in a good state of repair. The construction materials must be easily cleanable and maintained, and buildings and facilities should be designed to prevent cross-contamination, product mix-ups, or the accumulation of filth or contaminating materials, particularly when excipients are exposed to the environment. Adequate storage space must be available for excipients that are highly sensitizing or toxic, and dedicated facilities may be necessary. Adequate procedures should be in place to ensure the cleaning, maintenance, and use of buildings and facilities.

Receiving and dispatch bays should be designed to protect the facilities and excipients during loading and unloading during adverse weather conditions. Incoming bay areas should be designed and equipped to allow containers to be cleaned before storage. A pest-control system should be in place to ensure that materials are protected from infestation by insects, rodents, animals, birds, or other vermin. There should be written procedures defining the adequate holding and storage of excipients, including pest-control processes. The pest-control materials must be safe and must be known not to cause contamination. Approved pesticides, insecticides, and rodenticides should be used and documented. Excipients that may contain contamination must be controlled to prevent cross-contamination in holding areas or the spread of contamination to other areas of the facility.

## 3.2 Warehousing and Storage

Written procedures should describe the receipt, storage, dispatch, and other handling of excipients, as well as the security measures necessary to prevent theft of materials or the introduction of counterfeit or adulterated materials into the supply chain. Buildings should be adequately lighted and should have proper utilities for the intended activities. They should be dry and controlled to appropriate environmental conditions. Buildings and facilities should store excipients in the proper environmental conditions. Temperature-controlled and -monitored storage should be available as required for any building used for holding excipients.

Warehousing and storage conditions for excipients should comply with the monograph specifications, as reflected in the excipient's container label. When specific storage conditions are required for excipients (e.g., temperature and humidity control), they should be provided in a controlled manner, monitored (e.g., by an alarm system or manual control), and recorded. Any automated system(s) used to monitor the environmental conditions for areas where excipients are handled or stored must be validated. An approved document should indicate the location of each environmental monitoring device and the condition(s) it monitors. The locations for these devices or probes should reflect the extreme environmental conditions of the space as

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determined by an environmental mapping exercise. Excipients that present risks such as fire or explosion should be stored in safe, dedicated areas. Excipients that are sensitizing or toxic should be adequately and appropriately segregated, and warehouse and storage areas should be routinely cleaned, appropriately maintained, and free of pests.

Excipients should be stored in a manner that permits cleaning of the storage area and movement of materials. Pallets used to hold materials should not cause contamination, and required pallet quality and construction materials should be defined in writing. Pallets should be clean and in a good state of repair, and firms should appropriately track supplies to ensure adequate treatment of the wood materials. Wood pallets, if used, should comply with import requirements.

Organizations should have in place written procedures to ensure that the excipient will be supplied within its expiry or retest period and should have adequate controls to prevent the distribution of expired excipients. If no expiry date is applicable, the first-in-first-out principle should be used. Rejected excipients and other materials related to excipient quality (e.g., packaging components) should be so labeled or identified, and controls such as physical or electronic separation should prevent their use pending final disposition. During the warehousing or storage of excipients, any known broken or damaged containers should be withdrawn from usable stock, and the containers should be handled as rejected materials.

Materials quarantined pending a release decision should be labeled or identified (e.g., electronically) to prevent unauthorized use. These materials should be held from use, and written procedures should guide final disposition. There should be written procedures for the cleanup of any spillage to ensure complete removal of any risk of contamination.

#### 3.2.1 ENVIRONMENTAL CONTROLS

When excipients require specific storage conditions to preserve their integrity and quality during the retest/re-evaluation or expiry interval, the storage conditions required should be stated on the label, labeling, or other literature, e.g., the Excipient Information Package[4] or COA. Distributors should follow the information and requirements for environmental controls provided by the manufacturer and should provide appropriate controls and monitoring to ensure adherence to the stated storage conditions with appropriate documentation. Distributors should also maintain records to indicate the excipient was stored according to the manufacturer's recommendations and should conduct regular assessments to confirm that designated conditions are met.

If the manufacturer does not indicate specific storage conditions, the distributor should ensure that proper storage conditions are maintained to protect the packaging and labeling. Uncontrolled warehousing conditions vary with geographical location, particularly with latitude. If the excipient is shipped to geographical locations that have storage conditions well outside the conditions used in the manufacturer's stability study or justification for the absence of special storage conditions, then additional studies may be required to show stability at the new conditions. A warehouse-monitoring program should be established if the effects of the new environmental conditions are not known.

Outdoor storage of excipients (for example, bulk materials, flammable materials, acids, or other corrosive substances) is acceptable provided the containers give suitable protection against deterioration or contamination of their contents, identifying labels remain legible, discharge ports have adequate protective closures, and the exteriors of moveable containers are adequately cleaned before opening and use.

### 3.3 Equipment

Equipment used in bulk transport, repackaging, labeling, testing, or storage of the excipient should be maintained in a good state of repair and should be of suitable size, construction, and location to facilitate cleaning, maintenance, and correct operation. Equipment should be verified before use to ensure that it is constructed, installed, and functioning as required for the excipient. When equipment is located outdoors, there should be suitable controls (e.g., closed systems or protective encasements) to minimize environmental risks to excipient quality.

When possible, dedicated equipment (e.g., bulk trucks, packaging equipment, storage tanks, pipework, hoses, and pumps) should be used in direct contact with the excipient. When nondedicated equipment is used in direct contact with the excipient, validated cleaning procedures should be applied. A restricted prior-cargo list should be supplied to transport companies in case non-dedicated bulk transport equipment is used. Quality-critical measuring equipment and balances for the handling and testing of the excipient should be of appropriate range and precision. Such equipment should be identified.

#### 3.3.1 EQUIPMENT CONSTRUCTION

Equipment in contact with an excipient should be constructed so that contact surfaces are not reactive, additive, or absorptive and thus do not alter the quality of the excipient. Substances required for operation, such as lubricants or coolants, preferably should not come into contact with excipients and packaging materials. When contact is possible, distributors should use materials of suitable quality that will not affect product quality. The choice of such materials should be justified.

Equipment should be designed to minimize the possibility of contamination from the environment and direct operator contact during activities such as unloading bulk trucks, use of transfer hoses (particularly those used for transfer of excipients), sampling, repackaging, and cleaning. Distributors should consider the sanitary design of equipment in contact with excipients. They should assess the suitability and integrity of seals in order to minimize the risk of contamination. Piping should be appropriately labeled to indicate the content and direction of flow.

#### 3.3.2 EQUIPMENT MAINTENANCE

Documented procedures should be established and followed for maintenance of critical equipment used in the repackaging, labeling, testing, or storage of the excipient. Distributors should maintain records (e.g., logs, computer databases, or other

[4] IPEC. *The IPEC Excipient Information Package (EIP): Template and User Guide*. Arlington, VA: IPEC; 2013. Available at: http://ipecamericas.org/reference-center/document-depot (Accessed June 23, 2017).

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appropriate documentation) of quality-critical equipment use and maintenance. Defective equipment should be removed or appropriately labeled to avoid misuse.

Quality-critical measuring equipment and balances should be controlled on a scheduled basis. This control should include the following:

- Calibration of instruments or other appropriate verification at suitable intervals, according to an established documented program
- Establishment of the equipment's limits of accuracy and precision
- Provisions for remedial action in the event that accuracy or precision requirements are not met

Calibration standards should be traceable to recognized national or compendial standards as appropriate. Instruments and equipment that do not meet established specifications should not be used, and an investigation should be conducted to determine the validity of the previous results since the last successful calibration. The current calibration or verification status of quality-critical equipment should be known to users and should be verifiable.

#### 3.3.3 EQUIPMENT CLEANING

Cleaning equipment should be chosen and used so that it cannot be a source of contamination. Cleaning materials should be appropriate for the task, and their selection should be justified. Rotation of sanitizing and cleaning agents should be considered where appropriate. In order to avoid contamination with cleaning products or products previously processed in the equipment, written cleaning procedures should be established for equipment that comes in contact with the excipient. Cleaning procedures should contain sufficient detail to allow cleaning in a reproducible and effective manner. Cleaning and sanitation processes should be recorded, and evidence of their effectiveness should be provided, for example, by the following:

- Testing the final rinse after cleaning for residues of the previous product
- Checking the equipment after cleaning for residues of the previous product
- Testing each batch for residues of the previous product handled with the same equipment

### 3.4 Sampling, Repackaging, and Labeling

To minimize risks associated with repackaging and labeling, appropriate GMPs should be applied (see *Good Manufacturing Practices for Bulk Pharmaceutical Excipients* ⟨1078⟩). For completeness, certain key activities and the necessary precautions are discussed below.

#### 3.4.1 BLENDING, REPACKAGING, AND LABELING

Operations such as combining sublots into a homogeneous batch, repackaging, or labeling are manufacturing processes, and therefore distributors should follow appropriate GMPs (see ⟨1078⟩):

- Processes whereby the excipient's packaging is opened and the excipient is exposed to the environment (for example, transferring excipient from one container to another, including from bulk equipment to storage tanks/silos or from storage tanks/silos into containers) are critical handling steps related to the integrity of the finished product. If only the secondary packaging is modified, operators should take appropriate care to maintain the integrity of the primary packaging and the excipient.
- Excipients may degrade because of exposure to the repackaging atmosphere (e.g., oxygen, humidity, light, and temperature).
- Excipients can be contaminated by foreign matter such as lubricants, cleaning materials, or other substances.
- Transparency to the customer that relabeling, with or without opening the original excipient manufacturer's packaging, has occurred is critical to representation of the product quality and suitability for use.
- Transparency to the customer of data sources listed on certification documentation (labeling) is critical to representation of the product quality and suitability for use.

#### 3.4.2 REPACKAGING AND LABELING BATCHES

Staff in the excipient supply chain should give special attention to the following points:

- All repackaging and labeling requirements should be defined in written procedures.
- Contamination, cross-contamination, and mix-ups should be avoided by the use of suitable equipment and cleaning procedures and with adequate labeling.
- Environmental conditions and repackaging procedures should be designed to avoid contamination and to maintain the integrity of the excipient during repackaging and labeling.
- Operators should consider the use of filtered air in the repackaging area if necessary for the product. The standard of filtration should be justified.
- Labels should be printed using a controlled process (see *Section 3.4.9 Repackaging and Labeling*).
- Personnel involved in repackaging processes should wear clean protective apparel such as head, face, hand, and arm coverings, as necessary, and should practice appropriate personal hygiene (e.g., hand disinfection following health requirements, health monitoring, and removal of jewelry). Personnel should be trained about special hygiene requirements, and this training should be documented.
- Repackaging areas should be cleaned and sanitized regularly.

Batch numbers should be assigned according to documented procedures. When staff assigns new batch numbers, they should ensure traceability to original batch numbers by proper documentation. Assigning one batch number to containers of

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different batches that comply with the same specification is an unacceptable practice (see also *Sections 3.4.3 Excipient Batch Homogeneity* and *3.4.4 Blended Excipients*).

- As part of the batch record, a copy of the information on the original labels should be retained (e.g., a photocopy). A sample of the new label should also be kept.
- All repackaging and labeling processes should be designed and carried out to avoid commingling, contamination, and mix-up and to ensure full traceability of the excipients back to the original excipient manufacturer and traceability downstream to the final customer. Responsible personnel should sufficiently record every completed step, along with the name of the operator and the date and time each step was completed, e.g., in the master batch manufacturing record, or by means of computerized systems.

### 3.4.3 EXCIPIENT BATCH HOMOGENEITY

Mixing to form a homogeneous batch is a manufacturing step and should be defined in a written procedure. A batch can be homogeneous only when conforming materials are thoroughly mixed. The conformity of each batch with its specification should be confirmed before it is added. Mixing should always be controlled, and homogeneity should be verified and documented (see *Good Manufacturing Practices for Bulk Pharmaceutical Excipients* ⟨1078⟩). Blending of batches or lots of excipients that individually do not conform to specifications with other lots that do conform (in an attempt to salvage or hide adulterated or expired material) is not an acceptable practice. Only excipients from the same manufacturing site received by a distributor and shown to conform to the same specifications can be mixed. The customer should be informed that the material supplied is a mixture of the manufacturer's batches.

### 3.4.4 BLENDED EXCIPIENTS

The blending process should be verified to ensure that it does not influence the quality of the excipient. The blended excipient should be tested to ensure conformance to the specification and to provide data for the COA (see ▲*Significant Change for Bulk Pharmaceutical Excipients* ⟨1195⟩▲ (CN 1-May-2021)). Under certain circumstances and with appropriate controls, a COC can be used if the basis for the claim of conformity is traceable within the document. The blended batch referred to in the new certification document should be traceable to all the original certification documents and batch numbers (see ⟨1078⟩).

### 3.4.5 CERTIFICATES OF ANALYSIS

The original excipient manufacturer's COA should be retained and made available to the user on request. The batch referred to in the COA delivered to the end user should be traceable to the original excipient manufacturer's COA. Quality documents accompanying deliveries should be subject to an agreement between the distributor and the final customer. For retesting, analytical methods of the original excipient manufacturer or pharmacopeial methods should be applied. When other methods are applied, these should be agreed upon by both parties.

### 3.4.6 CONTAINER–CLOSURE SYSTEMS

For repackaged material, the repackager is responsible for justifying the shelf life and repackaging conditions. The original manufacturer and the distributer should share information and agree about repackaging conditions and primary packaging materials. They should establish primary container–closure system material and packaging configuration specifications, and they should develop a written procedure that clearly defines packaging for each individual excipient based on its stability.

If the same types of primary container–closure system and packaging configuration are used for repackaging, then the new container–closure system and packaging configuration should be equivalent to that used by the original excipient manufacturer. The repackager and distributor should consider exposure of the excipient to the repackaging environment, and both can rely on the manufacturer's stability evaluation and thus assign the same shelf life for the excipient.

When the repackager's primary container–closure system's packaging configuration differs significantly from that of the original manufacturer [e.g., in terms of desiccants, permeability of the protective barrier layer (which may be either the primary or secondary container–closure system), or the headspace], the repackager must demonstrate that the new system is adequate to protect the excipient from contamination and deterioration for the shelf life (retest or expiration period) defined by the excipient manufacturer. Otherwise, the shelf life defined by the manufacturer cannot be transferred to the repackaged material. The need for stability studies should be confirmed (see *Sections 3.4.14 Stability and Expiration Dates* and *3.5 Retesting and Shelf Life*).

The container–closure system for the pharmaceutical excipient should protect the material from the time of packaging until its final use by the drug product manufacturer. The container–closure system should be designed to help prevent theft or adulteration by counterfeiting.

Storage and handling procedures should protect containers and closures and minimize the risk of contamination, damage or deterioration, and mix-ups (e.g., between containers that have different specifications but are similar in appearance).

### 3.4.7 RETURNED AND REUSED CONTAINERS

Returned containers may have unknown residues from uses other than the intended one. Therefore, use of new containers is recommended for excipients. If containers are reused, a rationale for the extent of cleaning should be justified and documented for specific excipients and different types of containers. Repackagers should collect evidence that the quality of the material packed is not adversely affected by reuse of containers.

Distributors and customers should have an agreement defining the specific conditions for reuse (e.g., handling, sealing, and cleaning). If returnable excipient containers are reused, all previous labeling should be removed or obliterated.

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### 3.4.8 ENVIRONMENTAL CONTROLS

Environmental controls should ensure that temperature, humidity, and cleanliness of air and equipment are appropriate to avoid any contamination to or deterioration of the excipient. The necessary environmental conditions for the repackaging of each excipient should be defined. Environmental control is a specialist subject, and experts should be consulted (see also *Section 2.6 Handling of Nonconforming Materials*).

### 3.4.9 REPACKAGING AND LABELING

Repackagers should implement procedures to ensure that the correct quantity of labels is printed and issued and that labels contain the necessary information. Sufficient crosschecks should be in place to ensure proper data transfer. Procedures should be in place to avoid mislabeling, and printing and use of labels should be restricted. All labeling operations (e.g., generating, printing, storage, use, and destruction) should be recorded. Labeled containers should be inspected, and surplus labels should be destroyed to avoid any misuse. If labels are not printed immediately before each specific labeling operation, the security of the label stock should be controlled, and access limitations should be defined. Repackaging and labeling facilities should be inspected immediately before use to ensure that all materials that are not required for the next repackaging operation have been removed.

### 3.4.10 REPACKAGED EXCIPIENTS—ACCOMPANYING DOCUMENTATION

Deliveries of repackaged excipients should be accompanied by information about the original manufacturing site (name and address) and repackaging and labeling sites. This information should be provided in the supplier certification documentation (e.g., COAs) or by other means (see *Section 4.8 Traceability*). The supplier should provide this information to the customer via official communications.

### 3.4.11 TESTING OF REPACKAGED EXCIPIENTS

Appropriate testing of repackaged excipients should be performed to demonstrate consistent excipient quality. Testing to the complete monograph may not be necessary, but the recipient should test defined key quality parameters that could be affected by the repackaging process. Recipients should consider the manufacturer's recommendations for key quality parameters, and until these tests have been performed the repackaged materials should be kept under quarantine and should be identified as quarantined material. The materials should comply with the defined specifications before they are released for distribution.

Excipient testing and release should be performed under the responsibility of the quality unit and should conform to written specifications and analytical test requirements. Repackagers should ensure that test data are recorded and that results are evaluated before release of the repackaged or transferred excipient.

The excipient cannot be upgraded as a result of any repackaging process. It is unacceptable to upgrade nonpharmaceutical grades to pharmaceutical grades on the basis of conforming analytical results, i.e., by testing to pharmacopeial standards. Pharmaceutical grades can be achieved only when the excipient is originally produced and subsequently processed in accordance with GMPs (see *Good Manufacturing Practices for Bulk Pharmaceutical Excipients* ⟨1078⟩).

### 3.4.12 OFFICIAL PHARMACOPEIAL METHODS FOR RETESTING

For control of key parameters during repackaging or full retesting of excipients, official pharmacopeial methods or methods validated against the pharmacopeial methods should be used. Otherwise, repackagers should use the original excipient manufacturer's analytical methods. The methods used should be listed on the COA accompanying the excipient or should be made available to the customer by other documents. These documents should also reference any contract laboratory that is used to perform analyses. The COA should clearly identify which tests have not been performed on the repackaged or transferred batch but have been taken from the original manufacturer's COA.

### 3.4.13 SAMPLING

Excipient sampling must be done in a manner that prevents contamination, and dedicated sampling areas with adequate environmental controls are necessary. Areas for sampling should be designed to allow cleaning of the outside of the container before the container is opened. Adequate cleaning procedures should be in place for the sampling areas. Sampling tools should be dedicated to the sampling area and also to the specific material, or sampling tool cleaning must be validated to ensure no cross-contamination from the tool.

Any container opened for sampling should be marked with the date and name of the person who performs this operation. The amount of sample removed should be recorded.

If excipients are repackaged, processed, or packaged from bulk, retained samples representative of the excipient batch should be kept for at least one year after the expiration or re-evaluation date or for at least one year after distribution is complete, whichever is longer. The minimum sample size should be based on the amount required to perform at least two complete analyses. Sample storage conditions should prevent any contamination or deterioration and should comply with the label storage conditions (see general information chapter, *Bulk Powder Sampling Procedures* ⟨1097⟩).

### 3.4.14 STABILITY AND EXPIRATION DATES

Excipient stability and expiration dating of excipients are primarily the responsibility of the original manufacturer. Whenever the original manufacturer's packaging is opened, the repacker is responsible for providing evidence that the excipient manufacturer's stability and expiration dating are still applicable.

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If a distributor transfers an excipient to another container or repackages it, stability and shelf life (retest or expiry period) should be taken into account. The type of container, primary packaging materials, barrier packaging materials, packaging configuration, environmental exposure during repackaging, and storage conditions at the repackaging site should also be taken into account when the shelf life (retest or expiry period) is defined. The recommended expiration date provided by the original excipient manufacturer should not be extended without demonstrating sufficient stability to justify extended shelf life (retest or expiry period). If shelf life is extended beyond the original manufacturer's recommendation, the type of packaging, storage conditions, and stability-indicating analytical data should be clearly defined, and the repacker assumes the primary responsibility for the extension.

If special storage conditions (e.g., inert gas overlay, protection from light, heat, moisture, etc.) are needed, the restrictions should be indicated on the new labeling (see *Section 3.5 Retesting and Shelf Life*).

## 3.5 Retesting and Shelf Life

The organization's stated shelf life or retest/re-evaluation interval should be maintained for the excipient. Expiration or shelf life dates indicate the period beyond which the excipient should not be used or distributed. Retest/re-evaluation intervals indicate the period beyond which the excipient must be evaluated to determine continuing acceptability for use. The expiration/shelf life date provided by the original excipient manufacturer should not be extended without documentation from the manufacturer demonstrating sufficient stability to justify an extended shelf life. Such documentation should specify the type of container and storage conditions necessary to make this claim, and the distributor should have documentation that the excipient was stored in the stated container and under the necessary conditions.

Excipients without expiration, retest, re-evaluation, or shelf life dates should be accepted for use only if the manufacturing date can be confirmed and only if the excipient has been held and shipped under conditions that conform to the appropriate standards of GMP or GDP. Distribution of the excipient beyond the retest/re-evaluation period should be done only in consultation with the manufacturer and with the consent of the purchaser or recipient. If the distributor has the capabilities for sampling and performing the manufacturer's specified evaluation, then the distributor can perform the assessment. Sampled lots should be placed under quarantine to prevent shipping during the evaluation.

Distributors who do have capabilities for sampling according to the manufacturer's instructions but do not have testing or evaluation capabilities should send the samples to the manufacturer or a qualified third-party laboratory for retesting/re-evaluation. Excipient lots that conform to the manufacturer's criteria can be released from quarantine, and the distributor's supporting evaluation data should accompany the original excipient manufacturer's data to indicate the excipient's acceptability for use. If the distributor does not have the capability to sample or evaluate the excipient, it should not be shipped to customers beyond the end of the retest/re-evaluation interval. The excipient or a representative sample of the excipient can be returned to the manufacturer or a third party for retesting/re-evaluation. The excipient can be held by the distributor pending further results obtained from the representative sample.

If an excipient is transferred to another container or is repackaged by the distributor, the latter must conduct an assessment of the stability of the excipient to determine if the original excipient manufacturer's information can be carried forward. If the distributor uses the same type of packaging material that provides the same packaged environment (headspace, surface area, closure tightness, etc.) as that used by the original manufacturer and if the transfer or repackaging is performed in a manner that protects the excipient from adverse environmental effects that could affect the stability, then the original excipient manufacturer's shelf life/expiry date or retest/re-evaluation interval can be carried forward. If primary packaging material or barrier packaging material differs from the original excipient manufacturer's primary packaging material or if the packaged environment varies significantly, then an evaluation of the container and its closure system should demonstrate that it is adequate to protect the excipient from deterioration and contamination during the manufacturer's shelf life/expiry date or retest/re-evaluation interval. Otherwise, a stability assessment is necessary to determine the appropriate shelf life/expiry date or retest/re-evaluation interval for the repackaged excipient. Such assessments should be conducted according to the manufacturer's specifications and test methods.

## 3.6 Expiration Dates

Not all excipients have an expiration date, but if one is assigned it should be displayed on the container and should show the period during which the excipient is expected to remain within specifications if stored properly and after which it should not be used. It is established for every batch by adding the shelf life to the date manufacturing began. The expiration date is based on the type of container and storage conditions, so these parameters should be clearly defined. If special storage conditions are needed (e.g., protection from light, oxygen, heat, humidity, etc.), they should be indicated on the labeling because they could influence usability through the expiration date.[5]

The expiration dates for excipients should be established by documented stability tests or long-term stability data. Occasionally, the expiration date may be established by reference to historical data. Stability involves not only the compendial requirements but also changes in performance properties. Excipient stability tests should determine whether possible degradation, changes in molecular weight and distribution, moisture gain or loss, viscosity changes, microbiological contamination, or other possible changes in excipients could occur when the excipient is stored in a specific container–closure type at specific storage conditions. Stability for repackaged excipients can be found under *Section 3.5. Retesting and Shelf Life*.

[5] IPEC. *The IPEC Excipient Stability Program Guide 2010*. Arlington, VA: IPEC; 2010. Available at: http://ipecamericas.org/reference-center/document-depot (Accessed June 23, 2017).

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## 3.7 Labels, Icons, and Labeling

### 3.7.1 LABELS AND ICONS

Label-generating systems and processes should be secure, controlled, and documented. Appropriate verification records should be maintained, and each container should be appropriately identified and labeled. Labels applied to individual small containers should be clear, unambiguous, and permanently fixed in the company's established format. The information on the label should be indelible. Alternative methods can be used for bulk containers/transport and should be justified.

The label may include wording or depict icons to highlight storage and transportation handling requirements and hazards (e.g., avoid dropping, maintain specified environmental conditions, etc.). The use of symbols that are recognized by international organizations is recommended (see *Risks and Mitigation Strategies for the Storage and Transportation of Finished Drug Products* ⟨1079⟩ ). During international distribution, the proper language(s) should be used to ensure that handlers understand the requirements set forth on the label.

### 3.7.2 LABELING

The labeling (which includes both the label and any accompanying documents) should include at least the following information:

- Name of the excipient, including grade and reference to pharmacopeia, as relevant
- If applicable, the International Nonproprietary Name
- Amount (weight or volume)
- Batch number assigned by the original excipient manufacturer or the batch number assigned by the repacker if the material has been repacked and relabeled
- Retest date or expiry date (as applicable)
- Any specified storage conditions, as applicable
- Handling precautions, where necessary
- Identification of the original manufacturing site as agreed with the pharmaceutical customer (see *Section 4.8 Traceability*)
- Name and contact details of the suppliers

***Change to read:***

# SECTION 4: RETURNED GOODS, DISPATCH, TRANSPORT, IMPORTATION, ADULTERATION, AND TRACEABILITY

## 4.1 Returned Goods

### 4.1.1 GENERAL

Return of goods by users to suppliers should be reviewed on a case-by-case basis. The distributor should facilitate a root cause analysis and investigation of complaints.

[NOTE—Users should document the reason(s) for return of goods to the supplier.]

Before returning the goods, if the user identifies and confirms unacceptable product quality the user should provide the supplier with the user's supporting documentation, such as tests and investigation results. If requested, the user should also provide product samples used for tests and investigations. The supplier should be provided an opportunity to conduct thorough investigations to confirm the validity of the user's quality complaint. While the investigation proceeds, the user should quarantine the material in accordance with internal standard operating procedures and should store the material in an area specifically designated for returns, with limited access to operations, and well-separated from incoming or released raw materials.

Goods returned by the user because of excess inventory or other causes unrelated to quality can re-enter commerce within the specified shelf life, provided conditions of storage, transportation, and container integrity have been thoroughly reviewed by the supplier and the quality of the excipient has not been compromised in any way. A formal documented review of each returned container and container tamper-evidence device should be done to verify that these match the container configuration when the materials left the supplier's facility.

If the user opens a commercial packaging container for sampling or investigation (related or unrelated to quality issues) and whether any material was taken out or not, each container should be clearly labeled *Opened*. Written documentation should be provided to the supplier confirming that the container(s) were opened and resealed according to GMPs and describing the reasons for opening, amount withdrawn, and how the pack/container was resealed. Documentation of returned goods should contain a detailed description of all such events including repackaging. Returned excipient containers opened by the user should be clearly identified as such and should not be released as pharmaceutical excipients. In exceptional cases, the material can be released as excipient-grade product if a documented thorough investigation shows no risk of product contamination or deterioration. The quality department should release this material.

Users and suppliers should maintain records of all returned goods, including the product name (trade name and chemical name), batch or lot number, reason for the return, quantity returned, and investigation documentation when applicable. In addition, the supplier should record the final disposition of the material. If returned excipients have been held, stored, or shipped under conditions that could compromise product quality (including ingredients, containers, or labeling), the manufacturer

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should destroy the excipients. Exceptionally, manufacturers can release the excipients if their examination, testing, and investigations prove that the material meets suitable standards of identity, quality, and purity and that GMPs and GDPs have not been compromised.

#### 4.1.2 DISPOSITION OF RETURNED GOODS

The excipient manufacturer's and/or supplier's quality unit should assess the returned product. The options are the following:

- Return to commerce
- Reprocess
- Regrade to a less stringent standard such as technical or industrial grade (non-GMP use)
- Destroy

Only containers that have not been opened should be considered for return to commerce without further action.

If the quality assessment of returned goods leads to their final destruction and if associated batches are potentially implicated, an appropriate investigation should be conducted and documented to show that the quality of the associated batches is not affected.

#### 4.1.3 REPROCESSING

Reprocessing is a manufacturing step, and the requirements of *Good Manufacturing Practices for Bulk Pharmaceutical Excipients* ⟨1078⟩ apply. The requirements of ⟨1078⟩ apply only to those intermediate supply chain entities that undertake reprocessing.

### 4.2 Shipping and Transportation

#### 4.2.1 SHIPPING

The supplier (the manufacturer or distributor) of pharmaceutical excipients should ensure that the integrity of the pharmaceutical excipient is maintained by the appropriate storage and transport conditions as described in product labeling. After training, staff should follow written procedures for shipping pharmaceutical excipients. These procedures include the requirement to follow the recommended storage and transportation requirements including temperature, humidity, or other special handling precautions.

Actions should be documented when they are performed. Shipping records for pharmaceutical excipients should provide for the following information:

- Date of shipment
- Name and address of the entity that accepted the materials for the transportation
- Mode of transportation
- Name, address, and status of the consignee
- Material name
- Quantity shipped
- Batch number and expiry date
- Required storage and transport conditions (refrigeration, freezing, or controlled room temperature required)
- Shipping code or identification number of the delivery order

When regulatory actions such as FDA Field Alerts or drug product recalls occur, the excipient handler must be prepared to act promptly. Shipping documentation must be sufficient to allow adequate handling of any excipient associated with regulatory action. When reasonable, the shipping schedule for excipients should be documented, and responsibilities can be enumerated in a quality or collaborative agreement between entities to show ownership in the supply chain (Entity A to Entity B; Entity B to Entity C; etc.—see *Section 4.4 Packaging: Tamper-Evident Seals*). The buildings and facilities used to ship materials should be appropriate for their intended use in the storage and handling of excipients (see *Section 3.1 Buildings and Facilities*).

Before loading materials, shippers should inspect the container and vehicle to ensure cleanliness and other consignments (if the shipment is a part load) to ensure no form of contamination is likely to occur. This inspection should be documented according to a written procedure. Materials should not be offloaded into other containers or vehicles without the written permission of the material owner or consignee.

#### 4.2.2 TRANSPORTATION

Materials should be transported in a manner that will ensure the maintenance of controlled conditions as specified by the manufacturer. The transport process should not adversely affect the materials or integrity of the packaging. The supplier of transport services must be provided with the required information in order to maintain specified conditions.

The pharmaceutical excipient manufacturer or supplier should agree with the purchaser for arranging transportation. The need for temperature-controlled storage and transport should be determined using a risk-based approach, taking into account the nature of the excipient, results of stability assessments available from the excipient manufacturer or repackager, the supply chain, and the potential risks to the excipient. If temperature-controlled transportation is contracted, the shipper must have a mechanism for noting and reporting temperature excursions. Labeling on containers and transportation documents should detail the environmental conditions in a manner that provides the transporter or receiver with knowledge and immediate identification of these conditions, if required. The responsibility for ensuring that the proper storage conditions are met rests with each entity that handles, stores, or transports the materials.

Pharmaceutical excipients should be stored and transported in such a way that the identity and integrity of the material are retained, the material does not contaminate and is not contaminated by other materials, and adequate precautions are taken

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against spillage, breakage, misappropriation, and theft. The required storage conditions for pharmaceutical excipients should be maintained within acceptable limits during transportation.

Excipients that are potentially dangerous because of the risk of fire or explosion (e.g., combustible liquids, solids, and pressurized gases) should be stored and transported in safe, dedicated, and secure areas, containers, and vehicles. In addition, applicable international agreements and federal regulations should be followed.

## 4.3 Tampering or Damaged Materials

Materials that are suspected of being tampered with or damaged must be quarantined immediately, and the manufacturer or distributor should be notified. The disposition of the quarantined material should be determined by the excipient manufacturer or supplier, after consultation with the customer. The excipient may be returned to the manufacturer or supplier. Alternatively, arrangements could be made for local certified destruction of the quarantined material. The supplier should make every effort to prevent these materials from being used until an investigation is completed and the final disposition of the material is determined. Written procedures should guide treatment of excipients that have been tampered with or the identification and handling of damaged material.

## 4.4 Packaging: Tamper-Evident Seals

A tamper-evident package has one or more indicators or barriers to entry that, if breached or missing, can reasonably be expected to provide visible evidence that tampering has occurred. To reduce the likelihood of successful tampering and to increase the likelihood that any breach will be discovered, the package should be distinctive by design or should employ one or more indicators of or barriers to entry. The term *distinctive by design* means that the packaging cannot be duplicated with commonly available materials or by commonly available processes.

A tamper-evident package may involve an immediate container–closure system in direct contact with the contents (primary packaging), a secondary container–closure system not in direct contact with the contents (secondary packaging), or any combination of systems intended to provide visual evidence of package integrity. For primary packaging in direct contact with the excipient (e.g., paper bags), any leak or break should be considered tampering even if the leak or tear is simply accidental damage. For excipients shipped in bulk, using, e.g., tank cars or containers, other means may be appropriate. However, whatever methods are adopted should provide adequate assurance as to the integrity of the excipient being shipped.

Visual examination of the packaging at each stage in the supply chain should provide evidence of repackaging or tampering with commercial packaging. In addition, the manufacturer's name and address, net weight of the material, material name, batch or packaging number, date of manufacture, and date of retest should be identified on a packaging label. The label should be prominently placed on the package and should be unaffected if the tamper-evident feature of the package is breached or missing.

The tamper-evident feature for excipient packaging should be designed so that it remains intact when handled in a reasonable manner from the time of packaging at the site of manufacture and throughout the supply chain—including but not limited to warehouse storage during various phases of the supply chain, transport, distribution, receipt, and storage at the user's facility until use for drug product manufacture.

The manufacturer should communicate tamper-evident features to the downstream members of the supply chain. If the latter observe any evidence that the tamper-evident feature or other part of the package has been compromised in any way, they should quarantine the material immediately and inform the supplier. Appropriate arrangements should be made with the supplier to return the material promptly with a description of the packaging breach. Alternatively, arrangements can be made by the excipient manufacturer or supplier for the local certified destruction of the quarantined material. The user should ensure adequate protection of the breached packaging during shipment to the supplier and can send photographs of the breached packaging to aid the supplier's investigation.

The supplier is responsible for the integrity of packaging, including but not limited to its tamper-evident features, until ownership of the commercial packages is transferred to the user. Material returned because of breach in packaging should be thoroughly reviewed and investigated by the supplier. The material should not be returned to commerce until the supplier has established that the integrity, identity, quality, purity, and safety of the excipient have not been compromised. The documentation requirement should comply with GMP expectations as well as elements of documentation and investigation suggested in *Section 4.1 Returned Goods*.

## 4.5 Where Ownership Begins

The excipient user is responsible for purchased materials throughout the supply chain. The supply chain qualification is documented by audits and COAs for all parties involved in trade and distribution of the materials. Such supply chain qualification and documentation supports the Excipient Pedigree and ownership of the excipient. The pedigree includes documentation of suitable excipient GMPs applied by the excipient manufacturer and suitable GDPs.

Ownership of the materials begins with the original excipient manufacturer and transfers to an intermediary or customer according to agreed-upon terms for insurance costs, transportation, and risk assumption. Such agreements are defined according to International Chamber of Commerce terms (Incoterms).[6] Incoterms are a series of international sales terms that are used to divide transaction costs and responsibilities between buyer and seller and reflect state-of-the-art transportation practices.

[6] International Chamber of Commerce. Incoterms. http://www.iccwbo.org/incoterms (Accessed June 6, 2011).

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## 4.6 Adulteration

### 4.6.1 ADULTERATION

Adulteration is defined in the FD&C Act and 21 CFR in Sections 501(a)(2)(B) and 501(b)[7] and 21 CFR 211 for finished pharmaceuticals and Sections 402(a)(3) and (4)[8] and 21 CFR 110 for human food, and in 21 CFR 111 for dietary supplements. These laws and regulations establish the minimum current GMP (cGMP) necessary to prevent adulteration for finished pharmaceuticals, food products, and dietary supplements, respectively. Excipients for pharmaceutical use must be manufactured under appropriate GMPs and must meet the required chemical and physical specifications. In addition to specifications, excipient manufacturers and users have generally agreed quality attributes and limits defined by regulatory agencies, common industry practices, and pharmacopeial expectations. Adulteration or contamination of the products can be monitored and detected by many means including, but not limited to, compliance with these predefined quality expectations.

Adulteration can occur when any possible contamination of a product takes place, e.g., from foreign materials or undesirable microorganisms. The problem of adulteration can be addressed by standard practices supporting cGMPs, such as HACCP, Standard Operating Procedures, and staff training to control product safety and purity. This type of adulteration is the unforeseeable and unintentional type that can be controlled and, at worst, detected before the product leaves the manufacturer's site.

FDA specifies that a product can be considered adulterated when conditions *may* lead to adulteration because it is impossible to test every product for every conceivable contaminant. The safety and purity of substances require that manufacturers should build quality controls into the process rather than relying on QC testing.

### 4.6.2 INTENTIONAL ADULTERATION

Compared to unintentional adulteration, intentional adulteration is more specific because it requires a willful and knowing violation of regulations and standards designed to protect end user safety. It is the deliberate adulteration of an excipient. When an excipient is deliberately adulterated by substituting a lower-cost material for a material of higher cost, it is considered economically motivated intentional adulteration. Every participant in the supply chain should know and monitor their supply chain for any adulterated materials and take all reasonable precautions to prevent intentional adulteration.[9]

## 4.7 Importation

Excipients manufactured outside the United States are subject to US FDA and US Customs and Border Protection (CBP) regulations for importation into the United States. The Bioterrorism Act (Public Health Security and Bioterrorism Preparedness and Response Act of 2002, Title III—Protecting Safety and Security of Food and Drug Supply)[10] has further formalized the requirements for importation of foods and drugs into the United States.

Overseas manufacturers of excipients used in drugs, food, and dietary supplements intended for human or veterinary use who intend to export products into the United States are required to follow the FDA, CBP, and Bioterrorism Act regulations. A streamlined process for the importation of excipients used in pharmaceuticals, food, and dietary supplements must be followed and implemented before the imports are allowed into the United States.

The manufacturing facilities and the manufacturers who produce the excipients should be registered with FDA. An FDA registration number is required for importation. In addition, FDA requires information about Prior Notice (PN) of Imports. Upon receipt of the information, FDA grants a PN confirmation number. FDA must confirm PN before the products are shipped, and the PN confirmation number must appear on the customs declaration that accompanies the package. PN information can be submitted electronically to FDA's Prior Notice Systems Interface (PNSI), a free Internet application that allows facilities to provide information regarding the expected imports. PN information also can be submitted via CBP's Automated Commercial System (ACS), a system that processes imports and obtains information needed to make decisions regarding articles entering the United States.

CBP processes imports of all goods for entry into United States, including but not limited to pharmaceuticals, food, and dietary supplements. CBP inspects but does not release products regulated by the FD&C Act and delegates the final release responsibility at the port of entry to the FDA for such materials. After reviewing the PN information, FDA may determine that the regulated articles should not be allowed into the United States or may allow conditional import of articles subject to testing and release at the port of entry. In addition, during FDA review at the port the regulated articles must meet all requirements of the FD&C Act and 21 CFR before they are released by FDA to the importer.

Importers of record (individuals or companies) for excipients can contract with a broker to transmit PN information and other documents for them. In this case, the submitter is the person responsible for providing the information, but the broker is the transmitter. Brokers are licensed private individuals or companies regulated by CBP who aid importers and exporters in moving merchandise through CBP. Brokers provide the proper paperwork and payments to CBP for clients and charge a fee for this service. Before brokers apply for a license, they must pass the customs broker examination.

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[7] FD&C Act. Chapter V: Drugs and Devices. Sec. 501. [21 USC §351] Adulterated Drugs and Devices. http://www.fda.gov/RegulatoryInformation/Legislation/FederalFoodDrugandCosmeticActFDCAct/FDCActChapterVDrugsandDevices/ucm108055.htm.

[8] FD&C Act. Chapter IV: Food. Sec. 402. [21 USC §342] Adulterated Food. http://www.fda.gov/RegulatoryInformation/Legislation/FederalFoodDrugandCosmeticActFDCAct/FDCActChapterIVFood/ucm107527.htm (Accessed June 6, 2011).

[9] FDA. Public Meeting on Economically Motivated Adulteration. 2009. http://www.fda.gov/NewsEvents/MeetingsConferencesWorkshops/ucm163619.htm (Accessed June 6, 2011).

[10] FDA. *Guidance for Industry: Questions and Answers Regarding the Interim Final Rule on Prior Notice of Imported Food, (Edition 2); Availability.* 2004. http://www.gpo.gov/fdsys/pkg/FR-2004-05-03/pdf/04-10023.pdf (Accessed June 6, 2011).

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FDA currently uses its Operational and Administrative System for Import Support (OASIS)[11] for making its admissibility determinations to ensure the safety, efficacy, and quality of the foreign-origin products for which FDA has regulatory responsibility under the FD&C Act. OASIS is integrated with CBP's ACS and FDA's PNSI systems to receive information related to imported articles.

US Customs and FDA storage areas at the Port of Arrival may not strictly be in compliance with storage conditions required for certain excipients. Importers of record and brokers who represent importers must ensure that the products are released from Customs and FDA inspection as soon as possible. If release is delayed, FDA generally allows removal by Customs and FDA and quarantine in the importer's warehouse until release. FDA staff who review imports at the port of entry are trained to understand that pharmaceutical excipients must be stored under defined conditions. The manufacturer, the importer on record in the importing country, and brokers have a responsibility in working with Customs and FDA staff to ensure that the storage conditions do not adversely affect product quality during quarantine and review.

## 4.8 Traceability

### 4.8.1 TRACEABILITY

The pedigree of the excipient should be tracked from the manufacturer's storage through the final delivery to customers by means of recorded identification. The entire supply chain should provide full traceability (for example, via lot numbers and shipping documents) in order to allow fast and efficient investigation of any quality issue or product recall. Every entity in the supply chain should also take responsibility from the preceding supplier and pass the product to subsequent intermediaries down to the end user. Therefore, the original excipient manufacturer and subsequent handlers should always be traceable, and the information should be available both downstream and upstream in the supply chain. All parties to the excipient supply chain should ensure that the excipient is strictly handled according to GDP at every stage.

To ensure the integrity of the supply chain, intermediaries should use contracts, agreements, inspections, and audits downstream and upstream to monitor compliance with GDP principles. When multiple entities constitute the supply chain for each single batch of excipient, each entity should provide its own supplier's certification documentation (see *Appendix: Definitions and Acronyms*) that represents their manufacture or receipt of the excipient batch through release to the subsequent entity. The total of each entity's supplier certification documentation should represent the entire supply chain from original excipient manufacture through use in the final drug product.

### 4.8.2 TRACEABILITY-RELATED DOCUMENTS

To ensure traceability, all entities in the supply chain should have clear definitions about the shipping documents to be expected with every delivery. At a minimum the documents for every delivery should provide the following information:

- Name and grade of the excipient
- Lot number(s) assigned by the original excipient manufacturer (see *Section 3.4.2 Repackaging and Labeling Batches*)
- Quality and compliance data (e.g., COA) of the excipient
- Origin of the excipient (manufacturer and manufacturing site)
- Original excipient COA(s) (see *Section 3.4.5 Certificates of Analysis*)
- Entity and site of repackaging (when performed), including opening or relabeling the original excipient manufacturer's packaging for any purpose
- Date of shipment and carrier
- Consignor and consignee

A copy of the COA also should accompany the shipment (see ▲*Significant Change for Bulk Pharmaceutical Excipients* ⟨1195⟩ ▲(CN 1-May-2021) and *Bulk Pharmaceutical Excipients—Certificate of Analysis* ⟨1080⟩).

In the event of repackaging from the original excipient manufacturer's package into another container (including any breach or labeling that does not result in a new package), the identity and address of the repackaging entity should be included in the shipping documents.

Additional data resulting from analyses conducted by entities other than the laboratory of the original excipient manufacturer should be provided, along with a clear indication of the source. Quality documents should facilitate traceability back to the original manufacturer with contact information. The COA issued by the manufacturer should indicate which results were obtained by testing the original material and which results were obtained by other means. A distributor should not change the original title and data of the COA or other quality documents. Whenever possible, the original excipient manufacturer's documentation should be used, or data transcription should be verified. The original manufacturing site should be identified on the COA.

If any lot mixing is carried out, COAs from manufacturers are no longer valid, and the distributor should perform analyses in its own laboratory or at an approved and qualified contract laboratory. The distributor should supply a COC, and if the blended lot has not been retested, the distributor should inform the customer that the material is a mixture of different original excipient manufacturers' lots, provided that all other repackaging and storage activities are carried out according to GDP.

# SECTION 5: EXCIPIENTS USED IN PHARMACY COMPOUNDING

Although analytical testing of incoming components by the compounding pharmacy to confirm quality attributes stated in the COAs is ideal, generally because of resource limitations, compounding pharmacists rely upon the distributors of excipients for assurance of quality and pedigree. Additional guidance on the quality attributes of excipients received by compounding

[11] FDA. Operational and Administrative System for Import Support (OASIS). 2009.

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pharmacies can be found in *USP* general chapter *Pharmaceutical Compounding—Nonsterile Preparations* ⟨795⟩. In certain instances *USP–NF* handles compounded preparations differently than commercially manufactured lots. For example, expiration dates are assigned to commercially manufactured products, and beyond-use dates are assigned to compounded preparations (see *Labeling* ⟨7⟩*, Labels and Labeling for Products and Other Categories, Compounded Preparations*). A similar situation is needed for bulk pharmaceutical excipients as not all excipients that are useful in compounding are listed in official compendia (see *Pharmaceutical Compounding—Nonsterile Preparations* ⟨795⟩).

State boards of pharmacy regulate pharmacy compounding. USP standards are provided in *Pharmaceutical Compounding—Nonsterile Preparations* ⟨795⟩, *Pharmaceutical Compounding—Sterile Preparations* ⟨797⟩, and *Quality Assurance in Pharmaceutical Compounding* ⟨1163⟩.

## APPENDIX: DEFINITIONS AND ACRONYMS

**Acceptance Criteria:** The specifications and acceptance or rejection limits—such as acceptable quality level or unacceptable quality level with an associated sampling plan—that are necessary for making a decision to accept or reject a lot or batch of raw material, intermediate, packaging material, or excipient.

**ACS:** Automated Commercial System.

**Adulterated Material:** A material that fails to conform to its purported quality standard or is intentionally contaminated, diluted, or substituted for another substance or which was not manufactured, processed, packaged, distributed and held in conformance with current good manufacturing practice.

**Audit:** An assessment of a system or process to determine its compliance with the requirements of a particular standard of operation. See also External Audit, Internal Audit, and Third-Party Audit.

**Batch (Lot):** A defined quantity of processed excipient which can be expected to be homogeneous. In a continuous process, a batch corresponds to a defined portion of the production based on time or quantity (e.g., vessel's volume, one day's production, etc.).

**Batch Number (Lot Number):** A unique and distinctive combination of numbers and/or letters from which the complete history of the manufacture, processing, packaging, coding, and distribution of a batch can be determined.

**Batch Process:** A manufacturing process which produces the excipient from a discrete supply of raw materials processed through discrete unit operations in one mass.

**Batch Record:** Documentation that provides a history of the manufacture of a batch of excipient.

**Blending (Mixing):** Intermingling different conforming grades into a homogeneous lot.

**Broker:** An entity that acts as an intermediary between a buyer and a seller of products or services. Brokers neither buy nor take possession of the products or services.

**Calibration:** The demonstration that a particular instrument or measuring device produces results within specified limits by comparison with those produced by a reference or traceable standard over an appropriate range of measurements.

**CBP:** Customs and Border Protection.

**CEP (Certificate of Suitability to the *European Pharmacopoeia*):** Certification granted to individual manufacturers by the European Directorate for the Quality of Medicines when a specific excipient or active ingredient is judged to be in conformity with a *European Pharmacopoeia* monograph.

**CFR:** Code of Federal Regulations.

**CFR (Cost and Freight, Named Destination):** (Incoterm) Seller must pay the costs and freight to bring the goods to the port of destination. However, risk is transferred to the buyer once the goods have crossed the ship's rail (maritime transport only).

**cGMP:** Current good manufacturing practices.

**CIF (Cost, Insurance, and Freight, Named Destination):** (Incoterm) Same as CFR except that the seller must, in addition, procure and pay for insurance for the buyer.

**CIP (Carriage and Insurance Paid, Named Destination):** (Incoterm) The containerized transport or multimodal equivalent of CIF. Seller pays for carriage and insurance to the named destination, but risk passes when the goods are handed over to the first carrier.

**Closed-Container Distributor (Pass-Through Distributor):** A distributor who sells only products that are tested, packaged, and sealed in the containers provided by the original manufacturer.

**Closed System:** A system that is isolated from its surroundings by a boundary so that no material can be transferred across it.

**COA (Certificate of Analysis):** A document that reports the results of a test of a representative sample drawn from the batch of material that will be delivered.

**COC (Certificate of Conformance):** A document that certifies that the supplied goods or service meets the required specifications. Also known as Certificate of Conformity and Certificate of Compliance.

**Commissioning:** The introduction of equipment for use in a controlled manner.

**Compounding:** The preparation, mixing, assembling, altering, packaging, and labeling of a drug, drug-delivery device, or device in accordance with a licensed practitioner's prescription, medication order, or initiative based on the practitioner/patient/pharmacist/compounder relationship in the course of professional practice (defined in *USP* general chapter *Pharmaceutical Compounding—Nonsterile Preparations* ⟨795⟩).

**Consignee/Consignor:** Person or firm (usually the seller) who delivers a consignment to a carrier for transportation to a consignee (usually the buyer) named in the transportation documents.

**Contamination:** The undesired introduction of impurities of a chemical or microbiological nature or foreign matter into or onto a raw material, intermediate, or excipient during production, sampling, packaging or repackaging, storage, or transport.

**Continuous Process:** A manufacturing process that continually produces the excipient from a continuous supply of raw material.

**Contract Giver:** A person or organization letting a contract.

**Contract Acceptor:** A person or organization accepting the terms of a contract and thereby agreeing to carry out the work or provide the services as specified in the contract.

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**Critical:** A process step, process condition, test requirement, or other relevant parameter or item that must be controlled within predetermined criteria to ensure that the excipient meets its specification.

**Critical to Quality:** See *Quality, Critical*.

**Cross-Contamination:** Contamination of a material or product with another material or product.

**Customer:** The organization that receives the excipient once it has left the control of the excipient manufacturer; includes brokers, agents, and users.

**Deviation:** Departure from an approved instruction or established standard.

**Distributor:** An entity that buys products from a manufacturer, takes possession of those products, and resells them to another party or parties. An essential characteristic of a distributor is the order of these transactions. Distributors buy products (i.e., hold inventory) before making sales.

**Drug Master File (DMF):** Detailed information about the manufacture of an excipient that is submitted to the US FDA.

**Drug (Medicinal) Product:** The dosage form in the final immediate packaging intended for marketing.

**Drug Substance:** Any substance or mixture of substances that is intended for use in the manufacture of a drug product and that, when used in the production of a drug, becomes an active ingredient of the drug product. Such substances are intended to furnish pharmacological activity or other direct effect in the diagnosis, cure, mitigation, treatment, or prevention of disease, or to affect the structure or any function of the body of humans or animals.

**Economically Motivated Adulteration:** The fraudulent, intentional substitution or addition of a substance in a product for the purpose of increasing the apparent value of the product or reducing the cost of its production for economic gain.

**Electronic Signature:** A computer data compilation of any symbol or series of symbols, executed, adopted, or authorized by an individual and intended to be the legally binding equivalent of the individual's handwritten signature.

**Excipient:** Any substance, other than the active pharmaceutical ingredient or drug product, that has been appropriately evaluated for safety and is included in a drug delivery system to aid the processing of the drug delivery system during manufacture; to protect, support, or enhance stability, bioavailability, or patient acceptability; to assist in product identification; or to enhance any other attribute of the overall safety and effectiveness of the drug delivery system during storage or use.

**Excipient Pedigree:** Includes documentation of suitable excipient good manufacturing practices applied by the excipient manufacturer and suitable good distribution practices. See IPEC *Excipient Pedigree White Paper*.

**External Audit:** (See also *Audit, Internal*, and *Third-Party Audit*.) An audit carried out typically on behalf of an excipient manufacturer's customer by a person or organization that is not the manufacturer or the customer.

**Expiry (Expiration) Date:** The date designating the time during which the excipient is expected to remain within specifications and after which it should not be used.

**FCA (Free Carrier, Named Place):** The seller hands over the goods, cleared for export, into the custody of the first carrier (named by the buyer) at the named place. This term is suitable for all modes of transport, including carriage by air, rail, road, and containerized/multimodal transport (also called *roll on–roll off*).

**FDA:** Food and Drug Administration.

**FD&C Act:** Food, Drug, and Cosmetic Act.

**FOB (Free on Board, Named Loading Port):** The classic maritime trade term according to which the seller must load the goods on board the ship nominated by the buyer, and cost and risk are divided at ship's rail. The seller must clear the goods for export. The purchaser is then responsible for all further costs associated with transport, importation, and storage until the shipment reaches its destination. The term also is applied to air transport when the seller is not able to export the goods according to the time schedule detailed in the letter of credit. In this case the seller allows a deduction equivalent to the carriage by ship from the air carriage. FOB also can be qualified in other ways. For example, *FOB Factory Gate* means that title and responsibility change as soon as the shipment leaves the supplier's premises.

**Forwarding Agents (Freight Forwarders):** Agents who assist other organizations or individuals in moving cargo to a destination and are familiar with the import and export rules and regulations of their own and foreign countries, the methods of shipping, and the documents related to foreign trade.

**Freight Forwarder:** See *Forwarding Agent*.

**GDP:** Good distribution practices.

**GMP:** Good manufacturing practices.

**Headspace:** The volume left at the top of an almost-filled container before sealing.

**HACCP (Hazard Analysis Critical Control Point):** Hazard Analysis and Critical Control Points has seven principles established by the National Advisory Committee for Microbiological Criteria for Foods to control product safety.

**Importer:** Either the US owner or consignee at the time of entry into the United States or the US agent or representative of the foreign owner or consignee at the time of entry into the United States who is responsible for ensuring that goods offered for entry into the United States are in compliance with all laws affecting the importation.

**Impurity:** A component of an excipient that is not the intended chemical entity or a concomitant component but is present as a consequence of either the raw materials used or the manufacturing process and is not a foreign substance.

**Independent:** In the context of internal audits, the quality of being free from any influence, economic or otherwise, from the group, department, or organization under audit.

**In-Process Control:** Checks performed during production in order to monitor and if necessary to adjust the process to ensure that the material conforms to its specifications. The control of the environment or equipment also can be regarded as a part of in-process control.

**In-Process Control/Testing:** Checks performed during production to monitor and, if appropriate, to adjust the process to ensure that the intermediate or excipient conforms to its specification.

**Intermediate:** Material that must undergo further manufacturing steps before it becomes an excipient.

**Internal Audit:** An audit conducted by an employee of the organization or by an individual from outside the organization, but on behalf of the organization, to determine the effectiveness of a system. (See: *Audit*, *External Audit*, and *Third-Party Audit*).

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**International Nonproprietary Name:** International Nonproprietary Names (INN) facilitate the identification of pharmaceutical substances or active pharmaceutical ingredients. Each INN is a unique name that is globally recognized and is public property. A nonproprietary name also is known as a generic name.

**ISO:** International Organization for Standardization.

**Lot:** See *Batch*.

**Labeling:** The affixing to a container or vessel of a tag or document that contains information about that container and its contents.

**Manufacturer/Manufacturing Process:** All operations of receipt of materials, production, packaging, repackaging, labeling, relabeling, quality control, release, and storage of excipients and related controls.

**Master Production Instruction (Master Production and Control Record):** Documentation that describes the manufacture of the excipient from raw material to completion.

**Material:** A general term used to denote raw materials (starting materials, reagents, and solvents), process aids, intermediates, excipients, packaging, and labeling materials.

**Nonconforming Material:** A material that is deficient in a characteristic, product specification, process parameter, record, or procedure that renders its quality unacceptable, indeterminate, or not according to specified requirements.

**OASIS:** Operational and Administrative System for Import Support.

**Original Excipient Manufacturer:** Organization responsible for manufacturing, under appropriate GMPs, the excipient(s) distributed and addressed by this chapter.

**Packaging/Repackaging Distributor:** A distributor who transfers products from the original packaging or transport vessel(s) provided by the original manufacturer into alternative packaging and sells the products in the alternative packages. See *Distributor* and *Repackager*.

**Primary Container–Closure System:** The packaging components that come into direct contact with the excipient in the closed, sealed package during storage and transport.

**Packaging Material:** A material intended to protect an intermediate or excipient during storage and transport.

**Packaging:** The container and its components that hold the excipient for storage and transport to the customer.

**Pass-Through Distributor:** See *Closed-Container Distributor*.

**PN:** Prior notice.

**PNSI:** Prior Notice Systems Interface.

**Primary, Secondary Packaging:** See *Packaging/Repackaging Distributor* and *Primary Container—Closure System*. Packaging materials which do not come into contact with the excipient during the normal course of storage and transport of the excipient.

**Production:** Operations involved in the preparation of an excipient from receipt of raw materials through processing and packaging of the excipient.

**QbD (Quality by Design):** A systematic approach to pharmaceutical development that begins with predefined objectives and emphasizes product and process understanding and process control based on sound science and quality risk management. It means designing and developing products and manufacturing processes to ensure a predefined quality.

**QMS:** Quality management system.

**Quality Assurance (QA):** The total of the organized arrangements made to ensure that all excipients are of the quality required for their intended use and that quality systems are maintained. See *Quality Unit*.

**Quality Control (QC):** Checking or testing that specifications are met. See *Quality Unit*.

**Quality, Critical:** Describes a material, process step or process condition, test requirement, or any other relevant parameter that directly influences the quality attributes of the excipient and that must be controlled within predetermined criteria.

**Quality Management System (QMS):** Management system that directs and controls a pharmaceutical company with regard to quality.

**Quality Manual:** Describes the elements of the QMS and includes the quality organizational structure, written policies, procedures, and processes or references to them, and a description of departmental functions as they relate to the policies, procedures, and processes. Document specifying the quality management system of an organization.

**Quality Unit:** See also: *Quality Control* and *Quality Assurance*. A group within a larger organization that is responsible for monitoring and ensuring all aspects of quality. Current industry practice generally divides the responsibilities of the quality control unit (QCU), as defined in the cGMP regulations, between quality control (QC) and quality assurance (QA) functions. QC usually involves (1) assessing the suitability of incoming components, containers, closures, labeling, in-process materials, and the finished products; (2) evaluating the performance of the manufacturing process to ensure adherence to proper specifications and limits; and (3) determining the acceptability of each batch for release. QA primarily involves (1) review and approval of all procedures related to production and maintenance, (2) review of associated records, and (3) auditing and performing/evaluating trend analyses.

**Quarantine:** The status of materials isolated physically or by other effective means pending a decision about their subsequent approval or rejection.

**Raw Material:** A general term used to denote starting materials, reagents, and solvents intended for use in the production of intermediates or excipients.

**Recall:** See *Retrieval*.

**Record:** A document stating results achieved or providing evidence of activities performed. The medium can be paper, magnetic, electronic or optical, photographic, another medium, or a combination thereof.

**Reevaluation Date (Retest Date, Re-evaluation Interval):** The date when the material should be reexamined to ensure that it is still in conformity with the specification.

**Recommended Re-evaluation Date:** The date suggested by the supplier when the material should be re-evaluated to ensure continued compliance with specifications. It differs from the Expiration Date because the excipient can be re-evaluated to extend the length of time the material can be used, if supported by the results of the evaluation and appropriate stability data.

**Repackager:** A person or organization that takes an excipient from the original manufacturer's container and repackages it into different containers. See also *Distributor* and *Packaging/Repackaging Distributor*.

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**Repackaging:** Removal of the excipient from its original container (combination of secondary and/or primary packaging), and transfer to another container.

**Reprocessing:** Introduction of previously processed material that did not conform to standards or specifications back into the process and repetition of one or more necessary steps that are part of the normal manufacturing process.

**Retrieval (Recall):** Process for the removal of an excipient from the distribution chain.

**Reworking:** Subjecting previously processed material that did not conform to standards or specifications to processing steps that differ from the normal process.

**Secondary, Primary Packaging:** See *Primary, Secondary Packaging*.

**Senior Management:** See *Top Management*.

**Significant Change:** A change that alters an excipient's physical or chemical property from the norm or that is likely to alter the excipient's performance in the dosage form.

**Specification:** The quality parameters to which the excipient, component, or intermediate must conform and that serve as a basis for quality evaluation.

**Stability:** Continued conformity of the excipient to its specifications.

**Stable Process:** A process whose output, regardless of the nature of the processing (batch or continuous), can be demonstrated by appropriate means to show a level of variability that consistently meets all aspects of the stated specification (both USP and customer specifications) and thus is acceptable for its intended use.

**Subcontractor:** A person or organization that undertakes work or services on behalf of a different person or organization that in turn is contracted to undertake work or provide services from the original contract giver.

**Supplier's Certification Documentation:** Specific information and data associated with a single batch of an excipient. Its accuracy is certified by the business entity that has had control of the same single batch of excipient. Supplier's Certification Documentation includes both quality and supply chain data and information. The methods and processes that derive the included data and information should be understood and controlled, and all data and information sources should be traceable. All entities that take possession and responsibility for the excipient batch should provide Supplier's Certification Documentation including the original excipient manufacturer, all distributors, and all repackagers. Special attention and clarity should be applied within the Supplier's Certification Documentation in any event that breaches the original manufacturer's packaging and/or labels (including addition of new labels).

**Third-Party Audit:** An audit conducted by an individual from outside the organization and who is neither a supplier nor customer of the organization, e.g., a certification body, to determine the effectiveness of a system.

**Top Management:** Person or group of people who direct and control an organization at the highest level. The highest level can be at either the site level or the corporate level and depends on how the quality management system is organized.

**Traceability:** Ability to determine the history, application, or location that is under consideration, e.g., origin of materials and parts, processing history, or distribution of the product after delivery.

**Trader:** An entity that buys products from a manufacturer, may or may not take possession of the products, and resells them to another party or parties. [NOTE—In the case of traders, the sale usually is made before product purchase.]

**User:** A person or organization that uses pharmaceutical excipients to manufacture pharmaceutical intermediates or finished products.

**Validation:** A documented program that provides a high degree of assurance that a specific process, method, or system will consistently produce a result meeting predetermined acceptance criteria.

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# ⟨1207.1⟩ PACKAGE INTEGRITY TESTING IN THE PRODUCT LIFE CYCLE —TEST METHOD SELECTION AND VALIDATION

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## 1. INTRODUCTION

*Package Integrity Evaluation—Sterile Products* ⟨1207⟩ provides guidance in the integrity assurance of product packages. This chapter describes package integrity verification during three product life cycle phases: 1) package development, and package processing and assembly validation; 2) product manufacturing; and 3) commercial product shelf-life stability assessments. Further, this chapter provides information on how to select, develop, and validate leak test methods.

## 2. PACKAGE INTEGRITY TESTING IN THE PRODUCT LIFE CYCLE

Appropriate packaging for a sterile product can be determined on the basis of a knowledge-gaining effort conducted during the product's life cycle.

### 2.1 Development and Validation

#### 2.1.1 PACKAGE DEVELOPMENT

Package development begins with the preparation of a product–package profile (e.g., user requirements specification), which considers the product end use, stability requirements, and method of manufacture, as well as the anticipated storage, shipment, and distribution environments. This profile also defines the proper product–package quality requirements and the package's maximum allowable leakage limit (see *Package Integrity Evaluation Sterile Products* ⟨1207⟩*, Product–Package Quality Requirements and the Maximum Allowable Leakage Limit*). With this prospectively developed body of information, one can select each package component's materials of construction, choose suitable component sources, and establish critical physical attributes and component dimensional tolerances. Each component material, with its critical dimensional tolerances, directly affects the integrity of the final packaged product. Assurance of package integrity originates from the use of appropriate materials, accurate and optimum closing properties, matching dimensional fit, appropriate multi-component stack heights and tolerances, and consistent control of processes used to assemble the closed package.

The manner in which the package is processed, formed, or assembled is an important consideration in package integrity assurance. A preliminary assessment of package integrity at the end of the development phase under conditions representative of the marketed product manufacturing system is prudent.

These conditions include processes such as sealing operations and component sterilization. Where possible, processes are performed according to established and approved user requirement specifications. Establishing appropriate physical characteristic specifications for container materials, considering lot-to-lot variations, can ensure that the most extreme

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processing conditions anticipated (e.g., multiple sterilization cycles) do not physically damage materials in a manner that would adversely affect package integrity.

Finally, the robustness of the manufactured product–package system may be evaluated during the development phase by exposing a representative number of product samples to specified storage, shipment, distribution, and final product-use environments. These efforts may include studies that evaluate package integrity at the extremes of the finished product–package profile, not simply at optimal conditions. Given the complexity of some sterile packages, it may be useful for package integrity development studies to incorporate multi-point analyses of test packages manufactured within specified process parameters. Utilizing a common container–closure for which the producer has significant experience and process knowledge can supplement the overall development process and may help reduce the efforts needed. A battery of integrity and seal quality test methods may be employed during product–package development, starting with techniques able to measure the product's inherent package integrity. Inherent package integrity of a viable package system conforms to the product's maximum allowable leakage limit.

Test package quantities for inherent package integrity verification may vary on the basis of: 1) the complexity of the product–package, 2) the specifics of the user specification requirements, and 3) the prior experience of the producer. Test quantity choice is also influenced by the confidence that can be placed on the package integrity test results as well as the level of integrity assurance desired. In some cases inherent package integrity verification may be more readily and economically determined by using empty or placebo-filled container–closure systems, thereby enabling larger sample quantities to be tested by the most sensitive and quantitative leak test methods without the risk of product formulation interference with the test method.

The outputs of the packaging development phase include the final user requirement specifications, which form the basis of production purchasing specifications for package components. Also during the development phase, the final equipment user requirement specifications are developed for package material cleaning, sterilization, and forming; sealing or assembly equipment; and allied materials supply and component feed systems. These user requirement specifications provide purchase specifications for the acquisition of equipment or for the vetting of potential contract manufacturers.

#### 2.1.2 PACKAGE PROCESSING AND ASSEMBLY VALIDATION

Final confirmation of acceptable inherent package integrity is generally part of a larger process validation activity for the overall production process. The scope depends upon the product type and whether the organization has previous experience with the container–closure system.

All processes germane to the sterilization and formation of a package having integrity are to be evaluated against the user requirement specifications established in the package development phase, including likely process extremes. For example, inherent package integrity verification may consider extremes of package assembly variables such as line speed, heat-sealing temperature, screw-cap application torque, and vial-capping forces, as well as resterilization processes, labeling, and secondary and tertiary packaging processes. Validation test requirements and scope should fit the statistical requirements and capabilities of each process, taking into account both package and package-line complexity, as well as prior experience with similar product–packages.

Testing done during technical transfer from the product development site to the manufacturing sites will assist in determining whether the user requirement specification targets and the control ranges established in development require any modification when packages are made on a full manufacturing scale.

Integrity test methods for package processing and assembly validation studies are meant to verify the packages' continued conformance to the product's maximum allowable leakage limit. For some product–packages, the most fitting integrity test methods for this life cycle phase may have a detection limit greater than the maximum allowable leakage limit. Methods able to reject largely leaking packages such as those caused by defective or out-of-specification components, package damage, and/or package misassembly are appropriate. Seal quality tests suitable for use include those able to monitor package processing and/or assembly consistency.

Successful validation will result in a package that meets its user requirement specifications. The primary objective in package development and subsequent validation is to arrive at a quality product–package prepared using processes that reliably and consistently run within specified operating parameters as defined in the user requirement specifications, yielding critical package defects at a satisfactorily low rate. When performed, in-process and end-product package integrity testing should complement, not replace, thorough package development efforts.

## 2.2 Product Manufacturing

To ensure the quality of the manufactured product–package, it is critical to specify components of sufficient quality and to select vendors carefully. The factors to consider include:

- Acceptable results of the initial vendor or supplier evaluation
- Appropriate vendor acceptance quality limits and statistical sampling plan(s), or relevant certification
- Incoming component quality verification, including statistical assessment of quality against purchase specifications
- Appropriate procedure(s) for establishing corrective and preventive action when a vendor falls short of quality expectations

Package integrity re-evaluation is considered when changes occur in the product, package design, package materials, or manufacturing/processing conditions. The extent of change control efforts are evaluated on a case-by-case basis, while the level of testing required to support the change is determined based on impact assessment.

The product's package integrity profile is compiled over the course of commercial manufacture. This database of ongoing leak and seal quality test results serves to flag potentially harmful package integrity excursions that may be linked to operative variations in package component design/material and package assembly/processing. Not all packaged-product damage can be readily detected post-assembly; therefore, processing procedure controls and monitors may be relied upon to safeguard against such problems. The proper combination of package leak tests, complementary package seal quality tests, and visual inspection is largely based on package failure results observed in earlier production validation and package development studies.

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Testing production lot samples can provide a measure of package integrity confirmation, while entire testing by nondestructive means is able to yield an ongoing assessment of integrity assurance. In some cases regulatory requirement dictates the level of testing performed. For other product–packages, justification for the level of testing is based on statistical process control results generated during the validation phase, and later, on the basis of routine manufacturing product-quality trending analyses.

For example, glass or plastic ampules closed by heat fusion are customarily subjected to 100% nondestructive leak testing. Products sealed under vacuum require appropriate package assembly validation supplemented by testing over time to ensure that the vacuum is retained. Similarly, integrity assurance of packages that require a specific, non-reactive, inert gas headspace is based on appropriate package assembly validation along with testing for rise in reactive gas or water vapor content over time.

## 2.3 Commercial Product Stability

Container–closure integrity tests have been recommended as alternatives to sterility testing as part of commercial product stability programs. The goal is to ensure package integrity as a function of long-term product storage (*1*). [NOTE—Package integrity tests do not replace product release sterility tests.] Testing product for sterility is a poor measure of product–package integrity and also will not ensure product–package integrity over the shelf life of the product when performed as part of the stability program. In addition, a package may be in no danger of microbial ingress and yet be unable to maintain the gas headspace content necessary for product quality. Validated package integrity test methods using technologies such as those described in this chapter are more sensitive and reliable than product lot sterility testing for detecting a breach in package integrity that could lead to sterility loss or failure in relevant product physicochemical quality attributes.

Ideally, package integrity tests selected to support marketed-product stability studies are able to verify the absence of the smallest leaks of concern for a given product–package system. In other words, the product's maximum allowable leakage limit falls within the chosen test method leak detection range. However, the test methodology most appropriate for a particular product–package system may be unable to detect the very smallest leaks of concern. In some cases the package contents interfere with the ability of leak test methodologies to detect the smallest leaks. For example, proteinaceous ingredients or even salts may clog leak pathways, inhibiting leak detection by gas flow methods such as vacuum decay or mass extraction. It is prudent to understand the product's potential to interfere with the selected leak test method, both initially after package assembly and over time.

In situations such as those described above, the package integrity test chosen to support stability studies should have a detection limit as close as feasible to the product's maximum allowable leakage limit. An understanding of what the method is capable of evaluating and how this is applicable to microbial integrity assurance is warranted.

For products that demand package headspace content preservation, it is appropriate that the integrity test for stability studies verify the continued presence of specific headspace gases or subatmospheric pressure over time. Satisfactory results verify the absence of leaks that could jeopardize product sterility as well as relevant physicochemical quality attributes. The required duration for monitoring container vacuum or headspace gas content maintenance that equates to microbial barrier assurance and liquid product loss prevention can be predicted on the basis of gas flow kinetics, should a leak pathway exist that is large enough to allow microbial entry and product formulation loss.

Stability test samples intended for package integrity evaluation are kept at labeled storage conditions for the marketed product. Test sample quantities chosen for each testing time point are to be relevant and sufficiently representative of the purpose of stability testing, which is to indicate whether integrity is affected by the stability conditions. Sample quantity selection takes into account all prior development and validation testing.

As noted in reference (*1*), if an integrity test is nondestructive to the product or package, samples that pass package integrity testing may be further used in the stability testing for that specific test period or interval. However, samples should not be tested for package integrity at one time point (e.g., 12 months), then stored for further stability testing at a later time point (e.g., 24 months). While not specifically noted in reference (*1*), it is logical to assume that test samples earmarked for integrity verification over the course of the stability study could be checked for integrity before placement on stability if a nondestructive leak test method is used. This would be akin to the common practice of visually inspecting test samples prior to placing them on stability. In this way, subsequent integrity failures can be attributed to stability storage, rather than to other causes.

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# 3. TEST METHOD SELECTION CRITERIA

No single package leak test or package seal quality test method is applicable to all product–package systems. Test method selection is made on a product–package on a case-by-case basis. Often more than one test method is employed during a given product's life cycle. Package test method selection as a function of product life-cycle phase, along with important integrity considerations, are discussed in *Package Integrity Testing in the Product Life Cycle*. A broad discussion of additional selection criteria for leak test methods follows. Specific leak test method examples are provided for informational purposes but are not intended as recommendations. The attributes and general capabilities of tests can be found in *Package Integrity Leak Test Technologies* ⟨1207.2⟩ and are helpful in the method selection process.

## 3.1 Package Contents

When selecting a leak test method, the first determining factor is the nature of the package contents. Whether the package contains a liquid or solid formulation, and whether it contains a headspace of inert gas, air, vacuum, or even no headspace at all, will influence leak test method choice. For example, if testing liquid-filled packages by vacuum decay or mass extraction, test vacuum conditions may trigger some formulations to solidify inside leak paths, thereby blocking gas leakage flow and

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making the test ineffective. Alternatively, electrical conductivity and capacitance tests can be used, but only if the liquid product is more electrically conductive than the package materials.

## 3.2 Package Design, Materials of Construction, and Mechanics

Packages vary widely in their design, their materials of construction, and the mechanisms whereby package closure is affected. Each of these variables influences the leak test method choice, as the following examples illustrate.

### 3.2.1 RIGID OR FLEXIBLE PACKAGES

Rigid (nonporous) packages can tolerate the pressure or vacuum-challenge conditions required in several leak tests, including tracer liquid ingress, vacuum decay, mass extraction, and some tracer gas tests. In contrast, some flexible packages can tolerate differential-pressure test conditions only if special tooling is used to restrict package expansion and prevent subsequent seal damage. Package restriction is necessary to ensure consistent differential pressure conditions across the package barrier.

### 3.2.2 MOVEABLE OR FIXED COMPONENTS

Moveable package components, such as syringe stoppers (plungers or pistons), may require restraint to prevent their dislocation during the differential pressure test conditions required for a majority of leak test procedures (e.g., tracer liquid test, pressure or vacuum decay test, mass extraction test, bubble emission test, and microbial challenge by immersion test).

### 3.2.3 POLYMERIC MATERIALS

When exposed to test vacuum conditions, package materials such as plastics and some elastomers may outgas volatiles that raise vacuum-decay leak test results as well as mass extraction results, falsely implicating package leakage. Plastic packages that are highly permeable to tracer gases may not be compatible with helium tracer gas leak detection, as helium permeating through the package could be mistaken for package leakage or may mask small leaks. Special fixtures to limit tracer gas permeation effects and isolate tracer gas exposure to the seal area under test have been used to mitigate such difficulties.

### 3.2.4 METALLIC MATERIALS

Packages made of foil laminate materials may prove incompatible with electrical conductivity and capacitance leak detection, which works best with relatively nonconductive package materials. However, aluminum caps used to secure vials closed with elastomeric closures pose no hindrance to electrical conductivity and capacitance tests, even for finding leaks located between the closure and the vial finish.

### 3.2.5 TRANSPARENT OR OPAQUE MATERIALS

Packages made of transparent or translucent materials allow for visual inspection and electromagnetic wave passage. Therefore, transparent or translucent materials can be tested by laser-based gas headspace analysis techniques, as well as tracer-liquid ingress or microbial ingress. Opaque packages are incompatible with testing approaches that require visual inspection of the package contents.

## 3.3 Closure Type and Mechanics

Refer to *Package Integrity Evaluation—Sterile Products* ⟨1207⟩*, Closure Type and Mechanics* for a discussion of the closure type categories represented by the various product–package systems. The design of the closure system and its leakage restriction function (i.e., the maximum allowable leakage), plus the types of defects anticipated, influence the integrity test method choice.

## 3.4 Maximum Allowable Leakage Limit

The maximum allowable leakage limit as a function of the product–package quality requirement is discussed in *Package Integrity Evaluation—Sterile Products* ⟨1207⟩*, Product–Package Quality Requirements and the Maximum Allowable Leakage Limit*. The following integrity test method selection considerations are offered in light of that discussion.

### 3.4.1 STERILITY AND PRODUCT FORMULATION CONTENT MUST BE PRESERVED; GAS HEADSPACE PRESERVATION IS NOT REQUIRED

Integrity tests for this product quality category include those able to verify that the maximum allowable leakage limit that prevents liquid and microbial ingress is not exceeded. Tracer gas tests performed using the vacuum mode and laser-based gas headspace analysis test methods are two examples. Both have been shown to be sensitive enough to quantitatively analyze leakage through the smallest leak paths found to pose the smallest chance of liquid leakage or microbial ingress in rigid packaging. Such tests have also proven useful for defining relationships among package design, component fit, package assembly parameters, and leakage rate, even in the absence of package defects.

Leak testing of product-filled packages during later product-life-cycle phases often requires other tests. Leak test methods available for this phase of the product life cycle are able to be validated to reliably detect defects a few micrometers and larger. Examples of such physicochemical leak tests include vacuum or pressure-decay tests, mass extraction methods, electrical conductivity and capacitance tests, and liquid tracer tests.

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#### 3.4.2 STERILITY, PRODUCT FORMULATION CONTENT, AND GAS HEADSPACE CONTENT MUST BE PRESERVED

For this product quality category, leak test options include those that directly check for package headspace pressure and/or content, such as laser-based gas headspace analysis techniques. The detection limit for such methods is a function of the method's ability to accurately measure package headspace content or absolute pressure at the product acceptance limit, given the package headspace volume and the time lapse after package assembly. Such methods have broad application throughout the product life cycle.

#### 3.4.3 STERILITY MUST BE PRESERVED; PRODUCT ACCESS IS REQUIRED

The product quality subcategory relates to the additional integrity requirement for those products that are contained in multiple-dose packages once the product is accessed by the end user. As discussed in *Package Integrity Evaluation—Sterile Products* ⟨1207⟩, *Product–Package Quality Requirements and the Maximum Allowable Leakage Limit, Sterility Must Be Preserved; Product Access is Required*, physicochemical as well as microbiological test methods designed to characterize and verify the unique barrier properties specific for the given container–closure system and its end-use application may be required (*2*).

Elastomeric closures of multiple-dose parenteral products are meant to reseal, limiting microbial ingress and product leakage during and between product access via needle puncture. The test in ▲*Elastomeric Closure Functionality in Pharmaceutical Packaging/Delivery Systems* ⟨382⟩, *Functionality Tests, Self-Sealing Capacity*▲ (CN 1-Dec-2020) is a blue dye liquid tracer test intended to screen closures for their ability to prevent gross liquid leakage post puncture. Caution is advised when solely relying on this test to prove a given closure's ability to reseal. Other test method(s) may need to be designed and implemented that more fully characterize the closure's leak prevention capabilities for the given product–package system and product end-use application.

## 3.5 Deterministic or Probabilistic Methods

A "deterministic leak test method" is one in which the leakage event is based on phenomena that follow a predictable chain of events, and leakage is measured using physicochemical technologies that are readily controlled and monitored, yielding objective quantitative data. Most deterministic leak test methods rely on the predictable movement of gas that inevitably occurs through an open leak path, given specific differential pressure and/or partial pressure test conditions (e.g., tracer gas, laser-based gas headspace analysis, pressure decay, vacuum decay, and mass extraction). The electrical conductivity and capacitance test is a deterministic leak test approach that relies on the more predictable presence of liquid near a leak path, rather than the less predictable movement of liquid through a leak. Deterministic methods are characterized as being capable of reproducibly detecting leaks at clearly defined and predictable detection limits. Because the majority of deterministic leak test methods described in this chapter require no special test sample preparation, sample preparation error is eliminated.

A "probabilistic leak test method" is stochastic in nature in that it relies on a series of sequential and/or simultaneous events each associated with uncertainties, yielding random outcomes described by probability distributions. Thus, the findings are associated with uncertainties that necessitate larger sample sizes and rigorous test condition controls to obtain meaningful results. Typically, sample size and test condition rigor are inversely related to leak size. Therefore probabilistic leak test methods are more challenging to design, develop, validate, and implement, especially when used to find leaks near the upper and lower limits of the test method's detection range. Probabilistic methods include microbial challenge tests, as well as some physicochemical tests. These methods include bubble emission tests, tracer liquid tests (employing either qualitative or quantitative measurement methods), and tracer gas tests by the sniffer probe approach.

A deterministic leak test method having the ability to detect leaks at the product's maximum allowable leakage limit is preferred when establishing the inherent integrity of a container–closure system. Deterministic methods may also be chosen if test sample quantities are limited, when checking for rarely occurring leaks of concern and/or when the potential risk for failing to find leaks of a given size or type is too great. Probabilistic methods are best chosen when the method outcome requirements demand a specific probabilistic testing approach. Probabilistic tests are further discussed in *Method Outcome* and *Quantitative or Qualitative Methods of Analysis* below.

## 3.6 Physicochemical or Microbiological Methods

"Physicochemical leak test methods" are those that use physical and/or chemical analysis techniques to test for package integrity. Physicochemical methods include both deterministic and probabilistic leak test methods. "Microbiological leak test methods" are probabilistic methods of analysis that use viable microorganisms to evaluate test sample integrity. This chapter includes the microbial challenge test by immersion exposure.

The following section includes additional information on the test method outcome criterion that is related to the microbiological challenge test method.

## 3.7 Method Outcome

The outcome(s) sought from leak test results will often drive the test method choice. These desired outcomes may include:

- Leak path presence of detection
- Leak path location determination
- Leakage rate measurement
- Liquid egress/ingress potential
- Microbial ingress potential

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All leak test methods are meant to identify the leak path presence. Often, leak tests are able to provide additional outcome information. Methods that also provide evidence of leak path location include electrical conductivity and capacitance tests, bubble emission tests, tracer gas sniffer probe tests, and some tracer liquid tests. Methods that provide a measure of whole-package leakage rate include laser-based gas headspace analysis, mass extraction, pressure and vacuum decay methods, and tracer gas tests by the vacuum mode.

Microbial challenge tests provide information on the degree of protection afforded by the product–package against microbial ingress that occurs via active growth or motility through leak pathways and/or by liquid carrier passive transport through leak pathways. Microbiological challenge tests help to clarify the risks to product sterility posed by specific package materials, package designs, or potential package barrier breaches. Sterility risks linked to particular environmental exposure or product use conditions may also demand a microbiological challenge methodology. In summary, all leak test methods at minimum detect leaks; some methods may provide more information, but no single method alone can yield all four outcomes listed above.

## 3.8 Quantitative or Qualitative Methods of Analysis

The measure of analysis can play a part in leak test method selection. In other words, does the method yield quantitative data that allow for objective analysis, or are the data strictly qualitative and require more subjective interpretation? Leak test methods that use a "quantitative measure of analysis" include electrical conductivity and capacitance tests, laser-based gas headspace analysis, mass extraction, pressure and vacuum decay, tracer gas tests (especially when testing via the vacuum mode), and tracer liquid tests that use quantitative analysis (e.g., spectrophotometric analysis).

Conversely, a "qualitative measure of analysis" is based on subjective observation of a specific quality, attribute, or characteristic of the test sample, e.g., a visual check for turbidity when evaluating microbial challenge test samples. Bubble emission tests that report visible evidence of continuous bubbling, and tracer liquid tests that rely on visible evidence of dye migration are other examples of subjective and qualitative analysis. Because qualitative measurement results are subject to interpretation, they may be prone to human error. When method considerations permit, leak test methods that yield quantitative measurements are preferred.

## 3.9 Leak Test Detection Limit

The detection limit of a leak test is the smallest leakage rate or leak size that the method can reliably detect, given the product–package of interest. A large variety of measurement units are used to describe leakage rates and leak sizes when specifying the detection limits (and detection ranges) of leak test methods. This often leads to confusion when comparing the performance claims of various instrument manufacturers or examining test results generated by multiple methods.

To address this, *Table 1* presents the relationship between orifice size (assuming a perfect hole of negligible length) and the rate at which dry air would pass through such a hole when exposed to 1 atmosphere (atm) differential pressure at a specified temperature.

These leakage rates and leak sizes are theoretical approximations and are not definitive.

*Table 1* services two purposes. First, it is meant to help the reader better grasp the relationship between theoretical hole diameter and the gas leakage rate. Second, it provides a common measurement scale that can be referred to later in this chapter to more simply state leak detection limits for the various technologies described. For example, test technology X found in published studies to detect leaks as small as about 8 µm would be referred to in ⟨1207.2⟩ as having an approximate leak detection limit of row 4 in *Table 1*.

Leak detection limit should not be the only or perhaps even the primary basis for choosing a test method. Often, the best method for a given application is dictated by other factors. For example, a tracer gas leak test method having an extremely small leakage rate detection limit may be the proper choice for establishing the inherent package integrity of a stoppered glass vial as a function of capping machine parameters during package development. Yet this method would be an inappropriate choice for rapid on-line testing in routine manufacturing. Instead, an electrical conductivity and capacitance test with a larger leak detection limit, able to test product-filled packages at on-line speeds, may be the best option. Refer to *Detection Limit* for a discussion on how to determine the limit of detection.

**Table 1. Gaseous Leakage Rate versus Orifice Leak Size[a]**

| Row | Air Leakage Rate[b] (std · cm³/s) | Orifice Leak Size[c] (µm) |
|---|---|---|
| 1 | $<1.4 \times 10^{-6}$ | <0.1 |
| 2 | $1.4 \times 10^{-6}$ to $1.4 \times 10^{-4}$ | 0.1 to 1.0 |
| 3 | $>1.4 \times 10^{-4}$ to $3.6 \times 10^{-3}$ | >1.0 to 5.0 |
| 4 | $>3.6 \times 10^{-3}$ to $1.4 \times 10^{-2}$ | >5.0 to 10.0 |
| 5 | $>1.4 \times 10^{-2}$ to 0.36 | >10.0 to 50.0 |
| 6 | >0.36 | >50.0 |

[a] This table is not intended for ranking test methods but is offered as an aid for expressing test method leak detection capabilities in this chapter.
[b] Dry air leakage rate measured at 1 atm differential pressure across an orifice leak (i.e., leak inlet pressure of 1 atm versus outlet pressure of approximately 1 Torr) at 25°. The theoretical correlations of orifice sizes to air leakage rates were provided by Lenox Laser, Glen Arm, MD. Leakage rates are approximation ranges.
[c] Nominal diameter orifice sizes assume a leak path of negligible length. Orifice sizes are approximation ranges.

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### 3.10 Leak Test Method Range

Leak test method range is the interval between the smallest and largest leak size (or leakage rate) that can be detected by a given leak test method with a suitable level of accuracy and precision. All leak test methods have an optimum detection range. Therefore, it is possible that additional tests may be needed to catch those leaks of concern that fall outside the chosen leak test method's detection range. For example, a test method able to find the smallest leaks may miss gross leakage such as a missing package component. Vision systems that check for major package defects such as a missing package component or a package crack may be required. Finally, before using a test method to check for large leaks, the impact of gross defects on leak test instrumentation should be considered; some instruments may malfunction or become damaged upon exposure to a leaking product or damaged packages. Refer to *Range* for additional detection range information.

### 3.11 Nondestructive or Destructive Methods

The need to preserve the test product–packages may influence the decision to select a nondestructive leak test method, rather than a destructive one. Destructive test methods damage the test sample and/or expose it to potential contaminants; hence, the product is not recoverable. Only nondestructive test methods are appropriate for leak testing product–package units earmarked for commercial or clinical study distribution.

Examples of destructive leak test methods include tracer liquid ingress tests, bubble emission tests, and microbiological challenge tests. Tracer gas and pressure decay leak tests are considered destructive if they require that the package barrier be compromised in order to introduce tracer or pressurizing gas into the assembled package. Examples of nondestructive leak test methods include mass extraction and vacuum decay leak tests, as well as noninvasive gas headspace analysis tests. Electrical conductivity and capacitance leak tests are deemed nondestructive if it can be shown that electrical current exposure causes no harm to the product; in rare instances, exposure has triggered headspace ozone formation, causing product oxidation (*3*).

### 3.12 Off-Line or On-Line Methods

Off-line leak testing is generally performed on a stratified random sampling of the product lot, away from the manufacturing line. Off-line package evaluation allows for the use of any validated nondestructive or destructive leak test and seal quality test option compatible with the product–package. Off-line testing can accommodate slower test cycle times; for methods in which test time is a performance factor, a slower off-line test is often more sensitive than its on-line counterpart. Off-line tests typically are less costly to perform as they utilize bench-top or smaller scale equipment without the sophisticated product-handling machinery required to support higher speed on-line processes.

On-line leak testing is commonly integrated into a continuous fill and seal product–package manufacturing process. A prerequisite for an on-line leak test method for entire lot testing is that it be nondestructive to the package and its contents. On-line testing can potentially provide greater assurance that all packages have integrity and can yield instant feedback in the event of package misassembly or breakage, enabling real-time line corrections. For some test systems, incorporating large-scale leak detection equipment into a complex high-speed product–package filling and assembly line can be prohibitive. Higher line speeds leading to shorter leak detection test cycle times can limit test method detection capability. In addition, the impact of instrument downtime on the production run as a result of leaking packages or possible equipment malfunction is an important consideration. A separate leak testing line may be set up outside the sterile manufacturing suite to allow for full lot testing without the complications of leak test integration with package filling/sealing operations. A few examples of on-line leak test technologies include electrical conductivity and capacitance, vacuum decay leak, and noninvasive laser-based gas headspace analysis tests.

## 4. TEST INSTRUMENT QUALIFICATION, METHOD DEVELOPMENT, AND METHOD VALIDATION

Leak test methods are validated in order to demonstrate method effectiveness. Method validation is preceded by instrument/equipment qualification, followed by test method development. The following discussion specific to leak test methods is intended to supplement the guidance for analytical instrument qualification presented in *Analytical Instrument Qualification* ⟨1058⟩, plus the guidance for method validation provided in *Validation of Compendial Procedures* ⟨1225⟩.

### 4.1 Instrumentation and Equipment Qualification

The qualification of instruments or equipment to be used for leak testing includes: 1) evaluation of instrument/equipment functionality, and 2) determination of test system detection capabilities using appropriate calibration tools or reference standards to simulate with-leak test conditions.

### 4.2 Method Development and Validation

After successful instrument/equipment qualification, leak test method parameters are developed and optimized to ensure a leak test method is able to meet all relevant leak detection performance criteria specific for the test product–package system. The following properties of a valid test method are defined as they relate to package integrity tests.

#### 4.2.1 ACCURACY

"Accuracy" is a measure of the method's ability to correctly differentiate packages that leak above the claimed detection limit from those that leak below this limit (i.e., do not leak). Accuracy provides a measure of false positive and false negative

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occurrence. Alternatively, for those methods that deliver an outcome that is a direct quantitative measure of gas leakage rate (or of gas concentration or gas pressure), accuracy is a measure of the method's ability to produce an outcome comparable to a true standard. For example, helium mass spectrometry provides a direct measure of helium leakage rate. Accuracy is the closeness of the instrument reading to the certified leakage rate of a nationally recognized traceable standard.

#### 4.2.2 PRECISION

"Precision" is the ability of the method to yield reliable, repeatable data. Precision includes repeatability (e.g., repeat testing of a homogeneous test sample population), ruggedness (within laboratory tests performed, for example, by multiple operators on multiple days, using multiple instruments; also known as intermediate precision), and reproducibility (among laboratories tests). The level of precision to which a leak test method is validated is often a function of resource availability (e.g., one instrument versus multiple instruments) and intended test method application (e.g., use of the method at one test site only versus across multiple test sites).

#### 4.2.3 SPECIFICITY

"Specificity" is the ability of the method to accurately differentiate between leaking and nonleaking packages, despite interfering factors that may cause false detection. For example, when employing tracer gas leak detection using helium mass spectrometry (vacuum mode), excessive helium permeation through the package wall may mask small package leaks or may be falsely interpreted as leakage in no-defect packages.

#### 4.2.4 DETECTION LIMIT

Refer to *Leak Test Detection Limit* for an introduction to this topic. The detection limit of a leak test is specific for a given testing approach when performed using a specific instrument make/model in evaluating a given product–package system. Utilizing the principles described in ⟨1225⟩, detection limit is demonstrated by challenging packages with and without known defects by the leak test method for multiple test days by multiple operators. The intended application of the method will dictate the level of precision required (i.e., whether to incorporate multiple operators/instruments/laboratories, etc.).

A common challenge with small leak detection is the potential for interfering factors to be misconstrued as leakage presence. For example, vacuum decay leak tests measure the rise in pressure inside an evacuated chamber containing the test package. Package leakage causes chamber pressure to rise, but package material volatiles, test system moisture, and package expansion may also do the same. Gas permeating through a package wall detected by a tracer gas test may be mistaken for leakage. Bubbles emitted during a bubble test may actually be the result of package surface outgassing, volatilization of dissolved gases in the immersion fluid, or the release of trapped air between package components.

The absence of a leak detection signal may also be misinterpreted as the absence of leaks. For example, tracer liquid tests may fail to reliably detect small leaks due to any one of a number of factors including air locks, product, or debris in the leak path; liquid surface tension; leak path geometry; or insufficient differential pressure test conditions. The same is true for microbial ingress tests that are further subject to the inherent variability of living microorganisms.

In short, false negative results that miss leaks and false positive results that incorrectly suggest leak presence are possible with any leak test method. Therefore, test method detection limit is determined by comparing readings of intentionally defective packages to nondefective ones. Leak detection limit determination test units consist of a randomly ordered population mix of negative and positive control units (refer to *Negative and Positive Controls*). Nondestructive test methods can employ the same set of units for multiple test exposures, while destructive methods will require a new set for each test series. Control subset unit quantities are chosen based on several factors: 1) the deterministic or probabilistic nature of the outcome, 2) the inherent package-to-package variability that may influence test results, and 3) the statistical confidence level required by the test acceptance criterion. The positive control subset includes units with defects sized to the anticipated detection limit, in addition to units with leaks bracketing this size limit. If the detection range is to be established, controls having large defects are included. Positive controls representing a wide defect size range are especially important for probabilistic methods in order to clearly understand leak detection likelihood as a function of leak size.

Because of the many product–packages and leak-testing options available, the resultant leak detection limit is more meaningfully stated when the negative and positive control subsets used, the test precision level, and the test results are summarized. The following is an example of expressing a test method's limit of detection:

"The detection limit for method X was determined to be 5 ± 2 µm. Validation studies found defects of this nominal size were detected 95% of the time; all larger defects were detected 100% of the time. Studies included three replicate test series performed on multiple days by multiple operators in a single laboratory using one instrument. Detection limit was determined using product-filled packages. Test units in each series included a negative control subset of 300 units (each without defect) and a positive control subset of 90 units (each having a laser-drilled defect ranging in nominal size from 1.5 ± 0.6 µm to 15 ± 3 µm). Each defect was independently size-certified by comparing the dry air leakage rate at 1 atm differential pressure (leak inlet pressure of 1 atm versus outlet pressure of approximately 1 Torr) at 25° to that of standard orifice leaks."

[NOTE—This is one example of how test method detection limit could be expressed and is not to be considered compulsory or restrictive either in content or level of detail.]

Leak test technologies exist that are able to detect leaks even smaller than can be artificially created in a positive control test set. Two examples follow. In both cases, a limited number of positive controls can serve to verify that the instrument set-up is able to detect leaks of specific type and in specific package locations but not to determine leak size detection limit capability.

- Tracer gas tests by helium mass spectrometry performed in the vacuum mode can detect leaks as small as about $10^{-11}$ mbar · L/s, which is about 5 logarithmic leakage units smaller than leakage through a hole 0.2 µm in size. For such methods, leakage rate detection ability can only be verified by using nationally recognized gas leakage rate standards to introduce leaks into the test system. Because gas permeation can be mistaken for leaks, it is important to experimentally establish

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the relationship between tracer gas leakage rate and permeation rate as a function of test cycle time for a given package system.

- Laser-based gas headspace analysis is another approach that may be able to identify the presence of leaks smaller than can be artificially created. For such methods, the limit of detection can be mathematically predicted on the basis of gas flow kinetics and is a function of the time lapse between analyses, and the smallest gas content or pressure change that can be reliably detected by the instrument for the given package system.

#### 4.2.5 QUANTITATION LIMIT

"Quantitation limit" is that lowest leakage rate or leak size that can be determined with acceptable accuracy and precision under the stated experimental conditions. Laser-based gas headspace analysis is a method that may be evaluated for quantitation limit. For most leak test methods, detection limit is more meaningful.

#### 4.2.6 LINEARITY

"Linearity" is the ability of the method to elicit test results that are mathematically proportional to leak path size or leakage rate. Deterministic leak test methods that exhibit linearity include laser-based gas headspace analysis and tracer gas analysis (vacuum mode). Other methods such as vacuum decay, pressure decay, and mass extraction also produce results that correlate to leak size or leakage rate. However, test findings are generally intended to identify leak presence and perhaps to understand relative leak size; they are not typically relied upon for leak size or leakage rate quantitation. Electrical conductivity and capacitance tests and all referenced probabilistic methods are not validated for linearity.

#### 4.2.7 RANGE

Leak detection range is defined in *Leak Test Method Range*. Leak test method range is explored in test method development to better understand leak test method detection limitations. For methods being relied upon to detect leaks within a specified size range, detection at the upper range limit may be confirmed in validation. Range is evaluated by using sets of negative controls and appropriately sized larger-defect positive controls. The large-defect positive control subset may include defects of various types likely to occur for the given product-package system (refer to *Type Defects*).

#### 4.2.8 ROBUSTNESS

"Robustness" is the method's ability to accurately identify leaking versus nonleaking packages despite small but deliberate variations in procedural parameters, providing an indication of the method's suitability during normal usage. One way to evaluate robustness is to perform the test using test parameters bracketing optimal or normal test specifications. Parameters to be varied are those having the greatest impact on test results; variation should reflect instrument performance accuracy. For example, the robustness of a vacuum decay leak test with a test cycle time of 30 s (accurate to within 0.5 s) might be demonstrated during method development by verifying method performance at set test cycle times of 29.5 and 30.5 s.

### 4.3 System Suitability

One outcome of test method development and validation is the establishment of a system suitability test(s), also called a performance verification test(s). "System suitability" is a manner of ensuring that the leak test method, including all factors that may be subject to variability that may impact test results (e.g., instrumentation, analysts, test sample preparation, and the test environment), is adequately controlled and maintained in such fashion that the method is rugged and robust. System suitability is important for all leak test methods. Extra care may be required for probabilistic methods more prone to variability.

For example, a highly instrumental method such as a mass extraction test may be checked for system suitability by leak testing a master package unit both with and without the added challenge of external air introduced to the test system via a calibrated leak adjusted to the method's limit of detection. If the method requires preliminary test package preparation (e.g., a drying step), then it would be appropriate to demonstrate that an intact package prepared in the prescribed manner elicits the anticipated mass flow rate response. System suitability can be performed at the beginning and end of each testing sequence for added method assurance.

System suitability for a test more probabilistic in nature takes into account multiple test method variables and may require greater numbers of challenge samples (i.e., positive and negative controls) for adequate method assurance. For example, tracer gas tests via a sniffer probe may require a routine demonstration that the operator is able to successfully differentiate packages without defect from those with leaks (ranging in size from smallest to largest, located at various package positions) in a blinded challenge study. Alternatively, the operator could be challenged by randomly introducing defective samples (unknown to the inspector) among the test sample population during routine test procedures.

### 4.4 Microbial Ingress Risk Comparison

Appropriate leak test method validation practices mirror compendial analytical test validation guidelines. In the past, validation of physicochemical leak test methods used for pharmaceutical sterile product packages also routinely included a direct or indirect comparison of physicochemical leak test results to microbiological challenge data. While an understanding of the relationship between physicochemical leak test method capabilities and microbial ingress risk may be important, an experimental comparison may not be needed or useful in all cases. [NOTE—The reader may refer to *Package Integrity Evaluation—Sterile Products* ⟨1207⟩*, Product–Package Quality Requirements and the Maximum Allowable Leakage Limit* for a discussion of maximum allowable leakage limit and inherent package integrity.]

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The following situations illustrate when a comparison study of the microbial ingress or liquid leakage risk to physicochemical leak test method capability relationship is likely not needed. [NOTE—This is not intended to be a complete or exhaustive listing of all situations for which comparison studies would not be required or useful but is provided for illustration only.]

- If the validated physicochemical leak test method has a proven detection limit at or below the product-package maximum allowable leakage limit.
- If the validated physicochemical leak test method is not being used to verify the absence of all leaks of concern. Instead, the method is being used to find leaks notably larger than the maximum allowable leakage limit. For example, a rapid on-line test shown to reliably detect leaks of 25–150 µm in nominal diameter is being used to reject damaged or misassembled product-filled packages within the method's leak detection range.

The following situations illustrate when an experimental indirect or direct comparison of the microbial ingress risk (or liquid leakage risk) to physicochemical leak test method capability relationship may be useful. [NOTE—This is not intended to be a complete or exhaustive listing of all situations for which comparison studies could prove beneficial but is provided for illustration only. These examples are not compulsory but are provided for instruction only.]

- If the validated physicochemical leak test method is being relied upon to measure or confirm the inherent package integrity of a product–package system, but the method's limit of detection is notably greater than the maximum allowable leakage limit. For example, a package has a maximum allowable leakage limit of less than $6 \times 10^{-6}$ mbar · L/s as measured by helium mass spectrometry (equivalent to holes less than approximately 0.2 ± 0.1 µm in nominal diameter). This limit was chosen based on published literature references. But the leak test method of choice for testing product-filled packages placed on stability is able to detect leaks equivalent to holes 3 µm in nominal diameter and larger. A study correlating microbial ingress or liquid leakage risk to defect type/size can provide a measure of the likelihood of microbial ingress (and/or liquid leakage) at the leak test method detection limit, and thus can provide an understanding of ability of the stability leak test to identify leaks of concern.
- If the validated physicochemical leak test method is being used to measure or verify the inherent package integrity of a product–package system, but the maximum allowable leakage limit is either lacking or not well defined. For example, a unique package is being used for which the maximum allowable leakage limit that will ensure absence of product loss or microbial ingress has not been determined. A study comparing the risk of microbial ingress or liquid leakage to leak type/size, and in turn to the likelihood of detection by the physicochemical leak test method may be useful.

When performing microbial ingress risk assessment studies it is important to keep in mind the probabilistic nature of microbial ingress. To achieve the most meaningful data, large population sets of negative and positive controls should be used. Test protocols should be thorough and well designed, taking into consideration the multiple factors and variables that can influence results. As suggested above, liquid leakage risk assessment studies may substitute for microbial ingress risk studies assuming the risk of liquid leakage is equivalent to or greater than that of microbial ingress, with appropriate justification.

## 4.5 Negative and Positive Controls

"Negative controls" are packages with no known leak, and "positive controls" are packages with intentional or known leaks. Negative and positive controls are designed and assembled for use in method development and validation with consideration given to container–closure design, materials of construction, characteristics of anticipated package leaks, and impact of product contents on test results. Negative and positive controls should represent packages assembled in a typical manner as the product being tested using normally processed components, the exception being the intentionally created leak in each unit of the positive control subset. Some leak test methods may necessitate positive controls that simulate test product headspace and formulation contents as well. System suitability checks for some test methods employ negative and positive controls (refer to *System Suitability*).

Test blanks should not be confused with negative controls. For example, liquid tracer leak detection by spectrophotometric analysis may require a blank solution without the liquid tracer element to confirm instrument baseline performance.

A "master" is a type of negative control test unit. It is a package prototype, model, or facsimile made to simulate the test package in shape and design. Masters may be made of solid material such as plastic or metal, or they may be simply a designated container–closure unit. Masters are no-leaking mock packages often used in system suitability verification tests for leak tests to verify instrument performance, such as for vacuum decay or mass extraction testing.

### 4.5.1 DEFECT CREATION METHODS

The positive control set typically represents a range of package defect sizes and types. Numerous approaches have been used to create package defects. When creating positive controls, a fundamental awareness of leakage dynamics as a function of different defect types and materials of construction is important.

Placing a so-called "hole" or break in the package wall is one positive control creation approach. In this case the defect materials of construction are identical to the package itself, thus potential test method interference due to product exposure to the package material can be readily identified. An example of such interference is the clogging of small leak paths by product formulation observed during vacuum decay, mass extraction, or tracer gas (vacuum mode) tests.

Laser drilling is often used to create package defects commonly referred to as holes. However, these so-called holes are not pristine orifices but are non-cylindrical and asymmetric and may consist of a tortuous matrix of micro-cracks. While laser-drilled defects are not dimensionally ideal, they offer the advantages of closely simulating actual package defects (e.g., cracks) and do not require the introduction of foreign materials, such as wires, tubes, or epoxies, that may influence leakage dynamics. Laser-drilled defects are often sized for nominal diameter by comparing dry air leakage rate through the defect at specified conditions of pressure and temperature to flow rates through standard orifice leaks in thin metal plates. Currently, laser-drilled defects in rigid glass or plastic components can be made as small as about 2–3 µm in nominal diameter and to about 5–10 µm in nominal diameter in flexible thicker wall package materials. Smaller diameter defects tend to clog from handling, environmental debris, or flexible package wall deflection.

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Glass micropipettes can be used to simulate single-orifice defects as small as about 0.1 µm in diameter. The tip diameter can be nominally sized using air flow measurements. When creating positive controls, micropipettes are inserted through a break in the package wall, then an appropriate sealant is applied to the insertion site. Challenges to micropipette use include ensuring a complete seal between the micropipette perimeter and the package wall and avoiding micropipette tip damage.

In addition, air trapped in the pipette tube can interfere with leak test methods that depend on fluid flow through the leak path, such as tracer liquid tests and microbial challenge ingress tests.

Microtubes (also called microcapillaries) inserted through the package wall and fixed in place with sealant are another means of creating positive control defects. Microtubes can be made of a variety of materials, can be cut to any length and can be as narrow as 2 µm in cross-sectional diameter. Microtubes are often employed as a substitute for a smaller-bore, shorter-length leak path when performing leak tests that rely on gas flow measurements. However, caution is advised before choosing lengthy, larger-bore microtubes to simulate an orifice leak of smaller diameter for leak tests that rely on the passage of liquids or microorganisms. Fluid dynamic theory correlating fluid flow through capillary tubes to pass through smaller bore holes is based on the unimpeded passage of ideal liquids through capillaries at equilibrium pressure conditions. Liquid product formulation and aqueous media flow into and through a microtube is complicated by numerous factors including liquid surface tension, liquid viscosity, surface contact angle, airlocks, particulate blockage, and tube-wall and tube-end finishes. Microbial ingress through microtube defects relies more on the presence of liquid in the tube than on the physical barrier to passage or grow-through afforded by the tube diameter (*4*). Microtubes are a logical choice when creating defects representing channel defects. In this case microtube length should mimic as closely as possible the actual package barrier thickness (package wall or seal width). Microtube use challenges include effecting smooth, perpendicular cuts of microtube ends and adequately sealing microtubes into the test sample wall.

Other commonly used defect creation methods include inserting a needle through the package wall; placing a wire, microfilament, or film between sealing surfaces; and adhering a holed, thin metal plate onto package surfaces. It is important to note that defects made by using an object foreign to the package (e.g., needle, film, wire plate) may display gas, liquid, or microbial leakage dynamics markedly different from that of actual defects (*5*). Positive controls made by such means are easy and inexpensive approaches for creating larger size defects useful for test method feasibility studies and for exploring a test method's detection range upper limit.

#### 4.5.2 TYPE DEFECTS

"Type defects" are positive controls representing realistic package flaws. Type defects are important to include in method validation studies to explore a leak test method's practical application in detecting realistic package failures and flaws. A few types of defect examples are listed below:

- Heat sealed bag: 1) weak seal, 2) wrinkled seal, 3) seal gap, 4) seal channel, 5) product entrapment in seal
- Stoppered vial package: 1) vial finish channel defect, 2) loosely capped stopper, 3) product trapped between stopper and vial
- Prefilled syringe: 1) needle shield punctured by staked needle, 2) defective plunger
- Ophthalmic dropper bottle: 1) loose cap, 2) missing or poorly inserted dropper tip, 3) defective tip or cap

Assigning precise sizes to type defects is generally not meaningful or feasible because of their inherent irregularity and complexity. Type defects are defined in more qualitative, descriptive terms such as those listed above. Because no leak test method can find all possible defects in a given container–closure system, information collected from type defect tests can be used to identify alternative approaches to detecting or limiting the occurrence of critical imperfections not readily found by the chosen leak test method.

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# ⟨1207.2⟩ PACKAGE INTEGRITY LEAK TEST TECHNOLOGIES

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## 1. INTRODUCTION

The purpose of this chapter is to provide information guiding the selection and proper use of leak test technologies (also called methodologies, approaches, or methods). The leak test technologies described in this chapter were selected on the basis of relevant research study data published in peer-reviewed journals and/or precision and bias study data generated in support of recognized test method standards. When referencing standard test methods (e.g., ASTM), the reader is advised to refer to the most recent versions. In some cases, the scope of referenced standard test methods does not include the package types of the scope in *Package Integrity Evaluation—Sterile Products* ⟨1207⟩. In all cases, methods and literature studies are cited to provide benchmark information useful for pharmaceutical package leak test method development and validation.

The technologies described in this chapter are not prescriptive methods but represent testing concepts that may be applied when leak testing sterile product–packages. Test technologies vary in terms of their potential detection limits, reliability, and applications; therefore, none are universally appropriate for leak testing all product–packages. This chapter provides information to allow a thorough comparison of testing approaches so that the most appropriate technology for a given situation can be identified.

After a methodology has been selected for use, the test equipment operation and performance is qualified. Test method parameters are optimized during method development and confirmed during validation. Thus, a final leak test method is specific to a particular container–closure or product–package system.

The leak test methods included are divided into two categories: deterministic and probabilistic. Deterministic leak test methods (*Table 1*) are preferred over probabilistic methods when other key method selection criteria permit. Probabilistic leak test methods (*Table 2*) are best used when the product–package system proves incompatible with deterministic methods, or when method outcome requirements demand a particular probabilistic testing approach.

In this chapter's *Table 1* and *Table 2*, the "leak size detection limit" provided for each methodology refers to leakage rates/leak sizes listed by row in *Package Integrity Testing in the Product Life Cycle—Test Method Selection and Validation* ⟨1207.1⟩*, Table 1*. The reported leak detection limits were chosen on the basis of literature sources, as well as commonly accepted experience. This information is intended to aid in the selection of the test technology but should not be used as a definitive statement of test method performance for any specific leak test method applied to any given product–package system. Instead, leak detection limit and range should be established during leak test method development and validation for the respective product–package or container–closure system. For instance, an approach cited as capable of detecting row 6 leaks may be validated by the user to detect leaks as small as those in row 3. Conversely, a method described as capable of detecting leaks in row 3 may be determined by the user to detect leaks only as small as row 5.

This battery of testing technologies and the information provided are intended to aid, not limit, the selection, development, validation, and use of leak test methods. Unlisted methodologies shown by the user to meet the qualification and validation requirements for a satisfactory leak test may be used. In addition, listed technologies may demonstrate expanded testing capabilities beyond those currently identified.

**Table 1. Deterministic Leak Test Technologies[a]**

| Deterministic Leak Test Technologies | Package Content Requirements | Package Requirements | Leak Detection Limit[b] | Measurement Outcome and Data Analysis | Effect of Method on Package | Test Time Order of Magnitude |
|---|---|---|---|---|---|---|
| Electrical conductivity and capacitance (high-voltage leak detection) | Liquid (with no combustion risk) must be more electrically conductive than package.<br>Product must be present at leak site. | Less electrically conductive than liquid product. | **Row 3**<br>Varies with product–package, instrument, test sample fixtures, and method parameters. | Quantitative measure of electrical current passing through the test sample: provides an indirect determination of leak presence and leak location as shown by a drop in test sample electrical resistivity, with a resultant increase in voltage reading above a predetermined pass/fail limit. | Nondestructive, although impact of test exposure on product stability is recommended | Seconds |

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**Table 1. Deterministic Leak Test Technologies[a]** *(continued)*

| Deterministic Leak Test Technologies | Package Content Requirements | Package Requirements | Leak Detection Limit[b] | Measurement Outcome and Data Analysis | Effect of Method on Package | Test Time Order of Magnitude |
|---|---|---|---|---|---|---|
| Laser-based gas headspace analysis | Gas volume, path length, and content must be compatible with instrument's detection capability. | Allows transmission of near-IR light. | **Row 1** Varies as a function of time span between analyses. | Quantitative measure of gas headspace content of the test sample by laser-based gas analysis, for a product requiring a headspace low in oxygen, carbon dioxide, or water vapor concentration; and/or low in absolute pressure. Whole test sample leakage rate is determined by compiling readings as a function of time. | Nondestructive | Seconds |
| Mass extraction | Gas or liquid must be present at leak site. Presence of liquid at leak site requires test pressures below vapor pressure. Product must not clog leak path. | Rigid, or flexible with package restraint mechanism. | **Row 3** Varies with product–package, instrument, test fixtures/chamber, and method parameters. | Quantitative measure of mass flow rate resulting from test sample headspace escape or liquid product volatilization within an evacuated test chamber housing the test sample. Quantitative pressure readings early in the test cycle indicate larger leak presence. Whole test sample leakage rate is determined by comparing the test sample mass flow results to results using leak rate standards and positive controls. | Nondestructive | Seconds to minutes |
| Pressure decay | Gas must be present at leak site. Product (especially liquids or semi-solids) must not cover potential leak sites. | Compatible with pressure detection mode. Rigid, or flexible with package restraint mechanism. | **Row 3** Varies with product–package, instrument, and method parameters. | Quantitative measure of pressure drop within a pressurized test sample. Pressure drop readings are a measure of gas escape through leak paths. Whole test sample leakage rate is determined by comparing pressure decay results to results using leak rate standards and positive controls. | Nondestructive, unless the means used to access test sample interior compromises test sample barrier. | Minutes to days, depending on package volume and required leak limit of detection |
| Tracer gas detection, vacuum mode | Tracer gas must be added to package. Tracer gas must have access to package surfaces being tested for leaks. | Able to tolerate high-vacuum test conditions Rigid, or flexible with package restraint mechanism Limited tracer gas permeability | **Row 1** Varies with instrument capability and test sample fixtures. | Quantitative measure by spectroscopic analysis of tracer gas leak rate emitted from a tracer-flooded test sample positioned in an evacuated test chamber. Whole test sample leakage rate is calculated by normalizing the measured tracer leak rate by tracer concentration in the test sample. | Nondestructive, unless tracer gas introduction into the package compromises test sample barrier. | Seconds to minutes |
| Vacuum decay | Gas or liquid must be present at leak site. Presence of liquid at leak site requires test pressures below vapor pressure. Product must not clog leak path. | Rigid, or flexible with package restraint mechanism | **Row 3** Varies with product–package, instrument, test sample chamber, and method parameters. | Quantitative measure of pressure rise (vacuum decay) within an evacuated test chamber housing the test sample; vacuum decay readings are a measure of headspace escape from the test sample, or liquid product volatilization. Whole test sample leakage rate is determined by comparing vacuum decay results for the test sample to results of tests performed using leak rate standards and positive controls. | Nondestructive | Seconds to minutes |

[a] All methods apply to nonporous, rigid and flexible packages as per the scope of ⟨1207⟩.
[b] The leak detection limit cited for each technology refers to *Package Integrity Evaluation—Sterile Products* ⟨1207⟩, *Table 1* and is provided for information only. This information is intended to assist in early methodology selection. The validated leak detection limit for a product–package test method may deviate from these values.

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**Table 2. Probabilistic Leak Test Technologies[a]**

| Probabilistic Leak Test Technologies | Package Content Requirements | Package Requirements | Leak Detection Limit[b] | Measurement Outcome and Data Analysis | Effect of Method on Package | Test Time Order of Magnitude |
|---|---|---|---|---|---|---|
| Bubble emission | Gas must be present at leak site. Product (especially liquids or semi-solids) must not cover package surfaces to be leak tested. | Rigid, or flexible with package restraint mechanism. | **Row 4** Varies with product–package, test sample fixtures and positioning, method parameters, and analyst technique and skill. | Qualitative measure by visual inspection of bubble emission caused by escape of test sample headspace while sample is submerged and exposed to differential pressure conditions. Alternatively, sample surfaces may be exposed to surfactant. Continuous bubble emission indicates leak presence, location, and relative size. | Destructive | Minutes |
| Microbial challenge, immersion exposure | Growth-supportive media or product. Presence of liquid at the leak site required for method reliability. | Able to tolerate pressure and immersion challenge. Rigid, or flexible with package restraint mechanism. | **Row 4** Varies with container–closure, test sample fixtures and positioning, challenge condition severity, and inherent biological variability. | Qualitative measure by visual inspection of microorganism growth inside test samples filled with growth-supportive media or product, post immersion in heavily contaminated challenge media while exposed to differential pressure conditions, followed by incubation to encourage microbial growth. Growth in the test sample indicates the presence of test sample leak site(s) capable of allowing passive or active entry of microbes. | Destructive | Weeks |
| Tracer gas detection, sniffer mode | Tracer gas must be added to package. Tracer gas must have access to package surfaces to be tested for leaks. | Leak site accessible to probe. Limited tracer gas permeability. | **Row 2** Varies with test sample, method parameters, test sample fixtures, and analyst technique and skill. Smaller leak detection may be possible under optimum test conditions. | Quantitative measure by spectroscopic analysis of tracer gas near the outer surfaces of the tracer-flooded test sample, sampled using a sniffer probe. Tracer presence above a pass/fail limit indicates leak presence and location. | Nondestructive, unless tracer gas introduction to the package interior compromises test sample barrier. | Seconds to minutes |
| Tracer liquid | Contents must be compatible with liquid tracer. Product must not clog leak path. | Rigid, or flexible with package restraint mechanism. Able to tolerate liquid immersion. Compatible with liquid tracer detection mode. | **Row 4** Varies with container–closure, test sample fixtures and positioning, challenge condition severity, and tracer liquid content. Smaller leak detection may be possible under optimal test conditions employing chemical analysis tracer detection. | Measure of tracer in test sample previously submerged in tracer-charged liquid while exposed to differential pressure conditions. Alternatively, tracer-charged test samples may be submerged in tracer-free collection fluid. Tracer migration measurement may be quantitative (by chemical analysis; preferred approach for small leak detection) or qualitative (by visual inspection). Tracer presence indicates leak site(s) capable of allowing tracer passage. Tracer magnitude may indicate relative leak size (assuming a single-leak pathway). | Destructive | Minutes to hours |

[a] All methods apply to nonporous, rigid and flexible packages as per the scope of ⟨1207⟩.
[b] The leak detection limit cited for each technology refers to *Package Integrity Evaluation—Sterile Products* ⟨1207⟩, *Table 1* and is provided for information only. This information is intended to assist in early methodology selection. The validated leak determination limit for a product–package specific test method may deviate from these values.

# 2. DETERMINISTIC LEAK TEST TECHNOLOGIES

## 2.1 Electrical Conductivity and Capacitance (High-Voltage Leak Detection)

### 2.1.1 DESCRIPTION

The electrical conductivity and capacitance leak test (high-voltage leak detection, or HVLD) is an approach for detecting the presence, and potentially the location, of a leak(s) in the wall of a nonporous, rigid or flexible package containing liquid or semi-liquid product. Test analysis is based on quantitative electrical conductance measurements (*1*–*5*). HVLD leak tests are generally nondestructive to the package and to the product, although an evaluation of HVLD exposure impact on product physicochemical stability is advised.

The test is performed by first positioning the test sample (containing liquid product) onto an electrically grounded instrument test fixture. Alternatively, the test sample may be placed onto a transport system that will carry the test sample through an electrically grounded testing zone. Upon test start, an electrode uniquely designed for the product–package type under test exposes all or part of the test sample to a high-frequency, high-voltage, low-amperage current. The presence of a leak path in the proximity of an electrically conductive, liquid-formulation product results in a drop in the electrical resistance of the test

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sample, as shown by a spike in current passing through the test sample above a predetermined pass/fail limit established using negative controls.

#### 2.1.2 APPLICATION

Rigid or flexible packages of nonporous components containing liquid or semi-liquid product may be tested:

- Package components must be relatively electrically nonconductive.
- Product must be electrically conductive, relative to the package.
- Product must not be flammable (i.e., not a combustion risk).
- Product must be near or at the leak inspection location at the time of the leak test.
- Solidified, electrically conductive product that blocks leak paths may be detected.
- Metal caps used to seal stoppered vial or cartridge packages conduct current, improving the likelihood of finding leaks under the cap.

HVLD tests are rapid, requiring no more than several seconds for a full scan of the test sample, thus making them appropriate for off-line testing, or as an on-line, 100% product inspection test method. This technology is useful for any product life cycle phase.

#### 2.1.3 TEST EQUIPMENT

HVLD instrumentation comes equipped with tooling and/or a test sample transport system for proper test sample and probe/ground positioning, an internal high-voltage transformer, electrode voltage and ground potentiometer adjustment capabilities, and a test result output display. The design and materials of construction used for the electrode probe and electrical ground are product–package specific.

#### 2.1.4 TEST PARAMETERS

The following are test parameters for the electrical conductivity and capacitance leak test, also known as HVLD:

- Conductivity of test sample product relative to test sample package: a greater difference will improve leak detection sensitivity
- Test voltage set point: voltage is set high enough to ensure leak detection, but not so high that current will arc, falsely rejecting the test sample
- Test sensitivity set point (potentiometer or gain set point): sensitivity should be maximized to ensure leak detection without triggering a false reject result
- Package content proximity to potential leak paths: leak detection sensitivity is directly related to the proximity of product to the leak path
- Electrode probe position relative to potential leak paths: probe proximity to the leak is directly related to the test method sensitivity
- Speed at which the electrode passes over the test sample surface: although the test is very rapid, test speeds too rapid may cause leaks to be missed
- Moisture presence on the package: test sample surface condensation can potentially trigger a false reject reading

### 2.2 Laser-Based Gas Headspace Analysis

#### 2.2.1 DESCRIPTION

Gas headspace analysis via laser-based techniques provides a quantitative, nondestructive measure of oxygen content, water vapor content, and low internal pressure in the headspace of a nonporous, rigid or nonrigid package (*6*–*8*). Some instruments are capable of measuring headspace carbon dioxide concentration as well.

The test is performed by first placing the test sample in a fixture designed for precise test sample positioning. Upon test start, frequency-modulated spectroscopy is used to cause a near-infrared (IR) diode laser light to pass through the gas headspace region of the sealed test sample. Light is absorbed as a function of gas concentration and pressure. The absorption information is processed using phase-sensitive detection techniques; a mixer demodulates the signal. The output voltage, which is proportional to the absorption line shape, is digitally converted and further analyzed by a microprocessor, yielding test sample signal results. Final test sample readings are automatically generated based on a comparison of test sample signals to a calibration curve. This curve is pre-established by using control packages flooded with traceable gas reference standards. Gas headspace analysis, as a function of time, provides a quantitative measure of the total leakage rate of the test sample. Leakage rates are judged acceptable or unacceptable on the basis of predetermined limits, calculated to ensure proper gas headspace content maintenance over the product life cycle.

#### 2.2.2 APPLICATION

Rigid or flexible packages made of nonporous components (transparent or semi-transparent material, either amber or colorless) that allow transmission of near-IR diode laser light may be tested. Test samples require a minimum headspace volume and headspace path length. The requirements vary on the basis of the gas moiety to be tested and may be specific to the instrument as well as to the construction and design of the package materials.

Test samples that may be analyzed fall into these categories:

- Products that require low-oxygen or low-carbon-dioxide headspace content

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- Products that require low water vapor content (e.g., lyophilized or powdered products)
- Products that require low internal package pressure (e.g., lyophilized products)

Package integrity, or absence of leakage, is confirmed by replicating tests on a given test sample as a function of time. Longer time periods between tests are needed to detect smaller leaks. Mathematical models appropriate to leak flow dynamics may be used to predict the time required for detecting leaks of various sizes or rates.

Headspace analysis at a single time point provides the headspace content result, which may or may not be indicative of package integrity.

- A test result not meeting specification could be due to package leakage, or could result from improper package filling or assembly processes that caused the package headspace to be out of specification.
- A test result that meets specification may confirm package integrity if enough time has elapsed since product–package preparation for measurable leakage to have occurred, assuming that the initial preparation of the test sample met manufacturing standards.

Methods of laser-based gas analysis may be used during any phase of the product life cycle. Tests are rapid and are appropriate for off-line testing using lab-scale equipment (typical measurement time, 2 s) or as an on-line, 100% product inspection method (typical measurement time, 0.2 s).

#### 2.2.3 TEST EQUIPMENT

Test instrumentation for laser-based gas headspace analysis is capable of accurate and reproducible near-IR diode laser light emission, light detection, and signal analysis. Tooling specific to product–package test samples is used to properly position test samples, ensuring reproducible laser-light transmission and detection. Standards with components identical to the packages under test, in terms of both the materials of construction and the dimensions (at the point where light is to be transmitted), are required. These standards also need to contain headspace content that is representative of the gas mixture under test (i.e., oxygen, carbon dioxide, water vapor, pressure).

#### 2.2.4 TEST PARAMETERS

The following are test parameters for laser-based gas headspace analysis:

- Test sample position with respect to laser beam transmission and detection points: imprecise test sample handling, and/or dimensional irregularity of the package, can increase measurement standard deviation
- Test sample headspace volume: the volume must meet minimum requirements for test instrumentation
- Test sample headspace absolute pressure: headspace absolute pressure will influence the detection limit and range for all test types
- Test sample speed: increase in testing speed will increase standard deviation of measurement
- Test sample temperature: temperature can influence moisture and pressure test results (e.g., lower temperatures are associated with lower internal pressure of the test sample) (*9*)
- Test sample outer surface moisture: presence of moisture may hamper test performance
- Time allotted between the replicate tests: performing replicate tests over a period of time allows calculation of continuous package leakage

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### 2.3 Mass Extraction

#### 2.3.1 DESCRIPTION

The mass extraction test is a nondestructive, quantitative measurement approach for detecting leakage in nonporous, rigid or flexible packages (*10*). Leakage of package headspace gases and/or leakage below the product fill level may be detected, given appropriately designed equipment and test parameters.

The test is performed by first placing the test sample inside a test chamber that is pneumatically connected to a mass extraction leak test system equipped with a vacuum generator package. The test chamber is uniquely designed to contain the test package, which is fitted with appropriate tooling to limit movement or expansion of moveable or flexible components, respectively.

Upon test start, the chamber is quickly evacuated for a predetermined time to reach a predetermined vacuum level. A series of such evacuation cycles may be performed, each intended to identify smaller leakage rates. After each cycle, the test system is isolated from the vacuum source and measurements of absolute pressure, pressure decay rate, and/or gas mass flow rate are captured. Readings greater than predetermined limits that were established using negative controls are indicative of container leakage, triggering test cycle abort.

For those test samples passing all previous larger leak vacuum cycles, a final vacuum is drawn. The test system is then isolated from the vacuum source. With all flow from the test chamber directed through the mass flow sensor, the mass flow rate is measured. Mass flow above a predetermined limit established using negative controls is indicative of container leakage.

#### 2.3.2 APPLICATION

Nonporous, rigid or flexible packages may be tested. Packages containing gas, liquid, and/or solid materials can be tested:

- Flexible packages or packages with nonfixed components require tooling to restrict package expansion or movement, respectively, when exposed to test vacuum conditions. Tooling minimizes the seal stress of flexible packages and maintains consistent package volume and differential pressure conditions across the leak path.
- Gas headspace must be at atmospheric pressure or at a pressure notably greater than test vacuum conditions.

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- Package surfaces below the product-fill level may be leak tested for those solid-formulation products that do not block leak-site gas flow and for those liquid products that volatilize at test vacuum but do not solidify and so block leak paths.
- Packages ranging in volume from a few milliliters to several liters may be tested.

Tests require anywhere from several seconds to a few minutes to perform. Longer test times are necessary for testing larger-volume packages. Lengthening test cycles also allows for detection of smaller leaks.

Mass extraction leak tests are useful in any phase of the product life cycle. Tests may be performed in a laboratory setting or off-line in the production environment. Longer laboratory or off-line test cycle times are generally capable of detecting smaller leaks. Higher speed on-line tests are restricted to larger leak detection.

#### 2.3.3 TEST EQUIPMENT

Mass extraction test instrumentation consists of a system of conduits and valves that pneumatically connect a test chamber with a test system pressure sensor, micro-flow mass sensor, and vacuum generator package, including an external vacuum source. The instrument includes appropriate timers, electronic controls, and monitors. A fixed-size orifice is included for periodic system performance verification. The test chamber is uniquely designed to contain the test package, which is fitted with appropriate tooling to limit movement or expansion of moveable or flexible components, respectively.

#### 2.3.4 TEST PARAMETERS

The following are test parameters for mass extraction:

- Pressures
  - Test system pressure reading after initial gross leak check. At evacuation stage, pressure is a function of test system volume, time allotted for evacuation, and the vacuum source pressure level.
  - Test system pressure reading after the secondary evacuation stage(s): pressure above a predetermined limit is due to test package leakage. The pressure level above baseline is a function of leak size, available headspace volume, and/or volatile liquid in the test sample.
  - The final absolute pressure of the test cycle must be lower than the headspace pressure of the test package for detection of gas headspace leaks, and/or lower than the volatilization pressure of liquid product formulation for leaks located below the liquid-product fill level.
- Mass flow
  - Mass flow rate reading after secondary evacuation stage(s).
  - The mass extracted from the test sample is monitored for larger leak detection after the first secondary evacuation stage; mass extracted from the test sample is monitored for the smallest leak detection after the last evacuation stage.
  - Background flow level (i.e., the baseline flow or noise level) is the flow rate for packages without leaks. Baseline flow is a function of test package and system outgassing, test system volume, and the time allowed for evacuation.
  - The mass flow rate at steady-state conditions, when extracted from the test chamber, is equal to test sample leakage into the test chamber, assuming that outgassing from the sample and external leakage from the test system are insignificant. Leakage is identified once the mass flow rate notably exceeds the rate of negative controls.
- Times
  - Time allotted for system evacuation for gross leak detection: enough time is allotted to draw off most of the test chamber gases, without exhausting the headspace gases of the test package, or without drawing off the liquid contents from grossly leaking packages.
  - Time allotted for large-leak check through the mass flow sensor: a brief time is required for detection of large- and medium-sized defects.
  - Time allotted for system evacuation for small leak detection: enough time should be allowed to establish the desired vacuum equilibrium of the test chamber. Insufficient time will not adequately draw off gases sorbed onto package surfaces or entrapped between components.
  - Time allotted for mass flow to stabilize: after the secondary evacuation stage(s), monitor the flow as the flow rate approaches steady state. Enough time is allotted so that flow from the smallest allowed defect is statistically greater than baseline (no-leak) flow.
  - Time allotted for the final leak test by mass flow: enough time should be allotted so that the mass flow rate exceeds baseline readings for negative controls.

### 2.4 Pressure Decay

#### 2.4.1 DESCRIPTION

The pressure decay test is a quantitative measurement approach for detecting leakage in nonporous, rigid or flexible packages. The test is destructive if the introduction of pressurized gas creates a break in the package wall or seal. The test is nondestructive if the introduction of gas into the test sample does not compromise the package barrier. Pressure decay testing is intended for integrity testing of the gas headspace region of the test sample.

To perform the test, a dry air or inert gas pressure source is attached to the test sample that is fitted with an internal pressure monitoring device. The test sample is pressurized to a predetermined pressure, after which the pressure source is isolated from the test sample. The decay in pressure is monitored for a predetermined time. Pressure decay that exceeds a predetermined limit established using negative controls indicates container leakage.

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The referenced ASTM F2095 method (*11*) is intended for testing flexible packages (pouches and foil-sealed trays). Seals or surfaces being tested cannot be in contact with product such as water, oils, or other liquids. ASTM method A describes testing packages without use of a restraint mechanism. The method requires that the package reach a stable volume configuration (i.e., it stops stretching) to take a measurement. ASTM method B requires that the test sample is kept between restraining plates during the test to limit the volume of the pressurized package.

### 2.4.2 APPLICATION

Nonporous, rigid or flexible packages may be tested:

- Package surfaces that can be tested are those unobstructed by product (e.g., oils, water, or other liquids); small leaks below the liquid-fill level would not be detected by this method.
- Flexible packages or packages with nonfixed components require tooling to restrict package expansion or movement, respectively, when exposed to test pressure conditions. Tooling minimizes the seal stress of the flexible package and maintains consistent package volume and differential pressure conditions across the leak path.
- Pressure decay can be used for testing packages anywhere from a few milliliters in volume to large, bulk-storage vessels.

Tests require anywhere from a few seconds to a several hours to perform. Longer test times are necessary for testing larger-volume containers. Lengthening test cycles also allows for detection of smaller leaks.

Pressure decay tests are useful in any phase of the product life cycle.

Tests may be performed in a laboratory setting or off-line in the production environment. Laboratory or off-line test equipment that allows for longer test times is generally capable of detecting smaller leaks. Higher-speed, on-line pressure decay equipment may be used to check for defects in open packages before package filling and closure.

### 2.4.3 TEST EQUIPMENT

Pressure decay test instrumentation includes conduits to connect the test sample with test system pressure transducers (absolute, differential, or a combination of both) and a pressure source (*12*). Instrumentation includes appropriate timers, electronic controls, and monitors. Greatest test method sensitivity and reproducibility are achieved when the instrument is kept in a temperature-controlled environment; test samples (especially larger-volume samples) are kept at a controlled, constant temperature during test; and dry pressurizing gas at constant temperature is used. It is optional to use tooling uniquely designed to limit movement or expansion of moveable or flexible components, respectively, thereby keeping test sample volume constant and limiting seal stress.

### 2.4.4 TEST PARAMETERS

The following are test parameters for pressure decay:

- Test sample internal pressure after pressurization:
  - The initial pressure reached after sample pressurization is a function of the test system volume, time allotted for pressurization, pressure source capacity, and temperature of the test sample headspace.
  - Higher pressure creates the potential for more rapid and sensitive leak testing. However, the selection of maximum test pressure should take into consideration personnel safety risks and potential damage to equipment and package.
- Pressure decay baseline: the baseline pressure decay (i.e., noise level) is the pressure drop that occurs for packages without leaks.
  - Baseline pressure drop is a function of test package volume, temperature conditions, and the length of time allowed for pressure to rise.
  - Baseline pressure drop requiring longer time periods is affected by gas sorption onto test package surfaces, gas moisture content (dry gas should be used), and gas temperature. Techniques to limit baseline pressure drop include the use of dry gases, and keeping the test container and the pressurized gas at a constant temperature.
- Pressure decay due to test package leakage: the extent of pressure decay above baseline is a function of leak size, available headspace volume in the test sample, the initial pressure inside the test sample, temperature control, and the time allotted for pressure to rise.
- Times
  - Time allotted for test sample pressurization: enough time is allotted to establish the desired pressure inside the test sample.
  - Time allotted after pressurization for pressure decay: enough time should be allotted so that the pressure decay from the smallest leaks can be detected (i.e., baseline decay is exceeded).
  - A time lag may be incorporated before monitoring for pressure decay to allow for gas equilibrium within the container and test system.
- Temperatures
  - Temperature of the pressurized gas can significantly affect test method sensitivity and reliability, especially when testing larger-volume containers.
  - An increase in gas temperature causes a rise in pressure, and conversely, a rise in gas pressure triggers an increase in gas temperature. Therefore, upon initial test package pressurization, the gas temperature will spike, causing a further spike in pressure. The subsequent drops in temperature and pressure during system equilibrium may be mistaken for leakage.

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- Effects of temperature variation may be minimized by: 1) allowing the temperature in the pressurized system to come to equilibrium before starting the pressure decay test, 2) minimizing temperature variation outside the test system, 3) minimizing test duration, and/or 4) applying a mathematical correction to the pressure readings.

## 2.5 Tracer Gas Detection, Vacuum Mode

### 2.5.1 DESCRIPTION

The leak detection method for tracer gas detects leakage from nonporous, rigid or flexible packages. The method can be destructive or nondestructive, depending on the test approach used. The test requires the presence of tracer gas inside the test sample package. Helium is the most commonly used tracer gas, and hydrogen is also used. The leakage rate of tracer gas is quantitatively measured using a spectrometric analytical instrument specific for the tracer gas. Instruments are designed to check for tracer gas leaking out of a test package, either by means of a test chamber that can be evacuated to draw gas out of test sample leaks (the vacuum mode) or by use of a vacuum wand (the sniffer mode) for scanning the outer surfaces of the test package (see *Tracer Gas Detection, Sniffer Mode*). The vacuum-mode tracer gas test is used more commonly than the sniffer mode for integrity testing sterile pharmaceutical product–packages. The vacuum mode is both quantitative and deterministic and is used to capture and quantify leakage from an entire test package, or it can be used to test for leakage along a test package surface or seal, given proper sample fixtures. Both vacuum and sniffer testing modes using helium as the tracer gas are described in ASTM F2391 (*13*).

To perform the vacuum mode test, test samples that have been fully or partially flooded with tracer gas are placed inside an evacuation chamber that is pneumatically connected to the tracer gas analysis instrument. Alternatively, the test sample may be fitted in such a manner that only the surface or seal of interest is exposed to the instrument, allowing for targeted leak detection at one specific seal or surface. In some cases, test samples that cannot withstand the high-vacuum test conditions may be tested with the use of tooling to restrict package expansion or movement.

At test start, the instrument's vacuum pump evacuates the test chamber or fixture, drawing leaking tracer gas through the analyzer. The absolute leak rate of the test sample is calculated by normalizing test results by the partial pressure of the tracer gas within the test sample at the time of test. For accurate results, tracer gas concentration within the sample must be uniform and consistent at the time of test; also, there should be minimal tracer gas permeation out of the test sample that can mask test sample leakage. Calibration tracer gas reference standards can be used for understanding the relationship between true leak rates and measured leak rates under actual test conditions.

The vacuum-mode tracer gas leak test is a nondestructive test, unless tracer gas introduction into the test sample requires package wall compromise (e.g., piercing), or if the presence of tracer gas is detrimental to the package contents.

### 2.5.2 APPLICATION

Rigid or flexible packages made of nonporous components:

- Flexible packages or packages with nonfixed components may require tooling to restrict package expansion or movement, respectively.
- Tracer gas permeation through the package material must not be so great that the leakage rate of concern is masked.
- A wide range of package sizes may be tested.

Leak paths must be clear of liquid or solid materials that could potentially block tracer gas flow.

Caution is advised when testing liquid-filled packages, because vapors or liquid drawn into the test system can seriously damage instrumentation.

Detection capabilities range from large leaks to the smallest leaks.

- Method capability is related to the size of the unobstructed leak path.
- Large leaks in the smallest packages may be missed because of the rapid loss of tracer gas (e.g., during the evacuation phase of the vacuum mode test).
- Significant tracer gas permeation through the package itself can interfere with the test by swamping leakage rate.

The test is nondestructive if the tracer gas is introduced into the package at the time of package assembly or closure, but the inclusion of tracer gas may prevent introduction of these packages into commercial or clinical markets. The test is destructive if the introduction of tracer gas compromises assembled package integrity (e.g., package puncture). Following test sample preparation, the actual leak test generally takes less than 1 min.

Tracer gas leak test methods may find application in any product life cycle phase. They are generally used in a laboratory environment. Tracer gas methods can also be used in production as an off-line testing approach; they can be used on-line if tracer gas is introduced into the test samples before final package closure.

### 2.5.3 TEST EQUIPMENT

Analytical instrumentation specific for tracer gas detection (e.g., mass spectrometer for helium detection) is required, typically equipped with an internal leak rate standard for instrument calibration at time of use. Additional equipment needed includes external calibration reference standards of tracer gas, to be used for comparisons of true leak rate versus measured leak rate under actual test conditions; a tracer gas source with a means for introducing tracer gas into the test sample; a test chamber or fixture to pneumatically connect the test sample with the instrument; tooling to restrict package expansion or movement, as appropriate; a means for accessing and analyzing the test sample headspace for tracer gas partial pressure after execution of the vacuum-mode leak test; and ventilation to remove tracer gas from the test area, or a tracer gas recapture system.

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#### 2.5.4 TEST PARAMETERS

The following are test parameters for tracer gas detection, vacuum mode:

- Tracer gas partial pressure within the test sample at the time of test:
  - When added by soaking the intact package, the concentration of the tracer gas is dependent on the time allotted, the positive pressure applied, and the tracer gas leak rate, plus the rate of permeation into the package
  - When added before package closure, tracer gas concentration is dependent on the gas flooding rate and time, the tooling and/or enclosure used to concentrate gas inside the package, and the efficiency of package closure for preventing escape of tracer gas
  - When added after package assembly, tracer gas concentration is dependent on the tooling used to pierce, flush, and vent the package; the gas flooding rate and time; and the sealant material applied to reseal the puncture site
- Differential pressure applied to the test sample during the vacuum mode will drive tracer gas out of the test sample, increasing method sensitivity
- Time allotted to allow for leakage to reach steady state should not exceed tracer gas permeation lag time

### 2.6 Vacuum Decay

#### 2.6.1 DESCRIPTION

The vacuum decay test is a nondestructive, quantitative measurement approach for detecting leakage in nonporous, rigid or flexible packages. Leakage in the package headspace gas region and/or below the product-fill level may be detected given appropriately designed test parameters and if product properties allow (as detailed below).

To perform the test, the test sample is placed in a closely fitting evacuation test chamber pneumatically connected to the leak test system, which is equipped with an external vacuum source. The test chamber is uniquely designed to contain the test package. Test samples with moveable or flexible components require appropriate tooling to limit the movement or expansion of such components, respectively.

Upon test start, the test chamber plus test system dead space are evacuated for a predetermined period of time. The targeted vacuum level chosen for the test is predetermined on the basis of the test sample type, size, and content. The vacuum source is then isolated from the test system. After a short time has elapsed to allow for system equilibration, the rise in dead space pressure (i.e., vacuum decay) is monitored for a predetermined length of time using absolute and/or differential pressure transducers. A pressure increase that exceeds a predetermined pass/fail limit established using negative controls indicates container leakage. ASTM F2338 may be referenced (*14*).

#### 2.6.2 APPLICATION

Nonporous, rigid or flexible packages may be tested. Packages containing gas, liquid, and/or solid materials can be tested:

- Flexible packages or packages with nonfixed components require tooling to restrict package expansion or movement, respectively, when exposed to test vacuum conditions. Tooling minimizes flexible package seal stress and maintains consistent package volume and differential pressure conditions across the leak path.
- Product–package gas headspace must be at atmospheric pressure or at a pressure notably greater than test vacuum conditions.
- Package surfaces below the product-fill level may be tested for leaks for those solid dosage formulation products that do not block leak-site gas flow or for those liquid dosage form products that volatilize at test vacuum without solidifying and blocking leak paths.
- Packages ranging in volume from a few milliliters to several liters may be tested.

Vacuum-decay leak tests are useful in all phases of the product life cycle. Tests require anywhere from a few seconds to a few minutes to perform. Longer test times are necessary for testing larger-volume packages or for detection of the smallest leaks. Longer test times are more appropriately performed in a laboratory setting or off-line in the production environment. Higher-speed on-line equipment is generally used for detecting larger leaks.

#### 2.6.3 TEST EQUIPMENT

Vacuum-decay leak test instrumentation consists of a system of conduits and valves that pneumatically connect a test chamber with the test system pressure sensors and an external vacuum source. The instrument includes appropriate timers, electronic controls, and monitors. An external gas flow meter allows for periodic system performance verification. The test chamber is uniquely designed to closely contain the test package and may be fitted with tooling to limit movement or expansion of moveable or flexible package components, as appropriate.

#### 2.6.4 TEST PARAMETERS

The following are test parameters for vacuum decay:

- Pressures
  - Test chamber pressure after evacuation: the initial pressure reached during test chamber evacuation is a function of test system volume, time allotted for evacuation, and vacuum pump capacity. The absolute pressure of the test system must be lower than the headspace pressure of the test package for detection of gas headspace leaks, and/or lower than the volatilization pressure of the liquid-product formulation for leaks located below the liquid-product fill level.

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- Pressure rise baseline: the baseline pressure rise (i.e., noise level) is the pressure increase that occurs for packages without leaks. Baseline pressure rise is a function of test package and system outgassing, test system volume, and the time allowed for pressure rise.
- Pressure rise due to test package leakage: the extent of pressure rise above baseline is a function of leak size, test chamber vacuum level at test start, available headspace volume or volatile liquid in the test sample, and the time allotted for pressure rise.

- Times
  - Time allotted for system evacuation: enough time should be allowed for establishing the desired vacuum pressure level of the test chamber, plus draw off gases sorbed onto package surfaces or entrapped between components. Excessive time will evacuate headspace from largely leaking packages, risking that there will be insufficient headspace gas for leak detection. Times should not be so great that leaking package headspace gases are exhausted or the liquid product floods and contaminates the test system.
  - Time allotted after evacuation for pressure rise (vacuum decay): enough time should be allotted so that the pressure rise from the smallest leaks to be detected exceeds baseline. A time lag may be incorporated after evacuation and before vacuum decay monitoring to allow for gas equilibrium within the container and test system.

# 3. PROBABILISTIC LEAK TEST TECHNOLOGIES

## 3.1 Bubble Emission

### 3.1.1 DESCRIPTION

The bubble emission leak test is a destructive, qualitative measurement approach for detecting and locating leaks in nonporous, rigid or flexible packages containing headspace gas.

The test is performed in one of two ways. The first is an internal pressurization method referenced in ASTM F2096 (*15*) in which a positive pressure air source with pressure monitor is inserted into the test sample. The test sample is then submerged in water, and air pressure is applied to a predetermined level, for a predetermined time period. The second approach is referenced in ASTM D3078 (*16*). The intact test sample is submerged in water or other suitable submersion fluid contained in a vacuum chamber. Vacuum is established to a predetermined level, for a predetermined time period.

With both approaches, leakage can be observed as a continuous stream of bubbles emitted from the leak site. Bubble diameter and emission rate may provide some indication of relative leak size. An alternative to test sample submersion is coating the test sample with surfactant, in which case any leakage is seen as foaming or bubbling at the leak site. The surface tension of the submersion fluid or surfactant allows for smaller bubble formation, potentially improving test sensitivity. Use of submersion fluid with low gas solubility may also improve test sensitivity.

The bubble emission test is categorized as a probabilistic leak test method. Although this method relies on the predictable flow of gas through leak paths, escaping gas can become entrapped within or between package components; false-leak outgassing events may occur; gas emitted from small leaks may solubilize in the immersion fluid before bubble formation; and test sample set up may be inadequate to ensure sufficient differential pressure conditions and appropriate bubble visibility. The use of negative and positive controls along with test samples provides evidence of test method limit of detection.

### 3.1.2 APPLICATION

Nonporous, rigid or flexible packages with gas headspace may be tested by bubble leak methods:

- Packages must be able to tolerate wetting or submersion
- Flexible packages or packages with nonfixed components generally require tooling to restrict package expansion or movement, respectively, when exposed to vacuum conditions. Tooling minimizes the seal stress of the flexible package and maintains consistent package volume and differential pressure conditions across the leak path. However, tooling may block leak paths or hinder bubble emission visibility
- Only leak sites that are present in the gas headspace region of the package can be detected
- This test is generally used for testing smaller-volume packages that are less than a few liters in size

Bubble tests are applicable in any product life cycle phase. Bubble tests require several minutes or longer for test sample analysis and subsequent cleaning and/or drying. Bubble tests are most commonly used in laboratory settings as part of a research investigation to verify leak presence and location. They can also be used as an off-line production leak test. Bubble tests are also used for integrity testing of aerosol–package products in a research or production setting. In this application, the test may be considered nondestructive to the product–package test sample.

### 3.1.3 TEST EQUIPMENT

Bubble tests require a pressure or vacuum source (as appropriate) equipped with pressure monitors and controls; tooling to restrict expansion of flexible packages or movement of nonfixed components; submersion fluid or surfactant to be applied to the package surface; submersion vessel equipped for external vacuum or internal pressure test mode; and visual inspection aids such as lighting, magnification, and/or background, as needed.

### 3.1.4 TEST PARAMETERS

The following are test parameters for bubble emission:

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- Differential pressure: greater differential pressure evokes more rapid bubble emission. Differential pressure should not be so great that package seals are compromised or that gas escapes through large leaks so rapidly that it may be confused with package surface outgassing
- Time allotted during differential pressure application: longer test times allow for smaller leak detection
- Times allotted for inspection (pacing) and for inspection breaks to lessen operator fatigue
- Package positioning during inspection
- Package mode of restraint for moveable or flexible components
- Submersion fluid (or surfactant) surface tension: lower surface tension improves method sensitivity
- Inspection environment parameters: lighting intensity and angle, degree of magnification, background color

## 3.2 Microbial Challenge, Immersion Exposure

### 3.2.1 DESCRIPTION

The immersion exposure microbial challenge is a destructive, qualitative measurement approach for confirming leaks in nonporous, rigid or flexible packages.

The test is performed by first filling test samples with sterile, growth-supporting media, followed by incubation and visual inspection of samples to ensure sample sterility before microbial challenge. Samples are then immersed in a concentrated bacterial suspension for a predetermined time. Samples can be exposed during immersion to a predetermined vacuum for a predetermined time, followed by release of vacuum while the packages remain immersed at ambient pressure for a predetermined time. Samples are then incubated under growth-promoting conditions, followed by examination of package contents for evidence of microbial growth by visual inspection or other appropriate analytical means. Alternative approaches can include exposure of immersed test samples to positive pressure conditions, or to multiple cycles of vacuum and/or pressure conditions. Test sample leakage is evidenced by visible growth of the challenge microorganism(s) inside test samples. Immersion microbial challenge tests rely on the presence of a liquid carrier in the leak path that sweeps microorganisms into the package or provides a means whereby microorganisms can actively migrate and/or grow into the test sample. The use of negative and positive controls along with test samples provides evidence of test method limit of detection.

The microbial challenge by immersion test is categorized as a probabilistic leak test because of the multiple events that must occur sequentially and/or simultaneously for leak detection to take place. All such events are difficult to predict or control, especially for detection of smaller leaks. For example, the microorganisms must be physically present at the leak site. The necessary presence of liquid in the leak path, and/or flowing through the leak path, is influenced by the package materials of construction, leak path tortuosity and topography, media surface tension, and leak path blockage by product, extraneous debris, or air locks. Microorganisms must not be hindered from entering the package by getting trapped in a tortuous leak path, and enough microorganisms must enter the package to allow for sufficient growth that can be detected visually after test sample incubation.

### 3.2.2 APPLICATION

Microbial challenge tests by immersion are most useful when an appropriate and validated physicochemical leak test method does not exist, or when the test outcome demands direct evidence of the prevention of microbial entry.

Nonporous packages of rigid or flexible components may be tested by immersion microbial challenge methods:

- Packages must be able to tolerate submersion.
- Flexible packages or packages with nonfixed components may require tooling to restrict package expansion or movement, respectively. Tooling minimizes stress on flexible package seals and maintains consistent differential pressure conditions across the package seal.

Immersion microbial challenge tests are performed in a laboratory environment, not as an on-line test of the finished product.

A test requires several days to prepare; the test samples are pre-incubated before microbial challenge to ensure initial package content sterility. The challenge itself, plus post-challenge sample decontamination, may take several hours. Final incubation followed by sample inspection may take 1–2 weeks. The immersion microbial challenge test is primarily used in product–package development and validation studies.

### 3.2.3 TEST EQUIPMENT

The test chamber for conducting the immersion microbial challenge test is designed and equipped to maintain suspension uniformity and appropriate temperature while exerting the required differential pressure condition. Fixtures for restraining and/or positioning packages during immersion are also required. Small, motile microorganisms are preferred for the challenge; examples include *Brevundimonas diminuta* and *Serratia marcescens*. The immersion challenge media should support challenge microorganism growth to the desired concentration. Soybean-casein digest medium is one commonly used medium.

The media filled into test samples may match the immersion challenge media formulation. Alternatively, product that has been shown to support microbial growth may be used. Media should allow sufficient growth of the particular challenge organism so that package contamination can be detected in the positive controls. Verification of growth promotion in the immersion media and the test sample media should be performed each time the immersion challenge test is conducted (see *Sterility Tests* ⟨71⟩, *Growth Promotion Test of Aerobes, Anaerobes, and Fungi*). Use of an incubation chamber is required for test samples, both before and after exposure. A means for determining microbial growth inside test packages is required.

### 3.2.4 TEST PARAMETERS

The following are test parameters for microbial challenge, immersion exposure:

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- Microorganism concentration: in the immersion challenge media, microorganism concentration should meet appropriate predetermined levels throughout the challenge test (a minimum concentration of $10^5$ CFU/mL is commonly used).
- Media fill volume: the fill volume of media in test samples should be sufficient to ensure a liquid path at each potential leak site. Exposure of package seals and leak sites to the media may also be accomplished by test sample positioning during the immersion challenge. It is also necessary to have sufficient volume and correct composition of the package headspace to encourage growth of the selected challenge microorganism. Note that when the composition of the package headspace does not include oxygen, other test conditions (e.g., anaerobic) may be applicable.
- Vacuum/pressure conditions: exposing immersed test samples to differential pressure conditions is an important method parameter that serves multiple purposes:
  - Differential pressure helps eliminate trapped air and ensures the presence of liquid media between package components and at leak sites.
  - Differential pressure simulates the pressure changes incurred during air or land freight transport of the product. Absolute pressure conditions that correspond to various altitudes anticipated during land and/or air freight transport are provided in ASTM D6653/D6653M (*17*). Differential pressure conditions of the test may be modified on the basis of knowledge of the product–package shipping environment.
  - Differential pressure exposure can simulate conditions experienced by the product during some sterilization treatments.
- Test times: longer exposure times improve the likelihood of microbial ingress into defective test samples and positive controls. However, a possible decrease in the ability of media to support growth over time must be considered.
  - Time allotted for immersion exposure during differential pressure exposure (vacuum and/or pressure)
  - Time allotted for immersion exposure at ambient pressure conditions
- Temperature during challenge: temperatures sufficient to support microbial growth are recommended. Temperature cycling can also be used as a tactic to eliminate airlocks and promote the presence of liquid media at package seal sites.
- Pre- and post-challenge test incubation temperature and times: the temperature selected should allow sufficient microbial growth. Incubation times should be sufficient to ensure visualization of growth; these times are determined on the basis of positive controls and samples from growth-promotion studies.
- Parameters for detecting post-incubation microbial growth (e.g., lighting and background color for visual inspection, handling procedure, and pacing).

## 3.3 Tracer Gas Detection, Sniffer Mode

### 3.3.1 DESCRIPTION

The following information is specific to the tracer gas detection performed in the sniffer mode. (For additional information, see *Tracer Gas Detection, Vacuum Mode*.)

Tracer gas detection using a sniffer attachment is used to detect leak presence and location in nonporous, rigid or flexible packages. This is a nondestructive leak test, unless tracer gas introduction into the test sample requires package wall compromise (e.g., piercing) or if tracer gas presence is detrimental to package contents. The sniffer mode test using helium as the tracer gas is described in ASTM F2391, Procedure A (*13*). Briefly, test samples are flooded completely or partially with the tracer gas via one of several options. These options may include piercing a closed test sample to introduce pressurized tracer gas (sealant is applied to close the puncture site); flooding the test sample before package closure; or "soaking" a closed test sample by pressurizing with tracer gas (most applicable to larger leak detection). Test samples are checked for leakage by scanning the outer package surfaces using a vacuum wand that is pneumatically connected to the tracer gas analytical test instrument (e.g., a mass spectrometer for helium detection). Calibration reference standards of tracer gas can be used for understanding the relationship between true leak rates and measured leak rates under actual test conditions. The use of negative and positive controls along with the test samples provides evidence of test method limit of detection.

The sniffer mode of tracer gas leak testing is a probabilistic leak test method. This is because the presence of concentrated tracer gas near the test sample surface is not a well-defined or predictable event, and the sniffer scanning procedure is prone to variability related to human technique. The sniffer mode is generally chosen when the leak location is to be identified.

### 3.3.2 APPLICATION

Rigid or flexible packages made of nonporous components may be tested:

- Flexible packages or packages with nonfixed components may require tooling or manual manipulation to force tracer gas through leak paths.
- Tracer gas permeation through the package material must not be so great that the leakage rate of concern is masked.
- A wide range of package sizes may be tested, ranging from small packages to large multi-liter vessels.

Leak paths must be clear of liquid or solid materials that could potentially block tracer gas flow. The leak size detection capability is related to an unobstructed leak path.

The sniffer probe must not be allowed to draw liquid or hazardous vapors into the test system, as this would risk serious instrument damage.

Tracer gas leak test methods require time for the introduction of tracer gas into the test package, and up to several minutes to scan the package. Large leaks in the smallest packages may be missed because of the rapid loss of tracer gas.

Methods of tracer gas leak tests in the sniffer mode are generally used in the laboratory environment for locating package leaks. Tracer-gas sniffer mode tests are useful in any product life cycle phase.

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#### 3.3.3 TEST EQUIPMENT

Test equipment for tracer gas leak detection is described in *Tracer Gas Detection, Vacuum Mode*. However, a sniffer probe with an enclosure and/or tooling for concentrating tracer gas passing through smaller leaks is used, rather than the test chamber or test fixtures described in *Tracer Gas Detection, Vacuum Mode*.

#### 3.3.4 TEST PARAMETERS

The following are test parameters for tracer gas detection, sniffer mode:

- Differential pressure applied to the test sample will drive tracer gas out of the test sample, increasing the method's sensitivity. For flexible packages, differential pressure can be exerted by package compression.
- Tracer gas partial pressure within the package at the time of the test is discussed, with instructions, in the *Tracer Gas Detection, Vacuum Mode*.
- Aspects of the sniffer mode vacuum wand, such as sweeping speed and distance from the package surface, as well as the tooling or enclosure used for concentrating leaking gas.

### 3.4 Tracer Liquid

#### 3.4.1 DESCRIPTION

The tracer liquid test method is a destructive approach for detecting and potentially locating leaks in nonporous, rigid or flexible packages. Tracer liquid tests provide an indication of leak presence and may provide a measure of relative leak size. Tracer liquid tests using liquid submersion work by the diffusive flow of the tracer element through a liquid-filled leak path and/or the effusive flow of tracer solution through the leak path.

The liquid submersion test uses one of two basic approaches. In the first approach, test samples are submerged in a tracer-element solution formulation contained in an evacuation chamber. Examples of tracer elements include dyes, radionuclides, or metallic ions. In the second approach, test samples containing tracer formulation are submerged in tracer-free liquid contained in an evacuation chamber. For both approaches, test samples may be fitted with tooling to ensure proper positioning and to restrict flexible or moveable components. The submerged test samples are subjected to vacuum at a predetermined pressure level for a predetermined time. After vacuum release, test samples remain submerged for a predetermined time. Additional test options include the use of positive pressure exposure or multiple cycles of differential pressure conditions to encourage effusive flow of the tracer element through the leak path.

In the first approach, after the challenge is complete, test sample outer surfaces are cleaned, and the contents are checked for evidence of tracer ingress (*18*). In the second approach, after the challenge is complete, the immersion fluid is checked for evidence of tracer liquid egress out of the test sample. In both cases, measurement of tracer liquid migration may be performed in a quantitative manner by using chemical analysis techniques (preferred for small leak detection). Alternatively, the presence of leakage may be determined qualitatively by visual inspection if the tracer element can be discerned visually. The use of negative and positive controls along with test samples provides evidence of test method limit of detection.

Liquid tracer tests are categorized as probabilistic methods. Successful liquid tracer detection relies on a combination of tracer solution wicking, tracer solution effusion, and tracer element diffusion through a liquid-filled leak path and are events that are difficult to predict or control, especially for detection of smaller leaks. These events are influenced by numerous factors, including the package materials of construction, leak path tortuosity and topography, tracer liquid surface tension, and leak path blockage by product, extraneous debris, and air locks.

#### 3.4.2 APPLICATION

Rigid or flexible packages of nonporous components may be tested using tracer liquid submersion methods:

- Packages must be able to tolerate wetting or submersion.
- Flexible packages or packages with nonfixed components may require tooling to restrict package expansion or movement, respectively.
    - Testing by submerging a test sample in tracer liquid is used when the test sample allows for visual examination of the tracer (e.g., dye) ingress, or when the tracer element is to be contained within the test sample after testing.
    - Testing by submerging tracer-filled test samples in tracer-free liquid may be used when the sample interior cannot be visually examined or when the tracer element is best captured for analysis outside the test sample.

Tests may require up to 1 h or longer for test sample exposure, cleaning, and inspection or analysis. Tracer liquid tests are primarily used for laboratory testing or off-line product sample testing. Tracer liquid submersion tests can be used in any product life cycle phase.

#### 3.4.3 TEST EQUIPMENT

A test vessel equipped for challenge conditions of vacuum and/or positive pressure is required and is pneumatically connected to a pressure and/or vacuum source, as appropriate, and equipped with pressure monitors and controls (*19*). The tracer liquid formulation should be physicochemically compatible with the test sample components and the tracer-free solution to be filled into the test samples or used as the immersion bath, to ensure optimal tracer solution functionality. Formulation considerations include tracer element type and concentration; surfactant use, type, and concentration; and solvent system. Examples of tracer liquid incompatibilities apparent upon contact with the test product include tracer dye fading, tracer element precipitation, and tracer element sorption onto package components. Tooling is often needed to restrict flexible package expansion or

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nonfixed component movement upon differential pressure exposure. Tooling minimizes stress on flexible package seals and ensures consistent differential pressure conditions across the leak path.

Tracer detection requires the use of either analytical detection instrumentation [e.g., UV-Vis spectrophotometry (*20*), phase induction spectrophotometry, or other] or visual inspection aids. Analytical detection offers the advantage of minimizing the error that is inherent in visual discernment of low dye concentrations. Optimal visual inspection requires the use of controlled inspection conditions, such as background color, lighting, pacing, fatigue breaks, and negative controls for comparison.

### 3.4.4 TEST PARAMETERS

The following are test parameters for tracer liquid:

- Differential pressure conditions used (vacuum and/or pressure): greater pressure differentials encourages tracer liquid passage.
- Submersion times during and after differential pressure application: longer times allow for greater tracer liquid passage through leak paths.
- Holding time between tracer liquid challenge and final inspection: some tracer liquids visibly fade or are sorbed onto package surfaces over time.
- Tracer liquid surface tension: lower surface tension allows for smaller leak detection.
- Tracer detection parameters
  - Analytical detection
    - Test sample content extraction procedure
    - Method-specific performance parameters
  - Visual inspection
    - Inspection environment parameters: lighting intensity and wavelength, background color, viewing angle, and test sample visibility
    - Time allowed for inspection (pacing) and breaks to lessen operator fatigue
    - Comparison with negative controls

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# ⟨1207.3⟩ PACKAGE SEAL QUALITY TEST TECHNOLOGIES

1. INTRODUCTION
2. CLOSURE APPLICATION AND REMOVAL TORQUE
3. PACKAGE BURST
4. PACKAGE SEAL STRENGTH
5. RESIDUAL SEAL FORCE
6. AIRBORNE ULTRASOUND

## 1. INTRODUCTION

The purpose of this chapter is to briefly summarize test methods useful for characterizing and monitoring package seal quality and to guide the reader in their selection and use. These methods are not leak tests but provide additional data regarding package seal characteristics that may affect package integrity and leakage.

"Package seal quality tests" are checks used to characterize and monitor the quality and consistency of a parameter related to the package seal, providing some assurance of the package's ability to maintain integrity. Seal quality tests ensure that seal attributes, package materials, package components, and/or the assembly process are consistently kept within established limits, thus further supporting package integrity. Seal quality tests differ from leak tests in that they provide no information relative to actual package integrity; thus, a package that meets the requirements of a seal quality test may still be defective and leak. For example, a flexible pouch package that passes a seal strength test may leak through a puncture in the pouch face. A bottle that meets closure application and removal torque tests may have a scratch on the bottle finish surface that allows product leakage. In contrast, a pouch or bottle that is poorly assembled could pass leak tests at the time of product manufacture, yet develop leaks later, before reaching the end user.

Therefore, seal quality tests and leak tests work together to ensure package integrity. The package seal quality tests described in this chapter were selected for inclusion on the basis of data in peer-reviewed scientific publications and/or data regarding recognized standard tests (e.g., precision and bias study results). Standard test methods (e.g., ASTM) are referenced where applicable (the reader is advised to utilize the most recent versions). In some cases, the scope of referenced standard test methods does not include the package types of the scope in *Package Integrity Evaluation—Sterile Products* ⟨1207⟩. All methods and literature references are cited to provide benchmark information useful for the application and use of pharmaceutical package seal quality test methods.

Unlike package leak tests, seal quality tests are qualified for use rather than being fully validated. Qualification includes a demonstration of instrument performance and in some cases proof of appropriate instrument set-up specific for the package to be tested.

Finally, this chapter is not intended to provide an exhaustive listing of all seal quality technologies that could be used. Nor is the use of a methodology cited meant to be compulsory. Other qualified tests that are not included in this chapter may be used, as appropriate.

## 2. CLOSURE APPLICATION AND REMOVAL TORQUE

The closure application torque test measures the force exerted during the application of a screw-thread cap onto a threaded container. Conversely, the closure removal torque test measures the force required to initiate screw-cap removal. The container–closure systems of some ophthalmic solution products are examples of sterile product packaging closed with screw-thread caps.

Cap application torque is kept within an optimum range to prevent leakage from loose caps and to preclude component distortion and compromised seals from over-torqued caps. Cap removal torque is less than application torque due to stress relaxation and closure "back off" that may occur as a function of time and other environmental variables. A properly designed and applied cap will retain sufficient sealing force until the package is opened at the time of use. Caps that back off excessively during shipping, storage, or distribution increase the risk of product leakage. For multiple-dose packages, cap design and application forces should be such that the end-user population may be able to open and reclose the package in a manner that properly preserves the pharmaceutical product.

Reproducibility of application/removal torque test results is improved when tests are performed using automatic instrumentation that applies and removes caps at uniform speeds, with fixtures to lock the bottle and cap into proper test position. Application and removal torque tests are described in several standard test methods that have been written to support various continuous-thread and child-resistant cap designs, including the ASTM methods referenced (*1*–*7*).

## 3. PACKAGE BURST

The package burst test is performed by inserting a pressure source into a test package and applying pressure until the package seal(s) burst open. Packages having seals that could be compromised when exposed to a net positive pressure force inside the package may be tested by this method. Such package types include flexible bags and pouches formed by heat-sealing processes.

The package burst strength test result (reported in pressure units) provides an indication of relative seal strength and evaluates the most likely location and mode of package failure when the package is exposed to a pressure differential. However, note that this test method cannot provide a measure of package seal uniformity or overall package integrity.

Package burst tests are described in ASTM F2054 (*8*) and ASTM F1140 (*9*) (Test method A). Method ASTM F2054 uses a restraining plate to limit package expansion during inflation. A restraining plate ensures that the stress is uniformly applied to

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all seal areas, allowing for identification of the weakest point along the seal. Additional factors can influence the test results, including package inflation rate, tooling dimensions (including gap height), and pressure-sensing mechanisms.

Method ASTM F1140 uses no restraining plate; therefore, this approach provides a snapshot of package performance when challenged with differential pressures in a typical sterilization cycle or distribution environment, for example. However, without restraining plates, stress applied to the package is highest at the middle of the package where the package inflates to the greatest diameter; therefore, the weakest area of the seal may not be identified by this approach. Package inflation rate, as well as pressure-sensing mechanisms and their detection limits, can influence test results.

## 4. PACKAGE SEAL STRENGTH

The package seal strength test, commonly known as the peel test, measures the force required to peel apart two bonded surfaces. This test is applicable for testing seals between two bonded, flexible surfaces (e.g., a pouch or bag) or between a flexible material and a rigid material (e.g., a lidded tray). Force results provide a measure of seal strength between the bonded surfaces. Maximum seal force, as well as average force to open the seal, may be determined with this method. The results are also useful for monitoring the consistency of package assembly.

The test is performed using a universal stress-strain instrument set to extension force mode, with special tooling for correctly positioning and holding the test sample. The seal strength test is described in ASTM F88 (*10*). Test results are affected by the peel test fixture design, the peel angle, the pull direction, pull speed, and properties of the test sample itself.

## 5. RESIDUAL SEAL FORCE

The residual seal force (RSF) test provides an indirect measure of the compressive force exerted by an elastomeric closure onto a parenteral vial finish after package assembly (capping). RSF tests can be performed on glass or plastic vial packages of all dimensions. A consistent and sufficiently significant RSF value provides a useful indicator of capping process consistency. RSF is linearly related to closure compression: more tightly capped vials yield higher RSF values.

The RSF test is performed using a universal stress-strain instrument, set to compression force mode, and a metal tool (called a cap anvil) designed to fit on top of the sealed vial package. A constant rate of compressive strain is exerted onto the anvil, which rests on top of the vial package, yielding a typical plot of stress as a function of time (or compressive distance). The point in the stress-response curve immediately before the terminal slope change corresponds to the capped closure's RSF, reported in either newtons or pound-force units. RSF tests are nondestructive in terms of package integrity. Test results are influenced by cap anvil tooling design, test compression rate, closure viscoelastic properties, closure dimensions, and the inclusion of an aluminum seal plastic top (more reproducible results are possible if plastic tops are removed before testing). Research articles describing the development and application of this method are referenced (*11*–*14*).

## 6. AIRBORNE ULTRASOUND

The airborne ultrasound test checks seal quality by passing an ultrasound signal through the sealed area of a package or item. The signal strength transmitted through the package seal under test is compared to that previously established for so-called good package seals. Poorly sealed areas will not transmit as much ultrasonic energy as properly sealed areas.

The types of package seals that can be tested using airborne ultrasound include flexible pouch seals and rigid tray seals made of metallic or plastic laminates. This technology can be used to check for the presence and location of package seal defects such as an incomplete seal, a channel, a wrinkle, or extraneous material inclusion, as well as for package seal bond weakness. The method may be used off-line to perform an *x*–*y* coordinate scan of a seal area, or it may be used to linearly scan seals during on-line manufacturing processes. Airborne ultrasound is a noncontact testing technology that requires no package preparation and no coupling media, such as a liquid or gel, to propagate sound; thus, it is nondestructive to the package under test.

The airborne ultrasound method is described in ASTM F3004 (*15*). The ASTM method test system is composed of a transducer that provides an ultrasonic signal, a means of holding/transporting the item under test within an air gap between the two transducers, and the detection transducer, which captures the intensity of the signal that passed through the air gap and the item under test. The capability of airborne ultrasound to detect specific package seal quality problems will vary on the basis of package material of construction, seal structure (e.g., smooth versus textured surface), scanning speed, and scanning signal strength.

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# ⟨1207⟩ PACKAGE INTEGRITY EVALUATION—STERILE PRODUCTS

## 1. INTRODUCTION

This chapter provides guidance on the integrity assurance of nonporous packages intended for sterile pharmaceutical products. Background instruction is provided on the topics of leaks, leakage rate, and package sealing/closure mechanisms. Explanation is given as to how packages that conform to specified leakage limits help to ensure the contained product meets and maintains sterility and relevant physicochemical specifications. The integration of package integrity assurance as a key component of the entire product life cycle is stressed. Guidance in the selection, validation, and use of leak test methodologies as well as package seal quality tests is included. Detailed recommendations are presented in three subchapters listed below:

- *Package Integrity Testing in the Product Life Cycle—Test Method Selection and Validation* ⟨1207.1⟩
- *Package Integrity Leak Test Technologies* ⟨1207.2⟩
- *Package Seal Quality Test Technologies* ⟨1207.3⟩

At the end of this chapter, the *Glossary* section defines terms as they are used in the context of this set of four general information chapters.

The term product–package refers to the container–closure system plus the product contents. The container–closure system consists of the primary packaging components, those components that are or may be in intimate contact with the product, as well as secondary packaging components vital to ensuring correct package assembly, for example, the aluminum cap used to seal a stoppered vial package.

Suitable container–closure systems adequately store and protect the contained pharmaceutical product. Thus, sterile product–package integrity is the ability of a sterile product container–closure system to keep product contents in, while keeping detrimental environmental contaminants out. Specifically, leaks of concern for sterile product–packages include the following three categories described in *Table 1*. In other words, the leaks of concern for a given product–package are a function of the degree of package protection demanded by the product to ensure that all relevant product physicochemical and microbiological quality attributes are met through product expiry and use.

**Table 1. Product Quality Risks Posed by Leaks of Concern**

| Leaks of Concern | Product Quality Risks Posed by Leaks |
|---|---|
| Capable of allowing entry of microorganisms | Failure of product sterility quality attribute |
| Capable of allowing escape of the product dosage form or allowing entry of external liquid or solid matter | Failure of relevant product physicochemical quality attributes |
| Capable of allowing change in gas headspace content. For example, loss of headspace inert gases (e.g., nitrogen), loss of headspace vacuum, and/or entry of gases (e.g., oxygen, water vapor, air). | Failure of relevant product physicochemical quality attributes and/or hindrance of product access by the end-user |

Package integrity is synonymous with container–closure integrity; these terms are used interchangeably throughout this chapter. In the past, to say a sterile product–package has container–closure integrity commonly meant that the package either had passed or was capable of passing a microbiological challenge test. This guidance chapter defines the concept of container–closure integrity more broadly, encompassing the absence of all package leaks that risk product quality. By this definition, a package is considered to have integrity if it allows no leakage greater than the product–package maximum allowable leakage limit. In other words, the largest and smallest leaks of concern are absent.

Leakage differs from permeation. Leakage is the unintentional entry or escape of matter (solids, liquids, or gases) through a breach in a package wall or through a gap between package components. Leakage can also refer to the leaking matter itself. Leakage flow rate is a function of the absolute and/or partial pressure gradient of leaking matter that exists across the package barrier. In the context of this chapter, permeation is the passage of fluid (e.g., gas) into, through, and out of a nonporous package wall. Permeation, not leakage, occurs when only a small fraction of molecules is able to move through a barrier by way of any one hole. A nonporous package is able to permit permeation, but not the volumetric flow of air. Package permeation test methodologies fall outside of the chapter scope and are not described.

Package integrity tests are leak test methods. A leak test is a method that detects the presence of (and in some cases, the magnitude or location of) a package breach or gap.

Package seal quality tests are techniques used to characterize and monitor the quality and consistency of a package seal or closure system parameter, which can influence the package's ability to maintain integrity. An example is the peel force test widely used to test seal strength. Seal quality tests are not leak tests but can play a valuable role by monitoring a characteristic of the seal itself, the package materials, the package components, and/or the processes required to create the seal or closure mechanism.

Integrity test methods vary not only in their application and detection limit, but also in terms of detection range, precision, and specificity, for example. No one test is appropriate for all packages or for all leak testing applications. Selection criteria for package integrity test methods, as well as method comparison aids, are presented to guide the user in the selection process.

Leak tests, even many commonly recognized industry standard testing approaches, require optimization and validation for each product–package application. A science- and risk-based approach may allow some tests to be leveraged for broader application under certain circumstances. For example, small differences in product formulation or package design and materials may permit the use of one test for multiple product–packages. Package integrity verification plays an important role throughout the product life cycle, starting with product development and continuing through marketed product stability studies.

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## 2. SCOPE

The package integrity leak tests and seal quality tests outlined in this chapter apply to the testing of both large and small volume container–closure systems for sterile pharmaceutical dosage forms. Such package systems include, but are not limited to, the following examples.

- Vials or bottles closed with elastomeric closures or screw-thread caps
- Form-fill-seal plastic or glass ampules
- Syringes or cartridges
- Flexible bags or pouches
- Packages for some drug/device combination products (e.g., packages cased inside autoinjectors)

Outside the chapter scope are packaging systems and processing equipment used in the preparation, storage, and manufacture of sterile pharmaceutical products. Examples include containers for active pharmaceutical ingredients and containers for product intermediate or final bulk product.

Outside the chapter scope are packages used for sterile diagnostic products or medical devices, and some packages used for sterile drug/device combination products.

Outside the chapter scope are products having a primary package composed of porous barrier package materials, i.e., materials designed with pores or openings to allow volumetric air flow while preventing airborne microbial contamination.

## 3. LEAKS AND LEAKAGE RATE

Leaks are commonly conceptualized as holes of a defined diameter, or channels of distinctive diameter and length, although leaks that occur naturally are generally complex, multicavity tortuous paths and are rarely uniform in size or shape. Even artificially created leaks such as laser-drilled defects (also called holes) used for leak-test method development and validation are irregular in size, shape, and depth. When stating the size of a leak, it is important to define the measurement approach. In some cases leaks are measured dimensionally, but quite often, leak size is determined based on gaseous leakage rate. For example, a package wall laser-drilled defect having a nominal diameter of 5 ± 2 µm may have been size-certified by matching the airflow rate through the drilled defect to that of a 5 ± 2 µm hole present in a thin metal plate reference standard when pressurized with dry air at specified differential pressure and temperature conditions.

Gaseous leakage rate is a measure of the rate of gas flow (in mass or volume units) that passes through a leak path under specific conditions of temperature and the concentration or pressure differential across the barrier wall. Therefore, gaseous leakage rate has dimensions of pressure multiplied by volume, divided by time. The international standard SI nomenclature is pascal cubic meter per second ($Pa \cdot m^3 \cdot s^{-1}$). These leakage measurement units refer to the quantity of leaking gas ($Pa \cdot m^3$) per unit of time. When a leakage rate is described and no test conditions are noted, standard conditions of one standard atmosphere differential pressure with dry air at 25° are assumed. For a more complete discussion of gaseous leakage rates and units of measure refer to reference *1*.

Unintentional leaks in packages are often detected or sized using gas as a tracer element. Given a situation in which a tracer gas partial pressure difference exists across the package barrier wall and no absolute pressure difference exists, gaseous leakage is predominantly diffusional in nature as the tracer gas moves from a region of higher concentration to a region of lower concentration. For example, the headspace in a vial package low in oxygen concentration and at ambient pressure at time of closure will exhibit a rise in headspace oxygen concentration over time as a function of diffusion rate (relative to package leak size) plus permeation rate (relative to permeability through the package). Such a change can be monitored instrumentally, as is discussed later in the chapter.

Given the situation in which an absolute pressure difference exists across the package barrier wall, gas leakage through package gaps is more rapid (flux being primarily convective) as gas moves from the higher pressure region to one of lower pressure. For most package materials, the permeation rate of the gas of concern through the package wall is insignificant in comparison. In this example, the rate of leakage into the package is determined by monitoring the change in headspace absolute pressure as a function of time.

For many instrumental leak test methods described in this chapter, gas flow into or out of package leaks is induced by exerting a pressure gradient across the package. In this way, a wide range of leak types and sizes can be identified.

Liquid leakage rate is a measure of the volume of liquid that moves through a leak path as a function of time under specified conditions of temperature and absolute differential pressure across the barrier wall. Liquid movement through a leak path occurs only when leak size/shape, package materials of construction, the absence of leak obstruction, tracer liquid composition, and test parameters all work together. All sterile product–packages within the chapter scope are intended to prevent liquid leakage and block microbial entry, thus it is the absence of liquid leakage flow or the absence of liquid in the leak path, rather than the rate at which liquid leakage occurs that is typically verified in relevant package leak tests. Liquid leakage rate measurements are only useful in measuring larger size leaks.

## 4. CLOSURE TYPE AND MECHANICS

An understanding of closure mechanics makes it possible to better characterize, monitor, and test packages for integrity. This section discusses various closure systems and how each type functions to ensure package integrity.

### 4.1 Physically Mated Closures

Closure can be achieved by the close physical mating of two surfaces that often are dissimilar in material composition. Examples include the interference fit of a plunger inserted in a syringe barrel, the compression fit of an elastomeric closure

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capped onto a vial finish surface, and the application force of a screw-cap torqued onto a bottle. Physically mated surfaces are not bonded together; therefore, a tiny gap exists even between well-closed components. Nevertheless, when properly designed and assembled, closure systems fit together such that liquid leakage (and microbial ingress) is prevented and gas migration is limited. Regarding screw-thread closures such as those used for ophthalmic-product dropper bottles, the barrier to leakage is afforded by inner cap surfaces pressing against the package opening, in some cases aided by a secondary gasket or plug. The winding path afforded by the threads of a screw-cap does not provide an optimal barrier to gas or liquid leakage, or to microbial ingress in the event of liquid presence in the cap threads.

### 4.2 Physicochemically Bonded Closures

Seals are formed when two similar or dissimilar surfaces are physicochemically bonded together. One example is the formation and sealing of contiguous containers from a single material (e.g., glass or plastic ampules). In other form, fill, and seal systems, sheets of material are mated by means of a heat or ultrasonic welding process. Two dissimilar materials may be joined using an intermediate bonding material. Fully fused seals inherently block liquid leakage and microbial ingress; however, gaseous leakage and permeation may occur.

### 4.3 Multiple-Dose Package Closures

Multiple-dose package closures are designed to allow product access while limiting microbial ingress and product leakage between doses. For example, elastomeric closures for parenteral products requiring needle-puncture access are designed to offer reseal protection against microbial ingress and product leakage. Some multiple-dose ophthalmic dosage form package closures are designed with filters, plugs, or other mechanical means that allow the product to be dispensed while restricting microbial ingress and product leakage. For example, ophthalmic product–package closure systems have been designed to automatically pinch shut between intermittent dosing to limit microbial entry and product loss.

## 5. PRODUCT–PACKAGE QUALITY REQUIREMENTS AND THE MAXIMUM ALLOWABLE LEAKAGE LIMIT

As noted in *Introduction*, package integrity is necessary to maintain product critical quality attributes within physicochemical label-claim specifications and to ensure product sterility until time of use. Detrimental contaminants include microorganisms and any substances that threaten patient safety or product quality. Product leakage can cause a product to fail content or potency specification limits. For certain products to maintain product physicochemical stability, the package needs to maintain a headspace of nonreactive gases and/or low water vapor content, sealed under atmospheric or reduced-pressure conditions. Headspace vacuum conditions may also be necessary to facilitate product ease of use, e.g., product reconstitution using a diluent injected into the container.

Most package types display very low but definite gaseous leakage flow through the gap that exists even between well-fitted closures. Therefore, it is not practical to require that packages be absolutely leak-free. Rather, it is the significance of leakage in relation to product quality that needs to be considered. In other words, the package should not permit leakage beyond the product's maximum allowable leakage limit. Such leaks of concern should be absent.

Identifying the maximum allowable leakage limit for a product–package is a science- and risk-based decision. The smaller the leak path, the less likely the product can escape and the less likely microorganisms or other contaminants can enter. Eventually, leak paths may be so small that only headspace gas exchange is realistically possible. Package construction and assembly, package contents, and the range of environments a given product–package may be exposed to during its life cycle are to be considered when specifying the maximum allowable leakage limit.

There are two major product–package quality requirement categories used when specifying the maximum allowable leakage limit and one subcategory that applies only to multiple-dose packages. These are discussed below.

### 5.1 Sterility and Product Formulation Content must be Preserved; Gas Headspace Content Preservation is not Required

This category includes product–packages for which the maximum allowable leakage limit correlates to the prevention of product formulation escape, or product contamination by external liquid or solid matter or by microorganisms, while gas headspace preservation (i.e., ambient pressure air) is of no concern. The smallest leak paths that allow only limited gas exchange are irrelevant as they pose no real risk to product quality.

Considerable published research exists exploring rigid package leaks and their relationship to risk of aqueous solution leakage and/or microbial ingress. A key study series found orifice leaks of approximately 0.1 µm in nominal diameter (using micropipettes) placed in rigid packages demonstrate a small risk of aqueous liquid passage, while orifice leaks as small as approximately 0.3 µm in nominal diameter first demonstrate some risk of microbial ingress by liquid challenge (*2,3*). For a summary of similar studies comparing risks of microbial ingress and liquid leakage to leak size and type, the reader may refer to reference *4*.

Given this body of evidence, a maximum allowable leakage limit of less than $6 \times 10^{-6}$ mbar · L/s (measured by helium mass spectrometry in the vacuum mode) can be adopted for products in this category packaged in rigid container–closure systems. This leakage rate equates to the presence of an orifice of nominal diameter of between 0.1 and 0.3 µm. At this leakage rate, the probability of microbial ingress was determined to be <0.10 (*2*). Selecting this conservative maximum allowable leakage limit will ensure a low risk of microbial ingress and liquid leakage and can eliminate the need to perform additional microbial ingress or liquid challenge studies as a function of leak size.

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For other container systems such as those made using flexible materials or those with complex, lengthy closure/seal interfaces, or those meant to contain a product of markedly greater leakage potential, the risk of microbial ingress or liquid passage through leak paths is not as widely publicized or perhaps understood. In such cases, where the relationship between defect size/type to the risks of microbial ingress and/or liquid passage is less prescriptive, a study exploring these relationships could be useful. Test results can be used to establish a meaningful maximum allowable leakage limit for the given product–package system, which can be employed for package integrity verification by other validated leak test methods of choice. Once established, this maximum allowable leakage limit can be applied to similar product–packages with appropriate justification.

Finally, before classifying a product–package in this category, one may consider the potential impact of product life cycle processing, storage, distribution and use scenarios on package integrity. For example, elastomeric closures have been found to shrink and lose their viscoelastic properties during ultra-cold storage (≤–80°) to such an extent that gas influx into stoppered vial packages may occur. During warming, package closure is restored, trapping gases and notably raising internal package pressure. In such cases, gas headspace preservation may be a product quality concern, even if the product does not require specific headspace content or pressure for optimum stability.

### 5.2 Sterility, Product Formulation Content, and Gas Headspace Content must be Preserved

The second category includes product–packages for which the package should prevent product formulation escape and product contamination by external liquid or solid matter or by microorganisms, but in addition, the package must preserve the gas headspace content or absolute pressure. Preservation of headspace content and/or pressure is needed to maintain product stability within physicochemical specification limits, and/or to aid end-user product access. Thus, the maximum allowable leakage limit for such products is likely more stringent than that described in the first category. Although outside the chapter scope, the influence of gas permeation through the package itself may also need to be considered when establishing the maximum allowable leakage limit as permeation plus leakage can impact package headspace content.

The maximum allowable leakage limit for products in this category may be expressed in terms of the maximum allowable package headspace content or pressure change as a function of time. This limit, established for each product–package system, may be applied to other similar product–packages systems with appropriate justification.

### 5.3 Sterility must be Preserved; Product Access is Required

This subcategory represents a quality requirement applicable only to multiple-dose product–packages included in either of the above two categories. Once the product–package has reached the end-user and the closure has been activated or otherwise compromised to allow dosage access and delivery, the maximum allowable leakage limit at this life cycle phase (called the in-use maximum allowable leakage limit) is defined in terms of microbial ingress and product loss prevention between and during dosage access. For example, elastomeric closures of multiple-dose vials or cartridges containing antimicrobial-preserved parenteral products are designed to afford reseal protection against microbial ingress and product formulation leakage as product is accessed via needle puncture as well as between doses.

To establish the in-use maximum allowable leakage limit for multiple-dose package closure systems, a study will likely be required to explore the relationship between product access attempts, product loss risk and/or microbial ingress risk, versus leakage measurement by an alternative adequately sensitive leak test method of choice. These data can be used to establish a meaningful in-use maximum allowable leakage limit expressed in units of measure reflective of the preferred leak test method and that will assure that product loss and microbial ingress risk during use is minimal. The in-use maximum allowable leakage limit established for a given product–package may be applied to other similar product–packages systems with appropriate justification.

## 6. INHERENT PACKAGE INTEGRITY AND THE PACKAGE INTEGRITY PROFILE

Inherent package integrity is the leakage characteristic of a well-assembled container–closure system using no-defect package components. Inherent package integrity is first determined during product–package development and qualification and is a measure of the leak tightness of a container–closure system, given anticipated variables of material composition, dimension, processing, and assembly. Inherent package integrity may also be determined as a function of anticipated final product storage, distribution, and use. Acceptable inherent package integrity for a container–closure system conforms to the specific product–package maximum allowable leakage limit. Inherent package integrity is expressed in terms that allow a meaningful comparison to the maximum allowable leakage limit.

Confirmation that the inherent package integrity conforms to the maximum allowable leakage limit is the first step in product–package integrity verification. Verification of package integrity continues throughout the product life cycle. The package integrity profile is an ongoing database of product life cycle package leak and seal quality test results. This profile provides information regarding package integrity given operative variations in package component design and material; package assembly and processing; and product storage, distribution, and stability. These concepts are more fully explored in *Package Integrity Testing in the Product Life Cycle—Test Method Selection and Validation* ⟨1207.1⟩, *Test Instrument Qualification, Method Development, and Method Validation*.

In summary, a product–package system having integrity is one in which the inherent package integrity conforms to the required product–package maximum allowable leakage limit. Further, the package integrity profile database operates as a risk management tool to ensure that finished product container–closure systems are intact, able to block microbial ingress, restrict loss of product contents including critical headspace gases, and prevent entry of detrimental gases or other substances, thus ensuring that the product meets all relevant physicochemical and microbiological label-claim specifications through expiry and final end-use.

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## GLOSSARY

For definitions of container, materials of construction, packaging component, packaging system, primary packaging component, and secondary packaging component, see *Packaging and Storage Requirements* ⟨659⟩. In the context of this chapter and its subchapters, the following definitions relevant to packaging and package integrity methods, seal and closure mechanisms apply. For definitions of specific leak test and seal quality test methods, refer to ⟨1207.2⟩ and ⟨1207.3⟩, respectively.

**Accuracy:** The accuracy of a leak test method is a measure of the ability of the method to correctly differentiate packages that leak above the claimed detection limit from those that leak below this limit (do not leak); a measure of false positive and negative occurrence. Alternatively, for those methods that deliver an outcome that is a direct quantitative measure of gas leakage rate (or of gas content or pressure), accuracy is a measure of the method's ability to produce an outcome comparable to a true standard, such as a nationally recognized gas standard.

**Container–closure integrity:** Container–closure integrity is the ability of a package to prevent product loss, to block microorganism ingress, and to limit entry of detrimental gases or other substances, thus ensuring that the product meets all necessary safety and quality standards. Synonymous with *Package integrity*.

**Container–closure integrity test:** A container–closure integrity test is any package leak test (either physicochemical or microbiological) that detects the presence of a package breach or gap. Some tests may also be able to identify the leak magnitude and/or location. The term container–closure integrity test is synonymous with package leak test or package integrity test.

**Container–closure system:** See *Packaging and Storage Requirements* ⟨659⟩*, General Definitions, Packaging System.*

**Detection limit:** The leak test detection limit is a measure of test method sensitivity. The detection limit is the smallest leakage rate (or leak size) that a leak test method can reliably detect. Also called limit of detection.

**Deterministic leak test method:** A deterministic leak test method is one in which the leakage event being detected or measured is based on phenomena that follow a predictable chain of events. In addition, the measure of leak detection is based on physicochemical technologies that are readily controlled and monitored, yielding objective quantitative data.

**In-use maximum allowable leakage limit:** See *Maximum allowable leaking limit*.

**Inherent package integrity:** Inherent package integrity is the leakage rate (or leak size) of a well-assembled container–closure system using no-defect package components. Inherent package integrity is a measure of the leak tightness of a container–closure system, given anticipated variables of material composition, dimension, processing, assembly; package storage, distribution and use.

**Leak:** A leak is a breach in a package wall or a gap between package components that is capable of permitting the passage of gas, liquid, or solid matter. Leak is synonymous with leak path.

**Leakage:** Leakage is the unintentional escape or entry of matter (solid, liquid, or gas) through a breach in the package wall or through a gap between package components. Leakage can also refer to the substance that enters or escapes from a compromised package. For example, "Leakage from the cracked container stained the package label."

**Leakage rate:** Gaseous leakage rate is a measure of the rate of gas flow (in mass or volume units) that passes through a leak path under specific conditions of temperature and absolute pressure or concentration differential. Leakage rate has dimensions of pressure multiplied by volume, divided by time. For example, the international standard SI nomenclature is pascal cubic meter per second ($Pa \cdot m^3 \cdot s^{-1}$). Other common units of measure include standard cubic centimeters per second (std $\cdot$ $cm^3 \cdot s^{-1}$ or sccs) and mbar liters per second ($mbar \cdot L \cdot s^{-1}$). Liquid leakage rate is a measure of the volume of liquid that moves through a leak path as a function of time under specified conditions of temperature and absolute differential pressure across the barrier wall. Liquid leakage rate measurement, possible for larger leak tests, requires that leak size, materials of construction; absence of leak obstruction; tracer liquid composition; and test parameters work together to ensure liquid movement through the leak path.

**Linearity:** Leak test method linearity is the ability of the method to elicit test results that are mathematically proportional to leak path size or leakage rate.

**Master:** A master is a package prototype, model, or facsimile made to simulate an actual package in shape and design. Masters may be made of solid plastic or metal, or they may be simply a designated container–closure unit. Masters are designed to simulate a no-leaking package, and are often used in leak test system suitability tests to verify instrument performance.

**Maximum allowable leakage limit:** The maximum allowable leakage limit is the greatest leakage rate (or leak size) tolerable for a given product–package that poses no risk to product safety and no or inconsequential impact on product quality. The maximum allowable leakage limit for a sterile pharmaceutical dosage form package will ensure the content's sterility, preserve product contents, and prevent entry by detrimental gases or other substances, thus ensuring that the product meets relevant physicochemical and microbiological specifications through expiry and use. For multiple-dose product–packages, the in-use maximum allowable leakage limit is defined as the degree of protection demanded of the closure to limit microbial ingress and product formulation leakage between and during dosage access.

**Microbiological challenge test:** A microbiological challenge test is a package leak test whereby package integrity is evaluated by exposing containers filled with growth-supportive media to microorganisms suspended in submersion media (a

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liquid-borne challenge test). Leakage is evidenced by the subsequent growth of the challenge microorganisms in the package contents. Synonymous with microbial challenge test.

**Negative control:** A negative control is a package with no known leak. Negative controls used for leak test method development and validation studies represent packages optimally assembled using normally processed components. Negative controls should duplicate the container–closure system of the product under integrity investigation. For some methods, it may be necessary for negative controls to simulate test product headspace and formulation content as well.

**Nonporous:** Nonporous packaging does not have pores or openings to allow volumetric air flow. In other words, NMT a small fraction of molecules is able to pass through any one hole. Nonporous materials may be impermeable, semi-permeable, or permeable to the passage of fluid through the package barrier wall.

**Nominal diameter:** In the context of this chapter, nominal diameter is a means of expressing the size of a package leak. Package leaks are typically irregular tortuous paths or matrices that cross a package wall or barrier. Such leaks are difficult to size in accurate or meaningful terms. The nominal diameter of a leak path is defined as the width of an orifice of relatively short depth, through which gas (e.g., dry air) at equivalent airflow rate may pass when subject to one atmosphere differential pressure at controlled temperature conditions.

**Package integrity:** See *Container–closure integrity*.

**Package integrity test:** See *Container–closure integrity test*.

**Package integrity profile:** The package integrity profile is a database of product life cycle package leak and seal quality test results that denotes product–package integrity given operative variations in package component design and material; package assembly and processing; and product storage, distribution, and stability.

**Package leak test:** See *Container–closure integrity test*.

**Package seal quality:** Package seal quality relates to the consistency of a package seal's performance within required specification limits. Examples of package seal quality attributes include heat seal bond strength and capped vial package residual seal force.

**Package seal quality test:** A package seal quality test is used to characterize and monitor the quality of a product–package seal or closure system to ensure that package assembly is consistently kept within established limits. Package seal quality tests are not and cannot substitute for leak tests. However, they can provide some assurance of the package's ability to maintain integrity by monitoring a characteristic of the seal itself, the package materials, the package components, and/or the processes required to create the seal or closure mechanism. Examples include the seal peel force test and the capped vial package residual seal force test.

**Permeation:** Permeation is the passage of fluid into, through, and out of a nonporous package wall. Permeation, not leakage, occurs when the package barrier has no holes large enough for more than a small fraction of molecules to pass through any one hole.

**Physicochemical package integrity test:** A physicochemical package integrity test is a leak test that detects the presence of a package leak, or detects/measures package leakage rate, via physical or chemical means. All leak test methods that do not use microorganisms for leak detection are physicochemical leak test methods.

**Porous:** A porous package or package material has pores or openings to allow volumetric air flow while still blocking airborne microbial passage. Examples include coated paper or spun-bonded polyolefin barrier materials that permit package penetration by ethylene oxide gas or steam.

**Positive control:** A positive control is a package with a known, intentional defect. Positive controls used for leak test method development and validation studies should duplicate study negative controls in terms of materials of construction, package assembly, and component processing. Positive controls are used during leak test method development and validation. Some methods require the use of positive controls during routine testing as well.

**Precision:** Leak test method precision is a measure of the method's ability to yield reliable, repeatable data. Precision includes repeatability (repeat testing of a single homogeneous test sample population set), ruggedness (within laboratory tests performed by multiple operators on multiple days, using multiple instruments; also known as intermediate precision), and reproducibility (tests among multiple laboratories).

**Probabilistic leak test method:** A probabilistic leak test method is the converse of a deterministic leak test method, being stochastic in nature. Probabilistic tests rely on a series of sequential and/or simultaneous events, each associated with random outcomes described by probability distributions. Thus, the findings are associated with uncertainties that necessitate large sample sizes and rigorous test-condition controls to obtain meaningful results. Typically, sample size and test condition rigor are inversely related to leak size.

**Product:** The pharmaceutical product includes the pharmaceutical formulation as well as the packaged headspace, which may consist of ambient air or nonreactive gases with specified water-vapor content under full or sub-atmospheric pressure conditions.

**Product–package:** The product–package includes the primary package with critical secondary components (the container–closure system) plus the packaged product (the contents).

**Qualitative measure of analysis:** A qualitative measure of analysis for leak testing is a measurement approach based on a subjective evaluation of some quality, attribute, or characteristic of the test sample. Visual inspection is an example of a qualitative measure of analysis.

**Quantitation limit:** The quantitation limit is a leak test method characteristic defining the lowest leakage rate or leak size that can be differentiated with accuracy and precision under the stated experimental conditions.

**Quantitative measure of analysis:** A quantitative measure of analysis for leak testing is a measurement approach based on objective, numeric data that either directly or indirectly correlates with leak presence, leak location, or leakage rate. Examples include the mass-of-gas-per-time reading generated by the helium mass spectrometry tracer-gas leak test, or the pressure reading as a function of test time measurement produced by the vacuum-decay method.

**Range:** The range of a leak test method is that interval between the smallest to largest leak size (or leakage rate) that can be detected by a given leak test method with a suitable level of accuracy and precision.

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**Robustness:** Robustness of a leak test method is the method's ability to accurately identify leaking versus nonleaking packages despite small but deliberate variations in procedural parameters, providing an indication of the method's suitability during normal usage.

**Specificity:** The specificity of a leak test method is the ability of the method to accurately differentiate leaking and nonleaking packages, despite interfering factors that may cause false detection.

**System suitability:** System suitability is a manner of ensuring that the leak test method including all factors, which may be subject to variability, that may impact test results (such as instrumentation, analysts, test sample preparation steps, and the test environment) are adequately controlled and maintained in such a fashion that the method is rugged and robust.

**System suitability test:** A system suitability test is a test to verify that the leak test method and all key factors that may impact test results are correctly controlled and set prior to method performance.

**Tortuous path:** As applied to leaks, a tortuous path is a convoluted, complex leakage pathway. Most naturally occurring leaks, such as cracks and tears, are tortuous in nature, rather than pristine holes. As applied to sealing mechanisms, a sealing material that has tortuous barrier qualities can block microbial entry. [NOTE—The winding path afforded by the threads of a screw-cap (e.g., an ophthalmic dropper bottle closure) does not provide an optimal barrier to gas or liquid leakage, nor does it provide an optimal barrier to microbial ingress in the event of a liquid presence in the cap threads.]

**Type defect:** A type defect is a positive-control package that represents realistic package flaws. Type-defect positive controls may be included in leak test method feasibility and development studies before method validation. An example of a type defect is a heat seal wrinkle or a loose cap. Type defects are inherently irregular in size and shape and are often described qualitatively instead of being described in terms of leak size or leakage rate.

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# ⟨1208⟩ STERILITY TESTING—VALIDATION OF ISOLATOR SYSTEMS

This chapter provides guidelines for the validation of isolator systems for use in sterility testing of compendial articles. [NOTE—In the context of this chapter, "decontaminated" refers to an item or surface that has been subjected to a process that eliminates viable bioburden.]

Isolators—devices that create controlled environments in which to conduct Pharmacopeial sterility tests—have been used since the mid-1980s. An isolator is supplied with air through a HEPA or better air filter and is able to be reproducibly decontaminated. Closed isolators, which are systems with no direct opening to the external environment, are normally used for sterility testing, although open isolators which allow the egress of materials through a defined opening that precludes the entry of contamination by means of air overpressure may be used. Closed isolators use only decontaminated interfaces or a rapid-transfer port for the transfer of materials. Isolators are constructed of flexible plastics (such as polyvinyl chloride), rigid plastics, glass, or stainless steel.

Isolator systems protect the test article and supplies from contamination during handling by essentially eliminating direct contact between the analyst and the test articles. All transfers of material into and out of the isolator are accomplished in an aseptic fashion while maintaining complete environmental separation. Aseptic manipulations within the isolator are made with half-suits, which are flexible components of the isolator wall that allow the operator a full range of motion within the isolator, or by gloves and sleeves. Operators are not required to wear special clean-room clothing for conducting sterility tests within isolators; standard laboratory clothing is adequate, although a pair of sterile gloves is frequently worn under the isolator gloves as an added precaution against contamination entering the isolator enclosure and for hygiene purposes. The interior of the isolator is treated with sporicidal chemicals that result in the elimination of all viable bioburden on exposed surfaces.

## ISOLATOR DESIGN AND CONSTRUCTION

### Air Handling Systems

An isolator used for sterility testing is equipped with microbial retentive filters (HEPA filters or better are required). At rest, the isolator meets the particulate air-quality requirements for an ISO Class 5 area as defined in ISO 14644-1 through -3* (see *Microbiological Control and Monitoring of Aseptic Processing Environments* ⟨1116⟩). However, the isolator need not meet Class 5 conditions during an operation that may generate particulates, and no requirements for air velocity or air exchange rate exist. The isolator should be sealed well enough during decontamination that the dissemination of sporicidal vapors or gases into the surrounding environment is kept to appropriately low levels. When direct openings to the outside environment exist, constant air overpressure conditions maintain sterile conditions within the isolator. In general, both open and closed isolators are maintained at positive pressure relative to the surrounding environment, and overpressures of 20 Pa or more are typical. The user should never exceed the maximum pressure recommended by the isolator manufacturer. Airflow within isolators used for sterility testing is either unidirectional or turbulent.

### Transfer Ports and Doors

Isolators may be attached to a "pass-through" decontaminator or transfer isolator to enable the direct transfer of sterile media, sterile dilution fluids, and sterile supplies from the decontaminator into the isolator system. Rapid transfer ports (RTPs) enable two isolators, i.e., the work station and transfer isolator, to be connected to one another, so that supplies can be moved aseptically from one isolator to another. Aseptic connections between two isolators or an isolator and an RTP-equipped container can be made in unclassified environments using RTPs. The nonsterile surfaces of the RTP are connected using locking rings or flanges. A compressed gasket assembly provides an airtight seal, thereby preventing the ingress of microorganisms.

When the two RTP flanges are linked to form an airtight passage, a narrow band of gasket remains that could harbor microbial contamination. This exposed gasket should be routinely disinfected immediately after the connection is made, and before materials are transferred through the RTP. Good aseptic technique is used when transferring materials and care is taken not to touch the gasket with the materials being transferred or with the gloved hands.

Preventive maintenance and lubrication of the gasket assemblies on the flanges is performed according to the RTP manufacturer's recommendations. The RTP gaskets are changed at the recommended frequency and periodically checked for damage, because cut or torn gaskets cannot make a truly airtight seal.

### Selection of a Location for the Isolator

Isolators for sterility testing need not be installed in a classified clean room, but it is important to place the isolator in an area that provides limited access to nonessential staff. The appropriate location provides adequate space around the isolator for moving transfer isolators, staging of materials, and general maintenance. No environmental monitoring of the surrounding room is required.

Temperature and humidity control in the room is important to operator safety and comfort and is critical for the effective utilization of certain decontamination technologies. Uniform temperature conditions in the room are desirable when temperature-sensitive decontamination methods are employed. Care should be taken in locating the isolator so that cold spots are avoided that might result in excessive condensation when condensing vapors are used for decontamination.

* International Organization for Standardization (ISO) International Standards 14644-1, -2, -3, and -7

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# VALIDATION OF THE ISOLATOR SYSTEM

The isolator system must be validated before its use in sterility testing as part of a batch release procedure. To verify that the isolator system and all associated equipment are suitable for sterility tests, validation studies are performed in three phases: installation qualification (IQ), operational qualification (OQ), and performance qualification (PQ). The following sections contain points to consider in the validation of isolator systems for sterility testing. The assignment of test functions to a particular phase of the validation program (i.e., IQ, OQ, and PQ) is not critical, as long as proper function of the isolator is demonstrated and documented before its use in compendial *Assays*.

## Installation Qualification (IQ)

The IQ phase includes a detailed description of the physical aspects of the system, such as the dimensions, internal configuration, and materials of construction. The unit layout is diagrammed with interfaces and transfer systems clearly and dimensionally indicated. Compliance with design specifications for utility services, such as air supply, vacuum, external exhaust, and temperature and humidity control, is verified. Other equipment used with the isolator system is also described in detail; if any revisions to design specifications are made, these are included. Equipment manuals and copies are catalogued and stored where they can be retrieved and reviewed. Compliance of drawings to design specifications is verified. All drawings and process and instrumentation diagrams are catalogued, stored, and are retrievable.

All documentation is reviewed to verify that it precisely reflects the key attributes of the installed system. This establishes a general benchmark for the isolator system's compliance with design specifications and installation requirements.

Potential process-control or equipment problems that could cause system failure during operation are identified and documented during failure-mode analysis and hazard analysis. The system is modified, if necessary, to minimize the risk of failure, and critical control point methods are established.

The results of the IQ are summarized in an Installation Qualification Report. The following documentation is suggested.

### EQUIPMENT

The equipment is listed with its relevant design specifications. The IQ verifies that equipment meeting the appropriate design specifications was received and that it was installed according to the manufacturer's requirements.

### CONSTRUCTION MATERIALS

The construction materials of critical system components are checked for compliance with design specifications. The compatibility of the intended decontamination method with the construction materials is verified.

### INSTRUMENTS

System instruments are listed with their calibration status.

### UTILITY SPECIFICATIONS

All utilities required for operation—as defined in the operating manuals and process and instrumentation diagrams—are checked for availability and compliance with design specifications. Any connection between utility systems and the isolator system is inspected and conformance of these connections to specifications is verified.

### FILTER CERTIFICATION

HEPA filters and other microbial retentive filters are tested and certified; copies of test results and certificates are included in the IQ summary. Purchase orders are reviewed and conformance of the air filtration system to specifications is verified.

### COMPUTER SOFTWARE

All computer software associated with the isolator system is listed with its name, size, and file revision number. The master computer disks are checked for proper labeling and stored securely.

## Operational Qualification (OQ)

The OQ phase verifies that the isolator system operates in conformance to functional specifications.

### OPERATIONAL PERFORMANCE CHECK

This test verifies that all alert and alarm functions comply with their functional specifications. The system's ability to comply with all set points and adjustable parameters is verified.

### ISOLATOR INTEGRITY CHECK

The integrity of the isolator is maintained during all normal operating conditions. A leak test is performed to verify the compliance with the manufacturer's functional specifications and to ensure safety prior to charging the isolator with a

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decontaminating sporicidal chemical. To safeguard against adventitious contamination, isolators are operated at a suitable positive pressure during normal operation. Validation studies must show that the air pressure set point can be maintained and controlled during operation.

### DECONTAMINATION CYCLE VERIFICATION

A decontamination cycle that is the function of the decontamination equipment in concert with the isolator(s) is verified.

Different decontamination methods can be used to eliminate bioburden from isolator systems and supplies. Among the chemicals that have been used to treat isolators are peracetic acid, chlorine dioxide, ozone, and hydrogen peroxide; each has different requirements for exposure conditions and process control. It is critical to comply with the manufacturer's operational requirements for the selected decontamination method and to describe them in the functional specifications. The temperature inside the isolator is also important, particularly for hydrogen peroxide vapor decontamination, where it is critical to maintain the concentration relative to the condensation point. Some sterilization chemicals, such as chlorine dioxide and ozone, require the addition of moisture to the isolator prior to decontamination. When elevated relative humidity is required, the ability to control it must be verified during OQ.

It is also important to verify the concentration and distribution of the decontaminating chemical. When applied in gaseous or vapor form, the distribution may be evaluated using chemical indicators, spectroscopic methods, or electronic sensors.

Gas and vapor decontamination methods may require fans in the isolator to distribute the chemical evenly. The location and orientation of these fans are adjusted to ensure optimum air distribution. If the isolator utilizes a recirculating unidirectional airflow system, distribution fans may not be required, but this should be evaluated on a case-by-case basis. Because shelving units, equipment, glove-and-sleeve assemblies, and half-suits have an impact on distribution patterns, distribution checks are done with the isolator fully loaded with equipment and supplies, and the setup of these units is defined and documented.

Many installations use smaller transfer isolators as portable surface decontamination units. In these transfer isolators, test articles and supplies are treated chemically to eliminate bioburden before transfer through an RTP into the testing isolator. Its loading configuration is defined, and configuration drawings are reviewed and verified during the OQ. [NOTE—The decontaminating chemicals used in isolators work on the surfaces of materials; therefore, any surface that is occluded will not be treated and could contain viable bioburden. Special precautions should be in place for treating surfaces known to be occluded with a sporicide if such surfaces may be revealed during the conduct of sterility tests.]

Decontamination agents need to be removed from the isolator after the exposure period, which is accomplished by a current of fresh air provided either by the decontamination equipment or by utilizing the isolator air handling system. Aeration is accomplished either in an open loop, in which the gas is exhausted through a vent to the atmosphere, or in a closed loop, in which the chemical is removed and destroyed by the decontamination equipment. The aeration system is checked; if an open-loop configuration is used, the external exhaust system's flow and safety are checked.

### DECONTAMINATION CYCLE DEVELOPMENT

When the OQ is completed, decontamination cycle development is performed to establish the parameters necessary for process control during routine decontamination cycles. Any of the methods generally used in the industry for the validation of decontamination processes—including bioburden-based, fractional cycle, and overkill methods—are adequate. The decontamination process is challenged with biological indicators (BIs). The spore population and resistance of the BIs to the decontamination conditions being applied are known. Wherever possible, a D value estimate is done for each B1 system or, alternatively, a survivor curve for the BI system is obtained (see *Biological Indicators—Resistance Performance Tests* ⟨55⟩); it is acceptable to obtain the D value from the BI vendor.

## Performance Qualifications (PQ)

The PQ phase verifies that the system is functioning in compliance with its operator requirement specifications. At the completion of the PQ phase, the efficacy of the decontamination cycle and, if appropriate, the adequacy of decontaminating chemical venting are verified. All PQ data are adequately summarized, reviewed, and archived.

### CLEANING VERIFICATION

In general, cleaning is not critical for sterility testing applications. However, residual products are a concern in multiproduct testing, particularly for aggressive antimicrobial agents, because these materials could interfere with the ability of subsequent tests to detect low levels of contamination in the product. Concerns about contamination with the product are heightened when it is an inherently antimicrobial powder, because powders are more readily disseminated. Cleaning to a level at which no visible contamination is present is adequate for sterility test isolator systems and is a suitable operator requirement specification. The cleaning method, frequency, equipment, and materials used to clean the isolator are documented.

### DECONTAMINATION VALIDATION

The interior surfaces of the isolator, the equipment within the isolator, and the materials brought into the isolator are treated to eliminate all bioburden. The decontamination methods used to treat isolators, test articles, and sterility testing supplies are capable of reproducibly yielding greater than a three-log reduction against highly resistant biological indicators (see *Biological Indicators for Sterilization* ⟨1229.5⟩), as verified by the fraction negative or total kill analysis methods. Total kill analysis studies are suitable for BIs with a population of $10^3$ spores per unit, while fraction negative studies are suitable for BIs with a population of $10^5$ or greater. A sufficient number of BIs are used to prove statistical reproducibility and adequate distribution of the decontaminating agent. Particular attention is given to areas that pose problems relative to the concentration of the agent. A

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larger number of BIs may be required in isolators that are heavily loaded with equipment and materials. The ability of the process to reproducibly deliver a greater than three-log kill is confirmed in three consecutive validation studies.

The operator establishes a frequency for re-decontamination of the isolator. The frequency may be as short as a few days or as long as several weeks, depending on the sterility maintenance effort (see *Maintenance of Asepsis within the Isolator Environment*).

## PACKAGE INTEGRITY VERIFICATION

Some materials are adversely affected by decontaminating agents, which can result in inhibition of microbial growth. Of concern are the penetration of decontaminating agents into product containers; accessory supplies such as filter sets and tubing; or any material that could come in contact with product, media, or dilution fluids used in the sterility test. It is the responsibility of the operator to verify that containers, media, and supplies are unaffected by the decontamination process. Screw-capped tubes, bottles, or vials sealed with rubber stoppers and crimp overseals have proven very resistant to the penetration of commonly used decontaminating agents. Wrapping materials in metal foil or placing them in a sealed container will prevent contact with the decontaminating agent; however, these procedures may also result in some surfaces not being decontaminated. In some cases, the use of shorter duration decontamination cycles and reduced concentrations may be necessary to minimize penetration of decontaminating agents into the package or container. Cycles that provide a less than three-log kill of resistant BIs may be acceptable provided microbiological analysis of the environment proves that the isolator(s) are free of recoverable bioburden.

In many cases, the operator will choose to treat the surfaces of product containers under test with the decontaminating agent in order to minimize the likelihood of bioburden entering the isolator. It is the responsibility of the operator to demonstrate, via validation studies, that exposure of product containers to the decontaminating agent does not adversely affect the ability of the sterility test to detect low levels of contamination within these test articles. It is suggested that the ability of the package to resist contamination be examined using both chemical and microbiological test procedures. Bacteriostasis and fungistasis validation tests must be performed using actual test articles that have been exposed to all phases of the decontamination process (see *Sterility Tests* ⟨71⟩). This applies to medicinal device packages as well as pharmaceutical container and closure systems.

Validation studies determine whether both sterility test media and environmental control media meet the requirements for *Growth Promotion Test of Aerobes, Anaerobes, and Fungi* under *Sterility Tests* ⟨71⟩.

## MAINTENANCE OF ASEPSIS WITHIN THE ISOLATOR ENVIRONMENT

The ability of the isolator system to maintain an aseptic environment throughout the defined operational period must be validated. In addition, a microbiological monitoring program must be implemented to detect malfunctions of the isolator system or the presence of adventitious contamination within the isolator. Microbiological monitoring usually involves a routine sampling program, which may include, for instance, sampling following decontamination on the first day of operation and sampling on the last day of the projected maintenance of asepsis period. Periodic sampling throughout the use period can be performed to demonstrate maintenance of asepsis within the isolator.

The surfaces within the isolator can be monitored using either contact plates for flat surfaces or swabs for irregular surfaces. However, because media residues could impose a risk on isolator asepsis, these tests are generally best done at the end of the test period. If performed concurrently with testing, care is used to ensure that any residual medium is removed from isolator surfaces, and that those surfaces are carefully cleaned and disinfected. Active air samples and settling plates may be used, but they may not be sufficiently sensitive to detect the very low levels of contamination present within the isolator enclosure.

A potential route for contamination to enter the isolator is during the introduction of supplies and samples into the enclosure. Validating that all materials taken into the isolator enclosure are free of microbial contamination is critical, as is periodic inspection of gaskets to detect imperfections that could allow ingress of microorganisms. Gloves and half-suit assemblies are another potential source of microbial contamination. Gloves are of particular concern because they are used to handle both sterility testing materials and test articles. Resistance to puncture and abrasion should be considered in the selection of gloves and sleeves. Hypalon materials are resistant to both chemical sporicides used in the decontamination of isolators and to punctures and are available in several thicknesses to provide adequate tactile feel through the gloves while maintaining their integrity.

Very small leaks in gloves are difficult to detect until the glove is stretched during use. There are several commercially available glove leak detectors; the operator ensures that the detectors test the glove under conditions as close as possible to actual use conditions. Microbiological tests are used to supplement or substitute physical tests. [NOTE—Standard "finger dab plates" may not be sensitive enough to detect low levels of contamination. Submersion of the gloves in 0.1% peptone water followed by filtration of the diluent and plating on growth media can detect loss of integrity in the gloves that would otherwise go unnoticed.]

Continuous nonviable particulate monitoring within the isolator's enclosure is ideal, because it can quickly detect filter failure. A second choice is periodic monitoring using a portable particle counter. Sampling for particles must be done in a manner that poses no risk to the maintenance of asepsis within the isolator.

## INTERPRETATION OF STERILITY TEST RESULTS

A sterility test resulting in a false positive in a properly functioning and validated isolator is very unlikely if bioburden is eliminated from the isolator interior with a high degree of assurance; if gloves, sleeves, and half-suits are free of leaks; and if the RTPs are functioning properly. Nevertheless, isolators are mechanical devices and good aseptic techniques are still required. A

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decision to invalidate a false positive is made only after fully complying with the requirements of *Observation and Interpretation of Results* under *Sterility Tests* ⟨71⟩.

## TRAINING AND SAFETY

As with sterility testing conducted in conventional clean rooms, operators are trained in procedures that are specific to their isolator. Use of proper aseptic techniques is vital to the conduct of sterility tests in isolators, just as it is in clean rooms. Therefore, training in proper aseptic techniques is required for all sterility testing technicians. All training sessions and the evaluation of the operator's performance are documented in the individual's training record. Training of all personnel in the appropriate safety procedures necessary for the operation and maintenance of the isolation system is imperative.

Personnel safety in the use of a decontaminating agent must be assessed. Material Safety Data Sheets, or equivalent documents, are available in the immediate area where the decontaminating agent is being used. All storage and safety precautions are followed. An operational readiness inspection of the safety of the isolator and all associated equipment is performed and documented prior to placing the unit in service.

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***Add the following:***

# ▲⟨1210⟩ STATISTICAL TOOLS FOR PROCEDURE VALIDATION

1. INTRODUCTION
2. CONSIDERATIONS PRIOR TO VALIDATION
3. ACCURACY AND PRECISION
    3.1 Methods for Estimating Accuracy and Precision
    3.2 Combined Validation of Accuracy and Precision
4. LIMITS OF DETECTION AND QUANTITATION
    4.1 Estimation of LOD
    4.2 Estimation of LOQ
5. CONCLUDING REMARKS
REFERENCES

## 1. INTRODUCTION

This chapter describes utilization of statistical approaches in procedure validation as described in *Validation of Compendial Procedures* ⟨1225⟩. For the purposes of this chapter, "procedure validation" refers to the analytical procedure qualification stage of the method life cycle, following design and development and prior to testing.

Chapter ⟨1225⟩ explains that capabilities of an analytical procedure must be validated based on the intended use of the analytical procedure. Chapter ⟨1225⟩ also describes common types of uses and suggests procedure categories (I, II, III, or IV) based on the collection of performance parameters appropriate for these uses. Performance parameters that may need to be established during validation include accuracy, precision, specificity, detection limit [limit of detection, (LOD)], quantitation limit, linearity, and range. In some situations (e.g., biological assay), relative accuracy takes the place of accuracy. This chapter focuses on how to establish analytical performance characteristics of accuracy, precision, and LOD. For quantitative analytical procedures, accuracy can only be assessed if a true or accepted reference value is available. In some cases, it will be necessary to assess relative accuracy. In many analytical procedures, precision can be assessed even if accuracy cannot be assessed. The section addressing LOD can be applied to limit tests in Category II.

The other analytical performance characteristics noted in ⟨1225⟩, which include specificity, robustness, and linearity, are out of scope for this chapter.

Because validation must provide evidence of a procedure's fitness for use, the statistical hypothesis testing paradigm is commonly used to conduct validation consistent with ⟨1225⟩. Although some statistical interval examples are provided in *3. Accuracy and Precision*, these methods are not intended to represent the only approach for data analysis, nor to imply that alternative methods are inadequate.

*Table 1* provides terminology used to describe an analytical procedure in this chapter. The definitions for individual determination and reportable value are in alignment with *General Notices, 7.10 Interpretation of Requirements*.

**Table 1. Analytical Procedure Validation Terminology**

| Terminology | Description |
|---|---|
| Laboratory sample | The material received by the laboratory |
| Analytical sample | Material created by any physical manipulation of the laboratory sample, such as crushing or grinding |
| Test portion | The quantity (aliquot) of material taken from the analytical sample for testing |
| Test solution | The solution resulting from chemical manipulation of the test portion such as chemical derivatization of the analyte in the test portion or dissolution of the test portion |
| Individual determination (ID) | The measured numerical value from a single unit of test solution |
| Reportable value | Average value of readings from one or more units of a test solution |

Not all analytical procedures have all stages shown in *Table 1*. For example, liquid laboratory samples that require no further manipulations immediately progress to the test solution stage. Demonstration that a reportable value is fit for a particular use is the focus of analytical validation.

*Table 2* provides an example of the *Table 1* terminology for a solid oral dosage form.

**Table 2. Example for Coated Tablets**

| Terminology | Description | |
|---|---|---|
| Laboratory sample | 100 coated tablets | |
| Analytical sample | 20 tablets are removed from the laboratory sample and are crushed in a mortar and pestle | |
| Test portion | Replicate 1: 1 g of crushed powder aliquot from the analytical sample | Replicate 2: 1 g of crushed powder aliquot from the analytical sample |

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**Table 2. Example for Coated Tablets** *(continued)*

| Terminology | Description | | | |
|---|---|---|---|---|
| Test solution | Replicate 1: Test portion is dissolved in 1 L of solvent | | Replicate 2: Test portion is dissolved in 1 L of solvent | |
| ID | ID 1 of replicate 1: test solution | ID 2 of replicate 1: test solution | ID 1 of replicate 2: test solution | ID 2 of replicate 2: test solution |
| Reportable value | Average value of four readings | | | |

## 2. CONSIDERATIONS PRIOR TO VALIDATION

Procedure validation is a cornerstone in the process of establishing an analytical procedure. The aim of procedure validation is to demonstrate that the procedure, when run under standard conditions, will satisfy the requirement of being fit for use. To maximize the likelihood of a successful validation, it is imperative that all aspects of the procedure be well understood prior to the validation. Surprising discoveries (whether "good" or "bad") during validation should be carefully evaluated to determine whether the procedure was adequately developed. Moreover, pre-validation work can reveal suitable approaches to reduce the total size of the validation experiment without increasing the risk of drawing the wrong conclusion. General principles and plans for sample preparation, general principles, experimental design, data collection, statistical evaluation, and choice of acceptance criteria should be documented in a validation experimental protocol signed before initiation of the formal validation.

Questions considered prior to validation may include the following:

- What are the allowable ranges for operational parameters, such as temperature and time, that impact the performance of the analytical procedure?
  - Robustness of these ranges can be determined using a statistical design of experiments (DOE).
- What are the ruggedness factors that impact precision?
  - Factors such as analyst, day, reagent lot, reagent supplier, and instrument that impact the precision of a test procedure are called ruggedness factors. When ruggedness factors impact precision, reportable values within the same ruggedness grouping (e.g., analyst) are correlated. Depending on the strength of the correlation, a statistical analysis that appropriately accounts for this dependence may be necessary. Ruggedness factors can be identified empirically during pre-validation or based on a risk assessment.
- Are statistical assumptions regarding data analysis reasonably satisfied?
  - These assumptions may include such factors as normality, homogeneity of variance, and independence. It is useful during pre-validation to employ statistical tests or visual representations to help answer these questions. *Analytical Data—Interpretation and Treatment* ⟨1010⟩ provides information on this topic.
- What is the required range for the procedure?
  - The range of an analytical procedure is the interval between the upper and lower levels of an analyte that has been demonstrated to be determined with a suitable level of precision, accuracy, and linearity using the procedure as written.
- Do accepted reference values or results from an established procedure exist for validation of accuracy?
  - If not, as stated in International Council for Harmonisation (ICH) Q2, accuracy may be inferred once precision, linearity, and specificity have been established.
- How many individual determinations will compose the reportable value, and how will they be aggregated?
  - To answer this question, it is necessary to understand the contributors to the procedure variance and the ultimate purpose of the procedure. Estimation of variance components during pre-validation provides useful information for making this decision.
- What are appropriate validation acceptance criteria?
  - The validation succeeds when there is statistical evidence that the assay is no worse than certain pre-specified levels for each relevant validation parameter.
  - What defines the assay as fit for use, and how does this relate to acceptance criteria?
- How large a validation experiment is necessary?
  - Validation experiments should be properly powered to ensure that there are sufficient data to conclude that the accuracy and precision can meet pre-specified acceptance criteria. Computer simulation is a useful tool for performing power calculations.
  - Efficiencies (both cost and statistical) can be gained if assessment of linearity, accuracy, and precision can be combined.

On the basis of the answers to these and similar questions, one can design a suitable validation experimental protocol.

## 3. ACCURACY AND PRECISION

A useful model for representing a reportable value is:

$$Y = \tau + \beta + E \quad (1)$$

$Y$ = a reportable value
$\tau$ = true or accepted reference value
$\beta$ = systematic bias of the procedure

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$E$ = random measurement error

Both τ (tau) and β (beta) are fixed statistical parameters, and $E$ is a normal random variable with a mean of zero and standard deviation σ (sigma). The magnitude of σ depends on the number of individual readings averaged to obtain the reportable value.

Accuracy of an analytical procedure expresses the closeness of agreement between τ and $Y$. Closeness is expressed as the long-run average of $(Y - \tau)$. This long-run average is called the systematic bias and is represented with β. To estimate β, it is necessary to know the true value, τ. Chapter ⟨1225⟩ notes that a reference standard or a well-characterized orthogonal procedure can be used to assign the value of τ. Accuracy should be established across the required range of the procedure.

Precision of an analytical procedure is the degree of agreement among reportable values when the procedure is applied repeatedly (possibly under different conditions) to multiple test portions of a given analytical sample. The most common precision metric is the standard deviation σ. This is denoted in *Equation 4* with the variable $S$. The term $\sigma^2$ is called the variance. Precision improves as σ decreases. Many commonly used statistical procedures rely on the assumption of the normal distribution, for which σ is a natural descriptor of variability.

***Change to read:***

## 3.1 Methods for Estimating Accuracy and Precision

An example is provided to demonstrate the test procedure for lot release using statistical analysis. This example uses high-performance liquid chromatography (HPLC). The measured drug substance (DS) is a *USP* compendial substance, so information concerning τ is available (*1*). Three different quantities of reference standard were weighted to correspond to three different percentages of the test concentrations: 50%, 100%, and 150%. The unit of measurement on each reportable value is the mass fraction of DS expressed in units of mg/g and does not change as the level of concentration varies. The value of τ is 1000 mg/g for all three concentrations. The computed statistics from the validation data set include the sample mean ($\overline{Y}$), the sample standard deviation ($S$), and the number of reportable values ($n$). *Table 3* presents the $n = 9$ reportable values and the computed statistics.

**Table 3. Reportable Values for Experiment**

| Test Concentration (%) | Test Solution | Reportable Value (mg/g) |
|---|---|---|
| 50 | 1 | 996.07 |
| 50 | 2 | 988.43 |
| 50 | 3 | 995.90 |
| 100 | 4 | 987.22 |
| 100 | 5 | 990.53 |
| 100 | 6 | 999.39 |
| 150 | 7 | 996.33 |
| 150 | 8 | 993.67 |
| 150 | 9 | 987.76 |
| Sample mean ($\overline{Y}$) | | 992.81 |
| Sample standard deviation ($S$) | | 4.44 |

Several assumptions are made for purposes of this example, which allows analysis of the combined data set in *Table 3*:

1. All $n = 9$ reportable values are independent.
2. The standard deviation of the reportable value is constant across all three concentration levels. If this condition is not met, data transformations may still allow combination of all the data in *Table 3* (pooling). If transformations are not successful, each concentration level must be validated for precision separately.
3. The average reportable value is equal across concentration levels. If this condition does not hold, it is necessary to employ an analysis of variance model and validate accuracy for each concentration level separately.

The point estimator for unknown bias β is:

$$\beta = \overline{Y} - \tau \quad (2)$$

$\beta$ = systematic bias
$\overline{Y}$ = sample mean
$\tau$ = true or accepted reference value

where

$$\overline{Y} = \frac{\sum_{i=1}^{n} Y_i}{n} \quad (3)$$

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$\overline{Y}$ = sample mean
$Y_i$ = individual values
$n$ = number of reportable values

The point estimator for the unknown value of σ is

$$S = \sqrt{\frac{\sum_{i=1}^{n}\left(Y_i - \overline{Y}\right)^2}{n-1}} \quad (4)$$

$S$ = point estimator for the unknown value of σ
$Y_i$ = individual values
$\overline{Y}$ = sample mean
$n$ = number of reportable values

Because point estimators have uncertainty associated with them, best practice requires calculation of a statistical confidence interval to quantify the uncertainty. Statistical confidence intervals provide a range of plausible values for β and σ for a given level of confidence. A 100(1 – 2α)% two-sided confidence interval for the bias β is

$$\left(\overline{Y} - \tau\right) \pm t_{1-\alpha:n-1} \times \frac{S}{\sqrt{n}} \quad (5)$$

β = 100(1 – 2α)% two-sided confidence interval of bias
$t_{1-\alpha:n-1}$ = percentile of central $t$-distribution with area 1 – α to the left and ($n$ – 1) degrees of freedom
$S$ = result found from *Equation 4*
$n$ = number of reportable values

For example, with α = 0.05 and $n$ = 9, $t_{0.95:8}$ = 1.860 provides a 100(1 – 2 × ▲0.05▲ (ERR 1-Mar-2018))% = 90% two-sided confidence interval for β. Using the example data in *Table 3* with τ = 1000 mg/g, the 90% confidence interval on β is

$$\left(\overline{Y} - \tau\right) \pm t_{1-\alpha:n-1} \times \frac{S}{\sqrt{n}}$$
$$\left(992.81 - 1000\right) \pm 1.86 \times \frac{4.44}{\sqrt{9}} \quad (6)$$
$$\left[-9.94 \text{ to } -4.44\right] \text{mg/g}$$

β = 100(1 – 2α)% two-sided confidence interval of bias
$t_{1-\alpha:n-1}$ = percentile of central $t$-distribution with area 1 – α to the left and ($n$ – 1) degrees of freedom
$S$ = result found from *Equation 4*

For the standard deviation, one is concerned with only the 100(1 – α)% upper confidence bound since typically, it needs to be shown that the standard deviation is not too large. An upper 100(1 – α)% confidence bound for σ is

$$U = S\sqrt{\frac{n-1}{\chi^2_{\alpha:n-1}}} \quad (7)$$

$U$ = an upper 100(1 – α)% confidence bound for σ
$S$ = result found from *Equation 4*
$n$ = number of reportable values
$\chi^2_{\alpha:n-1}$ = a percentile of a central chi-squared distribution with area α to the left and ($n$ – 1) degrees of freedom

For example, if α = 0.05 and $n$ = 9, then $\chi^2_{0.05:8}$ = 2.73. Using the data in *Table 3*,

$$U = S\sqrt{\frac{n-1}{\chi^2_{\alpha:n-1}}}$$
$$U = 4.44\sqrt{\frac{9-1}{2.73}} = 7.60 \text{ mg/g} \quad (8)$$

The confidence intervals in *Equations 5* and *7* can be used to perform statistical tests against criteria included in the validation protocol. Use of point estimates only does not provide the required scientific rigor. In particular, the two-sided confidence interval in *Equation 5* can be used to perform a two one-sided test (TOST) of statistical equivalence (*2*). Assume in the present example that the accuracy requirement is validated if evidence demonstrates that the absolute value of β is NMT 15 mg/g. Since the computed confidence interval from –9.94 to –4.44 mg/g falls entirely within the range from –15 to +15 mg/g, the bias criterion is satisfied. Most typically, the TOST employs a type I error rate of α = 0.05. This error rate represents the maximum risk of declaring that the acceptance criterion is satisfied, when in truth it is not satisfied. Thus, with α = 0.05, the two-sided confidence interval in *Equation 5* is 100(1 – 2α)% = 90%.

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The upper bound in *Equation 7* is used to validate precision. Suppose the pre-defined acceptance criterion for precision requires σ to be <20 mg/g. The computed upper bound of 7.60 mg/g in *Equation 8* represents the largest value we expect for σ with 95% confidence. Since 7.60 mg/g is <20 mg/g, precision has been successfully validated with a confidence of 95%.

***Change to read:***

## 3.2 Combined Validation of Accuracy and Precision

When assessing whether an analytical procedure is fit for its intended purpose, it is often useful to consider the combined impact of bias and precision. The degree to which β impacts the usefulness of an analytical procedure depends in part on σ. That is, a procedure with a relatively small value of σ can accommodate a relatively greater value of β than a procedure with a greater value of σ. For this reason, it is useful to establish a single criterion that can be used to simultaneously validate both accuracy and precision. One such criterion is proposed in a series of articles by Hubert et al. (*3*–*5*) and seeks to ensure that

$$Pr(-\lambda < Y - \tau < \lambda) \geq P, \text{ or}$$

$$Pr(-\lambda + \tau < Y < \lambda + \tau) \geq P \quad (9)$$

$Pr$ = reference probability
$\lambda$ = acceptable limit
$Y$ = a reportable value
$\tau$ = true or accepted reference value
$P$ = desired probability value

*Equation 9* has a dual interpretation. It can be interpreted as either (i) the probability that the next reportable value falls in the range from (–λ + τ) to (λ + τ) is ≥*P*, or (ii) the proportion of all future reportable values falling between (–λ + τ) and (λ + τ) is ≥*P*. Accordingly, two statistical intervals have been proposed to demonstrate that *Equation 9* is true:

1. A prediction interval (also referred to as an expectation tolerance interval) is used to demonstrate (i).
2. A tolerance interval (also referred to as a content tolerance interval) is used to demonstrate (ii).

Hahn and Meeker (*6*) note that the prediction interval is also referred to as an expectation tolerance interval and that the tolerance interval is also referred to as a content tolerance interval. Because the inference associated with the tolerance interval concerns a larger set of values, the tolerance interval is always wider than the prediction interval. Selection of an interval will depend on the desire to validate ▲▲ (ERR 1-May-2018) either (i) or (ii) and a company's risk profile.

Either interval can be used in the following manner to evaluate accuracy and precision simultaneously through *Equation 9*.

1. Compute the appropriate statistical interval using *Equation 10* for the prediction interval and *Equation 11* for the tolerance interval.
2. If the computed interval falls completely in the range from (–λ + τ) to (λ + τ), the criterion in *Equation 9* is satisfied, and the procedure is validated for both accuracy and precision.

The prediction interval used to validate *Equation 9* is

$$\overline{Y} \pm t_{(1+P)/2:n-1} \times S\sqrt{1+\frac{1}{n}} \quad (10)$$

$\overline{Y}$ = sample mean
$t_{(1+P)/2:n-1}$ = percentile of a central *t*-distribution with area (1 + *P*)/2 to the left and (*n* – 1) degrees of freedom
$S$ = result found from *Equation 4*

The 100(1 – α)% tolerance interval used to validate *Equation 9* is

$$K = \sqrt{\frac{Z^2_{(1+P)/2} \times (n-1)}{\chi^2_{\alpha:n-1}} \times \left(1+\frac{1}{n}\right)} \quad (11)$$

$$\overline{Y} \pm K \times S$$

$\overline{Y}$ = sample mean
$K$ = result found from *Equation 11*
$S$ = result found from *Equation 4*
$Z^2_{(1+P)/2}$ = ▲the square of the standard normal▲ (ERR 1-May-2018) percentile with area (1 + *P*)/2 to the left
$n$ = number of reportable values
$\chi^2_{\alpha:n-1}$ = a chi-squared percentile with area α to the left and (n – 1) degrees of freedom

The formula for *K* is based on an approximation by Howe (*7*), although exact tabled values can be found in several sources. The approximation works well in practical situations if exact values are not available.

For the data in *Table 3* with *P* = 0.90, the interval for *Equation 10* is computed as

$$\overline{Y} \pm t_{(1+P)/2,n-1} \times S\sqrt{1+\frac{1}{n}}$$

$$992.81 \pm 1.86 \times 4.44\sqrt{1+\frac{1}{9}} \quad (12)$$

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$$[984.1 \text{ to } 1001.5] \text{ mg/g}$$

The interval for *Equation 11* with 1 – α = 0.90 and *P* = 0.90 is

$$K = \sqrt{\frac{Z^2_{(1+P)/2} \times \left(n-1\right)}{\chi^2_{\alpha:n-1}} \times \left(1+\frac{1}{n}\right)}$$

$$K = \sqrt{\frac{(1.64)^2 \times \left(9-1\right)}{3.49} \times \left(1+\frac{1}{9}\right)} = 2.63$$

$$\overline{Y} \pm K \times S$$

$$992.81 \pm 2.63 \times 4.44$$

$$[981.2 \text{ to } 1004.5] \text{ mg/g} \quad (13)$$

The exact value for *K* is 2.637, and the approximation is seen to work quite well. As predicted earlier, the interval for *Equation 13* is wider than the interval for *Equation 12*.

Suppose the criterion for *Equation 9* is designed to ensure that the difference between *Y* and τ is <2% of τ with a probability NLT *P* = 0.90. Thus,

$$-\lambda + \tau = \tau(-0.02 + 1) = 1000(0.98) = 980 \text{mg/g}$$
$$\tau + \lambda = \tau(1 + 0.02) = 1000(1.02) = 1020 \text{mg/g} \quad (14)$$

Since both *Equations 12* and *13* fall in the range from 980 to 1020 mg/g, the procedure is validated using either interval.

It is also possible to estimate *Pr*(–λ < *Y* – τ < λ) in *Equation 9* directly using either the confidence interval described by Mee (*8*) or a Bayesian approach. The validation criterion is thus satisfied if this estimated probability exceeds *P*. A Bayesian tolerance interval is provided in Wolfinger (*9*) and can be computed using the statistical software package WinBUGS (*10,11*). Bayesian analyses can be challenging, and the aid of an experienced statistician is recommended.

## 4. LIMITS OF DETECTION AND QUANTITATION

The LOD and limit of quantitation (LOQ) are two related quantities determined in the validation of Category II procedures of ⟨1225⟩. These are procedures for the determination of impurities or degradation products in DS and finished pharmaceutical products. Only one is needed for each use: LOQ for quantitative tests and LOD for qualitative limit tests. These limits are also known under other names, including detection limit (DL) for LOD and lower limit of quantitation (LLOQ) for LOQ.

The following definitions are consistent with ⟨1225⟩ and ICH Q2:

- The LOD is the lowest amount of analyte in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions.
- The LOQ is the lowest amount of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions.

Candidate values for LOD or LOQ are examined during pre-validation or based on a risk assessment. The candidate values must then be verified. This is particularly important for LOQ, since the formulas for determining candidate values do not address the acceptable accuracy and precision requirement. Verification of the candidate values is performed as part of the validation protocol.

### 4.1 Estimation of LOD

The basic approach to estimating LOD is based on an alternative definition adopted by the International Union of Pure and Applied Chemistry (IUPAC) and the International Organization for Standardization (ISO). This definition introduces the notion of false-positive and false-negative decisions, thus recognizing the risk elements in using the LOD for decision making, and the definition makes clear that these values are dependent on laboratory capability.

The IUPAC/ISO definition of LOD is based on the underlying concept of a critical value ($R_C$), defined as the signal readout exceeded with probability α when no analyte is present. That is,

$$R_C = B + Z_{1-\alpha}\sigma_E \quad (15)$$

$R_C$ = signal readout exceeded with probability α when no analyte is present
$B$ = estimated mean readout for blanks
$Z_{1-\alpha}$ = a standard normal quantile with area 1 – α to the left
$\sigma_E$ = true repeatability deviation

*Figure 1* presents this relationship graphically.

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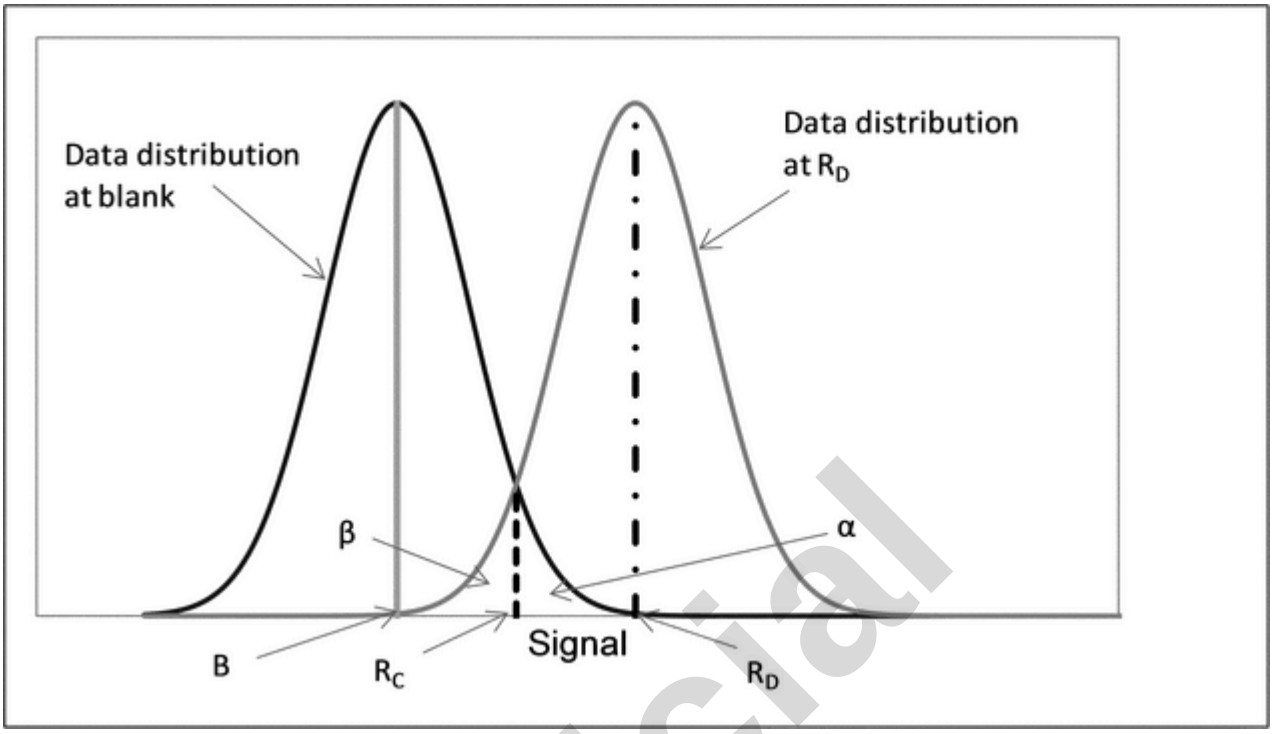


Figure 1. Determination of $R_C$ and $R_D$.

For example, if $\alpha = 0.05$, $1 - \alpha = 0.95$ and $Z_{0.95} = 1.645$. This determination depends on the distribution of values obtained when analyzing blanks. The LOD in the signal space ($R_D$) is defined as that value, which if true, is such that $R_C$ is exceeded with probability $1 - \beta$. That is,

$$R_D = R_C + Z_{1-\beta}\sigma_E \quad (16)$$

$R_D$ = LOD in the signal space
$R_C$ = critical value using IUPAC/ISO definition of LOD
$Z_{1-\beta}$ = standard normal quantile with area $1 - \beta$ to the left
$\sigma_E$ = true repeatability deviation

Solving *Equations 15* and *16* for $R_D$, we have

$$R_D = B + \left(Z_{1-\alpha} + Z_{1-\beta}\right)\sigma_E \quad (17)$$

$R_D$ = LOD in the signal space
$B$ = estimated mean readout for blanks
$Z_{1-\alpha}$ = a standard normal quantile with area $1 - \alpha$ to the left
$Z_{1-\beta}$ = a standard normal quantile with area $1 - \beta$ to the left
$\sigma_E$ = true repeatability deviation

Note that this definition allows for two values to be selected by the laboratory: α and β (which need not be equal). The α represents the type I or false-positive error rate, and β represents the type II or false-negative error rate. In *Figure 1*, $R_C$ and $R_D$ are illustrated with $\alpha = \beta = 0.05$ for normally distributed data so that $Z_{1-\alpha} = Z_{1-\beta} = 1.645$. Although the values of α and β need not be equal, this choice leads to a common rule for $R_D$, namely $B + 3.3\sigma_E$ ($3.3 \cong 2 \times 1.645$).

The LOD on the concentration scale is then found by converting the value in the signal scale, $R_D$, to one in the concentration scale, LOD, as shown in *Figure 2*.

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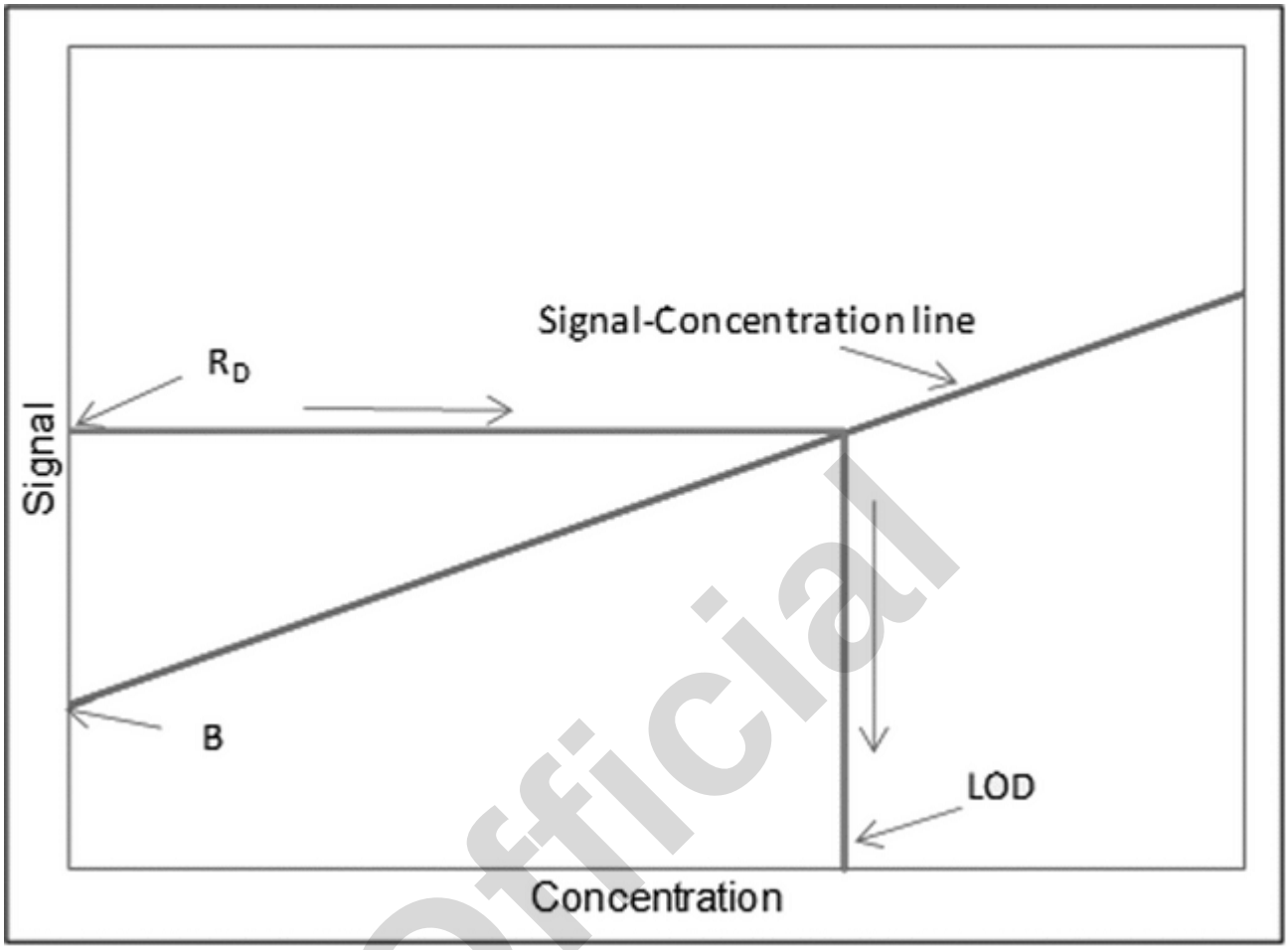


Figure 2. Determination of LOD from $R_D$.

This step requires that the signal ($R$) versus concentration ($X$) line, $R = B + mX$, as well as $\sigma_E$, be known exactly. The formulation provided in this section assumes the regression measurements are independent.

The LOD on the concentration scale is then calculated as

$$\text{LOD} = \frac{R_D - B}{m} = \frac{\left(Z_{1-\alpha} + Z_{1-\beta}\right)\delta_E}{m} \quad (18)$$

LOD = limit of detection
$R_D$ = LOD in the signal space
$B$ = estimated mean readout for blanks
$Z_{1-\alpha}$ = standard normal quantile with area $1 - \alpha$ to the left
$Z_{1-\beta}$ = standard normal quantile with area $1 - \beta$ to the left
$\sigma_E$ = true repeatability deviation
$m$ = slope

As a statistical procedure, the LOD definition in *Equation 18* is unsatisfactory for two reasons. First, since $\sigma_E$ is generally unknown, it must be determined how to best estimate this parameter. This is complicated because $\sigma_E$ is typically concentration dependent. Two common estimates are (i) the standard deviation of the blank responses and (ii) the standard deviation obtained from deviations about the regression line of signal on concentration. The choice needs to be the value that best represents $\sigma_E$ in the neighborhood of the LOD. Laboratories will often pick a worst-case value for $\sigma_E$. If the LOD is still suitable for its intended use, the laboratories are protected against understating the LOD. Understatement of the LOD results in an inflated type II error rate ($\beta$) and a deflated type I error rate ($\alpha$).

The second statistical concern with *Equation 18* is how to incorporate uncertainty due to the fact that the exact slope of the regression line of signal on concentration is unknown. Because the regression line is estimated, the definition of $R_D$ in *Equation 17* is itself an estimate. This is corrected by using a statistical prediction interval that takes into account the uncertainty in the estimated line as well as the variability associated with a future observation. The expanded formula for the critical value, $R_C$, originally defined in *Equation 15* that accounts for this uncertainty is

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$$R_C = B + t_{1-\alpha:n-2} \times S \sqrt{1 + \frac{1}{n} + \frac{\overline{X}^2}{\sum_{i=1}^{n} \left(X_i - \overline{X}\right)^2}}$$

$$S = \sqrt{\frac{\sum_{i=1}^{n} \left(R_i - B - mX_i\right)^2}{n - 2}} \quad (19)$$

$R_C$ = critical value using the IUPAC/ISO definition of LOD
$B$ = intercept of the fitted calibration line
$t_{1-\alpha:n-2}$ = percentile of a central $t$-distribution with area 1 – α to the left and ($n$ – 2) degrees of freedom
$S$ = standard error of regression line
$\overline{X}$ = average concentration
$n$ = number of observations used in the regression analysis
$X_i$ = concentration value used in determining the line
$R_i$ = reference interval
$m$ = slope

*Equation 19* differs from *Equation 15* because the *t*-distribution is used instead of the normal distribution for the multiplier, and two additional terms appear in the square root to capture the uncertainty of the regression line.

A second equation for $R_C$ answers the question, "Above which concentration can we be confident that we will obtain signals that are distinguishable from background?" This question is answered by using the lower 100(1 – β)% prediction bound of the calibration curve as shown in *Figure 3*.

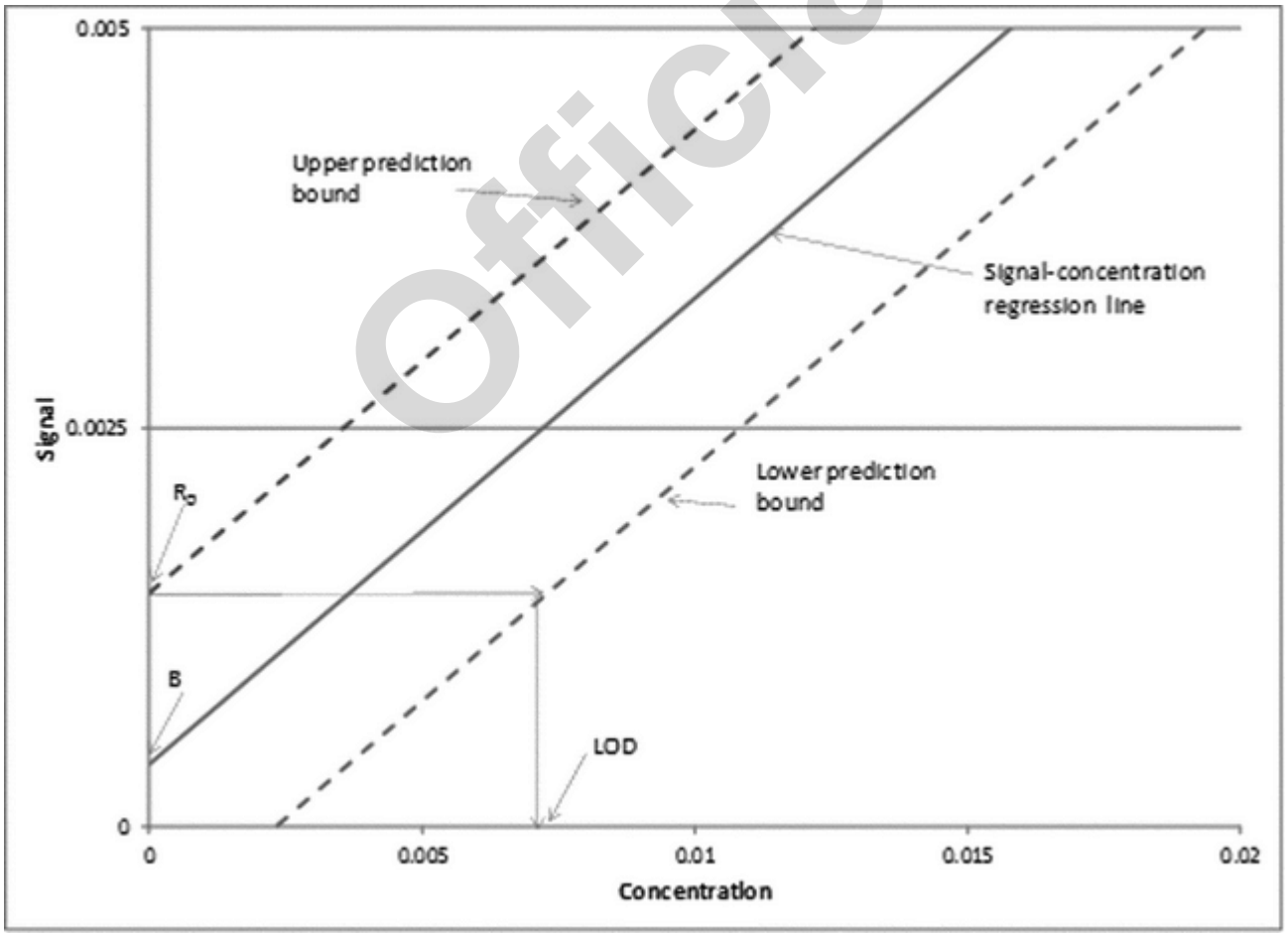


Figure 3. Determination of LOD using prediction bounds.

*Figure 3* is similar to *Figure 2*, but uses two dashed curves instead of the solid calibration line. Here

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$$R_C = B + LOD \times m - t_{1-\beta:n-2} \times S\sqrt{1 + \frac{1}{n} + \frac{(LOD - \overline{X})^2}{\sum_{i=1}^{n}\left(X_i - \overline{X}\right)^2}} \quad (20)$$

$R_C$ = critical value using the IUPAC/ISO definition of LOD
$B$ = estimated intercept of the fitted calibration line
LOD = limit of detection
$m$ = slope
$t_{1-\beta:n-2}$ = percentile of a central $t$-distribution with area 1 – β to the left and ($n$ – 2) degrees of freedom
$S$ = standard error of regression line
$\overline{X}$ = average concentration value squared
$n$ = number of observations used in the regression analysis
$X_i$ = concentration value used in determining the line

After equating *Equation 19* and *Equation 20*, and cancelling the *B* terms,

$$t_{1-\alpha:n-2} \times S\sqrt{1 + \frac{1}{n} + \frac{\overline{X}^2}{\sum_{i=1}^{n}\left(X_i - \overline{X}\right)^2}} = LOD \times m - t_{1-\beta:n-2} \times S\sqrt{1 + \frac{1}{n} + \frac{(LOD - \overline{X})^2}{\sum_{i=1}^{n}\left(X_i - \overline{X}\right)^2}} \quad (21)$$

$t_{1-\alpha:n-2}$ = percentile of a central $t$-distribution with area 1 – α to the left and ($n$ – 2) degrees of freedom
$S$ = standard error of regression line
$\overline{X}^2$ = average concentration value squared
LOD = limit of detection
$m$ = slope
$t_{1-\beta:n-2}$ = percentile of a central $t$-distribution with area 1 – β to the left and ($n$ – 2) degrees of freedom
$n$ = number of observations used in the regression analysis
$X_i$ = concentration value used in determining the line
$\overline{X}$ = average concentration value

*Equation 21* is a quadratic equation for LOD that can be solved exactly or by using iterative search tools available in spreadsheets. A slightly conservative (overly large) approximation for LOD that does not require a quadratic solution is obtained by assuming that LOD is negligible compared to $\overline{X}$ [i.e., $(LOD - \overline{X})^2$ is replaced with $\overline{X}^2$]. The resulting equation under this simplification is

$$LOD = \left(t_{1-\alpha:n-2} + t_{1-\beta:n-2}\right) \times \frac{S}{m}\sqrt{1 + \frac{1}{n} + \frac{\overline{X}^2}{\sum_{i=1}^{n}\left(X_i - \overline{X}\right)^2}} \quad (22)$$

LOD = limit of detection
$t_{1-\alpha:n-2}$ = percentile of a central $t$-distribution with area 1 – α to the left and ($n$ – 2) degrees of freedom
$t_{1-\beta:n-2}$ = percentile of a central $t$-distribution with area 1 – β to the left and ($n$ – 2) degrees of freedom
$S$ = standard error of regression line
$\overline{X}$ = average concentration value
$m$ = slope
$n$ = number of observations used in the regression analysis
$X_i$ = concentration value used in determining the line

which is similar in form to *Equation 18*. *Equations 18* and *22* both allow the two error probabilities, α and β, to differ. Often they are both taken as equal to 0.05.

The data in *Table 4* are used to demonstrate calculation of the LOD.

**Table 4. Data for LOD Example**

| Concentration $X$ (mg/mL) | Area (signal) |
|---|---|
| 0.01 | 0.00331 |
| 0.02 | 0.00602 |
| 0.05 | 0.01547 |
| 0.10 | 0.03078 |
| 0.15 | 0.04576 |
| 0.25 | 0.07592 |

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Fitting the linear regression to these data yields the regression line:

$$\text{Area} = 0.000235 + 0.03032 \times \text{Concentration} \quad (23)$$

so that $m = 0.3032$ and $B = 0.000235$. Values needed to compute LOD shown in *Equation 22* with $\alpha = \beta = 0.05$ are provided in *Table 5*.

**Table 5. Statistics Needed to Compute LOD in Concentration Units**

| Statistic | Value |
|---|---|
| $n$ | 6 |
| $m$ (slope) | 0.3032 |
| $S$ | 0.00019 |
| $t_{1-\alpha:n-2} = t_{0.95:4}$ | 2.132 |
| $t_{1-\beta:n-2} = t_{0.95:4}$ | 2.132 |
| $\overline{X}$ | 0.0967 |
| $\sum_{i=1}^{n} \left(X_i - \overline{X}\right)^2$ | 0.0419 |

The value of LOD computed from *Equation 22* is

$$LOD = \left(t_{1-\alpha:n-2} + t_{1-\beta:n-2}\right) \times \frac{S}{m}\sqrt{1 + \frac{1}{n} + \frac{\overline{X}^2}{\sum_{i=1}^{n}\left(X_i - \overline{X}\right)^2}}$$

$$LOD = \left(2.132 + 2.132\right) \times \frac{0.00019}{0.3032}\sqrt{1 + \frac{1}{6} + \frac{0.0967^2}{0.0419}} \quad (24)$$

$$LOD = 0.0032 \text{ mg/mL}$$

LOD = limit of detection
$t_{1-\alpha:n-2}$ = percentile of a central $t$-distribution with area $1-\alpha$ to the left and $(n-2)$ degrees of freedom
$t_{1-\beta:n-2}$ = percentile of a central $t$-distribution with area $1-\beta$ to the left and $(n-2)$ degrees of freedom
$S$ = residual standard error found from *Equation 4*
$\overline{X}^2$ = average concentration squared
$m$ = slope
$n$ = number of observations used in the regression analysis
$X_i$ = concentration value used in determining the line

## 4.2 Estimation of LOQ

As previously discussed, the important consideration in determining the LOQ is the estimation of what LOQ is required based on the intended use. The validation is designed to validate accuracy and precision in the neighborhood of the required LOQ.

In the absence of such knowledge, or where the laboratory wants to determine how low the LOQ might be (e.g., for potential other uses), then the laboratory can start with potential LOQ values greater than but near the LOD. Alternatively, methods for determining the LOD can be adapted to the LOQ as candidate starting values. Essentially, the formula used to compute LOD in *Equation 22* can be used to compute LOQ by replacing $(t_{1-\alpha:n-2} + t_{1-\beta:n-2})$ with 10. Values other than 10 can be used if justified. Once candidate values are obtained (typically during pre-validation), accuracy and precision are validated at these values.

All of the methods presented in this section are based on two assumptions: linearity and homogeneity of variance across the range of concentrations used in determining the calibration curve. Neither is a necessary condition. The calibration curve may be nonlinear, and a weighted least squares approach can be used to account for a lack of homogeneity. If the curve is nonlinear or the concentration variances vary greatly in the range of the LOD and LOQ, it is best to seek expert statistical help in defining LOD and LOQ. If variability about a straight line exists but is not large, an unweighted regression of the calibration curve will provide an average variability that can be used in the LOD and LOQ formulas.

Procedures other than those described above, such as signal-to-noise ratios, can be used to estimate LOD and LOQ. In either case, analysts should consider these values as preliminary and proceed to verify them, particularly if they fall below the concentration values used in determining the calibration curve. Verification involves analyzing samples with concentrations near the preliminary LOD and LOQ. Consideration should be given to how low must an LOD and LOQ be such that the procedure is suitable. For example, if data are already available at a level below the required LOD and a signal was detectable at that lower value, then that lower value may be taken as a verified LOD. There is little value in further verification—given the current requirement. There could still be value in verification of a lower value in case the requirement changes.

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## 5. CONCLUDING REMARKS

This chapter presented some simple statistical methods that can be used in procedure validation as described in ⟨1225⟩. These methods may not be applied in all situations, and other statistical approaches, both more and less sophisticated, may be appropriate for any particular situation.

Re-evaluation of a procedure should be considered whenever use of the procedure changes. For example, if a new product strength is introduced, the procedure is transferred to a new lab, samples are to be tested following a new type of stress test, or specifications change, a re-validation is most likely appropriate. In some situations, a re-assessment of existing data to revised acceptance limits is sufficient.

Finally, although not part of procedure validation, it is recommended that some type of statistical process control be used to monitor the performance of the procedure. Such a process provides early warning of "drift" in the analytical performance characteristics of accuracy and precision. Such changes in performance are not uncommon, and often occur as a result of worn equipment, change of routines, or aging reagents.

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# ⟨1211⟩ ▲STERILITY ASSURANCE▲ (USP41)

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## ▲INTRODUCTION

This informational chapter provides general information on the concepts and principles involved in the preparation of materials that must be sterile. Within the strictest definition of sterility, an item is deemed sterile only when it contains no viable microorganisms. However, this textual definition cannot be applied to actual items labeled as sterile because of irresolvable limitations in testing. Sterility cannot be demonstrated without the destructive testing of every sterile unit. In a real sense, microbiological safety is achieved through the implementation of interrelated controls that in combination provide confidence that the items are suitable for use as labeled. It is the controls that provide the desired assurance from microbiological risk rather than the results of any in-process or finished goods testing. The verification of safety of products labeled sterile is generally known as "sterility assurance" and that nomenclature will be used throughout this chapter.

The establishment of an effective sterility assurance program requires information about the material to be sterilized. An initial determination should be made regarding the potential for terminal sterilization of the material in its primary container applying the principles defined in *Sterilization of Compendial Articles* ⟨1229⟩. As described, the appropriate process provides a balance between conditions that are lethal to potential bioburden present in/on the item and those that preserve its essential quality attributes. Depending upon the results of that determination, sterility of the item may be achieved by either aseptic processing or terminal sterilization. The potential for a process that relies on both the protective measures inherent to aseptic processing and the lethal nature of terminal sterilization may offer advantages. Regardless of the process selection decision, the establishment of design, operation, process controls, and monitoring systems is essential to provide the necessary confidence in the outcome.

The production of sterile products is subject to numerous factors that influence the outcome (see *Figure 1*). The identified factors in the image should be considered for their impact on sterility of the final product, although not all of the influencing elements are depicted.

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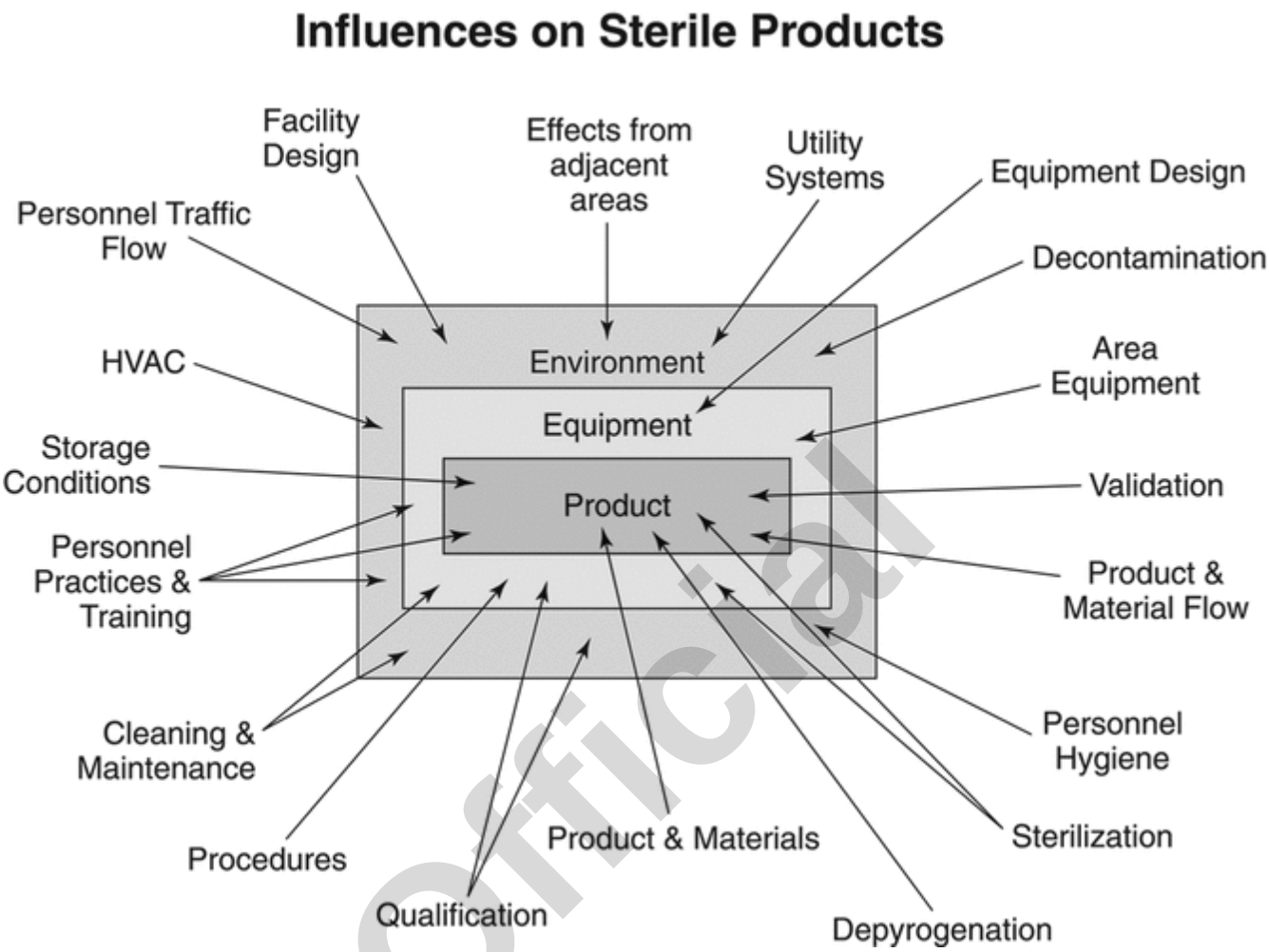


Figure 1. Influences on sterile products.

The decisions made relative to the influences shown in *Figure 1* will determine the success of the sterility assurance program. Poor choices, regardless of any successful process controls associated with them, must be acknowledged as fundamentally unsound. The process design objective is related to contamination controls intended to obviate risk of microbial ingress. This focus is appropriate regardless of whether the process used is aseptic processing or terminal sterilization.

Recognition that operating personnel are the most significant contributors of microbiological risk leads to design preferences and operating principles that should be adhered to with respect to sterile operations. This knowledge underscores the importance of separating personnel from the aseptic environment and limiting their interaction with sterilized components and product(s). The means for accomplishing these goals are embodied in two complementary practices (*1*):

- The use of automation technology—to reduce or eliminate personnel interventions and thus personnel-borne contamination
- The use of separative technologies—to eliminate, to the extent technically possible, human sourced contamination

Thus the implementation of appropriate contamination control procedures is paramount in design and operation of sterile product manufacturing systems.

Consideration of these principles adapts the Quality by Design (QbD) approach widely adopted in regulatory standards (*2*–*3*). Using QbD concepts in sterile operations is markedly different from the applications in the typical formulation, pharmaceutical, chemical, or biological synthesis process. The establishment of direct linkage between a monitored condition and process outcome with respect to sterile manufacturing is statistically difficult and analytically uncertain. The situation with respect to the definition of physical design elements is similar. Given the great variation in sterile product manufacturing with respect to scale, configuration, and complexity, it follows that the design alternatives and operating practices must also be flexible. Thus, the recommendations provided in this chapter are entirely non-numeric, because there are no ready means with which to demonstrate the suitability of specific values. Instead, QbD for sterile processing should be driven toward a singular goal of optimizing contamination control with a particular focus on the microbial risk impact of personnel. The specific means vary but should be of prime consideration in process design. *Figure 2* outlines the elements that contribute to sterility assurance, as described in the remaining sections of this chapter.

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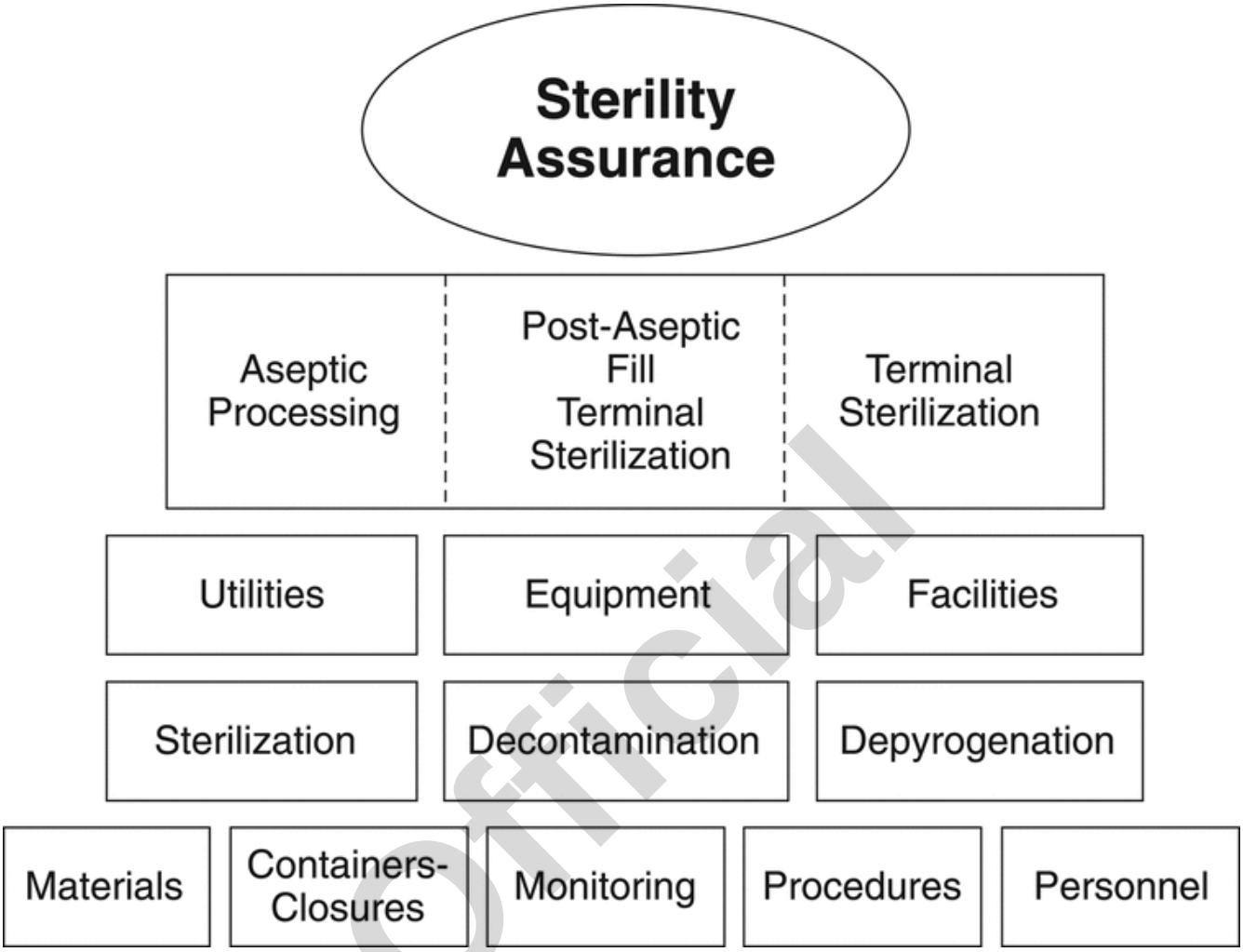


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Figure 2. Elements contributing to sterility assurance.

## Aseptic Processing

There are a substantial number of sterile products that cannot be terminally sterilized because of adverse impact on the product/package's essential properties and must be prepared by aseptic processing. Aseptic processes are designed to prevent the introduction of viable microorganisms into/onto separately sterilized materials during their assembly into a sealed sterile package. Aseptic processes can vary in complexity from comparatively simple filling/sealing to challenging and lengthy manufacturing sequences required for complex items. Regardless of process scale, all of the individually sterilized materials must be protected from contamination from the point of sterilization through closure of the primary package. This is accomplished through adherence to the principles described below in which an International Organization for Standardization (ISO) 5 condition is maintained when materials are exposed to the environment (see *Microbiological Control and Monitoring of Aseptic Processing Environments* ⟨1116⟩). Exclusive to aseptic processing is the execution of process simulations that support batch or campaign duration (*4*–*6*).

## Terminal Sterilization

Terminally sterilized products are the lowest risk category of sterile pharmaceutical products. Unlike products aseptically manufactured under conditions designed to prevent microbial ingress, terminally sterilized products are subjected to a sterilization process that imparts a quantifiable safety level. Terminal sterilization processes achieve this by delivering measurable physical conditions that correspond to microbial lethality. For terminally sterilized products, sterility assurance is defined in terms of the probability of nonsterility (PNS), or the probability of the terminal sterilization process generating a nonsterile unit (PNSU). Terminal sterilization processes must achieve a consistent validated performance of a PNSU of $\leq 10^{-6}$ (a probability of NMT 1 nonsterile unit in 1 million units produced) (see ⟨1229⟩). The convention by which terminal sterilization cycles are developed and validated ensures that the actual PNSU is typically much lower (better) than the minimum standard of $<10^{-6}$.

Chapter ⟨1229⟩ summarizes the common requirements for sterilization process design, development, validation, and process control. Terminal sterilization processes share common requirements of well-defined process parameters strictly controlled within defined operating limits. Terminal sterilization must be supported by a system of product disposition, which includes

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the assessment of critical physical process parameters, presterilization product parameters (e.g., bioburden, container–closure integrity), and environmental parameters. Terminal sterilization can rely on parametric release practices to obviate the need for sterility testing (see Terminally Sterilized Pharmaceutical Products—Parametric Release ⟨1222⟩).

## Post-Aseptic Processing Terminal Sterilization

An aseptic process followed by a terminal sterilization process provides superior control over the presterilization bioburden, such that the subsequent sterilization process can be designed with less overall lethality, thereby making it possible to substantially extend the use of terminal sterilization to products with greater sensitivity to the applied energy of the process. From a patient safety perspective, this approach has the following distinct advantages:

- An adventitious contaminant introduced during aseptic processing is easily killed by the terminal sterilization step, reducing the extent of in-process environmental monitoring performed
- Bioburden controls for the terminal process are simplified because all units have been aseptically filled
- Where a product is made using either process alone, the limitations of each (no terminal lethal component in aseptic, more degradation in terminal sterilization) would persist
- Where bioburden is controlled through aseptic processing, terminal sterilization can be applicable at lower lethality levels

Classical $F_0$, time-temperature, and radiation dose (kGy) targets for sterilization processes are arbitrarily selected and intended to simplify process validation, but in reality serve to reduce the use of terminal treatments. Physical lethality data based on fixed numerical values are inherently conservative and disregard the degradative impact of the sterilization process on the product. The focus must be on the ability of the process to kill bioburden organisms rather than biological indicators (see ⟨1229⟩ and *Moist Heat Sterilization of Aqueous Liquids* ⟨1229.2⟩).

While product quality attributes can be impaired by "standard" sterilizing conditions, the combined process can utilize less aggressive sterilizing conditions to minimize adverse effects upon the product and primary packaging materials. If the terminal treatment follows aseptic processing, then the sterilizing conditions need not be excessive as there is essentially no risk from presterilization bioburden in the filled containers. Sterilization process conditions would be dictated by the specifics of the product in parallel with the establishment of appropriate controls on presterilization bioburden derived from environmental and prefiltration isolates.

## Containers and Closures

The container and closure for a sterile formulation are integral parts of the sterile product. The container materials provide essential protection to the product throughout its shelf life and are chosen to minimize interaction with, preserve the quality attributes of, and facilitate dispensing of the sterile product. The container materials should be readily sterilizable, either separately prior to filling and/or together with the formulation in a terminal process. Containers and closures should be selected for:

- Reliability of container–closure integrity over the shelf life
- Absence of interaction with formulation materials
- Ease of handling in the processing environment and during administration
- Tolerance of variation in equipment and other components
- Cleanliness including freedom from particulates and absence of leachable or extractable chemicals
- Compatibility with the product
- Control over endotoxin content (where appropriate)
- Protection of components prior to use (where appropriate)
- Compatibility with preparation, sterilization, and depyrogenation processes (where appropriate)

The essential aspects of container–closure materials for sterile products are subject to numerous requirements elsewhere within *USP–NF*. The reader must consider the content provided in *Injections and Implanted Drug Products* ⟨1⟩, *Bacterial Endotoxins Test* ⟨85⟩, *Visible Particulates in Injections* ⟨790⟩, *Package Integrity Evaluation—Sterile Products* ⟨1207⟩, *Depyrogenation* ⟨1228⟩, and ⟨1229⟩.

## Decontamination

Decontamination is a broadly defined term used to describe a variety of processes that reduce microbial populations without an expectation for total kill.[1] It is not a substitute for sterilization; a sterilization process should be used wherever possible. A variety of chemical agents and methods are used that vary depending upon the application. Decontamination is used for bioburden reduction of materials, equipment, and environments in support of sterile product manufacture:

- For materials and surfaces that cannot be sterilized
- For materials and surfaces that do not require sterilization

Decontamination processes are ordinarily separated into two major categories based upon their effectiveness against spore-forming microorganisms (see *Disinfectants and Antiseptics* ⟨1072⟩). Sporicidal treatments are used in critical applications such as isolator decontamination, air-lock/pass-throughs, etc. Their toxicity to personnel and sometimes corrosive chemistry may preclude their exclusive use for microbial control. Non-sporicidal agents have fewer safety and material impact concerns, and the occasional use of a sporicidal agent is required to control spore populations. Applications for decontamination are diverse; among the more common uses are:

[1] Sterilization is preferred over decontamination and should be utilized wherever possible, consistent with minimization of handling post-sterilization.

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**Decontamination of controlled environments and non-product contact surfaces**

- In conventional cleanrooms, including restricted access barrier systems (RABS), this is predominantly a manual process performed after cleaning of the room/production line
- Decontamination of items upon transition into an environment of higher classification
- Isolators commonly use an automated process
- Periodic decontamination of operator gloves during processing

**Decontamination of product contact surfaces**

- Large equipment (e.g., stopper bowls) can be manually sanitized on a frequent basis in addition to sterilization to avoid the extensive manipulation required for their installation post-sterilization[2]
- Re-decontamination of sterilized equipment after aseptic assembly or intervention
- Periodic decontamination of previously sterilized utensils prior to interventions

## Depyrogenation

The minimization of pyrogen content is a requirement for injectable products. During the production of sterile products, depyrogenation processes are used in a variety of ways to minimize pyrogenic contamination of surfaces, materials, and products. Details on depyrogenation processes are provided in ⟨1228⟩.

## Equipment

Equipment used for sterile product manufacturing varies in its impact on the manufacturing process and on product quality and should have several important characteristics. For example, the equipment should:

- Operate reliably and produce products of consistent quality
- Not adversely impact essential product quality attributes
- Be easily cleanable and sterilizable, as necessary
- Minimize human intervention during set-up and operation through such features as physical separation, automation, and robotics
- Be tolerant of variations in container–closure materials
- Designed to minimize product exposure to the background environment

The extent to which the equipment interacts with process materials and the product affects the level of impact. Process equipment can influence the quality of the finished product in a variety of ways, and this can occur prior to and after sterilization and depyrogenation.

### EQUIPMENT IN DIRECT CONTACT WITH COMPONENTS, CONTAINERS, CLOSURES, AND STERILE PRODUCTS

This equipment category includes those items in direct contact with the drug substance, drug product, raw materials, and primary packaging components, including, for example, mixing and storage vessels, piping systems and tubing, filters, filling pumps, lyophilizer shelves, and feed hoppers. Product contact surfaces of this equipment are designed and may require additional treatment to minimize adverse impact (microbial, particulate, and chemical) on the contacted materials. The procedures used for the cleaning and sterilization of direct contact surfaces, including dirty, clean, and sterile hold times, must be validated to ensure they do not adversely impact essential product quality attributes as well as to verify the effectiveness of the cleaning procedure and that no microbial recontamination/proliferation occurs during equipment storage. Direct contact utensils are subject to the same considerations. With appropriate consideration of materials' compatibility, single-use disposable equipment (supplied sterile when necessary) may be utilized.

### EQUIPMENT HAVING INDIRECT CONTACT WITH COMPONENTS, CONTAINERS, CLOSURES, AND STERILE PRODUCTS

Equipment having a significant impact on product quality, that does not contact components, primary packaging materials, and sterile products, includes the electro-mechanical elements (non-product contact) of filling machines, stoppering machines, and sterilizers. The performance of this equipment can change fill weight, particle size, moisture level, content uniformity, container–closure integrity, and other essential quality attributes. This equipment is ordinarily located near exposed product contact equipment surfaces. For example, a pre-assembled filling set (product contact equipment) may be installed on a filling machine (significant impact without product contact), which provides control over the fill volume or weight. The surfaces of this equipment must be compatible with the cleaning and microbial decontamination and/or sterilization agents employed.

### OTHER EQUIPMENT

Some equipment has only an indirect impact on product quality, for example, conveyors, turntables, balances, air samplers, and carts. The influence of this equipment is largely on the environments in which the product is made. The exposed surfaces of this equipment must be compatible with the cleaning and decontamination agents used.

[2] Sterilization out-of-place would be used on a less frequent basis.

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## Facilities

Sterile manufacturing operations are supported by administrative, laboratory, maintenance, and warehouse functions and other activities. The impact of these operations on the location and overall design of the sterile manufacturing area must be considered. Emphasis should be given in facility design to the flows of materials, components, personnel, equipment, and waste streams throughout the facility and to the orderly transition of items between environments of different classifications to prevent mix-up and avoid product contamination. Facility environmental and utility systems must be designed to minimize microbial, chemical, and particulate contamination. The facility design must be supported by practices and procedures such as cleaning and decontamination, gowning, and material transfer. The architectural details of the facility infrastructure must consider the means for cleaning and disinfection. Detailed design recommendations can be found in the International Society for Pharmaceutical Engineering's *Baseline Guide: Sterile Product Manufacturing Facilities* (*7*).

The core activities for sterile product manufacture are carried out in classified environments operating in conformance with the ISO 14644 series of standards (*8*). A pressure cascade descending from the more critical areas to less critical is commonplace. In general, the more protection that materials have from potential sources of contamination during holding or processing, the less impact the facility has on the process outcome. Human operators within ISO 14644 classified cleanrooms used in aseptic processing are the greatest risk to product safety; therefore, no single risk mitigation factor in aseptic processing is more important than minimizing risk emanating from gowned operators.

Early-stage container–closure and equipment washing and preparation are carried out in lower classification areas (ISO 7–8). Nonsterile formulation is typically carried out in ISO 6–7 environments. The production materials are introduced into the processing area where subsequent steps are performed. The bioburden level of the materials influences the detailed design of the facility and its controls. Nonsterile materials (e.g., formulation, containers, closures, equipment, and utensils) require subsequent sterilization and, where necessary, depyrogenation. Sterile materials are introduced through airlocks and pass-throughs. The facility design controls for the background, and processing environments should be chosen to preserve the intended microbial attributes of the in-process and finished materials.

*Table 1* provides some examples of formulation and filling environments.

**Table 1. Examples of Environments for Processing**

| Processing Technology | Background Environment | Processing Environment | Reference |
|---|---|---|---|
| Conventional cleanroom | ISO 5–7 | ISO 5 | FDA AP 2004 (*9*), EMA Annex (*10*) |
| Closed RABS | ISO 7 | ISO 5 | ISPE (*11*), PHSS (*12*) |
| Open RABS | ISO 5 | ISO 5 | ISPE (*11*), PHSS (*12*) |
| Closed isolators | CNC[a] | ISO 5 | FDA AP 2004 (*9*), PDA TR No. 34 (*13*), ISPE (*7*) |
| Open isolators | CNC-ISO 8 | ISO 5 | FDA AP 2004 (*9*), PIC/S (*14*), PDA TR No. 34 (*13*), ISPE (*7*) |
| Blow-fill-seal/form-fill-seal | ISO 5–7 | ISO 5 | FDA AP 2004 (*9*), Baseman (*15*) |
| Closed systems | CNC | Not applicable[b] | PDA TR No. 28 (*5*); Agalloco, Hussong, et al. (*16*) |
| Terminal sterilization | ISO 8 | ISO 5–7 | EMA Annex (*10*) |

[a] Controlled non-classified—a non-classified controlled environment with filtered air supply.
[b] As the process occurs with a closed system, there is no separate processing environment.

[NOTE—Table values represent the operational condition and are adapted from the reference documents.]

### CONVENTIONAL CLEANROOM

The critical activities are performed in ISO 5 environments supported by surrounding ISO 5–7 environments where gowned personnel are normally located. There may be only limited separation between gowned personnel and sterile materials and product contact surfaces. The critical activities are performed within a unidirectional airflow environment. Decontamination of the cleanroom is commonly performed by personnel.

### RESTRICTED ACCESS BARRIER SYSTEMS

The typical RABS provides ISO 5 unidirectional air within the barrier and is situated in a conventional ISO 5–7 cleanroom. RABS may be designed to allow for opening of barriers to enable human intervention, or they may be designed to operate closed with the same operational restrictions regarding operator access that applies to isolators (see below). Air overspill from within the barrier is designed to prevent the ingress of contamination. Operator manipulation of sterile items is achieved using glove ports, and material transfers are accomplished without opening the system. A RABS that is opened mid-process should be treated as a conventional cleanroom (see above). RABSs require decontamination prior to use. This may be accomplished either manually or using automated systems.

### ISOLATORS

Isolators provide complete separation between personnel and the enclosed ISO 5 processing environment. A defined pressure differential is maintained between the ISO 5 environment and the surrounding area. Air overspill provides an aerodynamic seal

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at points where the product exits the isolator into an external environment of lesser classification or no classification. The use of unidirectional air is not required in isolators. Isolators are commonly decontaminated using automated systems.

#### BLOW-FILL-SEAL AND FORM-FILL-SEAL

These technologies form, fill, and seal flexible walled containers in an ISO 5 environment. Blow-fill-seal (BFS) and form-fill-seal (FFS) equipment configurations allow for installation in a variety of background environments. The critical activities are performed within a unidirectional airflow environment. Decontamination is performed as is common for the background environment.

#### CLOSED SYSTEMS

These systems provide for complete separation of production materials from personnel and surrounding environment. Closed systems can be single- or multiple-use vessels/chambers with means for materials ingress/egress. The designs avoid any human interaction or environmental contact with sterile materials. These systems vary in complexity and are sterilized either in situ or prior to use.

#### TERMINAL STERILIZATION

Filling systems and environments for containers to be terminally sterilized can be accomplished in ISO 7 or better environments. The critical activities are performed within a unidirectional airflow environment. Decontamination of the cleanroom is commonly performed by personnel.

## Materials (Active Pharmaceutical Ingredients, Excipients, and Process Aids)

The preparation of sterile products encompass a wide range of materials including active pharmaceutical ingredients (small and large molecules), excipients, solvents (usually water), process gases and processing aids, all of which contribute to the microbiological quality attributes of the product. Depending upon the product being manufactured, this can require consideration of bacterial, endotoxin, and particulate contamination. Specific microbiological quality testing requirements for inactive and active ingredients testing is often specified in a relevant *USP–NF* monograph. Requirements for microbiological testing for total aerobic bacteria, yeast and mold counts, and specified organisms are given in *Microbial Enumeration Tests* ⟨61⟩ and *Tests For Specified Microorganisms* ⟨62⟩, and the recommended but non-mandatory enumeration targets for microbiological testing are given in *Microbiological Examination of Nonsterile Products: Acceptance Criteria for Pharmaceutical Preparations and Substances for Pharmaceutical Use* ⟨1111⟩. Some specific products may require other testing for specified organisms, including viruses, as a requirement of their regulatory approval. Requirements for bacterial endotoxin testing in injectable products are covered in ⟨85⟩, which describes qualification of *Limulus* amebocyte lysate testing for acceptable levels of bacterial endotoxin. Raw material specifications must be appropriate to ensure that the manufacturing process consistently results, as demonstrated through process validation, in products conforming to the microbiological critical quality attributes.

Microbial contamination may be present on/in active pharmaceutical ingredients, excipients, and primary packaging materials. Controlling bioburden in materials and formulated product is a critical aspect of sterility assurance. The nature of the active pharmaceutical ingredient, the excipients, and the compounded product intended to be sterile are critical elements of the product knowledge. Recognizing the potential impact on critical quality attributes, the microbiological attributes of materials should be controlled through adequate supplier controls, shipping, receipt, sampling, handling, and storage. These controls should be commensurate with the microbiological risks to process controls and sterile product safety. Laboratory results should not be utilized to rationalize inadequate process controls on the part of the supplier.

The potential impact of the materials on the microbiological critical quality attributes of presterilized and finished product must be assessed by evaluating them with appropriate compendial assays or validated alternatives. Risk assessment should consider the origin of the material (e.g., fermentation, chemical synthesis, biologically derived, enzymatic, semi-synthetic synthesis, natural origin). Materials of biological origin may have higher inherent microbiological risk than materials derived solely from chemical synthesis. Materials may have inherent physicochemical properties that mitigate microbiological risks (e.g., low water activity, extreme pH, inherent antimicrobial properties) or increase microbiological risks (e.g., aqueous solution, growth supportive nutrients); this risk of supporting microbial proliferation must be assessed.

## Monitoring

Environmental monitoring is employed to qualitatively assess the effectiveness of the design and operational controls to provide suitable facility hygiene. It is neither a substitute for good facility, equipment, and process design, nor compensation for deficient practices and behavior. There are inherent limitations with all forms of viable and non-viable monitoring in terms of sample size, sample location, and recovery capability that preclude their use as anything more than an indication that a facility is operating within an acceptable state of control. Monitoring provides only a snapshot in time of the actual environmental conditions and excessive sampling due to its potentially intrusive nature can actually impair product safety or generate counts unrelated to process performance by increasing activity proximate to the critical zone.

Independent of air, surface, and personnel monitoring, media fills and sterility testing provide additional (albeit analytically and statistically limited) means to evaluate the robustness of the cleanroom design, performance, and effectiveness of cleaning/decontamination procedures, personnel gowning integrity, and aseptic practices.

Environmental control can be measured only by the monitoring performed. Satisfactory monitoring performance is the result of proper design and operation as described in this chapter and not a means to establish that condition. Performance criteria are established according to the classification of the room and its usage (see ⟨1116⟩).

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### VIABLE MONITORING

Viable monitoring consists of detecting and estimating the level of culturable microorganisms in the air, on surfaces, and on personnel. Sampling locations are defined following a risk assessment and sampling is executed by trained operators using a variety of methods including:

- Active air sampling
- Passive air sampling
- Viable particle counting using fluorescence technology
- Contact-plate sampling of surfaces, gloves, and gowns
- Swabbing of surfaces
- Personnel monitoring

### NON-VIABLE MONITORING

Non-viable monitoring measures the number and size of particulates present in the air. It can be used to initially classify the cleanroom in accordance with ISO 14644-1 and to assess routine manufacturing conditions (*8*). When used for the purposes of monitoring, it can be performed under static conditions (no activity) and/or dynamic conditions (routine operation). Non-viable particle monitoring is performed using calibrated particle counters.

### MEDIA FILLS/ASEPTIC PROCESS SIMULATIONS

Process simulations are exercises in which the performance of an aseptic activity is evaluated using a sterile growth medium. The medium can be directly substituted for the product or added to it. Aseptic process simulations are typically performed before the introduction of new or revised process components (e.g., products, facilities, equipment, personnel, containers and closures, and processes) and periodically thereafter (*17*). Process simulations should be fully representative of processing conditions and activities utilized during routine production.

### STERILITY TESTING

The sterility test is a harmonized compendial test. It must be understood that while execution of the test is required for the release of sterile products where parametric release has not been approved, it cannot prove the sterility of the materials tested. It should be recognized that parametric release is the default mode of sterile product release.

## Personnel

Personnel play an essential role in the preparation of sterile products. The essential activities they perform include cleaning, assembly, equipment operation, material transfer, environmental monitoring, and decontamination. While personnel are often necessary for the performance of these activities, the contamination derived from them must be prevented from entering the production materials before and after sterilization. The importance of the controls necessary to minimize exposure to and the release of human microbial contaminants in a sterile product manufacturing environment cannot be overstated.

The personnel involved in the preparation of sterile products must:

- Understand the principles of microbiology, sterilization/depyrogenation, aseptic processing, and contamination control
- Be proficient and diligent in gowning practices. Personnel required to wear aseptic gowning should periodically demonstrate their ability to properly gown
- Adhere to proper aseptic technique during all aseptic activities even when these are performed in a RABS or isolator. Periodic demonstration of these skills can be beneficial
- Be familiar with and adhere to standard operating procedures
- Practice good personal hygiene to minimize contamination potential
- Be trained in the proper and safe operation of necessary equipment
- Be monitored microbiologically after performing aseptic operations

## Procedures

Written procedures define the operations that have been determined through validation studies and experience to be effective in controlling and facilitating the manufacture and quality of pharmaceuticals and biopharmaceuticals. Procedures are especially important for the critical processes designed to assure the sterility of terminally sterilized and aseptically produced drug products. Procedures should be periodically reviewed and evaluated to ensure they are effective and current.

### INTERVENTIONS AND INTERVENTION PROCEDURES

There are two types of interventions associated with the aseptic production of sterile drug products. Inherent interventions are those activities that are an integral part of the aseptic process and are performed during the production of every batch. They include set-up, replenishment of components, weight and volume checks and adjustments, and environmental monitoring. Corrective interventions are those activities that correct problems and might not be performed during the production of every batch. They can be minimized and should be avoided through careful process design. Examples include stopper jams, broken and fallen glass, defective container seals, liquid leaks, and mechanical failures requiring manual correction.

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Each intervention, whether inherent or corrective, should be covered by written procedures sufficiently detailed to enable personnel to perform the intervention correctly, and to perform the intervention the same way each time regardless of whomever performs it. For example, procedures should specify the number of units, their locations, and how the units are to be removed, and personnel must be trained so they can correctly execute the procedures. No intervention should be permitted for which there is not a defined procedure. The following concepts should be considered during development, review, and implementation of intervention procedures.

1. Interventions performed during all forms of processing must be recognized as increasing the risk of contamination dissemination and are to be avoided or designed out of the process to the extent possible
2. Procedures for interventions should be critically reviewed to eliminate and/or simplify aseptic processes by reducing the frequency of inherent interventions and making all interventions easier to perform
3. Interventions should be designed for minimal risk of contaminating sterile and nonsterile materials
4. All interventions should be performed using sterilized tools whenever possible
5. Intervention procedures should be established in detail for all inherent interventions, and more broadly for corrective interventions (where some flexibility is necessary due to greater diversity)
6. Interventions should be incorporated in periodic media fills to evaluate the aseptic practices of the operators

Operators should initially, and periodically thereafter, be trained in all of the procedures they are expected to perform. Considerations for operator activity during the non-aseptic filling of containers should parallel those described above to minimize the potential for contamination ingress with somewhat less rigor than those needed for aseptic operations.

## Sterilization

The most effective means for the control of microbial population is sterilization, a process that either kills or removes viable microorganisms. In the production of sterile products, sterilization processes are used to prevent microbial contamination. Terminal sterilization processes that reproducibly destroy microorganisms in the final product container are the preferred means for the production of sterile products. Sterile products that cannot be terminally sterilized rely on individual sterilization processes (e.g., steam, radiation gas, filtration) for the various materials that comprise an aseptically processed sterile product. In addition, sterilization processes are used for product contact and other non-product contact items used in a variety of applications during the preparation of sterile products to provide absolute control of bioburden. Details on sterilization processes are provided in ⟨1229⟩.

## Utilities

The manufacture of sterile products requires utilities that can have a substantial impact on the final product. Some of the utilities in the facility can become an integral part of the formulated product (e.g., *Water for Injection*, *Nitrogen*) and appropriate design of the production and distribution system for these is essential. The systems for these are tightly controlled and frequent monitoring of the utilities produced is customary. These utilities may be also used in the process, and not become a part of the sterile product. Other utilities (e.g., clean steam, compressed air, *Purified Water*) that are used in the cleaning/decontamination of facilities, and/or preparation of equipment, containers, and closures can also be subjected to microbial control.

Utilities included in the product, in direct product contact, and in the preparation of equipment, containers, closures, and other items must meet the requirements defined in the appropriate *USP–NF* monograph. The systems for their preparation should be subject to formalized controls that maintain a controlled state over time. This is accomplished through a number of related practices essential for continued use of the system over an extended time. The essential practices to maintain controlled status of the utility systems include: calibration, change control, corrective and preventive maintenance, and ongoing process control.

There are other less impactful utilities (e.g., vacuum, cooling water) necessary for the operation of the facility and equipment. Although these non-product contact utilities may lack monograph requirements, their reliable operation is necessary for consistent production of sterile products.

# SUMMARY

The safety of products labeled sterile requires that their critical quality attributes consistently meet specifications. Sterility is the most essential quality attribute. Sterility is an unqualified concept in which an item is devoid of living microorganisms capable of reproduction. Monitoring of all types, environmental (viable and non-viable; air, surface, and personnel), media fills, and sterility tests are forms of microbiological analysis that have been historically employed as proof of “sterility”. These assessment tools cannot provide definitive evidence of either “sterility” or “nonsterility”, because the means to confirm either of those conditions non-destructively is not scientifically possible. Confidence in sterile product manufacturing is realizable only by a holistic approach in which all of the supportive elements of the operations are given due consideration and emphasis (*17*).

The absolute nature of sterility presents the practitioner with an inherent paradox—there is no ready means to demonstrate sterility of an item in the absolute sense regardless of the means used to provide it. Test methods including those defined in this compendium (*Sterility Tests* ⟨71⟩) utilize a number of samples taken from a large population to infer the “sterility” of the whole. Sterilization procedures including those validated for parametric release can deliver a low probability of a nonsterile unit, but not absolute assurance that “sterility” actually exists.

The uncertainty associated with proof of “sterility” notwithstanding, the means by which sterility assurance is provided are reasonably well defined. The “sterility” of any item is definitively established by the process controls summarized in this chapter rather than any form of monitoring or sampling. For terminally sterilized products, greater weight can be placed on the sterilization process utilized than on any form of testing. Confidence in aseptic processing is a result of sound design, reliable

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equipment, quality materials, effective procedures (including supportive sterilization processes), and personnel proficiency rather than through sampling dependent sterility testing, microbiological monitoring, and process simulation.

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***Add the following:***

# ▲⟨1212⟩ PROBE TACK TEST

## SCOPE

This general chapter is intended to provide general considerations and guidance on developing a probe tack test method for topical and transdermal delivery systems (collectively referred to as TDS) (*1*).[1]

## INTRODUCTION

Tack may be described, in the context of topical and transdermal systems, as the propensity of the pressure-sensitive adhesive layer to adhere quickly to a substrate under a light, brief application of force. Tack could also be described as quick stick, initial adhesion, or stickiness. Probe tack is an adhesive quality control test intended to ensure consistency from batch to batch. Probe tack quantitation is the measure of the force exerted by the adhesive on the probe during debonding and the displacement required to completely separate the probe from the adhesive. This test method involves bringing the tip of a clean probe of defined material into contact with the adhesive surface of a TDS under a fixed force for a fixed period of time and subsequently removing the probe from the surface at a given speed. The bond formed between the probe and the adhesive surface will break, and tack is expressed by the maximum force to break that bond or the area under the force per time to break that bond. Each test should result in clean removal of the probe with all adhesives remaining on the TDS (i.e., an indication of adhesive failure mode).

[NOTE—For more information, see ASTM D907-15, Standard Terminology of Adhesives and ASTM E6-15e4, Standard Terminology Relating to Methods of Mechanical Testing (*2,3*).]

## APPARATUS

An apparatus can include the following:

- A probe of defined material with inert surface, e.g., stainless steel, and defined surface, e.g., flat or minimally convex round with a defined diameter of, e.g., 5.0 mm (i.e., about 20 mm²).
- An annular ring whose dimensions are slightly larger than the probe diameter. The ring should have a low weight, e.g., about 20 g. A rigid metal plate/panel, e.g., stainless steel, brass, aluminum with predrilled holes may be substituted for the annular ring.

Choose combinations of probe surface area and annular ring weight that will result in a low pressure, e.g., about 10 kPa. Example: A 20-g ring (together with a specimen of negligible weight) will apply a pressure of about 10 kPa on a 20-mm² surface. Alternatively, a rigid plate instrument, with predrilled holes to be covered by the TDS, can be programmed to apply a specified threshold force.

Enabling either the probe or the sample to move, a test system should be capable of bringing the probe into contact with the adhesive surface at a specified approach speed, allowing the probe to adhere to the adhesive surface at a defined force and dwell time, and reversing the direction to detach the probe from the adhesive surface at the specified reverse speed.

The system should be capable of measuring the force when the probe pulls away from the adhesive and recording the maximum force.

A schematic example of an apparatus used in the probe tack test is shown in *Figure 1*. *Figure 2* illustrates a suitable alternate fixture for mounting the test TDS, a rigid plate with predrilled holes, in place of the annular ring.

[1] The primary ASTM reference used as the basis for developing a probe tack measurement of topical and transdermal delivery systems (ASTM D2979-16 Standard Test Method for Pressure-Sensitive Tack of Adhesives Using an Inverted Probe Machine) was withdrawn in 2019.

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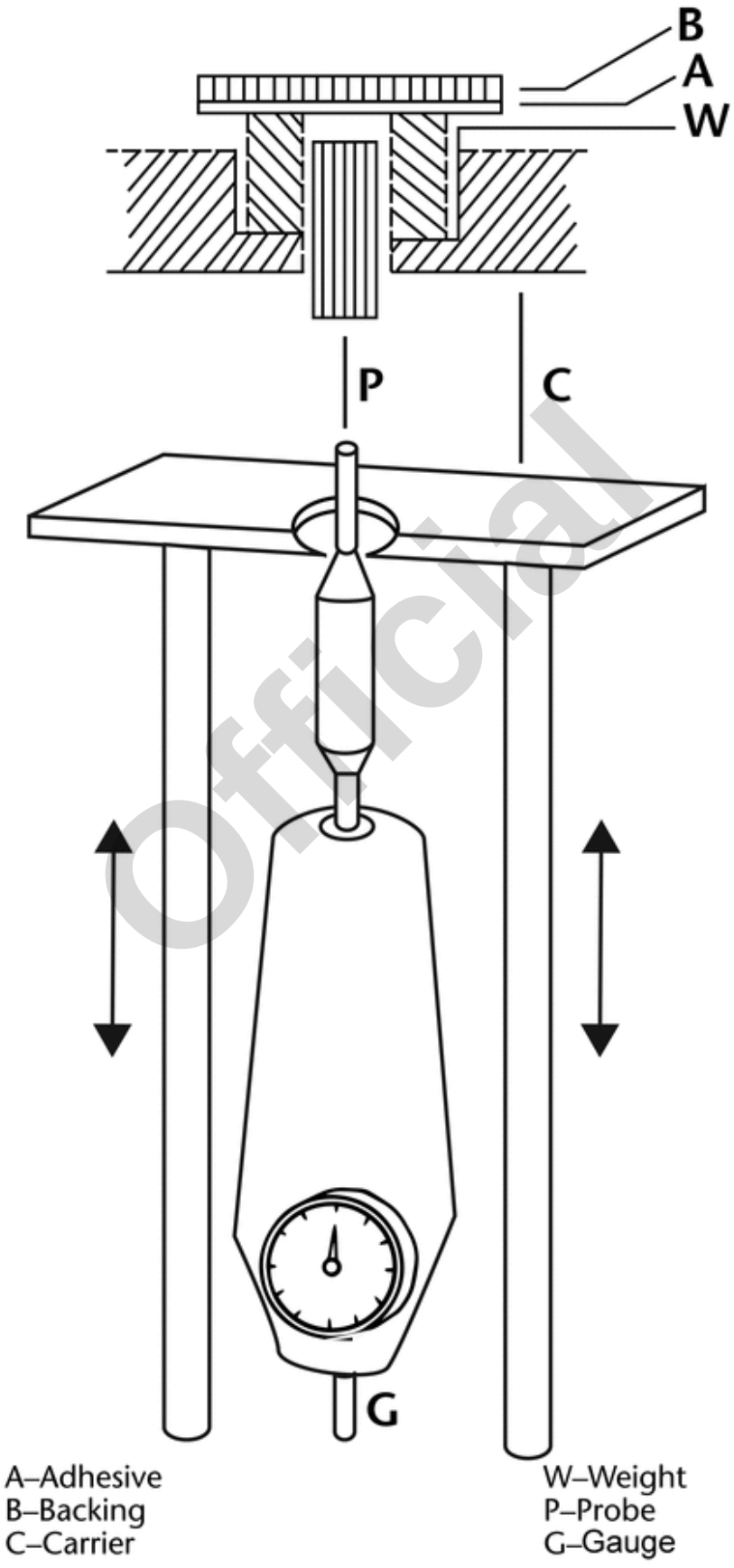


Figure 1. Schematic example of a probe tack testing apparatus.

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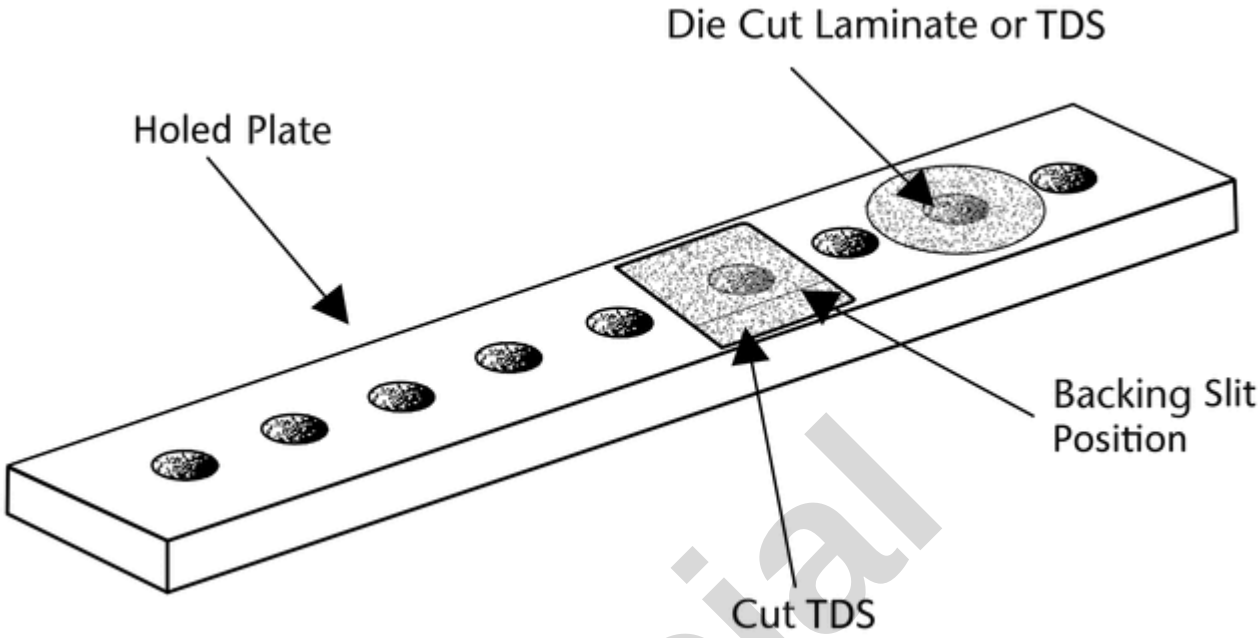


Figure 2. Alternate fixture: rigid plate with predrilled holes shown inverted for sample loading.

## PROCEDURE

Prior to performing the measurement, the test sample is conditioned at the specified testing conditions (temperature and humidity) for a specified duration.

Check the probe visually for any scratches or damages. Replace if necessary. Clean the probe head with a suitable solvent, removing any potential residue on the surface and without damaging the surface of the probe. Ensure the head is completely dry by allowing the solvent to evaporate or wiping with lint-free wipes.

Cut or punch out an appropriately sized specimen of a TDS, remove the release liner, and place the specimen, sticky side down (avoiding the location of the release liner slit), on the annular ring weight. The specimen must be large enough to cover the entire orifice and annular ring. Make sure there are no wrinkles on the TDS surface area at the site of the ring's orifice. Alternately (when using plates), clean the plate with a suitable solvent, and wipe to dryness with lint-free wipes. Ensure that the contact holes are free from objects or adhesive that would interfere with the probe. Remove the release liner and then place the sticky side of the TDS onto the rigid plate, centered around a predrilled hole (ensuring no wrinkles in the specimen and avoiding the location of the slit release liner (see *Figure 2*). A roller covered with silicone rubber with predefined weight and with a width enough to cover the TDS width may be used to apply constant force to the TDS to properly adhere onto the plate. Roll the roller without applying vertical force, allowing it to roll freely on the TDS. Turn the plate over so the sticky side of the TDS faces up through the predrilled holes, and place it on a fixture that secures the plate and allows for $x$ and $y$ plane adjustments. This will ensure that the probe will contact the exposed adhesive without contacting the plate.

Bring the probe and adhesive surface together at a constant speed (e.g., 10 mm/s) allowing the probe with a defined surface area to lift the specimen with the annular ring, thus applying a constant and low force. Alternatively, when using plates, program a constant force that the probe will apply to the specimen upon contact. Allow for a short and constant dwell time, e.g., 1 s. Separate the probe and the adhesive surface at a constant reversing speed, e.g., 10 mm/s. Record tack as the maximum force required to break the bond.

Perform at least $n = 5$ measurements with specimen from the same batch, taking one specimen per TDS. Check the probe visually in between replicate measurements and clean the probe head if appropriate. Tack is expressed by recording the maximum force to break the bond between the probe and the adhesive surface or the area under the force per time to break that bond. Clean the probe and store in a place protected from scratching.

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# ⟨1216⟩ TABLET FRIABILITY

***Change to read:***

This general information chapter has been harmonized with the corresponding texts of the *European Pharmacopoeia* and the *Japanese Pharmacopoeia*. The harmonized texts of these three pharmacopeias are therefore interchangeable, and the methods of the *European Pharmacopoeia* and/or the *Japanese Pharmacopoeia* may be used for demonstration of compliance instead of the present *United States Pharmacopeia* general information chapter method. These pharmacopeias have undertaken not to make any unilateral change to this harmonized chapter.

▲Portions of the chapter text that are national *USP* text, and are not part of the harmonized text, are marked with symbols (• •) to specify this fact. There are no local requirements or non-harmonized attributes in this chapter currently.▲ (USP 1-Aug-2023)

***Change to read:***

## ▲PURPOSE▲ (USP 1-AUG-2023)

This chapter provides guidelines for the friability determination of compressed, uncoated tablets. The test procedure presented in this chapter is generally applicable to most compressed tablets. The measurement of tablet friability supplements other physical strength ▲tests,▲ (USP 1-Aug-2023) such as tablet breaking force.

***Change to read:***

## ▲APPARATUS▲ (USP 1-AUG-2023)

Use a drum with an internal diameter between ▲283.0 and 291.0▲ (USP 1-Aug-2023) mm and a depth between ▲36.0 and 40.0▲ (USP 1-Aug-2023) mm, of transparent synthetic polymer with polished internal surfaces, and subject to minimum static build-up (see *Figure 1* for a typical apparatus). One side of the drum is removable. The tablets are tumbled at each turn of the drum by a curved projection with an inside radius between 75.5 and 85.5 mm that extends from the middle of the drum to the outer wall. The outer diameter of the central ring is between 24.5 and 25.5 mm. The drum is attached to the horizontal axis of a device that rotates ▲from 24 to 26▲ (USP 1-Aug-2023) rpm. Thus, at each turn the tablets roll or slide and fall onto the drum wall or onto each other.

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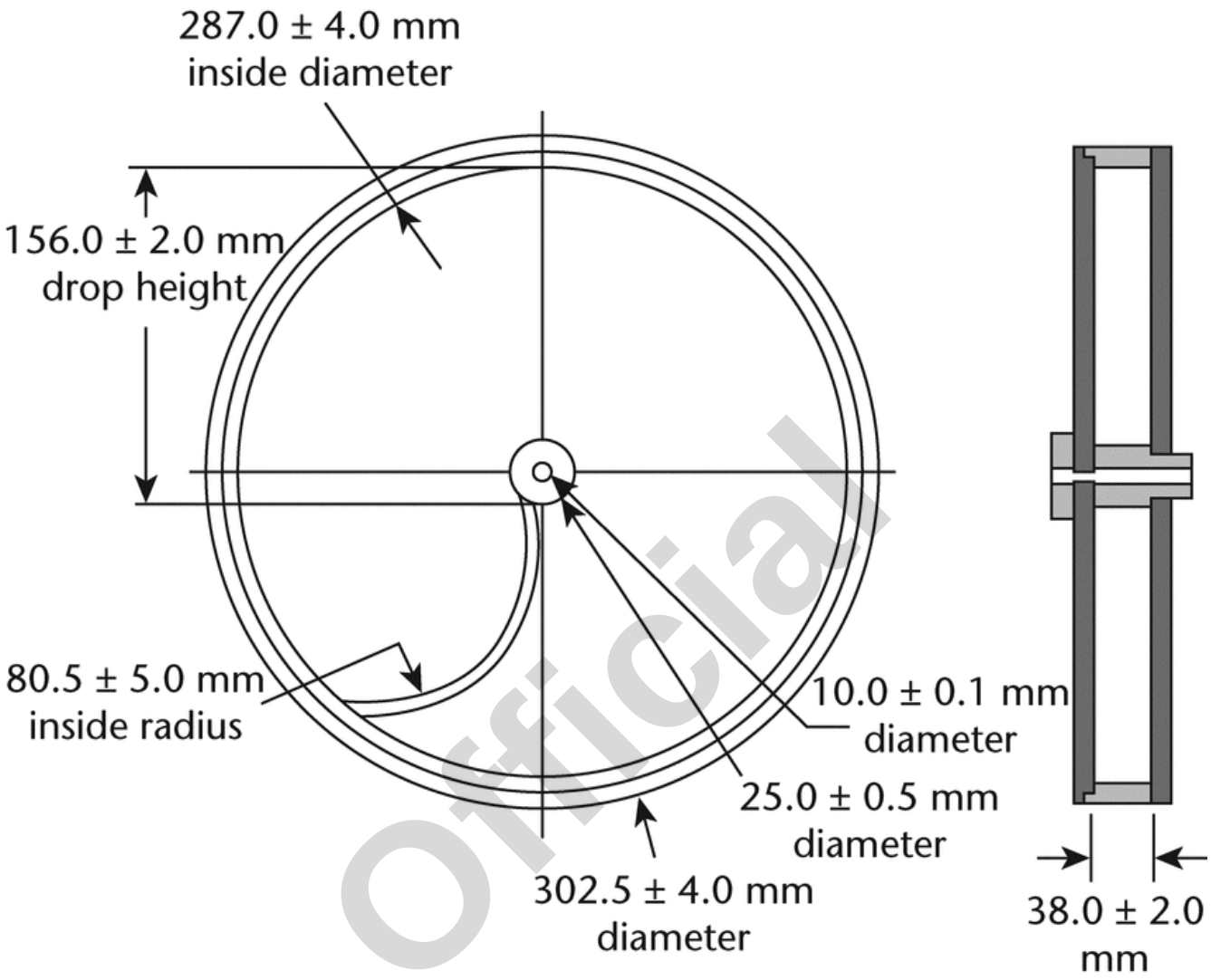


Figure 1. Tablet Friability Apparatus.

***Change to read:***

**▲PROCEDURE▲** (USP 1-AUG-2023)

For tablets with a unit weight equal to or less than 650 mg, take a sample of whole tablets corresponding as near as possible to 6.5 g. For tablets with a unit weight of more than 650 mg, take a sample of 10 whole tablets. The tablets should be carefully dedusted prior to testing. Accurately weigh the tablet sample, and place the tablets in the drum. Rotate the drum 100 times ▲using a speed from 24 to 26 rpm,▲ (USP 1-Aug-2023) and remove the tablets. Remove any loose dust from the tablets as before, and accurately weigh.

Generally, the test is run once. If obviously cracked, cleaved, or broken tablets are present in the tablet sample after tumbling, the sample fails the test. If the results are difficult to interpret or if the weight loss is greater than the target value, the test should be repeated twice and the mean of the three tests determined. A ▲▲ (USP 1-Aug-2023) weight loss from ▲a single test or the mean of three tests▲ (USP 1-Aug-2023) of not more than 1.0% is considered acceptable for most products. ▲Typically, in the case of effervescent and chewable tablets, the friability specification may be different.▲ (USP 1-Aug-2023)

If tablet size or shape causes irregular tumbling, adjust the drum base so that the base forms an angle of about 10° with the horizontal and the tablets no longer bind together when lying next to each other, which prevents them from falling freely.

In the case of hygroscopic tablets, an appropriate humidity-controlled environment is required ▲during▲ (USP 1-Aug-2023) testing.

Drums with dual scooping projections, or an apparatus with more than one drum, ▲designed to test▲ (USP 1-Aug-2023) multiple samples at ▲the same▲ (USP 1-Aug-2023) time, are also permitted.

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# ⟨1217⟩ TABLET BREAKING FORCE

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## INTRODUCTION

There are a variety of presentations for tablets as delivery systems for pharmaceutical agents, such as rapidly disintegrating, slowly disintegrating, eroding, ▲and▲ (USP 1-May-2019) chewable. ▲The concepts of this chapter are also applicable to lozenges.▲ (USP 1-May-2019) Each of these presentations places a certain demand on the bonding, structure, and integrity of the compressed matrix. Tablets must be able to withstand the rigors of handling and transportation experienced in the manufacturing plant, in the drug distribution system, and in the field at the hands of the end users (patients/consumers). Manufacturing processes such as coating, packaging, and printing can involve considerable stresses, which the tablets must be able to withstand. ▲For these reasons, several tests are available to assess the strength of the compact as well as the response to contact with water and other liquids. These tests include *Disintegration* ⟨701⟩, *Dissolution* ⟨711⟩, *Tablet Friability* ⟨1216⟩, and *Tablet Breaking Force*, the subject of this chapter. The mechanical strength of tablets is of considerable importance and is routinely measured. Tablet strength serves both as a criterion by which to guide product development and as a quality control specification. An acceptable value for the strength of the compact must consider the intended use as well as the mechanism of release of the active ingredient(s) from the dosage form.▲ (USP 1-May-2019)

One commonly employed test of the ability of tablets to withstand mechanical stresses determines their resistance to chipping and surface abrasion by tumbling them in a rotating cylinder. The percentage weight loss after tumbling is referred to as the friability of the tablets. Standardized methods and equipment for testing friability have been provided in ⟨1216⟩. ▲In addition to the loss of active drug, chipping and abrasion can each have a significant impact upon the success of subsequent manufacturing operations such as coating and packaging, and impact the consumer's perceived elegance of the dosage form.▲ (USP 1-May-2019)

Another measure of the mechanical integrity of tablets is their "breaking force", which is the force required to ▲produce failure (i.e., breakage)▲ (USP 1-May-2019) in a specific plane. The tablets are generally placed between two platens, one of which moves to apply sufficient force to the tablet to cause fracture. For conventional, round (circular cross-section) tablets, loading occurs across their diameter (sometimes referred to as diametral loading), and fracture occurs in that plane. ▲As previously stated, friability and breaking force measure different aspects of the compact strength. Depending upon the formulation, manufacturing method, and intended use, the results from one of the tests may be a better indicator of the quality of the dosage form than the other. This determination is best made at the time the formulation and manufacturing process are defined.▲ (USP 1-May-2019)

The breaking force of tablets is commonly called "hardness" in the pharmaceutical literature; however, the use of this term is misleading. In material science, the term "hardness" refers to the resistance of a surface to penetration or indentation by a small probe. The term "crushing strength" is also frequently used to describe the resistance of tablets to the application of a compressive load. Although this term describes the true nature of the test more accurately than does "hardness", it implies that tablets are actually crushed during the test, which often is not the case ▲nor the intent of the breaking force determination.▲ (USP 1-May-2019) Moreover, the term "strength" in this application can be questioned, because in the physical sciences that term is often used to describe a stress (e.g., tensile strength). Thus, the term "breaking force" is preferred and will be used in the present discussion.

▲During manufacture of compressed tablets, decisions must be made regarding tablet weight, thickness, friability, and breaking strength targets. Control of tablet weights must be given priority because weight control directly correlates with dosing accuracy. Once the desired weight is achieved, decisions must be made concerning the relative importance of the thickness, loss on drying, friability, and breaking strength. While thickness may be viewed as a physical parameter that only influences the appearance of the tablet, it also determines the pore volume of the compact (i.e., solid fraction, see also *Tablet Compression Characterization* ⟨1062⟩). While thickness, friability, and breaking strength are related, the relationships are not clear or easily predicted. As illustrated in ⟨1062⟩, the thickness of the tablet at constant weight may not be varied without influencing the breaking strength. It is therefore important for the manufacturer to identify which parameter most closely correlates with desired product performance.▲ (USP 1-May-2019)

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## TABLET BREAKING FORCE DETERMINATIONS

Early measuring devices were typically hand operated. For example, the Monsanto (or Stokes) hardness tester was based on compressing tablets between two jaws via a spring gauge and screw. In the Pfizer hardness tester, the vertically mounted tablet was squeezed in a device that resembled a pair of pliers. In the Strong-Cobb hardness tester, the breaking load was applied through the action of a small hydraulic pump that was first operated manually but was later motorized. Problems associated with these devices were related to operator variability in rates of loading and difficulties in proper setup and calibration. Modern testers employ mechanical drives, strain gauge-based load cells for force measurements, and electronic signal processing, and therefore are preferred. However, several important issues must be considered when using them for the analytical determination of breaking force; these are discussed below.

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## Platens

The platens should be parallel. Their faces should be polished smooth and precision-ground perpendicularly to the direction of movement. ▲In addition, the face of the platen should be perpendicular to the surface on which the tablet is placed to ensure the platen uniformly contacts the side of the tablet from top and bottom.▲ (USP 1-May-2019) Perpendicularity must be preserved during platen movement, and the mechanism should be free of any bending or torsion displacements as the load is applied. The contact faces must be larger than the area of contact with the tablet. ▲These considerations ensure that the applied load is operating over a consistent surface area of the dosage form.▲ (USP 1-May-2019)

## Rate and Uniformity of Loading

Either the rate of platen movement or the rate at which the compressive force is applied (i.e., the loading rate) should be constant. Maintaining a constant loading rate avoids the rapid buildup of compressive loads, which may lead to uncontrolled crushing or shear failure and greater variability in the measured breaking force. However, constant loading rate measurements may be too slow for real time monitoring of tablet production.

The rate at which the compressive load is applied can significantly affect results, because time-dependent processes may be involved in tablet failure (*1*). How a tablet matrix responds to differences in the loading rate depends on the mechanism of failure.

▲Additional discussion of the consolidation mechanism of materials is provided in ⟨1062⟩. Since different formulations of tablets exhibit varying amounts of strain rate sensitivity, it is important to conduct the breaking force test in a controlled manner and recognize that instruments that operate with different platen movement or loading rates may not give comparable results for the same product.▲ (USP 1-May-2019)

The test must be run consistently with equipment that has been routinely calibrated. Changing from testing units of different designs or from different manufacturers will require comparison of data to ensure that the two units are subjecting the dosage form to similar stress in a similar manner. Currently available equipment provides a constant loading rate of 20 newtons (N) or less per second or a constant platen movement of 3.5 mm or less per second. Controlled and consistent breaking is an important test procedure attribute. To ensure comparability of results, testing must occur under identical conditions of loading rate or platen movement rate. Since there are certain advantages to each system of load application, both are found in practice. Because the particular testing situation and the type of tablet matrix being evaluated will pose different constraints, there is also no basis to declare an absolute preference for one system over the other. This general chapter proposes consideration of both approaches.

The different methods may lead to numerically different results for a particular tablet sample, requiring that the rate of load application or displacement must be specified along with the determined breaking force.

## Dependence of Breaking Force on Tablet Geometry and Mass

Measurements of breaking force do not take into account the dimensions or shape of the tablet. ▲▲ (USP 1-May-2019) Tablet orientation and failure should occur in a manner consistent with ▲the orientation used and failure observed▲ (USP 1-May-2019) during the development of the dosage form. For direct comparisons (i.e., without any normalizations of the data), breaking force measurements should be performed on tablets having the same dimensions, ▲weights,▲ (USP 1-May-2019) geometry, and consistent orientation in test equipment.

## Tablet Orientation

Tablet orientation in diametral compression of round tablets without any scoring is unequivocal. That is, the tablet is placed between the platens so that compression occurs across a diameter. However, tablets with a unique or complex shape may have no obvious orientation for breaking force determination.

▲Because the breaking force for unembossed, noncircular cross-section tablets will likely depend on the tablet's orientation in the tester, it is best to settle on a standard orientation, preferably one that provides a consistent failure plane and that is readily reproduced by operators, to ensure comparability of results.▲ (USP 1-May-2019) In general, tablets are tested either across the diameter or parallel to the longest axis. Scored tablets have two orientation possibilities. When they are oriented with their scores perpendicular to the platen faces, the likelihood that tensile failure will occur along the scored line increases. This provides information about the strength of the matrix at the weakest point in the structure. When scored tablets are oriented with their scores parallel to the platen faces, more general information about the strength of the matrix is derived.

Capsule-shaped tablets or scored tablets may best be broken in a three-point flexure test (*2*). A fitting, which is either installed on the platens or substituted for the platens, supports the tablet at its ends and permits the breaking load to be applied to the opposite face at the unsupported midpoint of the tablet. The fittings are often available from the same source that supplies the hardness tester.

▲Since the orientation of the dosage form critically impacts the result of the test, it must be clearly defined in the test procedure and produce a consistent failure plane in the dosage form for the results to be comparable. The plane of failure should always be noted. If, while maintaining orientation, the plane of failure differs for a batch or sub-batch of tablets relative to previous results and direct comparison of the numerical value is not possible, this observation in itself can be an important indicator of a compression problem.▲ (USP 1-May-2019)

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## Units, Resolution, and Calibration

Modern breaking force testers are usually calibrated in kiloponds or newtons. The relationship between these units of force (*3*) is 1 kilopond (kp) = 1 kilogram-force (kgf) = 9.80 newtons (N). The test results should be expressed in standard units of force which facilitate communication. Some breaking force testers also will provide a scale in Strong-Cobb units (SCU), a carryover from the days when Strong-Cobb hardness testers were in common usage. The conversion between SCU and N or kp must be viewed with caution, because the SCU is derived from a hydraulic device and is a pressure.

Generally, contemporary breaking force testers use modern electronic designs with digital readouts. Some units also have an integral printer or may be interfaced with a printer. Breaking forces should be readable to within 1 N.

Breaking force testers should be calibrated periodically. The force sensor as well as the mechanics of the apparatus needs to be considered. For the force sensor, the complete measuring range (or, at a minimum, the range used for measuring the test sample) should be calibrated to a precision of 1 N, using either the static or dynamic method. Static calibration generally employs traceable counterweights; at least three different points are checked to assess linearity. Dynamic calibration makes use of a traceable reference-load cell that is compressed between the platens. The functional calibration of a breaking force test apparatus should also confirm that the velocity and the constancy of velocity for load application or displacement are within prescribed tolerances throughout the range of platen movement.

## Sample Size

▲Interpretation of the breaking force data must consider not only the mean value but the consistency of the test results for multiple tablets. The range of breaking force values may provide valuable information on the consistency of the tableting process. Excessive variability in values may reflect issues with die filling, weight control, or orientation of the dosage form in the test equipment. Additionally, the breaking strength result will reflect the orientation of the failure plane. For results to be compared, the tablets must fail in the same manner throughout the test. In cases where breaking force may be particularly critical, the average plus individual breaking force values should be reported.▲ (USP 1-May-2019)

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## TENSILE STRENGTH

▲Tensile strength is an intensive materials property, which by definition does not depend on the size or shape of the material being measured. The measurement of tensile strength provides a more fundamental measure of the mechanical strength of the compacted material and takes into account the geometry of the tablet. The application of a vertical force (*F*), across the diameter of a disc-shaped tablet generates a horizontal stress ($\sigma_X$), which is tensile and constant along the centerline of the tablet. The value of this stress is given by the following equation, which applies to diametral compression of cylindrical tablets:

$$\sigma_X = 2F/\pi DH$$

$\sigma_X$ = horizontal stress
$F$ = breaking force
$D$ = tablet diameter
$H$ = tablet thickness

The derivation of this equation may be found in standard texts (*4*–*6*); it is based on elastic theory and the following assumptions:

1. The tablet is an isotropic body
2. Hooke's law is obeyed
3. The modulus of elasticity in compression and in tension is the same
4. Ideal point loading occurs

If the force is increased to the point of tablet fracture and if the tablet breaks into two roughly equal halves, the corresponding tensile stress at failure is historically reported as the "tensile strength". However, recent studies (*7*) show that the tensile strength calculated using this method can be half or less of the actual tensile strength as measured in a three-point bend test. The discrepancy occurs because tablet fracture initiates under the intense shear stresses (*8*) that are present near the point of contact between the tablet and the compression platen. The intense shear stresses allow cracks to form under Mode III fracture conditions (out-of-plane shearing), even though $\sigma_X$ is still below the strength of the compact. Once fracture initiates, the cracks propagate along the center line by Mode I fracture (opening) and at tensile stresses well below the true tensile strength of a crack-free tablet. Despite these errors the diametrical strength is still useful in many applications (*9*). Moreover, the data will be normalized with respect to tablet dimensions, because both diameter and thickness are included in the equation.

The derivation has been extended to convex-faced tablets (*10*,*11*):

$$\sigma_X = (10F/\pi D^2) \times [(2.84H/D) - (0.126H/W) + (3.15W/D) + 0.01]^{-1}$$

$\sigma_X$ = tensile stress
$F$ = breaking force
$D$ = tablet diameter
$H$ = tablet thickness
$W$ = central cylinder thickness (tablet wall height)

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Other examples may be available in the literature (*12*).▲ (USP 1-May-2019)

Bending or flexure of tablets is another option for determining the tensile strength of tablets. Under ideal loading conditions, a breaking load applied to the unsupported midpoint of one face will result in the generation of pure tensile stress in the opposite face. If the tablets are right circular cylinders and are subjected to three-point flexure, the tensile strength may be estimated using the following equation (▲*13*▲ (USP 1-May-2019)):

$$\sigma_X = 3FL/2H^2D$$

$\sigma_X$ = tensile stress
$F$ = breaking force
$L$ = distance between supports
$H$ = tablet thickness
$D$ = tablet diameter

▲The value of *L* should be chosen judiciously to minimize errors in this calculation (*9*).▲ (USP 1-May-2019) The assumptions are the same as those for calculating tensile strength from diametral compression. However, tensile strengths determined by flexure and diametral compression may not agree because of likely nonideal loading and the induction of shear failure during testing.

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# ▲⟨1220⟩ ANALYTICAL PROCEDURE LIFE CYCLE

## INTRODUCTION

This general chapter holistically considers the validation activities that take place across the entire life cycle of an analytical procedure and provides a framework for the implementation of the life cycle approach.

The analytical procedure life cycle approach described here is consistent with the quality by design concepts described in International Council for Harmonisation (ICH) guidelines. The procedure life cycle approach emphasizes the importance of sound scientific approaches and quality risk management for the development, control, establishment, and use of analytical procedures. Total error is used in this chapter; however, measurement uncertainty can also be used.

The procedure life cycle approach is applicable to all types of analytical procedures, and the extent of effort should be consistent with the complexity of the procedure and the criticality of the quality attribute to be measured. The life cycle approach can be considered optional, and any of the elements can be applied on the basis of how the procedure is used. Elements of the life cycle approach can also be applied retrospectively if deemed useful or in early stages of development with the appropriate modifications.

Elements of life cycle management of analytical procedures are also discussed in *Analytical Procedures and Methods Validation for Drugs and Biologics* (Guidance for Industry, FDA 2015).

Validation of an analytical procedure is the process by which it is established, through laboratory studies, that the performance of the procedure meets the requirements for the intended analytical applications. Validation, or demonstration that a procedure is suitable for the intended purpose, takes place during the entire procedure life cycle, beginning during the initial procedure design activities and extending through routine use. These activities include the formal procedure validation, verification, and transfer of procedures, as well as establishing and assuring adherence to an appropriate set of procedure controls and system suitability requirements.

The procedure life cycle is comprised of the analytical target profile (ATP) and three stages, which are introduced below and shown in *Figure 1*.

The ATP defines the criteria for the procedure performance characteristics that are linked to the intended analytical application and the quality attribute to be measured. It applies to all stages of the procedure life cycle. For quantitative procedures, the ATP describes the required quality of the reportable value since the reportable value generated using a qualified analytical procedure provides the basis for key decisions regarding compliance of a test article with regulatory, compendial, and manufacturing limits. The acceptable level of risk of making an incorrect decision can also be considered when establishing ATP criteria.

**Stage 1:** Procedure design encompasses procedure development, which consists of the analytical technology and sample preparation. It includes understanding gained through knowledge gathering, systematic procedure development experiments, and risk assessments and associated lab experiments. The output of *Stage 1* includes:

1. A set of procedure conditions that minimizes procedure bias to a suitable level, can provide acceptable precision, and can meet the ATP criteria
2. An understanding of the effect of procedure parameters (e.g., temperature, wavelength, flow rate, etc.) on procedure performance
3. Optimization of performance characteristics of the analytical procedure such as accuracy, precision, the appropriateness of any calibration model, specificity and limit of quantitation (as far as applicable); this includes a preliminary replication strategy for samples and standards
4. An initial analytical control strategy (ACS), which is a set of controls (system suitability tests [SSTs] and other procedure-specific controls) needed to ensure proper performance

**Stage 2:** Procedure performance qualification consists of studies designed to demonstrate that the procedure is suitable for its intended purpose. This involves confirmation that the reportable values generated by application of the analytical procedure meet the ATP criteria as well as confirmation of procedure performance characteristics through the traditional validation, verification, or transfer studies. Data generated during *Stage 1* can be used if available and suitable. At the end of *Stage 2*, the replication strategy and the performance of the procedure is confirmed to meet the ATP and other criteria.

**Stage 3:** Ongoing procedure performance verification involves monitoring the analytical procedure during routine use and confirming that the performance continues to meet ATP criteria. Monitoring ensures that the performance of the procedure is maintained at an acceptable level over the procedure lifetime. It can also provide an early indication of potential performance issues or adverse trends and aid in identifying required changes for the analytical procedure. Confirming procedure performance after changes ensures that the modified procedure will produce reportable values that meet the criteria defined in the ATP.

More details about the procedure life cycle are described in the subsequent sections.

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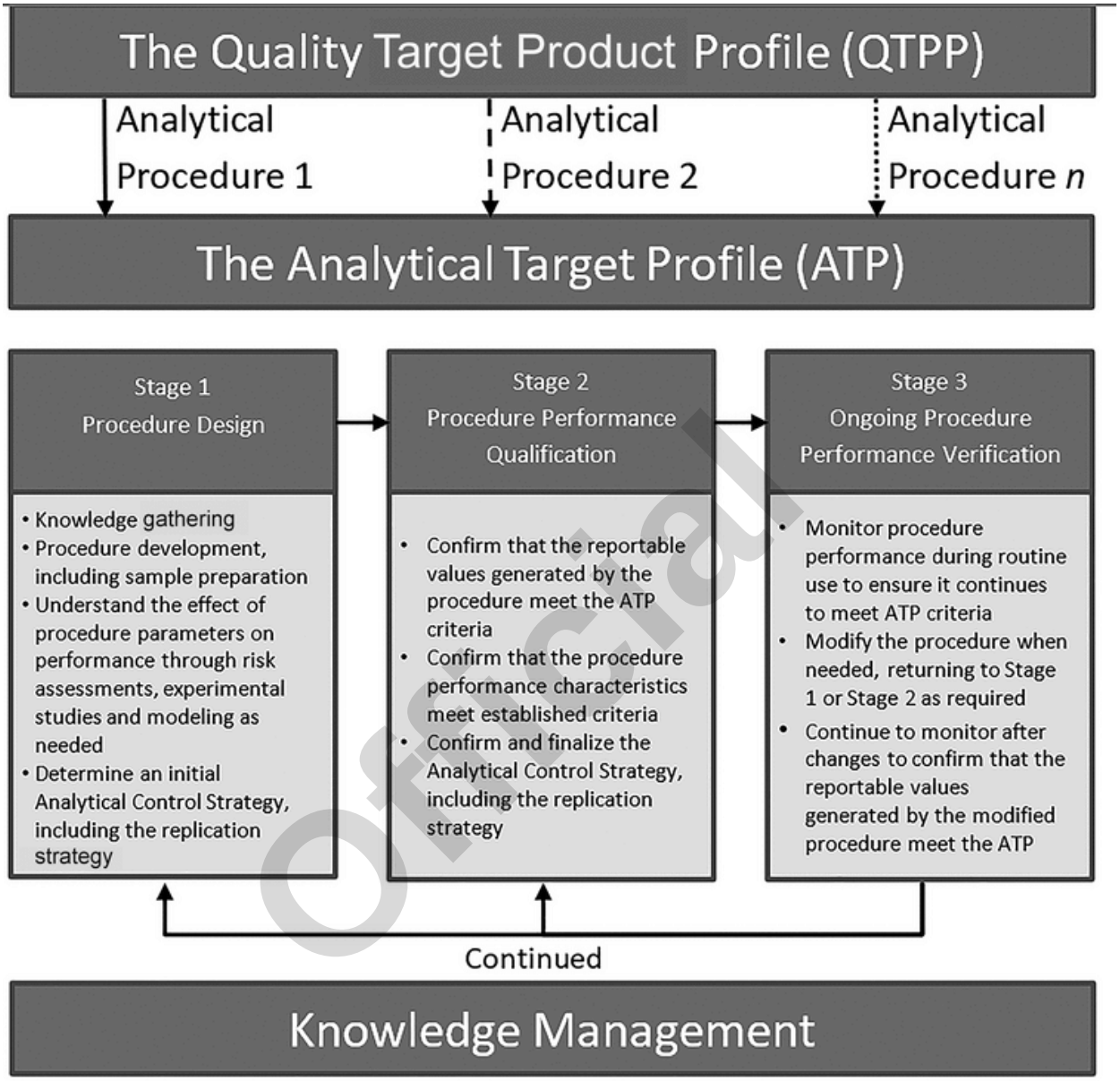


Figure 1. The three stages of the analytical procedure life cycle.

## ANALYTICAL TARGET PROFILE (ATP)

A fundamental component of the life cycle approach is the definition of the analytical target profile (ATP) for the analytical measurement. The ATP is a prospective description of the desired performance of an analytical procedure that is used to measure a quality attribute, and it defines the required quality of the reportable value produced by the procedure, aligned with the quality target product profile (QTPP). The ATP is based on the intended use for the procedure and, for quantitative or semi-quantitative procedures, should include upper limits on the precision and accuracy (bias) of the reportable value. The ATP focuses the design goals for a new analytical procedure, serves as a basis for procedure qualification criteria, and provides a guide for monitoring of the procedure during its life cycle. Ideally, the ATP should be specified in way that is independent of the measurement technology; however, in some cases it may be necessary to link it directly to a measurement technology. The ATP can be defined in many ways, but the overall focus of having a procedure with acceptable bias and precision should be part of the ATP.

Some examples of ATPs are shown below. The first example has separate criteria for accuracy and precision.

**Example 1:** The procedure must be able to accurately quantify [drug] in a range from [*A* units] to [*B* units] in the [description of test article] in the presence of [*x, y, z*] with an accuracy = 100% ± [*D*%] and a precision ≤ [*E*%] for the reportable value. The second example specifies that the combined accuracy and precision, or the distribution of the total analytical error (TAE), must fall within the desired level.

**Example 2:** The procedure must be able to quantify [analyte] in a range from [*A* units] to [*B* units] in the [description of test article] in the presence of [*x, y, z*] so that the distribution of the total analytical error of the reportable value falls within the total allowable analytical error range of ± [*C*%].

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These two examples are not exhaustive and other approaches to defining an ATP can be utilized.

## BIAS AND PRECISION

Procedure design, qualification, and performance verification often focus on specific procedure performance characteristics. For example, traditional procedure development is focused on achieving targets for accuracy, precision (repeatability and intermediate precision), detection/quantitation limit, and robustness. A close examination of these performance characteristics reveals that they focus on two primary aspects of the measurement:

1. Bias (accuracy): how close the measurement is, on average, to the true value that is being measured
2. Precision: how much the measurement will vary randomly under routine use

The link between specific procedure performance characteristics and the overall bias and precision should be considered during development. This can lead to a better understanding of the aspects that affect the bias and precision and to a better assessment and control of the risks associated with using the procedure.

Procedures should be developed to have levels of bias that are consistent with the ATP criteria, and the impact of bias on the reportable value should be considered. Appropriate limits for bias and precision in the ATP can be determined based on several factors, including:

- The criticality of the quality attribute being measured
- The risk that an unacceptable error could occur
- The width of the specification acceptance range for the quality attribute measured by the procedure
- The potential clinical safety or efficacy impact (if known) that an analytical error can have

After considering those factors, a maximum allowable combined bias and precision can be determined that provides appropriate performance for the procedure (see *Statistical Tools for Procedure Validation* ⟨1210⟩). Once this has been determined, the distribution of the reportable value should be within that combined range. This approach expands the bias and precision into a combined range for likely values for the measurement (*Figure 2*).

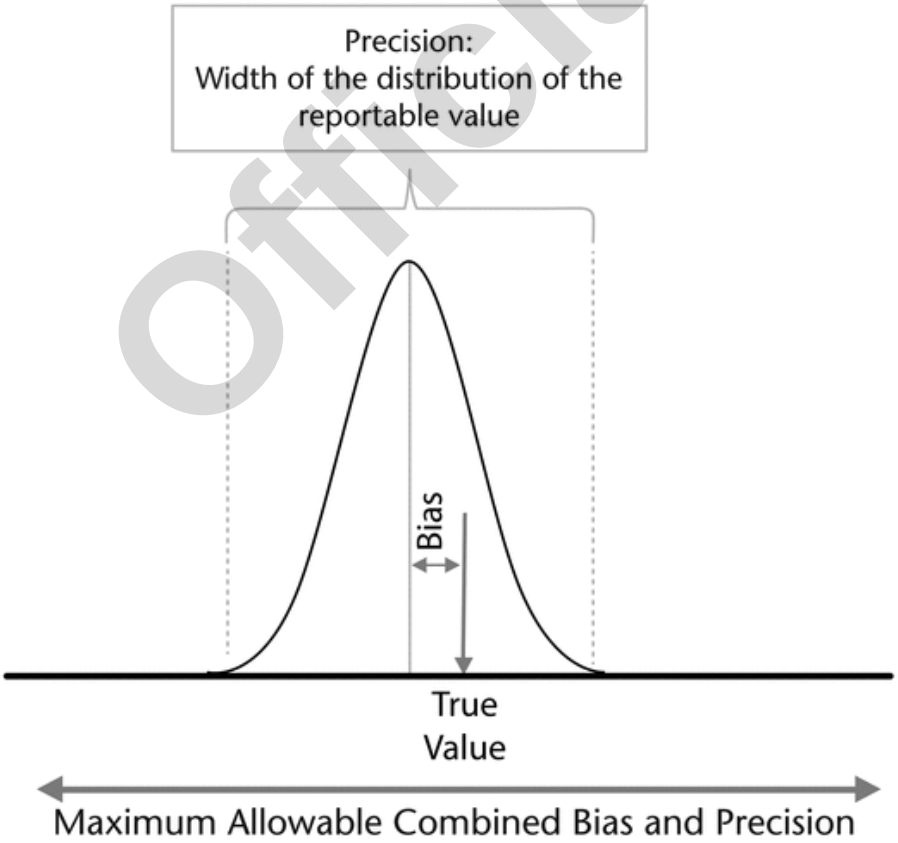


Figure 2. Relationship between the bias, precision, and the maximum allowable combined bias and precision.

## SPECIFICATION AND DECISION RULES

Deciding if a material conforms to specification relies on the use of a decision rule. A decision rule provides the acceptable level of the probability of making a wrong decision which can be used to define the maximum allowable combined bias and precision. There are several types of decision rules with the most common being the simple decision rule, where the material meets specification if the reportable value is within an acceptance range, and otherwise it does not meet specification (*Figure 3*).

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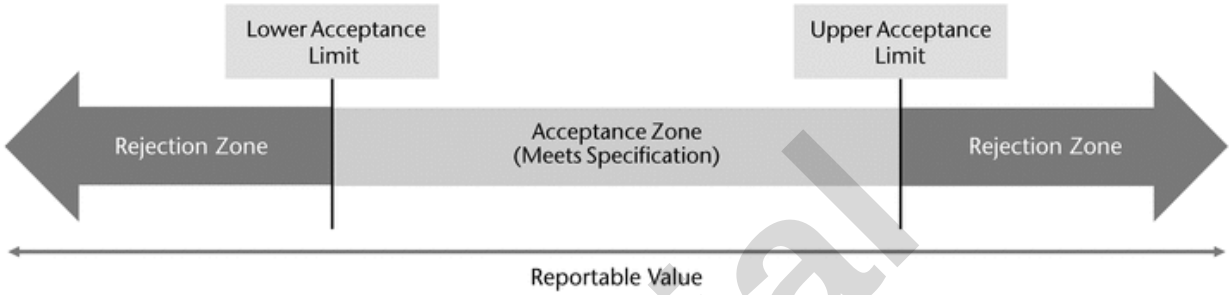


Figure 3. Use of a simple decision rule.

One consideration for determining the ATP criteria is understanding the impact that total analytical error has on the risk associated with the decision that a material conforms to specification. Total analytical error is used in this chapter; however, measurement uncertainty can also be used (see *Analytical Data—Interpretation and Treatment* ⟨1010⟩). The primary risk is that the decision rule produces the wrong decision; that is, that the material tested is determined to conform (or not conform) to specification limits when in fact the opposite is true. This risk may be strongly impacted by the procedure variability. The following are examples of risks associated with a specification that has an upper acceptance limit ($U_a$) under four scenarios (*Figure 4*):

**Scenarios 1 and 4:** In these scenarios, the decision is clear. The reportable value ($RV_1$ and $RV_4$) is well above or below the upper acceptance limit relative to the distribution of the total analytical error.

**Scenarios 2 and 3:** In these scenarios, it is less clear that the true quality characteristic is actually above or below the upper acceptance criterion and there is probability that the true value of the quality characteristic is actually inside (*Scenario 2*) or outside (*Scenario 3*) the specification acceptance range.

For *Scenario 1* and *Scenario 4*, the simple decision rule produces an outcome with very low risk of making the wrong decision. However, for *Scenario 2* and *Scenario 3*, the reportable value is close enough to the acceptance criterion to create some risk that the wrong decision could be made.

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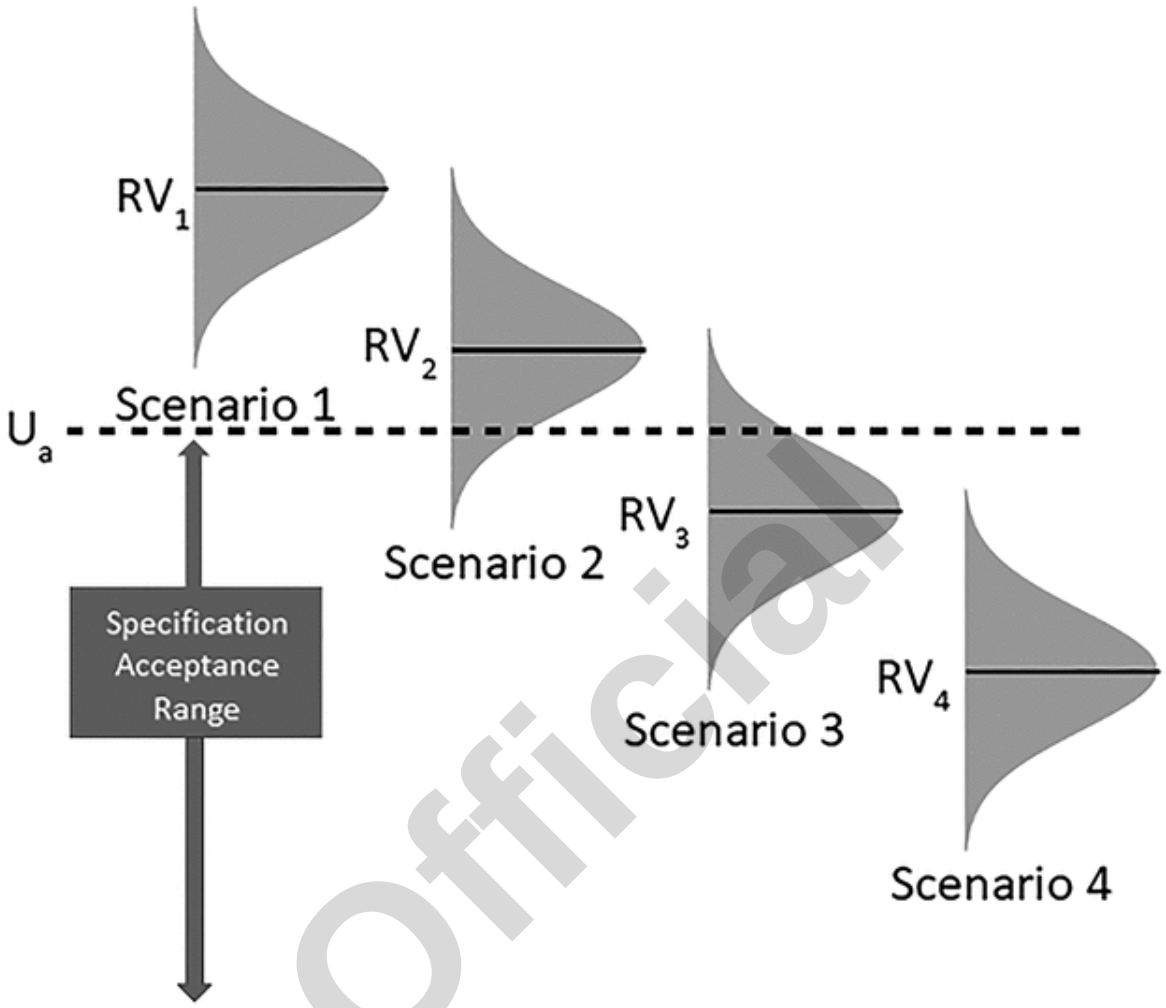


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Figure 4. The four possible scenarios when a reportable value (RV) is compared to the specification acceptance criteria. Each outcome shows a normal distribution curve with the reportable value at the center of the TAE distribution. (Adapted from *EURACHEM/CITAC Guide: Use of uncertainty information in compliance assessment*, First Edition, 2007.)

In all of these scenarios, what ultimately needs to be considered is the use of the material being tested and how the specifications are established. If the specification acceptance criteria are well within a safe and efficacious range, using the simple decision rule that the reportable value must be within the specification acceptance range provides adequate protection. However, the closer the acceptance range is to the safe and efficacious range, the larger the impact the distribution of the total analytical error can have on decision rule risks. Managing these risks may be achieved by altering the type of decision rule that is used.

In the situation where the safe and efficacious range is accurately known, guard bands can be applied to that range, based on the distribution of the total analytical error, to determine the acceptance range (*Figure 5*). In situations where additional risk reduction is desired, a multistage testing approach could be used with an indecision zone (*Figure 6*) for the initial stage of testing. When the reportable value is within the indecision zone, a predetermined supplementary sampling and testing plan and decision rule are used to determine conformance.

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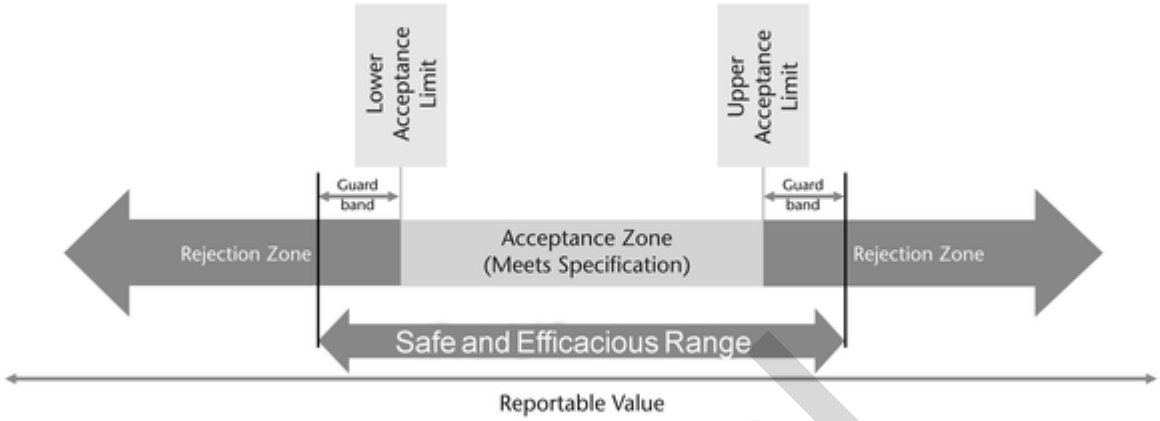


Figure 5. Decision rule using guard bands based on the total analytical error.

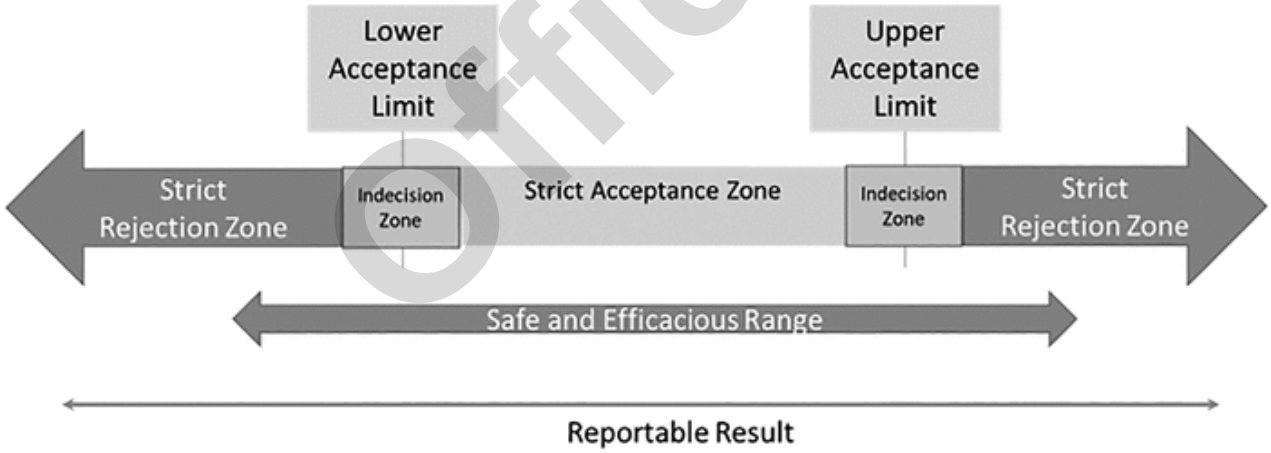


Figure 6. Decision rule using indecision zones based on the total analytical error.

## STAGE 1: PROCEDURE DESIGN

### Preparation for Analytical Procedure Development

When possible, ATPs should be established for quality attributes that require analysis and established before starting procedure design activities. Any technique that is capable of meeting the ATP criteria can be selected. Relevant prior knowledge can assist with the procedure development activities, which can include the physical and chemical properties of the analytes, information in the scientific literature, and any existing procedures for the analysis of the same material type or for similar material attributes. The availability of any relevant analytical technology and/or platform analytical procedures (applicable to materials of similar type) can also be considered, which may expedite procedure development activities.

Once the technology has been selected, and if development of a new analytical procedure is required, relevant information should be gathered prior to conducting development activities. Such information may include:

- Known chemical structures and their physical and chemical properties
- Reference standards, reagents, likely instrumentation and systems
- Any other relevant information linked to the operational requirements, such as instrumentation type/setup and sample preparation

### Analytical Procedure Development

The main purposes of procedure development are to identify conditions that minimize or avoid bias, to optimize variability, and to establish robust operating parameters that have the potential to meet the ATP. This can be carried out through

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the systematic screening and/or optimization of instrumental and sample preparation conditions when appropriate. This work begins with the process of understanding the influence of analytical procedure parameters on performance and their impact (directly or indirectly) on the ATP.

It is important to define the objective of development in terms of the desired levels of robustness to be achieved and the risks associated with the use of the procedure. This ultimately dictates the level of development needed in terms of the extent of experimentation and any modeling that may be used to optimize procedure performance.

The output of the procedure development process provides procedure operating conditions that represent the desired level of robustness.

**Quality Risk Management (QRM) and the Analytical Procedure**

QRM for an analytical procedure is a systematic process for the assessment, control, communication, and review of risk to the quality of the reportable value across the life cycle of the analytical procedure.

The aim of the QRM process is to assess the proposed procedure conditions and identify appropriate controls on the analytical procedure parameters and material attributes that will ensure the procedure meets the ATP. All variables associated with the analytical procedure should be considered, including sample and standard preparations, instrumental and equipment parameters, and environmental variables (e.g., laboratory temperature, humidity, etc.).

QRM activities can be applied during procedure development either formally or informally and major sources of bias and variability can be identified, reduced or even eliminated by ensuring the appropriate technology and procedure conditions.

The first step in the QRM process is the risk assessment, which starts with risk identification. To facilitate risk identification, the analytical procedure can be described using a process flow, map, or summary, and each part of the procedure can be broken down into detailed sub-steps. It is important to consider all steps, from sample and standard preparation to analyte testing to quantitation. The procedure process map can then be used to identify variables associated with the analytical procedure. Tools such as Ishikawa diagrams can be used in conjunction with the process maps to identify potential variables associated with each step in the analytical procedure. Prior knowledge related to the analytical technology can accelerate this step (e.g., generic Ishikawa diagrams and process maps may exist for common analytical techniques). *Figure 7* illustrates a range of potential variables associated with the sample preparation step from an HPLC analysis.

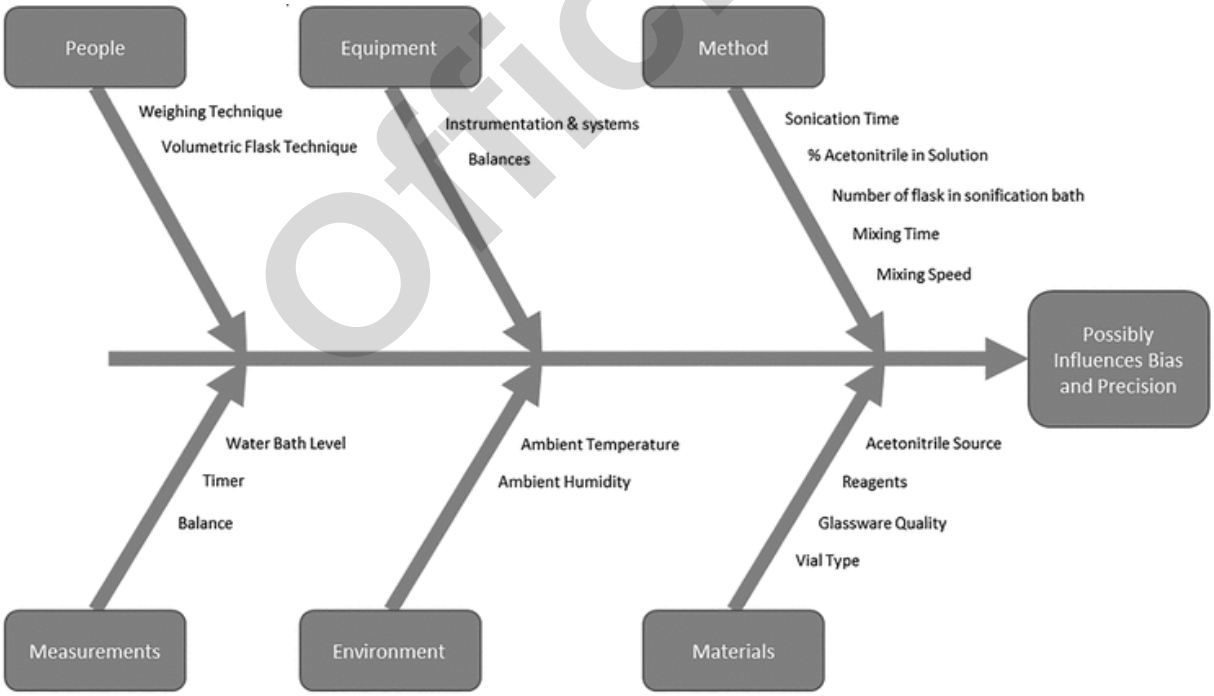


Figure 7. Ishikawa diagram used to identify potential variables.

Once potential variables have been identified, the risk associated with each variable can be estimated and ranked based on the ability to meet the ATP and other desired performance attributes. This assessment is driven by prior knowledge and scientific expertise, but some factors with unknown influence may need to be considered higher risk until further knowledge is available.

A *heat map* can be a valuable tool to support a qualitative assessment of risk. A heat map provides a visual indication of which variables are considered to have a potentially strong, medium, or minor impact on the performance of the analytical procedure (see *Table 1*).

**Table 1. Example of heat map capturing a qualitative risk assessment related to sample preparation and HPLC setup steps from an analytical procedure. Impact levels are strong (red), medium (amber), and minor (green).**

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| Analytical Unit Operation | Analytical Factor or Variable | Identified Potential Risk | RISK HEAT MAP | | Analytical Control Strategy |
|---|---|---|---|---|---|
| | | | Accuracy | Precision | |
| SAMPLE & REAGENT PREPARATION | Humidity of the laboratory | Moisture absorption by the sample can lead to incorrect weighing or degradation | | | Monitor environmental controls |
| | Analyst skill | Incorrect sample preparation; weighing & volumetric dilutions | | | Training program and records |
| | Sonication time | Lack of dissolution of the sample or degradation | | | Establish limit or conditions during development |
| | Composition of the solvent mixture used in sample preparation | Lack of complete dissolution of the sample | | | |
| INSTRUMENT & SYSTEM SET UP | % composition of the solvent in the mobile phase | Column performance, peak shape & retention times | | | Gravimetric preparation, SSTs |
| | Column temperature | | | | Establish operation within limits during instrument/system qualification; SSTs to confirm performance |
| | Batch of column packing material | | | | Establish variability during Stage 1 and design SSTs |
| | Quality of the solvent | Baseline drift and noise are wavelength dependent and may affect the peak shape | | | Specify required grade and transmittance characteristics |
| | Cleaning | Peaks from previous Injections | | | Establish cleaning protocol, SST |

Alternatively, each risk can be formally scored and ranked using set risk criteria, for example using failure mode and effects analysis (FMEA).

Once the risk associated with each factor and variable has been determined, planning for how to manage those risks occurs. The risk for those variables that are well understood may be mitigated by controlling them within a certain range (control variables). Other variables will be difficult or impractical to control, and the risks associated with them will need to be accepted (noise variables). For variables where there may be higher risk, one way to reduce risk is to gain additional knowledge about the influence of those parameters using modeling and/or experimentation.

Experimentation is a direct way of generating data that can be used to assess the impact of procedure parameters on performance, and the use of statistical design of experiments (DOE) is an effective way to do this. This approach is generally useful for exploring procedure parameters and optimizing the conditions of the procedure to obtain the desired performance. In a well-designed experiment, the way data are collected ensures that the effect of variables and their possible interactions on the quality attributes are studied. Specific recommendations on the types of DOEs to use are outside of the scope of this chapter; however, a design that examines combinations of factors and their possible interactions usually can better predict procedure performance compared to one-factor-at-a-time designs.

When available, mechanistic models can be used to understand the effect of procedure parameters on performance. Use of mechanistic models can reduce experimental work and provide a reliable estimate of the behavior of the analytes of interest.

Some procedures may not require modeling, for example if the technology is simple or if the overall risk of not meeting the ATP is low.

There are different modeling approaches that can be applied, including empirical and mechanistic, and either may be appropriate depending on the intended use of the analytical procedure and the desired model accuracy.

**Robustness and Method Operable Design Region (MODR)**

*Robustness* is a measure of the capacity of a procedure to remain unaffected by small but deliberate variations in parameters and to maintain suitability during normal usage. In some cases, it is helpful to demonstrate robustness of the procedure by developing models that describe the effect of parameters on the performance of the procedure, either through assessing performance against the ATP or other surrogate criteria. This knowledge also enables the determination of robust operation regions for procedure parameters and, if desired, a method operable design region (MODR).

The MODR is the multivariate space of analytical procedure parameters that ensure the ATP is fulfilled and therefore provide assurance of the quality of the measured value. The MODR should ideally be obtained through well-designed experiments that consider multivariate interactions. If the MODR relates to attributes that are linked to the elimination of bias (such as ensuring appropriate separation in chromatographic procedures) or minimization of variability (through operating at a suitable $\lambda_{max}$ wavelength), an impact on the final performance of the reportable value is not expected. Consequently, the validation performance characteristics do not need to be investigated completely within the MODR. When there are a number of noise factors associated with an analytical procedure, performance can vary over time

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among laboratories or instruments or other noise factors. In these cases, the MODR reflects the knowledge at a point in time and can be managed by the analytical life cycle (see *Stage 3*).

**Replication Strategy for the Reportable Value**

Once the target conditions and operating ranges for the procedure have been determined, an assessment can be performed to develop an understanding of the overall sources of variation contributed by the procedure. This can be accomplished in many ways, including propagation of error models and measurement system analysis experiments. Sources of variation may be grouped into general categories such as run-to-run and within-run variations. Those sources could also be broken down into component categories such as lab-to-lab, instrument-to-instrument, sample preparation, and sample introduction. If the amount of variation from these sources can be quantified, it may allow for the use of an optimized sample and standard replication strategy.

In general, replicating the aspects of the procedure that contribute to a large part of the overall variation followed by an appropriate analysis to generate the reportable value will lead to improved precision. Estimating the amount of variation associated with each step of the procedure during the procedure design phase can allow for determination of an optimal sample and standard replication strategy, which may enable the procedure to more easily meet the criteria of the ATP for the reportable value. Consideration should be given to including the acceptable variability among the individual results for the factors (e.g. sample preparation, sample injection etc.) being replicated in system suitability criteria (see *Figure 8* for an example of such a criterion).

**Analytical Control Strategy (ACS)**

The ACS is a set of controls needed to ensure the procedure performs as expected and plays a key role in ensuring that the ATP is realized throughout the life cycle. The preliminary ACS is identified during the procedure development process in *Stage 1* and includes SSTs and other environmental or procedure controls needed for the procedure to meet the ATP. It is derived from an understanding of the analytical procedure as a process and the requirements defined in the ATP. Critical procedure attributes, identified during development, should be controlled, and their acceptable conditions, materials, or criteria should be explicitly specified in the procedure.

The analytical procedure with its associated initial control strategy is the input to *Stage 2* of the life cycle.

## STAGE 2: ANALYTICAL PROCEDURE PERFORMANCE QUALIFICATION (APPQ)

*Stage 2* involves confirming (or qualifying) that the procedure meets the requirements of the ATP in the facility where the procedure will be routinely operated. This qualification activity takes place once the initial ACS has been defined.

The elements of *Stage 1* described above establish the analytical procedure, preliminary ACS, and replication strategy and conclude the initial development activities. Once the procedure is established, the procedure is ready to undergo analytical procedure performance qualification (APPQ). APPQ occurs in *Stage 2* of the life cycle and evaluates the procedure to determine if it is capable of consistently generating a reportable value that meets the defined ATP and is suitable for its intended purpose in the laboratory. APPQ is a term inspired by the FDA Guidance for Industry *Process Validation: General Principles and Practices* in which PPQ is used for process performance qualification that confirms the commercial manufacturing process design and performance is as expected. Here, APPQ encompasses all of the analytical procedure activities commonly referred to as qualification, verification, validation, and transfer described in other literature and guidances. This chapter expands these well-known activities to integrate them into the procedure life cycle and include them in a process that incorporates the ATP.

**Protocol and Study Design**

APPQ is an important milestone in the analytical life cycle and is thus a documented activity under protocol. The extent of the APPQ study design depends on several factors including the type of technology of the procedure being qualified.

The protocol for a qualification study generally includes (but is not limited to):

- The ATP.
- The acceptance criteria needed to meet the ATP (e.g., accuracy, precision, range) and procedure-specific performance characteristics (e.g., specificity, calibration model, quantitation limit). Also refer to other appropriate guidance related to procedure validation, verification, or transfer. Depending on the data collected in *Stage 1*, it may be appropriate to include by reference instead of repeating the experiment.
- A description of (or reference to) the procedure and its initial analytical procedure controls, including system suitability requirements.
- A description of the qualification experiments that will be performed and the statistical approach to be used to analyze the data. If appropriate, the experimental design can include varying noise factors in order to demonstrate acceptable performance under routine use.
- Acceptable performance and robustness under routine use.
- References to platform, universal, and general procedures that cross multiple products and analytes that may allow for reduced experiments based on previous qualification studies.
- Reference to data generated during *Stage 1* that will be used to demonstrate qualification (e.g., robustness).

**APPQ Results and Documentation**

Upon successful completion of the qualification study, an evaluation should be performed to determine if the relevant analysis meets the criteria outlined in the ACS, including system suitability criteria, defined in *Stage 1*. Additional controls or modification to system suitability criteria may be included to reduce sources of variability that are identified in the routine operating environment.

When the qualification experiments are complete and the results meet the predefined acceptance criteria and are justified to be acceptable, the study is documented in a report. The report summarizes the protocol study design and ATP, presents the data and calculation results, and draws conclusions about the procedure's suitability for intended use.

If the study did not meet predefined acceptance criteria, it may be appropriate to return to *Stage 1* and perform additional procedure development activities before re-entering *Stage 2*. *Stage 2* may also be reinitiated when significant changes to the analytical procedure operating conditions are needed or if there is a need to update the ATP.

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Analytical procedure transfer as well as verification of compendial procedures fall under *Stage 2* and are governed by the same documentation descriptions above. The extent of the study design for these activities should be based on the ATP and on an assessment of the risks to the procedure performance when a procedure is transferred or a compendial procedure is adopted.

The last steps in *Stage 2* are to finalize the documented procedure (including learnings from the APPQ) and to finalize the ACS. The replication levels (multiple sample and standard preparations, multiple injections, etc.) may be updated based on the precision of the reportable value found during the qualification. The impact of changing the replication strategy must consider its continued ability to meet the ATP.

After the completion of the *Stage 2* activities, the procedure is released for routine use; that is, it may be utilized for its intended purpose for testing.

**STAGE 3: ONGOING PROCEDURE PERFORMANCE VERIFICATION (OPPV)**

*Stage 3* of the procedure life cycle ensures that the analytical procedure remains in control during routine use and thus continues to meet the ATP criteria.

This stage includes both routine monitoring of data linked to the performance of the analytical procedure and evaluation of the procedure's performance after changes to determine if the analytical procedure continues to be fit for purpose. Since a large number of analyses may be performed over time during the application of the analytical procedure, the monitoring provides an excellent opportunity to obtain reliable performance data during the routine application. This in turn provides the opportunity to identify unusual behavior and lack of expected performance.

**Routine Monitoring**

Effective monitoring of an analytical procedure provides ongoing confidence that the reportable values generated are fit for purpose.

This stage includes an ongoing program to collect and analyze data that relate to analytical procedure performance. Monitoring may include tracking analytical performance attributes such as SSTs including SST failures, batch data trends including analytically caused out-of-specification or out-of-trend results, trends, and other attributes as appropriate. The extent of monitoring should be based on the risk associated with the respective quality attribute and with the analytical procedure. The monitored data and information should be evaluated periodically depending on the number of analyses performed. If there is an indication that the procedure is not in control, an investigation should be conducted and corrective and preventive actions taken.

**Analytical Control Attributes**

Data related to procedure performance can be extracted from the application of the analytical procedure itself or from additional analyses of control samples. Control samples can be those that are identical to or representative of the samples to be analyzed, a standard check result, or a standard addition to the sample. Such additional controls may be implemented on a risk basis, i.e., for complex procedures, or if indicated by performance failures in the monitoring program.

The ACS can be used as a source for identifying the relevant attributes used for ongoing monitoring, for example SST attributes such as system precision, signal-to-noise ratio, or peak symmetry. Operational attributes that are not directly linked to the ATP may not be well suited for the performance monitoring program. However, monitoring of such attributes, e.g., HPLC peak resolution or peak symmetry, may still provide benefits to proactively determine required activities, such as HPLC column replacement.

Direct monitoring of the reportable value with comparison to the ATP is best performed by analyzing control samples that are adequately representative of the test samples. As an alternate option, batch reportable values may be directly monitored. Although these values are comprised of both process- and analytical procedure-related variability, assessing their control charts allows the detection of changes and trends. Further investigations would be required to understand the cause, whether process or procedure related.

**Control Charts**

The use of control charts is a recommended practice for monitoring of procedure performance attributes and control sample results. A control chart is a plot of a response variable versus time with limits that are based on the distribution of expected values around a mean or target value (*Figure 8*). This approach is based on the premise that, no matter how well a procedure is designed, there exists a certain amount of natural variability in measurements. When the variation is due to random causes alone, the procedure is said to be statistically in-control, and values on the chart should generally stay within the control chart limits.

Control chart limits can be established to detect the presence of special causes of variation, sudden process shift, longer term drift, or excessive noise. When special cause variation is detected, an investigation can be conducted to determine the root cause and appropriate corrective actions.

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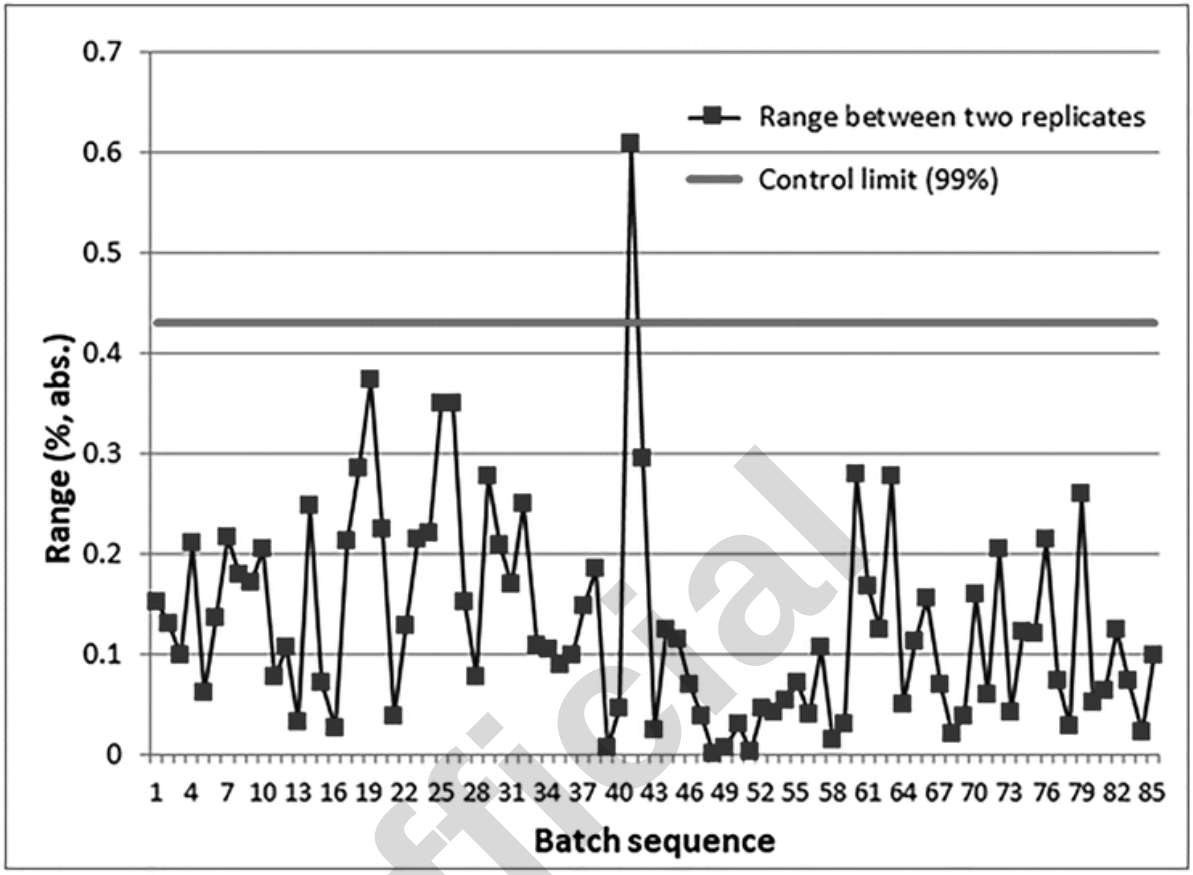


Figure 8. Example of a control chart for an API titration content range from two replicate determinations.

In addition to the monitoring of these analytical control attributes, average (pooled) performance attributes can be calculated, such as an average standard deviation, a standard deviation from duplicates, or precision of the reportable value from control samples. Monitoring analytical control attributes over time provides data that can be used for improved understanding of the true variation of components of the procedure and the overall procedure variation. This improved understanding can be valuable for determining necessary improvements to the procedure and for improving the ACS.

**Changes to an Analytical Procedure**

Changes to analytical procedures may be needed over the life cycle and can be prompted by several events including needed changes identified through a routine monitoring program, the adoption of new technology, or changes to the ATP. Such changes should be risk assessed for their impact to determine the appropriate activities required. In addition, appropriate change management approaches and documentation should be used when making changes to a procedure.

Depending on the degree of change made to the analytical procedure, the actions required to confirm the performance after the change will vary. The level of activities required to confirm that a changed analytical procedure is producing fit-for-purpose data will depend on an assessment of the risk associated with the change, the knowledge available about the procedure, and the effectiveness of the ACS.

**Examples of change**

- An adjustment to a procedure parameter to a value within the range that was previously qualified (e.g., a change within the MODR) may not require additional experimentation before implementation. However, any changes to the routine operating conditions within the MODR should be risk assessed against the ability to meet the ATP criteria and further studies performed as needed prior to implementation of the new operating conditions. An adjustment of the established monitoring limits also might be required.
- A change to a procedure variable to a value outside the set point or range that was previously qualified would require a risk assessment, which should consider the procedure performance characteristics that may be impacted by the change. These performance characteristics should then be considered in a PPQ study to confirm that the change does not impact the procedure's ability to meet the ATP.
- A change to a new laboratory (i.e., analytical transfer) would require review of the risk assessment and appropriate qualification activities to ensure that the implemented procedure meets the ATP requirements.
- A change to a new procedure/technique would require new or additional appropriate design and qualification activities (*Stage 1* and *Stage 2*) to demonstrate conformance of the new procedure to the ATP.

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- A change impacting the ATP, e.g., a specification acceptance limit change, would require a new ATP and review of the existing procedure design and qualification data (*Stage 1* and *Stage 2*) to determine whether the procedure will still meet the requirements of the new ATP.

## GLOSSARY

**Analytical Control Strategy (ACS):** A planned set of controls derived from current analytical procedure understanding that ensures the analytical procedure performance and the quality of the measured results and reportable value.

**Analytical Procedure Performance Qualification (APPQ):** General term for all activities performed in Stage 2 of the Analytical Procedure Life Cycle, i.e., to demonstrate that the procedure is suitable for routine application in the respective laboratory. This may include ICH validation, verification of compendial procedures, or transfer. [NOTE—Other definitions are also used for qualification of biological procedures, reference materials, and instrument qualification.]

**Analytical Procedure Validation:** All activities that confirm that a procedure is suitable for the intended purpose that take place over the entire life of the procedure. [NOTE—This interpretation is broader and more holistic than those in ICH. Within the context of the ICH Q2-Guidline, validation activities expected for a submission application are described. These activities take place in the lifecycle Stage 2, which also includes verification of compendial procedures, and analytical transfer.]

**Analytical Target Profile (ATP):** The ATP defines the required quality of the reportable value and is a description of the criteria for the procedure performance characteristics that are linked to the intended analytical application and the quality attribute to be measured.

**Bias:** Estimate of a systematic measurement error.

**Fit for purpose:** Demonstration of meeting the intended use as specified in the ATP through the analytical procedure lifecycle.

**Measurement Uncertainty:** A parameter associated with the result of a measurement, that characterizes the dispersion of the values that could reasonably be attributed to the measurand.[1]

**Method Operable Design Region (MODR):** A combination of analytical procedure parameter ranges within which the analytical procedure performance criteria are fulfilled and the quality of the measured result is assured.

**Ongoing Procedure Performance Verification (OPPV):** General term for all activities performed in Stage 3 of the Analytical Procedure Life Cycle. Assurance that an analytical procedure remains suitable during the routine application by verification of its ongoing suitability after changes, and by monitoring of performance parameters to identify potential performance changes or adverse trends.

**Platform analytical procedure:** A platform analytical procedure can be defined as a multi-product method suitable to test quality attributes of different products without significant change to its operational conditions, system suitability, and reporting structure. This type of method would apply to molecules that are sufficiently alike with respect to the attributes that the platform method is intended to measure.

**Quality Risk Management (QRM):** A systematic process for the assessment, control, communication and review of risks to the quality of the analytical procedure over the product lifecycle.

**Reportable Value:** The reportable value is the end result of a completed measurement procedure, as documented. The reportable value, which often is a summary value for several individual determinations, is compared with the acceptance criteria (see *General Notices 7.10 Interpretation of Requirements*).

**Total analytical error (TAE):** Total analytical error (TAE) represents the overall error in a test result that is attributed to imprecision and inaccuracy; TAE is the combination of both systematic error of the procedure and random measurement error.

**Transfer of analytical procedures (TAP):** Documented process that qualifies a laboratory (the receiving unit) to use an analytical test procedure that originated in another laboratory (the transferring unit), thus ensuring that the receiving unit has the procedural knowledge and ability to perform the transferred analytical procedure as intended (USP ⟨1224⟩).

**Verification of compendial procedures:** Documented assessment of whether the compendial procedure can be used for its intended purpose, under the actual conditions of use for a specified drug substance and/or drug product matrix (USP ⟨1226⟩).▲ (USP 1-May-2022)

[1] USP *PF* 44(1) Measurement Uncertainty for the Pharmaceutical Industry.

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# ⟨1222⟩ TERMINALLY STERILIZED PHARMACEUTICAL PRODUCTS—PARAMETRIC RELEASE

***Change to read:***

## INTRODUCTION

Parametric release is a practice to release finished product that relies on process control in lieu of end product testing to establish that a product is safe, pure, efficacious, and of suitable strength for commercial or clinical use. Parametric release is based on demonstrating that in-process conditions relevant to the establishment of key product quality attributes were attained and maintained throughout the relevant manufacturing steps. One attribute for which in-process controls would replace end product testing is sterility. The radiation sterilization of articles described in *Radiation Sterilization* ⟨1229.10⟩ performed in accordance with International Organization for Standardization (ISO) 11137 was the first sterilization process to use in-process controls and monitoring to replace sterility testing. The application of parametric release specifically to the attribute of sterility for commercial use requires prior regulatory approval.

Appropriately designed, validated, and controlled sterile product manufacturing systems are capable of exceptionally consistent performance in the preparation of products that have a probability of a nonsterile unit (PNSU) of $\leq 10^{-6}$. The exceptionally low probability of microbial presence in products manufactured using these systems renders the analytical methods described in *Sterility Tests* ⟨71⟩ statistically ineffectual.

While theoretically the limit of detection of the sterility tests is one viable cell, in actuality the limit of detection is likely far greater. A major limitation of ⟨71⟩ is that it is based on a limited sample. The probability of failing a sterility test given a contamination rate of 0.1% (an unacceptably high level of contamination) is 2% (where $n = 20$). For the contamination control capabilities achieved by well-controlled manufacturing systems, the probability of a sterility test detecting that level of contamination would likely be well below the limit of detection of the procedure. For these reasons, parametric release is the default mode of product release and should be used in lieu of sterility testing unless parametric release is not feasible.

In a parametric release program, sterility assurance is achieved by process, facility, and systems engineering; the establishment of appropriate risk-based user requirement specifications; and is conclusively demonstrated by the establishment, control, and monitoring of process parameters that confirm those user requirements are met. All modalities of sterile manufacturing should achieve ▲▲ (ERR 1-Nov-2019) a PNSU of $\leq 10^{-6}$. Parametric programs of product release should ensure all elements referenced in *Sterilization Assurance* ⟨1211⟩ consistently support the highest level of control.

Conditionally, terminal sterilization processes for the manufacture of products labeled sterile are candidates for parametric release. Terminal sterilization is the application of a suitable sterilization technology to a product, in its final sealed container or packaging, in a manner that achieves ▲▲ (ERR 1-Nov-2019) a PNSU of $\leq 10^{-6}$. Terminal sterilization may be conducted using any sterilization method that can be demonstrated to achieve a PNSU of $\leq 10^{-6}$ while at the same time retaining all other required product quality attributes. Examples of sterilization methods that have been successfully used to sterilize drug products or medical devices in their primary package are moist heat, dry heat, gas, and radiation. Processing parameters, critical processing parameters, and operational ranges ensuring the requisite microbial destruction should be understood, defined, measured, and controlled to support parametric release.

## USER REQUIREMENTS

Establishing precise, clearly defined requirements that demonstrate in-process conditions are attained and maintained throughout the relevant manufacturing steps is essential for parametric release. Therefore, organizations wishing to develop and validate a candidate parametric release program must define the necessary user requirements specification (URS) and ensure that all conditions within that specification are met. Process control conditions and validation within the URS should be acceptable to relevant competent regulatory authorities.

The URS should include all critical functions of the technology, equipment, manufacturing process, environmental requirements, operational requirements, and other important characteristics necessary for ensuring provision of a sterile product. Since these requirements will be specific to the organization, the sterilization process utilized, the product and its intended use, the URS should be generated by the user.

In developing and validating a parametric release program, critical quality attributes and process parameters should be included in the URS:

**Critical quality attributes:** Criteria immediately before and after sterilization should be achieved to ensure that the sterility of every unit is sustained through expiry.

**Process parameters:** These should be accurate predictors of the assurance of product sterility. The operating ranges are developed based on the sterilization process, process capability, calibration tolerance limits, and process parameter criticality. Process parameters necessarily should be based upon a thorough process knowledge and understanding of risk. Control of critical process parameters within their validated operating ranges is necessary to assure the sterility of product manufactured within a parametric release program. Any failure to adequately control critical process parameters within the established, validated operating ranges will result in the disposal of the processed product.

**Presterilization critical quality attributes and process parameters:** Presterilization process parameters are important to ensure a consistent and minimized microbiological challenge to the sterilization process. For a parametric release program, the level of control of the environment and presterilization product intermediate bioburden (amount, frequency of recovery, and species) should be within validated ranges and ensure the efficacy of the subsequent sterilization. Recovery of species or forms of microorganisms that are generally recognized as representing an increased challenge to

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the sterilization process (e.g., spores for moist heat sterilization) should be infrequent as demonstrable by historical data. The sterilization process might not effectively control pyroburden (which is usually endotoxins). Therefore, pyroburden should be appropriately controlled throughout the manufacturing process. *Table 1* summarizes the minimum presterilization quality attributes and associated process parameters.

**Table 1. Presterilization Critical Quality Attributes and Associated Process Parameters**

| Critical Quality Attributes | Process Parameters |
|---|---|
| Bioburden and the absence of specific microorganisms exhibiting an increased challenge to the mode of sterilization | • Pre-container-filling filter integrity[a]<br>• Hold times (formulated product and filled product units)[a]<br>• Cleaning and sterilization (where appropriate) of product contact surfaces[a]<br>• Environmental and personnel control performance<br>• Bioburden of excipient, raw material, drug substance, container, closure |
| Pyroburden | • Hold times (formulated product and filled product units)[a]<br>• Cleaning and sterilization (where appropriate) of product contact surfaces[a]<br>• Pyroburden of excipient, raw material, drug substance, container, closure |
| Filled container and closure integrity | • Filling and sealing control performance[a]<br>• Container and closure component dimensions and material properties |

[a] Critical process parameters.

**Sterilization process parameters:** Control of process parameters during the sterilization process is important to ensure a consistent and predictable level of product sterilization within the process' validated state. During the sterilization process, critical process parameters are monitored and recorded. Appropriate chemical, physical, or biological load monitors should be used to demonstrate lethality imparted upon the load. For example, the parametric release of most heat terminally sterilized products should use Class 5 indicators or, in certain circumstances, Class 3 (ISO 15882:2008) load monitors. Load monitors are located at specific locations in the load determined from development and qualification data to ensure each unit receives the required minimum sterilizing conditions. Certain container–closure configurations may possess sites that are less accessible to the physical or chemical agent used to terminally sterilize (e.g., the obscured interface between a container and its closure). In these circumstances, biological indicators and heat penetration probes (where applicable) should be placed at such locations during development and qualification studies to determine the required process parameters. For a parametric release program, the risk of human error should be minimized by use of electronic and automated systems to ensure the control of the sterilization cycle. In addition to qualified personnel, an automated electronic assessment of the sterilization cycle should assess the critical process parameters against the validated values. The assessment should be recorded and evaluated for the disposition of product. The following are examples of sterilization critical process parameters:

- Distribution (maximum, minimum, profile) of the physical or chemical agent throughout the entire cycle
- Time duration (minimum and maximum for any phases of the cycle)

**Post-sterilization:** If all presterilization critical quality attributes and sterilization process parameters are within their validated ranges, then all product units should be accepted as sterile. Failure to adequately control and measure critical process parameter data within the established, validated operating ranges of the sterilization process results in the disposal of the processed product. Under these circumstances, the application of a sterility test to ensure sterility is not permitted. Repeat sterilization of product from prior processing that failed to meet critical process parameters is only acceptable when supported with appropriate validations and regulatory approvals.

Subsequent to processing, successfully sterilized product should be physically segregated from nonsterilized and nonsterile product. Facility and/or equipment design may ensure physical segregation. Furthermore, an electronic or automated system should be used to reduce or remove the potential for mix-up due to human error.

## RISK ASSESSMENT

A well-designed parametric release process is superior because the sum aggregate of risks associated with elements of the program are lower compared to those of a non-parametric program reliant upon the sterility test. Therefore, a parametric release program is founded upon a thorough assessment, understanding, and management of risk. The multiple requisite elements of the program addressing engineering, process, procedural, and human factors require several assessments of risk to account for the different failure modalities and risk factors for severity and probability. All risk assessments should include the process parameters, critical process parameters, and the means of mitigating risks that may contribute to the failure to achieve critical quality attributes. These risk assessments are required to ensure that every product unit in every sterilization load within every manufactured batch achieves the required level of sterility assurance. For new products or sterilization processes, a risk assessment should be conducted during process development. For existing product or processes, the risk assessments should include historical data evaluation.

The following risk assessments are necessary to support a parametric release program:

**Presterilization product bioburden:** Assessing the potential risk of high bioburden or highly resistant microorganisms (e.g., spore formers), the means of enumeration and characterization, and opportunities for and modalities of product contamination. The risk assessment should additionally include other bioburden-related risks that may not be controlled by the sterilization process, such as pyrogens, and their potential to detrimentally alter the formulation chemistry of the product.

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**Loading patterns:** Evaluating the positioning, stacking, and distribution of product units within the sterilizer load, including the potential for damage during the loading of each load and during the sterilization process. Include the risk of product units failing to achieve sterility due to the aforementioned factors.

**Container and closure:** The container and closure system is an important material attribute in which risk assessment should evaluate the risk of non-integrity before, during, and after sterilization. This includes secondary packaging and the potential for damage, and event-related or time-related loss of integrity which could potentially allow microbial ingress.

**Sterilization cycle:** Evaluating the controls, real-time measurement (including the number, type, and position of load monitors), data acquisition, data handling, automated and electronic systems of process control and monitoring.

**Product segregation:** Risk assessment of product after sterilization should evaluate the means of product management and reconciliation (manual or electronic) and the physical segregation (manual or automated) of sterilized product from presterilized and nonsterilized product.

## REFERENCES

1. International Organization for Standardization. ISO 15882:2008: Sterilization of health care products—Chemical indicators—Guidance for selection, use and interpretation of results.

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# ⟨1223.1⟩ VALIDATION OF ALTERNATIVE METHODS TO ANTIBIOTIC MICROBIAL ASSAYS

## INTRODUCTION

Microbiological assay methods have traditionally been used to quantify the potency, or antimicrobial activity, of antibiotics. These microbiological procedures were historically used to certify antibiotics on a lot-by-lot basis to ensure sufficient activity. Before 1998, monographs for several approved antibiotics were published in FDA's Code of Federal Regulations, 21 CFR. These regulatory antibiotic assay procedures were later published in *USP–NF* as the official referee methods to determine the potency of antibiotics. The details of the microbial assay procedures for individual antibiotics, including the challenge organisms and test parameters, are described in *Antibiotics—Microbial Assays* ⟨81⟩. Microbial assays provide a direct measure of the effectiveness of the antibiotic against a reference microorganism. Although these microbiological methods have continued to serve as the official compendial referee methods since their publication in *USP–NF*, many manufacturers have replaced these microbiological analyses with high-performance liquid chromatography (HPLC) methods.

Although the microbiological methods provide direct proof of antimicrobial effectiveness and can integrate all moieties that contribute to antimicrobial effects in a formulation, these methods are less precise, more complex to perform, and slower to complete than alternative methods such as HPLC. Microbial assays also have limited selectivity and are not appropriate for evaluating organic impurities. The specific skill sets required to perform the microbiological antibiotic assays, their unique equipment requirements, and their comparative complexity deter many stakeholders from using these methods.

There are numerous reasons for replacing the microbiological antibiotic assays with chemical assays that use purity or content as surrogates for the measurement of biological activity. The advantages of chemical-based analytical methods have been described previously for simple, single-component antibiotics, as well as complex, multicomponent antibiotics (*1*). Physicochemical procedures, such as HPLC, allow for simpler preparation and rapid data acquisition with improved precision, accuracy, selectivity, and specificity. HPLC methods can be used effectively for both potency assignment and organic impurity testing. Additionally, because modern instruments and the expertise to use such equipment are widely available, the conversion to alternative methods may be economically advantageous.

This general chapter provides points to consider for manufacturers who want to use physicochemical alternatives instead of the microbial assay methods described in ⟨81⟩. Given the widespread use of HPLC as an alternative to microbial assay methods, this chapter focuses on HPLC methods. However, the principles set forth in this chapter are applicable to any alternative physicochemical procedure.

***Change to read:***

## GENERAL CONSIDERATIONS FOR ALTERNATIVE METHOD DEVELOPMENT

Multiple important factors need to be considered when replacing microbial methods with HPLC or other chemical techniques. This is because of the specific characteristics of antibiotics.

1. It is essential to know whether the antimicrobial activity manifested by a preparation results from a single, active ingredient or arises from multiple, often related, moieties. Where technically possible, the analyst should determine the contribution of major moieties ▲and the potential impact of their interaction▲ (USP 1-Aug-2022) to antibiotic effectiveness. For the purposes of this chapter, major moieties are defined as those that contribute more than 1% of the antibiotic potency. Where multiple moieties contribute to antibiotic activity, the chemical assay should be able to resolve all major moiety peaks in the formulation.
2. ▲To the extent possible, the individual activities of process impurities and degradation products that contribute more than 1% of the antimicrobial activity of an antibiotic preparation should be evaluated. This may not be necessary where evidence exists that each of the process impurities and degradation products contributes less than 1% of the total antimicrobial activity of a preparation.
3. As an alternative approach, testing a high number of lots of variable potency can be considered. A model taking into account the contribution of the process impurities and degradation products to the activity can be evaluated and validated. This approach may allow the demonstration of equivalency between the microbial assay and the alternative procedure without knowing the activity of individual moiety and impurity.▲ (USP 1-Aug-2022)
4. General chapter ⟨81⟩ is the referee standard in any procedure comparison. Therefore, the currently official USP procedure should be performed by the manufacturer (or under that manufacturer's direction) to establish reference assay values within the test range. It may be necessary to evaluate data using the guidelines in *Analysis of Biological Assays* ⟨1034⟩. ▲▲ (USP 1-Aug-2022) Manufacturers should establish appropriate limits for precision and accuracy of the microbial assay procedure based upon their product knowledge.
5. The candidate alternative HPLC or other chemical method should be fully validated according to *Validation of Compendial Procedures* ⟨1225⟩.
6. The value of any method comparison is dependent upon the precision and accuracy of the assay test results obtained from both methods. No statistical comparison should be undertaken if the assay data from either method does not meet predetermined acceptance criteria for method validation.
7. The guidelines in this chapter do not apply to already marketed products for which the manufacturer has already received regulatory approval to use an alternative method. In all cases, the applicability of an alternative method can only be determined through submission and review by the relevant regulatory authority.

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8. Any alternative to a compendial procedure must be validated and ▲must produce comparable results to the compendial method or procedure within allowable limits established on a case-by-case basis▲ (USP 1-Aug-2022) (*2*). A *Stimuli* article published in *PF* 35(3) [May–June 2009] discusses the "equivalent or better" approach to evaluating alternatives to compendial procedures (*3*). It is expected that such a comparison will use appropriate statistical analysis. Examples of the recommended types of statistical analyses are outlined in this chapter.

***Change to read:***

## TECHNICAL CONSIDERATIONS

▲The microbial assay and the candidate alternative method should be performed as described in the appropriate general chapter (e.g., ⟨81⟩ for microbial assays and *Chromatography* ⟨621⟩ for HPLC procedures); other physicochemical methods may be used (e.g., capillary electrophoresis) and should be conducted according to the specific general chapter. Stability-indicating alternative procedures are recommended.

Complex antibiotics, which have more than one active moiety, generally require additional data compared to simple antibiotics, where all or almost all antimicrobial activity is contributed by a single moiety.▲ (USP 1-Aug-2022)

***Change to read:***

## BRIDGING STUDIES

Bridging studies are used to compare the data obtained from candidate alternative procedures to the microbial assay data to determine whether the alternative procedure is an acceptable substitute. ▲Two approaches for developing the alternative procedure are described in detail below.

### Approach I

1. Validate the candidate alternative procedure according to ⟨1225⟩. The procedure should be specific, linear, precise, accurate, sensitive, robust, and stability indicating.
2. Where process impurities or degradation products contribute more than 1% of the antimicrobial activity of an antibiotic preparation, the process impurities or degradation products should be evaluated whenever possible. Each antimicrobial moiety that has the potential to contribute more than 1% of the antimicrobial activity of the antibiotic preparation should be isolated, and values for the relative microbial activity factor (*F*) against the USP Reference Standard should be established. Note that purification of moieties present at low levels could be a difficult and time-consuming work. Also, evaluation of the activity of minor moieties can be difficult due to limited quantities available and precision associated with the purity of the samples. Active moieties including process impurities and degradation products at levels not more than 1% of antimicrobial activity may be disregarded. For simple antibiotics, this step may be skipped.
3. Evaluate a suitable number of production lots of the antibiotic to determine the variation range of process impurities and degradation products of the antibiotic. When available, lots representing the specification range should be evaluated. This step is done before testing the samples using the two methods to evaluate the variation of the production and help the selection of representative batches for the bridging study.▲ (USP 1-Aug-2022)
4. ▲▲ (USP 1-Aug-2022) Test a minimum of three separate lots of the ▲sample against▲ (USP 1-Aug-2022) the USP Reference Standard using the microbial assay method and the candidate alternative procedure. ▲It is important to select lots that cover the activity and purity specification range. Testing lots with similar activity and purity will not demonstrate method comparability. As a consequence, more than three lots will usually be tested. Successful method comparability necessitates testing lots with variation in activity and purity. Stability samples with varying amounts of active moieties should have a change in potency that directly correlates to the alternative method. Forced degradation products or out-of-specification (OOS) lots may be introduced in the study to strengthen its robustness.
5. A minimum of three replicate samples per lot using both the candidate alternative method and the microbial assay procedures should be tested. One replicate is defined as the test measurement of one sample preparation (i.e., one sample stock solution). When a limited number of similar lots is used, it▲ (USP 1-Aug-2022) may be necessary to increase the number of replicates based on the maximum allowed percentage difference (see *Data Evaluation*) and the standard deviation of the method (*4*). ▲When a complex antibiotic displays significant composition variability, more lots may be used in the study with reduction in the number of replicates. The number of lots and replicates should be balanced according to the precision of each method and the complex antibiotic composition variability to ensure the maximum potential causes of variability are taken into account. If the HPLC assay has better precision than the microbial assay, fewer replicates may be needed. Manufacturers may work with the relevant regulatory authority to decide whether the replicates and lots are sufficient to demonstrate the method equivalency. *Appendix 4* presents one methodology for determining sample size for bridging studies.▲ (USP 1-Aug-2022) If possible, prepare standard stock and sample solutions and subdivide them for use with the microbial assay and candidate alternative assay procedures. This provides paired data that can be analyzed (see *Appendix 2*).
6. Use the relative microbial activity factor (*F*) to convert the percentage purity values for each moiety, and then sum them to determine a combined potency value. ▲For example, potency= $\Sigma x_i F_i$, where $x_i$ is the content of moiety *i* and $F_i$ is its relative microbial activity factor.
7. Apply the appropriate analysis on the results obtained by the two methods to determine whether the candidate alternative and the microbial assay procedures yield comparable results. If necessary,▲ (USP 1-Aug-2022) apply appropriate outlier (see *Analytical Data—Interpretation and Treatment* ⟨1010⟩ and ⟨81⟩) and comparison tests (see *Data Evaluation*).

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▲The number of outliers should be limited and justification for their rejection should be provided in the bridging study report.▲ (USP 1-Aug-2022)

8. ▲▲ (USP 1-Aug-2022) If the bridging study fails, it may be necessary to include potency contributions from ▲process▲ (USP 1-Aug-2022) impurities ▲and degradation products▲ (USP 1-Aug-2022) that make a contribution below 1% ▲or take into account synergistic activity. Additional analysis may also be required to evaluate the accuracy of measured purity of isolated impurity samples.

## Approach II

In the case of complex antibiotics, it can be difficult, time-consuming, or even technically infeasible to separate and purify all active antimicrobial moieties and test their activity. An alternative approach that tests a significant number of lots of samples may be considered. An example of this approach implemented for proteins can be found in a *Stimuli* article published in *PF* 40(4) [July–Aug. 2014] (*5*).

1. Validate the candidate alternative procedure according to ⟨1225⟩. The procedure must be specific, linear, precise, accurate, sensitive, robust, and stability indicating.
2. Identify moieties that may contribute to antimicrobial activity. Active moieties including process impurities and degradation products at levels below 1% of antimicrobial activity may be disregarded.
3. Test an appropriate number of lots, with varying amounts of active moieties covering the specification range, by both methods. Forced degradation products and/or OOS lots may be used in the study to demonstrate robustness. These are called "training lots". The number of replicates and lots should be based on the maximum allowed percentage difference (see *Data Evaluation*) and the standard deviation of the method (*4*). *Appendix 4* presents one methodology for determining sample size for bridging studies. Manufacturers may work with the relevant regulatory authority to decide whether the replicates and lots are sufficient to demonstrate the method equivalency and whether the model is appropriate. When possible, prepare standard stock and sample solutions and subdivide them for use with the microbial assay and candidate alternative assay procedures. This provides paired data that can be analyzed (see *Appendix 2*).
4. Identify an appropriate model to convert the sum of percentage purity values for each moiety to a combined potency value by applying conversion factors (*F*). For example, potency = $\Sigma x_i F_i$, where potency is the microbial activity from the microbial test; $x$ is the content of moiety from the physicochemical method; $F$ is the conversion factor. $F$ is determined in step 5. More complex models may be used.
5. Apply appropriate statistical analysis on the results obtained by the two methods to determine the conversion factors (*F*) so that the candidate alternative procedure and the microbial assay procedure yield equivalent results to find out the correlation between the two methods. Confirm the validity of the model obtained by comparing the predicted and obtained potency on another set of lots called "validation lots". The involvement of a statistician to help design the study, determine the relevant variables, analyze the results, and define the model is highly recommended. Outlier analysis in step 7 of *Approach I* can be applied.
6. Stability samples with varying amounts of active moieties should have a change in potency that directly correlates to the alternative method.
7. If the bridging study fails, it may be necessary to test the relative microbial activity for each moiety as stated in *Approach I*.▲ (USP 1-Aug-2022)

***Change to read:***

## DATA EVALUATION

Described below are USP's recommendations for data evaluation. Alternative approaches such as Deming regression (*6*) ▲▲ (USP 1-Aug-2022) may also be used. For an antibiotic with a comparatively narrow assay range (80%–125% or narrower), follow step 1. For an antibiotic with a wide assay range (80%–125% or wider), follow steps 1 and 2.

1. Demonstrate the equivalence of results at the targeted potency of 100% using a two one-sided test (TOST) to test for equivalence. TOST offers several advantages over the *t*-test, which looks for differences (*7–10*). This requires the laboratory to set a maximum allowed percentage difference (such as 3%, 4%, or 5%), denoted by *k* in the appendices. Use the formulas in *Appendix 1* for independent samples (different samples used for the two procedures) or *Appendix 2* for paired samples (a set of samples each of which is assayed by both procedures).
2. Compare the chapter microbial assay and candidate alternative assay procedures using ▲▲ (USP 1-Aug-2022) samples that cover the full monograph range of activity values. Prepare a Bland–Altman (BA) plot (*Appendix 3*). There should be no evidence of an important trend and the BA 95% agreement limits should not extend outside a predetermined maximum difference established by the laboratory.

***Change to read:***

## APPENDIX 1: TOST FORMULAS FOR INDEPENDENT SAMPLES

On the basis of the knowledge of the product, the laboratory must establish the maximum allowed percentage difference between the average result for the candidate alternative assay and the average ▲result▲ (USP 1-Aug-2022) for the microbial assay. ▲▲ (USP 1-Aug-2022) A difference that meets the requirements indicates that the alternative method provides acceptable results in the specified compendial range as compared with the microbial assay. In statistical notation, what must be demonstrated is:

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$$100\left|\frac{\mu_{HPLC}}{\mu_{Microbial}} - 1\right| < 100k \quad \text{Equation 1}$$

▲$\mu_{HPLC}$ = mean value for alternative method (HPLC)
$\mu_{Microbial}$ = mean value for the microbial assay▲ (USP 1-Aug-2022)
$k$ = a small positive number such as 0.03 (for an allowed 3% difference; ▲when determining $k$, factors such as the variability of the microbial assay, product specification, and intended use of the product can be considered)▲ (USP 1-Aug-2022)

These are the two values to be compared.
Rearranging, this becomes:

$$-k\mu_{Microbial} < \mu_{HPLC} - \mu_{Microbial} < k\mu_{Microbial} \quad \text{Equation 2}$$

The idea of TOST is to consider the two inequalities of *Equation 2* separately. That is, to demonstrate the hypothesis in *Equation 2* at the 5% level, one must demonstrate both of the inequalities in *Equation 3* at the 5% level:

$$\mu_{HPLC} - (1 + k)\mu_{Microbial} < 0 \text{ and } \mu_{HPLC} - (1 - k)\mu_{Microbial} > 0 \quad \text{Equation 3}$$

For a comparison of means, the TOST is equivalent to considering two-sided 90% confidence bounds. If the bounds satisfy the inequalities in *Equation 3*, then equivalence has been demonstrated. When the samples for the two assays under comparison are different ("independent samples"), determine the following upper ($U$) and lower ($L$) confidence bounds:

$$U = \overline{X}_{HPLC} - (1 + k)\overline{X}_{Microbial} + t_{0.05,\,df}\sqrt{S^2_{HPLC}/N_{HPLC} + (1 + k)^2 S^2_{Microbial}/N_{Microbial}} \quad \text{Equation 4}$$

$$L = \overline{X}_{HPLC} - (1 - k)\overline{X}_{Microbial} - t_{0.05,\,df}\sqrt{S^2_{HPLC}/N_{HPLC} + (1 - k)^2 S^2_{Microbial}/N_{Microbial}} \quad \text{Equation 5}$$

▲$\overline{X}_{HPLC}$ = sample average for the alternative method (HPLC)
$k$ = a small positive number such as 0.03 (for an allowed 3% difference; when determining $k$, factors such as the variability of the microbial assay, product specification, and intended use of the product can be considered)
$\overline{X}_{Microbial}$ = sample average for the microbial assay
$t$ = one-sided 5% value for a $t$-distribution
$S_{HPLC}$ = sample standard deviation for the alternative method (HPLC)
$N_{HPLC}$ = sample size for the alternative method (HPLC)
$S_{Microbial}$ = sample standard deviation for the microbial assay
$N_{Microbial}$ = sample size for the microbial assay▲ (USP 1-Aug-2022)

For the number of degrees of freedom ($df$), use:

$$\frac{\left(\frac{S^2_{Microbial}}{N_{Microbial}} + \frac{S^2_{HPLC}}{N_{HPLC}}\right)^2}{\left[\left(\frac{1}{N_{Microbial} - 1}\right)\left(\frac{S^2_{Microbial}}{N_{Microbial}}\right)^2\right] + \left[\left(\frac{1}{N_{HPLC} - 1}\right)\left(\frac{S^2_{HPLC}}{N_{HPLC}}\right)^2\right]} \quad \text{Equation 6}$$

This is an approximation that assumes $k$ is small. If using software that only allows for integer degrees of freedom (e.g., Excel), use linear interpolation to obtain the $t$-value. Conclude that the two procedures are equivalent (i.e., any difference on average is acceptably small) for the given lot ($L$) if:

$$L > 0 \text{ and } U < 0 \quad \text{Equation 7}$$

Example 1 ▲(Results for one sample lot):▲ (USP 1-Aug-2022)

| Microbial Assay Data | HPLC Assay Data |
|---|---|
| 72.02 | ▲72.38▲ (USP 1-Aug-2022) |
| 67.3 | ▲71.92▲ (USP 1-Aug-2022) |
| 71.79 | ▲72.25▲ (USP 1-Aug-2022) |
| 71.16 | — |
| 69.06 | — |
| 75.56 | — |
| 74.7 | — |

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| Microbial Assay Data | HPLC Assay Data |
|---|---|
| 74.16 | — |
| 76.48 | — |

Following the above formulas with $k = 0.03$:

$N_{HPLC} = 3$; $\overline{X}_{HPLC} = {}^{\blacktriangle}72.18;{}^{\blacktriangle}$ (USP 1-Aug-2022) $S_{HPLC} = {}^{\blacktriangle}0.237{}^{\blacktriangle}$ (USP 1-Aug-2022)
$N_{Microbial} = 9$; $\overline{X}_{Microbial} = {}^{\blacktriangle}72.47;{}^{\blacktriangle}$ (USP 1-Aug-2022) $S_{Microbial} = 3.045$
Degrees of freedom ($df$) = ${}^{\blacktriangle}8.283;{}^{\blacktriangle}$ (USP 1-Aug-2022) interpolated $t$-value = ${}^{\blacktriangle}1.860{}^{\blacktriangle}$ (USP 1-Aug-2022)
$L = {}^{\blacktriangle}0.039{}^{\blacktriangle}$ (USP 1-Aug-2022) and $U = {}^{\blacktriangle}-0.500,{}^{\blacktriangle}$ (USP 1-Aug-2022) so the 3% equivalence criterion is satisfied.

***Change to read:***

## APPENDIX 2: TOST FORMULAS FOR PAIRED SAMPLES

When the samples for the two assays are the same, the data are considered "paired". The hypotheses are the same as those in *Appendix 1*. Because of the pairing of samples, the standard deviation calculations differ.

Determine the confidence bounds, $U$ and $L$, as follows:

$$U = \overline{X}_{\text{HPLC}} - \left(1 + k\right)\overline{X}_{\text{Microbial}} + t_{0.05,\,df}\sqrt{S_U^2/N} \quad \text{Equation 8}$$

$$L = \overline{X}_{\text{HPLC}} - \left(1 - k\right)\overline{X}_{\text{Microbial}} - t_{0.05,\,df}\sqrt{S_L^2/N} \quad \text{Equation 9}$$

$N$ = number of samples for each procedure
$df$ = $N - 1$

$S_L$ and $S_U$ are calculated as follows:

$${}^{\blacktriangle}S_U^2 = \frac{1}{N-1}\sum_{j=1}^{N}\left[X_{\text{HPLC},\,j} - \left(1 + k\right)X_{\text{Microbial},\,j} - \Delta\overline{X}_U\right]^2 \quad \text{Equation 10}$$

in which, $\Delta\overline{X}_U = \frac{1}{N}\sum_{j=1}^{N}\left[X_{\text{HPLC},\,j} - \left(1 + k\right)X_{\text{Microbial},\,j}\right]{}^{\blacktriangle}$ (USP 1-Aug-2022)

$${}^{\blacktriangle}S_L^2 = \frac{1}{N-1}\sum_{j=1}^{N}\left[X_{\text{HPLC},\,j} - \left(1 - k\right)X_{\text{Microbial},\,j} - \Delta\overline{X}_L\right]^2 \quad \text{Equation 11}$$

in which, $\Delta\overline{X}_L = \frac{1}{N}\sum_{j=1}^{N}\left[X_{\text{HPLC},\,j} - \left(1 - k\right)X_{\text{Microbial},\,j}\right]{}^{\blacktriangle}$ (USP 1-Aug-2022)

Conclude that the two procedures are equivalent (i.e., any difference on average is acceptably small) for the given lot if:

$$L > 0 \text{ and } U < 0 \quad \text{Equation 12}$$

Example 2 ${}^{\blacktriangle}$(Results for multiple sample lots):${}^{\blacktriangle}$ (USP 1-Aug-2022)

| Microbial Assay Data | HPLC Assay Data |
|---|---|
| 1011 | 980.9 |
| 990 | 981.4 |
| 960 | 978.3 |
| 1000 | 974.3 |
| 970 | 966.7 |

Following the above formulas with $k = 0.03$:

$N = 5$; $\overline{X}_{HPLC} = 976.3$; $\overline{X}_{Microbial} = 986.2$
$S_L = 18.749$; $S_U = 19.958$
$df = 4$; $t$-value = 2.132
$L = 1.830$ and $U = -20.438$, so the 3% equivalence criterion is satisfied.

***Change to read:***

## APPENDIX 3: BLAND–ALTMAN PLOTS

*Figure 1* shows the data plotted as HPLC assay ($x$-axis) versus microbial assay ($y$-axis). In *Figure 2*, the $x$-axis represents the average response obtained using the alternative and microbial assay methods. The $y$-axis represents the difference in responses measured with the alternative and microbial assay methods.

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The bias for each data set is represented by the average difference.

95% limits of agreement are represented by bias ± 2*S*.

Data points outside the limits of agreement are considered outliers.

The BA plot (*11–12*) should show data points scattered within the limits of agreement with no obvious pattern. If a pattern exists (e.g., proportional pattern or funneling outwards with increasing average), perform a log transformation. If performing a log transformation, take the antilog of results for analysis using the original scale of measurement.

Example 3:

| ▲Lot | Microbial Assay Data | HPLC Assay Data | Microbial Assay–HPLC Assay |
|---|---|---|---|
| 1 | 1213 | 1226 | –13 |
| | 1253 | 1284 | –31 |
| | 1276 | 1285 | –9 |
| | 1293 | 1354 | –61 |
| | 1222 | 1240 | –18 |
| 2 | 1155 | 1181 | –26 |
| | 1108 | 1131 | –23 |
| | 1118 | 1169 | –51 |
| | 1101 | 1082 | 19 |
| | 1136 | 1134 | 2 |
| 3 | 893 | 881 | 12 |
| | 805 | 790 | 15 |
| | 827 | 811 | 16 |
| | 821 | 812 | 9 |
| | 867 | 839 | 28 |
| 4 | 1085 | 1096 | –11 |
| | 1032 | 1050 | –18 |
| | 1046 | 1052 | –6 |
| | 1019 | 1024 | –5 |
| | 1048 | 1035 | 13 |
| 5 | 1000 | 977 | 23 |
| | 920 | 918 | 2 |
| | 978 | 964 | 14 |
| | 906 | 924 | –18 |
| | 903 | 898 | 5 |

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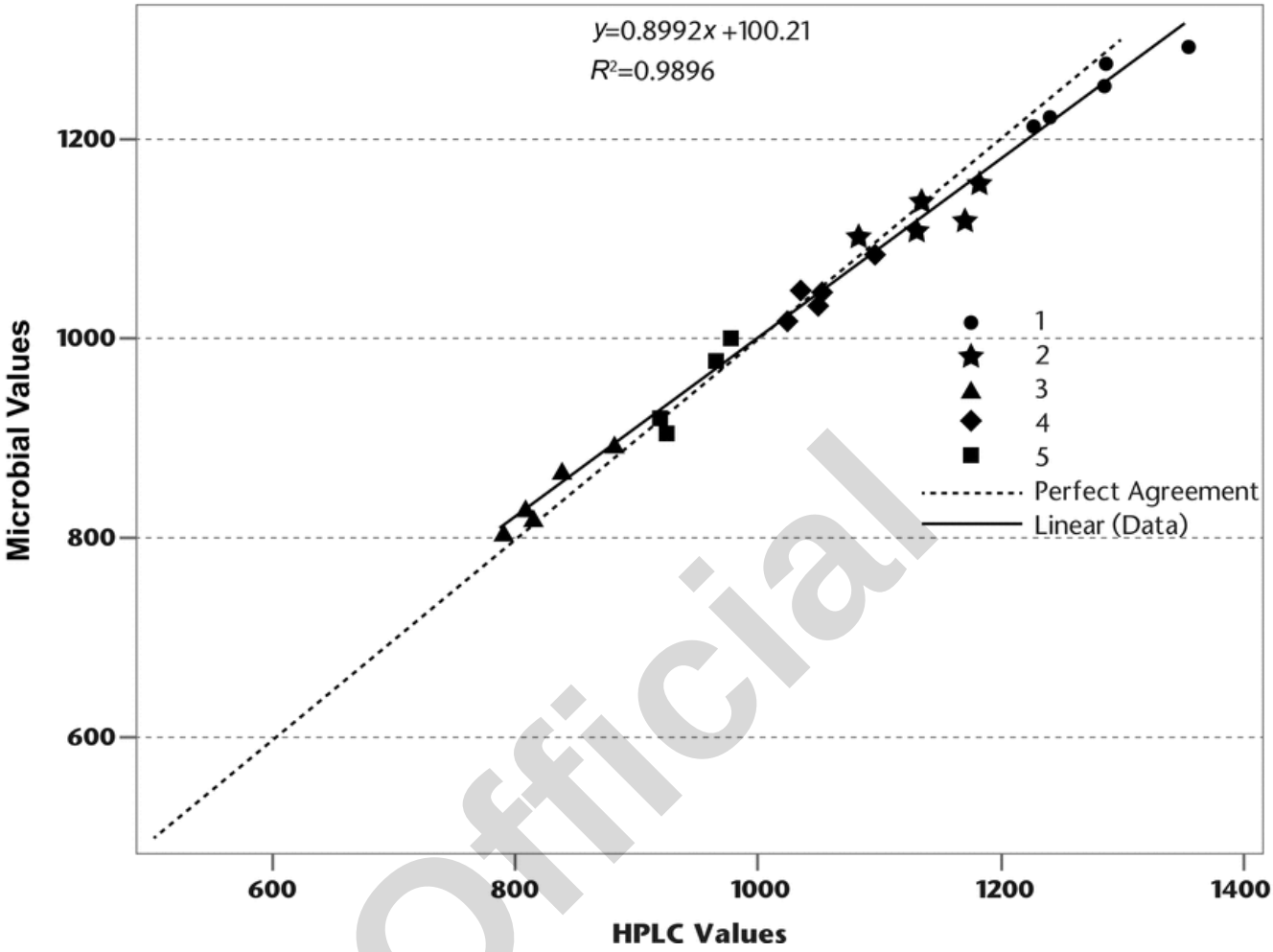


Figure 1. Example data plotted as microbial assay data versus HPLC assay data. ($R^2$= coefficient of determination)

▲ (USP 1-Aug-2022)

*Figure 1* shows the data plotted as microbial (*y*-axis) versus HPLC (*x*-axis). This appears to be in good agreement. *Figure 2* shows what happens when the same data are instead plotted as recommended by Bland–Altman.

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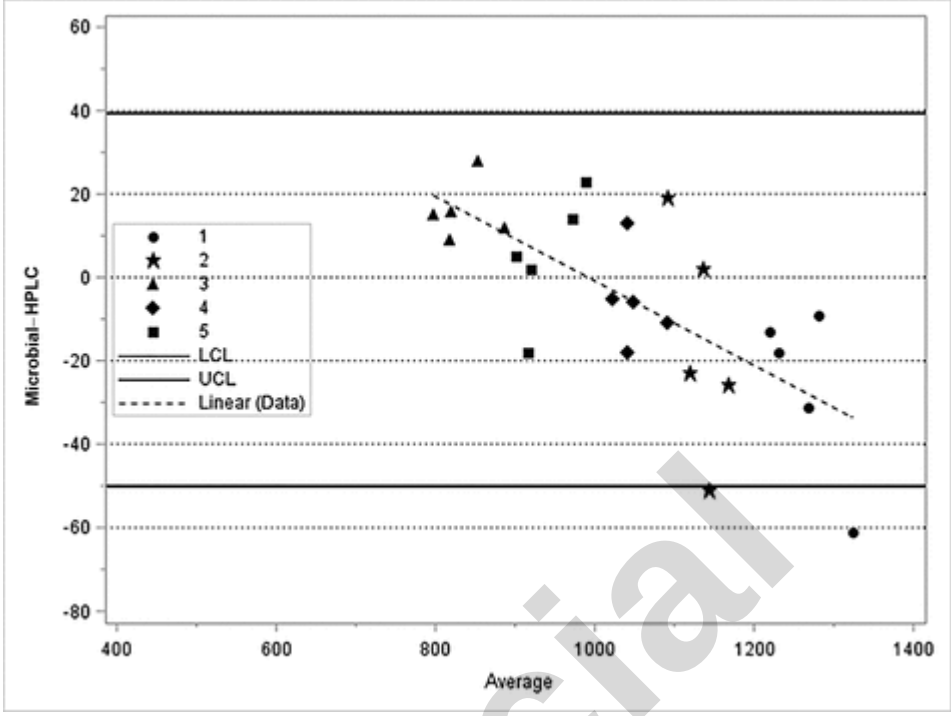


Figure 2. Example data in a BA plot. (LCL = lower control limit; UCL = upper control limit.)

▲ (USP 1-Aug-2022)

Calculate the bias, estimated by the mean difference ($d$) and standard deviation (SD) of the difference:

$d = -5.28$
SD of difference = 22.36

Most of the differences are expected to lie between $d$ – 2SD and $d$ + 2SD (if normally distributed, 95% will lie in these limits). If differences within $d \pm 2SD$ (limits of agreement) are not important, the two measurement methods can be used interchangeably:

LCL ($d$ – 2SD) = –49.95 Equation 13
UCL ($d$ + 2SD) = 39.39 Equation 14

The difference between results from the two procedures has a strong trend. This trend can be seen in *Figure 1*, but the BA plot in *Figure 2* makes it clearer.

***Add the following:***

## ▲APPENDIX 4: SAMPLE SIZE JUSTIFICATION FOR ANTIBIOTIC BRIDGING STUDIES

The methodology for determining sample size for antibiotic bridging studies presented in ⟨1010⟩ for the estimate to reflect the equivocal zone can be used. *Table 1* lists the sample sizes corresponding to different choices for three parameters in *Equation 15*.

$$N \geq \frac{2\sigma^2}{\delta^2}\left(Z_\alpha + Z_{\beta/2}\right)^2 \quad \text{Equation 15}$$

$N$ = sample size
$\sigma$ = standard deviation of the microbial assay [assuming 1000 µg/mg, $\sigma = 10 \times$ CV (coefficient of variation)]
$\delta$ = difference (±) from the true value that is considered equivalent
$Z_\alpha$ = Type I errors ($Z_\alpha$ is usually set at 95%)
$Z_{\beta/2}$ = Type II errors

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**Table 1**

| Power | $Z_{\beta/2}$ | δ | σ | *N* |
|---|---|---|---|---|
| 80% | 0.842 | 30 | 50 | 35 |
| | | 40 | 50 | 20 |
| | | 50 | 50 | 13 |
| | | 60 | 50 | 9 |
| | | 70 | 50 | 7 |
| | | 80 | 50 | 5 |
| 90% | 1.282 | 30 | 50 | 48 |
| | | 40 | 50 | 27 |
| | | 50 | 50 | 18 |
| | | 60 | 50 | 12 |
| | | 70 | 50 | 9 |
| | | 80 | 50 | 7▲ (USP 1-Aug-2022) |

***Change to read:***

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# ⟨1223⟩ VALIDATION OF ALTERNATIVE MICROBIOLOGICAL METHODS

## INTRODUCTION

This chapter provides guidance on the selection, evaluation, and use of microbiological methods as alternatives to compendial methods. To properly implement alternative methods, one must consider a number of important issues before selecting the analytical technology and qualifying that method with the actual product. These issues include, but are not limited to, identification of suitable alternative methodology, development of user specifications for equipment selection, demonstration of the applicability of the method as a replacement for a standard compendial method, and qualification of the method in the laboratory.

This chapter outlines:

- User requirements
  - Instrument qualification
  - Validation of alternate technologies
  - Method suitability
- The limitations of the use of CFU as a standard signal for microbiological methods.
- Four novel options for demonstrating equivalence
  - Acceptable procedures
  - Performance equivalence
  - Results equivalence
  - Decision equivalence
- Application of the concept of non-inferiority to method validation
- Guidance on statistical methods that may be employed in method validation

A glossary of the terms used in the chapter is provided at the end of this chapter.

Microbiological methods, other than microbial identification and strain typing methods (discussed in *Microbial Characterization, Identification, and Strain Typing* ⟨1113⟩), described in the compendia fall into two general categories:

1. Qualitative methods (not enumerative) that are used to assess the general microbial quality of compendial articles. This category includes assays that are intended to demonstrate the presence or absence of microorganisms in a compendial article.
2. Quantitative methods that yield a numerical (enumerative) result in terms of the microbial content of a compendial article.

There are inherent analytical factors that must be considered in the implementation of microbiological methods and in the comparison of a candidate alternative method to an existing compendial method. With respect to qualitative (“absence of”) analysis, it is critical to consider that in microbiology, the finding of “no microorganisms present” does not mean in absolute terms that zero cells are actually present in the compendial article. A result of “no growth” in a current compendial method is properly interpreted as “no growth was detected in the test sample from the compendial article under the specified conditions”.

The actual limits of detection of compendial microbiological methods have never been established quantitatively, and it is understood that many variables can affect the recovery of microorganisms. These variables include selection of growth media, incubation conditions, nutritional requirements of microorganisms that may be present, physical condition of microorganisms, and characteristics of the compendial article under test. Studies on the recovery of microorganisms from potable and environmental waters have demonstrated that traditional plate-count methods reporting cell count estimates as colony-forming units (cfu) may recover 0.1%–1% of the actual microbial cells present in a sample (1), in comparison to alternative methods that use flow cytometry and therefore yield a different signal (cell count). The presence of a greater number of cells based on an alternative method with a signal other than cfu has not correlated with more user risk or a higher likelihood of pathogens being present when there is an established safety record. These results do indicate that in some types of samples, the mean estimated cell count recorded using a growth-based compendial assay may result in a very different mean value than a cell count estimate derived from an alternative microbiological method that relies on a signal other than the cfu. Also, one must consider that in analytical microbiology the concept of false positive or false negative results are both scientifically and conceptually difficult. It would not be appropriate, for example to consider in a comparison between a standard compendial method and a candidate alternative method that a negative result in the standard method meant that positives observed in alternative method were false positives. It is a normal characteristic of a conventional growth-based method to recover some species well and yet to be unable to recover others. While it is not necessary for an alternative method and the conventional method to produce a match in terms of result, what is important is that the candidate method be capable of allowing a microbiologist to make an equivalent decision regarding product quality consistently.

It is extremely important in the application of this chapter that users take into account that microbiology is a logarithmic science. While we can distinguish between 100 and 1000 cfu (a difference of 1 $\log_{10}$), it may be not possible to discern smaller differences (less than 0.3–0.5 $\log_{10}$). The inherent variability of these methods is substantially greater than analytical chemistry methods. This inherent analytical variability must always be considered in the selection, development, and validation of alternative methods. The expectation of a degree of agreement between alternate microbiological methods and traditional growth-based methods beyond what is technically feasible could complicate the implementation of newer analytical technologies regardless of their specific mode of analysis.

Achieving the level of characterization (variability of the method) that is possible using modern chemical methods (e.g., high-performance liquid chromatography with a precision of 1%–2% relative standard deviation) is not possible in microbiology. It is reasonable to consider that the typical level of precision will typically be on the order of 15%–35% relative

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standard deviation, although results outside this range both on the high and low sides are certainly possible. Also, the enormous numbers and diversity of potential microorganisms as well as the inherent variability of metabolic activity levels in nature can complicate recovery. The advent of alternative microbiological methods, which in some cases may recover higher cell counts than typically observed using existing compendial methods, should not be taken to mean that new patient risks now exist that had not been heretofore recognized.

## *USP* Perspective on Implementation of Alternative Methods or Procedures

The *U.S. Pharmacopeia* (*USP*) has long provided mechanisms for the implementation of alternative assay methods or procedures to analyze compendial articles. *General Notices, 6.30 Alternative and Harmonized Methods and Procedures* states, "Alternative methods and/or procedures may be used if they provide advantages in terms of accuracy, sensitivity, precision, selectivity, or adaptability to automation or computerized data reduction, or in other special circumstances." This statement allows considerable user latitude in the decision to use an alternative procedure for routine product release, provided that proper technical and scientific attention is paid to the selection, qualification, and implementation of the method. If a product has proven safe in widespread use when released or controlled using current methods, the implementation of an alternative method which can be well-correlated to the existing method should be straightforward.

## *USP* Methods and Procedures as Referee Tests

All methods and procedures described in the *USP* general chapters on microbiological tests, which are numbered below ⟨1000⟩, are intended to be referee tests for any product legally marketed in the United States. This means that in the event a dispute should occur for any reason, only the result obtained using the method or procedure published in *USP* is conclusive. Thus, alternative methods or procedures implemented and qualified by a user will not serve as a legal replacement for the official *USP* method, which will continue to serve as the referee test in the case of a dispute.

## General Considerations Regarding Quality Control Product-Release Assays

Although the methods and procedures described in the *USP* general chapters are intended to be official referee test methods, it is recognized that these tests may not function optimally as quality control tests for specific compendial articles without modification. It is expected that the official *USP* methods will require evaluations to determine their suitability for use when applied to specific products, and that such evaluations will be conducted on the basis of a user's specific product knowledge. Procedures for demonstration of this method suitability (verification; inhibition/enhancement) are provided in the relevant compendial test chapters. For example, some compendial articles may have inherent antimicrobial properties that could, if not modified (or neutralized), adversely affect the suitability of a given compendial method or procedure. When these methods or procedures are included in regulatory filings and the product is approved, they will be used as product-release and shelf-life tests.

## Harmonized Chapters

Some *USP* microbiological methods or procedures contain a statement that they have been harmonized with corresponding methods or procedures found in the *European Pharmacopoeia* and the *Japanese Pharmacopoeia*. In chapters containing this statement, the text describing the method or procedure is considered interchangeable (for additional information, see *Pharmacopeial Harmonization* ⟨1196⟩). If a compendial method or procedure is conducted using harmonized text as written in the *USP*, *European Pharmacopoeia*, or *Japanese Pharmacopoeia*, it is considered legally interchangeable. However, the implementation of an alternative method as a quality control test to replace a method described in the *USP* does not mean this method or procedure would be interchangeable from the perspective of all relevant jurisdictions unless it meets the criteria for alternative methods as specified by the other compendia.

## Submission of Alternative Methods or Procedures to *USP*

In the *USP*, it is stipulated by *General Notices, 6.30 Alternative and Harmonized Methods and Procedures* that alternate methods should be submitted to the *USP* for evaluation, if an organization wishes to have the alternative method to be considered for inclusion as a compendial method. This opportunity to advance microbiological testing is often overlooked by USP stakeholders. Submission of alternative methods or procedures allows USP to consider any such method as an addition to or replacement for an existing, standard method or procedure. A submission of an alternative method must include complete analytical and equipment details, as well as detailed analytical data from relevant qualification trials.

For any method or procedure to be considered as a replacement or additional referee method, it must not be a patented method or procedure with reagents or instrumentation available from only a single source. Also, any candidate replacement or additional method must have broad applicability, suitable for routine use and must be compatible with a broad spectrum of relevant compendial articles.

## Additional *USP* Chapters Germane to the Implementation of Alternative Microbiological Methods

Several *USP* general information chapters provide usual information regarding the implementation of alternative methods and procedures. *Validation of Compendial Procedures* ⟨1225⟩ provides detailed information on submissions of alternative methods to the compendia as well as general guidance on validation and the evaluation of analytical performance characteristics. Useful information regarding the validation of methods used for the recovery of viable microorganisms from compendial articles

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(neutralization of antimicrobial properties of articles) appears in *Validation of Microbial Recovery from Pharmacopeial Articles* ⟨1227⟩. Information on the qualification of microbial identification methods may be found in ⟨1113⟩. Finally, information on the qualification of analytical equipment can be found in *Analytical Instrument Qualification* ⟨1058⟩.

Although *Analysis of Biological Assays* ⟨1034⟩ is primarily concerned with the determination of product potency by utilizing bioassays, it also discusses fundamental principles regarding variance, error, and biometrics that may be informative to those who are developing and validating alternative microbiological methods or procedures.

## Other Information and Regulation Regarding the Use of Alternative Methods

In the U.S. Food and Drug Administration (FDA) Current Good Manufacturing Practices regulations, 21 CFR Part 211.194 describes requirements for test methods utilized to assess the compliance of pharmaceutical articles with approved specifications. The regulations state that test methods must have suitable capability regarding accuracy and reliability. This subsection of the regulations also recognizes the legal basis of *USP* and the *National Formulary* (*NF*) standards and makes it clear that it is the responsibility of the user to validate methods or procedures that differ from those standardized in the compendia.

The International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) document Validation of Analytical Procedures: Text and Methodology [Q2(R1)] also may be a useful reference in the development and validation of alternative methods or procedures. This ICH document provides information useful in the submission of analytical procedures in association with product registration applications submitted within the United States as well as Japan and Europe.

# USER REQUIREMENTS

Determining the precise technical requirements necessary for an alternative microbiological method is essential before one can select the appropriate technology and equipment that meet the relevant assay requirements. Therefore, it is suggested that organizations wishing to develop and validate a candidate alternative method produce a user requirement specification (URS) document. This document should include all critical functions of the technology, critical user interface requirements, space requirements, environmental requirements, operational requirements, and all other important characteristics of an alternative method for the intended use. These requirements will be specific to the company or organization, as well as to the alternative method's intended use, and therefore the requirements should be generated by the user.

In generating this URS document, three separate components of the alternative microbiological method validation must be considered:

1. *Instrument qualification*. Most alternative microbiological methods will depend on specific equipment. This analytical equipment is subject to industry standard instrument qualification requirements (see ⟨1058⟩ for further information).
2. *Validation of alternate technologies*. The basic rationale for using an alternative methodology is to improve on some aspect of the existing technology of the current compendial method without sacrificing essential characteristics of that technology (e.g., plate count and membrane filtration). The current technology for compendial microbiology methods consists of detection of the growth of viable microorganisms on (or in) a nutrient medium. The alternative technology must be at least equivalent to the current technology in terms of performance for the intended use. Much of the technical support for equivalence may come from the peer-reviewed scientific literature or from a prior regulatory submission (e.g., a vendor submitted the Drug Master File to the FDA, or prior submission from a company on this technology), but this must be confirmed, as appropriate for the intended use.
3. *Method suitability*. This consideration must address both the technology's suitability to the specific test and the lack of product inhibition and enhancement on the test results.
    A. Suitability of the technology to the specific test. Many compendial microbiological tests have mandated test requirements. An example of this would be sample plans consisting of the quantity of material to be tested (e.g., 10 g or 20 units of a specific volume). Because the test results are frequently used to determine compliance with finished product specifications, and the specifications are dependent on sample volume or quantity, the alternative technology must be able to satisfy sample volume requirements as required in the general test method. The use of a lesser volume or sample size is not recommended and would need to be fully justified by the user on a case-by-case basis. The alternative technology is considered suitable if it can meet all critical parameters of the compendial test.
    B. Inhibition and enhancement. Specific products may interfere or enhance the signal of different measurement technologies to the specific signal of interest (see ⟨1227⟩). This component of alternative microbiological method validation (i.e., suitability) must be demonstrated for each product tested.

## Components of Data Quality

General information chapter ⟨1058⟩ describes four different components of data quality. The most fundamental component is qualification of the instrument; that is, a demonstration that the instrument is functioning as designed. Next in significance is the method validation; a demonstration that the technology is functioning as expected. For instance, this might be a demonstration that an alternative microbiological method is at least as suitable for its role in the test method as was the traditional plate count or recovery in nutrient broth. Next in importance is the inclusion of relevant controls in the test to demonstrate the ongoing suitability of the test system. The final component of data quality is the use of quality control samples, a practice not commonly used in microbiology because analysts strive to exclude live cultures from a product testing area. These different components of data quality are an important consideration in validating an alternative microbiological test as they help frame the URS.

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## Classical Microbiological Methods

The cfu has been in use for about 125 years and continues to be specified as the unit of microbial enumeration in all current *USP* monographs. However, it is important to understand that the cfu has always been an *estimation* of microorganisms present, rather than an actual count. The conceptualization of cfu as a signal requires a fundamental grasp of the process of plating bacteria, yeast, or mold on solid media, as well as knowledge of what is required to produce a single colony.

The plate count method provides an estimate of the number of microorganisms present based on the growth of discrete countable colonies on an individual plate; thus, the plate count is not a true cell count. Although it is theoretically possible for a single viable cell to give rise to a cfu, a single cell growing into a colony on a plate is unlikely to happen in nature. "Viability of a cell" is defined as the ability to multiply by binary fission such that a colony appears. For a colony to appear, viable cells must find specific conditions of nutrient growth medium, incubation, and time. Individual cells, however, are a rarity in nature, and it is far more likely that any colony growing on solid media arose from a clump, chain, or mass of cells deposited together. The cfu signal then is prone to underestimate the actual number of cells present in a sample. The extent of underestimation will vary, depending on the nature of the microorganism and the way in which the sample was prepared.

The cfu signal is also completely dependent upon recovery of microorganisms from environmental conditions, which produce stress on the organism's ability to survive. It is important to note that all organisms that are outside their preferred environmental niche will be to one degree or another stressed. Furthermore, outside laboratory culture microorganisms are unlikely to be in the exponential growth phase most optimal for transfer onto microbiological media for recovery and growth. In a very real sense most microorganisms present in compendial articles, in dry, nutrient-free environments, at elevated temperatures, high ionic strength, pH extremes, or in the presence of antimicrobial chemicals are severely stressed and may prove difficult or impossible to recover. Clearly these stress factors play a role in the plate count anomaly mentioned above. If the growth, nutritional, or incubation conditions presented to microorganisms are not sufficient to result in recovery and the growth of colonies, the signal may be 0 cfu, or no growth, even when viable cells are present. These stress factors may not be important considerations with nongrowth-based alternate methods and such methods may produce signals from cells that will not grow on media. Precision can be compromised further when organisms are present in large clumps, often associated with organic material, and are broken into smaller units during preparation. In this case, depending on the processing or handling of the sample, a clump could appear as a single colony or multiple colonies. Furthermore, the number of cfus on a plate must be in a countable range, for example bacteria, 25–250, for reasonably reliable enumeration.

Thus, the methods of growth-based microbiology represent a logarithmic science with a signal of enumeration (cfu) that is truly an estimate rather than a precise cell count. Understanding the strengths and weaknesses of the cfu as a signal is vital in the validation of an alternative method that uses an alternative signal. Therefore the cfu cannot be considered the only unit of microbiological enumeration.

## Signals from Alternate Microbiological Methods

Rapid or modern microbiological methods typically produce signals in units other than cfu for microbial estimation and enumeration. These signals are often processed via instruments rather than visually. Extensive studies have been conducted on the capabilities of the various methods that can be applied to microbial assessment of compendial articles, and in most cases the prospective user will know the characteristics of the method and the signal it produces before selecting that method as an alternative. Guidance on method selection is provided in the section on *Validation of Alternate Technologies* and in peer-reviewed scientific publications.

Most of the rapid microbiological methods are, to some extent, direct cell count methods. They, therefore, may provide a higher cell count estimate than the cfu method for a given sample, depending on how the method is used and which compendial article is under evaluation.

Some alternate or rapid methods detect and estimate cell counts on the basis of metabolic activity, which gives rise to a signal that can be measured instrumentally. Examples of these types of signals include adenosine triphosphate (ATP) content (bioluminescence), laser-induced fluorescence, enzymatic activity, and physiochemical changes to the composition of a nutritional broth or the headspace above the broth.

Alternative methods may also be based on vital staining, in which cells are stained or exhibit autofluorescence (based on cell components) and then are directly counted, either microscopically or instrumentally. To increase the probability that only living cells will be counted, multiple stains may be used, which can (1) increase sensitivity based on cell membrane function, (2) enhance reaction with nucleic acids, or (3) improve detection of metabolic activity.

There are also nucleic acid based methods that can be used, as well as a range of other physicochemical methods of analysis that have been utilized in pharmaceutical, biopharmaceutical, clinical, and food microbiology. These methods may target, amplify, detect, or quantify a nucleic acid sequence, and it is important to understand the type of signal that results from the analytical method. In addition, one should understand the physiological characteristic of the microorganism that gives rise to the signal, which then makes it possible to enumerate the cells.

### SUCCESS CRITERIA

Alternative methods for obtaining a cell count may provide higher or lower cell counts than those provided by traditional compendial methods during the enumerative analysis of compendial articles. However, whether the cell counts are higher or lower with the alternative method, it is generally possible to detect adverse trends in comparison with the estimates obtained using a compendial method.

Observations of cell counts that differ from cfu results are not a concern if the different methods and their different signals of cell presence are equivalent to or are non-inferior to referee methods in terms of assessing the microbiological safety of an article. Higher cell counts must not be considered as necessarily indicative of greater risk given the inherent variability of standard growth methods and the physical and chemical nature of compendial articles subject to analysis. This is especially true when

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enumerating microorganisms in articles that have a long history of safe and effective use. In such cases the discovery that an article contains a higher cell count then previously known does not mean that its safety has deteriorated.

With qualitative methods, i.e., presence or absence, comparisons of false negative and positive results obtained in controlled studies with the compendial and alternative microbiological method may be a measure of equivalence. There are commercially available enhancements to growth-based methods that allow colonies on solid media to be read more quickly, with substantially less incubation time, than is possible using only the unaided eye. These methods still require the growth of organisms on or in media. Therefore, many of these methods are not in the strictest sense different from the existing compendial methods, but are instead merely enhancements providing a more rapid detection of colonies. In the implementation of these enhanced methods for the detection of colony growth only the detection capability of the method requires verification.

SAMPLE SIZE

Any alternative microbiological test method (within its intended purpose) may use any sample size and number of tests that is sufficient to produce an equivalent decision (or better) regarding microbiological quality as compared to the reference method. It may be simpler, for many if not most alternative methods, to comply with the sampling instructions that are provided in the official compendial method. In case the sampling approach defined in the official compendial method is utilized no justification of the sample size is required.

### Statistics and Alternative Methods

Attempts to use statistics to compare the cfu results to signals arising from biochemical, physiological, or genetic methods of analysis may have limited value. Given the differences among these methods, they cannot be expected to yield signals that could be compared statistically in terms of mean values and variability. Thus, the enumerative values, given as cfu results in association with reference methods, typically cannot be used as acceptance criteria for the assessment of articles via candidate alternative methods. Instead, it is the users' responsibility to propose values, supported where necessary by scientific literature, that they can demonstrate are appropriate for the method that they have chosen and validated. This can be done independently of existing standards expressed in terms of cfu.

## INSTRUMENT QUALIFICATION

Instrument qualification should follow, at least in general terms, the discussion in ⟨1058⟩. The instrument qualification for equipment critical to the functioning of an alternative microbiological method involves four distinct phases:

1. Establishment of User Requirement Specifications for critical method attributes which should be formalized in a URS document.
2. Installation qualification—Was the instrument installed correctly?
3. Operational qualification—Does the instrument meet the manufacturer's specifications for correct operation?
4. Performance qualification—Does the instrument meet the URS for performance?

## VALIDATION OF ALTERNATE TECHNOLOGIES

### User Requirements Specification

Preparation of the URS document should involve input from all stakeholders for the microbiological test method. These stakeholders may include representatives from the Microbiology, Quality, Regulatory Affairs, and Operations groups, as well as others. The time spent on this step should be considered an investment in reaching a clear understanding of the company's needs before equipment is purchased, which will drive the performance qualification. At minimum, this document should include the following:

- Purpose and intended use (defined need for instrument)
- Description of who will use the equipment
- Operational requirements (data format, user interfaces, and operating environment)
- Constraints (timetables, downtime, maintenance, user skill levels, product compatibility, limit of detection, accuracy, and rapidity)
- Life cycle (development, testing, delivery, validation, training, and obsolesce)
- Capability (turnaround time, test capacity and throughput, and labor requirements)
- Sustainability (consumables, calibration, validation, and preventative maintenance)

See ⟨1058⟩ for information on the qualification of analytical instrumentation. The principles outlined in ⟨1058⟩ are generally applicable to the qualification of instruments used to conduct alternative microbiological analysis. The user may need to tailor the specific recommendations in ⟨1058⟩ to their particular instrument qualification specifications.

### Validation Criteria

The validation parameters generally recommended for qualitative and quantitative microbiological tests are shown in *Table 1*. Examples of qualitative tests are the sterility test and the test for absence of specified microorganisms. A quantitative test would be microbial enumeration. Note that qualitative testing is binary, and for this reason there is generally no need to define equivalency of units of measure, only equivalency of outcome.

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**Table 1. Validation Parameters by Type of Microbiological Test**

| Validation Parameter | Qualitative Tests | Quantitative Tests |
|---|---|---|
| Accuracy | No | Yes |
| Precision | No | Yes |
| Specificity | Yes | Yes |
| Limit of detection | Yes | Yes |
| Limit of quantification | No | Yes |
| Linearity | No | Yes |
| Operational (dynamic) range | No | Yes |
| Robustness | Yes | Yes |
| Repeatability | Yes | Yes |
| Ruggedness | Yes | Yes |
| Equivalency | Yes | Yes |

## SPECIFICITY

**Definition:** The specificity of an alternate qualitative microbiological method is defined as its ability to detect a range of challenge microorganisms specific to the technology. "Range of microorganisms" may be defined as a limited number of microorganisms representing risk to patient or product, microorganisms found in the manufacturing environment and product failures, microorganisms that are appropriate for measuring the effectiveness of the alternative method, and microorganisms that are representative in terms of morphological and physiological attributes appropriate for the method and the product.
**Demonstration:** Specificity is demonstrated by comparable recovery of the challenge panel of microorganisms in both the compendial and alternate methods. The microbial challenge is above the limit of detection or quantification but at a level that provides a measure of efficacy of the methods.

*Growth based*—Add low numbers (around 100 cfu) of each microorganism on the panel and perform both the compendial and alternative methods to demonstrate recovery of the microorganism.

*Nongrowth based*—Use suitable negative and positive controls to demonstrate that extraneous matter that may be in the system (e.g., extracellular ATP, DNA, or inhibition and enhancement factors) does not interfere with the detection of the defined range of microorganisms.

All challenge microorganisms should be recovered and identified in growth-based methods. For nongrowth-based methods microorganisms should be recovered and identified where possible.

## LIMIT OF DETECTION

**Definition:** The limit of detection (LOD) of an alternate microbiological method is defined as lowest number of microorganisms in a defined volume of sample that can be detected, but not necessarily quantified, under the stated experimental conditions. This should be conducted with the quality control organisms cited in *Antimicrobial Effectiveness Testing* ⟨51⟩, *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* ⟨61⟩, *Microbiological Examination of Nonsterile Products: Tests for Specified Microorganisms* ⟨62⟩, *Mycoplasma Tests* ⟨63⟩, and *Sterility Tests* ⟨71⟩ as appropriate to the alternative method.
**Demonstration**
*Method 1*

- Inoculate a suitable diluent solution with a serial dilution range of each challenge microorganism, appropriate for the intended use of the method and the technology. In most cases, the compendial media growth promotion test panel may be sufficient.
- The level of inoculation should be adjusted to a target of 50% of the dilution samples that show growth in the compendial test.
- Perform both the compendial and alternate tests.
- Tests should be repeated a sufficient number of times (statistically significant alpha risk: 0.05; beta risk: 0.20) for both the compendial and alternate tests.
- *Statistics:* Use the chi-square test or another appropriate approach to demonstrate equivalent recovery of the microorganism challenges.
- Alternately, use *Method 2*.

*Method 2 (MPN Method)*

- Create a dilution series of the challenge organisms in a suitable diluent solution to include at least the range of $10^{1}$ cfu to $10^{-2}$ cfu (for a 10-fold series) or 5 cfu to $10^{-1}$ cfu/inocula volume (for a 2-fold dilution series).
- Perform both the compendial and alternate tests with at least 5 simultaneous replicates of each dilution from the chosen series.
- Determine the most probable number (MPN) from three dilutions in series that provide both positive and negative growth (or signal).
- Statistics: Use the chi-square test or another appropriate approach to demonstrate equivalent recovery of the microorganism challenges.

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ROBUSTNESS

**Definition:** A capacity of the method to remain unaffected by small but deliberate variations in method parameters, e.g., reagent volume, incubation time, or ambient temperature providing an indication of its reliability during normal usage. A measure of robustness is not a comparison between the compendial and alternate methods; rather, it is a necessary component of validation of the alternate method so that the user understands the limits of the operating parameters of the method. The user may rely on data supplied by test method manufacturer.

RUGGEDNESS

**Definition:** The degree of precision of test results obtained by the analysis of the same samples under a variety of typical test conditions such as different analysts (for example, three), instruments, and reagent lots (the method for demonstration may follow instrument or materials supplier recommendations, or it could be based solely on data supplied by test method manufacturer).

For the definition of other validation parameters see *Glossary*.

## METHOD SUITABILITY

For each new product to be tested using the validated alternate microbiological method, perform the suitability test as described in general test methods (see ⟨51⟩, ⟨61⟩, ⟨62⟩, ⟨63⟩, and ⟨71⟩), using the number of unit and quantities prescribed and the sample preparation appropriate for the product and the required test sensitivity to determine the absence of a product effect that would obscure the signal of the method.

Method suitability may be demonstrated using three independent tests. Only the accuracy and precision validation parameters are required for quantitative methods. For qualitative methods, recovery of challenge organisms as indicated in ⟨62⟩, ⟨71⟩, and ⟨1227⟩ is sufficient.

After an alternative method has been shown to be equivalent to the compendial test with one product, it is not necessary to repeat the equivalency parameters for every new product; it is merely necessary to verify the method suitability for each additional product. For example, when employing a nucleic acid based method, with each new product, one must demonstrate that residual product does not interfere with the concentration, extraction, purification, and recovery of the target nucleic acid, or the polymerase chain reaction (PCR) amplification and chemical probe detection of the target ribosomal ribonucleic acid (rRNA) gene sequence.

## EQUIVALENCY

All microbiological tests are performed to enable informed decision making regarding the microbiological quality of a product, raw material, component, or process step. In this respect, the intended purpose of microbiological tests may be to either evaluate for the presence or absence of microorganisms (as in the sterility test) or to estimate the number of organisms present. The technological means by which microbiological test methods assess microbiological quality and enable a product-quality decision may differ from the growth-based means typical of reference methods. The units of measurement (signal) of a microbiological quality assessment performed using alternative microbiological test methods will generally not be a cfu, but rather a different approach to obtaining a cell count estimate. Therefore, the validation of alternative microbial methods should involve two components: (1) equivalence demonstration and (2) analytical method and equipment qualification.

### Equivalence Demonstration

*General Notices, 6.30 Alternative and Harmonized Methods and Procedures* states that alternate methods may be used if they provide advantages in terms of accuracy, sensitivity, precision, selectivity, or adaptability to automation or computerized data reduction. It further stipulates that alternative methods can be implemented in other special circumstances. Such alternate methods shall be validated as described in ⟨1225⟩ and must be shown to produce equivalent or better results than the referee method for any given quality attribute. When comparing two test procedures to show equivalent or better performance, statistical evidence is assembled to show equivalence or, in statistical terms, non-inferiority. For example, with microbial enumeration, equivalency may be shown if there is no statistically significant difference between the two means generated when enumerating with the compendial and alternative methods. However, this may not be possible when the two methods yield different signals. Examples of this situation are when the microbial enumeration method uses vital staining of microbial cells or measurement of genomic material in place of cfus.

Similarly, the FDA *Guidance for Industry* document *Analytical Procedures and Methods Validation: Chemistry, Manufacturing, and Controls Documentation* states that a validated alternative analytical procedure should be submitted only if it is shown to have performance equal to or better than the regulatory analytical procedure. Also, section 2.7 of the ICH document *Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Chemical Substances (Q6A)* states that alternative procedures are those that may be used to measure an attribute when such a procedure controls the quality of a drug substance or drug product to an extent that is comparable to or superior to the official method. However, other options to demonstrate equivalence are available and are discussed in *Demonstration of Equivalency*.

### Demonstration of Equivalency

Four options are available to establish the equivalence of a candidate alternative analytical method: (1) acceptable procedures (i.e., merely meeting a minimum performance or acceptance requirement without a need to demonstrate equivalence to the

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compendial method); (2) performance equivalence to the compendial method; (3) results equivalence to the compendial method; and (4) decision equivalence to the compendial method (*1*). A comparison of these four equivalence options is given in *Table 2*. The multiple equivalence options reflect the diversity in the technology and applications of the alternative test methodologies and may be viewed as a paradigm shift in *Equivalence Demonstration*.

**Table 2. Equivalence Option Matrix**

| Option | Demonstration | Comparison to Official Compendial Method | Based on Numerical Results or Conclusion | Number of Characteristics |
|---|---|---|---|---|
| 1. Acceptable procedures | Acceptable | No | Results | Multiple |
| 2. Performance equivalence | Equivalent | Yes | Results | Multiple |
| 3. Results equivalence | Equivalent | Yes | Results | Single |
| 4. Decision equivalence | Equivalent | Yes | Conclusions | Single |

### OPTION 1: ACCEPTABLE PROCEDURE

This is not strictly an equivalence option that requires direct comparison between the candidate alternative method and an official compendial method. With this option, a reference material with known properties may be used, such as a standard inoculum of a specific microorganism, a quantity of highly purified bacterial genome, an ATP level or another appropriate signal. In some cases, it could be required that the alternative method measure the signal in the presence of the test sample, with validation criteria that are consistent with the capability of the technology, as described in the scientific literature.

### OPTION 2: PERFORMANCE EQUIVALENCE

Performance equivalence requires the demonstration of equivalent or better results with respect to validation criteria—such as accuracy, precision, specificity, limit of detection, limit of qualification, robustness, and ruggedness—that may be appropriate for the intended use of the alternative qualitative or quantitative method. It is possible that the alternative method may not conform to some of the validation parameters listed compared with the official method and still be acceptable because of the advantages of the alternative method. This may be the case if the alternative method has any of the advantages stated in the *General Notices, 6.30 Alternative and Harmonized Methods and Procedures*. Other special circumstances would include improvements in time to obtain a result or the cost of running the test. If a candidate alternative method is suitable for assessing the quality of the material tested, it may be still acceptable, even if it differs from the official method in one or more validation parameters. The final analytical qualification criteria should reflect only the criteria that the microbiologist deems necessary to achieve performance equivalence.

### OPTION 3: RESULTS EQUIVALENCE

When results equivalence is required, the hypothesis to be tested is that the alternative and compendial test methods give equivalent numerical results. This contrasts with the evaluation of the validation parameters, as is done in performance equivalence. Because the same sample cannot be tested in microbiology, typically a tolerance interval is established when comparing the two methods, with the alternative method determined to be numerically superior or non-inferior. Reports on the use of alternative non-growth-based methods have shown that they may produce significantly higher cell count estimates than a growth method that reports outcomes in cfu. In this case, the analyst could use a calibration curve showing a correlation between the two methods in the product specification range.

### OPTION 4: DECISION EQUIVALENCE

A decision equivalence is similar to results equivalence but differs in that a numerical result is not generated; instead a pass/fail result is obtained. With this approach, the frequency of positive and negative results generated should be no worse than with the compendial method. This non-inferiority requirement is based on the long history of product quality tested by and released with the referee compendial test. For the purposes of qualification, laboratory studies involving spiking low levels of microorganisms may be considered. The following sections provide suggested approaches for demonstrating that the alternative procedure is equivalent to or better than the compendial procedure. Users may use other valid methods for demonstration of equivalence with supporting scientific justification.

## Equivalence Demonstration for Alternative Qualitative Microbiological Procedures

Results obtained by procedures in ⟨62⟩, ⟨63⟩, and ⟨71⟩ are indicative of the presence or absence of microorganisms in the sample tested. These tests do provide a decision (i.e., the compendial article either passes or fails the test). This type of data fits in the decision equivalence category as described in the *Stimuli* article (*2*). *Approach 1* (see below) is based on demonstrating decision equivalence. *Approach 2* (see below) is an alternative that converts the qualitative results to quantitative ones by using the most probable number (MPN) procedure. Both approaches use a non-inferiority hypothesis (*3*).

To demonstrate the acceptability of the alternate procedure relative to the current microbiological procedure, the laboratory must demonstrate that the new procedure is as good as or better than the current procedure in terms of the ability to detect the presence of microorganisms. In general, a recommended approach for comparing the alternate procedure to the compendial procedure is to use a non-inferiority test (one-sided, as in non-inferiority tests conducted in clinical trials for new drug products) (*4*) rather than two-sided equivalence [as in bioequivalence (*5*)]. Non-inferiority is an appropriate approach for

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two reasons. First, from a patient perspective, it is beneficial to promote an alternative procedure that is potentially more sensitive than the referee procedure. In contrast, a two-sided approach penalizes better recovery of microorganisms. It is important to note that those implementing the alternate method will need to assess the risk associated with the change in procedure, because a more sensitive procedure may generate more positive results. Second, the alternate procedure has benefits (principally, reduced time to a result) that make it preferable to the compendial procedure, even if it is not as sensitive, as long as it allows for a quality decision on the product that is non-inferior to the compendial method.

## APPROACH 1: USE PRESENCE AND ABSENCE RESULTS

The non-inferiority hypothesis for this approach is that the proportion of samples that produce a signal for the new procedure ($P_N$) is NMT some amount ($\Delta > 0$) less than the proportion for the current, compendial procedure ($P_C$) (*6*):

$$\text{Result} = P_N - P_C \geq -\Delta$$

The $\Delta$ is the non-inferiority margin. Unless the laboratory requires a tighter margin, use $\Delta = 0.20$ in the experiments described below. Calculate a one-sided 90% confidence interval for $P_N - P_C$ (*7*). Non-inferiority is concluded if the lower confidence limit exceeds –0.20. If the experiment is able to conclude in favor of the non-inferiority hypothesis, then it can be stated, with 95% confidence, that $P_N \geq P_C - 0.20$ at the bioburden level studied.

This evaluation should be conducted using types of microorganisms selected by the laboratory as representative of the general types of microorganisms encountered. The choices can follow ⟨71⟩ or appropriate suitability test organisms, organisms recovered from product testing and/or microorganisms representative of those that may convey risk to patients given a product's route of administration.

The laboratory should conduct an evaluation to determine whether the alternate procedure can be shown to be non-inferior to the microbiological procedure in terms of sensitivity as measured by the proportion of samples returning a positive result for microbial recovery and growth. For each organism in a qualitative test, conduct three evaluations. The first uses samples prepared by serial dilution to be at or around $10^0$, i.e., 1 cfu, where no growth is likely to be observed (hence no signal will be detected by the growth-based microbiological procedure) to characterize the sensitivity of the new procedure at this level. The second uses samples at or around $10^2$ (100–200 cfu), where the microbiological procedure would be expected to detect growth at a relatively high percentage of about 75% or greater, to determine the acceptability of the new procedure. The third is a comparison of the two procedures at a burden where 50%–75% of samples would be expected to grow colonies [often a serial dilution to around $10^1$ (10–50 cfu)] to test the non-inferiority hypothesis as described above. In the non-inferiority experiment for qualitative microbiology tests, a minimum of 75 samples should be tested on each procedure. Using 75 samples provides approximately 80% power. Should the laboratory find that a higher statistical power is necessary given the requirements of the analysis for a given product, increasing the number of samples analyzed to 100 will result in a power of approximately 90%. These procedures are appropriate for the purposes of concluding non-inferiority if the two procedures are actually equally sensitive using $\Delta = 0.20$. If the laboratory concludes their new procedure is less sensitive than the compendial procedure, a larger number of samples will be required to maintain these power levels.

**Independent samples:** Suppose that $N_A$ samples have been tested with the candidate alternative procedure, of which $X_A$ samples are positive, and that $N_C$ samples (not the same as those tested with the candidate) have been tested with the compendial procedure and that $X_C$ samples are positive. Calculate the following:

$$\hat{p}_A = X_A/N_A \quad \hat{p}_C = X_C/N_C$$
$$\theta = N_C/N_A$$
$$a = 1 + \theta$$
$$b = -\left[R\left(1 + \theta\hat{p}_C\right) + \theta + \hat{p}_A\right]$$
$$c = R\left(\hat{p}_A + \theta\hat{p}_C\right)$$
$$\tilde{p}_A = \left[-b - \left(b^2 - 4ac\right)^{1/2}\right]/(2a)$$
$$\tilde{p}_C = \tilde{p}_A/R$$
$$V = \frac{\tilde{p}_A\left(1 - \tilde{p}_A\right)}{N_A} + R^2\frac{\tilde{p}_C\left(1 - \tilde{p}_C\right)}{N_C}$$
$$Z = \left(\hat{p}_A - R\hat{p}_C\right)/\sqrt{V}$$

where $R$ is the ratio of variances at which to determine power.

Conclude non-inferiority if $Z > z_\alpha$ where $z_\alpha$ is the upper $\alpha$ percentage point of a standard normal distribution.

**Paired samples:** Suppose that $N$ samples have been tested by both the candidate alternative and compendial procedures. The results can be displayed in a 2 × 2 table (see *Table 3*).

**Table 3. Results for Paired Sample**

| Alternative Procedure | Compendial Procedure | | Row Totals |
|---|---|---|---|
| | Positive | Negative | |
| Positive | $X_{11}$ | $X_{10}$ | $X_A$ |
| Negative | $X_{01}$ | $X_{00}$ | $N - X_A$ |

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**Table 3. Results for Paired Sample** *(continued)*

| Alternative Procedure | Compendial Procedure | | Row Totals |
|---|---|---|---|
| | Positive | Negative | |
| Column totals | $X_C$ | $N - X_C$ | $N$ |

Compute the following:

$$L = \left[X_{10} - RX_{01} + (1-R)X_{11}\right]/N$$
$$V = X_A\left(X_{10} + X_{01}\right)/X_C^3$$
$$Z = L/\sqrt{V}$$

Conclude non-inferiority if $Z > z_\alpha$ where $z_\alpha$ is the upper α percentage point of a standard normal distribution.

APPROACH 2: COMPARE MPN RESULTS

For the compendial reference and the alternative procedures, conduct an MPN comparative study using standard procedures for MPN for each of the *N* samples. Ideally, the same samples are used for the two procedures, but this is not a necessity.

For *Approach 2*, the non-inferiority hypothesis is

$$\mu_A - \mu_C \geq \log(R) \text{ or } \mathit{anti}\log(\mu_A - \mu_C) \geq R$$

where $\mu_A$ and $\mu_C$ are the means in the log scale for the alternative and compendial procedures, respectively.

**Independent samples:** Determine MPN for $N_A$ samples by the alternative procedure, convert all values to logs, and determine the sample mean of the log values ($\bar{x}_A$) and sample variance of the log values ($S^2_A$). Similarly, determine $\bar{x}_C$ and $S^2_C$ from the logs of $N_C$ samples tested with the compendial procedure. Determine the following:

$$L_{low} = \bar{X}_A - \bar{X}_C - t_{\alpha,df}\sqrt{\frac{S_A^2}{N_A} + \frac{S_C^2}{N_C}}$$

where $t_{\alpha, df}$ is the upper α percentage point of the *t* distribution with *df* degrees of freedom and

$$df = \frac{\left(S_A^2/N_A + S_C^2/N_C\right)^2}{\frac{\left(S_A^2/N_A\right)^2}{N_A - 1} + \frac{\left(S_C^2/N_C\right)^2}{N_C - 1}}$$

If using software that only allows for integer degrees of freedom (e.g., Excel), use linear interpolation to obtain the *t* value. Conclude non-inferiority if $\mathit{anti}\log(L_{low}) \geq R$.

**Paired data:** Determine MPN for *N* samples by the alternative procedure and for the same *N* samples by the compendial procedure, convert all 2*N* values to logs, and determine the sample mean ($\bar{x}$) and variance ($S^2$) of the differences of log alternative value minus log compendial value. Determine the following:

$$L_{low} = \bar{x} - t_{\alpha, N-1}\, S/\sqrt{N}$$

where $t_{\alpha, N-1}$ is the upper α percentage point of the *t* distribution with $N - 1$ degrees of freedom. Conclude non-inferiority if $\mathit{anti}\log(L_{low}) \geq R$.

## Equivalence Demonstration for Alternative Quantitative Microbiological Procedures

A key characteristic of some alternative quantitative procedures is that their signal may differ significantly from the cfu of the compendial microbiological procedure. As a consequence, equivalence as it is typically understood cannot be shown; that is, the numerical results are expected to differ in magnitude and units. Instead, this chapter suggest two criteria for the verification of candidate alternative quantitative procedures:

1. Results from the candidate procedure have at least acceptable precision (repeatability).
2. The results from the candidate procedure are highly correlated with those from the compendial procedure. A high correlation is taken to indicate that quantitative acceptance criteria expressed in cfu can be calibrated to criteria in the units of the alternative procedure.

PRECISION

Prepare a minimum of six samples at a minimum of two bioburden levels near specification limits relevant to the laboratory. Run the candidate alternative procedure for the prepared samples. [NOTE—This is to correspond to repeatability conditions; see ⟨1225⟩.] At each level, determine the sample variance ($S^2$) of the logarithms ($\log_{10}$) of sample results. Calculate the following:

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$$UL = 100 * \left[ antilog\left( \sqrt{\frac{(n-1)S^2}{\chi^2_{.05,\ n-1}}} \right) - 1 \right]$$

Where $n$ is the number of samples ($n \geq 6$) and $\chi^2_{.05,\ N-1}$ is the lower 5% value of a chi-square distribution with $n-1$ degrees of freedom. Precision is acceptable if *UL ≤ σ, where σ is the predetermined maximal acceptable repeatability percent geometric coefficient of variation, %GCV. (For small values, the %GCV will be approximately the %RSD.)

The greater the number of samples ($n$) the greater the likelihood (power, in statistical terms) that a procedure, the precision of which is actually acceptable, will yield data that meet this criterion and thus be declared acceptable. The laboratory may use prior data to determine a value of $n$ that meets their needs.

**Example:** For the data for the alternative procedure in *Table 4*, calculate the following:

$$n = 10$$

$$S^2 = 0.000241, \text{ and}$$

$$\chi^2_{.05,\ 9} = 3.325113, \text{ so}$$

$$UL = 6.06\%$$

This alternative procedure thus has acceptable repeatability precision as long as the prespecified success criterion σ had been at least 6.06%.

## CORRELATION (LINEARITY)

Prepare a minimum of two samples at each of four different bioburden levels covering the range from near limit of qualification (LOQ) to one log above the specification limit defined in the standard compendial assay to which the candidate alternative method is being compared. Determine the activity for all samples using both the candidate alternative and compendial procedures. Plot and determine the correlation between the log of values from the candidate alternative procedure ($y$) and the log of values from the compendial procedure ($x$). The correlation is acceptable if at least 0.95 (or $R^2$ at least 0.9025).

Although a linear relationship between the two sets of results is typically expected, a nonlinear relationship can be acceptable. In the case of a nonlinear relationship, use the Spearman (nonparametric) correlation instead of the Pearson correlation.

**Table 4**

| Compendial (cfu) | Alternative (cell count) |
|---|---|
| 70 | 970 |
| 71 | 965 |
| 75 | 950 |
| 92 | 990 |
| 100 | 1000 |
| 105 | 1051 |
| 116 | 1046 |
| 123 | 1039 |
| 127 | 985 |
| 130 | 1020 |

*Figure 1* shows the plot of these data after conversion to base 10 logs. Because $R^2$ does not meet the stated requirement, the results from these two procedures are not sufficiently correlated.

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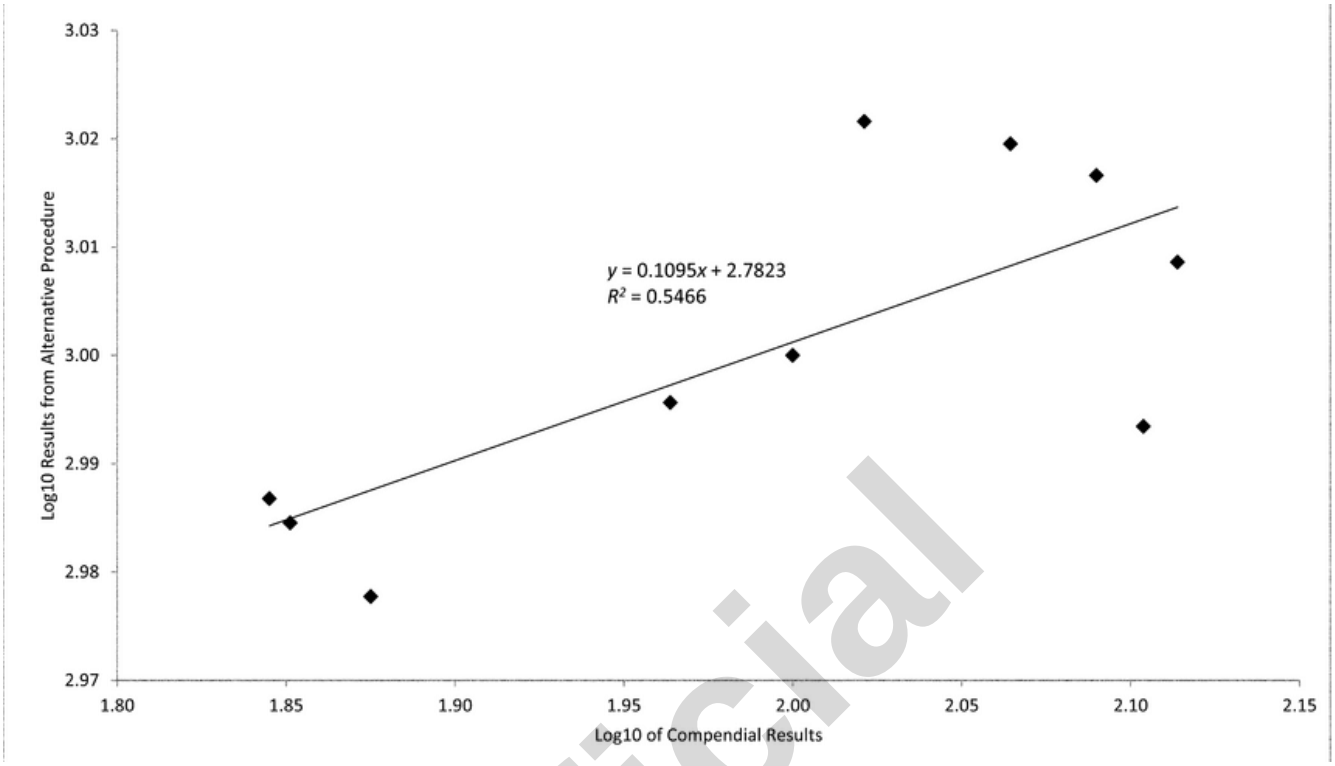


Figure 1.

Candidate alternative procedures that may be suitable for making decisions about the microbiological quality of a sample (as in *Figure 1*) may not correlate well enough with the compendial procedure to meet the above correlation requirement. In that case, the other option is to apply the decision equivalence approach as described earlier for qualitative tests. During procedure development, the laboratory should determine a specification for the alternative method to correspond to the compendial specification for the required level of microbiological quality. For example, if the required level of microbiological quality is NMT $10^2$ cfu, for which the compendial maximum acceptable count is 200 cfu, the laboratory will need to determine an acceptance criterion for the candidate alternative procedure that will match that value from the perspective of making a decision regarding microbial quality. Then, the validation experiment to confirm this choice proceeds as described earlier for qualitative tests.

## GLOSSARY

**Accuracy:** Closeness of the test results obtained by the alternative test method to the value obtained by the compendial method, to be demonstrated across the dynamic (operational) range of the method.

**Alternative microbiological method:** A modern or rapid microbiological test procedure (MMM or RMM) that is different from the traditional growth-based method, such as the plate count or recovery in liquid broth. The alternative or rapid method may use different technologies, instrumentation and software to manage the testing and analyses of data and may provide quantitative (enumeration) or qualitative (detection) microbial test results.

**Colony-forming unit (cfu):** An estimate of the number of microorganisms obtained by traditional plate count methods. The enumeration is dependent on the ability of the microorganisms in the sample to grow on the microbiological culture media under the conditions of incubation. Because it is uncertain whether a colony was derived from the growth of one or even one thousand cells, the results are reported as cfu/mL (for a liquid) or cfu/g (for a solid) and not as cells/mL or cells/g.

**Conventional microbiological method:** A classical or traditional growth-based method, such as enumeration on an agar plate or detection in a liquid broth when incubated for a specified time and temperature. These methods are used in ⟨51⟩, ⟨61⟩, ⟨62⟩, ⟨63⟩, and ⟨71⟩.

**Equivalence:** When the test results from two procedures are sufficiently close for the intended use of the procedures. Demonstration of equivalence requires a prespecified measure of how similar the test results need to be.

**False negative:** A test result that is incorrectly determined as negative (e.g., the absence of a viable microbial detection result when viable microorganisms are present). A type II error, also known as an error of the second kind, occurs when the null hypothesis is false but erroneously fails to be rejected. It is failing to assert what is, in fact, present—a miss. A type II error may be compared with a so-called false negative in a test (and seen as a "miss") that is checking for a single condition with a definitive result of true or false. The rate of the type II error is denoted by the Greek letter β and is related to the power of a test (which equals 1 − β).

**False positive:** A test result that is incorrectly determined as positive (e.g., a viable microbial detection result when viable microorganisms are not present). In statistical test theory, the idea of a statistical error is an integral part of hypothesis testing. These are described as type I and type II errors. A type I error, also known as an error of the first kind, occurs when the null hypothesis (H0) is true but is rejected. It is asserting something exists that is, in fact, absent (i.e., a false hit). A type I error may

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be compared with a so-called false positive (a result that indicates that a given condition is present when it actually is not present) in tests where a single condition is tested. The rate of the type I error is called the "size" of the test and denoted by the Greek letter α. It usually equals the significance level of a test. In the case of a simple null hypothesis, α is the probability of a type I error.

**Independent samples:** Samples selected from the same population or different populations that have no effect on one another. That is, no correlation exists between the samples.

**Limit of detection (LOD):** The lowest concentration of microorganisms in a test sample that can be detected, but not necessarily quantified, under defined experimental conditions.

**Limit of quantification (LOQ):** The lowest number of microorganisms in a test sample that can be enumerated with acceptable accuracy and precision under defined experimental conditions.

**Linearity:** The ability to produce results that are proportional to the concentration of microorganisms present in the sample within a given range.

**Method suitability:** Demonstration of lack of enhancement or inhibition by the product on the signal generated by the method.

**Non-inferiority:** Demonstration that the alternate method is not worse than the compendial method by more than a small prespecified amount. This amount is known as the non-inferiority margin or δ. Non-inferiority is different from equivalence in that in an equivalence trial, the desired conclusion is that two microbiological methods are not unacceptably different from each other. In a non-inferiority test the objective is to demonstrate that a new product is not unacceptably worse than an older one.

**Paired samples:** A sample of matched pairs of similar units.

**Range-Dynamic or Operational:** The interval between the upper and lower levels of microorganisms that have been demonstrated to be determined with specified accuracy, precision, and linearity.

**Repeatability precision:** The degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of the same suspension of microorganisms and uses different suspensions across the range of the test. Also known as "repeatability".

**Robustness:** A method's capacity to remain unaffected by small but deliberate variations in method parameters, such as, reagent volume, time or temperature of incubation providing an indication of its reliability during normal usage.

**Ruggedness:** Intermediate (within laboratory) precision associated with changes in operating conditions. Factors contributing to intermediate precision involve anything that can change within a given laboratory and that may affect the assay (e.g., different days, different analysts, different equipment).

**Specificity:** The ability to detect a range of microorganisms, which demonstrate that the method is fit for its intended use. These microorganisms may include a limited number of microorganisms representing risk to patient or product, microorganisms found in the manufacturing environment and product failures, microorganisms that are appropriate for measuring the effectiveness of the alternative method, and microorganisms that are specified in the relevant compendial tests, that are appropriate for the method and the product.

**User Responsibility:** The responsibility for the installation and operational, and performance qualification may be a joint responsibility of the instrument manufacturer and the user of the alternative microbiological method. The method validation may be conducted by the instrument manufacturer, when justified. The method suitability testing conducted for each specific product must be solely the user's responsibility.

**Validation:** The process of demonstrating and documenting that the performance characteristics of a procedure and its underlying method meet the requirements for the intended application and that the procedure is thereby suitable for its intended use. Formal validations are conducted prospectively according to a written plan that includes justifiable acceptance criteria on validation procedures.

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# ⟨1224⟩ TRANSFER OF ANALYTICAL PROCEDURES

## INTRODUCTION

Testing to the specification of an ancillary material, intermediate, and/or ingredient and product is critical in establishing the quality of a finished dosage form. The transfer of analytical procedures (TAP), also referred to as method transfer, is the documented process that qualifies a laboratory (the receiving unit) to use an analytical test procedure that originated in another laboratory (the transferring unit), thus ensuring that the receiving unit has the procedural knowledge and ability to perform the transferred analytical procedure as intended.

The purpose of this general information chapter is to summarize the types of transfers that may occur, including the possibility of waiver of any transfer, and to outline the potential components of a transfer protocol. The chapter does not provide statistical methods and does not encompass the transfer of microbiological or biological procedures.

## TYPES OF TRANSFERS OF ANALYTICAL PROCEDURES

TAP can be performed and demonstrated by several approaches. The most common is comparative testing performed on homogeneous lots of the target material from standard production batches or samples intentionally prepared for the test (e.g., by spiking relevant accurate amounts of known impurities into samples). Other approaches include covalidation between laboratories, the complete or partial validation of the analytical procedures by the receiving unit, and the transfer waiver, which is an appropriately justified omission of the transfer process. The tests that will be transferred, the extent of the transfer activities, and the implementation strategy should be based on a risk analysis that considers the previous experience and knowledge of the receiving unit, the complexity and specifications of the product, and the procedure.

### Comparative Testing

Comparative testing requires the analysis of a predetermined number of samples of the same lot by both the sending and the receiving units. Other approaches may be valid, e.g., if the receiving unit meets a predetermined acceptance criterion for the recovery of an impurity in a spiked product. Such analysis is based on a preapproved transfer protocol that stipulates the details of the procedure, the samples that will be used, and the predetermined acceptance criteria, including acceptable variability. Meeting the predetermined acceptance criteria is necessary to assure that the receiving unit is qualified to run the procedure.

### Covalidation Between Two or More Laboratories

The laboratory that performs the validation of an analytical procedure is qualified to run the procedure. The transferring unit can involve the receiving unit in an interlaboratory covalidation, including them as a part of the validation team at the transferring unit and thereby obtaining data for the assessment of reproducibility. This assessment is made using a preapproved transfer or validation protocol that provides the details of the procedure, the samples to be used, and the predetermined acceptance criteria. The general chapter *Validation of Compendial Procedures* ⟨1225⟩ provides useful guidance about which characteristics are appropriate for testing.

### Revalidation

Revalidation or partial revalidation is another acceptable approach for transfer of a validated procedure. Those characteristics described in ⟨1225⟩, which are anticipated to be affected by the transfer, should be addressed.

### Transfer Waiver

The conventional TAP may be omitted under certain circumstances. In such instances, the receiving unit is considered to be qualified to use the analytical test procedures without comparison and generation of interlaboratory comparative data. The following examples give some scenarios that may justify the waiver of TAP:

- The new product's composition is comparable to that of an existing product and/or the concentration of active ingredient is similar to that of an existing product and is analyzed by procedures with which the receiving unit already has experience.
- The analytical procedure being transferred is described in the *USP–NF*, and is unchanged. Verification should apply in this case (see ⟨1226⟩).
- The analytical procedure transferred is the same as or very similar to a procedure already in use.
- The personnel in charge of the development, validation, or routine analysis of the product at the transferring unit are moved to the receiving unit.

If eligible for transfer waiver, the receiving unit should document it with appropriate justifications.

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## ELEMENTS RECOMMENDED FOR THE TRANSFER OF ANAYTICAL PROCEDURES

Several elements, many of which may be interrelated, are recommended for a successful TAP. When appropriate and as a part of pretransfer activities, the transferring unit should provide training to the receiving unit, or the receiving unit should run the procedures and identify any issues that may need to be resolved before the transfer protocol is signed. Training should be documented.

The transferring unit, often the development unit, is responsible for providing the analytical procedure, the reference standards, the validation reports, and any necessary documents, as well as for providing the necessary training and assistance to the receiving unit as needed during the transfer. The receiving unit may be a quality control unit, another intracompany facility, or another company such as a contract research organization. The receiving unit provides qualified staff or properly trains the staff before the transfer, ensures that the facilities and instrumentation are properly calibrated and qualified as needed, and verifies that the laboratory systems are in compliance with applicable regulations and in-house general laboratory procedures. Both the transferring and receiving units should compare and discuss data as well as any deviations from the protocol. This discussion addresses any necessary corrections or updates to the final report and the analytical procedure as necessary to reproduce the procedure.

A single lot of the article may be used for the transfer, because the aim of the transfer is not related to the manufacturing process but rather to the evaluation of the analytical procedure's performance at the receiving site.

## PREAPPROVED PROTOCOL

A well-designed protocol should be discussed, agreed upon, and documented before the implementation of TAP. The document expresses a consensus between the parties, indicating an intended execution strategy, and should include each party's requirements and responsibilities. It is recommended that the protocol contain the following topics as appropriate: objective, scope, responsibilities of the transferring and receiving units, materials and instruments that will be used, analytical procedure, experimental design, and acceptance criteria for all tests and/or methods included in the transfer. Based on the validation data and procedural knowledge, the transfer protocol should identify the specific analytical performance characteristics (see ⟨1225⟩ and ⟨1226⟩) that will be evaluated and the analysis that will be used to evaluate acceptable outcomes of the transfer exercise.

The transfer acceptance criteria, which are based on method performance and historical data from stability and release results, if available, should include the comparability criteria for results from all study sites. These criteria may be derived using statistical principles based on the difference between mean values and established ranges and should be accompanied by an estimation of the variability (e.g., percent relative standard deviation [%RSD] for each site), particularly for the intermediate precision %RSD of the receiving unit and/or a statistical method for the comparison of the means for assay and content uniformity tests. In instances of impurity testing, where precision may be poorer such as in the case of trace impurities, a simple descriptive approach can be used. Dissolution can be evaluated by a comparison of the dissolution profiles using the similarity factor $f_2$ or by comparison of data at the specified time points. The laboratories should provide appropriate rationale for any analytical performance characteristic not included. The materials, reference standards, samples, instruments, and instrumental parameters that will be used should be described.

It is recommended that expired, aged, or spiked samples be carefully chosen and evaluated to identify potential problems related to differences in sample preparation equipment and to evaluate the impact of potential aberrant results on marketed products. The documentation section of the transfer protocol may include report forms to ensure consistent recording of results and to improve consistency between laboratories. This section should contain the additional information that will be included with the results, such as example chromatograms and spectra, along with additional information in case of a deviation. The protocol should also explain how any deviation from the acceptance criteria will be managed. Any changes to the transfer protocol following failure of an acceptance criterion must be approved before collection of additional data.

## THE ANALYTICAL PROCEDURE

The procedure should be written with sufficient detail and explicit instructions, so that a trained analyst can perform it without difficulty. A pretransfer meeting between the transferring and receiving units is helpful to clarify any issues and answer any questions regarding the transfer process. If complete or partial validation data exist, they should be available to the receiving unit, along with any technical details required to perform the test in question. In some cases it may be useful for the individuals who were involved with the initial development or validation to be on site during the transfer. The number of replicates and injection sequences in the case of liquid or gas chromatography should be clearly expressed, and, in the case of dissolution testing, the number of individual dosage units should be stipulated.

## TRANSFER REPORT

When the TAP is successfully completed, the receiving unit should prepare a transfer report that describes the results obtained in relation to the acceptance criteria, along with conclusions that confirm that the receiving unit is now qualified to run the procedure. Any deviations should be thoroughly documented and justified. If the acceptance criteria are met, the TAP is successful and the receiving unit is qualified to run the procedure. Otherwise, the procedure cannot be considered transferred until effective remedial steps are adopted in order to meet the acceptance criteria. An investigation may provide guidance about the nature and extent of the remedial steps, which may vary from further training and clarification to more complex approaches, depending on the particular procedure.

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# ⟨1225⟩ VALIDATION OF COMPENDIAL PROCEDURES

Test procedures for assessment of the quality levels of pharmaceutical articles are subject to various requirements. According to Section 501 of the Federal Food, Drug, and Cosmetic Act, assays and specifications in monographs of the *USP–NF* constitute legal standards. The Current Good Manufacturing Practice regulations [21 CFR 211.194(a)] require that test methods, which are used for assessing compliance of pharmaceutical articles with established specifications, must meet proper standards of accuracy and reliability. Also, according to these regulations [21 CFR 211.194(a)(2)], users of analytical methods described in *USP–NF* are not required to validate the accuracy and reliability of these methods, but merely verify their suitability under actual conditions of use. Recognizing the legal status of *USP* and *NF* standards, it is essential, therefore, that proposals for adoption of new or revised compendial analytical procedures be supported by sufficient laboratory data to document their validity.

The text of this information chapter harmonizes, to the extent possible, with the International Council for Harmonisation (ICH) tripartite guideline *Validation of Analytical Procedures* and the *Methodology* extension text, which are concerned with analytical procedures included as part of registration applications submitted within the EC, Japan, and the USA.

## SUBMISSIONS TO THE COMPENDIA

Submissions to the compendia for new or revised analytical procedures should contain sufficient information to enable members of the USP Council of Experts and its Expert Committees to evaluate the relative merit of proposed procedures. In most cases, evaluations involve assessment of the clarity and completeness of the description of the analytical procedures, determination of the need for the procedures, and documentation that they have been appropriately validated. Information may vary depending upon the type of method involved. However, in most cases a submission will consist of the following sections.

### Rationale

This section should identify the need for the procedure and describe the capability of the specific procedure proposed and why it is preferred over other types of determinations. For revised procedures, a comparison should be provided of limitations of the current compendial procedure and advantages offered by the proposed procedure.

### Proposed Analytical Procedure

This section should contain a complete description of the analytical procedure sufficiently detailed to enable persons "skilled in the art" to replicate it. The write-up should include all important operational parameters and specific instructions such as preparation of reagents, performance of system suitability tests, description of blanks used, precautions, and explicit formulas for calculation of test results.

### Data Elements

This section should provide thorough and complete documentation of the validation of the analytical procedure. It should include summaries of experimental data and calculations substantiating each of the applicable analytical performance characteristics. These characteristics are described in the following section.

## VALIDATION

Validation of an analytical procedure is the process by which it is established, by laboratory studies, that the performance characteristics of the procedure meet the requirements for the intended analytical applications. Typical analytical performance characteristics that should be considered in the validation of the types of procedures described in this document are listed in *Table 1*. Because opinions may differ with respect to terminology and use, each of the performance characteristics is defined in the next section of this chapter, along with a delineation of a typical method or methods by which it may be measured. The definitions refer to "test results". The description of the analytical procedure should define what the test results for the procedure are. As noted in ISO 5725-1 and 3534-1, a test result is "the value of a characteristic obtained by carrying out a specified test method. The test method should specify that one or a number of individual measurements be made, and their average, or another appropriate function (such as the median or the standard deviation), be reported as the test result. It may also require standard corrections to be applied, such as correction of gas volumes to standard temperature and pressure. Thus, a test result can be a result calculated from several observed values. In the simple case, the test result is the observed value itself." A test result also can be, but need not be, the final, reportable value that would be compared to the acceptance criteria of a specification. Validation of physical property methods may involve the assessment of chemometric models. However, the typical analytical characteristics used in method validation can be applied to the methods derived from the use of the chemometric models.

**Table 1. Typical Analytical Characteristics Used in Method Validation**

| |
|---|
| Accuracy |
| Precision |

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**Table 1. Typical Analytical Characteristics Used in Method Validation** *(continued)*

| |
|---|
| Specificity |
| Detection limit |
| Quantitation limit |
| Linearity |
| Range |
| Robustness |

The effects of processing conditions and potential for segregation of materials should be considered when obtaining a representative sample to be used for validation of procedures.

In the case of compendial procedures, revalidation may be necessary in the following cases: a submission to the USP of a revised analytical procedure or the use of an established general procedure with a new product or raw material (see below in *Data Elements Required for Validation*).

The ICH documents give guidance on the necessity for revalidation in the following circumstances: changes in the synthesis of the drug substance, changes in the composition of the drug product, and changes in the analytical procedure.

This chapter is intended to provide information that is appropriate to validate a wide range of compendial analytical procedures. The validation of compendial procedures may use some or all of the suggested typical analytical characteristics used in method validation as outlined in *Table 1* and categorized by type of analytical method in *Table 2*. For some compendial procedures the fundamental principles of validation may extend beyond characteristics suggested in this chapter. For these procedures the user is referred to the individual compendial chapter for those specific analytical validation characteristics and any specific validation requirements.

## Analytical Performance Characteristics

### ACCURACY

**Definition:** The accuracy of an analytical procedure is the closeness of test results obtained by that procedure to the true value. The accuracy of an analytical procedure should be established across its range. [A note on terminology: The definition of accuracy in this chapter and ICH Q2 corresponds to unbiasedness only. In the International Vocabulary of Metrology (VIM) and documents of the International Organization for Standardization (ISO), “accuracy” has a different meaning. In ISO, accuracy combines the concepts of unbiasedness (termed “trueness”) and precision.]

**Determination:** In the case of the assay of a drug substance, accuracy may be determined by application of the analytical procedure to an analyte of known purity (e.g., a Reference Standard) or by comparison of the results of the procedure with those of a second, well-characterized procedure, the accuracy of which has been stated or defined.

In the case of the assay of a drug in a formulated product, accuracy may be determined by application of the analytical procedure to synthetic mixtures of the drug product components to which known amounts of analyte have been added within the range of the procedure. If it is not possible to obtain samples of all drug product components, it may be acceptable either to add known quantities of the analyte to the drug product (i.e., “to spike”) or to compare results with those of a second, well-characterized procedure, the accuracy of which has been stated or defined.

In the case of quantitative analysis of impurities, accuracy should be assessed on samples (of drug substance or drug product) spiked with known amounts of impurities. Where it is not possible to obtain samples of certain impurities or degradation products, results should be compared with those obtained by an independent procedure. In the absence of other information, it may be necessary to calculate the amount of an impurity based on comparison of its response to that of the drug substance; the ratio of the responses of equal amounts of the impurity and the drug substance (relative response factor) should be used, if known.

Accuracy is calculated as the percentage of recovery by the assay of the known added amount of analyte in the sample, or as the difference between the mean and the accepted true value, together with confidence intervals.

The ICH documents recommend that accuracy should be assessed using a minimum of nine determinations over a minimum of three concentration levels, covering the specified range (i.e., three concentrations and three replicates of each concentration).

Assessment of accuracy can be accomplished in a variety of ways, including evaluating the recovery of the analyte (percent recovery) across the range of the assay, or evaluating the linearity of the relationship between estimated and actual concentrations. The statistically preferred criterion is that the confidence interval for the slope be contained in an interval around 1.0, or alternatively, that the slope be close to 1.0. In either case, the interval or the definition of closeness should be specified in the validation protocol. The acceptance criterion will depend on the assay and its variability and on the product. Setting an acceptance criterion based on the lack of statistical significance of the test of the null hypothesis that the slope is 1.0 is not an acceptable approach.

Accuracy of physical property methods may be assessed through the analysis of standard reference materials, or alternatively, the suitability of the above approaches may be considered on a case-by-case basis.

### PRECISION

**Definition:** The precision of an analytical procedure is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of a homogeneous sample. The precision of an analytical procedure is usually expressed as the standard deviation or relative standard deviation (coefficient of variation) of a series of measurements. Precision may be a measure of either the degree of reproducibility or of repeatability of the analytical procedure under normal operating conditions. In this context, reproducibility refers to the use of the analytical procedure in different laboratories, as

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in a collaborative study. Intermediate precision (also known as ruggedness) expresses within-laboratory variation, as on different days, or with different analysts or equipment within the same laboratory. Repeatability refers to the use of the analytical procedure within a laboratory over a short period of time using the same analyst with the same equipment.

**Determination:** The precision of an analytical procedure is determined by assaying a sufficient number of aliquots of a homogeneous sample to be able to calculate statistically valid estimates of standard deviation or relative standard deviation (coefficient of variation). Assays in this context are independent analyses of samples that have been carried through the complete analytical procedure from sample preparation to final test result.

The ICH documents recommend that repeatability should be assessed using a minimum of nine determinations covering the specified range for the procedure (i.e., three concentrations and three replicates of each concentration) or using a minimum of six determinations at 100% of the test concentration.

## SPECIFICITY

**Definition:** The ICH documents define specificity as the ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation products, and matrix components. Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedures. [NOTE—Other reputable international authorities (IUPAC, AOAC-I) have preferred the term "selectivity", reserving "specificity" for those procedures that are completely selective.] For the tests discussed below, the above definition has the following implications.

*Identification tests:* Ensure the identity of the analyte.

*Purity tests:* Ensure that all of the analytical procedures performed allow an accurate statement of the content of impurities of an analyte (e.g., related substances test, heavy metals limit, or organic volatile impurities).

*Assays:* Provide an exact result, which allows an accurate statement on the content or potency of the analyte in a sample.

**Determination:** In the case of qualitative analyses (identification tests), the ability to select between compounds of closely related structure that are likely to be present should be demonstrated. This should be confirmed by obtaining positive results (perhaps by comparison to a known reference material) from samples containing the analyte, coupled with negative results from samples that do not contain the analyte and by confirming that a positive response is not obtained from materials structurally similar to or closely related to the analyte.

In the case of analytical procedures for impurities, specificity may be established by spiking the drug substance or product with appropriate levels of impurities and demonstrating that these impurities are determined with appropriate accuracy and precision.

In the case of the assay, demonstration of specificity requires that it can be shown that the procedure is unaffected by the presence of impurities or excipients. In practice, this can be done by spiking the drug substance or product with appropriate levels of impurities or excipients and demonstrating that the assay result is unaffected by the presence of these extraneous materials.

If impurity or degradation product standards are unavailable, specificity may be demonstrated by comparing the test results of samples containing impurities or degradation products to a second well-characterized procedure (e.g., a pharmacopeial or other validated procedure). These comparisons should include samples stored under relevant stress conditions (e.g., light, heat, humidity, acid/base hydrolysis, and oxidation). In the case of the assay, the results should be compared; in the case of chromatographic impurity tests, the impurity profiles should be compared.

The ICH documents state that when chromatographic procedures are used, representative chromatograms should be presented to demonstrate the degree of selectivity, and peaks should be appropriately labeled. Peak purity tests (e.g., using diode array or mass spectrometry) may be useful to show that the analyte chromatographic peak is not attributable to more than one component.

For validation of specificity for qualitative and quantitative determinations by spectroscopic methods, chapters related to topics such as near-infrared spectrophotometry, Raman spectroscopy, and X-ray powder diffraction should be consulted.

## DETECTION LIMIT

**Definition:** The detection limit is a characteristic of limit tests. It is the lowest amount of analyte in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions. Thus, limit tests merely substantiate that the amount of analyte is above or below a certain level. The detection limit is usually expressed as the concentration of analyte (e.g., percentage or parts per billion) in the sample.

**Determination:** For noninstrumental procedures, the detection limit is generally determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected.

For instrumental procedures, the same approach may be used as for noninstrumental procedures. In the case of procedures submitted for consideration as official compendial procedures, it is almost never necessary to determine the actual detection limit. Rather, the detection limit is shown to be sufficiently low by the analysis of samples with known concentrations of analyte above and below the required detection level. For example, if it is required to detect an impurity at the level of 0.1%, it should be demonstrated that the procedure will reliably detect the impurity at that level.

In the case of instrumental analytical procedures that exhibit background noise, the ICH documents describe a common approach, which is to compare measured signals from samples with known low concentrations of analyte with those of blank samples. The minimum concentration at which the analyte can reliably be detected is established. Typically acceptable signal-to-noise ratios are 2:1 or 3:1. Other approaches depend on the determination of the slope of the calibration curve and the standard deviation of responses. Whatever method is used, the detection limit should be subsequently validated by the analysis of a suitable number of samples known to be near, or prepared at, the detection limit.

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## QUANTITATION LIMIT

**Definition:** The quantitation limit is a characteristic of quantitative assays for low levels of compounds in sample matrices, such as impurities in bulk drug substances and degradation products in finished pharmaceuticals. It is the lowest amount of analyte in a sample that can be determined with acceptable *Precision* and *Accuracy* under the stated experimental conditions. The quantitation limit is expressed as the concentration of analyte (e.g., percentage or parts per billion) in the sample.
**Determination:** For noninstrumental procedures, the quantitation limit is generally determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be determined with acceptable *Accuracy* and *Precision*.

For instrumental procedures, the same approach may be used as for noninstrumental procedures. In the case of procedures submitted for consideration as official compendial procedures, it is almost never necessary to determine the actual quantitation limit. Rather, the quantitation limit is shown to be sufficiently low by the analysis of samples with known concentrations of analyte above and below the quantitation level. For example, if it is required that an analyte be assayed at the level of 0.1 mg/tablet, it should be demonstrated that the procedure will reliably quantitate the analyte at that level.

In the case of instrumental analytical procedures that exhibit background noise, the ICH documents describe a common approach, which is to compare measured signals from samples with known low concentrations of analyte with those of blank samples. The minimum concentration at which the analyte can reliably be quantified is established. A typically acceptable signal-to-noise ratio is 10:1. Other approaches depend on the determination of the slope of the calibration curve and the standard deviation of responses. Whatever approach is used, the quantitation limit should be subsequently validated by the analysis of a suitable number of samples known to be near, or prepared at, the quantitation limit.

## LINEARITY AND RANGE

**Definition of linearity:** The linearity of an analytical procedure is its ability to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range. Thus, in this section, "linearity" refers to the linearity of the relationship of concentration and assay measurement. In some cases, to attain linearity, the concentration and/or the measurement may be transformed. [NOTE—The weighting factors used in the regression analysis may change when a transformation is applied.] Possible transformations may include log, square root, or reciprocal, although other transformations are acceptable. If linearity is not attainable, a nonlinear model may be used. The goal is to have a model, whether linear or nonlinear, that describes closely the concentration–response relationship.
**Definition of range:** The range of an analytical procedure is the interval between the upper and lower levels of analyte (including these levels) that have been demonstrated to be determined with a suitable level of precision, accuracy, and linearity using the procedure as written. The range is normally expressed in the same units as test results (e.g., percent or parts per million) obtained by the analytical procedure.
**Determination of linearity and range:** Linearity should be established across the range of the analytical procedure. It should be established initially by visual examination of a plot of signals as a function of analyte concentration of content. If there appears to be a linear relationship, test results should be established by appropriate statistical methods (e.g., by calculation of a regression line by the method of least squares). Data from the regression line itself may be helpful to provide mathematical estimates of the degree of linearity. The correlation coefficient, *y*-intercept, slope of the regression line, and residual sum of squares should be submitted.

The range of the procedure is validated by verifying that the analytical procedure provides acceptable precision, accuracy, and linearity when applied to samples containing analyte at the extremes of the range as well as within the range.

ICH recommends that, for the establishment of linearity, a minimum of five concentrations normally be used. It is also recommended that the following minimum specified ranges should be considered:

*Assay of a drug substance (or a finished product):* From 80% to 120% of the test concentration

*Determination of an impurity:* From 50% to 120% of the acceptance criterion

*For content uniformity:* A minimum of 70%–130% of the test concentration, unless a wider or more appropriate range based on the nature of the dosage form (e.g., metered-dose inhalers) is justified

*For dissolution testing:* ±20% over the specified range (e.g., if the acceptance criteria for a controlled-release product cover a region from 30% after 1 h, and up to 90% after 24 h, the validated range would be 10%–110% of the label claim).

The traditional definition of linearity, i.e., the establishment of a linear or mathematical relationship between sample concentration and response, is not applicable to particle size analysis. For particle size analysis, a concentration range is defined (instrument- and particle size-dependent) such that the measured particle size distribution is not affected by changes in concentration within the defined concentration range. Concentrations below the defined concentration range may introduce an error due to poor signal-to-noise ratio, and concentrations exceeding the defined concentration range may introduce an error due to multiple scattering.

## ROBUSTNESS

**Definition:** The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in procedural parameters listed in the procedure documentation and provides an indication of its suitability during normal usage. Robustness may be determined during development of the analytical procedure.

## SYSTEM SUITABILITY

If measurements are susceptible to variations in analytical conditions, these should be suitably controlled, or a precautionary statement should be included in the procedure. One consequence of the evaluation of *Robustness* and ruggedness should be that a series of system suitability parameters is established to ensure that the validity of the analytical procedure is maintained whenever used. Typical variations are the stability of analytical solutions, different equipment, and different analysts. In the case

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of liquid chromatography, typical variations are the pH of the mobile phase, the mobile phase composition, different lots or suppliers of columns, the temperature, and the flow rate. In the case of gas chromatography, typical variations are different lots or suppliers of columns, the temperature, and the flow rate.

System suitability tests are based on the concept that the equipment, electronics, analytical operations, and samples to be analyzed constitute an integral system that can be evaluated as such. System suitability test parameters to be established for a particular procedure depend on the type of procedure being evaluated. They are especially important in the case of chromatographic procedures. Submissions to the USP should make note of the requirements in *Chromatography* ⟨621⟩, *System Suitability*.

## Data Elements Required for Validation

Compendial test requirements vary from highly exacting analytical determinations to subjective evaluation of attributes. Considering this broad variety, it is only logical that different test procedures require different validation schemes. This chapter covers only the most common categories of tests for which validation data should be required. These categories are as follows:

CATEGORY I

Analytical procedures for quantitation of major components of bulk drug substances or active ingredients (including preservatives) in finished pharmaceutical products.

CATEGORY II

Analytical procedures for determination of impurities in bulk drug substances or degradation compounds in finished pharmaceutical products. These procedures include quantitative assays and limit tests.

CATEGORY III

Analytical procedures for determination of performance characteristics (e.g., dissolution, drug release, and others).

CATEGORY IV

Identification tests.

For each category, different analytical information is needed. Listed in *Table 2* are data elements that are normally required for each of these categories. [NOTE—For detailed information regarding the validation of dissolution procedures, see *The Dissolution Procedure: Development and Validation* ⟨1092⟩.]

**Table 2. Data Elements Required for Validation**

| Analytical Performance Characteristics | Category I | Category II | | Category III | Category IV |
|---|---|---|---|---|---|
| | | Quantitative | Limit Tests | | |
| Accuracy | Yes | Yes | [a] | [a] | No |
| Precision | Yes | Yes | No | Yes | No |
| Specificity | Yes | Yes | Yes | [a] | Yes |
| Detection limit | No | No | Yes | [a] | No |
| Quantitation limit | No | Yes | No | [a] | No |
| Linearity | Yes | Yes | No | [a] | No |
| Range | Yes | Yes | [a] | [a] | No |

[a] May be required, depending on the nature of the specific test.

Already established general procedures (e.g., titrimetric determination of water or bacterial endotoxins) should be verified to establish their suitability for use, such as their accuracy (and absence of possible interference) when used for a new product or raw material.

When validating physical property methods, consider the same performance characteristics required for any analytical procedure. Evaluate use of the performance characteristics on a case-by-case basis, with the goal of determining that the procedure is suitable for its intended use. The specific acceptance criteria for each validation parameter should be consistent with the intended use of the method.

Physical methods may also be classified into the four validation categories. For example, validation of a quantitative spectroscopic method may involve evaluation of *Category I* or *Category II Analytical Performance Characteristics*, depending on the method requirements. Qualitative physical property measurements, such as particle size, surface area, bulk and tapped density, which could impact performance characteristics, often best fit in *Category III*. *Category IV Analytical Performance Characteristics* usually applies to validation of qualitative identification spectroscopic methods. However, the various techniques may be used for different purposes, and the specific use of the method and characteristics of the material being analyzed should be considered when definitively applying a category to a particular type of method.

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The validity of an analytical procedure can be verified only by laboratory studies. Therefore, documentation of the successful completion of such studies is a basic requirement for determining whether a procedure is suitable for its intended application(s). Current compendial procedures are also subject to regulations that require demonstration of suitability under actual conditions of use (see *Verification of Compendial Procedures* ⟨1226⟩ for principles relative to the verification of compendial procedures). Appropriate documentation should accompany any proposal for new or revised compendial analytical procedures.

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# ⟨1226⟩ VERIFICATION OF COMPENDIAL PROCEDURES

The intent of this chapter is to provide general information on the verification of compendial procedures that are being performed for the first time to yield acceptable results utilizing the personnel, equipment, and reagents available. This chapter is not intended for retroactive application to already successfully established laboratory procedures. *Validation of Compendial Procedures* ⟨1225⟩ provides general information on characteristics that should be considered for various test categories and on the documentation that should accompany analytical procedures submitted for inclusion in *USP–NF.* Verification consists of assessing selected analytical performance characteristics, such as those that are described in ⟨1225⟩, to generate appropriate, relevant data rather than repeating the validation process.

Users of compendial analytical procedures are not required to validate these procedures when first used in their laboratories, but documented evidence of suitability should be established under actual conditions of use. In the United States, this requirement is established in 21 CFR 211.194(a)(2) of the current Good Manufacturing Practice regulations, which states that the "suitability of all testing methods used shall be verified under actual conditions of use."

Verification of microbiological procedures is not covered in this chapter because it is covered in *Antimicrobial Effectiveness Testing* ⟨51⟩, *Microbial Enumeration Tests* ⟨61⟩, *Tests for Specified Microorganisms* ⟨62⟩, *Sterility Tests* ⟨71⟩, and *Validation of Microbial Recovery from Pharmacopeial Articles* ⟨1227⟩.

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## VERIFICATION PROCESS

The verification process for compendial test procedures is the assessment of whether the procedure can be used for its intended purpose, under the actual conditions of use for a specified drug substance and/or drug product matrix.

Users should have the appropriate experience, knowledge, and training to understand and be able to perform the compendial procedures as written. Verification should be conducted by the user such that the results will provide confidence that the compendial procedure will perform suitably as intended.

If the verification of the compendial procedure is not successful, and assistance from USP staff has not resolved the problem, it may be concluded that the procedure may not be suitable for use with the article being tested in that laboratory. It may then be necessary to develop and validate an ▲alternative▲ (USP 1-Dec-2019) procedure as allowed in the *General Notices, 6.30 Alternative and Harmonized Methods and Procedures*. The ▲alternative▲ (USP 1-Dec-2019) procedure may be submitted to USP, along with the appropriate data, to support a proposal for inclusion or replacement of the current compendial procedure.

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## VERIFICATION REQUIREMENTS

Verification requirements should be based on an assessment of the complexity of both the procedure and the material to which the procedure is applied. Although complete revalidation of a compendial method is not required to verify the suitability of a procedure under actual conditions of use, some of the analytical performance characteristics listed in *Validation of Compendial Procedures* ⟨1225⟩, *Table 2*, may be used for the verification process. Only those characteristics that are considered to be appropriate for the verification of the particular procedure need to be evaluated. The process of assessing the suitability of a compendial analytical test procedure under the conditions of actual use may or may not require actual laboratory performance of each analytical performance characteristic. The degree and extent of the verification process may depend on the level of training and experience of the user, on the type of procedure and its associated equipment or instrumentation, on the specific procedural steps, and on which article(s) are being tested.

Verification should assess whether the compendial procedure is suitable for the drug substance and/or the drug product matrix, taking into account the drug substance's synthetic route, the method of manufacture for the drug product, or both, if applicable. Verification should include an assessment of elements such as the effect of the matrix on the recovery of impurities and drug substances from the drug product matrix, as well as the suitability of chromatographic conditions and column, the appropriateness of detector signal response, etc.

As an example, an assessment of specificity is a key parameter in verifying that a compendial procedure is suitable for use in assaying drug substances and drug products. For instance, acceptable specificity for a chromatographic method may be verified by conformance with system suitability resolution requirements (if specified in the procedure). However, drug substances from different suppliers may have different impurity profiles that are not addressed by the compendial test procedure. Similarly, the excipients in a drug product can vary widely among manufacturers and may have the potential to directly interfere with the procedure or cause the formation of impurities that are not addressed by the compendial procedure. In addition, drug products containing different excipients, antioxidants, buffers, or container extractives may affect the recovery of the drug substance from the matrix. In these cases, a more thorough assessment of the matrix effects may be required to demonstrate suitability of the procedure for the particular drug substance or product. Other analytical performance characteristics such as an assessment of the limit of detection or quantitation and precision for impurities procedures may be useful to demonstrate the suitability of the compendial procedure under actual conditions of use. ▲It is the user's responsibility to demonstrate the long term (more than 24 h) stability and storage conditions of Standard and sample preparations throughout the duration of the procedure during the verification of compendial procedures.▲ (USP 1-Dec-2019)

Verification is not required for basic compendial test procedures that are routinely performed unless there is an indication that the compendial procedure is not appropriate for the article under test. Examples of basic compendial procedures include, but are not limited to, loss on drying, residue on ignition, various wet chemical procedures such as acid value, and simple instrumental determinations such as pH measurements. However, for the application of already established routine procedures

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to compendial articles tested for the first time, it is recommended that consideration be given to any new or different sample handling or solution preparation requirements.

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# ⟨1227⟩ VALIDATION OF MICROBIAL RECOVERY FROM PHARMACOPEIAL ARTICLES

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## INTRODUCTION

▲This chapter provides guidelines for the validation of recovery methods for the estimation of the number of viable microorganisms, the detection of indicators or specified microorganisms, and the sterility testing of pharmacopeial articles. The test procedures in *Antimicrobial Effectiveness Testing* ⟨51⟩, *Sterility Tests* ⟨71⟩, *Microbial Enumeration Tests* ⟨61⟩, and *Tests for Specified Microorganisms* ⟨62⟩ are considered validated. However, use of compendial methods requires establishment of suitability of the method demonstrating recovery of the challenge organisms in the presence of the product. Alternatives/modifications to these recovery procedures beyond what are described in these chapters (such as dilution, chemical or enzymatic neutralization, and membrane filtration) require validation.▲ (USP 1-Dec-2019) It is generally understood that if a product possesses antimicrobial properties because of the presence of a specific preservative or because of its formulation, this antimicrobial property must be neutralized to recover viable microorganisms. This neutralization may be achieved by the use of a specific neutralizer, by dilution, by a combination of ▲▲ (USP 1-Dec-2019) dilution, ▲filtration, and rinsing,▲ (USP 1-Dec-2019) or by any combination of these methods. ▲When the product displays intrinsic antimicrobial activity for a given microorganism and, given this antimicrobial activity, the risk of microbial contamination is low, the method could be considered as fit for the purpose of providing a strong rationale.▲ (USP 1-Dec-2019)

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## INFLUENTIAL FACTORS

Several factors affect the measurement of a test solution's antimicrobial activity, and these must be considered in the validation design. They include the nature of the microorganisms used as challenge organisms, preparation of the inoculum of challenge organisms, specific conditions of the test, and conditions of recovery. These factors also affect the validation of recovery methods for aqueous or nonaqueous products, irrespective of their antimicrobial properties; thus, all test methods should be validated with these factors in mind.

The nature of the challenge microorganism exerts a strong effect upon the response to the antimicrobial agent, and so upon the neutralization required for recovery. Represented among these organisms in compendial tests are gram-positive bacteria, gram-negative bacteria, ▲anaerobic bacteria,▲ (USP 1-Dec-2019) yeasts, and molds. Each organism to be used in the test must be included in the validation.

The preparation of the inoculum of challenge microorganisms also affects the testing of products having antimicrobial properties. The growth and preparation of the challenge organism determines the physiological state of the cell. This state has a direct influence on the results of any test of antimicrobial efficacy. Microbial tests do not use individual cells; rather, populations of cells are harvested for study. The data generated from these studies are less variable if the cell populations are homogeneous. Liquid cultures or confluent growths on solid medium are best suited for reproducible culture preparation. The conditions of organism preparation and storage must be standardized for the neutralizer evaluation and should reflect the conditions of the antimicrobial assay.

The specific conditions of the test, including buffers used, water, light conditions, and temperature, must be reproduced in the validation study. All test conditions also should be standardized and performed in the validation study exactly as performed in the test.

The conditions of microbial recovery are among the most crucial in accurately estimating the number of microorganisms present in a test solution. The first consideration is the recovery medium used to support the growth of survivors. This concern is discussed in detail below. The second consideration is the incubation conditions. Optimal conditions for growth must be present to ensure complete growth and reproducible results.

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## METHODS OF NEUTRALIZING ANTIMICROBIAL PROPERTIES

Three common methods are used to neutralize antimicrobial properties of a product: 1) chemical inhibition, 2) dilution, and 3) filtration and ▲rinsing.▲ (USP 1-Dec-2019)

### Chemical ▲Neutralization▲ (USP 1-Dec-2019)

*Table 1* shows known neutralizers for a variety of chemical antimicrobial agents and the reported toxicity of some chemical neutralizers to specific microorganisms. However, despite potential toxicity, the convenience and quick action of chemical inhibitors encourage their use. Chemical ▲neutralization of antimicrobial agents▲ (USP 1-Dec-2019) is the preferred method for the antimicrobial efficacy test. The potential of chemical ▲neutralizers▲ (USP 1-Dec-2019) should be considered in the membrane filtration and the direct ▲inoculation▲ (USP 1-Dec-2019) sterility tests. Antibiotics may not be susceptible to neutralization by chemical means, but rather by enzymatic treatment (e.g., penicillinase). These enzymes may be used where required.

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**Table 1. Some Common Neutralizers for Chemical ▲Antimicrobial Agents▲ (USP 1-Dec-2019)**

| Neutralizer | ▲Antimicrobial▲ (USP 1-Dec-2019) Class | Potential Action of ▲Antimicrobial Agents▲ (USP 1-Dec-2019) |
|---|---|---|
| Bisulfate | Glutaraldehyde, mercurials | Non-sporing bacteria |
| Dilution | Phenolics, alcohol, aldehydes, sorbate | — |
| Glycine | Aldehydes | Growing cells |
| Lecithin | Quaternary ammonium compounds (QACs), parabens, bis-biguanides | Bacteria |
| $Mg^{+2}$ or $Ca^{+2}$ ions | EDTA | — |
| Polysorbate | QACS, iodine, parabens | — |
| Thioglycollate | Mercurials | Staphylococci and spores |
| Thiosulfate | Mercurials, halogens, aldehydes | Staphylococci |

## Dilution

A second approach to neutralizing antimicrobial properties of a product is by dilution, because the concentration of a chemical ▲antimicrobial agent▲ (USP 1-Dec-2019) exerts a large effect on its potency. The relationship between concentration and antimicrobial effect differs among bactericidal agents but is constant for a particular antimicrobial agent. This relationship is exponential in nature, with the general formula:

$$C^{\eta}t = k$$

$C$ = concentration ▲of the antimicrobial agent▲ (USP 1-Dec-2019)
$\eta$ = concentration exponent ▲(dilution coefficient),▲ (USP 1-Dec-2019) the slope of the plot of log $t$ versus log $C$
$t$ = time required to kill a standard inoculum
$k$ = a constant

Antimicrobial agents with high $\eta$ values are rapidly neutralized by dilution, whereas those with low $\eta$ values are not good candidates for neutralization by dilution ▲(see *Table 2*).

**Table 2. Concentration Exponents for Some Common Antimicrobial Agents**

| Representative Antimicrobial Agent | η Values | Increased Time Factor (*x*) to Kill Microorganisms When the Concentration Is Reduced to: | |
|---|---|---|---|
| | | One-Half | One-Third |
| Phenolics | 6 | 64 | 729 |
| Alcohol | 10 | 1024 | 59,000 |
| Parabens | 2.5 | 6 | 16 |
| Chlorhexidine | 2 | 4 | 8 |
| Mercury compounds | 1 | 2 | 3 |
| Quaternary ammonium compounds | 1 | 2 | 3 |
| Formaldehyde | 1 | 2 | 3▲ (USP 1-Dec-2019) |

## Membrane Filtration

An approach that is often used, especially in sterility testing, is neutralization by membrane filtration. This approach relies upon the physical retention of the microorganism on the membrane filter, with the antimicrobial agent passing through the filter into the filtrate. The filter is then incubated for recovery of viable microorganisms. However, filtration alone may not remove sufficient quantities of the ▲antimicrobial▲ (USP 1-Dec-2019) agent to allow growth of surviving microorganisms. Adherence of residual antimicrobial agents to the filter membrane may cause growth inhibition. Filtration through a low-binding filter material, such as polyvinylidene difluoride, helps to minimize this growth inhibition. Additionally, the preservative may be diluted or flushed from the filter by rinsing with a ▲non-toxic▲ (USP 1-Dec-2019) fluid, such as diluting *Fluid A* (see *Sterility Tests* ⟨71⟩, *Diluting and Rinsing Fluids for Membrane Filtration* for diluting fluid compositions). Chemical neutralizers in the rinsing fluid can ensure that any antimicrobial residue on the membrane does not interfere with the recovery of viable microorganisms.

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# VALIDATION OF NEUTRALIZATION METHODS—RECOVERY COMPARISONS

A validated method for neutralizing the antimicrobial properties of a product must meet two criteria: neutralizer efficacy and neutralizer ▲non-toxicity.▲ (USP 1-Dec-2019) The validation study documents that the neutralization method employed is effective in inhibiting the antimicrobial properties of the product (neutralizer efficacy) without impairing the recovery of viable microorganisms (▲lack of▲ (USP 1-Dec-2019) neutralizer toxicity). Validation protocols may meet these two criteria by comparing recovery results for treatment groups.

The first is the test group, in which the product is subjected to the neutralization method, then a low level of challenge microorganism [less than 100 colony-forming units (cfu)] is inoculated for recovery. The second is the peptone control group, in which the neutralization method is used with peptone, or diluting *Fluid A* (see *Sterility Tests* ⟨71⟩*, Diluting and Rinsing Fluids for Membrane Filtration*), as the test solution. The third is the viability group, in which the actual inoculum is used without exposure to the neutralization scheme. Similar recovery between the test group and the peptone group demonstrates adequate neutralizer efficacy; similar recovery between the peptone group and the viability group demonstrates adequate neutralizer ▲non-toxicity.▲ (USP 1-Dec-2019)

In principle, the protocol must show that recovery of a low inoculum (less than 100 cfu) is not inhibited by the test sample and the neutralization method. Validation protocols may meet these two criteria by comparing recovery among three distinct test groups: 1) neutralized product with inoculum, 2) challenge inoculum control in buffered solution, and 3) inoculum in the absence of product or neutralizer. This can be established by directly comparing the result in the treated solution (1) to the inoculum (3) above. If the growth on the treated solution is not comparable to the growth on the inoculum group, it should be determined whether the neutralization method itself is toxic to the microorganisms.

## Recovery on Agar Medium

In the tests under ⟨51⟩ and ⟨61⟩, ▲▲ (USP 1-Dec-2019)the number of viable challenge microorganisms in the product is estimated ▲▲ (USP 1-Dec-2019) by calculating the concentration of cfu per milliliter by the plate count method. A design for validating neutralization would incorporate the treatment groups as described under *Validation of Neutralization Methods—Recovery Comparisons*. At least three independent replicates of the experiment should be performed, and each should demonstrate ▲a mean count of any of the test organisms not differing by a factor greater than 2, i.e., 50%–200% recovery, from the value of the control in the absence of product. If it is necessary to solubilize the test ▲sample,▲ (ERR 1-Dec-2019) the effects of the solubilization method on viable microorganisms must be determined. This situation can occur when testing ointments, suspensions, or other articles.▲ (USP 1-Dec-2019)

If a greater number of replicates is required in the validation study, the comparisons may be evaluated by transforming the numbers of cfu to their logarithmic values and analyzing the data statistically by the Student *t* test (pairwise comparisons) or by analysis of variance (ANOVA) (for comparing all groups). If ANOVA is used, and significant differences among the populations are determined, a test such as Dunnett's test may be used, with the peptone group used as the control group.

## Recovery by Membrane Filtration

This validation follows the procedure described in ▲*Sterility Tests* ⟨71⟩*, Method Suitability Test*,▲ (USP 1-Dec-2019)with the exception of plating on solid medium to quantitate recovery. ▲It should be emphasized that quantitative recovery is not required to demonstrate sterility test suitability. It only requires a qualitative assessment (visual turbidity).▲ (USP 1-Dec-2019) Three 100-mL rinses are assumed, but the volume and number of rinses are subject to validation. ▲A maximum of five 100-mL washes should be used for routine testing even if during method suitability it has been demonstrated that such a cycle does not fully eliminate the antimicrobial activity.▲ (USP 1-Dec-2019)Each validation run should be performed independently at least three times.

In the test solution group, the product is passed through the membrane filter, followed by two 100-mL portions of diluting-neutralizing fluid. After the second rinse has been filtered, a final 100-mL portion containing less than 100 cfu of the specific challenge microorganism is passed through the filter. This filter is then placed on the appropriate agar recovery medium and incubated for recovery.

The inoculum is directly plated onto the solid medium. It is possible that filtration will lead to reduced recovery of the challenge microorganism, either through inherent toxicity of the membrane or by adherence of the microorganism to the filtration vessel walls. A control group can be used to evaluate this component of membrane filtration validation. Diluting *Fluid A* is used as the dilution medium without exposing the filter to the product. After addition of the low-level inoculum to the final rinse, the filter is plated as above. Technique-specific loss of microorganisms can be estimated by comparing the recovery in the diluting *Fluid A* (see *Sterility Tests* ⟨71⟩*, Diluting and Rinsing Fluids for Membrane Filtration*) group to the inoculum count.

It is assumed in this discussion that the test sample can be filtered. If it is necessary to solubilize the test sample, the effects of the solubilization method on viable microorganisms must be determined. This situation can occur when testing ointments, suspensions, or other articles.

The method can be considered validated if the recovery rate in the three independent replicates is similar for the test solution and the diluting *Fluid A* (see *Sterility Tests* ⟨71⟩*, Diluting and Rinsing Fluids for Membrane Filtration*) control.

## Recovery in Liquid Medium

It is assumed in *Sterility Tests* ⟨71⟩*, Test for Sterility of the Product to be Examined, Direct Inoculation of the Culture Medium* that the recovery medium will allow for growth of all surviving microorganisms. The broth in that test must serve both to neutralize any antimicrobial properties of the test solution and to support the growth of the microorganisms. The treatment groups

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described under *Validation of Neutralization Methods—Recovery Comparisons* can be used for validation of the recovery method, with the proportions of product and recovery medium varied to achieve adequate neutralization. The method can be considered validated if all groups show ▲clearly visible growth visually comparable to that in the control vessel without product within the indicated time period in ⟨71⟩.▲ (USP 1-Dec-2019)

## RECOVERY OF INJURED MICROORGANISMS

The validation studies described above use challenge microorganisms that have never been exposed to antimicrobial agents, and thus are not identical to organisms seen in antimicrobial effectiveness testing or when a sterility test is performed on a preserved product. If the use of alternative media is desired, the recovery of injured microorganisms should be addressed in the validation study. This may be done by directly comparing the recovery of each challenge microorganism on the preferred medium and on the alternative medium, after exposure to the product. This exposure should include at least two time periods showing survival of less than 100 cfu/mL, unless the rate of kill of the antimicrobial agent is such that no recovery is possible even if the microorganism is plated within minutes of exposure. This comparison should be performed at least three times. The alternative medium is validated if the recovery seen on that medium is no less than that seen on the preferred medium, within an error of 0.5 log units.

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## ESTIMATING THE NUMBER OF COLONY-FORMING UNITS

The accuracy of any estimate of viable cfu is affected by the number plated. As the number of viable cells plated increases, crowding effects decrease the accuracy of the count, reducing the estimate. As the number decreases, random error plays an increasing role in the estimate.

The accepted range for countable colonies on a standard agar plate is between 25 and 250 for most bacteria and *Candida albicans.* This range was established in the food industry for counting coliform bacteria in milk. This range is acceptable for compendial organisms, except for fungi. It is not optimal for counting all environmental isolates. The recommended counting range for *Aspergillus* ▲*brasiliensis*▲ (USP 1-Dec-2019) is between 8 and 80 cfu/plate.

▲▲ (USP 1-Dec-2019)

Lower counting thresholds for the greatest dilution plating in series must be justified. Numbers of colonies on a plate follow the Poisson distribution, so the variance of the mean value equals the mean value of counts. Therefore, as the mean number of cfu per plate becomes lower, the percentage error of the estimate increases (see ▲*Table 3*). For example,▲ (USP 1-Dec-2019) 3 cfu/plate at the $10^{-1}$ dilution provide an estimate of 30 cfu/mL, with an error of 58% of the estimate.

**▲Table 3.▲ (USP 1-Dec-2019) Error as a Percentage of Mean for Plate Counts**

| cfu/Plate | Standard Error | Error as % of Mean |
|---|---|---|
| 30 | 5.48 | 18.3 |
| 29 | 5.39 | 18.6 |
| 28 | 5.29 | 18.9 |
| 27 | 5.20 | 19.2 |
| 26 | 5.10 | 19.6 |
| 25 | 5.00 | 20.0 |
| 24 | 4.90 | 20.4 |
| 23 | 4.80 | 20.9 |
| 22 | 4.69 | 21.3 |
| 21 | 4.58 | 21.8 |
| 20 | 4.47 | 22.4 |
| 19 | 4.36 | 22.9 |
| 18 | 4.24 | 23.6 |
| 17 | 4.12 | 24.3 |
| 16 | 4.00 | 25.0 |
| 15 | 3.87 | 25.8 |
| 14 | 3.74 | 26.7 |
| 13 | 3.61 | 27.7 |
| 12 | 3.46 | 28.9 |
| 11 | 3.32 | 30.2 |
| 10 | 3.16 | 31.6 |

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**▲Table 3.▲** (USP 1-Dec-2019) **Error as a Percentage of Mean for Plate Counts** *(continued)*

| cfu/Plate | Standard Error | Error as % of Mean |
|---|---|---|
| 9 | 3.00 | 33.3 |
| 8 | 2.83 | 35.4 |
| 7 | 2.65 | 37.8 |
| 6 | 2.45 | 40.8 |
| 5 | 2.24 | 44.7 |
| 4 | 2.00 | 50.0 |
| 3 | 1.73 | 57.7 |
| 2 | 1.41 | 70.7 |
| 1 | 1.00 | 100.0 |

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# ⟨1228.1⟩ DRY HEAT DEPYROGENATION

## INTRODUCTION

Dry heat is the method most frequently used for the depyrogenation of heat stable materials. Dry heat depyrogenation is dependent upon two parameters: time and temperature, which equates to a thermal input. As a result, dry heat depyrogenation processes can be easily monitored/controlled and are highly reproducible. Depyrogenation processes typically operate at a range of temperatures from approximately 170° up to about 400°.

The most prevalent pyrogenic agents in parental manufacturing that are of concern relative to patient safety are bacterial endotoxins, found in the outer cell walls of Gram-negative bacteria. The destruction of bacterial endotoxins (depyrogenation) by dry heat has been studied extensively and has been shown to follow first order kinetics. The well-defined kinetics of inactivation makes it possible to predict the efficacy of dry heat processes operating at different times and temperatures by understanding the total thermal input ($F_D$).

The range of temperatures used for dry heat depyrogenation overlaps the upper range of temperatures used for dry heat sterilization (see *Dry Heat Sterilization* ⟨1229.8⟩). This is because bacterial endotoxins are more resistant to the effects of dry heat than the most heat-resistant bacterial spores. This chapter provides an overview of the process of dry heat depyrogenation, its control, and validation.

## TECHNOLOGIES USED FOR DEPYROGENATION BY DRY HEAT

Although all dry heat depyrogenation processes rely strictly on time of exposure and temperature to assure effectiveness, the equipment used typically falls into two categories: the dry heat "batch" oven and continuous tunnel systems. Batch ovens are routinely used for the depyrogenation of product containers, most often glass, but also other heat stable product contact parts or laboratory equipment. Continuous tunnels, on the other hand, are used primarily to depyrogenate glass product containers.

### Batch Ovens

Circulating heated air is used to heat the load items, which may be individually covered or wrapped in a material that is unaffected by the temperature used, or placed in a lidded container for protection during pre- and post-process handling. When depyrogenation and sterilization are to be achieved in the same process, air supplied to the oven is passed through one or more high efficiency particulate air (HEPA) filter(s) to maintain sterility within the oven after completion of the dwell period. These forced air ovens typically operate at a positive air pressure differential relative to the surrounding room. This design results in particulate air quality that can meet ISO 5 requirements to reduce particulate matter and microbial contamination risk throughout processing.

In order to ensure sufficient lethality and process control, oven control probe(s) must maintain a predefined temperature for a predefined time period prior to cooling. The limited heat transfer capacity of air requires that items in the oven be placed in fixed locations confirmed acceptable during the cycle development/validation effort. Caution should be exercised in defining variable load patterns as minimum load sizes may result in inadvertent slower heating of the load and greater temperature variability. Smaller facilities may use a single door oven, but the principles of operation and validation are the same as with larger double door production units. The important batch oven process variables are set-point temperature, duration of dwell period, load type and configuration, airflow characteristics, and container size.

### Continuous Tunnels

The use of tunnels for dry heat depyrogenation of glass containers on a moving conveyor allows for substantially higher throughput and packing densities than the batch process, reduces handling, and is ordinarily integrated with a washing and filling system. Tunnels typically use forced heated air systems or radiant IR systems that recirculate air through a battery of HEPA filters. Load items in tunnels are typically fed directly from an integrated container washing system.

Depyrogenation tunnels have separate zones for heating and cooling, allowing for continuous in-feed and discharge at temperatures appropriate for production purposes. The tunnel is maintained at constant airflow and temperature conditions during use, and as glass passes through the tunnel it is heated to depyrogenating temperatures and cooled before exiting. Although the conditions within the tunnel are essentially constant and well controlled, the temperature of the glass as it passes through the tunnel on the conveyor will change with its location. Dwell time is controlled by adjusting the conveyor speed,[1] which in the depyrogenation tunnel is the process parameter that governs exposure time.

The air in the tunnel is most commonly heated using electrical coils but other heat sources, such as infrared or high-pressure steam, have been used. For energy conservation, heated air in depyrogenation tunnels is often recirculated. The important continuous tunnel process variables are heating zone temperature, cooling zone temperature, belt speed, and container mass per unit.

[1] Not all tunnels have a variable speed capacity.

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## DRY HEAT DEPYROGENATION FUNDAMENTALS

Dry heat depyrogenation uses air first to heat and then to cool the items. The limited heat capacity of dry air results in relatively slow heating and cooling of the load items. Variability in temperature distribution in dry heat ovens and tunnels is typically much higher than that observed in moist heat systems. The limited heat capacity of air requires that items in ovens be placed in the same locations as confirmed acceptable in the cycle development/validation effort. Packing and thermal mass will also play critical roles in temperature management. Caution must also be exercised with varying load mass and distribution as in some instances (resulting from oven design, air flow characteristics, and control probe position) minimum load sizes may result in process variability.

## DEPYROGENATION PROCESS CONTROL

Process temperatures in dry heat depyrogenation are controlled by calibrated temperature sensors placed at specific locations within the equipment. The exposure portion of the process is designed to attain a minimum dwell time at a predefined minimum temperature ensuring that depyrogenation conditions are adequately uniform. The defined dwell time is determined by using measurement devices (e.g., thermocouples) directly in contact with the items to be depyrogenated during development. The inactivation of bacterial endotoxins by dry heat involves the control of only two parameters: time and temperature.

The simplicity of process control for these parameters provides a predictable depyrogenation effect. Once validated, a clear understanding of the inputs to the system, meaning resident endotoxin load on incoming materials, and outputs of the system, meaning reduction of resident levels of endotoxin to safe levels, is more meaningful than spiking with unnaturally high levels of challenge material and looking for a prescriptive requirement for log reduction. The dosimetric measurement for dry heat depyrogenation processes is the $F_D$ unit. An $F_D = 1$ is defined as the depyrogenation effect achieved by 1 min of heating at 250°. The $F_D$-value enables the integration of temperature over the process duration (time). By convention, the rate at which depyrogenation destruction rate (*D*-value) varies as a function of temperature change is defined as the *z*-value. The *z*-value for dry heat depyrogenation has been shown to be in the range of 45°–55°. For the purposes of this chapter, 50° is used as a standard *z*-value. Other values may be used. (*1,2*)

The $F_D$ approach is used as a means to compare dry heat depyrogenation effects produced by processes that operate at varying temperatures. Basic mathematics can be used to calculate the depyrogenation effect produced at temperatures other than 250° to determine equivalence to that provided at 250°.

Using a reference temperature of 250° and an assumed *z*-value of 50°, the $F_D$ calculation can be determined:

$$F_D = \int_{t_1}^{t_2} 10^{\left(\frac{T-250}{50}\right)} dt = \sum_{t_1}^{t_2} 10^{\left(\frac{T-250}{50}\right)} \Delta t$$

$F_D$ = accumulated destruction
$t_1$ = process start time
$t_2$ = process end time
$T$ = temperature at each time increment
$\Delta t$ = time interval between temperature measurements

Summing the instantaneous temperature contributions over the entire depyrogenation process allows for the calculation of the overall process efficacy or $F_D$ delivered over the course of the process. Many commercial data loggers are equipped with software that enables them to make this calculation and integrate the total $F_D$ accumulated during a process. The $F_D$ calculation is used during initial validation, validation maintenance, and change control. The mathematical principles of the $F_D$ calculation are essentially the same as those used to calculate lethality ($F_0$) values in moist heat sterilization. $F_D$ values are used to confirm process consistency over time as correlation to endotoxin destruction is rarely possible.

## VALIDATION

Because dry heat depyrogenation is appropriate only for heat stable materials, a high margin for safety is always attainable. Times and temperatures used for the purpose of destroying challenge materials can result in extreme challenges to material integrity and stability. Attention to depyrogenation processes, including an understanding of the resident endotoxin load on incoming materials and reduction to levels needed to assure patient safety, should take place during drug product development, prior to validation.

### Equipment Qualification

Equipment Qualification (EQ) is a predefined program that focuses on the processing equipment to confirm that it has been properly installed and operates as intended prior to evaluation of the process. In some companies, EQ may be separated into installation qualification (IQ) and operational qualification (OQ), or combined together under a joint terminology of installation/

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operational qualification (I/OQ). Equipment qualification provides a baseline for preventive maintenance and change control assuring reproducibility of equipment operation over time.

## Empty Chamber Temperature Distribution for Ovens

The oven should be evaluated for empty chamber temperature distribution. This is assessed by measurement of temperature at each corner of oven, near the controlling probe(s) and other locations as justified. Differences in the cycle dwell period can be discounted in this evaluation, as only the shortest dwell period need be evaluated. The evaluation is best performed over the last few minutes of the dwell period once the system has fully stabilized. The acceptance criteria for this test vary with the oven's design and operating conditions; however, temperature distribution is typically substantially less uniform than observed in autoclaves and may be ±15° or more. Depyrogenation ovens that are located at floor level may have even greater ranges in temperature. The temperature distribution measurement may be of value in the evaluation of changes to the oven.

## Empty Temperature Distribution in Tunnels

While these studies are often done, they are actually of limited value. Unloaded depyrogenation tunnels will always produce far more variability in temperature distribution than will a fully loaded tunnel. Therefore, for depyrogenation tunnels, temperature studies under fully loaded conditions only are indicated. Important to the proper operation of the dry heat tunnel is the establishment of the required air flow balance between the tunnel and the adjoining areas. Improper air flow can cause uneven heating across the load being processed. The temperature distribution measurement may be of value in the evaluation of changes to the tunnel.

## Component Mapping

The ability of dry heat to penetrate load items and to bring them to the required temperature should be determined.

Load items that are complex, of significant mass, with enclosed volumes and product contact surfaces that must be depyrogenated, should be subjected to component mapping to determine internal cold spots. All load items should be prepared, wrapped (if that is the practice), and oriented in a manner consistent with how they will be processed. Glass typically enters the depyrogenation process (whether tunnel or oven) wet and must be evaluated wet to properly determine the effect on overall thermal input. Mapping of glass components to be processed in tunnels is not necessary; all monitoring of temperature in tunnels is accomplished with probes in contact with the bottom of the container.

## Load Mapping in Ovens

Fixed loading patterns are necessary in oven depyrogenation because of the limited heat capacity of the air; fully packed conditions because of their greater mass ordinarily result in the best process temperature uniformity. Load mapping assures that items placed throughout the load attain the desired depyrogenation conditions. Identification of cold zones within the oven should be established during depyrogenation cycle development. Information from the load mapping is used to adjust cycle timing to assure appropriate efficacy across the entire load. It may be possible to validate maximum and minimum loads (as determined by either the number of items or their mass).

$F_D$ is calculated from the temperature data at all monitored locations within the load pattern.

## Load Mapping in Tunnels

Load mapping can be assessed using sets of calibrated sensors (i.e., trailing or wireless temperature sensors) positioned within the glass pack as it moves through the tunnel. Temperature sensors should be placed into direct contact with the glass item at the bottom of the container. Temperature measurements should be made on the leading edge, the middle (highest density), and the trailing edge of the glass pack across the width of the conveyor belt. There should be NLT 5 temperature sensors positioned across the belt in each section of the load. $F_D$ is calculated from the temperature data at all monitored locations. Studies should be performed using all container sizes to determine the lowest $F_D$ locations. The $F_D$ results can be used to support the selection of containers/conditions to be evaluated in the confirmation studies (see below). There is no requirement to perform temperature heat distribution measurements during these studies.

## Confirmation of Depyrogenation

The materials/glass components to be depyrogenated should be assessed for their incoming endotoxin content prior to the validation study. This would include glass as received and immediately after washing. All tested materials should be handled and prepared using defined procedures. Materials and glass prepared in the same manner are used in the depyrogenation validation studies. The addition of challenge material to the load items, including a requirement to demonstrate a 3-log reduction, may not be required if time and temperature studies consistently indicate that depyrogenation conditions are met. Temperature monitoring as described above must be done simultaneously with the depyrogenation confirmation studies. The confirmation studies should be performed at reduced time-temperature conditions from those utilized in routine processing and deliver lower $F_D$ results when compared to those determined in the mapping studies and are considered "worst case" confirmation of depyrogenation process efficacy. There is no requirement to perform temperature heat distribution measurements during these studies.

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OVENS

A minimum of five (5) samples should be taken in proximity at NLT 10 temperature-monitored locations (including those determined to be the coldest from the oven load mapping study) in the oven and tested for endotoxin content post-processing. The process is considered acceptable if the amount of endotoxin per sample is NMT 0.1 EU.

TUNNELS

A minimum of five (5) samples should be taken in proximity to each monitored position within the tunnel load (including those determined to be the coldest from the tunnel load mapping study) and tested for endotoxin content post-processing. The process is considered acceptable if the amount of endotoxin per sample is NMT 0.1 EU.

## ROUTINE PROCESS CONTROL

As with all processes, after the dry heat depyrogenation process has been validated, it must be subject to ongoing controls that maintain it within the validated state at all times. Temperature and exposure time, which are the important dry heat depyrogenation parameters, can be used to confirm performance on a routine basis. Where direct assessment of $F_D$ is not possible, assuring that the temperature and exposure time conditions were met results in an equivalent confidence that the depyrogenation system operated in a validated state of control.

*Depyrogenation* ⟨1228⟩ details the general practices that are appropriate for all depyrogenation systems. This is accomplished by a number of related practices that are essential for the continued use of the process over an extended period of time. The essential practices to maintain validated status include calibration, physical measurements, periodic endotoxin assessment on incoming materials, ongoing process control, change control, preventive maintenance, and periodic reassessment and training.

## APPENDIX

### Additional Sources of Information

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# ⟨1228.3⟩ DEPYROGENATION BY FILTRATION

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    2.2 Reverse Osmosis
    2.3 Ultrafiltration
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    2.6 Membrane Adsorbers
3. VALIDATION

## 1. INTRODUCTION

Depyrogenation by filtration involves the physical removal of endotoxins from pharmaceutical solutions by adsorption and size exclusion. Endotoxins are responsible for making up the majority of pyrogens, which must be removed from pharmaceutical products including injectable biologics. There are many factors to be considered when designing a depyrogenation filtration process for solutions containing proteins and peptides: type of target protein and its concentration, electrolyte concentration, pH and buffer system, protein molecular weight and isoelectric point (pI), filtration parameters (e.g., flow rate), and interactions with other components causing aggregation. In general, a combination of these factors determines the most effective depyrogenation method.

Depyrogenation of liquids may be accomplished by means of filtration through various types of filter media including microporous membranes, reverse osmosis (RO) membranes, ultrafilters, charge-modified depth filters, activated carbon, and membrane absorbers. Depyrogenation filtration processes are not intended to remove microorganisms from a process stream; however, by their nature, filters selected for use in depyrogenation processes may also be capable of retaining many types of microorganisms.

## 2. TECHNOLOGIES USED FOR DEPYROGENATION BY FILTRATION

### 2.1 Microporous Membrane Filtration

Microporous membranes (typically with pore size or retention ratings between 1.0 and 0.1 µm) can be very effective in removing intact bacteria via size exclusion and adsorption within flow pathways. The use of microporous membranes on a freshly prepared solution to be filtered can effectively prevent bacterial proliferation in the solution, along with any potential subsequent endotoxin formation. Endotoxin, however, is composed of fragments of bacterial cell wall, often <0.025 µm (*1*) that may easily penetrate most bacteria-retentive membrane filters. These negatively charged particles with endotoxin activity can be removed via adsorption by positively charged membranes (*2*). Adsorption of endotoxin has also been shown by hydrophobic membranes, where it is thought that a hydrophobic interaction occurs between the Lipid A core and hydrophobic sites on the membrane flow path surfaces (*3*). Reduction or removal of endotoxin activity by adsorption to microporous membranes can be dependent on flow rate, pH, concentration, and fluid and membrane surface properties. Once the effective binding capacity of the membrane approaches saturation under applied conditions, remaining endotoxin will pass through the membrane.

### 2.2 Reverse Osmosis

RO membranes are the tightest membranes in size separation. They can separate dissolved salts and sugars from water. Pyrogens, and essentially everything else, are removed from water via size exclusion. RO systems are operated most efficiently at high pressure (200–1000 psi) to overcome osmotic pressure. RO membrane rating or tightness is measured and expressed with retention or rejection of marker salts such as sodium chloride or magnesium sulfate.

RO membranes may be composites (thin film coated on top of ultrafiltration membranes) or cast as a single layer (cellulose acetate type). Configuration of RO membrane modules can be flat sheet, tubular, or hollow fiber. All commercially available RO membranes are polymeric, and most are of a spiral-wound, flat-sheet format.

RO systems are not intended to remove all bacteria, and because they are run at ambient temperatures, microbiological contamination is a concern. Ultraviolet (UV) light may be used in the system downstream from the RO units to control microbiological contamination.

### 2.3 Ultrafiltration

Ultrafiltration (UF) is a process whereby a fluid is passed through membranes with pore sizes nominally between about 1 and 100 nm under pressure. The filters are usually not rated by the pore size but by the molecular weight cut-off (MWCO). The methods to determine the MWCO vary by the manufacturer and usually involve measuring passage of molecules of a certain size, such as a solution of mixed dextrans, polyethylene glycol, or proteins to assign a numerical rating (*4*).

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UF membranes are usually polymeric porous structures, manufactured from a range of materials, most commonly regenerated cellulose or polyether sulfone, but also ceramics. UF membranes may be produced as flat sheet, hollow fibers, or ceramic tubes.

UF is generally operated in tangential/cross flow mode, which separates the starting (feed) solution into two components: permeate (the portion of solution going through the membrane) and retentate (the concentrated solution that is passed over the membrane). UF membranes need to be encased in a suitable integral device to enable practical operation. Heat sealing, over-molding, and resin-potting are all used to assemble membrane devices and ensure integral flow paths. Ceramic tubes are sealed by gaskets within tubular cylinders.

It is generally assumed that the basic subunit of lipopolysaccharide (LPS) is about 10–20 kDa (*5*). Membranes of 6–10 kDa cut-off are often used for depyrogenation by size exclusion. However, monomeric LPSs are rarely found in solution because of their poor solubility in water. LPS is usually present in aggregated forms, such as vesicles ranging in molecular weight from 300 to 1000 kDa. Thus, endotoxin can be successfully removed by higher flux membranes, with MWCOs of 30–100 kDa (*6*).

Adsorption, in addition to size exclusion, also can be a mechanism of endotoxin removal by UF. Several hollow-fiber membrane materials have been evaluated, and the best removal was obtained with more hydrophobic membranes. Endotoxin removal was correlated to the degree of endotoxin adsorption on the membranes in an equilibrium experiment (*7*).

UF has been used successfully to depyrogenate small molecule drugs, buffers, electrolytes, antibiotics, and antifungal agents (*8*). UF is generally not recommended for endotoxin removal from solutions containing larger molecules such as proteins.

## 2.4 Charge-Modified Depth Filters

Depth filters exhibit two primary clarification mechanisms because of their structural and chemical composition: size exclusion, either through sieving or entrapment; and adsorption, either through electrokinetic (positive zeta potential) or hydrophobic interactions.

Size exclusion of particles is a function of the tortuous flow path through the media as well as the depth or length of the flow path in relation to the size distribution of the contaminate loading, e.g., cellular debris, including LPS from cell walls and hard particles. Depth filtration efficiency depends on many factors, including the filter media characteristics, materials of construction (e.g., cellulose, filter aids, binding resins), the fluid characteristics (e.g., viscosity, dirt load, cell debris, temperature), as well as the particle characteristics (e.g., solid/hard, pleomorphic, proteinaceous, colloidal). Electrokinetic adsorption is attributed to the resin binders and filter aids that impart a net-positive charge, positive zeta potential, to the filter medium. Adsorption is a complex mechanism that will vary based on a combination of parameters including positive zeta potential, hydrophobic adsorption, particle surface charge, pH, and ionic strength of process fluids. This positive zeta potential can remove negatively charged particles smaller than the nominal rating of the depth filter medium. The adsorptive mechanism results in high removal efficiencies for fine particles, colloidal and cellular materials, e.g., bacterial endotoxins, nucleic acids, and removal of negatively charged trace contaminants, whereas the depth medium porosity influences operating parameters such as pressure differentials, flow rates, dirt load capacity, and throughput.

Most cellulose-based depth filters contain a filter aid to enhance particle retention and flow characteristics. Filter aids are available in various particle sizes and levels of purity. Common filter aids include diatomaceous earth, perlite (volcanic origin), carbon (natural sources), and silica- and/or metallic-based materials.

The cartridges and capsule configurations are constructed of primarily polypropylene and other common elastomers and polymers, e.g., nylon, polycarbonate, polysulfone. Depth filters are available in standard filter cartridge/capsule configurations; lab-scale discs (47 mm/90 mm), flat stock sheets, lenticular cartridges (stacked discs), and capsules.

In general, the charge-modified depth filters showed lower endotoxin breakthrough levels at charge exhaustion as compared to charge-modified membrane filters.

Membranes demonstrated total endotoxin breakthrough once the charge capacity of the membrane is saturated. Generally, charge-modified depth filter media demonstrate lower endotoxin unit (EU) levels that increase slowly at the point of first endotoxin detection as compared to membranes that exhibit complete breakthrough.

Benefits of charge-modified depth filters include removal of bacterial endotoxin [4–5 log reduction value (LRV)] (*9–11*), DNA fragments, host cell protein, reduction of viruses, and economical throughput with low extractable levels. System flow rate determinations are necessary to optimize residence time to maximize adsorptive capture. This parameter is especially important for the removal of colloids and endotoxins.

Cellulosic depth filters commonly contain extractable *Limulus* amebocyte lysate (LAL)-reactive materials that are often determined to be β-1,3-glucans. β-1,3-Glucans activate an alternative LAL pathway, Factor G. The activation of Factor G by β-1,3-glucans will induce the proclotting enzyme, causing a non-endotoxin-positive LAL result (or enhanced result). To reduce the risk of β-1,3-glucan extractables from cellulosic depth filters, it is important to follow the recommended rinse conditions of the specific depth filter. An alternative to reduce the effects of β-1,3-glucans is to select LAL reagents tolerant of β-glucans or to add a β-glucan blocking buffer to LAL samples.

To some users, the most important attribute of charge-modified depth filters is their effectiveness as prefilters. In more difficult filtrations, such as those containing colloids, bacteria, or endotoxins, the user can realize substantial cost savings.

## 2.5 Activated Carbon Depth Filters

Depth filtration, using activated carbon as a filter aid adsorbent, removes color, odor, and bacterial endotoxins and nucleic acids. Activated carbon is derived from organic materials, e.g., peat, wood, coconut, bone, lignite coal. The microstructure of the carbon contains millions of pores that create a highly adsorptive material with a vast internal effective surface area as compared to polymeric microporous structures. These carbon filter aids are typically activated by steam or chemical treatment such as acid. Although highly effective in reducing endotoxin (4–5 log reduction) and other undesirable contaminants, active carbon may, because of this highly adsorptive characteristic, remove other process components and target molecules due to this nonspecific adsorption property. The high loading capacity and strong adsorptive attributes make activated carbon depth

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filtration an attractive alternative to conventional filtration methods or addition of bulk carbon, where care must be taken to remove fine carbon particulates in the effluent.

### 2.6 Membrane Adsorbers

When the target protein in the solution is in the same molecular weight range as that of the endotoxins (10–20 kDa for endotoxin monomers), the target proteins cannot be separated by UF. Ion exchange chromatography is the most common depyrogenation method for proteins; however, it has some drawbacks, which limit its usefulness as a depyrogenation step. This includes handling and usage problems such as packing, channeling, low flow rates, long regeneration times, compressibility, and limited chemical stability. Charge-modified membrane adsorbers with ion exchange ligands functionalized on the membrane surface can provide the required performance needed for depyrogenation from the laboratory up to process scale. Generally, two strategies can be used for removal of endotoxin from solutions with such membrane adsorber devices. Using the strong basic anion exchanger of quaternary amine (Q) type in a buffer with pH lower than the pI of the protein, endotoxin will bind to the charged membrane substrate, and protein will pass through the membrane (negative chromatography). Alternatively, a strong acidic ion exchanger type S also can be used with a buffer pH lower than the pI of the protein. In this case, the endotoxin will pass through, and the protein will be bound to the charged membrane substrate, which can be subsequently eluted using appropriate buffers in the next step.

Such membrane adsorbers have been used as validated endotoxin clearance steps in downstream processing of monoclonal antibody (mAb) or recombinant protein manufacturing. Typical log reduction values (LRVs) reported are >4 (*12*) based on lab scale testing.

Another solution is to use mixed-mode membrane adsorbers exhibiting both anionic and hydrophobic chemistries. Endotoxins (hydrophobic and negatively charged) tightly bind onto the membrane surfaces. By adjusting the concentration of salt or pH appropriately, proteins flow through the mixed-mode membrane adsorber by charge repulsion, while endotoxins remain bound. Mixed-mode membrane adsorbers allow the depyrogenation of protein solution or buffers with higher concentrations of salt (e.g., 100–500 mM) than with the Q adsorber. Such membrane adsorbers have been used as validated endotoxin clearance steps in downstream processing of mAb or recombinant protein manufacturing. Typical LRVs reported are in the 3–4 range based on lab scale testing.

## 3. VALIDATION

See *Depyrogenation* ⟨1228⟩ for a comprehensive discussion of depyrogenation process validation and the use of endotoxin standards.

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# ⟨1228.4⟩ DEPYROGENATION BY RINSING

## INTRODUCTION

The production of parenteral products requires that products be sterile, but also that they are free from harmful levels of pyrogens, or fever causing agents. For the purposes of the ⟨1228⟩ series, the term "depyrogenation" refers to the destruction or removal of bacterial endotoxins, the most prevalent and quantifiable pyrogen in parenteral preparations. Effective depyrogenation depends on the product and the method of removal and/or destruction. Although depyrogenation of heat-stable articles may be best accomplished by dry heat, heat-labile equipment, components, ingredients, or materials such as drug product containers and closures, and some medical devices may be depyrogenated through physical means such as rinsing. This chapter provides an overview of the depyrogenation process, its validation, and routine process control.

## RINSING PROCESS

Rinsing is the most common means of reduction or removal of bacterial endotoxins on closures (such as elastomeric stoppers), medical devices, and other materials that are not compatible with the temperatures used in dry heat depyrogenation. The mechanism for this method of depyrogenation is removal of the endotoxin, followed by dilution (*1*). The process of general rinsing to remove pyrogens is accomplished by using high-purity water such as *Water for Injection* preferably above 60°. Multiple rinses may be necessary with proper controls to ensure that the *Water for Injection* does not become contaminated with Gram-negative bacteria and bacterial endotoxins during processing.

The use of high-purity rinse water is often the key to successful reduction of bacterial endotoxin activity on the surfaces of the materials being processed. At a minimum, the rinse water quality should meet the bacterial endotoxin limit of *Water for Injection*, which is <0.25 USP Endotoxin Units (EU)/mL. Using water that risks having bacteria grow in it may result in the deposition of bacterial endotoxins onto the items, or in the case of depyrogenated articles, the re-deposition (re-pyrogenation) of endotoxins from the rinse water itself. The advent of *Water for Injection* for use in the healthcare manufacturing industry has ensured that the most prevalent ingredient in most of our products is no longer a source of endotoxin. Additionally, re-pyrogenation of articles due to subsequent microbial proliferation on the rinsed items should be minimized either by a prompt subsequent sterilization or by drying the items after rinsing.

It is important to note that starting with *Water for Injection*, while essential, does not ensure that such water remains suitable for use through the duration of the depyrogenation process. When cooled from the elevated circulation temperatures used for its distribution, *Water for Injection* is vulnerable to microbial proliferation. *Water for Injection* held at temperatures below 55° and above 8° should be considered at risk for microbial contamination unless sterilized and held in a sterile vessel. Therefore, holding water for more than 3–4 h within this temperature danger zone is an unsuitable practice. Systems that recirculate *Water for Injection* can be effective but process conditions and water storage times and temperatures should be carefully controlled and validated.

Solvents other than *Water for Injection*, such as caustic alkali or detergents, have been used for depyrogenation by rinsing. The concern with these solvents is the removal of residuals that may ultimately be harmful to patients or the product, so care must be taken to test for residuals during validation of the method.

For any process that purports to depyrogenate by rinsing, there are a number of critical factors that must be defined and controlled during the validation study and beyond:

1. Solvent description, including normality or concentration if caustic alkali or detergents are used; if high-purity water is used, the source should be clearly described
2. Solvent temperature
3. Solvent pressure, particularly where rinsing is used to depyrogenate glass or other articles that remain stationary during the process
4. Solvent flow rate through the system
5. A justification for the recirculation of solvents

## VALIDATION

Validation of depyrogenation by physical means is not different, in principle, from the validation of other depyrogenation methods. Endotoxin indicators, which are articles representative of the material to be depyrogenated spiked with a known amount of endotoxin, are prepared in a laboratory (see *Endotoxin Indicators for Depyrogenation* ⟨1228.5⟩). Quantitation of endotoxin activity prior to and subsequent to the rinsing process will demonstrate the effectiveness of the process in removing endotoxins. To prepare endotoxin indicators, inoculate each indicator with a known level of activity of Control Standard Endotoxin (CSE), USP Endotoxin RS (which, by convention, is abbreviated as RSE), or Naturally Occurring Endotoxin (NOE), which is calibrated against RSE before processing. Generally speaking, a small volume (e.g., <100 µL) of a highly concentrated analyte (purified LPS or NOE) is inoculated onto a section of the article that is the most difficult for the rinse solvent to reach. For example, stoppers are generally inoculated onto the product contact portion of the stopper. A low volume of inoculum is used to ensure rapid drying. The inoculated indicators may be dried in a unidirectional air flow cabinet or other validated means to hasten drying and limit the possibility of extrinsic microbial contamination that can arise in uncontrolled environmental conditions, particularly with moisture present. Historically, 1000 EU/article has been the target spike value, but depending on 1) the level of endotoxins historically found on the material, 2) the amount of recoverable endotoxins observed after the drying

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process, and 3) the "safe" level of endotoxin activity that must be attained post-processing, an inoculum at a level other than 1000 EU may be justified.

Because the prepared endotoxin indicators may be mixed with many uninoculated articles (e.g., stoppers), it is essential to provide a means to identify the indicators. For example, if the stoppers used by the company are gray, an identical stopper that is a different color but has the same elastomeric material, or a stopper of the same composition but a different shape, could be used for the indicators. If a stopper of different color or other identifying mark is not available, the inoculated indicator stoppers might be placed in individual small mesh bags. For glass containers, the indicator containers might be amber rather than clear, or they may be marked with indelible or heat resistant ink. For metal articles, a heat-resistant marker might be used to identify the indicators. In any event, the endotoxin indicators must be identical in materials of composition to the other articles in the study, but must be prepared so that they can be easily retrieved for analysis.

Once dry, the endotoxin indicators are analyzed for the recoverable level of endotoxins activity using the *Limulus* amoebocyte lysate (LAL) assay. To determine the recoverable activity, follow the process for extracting endotoxin from medical devices (see *Medical Devices—Bacterial Endotoxin and Pyrogen Tests* ⟨161⟩). Submerge the article or a number of articles in Water for Bacterial Endotoxins Test (BET; see *Bacterial Endotoxins Test* ⟨85⟩*, Reagents and Test Solutions, Water for Bacterial Endotoxins Test*) prewarmed to 37°. Allow the article(s) to remain in contact with the Water for BET for an hour. Agitation such as intermittent vortexing or sonication may be added to enhance recovery. If methods other than immersion in Water for BET are used, they must be validated to demonstrate that they do not result in a loss of endotoxin activity and identified in the protocol so that subsequent studies use the same process. Regardless of the method used, a statistically significant number of samples representing the load under study should be evaluated to ensure adequate reproducibility.

## Controls

Two sets of controls are recommended for validation studies:

1. Testing of uninoculated indicators—provide data on the resident level of endotoxin activity on the lot of stoppers to be tested.
2. An appropriate number of endotoxin indicators should be retained by the laboratory and used as positive controls. After the depyrogenation process is completed, extract both the processed units and positive control indicators as described above and test using a qualified BET method.

Any activity detected on the retained positive controls is called “recoverable” activity and activity detected on the processed indicators is called “residual” activity (*2*). The following factors should be considered during the extraction of endotoxin activity:

1. Inoculated units should not be pooled for analysis. The conceptual basis for this study design is to determine the depyrogenation capability of the process, and pooling extracts from a number of units would not allow evaluation of reproducibility and also bias the results. However, there may be a unique circumstance in which accurate or valid results may not be possible because of a small sample size or other logistical or practical considerations. If articles are pooled, the reason for pooling should be documented in the body of the protocol.
2. The test result must account for all sources of dilution. For example, the units for an LAL test are EU/mL. If the extraction volume is 1 mL/stopper, then the result of the test is really EU/stopper. But, for example, if the extraction volume is 5 mL/stopper, then each milliliter of the extract is a 1:5 dilution of any endotoxin activity that has been extracted from the indicator.

The log reduction is calculated using the following formula:

$$\text{Log}_{10}\text{ reduction} = (\log_{10}\text{ recoverable activity}) - (\log_{10}\text{ residual activity})$$

For example, if a laboratory detects 4500 recoverable EU/stopper in the controls and 0.3 residual EU/stopper, the log reduction is calculated as:

$$\text{Log}_{10}\text{ reduction} = (\log_{10} 4500\text{ EU}) - (\log_{10} 0.3\text{ EU})$$

$$\text{Log}_{10}\text{ reduction} = (3.65) - (-0.52) = 4.17$$

Historically, laboratories added sufficient inoculum to each endotoxin indicator unit so that at least 1000 EU could be recovered in the retained controls. However, there are factors that the analyst and the validation team should be aware of in conducting these studies. Foremost among these factors is an understanding that the lipopolysaccharides extracted via the Westphal procedure, of which CSE and RSE consist, have physicochemical properties that can make achieving a uniform dispersion of a highly concentrated quantity of this material difficult or impossible. Also, CSE tends to adsorb to surfaces and therefore may be difficult to remove from some materials resulting in control recoveries of less than 50%. In some cases, the recovery may be substantially less than 50%. There may also be considerable variability in recovery from test to test or even within a single test run. This is to be expected and history has taught us that this does not impact the usefulness or effectiveness of a depyrogenation study.

A 3-log reduction of the recoverable endotoxin is a common target for acceptable depyrogenation effectiveness (*3*). However, this may not be attainable in all test systems or under all test conditions nor may it be necessary. Where a 3-log reduction is not attainable it is important to carefully evaluate endotoxin risk in the process based extensively on inherent endotoxin burden. Rather than strict reference to a 3-log reduction, the depyrogenation program must prove that endotoxin activity at levels below a scientifically established target can be consistently attained. The target should consider the use of the article and the potential contribution to the final endotoxin limit established for the drug product. Although the endotoxin limit is established based on calculations of active pharmaceutical ingredient (API) dosage contained in the drug product, any contributions by the excipients and container/closure must be considered in any validation study, and the sum of all these contributions must meet this limit.

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Elastomeric closures are generally received with little or no detectable endotoxin activity. It is possible that a 2-log reduction by rinsing could be sufficient for some packaging components, excipients, or other materials. An important consideration in making this determination is the incoming pyroburden and bioburden presented by any material to the depyrogenation process.

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## ROUTINE PROCESS CONTROL

Proactive control of Gram-negative bacterial contamination is essential to the production of safe parenteral products. The use of properly generated, stored, and distributed ▲*Water for Injection*▲ (ERR 1-Nov-2019) ensures that the most prevalent ingredient in most products is not a significant source of endotoxin.

Once a depyrogenation process has been effectively developed and validated, it must be maintained in that state to ensure continued acceptability. Chapter ⟨1228⟩ details the general practices that are appropriate for all depyrogenation systems. This is accomplished by a number of related practices that are essential for the continued use of the process over an extended period of time. The essential practices to maintain validated status include calibration, physical measurements, periodic endotoxin assessment on incoming materials, ongoing process control, change control, preventive maintenance, and periodic reassessment and training.

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# ⟨1228.5⟩ ENDOTOXIN INDICATORS FOR DEPYROGENATION

1. INTRODUCTION
2. ENDOTOXIN AND LPS
3. APPLICATION OF ENDOTOXIN INDICATORS
4. PREPARATION AND USE OF ENDOTOXIN INDICATORS
    4.1 Methodology to Create a Laboratory-Prepared Endotoxin: Principles to Consider
    4.2 Inoculation of EIs
    4.3 Recovery of Endotoxin from EIs
    4.4 Choice of Test Methodology for the Analysis of EIs
5. ANALYSIS OF RESULTS OF DEPYROGENATION STUDIES

## 1. INTRODUCTION

Depyrogenation is defined as destruction or removal of pyrogens (see *Depyrogenation* ⟨1228⟩). For the purposes of this chapter, the terms "bacterial endotoxin" or "endotoxin" refer to a component of the outer cell membrane of Gram-negative bacteria, which is known to induce a febrile response in humans and other mammals. The endotoxin complex contains many cell wall components including, but not limited to, phospholipids, lipoproteins, and lipopolysaccharides. Lipopolysaccharide (LPS) is the biologically active portion of both naturally occurring and laboratory-prepared endotoxin complexes. The USP Endotoxin Reference Standard (which, by convention, is abbreviated as RSE) and control standard endotoxin (CSE) preparations purchased from lysate manufacturers and other third-party vendors are not endotoxins but rather are preparations of purified LPS.

"Endotoxin indicators" (EIs) are tools that are used (where required) in conjunction with physical measurements to analyze the effectiveness of a depyrogenation process. EIs used to determine the effectiveness of dry heat depyrogenation processes are commonly purchased as glass vials that are inoculated with a known level of LPS activity. This chapter expands the definition of EI to include any carrier, including glass vials, inoculated with endotoxin or LPS that is used to challenge a depyrogenation process. EIs can be used to analyze the effectiveness of endotoxin removal by washing, rinsing, cleaning, or by using separation technologies, such as filtration or chromatography. Carriers can be a variety of materials, including rubber stoppers to assess stopper-washing processes, bulk product to assess and validate processing steps, and stainless steel coupons to assess the cleaning of production vessels.

Purified LPS, such as CSE obtained from lysate manufacturers or other third-party vendors, has historically been a convenient choice for use as the analyte used in the preparation of EIs. However, EIs prepared in-house using laboratory-derived endotoxin more closely mimic product contamination, and as a result can provide a more realistic assessment of the depyrogenating capability of various production processes than does highly purified LPS. This chapter provides information on the preparation and use of these more specialized indicators to assure both consistency and comparability of data among method development and validation studies.

## 2. ENDOTOXIN AND LPS

A bacterial endotoxin is defined in the *Introduction*. LPS is the biologically active portion of the naturally occurring and laboratory-prepared endotoxin complex. Highly purified LPS, extracted from the natural endotoxin complex, is used to prepare the primary compendial RSE or secondary CSE preparations, such as those purchased from *Limulus* amebocyte lysate (LAL) reagent manufacturers. LPS consists of three distinct regions:

1. The structure of the hydrophobic lipid A portion of the molecule is the most highly conserved among Gram-negative species and is responsible for most, if not all, of the biological activity of endotoxins
2. A core oligosaccharide links the lipid A to the hydrophilic *O*-specific side chain or *O*-antigen
3. The hydrophilic *O*-antigen is a highly variable region that confers serological specificity to the organism and is often used to distinguish strains of Gram-negative bacteria

When drug products and devices are contaminated with endotoxin, the contaminant is not purified LPS but rather whole Gram-negative cells and/or cell wall fragments containing LPS. LPS and endotoxin are therefore dissimilar in many respects.

The amphipathic nature of the LPS molecule [i.e., having both a polar (hydrophilic) end and a nonpolar (hydrophobic) end] enables it to form complicated, three-dimensional, aggregated structures in solution. The aggregated forms of LPS have the capacity to adsorb, or "stick", to surfaces, and depending on the LPS formulation and the surface, extraction and detection may prove difficult using conventional extraction methods (see below). The degree of aggregation of the purified molecule is also affected by the conditions to which the LPS is exposed. Factors such as temperature, pH, salt concentration, divalent cation concentration, chelating agents, and detergents can have a profound effect on the biological activity and stability of LPS in solution. Purified LPS preparations used for depyrogenation studies should not contain any "fillers" or excipients. The excipients that are commonly used in the formulation of CSE have been shown to reduce the heat resistance of LPS (*1*) and may interfere in the recovery of LPS because of a caramelized excipient that has been post-processed by dry heat.

Endotoxins contaminating parenteral products may exhibit greater stability of activity in solution and less surface adsorption than purified LPS. As well, the detection of endotoxin may be less influenced than LPS by aggregation, disaggregation, or other conformations induced by some product matrices. Information on principles to consider when preparing endotoxin in the laboratory can be found below.

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## 3. APPLICATION OF ENDOTOXIN INDICATORS

The choice of an EI should be relevant to the process being validated. For physical depyrogenation, such as dry heat, the carrier material for the EI may be a surface such as a glass vial or appropriate coupon material with documented heating characteristics similar to the materials being processed, and onto which a known quantity of LPS or endotoxin has been inoculated. For stopper washing/depyrogenation studies, stopper carriers are inoculated with known levels of LPS or endotoxin. For raw materials or process intermediates that are inherently contaminated with assayable levels of endotoxin activity, there may not be a need to add LPS or endotoxin to validate endotoxin reduction in the manufacturing process, as the level of contamination may be sufficient to accurately measure activity upstream and downstream of the depyrogenating step(s).

For processes using raw materials or for upstream intermediates that are not contaminated with endotoxins, the use of either the USP RSE or CSE, which are both highly purified preparations, may not reflect the actual removal or reduction potential of the product stream depyrogenation step(s) under challenge. For these purposes, endotoxins harvested from Gram-negative cultures may be more suitable for depyrogenation processes typically found in biopharmaceutical product streams. The cell wall fragments and outer membrane constituents associated with these endotoxins represent realistic challenges to process operations such as ultrafiltration, affinity chromatography, and the use of charged media membranes or columns.

Challenge studies for LPS or endotoxin removal in process streams should be conducted at the laboratory or pilot scale so as not to introduce high levels of endotoxin or LPS into the actual production environment.

## 4. PREPARATION AND USE OF ENDOTOXIN INDICATORS

### 4.1 Methodology to Create a Laboratory-Prepared Endotoxin: Principles to Consider

Glass vial EIs purchased from third-party vendors do not need further preparation before use. These indicators are labeled with a nominal value of inoculated LPS, and the label claim should be confirmed upon receipt to assure that there is sufficient activity (endotoxin unit, or EU) available for the study.

- There is not one “best” or “standard” method for preparing endotoxin in the laboratory, but one example of a published method for the preparation of laboratory-prepared endotoxin may be found in Bowers and Tran (*2*). Regardless of the methodology for preparation, the following recommendations should be considered to properly and consistently produce, identify, and maintain laboratory-prepared endotoxin for use as a tool for depyrogenation studies. An appropriate Gram-negative bacterial strain from a recognized culture collection is a good choice for preparing a laboratory-derived endotoxin. Alternatively, a Gram-negative organism isolated from a facility, water system, raw material, or product that is identified to the species level, that has been shown to be genetically stable and that is properly maintained, may also be considered. Establishing the identity and baseline genetic fingerprint of an environmental organism will assure that subsequent preparations are consistent.
- The laboratory should create detailed procedures or laboratory work instructions for culture maintenance and endotoxin preparation to assure consistency between batches of endotoxin. For example, endotoxin may be isolated from a culture of Gram-negative bacteria, according to the method of Bowers and Tran (*2*) or a well-documented variation of that method. Whatever the methodology for growth and endotoxin isolation that is developed by the laboratory, the methodology should be documented and used consistently.
- Consistent with good microbiological practice, the culture and maintenance of the cells used to produce a laboratory-prepared endotoxin should be consistent with *Microbiological Best Laboratory Practices* ⟨1117⟩. Instructions on 1) the proper maintenance of the organism; 2) growth conditions, including any requirements to prepare media, nutrient requirements, and time/temperature of incubation; 3) methods for cryopreservation or lyophilization for master cell banks and working cell banks; 4) storage of the endotoxin, once prepared including concentration, vessel type, and volume; and 5) master batch production records to assure consistency in subsequent studies should be written, managed via change control, and followed.
    - Once isolated, the relative activity of the endotoxin preparation should be established by comparing its activity to a known LPS standard such as RSE, or a CSE that has been standardized against the RSE. Determination of activity involves diluting the endotoxin preparation and assaying the dilutions against an LPS standard curve such that the result of the dilution falls within the range of the referenced standard curve. As with the CSE standard used in the bacterial endotoxins test (BET) assay from *Bacterial Endotoxins Test* ⟨85⟩, the activity of the endotoxin may vary, depending on the lot of lysate and lot of LPS used for the analysis. It is recommended, consistent with the assignment of potency for the CSE, that activity of an endotoxin preparation be evaluated for each lysate manufacturer, lysate lot, and test method (gel, kinetic turbidimetric, or kinetic chromogenic) in use in the laboratory.
      The activity of the stock endotoxin preparation in EU/mL is reported as:

      (Test result in EU/mL) × (dilution factor) = EU/mL of the starting endotoxin preparation
    - Once activity has been determined, and if applicable to the study design, a standard series of dilutions of the newly prepared endotoxin should demonstrate onset times that result in slope and *y*-intercept values that are consistent with the standard curve parameters of the RSE/CSE standard using the same lot of lysate. This demonstrates that the activity of endotoxin preparation dilutes and reacts with the lysate in a manner that is similar to LPS.
    - Characterization of the endotoxin preparation should also include data on the stability of the preparation, because stability is critical to the comparison of data from one study to the next. If the endotoxin preparation is stored, storage parameters including the concentration of the preparation in EU/mL, the composition of the vessel, the temperature of storage, and the length of storage, should be defined. An expiration date should be assigned based on determined stability.

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## 4.2 Inoculation of EIs

To prepare an EI in house, inoculate endotoxin or LPS onto an article (carrier) that will serve as the substrate for the EI. Carriers for EIs can be anything that is subject to depyrogenation such as: vials (for dry heat depyrogenation), stoppers (for stopper washing), stainless steel coupons (for vessel cleaning), or product (for depyrogenation of process streams).

The simplest way to inoculate these indicators is to add a small volume of a highly concentrated solution of endotoxin or LPS to the carrier. The volume and concentration of added endotoxin or LPS should be calculated to add at least 1000 EU to the carrier, although higher or lower concentrations may be justified based on historical data on the endotoxin content of the material. [NOTE—The remaining discussion assumes a 1000-EU inoculum, but the principles hold for any level of initial inoculum.] For nonliquid carriers, the endotoxin is "fixed" or dried onto the carrier substrate. This fixing step is most easily accomplished by drying in a unidirectional air flow cabinet or hood, although other drying methods including vacuum drying, lyophilization, and other fixation methods could be used. In depyrogenation challenge studies, once a fixing method is chosen, it should not change in subsequent studies to assure comparability of results. Before using the EIs, a recovery procedure, consisting of a reconstitution or extraction method, should be developed and verified for consistency (*3*).

For liquid carriers such as bulk product, the level of inoculation in EU/mL should be justified based on "worst case" challenge for the depyrogenation step under study, meaning that the highest concentration of endotoxin that could be in the upstream product, based on process knowledge and historical endotoxin values, should be used. Such justification should take all contributing factors into account, including but not limited to: Gram-negative bioburden in raw materials and bulk; endotoxin content in raw materials including water, contributions by product contact surfaces; and the effect of hold times, particularly for nonsterile bulk.

## 4.3 Recovery of Endotoxin from EIs

To use EIs, it is necessary to recover and quantify the activity of the endotoxin or LPS from both unprocessed indicators (controls) and from processed indicators (i.e., those that have been through the depyrogenation process). LPS tends to adsorb to surfaces and may aggregate or disaggregate in some product matrices; therefore, recovery of activity from EIs made with LPS is often not 100% of the nominal or measured spike value. This section addresses the methodology for recovery and possible strategies for addressing recoveries that may be observed in challenge testing.

In the case of commercially available EIs prepared with LPS, the manufacturer's directions for extraction and recovery should be followed. With such products, there should be little difficulty in achieving recovery within a factor of 2 of the labeled LPS concentration. If recoveries within the specified range cannot be achieved, the manufacturer should be contacted for technical assistance.

For EIs made in-house using LPS, the composition of carriers, such as plastics, can affect recovery or result in inconsistent recovery because of adsorption. For these carriers, there is no prelabeled concentration to verify. In this case, the expected recovery should be based on the measure of the activity of endotoxin or LPS added to the article and the volume of extraction fluid used to recover it. The actual (measured) activity in the extract should then be compared to the measured activity of the endotoxin or LPS added to determine the percentage of recovery.

For example, consider a stock endotoxin or LPS preparation containing a measured activity of 100,000 EU/mL that is used to prepare in-house EIs. If a volume of 50 µL of this preparation is dried on the surface of each of a number of 10-mL vials, the known amount of activity added is 5000 EU. If the recovery/extraction is performed in 5 mL of water for BET, and the recovery is 100%, the expected activity in the extract solution is 1000 EU/mL.

$$\frac{5{,}000\ \text{EU/vial}}{5\ \text{mL extraction solution/vial}} = 1000\ \text{EU/mL}$$

If, however, the measured activity after extraction is 200 EU/mL as opposed to the expected 1000 EU/mL, the efficiency of the extraction method is 20%.

Recovery of endotoxin or LPS from nonliquid EIs prepared in-house can follow recommendations for the extraction of medical devices in preparation for LAL testing. *Medical Devices—Bacterial Endotoxin and Pyrogen Tests* ⟨161⟩ states, "The standard extraction method is to soak or immerse the device or flush the fluid pathway with extracting fluid that has been heated to 37 ± 1.0°, keeping the extracting fluid in contact with the relevant surface(s) for NLT 1 h." The volume used for reconstitution or extraction should be appropriate for the material, size, and shape of the EI, recognizing that a volume too low may not efficiently recover the endotoxin or LPS and that excessive volumes will unnecessarily dilute the endotoxin or LPS that has been extracted.

If the recovery of added endotoxin or LPS is variable, an alternate extraction method may be developed and validated. This may include agitation or mixing, sonication or alternative extraction solutions. A combination of extraction in 0.01% sodium laurel sulfate, sonication, and vortex mixing is one such approach that has been reported to be more effective than extraction in water for medical devices (*4*–*6*). Other extraction methods are summarized by Bryans et al. (*7*) and in ANSI/AAMI standard ST72:2011 (*8*).

Another situation concerns liquid endotoxin or LPS preparations that are used either to validate a depyrogenation process in a process stream or to investigate the destruction or removal of endotoxin or LPS in a manufacturing process. In these cases, the initial concentration of the stock liquid endotoxin or LPS solution should be measured before it is added to the system or process. If some of this preparation is added ("spiked") to a bulk process solution that is then subject to a particular process or treatment, the activity of endotoxin or LPS in this bulk solution should be measured and recorded as the starting activity. It is important to determine whether changes in the endotoxin or LPS activity of the processed solution are due to effects of the process and not to instability of the LPS or endotoxin in the solution. The stability of the activity of the LPS or endotoxin in these preparations should be verified over a period appropriate to the proposed use of the preparation.

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As with the spiking method (choice of endotoxin/LPS and "fixing" process), whichever reconstitution/extraction procedure is chosen should be verified for consistency and should be used for all subsequent studies to assure comparability of results.

### 4.4 Choice of Test Methodology for the Analysis of EIs

Any of the test methods described in ⟨85⟩ can be used for the analysis of processed and unprocessed EIs. As with the rest of the methodology, it is highly recommended that an assay (kinetic turbidimetric, kinetic chromogenic, or gel clot assay) be chosen during method development and used consistently throughout the initial study and in subsequent studies to assure that data are comparable. The use of alternate assays is permissible, provided that they are validated to assure that they are equivalent to or non-inferior to the standard compendial assays.

## 5. ANALYSIS OF RESULTS OF DEPYROGENATION STUDIES

To evaluate the effectiveness of a depyrogenation process, the residual activity that is recovered from processed indicators is compared to the endotoxin or LPS activity of unprocessed controls. Typically, the $\log_{10}$ of the endotoxin or LPS activity measured for the processed EI (or solution) is subtracted from the $\log_{10}$ of the measured endotoxin or LPS activity of unprocessed control indicators. The result of the subtraction is the log reduction that is attributable to the depyrogenation process. If there are multiple controls and/or samples of processed material (and there usually are), the most conservative approach is to subtract the highest $\log_{10}$ concentration recovered from the processed EIs (or solution samples) from the lowest $\log_{10}$ unprocessed control endotoxin activity. For example:

- The activities in three unprocessed EIs are 1286, 1000, and 1532 EU/mL
- The activities in three processed EIs are 0.634, 0.512, and 0.496 EU/mL

The log reduction is calculated as:

$$\log_{10}(1000) - \log_{10} 0.634 = 3 - (-0.198) = 3.198 \text{ log reduction}$$

Historically, a ≥3-log reduction has been required by regulatory/compliance guidance. However, depending on the process and historical data, a 3-log reduction may be either excessive or inadequate. For example, for glass vials with a low or nonmeasurable endotoxin content upon receipt, the requirement to continually and repeatedly revalidate with an acceptance criterion of a 3-log reduction of the endotoxin spike of >1000 EU is excessive. Alternatively, a fermentation process with an endotoxin content of $>10^7$ EU/mL in the clarified culture supernatant will require more than a 3-log reduction to achieve safe levels of endotoxin in the drug substance or drug product. Additionally, given the sensitivity of BET assays, it may not be necessary to spike with 1000 EU to demonstrate a 3-log reduction. For example, if the assay sensitivity is 0.005 EU/mL, one may choose to spike with 50 EU and demonstrate an ultimate recovery in the test articles of less than 0.05 EU/mL, which calculates to greater than a 3-log reduction. In any event, the design of experiments including an appropriate specification for the log reduction of processed indicators and required test sensitivity to demonstrate the specified log reduction should be established and justified in a preapproved protocol for the study. The total reduction, of course, may be achieved over several steps in a purification process. Thus, the necessary reduction is often achieved additively over the course of multiple purification steps.

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# ⟨1228⟩ DEPYROGENATION

## INTRODUCTION

The production of parenteral products requires not only that products be sterile, but that they are also free from harmful levels of pyrogens. Depyrogenation is defined as the direct and validated destruction or removal of pyrogens. For the purposes of this and subsequent chapters of the ⟨1228⟩ series, the term "depyrogenation" refers to the destruction or removal of bacterial endotoxins, the most prevalent and quantifiable pyrogen in parenteral preparations. The chapters in this series discuss depyrogenation procedures that are applicable to product streams, equipment, and drug product containers and closures.

The ⟨1228⟩ series builds on the tenets described in *Sterility Assurance* ⟨1211⟩, first published in *USP 20–NF 15*. In the time since that publication, the science of depyrogenation has advanced, as has control over manufacturing processes. Manufacturers are very aware of the impact that facility, equipment, and process design has on endotoxin levels and depyrogenation, and they are mindful of the effects of elevated endotoxin levels in raw materials and water systems. Risk management tools, including process evaluation tools such as Hazard Analysis Critical Control Point (HACCP), are helping companies to identify critical control points (CCP) for the control of endotoxin and other impurities or contaminants in the manufacturing process. As a result of these advances in science, technology, and philosophy, the ⟨1228⟩ series will also offer alternatives to historical or traditional thinking.

## BACTERIAL ENDOTOXIN AND LIPOPOLYSACCHARIDE

Bacterial endotoxin is a component of the outer cell membrane of Gram-negative bacteria. The natural endotoxin complex contains many cell wall components including phospholipids, lipoproteins, and lipopolysaccharide (LPS), which is the biologically active component of endotoxin. Purified endotoxin is chemically defined as a LPS. LPS consists of three distinct regions:

1. The hydrophobic lipid A portion of the molecule is highly conserved among Gram-negative bacteria, and is largely responsible for most, if not all, of the biological activity of endotoxin.
2. A core oligosaccharide links the lipid A to the hydrophilic *O*-specific side chain or *O*-antigen.
3. The hydrophilic *O*-antigen is a highly variable region that confers serological specificity to the organism and is often used to distinguish strains of Gram-negative bacteria.

Due to the amphipathic nature of the LPS molecule [i.e., having both a polar (hydrophilic) end and a nonpolar (hydrophobic) end], purified LPS preparations, such as reference standard endotoxin (RSE) and control standard endotoxin (CSE) tend to form bilayers, micelles, ribbons, and other conformations when in solution, and they may adsorb, or "stick", to surfaces, making them difficult to extract and detect. The degree of adsorption of LPS to solid surfaces is affected by the composition and finish of the material to be depyrogenated. The extent of aggregation of LPS in solution is affected by a host of formulation matrix attributes to which it is exposed, such as temperature, pH, salt concentration, divalent cation concentration, detergents or emulsifiers, and chelating agents.

When parenteral products are contaminated with endotoxin, the contaminant is not purified LPS, but rather whole cells or cell wall fragments generated during the normal growth cycle of the bacteria or disruption of bacteria, where the LPS remains embedded in or associated with other cell wall components. Purified LPS and native endotoxin are dissimilar in many respects, and the two terms should not be used interchangeably. Depending on the materials of construction or the formulation of the article to be depyrogenated, the use of native endotoxin as a challenge material in depyrogenation studies may be a consideration because a native endotoxin preparation better reflects operational reality, particularly for the depyrogenation of product streams, and because LPS molecules in natural endotoxin are embedded in cell wall complexes, they may be much less prone to the aggregation and adsorption issues seen with purified LPS.

For the purposes of the ⟨1228⟩ series, the term "challenge material" will be used to generically describe material (endotoxin or LPS) used as a spiking analyte for depyrogenation studies. "Endotoxin" will refer to the moiety in its natural state, meaning pieces of Gram-negative cell wall from a well-characterized source. LPS will refer to the purified material.

## MEASURING ENDOTOXIN PRE- AND POST-PROCESSING

The primary procedure used for the measurement of bacterial endotoxin is the *Bacterial Endotoxins Test* ⟨85⟩ (BET). A well-controlled BET assay can provide assurance of accurate readings for the calculation of the reduction in challenge material activity pre- and post-processing, as well as provide consistent quantitation of levels of native endotoxin in raw materials, at CCP in the manufacturing process, and in finished products.

There are many variables in study structure and test method that can affect the outcome of a depyrogenation study. Development of a test method depends on the material under test, the identification of an appropriate challenge preparation, and the method used to extract recoverable activity prior to processing and residual activity after processing. Once a test system is developed that includes the identification of a source for challenge material preparations, inoculation of the articles to be depyrogenated (including drying procedures), extraction or recovery methods, and appropriate BET test methodology and sensitivity, it is recommended that subsequent tests should use the same conditions to ensure the comparability of test results. Points to consider when constructing a depyrogenation study include the following:

1. **The challenge material**: Consider the source of the endotoxin (purified or natural). When using purified LPS, choose a preparation with no fillers, because the presence of these fillers can add to the variability and therefore decrease the

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accuracy of the assay. Once challenge material preparation is chosen, it is recommended to use material from the same source in subsequent studies to reduce variability.[1]

2. **The characteristics of the material being depyrogenated**: It is important to understand the characteristics of the material being tested. For example, LPS may adsorb to plastics, and although two objects may be made of the same plastic, surface finish, surface area, and conformation differences may affect extraction efficiency and LPS recovery. For solutions, formulation matrices may affect aggregation of purified LPS in that pH, salt concentration, chelating agents, surfactants, and the presence of divalent cations may all have an impact on the recovery of the challenge material. The use of natural endotoxin may mitigate some of these recovery issues. For materials that are received with low or undetectable levels of endotoxin, depyrogenation studies using endotoxin or LPS challenge materials may be unnecessary if control is demonstrated and decisions are scientifically justified.
3. **The level of activity needed for the study**: How much pre-processing activity do you need to execute the study? The current industry standard is to add enough endotoxin to the system so that at least 1000 EU can be recovered prior to depyrogenation. However, depending on the test system, 1000 EU may be either excessive or insufficient. For example, when designing a study for the depyrogenation of a product stream that normally contains <1 EU in a certain volume, a spike of 1000 EU of a CSE in that same volume may be excessive. For solid-surface materials, the level of activity in the challenge material should be established taking into account the materials of construction and finish as they may contribute to LPS adsorption. Knowledge of historical levels of endotoxin in or on the surface, the efficiency of the depyrogenation processes, the efficiency of the challenge material extraction or recovery method, and the log reduction or safety level target acceptance criterion are all important to the setting of a pre-processing activity requirement. If a reduction study for a product stream is required, it may be more appropriate to add an amount of naturally occurring endotoxin to the product consistent with the maximum expected endotoxin load ("worst case"), based on known endotoxin contributors (e.g., raw materials and water) and process capability to demonstrate reduction to safe levels. Whatever the procedure, the logic and methodology for endotoxin reduction studies should be justified and documented. For those materials that routinely contain a level of endotoxin, such as fermentation broths where there are high levels of activity to begin with, it may not be necessary to add additional challenge material.
4. **Preparation of test samples**: The method used to affix challenge materials to the surface of materials to be depyrogenated may affect its removal or recovery. Air-drying is the most convenient method of affixing challenge material to hard surfaces, but freeze drying and vacuum drying also have been used. To improve drying efficiency, it is suggested that a small volume of a highly concentrated activity of challenge material be used. This volume may be added to the surface of the item in an area that has been defined as "hardest to depyrogenate" or may be dispersed to represent the more likely natural occurrence. Inoculation methodology must be well defined for comparability across studies. Decisions regarding the design of studies must be documented and justified.
5. **Recovery methods**: Although there are standard methods for the recovery of endotoxin from medical devices, there is no standard method for the recovery of endotoxin dried onto solid surfaces that are used as indicators in depyrogenation studies. Although it is most convenient to adopt the standard extraction methods described in *Medical Devices—Bacterial Endotoxin and Pyrogen Tests* ⟨161⟩ and ANSI/AAMI ST72:2011,[2] a laboratory may choose to develop and validate a method that better suits the material under test. The efficiency of the recovery of endotoxin will depend on the attributes of the material under test, the composition of the challenge material, the concentration of the challenge material spike, and the method of drying. Recoveries of less than 100% of the challenge material nominal spike in positive controls are not uncommon. Perhaps more important than the percent recovery in positive controls is consistent recovery across lots of the same material and across depyrogenation studies.[3, 4]
6. **Test method**: Lysate formulations differ, and they may be subject to different interferences such as leachables, chelators, and salts. If interferences are encountered during method development that are difficult to overcome, consider trying another test method (e.g., gel clot, kinetic chromogenic, kinetic turbidimetric, endpoint chromogenic) or a different source of reagent.
7. **Depyrogenation method**: Variability in the depyrogenation treatment may impact recovery. Treatments vary in efficiency, both within and between treatment types. For example, a large difference in efficiency will be seen when comparing the depyrogenation of glass vials using dry heat versus rinsing in water for injection (WFI), and differences may be seen in the reduction of challenge material by the filtration of a solution depending on the type of filter chosen. Likewise, variability can be seen when using a single depyrogenation methodology with two different materials, such as the filtration of two different solutions using the same type of filter medium.

The efficiency of a depyrogenation process has been historically measured in terms of a logarithmic (log) reduction of a large "spike" or bolus of purified endotoxin that is added to or dried onto a material prior to treatment. Although log reduction is a convenient measurement benchmark, the more relevant and pragmatic indicator of depyrogenation efficiency is one based on process capability and patient safety, which is the reduction of the measured or anticipated worst case natural levels of contaminating endotoxin to safe levels as defined by reference to calculated endotoxin limits for the material.

With the implementation of the principles of quality by design (QbD) and risk management, the 3-log reduction that was first introduced in 1984 may be inappropriate as a universal benchmark for modern depyrogenation processes. For example, materials with a high level of native endotoxin, such as fermentation broths, could require more than a 3-log reduction to reach a safe level, whereas dry heat depyrogenation of materials with normally low or undetected levels of native endotoxin, such as washed glass vials, will require substantially less than a 3-log reduction. The appropriate endotoxin log reduction for the process should be determined by the user based on a full understanding of the product and process capability including

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input sources, levels of endotoxin, efficiency of depyrogenation methods, and output (product- or process-specific) endotoxin requirements. Effective process control requires knowledge of input, in-process (where appropriate), and output endotoxin levels. Under these circumstances, with appropriate process development, justification for reduced endotoxin challenges or the elimination of endotoxin challenges may be made based on historical data and demonstration of continued control.

## CONTROL OF ENDOTOXIN IN PARENTERAL PRODUCTS

The best control of endotoxin levels in parenteral products is the control of Gram-negative bioburden in raw materials, equipment, process streams, and manufacturing environment and operators. Parenteral manufacturers may exercise three categories of control to keep endotoxin content in drug products at safe levels.

The first category is “indirect control”, which is comprised of a series of preventive measures that control bioburden, the potential endotoxin contribution by formulation components (e.g., raw materials, APIs, excipients), water, primary packaging components, equipment, and the manufacturing environment, including personnel.

The second category is “process control”, in which endotoxin is monitored at CCP during processing to ensure that there is no increase in endotoxin. These process control elements are subject to validation or qualification.

The third category is “direct control”, or the direct destruction or removal of endotoxins from product streams, equipment, and primary packaging materials. As with controls on processing, direct measures of endotoxin destruction or removal must be validated.

## INDIRECT CONTROL

Reducing opportunities for Gram-negative microbial proliferation at any stage of manufacturing will reduce the likelihood of endotoxin contamination in the following ways:

- Exercising control over the endotoxin content of incoming materials, particularly materials derived from natural sources or those with high water activity, will reduce the opportunity for Gram-negative microbial proliferation and therefore reduce or eliminate the need for endotoxin removal downstream. Because of their manufacturing processes, glass and plastic containers as well as elastomeric closures are often received with very low or undetectable levels of endotoxin. Qualification of primary packaging suppliers should include an audit that examines and confirms the supplier’s consistent and documented control over applicable manufacturing processes.
- Bioburden and endotoxin control should be a component of a vendor audit and supplier qualification program for formulation materials that could potentially contribute endotoxin to parenteral products.
- Water is the most ubiquitous raw material in the manufacturing of parenteral products, but unless the generation and distribution of high-quality water is properly validated and controlled, the system will be prone to contamination by Gram-negative bacteria and the establishment of biofilms that can contribute significantly to the endotoxin load of the product (see *Water for Pharmaceutical Purposes* ⟨1231⟩ for a discussion of the types of waters used in pharmaceutical manufacturing and guidance for validation, maintenance, sampling, and testing of systems).

## PROCESS CONTROL

Product-specific process control requires the identification of CCP for the introduction or removal of endotoxin. Process control requires good process and equipment design consistent with QbD using a risk management tool such as HACCP. Process control measures include but may not be limited to the following:

- Control of manufacturing practices is essential to endotoxin control. Endotoxin control should be a part of validated cleaning procedures. The use of product contact materials for which endotoxin control cannot be established should be avoided. Clean product-contact equipment should be stored dry to avoid bioburden and Gram-negative bacterial proliferation.
- Hold times during manufacturing, particularly for nonsterile bulk in-process materials and drug product, should be validated to ensure that the hold conditions do not support microbial proliferation and therefore potential endotoxin production by Gram-negative bacteria.
- Environmental control, including good manufacturing practices (aseptic, as appropriate), is essential to process management of endotoxin. Operators should be properly garbed and trained. Housekeeping and disinfecting practices should be established to reduce the possibility of microbial proliferation in critical areas. Cleaning regimens should emphasize that standing water be removed at the end of the cleaning process.
- Endotoxin control and monitoring will be covered in a forthcoming chapter.

## DIRECT CONTROL

These processes require validation to ensure that endotoxins are removed or reduced to safe levels. Direct control may be accomplished by a variety of methods and processes that may be combined to ensure endotoxin reduction to a safe level. The most commonly used depyrogenation processes and the associated control measures are the subject of the ⟨1228⟩ series.

## SELECTION OF AN APPROPRIATE DEPYROGENATION METHOD

The basic principles for the control and validation of a depyrogenation process using a life cycle approach include:

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- Assessment of manufacturing processes to identify materials and process components that are essential to the control of endotoxin
- Focused depyrogenation process development that is consistent with the material to be depyrogenated, the resident endotoxin level of the material to be depyrogenated, and the limit of endotoxin for the finished article
- Adequate validation studies
- Ongoing monitoring of process controls to ensure continued efficacy of the depyrogenation process
- Identifying and documenting changes (a change control program) to the depyrogenation processes over time

Known or anticipated levels of endotoxin can be determined so that appropriate indirect or direct measures of control, consistent with the product or materials of construction, will ensure that endotoxin is eliminated or reduced to levels that ensure product and patient safety.

Should direct methods of depyrogenation be required for the control of endotoxin in or on the article, an important consideration during manufacturing process development is the selection of an appropriate method from the possible alternatives: dry heat, chemical, filtration, or physical removal. In some instances this selection is limited by the potential effects of the depyrogenation treatment may have on the materials themselves. The choice of the appropriate process for a given item requires knowledge of depyrogenation techniques and information concerning effects of the process on the material being processed. The selection of a particular treatment (and the details of its execution) often represents a compromise between those conditions required to destroy the endotoxin or remove it to the desired level and the effect of the process on the materials. Depyrogenation processes should be no more aggressive than required for effective process control to avoid adverse consequences to material quality attributes.

## VALIDATION OF A DEPYROGENATION METHOD

The validation program comprises several formally documented stages to establish that the depyrogenation process is capable of operating within prescribed parameters for process equipment, that independent measurements of critical parameters are possible and accurate, and that acceptance criteria for challenge material removal or destruction are met.

- The development stage investigates and establishes the operating parameters that define the controls to be used for the depyrogenation process.
- The installation qualification (IQ) stage establishes that equipment controls and other instrumentation needed to execute depyrogenation processes and measure the results of the depyrogenation process are properly designed and calibrated. Documentation should be available to demonstrate the acceptability of any required utilities such as steam, water, and air.
- The operational qualification (OQ) stage confirms that the equipment and other processes components function within the defined depyrogenation parameters.
- The performance qualification (PQ) stage of the validation program directly evaluates the depyrogenation of materials or articles. Wherever possible, these studies should employ or simulate the actual conditions of use, including the use of real or simulated product material. Worst case conditions, for example, might include the bracketing of critical parameters such as time/temperature and belt speed for dry heat depyrogenation of glass vials, flow rate for depyrogenation by filtration of solutions, and maximum measured, anticipated, or defined endotoxin loads for any material. “Worst case” should be defined and justified in the validation protocol. Endotoxin indicators may be utilized to support physical measurements in the validation of the depyrogenation process. Although the “rule of three” suggests that three consecutive successful validation runs be executed, perform sufficient replicate studies to demonstrate the capability and efficiency of the depyrogenation process, including the validation of operational ranges of any equipment used in such processes. The number of replicate studies chosen should be scientifically based and justified. At the end of the PQ, a report is written to establish operating parameters.

## ROUTINE PROCESS CONTROL

Once a depyrogenation process has been validated it must be maintained in that state to ensure the continued acceptability of its operation. This is accomplished through a number of related practices essential for continued use of the process.

- **Physical measurements**: Data reported by the equipment sensors and recorders must be verified after the completion of each depyrogenation cycle.
- **Calibration**: Any equipment used in the control or quantitative assessment of parameters required for a depyrogenation process must have its measurement accuracy verified against a traceable standard on a periodic basis.
- **Preventive maintenance**: There should be a defined maintenance schedule for each piece of process or testing equipment required for depyrogenation that is consistent with the manufacturer’s written recommendation.
- **Ongoing process control verification**: Depending on the specifics of the particular depyrogenation process, there may be additional requirements for ongoing confirmation of process efficacy. These can include the testing of raw materials, water supplies, and in-process sampling. These performance parameters are monitored against assigned limits designed to ensure that finished products meet acceptable endotoxin levels. Monitoring of operating parameters and controls plays an important role in maintaining the depyrogenation process in a validated state.
- **Periodic reassessment**: It is expected that the effectiveness of depyrogenation processes be reconfirmed on a periodic basis. A reassessment schedule should be formalized to assess the potential impact of de minimis or undetected changes to maintain the process in a validated state.
- **Change control**: In order to remain in a validated state, the various material, procedure, and equipment elements impacting the depyrogenation process should be carefully monitored to ensure that changes are properly evaluated for their potential impact on the process. The scope of the change control program must include materials being processed,

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process equipment, processing parameters, and process holding time limits. The extent of the effort required to support a change will vary with the potential impact of the change on the process outcome.

- **Training**: Depyrogenation processes rely heavily on scientific principles for the effective destruction or removal of endotoxins. Scientists and engineers well-grounded in the principles of endotoxin removal and testing develop processes to ensure effective depyrogenation. Individuals involved in the development of depyrogenation processes require a background in microbiology, physics, chemistry, and engineering, and they must be familiar with good manufacturing principles and regulations. Depyrogenation is an interdisciplinary activity where the combined knowledge of a group of individuals is generally required for the establishment of a reliable process. In addition to the depyrogenation process development team, individuals responsible for the maintenance and operation of depyrogenation processes must also be trained appropriately to ensure that their actions contribute to success. The operators are often the first to identify changes in process performance because of their intimate involvement with it. Effective training programs should be established and documented. Training programs should emphasize depyrogenation principles, adherence to established processes and procedures, and the importance of documenting deviations from normal operations.

## ROUTINE TESTING

Testing is not a control mechanism but rather a tool to assess the effectiveness of control measures. Depending on the specifics of the particular depyrogenation process, there may be additional requirements for ongoing confirmation of process efficacy.

These requirements can include the testing of raw materials, water supplies, and in-process sampling. These performance parameters are monitored against assigned limits based on historical data, and are designed to ensure that finished products meet acceptable endotoxin levels. Monitoring of operating parameters and controls plays an important role in maintaining the depyrogenation process in a validated state.

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# ⟨1229.1⟩ STEAM STERILIZATION BY DIRECT CONTACT

## SCOPE AND BACKGROUND

Steam sterilization is perhaps the most common of all sterilization processes. It is used in settings ranging from practitioner offices to large-scale manufacturing facilities. The diversity of practices that use steam sterilization is reflected in the range and sophistication of the equipment used. This general information chapter addresses sterilization in which saturated steam comes into direct contact with the load items (whether wrapped or unwrapped) and provides an overview of the basic concepts of this mode of sterilization, including its validation. The load items in this sterilization process are variously termed parts, components, hard goods, wrapped goods, or porous goods. These items may be metallic, glass, ceramic, elastomeric, or polymeric materials that have little or no sensitivity to thermal degradation at the sterilizing temperatures. For steam sterilization by direct contact, it is customary to sterilize items using an overkill method.

Sterilization of liquid-filled containers may be substantially different. *Moist Heat Sterilization of Aqueous Liquids* ⟨1229.2⟩ provides information about applications in which steam is a heating medium but is not in contact with the sterilization target, the liquid in the container.

## SATURATED STEAM

Saturated steam is a biphasic mixture of $H_2O$ in gas and liquid phases in thermal equilibrium. Saturated steam has a singular temperature–pressure relationship in which both phases are present, and at a given temperature only one pressure is possible for saturation. The importance of using saturated steam for sterilization arises primarily from two attributes. First, saturated steam rapidly kills microorganisms because of the presence of liquid water. Steam heated above saturation, also termed superheated steam, lacks liquid water, and although it is higher in temperature than saturated steam it is substantially less lethal to microbes. Second, when steam changes phase from gas to liquid, it releases thermal energy (2202 kJ/kg at 121°) that is transferred to the load items, facilitating sterilization of their exposed surfaces.

The initial objective for saturated steam sterilization is that the air in the sterilizing chamber must be replaced by saturated steam. Residual air within the sterilizer chamber and load items acts as both an insulator and an obstacle to steam penetration to all surfaces of the load items, and its removal is essential for effective sterilization. The presence of residual air in the chamber negates the singular temperature–pressure relationship of saturated steam. In the absence of saturation, physical measurements may not provide assurance of lethality.

## GRAVITY DISPLACEMENT CYCLES

In the simplest autoclave cycles, air removal is accomplished by gravity displacement. Because steam is hotter and less dense than air, it rises to the top of the autoclave, and the colder air exits at the bottom of the chamber. Saturated steam entering the chamber changes into liquid condensate as it contacts the colder surfaces of the autoclave chamber and load items. Retention of condensate within the load reduces cycle effectiveness because it is a barrier to steam contact, and additional steam is needed to maintain the saturated steam at the sterilizing temperature. The load items, wrapping materials, and load arrangement should be designed to facilitate air removal and condensate drainage. In gravity displacement cycles, the load slowly reaches the desired sterilizing temperature because air removal is relatively slow compared to cycles in which its removal is mechanically assisted. During the exposure segment of the cycle, a thermostatic trap at the bottom of the chamber drain allows the removal of condensate (and any residual air) from the sterilizer while maintaining sterilizing conditions. At the conclusion of the dwell period, the chamber is returned to atmospheric pressure.

## PREVACUUM CYCLES

To remove air more effectively from the chamber and the load items, sterilizers may employ multiple evacuation/pressure pulses in which air is replaced by steam. The number and depth of these pulses may vary. Because the alternating vacuum and pressure pulses may stress wrapping materials, the latter must be chosen carefully. The operation of the sterilizer during the exposure segment is similar to that of the gravity displacement cycle previously described. The vacuum system can be used at the end of the process to remove residual steam and condensate from the load items. The selection of a specific cycle and its associated sterilization parameters for a given item depends on a number of factors, including the heat lability of the material, heat penetration into the article, the item's mass, difficulties with air/condensate removal, and other factors described in the validation program (see below).

## STERILIZATION CYCLE CONTROL

Sterilizers are controlled by computerized/automated systems that manage the overall process execution and data reporting. The systems for steam sterilization may be controlled by calibrated temperature and/or pressure sensors on the equipment. During the exposure portions of the cycle, a minimum dwell time at a predefined temperature is required to ensure the method lethality target (minimum time–temperature or $F_0$) is met. Cycle efficacy for steam sterilization often is measured using $F_0$, which is defined as the equivalent exposure time at 121°. $F_0$ is a means for quantifying steam sterilization effectiveness by determining the equivalent sterilization time in minutes relative to a base temperature of 121° and a z-value of 10°; z-value is defined as the

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number of degrees of temperature change necessary to change the D-value by a factor of 10. The $F_0$ method is used to evaluate sterilization processes operated at varying temperature conditions to a single standard.

The process lethality at temperatures other than 121° can be calculated to determine lethality equivalent to that provided at 121°. Moist heat sterilization process efficacy is not intrinsically linked to a target temperature of 121°, which is simply the Celsius conversion of 250°F, and other temperatures can be used. Sterilizer control systems for direct sterilization typically provide a minimum time at a defined set point temperature after the initial air/condensate removal. Steam sterilizers are controlled using temperature sensors located in the drain line before the thermostatic trap, although other control schemes may be used. The temperature at this location typically is recorded for permanent documentation of sterilizing conditions. In sterilization by direct contact, exceeding the minimum time–temperature requirements or $F_0$ is acceptable because of minimal adverse consequences to the materials being sterilized.

Total lethality can be calculated over the course of the process. For the specific reference temperature of 121° and a z-value of 10°, the total accumulated $F_0$ can be determined by the following equation:

$$F_0 = \int_{t_1}^{t_2} 10^{\left(\frac{T-121}{10}\right)} dt = \sum_{t_1}^{t_2} 10^{\left[\frac{T-121}{10}\right]} \Delta t$$

where

$t_1$ = start time

$t_2$ = end time

$T$ = temperature

Summing the instantaneous lethality contributions over the entire sterilization process allows the calculation of the overall process lethality or $F_0$ delivered. The $F_0$ calculation should begin at 100° and should continue through the end of the dwell period provided that saturated steam conditions are maintained.

## VALIDATION OF STERILIZATION BY DIRECT CONTACT

The predominant approach for steam sterilization by direct contact is the overkill method defined in *Sterilization of Compendial Articles* ⟨1229⟩. Overkill sterilization is a method in which the destruction of a high concentration of a resistant microorganism is correlated with the destruction of reasonably anticipated bioburden present during routine processing. That objective can be demonstrated by attaining any of the following: a defined minimum lethality ($F_0$), a defined set of physical conditions, or confirmation of a minimum log reduction of a resistant biological indicator.

The validation requirements for the overkill method are less onerous than those for other methods such as those based on bioburden or bioburden/biological indicators. When the load items can withstand substantial heat without adverse consequence, overkill is the method of choice for steam sterilization because of its ease of execution, reduced considerations for bioburden control, and overall simplicity.

### Equipment Qualification

Equipment qualification is a predefined program that examines the equipment to confirm that it has been properly installed and operates as intended before the sterilization process. Equipment qualification can be separated into installation qualification and operational qualification, or can be considered joint installation and operational qualification. The qualification effort provides a baseline for the sterilizer's preventive maintenance and change control.

### Empty Chamber Temperature Distribution

A common procedure to evaluate steam sterilizer installation is the evaluation of the empty chamber's performance. Each air removal method used in the sterilizer is evaluated by temperature measurement near the corners of the sterilizer chamber, near the controlling probe, and other locations as appropriate. The distribution of temperatures in the empty chamber should be determined only by sensors located in the chamber, and the temperatures of the chamber drain or outside the chamber proper are not directly relevant in this validation activity. Differences in the cycle dwell period can be ignored because only the shortest dwell period for each air removal method must be evaluated. The acceptance criteria for this test vary with the sterilizer's capabilities and customary use. Biological indicators are not required in the evaluation of empty chamber temperature distribution.

### Component Mapping

Items that are steam sterilized can be quite complex and may have interior void volumes, obscured surfaces, crevices, and difficult-to-reach product contact surfaces that must be sterilized. The ability of saturated steam to penetrate the wrapping materials or containers and to reach the surfaces should be established for each item. Although this is relatively easy for simple items such as spatulas, beakers, and other simple geometric shapes, it can be substantially more difficult for filling assemblies, filter housings, tubing, and hoses. Analysts should conduct studies to determine cold spots in items to ensure that heat penetration takes place throughout the load items using thermocouples in contact with the item's surface. These studies can be performed in a laboratory setting and need not be repeated when the same item is sterilized in multiple autoclaves. During this evaluation, all load items should be wrapped and oriented in a manner that facilitates steam ingress and air and condensate removal. Items must be wrapped and oriented in an essentially identical manner for reproducible sterilization.

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## Load Mapping

The determination of loading patterns is an essential practice for terminal sterilization of aqueous liquids by moist heat (see *Moist Heat Sterilization of Aqueous Liquids* ⟨1229.2⟩), but this practice is not a critical concern regarding direct sterilization of items because differences between components play a greater role than location within the load.[1] Loads for direct steam sterilization can be validated using a maximum and minimum load as determined by either the number of each item or their mass. Best practices include placing larger items on the lower shelves, allowing condensate from these items to exit the sterilizer with minimal contact with other load items.

## Biological Indicators

The commonly used biological indicator for steam sterilization by direct contact contains spores of *Geobacillus stearothermophilus* (ATCC 12980 or ATCC 7953), a thermophilic microorganism with a moist heat resistance substantially greater than that of most vegetative microorganisms. The spore challenge can be placed on a substrate within or on a load item, or the challenge can be a load item that is inoculated with a spore suspension. When biological indicators are used according to the manufacturer's directions, the resistance information provided by the vendor can be used. End users must determine the population and resistance of inoculated items they prepare.

## Heat Penetration and Microbiological Challenge

The goal of the validation activity is the confirmation of acceptable heat penetration using temperature measurements and biological indicator challenges. Customarily this study is performed under conditions where the exposure time and/or temperature are reduced slightly from the routine set points. Thermocouples and biological indicators should be placed with load items at the locations determined to be most difficult to heat during component mapping. Thermocouples should be in contact with the item's surface. Analysts must take care in the insertion of thermocouples and biological indicators so they do not alter the ability of the steam to enter the objects being challenged. This difficulty can be overcome with special fittings for thermocouple entry or by placement of temperature probes in units placed near the units that contain biological indicators. In the latter case, replicate studies provide proof of cycle efficacy when both the biological indicators are killed and the physical measurements correspond to the expected time–temperature values or $F_0$. If the microbial and physical measurements do not meet predefined acceptance criteria, an investigation is required and corrective action is necessary to rectify the discrepancy.

## Routine Process Control

As with all sterilization processes, after validation, steam sterilization must be subject to formal controls that maintain it in a validated state over time. *Sterilization of Compendial Articles* ⟨1229⟩ outlines the general requirements for all sterilization processes including training, calibration, physical measurements, physical integrators or indicators, ongoing method control, change control, preventive maintenance, and periodic reassessment.

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# ⟨1229.2⟩ MOIST HEAT STERILIZATION OF AQUEOUS LIQUIDS

## INTRODUCTION

Steam sterilization of aqueous liquids (including both suspensions and emulsions with mixing), also known as sterilization of nonporous loads, is the method of choice for aqueous parenteral products, in-process aqueous liquids, laboratory media, and biological waste materials. This type of sterilization is accomplished primarily in closed containers. During steam sterilization by direct contact (also called steam sterilization of parts, hard goods, or porous items) the steam in the chamber directly contacts the surface of load items to effect sterilization (see *Steam Sterilization by Direct Contact* ⟨1229.1⟩). In contrast, sterilization of liquids in containers is accomplished by application of heat to the container, heating of the container wall, and ultimately heating of the internal liquid volume. This can be accomplished using steam, superheated water, and air in various combinations. Some aqueous liquids are susceptible to over-processing that could render them unfit for their intended use. Manufacturers should consider the influence of these differences when they establish a suitable process.

During the sterilization of liquid-filled containers, differential pressures between the interior of the containers and the sterilization chamber may potentially impact container integrity. Air over-pressure is used to minimize the pressure differential between the container and the sterilizer to protect the integrity of the container, especially prefilled syringes and plastic containers. Before sterilization of product containers, manufacturers should consider the potential adverse consequences of excess heat on the materials. In order to ensure sterility as well as functionality, the process definition and validation method used must incorporate both lower and upper temperature and time limits on the process conditions.

When the overkill method can be used for sterilization of sealed liquid containers, it is the preferred method and is described in ⟨1229.1⟩. When product quality attributes can be impaired by excessive heat, the sterilization process should use less time or a lower temperature to minimize the adverse effect on the materials. Sterilization time–temperature or $F_0$ conditions ($F_0$ is defined as the equivalent sterilization time relative to a base temperature of 121°) include both lower (sterility-related) and upper (stability-related) limits.[1] Manufacturers commonly employ the bioburden/biological indicator (BB/BI) or bioburden methods when constraints on the material's ability to withstand the process require the use of less aggressive conditions. This approach requires appropriate controls on presterilization bioburden and/or product-related *D*-values in conjunction with bioindicators of lower spore count or resistance to ensure sterilization.

### Terminal Sterilization of Products

The maintenance of product attributes may require the use of sterilizing conditions that are less aggressive and sterilization equipment, cycles, and validation methods adapted to these more restricted circumstances. The substantial variations in equipment designs and methods for terminal sterilization preclude a singular description of a typical cycle. All terminal sterilizers heat the load, but they accomplish this in varying ways: saturated steam; steam–air mixtures, steam–air–water mixtures, and superheated water. Air over-pressure for maintenance of container integrity and cooling containers and water for heating/cooling of the load may be present depending upon the autoclave size, throughput expectations, and container.

### In-Process Fluids

In-process fluids are used for pH adjustment, dilution to a specified volume, lubrication, and other purposes. In many instances these liquids are sterilized in conjunction with items that must be sterilized by direct steam contact, and the sterilization process must ensure that all items are adequately sterilized.

### Laboratory Media

Laboratory media often are sterilized in standard steam autoclaves with minor adaptations. Provision for slow exhaust (to reduce stress on container integrity and minimize boil over) and jacket cooling can help improve the basic steam sterilizer design and operation to better accommodate the materials. The sterilization process may be specific for media containers or a combination of both liquid-filled containers and hard goods. This process may resemble the methods used for terminally sterilized products (see above). The sterilization of laboratory media may entail the processing of a number of different containers that contain different materials. Manufacturers should be aware of the potential for under- and over-processing across the load and must consider container size, container contents, and position. When liquid-filled containers are combined in the same load with hard goods, manufacturers must consider the unique concerns of each to ensure all items are properly sterilized. Because laboratory media are considered self-indicating with respect to sterility, the use of internal biological indicators during validation is not required.

### Biowaste Sterilization

The sterilization of biowaste in sealed containers from laboratory or production use is similar to parts sterilization. The process is defined to ensure a minimum time–temperature exposure or attainment of a specified $F_0$ value throughout all items of the load. Depending on the potential contaminants present, the autoclave design may incorporate condensate collection/sterilization or sterilizable exhaust filters to ensure that pathogens are adequately contained. Because the objective of biowaste sterilization is to render the materials safe for contact and disposal, the overkill method described in ⟨1229.1⟩ is employed.

[1] Degradation kinetics may differ from those of microbial kill, and $F_0$ values may not be sufficient to fully evaluate "worst case" effects.

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## BIOBURDEN/BIOLOGICAL INDICATOR METHOD

Application of the BB/BI method requires a thorough understanding of the bioburden type, population, and resistance typically present in the presterilized product-filled container. The method relies on substantial differences between moist heat resistance and the population of the bioburden present and the biological indicator used during validation (*Figure 1*).

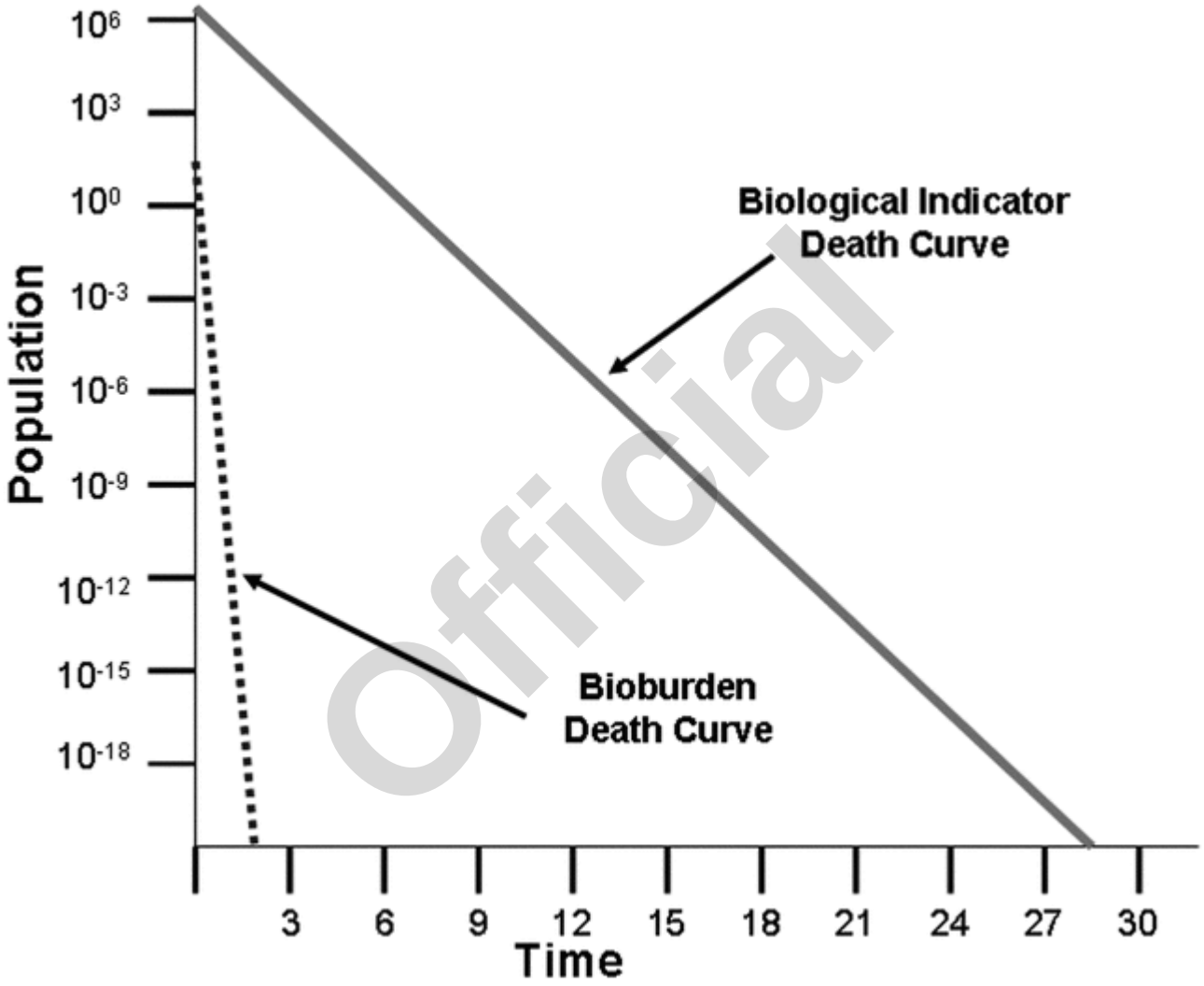


Figure 1. Relative resistance and population of typical bioburden and biological indicator microorganisms.

BB/BI is a method in which the incomplete destruction (or destruction of a modest population) of a resistant biological indicator can be used to demonstrate the capability of the process to reliably destroy any bioburden. This is accomplished using detailed knowledge of the BB and BI populations and their relative resistance.

Typical BB microorganisms have only minimal resistance in comparison to BIs, and this can be confirmed by heat screening of BB isolates. The BB population is controlled by filtration steps for the fluid, process time limits, environmental controls, gowning systems, and other means. The conventional BIs for terminal sterilization using the BB/BI method are *Clostridium sporogenes* ATCC 7955 and *Bacillus subtilis* ATCC 5230, although other strains can be used. The use of *Geobacillus stearothermophilus* for terminal sterilization is uncommon with the BB/BI method because the organism's strong resistance to moist heat makes it poorly suited for this application.

Confirmation of an acceptable probability of a nonsterile unit (PNSU) can be accomplished using physical measurements and BI response (which define the lethality of the process) in conjunction with processing limits for the BB population and resistance (which define the $N_0$ and $D$-value). $D$-value is the time (customarily in minutes) required to reduce the microbial population by 90% or 1 $\log_{10}$ cycle (i.e., to a surviving fraction of 1/10) and must be associated with the specific lethal conditions at which it was determined. For example, $D_{121}$ is the $D$-value at 121°. Articles intended to be sterile must attain a $\leq 10^{-6}$ PNSU, i.e., less than or equal to 1 chance in 1 million that viable bioburden microorganisms are present. The PNSU can be determined from *Equation 1*.

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$$\log N_u = -F/D + \log N_0 \text{ [1]}$$

$N_u$ = PNSU
$F_0$ = $F_0$-value of the process (lethality)
$D$ = $D$-value of the natural bioburden
$N_0$ = bioburden population per container

The following example indicates the resulting PNSU under the defined conditions of validation and routine operation (*Table 1*).

**Table 1. Examples of PNSU Calculation**

| Validation | Routine Usage |
|---|---|
| $F_0$ = 8.0 min | $F_0$ = 8.0 min |
| $D_{121}$ of BI = 0.5 min | $D_{121}$ of bioburden = 0.005 min |
| $N_0$ of BI = $10^6$ | $N_0$ of bioburden = 100 (or $10^2$ ) |
| PNSU for BI = $10^{-10}$ | PNSU for BB = $10^{-1598}$ |

Determining the resistance of the bioburden is accomplished using a heat-screening process during which a pure culture (a laboratory culture containing a single species of organism spores with minimal vegetative cells) is boiled at 100° for various periods. If the bioburden microorganism is viable after exposure, its resistance at 121° can be estimated for use in the PNSU calculation (▲see *Methods for Determination of Resistance of Microorganisms to Sterilization Processes* ⟨56⟩▲ (USP 1-Aug-2022)).
▲▲ (USP 1-Aug-2022)

## Bioburden Method

In most respects the BB method is similar to the BB/BI indicator method. The difference lies in the isolation and characterization of the most-resistant bioburden microorganism. The worst-case isolate is used as the biological indicator in the evaluation of the process. For use in this manner, it must be cultured to produce a suitable challenge population. When this method is used, the bioburden of each process cycle must be closely controlled with respect to population and must be monitored for resistance.

## Sterilization Cycle Control

Process equipment for terminal sterilization typically is controlled by calibrated and pressure sensors in/on the chamber/equipment. During the exposure portions of the cycle, attainment of a minimum dwell time at a predefined temperature is used to support process lethality. Cycle lethality for terminal sterilization customarily is measured using $F_0$, which is defined as an actual exposure time at a variable process temperature that is equivalent to an exposure at 121° based on an ideal microorganism with a $z$-value of 10°. This can include lethality delivered during the heat-up and cooling phases of the sterilization process. A $z$-value is defined as the number of degrees of temperature change necessary to change the $D$-value by a factor of 10. The $F_0$ approach is used to evaluate to a single standard sterilization processes that are operated at varying temperature conditions. The process lethality at temperatures other than 121° can be calculated to determine lethality equivalent to that provided at 121°. Sterilizer control systems for terminal sterilization deliver conditions within a predefined time–temperature or $F_0$ range to avoid over-processing.

Simple mathematics can be used to calculate the total lethality over the course of the process. For the specific reference temperature of 121° and a $z$-value of 10.0°, the $F_0$ calculation can be determined by *Equation 2*:

$$F_0 = \int_{t_1}^{t_s} 10\left(\frac{T - 121}{10}\right) dt = \sum_{t_1}^{t_s} 10\left(\frac{T - 121}{10}\right) \Delta t \text{ [2]}$$

$t$ = time
$T$ = temperature

Summing the instantaneous lethality contributions over the entire sterilization process allows the calculation of the overall process lethality or $F_0$ delivered over the course of the entire process at varying conditions.

## Validation of Liquid-Filled Container Sterilization

As previously noted, the preferred method for steam sterilization is the overkill method as defined in *Sterilization of Compendial Articles* ⟨1229⟩ and ⟨1229.1⟩. However, when the processed materials are susceptible to damage by moist heat at the overkill conditions, the BB/BI method is better suited because it results in reduced heat input while affording the same degree of process efficacy but with different controls. As noted above, terminal sterilization processes require greater consideration of the effects of the treatment on material properties. This has implications for many of the elements of the qualification and validation exercises as indicated below. The validation requirements for the BB/BI and BB methods are more rigorous than those associated with the overkill method. Although the overkill method can be confidently used without detailed consideration of the

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presterilization bioburden, application of the BB/BI and BB methods require continued monitoring and control of the bioburden, specifically the population and resistance. This is accomplished by testing of filled containers just before sterilization and measuring the number of colony-forming units per container and confirming the absence of resistant BB isolates. When resistant isolates are found, their thermal resistance in the fluid should be determined.

## EFFECT OF THE STERILIZATION PROCESS

A preliminary determination of the liquid and the container–closure system's ability to withstand the expected sterilizing conditions should be made during product development. This can be accomplished by sterilization at conditions slightly in excess of the maximum expected and evaluating the effect on the material. The evaluation should encompass the essential quality attributes with attention focused on known and potential new impurities. Appearance and other physical properties also should be evaluated as a part of this effort.

## EQUIPMENT QUALIFICATION

Equipment qualification is a predefined program that confirms the equipment has been properly installed and that it operates as intended. Qualification of the sterilizing equipment provides a baseline for preventive maintenance and change control for the sterilizer. The sterilization equipment may require qualification of air, water, utility, and other systems that impact the sterilization equipment's performance.

## EMPTY CHAMBER TEMPERATURE DISTRIBUTION

The dual considerations of sterility and stability commonly associated with sterilization of liquids require that equal attention be paid to potential under- and over-processing of the load. For this reason the temperature gradient across the sterilizer may require substantially tighter control than that expected in sterilization by direct contact. The objective is to minimize the time–temperature or $F_0$ differences across the load throughout the process. Biological indicators are not required in the evaluation of empty chamber temperature distribution.

## BIOLOGICAL INDICATORS

The selection of a BI must be considered carefully because of the balance that must be maintained between attaining sterilization and maintaining the sterilized material's essential quality attributes. The biological challenge is either directly inoculated into a liquid-filled container or is introduced via self-contained units provided there is adequate correlation between their resistance and the resistance that would occur in the process fluid. The liquid can be either the product or a surrogate fluid. The resistance of the indicator in the product (and surrogate fluid, where used) must be known. The surrogate's physical properties should approximate those of the product. If there are surfaces within the container that are not presterilized, biological challenge of those surfaces may be required.

## LIQUID *D*-VALUE DETERMINATION

Determination of the thermal resistance (*D*-value and *z*-value) for the biological indicator in the liquid is required. This must be performed in a Biological Indicator Evaluation Resistometer (BIER) in replicate. The thermal resistance of each BI lot in the liquid should be determined. When a surrogate liquid is used for convenience (e.g., a master solution approach) or because of microbial inhibition of the BI by the liquid, the thermal resistance in the surrogate must be determined.

## CONTAINER MAPPING

Liquid-fill containers with volumes greater than or equal to 100 mL should be mapped to determine internal cold spots. The mapping should be performed with product containers oriented as they would be within the load. The temperature probes should be introduced into the containers using methods that maintain container integrity. Internal supports (of minimally heat-conductive materials) may be required to ensure proper positioning of the probe within the container. After these locations are determined, they are used as either monitoring locations or are correlated to external conditions on the container during validation and routine processing. Smaller containers (less than 100 mL) have fewer discernible cold spots, the importance of which is reduced as container size decreases. Smaller containers (less than 100 mL) can be monitored with temperature probes secured to their exterior. The "cold spot" should be considered a "region" and not a single point in the container.

## LOAD POSITIONING AND MAPPING

A fixed loading position within the sterilizer may be necessary for proper sterilization to ensure uniformity of heating and cooling in routine use. Once the load is positioned properly, its size can vary within a defined range. Load-mapping studies should be performed to determine the coldest and hottest locations within the load. These locations may not be specific individual containers but rather regions. This ensures that the containers are neither under- nor over-processed in routine operation of the sterilizer. Validation of variable-size load patterns is accomplished using a bracketing approach for which success with maximum and minimum loads (avoiding both under- and over-processing) establishes the acceptability of intermediate-size loads. However, evaluation of intermediate load sizes may be beneficial. In product sterilization, only a single-size container with a single product lot is processed concurrently.

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### HEAT PENETRATION AND MICROBIOLOGICAL CHALLENGE

The core of the validation activity is confirmation of acceptable heat penetration using temperature measurements and microbial challenge inactivation. Temperature probes and biological indicators are placed within the load at worst-case locations (e.g., the coldest portions of the loaded chamber). Introduction of the thermocouples must not alter the integrity of the container. Biological challenges are placed in containers adjacent to those that contain heat penetration probes (or the same unit with external temperature measurement).

Proof of cycle efficacy is provided by replicate studies in which the BIs perform as expected and the physical measurements correspond to the expected values of time and temperature or $F_0$. If the microbial and physical measurements do not correlate, an investigation is in order, and corrective action must be taken to rectify the discrepancy. Samples from the hottest regions of the load are used for evaluation of material stability and quality.

### PRODUCT QUALITY AND STABILITY EVALUATION

Manufacturers must conduct ongoing evaluation of the product's ability to withstand the routine sterilizing conditions. The evaluation should encompass the essential quality attributes with attention focused on known and potential new impurities and those materials that receive the most heat input. Manufacturers also should evaluate appearance, other physical properties, and container–closure integrity as a part of this effort. For microbiological media, the ability of the media to meet growth promotion and other requirements is required as indicated in the appropriate test chapter(s) (e.g., *Microbial Enumeration Tests* ⟨61⟩, *Tests for Specified Microorganisms* ⟨62⟩, and *Sterility Tests* ⟨71⟩).

## Routine Process Control

All sterilization processes should be subject to formalized practices that maintain them in a controlled state. The practices outlined in ⟨1229⟩ include the general requirements appropriate for all sterilization systems. This is accomplished by a number of related practices that are essential for continued use of the process over an extended period of time. The practices include: calibration, physical measurements, physicochemical integrators, indicators for sterilization, monitoring of bioburden, ongoing process control, change control, preventive maintenance, periodic reassessment, and training.

The use of parametric release is common in the terminal sterilization of finished product containers. This subject is addressed in *Terminally Sterilized Pharmaceutical Products—Parametric Release* ⟨1222⟩.

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# ⟨1229.3⟩ MONITORING OF BIOBURDEN

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## INTRODUCTION

Monitoring of in-process bioburden of pharmaceutical components and products is an essential element of the overall contamination-control program for appropriate sterilization process control. Bioburden monitoring should be designed for the recovery of a broad range of microorganisms that are likely to be present in the material being processed. Sterilization processes are implemented in order to eliminate bioburden in materials and the products, ensuring both adequate process control and end-user safety.

Bioburden is a potential risk to the patient not only because the sterilization process might not be completely effective, but also post-processing because of the possible presence of residual materials such as allergens, endotoxins, and exotoxins. It may also have adverse impact on product quality and stability. Therefore, although bioburden may be confidently killed by destructive sterilization processes or removed by retentive processes (filtration), as summarized in the ensuing sections, its proliferation before sterilization should be avoided. Process controls and cleaning, sanitization, and disinfection programs provide active means for the control of bioburden population and support the sampling, enumeration, and characterization of bioburden necessary to ▲ensure▲ (USP 1-Aug-2022) that sterilization processes are effective.

### Destructive Processes

Destructive sterilization processes, e.g., moist and dry heat sterilization, are developed and validated to kill microorganisms. It is critically important to the quality control aspects of the sterilization program to fully understand the bioburden. The microorganisms that are most resistant to widely used destructive processes are spores of certain Gram-positive bacteria (note that mold spores are less resistant to these destructive processes than are bacterial spores). As a result of the resistance properties of these organisms, some species are commonly used as biological indicators for the evaluation of sterilization process performance. In their vegetative state, microorganisms that form spores do not exhibit extraordinary resistance to sterilization processes.

Processes that rely on ionizing radiation are an exception to typical resistance profiles because some non-spore-forming microorganisms exhibit higher resistance than do spore formers. Bioburden within materials and products subjected to radiation processes is evaluated as part of dose setting activities and is explained in the *Radiation* section that follows.

The sterilization process, the development of a thorough understanding of the process, and its microbiological impact are major elements in confirming that an acceptable level of sterility assurance is established. Understanding the inactivation kinetics of the typical bioburden as well as other potential bioburden microorganisms is critical to successful implementation. The new process should have a documented risk assessment outlining its monitoring and control aspects. The establishment of a new sterilization process requires the evaluation of the suggested biological indicator resistance (*1,2*).

### Retentive Processes

The microorganisms least likely to be retained by a sterilizing filtration process are those that are potentially smaller than the smallest pores in the filter matrix. Although size exclusion is an important factor, filters do not retain microorganisms solely by sieve retention. Adsorption, wherein microorganisms are retained within the filter matrix by entrapment or electrostatic forces, also is an important retention mechanism. The control of prefiltration bioburden is an important risk-mitigation factor in retentive processes, and this is particularly true when adsorption may be a significant retention mechanism. Bioburden removal capability therefore depends on the size and number of the bioburden microorganisms, the pore size distribution of the filter, the properties of the solution being filtered, and the filtration process parameters.

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## MONITORING AND SAMPLING

Statistical and analytical limitations complicate the evaluation of bioburden from both liquid and solid materials. Many products are inherently antimicrobial, and some formulations contain antimicrobial preservatives, both of which can limit bioburden recovery. Products that are outside of the pH range of approximately 4–9, are strongly hypertonic or strongly hypotonic, or have low water activity, may reduce the level of recoverable microorganisms.

The collection of multiple samples from the same bulk often gives varying results due to temporal and spatial differences in microbial distribution. Samples from varied components and different formulations will give different results because of different nutritional and cultural requirements, environmental stress to microorganisms, sampling methods, sample size, sampling pattern, species heterogeneity, and shifting microbial populations. Because of the technical challenges associated with bioburden analysis, results will be dependent upon sample-specific and method-specific variables when attempting to recover, grow, or enumerate organisms. Sampling frequency and maximum time delay between sampling and testing should be established based on previous data.

Presterilization bioburden analysis should be conducted on samples that are representative of materials produced during routine preparation and processing. Sampling frequency should be established based on previous data, known variability, batch size, material, process, and environmental influences. Bioburden should be recovered and enumerated from samples that are representative of the process material. When the material is stored in separate containers, analysts should consider testing

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multiple or composite samples. Bioburden evaluation should focus on microorganisms that represent a greater concern in the sterilization process. Total count methods should consider the properties of both the product under evaluation and the characteristics of the process that may affect recovery. Cultivation methods, diluents, and media selection must be based on past experience with the manufacturing process and test material (*3*). The methods listed in *Microbial Enumeration Tests* ⟨61⟩ and *Mycoplasma Tests* ⟨63⟩ may be appropriate for bioburden evaluation, although they may require modification to the methods and qualifications in order to meet the requirements of specific test materials.

## Bioburden Screening

Materials and products that are to be sterilized should be examined to determine the level of bioburden in the article. Determining the microbial population level is an essential element in ensuring process efficacy. For lethal processes other than radiation, the presence of gram-positive spore-formers represent the greatest potential for survival. The potential resistance of the bioburden to the specific sterilization process is an important consideration and can be evaluated by a screening process. Isolates that are identified as gram-negative rods have the potential to cause high levels of endotoxin. *Figure 1* illustrates one possible bioburden screening program.

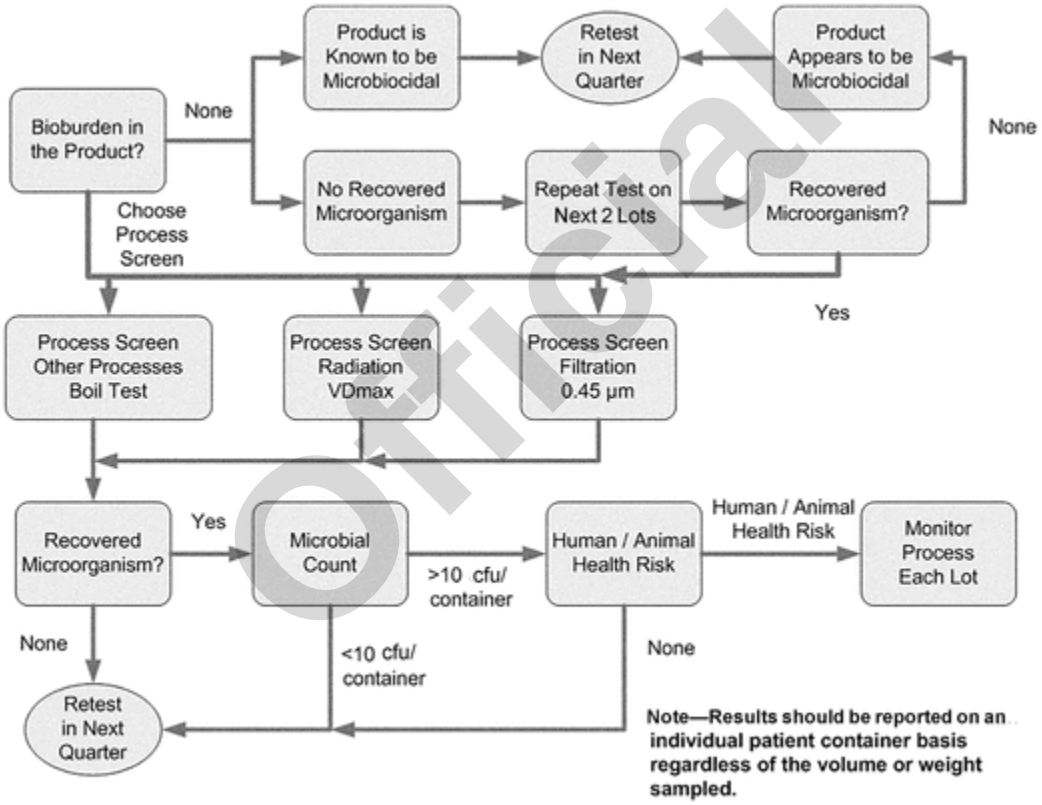


Fig. 1. Typical bioburden screening program.

## Destructive Processes (except Radiation)

Microorganisms can be recovered after exposure to heat screening or shock at 100°, which eliminates vegetative cells and also triggers the germination process in spore-forming bacteria ▲(see *Methods for Determination of Resistance of Microorganisms to Sterilization Processes* ⟨56⟩)▲ (USP 1-Aug-2022) (*4*). Heat shock treatment is an effective means to reduce the amount of bioburden microorganisms to be evaluated because spore-formers are the most resistant to all lethal processes other than radiation. Heat screening eliminates vegetative cells that lack resistance to sterilization and encourages germination, thus making it easier to isolate spore formers.

Published reports compare the resistance of recovered microorganisms to that of predefined bioindicators for many common (*4,5*) sterilization processes. Analysts should evaluate the isolated spore-forming bacteria to determine their resistance *D*-value to specific lethal sterilization processes and thus to ensure the validity of the sterilization method. The available data describe the lack of resistance of medically and environmentally isolated bacteria, mold, and yeast species to the more commonly used sterilization methods, so analysts may find only limited value in repeating these resistance studies.

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## Radiation

Sterilization by radiation processes is based on the bioburden in the presterilized material. Analysts commonly use bioburden-based sterilization processes as described in ANSI/AAMI/ISO 11137 (*6*). To ensure consistent sterility assurance, lot-to-lot variability of bioburden must be controlled. Data should be collected and analyzed for raw materials, intermediates, and/or products to ensure process control. ANSI/AAMI/ISO 11137 provides detailed information about radiation sterilization practices, including cycle development, validation approaches, and dose-auditing methodologies including expectations for initial and periodic bioburden assessment. ANSI/AAMI/ISO 11737 provides guidance for establishing methods to estimate bioburden levels on medical devices prior to irradiation (*7*).

## Sterilizing Filtration

Bioburden screening for sterilizing filtration processes not yet subjected to formal validation can be performed by passing the material through a 0.45-µm-rated filter and examining the filtrate for viable microorganisms. Only those microorganisms that pass the 0.45-µm filter are of interest because they present the greatest potential challenge to the sterilizing filtration process. They should be evaluated against the upstream bioburden. Bioburden of greatest concern includes *Pseudomonas*, *Brevundimonas*, *Ralstonia*, and *Mycoplasma*.

## Identification

Recovered microorganisms can be identified to an appropriate level using the methods described in *Microbial Characterization, Identification, and Strain Typing* ⟨1113⟩. Only those microorganisms that present a potential risk to the product or the patient require identification to the species level or beyond. For a detailed discussion of the methods used to grow and identify microorganisms, see ⟨1113⟩. It is not necessary for the purposes of evaluating presterilization bioburden to identify all isolates to the species level, although this can be helpful in some investigations. Analysts must conduct all evaluative work with pure cultures, and they must apply normal microbiology laboratory procedures for the selection and maintenance of pure cultures.

## Bioburden Control

A typical bioburden-control program includes review and analysis of potential sources of contamination as well as sound process design and preventive and monitoring measures. The microbiological contamination-control program should be developed to identify and control bioburden and to assess product risk based on a formal assessment of risk modalities. The bioburden risk assessment should result in the establishment of critical control points and should include consideration of the following elements:

- Microbiological attributes of materials before sterilization and the manufacturing process used for the materials (if applicable)
- Inherent antimicrobial properties of the materials
- Time limits for process execution
- Water activity of the material
- Environmental conditions within the facility
- Equipment design and cleaning
- Sanitization, decontamination, and other active microbial control processes (such as prefiltration, temperature, pH, osmolarity, etc.)

Controlling the bioburden of materials and products to be sterilized will ensure conformance to the levels required by the sterilization process validation. Additionally, controlling the bioburden levels of the items to be sterilized assures that residuals (e.g., allergens, endotoxins, and exotoxins) from that population will also be controlled. This is important because direct detection of these materials is challenging.

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# ⟨1229.4⟩ STERILIZING FILTRATION OF LIQUIDS

## INTRODUCTION

Sterilization processes are divided broadly into two categories: destruction of microorganisms and their physical removal from the material to be sterilized. Autoclaving is an example of the former, and sterilizing filtration is an example of the latter. The physical removal of microorganisms depends on the bioburden of the solution to be filtered, the properties of the solution, the filtration conditions, and the filter itself.

Sterilizing filtration is a process that can be validated to consistently yield filtrates that are sterile, as defined in *Sterilization of Compendial Articles* ⟨1229⟩. This chapter provides an overview of (1) various factors that affect the filtration process, (2) the filter integrity test and when to perform it, (3) prefiltration bioburden control, (4) responsibilities of the filter manufacturer and user, and (5) troubleshooting the filtration process.

Multiple factors contribute to the effectiveness of any sterilizing filtration process. These include the type and number of microorganisms, the properties of the liquid, the filter design and membrane polymer, and the filtration process parameters. Properties of the liquid that influence filtration effectiveness include its chemistry, viscosity, surface tension, pH, osmolarity, ionic strength, and temperature, as well as the presence of insoluble materials. Aspects of the filter that affect the filtration include effective filter area, nominal pore size, pore-size distribution, membrane thickness, porosity, membrane polymer, filter pleat density, nonwoven support layers, electrostatic charge, and the hydrophilic nature of the filter membrane. The filtration process parameters that influence microbial retention include temperature, flow rate, volume, filtration time, differential pressure, and pressure pulsations.

Additionally, effective sterilizing filtration depends on (1) the production controls and quality assurance systems used by the filter manufacturer to ensure the quality and uniformity of the filter membranes and fabricated filters, (2) the qualification and validation studies conducted by, or for, the filter user to demonstrate that the chosen sterilizing filtration process achieves a sterile filtrate, (3) effective controls to ensure that prefiltration bioburden and operating parameters remain within the validated ranges, and (4) filter integrity. Filter users should ensure that the filtrate remains sterile by using validated sterilization processes for the filtration assembly and all downstream manufacturing equipment and effective aseptic handling of the sterilized materials. Filter users should carefully consider placement of the filter (e.g., proximity to the filling line or hold tank) to minimize the possibility of postfiltration contamination.

### Sterilizing-Grade Filters

A sterilizing-grade filter is one that is capable of retaining a minimum $1 \times 10^7$ cfu of *B. diminuta* (ATCC 19146) per square centimeter of effective filter area when tested in accordance with ASTM F838-05 (2013), *Standard Test Method for Determining Bacterial Retention of Membrane Filters Utilized for Liquid Filtration (2)*.

The designation "sterilizing grade" implies a sterilizing action only if other conditions are met, including the integrity test specification established by the filter manufacturer and validated by the user (see *Validation*, below).

Sterilizing-grade filters typically are microporous membranes that have nominal pore-size ratings of about 0.2 µm. These membranes are fabricated with various materials, have relatively narrow pore-size distributions, and can be integrity tested. The integrity test results can be correlated with microbial retention. Membrane filters that have a nominal pore size of 0.45 µm can be validated to produce sterile filtrates under some conditions; for example, some liquids require high differential pressures to achieve useful flow rates, and these pressures are not suitable for use with 0.2-µm–rated filters. When manufacturers use 0.45-µm filters, they should ensure particularly stringent control of presterilization bioburden.

### Retention Mechanisms

Microporous membranes remove microorganisms from the liquid by two primary mechanisms: sieve retention, which relies on physical blockage of particles that are larger than the pores they encounter, and adsorption, which is a charge-related phenomenon whereby particles are bound to the membrane surfaces. Both mechanisms should be considered during the development, qualification, and validation of sterilizing filtration processes.

Sieve retention, for the most part, is independent of filtration conditions and the microbial challenge level, but the composition of the liquid, temperature, and filtration conditions can affect sieve retention under certain conditions.

Adsorption is a charge-related phenomenon that is influenced by the composition of the membrane, the properties of the filtered liquid, the filtration conditions, and the number and type of microorganisms present in the liquid.

The number and type of microorganisms and other particles present in the material to be filtered can affect retention. Sieve retention implies that every microorganism is larger than the largest pore in the filter. Microorganisms and filter pores are not uniform in size, and some microorganisms may be smaller than the largest pores. Even if a microorganism is smaller than the pore it encounters, it may be retained if a site is available and the filtration conditions are conducive to adsorption. Retention probability is related to the number of microorganisms in the upstream bioburden *(4,5)*.

## FACTORS THAT AFFECT RETENTION

### Nature of Pores

Microporous membranes consist of a polymer matrix that is penetrated by interstices commonly referred to as pores. Compared with those in depth filters, the pores in microporous membranes have a relatively narrow size distribution. The size,

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number, and shape of the pores determine the filter's retention capabilities. With the exception of the pores in track-etched filter membranes, pores are not cylindrical; they are made up of a series of pseudopolyhedral structures with varying internal diameters *(6)*.

The pore size, pore-size distribution, and membrane porosity are a function of the manufacturing process. Careful design and control of that process are necessary to ensure that the resulting membrane has the desired integrity test value, microbial retention capability, and uniformity.

## Nature of Microorganisms

Microorganisms have a variety of shapes and sizes. If the microorganism encounters a membrane pore that is smaller than its smallest diameter, the microorganism likely will be captured by sieve retention. If, however, the microorganism is smaller than the pore it encounters, it may be retained by adsorption if the residence time, electrostatic charge, pH, fluid chemistry, and membrane material are conducive to adsorption.

Some genera of microorganisms are deformable, so that at high-pressure differentials or flow rates a microorganism may be forced through a filter pore that is only slightly smaller than the organism *(7)*. Mollicute bacteria lack cell walls and thus are small and pliable enough to pass through filter membranes under certain conditions.

Grow-through (i.e., passage of microorganisms through a filter as a function of time) may result from one or more scenarios. The microorganism may penetrate as it multiplies: a parent cell divides into two smaller daughter cells that negotiate pore passageways. Penetration can occur with time, because the increasing number of microorganisms overwhelms the few larger pores that are encountered. However, because of the limited time periods typically involved, the controls on bioburden, and the often limited availability of nutrients, grow-through is considered a rare phenomenon in pharmaceutical processes (see *Monitoring of Bioburden* ⟨1229.3⟩) *(8)*.

## Composition and Structure of the Filter Matrix

Several factors related to the filter matrix can affect microbial retention. These include the material from which the filter is made, the pore size and pore-size distribution, whether the membrane is isotropic or anisotropic (i.e., whether membrane pore structure is uniform from face to face or "tapers" from one face to the other), membrane thickness, and whether the filter consists of single or multiple layers.

The membrane material is especially important if adsorption is a significant mechanism in a particular filtration scheme. For example, polyamide exhibits stronger microbial adsorption than does cellulose ester *(3)*.

Microporous membranes that have a relatively wide pore-size distribution are less likely to retain microorganisms, particularly at high challenge levels, than membranes of comparable pore-size ratings with narrower pore-size distributions. This relates to the probability of a microorganism's encountering a pore larger than itself.

Thicker membranes generally are more retentive than thinner membranes of the same type and pore-size rating, owing to the higher probability of entrapment or adsorption within the pore structure because of the increased distance that bacteria must travel in thicker membranes. This distance favors entrapment, as does increased residence time within the pore, which favors adsorptive retention *(3)*.

Multi-layered membrane filters exhibit a higher probability of retention than do single-layered membrane filters of the same thickness because the small number of largest pores is the factor affecting retention and the probability of a large pore in one layer being congruent to a large pore in the adjoining layer of a double-layer filter is negligible *(3)*.

## Composition of the Filtered Solution

The composition of the filtered solution can adversely affect the membrane material if an incompatibility exists, causing damage to the membrane and affecting both retention and physical integrity, unless detected before the filter is selected for use. In addition, if adsorption is a significant retention mechanism, then solution properties such as pH and the presence of surfactants become important.

Surface charge and ionic strength are important variables. Bacterial and membrane surfaces in aqueous media are negatively charged, resulting in repulsion. The repulsive force is balanced by attractive forces, which include hydrophobic surface energy minimizing forces, operative only over short distances, and hydrogen bonding. High ionic strength allows the surfaces to close —because of discharge through the electrolyte—to the point where hydrophobic adsorption can occur *(3)*. Also, high ionic strength can draw water out of the cell, reducing its size, which may lower the probability of retention, depending upon the composition of the prefiltration bioburden. Surface tension and the presence of surfactants influence retention. Adsorption of surfactant by the filter and the microorganism creates repulsion, leading to a decreased probability of retention. Surfactants in concentrations as low as 0.05% have been shown to inhibit adsorption and decrease the retention of latex spheres *(9)*.

## Filtration Conditions

Differential pressure, flow rate, and temperature are among the factors that can affect microbial retention. Hydraulic shock should be avoided, not only because it affects pressure differential and flow rate but also because it can damage the filter. Flow rate is proportional to differential pressure, and higher flow rates reduce adsorption because the contact time is reduced *(10)*.

Microbial retention may be reduced at higher temperatures when these result in higher flow rates due to decreased solution viscosity. Temperature effects are not significant in filters where sieve retention is the primary removal mechanism.

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## Filter Efficacy: Log Reduction Value

Filtration efficacy can be defined in terms of a log reduction value, which is the logarithm of the quotient produced by dividing the upstream challenge population by the recovered downstream population.

The log reduction value is influenced by the number and size of the challenge microorganisms, the filter design and membrane polymer, the filtration process parameters, and the properties of the solution.

To determine the specific log reduction value of a filter, the challenge test should permit some passage of the test microorganism through the filter in order to produce a denominator. Sterilizing-grade membrane filters should not permit passage of the specified challenge microorganisms for that filter rating. For this reason the log reduction value of sterilizing-grade filters is described as equal to or greater than the log of the challenge population.

## Validation

As noted, microbial retention in sterilizing filtration relies on a combination of sieve retention and adsorption. Validation of sterilizing filtration therefore requires determination of the effect of the liquid on the filter, determination of the effect of the filter on the liquid, and demonstration that the filter can consistently yield sterile solutions under the intended conditions of use. The liquid to be filtered can affect the pore structure of the membrane, can have electrostatic properties different from the standard challenge suspension used to establish integrity test specifications, and can change the size and shape of the challenge microorganisms. Factors that should be considered when developing a sterilizing-filtration validation protocol include the surface tension, pH, temperature, and osmolarity of the liquid to be filtered; the compatibility of the material or solution components with the filter itself; the pressures, flow rates, and hydraulic shock likely to be encountered; and the maximum filtration time and volume to be filtered. The effect of sterilization (steam, radiation, or gas) on the filter's retention capability also should be considered.

*B. diminuta* (ATCC 19146) is used as the challenge organism unless it is not viable in the liquid to be filtered. Viability studies should be used to confirm that the liquid has no adverse effects on the challenge organism. If the challenge organism is viable in the liquid to be filtered, the liquid should be inoculated to achieve a challenge level of $1 \times 10^7$ cfu/cm$^2$, and the filtrate should be evaluated for the presence of the challenge organism. If *B. diminuta* is not viable in the liquid, several options are available, and analysts can (1) modify the liquid to ensure the viability of the challenge organism (e.g., adjust pH or remove the bactericidal component), (2) reduce the exposure time to ensure that the challenge organism remains viable, or (3) change the challenge organism from *B. diminuta* to one that has been isolated from the liquid to be filtered. These studies should employ production process pressure differentials or process flux values as appropriate.

If possible, the liquid to be filtered should be used because in some instances the challenge organism has penetrated a filter in contact with the liquid but has been retained by the same filter when inoculated into a surrogate fluid *(11)*.

Three different lots of filter membranes should be used for the microbial retention studies. The membranes should have preuse integrity test values that are near the filter manufacturer's specification in order to minimize the possibility that production filters will fail to meet the integrity test value established during the validation exercise. Successful microbial challenge studies result in no microorganisms detectable in the filtrate. The sterilization process for the filter, its housing, and associated equipment should be validated. The filter should be sterilized within its housing rather than relying on aseptic assembly following sterilization of the filtration system components. The assembled filtration apparatus can be steam sterilized in an autoclave, using a vacuum cycle, with particular attention to the orientation and wrapping of the housing and any associated tubing to allow condensate drainage and steam penetration. Validated steam sterilization-in-place cycles also can be used.

Sterilization methods and cycles should be carefully chosen and designed to preclude damage to the filter. Pre- and poststerilization integrity testing can be used to confirm that the sterilization procedure does not change the integrity test value and to demonstrate that the sterilization process does not damage the filter.

Finally, the scale of the validation approach should be considered. Two approaches are common. One uses a small section of membrane material, typically a 47-mm disk, to represent the filter. This approach validates the microbial retention capability of the membrane. A second approach uses the intended filter configuration, typically a 10-inch cartridge in its housing. This approach validates the filter system performance in addition to the retention capability of the membrane material. Analysts should consider the volume of the test liquid and operational considerations when they choose which approach to use.

## Integrity Testing Principles and Methods

Integrity testing can be used to show that a filter has the correct pore-size rating, is installed properly in its housing, and has not been damaged by the process used to sterilize it. These integrity test methods generally rely on detecting gas flow caused by pressure differential across a wetted membrane. *Figure 1* shows the relationship between gas flow and pressure differential for high-surface-area and low-surface-area membranes (a 47-mm membrane disk can be considered low surface area, and a multicartridge array of 10-inch cartridge filters can be considered high surface area).

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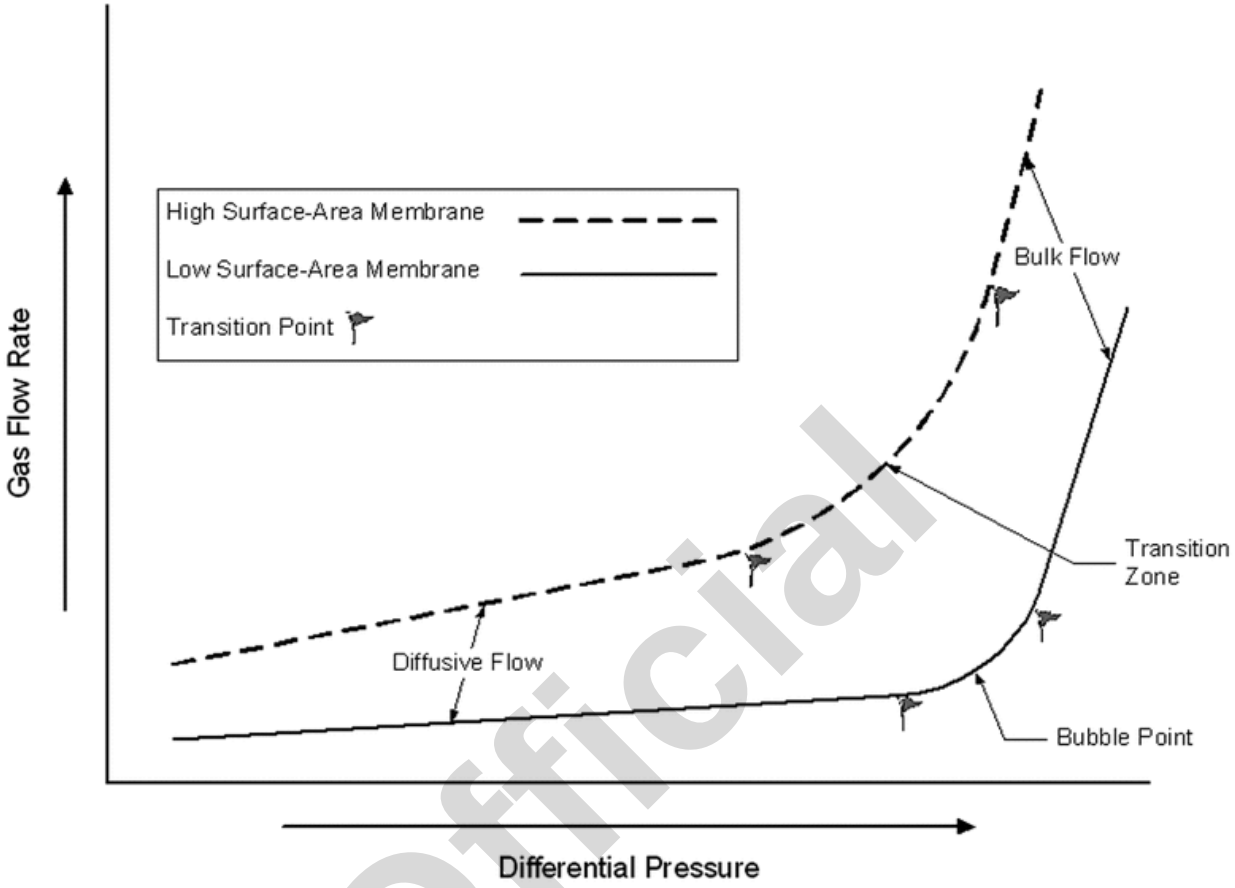


Figure 1. Relationship between gas flow and pressure differential for high-surface-area and low-surface-area membranes (see the section *Integrity Testing Principles and Methods* for definitions).

A filter that successfully passes an integrity test based on specifications developed during an effective sterilizing filtration validation study is capable of producing a sterile filtrate.

Integrity test methods for microporous membrane filters include bubble point, diffusive flow, and pressure hold. Membrane type and size and the particular filtration process influence the choice of an appropriate integrity test method. The sensitivity of the bubble point test decreases with increasing filter area because of increasing diffusive flow. Bubble point, diffusive flow, and pressure-hold integrity tests are used for hydrophilic microporous membrane filters. As shown in *Figure 1*, diffusive flow occurs at pressures below the bubble point, and bulk flow takes place above the bubble point. The bubble point marks the transition between diffusive flow and bulk flow. The exact bubble point is difficult to detect in high-surface-area filters, because the high membrane surface area generates significant diffusion. Integrity testing may be performed manually or by automated equipment designed specifically for that purpose.

## BUBBLE POINT

The bubble point occurs when a gas displaces a wetting liquid from the largest membrane pores, resulting in bulk gas flow through those pores. The flow is evidenced by a steady stream of bubbles through a column of water on the downstream side of the membrane.

The bubble point relates to the pore size of the membrane and the contact angle that the wetting liquid makes with the pore wall. Bubble point is indirectly proportional to membrane pore size and is directly proportional to the surface tension of the wetting liquid; that is, filters with smaller pores have higher bubble points than those with larger pores, and liquids that easily wet the membrane exhibit higher bubble points than those that do not. Bubble point is defined as:

$$P = 4 \times \gamma \times \cos\Theta / D$$

$P$ = pressure to evacuate the pore
$\gamma$ = surface tension
$\Theta$ = angle of wetting
$D$ = pore diameter

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Microbial retention and bubble point correlate: numerous studies have demonstrated that when the microbial reduction ratio is plotted against the bubble point, a line of constant slope results *(12)*.

The actual bubble point is independent of the membrane surface area, but diffusive flow through high-surface-area filters can mask the true bubble point. The bubble point test is easy to perform on small- to medium-scale filters, the test time is short, and temperature effects are minor. Manual bubble point testing requires manipulation of the downstream side of the filter and is subjective.

### DIFFUSIVE FLOW

In diffusive flow testing a wetted filter provides a liquid layer across which gas can flow by means of diffusion. Diffusive flow is measured directly at constant pressure.

Diffusive flow is proportional to the differential pressure of the test gas, the diffusivity of the test gas in the wetting liquid, the thickness (depth) of the wetting liquid, the porosity (i.e., void volume) of the membrane, and the effective filtration area. Diffusive flow is defined by Fick's Law of Diffusion, shown as:

$$N = [D \times H \times (p_1 - p_2)]/(L \times \rho)$$

$N$ = permeation rate (moles of gas per unit time)
$D$ = diffusivity of the gas in the liquid
$H$ = solubility coefficient of the gas
$p_1 - p_2$ = transmembrane pressure (differential pressure)
$L$ = thickness of liquid in the membrane
$\rho$ = void volume (porosity) of the membrane

Unlike the bubble point test, diffusive flow testing measures gas flow through all the wetted pores, and thus diffusive flow does not correlate directly with microbial retention. However, bacterial challenge tests with a series of filters that have decreasing diffusion rates show that a gas diffusion rate exists below which sterile filtrates are obtained.

Diffusive flow testing is highly sensitive, especially for higher-surface-area membranes. Larger pores or flaws can be detected by a thinning of the liquid layer and correspondingly higher diffusive flow rates. Diffusive flow testing is useful for membranes with small pore sizes (e.g., 0.1 µm and smaller) because of the high pressures required for bubble point testing. Diffusive flow testing measures flow across the total pore volume, which may mask a flaw, especially in high-surface-area multiple-cartridge arrays *(13)*. This test also is highly sensitive to temperature.

### PRESSURE HOLD

The pressure hold integrity test is a variation of the diffusive flow test. The wetted membrane provides a liquid layer across which gas can flow by means of diffusion. The gas flow is proportional to differential pressure and is measured by pressure decay on the upstream side of the membrane.

The rate of pressure decay is influenced by upstream volume of the particular holder–filter combination, valve placement, and tubing volume. Temperature should remain constant because of the relationship between temperature and pressure defined by the ideal gas law.

Conversion of pressure decay test results to diffusive flow values allows correlation with microbial retention. Analysts establish the relationship between pressure decay and diffusive flow by calculating diffusive flow on the basis of pressure drop per unit time, with a known upstream volume and reference pressure. Diffusive flow as it relates to pressure decay is shown as

$$D = [(p_1 \times V_1)/(p_0 \times t)] \times ln[p_1/(p_2 - \Delta p)]$$

$D$ = diffusion
$p_1$ = starting test pressure
$V_1$ = upstream volume of filter system
$p_0$ = atmospheric pressure
$t$ = test time
$p_2$ = ending test pressure
$\Delta p = p_1 - p_2$

The advantages and disadvantages of the pressure-hold test are similar to those of the diffusive flow test. The pressure-hold test has the additional abilities of revealing imperfections in the assembly and sealing of the housing and filter seating and avoiding downstream manipulation. Its disadvantages are that it is strongly influenced by temperature and that accurate measurement of the upstream volume is required *(14)*.

## When to Test Integrity

The decision not to perform preuse, presterilization integrity testing should be based on a formal risk assessment.

Preuse, poststerilization integrity testing may create an unnecessary risk for microbial contamination of the filter and associated downstream tubing and equipment. Preuse, poststerilization integrity testing is unnecessary if effective validation studies have demonstrated that the process for sterilizing the filter does not affect the integrity test value of the filter.

Postfiltration integrity testing should be conducted to ensure that the filter was not damaged during the filtration process.

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## PREFILTRATION BIOBURDEN CONTROL

The bioburden removal capability depends on the available filter retention capacity, which is a function of the inherent bioburden load present in the entire volume of the liquid to be filtered and the effective filter surface area. Studies have demonstrated that microbial retention in sterilizing filtration is a function of the upstream bioburden *(3,15,16)*. Also, nonviable particulate matter that may be present in the solution can influence the retentive capacity of the filter *(17)*. Therefore, the prefiltration bioburden and particulate levels of the solution should be minimized and controlled before the final sterilizing filtration step.

Various filter configurations and processes can be used to control the bioburden and nonviable particulate levels presented to the final, sterilizing-grade filter. One configuration is a multifilter arrangement that consists of two sterilizing-grade filters (or a bioburden reduction filter followed by a sterilizing-grade filter) connected in series (*Figure 2*).

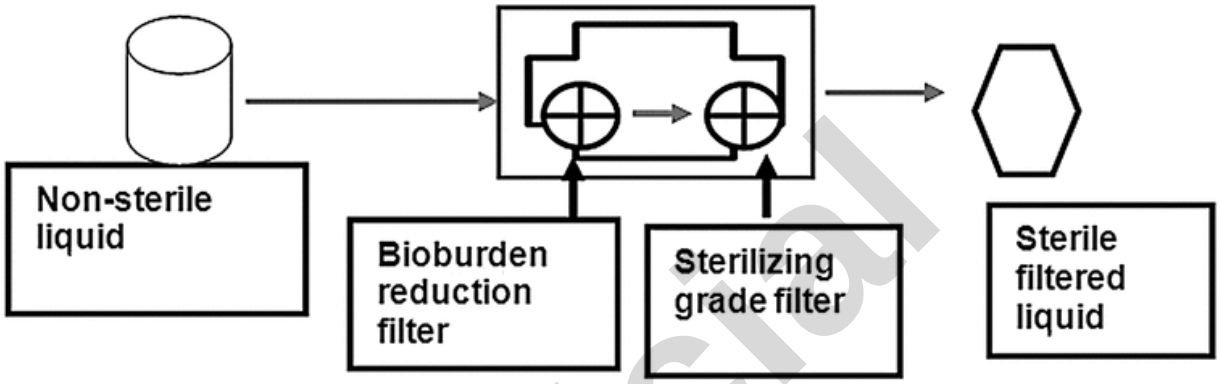


Figure 2. Multifilter configuration to control bioburden and nonviable particulates.

Another configuration appropriate for prefiltration bioburden control uses two filtration steps separated in time: the liquid is sterile-filtered into a sterilized tank, where it is then held before a final, sterilizing filtration (*Figure 3*).

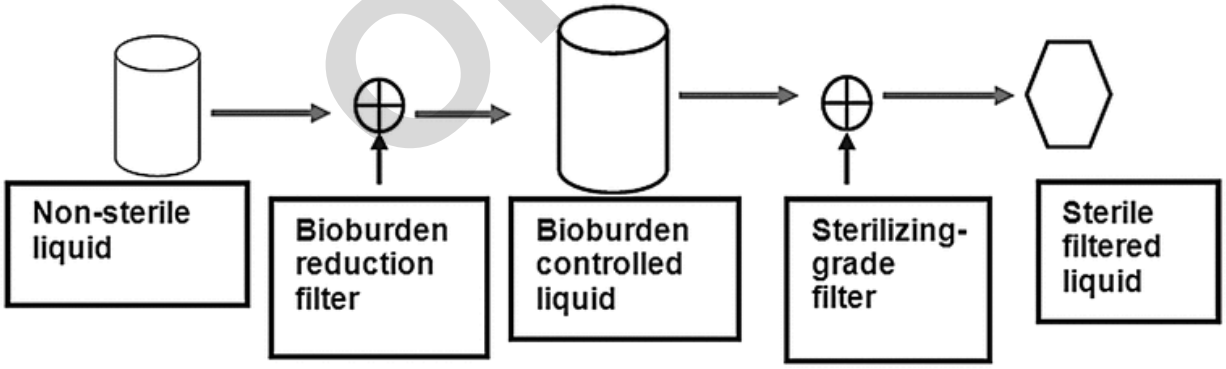


Figure 3. Bioburden control using two filtration steps.

In each of these scenarios, the bioburden and particulate levels presented to the final, sterilizing-grade filter are low and are controlled by prefiltration.

When the process results in a consistently low and controlled prefiltration bioburden, use of a single sterilizing-grade filter is appropriate.

Irrespective of the strategy employed, validation studies should demonstrate the capability to consistently achieve the requisite levels of prefiltration bioburden and particulate level reduction and control.

## RESPONSIBILITIES

### Filter Manufacturer

The filter manufacturer is responsible for ensuring that the filter production process has been validated and is well controlled and that the sterilizing-grade filters meet the requirements of ASTM F838-05 (2013). The filter manufacturer determines the

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integrity test specification for the filters, usually adding a safety factor to ensure that each filter will meet that specification. The filter manufacturer conducts extractable and leachable studies to ensure that the filter does not release objectionable levels of these materials into the solvent systems typically employed in pharmaceutical manufacturing. The filter manufacturer conducts cleanliness tests to assure that the filter does not adversely affect the USP particulate requirements of the product. The filter manufacturer provides technical support and troubleshooting advice if the filter user encounters a problem.

### Filter User

The filter user is responsible for establishing microbial retention at the validated integrity test value, establishing microbial retention in the liquid to be filtered, and validating the use and sterilization of the filter and housing. The filter user is responsible for determining that the filter is not additive or extractive to the extent that the filtered liquid is adversely affected.

## TROUBLESHOOTING

Failure of a filter to pass an integrity test may mean that the filter is damaged, is improperly sealed in the housing, is incompletely wetted, or is nonintegral. It also could mean that the filter is incorrectly labeled (e.g., has the wrong pore size) or that the integrity test apparatus has been improperly set up or calibrated.

The cause of an integrity test failure can be determined by evaluating the test setup, test parameters, wetting fluid, and wetting procedure; ensuring that the system is leak-free and the temperature is constant; and ensuring that the test equipment has been properly calibrated.

If the cause of the failure cannot be determined, analysts can rewet the filter and repeat the integrity test, increasing flush time, flush volume, and pressure differential. It may be beneficial to use a lower surface tension reference fluid (e.g., 70% isopropyl alcohol). If the filter fails the integrity test using the reference fluid, it should be considered nonintegral. In addition, the filter can be returned to the manufacturer for a full analysis to further elucidate the cause of the integrity test failure.

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# ⟨1229.5⟩ BIOLOGICAL INDICATORS FOR STERILIZATION

## INTRODUCTION

A biological indicator (BI) is a well-characterized preparation of a specific microorganism that has known resistance to a specific sterilization process.[1] BIs are used to demonstrate the effectiveness of processes that render a product sterile in its final package or container, as well as the effectiveness of the sterilization of equipment, product contact materials, and packaging components as required. BIs may also be used to monitor established sterilization cycles and are used for periodic reassessment of sterilization process effectiveness. BIs are process aids and can support the correlation of physical parameters to microbiological destruction. Microorganisms recognized as suitable for BIs are spore-forming bacteria; the spores of these microorganisms are significantly more resistant than the vegetative cells that comprise the majority of bioburden in or on materials.

## PROPER USE OF BIOLOGICAL INDICATORS

BIs provide microbiological evidence of process effectiveness that should be correlated to physical measurements (see *Sterilization of Compendial Articles* ⟨1229⟩). Microbiological resistance to sterilization varies with the physical conditions; however, there are no established means for accurately predicting microbial destruction based solely on physical measurements, with the exception of radiation sterilization. BI placement locations within or on materials to be sterilized are chosen to confirm that the desired sterilizing conditions have been attained.

BIs are typically spore-forming bacteria of the genera *Geobacillus*, *Bacillus*, and *Clostridium*. Preference in BI selection should be given to well-characterized strains.

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## RESPONSIBILITIES

### BI Manufacturer's Responsibility

The responsibility for determining the performance characteristics of each BI lot resides with the BI manufacturer. The manufacturer should provide, with each lot of BIs, a certificate of analysis that attests to the validity of BI performance claims. The manufacturer should provide information concerning the microbial population and resistance (*D*- and *z*-values, respectively, where appropriate) as well as storage and expiry information. The BI manufacturer ▲should▲ (USP 1-Aug-2022) include survival ▲and▲ (USP 1-Aug-2022) kill times for the BI in their documentation ▲and this should be verified by the end user.▲ (USP 1-Aug-2022) The resistance of the BI should be determined by the manufacturer under defined conditions. The manufacturer should provide directions for use, including the medium and conditions used for the recovery of microorganisms after exposure to the sterilization process. Disposal instructions also should be provided by the manufacturer of the BI.

### BI User's Responsibility

When BIs are purchased, their suitability for use in a specific sterilization process must be established. The BI user should obtain a certificate of analysis for each lot of BIs and verify the manufacturer's label claims for spore population (see *Biological Indicators—Resistance Performance Tests* ⟨55⟩). When a BI is used in accordance with the BI manufacturer's directions, the resistance of the BI need not be reconfirmed.

### User-Prepared Biological Indicators

A user of BIs may elect to propagate spore crops of a single species for use as a suspension. Alternatively, these spore suspensions may be purchased from a BI manufacturer. When liquid suspensions are applied to a substrate, it is the user's responsibility to determine the population and resistance of the microorganism used. The resistance determined for liquid suspensions relates only to other lots of the same suspension and is not representative of how that microorganism will perform on a substrate or in a different suspending medium. In these circumstances, the BI resistance and population should be re-established (see ⟨55⟩).

## CHARACTERIZATION OF BIOLOGICAL INDICATORS

The use of BIs should include procedures for their acceptance and control. The following elements outline the major considerations. Resistance performance is addressed separately in ⟨55⟩, which provides methods for evaluating BI resistance.

[1] Microbial retention challenges as described in *Sterilizing Filtration of Liquids* ⟨1229.4⟩ are not BIs.

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## Packaging and Storage

Store under the conditions recommended on the label or under validated conditions, and protect from light, toxic substances, excessive heat, and moisture.

## Expiration Date

Use within the BI's labeled or determined expiration date.

## Identification

Where identification of the BI species is deemed necessary, as in the course of an investigation into unusual results, use either a phenotypic or genotypic identification method (see *Microbial Characterization, Identification, and Strain Typing* ⟨1113⟩ for additional information).

## Purity

By examination of the colonies derived from the spores on a suitable plate culture medium, determine that there is no evidence of contamination with other microorganisms.

## Disposal

Prior to discarding used spores, sterilize using a method recommended by the BI manufacturer or other equivalent means.

# TYPES OF BIOLOGICAL INDICATORS

A BI is a well-characterized preparation of a specific bacterial spore of known resistance to a specific sterilization process. Some BIs may contain two different species and concentrations of bacterial spores for use in the evaluation of two different sterilization processes.

One form of BI preparation includes spores that are placed on a carrier (e.g., a disk or strip of paper, glass, plastic, metal, or other material) and may be packaged to maintain the integrity and viability of the spores inoculated onto the carrier. The carrier and primary packaging should not be damaged or degraded by the specific sterilization process. Another preparation of BIs is a spore suspension that is inoculated on or into representative units of the article to be sterilized. A surrogate article may be used if it is not practical to inoculate the actual article. A surrogate article is a preparation that differs in one or more ways from the actual article but performs as the actual article during cycle development, validation, and routine use. The physical design of actual or surrogate articles can affect the resistance of spore suspensions that are inoculated on or into an article (see ⟨55⟩). In the case of liquid inoculated products, it is essential to determine the population, *D*-value (and, in terminal sterilization applications, *z*-value) of the relevant BI spore in the liquid product, and any simulated product substrate (if utilized).

A third form of BI is a sealed system that includes the growth medium (either in direct contact with the BI during the sterilization or placed in contact with the BI after sterilization) for recovery of process-exposed BI microorganisms. Some BI systems may contain a growth indicator or sensor in addition to growth media.

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# SELECTION FOR SPECIFIC STERILIZATION PROCESSES

The selection of a BI requires knowledge of the resistance of the BI system to the specific sterilization process. It must be established that the BI system provides a challenge to the sterilization process greater than the resistance of the native bioburden. The recommendations for BI with each sterilization process are not exclusive; they represent only the more common choices.

## Steam Sterilization by Direct Contact

For steam sterilization by direct contact, the commonly used BI contains spores of *G. stearothermophilus* (ATCC 12980 or ATCC 7953), a thermophilic microorganism with a moist heat resistance substantially greater than that of most vegetative microorganisms (see *Steam Sterilization by Direct Contact* ⟨1229.1⟩).

## Moist Heat Sterilization of Aqueous Liquids

Heat-resistant spore-forming microorganisms such as *C. sporogenes* (ATCC 7955), *B. subtilis* (ATCC 35021), or *B. atrophaeus* (ATCC 9372) are used. *B. subtilis*, *B. atrophaeus*, and *C. sporogenes* are preferred for use in sterilization of aqueous solutions or where their lower thermal resistance is more appropriate.

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## Dry Heat Sterilization

For dry heat sterilization, spores of *B. atrophaeus* (ATCC 9372) are typically used (see *Dry Heat Sterilization* ⟨1229.8⟩). Where dry heat depyrogenation has been demonstrated (*Dry Heat Depyrogenation* ⟨1228.1⟩), sterilization by dry heat need not be confirmed, and a BI is not required. The elevated temperatures required to depyrogenate materials are more than sufficient to sterilize the materials at the same time.

## Ionizing Radiation

The use of a resistant BI is unnecessary for the evaluation of radiation sterilization processes. Dose setting involves the evaluation of preirradiation bioburden as well as dosimetric evaluation and allied tests as defined in ISO 11137-1, ISO 11137-2, and ISO 11137-3 ▲(*1–3*),▲ (USP 1-Aug-2022) as well as in *Radiation Sterilization* ⟨1229.10⟩.

## Gas Sterilization

For ethylene oxide sterilization, spores of *B. atrophaeus* are commonly used. For other gaseous agents, spores of *G. stearothermophilus* or *B. atrophaeus* are commonly used (see *Gaseous Sterilization* ⟨1229.7⟩).

## Chemical Sterilization

The sterilization of items using a liquid sterilant is accomplished using spores of an appropriate strain such as *B. atrophaeus*, *B. subtilis*, or other appropriate spore-forming species, as determined by the user. Whichever strain is chosen for this purpose should have greater resistance than does the bioburden.

## Vapor Phase Sterilization

The biphasic nature of these materials precludes the accurate determination of specific lethal conditions (for establishment of *D*-values, see *Vapor Phase Sterilization* ⟨1229.11⟩). BIs using either *G. stearothermophilus* or *B. atrophaeus* have been utilized in the evaluation of these processes.

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# ⟨1229.6⟩ LIQUID-PHASE STERILIZATION

## INTRODUCTION

Microorganisms are subject to destruction in a variety of ways. Aside from the classical methods of steam, dry heat, and radiation, destructive sterilization may also occur by immersion in a chemical solution. This is termed liquid-phase sterilization *(1)*. A number of chemical agents, such as aldehydes, acids, bases, and strong oxidants in solution, under the appropriate conditions, are capable of destroying bacteria and fungi, including both vegetative cells and spores in a quantitative fashion *(2,3)*. Objects to be sterilized are immersed in the solution of the chemical agent, after which the agent must be removed in a manner that preserves the sterilized object from recontamination. Removal of the chemical sterilant from the exposed surfaces that have been sterilized must be accomplished in a manner that maintains the sterility of the item postprocessing. Recontamination falls outside the scope of usual consideration for sterilization processes. However, in liquid chemical sterilization it is customary to include the agent's removal (whether this is accomplished by physical or chemical means) in the overall process, together with any needed additional steps to avoid recontamination.

A substantial number of liquids in aqueous solution are capable of sterilizing articles during immersion. Examples include the following:

- Aldehydes—glutaraldehyde, formaldehyde
- Acids—peracetic acid, nitric acid, sulfuric acid
- Bases—sodium hydroxide, potassium hydroxide
- Oxygenating compounds—hydrogen peroxide, ozone, chlorine dioxide
- Halides—sodium hypochlorite, chlorine

As is the case for gas sterilization, the effectiveness of chemical sterilants varies with concentration and temperature. Other factors that affect antimicrobial activity include pH, extent of mixing (if present), and presence of cellular or other debris. Because of the limited number of variables, process control for sterilization by liquids is relatively simple.

Because there are no widely accepted biological indicators for sterilization by liquids, the use of a common mesophilic sporeformer such as *Bacillus atrophaeus* or *B. subtilis* is commonplace because these are the likely worst-case bioburden isolates.

The agents used for sterilization by liquids vary with respect to sterilant stability, effective pH range, concentration, temperature, contact time required, and potential interaction with the materials. When selecting the most appropriate sterilant, manufacturers must consider its effect on the materials, package components, and equipment, as is the case with all other sterilizing processes. The variety of agents, process diversity, and potential applications preclude a material-by-material review of these agents in this chapter. Manufacturers should note that these agents are highly toxic, and appropriate safety measures should be practiced at all times during cycle development, validation, and routine operation.

## VALIDATION OF STERILIZATION BY LIQUIDS

Experimental evidence has shown that first-order kinetics is appropriate for microbial destruction, which makes validation a simple exercise. The validation of sterilization by liquids can be accomplished using either the half-cycle approach or the bracketing method.

### Half-Cycle Approach

The half-cycle approach described below is a modification of the method described in *Gaseous Sterilization* ⟨1229.7⟩ (see *Figure 1*). It is a method that requires the destruction of a resistant microorganism at defined lethal conditions. In routine operation, the process dwell period is arbitrarily doubled and supports a theoretical reduction of the biological indicator (and thus the bioburden) to a probability of a nonsterile unit of at least $10^{-6}$ for a full cycle.

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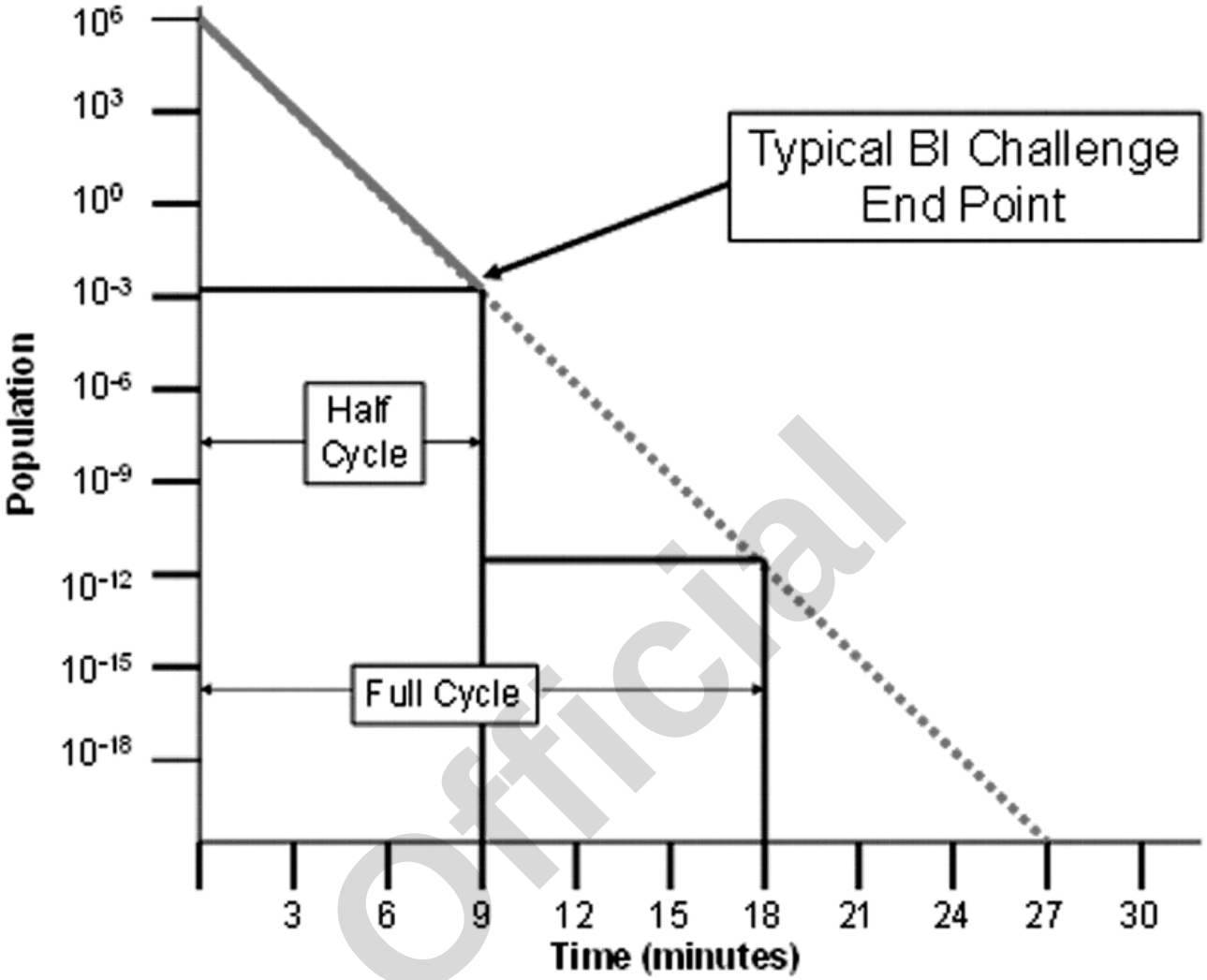


Figure 1. Half-cycle sterilization validation.

The half-cycle method originally was used for ethylene oxide sterilization when the relationship between the microorganisms and the delivered process parameters was less certain.

## Bracketing Approach

In this method (see *Figure 2*), analysts evaluate conditions of concentration and temperature that bracket the defined process condition to support both over- and under-treatment of the materials and bioburden, respectively. Users can establish the death rate for the microbial population for each of the conditions that bracket the routine process.

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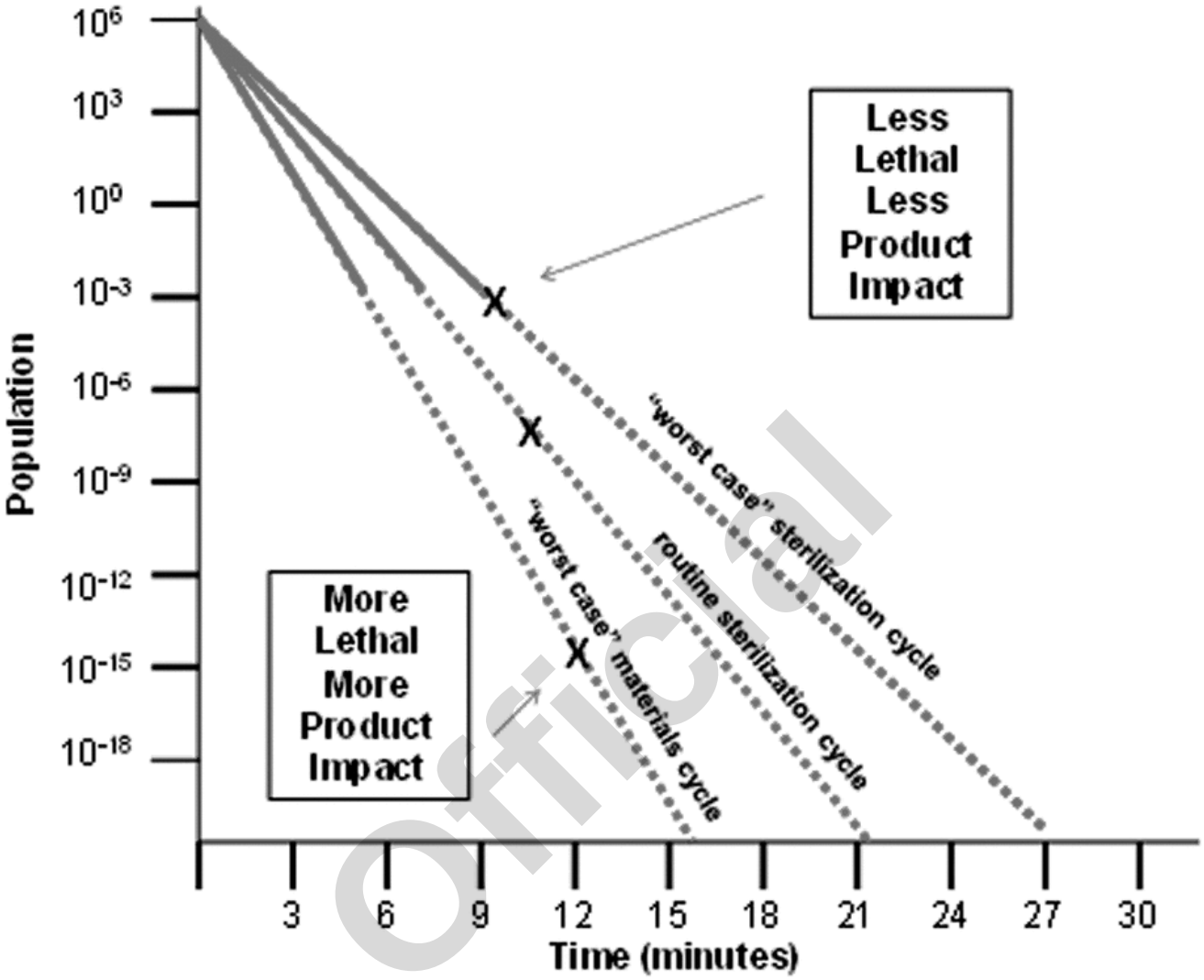


Figure 2. Bracketing method.

Regardless of the approach used, in order to complete the cycle development and validation manufacturers must identify a rapid neutralization method that inactivates the chemical agent to allow microbial quantification after fractional kill exposure. The exposure periods may need to be brief because many of these agents have rapid kill rates.

## Equipment Qualification

Equipment qualification is a predefined program that examines the equipment to confirm that it has been properly installed and operates as intended before the sterilization process. This activity for sterilization by liquid chemicals is simple, because the equipment used is rarely complex. Temperature control and agitation/recirculation rates are the essential considerations.

## Component and Load Definition

Sterilization by liquids is a surface phenomenon, and all surfaces of the materials must be immersed in the sterilant. Treatment uniformity can be ensured by recirculation or mixing of the sterilant during the process. Penetration into needle lumens, closely fitted parts, and porous materials should be confirmed. The use of a maximum load per defined vessel or container represents the worst case because it provides the maximum surface area to be sterilized.

## Biological Indicators

The common indicator organisms for chemical sterilization are *B. atrophaeus* ATCC 9372 or *B. subtilis* ATCC 6633. The spore challenge is inoculated directly onto the items. End users should determine the populations of inoculated items. Manufacturers should place indicators within loads at locations believed to be hardest for the agent to reach, on the basis of visual examination.

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## Process Confirmation/Microbiological Challenge

The core of the validation activity is confirmation of acceptable process parameters and inactivation of the microbial challenge. The end user should expect a linear death curve for the spore challenge and require total death of the challenge. The end user can consider adjustment in chemical sterilant concentration, process time, agitation, and other factors. Proof of cycle efficacy is provided in replicate studies in which the biological indicators are killed, and physical measurements are taken as documentation.

## Agent Neutralization/Removal

After exposure, the sterilizing agent must be adequately removed from the items or neutralized before further processing. This segment of the process uses chemical neutralization or physical removal and must be executed in a manner that preserves the sterility of the items. Aseptic processing with appropriate capability demonstration should be provided. Process simulation beginning with the completion of sterilization through placement into a sealed sterile container is expected. See *Sterility Assurance* ⟨1211⟩ for additional information.

## Routine Process Control

Liquid-phase sterilization must be subject to controls that maintain the validated state. The practices outlined in *Sterilization of Compendial Articles* ⟨1229⟩ address the general requirements for all sterilization systems. Sterilization is accomplished by means of a number of related practices that are essential for continued use of the process over an extended period of time, including calibration, chemical and physical measurements, ongoing process control, change control, preventive maintenance, periodic reassessment, and training.

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# ⟨1229.7⟩ GASEOUS STERILIZATION

## INTRODUCTION

The use of sterilizing gases for the preparation of materials and equipment is commonly used for items that are susceptible to damage by heat or radiation processes. Many polymeric materials, especially medical devices, are surface sterilized in this manner, as is nonpressure-rated process equipment. The sterilization of dry powders using gases is inappropriate due to the inability of gases to penetrate solid materials. The majority of gas sterilization processes employ ethylene oxide (EO), and procedures for use with other gases generally are patterned after EO practices. Ozone, mixed oxides of nitrogen, and chlorine dioxide are some of the other gaseous sterilants used. [Systems that can exist in liquid and gas phase at the operating temperatures (e.g., hydrogen peroxide, peracetic acid, and paraformaldehyde) are excluded from consideration in this chapter.] EO's ability to penetrate through polymers, cellulosics, and other materials allows it to be used for the terminal sterilization of medical devices in their final packaging. The other sterilizing gases may be suitable for similar applications.

Process control for gas sterilization equipment is accomplished by control of sterilant gas concentration, relative humidity, temperature, and system pressure. Mixing of the gas in the sterilization chamber may be beneficial. EO sterilization may be used for parametric release as described in *Terminally Sterilized Pharmaceutical Products—Parametric Release* ⟨1222⟩.

Gas sterilization differs markedly from processes during which the agent used can condense during the operation. Vapor sterilization processes will be addressed separately in *Vapor-Phase Sterilization* ⟨1229.11⟩.

As outlined in *Sterilization of Compendial Articles* ⟨1229⟩, analysts must take care in ensuring sterility and demonstrating that the essential quality attributes of the materials are not adversely affected by the process. With respect to gas processes, key considerations include the immediate effects of sterilizing gas on the materials or equipment being sterilized, residual sterilant, sterilant byproducts, and potential chemical reactions. The common gas processes differ slightly with respect to process execution and material concerns and thus are described individually.

## ETHYLENE OXIDE

EO is a powerful alkylating agent that destroys microorganisms by chemical reaction, primarily with cell DNA. The destructive mechanism largely follows first-order kinetics and depends on concentration, humidity, and temperature. The use of EO for medical devices in their final packaging has, to a large extent, shaped EO sterilization processes (and, to a lesser extent, all gas sterilization) for other applications (*2,3*). The usual EO process follows a sequence of prehumidification, air removal, rehumidification in the chamber, gas exposure, gas removal from the chamber, and postexposure aeration. The preexposure steps ensure that adequate moisture is present on and within the items being sterilized. The postexposure steps provide time for the diffusion of EO and its byproducts out of the materials and packaging. When EO is used for nonporous equipment the process can be streamlined, which eliminates many of the pre- and postexposure steps because of the need only for surface sterilization. During EO sterilization the gas is introduced at the beginning, and only minimal additions are necessary later to maintain pressure as the gas is absorbed into the material/sterlization load within the vessel. Humidity adjustment during the process also may be required. In some instances, EO reacts with materials in the load to form ethylene chlorohydrin and ethylene glycol. These compounds, including EO, must be reduced to safe levels before the items can be used by patients (*4*,*5*). EO processing requires strict worker safety and environmental controls because it is associated with carcinogenicity, mutagenicity, and neurotoxicity. In addition, EO is explosive in concentrations of greater than 2.6% by volume in air, therefore, inert gases are often used to minimize flammability. The commonly accepted biological indicator (BI) strain is *Bacillus atrophaeus* (formerly *B. subtilis* var. *niger*).

## OZONE

Ozone is a potent oxidizing agent produced by passing a stream of oxygen or air through a high-voltage electrical field. Ozone is an effective biocidal agent for treatment of water supplies and has demonstrated lethality at concentrations from 2%–10% in air. Optimal microbial destruction is accomplished when the relative humidity is above 80% at room temperature. Ozone degrades to oxygen in the presence of moisture and metals and therefore usually is generated in situ. Ozone does not penetrate porous materials to the same extent as EO does. Process systems that use ozone for gas sterilization have the advantage of simplicity. Its generation and destruction (using a catalytic converter) are accomplished without moving parts or consumables other than the supplied oxygen. The sterilization process uses a sequence of humidification, injection, exposure, and ventilation to remove the ozone from the chamber at the end of the cycle. The common BIs identified for ozone are *Geobacillus stearothermophilus* and *Bacillus atrophaeus*.

## CHLORINE DIOXIDE

Chlorine dioxide is an effective sterilizing gas. Pure chlorine dioxide is metastable and therefore is generated as needed. Chlorine dioxide is noncarcinogenic, nonflammable, and effective at ambient temperatures. Its ability to penetrate materials may be less than that of EO.

A typical chlorine dioxide sterilization process uses a sequence of preconditioning, conditioning dwell period, charge, and exposure, followed by aeration.

The BI most commonly used is *Bacillus atrophaeus*.

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## NITROGEN DIOXIDE

Nitrogen dioxide is a sterilizing gas effective at ambient temperature. Liquid nitrogen dioxide is converted to a gas on introduction to the target chamber. Nitrogen dioxide is nonexplosive and its residues are noncarcinogenic, noncytotoxic, and nonteratogenic. It has a limited ability to penetrate polymeric materials in comparison to EO, which makes postcycle aeration more rapid. It is incompatible with cellulosic materials such as paper and cardboard. The suggested BIs for nitrogen dioxide are *G. stearothermophilus* and *B. atrophaeus*.

## VALIDATION OF GAS STERILIZATION

The validation of gaseous sterilization generally begins with the establishing of a "minimum lethal process dwell time" through the use of fractional exposure studies. These fractional studies establish that exposure time, under standard process conditions, where the biological indicator is fully inactivated. This minimum exposure time then becomes the basis for the application of the half-cycle approach for validating the sterilization cycle. The absence of information relating the effect of varying gas concentration, humidity, and temperature on microorganisms resulted in a conservative assumption that the bioburden is equal in antimicrobial resistance and population to that of the biological indicator. The half-cycle method can be defined as follows.

The half-cycle validation method requires the destruction of a high concentration (NLT $10^6$ spores) of a resistant microorganism under defined, minimum conditions for complete kill. This establishes the minimum lethal process dwell time. In routine operation, the process dwell period is arbitrarily doubled and supports a theoretical reduction of the biological indicator (and thus the bioburden) to a probability of a nonsterile unit (PNSU) of $10^{-6}$ (for definitions of terms in this chapter, see *Sterilization of Compendial Articles* ⟨1229⟩).

The half-cycle method used for gas sterilization is shown in *Figure 1*.

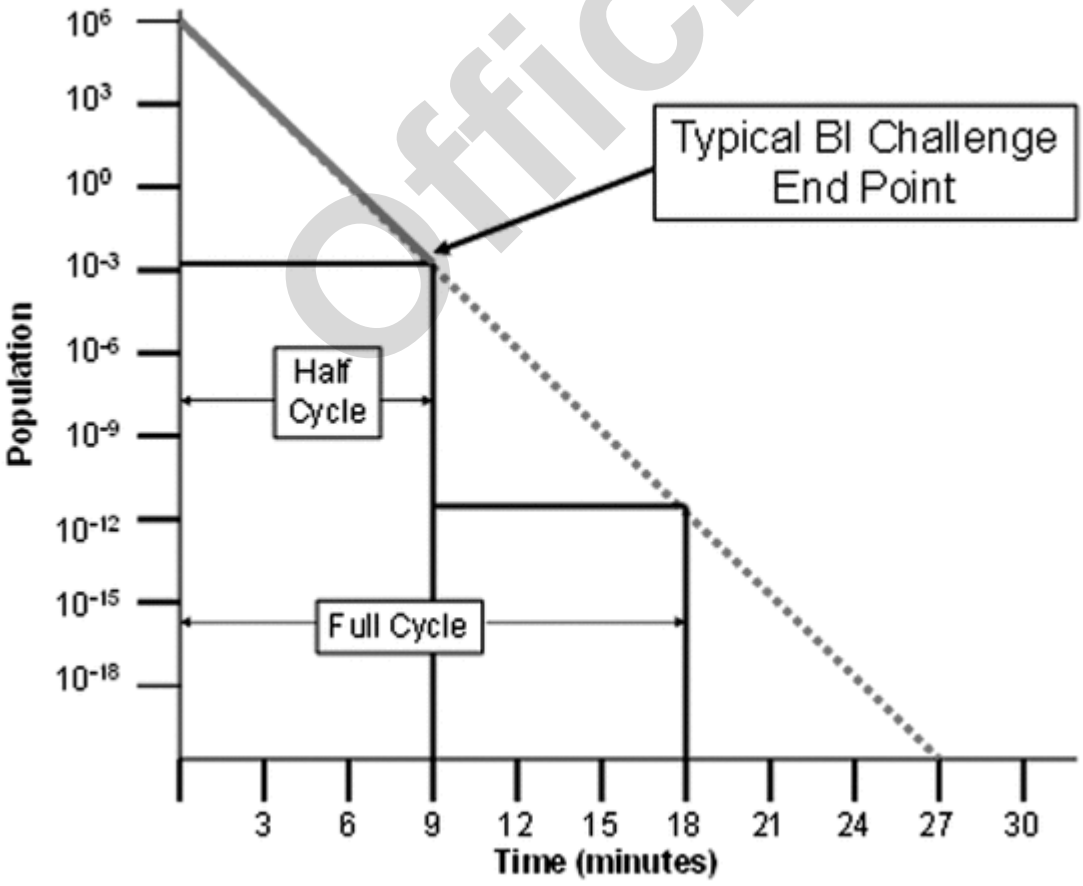


Figure 1. Half-cycle sterilization validation.

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Alternative approaches to cycle validation are available. Gillis and Mosley developed a means for parametric evaluation of EO sterilizing conditions that may result in greater use of other validation methods (*6*). A bracketing approach (see *Figure 2*) that better supports the process operating ranges for the critical parameters relative to the half-cycle method has also been used. In the bracketing method, one evaluates conditions that bracket the defined process condition in order to establish parameters for the minimum and maximum effects on the materials and bioburden. The minimum lethal process dwell time (see half-cycle description above) establishes the worst case for microbial kill. Incremental increases in process dwell time beyond the minimum lethal process dwell time are used to establish the routine and maximum exposure periods, the latter of which imparts the greatest effect on materials. In addition, adjustments to agent concentration and relative humidity are utilized to further enhance the bracketing approach. By this method, the routine process conditions may be established between the minimum and maximum process conditions to assure complete microbial kill while maintaining the integrity of the materials.

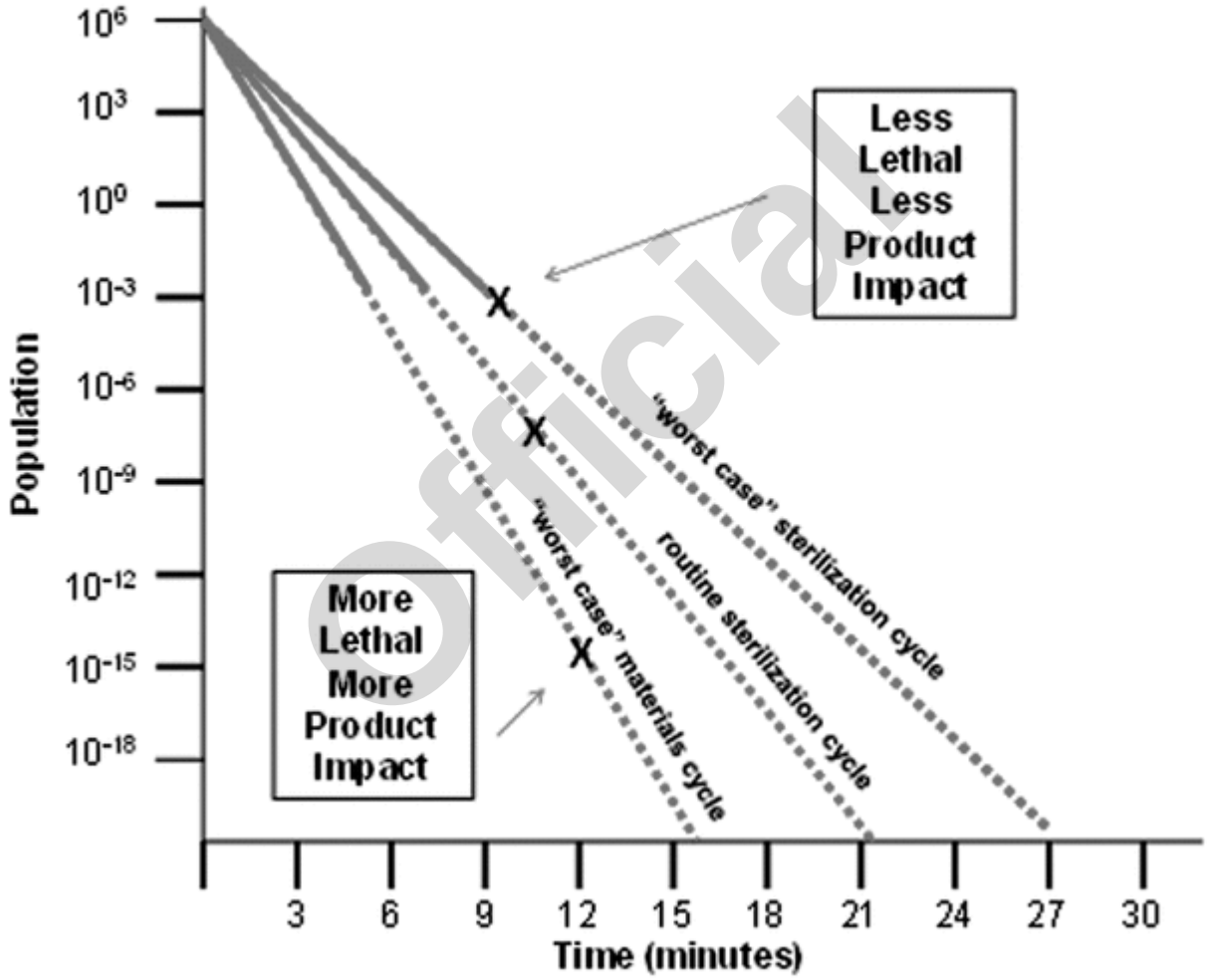


Figure 2. Bracketing method.

## Equipment Qualification

The equipment qualification for gas sterilization should include both pre- and postcycle systems to confirm that the equipment has been properly installed and operates as intended.

## Empty Chamber Parameter Distribution

Despite the use of true gases, evaluation of parameter uniformity across the chamber is a common activity. This ensures that the gas and humidity introduction methods provide consistency throughout the chamber and can be correlated to the routine monitoring location(s), when present. Biological indicators are not required in the evaluation of the empty chamber uniformity.

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### Component and Load Mapping

Component and load mapping using invasive sampling are not a part of gas sterilization because sampling systems placed within the load items would alter gas and humidity penetration. Evaluation of lethal conditions with individual items and across loading patterns is best provided by biological indicators or process challenge devices placed within the load items and distributed within the load. Indicators or process control devices are placed within the items and load at locations believed to be hardest for the gas and humidity to penetrate.

### Biological Indicators

The biological indicator of choice for gas sterilization varies, as noted above. *B. atrophaeus* (ATCC 9372) is used with EO and chlorine dioxide, and ozone sterilization is monitored with *G. stearothermophilus* (ATCC 12980 or 7953). D-values for the biological indicator can be used to establish exposure periods for the sterilization process to ensure adequate process efficacy. When positioning biological indicators within items it is important to ensure that the placement of the BI does not occlude gas passage or otherwise interfere with the distribution/penetration of the sterilant within the item.

### Process Confirmation and Microbiological Challenge

The core of the validation activity is the confirmation of acceptable process parameters with simultaneous physical and chemical measurement and microbial challenge. Sensors are placed in the chamber, or biological indicators are positioned within the load items. Proof of cycle efficacy is provided in replicate studies in which the biological indicators are killed and the physical measurements correspond to the expected values.

## ROUTINE PROCESS CONTROL

Gas sterilization is subject to formal controls that maintain a validated state over time. The practices outlined in ⟨1229⟩ include the general requirements appropriate for all sterilization systems. Sterilization is accomplished by a number of related practices that are essential for continued use of the process over an extended period of time. The essential practices to maintain validated status include calibration, physical measurements, ongoing process control, change control, preventive maintenance, periodic reassessment, and training. When parametric release has not been established, biological indicators positioned within the load are used for routine release of each sterilization load, along with a review of documentation from the sterilizer control system.

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# ⟨1229.8⟩ DRY HEAT STERILIZATION

Dry heat sterilization is a process utilized for heat-stable items (glass, stainless steel, nonaqueous liquids, powders, etc.) that are unsuited for steam sterilization because of either an absence of water (nonaqueous liquids and powders) or requirements for absolute dryness following processing (product contact parts for nonaqueous products). Because dry heat relies on air for the transfer of heat to and from the load items, the process takes longer than a steam process for a comparable size item or load. Lengthy heating and cooling periods require that the load items be unaffected by heat over a long period of time and also require the use of the overkill method for cycle development and validation.

Dry heat sterilization is typically performed in the range of 160°–190° where the objective is sterilization rather than depyrogenation. (Depyrogenation will be covered separately in *Dry Heat Depyrogenation* ⟨1228.1⟩). In dry heat sterilization, hot air is in direct contact with the load items (whether wrapped or unwrapped) and transfers some of its thermal energy. Unlike steam sterilization, in dry heat sterilization there is no phase change of the heating medium, and thus heat transfer is less efficient. The items can be stainless steel, glass, ceramic, or other heat-stable materials and may be wrapped or covered with aluminum foil to protect them during pre- and postprocess handling. Dry heat sterilization is commonly used for heat-stable materials (e.g., petrolatum or powders).

The limited heat transfer capacity of air requires that items in the oven be placed in locations that were confirmed to be acceptable during the validation effort. Manufacturers should exercise caution with varying load sizes because in some instances (resulting from system design and control probe positioning) minimum load sizes may present a worst case.

## STERILIZATION CYCLE CONTROL

Process equipment for dry heat sterilization is controlled by calibrated temperature sensors. During the exposure portions of the cycle, attainment of a minimum dwell time at a predefined temperature is used to document process lethality. Cycle efficacy for dry heat sterilization customarily is measured using $F_H$, which typically is defined as the amount of time the load receives the equivalent of exposure at 170°. The $F_H$ approach is used to compare sterilization processes that operate at varying temperature conditions to a single standard. The process lethality at temperatures other than 170° can be calculated to determine lethality equivalent to that provided at 170°. Sterilizer control systems must deliver conditions within a predefined time–temperature or $F_H$ range. Simple mathematics can be used to calculate the total lethality over the course of the process. For the specific reference temperature of 170° and a *z*-value (for definitions see *Steam Sterilization by Direct Contact* ⟨1229.1⟩) of 20°, the $F_H$ calculation can be determined by the following equation:

$$F_H = \int_{t_1}^{t_2} 10^{\left(\frac{T-170}{20}\right)} dt = \sum_{t_1}^{t_2} 10^{\left(\frac{T-170}{20}\right)} \Delta t$$

$F_H$ = accumulated lethality
$t_2$ = end time
$t_1$ = start time
$T$ = temperature

Accumulation of the lethality ($F_H$) for the sterilization process across the entire cycle (heat-up and cool-down segments included) includes the contribution of those segments and allows the cycle to be defined by a targeted lethality rather than by a time at a defined minimum temperature.

## VALIDATION OF DRY HEAT STERILIZATION

Because dry air has limited heat capacity and dry heat conditions are more variable than those encountered with other thermal sterilization methods, analysts routinely validate their dry heat sterilization procedures using the overkill method as defined in *Sterilization of Compendial Articles* ⟨1229⟩.

Overkill sterilization can be defined as a method in which the destruction of a high concentration of a resistant microorganism supports the destruction of reasonably anticipated bioburden that could be present during routine processing. That objective can be demonstrated by attaining any of the following: a defined minimum lethality; a defined set of method conditions; or confirmation of minimum log reduction of a resistant biological indicator.

The validation requirements for the overkill method are less onerous than those of the other sterilization approaches. Because the load items can withstand substantial amounts of heat without adverse consequence, the greater lethality provided by the overkill method clearly is justifiable.

### Equipment Qualification

Equipment qualification is a predefined program that focuses on the sterilizing equipment to confirm that it has been properly installed and operates as intended before evaluation of the sterilization process. In some companies, equipment qualification is separated into installation qualification and operational qualification or is lumped together under a joint terminology of installation/operational qualification. The major use of qualification of the sterilizing equipment is to provide a baseline for preventive maintenance and change control, ensuring reproducibility of operation over time and assurance that the sterilization process is constantly and accurately performed.

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## Empty Chamber Temperature Distribution

The equipment should be evaluated for empty chamber temperature distribution. The oven or tunnel should be evaluated to determine the range of temperatures within the system, and the cycle parameters should be determined to ensure adequate lethality across the expected load. The acceptance criteria for empty chambers can vary with the equipment capabilities and customary use, but it is typically less uniform than observed in steam sterilizers. Biological indicators are not required during the evaluation of empty chamber temperature distribution.

## Component Mapping

Load items that are complex and feature enclosed volumes and product contact surfaces should be subjected to component mapping to determine internal cold spots. This is particularly important in powder sterilization. For each load item, manufacturers should establish the ability of heat to penetrate the items or containers and to bring them to the required temperature. These studies can be performed in a laboratory setting and need not be repeated when the same item is sterilized in other equipment. Thermocouples should be placed into direct contact with the item(s) being evaluated. During component mapping load items should be prepared and oriented in a manner that is consistent with how they will be processed.

## Load Mapping

Fixed loading patterns for dry heat sterilization in batch ovens are preferable because the limited heat capacity of the air allows substantial temperature differences across the load. It may be possible to validate maximum and minimum loads as determined by either the number of items or their mass within the oven. Loading in a continuous tunnel process is typically well defined by the limitations of the conveying system. Load and component mapping ensures that all load items attain the required temperature. Information from the load mapping is used to adjust cycle timing to ensure appropriate lethality. System control must consider the relationship between load position and size relative to temperature control locations.

## Biological Indicators

The biological indicator (BI) for dry heat sterilization is *Bacillus atrophaeus* (ATCC 9372), a thermophilic spore-former with high resistance to dry heat. The spore challenge is placed on a substrate positioned within the load or on a load item. If spores are used as intended by the BI manufacturer, the population and resistance information provided by the vendor can be used. End users should determine the population and resistance of their biological indicator used when inoculating their own items.

## Heat Penetration and Microbiological Challenge

The core of the validation activity is the confirmation of acceptable heat penetration using temperature measurements and microbial challenges. Thermocouples and BIs are placed within the load items at the locations determined during the component and load mapping to present the worst case. Thermocouples should be placed into direct contact with the item(s) being monitored. Proof of cycle efficacy is provided by replicate studies in which the BIs are killed and the physical measurements correspond to the expected values of time–temperature or $F_H$. If the microbial and physical measurements do not correlate, manufacturers should conduct an investigation and should take corrective action to rectify the discrepancy. This study customarily is performed slightly subminimal to the lower specification limits for time, temperature, and/or cumulative lethality.

# ROUTINE PROCESS CONTROL

As with all sterilization processes, after the dry heat sterilization process has been validated, it must be subject to formalized controls that keep it in a validated state over time. General chapter *Sterilization of Compendial Articles* ⟨1229⟩ details the general practices that are appropriate for all sterilization systems. This is accomplished by a number of related practices that are essential for the continued use of the process over an extended period of time. The essential practices to maintain validated status include calibration, physical measurements, physical integrators and indicators, ongoing process control, change control, preventive maintenance, and periodic reassessment and training.

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# ⟨1229.9⟩ PHYSICOCHEMICAL INTEGRATORS AND INDICATORS FOR STERILIZATION

## INTRODUCTION

Physicochemical integrators provide some assessment of sterilization process efficacy and may be used in cases where validation of a sterilization process is not required—an exception is the validation and monitoring of radiation sterilization with dosimetry. The physicochemical indicator provides an immediate visual confirmation that an item has been exposed to a sterilization process. Performance standards both within and between lots of physicochemical integrators or indicators from a given manufacturer should be consistent. Integrators or indicators should not interact physically or chemically with any container or product when placed in the sterilizer load, and should not alter the strength, quality, or purity of the sterilized article. The integrator or indicator should be positioned such that it does not alter the effectiveness of the sterilization process. The principal usage of physicochemical integrators and indicators is to provide a rapid means of confirmation of sterilization cycle completion. This is especially important with single door sterilization chambers where a potential mix-up of nonsterilized and sterilized items is more likely. Aside from radiation sterilization where dosimetric data is accepted as definitive they should not be used as the sole proof of cycle efficacy (see *Radiation Sterilization* ⟨1229.10⟩).

## PHYSICOCHEMICAL INTEGRATORS

A physicochemical integrator is defined as a device that responds to one or more sterilization process critical parameters, which results in a measurable value that can be correlated to microbial lethality. The manufacturers of physicochemical integrators should provide data to demonstrate that the labeled performance characteristics tests of the integrators are met.

Physicochemical integrators require precautions for use and the appropriate interpretive criteria to define their performance characteristics. Performance of the sterilization apparatus must be ascertained from records generated by calibrated instruments (temperature, pressure, exposure time, gas concentration, and others, as applicable). The integrator can demonstrate only inadequate or adequate exposure to a combination of sterilization parameters.

Physicochemical integrators for radiation sterilization are designed to react predictably to the delivered radiation dose and can provide primary evidence of sterilization process effectiveness. The use of dosimeters in radiation sterilization cycle development and routine process control is addressed in ANSI/AAMI/ISO 11137-3, Sterilization of health care products—Radiation—Part 3: Guidance on dosimetric aspects (*1*).

## PHYSICOCHEMICAL INDICATORS

A physicochemical indicator is defined as a device that provides visual evidence of exposure to one or more critical sterilization parameters. Physicochemical indicators cannot provide primary evidence of sterilization efficacy.

## REFERENCE

1. ANSI/AAMI/ISO 11137-3:2006/(R)2010, Sterilization of health care products—Radiation—Part 3: guidance on dosimetric aspects. New York: American National Standards Institute; 2010.

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# ⟨1229.10⟩ RADIATION STERILIZATION

## INTRODUCTION

Radiation sterilization utilizes the lethal effect of various forms of radiation as a means of microbial destruction. Ionizing radiation (gamma, x-ray, or beam) sterilization is used extensively for the sterilization of medical devices and for a variety of other materials and products. Nonionizing sterilization methods such as microwave, infrared, x-ray, and ultraviolet light may be useful but have more restricted application, and are outside the scope of this chapter. This chapter provides an overview of sterilization using ionizing radiation and its validation, including dose setting, material compatibility, and dose verification.

The effects of radiation on materials can be substantial and are a major consideration when manufacturers select radiation as a processing method. The advantages of sterilization by irradiation include simplicity, absence of mechanical complexity, reproducibility, and overall efficiency. In fact, radiation sterilization is unique because the basis of control essentially is the absorbed radiation dose, which can be precisely measured. Methods used to establish appropriate radiation doses to achieve the desired sterility assurance level are defined in ISO 11137-1 Sterilization of Health Care Products; ISO 11137-2 Sterilization of Health Care Products—Radiation—Part 2: Establishing the Sterilization Dose; and ISO TS 13004: 2013 Sterilization of Health Care Products—Radiation—Substantiation of Selected Sterilization Dose: Method VDmaxSD. These methods include Method 1, Method 2A, Method 2B, and Method $VD_{max}$, which differ in the specific testing scheme and the number of articles that are needed for testing and are based on certain assumptions about bioburden. The use of a biological indicator is inappropriate during radiation sterilization validation because (a) there are accurate correlations between dose measurement and microbial destruction for a wide range of microorganisms and (b) the established dose setting methods are based on the material's bioburden in its natural state. These correlations have been developed by the medical device industry and provide a direct methodology for process control. Dosimetry plays a central role in radiation sterilization and serves as a direct means for affirming process lethality. The radiation dose measured in kGy (formerly MRads) is directly related to the lethal effects of the radiation on microorganisms. The measured dose has the same utility as $F_0$ in steam sterilization. Routine process control for radiation sterilization is provided by one or more reference dosimeters on the exterior of the packages (after dosimeters have been correlated during validation with dose measurement inside the package). The robustness and reliability of the absorbed dose of the article to be sterilized can support parametric release, as described in *Terminally Sterilized Pharmaceutical Products—Parametric Release* ⟨1222⟩, for many items.

## GAMMA STERILIZATION

Gamma sterilization entails the use of a specifically designed facility where items to be sterilized are exposed to a $Co^{60}$ radiation source in a manner that ensures uniform dosing. Highly penetrating photons (gamma rays) are emitted from $Co^{60}$ as it decays to $Ni^{60}$. The half-life for this isotope is 5.27 years, which means that over the course of each year the source loses about 12% of its radioactivity. This steady reduction in radioactivity requires that radiation process operators adjust their process controls (typically exposure time) to maintain the established dose required. Periodically, additional $Co^{60}$ is required to maintain practical throughput.

## X-RAY STERILIZATION

X-ray sterilizers generate highly penetrative photons similar to the gamma photons from $Co^{60}$ irradiators. X-ray photons are generated when accelerated electrons impact a target such as tantalum. These systems rely on scanning of materials with x-ray photons in order to sterilize them. Properly maintained, these systems are able to deliver a constant dose over time. No local radioactive source is required for x-ray sterilization systems.

## E-BEAM STERILIZATION

Electron beam systems rely on scanning of objects with focused electrons to sterilize the items within a defined radiation field. Properly maintained and controlled, these systems deliver a constant dose, so there is no change in dose with respect to time. The principal advantages of electron beam sterilization are a much higher dose rate and the absence of a localized radioactive source. These systems can be installed and operated by the end user. Electron beam penetration is substantially less than that obtained with photons, and therefore dose mapping is critical to ensure that items of varying density and complexity are properly sterilized. Because of the high dose rates used with electron beam sterilization, some materials can experience significantly higher temperatures than the same materials would experience in $Co^{60}$ irradiation.

## VALIDATION OF RADIATION STERILIZATION

Cycle development for radiation requires the identification of an appropriate radiation dose for the objects and confirmation that the dose does not adversely affect the material's essential quality attributes. In other words, analysts should identify the minimum sterilization dose as well as the maximum dose the material can withstand without negative effects. With this information analysts can set the dose for a specific radiation sterilization application.

Dose setting or dose establishment typically is achieved by following one of the ISO methods. These are Method 1, Method 2A, Method 2B, and Method $VD_{max}$. The choice of the most appropriate method depends chiefly on production batch size,

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knowledge of the normal bioburden, and the material's sensitivity to radiation. Dose mapping plays an important role through the cycle development and dose-setting exercise.

## Dose Setting

Method 1 is based upon the assignment and verification of a sterilization dose based on a microbial population. The resistance of the microbial population is not determined, and dose setting is based on a standard radiation resistance assigned to the microbial population, derived from data obtained from medical device manufacturers and from the literature. This analysis assumes that the distribution of standard resistance represents a more severe challenge than the natural microbial population on the material to be sterilized. A verification dose study should confirm the relative resistance assumption. The $VD_{max}$ method is similar to Method 1 (it requires both bioburden and dose verification testing) but relies on bioburden ranges (e.g., <1000 CFUs per item for a 25-kGy sterilization dose and, for example, 0.1–1.5 CFUs per item for a 15-kGy sterilization dose).

The more complex Method 2 does not require the enumeration of the microbial population for the purpose of setting the sterilization dose (although it is required for routine monitoring and control) but uses a series of incremental dose exposures to establish a dose at which approximately 1 out of 100 samples irradiated at that dose will be nonsterile. This is not the sterilization dose, but it provides the basis to determine the sterilization dose by extrapolation from this information.

## Material Compatibility

Once the required dose level has been established, the maximum dose should be established. Analysts typically establish the maximum dose by evaluating the highest likely dose that might be seen during the sterilization process, adding a safety factor, and evaluating the item for immediate and long-term effects of the radiation exposure. Some materials may appear unchanged initially, and the effects may become evident only over time. The evaluation should consider all of the materials exposed to the radiation processing, especially the drug product and its primary container. Product stability, safety, and functionality should be confirmed over the product's intended use period.

## Dose Verification

The methods for cycle development and dose setting rely on the bioburden approach. Analysts use defined presterilization bioburden controls and periodic evaluation of the process effects on the bioburden to maintain cycle efficacy. Establishing the required dose for microbial destruction during cycle development uses the bioburden's natural resistance; analysts then extrapolate the dose-setting algorithms to establish a dose that is capable of delivering a probability of a nonsterile unit (PNSU, a standard measurement) of $1 \times 10^{-6}$. The results of the dose-setting approaches for initial bioburden with different populations and resistance to radiation sterilization are depicted in *Figure 1*.

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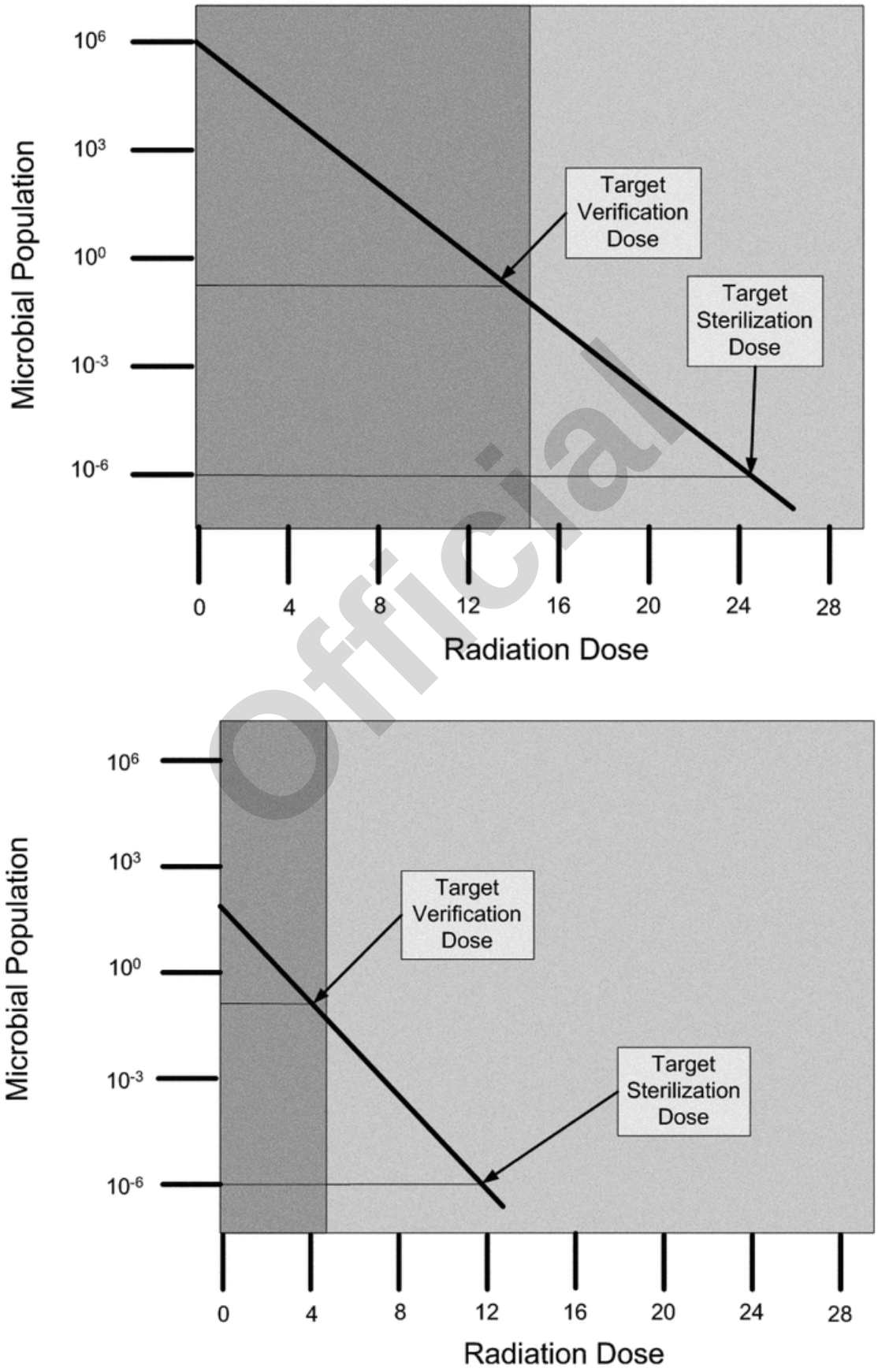


Figure 1. Results of radiation dose setting using $VD_{max}$. (top) Higher bioburden population, higher resistance to radiation

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sterilization. (bottom) Lower bioburden population, lower resistance to radiation sterilization.

## Validation Activities

Confirmation of appropriate dose delivery when using the sterilization dose requires a number of supportive activities.

### EQUIPMENT QUALIFICATION

The use of gamma sterilization requires initial and periodic assessment of equipment controls and parameters necessary to establish the system's capability. Sterilization systems that deliver directed beams or rays have controls for scan speed, source intensity, and system timers. The other elements of radiation sterilization equipment largely are related to material transport and are easily qualified. Qualification of safety controls, devices, and software is required.

### EMPTY CHAMBER DOSE MAPPING

This optional exercise entails mapping the target area for radiation dose in the absence of a load and is a possible means to evaluate a focused beam or ray system. It provides a baseline of performance that may be useful over time.

### LOAD DOSE MAPPING

The arrangement of items in irradiation containers, carriers, or pallets is an essential part of the initial validation exercise. The goal of the mapping is to define the distribution of a dose throughout the load items and establish a configuration that minimizes dose variation across the materials. The items are mapped using multiple dosimeters positioned internally and externally. Identification of maximum dose location is important in evaluating the effects of the radiation on the load items. The location of minimum and maximum dose can be identified from the dose mapping data for monitoring in routine sterilization of materials.

### BIOLOGICAL INDICATORS

The use of biological indicators for radiation sterilization is not indicated because the physical and dosimetric measurements employed are more reliable, reproducible, and robust than biological systems.

### DOSIMETRY

Process control for radiation sterilization relies heavily on dosimetry for both initial development and ongoing verification. For guidance on the selection and use of a dosimetry system for use in radiation sterilization refer to ASTM E2628 Practice for Dosimetry in Radiation Processing. The dosimeters and the instruments used with them should be calibrated according to ISO/ASTM 51261 Practice for Calibration of Routine Dosimetry Systems for Radiation Processing.

### PROCESS CONFIRMATION

The core of the validation activity is the confirmation of acceptable lethality using dosimeters that are positioned across the material as it is processed through the radiation-sterilizing equipment. Proof of sterilization cycle efficacy is provided in replicate studies in which the dosimetry results correspond to the required minimum value for sterility assurance and demonstrate that the maximum value has not been exceeded.

## Routine Process Control

Radiation sterilization should be subject to formal controls that maintain the validated status. The practices outlined in *General Principles of Sterilization of Compendial Articles* ⟨1229⟩ provide the general requirements appropriate for all sterilization systems. This is accomplished by a number of related practices that are essential for the continued use of the process over an extended period of time. The practices that are essential to maintain validated status for radiation include training, calibration, physical measurements, bioburden monitoring, change control, preventive maintenance, and periodic dose audits.

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2. Herring, C., & Saylor, M., Sterilization with Radioisotopes, chapter in Morrissey, R., & Phillips, G. B. (eds.), *Sterilization Technology—A Practical Guide for Manufacturers and Users of Health Care Products*, Van Nostrand Reinhold, New York, 1993.
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# ⟨1229.11⟩ VAPOR PHASE STERILIZATION

## INTRODUCTION

Sterilization can be accomplished using sporicidal agents suspended in air (i.e., vapor). Sterilizing agents that operate in this fashion include hydrogen peroxide ($H_2O_2$), peracetic acid ($CH_3CO_3CH$), formaldehyde ($CH_2O$), and glutaraldehyde [$CH_2(CH_2CHO)_2$] in aqueous solution. At room temperature these are liquids or solids that can be vaporized for introduction into a vessel or chamber. They differ from sterilizing gases and liquids in that there are multiple phases within the vessel during sterilization. Vapor sterilization systems are well suited for heat-sensitive materials and surface sterilization. Items exposed to the process should have their surfaces exposed to the greatest extent possible. Vapor sterilization processes require appropriate sterilant concentration, temperature, and relative humidity, all of which may be variable during the exposure period. Because the agent is ordinarily supplied as an aqueous solution, moisture is introduced with the agent. The consequences of variation in these parameters may be localized differences in relative humidity, agent concentration, and condensation rates on the surfaces to be treated, resulting in variations in process lethality. The parameters to be established include sterilant amount (usually derived from injection quantities), relative humidity, and temperature. There is no demonstrated correlation between gas phase conditions, surface conditions, and microbial kill. For this reason, online monitoring of vapor phase concentration is not widely utilized as a control parameter. Efforts to develop a standardized biological indicator for vapor systems have been hampered by the multiphasic nature of these sterilants. Selection of the appropriate biological indicator (BI) and resistance should be based on experimentation within the user's system. Only under well-defined, specific conditions (e.g., agent concentration, humidity level, temperature, substrate, and phase) can a reliable D-value be established (for a definition of D-value, see *Sterilization of Compendial Articles* ⟨1229⟩).

This chapter will briefly review hydrogen peroxide and peracetic acid sterilization systems, because they are more widely used than other vapor phase sterilizing agents in the pharmaceutical and medical device industries.

## HYDROGEN PEROXIDE

The efficacy of hydrogen peroxide as a liquid sterilant has been long established.[1] There are several effective approaches to hydrogen peroxide ($H_2O_2$) injection, including continuous, intermittent, or all at once. Some of the systems utilize an evacuation or drying step prior to introduction of the hydrogen peroxide ($H_2O_2$) to allow for increased concentration without excess condensation. Alternatively, hydrogen peroxide ($H_2O_2$) can be introduced as a liquid, followed by target heating. Following the exposure period, the chamber or target is aerated to an acceptable level for further processing of materials and/or personnel exposure (whichever is lowest) prior to opening and removing the sterilized article.

## PERACETIC ACID

Peracetic acid ($CH_3CO_3CH$), alone or mixed with hydrogen peroxide, is a sporicidal agent that has been proven effective.[1] Peracetic acid is introduced as a liquid through an atomizer, resulting in the presence of liquid and vapor phases within the chamber. After the process dwell period, it is removed by evaporation.

## VALIDATION OF VAPOR STERILIZATION

Standard sterilizing conditions have not been defined due to the varying phase and multiphase nature of the sterilant during sterilization processes. Therefore, no standardized BIs with D-values that may be used for conventional predictive analysis of kill rates exist. In the absence of BI D-values, and due to the variation in vapor sterilization cycle parameters, an empirical approach must be used. The kill rates in the gas and liquid phases that constitute the vapor may be substantially different (liquid kill rates are considered greater than gaseous kill rates). Destruction of BIs distributed across the system or load demonstrates lethality regardless of which phase effects the kill. Sterilization process parameters (usually time) that do not kill the BIs may be adjusted until a complete kill is achieved. This establishes the minimum conditions necessary for a complete kill. Vapor sterilization may be validated using a half-cycle, bracketing, or other suitable approach as defined in ⟨1229⟩ and *Gaseous Sterilization* ⟨1229.7⟩.

The half-cycle validation method requires the destruction of a suitable concentration of a resistant microorganism under defined, minimum conditions for a complete kill. Then, in routine operation, the minimum lethal time period is arbitrarily doubled, which supports a doubling of the spore log reduction of the BI, and is more than sufficient to inactivate the bioburden.

A bracketing approach, which better supports process operating ranges for the critical parameters than does the half-cycle method, also can be employed. In the bracketing method, one evaluates conditions that bracket the defined process condition in order to establish parameters for the minimum and maximum effects on the materials and bioburden. The minimum lethal process dwell time establishes the worst case for microbial kill. Incremental increases in process dwell time beyond the minimum lethal time period are used to establish the maximum exposure periods, which impart the greatest effect on materials. In addition, adjustments to the quantity of agent introduced, operating temperature, and relative humidity are utilized to further enhance the bracketing approach. By this method, the routine process conditions may be established between the minimum and maximum process conditions to ensure complete microbial kill while maintaining the integrity of the materials.

[1] Block SS, editor. *Disinfection, Sterilization, and Preservation.* 5th ed. Philadelphia: Lippincott Williams & Wilkins; c2001. Chapter 9, Peroxygen compounds; pp. 185–204.

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The following activities are defined for a batch process, and therefore appropriate adaptation is necessary when they are applied to intermittent or continuous sterilization processes.

- *Equipment qualification*—The equipment qualification for vapor sterilization mimics that of other sterilization processes in order to confirm that the equipment has been properly installed and operates as intended.
- *Empty chamber parameter distribution*—Although multipoint measurement is possible, it lacks correlation to surface microbial kill. Humidity and temperature measurements, along with chemical indicators, can provide a limited indication of sterilant distribution. BIs are not required in the evaluation of the empty chamber.
- *Component mapping*—For surface sterilization, internal mapping of load items is not required. Although vapors are primarily used as surface sterilants, when they are used for packaged articles with internal surfaces and volumes that need to be sterilized, internal mapping should be performed, with BIs placed in difficult-to-penetrate locations to confirm process lethality.
- *Load mapping*—Humidity and temperature measurements, along with chemical indicators, can provide a limited indication of sterilant distribution on component surfaces. BIs are not required. Effects of load size and patterns should be assessed.
- *Biological indicators*—The use of multiple BIs at each test location is recommended to more adequately support the process lethality.
- *Process confirmation and microbiological challenge*—The core of the validation activity is the confirmation of acceptable process parameters and inactivation of the microbial challenge. Proof of cycle efficacy is provided in replicate studies in which the BIs are killed and chemical or physical measurements are utilized.

## ROUTINE PROCESS CONTROL

Vapor sterilization is subject to controls that maintain a validated state over time. The practices outlined in ⟨1229⟩ describe the general requirements appropriate for all sterilization systems. The essential practices required to maintain validated status include calibration, physical measurements, use of BIs, physical or chemical integrators and indicators, ongoing process control, change control, preventive maintenance, periodic reassessment, and training.

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# ⟨1229.12⟩ NEW STERILIZATION METHODS

## INTRODUCTION

Sterilization processes are developed for the elimination of viable microorganisms while preserving the essential physical, chemical, and biological properties of the materials subjected to them. Where this cannot be accomplished by the sterilization methods described in *Sterilization of Compendial Articles* ⟨1229⟩, it may be possible to sterilize by using a proposed method not commonly used. When doing so, it is the end user's responsibility to demonstrate that the proposed new method can be used safely and effectively.

## POINTS TO CONSIDER FOR A NEW STERILIZATION METHOD

The major steps in the implementation of a new sterilization method include the following:

- Elimination of any established method through experimental evidence and/or comprehensive literature review of any materials used
- A literature review to identify supportive information on the proposed method
- Identification and confirmation of reproducible lethality against a broad range of microorganisms, including bacterial spore-formers
- Identification and definition of critical process parameters necessary to ensure sufficient lethality. The effective range of these parameters should be explored to identify necessary conditions for the proposed sterilization process. Among the parameters to be considered, depending upon the nature of the process under consideration, are process dwell time, temperature, concentration, energy or power, and relative humidity. This evaluation should include positive and negative controls to ensure that the proposed method is in fact responsible for microbial destruction.
- The selection of a biological indicator (usually a spore-forming microorganism) with increased resistance to the sterilization method
- Evaluation of the proposed method against anticipated bioburden microorganisms and comparison of relative resistance of the bioburden microorganism to that of the chosen biological indicator
- The identification of in-process and/or post-process measurements and/or analysis that can reliably confirm the effectiveness of the proposed sterilization process.

Where the proposed new method is used for materials intended for human and/or veterinary use, the relevant regulatory authorities should be contacted to secure their acceptance before either investigational or clinical usage. Validation of the proposed new method should be completed before use on a commercial basis.

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# ⟨1229.13⟩ STERILIZATION-IN-PLACE

## INTRODUCTION

Sterilization-in-place (SIP) can be defined as the sterilization of a system or piece of process equipment in situ. The purpose of SIP[1] is to eliminate, or greatly reduce, the need for post-sterilization handling, including that necessary to make aseptic connections. Mobile process equipment (e.g., portable tanks, storage vessels, and other equipment), once sterilized in this manner, may be relocated. The SIP process can be carried out by using any of the following physical methods: moist heat, dry heat, gas, liquid, or vapor (described below) according to the approaches described in *Sterilization of Compendial Articles* ⟨1229⟩, as adapted for use with the specific equipment or system.

## COMMON ELEMENTS OF SIP PROCESSES

There are a number of considerations appropriate for the design and use of SIP procedures that apply to all of the sterilization methods:

- The use of an SIP method is normally associated with a "closed system." Closed systems are almost always sterilized in situ, and the design elements of the typical closed system are consistent with many SIP process needs.
- For large systems, it may be necessary to sterilize in portions. The individual sterilization processes should overlap to ensure treatment of all internal surfaces.
- The focus of the SIP procedure is sterilization of the product contact surfaces (the interior of the system). Demonstration of process lethality relies upon physical measurements and biological indicators. This confirmation should extend to the "sterile boundary" of the system, including vessel headspace, connections to other vessels/equipment, and other parts of the system. The interior surfaces of the process equipment, irrespective of their materials of construction, should be exposed to lethal conditions sufficient to sterilize the system and confirmed as lethal with an appropriate biological challenge.
- SIP is accomplished almost exclusively using the overkill approach to sterilization. The components of the physical equipment should be chosen based upon their ability to withstand the sterilizing conditions to be used. Filters in the process system, whether membrane or high-efficiency particulate air (HEPA), are typically susceptible to damage during SIP, and care must be taken to preserve their integrity. Filter manufacturers can provide guidance on acceptable sterilization methods and parameters.
- The absence of specifically designed equipment in which the sterilization process is performed places the bulk of responsibility for design onto the user. SIP systems ordinarily cannot be purchased directly in the way one purchases a steam sterilizer or a dry heat oven. Instead, the system, which was designed for the operating process, may require modification to accommodate the SIP process to be used. The user must assume the role of designer for the process, equipment, and control system.
- The system design and operating procedures must provide for an efficient means of introducing and removing the sterilization agent. Sterilization agent removal must consider the potential effects of residual sterilant on the materials to be processed. Establishment of a reliable process sequence is a critical part of the cycle development exercise. The sterilizing agent is normally introduced through a filter on the system that may also serve as a process, purge gas, or vent filter.
- At the conclusion of the sterilization process sequence and until ready for use, the system should be pressurized with a purge gas (sterile air or nitrogen are the most common) to prevent the introduction of contaminants to the sterilized system.
- The critical process parameters for the SIP process should be recorded as the process is executed. The important parameters may include temperature, pressure, concentration, flow rate, humidity, and time, among others.

## CLOSED SYSTEMS

In pharmaceutical manufacturing operations, closed systems are used for various applications including maintenance of large quantities of materials (liquids or powders) in a sterile state; manufacture of biological and synthetic organic active ingredients (especially where microbial absence is essential); and preparation of process equipment for use in sterile drug product manufacturing and filling. The use of closed systems provides superior separation of sterile materials from the surrounding environment. Typically, closed systems are maintained under positive pressure at all times. The characteristics of a closed system that establish its designation as "closed" include the following:[2]

- It maintains integrity during all operating periods and under all conditions.
- It is sterilized-in-place or sterilized while closed before use.
- It can be adapted for materials transfer in and/or out while maintaining its sterile state.
- It can be connected to other closed systems while maintaining the integrity of all systems.
- It is subject to scheduled preventive maintenance.
- It uses sterilizing-grade filters for sterilization of liquid and gas process streams.

[1] AAMI/ISO 13408-5. Aseptic processing of health care products—part 5: sterilization in place; 2008.
[2] Parenteral Drug Association, Technical Report No. 28, Revised. Supplement Volume 60, No. S-2, Process simulation testing for sterile bulk pharmaceutical chemicals. Bethesda, MD: PDA; 2005.

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## SIP METHODS

### Moist Heat

The use of saturated steam is the most prevalent method for SIP of large systems. The majority of installations use gravity displacement cycles adapted from those originally used in steam sterilizers (the size and complexity of many systems preclude the use of prevacuum cycles). Important considerations include the provision for air removal, condensate discharge, and steam removal post-dwell.[3, 4] This method is commonly used for bioreactors, sterile bulk production, holding tank and delivery lines, and other large systems.

### Superheated Water

Systems used for *Water for Injection* and *Purified Water* can be sterilized by using superheated water (water that is heated above its boiling point and pressurized to maintain it as a liquid) circulating through the system. This method has the ability to sterilize vessels, filters, and other wetted components at the same time.[5] Removal of residual water subsequent to the sterilization phase is recommended.

### Dry Heat

Dry heat has been used for SIP of spray dryers and their associated material collection systems. The air supply for sterilization in these systems is provided through HEPA filters.

### Gas

Gas-phase SIP has been used for non- and low-pressure-rated process equipment, such as freeze dryers, prefreezers, process vessels, and other equipment.

### Liquid

Liquid chemical sterilization is best suited for liquid-handling systems and can be used only for fully wetted surfaces. This process is similar to those using superheated water except the lethal modality is chemical rather than thermal.

### Vapor

Sterilizing vapors have been used for the in situ sterilization of the same types of process equipment as those treated with sterilizing gases. The precautions associated with vapor sterilization described in *Vapor Phase Sterilization* ⟨1229.11⟩ are required.

## ROUTINE PROCESS CONTROL

SIP processes are subject to formal controls that maintain a validated state over time. The practices outlined in ⟨1229⟩ include the general requirements appropriate for all sterilization systems as well as those specific to an individual sterilization method. Sterilization is accomplished by a number of related practices that are essential for continued use of the process over an extended period of time. The essential practices to maintain validated status include calibration, physical measurements, physical integrators or indicators, ongoing process control, change control, preventive maintenance, periodic reassessment, and training.

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[3] Agalloco J. Steam sterilization-in-place technology and validation. In: Agalloco J, Carleton FJ, editors. Validation of pharmaceutical processes. 3rd ed. New York: Informa USA; 2007.
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[5] Haggstrom M. Sterilization-in-place using steam or superheated water. In: Proceedings of the PDA Basel conference. Bethesda, MD: PDA; 2002.

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# ⟨1229.14⟩ STERILIZATION CYCLE DEVELOPMENT

## INTRODUCTION

The proper development and implementation of a sterilization process requires a number of important sequential steps to ensure that an appropriate sterilization process results in materials and products that are both microbiologically safe and suitable for their intended use.[1] Sterilization technologies that rely on heat, ionizing radiation, and chemicals often have the potential to alter the physicochemical properties of materials to which they are applied. In some cases, sterilization could either leave toxic residues on materials that were sterilized or result in impurities (i.e., leachables) being more readily released into products that contact them. Thus, it is necessary to consider sterilization not only as a means of eliminating microorganisms but also as a process that has the potential to change materials in a manner that impacts usefulness, safety, or both. A balance between the microbial lethality of a sterilization process and other aspects of patient safety must be established. A robust sterilization process that satisfies both is the desired objective of the cycle development activities.

## PROCESS SCREENING

### Radiation

The methods for establishment of radiation sterilization are described in detail in ISO 11137, and adherence to the most recent version of that standard is recommended.

### Sterilizing Filtration

Filtration sterilization processes rely on exclusion of microorganisms and differ substantially from those that rely on lethality (see *Sterilizing Filtration of Liquids* ⟨1229.4⟩). Process development for filtration must consider many factors, such as adverse effects of the fluid on the filter (e.g., chemical compatibility) and of the filter on the fluid (e.g., extractables and leachables), as well as adsorption of fluid components. Physical interactions such as pressure differential, hydraulic shock, flow rate, temperature, and filtration capacity must also be considered. See ⟨1229.4⟩ for additional information.

### Other Sterilization Methods

The first step in the development of a sterilization cycle is exposing the materials to the sterilization process and evaluating the impact that the process has on them. Initially, this determination can be a general screening process in which gross changes in appearance may be sufficient to confirm incompatibility. Where appearance is unchanged, the material should be evaluated for changes to its physical and chemical attributes. If the results are marginal, reductions in those parameters that most impact lethality and/or material effects may be beneficial. Tests conducted over a range of process lethality that would result in microbiological destruction should be performed. At this stage of development, the final process conditions required are not yet established, so flexibility in the preliminary evaluation is necessary. Common sense should be applied when considering sterilization process alternatives. For example, thermal treatments are inappropriate for refrigerated materials, items cannot be heated close to their melting point, sterilizing gases and liquids cannot penetrate sealed containers or nonporous materials, and other obvious restrictions may also be present. In the evaluation of items made of many materials (i.e., sealed vials, permeable pouches, etc.), consider the effect on all material components of the item being sterilized. Altering one or more components of the item may result in a favorable outcome. In many projects involving the sterilization of a custom-designed component, the sterilization process evaluation is an essential element of proof-of-principle studies. No component selection and manufacturing process design for a sterile material can be considered complete until a suitable sterilization process has been identified and fully evaluated, and renders the material sterile while having minimal effects on its physical and chemical attributes.

The following conditions are merely examples for consideration when evaluating materials for compatibility with the sterilization processes indicated:

1. Moist heat—at conditions sufficient to reliably kill the expected bioburden (see *Steam Sterilization by Direct Contact* ⟨1229.1⟩ and *Moist Heat Sterilization of Aqueous Liquids* ⟨1229.2⟩)
2. Dry heat—check for the lowest melting point of materials and process at 25° below the lowest melting temperature (see *Dry Heat Sterilization* ⟨1229.8⟩)
3. Liquids, gases, and vapors—at conditions sufficient to reliably kill the expected bioburden (see *Liquid-Phase Sterilization* ⟨1229.6⟩, *Gaseous Sterilization* ⟨1229.7⟩, and *Vapor Phase Sterilization* ⟨1229.11⟩)

Other conditions can be utilized in this preliminary evaluation, and multiple methods may need to be explored. Use information in the literature or provided by the suppliers to facilitate this effort. Materials should be changed if the initial materials are suspected of being incompatible with an otherwise acceptable method. The selected sterilization method should be the process that has the least impact on the essential quality attributes of the material and delivers lethality sufficient to exceed the desired minimum probability of a nonsterile unit (PNSU).

[1] The term "materials", as used throughout this chapter, includes drug substances, drug products, in-process materials, components, containers, closures, laboratory media, utensils, product contact materials, and other items subject to sterilization processes.

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## BIOBURDEN EVALUATION

In parallel with the process described above, an assessment of the bioburden present in or on the items to be sterilized should be conducted (see *Monitoring of Bioburden* ⟨1229.3⟩). The initial evaluation should include an estimation of bioburden population and resistance. A control strategy for periodic bioburden monitoring for resistance and population should be instituted regardless of the results of the initial study (see ⟨1229.3⟩).[2] For radiation sterilization processes, the expectations for bioburden evaluation are established in ISO 11137.

### Bioburden Resistance

For the remaining sterilization processes, the execution of a boil test (100° for 10 min) can serve as a basic screen for spore-forming microorganisms (see ⟨1229.2⟩). The absence of survivors in a boil test greatly simplifies the sterilization cycle development as the sterilizing conditions for non–spore-formers are less stressful on the production materials. If microorganisms are determined to survive the boil test, their resistance to the chosen sterilization process should be established. The boil test allows for estimation of a D value for a moist heat process. For other processes, a literature reference or laboratory study is required (see ISO 18472 *Sterilization of Health Care Products—Biological and Chemical Indicators—Test Equipment*).

### Bioburden Population

If no microorganisms are recovered or only non–spore-formers are recovered, an estimation of bioburden population can be utilized to expedite selection of the appropriate process duration. As spore-forming bacteria are ubiquitous, the potential presence of spores in the bioburden should be given consideration.

## ESTIMATION OF PROCESS DURATION

The minimum duration of the sterilization dwell period can be determined once the sterilizing method has been selected and the bioburden (as determined or assumed) information has been obtained. This is accomplished by rearranging *Equation 1*, used to estimate the PNSU (see *Sterilization of Compendial Articles* ⟨1229⟩).

$$\log N_U = \frac{-F}{D} + \log N_0 \qquad (1)$$

Rearrangement of the equation and solving for *F* results in *Equation 2*.

$$F = D\left(\log N_0 - \log N_U\right) \qquad (2)$$

$N_U$ = assumed PNSU (NMT 1 positive unit in $10^6$ units)
$F$ = dwell time of the sterilization process at a defined condition expressed in minutes[3]
$D$ = estimated D value of the bioburden in minutes
$N_0$ = estimated maximum population of the bioburden

The calculated *F* value represents the minimum duration of the process dwell period to reduce the assumed initial microbial population to less than the desired PNSU. Safety factors can be added to the assumed D value of the bioburden, the minimum desired PNSU, and the bioburden population estimate. The use of a safety factor reduces contamination risk as it increases the process duration, but it may adversely affect the materials being sterilized. With or without safety factors, determination of the estimated process duration under sterilizing conditions is a prerequisite to the subsequent activities.

Depending on the *F* value (or process dwell time) selected, the validation approach for sterilization can be determined. The preferred, but not required, method is the use of an overkill approach (see ⟨1229⟩) where the *F* value allows for complete inactivation of a resistant biological indicator. Where material limitations restrict the use of lengthy sterilizing conditions, the bioburden/biological indicator or bioburden methods may be more appropriate (see ⟨1229⟩).

## FORMAL MATERIAL EVALUATION

Once the appropriate sterilization process has been chosen, the effects of that process on the materials should be reconfirmed (*1*). The requirements for this can vary from simple physical examinations for materials expected to be largely unaffected by the sterilization process, such as stainless steel or glass with steam, to comprehensive stability examinations for formulated products in their final container-closure configuration. The criteria for these studies should be objective wherever possible. It is useful to do a literature search for materials about which there is minimal experience, and to search and discuss potential material effects with suppliers. The preparation and sterilization of the test units for these formal studies can be conducted in a laboratory setting and must be fully documented.

[2] Native bioburden must be routinely tested and trended with appropriate screening in place for organisms that may exceed the worst case in terms of population and/or survivability. If a more resistant organism and/or abnormally high population has been isolated through a screening procedure, the impact on the sterilization process to achieve the required sterility assurance level must be determined.

[3] The *F* value is equivalent to $F_0$ for moist heat, $F_H$ for dry heat, or process dwell time *L* for other sterilization processes and is typically expressed in minutes.

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When sterilizing using chemical agents, the sterilized materials should be tested for the presence of residual sterilant and its known degradation by-products. Post–sterilization cycle aeration or other treatments may be necessary to reduce these to acceptable levels.

## BIOLOGICAL INDICATOR SELECTION

A biological indicator should be selected that is appropriate for the chosen sterilization process and its duration, as well as the intended validation approach (see *Biological Indicators for Sterilization* ⟨1229.5⟩). The chosen biological indicator provides a means to measure the lethality of the process through the selection of biological indicator population and resistance. The sterilization process duration should not be adapted to accommodate the specifics of any biological indicator.

## CONTAINER AND ITEM MAPPING

Studies should be performed to determine the location within the materials to be sterilized that is least likely to achieve sterilizing conditions. The mapping can be conducted using thermocouples, dosimeters, biological indicators, and/or chemical indicators placed within the materials. The materials to be sterilized should be wrapped or packaged and oriented as intended for routine use of the sterilization process. The wrapping and packaging materials and methods should be controlled to ensure reproducibility. It is essential when conducting these studies that the introduction of the measuring devices be accomplished in a manner that does not alter (positively or negatively) the penetration of the sterilant through any wrapping and packaging or through the material itself. If the materials to be sterilized have simple geometry, with minimal internal volume, and are sterilized with other more complex items, mapping of the simpler materials can be omitted. Interpolation of volume, density, dimension, and mass may be possible to reduce the extent of studies required. Mapping studies should be documented for future reference.

## LOAD MAPPING

The initial use of a sterilization process should include confirmation that the sterilization conditions can be delivered throughout the entire load. Additional guidance on this, and the subsequent aspect of sterilization validation and process control, can be found in other chapters within the ⟨1229⟩ family of chapters.

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# ⟨1229.15⟩ STERILIZING FILTRATION OF GASES

## INTRODUCTION

Gases, such as compressed air, nitrogen, and atmospheric gases that contact sterile components, containers and closures, and product contact surfaces of processing equipment, such as tanks and piping, must be sterile to prevent contamination of pharmaceuticals and biopharmaceuticals. The sterilization of gases is typically accomplished by passing the gas through one or more sterilizing-grade membrane filters. The filtration process must be designed to ensure that the filtered gas has been sterilized.

## GAS VERSUS LIQUID STERILIZING FILTRATION

*Sterilizing Filtration of Liquids* ⟨1229.4⟩ contains information that is applicable to this chapter, including but not limited to sterilizing-grade filters, retention mechanisms and factors affecting retention, validation, integrity-testing principles and methods, and prefiltration bioburden control. The significant differences between liquid and gas filtration will be discussed in this chapter.

## MEMBRANES FOR GAS STERILIZATION

Filters used for sterilization of gases are commonly hydrophobic membranes, although hydrophilic membranes can be employed where membrane wetting during use is not an issue. Wetting may be a factor with filters used for autoclave venting and where vent filters are required to maintain the sterility of tank headspace. Microporous filter membranes that have a pore-size rating of NMT 0.2 µm, which meets the ASTM International standard (see F838-15a *Standard Test Method for Determining Bacterial Retention of Membrane Filters Utilized for Liquid Filtration*), are suitable for the sterilization of gases; in fact, these filters are considerably more retentive for gases than for liquids (*1*). Additionally, the filters must be chemically compatible with the gas to be filtered and physically compatible with the filtration conditions (e.g., pressure differentials and flow rates).

## RETENTION MECHANISMS

The primary retention mechanisms of membrane filters used in gas sterilization are size exclusion, impaction, diffusional interception, and electrostatic attraction. Size exclusion is effective for particles (microorganisms) that are too large to pass through the pore structure of the filter membrane and is essentially independent of the velocity of gas flowing through the filter. Retention mechanisms other than size exclusion can be effective for particles smaller than the pores they encounter. Retention due to impaction, diffusional interception, and electrostatic attraction is dependent on particle size, flow rate, and relative humidity. For example, particle retention efficiency due to impaction is proportional to the mass of the particle; heavier particles have a greater momentum than lighter ones and are therefore more likely to contact pore walls and subsequently be retained by the filter during tortuous flow. In contrast, the capture efficiency of diffusional interception is inversely proportional to particle size and velocity. Smaller particles influenced by Brownian motion can exit the streamlines, contact the filter matrix, and be retained, and lower velocity increases potential contact time and improves capture efficiency. Electrostatic attraction of particles is reduced by a high relative humidity in the filtered gas. Adsorption, which can be important in liquid filtration, is not a retention mechanism for gases.

## VALIDATION

Validation of the processes used in sterilizing filtration for gases, in general, may be divided into two parts: sterilization of the filter and its housing, and the ability of the filter to remove microorganisms from the filtered gas.

Sterilization of the filter and its housing has been addressed specifically in ⟨1229.4⟩ and *Sterilization-in-Place* ⟨1229.13⟩, and generally in *Sterilization of Compendial Articles* ⟨1229⟩.

Membrane filters meeting the requirements of ASTM F838-15a are suitable for sterilizing filtration of gases in terms of microbial retention. Liquid bacterial challenge testing represents a worst-case condition for sterilizing gas filters because the retention efficiency in liquids is lower than in gases (*2*).

## INTEGRITY TESTING

Integrity testing requirements and techniques are discussed in ⟨1229.4⟩. The hydrophobic filters typically used for gas filtration can be integrity tested using bubble-point, diffusive-flow, and pressure-hold tests that use a liquid of low surface tension to completely wet the pore structure of the filter membrane. Additionally, integrity testing can be performed by measuring the membrane's resistance to wetting with water as a function of pressure. Water intrusion testing is an example of this type of integrity testing.

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***Add the following:***

# ▲⟨1229.16⟩ PRION INACTIVATION

## INTRODUCTION

Prions are transmissible agents comprised of abnormal proteinaceous materials that lead to the precipitation of healthy protein within eukaryotic cells, leading to cell death. Prions have been associated with transmissible spongiform encephalopathies (TSE), fatal neurological diseases, that occur in humans and other animals. Some examples of TSE are Creutzfeldt-Jakob disease (CJD) in humans, scrapie in sheep, and bovine spongiform encephalopathy (BSE) in cattle. Prions are considered highly resistant to most physical and chemical destruction agents, and in many cases are more resistant than bacterial spores to these agents (*1*). Prions are potential contaminants in materials and cell lines of mammalian origin and their inactivation/removal is required where their presence can be expected. They can also be found on equipment and medical devices exposed to contaminated materials. Empirical confirmation is recommended in the application of all prion inactivation processes as there are different contaminants, conflicting statements regarding performance, and limited information on many of the potential processes.

The removal of proteinaceous materials including prions is made more difficult if these are allowed to dry on surfaces before treatment. For that reason all surfaces should be kept water wet until inactivation begins.

The process conditions and agents used for prion inactivation must consider patient safety as part of the risk assessment during product manufacture and reprocessing of equipment and medical devices. The effect of the process/agent on the materials being processed must be considered.

When the items/materials to be treated are intended for disposal, incineration is considered to be the most effective means of prion inactivation.

## METHODS OF DESTRUCTION

### Chemical Methods

Physical removal and prion degradation (or fragmentation) have been demonstrated using a variety of alkaline cleaning formulations, typically at pH >9. The usefulness of these harsh solutions varies considerably depending on the formulation, concentration, pH, and cleaning process conditions (e.g., exposure time, temperature). Reported results regarding the chemical inactivation of prions have been inconsistent because of numerous variables involving the prion (e.g., strain, concentration, underlying tissue, test species) and the chemical treatment (e.g., agent, concentration, process parameters). Effective prion-inactivating chemical agents include chlorine (delivered in various forms), phenolic compounds, guanidine thiocyanate, and sodium hydroxide, among others (*2*). Chemical methods (with or without heat) are better suited for surface treatment.

### Thermal Methods

The destruction of prions by moist heat has been reported across a wide range of temperatures (from 100°/15 min to 138°/1 h), which reflects the diversity of the challenge presented (*2*). Thermal treatments can be used for raw materials, fluids, and equipment.

### Combination Methods

The combination of chemical and thermal methods provides the greatest confidence in prion titer reduction for equipment. A common process for prion decontamination is moist heat (or superheating under pressure) over time (e.g., 121° for ≥1 h, 132°–136° for ≥18 min) immersed in concentrated alkaline solutions such as sodium hydroxide (NaOH) at 1–2 N. Lower concentrations of sodium hydroxide and potassium hydroxide have been shown to be effective against surface prion contamination when used in combination with surfactants supplemented with other chemical additives. An example is a disinfectant formulation containing 0.2% sodium dodecyl sulfate (SDS), 0.3% sodium hydroxide, and 20% *n*-propanol; this combination has been shown to be effective against prions, bacteria, and viruses. The effects of alkali and steam sterilization on prions have been studied and shown to cause protein and peptide fragmentation over time; these effects can be enhanced at increased concentrations of the alkali solution and/or higher temperatures (*1*). When working with hazardous chemical methods, there are practical problems that need to be considered, such as risks to personnel while handling harsh chemical agents, the eventual deleterious effects on the autoclave chamber, and the potential detrimental effects on medical devices due to surface reactive damage to metals and plastics. In addition to sodium hydroxide, sodium hypochlorite (chlorine) solutions are widely used as anti-prion chemicals (*3*).

## METHODS THAT ARE NOT EFFECTIVE

The following disinfectants or disinfection processes have been shown to be ineffective for prion decontamination: formaldehyde, glutaraldehyde, alcohols, radiation (ionizing or nonionizing), dry heat, ethylene oxide, quaternary ammonium compounds, pasteurization, and boiling (*4*).

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# ▲⟨1229.17⟩ MYCOPLASMA STERILIZATION

## INTRODUCTION

Mycoplasmas, Acholeplasmas, and Ureaplasmas are small prokaryotes, all belonging to the Mollicutes class—a class of bacteria lacking a cell wall and surrounded by a deformable membrane. Mycoplasmas are contaminants found in biopharmaceutical, biologic, and mammalian cell culture processes that may infect eukaryotic cells, and, as such, they are less likely to contaminate small-molecule pharmaceutical processes and products. Mycoplasmas can be associated with microbiological growth media such as Soybean–Casein Digest Medium (SCDM). Mycoplasmas are easily inactivated by heat and radiation and are therefore not a concern where process streams are terminally sterilized. However, where sterilizing filtration is employed, the presence of mycoplasmas must be considered in designing filter validation studies. The small size (0.15–0.3 µm) and flexibility of the organism allows its passage through the 0.2-µm-rated microporous membrane filters commonly used to sterilize liquid pharmaceutical process streams.

## INACTIVATION

Mycoplasmas are inactivated by sterilization processes such as autoclaving and irradiation.[1] Where mycoplasma contamination is a concern, raw materials and process streams can be subjected to lethal sterilization processes prior to use in biopharmaceutical product manufacture in order to minimize the presence of mycoplasmas prior to final sterilizing filtration, thereby reducing their population in the prefiltration bioburden and improving retention efficiency.

## REMOVAL BY FILTRATION

The principles outlined in *Sterilizing Filtration of Liquids* ⟨1229.4⟩ are applicable to the removal of mycoplasmas from biopharmaceutical process streams. The small size and deformability of the organisms require the use of sterilizing filters with a pore-size rating finer than 0.2 µm, and mycoplasma-retentive filters having pore size ratings of 0.1 µm are available for this purpose.[2]

As with conventional sterilizing filtration, many factors influence the retention of mycoplasmas, including filter type and pore size rating; organism(s), concentration, and nutritive state; composition and viscosity of the filtered fluid; and filtration conditions, including differential pressure, contact time, and volume filtered. For example, nutritional conditions can affect the size of *Acholeplasma laidlawii*.[3] High differential pressure can force deformable mycoplasmas through microporous filter membranes.[4] These factors must be considered when designing and conducting validation studies for mycoplasma removal.

Parenteral Drug Association (PDA) Technical Report No. 75[5] is a consensus method that can be used as the basis for determining the retentivity of the chosen mycoplasma removal filter under controlled conditions comparable to those specified in American Society for Testing and Materials (ASTM) Standard F838 for bacterial retention.[6] Mycoplasma retentivity in the consensus method is determined using a challenge of *A. laidlawii* at a concentration of $\geq 1 \times 10^7$ cfu/cm$^2$. The chosen filter must then be validated for mycoplasma retention under actual conditions of use with *A. laidlawii* as the challenge organism in the process fluid or product, as appropriate. If complete retention is not achievable, a log reduction value (LRV) may be calculated and used with a safety factor based on prefiltration mycoplasma bioburden to ensure sufficient mycoplasma removal.▲ (USP 1-Aug-2020)

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***Add the following:***

# ▲⟨1229.18⟩ VIRAL CLEARANCE METHODS

Viruses are infectious agents that cannot grow or reproduce apart from a living cell. They are substantially smaller than bacteria and cannot be removed by the typical sterilizing grade filter (see *Sterilizing Filtration of Liquids* ⟨1229.4⟩). Viruses are composed of genetic material, either DNA or RNA, and are surrounded by a protein coat and, in some viruses, by a membranous envelope. Viruses can introduce their DNA/RNA into living cells where they can replicate to spread the infection. Viruses cause a variety of diseases, e.g., common cold, influenza, warts, AIDS, Ebola, and smallpox. To minimize the risk to patients, sterile materials must be produced in a manner that minimizes the potential for viral contamination. Detailed treatment of this subject can be found in *Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin* ⟨1050⟩ and *Design, Evaluation, and Characterization of Viral Clearance Procedures* ⟨1050.1⟩.

Viruses are extremely diverse, vary widely in complexity, and can evolve rapidly, making their removal and destruction problematic. The greatest concern for viral removal is with the use of materials of an animal origin, such as biological processes where mammalian cells are used for the production of therapeutic proteins. Virus removal is of concern due to susceptibility of mammalian cells to viral contamination, difficulty in virus removal from mammalian cell culture processes specifically, and potential destruction of the biological product by removal/inactivation techniques. There are three major components to the prevention of viral contamination in biological processing:

1. Avoidance: Using cell lines and biological materials free of viral contamination to avoid initial ingress
2. Removal/Inactivation: Inclusion of viral clearance steps in the downstream processing of biologics to eliminate those present in the process and ancillary materials
3. Confirmation: Testing of in-process and finished materials to confirm absence of viral contaminants

Consideration for viral safety, the means for viral analysis, and the subsequent virus group selection for use in viral clearance studies can be found in ⟨1050⟩. The focus of this chapter is viral clearance methods:

"The objective of viral clearance studies is to assess process step(s) that can be considered to be effective in inactivating/removing viruses and to estimate quantitatively the overall level of virus reduction obtained by the process. This should be achieved by the deliberate addition ("spiking") of significant amounts of a virus to the crude material and/or to different fractions obtained during the various process steps and demonstrating its removal or inactivation during the subsequent steps" (*1*).

In the execution of viral clearance studies, the selection of appropriate viral challenges must consider the expected viral contamination (as determined from the material origin) and non-specific model viruses representing other potential viral contaminants. Biologic manufacturing processes typically include multiple viral clearance steps relying on different clearance modalities to ensure effective removal of different types of viruses (*2*). The clearance performance of each method is demonstrated independently in a scale-down model of the manufacturing process. Regardless of the viral clearance methods utilized, testing against a panel of potential virus contaminants is expected. The viruses used in the challenge studies should include both endogenous and non-endogenous viruses as appropriate to assess potential specific and nonspecific viral contamination (*1,3*). Viral clearance capability of the various methods varies greatly and is commonly reported as a log reduction value (LRV).

The expectations for virus clearance vary with the specifics of the product and process. The expected overall LRV must consider the initial viral population and an additional margin of safety. Considerations in establishing the minimum LRV should include:

- The appropriateness of the test viruses used
- The design of the clearance studies
- The log reduction achieved
- The time dependence of inactivation
- The potential effects of variation in process parameters on virus inactivation and removal
- The limits of assay sensitivities
- The possible selectivity of inactivation and removal procedure(s) for certain classes of viruses (*1*)

There are varied means of virus removal and clearance. The most widely used methods are filtration, chromatography, thermal, chemical (solvent/detergent combinations), and radiation treatments.

## FILTRATION AND CHROMATOGRAPHIC METHODS

Various forms of filtration including ultrafiltration and nanofiltration can be used to capture larger viruses while allowing smaller materials to pass through the filter. The retention of viruses by filtration relies on principles similar to those for sterilizing filters as described in ⟨1229.4⟩. The primary mechanism for virus filtration relies on a sieving mechanism using filters with pore sizes smaller than the expected viral contamination as they may not be removed by the typical sterilizing grade filter. The filters used for viral removal may be rated by their approximate molecular weight cutoff (MWCO) or their average pore size and typically provide multiple log reductions in virus concentration. The filters used for viral removal must be evaluated to confirm their compatibility with the fluid being processed, which typically varies with its position in the manufacturing process.

Chromatography steps common in biologic manufacturing processes are also able to remove viral contamination. Size exclusion and other chromatographic processes can result in multiple log reduction of viruses (*4–5*). The effectiveness of viral retention varies with the virus type and the specific chromatography process.

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## THERMAL METHODS

Some viruses are susceptible to destruction by thermal means. An in-line high-temperature short-time (HTST) process may be able to preserve the essential quality attributes of protein materials (*6*). HTST treatment is one of the proven techniques for the treatment of commercial-scale cell culture medium to mitigate the potential risk of contamination by many viruses. Medium precipitates may have an adverse effect on virus inactivation due to clogging of the HTST system and altering the HTST parameters, affecting medium composition and cell growth. Therefore, it is essential to validate the HTST parameters (*7*–*8*). Confirmation of efficacy must be demonstrated empirically as there are no defined thermophysical conditions for inactivation of viruses.

## CHEMICAL METHODS

The common chemical means for viral clearance can include acidic or alkaline pH adjustments and solvent/detergent treatments. For example, low pH exposure (<4.0) is effective for the destruction of many enveloped viruses (*9*). Solvent/detergent processes can use incubation of materials in an organic solvent (e.g., tri-*N*-butyl phosphate) and detergent combination both of which must be removed afterward (*10*).

## RADIATION METHODS

Animal-derived materials pose a risk of introducing viruses and *Mycoplasma* and *Acholeplasma* into biological harvest fluid during the upstream manufacturing process using mammalian cell substrates. Available data indicates that gamma radiation (25–40 kiloGrays) causes complete inactivation of target viruses and *Mycoplasma*. Although the gamma irradiation is highly effective, the efficacy and viral kill kinetics in serum appear to be dependent on the size of the target virus (*11*).

The diversity of viruses and the potential for adventitious contamination have led to biological processes that typically include a variety of different viral clearance steps to provide the greatest margin of safety. The demonstration is commonly performed empirically using a virus panel representative of both potential intrinsic and extrinsic contaminants as there is limited ability to predict the effectiveness of viral removal and destruction processes. As with any other processes included in the ⟨1229⟩ series, it should be demonstrated that the sum of the viral clearance efforts do not adversely affect the biological drug substance.

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# ⟨1229⟩ STERILIZATION OF COMPENDIAL ARTICLES

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## BACKGROUND AND SCOPE

This general information chapter provides an overview of the concepts and principles involved in sterilization (by various modes) of compendial articles that must be sterile. It includes information about supportive sterilization processes utilized in their preparation.[1] More detailed recommendations are presented in specific information chapters for each sterilization mode:

- *Steam Sterilization by Direct Contact* ⟨1229.1⟩
- *Moist Heat Sterilization of Aqueous Liquids* ⟨1229.2⟩
- *Monitoring of Bioburden* ⟨1229.3⟩
- *Sterilizing Filtration of Liquids* ⟨1229.4⟩
- *Biological Indicators for Sterilization* ⟨1229.5⟩
- *Liquid-Phase Sterilization* ⟨1229.6⟩
- *Gaseous Sterilization* ⟨1229.7⟩
- *Dry Heat Sterilization* ⟨1229.8⟩
- *Physicochemical Integrators and Indicators for Sterilization* ⟨1229.9⟩
- *Radiation Sterilization* ⟨1229.10⟩
- *Vapor Phase Sterilization* ⟨1229.11⟩
- ▲*New Sterilization Methods* ⟨1229.12⟩
- *Sterilization-in-Place* ⟨1229.13⟩
- *Sterilization Cycle Development* ⟨1229.14⟩
- *Sterilizing Filtration of Gases* ⟨1229.15⟩
- *Prion Inactivation* ⟨1229.16⟩
- *Mycoplasma Sterilization* ⟨1229.17⟩
- *Viral Clearance Methods* ⟨1229.18⟩▲ (USP 1-Aug-2022)

In the strictest definition of sterility, a specimen is deemed sterile only when there is a complete absence of viable microorganisms (bacteria, yeasts, and molds), but sterility cannot be demonstrated with respect to compendial articles and other items because of the inherent limitations of the current test (see *Sterility Tests* ⟨71⟩). Sterility, therefore, is defined in probabilistic terms that establish an acceptable level of risk. Sterility can be accomplished only by the use of a validated sterilization process under appropriate current good manufacturing practices and cannot be demonstrated by reliance on sterility testing alone. The basic principles for control of sterilization processes, including method development, validation, and ongoing assurance, are as follows:

1. Sterilization process development that includes evaluation of the stability and compatibility of materials, container integrity, expected presterilization bioburden, equipment method control parameters, etc.
2. Identification of sterilization process parameters that preserve the inherent properties of the materials yet inactivate or remove microorganisms.
3. Demonstration that the sterilization process and equipment are capable of operating within the prescribed parameters and corresponding to independent measurements of the critical parameters.
4. Performance of replicate studies that represent the operational range of the equipment and employ actual or simulated product. The use of biological indicators for correlation between the measured physical parameters, and the expected lethality is recommended wherever possible.
5. Maintenance and monitoring of the validated process during routine operation.
6. Assurance that the bioburden (number and type) of the materials is acceptable and is maintained within predetermined limits during routine operation.

## VALIDATION OF STERILIZATION PROCESSES

Validation of sterilization processes requires knowledge of sterilization technology and use of the appropriate instrumentation and equipment to control and verify critical sterilization process parameters. An important aspect of the sterilization validation program involves the use of biological indicators when appropriate. All sterilization processes should be maintained in a state of validation that includes periodic requalification. The validation program for each type of sterilization comprises several formally documented stages.

The general principles of validation programs are applicable to all sterilization procedures. Individual details are presented in the specific *USP* informational chapters for each sterilization mode.

The process development stage investigates and establishes the operating parameters that define the controls that will be used for the sterilization process. Portions of the cycle development can be performed in a laboratory setting. The *installation qualification* stage establishes that equipment controls and other instrumentation are installed as specified and calibrated. Documentation should demonstrate the acceptability of the required utilities such as steam, water, and air. The *operational*

[1] These processes may also provide depyrogenation, the extent of which depends on the actual sterilization conditions. (Depyrogenation by various means ▲are addressed in the ⟨1228⟩ series of chapters.▲ (USP 1-Aug-2022))

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*qualification* stage confirms that the equipment functions within the defined sterilization parameters. The *performance qualification* stage of the validation program directly evaluates the sterilization of materials or articles. This determination requires independent parameter measurement during the sterilization process, as well as biological challenges in operational configurations. Correlation between the physical measurements and the demonstrated microbiological lethality or removal capability for sterilizing filtration methods supports the effectiveness of the sterilization process. The *routine process control* stage of the sterilization process requires a number of supportive practices and is outlined in detail below.

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## ESTABLISHING AND JUSTIFYING STERILIZATION PROCESSES THAT RELY ON MICROBIAL INACTIVATION

Articles intended to be sterile must attain a $\leq 10^{-6}$ probability of a nonsterile unit, i.e., less than or equal to one chance in one million that viable *bioburden* microorganisms are present. [NOTE—This is also called the sterility assurance level. The term probability of a nonsterile unit (PNSU) is used throughout this chapter because it is descriptive and substantially easier to understand.] This PNSU can be accomplished by balancing the method effect on the materials and the destruction of the bioburden (see *Figure 1*). Three methods are currently in use: overkill, bioburden/biological indicator, and bioburden-based methods. These methods are described in greater detail below. Regardless of the method chosen, the objective is a maximum PNSU of $\leq 10^{-6}$ for the bioburden. An overkill method is the simplest method to establish but has the greatest impact on materials. The bioburden-based method requires the most method control but subjects the materials to the least stressful conditions. Confidence in the process's lethality is the same, regardless of the method utilized.

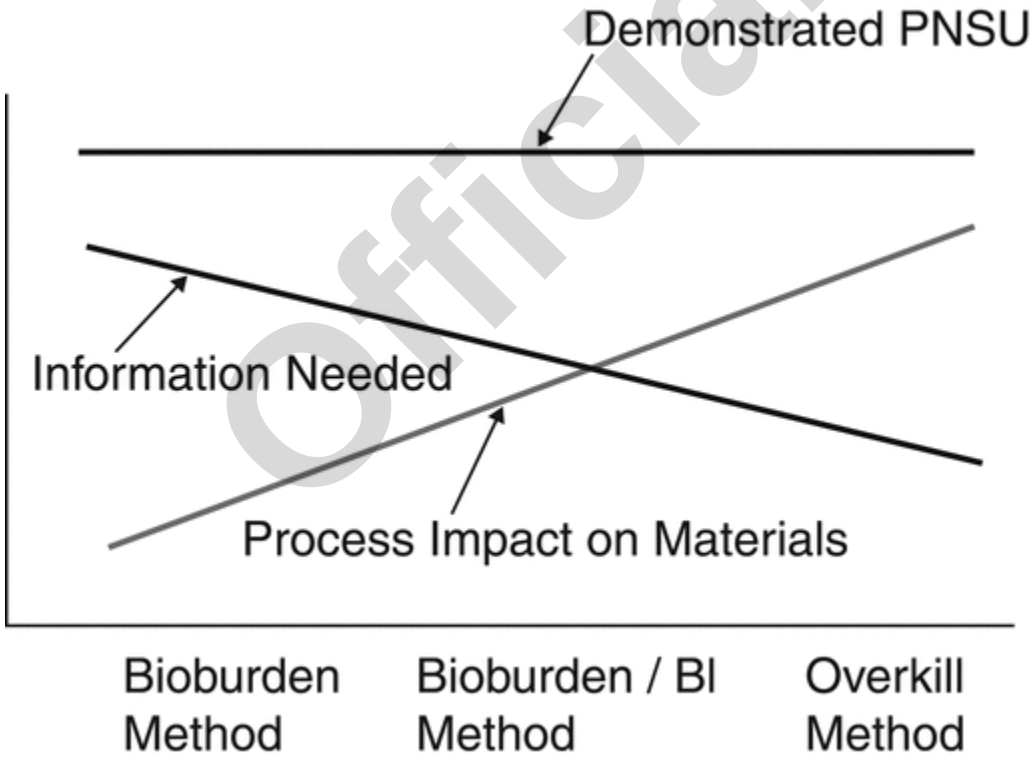


Figure 1. A basic comparison of various validation methods.

For items that are essentially unaffected by the sterilization process, the overkill method is preferred because of its simplicity. Overkill sterilization can be defined as a method in which the destruction of a high concentration of a resistant microorganism supports the destruction of reasonably anticipated bioburden present in routine processing. That objective can be demonstrated by attaining any of the following: a defined minimum lethality; a defined set of method conditions; or confirmation of minimum log reduction of a resistant biological indicator.

If articles could be damaged by extended exposure to the sterilization process, it may not be feasible to employ an overkill method. In these instances, demonstrating the effectiveness of the sterilization cycle requires not only information about the delivered method conditions but also knowledge about resistance and control of the population of the materials' bioburden. This method is widely used for the terminal sterilization of heat-labile solutions and laboratory media. This sterilization strategy is variously called the bioburden/biological indicator or combination based method and is defined thus:

> Bioburden/biological indicator based sterilization is an approach in which the incomplete destruction (or destruction of a modest population) of a resistant biological indicator can be used to demonstrate the capability of the method to reliably destroy the bioburden present. This is accomplished using detailed knowledge of the bioburden and biological indicator populations and their relative resistance.

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The bioburden-based method is used when material stability is limited or when there are no suitable biological indicator microorganisms available to use with the sterilizing process. Customarily, radiation sterilization is validated using the bioburden based method. The bioburden-based method can be defined as:

> A method in which bioburden samples from the material are routinely evaluated for resistance to the sterilization process and may be utilized to demonstrate the effectiveness of the process. Routine monitoring of the bioburden population, and its resistance to the sterilization process is mandatory.

Filter sterilization of liquids and gases differs from other sterilization modes because filtration relies on removal of microorganisms from the fluid rather than inactivation by chemical or physical means.

## *D*-Value and Microbial Resistance

The *D*-value is the time (customarily in minutes) or radiation dose (customarily in kiloGrays) required to reduce the microbial population by 90% or 1 $\log_{10}$ cycle (i.e., to a surviving fraction of 1/10) and must be associated with the specific lethal conditions at which it was determined (see *Figure 2*). For steam and dry heat, the *D*-value is a function of temperature. In gas sterilization (ethylene oxide, chlorine dioxide, or ozone), *D*-values are a function of the chemical concentration, relative humidity, and temperature. Similarly, for liquid chemical sterilization the *D*-value is a function of the temperature and sterilant concentration. [NOTE—Determining the *D*-value for vapor (condensing) systems such as hydrogen peroxide, hydrogen peroxide plasma, and peracetic acid is complex because of the biphasic nature of the materials. Radiation and filtration sterilization are validated using unique methods. These processes are validated by methods that differ from those in this introductory chapter.]

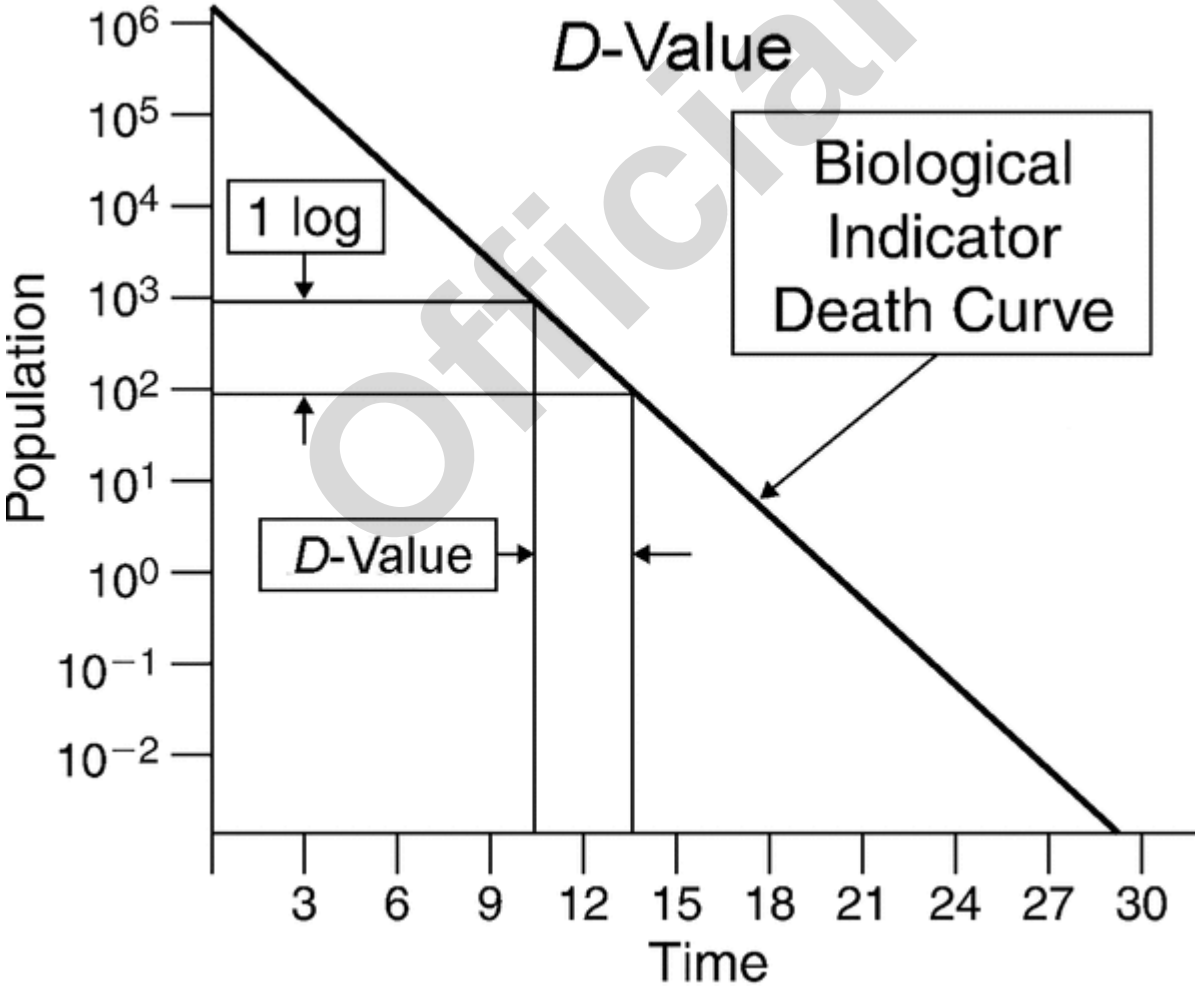


Figure 2. Graphical representation of *D*-value.

The *D*-value is not an inherent attribute of the microbe only, so the influence of other factors such as substrate, matrix, recovery media, and test methodology must be considered in *D*-value determination. The resistance of a biological indicator is defined for the indicator as a system. Accurate assessment and comparison between *D*-values requires standardization of test methods. To properly evaluate the effectiveness of a sterilization process, analysts must evaluate the resistance of the bioburden experimentally or via a literature search.

The death curve for microorganisms subjected to a sterilization process is comprised of three distinct regions (see *Figure 3*):

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1. *Survivor curve region*—Where viable microorganisms can be recovered and counted to determine the slope of the death curve. Using survivor counts in short exposure periods, the first section of the death curve can be drawn to where the population is approximately 10 cfu.
2. *Fraction negative region*—Where replicate studies with multiple biological indicators are used to estimate the slope. This can extend the demonstrable portion of the death curve to approximately $10^{-2}$to $10^{-3}$.
3. *Estimated region*—Where the death rate curve established by either the survivor curve method or fraction negative method is extrapolated to the desired PNSU. Below $10^{-3}$ the death curve is assumed to be linear and is depicted in *Figure 3* by the dashed line beyond the point assuming that the death of microorganisms continues at the same rate.

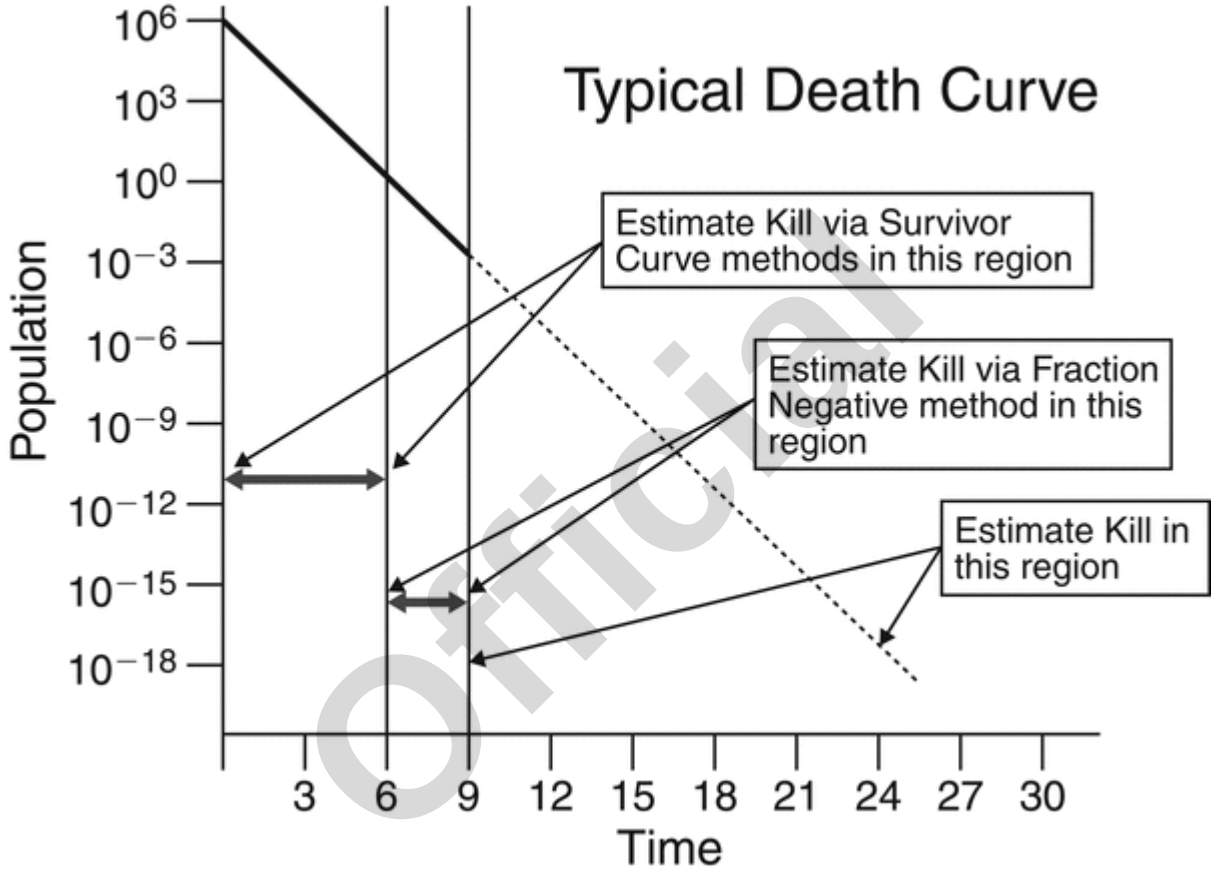


Figure 3. Death curve showing the various regions.

As stated earlier, the goal of the sterilization process is inactivation of the bioburden without adversely affecting product quality attributes. Demonstration of the lethality of a sterilization process under routine operation relies on differences in the relative resistance and population of the bioburden relative to the biological indicator (see *Figure 4*). Where the overkill method is used, bioburden controls can be less rigorous.

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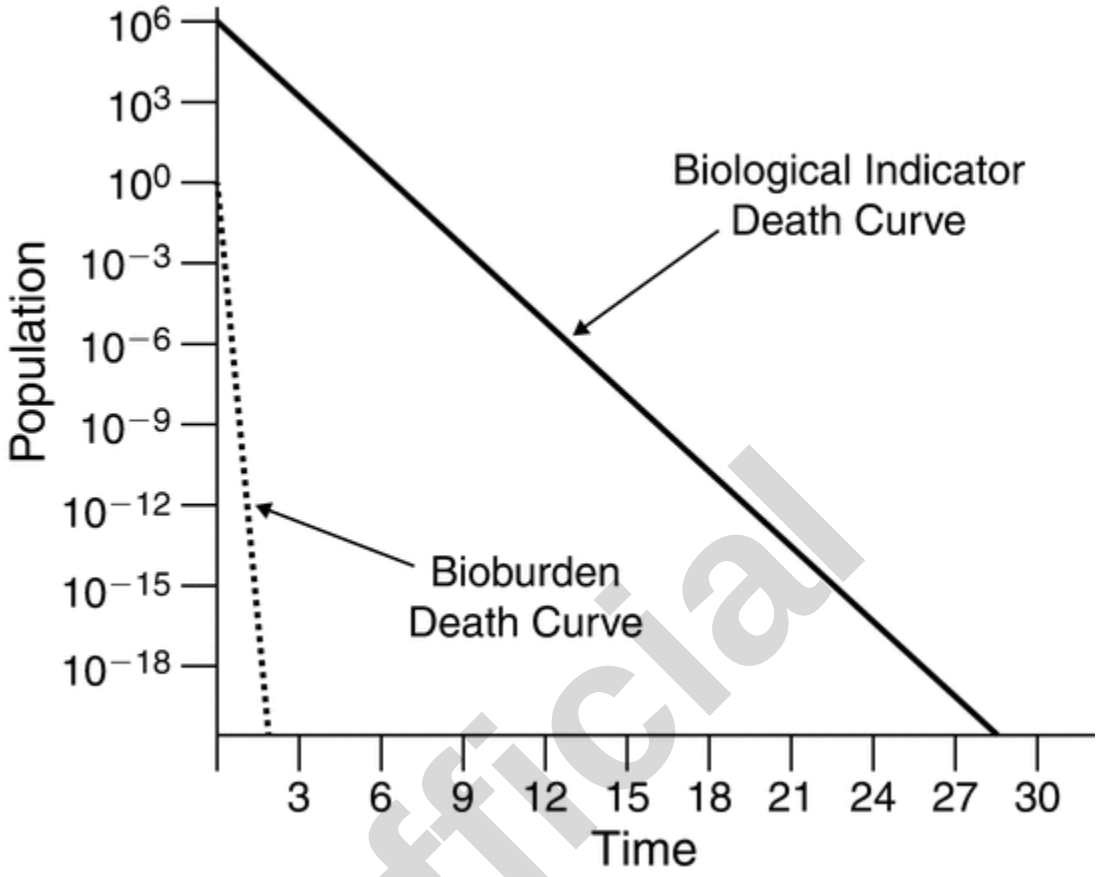


Figure 4. Relative resistance and population of typical biological indicator and bioburden microorganisms.

Validation of sterilization processes links physical measurements with biological indicator performance to establish method lethality. [NOTE—In radiation sterilization bioburden response is linked to physical irradiation dosage measurements.] Knowledge of the method's effectiveness coupled with bioburden controls on the materials under processing and information on bioburden resistance allow determination of the probability of a nonsterile unit.

Analysts must know the resistance of the biological indicator to the process in order to ensure that the organism's response to the process is properly understood. Equally important is an understanding of the bioburden present during routine use of the sterilization process and its possible resistance to the chosen process.

### Biological and Physical Data

The biological indicator, when used, is a convenient means to simplify the sterilization validation effort. Biological indicators customarily are bacterial spores that have established resistance to the sterilization process under evaluation. When supplied as spores (on a substrate or as a suspension) with known initial population and resistance, their response to the method can be correlated to the measured physical conditions. Spores are preferable as biological indicators because their resistance and population are predictable and stable when they are handled, stored, and used as recommended. ▲Additional details about biological indicators can be found in *Biological Indicators for Sterilization* ⟨1229.5⟩.▲ (USP 1-Aug-2022)

The spores can be placed in the sterilization load in locations where physical parameter measurements such as temperature or gas concentration cannot be easily obtained (e.g., within needle lumens, syringes, and ampules) or where measurement may alter the delivered conditions (e.g., sampling of the lethal gas). The biological indicator provides a means to directly assess the sterilizing effect of the method in a manner not possible by physical measurements. The lethality-based physical measurement and biological inactivation data from a sterilization process should be in reasonable agreement. When this is not the case, an investigation should be considered.

## STERILIZATION INDICATORS AND INTEGRATORS

The execution of sterilization processes can be supported by physical and chemical indicators and integrators that provide an indication that processing is completed. These are available in many different forms for use in conjunction with many common sterilization processes.

Sterilization indicators respond to sterilization process parameters in a nonquantitative fashion; i.e., they show passing or failing results. They are useful in an operating environment as a means to identify whether an item has been exposed to a

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sterilization process. They are of minimal use in directly establishing process efficacy. Sterilization integrators are more sophisticated devices that react quantitatively in response to one or more of the critical sterilization parameters and yield a result that can be correlated to lethality. The most sophisticated integrators are radiation dosimeters that are so accurate and robust that their use has displaced the use of biological indicators for the validation of radiation sterilization. Additional detail about integrators and indicators can be found in *Physicochemical Integrators and Indicators for Sterilization* ⟨1229.9⟩.

## STERILIZATION PROCESS DEVELOPMENT

An important consideration in any sterilization activity is the selection of an appropriate process from the many possible alternatives: steam, dry heat, gas, radiation, vapor, chemical, or filtration. The choice of the appropriate method for a given item requires knowledge of the sterilization process and information concerning effects of the process on the material being sterilized. The selection of a particular sterilizing treatment and the details of its execution often represent a compromise between the conditions required to destroy or remove the bioburden to the desired level and the impact of the sterilization process on the materials being processed. Sterilization processes should be sufficiently robust for certainty of microbial inactivation while avoiding adverse consequences to material quality attributes.

The overkill method employs conditions that are capable of destroying a high concentration of a resistant biological indicator and thus are a greater challenge to material integrity and stability. Overkill is employed only where the items being sterilized can withstand extended exposure to the sterilizing process and is used most commonly for metal, glass, and other items that are unaffected by process exposure. Its use is always preferable where materials can tolerate the more aggressive conditions utilized.

The half-cycle validation method is a special case of the overkill method in which a lethal cycle to the biological indicator is arbitrarily doubled. Its use unnecessarily exposes materials to harsh conditions, and it should be avoided.

Bioburden/biological indicator (or combination) methods are appropriate when the product has some sensitivity to the sterilizing conditions. Analysts commonly use it for large- and small-volume parenterals, in-process solutions, and laboratory media for which the material properties would be impaired by a lengthy exposure to the sterilizing conditions. The proper use of the method requires control over the presterilization bioburden levels and confidence that the bioburden's resistance is such that it will be destroyed during processing. The complete destruction of or the use of a high population of the bioburden/biological indicator is not necessary for use of this method because it relies on differences in the relative resistance and population of the biological indicator and bioburden microorganisms.

The bioburden method bases the sterilization duration solely on the expected population and resistance of the bioburden on the materials. This is the method of choice for all radiation sterilization. It relies on periodic bioburden monitoring and resistance screening to establish confidence in the method. The bioburden method does not require use of a biological indicator.

Filtration is used for liquids and gases that either will not withstand heat, radiation, or chemical sterilization processes or are more conveniently sterilized in-line.

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## ROUTINE PROCESS CONTROL

After a sterilization process has been initially validated, it must be maintained in that state to ensure continued acceptable operation. This is accomplished by a number of related activities that are essential for continued use of the method.

*Calibration*—Equipment instrumentation must be periodically calibrated against a traceable standard. This includes recording as well as controlling instruments that regulate the operation of the equipment.

*Physical measurements*—Data reported by the equipment sensors and recorders must be verified after the completion of each sterilization cycle. The records from the sterilization equipment are an essential part of the documentation.

*Physical integrators/indicators*—When they are used, integrators, and to a lesser extent indicators, provide an immediate indication of method execution and differentiate between processed and unprocessed materials. When these integrators provide a direct indication of method lethality (e.g., radiation dosimeters), they can be used for material release.

*Parametric release*—The release of finished products without sterility testing is addressed in *Terminally Sterilized Pharmaceutical Products—Parametric Release* ⟨1222⟩.

*Additional considerations*—Depending on the specifics of the particular sterilization process, there may be additional requirements for confirming method efficacy. These can include bioburden sampling, bioburden resistance determination ▲(see *Methods for Determination of Resistance of Microorganisms to Sterilization Processes* ⟨56⟩),▲ (USP 1-Aug-2022) biological indicator resistance determination, and supplier audits. As applicable, these activities should be carried out to maintain the sterilization process in a validated state.

*Change control*—To maintain a validated state, materials, procedures, and equipment that influence the sterilization process should be monitored to ensure that all changes are recorded and evaluated in terms of their potential impact. To accomplish this, analysts must establish a formalized system for change control.

*Preventive maintenance*—The user should establish a defined preventive maintenance program for each piece of sterilizing equipment in accordance with the equipment manufacturer's written recommendation. Preventive maintenance represents a special class of predefined changes that have no adverse effects on the operation of the system and thus do not require evaluation under change control.

*Periodic reassessment*—In the absence of change to the materials, method, or equipment, the effectiveness of sterilization processes should be reconfirmed on a periodic basis. This system should be formalized and should address the potential impact of a number of de minimis changes or undetected changes to the validated system. In the absence of change, the amount of information required to support a sterilization process is less than that required for initial acceptance of the sterilization process.

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*Training*—Sterilization processes rely heavily on scientific principles for the effective destruction of microorganisms. Scientists and engineers well-grounded in the principles of microbial death and removal develop processes to ensure effective sterilization. Individuals involved in the development of sterilization processes require a background in microbiology, physics, chemistry, and engineering, and they must be familiar with good manufacturing principles and regulations. Sterilization is an interdisciplinary activity where the combined knowledge of a group of individuals is generally required for the establishment of a reliable process. In addition to the sterilization process development team, individuals responsible for maintenance and operation of sterilization processes must also be trained appropriately to ensure that their actions contribute to success. These individuals are often the first to identify upsets and shifts in process performance because of their intimate involvement with it. Effective training programs should be established and documented. Training programs should emphasize sterilization principles, adherence to established processes and procedures, and the importance of documenting deviations from normal operations.

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# ⟨1230⟩ WATER FOR HEMODIALYSIS APPLICATIONS

## GENERAL PURIFICATION CONSIDERATIONS

Chemical and microbial components that can be found in drinking water meeting U.S. Environmental Protection Agency National Primary Drinking Water Regulations (or equivalent) may have the potential to produce significant negative effects in patients undergoing hemodialysis. It is, therefore, necessary to subject the water to further treatment to reduce these components to acceptable levels. The *Water for Hemodialysis* monograph provides bacterial and chemical tests that are required to ensure patient safety. Additional testing is recommended as follows:

1. Excess levels of aluminum, fluorides, and chlorine may be found seasonally in drinking water as a result of chemicals used in water treatment. These components should be monitored in Water for Hemodialysis being produced in accordance with established standard operating procedures. The maximum acceptable levels of these and other elements and compounds, as proposed by AAMI (Association for the Advancement of Medical Instrumentation) are listed in *Table 1*. These attributes should be periodically monitored to ensure they are being controlled by the routine testing performed in accordance with the *Water for Hemodialysis* monograph.
2. A comprehensive validation testing of the system producing Water for Hemodialysis should be performed initially and periodically thereafter to ensure that the water treatment equipment and system sanitization processes are functioning properly.

**Table 1. Maximum Allowable Chemical Levels in Water for Hemodialysis** (water used to prepare dialysate and concentrates from powder at a dialysis facility and to reprocess dialyzers for multiple use)*

| Element or Compound | Maximum Concentration (mg/L) |
|---|---|
| *Contaminants with documented toxicity in hemodialysis* | |
| Aluminum | 0.01 |
| Chloramines | 0.1 |
| Free chlorine | 0.5 |
| Copper | 0.1 |
| Fluoride | 0.2 |
| Lead | 0.005 |
| Nitrate (as N) | 2 |
| Sulfate | 100 |
| Zinc | 0.1 |
| *Contaminants normally included in dialysate* | |
| Calcium | 2 (0.1 mEq/L) |
| Magnesium | 4 (0.3 mEq/L) |
| Potassium | 8 (0.2 mEq/L) |
| Sodium | 70 (3.0 mEq/L) |
| *Other contaminants* | |
| Antimony | 0.006 |
| Arsenic | 0.005 |
| Barium | 0.1 |
| Beryllium | 0.0004 |
| Cadmium | 0.001 |
| Chromium | 0.014 |
| Mercury | 0.0002 |
| Selenium | 0.09 |
| Silver | 0.005 |
| Thallium | 0.002 |

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The chemical limits included in *Table 1* have been recognized by federal government agencies as standards for Water for Hemodialysis. Written standard operating procedures for water testing should be established by the physician in charge or the designated facility manager. The test frequency decision should be based upon historical data analysis, the quality of the source water as reported by the municipal water treatment facility or public health agency in the area, etc. Records should be maintained to document levels and any necessary remedial action taken promptly.

Chemical analysis of water components listed should be performed using methods referenced in the American Public Health Association's *Standard Methods for the Examination of Water and Wastewater,* 21st Edition,[1] those referenced in the U.S. Environmental Protection Agency's *Methods for the Determination of Metals in Environmental Samples,*[2] or equivalent methods as referenced in ANSI/AAMI RD 62:2006.

## MICROBIAL CONSIDERATIONS

The *Water for Hemodialysis* monograph includes total aerobic microbial count (TAMC) limits of 100 cfu/mL and endotoxin limits of 1 USP Endotoxin Unit/mL. In addition, the absence of *Pseudomonas aeruginosa* should be routinely determined because this is an opportunistic pathogen hazardous to acutely ill hemodialysis patients. Both the high microbial counts and the presence of *Pseudomonas aeruginosa* can be associated with inadequate water system maintenance and sanitization. Sampling the water should be done at all use points where the water enters the dialysis equipment. Samples should be assayed within 30 minutes of collection or immediately refrigerated and then assayed within 24 hours of collection. The microbial enumeration and absence tests are performed using procedures found in the USP general test chapters *Microbial Enumeration Tests* ⟨61⟩ and *Tests for Specified Microorganisms* ⟨62⟩. Quantification of bacterial endotoxins is performed using procedures found in the USP general test chapter *Bacterial Endotoxins Test* ⟨85⟩.

Because of the incubation time required to obtain definitive microbiological results, water systems should be microbiologically monitored to confirm that they continue to produce water of acceptable quality. "Alert" and "Action Levels" are therefore necessary for the monitoring and control of the system. An Alert Level constitutes a warning and does not require a corrective action. An Action Level indicates a drift from normal operating conditions and requires that corrective action be taken to bring the process back into the normal operating range. Exceeding an Alert or Action Level does not imply that water quality has been compromised. The maximum recommended Action Level for a total viable microbial count in the product water should be no greater than 25 cfu/mL, and the maximum recommended Action Level for bacterial endotoxins should be no greater than 0.25 USP Endotoxin Unit/mL. As with all process control values, Action and Alert Levels should be established from normal system monitoring trends and process capabilities in a fashion that allows remedial actions to occur in response to process control level excursions well before specifications are exceeded (also see *Microbial Considerations* under *Water for Pharmaceutical Purposes* ⟨1231⟩).

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[1] American Public Health Association, Washington, DC 20005.
[2] U.S. Environmental Protection Agency Publication EPA-600-R-94-111, Cincinnati, OH.

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# ⟨1231⟩ WATER FOR PHARMACEUTICAL PURPOSES

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## 1. INTRODUCTION

Water is widely used as a raw material, inactive ingredient, medicinal vehicle, and solvent in the processing, formulation, and manufacture of pharmaceutical products (dosage forms), active pharmaceutical ingredients (APIs), API intermediates, compendial articles, and analytical reagents as well as in cleaning applications.

This informational chapter on pharmaceutical water topics includes some of the chemical and microbiological concerns unique to water and its preparation and uses. The chapter provides information about water quality attributes (that may or may not be included within a water monograph) and processing techniques that can be used to improve water quality. It also discusses water system validation and gives a description of minimum water quality standards that should be considered when selecting a water source including sampling and system controls. It is equally important for water systems to be operated and maintained in a state of control to provide assurance of operational stability and therefore the capability to provide water that meets established water quality standards.

This chapter is intended to be educational, and the user should also refer to existing regulations or guidelines that cover US and international [International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) or World Health Organization (WHO)] good manufacturing practice (GMP) issues, as well as operational and engineering guides and/or other regulatory guidance for water [e.g., from the US Food and Drug Administration (US FDA), US Environmental Protection Agency (EPA), or WHO]. This chapter is not, and should not be considered, an all-inclusive document on pharmaceutical waters. It contains basic information and points to be considered for the processing, holding, monitoring, and use of water. It is the user's responsibility to ensure that:

1. The selection of the type and specifications of water is appropriate for its intended use.
2. Water production and quality meet applicable governmental regulations and guidance.
3. The pharmacopeial specifications for the types of water used in monographed articles are met.
4. Water used in the preparation of reagents for analysis or the performance of required tests meets USP requirements.

Control and monitoring of the chemical and endotoxin purity of waters are important for complying with the requirements of the monographs in this compendium. Attributes listed in *USP* monographs should be considered the "minimum" requirements. More stringent requirements may be needed for some applications to ensure suitability for particular uses. Basic guidance on the appropriate applications of waters can be found in the monographs and is also discussed further in this chapter.

Control of the microbiological quality of water is also important for many of its uses. This attribute is intentionally not specified in most water monographs. Microbiological control is discussed throughout this chapter, but especially in the following sections: *4. Validation and Qualification of Water Purification, Storage, and Distribution Systems*; *5. Design and Operation of Purified Water and Water for Injection Systems*; *6. Sampling*; *8. Microbial Evaluations*; and *9. Alert and Action Levels and Specifications*.

This chapter contains various chemical, microbiological, processing, and engineering concepts of importance to users of water. Water system validation, process control (PC) levels, and specifications are also presented later in this chapter.

## 2. SOURCE WATER CONSIDERATIONS

Source water is the water that enters the facility. The origin of this source water can be from natural surface waters like rivers and reservoirs, deep-bed well waters, sea waters, or some combination of these, potentially including multiple locations of each type of source water. Thus, source water can be supplied from these various origins (public or private), from municipalities' on-site water sourcing, or by external delivery such as a truck. It is possible that source water may not be potable and safe to drink. Such water may require pretreatment to ensure that it meets drinking water standards. It is the responsibility of the users of any source water to ensure that the water used in the production of drug substances (or API), as well as water for indirect drug product contact or for purification system feed water purposes meets, at a minimum, drinking (i.e., potable) water standards as defined by the requirements of the National Primary Drinking Water Regulations (NPDWR) (40 CFR 141) issued by the US EPA or the drinking water regulations of the European Union (EU) or Japan, or the WHO drinking water guidelines

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(see *3.4 Drinking Water*). These regulations establish limits on the types and quantities of certain chemical and microbiological contaminants and ensure that the water will contain safe quantities of chemical and microbial species.

Where water supplies are from regulated water utility companies, less stringent monitoring may be possible because the attributes may be tested regularly and ensured by the supplier (see *9.4.5 Source Water Control*). Water being withdrawn from a nonregulated supply should be sampled and tested appropriately at a suitable frequency that takes into account local environmental and seasonal changes and other quality fluctuations. Testing should ensure conformance with one of the drinking water standards discussed above.

The use of water complying with one of these designated drinking waters as a source water allows water pretreatment systems to only be challenged to remove small quantities of potentially difficult-to-remove chemicals. Control of objectionable chemical contaminants at the source water stage eliminates the need to specifically test for some of them [e.g., trihalomethanes and elemental impurities (see *Elemental Impurities—Limits* ⟨ 232⟩ )] after the water has been further purified, assuming there is no opportunity for recontamination.

Source waters can be used for nonproduct contact purposes such as for non-contact cooling systems. Such water may not normally be required to meet drinking water standards. Under such circumstances, the quality standards for this water when used in a pharmaceutical facility should be subject to quality standards established by the user and defensible to regulatory agencies.

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## 3. WATERS USED FOR PHARMACEUTICAL MANUFACTURING AND TESTING PURPOSES

There are many different grades of water used for pharmaceutical purposes. Several are described in *USP* monographs that specify uses, acceptable methods of preparation, and quality attributes. These waters can be divided into two general types: bulk waters, which are typically produced on-site where they are used; and sterile waters, which are produced, packaged, and sterilized to preserve microbial quality throughout their packaged shelf life. There are several specialized types of sterile waters that differ in their designated applications, packaging limitations, and other quality attributes. Monographed waters must meet the quality attributes as specified in the related monographs, and any *Notes* appearing in those monographs should be considered and addressed.

With the exception of Bacteriostatic Water for Injection, the monographed bulk and sterile waters have a statement indicating that there are no added substances, or no added antimicrobial agents. In the case of antimicrobial agents, the purpose is to ensure that the sterile water product is rendered sterile based solely on its preparation, packaging, and storage. In the case of the more general statement, “no added substances”, this requirement is intended to mean “no added substances that aren’t sufficiently removed”. Two specific examples support this intention, but there are many examples. First, the use of softeners is commonplace. A softener replaces calcium and magnesium ions (also known as hardness ions) with sodium, so technically two sodium ions are added for each ▲hardness▲ (USP 1-Dec-2021) ion. The purpose of sodium displacement is to protect downstream equipment from the hard water. The sodium ions are eventually removed sufficiently, and this is proven when the water sample passes the test in *Water Conductivity* ⟨ 645⟩ . Another specific example is the use of ozone as a ▲sanitizing agent▲ (USP 1-Dec-2021) that is added to the storage tank for microbial control. This could be considered an added substance, unless the ozone is destroyed before use, as is normally the case. Other notable examples include the addition of chlorine to kill bacteria in the pretreatment system, use of bisulfite to chemically reduce chlorine to chloride and protect downstream equipment, and use of a nitrogen blanket for protection from atmospheric contamination.

There are also other types of water for which there are no monographs. These are waters with names given for descriptive purposes only. Many of these waters are used in specific analytical methods. The descriptive titles may imply certain quality attributes or modes of preparation, but these non-monographed waters may not necessarily adhere strictly to the stated or implied modes of preparation or specified attributes. Waters produced by other means or controlled by other test attributes, or even a monographed water, may equally satisfy the intended uses for these waters. It is the user’s responsibility to ensure that such waters, even if produced and controlled exactly as stated, are suitable for their intended use. Wherever the term “water” is used within this compendium without other descriptive adjectives or clauses, the intent is that water of no less purity than USP *Purified Water* be used (see *3.1.1 Purified Water*). A brief description of the various types of waters commonly associated with pharmaceutical applications and their significant uses or attributes follows.

*Figure 1* may be helpful in understanding some of the various types of waters, their preparation, and uses.

[a] Complying with US EPA NPDWR or the Drinking Water regulations of EU, Japan, or WHO.

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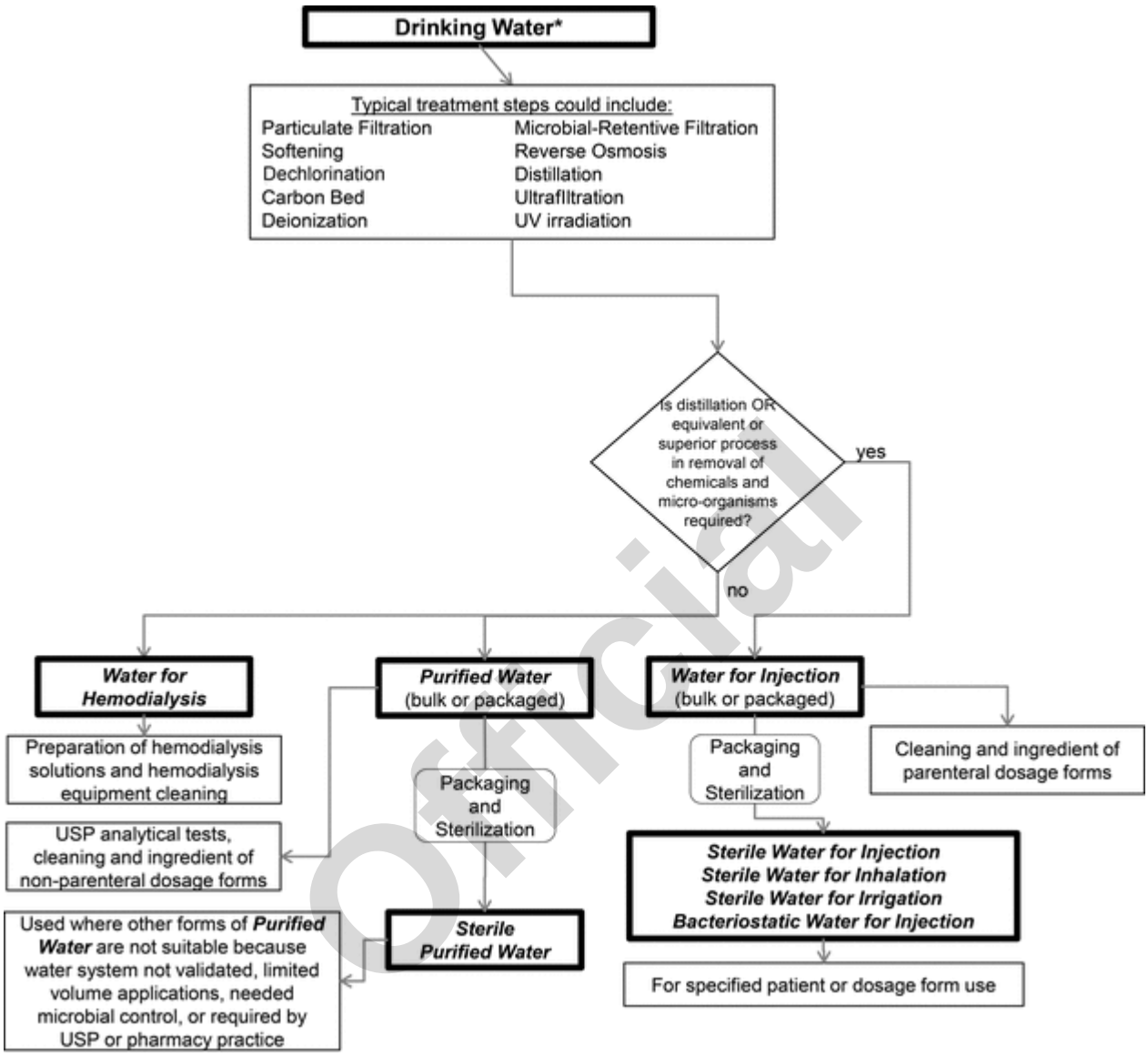


Figure 1. Water for pharmaceutical purposes.[a]

## 3.1 Bulk Monographed Waters and Steam

The following waters are generally produced in large volumes using a multiple-unit operation water system. These waters are typically distributed in a piping system for use at the same site.

### 3.1.1 PURIFIED WATER

*Purified Water* (see the *USP* monograph) is used as an excipient in the production of nonparenteral preparations and in other pharmaceutical applications, such as the cleaning of nonparenteral product-contact components and equipment. Unless otherwise specified, *Purified Water* also is to be used as the minimum water quality for all tests and assays in which "water" is indicated (see *General Notices, 8.230.30. Water in a Compendial Procedure*). This applies regardless of the font and letter case used in its spelling.

The minimal quality of source water for the production of Purified Water is drinking water whose attributes are prescribed by the US EPA, EU, Japan, or WHO. This source water may be purified using unit operations that include deionization, distillation, ion exchange, reverse osmosis, filtration, or other suitable purification procedures. Purified Water must meet the requirements for ionic and organic chemical purity and must be protected from microbial contamination. Purified Water systems must be validated to reliably and consistently produce and distribute water of acceptable chemical and microbiological quality. Purified Water systems that function under ambient conditions are particularly susceptible to the establishment of biofilms of microorganisms, which can be the source of undesirable levels of viable microorganisms or endotoxins in the water. These ambient Purified Water systems require frequent sanitization and microbiological monitoring to ensure that the water reaching the points of use has appropriate microbiological quality.

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The *Purified Water* monograph also allows bulk packaging for commercial use elsewhere. In contrast to Sterile Purified Water, packaged Purified Water is not required to be sterile. Because there is potential for microbial contamination and other quality changes in this packaged nonsterile water, this form of Purified Water should be prepared and stored in a manner that limits microbial growth, and/or should be used in a timely fashion before microbial proliferation renders it unsuitable for its intended use. Also, depending on the material used for packaging, extractable compounds could be leaching into the water from the packaging. Although this article is required to meet the same chemical purity standards as the bulk water, extractables from the packaging will likely render the packaged water less chemically pure than the bulk water. The nature of these impurities may even render the water an inappropriate choice for some applications. It is the user's responsibility to ensure fitness for use of this packaged article when it is used in manufacturing, clinical, or analytical applications where the purer bulk form of the water is indicated.

### 3.1.2 WATER FOR INJECTION

*Water for Injection* (see the *USP* monograph) is used as an excipient in the production of parenteral and other preparations where product endotoxin content must be controlled, and in other pharmaceutical applications, such as the cleaning of certain equipment and parenteral product-contact components.

The minimal quality of source water for the production of Water for Injection is drinking water whose attributes are prescribed by the US EPA, EU, Japan, or WHO. This source water may be treated to render it suitable for subsequent final purification steps, such as distillation (or whatever other validated process is used, according to the monograph). The finished water must meet all of the chemical requirements specified in the monograph, as well as an additional bacterial endotoxin specification. Because endotoxins are produced by the kinds of microorganisms that are prone to inhabit water systems, the equipment and procedures used by the system to purify, store, and distribute Water for Injection should be designed to control microbial contamination and must be designed to remove incoming endotoxins from the source water. Water for Injection systems must be validated to reliably and consistently produce and distribute this quality of water.

The *Water for Injection* monograph also allows bulk packaging for commercial use. In contrast to Sterile Water for Injection, packaged Water for Injection is not required to be sterile. However, to preclude significant changes in its microbial and endotoxins content during storage, this form of Water for Injection should be prepared and stored in a manner that limits microbial introduction and growth and/or should be used in a timely fashion before microbial proliferation renders it unsuitable for its intended use. Also, depending on the material used for packaging, extractable compounds could be leaching into the water from the packaging. Although this article is required to meet the same chemical purity standards as the bulk water, extractables from the packaging will likely render the packaged water less chemically pure than the bulk water. The nature of these impurities may even render the water an inappropriate choice for some applications. It is the user's responsibility to ensure fitness for use of this packaged article when it is used in manufacturing, clinical, or analytical applications where the purer bulk form of the water is indicated.

### 3.1.3 WATER FOR HEMODIALYSIS

*Water for Hemodialysis* (see the *USP* monograph) is used for hemodialysis applications, primarily the dilution of hemodialysis concentrate solutions. The minimal quality of source water for the production of Water for Hemodialysis is drinking water whose attributes are prescribed by the US EPA, EU, Japan, or WHO. Water for Hemodialysis has been further purified to reduce chemical and microbiological components, and it is produced and used on site. This water contains no added antimicrobial agents, and it is not intended for injection. Water for Hemodialysis must meet all of the chemical requirements specified in the monograph as well as an additional bacterial endotoxin specification. The microbial limits attribute for this water is unique among the "bulk" water monographs but is justified on the basis of this water's specific application, which has microbial content requirements related to its safe use. The bacterial endotoxins attribute is likewise established at a level related to its safe use.

### 3.1.4 PURE STEAM

*Pure Steam* (see the *USP* monograph) is also sometimes referred to as "clean steam". It is used where the steam or its condensate would directly contact official articles or article-contact surfaces, such as during their preparation, sterilization, or cleaning where no subsequent processing step is used to remove any impurity residues. These Pure Steam applications include, but are not limited to, porous load sterilization processes, product or cleaning solutions heated by direct steam injection, or humidification of processes where steam injection is used to control the humidity inside processing vessels where the official articles or their in-process forms are exposed. The primary intent of using this quality of steam is to ensure that official articles or article-contact surfaces exposed to it are not contaminated by residues within the steam.

The minimal quality of source water for the production of Pure Steam is drinking water whose attributes are prescribed by the US EPA, EU, Japan, or WHO, and which has been suitably treated. The water is then vaporized with suitable mist elimination and distributed under pressure. The sources of undesirable contaminants within Pure Steam could arise from entrained source water droplets, anticorrosion steam additives, or residues from the steam production and distribution system itself. The chemical tests in the *Pure Steam* monograph should detect most of the contaminants that could arise from these sources. If an official article is exposed to Pure Steam and it is intended for parenteral use or other applications where the pyrogenic content must be controlled, the Pure Steam must additionally meet the specification for *Bacterial Endotoxins Test* ⟨ 85⟩ .

These purity attributes are measured in the condensate of the article, rather than the article itself. This, of course, imparts great importance to the cleanliness of the process for Pure Steam condensate generation and collection, because it must not adversely impact the quality of the resulting condensed fluid.

Other steam attributes not detailed in the monograph, particularly the presence of even small quantities of noncondensable gases or the existence of a superheated or dry state, also may be important for applications such as sterilization. The large release of energy (latent heat of condensation) as water changes from the gaseous to the liquid state is the key to steam's sterilization efficacy and its efficiency, in general, as a heat transfer agent. If this phase change (condensation) is not allowed to happen

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because the steam is extremely hot and is in a persistent superheated, dry state, then its usefulness could be seriously compromised. Noncondensable gases in steam tend to stratify or collect in certain areas of a steam sterilization chamber or its load. These surfaces thereby would be at least partially insulated from the steam condensation phenomenon, preventing them from experiencing the full energy of the sterilizing conditions. Therefore, control of these kinds of steam attributes, in addition to its chemical purity, also may be important for certain Pure Steam applications. However, because these additional attributes are use-specific, they are not mentioned in the *Pure Steam* monograph.

Note that lower-purity "plant steam" may be used in the following applications: 1) for steam sterilization of nonproduct-contact nonporous loads, 2) for general cleaning of nonproduct-contact equipment, 3) as a nonproduct-contact heat-exchange medium, and 4) in all compatible applications involved in bulk pharmaceutical chemical and API manufacture.

Finally, because Pure Steam is lethal to microbes, monitoring of microbial control within a steam system is unnecessary, as is microbial analysis of the steam condensate.

## 3.2 Sterile Monographed Waters

The following monographed waters are packaged forms of either Purified Water or Water for Injection that have been sterilized to preserve their microbiological properties. These waters may have specific intended uses as indicated by their names and may also have restrictions on the packaging configurations related to those uses. In general, these sterile waters may be used in a variety of applications in lieu of the bulk forms of water from which they were derived. However, a substantial difference exists between the acceptance criteria for the chemical purities of these bulk waters versus sterile waters. The specifications for sterile waters differ from those of bulk waters to accommodate a wide variety of packaging types, properties, volumes, and uses. As a result, the inorganic and organic impurity specifications are not equivalent for bulk and packaged waters. The packaging materials and elastomeric closures are the primary sources of these impurities, which tend to increase over the shelf life of these packaged articles. Therefore, due consideration must be given to the chemical purity suitability at the time the sterile forms of water are used, when they are used in manufacturing, analytical, and cleaning applications in lieu of the bulk waters from which they were derived. It is the user's responsibility to ensure the fitness for use of these sterile packaged waters in these applications. Nevertheless, for the applications for each sterile water discussed below, their respective purities and packaging restrictions generally render them suitable by definition.

### 3.2.1 STERILE PURIFIED WATER

*Sterile Purified Water* (see the *USP* monograph) is Purified Water packaged and rendered sterile. It can be used in the preparation of nonparenteral compendial dosage forms or in analytical applications requiring Purified Water where 1) access to a validated Purified Water system is not practical; 2) only a relatively small quantity is needed; 3) Sterile Purified Water is required by specific monograph or pharmacy practice; or 4) bulk-packaged Purified Water is not suitably controlled for the microbiological quality for its intended use.

### 3.2.2 STERILE WATER FOR INJECTION

*Sterile Water for Injection* (see the *USP* monograph) is Water for Injection packaged and rendered sterile. It is used for extemporaneous prescription compounding and as a sterile diluent for parenteral products. ▲Water used for dilution or reconstitution of parenteral drug products must meet the requirements of Sterile Water for Injection (see *3.2.3 Bacteriostatic Water for Injection* for similar uses). This water at times is also called "Sterile Water for Dilution", "Sterile Water for Reconstitution", and similar terms.▲ (USP 1-Dec-2021) It also may be used for other applications where bulk Water for Injection or Purified Water is indicated but access to a validated water system is not practical, or where only a relatively small quantity is needed. Sterile Water for Injection is packaged in single-dose containers not larger than 1 L.

### 3.2.3 BACTERIOSTATIC WATER FOR INJECTION

*Bacteriostatic Water for Injection* (see the *USP* monograph) is Water for Injection packaged and rendered sterile, to which has been added one or more suitable antimicrobial preservatives. It is intended to be used as a diluent in the preparation of parenteral products, most typically for multi-dose products that require repeated content withdrawals. It may be packaged in single-dose or multiple-dose containers not larger than 30 mL.

### 3.2.4 STERILE WATER FOR IRRIGATION

*Sterile Water for Irrigation* (see the *USP* monograph) is Water for Injection packaged and sterilized in single-dose containers that may be larger than 1 L and allow rapid delivery of their contents. Because of its usage, Sterile Water for Irrigation is not required to meet *Particulate Matter in Injections* ⟨ 788⟩ . It also may be used in other applications that do not have particulate matter specifications, where bulk Water for Injection or Purified Water is indicated but where access to a validated water system is not practical, or where somewhat larger quantities are needed than are provided as Sterile Water for Injection.

### 3.2.5 STERILE WATER FOR INHALATION

*Sterile Water for Inhalation* (see the *USP* monograph) is Water for Injection that is packaged and rendered sterile and is intended for use in inhalators and in the preparation of inhalation solutions. This monograph has no requirement to meet ⟨ 788⟩ ; it carries a less stringent specification for bacterial endotoxins than Sterile Water for Injection, and therefore is not suitable for parenteral applications.

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## 3.3 Drinking Water

In addition to the bulk monographed waters described above, non-monographed waters also can be used in pharmaceutical processing steps such as cleaning and synthetic steps, and also as a starting material for further purification or testing purposes. Unless otherwise specified in the compendium, the minimum quality of water is *Purified Water*. [NOTE—The information in this chapter is not an all-inclusive discussion of all non-monographed waters identified in the *USP–NF*.]

Drinking water is an non-monographed water that can be referred to as "potable water" (meaning drinkable or fit to drink), "National Primary Drinking Water", "primary drinking water", or "US EPA Drinking Water". Except where a singular drinking water specification is stated (such as the US EPA NPDWR, as cited in 40 CFR Part 141), this water must comply with the quality attributes of either the NPDWR or the drinking water regulations of the EU or Japan, or the *WHO Guidelines for Drinking-Water Quality*. Drinking water may originate from a variety of sources including a public water supply, a private water supply (e.g., a well), or a combination of these sources (see *2. Source Water Considerations*).

Drinking water may be used in the early stages of cleaning pharmaceutical manufacturing equipment and product-contact components. Drinking water is also the minimum quality of water that should be used for the preparation of official substances and other bulk pharmaceutical ingredients. Where compatible with the processes, the contaminant levels allowed in drinking water are generally considered safe for use in preparing official substances and other drug substances. However, where required by the processing of the materials to achieve their required final purity, higher qualities of water may be needed for these manufacturing steps, perhaps even water as pure as Water for Injection or Purified Water. Such higher-purity waters, however, might require only selected attributes to be of higher purity than drinking water (see *Figure 2a* and *Figure 2b*). Drinking water is the prescribed source or feed water for the production of bulk monographed pharmaceutical waters. The use of drinking water specifications establishes a reasonable set of maximum allowable levels of chemical and microbiological contaminants with which a water purification system will be challenged. Because seasonal variations in the quality attributes of the drinking water supply can occur, it is important to give due consideration to its uses. The processing steps in the production of pharmaceutical waters must be designed to accommodate this variability.

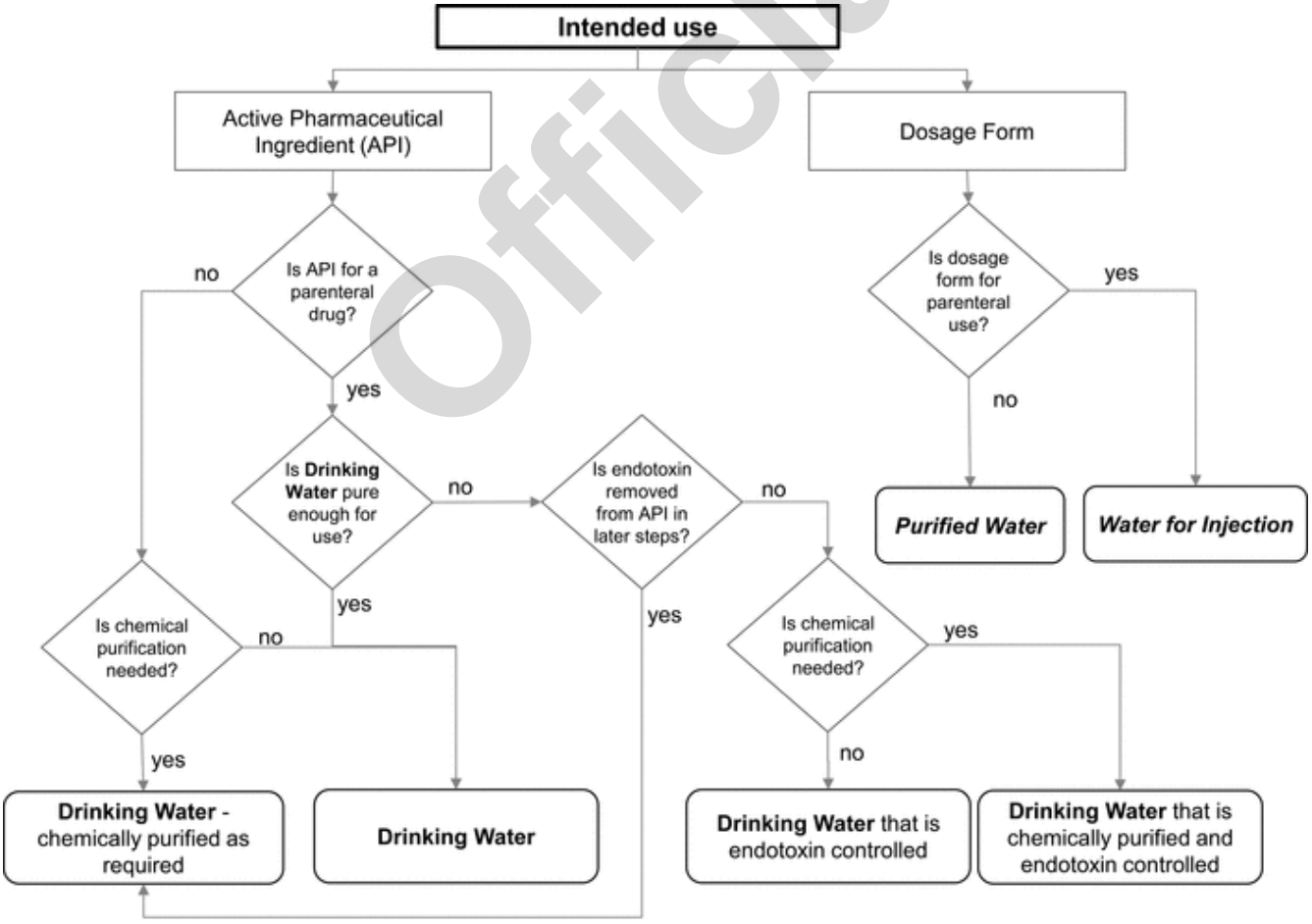


Figure 2a. Selection of water for pharmaceutical purposes: APIs and dosage forms.

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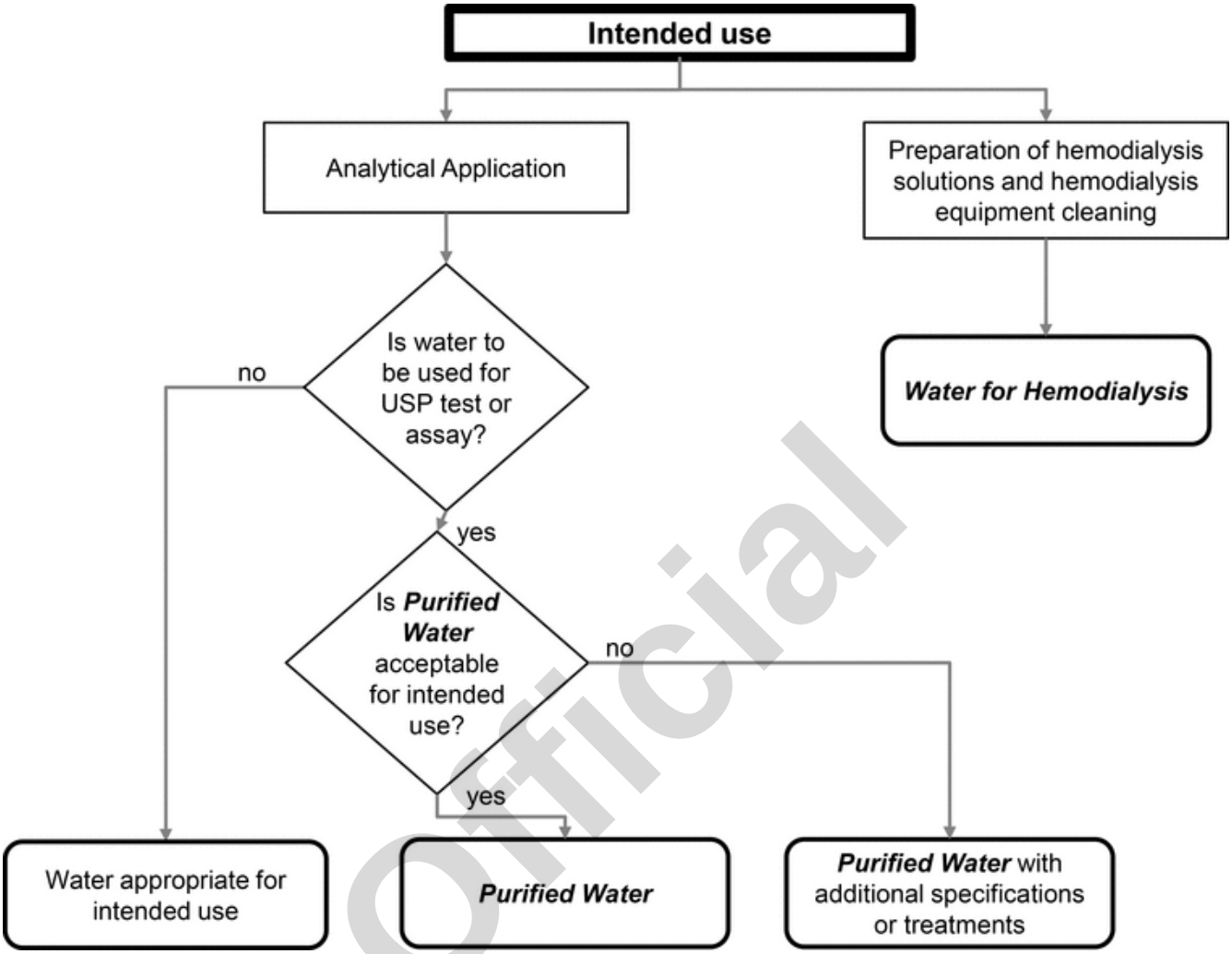


Figure 2b. Selection of water for pharmaceutical purposes: Analytical reagents.

## 3.4 Other Non-Monographed Waters

In addition to drinking water, this compendium discusses non-monographed waters with various other designations. These include waters of various quality levels for special uses such as, but not limited to, cleaning and testing purposes.

Both *General Notices* (see *General Notices, 8.230.30 Water in a Compendial Procedure*) and *Reagents, Indicators, and Solutions* clearly state that where the term "water" is indicated for use in analyses without grammatical qualification or other specification, the quality of the water must be *Purified Water*. However, numerous ▲▲ (USP 1-Dec-2021) qualifications do exist. Some of these qualifications involve adjectives describing methods of preparation, ranging from specifying the primary purification step to specifying additional purification. Other qualifications call for specific attribute "absences" to be met that might otherwise interfere with analytical processes. In most of these cases, the required attribute absences are not specifically tested. Sometimes, a further "purification process" is specified that ostensibly allows the water to adequately meet this required "absence attribute".

However, preparation instructions for many reagents were carried forward from the innovator's laboratories to the originally introduced monograph for a particular *USP–NF* article or general test chapter. The quality of the reagent water described in these tests may reflect the water quality designation of the innovator's laboratory. These specific water designations may have originated without the innovator's awareness of the requirement for *Purified Water* in *USP–NF* tests. Regardless of the original reason for the creation of these numerous special analytical waters, it is possible that the attributes of these special waters now could be met by the basic preparation steps and current specifications of *Purified Water*. In some cases, however, some of the cited post-processing steps are still necessary to reliably achieve the required attributes.

Users are not obligated to utilize specific and perhaps archaically generated forms of analytical water where alternatives with equal or better quality, availability, or analytical performance may exist. The consistency and reliability of operations for producing these alternative analytical waters should be verified so that the desired attributes are produced. In addition, any alternative analytical water must be evaluated on an application-by-application basis by the user to ensure its suitability. The following is a summary of the various types of non-monographed analytical waters that are cited in the *USP–NF*. This is not an exhaustive listing. Those listed are used in multiple locations. Several non-monographed analytical waters are not included below because they are only found in one or perhaps two locations within this compendium.

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Note that the names of many of the waters listed imply a very low chemical impurity level. For example, "deionized water" implies that all the ions have been removed. However, in most cases discussed below, exposure of the water to air will result in the ingress of carbon dioxide ($CO_2$), leading to the formation of bicarbonate and hydrogen ions. Therefore, the removal of ions cannot be completely maintained for most analytical applications.

### 3.4.1 AMMONIA-FREE WATER

From a functional standpoint, ammonia-free water must have a negligible ammonia concentration to avoid interference in tests sensitive for or to ammonia. Due to the nature of the uses of this water, *Purified Water* could be a reasonable alternative for these applications.

### 3.4.2 CARBON DIOXIDE-FREE WATER

Carbon dioxide-free water is a reagent defined in the *USP–NF* as Purified Water that has been vigorously boiled for NLT 5 min, and then cooled and protected from absorption of atmospheric carbon dioxide. Alternatively, this could be *Purified Water* that has a resistivity of NLT 18 Mohm-cm at 25°.

Because the absorption of atmospheric carbon dioxide lowers the pH of high-purity waters, most of the uses of carbon dioxide-free water are either associated as a solvent in pH-related or pH-sensitive determinations or as a solvent in bicarbonate-sensitive reagents or determinations.

The term "carbon dioxide-free water" is sometimes used improperly. Besides its use for pH or acidity/alkalinity tests, the purpose for using this water is not always clear. The intention could be to use water that was deaerated (free of dissolved air) or deionized (free of extraneous ions), or even Purified Water with an additional boiling step. Although boiling is highly effective for removing carbon dioxide as well as all other dissolved gasses, these gases are readily re-absorbed unless the water is protected. Even with protection, such as use of a stoppered container, re-absorption will occur over time as air will readily transmit through seals and diffuse through most materials. Deionization is also an efficient process for removing dissolved carbon dioxide. Carbon dioxide forms ionic bicarbonate in water and will be subsequently removed by ion-exchange resins. However, the same problem of carbon dioxide re-absorption will occur after the deionized water is exposed to air. Also, the deionization approach for creating carbon dioxide-free water does not deaerate the water or remove other dissolved gases such as oxygen ($O_2$); it only removes carbon dioxide and other ions.

Depending on the application, *Purified Water* may meet the requirements where carbon dioxide-free water is called for. This could also include pH or acidity or alkalinity tests. The pH of a sample of pure deionized water is, by definition, 7.0. When that same sample is exposed to typical environmental atmospheric conditions, the water sample will absorb carbon dioxide and result in a pH range of approximately 5.4–6.2 ($[H^+]$ is in the range of $4.0 \times 10^{-6}$ M to $6.3 \times 10^{-7}$ M). The added acidity caused by carbon dioxide absorption may be insignificant compared to the material being analyzed.

### 3.4.3 DISTILLED WATER

Distilled water is produced by vaporizing drinking water or a higher quality of water and condensing it into a purer state. It is used primarily as a solvent for reagent preparation, and it is also specified in the execution of other aspects of tests, such as for rinsing an analyte, transferring a test material as a slurry, as a calibration standard or analytical blank, and for test apparatus cleaning. Distilled water is also cited as the starting water to be used for making high-purity water (see *3.4.8 High-Purity Water*). Because none of the cited uses of this water imply a need for a particular purity attribute that can only be derived by distillation, water meeting the requirements for *Purified Water* derived by other means of purification or *Water for Injection* could be equally suitable where distilled water is specified. It is the user's responsibility to verify the suitability of Purified Water or Water for Injection.

### 3.4.4 FRESHLY DISTILLED WATER

Freshly distilled water or "recently distilled water" is produced in the same manner as distilled water and should be used soon after its generation. This implies the need to avoid endotoxin contamination, as well as any other forms of contamination from the air or containers, that could arise with prolonged storage. Freshly distilled water is used for preparing solutions for subcutaneous test-animal injections and for a reagent solvent in tests for which there appears to be no particularly high-water purity needed that could be ascribable to being "freshly distilled". In the test-animal application, the term "freshly distilled" and its testing use imply a chemical, endotoxin, and microbiological purity that could be equally satisfied by *Water for Injection* (although no reference is made to these chemical, endotoxin, or microbial attributes or specific protection from recontamination). For non-animal uses, water meeting the requirements for *Purified Water* derived by other means of purification and/or storage periods could be equally suitable where "recently distilled water" or freshly distilled water is specified. It is the user's responsibility to verify the suitability of Purified Water or Water for Injection.

### 3.4.5 DEIONIZED WATER

Deionized water can be produced by starting with either drinking water or Purified Water, depending upon monograph or testing procedures defined in the compendia. Deionized water is produced by an ion-exchange process in which the cations and anions are replaced with H+ and OH– ions by use of ion-exchange resins. Similar to distilled water, deionized water is used primarily as a solvent for reagent preparation, but it is also specified in the execution of other aspects of tests, such as for transferring an analyte within a test procedure, as a calibration standard or analytical blank, and for test apparatus cleaning. Also, none of the cited uses of this water imply any needed purity attribute that can only be achieved by deionization. Therefore, water meeting the requirements for *Purified Water* that is derived by other means of purification could be equally suitable where deionized water is specified. It is the user's responsibility to verify the suitability of Purified Water.

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### 3.4.6 DEIONIZED DISTILLED WATER

Deionized distilled water is produced by deionizing distilled water (see *3.4.5 Deionized Water*). This water is used as a reagent in a liquid chromatography test that requires a low ionic or organic impurity level. Because of the importance of this high purity, water that meets the requirements for *Purified Water* may not be acceptable. High-purity water (see *3.4.8 High-Purity Water*) could be a reasonable alternative to this water. It is the user's responsibility to verify the suitability of the alternative water used.

### 3.4.7 FILTERED WATER

Filtered water is Purified Water that has been filtered to remove particles that could interfere with the analysis where this water is specified. The term is sometimes used synonymously with "particle-free water" and "ultra-filtered water" and is cited in some monographs and general chapters as well as in *Reagents, Indicators, and Solutions*. Depending on its referenced location in *USP–NF*, it is variously defined as water that has been passed through filters rated as 1.2, 0.2, or 0.22 µm, or filters with an unspecified porosity rating. Even though the water names and the filter ratings used to produce these waters are defined inconsistently, the use of 0.2-µm or 0.22-µm filtered *Purified Water* should be universally acceptable for all applications where particle-free water, filtered water, or ultra-filtered water is specified.

### 3.4.8 HIGH-PURITY WATER

▲High-purity water is used as a reagent, as a solvent for reagent preparation, and for test apparatus cleaning in which less stringent water purity would not be considered acceptable. However, if a user's routinely available Purified Water meets or exceeds the conductivity parameters described in the following two paragraphs for high-purity water, it could be used in lieu of high-purity water. High-purity water is typically prepared by deionization (see *3.4.5 Deionized Water*) and protected from atmospheric contamination. Use water that is freshly prepared and is not stored as this may introduce contaminants.

For assays that require low-conductivity water (e.g., *Ion Chromatography* ⟨ 1065⟩ and *Plasma Spectrochemistry—Theory and Practice* ⟨ 1730⟩ ), a conductivity value of NMT 0.0556 µS/cm (NLT 18.0 Mohm-cm) may be appropriate. If the water of this purity makes contact with the atmosphere even briefly as it is being used or drawn from its purification system, its conductivity will immediately increase by as much as about 1.0 µS/cm at 25° as atmospheric carbon dioxide dissolves into the water and immediately dissociates and equilibrates to form hydrogen ($H^+$) and bicarbonate ($HCO_3^-$) ions. Therefore, if the analytical use requires that water conductivity remains as low as possible or the bicarbonate/carbon dioxide levels be as low as possible, the water should be protected from atmospheric exposure. If high-purity water is used for *Elemental Impurities—Procedures* ⟨ 233⟩ , exposure to carbon dioxide does not affect the analysis and a higher conductivity may be acceptable.

If the water is used for ⟨ 233⟩ , transport it in containers that do not leach elemental impurities. To definitely ensure that this water does not contain elemental impurities, an in-line conductivity of NMT 0.0571 µS/cm (NLT 17.5 Mohm-cm) at 25° is needed. However, dependent upon the impurity that will be analyzed, the presence of contaminating ions from other non-elemental impurity sources may result in a higher conductivity being observed that does not affect the performance of ⟨ 233⟩ .▲ (USP 1-Dec-2021)

### 3.4.9 DEAERATED WATER

Deaerated water or "degassed water" is Purified Water that has been treated to reduce the content of dissolved air by "suitable means" such as boiling, sonication, and/or stirring during the application of a partial vacuum, followed by immediate use or protection from air re-absorption.

### 3.4.10 OXYGEN-FREE WATER

Oxygen-free water is Purified Water that has been treated to remove or reduce dissolved oxygen. Such treatment could involve deaerating by boiling or sparging with an inert gas such as nitrogen or helium, followed by inert gas blanketing to prevent oxygen re-absorption. Any procedure used for removing oxygen should be verified as reliably producing water that is fit for use.

### 3.4.11 WATER FOR BACTERIAL ENDOTOXINS TEST

Water for Bacterial Endotoxins Test (BET) is also referred to as Limulus Amebocyte Lysate (LAL) Reagent Water. This type of water is often Water for Injection, which may have been sterilized. It is free from a level of endotoxin that would yield any detectable reaction or interference with the LAL reagent used in the BET (see ⟨ 85⟩ ).

## 4. VALIDATION AND QUALIFICATION OF WATER PURIFICATION, STORAGE, AND DISTRIBUTION SYSTEMS

### 4.1 Validation Requirement

Establishing the reliability of pharmaceutical water purification, storage, and distribution systems requires demonstrating control of the process through an appropriate period of monitoring and observation. Finished water is typically continuously produced and used, while product and process attributes may only be periodically assessed. The quality of bulk finished water cannot be established by only testing monograph attributes. The unit operations in the pharmaceutical water system need to demonstrate that they are in control through monitoring of the process parameters and water quality. The advent of using conductivity and total organic carbon (TOC) to define chemical purity allows the user to more quantitatively assess the water's

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chemical purity and its variability as a function of routine treatment system maintenance and regeneration. Treatment processes must also demonstrate control of microbial attributes within the overall system. Some unit operations that are needed for chemical treatment may significantly increase microbial and bacterial endotoxin levels. These are later controlled by downstream unit operations. Knowledge of the treatment system processes and the effectiveness of control measures is needed to ensure that the pharmaceutical waters are acceptable for use.

Efficacy of the design, operation, sanitization, and control of the pharmaceutical water system is demonstrated through the monitoring of chemical and microbial attributes. A typical water system validation program involves an initial increased frequency of monitoring of the treatment system process parameters and sampling and testing of major process points to demonstrate the ability to produce the acceptable water and to characterize the operation of the system. This is followed by a life cycle approach of validation maintenance and monitoring.

## 4.2 Validation Approach

Validation is the program of documenting, to a high level of assurance, that a specific process is capable of consistently delivering product conforming to an established set of quality attributes. A validation program qualifies and documents the design, installation, operation, and performance of the system. A graphical representation of a typical water system validation life cycle is shown in *Figure 3*.

The validation protocol should be based on the boundaries of the water system and the critical water quality and process attributes needed to maintain consistent performance. The system boundary may stop at the point of use or may include the water transfer process. If the transfer process from the distribution system outlets to the water use locations (typically either with hoses or hard-piped equipment connections) is defined as outside the water system boundary, then this transfer process still needs to be validated to not adversely affect the quality of the water as it is delivered for use. Because routine quality control (QC) microbial monitoring is performed for the same transfer process and components (e.g., hoses and heat exchangers) as that of routine water use (see *6.1.2 Quality Control Sampling*), there is some logic to include this water transfer process within the distribution system validation.

### 4.2.1 VALIDATION ELEMENTS

Validation is accomplished through the use of a structured, documented process. The phases of this process include Design Qualification (DQ), Installation Qualification (IQ), Operational Qualification (OQ), Performance Qualification (PQ), and Validation Maintenance. The process is documented in a validation protocol. The elements may be in individual protocols for each phase or integrated into variations of a DQ/IQ/OQ/PQ combined document format. The protocols are formally approved quality documents. Factory Acceptance Testing (FAT), Site Acceptance Testing (SAT), and commissioning testing of the system may supplement qualification tests for IQ or OQ provided that they are properly documented and reviewed, and if the system functionality is shown as unaffected by the transport and installation.

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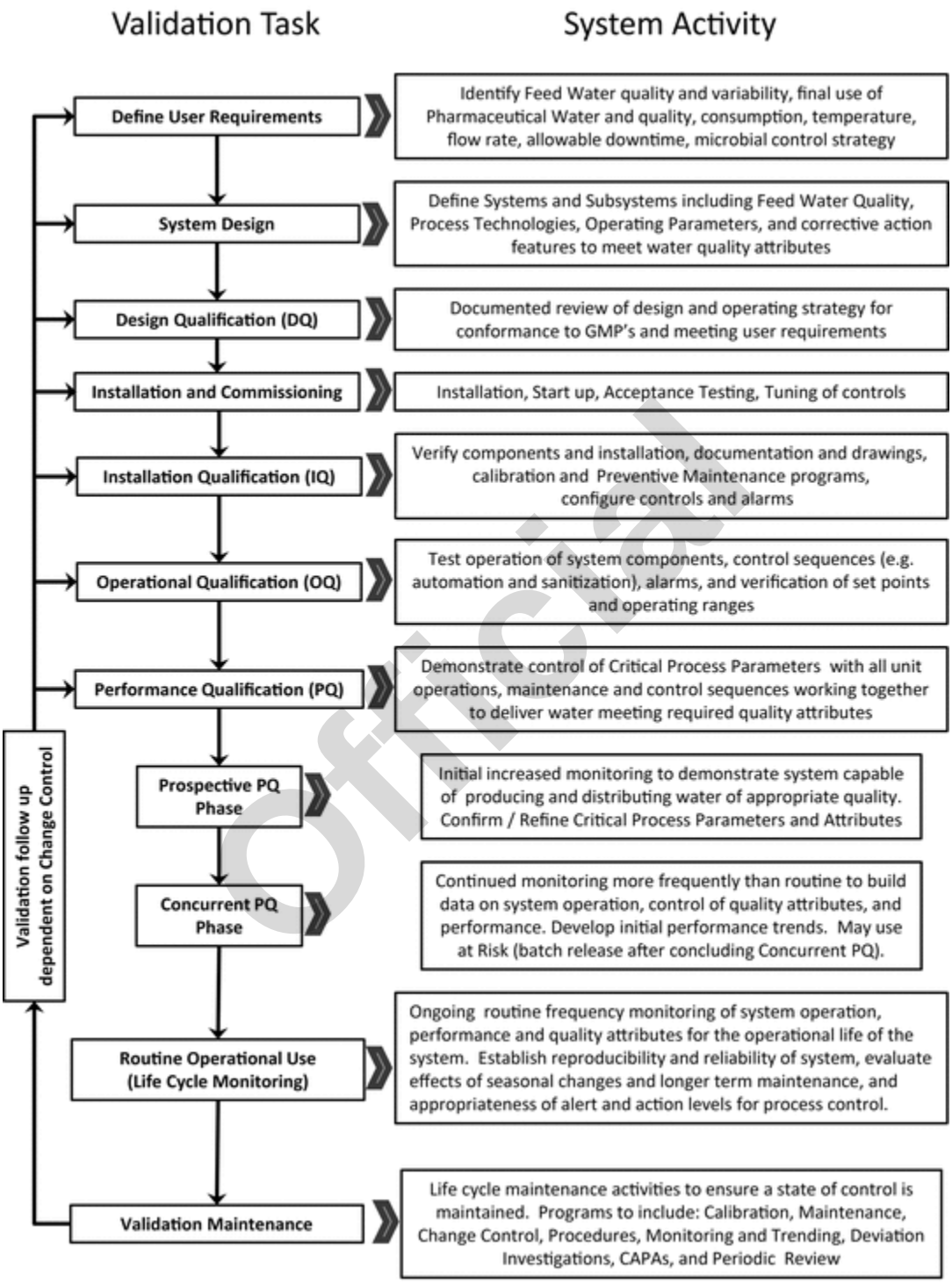


Figure 3. Water system validation life cycle.

### 4.2.2 USER REQUIREMENTS SPECIFICATION AND DESIGN QUALIFICATION

The user requirements for the water system should identify the design, operation, maintenance, and quality elements needed to produce the desired water type from the available source water, including its anticipated attribute variability. The essential elements of quality need to be built in at this stage and any GMP risks mitigated to an acceptable level.

The review of the specifications, system design, components, functions, and operation should be performed to demonstrate that the system complies with GMPs and verify that the design meets the user requirements. This documented review may be performed as part of the overall design process or as a separate DQ.

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#### 4.2.3 INSTALLATION QUALIFICATION

An IQ protocol for a water system confirms that the system has been properly installed and documented. This may include verification of components, piping, installation, and weld quality; documentation of the specifications for all system components present; inspections to verify that the drawings accurately depict the final configuration of the water system; and, where necessary, special tests to verify that the installation meets the design requirements. Additionally, the water system is readied for operational testing, including calibration of instruments, configuration of alarm levels, and adjustment of operating parameters (e.g., flow rate, pressure).

#### 4.2.4 OPERATIONAL QUALIFICATION

The OQ phase consisting of tests and inspections to verify that the equipment, system alerts, and controls are operating reliably and that appropriate Alert and Action Levels are established (this phase of qualification may overlap with aspects of IQ and PQ). During this phase of validation specific testing is performed for alarms, verifying control sequences, equipment functional checks, and verification of operating ranges. Standard operating procedures (SOPs) for all aspects of water system operation, maintenance, water use, water sampling, and testing, and other requirements should be in place and operator training completed. At the completion of the OQ, the water system has demonstrated that the components are operational and the system is producing suitable water.

#### 4.2.5 PERFORMANCE QUALIFICATION

The prospective PQ stage considers two aspects of the water system: critical process parameters and critical water attribute parameters. These are evaluated in parallel by monitoring the water quality and demonstrating acceptable quality attributes while demonstrating control of the process parameters (see *6.3 Validation Sampling Plans*). The initial PQ stage may result in refinement of process parameters to yield appropriate water quality. This PQ stage includes an increased frequency of monitoring for approximately 2–4 weeks, or sufficient time to generate adequate data to demonstrate that water meeting the appropriate quality attributes is produced and distributed. One of the reasons for this duration is that biofilm, the source of planktonic organisms in water samples, takes time to develop and to determine if the sanitization unit operations and processes are adequate to control microbial proliferation. The chemical control program adequacy is typically apparent in less time than it takes to see microbial control adequacy. However, chemical purification can be compromised by poor microbial control and, to a lesser degree, vice versa.

Once a level of control of microbial and chemical attributes has been demonstrated, the next phase of PQ is to continue the frequency of monitoring for approximately 2–4 weeks at a somewhat reduced level that will still give adequate data on system performance while using the pharmaceutical water. The water may be used for manufacturing at risk, and the associated products may be released only after water quality attributes have been determined to be acceptable and this validation phase has been completed. At the completion of the second phase, the data should be formally reviewed and the system approved for operational use.

### 4.3 Operational Use

When the water system has been placed into operational use, monitoring of the water quality attributes and the system process parameters is performed at a routine frequency (see *6.4 Routine Sampling Plans*) to ensure that they remain with a state of control during long-term variability from seasonal variations in source water quality, unit operation maintenance, system sanitization processes, and earlier-established Alert and Action Levels.

The water system should continue to be monitored and evaluated on an on-going basis following a life cycle approach using online instruments or samples for laboratory-based testing. The use of online instruments and process automation technology, such as conductivity, TOC, temperature, flow rate, and pressure can facilitate improved operational control of the attributes and parameters and for process release. Manual observation of operating parameters and laboratory-based testing is also appropriate and acceptable for monitoring and trend evaluation.

#### 4.3.1 MONITORING

The frequency of routine monitoring should be based on the criticality of the finished water, capabilities of the process, and ability to maintain product water quality trends. Monitoring may be adjusted from the initial validation monitoring program when there is sufficient data to support a change (see *6.4 Routine Sampling Plans*).

#### 4.3.2 VALIDATION MAINTENANCE

Maintaining the validated state of control requires a life cycle approach. After the completion of the PQ and release of the water system for use, ongoing activities and programs have to be in place to maintain the validated state of control after the system has been validated and placed into service (see *5.4 Operation, Maintenance, and Control*). This includes unit operation, calibration, corrective maintenance, preventive maintenance, procedures, manuals and drawings, standardization of instruments, process parameter and quality attribute trending, change control, deviations, corrective and preventive actions (CAPA), training, records retention, and maintaining logbooks.

#### 4.3.3 CHANGE CONTROL

Identification and control of changes made to unit operations and other system components, operation parameters, system sanitization, and laboratory processes or procedures need to be established. Not all changes will require validation follow up,

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but even minor ones, such as gasket elastomer changes could have an affect on quality attributes. The impact of the change on process parameters and quality attributes must be identified, evaluated and remediated. This may result in a selective validation activity to demonstrate the ongoing state of control for the system and ability to maintain water quality attributes.

Certain calibration and preventive maintenance activities may be considered routine tasks if they do not affect on system operation or water quality. Replacement of components needs to be carefully evaluated. Replacement of components using exact parts generally does not affect system operation or control. Replacement of components with ones that are not exact parts but have similar functional specifications can be performed at risk with the critical specifications (e.g., material of construction, dimensions, flow rate, response factors) having been evaluated and the differences determined to be acceptable and documented within the change control system.

#### 4.3.4 PERIODIC REVIEW

The water system qualification, maintenance history, calibration records, quality and process data, issues with the unit operations and any process variability, change control, and other validation maintenance data should be assessed periodically to determine impact on the state of control.

The review may result in adjustments to operating or sanitization processes, calibration or maintenance plans, or monitoring plans. This may also result in additional testing or repeating certain qualification tasks (e.g., re-qualification).

***Change to read:***

## 5. DESIGN AND OPERATION OF PURIFIED WATER AND WATER FOR INJECTION SYSTEMS

The design, installation, and operation of systems to produce Purified Water and Water for Injection include similar components, control techniques, and procedures. The quality attributes of the two waters differ in their bioburden expectation, the presence of a bacterial endotoxin requirement for Water for Injection, and in their methods of preparation. The similarities in the quality attributes provide considerable common ground in the design of water systems to meet either requirement. The critical difference is the degree of control of the system and the final purification steps needed to ensure removal of bacteria and bacterial endotoxins and reductions in opportunities for biofilm re-development within those purification steps that could become in situ sources of bacteria and endotoxin in the finished water.

Many aspects of system design and operation relate to control and elimination of biofilm. Unit operations can cause the deterioration of water microbial attributes and the formation of biofilm on unit operation surfaces, even when properly maintained (see *8.2 Biofilm Formation in Water Systems*).

Production of pharmaceutical water involves sequential unit operations (processing steps) that address specific water quality attributes and protect the operation of subsequent treatment steps. A typical evaluation process for selecting an appropriate water quality for a particular pharmaceutical purpose is shown in the decision trees in *Figure 2a* and *Figure 2b*. These diagrams may be used to assist in defining requirements for specific water uses and in the selection of unit operations. The final unit operation used to produce Water for Injection is limited to distillation or other processes equivalent or superior to distillation in the removal of chemical impurities as well as microorganisms and their components, such as bacterial endotoxins. Distillation coupled with suitable pretreatment technologies has a long history of generally reliable performance (though not completely infallible) and can be validated as a unit operation for the production of Water for Injection. Other combinations of purification technologies also may be suitable in the production of Water for Injection if they can be shown through validation to be as effective and reliable as distillation in the removal of chemicals and microorganisms. The development of new designs and materials of construction for other technologies (e.g., reverse osmosis, electrodeionization, and ultrafiltration) that allow intermittent or continuous operation at hot bactericidal conditions show promise for a valid use in producing Water for Injection.

### 5.1 Unit Operations Considerations

To achieve the quality attributes for pharmaceutical waters, multiple-unit operations are required. The design of the water purification system needs to take into consideration different aspects, including the source water quality, sanitization, pharmaceutical water quality attributes, uses of the water, and maintenance programs. Each unit operation contributes specific purification attributes associated with chemical and microbiological parameters.

The following sections briefly describe selected unit operations and the design, installation, operation, maintenance, and monitoring parameter considerations associated with them. Not all unit operations are discussed, nor are all potential shortcomings addressed.

#### 5.1.1 PREFILTRATION

The purpose of prefiltration—also referred to as initial, coarse, particulate, or depth filtration—is to remove solid contaminants from the incoming source water supply and protect downstream system components from particulates that can inhibit equipment performance and shorten their effective life. This coarse filtration technology primarily uses sieving effects for particle capture and a depth of filtration medium that has a high “dirt load” capacity. Such filtration units are available in a wide range of designs and for various applications. Removal efficiencies and capacities differ significantly, from granular bed filters such as multimedia or sand for larger water systems, to depth cartridges for smaller water systems. Unit and system configurations vary widely in the type of filtering media and the location in the process. Granular or cartridge prefilters are often situated at the beginning of the water purification system prior to unit operations designed to remove the source water disinfectants. Cartridge-type coarse filters also may be used to capture fines released from granular beds such as activated carbon and deionization beds. These locations, however, do not preclude the need for periodic microbial evaluation.

Design and operational issues that may affect the performance of depth filters include channeling of the filtering media, blockage from silt, microbial growth, and filtering-media loss during improper backwashing. Control methods involve pressure

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and flow monitoring during use and backwashing, sanitizing, and replacing filtering media. An important design concern is sizing the filter to prevent channeling or media loss resulting from inappropriate water flow rates as well as proper sizing to minimize excessively frequent or infrequent backwashing or cartridge filter replacement.

### 5.1.2 ACTIVATED CARBON

Activated carbon beds, depending on the type and placement, are used to adsorb low-molecular-weight organic material, bacterial endotoxins, and oxidizing additives such as chlorine and chloramine compounds, removing them from the water. They are used to achieve certain quality attributes and to protect against reactions with downstream unit operations, stainless steel surfaces, resins, and membranes.

The chief operating concerns regarding activated carbon beds include the propensity to support bacterial growth, the potential for hydraulic channeling, the organic adsorption capacity, and insufficient contact time. Operation deficiencies may result in the release of bacteria, endotoxins, organic chemicals, and fine carbon particles.

Control measures may involve monitoring water flow rates and differential pressures, sanitizing with hot water or steam, backwashing, testing for adsorption capacity, and frequent replacement of the carbon bed. Monitoring of carbon bed unit operation may also include microbial loading, disinfectant chemical reduction, and TOC if used for TOC reduction. The use of hot water or steam for carbon bed sanitization is ineffective if there is channeling rather than even permeation through the bed. Channeling can be mitigated through design and proper flow rates during sanitization.

Microbial biofilm development on the surface of the granular carbon particles can cause adjacent bed granules to agglomerate. This may result in ineffective removal of trapped debris and fragile biofilm during backwashing, and ineffective sanitization.

Alternative technologies to activated carbon beds can be used to avoid their microbial challenges. These include disinfectant-neutralizing chemical additives and intense ultraviolet (UV) light for removal of chlorine, and regenerable organic scavenging deionizing resins for removal of organics.

### 5.1.3 ADDITIVES

Chemical additives are used in water systems to 1) control microorganisms by use of sanitizing agents, such as chlorine compounds and ozone; 2) enhance the removal of suspended solids by use of flocculating agents; 3) remove chlorine compounds; 4) avoid scaling on reverse osmosis membranes; and 5) adjust pH for more effective removal of carbonate and ammonia compounds by reverse osmosis. These additives do not constitute “added substances” as long as they are either removed by subsequent processing steps or are otherwise absent from the finished water. Control of additives to ensure a continuously effective concentration and subsequent monitoring to ensure their removal should be designed into the system and included in the monitoring program.

### 5.1.4 ORGANIC SCAVENGERS

Organic scavenging devices use macroreticular, weakly basic anion-exchange resins capable of removing negatively charged organic material and endotoxins from the water. Organic scavenger resins can be regenerated with appropriate biocidal caustic brine solutions. Operating concerns are associated with organic scavenging capacity; particulate, chemical, and microbiological fouling of the reactive resin surface; flow rate; regeneration frequency; and shedding of fines from the fragile resins. Control measures include TOC testing of influent and effluent, backwashing, monitoring hydraulic performance, and using downstream filters to remove resin fines.

### 5.1.5 SOFTENERS

Water softeners may be located either upstream or downstream of disinfectant removal units. They utilize sodium-based cation-exchange resins to remove water-hardness ions, such as calcium and magnesium, that could foul or interfere with the performance of downstream processing equipment such as reverse osmosis membranes, deionization devices, and distillation units. Water softeners also can be used to remove other lower affinity cations, such as the ammonium ion, that may be released from chloramine disinfectants commonly used in drinking water. If ammonium removal is one of its purposes, the softener must be located downstream of the disinfectant removal operation. Water softener resin beds are regenerated with concentrated sodium chloride solution (brine).

Concerns include microorganism proliferation, channeling, appropriate water flow rates and contact time, ion-exchange capacity, organic and particulate resin fouling, organic leaching from new resins, fracture of the resin beads, resin degradation by excessively chlorinated water, and contamination from the brine solution used for regeneration.

Control measures involve recirculation of water during periods of low water use, periodic sanitization of the resin and brine system, use of microbial control devices (e.g., UV light and chlorine), locating the unit upstream of the disinfectant removal step (if used only for softening), appropriate regeneration frequency, effluent chemical monitoring (e.g., hardness ions and possibly ammonium), and downstream filtration to remove resin fines. If a softener is used for ammonium removal from chloramine-containing source water, then the capacity, contact time, resin surface fouling, pH, and regeneration frequency are very important.

### 5.1.6 DEIONIZATION

Deionization and continuous electrodeionization (CEDI) are effective methods of improving the chemical quality attributes of water by removing cations and anions. Deionization systems have charged resins that require periodic regeneration with an acid and base. Typically, cation resins are regenerated with either hydrochloric or sulfuric acid, which replace the captured positive ions with hydrogen ions. Anion resins are regenerated with sodium hydroxide or potassium hydroxide, which replace captured negative ions with hydroxide ions. Because free endotoxin is negatively charged, some removal of endotoxin is

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achieved by the anion resin. The system can be designed so that the cation and anion resins are in separate or "twin" beds, or they can be blended together to form a "mixed" bed.

The CEDI system uses a combination of ion-exchange materials such as resins or grafted material, selectively permeable membranes, and an electric charge, providing continuous flow (of product and waste concentrate) and continuous regeneration. Water enters both the resin section and the waste (concentrate) section. The resin acts as a conductor, enabling the electrical potential to drive the captured cations and anions through the resin and appropriate membranes for concentration and removal in the waste water stream. As the water passes through the resin, it is deionized to become product water. The electrical potential also separates the water in the resin (product) section into hydrogen and hydroxide ions. This permits continuous regeneration of the resin without the need for regenerant additives. However, unlike conventional deionization, CEDI units must start with water that is already partially purified because they generally cannot achieve the conductivity attribute of Purified Water when starting with the heavier ion load of source water.

Concerns for all forms of deionization units include microbial and endotoxin control, chemical additive impact on resins and membranes, and loss, degradation, and fouling of resin. Issues of concern specific to deionization units include regeneration frequency and completeness, channeling caused by biofilm agglomeration of resin particles, organic leaching from new resins, complete resin separation for mixed bed regeneration, and bed fluidization air contamination (mixed beds).

Control measures may include continuous recirculation loops, effluent microbial control by UV light, conductivity monitoring, resin testing, microporous filtration of bed fluidization air, microbial monitoring, frequent regeneration to minimize and control microorganism growth, sizing the equipment for suitable water flow and contact time, and use of elevated temperatures. Internal distributor and regeneration piping for deionization bed units should be configured to ensure that regeneration chemicals contact all internal bed and piping surfaces and resins.

Rechargeable canisters can be the source of contamination and should be carefully monitored. Full knowledge of previous resin use, minimum storage time between regeneration and use, and appropriate sanitizing procedures are critical factors for ensuring proper performance.

### 5.1.7 REVERSE OSMOSIS

Reverse osmosis units use semipermeable membranes. The "pores" of reverse osmosis membranes are intersegmental spaces among the polymer molecules. They are big enough for permeation of water molecules, but they limit the passage of hydrated chemical ions, organic compounds, and microorganisms. reverse osmosis membranes can achieve chemical, microbial, and endotoxin quality improvement. Many factors, including pH, temperature, source water hardness, permeate and reject flow rate, and differential pressure across the membrane, affect the selectivity and effectiveness of this permeation. The process streams consist of supply water, product water (permeate), and waste water (reject). Depending on the source water, pretreatment and system configuration variations and chemical additives may be necessary to achieve the desired performance and reliability. For most source waters, a single stage of reverse osmosis filtration is usually not enough to meet Purified Water conductivity specifications. A second pass of this permeate water through another reverse osmosis stage usually achieves the necessary permeate purity if other factors such as pH and temperature have been appropriately adjusted and the ammonia from source water that has been previously treated with chloramines is removed.

Concerns associated with the design and operation of reverse osmosis units include membrane materials that are sensitive to sanitizing agents and to particulate, chemical, and microbial membrane fouling; membrane and seal integrity; and the passage of dissolved gases, such as carbon dioxide and ammonia. Failure of membrane or seal integrity will result in product water contamination. Methods of control involve suitable pretreatment of the influent water stream; appropriate membrane material selection; membrane design and heat tolerance; periodic sanitization; and monitoring of differential pressures, conductivity, microbial levels, and TOC.

The development of reverse osmosis units that can tolerate sanitizing water temperatures and also operate efficiently and continuously at elevated temperatures has added greatly to their microbial control ability and to the avoidance of biofouling. Reverse osmosis units can be used alone or in combination with deionization and CEDI units, as well as ultrafiltration, for operational and quality enhancements.

### 5.1.8 ULTRAFILTRATION

Ultrafiltration is a technology that is often used near the end of a pharmaceutical water purification system for removing endotoxins from a water stream though upstream uses are possible. Ultrafiltration can use semipermeable membranes, but unlike reverse osmosis units, these typically use polysulfone with intersegmental "pores" that have been purposefully enlarged. Membranes with differing molecular weight "cutoffs" can be created to preferentially reject molecules with molecular weights above these ratings.

Ceramic ultrafilters are another molecular sieving technology. Ceramic ultrafilters are self-supporting and extremely durable; they can be backwashed, chemically cleaned, and steam sterilized. However, they may require higher operating pressures than do membrane-type ultrafilters.

All ultrafiltration devices work primarily by a molecular sieving principle. Ultrafilters with molecular weight cutoff ratings in the range of 10,000–20,000 Da are typically used in water systems for removing endotoxins. This technology may be appropriate as an intermediate or final purification step. As with reverse osmosis, successful performance is dependent upon pretreatment of the water by upstream unit operations.

Issues of concern for ultrafilters include compatibility of membrane material with heat and sanitizing agents, membrane integrity, fouling by particles and microorganisms, and seal integrity. Control measures involve filter membrane composition, sanitization, flow design (dead end versus tangential), cartridge replacement, elevated feed water temperature, and monitoring TOC and differential pressure.

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### 5.1.9 MICROBIAL-RETENTIVE FILTRATION

Microbial-retentive membrane filters have a larger effective "pore size" than ultrafilters and are intended to prevent the passage of microorganisms and similarly sized particles without unduly restricting flow. This type of filtration is widely employed within water systems for filtering the bacteria out of both water and compressed gases as well as for vent filters on tanks and stills and other unit operations.

In water systems, a filter's microbial retention characteristics exhibit different phenomena than in other aseptic filtration applications.

The following factors interact to create the retention phenomena for water system microorganisms: the variability in the range and average pore sizes created by the various membrane fabrication processes; the variability of the surface chemistry and three-dimensional structure related to the different polymers used in these filter matrices; and the size and surface properties of the microorganism intended to be retained by the filters. In some situations, the appearance of water system microorganisms on the downstream sides of some 0.2- to 0.22-µm rated filters after a period of use (days to weeks) seems to support the idea that water-borne microorganisms can penetrate the 0.2- to 0.22-µm rated filters. It is not known whether this downstream appearance is caused by exceeding the retentive capabilities of the filters due to high prefiltration bioburden levels of water-borne microorganisms and extended filtration times. These conditions can lead to a "pass-through" phenomenon resulting from tiny cells or less cell "stickiness", or perhaps by a "grow-through" phenomenon in which cells hypothetically replicate their way through the pores to the downstream side. Whatever the penetration mechanism, 0.2- to 0.22-µm rated membranes may not be the best choice for some water system uses (see *Sterility Assurance* ⟨1211⟩).

Nevertheless, microbial retention success in water systems has been reported with the use of filters rated as 0.2 or 0.1 µm. There is general agreement that, for a given manufacturer, its 0.1-µm rated filters are tighter than its 0.2- to 0.22-µm rated filters. However, comparably rated filters from different manufacturers may not have equivalent performance in water filtration applications because of the different filter materials, different fabrication processes, and nonstandardized microbial retention challenge processes currently used for defining the 0.1-µm filter rating. It should be noted that filters with a 0.1-µm rating may result in a lower flow rate compared to 0.2- to 0.22-µm filters, so whatever filters are chosen for a water system application, the user must verify that they are suitable for their intended application, use period, and use process, including flow rate.

For microbial retentive gas filtrations, the same sieving and adsorptive retention phenomena are at work as in liquid filtration, but the adsorptive phenomenon is enhanced by additional electrostatic interactions between the particles and filter matrix. These electrostatic interactions are so strong, particle retention for a given filter rating is significantly more efficient in gas filtration than in water or product-solution filtrations. These additional adsorptive interactions render filters rated at 0.2–0.22 µm unquestionably suitable for microbial retentive gas filtrations. When microbial retentive filters are used in these applications, the membrane surface is typically hydrophobic (non-wettable by water). A significant area of concern for gas filtration is blockage of tank vents by condensed water vapor, which can cause mechanical damage to the tank. Control measures include electrical or steam tracing and a self-draining orientation of vent filter housings to prevent accumulation of vapor condensate. However, a continuously high filter temperature will take an oxidative toll on polypropylene components of the filter, so sterilization of the unit prior to initial use and periodically thereafter, as well as regular visual inspections, integrity tests, and filter cartridge changes, are recommended control methods.

In water applications, microbial retentive filters may be used downstream of unit operations that tend to release microorganisms or upstream of unit operations that are sensitive to microorganisms. Microbial retentive filters also may be used to filter water feeding the distribution system. It should be noted that regulatory authorities allow the use of microbial retentive filters within distribution systems or even at use points if they have been properly validated and are appropriately maintained. A point-of-use filter should only be intended to "polish" the microbial quality of an otherwise well-maintained system and not to serve as the primary microbial control device. The efficacy of system microbial control measures can only be assessed by sampling the water upstream of the filters. As an added measure of protection, in-line UV lamps, appropriately sized for the flow rate (see *5.3 Sanitization*), may be used just upstream of microbial retentive filters to inactivate microorganisms prior to their capture by the filter. This tandem approach tends to greatly delay potential microbial penetration phenomena and can substantially extend filter service life.

### 5.1.10 ULTRAVIOLET LIGHT

The use of low-pressure UV lights that emit a 254-nm wavelength for microbial control is discussed in *5.3 Sanitization*, but the application of UV light in chemical purification is also emerging. This 254-nm wavelength is also useful in the destruction of ozone. At wavelengths around 185 nm (as well as at 254 nm), medium-pressure UV lights have demonstrated utility in the destruction of the chlorine-containing disinfectants used in source water as well as for interim stages of water pretreatment. High intensities of 185 nm alone or 254 nm in combination with other oxidizing ▲sanitizing agents,▲ (USP 1-Dec-2021) such as hydrogen peroxide, have been used to lower TOC levels in recirculating distribution systems. The organics are typically converted to carbon dioxide, which equilibrates to bicarbonate, and incompletely oxidized carboxylic acids, both of which can easily be removed by polishing ion-exchange resins.

Areas of concern include inadequate UV intensity and residence time, gradual loss of UV emissivity with bulb age, gradual formation of a UV-absorbing film at the water contact surface, incomplete photodegradation during unforeseen source water hyperchlorination, release of ammonia from chloramine photodegradation, unapparent UV bulb failure, and conductivity degradation in distribution systems using 185-nm UV lights.

Control measures include regular inspection or emissivity alarms to detect bulb failures or film occlusions, regular UV bulb sleeve cleaning and wiping, downstream chlorine detectors (when used for dechlorination), downstream polishing deionizers (when used for TOC reduction), and regular (approximately yearly) bulb replacement. UV lamps generate heat during operation, which can cause failure of the lamps or increase the temperature of the water. Precautions should be in place to ensure that water flow is present to control excessive temperature increase.

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### 5.1.11 DISTILLATION

Distillation units provide chemical and microbial purification via thermal vaporization, mist elimination, and water vapor condensation. A variety of designs is available, including single effect, multiple effect, and vapor compression. The latter two configurations are normally used in larger systems because of their generating capacity and efficiency. Source water controls must provide for the removal of hardness and silica impurities that may foul or corrode the heat transfer surfaces, as well as the removal of those impurities that could volatilize and condense along with the water vapor. In spite of general perceptions, even the best distillation process does not ensure absolute removal of contaminating ions, organics, and endotoxins. Most stills are recognized as being able to accomplish at least a 3–4 log reduction in these impurity concentrations. They are highly effective in sterilizing the feed water.

Areas of concern include carryover of volatile organic impurities such as trihalomethanes (see *2. Source Water Considerations*) and gaseous impurities such as ammonia and carbon dioxide, faulty mist elimination, evaporator flooding, inadequate blow down, stagnant water in condensers and evaporators, pump and compressor seal design, pinhole evaporator and condenser leaks, and conductivity (quality) variations during start-up and operation.

Methods of control may involve the following: preliminary steps to remove both dissolved carbon dioxide and other volatile or noncondensable impurities; reliable mist elimination to minimize feed water droplet entrainment; visual or automated high-water-level indication to detect boiler flooding and boil over; use of sanitary pumps and compressors to minimize microbial and lubricant contamination of feed water and condensate; proper drainage during inactive periods to minimize microbial growth and accumulation of associated endotoxin in boiler water; blow down control to limit the impurity concentration effect in the boiler to manageable levels; on-line conductivity sensing with automated diversion to waste to prevent unacceptable water upon still start-up or still malfunction from getting into the finished water distribution system; and periodic testing for pinhole leaks to routinely ensure that condensate is not compromised by nonvolatilized source water contaminants.

### 5.1.12 STORAGE TANKS

Storage tanks are included in water distribution systems to optimize processing equipment capacity. Storage also allows for routine maintenance within the purification system while maintaining continuous supply to meet manufacturing needs. Design and operation considerations are needed to prevent or minimize the development of biofilm, to minimize corrosion, to aid in the use of chemical sanitization of the tanks, and to safeguard mechanical integrity.

Areas of concern include microbial growth or corrosion due to irregular or incomplete sanitization and microbial contamination from unalarmed rupture disk failures caused by condensate-occluded vent filters.

Control considerations may include the use of closed tanks with smooth interiors, the ability to spray the tank headspace using spray balls on recirculating loop returns, and the use of heated, jacketed/insulated tanks. ▲These minimize▲ (USP 1-Dec-2021) corrosion and biofilm development and ▲aid▲ (USP 1-Dec-2021) in thermal or chemical sanitization. Storage tanks require venting to compensate for the dynamics of changing water levels. This can be accomplished with a properly oriented and heat-traced filter housing fitted with a hydrophobic microbial retentive membrane filter affixed to an atmospheric vent. Alternatively, an automatic membrane-filtered compressed gas blanketing system may be used. In both cases, rupture disks equipped with a rupture alarm device should be used as a further safeguard for the mechanical integrity of the tank.

### 5.1.13 DISTRIBUTION SYSTEMS

Distribution system configuration should allow for the continuous flow of water in the piping by means of recirculation. Use of no recirculating, dead-end, or one-way systems or system segments should be avoided whenever possible. If not possible, these systems should be flushed periodically and monitored more closely. Experience has shown that continuously recirculated systems are easier to maintain. Pumps should be designed to deliver fully turbulent flow conditions to facilitate thorough heat distribution (for hot-water sanitized systems) as well as thorough chemical ▲sanitizing agent▲ (USP 1-Dec-2021) distribution. Turbulent flow also appears to either retard the development of biofilms or reduce the tendency of those biofilms to shed bacteria into the water. If redundant components, such as pumps or filters, are used, they should be configured and used to avoid microbial contamination of the system.

Components and distribution lines should be sloped and fitted with drain points so that the system can be completely drained. In distribution systems, dead legs and low-flow conditions should be avoided, and valved tie-in points should have length-to-diameter ratios of six or less. In systems that operate at self-sanitizing temperatures, precautions should be taken to avoid cool points where biofilm development could occur. If drainage of components or distribution lines is intended as a microbial control strategy, they also should be configured to be dried completely using dry compressed gas because drained but still-moist surfaces will still support microbial proliferation. Water exiting from the distribution system should not be returned to the system without first passing through all or a portion of the purification system.

The distribution design should include the placement of sampling valves in the storage tank and at other locations, such as in the return line of the recirculating water system. Direct connections to processes or auxiliary equipment should be designed to prevent reverse flow into the controlled water system. Hoses and heat exchangers that are attached to points of use to deliver water must not chemically or microbiologically degrade the water quality. The distribution system should permit sanitization for microorganism control. The system may be continuously operated at sanitizing conditions or sanitized periodically.

### 5.1.14 NOVEL/EMERGING TECHNOLOGIES

New water treatment technologies are being developed continuously. Before these technologies are utilized in pharmaceutical water systems, they should be evaluated for acceptable use in a GMP environment. Other considerations should include the treatment process, reliability and robustness, use of added substances, materials of construction, and ability to validate. Consideration should be given to recognize the areas of concern during the evaluation and to identify control measures for the technology. This should include impact on chemical and microbial attributes.

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## 5.2 Installation, Materials of Construction, and Component Selection

Installation techniques are important because they can affect the mechanical, corrosive, and sanitary integrity of the system. Valve installation should promote gravity drainage. Pipe supports should provide appropriate slopes for drainage and should be designed to support the piping adequately under worst-case thermal and flow conditions. The methods of connecting system components—including units of operation, tanks, and distribution piping—require careful attention to preclude potential operational and microbial problems.

Stainless steel welds should provide reliable joints that are internally smooth and corrosion-free. Low-carbon stainless steel, compatible wire filler where necessary, inert gas, automatic welding machines, and regular inspection and documentation help to ensure acceptable weld quality. Follow-up cleaning and passivation of metal surfaces after installation are important for removing contamination and corrosion products and to re-establish the passive corrosion-resistant surface.

Plastic materials can be fused (welded) in some cases, and also require smooth, uniform internal surfaces. Adhesive glues and solvents should be avoided due to the potential for voids and organic extractables. Mechanical methods of joining, such as flange fittings, require care to avoid the creation of offsets, gaps, penetrations, and voids. Use of plastic materials may contribute to TOC levels.

Control measures include good alignment, properly sized gaskets, appropriate spacing, uniform sealing force, and the avoidance of threaded fittings.

Materials of construction should be selected to be compatible with control measures such as sanitizing, cleaning, or passivation. Temperature rating is a critical factor in choosing appropriate materials because surfaces may be required to handle elevated operating and sanitization temperatures. If chemicals or additives will be used to clean, passivate, or sanitize the system, materials resistant to these chemicals or additives must be utilized. Materials should be capable of handling turbulent flow and elevated velocities without erosion of the corrosion-resistant film (e.g., the passive chromium oxide surface of stainless steel) or reduction in wall thickness for plastics. The finish on metallic materials such as stainless steel, whether it is a refined mill finish, polished to a specific grit, or an electropolished treatment, should complement the system design and provide satisfactory corrosion and microbial activity resistance. The finish also should be a material that can be chemically sanitized. Auxiliary equipment and fittings that require seals, gaskets, diaphragms, filter media, and membranes should exclude materials that permit the possibility of extractables, shedding, and microbial activity. Insulating materials exposed to stainless steel surfaces should be free of chlorides to avoid the phenomenon of stress corrosion cracking that can lead to system contamination and the destruction of tanks and critical system components.

Specifications are important to ensure proper selection of materials and to serve as a reference for system qualification and maintenance. Information such as the manufacturer's metallurgical reports for stainless steel and reports of composition, ratings, and material-handling capabilities for nonmetallic substances should be reviewed for suitability and retained for reference. Component (auxiliary equipment) selection should be made with assurance that it does not create a source of contamination intrusion. Heat exchangers should be constructed to prevent leakage of heat transfer medium into the pharmaceutical water and, for heat exchanger designs where prevention may fail, there should be a means to detect leakage. Pumps should be of sanitary design with seals that prevent contamination of the water. Valves should have smooth internal surfaces with the seat and closing device exposed to the flushing action of water, such as occurs in diaphragm valves. Valves with pocket areas or closing devices (e.g., ball, plug, gate, globe) that move into and out of the flow area should be avoided.

## 5.3 Sanitization

Microbial control in water systems is achieved primarily through sanitization practices. Systems can be sanitized using either thermal chemical or photochemical means.

### 5.3.1 THERMAL SANITIZATION

Thermal approaches to system sanitization include periodic or continuously circulating hot water and the use of steam. Temperatures of 65°–80° are most commonly used for thermal sanitization. Continuously recirculating water of at least 65° at the coldest location in the distribution system also has been used effectively in stainless steel distribution systems when attention is paid to uniformity and distribution of such self-sanitizing temperatures. These techniques are limited to systems that are compatible with the higher temperatures needed to achieve sanitization. Frequent use of thermal sanitization at appropriate temperatures should eliminate the need for other sanitization methods.

The use of thermal methods at temperatures above 80° is contraindicated because it does not add to microbial control of the system or reduction of biofilm. Some methods (e.g., steam sanitizing, hot water circulation at temperatures ≥100°) can be less effective or even destructive because of the need to eliminate condensate or manipulate system components, stress materials of construction, deform filters, and its adverse impact on instrumentation.

Although thermal methods control biofilm development by either continuously inhibiting its growth or, in intermittent applications, by killing the microorganisms within developing biofilms, they are not effective in removing established biofilms. Killed but intact biofilms can become a nutrient source for rapid biofilm regrowth after the sanitizing conditions are removed or halted. In cases of infrequent thermal sanitizations that allow biofilm development between treatments, a combination of routine thermal treatment and periodic supplementation with chemical sanitization may be more effective. The more frequent the thermal sanitization, the more likely it is that biofilm re-development can be eliminated.

### 5.3.2 CHEMICAL SANITIZATION

Chemical methods, where compatible, can be used on a wider variety of construction materials. These methods typically use oxidizing agents such as ozone, hydrogen peroxide, peracetic acid, or combinations thereof. Halogenated compounds can be effective sanitizers but are less aggressive oxidizing agents and may be difficult to flush from the system. Chemical agents may not penetrate the full biofilm matrix or extend into all biofilm locations (e.g., crevices at gasketed fittings) and may leave

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biofilms incompletely inactivated. Compounds such as ozone, hydrogen peroxide, and peracetic acid oxidize bacteria and biofilms with reactive peroxides and by forming very reactive free radicals (notably hydroxyl radicals). The short half-life of ozone in particular, and its limitation on achievable concentrations, require that it be added continuously during the sanitization process. Hydrogen peroxide and ozone rapidly degrade to water and/or oxygen, and peracetic acid degrades to oxygen and acetic acid. The ease of degradation of ozone to oxygen using 254-nm UV lights in circulating loops allows it to be used effectively on a continuously sanitizing basis in holding tanks and on an intermittent basis (e.g., daily or weekly) in the distribution loops. The highly reactive nature of ozone requires the use of system materials and components that are even more oxidation resistant than those typically used with the other oxidizing agents.

It is important to note that microorganisms in a well-developed biofilm can be extremely difficult to kill, even by using aggressive oxidizing chemicals. The less developed and therefore thinner the biofilm, the more effective the biofilm inactivation. Therefore, optimal microbial control is achieved by using oxidizing chemicals at a frequency that does not permit significant biofilm development between treatments.

Validation of chemical sanitization requires demonstration of adequate chemical concentrations throughout the system, exposure to all wetted surfaces including the body of use point valves, and complete removal of the ▲sanitizing agent▲ (USP 1-Dec-2021) from the system at the completion of treatment. Methods validation for the detection and quantification of residues of the ▲sanitizing agent▲ (USP 1-Dec-2021) or its objectionable degradants is an essential part of the validation program.

#### 5.3.3 UV SANITIZATION

In-line UV light at a wavelength of 254 nm also can be used to continuously “sanitize” only the water circulating in the system, but these devices must be properly sized for the water flow. Such devices inactivate a high percentage (but not 100%) of microorganisms that flow through the device but cannot be used to directly control existing biofilm upstream or downstream of the device. However, when coupled with conventional thermal or chemical sanitization technologies or located immediately upstream of a microbially retentive filter, UV light is most effective and can prolong the interval between needed system re-sanitizations.

#### 5.3.4 SANITIZATION PROCEDURES

Sanitization steps require validation to demonstrate the ability to reduce and hold microbial contamination at acceptable levels. Validation of thermal methods should include a heat distribution study to demonstrate that sanitization temperatures are achieved throughout the system, including the body of use point valves; sampling ports; instrument side branches; and fittings, couplings, and adapters, relying on water convection and thermal conduction through system materials for heat transfer to wetted surfaces.

The routine frequency of sanitization should be supported by the results of system microbial monitoring. Conclusions derived from trend analysis of the microbiological data should be used as the alert mechanism for the need for extraordinary maintenance. The routine frequency of sanitization should be established in such a way that the system operates in a state of microbiological control and does not regularly exceed Alert and Action Levels (see *9.4 Defining Alert and Action Levels and Specifications*).

### 5.4 Operation, Maintenance, and Control

A preventive maintenance program should be established to ensure that the water system remains in a state of control. The program should include 1) procedures for operating the system, 2) monitoring programs for critical quality attributes and operating conditions including calibration of critical instruments, 3) schedule for periodic sanitization, 4) preventive maintenance of components, and 5) control of changes to the mechanical system and to operating conditions.

#### 5.4.1 OPERATING PROCEDURES

Operating procedures for the water system and for performing routine maintenance and corrective action should be written, and they should also define the point when action is required. The procedures should be well documented and should detail the function of each job, assign who is responsible for performing the work, describe how the job is to be done, and identify acceptable operating parameters. The effectiveness of these procedures should be assessed during water system validation.

#### 5.4.2 PROCESS MONITORING PROGRAM

A process-monitoring program should establish the critical quality attributes and operating parameters that are documented and monitored. The program may include a combination of in-line sensors and/or automated instruments (e.g., for temperature, TOC conductivity, hardness, and chlorine), automated or manual documentation of operational parameters (e.g., flow rates or pressure drop across a carbon bed, filter, or reverse osmosis unit), and laboratory tests (e.g., total microbial counts). The frequency of sampling, the requirement for evaluating test results, and the necessity of initiating corrective action should be included.

#### 5.4.3 ROUTINE MICROBIAL CONTROL

Sanitization may be integral to operation and maintenance, and necessary on a routine basis, depending on system design and the selected units of operation, to maintain the system in a state of microbial control. Technologies for sanitization are described above in more detail in *5.3 Sanitization*.

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#### 5.4.4 PREVENTIVE MAINTENANCE

A preventive maintenance program should be in effect. The program should establish what preventive maintenance is to be performed, the frequency of maintenance work, and how the work should be documented.

#### 5.4.5 CHANGE CONTROL

The mechanical configuration, operating conditions, and maintenance activities of the water system must be controlled. Proposed changes should be evaluated for their impact on the whole system. The need to requalify the system after changes are made should be determined. After a decision is made to modify a water system, the affected drawings, manuals, and procedures should be revised. Portions or operations of the water system that are affected by the modification should be tested to demonstrate a continued state of control. The extent and duration of testing should be related to the risk impact of the change to the system.

## 6. SAMPLING

The testing of water samples from a water system is critical to the ongoing control of the system and assessment of the quality of the water being used. If improperly collected, a sample could yield a test result that is unrepresentative of the sample's purpose. This could lead to inaction when remediation is needed or to unnecessary remediation when none is necessary. It could also lead to misinterpretations of product impact. Therefore, properly collecting water samples, understanding their purpose, and establishing appropriate water system sampling plans are essential to water QC and system control.

### 6.1 Purposes and Procedures

To assess a particular water attribute, a sample of the water usually must be removed from a water system for specific attribute testing. The sample needs to be obtained from specific locations that are representative for the purpose being monitored. This sample may be analyzed by in-line/on-line instruments or it may be completely removed from the system as a "grab sample" in a container for off-line testing. In-line/on-line testing avoids the exogenous contamination potential of grab samples that could lead to artifactually variable data trends and incorrect decisions on system performance, maintenance, and utilized water, as well as initiating fruitless causative investigations. Grab samples may be appropriate where the water in the system is not homogeneous for certain attributes.

The data from water testing are generally used for one of two purposes: for PC of the water purification and distribution system or for release or QC of the water being drawn from the system for some application or use. In many cases, depending on the sampling location and sampling process, the resulting data can be used for both PC and QC purposes.

#### 6.1.1 PROCESS CONTROL SAMPLING

Because PC sampling is intended to reflect the quality of the water behind the valve and within the distribution system, coming from the purification system, or between its purification steps, efforts should be made to avoid contaminating the water as it is drawn from the system so that its test results accurately reflect the water quality within the system at that location. This may require the use of strategically located sampling ports, in addition to points of use.

If microbial testing is needed for PC purposes, the sampling valve should have a properly installed, sanitary design that uses vigorous pre-sampling flushing. This flushing shears off fragile biofilm structures growing on surfaces within the valve and water path before the sample is collected. This avoids biasing the microbial count of perhaps pristine water in the system behind that valve. A fully open valve flush (at >8 ft/s velocity within the valve and connector) for at least 30 s typically provides sufficient shear forces to adequately remove any fragile biofilm structures. Additional control measures for preventing sample contamination could also include stringent pre- and post-sampling outlet sanitation, the use of sterile hoses and gaskets or other connectors to direct the water flow, and other measures.

The data from PC sampling indicate how well the system is maintaining the water quality at that sampling location. These data are subsequently used to signal when some extraordinary intervention might be needed, in addition to normal maintenance and system sanitization operations, to restore the system to the expected level of purity.

PC sampling can only be used to indicate the quality of the water being delivered to the points of use (for QC purposes) if it has been shown to be representative of that point-of-use quality. This may be possible with chemical attributes that are typically not affected by the fluid path of the water delivery process, but is generally not possible with microbial attributes, which can be greatly affected by localized biofilms along that fluid path. If this fluid path is not utilized for PC sampling, then the resulting data typically cannot be used for QC purposes.

#### 6.1.2 QUALITY CONTROL SAMPLING

QC sampling is intended to reflect the quality of water that is being used. These samples should be collected at the true point of use; that is, where the water is delivered for use, not where it leaves the water system. QC sampling must utilize that same delivery path and components utilized for a water transfer during actual water use. This includes the same valves, hoses, heat exchangers, flow totalizers, hard-piped connections, and other components utilized during water use.

In addition to the water transfer components, QC sampling must also use the same water transfer process employed during water use, including the same pre-use outlet and delivery path flushing procedure and the same outlet, fitting, and hose sanitization practices employed during actual water use. The water delivery process and components used for QC sampling must be identical to manufacturing practices at every system outlet for the QC sample to mimic the quality of water being used by accumulating the same chemical and microbial contaminant levels it would during actual use from that outlet location.

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Where permanent connections from the water system to equipment are present, accommodation should be made in the design to collect samples from locations as close to the equipment as possible. For example, samples can be collected from special sample ports or other valves near the equipment connection that allow the collected water sample to accurately reflect the water quality that is used. Where the water transfer conduit is designed and/or definitively treated to eliminate all contaminating influences prior to water transfer through that conduit, PC sampling locations within the distribution system can reflect the quality of the water that is actually used for QC purposes at those permanent connections. However, the success of the design and treatments intended to eliminate these contaminating influences must be verified. This is typically done during water system validation.

Where routine water use practices involve contamination-prone activities, such as no pre-use flushing or poor hose storage/sanitization/replacement practices, these water use practices should be improved to reduce the potential for delivering contaminated water from the water system and for unacceptable QC sample testing results that reflect that same contamination.

## 6.2 Attributes and Sampling Locations

The tests being performed on the samples are relevant to the sampling location and purpose of the sample. In-process monitoring of non-monograph attributes may be indicated for specific unit operations. For instance, before and after a softener, it may be important to determine water hardness to verify softener efficacy. Before and after an activated carbon bed/filter, it may be important to verify chlorine or TOC removal and/or reduction or test for an increase in microbial count. Before a distillation unit, it may be important to quantitate the incoming bacterial endotoxin level to ensure that the still is not being over-challenged beyond its typical 3–4 log purification capability. However, once the water is in the distribution system, the compendial attributes of importance typically include at least conductivity, TOC, and microbial count. In Water for Injection systems and other systems or system locations where bacterial endotoxin control is important, endotoxin is also assayed. Other tests may be necessary depending on the intended uses of the water.

### 6.2.1 CHEMICAL ATTRIBUTES

Dissolved chemical contaminants detected by conductivity or TOC testing tend to be uniformly distributed in the water throughout the water system. However, there are exceptions where localized chemical contamination sources can occur, such as from a coolant-leaking heat exchanger in a sub-loop, or at a point of use, or within a dead leg. These chemical contaminants may only be seen at the associated outlets and not systemically. However, in the absence of localized contamination influences, chemical attributes are candidates for on-line testing at fixed strategic locations within the distribution system, such as near a circulating loop return, and are generally reflective of the same chemical quality at all locations and points of use within the distribution system. Nevertheless, the suitability of the on-line locations of these instruments for QC release purposes must be verified as being representative of the use-point water quality. This is usually done during water system validation.

### 6.2.2 MICROBIAL ATTRIBUTES

The same uniformity scenario cannot be assumed for microbial attributes. Planktonic organisms in a water sample could have originated from biofilms in the purification or distribution systems releasing more or less uniform levels of planktonic organisms into the circulating water, as detectable in samples from all outlets. However, a local biofilm developing within a water delivery conduit (e.g., a use-point outlet valve and transfer hose) in an otherwise pristine biofilm-free water system could release planktonic organisms detectable only in water delivered through that conduit. Therefore, QC release samples for assessing the quality of water that is delivered by the system during water use must be collected after the water has traversed the same fluid conduit (including the same preparatory activities such as outlet sanitization and pre-flushing) from the water distribution system to the specific locations where the water is used.

On-line microbial water sampling/testing has value in pharmaceutical water systems only for PC purposes unless the water is taken from the point of use in the same manner as routine water usage, in which case the data can also have a QC release purpose. Microbial counts detected from strategic sampling ports continue to have PC and investigational value, but generally cannot be substituted for QC release testing except in certain scenarios, as described in *6.1.2 QC Sampling*.

## 6.3 Validation Sampling Plans

The initial sampling plan for a pharmaceutical water system is usually developed for a validation program (see *4. Validation and Qualification of Water Purification, Storage, and Distribution Systems*). This strategy is for characterization of the system's ability to purify, distribute, and deliver pharmaceutical water. Typically, the initial validation sampling is for a short duration (e.g., at least 2–4 weeks) at a high sampling frequency to generate a significant body of data that will allow detection of short-term or localized chemical or microbial quality deviations from all outlets. These data provide an initial assessment of system performance to guide decisions about using the water for operational purposes.

The initial validation sampling plan is re-evaluated when the pharmaceutical water is placed into operation, typically to reduce the amount of data being generated while not compromising the ability to identify anomalous operations/events, especially during the early life cycle of the water system. In the absence of such quality deviations during the initial sampling period, the sampling frequency can be lessened for a period of time (e.g., at least 2–4 additional weeks) to ensure that somewhat longer-term adverse quality trends are not apparent. During this second period of time, the water may be considered for at-risk routine use, pending the acceptable completion of the second validation sampling period. After successful completion, monitoring can be eventually lessened again to what will become the routine sampling plan.

Periodic review of the water system operation and monitoring needs to be performed to assess seasonal source water variability, effectiveness of sanitization, and routine maintenance events. Periodic review should be performed during the complete life cycle of the water system, typically annually, for evidence of longer-term data trends and quality deviations.

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The routine sampling plan should be re-evaluated periodically based on the available data to determine the appropriate frequency and sample locations. This review offers an opportunity to improve data evaluation and reduce workloads based on what that data indicate relative to process and QC. The routine sampling plan should have a rationale for the frequency and locations that are selected to justify how the resulting data will be used to characterize the overall operation of the system and the release of the water for use.

## 6.4 Routine Sampling Plans

### 6.4.1 SOURCE WATER SAMPLING

As mentioned in earlier sections, the source water for pharmaceutical water systems must comply with the standards for one of the drinking waters listed in the associated compendial water monograph or in *General Notices*. When a municipality or other water authority is providing this drinking water, they are required to comply with the local drinking water regulations for the water supplied to a drinking or potable water distribution piping grid for that region. The quality of that water by the time it reaches the pharmaceutical user is dependent on a number of factors, including distance from the input source, duration of travel within the piping, and condition of the piping in that potable water distribution grid, any of which could have adversely affected some of its initial chemical and/or microbial attributes. Based on a risk assessment, it may be prudent to verify full compliance with regulations using water collected from sample ports prior to the pretreatment system, or other equivalent drinking water outlets within the facility. If the water complies, then continued assurance of compliance could be verified using drinking water regulation test results provided by the water authority or by periodic retesting of selected or all the drinking water attributes by the user or by both the user and the water authority. If private sourced water is utilized, it is the user's responsibility to demonstrate full drinking water regulation compliance, using water samples from such sampling ports on a periodic basis as determined by a risk analysis.

These pre-pretreatment sampling ports could be used, at the user's discretion, to periodically monitor other source water attributes that could affect specific pretreatment or purification unit operations. Depending on the user's source water quality consistency and a risk assessment of its potential impact on the purification process, the periodically monitored attributes could include microbial count, absence of coliforms, bacterial endotoxin levels, conductivity, TOC, pH, hardness, chlorine, silica, turbidity or silt density index, and others. These data could be useful in investigations and for operational adjustments to critical unit operation parameters and maintenance procedures, or for feedback to the potable water provider if unusual trends are observed.

### 6.4.2 PRETREATMENT AND PURIFICATION SYSTEM SAMPLING

The location and frequency of sampling from ports within the pretreatment and purification systems may be selected based on a risk analysis of unit operation purpose. For example, the purpose of this sampling for PC is primarily to ensure maintenance of acceptable unit operation performance, to assess maintenance procedure efficacy, and to investigate the need for remedial action. Quality deviations in the early portions of the purification process can affect unit operation efficiency but usually do not affect the finished water quality or acceptable use.

### 6.4.3 PURIFIED WATER DISTRIBUTION SYSTEM SAMPLING

Purified Water distribution system sampling is intended to provide continuing assurance of ongoing PC and compliance with the user's finished water chemical and microbiological requirements. Generally, the locations for that sampling and the frequency of testing the specific attributes are a matter of process and QC consistency, as well as risk tolerance in the event of a deviation.

Depending on the water system design, the chemical attributes of a water system tend to be relatively constant and more uniformly distributed than the microbiological attributes. Therefore, less frequent sampling at only selected locations could be justified for chemical testing based on familiarity with system design and the existence of historically consistent operational data. However, with some purification system designs, the chemical quality could change dramatically in a short period of time (e.g., from the exhaustion of deionization beds), so frequent or even continuous in-line/on-line monitoring of the chemical attributes would be advisable to be able to recognize and correct the cause of the problem before non-compliant water is produced and used.

For microbial testing, all use points and critical sample ports in a distribution system are typically sampled routinely, including those that are infrequently used by manufacturing. There is no prescribed sampling frequency for Purified Water system outlets, so typical outlet sampling frequencies vary from daily to monthly, with sampling occurring somewhere in the system at least at weekly intervals.

A risk analysis is suggested for determining the sampling plan for a Purified Water system. Factors in this analysis could include (but are not limited to) the test result history for the entire water system as well as specific outlets, the criticality of specific outlets to manufacturing, the usefulness of selected sample ports as indicators of ongoing system control, and the scope of impact on products and activities should an unfavorable test result occur. For the scope of impact, the less frequent the sampling, the more products and processes will be affected by an unfavorable test result.

### 6.4.4 WATER FOR INJECTION DISTRIBUTION SYSTEM SAMPLING

The sampling plans for Water for Injection distribution systems (as well as any water system where some level of bacterial endotoxin control is needed) utilize the same general sampling approaches as do Purified Water systems. However, the regulatory expectations for Water for Injection distribution system sampling plans are more prescriptive because microbial control must be much more stringent as it is related to the bacterial endotoxin attribute. In general, water sampling for microbial

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and bacterial endotoxin testing is expected to occur daily somewhere in the system, with each outlet being sampled periodically, based on a risk assessment, to characterize the quality of the water.

### 6.5 Non-Routine Sampling

Non-routine sampling also can be performed on the water system for episodic events or reasons for which the routine sampling plans are insufficient to capture the needed information. Examples include change control purposes such as evaluating potential changes to sampling, testing, maintenance procedures, or system design; data or event excursion investigation purposes; or simply for long-term informational purposes and establishing baselines for future investigational value. The purpose of the non-routine sampling dictates the sampling procedures to be used, the attributes to be tested, and the location and repeating occurrence (if any) of that testing. It also should be noted that such non-routine sampling may be done from sampling ports that may or may not be routinely tested. Sampling ports can be positioned in a water system purely for investigational, non-routine sampling, and as such, they do not need to be part of a routine sampling plan.

***Change to read:***

## 7. CHEMICAL EVALUATIONS

### 7.1 Chemical Tests for Bulk Waters

The chemical attributes of *Purified Water* and *Water for Injection* that were in effect prior to *USP 23* were specified by a series of chemistry tests for various specific and nonspecific attributes with the intent of detecting chemical species indicative of incomplete or inadequate purification. Although these methods could have been considered barely adequate to control the quality of these waters, they nevertheless stood the test of time. This was partly because the operation of water systems was, and still is, based on on-line conductivity measurements and specifications generally thought to preclude the failure of these archaic chemistry attribute tests.

In 1996, USP moved away from these chemical attribute tests, switching to contemporary analytical technologies for the bulk waters Purified Water and Water for Injection. The intent was to upgrade the analytical technologies without tightening the quality requirements. The two contemporary analytical technologies employed were TOC and conductivity. The TOC test replaced the test for *Oxidizable Substances* that primarily targeted organic contaminants. A multi-staged conductivity test that detects ionic (mostly inorganic) contaminants replaced, with the exception of the test for *Heavy Metals*, all of the inorganic chemical tests (i.e., *Ammonia*, *Calcium*, *Carbon Dioxide*, *Chloride*, *Sulfate*).

Replacing the heavy metals attribute was considered unnecessary because 1) the source water specifications (found in the US EPA NPDWR) for individual heavy metals were tighter than the approximate limit of detection of the *Heavy Metals* test for *USP XXII* Water for Injection and Purified Water (approximately 0.1 ppm), 2) contemporary water system construction materials do not leach heavy metal contaminants, and 3) test results for this attribute have uniformly been negative. There has not been a confirmed occurrence of a singular test failure (failure of only the *Heavy Metals* test with all other attributes passing) since the current heavy metal drinking water standards have been in place.

*Total Solids* and *pH* were the only tests not covered by conductivity testing. The test for *Total Solids* was considered redundant because the nonselective tests of conductivity and TOC could detect most chemical species other than silica, which could remain undetected in its colloidal form. Colloidal silica in Purified Water and Water for Injection is easily removed by most water pretreatment steps, and even if present in the water, it constitutes no medical or functional hazard except in extreme and rare situations. In such extreme situations, other attribute extremes are also likely to be detected. It is, however, the user's responsibility to ensure fitness for use. If silica is a significant component in the source water, the purification unit operations could fail and selectively allow silica to be released into the finished water (in the absence of co-contaminants detectable by conductivity);therefore, either silica-specific testing or a total-solids type testing should be utilized to monitor for and control this rare problem.

The *pH* attribute was eventually recognized to be redundant to the conductivity test (which included *pH* as an aspect of the test and specification); therefore, *pH* was discontinued as a separate attribute test.

The rationale used by USP to establish its Purified Water and Water for Injection conductivity specifications took into consideration the conductivity contributed by the two least-conductive former attributes of *Chloride* and *Ammonia*, thereby precluding their failure had those wet chemistry tests been performed. In essence, the *Stage 3* conductivity specifications (see ⟨ 645⟩ , *Bulk Water, Procedure, Stage 3*) were established from the sum of the conductivities of the limit concentrations of chloride ions (from pH 5.0 to 6.2) and ammonia ions (from pH 6.3 to 7.0), plus the unavoidable contribution of other conductivity-contributing ions from water ($H^+$ and $OH^-$), dissolved atmospheric carbon dioxide [as bicarbonate ($HCO_3^-$)], and an electro-balancing quantity of either sodium ($Na^+$) or chlorine ($Cl^-$), depending on the pH-induced ionic imbalance (see *Table 1*). The *Stage 2* conductivity specification is the lowest value in this table, 2.1 µS/cm. The *Stage 1* specifications, designed primarily for on-line measurements, were derived by essentially summing the lowest values in individual ($H^+$, $OH^-$, $HCO_3^-$) and group ($Cl^-$, $Na^+$, $NH_4^+$) contributing ion columns for each of a series of tables similar to *Table 1*, created for each 5° increment between 0° and 100°. For purpose of an example, the italicized values in *Table 1*, the conductivity data table for 25°, were summed to yield a conservative value of 1.3 µS/cm, the *Stage 1* specification for a nontemperature-compensated, nonatmosphere-equilibrated water sample that actually had a measured temperature of 25°–29°. Each 5° increment in the table was similarly treated to yield the individual values listed in the *Stage 1* specifications (see ⟨ 645⟩ , *Table 1*).

▲Although *Stage 1* is intended for on-line measurement, it can be used off-line provided it is understood that the sample is subject to contamination by absorption of atmospheric carbon dioxide ($CO_2$), which will result in higher conductivities than on-line measurements. This absorption results in the formation of conductive ions, hydrogen ($H^+$) and bicarbonate ($HCO_3^-$). The quantity of the increase is dependent on the temperature, surface exposure, ambient carbon dioxide concentration, and amount of time the water sample is exposed to air, but the absorption begins immediately upon contact with air. The

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conductivity will vary with time unless a steady state is achieved. These higher conductivities may not pass the *Stage 1* test requirements, which in turn would require the performance of *Stage 2* and possibly *Stage 3* testing to achieve a passing result. If the off-line tested sample passes *Stage 1*, no further work is needed because *Stage 1* is not subject to false negatives—only false positives. Consequently, because of the variable influence of atmospheric carbon dioxide from sample to sample, these off-line *Stage 1* test results should not be used for trending purposes because they do not accurately reflect water system performance.▲ (USP 1-Dec-2021)

**Table 1. Contributing Ion Conductivities of the Chloride-Ammonia Model as a Function of pH (in atmosphere-equilibrated water at 25°)**

| | Conductivity (µS/cm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| pH | $H^+$ | $OH^-$ | $HCO_3^-$ | $Cl^-$ | $Na^+$ | $NH_4^+$ | Combined Conductivities | Stage 3 Limit |
| 5.0 | 3.49 | *0* | *0.02* | *1.01* | *0.19* | *0* | 4.71 | 4.7 |
| 5.1 | 2.77 | 0 | 0.02 | 1.01 | 0.29 | 0 | 4.09 | 4.1 |
| 5.2 | 2.20 | 0 | 0.03 | 1.01 | 0.38 | 0 | 3.62 | 3.6 |
| 5.3 | 1.75 | 0 | 0.04 | 1.01 | 0.46 | 0 | 3.26 | 3.3 |
| 5.4 | 1.39 | 0 | 0.05 | 1.01 | 0.52 | 0 | 2.97 | 3.0 |
| 5.5 | 1.10 | 0 | 0.06 | 1.01 | 0.58 | 0 | 2.75 | 2.8 |
| 5.6 | 0.88 | 0 | 0.08 | 1.01 | 0.63 | 0 | 2.60 | 2.6 |
| 5.7 | 0.70 | 0 | 0.10 | 1.01 | 0.68 | 0 | 2.49 | 2.5 |
| 5.8 | 0.55 | 0 | 0.12 | 1.01 | 0.73 | 0 | 2.41 | 2.4 |
| 5.9 | 0.44 | 0 | 0.16 | 1.01 | 0.78 | 0 | 2.39 | 2.4 |
| 6.0 | 0.35 | 0 | 0.20 | 1.01 | 0.84 | 0 | 2.40 | 2.4 |
| 6.1 | 0.28 | 0 | 0.25 | 1.01 | 0.90 | 0 | 2.44 | 2.4 |
| 6.2 | 0.22 | 0 | 0.31 | 1.01 | 0.99 | 0 | 2.53 | 2.5 |
| 6.3 | 0.18 | 0 | 0.39 | 0.63 | 0 | 1.22 | 2.42 | 2.4 |
| 6.4 | 0.14 | 0.01 | 0.49 | 0.45 | 0 | 1.22 | 2.31 | 2.3 |
| 6.5 | 0.11 | 0.01 | 0.62 | 0.22 | 0 | 1.22 | 2.18 | 2.2 |
| 6.6 | 0.09 | 0.01 | 0.78 | 0 | 0.04 | 1.22 | 2.14 | 2.1 |
| 6.7 | 0.07 | 0.01 | 0.99 | 0 | 0.27 | 1.22 | 2.56 | 2.6 |
| 6.8 | 0.06 | 0.01 | 1.24 | 0 | 0.56 | 1.22 | 3.09 | 3.1 |
| 6.9 | 0.04 | 0.02 | 1.56 | 0 | 0.93 | 1.22 | 3.77 | 3.8 |
| *7* | *0.03* | 0.02 | 1.97 | 0 | 1.39 | 1.22 | 4.63 | 4.6 |

As stated above, this rather radical change to utilizing a conductivity attribute as well as the inclusion of a TOC attribute allowed for on-line measurements. This was a major philosophical change and allowed industry to realize substantial savings. The TOC and conductivity tests also can be performed off-line in the laboratories using collected samples, although sample collection tends to introduce opportunities for adventitious contamination that can cause false high readings. The collection of on-line data is not, however, without challenges. The continuous readings tend to create voluminous amounts of data, where previously only a single data point was available. As stated in *6. Sampling*, continuous in-process data are excellent for understanding how a water system performs during all of its various usage and maintenance events in real time, but this is too much data for QC purposes. Instead, one can use a justifiable portion of the data (at a designated daily time or at the time of batch manufacturing) or the highest value in a given period as a worst case representation of the overall water quality for that period. Data averaging is generally discouraged because of its ability to obscure short-lived extreme quality events.

## 7.2 Chemical Tests for Sterile Waters

Packaged/sterile waters present a particular dilemma relative to the attributes of conductivity and TOC. The package itself is the major source of chemicals (inorganics and organics) that leach over time into the packaged water and can easily be detected by the conductivity and TOC tests. The ▲historic test for▲ (USP 1-Dec-2021) "organic purity" ▲in▲ (USP 1-Dec-2021) bulk and packaged ▲waters was the *Oxidizable Substances* test. The▲ (USP 1-Dec-2021) insensitivity of that test to many of the organic leachables from plastic and elastomeric packaging materials was largely unrecognized, allowing organic levels in ▲some▲ (USP 1-Dec-2021) packaged/sterile waters to be ▲historically▲ (USP 1-Dec-2021) quite high (possibly ▲as much as 10- to 100-fold higher than▲ (USP 1-Dec-2021) the TOC specification for bulk water).

Similarly, glass containers can also leach inorganics, such as sodium, which are easily detected by conductivity ▲and not▲ (USP 1-Dec-2021) detected by the former wet chemistry attribute tests. Most of these ▲inorganic▲ (USP 1-Dec-2021) leachables are considered harmless based on current perceptions and standards at the ▲low▲ (USP 1-Dec-2021) concentrations present.

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Nevertheless, ▲both organic and inorganic leachables▲ (USP 1-Dec-2021) degrade the quality of the high-purity waters placed into these packaging systems. Some packaging materials contain more leachables than others and may not be as suitable for ▲some applications as other packaging systems. Not all packaging materials are suitable for holding bulk water.

Because Sterile Water for Injection is Water for Injection that has already met its monographed requirements for low conductivity and TOC, the Sterile Water for Injection▲ (USP 1-Dec-2021) attributes of conductivity and TOC ▲▲ (USP 1-Dec-2021) reveal more about the packaging leachables than they do about the water's original purity. ▲The historically ▲ (USP 1-Dec-2021) "allowed" leachables ▲(due to the insensitivity and selective reactivity of the historic water purity tests)▲ (USP 1-Dec-2021) could render the sterile packaged ▲waters▲ (USP 1-Dec-2021) unsuitable for many uses where the bulk waters are perfectly adequate. ▲Therefore, usage of these packaged waters for analytical, manufacturing, and cleaning applications should be only exercised after the purity of the water for the application has been confirmed and documented as suitable for its intended use.

### 7.2.1 CONDUCTIVITY FOR STERILE WATERS

To better control the ionic packaging leachables, ⟨ 645⟩ is divided into two sections. The first, ⟨ 645⟩ , *Bulk Water*, applies to *Purified Water*, *Water for Injection*, *Water for Hemodialysis*, and *Pure Steam*, and includes the three-stage conductivity testing instructions and specifications. The second, ⟨ 645⟩ , *Sterile Water*, applies to *Sterile Purified Water*, *Sterile Water for Injection*, *Sterile Water for Inhalation*, and *Sterile Water for Irrigation*. The *Sterile Water* section includes a conductivity testing approach similar to ⟨ 645⟩ , *Bulk Water, Procedure, Stage 2* because it is intended as a laboratory test. Because these sterile waters were made from bulk water that already complied with the 3-stage conductivity test, the packaging leachables are the primary target analytes of the conductivity specifications in ⟨ 645⟩ , *Sterile Water*.The effect on potential leachables concentrations as a function of surface area-to-volume ratio differences of different container sizes is the rationale for having two different specifications: one for small packages containing nominal volumes of 10 mL or less and another for larger packages. These conductivity specifications are harmonized with the *European Pharmacopoeia* conductivity specifications for Sterile Water for Injections. All *USP* monographed waters, except Bacteriostatic Water for Injection, have a conductivity specification that directs the user to either ⟨ 645⟩ , *Bulk Water* or ⟨ 645⟩ , *Sterile Water*. For the sterile packaged water monographs, this water conductivity specification replaces the previous wet chemistry limit tests intended for inorganic contaminants that had been previously specified in these monographs prior to *USP 32* (May 1, 2009).

### 7.2.2 TOTAL ORGANIC CARBON FOR STERILE WATERS

Controlling the organic purity of these sterile packaged waters, particularly those in plastic packaging, is more challenging. The TOC test can better detect these impurities and therefore can be better used to monitor and control these impurities than the previous *Oxidizable Substances* test prior to *USP 23 Supplement 5*.

Therefore, to better control the organic packaging leachables, *Total Organic Carbon* ⟨ 643⟩ is divided into two sections, similar to ⟨ 645⟩ . The first section, ⟨ 643⟩ , *Procedures, 1. Bulk Water*, applies to *Purified Water*, *Water for Injection*, *Water for Hemodialysis*, and condensate form of *Pure Steam*. The second section, ⟨ 643⟩ , *Procedures, 2. Sterile Water*, applies to *Sterile Purified Water*, *Sterile Water for Injection*, *Sterile Water for Inhalation*, and *Sterile Water for Irrigation*.

The effect on potential leachables concentrations as a function of surface area-to-volume ratio differences of different container sizes is the rationale for having different container volume-based specifications for TOC. Low volume containers (<5 mL) that are intended for single administration (e.g., syringes) have a higher surface area-to-volume ratio than large volume packages (e.g., intravenous packages). This results in potentially high concentrations of leachables appearing in the smaller doses/packages. Additionally, packages like prefilled syringes may have innocuous organic silicone-based plunger lubricants contributing to the water TOC. A volume breakpoint was also created for intermediate-sized containers at 100 mL [for containers between 5 and 100 mL] as one group and then for large volume parenteral (LVP) containers larger than 100 mL. Therefore, there are three TOC specifications that are related to the volume of the container and created by the volume ranges for breakpoints at 5 mL and again at 100 mL.

The rationale for establishing staged limits for organic carbon concentration from low to high volume containers is based on the smaller patient doses being administered from the smaller containers. (Dose is proportional to the container size.) The actual dose of leachables administered to a patient would be higher from the large volume containers.

The specification for each nominal container volume has two limit response levels. This is to limit the maximal potential leachables for a particular packaging type and volume that could be considered acceptable. If the TOC response is NMT limit response 1, the sample meets the test requirements with no further justification of acceptability needed. If the TOC response exceeds limit response 1, it is compared to limit response 2. If the response exceeds limit response 2, the sample does not meet the test requirements, the test is ended, and the sample fails.

If the response is more than limit response 1 and less than limit response 2, then a further justification of suitability is needed. That justification requires the identification and quantification of each individual organic impurity whose carbon composition exceeds a concentration of 0.20 mg/L of carbon. If no individual impurities exceed 0.20 mg/L of carbon, the water sample meets the test requirements.

For individual impurities that exceed 0.20 mg/L of carbon, their dose to the patient must be evaluated for its impact on patient safety based on toxicological studies whose publications must be cited. The water sample meets the test requirements if all such impurities are identified and quantified and their patient doses are deemed safe and documented. The water sample does not meet the test requirements if one of the following two results occur:

1. Any of the impurities at greater than 0.20 mg/L of carbon are not identified or quantified
2. Any of such impurities do not have referenced toxicological/safety documentation for the patient doses received

These test requirements apply to individual batches of product. They cannot be broadly applied to a given product or container type or grandfathered as acceptable by previously approved packaging configurations. The rationale for this requirement lies in the composition of the leachables that can be variable from batch to batch and even unit to unit of a given batch of product, depending on the PC used for the packaging fabrication and component assembly processes. In addition, there can be variability in the polymeric resin raw material composition used in some of the packaging components based on

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batch-to-batch variability or variability between suppliers of the same raw material. Therefore, the product batch sampling plan must be able to detect these potential variabilities, if present.

The system suitability for the TOC testing must be performed using the concentration that corresponds to the highest TOC concentration for the container volume that could be considered acceptable for a given batch of product. This corresponds to limit response 2 for the corresponding nominal container volume.

Additional testing considerations include the following:

1. Vigorous shaking of the container is needed prior to withdrawing a sample in order to release superficial organic residues from the internal surfaces of the containers.
2. Each sampled container must be individually tested and compared to the specification.
3. Pooling of container contents may be needed *only* when individual containers do not contain enough volume to be tested individually. In such cases, the lowest number of containers needed to achieve the minimal pooled volume required by the testing apparatus should be used.▲ (USP 1-Dec-2021)

## 7.3 Storage and Hold Times for Chemical Tests

Because of the homogeneous nature of chemical impurities in water, the storage requirements and impact of holding times are very practically determined, unlike the challenges of microbial impurities. In general, the chemical purity of high-purity water samples can only degrade over time, possibly generating a failed result of the sample that would have passed if it were tested immediately or on-line. The general fact is that the longer samples are stored, the greater the potential to be adversely affected by containers or conditions.

For off-line chemical tests ▲for▲ (USP 1-Dec-2021) waters, there are no compendial requirements for storage time and conditions. However, the general recommendation is to perform testing as soon as practical to avoid false adverse results. Where possible, store ▲water samples in▲ (USP 1-Dec-2021) cool ▲conditions▲ (USP 1-Dec-2021) and measure as quickly as practical. This reduces the chances that a water sample gets contaminated over time, and this would reduce unwarranted and unnecessary investigations of false positives.

### 7.3.1 CONTAINERS

When sampling water for off-line analysis, the selection and cleanliness of the container play a significant part in obtaining accurate data. For samples to be tested for chemical impurities according to ⟨ 643⟩ and ⟨ 645⟩ , the proper container should be one that does not contaminate the sample during the storage/hold time. For example, the use and preparation of glass containers could be very acceptable for storing samples for TOC testing, but some glass containers do leach ions over time (hours and days), and they can adversely affect a conductivity test by creating a false positive result—if the storage time is too long. Likewise, there are some polymer materials that can adversely affect the TOC chemical impurity in water. However, many polymer materials are very inert.

In any case, cleanliness of the container is crucial because trace quantities of soaps and fingerprints will adversely affect the chemical purity of the water. Properly cleaned containers are acceptable because chemical impurities are easily rinsed away. Extensive chemical cleaning methods such as acid or caustic rinsing should never be needed. If they are needed, consider replacing the containers.

### 7.3.2 STORAGE TIME AND CONDITIONS

There are no specific recommendations for storage of samples for water analyses. If there is some trace interaction of the container and water, then generally colder and shorter storage times are better than warmer and longer storage times. Chemical dissolution and reactivity are usually enhanced by increased temperature. Furthermore, time is always an element because the water sample can only get worse in a container, and it never gets better with time.

## 7.4 Elemental Impurities in Pharmaceutical Waters

Elemental impurities (EI) have the most restrictive limits for Water for Injection used in manufacturing parenterals, in particular large-volume injections (see *Injections and Implanted Drug Products* ⟨ 1⟩ for a definition of large-volume injections) because of the large dose. The most restrictive permissible daily exposure (PDE) of EI resides with lead, mercury, cadmium, and arsenic. Other EI listed in ⟨ 232⟩ permit a substantially higher PDE, and are therefore less restrictive.

Water that meets US EPA NPDWR or WHO Drinking Water Guidelines that has been purified by conventional technologies used to produce Water for Injection can comply with ⟨ 232⟩ for parenterals.

*Table 2* shows that source water that meets US EPA NPDWR or WHO Drinking Water Guidelines has maximum contaminant levels (concentration) for lead, mercury, cadmium, and arsenic that are NMT 10 times (1-log) higher than the EI limits for parenterals, based on a daily dose of 2000 mL. For a smaller volume injection, the allowed parenteral daily dose of EI is correspondingly higher. The purification technologies needed to produce Water for Injection that reduce the impurities by a factor of 100 to 1000 will assure compliance with ⟨ 232⟩ , provided no elemental impurities are added during processing, packaging, delivery, or storage.

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**Table 2. Elemental Impurity Limits for Drug Products and Their Water Components per** ⟨ 232⟩

| Element | Parenteral PDE (µg/day) | Parenteral Daily Dose (µg/mL[a]) | US EPA National Primary Drinking Water Regulations (µg/mL[b]) | WHO Drinking Water Guidelines (µg/mL[b]) | Result of 2-Log Reduction of EI Concentration for Water for Injection (µg/mL[c]) |
|---|---|---|---|---|---|
| Cadmium | 2 | 0.001 | 0.005 | 0.003 | 0.00005 |
| Lead | 5 | 0.0025 | 0.015 | 0.01 | 0.00015 |
| Inorganic arsenic | 15 | 0.0075 | 0.01 | 0.01 | 0.0001 |
| Inorganic mercury | 3 | 0.0015 | 0.002 | 0.006 | 0.00006 |

[a] Concentration based on a daily dose of 2000 mL, and all drug product elemental impurities coming from the water component.
[b] Drinking Water regulations state these Maximum Contaminant Levels (MCLs) as mg/L, which equals µg/mL or ppm.
[c] Determined from the greater of the US EPA regulations column and WHO guidelines column for each element, and then divided by 100 (2-log).

Chemical purification technologies for Purified Water are similarly efficient in removing EI as those for Water for Injection production. Because all sterile waters are prepared from Purified Water or Water for Injection, the assurance of compliance to ⟨ 232⟩ extends to sterile waters, provided no elemental impurities added are during processing, packaging, delivery, or storage.

Further discussion can be found in *Pharmacopeial Forum* (*1*).

***Change to read:***

# 8. MICROBIAL EVALUATIONS

This section presents a discussion about the types and sources of microorganisms and whether certain microbes are prone to colonize pharmaceutical water systems. This section also addresses microbiological examination of water samples, including a discussion on recovery methods.

## 8.1 Microorganism Types

Microorganisms are ubiquitous and their natural habitats are extremely diverse. Based on comparative ribosomal RNA sequencing, the phylogenetic tree of life consists of three domains: Bacteria and Archaea (both prokaryotes), and Eukarya (eukaryotes). Most microorganisms that contaminate pharmaceutical products are prokaryotic bacteria and eukaryotic fungi (yeasts and molds). These microbes are typical isolates from pharmaceutical environments, including the associated personnel, and a few are frank or opportunistic pathogens. Contamination with viruses is a concern in bioprocessing that uses animal cells.

### 8.1.1 ARCHAEANS

Microbes from the domain Archaea are phylogenetically related to prokaryotes but are distinct from bacteria. Many are extremophiles, with some species capable of growing at very high temperatures (hyperthermophiles) or in other extreme environments beyond the tolerance of any other life form. In general, most extremophiles are anaerobic or microaerophilic chemolithoautotrophs. Because of their unique habitats, metabolism, and nutritional requirements, Archaeans are not known to be frank or opportunistic pathogens, and they are not capable of colonizing a pharmaceutical water system.

### 8.1.2 BACTERIA

Bacteria are of immense importance because of their rapid growth, mutation rates, and ability to exist under diverse and adverse conditions; some of them are human pathogens. Some are very small and can pass through 0.2-µm rated filters. Others form spores, which are not part of their reproductive cycle. Bacterial spore formation is a complex developmental process that allows the organisms to produce a dormant and highly resistant cell in times of extreme stress. Bacterial endospores can survive high temperatures, strong UV irradiation, desiccation, chemical damage, and enzymatic destruction, which would normally kill vegetative bacteria.

Using a traditional cellular staining technique based on cell wall compositional differences, bacteria are categorized into gram positive and gram negative, although many sub-groups exist within each category based on genomic similarities and differences.

**8.1.2.1 Gram-positive bacteria:** Gram-positive bacteria are common in a pharmaceutical manufacturing environment but not in water systems. This is because they are generally not suited to surviving in a liquid environment that has the chemical purity of a pharmaceutical-grade water system. Gram-positive bacteria include the spore-forming bacteria from the genus *Bacillus*, which are common soil and dust microorganisms, and the non-sporulating bacteria from the genera *Staphylococcus*, *Streptococcus*, and *Micrococcus*, which normally colonize human skin and mucous membranes. Other types of gram-positive bacterial microorganisms include organisms from the genera *Corynebacterium*, *Mycobacterium*, *Arthrobacter*, *Propionibacterium*, *Streptomyces*, and *Actinomyces.* This latter group of microbes can be found in various natural habitats including the human skin and soil.

Although gram-positive bacteria can be detected in pharmaceutical water samples, their recovery is often associated with faulty aseptic technique during sampling or testing, or associated with exogenous contamination sources. Although these non-aquatic microorganisms could be present in source water and could, in rare circumstances, make their way into the early stages of a water purification unit operation, gram-positive bacteria are not known to colonize water systems. In addition, these

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microbes will likely be removed by one or more of the purification unit operations prior to the ultimate creation of the pharmaceutical-grade water.

**8.1.2.2 Gram-negative bacteria:** These types of bacteria are found in soil, water, plants, and animals. Gram-negative bacteria are relevant to pharmaceutical manufacturers, primarily due to their production of endotoxins as well as their ability to populate water systems, a topic discussed in *8.4 Endotoxin*. Some gram-negative bacteria prefer aquatic habitats and tend to colonize water systems and other wet environments as biofilms, a topic discussed in *8.2 Biofilm Formation in Water Systems*.

**8.1.2.3 Mycoplasma:** Organisms from the genus Mycoplasma are the smallest of the bacteria. Unlike other bacteria, these organisms do not have a cell wall and many exist as intracellular or animal/plant parasites. Mycoplasmas also may require specific nutrients for survival, including sterols, and they cannot survive in a hypotonic environment such as pure water. Based on these facts, this type of bacteria is not a concern for pharmaceutical-grade water systems.

### 8.1.3 FUNGI

Fungi are mainly aerobic mesophilic microbes. They exist as unicellular (yeast) and multicellular filamentous (mold) organisms. Molds are often found in wet/moist ▲environments▲ (USP 1-Dec-2021) but usually non-aquatic environments, such as soil and decaying vegetation. Yeasts are often associated with humans and vegetation, and both yeasts and molds also can be found in pharmaceutical environments. As mold matures it develops spores, which, unlike bacterial spores, are part of its reproductive cycle and are less resistant to adverse conditions. Mold spores are easily spread through air and materials, and could contaminate water samples.

Neither yeasts nor molds are suited for colonization or survival in pharmaceutical water systems. Their recovery is often associated with faulty aseptic technique during sampling or testing, or associated with exogenous contamination sources. These non-aquatic microorganisms, if present in source water, could make their way into the early stages of a water purification system; however, they will likely be removed by one or more of the purification unit operations.

### 8.1.4 VIRUSES

A virus is a small infectious agent unlike eukaryotes and prokaryotes. This is because viruses have no metabolic abilities of their own. Viruses are genetic elements containing either DNA or RNA that replicate within host cells. Human pathogenic viruses, especially those of fecal origin, could be present in source water. However, they are easily neutralized by typical water purification treatments, such as chlorination. Therefore, it is unlikely that human pathogenic viruses will be present or will proliferate (due to the absence of host cells) in pharmaceutical-grade waters.

### 8.1.5 THERMOPHILES

Thermophiles are heat-loving organisms and can be either bacteria or molds. Thermophilic and hyperthermophilic aquatic microorganisms (see *8.1.1 Archaeans*) require unique environmental and nutritional conditions to survive (e.g., presence of specific inorganic or organic nutrients and their concentrations, extreme pH, presence or absence of oxygen). These conditions do not exist in the high-purity water of pharmaceutical water systems, whether ambient or hot, to support their growth. Bacteria that are able to inhabit hot pharmaceutical water systems are invariably found in much cooler locations within these hot systems (e.g., within infrequently used outlets, ambient subloops off of hot loops, use-point and sub-loop cooling heat exchangers, transfer hoses and connecting pipes, or dead legs). These bacterial contaminants are the same mesophilic (moderate temperature-loving) types found in ambient water systems and are not thermophiles. Based on these facts, thermophilic bacteria are not a concern for hot pharmaceutical-grade water systems.

## 8.2 Biofilm Formation in Water Systems

A biofilm is a three-dimensional structured community of sessile microbial cells embedded in a matrix of extracellular polymeric substances (EPS). Biofilms form when bacteria attach to surfaces in moist environments and produce a slimy, glue-like substance, the EPS matrix, while proliferating at that location. This slimy matrix facilitates biofilm adhesion to surfaces as well as the attachment of additional planktonic cells to form a microbial community.

The EPS matrix of biofilms that colonize water systems also facilitates adsorption and concentration of nutrients from the water and retains the metabolites and waste products produced by the embedded biofilm cells, which can serve as nutrients for other biofilm community members.

This EPS matrix is also largely responsible for biofilm's resistance to chemical sanitizers, which must penetrate completely through the matrix to contact and kill the biofilm cells within the matrix. Heat sanitization approaches do not generally have these EPS matrix penetration difficulties, so they are usually considered superior to chemicals in killing biofilms where materials of construction allow.

The three-dimensional structure of a well-developed biofilm, as well as the biofilm's creation and release of small, motile "pioneer cells" for further colonization, are facilitated through gene expression modulating "quorum sensing" chemicals released in tiny amounts by individual biofilm cells and concentrated to a functional level within this same EPS matrix. So, the EPS matrix of biofilms is primarily responsible for the biofilm's success in colonizing and proliferating in very low nutrient-containing high-purity water systems. The EPS matrix also explains the difficulty in killing and/or removing biofilms from water purification and distribution system surfaces.

### 8.2.1 BIOFILM-FORMING BACTERIA IN WATER SYSTEMS

Common microorganisms recovered from water system samples include gram-negative bacteria from the genera *Pseudomonas*, *Ralstonia*, *Burkholderia*, *Stenotrophomonas*, *Comamonas*, *Methylobacterium*, and many other types of *Pseudomonas*-like organisms known collectively as pseudomonads (members of the family *Pseudomonadaceae*). These types of

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microbes, found in soil and source water, tend to colonize all water system distribution and purification system surfaces including activated carbon beds, deionizing resin beds, reverse osmosis systems, membrane filtration modules, connecting piping, hoses, and valves. If not controlled, they can compromise the functionality of purification steps in the system and spread downstream, possibly forming biofilms on distribution system surfaces (e.g., tanks, piping, valves, and hoses), from where they can be sheared or otherwise released into the finished water used in processes and products.

Some of the biofilm pseudomonads are opportunistic human pathogens and may possess resistance to commonly used pharmaceutical product preservatives, particularly when embedded in EPS matrix flocs sheared from water system biofilms. Several pseudomonads are also capable of utilizing a wide variety of carbon sources as nutrients, allowing them to colonize austere, adventitious nutrient environments such as water systems. This nutritional diversity also makes them capable of growing to very high numbers in some pharmaceutical products and raw materials, thus leading to product adulteration and potential risk to patient health. Given that these bacteria are commonly found in aqueous environments, endotoxin control for Water for Injection systems (and some Purified Water systems) through biofilm control becomes critical.

#### 8.2.2 NON-BIOFILM-FORMING BACTERIA IN WATER SYSTEMS

Other types of non-pseudomonad gram-negative bacteria, such as the genera *Escherichia*, *Salmonella*, *Shigella*, *Serratia*, *Proteus*, *Enterobacter*, and *Klebsiella*, are used as indicators of fecal contamination. Although some of these bacteria are also plant pathogens, others can be human enteric pathogens and can contaminate potable water supplies. These non-pseudomonads are not suited to colonizing or surviving in pharmaceutical water systems owing to the water's chemical purity. In fact, non-pseudomonad enteric bacteria are extremely unlikely contaminants of pharmaceutical water systems unless local sewage and source water controls are not in place. Such controls are required in order to comply with the source water requirements for making USP-grade waters as described in their respective monographs.

## 8.3 Microorganism Sources

#### 8.3.1 EXOGENOUS CONTAMINATION

Exogenous microbial contamination of bulk pharmaceutical water comes from numerous possible sources, including source water. At a minimum, source water should meet the microbial quality attributes of drinking water, which is the absence of fecal coliforms (*Escherichia coli*). A wide variety of other types of microorganisms, chiefly gram-negative bacteria, may be present in the incoming water. If appropriate steps are not taken to reduce their numbers or eliminate them, these microorganisms may compromise subsequent water purification steps.

Exogenous microbial contamination can also arise from maintenance operations, equipment design, and the process of monitoring, including the following:

- Unprotected, faulty, or absent vent filters or rupture disks
- Backflow from interconnected equipment
- Non-sanitized distribution system openings for component replacements, inspections, repairs, and expansions
- Inadequate drain air-breaks
- Innate bioburden of activated carbon, ion-exchange resins, regenerant chemicals, and chlorine-neutralizing chemicals
- Inappropriate rinsing water quality after regeneration or sanitization
- Poor sanitization of use points, hard-piped equipment connectors, and other water transfer devices such as hoses
- Deficient techniques for use, sampling, and operation

The exogenous contaminants may not be normal aquatic bacteria but rather microorganisms of soil, air, or even human origin. The detection of non-aquatic microorganisms may be an indication of sampling or testing contamination or a system component failure, which should trigger investigation and remediation. Sufficient care should be given to sampling, testing, system design, and maintenance to minimize microbial contamination from exogenous sources.

#### 8.3.2 ENDOGENOUS CONTAMINATION

Endogenous sources of microbial contamination can arise from unit operations in a water purification system that is not properly maintained and operated. Microorganisms present in source water may adsorb to carbon bed media, ion-exchange resins, filter membranes, and other equipment surfaces, and initiate the formation of biofilms.

Downstream colonization can occur when microorganisms are shed from existing biofilm-colonized surfaces and carried to other areas of the water system. Microorganisms may also attach to suspended particles such as carbon bed fines or fractured resin particles. When the microorganisms become planktonic, they serve as a source of contamination to subsequent purification equipment and to distribution systems.

Another source of endogenous microbial contamination is the distribution system itself. Microorganisms can colonize pipe surfaces, rough welds, misaligned flanges, valves, and dead legs, where they proliferate and form biofilms. Once formed, biofilms can become a continuous source of microbial contamination, which is very difficult to eradicate. Therefore, biofilm development must be managed by methods such as frequent cleaning and sanitization, as well as process and equipment design.

## 8.4 Endotoxin

Bacterial endotoxin is a lipopolysaccharide (LPS) that is a component of the outer cell membrane of gram-negative bacteria. Endotoxins may occur as collections of LPS molecules associated with living microorganisms, fragments of dead microorganisms, the EPS matrix surrounding biofilm bacteria, or free molecular clusters or micelles containing many

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lipopolysaccharide molecules. The monomeric form of the endotoxin molecule does not exist in high-purity water because of the molecule's amphipathic nature. Some grades of pharmaceutical waters, such as those used in parenteral applications (e.g., Water for Injection, Water for Hemodialysis, and the sterilized packaged waters made from Water for Injection) strictly limit the amount of endotoxins that may be present because these compounds are pyrogenic.

#### 8.4.1 SOURCES

Endotoxins may be introduced into the system from the source water or may be released from cell surfaces of bacteria in water system biofilms. For example, a spike in endotoxin may occur following sanitization as a result of endotoxin release from killed cells. Endotoxin quantitation in water samples is not a good indicator of the level of biofilm development in a water system because of the multiplicity of endotoxin sources.

#### 8.4.2 REMOVAL AND CONTROL

To control endotoxin levels in water systems, it is important to control all potential sources of contamination with gram-negative bacteria as well as free endotoxin in the water. Contamination control includes the use of upstream unit operations to reduce bioburden from incoming water, as well as engineering controls (e.g., heat sanitization, equipment design, UV sanitizers, filters, material surface, and flow velocity) to minimize biofilm development on piping surfaces and to reduce re-inoculation of the system with free-floating bacteria.

Endotoxin remediation may be accomplished through the normal exclusion or removal action afforded by various unit operations within the treatment system. Examples of endotoxin removal steps in a water purification train include reverse osmosis, deionization, ultrafilters, distillation, and endotoxin-adsorptive filters.

## 8.5 Test Methods

Microbes in water systems can be detected as exampled in this section or by methods adapted from *Microbial Enumeration Tests* ⟨ 61⟩ , *Tests for Specified Microorganisms* ⟨ 62⟩ , or the current edition of *Standard Methods for the Examination of Water and Wastewater* from the American Public Health Association. This section describes classical culture approaches to bioburden testing, with a brief discussion on rapid microbiological methods.

Every water system has a unique microbiome. It is the user's responsibility to perform method validation studies to demonstrate the suitability of the chosen test media and incubation conditions for bioburden recovery. In general, users should select the method that recovers the highest planktonic microbial counts in the shortest time, thus allowing for timely investigations and remediation. Such studies are usually performed before or during system validation.

The steady state condition can take months or even years to be achieved, and can be affected by a change in source water quality, changes in finished water purity by using modified or increasingly inefficient purification processes, changes in finished water use patterns and volumes, changes in routine and preventative maintenance or sanitization procedures and frequencies, or any type of system intrusion (e.g., component replacement, removal, or addition).

#### 8.5.1 MICROBIAL ENUMERATION CONSIDERATIONS

Most microbial contaminants in water systems are found primarily as biofilms on surfaces, with only a very small percentage of the microbiome suspended in the water, or planktonic, at any given time. Although it would seem logical to directly monitor biofilm development on surfaces, current technology for surface evaluations in an operating water system makes this impractical in a GMP environment. Therefore, an indirect approach must be used: the detection and enumeration of planktonic microorganisms that have been released from biofilms. This planktonic microbiome will affect the processes or products where the water is used.

The detection and enumeration of the planktonic microbiome can be accomplished by collecting samples from water system outlets. Planktonic organisms are associated with the presence of biofilms as well as free-floating bacteria introduced into the system (pioneer cells), which may eventually form new biofilms. Therefore, by enumerating the microorganisms in water samples, the overall state of control over biofilm development can be assessed. This assessment has historically been accomplished with classical cultural techniques, which are viewed as the traditional method. However, nutritional limitations of the growth media may not satisfy growth requirements of organisms present in the water system that originated from a biofilm. As a result, traditional cultural methods may only detect a fraction of the biofilm bacteria present in the water sample. Other options are available, such as rapid microbiological methods.

There is no ideal cultural enumeration method that will detect all microorganisms in a water sample, although some media or incubation temperatures may be better than others. However, from a PC perspective, this limitation is acceptable because it is the relative changes in the trends for water sample microbial counts that indicate the state of PC.

Consideration also should be given to the timeliness of microbial testing after sample collection. The number of detectable organisms in a sample collected in a sterile, scrupulously clean sample container will usually decrease as time passes. The organisms within the sample may die or adhere to the container walls, reducing the number that can be withdrawn from the sample for testing. The opposite effect can also occur if the sample container is not scrupulously clean and contains a low concentration of nutrients that could promote microbial growth. Because the number of organisms in the water can change over time after sample collection, it is best to test the samples as soon as possible. If it is not possible to test the sample within 2 h of collection, the sample should be held at refrigerated temperatures (2°–8°) and tested within 24 h. In situations where even 24 h is not possible (such as when using off-site contract laboratories), it is particularly important to qualify the microbiological sample hold times and storage conditions to avoid significant changes in the microbial population during sample storage.

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## 8.5.2 THE CLASSICAL CULTURAL APPROACH

Classical cultural approaches for microbial testing of water include but are not limited to pour plates, spread plates, membrane filtration, and most probable number (MPN) tests. These methods are generally easy to perform, and provide excellent sample processing throughput. Method sensitivity can be increased via the use of larger sample sizes. This strategy is used in the membrane filtration method. Cultural approaches are further defined by the type of medium used in combination with the incubation temperature and duration. This combination should be selected according to the monitoring needs of a specific water system and its ability to recover the microorganisms of interest; that is, those that could have a detrimental effect on the products manufactured or process uses, as well as those that reflect the microbial control status of the system.

**8.5.2.1 Growth media:** The traditional categorization is that there are two basic forms of media available: "high nutrient" and "low nutrient". Those media traditionally categorized as high-nutrient include Plate Count Agar (TGYA), Soybean Casein Digest Agar (SCDA or TSA), and m-HPC Agar (formerly m-SPC Agar). These media are intended for the general isolation and enumeration of heterotrophic or copiotrophic bacteria. Low-nutrient media, such as R2A Agar and NWRI Agar (HPCA), have a larger variety of nutrients than the high-nutrient media. These low-nutrient media were developed for use with potable water due to their ability to recover a more nutritionally diverse population of microorganisms found in these environments. The use of R2A may not be the best choice for high-purity water systems. Even though high-purity water creates an oligotrophic environment, it has been shown empirically that in many high-purity compendial waters, the microbial count disparity between low- and high-nutrient media is dramatically less to nil, as compared to potable water. Nevertheless, using the medium that has been demonstrated as acceptable through comparative media analysis is recommended.

**8.5.2.2 Incubation conditions:** Duration and temperature of incubation are also critical aspects of microbiological testing. Classical compendial methods (e.g., ⟨ 61 ⟩) specify the use of high-nutrient media, typically incubated at 30°–35° for NLT 48 h. Given the types of microbes found in many water systems, incubation at lower temperatures (e.g., ranges of 20°–25° or 25°–30°) for longer periods (at least 4 days) could recover higher microbial counts than classical compendial methods. Low-nutrient media typically require longer incubation conditions (at least 5 days) because the lower nutrient concentrations promote slower growth. Even high-nutrient media can sometimes yield higher microbial recovery with longer and cooler incubation conditions.

**8.5.2.3 Selection of method conditions:** The decision to test a particular system using high- or low-nutrient media, higher or lower incubation temperatures, and longer or shorter incubation times should be based on comparative cultivation studies using the native microbiome of the water system. The decision to use media requiring longer incubation periods to recover higher counts also should be balanced with the timeliness of results. Detection of marginally higher counts at the expense of a significantly longer incubation period may not be the best approach for monitoring water systems, particularly when the slow growers are not new species but the same as those recovered within shorter incubation times. Some cultural conditions using low-nutrient media lead to the development of microbial colonies that are much less differentiated in colonial appearance, an attribute that microbiologists rely on when selecting representative microbial types for further characterization. The nature of some of the slow growers and the extended incubation times needed for their development into visible colonies also may lead to those colonies becoming dysgonic and difficult to subculture. That could limit their further characterization, depending on the microbial identification technology used. The selection of method parameters should provide conditions that adequately recover microorganisms from the water system, including those that are objectionable for the intended water use.

## 8.5.3 SUGGESTED CLASSICAL CULTURAL METHODS

Example methods are presented in *Table 3*.

**Table 3. Example Culture Methods**

| Type of Water | Method |
|---|---|
| Drinking water | Pour plate method or membrane filtration method[a] |
| | Suggested sample volume: 1.0 mL[b] |
| | Growth medium: Plate Count Agar[c] |
| | Incubation time: 48–72 h[d] |
| | Incubation temperature: 30°–35°[e] |
| Purified Water | Pour plate method or membrane filtration method▲[a]▲ (USP 1-Dec-2021) |
| | Suggested sample volume: 1.0 mL for pour plate or up to 100 mL for membrane filtration[b] |
| | Growth medium: Plate Count Agar[c] |
| | Incubation time: 48–72 h[d] |
| | Incubation temperature: 30°–35°[e] |
| Water for Injection | Membrane filtration method[a] |
| | Suggested sample volume: 200 mL[b] |
| | Growth medium: Plate Count Agar[c] |
| | Incubation time: 48–72 h[d] |
| | Incubation temperature: 30°–35°[e] |

[a] A membrane filter with a rating of 0.45 µm is generally considered preferable to smaller porosity membranes.
[b] Sample size must be appropriate for the expected microbial count of the water in order to derive statistically valid colony counts.

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[c] For optimum recovery, an alternative medium may be more appropriate (e.g., m-HPC, TSA/SCDA, R2A).
[d] For optimum recovery, alternative incubation times may be needed.
[e] For optimum recovery, alternative incubation temperatures may be needed.

For media growth promotion, use at a minimum *Pseudomonas aeruginosa* ATCC 9027 and *Bacillus subtilis* ATCC 6633. Additional organisms should be used to represent those that are considered objectionable and/or typically isolated from the water system (house isolates).

#### 8.5.4 MICROBIAL IDENTIFICATION

In addition to the enumeration of the bioburden in the water, there is a need to identify and/or select certain microbial species that could be detrimental to products or processes. Some bacteria also may be resistant to preservatives and other antimicrobial chemicals used in nonsterile liquid and semi-solid products, thus leading to potential product spoilage. For example, *Pseudomonas aeruginosa* and *Burkholderia cepacia*, as well as some other pseudomonads, are known opportunistic pathogens under certain conditions. As such, it may be appropriate to consider these species as objectionable microorganisms for the type of water used to manufacture nonsterile liquid and semi-solid products. There is a higher risk of infection if these organisms are found in products targeted for susceptible patient populations (e.g., the very young, the very old, and the immunocompromised) or products contacting highly susceptible tissues (e.g., inhaled products or some topical products). However, if the product where the water is used carries an absence specification for a particular pathogenic species that is not capable of living in a high-purity water system (e.g., *Staphylococcus aureus* or *E. coli*), then these non-aquatic species should not be candidates for routine recovery testing from water samples. For more information, see *Microbiological Examination of Nonsterile Products: Acceptance Criteria for Pharmaceutical Preparations and Substances for Pharmaceutical Use* ⟨ 1111⟩ , *Microbial Characterization, Identification, and Strain Typing* ⟨ 1113⟩ , and *Microbiological Best Laboratory Practices* ⟨ 1117⟩ .

For PC and QC, it is valuable to know the microbial species present in the normal microbiome of a water system, even if they are not specifically objectionable. If a new species is detected, it may be an indication of a subtle process change or an exogenous intrusion. The identity of the microorganism may be a clue as to its origin and can help with implementation of corrective or preventive action. Therefore, it is industry practice to identify the microorganisms in samples that yield results exceeding established Alert and Action Levels. It is also of value to periodically identify the normal microbiome in a water system, even if counts are below established Alert Levels. This information can provide perspective on the species recoveries from Alert and Action Level excursion samples, indicating whether they are new species or just higher levels of the normal microbiome. Water system isolates may be incorporated into a company culture collection for use in tests such as antimicrobial effectiveness tests, microbial method validation/suitability testing, and media growth promotion. The decision to use water isolates in these studies should be risk-based because many such isolates may not grow well on the high-nutrient media required. And because once adapted to laboratory media, they may not perform like their wild type progenitors.

#### 8.5.5 RAPID MICROBIOLOGICAL METHODS

In recent years, new technologies that enhance microbial detection and the timeliness of test results have been adopted by pharmaceutical QC testing labs. Rapid microbiological methods (RMM) are divided into four categories: growth-based, viability-based, metabolite-based, and nucleic acid-based. Examples of RMM used for the evaluation of microbial quality of water systems include the following:

- Microscopic visual epifluorescence membrane counting techniques
- Automated laser scanning membrane counting approaches
- Early colony detection methods based on autofluorescence, adenosine triphosphate (ATP) bioluminescence, or vital staining
- Genetic-based detection/quantitation

See *Validation of Alternative Microbiological Methods* ⟨ 1223⟩ for further information on rapid microbiological methods.

## 9. ALERT AND ACTION LEVELS AND SPECIFICATIONS

### 9.1 Introduction

Establishment of Alert and Action Levels for any manufacturing process facilitates appropriate and timely control. In the case of a pharmaceutical water system, the key PC parameters can be specific chemical, physical, and microbiological attributes of the water produced. Typically, most chemical attributes can be determined in real time or in the lab within a few minutes after sample collection. Physical attributes such as the pressure drop across a filter, temperature, and flow rate—which are sometimes considered critical for operation or sanitization of the water system—must be measured in situ during operation. Obtaining timely microbial data is more challenging compared to chemical and physical attributes, often taking several days. This limits the ability to control microbial attributes in a timely manner, and therefore requires a more challenging evaluation of the test results and conservative implementation of PC levels. This section provides guidance on the establishment and use of Alert and Action Levels, as well as Specifications to assess the suitability of the water and the water system for use in production.

### 9.2 Examples of Critical Parameter Measurements

Examples of measurements and parameters that are important to water system processes and products are described below. The list, which is not intended to be exhaustive or required, contains some examples of parameters that could be measured to demonstrate that the system is in a state of control.

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Examples of measurements that could be critical to the purification or sanitization process include the following:

- Temperature, for thermally sanitized systems
- Percent rejection of a reverse osmosis system
- Endotoxin levels of feed water to a distillation system
- Chlorine presence immediately prior to a reverse osmosis system

Examples of measurements that could be critical to the water distribution process include the following:

- Return/end-of-loop line pressure, to forewarn of the potential to aspirate air or fluids because of simultaneous use of too many outlets
- Temperature to assure the self-sanitizing conditions are maintained for a hot water system
- Flow rate, to ensure that sufficient water is available for operations

Examples of measurements that could be critical to final water quality include the following:

- Conductivity
- TOC
- Endotoxin—for Water for Injection systems
- Bioburden
- Ozone or other chemicals—for chemically sanitized systems

## 9.3 Purpose of the Measurements

Although the purpose of each measurement varies, the results can be used to provide system performance feedback, often immediately, serving as ongoing PC and product quality indicators. At the same time, the results provide information necessary for making decisions regarding the immediate processing and usability of the water (see *6.1 Purposes and Procedures*). However, some attributes may not be monitored continuously or may have a long delay in data availability (e.g., microbial data). Regardless, both real-time data and data with longer cycle times can be used to properly establish Alert and Action Levels, which can serve as an early warning or indication of a potentially approaching quality shift.

As PC indicators, Alert and Action Levels are trigger points for the potential need for investigation and/or remedial action, to prevent a system from deviating from normal conditions and producing water unsuitable for its intended use. This “intended use” minimum quality is sometimes referred to as a “Specification” or “Limit”, and may include limits for conductivity and TOC listed in water monographs, or other specifications required for these waters that have been defined by the user internally.

In all cases, the validity of the data should be verified to ensure that the data are accurate and consistently representative of the water quality in the system, regardless of whether the sample was collected from a sampling port or use point. The resulting data must not be unduly biased, positively or negatively, because of the sampling method, the environment in the vicinity of the sampling location, the test procedure, instrumentation, or other artifacts that could obscure or misrepresent the true quality of the water intended by the purpose of the sampling (i.e., for PC or for QC).

## 9.4 Defining Alert and Action Levels and Specifications

Data generated from routine water system monitoring should be trended to ensure that the system operates in a state of chemical and microbiological control. To assist with the evaluation of system performance, companies should establish in-process control levels based on historical data or a fraction of the water Specifications (as long as this latter approach yields values with relevance to process performance).

When establishing Alert and Action Levels and Specifications, a two- or three-tier approach is typically used. In a three-tier approach, the typical structure is to establish in-process controls using “Alert Level”, “Action Level”, and “Specifications”. Alert and Action Levels are used as proactive approaches to system management prior to exceeding Specifications. The criteria for defining and reacting to adverse trends should be set by the user. These levels should be set at values that allow companies to take action to prevent the system from producing water that is unfit for use. Water Specifications or Limits represent the suitability for use of the water.

In a two-tier approach, a combination of the above terminology is used, depending on the parameter to be monitored. For example, if the attribute does have a monograph specification, the two tiers are Alert Level (or Action Level) and Specification. If the attribute does not have a limit or specification, the two tiers are usually Alert Level and Action Level.

A single-tier approach is possible, but this is risky and difficult to manage. With this approach, where the water/system is either acceptable or not acceptable, the single-tier method does not allow for any adjustment, correction, or investigation prior to stopping production.

However, certain sampling locations, such as sampling ports that are not used for manufacturing products or processes, do not represent the finished water quality where a Specification could be applied. In these locations, a two-tier approach (Alert and Action Levels only) could be applied. In some sampling locations, a single PC level might possibly be appropriate, depending on the attribute.

### 9.4.1 ALERT LEVEL

An Alert Level for a measurement or parameter should be derived from the normal operating range of the water system. Specifically, Alert Levels are based on the historical operating performance under production conditions, and then are established at levels that are just beyond the majority of the normal historical data. The Alert Level for a parameter is often a single value or a range of values, such as the following:

- Higher than typical conductivity or TOC
- Higher than typical microbial count

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- Higher than typical endotoxin level
- Low temperature during thermal sanitization
- pH range control prior to a reverse osmosis
- Ozone concentration in a storage tank

Various methods, tools, and statistical approaches are available for establishing Alert Levels, and the user needs to determine the approaches that work for their application. Some numerical examples are two or three standard deviations (or more) in excess of the mean value, or some percentage above the mean value but below a Specification. An event-based example could be the appearance of a new microorganism or a non-zero microbial count where zero is the norm.

When an Alert Level is exceeded, it indicates that a process or product may have drifted from its normal operating condition or range. Alert Level excursions represent a warning and do not necessarily require a corrective action. However, Alert Level excursions may warrant notification of personnel involved in water system operation, as well as the quality assurance (QA) personnel. Alert Level excursions may also lead to additional monitoring, with more intense scrutiny of the resulting and neighboring data as well as other process indicators.

### 9.4.2 ACTION LEVEL

An Action Level is also based on the same historical data, but the levels are established at values (or ranges) that exceed the Alert Levels. The values/ranges are determined using the same types of numerical or event-based tools as the Alert Levels, but at different values/ranges.

In a three-tier approach, it is good practice to select an Action Level that is more than the Alert Level, but less than the Specification to allow the user to make corrective actions before the water would go out of compliance.

Exceeding a quantitative Action Level indicates that the process has allowed the product quality or other critical parameter to drift outside of its normal operating range. An Action Level also can be event-based. In addition to exceeding quantitative Action Levels, some examples of event-based Action Level excursions include, but are not limited to, the following:

- Exceeding an Alert Level repeatedly
- Exceeding an Alert Level in multiple locations simultaneously
- The recovery of specific objectionable microorganisms
- A repeating non-zero microbial count where zero is the norm

An Action Level being exceeded should prompt immediate notification of both QA staff and the personnel involved in water system operations and use so that corrective actions can be taken to restore the system back to its normal operating range. Such remedial actions should also include investigative efforts to understand what happened and eliminate or reduce the probability of recurrence. Depending on the nature of the Action Level excursion, it may be necessary to evaluate its impact on the water uses during the period between the previous acceptable test result and the next acceptable test result.

### 9.4.3 SPECIAL ALERT AND ACTION LEVEL SITUATIONS

In new or significantly altered water systems, where there is limited or no historical data from which to derive trends, it is common to establish initial Alert and Action Levels based on equipment design capabilities. These initial levels should be within the process and product Specifications where water is used. It is also common for new water systems, especially ambient water systems, to undergo changes, both chemically and microbiologically, over time as various unit operations (e.g., reverse osmosis membranes) exhibit the effects of aging. This type of system aging effect is most common during the first year of use. As the system ages, a steady state microbiome (microorganism types and levels) may develop due to the collective effects of system design, source water, maintenance, and operation, including the frequency of re-bedding, backwashing, regeneration, and sanitization. This established or mature microbiome may be higher than the one detected when the water system was new. Therefore, there is cause for the impurity levels to increase over this maturation period and eventually stabilize.

Some water systems are so well controlled microbially—such as continuously or intermittently hot Water for Injection distribution systems—that microbial counts and endotoxin levels are essentially nil or below the limit of reasonable detectability. This common scenario often coincides with a very low Specification that is poorly quantifiable due to imprecision (as much as two-fold variability) of the test methods that may be near their limits of detection. In such systems, quantitative data trending has little value, and therefore, quantitative PC levels also have little value. The non-zero values in such systems could be due to sporadic sampling issues and not indicative of a water system PC deviation; however, if these non-zero values occur repeatedly, they could be indicative of process problems. So, an alternative approach for establishing Alert and Action Levels with these data could be the use of the incident rate of non-zero values, with the occasional single non-zero “hit” perhaps being an Alert Level (regardless of its quantitative value), and multiple or sequential “hits” being an Action Level. Depending on the attribute, perhaps single hits may not even warrant being considered an Alert Level, so only a multiple-hit situation would be considered actionable. It is up to the user to decide on their approach for system control (i.e., whether to use one, two, or three levels of controls for a given water system and sampling location, and whether to establish Alert and Action Levels as quantitative or qualitative hit-frequency values).

### 9.4.4 SPECIFICATIONS

Water Specifications or Limits are set based on direct potential product and/or process impact and they represent the suitability for use of the water. The various bulk water monographs contain tests for *Conductivity*, *TOC*, and *Bacterial Endotoxins* (for *Water for Injection*). Aside from the monographs for *Water for Hemodialysis* and multiple sterile waters, microbial specifications for the bulk waters are intentionally not included in their monograph tests.

The need for microbial specifications for bulk waters (*Purified Water* and *Water for Injection*) depends on the water use(s), some of which may require strict control (e.g., very low bioburden, absence of objectionable organisms, or low ionic strength) while others may require no specification due to the lack of impact. For example, microbial specifications are appropriate and

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typically expected for water that is used in product formulations and final equipment rinses. Where the water is used for analytical reagent preparations and the analytical method is not affected by microbial contaminants, or for cleaning processes that conclude with a final antimicrobial heat drying or solvent rinsing step, the microbial quality of the water is likely less of a concern. The decision to establish microbial Specifications for bulk pharmaceutical waters should be based on a formal risk assessment of its uses and justified by scientific rationale.

It is very important to understand the chemical and microbial quality of the water in its final form as it is delivered from a water system to the locations where it is used in manufacturing activities and other points of use. The quality of the water within the water system could be compromised if it picks up chemical or microbial contaminants during its delivery from the system to the points of use. These points of use, where cumulative contamination could be present, are the locations where compliance with all the water Specifications is mandated.

As discussed above, compliance with chemical Specifications can be confirmed periodically between uses, immediately prior to use, or even while the water is being utilized in product manufacturing. Although the use of RMM may provide for timely microbial data, the use of conventional cultivative microbiological testing usually delays confirmation of microbial compliance until after the water has been used. However, for some applications, this logistical limitation should not eliminate the need for establishing microbial Specifications for this very important raw material.

The manufacturing risk imposed by these logistics accentuates the value of validated microbial control for a water system. It also emphasizes the value of unbiased sampling for microbial monitoring (e.g., influences from technique, hoses, flushing) of samples collected from pertinent locations, with evaluation of the resulting data against well-chosen and preferably trend-derived Alert and Action Levels, which can facilitate remedial PC to preclude Specification excursions.

Users should establish their own quantitative microbial Specifications suited to their water uses. But these values should not be greater than 100 cfu/mL for *Purified Water* or 10 cfu/100 mL for *Water for Injection* unless specifically justified, because these values generally represent the highest microbial levels for pharmaceutical water that are still suitable for manufacturing use.

A Specification excursion should prompt an out-of-specification (OOS) investigation. The investigation is performed to determine 1) the root cause of the excursion so that CAPA may be taken for remediation purposes, and 2) assess the impact on affected processes and finished products where the water was used. Product disposition decisions must be made and are dependent on factors that could include the following:

- Role of water in the product or in-process material
- Chemical or microbial nature of the attribute whose Specification value was exceeded
- Level of product contamination by the water
- Presence of objectionable microorganisms
- Any downstream processing of affected in-process materials that could mitigate the OOS attribute
- Physical and chemical properties of the finished product where the water was used that could mitigate the OOS attribute
- Product administration routes and potentially sensitive/susceptible users

### 9.4.5 SOURCE WATER CONTROL

The chemical and microbial attributes of the starting source water are important to the ability of the water system to remove or reduce these impurities to meet the finished water Specifications (see *2. Source Water Considerations*). Using the example microbial enumeration methods in *Table 3* a reasonable maximum bacterial Action Level for source water is 500 cfu/mL. This number is derived from US EPA NPDWR where it is used as an Action Level for the water authority indicating the need for improving disinfection and water filtration to avoid the penetration of viral, bacterial, and protozoal pathogens into the finished drinking water. It is not, however, a US EPA heterotrophic plate count Specification or Maximum Contaminant Level (MCL) for Drinking Water.

Nevertheless, of particular importance could be the microbial and chemical quality of this starting water because the water is often delivered to the facility at a great distance from its source and in a condition over which the user has little or no control. High microbial and chemical levels in source water may indicate a municipal potable water system upset, a change in the supply or original water source, a broken water main, or inadequate disinfection, and thus potentially contaminated water with objectionable or new microorganisms or coincidental chemical contaminants may be present.

Considering the potential concern about objectionable microorganisms and chemical contaminants in the source water, contacting the water provider about the problem should be an immediate first step. In-house remedial actions also could be needed, including performance of additional testing on the incoming water (as well as the finished water in some cases) or pretreating the water with additional microbial and chemical purification operations (see *5.1 Unit Operations Considerations*).

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# ⟨1234⟩ VACCINES FOR HUMAN USE—POLYSACCHARIDE AND GLYCOCONJUGATE VACCINES

## INTRODUCTION

This chapter describes best practices for production, conjugation, and characterization of polysaccharide and glycoconjugate vaccines. It describes key quality attributes at each step of the process and suggests best methods to assess these attributes. The scope of this chapter includes vaccines consisting of one or more purified polysaccharides (such as pneumococcal, meningococcal, and Typhoid Vi vaccines) and components involved in their production, and vaccines consisting of one or more glycoconjugate immunogens in which a saccharide has been covalently attached to a suitable carrier protein. The latter category includes *Haemophilus influenzae* type b (Hib), meningococcal, and pneumococcal conjugate vaccines. The chapter does not include combination vaccines in which Hib conjugates are combined with unrelated immunogens against diphtheria, tetanus, and pertussis.

## BACKGROUND

### Occurrence of Capsular Polysaccharides

Many pathogenic bacteria possess a polysaccharide capsule that encloses the cell, modulates the flow of nutrients to the cell surface, and protects against dehydration. When a bacterium establishes an infection in a mammalian host, the polysaccharide capsule hides cell surface components from elements of the mammalian immune system, such as antibodies and complement proteins that otherwise would activate mechanisms to kill the pathogen. Although polysaccharide capsules are themselves immunogenic in children and adults, development of a protective immune response may be too slow to defend the host against disease. In many cases, antibodies directed against the capsular polysaccharide are protective, and the prior existence of these antibodies prevents establishment of the infection; this is the basis for their use as vaccines or components of conjugate vaccines. Many bacterial species can be divided into serogroups or serotypes that express structurally and immunologically distinct capsular polysaccharides. The number of known serotypes differs between organisms. There are six known *Haemophilus influenzae* serotypes and more than 90 *Streptococcus pneumoniae* serotypes. Different serotypes (or serogroups) of the same organism may have different infectivities. For instance, the large majority of disease caused by *H. influenzae* is due to the b serotype. Different serotypes may cause disease in different geographical regions, may produce a different spectrum of disease, or may be prevalent in different age groups. The serotype-specific pattern of disease may change with time within a given geographical region. For these reasons, many polysaccharide and conjugate vaccines contain multiple saccharide immunogens.

### Structures of Capsular Polysaccharides

Capsular polysaccharides are high molecular weight polymers that contain a strict repeat unit. This repeat unit can be a single monosaccharide unit or can be an oligosaccharide unit that contains as many as eight sugar residues. The repeat units can be linear or branched, and are sometimes linked together by phosphodiester bonds. Bacterial polysaccharides often contain unusual sugar residues that are not found elsewhere, and may be substituted with a wide range of acylating groups (*O*-acetyl groups are the most common) and phosphorylated substituents such as phosphoglycerol. Incomplete *O*-acetylation and migration of *O*-acetyl groups between different hydroxyl groups leads to a degree of heterogeneity in the polysaccharides.

### Immune Responses to Capsular Polysaccharides

Capsular polysaccharides are T-cell–independent type 2 immunogens. These immunogens do not evoke antibody isotype switching, affinity maturation, or immunological memory. Because of the relatively late development of the relevant arm of the human immune system, unconjugated polysaccharide vaccines usually induce only a poor immune response in infants under the age of two and thus are not used for this population group. Adjuvants in polysaccharide vaccines do not improve the immune response. In a typical adult target population only a single dose is required to induce a protective immune response. In the absence of immunological memory, regular revaccination is required, often at five-year intervals. Repeated vaccination can lead to hyporesponsiveness to the vaccine.

### Conjugation of Polysaccharides to Carrier Protein

Covalent attachment of a capsular polysaccharide, or an oligosaccharide derived from it, to a protein carrier creates a conjugate vaccine. Immunization with a conjugate vaccine induces humoral immunity by means of a different molecular mechanism that does not require cross-linking of immune cell surface proteins. For this reason, effective conjugate vaccines can be produced using oligosaccharide haptens that may be derived from a capsular polysaccharide or from inherently lower molecular weight polysaccharides such as the *O*-chain of a lipopolysaccharide. Conjugates can also be produced using naturally high-mass polysaccharides or by controlled size reduction to shorter chains. Depending on the manufacturing process there are three basic structural models for conjugate vaccines:

- conjugates in which a carrier protein is modified with multiple oligosaccharide chains that have one or two activation sites to allow attachment to a carrier protein, resulting in a monomeric glycoconjugate or a glycoconjugate with limited cross-linking

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- cross-linked conjugates in which multiple activated polysaccharide chains and carrier proteins couple to multiple polysaccharide chains, creating a cross-linked network of proteins and glycans
- conjugates in which a size-reduced polysaccharide is covalently attached to a complex of proteins, typically bacterial outer proteins, via multiple attachments.

### The Carrier Protein

The most widely used carrier proteins are related to bacterial toxins that are detoxified by chemical or genetic means. Conjugate vaccines induce a T-cell–dependent response that is developed early in life and leads to immunological memory and boosting of the response by further doses of the vaccine, thus they are suitable for infant immunization. The role of the carrier protein in modulating the immune response is discussed below.

### Conjugation Chemistry

Polysaccharides can be covalently attached to proteins, although activation of the polysaccharide is required. Sometimes the carrier protein is also activated to create compatible reactive groups. Polysaccharides can be activated initially by creation of reactive aldehyde groups, by periodate oxidation or uncovering of the reducing terminal of sugars, by reaction of hydroxyl groups with highly reactive reagents such as cyanogen bromide (or 1-cyano-4-dimethylamino-pyridinium tetrafluoroborate [CDAP] as a crystalline alternative) or carbonyldiimidazole, by use of carboxylic acid groups, or, if available, by free amino groups or phosphate groups. Periodate oxidation, dilute acid hydrolysis, and some other approaches to polysaccharide activation may, depending on the structure of the polysaccharide, lead to depolymerization of oligosaccharides, which are typically fractionated by size and an appropriate fraction coupled to the carrier. The "natural" reactive groups on proteins include lysine ε-amino groups, *N*-terminal amino groups, carboxylic acids in aspartate or glutamate, or thiol groups. Carrier protein activation may also involve the creation of hydrazide groups or thiols for linkage. Covalent attachment between the activated polysaccharide and the carrier protein, activated or not, can take place via the use of activated esters, sometimes created *in situ* with a water-soluble carbodiimide reagent, by reduction of Schiff's bases, by reaction of a thiol with maleimide, or by elimination of bromide. Conjugation can either be direct, as in reductive amination, or via the introduction of a suitable bifunctional linker. In general, the choice of conjugation chemistry is defined by either the structure of the polysaccharide repeat unit or the desire to produce a conjugate of a specific structural family.

### Immune Responses to Conjugate Vaccines

The immune response to the saccharide component of conjugate vaccines is T-cell–dependent and is similar to the response for proteins, although this process is still not fully understood with respect to glycoconjugates. Because this immunological pathway is in place even in infants, conjugate vaccines were developed initially for use in infants. Following interaction with antigen-presenting cells such as dendritic cells, macrophages, and B-cells, glycoconjugate vaccines are internalized and processed into small peptides and glycopeptides that then are re-exposed and presented to T-lymphocytes in association with the major histocompatibility complex class II molecules. Multiple immunizations of an immunologically naive infant are normally required to raise an antibody response, but processes such as isotype switching and affinity maturation take place and immunological memory is stimulated. In general terms, these processes result in induction of long-lasting high-affinity antibodies that are effective at preventing bacterial pathogens from establishing an infection. Adjuvants in glycoconjugate vaccines are effective at boosting immune responses. These vaccines have also been shown, in the cases of Hib, meningococci, and pneumococci, to eliminate nasopharyngeal carriage of organisms, thus an important aspect of their effectiveness arises from suppressing transmission of infectious serotypes between individuals, called a *herd effect*.

## KEY QUALITY PARAMETERS FOR BULK POLYSACCHARIDES

Bulk monovalent polysaccharide is purified from bacterial cell culture and is a key stable intermediate in the manufacture of both polysaccharide and conjugate vaccines. Many quality parameters for final vaccines can be initially assessed by analysis of bulk monovalent polysaccharides or by using critical in-process tests supporting the manufacturing process. Important quality parameters for purified polysaccharide include identity, purity, composition, and molecular size, and depend on the type and extent of further processing. The purification process is validated to consistently produce compliant material.

The purity of the polysaccharides depends on the purification steps, including harvest methods, clarification, and downstream purification processes. For purification, a combination of precipitation, filtration, and chromatographic procedures can be used, depending on the chemical nature of the polysaccharide. The final purification step can consist of buffer exchange and filtration followed by storage of purified polysaccharide (frozen), or additional precipitation and washing of the precipitate with solvent before drying, followed by storage. Drying of polysaccharides can be performed in dessicators and can include several steps of grinding or fluffing and return to the dessicators for further drying. Manufacturers should take care during these steps because mechanical handling of the polysaccharide can reduce its molecular size. The purified polysaccharide is stored at a suitable temperature in conditions that avoid the uptake of moisture. Lyophilization of polysaccharides is also possible. The stability of the polysaccharide under specified storage conditions should be demonstrated; this may include assessment of the optimal moisture content of the dried material.

Differentiating which tests should be used for polysaccharide release and which are better suited to in-process testing to ensure process consistency depends on the process and how the polysaccharide will be further processed. Final decisions about process parameters typically take place after discussions with regulatory authorities.

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## Dry Weight

Because dry weight is used both to calculate the results of certain tests with reference to the dried substance and to calculate amounts for subsequent processes, volatile matter, including water, in the purified polysaccharide is determined by a combination of suitable methods including the following:

- thermogravimetry (see *Loss on Drying* ⟨731⟩)
- Karl Fischer (water only: see *Water Determination* ⟨921⟩)
- residual solvents determined by gas chromatography (see *Residual Solvents* ⟨467⟩ and *Chromatography* ⟨621⟩) and by nuclear magnetic resonance (NMR) spectroscopy (see *Nuclear Magnetic Resonance Spectroscopy* ⟨761⟩) or colorimetric methods.

## Polysaccharide Identity

The manufacturer is required to confirm the identity of the active component present in the purified polysaccharide. If other polysaccharides are produced at the same manufacturing site, the method should be validated to show that it distinguishes the desired polysaccharide from all other polysaccharides produced in that facility (see 21 CFR 610.14). The identity is determined by a prespecified combination of suitable methods such as:

- Immunological test methods: These methods require access to highly specific antisera that are able to distinguish between closely related polysaccharide antigens. Commonly used formats for this purpose are immunoprecipitation, immunoelectrophoresis, and enzyme-linked immunosorbent assays (ELISA). The (serological) specificity of the antiserum should be demonstrated by the absence of cross-reactivity with heterologous polysaccharides manufactured in the same facility. More information regarding these methods can be found in *Immunological Test Methods—General Considerations* ⟨1102⟩, *Immunological Test Methods—Enzyme–Linked Immunosorbent Assay (ELISA)* ⟨1103⟩, and *Immunological Test Methods—Immunoblot Analysis* ⟨1104⟩.
- NMR spectroscopy (see ⟨761⟩): NMR methods require access either to authentic samples of the polysaccharide or to reference spectra. Comparison is made visually in terms of the positions, relative intensities, and multiplicities of significant resonances, or by use of other objective methods.
- Polysaccharide identity can also be assessed by use of a matrix of compositional assays, usually with colorimetric or chromatographic readouts, that define factors such as the amounts of different sugar types (e.g., amino sugars, uronic acids, or methyl pentoses depending on the polysaccharide), the amount of substituents (e.g., *O*-acetyl groups), and the content of nitrogen and phosphorus.

## Polysaccharide Purity and Quantity

### COLORIMETRIC ASSAYS

Historically, polysaccharide content compared to dry weight (purity) was estimated using a range of colorimetric assays for functional groups specific to the polysaccharide antigen. Because the reference standard is typically a pure monosaccharide or similar model compound, and because assays were typically determined before most structures were known, component quantification may not parallel true stoichiometry. Thus, manufacturers should develop correlations among alternative approaches. Purity can be calculated based on the method employed and the salt form present. *Table 1* lists colorimetric tests that may be appropriate to determine the composition of a particular polysaccharide within a vaccine. The response factors for the sugar units in the polysaccharide may differ from those of a pure monosaccharide reference standard. Manufacturers should address these concerns during method validation.

**Table 1. Colorimetric Assays for Polysaccharide Composition and Quantity**

| | Orcinol Assay (Bial Reagent) | Molybdate (Chen) Assay | Resorcinol Assay (Seliwanoff Reagent) | Sulfuric Acid Digestion | Carbazole Assay | Dimethylaminobenzaldehyde Assay | Cysteine Sulfuric Acid Assay | Hestrin Assay[a] | Anthrone-Sulfuric Acid Assay |
|---|---|---|---|---|---|---|---|---|---|
| PS Antigen | Ribose | Phosphate | Sialic Acid | Total Nitrogen | Uronic Acids | Hexosamines | Methylpentoses | *O*-Acetyl | Total Sugar |
| Hib PRP | X | X | | | | | | | |
| MenA | | X | | | | | | X | |
| MenC | | | X | | | | | X | |
| MenY | | | X | | | | | X | X |
| MenW135 | | | X | | | | | X | X |
| Pneumo (Serotype Specific) | | X | X | X | X | X | X | X | X |
| Vi | | | | | | | | X | |

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[a] The hestrin assay is appropriate for compositional analysis and identity, but because the degree of *O*-acetylation can vary between polysaccharide batches it is not normally a suitable assay for polysaccharide quantitation.

### HYDROLYSIS AND COMPOSITION ANALYSIS BY CHROMATOGRAPHY

Acid or base hydrolysis depolymerizes polysaccharides into oligosaccharides, monosaccharides, or smaller fragments that are polysaccharide-specific for the optimized hydrolysis conditions employed. Aggressive hydrolysis conditions can destroy some components of the polysaccharide. These fragments can be quantified directly by use of, for example, high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) or conductivity detection for ions (HPAEC-CD), or by reversed phase high-performance liquid chromatography (HPLC) of fluorophore-labeled sugars. Alternatively, the hydrolysate can be derivatized and analyzed by gas chromatography with flame ionization (GC-FID) or mass spectrometric detection (GC-MS). Suitable polysaccharide reference materials or monosaccharides are required, and the hydrolysis conditions are product-specific. *Table 2* summarizes the type of hydrolysis applied, the analytical methods, and the analytes that have been quantified for different polysaccharides.

**Table 2. Chromatographic Methods for Compositional Analysis and Quantification of Vaccine Polysaccharides[a]**

| PS Antigen | Acid Hydrolysis and HPAEC | Acid Hydrolysis, Fluorophore Labeling, and HPLC | HF Hydrolysis and HPAEC | Base Hydrolysis and HPAEC | Meth - anolysis and GC or HPAEC |
|---|---|---|---|---|---|
| PRP | Ribitol | — | Phosphate | PRP monomer | — |
| MenA | ManN-6-P | — | Phosphate | *O*-acetyl | — |
| MenC | Neu5Ac | — | — | *O*-acetyl | — |
| MenY | Glc, Neu5Ac | Neu5Ac | — | *O*-acetyl | — |
| MenW135 | Gal, Neu5Ac | Neu5Ac | — | *O*-acetyl | — |
| Pneumo (Serotype Specific) | Alditols, methylpentoses, hexoses, hexosamines, uronic acids, Pyruvate | — | Phosphate | *O*-acetyl | Alditols, methylpentoses, hexoses, hexosamines, uronic acids |
| Vi | — | — | — | *O*-acetyl Vi monomer fragment | — |

[a] PS = polysaccharide; HF = hydrofluoric acid; PRP = polyribosylribitol phosphate; MenA = Meningococcus group A; MenC = Meningococcus group C; MenY = Meningococcus group Y; MenW135 = Meningococcus group W135; Pneumo = Pneumococcus; Man = mannose; Neu = neuraminic acid; Ac = acetyl; Glc = glucosamine; Gal = galactose.

### CAPILLARY ELECTROPHORESIS

Capillary zone electrophoresis has been used for the identification and quantification of meningococcal polysaccharides without depolymerization.

### IMMUNOCHEMICAL ASSAYS

Immunochemical assays such as immunonephelometry or ELISA require access to specific antisera that must be calibrated to reference materials. The response may be modified by the matrix or size of the polysaccharide.

### NMR SPECTROSCOPY

The relative intensities of characteristic resonances can confirm the proportions of different sugar residue types and substituents such as *N*- or *O*-acetyl or pyruvate that are present in the polysaccharide. Quantification can be achieved by comparison of these intensities to that of an added internal standard (see ⟨761⟩).

Regarding phosphorus determination, one should remember that a number of bacterial polysaccharides contain phosphodiester linkages. The polysaccharide may be quantified based on its phosphorus content by colorimetric assays or instrumental approaches such as inductively coupled plasma–optical emission (ICP-OES) or ICP-mass spectrometry (ICP-MS).

### COUNTERIONS

If the percentage purity of polysaccharide bulks (compared to dry weight) is part of their release program, the amount and type of counterion present must be considered and can be determined by, for example, ICP-MS. This is normally an in-process control step.

## Polysaccharide Molecular Size Distribution

The molecular size distribution is generally evaluated by liquid chromatography using soft gel–filtration procedures or size exclusion–high-performance liquid chromatography (SEC-HPLC) equipped with in-line refractive index (RI). The results are reported as the distribution coefficient ($K_D$) determined from the main peak of the elution curve or as the percentage of material that elutes before a defined $K_D$ cut-off value. The absolute molecular weight of the polysaccharide and its hydrodynamic (gyration) radius can be determined by coupling static light scattering and RI detectors to the SEC-HPLC column, and measuring

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the RI increment (*dn*/*dc*) using reference polysaccharides. Requirements based on molecular weight can be expressed in terms similar to those based on molecular size, and related either to peak values or to the proportion that elutes before a defined cut-off value.

### Level of Protein Contamination

The residual protein content of the polysaccharide should be determined by an appropriate assay and should be shown to be below the approved specification. These specifications typically vary by polysaccharide and serotype. Method validation should assess the need for sample pretreatment before protein determination and specific interference by the polysaccharide in the protein assay. Further, method validation should demonstrate that the assay sensitivity is appropriate for the specification. *Biotechnology-Derived Articles—Total Protein Assay* ⟨1057⟩ contains information regarding these assays. Methods that are typically applied to polysaccharides include colorimetric assays (e.g., Lowry, Biuret, bicinchonic acid, or Bradford assays) and UV absorbance.

### Level of Nucleic Acid Contamination

The nucleic acid content of the purified polysaccharide should be determined and should be shown to meet specifications, which are typically less than 1% w/w. *Nucleic Acid–Based Techniques—Approaches for Detecting Trace Nucleic Acids (Residual DNA Testing)* ⟨1130⟩ provides additional information about these methods.

### Quantification of Process- and Product-Related Impurities

Depending on in-process testing or release requirements, chromatographic and spectroscopic methods can be used to quantify residuals from the fermentation and isolation/purification steps. These residuals may include antifoaming agents, phenol, cetyltrimethyl ammonium bromide, ethanol, and other residual solvents. Other impurities should also be quantified using appropriate tests, including:

- bacterial endotoxins (see *Bacterial Endotoxins Test* ⟨85⟩)
- pyrogens (see *Pyrogen Test* ⟨151⟩)
- sterility (see *Sterility Tests* ⟨71⟩), if required
- bioburden (see *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* ⟨61⟩), where appropriate.

As an in-process control, the proportion of pneumococcal C polysaccharide in pneumococcal capsular polysaccharides can be determined by a combination of $^{1}H$ and $^{31}P$ NMR spectroscopy or by HPAEC-PAD analysis of ribitol. Alternatively, it can be derived from the compositional analysis.

### Stability-Indicating Tests

The polysaccharide may lose integrity because of gradual hydrolysis, resulting in a reduced molecular size, and this degradation can be monitored by size exclusion chromatography or by high-performance size exclusion chromatography (HPSEC) with static light scattering detectors for molecular weight. Loss of *O*-acetyl groups can be monitored by HPLC methods. Both the loss or migration of *O*-acetyl groups and the integrity of phosphate-containing polysaccharides can be tracked by NMR spectroscopy. Immunochemical methods can also be used to monitor polysaccharide integrity but should be validated for that purpose.

## KEY QUALITY PARAMETERS FOR BULK FORMULATED POLYSACCHARIDE VACCINE

The bulk formulated polysaccharide vaccine refers to a solution that contains a blend of appropriate amounts of the required monovalent bulk polysaccharides, as well as any buffer salts, excipients, adjuvants, and antimicrobial preservatives present in the vaccine product. Typically, the vaccine is sterilized, usually by filtration, and is ready for filling in the final dosage forms.

### Antimicrobial Preservative

If an antimicrobial preservative is present and is not assayed at a later stage, its concentration can be assayed at this stage using an appropriate validated chemical, physicochemical, chromatographic, or spectroscopic assay. While the specification will depend on the regulatory agency, typically the amount of preservative should not exceed 120% of the expected value, and the manufacturer must demonstrate that the preservative is effective at that concentration (see *Antimicrobial Effectiveness Testing* ⟨51⟩).

### Sterility

At this stage, if the expectation is that the material is sterile, then sterility should be demonstrated using an appropriate validated assay such as one of those described in ⟨71⟩. For other manufacturing processes, measurement of some combination of bioburden, endotoxin count, or pyrogenicity may be sufficient.

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### Polysaccharide Quantity

To ensure correct dilution of the bulk before final filling, and unless other control mechanisms are in place, determination of the content of individual serotypes or total polysaccharide content may be required using appropriate validated physicochemical or immunochemical methods as defined in the bulk polysaccharide section.

### pH, Osmolarity/Isotonicity, and Excipients

The final format of a vaccine may be liquid or lyophilized. If the format in this stage does not change during the final fill and is not analyzed later then manufacturers should assay this bulk material for:

- pH (see *pH* ⟨791⟩)
- possibly osmolarity or isotonicity (see *Osmolality and Osmolarity* ⟨785⟩). If approved by a regulatory agency, routine osmolarity testing may be omitted if the manufacturer demonstrated consistency in development and clinical lots.
- excipient content, if present.

## KEY QUALITY PARAMETERS FOR POLYSACCHARIDE VACCINES: FINAL FILLS

In accordance with 21 CFR 610.1, "No lot of any licensed product shall be released by the manufacturer prior to the completion of tests for conformity with standards applicable to such product. Each applicable test shall be made on each lot after completion of all processes of manufacture which may affect compliance with the standard to which the test applies. The results of all tests performed shall be considered in determining whether or not the test results meet the test objective, except that a test result may be disregarded when it is established that the test is invalid due to causes unrelated to the product." Test methods should be appropriately verified and validated.

### Description and Solubility

Liquid vaccines are typically clear colorless liquids that are essentially free from visible particles. Lyophilized products are typically white or cream-colored powders or pellets that are freely soluble in water and yield clear colorless liquids that are free from visible particles.

### Polysaccharide Identity

The manufacturer should demonstrate that all the expected polysaccharides are present in the final fill. The test may be immunochemical, physicochemical, or chemical. Regulation 21 CFR 610.14 requires that the identity test should distinguish the product from other products handled in the same facility. In some cases specific quality attributes of the polysaccharide relating to identity, such as *O*-acetyl content, may also be specified, and should be assayed at this final fill stage if not assessed at an earlier stage. Often polysaccharide identity and quantity can be confirmed and determined by the same assay.

### Polysaccharide Quantity

The content of each polysaccharide present in the final lot should be determined by a suitable validated immunochemical or physicochemical method. Typically, Vi and pneumococcal polysaccharide vaccines contain 25 µg of each serotype in a single human dose, whereas meningococcal polysaccharide vaccines contain 50 µg of each serogroup in a single human dose. When immunochemical methods are used, the antisera should be specific for each polysaccharide in the vaccine, including, in the case of the pneumococcal serotypes, immunologically cross-reactive species. Specifications are established on a case-by-case basis, but typically the content of each polysaccharide in the vaccine should be either between 70% and 130% or between 80% and 120% of the label claim.

### Polysaccharide Structural Integrity and Molecular Size

In the absence of suitable validation data showing that no changes occur during filling and storage, the structural integrity and molecular size of the individual polysaccharide components should be assessed as far as is possible following the final fill. Depending on the nature of the polysaccharide and the complexity of the vaccine, the integrity of individual serotypes or serogroups can be established by a combination of immunochemical stability-indicating measurements (e.g., ELISA, rate nephelometry, or physicochemical methods), by size or molecular weight determination alone or in combination with serotype-specific assays, or by quantification of specific groups such as *O*-acetyl groups that have been shown to be critical for immunogenicity. Together with the serotype-specific quantification, these assays act as a surrogate for a potency assay.

### pH

The pH of the final fill should be determined for liquid products or for redissolved lyophilized products according to ⟨791⟩ and should meet the requirements of the relevant licensing authority. This attribute should be included in stability-testing programs.

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### Antimicrobial Preservative

Where applicable, the amount of antimicrobial preservative should be determined by a suitable validated approach. Typically, the value should not exceed 120% of the quantity stated on the label. The approved lower limit should not be lower than the minimum amount shown to be effective throughout the product's shelf life.

### Process Impurities

Unless the product has been tested at an earlier manufacturing stage, and depending on the manufacturing process used, process impurities or residuals such as phenol or formaldehyde should be tested by appropriate validated assays. For more information about allowable process impurities, see *Vaccines for Human Use—Bacterial Vaccines* ⟨1238⟩.

### Sterility

The sterility of each lot should be ensured according to procedures described in ⟨71⟩ and 21 CFR 610.12. The product should comply with the requirements of the tests.

### Pyrogens or Bacterial Endotoxin

Depending on regulatory requirements, the product's endotoxin content or pyrogenicity should be determined and should be shown to meet the relevant specifications. Methods for endotoxin testing are found in ⟨85⟩. Methods for pyrogen testing are found in ⟨151⟩.

### Osmolarity and/or Isotonicity, Excipients, and Moisture Content

If the vaccine is a liquid preparation, the pH and osmolarity/isotonicity of each final lot should be tested and shown to be within the pre-approved specifications. For a lyophilized preparation, analysts should measure the pH after reconstitution with the appropriate diluent.

Excipient functional category (sometimes referred to as *functionality*) is a broad, qualitative, and descriptive term for the purpose or role an excipient serves in a formulation. Of greater importance, however, are the quantitative performance requirements (i.e., critical material attributes) of excipients that must be evaluated and controlled to ensure consistent performance throughout the product life cycle. Manufacturers should anticipate lot-to-lot and supplier-to-supplier variability in excipient properties and should have in place appropriate controls if needed to ensure consistent excipient performance (refer to *Excipient Performance* ⟨1059⟩ for guidance).

Regarding moisture content in lyophilized products, as specified in 21 CFR 610.13 each lot of dried product must be tested for residual moisture and must meet and not exceed established limits as specified by an approved method (see also ⟨731⟩ and ⟨921⟩).

### Diluent for Lyophilized Products

Data should be provided to support diluent sterility (see ⟨71⟩) and to ensure that adventitious microbial contamination does not occur under the reconstitution conditions (i.e., diluents should not introduce contamination) or during storage conditions as described in the package insert. If an antimicrobial preservative is used (as is the case normally only in multidose products), testing according to ⟨51⟩ is recommended to demonstrate acceptability. Testing may not be required on all lots once process control and consistency have been established.

### General Safety

Depending on regulatory requirements, a general safety test may be necessary as set out in 21 CFR 610.11(g), and the product should meet the specifications.

## KEY QUALITY PARAMETERS FOR CARRIER PROTEIN

A number of protein carriers have been used in preclinical and clinical evaluation of conjugate vaccines. Proteins such as diphtheria and tetanus toxoids, which derive from the respective toxins after chemical detoxification with formaldehyde, initially were selected as carriers because of the safety track record accumulated with tetanus and diphtheria vaccination, and these proteins are used today as carriers for meningococcal, Hib, and pneumococcal vaccines in a number of countries worldwide. CRM197, a nontoxic mutant of diphtheria toxin, is also used as a carrier for licensed Hib, pneumococcal, and meningococcal conjugate vaccines and for other vaccines being developed. An outer membrane protein complex (OMPC) of serogroup B meningococcus is the carrier for a licensed Hib conjugate vaccine. An Hib-related protein, Protein D, is the carrier for most of the polysaccharides included in one licensed conjugated pneumococcal vaccine.

### Key Quality Parameters for Carrier Protein

Five carrier proteins currently are used for conjugate vaccines approved for use by various regulatory authorities: Diphtheria Toxoid, Tetanus Toxoid, CRM197, *Haemophilus* Protein D, and OMPC. When the carrier protein is a component of an approved

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vaccine like diphtheria and tetanus toxoids, the first key quality parameters are those defined by the release tests on the concentrated bulks for these components. Other quality parameters include the level of oligomerization (monomer vs. multimeric forms). For carrier proteins that are not licensed as stand-alone vaccines, the list of key quality attributes should at least include identity, sterility or bioburden (depending on the manufacturing process), endotoxins, and purity. In some specific cases, additional quality attributes may require measurement.

#### CARRIER PROTEIN IDENTITY

The identity of the carrier proteins can be assessed by suitable methods that can be divided into two categories:

- Immunochemical methods: immunoprecipitation (flocculation, radial immunodiffusion, and nephelometry), immunoelectrophoretic methods (rocket immunoelectrophoresis), and immunoenzymatic methods (immunoblots and ELISA)
- Standard physicochemical methods used for other purified proteins: mass spectrometry, peptide mapping, and molecular mass determination.

Using these methods, a sample preparation is compared to a reference preparation to demonstrate consistency. The tests listed here may not be appropriate for toxoid proteins.

#### STERILITY OR BIOBURDEN

Sterility and bioburden are determined according to procedures described in ⟨71⟩ and ⟨61⟩, respectively, and 21 CFR 610.12.

#### ENDOTOXINS

To ensure an acceptable level of endotoxin in the final product, manufacturers can determine the endotoxin content of the carrier protein according to ⟨85⟩ and thus can show that endotoxin levels are within acceptable limits. For some products, rabbit pyrogenicity testing ( ⟨151⟩) may be a more relevant test.

## Diphtheria Toxoid

#### ANTIGENIC PURITY

Depending on the manufacturing process diphtheria toxoid preparation can show different degrees of purity. Typically, antigenic purity for diphtheria toxoid as determined by the flocculation test should be at least 1500 Lf (limit of flocculation) units/mg of protein.

#### MONOMER, DIMER, OR AGGREGATE CONTENT

Diphtheria toxin is characterized by the presence of dimeric and multimeric aggregation forms that are also present in the corresponding detoxified preparations. Regarding this parameter, analysts can monitor the manufacturing consistency of diphtheria toxoid by determining the content of monomers vs. dimers and other aggregates using a suitable method such as SEC-HPLC coupled with a static light scattering detector. In some cases, because of the low purity of the preparation the HPLC profile may result in a broad peak that cannot be resolved into the contribution of the individual species.

## Tetanus Toxoid

#### ANTIGENIC PURITY

Typically, the antigenic purity of tetanus toxoid as determined by the flocculation test should be at least 1500 Lf units/mg of protein.

#### MONOMER, DIMER, OR AGGREGATE CONTENT

The detoxification process for tetanus toxin results in oligomerization to an extent that depends on the process conditions. Similar to diphtheria toxoid, the physicochemical consistency of tetanus toxoid can be monitored by the determination of monomeric vs. dimeric forms and other aggregates using suitable methods like SEC-HPLC coupled with static light scattering detection. Other methods like ultracentrifuge analysis can be applied but may be less suitable for routine testing.

## CRM197

CRM197 protein is a nontoxigenic diphtheria toxin isolated from the supernatant of cultures of *Corynebacterium diphtheriae* C7(β197)$^{tox-}$ and purified by a sequence of chromatographic and diafiltration steps. A guideline for production and control of bacterial proteins used in vaccine manufacturing is given in ⟨1238⟩. Recombinant CRM197 should meet requirements for nonrecombinant material, although additional characterization appropriate for recombinant proteins may be required.

#### PURITY

The purity of CRM197 batches should be determined via suitable methods, e.g., HPLC (see ⟨621⟩), sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; see *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* ⟨1056⟩), or

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*Capillary Electrophoresis* ⟨1053⟩. Typical expectations are that the purity of CRM197 should be at least 90% and often greater than 95%.

DEGREE OF NICKING

CRM197 contains an exposed loop of three arginine residues that is clipped by proteases present in the culture medium, resulting in a so-called nicked form. The manufacturing process should demonstrably be able to regularly produce CRM197 with a consistently low degree of nicking. In the presence of a reducing agent like dithiothreitol, the nicked form breaks down into two distinct polypeptides called fragments A and B that can be easily detected by SDS-PAGE, which accordingly is a suitable method to determine the degree of nicking. In a validated process testing may be needed as an in-process control.

### *Haemophilus* Protein D

*Haemophilus* Protein D is obtained as a recombinant protein from *E. coli* fermentation that, after extraction from the cells, is purified by a series of chromatographic and diafiltration steps and finally is sterile filtered. Routine release tests for protein D include identity, purity, sterility, protein content, and endotoxin content. Host cell proteins and host cell DNA should also be tested unless process validation has shown consistent clearance.

PURITY

Purity should be monitored with an appropriate test such as HPLC, SDS-PAGE, or capillary electrophoresis (CE).

### Outer Membrane Protein Complex

The OMPC of *Neisseria meningitidis* group B is derived from meningococcal serogroup B bacterial cells by extraction with buffer-containing detergent. Analysts can monitor manufacturing consistency by determining the OMPC composition with SDS-PAGE or another suitable method.

ENDOTOXIN CONTENT OR PYROGENICITY

To ensure an acceptable level of endotoxin in the final product, analysts can determine the endotoxin content of the carrier protein and can show that it falls within acceptable limits according to ⟨85⟩. Alternatively, OMPC preparations should pass the rabbit pyrogenicity test following injection into rabbits at, typically, 0.25 µg/kg of body mass (see ⟨151⟩).

MENINGOCOCCAL OMPC REQUIREMENTS—PURITY AND LIPOPOLYSACCHARIDE CONTENT

The composition of meningococcal OMPC carrier should be monitored for consistency by SDS-PAGE or by another suitable method. Typically, the lipopolysaccharide (LPS) content should not exceed 8% by weight. Suitable methods for LPS determination include HPLC, colorimetric analyses, SDS-PAGE, and GC-MS.

## KEY QUALITY PARAMETERS FOR ACTIVATED INTERMEDIATES

Different chemical strategies can be applied to the synthesis of glycoconjugate vaccines. Two main approaches have traditionally been employed for glycoconjugate vaccines preparation: one is based on random chemical activation along the chain of the native or slightly size-reduced polysaccharide, followed by conjugation; the other is based on selective activation of end groups of oligosaccharides generated by controlled fragmentation of the native polysaccharide and subsequently coupling to the protein carrier. Depending on the conjugation chemistry, a chemical spacer can be used to facilitate the coupling of the protein to the saccharide antigens, and, in some cases, prior derivatization of the protein carriers is also required. In some cases the activated or derivatized polysaccharide or oligosaccharide is isolated and represents an intermediate of the glycoconjugate vaccine manufacturing process. In order to ensure a reproducible product by means of consistent application of the appropriate conjugation stoichiometry, manufacturers should use appropriate methods to determine the degree of activation or derivatization of the poly- or oligosaccharide and of the carrier protein.

### Degree of Activation of Activated Polysaccharide

If appropriate, different activation or derivatization strategies can be applied to poly- or oligosaccharides in order to make them suitable for covalent coupling to the carrier protein. In some cases the hydroxyl groups of the polysaccharide first are reacted with cyanogen bromide or CDAP or carbonyldiimidazole to form active esters. These active intermediates then can be reacted *in situ* with adipic acid dihydrazide or other bifunctional amines to introduce an amino linker. Some bacterial polysaccharides possess carboxyl or phosphate groups that might be used for introduction of an amino linker using a carbodiimide-mediated chemistry. Subsequent coupling to the carboxyl groups of the carrier protein to obtain the desired glycoconjugate can be performed using, for example, a soluble carbodiimide-mediated chemistry. Alternatively, the amino linker incorporated into the polysaccharide structure can be further derivatized to obtain a bromo-acyl or a maleimido function that is amenable to coupling with a thiol group that is present or previously was incorporated into the carrier protein.

In some other cases aldehyde groups can be introduced into the polysaccharide structure by reaction of vicinal hydroxyl groups with the oxidizing reagent sodium metaperiodate ($NaIO_4$). Depending on the polysaccharide structure, the $NaIO_4$

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treatment can also be used for simultaneous controlled depolymerization and aldehyde group generation. The poly- or oligosaccharides-containing aldehyde groups then can be covalently coupled to lysine residues and the *N*-terminal amino groups of the carrier protein by reductive amination in the presence of sodium cyanoborohydride ($NaBH_3CN$) or other reducing agents that are selective for Schiff bases.

Some manufacturing strategies are based on the controlled hydrolysis of the native polysaccharides to produce oligosaccharides that can be specifically derivatized by a sequence of steps that lead to the introduction of an active ester function at their reducing ends. The desired conjugate is then obtained by reaction of the activated oligosaccharides with the lysine residues and *N*-terminal amino groups of the carrier protein.

To ensure a consistent conjugation stoichiometry, and therefore a consistent manufacturing process, manufacturers should determine the level of derivatization or activation of the poly- or oligosaccharide intermediates. Appropriate methods for determination of the newly introduced chemical functions into the saccharide structures should be in place and could include, for example, colorimetric or other suitable methods. In cases where the activated polysaccharide is conjugated without isolation, consistency in the degree of polysaccharide activation may also be demonstrated as part of process validation or reflected by characteristics of the final conjugate bulk.

The calculation of the degree of poly- or oligosaccharide derivatization may require determination of the total saccharide quantity that can be achieved by applying, for example, HPAEC-PAD, colorimetric assays, or other suitable methods. In a validated process where production consistency has been established, and depending on the conjugation chemistry used and the results of clinical trials, testing may be used as an in-process control. Residual unconjugated linker that could interfere with subsequent steps should be controlled via measurement or process validation.

### Molecular Sizing of Activated, Derivatized, or Processed Polysaccharide

The molecular size and degree of polymerization of the poly- or oligosaccharide intermediates depends on the particular manufacturing process and should be measured because these attributes can affect the consistency of the conjugation process. Suitable tests should be applied to intermediate pools that are selected on the basis of the different manufacturing processes. Examples of suitable methods for profiling molecular sizes and determining the degree of polymerization of poly- and oligosaccharides are: SEC coupled with UV, RI, or static light scattering detectors; colorimetric assays based on total and end group determination; HPAEC-PAD; or NMR spectroscopy. In a validated process, testing can be used as an in-process control.

### Degree of Activation of Activated Carrier Protein

As mentioned above, some manufacturing procedures for glycoconjugate vaccines may also require activation of the protein carrier. This process step introduces into the protein side chains additional functional groups that react with the poly- or oligosaccharide intermediates activated with the proper functional group. In general, such functional groups are introduced by derivatization of protein amino acid side chains like glutamic or aspartic acid with a bifunctional reagent (e.g., adipic acid dihydrazide or hydrazine) so that a highly nucleophilic hydrazide group becomes available for coupling with the polysaccharide. In other manufacturing strategies, the lysine side chains of the carrier protein can be derivatized to introduce different reactive groups (e.g., bromo-acyl, thiol, or maleimido groups). Appropriate methods for determination of the newly formed chemical functions introduced into the carrier proteins should be in place and may include spectrophotometric assays and mass spectrometry. The calculation of the degree of activation or derivatization of the carrier protein may also require the determination of the total protein quantity (e.g., by colorimetric assays or other appropriate methods). In a validated process where production consistency has been established, and depending on the conjugation chemistry used and the results of clinical trials, testing may be used as an in-process control. Depending on the conjugation chemistry used (i.e., immediate conjugation after activation), consistency in degree of carrier protein activation may also be demonstrated as part of process validation or reflected by characteristics of the final conjugate bulk.

### Carrier Protein Monomer Content

In some cases the procedures for protein carrier activation or derivatization may result in a certain degree of covalent aggregation of the carrier itself, and this should be monitored with appropriate tests like SEC-HPLC coupled with static light scattering detection, SDS-PAGE, matrix-assisted laser desorption–ionization mass spectrometry, or other suitable tests.

## KEY QUALITY PARAMETERS FOR MONOVALENT BULK CONJUGATE (DRUG SUBSTANCE)

### Conjugation

Conjugation of the polysaccharide antigen to the carrier protein or protein complex is the critical component of the manufacturing process for conjugate vaccines. A general overview of a conjugation process is presented in ⟨1238⟩. In addition to developing a description of critical processing equipment, reagents, and processing steps, manufacturers should provide the rationale for the conjugation chemistry selected and the purification steps, if any, used to remove unwanted reaction components. Clearance of product-related impurities (e.g., unconjugated polysaccharide or unconjugated carrier protein) should be monitored and controlled.

Depending on the nature of the manufacturing process, the monovalent conjugate bulks can be considered to be the drug substance or can be considered a process intermediate.

During conjugation, the reactive functional groups present on the polysaccharide antigen are reacted with the functional groups located on the carrier protein or carrier complex to form stable, covalent bonds. Many different types of chemistries are used, including reductive amination, thio-alkylation, or CDAP chemistry. The choice of chemical method should be based on

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the availability of functional groups, either naturally occurring or introduced via an activation or side chain loading process, and the ability to control the manufacturing process to produce a consistent and stable product.

Although the conjugation process may be conceptually straightforward, the process should be well controlled. The process typically consists of mixing the activated polysaccharide with the selected carrier and allowing the components to react. Depending on the nature of the chemical reaction, an additional chemical reactant may be needed to complete the reaction or stabilize the conjugate product. For example, in the case of reductive amination, it may be necessary to add a reducing agent to convert the linkage from a relatively unstable Schiff base to a more stable secondary amine. Chemical deactivation or capping of residual reactive groups may also be required. Finally, residual levels of unreacted components such as free protein, free polysaccharide, chemical reagents, and byproducts should be removed from the process via validation or should be monitored by testing. Regulatory authorities may request stability evaluation of these intermediates because the data may support stability predictions for multivalent vaccines for which data collection is more difficult.

To define and control the conjugation process, the manufacturer should establish targets for process parameters and tolerances for all critical process steps where possible, including extent of activation, charge ratios for each reaction component, reaction time, reaction temperature, reaction pH, and mixing conditions. Additionally, tolerances for the purity of each of the reaction components should be established, including the polysaccharide, carrier protein, and any chemical components as noted above.

The common key quality attributes for monovalent conjugates, the rationale for monitoring these parameters, and suitable test methods are described below.

## Polysaccharide Identity

Polysaccharide identity confirms that the correct antigen was used during the manufacturing process and that no critical epitope was lost during conjugation. Polysaccharide identity should be confirmed using a suitable immunological or chemical method. Examples of immunological methods include ELISA, immunoblot analysis, and rate nephelometry. The specificity of the test method must be ensured by selection of appropriate reagents. Alternatively, the identity of the polysaccharide can be confirmed using a chemical or physical method such as HPLC, HPAEC-PAD, GC, or NMR if acceptable specificity can be demonstrated and it can be shown that the carrier protein does not substantially interfere with the identification of the polysaccharide.

## Carrier Protein Identity

Depending on the nature of the manufacturing process and the manufacturing controls, it may be necessary to confirm the identity of the carrier protein, e.g., during a manufacturing process for a multivalent product in which different antigens are conjugated to different carrier proteins within the same facility. Carrier protein identification can also be performed using an immunological method such as ELISA or, if possible, an appropriate chemical method such as peptide mapping (see *Biotechnology-Derived Articles—Peptide Mapping* ⟨1055⟩). If appropriate, the carrier identity can be evaluated in the same assay that is used for the identity of polysaccharide.

## Polysaccharide Quantity

Polysaccharide quantity or concentration must be confirmed for all lots of monovalent conjugate because it is directly related to the product dose. Polysaccharide yield can also be a useful marker for process consistency. A variety of methods are available and suitable for use in determining the polysaccharide concentration. These include colorimetric methods such as the phenol–sulfuric acid, orcinol, and anthrone–sulfuric acid assays and monosaccharide analysis following hydrolysis by HPAEC-PAD, HPLC with fluorescence detection (HPLC-FD), or GC. Immunological methods that may be suitable include ELISA or rate nephelometry. The suitability of these methods depends on the availability of appropriate reagents. The choice of method should be made on the basis of precision and accuracy. Interference from the carrier protein must be avoided. Additionally, the chemistry of the polysaccharide antigen should be considered when analysts select the method. For example, the phenol–sulfuric acid assay may not be suitable for use if the antigen is composed largely of amino sugars or sialic acid. Suitable methods for polysaccharide quantification are listed in previous sections of this chapter.

## Carrier Protein Quantity

The concentration of the carrier protein must be confirmed for all lots of monovalent conjugate. Conjugate vaccines are typically formulated based on the polysaccharide concentration, not the carrier protein concentration. However, the concentration of the carrier protein is needed to determine the polysaccharide–protein ratio, a key indicator of process consistency.

Analysts should select a test method that is specific for the carrier protein and does not suffer from interference from the polysaccharide components. Suitable methods may include amino acid analysis (see *Biotechnology-Derived Articles—Amino Acid Analysis* ⟨1052⟩), colorimetric protein tests such as the bicinchoninic acid assay or UV absorbance (see ⟨1057⟩ for both types of methods), or the protein-specific output from HPSEC with static light scattering, RI, or UV detection.

## Polysaccharide–Protein Ratio

As noted in *Carrier Protein Quantity,* the polysaccharide–protein ratio may be an indicator of process consistency. Therefore, tolerances should be established for two of the three parameters: polysaccharide concentration, protein concentration, and polysaccharide–protein ratio. It is not necessary to establish limits for all three parameters because the polysaccharide–protein

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ratio is typically calculated from the measured polysaccharide and carrier protein concentrations. Because the downstream processing and dilutions usually are based on the amount of polysaccharide present in the monovalent bulks, the limit for the polysaccharide content can be based on the minimum concentration required for downstream processing.

## Molecular Size Distribution or Integrity or Proof of Covalency

The molecular size of the conjugate is a key indicator of process consistency. Unusually small conjugates may indicate incomplete conjugation whereas unusually large conjugates may indicate aggregation and may result in a loss of yield during downstream filtration steps. The average molecular size and the size distribution should be measured using appropriate sizing methods such as SEC, HPSEC, HPSEC with static light scattering or RI detection, or analytical ultracentrifugation. The choice of the method should be made on the basis of the expected size of the conjugate and the availability of a chromophore for detection.

## Proportion of Free (or Unconjugated) Polysaccharide

The proportion of unconjugated polysaccharide must be monitored for each lot of monovalent conjugate because of the possibility that the presence of a large amount of unconjugated polysaccharide may suppress the immune response to the antigen. Additionally, the presence of free polysaccharide is a key indicator of process consistency and is an indirect measure of covalent attachment to the carrier. Measurement of the proportion of unconjugated polysaccharide can be used as a stability-indicating test if appropriately validated.

To measure the level of unconjugated polysaccharide, analysts must separate the unconjugated polysaccharide from the conjugated polysaccharide. This can be achieved chromatographically, or by chemical precipitation of the conjugate with acid or detergents, aluminum adsorption, capillary electrophoresis, gel filtration, centrifugal ultrafiltration, solid-phase extraction, or immunoprecipitation. The amount of free polysaccharide then can be quantitated using the method that was used to quantitate the total polysaccharide level, if that method is sufficiently sensitive, by UV detection if a chromophore is present, or by immunological or appropriate physicochemical methods.

The level of unconjugated polysaccharide must be measured at release and during stability testing because deconjugation is a potential degradation mechanism.

## Proportion of Unconjugated Carrier Protein

The level of unconjugated carrier protein must be monitored for each lot of monovalent conjugate because this level is a key marker of process consistency and is an indirect measure of covalency. To measure the amount of unconjugated protein, the unconjugated protein must be separated from the conjugate. This can be done chromatographically or by electrophoresis (slab or capillary). Once it is separated, the amount of unconjugated protein can be monitored by UV or by a colorimetric method (see ⟨1057⟩). Method selection should be based on sensitivity, precision, and specificity for unconjugated protein.

## Unreacted Functional Groups

During the conjugation process, reactive functional groups on the polysaccharide react with functional groups on the carrier protein. However, the reaction is typically not driven to completion, and a process of capping of remaining reactive groups may be required, depending on the nature of the residual reactive groups, the conjugation chemistry used, and manufacturing process optimization. The method chosen to cap reactive groups depends on the conjugation chemistry employed. Even so, some reactive groups may still remain on the conjugate even after the reaction is quenched by reduction, or after remaining reactive groups have been chemically capped. Safety concerns, if any, depend on the nature of the reactive groups and the level of reactive groups that remain. The level of residual reactive groups should be monitored as a measure of process consistency unless process validation has shown that unreacted functional groups detectable at this stage are removed during subsequent manufacturing processes. Additionally, the presence of residual reactive groups may affect product stability during storage.

The test method used to evaluate residual reactive groups depends on the activation chemistry that is used and the nature of the polysaccharide antigen. Appropriate methods may include gas chromatography, HPLC with fluorescence, or UV detection following hydrolysis.

## Residual Reagents

Consistency in the amount of residual reagents from the conjugation chemistry can be demonstrated during process development, and the process can be validated for their clearance. This validation includes not only unconjugated polysaccharide and protein but also buffers, salts, small-molecule reaction components, and byproducts generated during conjugation. Provided that consistent levels of residual solvents are recovered, such testing may serve as an in-process control.

## Sterility or Bioburden

Depending on the manufacturing process, the monovalent conjugate bulks should be tested for bioburden or sterility. If the monovalent conjugate bulks are subjected to additional process steps with no process holds, in some cases it may be appropriate to perform the sterility or bioburden test at a downstream step.

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### Bacterial Endotoxins

The monovalent conjugate bulks must be tested for bacterial endotoxins. If the monovalent conjugate bulks are subjected to additional process steps with no process holds, in some cases it may be appropriate to perform the endotoxin test at a downstream step.

## FORMULATED AND ADJUVANTED (IF APPROPRIATE) CONJUGATE BULKS

Monovalent conjugate bulks can be individually adsorbed and formulated as monovalent bulks before mixing during preparation of the final vaccine.

### Adjuvant Content

If an adjuvant has been added to the conjugate bulk, its content should be determined by an appropriate method. If aluminum is used as an adjuvant, typical maximum values are 0.85 mg of aluminum per dose, although higher limits up to 1.25 mg of aluminum per dose may be accepted if justified, and lower limits may apply according to governing agency requirements.

### Polysaccharide Content

Assessment of polysaccharide content can be required but is often difficult at this stage because of the presence of adjuvants or excipients. Several methods are available and are suitable for use in determining the polysaccharide concentration. These include colorimetric methods such as the phenol–sulfuric acid, orcinol, and anthrone–sulfuric acid assays; and post-hydrolysis monosaccharide analysis by HPAEC-PAD, HPLC-FD, or GC. Suitable immunological methods include ELISA or rate nephelometry. If possible, the same method must be used for both this step and the conjugate bulk step. The choice of method should be based on precision, accuracy, and specificity. Interference from the matrix must be avoided. Additionally, the chemistry of the polysaccharide antigen should also be considered when analysts select the method.

### Free (or Unconjugated) Polysaccharide

Free polysaccharide content must be measured as a release test for monovalent formulated bulk if the free polysaccharide cannot be accurately or precisely measured in the final product. For most applications, polysaccharide testing is a marker of consistency; i.e., it is monitored for each bulk in order to establish production consistency. The test may be omitted when manufacturing consistency has been demonstrated, or the test can be used as an in-process control. If free polysaccharide could be adsorbed on adjuvant, a desorption step using, for example, phosphate buffer, may be required. The unconjugated polysaccharide must be separated from adsorbed and nonadsorbed conjugated polysaccharide. Adsorbed conjugate can be removed by centrifugation, and nonadsorbed conjugate can be eliminated chromatographically; by chemical precipitation of the conjugate with acid or detergent precipitation or aluminum adsorption; by immunochemical precipitation with anti–carrier antibodies; by capillary electrophoresis; or by gel or membrane filtration or ultrafiltration. The amount of free polysaccharide then can be quantitated using the same method that was used to quantitate the total polysaccharide level if the latter is sufficiently sensitive and accurate. Quantitative tests for polysaccharide include colorimetric methods such as the phenol–sulfuric acid, orcinol, and anthrone–sulfuric acid assays; and monosaccharide analysis following hydrolysis by HPAEC-PAD, HPLC-FD, or GC. Suitable immunological methods include ELISA or rate nephelometry.

Results should be expressed as the percentage of unconjugated polysaccharide vs. the total content experimentally determined, or, if this value cannot be determined experimentally, it may be possible to calculate it from a theoretical value.

The level of unconjugated polysaccharide must be measured during stability studies because deconjugation is a potential degradation mechanism.

### Level of Adsorption to Adjuvant

The level of adsorption in monovalent formulated bulks must be performed as a release test if it cannot be performed on the final product. If it is not a release test then the adsorption test is a marker of consistency, and the test is performed on each bulk in order to establish production consistency. After consistent production has been demonstrated, the test can be omitted.

After product centrifugation, nonadsorbed conjugate is quantified in the supernatant. The adsorbed conjugate then is quantified by a suitable validated method, which can be the same test used for the total conjugate content. If the physical or chemical method used for the quantification is not specific for the conjugated form of the saccharide, the amount of free polysaccharide is subtracted from the total polysaccharide in order to determine the quantity of adsorbed conjugate. Otherwise an immunologically specific method can be used (e.g., ELISA). Results can be expressed as the percentage of nonadsorbed conjugate vs. the total content experimentally determined. If it is not possible to determine the amount of nonadsorbed conjugate experimentally then it may be possible to calculate it from a theoretical value. The level of adsorption to adjuvant must be measured during stability testing.

### Sterility

The sterility of each lot should be measured according to procedures described in ⟨71⟩ and 21 CFR 610.12.

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# KEY QUALITY PARAMETERS FOR CONJUGATE VACCINE DRUG PRODUCT

## Description and Solubility

Each container in each final fill or drug product should be inspected visually (manually or with automatic inspection systems), and containers that show abnormalities such as improper sealing, lack of integrity, or turbidity should be discarded. Similarly, the presence of clumping or particles may indicate a product failure.

## Polysaccharide Identity

Polysaccharide identity tests confirm that the correct antigen was used during the manufacturing process. Polysaccharide identity should be confirmed using a suitable immunological or physicochemical method, e.g., ELISA, immunoblots, or rate nephelometry. The specificity of the test method must be ensured by use of appropriate reagents. Acceptable specificity must be demonstrated, and tests must show that the carrier protein does not substantially interfere with the identification of the polysaccharide. Assays based on hydrolysis and chromatographic identification of saccharide components (e.g., HPAEC) after polysaccharide hydrolysis may be acceptable.

## Polysaccharide Quantity

Typically, monovalent conjugate vaccines contain 10 µg of saccharide, and multivalent vaccines contain between 1 and 10 µg of each serotype or serogroup per single human dose. Assessment of the content of polysaccharide may be difficult because of the presence of adjuvant or excipients, especially when multiple components are present. The amount of each polysaccharide may be required in order to calculate the free polysaccharide content and the proportion of unadsorbed conjugate, or this information may be used during processing of the vaccine's final formulation. Polysaccharide quantity or concentration must be confirmed for all types of monovalent conjugates because it is directly related to the product dose. A variety of methods are available and suitable for use in determining the polysaccharide concentration [see discussion of polysaccharide quantitation in the *Key Quality Parameters for Monovalent Bulk Conjugate (Drug Substance)* section above].

## Carrier Protein Identity (if Appropriate)

Depending on the nature of the manufacturing process and the manufacturing controls, if the identity of the carrier protein has not been confirmed at an earlier stage, it may be necessary to do so before product release. For example, an identity test for the carrier protein could be necessary during the manufacturing process for a multivalent product for which different antigens are conjugated to different carrier proteins within the same facility. Carrier protein can be identified using an immunological method such as an immunoblot or ELISA, or using an appropriate chemical method such as peptide mapping. If appropriate, the carrier's identity can be evaluated in the same assay used to identify the polysaccharide.

## Molecular Size (if Feasible or Appropriate)

If the molecular size distribution has not been established for the individual monovalent bulk conjugates used in the drug product formulation, the molecular size distribution of the conjugates must be determined in the final fill or drug product. The molecular size of the conjugate is a key indicator of process consistency. The average molecular size and the size distribution should be measured using appropriate sizing methods such as SEC, HPSEC, HPSEC with static light scattering or RI detection, analytical ultracentrifugation, or dynamic light scattering. The choice of the method should be based on the expected size of the conjugate and the availability of a chromophore or fluorophore for detection. In the case of molecular size distribution determinations at the final fill or in drug products composed of multivalent polysaccharides, serotype-specific detection methods may be required for the individual monovalent conjugates. Molecular size is a sensitive indicator of conjugate stability, and, where possible, it should be measured during stability studies.

## Proportion of Free (or Unconjugated) Polysaccharide

To measure the level of unconjugated polysaccharide, analysts must separate the unconjugated polysaccharide from the adsorbed and nonadsorbed conjugated polysaccharide and interfering substances. If unconjugated polysaccharide may be adsorbed to adjuvant, prior desorption with, for example, phosphate buffer is required. Methods for separation and measurement of conjugated and unconjugated polysaccharide are described above in *Formulated and Adjuvanted Conjugate Bulks.* The level of unconjugated polysaccharide is a stability-indicating measurement because deconjugation is a potential degradation mechanism and should be measured during stability studies. However, assessing the level of unconjugated polysaccharide and the stability of complex multivalent products is technically demanding, and alternative approaches to assessing antigen integrity include molecular size, *O*-acetyl content, or immunological measurement. Specific assays may provide partial but overlapping information and should be matched to the product. Free saccharide data obtained for individual monovalent conjugates may also prove valuable and predictive.

## pH, Osmolarity/Isotonicity, and Excipients

If the vaccine is a liquid preparation, the pH and osmolarity/isotonicity of each final lot should be tested and shown to be within the pre-approved specifications. For a lyophilized preparation, the pH should be measured after reconstitution with the

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appropriate diluent. Residual moisture in lyophilized products should be determined. See comments above regarding excipient functionality.

## Adjuvant Quantities (if Appropriate)

If an adjuvant has been added to the conjugate bulk, its content should be determined by an appropriate method. If aluminum compounds (such as aluminum hydroxide or hydrated aluminum phosphate) are used as adjuvants, the amount of aluminum should not exceed 1.25 mg per single human dose or as otherwise required by the governing agency (see also 21 CFR 610.15).

## Antimicrobial Preservative (if Appropriate)

During product development manufacturers should consider the stability of the chosen preservative and possible interactions between the vaccine components and the preservative. If a preservative has been added to the vaccine, the content of preservative should be determined by an appropriate method (see *Antimicrobial Agents—Content* ⟨341⟩). The amount of preservative in the vaccine dose should be shown neither to have any deleterious effect on the antigen nor to impair the safety of the product in humans. If present, the amount must be NLT the minimum amount shown to be effective and typically should be NMT 120% of the amount stated on the label.

## Moisture Content (Lyophilized Products)

If the vaccine is freeze-dried, the average moisture content should be determined by an appropriate method. Values should be within limits established during the product's stability studies. Typically, the average residual moisture content should be NMT 2.5%, and no vial should be found to have a residual moisture content of 3% or greater.

## Sterility

The sterility of each lot should be determined according to procedures described in ⟨71⟩ and 21 CFR 610.12.

## Pyrogens and Endotoxins

Endotoxin (see ⟨85⟩) content or pyrogenic activity (see ⟨151⟩) should be within approved product specifications.

## Diluent for Reconstitution of Lyophilized Vaccines

Manufacturers should generate data that show that adventitious microbial contamination does not grow under the reconstitution conditions (e.g., with diluents that will be used for reconstitution) or under specified storage conditions. A preservative is not normally required for single-dose vials when the product will be used soon after reconstitution, but multidose vials do require preservatives. Testing in alignment with ⟨51⟩ is recommended to demonstrate acceptability, but testing may not be required routinely after process control and consistency have been established.

## General Safety or Abnormal Toxicity

The general safety or abnormal toxicity for vaccines should be established by appropriate evaluation and should be consistent with levels found to be acceptable in vaccine lots that were used in clinical trials.

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# ⟨1235⟩ VACCINES FOR HUMAN USE—GENERAL CONSIDERATIONS

***Add the following:***

▲INTRODUCTION
Regulations and Standards
OVERALL MANUFACTURING PLAN
Manufacturing Facilities and Systems
Manufacturing Process
Manufacturing Surveillance
RAW MATERIALS, FERMENTATION, AND CELL CULTURE MEDIA
MATERIALS OF ANIMAL ORIGIN
SEED LOT SYSTEMS AND CELL BANKING
PROPAGATION AND HARVEST
VACCINES PRODUCED BY RECOMBINANT TECHNOLOGY
PURIFICATION
Biotechnology-Engineered Cells
INTERMEDIATES
Production of Intermediates
Tests for Intermediates
FILTERED BULK OR DRUG SUBSTANCE
FINAL BULK
Production of Final Bulk
Tests for Final Bulk
Stability Test for Final Bulk
FINAL CONTAINER
Excipients and Other Additives
EVALUATING THE STABILITY OF VACCINES
Stability Protocols
Analytical Measurements
Formal Evaluation of Stability Data and Product Expiry Dating
NOMENCLATURE
CONTAINER
LABELING
Container Label
Package Label
Prescribing Information
LOT RELEASE TESTING
General Principles
Common Tests
Permissible Combinations
Quality
Alternative Tests
RETENTION SAMPLES AND OTHER REQUIREMENTS
GLOSSARY
APPENDIX▲ (USP 1-May-2021)

***Change to read:***

## INTRODUCTION

Vaccines have been used for centuries to immunize individuals against pathogenic organisms with the goal of preventing the associated diseases. Vaccines are biological products that contain antigens capable of inducing a specific and active acquired immune response in the body. Antigens present in vaccines are processed by specialized cells in the body's immune system, resulting in the development of blood proteins known as antibodies (i.e., humoral immunity), specialized lymphocytes (i.e., cell-mediated immunity), or both. Therefore, immune responses may be antibody mediated, cell mediated, or both. Antigens are ▲the components responsible▲ (USP 1-May-2021) for vaccine function and generally consist of a portion of the pathogenic organism, ▲such as an inactivated or▲ (USP 1-May-2021) attenuated form of the whole microorganism, ▲or are produced recombinantly from a non-infectious organism engineered to express antigens from a pathogenic species or recombinant protein expressed in genetically engineered cell line. In the case of vaccines derived from recombinant technology, the antigen is expressed in a host cell of an unrelated microorganism and then used for vaccine manufacture.▲ (USP 1-May-2021) In the case of

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DNA-based vaccines (currently under development), the vaccine would contain nucleotide sequences (genetic material) that encode microbial antigens.

▲▲ (USP 1-May-2021) A list of vaccines currently licensed in the US is posted ▲on the FDA website (see *Appendix*). There are▲ (USP 1-May-2021) various types ▲of vaccines, which can be classified▲ (USP 1-May-2021) depending on their design and the processes involved in their manufacture ▲(see *Table 1*).▲ (USP 1-May-2021) Vaccines for human use may contain whole killed or attenuated organisms (e.g., bacteria or viruses) or antigens derived from portions of a pathogen (either by partitioning and purification or derived using recombinant technology, ▲or they may be engineered to be expressed by non-infectious viruses.▲ (USP 1-May-2021) Some polysaccharide vaccines are conjugated to a carrier in order to enhance their immune response.

**▲Table 1. Bacterial and Viral Vaccines**

| Bacterial Vaccines | Viral Vaccines |
|---|---|
| Carbohydrate subunit vaccines[a] | Recombinant virus-like particles (VLPs)[b] |
| Purified polysaccharide vaccines | Isolated (virus-derived) viral proteins[a] |
| Glycoconjugate vaccines | Recombinant (monomeric) viral proteins[b] |
| Protein subunit vaccines | — |
| Toxoid vaccines | — |
| Isolated bacterial proteins | — |
| Recombinant bacterial proteins[b] | — |
| Bacterially derived protein-containing lipid vesicles[c] | — |
| Inactivated bacterial vaccines[d] | Inactivated viral vaccines[d] |
| Live attenuated bacterial vaccines | Recombinant chimeric viral vaccines |
| — | Live attenuated viral vaccines[e]▲ (USP 1-May-2021) |

[a] Subunit vaccines are extracts from inactivated/killed viruses or bacteria and generally undergo some degree of purification.
[b] Recombinant protein viral and bacterial vaccines are derived from host cells that have been transformed with expression vectors that carry genes that encode antigenic material from infectious agents. The expression cells are grown in bioreactors to produce the recombinant antigenic material.
[c] Protein-containing vesicles are semi-purified native bacterial membrane fragments or artificial liposomes containing specific semi-purified native bacterial proteins or recombinantly expressed antigenic bacterial proteins. Processing and semi-purification renders the preparations non-viable while retaining their immunogenicity.
[d] Inactivated bacterial and viral vaccines are produced by growing cells of disease-causing bacteria or viruses in cell substrates and subsequently inactivating them to prevent replication in the recipient. These preparations may or may not be partially or completely purified.
[e] Live attenuated bacterial or viral vaccines are weakened (attenuated) forms of a pathogen. They contain antigens that are similar to disease-causing microbes. They may be derived from the pathogen itself, or from a different organism that contains antigens that cross-react with the virulent microbe (e.g., vaccinia and variola).

In addition to antigen(s), vaccines may contain ▲▲ (USP 1-May-2021) other components, such as adjuvants that enhance the immune response to the vaccine antigen, preservatives to prevent bacterial or fungal contamination of multiple-dose vials, or other excipients needed for pharmaceutical manufacturing or vaccine stabilization. Residual components from the manufacturing process also may be present in vaccine preparations. Examples of these categories are listed in *Table 2*.

**Table 2. Vaccine Components**

| | |
|---|---|
| Antigens | Whole organisms |
| | Components/subunits |
| | Recombinant proteins |
| Adjuvants▲, CpG oligonucleotides▲ (USP 1-May-2021) | Aluminum salts |
| | ▲Monophosphoryl Lipid A, saponins▲ (USP 1-May-2021) |
| | Squalene▲, CpG oligonucleotides▲ (USP 1-May-2021) |
| Antimicrobial preservatives | Thimerosal |
| | 2-Phenoxyethanol |
| | Benzethonium chloride |
| | Phenol |
| Stabilizers | Salts |
| | Amino acids |
| | Sugars |
| | Proteins |
| | ▲Surfactants▲ (USP 1-May-2021) |

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**Table 2. Vaccine Components** *(continued)*

| | |
|---|---|
| Manufacturing residuals | Cell-derived residuals |
| | Materials of animal origin |
| | ▲Residual conjugation reagents▲ (USP 1-May-2021) |
| | Antibiotic residuals |
| | Inactivating chemical agents |
| ▲Containers | Vial and stopper |
| | Syringe and plunger▲ (USP 1-May-2021) |

Different vaccine antigens are often combined in one final formulation in order to elicit immunity against multiple diseases and to reduce the number of separate administrations needed to achieve immunity to the various vaccine antigens.

Despite the multiple forms vaccines may take, several common features characterize the manufacture and testing of vaccines. This chapter focuses on commonalities throughout the manufacturing process, from raw material qualification to final release tests.

## Regulations and Standards

Vaccines are regulated by the US FDA as biological products. The general requirements are listed in national laws and international guidances. For the US, national requirements are codified in 21 Code of Federal Regulations (CFR) §200 and 21 CFR §600, with additional recommendations available in the FDA's *Points to Consider* and guidance documents ▲(see *Appendix*).▲ (USP 1-May-2021) [International guidances are available from the International Council for Harmonisation (ICH) ▲(see *Appendix*)▲ (USP 1-May-2021) and the World Health Organization.] New methodologies are continually being developed and validated and will be included in the *USP* as they become available. Reference standards are available from USP and the FDA.

***Change to read:***

## OVERALL MANUFACTURING PLAN

When considering the overall plan for manufacturing a vaccine, manufacturers need to consider the following factors:

- Physical facilities ▲and design, qualification, and maintenance of utilities and equipment▲ (USP 1-May-2021)
- Raw materials and process aids
- ▲▲ (USP 1-May-2021)Manufacturing process▲▲ (USP 1-May-2021)
  - Initial process (production of virus/bacteria and recombinant materials)
  - Downstream processes (purification or chemical modification, if applicable)
- Antigen modifications such as conjugation or toxoiding
- Storage of process intermediates and final bulk
- In-process and final product testing regimens and control schemes
- Addition of adjuvants, if applicable
- Formulation and filling
- Container–closure system
- Stability program that supports the ▲expiry▲ (USP 1-May-2021) dating ▲▲ (USP 1-May-2021) of the product

Quality systems are needed to support the following manufacturing process development: specifications for raw materials, process ▲controls and process▲ (USP 1-May-2021) intermediates, and final product ▲quality, stability, and shelf-life studies; analytical development and validation;▲ (USP 1-May-2021) change control; ▲facility, utility, and equipment qualification; cleaning, maintenance, and potential change-over protocols;▲ (USP 1-May-2021) and failure investigations and complaints. All of these elements are important in the life cycle of the vaccine product.

The overall goal of a comprehensive manufacturing program is to consistently ▲and continuously▲ (USP 1-May-2021) produce a vaccine that is safe and effective. Concurrently with clinical development of the vaccine, the manufacturing process is refined, ▲the process is validated for consistency,▲ (USP 1-May-2021) and the ▲test▲ (USP 1-May-2021) methods are validated for ▲their intended use.▲ (USP 1-May-2021) This includes systems to control changes to the process or inputs. Manufacturers should expect that changes will be required during the vaccine's manufacturing life cycle, and manufacturers necessarily will use data from development and routine manufacturing to assess the process as well as proposed changes. The manufacturers should adopt systems that continually evaluate all aspects of manufacturing to identify unanticipated changes in vaccine quality and to assess them as quickly as possible.

## Manufacturing Facilities and Systems

Manufacturers should ▲document the▲ (USP 1-May-2021) general layout of the manufacturing facilities, including diagrams that show the following: flow of raw materials and process inputs; movement of product, intermediates, waste streams, and personnel; and air flows and pressurization levels. These diagrams assist in minimizing the risk of potential product contamination from various sources. These sources can include cross-contamination from other products, contamination from different batches of the same product, and extraneous contamination from microorganisms ▲or foreign material.▲ (USP 1-May-2021)

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Evaluation of the flow diagrams can assist with strategies for the development of engineering controls, personnel procedures, and monitoring systems to enable compliance with ▲current▲ (USP 1-May-2021) good manufacturing practices (▲cGMPs).▲ (USP 1-May-2021) Analysis of potential risks may also provide insights about what information should be recorded in batch documentation to facilitate consistent manufacture and failure investigations. Together, physical facilities, ▲utilities, equipment,▲ (USP 1-May-2021) procedures, personnel, training, and quality systems make up the GMP environment in which a vaccine will be produced.

## Manufacturing Process

The manufacturing process includes process inputs such as raw materials and processing aids and unit operations comprising both the initial and downstream processing steps. A process flow map for the manufacturing process is useful and assists in validation of the manufacturing process. This map shows all unit operations, the inputs to each operation, and the outputs to subsequent manufacturing steps. Analytical testing done at relevant steps and the specifications required to proceed to the next stage of processing may be added to the map. A process map also supports a processing space to facilitate a rugged process (i.e., one based on suitable characterization studies to establish boundaries within which manufacturing can occur to promote unchanged safety and efficacy outcomes).

The process flow map should include all steps from making the seed/cell bank ▲▲ (USP 1-May-2021) to formulation, filling, ▲and packaging▲ (USP 1-May-2021) of the final product. The validation strategy should include the steps that require validation, along with identification of the process space, associated critical process parameters (CPPs), and critical quality attributes (CQAs). The CPPs are those that directly affect the core quality attributes needed to successfully manufacture a batch of product. Some manufacturers identify other processing parameters that are important for processing but do not affect CQAs. These important but noncritical factors help identify the process development space, can contribute to the development of a rugged process, ▲and▲ (USP 1-May-2021) can be useful when the company assesses processing deviations. The concepts of quality by design and exploration of the process space are relatively new to the biologics/vaccine industry but are becoming considerations for the overall development-planning process).

## Manufacturing Surveillance

Manufacturing surveillance is the continual observation of how the process and the resulting product are performing. This section is not exhaustive; rather, the points raised here outline the types of considerations recommended for a manufacturer during the development of a vaccine. Manufacturing surveillance includes the following:

- Periodic review of the performance of the manufacturing process
- Analytical assays
- Stability programs
- ▲Facility evaluation through environmental monitoring▲ (USP 1-May-2021)
- Product complaints
- Adverse event reports
- Product failure investigations
- Atypical or deviation events

Taken as a whole, these activities allow a manufacturer to assess the state of the process and product and to evaluate which, if any, operations need to be modified. These same systems also provide a surveillance matrix to evaluate changes. In any of these programs, it is also valuable to develop additional characterization assays that are not used for process intermediate or product release purposes but may be used for further evaluation when additional information is needed or desired. These additional assays for characterization are often based on different underlying analytical procedures to provide different ways to evaluate materials.

Routine surveillance processes are increasingly implemented to attempt to detect changes in processes before any CQAs are adversely affected. Not all vaccine processes can be characterized to the same extent or level (e.g., a live virus vaccine vs. a recombinant protein vaccine), and statistical tools are often used to determine alert or action levels in surveillance programs. Exceeding these levels requires the manufacturer to evaluate the situation but does not necessarily signal product failure.

GMP manufacturing entails facility, ▲utilities, and equipment▲ (USP 1-May-2021) design; process development; quality systems; and manufacturing surveillance. Together, these systems help the manufacturer to control the production of a vaccine. As noted, many types of vaccines are marketed, and each has its unique features and therefore requires different plans for each of the steps mentioned in this section.

***Delete the following:***

## ▲SEED LOT SYSTEMS

Seed lots are the stocks of specific strains of bacteria, viruses, or biotechnology-engineered cells used to express vaccine antigens. All seed lots should be documented in terms of their isolation, derivation (or construction, in the case of recombinant vector or engineered cells), and passage history. The purpose of a seed-lot system, which typically includes master and working stock seeds, and associated master and working cell banks, is to help ensure the consistency of vaccine manufacturing. The use of master and working seed lots provides a method to limit the replication of the seed and to minimize the possibility of genetic variation.

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A master seed lot is a physically homogeneous preparation derived from an original seed processed at one time and passaged for a limited number of times. The master seed lot is characterized for its biological, biochemical and genetic characteristics, and to ensure its purity, its freedom from adventitious agents, and its clinical ability to produce an effective vaccine.

Cultures from the working seed lot should have the same characteristics as the master seed lot from which they are derived. For influenza vaccines, which may be reformulated with new virus antigens each year, certified seed lots can be obtained from national regulatory agencies.

A working seed lot is derived from the master seed within a limited number of passages. The working seed is tested to ensure its purity, freedom from adventitious agents, and biochemical properties. The working seed is used for production of vaccine without intervening passages.

## Bacterial Vaccine Seed Lot System

In the bacterial seed lot system, a master seed is subcultured to produce a working seed one passage beyond the master seed. An aliquot of the working seed is then expanded to produce a vaccine lot. The strain(s) used for the master seed lots are identified by historical records that include information about their origin. Information about the bacterial seed lot system should include source, passage history, and raw materials to which it was exposed, with specific emphasis on raw materials of ruminant origin. Seeds should be stored at an appropriate temperature in more than one location within a facility or at a distant site in order to decrease catastrophic risk.

Identity tests may include inoculation onto suitable biochemical media, Gram stains, genotype, and serological identification with suitable specific antisera. Special tests may be added, for example, to show culture viability but also lack of virulence.

Purity of the bacterial strains used for seed lots is verified by methods of suitable sensitivity to ensure that no adventitious agents are present. These purity tests often are performed in the presence of the seed under conditions where growth is inhibited by the presence or the absence of specific nutrients. Streaking can also be used to show that the cultured seed is a pure culture.

## Viral Vaccine Seed Lot System

The derivation and passage history of viral seeds should be recorded in detail. Any manipulation of the viral phenotype (e.g., cold adaptation, development of temperature sensitivity, or attenuation of virulence) or intentional genetic manipulations (e.g., reassortment or recombination) should be documented.

These viral seeds are commonly differentiated into a master viral seed and working viral seeds or working viral stock. Viral seeds should be stored at cryogenic temperatures to promote stability and in more than one location within a facility or at a distant site to decrease catastrophic risk. Manufacturers should assess the following characteristics of the viral seed stock:

- Growth characteristics on the intended production cell substrate,
- Tissue tropism;
- Genetic markers;
- Identity (for recombinant vectors);
- Viability during storage,
- Genetic stability through production;
- Attenuation properties;
- Purity;
- Absence of adventitious agents. If attenuation or derivation is achieved by passage through different species, the viral seed should be assessed for absence of adventitious agents common to those species.

The master viral seed should be extensively characterized to demonstrate the stability of genotype and phenotype for a number of passages beyond the level used in production. Generally, during assessment of genetic stability, a master seed undergoes a minimum of five passes beyond the passage that will produce the final vaccine.

Tests should be performed for identity (e.g., sequencing the entire virus or a portion of it), adventitious agents, viral phenotype, genetic stability, and, if applicable, agents that might be present in the seed as a result of its passage history. Viral phenotype can be assessed further for tissue tropism, attenuation properties, and temperature sensitivity. Not all of these tests may be necessary for every viral seed strain.

In some cases the viral seeds may have a broad host range and therefore may require neutralization of the vaccine virus before they are tested for adventitious agent(s). If possible, testing for adventitious agents should be done without neutralization in order to avoid an antiserum that may inadvertently neutralize an adventitious agent present in the seed. Sometimes it is not possible to effectively neutralize a viral seed, and in such cases alternative strategies can be used. For example, the test can be performed in a cell substrate that does not permit replication by the vaccine virus. However, such a substitution of the substrate cell may compromise the test's sensitivity for detection of other adventitious agents. Therefore, the tests may be supplemented with use of polymerase chain reaction (PCR) assays.

Assessment of neurovirulence may be appropriate if the virus is known to be neurotropic. Manufacturers should consult with regulators about appropriate animal models, methods, and scoring systems for this assessment before they initiate such studies. For viruses that are neurovirulent or may revert to neurovirulence (e.g., polioviruses), it may be necessary to assess neurovirulence beyond the master seed.

If the master viral seed is well characterized, the working viral seed may not require extensive characterization. For example, it may not be necessary to repeat testing for all the relevant viruses from the derivation history.

## Systems for Biotechnology-Engineered Vaccines

For a vaccine produced via a biotechnology-engineered cell-expression system, a master seed lot or a master cell bank will be established during product development. The seed lot or cell banks should be homogenous, which is often accomplished

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by limiting dilutions. The seed lot or cell bank system should be characterized in a manner analogous to that used for the cell substrate discussed in the next section, and additional tests can be used to demonstrate the genetic stability of the expression system.▲ (USP 1-May-2021)

***Change to read:***

## ▲RAW MATERIALS,▲ (USP 1-MAY-2021) FERMENTATION, AND CELL CULTURE MEDIA

▲Raw materials can directly affect the identity, strength, purity, and quality of vaccines. A consistent manufacturing process critically depends on the use of consistent raw materials (e.g., during seed banking, propagation, harvest, purification, and formulation). Accurate records of the composition and source of the culture medium used in seed banking and routine fermentation should be maintained, and the release criteria for raw materials or components should also be documented.▲ (USP 1-May-2021)

A medium is the material in which an organism is grown and amplified in quantity to produce mass material for vaccine production. Its composition is diverse and depends on the cell types that the medium supports, ranging from well-defined chemical media to chemically undefined media that contain natural components such as sera from animal origin (see *Bovine Serum* ⟨1024⟩). ▲Raw materials for bacterial growth media typically consist of both well-defined chemical entities (e.g., amino acids, carbohydrates, vitamins, minerals) and more complex components (e.g., protein hydrolysates, yeast extracts, peptones). Manufacturers should consider the source of each of these raw materials to ensure that they come from reliable vendors who adhere to cGMP quality standards and can assure a long-term supply. Manufacturers should communicate with raw material vendors in order to avoid any changes in the sourcing or manufacture of components and to avoid supply shortages. Without such communications, the consistency of the propagation processes and the supply of the vaccine can be adversely affected. Consistent raw materials are particularly critical for more complex fermentation components such as fetal calf serum, yeast extracts, or peptones, for which changes may be difficult to detect but are likely to have a direct effect on fermentation. In general, the use of animal-derived raw materials should be minimized because they pose additional risks and are more prone to variability. The section below, *Materials of Animal Origin*, describes additional considerations specific to animal-origin raw materials.

The use of antibiotics should be minimal or should be avoided to ensure that no unwanted antibiotics are included in the drug product unless they are intentionally used in manufacturing (e.g., as selective markers).

As manufacturers scale up fermentation to pilot production (i.e., within 10-fold of final manufacturing scale), they should also ensure, to the extent possible, the availability of multiple sources for all raw materials. This will ensure that supply or business instabilities at one vendor do not become the limiting factor in vaccine manufacture.

Specific requirements relating to bacterial vaccines are covered in *Vaccines for Human Use—Bacterial Vaccines* ⟨1238⟩, and aspects specific to virus-derived and recombinant viral vaccines are covered in *Vaccines for Human Use—Viral Vaccines* ⟨1239⟩.▲ (USP 1-May-2021)

***Add the following:***

## ▲MATERIALS OF ANIMAL ORIGIN

Some raw materials and reagents, such as gelatin, calf serum (see ⟨1024⟩), or trypsin for vaccine manufacturing raise concerns regarding the potential presence of adventitious agents. Gelatin or processed gelatin also is used as a vaccine stabilizer. The gelatin source may be either bovine or porcine. Although the conditions of manufacturing gelatin are harsh (i.e., the product is subjected to extremes of heat and pH), there remains a concern with bovine sources about the presence of the transmissible spongiform encephalopathy (TSE) agent, because this agent is known to resist such conditions. Therefore, if gelatin added to a vaccine or used in manufacturing is from a bovine source, the material should have the appropriate documentation certifying that it comes from a country or region that is in compliance with TSE guidance for industry. In case such a certification cannot be provided, tests should be performed on the raw material to demonstrate that it is TSE-free. If additives from animal sources are added to the culture medium, they should be certified to be free from contaminants and adventitious agents, or raw materials should be sourced from countries acceptable to the FDA. Vendors/manufacturers should provide information about the identity and source of additives and should test for adventitious agents. Additionally, manufacturers should test these materials when possible to minimize the risks of contamination with adventitious agents, such as those that cause bovine spongiform encephalopathy or TSE. Reduction of serum components [e.g., bovine serum albumin (BSA)] should be considered in processing.▲ (USP 1-May-2021)

***Add the following:***

## ▲SEED LOT SYSTEMS AND CELL BANKING

Cell banking is described in detail in *Cell Banking* ⟨1042⟩. Aspects specific to bacterial vaccines are included in ⟨1238⟩, and aspects related to viral vaccines (such as virus seed stocks) are described in ⟨1239⟩.▲ (USP 1-May-2021)

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***Change to read:***

## PROPAGATION AND HARVEST

The propagation and harvest phases follow the manufacturing process from the initiation of cell growth in the working cell bank to the separation of the crude drug substance. In addition, in these manufacturing process steps, raw materials, media, and solutions should be qualified for their intended use. Batch numbers should be clearly assigned as needed, and the relationship between component harvests and batches of individual drug substances should be recorded clearly.

▲Propagation and harvest for bacterial vaccines are described in detail in ⟨1238⟩ and in specific product class chapters. Propagation and harvest for viral vaccines are described in detail in ⟨1239⟩.▲ (USP 1-May-2021)

***Add the following:***

## ▲VACCINES PRODUCED BY RECOMBINANT TECHNOLOGY

The production of antigen, either monomeric or in self-assembling aggregates such as virus-like particles, is increasingly being performed using recombinant technology in a heterologous production cell line. In general, the requirements for the production of these products follow the requirements for other recombinant protein products.▲ (USP 1-May-2021)

***Change to read:***

## PURIFICATION

The objective of the purification steps is to remove as much as possible of the impurities in the initial harvest and to maximize the purity of the final vaccine product. Process residuals may consist of materials from the culture medium and/or cellular components. Purification procedures should be optimized and validated. When applicable, viral clearance steps (viral removal or inactivation) should be included and validated using relevant model viruses. Special considerations are observed depending on the types of vaccines and production system used.

▲Information specific to the purification of bacterial vaccines is presented in ⟨1238⟩, material specific to bacterial polysaccharide and glycoconjugate vaccines is presented in *Vaccines for Human Use—Polysaccharide and Glycoconjugate Vaccines* ⟨1234⟩, and information specific to viral vaccines is presented in ⟨1239⟩.▲ (USP 1-May-2021)

### Biotechnology-Engineered Cells

Of ▲particular▲ (USP 1-May-2021) concern in the purification of recombinant-derived vaccine components, ▲including virus-like protein vaccines,▲ (USP 1-May-2021) is the issue of residual host cell components that could produce an adverse immunogenic response in patients. This response could be exacerbated by the presence of vaccine adjuvants.

▲▲ (USP 1-May-2021)

***Change to read:***

## INTERMEDIATES

Intermediates are defined here as the unformulated active (immunogenic) drug substances that are ▲further▲ (USP 1-May-2021) processed before final formulation and can be stored ▲▲ (USP 1-May-2021) before further processing. Those intermediates ▲that will▲ (USP 1-May-2021) be stored should be included in a formal stability program ▲to assess maximum storage times.▲ (USP 1-May-2021) Examples of intermediates include bulk polysaccharides, purified recombinant proteins (concentrates), ▲toxoids, purified and inactivated virus harvests, and polysaccharide activation and carrier-protein conjugation intermediates.▲ (USP 1-May-2021)

### Production of Intermediates

Intermediates are manufactured from starting materials by one or a combination of different processes (e.g., fermentation, cultivation, isolation, or synthesis). Subsequent steps of the procedure involve preparation, characterization, and purification, eventually resulting in the drug substance ▲intermediate.▲ (USP 1-May-2021) Quality systems documents are adopted for ▲process development, in-process controls, and▲ (USP 1-May-2021) production and all applicable information should be recorded in a controlled document (i.e., a batch record). When applicable, stability studies ▲should be available▲ (USP 1-May-2021) and release tests should be performed before proceeding to the next ▲processing▲ (USP 1-May-2021) steps (see below).

### Tests for Intermediates

The quality attributes of the intermediate are commonly tested in conjunction with further ▲batch▲ (USP 1-May-2021) processing. Characterization beyond release testing should be considered. Characterization methods can use appropriately qualified procedures. Depending on the individual vaccine, some tests are routinely performed before the intermediates are converted to the final bulk.

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If intermediates need to be stored and/or subsequently shipped to a different location for further processing, the stability of these materials should be demonstrated. Stability tests can be a combination of both physicochemical analysis and biological assays.

***Add the following:***

## ▲FILTERED BULK OR DRUG SUBSTANCE

The final step in the manufacturing of antigen intermediates is the preparation of filtered bulk or drug substance. It is acceptable to have a low-bioburden drug substance, provided that sterile filtration is performed prior to use of this material to prepare formulated bulk. Preparation of low-bioburden drug substance involves filtration using 0.22-µm sterilizing grade filter membranes. For some antigens, which are not amenable to filtration, the process of manufacturing should be an aspectic process demonstrated through the use of media-fill tests. According to the FDA guideline (see *Appendix*) and industry standards, filters used for the final filtration to prepare sterile filtered bulk should be validated to reproducibly remove microorganisms from a carrier solution containing bioburden of a high concentration of at least $10^7$ colony forming units (cfu)/$cm^2$ of effective filter area (EFA). Thus, the retention capacity of a validated sterilizing-grade filter with an EFA of A ($cm^2$) is at least A × $10^7$ cfu. However, the currently used 0.22-µm sterile filter membranes can withhold much higher microbial challenges (unpublished company results for 0.22-µm polyvinylidene fluoride membranes demonstrated that a bacterial challenge concentration as high as $10^9$ cfu/$cm^2$ can be validated).▲ (USP 1-May-2021)

***Change to read:***

## FINAL BULK ▲(FORMULATED BULK)▲ (USP 1-MAY-2021)

Final bulk is the ▲formulated▲ (USP 1-May-2021) drug product that contains the drug substance(s), excipients, and other ingredients at the desired concentrations and is ready for filling into individual ▲dosage containers from which the vaccine would be administered.▲ (USP 1-May-2021)

### Production of Final Bulk

Appropriately controlled amounts of all ingredients are blended to uniformity to produce the final bulk. The processing may include one or more steps such as buffer exchange and addition of diluents, bulking agents, stabilizing excipients, adjuvants, and preservatives. Final bulk may be prepared aseptically or processing may include a sterilization step.

### Tests for Final Bulk

The quality attributes of the final bulk should be tested. Appropriate testing should be performed with respect to identity, purity, potency, sterility (see *Sterility Tests* ⟨71⟩), and antimicrobial effectiveness (see *Antimicrobial Effectiveness Testing* ⟨51⟩). Tests demonstrating safety ▲and potency,▲ (USP 1-May-2021) if applicable, are performed.▲▲ (USP 1-May-2021)

Testing is required for specific process-related and product-related impurities, depending on the vaccines being manufactured. In addition, tests are required for the bulking agent, stabilizing excipients, adjuvants, and/or preservatives, if used. All the testing should be done according to respective standard operating procedures (SOPs), and all tests should have specifications (or provisional specifications, where applicable).

### Stability Test for Final Bulk

If final bulks are stored and/or subsequently shipped to a different location for further processing, the stability of these materials should be demonstrated. Stability tests can be a combination of both physicochemical analysis and biological assays. Implementation of a stability program is required for formal stability studies, and the studies should be executed according to a protocol that contains detailed information about the types of tests, including specifications, ▲storage condition,▲ (USP 1-May-2021) testing intervals, and data and analysis.

***Change to read:***

## FINAL CONTAINER

A final container of vaccine contains the active ingredient(s) [i.e., antigen(s)] as well as additional components, such as stabilizers, adjuvants, ▲and/▲ (USP 1-May-2021)or antimicrobial preservatives. They also may include residual materials from the manufacturing process.

### Excipients and Other Additives

In addition to specific antigens, vaccines often include excipients and other additives that are intentionally added to the vaccine by the manufacturer for a specific purpose. These include adjuvants, antimicrobial preservatives, and stabilizers. Vaccines also contain manufacturing residuals, which are trace amounts of various components used during manufacturing. Thus, the

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combinations of these components comprise and define the complete vaccine product. Manufacturers must adhere to regulations governing permissible limits of such components, as indicated in the product's license.

## ADJUVANTS

Adjuvants are agents incorporated into vaccine formulations to enhance and increase the immune responses generated by the vaccine antigens. Specifically, they can increase the amount of antibody produced, direct the immune response (Th1 or Th2), increase the duration of antibody presence (persistence), or produce a combination of these effects.

Aluminum compounds have long been the most widely used adjuvants worldwide. Two methods traditionally have been used for combining aluminum adjuvant to antigen to form aluminum-adsorbed vaccines. The first involves the addition of the antigen solution to preformed aluminum precipitate. The second involves the addition of an antigen to aluminum in solution and the addition of a compound that will coprecipitate the aluminum salt and the antigen in situ. Solutions of aluminum potassium sulfate, known as alum or aluminum chloride, have been used together with phosphate salts as precipitating agents. A number of aluminum adjuvant formulations are used in vaccines.

Tests for aluminum are based on metal detection tests described in *Aluminum* ⟨206⟩. Regulations ▲[see 21 CFR §610.15(a), *Ingredients, preservatives, diluents, adjuvants*]▲ (USP 1-May-2021) limit the amount of aluminum permitted in a dose of vaccine.

▲Other adjuvants have recently been used in vaccine products licensed in the US, including squalene and monophosphoryl lipid A (MPLA) (in combination with an aluminum-based adjuvant). MPL is quantified either through degradation to fatty acid methyl esters and quantification by gas chromatography, or through quantification of the glucosamine content. Squalene is quantitated by HPLC. Squalene adjuvants should be characterized for both the mean particle size and the number of large particles. The surfactants (e.g., sorbitan trioleate, Tween 80) used to formulate the squalene adjuvant can also be quantitated using HPLC. Specific tests to monitor the stability of bulk adjuvant should be developed.▲ (USP 1-May-2021)

Note that adjuvants are not licensed by themselves; they do not constitute a product. Rather, a vaccine consisting of specific antigen(s) and an adjuvant are licensed together as a drug product.

## ANTIMICROBIAL PRESERVATIVES

In the case of multiple-dose containers, antimicrobial preservatives are added to inhibit the growth of microorganisms that may be introduced from repeated puncture of multidose vials. With certain exceptions, a preservative is required to be present in vaccines marketed in multidose containers [21 CFR §610.15(a)]. Exceptions include yellow-fever vaccine; measles, mumps, and rubella ▲vaccine;▲ (USP 1-May-2021) and dried vaccines when the accompanying diluent contains a preservative.

The microbial preservatives currently used in vaccines are thimerosal, 2-phenoxyethanol, benzethonium chloride, and phenol. These agents must pass the appropriate antimicrobial effectiveness test, as described in ⟨51⟩. Antimicrobial test challenges should be conducted as part of the normal formal stability program, including at the expiration date. Various tests for preservatives can be found in *Antimicrobial Agents—Content* ⟨341⟩.

## STABILIZERS

The primary purpose of stabilizers is to protect certain vaccines from adverse conditions such as heat or to serve as a cryopreservative during the lyophilization process, usually during the freezing step. The particular materials chosen for this purpose include sugars (e.g., sucrose or lactose), amino acids [e.g., glycine or glutamic acid (monosodium salt)], glycerol, and proteins [e.g., human serum albumin (HSA) or gelatin]. Materials should be customized to a specific vaccine formulation and selected with patient safety in mind.

When a protein is chosen as a stabilizer, two main safety concerns arise. One stems from the source of the protein: animal or human origin raises the possibility of the presence of an adventitious agent. The second concern is the possibility of an allergic reaction in persons sensitized to that protein. This should be evaluated as part of the clinical program during vaccine development. At present, two proteins are used as stabilizers for vaccines: HSA and gelatin. The FDA requires that any serum-derived albumin used in manufacturing be US-licensed HSA. FDA guidance further recommends that a statement indicating the source and related risks appear in the "Warnings" section of the labeling for HSA-containing products.

▲In case such a certification cannot be provided, tests should be performed on the raw material to demonstrate that it is TSE-free.▲ (USP 1-May-2021)

## MANUFACTURING RESIDUALS

Vaccines may contain residual amounts of any of the materials used in the manufacturing process. These materials are termed manufacturing residuals. As a general principle, it is not possible to remove a particular substance completely, nor is it possible to conclusively demonstrate that a particular substance has been completely removed. Therefore the goal is to reduce these substances to an undetectable level, using a sensitive and validated analytical methodology. Some products are tested for pyrogenic substances as a manufacturing residual (see *Pyrogen Test* ⟨151⟩); and, if the product is freeze-dried, it should be tested for residual moisture (see *Loss on Drying* ⟨731⟩). Residual levels of manufacturing materials, including inactivating agents if applicable, should be justified. The release specifications of these components are required as part of the approved license ▲and can be validated out as manufacturing experience increases.▲ (USP 1-May-2021)

## CELL-DERIVED RESIDUALS

Live attenuated bacterial vaccines are not usually subject to a high degree of post-expansion purification. However, killed bacterial component vaccines typically undergo significant purification to reduce cell-derived residuals. Common cellular components to be reduced are proteins, nucleic acids, and polysaccharides. Assays for these components are routinely conducted, if appropriate, to ensure purity. A common residual in bacterial vaccines made from gram-negative bacteria is

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lipopolysaccharide (LPS), commonly known as endotoxin. Endotoxin testing is performed during the manufacturing process for any gram-negative bacterial vaccine. In the case of gram-positive bacterial vaccines, the endotoxin testing should be conducted to ensure that no contaminants from gram-negative bacterial growth are present. Also, there must be a release specification for this residual. Two tests are currently used to detect LPS in biological products, the *Limulus* amebocyte lysate (LAL) test (see *Bacterial Endotoxins Test* ⟨85⟩) and the rabbit pyrogen test (see ⟨151⟩). The *Limulus* lysate that is used to test for bacterial endotoxin in FDA-regulated products is itself a US-licensed product. The rabbit pyrogenicity test requires the use of animals and is more difficult to perform; therefore, it is not employed to the ▲same▲ (USP 1-May-2021) extent as the LAL test.▲▲ (USP 1-May-2021)

Viral vaccine manufacturing requires cell substrates to produce the viruses, which are then taken through purification processes. Generally, killed viral vaccines are more highly purified than live attenuated ones. Depending on the method used to manufacture the vaccine, manufacturers work with the FDA to develop prudent specifications for the final vaccine. Animal-derived host cells have been used extensively in vaccine manufacturing, particularly for viral vaccines. For example, influenza and yellow fever vaccines are produced in egg allantoic fluid and chicken embryos, respectively. Mumps, measles, and some rabies vaccines are produced in chick embryo cells. The labels of these products must state that residual chicken proteins may be present in the final vaccine, and the label may indicate how much is present. Further, the label also urges practitioner caution when vaccinating a person with a known hypersensitivity to eggs.

▲Some▲ (USP 1-May-2021) hepatitis B vaccines are based on recombinant DNA-derived proteins expressed in yeast cultures. In ▲these▲ (USP 1-May-2021) cases, the labels notify health care professionals that yeast protein may be present in the vaccine and recommend that suitable precautions be exercised. In the case of live viral vaccines, considerations may be given to the reduction of cellular residual materials (e.g., host DNA, proteins). ▲▲ (USP 1-May-2021)

### ANTIBIOTIC RESIDUALS

Some antibiotics (but not penicillin) can be used in minimal amounts in the manufacturing process for viral vaccines, according to 21 CFR §610.15(c). Those that have been used include gentamicin, streptomycin, neomycin, and polymyxin B. There is no requirement for tests of residual levels of these antibiotics in the final vaccine. However, according to 21 CFR §610.61(m), the calculated amount expected to remain as a residual in the final vaccine, based on the amount added and the dilution factor in the manufacturing process, must be stated on the product label.

### INACTIVATING CHEMICAL AGENTS

Several chemical agents have been used to inactivate bacteria and viruses or to detoxify toxins in vaccine production processes. Formaldehyde and β-propiolactone are the most commonly used inactivating agents. Other less often used inactivating agents include glutaraldehyde and hydrogen peroxide. As a manufacturing residual, the inactivating agent should be removed from the final product as thoroughly as possible. The upper limit for formaldehyde is generally 0.02%, equivalent to 0.1 mg per 0.5-mL vaccine dose. The limit for β-propiolactone should be below the limit of detection.

***Change to read:***

## EVALUATING THE STABILITY OF VACCINES

The stability of vaccine products depends on the nature of the vaccine antigen, the product formulation, ▲the container–closure system,▲ (USP 1-May-2021) and the control of vaccine storage prior to use.

Vaccine products are evaluated using programs that include real-time long-term storage under prescribed conditions. The use of extreme temperatures ▲or other factors▲ (USP 1-May-2021) to potentially accelerate degradation may help manufacturers understand the stability of the product.

Vaccine products, like all pharmaceutical products, should be evaluated to define suitable conditions for storage (21 CFR §610.50 and §610.53). General principles of stability testing for biological products are described in *Quality of Biotechnological Products: Stability Testing of Biotechnological/Biological Products* ⟨1049⟩. Typically, these concerns are focused on the final vaccine product, but evaluations are also needed for bulk intermediates to justify the conditions under which they are held. In both cases, manufacturers define in advance the conditions to which the product will be exposed (e.g., temperature, light, and humidity) and the time range during which the product will be exposed to those conditions. Stability studies should evaluate all storage conditions to which the product or intermediate is likely to be exposed during production, handling, shipping, and storage so that appropriate time limits can be placed on the exposure to those conditions.

The primary criteria for defining the storage conditions for these intermediates and the final products are generally focused on acceptable maintenance of potency; however, as discussed below, there often are other attributes that need to be considered.

Evaluation of the stability of vaccine products has three general purposes. First, the products are shown to maintain an acceptable analytical profile throughout manufacture and use to preserve safety and effectiveness. Second, stability studies across several product batches provide an effective way to characterize the inherent properties of the product. This in turn leads to the third use, demonstrating manufacturing consistency in the product. ▲Higher-temperature stability studies are usually performed to generate data in support of excursions and investigations and are not expected to be performed on every batch that is placed on stability.▲ (USP 1-May-2021)

### Stability Protocols

The overall experimental plan for evaluating the stability profile of a given set of product or intermediate batches typically specifically defines the conditions under which the samples will be stored, why these conditions are relevant, the length of time

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the samples will be stored at each condition, when samples will be tested during this time course, and the analytical measurements at each time point. Additionally, these stability protocols itemize the analytical procedures to be used. For stability studies that occur early in product development, the studies may be conducted to confirm the suitability of the product formulation and/or storage conditions. Later in development, stability studies are typically conducted to provide data supporting the product ▲expiry dating▲ (USP 1-May-2021) or intermediate hold time, to provide more elaborate product characterization, and to evaluate manufacturing consistency. These latter studies define the product end–expiry specifications that allow definitions of acceptable and unacceptable product. Unacceptable product is defined as product that is no longer acceptable for use in clinical studies or for commercial use (e.g., because of degradation or loss of potency). Stability studies should be conducted over a duration sufficient to determine the point of loss of acceptable potency or other relevant parameters.

## Analytical Measurements

Manufacturers should consider the rigor of the analytical method(s) used to evaluate the stability of complex products and improve their understanding of the parameters that are critical to immunogenicity (including stability-indicator parameters). Selection of the stability-indicator parameters varies with the unique characteristics of each vaccine.

The primary parameter that reflects stability for most vaccines is the potency assay (see *Potency Tests* below). This assay can take many forms, depending on individual vaccines (e.g., an infectivity assay for a live virus vaccine or a measure of the ▲quantity▲ (USP 1-May-2021) of conjugated ▲saccharide in a glycoconjugate▲ (USP 1-May-2021) vaccine). The potency assay is generally the key analytical result predicting whether a vaccine will remain suitable for use and whether it will produce the expected clinical response. Other analytical measurements can provide important supplemental data, particularly those that have a clear link to the potency of the product. Examples include the degradation profile, dissociation of a carrier protein from conjugated vaccines, and dissociation of an adjuvant from an antigen complex. Additionally, other common assays typically are performed as part of the stability study and may address physical or chemical changes in the product that may or may not affect its potency (e.g., general safety, degree of aggregation, pH, moisture, container, preservative, and enclosure).

## Formal Evaluation of Stability Data and Product Expiry Dating

Vaccines must remain within potency specifications at the expiration date, provided that the product was stored under the normal conditions specified. Manufacturers should conduct stability studies to determine those storage conditions and that ▲expiry dating▲ (USP 1-May-2021) to demonstrate that the product remains within the potency specifications. Manufacturers should conduct stability studies on a continuing basis. If a major manufacturing process changes, additional stability studies should be conducted to verify that there is no adverse impact on the stability profile. ▲▲ (USP 1-May-2021) An accelerated study involving temperatures both higher and lower than routine can evaluate the impact of temperature excursions on products. A similar evaluation should be done for product intermediates to establish how long a given intermediate can be held under defined conditions before it is processed further or discarded.

# NOMENCLATURE

There are no uniform systems for naming new vaccines. 21 CFR §299 describes the cooperation of the FDA and the US Adopted Names Council (USAN) in naming drugs, including vaccines. USAN is a private organization sponsored by the American Medical Association, USP, and the American Pharmacists Association. Section 262 in Title 42 of the Public Health Service Act requires that each package of the biological product be plainly marked with the proper name (name designated in the license 21 CFR §600.3) of the biological product contained in the package.

***Add the following:***

# ▲CONTAINER

The final container for the drug product is typically a vial with a rubber stopper and seal, or a prefilled syringe with a glass barrel and rubber plunger. Adsorption of the drug product on the container is prevented by selecting appropriate liquid formulation components, which may include proteins or mild non-ionic detergents; they also prevent virus aggregation and promote a more consistent product. Plastic containers have been introduced recently. Stopper and plunger components are typically butyl rubber compounds, which can be polytetrafluoroethylene- or silicone-coated to minimize vaccine adsorption, leaching of extractable components, or both. Sterilization methods should be characterized to ensure that they do not alter the container material properties with respect to vaccine interactions; for example, moisture absorbed during steam sterilization may add moisture over time to lyophilized vaccines, and ionizing radiation may alter the surface chemistry of polymer compounds. All containers and product-contact components should meet USP Class VI extractables testing (see *The Biocompatibility of Materials Used in Drug Containers, Medical Devices, and Implants* ⟨1031⟩) for the formulation chemistry and sterilization methods of interest and be compliant to relevant biocompatibility testing according to the risk of the intended use.▲ (USP 1-May-2021)

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***Change to read:***

## LABELING

Vaccine product labeling is regulated in compliance with 21 CFR §201 and §610. Requirements are set for container labeling and package labeling.

### Container Label

Provisions are made for the following labels:

- Full label
- Partial label
- No label on the container itself when the containers cannot support a label that includes all required information; such products should be placed in a package that does include all required information

The label should be affixed to the container in a manner that allows visual inspection of the contents for the full length or circumference of the container. If no package exists, the container bears all of the information required for the package label.

The full container label normally contains the following:

- Proper name of the product
- Name, address, and license number of the manufacturer
- Lot number or other lot identification
- Expiration date
- Recommended individual dose, for multiple-dose containers
- The phrase “Rx only” for prescription biologicals
- ▲Storage conditions
- Instructions related to resuspension or shaking, if appropriate▲ (USP 1-May-2021)
- Any applicable cautionary statements.

### Package Label

In addition to the information required on the container label, the package label should describe the following:

- Any preservative used and its concentration, or the words “no preservative” if no preservative is used and its absence is a safety factor
- Number of containers, if more than one; or amount of product in the container, expressed as number of doses, volume, units of potency, weight, and equivalent volume (for dried product to be reconstituted); or a combination of the above to provide an accurate description of the contents, as applicable
- Recommended storage temperature
- The words “shake well”, “do not freeze”, or the equivalent, as well as other instructions when indicated by the character of the product
- Recommended individual dose, for multiple-dose containers;
- Recommended route of administration, or reference to such directions in an enclosed circular
- Presence of known sensitizing substances;
- Type of antibiotics added during manufacture and the amount calculated to remain in the final product
- Inactive ingredients, when they constitute a safety factor or are referenced to an enclosed circular
- Adjuvant, if present
- Source of the product, when this may be a factor in safe administration
- Identity of each microorganism used in manufacture and, if applicable, the production medium and the method of inactivation or reference to an enclosed circular
- Minimum potency in terms of official standard of potency, or the words “no US standard of potency”.

### Prescribing Information

Detailed information about a vaccine appears in its prescribing information, commonly called the package insert. Increasingly, vaccines are distributed with patient package inserts written in lay language. Prescribing information (21 CFR §201.56 and §201.57) includes the following:

- Highlights of prescribing information
- Product names, other required information
- Boxed warning
- Recent major changes
    - Indications and usage
    - Dosage and administration
    - Dosage forms and strengths
    - Contraindications

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- Warnings and precautions
- Adverse reactions
- Drug interactions
- Use in specific populations (e.g., pregnancy, nursing mothers, pediatric, geriatric)
- Drug abuse and dependence
- Overdosage
- Description
- Clinical pharmacology
- Nonclinical toxicology
- Clinical studies
- References
- How supplied/storage and handling
- Patient counseling information

***Change to read:***

## LOT RELEASE TESTING

### General Principles

▲Drug product is defined in 21 CFR §210.3(b)(4) as "a finished dosage form, for example, tablet, capsule, solution, etc., that contains an active drug ingredient generally, but not necessarily, in association with inactive ingredients. The term also includes a finished dosage form that does not contain an active ingredient but is intended to be used as placebo." In compliance with 21 CFR §600, *Biological products: general*, and in particular, 21 CFR §601.2, *Applications for biologics licenses; procedures for filing*, the manufacturer "shall submit data derived from nonclinical laboratory and clinical studies which demonstrate that the manufactured product meets prescribed requirements of safety, purity, and potency…" Documentation of the vaccine drug product includes the following: components used in manufacture; composition of the drug product; specification for each component; manufacturing and packaging procedure and in-process controls for the drug product; and specifications necessary to ensure identity, strength, quality, purity, and potency in accordance with 21 CFR §211, *Current good manufacturing practice for finished pharmaceuticals*.▲ (USP 1-May-2021)

Manufacturers perform all appropriate tests for the licensed specifications for the product, according to 21 CFR §610.1 and §610.2. ▲Some tests that are performed on the final bulk need not be repeated on filled vials (e.g., when one final bulk is used to fill multiple batches of filled vials).▲ (USP 1-May-2021) Samples of each licensed lot and protocols ▲for vaccines▲ (USP 1-May-2021) containing ▲all appropriate tests from▲ (USP 1-May-2021) the manufacturers' test results are submitted to the FDA ▲for review, testing, or both.▲ (USP 1-May-2021) After the FDA evaluates the protocol to ensure that the product specifications are met, and after satisfactory confirmatory testing, the FDA approves the release of the lot if all tests meet the standards of safety, purity, and potency established for the particular vaccine product. After approval is granted, the manufacturer distributes and markets ▲that lot of product. The lot release protocol for each vaccine includes the specific potency test, as well as common tests, such as the visual inspection of final vials, and safety, sterility, and purity testing for each lot.▲ (USP 1-May-2021)

Guidelines are available regarding alternatives to lot release and a surveillance system. All of these variations are subject to the regulations in 21 CFR §610.2 that allow the FDA to require that samples of any lot of licensed product (e.g., vaccine), together with the protocols showing results of applicable tests, be sent to the FDA.

### Common Tests

The tests common to all lots of all products include tests for potency, general safety, sterility, purity, identity, and constituent materials. The manufacturer completes these tests for conformity with standards applicable to each product. The results of all tests are considered, except when a test has been invalidated as a result of causes unrelated to the product (21 CFR §610.1).

#### POTENCY TESTS (VACCINE-SPECIFIC)

The basic definition and requirements for vaccine potency and potency assays are provided in 21 CFR §600.3 and §610.10. A vaccine potency assay should indicate the therapeutic activity of the drug product as indicated by appropriate laboratory tests or by adequately developed and controlled clinical data. Potency may be expressed in terms of units by reference to a standard ▲("relative potency").▲ (USP 1-May-2021) Product potency tests vary with vaccine product types (e.g., viral, bacterial, live attenuated, inactivated, or polysaccharide). As a result, potency assays for vaccines ▲are based on a wide range of analytical approaches.▲ (USP 1-May-2021) In vitro potency tests for live virus may include plaque formation assays, endpoint dilution assays [e.g., the tissue culture infective dose ($TCID_{50}$)], virus neutralization assays, or quantitative polymerase chain reaction (PCR) assays. Quantitative colony formation assays are used for live attenuated bacterial vaccines. Animal challenge tests for immunogenicity assays of potency, such as those for diphtheria and tetanus (US Department of Health, Education, and Welfare, 1953; see ▲*Appendix*),▲ (USP 1-May-2021) or rabies and anthrax show in vivo response. ▲Antigen quantification▲ (USP 1-May-2021) assays use enzyme-linked immunosorbent assays (ELISA; e.g., with hepatitis A) or rate nephelometry and rocket immunoelectrophoresis (e.g., with pneumococcal polysaccharides). The potency tests for bacterial vaccines, such as the meningococcal polysaccharides, pneumococcal polysaccharides, or *Haemophilus b* protein conjugate vaccines use chemical and physical chemical assays. In the case of pure polysaccharide vaccines, the concentration or quantity of the vaccine component (polysaccharide) and its quality (e.g., size) have been shown to be indicative of the human immune response.

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Assay precision and reproducibility vary with the different methodologies that are used in potency assays, ranging from the high accuracy and precision of chemical tests at one end of the spectrum to bioassays at the other end. *Design and Analysis of Biological Assays* ⟨111⟩ provides guidance for bioassays and applies to vaccine potency assays. Other tests should be validated as described in *Validation of Compendial Procedures* ⟨1225⟩.

### RELEASE TESTS

Official release of vaccines by the vaccine regulatory authority may be based on either the bulk or the final container. It is highly desirable to perform potency tests on the final container. However, under certain circumstances this may not be practical or even possible ▲(e.g., for some combination or multivalent vaccines);▲ (USP 1-May-2021) thus, a case-by-case approach ▲is sometimes▲ (USP 1-May-2021) required. The choice of whether to test the bulk or the final container derives from a number of considerations, such as the quantity of vaccine available for tests at the different manufacturing stages. For certain vaccines, both bulk and final container receive official release.▲▲ (USP 1-May-2021)

### GENERAL SAFETY

For biological products that are intended for administration to humans, manufacturers perform a general safety test in order to detect any extraneous toxic contaminants. Procedures and exceptions are specified in 21 CFR §610.11.

### STERILITY

A sterility test of each lot of each product is conducted according to procedures described in ⟨71⟩ and 21 CFR §610.12 for ▲▲ (USP 1-May-2021) bulk and▲/or▲ (USP 1-May-2021) final container material▲, as appropriate.▲ (USP 1-May-2021)

### BACTERIAL ENDOTOXINS

Each lot of final containers of a vaccine intended for use by injection is tested for bacterial endotoxins ▲when appropriate,▲ (USP 1-May-2021) as indicated in ⟨85⟩.

### PURITY

Vaccines need to be ▲as▲ (USP 1-May-2021) free of extraneous material ▲as possible.▲ (USP 1-May-2021) Approved vaccine license applications indicate extraneous materials that are unavoidable in the manufacturing process for a specific product. The application may indicate test results and allowable limits for such materials, according to procedures described in 21 CFR §610.13.

### RESIDUAL MOISTURE

Each lot of dried product is tested for residual moisture [see 21 CFR §610.13 (a), ⟨731⟩, and FDA's *Guideline for the Determination of Residual Moisture in Dried Biological Products* (see ▲*Appendix*)].▲ (USP 1-May-2021)

### PYROGENS

Each lot of final containers of a vaccine intended for use by injection is tested for pyrogenic substances, as indicated in ⟨151⟩ ▲as appropriate,▲ (USP 1-May-2021) and 21 CFR §610.13(b).

### IDENTITY

The contents of a final container of each filling of each lot are tested for identity after labeling is completed. Identity is established by the physical or chemical characteristics of the vaccine, inspection by macroscopic or microscopic methods, specific cultural tests, or in vivo or in vitro immunological tests. In large part, identity tests are performed to distinguish the subject vaccine from other materials manufactured at the same site (21 CFR §610.14).

### CONSTITUENT MATERIALS

Ingredients, preservatives, diluents, adjuvants, extraneous protein, cell culture-produced vaccines, and antibiotics are tested according to 21 CFR §610.15.

## Permissible Combinations

Formulations that combine several vaccines must be licensed as combinations (21 CFR §610.17). The potency of each vaccine in the combination is individually tested and must meet the specifications in the context of the final combined product; other appropriate quality tests also apply. For vaccines that are physically combined in clinical locations just before administration to a patient, prescribing information should describe specific procedures to follow in those settings.

## Quality

In general, quality control systems for vaccine manufacture are identical to those routinely employed for the production of other pharmaceuticals. These include raw material testing and release, manufacturing, process-control documentation, and

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aseptic processing. Manufacturers formally assign responsibility to designated staff for maintaining the continued safety, purity, and potency of the product and for ensuring compliance with applicable product and establishment standards, along with compliance with cGMPs. Analysts use reference standards and validated methods to determine active ingredients, residuals, and impurities. Manufacturers determine product safety in a variety of ways that may include the use of experimental animals, procedures to demonstrate product sterility, and tests to ensure product potency. The complexity of the quality control systems for vaccines lies in the variety of methods used to produce and control production. Lot release testing proceeds according to 21 CFR §610.2 and involves evaluating lots for safety, purity, and potency before release. Manufacturers follow FDA and applicable international standards for testing and validation. The basic considerations for validation are included in ⟨1225⟩, in addition to guidance documents issued by the FDA and the ICH (see ▲*Appendix*).▲ (USP 1-May-2021)

## Alternative Tests

Modification of test methods or manufacturing processes as licensed may be permitted if the regulatory authority can be assured that the modifications cause no reduction in the safety, purity, potency, and effectiveness of the biological product. It may be necessary for the manufacturer to file the proposed changes prior to implementation (21 CFR §601.12 and 21 CFR §610.9).

***Add the following:***

## ▲RETENTION SAMPLES AND OTHER REQUIREMENTS

Retention samples are held by the manufacturer for at least 6 months after the expiration date. An adequate quantity of material from each lot of each product is held for examination and testing for safety and potency (see 21 CFR §600.13).

Records are maintained concurrently with each step in the manufacture and distribution of the product such that at any time successive steps of manufacture and distribution may be traced (see 21 CFR §600.12).

For storage conditions, see 21 CFR §610.50 and §610.53.

For shelf life/expiry date, see 21 CFR §610.50 and §610.53.▲ (USP 1-May-2021)

***Change to read:***

## GLOSSARY

**Acceptance criteria:** The product specifications and acceptance or rejection criteria, with an associated sampling plan, necessary for making a decision to accept or reject a lot or batch (or any other convenient subgroups of manufactured units).

**▲Adjuvant:** A component added to a vaccine which, although not pharmacologically active in its own right, enhances or modifies the patient's response to the vaccine immunogens.▲ (USP 1-May-2021)

**Adventitious agent:** A microorganism (e.g., bacteria, fungi, mycoplasma, spiroplasma, mycobacteria, rickettsia, viruses, protozoa, parasites, TSE agent) that is inadvertently introduced into the production of a biological product.

**▲Ancillary raw material:** Ancillary raw materials are components or reagents used during the manufacture of vaccines and biologicals that exert an effect on the product but are not intended to be part of the final product.▲ (USP 1-May-2021)

**Batch:** A specific quantity of a drug or other material intended to have uniform character and quality, within specified limits, and produced according to a single manufacturing order during the same cycle of manufacture.

**Biological product:** Any virus, therapeutic serum, toxin, antitoxin, or analogous product applicable to the prevention, treatment or cure of diseases or injuries of man.

▲▲ (USP 1-May-2021)

**Characterization:** Determination of the properties of a substance.

**Component:** Any ingredient intended for use in the manufacture of a drug product, including those that may not appear in such drug product.

**Container** (also final container)**:** The immediate unit, bottle, vial, ampule, tube, or other receptacle containing the product as distributed for sale, barter, or exchange.

**Control:** Having responsibility for maintaining the continued safety, purity, and potency of the product and for compliance with applicable product and establishment standards, and for compliance with current GMPs.

**Control cells:** Cells that are split off from the production culture and maintained in parallel under the same conditions and using the same reagents (e.g., culture medium) to perform quality control tests on cells that have not been exposed to the vaccine virus (which may interfere with some tests).

**▲Expiry dating:**▲ (USP 1-May-2021) The period beyond which the product cannot be expected beyond reasonable doubt to yield its specific results.

▲▲ (USP 1-May-2021)

**Drug product:** A finished dosage form (e.g., solution, suspension) that contains an active drug ingredient generally in association with inactive ingredients ▲in the final container–closure.▲ (USP 1-May-2021)

**▲Excipient:** Excipients are substances other than the active pharmaceutical ingredients (API) that are appropriately evaluated for safety and enable the drug substance to be delivered to the patient in the right form and supports the way and place of action without being active themselves.▲ (USP 1-May-2021)

**Expiration date:** The calendar month and year, and where applicable, the day and hour, that the ▲expiry dating▲ (USP 1-May-2021) ends.

**Filling:** A group of final containers, identical in all respects, that have been filled with the same product from the same bulk lot without any change that will affect the integrity of the filling assembly.

**Final bulk:** The stage of vaccine production directly prior to filling of individual vials.

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**Free of and freedom from:** For a substance to be considered free of a contaminant, an assay must demonstrate that a defined quantity of the substance is negative for that contaminant to a defined level of sensitivity. The level of assay sensitivity is defined by the choice of assay and can be determined experimentally using standardized reagents. Alternatively, a validated process that is known to remove a contaminant to a defined level may be used to demonstrate freedom from that contaminant.

**Harvest:** Collection of material at the end of vaccine virus propagation in cell culture, from which vaccine will be prepared. This material may be the culture supernatant, the cells themselves (often in disrupted form), or some combination thereof.

**Inactive ingredient:** Any component other than an active ingredient ▲or an adjuvant.▲ (USP 1-May-2021)

**In-process material:** Any material fabricated, compounded, blended, or derived by chemical reaction that is produced for, and used in, the preparation of the drug product.

**Intermediates:** Unformulated active ingredients that are processed before final formulation and can be stored ▲▲ (USP 1-May-2021) before further processing.

**Label:** Any written, printed, or graphic matter on the container or package or any such matter clearly visible through the immediate carton, receptacle, or wrapper.

▲▲ (USP 1-May-2021)

**Lot:** A batch, or a specific identified portion of a batch, having uniform character and quality within specified limits; or, in the case of a drug product produced by continuous process, a specific identified amount produced in a unit of time or quantity in a manner that assures its having uniform character and quality within specified limits.

**Lot number, control number, or batch number:** Any distinctive combination of letters, numbers, or symbols, or any combination thereof, from which the complete history of the manufacture, processing, packing, holding, and distribution of a batch or lot of drug product or other material can be determined.

**Manufacture:** All steps in the propagation or manufacture and preparation of products including, but not limited to, filling, testing, labeling, packaging, quality control, and storage by the manufacturer.

**Manufacturer:** Any legal person or entity engaged in the manufacture of a product subject to license under the Public Health Service (PHS) Act. Also includes any legal person or entity who is an applicant for a license where the applicant assumes responsibility for compliance with the applicable product and establishment standards.

▲▲ (USP 1-May-2021)

**Master virus seed:** A viral seed of a selected vaccine virus from which all future vaccine production will be derived, either directly or via working virus seeds.

▲▲ (USP 1-May-2021)

**Package:** The immediate carton, receptacle, or wrapper, including all labeling matter therein and thereon, and the contents of the one or more enclosed containers. If no package is used, the container will be deemed to be the package.

▲▲ (USP 1-May-2021)

**Potency:** The therapeutic activity of the drug product as indicated by appropriate laboratory tests or by adequately developed and controlled clinical data. Potency may be expressed in terms of units by reference to a standard.

▲▲ (USP 1-May-2021)

**Process:** A manufacturing step performed on the product itself that may affect its safety, purity, or potency, in contrast to manufacturing steps that do not intrinsically affect the safety, purity, or potency of the product.

**Proper name:** The name, designated in the license, to be used on each package of the product.

**Purity:** Relative freedom from extraneous matter in the finished product, whether or not harmful to the recipient or deleterious to the product. Purity includes, but is not limited to, relative freedom from residual moisture or other volatile substances and pyrogenic substances.

**Qualification:** Determination of the suitability of a material for manufacturing based on its characterization.

▲**Residual impurity:** Residual impurities are unwanted chemicals or biologicals present in traces in the final product and arise from a normal manufacturing process. They have no therapeutic value and are potentially harmful. Therefore, they need to be controlled during the manufacturing process.▲ (USP 1-May-2021)

**Safety:** The relative freedom from harmful effect to persons affected, directly or indirectly, by a product when prudently administered, taking into consideration the character of the product in relation to the condition of the recipient at the time.

**Specification:** The quality standard (i.e., tests, analytical procedures, acceptance criteria) provided in an approved application to confirm the quality of products, intermediates, raw materials, reagents, components, in-process materials, container–closure systems, and other materials used in the production of a product.

**Standards:** Specifications and procedures applicable to an establishment or to the manufacture or release of products, which are prescribed in this chapter or established in the biologics license application and designed to ensure the continued safety, purity, and potency of such products.

**Sterility:** Freedom from viable contaminating microorganisms, as determined by tests prescribed by the FDA.

▲▲ (USP 1-May-2021)

**Unacceptable product:** Product that is no longer acceptable for use in clinical studies or for commercial use (e.g., because of degradation or loss of potency).

**Validation:** The performance characteristics of an analytical procedure, based on the demonstration that the procedure is suitable for its intended purpose or use. Validation of a process is the determination of the extent to which a process meets the requirements for the various performance characteristics and the demonstration that the process uniformly performs to defined characteristics. Validation is generally performed in accordance with ⟨1225⟩ and the relevant ICH guidelines.

**Viral clearance:** The combination of the physical removal of viral particles and the reduction of viral infectivity through inactivation.

**Virus seed or viral seed:** A live viral preparation of uniform composition (not necessarily clonal) derived from a single culture process, aliquoted into appropriate storage containers, and stored under appropriate conditions.

▲▲ (USP 1-May-2021)

**Working virus seed:** A viral seed derived by propagation of virus from the master virus seed under defined conditions and used to initiate production cell cultures lot-by-lot.

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## ▲APPENDIX▲ (USP 1-MAY-2021)

### Selected Regulatory Documents

- ▲Food and Drug Administration. US Code of Federal Regulations.
  - 21 CFR §201 (Labeling)
  - 21 CFR §299 (Drugs; official names and established names)
  - 21 CFR §600 (Biological products: general)
  - 21 CFR §610 (General biological products standards)
  - 42 USC §262 (Regulation of biological products)▲ (USP 1-May-2021)
- Food and Drug Administration. Guidance for industry. Revised preventive measures to reduce the possible risk of transmission of Creutzfeldt-Jakob disease (CJD) and variant Creutzfeldt-Jakob disease (vCJD) by blood and blood products. Rockville, MD: Food and Drug Administration; January 2002. ▲https://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Blood/UCM307137.pdf.▲ (USP 1-May-2021)
- Food and Drug Administration. Guideline for the determination of residual moisture in dried biological products; January 1990.▲▲ (USP 1-May-2021)
- Food and Drug Administration. Guidance on alternatives to lot release for licensed biological products. *Federal Register*. 1993;58(137):38771–38773. http://www.fda.gov.
- Food and Drug Administration. Guidance for industry. Characterization and qualification of cell substrates and other biological starting materials used in the production of viral vaccines for the prevention and treatment of infectious diseases. Rockville, MD: Food and Drug Administration; September 2006. ▲https://www.fda.gov/regulatory-information/search-fda-guidance-documents/characterization-and-qualification-cell-substrates-and-other-biological-materials-used-production.▲ (USP 1-May-2021)
- Department of Health, Education, and Welfare (now the National Institutes of Health). *Minimum Requirements for Immune Serum Globulin (Human).* 3rd rev. Bethesda, MD: Department of Health, Education, and Welfare, 1953.
- International Council for Harmonisation. Q2(R1) Validation of analytical procedures: text and methodology. ▲Geneva, Switzerland: ICH, 2005. http://www.ich.org/products/guidelines/quality/quality-single/article/validation-of-analytical-procedures-text-and-methodology.html.
- Food and Drug Administration. Guidance for industry. Pyrogen and endotoxins testing: questions and answers. Rockville, MD: Food and Drug Administration; June 2012.
- Food and Drug Administration. Guidance for industry: sterile drug products produced by aseptic processing—current good manufacturing practice. Rockville, MD: Food and Drug Administration; 2004. https://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM310098.pdf.
- Food and Drug Administration. Guidance, compliance & regulatory information (biologics). https://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/default.htm.
- Food and Drug Administration. Vaccine and related biological product guidances. https://www.fda.gov/vaccines-blood-biologics/biologics-guidances/vaccine-and-related-biological-product-guidances.
- Food and Drug Administration. Vaccines listed for use in the United States. http://www.fda.gov/BiologicsBloodVaccines/Vaccines/ApprovedProducts/ucm093833.htm.
- International Council for Harmonisation (ICH) website. http://www.ich.org.▲ (USP 1-May-2021)

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# ⟨1236⟩ SOLUBILITY MEASUREMENTS

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- Bovine Simulated Ruminal Fluids

GLOSSARY
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## INTRODUCTION[1]

Solutes may differ in both the extent and the rate at which they dissolve in a solvent. Solubility is the capacity of the solvent to dissolve a solute whereas dissolution rate is how quickly the solubility limit is reached. Equilibrium solubility is the concentration limit, at thermodynamic equilibrium, to which a solute may be uniformly dissolved into a solvent when excess solid is present. The apparent solubility may be either higher or lower than the equilibrium solubility due to transient supersaturation or incomplete dissolution due to insufficient time to reach equilibrium. Equilibrium can be defined as sufficiently converged when it no longer changes significantly during a certain time frame. Solubility may be stated in units of concentration such as molality, molarity, mole fraction, mole ratio, weight/volume, or weight/weight.

Solubility can be expressed in absolute as well as relative terms. One method of describing the absolute solubility is the descriptive solubility defined in *General Notices, 5.30 Description and Solubility*. Relative measures of the solubility are important for predicting the drug delivery characteristics of a dosage form and characterizing a drug as either high solubility or low solubility in the biopharmaceutics classification system (BCS) (*1*).

Accurate determination of the solubility of pharmaceutical materials is important for understanding both quality control and drug delivery issues for pharmaceutical formulations. The apparent solubility (see the *Glossary*) of a material is affected by the physicochemical properties of the material (e.g., surface area, particle size, crystal form), the properties of the solubility media (e.g., pH, polarity, surface tension, added surfactants, co-solvents, salts), and the control of the solubility measurement parameters (e.g., temperature, time, agitation method). Additionally, the apparent solubility may be comprised of the intrinsic solubility of the uncharged moiety, the solubility of the ionized compound, and the effect of solubilizers and multiple crystal forms or salt forms. Control of these experimental factors during solubility measurements is key to obtaining accurate, reliable values for the equilibrium solubility of a material.

This chapter will begin with a discussion of the concepts and equations that are relevant to solubility measurements. Understanding these relationships is fundamental to accurate evaluation of solubility. This will be followed by a brief description of typical experimental methods used to assess solubility of pharmaceutical materials. Finally, the use of solubility measurements to obtain biorelevant solubility (for human products) and species-dependent solubility (for veterinary products) will be discussed.

[1] The terms used in this chapter are defined in the *Glossary*.

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# BACKGROUND

## Thermodynamic Equilibrium and Solubility

Dissolution of a crystalline solid solute can be modeled by the two-step process of melting the crystal into a pure liquid solute followed by mixing the liquid solute into the solvent. The Gibbs free energy of mixing determines whether, and to what extent, two compounds mix to form a homogeneous phase.

$$\Delta G_{mix} = \Delta H_{mix} - T\Delta S_{mix}$$

$\Delta G_{mix}$ = Gibbs free energy of mixing
$\Delta H_{mix}$ = enthalpy of mixing; indicates if the mixing is an endothermic or exothermic process
$T$ = temperature in degrees Kelvin
$\Delta S_{mix}$ = change in entropy (disorder) that results from mixing

If the change in the Gibbs free energy is negative, the mixing will be thermodynamically favored. When equilibrium is reached, $\Delta G$ will be equal to zero. The enthalpy of mixing is due to the breaking of cohesive interactions (solute–solute, solvent–solvent) and the creation of adhesive interactions (solute–solvent). In other words:

$$\Delta H_{mix} = H_{UU} + H_{VV} - H_{UV}$$

$\Delta H_{mix}$ = enthalpy of mixing
$U$ = solute
$V$ = solvent

This enthalpy change is also equivalent to the work done by removing a volume of pure solvent and a volume of pure solute and exchanging them. The enthalpy change is equal to the newly created interfacial surface energy according to:

$$\Delta H_{mix} = \gamma_{UV}A_U = \sum_i \gamma_{iV}A_i$$

$\gamma_{UV}$ = solute-solvent interfacial surface tension
$A_U$ = interfacial surface area
$\gamma_{iV}$ = group-solvent interfacial surface tension for group $i$
$A_i$ = surface area of group $i$

This equation also shows how the total surface energy can be broken into $i$ smaller groups each with its own surface area, $A_i$, and corresponding group-water interfacial tension $\gamma_{iV}$.

For an ideal system, $\Delta H_{mix}$ is zero because the interactions between the ideal solute and ideal solvent are identical. The entropy of mixing for an ideal solution always increases as a result of mixing and is given by:

$$\Delta S_{mix}^{ideal} = R(X_U \ln X_U + X_V \ln X_V)$$

which under dilute conditions can be simplified to:

$$\Delta S_{mix}^{ideal} = R \ln X_U$$

For an ideal system under dilute conditions when a crystalline solid is in equilibrium with a saturated solution of the same solute:

$$\Delta G_U^{ideal} = -T\Delta S_U^{ideal} = -RT \ln X_U$$

$$\ln X_U = \frac{\Delta G_m}{RT}$$

$R$ = gas constant
$X_U$ = concentration of the solute expressed as a mole fraction
$\Delta G_m$ = the free energy change from melting the crystalline solid

Which illustrates how the solubility of the substance can be correlated with the melting point. For a real solution, the solute may also affect (reduce) the disorder in the solvent by inducing structure to the solvent. Therefore, for a real solution, this yields:

$$\Delta G_{mix}^{real} = \sum_i \gamma_{iV}A_i - T(\sum_i h_i A_i + \Delta S_{mix}^{ideal})$$

$h_i$ = entropic effect on solvent of group $i$ with area $A_i$

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This model allows the total solute surface area ($A_U$) to be broken into smaller pieces ($\sum_{Ai}$) and the contribution of these groups to the enthalpic and entropic contributions to the free energy can be estimated.

## Methods of Estimating Aqueous Solubility

Yalkowsky demonstrated (*2*,*3*) that a relatively simple general solubility equation (GSE) may be used to empirically estimate the intrinsic solubility of compounds in water.

$$\log S_0 = 0.5 - 0.01(MP - 25) - \log K_{OW}$$

$S_0$ = intrinsic solubility (of the unionized molecule)
$MP$ = melting point of the crystalline solid (in degrees Celsius)
$K_{OW}$ = octanol–water partition coefficient; the temperature of the water is 25°

The GSE indicates that the aqueous solubility will be reduced for compounds with a higher melting point and compounds with a higher tendency to partition into an oil phase (octanol). The logarithm of the octanol–water partition in the GSE accounts for the difference between an ideal solution and an aqueous solution due to the enthalpy of mixing (*3*). The GSE can also be used to predict the solubility of ionizable compounds by combining it with the Henderson–Hasselbalch equation if the p$K_a$ is known (see *Effect of pH*).

Use of GSE requires the measurement of the melting point and partition coefficient (and p$K_a$ for ionizable compounds). There are several computer programs that will support the estimation of the partition coefficient and the p$K_a$ for compounds based on structure (*4*), but this is not the case for melting points. Efforts to develop computational methods to predict aqueous solubilities have relied on training sets of molecules to search for correlations with properties that can be more easily predicted from the structure (e.g., molecular weight, solvent-accessible surface area, number of rotatable bonds, etc.) (*5*). The success of these computational approaches is often limited to molecules that are similar to the training set. These calculational methods are adequate for providing assistance in prescreening synthetic candidates, but are not sufficiently accurate to substitute for experimental solubility.

## Factors that Affect Solubility and Solubility Measurements

### EFFECT OF pH

The solubility of ionizable acids and bases is pH dependent because the charged species have a higher affinity for the aqueous environment than the neutral form. The total solubility of the ionizable acid or base is the sum of the intrinsic solubility and the amount of ionized solute present at that pH. The Henderson–Hasselbalch equation relates the increase in the solubility to the pH of the solution relative to the p$K_a$ (acidic) or p$K_a$ (basic) of the ionizable acid or base.

$$\mathrm{pH} = \mathrm{p}K_a + \log\frac{[A^-]}{[HA]};\ S_{tot} = S_0\left(1 + 10^{\left(\mathrm{pH} - \mathrm{p}K_a\right)}\right)$$

p$K_a$ = –log($K_a$)
$K_a$ = acid dissociation constant
$[A^-]$ = molar concentration of the acid's conjugate base
$[HA]$ = molar concentration of the undissociated weak acid
$S_{tot}$ = total solubility of weak acid
$S_0$ = intrinsic solubility of uncharged moiety

$$\mathrm{pH} = \mathrm{p}K_a + \log\frac{[B]}{\left[BH^+\right]};\ S_{tot} = S_0\left(1 + 10^{\left(\mathrm{p}K_a - \mathrm{pH}\right)}\right)$$

p$K_a$ = –log($K_a$)
$K_a$ = base dissociation constant
$[B]$ = molar concentration of the base's conjugate base
$[BH^+]$ = molar concentration of the dissociated base
$S_{tot}$ = total solubility of weak base
$S_0$ = intrinsic solubility of uncharged moiety

The Henderson–Hasselbalch equation helps explain the increase in solubility at the first p$K_a$, but is not useful for modeling the behavior of polyprotic acids over a pH range incorporating additional p$K_a$ values. Because ionizable molecules can differ in the number and type of ionizable groups, it is important to explore solubility across a range of pH values. *Figure 1* illustrates this pH dependence of the solubility for a molecule with two ionization constants of 5.6 and 11.7. The molecule is charged below pH 5.6 and above 11.7 and is neutral between these two pH values. Where the molecule is unionized, the solubility is equal to the intrinsic solubility. For the ionized molecule, the solubility increases on a log scale as the pH changes. The formation of a salt may limit the solubility at a low or high pH (see *Figure 1*). If the acid used to adjust the pH contributes the counter-ion for the salt, the common-ion effect will further suppress the solubility as the counter-ion concentration is increased (see *Figure*

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*1*). If the salt is dissolved at higher pH, the salt may supersaturate the solution initially, but will eventually precipitate as whatever solid form has lower solubility at that pH (*6*).

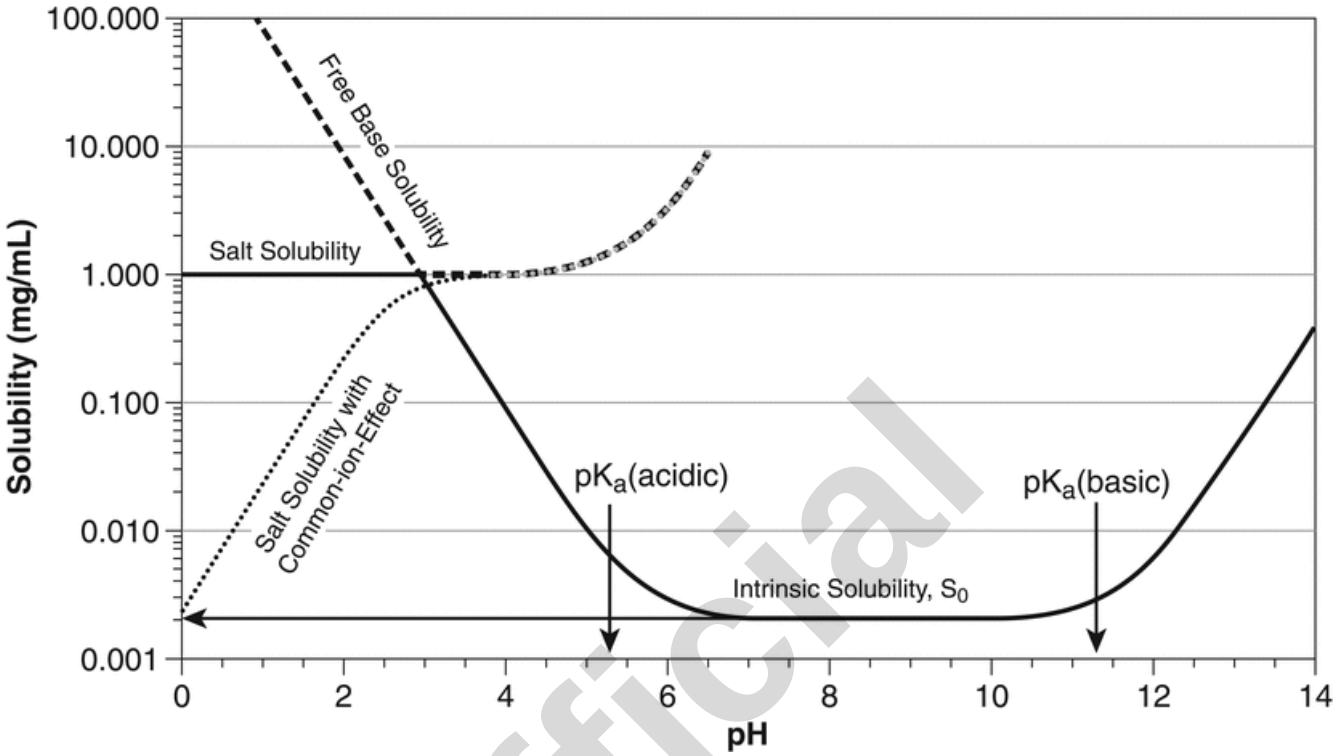


Figure 1. Effect of pH on solubility for an ionizable compound. When the molecule is unionized, the solubility is equal to the intrinsic solubility. For the ionized molecule, the solubility increases on a log scale as the pH changes. The salt solubility limits the solubility at a low pH. If the acid used to adjust the pH contributes the counter-ion for the salt, the common-ion effect will further suppress the solubility as the counter-ion concentration is increased (apparent at pH <2 in the figure).

## EFFECT OF SALTS AND COUNTER-IONS

Ionizable compounds can also form salts with an oppositely charged counter-ion (*6*). In a solution, in the presence of the charged counter-ion, the solubility product describes this equilibrium reaction as follows:

For the salt dissolution, $A_nB_m(s) \rightarrow nA^+(aq) + mB^-(aq)$
solubility product, $K_{sp} = [A^+]^n[B^-]^m$

The maximum solubility of the salt in a solution is also illustrated in *Figure 1*. As a result of the formation of the salt, the actual solubility of the charged molecule is seen to plateau (at a pH below the p$K_a$ of the drug in this example) instead of continuing to increase as predicted by the Henderson–Hasselbalch equation. Because the solubility product, $K_{sp}$, is a constant, the solubility of the ionizable moiety may drop even further if the acid being used to adjust the pH increases the concentration of the oppositely charged counter-ion. The reduction in the solubility of the charged molecule as the counter-ion concentration is increased is referred to as the common-ion effect (*6*). This is frequently seen when hydrochloric acid (HCl) is used to reduce the pH, and the solubility of the chloride salt is reduced due to increasing chloride concentration (e.g., at pH <2). Although not illustrated in *Figure 1*, salts may also limit the solubility on the basic side of the plot (e.g., a sodium salt of an acid moiety), and the common-ion effect may similarly affect the solubility at a high pH when the compound used to adjust the pH has a common-ion (e.g., sodium hydroxide).

## EFFECT OF CO-SOLVENTS

Water is often a poor solvent for many pharmaceutical ingredients, but water is miscible with other solvents that may provide good solubility for these substances (e.g., ethanol, propylene glycol, polyethylene glycol, etc.). According to the log-linear model (*2*), the log *S* of the solute can generally be linearly interpolated between two miscible co-solvents. This relationship is illustrated in *Figure 2*. When this solubility plot is switched to a linear scale, it becomes evident that even low concentrations of the poor solvent (typically water) in the co-solvent mixture can dramatically reduce the solubility for the solute. For this reason, solutions containing co-solvents are particularly prone to precipitation when diluted due to the significant change in solubility. [NOTE—This simple model, as depicted in *Figure 2*, assumes that maximum solubility occurs at 100% of the good solvent and this may not be the case for all co-solvent systems.]

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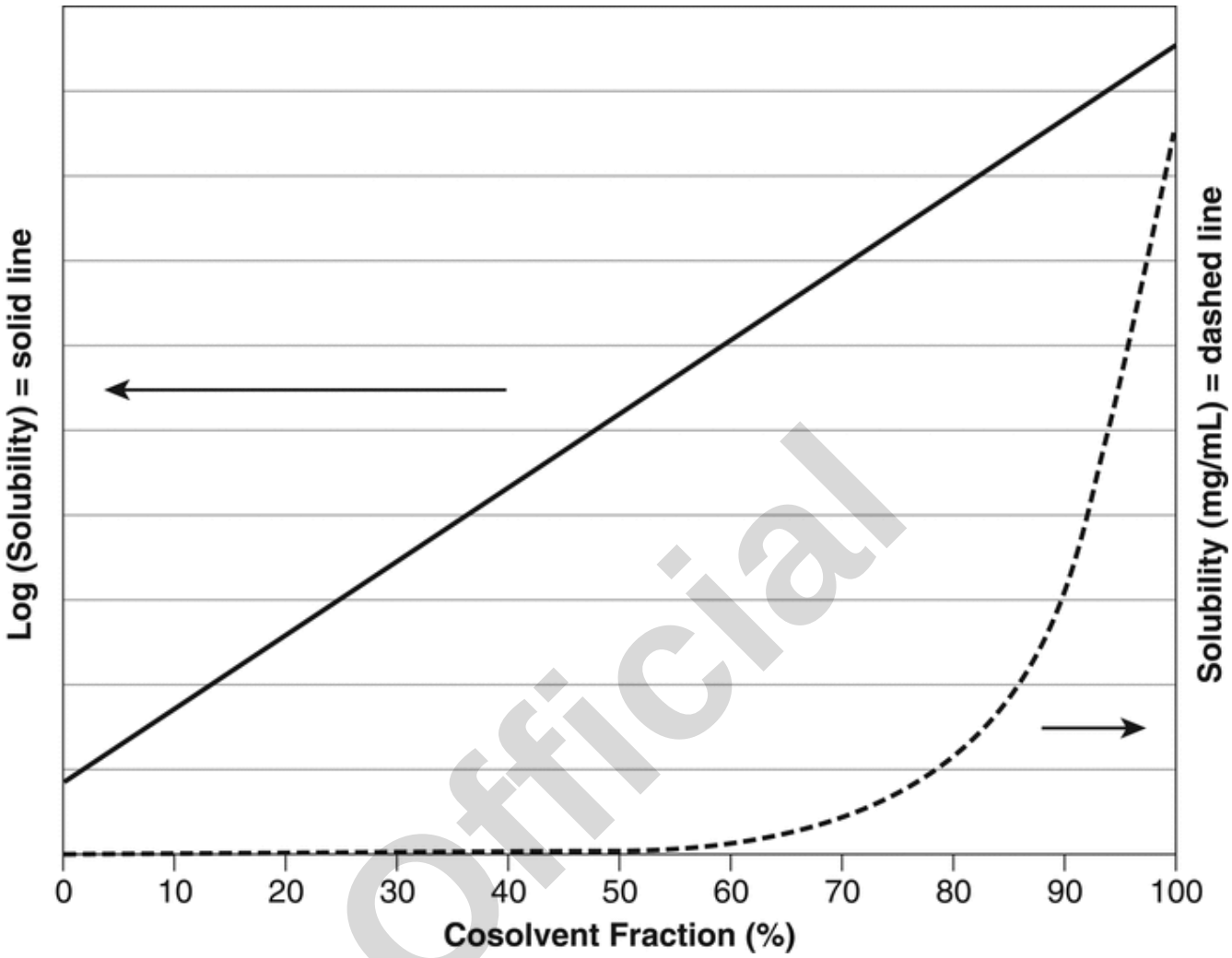


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Figure 2. Illustration of the log-linear model of Yalkowsky and co-workers (*2*). The log of solubility appears to be linear as a function of the co-solvent fraction. When plotted on a linear scale, it is apparent that the solubility drops exponentially as the poor solvent is added to the better solvent.

## EFFECT OF SURFACTANTS

Surfactants are amphiphiles, which are characterized by polar and nonpolar regions. When placed in water, a surfactant prefers to reside at the air-water interface and orients its polar region in water and its nonpolar region to the less polar interface (air). When the air-water interface is saturated with adsorbed surfactant, additional surfactant molecules aggregate into a spherical micelle with a polar surface and a nonpolar core. This point when micelles form is known as the critical micelle concentration (CMC). Above the CMC, the number of micelles in a solution increases linearly as the concentration of surfactant increases. If a pharmaceutical material is able to partition into the micelle, its solubility will increase linearly as the number of micelles increases (see *Figure 3*). The CMC for surfactants is dependent on several factors including temperature, ionic strength, and pH. As an example, the CMC for sodium lauryl sulfate is 6 mM, and the CMC for polysorbate 80 is 0.012 mM, in pure water at 25°. The solubilization of a molecule by a surfactant can be evaluated based on two descriptors: the molar solubilization capacity, and the micelle-water partition coefficient. The micelle-water partition coefficient is the ratio of drug concentration in the micelle to the drug concentration in water for a particular surfactant concentration (*7*).

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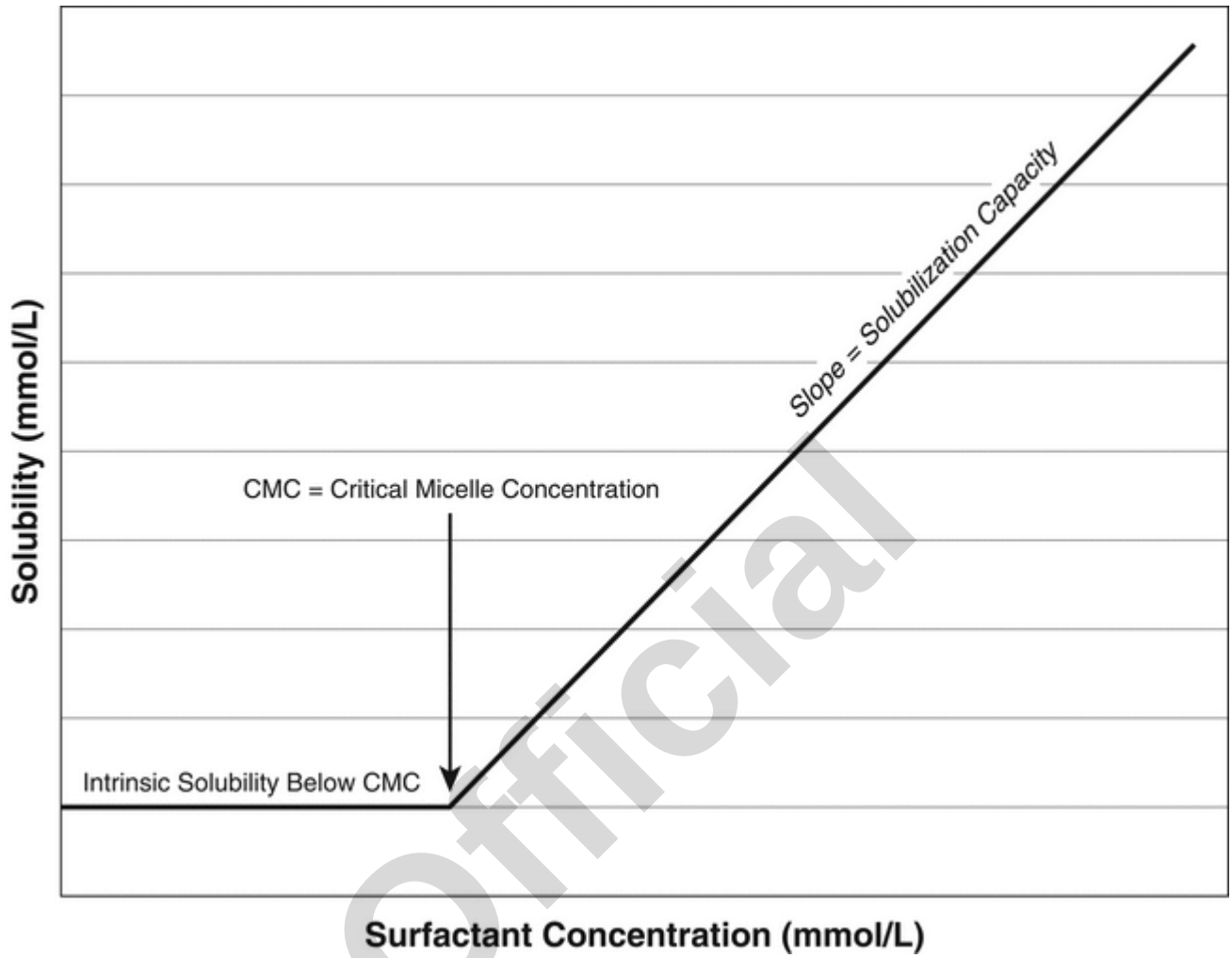


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Figure 3. Solubility enhancement by surfactants. Solubilization requires the formulation of micelles. Below the CMC, added surfactant results in monomers of surfactant dissolved in the solvent and no solubility enhancement occurs. Above the CMC, the solubility increases linearly. The slope of this linear increase indicates the solubilization capacity of the micelles.

As illustrated in *Figure 3*, the solubility in the presence of surfactants is the additive combination of the amount dissolved in the aqueous phase plus the amount solubilized by the micelles. The micelles will be larger than the solute and will diffuse more slowly than the solute. Drug delivery in the presence of micelles will be due to the absorption of the free drug in solution as well as drug delivery by micelle-mediated transport (*5*,*6*). Therefore, solubilization by surfactants may not result in an enhancement in drug delivery that is directly proportional to the increase in aqueous solubility (*5*,*6*).

## EFFECT OF COMPLEXING AGENTS

Complexing agents may form intermolecular complexes with low-solubility materials and enhance the solubility. Aqueous solubility of nonpolar molecules in the presence of complexing agents is improved as the nonpolar molecules and the nonpolar region of the complexing agent are sequestered out of water. When this occurs, the aqueous solution can accommodate more of the nonpolar molecules. Regardless of the ratio of ligand to solute in these complexes (e.g., 1:1, 2:1, 3:1, etc.), the solubility enhancement is expected to increase as the concentration of the complexing agent increases (*8*). This is very similar to solubilization by surfactants except there will not be a minimum concentration of the complexing agent required. Complexes with high stability constants may bind solutes strongly enough to enhance aqueous stability. Cyclodextrins are often used to enhance solubility by forming complexes with drug substances.

## EFFECT OF SURFACE AREA (DISSOLUTION RATE)

The Noyes–Whitney equation (as expressed by Nernst and Brunner) states:

$$\text{Rate} = \frac{\partial C}{\partial t} = \frac{DA\left(C_S - C\right)}{h}$$

$C$ = concentration of the solute in the solvent at time, $t$

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$D$ = diffusivity of the solute
$A$ = surface area of the solute particles
$C_S$ = saturated solubility of the solute
$h$ = thickness of the diffusion layer

In unstirred solutions, the diffusion layer thickness, *h,* may be large and is primarily affected by the diffusivity of the solute; however, good mixing may significantly reduce the diffusion layer thickness. For small particles in well-mixed solutions, the diffusion layer thickness, *h*, was found (*9*) to be proportional to the square root of the particle radius. This equation indicates that the smaller particles will have greater surface area and will dissolve more quickly. In order to reach the equilibrium solubility as quickly as possible, the surface area should be kept as high as possible (i.e., smaller particles) and the diffusion layer thickness kept as small as possible (i.e., good mixing) (*9*).

For spherical particles, the surface area, *A*, can be expressed as a function of the total mass, *M*:

$$\text{For spherical particles, } A = \frac{6M}{\rho d}$$

$\rho$ = density
$d$ = particle diameter

The dissolution rate of a material will not affect the equilibrium solubility, but will affect how quickly this equilibrium is achieved.

$$\text{Rate} = \frac{\partial C}{\partial t} = \frac{6DM}{\rho dh}(C_S - C)$$

### EFFECT OF SURFACE ENERGY

The surface energy of the particle may affect the solubility. According to the Kelvin equation, smaller particles have higher solubility than larger particles due to the effect of the surface energy on the total Gibbs free energy of the system. Typically, this effect on solubility only becomes significant for particles smaller than 1 micron. The Kelvin equation quantifies this as:

$$\ln\frac{S}{S_0} = \frac{4\gamma V_m}{RTd}$$

$S$ = apparent solubility
$S_0$ = solubility of an infinitely large particle
$\gamma$ = surface energy of the solute
$V_m$ = molecular volume of the solute
$R$ = gas constant
$T$ = temperature
$d$ = diameter of the particle

The solubility difference between smaller and larger particles leads to so-called Ostwald ripening in polydisperse suspensions. The small particles dissolve and result in a solution that is supersaturated relative to the solubility of the larger particles. This leads to recrystallization on the surface of the larger particles. The larger particles grow in size while the smaller particles dissolve resulting in an increase in the mean particle size of the suspension (*10*).

## EXPERIMENTAL METHODS

## Methods for Determination of Equilibrium Solubility

### SATURATION SHAKE-FLASK METHOD

The shake-flask method is based on the phase solubility technique that was developed 40 years ago and is still considered by most to be the most reliable and widely used method for solubility measurement today (*11–18*). The shake-flask method should be used when equilibrium solubility needs to be determined. Other methods may be used to evaluate apparent solubility, but are not considered suitable for evaluation of true equilibrium solubility.

The solubility medium selected for solubility measurements should be selected to be relevant to the application with efforts made to control the surfactant type and concentration, the ionic strength of the buffer, and the types of counter-ions present in the buffer. When the results are intended to predict absorption or bioavailability, it is recommended that one of the biorelevant media solutions be used (see *Solubility Measurements in Biorelevant Media*). When the results are intended to support the development of dissolution tests, it is recommended that the dissolution medium be used. For research purposes, when the pH dependence of the compound is being evaluated, a buffer that allows control of ionic strength and counter-ion types over a wide pH range is recommended (e.g., Britton–Robinson or Sörensen buffers). For solubility measurements that will be used for BCS classification, USP-recommended buffers should be used (*19*).

**Sample preparation:** The test substance is typically prepared by adding an excess of solid to the solubility medium, which is in a stoppered flask or vial. The amount of medium in the flask or vial does not need to be measured accurately. It is recommended that the solid be added to the solubility medium at about 1–2 mg/mL in excess of the estimated solubility. (For

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low-solubility compounds, a concentration of 1–2 mg/mL may be sufficient.) The surface area of the solid may be increased by grinding (e.g., in a mortar and pestle) of the sample prior to addition to the medium or by sonication of the sample after addition to the medium. [**CAUTION**—It is advised to use caution when employing high-energy methods to increase the surface area because it may alter the solid form of the solute.] It is recommended that sample preparation be performed in triplicate to provide at least 3 solubility results for each test condition.

**Equilibration of solution:** To facilitate dissolution of the solid, the suspension should be actively mixed or agitated. As a good initial time of incubation, 24 h is recommended; however, the suitability of the selected equilibration time must be verified. The temperature of the suspension should be well controlled during this dissolution phase (±0.5°). Following the dissolution phase, it is recommended that the excess solid be allowed to sediment completely. Sedimentation and decantation is recommended as the safest method for separation of the solid from the saturated solution. For non-clarifying colloid solutions, centrifugation can be used. Sampling of the supernatant should avoid incorporating any undissolved solid, as this will significantly affect the solubility result. The transfer pipet needs to be pretreated with sample solution before use, so that surface adsorption is not altering the transferred solution. If filtration cannot be avoided, then it is essential that the proper filter type is selected. For polar, ionized species, hydrophobic type filters (nylon) are recommended; while for unionized species the hydrophilic type filters [e.g., polyvinylidene difluoride (PVDF) or polyethersulfone (PES)] are recommended. The filtration should be done after sedimentation, and not directly after agitation. Presaturation of the filter is necessary (i.e., the initial portions of filtrate should be discarded). The temperature of the suspension during the sedimentation and centrifugation steps must also be well controlled (±0.5°) and be equivalent to the temperature at which solubilization occurred.

Saturation (equilibrium) has been reached when multiple samples, assayed after different equilibration time periods, yield equivalent results (e.g., change by less than 5% over 24 h, or less than 0.2%/h). To confirm that the apparent solubility is the equilibrium solubility, it is recommended that the same suspension be re-equilibrated via the same procedure (e.g., mix for an additional 24 h).

**Analysis of solution:** The requirements for the analytical method used to quantitate the concentration of the solute and the level of analytical validation required should be commensurate with the intended use of the solubility data. In general, the method should be linear and specific. The supernatant solution may need to be diluted before analysis to be within the linearity of the analytical method and to avoid possible precipitation. The solution may be analyzed by UV-Vis spectrometry or by liquid chromatography methods to determine the soluble concentration. The advantage of HPLC is that it can detect instability by resolving drug-related impurities (*13*,*20*).

It is recommended that the excess solid in the suspension be analyzed at the end of the solubility measurement to verify that the solid form has not changed. In cases where the solid form has changed, it is likely that the new solid form has a lower solubility than the initial solid form and that the observed solubility is due to the new lower-solubility form; however, this should be evaluated on a case-by-case basis. Powder X-ray diffraction (PXRD), Raman or near infrared (NIR) spectrometry, or evaluation of the melting point by differential scanning calorimetry (DSC) are examples of techniques that can be used to evaluate the solid form. Solutes that are unstable (either chemically or physically) during the equilibration time are not suitable for equilibrium solubility measurements by the shake-flask method. For example, amorphous drugs that will convert to lower solubility salts or polymorphs should be analyzed using one of the apparent solubility methods.

**Reporting of solubility results:** If a non-standard composition for the media is used in the solubility determination, the details of the composition should be reported. The ionic strength of the media used in the solubility determination should be calculated and reported with the solubility result. The pH of the supernatant solution should be recorded (at the temperature of the solubility measurement) when the sample is withdrawn for analysis. When using well-defined, standard media, it is recommended that the pH of the media not be adjusted to compensate for alteration of the pH by the dissolving species; rather, the solubility value should be reported at the pH value and temperature observed at the end of the equilibration step (*12*,*18*). If the pH of the media is significantly affected by the dissolving species and solubility at a particular pH is desirable, it is recommended to perform an additional solubility measurement in a higher buffer-capacity medium. Report the mean temperature and the precision of temperature control during the equilibration.

The precision of the reported equilibrium solubility should reflect the level of agreement between the measurements rather than the precision of the solubility analysis. Standard deviations in the measured solubility (based on averaging 3 or more independent samples) should be included.

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## METHODS FOR DETERMINATION OF APPARENT SOLUBILITY

### Intrinsic Determination (Rotating Disk)

The measurement of intrinsic dissolution is described more fully in ▲*Intrinsic Dissolution Testing Procedures for Rotating Disk and Stationary Disk* ⟨1087⟩▲ (CN 1-Dec-2020). This measurement technique may also be used to assess the solubility.

To apply this method to the measurement of solubility, the dissolution experiment must be continued to the point that the rate of dissolution is insignificant (e.g., less than 5%/24 h or less than 0.2%/h).

All the requirements discussed for the shake flask method in *Analysis of solution* and *Reporting of solubility results* will also apply to measurements using the intrinsic dissolution apparatus.

### Potentiometric Titration

The potentiometric acid–base titration for solubility measurements is based on a characteristic shift in the middle of the titration curve that is caused by precipitation (*21*). For the titration, accurate volumes of a standardized acid or base are added to a solution containing an ionizable substance and a salt, for example, 0.15 M potassium chloride (KCl), which is included to increase the accuracy of the measurements. Sparging (a technique that involves bubbling a chemically inert gas such as

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nitrogen, argon, or helium through a liquid) with argon prevents carbon dioxide ($CO_2$) from the atmosphere from influencing the pH value. A glass electrode is used to monitor the pH value continuously. The potentiometric titration curve is obtained by plotting the pH value against the consumed volume of acid/base (*21*).

### Turbidimetry

Turbidimetry involves the dissolution of a compound in an organic solvent, for example, dimethyl sulfoxide (DMSO). The resulting solution is added to a buffer solution in intervals adequate to characterize changes in turbidity. Further aliquots of the solution are added after the first detection of turbidity by light scattering. Subsequently, the volume added can be plotted against the turbidity. The solubility is then estimated by back-extrapolation to the point where precipitation began. This method can be used to measure as many as 50–300 samples per day. When using solvents such as DMSO, drawbacks include the increase in solubility of the drug substance for the short duration of the experiment, which leads to a kinetic rather than thermodynamic solubility, the formation of a supersaturated solution, and the undefined crystalline form of the precipitated solid (unless it is removed from the suspension and characterized).

### Physical Assessment of Solubility

For compounds with extremely high solubility, as well as for biologics and other molecules that lack chromophores or are not easy to quantitate in solution, a physical assessment of solubility may be used to assess apparent solubility. In this case, the principle of measurement is the loss of solid material to the solution phase. Equilibrium may be assessed by both the stability of the weight loss as well as the stability of the change in the physical properties of the resulting solution (e.g., refractive index, density, osmolality, etc.). Because the physical assessment of solubility does not involve a specific or stability-indicating assay, it is recommended that some attempt be made to verify the stability and purity of the solute. Also, evaporation of the solvent should be carefully monitored and controlled during the solubility measurement performed by this method.

## SOLUBILITY MEASUREMENTS IN BIORELEVANT MEDIA (*22–26*)

The use of simple aqueous buffers to evaluate the aqueous solubility of a drug substance as a function of pH may underestimate the bioavailability (*22*,*23*). The media recipes presented here are examples that may be used to evaluate the solubility in simulated human, canine, and bovine (ruminant) fluids to make improved estimates of bioavailability.

The temperature of the solubility medium should be controlled at ±0.5° during biorelevant solubility measurements. Biorelevant solubility measurements should follow the shake-flask method, including solubility measurements at multiple time points to confirm that equilibrium has been achieved. The salt form of a drug added to the solubility medium may significantly affect the composition of the medium (i.e., ionic strength, pH, etc.). Therefore, the solubility of a salt and a free base of the same drug should not be assumed to be equivalent unless proven through independent measurements starting with the different crystalline solids.

### Human Fasted-State Simulated Gastric Fluid (FaSSGF) (*24*)

The pH is 1.6 at 37°. See *Table 1* for the media composition.

**Table 1**

| Ingredient | Concentration (mM) | Concentration (g/L) |
|---|---|---|
| Hydrochloric acid | ~31.3 (q.s. to pH 1.6) | ~1.14 (q.s. to pH 1.6) |
| Sodium chloride | 34.2 | 2.00 |
| Sodium taurocholate | 0.08 | 0.047 |
| Lecithin | 0.02 | 0.015 |
| Pepsin | — | 0.1 |

### Human Fed-State Simulated Gastric Fluid (FeSSGF) (*24*)

The pH is 5 at 37°. See *Table 2* for the media composition.

**Table 2**

| Ingredient | Concentration (mM) | Concentration (g/L) |
|---|---|---|
| Hydrochloric acid | (q.s. to pH 5) | (q.s. to pH 5) |
| Sodium hydroxide | (q.s. to pH 5) | (q.s. to pH 5) |
| Sodium chloride | 237.0 | 13.85 |
| Sodium acetate | 29.75 | 2.441 |

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**Table 2** *(continued)*

| Ingredient | Concentration (mM) | Concentration (g/L) |
|---|---|---|
| Acetic acid | 17.12 | 1.028 |
| Milk, whole | 1:1 | 1:1 |

Prepare buffer and then mix 1:1 with milk. Adjust to a pH of 5, if necessary.

## Human Fasted-State Simulated Intestinal Fluid (FaSSIF-V2) (*24*)

The pH is 6.5 at 37°. See *Table 3* for the media composition.

**Table 3**

| Ingredient | Concentration (mM) | Concentration (g/L) |
|---|---|---|
| Maleic acid | 19.12 | 2.219 |
| Sodium hydroxide | 34.8 | 1.392 |
| Sodium chloride | 68.62 | 4.010 |
| Sodium taurocholate | 3.0 | 1.766 |
| Lecithin | 0.2 | 0.165 |

## Human Fed-State Simulated Intestinal Fluid (FeSSIF-V2) (*24*)

The pH is 5.8 at 37°. See *Table 4* for the media composition.

**Table 4**

| Ingredient | Concentration (mM) | Concentration (g/L) |
|---|---|---|
| Maleic acid | 55.02 | 6.338 |
| Sodium hydroxide | 81.65 | 3.226 |
| Sodium chloride | 125.5 | 7.460 |
| Sodium taurocholate | 10 | 5.89 |
| Lecithin | 2.0 | 1.65 |
| Glyceryl monostearate | 5.0 | 1.79 |
| Sodium oleate | 0.8 | 0.24 |

## Human Simulated Colonic Fluid—Proximal Colon (SCoF2) (*25*)

The pH is 5.8 at 37°. See *Table 5* for the media composition.

**Table 5**

| Ingredient | Concentration (mM) | Concentration (g/L) |
|---|---|---|
| Sodium hydroxide | ~159 (q.s. to pH 5.8) | ~5.4 (q.s. to pH 5.8) |
| Acetic acid, glacial | 170 | 10.2 |

## Human Simulated Colonic Fluid—Distal Colon (SCoF1) (*25*)

The pH is 7.0 at 37°. See *Table 6* for the media composition.

**Table 6**

| Ingredient | Concentration (g/L) |
|---|---|
| Potassium chloride | 0.2 |
| Sodium chloride | 8 |
| Potassium phosphate, monobasic | 0.24 |
| Sodium phosphate, dibasic | 1.44 |

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## Canine Fasted-State Simulated Gastric Fluid (FaSSGFc pH 1.2–2.5) (*26*)

Canine gastric pH can vary substantially. Due to interstudy variations in the canine gastric pH estimates, solubility should be evaluated over a range of 1.2–6.5. The pH of this fluid is adjusted by altering the amount of hydrochloric acid so that pH values in the range of 1.2–2.5 may be achieved. The pH is 1.2–2.5 at 37°. See *Table 7* for the media composition.

**Table 7**

| Ingredient | Concentration (mM) | Concentration (mg/mL) |
|---|---|---|
| Hydrochloric acid | ~3.6–82 (q.s. to pH 1.2–2.5) | ~0.13–3 (q.s. to pH 1.2–2.5) |
| Sodium chloride | 14.5 | 0.847 |
| Sodium taurocholate | 0.10 | 0.055 |
| Sodium taurodeoxycholate | 0.10 | 0.054 |
| Lecithin | 0.025 | 0.019 |
| Lysolecithin | 0.025 | 0.012 |
| Sodium oleate | 0.025 | 0.0076 |

## Canine Fasted-State Simulated Gastric Fluid (FaSSGFc pH 2.5–6.5) (*26*)

Canine gastric pH can vary substantially. Due to interstudy variations in the canine gastric pH estimates, solubility should be evaluated over a range of 1.2–6.5. The pH of this fluid is adjusted by altering the amount of sodium hydroxide so that pH values in the range of 2.5–6.5 may be achieved. The pH is 2.5–6.5 at 37°. See *Table 8* for the media composition.

**Table 8**

| Ingredient | Concentration (mM) | Concentration (g/L) |
|---|---|---|
| Maleic acid | 21.68 | 2.516 |
| Sodium hydroxide | ~14.5–40 (q.s. to pH 2.5–6.5) | ~0.58–1.6 (q.s. to pH 2.5–6.5) |
| Sodium chloride | 18.81 | 1.099 |
| Sodium taurocholate | 0.10 | 0.055 |
| Sodium taurodeoxycholate | 0.10 | 0.054 |
| Lecithin | 0.025 | 0.019 |
| Lysolecithin | 0.025 | 0.012 |
| Sodium oleate | 0.025 | 0.0076 |

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## Canine Fasted-State Simulated Intestinal Fluid (FaSSIFc) (*26*)

The pH is 7.5 at 37°. See *Table 9* for the media composition.

**Table 9**

| Ingredient | Concentration (mM) | Concentration (mg/mL) |
|---|---|---|
| Sodium dihydrogen phosphate, monohydrate | 28.65 | 3.953 |
| Sodium hydroxide | 21.66 | 0.866 |
| Sodium chloride | 59.63 | 3.485 |
| Sodium taurocholate | 5.0 | 2.8 |
| Sodium taurodeoxycholate | 5.0 | 2.7 |
| Lecithin | 1.25 | 0.96 |
| Lysolecithin | 1.25 | 0.62 |
| Sodium oleate | 1.25 | 0.38 |

## Bovine Simulated Ruminal Fluids (*27–29*)

The media defined here are suitable to represent bovine as well as other ruminant species. The normal pH of a healthy reticulo-rumen is in the 5.5–6.8 range. High-grain diets typically result in a lower ruminal pH (~5.5), whereas high-forage diets result in a higher ruminal pH (~6.8).

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In the abomasum (true stomach), the pH is about 2–3 and is similar to conditions observed in monogastrics and humans. To represent the abomasum, one may use 0.01 M hydrochloric acid (pH 2), 0.0033 M hydrochloric acid (pH 2.5), or 0.001 M hydrochloric acid (pH 3). Intestinal pH in ruminants is similar to that observed in monogastrics and humans. The pH at the pylorus is about 3.0 and increases to about 7.5 in the ileum. To represent the bovine intestinal fluids, one may use one of the simulated intestinal fluids defined for human or canine above.

The temperature is set for 39°. [NOTE—The pH of media should be confirmed at 39°.] See *Table 10* for the media composition.

**Table 10**

| Ingredient | High-Grain Diet Simulated Rumen | High-Forage Diet Simulated Rumen |
|---|---|---|
| Tryptone | 2.5 g/L | 2.5 g/L |
| Sodium bicarbonate | 4.0 g/L (47.6 mM) | 8.75 g/L (104 mM) |
| Potassium phosphate monobasic anhydrous | 1.55 g/L (11.4 mM) | 1.55 g/L (11.4 mM) |
| Sodium phosphate dibasic anhydrous | 1.425 g/L (10.0 mM) | 1.425 g/L (10.0 mM) |
| Ammonium bicarbonate | 1.0 g/L (12.6 mM) | 1.0 g/L (12.6 mM) |
| Magnesium sulfate heptahydrate | 0.15 g/L (0.6 mM) | 0.15 g/L (0.6 mM) |
| Acetic acid | 2.40 g/L (40 mM) | 1.42 g/L (24 mM) |
| Propionic acid | 2.59 g/L (35 mM) | — |
| Butyric acid | 1.32 g/L (15 mM) | — |
| Sodium acetate trihydrate | — | 6.26 g/L (46 mM) |
| Sodium propionate | — | 1.44 g/L (15 mM) |
| Sodium butyrate | — | 1.10 g/L (10 mM) |
| Micromineral stock solution (see *Table 11*) | 125 µL | 125 µL |
| pH | 5.5 | 6.8 |
| Surface tension | ~50 dynes/cm$^2$ | ~50 dynes/cm$^2$ |
| Ionic strength | 0.102 mol/L | 0.240 mol/L |
| Buffer capacity | 47 mmol/L/pH | 49 mmol/L/pH |

**Table 11**

| Ingredient | Micromineral Stock Solution Concentration (g/100 mL) |
|---|---|
| Water | q.s. to 100 mL |
| Calcium chloride dihydrate | 13.2 |
| Manganese chloride tetrahydrate | 10.0 |
| Iron (III) chloride hexahydrate | 8.0 |

## GLOSSARY

[NOTE—The following definitions are provided to clarify the use of these terms in the context of this chapter. These definitions are not intended to supersede or contradict definitions found elsewhere in the *USP–NF*.]

**Apparent solubility:** The empirically determined solubility of a solute in a solvent system where insufficient time is allowed for the system to approach equilibrium or where equilibrium cannot be verified. The apparent solubility may be either higher or lower than the equilibrium solubility due to transient supersaturation or incomplete dissolution and insufficient time to reach equilibrium.

**Aqueous solubility:** Solubility in a medium that is primarily comprised of water but may also contain solubilization enhancement from co-solvents, surfactants, complexing agents, pH, or other co-solutes. The term "aqueous solubility" is very general and should not be confused with the water solubility. The aqueous solubility is significantly affected by the composition of the aqueous medium.

**Dissolution:** The non-equilibrium process of approaching the solubility limit at thermodynamic equilibrium (i.e., the solute and solvent forming a uniformly mixed solution). The dissolution rate will affect the time required to reach equilibrium and may affect the apparent solubility, but will not affect the final equilibrium solubility.

**Equilibrium solubility:** The concentration limit, at thermodynamic equilibrium, that a solute can dissolve into a saturated solution when excess solid is present. Equilibrium can be defined as sufficiently converged when it no longer changes significantly during a certain time frame.

**Intrinsic solubility:** The solubility of the uncharged (neutral) moiety. Intrinsic solubility can only be accurately measured in pH ranges where the distribution of species is dominated by the uncharged molecule. For some compounds, it may be impossible to directly measure the intrinsic solubility and it must be determined by fitting the solubility data as a function of pH.

**Solubility:** The extent to which a solute may be uniformly dissolved into a solvent. This may be referred to as equilibrium (saturated) solubility to differentiate it from apparent solubility. Solubility may be stated in units of concentration such as molality, mole fraction, mole ratio, weight/volume, and weight/weight.

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**Water solubility:** Solubility in pure water. Measurements of solubility in pure water are problematic due to poor control of pH and ionic strength.

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# ⟨1237⟩ VIROLOGY TEST METHODS

## INTRODUCTION

This chapter describes virology test methods applicable to the development of biological product drugs, such as recombinant proteins, subunit vaccines, therapeutic monoclonal antibodies, and growth hormones. Several topics are excluded from the scope of this chapter:

- Blood- and plasma-derived products as well as whole blood and plasma products used directly in transplantation or infusion. However, the basic principles, strategies, and testing methods for ensuring virus-free products are applicable.
- Methodologies for the safety testing of live viral vaccines.
- Specific methods for viral clearance studies, which are described in the *USP* general information chapter *Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin* ⟨1050⟩.

Virology test methods have historically been employed in the clinical settings of disease diagnosis, intervention, and containment; but the development of biological (biologics and biotechnology-derived) products and therapies for human or animal use has created the need for sensitive viral detection assays for use in the GMP production and testing of biological products. This need is not limited to the production of viral vaccines, but also applies to the development and manufacture of recombinant proteins, cell and gene therapies, and other products.

Sensitive virology test methods for quality control of biological products are necessary for several reasons. The production of biological products often requires a variety of raw materials and processing reagents of animal origin that have varying potential for introducing viral contaminants. The production of biological products may allow the replication of adventitious agents during processing, and therefore these materials must be prescreened to avoid the opportunity for contamination of the product. Another point to consider regarding screening these materials is that the product may not be compatible with processing methods used to eliminate or inactivate these adventitious agents. Because of the nature of the biological products, the production process needs to include appropriate testing regimens that monitor the possible introduction of adventitious agents and/or viral agents into the systems used. For these reasons, sensitive viral detection methods are required not only for the release testing of biological drug products, but also during the intermediate stages of processing, process development, and routine manufacture. Important stages for consideration include the development of cell substrates and banks, raw materials of animal origin, process intermediates, and critical excipients when derived from animal tissues. This strategy should be augmented with viral clearance and inactivation studies whenever possible.

For products intended to contain live viruses (e.g., infectious oncolytic viruses and live viral vector products used for gene therapy), the cell- and animal-based infectivity methods discussed in this chapter may be useful only following neutralization of the specific viral entity contained in the product for any product that is intended to contain live viruses (e.g., infectious oncolytic viruses and live viral vector products used for gene therapy). Alternatively, selection of appropriate indicator cell lines or animal models in which the specific viral entity is known not to replicate can be considered. It should also be expected that assay systems based on detection of viral particles or viral components will indicate the presence of the viral entity itself in such products, but may not indicate the viability of the virus. The remainder of the chapter is divided into three sections discussing assays for the three topics: (1) *Detection of Viable Viruses,* (2) *Detection of Viral Components,* and (3) *Detection of Antibodies to Viral Antigens.* The chapter covers the classic virology methods that are still routinely used, as well as modern molecular and immunological approaches. The methods described in these sections may possess different sensitivities to diverse viruses; they are therefore intended to complement each other to provide a science-based foundation for the detection of adventitious viruses. Multiple methods may be used in complementary fashion to improve the pathogen safety margin of a product. Identification of viruses detected in cell-based assays on the basis of cytopathic effects often depends on the use of molecular and immunological analyses; these analyses are therefore relevant both to viral detection and to subsequent viral identification. The chapter provides an overview of the detection and analysis of the most important groups of viruses as well as the most commonly used techniques. Tests specific to individual vaccines or biological products are excluded, because they are expected to be included in monographs for such products.

Methods that are well established with little variation in practice are described in more detail, whereas methods that are more flexible are described in general terms, both in the performance of the tests and in considerations for acceptance. Relevant regulatory references are given in the *Appendix.* Relevant USP general chapters should be consulted with regard to bioassay design, data analysis, interpretation, and assay validation.

## DETECTION OF VIABLE VIRUSES

Infectious virus particles contaminating biologics and biotechnology-derived products are of great safety concern, because they have the potential for causing serious, possibly life-threatening, infections in the patients treated. This is particularly true if the patients are immunocompromised. Although complete assurance of viral safety for finished biological products can never be realized, a significant safety margin can be established through viral detection methods applied to unprocessed bulk and raw materials before purification in combination with purification processes that demonstrate the ability to inactivate or remove potential viral contaminants present at levels too low to detect. (See *Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin* ⟨1050⟩ for information on viral clearance and inactivation methods.) For cell and gene therapy products that lack extensive purification steps, final products may be directly tested for the presence of relevant contaminating viruses. This section describes two broad systems for the detection of infectious virus: cell culture–based infectivity assays and in vivo infectivity assays. These systems may possess complementary sensitivities for viruses, and as a result, both methods may be used as limit tests for cell bank and raw material characterization and for lot release testing of biologics and biotechnology-derived products. Considerations for optimizing sample preparation for these tests are discussed, followed

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by a description of the more commonly employed detection assays. Finally, to ensure the reliability of experimental results, quality control issues in general and detection limit estimation in particular are discussed.

## Sample Selection of and Preparation for Cell- and Animal-Based Virus Detection Assays

The requirements for selection, preparation, and storage of test samples for viral detection methods (cell- and animal-based) are dictated by the lability of the viruses being detected. The ability of a virus to remain infectious in the absence of a host cell is highly variable. Virus infectivity also may differ in sensitivity to repeated freezing and thawing cycles.

Sample preparation typically involves storage of test samples at low temperatures (ideally –60° or below) as soon as practicable upon collection. When intended for use in a viral screening assay, aliquots of samples should be prepared to avoid multiple freezing and thawing. Samples intended for viral infectivity assays are typically shipped with sufficient dry ice to last several days more than the expected time required for transit. When received at the testing laboratory, the sample should be examined to verify that it is still frozen, and appropriate documentation should be completed. For any storage or hold condition, the impact of the condition on viral viability should be empirically assessed and sufficient cold chain management ensured.

Typical sample types for viral detection assays are described below.

### CELL LYSATES

Test samples derived from cell substrates (master and working cell banks, end-of-production cell samples) are prepared in a manner that allows sampling of both the cells (for cell-associated viruses) and the conditioned medium (for virus shed into the medium). To achieve this, a culture of the cells is sampled. A cell suspension of ~$10^7$ cells per mL in conditioned medium is prepared and frozen (ideally at –60° or below). Because this medium does not contain cryopreservative, the majority of the cells will lyse upon thawing of the sample, releasing the cell-associated virus. Low-speed centrifugation will remove larger cellular debris and yield a supernatant that may be inoculated directly onto detector cells in cell-based viral infectivity assays. A similar sample is prepared for in vivo viral adventitious agent testing. In this case, however, the test sample is thawed and injected without clarification into the various animal systems via the various described routes.

### BIOTECHNOLOGY BULK HARVEST (UNPROCESSED BULK HARVEST) SAMPLES

Routine lot testing of bulk harvest samples is mandatory for most types of biologics (see *Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin* ⟨1050⟩). The sampling must be done at the unprocessed bulk harvest stage, because downstream purification processes may remove or inactivate any viruses that might contaminate the starting materials. The harvest sample from the bioreactor should be collected and stored without further manipulation, as soon as practicable, at –60° or below. To prevent multiple freeze and thaw cycles, individual aliquots should be prepared for each individual assay to be performed. Additional aliquots should be retained in case repeat testing is required. Depending on the nature of the manufacturing process, the bulk harvest samples may contain varying quantities of the production substrate cells. Because the bulk harvest does not contain cryopreservatives, the majority of the cells present will lyse upon thawing of the sample, releasing the cell-associated virus. Low-speed centrifugation to clarify the sample will result in a supernatant that may be inoculated directly onto detector cells in cell-based viral infectivity assays. There may be instances where the test sample is cytotoxic to the detector cells of the cell-based assays and procedural modifications may be required to deal with this. A similar sample is prepared for in vivo viral safety testing. In this case, however, the test sample is thawed and injected without clarification into the various animal systems via the various described routes.

### RAW MATERIALS OF ANIMAL ORIGIN

Ingredients of animal origin used in the manufacture of biological products for human or veterinary use must be tested for species-specific viruses of concern as described in 9 CFR 113.53 (see also the *USP* general information chapter *Bovine Serum* ⟨1024⟩, being prepared for future publication). The raw materials may be stored under a variety of conditions, as appropriate to the raw material. Sample preparation and method of application to the test system depend on the nature of the sample. The possibility that animal-derived raw materials may contain bacterial or fungal contaminants should be considered. In some cases, it may be necessary to treat the samples with antibiotics or to filter the samples (0.22 or 0.45 micron pore size) prior to inoculation in order to prevent bacterial or fungal outgrowth in the test system. Animal sera are typically received frozen and are thawed and incorporated into the growth medium at an appropriate concentration (typically 15%, v/v) as a means of exposing the detector cells. Powdered trypsin (not less than 5 grams, as per 9 CFR 113.53) is suspended in a suitable diluent, such as phosphate-buffered saline, and is then subjected to high-speed centrifugation to pellet any virions that may be present. The concentrated pellet is resuspended in phosphate-buffered saline, and the resulting material is used to inoculate appropriate detector cells. Medium additives, such as bovine thrombin, may be incorporated into the growth medium at a predetermined multiple of the nominal concentration to be used in the manufacturing process. The resulting growth medium containing the additives is then used as a means of exposing the detector cells to the test material. The exact multiple to be used in such testing may be limited by such factors as solubility in growth medium or cytotoxicity to the detector cells. These factors should be assessed in advance of testing. The principle of using higher concentrations in the detection method than during processing should be followed, within the bounds of indicator cell toxicity, as a means to increase sensitivity to detection.

### WHOLE CELLS

Intact viable cells are used as the test sample in certain viral detection assays. Because the test cells may attach and proliferate in the culture vessel along with the detector cells, assays using this type of sample are referred to as cocultivation assays. The requirements for the specific assay may vary in relative proportions of detector and test cells, viability of the test cells, or the confluency of test cells at the time of collection.

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# Cell Culture-Based Viral Detection Methods

To ensure the absence of adventitious viral agents, cell culture–based viral detection assays are used for a variety of purposes, including but not limited to clinical diagnostic procedures; evaluation of raw materials and cell substrates; assessments of the viral identity, the purity, and the potency of virus seed stocks; and lot release testing of unprocessed bulk harvests during biologics production. An important distinction between cell-based assays and direct detection assays (see the section *Detection of Viral Components*) is that the former will detect only replicating virus, whereas the latter will detect viral antigens, viral genomic material, and the like, which may or may not be indicative of the presence of replicating virus. Similarly, detection of circulating antibodies directed against viral antigens (discussed in the section *Detection of Antibodies to Viral Antigens*), may be indicative of either a current or a past infection of an animal and does not necessarily indicate that the animal is currently harboring an infection.

Infectious viruses detected in cells or in cell-derived materials fall into two broad categories, based on the expectations of the analyst. Endogenous viruses are those normally detected in the cells as a result of the integration of the viral genomic material into the host cell DNA. Exogenous viruses are those not normally present in the cells but found as a result of a viral infection of the cells.

The underlying assumption for all cell-based viral detection methods is the ability of viruses to replicate in an appropriate host cell. Viruses lack the cellular machinery required for producing their own genomic material and structural proteins, and they must therefore enter and subordinate a host cell for this purpose. Cell-based viral infectivity assays use indicator (detector) cells that serve as host cells for viable virions present in test samples.

Cell-based infectivity assays may be placed in three broad categories on the basis of types of viruses to be detected: (1) retroviral assays, (2) virus-specific assays, and (3) viral screening assays. The types of endpoints used to detect the viruses may differ by category. Although screening assays are typically not optimized for single viral entities, the virus-specific assays and titration assays, as well as some of the retroviral assays, may be optimized to some extent for specific viruses. Accurate titration of stock viruses that are used as positive controls or are used to determine the detection limit of an assay is critical.

The regulatory guidance underlying the various viral safety tests depends on the nature of the samples to be evaluated, and analysts are referred for more detail to documentation relevant to their own regulatory environments.

## General Requirements for Cell Culture–Based Assays

### DETECTOR CELLS AND THE CONCEPT OF VIRAL HOST RANGE

The range of viruses detectable using a cell-based infectivity assay depends on a number of factors, including the type of host cell(s) used as the indicator (detector) cultures and the detection endpoints used in the assay. Viruses differ in their abilities to infect specific host cell types. Most viruses exhibit at least some degree of host cell tropism (i.e., ability to infect a specific species or tissue type). This attribute is typically due to a requirement for interaction of a virion with a specific cell membrane receptor during the process of infection of the host cell. A cell susceptible to infection and capable of production of progeny by a given virus is referred to as *permissive* for that virus; cells not supporting viral proliferation are referred to as *nonpermissive,* or *restricted,* for that virus. As a consequence of the differences in host cell tropism, assays intended to screen for a wide range of viruses must include multiple detector cell types. For the same reasons, design of a cell-based infectivity assay for a specific virus must include a detector cell known to be permissive for that virus.

### VIRUS SUSCEPTIBILITY OF COMMON CELL LINES

For most endpoint assays used to determine whether a host cell is infected with a virus, a monolayer culture is preferable to a semiadherent or suspension culture. For instance, cytopathic effect and hemadsorption are visualized microscopically. Cells that are not adherent have little morphology to evaluate, and hemadsorption cannot be properly evaluated in a suspension culture. For this reason, some regulatory documents pertaining to cell-based virus infectivity assays stipulate the use of monolayer detector cultures.

A list of commonly employed indicator cell lines and their application in viral screening assays is provided in *Table 1.* Regarding the viral tropism of these cells, "Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals" (1993) and ICH's Q5A (R1) guidelines (for these references, see *Appendix*) require that human diploid cells such as MRC-5 and WI-38, which are permissive for a range of viruses of human concern, and monolayer cultures of the same species as that of the cell substrate used to produce the product are included in the viral screening test for biologics destined for use in humans.

**Table 1. Indicator (Detector) Cell Lines Used for Adventitious Viral Screening Assays**

| Cell Line[a] | Origin | Endpoint(s)[b] | Target virus(es) |
|---|---|---|---|
| *Cell lines with relatively broad viral tropism:* | | | |
| BHK-21 | Syrian hamster | CPE, HAd, HA | Insect-borne viruses (arboviruses) |
| Vero | African green monkey | CPE, HAd, HA | Viruses infectious to humans, primates |
| *For processes involving human cell substrates:* | | | |
| HeLa | Human | CPE, HAd, HA | Viruses infectious to humans |
| MRC-5 | Human | CPE, HAd, HA | Viruses infectious to humans |
| *For processes involving Chinese hamster cell substrates:* | | | |
| CHO-Kl | Chinese hamster | CPE, HAd, HA | Viruses infectious to Chinese hamsters |

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**Table 1. Indicator (Detector) Cell Lines Used for Adventitious Viral Screening Assays** *(continued)*

| Cell Line[a] | Origin | Endpoint(s)[b] | Target virus(es) |
|---|---|---|---|
| *For processes involving mouse cell substrates:* | | | |
| MEF | Mouse | CPE, HAd, HA | Viruses infectious to mouse cells |
| NIH/3T3 | Mouse | CPE, HAd, HA | Viruses infectious to mouse cells |
| *For processes involving bovine cell substrates or bovine raw materials:*[c] | | | |
| MDBK | Bovine | CPE, HAd, HA | Bovine viruses |
| BT | Bovine | CPE, HAd, HA | Bovine viruses |
| EBTr | Bovine | CPE, HAd, HA | Bovine viruses |

[a] Examples of cell lines used for viral screening assays are shown. MRC-5 and Vero, or cells with similar host ranges, are used in all assays. Depending on the cell substrate used to manufacture a biologic, additional cell lines are also used in the screening assay. In addition, a bovine cell might be included if bovine serum was used in the manufacturing process.
[b] CPE, cytopathic effect; HAd, hemadsorption; HA, hemagglutination (optional).
[c] Inclusion of a bovine cell in a virus screen should not be construed as a replacement for or alternative to a raw materials test. Raw materials testing is driven in the U.S. by 9 CFR 113.47 and 113.52, and cell lines used for this testing are described in *Table 2*.

A list of commonly employed indicator cell lines and their application in raw materials testing assays is provided in *Table 2*.

**Table 2. Indicator (Detector) Cell Lines Used in Raw Material Testing**

| Cell Line[a] | Assay Type | Endpoint(s)[b] | Animal Origin of Raw Material |
|---|---|---|---|
| Vero | Isolation/detection | CPE, HAd, IFA | All sources |
| BT | Isolation/detection | CPE, HAd, IFA | Bovine; all sources (BVDV)[c] |
| EBTr | Isolation/detection | CPE, HAd, IFA | Bovine; all sources (BVDV)[c] |
| MDBK | Isolation/detection | CPE, HAd, IFA | Bovine; all sources (BVDV)[c] |
| PT-1 | Isolation/detection | CPE, HAd, IFA | Porcine |
| PK-1 | Isolation/detection | CPE, HAd, IFA | Porcine |
| MDCK | Isolation/detection | CPE, HAd, IFA | Canine |
| GT | Isolation/detection | CPE, HAd, IFA | Caprine |

[a] The requirement (9 CFR 113.47 and 113.52) for evaluating raw materials of animal origin is to use (1) Vero cells, (2) a bovine cell for detecting BVDV, and (3) a cell line of the same species of origin as the raw material for detecting viruses of concern from that species. Examples are given of some cell lines that are used in the industry.
[b] CPE, cytopathic effect; HAd, hemadsorption; IFA, immunofluorescent antibody staining.
[c] As per 9 CFR 113.47, raw materials of any animal origin are to be tested for bovine viral diarrhea virus (BVDV).

There may be very specific requirements for detector cells for certain viruses. For instance, assays intended to detect infectious HIV use human peripheral blood lymphocytes and involve a p24 antigen capture enzyme immunoassay endpoint. A list of commonly employed indicator cell lines and their application in the detection of specific viruses is provided in *Table 3*.

**Table 3. Indicator (Detector) Cell Lines Used for Detection of Specific Virus(es)**

| Cell Line[a] | Assay Type | Endpoint(s)[b] | Target Virus |
|---|---|---|---|
| 324K | Isolation/detection | CPE, HAd, IFA | Murine minute virus |
| A9 | Isolation/detection | CPE, HAd, IFA | Murine minute virus |
| BHK-21 | Isolation/detection | CPE, HAd | Arboviruses[c] |
| MRC-5[d] | Isolation/detection | CPE | Human cytomegalovirus |

[a] Examples of cell lines used for optimizing the detection of specific viruses or virus types are shown. In many cases, the assay methodologies must also be optimized for detection of the target viruses.
[b] CPE, cytopathic effect; HAd, hemadsorption; IFA, immunofluorescent antibody staining.
[c] Insect-borne viruses as a group are referred to as arboviruses. This term has no taxonomic significance.
[d] Other human diploid cell lines such as WI-38 are also suitable. Assay duration must be 28 days at a minimum.

## GROWTH REQUIREMENTS FOR DETECTOR CELLS

Viral proliferation within a permissive host cell may be dependent on the rate of host cell proliferation. This is especially true for viruses that display cell-cycle dependence for generation of viral progeny. For most detection assays, detector cultures are seeded at a density intended to achieve a cell monolayer in exponential growth. This corresponds to a cell confluency of 50% or less (optimal cell densities may depend on the assay type and the detector cell to be used) at the time of inoculation of the cultures with virus or test sample. For the same reasons, the assay design may include provision for detector cell subculture (the collection of cells from the original culture and seeding of a predetermined fraction of these into a new flask). Alternatively, a

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passage may be performed, consisting of collection of conditioned medium from the original culture and inoculation of this material onto a secondary detector cell culture that is in log-phase growth. The frequency of subculture/passage required in an assay is determined largely by the rate of growth of the detector cell. Incorporation of these steps into detection assay designs helps to ensure that the conditions remain optimal for amplification of viral progeny within the host cells.

### NEED FOR DETECTOR CELL IDENTIFICATION AND BANKING

Detector cells used for cell-based viral detection assays are a critical reagent for ensuring viral safety, viral potency, viral identity, and viral clearance capacity in purification schemes. In many cases this testing is intended to support GMP processes; therefore, the detector cell banks may need to be prepared and qualified in much the same manner as other critical reagents. The details of the viral safety evaluation methods are described in *Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin* ⟨1050⟩. Regardless of specific compliance requirements, periodic identification and qualification of the detector cell banks to be used subsequently for viral infectivity assays is good practice. The following should be regarded as minimal quality control testing for such detector cell banks: sterility, mycoplasma and viral screening, and cell identity by DNA fingerprinting, karyology, or isoenzyme analysis. In addition, specific assays for bovine and porcine viruses may be required if bovine or porcine raw materials were used in preparation of the banks. *Bovine Serum* ⟨1024⟩ and relevant serum-type specific ancillary materials monographs should be consulted when serum products are used in cell growth media.

## Endpoints for Detection of Viral Infection

The various endpoints used to identify infection of a detector cell with a virus include the following:

- Visual observation of cytopathic effects
- Hemagglutination or hemadsorption of erythrocytes
- Immunofluorescent staining
- Cocultivation with other types of detector cells
- Quantitative polymerase chain reaction (qPCR) for direct detection of viral genomic sequences
- Electron microscopic analysis of viral pellets or fixed cells for visual observation of viral particles
- Biochemical endpoints such as reverse transcriptase assays, which detect virus-specific enzymatic activity

These various endpoints are used in a complementary fashion, because a given virus may not cause a positive response in each endpoint. For instance, some viruses can grow to high titers without producing visible cytopathic effects and so must be detected using other endpoints. Polymerase chain reaction (PCR) and electron microscopic analysis per se are not capable of distinguishing viable from nonviable viruses. However, when used in conjunction with cell culture growth kinetics, these approaches can be powerful orthogonal detection methods to demonstrate the increase of viral replication and therefore viable virus. The failure to observe viral particles in electron microscopic analysis of fixed cells should not be considered absolute proof of the absence of infectious virus in the cells. In a general sense, the same is true for each of the detection endpoints discussed above. Each endpoint has a detection limit below which a virus may be present but not detected.

### VIRAL CYTOPATHIC EFFECTS

Visually observable manifestations of the infection of susceptible host cells with certain types of viruses are collectively referred to as viral cytopathic effects (CPE). Although CPE may be considered an indirect detection of viral infection, in the context of specific host cells they can have distinctive morphological manifestations. These may include the appearance of inclusion bodies, abnormal cell morphology, changes in culture confluency, cell death and cell lysis, and others. The nature of the CPE observed may depend on the host cell and the infecting virus. In addition, for a virus that normally causes CPE, there may exist variants that do not cause CPE. CPE can be differentiated from cytotoxic effect by the tendency of the former to exhibit progression irreversibly with time, whereas the latter may be reversible. In some cases structural proteins of viruses may cause cytotoxic effects similar to the cytopathic effects of the infectious virus. Differentiating the cytotoxic effect of such proteins from the cytopathic effect of infectious virus may require observation of the culture over time to determine whether the effect progresses or the cells appear to recover. Alternatively, cell-free passage of the original culture onto fresh detector cells can be used to differentiate these two apparently similar manifestations. The cytotoxicity associated with the structural proteins in the absence of infectious virus would not be expected to pass to the secondary culture.

Giemsa staining may optimize the ability of the operators to visualize certain inclusion bodies (clusters of viral particles) that are characteristic of viral cytopathic effect and is required by 9 CFR 113.53 in assays used to demonstrate that bovine, porcine, equine, and ovine raw materials are free of species-specific viruses.

### DETECTION OF HEMAGGLUTINATING VIRUSES

A characteristic of certain viruses (referred to as hemagglutinating viruses) is that one or more of their viral proteins cause hemagglutination of one or more types of erythrocytes. Hemagglutination is an interaction between viral proteins or *hemagglutinins* and erythrocytes, leading to adhesion of the erythrocytes to surfaces, cells, and each other. This property forms the basis of two endpoint procedures that are employed in cell-based viral infectivity assays: *hemadsorption* and *hemagglutination.*

Hemadsorption is performed by adding a suspension of one or more erythrocyte types directly to the monolayer culture of detector cells. If viral proteins of a hemagglutinating virus are expressed from infected cell membranes, the susceptible erythrocytes will bind tightly to the cell membranes. Noninfected cells do not display this binding; therefore, the technique can be used to visualize a focus of infected cells against a background of uninfected cells. For this reason, this particular endpoint may display greater detection sensitivity than other assay endpoints, such as cytopathic effect or hemagglutination. In the

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advanced stages of infection, binding of erythrocytes to cells, to each other, and to open plastic surfaces in the culture vessel may be observed.

The hemagglutination procedure is performed on the conditioned medium and is essentially an evaluation for free virus or viral hemagglutinins in solution. An aliquot of the conditioned medium from a detector culture is combined, in a microwell plate having v-bottomed wells, with one or more types of erythrocytes. After an appropriate amount of time the plates are evaluated. Absence of hemagglutination is reflected by a well-defined pellet (button) of erythrocytes sedimenting to the bottom of the well. In comparison, hemagglutination is reflected by the absence of a button, or by a button with irregular shape. Scoring the latter represents an opportunity for operator subjectivity. In addition, this endpoint can be considered the least sensitive in detection assays, because it is dependent on achieving a sufficient concentration of viral hemagglutinins in solution. For these reasons, hemadsorption is typically viewed as the more useful and reliable of the techniques for detecting hemagglutinating viruses.

The responses obtained in detection assays using hemadsorption and hemagglutination endpoints are highly dependent on the virus being assayed, as well as on the types of erythrocytes used. Many of the viruses of concern to the biotechnology industry do not cause hemadsorption and hemagglutination, or their hemagglutinins react with red blood cell types not commonly used in detection assays.

### DETECTION BY IMMUNOFLUORESCENT ANTIBODY (IFA) STAINING

Certain cell-based viral detection assays are intended to detect specific viral entities and are required for raw materials tests derived from bovine-, porcine-, equine-, and ovine-derived materials (9 CFR 113-53). In order to achieve this, the assays must be optimized with respect to host cell selection, study design, and sample preparation. Specificity of detection is also conferred through use of IFA staining techniques. Primary antisera or monoclonal antibodies directed against the viral antigens of interest are used, either in direct staining applications or in conjunction with a fluorochrome-conjugated secondary antiserum. The immunostained detector cell monolayers are then visualized with an epifluorescence microscope to reveal the presence of reactive infected cells.

## Design of Cell-Based Viral Assays

### VIRAL DETECTION ASSAYS

Detector cell cultures are seeded and allowed to incubate for the appropriate amount of time. Viral samples may be inoculated directly into the medium of the mitotic phase of cell culture, but more typically the medium is removed from the overnight detector cell culture and is replaced with the test sample. For the latter method, the test sample must therefore be approximately isotonic, and cytotoxic agents such as selection agents must be maintained within levels tolerable to the detector cell. For test samples comprised of live cells, the sample must be subjected to freeze-thaw before inoculation in order to lyse the sample cells. If the cells are not lysed, a cocultivation involving the detector and sample cells will result. The latter is part of the design for cocultivation assays. But for most of the cell-based infectivity assays such a cocultivation is not intended and could adversely impact the sensitivity of the assay. The test sample is typically allowed to adsorb to the detector cell monolayers for an appropriate amount of time. It is then removed and replaced with the growth medium suitable for the detector cell. Once inoculated, the detection assay involves incubation of the detector cells for a prescribed amount of time, with periodic refeeding as necessary. Endpoint evaluations as described above are performed according to the study design. Variations of the given procedure may be used for exposing detector cells to raw materials. For raw materials, detector cell exposure may consist of incorporation of the test materials into the growth medium used to maintain the detector cells throughout the assay.

### VIRAL TITRATION ASSAYS

Viral titration assays are designed to generate quantitative information about the virus of interest. These assays do not quantify absolute numbers of viral particles; rather, the results are expressed in terms of infectious units. An infectious unit is the amount of virus required to establish a productive infection, and several categories of titration assays are used. They vary on the basis of the endpoint used to demonstrate infection and include viral plaque titration or plaque-forming units (units: PFU per mL); 50% tissue culture infectious dose (units: $TCID_{50}$ per mL); and 50% fluorescent antibody infectious dose (units: $FAID_{50}$ per mL). The amount of a hemagglutinating virus in a sample can also be expressed in terms of hemagglutinating titer (units: endpoint dilution, i.e., the greatest dilution of the sample which still results in a positive hemagglutination, or HA, response).

Regardless of the endpoint used, a typical titration assay design consists of a sequence of sample dilutions based on 0.5 $\log_{10}$ or 1 $\log_{10}$ increments. The various dilutions of the sample are then applied to an appropriate number of replicate permissive detector cell monolayers. After a suitable incubation time, the monolayers are scored directly for cytopathic effect ($TCID_{50}$ assay), fixed and processed for immunostaining and scored for reactive cells ($FAID_{50}$ assay), or overlaid with agarose and processed for plaque generation PFU assay. $TCID_{50}$ and $FAID_{50}$ titers are typically calculated using published formulas, such as Spearman-Kärber and Reed-Muench. Assay controls for such quantitative assessments should routinely include a reference sample of known potency.

## Detection of Retroviruses

Retroviruses represent a special case for cell-based viral detection assay because of the occurrence of retroviral infection in the absence of responses to the typical endpoints discussed. In order to detect retroviruses, scientists can employ a number of different endpoints. A list of commonly employed indicator cell lines and associated endpoints, and their application in retrovirus detection assays, is provided in *Table 4.*

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**Table 4. Indicator (Detector) Cell Lines Used in Retrovirus Infectivity Testing**

| Cell Line | Assay Type | Endpoint(s)[a] | Target Virus |
|---|---|---|---|
| SC-1/XC | Isolation/direct detection | Plaques (XC); RT | Ecotropic murine retrovirus |
| Balb/C | Isolation/direct detection | Plaques (XC); RT | B-Tropic murine retrovirus |
| NIH/3T3 | Isolation/direct detection | Plaques (XC); RT | N-Tropic murine retrovirus |
| Mink Lung | Isolation | RT; mink S+L- | Xenotropic, amphotropic murine retrovirus |
| Mink S+L– | Direct detection | Foci | Xenotropic, amphotropic murine retrovirus |
| Feline S+L– | Direct detection | Foci | Xenotropic, amphotropic murine retrovirus, gibbon ape leukemia virus (GALV), and RD-114 feline retrovirus |
| *Mus dunni* | Isolation, cocultivation | RT; S+L– | Murine retroviruses and retroviruses infectious to humans |
| QT6[b] | Isolation, cocultivation | RT | Avian retroviruses |
| RD | Isolation, cocultivation | RT; S+L– | Retroviruses infectious to humans |
| MRC-5 | Isolation, cocultivation | RT; S+L– | Retroviruses infectious to humans |
| WI-38 | Isolation, cocultivation | RT; S+L– | Retroviruses infectious to humans |
| 293 | Isolation, cocultivation | RT; S+L– | Retroviruses infectious to humans |
| A549 | Isolation, cocultivation | RT; S+L– | Retroviruses infectious to humans |
| Raji | Isolation, cocultivation | RT | Retroviruses infectious to humans |
| Human PBMC | Isolation, cocultivation | RT, HIV p24 EIA | HIV and other human retroviruses |

[a] The endpoint assays include the following: RT, reverse transcriptase; PERT, product enhanced reverse transcriptase (including also product enhanced reverse transcriptase and real-time quantitative product enhanced reverse transcriptase assays); and EIA, enzyme immunoassay.
[b] A quail cell; primary fibroblast cultures of chicken or turkey origin are also sometimes used.

Retroviral infection is dependent on the presence of receptors on the host cell membranes. The presence of such receptors confers host cell tropism.

## DESIGN FOR RETROVIRUS INFECTIVITY ASSAYS

Two types of infectivity assays are used, depending on the nature of the test material. For materials other than intact cells, the test material is inoculated onto one or more of a variety of detector cells, and the latter are then passaged as required to amplify any virus present. Because of the nature of retroviral replication, cytopathic effects typically do not occur during infection, although there are some exceptions. Before the first subculture and at the end of the final passage, one or more endpoint assays are employed to detect the presence of a retrovirus. For test materials in the form of intact cells, detector cells are seeded and subsequently inoculated with the test cells, resulting in a cocultivation. The cultures are passaged five or more times. Before the first subculture and at the end of the final passage, one or more endpoint assays are employed to detect the presence of a retrovirus.

## ENDPOINT ASSAYS FOR RETROVIRUS DETECTION

Endpoint assays may be classified as direct, which lead to distinct morphological changes in the detector cells; or indirect, as measured by the detection of biochemical, molecular, or immunological markers for infection.

**XC-Plaque Assay**—The XC-plaque assay was developed as a direct means of detecting infectious murine retroviruses. Detection of the retrovirus is accomplished by UV-irradiating the detector cells used to amplify the virus and overlaying the irradiated detector cells with a specific rat cell (XC). The presence of infectious murine retroviruses in the detector cells is reflected by the formation of distinctive syncytia in the XC monolayer, which are easily visualized when the cultures are fixed and stained with a suitable dye such as crystal violet.

The N/B tropism of an ecotropic murine virus may be determined by inoculating Balb/c and NIH Swiss detector cells with the isolate, performing one or two passages on each cell line, and comparing the XC-plaque titer post passage to that determined for the initial isolate.

**Mink and Feline S+L– Focus Assays**—The S+L– focus endpoint was developed to facilitate direct detection of infectious murine xenotropic and amphotropic viruses. The test sample may be inoculated directly into cultures of the S+L– cells, or, alternatively, may be amplified first by inoculation into mink lung, human, or *Mus dunni* detector cells. Cell-free supernatants from the detector cell cultures are used to inoculate the S+L– cells. The latter are infected with a sarcoma virus that is replication-defective, requiring the presence of a helper leukemia virus to render it capable of causing transformation of the host cell. The presence of infectious retrovirus virus in the detector cultures is reflected by the formation of characteristic focal areas of cell transformation in the S+L– cells caused by the rescued sarcoma virus.

**Detection of Retroviral Reverse Transcriptase**—Assays designed to measure reverse transcriptase (RT) activity are useful as an indirect detection method, because the enzyme is indicative of the presence of all retroviruses, whether infectious or not. The RT enzyme is encoded for in the retroviral genome and is used by the virus to transcribe genetic information in viral genomic RNA into proviral DNA.

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*Radiolabeled Nucleotide Incorporation Assay*—The earliest methods for measuring RT activity were based on the measurement of $^{32}P$- or $^{3}H$-labeled nucleotide incorporation into the complementary cDNA product, using an appropriate RNA template. Incorporation of radiolabeled nucleotide at levels higher than a predetermined threshold is interpreted as evidence of the presence of retroviral RT activity. The contributions of cellular DNA polymerases can be ruled out through use of a dual template assay (having both RNA and DNA templates) or inclusion of activated calf thymus DNA.

*Product-Enhanced Reverse Transcriptase (PERT) or Quantitative PERT (Q-PERT)*—Polymerase chain reaction amplification has been used to increase the sensitivity of RT activity measurement. RT activity is detected by PCR amplification of complementary DNA, newly synthesized from an RNA template by reverse transcriptase. The assay may be performed with a gel endpoint (PERT) or as a quantitative assay (Q-PERT). This method has increasingly gained acceptance by regulatory agencies. For more details on PCR-based techniques, see the USP general information chapter *Nucleic Acid Based Techniques—Amplification* ⟨1127⟩.

### ELECTRON MICROSCOPY

Transmission electron microscopy (TEM) may be used to detect and enumerate viral particles within cells. In addition, the technique allows for differentiation of types A, B, C, and D retroviruses based on morphological considerations and can be used to localize viral particles within the cell. As a technique for identification of viruses (including RNA and DNA viruses in general), TEM of sectioned cells is extremely valuable. The cells, typically sampled during the log phase of growth, are pelleted by low-speed centrifugation, and the cell pellet is fixed with a suitable fixative. The fixed cell pellet is embedded, sectioned, stained, and observed with TEM. Size (diameter) of the particles, morphology, presence or absence of surface features such as envelopes and spikes, and location within the cell can be determined with this technique. Such information is important for the identification of a virus. However, failure to observe viral particles with this method does not conclusively demonstrate the lack of viral contamination in the sample.

Biological fluids may also be evaluated by TEM, primarily to determine particle size and concentration. The cell-free supernatant is subjected to ultracentrifugation to pellet any virus present. The resulting pellet is fixed with a suitable fixative. A predetermined number of grid spaces containing representative areas of thin sections of the pellet are evaluated for particles. The enumeration results obtained may show a high degree of variability, and failure to observe particles does not imply that none were present in the sample. Molecular (quantitative PCR and quantitative PERT) endpoints have also been used as alternative methods for estimation of viral particle load in samples.

### ANTIGEN-CAPTURE ENZYME IMMUNOASSAY

Specific viral proteins (e.g., HIV p24 antigen or avian leucosis viral envelope proteins) may be detected as a means of determining the presence of a retrovirus. Viral antigens are captured by specific antibodies coated onto microtiter plate wells and are detected by the addition of a second labeled antibody and appropriate substrate.

## Assays Designed to Detect Specific Viruses

Additional methodologies have been developed to allow detection of specific viruses or groups of viruses. These types of assays are often used for raw material evaluation. In some cases, these specific assays were developed because the target viruses do not cause endpoint responses in the viral screening assays. In contrast to screening assays, specific virus assays are typically optimized for detection of the target virus or viruses. This optimization takes into account the lability of the virus, the host range, the possible endpoint responses elicited, and any special requirements of the target virus. The use of well-characterized viruses as positive controls in such assays provides assurance that the methodologies are suitable for the target virus or viruses. Spiking of the test sample matrix with the positive control virus enables the investigator to assess the potential for matrix interference and to assess the limit of detection for the method. Such considerations are not applicable to screening assays. Specific virus testing for bovine- and porcine-derived raw materials is discussed below. Evaluation of caprine, ovine, equine, canine, and feline raw materials is also stipulated in 9 CFR section 113.47. This section should be consulted with respect to the viruses of concern, and 9 CFR 113.52 should be consulted for methodology. A list of commonly employed indicator cell lines and their application in raw materials testing assays is provided in *Table 2.* A list of commonly employed indicator cell lines and their application in detection of specific viruses is also provided (see *Table 3*).

### DETECTION OF BOVINE VIRUS CONTAMINATION

Raw materials of bovine origin include such commonly employed medium components as fetal bovine and calf serum, serum albumin, collagen, thrombin, and trypsin. Each of these additives represents a route of entry for adventitious viral contaminants into a cell culture or manufacturing process. Requirements for evaluation of such materials ensure the absence of contaminating viruses.

For the details on testing for bovine serum and its derivatives, see future general chapter *Bovine Serum* ⟨1024⟩.

### DETECTION OF PORCINE VIRAL CONTAMINANTS

Raw materials of porcine origin include trypsin as well as other cell culture reagents. The specific porcine viruses of concern in the United States are stipulated in 9 CFR 113.47 and include porcine parvovirus, porcine adenovirus, transmissible gastroenteritis virus, and porcine hemagglutinating encephalitis virus. In addition, porcine raw materials must also be evaluated for the presence of bovine viral diarrhea virus (BVDV), reovirus, and rabies virus. Porcine tissues intended for xenotransplantation into humans also are routinely evaluated for the porcine endogenous retrovirus (PERV). The host cells typically used in the detection of porcine viruses are porcine testicle or porcine kidney, a bovine cell, and Vero cells. The methodology described in 9 CFR 113.52 is analogous to that for evaluation of bovine raw materials and includes provision for multiple subcultures, for Giemsa staining of fixed cells, for hemadsorption testing, and for use of specific immunostaining of fixed cells.

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### CELL-BASED DETECTION OF MURINE MINUTE VIRUS

Murine minute virus (MMV) is a mouse parvovirus that has been detected in biologics manufacturing involving Chinese hamster cell substrates. As with other parvoviruses, MMV represents a special case in that the virus is difficult to inactivate using typical cleaning agents and is capable of surviving for prolonged periods of time on surfaces. Cell-based assays for MMV involve detector cell lines that are especially susceptible to this virus, such as 324K (a human cell) and A9 (a murine cell). Optimization for detection of a parvovirus also includes provision for detector cell subcultures to remain in log-phase division for a significant portion of the incubation period. Endpoints for detection of MMV include one or more of the following: cytopathic effect, hemagglutination of mouse and guinea pig erythrocytes, immunostaining, and polymerase chain reaction.

### CELL-BASED DETECTION OF INSECT-BORNE VIRUSES

Insect-borne viruses include both viruses infectious only for insect cells (e.g., baculovirus) and those transmitted to mammalian cells via insect vectors (arboviruses). Detection of the former may be accomplished using an insect cell as a detector cell. Suitable substrates might include cells of *Spodoptera, Trichoplusia, Drosophila*, mosquito, or other insect origin. Such cells are typically cultured at lower temperatures (25° to 28°) relative to mammalian cells, and many of these cultures are suspension or semiadherent at best. Endpoints may include cytopathic effect, electron microscopy, and PCR.

Of more relevance to patient safety is the detection of arboviruses (insect-borne viruses infectious to animals and humans). This may be accomplished using a suitable mammalian detector cell. The Syrian hamster kidney cell (BHK-21) is a cell line that has shown susceptibility to a wide range of arboviruses. This cell line grows in a monolayer culture, and the endpoints that may be used include cytopathic effect, hemadsorption and hemagglutination, and PCR.

### CELL-BASED DETECTION OF HUMAN CYTOMEGALOVIRUS

Human cytomegalovirus (CMV) is a slow-growing virus of special concern for biologics produced using human cell substrates. It may be detected in cell-based assays using human diploid detector cells such as WI-38 or MRC-5, provided that sufficiently long durations of incubation are employed (28 or more days). The endpoints include cytopathic effects and immunostaining and/or PCR.

## In Vivo Methods

Intact and susceptible animals may serve as potential host organisms for detecting viruses in test samples. In this case, viral proliferation in the tissues of the host animal may be reflected as adverse health effects (including death) that can be monitored and recorded. Viral detection assays based on intact animals are intended to complement in vitro assays, because some viruses that do not cause a response in the in vitro assays may be detectable in the animal systems (and vice versa). Viral safety studies employing live animals must be performed in accordance with applicable regional guidelines for the ethical use of animals, using laboratories that are accredited for the housing of the animals.

### IN VIVO VIRAL SCREEN

The in vivo viral screen is used primarily for cell bank, viral seed stock, and viral vaccine testing and is considered to complement the in vitro virus screening assay. Multiple animal species, as well as multiple injection routes, are employed to provide a broad range of host tissues and possible responses. A list of commonly used host animals, routes of inoculation, and target viruses are shown in *Table 5*.

**Table 5. In Vivo Viral Screening Assays**

| Host Animal | Route of Inoculation | Target Virus |
|---|---|---|
| Suckling mouse | Intraperitoneal injection | Arboviruses |
| | Intracranial injection | Coxsackie A and B |
| | *Per os* injection | Herpes simplex Type 1 and 2 |
| | | Togaviruses |
| | | Junin |
| | | Herpes B |
| Adult mouse | Intraperitoneal injection | Rhabdoviruses |
| | Intracranial injection | Togaviruses |
| | *Per os* injection | Lymphocytic choriomeningitis virus (LCMV) |

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**Table 5. In Vivo Viral Screening Assays** *(continued)*

| Host Animal | Route of Inoculation | Target Virus |
|---|---|---|
| Guinea pig | Intraperitoneal injection | Rhabdoviruses |
| | Intracranial injection | LCMV |
| | | Lassa |
| | | Junin |
| | | Marburg |
| | | Ebola |
| | | Vaccinia viruses |
| Embryonated hens' eggs | Allantoic | Arboviruses |
| | Yolk sac | Equine encephalomyelitis viruses |
| | Chorio-allantoic membrane | Herpes viruses |
| | | Influenza |
| | | Mumps |
| | | Newcastle disease |
| | | Parainfluenza Types 1 and 2 |
| | | Rabies |
| | | Vaccinia |
| | | Variola |
| | | Lymphogranuloma venereum |
| | | Ornithosis |

Following injection of the test sample, each animal model is monitored for an appropriate period of time that allows for the observation of clinical signs of viral infection. Any abnormality is investigated to determine the cause of the effect.

The suckling mice are observed for an appropriate period of time. Pooled homogenates from any surviving animals are then passaged into additional litters of suckling mice. The latter are observed for an additional period of time.

The guinea pigs are observed for clinical signs of viral infection and for injection site lesions. Necropsy for gross tubercular lesions is performed for certain types of test samples.

Allantoic fluids from eggs can be tested for hemagglutination of chicken, guinea pig, and human type-O erythrocytes. Additional fluids are pooled for each treatment group (test article and control), and these are passaged (inoculated) into a new group of embryonated eggs. Following an appropriate incubation period (typically measured in days), the allantoic fluids are again tested for hemagglutination of chicken, guinea pig, and human type-O erythrocytes. Following injection by the yolk sac route, the eggs are incubated for at least 9 days and are assessed for viability. The yolk sacs are then harvested and pooled for each group (test article and control), and a 10% solution of the resulting material is inoculated by the same route into a new group of embryonated eggs. The eggs are again incubated for an appropriate period of time (days) and are assessed for viability.

## IN VIVO ASSAYS INTENDED TO DETECT SPECIFIC VIRUSES

Some in vivo assays are designed to detect, if not specific viruses, at least specific sets of viruses. The antibody production assays use the production of a humoral immune response in susceptible host animals inoculated with test samples. Viral antibody-free animals of the various species are injected with the test sample. At the end of an appropriate incubation period, one or more of a variety of endpoint assays may be performed to detect the generation of a humoral antibody response in the animal sera. Production in the animal of antibodies directed against a specific virus provides evidence of the presence of viral antigen or infectious virus in the test sample. This type of assay is typically used to ensure that rodent cell banks and viral seed stocks are free of adventitious viruses. Three antibody production assays, along with the route of injection and target viruses, are summarized in *Table 6*.

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**Table 6. In Vivo Antibody Production Assays**

| Antibody Production Assay | Route of Injection | Target Virus |
|---|---|---|
| Mouse antibody production (MAP) assay | Intranasal | Ectromelia |
| | Intraperitoneal | Hantaan |
| | Intracranial | Mouse K |
| | | Lactate dehydrogenase elevating virus |
| | | Lymphocytic choriomeningitis virus (LCMV)* |
| | | Murine minute virus |
| | | Mouse adenovirus |
| | | Mouse cytomegalovirus |
| | | Mouse encephalomyelitis virus type II |
| Mouse antibody production (MAP) assay | | Mouse hepatitis virus |
| | | Epizootic diarrhea of infant mice |
| | | Pneumonia virus of mice |
| | | Polyomavirus |
| | | Reovirus type 3 |
| | | Sendai |
| | | Mouse thymic virus |
| Hamster antibody production (HAP) assay | Intranasal | Lymphocytic choriomeningitis virus (LCMV)* |
| | Intraperitoneal | Polyomavirus |
| | Intracranial | Reovirus Type 3 |
| | | Sendai |
| | | Simian virus 5 |
| Rat antibody production (RAP) assay | Intranasal | Hantaan |
| | Intraperitoneal | Kilham rat virus |
| | Intracranial | Mouse encephalomyelitis virus type II |
| | | Polyomavirus |
| | | Reovirus type 3 |
| | | Sendai |
| | | Toolan's H1 virus |
| | | Rat coronavirus/sialodacryoadenitis virus |

* A group of test sample–injected mice is challenged with a known lethal dose of authentic LCMV. If this group of mice does not die from the challenge dose, a second group with twice the number of mice is used for a repeat challenge. If there are survivors in this group, the test sample is considered positive for LCMV.

## Considerations for Validation, Matrix Qualification, and Quality Control of Cell- and Animal-Based Test Systems

Viral detection assays used to ensure the viral safety of human and animal therapeutics are expected to have undergone validation. The approach to the validation depends on the nature of the assay and associated regulatory compliance level.

Any assay should be sufficiently developed that it can be performed with an appropriate set of predetermined system suitability and acceptance criteria. These criteria usually include the use of relevant negative and positive controls but may also include requirements for linearity and meeting of a predetermined detection limit. The results constituting a positive or negative response in the assay should be established prior to execution of the validation. An assay used under GMP compliance is expected to have been validated according to appropriate guidelines.

An assay used to ensure the safety of a commercially marketed biological product must be further characterized for suitability in the presence of the specific product matrix. The matrix qualification study should address the potential for specific interference with the viral detection endpoints used in the assay, and typically involves spiking of one or more model viruses into the product matrix at levels approaching the limit of detection to ensure the absence of interference.

For quantitative detection assays, the detection limit should be probed. This usually involves spiking of the model virus(es) at decreasing amounts into medium or the product matrix. The lowest spiking level of the virus reliably detected is used as an approximation of the actual limit of detection of the assay. Experimental error for these cell culture-based assays is usually expected to be in the range of 0.5 to 1 $\log_{10}$. The determination of a detection limit is less meaningful for limit tests and viral screening assays in general. For the latter, knowledge of the detection limit for one virus does not imply a similar limit for another

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virus. Since screening assays are not optimized for a specific virus, the limit of detection for the assay can vary greatly from one virus to another.

Animal-based viral detection systems are generally not subject to the requirements for validation, matrix qualification, use of positive controls, and determination of detection limit that regulatory agencies expect of cell-based and biochemical tests. The use of animals for safety testing is subject to the regional guidelines for the ethical use of animals, and the kinds of activities listed above generally are not considered appropriate use of animals. However, negative control animals are included in these assays, and retrospective validation or gap analysis based on historic incidence of system suitability failures or positive findings is sometimes possible.

## DETECTION OF VIRAL COMPONENTS

Direct detection of viral components can provide a direct measurement of viral levels in a sample preparation. It has also become primarily important for detection or identification of viruses in biological products or in the raw materials used in their manufacture. Systems capable of identifying components unique to specific phases associated with viral latency and replication are now available. During interaction with their host cells, viruses may incorporate modified host molecules during the production of new intact virus particles, or they may induce discernable changes in host cell makeup or function.

Most immunological methods and reagents currently available detect the constituents of intact virions. It is the relative abundance of these proteins that makes them most amenable to the development of antibody-based reagents. Abundance also makes them optimal targets for detection of the virus. Recently developed targeting and detection reagents are aimed at minor viral components that may be found only during specific phases of replication. These allow a more detailed analysis of the stage of viral infection. The basic methodology for the detection of viral antigens is well established, but more recent innovations in materials and reagent development have broadened its application.

Developments in the targeting and detection of viral nucleic acid components have led to enzyme-based systems for the amplification of nucleic acids in vitro and in situ (see *Nucleic Acid-Based Techniques—Amplification* ⟨1127⟩). The potential specificity of this detection method allows the examination of biological systems with a high degree of confidence for the presence or absence of a specific targeted virus. A wide variety of reagents, technology platforms, and methodologies are available. The aim of this section is to elaborate on the most common practices and platforms used in the detection of viral components.

### Sample Selection and Preparation for the Detection of Viral Components

This subsection addresses general considerations for various types of test samples and the most common assay targets (viral proteins and nucleic acids). The target proteins may have varying levels of posttranslational modification (e.g., glycosylation, phosphorylation), and the target nucleic acids may be either RNA or DNA, single or double stranded. Therefore, it is important to have a basic understanding of the physicochemical nature of the virus under study so that the sample handling procedures support the detection of the target component. For detailed considerations regarding the extraction of nucleic acids, see the USP general information chapter *Nucleic Acid-Based Techniques—Extraction, Detection, and Sequencing* ⟨1126⟩.

#### Types of Samples

##### CELLULAR

When viral components are associated with intact cells, samples must be generated either as whole cell lysates or as subcellular fractions. Maintaining the temperature of the test samples at or near freezing (0° to 4°) during processing and limiting the time that samples are held in an unfrozen state will reduce the potential for loss of target antigens and nucleic acids due to cytosolic enzymes (proteases, nucleases) present in the cell lysates. Reagents that can inactivate or limit the activity of such enzymes may be used to prevent degradation of the target components, especially when exceptionally labile samples must be handled at room temperature. Centrifugation of intact cells allows for some additional manipulation of the sample matrix. The growth medium can be discarded and the cells suspended in a buffer formulated to enhance the recovery and detection of the targeted viral component. Collection and storage parameters should also account for the presence of cellular DNA. This can increase the viscosity of the sample, rendering it difficult to pipet.

##### TISSUE CULTURE SUPERNATANT

Depending on the stage of the infection and the type of virus involved, the conditioned medium may represent a preferred test sample. An advantage is that the presence of cellular debris can usually be reduced through use of a low-speed centrifugation (clarification) step. The main disadvantage is the potentially low concentration of the analyte, and therefore concentration of the sample may be required.

##### PROCESS INTERMEDIATE (UNPROCESSED BULK HARVEST)

In general, viral safety lot release testing is done at the bulk harvest stage prior to any purification. This is true regardless of whether the assay detects infectious virus or viral components. The presence of host cell DNA may need to be assessed in the case of biologics manufactured in animal cells.

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## Sample Stability and Matrix Effect

Sample stability is a key element in the successful detection of viral components. Protein structure can be altered by numerous environmental factors, including pH, ionic strength, solvents, detergents, temperature, and free radicals. In addition, complex biological matrices frequently contain proteolytic enzymes that can alter or destroy key antigenic features of a protein or peptide. Sample collection, storage, and handling must allow maintenance of the antigenic features targeted by reagent antibodies.

Conformational changes affecting the opportunity for antigen detection are difficult to address. Depending on the reagents required for detection, conformational changes may be required for antigen detection. For example, if antibodies are produced for an antigen detection system using native viral antigen, then unmasking and maintaining the conformation of the antigen throughout the sample preparation is essential. Conversely, if peptide fragments are used to produce antibody, then a denaturation step may be required to allow for effective antigen detection.

Conditions associated with sample preparation must be investigated in a combinatorial fashion whereby one parameter or component is varied while all others remain fixed. In this way, the formulation of lysis and processing buffers can be optimized for pH, ionic strength, and types of detergents and denaturants. The sample preparation steps must condition the targeted antigen in order to obtain the form most readily recognized by the reagent antibody.

The stability of nucleic acids in test samples is largely affected by nuclease activities present in the sample and the degree of protection provided by the intact structure of the virus particle. Encapsidated nucleic acids are particularly stable as long as the integrity of the capsid is maintained. Viral capsids are vulnerable to proteolytic digestion. Virus particles stored at ambient temperature as part of a complex biological matrix are especially susceptible to degradation by proteases. Storage at refrigerated temperatures (2° to 8°) for short periods of time or at temperatures below freezing can be used to limit proteolytic activity. When samples are stored at frozen temperatures, freeze-thaw cycles should be limited. Under conditions where an individual sample must be accessed multiple times, preparation of aliquots is advisable.

### SAMPLE COLLECTION

In a biotechnology setting, sample collection is dictated by sampling plans that are established to meet regulatory requirements. Nucleic acid testing in association with an amplification step has the potential of detecting a virus at the earlier stages of infection. The use of nucleic acid amplification methods reduces the dependence on timing and the amount of material required, because the amplification process effectively boosts assay sensitivity by increasing the amount of target relative to background.

General aspects of nucleic acid sample preparation and stability are discussed in *Nucleic Acid-Based Techniques—Extraction, Detection, and Sequencing* ⟨1126⟩. The following section specifically addresses the unique aspects of viral sample selection and preparation.

### SAMPLE STORAGE

Conditions for sample storage should be consistent with maintaining the antigenic properties of targeted viral proteins and/or preserving the nucleic acid content of the sample. The duration and temperature of storage is dictated also by cycle times associated with testing.

### IMMUNE COMPLEX DISRUPTION

The masking of antigen epitopes may occur when other proteins associate at or near the epitope targeted by reagent antibodies. For viral antigens this may occur when the antigen comprises the structural component of the virus. Such viral antigens are likely to retain strong affinities for other viral proteins or the ability to exist in multimeric form under normal conditions for detection. Another obstacle for epitope recognition is the naturally occurring immune complex when reagent antibodies have been developed to detect native protein in a blood plasma matrix. Immune complexes consisting of viral antigens and host antibodies are normal in such physiologic samples. The stronger the host immune response, the more likely masking of antigen due to immune complex formation will occur. Methods aimed at the preparation of blood and plasma samples for detection should address the presence of preexisting immune complexes and incorporate steps designed to disrupt such complexes to improve the opportunity for viral antigen detection.

## Detection of Viral Antigens

Viral capsid proteins are common targets for antigenic detection methods. Structural proteins that make up the framework of the viral core are often some of the most abundant viral proteins produced during viral replication. In nonenveloped viruses, the core structural proteins are likely to provide the dominant antigenic features. When the virus is enveloped, proteins associated with the envelope often provide key antigenic features. This section examines methods commonly used to detect viral antigens and addresses considerations aimed at optimizing formation of the appropriate immune complex.

### ASSAYS USED FOR THE DETECTION OF VIRAL ANTIGENS

Immunologic methodologies used to detect viral antigens are based on the specificity and affinity of the antibody and viral antigen interaction. Of the various platforms available, one commonly employed in viral safety testing is immunofluorescent antibody staining. This lends some degree of viral specificity to the cell-based methods described in the first part of this chapter. Other techniques include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay, and Western blotting. The principles and general methods for these assays will be described in the USP general information chapters *Immunological Test Methods—General Considerations* ⟨1102⟩, *Immunological Test Methods—Reagent Development* ⟨1103⟩, *Immunological Test Methods—Immunoassay Methodologies* ⟨1104⟩, and *Immunological Test Methods—Assay Design, Quality Control, and Data*

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*Analysis* ⟨1105⟩, being prepared for future publication. The following sections will generally address aspects specific to their use in virology.

**Immunofluorescence Assay**—The immunofluorescence assay (also referred to as immunofluorescent antibody staining) is used to detect viral proteins expressed in various cellular compartments. Since the technique can detect viral antigens within single cells, it confers a high degree of sensitivity and enables infection to be detected at a very early stage. The technique is often employed as a detection endpoint for cell-based viral infectivity assays to provide additional sensitivity and specificity (see tests for raw materials as described in 9 CFR 113.53). In addition, the technique is employed for verifying the identity of viral stocks and is useful as a means of identifying viruses detected in viral screening assays.

**Enzyme-Linked Immunosorbent Assay (ELISA)**—ELISA is best suited for detection of soluble antibodies and antigens in a variety of test samples. Sensitivity, quantification, robustness, ease of experimentation, and readily available inexpensive reagents make it adaptable to a high throughput environment. For viral antigen detection, a sandwich ELISA assay is commonly used. A viral antigen specific antibody (preferably a monoclonal antibody with high affinity) is first immobilized onto a solid phase. The test sample is then incubated for a predetermined period under appropriate conditions. After washing, a second antibody that recognizes the viral antigen is incubated. The second antibody is linked to either an enzyme or a chromomeric reagent that emits signal with an appropriate substrate and can be recorded with an appropriate instrument. The assay can be performed in a qualitative or a quantitative manner. For a qualitative ELISA assay, sufficient replicates of both positive and negative control samples are required in order to determine the appropriate cut-off value and the assay acceptance criteria. A mean value of a test sample that is equal to or greater than the cut-off value is considered positive. For a quantitative ELISA assay, an additional standard curve with a positive reference standard material of known quantity must be established. The number of replicates should be adequate to determine the assay variation and linearity. The quantity of test sample can be calculated against the standard curve.

**Radioimmunoassay (RIA)**—The radioimmunoassay is a versatile quantitative immunoassay that can be used to detect substances including viral antigens and antibodies. It even can be applied to nonprotein molecules as long as an antibody that specifically binds the test substance is available. Radioimmunoassays can be customized in different formats to suit specific test requirements. Many of the considerations taken into account with other immunological assays are applicable to the RIA. In general, radioimmunoassays can be divided into two major categories: solution (homologous) and solid-phase radioimmunoassays. Both methods have been successfully used to detect and quantify a variety of viral antigens or components of viruses, such as hepatitis A, B, and C; human and murine retroviruses; adenovirus; avian C-type virus; rubella virus; and respiratory syncytial virus.

**Western Blotting (Immunobloting)**—Western blotting, also known as immunoblotting, is used to identify specific antigens in the presence of other, potentially cross-reactive antigens. In this case, the specificity required is obtained by combining the antigen-antibody reaction with some form of separation (typically electrophoresis). Depending on the visualization methods employed (including digital methods such as densitometry), this method can be quite sensitive and even semiquantitative. One advantage of this approach is that in addition to detecting the antigen using an immunological approach, data on the approximate mass of the target protein may be obtained. A potential caveat is that most test proteins are denatured during this procedure and that antibody that depends on epitope conformation may not recognize the linear epitopes.

## Detection of Viral Nucleic Acids

The detection of viral nucleic acids provides another route for the determination of viral loads and for establishing the identity of a contaminant. Nucleic acids, like protein antigens, are essential components of viruses, and detectable quantities are usually indicative of viral presence. Detection assays can be designed and developed in some cases to parse viremia into phases, especially when the differentiation of nucleic acids along functional forms and configurations can provide clear insight into viral activity. Assays can be designed to determine whether viral DNA has been integrated into the host genome or still is encapsidated. Early viremia may be detected as viral mRNA transcripts prior to the accumulation of detectable viral particles. Nucleic acids may be the only detectable viral component of viruses that do not replicate well in tissue culture systems. Such systems may fail to produce mature virus particles, but the detection of viral transcripts can provide insight into whether the virus has the ability to infect the cell. Nucleic acid testing represents the most useful endpoint for the detection of certain viruses failing to cause responses using typical endpoints.

### SAMPLE PREPARATION: SPECIAL CONSIDERATIONS FOR NUCLEIC ACID TESTING

The degradation of nucleic acids in samples can be limited through proper handling and storage practices and even enhanced by closely linking sample collection and preparation steps. In addition to preparing nucleic acids for further processing, denaturation is an important step toward stabilizing nucleic acids where storage temperatures extend above 0°.

**Denaturation and Dissociation of Virions (Viral Lysis)**—Chaotropic detergents and salts can be important agents for disrupting and removing viral proteins that make up the viral capsid. Their addition can provide a useful first step when concentration of virions is not necessary or even possible. In sufficient quantity they rapidly denature the entire contents of a biological sample, essentially fixing nucleic acid content through the inactivation of nucleases and other proteins that may affect sample stability. Saturated solutions containing guanidium salts, such as guanidine hydrochloride or guanidine isothiocyanate, are commonly used for the dissociation of viral nucleic acids from protein components. These solutions may be used alone or in combination with ionic detergents and other denaturants such as phenol. The main advantage of guanidinium salts is that they are readily removed during the concentration of viral nucleic acids using ethanol or isopropanol. Urea may also be used as a mild denaturing agent, although it does not perform as effectively as guanidine in its ability to disrupt virus particles.

**Deproteinization**—The removal of proteins during the processing of samples for the detection of viral nucleic acids is helpful in ensuring the reproducibility and robustness of assays, particularly those that rely on amplification to detect exceptionally low quantities of nucleic acids. Several strategies may be used to facilitate deproteinization of the sample; they are discussed in *Nucleic Acid-Based Techniques—Extraction, Detection, and Sequencing* ⟨1126⟩.

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**Recovery of Viral Nucleic Acids**—Separation and recovery of extracted viral nucleic acid are important steps in the testing of nucleic acids. Nucleic acid yield and purity obtained at this step are critical determinants of assay robustness. Poor nucleic acid recovery and limited purification may inhibit amplification and detection reaction resulting in poor assay sensitivity. The development of high-yield, high-purity recovery steps is an important goal in the optimization of nucleic acid detection methods. Details on the general aspects of extraction and detection of nucleic acids are discussed in *Nucleic Acid-Based Techniques—Extraction, Detection, and Sequencing* ⟨1126⟩. However, if no inhibitory components are identified in the system, direct testing on an aliquot of a sample may increase the assay sensitivity.

**Isolation of Viral DNA**—Integrated viral genomes must be recovered and processed along with the genome of the host cell. The viral genome is analyzed within the context of the host genome, and therefore similarities between virus and host genomes must be accounted for to ensure that assay results are specific for the virus and do not simply reflect the presence of cross-reacting host genome sequences. Viral genomes that exist as episomal entities require similar consideration, but there may be opportunities during sample preparation to limit the amount of host nucleic acid present in the preparation. Sedimentation gradients or silica-based separation chromatography can sometimes be used to enrich episomal nucleic acids through size-related exclusion or partitioning of processed nucleic acids.

The recovery of encapsidated viral genomes may allow for larger amounts of material, especially if the target virus has accumulated in large quantities in the system being monitored. When the amount of material available for recovery is low or the processing of large amounts of the material is impractical, the process of isolating and conditioning the virus particles, and subsequently the encapsidated genome, must be compatible with the assay system that will be used. Portions of the viral genome may need to be amplified, or the genome itself may be captured in an elaborate process that allows detection through the generation of an amplified signal. A standard method for isolating viral genomes may need to be modified appropriately to ensure that materials used in the preparation do not interfere with steps conducted later in the process.

Viral genomes that consist of RNA are typically converted to a DNA intermediate that is easier to handle and store. Methods aimed at the isolation of viral genomes consisting of RNA, however, require similar considerations concerning the localization of the genome: replicating RNA viral genomes may be recovered as part of a preparation of host total RNA or even mRNA if the genome contains polyA sequences. RNA lends itself more effectively to hybrid capture methods during isolation, and hybrid capture can be used to enrich RNA preparations specifically for RNA viral genomes. RNA genomes can be extracted from virus particles in much the way that DNA viral genomes are extracted. RNA genomes are converted to complementary (cDNA) sequences using retroviral RT. Storage is of greater concern for naked viral RNAs. Storage of RNA usually requires temperatures below –20°.

## Detection of Viral Genome Versus Viral Transcripts

Viral genomes exist as either DNA or RNA, or sometimes both: in the case of retroviruses the integrated genome is DNA, whereas the encapsidated form is RNA. The ability to differentiate among the various forms of viral nucleic acids can help to elucidate the course of specific viral infections. Assays for nucleic acid activity can differentiate readily between integrated and encapsidated genomes when the form of the viral nucleic acid varies between states, as in the case of retroviruses. Incorporation of specific nucleases into the assay methodology can be used to reduce or eliminate one form over the other. If viral genomes are known to integrate at specific sites within the host genome, primers and probes can be developed around the integration site and incorporate significant elements of both host and viral genomes. Some viral mRNAs contain splice sites, and the differentiation of spliced nucleic acid sequences from unspliced sequences creates a unique mechanism for determining the status of nucleic acid localization and infection.

### CHARACTERIZATION OF DNA VIRAL GENOMES

Methods for the recovery and preparation of viral genomes for characterization depend on the state of the viral genome. If the genome has been incorporated into a cellular compartment, the recovery and preparation strategy must take into account the cellular components that make up the sample matrix. If the viral genome targeted for analysis is the encapsidated form, the methods must focus on recovery of the virus particle and must include additional steps aimed at extracting the nucleic acid from the individual particles. Identification and characterization of viral genomes require specific complementary nucleic acid probes and primers whose sequence will be dictated by available information about the sequence of the targeted viral nucleic acids and the type of assay that will be used. Determination of the sequence of the viral nucleic acid of interest usually provides the most unambiguous means for characterization. However, a number of methods can be used as simple indicators for the presence or absence of specific sequence-based characteristics. For example, melting curve profiles using short oligonucleotide sequences can be used to establish whether a specific viral genotype is present.

### IDENTIFICATION AND GENOTYPE ANALYSIS

Nucleic acid testing is often used to identify viral isolates obtained from viral screening assays or to provide identity for viral stocks. Methods used for the identification of viral genomes are not unique to other applications in the field of molecular biology. Typically, an amplification step is required in order to achieve quantities for analysis. Amplified sequencing of the amplicons or application of a standard hybridization technique may be employed for more detail as to the nature of the amplified signal. For more details, refer to *Nucleic Acid-Based Techniques—Amplification* ⟨1127⟩.

### HYBRIDIZATION TECHNIQUES

A variety of hybridization techniques are used to detect viral nucleic acid sequences, including Southern blot, Northern blot, DNAse/RNase protection, in situ hybridization, microarray technology, and other techniques. The description of these methods, which is well beyond the scope of this chapter, can be found in *Nucleic Acid-Based Techniques—Extraction, Detection, and Sequencing* ⟨1126⟩.

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# DETECTION OF ANTIBODIES TO VIRAL ANTIGENS

A variety of methods are available for detection and quantification of antibodies to viral agents, including neutralization, complement fixation, and immunoassays based on enzyme- or fluorescently labeled reagents. Although many details of the immunological methods mentioned above are beyond the scope of the chapter, this section addresses specific application aspects of viral antibody detection, including preparation and storage of test samples, common assay methods and platforms available, and specific examples of how these assays may be used to measure antibodies to specific viral agents.

## Viral Structure Relative to Antigenic Composition and Selection of Antibody Assay

Mammalian viruses vary considerably in their nucleic acid content and thus the number of antigenic, virus-specific proteins produced.

Many viral proteins or glycoproteins are highly antigenic and induce a potent humoral immune response during natural infection, whether in humans or in animal models. In most cases, the immune system responds when the virus and its antigens appear in the extracellular fluid or on the infected cell membranes. The degree to which viral antigens are expressed is governed by the intracellular replication and protein synthesis of viruses in host organ tissues and by the several possible types of virus–host cell interaction. Antibodies produced as a result of natural viral infection are likely to represent the broadest response to antigens in their native state.

When selecting, developing, or evaluating an assay method for measurement of antibodies to viral proteins, the analyst must take into account the source of the antibodies and the method by which they were obtained or prepared. Details will be presented in *Immunological Test Methods—Reagent Development* ⟨1103⟩ and *Immunological Test Methods—Immunoassay Methodologies* ⟨1104⟩.

## General Considerations Regarding Sample Preparation for Antibody Detection of Viral Antigens

Antibodies are relatively stable, but care must be taken to ensure the integrity of the test antibodies during sample preparation and storage. Serologic tests may be developed to measure antibodies to viral agents in unfractionated biological fluids. The possibility for matrix interference with the antibody detection method should be considered.

In general, biological test samples should be clarified by centrifugation or filtration, depending on their intended use. Serum samples should not be hemolyzed, lipemic, or icteric. In some cases the specimen should also be heat-treated to inactivate endogenous complement and other components.

Test samples should be processed as soon as possible. When it is necessary to store samples, most test samples should be stored at –20° for short-term storage and below –80° for long-term storage. For all samples, the stability of the material needs to be assessed experimentally. Aliquots of appropriate volume should be prepared in accordance with test procedures to avoid unnecessary freeze-thaw cycles.

## Antibody Methods

This section discusses the primary methods for detecting antibodies directed against viral antigens. The assay methods often include a variety of alternative formats for the detection of antibody. Only the more commonly used formats for antibody detection are discussed in this section. Some methods, including fluorescent antibody assays and enzyme immunoassays, are widely applicable to the detection of antibodies to many different viral agents; others are limited to selected viruses having certain properties (e.g., hemagglutinins).

### IMMUNOFLUORESCENCE MICROSCOPY FOR ANTIBODY DETECTION

When fluorescein isothiocyanate (FITC) is chemically coupled to an antibody molecule, the resulting *FITC-labeled antibody* can be used as a secondary antibody probe to detect the presence of a primary, virus-specific antibody bound to a virus-infected cell on a microscope slide (indirect immunofluorescence).

The indirect immunofluorescence or indirect fluorescent antibody (IFA) assay is one of the most basic and useful methods for detection of antibodies to viruses. The assay can be used to detect both virus-specific IgG- and IgM-class antibodies. When the assay is used to detect IgM antibodies, it usually requires the physical removal or inactivation/binding of IgG-class antibodies. In the absence of this step, the presence of IgM-specific antibody may be masked by excess IgG-specific antibody competing for primary binding sites on the substrate surface. IFA assays may be qualitative or quantitative.

The IFA for antibody to viral agents requires the use of virus-infected cells expressing viral antigens in cellular membranes. Viral stocks are prepared, titered, and used to infect permissive cells in tissue culture. The cells are harvested at appropriate times, washed, and spotted onto multiwell microscope slides at an appropriate density. Control slides are also prepared with noninfected cells. The slides are allowed to air-dry and then fixed in cold acetone. The fixed slides can then be stored under appropriate conditions for extended time periods. The stability of the viral antigens over time should be confirmed.

The test article to be examined for the presence of virus-specific antibodies can be applied to the slide, followed by an appropriate secondary antibody conjugated with a fluorescent tag that can be visualized under a fluorescent microscope. IgG- or IgM-specific antibodies can be distinguished by using the appropriately prepared secondary antibody.

Reading and correctly interpreting endpoints of IFA slides for antibody detection requires an experienced analyst, particularly when cellular location and fluorescent-staining patterns are critical for a specific virus. Such interpretation requires the use of appropriate controls and scoring or intensity of fluorescence. This is highly dependent on the quality of reagents, the consistency of the fluorescent microscopy and light source being used, and the experience of the analyst.

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## ENZYME IMMUNOASSAY FOR ANTIBODY DETECTION

The EIA and variations of it are the most widely used methods for the detection of viral antibodies in serum and other biological products. The most commonly used EIA for antibody detection is referred to as a noncompetitive solid phase EIA for antibody detection. The typical configuration of an EIA for antibody involves coating tubes or microwell plates with viral antigen(s), the addition of test serum or product to the tubes or wells, the binding of specific antibody in serum or product to antigen, and the detection of bound antibody by addition of a second antibody with binding affinity to the primary antibody, which is labeled to allow for its detection.

The assays can be specific to IgG- or IgM- class antibody or may detect total antibody. Assays for IgM may achieve improved specificity when performed as *IgM-capture* assays. These assays involve the use of plates or wells coated with anti-IgM antibody to capture total IgM in serum or product as a first step. Subsequently, viral antigen is added; it binds to the plate only if virus-specific IgM antibody has initially been captured, and it is detected by addition of a second labeled antibody specific to viral antigen. The assay is most often performed as a qualitative measure of the presence of an antibody for a specific virus. Sufficient replicates of both positive and negative control samples are required in order to determine the appropriate cut-off value and the assay acceptance criteria. A mean value of a test sample equal to or greater than the cut-off value is considered positive.

## COMPLEMENT FIXATION TEST

Complement fixation has selective value in allowing for simultaneous assay of antibodies to a wide variety of viral agents. The procedure involves multiple variables consisting of two pairs of antigen–antibody reactions. The first reaction, between a known virus antigen and a specific antibody in the test sample, takes place in the presence of a predetermined amount of exogenous complement. The complement is removed by the antigen–antibody complex. The second antigen–antibody reaction consists of sheep red blood cells (SRBCs) and hemolysin (antibody against SRBC). When this indicator system is added to the reaction mixture, the sensitized SRBCs will lyse only in the presence of free complement. The extent of lysis of SRBCs is inversely correlated with the amount of the antibody in the test article.

The experimental procedure involves the optimal titration of concentrations of hemolytic serum, complement, and viral antigen, using chessboard format. If used as the test sample, human serum should be inactivated at 56° for 30 minutes to inactivate the endogenous complement activity. A number of important controls must be run along with the test, and results must be within limits before the test can be properly interpreted. These include the sensitivity of SRBCs to lysis and complement concentration used. The relative amount of virus-specific antibody present can be determined by testing serial dilutions of the serum or product. The *complement-fixing titer* is the reciprocal of the highest dilution that prevents 50% hemolysis.

## NEUTRALIZATION FOR ANTIBODY DETECTION

Neutralization for the measurement of antibodies to viral agents is still one of the most valuable assays available because of its high specificity and its ability to detect neutralizing antibodies. Neutralization is defined as the loss of viral infectivity through the binding of specific antibodies to viral coat proteins (or envelope glycoproteins) on the surface of the infectious viral particle. The assay may be used to measure the presence of antibodies to a known virus in a serum or product sample, or conversely to identify an unknown virus by using a serum or product sample containing known antibodies.

Before performing a neutralization assay to measure the presence of antibodies in serum or product, a known virus must first be grown and titrated in the test system in which the neutralization assay will be performed. For viruses prepared in cell culture, this usually involves inoculating susceptible cultures with relatively low multiplicity of infection (MOI; <1 PFU per cell) and harvesting the infected cells when about 50% to 75% cytopathic effect (CPE) is demonstrated. The virus preparation is then titrated by preparing serial multifold dilutions and inoculating replicate tubes or plate cultures with a fixed volume of the virus preparation. The endpoint of the titration is the dilution of the virus that will infect 50% of the cell cultures inoculated. This endpoint is said to contain one 50% tissue culture infective dose ($TCID_{50}$) in the volume used. If the test system involves animal lethality, the endpoint is referred as one 50% lethal dose ($LD_{50}$). The amount of virus used in the neutralization assay to follow is typically standardized to contain 100 $TCID_{50}$ or $LD_{50}$.

The test or host system used in neutralization assays is chosen on the basis of the specific virus to be tested and its ability to replicate in the system. The commonly used host systems include cell culture, embryonated chicken eggs, and mice. Cell culture is usually the preferred test system, because the viruses used in the neutralization assay usually readily replicate and produce CPE. Susceptible host cells are grown in monolayers in dishes or multiwell plate cultures. After the virus/neutralizing serum mixture is added, the cultures are overlaid with agar-containing medium to restrict spread of CPE and allow development of viral plaques. The prevention of plaque development is indicative of the presence of neutralizing antibody. Alternatively, neutralization can be performed in tube monolayer cultures or even in suspension tissue culture. Embryonated eggs may be used when the virus to be used or tested does not produce plaques in tissue culture systems. The route of inoculation and the endpoint depend on the virus.

Neutralization assays may be set up in various ways, depending on the specific virus of interest and the serum or product to be tested for neutralizing activity. In general, a fixed amount of infectious virus is preincubated with undiluted and serial dilutions of serum or product to be tested for neutralizing activity and separately with preimmune serum or control product; this approach is referred to as the *constant virus–varying serum* method. Following preincubation, the mixtures are separately injected or added to the test system. Reduction in infectivity between test and control serum or product is scored in various ways, depending on the test system. The endpoint of the assay is generally defined as the highest dilution of the serum or product that neutralizes one-half of the initial viral inoculum, as calculated by Reed-Muench or the Spearman-Kärber method.

The titer of neutralizing antibody in the test serum or product is the reciprocal of the highest dilution that completely inhibits CPE or other virus effect in the test system. This dilution is said to contain 1 neutralizing antibody unit per unit volume used in the titration. When a serum or product known to contain neutralizing antibody is used in an assay to determine the identity of

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an unknown virus, 20 neutralizing antibody units in a fixed volume are generally used in the assay. Positive and negative control sera must give expected reactivity in the assay.

### HEMAGGLUTINATION INHIBITION (HAI)

A number of enveloped viruses, including the influenza and parainfluenza viruses, acquire protein receptors capable of binding RBCs (hemagglutinins) of various animal species on their surface as they bud through infected cell plasma membranes during viral maturation. In addition, some nonenveloped viruses such as adenoviruses and certain enteroviruses have hemagglutinin proteins in their outer capsid. This property allows for detection of a specific virus in a sample if a known specific antibody to the virus is available. Alternatively, the presence of antibody specific to the virus can be detected and quantitated by its ability to inhibit hemagglutination. This is the principle of the hemagglutination inhibition (HAI) test.

The HAI test for antibody is performed by making serial dilutions of the specimen to be tested and mixing the dilutions with a fixed amount of the virus or specific viral hemagglutinin protein in a tube or microtitration plate format. Indicator RBCs from the appropriate animal species are added, the suspension is mixed, and the tubes or plates are allowed to stand for a predetermined period. If specific antibody is present, the virus will bind and the RBCs will not agglutinate; they will settle to the bottom of the tube or plate and form an RBC "button". If specific antibody is absent, the RBCs will be agglutinated by the virus and form a diffuse film. The titer of the serum or product is the reciprocal of the dilution that completely inhibits agglutination.

HAI is very useful for subtyping influenza virus isolates. A number of factors contribute to the potential variability of the HAI test. Certain serum samples and products may contain nonspecific inhibitors of RBC agglutinins, which may yield false-positive results. A number of procedures have been developed to remove such inhibitors, including adsorption and heat inactivation procedures. Specimens may also contain RBC agglutinins other than specific antibody, and these may contribute to false-negative results. Appropriate preparation and titration of reagents, including RBCs and viral hemagglutinin stocks and suspensions, is critical. In addition, controls for nonspecific agglutination or inhibitors of agglutination must be included in every assay.

### WESTERN BLOT (OR IMMUNOBLOT) ASSAY FOR ANTIBODY DETECTION

The immunoblot, or Western blot, assay is a technique for the simultaneous detection of antibodies to various protein antigens of a given virus. The term *recombinant immunoblot assay* (RIBA) is applicable when the starting protein mixtures are recombinant proteins obtained from prokaryotic or eukaryotic expression systems instead of crude or partially purified virus from infected cells. The method is often used diagnostically as a supplementary or confirmatory test in situations where an initial assay for antibody lacks sufficient specificity or is known to be prone to false-positive results. This is especially important when the test is being used to diagnose an infection of clinical significance such as HIV or HCV infection.

A number of commercial immunoblot kits are available, particularly for viruses such as HIV and HCV; several have regulatory approval for diagnostic use. Alternatively, viral antigen preparations may be produced in-house or purchased, along with other reagents required for the assays. Careful control and/or sourcing of these reagents are critical to ensuring that compliance requirements are maintained.

For selected viral agents, there are generally accepted interpretive standards for the analysis of reactivity or positive results in an immunoblot assay. However, the presence of nonspecific bands may be due to antibody reactivity to cellular protein antigens caused by autoimmune diseases and/or the use of crude virus-infected cell proteins as antigen in the assay. Indeterminate reactions may also occur if only a limited number of specific antibody bands are observed.

Appropriate positive and negative control sera must be included in each assay and reactivity must be scored for both the presence and the intensity of expected protein bands.

### APPLICATION OF THE ANTIBODY DETECTION METHODS TO SPECIFIC VIRUSES

Human blood-borne pathogens that may be present in infectious form in human donated blood used directly in the production of biological products are a concern because they may present a risk of transmission to others. Testing for virus-specific antibodies in donated blood serves as a screening procedure for the elimination of suspect units. Alternatively, the viruses may represent important agents for which human vaccines have been or are being developed. Thus the ability to detect virus-specific antibodies in an immunized individual or animal model may be important for demonstrating the efficacy of the vaccine. Currently, in the United States, a number of FDA-approved screening or definitive tests may be conducted on donated units of blood for evidence of the presence of agents of infectious diseases, including hepatitis B and C viruses, human immunodeficiency virus, and West Nile virus. In addition, plasma sent for fractionation before production of plasma-derived products is required to be tested for hepatitis A virus (HAV) and human parvovirus B-19.

## GLOSSARY

**Acceptance Criteria:** Anticipated results, which may be numerical limits, ranges, or other characterization for the tests described. They establish the standards to which a drug substance or drug product should conform in order to be considered acceptable for its intended use.

**Adventitious Agent:** Acquired accidental contaminant in a cell line such as viruses and toxins; the agent is often infectious.

**Amplicon:** A segment of DNA generated by the PCR process whose sequence is defined by forward and reverse primers.

**Antibody:** An infection-fighting protein molecule that binds, neutralizes, and helps destroy foreign microorganisms or toxins. Also known as immunoglobulins, antibodies are produced by the immune system in response to antigens.

**Antigen:** Any agent that induces the production of an antibody and reacts specifically with it.

**Assay Validation:** A formal, archived demonstration of the analytical performance of an assay that provides justification for use of the assay for an intended purpose and a range of acceptable potency values.

**Bioassay:** Analytical method that uses living animals, cells, tissues, or organisms as test subjects.

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**Biologics:** Products such as antitoxins, antivenins, blood, blood derivatives, immune serums, immunologic diagnostic aids, toxoids, vaccines, and related articles that are produced under license in accordance with the terms of the federal Public Health Service Act (58 Stat. 682) approved July 1, 1944, as amended, have long been known as "biologics." However, in Table III, Part F, of the Act, the term "biological products" is applied to the group of licensed products as a whole. For Pharmacopeial purposes, the term "biologics" refers to those products that must be licensed under the Act and comply with Food and Drug Regulations —Code of Federal Regulations, Title 21 Parts 600–680, pertaining to federal control of these products (other than certain diagnostic aids), as administered by the Center for Biologics Evaluation and Research or, in the case of the relevant diagnostic aids, by the Center for Devices and Radiological Health of the federal Food and Drug Administration. [Definition from *Biologics* ⟨1041⟩*, USP–NF* vol. 30 (2007), p. 414.]

**Biotechnology-Derived Product:** Macromolecular article derived from biotechnology processes such as recombinant DNA (rDNA) technology, hybridoma technology, and the like.

**Bulk Harvest:** See *Unprocessed Bulk Harvest.*

**Capsid:** The outer protein shell of a virus particle.

**Cell Bank:** A defined population of cells, such as an immortalized cell line, grown by a defined process and cryopreserved in a defined process and within a defined passage number range. The assumption is that each vial from a cell bank is comparable and that when thawed and added to a manufacturing vessel (or an analytical assay), it will perform in a consistent way.

**Chaotropic:** A reagent that causes molecular structure to be disrupted; in particular, those formed by noncovalent forces such as hydrogen bonding, van der Waals interactions, and the hydrophobic effect.

**Complement:** A group of proteins in the blood that work in concert with other immune system proteins and cells (such as antibodies) in attaching foreign substances.

**cDNA:** Complementary DNA. Two strands of nucleic acid that can hybridize by specific base pairing between the nucleotides.

**Confluency:** Refers to the point when 100% of the surface area of the vessel is covered in cells.

**Cryopreservative:** Reagent used to keep a cell alive in deep-frozen condition (usually in liquid nitrogen).

**Cytopathic:** Damaging to cells, causing them to exhibit signs of disease or cell death.

**ELISA:** Enzyme-linked immunosorbent assay. A biochemical technique used to detect the presence of an antibody or an antigen in a sample.

**Endpoint Assay:** An analytical method that measures the amount of accumulated product at the end of the assay.

**Epitope:** A molecular region on the surface of an antigen that is recognized by an antibody and can combine with the specific antibody produced by such a response; also called a determinant or an antigenic determinant.

**Glycoprotein:** Protein that contains sugar side chains added as a posttranslational process; the presence of sugar side chains often affects activity, antigenicity, and in vivo stability.

**Host Cell Tropism:** The range of susceptible cells that a particular microorganism can infect.

**ICH:** The International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use.

**Limit of Detection (LOD):** The lowest concentration of an analyte in a sample that can be detected, not quantitated. It is a limit test that specifies whether or not an analyte is above or below a certain value. It is expressed as a concentration at a specified signal-to-noise ratio, usually 2 or 3.

**Mycoplasma:** Parasitic microorganism that infects mammalian cells, possessing some characteristics of both bacteria and viruses. Prokaryotic microorganisms belong to the family *Mycoplasmataceae,* with no cell walls. They may grow attached or close to cell surfaces in the cytoplasm and subtly change the properties of the cells.

**Passage:** An operational procedure used to feed cultured cells, usually by providing fresh medium and dilution of cells in a new culture vessel. The number of such operations is referred to as the passage number. It is not the same as cell generation number, which is strictly related to cell doubling time.

**qPCR:** Quantitative polymerase chain reaction. A modification of the polymerase chain reaction used to measure the quantity of DNA, complementary DNA, or ribonucleic acid present in a sample. Like other forms of polymerase chain reaction, the process is used to amplify DNA samples via the enzyme DNA polymerase.

**Raw Materials:** All components used to manufacture a drug substance or drug product; regulated by 21 CFR 211.

**RT-PCR:** Reverse transcriptase polymerase chain reaction. A variation of the PCR technique in which cDNA is made from RNA via reverse transcription. The cDNA is then amplified using standard PCR protocols.

**Serotype:** The kind of microorganism as characterized by testing for recognizable antigens on the surface of cells of the microorganism.

**Spiking:** Adding a known amount of analyte from a laboratory standard acting as a tracer to check a method for recovery or accuracy.

**Syncytium:** A multinucleated mass of cytoplasm that is not separated into individual cells.

**System Suitability:** The checking of a system to ensure system performance before or during the analysis of unknowns. Parameters such as plate count, tailing factors, resolution, and reproducibility are determined and compared against the specifications set for the method. These parameters are measured during the analysis of a system suitability *sample,* which is a mixture of main components and expected by-products.

**$TCID_{50}$:** 50% tissue culture infective dose. The level of dilution of a virus at which half of a series of laboratory wells contain active, growing virus.

**Unprocessed Bulk Harvest:** The pooled harvests of cell culture fluids that constitute a homogeneous mixture for manufacture into a unique lot of product.

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# APPENDIX

## Relevant Regulatory References

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# ⟨1238⟩ VACCINES FOR HUMAN USE—BACTERIAL VACCINES

***Add the following:***

▲INTRODUCTION
RAW MATERIALS AND MATERIALS OF ANIMAL ORIGIN
CELL BANKS
- Source and History
- Cell Bank Manufacture
- Cell Bank Testing

PROPAGATION AND HARVEST
- Fermentation Starting Materials: Cell Inoculum
- Fermentation Hardware
- Harvest Hardware
- Process Development
- Product Requirements
- Process Design
- Early Development Considerations
- Process Monitoring
- Scale-Up

PURIFICATION
- Polysaccharide Purification
- Protein Purification

INTERMEDIATES
FILTERED BULK (DRUG SUBSTANCE)
DRUG PRODUCT AND LOT RELEASE
- Other Vaccine Components and Vaccine Properties▲ (USP 1-May-2021)

***Change to read:***

## INTRODUCTION

An overview of vaccines for human use is presented in *Vaccines for Human Use—General Considerations* ⟨1235⟩, ▲which includes a glossary of terms.▲ (USP 1-May-2021) Bacterial vaccines can be derived from whole cells, either killed or attenuated in their ability to cause disease, or from some component(s) of the intact cell that is important for virulence or damage to the host ▲(see *Table 1* for examples). In some cases, the antigen may be recombinant and produced in a non-pathogenic organism.▲ (USP 1-May-2021) Another subset of bacterial vaccines, derived from toxins, is the toxoids. Bacterial vaccine products can be mixtures of components from different species, from different strains or different serotypes of the same species, or from different components from cells of the same species.

**▲Table 1. Bacterial Vaccines Currently Licensed in the US**

| | |
|---|---|
| Polysaccharide-based vaccines<br>• *Haemophilus influenzae* type b (Hib) glycoconjugate[a, b]<br>• Pneumococcal polysaccharides[a]<br>• Pneumococcal glycoconjugates[a]<br>• Meningococcal polysaccharides[a]<br>• Meningococcal glycoconjugates[a]<br>• *S. enterica* ser. Typhi Vi polysaccharide[a] | Bacterial toxoids[b]<br>• Tetanus toxoid[b]<br>• Diphtheria toxoid[b] |
| Protein subunit vaccines<br>• Acellular pertussis[b]<br>• Anthrax<br>• Meningococcal protein-containing vesicles in combination with recombinant antigens | Live attenuated bacterial vaccines[c]<br>• *S. enterica* ser. Typhi Ty21a<br>• Bacillus Calmette–Guérin (BCG)<br>• *Vibrio cholerae* serogroup O1<br>▲ (USP 1-May-2021) |

[a] See *Vaccines for Human Use—Polysaccharide and Glycoconjugate Vaccines* ⟨1234⟩ for further information.
[b] Frequently administered with other antigens in a combination vaccine.
[c] Currently there are no inactivated bacterial vaccines licensed in the US.

The simplest bacterial vaccines consist of the purified cell-surface capsular polysaccharides (CPS) from organisms such as *Salmonella enterica* serovar Typhi, various meningococcal serogroups, or pneumococcal serotypes that cause meningitis, otitis media, acute respiratory infections, and pneumonia. Although the typhoid vaccine consists of a single polysaccharide, the meningococcal vaccines contain as many as four serogroup-specific CPS, and the pneumococcal vaccine contains 23 serotypes.

The immunological response to meningococcal and pneumococcal polysaccharides, and to the CPS from *Haemophilus influenzae* type b (Hib), is improved by covalent attachment of the CPS or an oligosaccharide derived from it to a suitable carrier

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protein, ▲often a bacterial toxoid or naturally occurring non-toxic mutant toxin.▲ (USP 1-May-2021) The immunological response to these glycoconjugate vaccines is elicited via immunologic pathways different from those induced by purified polysaccharides, creates a T-cell-dependent response, and establishes immunological memory. ▲These vaccines are highly effective in infants. Detailed information on polysaccharide and glycoconjugate vaccines is provided in *Vaccines for Human Use—Polysaccharide and Glycoconjugate Vaccines* ⟨1234⟩.▲ (USP 1-May-2021)

Many bacterial pathogens, including those that cause diphtheria and tetanus, produce toxins that kill tissue. Immunological neutralization of these toxins is sufficient to prevent disease. These subunit vaccines consist of chemically detoxified toxins (toxoids) purified from culture supernatant and are capable of eliciting neutralizing antibodies against the native toxin. ▲▲ (USP 1-May-2021)

Although earlier pertussis vaccines consisted of myriad chemically inactivated whole-cell and toxin components, current acellular products contain various combinations of specific purified proteins, sometimes toxoided (e.g., with fimbriae and other cell-surface protein components). Compared to older products, these vaccines apparently produce protection by a different mode of action but have a lower incidence of adverse events. A combination of diphtheria and tetanus toxoids and an acellular pertussis vaccine form the core components of many polyvalent pediatric and adult combination vaccines. Hib glycoconjugate, hepatitis B, and/or inactivated poliovirus immunogens may also be added to these vaccines. ▲No inactivated whole-cell bacterial vaccines are currently licensed in the US.

Another type of meningococcal bacterial subunit vaccine is based upon pathogen-derived lipid vesicles containing a wide range of immunogenic outer membrane proteins. These vaccines may also contain individually produced recombinant immunogens.▲ (USP 1-May-2021)

Live attenuated bacterial vaccines are currently limited to Bacillus Calmette–Guérin (BCG), which protects against tuberculosis when administered through the skin, and the *S. enterica* ser. Typhi Ty21a construct, which is an oral vaccine against typhoid fever.

The immune response against these ▲▲ (USP 1-May-2021) antigens can be increased by the inclusion of adjuvants. ▲Adjuvants included in▲ (USP 1-May-2021) licensed ▲bacterial vaccines▲ (USP 1-May-2021) in the US are based on aluminum salts such as aluminum hydroxide and aluminum phosphate, although the development and characterization of new adjuvants is an active area of research.

***Change to read:***

## RAW MATERIALS ▲AND MATERIALS OF ANIMAL ORIGIN▲ (USP 1-MAY-2021)

Raw materials can directly affect the identity, strength, purity, and quality of bacterial vaccines. ▲To achieve▲ (USP 1-May-2021) a consistent manufacturing process, it is critical to use consistent raw materials (e.g., during seed banking, fermentation, harvest, and purification). ▲▲ (USP 1-May-2021) Raw materials for bacterial growth media typically consist of both well-defined chemical entities (e.g., amino acids, carbohydrates, vitamins, minerals) and more complex components (e.g., protein hydrolysates, yeast extracts, peptones). ▲Expectations for the sourcing, quality, and control of raw materials are highlighted in ⟨1235⟩ and in *Cell Banking* ⟨1042⟩.▲ (USP 1-May-2021)

***Change to read:***

## CELL BANKS

### Source and History

▲The development, characterization, stability testing, and storage of master cell banks (MCBs) and working cell banks (WCBs) is a unit operation and is described in detail in ⟨1042⟩.▲ (USP 1-May-2021)The source should generate a sufficient ▲quantity▲ (USP 1-May-2021) of antigen(s) to meet the medical need.▲▲ (USP 1-May-2021) For microbial expression systems such as *E. coli*, ▲▲ (USP 1-May-2021) the manufacturer should describe the method used to prepare the DNA coding for the protein, including the cell and origin of the source nucleic acid.

▲▲ (USP 1-May-2021) The steps in the assembly of the expression construct must be described in detail. This description should include the source and function of the component parts of the expression construct (e.g., origins of replication, antibiotic resistance genes, promoters, enhancers, and whether the protein is synthesized as a fusion protein). Manufacturers should provide restriction endonuclease digestion maps that illustrate the sites used in preparing the expression construct and sites used in the identification of DNA fragments.

A complete nucleotide sequence analysis of the expression construct's coding region for the protein of interest should be performed. The sequence analysis should be provided and should include a complete annotation designating all of the important sequence features. The copy number and physical state of the expression construct should be determined.

▲Requirements for defining and reporting the origins and genealogy of the cells used to construct the cell bank are provided in ⟨1042⟩.▲ (USP 1-May-2021)

### Cell Bank Manufacture

▲▲ (USP 1-May-2021)

For microbial expression systems, a single host cell that contains the expression construct is propagated to generate the MCB. Manufacturers should document the cell cloning history and method of transferring the expression construct into the host cell. They also should describe completely the methods and criteria used to amplify the expression construct and to select the cell clone for production. ▲See ⟨1042⟩ for further details.▲ (USP 1-May-2021)

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## Cell Bank Testing

▲▲ (USP 1-May-2021)

Additionally, for recombinant bacterial expression cell lines (e.g., *E. coli*), molecular characterization testing can include DNA sequencing of the target gene sequence along with the flanking regions, expression construct retention, and plasmid copy number. Analysis of the expression construct at the nucleic acid level should be performed with consideration that this verifies only the coding sequence of a recombinant gene. Restriction endonuclease mapping or other suitable techniques should be used to analyze the expression construct for insertions or deletions and for the number of integration sites. For extra-chromosomal expression systems, the percent of host cells that retain the expression construct should be determined under selected and nonselected growth conditions. For cells with chromosomal copies of the expression construct, the nucleotide sequence encoding the product could be verified by recloning and sequencing of chromosomal copies.

▲▲ (USP 1-May-2021)

Characteristics of the recombinant protein product can also be applied (see below) as another means of defining the ultimate output of the cell line.

▲▲ (USP 1-May-2021) If a new MCB is to be generated by expression construct transfer into host cells followed by clonal selection, then acceptance criteria for both the new clone and the protein produced by the clone should be described and justified.

▲Requirements for validation, testing, storage, and stability assessment of the cell banks are provided in ⟨1042⟩.▲ (USP 1-May-2021)

***Change to read:***

## ▲PROPAGATION AND HARVEST▲ (USP 1-MAY-2021)

Production of the drug substance for a bacterial vaccine requires a fermentation process that is consistent and is ▲sufficient▲ (USP 1-May-2021) to support commercial production. The approach to achieving this has become fairly standardized and provides a relatively high probability of success for early batch production to support a development program. It is still a significant challenge to achieve sufficient productivity to support commercial manufacture of a licensed product. Directly after any fermentation process is the harvest process, which serves as a transition step between biomass expansion and downstream process steps. For the purposes of this chapter, harvest will be considered as an extension of the fermentation process.

### Fermentation Starting Materials: Cell Inoculum

The cell inoculum for the fermentation process is the single most important component for establishing a reproducible fermentation process. In early development before finalizing the fermentation conditions, manufacturers typically must generate an interim source of this inoculum, a process development cell bank (PDCB). The origin of the PDCB should be a clonal isolate of the original transfected or isolated strain that demonstrates suitable growth properties and produces the antigen of interest in sufficient quantity and quality for the intended purpose. The use of a clonal isolate ensures that the genetic starting point for each batch is the same and that subtle variations in process conditions will not inadvertently allow one population versus another to dominate the culture. That is, the PDCB is used for fermentation development to ensure that variations in the fermentation conditions can be interpreted without the overlay of competition between populations of transfectants.

Initial development of the fermentation process, preferably with the PDCB, typically precedes production of the MCB and WCB. Best practice is to derive these cell banks from the same clonal isolate as the PDCB to reduce the need for a second cycle of fermentation development when the WCB is deployed. Substitution of a WCB for the PDCB at the final stages of fermentation development is common practice, but care must be taken to constrain such experiments to optimization of the fermentation process. More detail is found in *Cell Banks* above.

### Fermentation Hardware

The biomass production process typically begins with a small-volume inoculum in an initial fermentation volume that is 20- to 100-fold larger than the initial inoculum volume. This initial passage is often followed by one or more intermediate fermentations that expand the production volume by 20- to 100-fold at each step until the production fermentation volume (typically 500–3000 L) is reached. Routine manufacture at these scales requires well-controlled fermentation conditions and physical facilities that meet the economic and current good manufacturing practices (cGMP) ▲requirements▲ (USP 1-May-2021) for a successful product.

Bacterial fermentations have traditionally been carried out in glass, glass-lined, or passivated stainless steel fermenters that comply with cGMP requirements, particularly when using large fermenters (e.g., those with >1000 L working volume) because of containment issues with such large volumes of liquid. Traditional fermentation systems require hard-piped control systems that meet the need for clean-in-place and steam-in-place capability. The bioburden and complexity of the facility will increase if the fermentation operations must also accommodate multiple product lines.

Smaller fermentation batches are increasingly performed in disposable bioreactors such as single-use bags with completely disposable product contact surfaces, including sensors and probes. These systems are becoming readily available, are less expensive, and are more flexible than fixed equipment and they meet the needs of the competitive business and evolving cGMP expectations and requirements. A note of caution is warranted, however, because this disposable technology can lead to changes in the material of product contact surfaces. Such changes then require re-evaluation and sometimes revalidation of the manufacturing process for late-stage development and commercial products. Thus, the reduced cleaning burden may ▲be accompanied by▲ (USP 1-May-2021) an increased need for extractability and leachability studies.

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## Harvest Hardware

Harvest of the fermentation product can focus on recovery of either the wet cell mass from which the product will be extracted or the fermentation broth from which the product will be directly purified. In the former case, centrifugal separation is typically employed. Production-scale centrifuges can be either closed operations with a fixed volume of input and manual recovery of the pellet, or continuous-flow operations that automatically eject and recover the clarified supernatant and/or the accumulated pellet. Although centrifuges are efficient in harvesting a fermentation product, shear forces can have significant effects on the product stream (e.g., lysed cells, sheared molecules in solution). Alternatively, and particularly when the product is secreted into the solution rather than retained in the cells, membrane filtration systems may be used to clarify the product stream for subsequent purification. Tangential-flow and depth filtration systems can be effective for recovering soluble product with less concern about shear forces.

In all cases, monitoring the processing of the fermentation output and solids removal from the liquid matrix can be a simple yet effective approach to monitor process consistency and comparability. Off-line tools such as HPLC, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), or Western blot analyses can track product integrity issues such as aggregation or proteolysis.

## Process Development

A productive, robust fermentation process is the result of careful consideration of a broad collection of variables, considered alone as well as in conjunction with product design and downstream processing. Fermentation process variables include chemical inputs (e.g., carbon sources, minerals, vitamins, trace elements, antifoam, and gases), physical inputs (e.g., temperature, mixing, and pH), and biological processes [e.g., nutrient utilization rates, metabolite levels, and inductors (quantity/type/addition duration)].

## Product Requirements

A critical consideration in process development is anticipating how much of the product will be needed. Too little product caused by operating at too small a scale imposes supply constraints and often post-licensure urgency to scale up the process. In contrast, too much product results in excess inventory, expiring lots, infrequent manufacturing, ▲▲(USP 1-May-2021) and generally poor economics. A clear market evaluation is needed before designing the manufacturing process or committing to a process for scale-up or -down.

## Process Design

If one has a reasonably defined production need and an initial estimate of product yield, it is possible to extrapolate the scale of the fermentation from the volume (yield), production frequency, and expected productivity. Commercial fermentation of bacterial cultures is routinely carried out in volumes as large as 3000 L, but larger volumes are also used. A few large lots per year can be advantageous for a very robust process but may be limited by downstream process capabilities and/or the stability of the fermentation product as a production intermediate. An additional consideration may be the difficulty in generating enough lots to ensure that the fermentation process is indeed robust. Failure of a large lot carries important financial and inventory risks.

A large number of lots can impose logistical problems if the turnaround time is too tight or the coordination of downstream events becomes too complex. The logistics include quality control testing, which depends on the number rather than the size of lots. Production that involves a large number of smaller lots can require blending of multiple intermediate lots in order to produce a final drug product lot. This can cause challenges if product-related problems occur and may entail root-cause investigations. In general, appropriate fermentation sizing results in a process that has a turnaround time of less than 1 week, that can be accommodated with one or a few purification runs, and that results in one to several fills of final product after each purification cycle.

## Early Development Considerations

During early development of a biological product, the most important fermentation considerations are an appropriate, well-defined MCB and a fermentation process that is reasonably productive, reproducible, and scalable. The fermentation process is often underestimated when one considers the physical, chemical, and biological control of the process as process volumes change by orders of magnitude.

The mechanics of the fermentation process are an important consideration. Fermentations are typically studied in shake flask experiments or even microscale reactors that can readily accommodate many experiments conducted in parallel. Although this is attractive for the initial identification of process conditions, the ultimate culture vessel should be a controlled fermenter where growth conditions can be controlled and monitored in a more rigorous and comprehensive manner. Manufacturers should begin work in small-scale fermenters as early as possible to ensure that robust, controlled experiments can be run to refine the initial fermentation conditions.

Fermentation harvest processes also should be scalable. Although it is possible to scale centrifugation conditions, it is a challenge to maintain equivalent centrifugal conditions, particularly in a flow-through mode. Filtration processes can usually be scaled more predictably, provided that the membrane manufacturer is anticipating the needs of the process development scientist.

When manufacturers define a process, they should evaluate its robustness by purposeful deviations such as changes in the sources of raw materials and the time and temperature limits of unit operations. Such evaluations better define the rationale

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for setting process limits and for identifying which ▲parameters▲ (USP 1-May-2021) are most critical to the success of the manufacturing process.

## Process Monitoring

On the basis of early development process characterization data, manufacturers should be able to identify key analytical measures that, if applied to all lots, can either verify the correct progression of the process or serve as a sentinel to determine whether a specific batch is showing signs of deviation from the typical profile. In the absence of such data, an aberrant process may go unnoticed or may not be detected until testing of a process intermediate shows either an out-of-trend or out-of-specification result.

For a fermentation process, many critical variables (e.g., optical density, pH, and specific nutrient levels) can be measured online and in real time to potentially allow the intervention to bring a given process back into the normal range, or at least to identify the point in the process at which the deviation occurred. Such data can be valuable in identifying potential process improvements. Conversely, in the absence of such data, troubleshooting can be a challenging and protracted process.

## Scale-Up

Just as early development requires a focus on small-scale operations, scale-up becomes essential at some point to ensure that sufficiently large lots can be made to meet program needs. As these needs become increasingly complex, larger lots are essential to ensure that multiple experiments and observations can be tied to the same lot of product, which in turn is ▲essential▲ (USP 1-May-2021) to understanding critical process and product variables. If proper process engineering considerations were taken into account at the smaller production scale, scale-up can usually be done in 10-fold increments in volume with a reasonable expectation that significant process performance or product changes will not be seen. This approach may require adjustments at an intermediate scale if the initial fermentation was based on too small a volume or if the final production scale is very large. Again, process monitoring data can be very helpful in evaluating the success of the scaled-up process.

If clinical development studies are performed at less than full manufacturing scale, as they usually are, manufacturers will be obliged to relate the comparability of the process performance and the product characteristics at the different scales. Analytical data can be compelling, but in their absence or ▲when scale-related differences are present,▲ (USP 1-May-2021) manufacturers must demonstrate that these differences are not clinically significant. However, the use of comparability protocols for scale changes will ▲need▲ (USP 1-May-2021) to be approved by the local regulatory authority. ▲▲ (USP 1-May-2021) Analysts must take care to ▲separate▲ (USP 1-May-2021) differences caused by fermentation scale-up from changes caused by harvest or purification scale-up. One way to accomplish this is to compare process intermediates obtained, when possible, during the fermentation and harvest processes. As an example, online monitoring of fermentation conditions such as pH or glucose level can be used to demonstrate similarity during the time course of the fermentation. Similarly, measurements ▲obtained▲ (USP 1-May-2021) at the end of the fermentation process (e.g., final cell density, cell viability) and intermediate measurements during harvest (e.g., turbidity of clarified broth, wet cell mass in the pellet) provide useful information for evaluating the similarity or differences during scale-up.

***Change to read:***

# PURIFICATION

A general overview of purification for ▲▲ (USP 1-May-2021) vaccines is presented in ⟨1235⟩. In addition to a description of critical processing equipment, reagents, and processing steps, manufacturers should provide the rationale for the purification process. ▲▲ (USP 1-May-2021) As with the other processes, analysts should consider the source of all raw materials and ensure that they come from reliable vendors who adhere to cGMP and can ensure a long-term supply. The cGMPs will apply to late-stage clinical supplies and commercial materials. The removal of non-product-related impurities (e.g., processing reagents, endotoxins, contaminating cell proteins or nucleic acids, and other residual contaminants) should be verified.

The drug substance can be one of several types of compounds, e.g., polysaccharides (wild type or modified), proteins (wild type, mutant, toxoids, or recombinant), products of conjugation of polysaccharides and proteins, or products of conjugation of peptides and proteins.

To define and control purification processes for drug substances and drug products, the manufacturer should establish targets for process parameters and tolerances for all critical process steps including yields, activity, and purity to ensure the efficacy, safety, and consistency of the final product. Requirements for pooling, if applicable, should be established. The requirements and conditions for storage of intermediates, bulks, and final containers must be established by an official stability program. The use, reuse, regeneration, and cleaning of all drug product/drug substance contact equipment (e.g., filters, chromatographic columns and resins, tanks, and process lines) should be validated. In addition, extractable/leachable studies should be performed for all product contact equipment (e.g., disposable bag systems, chromatographic column resins, and process lines).

## Polysaccharide Purification

▲Methods for the purification and in-process control of the quality of polysaccharide antigens used as vaccines or for the manufacture of glycoconjugate vaccines are presented in ⟨1234⟩.▲ (USP 1-May-2021)

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## Protein Purification

The classes of bacterial protein vaccines include toxoids, nontoxoid ▲antigens▲ (USP 1-May-2021) (e.g., pertussis antigens), engineered recombinant products, and naturally occurring ▲variants▲ (USP 1-May-2021) (e.g., CRM197) used as carrier proteins ▲in glycoconjugates.▲ (USP 1-May-2021)

### TOXOIDS

At the end of fermentation, the toxin-containing culture medium should be separated aseptically from the bacterial mass as soon as possible or placed in a cold room until separation can be effected. The toxin content, ▲expressed as limit of flocculation▲ (USP 1-May-2021) (Lf/mL), is checked using a flocculation assay with the appropriate antitoxin standard to monitor production consistency (the culture should contain NLT 40 Lf/mL). The toxin is purified first to remove any components that could cause adverse reactions in humans. A typical process includes depth filtration followed by 0.2-µm filtration to assist in the removal of cellular debris. Following preliminary purification, the toxin is then detoxified with formaldehyde or glutaraldehyde or any suitable chemical reagent by a method that avoids both destruction of the immunogenic potency of the toxoid and reversion of the toxoid to toxin, particularly upon exposure to heat. Some toxoids require a single addition of formaldehyde, but others can require multiple additions. Alternatively, the toxin could be detoxified and then purified or partially purified by depth filtration, detoxified by the addition of an appropriate aldehyde, filtered using 0.2-µm filtration, and then pooled. The pooled toxoid solution is further purified by clarification with activated carbon, followed by multiple ammonium sulfate precipitation steps that further fractionate and concentrate the toxoid. Typical additional purification steps include concentration, diafiltration, and/or chromatography. Purification before detoxification results in a purer product and can be advantageous if the toxoid is to be used as the protein component of a protein–carbohydrate conjugate (because copurifying high-molecular-weight glycans will be removed before detoxification).

During detoxification and purification, ▲testing must be performed to control and ensure the consistency of the purification process, including▲ (USP 1-May-2021) endotoxin testing according to *Bacterial Endotoxins Test* ⟨85⟩, ▲testing for▲ (USP 1-May-2021) formaldehyde and protein ▲content, and testing of the▲ (USP 1-May-2021) irreversibility ▲of toxoiding.▲ (USP 1-May-2021) If the material must be sterile, ▲the sterility test described in▲ (USP 1-May-2021) *Sterility Tests* ⟨71⟩ can be performed.

### PROTEINS/RECOMBINANT PROTEINS

Proteins used to make vaccines can be recombinant (in their native state or engineered to modify certain amino acids) or they can be naturally occurring ▲variants▲ (USP 1-May-2021) that have no wild-type activity but are capable of inducing the appropriate immune response. Proteins are harvested from the fermenter and are extracted (for example, by mechanical and chemical disruption) and then purified by suitable methods, typically consisting of filtration–concentration steps (e.g., ultrafiltration, tangential-flow filtration, diafiltration, centrifugation, selective precipitation, and even direct capture using expanded-bed chromatography or big-bead technologies). The enriched protein solution can be further purified using appropriate filtration and chromatographic steps. For all equipment that contact drug substances (e.g., chromatographic resins, membranes, disposable bag systems, or process lines), manufacturers should assess extractables and leachables. Analysts should determine the column resin life for all chromatographic systems used in the purification (including number of uses, reconditioning requirements, and storage conditions).

The type of chromatography used to purify proteins depends on the physical/chemical properties of the desired protein as well as those of other molecular entities in the harvest culture. As an example, $CRM_{197}$ can be purified using a multi-step chromatographic process: production material is first diafiltered and then separated by ion-exchange chromatography [diethylaminoethyl (DEAE-Sepharose)] in order to purify the target protein from other molecular entities present in the purification stream. The peak of interest is collected and ammonium sulfate is added, followed by 0.22-µm filtration to condition the material before loading on the hydrophobic-interaction chromatography column (phenyl-Sepharose) for purification of the target protein based on its surface hydrophobicity. The peak fraction is then diluted with Water for Injection and is separated onto a ceramic hydroxyapatite column to further purify the target protein based on its surface charge. The eluted peak is then buffer exchanged into the storage buffer by ultrafiltration/diafiltration using cross-flow membrane filtration, followed by 0.22-µm filtration to yield the sterile purified concentrate.

In-process control of protein purification includes monitoring specific protein content and critical process steps as well as monitoring the removal of unwanted fermentation and purification components. The pH is critical for ion-exchange chromatography, and therefore pH should be monitored. For steps designed to remove endotoxin, procedures in ⟨85⟩ are used to monitor column eluants. Bioburden is monitored according to *Microbial Enumeration Tests* ⟨61⟩ after filtration and chromatography steps. If the material must be sterile, the sterility test in ⟨71⟩ should be performed.

### POLYSACCHARIDE–PROTEIN CONJUGATES

▲An overview of the production, purification, and in-process testing of glycoconjugate vaccines is presented in ⟨1234⟩.▲ (USP 1-May-2021)

***Change to read:***

## INTERMEDIATES

An intermediate or process intermediate in vaccine manufacture is the reaction product of each step in the process except the last one, which forms the final product. Examples of intermediates are bulk-purified polysaccharides, proteins, and activated polysaccharides that conjugate to protein.

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Most vaccine production processes are stepwise and take more than one elementary step to complete. An intermediate is produced from raw materials at one or more process steps (e.g., bacterial growth, extraction and purification, and▲/or▲ (USP 1-May-2021) chemical modification), eventually resulting in the drug substance. The identification of the key intermediates, their production, and sampling for analytical tests must be defined in controlled documents (e.g., batch records, analytical protocols).

Intermediates, which can ▲sometimes▲ (USP 1-May-2021) be stored for considerable periods of time before further processing, ▲must▲ (USP 1-May-2021) be included in a formal stability program. ▲▲ (USP 1-May-2021) Stability studies in normal or accelerated conditions should be performed to define the maximal hold time for intermediates and ▲also should be performed▲ (USP 1-May-2021) when significant process changes are implemented.

From raw material to finished drug substance, testing throughout the process ensures a quality product. Testing of intermediates is a key quality control step to ensure their identity and purity. The quality attributes of the intermediate are commonly tested in conjunction with further processing, and their release testing should be considered. Standard operating procedures (SOPs) must be properly defined for the analytical control tests. Because of their critical role in the production process, some key intermediates could be included in formal release testing, in addition to the intermediates identified for in-process testing.

▲▲ (USP 1-May-2021)

Examples of tests for the structural characterization and purity estimation of protein-based intermediates include the following:

- Identity and molecular size [(SDS–PAGE), Western blot, size-exclusion chromatography–ultraviolet (SEC–UV), SEC–fluorescence, size-exclusion chromatography–multi-angle laser light scattering (SEC-MALLS), reversed-phase chromatography]
- Endotoxin content (see ⟨85⟩)
- Residual nucleic acids (colorimetric assay; see *Nucleic Acid-Based Techniques—Approaches for Detecting Trace Nucleic Acids (Residual DNA Testing)* ⟨1130⟩)
- Bioburden (total viable aerobic count of microbial contamination; see ⟨61⟩)
- Other proteins as impurities (SDS–PAGE, Western blot, SEC–UV, SEC–fluorescence, reversed-phase chromatography)

The tests previously ▲described▲ (USP 1-May-2021) for protein-based intermediates are also applicable when the protein-based product is defined as the drug substance. In addition to the examples above, many other methodologies can be applied for the identity and purity evaluation. ▲Appropriate tests for saccharide and glycoconjugate intermediates are discussed in ⟨1234⟩.▲ (USP 1-May-2021)

Stability tests for intermediates can include physicochemical methods (see *Intermediates* above), which are formally included within an analytical panel for the stability study. In addition, biological and immunochemical tests [e.g., enzyme-linked immunosorbent assay (ELISA)] can be included. Bioburden and endotoxin testing may not be required at each level (each intermediate, drug substance) provided that testing is performed at a sufficient number of steps in the overall production process. Bioburden is typically performed prior to sterile filtration via in-process testing. If intermediates must be stored and/or subsequently shipped to a different location for further processing, the stability of these materials must be demonstrated.

***Change to read:***

## ▲FILTERED BULK▲ (USP 1-MAY-2021) (DRUG SUBSTANCE)

▲The final step in the manufacturing of antigen drug substance is the preparation of filtered bulk or drug substance.▲ (USP 1-May-2021)

The drug substance is the final product of the antigen manufacturing process, before the formulation of the final vaccine dosage. The final bulk may be prepared aseptically or may include a sterilization step. Sampling for analytical tests for release and stability studies ▲▲ (USP 1-May-2021) must be defined in controlled documents (e.g., batch records, analytical protocols).

A drug substance can be stored ▲▲ (USP 1-May-2021) before further processing, but if it is stored the drug substance must be included in a formal stability program. ▲▲ (USP 1-May-2021) Stability studies in normal or accelerated conditions should be performed to define maximal hold times. A stability program is required for formal stability studies, and the studies must be executed according to a protocol that contains detailed information about the types of tests, specifications, ▲storage conditions,▲ (USP 1-May-2021) testing intervals, and time points.

Testing of the drug substance must be performed to ensure its identity and purity. All testing must be done according to established SOPs, and all tests must have specifications (or provisional specifications, where applicable).

▲Many▲ (USP 1-May-2021) methodologies can be applied for identity and purity evaluation. For instance, the specific impurities that must be measured are determined based on negotiations between manufacturers and the national drug regulatory agency during the licensure process. Bioburden and endotoxin testing may not be required at each level (each intermediate, drug substance) provided that testing is performed at sufficient steps in the overall production process. Bioburden is typically performed prior to sterile filtration via in-process testing.

All of the results must be reported in a controlled document. Stability tests can include both physicochemical methods (see stability information, above) and biological/immunochemical tests (e.g., ELISA and serum bactericidal activity; see *Quality of Biotechnological Products: Stability Testing of Biotechnological/Biological Products* ⟨1049⟩).

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## DRUG PRODUCT AND LOT RELEASE

General principles are described in ⟨1235⟩, which outlines the lot release procedure in accordance with 21 Code of Federal Regulations (CFR) §610.1 and 21 CFR §610.2. For products that will be used in the US, samples and protocols containing all appropriate tests must be submitted to the FDA for review and/or testing. If the FDA determines that the lot meets the standards of safety, purity, and potency required for the particular vaccine, the ▲manufacturer distributes and markets the product.▲ (USP 1-May-2021)

Tests required for each lot-release protocol include potency, general safety, sterility, purity, identity, and constituent materials. Potency and potency-related tests are different for each bacterial vaccine. The inclusion of these tests makes each bacterial vaccine lot-release protocol unique.

The contents of a final container of each filling of each lot are tested for identity after labeling is completed. Identity is established based on either physical or chemical characteristics of the vaccine, inspection by macroscopic or microscopic methods, specific culture tests, or in vivo or in vitro immunological tests. In large part, identity testing is performed to distinguish the vaccine from other materials that are manufactured at the same site (21 CFR §610.14). The same tests that establish identity may also be appropriate for defining the quantity of immunogen present in the final vial. This is especially important for carbohydrate-based vaccines that are dosed by mass and for which physicochemical measures of antigen quality are used.

Immunochemical methods, which include immunoprecipitation methods and immunoelectrophoretic methods, have been useful. Immunoprecipitation methods, such as flocculation and precipitation, can be carried out in solution or in a gel matrix and involve mixing the antigen with an appropriate antibody, leading to the formation of flocculating or precipitating aggregates that can be detected visually or by light scattering (▲e.g.,▲ (USP 1-May-2021) nephelometry). The ratio of reactants must be varied to optimize the detected response. In solution this can be achieved by titrating one reactant with the other, and increased sensitivity can be obtained by using antigen- or antibody-coated particles (e.g., latex) as reactants. In gel systems, a gradient is created as one or more of the reactants diffuse, creating a visible line where precipitation occurs. Immunoelectrophoresis (IE) is a qualitative technique that combines two methods: gel electrophoresis followed by immunodiffusion. Crossed IE is a modification of the IE method that is suitable both for qualitative and quantitative analysis. Visualization and characterization of immunoprecipitation lines can be performed using selective or nonselective stains, fluorescence, enzyme or isotope labeling, or other relevant techniques. Selective staining methods are usually performed to characterize nonprotein substances in the precipitates. In translucent gels, such as agar or agarose, the precipitation line becomes clearly visible in the gel provided that the concentration of each of the reactants is appropriate.

Where multiple active components are present as a result of copurification (e.g., certain acellular pertussis vaccines), the manufacturer must demonstrate that the composition of the product is consistent between batches, unless this has been validated during the development of the manufacturing process.

▲For toxoid vaccines or toxoid carriers in glycoconjugate vaccines,▲ (USP 1-May-2021) manufacturers should demonstrate that reversion to toxicity has not occurred (and will not occur over the shelf life) for a product derived from or containing a toxoid material, unless the contrary has been validated. This may require the use of a cell line or an in vivo test, although enzymatic approaches are being validated.

An antigenic purity test is an assay that assesses the ▲quality▲ (USP 1-May-2021) of antigen and is used for diphtheria and tetanus toxoid vaccines. The antigen content is determined by a flocculation assay ▲and is typically expressed in Lf/mg.▲ (USP 1-May-2021)

The manufacturer should prove a high and consistent level of immunogen adsorption to any solid-phase adjuvant (such as aluminum phosphate or aluminum hydroxide) that is consistent with the release specification.

For certain vaccines such as the anthrax vaccine and toxoid vaccines, the manufacturer is required to demonstrate that the vaccine is protective against disease or death in animal models challenged with a predefined dose of the target pathogen. This generally requires definition of the animal model, route of administration, vaccine dilutions required, a means to observe effects, and a reference vaccine against which effects are compared. The data should be analyzed appropriately (see *Analysis of Biological Assays* ⟨1034⟩).

Stability-indicating assays are those used to determine the stability of the product. Of primary importance is the potency assay, although glycan degradation may be important ▲when evaluating▲ (USP 1-May-2021) glycoconjugate vaccines.

### Other Vaccine Components and Vaccine Properties

Aluminum compounds are the primary adjuvants used in vaccines in the US. Chapter ⟨1235⟩ provides provisions of 21 CFR §610.15 governing the use of aluminum and amounts allowed. The adjuvants widely used in bacterial vaccines include aluminum potassium sulfate (alum), aluminum phosphate, aluminum hydroxide, and combinations of these compounds. Bacterial vaccines formulated with such adjuvants are referred to as adsorbed vaccines, and this term may be included in the official name of the vaccine. Other adjuvant systems may be evaluated. Aluminum is quantitated using colorimetry, titrimetry, emission or atomic absorption spectroscopy, or inductively coupled plasma–mass spectrometry.

For regulations regarding residual manufacturing reagents, see the FDA's 1999 *Guidance for Industry: Content and Format of Chemistry, Manufacturing, and Controls Information and Establishment Description Information for a Vaccine or Related Product*. Manufacturing reagents such as formaldehyde and glutaraldehyde are sometimes used in inactivation, the toxoid-making processes, or elsewhere during manufacture and may be present in residual amounts in the final product. Limits for formaldehyde and other residuals must be minimized in accordance with the approved product license.

Common preservatives used in bacterial vaccines include thimerosal, phenol, 2-phenoxyethanol, and benzalkonium chloride. 21 CFR §610.15 provides additional information about the minimization of thimerosal content and the production of thimerosal-free vaccines. Limits and content specifications are set for each bacterial vaccine in the product license.

Each lot of final containers of a vaccine intended for use by injection is tested for bacterial endotoxins ▲(when required),▲ (USP 1-May-2021) as indicated in ⟨85⟩.

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Each lot of final containers of a vaccine intended for use by injection may be tested for pyrogenic substances as indicated in *Pyrogen Test* ⟨151⟩ and 21 CFR §610.14.

Each lot of dried product ▲must▲ (USP 1-May-2021) be tested for residual moisture [see *Loss on Drying* ⟨731⟩ and FDA's *Guideline for the Determination of Residual Moisture in Dried Biological Products* (January 1990)]. Residual moisture should be determined for lyophilized vaccines.

▲In some cases, a▲ (USP 1-May-2021) general safety test is performed on biological products intended for administration to humans with the purpose of detecting extraneous toxic contaminants. Procedures and exceptions are specified in 21 CFR §610.11.

▲Excipients, preservatives, diluents, and adjuvants are tested to meet generally accepted standards of purity and quality per 21 CFR §610.16 and/or applicable guidance documents at an appropriate stage of manufacture.▲ (USP 1-May-2021)

The sterility of each lot of each product is ▲evaluated▲ (USP 1-May-2021) according to procedures described in ⟨71⟩ and 21 CFR §610.12 for both bulk and final container material.

▲Information on retention samples, record keeping, storage conditions, and expiry dates, as well as guidelines for the package insert, is included in ⟨1235⟩.▲ (USP 1-May-2021)

***Delete the following:***

## ▲OTHER REQUIREMENTS

Retention samples are held by the manufacturer for at least six months after the expiration date. Enough material of each lot of each product is held for examination and testing for safety and potency (see 21 CFR 600.13).

Records are maintained concurrently with each step in the manufacture and distribution of product such that at any time successive steps of manufacture and distribution may be traced (see 21 CFR 600.12).

For storage conditions, see 21 CFR 610.50 and 53.

For shelf life/expiry date, see 21 CFR 610.50 and 53.▲ (USP 1-May-2021)

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***Add the following:***

# ▲⟨1239⟩ VACCINES FOR HUMAN USE—VIRAL VACCINES

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## INTRODUCTION

Vaccines for the prevention of at least 15 human viral diseases have been commercialized in the United States (see *Table 1*); others have been licensed outside the US. Together, these vaccines have formed a primary public health measure for the prevention of these viral diseases worldwide. For the purposes of this chapter, a viral vaccine is a vaccine that prevents a viral disease; this includes products derived from cultured viruses and those derived from recombinant expression of viral antigens. General considerations for both viral and bacterial vaccines are provided in *Vaccines for Human Use—General Considerations* ⟨1235⟩.

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**Table 1. Viral Vaccines Licensed in US for Prevention of Human Diseases**

| Live and Attenuated Viral Vaccines | Nonreplicating Viral Vaccines[a] |
|---|---|
| | **Inactivated or Killed** |
| • Adenovirus types 4 and 7[a]<br>• Influenza A and B<br>• Measles<br>• Mumps<br>• Rotavirus<br>• Rubella<br>• Smallpox<br>• Yellow fever<br>• Varicella-zoster (separately, chickenpox and shingles)<br>• Dengue<br>• Ebola Zaire | • Hepatitis A<br>• Poliomyelitis types 1, 2, and 3<br>• Rabies<br>• Japanese encephalitis |
| | **Split or Subunit Virus Vaccines: Recombinant or Virus-Derived** |
| | • Influenza A and B (virus-derived)<br>• Influenza A and B (recombinant) |
| | **Virus-Like Particle Vaccines** |
| | • Hepatitis B<br>• Human papillomavirus bivalent (types 16 and 18)<br>• Human papillomavirus quadrivalent (types 6, 11, 16, and 18)<br>• Human papillomavirus 9-valent (types 6, 11, 16, 18, 31, 33, 42, 52, and 58) |
| | **Nonreplicating Viral Vaccines** |
| | • Human papillomavirus 9-valent (types 6, 11, 16, 18, 31, 33, 42, 52, and 58) |

[a] Typically referred to as inactivated.

## General Categories of Viral Vaccines

There are currently two general categories of viral vaccines that have received regulatory approval: 1) live and attenuated and 2) nonreplicating. The nonreplicating viral vaccines contain three subgroups: virus-derived (killed/inactivated), split/subunit vaccines, and virus-like particles (VLP). Other strategies, including DNA and RNA-based vaccines, are under development. The characteristics, advantages, and challenges of viral vaccines are listed in *Table 2*.

**Table 2. Characteristics, Advantages, and Challenges of Viral Vaccines**

| Live and Attenuated Viral Vaccines | | |
|---|---|---|
| **Characteristics** | **Advantages** | **Challenges** |
| • Able to replicate in the host<br>• Attenuated in pathogenicity | • May elicit broader immune responses than other vaccine types (i.e., both humoral and cellular)<br>• May require fewer doses<br>• Highly effective<br>• Protection is generally longer lasting | • Limited understanding of mechanism of action and stability of attenuation<br>• Ensuring stability<br>• Characterization<br>• Manufacturing<br>• Possible transmission to another person via "shedding"<br>• Controlling reactogenicity |
| **Nonreplicating, Subunit, and Recombinant VLP Viral Vaccines** | | |
| **Characteristics** | **Advantages** | **Challenges** |
| • Unable to replicate in the host<br>• Not pathogenic<br>• Elicit mostly humoral immune responses | • Cannot multiply or revert to pathogenicity<br>• Generally less reactogenic<br>• Not transmissible to another person<br>• More stable than live viral vaccines | • Ensuring sufficient immunogenic potency<br>• May require adjuvant to enhance immune responses<br>• Stimulating cell-mediated immune responses<br>• Variable efficacy |

### LIVE AND ATTENUATED VIRAL VACCINES

Live viral vaccines contain live viruses or live viruses that have been attenuated (weakened) to reduce or eliminate their virulence (i.e., their ability to cause disease). Attenuation results from reassortment or from mutations induced by the passage of the virus in embryonated eggs, tissue culture using semi-permissive or atypical animal cells, or under altered growth conditions (e.g., at temperatures lower than the host's body temperature), or a combination of these mechanisms. Alternatively, live viral vaccines can be produced using recombinant technology. Attenuated vaccine strains are still immunogenic and replicate at a reduced rate compared to the wild type strain in their original host. The development of live viral vaccines is not standardized and the passage and propagation history varies between different vaccine viruses. Viruses for this group of vaccines are grown in human (e.g., MRC-5, WI-38) and non-human (e.g., primary chick embryo cells, Vero cells) cells, and do not undergo an inactivation step in their manufacturing process. Therefore, contamination by extraneous viruses and other adventitious agents already present in these substrates or introduced during manufacturing (e.g., raw material contamination) is a potential risk. This contamination risk requires a control strategy for manufacturing and testing that ensures vaccine purity, quality, and safety. Some live viral vaccines (e.g., yellow fever or live influenza) are produced using specific pathogen-free (SPF)

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eggs. These eggs are produced by chicken flocks that are regularly screened for avian pathogens (e.g., avian leukosis virus) and are maintained using appropriate animal husbandry practices.

### NONREPLICATING VIRAL VACCINES

**Inactivated or killed viral vaccines:** Inactivated (or killed) viral vaccines are produced by culturing and isolating the virus and then killing it chemically or with heat. The goal is to destroy the viruses' replicative function while keeping the outer virion coat intact. Nonreplicating viral vaccines may or may not be purified after harvest and before inactivation. Although many such vaccines have been successful in controlling viral diseases, not all viral proteins are equally immunogenic, and an unbalanced presentation (greater number of less immunogenic proteins) can dampen the immune response to protective antigens. Modification of the primary protective antigens can induce atypical immune responses that may enhance disease upon natural infection. This phenomenon was observed for formalin-inactivated measles virus and respiratory syncytial virus in early vaccines developed in the 1960s. Because of this experience, the use of attenuated measles vaccines is now recommended.
**Subunit or split virus vaccines:** Subunit vaccines are composed of viral gene products, which may be extracted from a cultured virus or produced via recombinant technology, e.g., in insect cell lines using baculoviruses. Traditional inactivated influenza A and B vaccine components are grown in embryonated eggs; and antigens of interest [e.g. hemagglutinin (HA) and neuraminidase (NA)] are purified following membrane solubilization (splitting). Recombinant HA antigens can be produced using the baculovirus expression vector system (BEVS), leveraging the natural infection process of insect cells by baculoviruses. For example, in one such vaccine, r-hemagglutinin proteins are produced in a continuous insect cell line derived from Sf9 cells of the fall armyworm, *Spodoptera frugiperda* (related to moths, caterpillars, and butterflies).
**Recombinant virus-like particle vaccines:** VLP vaccines, in which recombinant viral antigens are assembled (or self-assembled) into particles or are incorporated into a lipid vesicle, have been developed for human papillomavirus (HPV) and hepatitis B vaccines. The most common recombinant platforms are yeast (*Saccharomyces cerevisiae* for hepatitis B) and insect cell lines (*Spodoptera frugiperda* or *Trichoplusia ni* for HPV). Although they are more immunogenic than the monomeric antigen, some currently manufactured VLP vaccines based on recombinant protein technology require the addition of adjuvants [e.g., aluminum salts, monophosphoryl lipid A (MPLA), squalene emulsions] to optimize immunogenicity. These vaccines have similar advantages and challenges to nonreplicating viral vaccines.

## RAW MATERIALS AND MATERIALS OF ANIMAL ORIGIN

General guidelines for viral vaccines are found in ⟨1235⟩.

Culture ingredients must be selected carefully, especially for enveloped viruses since many of these can only be partially purified before losing infectivity or antigenicity. In such cases, the suitability of raw material for human administration can be a key criterion. A formal risk analysis of all raw materials should be performed to assess any risk of low-level contamination by unwanted entities. Media components for cell culture of viral vaccines (both during isolation and subsequent culture) need to be documented in detail. Chemically defined media without materials of animal origin are preferred. The cell culture medium should be suitable for the cells used in production. If human albumin is used, it must be licensed by the FDA for use in vaccines. The final product should be within specified limits of residual medium components such as serum, antibiotics, selection agents, or reagents added for growth enhancement. In the case of recombinant technology-based viral vaccines, fermentation media may consist of materials like proteins, sugars, inorganic trace elements, amino acids, and vitamins needed for cell growth. The protein component may be as simple as free casein (milk protein), or it can be as complex as extracts from bacterial, plant, or animal sources. The composition of a medium is often customized to optimize product quality attributes. Medium components that are known to cause allergic reactions should be avoided (e.g., eggs, gelatin).

For animal-derived raw materials it is particularly important to guard against the entry of adventitious agents into the process. Many vaccine viruses cannot be separated from environmental contaminants, and therefore prevention of environmental contaminant entry is required to ensure the safety of the product during the manufacturing operation. Animal raw materials are controlled in several ways. The origin of the herd(s) or flock(s) used is documented, controlled or isolated, and monitored to ensure the absence of disease. The material is harvested from the animals under controlled conditions using standardized, documented practices designed for pharmaceutical use to avoid contamination of the raw material with other viruses or microbes. The material is processed and stored accordingly and is often treated to destroy or remove any microbes that may have been introduced from the source or during processing (e.g., by sterile filtration, UV or gamma irradiation, detergents, heat, or a combination thereof). If additives from animal sources are added to the culture medium, they should be carefully sourced and certified to be free from contaminants and adventitious agents such as those that cause bovine spongiform encephalopathy or transmissible spongiform encephalopathy. To ensure an appropriate level of product safety, quantitative risk analysis with careful documentation should be performed on all factors that impact viral safety. For specific guidance on bovine serum quality attributes, sourcing, and testing, see *Bovine Serum* ⟨1024⟩ and *Fetal Bovine Serum—Quality Attributes and Functionality Tests* ⟨90⟩.

## EGGS AND PRIMARY CELL LINES USED IN VIRAL VACCINE PRODUCTION

The embryonated chicken egg is a highly productive growth substrate for certain viruses, such as those used to make vaccines for yellow fever and several influenza vaccines. In the case of influenza vaccines, the vaccine virus is harvested from egg allantoic fluid. In the case of yellow fever vaccine, the vaccine virus is harvested from embryo tissues. Therefore, residual egg or embryo components are special considerations in vaccine purification.

Egg-based vaccine production, like all biomass expansions, requires care and quality control of the virus seed lots and egg substrates to avoid contamination with other organisms. For example, chicken flocks used to prepare chicken embryo cells undergo extensive serological testing for adventitious agents before the flock can be used to prepare the cells. For vaccines

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produced in embryonated eggs the eggs may be either SPF (for viral seed production) or may be from healthy flocks (for vaccine production) monitored using methods approved by local animal health authorities.

Appropriate records must be retained for documentation [21 CFR 610.18(d)].

### Maintenance of Flocks

For in ovo cultured vaccines, eggs are derived from isolated healthy flocks of birds that are established for several generations and tested to ensure absence of common transmissible pathogens. Potential contaminants of the avian flocks (including those that infect nonhuman species) are routinely tested for and monitored. Flock origin and husbandry, isolation and management practices, health history, and testing should be documented clearly.

### Handling of Eggs

The number of eggs used for vaccine production varies according to the validated process in the manufacturers' license. Upon arrival at the facility, the eggs are first candled for egg selection and held up to light to view the embryo to determine its viability and lack of contamination. The eggs are then sanitized and injected with the vaccine virus of interest, which is allowed to propagate until harvest. Embryo age, candling procedures, sanitization methods, and injection and incubation methods are all critical manipulations performed under cGMP.

### Primary Avian Embryo Cell Lines

For vaccines produced in primary avian embryo cells, the practices described above should be followed and described up to the step of sanitization. Embryo retrieval, tissue dissection, and dissociation methods before in vitro culture should be conducted under highly controlled environmental conditions and with reagents of appropriate quality. The methods and raw materials used should be described. For these cultures, a substantial portion of cells is held aside uninfected for several weeks after the virus culture has been completed to ensure detection of any slow-growing microbes or viral agents. These uninfected control cell cultures are propagated and handled following the same process used for the virus-inoculated cells to help ensure that the cells used for vaccine production are free of adventitious agents.

### Primary Cell Lines from Other Animal Sources

Primary cell cultures from tissues can be cultured in vitro and used for vaccine production. Animals used to generate the primary cells or cell bank must be screened for potential viruses or have their serum tested to detect specific antibodies (see *Virology Test Methods* ⟨1237⟩ for specific guidance). Use of controlled colonies, herds, or flocks is recommended to monitor for specific pathogens. The use of primary cells requires additional testing from each manufacturing run to ensure the absence of contamination by infectious agents. To support the reduction of animal use, diploid and continuous cell lines are recommended. Continuous cell lines can be characterized extensively (including tumorigenicity testing, if required), and the culture conditions can be optimized and standardized. The Cell Banking system will provide production consistency. Continuous cell lines that express endogenous virus should be tested with tests capable of detecting such agents (see FDA Guidance for Industry-Characterization of Cell Substrates and Other Biological Materials Used in the Production of Viral Vaccines for Infectious Disease Indications and ICH Q5A).

Human diploid cells are used in the manufacturing process of several vaccines. The two most commonly used diploid cell lines, MRC-5 and WI-38, are derived from human embryonic cells and have the normal diploid number of human chromosomes. They are widely used to manufacture vaccines because they have been shown to have no tumorigenic or oncogenic potential and have been shown to be susceptible to a wide range of human viruses. However, unlike continuous cell lines that can be passaged indefinitely, human diploid cell lines can only be passaged a limited number of times, after which they experience a rapid decline in their ability to proliferate. This issue is managed by freezing multiple aliquots of master and working cell banks.

## CELL BANKS AND SEED LOT SYSTEMS

To produce vaccines in cell culture, two seed bank systems are required: one for the cell substrates and another for the viral seed lots. General information on the development, characterization, testing, and production of mammalian and bacterial cell banks and the related documentation requirements is provided in *Cell Banking* ⟨1042⟩ and ⟨1235⟩. Routine testing for master cell bank (MCB), working cell bank (WCB), control cells, and end of production cells (EOPC) is presented in *Table 3* below. A risk assessment for adventitious agents testing should be performed to determine if additional tests are needed (see note below *Table 3*). In cases where recombinant technology is used to develop the seed, the applicable guidance for information that should be supplied regarding the characterization and expression of such recombinant vectors can be found in *Quality of Biotechnological Products: Analysis of the Expression Construct in Cells Used for Production of r-DNA Derived Protein Products* ⟨1048⟩. Stability of cell-line viability under cryopreservative storage conditions must be demonstrated using real-time testing; see *Cryopreservation of Cells* ⟨1044⟩ for guidance on cryopreservation of cell lines.

### Viral Seed Lots

New master viral seed preparations are established under current good manufacturing practices (cGMP) conditions in a manner that is similar to the preparation of the cell banking process, as described in ⟨1235⟩ and ⟨1042⟩. The derivation and passage history of viral seeds (including the sourcing of all biological materials used: plasmids, animal-sourced raw materials, donor isolate and strain) are recorded in detail [21 Code of Federal Regulations (CFR) §610.18, *Cultures*]. Any manipulation of

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the viral phenotype (e.g., cold adaptation, development of temperature sensitivity, or attenuation of virulence) or intentional genetic manipulations (e.g., reassortment or recombination) should be documented (*e.g.* viral sequence level).

Viral seeds are commonly differentiated into a master viral seed and working viral seeds or working viral stock. Manufacturers should assess the following characteristics of the viral seed stock (characteristics assessed will depend on type of seed stock/ production process *i.e.* egg or cell line):

- Growth characteristics on the intended production cell substrate
- Tissue tropism
- Genetic markers
- Identity (for recombinant vectors)
- Viability during storage
- Genetic stability through production
- Attenuation properties
- Purity
- Microbial contamination

Absence of adventitious agents; if attenuation or derivation is achieved by passage through different species, the viral seed should be assessed for absence of adventitious agents also common to those species as well as for adventitious agents for the species in raw materials used.

The master viral seed should be extensively characterized to demonstrate the stability of the relevant genotype to a passage level comparable to a production batch. Cultures from the working seed lot should have the same relevant characteristics as the master seed lot from which they are derived. For influenza vaccines, which may be reformulated with new virus antigens each year, certified seed lots can be obtained from national regulatory agencies. A working seed lot is derived from the master seed within a limited number of passages. The working seed is tested to ensure its purity, freedom from adventitious agents based on viral risk assessment, and appropriate biochemical properties. The working seed is used for vaccine production without intervening passages. Reagents used for viral seed processing must be of appropriate quality.

Tests should be performed for identity (e.g., sequencing the entire virus or a portion of it), adventitious agents, viral phenotype, genetic stability, and, agents that might be present in the seed as a result of its passage history if applicable. Viral phenotype can be further assessed for tissue tropism, attenuation properties, and temperature sensitivity. Not all of these tests may be necessary for every viral seed strain.

Tests for identity, microbial contamination, mycoplasma, and adventitious agents should be performed based on established compendial and regulatory requirements (regulatory requirements vary and may be different outside the US). Depending on past treatments of the virus isolate (raw materials, agents, and biological reagents that the seed was in contact with), specific tests for additional adventitious agents that could potentially be present must also be carried out on the master viral seed preparation based on a risk analysis approach. The master viral seed bank is used to produce a working viral seed bank. The subsequent testing of the working viral seed preparation is dependent on the extent of testing carried out on the master seed, as well as the process and raw materials used to produce the working viral seed preparation. In the case of live and attenuated viral vaccines, the following tests should be included either on master or working viral seed:

- Safety test (microbial contamination, mycoplasma, mycobacteria)
- Whole genome sequencing
- Phenotypic, genotypic or any other attenuation test
- Genetic stability of seed virus
- Neurovirulence test (if virus is neurotropic, e.g., monkey neurovirulence test for yellow fever virus)

[NOTE—Assessment of neurovirulence should be performed if the virus is known to be neurotropic. Manufacturers should consult with regulators about appropriate animal models, methods, and scoring systems for this assessment before they initiate such studies. For viruses that are neurovirulent or may revert to neurovirulence (e.g., polioviruses), it may be necessary to assess neurovirulence beyond the master seed.]

**Table 3. Example of routine testing scheme for cell banks and viral seeds**

| TEST | MCB | WCB | Control Cells | Control Eggs | EOPC/ Extended CB | MVS | WVS |
|---|---|---|---|---|---|---|---|
| Identity | + | ± | + | — | ± | + | + |
| Sterility (bacterial/ fungal) | + | + | + | + | + | + | + |
| Mycobacteria | ± | ± | ± | — | ± | ± | ± |
| Mycoplasma | + | + | — | — | — | + | + |
| Spiroplasma[a] | + | + | — | — | — | + | + |
| Cytopathic effects | — | — | + | — | — | — | — |
| Hemadsorbing virus | — | — | + | — | — | — | — |
| Hemagglutinating agents | — | — | — | + | — | — | — |
| In vitro adventitious agents | + | + | ± | — | + | + | + |

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**Table 3. Example of routine testing scheme for cell banks and viral seeds** *(continued)*

| TEST | MCB | WCB | Control Cells | Control Eggs | EOPC/ Extended CB | MVS | WVS |
|---|---|---|---|---|---|---|---|
| In vivo adventitious agents[b] | + | — | ± | — | + | + | + |
| Specific virus by NAT | + | + | — | — | — | + | + |
| Tests for virus using broad molecular methods[c] | + | + | — | — | + | + | + |
| Retroviruses/ retroviral elements | + | — | ± | — | + | + | + |
| Transmission electron microscopy (TEM) | + | — | — | — | + | — | — |
| Antibody production assay | ± | — | — | — | — | ± | — |
| Bovine viruses[b] | ± | ± | ± | — | ± | ± | ± |
| Porcine viruses[b] | ± | ± | ± | — | ± | ± | ± |
| Avian viruses | — | — | — | — | — | + | + |
| Avian leucosis virus | — | — | + | + | — | — | — |
| Insect viruses[a] | + | + | — | — | — | + | + |
| Tumorigenicity | + | — | — | — | + | — | — |
| Oncogenicity | — | — | — | — | ± | — | — |
| + = *required stage for testing* | | | | | | | |
| ± = *alternative stage for testing* | | | | | | | |

[a] If insect cells or plant source raw materials used.
[b] If applicable.
[c] As alternatives to in vivo and specific NAT or as supplement/alternative to in vitro culture based on risk assessment as agreed with the competent authority.

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## Cell Culture

In some cases, the viral seeds may have a broad host range and therefore may require neutralization of the vaccine virus before they are tested for adventitious agent(s). The neutralization procedure must be demonstrated to not interfere with detection of adventitious agents. If possible, testing for adventitious agents should be done without neutralization to avoid an antiserum that may inadvertently neutralize an adventitious agent present in the seed.

A strategy for testing adventitious agents in viral vaccines must be developed based on a risk assessment following the principles of viral contamination risk. This strategy includes a full range of suitable tests able to detect different families of adventitious agents that may infect the source of virus strains including cell substrates and raw material of animal or plant origin. The capacity of the manufacturing process to remove or inactivate viruses is evaluated as part of the risk assessment. The list of tests must be adapted depending on the extraneous agents that have the potential to contaminate the product: for in vitro tests, the risk assessment may allow, with the agreement of the competent authority, the use of other permissive cell lines or molecular biology methods depending on the manufacturing process and the incubation temperature for the growth of particular viruses. If in vivo tests are more relevant than in vitro tests for the detection of some adventitious viruses (e.g. suckling mice for the vesicular stomatitis virus and fertilized SPF eggs for the influenza virus) the decision to maintain or to introduce such in vivo assays in a testing strategy must be justified by the risk assessment.

New, sensitive molecular methods with broad detection capabilities are available. These new approaches include high-throughput sequencing (HTS) methods, nucleic acid amplification techniques (NAT) (e.g. polymerase chain reaction (PCR), reverse transcriptase PCR (RT-PCR), product-enhanced reverse transcriptase (PERT) assays) for whole virus families or random-priming methods (associated or not with sequencing), hybridization to oligonucleotide arrays, and mass spectrometry with broad-spectrum PCR. These methods may be used either as an alternative to in vivo tests and specific NAT or as a supplement/alternative to in vitro culture tests based on the risk assessment and with the agreement of the competent authority.

If the master viral seed is well characterized, the working viral seed may not require extensive characterization. For example, it may not be necessary to repeat testing for all the relevant viruses from the derivation history.

## Seed Storage

To ensure consistent production of the viral vaccine product, a well-defined storage system is required. Viral seeds should be stored at temperatures to maintain their stability (i.e. in liquid nitrogen or ultra-low temperature freezers) and in more than one location within a facility or at a distant site (if possible) to decrease risk of loss in case of a catastrophic event. Manipulation

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of all vials must be controlled and documented to ensure the consistency of viral seed stock inventory. Viral seeds are stored under validated temperatures that support their stability.

A strategy to demonstrate the stability of the master and working viral seed preparations under the frozen storage conditions should be established, preferentially using real-time testing. Virus replication (infectious titer) can be determined during routine production. Trending the infectivity titer will alert the manufacturer to any stability issues and the need to produce a new working viral seed preparation. The master viral seed preparation should also be monitored in real time during the preparation of a new working viral seed. In the event that the master viral seed is not required for an extended period of time, a stability protocol with defined testing intervals should be implemented. If the infectious titers of the working viral seed are not decreasing, and the viral seed was produced and stored in a manner similar to the master viral seed preparation, stability of the master viral seed preparation may be inferred, and its testing may be reduced to conserve the supply.

### Recombinant Cell Lines

To date, the production of recombinant viral antigens has used yeast and insect cell lines. The requirements, as for any other recombinant expression system, are outlined in ⟨1235⟩. For cell lines containing exogenously assembled expression constructs (recombinant cell lines), characterization of nucleotide and amino acid sequences should be performed. Extensive characterization of cell banks should use the most relevant qualified technology. Chapters ⟨1042⟩ and ⟨1048⟩ provide advice on the characterization of the engineered cell line and expression construct.

## PROPAGATION AND HARVEST

Manufacturing of live and inactivated viral vaccines using eukaryotic cell culture is a two-phase production process. The first phase is the expansion of the cell cultures used as a substrate for viral replication. The second phase includes the initial virus infection and subsequent replication and virus production. Note that production of nonreplicating, recombinant viral antigens in yeast or insect cell lines does not involve infection of the growing cell and the requirements for these vaccines are essentially the same as the requirements for other recombinant proteins, described in the relevant compendial guidance for recombinant protein products.

## CELL SUBSTRATE GROWTH PHASE

The cell substrate expansion process for viral production is the phase designed to prepare the cells in an appropriate physiological state to sustain virus growth. Cell substrates often require complex animal-derived supplements such as serum. The source and testing requirements of bovine serum are subject to regulatory requirements (also see ⟨1024⟩).

## VIRUS PRODUCTION PHASE

Relatively few cell types have been used as substrates in US-licensed viral vaccines. Vaccine manufacturers have optimized nutrient requirements, growth factors, and serum concentration to support robust growth and strong virus productivity for these cell lines.

### In Ovo Cultivation

The embryonated chicken egg is a highly productive growth substrate for certain viruses, such as those used to make vaccines for yellow fever and several influenza vaccines. The culture of viruses in ovo is fairly straightforward. In the case of yellow fever vaccine, the vaccine virus is harvested from embryo tissues. In the case of influenza vaccines, the vaccine virus is harvested from egg allantoic fluid. Egg-based vaccine production, like all biomass expansions, requires care and quality control of the viral seed lots and egg substrates to avoid contamination with other organisms. Flock genetics, nutrition, and age can all affect in ovo virus propagation, as can egg sanitization procedures that may compromise the allantoic membrane cells. Optimization of the parameters should be documented to control the viral production process. Common egg culture variables include: egg age, amount of virus inoculated per egg, mode of inoculation, and harvest timing. Depending on the virus strain, the incubation temperature can also be critical. These factors have to be optimized for each individual serotype and master/working seed (for influenza viruses in particular), and the impact of all these factors should be investigated and documented.

### Establishment of Primary Cell Cultures

Procedures for recovery of primary cells should be described from organ recovery through dispersed cell suspension, with attention paid to the reproducibility of the cell substrate's properties in culture. This analysis may involve subsequent growth reproducibility, or if minimal growth is involved, it should be aimed at reproducibility of metabolism and viral receptivity and productivity. In cases where whole organism harvest is involved (e.g., whole avian embryo dissociation), only a fraction of the resulting cells may be susceptible to viral infection. Other cells from the harvest provide a supporting matrix and other factors and can potentially inhibit virus replication. Preferably, modern analytical methods would be used to characterize the spectrum of cells harvested. Under conditions suitable for viral replication, little or no cell growth is expected.

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## Cell Culture

Culture for live virus vaccine production generally consists of two phases: a cell growth phase and a virus production phase. These processes are often complicated due to the diversity of cell types and their general dependence on a surface for growth. Virus properties—including replication cycle speed, yield of virus from the cells, and stability of infectious virus or antigen under culture conditions—also exert major influences on process technology, configuration, and operation in the manufacturing setting. Reproducibility of the cell processing steps is critical, and therefore good documentation of each manipulation ensures consistency of key parameters for product manufacturing. The virus culture process begins with the establishment of a receptive cell mass of sufficient size to produce a batch, which is typically about $10^5$–$10^6$ doses of vaccine. Generally, about $10^6$–$10^8$ cells are cryopreserved in one or more aliquots, whereas about $10^8$–$10^{12}$ cells may be required for a production batch. Historically, processes have also used secondary or tertiary cultures, in which primary tissues are grown for several cell divisions to either increase the biomass size and batch size, or to minimize the amount of primary tissue required per batch, or both.

Recovery of cells from cryopreservation involves thawing the cells and diluting out the cryoprotective (banking) medium such that maximum cell viability is preserved and reproducible cell growth is ensured. Practices should be documented and justified on the basis of development experiments; typical parameters investigated are thaw rates and temperatures, dilution rates, and dilution factors. A protocol must be devised that results in reproducible cell numbers in a state conducive to virus production. This requires passaging the cells and providing a larger surface area for growth for attachment-dependent cells. Cell expansion output:input ratios for a passage are typically 2:1–20:1, depending on the cell type and growth medium. In certain contexts, cells require a minimum threshold concentration to provide each other with growth factors, and this requirement may reduce the passage ratio. Other cell types, or media that provide these growth factors, can allow much lower inocula and larger ratios.

Two typical objectives for expansion passaging reproducibility are: 1) ensuring that cells experience a minimal “lag phase” (sometimes caused by the need for sufficient production and accumulation of growth factors and cellular attachment factors) and 2) ensuring that cells do not grow to the point of stagnation/death (often caused by nutrient exhaustion, or by crowding on the surface that causes “contact inhibition” of growth). Either of these issues can lead to a lag phase in the next passage. Early cell expansion is usually performed in a separate production area to avoid the potential for cross-contamination. The earliest, small-volume passages are often conducted in open laboratory vessels (e.g., flasks, roller bottles). However, it is highly desirable to use closed containers to facilitate aseptic handling of containers and to minimize or eliminate operator exposure risk. Control of the environment and aseptic handling practices are written into the procedures for use during development and are maintained during manufacturing.

The final cell expansion is typically an integral part of virus production, with goals of achieving the final biomass required for production and producing cells that are highly productive for virus. In batch or fed-batch cultures, a “cell density effect” may exist, whereby growth to higher cell concentrations correlates with lower virus productivity on a per-cell basis. This can be caused by many factors and should be characterized to ensure that the process operates in a robust and predictable manner.

## Virus Culture

The final cell expansion and virus culture are typically a biphasic process in which the cells are grown in the final production vessel(s) and then are inoculated with virus. The viral inoculation often coincides with medium replacement or addition, as well as alteration of other culture parameters such as temperature. If cells are grown in a serum-containing medium, the medium for viral production is usually switched to a serum-free medium to minimize serum carryover into the harvest. If the virus is secreted into the medium and cannot be purified rigorously after harvest, extensive washing may be required to reduce residual serum to acceptable levels (see 21 CFR §610.15, *Constituent materials*). Documentation of washing effectiveness should be developed. Replacement of serum may be required for maximal virus yield, and this may entail the addition of other protein sources such as albumin, transferrin, or insulin.

Virus infection results are known to be sensitive to cell concentration; time of harvest; future cell substrate growth trajectory at the point of infection; and the “multiplicity of infection” (MOI), which is the number of infectious virus particles inoculated per cell. These factors, with the duration of viral culture, affect the quantities of infectious virus and total virus and can govern the quality of a vaccine via the ratio of infectious to total virus, the genetic stability, coupled with the degradation products that may have reduced immunogenicity. In addition to assessing titers over the duration of infection, it is useful to characterize virus stability under culture conditions with cell-free culture broth samples, because this can inform whether titer changes are due to changes in synthesis or degradation. High MOIs that occur over repeated passages are generally undesirable, especially for RNA viruses, because of the propagation of defective genomes that are trans-complemented by co-infection of a cell by another viral genome. Genetic stability of the vaccine virus should be well characterized. During development, the infection parameters need extensive characterization against all of the key quality attributes for the vaccine to ensure a robust process; thus, these parameters should be described.

Virus disposition upon replication is a significant determinant of the infection process configuration. Enveloped viruses are typically 1) secreted into the culture medium as they acquire their lipid envelope at the cell membrane or 2) fusing as secretory vesicles with the plasma membrane. For non-lytic viruses, multiple batch-wise harvests of the culture supernatant are common and re-feeding the culture upon harvest can promote higher yields. Perfusion processes are not used often because of product dilution and the difficulty, as well as added expense, of downstream concentration. For lytic viruses, destruction of the cells allows only for a single harvest; depending on the time elapsed from infection to lysis, feeding of the culture may maximize yields. Other viruses remain intracellular or cell associated, and processes for these may allow for extensive feeding to accumulate virus product. If the cell integrity is not compromised, washing to remove undesirable medium constituents may be performed at the end of the culture.

Many parameters of the culture environment also affect virus growth and stability, and it is expected that all relevant process parameters will be controlled to meet product quality expectations, as well as their acceptable ranges (“operating space”) as defined in the International Council for Harmonisation (ICH)’s *Q8(R2) Pharmaceutical development*. It is important to note that there is biological variability in cell growth, virus–host cell interactions are not well defined, and the analytics for potency and

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other parameters are highly variable and should be defined. Consequently, despite efforts to characterize viral vaccines and their processes, it is not uncommon to experience root mean square variances of 50%–100% in process output, even for a well-controlled process, unlike some other biological medicines.

## Control Cells During Production

Control cells are recommended for use when it is not feasible to directly test cells or product at various production stages. Control cells are tested for adventitious agents. For vaccines produced in cell cultures (e.g., live, attenuated, nonreplicating, and recombinant vaccines manufactured in insect cell lines), control cells may be evaluated for the presence of adventitious agents in conjunction with product testing using the same tests that are performed on the production culture and at the same time as a similar test would be performed on a manufactured product.

The national regulatory authority guidance-recommended volume of cell suspension used for seeding vaccine production culture should be used for the preparation of control cells. For static cell culture (using flask, roller bottle, cell stacks, or cell factory), the World Health Organization guideline recommends using a fraction equivalent to at least 5% of the total or 500 mL of cell suspension, or 100 million cells to prepare uninfected control cell cultures. For large scale production using a bioreactor, the volume of control cells should be as agreed with regulatory authority. The control cell culture should be treated similar to the production cell culture except the control is not infected with the vaccine virus under production; otherwise, the control cell culture should be maintained in conditions that are equivalent to those used for production culture, including the same batches of media and media changes, etc. The control cell should be incubated under the same condition of the production cell culture for 14 days or until the last virus harvest from production culture, whichever happens later. The control cells should be tested for identity and observed for cytopathic and morphological changes during this incubation period. For the test to be valid, at least 80% of control cells should not show any cytopathic or morphological changes during the observation period. Control cells should be tested for hemadsorbing viruses at the end of the observation period or at the time of viral harvest (whichever occurs later). At least 25% of the control cells are tested. If red blood cells to perform hemadsorption were stored, storage should be at 2°–8° and should not exceed 7 days. Calcium and magnesium ions should be absent from the medium for hemadsorption testing. The control cell culture supernatant should be tested for extraneous agents based on risk assessment aligned with the analytical testing strategy. For testing other extraneous agents, indicator cell lines (cell lines from simian origin and from human origin; production cell of a different batch if production cell is not of simian or human origin) should be inoculated with a control cell supernatant and incubated under the same conditions of the production cell culture for a period of 14 days. If any cytopathic changes due to extraneous agents occur in any of the cultures, the virus harvest produced from the batches of cells from which the control cells were taken should be discarded.

If avian tissues are used for propagation of a vaccine virus, avian leucosis virus (ALV) tests should be carried out using control cell culture supernatant. This testing may be carried out directly on the cell substrate. In some cases, detailed documentation of the health of a specific pathogen free flock may be sufficient to ensure absence of ALV.

For live attenuated vaccines that are produced in eggs, a risk-based control strategy for extraneous agents should be used. For example, control eggs may be tested for extraneous viruses. A portion of uninoculated embryonated eggs from the same batch of eggs used for vaccine production should be incubated under the same condition as those maintained for production and for the same incubation period. Allantoic fluid from control eggs should be checked for hemagglutinating agents and extraneous agents e.g., ALVs.

## Culture Process Technologies and Facility

Because of safety concerns regarding the use of tumorigenic cell types, the cells used for viral vaccine culture are almost uniformly dependent on an attachment surface for growth, with few anchorage-independent exceptions (e.g., Madin–Darby canine kidney cells for influenza). The attachment requirement creates a need to supply not only soluble nutrients but also an attachment surface for the common cell types (e.g., MRC-5 and Vero). Given the large variability in virus yields and production volumes, surface area requirements can differ substantially. This has created a situation in which a variety of technologies are used for cultivation. For small-scale operations, simple adaptation of laboratory vessels may suffice, and scale-up consists of scale-out (i.e., increasing the number of vessels). Beyond a certain point, aseptic risk precludes further scale-out. Intermediate scales of culture involve the use of specialized culture vessels, along with robotics where scale-out strategies are still used. Many of these technologies use disposable vessels and reactors with a focus on: 1) minimizing the risk of contamination and 2) the compatibility of product contact surfaces with the cells and virus. An extractable and leachable assessment should be performed for each disposable system of novel components used (see *The Biocompatibility of Materials Used in Drug Containers, Medical Devices, and Implants* ⟨1031⟩).

Large-scale production is performed in engineered bioreactor systems that use agitation technology similar to the technology for bacterial vaccines and other biologicals. In the most common reactors using agitation, attachment-dependent cells use "microcarrier" technology, which consists of small beads of about 150-µm in diameter that are easily suspended by agitation and provide a very large surface area in a given volume of culture. Other configurations with packed or static beads are also used for these systems. The vessels used can be made of either disposable plastics or stainless steel. Compatibility of the material with the medium and the cells, and the non-impact of leaching, should be evaluated for either type of vessel, as well as for fittings and probes used in monitoring and controlling the cultures. For nondisposable equipment, cleaning and sterilization procedures must be established, validated, and well documented. For reactors with loose or suspended carriers, the carriers should be readily excluded from the product stream and should not shed pieces or chemicals that are not suitable for human administration.

For all systems, the environment should be designed appropriately, reflecting the need for aseptic manipulations if they are required for either culturing or bioreactor set-up. In addition, the potential for spills or breakage and the risk of operator exposure need to be taken into account, especially when culturing wild-type virus strains. In a multi-product facility, changeover procedures that minimize the potential for cross-contamination and validation of inactivation should be established and

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documented. Operator training and facility management procedures must be established for handling cultures and avoiding contamination of the product, the facility, and the operators themselves.

## PURIFICATION

A general overview of purification of vaccines is presented in ⟨1235⟩. Special considerations are observed depending on the types of vaccines and production system used, as discussed below.

For live, attenuated, and nonreplicating virus vaccines, the desired antigen is a virus entity that is amplified from a working viral seed, in a complex biological substrate, and generally with minimal downstream processing to prepare the drug substance. Although nonreplicating viral vaccines also require viral seed expansion, virus inactivation and purification are more complex to ensure complete removal of infectious virus, removal of inactivating agents, and maintenance of the desired antigenic properties. Many factors should be considered in developing a downstream purification strategy, with the understanding that all processing steps will result in some loss of viable virus. Downstream processing steps are designed to: 1) remove or reduce cellular debris (e.g., host cell protein and DNA) from the vaccine harvest; 2) remove impurities or reduce their levels; 3) concentrate the virus; 4) add a virus stabilizer; and 5) remove or reduce potential microbial contamination from the virus preparation to meet a specified level of adventitious agent removal by filtration. The ability to do this may be limited in the case of live virus vaccines.

If a continuous cell line (e.g., Vero) is used for vaccine production, a validated filtration step is necessary to separate the virus from intact cells. The quantity and size of any residual host cell DNA should also be determined (see *Nucleic Acid-Based Techniques—Approaches for Detecting Trace Nucleic Acids (Residual DNA Testing)* ⟨1130⟩). Currently, NMT 10 ng of host cell DNA is permitted per dose of a parenterally administered vaccine, and regulatory agencies continue to consider the level of risk posed by host cell DNA for vaccines that are administered by other routes (e.g., nasal or oral) on a case-by-case basis. Multiple purification methods to reduce the size and amount of residual host cell DNA present in the vaccine are desirable and include steps such as treatment with DNase, diafiltration, ultrafiltration, and column chromatography.

### Viral Inactivation

Because of the pathogenic nature of the viruses (e.g., hepatitis A, Japanese encephalitis, rabies, and poliomyelitis), many viral antigens must be inactivated while maintaining their antigenic properties. Inactivation may involve the use of chemicals, detergents and surfactants, or irradiation, or various combinations thereof. Removal or reduction of the inactivating chemicals, detergents, and surfactants from the antigen to acceptable levels may be achieved by centrifugation, chromatography, ultrafiltration, diafiltration, or combinations of the above. The viral inactivation process should be optimized, validated, and well-documented. Inactivation is confirmed by carrying out a test for residual infectious viral particles on each lot.

### Live, Attenuated, and Nonreplicating Viral Vaccines

To preserve the infectivity and antigenic integrity of the vaccine viruses while removing egg-derived components, relatively simple, mild methods (e.g., zonal sucrose gradient centrifugation and diafiltration) are used for vaccine virus concentration, purification, and buffer exchange. After removal of cellular debris, further purification can be accomplished by using zonal centrifugation on a density gradient or by using column chromatography. Nucleic acid in Vero cell-produced vaccines can be reduced by treatment with suitable endonucleases, and removed by ultrafiltration using a 50-kDa membrane or by ion-exchange chromatography. The resulting drug product may be stabilized via cryoprotectant or lyophilization formulation (e.g., combinations of materials such as sucrose, phosphate, glutamate, and processed gelatin as stabilizers), filtration (using a filter of 0.22-µm pore size), for low bioburden bulk, and may be stored frozen at a temperature validated to support stability.

Some viruses may be heat labile, e.g., varicella virus, therefore precaution should be taken throughout manufacturing and distribution to maintain viral viability.

Viruses for nonreplicating vaccines can be produced using non-SPF eggs because of required chemical inactivation steps in the manufacturing process. Because the vaccine virus needs to be retained intact while removing egg-derived components and inactivating chemicals, relatively mild purification and concentration methods (e.g., zonal sucrose gradient centrifugation) are used. If chemical agents are used in the process, they should be minimized in the final product to below prespecified levels.

In some cases (e.g., the Varicella live vaccine) viruses are not released in the culture medium but remain associated to the cell substrate. In this case, the infected cells constituting a single harvest are rinsed, removed from the attachment surface, and subsequently disrupted by sonication to release the virus.

### Virus-Derived Subunit Vaccines

Although most seasonal influenza vaccines (influenza A and B) are prepared either in embryonated eggs or via cell culture, the derived virus is processed using different manufacturing methods to make the drug substance. The drug substances can be live or attenuated viruses (whole virus), a detergent-solubilized "split vaccine", or a subunit vaccine resulting from purification of the surface glycoproteins (e.g., HA and NA in the case of influenza virus) from the internal nucleocapsids. To produce the split virus and subunit vaccines, the whole virus is subjected to disruption with a surfactant, which solubilizes the viral membrane. For subunit vaccines, the internal subviral core of the virus is separated from the surface proteins on the basis of their differing sedimentation rates. With split-virus vaccines, the choice and use of surfactant ensures that the subviral core itself is disassembled. Diafiltration is normally used to remove the surfactant and other media components from the product, and then the process is terminated with a sterile filtration step to produce a monovalent bulk solution.

Viruses for split virus and purified subunit influenza vaccines are produced in embryonated eggs. Inactivation and purification of vaccine viruses are achieved by chemical treatment (e.g., formaldehyde or β-propiolactone) and zonal sucrose gradient

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centrifugation, respectively. Split virus vaccines are prepared by the disruption of vaccine virus particles using a detergent (e.g., sodium deoxycholate) that preserves antigenic integrity.

### Recombinant or VLP Vaccines

Recombinant proteins that are components of VLP vaccines, such as hepatitis B and HPV, undergo stronger, more stringent purification processes. As with nonreplicating viral vaccines, the purification methods must maintain the desired antigenic properties. After cell culture propagation of recombinant proteins or VLPs, the cells are harvested and purified by downstream processing. This may involve chromatography, precipitation, microfiltration, ultrafiltration, or other techniques that will be specific for the vaccine being produced by each manufacturer.

The first licensed human vaccine using recombinant DNA technology was the hepatitis B VLP vaccine. The hepatitis B VLP vaccine is derived from non-infectious hepatitis B virus particles. The vaccine drug substance consists of spherical particles of the recombinant hepatitis B virus surface antigen (HBsAg), a lipidated protein whose viral gene component was cloned into the yeast *Saccharomyces cerevisiae*. The HBsAg particles consist of approximately 30% lipid by mass. For hepatitis B VLP production it is necessary to express the particles in host cells that have the appropriate environment and lipid composition for the HBsAg to self-assemble into spherical particles of the correct size.

In the case of the HPV vaccine, formation of VLPs can coincidentally result in the incorporation of host cell components (e.g., DNA) into the quaternary structure of the molecular assembly, resulting in a class of impurities that has a tight association with the active pharmaceutical ingredient. As a result, in some cases modern approaches to VLP production include a disassembly step that dissociates impurities from the viral proteins. This procedure is followed by a reassembly step that reforms the VLPs in the absence of the host components. Liquid-phase extractions and chromatographic procedures can be used to provide high-purity components for use in vaccine products with no substantial risk of carrying over significant residual host components. One HPV vaccine is bivalent and consists of recombinant proteins expressed and purified from insect cells infected with recombinant baculovirus (the vaccine contains no viral genetic material). The second HPV vaccine is tetravalent and consists of recombinant proteins purified from yeast cells. After purification, the VLPs may be mixed or absorbed onto an adjuvant to increase the immune response.

Recombinant influenza virus can be produced in continuous insect cell cultures which are grown in a serum-free medium composed of chemically-defined lipids, vitamins, amino acids, and mineral salts. Each of the four HAs is expressed in this cell line using a baculovirus vector (*Autographa californica* nuclear polyhedrosis virus), extracted from the cells with Triton X-100 and further purified by column chromatography. The purified HAs are then blended and filled into single-dose syringes. Manufacturers must also ensure that residual host cell components are sufficiently removed during the purification of recombinant-derived vaccine components.

Characterization of the recombinant monomeric antigens follows the typical requirements for recombinant proteins and would include peptide mapping coupled with mass spectrometry to assess the integrity of the primary structure, as well as sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and other appropriate assays. Characterization of the intact VLPs will focus on assessing the size distribution of the particles using techniques such as transmission electron microscopy (TEM) or cryo-electron microscopy (cryoEM) and dynamic light scattering (DLS).

### In-Process Controls

Manufacturers identify critical process parameters and perform appropriate tests to monitor the purification process. Among the latter are filter integrity tests, microbial enumeration tests (see *Microbial Enumeration Tests* ⟨61⟩ and *Bacterial Endotoxins Test* ⟨85⟩), and other suitable tests for known process residues of concern. Process residues include raw materials, culture medium components (e.g., serum and antibiotics), cell substrates (e.g., proteins and DNA), buffer components (e.g., EDTA), and other materials (endonucleases, protamine sulfate, detergents/surfactants) used during purification. In addition to in-process control tests, manufacturers may perform in-process monitoring tests to monitor total protein, specific antigens, or ancillary materials at various steps in the purification process of intermediates. These in-process monitoring tests are carried out to gather product and process knowledge to evaluate consistency in the manufacturing process.

To demonstrate process performance and reliability, manufacturers should characterize inherent process-related impurities (e.g., protein, DNA, and lipids). Process validation studies may be carried out to demonstrate sufficient and consistent removal of certain residuals to an acceptable level such that routine testing is no longer required. If material must be sterile, analysts can perform *Sterility Tests* ⟨71⟩.

## INTERMEDIATES

Intermediates are defined as the unformulated active drug substances that are further processed before final formulation and can potentially be stored for long periods of time (see ⟨1235⟩). If intermediates are to be stored, hold times should be validated through a formal stability study or program. Stored intermediates are often suitable for more detailed characterization studies than are harvest bulks or final products (see, for example, *Table 4*). Some release tests are routinely performed before the intermediates are converted to the final bulk; this varies depending on the individual vaccines.

### Tests for Intermediates

In general, viral safety lot release testing is done at the bulk harvest stage before any purification. This is true regardless of whether the assay detects the infectious virus or viral components. Typically, the prefiltered harvest stage is the most concentrated due to the limited processing that has occurred; therefore, it is likely to be the best stage of production for testing for adventitious agents. Depending on the specific vaccine, different intermediates than the unprocessed harvest may be

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preferred for viral safety, for example, in cases where cytotoxicity of the unprocessed bulk harvest may be a concern. The presence of host cell DNA may need to be assessed in the case of biologics manufactured in animal cells. A hold step may be introduced at this processing step, for example, to combine two or more single virus harvests for further processing.

**Table 4. Example list of generic tests used for unprocessed bulk harvest (not required for all vaccine products). A risk-based approach for control should be performed and agreed to with the competent authority.**

| Assay | Purpose | Method |
|---|---|---|
| Adventitious agent | Verify absence of viral contamination | Cell culture with cytopathic effect (CPE) and hemadsorption endpoints |
| Mycoplasma | Detect mycoplasma | Cell culture, PCR, growth, or fluorescent staining as endpoints (*Mycoplasma Tests* ⟨63⟩) |
| Microbiological purity | Find evidence of microbial or fungal contamination | Bioburden/absence of bacterial and fungal contamination, other as appropriate aligned with manufacturing conditions |
| Potency | Measure quantity of infectious virus or antigen | Cell culture with plaque or tissue culture infective dose ($TCID_{50}$) endpoints; RNA quantification, egg infective dose ($EID_{50}$), plaque assay, single radial immunodiffusion (SRID), immunochemical method |

In-process adventitious agent inactivation steps are not included for live viral vaccines, because these steps could compromise the live nature of the vaccine itself. As a result, it may not be possible to validate clearance of any adventitious agents. Therefore, comprehensive testing for adventitious agents and qualification and control of the vaccine source materials (including cell substrate) are essential as part of vaccine safety control. Bioburden is monitored using appropriate microbiological methods based on technical principles described in, for example, ⟨61⟩ or ⟨71⟩.

Other product-specific testing may be appropriate. For example, for live influenza vaccines, each lot of viral harvest is tested for the cold-adapted phenotype (can grow at 25°) and the temperature-sensitive phenotype (restricted replication at 37°–39°). [NOTE—The attenuation phenotype (restricted replication in the upper and lower respiratory tract of ferrets) is tested at viral seed stage.] Each lot of viral harvest is also tested extensively by in vitro methods for contaminating agents. Viral harvests from the A and B strains are subsequently blended and diluted as required to the desired potency to produce bulk vaccine. If intermediates are stored prior to drug substance manufacturing, stability over the hold time should be established.

## DRUG SUBSTANCE

The drug substance is normally the bulk antigen stored in an appropriate container and may be partially formulated in anticipation of subsequent steps. In some cases, the drug substance might already have been combined with adjuvants and excipients to be exceptionally agreed with the National competent authority. The drug substance may have been filtered, sterile filtered, or aseptically processed and then tested respectively for bioburden with adequate acceptance limits or for sterility.

Drug substances can be stored in containers at temperatures that ensure their stability before further processing. Storage times for drug substances should be validated through a formal stability study or program. Purity testing may occur with the drug substance in lieu of drug product. Product- and process-related impurities are monitored to confirm that their levels meet the requested specifications. Because viral vaccine drug substances generally have high potency, and subsequent dilution to the drug product potency may be significant, measurements of process impurities are best obtained from the drug substance rather than from the final container.

Examples of release tests for the drug substance may include:

- Appearance
- Identity of antigen
- Potency and activity, or quantity of epitope or antigen
- Infectious titer (live, attenuated viral vaccines)
- Protein integrity and purity [e.g., by SDS–PAGE, size exclusion–high-performance liquid chromatography (SEC–HPLC), cation exchange (CEX)]
- Monomer content in multimeric VLP vaccines
- Impurities
- Endotoxins content
- Bioburden or sterility
- Mycobacteria
- Mycoplasma and spiroplasma

In addition to the examples listed above, other methodologies can be applied for identity and purity evaluation. For instance, specific impurities that must be measured are agreed upon between manufacturers and the licensing authority during the licensure process.

## DRUG PRODUCT

General guidelines for vaccine products are provided in ⟨1235⟩. This includes information regarding labeling and assessing the stability of vaccines.

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Mechanically, the processes of formulation and filling for viral vaccines have changed little over time. Formulation is usually a simple dilution of high-potency drug substance to the appropriate strength for filling that takes into account yield losses expected through subsequent temperature exposures (including freeze–thaw cycles) and, where applicable, losses due to lyophilization. Some nonreplicating, subunit, and recombinant VLP vaccines are produced as formulations that are adjuvanted, alum adsorbed, or both. It is important to note that production of drug product lots involves filling containers into the tens or hundreds of thousands, and special attention must be paid to ensuring homogeneity of product over so many containers with volumes ranging from 0.1 to 10 mL. For live products, thermal degradation can occur over the course of the filling operation, which should be considered in process design. For products containing aluminum suspensions, homogeneity of the suspension is a significant concern. Generally, upon agreement with the relevant licensing authority, liquid product is "overfilled", both with respect to volume (to ensure the ability to withdraw a full dose) and with respect to potency (to ensure a statistically acceptable probability that each container receives the declared potency at the end of expiry), given the variability of the process and assays used.

## LOT RELEASE TESTS

General requirements for drug product lot release testing of vaccines are detailed in ⟨1235⟩.

### Potency Assays

General requirements for potency tests are described in ⟨1235⟩. Many types of potency-indicating tests exist for viral vaccines and the selected potency test depends on the type of vaccine. Examples of viral vaccine potency assays include:

- Specific virus neutralization assay
- Quantitative PCR
- Viral antigen quantitation
- Viral titre
- Quantitative plaque formation assay
- Immunogenicity assay

### Common Tests

Tests for sterility, purity, residual moisture (lyophilized product), bacterial endotoxins, identity, excipients, and manufacturing residuals are described in ⟨1235⟩, except as described below. Viral and rickettsial vaccines and antigens are generally exempted from a requirement for a pyrogenicity test.

### Manufacturing Residuals

Expectations for the control of manufacturing residuals, including surfactants and those chemicals used in the inactivation process(es) are described in ⟨1235⟩. Formaldehyde and other chemicals used to manufacture vaccines must be minimized by purification steps in the manufacturing process. Limits are set on their residual content in the final vaccine, and content specifications are defined in the approved product license application.

Requirements to control residual material from cell substrates are described in ⟨1235⟩, including the steps that should be taken to prevent potential complications for hyper responsive recipients of these vaccines.

### Preservatives

Chapter ⟨1235⟩ provides information on the acceptable use of antimicrobial preservatives, the types of preservatives used, the minimization of thimerosal content as far as possible, and the production of thimerosal-free vaccines. Limits and content specifications are set for each vaccine in the product license.

### Excipients

Formulation chemicals for viral vaccines are often introduced during drug substance production, during the infection phase, or at harvest to enhance process productivity, particularly for enveloped live virus vaccines. Non-enveloped viruses or vaccines with extensive purification may introduce formulation chemicals only at the end of the purification process. A notable exception is formulation for combination viral vaccines, where the individual viruses may need stabilizers that are different from the global optimum for the mixture. In these cases, the final formulation of chemicals may be introduced late in the process to preserve maximum potency.

Formulation components may be included to minimize oxidation, to compete for other reactions that would damage the product, to provide pH buffering or tonicity, and in the case of lyophilized formulations, to replace water that would otherwise bind to proteins or provide other functions in the lyophilization process (e.g., cake formation/integrity). Chemically defined components are desirable and all components should meet appropriate pharmacopeial standards. In some instances, macromolecules such as albumin or dextran or fragments thereof (such as hydrolyzed gelatin) may be included in formulations to function as highly effective stabilizing agents. For those that originate from animal or plant sources or for new excipients, risk assessment should be conducted on the origin and processing of the materials to ensure freedom from infectious agents, and for other biological safety aspects (see *Excipient Biological Safety Evaluation Guidelines* ⟨1074⟩). Where pharmacopeial monographs exist, the excipients should conform. In addition to stabilizing agents, viral vaccines may contain preservatives if

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the final container contains multiple doses. Preservatives should meet antimicrobial effectiveness testing requirements in the formulation (see *Antimicrobial Effectiveness Testing* ⟨51⟩). It is common for preservatives to reduce the sensitivity of drug product sterility testing. This needs to be addressed during sample qualification for release testing to ensure adequate safety (see *Validation of Microbial Recovery from Pharmacopeial Articles* ⟨1227⟩). In addition, some formulations may be incompatible with other in vitro or in vivo safety testing and may require alterations of conditions or sample pretreatments to achieve valid test results.

### Adjuvants

Aluminum compounds including aluminum potassium sulfate (alum), aluminum phosphate, aluminum hydroxide, and combinations of these compounds are currently approved adjuvants for vaccine use. Chapter ⟨1235⟩ outlines sections of the 21 CFR §610.15 regulation governing the use of aluminum and the allowed amounts. Aluminum is quantified using colorimetric, titrimetric, or emission or atomic absorption spectroscopic methods of analysis (see *Aluminum* ⟨206⟩). The degree of adsorption of protein components to the adjuvant should be determined.

The adjuvants MPLA and squalene emulsions (AS03 and MF59 C.1) are licensed for vaccine use. MPL is quantified either through degradation to fatty acid methyl esters (FAMEs) and quantification by gas chromatography, or through quantification of the glucosamine content. Squalene is usually quantitated by HPLC.

## OTHER INFORMATION

Additional guidelines for viral vaccines covering product retention, shelf life, expiry dates, storage, container, and labeling are detailed in ⟨1235⟩.

## APPENDIX

### Selected Regulatory Documents

- 21 CFR §600.3 (Definitions)
- 21 CFR §610.2 (Lot release)
- 21 CFR §610. 9 (Alternative tests implementation)
- 21 CFR §610.10 (Potency)
- 21 CFR §610.12 (Sterility)
- 21 CFR §610.13 (Purity, moisture, pyrogens)
- 21 CFR §610.14 (Identity)
- 21 CFR §610.15 (Constituent materials)
- 21 CFR §610.17 (Combinations)
- 21 CFR 610.18(c)(1)(ii) [Tumorigenicity]
- 21 CFR 610.18 (d) (Egg records)
- 21 CFR §610.30 (Mycoplasma)▲ (USP 1-May-2021)

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# ⟨1240⟩ VIRUS TESTING OF HUMAN PLASMA FOR FURTHER MANUFACTURE

## SCOPE

The scope of this chapter is limited to the virus testing performed on human plasma for the further manufacture of pharmaceuticals, which are referred to as plasma-derived products (see *Virology Test Methods* ⟨1237⟩ for virus testing of other therapeutic products). These types of plasma include either source plasma collected by apheresis or recovered plasma obtained from whole blood collection or as a byproduct in the production of blood components. In all cases, the source material is obtained through voluntary donations. The following topics are specifically excluded from the scope of this chapter:

- Virus testing of nonhuman blood or plasma; for example, fetal bovine serum (see *Bovine Serum* ⟨1024⟩ for more information on testing this material), which may be used in the production of biological or recombinant therapeutics
- Virus testing of human-derived whole blood, blood components used for transfusion, and materials in tissue and organ banks
- Testing for nonviral organisms; for example, bacteria, fungi, and parasites (some of these topics are discussed in *Sterility Tests* ⟨71⟩), or the causative agent of transmissible spongiform encephalopathy.

This chapter introduces the virus testing that is performed on plasma used for the production of therapeutic proteins. Topics that are addressed include:

1. The rationale for implementing tests for viruses
2. The types of testing applied to plasma donations destined for further manufacture
3. The current regulatory environment for such virus testing

The chapter also includes an *Appendix* that contains pertinent regulatory guidances and supporting references.

## INTRODUCTION

Human-plasma-derived products are used to treat coagulation disorders, primary immune deficiency, and congenital emphysema as well as other diseases (see *Human Plasma* ⟨1180⟩). Because these products are manufactured from pooled human plasma donations, the presence of blood-borne viral pathogens from individual plasma donations can potentially contaminate the resulting final products manufactured from a large pool of donations and thus transmit the virus to many recipients. Sufficient measures must be taken to ensure that these products are as safe as possible. In order to minimize the risk of transmission of viruses by these products, manufacturers use several strategies, which include:

- Selection and management of the donors (see also ⟨1180⟩)
- Selection and management of donations or units (see also ⟨1180⟩)
- Testing for infectious viral pathogens in plasma in the form of samples of individual or pooled donations and fractionation pools (defined for the purposes of this document as the first homogenous pool or early production intermediate suitable for testing and representative of the material to be used for product manufacturing)
- Donor-screening methods, which also include a look-back procedure for the quarantine and destruction of unused, previously donated units from an infected donor (see also ⟨1180⟩)
- Incorporation of validated virus inactivation and removal steps (pathogen-reduction steps) into the manufacturing processes (see also ⟨1180⟩)
- Monitoring and investigating adverse events in recipients of final products, both hemovigilance and pharmacovigilance (see also ⟨1180⟩)
- Adherence to Good Manufacturing Practices at all levels of the production process as a strategy to reduce risk of virus transmission (see GAO-HEHS-98-205, 21 CFR Part 606, and WHO Technical Report Series 941 cited in the *Appendix*).

Plasma used for further manufacture can be either source plasma or recovered plasma. In the United States, licensed human plasma products are derived mainly from source plasma. Because plasma for further manufacture is obtained by pooling a large number of donations, there is a risk of viral contamination of the pool, thus resulting in a much higher potential risk of virus transmission to multiple recipients than is the case for blood for transfusion. The manufacturing process, which is used to purify and concentrate the desired protein, is not capable of completely removing the viral load, and therefore validated virus-reduction steps capable of effectively reducing transfusion-transmissible viruses in the starting material are included in the manufacturing process. A detailed discussion of virus inactivation and removal procedures for pathogen reduction can be found in the 2004 WHO Technical Report Series 924 cited in the *Appendix*.

Approaches for screening plasma for further manufacture can be categorized into two groups: donor-screening and in-process testing methods. The donor-screening method takes into account not only the plasma-derived end product but also the plasma donor. This category of testing typically is required for blood-transmissible viruses such as human immunodeficiency virus (HIV), hepatitis C virus (HCV), and hepatitis B virus (HBV). Virus transmission is a major public safety concern, because infections with these highly pathogenic viruses typically progress to chronicity. During donor screening the objectives of the test laboratory or manufacturer are not only to identify positive units for destruction before production pooling but also to identify and notify infected donors. Donors who test positive for HCV or HIV are permanently deferred from donating both blood and plasma. Although fewer than 5% of HBV-infected adults develop persistent asymptomatic infection (i.e., a carrier state), HBV-positive donors are deferred permanently. Collection of source plasma from donors who are convalescing from HBV is sometimes permitted for further manufacturing into plasma-derived products such as Hepatitis B Immune Globulin (Human) [21 CFR 610.41(3)].

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In contrast to viruses that are associated with donor screening, viruses such as hepatitis A virus (HAV) and parvovirus B19 (B19V) usually cause self-limiting infections in immunocompetent individuals, and thus manufacturers use in-process nucleic acid amplification technology (NAT) testing that results in only the removal of plasma units with high levels of virus before pooling for production. In these cases, there is no donor-management procedure and hence no requirement to inform the donor of the result. This approach focuses primarily on the product, not the donor, because some recipients of these products are susceptible to an infection that may occur if the pathogens were present in the plasma-derived product. Donor and donation-management procedures are well developed in the blood and plasma industry, and more details on these specific topics can be found in ⟨1180⟩. Other permanent or temporary donor-deferral criteria are in place to avoid donations from potentially infected donors based on the epidemiological surveillance of a country or region or donor population for transfusion-transmissible infections relevant to the safety of blood components.

Viruses that greatly affect public health, such as HIV, HBV, and HCV, are detected by serological assays that measure either a viral antigen, such as hepatitis B surface antigen (HBsAg), or an antibody, such as anti-HCV or anti-HIV-1/2 antibodies, in infected donors and associated donations. These immunoassays for detecting viral markers in plasma donations must be sensitive and able to detect a viral infection as early as possible following infection in order to identify and exclude potentially infectious donations.

There is a finite time period, known as the window period, between the infection of a donor and the time at which the test method can detect the antibody response to the virus, the viral antigens, or the viral nucleic acid. This window period varies from disease to disease as well as from person to person. The window period can be effectively "shortened" by changing from a test based on detecting antibodies to one based on detecting the virus directly, namely the viral antigen or, especially, the viral nucleic acid (see *Figure 1*), thereby interdicting donations that contain transfusion-transmissible viruses. Tests for detecting the viral nucleic acids (i.e., NAT tests) were introduced in the 1990s. NAT tests are sensitive and can considerably shorten the window period (see *Figure 1*). In a later development after B19V transmission incidents involving plasma-derived products, NAT tests were initiated to interdict high-titer donations, thereby decreasing the B19V virus load in manufacturing pools.

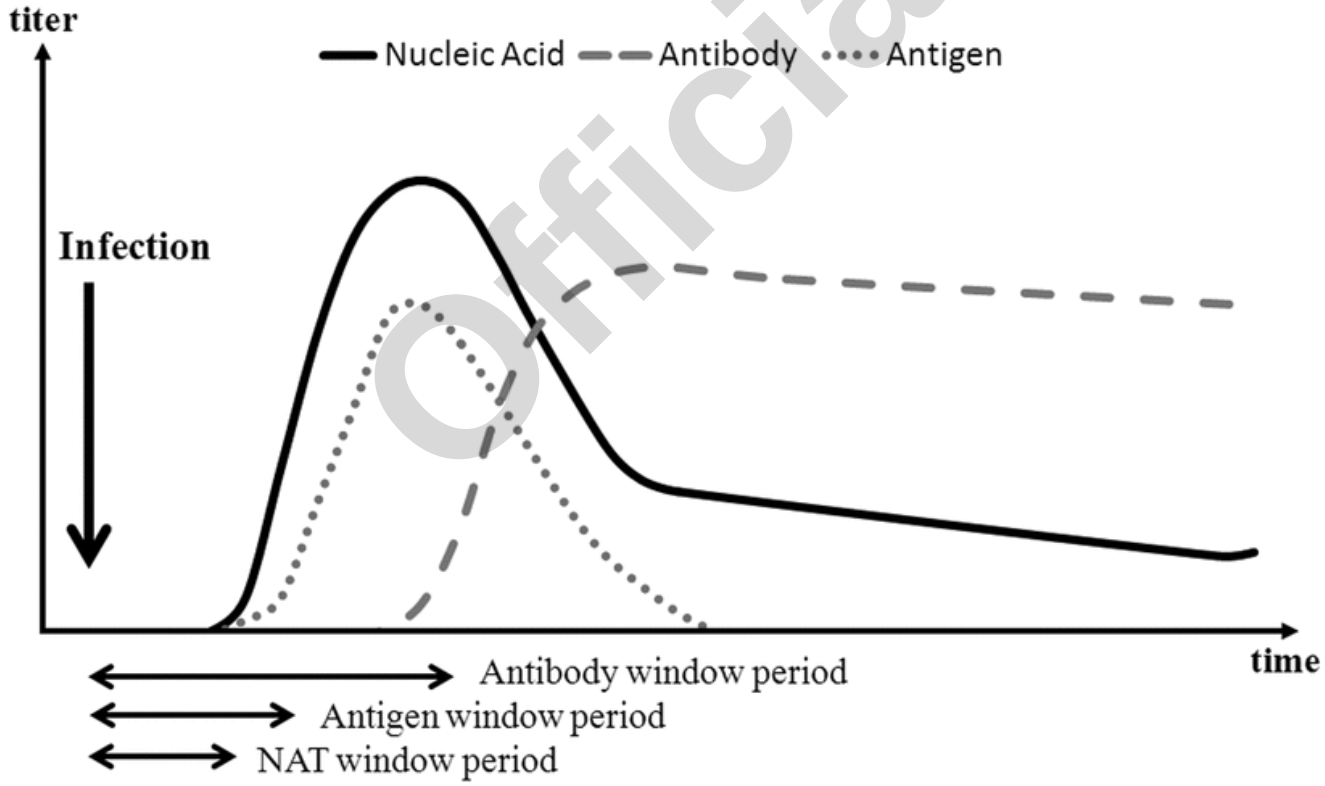


Figure 1. Dynamics of virus replication and detection of an infection.

Both source plasma and recovered plasma are tested by serological and NAT tests that are approved by competent regulatory authorities or, in the case of in-process testing, are validated to manufacturers' requirements. Plasma units found acceptable by these tests are combined into large pools, called fractionation pools, for manufacturing of plasma-derived products. The fractionation pool size can vary from several hundred donations (typically used for the production of specific immunoglobulins) to several thousand donations (used, for example, for the manufacture of albumin). Finally, the fractionation pools are retested for the target viruses. Testing of the plasma donations, at the individual or minipool level, and the fractionation pools are two of the elements that manufacturers put into place to maintain the safety margins of these products. Serology testing is performed on the individual donations. In contrast to serology tests, current NAT tests are highly sensitive and specific; therefore, manufacturers, in addition to testing individual donations, test minipools made up of equal volumes of each donation. Currently, the minipool size used for NAT testing varies from 6 to 512 donations. The high sensitivity of NAT tests also allows earlier virus detection compared to an antigen- or antibody-based test, thereby reducing the average length of the window period.

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Plasma-derived products are produced from tested fractionation pools and are further manufactured by using a combination of fractionation and purification steps. These steps may have some inherent potential to remove or inactivate viruses and thus reduce viral contaminants that may have been present in the starting plasma. Nevertheless, manufacturing of plasma-derived products also includes dedicated steps designed solely to inactivate (e.g., by solvent-detergent treatment) or remove (e.g., by virus filtration) potential viral contaminants.

## RATIONALE FOR VIRUS TESTING OF PLASMA FOR FURTHER MANUFACTURE

Historically, virological test methods have been used for detecting viral antigen or antibodies in clinical settings for disease diagnosis, intervention, and containment. Subsequently, these methods were adapted to screen blood and plasma donations with high sensitivity and specificity for transfusion-transmissible viruses. In order to develop a new virus screening test, scientists must know the biochemical properties of a new emerging pathogen (e.g., the nucleic acid sequence) for the development of an NAT test or the protein (e.g., antigen) for immunological tests. When implementing such a test for a given pathogen, the public health implications of positive test results and the potential for early intervention and treatment of the disease have to be considered. In addition, the availability of plasma for further manufacture and the effects of the virus on the safety of the finished product should be taken into consideration.

The emergence of a viral pathogen in the donor population could result in a considerable virus load in the plasma donations and in the resulting fractionation pools. For viruses such as HBV, HCV, HIV-1, and HIV-2 that can cause chronic diseases with potential public health effects, all donations positive for one or more of these viruses, irrespective of the virus titer, must be removed, and the donor must be informed. Some viruses such as B19V are prevalent in the population (as many as 1 in 5000 individuals may be infected during an epidemic period) and can be present at high virus titers in infected individuals. Thus, the removal of all B19V-positive donations could lead to a shortage of plasma. Instead, in-process NAT testing is done to interdict high-titer donations and thereby limit the B19V load in the manufacturing pool. The rationale for such screening is that B19V causes a self-limiting infection in most immunocompetent individuals. Following recovery, such individuals have neutralizing antibodies to B19V. Seroconversion occurs early in life, because B19V infection is common in childhood, and approximately 50% of 15-year-old adolescents have B19V antibodies. Infection of susceptible individuals continues throughout adult life, and B19V seroprevalence increases with age. The B19V neutralizing antibodies present in a plasma pool and the validated virus reduction steps included in the manufacturing process ensure that the inclusion of such donations does not compromise either the safety of the plasma-derived products or the availability of plasma for further manufacture.

In some instances it may not be necessary or feasible to test for a blood-borne virus. For example, testing of plasma for cell-associated viruses such as Human T Lymphotropic Virus (HTLV) types I and II, which present with no or with only limited virus load in plasma (but with a considerable virus load in whole blood donations) is not required. Similarly, testing for West Nile Virus (WNV), a member of the Flaviviridae family, is unnecessary because the virus load is low during the asymptomatic window period, the prevalence in the donor population is low (resulting in a low virus load in a plasma pool for fractionation), and WNV can be effectively inactivated by the manufacturing process as demonstrated by validation studies using relevant Flaviviridae model viruses. Therefore, WNV NAT testing for plasma (source and recovered) for further manufacture is not required. However, in the United States, the Food and Drug Administration (FDA) recommends WNV NAT testing for blood and blood components for transfusion because of the epidemiological situation and the risk of WNV transmission by blood components (see the FDA Guidance for Industry cited in the *Appendix*).

Other pathogenic viruses such as influenza viruses and severe acute respiratory syndrome coronavirus (SARS-CoV) are associated with clinical disease after a short incubation period and have a low or no virus load during the asymptomatic window period. No transmission by blood transfusion or plasma-derived products has been reported for these viruses. Furthermore, the manufacturing process for plasma-derived products has been shown to effectively inactivate influenza viruses. Therefore, NAT testing of plasma is not required for these viruses.

For a virus with a high prevalence in the donor population but without known clinical implications, no screening program, neither NAT nor serology, is required because the majority of donors would no longer be eligible to donate, thereby threatening the supply of blood and plasma and of plasma-derived products. Viruses that fall in this category are Torque Teno Virus (TTV), which is present in greater than 80% of the general population, and GB virus C (GBV-C, previously known as hepatitis G virus, HGV).

Newly emerging pathogens such as hepatitis E virus (HEV) can potentially enter the blood and plasma donor population, resulting in viral infections in recipients of blood and plasma-derived products. Monitoring the emergence of such agents is a continuous effort that involves academia, public organizations that monitor health and develop early warning systems, regulatory agencies, and industry. Currently, several epidemiological surveillance systems are in place and include hemovigilance or biovigilance to address the potential risk of emerging pathogens to the recipients of blood and plasma-derived products. This risk can be mitigated by appropriate measures, such as donor deferral because of geographic risk and risk behaviors, the testing of donations if appropriate, and the inclusion of virus-reduction steps for a wide range of enveloped and nonenveloped viruses during the manufacturing process.

## APPROACHES TO TESTING

Virological screening assays are designed to detect antibodies, antigens, or nucleic acid sequences of the infectious virus via serological and NAT testing. Sensitive virological test methods are a prerequisite for the quality control of fractionation pools in order to interdict and discard infected donations before manufacturers process these donations into pools to produce plasma-derived products.

All assays used to screen blood or plasma donations should be designed for their intended use and should meet the performance requirements specified by the Clinical and Laboratory Standards Institute (CLSI) guidelines for qualitative and quantitative tests. Assays also should comply with guidance from regulators, such as the European Common technical

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specifications for in vitro diagnostic assays (see *Appendix*). Associated calibrators or control materials must be traceable to reference material of a higher order or to reference measurement procedures. The *Appendix* includes FDA guidance documents pertaining to the manufacture and clinical evaluation of these assays and the use of controls. U.S. and EU requirements or recommendations for tests for screening plasma for further manufacture are described in the *Regulatory Environment* section.

### Serological (Immunological) Assays

Serological assays detect antibodies, antigen, or a combination of both. Antibody-detection assays usually are performed by incubating an immobilized virus antigen (virus lysate or, more common currently, virus proteins produced by recombinant protein technology) with a plasma sample. If antibodies specific to the viral protein are present in the sample, they bind to the target antigen. The virus-specific, bound antibody is in turn incubated with a labeled secondary antibody that is specific for the virus-specific, bound antibody. The label yields a signal that then is detected. Alternatively, for the measurement of a viral antigen present in a sample, immobilized antibodies specific for the viral antigen first are incubated with a plasma sample, a labeled antibody (often a monoclonal antibody) against the virus protein is added, and the mixture is incubated. For more details about these assays, see *Immunological Test Methods—Enzyme-Linked Immunosorbent Assay (ELISA)* ⟨1103⟩.

### Nucleic Acid Amplification Technology Tests

NAT is a collective term for the various methods that are used to amplify and detect the specific genomic sequences in various sample types. These methods include polymerase chain reaction (PCR), transcription-mediated amplification (TMA), and branched DNA (bDNA) and are detailed in *Nucleic Acid-Based Techniques—General* ⟨1125⟩, *Nucleic Acid-Based Techniques—Extraction, Detection, and Sequencing* ⟨1126⟩, and *Nucleic Acid-Based Techniques—Amplification* ⟨1127⟩. NAT tests typically use gene-specific oligonucleotides (e.g., primers, probes), enzymatic amplification reagents (e.g., buffers, nucleotides, enzymes, cofactors), and a method that allows the detection of the resulting amplification products. Currently, NAT tests for HBV, HCV, HIV, B19V, and, in some cases, HAV, are used to screen plasma for further manufacture.

For high-throughput testing, which is desired for the testing of plasma donations used for further manufacture, NAT offers distinct advantages over serological testing. First, because of the high sensitivity of these methods, samples of plasma donations can be combined into pools (minipools) that allow simultaneous testing of multiple samples, in contrast to serological assays that are performed on individual samples. Although the operational logistics used in donation minipool testing are more complicated than those involved in testing individual donations, the minipool approach generally allows an improved turnaround time for the release of negative samples compared to the traditional nonpooling method. A reactive minipool is deconstructed to identify any positive donation(s). However, because of the dilution of virus in any given donation, minipool testing has an inherently decreased sensitivity compared to individual donation testing.

*Table 1* summarizes the potential viral load, which could be avoided by NAT testing, in a fractionation pool caused by the inclusion of a single serological-window-period donation for the five major transfusion-transmitted viruses.

**Table 1**

| Virus | Potential Viral Load in Fractionation Pool Caused by Contamination with One Window-Period Donation (Approximate Values)[a] |
|---|---|
| HBV | $8 \times 10^5$ IU |
| HCV | $8 \times 10^{10}$ IU |
| HIV-1 | $8 \times 10^9$ IU |
| HAV | $8 \times 10^9$ IU |
| B19V | $8 \times 10^{14}$ IU |

[a] Assuming one plasma donation is approximately 800 mL. References supporting these values are found in the *Appendix*.

## REGULATORY ENVIRONMENT

Regulatory agencies have the goal of ensuring that plasma-derived products are safe with respect to risk from blood-borne pathogens. In addition to regulating the final products, regulators also oversee the assays used to test for infectious agents and set policies about how those tests will be used. Although regulatory agencies in the United States and Europe share the common goal of safety, they use different legal structures and strategies. In general, the hierarchy of regulatory documents is similar. Both start with laws that set the definitive requirements for donor selection and plasma screening.

In the United States, the primary laws that regulate plasma-derived products and the assays that test their safety are the Public Health Service (PHS) Act and the Federal Food, Drug, and Cosmetic (FD&C) Act. The PHS Act addresses biologics and communicable disease controls, and the FD&C Act addresses drugs and medical devices. Donor-screening tests are licensed under the PHS Act rather than being cleared or approved under the medical device provisions of the FD&C Act. Testing requirements for communicable disease agents, including viral pathogens such as HBV, HCV, and HIV, are required under 21 CFR, including not only test requirements (21 CFR 610.40) but also donor deferral (21 CFR 610.41) and look-back requirements (21 CFR 610.46 through 610.48). If a plasma or blood donation is reactive in one of the screening tests, especially in the donor-screening tests for HBV, HCV, or HIV, supplementary or confirmation tests should be conducted to clarify whether the donor is infected [21 CFR 610(b)]. The donor must be informed (21 CFR 630.6) and should be excluded from donating blood or plasma (temporarily or permanently according to 21 CFR 630.6), and manufacturers should initiate a look-back procedure (21 CFR 610.40–48). For HIV and HCV, the procedure includes not only the quarantine and destruction of unused,

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previously donated units from an infected donor, but also the further testing of the donor and notification of the recipients of the blood and blood components (21 CFR 610.47–48). The look-back period can be as long as 1 year (21 CFR 610.46–48).

The responsibility for legislation of the European Union (EU) is shared between the EU and the European Member States. The European Commission (EC) is responsible for the regulation of the common European market and thus is responsible for medicinal products for human use, whereas the Member States are responsible for health care, which includes the supply of hospitals with blood components such as plasma and cellular components for transfusion. Because the EC is responsible for the regulation of medicinal products derived from human blood or plasma, it is, as a consequence, also responsible for the regulation of plasma for further manufacture. The laws of the EU are found in regulations and directives from the EC and in the binding monographs of the *European Pharmacopoeia* (*Ph. Eur.*).

Additional tests and specifications for plasma for further manufacture have been developed as part of the Plasma Product Therapeutics Association (PPTA) Voluntary Standards Program. The PPTA Quality Standard for Excellence, Assurance, and Leadership (QSEAL) includes additional routine testing of blood and plasma donations or plasma pools for HCV RNA, HIV RNA, HBV DNA, HAV RNA, and B19V DNA. Companies certified by PPTA under the QSEAL program have implemented this testing.

## Testing of Plasma for Further Manufacture

In the United States, all virus tests intended for donor screening, such as HBV, HCV, and HIV, are regulated as biologics and are subject to clinical validation and licensure by FDA's Center for Biologics Evaluation and Research (CBER). Clinical specificity must be evaluated and demonstrated with healthy donors and follow-up testing when applicable, and clinical sensitivity should be evaluated and demonstrated with high-risk donors and follow-up testing. Use of reference panels (from FDA or a designated source) is needed for release of each lot of kits intended for market distribution. In-process tests such as NAT testing for HAV or B19V do not require clinical trials to demonstrate assay effectiveness. However, the manufacturers of plasma-derived products should perform preclinical validation and should submit data for review and approval by CBER as analytical procedures for plasma-derived products.

In the European Union, donation screening tests are regulated as medical devices. Specifically, Directive 98/79/EC outlines requirements for the approval of tests or test kits, which require the CE mark before marketing and use for testing. The CE mark confirms that the test or the test kit meets specified quality criteria. Screening tests for manufacturing pools must be validated by the end user following specific guidelines. As in the United States, in-process tests are not licensed, and the end user is responsible for validating the test.

In Europe, in addition to virus screening of plasma pools by the manufacturers of plasma-derived products, screening for defined viruses is part of the official batch-release procedure. The EC and the Council of Europe agreed in May 1994 to create a network of Official Medicines Control Laboratories (OMCLs). The OMCLs perform tests on each batch of plasma-derived medicinal product, including virus testing of the fractionation pool used to produce the batch. All required tests are performed and documented in the European Batch Release Certificate that is accepted by each Member State as the basis for placing the product on the market. In order to comply with the sensitivity limits set for NAT testing, minipools of various sizes (6–512 donations) are tested by the manufacturer or by the blood donation centers where the collection and testing of blood or plasma is performed. Only donations that meet the requirements are used for pooling.

## Serological Tests

### FDA REQUIREMENTS OR RECOMMENDATIONS

An individual donation of source plasma or recovered plasma derived from whole blood must be tested for HBsAg, anti-HIV-1, anti-HIV-2, and anti-HCV, but not for anti-HBc, anti-HTLV-I, and anti-HTLV-II by FDA-licensed serological tests intended for donor screening (*Table 2*). A reactive donation must be further tested by a supplemental (i.e., additional, more specific) test that has been approved for such use. Even with the implementation of corresponding NAT tests, serological testing of each donation still must be performed.

Currently, FDA recommends using licensed donor-screening kits that are capable of detecting anti-HBsAg, the antibody capable of neutralizing HBV, at 0.5 ng/mL or less. Whole blood sometimes is tested for anti-HBc, but because anti-HBsAg often occurs with anti-HBc, plasma for further manufacture is not required to be tested for anti-HBc. Therefore, although recovered plasma is derived from whole-blood donations that might have been tested for anti-HBc, it can be shipped for further manufacturing regardless of the test results.

FDA first recommended standardized anti-HIV-1 donor-screen tests in 1989 in a draft Points to Consider document that described test kit manufacture and the preclinical and clinical studies needed for licensure, and this recommendation generally can be applied to other serological tests. Since the availability in 1991–1992 of licensed serological kits for simultaneous detection of antibodies to HIV-1 and HIV-2, FDA further recommends the use of either a licensed combined test or two separate licensed tests for donor screening.

FDA licensed an anti-HCV test containing multiple recombinant antigens in 1992, and a subsequent guidance recommended that all donations for blood and blood components intended for transfusion and source plasma intended for further manufacture be screened by an FDA-licensed test for anti-HCV.

### EU REQUIREMENTS

Requirements for collection of blood and plasma, for selection of donors, and for testing of donations in Europe were released in 2003. Directives 2002/98/EC and 2003/63/EC contain donor-selection criteria and testing requirements for blood and plasma independent of its use. The requirements of the directives are standards for plasma for further manufacture but can be extended by a Member State for the regulation of blood components. An overview is provided in the Reports of the European Committee (Partial Agreement) on Blood Transfusion (CD-P-TS). The report indicates that in addition to the serological standard tests that are obligatory for the testing of plasma for fractionation (summarized in *Table 2*), testing for HIV antigen, anti-HBc antibodies,

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HCV antigen, anti-HTLV-I, and anti-HTLV-II antibodies is required in some Member States, and testing for antibodies against cytomegalovirus is performed in certain cases. However, these additional rules are applicable only if blood components for transfusion (erythrocytes, platelets, or plasma) are produced.

The test regime required for donations used for production of plasma derivatives are summarized in the *Ph. Eur.* monograph *Human Plasma for Fractionation (0853)*. Only licensed tests or test kits can be used for donor screening. Licensed tests have a CE mark, which confirms that the quality of the test meets predefined criteria (e.g., an HBV screening test must detect HBsAg in a concentration of 0.5 ng/mL or less). The most important quality attributes, namely specificity and sensitivity, are tested in clinical trials using donor samples (minimum of 5000 samples) and clinical samples (minimum of 200 samples). Sensitivity of the tests must be demonstrated with positive samples (minimum of 400 samples) and with seroconversion panels (minimum of 20 panels).

HBsAg tests and antibody tests against HIV Types 1 and 2 also are used for the testing of fractionation pools. The plasma manufacturer should demonstrate that the test is qualified for this use and meets the requirements laid down in the appropriate guidelines of the European Medicines Agency.

The current test regime is discussed regularly among EU Member States and may be subject to change if necessary because of the epidemiological situation. If changes in the requirements are made, the *Ph. Eur.* monograph *Human Plasma for Fractionation (0853)* and the product-specific monographs will be adopted accordingly (e.g., a change in the monograph *Human Plasma (Pooled and Treated for Virus Inactivation) (1646)* is proposed; it requires testing for HEV by NAT).

## NAT Tests

As described previously, only licensed serological tests that use antibody- or antigen-detection technology are required to screen plasma in single-donation format. However, NAT testing generally can detect evidence of viral infection at an early stage, and FDA licensed NAT tests for HIV-1 and HCV in 2001 for source plasma donors and in 2002 for whole-blood collections. Thus NAT tests are used to screen plasma donations, generally in a minipool format, using pool sizes that depend on the analytical sensitivity of the NAT test and, in some cases (HCV and B19V), the fractionation pool. In general, plasma donations are screened in a minipool format in which the pool size depends on the analytical sensitivity of the NAT test. The size of the minipool used for source plasma donations generally is much larger than that for blood donation testing (as large as 512 compared with 96 for blood donations). The turnaround time required for retesting a reactive pool in order to identify the reactive donation is less critical for plasma compared with that for blood for transfusion because some blood components such as platelets have a short shelf life.

### FDA REQUIREMENTS OR RECOMMENDATIONS

To adequately and appropriately reduce the risk of transmissions of HIV-1, HCV, and HBV, FDA-licensed NAT tests are required for donor screening. A list of FDA-licensed, donor-screening NAT tests and serological tests is updated as needed and is available on the FDA website.

FDA's initial guidance for HIV NAT in 1999 recommended standards for the manufacture and clinical evaluation of tests to detect nucleic acid sequences of HIV-1 and HIV-2 for licensure. This guidance provided some of the major regulatory and scientific guidance for NAT assays not only for HIV but also for other transfusion-transmitted viruses. Since then FDA has revised the requirements for the analytical sensitivity of HIV-1 and HCV NAT tests as 100 IU/mL for HIV-1 RNA and HCV RNA when tested in a minipool or as 10,000 IU/mL HIV-1 RNA or 5000 IU/mL HCV RNA when tested in an individual donation. FDA's 2004 guidance on NAT screening of HIV-1 and HCV in donor whole blood, blood components, and source plasma and a further guidance in 2010 contain recommendations about testing, product disposition, and donor deferral and reentry. The latter supersedes earlier recommendations for reentry of donor deferral and reentry because of serological testing results for anti-HIV-1 and anti-HCV.

The source plasma industry has voluntarily implemented HBV NAT testing in minipool format. Several FDA-licensed HBV NAT tests for donor screening are available. In 2012 FDA finalized a guidance recommending the use of HBV NAT on pooled and individual samples from donors of whole blood and blood components for transfusion or for further manufacture, including recovered plasma and source plasma. The guidance recommends an NAT test sensitivity of 100 IU/mL for testing individual donations of whole blood and blood components intended for transfusion. Because of the virus-reduction step(s) used during the manufacturing of plasma-derived products and the presence of neutralizing anti-HBsAg in the manufacturing pools, FDA recommends a NAT test sensitivity of 500 IU/mL for individual donations when manufacturers test minipools of plasma for further manufacture. The guidance also contains recommendations about product testing and disposition, donor management, methods of donor requalification, and product labeling. The guidance also supersedes the relevant recommendations based on HBsAg and anti-HBc serological testing results.

In 2009 FDA issued a final guidance for B19V NAT testing following a postmarket surveillance study report of a B19V transmission incident associated with solvent and detergent-treated (S/D-treated) pooled plasma. The guidance recommends the use of B19V NAT as an in-process test for plasma for further manufacturing to ensure that the level of B19V DNA in fractionation pools does not exceed $10^4$ IU/mL. The guidance document recommends that the primers and probes selected for a B19V NAT test should detect all known genotypes of the virus. The WHO International B19 Genotype Panel containing three genotypes is available for validation purposes. Currently, in-process HAV NAT testing is widely implemented by fractionators who use source plasma as starting plasma, but FDA has not issued a relevant guidance document. Because the in-process B19V NAT test is used to limit the virus load in the plasma pool, these tests must be capable of B19V DNA quantitation (unlike the NAT tests for HBV, HCV, HIV, and HAV, which are qualitative NAT tests).

### EUROPEAN REQUIREMENTS

NAT tests used for donor screening are subject to licensing and receive the CE mark if a test meets the predefined test specifications. NAT tests for plasma pool samples must be validated according to the *Ph. Eur.* general test *Nucleic Acid*

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*Amplification Techniques (20621)*. Currently, plasma for manufacture (plasma pools for fractionation) must be tested for HCV RNA by a NAT test, but there are no requirements for NAT testing for HBV DNA and HIV RNA although most plasma manufacturers voluntarily test for all three viruses. The guideline requires that a test should be able to detect all HCV genotypes. However, in view of the difficulty of obtaining rare HCV genotypes, it is sufficient that at least the most prevalent genotypes (in Europe, genotypes 1 and 3) are detected at a suitable level. Plasma should be negative when screened with a test that can detect a sample containing 100 IU/mL of HCV RNA (calibrated against the WHO HCV International Standard).

Testing for B19V DNA generally is not required but must be performed for products that are seen as a higher risk for patients if this virus is present (e.g., anti-D immunoglobulin products and plasma that is pooled and inactivated by S/D treatment). In addition, the latter product also must be tested and found nonreactive for HAV RNA, and in the future it also should be nonreactive for HEV RNA. Unlike testing for HCV and HAV where the fractionation pool should be nonreactive for these viruses, for B19V testing the virus load in the fractionation pool and pooled S/D-treated plasma should not exceed 10 IU/µL B19V DNA. The requirements are detailed in the product-specific *Ph. Eur.* monographs. In order to avoid a reactive pool, which would have to be discarded, NAT testing also is performed on single donations or preferentially on minipools comprising 16–512 donations. Although the requirement for B19V and HAV NAT testing is applicable only to plasma used for the manufacture of pooled S/D-treated plasma and anti-D immunoglobulin products, most plasma manufacturers voluntarily test plasma destined for manufacture of all plasma-derived products to reduce the virus load in the fractionation pools.

The current FDA and EU requirements for testing plasma for further manufacture are summarized in *Table 2* and *Table 3*.

**Table 2. FDA and EU Serology Testing Requirements for Plasma for Further Manufacture**

| Screening Test | FDA | EU |
|---|---|---|
| **Serological Testing of Individual Plasma Donations (Recovered and Source)** | | |
| HBsAg | Required | Required |
| Anti-HBc | Not required | Not required |
| Anti-HIV-1/Anti-HIV-2 | Required | Required |
| Anti-HTLV-I/II | Not required | Not required |
| Anti-HCV | Required | Required |
| **Serological Testing of the Fractionation Pool** | | |
| HBsAg | Not required but widely implemented by plasma fractionators | Required |
| Anti-HIV | Not required but widely implemented by plasma fractionators | Required |

**Table 3. FDA and EU NAT Testing Requirements for Plasma for Further Manufacture**

| Screening Test | FDA | EU |
|---|---|---|
| **NAT Testing of Plasma Donations in Minipool Format** | | |
| HIV-1 RNA | Required, using tests with a sensitivity of 10,000 IU/mL for the individual donation | Not required but widely implemented by plasma fractionators |
| HCV RNA | Required, using tests with a sensitivity of 5000 IU/mL for the individual donation | Not required but recommended in order to avoid unnecessary loss of a fractionation pool (see below) |
| WNV RNA | Not required | Not required |
| HBV DNA | Required, using tests with a sensitivity of 500 IU/mL for the individual donation | Not required but widely implemented by plasma fractionators |
| B19V DNA | Required with a manufacturing pool limit of $\leq 10^4$ IU/mL B19V DNA | Required for specific products (anti-D immunoglobulin and pooled S/D-treated plasma); a manufacturing pool limit of B19V DNA $\leq$10 IU/µL is required. This limit is voluntarily implemented by most plasma fractionators for all products. |
| HAV RNA | Not required but widely implemented by plasma fractionators | Required only for S/D-treated plasma; not required for other products but widely implemented by most plasmafractionators |
| HEV | Currently not required | Testing not yet required; testing requirements will be introduced for a specific product only (pooled S/D-treatedplasma). |
| **NAT Testing of the Fractionation Pool** | | |
| HIV-1 RNA | Not required but widely implemented by plasma fractionators | Not required but widely implemented by plasma fractionators |
| HCV RNA | Not required but widely implemented by plasma fractionators | Required; the fractionation pool must be nonreactive using a test that detects 100 IU/mL of HCV RNA. |
| WNV RNA | Not required | Not required |
| HBV DNA | Not required but widely implemented by fractionators | Not required but widely implemented by fractionators |
| B19V DNA | Required; a limit of $\leq 10^4$ IU/mL B19V DNA for fractionation pools is required. | Required for specific products (anti-D immunoglobulin and pooled S/D-treated plasma); a limit of B19V DNA $\leq$10 IU/µL for fractionation pools is required. This limit is voluntarily implemented by most plasma fractionators for all products. |

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**Table 3. FDA and EU NAT Testing Requirements for Plasma for Further Manufacture** *(continued)*

| Screening Test | FDA | EU |
|---|---|---|
| HAV RNA | Not required but widely implemented by plasma fractionators | Required only for a specific product (pooled S/D-treated plasma); the fractionation pool must be nonreactive using a test that detects 100 IU/mL. |
| HEV | Not required | Testing not yet required; testing requirements will be introduced for a specific product only (pooled S/D-treated plasma). After the requirement is implemented, the plasma pool must be nonreactive using a test that can detect 2.5 $\log_{10}$ IU/mL of HEV RNA[a]. |

[a] The new monograph will be implemented soon. See the draft *Ph. Eur.* monograph *Human Plasma (Pooled and Treated for Virus Inactivation (1640)).* http://pharmeuropa.edqm.eu/TextsForComment/NetisUtils/srvrutil_getdoc.aspx/2L3OqDZGmCLmnDZGsHIveT6q0//1646E.pdf. Accessed 20 February 2013.

## CONCLUSIONS

All the measures discussed in this chapter, along with virus-reduction steps included during the manufacturing process, ensure the safety of plasma-derived products. However, testing of plasma for further manufacture is only one of the steps taken to ensure the safety of the final plasma-derived products. Both manufacturers and regulators face continuing challenges because of the emergence of new blood-borne viruses, mutants, and variants of existing viruses not detected by current serological/NAT technology. The development of new screening tests and regulatory guidance documents depends on whether the emerging virus is a risk to the safety of plasma-derived products.

## APPENDIX

### Regulatory Guidances


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# ⟨1241⟩ WATER–SOLID INTERACTIONS IN PHARMACEUTICAL SYSTEMS

## INTRODUCTION

This general chapter is harmonized with the corresponding texts of the *European Pharmacopoeia* and/or the *Japanese Pharmacopoeia.* These pharmacopeias have undertaken not to make any unilateral change to this harmonized chapter. Portions of the present general chapter text that are national USP text, and therefore not part of the harmonized text, are marked with symbols (•,•) to specify this fact.

Pharmaceutical solids as raw materials or as constituents of dosage forms most often come in contact with water during processing and storage. This may occur (a) during crystallization, lyophilization, wet granulation, or spray drying; and (b) because of exposure upon handling and storage to an atmosphere containing water vapor or exposure to other materials in a dosage form that contain water capable of distributing it to other ingredients. Some properties known to be altered by the association of solids with water include rates of chemical degradation in the "solid-state", crystal growth and dissolution, dispersibility and wetting, powder flow, lubricity, powder compactibility, compact hardness, and microbial contamination.

Although precautions can be taken when water is perceived to be a problem, i.e., eliminating all moisture, reducing contact with the atmosphere, or controlling the relative humidity of the atmosphere, such precautions generally add expense to the process with no guarantee that during the life of the product further problems associated with moisture will be avoided. It is also important to recognize that there are many situations where a certain level of water in a solid is required for proper performance, e.g., powder compaction. It is essential for both reasons, therefore, that as much as possible is known about the effects of moisture on solids before strategies are developed for their handling, storage, and use.

Some of the more critical pieces of required information concerning water–solid interactions are:

- total amount of water present;
- the extent to which adsorption and absorption occur;
- whether or not hydrates form;
- specific surface area of the solid, as well as such properties as degree of crystallinity, degree of porosity, and glass transition and melting temperature;
- site of water interaction, the extent of binding, and the degree of molecular mobility;
- effects of temperature and relative humidity;
- essentially irreversible hydration;
- kinetics of moisture uptake;
- various factors that might influence the rate at which water vapor can be taken up by a solid;
- for water-soluble solids capable of being dissolved by the sorbed water, under which conditions dissolution will take place.

## PHYSICAL STATES OF SORBED WATER

Water can physically interact with solids in different ways. It can interact at the surface (adsorption) or it can penetrate the bulk solid structure (absorption). When both adsorption and absorption occur, the term sorption is often used. Adsorption is particularly critical in affecting the properties of solids when the specific surface area is large. Large values of specific surface area are seen with solids having very small particles, as well as with solids having a high degree of intraparticle porosity. Absorption is characterized by an association of water per gram of solid that is much greater than that which can form a monomolecular layer on the available surface, and an amount that is generally independent of the specific surface area.

Most crystalline solids will not absorb water into their bulk structures because of the close packing and high degree of order of the crystal lattice. Indeed, it has been shown that the degree of absorption into solids exhibiting partial crystallinity and partial amorphous structure is often inversely proportional to the degree of crystallinity. With some crystalline solids, however, crystal hydrates may form. These hydrates may exhibit a stoichiometric relationship, in terms of water molecules bound per solid molecule, or they may be non-stoichiometric. Upon dehydration, crystal hydrates may either retain their original crystal structure, lose their crystallinity and become amorphous, or transform into a new anhydrous or less-hydrated crystal form.

Amorphous or partially amorphous solids are capable of taking up significant amounts of water because there is sufficient molecular disorder in the solid to permit penetration, swelling, or dissolution. Such behavior is observed with most amorphous polymers and with small-molecular-mass solids rendered amorphous during preparation, e.g., by lyophilization, or after milling. The introduction of defects into highly crystalline solids will also produce this behavior. The greater the chemical affinity of water for the solid, the greater the total amount that can be absorbed. When water is absorbed by amorphous solids, the bulk properties of the solid can be significantly altered. It is well established, for example, that amorphous solids, depending on the temperature, can exist in at least one of two states: "glassy" or "fluid"; the temperature at which one state transforms into the other is the glass transition temperature, $T_G$.

Water absorbed into the bulk solid structure, by virtue of its effect on the free volume of the solid, can act as an efficient plasticizer and reduce the value of $T_G$. Because the rheological properties of "fluid" and "glassy" states are quite different, i.e., the "fluid" state exhibits much less viscosity as the temperature rises above the glass transition point, it is not surprising that a number of important bulk properties dependent on the rheology of the solid are affected by moisture content. Because amorphous solids are metastable relative to the crystalline form of the material, with small-molecular-mass materials, it is possible for absorbed moisture to initiate reversion of the solid to the crystalline form, particularly if the solid is transformed by the sorbed water to a "fluid" state. This is the basis of "cake collapse" often observed during the lyophilization process. An additional phenomenon noted specifically with water-soluble solids is their tendency to deliquesce, i.e., to dissolve in their own sorbed water, at relative humidities, $RH_i$, in excess of the relative humidity of a saturated solution of the solid, $RH_0$. Deliquescence arises

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because of the high water solubility of the solid and the significant effect it has on the colligative properties of water. It is a dynamic process that continues to occur as long as $RH_I$ is greater than $RH_0$.

The key to understanding the effects water can have on the properties of solids, and vice versa, rests with an understanding of the location of the water molecule and its physical state. More specifically, water associated with solids can exist in a state that is directly bound to the solid, as well as in a state of mobility approaching that of bulk water. This difference in mobility has been observed through such measurements as heat of sorption, freezing point, nuclear magnetic resonance, dielectric properties, and diffusion.

Such changes in mobility have been interpreted as arising because of changes in the thermodynamic state of water as more and more water is sorbed. Thus, water bound directly to a solid is often thought as unavailable to affect the properties of the solid, whereas larger amounts of sorbed water may become more clustered and form water more like that exhibiting solvent properties. In the case of crystal hydrates, the combination of intermolecular forces (hydrogen bonding) and crystal packing can produce very strong water–solid interactions. Recognizing that the presence of water in an amorphous solid can affect the glass transition temperature and hence the physical state of the solid, at low levels of water, most polar amorphous solids are in a highly viscous glassy state because of their high values of $T_G$. Hence, water is "frozen" into the solid structure and is rendered immobile by the high viscosity, e.g., $10^{13}$ Pa · s. As the amount of water sorbed increases and $T_G$ decreases, approaching ambient temperatures, the glassy state approaches that of a "fluid" state and water mobility along with the mobility of the solid itself increases significantly. At high RH, the degree of water plasticization of the solid can be sufficiently high so that water and the solid can now achieve significant amounts of mobility. In general, therefore, this picture of the nature of sorbed water helps to explain the rather significant effect moisture can have on a number of bulk properties of solids such as chemical reactivity and mechanical deformation. It suggests strongly that methods of evaluating chemical and physical stability of solids and solid dosage forms take into account the effects water can have on the solid when it is sorbed, particularly when it enters the solid structure and acts as a plasticizer.

### Rates of Water Uptake

The rate and extent to which solids exposed to the atmosphere might either sorb or desorb water vapor can be a critical factor in the handling of solids. Even the simple act of weighing out samples of solid on an analytical balance and the exposure, therefore, of a thin layer of powder to the atmosphere for a few minutes can lead to significant error in, for example, the estimation of loss on drying values. It is well established that water-soluble solids exposed to relative humidities above that exhibited by a saturated solution of that solid will spontaneously dissolve via deliquescence and continue to dissolve over a long time period. The rate of water uptake in general depends on a number of parameters not found to be critical in equilibrium measurements because rates of sorption are primarily mass-transfer controlled with some contributions from heat-transfer mechanisms. Thus, factors such as vapor diffusion coefficients in air and in the solid, convective airflow, and the surface area and geometry of the solid bed and surrounding environment, can play an important role. Indeed, the method used to make measurements can often be the rate-determining factor because of these environmental and geometric factors.

## DETERMINATION OF SORPTION–DESORPTION ISOTHERMS

### Principle

The tendency to take up water vapor is best assessed by measuring sorption or desorption as a function of relative humidity, at constant temperature, and under conditions where sorption or desorption is essentially occurring independently of time, i.e., equilibrium. Relative humidity, RH, is defined by the following equation:

$$RH = (P_C \times 100)/P_0$$

$P_C$ = pressure of water vapor in the system

$P_0$ = saturation pressure of water vapor under the same conditions

The ratio $P_C/P_0$ is referred to as the relative pressure. Sorption or water uptake is best assessed starting with dried samples and subjecting them to a known relative humidity. Desorption is studied by beginning with a system already containing sorbed water and reducing the relative humidity. As the name indicates, the sorption–desorption isotherm is valid only for the reference temperature, hence a special isotherm exists for each temperature. Ordinarily, at equilibrium, moisture content at a particular relative humidity must be the same, whether determined from sorption or desorption measurements. However, it is common to see sorption–desorption hysteresis.

### Methods

Samples may be stored in chambers at various relative humidities. The mass gained or lost for each sample is then measured. The major advantage of this method is convenience, while the major disadvantages are the slow rate of reaching constant mass, particularly at high relative humidities, and the error introduced in opening and closing the chamber for weighing. Dynamic gravimetric water sorption systems allow the on-line weighing of a sample in a controlled system to assess the interaction of the material with moisture at various programmable levels of relative humidity at a constant temperature. The major benefit of a controlled system is that isothermal conditions can be more reliably established and that the dynamic response of the sample to changing conditions can be monitored (see *Figure 1*).

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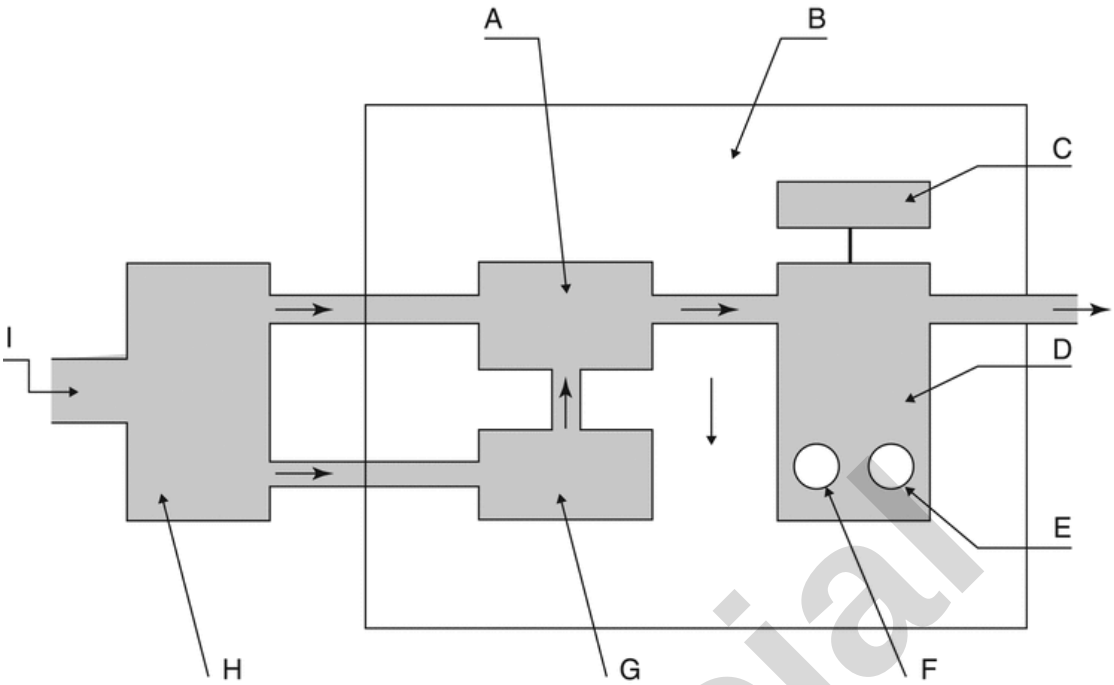


A. Humidity controller
B. Temperature controlled chamber
C. Balance module
D. Humidity regulated module
E. Reference
F. Sample
G. Vapour humidifier
H. Flow control module
I. Dry gas

Figure 1. Example of an apparatus for the determination of the water sorption (other designs are possible).

Data points for the determination of the sorption isotherm (e.g., from 0% to approximately 95% RH, noncondensing) are only taken after a sufficiently constant signal indicates that the sample has reached equilibrium at a given level of humidity. In some cases (e.g., deliquescence), the maximum time may be restricted although the equilibrium level is not reached. The apparatus must adequately control the temperature to ensure a good baseline stability as well as accurate control of the relative humidity generation. The required relative humidities can be generated, e.g., by accurately mixing dry and saturated vapor gas with flow controllers. The electrostatic behavior of the powder must also be considered. The verification of the temperature and the relative humidity (controlled with, for example, a certified hygrometer, certified salt solutions, or deliquescence points of certified salts over an adequate range) must be consistent with the instrument specification. The balance must provide a sufficient mass resolution and long-term stability.

It is also possible to measure amounts of water uptake not detectable gravimetrically using volumetric techniques. In the case of adsorption, to improve sensitivity, the specific surface area of the sample can be increased by reducing particle size or by using larger samples to increase the total area. It is important, however, that such comminution of the solid does not alter the surface structure of the solid or render it more amorphous or otherwise less ordered in crystallinity. For absorption, where water uptake is independent of specific surface area, only increasing sample size will help. Increasing sample size, however, will increase the time to establish some type of equilibrium. To establish accurate values, it is important to get desolvation of the sample as thoroughly as possible. Higher temperatures and lower pressures (vacuum) facilitate this process; however, care must be taken to note any adverse effects this might have on the solid such as dehydration, chemical degradation, or sublimation. Using higher temperatures to induce desorption, as in a thermogravimetric apparatus, likewise must be carefully carried out because of these possible pitfalls.

## Report and Interpretation of the Data

Sorption data are usually reported as a graph of the apparent mass change in percent of the mass of the dry sample as a function of relative humidity or time. Sorption isotherms are reported both in tabular form and as a graph. The measurement method must be traceable with the data.

Adsorption–desorption hysteresis can be interpreted, for example, in terms of the porosity of the sample, its state of agglomeration (capillary condensation), the formation of hydrates, polymorphic change, or liquefying of the sample. Certain types of systems, particularly those with microporous solids and amorphous solids, are capable of sorbing large amounts of water vapor. Here, the amount of water associated with the solid as relative humidity is decreased, is greater than the amount that originally sorbed as the relative humidity was increased. For microporous solids, vapor adsorption–desorption hysteresis is

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an equilibrium phenomenon associated with the process of capillary condensation. This takes place because of the high degree of irregular curvature of the micropores and the fact that they "fill" (adsorption) and "empty" (desorption) under different equilibrium conditions. For nonporous solids capable of absorbing water, hysteresis occurs because of a change in the degree of vapor–solid interaction due to a change in the equilibrium state of the solid, e.g., conformation of polymer chains, or because the time scale for structural equilibrium is longer than the time scale for water desorption. In measuring sorption–desorption isotherms, it is therefore important to establish that something close to an equilibrium state has been reached. Particularly with hydrophilic polymers at high relative humidities, the establishment of water sorption or desorption values independent of time is quite difficult, because one is usually dealing with a polymer plasticized into its "fluid" state, where the solid is undergoing significant change.

In the case of crystal hydrate formation, the plot of water uptake versus pressure or relative humidity will in these cases exhibit a sharp increase in uptake at a particular pressure and the amount of water taken up will usually exhibit a stoichiometric mole:mole ratio of water to solid. In some cases, however, crystal hydrates will not appear to undergo a phase change or the anhydrous form will appear amorphous. Consequently, water sorption or desorption may appear more like that seen with adsorption processes. X-ray crystallographic analysis and thermal analysis are particularly useful for the study of such systems.

For situations where water vapor adsorption occurs predominantly, it is very helpful to measure the specific surface area of the solid by an independent method and to express adsorption as mass of water sorbed per unit area of solid surface. This can be very useful in assessing the possible importance of water sorption in affecting solid properties. For example, 0.5% m/m uptake of water could hardly cover the bare surface of 100 $m^2/g$, while for 1.0 $m^2/g$ this amounts to 100 times more surface coverage. In the case of pharmaceutical solids that have a specific surface area in the range of 0.01 $m^2/g$ to 10 $m^2/g$, what appears to be low water content could represent a significant amount of water for the available surface. Because the "dry surface area" is not a factor in absorption, sorption of water with amorphous or partially amorphous solids can be expressed on the basis of unit mass corrected for crystallinity, when the crystal form does not sorb significant amounts of water relative to the amorphous regions.

## DETERMINATION OF THE WATER ACTIVITY

### Principle

Water activity, $A_W$, is the ratio of vapor pressure of water in the product (P) to saturation pressure of water vapor ($P_0$) at the same temperature. It is numerically equal to 1/100 of the relative humidity (RH) generated by the product in a closed system. RH can be calculated from direct measurements of partial vapor pressure or dew point, or from indirect measurement by sensors whose physical or electric characteristics are altered by the RH to which they are exposed. Ignoring activity coefficients, the relationship between $A_W$ and equilibrium relative humidity (ERH) are represented by the following equations:

$$A_W = P/P_0$$

$$ERH(\%) = A_W \times 100$$

### Method

The water activity is determined by placing the sample in a small airtight cup inside which the equilibrium between the water in the solid and the headspace can be established. The volume of the headspace must be small in relation to the sample volume in order not to change the sorption state of the sample during the test. The equilibration as a thermodynamic process takes time but may be accelerated by forced circulation within the cell. The acquired water activity value is only valid for the simultaneously determined temperature. This requires a precise temperature-measuring device as part of the equipment. Furthermore, the probe must be thermally insulated to guarantee a constant temperature during the test. The sensor measuring the humidity of the headspace air above the sample is a key component. Theoretically, all types of hygrometers can be used, but for analytical purposes miniaturization and robustness are a precondition. The $A_W$ measurement may be conducted using the dew point/chilled mirror method.[1] A polished, chilled mirror is used as a condensing surface. The cooling system is electronically linked to a photoelectric cell into which light is reflected from the condensing mirror. An air stream, in equilibrium with the test sample, is directed at the mirror which cools until condensation occurs on the mirror. The temperature at which this condensation begins is the dew point from which the ERH is determined. Commercially available instruments using the dew point/chilled mirror method or other technologies need to be evaluated for suitability, validated, and calibrated when used to make water activity determinations.

These instruments are typically calibrated over an adequate range, for example, using some saturated salt solutions at 25° such as those listed in *Table 1*.

**Table 1. Standard Saturated Salt Solutions**

| Saturated Salt Solutions at 25° | ERH (%) | $A_W$ |
|---|---|---|
| Potassium sulfate ($K_2SO_4$) | 97.3 | 0.973 |
| Barium chloride ($BaCl_2$) | 90.2 | 0.902 |
| Sodium chloride (NaCl) | 75.3 | 0.753 |

[1] AOAC International Official Method 978.18.

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**Table 1. Standard Saturated Salt Solutions** *(continued)*

| Saturated Salt Solutions at 25° | ERH (%) | $A_w$ |
|---|---|---|
| Magnesium nitrate ($Mg(NO_3)_2$) | 52.9 | 0.529 |
| Magnesium chloride ($MgCl_2$) | 32.8 | 0.328 |
| Lithium chloride ($LiCl_2$) | 11.2 | 0.112 |

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# ⟨1251⟩ WEIGHING ON AN ANALYTICAL BALANCE

## INTRODUCTION

Weighing is a frequent step in analytical procedures, and the balance is an essential piece of laboratory equipment. The general information described here applies directly to electronic balances used in analytical procedures. Although many portions of the chapter are applicable to all balances, some are applicable only to analytical balances. This chapter should not be considered all-inclusive, and other sources of information (e.g., the US National Institute of Science and Technology and balance manufacturers) may be useful and applicable when analysts perform a weighing operation or implement a weighing procedure. The information given in this chapter is applicable not only to balances used for materials that must be accurately weighed (see *Balances* ⟨41⟩) but also to balances used in all analytical procedures.

## QUALIFICATION

Users should consult *Analytical Instrument Qualification* ⟨1058⟩, standard operating procedures, and recommendations from manufacturers when they devise qualification plans.

### Installation

The balance's performance depends on the conditions of the facility where it is installed. Analysts should consult information provided by the manufacturer before they install a balance.

#### SUPPORT SURFACE

The balance should be installed on a solid, level, nonmagnetic surface that minimizes the transmission of vibration (e.g., a floor-mounted, granite weigh bench). If a metallic support surface is used, the surface should be grounded in order to prevent the buildup of static electricity.

#### LOCATION

If possible, the balance should be located in a room that is temperature and humidity controlled. The location should have a clean, consistent electrical power supply. The location should be free of drafts and should not be near ovens, furnaces, air conditioner ducts, or cooling fans from equipment or computers. The balance should be positioned away from outside windows so that direct sunlight does not strike the balance. The balance should not be installed near sources of electromagnetic radiation such as radio-frequency generators, electric motors, or hand-held communication devices (including cordless telephones, cellular telephones, and walkie-talkies). The balance should not be located near magnetic fields induced by laboratory instrumentation or other equipment.

The performance of the balance should be assessed following installation and before use in order to demonstrate adequate performance. In some situations, it may not be possible to position the balance in an optimum environment. Examples of potential facility issues include the following:

1. Air currents sometimes are present in the laboratory.
2. Temperatures in the laboratory vary excessively (check the manufacturer's literature about temperature sensitivity).
3. Humidity is either very low or very high. Either condition may increase the rate at which the sample weight varies because of pickup or loss of water. Low humidity increases the buildup of static electricity.
4. Adjacent operations are causing vibration.
5. Corrosive materials are used nearby or are routinely weighed.
6. The balance is located within a fume hood because it is used to weigh corrosive or hazardous materials.
7. The balance is adjacent to equipment that produces a magnetic field (e.g., a magnetic stirrer).
8. Direct sunlight strikes the balance.

In situations when the balance is located near equipment or systems that induce vibration, drafts, electromagnetic radiation, magnetic fields, or changes in temperature or humidity, the assessment should be conducted with those systems operating in order to duplicate a worst-case scenario.

### Operational Qualification

An operational qualification should be performed either by the user or by a qualified third-party vendor after the equipment has been installed.

As a minimum, the power should be turned on and the balance should be allowed to equilibrate according to the manufacturer's instructions (1–24 h, depending on the type of balance) before use. Depending on the balance, analysts should include the following procedures in the operational qualification:

1. Mechanical mobility of all moveable parts
2. Control of stable indication
3. Manually triggered or automatic adjustment by means of built-in weights

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4. Operation of ancillary equipment
5. Tare function
6. Initial calibration

Several types of electronic analytical balances use built-in weights for manually triggered or automatic adjustment. This adjustment usually is applied to reduce the drift of the balance over time and to compensate for drifts caused by variations in the ambient temperature.

Calibration normally is performed as part of the operational qualification, but it also can be performed periodically thereafter. Calibration should be performed at the location where the balance is used in normal operation.

## Performance Qualification

*Table 1* provides a list of the most important balance properties that should be assessed during performance qualification. Depending on the risk of the application and the required weighing process tolerance, some of these tests may be omitted. Tests also can be omitted if there is evidence that the property in question has only minimal effect on the weighing performance. Any procedures used should be consistent with in-house standard operating procedures, applicable for the specific balance, and adequately justified. Performance qualification should be performed periodically as described in standard operating procedures, and the frequency of each of the individual tests can vary depending on the criticality of the property.

The weights that are used to perform the tests should be stored and handled in a manner that minimizes contamination. Before executing the tests, the technician should place the weights in the vicinity of the balance for an appropriate time to reach sufficient thermal equilibrium. If possible, tests should be carried out with a single test weight in order to minimize handling errors, but multiple test weights are permitted.

The tests should be recorded in such a manner that the data can be used to easily track balance performance and to assist in laboratory investigations as needed. Meaningful acceptance criteria can be set depending on the required weighing tolerance, i.e., the maximum allowed deviation permitted by specifications, regulations, etc., of a quantity to be weighed from its target value. Procedures should be in place to address test results that are outside acceptable ranges and to provide assurance that balance cleanliness and environment have not affected the result. Also, a procedure should be in place for removing a balance from operation when observed results fall outside acceptable ranges.

**Table 1. Suggested Performance Tests and Acceptance Criteria**

| Property | Definition | Examples | Acceptance Criteria |
|---|---|---|---|
| Sensitivity | Change in the displayed value divided by the load on the balance, which causes this change. | The test load at or sufficiently close to the capacity of the balance. | NMT 0.05% deviation from 1 (the sensitivity of a correctly adjusted balance), where ⟨41⟩ is applicable. For other uses, respective tolerance requirement divided by 2. |
| Linearity | Ability of a balance to follow the linear relationship between a load and the indicated weighing value. Nonlinearity usually is expressed as the largest magnitude of any linearity deviation within the test interval. | From 3 to 6 points over the range of the balance. | NMT 0.05% deviation where ⟨41⟩ is applicable. For other uses, respective tolerance requirement divided by 2. |
| Eccentricity | Deviation in the measurement value caused by eccentric loading—in other words, the asymmetrical placement of the center of gravity of the load relative to the load receiver. Eccentricity usually is expressed as the largest magnitude of any of the deviations between an off-center reading and the center reading for a given test load. | Performed in the center of gravity and the four quadrants (for rectangular platter shapes) or at analogous locations for other platter shapes. Test load usually should be 30% of the capacity of the balance or higher (refer to the manufacturer's manual for any possible upper limit). | NMT 0.05% deviation where ⟨41⟩ is applicable. For other uses, respective tolerance requirement divided by 2. |
| Repeatability | Ability of a weighing instrument to display identical measurement values for repeated weighings of the same objects under the same conditions, e.g., the same measurement procedure, same operator, same measuring system, same operating conditions, and same location over a short period of time. Repeatability usually is expressed as the standard deviation of multiple weighings. | 10 replicate weighings (using a test weight that is a few percent of the nominal capacity of the balance). | Requirement from ⟨41⟩ where applicable. For other uses, user specified requirements will apply. |

Sensitivity, linearity, and eccentricity all account for systematic deviations; i.e., they limit the accuracy of the balance (based on the definition of accuracy in *Validation of Compendial Procedures* ⟨1225⟩ and ICH Q2). In the International Vocabulary of Metrology (VIM) and documents of the International Organization for Standardization, this concept is referred to as trueness. Because deviations are largely independent from each other, it is not likely that all deviations occur simultaneously and have the same algebraic sign. Therefore the arithmetic addition of all individual deviations to assess the balance accuracy would constitute a rather conservative approach. A quadratic addition of the individual deviations is a more realistic approach. By allocating 50% of the weighing tolerance budget to the acceptance criteria of the individual properties, e.g., sensitivity, linearity, and eccentricity, analysts ensure adherence to the required weighing tolerance. Therefore, the acceptance criteria for the individual properties that account for the systematic deviations are set to weighing tolerance divided by 2. These properties—or a subset of them—also can be taken to fulfill the accuracy requirement described in ⟨41⟩. In this case the acceptance criteria thus allow a maximum deviation of 0.05% for sensitivity, linearity, and eccentricity. Repeatability preferably is tested with a test weight of a few percent of the balance capacity. At the lower end of its measurement range, the performance of laboratory

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balances is limited by the finite repeatability, and limitations induced by systematic deviations normally can be neglected. Therefore, the whole weighing tolerance budget can be allocated to the acceptance criterion of the repeatability test.

For the sensitivity and linearity tests as described above, the analyst should use certified weights with an appropriate weight class (e.g., according to International Organization of Legal Metrology R111 or American Society for Testing and Materials E617, available from www.oiml.org and www.astm.org, respectively). [NOTE—If a differential method is used for the linearity test, certified weights may not be required.]

Depending on the acceptance criterion, it may be sufficient to consider only the nominal weight value of the test weights. If the nominal value of the test weight is considered, analysts should ensure that the maximum permissible error does not exceed one-third of the acceptance criterion. Alternatively, if the certified value of the test weight is considered, its calibration uncertainty should not exceed one-third of the acceptance criterion. If more than one weight is used to perform the test, the calibration uncertainties of the weights must be summed and the sum should not exceed one-third of the acceptance criterion. For tests such as eccentricity or repeatability, the use of certified weights is optional, but analysts must ensure that the mass of the weight does not change during the test.

The tests described above also can be included in formal periodic calibration in order to fulfill applicable cGMP requirements.

## Balance Checks

A balance check using an external weight helps ensure that the balance meets weighing tolerance requirements. The balance check is performed at appropriate intervals based on applicable standard operating procedures. The frequency of the balance check depends on the risk of the application and the required weighing tolerance. Checks with external weights can be replaced partially using automatic or manually triggered adjustment by means of built-in weights. When analysts perform the balance check with an external weight, the same acceptance criteria may apply as described in the sensitivity test above.

## Minimum Weight

The minimum net sample weight, in short, minimum weight, $m_{min}$, of an analytical balance can be expressed by the equation:

$$m_{min} = k \times s/\text{required weighing tolerance}$$

where $k$ is the coverage factor (usually 2 or larger) and $s$ is the standard deviation (in a mass unit, e.g., in mg) of NLT 10 replicate measurements of a test weight. If the standard deviation obtained is less than $0.41d$, where $d$ is the scale interval, the standard deviation is replaced by $0.41d$. The lower limit of $0.41d$ for the standard deviation results from the rounding error of the digital indication of a weighing instrument. The rounding error that is allocated to a single reading is calculated as $0.29d$. Note that a weighing always consists of two readings, one before and one after placing/removing the sample on/from the pan, with the difference between the two indications being the net sample weight. The two individual rounding errors are usually added quadratically, leading to $0.41d$. Taring the instrument after placing the tare container on the pan does not affect the rounding error as the zero indication is also rounded. The minimum weight describes the lower limit of the balance below which the required weighing tolerance is not adhered to. The equation above takes into account that the performance of analytical balances at the lower end of the measurement range is limited by the finite repeatability.

For materials that must be accurately weighed, ⟨41⟩ stipulates that repeatability is satisfactory if two times the standard deviation of the weighed value, divided by the desired smallest net weight (smallest net weight that the users plan to use on that balance), does not exceed 0.10%. For this criterion the equation above simplifies to:

$$m_{min} = 2000 \times s$$

If the standard deviation obtained is less than $0.41d$, where $d$ is the scale interval, the standard deviation is replaced by $0.41d$.

If not subject to the requirements of ⟨41⟩, the minimum weight value may vary depending on the required weighing tolerance and the specific use of the balance.

To facilitate handling, the test weight that is used for the repeatability test does not need to be at the minimum weight value but can be larger because the standard deviation of repeatability is only a weak function of the test weight value.

In order to satisfy the required weighing tolerance, when samples are weighed the amount of sample mass (i.e., the net weight) must be equal to or larger than the minimum weight. The minimum weight applies to the sample weight, not to the tare or gross weight.

Factors that can influence repeatability while the balance is in use include:

1. The performance of the balance and thus the minimum weight can vary over time because of changing environmental conditions.
2. Different operators may weigh differently on the balance—i.e., the minimum weight determined by different operators may be different.
3. The standard deviation of a finite number of replicate weighings is only an estimation of the true standard deviation, which is unknown.
4. The determination of the minimum weight with a test weight may not be completely representative for the weighing application.
5. The tare vessel also may influence minimum weight because of the interaction of the environment with the surface of the tare vessel.

For these reasons, when possible, weighings should be made at larger values than the minimum weight, i.e., the desired smallest net weight that the users plan to use on that balance should be larger than the minimum weight.

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# OPERATION OF THE ANALYTICAL BALANCE

Select the appropriate balance for the quantity and performance needed. General chapter ⟨41⟩ provides requirements for balances used for materials that must be accurately weighed. The balance user should check the balance environment (vibration, air currents, and cleanliness) and status of calibration before use.

## Receivers

To ensure suitable performance in measuring the weight of a specimen, analysts should consider selection of a proper receiver for the material.

### GENERAL CHARACTERISTICS

All receivers must be clean, dry, and inert. The total weight of the receiver plus the specimen must not exceed the maximum capacity of the balance. With a properly maintained and adjusted laboratory balance, weighing uncertainty for small samples, i.e., net weights with a mass not exceeding typically a few percent of the capacity of the balance, essentially is determined by the repeatability. However, repeatability depends on the size and surface area of the weighed object. For this reason large or heavy receivers introduce a deviation from the conditions under which the repeatability was determined without considering the receivers. Therefore, either receivers of a low mass and small surface should be used (especially in cases when specimens of low weight are being measured) or the repeatability test should be performed with the receiver placed on the weighing pan as a preload. Receivers should be constructed from nonmagnetic materials in order to prevent magnetic interference with electronic balance components. Receivers should be used at ambient temperature in order to prevent the formation of air currents within the weighing chamber.

### SOLID SAMPLES

Receivers for weighing solid materials include weighing paper, weighing dishes, weighing funnels, or enclosed vessels, including bottles, vials, and flasks. Hygroscopic papers are not recommended for weighing because they may have a detrimental effect on the observed results.

Weighing dishes typically are constructed from a polymer or from aluminum. Antistatic weighing dishes are available for measuring materials that retain static electricity. Weighing funnels typically are constructed from glass or from a polymer. The design of this type of receiver combines attributes of a weighing dish and a transfer funnel, which can simplify the analytical transfer of a weighed powder to a narrow-necked vessel such as a volumetric flask. For solid samples that are volatile or deliquescent, analysts must weigh the material into an enclosed vessel. Where practical, analysts should use an enclosed vessel with a small opening in order to reduce sample weight loss from volatilization or weight gain from the adsorption and absorption of atmospheric water.

### LIQUID SAMPLES

Receivers for liquid samples typically are inert, enclosed vessels. For liquid samples that are volatile or deliquescent, analysts should use an enclosed vessel with a small opening, and the enclosure should be replaced rapidly following material transfer. Special precautions should be taken to be certain that the receiver and the enclosure are constructed from a material that is compatible with the liquid sample. The receiver and enclosure must have a seal that is sufficient to prevent leaks from a liquid that is of low viscosity or has low surface tension or a low boiling point.

## Types of Weighing

### WEIGHING FOR QUANTITATIVE ANALYSIS

The initial step for many quantitative analyses is to accurately weigh a specified amount of a sample. *General Notices, 6.50.20 Solutions* stipulates that solutions for quantitative measures must be prepared using accurately weighed analytes: i.e., analysts must use a balance that meets the criteria in ⟨41⟩. Errors introduced during the weighing of a sample can affect the accuracy of all subsequent analytical measurements.

### ADDITION WEIGHING

Addition weighings typically are used for solid samples or liquid samples for which volatility is not an issue. The receiver is placed on the balance. After the balance display stabilizes, the analyst should tare the balance; add the desired amount of material to the receiver; allow the balance display to stabilize; record the weight; and quantitatively transfer the material to an appropriate vessel or, if it cannot be guaranteed that the entire amount has been transferred, weigh the receiver again and note the weight difference.

### DISPENSE WEIGHING

Dispense weighing typically is used for weighing emulsions or viscous liquids such as ointments. In these situations it is not practical to weigh the material into a typical receiver. Accordingly, the analyst should tare the balance; place the sample on the balance in a suitable container (e.g., a bottle, tube, transfer pipet, or syringe) that has been wiped clean on the outside; record the weight after the balance display stabilizes; transfer the desired amount of sample to an appropriate receiving vessel, such

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as a volumetric flask; and place the pipet or syringe back onto the balance. The difference in the two weighings is equal to the weight of the transferred specimen.

#### GRAVIMETRIC DOSING

Gravimetric dosing typically is used for sample and standard preparations or capsule filling. For such weighing the analyst places the volumetric flask, vial, or capsule shell on the balance; tares the balance after the balance display stabilizes; adds the solid or liquid components into the receiver by means of dosing units; and records the respective weights.

## Problem Samples

#### ELECTRICALLY CHARGED SAMPLES AND RECEIVERS

Dry, finely divided powders may be charged with static electricity that can make the powder either attracted to or repelled by the receiver or the balance, causing inaccurate weight measurements and specimen loss during transfer. A drift in the balance readings should alert the operator to the possibility that the material has a static charge. Commercially available balances with a built-in antistatic device can be used to remedy the problem. Such devices may use piezoelectric components or a very small amount of a radioactive element (typically polonium) to generate a stream of ions that dissipate the static charge when passed over the powder being weighed. Antistatic weigh boats, antistatic guns, and antistatic screens also are commercially available. The static charge depends also on the relative humidity of the laboratory, which in turn depends on atmospheric conditions. Under certain conditions, static charge is caused by the type of clothing worn by the operator and this charge can cause large errors in the weighing. Borosilicate glassware and plastic receivers have a well-known propensity for picking up static charge, especially at low relative humidity. The gloves used to protect the operator also may increase the potential for a static charge problem. Placing the container in a metal holder may help to shield the static charge, and antistatic gloves also can help to alleviate the problem.

#### VOLATILE SAMPLES

When weighing a liquid that has a low boiling point, analysts must receive the specimen in a vessel with a gas-tight enclosure of small diameter. The analyst then tares the vessel and enclosure, adds the desired amount of sample, and replaces the enclosure. After the balance display stabilizes, the analyst records the specimen weight.

#### WARM OR COOL SAMPLES

Samples that are warm or cool should be equilibrated in the laboratory, or the weight readings may be erroneous. With regard to warm samples, the apparent weight is smaller than the true weight because of heat convection. For example, a flask that is warmer than ambient air warms up this air, which then flows upward along the flask and reduces the apparent weight of the contents by viscous friction.

#### HYGROSCOPIC SAMPLES

Hygroscopic materials readily absorb moisture from the atmosphere and steadily gain weight if left exposed. Therefore, hygroscopic samples must be either weighed promptly or placed in a vessel with a gas-tight enclosure. For a gas-tight vessel, analysts should tare the vessel and enclosure, add the desired amount of sample, and replace the enclosure. After the balance display stabilizes, the analyst can record the specimen weight.

#### ASEPTIC OR BIOHAZARDOUS SAMPLES

The weighing of sterile or biohazardous samples should take place within the confines of a clean bench, biosafety cabinet, isolator, or similar containment device. Air flow within the hood potentially can cause balance instability, so after a balance has been installed under a hood, analysts should perform a rigorous qualification study with suitable weight artifacts (see ⟨41⟩) in order to determine the acceptability of the balance performance in this environment.

#### WEIGHING CORROSIVE MATERIALS

Many chemicals, such as salts, are corrosive, and materials of this nature should not be spilled on the balance pan or inside the balance housing. Extra care is essential when materials of this nature are weighed. Analysts should consider the use of sealed containers such as weighing bottles or syringes. In the event of a spill, requalification of balance may be necessary, depending on the nature of the spill.

## Safety Considerations When Weighing

During a weighing, the analyst may be exposed to high concentrations of a pure substance. The analyst must carefully consider this possibility at all times and should be familiar with the precautions described in the substance's Material Safety Data Sheet before weighing it. Hazardous materials should be handled in an enclosure that has appropriate air filtration. Many toxic—and possibly allergenic—substances present as liquids or finely divided particles. When weighing these substances, analysts should use a mask that covers the nose and mouth to prevent any inhalation of the substance, and they should use gloves to prevent any contact with the skin. [NOTE—The use of gloves is good practice for handling any chemical. If it is necessary

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to handle the container being weighed, the analyst should wear gloves not only for self-protection but also to prevent moisture and oils from being deposited on the weighed container.]

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# ⟨1265⟩ WRITTEN PRESCRIPTION DRUG INFORMATION—GUIDELINES

The purpose of these guidelines—comprising format, content, and accessibility of prescription drug leaflets—is to help ensure that leaflets are useful. In this context, "useful" means that recipients receive, understand, and are motivated to apply written information about their medicines to achieve maximum benefit and minimize harm. Dispensers, prescribers, health care providers who counsel patients about their medicines, and the patients themselves are intended to be the primary beneficiaries for these guidelines.

## CRITERIA (FROM THE KEYSTONE ACTION PLAN[1])

Written prescription medicine information should be based on the following criteria:

1. Scientifically accurate,
2. Unbiased in content and tone,
3. Sufficiently specific and comprehensive,
4. Presented in an understandable and legible format that is readily comprehensible to consumers,
5. Timely and up-to-date, and
6. Useful.

## FORMAT GUIDELINES

1. Group all information from the same category, using brief, clear titles and bullets or subheadings as needed. Avoid symbols and subheadings not directly connected to the information they mark.
2. Be consistent in the placement and labeling of categories of information in all leaflets.
3. Provide information at the sixth-grade reading level or below, if possible (never above eighth-grade level). Do not exclude information to achieve a lower reading level.
4. Use simple, common, accurate terms (for example, use "noise in the ears", not "tinnitus").
5. Use direct language that avoids words with opposite meanings (for example, use "decrease blood pressure", not "increase low blood pressure effect").
6. Provide reasons for instructions (for example, "take with food to avoid upset stomach").
7. Emphasize the most important information. Clearly distinguish warnings from instructions or from other text that may be misinterpreted as warnings.
8. Accompany each pictogram, if used, with corresponding text placed close to the pictogram. Use the simplest pictograms possible. For pictograms intended to prompt patients to ask questions or inform health care providers, add text such as "Tell Doctor" or "Ask Pharmacist".
9. Make text readable by using 12-point or larger type, both uppercase and lowercase letters, an easy-to-read font (for example, a serif font), and adequate space between lines and paragraphs. To call attention to important information, use a larger, boldface type.
10. Evaluate format by performing tests of readability, comprehension, memory, problem solving, and behavioral efficacy and intention, using representative samples of the target population.

## CONTENT GUIDELINES

1. Provide enough detail to facilitate correct use, achieve maximum benefit, and minimize harm, including a statement that identifies activities (such as driving or sunbathing) that the patient should avoid.
2. Write text that is unbiased in content and tone and scientifically accurate. The uses described should be consistent with FDA-approved labeling or otherwise permitted by FDA, or should appear in federally recognized drug compendia. Distinguish unlabeled from labeled use.
3. For drugs sold under a brand name, provide both brand and generic names, and include a pronunciation guide for each.
4. Describe the drug and its dosage form. Include indications and contraindications, specific directions for use, what to do if a dose is missed, and what to do in the event of an overdose or poisoning.
5. Do not use abbreviations.
6. Indicate the intended type of benefit (for example, "cure", "prevention", "to help relieve symptoms"). Indicate how—and how soon—the patient should recognize the benefit and what to do if none is observed.
7. Give a balanced evaluation of risks and benefits.
8. List side effects, in order of severity, such as "serious", "most common", and other similar type groupings. It may not be appropriate to provide sufficient detail for the patient to be able to monitor serious or common side effects. Provide guidance to consult the doctor or pharmacist, and indicate that not all the side effects are listed.

[1] In December 1996, the "Action Plan for the Provision of Useful Prescription Medicine Information" was presented to the Secretary of Health and Human Services. The plan, commonly known as the "Keystone Plan," described certain criteria for written prescription medicine information. These criteria are described in detail in the action plan, which can be found at www.fda.gov/cder/offices/ods/keystone.pdf.

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9. List sufficiently specific and comprehensive information that includes the provision of all important risk information. Patients should be advised to be sure to inform the provider about all the medicines they are taking.
10. Indicate the potential for therapeutic duplication if the drug is available under multiple names or over-the-counter, or if the active ingredient is contained in other products.
11. If known, include a statement concerning the safety of use in the presence of other conditions and during pregnancy or breast-feeding. Direct affected patients to discuss their condition with health care providers. If the safety of use during pregnancy or breast-feeding has not been established, say so.
12. State whether safety and efficacy have been established in pediatric, geriatric, and other special populations. Patients should be encouraged to discuss with their health care provider any recommendations for dosage adjustment.
13. Illustrate information with diagrams when appropriate. Label the diagram components (for example, device parts) if they are not obvious. The words on the label should be prominently placed thereon with such conspicuousness and in such terms as to render them likely to be read and understood by the ordinary individual under customary conditions of purchase and use.
14. Include the following:
    A. A statement that the product is to be used only by the person for whom it was prescribed,
    B. Storage information,
    C. A completeness disclaimer advising the patient to discuss this issue with the health care provider,
    D. The publisher of the leaflet and the date the leaflet was developed or revised,
    E. Sources of in-depth information and answers to questions, and
    F. Other relevant general statements.
15. The patient should be advised about risks of developing dependence on, or tolerance to, the medication.

## ACCESSIBILITY GUIDELINES

1. Write text that is relevant to the intended use of the drug.
2. Design the leaflets to be easy to recognize, consistent in format, and easy to store and retrieve.
3. Supplement the leaflets with oral counseling of patients, including children, the elderly, and caregivers.
4. Include a statement asking the patient to reread the leaflet.
5. Distribute the leaflets with all prescription medicines to consumers (namely, persons independently responsible for any aspect of medicine use or for giving medicines to others).
6. Produce leaflets in Spanish, English, or other languages; and establish criteria for producing them in other languages and for special populations (for example, children, visually handicapped) [NOTE—Ideally, prescription drug information leaflets would be customized for the patient's condition and for other relevant information (for example, gender, age, or physical limitations), and would be available in the patient's primary language. Currently, such customization is neither feasible nor practical, but it remains a goal.]

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# ⟨1285.1⟩ HEMATOXYLIN AND EOSIN STAINING OF SECTIONED TISSUE FOR MICROSCOPIC EXAMINATION

## INTRODUCTION

Histologic methods involve the preparation of organs, tissues, or cells for microscopic analysis of constituent elements that have been exposed to particular chemistries or immunochemical staining procedures. Histologic methods have many goals, and are often used to evaluate native and processed therapeutic tissue-based products. For example, these methods can be used to establish the integrity of the tissue with regard to cellular content and extracellular constituents. Nuclear staining intensity and nuclear shape may be altered if tissue recovery or handling is compromised. Furthermore, extracellular structural and accessory components such as collagen, elastin, and glycosaminoglycans can be specifically stained so their presence and location can be determined.

Histologic methods are particularly useful for detecting trends in content (e.g., cell numbers and amounts of tissue constituents). Therefore, they have been valuable in the development and verification of tissue-processing methods intended to reduce or eliminate cellular content (decellularization detected by reduced nuclear staining, for example with hematoxylin) while maintaining extracellular matrix structure and components (e.g., retention of glycosaminoglycans or other carbohydrates assessed by persistence in Alcian blue staining).

Tissue histologic analysis typically begins with a routine staining procedure using hematoxylin and eosin (H&E) to detect cellular chromatin and cytoplasm and extracellular structures (e.g., collagen and muscle). Hematoxylin acts as a basic dye. In a metal complex it binds to acidic structures such as cellular nucleic acids (DNA or RNA), producing the blue–purple stain typically noted as nuclear staining. Additionally, in calcified tissues it forms a blue or purple precipitate. Eosin acts as an acid dye and stains basic materials red (muscle) to pink (collagen). Eosin staining is responsible for the detection of the cytoplasm and intracellular and extracellular proteins, but it does not allow identification of specific intracellular structures or proteins. H&E does not stain fat itself, but fatty areas can be identified by the staining of the stroma, yielding an outline of adipocytes.

H&E staining is often used to demonstrate the reduction of nuclei following tissue decellularization processes, but H&E staining also provides structural information. H&E staining reveals that Type I collagen fibrils have a particular arrangement and wave pattern depending on the tissue observed and the orientation of the specimen. Altered collagen fibril patterns, e.g., smearing and loss of fibrils, may suggest disruption of the native structure of the tissue. Decellularization methods, which can alter the structural integrity of tissue, may reduce the staining intensity of collagen. Alternatively, the staining intensity can be artificially increased if residual charged processing chemicals remain in the tissues. H&E staining can help identify noncollagen protein structures. For example, elastin fibers are not specifically stained by this method, but thick elastin fibers such as the internal elastic lamina of arteries are easily detected by their profound waviness and subintimal location shown by staining. A mineralized matrix (e.g., in bone) can be seen as dark red to purple (depending on the hematoxylin choice) granular staining within fields of structural protein fibers.

H&E staining is a starting point for more detailed histologic analysis. Differential staining of collagen (typically colored blue) is achieved with Masson Trichrome stain, and Verhoeff's Van Gieson and Movat Pentachrome stains highlight elastin (black). The other major components of connective tissues are the glycosaminoglycans, and these can be detected and to some extent differentiated with various cationic dyes such as Alcian blue in Movat's Pentachrome stain, for example.

More detailed compositional analysis of tissues and observations of the effects of decellularization methods require structure-specific probes. At the level of light microscopy, this specificity is provided by immunohistochemical staining using antibodies to targeted structural proteins and extracellular matrix constituents or by using labeled lectin probes to detect certain sugar structures. The latter are of particular utility for evaluating the removal of important xeno-antigenic epitopes.

The purpose of this chapter is to identify the factors that should be controlled to optimize the consistency of the results of tissue staining with H&E. In addition, the chapter provides a common H&E staining procedure.

## BASIC PRINCIPLES

Hematoxylin is oxidized to hematein, which complexes with a metal cation, binds to negatively charged groups, and stains basophilic substances such as nucleic acids. The most common cation for this application is aluminum (III), but iron (III) and tungsten also are important. Hematoxylin also stains calcium deposits. The most common metal used in hematoxylin staining is aluminum, and the resulting stain produces a blue color. This aluminum salt form of hematoxylin requires a pH >5 to form an insoluble blue aluminum hematein complex. The high pH is obtained in a bluing step using a weak alkali solution such as ammonium water. Because anionic materials often are used to process tissue (e.g., anionic detergents), these materials should be removed from the tissue first, or nonspecific hematoxylin staining may be seen.

Eosin is an acidic analine dye with affinity for cationic amino acids such as arginine and lysine in proteins. Cytoplasm, muscle, connective tissue, colloid, red blood cells, and decalcified bone matrix all stain pink to pink/orange/red with eosin. Eosin methods use solutions containing various proportions of water, ethanol, and acetic acid. The uptake of eosin in cells is promoted by higher eosin concentration, a higher proportion of water than ethanol, and the inclusion of acetic acid.

## PROCEDURES—POINTS TO CONSIDER

It is important to control the consistency and quality of the stain reagents and process. Each new lot of stains should be qualified with known control specimens to ensure consistent staining processes. Solution color should be monitored, and if colorless solutions become colored they should be discarded (especially the alcohols and xylene). Staining solutions can age,

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and their staining effectiveness changes over time. Thus analysts should establish or assign a shelf life to critical staining reagents. Some reagents form precipitates or undergo overt color changes over time. Best practices indicate that such solutions should be replaced. Labeled expiration dates of commercial stains should be followed closely. If reagents are mixed in the laboratory, analysts should monitor their performance over time using control specimens to assess staining intensity and differentiation. Expiration dates should be added for each laboratory reagent. Best practices require that to the extent possible samples to be compared should be stained at the same time. To facilitate comparison, staining should be monitored and corrected if necessary. Regressive staining can be performed if overstaining with hematoxylin occurs. In this case, excess stain is removed with acid (e.g., 0.5% HCl in 70% ethanol) in a step called differentiation. Excessive hematoxylin staining can block eosin staining. Ideally, progressive staining, or real-time monitoring of the staining process to the desired intensity, should occur.

As with any tissue-staining method, H&E staining intensity may be affected by steps taken before the staining procedure, the chemicals used, and the conduct of the staining itself. Section thickness can affect the overall specimen depth and can influence the apparent stain. Inadequate removal of paraffin wax can prevent interaction of the aqueous solutions of the staining steps with the tissue. The color can be too strong if certain hematoxylin reagents are not diluted before use, if the stain is applied for too long, if the differentiation step is too short, or if the acid is too dilute. Weak staining can be the result of exhausted hematoxylin, short staining time, overdifferentiation, or low pH during the bluing step.

## SYSTEM SUITABILITY

Control tissue should be used during each run to ensure that all the reagents are working appropriately and the processing regime does not adversely affect staining. Untreated fresh tissue makes a good positive control, because there are no processing reagents that could affect the staining process. An appropriate negative control is an unstained sample from a previous lot that met all the product quality criteria of the H&E analysis. Controls are important because processing methodologies such as decellularization or cross-linking may positively or negatively affect the ability of the reagents to stain the tissue as expected and can contribute to lot-to-lot variability in staining intensity.

## QUANTITATION

A physical count of positively stained cells in a given area (cells/mm$^2$) is preferable to simply comparing a sample to a previously submitted visual reference, particularly for products that undergo a decellularization process that yields a greatly reduced (yet nonzero) number of positively stained cells. Many, though not all, samples of tissues that have undergone a process of cellular removal still have identifiable cellular debris after processing. It is difficult to compare these nonzero samples to a visual reference, so a standardized count per area is a better comparison of process effectiveness.

Software-based automated cell-counting systems that are available on many microscopy systems must be validated to confirm that the software appropriately counts cells and sample area. When the tissue has not been decellularized, there should be a high value of positive signals, and the system should be able to accurately differentiate that tissue from other samples with low cell counts. Validation is important when analysts attempt to quantify the number of cells in a given area on irregularly shaped samples because the algorithm used to estimate areas may have limitations when applied to these types of samples. Sample area should be measured accurately when there is a low cell count, because a large error in the denominator can cause a significant error in the reported cell density.

## EXAMPLE PROTOCOL

A common H&E method is described below. Analysts can follow this example after preparing and deparaffinizing tissue sample slides as described in *Preparation of Biological Specimens for Histologic and Immunohistochemical Analysis* ⟨1285⟩. This protocol is commonly used to study the effectiveness of tissue decellularization.

**Bluing reagent:** 3 mL of 28% ammonium hydroxide in 1 L of *Purified Water*

**Staining method:** After deparaffinizing tissue-containing slides, place the slides in a hematoxylin solution[1] for 1.5–3 min. [NOTE—Staining times can vary depending on hematoxylin brands (and type) and the tissues being stained, so optimization of staining time is required in this step. For this reason, laboratories should track the reagent sources to ensure consistency.] Rinse slides under running tap water for 0.5–3 min. If necessary, perform a differentiation step to remove excess stain so the desired element or structure is left stained by placing the slide in a clarifying solution[2] for 30–60 s followed by rinsing in tap water for 30–60 s. Next, place slides in *Bluing reagent* for 1–2 min. Dip slides 12 times in *Purified Water*. Rinse thoroughly in tap water for 0.5–2 min to remove the *Bluing reagent*. [NOTE—Insufficient rinsing affects subsequent staining because of a change in pH.] Next, place the slides in the following solutions for the specified times: 95% ethanol for 30 s to 1 min [NOTE—The time will vary depending on eosin type and components.] then place the slides in eosin solution[3] for 45 s to 3 min, 70% ethanol for 3 min, 95% ethanol for 3 min, and finally 100% ethanol for 3 min. [NOTE—Alcohol washing must be closely monitored to avoid excessive removal of eosin stain. Excessive removal of eosin from the extracellular matrix can be seen as pale pink staining.] Place slides in three changes of fresh xylene or xylene substitute for 1–3 min each. Place a couple drops of mounting medium on the slide, and apply the cover slip.

**Expected results:** Nuclei should be stained blue. Cartilage and calcium deposits should stain various shades of blue. Cytoplasm and other tissue constituents stain various shades of pink. Erythrocytes and eosinophilic granules stain bright pink to red.

[1] Richard Allan Scientific #7231 or suitable equivalent.
[2] Clarifier 2 from Richard Allan Scientific or suitable equivalent.
[3] Richard Allan Scientific #7111 or suitable equivalent.

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## CONCLUSIONS

It is critically important to test and verify staining conditions for each new tissue being studied. Staining intensity may be altered by tissue treatment before processing. A notable example is the treatment of tissues to decellularize or antigenically modify them, because these procedures may affect tissue components and may alter the staining of the remaining constituents.

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# ⟨1285⟩ PREPARATION OF BIOLOGICAL SPECIMENS FOR HISTOLOGIC AND IMMUNOHISTOCHEMICAL ANALYSIS

## INTRODUCTION

Histology and immunohistochemistry (IHC) are commonly used to visualize the cellular and biochemical constituents of tissues. Whether the attributes to be probed are detected based on chemical reactivities (histochemistry) or are detected using antibodies (IHC) or lectins with known specificities, the preparation of the specimen for staining is a critical phase of the analysis. Preparation typically involves (1) tissue fixation, (2) mounting the tissue in an embedding medium to permit sectioning that is sufficiently thin for the tissue to be viewed microscopically, and then (3) removal of any embedding medium before histochemical or IHC evaluation.

The purpose of this chapter is to identify factors that should be controlled to optimize the consistency of tissue staining results. The following sections address tissue fixation used to prevent degradation and to prepare the tissue for sectioning and staining. Since these methods are often used to characterize tissue-based therapeutic products and the adequacy of their process methods, USP sometimes includes visual tools to support those product monographs. USP Authentic Visual References (AVRs) are often histology images that have been prepared as described in this chapter and are used as reference standards associated with product monographs. The purpose of histologic analysis is to generate visual images that can be used either for illustration or as a visual reference. When used as AVRs, the image set should include representative images of both failed and passing samples at different magnifications. AVRs can be used to better clarify specifications and acceptance criteria related to, for example, cell content, collagen structure, or integrity.

## BASIC PRINCIPLES

The sample tissue must be properly and adequately treated or fixed to limit changes to the extracellular matrix elements or to specific constituents such as cells or proteins. Tissue fixation typically employs a chemical that can rapidly permeate the tissue to effectively cross-link proteins and limit degradation caused by either chemical or enzymatic action. Standard fixation methods typically use 10% neutral buffered histological-grade formalin and are adequate for most, if not all, histochemical analyses. Formalin fixation provides material suitable for IHC analysis provided that proper attention is paid to postfixation treatments (collectively known as antigen retrieval). Once tissue is properly fixed, it can be embedded.

The tissue is embedded in a medium that is sufficiently stiff to maintain sample geometry and the desired orientation for sectioning but is soft enough to be easily and rapidly cut through without distortion. The most common medium used is paraffin, although it is not suitable for nondecalcified hard tissues and other stiff materials (e.g., hard polymers or metals). Because paraffin is not water soluble, tissue generally is passed through a graded series of increasing concentrations of ethanol and finally into xylene (or a suitable substitute) in which the paraffin wax is soluble. Sufficient time is allowed for the paraffin to permeate the tissue, and then the block is sectioned to the desired thickness. Hard tissues commonly are embedded in hard plastics such as poly(methyl methacrylate).

After sectioning, the samples are floated on warm water with or without gelatin so they can be transferred to microscope slides. If the sections are to be used for IHC, it is recommended that gelatin not be used in the water because it can contribute significantly to background staining. Positively charged slides also are recommended to aid in sample retention throughout the IHC processing regime. The sections are allowed to dry in air, in a slide warmer, or in an oven at no more than 50°. Because most histochemical and IHC procedures are carried out under aqueous conditions, the paraffin within the mounted tissue sections must be removed. This is accomplished by reversing the xylene and alcohol rinses until the samples are rinsed in water immediately before staining.

The mounted sections are now ready for histochemical staining. If an immunological staining method is to be used, in most cases an antigen retrieval step will be necessary to break the protein cross-links formed by formalin fixation. Many procedures are available to accomplish this, and although heat treatment in acid vapor appears to be effective in recovering the reactivity of most targets in tissues, the ultimate choice may depend on the characteristics of the target to be evaluated.

## PROCEDURES—POINTS TO CONSIDER

### Fixation

The most common fixatives are aldehydes, alcohols, and oxidizing agents. Each has advantages and disadvantages depending on the particular purpose (see *Table 1*).

**Table 1. Attributes of Common Tissue Fixatives**

| Fixative Name | Mechanism | Advantages | Disadvantages | Notes |
|---|---|---|---|---|
| Formaldehyde (e.g., 10% neutral buffered formalin) | Cross-links protein | Minimal alteration of protein structure and antigenicity; good penetration | Buffered version is preferred to counteract oxidation of formaldehyde to formic acid, which tends to cause a brown artifact. | Unbuffered precipitates can form, and will be acidic (pH 3–4.6). |

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**Table 1. Attributes of Common Tissue Fixatives** *(continued)*

| Fixative Name | Mechanism | Advantages | Disadvantages | Notes |
|---|---|---|---|---|
| Glutaraldehyde (e.g., 0.25%–4%) | Cross-links protein | Morphology suitable for electron microscopy | Deforms α-helix structure of protein, so not good for IHC; slow penetration | Use with 2–3 µm thick sections. Opens structures such as blood vessels to improve access to all tissue surfaces and to prevent opposing surfaces from bonding to each other |
| Alcohols (e.g., methanol or ethanol) | Denature proteins | Best applied to cytologic smears; good penetration | Can cause tissue hardness and brittleness, making sectioning difficult | |
| Permanganates, dichromates, or osmium tetroxide | Oxidizing agents that cross-link proteins | Fixation of cytoplasm without precipitation; fixation of lipids, especially phospholipids | Causes substantial denaturation; uncommon | |
| Picrates | Oxidizing agents that cross-link proteins | Good retention of nuclear structure with limited tissue hardening | Explosion hazard; causes cell shrinkage | |

Tissue that is not appropriately fixed will not embed and section well, and subsequently will not stain well. Adequate fixation time is of primary importance in quality assurance. Fixatives usually are delivered at a 15:1 to 20:1 ratio of fixative volume to tissue mass. Alternatively, multiple changes of fixative can be used with agitation. It is important to expose tissues to fixatives for a sufficient time, making sure that the sample does not dry before or during this process. Even small tissue pieces may require 12–24 h of exposure time. Tissue handling also is important because areas that are physically gripped may not be fixed as rapidly as areas that are not gripped.

Typical concentrations for some fixatives are shown in *Table 1*. Note that if fixation concentrations are too high or if fixation time is too long, structural artifacts can form. The fixation temperature can be increased somewhat but not to the extent that will cause protein denaturation.

Buffers also are important for high-quality fixation. A pH range of 6–8 usually is best because it will most likely maintain the native tissue structure. Buffering must be sufficient to overcome acidification caused by hypoxia and to prevent black deposits of formalin–heme.

## Tissue Processing

### DEHYDRATION

Once the tissue is properly fixed, it is ready for dehydration and the embedding process. The most common dehydrants are alcohols, but sometimes acetone is used because it rapidly fixes the tissue (although it is a fire hazard). Tissues may be physically damaged if they are insufficiently dehydrated, often because of water contamination of ethanol wash solutions. After dehydration, the tissue is placed in a clearing agent before embedding. Some common clearing agents are the following: xylene, toluene, chloroform, methyl salicylate, limonene (a volatile oil found in citrus peels), or some commercial xylene substitutes.[1]

### EMBEDDING

Depending on the tissue type and subsequent staining protocol, there are several embedding and mounting-media options. Paraffin is most commonly used to embed tissues. Its density is similar to that of most tissues, which facilitates sectioning, and multiple paraffin types with different melting points and hardness can be evaluated if tissue sections do not cut smoothly. If very thin sections are needed or if harder tissues (e.g., bone) will be embedded, plastics are good options (e.g., methacrylate, glycol methacrylate, araldite resin, or epon). Once the mounting medium is selected, the specimen should be carefully aligned and oriented in the medium relative to the desired cutting plane. Tissues that are properly fixed and embedded, if stored appropriately, can stay in this form indefinitely before staining.

Tissues can be embedded manually or with automated systems. Some embedding systems have a chamber for keeping molds and samples hot and ready for embedding. They also may have a separate chamber to melt paraffin, an attached dispenser for pouring paraffin into embedding molds, and a separate cold plate to cool the recently embedded tissues. Both manual and automated station methods are essentially the same and are well accepted as tissue embedding methods. The only difference is that the latter are semi-automated so that the system is operational within an hour or less (i.e., paraffin is melted, heating chambers are warmed, and the cold plate is cooled). Manual methods are somewhat slower. Regardless of the system used, the integrity of the histological section must not be compromised.

### SECTIONING

When tissue blocks are ready for staining, sections are cut with a sharp knife or disposable blades. Paraffin-embedded tissue sections typically are 4–8 µm thick, but plastic-embedded tissues usually are 2 µm thick. The tissue sections are placed on slides and can be stored in this state. The slides are cleared again (to dissolve the paraffin) and are rehydrated before staining or IHC. In all cases analysts should be aware of the importance of each step in order to avoid producing artifacts (e.g., fixation-related shrinkage or nicks, folds, or tears in the tissue sections caused by improper microtome work or a knife that is too dull). Once the staining procedure is complete, analysts should apply sufficient mounting medium over the tissue sections in order to avoid entrapment of air bubbles when a cover slip is applied.

[1] For example, Clear Rite, Pro Par Clearant.

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## Example Protocol

The following is an example of a fixation, paraffin-embedding, and processing method that is commonly used before hematoxylin and eosin (H&E) staining to study the effectiveness of tissue decellularization processes.

**Fixation solution:** 0.1 M sodium phosphate, pH 7, containing 10% formalin

**Paraffin:** Melt paraffin at 60° for 3 h before analysis. [NOTE—For proper infiltration and success in later steps, it is best to keep the *Paraffin* fresh (free of clearing agent) with frequent changes of paraffin (at least two changes). The temperature of the *Paraffin* must be NMT 2°–4° above its melting point; otherwise, the tissues exposed to overheated *Paraffin* will overharden.]

**Paraffin solution 1:** *Paraffin* and xylene (1:3) or a xylene substitute[2]

**Paraffin solution 2:** *Paraffin* and xylene (1:1) or a xylene substitute

**Paraffin solution 3:** *Paraffin* and xylene (3:1) or a xylene substitute

**Fixation and dehydration, clearing, and paraffin infiltration of the tissue (DCI):** Place each tissue sample (NMT 1 $cm^3$) in *Fixation solution* for a minimum of 6–8 h. The volume of *Fixation solution* should be NLT 15–20 times the volume of the tissue sample. [NOTE—When processing bone samples, thoroughly fix before decalcification and DCI. In this case, after fixation, rinse the sample with Purified Water, and place in a rapid decalcifier solution[3] for the recommended time. After decalcification, rinse the bone samples with Purified Water again, and place them back in the *Fixation solution*.] Place each tissue sample in an embedding cassette, and label the cassette with a pencil or histology pen. Place cassettes in the following series of solutions for 30 min each: 2 changes of 70% ethanol, 2 changes of 95% ethanol, and 3 changes of 100% ethanol. [NOTE—An optional approach is to transfer cassettes directly from 100% ethanol to 100% xylene for 3 changes. Place cassettes in *Paraffin* heated to 2°–4° above its melting point for 1–3 changes for NLT 90 min and NMT 180 min. Exposure to prolonged heat causes shrinkage and hardening of tissues. Avoid overnight treatment.] Next, place cassettes in the following series of solutions for 60 min each at 60°: *Paraffin solution 1, Paraffin solution 2,* and *Paraffin solution 3*.

**Tissue embedding:** [NOTE—This example is a manual method, but an automated embedding method can be substituted.] Move the container with the cassettes described above from the incubator into a 60° water bath to prevent the infiltrated paraffin from solidifying during the embedding procedure. [NOTE—For easier release later from the embedding molds, molds can be sprayed with a diluted mold-release concentrate.] Place a small amount of *Paraffin* into a room-temperature base mold. Using forceps, remove a tissue-containing cassette from the liquid *Paraffin*, and position the tissue section with the desired orientation in the mold before the *Paraffin* solidifies. [NOTE—If it is not positioned properly, the tissue sample can be placed back into the tissue cassette containing the liquid *Paraffin,* and then a new mold can be prepared and tissue positioning can be attempted again.] Once the tissue is in place, place the labeled portion of the tissue cassette onto the base mold, and add *Paraffin* to a level just below the lip of the cassette. Place the cassette mold assembly onto a frozen cold plate. When the *Paraffin* begins to solidify on top, transfer the assembly to an ice bath until the entire block is solidified (about 20 min). Once the *Paraffin* is solidified, carefully separate the base mold from embedded tissue without using excessive force. Store blocks at 4° until needed for sectioning. Blocks can be sectioned immediately, but it is advisable to wait at least 24 h for best results.

**Sectioning paraffin-embedded tissue:** Using an appropriate marker, label glass slides with a tissue-sample identifier. Select an embedded tissue block, and trim any excess wax remaining around the edges of the block. Fill a flotation bath with Purified Water, with or without gelatin (histology samples only), and warm to 36°–46°. Carefully place a new microtome blade into the blade holder. Ensure that the blade is locked into place. If available, select the manual or motorized mode of the microtome. Place the tissue block in the microtome cassette holder, and adjust its orientation using the adjusting screws until the block is correctly positioned vertically and horizontally in relation to the blade. Select the *trim* setting. [NOTE—The trim cut usually is set to 15–16 µm, but this can be increased if a large amount of trimming is necessary, or can be decreased if only a small amount of tissue is present in the block. In addition, blocks can be refrigerated or placed on ice before sectioning to keep them cool and firm.] Advance the block forward to the desired position. Once the block is positioned, start trimming or "facing" the block. After the tissue is visible in the sections, set an appropriate sectioning depth and begin collecting tissue sections. [NOTE—Routine H&E sections should be cut at approximately 6 µm, but the section thickness can be adjusted between 4 and 8 µm as necessary if there is difficulty obtaining good-quality sections.] Using forceps, transfer the sections to the tissue flotation bath. Gently place the ribbon of sections onto the water surface while gently pulling to eliminate any wrinkles from the ribbon. [NOTE—If the water in the flotation bath becomes too hot, the tissue sections will fragment. If this occurs, decrease the heat setting on the bath, and allow the water to cool before proceeding.] Next, separate one or two adjacent, intact, full sections from the ribbon with a sharp dissecting probe or forceps. Using a glass slide placed at approximately a 45° angle beneath the section, lift the sections onto the slide. Gently tap off the excess water, and allow the sample to dry. As needed, repeat the sectioning process for additional samples. After all slides have been prepared and before staining, allow the slides to air-dry for NLT 15 min or until no water droplets are visible. Remove the wax blocks from the microtome, and store appropriately.

**Deparaffinization and rehydration of histological slides:** Before staining, place slides with mounted tissue specimens in either Coplin jars, reagent buckets, or staining racks. Place slides in the following solutions for at least 3 min each: 3 changes of xylene (or a xylene substitute), then 100% ethanol, then 95% ethanol, then 70% ethanol, then 2 changes of Purified Water. Proceed with the desired staining procedure. [NOTE—Slides can be left in the water for several hours but then must be stained.]

[2] For example, CitriSolv, Clear Rite 3, Pro Par Clearant, or equivalent.
[3] For example, Decalcifying Solution, Richard-Allen.

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## METHOD DEVELOPMENT AND VALIDATION: POINTS TO CONSIDER

Histological methods sometimes are used to demonstrate that cells within a tissue sample are still viable (e.g., when evaluating a cryopreservation method's performance). In other circumstances, the method is used to evaluate the absence of viable cells following a decellularization process. Each case presents specific validation challenges.

H&E staining provides a useful tool for determining whether significant quantities of cells are still viable after preservation. During method validation, immunohistological stains can be used to ensure that specific cell types are still present in the preserved samples. Testing samples for all expected cell types during the validation phase provides support for the less specific H&E staining (that will stain all cells and not just specific cell types) during routine monitoring.

When validating any histological method, analysts should demonstrate that different observers can detect the same quality (or quantity) of an object, tissue type, cell body, nucleus, etc., and that the characterization by different observers can be replicated in several specimens following the same process. Samples for evaluation may consist of multiple types of cells, extracellular matrix proteins, and glycosaminoglycans, or other components. Some of these components may be resistant to decellularization processes or may be difficult to stain. During assessment of method reproducibility, analysts should evaluate multiple samples throughout the sample to ensure that they are representative of the types of materials expected to be found in the matrix. In addition, because inadequate decellularization procedures may not remove all cells throughout the material, it is important to take samples from multiple locations in the tissue so that remaining cells are not underestimated. Intra-operator reproducibility also should be a part of the assessment because the relatively weaker signal from fewer positively stained cells may make visualization more challenging for some technicians. The method validation should use samples that underwent worst-case decellularization processing conditions as determined by statistical modeling to ensure that residual cells can still be identified and quantified in these marginal samples. Finally, the validation of the method should include control samples to confirm the suitability of the staining system (see *Hematoxylin and Eosin Staining of Sectioned Tissue for Microscopic Examination* ⟨1285.1⟩).

The histological assessment of decellularized tissue-based matrix products is one tool to characterize the matrix but cannot be the sole indicator of product quality. On the one hand, processing may generate decellularized matrix products that have no detectable cells but may not possess other critical quality attributes. On the other hand, tissue matrix products undergoing other decellularization processes may result in products with greatly reduced cellular content (although some cells may remain) that retain the necessary performance characteristics for that therapeutic purpose.

### Sample Selection for the Analysis of a Decellularization Process

Samples cannot always be taken from a random location within the product. For example, it is not practical to pull a sample from the center of a sheet of decellularized pericardium without creating a defect that would make the end product undesirable. If routine sampling plans require sampling from the edges or waste portion of a given matrix, analysts should conduct an initial validation study that confirms that such a sample is representative of the whole. This is important because the geometry of the matrix may affect the exposure of the matrix to the decellularization processing solutions. For example, if the tissue matrix is tubular, e.g., a nerve segment, and a sample is taken from the end for histological assessment, then this sample will have different exposure to solutions than a sample taken from the center of the nerve segment. Thus, the sampling may not represent a worst-case assessment, and the suitability of the sample should be determined by means of a validation study.

## CONCLUSIONS

This chapter outlines common steps to prepare tissue or organ specimens for light microscopic analysis following either staining or treatment with structure-specific probes. Each of these steps can produce specimen artifacts that either interfere with the observation or actually introduce structures that were not present in the original sample (e.g., certain well-known chemical artifacts). Therefore, proper attention and consistent execution of each step are critical factors in generating sections that reflect the character of the tissue and that stain in a reproducible manner.

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***Add the following:***

# ▲⟨1381⟩ ASSESSMENT OF ELASTOMERIC COMPONENTS USED IN INJECTABLE PHARMACEUTICAL PRODUCT PACKAGING/DELIVERY SYSTEMS

1. INTRODUCTION
2. SCOPE
3. DESCRIPTION OF ELASTOMERIC COMPONENTS IN PACKAGING SYSTEMS
4. ELASTOMERIC COMPONENTS—MATERIALS OF CONSTRUCTION
    4.1 Thermoset Elastomeric Components
    4.2 Thermoplastic Elastomeric Components
    4.3 Surface Coatings and Treatments
    4.4 Compounds of Concern
5. ELASTOMERIC COMPONENTS—MANUFACTURING TECHNOLOGY AND STERILIZATION PROCEDURES
    5.1 Generic Manufacturing
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6. IDENTIFICATION TESTS
7. TEST REQUIREMENTS AND RESPONSIBILITIES
8. SUMMARY OF ⟨381⟩ PHYSICOCHEMICAL TESTS
    8.1 Test Samples
    8.2 Physicochemical Tests

## 1. INTRODUCTION

Risk to drug product quality may exist when elastomeric components come into direct or indirect contact with pharmaceutical products. Elastomeric components used in pharmaceutical packaging/delivery systems must be proven suitable for their intended use based on aspects of protection, compatibility, safety, and performance.

Tests and acceptance criteria applicable to elastomeric components used in packaging/delivery systems for injectables are referenced in conjunction with *Injections and Implanted Drug Products* ⟨1⟩.

*Elastomeric Components in Injectable Pharmaceutical Product Packaging/Delivery Systems* ⟨381⟩ presents a body of test procedures and acceptance criteria for elastomeric components employed in injectable product packaging/delivery systems. The biological reactivity tests and physicochemical tests provide baseline information on a component's suitability. The test methods and acceptance criteria are detailed in their description but lack an explanation as to their relevance to the composition of the elastomers. Due to the large and diverse nature of the pharmaceutical marketplace, it may not be intuitive to stakeholders as to the proper use and application of ⟨381⟩. Therefore, a primary purpose of this chapter is to communicate the key concepts that form the foundation of ⟨381⟩ and to establish and clarify its application and applicability.

Beyond the baseline requirements provided in ⟨381⟩, elastomers will need to be qualified for intended use commensurate with the level of risk to drug product quality. These evaluations would encompass studies for extractables and leachables. Recommendations for conducting these studies are found in *Assessment of Extractables Associated with Pharmaceutical Packaging/Delivery Systems* ⟨1663⟩ and *Assessment of Drug Product Leachables Associated with Pharmaceutical Packaging/Delivery Systems* ⟨1664⟩.

Finally, elastomeric component functional suitability as part of the finished product packaging/delivery system must be demonstrated. Relevant information is found in *Elastomeric Component Functional Suitability in Parenteral Product Packaging/Delivery Systems* ⟨382⟩ and *Assessment of Elastomeric Component Functional Suitability in Parenteral Product Packaging/Delivery Systems* ⟨1382⟩.

## 2. SCOPE

This chapter serves as a supplement to the elastomeric component baseline requirements in ⟨381⟩. This chapter seeks to 1) describe elastomeric components and their materials of construction for use in injectable pharmaceutical packaging/delivery systems; 2) provide a high-level introduction to elastomer chemistry, manufacturing technology, and the post-processing of components; 3) designate and expand on baseline requirements; 4) discuss identification testing; 5) discuss test requirements and responsibilities; and 6) provide a summary of required and recommended test methods.

## 3. DESCRIPTION OF ELASTOMERIC COMPONENTS IN PACKAGING SYSTEMS

Certain components used in pharmaceutical packaging/delivery systems must have elastic properties for the system to function properly. Elastomers are a unique family of polymers with properties including the ability to recover from being stretched or deformed beyond their original state. This allows components to be flexible, maintain a seal, and be able to reseal after puncturing. In the following sections, typical elastomer materials, compositions, and physical attributes are summarized.

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Various compounding ingredients, including curing systems, are required to produce optimal elastomeric performance. In thermoset elastomers, the physical attributes will depend on polymer cross-linking, achieved during the vulcanization process. The cured polymer will produce a chemical reaction and by-products that will impact the chemical makeup of the elastomeric formulation. The compounding ingredients, reaction by-products, and post-processing steps, such as sterilization, will influence the outcome of the component's chemical characterization.

Given the complex nature of packaging systems and their manufacturing and development processes, multiple testing procedures are needed to establish their suitability for use with a specific pharmaceutical product. The logical manufacturing and development process for packaged drug products—starting with the packaging system's materials of construction, continuing with the packaging system itself, and ending with the packaged drug product—forms the basis of the following three-stage approach to packaging/delivery systems qualification:

- **Component screening:** The baseline requirements for biological reactivity and physicochemical testing described in this chapter.
- **Controlled extraction studies:** Studies as described in ⟨1663⟩ to create extractables profile(s) of particular injectable pharmaceutical packaging/delivery systems, packaging components, or materials of construction.
- **Pharmaceutical product assessment:** Actual-case measurement of confirmed leachables in the injectable pharmaceutical product in the packaging/delivery system intended for the commercial market. (For additional information, see ⟨1664⟩).

Assessment of elastomeric component functionality is performed within the context of the intended product packaging system. (For additional information, see ⟨382⟩ and ⟨1382⟩.)

# 4. ELASTOMERIC COMPONENTS—MATERIALS OF CONSTRUCTION

## 4.1 Thermoset Elastomeric Components

### 4.1.1 THERMOSET COMPOSITION (TYPICAL)

A thermoset elastomer is a polymer system in which the elastomeric properties are derived from chemical cross-linking that is irreversible. This cross-linking is created between a curative (cross-linking agent) and a polymer (elastomer) when the materials are subjected to heat and pressure. In thermoset elastomers de-cross-linking is not possible without destruction of the material. The ingredients that typically are part of the thermoset elastomeric (rubber) formulation are shown in *Table 1*.

**Table 1. Thermoset Elastomeric Components: Typical Elastomeric Ingredients**

| Ingredient | Function |
|---|---|
| Polymers (elastomers) | Provide elastic properties after curing |
| Curatives (cross-linking agents) | Form cross-links to provide elasticity and strength |
| Fillers/extenders | Impart hardness, modulus/deformation, strength, reinforcement |
| Processing aids | Impart flexibility, fatigue resistance, mold flow |
| Antioxidants/antiozonants | Provide stabilization, i.e., protection against UV, oxygen, ozone |
| Plasticizers | Aid processing by providing flexibility, hardness |
| Dyes/coloring materials | Impart color |

### 4.1.2 POLYMER TYPES AND ATTRIBUTES

Typical polymers used in elastomeric components for packaging/delivery systems for injections, along with characteristic physical attributes and component examples, are shown in *Table 2*. Different types of elastomers may be combined in a single formulation in order to combine physical attributes.

**Table 2. Typical Elastomers for Thermoset Elastomeric Components**

| Elastomer | Physical Attributes | Typical Components |
|---|---|---|
| Isobutylene/isoprene copolymer (butyl) (IIR); Brominated isobutylene isoprene (BIIR); Chlorinated isobutylene isoprene (CIIR); Brominated isobutylene para methylstyrene terpolymer (BIMS) | Gas barrier; Aging resistance | Stoppers, plungers, lined seals |
| Natural polyisoprene (NR);[a] Synthetic polyisoprene (IR) | Good coring and reseal behavior; Abrasion resistance; Higher gas permeability | Stoppers, plungers for single-use syringes, septa, needle shields, tip caps |
| Styrene butadiene rubber (SBR) | Higher gas permeability | Needle shields, tip caps |
| Ethylene propylene diene monomer rubber (EPDM) | Chemical resistance; Aging resistance | O-rings |
| Acrylonitrile butadiene rubber (nitrile) (NBR) | Chemical resistance (e.g., mineral oils) | Stoppers, plungers, gaskets, O-rings |
| Polychloroprene (neoprene) (CR); Epichlorohydrin (ECO/CO) | Chemical resistance | O-rings, gaskets |

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**Table 2. Typical Elastomers for Thermoset Elastomeric Components** *(continued)*

| Elastomer | Physical Attributes | Typical Components |
|---|---|---|
| Polysiloxane (VMQ, PMQ, PVMQ) | Heat resistance | Stoppers, plungers, tubing, gaskets, O-rings |

[a] NR must be labeled appropriately per 21 CFR §801.437; see www.fda.gov/downloads/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/ucm070929.pdf.

## 4.2 Thermoplastic Elastomeric Components

### 4.2.1 THERMOPLASTIC COMPOSITION (TYPICAL)

A thermoplastic elastomer (TPE) is a polymer system with a different nature. Elasticity is incorporated in a different way (see below), and, more importantly, thermoplastic elastomers have a thermal behavior that is comparable to plastics. Thermoplastic elastomers when heated lose their elasticity and become deformable like a plastic. Cooled down again, they regain their elasticity. This cycle can be repeated. Unlike thermoset elastomers, the elastic behavior of thermoplastic elastomers is reversible.

TPEs, like thermoset elastomers, owe their elasticity to a polymer network that has a certain degree of structure. However, for thermoset elastomers, the structure occurs through chemical cross-linking with covalent bonding, whereas TPEs use a different mechanism. For example, in styrenic block copolymers, hard polystyrene blocks serve as physical cross-links in a three-dimensional network of softer chains of a different polymer such as polyisoprene or ethylene/butylene copolymer.

In a different class of thermoplastic elastomer named thermoplastic vulcanizates, a hard plastic phase and a soft elastomeric phase are present. The hard phase can use material such as polyolefin, while the soft phase is a thermoset elastomer phase with a high degree of chemical cross-linking. The curing of the elastomer phase is done in a process known as “dynamic vulcanization”. During this process, chemical cross-linking takes place during the mixing of the elastomer with the vulcanization system and other ingredients, such as polyolefin. Additional ingredients that may be present in TPEs are fillers, antioxidants, antiozonants, and plasticizers.

## 4.3 Surface Coatings and Treatments

After manufacturing, elastomeric components may not fulfill all the properties that are required for their application. An additional coating or surface treatment may be necessary. The most common surface treatment is siliconization, which is used to overcome the inherent tackiness of components and to provide lubricity. Tackiness will negatively impact component processing (e.g., sterilization, machinability at filling) and may introduce permanent component deformation. Chlorination is a less frequently used surface treatment. In this treatment, the component surface is exposed to chlorine, resulting in non-tacky and somewhat lubricious parts. In order to provide non-tackiness and lubricity, but at the same time reduce extractable levels (i.e., barrier effect), components may be covered, on part of their surface or on their entire surface, with lubricious barrier materials that are applied either by coating or by film lamination. Polymeric coatings and surface treatments are shown in *Table 3*.

**Table 3. Surface Coatings, Films, and Treatments for Elastomeric Components**

| Coating or Treatment | Physicochemical Effect | Typical Components |
|---|---|---|
| Silicone: silicone oil or emulsion, cross-linked silicone oil | Non-tackiness, lubricity | All components |
| Chlorination | Non-tackiness | Small, thin-walled components |
| Coating deposited by chemical vapor deposition | Barrier, non-tackiness, lubricity | Stoppers, plungers |
| Fluoropolymer coating | Barrier, non-tackiness, lubricity | Stoppers, plungers |
| Fluoropolymer lamination [ethylene tetrafluoroethylene (ETFE), fluorinated ethylene propylene (FEP)] | Barrier, non-tackiness, lubricity | Stoppers, plungers |

## 4.4 Compounds of Concern

Elastomeric components are made of various materials of construction. Some of these materials may raise quality or safety concerns. For example, *Table 4* lists several ingredients in elastomeric components that are carcinogens. Whenever possible, use of elastomeric components containing these ingredients should be avoided. It is recommended that the user evaluate the presence of such materials in components, either by direct use or by use in the manufacturing processes of the materials of construction. An overview of such materials, together with the associated concerns, is shown in *Table 4*.

**Table 4. Elastomeric Components: Compounds of Concern**

| Compound of Concern | Source | Risk |
|---|---|---|
| Latex | Associated with compounds containing dry natural rubber or derivatives | Associated with anaphylaxis in individuals allergic to natural rubber latex proteins |
| Materials of animal origin | Fatty acids and their metal salts used as processing aids or slip agents in polymers | Potential sources of transmissible spongiform encephalopathies (TSEs) in pharmaceutical products before manufacturing[a] |

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**Table 4. Elastomeric Components: Compounds of Concern** *(continued)*

| Compound of Concern | Source | Risk |
|---|---|---|
| 2-Mercapto-benzothiazole (MBT) | Vulcanization accelerator used in the production of elastomers | Potential carcinogen |
| *N*-Nitrosamines | Associated with the use of certain secondary amines in the cure system | Potential carcinogens |
| Phthalates [e.g., orthophthalates such as bis(2-ethylhexyl) phthalate (DEHP)] | Used as a plasticizer or softener in polymers | Associated with developmental, reproductive, and endocrine health effects |
| Polycyclic aromatic hydrocarbons (PAHs) | Associated with carbon black (colorant or reinforcing agent) | Potential carcinogens |

[a] When tallow is derived from TSE-relevant starting material, a rigorous manufacturing process should eliminate or reduce any TSE risk.

**Latex:** Contact with elastomers or packaging/delivery systems containing natural rubber latex, dry natural rubber, or synthetic derivatives of natural rubber latex has been associated with allergic reactions, including anaphylaxis, in individuals allergic to natural rubber latex proteins. There is no one threshold level of exposure that can be considered safe; factors include the exposure route and the immune status of the individual. Information regarding suitable labeling can be found in the FDA Guidance for Industry *Recommendations for Labeling Medical Products to Inform Users that the Product or Product Container is not Made with Natural Rubber Latex* (December 2, 2014).

**Materials of animal origin:** Pharmaceutical products having raw materials derived from animal sources have a risk of transmissible spongiform encephalopathies (TSEs). Cattle infected with bovine spongiform encephalopathy (BSE) can transmit to humans in the form of Creutzfeldt-Jakob disease (CJD) and the variant (vCJD). This disorder is caused by a prion (protein) that causes the brain to be porous or spongy. Manufacturers should use materials from "non TSE-relevant animal species" or non-animal origin where possible to minimize the risk. When tallow is derived from TSE-relevant starting material, a rigorous manufacturing process should eliminate or reduce any TSE risk.

**2-Mercapto-benzothiazole (MBT):** 2-Mercapto-benzothiazole (MBT) is used as a non-volatile vulcanization accelerator in the production of elastomers. MBT reacts with zinc oxide and sulfur to cross-link elastomers. It can be bonded in the vulcanizate or may be present as a substance incorporated in the polymer. The amount of free MBT in the final product will be dependent on the vulcanization conditions.

***N*-Nitrosamines:** *N*-Nitrosamines are organic nitrogen-containing compounds that, under certain reaction conditions, are yielded as reaction products of nitrosating agents (e.g., certain oxides of nitrogen and nitrosatable secondary amines). Most secondary amines, but not all, may lead to carcinogens. There are 6 *N*-nitrosamines of concern that are listed in *Orally Inhaled and Nasal Drug Products* ⟨1664.1⟩.

**Phthalates:** Phthalates are a group of chemicals (orthophthalates and terephthalates) used in many polymer products to make plastics more flexible or as polymer-solvating agents. Phthalate esters are prepared by the esterification of phthalic anhydride with alcohols (C1/C2-C13). They are broadly divided into two distinct main groups with very different applications, toxicological properties, classification, and legal requirements. Orthophthalates used in food packaging are being reviewed by the FDA due to concerns for reproductive and endocrine health effects. More information can be found in the US *Federal Register* proposed rules (Vol. 83 No. 220, November 14, 2018).

**Polycyclic aromatic hydrocarbons:** Polycyclic aromatic hydrocarbons (PAHs), also known as polynuclear aromatic hydrocarbons (PNAs), are organic compounds with two or more fused aromatic (benzene) rings and are found as contaminants in oils used in elastomeric formulations and in carbon black used as a colorant or reinforcing agent. Carbon black is a material produced by the incomplete combustion of heavy petroleum products (i.e., ethylene cracking or coal tar). There are different types of carbon black and those with lower surface-area-to-volume ratios will have fewer PAHs absorb onto the surface during the manufacturing process. The PAHs are firmly bound to the carbon black surface under normal handling and use. They can only be extracted from the surface of the carbon black under rigorous laboratory conditions with strong solvents at elevated temperatures. There are 17 PAHs of concern that are listed in ⟨1664.1⟩.

# 5. ELASTOMERIC COMPONENTS—MANUFACTURING TECHNOLOGY AND STERILIZATION PROCEDURES

## 5.1 Generic Manufacturing

### 5.1.1 THERMOSET ELASTOMERS

The overall process of manufacturing thermoset elastomers consists of an elastomeric compound held in a heated mold under pressure. During this process, the elastomer is "cured" by undergoing a chemical reaction resulting in an elastic polymer network. The basic steps in manufacturing such components for pharmaceutical use are as follows:

- **Weighing of elastomeric ingredients:** Portions of the various ingredients are weighed according to instructions that reflect the elastomeric compound formulation.
- **Mixing:** The weighed portions of the various materials of construction are homogeneously mixed.
- **Preforming:** The mixed elastomer is brought into a physical shape that allows easy handling in the subsequent step.
- **Molding:** Elasticity is introduced and the components are shaped by the curing reaction. The products are not individually shaped but are attached to a web.
- **Die-trimming:** The components are separated from the web by die-trimming.

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- **Washing, lubrication, and drying:** The components are brought into their final state of microbiological and particulate cleanliness before packing. Lubrication, most often in the form of siliconization, typically is combined with washing and drying.
- **Packing:** The components are packed in suitable packaging material.

#### 5.1.2 THERMOPLASTIC ELASTOMERS

TPEs are processed like plastic materials. They are injected, as a hot mixture, into a cooled mold. Unlike thermoset elastomers, a thermoplastic elastomer does not involve curing agents during molding. TPE components are not attached to a web, so they do not need die-trimming. Co-injection of a plastic material with a TPE is a way to create a two-material component, where the TPE part has a sealing and/or resealing function.

### 5.2 Sterilization Procedures

Elastomeric components for injections undergo sterilization as individual components prior to the filling process and then may be sterilized a second time as part of an assembled packaging system after filling. Sterilization of an individual component may be done using ethylene oxide, ionizing radiation, or steam; method selection is dependent on the elastomeric formulation and system design. For example, depending on the irradiation dose, some elastomeric formulations may not withstand ionizing radiation. Sterilization of the filled packaging system is usually done using steam. The desired outcome is a sterile component with no change to its critical parameters such as material chemical profile (extractables), functional performance, or drug product compatibility. For guidance on sterilization procedures, refer to the following suite of chapters: *Sterilization of Compendial Articles* ⟨1229⟩, *Steam Sterilization by Direct Contact* ⟨1229.1⟩, *Gaseous Sterilization* ⟨1229.7⟩, and *Radiation Sterilization* ⟨1229.10⟩. For products regulated as medical devices (e.g., unfilled syringes or infusion administration sets), different standards and requirements may apply.

**Ethylene oxide sterilization:** Ethylene oxide (EtO) can be used to sterilize elastomeric components, but the method has the drawback of requiring an outgassing time period to allow the levels of residual compounds to fall below the regulatory limits. Most EtO sterilization processes involve three different stages, which are preconditioning, sterilization, and degassing. The preconditioning stage includes a dwell time, under controlled temperature and humidity, which may decrease the resistance of microorganisms to inactivation and reduce the sterilization cycle time. Sterilization is completed by the introduction of EtO gas followed by an aeration phase that removes residual EtO. The aeration phase also allows time for removal of the common EtO degradants, ethylene chlorohydrin and ethylene glycol, from the elastomer. The degassing time required depends on factors such as the composition and size of the elastomeric part.

**Ionizing radiation sterilization:** Ionizing radiation can use either electron beam (e-beam) or gamma radiation. Cobalt 60 is a frequently used source of gamma rays, which are very penetrating. An e-beam produces accelerated electrons, which do not have the ability to penetrate materials to the same depth achieved by gamma rays. The energy provided by these two methods is sufficient to deliver a sterilizing dose but is also capable of exciting and dissociating polymer bonds. The free radicals produced within polymer structures initiate a series of complex chemical reactions (e.g., chain scission or cross-linking) that may continue for a period of time after irradiation is completed. Inhibitors and stabilizers can be added to polymer formulations and are designed either to absorb energy or react with the free radicals. Even so, not all elastomeric formulations are deemed to be suitable for radiation sterilization.

**Steam sterilization:** Steam sterilization is typically performed in an autoclave under saturated steam conditions. Commonly used cycles are 121°–122° for 30–60 min. The components are dried following sterilization, not only to remove surface water but also to remove residual moisture that has entered the matrix of the elastomer. Drying procedures need to be optimized based on a number of factors including the length of the sterilization cycle, the elastomeric formulation, and the elastomer size and shape. Particular care should be taken when the elastomeric components are to be used to seal a lyophilized or powder-filled product, as residual moisture in the component can migrate into the formulation over time.

## 6. IDENTIFICATION TESTS

Components are made of a wide variety of elastomeric materials and optional polymeric coatings. For this reason, it is beyond the scope of ⟨381⟩ to specify identification tests that encompass all possible component presentations. However, it is the responsibility of the component supplier and the drug product manufacturer to verify the component's elastomeric formulation and any coating or laminate material used according to suitable identification tests. Examples of some of the analytical test methodologies that may be used include specific gravity, percentage of ash analysis, sulfur content determination, Fourier-transform infrared spectroscopy–attenuated total reflectance (FTIR–ATR) test, chromatography of an extract, UV absorption spectrophotometry of an extract, or infrared absorption spectrophotometry of a pyrolysate.

## 7. TEST REQUIREMENTS AND RESPONSIBILITIES

Elastomeric components provided by a supplier to a drug product manufacturer may demonstrate compliance with specific (e.g., biological and/or physicochemical) acceptance criteria as required by the drug product manufacturer in accordance with requirements established by the manufacturer for storage and/or delivery of a unique drug product. Processing such as sterilization may be performed on finished elastomeric components by the supplier prior to delivery to the drug product manufacturer, or by the drug product manufacturer before use, as agreed upon by both the supplier and the drug product manufacturer.

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For elastomeric components that are subsequently processed or sterilized by the drug product manufacturer after receipt from a supplier, the drug product manufacturer is responsible for ensuring that the components are compliant with appropriate acceptance criteria required for storage and/or delivery of a unique drug product after the application of such processing and/or sterilization conditions (i.e., in their ready-to-use state). This is of particular importance if elastomeric components are processed under conditions that may impact the biological, physicochemical, or functionality characteristics of the component.

## 8. SUMMARY OF ⟨381⟩ PHYSICOCHEMICAL TESTS

### 8.1 Test Samples

Elastomeric components are comprised of multiple materials that have unique attributes for each configuration, application, and change resulting from any treatment that occurs after formation (post-processing). This can be a challenge for establishing elastomeric baselines due to multiple combinations of materials and post-processing treatments by the component manufacturer and the drug product manufacturer.

Elastomeric components may be classified into two types: Type I and Type II. Type I elastomeric components meet the strictest requirements and are preferred because they have lower levels of extractables. Type I elastomers, however, may not exhibit sufficient elasticity to be suitable for special uses (e.g., multiple piercing, which requires reseal capacity and lower fragmentation rates). In such cases, the solution consists of using elastomers that do not respect all Type I physicochemical acceptance limits because they have properties that allow the component to meet sealability and fragmentation requirements. An allowance is made for Type II elastomeric components to meet less stringent alternative acceptance criteria for physicochemical testing. Some Type II components may fail to pass *Biological Reactivity Tests, In Vitro* ⟨87⟩ requirements but will meet *Biological Reactivity Tests, In Vivo* ⟨88⟩ requirements, thus complying with biological reactivity requirements.

Test samples described in ⟨381⟩ are to mimic the finished, processed component intended for the final product and its packaging/delivery system. It is the drug product manufacturer who is ultimately responsible for the quality and safety of the finished pharmaceutical product. Therefore, the drug product manufacturer is responsible for ensuring component conformance to the baseline requirements of ⟨381⟩.

### 8.2 Physicochemical Tests

**Appearance (turbidity/opalescence):** This is a nonspecific test for all the extractable species in an elastomeric formulation that are not soluble in an aqueous solution. A high turbidity is the indication of a high extractable potential. Species promoting turbidity have numerous origins in an elastomeric formulation, including fatty-acid derivatives, residues of curing systems, and oligomers from the elastomer.

**Color:** This is a nonspecific test indicative of the presence of extractable species in an elastomeric formulation that have the capacity of attributing color to an aqueous solution. Species that cause color may have several origins in an elastomeric formulation. Aqueous solutions are common in pharmaceutical packaging/delivery systems.

**Acidity or alkalinity:** This is a nonspecific test indicative of the acidic, basic, or buffering power of the aqueous extractables from the elastomeric formulation. High values in the acidity/alkalinity test may need to be evaluated in conjunction with the specifics of a drug solvent vehicle and anticipated acceptance criteria for the pH of the drug product.

**Absorbance:** This UV spectrum test of an aqueous extract from an elastomeric formulation is indicative of the unsaturated or aromatic character of the chemical species extracted. Unsaturated compounds in the extracts may originate from many raw materials and additives of an elastomeric formulation such as antioxidants and curing or dying agents.

**Reducing substances:** This is a nonspecific test. Extracted species from an elastomeric formulation with potential reducing power may originate from most raw materials of an elastomeric formulation (polymers, curing systems, antioxidants, etc.).

**Volatile sulfides:** This test is specific for elastomeric formulations containing sulfur. Sulfur and sulfur precursors are often used as components of curing systems for elastomers.

**Ammonium:** This test is specific for elastomeric formulations with nitrogen-containing raw materials. Ammonium ions can be generated during the curing process. Thiurams and thiazoles are examples of nitrogen-containing curing systems used.

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***Add the following:***

# ▲⟨1382⟩ ASSESSMENT OF ELASTOMERIC COMPONENT FUNCTIONAL SUITABILITY IN PARENTERAL PRODUCT PACKAGING/DELIVERY SYSTEMS

1. INTRODUCTION
2. EARLY PACKAGING/DELIVERY SYSTEM SELECTION AND DEVELOPMENT: FUNCTIONAL SUITABILITY ASSESSMENT CONSIDERATIONS
3. FINAL PRODUCT PACKAGING/DELIVERY SYSTEM FITNESS-FOR-INTENDED-USE SUITABILITY ASSESSMENT
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    4.1 Test Samples
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5. CONCLUSION

## 1. INTRODUCTION

This chapter contains information and guidance to assist in the functional suitability assessment of elastomeric components as part of packaging/delivery systems intended for parenteral dosage forms contained in *Elastomeric Component Functional Suitability in Parenteral Product Packaging/Delivery Systems* ⟨382⟩. Such components include primary packaging/delivery system components that are partially or completely made of elastomeric material. Excluded from this chapter are products and their packaging that are regulated as medical devices (e.g., unfilled syringes, infusion administration sets, delivery systems for drug-eluting stents). The proper selection and design of functional suitability assessment studies is based on sound and justifiable scientific principles provided in ⟨382⟩, *1. Introduction*.

## 2. EARLY PACKAGING/DELIVERY SYSTEM SELECTION AND DEVELOPMENT: FUNCTIONAL SUITABILITY ASSESSMENT CONSIDERATIONS

Early in the packaging/delivery system selection and development process, the final packaging/delivery system and its components may not be fully defined and functional suitability requirements may not be established. This is especially true if the packaging/delivery system or the drug product is novel to the drug product applicant. At this phase of the product life cycle, functional suitability assessments are performed to better understand packaging/delivery system performance and/or to screen potential elastomeric component and container candidates. To that end, *Table 1* lists elastomeric component functional suitability tests in standards published by the International Organization for Standardization (ISO). The list should not be considered all-inclusive. The most recent standards should be referenced.

The terms "standards" and "recognized standards" used throughout this chapter and ⟨382⟩ refer to those published by ISO.

Although not mandated, testing components and/or closure packaging/delivery systems according to such standards may provide useful information, especially during early product packaging/delivery system development. Other relevant internationally recognized standards deemed scientifically appropriate may be used instead of, or in addition to, those listed in *Table 1*. Standard test data may be made available by elastomeric component or packaging/delivery system suppliers.

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**Table 1. ISO Standards: Functional Suitability Tests for Elastomeric Components**

| ISO Standard (listed in numeric order) | | Functional Suitability Tests |
|---|---|---|
| Sterile hypodermic syringes for single use[a] | 7886-1 | **Syringes for manual use**<br>• Freedom from air and liquid leakage past plunger stopper<br>• Force to operate the plunger (also called the piston)<br>• Fit of plunger stopper/plunger in barrel |
| | 7886-2 | **Syringes for use with power-driven syringe pumps**<br>• Freedom from air and liquid leakage past piston<br>• Flow characteristics<br>• Plunger movement forces |
| | 7886-3 | **Auto-disable syringes for fixed-dose immunization**<br>• Freedom from air and liquid leakage past piston<br>• Auto-disable feature<br>• Performance after shipping |
| | 7886-4 | **Syringes with re-use prevention feature**<br>• Freedom from air and liquid leakage past piston<br>• Re-use prevention feature<br>• Performance after shipping |
| Injection containers and accessories | 8362-2 | **Closures for injection vials**<br>• Penetrability<br>• Fragmentation<br>• Self-sealing and aqueous solution tightness test<br>• Dye solution tightness test |
| | 8362-5 | **Freeze-drying closures for injection vials**<br>• Penetrability<br>• Fragmentation<br>• Self-sealing and aqueous solution tightness test<br>• Aqueous solution tightness test<br>• Moisture barrier |
| Infusion equipment for medical use (closures used in combination with bottles and intended to be pierced with an injection needle or spike) | 8536-2 | **Closures for infusion (glass) bottles**<br>• Fragmentation<br>• Spike penetration force<br>• Spike retention/sealability |
| | 8536-6 | **Freeze-drying closures for infusion bottles**<br>• Fragmentation<br>• Spike penetration force<br>• Spike penetration/sealability |
| Sterile single-use syringes, with or without needle, for insulin | 8537 | • Fit of plunger stopper in barrel (forces to operate piston)<br>• Freedom from leakage at needle<br>• Freedom from leakage past plunger stopper |
| Elastomeric parts for parenterals and for devices for pharmaceutical use (used in combination with vials and intended to be pierced with an injection needle) | 8871-5 | **Functional requirements and testing**<br>• Penetrability<br>• Fragmentation<br>• Self-sealing and aqueous solution tightness<br>• Aqueous solution tightness |

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**Table 1. ISO Standards: Functional Suitability Tests for Elastomeric Components** *(continued)*

| ISO Standard (listed in numeric order) | | Functional Suitability Tests |
|---|---|---|
| Prefilled syringes | 11040-2 | **Plunger stoppers for dental local anesthetic cartridges**<br>• Freedom from leakage<br>• Sliding characteristics |
| | 11040-3 | **Seals for dental local anesthetic cartridges**<br>• Fragmentation<br>• Freedom from leakage |
| | 11040-4 | **Glass barrels for injectables and sterilized subassembled syringes ready for filling**<br>• Closure system allowance for sterilization<br>• Extrusion force<br>• Closure system liquid leakage past needle shield or tip cap<br>• Luer lock rigid tip cap unscrewing torque<br>• Pull-off force of tip cap or needle shield<br>• Closure system barrel integrity (dye solution tightness test) |
| | 11040-6 | **Plastic barrels for injectables and sterilized subassembled syringes ready for filling**<br>• Closure system allowance for sterilization<br>• Extrusion force<br>• Closure system liquid leakage past needle shield or tip cap<br>• Luer lock rigid tip cap unscrewing torque<br>• Pull-off force of tip cap or needle shield<br>• Closure system barrel integrity (dye solution tightness test) |
| | 11040-8 | **Requirements and test methods for finished prefilled syringes**<br>• Break-loose and extrusion forces<br>• Liquid leakage beyond plunger<br>• Pull-off force of tip cap or needle shield<br>• Luer lock rigid tip cap unscrewing torque<br>• Container closure integrity |
| Needle-based injection systems for medical use (cartridges for dental use not included) | 11608-3 | **Finished containers**<br>• Plunger force<br>• Freedom from leakage<br>• Resealability<br>• Coring |
| Pen systems | 13926-2 | **Plunger stoppers for pen injectors for medical use**<br>• Freedom from leakage past plunger stopper under axial pressure<br>• Initiating and sustaining forces (break-loose and extrusion forces) |
| | 13926-3 | **Seals for pen injectors for medical use**<br>• Fragmentation<br>• Freedom from leakage past seals under axial pressure<br>• Resealability |
| Plastic containers for intravenous injections (blow-molded bottles, film bags) | 15747 | • Resistance to temperature stability, pressure, and leakage<br>• Penetration ability<br>• Adhesion strength of the infusion device and impermeability of the insertion point<br>• Tightness of the injection point |
| Medical infusion equipment—Plastic caps with inserted elastomeric liners for containers manufactured by the blow-fill-seal (BFS) process | 15759 | **Physical requirements and testing for liners**<br>• Fragmentation<br>• Penetration force<br>• Dynamic spike-retention capability<br>• Static spike-retention capability of the liner and leak resistance of the piercing area<br>• Resealability |

[a] Single-use device: A single-use device, also referred to as a disposable device, is intended to be used on one patient during a single procedure. It is not intended to be reprocessed (cleaned, disinfected/sterilized) and used on another patient.

## 3. FINAL PRODUCT PACKAGING/DELIVERY SYSTEM FITNESS-FOR-INTENDED-USE SUITABILITY ASSESSMENT

Evidence of the elastomeric component's ability to satisfactorily function as part of the final packaging/delivery system according to its intended product use is required to support commercial market approval of a finished drug product. Internationally recognized standards, such as those listed in *Table 1*, can provide a useful benchmark when designing appropriate elastomeric component functional suitability assessment studies. However, the drug product applicant is advised to exercise caution before prescriptively adopting standardized tests. Such tests may not provide a complete or adequate assessment of the elastomeric component's ability to meet the final packaging/delivery system's product-specific functional demands and instead should be considered a precursor to design verification and validation of the final finished combination product. Standardized tests may not be adequate or appropriate in specific situations for many reasons, for example:

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- The final packaging/delivery system components may differ in design or dimension from the description in the standard.
- The manner in which the final packaging/delivery system's components are processed, reprocessed, and/or assembled may differ from the description in the standard.
- The manner in which a liquid, such as a diluent, must be added to the packaging/delivery system for final dosage form preparation (such as for lyophilized product reconstitution, powdered product constitution, product dilution, or admixture) may differ from the description in the standard.
- The manner in which the packaged product is to be accessed at the time of use may differ from the description in the standard. Differences may include needle or spike design, material, or lubricity; needle puncture speed; and the number of penetrations per closure.
- The standard's test procedure or analysis method may provide an inadequate measure of packaging/delivery system functional suitability given the specific rigorous demands placed on the final packaging system. For instance, bench test conditions may not sufficiently mirror the rigors imposed on the marketed product during actual storage, distribution, and usage conditions.
- The test method may lack sufficient sensitivity or precision to provide an adequate measure of packaging/delivery system functional suitability. For example, a container-closure seal integrity test that relies on dye ingress observation may be too insensitive to provide an accurate picture of a lyophilized dosage form package's ability to meet the maximum allowable leakage limit demanded by the finished product packaging/delivery system.
- The standard test's acceptance criterion may not reflect the functional performance demanded of the final packaging/delivery system for the particular intended use. For example, the maximum allowable needle penetration force for a closure dictated by a standard may result in damage to the penetration needle supplied with the intended delivery device.
- The standard test's sample size may not reflect the reliability demanded of the final packaging/delivery system. A larger sample size will be expected in design verification and validation of the final packaging/delivery system.

Therefore, the drug product applicant is tasked with developing a body of elastomeric component functional suitability assessment tests that logically and most appropriately assess the final packaging/delivery system. Challenges placed on the final product's packaging/delivery system during testing should mimic challenges the product is likely to encounter through storage/distribution, product expiry, and clinical in-use condition. These testing challenges should provide clear and definitive measures of packaging/delivery system performance.

## 4. GENERAL CHAPTER ⟨382⟩ BACKGROUND AND GUIDANCE

Chapter ⟨382⟩ offers drug product packaging/delivery system fitness-for-intended-use functional suitability assessment procedures and acceptance criteria that are reflective of current best practices. These best practices take into consideration all the recognized ISO standards in *Table 1* as well as USP tests and guidances (e.g., *Biological Reactivity Tests, In Vitro* ⟨87⟩ and *Biological Reactivity Tests, In Vivo* ⟨88⟩). Specific sources for the various tests are detailed in the following sections. These tests are not intended to be exhaustive. The drug product applicant may require additional tests to adequately assess the component's functional suitability as part of a particular packaging/delivery system.

Re-evaluation of functional suitability may be required when changes occur in components, processes, or even the commercialized product itself during its life cycle. The functional suitability of a drug product packaging/delivery system is the responsibility of the drug product applicant.

The following sections contain guidance and background information relevant to tests in ⟨382⟩.

### 4.1 Test Samples

The following relates to ⟨382⟩, *3. General Test Requirements, 3.1 Test Samples*. Functional suitability of elastomeric components cannot be tested for in isolation from the intended packaging/delivery system. Furthermore, a component's functional performance assessment outcome can be affected by the design, mating interfaces, processing, and assembly of that packaging/delivery system. For example, closure processing parameters and vial package sealing forces directly impact parenteral vial packaging integrity results. Plunger break-loose and extrusion forces are directly related to the design, material of construction, and lubricity of the syringe barrel.

Test samples employed for each functional suitability test are to mirror the components and packaging/delivery system of the intended product as closely as possible, because component functionality is connected to the packaging/delivery system. Components are to be prepared, processed, and assembled as defined for the final product packaging/delivery system, especially if such steps are believed to have a potential impact on component functionality.

The following examples are offered as illustration:

- Components are to be washed, lubricated, and sterilized according to intended product protocol.
- Components are to be laminated or coated according to the requirements of the intended product packaging/delivery system.
- Vial and bottle closures are to be optimally capped onto vials in a manner reflective of the intended finished product packaging/delivery system.
- Syringe/cartridge barrels are to be prepared and lubricated according to the requirements of the intended pharmaceutical product.
- Syringe/cartridge plungers are to be inserted into syringe barrels according to production practices (e.g., by use of a vent tube or vacuum insertion). Manual placement can be utilized for feasibility assessment.
- Cartridge line seal crimping pressure is to be reflective of the intended finished product packaging/delivery system.

Some tests, such as ⟨382⟩, *5. Needle and Spike Access Functional Suitability Tests, 5.1 Fragmentation*, state that the test samples are to be filled with water. However, in cases where package contents can influence test outcome, such as ⟨382⟩, *6. Plunger*

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*Functional Suitability Tests, 6.1 Plunger Break-Loose and Extrusion Forces* tests, it is recommended that test samples be filled with product or a product proxy so that the test outcome better reflects packaging/delivery system intended use. Alternatively, content material that brackets the characteristics of multiple products may be chosen.

Some flexibility in test sample preparation and content is permitted if the variation is judged to have little or no impact on test outcome. With appropriate justification, test samples may bracket relevant parameters of packaging/delivery system design, intended interfaces, dimensional analysis, component processing, package assembly, and product contents. Bracketing may be employed to allow a functional suitability assessment program that addresses a wider spectrum of packaging/delivery systems and/or products.

The selection, design, preparation, assembly, and contents of test samples should follow sound scientific principles so that the final product packaging/delivery system can be comprehensively evaluated for functional suitability.

## 4.2 Test Sample Population Size

As described in ⟨382⟩ *3. General Test Requirements, 3.2 Test Sample Population Size*, test sample quantity should provide a reasonable measure of confidence of the packaging/delivery system elastomeric component functionality. Current recognized standards inform the test sample population sizes specified in chapter tests. For this reason, sample sizes vary across test categories and even among package types within a test category. For example, ⟨382⟩*, 5. Needle and Spike Access Functional Suitability Tests, 5.1 Fragmentation* vial system tests require 12 test samples (as per ISO 8362-2, 8362-5, and 8871-5), whereas bottle system tests require 10 test samples (as per ISO 8536-2 and -6). In the case of fragmentation tests, it was deemed important to respect the historical particulate findings generated using these varied sample population sizes. On the other hand, ⟨382⟩*, 5. Needle and Spike Access Functional Suitability Tests, 5.3 Needle Self-Sealing Capacity* tests require 30 test samples regardless of the packaging/delivery system to ensure more meaningful leakage findings even though recognized standards require fewer samples.

Test sample population sizes cited in chapter test methods represent minimal test sample population size requirements but may be inadequate depending on risk assessments and whether the data generated will be attribute or variable. Sample population sizes that are larger than those specified in test procedures are encouraged to provide greater assurance of packaging/delivery system performance and to minimize the risk of product failure during commercial use.

## 4.3 Packaging/Delivery System Integrity Tests

The verification of packaging/delivery system integrity is required according to ⟨382⟩*, 4. Packaging/Delivery System Integrity Tests*. All components that are intended to seal or affect container closure must adequately protect and contain the packaging/delivery system contents. In this context, all such components are termed closures. All closures are required to ensure adequate system integrity; therefore, all packaging/delivery systems within the scope of the chapter are required to meet an appropriate system integrity functional suitability assessment. This section does not apply to closures after they have been breached by a needle, spike, or other access device.

Packaging/delivery system integrity refers to the ability of a packaging/delivery system to keep product contents in, and to keep detrimental environmental contaminants out. All packaging/delivery systems for parenteral products closed with elastomeric components are required to demonstrate integrity, as defined by the level of protection necessary for product quality maintenance. All systems with elastomeric closures mechanically fitted to the container demonstrate some gaseous leakage past the seal interface, even when optimally assembled. Leaks of concern for sterile product packaging/delivery systems are those that pose an unacceptable level of risk to relevant product physicochemical, biological, and microbiological quality attributes.

Specifically, all parenteral product packaging/delivery systems must: 1) prevent microbiological ingress to ensure that product sterility is met; and 2) prevent product escape or entry of external liquid or solid matter to ensure that relevant product physicochemical quality attributes are met. In addition, some products require the maintenance of package headspace content in a manner that ensures relevant product physicochemical quality attributes are met and/or allows for ease of product access by the end user. As some packaging/delivery systems employ more than one closure, and each closure may provide a different level of product protection, different integrity tests may be required to effectively evaluate the inherent integrity of the various closure types.

See *Package Integrity Evaluation—Sterile Products* ⟨1207⟩, as well as its subchapters, for further guidance on the concepts of inherent package integrity and maximum allowable leakage limit, and for guidance on the proper selection, development, validation, and utilization of appropriate leak test methods.

## 4.4 Needle and Spike Access Functionality Tests

Information in ⟨382⟩*, 5. Needle and Spike Access Functional Suitability Tests* applies to packaging/delivery system closures that allow for drug product access by hypodermic needle, spike, or other closure penetration device. Packaging/delivery systems intended for parenteral products that permit dosage form access by insertion of a closure piercing device are required to allow for safe and effective product access, without damaging the packaging/delivery system or the drug product and without risking harm to either the patient receiving the medication or the individual accessing and/or administering the product.

Traditionally, needle and spike access functionality tests have not accounted for the possibility of an additional closure penetration commonly performed to introduce liquids into the product packaging/delivery system for lyophilized product reconstitution, powdered product constitution, product admixture, or product dilution. Closure penetration practices for dosage form preparation vary widely. The piercing device employed for dosage form preparation may be very different in size or design from the needle or spike used for subsequent product access and withdrawal. There is little published information on the impact of such initial closure penetrations on closure performance or product quality. To address this gap, needle and spike

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access functional suitability tests have been expanded to incorporate a first closure piercing for products requiring final dosage form preparation in addition to the closure piercing(s) for product access and withdrawal.

Tests dictate that all piercings be performed using the dosage form preparation piercing device(s) and/or the product access piercing device(s) intended or recommended by the drug product applicant. For example, if the intent is to provide or to specify a needle or other piercing device with the marketed product for a given purpose, then this same item or a facsimile is to be used. If a piercing device will neither be specified nor provided (i.e., not designated), the procedures cite dosage form preparation and drug product access devices to be used for the tests. Drug product access needles/spikes cited align with current reference standards. Dosage form preparation needles cited in the procedures are unique to USP.

The following tests are included in this functional suitability assessment category.

## FRAGMENTATION

See ⟨382⟩, *5. Needle and Spike Access Functional Suitability Tests, 5.1 Fragmentation*. Also called coring, fragmentation is a measure of the packaging/delivery system's tendency to fragment or core when penetrated by a dosage form preparation piercing device (if applicable) and by a product access piercing device. If injected, such closure fragments may pose a health risk to the patient.

The penetration tests largely align with fragmentation tests in ISO standards in the piercing devices used and the number of piercings per test sample. Specifically, *Procedure A* in ⟨382⟩, *5. Needle and Spike Access Functional Suitability Tests, 5.1 Fragmentation, Vial and bottle systems* was informed by ISO 8362-5 and 8871-5. *Procedure B* was informed by ISO 8536-2 and -6. The ⟨382⟩, *5. Needle and Spike Access Functional Suitability Tests, 5.1 Fragmentation, BFS systems* test method was informed by ISO 15759. *Procedure A* of ⟨382⟩, *5. Needle and Spike Access Functional Suitability Tests, 5.1 Fragmentation, Cartridge systems* was informed by ISO 13926-3 and *Procedure B* was informed by ISO 11608-3.

Unlike ISO tests, an extra initial piercing option representing a dosage form preparation piercing is included in the vial and bottle systems as well as the BFS systems test procedures.

Also unlike ISO tests, fragmentation count analysis is to be performed according to *Particulate Matter in Injections* ⟨788⟩, *Method 2 Microscopic Particle Count Test*, with noted modifications. Modifications include allowances for microscope magnification range adjustment and the use of the linear graticule for particle sizing. Test procedures require particle sizing based on the longest linear dimension of the particle.

Acceptance criteria are defined according to the quantity of detected particles ≥150 µm in the longest linear dimension. Smaller particles are not reported. Recognized standards define fragmentation acceptance criteria according to the number of particles visibly detected, i.e., particles ≥50 µm (e.g., ISO 8871-5). ⟨382⟩, *5. Needle and Spike Access Functional Suitability Tests, 5.1 Fragmentation* has adopted the same particle quantity limits, as per the recognized standards, but counted particles are ≥150 µm, sized microscopically, defined by the longest linear dimension. This change was based on more recent data suggesting visibly detected particles are closer to 150 µm in size, as per *Visible Particulates in Injections* ⟨790⟩.

## PENETRATION FORCE

See ⟨382⟩, *5. Needle and Spike Access Functional Suitability Tests, 5.2 Penetration Force*. Also called penetrability, penetration force is the maximum force necessary to penetrate the closure using a dosage form preparation piercing device (if applicable) and a product access piercing device. Penetration force tests also confirm the ability of the closure to remain in place without being forced into the container during piercing.

Test procedures and acceptance criteria were informed by current reference standards. In some cases details were modified so all packaging/delivery systems could be tested similarly. Details are included below.

*Procedure A* and *Procedure B* of ⟨382⟩, *5. Needle and Spike Access Functional Suitability Tests, 5.2 Penetration Force, Vial and bottle systems* evaluate the penetration force required for a dosage form preparation hypodermic needle and for a dosage form access hypodermic needle, respectively. *Procedure C* evaluates the penetration force required for spike access. ISO standard vial package closure penetration tests do not specify the needle insertion rate or the use of a mechanical testing machine (ISO 8362-2, 8362-5, 8871-5). Standard bottle spike penetration force tests specify use of a mechanical testing machine, operated at an insertion rate of 200 mm/min with a load cell accuracy of ±2 Newtons (N) (ISO 8536-2 and -6). For consistency among chapter tests, the vial and bottle systems tests specify the use of a mechanical testing machine operated at the penetration rate of 200 mm/min with a data acquisition rate of NLT 100 Hz for both vials and bottles penetrated by either needles or spikes.

*Procedure A* and *Procedure B*, which use a needle for penetration, require a load cell accuracy of ±0.25 N in anticipation of results less than 10 N. *Procedure C*, which uses a spike, anticipates higher penetration forces, therefore, a load cell accuracy of ±2 N is specified, similar to the corresponding ISO standards.

There exists no published data or ISO standard for dosage form preparation penetration force for vial and bottle systems; therefore *Procedure A* does not include quantitative acceptance limits. The end user is responsible for establishing a limit appropriate for the system and its intended use.

The penetration force acceptance limit for BFS systems is also not specified. The penetration force limit in ISO 15759 only evaluates the penetration force through the closure, not the force required to penetrate the closure combined with the underlying plastic layer in a fully assembled BFS system. For this reason, the end user is responsible for establishing a limit that is appropriate for the system and its intended use.

The penetration force acceptance limit for plastic systems of no more than 200 N is the highest for all package types. This high value is informed by ISO 15747 and includes the friction force between the spike and the access port tube.

## NEEDLE SELF-SEALING CAPACITY

See ⟨382⟩, *5. Needle and Spike Access Functional Suitability Tests, 5.3 Needle Self-Sealing Capacity*. Also called reseal capacity, injection port tightness, or in-use leakage tests, this term applies to product packaging/delivery system closures required to ensure adequate package integrity during in-use conditions of multiple breaches by a needle(s). Such systems include

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multiple-dose product packaging/delivery systems, as well as systems with closures that must be penetrated more than once to permit dosage form preparation followed by penetration(s) for product access and administration.

Test samples are challenged prior to packaging/delivery system in-use integrity verification by exposing the closures to worst case (i.e., the most challenging) piercing conditions anticipated for the intended product. In cases where the largest gauge piercing device(s) and/or the maximum penetration quantity conditions are undefined, ⟨382⟩ lists challenge conditions to be employed, similar to self-sealing test requirements corresponding to recognized standards.

The ⟨382⟩ test requires a minimum of 30 test samples for all packaging/delivery system types. This quantity is greater than the specification in ISO's standards for resealability tests. However, the requirement for 30 test samples does align with the requirements of ⟨382⟩, *4. Packaging/Delivery System Integrity Tests*. A smaller quantity was viewed to be less likely to yield meaningful leakage information.

No leak test procedure is mandated for self-sealing capacity. Tests described in corresponding recognized standards may be consulted. However, such tests may not accurately or appropriately measure the integrity of the compromised closure for the intended product and its use. Therefore, the reader is referred to *Package Integrity Evaluation—Sterile Products* ⟨1207⟩ and its subchapters for guidance in test method selection, development, validation, and utilization.

SPIKE RETENTION AND SEALABILITY CAPACITY

See ⟨382⟩, *5. Needle and Spike Access Functional Suitability Tests, 5.4 Spike Retention and Sealability Capacity*. This test is a measure of a closure system's ability to be fully penetrated by a spike (without pushing the closure into the container); to block visible evidence of liquid product leakage between the spike and the closure during the product-dosing time period; and to retain the spike during this time period.

Test samples are to be challenged by exposing the closures to worst case (i.e., the most challenging) piercing conditions anticipated for the intended product. In cases where the spike piercing device is undefined, utilize the device described in the corresponding ISO standards. Note that the ⟨382⟩ test sample size and test conditions are similarly based on recognized standard procedures for the respective packaging/delivery system categories.

## 4.5 Plunger Functional Suitability Tests

Information in ⟨382⟩, *6. Plunger Functional Suitability Tests* applies to packaging/delivery systems that incorporate a plunger (also called a piston), namely cartridge systems and syringe systems. This section include tests to evaluate plunger break-loose and extrusion forces as well as plunger seal integrity.

Packaging/delivery systems designed to allow for product elution via a plunger are required to allow for complete, safe, and effective product delivery without damaging the system and without risking harm to either the patient receiving the medication or the individual accessing and/or administering the product. The plunger is also required to ensure adequate product containment from the time the product is filled until product delivery to the patient is complete.

PLUNGER BREAK-LOOSE AND EXTRUSION FORCES

See ⟨382⟩, *6. Plunger Functional Suitability Tests, 6.1 Plunger Break-Loose and Extrusion Forces*. This test evaluates plunger break-loose force (also known as the initiating force), which is the force required to initiate the movement of the plunger. It also evaluates plunger extrusion force (also known as the sustaining force), which is the force required to sustain the movement of the plunger to expel the content of the syringe or cartridge. This test allows an analysis of the ease with which product delivery may be performed.

Many variables can affect these forces. For example, product characteristics of fluid viscosity, density, and fluid surface tension can directly impact break-loose and extrusion forces. These forces are also influenced by the interference fit between the plunger and the barrel, the plunger rib design and quantity, the lubrication of the plunger and the inner surface of the barrel, the fit of the plunger rod into the threaded plunger, the needle internal diameter, the inside diameter of the syringe or cartridge, and the target injection time. The use of connecting devices with the test sample can significantly influence forces such as extrusion forces.

Furthermore, it is noteworthy that break-loose force can increase over time to an unacceptable level at which point it is difficult to initiate plunger movement by hand. If the plunger is operated by a spring, the break-loose force required may be greater than the capability of the spring. The drug product applicant is advised to consider this possibility when designing drug product shelf-life stability assessment studies. Finally, component processing can also impact plunger function. For example, an irregular extrusion force, or one that rises significantly towards the syringe nozzle, can indicate non-homogeneous lubrication of the barrel due to improper application or degradation.

The performance requirements of the packaging/delivery system should consider the intended use of the syringe or cartridge. For example, if the product is to be manually delivered, break-loose force, extrusion forces, and range of possible rates of delivery should accommodate the skill set of the population responsible for product administration. If the product is to be delivered using an automatic pen injector, forces must be appropriate for the delivery system hardware.

In conclusion, a wide number of variables can impact break-loose and extrusion force results. The many combinations of products, packaging/delivery systems, and intended uses influence acceptable functional performance criteria. Therefore, a single test method cannot be defined, nor can acceptance criteria be specified, for all relevant packaging/delivery systems, products, and intended-use applications. The user is responsible for following the generic test method outlined in ⟨382⟩ and for establishing meaningful quantitative acceptance criteria that best represent the demands of the specific product packaging/delivery system.

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PLUNGER SEAL INTEGRITY

See ⟨382⟩, *6. Plunger Functional Suitability Tests, 6.2 Plunger Seal Integrity*. This test is designed to apply a fixed force to the plunger of a sealed syringe or cartridge containing liquid product in order to detect leakage past the plunger. *Procedure A* applies to manually operated prefilled and single-use syringe systems. This procedure and acceptance criteria were informed by ISO 7886-1. *Procedure B* applies to non-manually operated prefilled syringe systems that function as part of an auto-injector system such as a spring-driven or power-driven delivery device. This procedure and acceptance criteria were informed by ISO 11040-8. *Procedure C* is specifically for cartridge systems used for dental local anesthesia products and was informed by ISO 11040-2 and -3. *Procedure D* is for all cartridge systems, excluding those for dental local anesthesia products. The procedure and acceptance criteria were informed by ISO 13926-2 and -3. For all procedures, satisfactory plunger seal tightness will not permit visible leakage of liquid product past the rear rib or final seal of the plunger when forces simulating product delivery are applied. Inspect the primary components (syringe and plunger) prior to the insertion of the plunger to avoid trapping liquid between the plunger ribs and the container. For cartridge containers that are also closed with a stopper or septum (without a needle), acceptance criteria also require the absence of visible leakage past this closure.

### 4.6 Tip Cap and Needle Shield Functional Suitability Tests

⟨382⟩, *7. Tip Cap and Needle Shield Functional Suitability* tests examine the functional suitability of tip caps and needle shields. These components are intended to protect the needle or nozzle (Luer end) of the syringe, allow for nozzle/needle sterilization, and maintain the sterility of the contents of the syringe container. The functional suitability tests examine the forces required to remove the tip cap or needle shield prior to dose administration. *Procedure A* measures the axial force used to pull off needle shields and tip caps. *Procedure B* is a torque test that measures the force use to unscrew and remove the Luer lock rigid tip caps. A packaging/delivery system is satisfactory if the force needed to remove the component allows for the manual removal of the tip cap or needle shield with relative ease but prevents the accidental loss of these components during storage or transit.

When selecting acceptance criteria, the user should consider the various factors that can influence removal forces. For example, processes applied to the components pre- and post-assembly, such as lubrication and sterilization, can impact test results. Other factors include elastomeric component age, age of the assembled system, and possibly finished product storage conditions.

The procedures and acceptance criteria in ⟨382⟩, *7. Tip Cap and Needle Shield Functional Suitability* were influenced by ISO 11040-4 and -8.

## 5. CONCLUSION

In summary, the functional performance of elastomeric components is to be evaluated in a manner that ensures the elastomeric component is suitable. Although this chapter's scope does not include functional suitability tests relevant to other components of the packaging/delivery system, or to all aspects of the system itself, such tests are nevertheless important. Consideration should be given to the full scope of packaging/delivery system functional demands inherent in the manufacture, safety, use, and marketability of the intended product. To this end, the applicant may seek direction in other relevant packaging/delivery system guidances and standards. Determination of a suitable elastomeric component requires that the applicant employ a science- and risk-based approach to accomplish a thorough and complete functional suitability assessment verifying the entire packaging/delivery system's fitness for intended use.▲ (USP 1-Dec-2020)

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***Add the following:***

# ▲⟨1430.1⟩ ANALYTICAL METHODOLOGIES BASED ON SCATTERING PHENOMENA—STATIC LIGHT SCATTERING

1. INTRODUCTION
2. THEORY
3. APPLICATIONS
    3.1 Low-Angle Light Scattering Determination of Molecular Weight
    3.2 Multi-Angle Light Scattering
    3.3 Combination of Light Scattering with Size-Fractionation Techniques
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## 1. INTRODUCTION

The phenomena observed when photons [i.e., an electromagnetic (EM) wave] strike or interact with a small object (a particle or a molecule) and thereby change direction is called light scattering (LS).

In static light scattering (SLS), the inevitable short-term temporal fluctuations in scattering intensity due to Brownian motion are averaged over a range of time scales from several hundredths to several tenths of a second. SLS measures the time average intensity of scattered light from particles in a suitably prepared sample; the detector signal is, therefore, time independent or "static".

In SLS, the scattered light is detected and measured as a function of the angle between the detector and the incident beam direction. Light scattering can be either at a single fixed angle, as in low-angle light scattering (LALS) or right-angle light scattering (RALS); or over a range of angles, as in multi-angle light scattering (MALS).

SLS provides information about molecular weight, particle size, particle shape, and molecular interactions.

This chapter provides guidance and procedures for LALS and MALS. These SLS methodologies are based on the Rayleigh approximation of classical Mie scattering theory. In these scattering methods, the particles are assumed to be present in solution (or gas) and free to move; thus, the particles are inherently in an unordered state. Other scattering phenomena such as reflection, refraction, and diffraction require the scattering particles to be highly ordered and are out of the scope of this text.

## 2. THEORY

SLS techniques are based on measurement(s) of the scattered light intensity ($I_S$) itself, as opposed to indirect measurements (e.g., inferring the scattered intensity from the attenuation of transmitted light).

In order to obtain a measure that relates the material itself to the scattering intensity, independent of equipment characteristics, one defines the Rayleigh ratio, $R(\Theta)$, for the sample under investigation (*Equation 1*):

$$R(\theta) = \frac{I_S(\theta) r^2}{I_0 V_S(\theta)} \qquad (1)$$

$R(\Theta)$ is defined as the scattered intensity per unit solid angle, scattering volume ($V_S$), and incident intensity ($I_0$) that is in excess of that scattered by solvent alone, and $r$ is the distance of the scattering volume from the detector.

*Figure 1* shows that the scattering of light on the particle changes the wave vector from $k_{incident}$ to ~$k_{scatter}$. For sufficiently small particles, only the direction, but not the wavelength, changes as a result of the collision. Hence, the length of the wave vector $\vec{k}$, remains unchanged.

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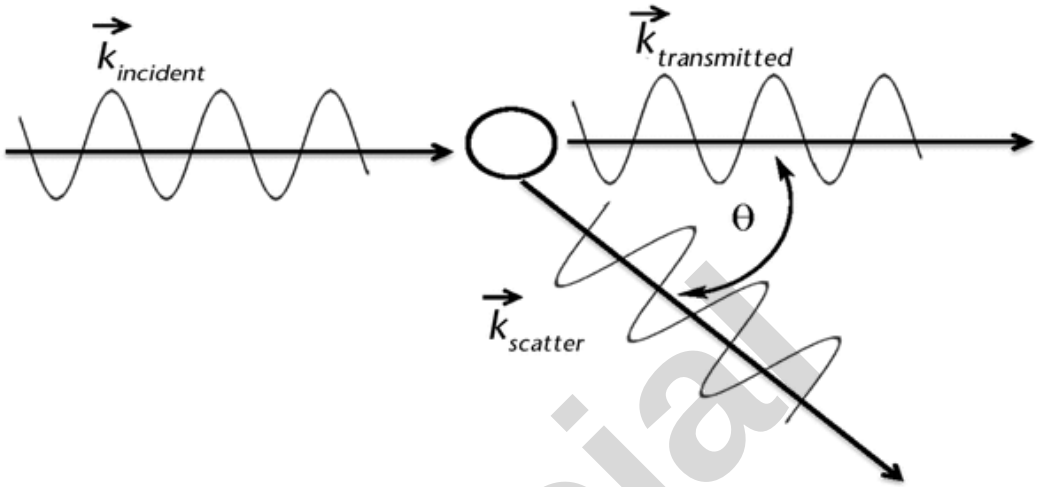


Figure 1. Scattering of light at an angle Θ.

As the EM wave interacts with the electron orbits of the constituent molecules, the orbits are perturbed periodically at the same frequency as the electric field of the EM wave. The oscillations of bound and free charges in turn generate EM waves inside and outside of the particle.

Although Maxwell's equations for an EM light wave interacting with a sphere completely describe these interactions, proper boundary conditions for electric and magnetic fields are needed to find the exact solutions for these equations, along with the values of the quantities of interest. Moreover, such solutions are difficult to find.

One of the rare exact solutions for the scattering of a plane wave by a uniform sphere is obtained from the Mie theory of scattering (*1*). Mie theory has no size limitations; it converges to the limit of geometric optics for large particles. Mie theory, therefore, may be used to describe most spherical particle scattering systems. However, difficulties in finding exact solutions for the wave equations have led to the development of approximate methods of solving the scattering problem. One class of these approximations, known as Rayleigh scattering theory, describes light scattering by particles that are small compared to the wavelength of the light (*2*,*3*). Due to the complexity of the Mie scattering solution, the Rayleigh scattering theory is generally preferred, if applicable.

When the scattering particles are much smaller than the wavelength of the light (approximately 1/20 of the wavelength or less), they are considered to be at the Rayleigh scattering regime. The EM field acting on the particle in the Rayleigh scattering regime is effectively homogenous. This is a very important approximation for biomedical optics because many of the structures from which cell organelles are built (e.g., the tubules of the endoplasmic reticulum, cisternae of the Golgi apparatus) fall into this category.

Therefore, the time for penetration of the electric field is much shorter than the period of oscillation of the EM wave. The particle behaves like a dipole and therefore radiates (scatters) light isotropically, i.e., at an equal intensity in all directions relative to the direction of the incident light. With larger particles and/or shorter wavelengths, the scattered light begins to exhibit additional interference effects from other atoms within the molecule.

In addition, the scattered light has the same wavelength as the incoming light, a process referred to as elastic scattering. Moreover, the intensity of the scattered light is independent of the particle size.

At the Rayleigh limit, the Rayleigh ratio is transformed to *Equation 2*:

$$\frac{K^* \times c}{R(\theta)} = \frac{1}{M_w \times P(\theta)} + 2A_2 \times c \qquad (2)$$

The variables are defined as follows:

$R(\Theta)$ = Rayleigh ratio

$K^*$ = optical contrast parameter = $[4\pi^2 n_0^2 (dn/dc)^2]/(\lambda_0^4 N_A)$ and $K^* = K \times (dn/dc)^2$ where $K$ is a constant, $K = \frac{4\pi^2 n_0^2}{\lambda_0^4 N_A}$

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[NOTE—$K^* \neq 0$ only when $n \neq n_0$, i.e., $dn/dc \neq 0$. Modern instruments rely on laser light, and the incident light is polarized. For unpolarized light, the constant factor 4 is replaced with 2.]

$P(\Theta)$ = form factor = $1 + (16\pi^2/3\lambda^2)(r_g^2)\sin^2(\theta/2)$ captures the decrease in scattered intensity resulting from interference between individual scattering centers within a large molecule. At a small $\Theta$, $P(\Theta) \approx 1$, because $\sin^2(\theta/2) \rightarrow 0$.

$A_2$ = second virial coefficient; describes interactions between molecules. In an ideal system/solution, $A_2 = 0$ (i.e., there are no interactions between molecules) and the intensity of scattered light will increase linearly with the concentration. Because interactions between molecules become rarer at lower concentrations ($c$), at the limit $c \rightarrow 0$ it is reasonable to assume that $A_2 = 0$.

$M_w$ = weight-average molecular weight of the analyte/solute

$r_g$ = radius of gyration, the equivalent spherical particle radius. At the Rayleigh limit, $r_g << \lambda_0$, for example in the case of visible light in which $\lambda_0 > 400$ nm, then $r_g < 15$–$20$ nm. This is true for folded proteins with $M_w < 5 \times 10^7$.

$c$ = analyte concentration

$\lambda$ = relative scattering wavelength: $\lambda = \lambda_0/n$

$\lambda_0$ = incident wavelength

$n$, $n_0$ = real refractive indices of the particle and solvent [refractive index (RI) in the absence of absorption]

$dn/dc$ = the differential RI (concentration) increment, i.e., the change of $n$ with the analyte concentration, needs to be known in advance. It should be determined separately, either from literature data or experimentally. It has a constant value for many proteins in water-based buffers and for polysaccharides:

- Proteins = 0.18–0.20 mL/g
- Polysaccharides = 0.14–0.15 mL/g

For glycosylated proteins, $dn/dc$ may not be the same for all glycoforms.

$N_A$ = Avogadro constant

# 3. APPLICATIONS

## 3.1 Low-Angle Light Scattering Determination of Molecular Weight

Depending on the experimental conditions, it is possible to further simplify *Equation 2*, thereby making it amenable to practical applications. In particular, at small scattering angles, $\Theta \rightarrow 0$, $P(\Theta) \rightarrow 1$. Therefore, at small angles, scattering is independent of $\Theta$. This observation forms the basis of LALS. Note that measurements at $\Theta = 0$ are impractical because the incident (laser) light would interfere with the measurement.

### 3.1.1 MEASUREMENTS AT LOW CONCENTRATION

Further, at the limit of low concentrations, the term $A_2 \times c$ approaches 0. *Equation 2* then simplifies to *Equation 3*, as shown below:

$$R_{LS}(\theta) = K_{LS} \times c \times M_w \times (dn/dc)^2 \qquad (3)$$

A prerequisite prior to applying the above equation is an accurate determination of the value of $dn/dc$, which is dependent on both the solvent and the sample/analyte. The value of $dn/dc$ can be determined experimentally by measuring the RI of the sample in a known solvent at various concentrations. The slope of the regression line of $n$ versus $c$ is then $dn/dc$.

For example, for a protein or complex that contains no carbohydrate, $dn/dc$ is constant (often $\approx 0.185$ mL/g) and nearly independent of the amino acid composition of the protein. However, it is advised to measure the actual $dn/dc$ for each material in order to obtain an accurate result.

### 3.1.2 MEASUREMENTS AT MULTIPLE CONCENTRATIONS

When data are available at several concentrations, the second virial coefficient, $A_2$, may be determined (see *Equation 2*).

To determine the molecular weight, $M_w$, from measurements at different concentrations and at low angles [at which $P(\Theta) \approx 1$], use *Equation 2*:

$$\frac{K^* \times c}{R(\theta)} = \frac{1}{M_w} + 2A_2 \times c \; (s)$$

By extrapolating the function $\frac{K^* \times c}{R(\theta)}$ to zero concentrations, the linear regression yields $M_w$ as well as $A_2$:

$$Intercept = 1/M_w \; Slope = 2 \times A_2$$

However, the molecular weight obtained by this approach is very sensitive to the molecular polydispersity of the sample as well as interference by impurities.

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#### 3.1.3 COMBINATION WITH OTHER DETECTION TECHNIQUES

Determination of $M_w$ by LALS stipulates that $dn/dc$ is known. To this end, the RI increment, $dn/dc$, has to be measured for several different concentrations to generate a plot of $dn/dc$ versus $c$, the $y$-intercept of which is $dn/dc$. Alternatively, when $dn/dc$ is constant, the slope of the regression line of $n$ versus $c$ is $dn/dc$. Determination of $dn/dc$ is facilitated by the coupling of LALS with an RI detector, and *Equation 4* is used for the RI signal, as shown below:

$$R_{RI}(\theta) = K_{RI}cM_w(dn/dc) \qquad (4)$$

Combining *Equation 3* and *Equation 4* leads to *Equation 5*, as shown below:

$$M_w = K'(LS)/(RI) \qquad (5)$$

where $K' = K_{RI}/[K_{LS}(dn/dc)]$ is the instrument calibration constant. The molecular weight $M_w$ is then derived from the ratio of the *LS* versus the *RI* signal. This assumes that $dn/dc$ is constant.

When $dn/dc$ is no longer constant, for example in the case of carbohydrate-containing proteins, an additional detector is needed. For samples detectable by UV, a wavelength is chosen at which ε for the carbohydrate but not the protein part is = 0. Then the ε in *Equation 6* below is effectively that of the protein:

$$UV = K_{UV}C\varepsilon \qquad (6)$$

The value of the specific refractive increment, $dn/dc$, can then be assumed to be proportional to $(RI)A/(UV)$. The outputs of the three detectors, designated *LS*, *RI*, and *UV*, respectively, are related to the molar mass of a sample according to *Equation 7*, as shown below:

$$M_w = \frac{K_{RI}^2}{K_{LS}K_{UV}} \times \frac{(LS)(UV)}{\varepsilon(RI)^2} \qquad (7)$$

ε is a material but not an equipment constant and usually must be determined separately.

### 3.2 Multi-Angle Light Scattering

In MALS, measurements are made at multiple angles (at least three). To obtain the values of the three unknowns on the right-hand side of *Equation 2* ($M_w$, $A_2$, and the mean square radius, $r_g^2$), measurements of $R(\Theta)$ at three different angles are needed in order to obtain three independent expressions of *Equation 2*. The number of angles at which measurements are made (i.e., the quantity of data collected) determines the precision (reproducibility) as well as the accuracy of the measurement.

By measuring the angular dependence of the signal, it is possible to calculate the molecular size ($r_g$) from the initial slope (at small angles) of the angular dependence in *Equation 8*, as shown below:

$$\frac{d_y}{d_x} = \frac{16\pi^2 n_0^2 r_g^2}{3\lambda_0^2 M_w} \qquad (8)$$

where $n_0$ is the RI of the solvent, $r_g$ is the root mean square radius (radius of gyration, molecular size), $\lambda_0$ is the vacuum wavelength of the laser, and $M_w$ is the molecular weight.

In addition, measuring the signal over multiple angles provides information on the three-dimensional shape of the analyte, either by using non-linear regression analysis or graphically by using Zimm plots (*4*).

### 3.3 Combination of Light Scattering with Size-Fractionation Techniques

A more robust approach to molecular weight determination is based on pre-fractionation of poly-disperse protein mixtures or analytes into “slices” of uniform molecular weight. The coupling of fractionation and detection techniques affords more information than either method can provide on its own.

Size-fractionation techniques include size exclusion chromatography (SEC), field-flow fractionation (FFF) techniques, and other separation modes. By applying light scattering in combination with another concentration detector such as RI or UV, a more complete description of the molecular weight distribution can be realized.

*Figure 2* shows an example of this for a sample of Dextran with a known molecular weight of approximately 40 kDa. The diagonal line shows the molecular weight across the SEC curve, and the entire distribution can be used to calculate molecular weight moments; the most obvious one is $M_p$, which is the molecular weight at the peak of the distribution. In addition, three other parameters can be calculated:

1. Number-average molar mass:

$$\overline{M_n} = \frac{\Sigma n_i M_i}{\Sigma n_i} = \frac{\Sigma c_i}{\Sigma c_i/M_i}$$

2. Weight-average molar mass:

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$$\overline{M_w} = \frac{\Sigma n_i M_i^2}{\Sigma n_i M_i} = \frac{\Sigma c_i M_i}{\Sigma c_i}$$

3. *Z*-average molar mass:

$$\overline{M_Z} = \frac{\Sigma n_i M_i^3}{\Sigma n_i M_i^2} = \frac{\Sigma c_i M_i^2}{\Sigma c_i M_i}$$

The value of $n_i$ is the number of molecules with molar mass $M_i$, and $c_i$ is the weight concentration of molecules with molar mass $M_i$ (as determined by the RI detector).

In the case of 40 kDa Dextran, the measured parameters are shown in *Table 1*, along with a calculation of the polydispersity ($I_p = M_w/M_n$).

**Table 1. Measured Molar Mass Parameters for 40 kDa Dextran**

| Parameter | Value |
|---|---|
| $M_n$ | 29,420 |
| $M_p$ | 34,650 |
| $M_w$ | 40,760 |
| $M_z$ | 55,960 |
| $I_p$ (= $M_w/M_n$) | 1.385 |

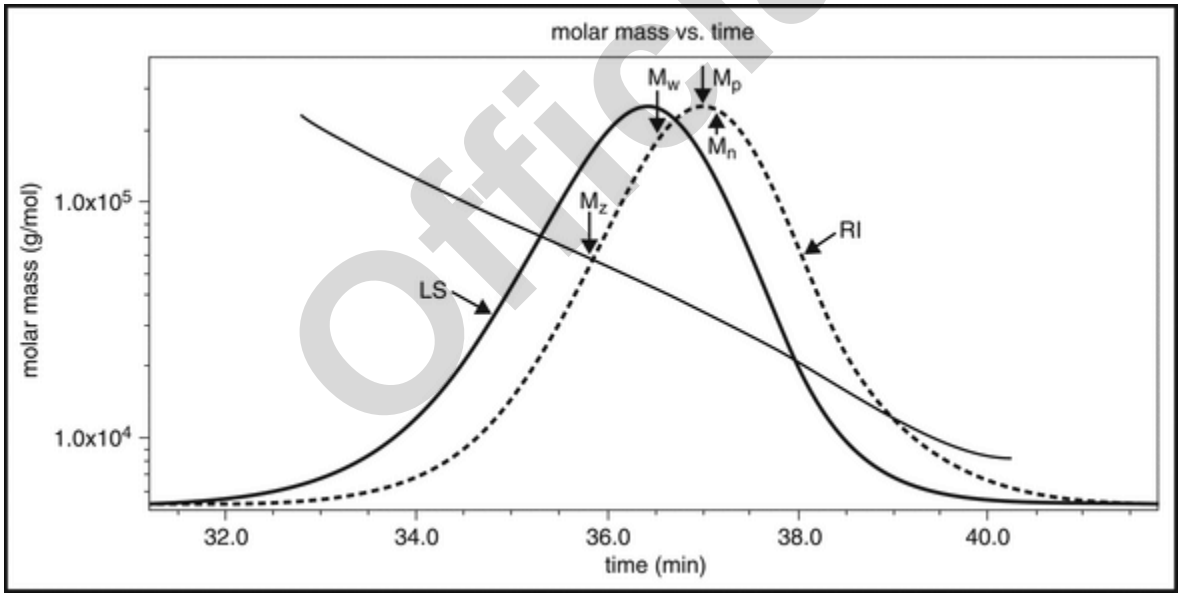


Figure 2. Molecular weight distribution for 40 kDa Dextran.

## 4. INSTRUMENTATION

Modern light scattering spectrometers use laser light because of the following advantages that lasers offer over conventional light sources:

- Reliability
- Beam collimation
- Single wavelength (scattering depends on the wavelength of the scattered light)
- Equipment durability and compactness

*Figure 3* shows a typical setup for the measurement of SLS. A monochromatic light source (i.e., a light source emitting light of only one wavelength; usually a laser) shines light on the sample. The intensity or the power of the scattered light is being measured at a known scattering angle Θ by a detector.

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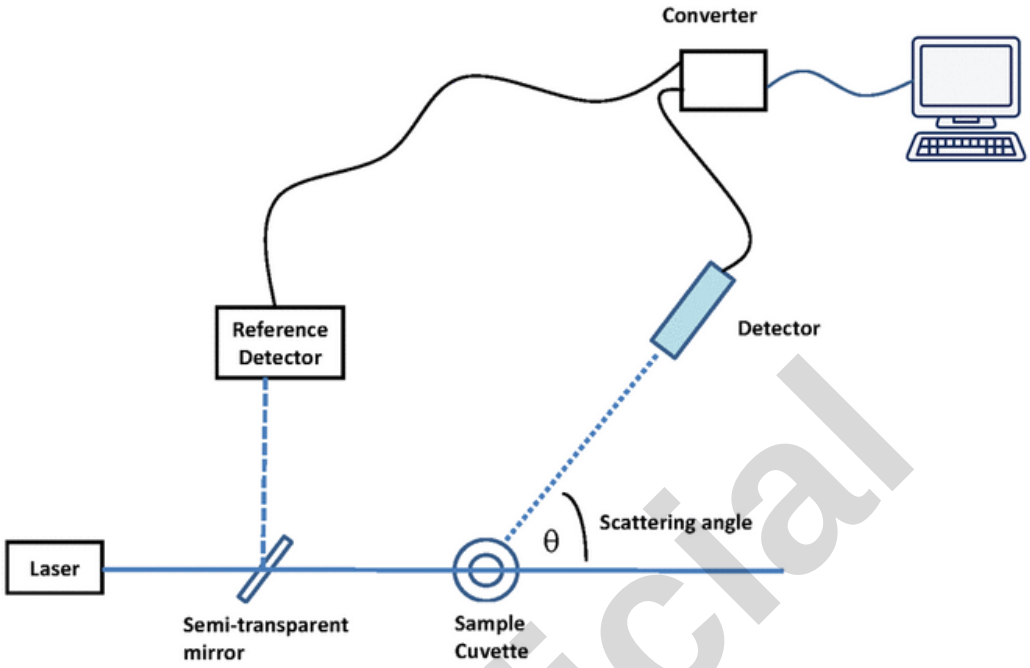


Figure 3. A typical setup of a static light-scattering instrument.

Historically, the measurements at different angles were made using a scanning photometer (single detector, variable angle). More recently, photometers containing a fixed array of 3–18 detectors have come into use.

## 5. LOW-ANGLE LIGHT SCATTERING VERSUS MULTI-ANGLE LIGHT SCATTERING

A major advantage of LALS is the exact measurement of high molecular weights (>10 million g/mol) without the principle-related errors that often occur with extrapolations, which are necessary for measurements at higher angles (but not with the use of many angles).

The major drawbacks of LALS when compared to MALS are as follows:

- **Higher noise:** Low angles are always noisier than high angles. Although using in-line filter(s) removes the scattering effects of debris from the low-angle data, it also removes much of the signal. Further, the measurement precision is roughly proportional to the square root of the number of detectors.
- **Less information:** Multi-angle data are needed to solve the Rayleigh equations.
- Inability to obtain information on molecular shape (see *3.2 Multi-Angle Light Scattering*).

LALS techniques and systems may be preferred for their simplicity and have been optimized to deal with their inherently lower information content (e.g., linearization strategies of multi-concentration data such as Zimm plots). Low-angle systems will be much cheaper and less dependent than the more complex equipment and (proprietary) fitting algorithms used in multi-angle systems. Finally, with the use of additional coupled simple detector systems such as RI and UV, the determination of molecular weight using LALS is a feasible, robust option.

A combination of the simplicity of LALS with the improved signal-to-noise ratio of higher scattering angles, offered by modern detectors, is achieved when performing measurements at $\Theta = 90°$, the angle with the highest signal-to-noise ratio. Also, except for proteins of high molecular weight, there will be no significant angular dependence of the scattering with this configuration. However, for high molecular weight proteins, the light scattering signal has to be corrected with a viscosity detector, which requires making additional theoretical assumptions that are difficult to verify for routine applications.

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## 6. PRACTICAL CONSIDERATIONS

Many SLS experiments, particularly those aimed at elucidating properties of proteins and other polymers in solution, will fall into the Rayleigh–Gans–Debye (RGD) scattering regime. *Table 2* summarizes the governing equations for four typical experimental situations: 1) low-angle scattering at a low concentration, which provides information on the molecular weight of the sample; 2) low-angle scattering at multiple concentrations, which provides information on molecular weight and solution non-ideality; 3) multi-angle scattering at a low concentration, which provides information on molecular weight and molecular size; and 4) multi-angle scattering at multiple concentrations, which provides information on molecular weight, molecular size, and solution non-ideality.

**Table 2. Most Common Governing Equations**

| | Low Concentration | Multiple Concentrations |
|---|---|---|
| Low-angle light scattering (LALS) | $\frac{K^*c}{R(\theta)} = \frac{1}{M_w}$ Output: $M_w$ only | $\frac{K^*c}{R(\theta)} = \frac{1}{M_w} + 2A_2c$ Output: $M_w$ and $A_2$ |
| Multi-angle light scattering (MALS) | $\frac{K^*c}{R(\theta)} = \frac{1}{M_w}\left(1 + \frac{q^2 r_g^2}{3}\right)$ Output: $M_w$ and $r_g^2$ | $\frac{K^*c}{R(\theta)} = \frac{1}{M_w}\left(1 + \frac{q^2 r_g^2}{3}\right) + 2A_2c$ Output: $M_w$, $r_g^2$, and $A_2$ |

Additional considerations:

- To obtain a reliable estimate for the excess Rayleigh ratio, one must use a calibration standard to obtain an "absolute" scattered intensity. Pure organic solvents (e.g., toluene) can be used for this purpose. It is important to note that the Rayleigh ratio of the solvent is a function of wavelength of the incident light. Alternatively, one can calibrate the system using a standard of known molecular weight (e.g., bovine serum albumin).
- For the above RGD equations to hold, samples must not absorb light in the wavelength range of the incident beam. Because laser light in the visible range is most common, samples should be colorless. If the samples are colored, more advanced data analysis techniques are required. Otherwise, the results will be unreliable.
- Likewise, cuvettes in the instrument systems with removable cuvette should not absorb light in the wavelength range of the incident beam. Care should be taken to ensure that the cuvette is free from smudges, scratches, or any other optical defect that could interfere with the measurement.
- Scattered intensity depends on the molecular size. Very small levels of large particles can dominate the signal and compromise the measurement. Samples should be appropriately prepared to minimize contamination from environmental or other sources. Sample preparation may include filtration, centrifugation, or other appropriate separation techniques. Care should be taken to ensure that the method of sample preparation itself does not introduce experimental artifacts (e.g., the analyte of interest adsorbing to a filter membrane). It is good practice to examine samples by dynamic light scattering to verify the absence of large contaminating species prior to conducting SLS measurements.

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***Add the following:***

# ▲⟨1430.2⟩ ANALYTICAL METHODOLOGIES BASED ON SCATTERING PHENOMENA—LIGHT DIFFRACTION MEASUREMENTS OF PARTICLE SIZE

1. INTRODUCTION
2. THEORY
3. INSTRUMENTATION
4. APPLICATIONS
5. METHOD DEVELOPMENT
6. METHOD VALIDATION STRATEGY
7. DATA INTERPRETATION AND REPORTING

## 1. INTRODUCTION

Light scattering measurements may be used for the determination of particle size distributions because light scattered from particles has an angular dependence on the size of the particles. In principle, this application makes use of the same phenomenon as the low-angle light scattering (LALS) and multi-angle light scattering (MALS) techniques. The difference in the particle size measurement method lies in the higher spatial or angular resolution needed for the detection of scattered light. Light diffraction measurements allow for the derivation of a full-size distribution, with moderate to high resolution, rather than a single characteristic size. Specialized instrumentation and software have been developed for the specific needs of this application. Additional details on method and instrument requirements are provided in *Light Diffraction Measurement of Particle Size* ⟨429⟩.

Light scattered by particles in the near-forward direction is analogous to diffraction of light through an aperture. As this application makes use of the full pattern of diffracted light, it is properly described as light diffraction. It is also often referred to as laser diffraction due to the almost universal use of laser light sources.

The range of application, speed, minimal sample restrictions, and ease of use has made this the most broadly applied and commonly used technique for particle size analysis. Distributions with a resolution on the order of 100 independent channels are common. Measurements can be performed in seconds or less. Depending on the instrument configuration, the method is applicable to sizes ranging from about 0.01 µm to greater than 3000 µm. Particles may be measured in almost all matrices through which light can be transmitted, including liquids, gases, vacuums, and, potentially, solids.

The discussion in this chapter will focus on the application of laser light diffraction to materials of pharmaceutical interest. This still covers a wide range of materials, including excipients, active pharmaceutical ingredients (APIs), intermediates, starting materials, raw materials, in-process formulations, and drug products. Materials may be liquid, solid, inorganic, organic, crystalline, amorphous, single- or multi-phase, and combinations of these materials.

## 2. THEORY

The complete description of light scattering by spheres is given by the Mie theory, which is based on the solution to Maxwell's equation for the interaction of light with an optically homogeneous sphere. This accounts, not only for light diffracted by the particles, but also for light passing through the particles, i.e., refracted light. The Fraunhofer model represents a special case of the Mie theory that does not account for refraction. The assumptions here are that the refractive index (RI) of the particle material relative to the matrix and the absorptivity are high, so that effectively no light passes through. It also assumes that particles are large relative to the wavelength of the incident light. Thus, it is most properly applicable to particles greater than several micrometers for visible light sources. Though the Fraunhofer model can be applied when these conditions are not met, it can produce known artifacts and errors. However, it can still be useful as a default when the RI is unknown. The errors are often negligible for larger particles. A more detailed description of the Mie and Fraunhofer models can be found in ISO 13320:2009—*Particle size analysis—Laser diffraction methods*.

Some limitations in Mie theory are that it assumes (among other conditions) that particles are spheres with smooth surfaces and that they are optically homogeneous. These conditions are not generally met for most real particles of pharmaceutical materials. An additional complication is that most measurements are performed with the particles in motion. Thus, non-spherical particles may present a range of aspects to the incident light axis during the measurement, in effect appearing as multiple particles. Consequences of these deviations from theory are discussed in *7. Data Interpretation and Reporting*.

RI includes both real and complex components. The real component is commonly determined by either a microscopy method (Becke line technique) or from the RI of solutions of known concentration.

In the Becke line technique, particles are immersed in oils of known RI and observed with transmitted light. As the focus is moved above the plane of the particle, a bright ring may be observed moving into the region of higher RI, i.e., either into or away from the particle boundary. Using different oils, the particle RI may be determined well within the needed accuracy. Using polarized light, the RI of the individual crystalline axes may also be determined. In that case, an averaged value may be used in the Mie model.

In the solution method, an Abbe refractometer is used to measure the RI of a solution for which the concentration of the material is known. Extrapolating the solution RI, from a series of known concentrations of the material, to that of the pure substance yields a good approximation. This may require knowledge of the true density of the material.

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The imaginary component of the RI is the absorptivity value for the wavelength of light used in the light diffraction measurement. For most non-colored materials, this will be very low. This value is not typically measured.

For both components, the values should be considered as initial approximations. Although the Mie model results are not especially dependent on the chosen values, it can sometimes yield unrealistic results. The sensitivity of the measurement to small changes in the assumed values should be assessed. This requires only the recalculation of an existing measurement using different RI values. Final RI and absorbance values should be chosen to avoid being too near an area of high sensitivity. The resulting size distributions should always be evaluated to assure that they realistically represent the sample.

## 3. INSTRUMENTATION

Instrumentation consists of light source(s), a measurement region, optics to collect the scattered and unscattered light, multi-element detector(s), and necessary hardware and software for the computations. Often more than one light source may be used. Light sources are usually lasers, though not always. Sources of different wavelengths are mainly used to extend the size range, especially to better handle samples in the submicron region.

The measurement region is where the sample is measured. Depending on the specific application, this is typically in a well-defined position relative to the detector and optics, and comprises windows that physically contain the sample dispersion. Associated sample dispersion equipment is also part of the instrument. (An exception would be for instruments specifically designed for sprays or on-line use.)

The detectors must provide adequate spatial resolution needed to best define the diffraction pattern. To best resolve the variations in diffraction patterns in the applicable size range, most elements are within a relatively low forward angle. Additional detectors at wider angles are often incorporated to extend the range to smaller sizes. Depending on the instrument design, different lenses may be used for specific size ranges. This is not common with the reverse Fourier configuration.

Further descriptions and illustrations of common instrumentation configurations are shown in ⟨429⟩ and ISO 13320.

## 4. APPLICATIONS

In principle, the light diffraction technique may be applied to any particulate system that allows for sufficient transmission of light: solid in liquid, liquid in liquid, gas in liquid, solid in gas, and liquid in gas. However, it is most commonly applied to solids dispersed in either liquids or gas (air), and to sprays of liquids or solids in air.

For solid powders, dispersion in either liquid or gas both offer advantages and disadvantages. Dispersion in liquids generally allows better control of the degree of dispersion of the material, which is important for understanding the desired characteristics of the powder. It also offers the advantage of being able to sample the dispersion's particles from the instrument dispersion outflow for observation by microscopy. Microscopy permits observation of the particles in the same state they exist during the light diffraction measurement. Obtaining particles from a dry dispersion as they exist in the dispersion would be difficult, if not impossible.

Disadvantages of liquid dispersion include possible changes in the material characteristics due to dissolution or flocculation. Selection of the dispersant system, including use of surfactants, is critical to developing a suitable method.

The advantages of dry or air dispersion of solid powders include the absence of solubility concerns and minimal sample preparation. A significant disadvantage is decreased control of particle dispersion. Though a range of dispersion energy can be applied by controlling the shearing air pressure, there is typically a reduced ability to separate deagglomeration from particle fracture processes. The occurrence of artifacts appearing as larger particles has also been observed, possibly due to material flaking from the surfaces of the dispersion unit. Larger sample sizes may also be needed.

Dry dispersion also requires adequate filtration and containment of the vacuum exhaust. Highly potent or toxic materials may not be suited for this analysis.

The use of dry dispersion is most appropriate for materials used in a similarly dispersed state, for example, the analysis of liquid or powder sprays from drug products and devices.

## 5. METHOD DEVELOPMENT

The fundamental step in starting the development of a method is to define the desired state of dispersion for the sample. Almost all powder samples will exhibit some degree of agglomeration. Attractions due to dispersion or van der Waals forces, electrostatics, or physical attachment, etc., are present in any sample. The strength of the attachments varies widely and depends on the particles' size, composition, and prior processing.

The terms agglomerate and aggregate will not be defined here. Within the scope of pharmaceutical materials, there can be an essentially continuous range of particle association strengths. Agglomerates or associations can range from those that are very weak and easily disrupted with minimal agitation, to those that are very strong and physically bound particles. Hence, the terms agglomerate and aggregate are sometimes used interchangeably, depending on the process used to create the material.

It is critical that the intended use of the particle size information be understood to determine the appropriate degree of dispersion. For example, measurement of primary, i.e., fully deagglomerated particles may be more informative for understanding the particle formation process or inherent dissolution behavior of a powder. Alternatively, including agglomerates in the measured distribution may be more useful for understanding issues of content uniformity and powder flow. A good understanding of the intended use of the material and expected forces encountered during further processing will help to determine the appropriate dispersion. Optical microscopy is an important tool to characterize the sample dispersion.

A typical dispersion study (assuming a solid in liquid dispersion) would first consist of analyzing a suspension created with minimal dispersion energy, e.g., by stirring or vortexing. Subsequent analyses of the suspension are performed after application of increasing dispersion energy, e.g., with added sonication times. If this is not possible with a single preparation, multiple preparations may be used. At each stage, the stability of the sample should be examined, e.g., with a stirring study. Comparing

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the resulting distributions with consideration of the sample behavior and supporting microscopy reveals the suitability of the dispersion conditions.

For dry dispersions, analyses using different shear air pressures and feed rates are done. This is often referred to as an air pressure titration study.

Once the intended state of dispersion is determined, method development will focus on how to best achieve and maintain that state reproducibly and reliably. For powders, dispersion either in air or a liquid is necessary. For liquids, the dispersant must support the dispersion of the particles to the desired extent, with no dissolution or other physical change or re-agglomeration. Though dissolution is not an issue for air or gas dispersion, achieving the proper degree of dispersion may be a greater issue.

The amount of incident light scattered or absorbed by the sample, expressed in terms of obscuration, transmittance, or optical concentration range of the sample, is related to the sample concentration and serves as a convenient proxy for relative concentration. It is expected that this will affect the measured size, though a range of values where the sensitivity of the results is low should be possible. This is generally a function of the overall instrument design and not material-specific. The lowest concentration must be sufficient to minimize the effects of noise, and the upper limit is needed to minimize the effects of multiple scattering. Suitable ranges may be recommended by the instrument manufacturer. Restricting the range may improve reproducibility.

## 6. METHOD VALIDATION STRATEGY

Chapter ⟨429⟩ provides guidance to demonstrate the required accuracy and precision for the instrument and methods of the laser light diffraction technique.

The International Council for Harmonisation (ICH) guidelines on method validation do not fully account for characterization methods such as particle size analysis. However, the fundamental concerns about method precision and robustness still apply.

As described in *Validation of Compendial Procedures* ⟨1225⟩, precision should be addressed by assessing repeatability and intermediate precision. Additional assessments of reproducibility could include multiple instruments and laboratory location.

The robustness assessment should address the sensitivity of the method to other likely sources of variation and should establish a range of acceptable conditions. Factors to consider will depend on the type of dispersion. For liquid dispersions, in addition to the classic stability considerations, this should include an assessment of the stability of the dispersion in the instrument over potential analysis times. Sample concentration, as indicated by obscuration, is an important factor, as it is most likely to have some effect. Other typical factors could include stability of the predispersion, recirculation or stir rate, dispersion energy, equilibration or stir time before initiating the measurement, and measurement time. For dry dispersion, measurement time, sample amount, air pressure, and feed rate to achieve target obscuration may be most important. These factors may be examined using an experimental design. For example, two or more factors could be evaluated by using high and low values at about the nominal value and determining the main effects.

Overall, the greatest sources of variations in the light diffraction technique are the sample properties, the sample preparation, and sampling. These far exceed any variations in the measurement process.

Given the fundamental aspects of the measurement process and the technique-dependent definition of size, the assessment of the accuracy is not simple. Comparison to an orthogonal technique is not always possible, especially given the different effects the particle shapes may have. A subjective assessment using optical or electron microscopy is recommended to compare the range of particle sizes observed with the reported light diffraction results.

## 7. DATA INTERPRETATION AND REPORTING

The ease of acquiring particle size distribution information by light diffraction belies potential pitfalls in its interpretation. Other than for perfect spheres, the concept of accuracy in describing the size of a single particle, much less a collection of varied particles, is elusive. The chosen optical model, particle shapes, and optical properties of the sample all influence the results of the measurement.

Calculations are based not on the sizing of any single particle but rather on the average diffraction pattern from the ensemble of particles passing through the measurement region over time. During this time, the particles can present multiple orientations.

The calculated size distribution is also not directly calculated from the diffraction pattern. Rather it is the result of comparing the cumulative theoretical patterns of individual size fractions, calculated using the chosen optical model, to the measured diffraction pattern. The relative proportions of the individual size are varied until the resulting calculated pattern best matches the measured one.

Thus the size distribution of a sample is best described as the size distribution of spheres with defined optical properties that produces a diffraction pattern equivalent to that of the sample. Interpreting the measured size distribution in terms of specific physical dimensions, e.g., length or width, is a mistake. Doing so has important implications for the interpretation of the reported size distribution, especially when considering the effects of particle shape on the results.

In some cases, the distribution of the modeled spheres may be restricted to a certain defined shape, e.g., a normal, log-normal, or Rosin–Rammler distribution. This was more common during the early stages of the instrument development, when computational abilities were more limited. This approach is not commonly used today, though the option may be available in the instrument software.

Because of how the results are derived, the particle size distribution from light diffraction measurement is best considered as the cumulative fraction of volume (or mass, assuming a uniform density) that is less than the defined sizes. This cumulative distribution is the basis for deriving the most commonly used statistics. These include percentile sizes such as ×50 and ×90 (the sizes for which 50% and 90%, respectively, of the sample volume are less than), as well as size quantiles such as Q10 (the fraction less than 10 µm). These are derived easily by interpolation from the cumulative distribution. For other statistics, such as various mean sizes, central values of the individual size bins must be assumed.

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In plotting particle size distributions, if represented as a scatter plot, it should be realized that it is more properly considered as a histogram, with the relative height of each band representing the volume fraction within the size band, i.e., between the upper and lower limit. Unless the bins are evenly distributed on the size scale, the distribution density function versus size is what should actually be plotted. The distribution density is the volume fraction divided by the width of the size bin. This allows for the properly proportioned representation of a distribution when the size bands are of different widths. It will also permit measurements from different sources, with different numbers of bins to be overlaid on the same plot. One caution is that this quantity will change if the scale basis of the size axis is changed, e.g., from linear to logarithmic.

Further descriptions of particle size distribution statistics can be found in ISO 9276-1:1998—*Representation of results of particle size analysis—Part 1: Graphical representation* through ISO 9276-6:2008—*Representation of results of particle size analysis—Part 6: Descriptive and quantitative representation of particle shape and morphology*.▲ (USP 1-Dec-2019)

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***Add the following:***

# ▲⟨1430.3⟩ ANALYTICAL METHODOLOGIES BASED ON SCATTERING PHENOMENA—DYNAMIC LIGHT SCATTERING

1. INTRODUCTION
2. APPLICATIONS
3. THEORY
    3.1 Correlation Function Analysis
4. INSTRUMENTATION
5. MEASUREMENT
    5.1 Factors That Affect the Measurement
    5.2 Sample Preparation
    5.3 Test Procedures
    5.4 Data Interpretation
6. ADVANTAGES AND LIMITATIONS
7. ADDITIONAL SOURCES OF INFORMATION

## 1. INTRODUCTION

Dynamic light scattering (DLS)—also referred to as quasi-elastic light scattering (QELS)—is a nondestructive, light-scattering technique for particle characterization of colloidal dispersions, biological solutions, and solutions of macromolecules. DLS has also been referred to in the literature as photon correlation spectroscopy (PCS), which is one of the DLS measurement methods such as frequency analysis and cross-correlation spectroscopy. It is often used for measuring average particle diameter and the broadness of the size distribution of submicron-sized particles that are dispersed in a liquid and are free from the influence of sedimentation. Compared to other light-scattering techniques, DLS can provide size information in the nanometer (nm) range, which is out of the primary range of static light scattering (e.g., laser diffraction). In addition, DLS can also provide information on the stability of suspensions/emulsions as it is highly sensitive to aggregation and to the behavior of suspensions/emulsions under different dispersing conditions.

## 2. APPLICATIONS

Typical applications for particle sizing by DLS include those measuring any particle dispersed in a liquid (suspensions or emulsions) that is influenced by Brownian motion. These applications include, but are not limited to:

- Measuring micronized water insoluble active pharmaceutical ingredients (APIs) for injectable, oral, or other use
- Measuring droplet size of oil in water emulsions where the API is dissolved in the oil
- Monitoring liposome formation to ensure a desired size is attained
- Monitoring the quality and uniformity of a suspension used in a coating process, such as time-release coatings
- Monitoring the size of macromolecules, such as proteins or protein conjugates
- Any additional step in a process or finished product where a suspension is used

## 3. THEORY

The application of DLS applies the concept of random motion of particles in a colloidal system, commonly referred to as Brownian motion, in the particle size measurement. Dispersed particles scatter incident light proportional to the radii and inversely proportional to the light wavelength, indicating that the large particles scatter much more light than the small particles, and the short wavelength light is scattered stronger than the long wavelength light from the same particle. If the diameter of the particle is less than 1/10th of the wavelength of the incident light, the scattering is commonly referred to as Rayleigh scattering. However, if the particles are greater than 1/10th of the wavelength of the incident light, then the scattering process can be described by the Mie theory. In that case, the scattering is angle dependent and the scattered light is more intensely concentrated toward the direction of the incident light.

As the particles scatter light in DLS, the time-dependent fluctuations of the intensity of the scattered light resulting from random motion of the particles is measured. The fluctuations of intensity in scattered light are correlated with short decay intervals ($\tau$). One of the approaches to mathematically describe the time variation of such a random motion is the application of the correlation function. For monodisperse systems, the intensity autocorrelation function (ACF) is expressed as a mono-exponential function [$G(\tau)$] as shown in *Equation 1*:

$$G(\tau) = 1 + be^{-2D_t q^2 \tau} \quad (1)$$

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where $b$ is a constant, depending upon the instrument and settings of optics; $D_t$ is the translational diffusion coefficient; and $q$ is the scattering vector.

The scattering vector is further expressed as shown in *Equation 2*:

$$|q| = 4\pi n_0/\lambda_0 \cdot \sin(\theta/2) \quad (2)$$

where $n_0$ is the refractive index (RI) of the medium, $\lambda_0$ is the light wavelength in a vacuum, and $\theta$ is the scattering angle.

The intensity autocorrelation function [$G(\Pi)$], often expressed as $G_2(\tau)$, is expressed as a function of the field correlation function, $G_1(\tau)$, as shown in *Equation 3*:

$$G_2(\tau) = 1 + G_1(\tau)^2 \quad (3)$$

In DLS, a correlation is generated where raw correlation function (RCF) is plotted against delay time ($\tau$) as shown in *Equation 4*:

$$\text{RCF} = G_2(\tau) - 1 = G_1(\tau)^2 \quad (4)$$

$D_t$ is calculated by data analysis of the autocorrelation function using *Equation 1*.

The diffusion coefficient ($D$) is then related to the radius ($R$) of the particles by means of the Stokes–Einstein equation as shown in *Equation 5*:

$$D = k_B T/6\pi\eta R \quad (5)$$

where $k_B$ is the Boltzmann constant, $T$ is the absolute temperature, and $\eta$ is the viscosity of the dispersion medium. The size of spherical particles is calculated from the Stokes–Einstein equation if the viscosity of the medium is known at a given temperature. As can be seen from *Equation 5*, temperature is an important factor impacting the diffusion coefficient both directly ($T$ in the numerator) and indirectly as viscosity ($\eta$ in the denominator) is an inverse function of temperature. The particle size determined by DLS is the apparent hydrodynamic radius, $R_h$(app), and can change with the particle concentration ($c$) as shown in *Equation 6*, where $D(c)$ indicates that the measured diffusion coefficient is a function of $c$:

$$R_h(\text{app}) = \frac{k_B T}{6\pi\eta D(c)} \quad (6)$$

## 3.1 Correlation Function Analysis

Intensity correlation functions are commonly normalized so that $G(\infty)$ equals 1. Normalization of autocorrelation coefficient results in a baseline limit of 1 for $G(\infty)$ for monochromatic light, with an upper correlation limit of 2 for $G(0_0)$.

The time dependence of the fluctuation intensity is typically evaluated by employing a digital correlator. Each data point in the measured intensity correlation function is the average of the multiples of the number of photons counted in one sampling time and another sampling time with an interval between these two sample times called delay time. The entire intensity correlation function is formed by varying the delay time. Correlation is high at short delay times, and, as particles diffuse over time, correlation reduces to 0. The exponential decay of the correlation function is a characteristic feature of the diffusion coefficient.

The $z$-averaged decay rate ($\Gamma$) and the polydispersity index (PI) are commonly obtained by fitting a polynomial of third degree to the logarithm of the intensity correlation function per the cumulant method.

The decay rate is directly related to the diffusion coefficient ($D$) as shown in *Equation 7*:

$$\Gamma = q^2 D \quad (7)$$

where $q$ is the modulus of the scattering vector.

The cumulant analysis determines the mean diameter and a distribution width only. For polydisperse samples, more complex methods such as the CONTIN method are required in order to determine the size distribution function. Modern dynamic light scattering instruments perform cumulant analysis and size distribution function analysis automatically. The quality of the result, however, depends significantly on the quality of the data and the constraint settings of the fitting procedure.

The area under each peak of the intensity particle size distribution is proportional to the relative scattering intensity of each group of particles. The scattering intensity is weighted by molecular weight and radius; thus, the intensity distribution is more skewed toward the larger particles. Therefore, a change of the intensity to a mass or volume distribution can be achieved by means of the Mie theory. The underlying assumptions are as follows:

1. All particles exhibit an equal and uniform density.
2. The particles are spherical.
3. No inaccuracy exists in the intensity particle size distribution.

Although the first two assumptions can be applicable, the third assumption is not accurate due to the ineffective correlogram fitting in the DLS method, indicating that, no matter how monodisperse a sample is, the particle size distribution as measured by the DLS method will have intrinsic polydispersity and will not have a single band distribution.

Historically DLS studies were usually performed at an angle of 90°, which required very dilute samples to avoid multiple scattering phenomena and to have enough scattering intensity. With current instruments, it is now possible to perform DLS measurement on higher concentrated samples, up to 40% (w/v), with the advancement of backscatter technology.

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The DLS technique provides size and polydispersity information at relatively low to moderate concentrations. At a high concentration DLS measurements become more challenging. These issues have been resolved to a great extent by the recent advancements in DLS backscattering and 3-D cross-correlation. Backscattering measurements allow detection of scattered light at a higher angle (typically 160°–180°), which is measured close to the cuvette wall. In 3-D cross-correlation technology, the sample is analyzed by two detectors simultaneously at the same scattering vector. Such measurements allow cross-correlating the signals from both detectors and, thereby, suppressing the contribution of multiple scattering.

Dynamic light scattering is typically used for measurements in the submicron range, although this technology may measure particle size up to a few microns. The upper size limit of DLS depends on sample density, implying that the upper limit of measurement is the point where the sedimentation of the particles overcomes the diffusion process. The lower limit of particle size measurement depends on the excess scattered light produced by the scattering particles relative to that produced by the suspension medium. In addition, sample concentration, relative RI of the particle and medium, laser power and wavelength of light, and detector configuration will affect the lower size limit measured by DLS. The major advantages of this method include its nondestructive nature, small amount of sample requirement, and ease of sample preparation. The accuracy of results determined by DLS may be compromised by sample contamination during the sample preparation. In addition, air bubbles and silicone droplets may be erroneously regarded as particles, leading to inaccurate results. Measurements of nonspherical particles can also be a challenge for DLS and may lead to inaccurate estimates since DLS assumes that particles are spherical and the diffusion is only translational. Nonspherical particles, especially very large and dissymmetrical (rod-like shape) ones, have also a rotational diffusion component in addition to the translational one. The rotational diffusion component introduces a time-dependent anisotropic effect to the measured fluctuations of the scattered light, which results in inaccurate size estimates. Lastly, the accuracy of the results of DLS measurements is affected also by changes in temperature and viscosity of samples.

## 4. INSTRUMENTATION

The instrumentation generally comprises a laser as the light source, sample holder or cell, suitable optics, detector, correlator or spectrum analyzer, and a computation unit. Several configurations of signal detection and analysis are possible (see *Figure 1*).

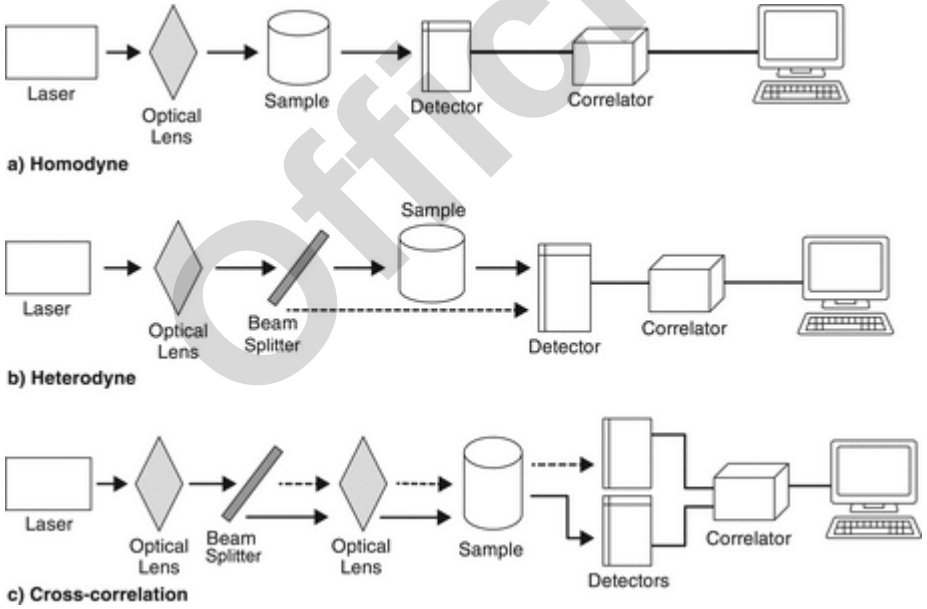


Figure 1. Typical optical arrangement for DLS.

Homodyne detection uses only the light scattered from the incident beam. In heterodyne detection, a portion of the incident beam is mixed with the scattered light as a reference. A cross-correlation arrangement involves the combination of two simultaneous homodyne measurements.

A laser light source provides a monochromatic, coherent light source to illuminate the sample. The laser is generally polarized with the electric field component perpendicular to the plane of the incident and scattered light paths, usually vertically.

The sample is contained in a holder or cell, which must be capable of maintaining the temperature of the sample to within ±0.3°.

Optics focus the beam to define the measurement region and collect the scattered light for analysis. These may be external to the sample cell or, with fiber optics, may be inserted into the sample dispersion. A polarizing analyzer may be included to maximize transmission of the vertically polarized light.

Scattered light collected by the optics is measured by a detector capable of responding to the temporal fluctuations of the light intensity and providing an output proportional to the intensity. The detector is positioned at a fixed angle relative to the incident light, generally between 90° and 180° (side scattering and backscattering). Some instruments that include the optics to perform zeta potential determination allow for size to be determined by DLS using a forward-scattering angle, such as 10°–15°.

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The time-dependent signal from the detector is processed by the correlator or spectrum analyzer. Further computations are then carried out to determine the results: the average hydrodynamic diameter and the PI or the particle size distribution depending on the analysis methods used.

Further details about the recommended instrumentation and possible configurations may be found in ISO 22412:2017—*Particle size analysis—Dynamic light scattering (DLS)* or in *Particle Size Analysis by Dynamic Light Scattering* ⟨430⟩.[1]

As the measurement depends on first principles, including knowledge about the suspending fluid, there is no calibration required. However, proper instrument function should be confirmed periodically using suitably characterized standard particles. Standards may be available from the instrument vendors or from a national standards organization such as the National Institute of Standards and Technology. Requirements for the instrument suitability are specified in ⟨430⟩.

## 5. MEASUREMENT

### 5.1 Factors That Affect the Measurement

**Concentration:** The concentration must be sufficiently high to maintain constant time-averaged intensity but at the same time very low to prevent multiple scattering and/or particle–particle interaction.

**Measurement duration:** The typical measurement time is from 1–10 min.

**Multiple scattering:** At high concentrations, scattered photons may undergo multiple scattering, which results in a correlation function that decays faster and results in an apparent smaller particle size. Therefore, sample concentrations should be low enough that the optical paths remain to avoid multiple scattering.

**Particle–particle interaction:** In order to obtain an accurate measurement, the signal from all scattering particles freely experiencing Brownian motion within the scattering volume need to be measured. However, it may be difficult to always ensure the above requirement since at certain concentrations a portion of the scattering particles may be subjected to a constrained Brownian motion.

**Sample preparation:** It is absolutely critical to avoid large particles, commonly called “dust”, to avoid inaccurate signals and erroneous particle size measurements.

**Size range:** The size range commonly determined by DLS is 2–2000 nm. The instrument optics and engineering determine the lower size limit, while the upper limit is determined by the sedimentation and number fluctuations due to the limited number of large particles in a small scattering volume.

**Stray light:** Stray light must be minimized under all conditions.

**Temperature and Viscosity:** Temperature and viscosity of the sample must be controlled within a certain range in order to have an accurate measurement and reliable results.

### 5.2 Sample Preparation

As DLS monitors the intensity fluctuations in the light scattered from the sample due to the Brownian motion observed, different liquid environment conditions will affect the observed particle size. The conditions include: pH, conductivity, surfactants, or other chemicals or materials dissolved in the base liquid. This is why size data from DLS will differ from microscopic images taken with scanning electron microscopy or transmission electron microscopy. Microscopic characterization of nanomaterials involves drying the sample and placing it under vacuum while DLS requires the material to be dispersed in a liquid. DLS will give information on how the nanomaterial will behave in a liquid and report this behavior in terms of size.

It is best practice to analyze a sample suspension that is as close as possible to the concentration and conditions where the suspension will be used. In cases where the sample may be too concentrated, it is important to 1) perform dilutions with the same base liquid and 2) perform the dilution by mass. The solvents/buffers used as base liquid must be properly filtered. This will ensure that the DLS results are repeatable and that the data from any subsequent samples analyzed will be comparable. If the dilution is not performed in this manner or is performed differently each time, the results may become unreliable.

### 5.3 Test Procedures

The test procedures in a typical DLS instrument take into consideration the following aspects:

1. **Type of cell/cuvette:** Typically plastic or glass. (Quartz is not necessary unless specified by the manufacturer and is reserved for work in the ultraviolet light range, whereas most DLS instruments operate in the visible light range.)
2. **Temperature setting:** Typically controlled by a Peltier thermoelectric controller.
3. **Equilibration time:** If the sample is not at room temperature prior to analysis or if the temperature setting is set higher or lower than room temperature, it is important to allow the suspension temperature to equilibrate.
4. **Accumulation time:** The number of accumulations represents the number of individual measurements of the light fluctuations to build the ACF. Increasing the number of accumulations increases resolution and quality of the ACF, while decreasing the number of accumulations decreases analysis time. Most manufacturers have a recommended accumulation time that is a balance of resolution and analysis time.
5. **Selection of base liquid:** This is the base liquid with no particles present. In most cases, this is simply water. It is not necessary to add pH or salt concentration information since these characteristics are considered additives or conditions of the water that will have a negligible effect on viscosity and RI.

[1] This chapter will be published in a future *Pharmacopeial Forum* (*PF*) issue.

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6. **Sample ionic strength optimization:** To obtain a particle diameter close to its real dimension the PCS measurement has to be performed at a suitable ionic strength supplied by the presence of sufficient electrolyte (e.g., by a monovalent salt). A conductivity of greater than 1 millisiemens/cm is generally recommended. The stability of the suspension has to be closely monitored during the ions' addition to avoid the colloidal system collapse when the measurement is performed.
7. **Number of repetitions:** This is the number of times to repeat the analysis on the different aliquot from the same sample.

Additional procedures, calculation algorithms, and other items can be added based on the availability of the features by the instrument manufacturer.

### 5.4 Data Interpretation

The primary size result reported by DLS is the average hydrodynamic diameter (cumulant average or *z*-average) and the polydispersity index (PI). The PI is a measure of the broadness of the size distribution. For monodispersed materials, these two values are sufficient to evaluate the size of the particles.

For polydisperse systems, it is possible to calculate a particle size distribution using a Laplace inversion of the ACF and distribution algorithms. In practice, the general acceptance criteria for a Laplace inversion is that the PI is less than 0.7. The typical size distribution algorithms available are CONTIN and non-negative least squares (NNLS). Other algorithms may be available from the instrument manufacturer. The primary output for the size distribution data is an intensity weighted distribution. Number and volume-weighted distributions can be calculated from the intensity distribution. However, converting to number distribution should be done with caution.

Due to the low resolution of DLS and the error from the mathematical conversion, the values of $D_{10}$, $D_{50}$, and $D_{90}$ should not be used to describe the distribution results.

## 6. ADVANTAGES AND LIMITATIONS

DLS gives size information in the nanometer range, which is difficult to achieve using some other particle analyzing techniques. DLS also gives information on the stability of a suspension as well as interactions with the liquid environment, as the size determined is the hydrodynamic diameter.

DLS is not a very high-resolution technique for broad size distributions. The presence of any larger particles will influence the size calculation at the smaller end of the distribution. In some cases, filtration of the aliquot under analysis may be needed to ensure aggregates, agglomerates, or large particles are removed.

The assumption is made that the particles in the suspension are only in Brownian motion and no other influences. If the particles are actively flocculating or settling, this motion can be detected and the instrumentation will assume that it is Brownian motion and give inconsistent results. This is why the upper size limit is dependent on the density of the particles and the viscosity of the liquid. It is not always correct to assume that using a higher viscosity liquid will limit settling and yield better results because this will also limit Brownian motion.

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# ▲⟨1430.4⟩ ANALYTICAL METHODOLOGIES BASED ON SCATTERING PHENOMENA—ELECTROPHORETIC LIGHT SCATTERING (DETERMINATION OF ZETA POTENTIAL)

1. INTRODUCTION
2. THEORY
3. INSTRUMENTATION
4. APPLICATIONS
5. DATA ANALYSIS
    5.1 Calculation of Electrophoretic Mobility
    5.2 Calculation of Zeta Potential
6. DATA INTERPRETATION
7. ADDITIONAL SOURCES OF INFORMATION

## 1. INTRODUCTION

Zeta potential is a parameter that is primarily used to characterize the stability of suspensions and emulsions. It can also be used to study surface morphology and adsorption on particles and other surfaces in contact with a liquid. Zeta potential is indirectly determined by the appropriate theoretical models from experimentally determined parameters, such as electrophoretic mobility. Electrophoretic light scattering (ELS) is the most general and common way to determine zeta potential of colloidal systems.

## 2. THEORY

A suspension of particles that has a given electrokinetic charge is placed in a cell that has a pair of electrodes placed apart at a known distance. A potential of known strength is generated between the electrodes and, by electrophoresis, results in the particles migrating toward the electrode of opposite charge. To detect and quantify this migration, particles are illuminated by a laser and the Doppler shifts of the scattered light from particles are recorded. The difference between the frequencies of the incident light and the scattered light is used to determine the velocity and direction of the particle migration. The particle velocity is then used to calculate the electrophoretic mobility by taking into account the strength of the electric field. Then, taking into account the viscosity and permittivity of the base liquid, the electrophoretic mobility is used to calculate the zeta potential.

## 3. INSTRUMENTATION

ELS, zeta potential instruments are light-scattering instruments that have either a reference beam optics alignment (most common) or a cross-beam optics alignment (less common). An example of a typical small-angle, light-scattering, zeta potential instrument (from ISO 13099-2:2012[1]) is shown in *Figure 1*.

[1] ©ISO. This material is reproduced from ISO 13099-2:2012 with permission of the American National Standards Institute (ANSI) on behalf of the International Organization for Standardization. The complete standard can be purchased from ANSI at webstore.ansi.org. All rights reserved.

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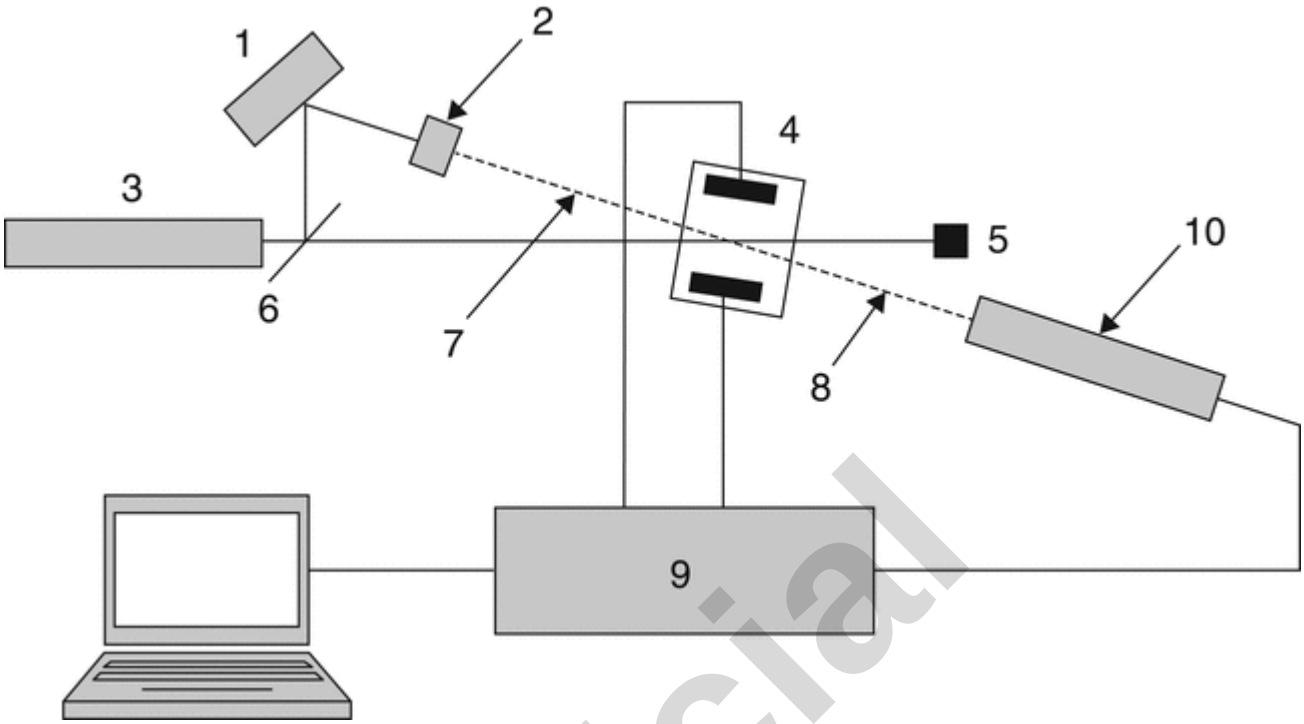


Figure 1. An example of a small-angle light scattering zeta potential instrument with reference beam optic arrangement.

| Key | |
|---|---|
| 1. Optical modulator | 6. Beam splitter |
| 2. Attenuator | 7. Reference beam |
| 3. Laser | 8. Scattered or reference light |
| 4. Sample cell with electrodes | 9. Processor |
| 5. Beam stop | 10. Photoelectric detector |

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The cross-beam optical alignment instruments differ from the reference beam instruments in the fact that the laser beam is split into two beams of equal intensity that cross symmetrically (at a fixed angle between them) enter the sample cell from the same side.

A typical zeta potential measurement instrument is comprised of the following components:

1. A sample cell to contain the test suspension between two electrodes so that an electric field can be applied.
2. A suitable laser to illuminate the particles within the sample.
3. A suitable detector to capture the scattered light and the frequency shifts in the scattered light when the electric field is applied.
4. A software and computer system to process the collected scattering data and compute the mobility and zeta potential using appropriate models and algorithms.

The primary experimental technique used to determine zeta potential of colloidal systems is ELS. In this technique, a suspension is placed between two electrodes and an electric field is generated causing the ions and charged particles to migrate toward the oppositely charged electrode. When a laser is focused on the sample, this migration causes a Doppler shift in the frequency of the light scattered by the solution. An intensity or spectrum analysis of the shift in frequency is used to determine the electrophoretic mobility of the particles. Electroosmosis, the migration of the liquid along the sides of the cell flowing in the opposite direction from the particle migration, may affect the electrophoretic mobility measurement. A mechanism to correct for electroosmosis is often used during the measurement.

In cases where the Doppler shift is very small, the frequency analysis may not have enough resolution to determine the change. In such cases, phase analysis light scattering-based systems (PALS) can be used to perform the phase analysis of the shift. A typical schematic example of a PALS-based instrument is shown in *Figure 2*.

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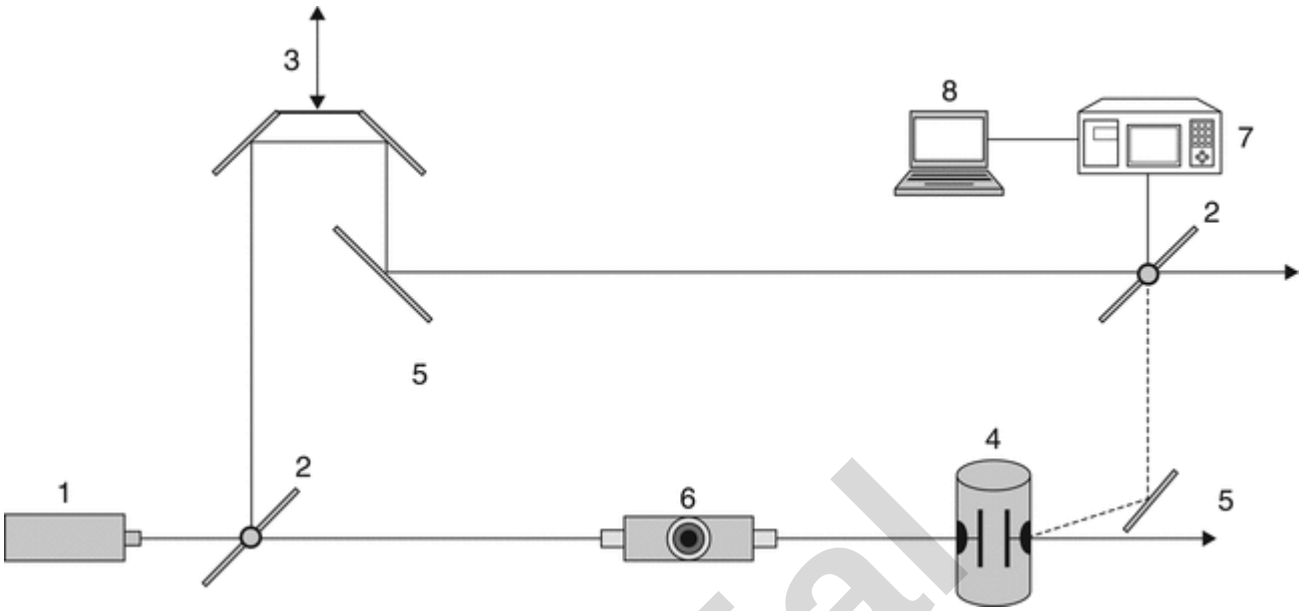


Figure 2. An example of PALS-based arrangement set-up of a zeta potential instrument.

| Key | |
|---|---|
| 1. Laser | 5. Mirror |
| 2. Beam splitter | 6. Variable attenuator |
| 3. Pair of mirrors | 7. Scattering detector |
| 4. Sample cell with electrodes | 8. Computer |

The primary advantage of PALS is that it has higher resolution and can therefore detect very small Doppler shifts. This is typically needed for suspensions in nonpolar solvents or in aqueous solvents of high ionic strength in order to reduce Joule heating of the electrode surface. Elevated temperatures can cause changes in solution viscosity, which convolutes the measurement. Additionally, sample degradation can occur in some cases. The reported result from PALS is only the mean value of electrophoretic mobility. It does not provide any distribution information. Some current implementations combine PALS and spectrum analysis to obtain distribution information.

## 4. APPLICATIONS

In general, ELS is used to characterize the charge distribution (or average charge) and stability of suspensions and emulsions. ELS has been used widely for characterizing colloidal systems where it is important to control their stability (e.g., flocculation of submicron materials suspended in a liquid). Examples include preparations of pharmaceutical suspensions and emulsions where flocculation could affect appearance, efficacy, and bioavailability. In biological applications and in the biotechnology industry, ELS was used early on to characterize the surface properties of cells. More recently, the biopharmaceutical industry has adopted ELS measurements during development of formulations for the evaluation of degradation pathways and the characterization of compositional variants.

## 5. DATA ANALYSIS

### 5.1 Calculation of Electrophoretic Mobility

The ELS method is used to determine the electrophoretic mobility (μ) by measurement of the Doppler frequency shift (Δω) of the scattered light in an applied electric field (*E*) using either *Equation 1* (for the reference beam optical alignment) or *Equation 2* (for the cross-beam optical alignment):

$$\mu = \frac{\Delta\omega\lambda}{4\pi n E \sin\left(\frac{\theta}{2}\right)\sin\left[\left(\frac{\theta}{2}\right)+\xi\right]} \qquad (1)$$

$$\mu = \frac{\Delta\omega\lambda}{4\pi n E \sin\left(\frac{\theta'}{2}\right)} \qquad (2)$$

Δω = Doppler frequency shift
λ = laser wavelength in vacuum

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$n$ = refractive index of the medium (or base liquid)
$E$ = electric field strength
$\theta$ = angle between the incident light and the scattered light
$\xi$ = angle between the scattered light and the orientation of the electric field
$\theta'$ = angle between the two beams

## 5.2 Calculation of Zeta Potential

For non-conductive spheres, zeta potential ($\zeta$) is commonly calculated using an extension of the Henry equation for its relationship with electrophoretic mobility ($\mu$) (*Equation 3*).

$$\mu = \frac{2\zeta\varepsilon}{3\eta_0} f(\kappa\alpha) \qquad (3)$$

$\varepsilon$ = dielectric permittivity of the medium (or base liquid)
$\eta_0$ = viscosity of the medium (or base liquid)
$\kappa$ = reciprocal of the Debye double-layer thickness
$\alpha$ = sphere radius
$f(\kappa\alpha)$ = a monotonic function varying from $f(\kappa\alpha) = 1$, as $\kappa\alpha \to 0$, to $f(\kappa\alpha) = 3/2$, as $\kappa\alpha \to \infty$

When $\kappa\alpha >> 1$, typical for large particles in aqueous suspensions, $f(\kappa\alpha) = 3/2$ in the above formula, leading to the Smoluchowski equation (*Equation 4*).

When $\kappa\alpha << 1$, typical for small particles in organic liquids, $f(\kappa\alpha) = 1$ in the above formula, leading to the Hückel equation (*Equation 5*).

$$\zeta = \frac{\eta_0\mu}{\varepsilon_r\varepsilon_0} \qquad (4)$$

$$\zeta = \frac{3\eta_0\mu}{2\varepsilon_r\varepsilon_0} \qquad (5)$$

Thus, depending on the nature of the particles and suspending medium, zeta potential ($\zeta$) is calculated from the electrophoretic mobility ($\mu$) utilizing either the Smoluchowski equation (*Equation 4*) or the Hückel equation (*Equation 5*).

The Smoluchowski equation is used when large solute particles (the hydrodynamic radius is large compared to the thickness of the electrical double layer) are probed, whereas the Hückel equation is preferred when small solute particles (the hydrodynamic radius is small compared to the thickness of the electrical double layer) are probed.

# 6. DATA INTERPRETATION

Zeta potential, expressed in units of millivolts (mV), is primarily used to characterize the physical stability of a suspension/emulsion. An unstable suspension may result in flocculation and sedimentation, and an unstable emulsion may collapse and result in phase separation. In general, as the absolute value of zeta potential decreases in magnitude, the suspension may become less stable and vice versa. The stability evaluation must be performed in conjunction with other relevant techniques. Although the relative stability change of the suspension may be indicated by changes in the value of the zeta potential, there is no direct link between zeta potential and absolute stability. Moreover, multiple determinations of different lots or preparations may be necessary to make general conclusions regarding the stability of different suspensions.

Although zeta potential is commonly used to quantitatively characterize the stability of a suspension, it is not intended to be the sole indicator of stability. Typically, zeta potential data are coupled with other quantitative or qualitative characterization techniques to monitor and characterize suspension stability, such as monitoring appearance or turbidity over time.

Zeta potential is also relative to the composition of the suspension. Typically, there is no distinct value of zeta potential that can be used as a threshold of stability.

It is important to note that zeta potential is a characteristic of a suspension, and not just of the particles. This includes the particles and the surrounding liquid environment with any ions or chemicals present in equilibrium. If care is not taken, performing multiple dilutions may alter this environment and change the zeta potential. It is also important to note that since the measurement relies on the migration of particles in an electric field, performing repeat measurements (NMT 3–5 depending on the sample) on the same aliquot may cause a shift in the zeta potential. If significant change is observed, an orthogonal method may be used to verify the integrity of the sample. Also note that a change of the zeta potential of a suspension may also change the observed hydrodynamic particle diameter by dynamic light scattering.

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# ▲⟨1430.5⟩ ANALYTICAL METHODOLOGIES BASED ON SCATTERING PHENOMENA—SMALL ANGLE X-RAY SCATTERING AND SMALL ANGLE NEUTRON SCATTERING

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4. CONTRAST MATCHING
5. INSTRUMENTATION
6. EXPERIMENTAL CONSIDERATIONS
    6.1 Incident Flux
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    6.3 Instrument Calibration
    6.4 Sample Quality
    6.5 Sample Concentration
    6.6 Sample Containers
    6.7 Backgrounds
    6.8 Counting Times
    6.9 Sample Transmission
    6.10 Multiple Scattering
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## 1. INTRODUCTION

Small-angle X-ray scattering (SAXS) and small-angle neutron scattering (SANS) make up a sub-family of small-angle scattering (SAS) diffraction techniques suitable for probing the size, shape, ordering, and interactions of molecules and molecular assemblies on length scales that are typically from around 1 nanometer (nm) to a few hundred nm. This size range overlaps with the lower range probed using the laser light diffraction technique (see *Analytical Methodologies Based on Scattering Phenomena—Light Diffraction Measurements of Particle Size* ⟨1430.2⟩).

Because these length scales are much larger than inter-atomic distances, in crystallographic terms SAS is considered low-resolution diffraction. Nonetheless, it is an extremely useful and versatile technique capable of providing structural information complementary to cryogenic transmission electron microscopy (cryo-TEM), high-resolution nuclear magnetic resonance (NMR), and analytical ultracentrifugation.

SAS differs from dynamic light scattering (DLS; see *Analytical Methodologies Based on Scattering Phenomena—Dynamic Light Scattering* ⟨1430.3⟩) in that it is a direct probe of size and shape.

### 1.1 Applications

SAS is routinely performed on solids, liquids, and dispersion samples (both aqueous and nonaqueous). Some types of SAS have been used to study foams.

In terms of pharmaceutical applications, SAS has been used to study topical creams, intravenous emulsions, hydrogels, polymer-drug conjugates, proteins, enzymes, and antibodies (*1*–*5*). Some types of SAS are particularly well suited to studying the physical chemistry underlying formulation problems.

SAS can be used in various applications to evaluate the size, shape, and ordering of molecules. For example, SAS can provide information on the size and dispersity of droplets, macromolecules, micelles, liposomes, nanoparticles, crystalline domains, and pores (including in mixtures of these and other multi-phase systems). With regard to shape, SAS can be used to determine the shape of micelles and nanoparticles; the tertiary structure of proteins, enzymes, and antibodies (using SAS-constrained modeling); and the conformational distribution of adsorbed layers. Finally, examples of the use of SAS to assess molecular order include determining the core-shell structure of micelles and sterically stabilized nanoparticles, the bilayer structure in liposomes, and the liquid crystalline structure (e.g., lattice parameters, space groups).

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## 2. TYPES OF SAS

SAS is a specialized, but nonetheless highly useful and versatile, technique. Although a detailed discussion of SAS is beyond the scope of this chapter, the intention of this chapter is to raise awareness and then direct the reader to authoritative sources for further information.

SAS is most commonly performed using light [small-angle light scattering (SALS)], X-rays (SAXS), or neutrons (SANS). It is the wavelength of these different radiation sources, and the way that the radiation interacts with the atoms in a sample, that determines what type of information is obtained in a particular SAS experiment. Importantly, data from all of these variants of SAS can be analyzed using common formulae and software packages. *Table 1* summarizes some of the key aspects of the different types of SAS.

**Table 1. Key Aspects of Different Types of Small-Angle Scattering**

| | SALS | Lab-SAXS | Synchrotron-SAXS[a] | SANS[a] |
|---|---|---|---|---|
| Radiation scattered by | Electrons | Electrons | Electrons | Nuclei |
| Radiation velocity (m/s) | $3 \times 10^8$ | $3 \times 10^8$ | $3 \times 10^8$ | $10^2$–$10^3$ |
| Accessibility | Laboratory | Laboratory | Synchrotron source | Neutron source |
| Relative brilliance | Medium | Medium | High | Low |
| Wavelengths (nm) | 400–700 | 0.07–0.23 | 0.06–0.33 | 0.15–2.5 |
| Length scales probed (nm) | 250–25,000 | ≤1–350 | 0.1–2500 | 0.2–1000+ |
| Impact of dust contamination | Significant | Negligible | Negligible | Negligible |
| Effect of hydrogen (H)/deuterium (D) substitution | None | None | None | Large |
| Radiation/heat damage | Negligible | Possible | Very likely | Negligible |
| Absolute intensity calibration | Possible | Possible | Possible | Routine |
| Sample volumes (mL) | 0.05–5 | 0.007–0.1 | 0.0001–0.5 | 0.1–5.5 |
| Study optically opaque samples | No | Yes | Yes | Yes |
| Typical container materials | Glass, quartz | Polyimide (Kapton), polycarbonate, beryllium, silica/quartz, silicon nitride | Polyimide (Kapton), polycarbonate, beryllium, silica/quartz, silicon nitride | Silicon, silica/quartz, aluminum, titanium, vanadium, copper silicon nitride |

[a] Access to national and international large facilities is usually obtained through competitive peer review, but experimental time for successful proposals is then free, provided that the results are placed in the public domain. Where this route of access is unsuitable, for example, for commercial customers who require confidentiality or a guarantee of experimental time, a range of paid industrial access mechanisms will typically be available.

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## 3. BASIC THEORY

When a beam of radiation is directed at a sample, a fraction of the beam will be transmitted, a fraction will be absorbed (and in some experiments may then be re-radiated), and the remainder will be diffracted through a range of angles, Θ. In a typical SAS experiment, $0.01° < \Theta < 10°$; this value of Θ is different from that in wide-angle and classical crystallographic diffraction experiments, where $\Theta > 10°$. On some instruments, it may be possible to record the small- and wide-angle data concurrently.

Radiation scattered through the same angle but originating from different points in the sample may then superimpose constructively or destructively at the detector to construct a "scattering pattern". This reflects spatial fluctuations in the refractive index (RI) (SALS), electron density (SAXS), or scattering length density (SANS) of the sample, which in turn arise from differences in the size, shape, and order of the constituent molecules.

The length scale, *d*, of the spatial fluctuations that may be accessed in a SAS experiment is determined by Bragg's Law:

$$d = \frac{\lambda}{2\sin(\theta)}$$

where λ is the wavelength. Experimentally, it is more typical to express this in terms of (the modulus of) a quantity called the "scattering vector", *q*:

$$q = \frac{4\pi n}{\lambda}\sin(\theta) = |q| = |k_s - k_i|$$

from which it follows that:

$$d = \frac{2\pi n}{q}$$

where $k_i$ and $k_s$ are the incident and scattered wave vectors and *n* is the RI of the medium. Conveniently, for neutrons and for X-rays, *n* may be assumed to be unity. Note that *q* is an inverse length scale. The geometry of a SAS experiment is illustrated in *Figure 1*.

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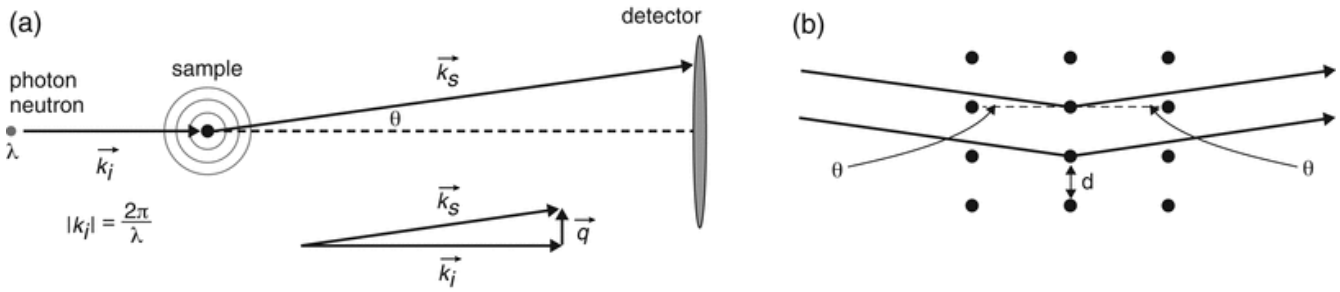


Figure 1. (a) The geometry of an SAS experiment, where $k_i$ and $k_s$ are the incident and scattered wave vectors and $q$ is the resultant scattering vector. (b) Schematic representation of diffraction from atoms in a sample.

The number of photons/neutrons of a given wavelength, scattered through a particular angle, that arrive at a detector pixel in a unit time, $N(\lambda,q)$, may be expressed as:

$$N(\lambda,q) = N_0(\lambda)\Delta\Omega\ \eta(\lambda)T_r(\lambda)V_S I(q)$$

where $N_0$ is the incident flux of photons/neutrons, $\Delta\Omega$ is the solid angle element defined by the size and distance of the detector pixel from the sample, $\eta$ is the detector efficiency, $T_r$ is the transmission of the sample (i.e., 1 – absorbance), $V_S$ is the volume of the sample illuminated by the beam, and $I(q)$ is the scattering function. Note that the first three terms are instrument-specific, whereas the last three terms are sample-dependent. The scattering function, the quantity determined in an SAS experiment, is given by:

$$I(q) = \Phi V(\Delta\rho)^2\ P(q)S(q) + B$$

where $\Phi$ is the volume fraction of the scattering objects in the sample (e.g., the volume fraction of nanoparticles in a dispersion); $V$ is the volume of one scattering object; $\Delta\rho = (\rho_{object} - \rho_{matrix})$ is the contrast of the sample (discussed below); the form factor $P(q)$ describes how the scattering is modulated by the size, shape, and dispersity of the scattering objects; the structure factor $S(q)$ describes how the scattering is modulated by interactions; and $B$ is a residual background signal normally taken to be $q$-independent. $P(q)$ has a value between 0 and 1. As $\Phi \rightarrow 0$, so $S(q) \rightarrow 1$.

For a solute in solution:

$$\Phi V = \frac{cM}{N_A\delta^2}$$

where $c$ is the mass concentration of the solute, $M$ is its formula weight, and $\delta$ is its bulk density. The objective of an SAS experiment is to extract information from $P(q)$ and/or $S(q)$. How this is achieved is discussed later. However, every component in the sample, including the container, will contribute to the overall scattering from the sample. So in addition to obvious background correction measurements (e.g., empty container; container + solvent), it is often useful to manipulate the various other individual contributions.

## 4. CONTRAST MATCHING

From the discussion in the preceding section, it is apparent that if the contrast term is zero, there will be no SAS. This condition is known as "contrast matching" and is potentially extremely useful because it permits the simplification of the scattering from complex multi-component samples and the study of individual components.

In SALS, contrast matching requires that two or more components have the same RI. The reader may have experienced this phenomenon when washing a glass in a bowl of water; the glass can seem to disappear because the glass and the water have similar RIs. In many experiments, however, RI matching of the components in a sample is often difficult to achieve in a useful manner. Some SALS and DLS instruments, however, use index-matching baths around the sample cell to reduce scattering from the outer wall of the cell.

In SAXS, contrast matching requires that two or more components have the same electron density. Because the electron density of a molecule is strongly influenced by the atomic number of the elements it contains, contrast matching a molecule with a heavy atom, such as a cationic surfactant, to an aqueous medium is not possible unless an additional solute is added to bolster the electron density of the medium. However, this course of action may change the pristine characteristics of the analyte and its chemical environment.

Contrast matching is more useful in SANS. Here, contrast matching is achieved if two or more components have the same scattering length density, $\rho$:

$$\rho = \frac{\delta N_A}{M}\Sigma_i b_i$$

where $b_i$ is the (bound) "coherent neutron scattering length" of nucleus $i$, and the other variables are as defined previously. The summation only needs to be performed over the empirical formula for a component.

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Scattering length density calculators can be found online or as tools in some SAS analysis programs. A compendium of neutron scattering lengths is available online (*6*).

An example of a calculation for poly(ethylene oxide),–[$CH_2$-$CH_2$-O]$_n$–:

$$C_2H_4O,\ M = 44\ \text{g/mol},\ \delta = 1.127\ \text{g/cm}^3,\ b\ \text{in cm}$$

$$\rho = \frac{1.127 \times 6.023 \times 10^{23}}{44} \times \{(2 \times 6.646 \times 10^{-13}) + (4 \times -3.739 \times 10^{-13}) + 5.803 \times 10^{-13}\}$$

$$\rho = 0.64 \times 10^{10}\text{cm}^{-2} \text{ or } 0.64 \times 10^{-6}\text{Å}^{-2}$$

Importantly, because they depend on the neutron–nucleus interaction, scattering lengths are isotope-dependent. The same calculation for perdeuterated poly(ethylene oxide), for example, would yield $\rho = 6.46 \times 10^{-10}$ cm$^{-2}$.

[NOTE—It is a coincidence that this value appears to be 10 times the hydrogenous value.]

Thus, D–for–H exchange offers significant opportunities for exploiting contrast matching in soft-matter SANS experiments. *Table 2* lists some illustrative scattering length densities. These may sometimes be found expressed in the equivalent but non-SI units of $10^{-6}$ Å$^{-2}$.

**Table 2. Neutron Scattering Length Densities of Pharmaceutically Relevant Molecules**

| Molecule | $\rho(10^{10}\text{cm}^{-2})$ | Molecule | $\rho(10^{10}\text{cm}^{-2})$ | Molecule | $\rho(10^{10}\text{cm}^{-2})$ |
|---|---|---|---|---|---|
| $H_2O$ | –0.56 | $H_{25}$–sodium dodecyl sulfate (SDS) | 0.33 | Lipids | 0.1–0.4 |
| $D_2O$ | 6.37 | $D_{25}$–SDS | 5.83 | Proteins | 2.2–2.6 |
| $H_8$–toluene | 0.94 | $H_{60}$–Triton ×100 | 0.57 | Carbohydrates | 2.7 |
| $D_8$–toluene | 5.66 | $H_{31}$–dodecyldimethylamine oxide (DDAO) | –0.20 | DNA | 4.0 |
| Perfluorodecalin | 4.21 | $H_{39}$–deoxycholate Na | 0.66 | RNA | 4.5 |

Several useful characteristics of scattering length densities are evident in the table. First, they can be positive, negative, or, in principle, zero. Second, different classes of biological molecules span a wide range of scattering length densities. Lastly, it is usually possible to find a solvent, or preferably a mixture of solvents, that will contrast match any solute. So, for example, DNA can be contrast matched in a mixture of 34.1% $H_2O$: 65.9% $D_2O$ by volume, and a perfluorodecalin-in-water emulsion would not scatter neutrons if the oil was dispersed in a mixture of 31.1% $H_2O$: 68.9% $D_2O$ by volume. It is even possible to make an $H_2O$:$D_2O$ mixture that does not scatter (i.e., $\Delta\rho = 0$). *Figure 2* shows how contrast matching could be used in a SANS study.

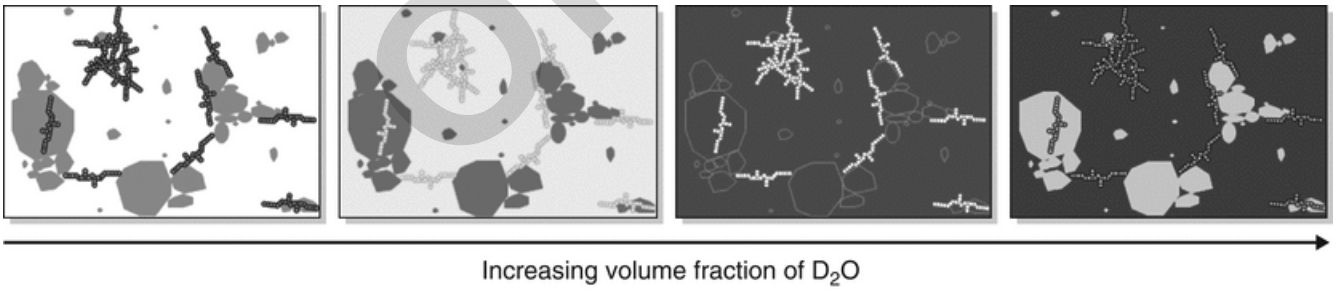


Figure 2. Schematic illustration of how contrast matching might be used in a hypothetical SANS study of a dispersion containing polymer/surfactant and drug particles. The scattering from all four figures would be different. The first and last would differ only in overall intensity, but the form of the scattering from these would differ from the forms of the second and third figures, which highlight different components of the sample.

When calculating scattering length densities, it is important to use as accurate a value as possible for the bulk density, $\delta$. However, sometimes determining $\delta$ can be problematic for deuterated compounds. In such instances, a useful rule of thumb for solvents and relatively simple molecules is $\delta_D = \delta_H \times 1.1$. Also, it is important to remember that $\delta$ is temperature-dependent.

Molecules containing hydroxyl groups, carboxylic acid groups, and even some amine groups will readily exchange those functional protons for deuterons when exposed to $D_2O$. This means that the scattering length density of some molecules can be pH-dependent; proteins are an obvious example. Some scattering length density calculators include a degree of exchange parameter to compensate for this pH dependence.

## 5. INSTRUMENTATION

The components of a SAS system (see *Figure 3*) are common across the different forms of the technique.

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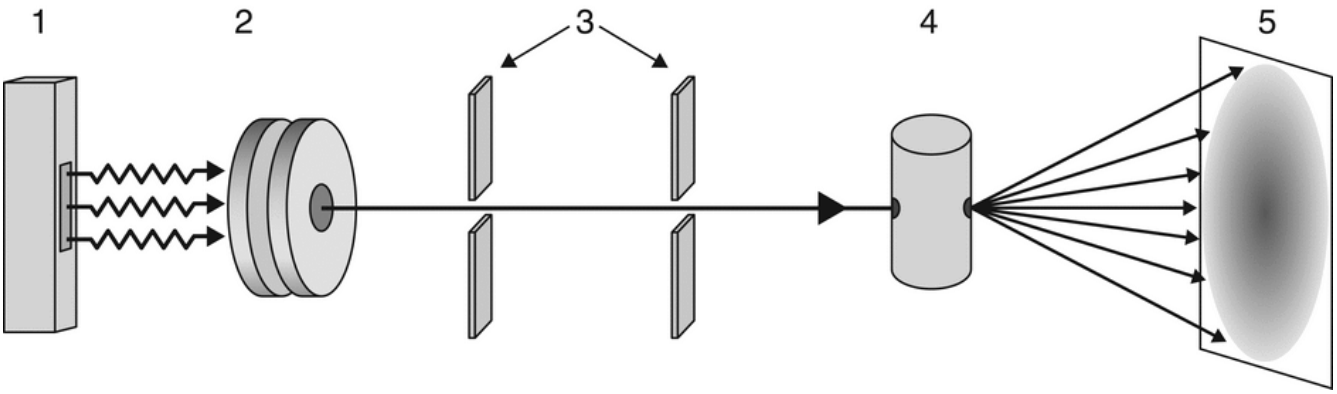


Figure 3. Schematic representation of a generic SAS instrument.

| Key | |
|---|---|
| 1. Source<br>2. Optics<br>3. Slits | 4. Sample<br>5. Detector |

SALS and SAXS are performed using monochromatic radiation, which is collimated with slits or pinholes to the desired profile regarding size (diameter or width/height) and shape (line or point). The wavelength of monochromatic radiation is determined by the choice of source (replacing one type of anode material with another), but may also be varied by changing optical filters, or adjusting the synchrotron monochromator. SANS at reactor neutron sources is also performed in this manner, with the wavelength adjustment performed by a rotating device called a velocity selector. In these fixed-wavelength measurements, altering the dynamic range in $q$ of the instrument requires changing the sample-to-detector distance (either along the incident beam direction or off-axis) in order to change the range of scattering angles subtended to the sample.

However, SANS can also be performed at pulsed neutron sources (based on particle accelerators—so-called spallation sources—or pulsed reactors). At these sources, a "polychromatic" ("white") beam of neutrons is directed at the sample, and the time of flight (TOF) of the scattered neutrons to the detector is recorded. This establishes the neutron velocity and thereby its wavelength. The dynamic range in $q$ of these TOF-SANS instruments is much broader than that of their fixed-wavelength counterparts because every wavelength that reaches a pixel on the detector corresponds to a different $q$ value, although a given pixel on the detector still represents a single scattering angle. Moreover, the intrinsic wavelength resolution, and therefore $q$-resolution, of TOF-SANS is much better than that of fixed-wavelength SANS.

The scattering from a sample actually occurs over 4πspace, but because an SAS instrument is linear, the detector only intercepts a conic section of the total scattering. Because most soft matter samples have some degree of intrinsic disorder (referred to as "polycrystallinity" by crystallographers), the detector records multiple rings of varying intensity rather than discrete spots (reflections) (see *Figure 4*).

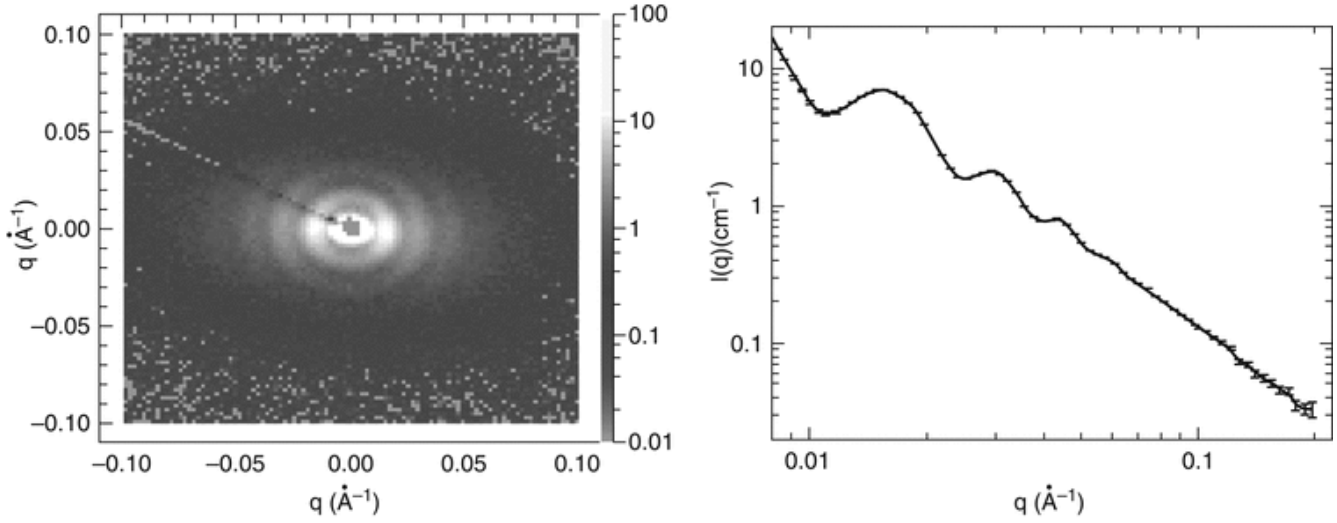


Figure 4. Two representations of the SAS (in this case, SANS) from the same aqueous dispersion of elongated surfactant micelles. The sample has gelled to form a lyotropic liquid crystalline phase exhibiting lamellar order (at least 4 orders of diffraction are visible as rings or peaks). The lamellae have then aligned vertically (giving rise to an anisotropic 2D pattern along the equatorial direction on the detector) as a result of the shear field imposed when the sample was injected into the sample cuvette.

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The scattered radiation will usually be detected by solid-state charge-coupled device (CCD) cameras (SALS/SAXS), gas detectors (SAXS/SANS), or scintillator detectors (SANS). Often these will be highly pixelated two-dimensional ("area") detectors, which are extremely useful for resolving anisotropic scattering from oriented samples. A state-of-the-art SAXS detector for laboratory applications may have 1 thousand (for one-dimensional detectors) to more than 1 million pixels (for two-dimensional detectors), and more for large-facility instruments. Consequently, the data volumes generated during an experiment may be quite large (gigabytes to terabytes).

In order to allow them to access smaller angles, and because they require more substantive radiation shielding, SAXS/SANS instruments at large facilities may extend several tens of meters from the source. Even a state-of-the-art laboratory SAXS instrument is typically 3–5 m in length. While in principle one could attain ever-smaller $q$ values by building very long instruments, in practice the count rate at the detector declines as the inverse-square of the sample–detector distance; thus, measurements become impractically slow. To circumvent this limitation and permit very-small-angle scattering (VSAXS/VSANS) or ultra-small-angle scattering (USAXS/USANS) measurements, alternative strategies are employed, such as using focusing optics or slit-collimated Bonse–Hart "double–crystal" geometries. These instruments can access length scales ($d$) into the micron scale, i.e., the same realm accessible by optical microscopy. The type of source collimation, point-source (pinhole) versus slit, has implications for data processing. Very recently, a new form of SANS has emerged wherein the scattering angle is encoded in the precession of polarized neutrons. This technique, spin-echo (modulated) SANS (SESANS/SEMSANS), can also access micron-length scales but measures in $d$-space rather than in $q$-space. Its advantages over Bonse–Hart VSANS are that it retains good count rates and is much less susceptible to multiple scattering from the sample. Further discussion of these extended SAS techniques is beyond the scope of this chapter, but the reader should be aware of their existence.

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# 6. EXPERIMENTAL CONSIDERATIONS

Performing a successful SAS experiment depends on many factors, including but not limited to the following factors described in subsections *6.1–6.12* (*7–9*).

## 6.1. Incident Flux

Incident flux is the number of photons/neutrons per unit area per second delivered to the sample. It is a function of the brilliance of the source (see *Table 1*) and the wavelength selection/collimation employed for the experiment. Kinetic or dynamic measurements, or measurements on very weakly scattering samples, will fare better on higher-flux instruments.

## 6.2 Resolution

There are two types of resolution of relevance to an SAS experiment:

1. Size resolution of the instrument
2. $q$-resolution of the measurement

### 6.2.1 SIZE RESOLUTION

The maximum, $d_{max}$, and minimum, $d_{min}$, length scales of the structure to be probed need to lie inside the measurement limits of the instrument, i.e.:

$$q_{min} < \frac{\pi}{d_{max}} \blacktriangle\blacktriangle \text{ (ERR 1-Dec-2019)}$$

and

$$q_{max} > \frac{\pi}{d_{min}}$$

This principle is illustrated in *Figure 5*.

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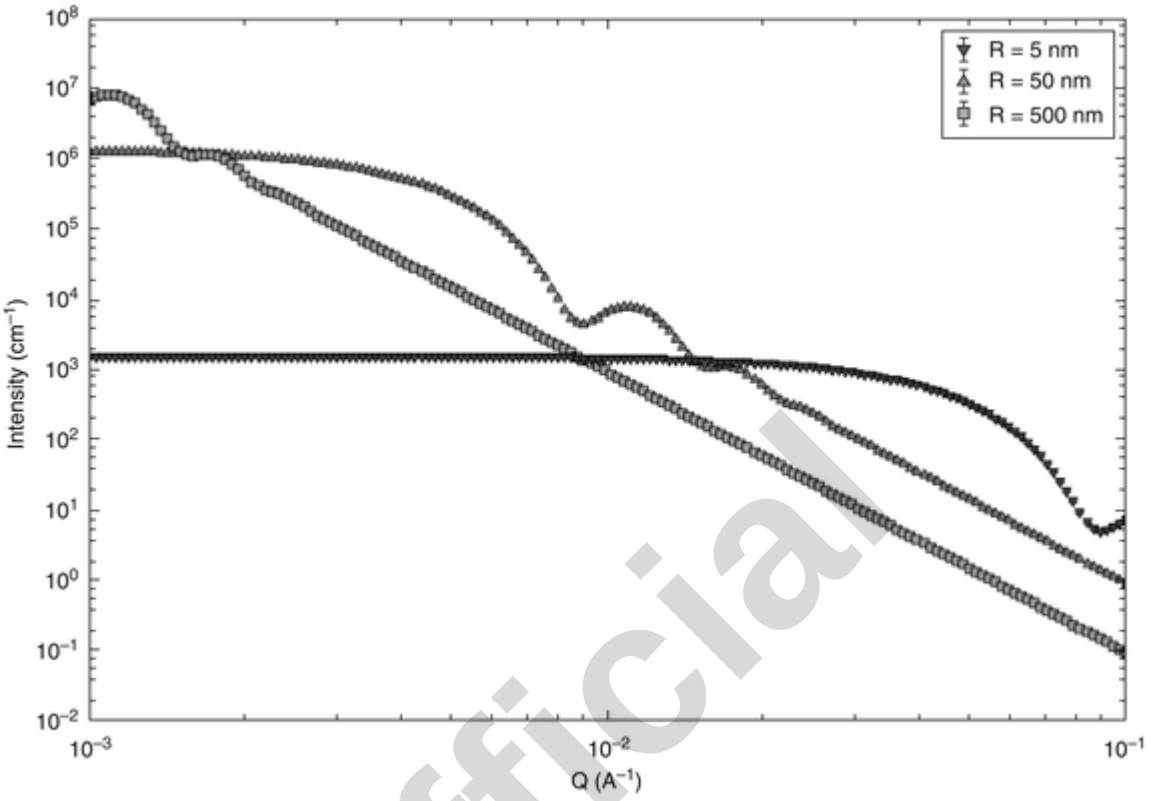


Figure 5. Calculated scattering for three dispersions of perfectly spherical homogeneous particles of the same material in the same matrix, differing only in the particle radius, *R*. A 10% log-normal particle size distribution has been applied to aid realism. The curves are displaced vertically to aid clarity. Only the scattering from the *R* = 50 nm particles is fully within the measurement window of this simulated instrument, as shown by the limiting behavior of the form factor at low Q and multiple interference fringes at intermediate Q. Also note the *q*-4 dependence of the high-*q* scattering, a manifestation of Porod's Law arising from the particle surfaces. Down triangles, *R* = 5 nm; up triangles, *R* = 50 nm; squares, *R* = 500 nm.

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#### 6.2.2 *q*-RESOLUTION

The *q*-resolution is the precision with which adjacent *q*-points can be distinguished, and therefore it governs the level of fine detail in the measured scattering data that can be resolved (for example, the peaks in *Figure 4*).

The *q*-resolution is primarily determined by the wavelength resolution of the instrument, then by the geometric resolution arising from the selected instrument collimation, and then by a range of other less significant factors.

Laser light sources and X-ray sources have the best wavelength resolution, with $\Delta\lambda/\lambda$ values as low as 0.01%. Conventional light sources depend on the use of a bandpass filter for which $\Delta\lambda/\lambda$ may be ~1%. Although good wavelength resolution is an intrinsic property of a laser, in the case of X-rays, it is determined by the monochromator in use. The wavelength resolution of fixed-wavelength SANS instruments depends on the specification of the neutron velocity selector employed. Typically, this resolution will be between 8% and 20%. However, in TOF-SANS the wavelength resolution is determined by the precision with which the neutron TOF (typically a few thousand microseconds) can be measured, and thus $\Delta\lambda/\lambda$ values of approximately 5% would not be uncommon. In most instances, better wavelength resolution can always be traded for reduced flux and vice versa.

The geometric resolution is essentially governed by the beam size at the sample; a smaller size is better. Laser-based SALS and SAXS instruments with submillimeter beam footprints fare much better than SANS instruments in this regard, where a relatively small beam may still be 6–8 mm in diameter. This difference is further compounded by the fact that because neutrons cannot be easily focused, reducing the beam footprint also decreases the flux on the sample.

### 6.3 Instrument Calibration

SAS instruments require two separate calibrations, but with an astute choice of calibration sample it might be possible to perform both calibrations in one measurement:

1. Calibration of the *q*-scale
2. Calibration of the intensity scale

An instrument technician or, at large facilities, the assigned experiment local contact, will normally perform these calibrations. However, if one's plan is to use several different SAS instruments it is advisable to acquire and measure one's own calibrants to ensure confidence in the local calibrations and ensure that all of one's data are on self-consistent scales.

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#### 6.3.1 *q*-CALIBRATION

It is possible to omit the *q*-calibration on a fixed-geometry SAS instrument where the sample-to-detector distance is constant/known. In all other instances, it is necessary to measure a sample with a characteristic *q*–dependence related to structure that can be unambiguously verified by other means [e.g., TEM, atomic force microscopy (AFM), etc.]. Alternatively, if an SAXS instrument has an energy-responsive detector, and an accurate determination of scattering angle is possible, then an absorption edge can be used to verify *q*. Two commonly used *q*-calibrants for SAXS/SANS are silver behenate (AgBH) (see *Figure 6*) and rat tail collagen (RTC).

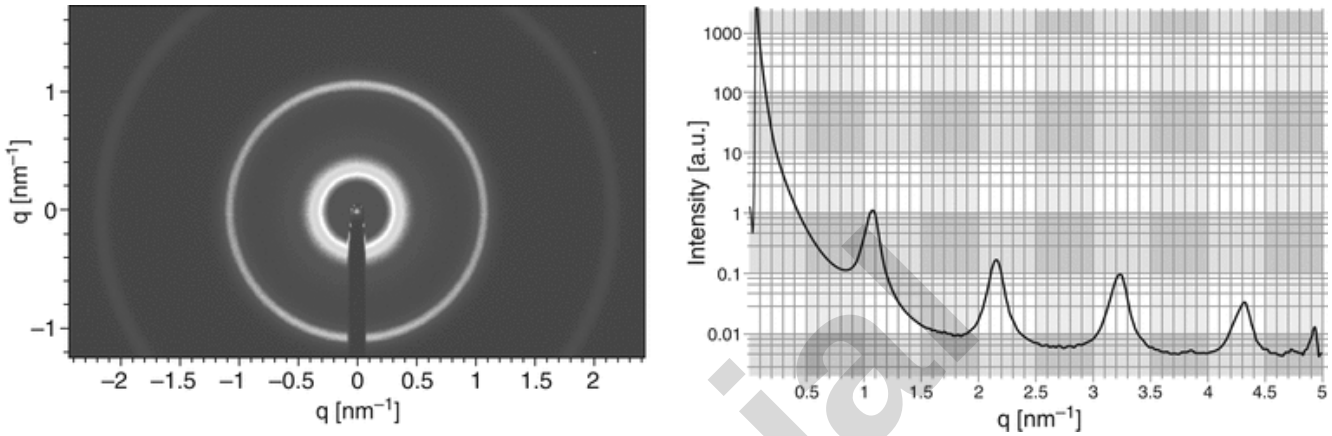


Figure 6. Two representations of the SAS (in this case, SAXS) from the same sample of polycrystalline silver behenate.

#### 6.3.2 INTENSITY CALIBRATION

Intensity calibration relies on the fact that for some types of material, $I(q = 0)$ can be related to molecular parameters that can be calculated with reliability or determined using other techniques, for example, the molecular weight of a polymer, the surface area-to-volume ratio of a porous solid, or the second virial coefficient or isothermal compressibility of a pure liquid. However, because an SAS instrument does not physically collect data at $q = 0$, an extrapolation is required. Extrapolation is only possible, with the necessary precision, if the scattering function for the chosen calibrant has a definitive form. Commonly used intensity calibrants include water (SAXS; *10*), Lupolen (SAXS), blends of hydrogenated and perdeuterated homopolymers (SANS; *11,18*), porous solids (SAXS/SANS; *18*), glassy (vitreous) carbon (SAXS/SANS; *12,18*), and vanadium (SANS).

### 6.4 Sample Quality

As in any experiment, the best results will be obtained using carefully prepared samples. In particular, because the SAS from an object scales as the sixth power of its size, the presence of even a relatively few large objects in a sample can disproportionately interfere with the quality of the data, all else being equal. SALS is particularly affected by the presence of large particles because of the aforementioned difficulties in RI matching. For this reason, all solvents used for SALS experiments should be rigorously filtered before use to exclude the presence of dust particles. SAXS and SANS (but not USAXS/USANS) are much less affected by dust because they probe shorter length scales (dust particles are micron-sized). However, the presence of unwanted, unintended, or unexpected aggregation of components of the sample can be just as troublesome. Prescreening for aggregation in biological samples destined for SAXS or SANS instruments (for example, by DLS) should be considered *de rigour*. Poor contrast in a sample can also be problematic, although in some instances (for example, in SANS contrast matching experiments), it is unavoidable.

### 6.5 Sample Concentration

Although the magnitude of $I(q)$, and the precision with which it is measured, are both generally improved by using more concentrated samples, this comes with disadvantages as well, i.e., lower transmissions (see *6.9 Sample Transmission*) and the possible introduction of atomic pair correlations manifesting themselves in an $S(q)$ contribution to the scattering function. Whether this is an issue depends on the sample being studied and the intended method of data analysis. Some relatively simple modeling of the scattering before the experiment may help to establish the viable concentration envelope.

Of course, the converse is also true, and very dilute samples will likely suffer from a poor signal-to-noise ratio and necessitate longer experimental counting times, although this longer experimental time might be mitigated by the use of a high-flux instrument. However, as an illustration, most SAXS/SANS instruments can handle protein/enzyme solutions at concentrations as low as $\Phi \sim 0.1\%$ (1 mg/mL), whereas $S(q)$ is not usually a concern in a colloidal dispersion at $\Phi < 5\%$. For investigations of $S(q)$, relevantly higher concentrations are used.

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## 6.6 Sample Containers

Unless one is working with a self-supporting sample, the sample will need to be contained. The problem with sample containers is that they also scatter, so the material from which they are constructed is an important consideration. Suitable container window materials combine the mechanical strength and chemical resistance with high transmittance and scattering features, which do not interfere with those of the sample. As such, multiple window materials may be necessary to cover the entire range over which a sample is to be measured.

For SALS and SAXS, it is quite common to use glass sample containers. Glass is composed only of light atoms (an important consideration for SAXS), and its transparency is as useful for observing the sample as it is a prerequisite for SALS. However, because glass contains boron (a neutron absorber) it is not suitable for SANS.

Amorphous synthetic quartz (SiO2, also called fused silica) is a good choice of material for SALS, SAXS, and SANS. It can be cut, polished, and fabricated and is also transparent and only composed of light atoms; however, it is boron-free. It also has good thermo-mechanical stability over an experimentally useful range of temperatures and is chemically inert to all but concentrated bases and hydrofluoric acid. Material tradenames include Spectrosil and Suprasil, but in principle anything suitable for far-UV (not IR) spectrophotometry should suffice.

For SAXS, it is possible to utilize sample containers constructed from amorphous engineering polymers such as poly(carbonate) (PC), poly(etheretherketone) (PEEK), or poly(imide) (Kapton). Poly(amides) (nylons) are not suitable because they are semi-crystalline. All hydrogenous polymers are wholly unsuitable for use as SANS sample containers.

The greater penetrating ability of neutrons means that SANS sample containers can be constructed from metals. Pure metals should be used where possible (as SANS is an excellent technique for probing the nanostructure of metal alloys), or when a prior assessment of suitability has been conducted. Good choices of metals for use with neutrons are aluminum, titanium, vanadium, and copper. These metals can activate on prolonged exposure to neutron radiation but not to levels that make them impractical to use. Beryllium, being of low atomic number, is however a practical choice for SAXS.

In most experiments, the sample will simply be contained in the quiescent state in a capillary or cuvette. However, it is important to understand that quiescent conditions are not necessary. Indeed, the ability of SAS to probe a sample under changing conditions (pH, temperature, pressure) or applied fields (electric, magnetic, shear) is usually where the greatest information is ultimately derived. The use of cryostats, furnaces, pressure cells, rheometers, tensile stages, and stop-flow cells with SAXS/SANS instruments is very common. The use of flow-through cells to mitigate radiation damage (see *6.11 Radiation Damage*) is very common on high-flux SAXS instruments.

Sample containers will typically have path lengths ranging from 0.1–2 mm (SAXS) to 1–5 mm (SANS) to 10 mm or more (SALS). Multiplying this value by the incident beam size—a few micrometers (SAXS) to a few millimeters (SALS/SANS)—reveals that the required sample volume is rarely more than a couple of milliliters, and potentially less.

## 6.7 Backgrounds

In the same way that the sample container contributes to the measured scattering, so does the matrix containing the sample, for example, the dispersion medium. For this reason, it is typical to measure the scattering from the pure matrix, in the same or an identical sample container under the same experimental conditions, and then to subtract this matrix scattering from the sample scattering. This procedure should also correct for other sources of "sample-independent" background (for example, the container, vacuum windows, detector response, etc.).

Difficulties can arise if the sample component of interest occupies a substantial proportion of the overall sample volume because subtracting 100% of the matrix may no longer be appropriate, leading to over-correction of the data. In these instances, a volume fraction-weighted background correction may be appropriate.

Arguably, the most insidious form of "sample-independent" background is multiple scattering (discussed in *6.10 Multiple Scattering*).

## 6.8 Counting Times

Experimental measuring times for an SAS experiment may vary from a fraction of a second (synchrotron-SAXS) to many minutes or hours (SANS). However, because SAS measurements are Poisson counting processes, to halve the statistical uncertainty on an $I(q)$ point requires four times as much data. This calculation is further complicated by the fact that when a background is subtracted, the statistical errors on the sample and background are added in quadrature, as shown here:

$$Error_{sample-background} = \sqrt{\left\{\left(Error_{sample}\right)^2 + \left(Error_{matrix}\right)^2\right\}}$$

Thus the final data quality may not be apparent until after the experiment has finished. This means that invariably one ends up making a judgment call about the statistical quality of the data before moving on to the next sample. Therefore, counting times are often based on previous experience or simple trial-and-error, or a combination of both. A good rule of thumb is to measure each background for the same number of photons/neutrons as the sample it is intended to correct.

## 6.9 Sample Transmission

The sample transmission, $T_r$, is a dimensionless number ranging between 0 and 1 and is a measure of the balance between the scattering power of the sample and absorption as described by the Beer–Lambert Law:

$$T_r = exp(-\mu t) = 1 - A$$

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where $t$ is the pathlength (in most instances, the thickness) of the sample, $\mu$ is an absorption coefficient, and $A$ is the absorbance; $\mu$ is dependent on the type of radiation in use.

Despite the simple appearance of this expression, accurately calculating $T_r$ for a sample is fraught with difficulties because $\mu$ may be unknown or difficult to determine. The best course of action is to measure $T_r$ at the time the SAS is recorded, simultaneously, if possible. Note that in TOF–SANS, $T_r$ is a function of wavelength.

A good rule of thumb is to keep $T_r > 0.37$ (i.e., $t < 1/\mu$), although this will have consequences for multiple scattering (see *6.10 Multiple Scattering*).

## 6.10 Multiple Scattering

Multiple scattering is where a photon/neutron that has been scattered once by the sample is scattered again (possibly several times). Multiple scattering should be avoided, if possible, because a routine method for correcting SAS data for multiple scattering has not been developed, except in some limited circumstances. The essence of the problem is that the history of multiply scattered photons/neutrons is unknown, and thus one cannot know whether or not to use them.

It is, however, quite straightforward to estimate the extent of multiple scattering from a sample. To a first approximation, the average number of times a photon/neutron has been scattered, $\tau$, is:

$$\tau = \mu t = -\ln T_r \propto t\lambda^2$$

Thus, even for $T_r = 0.9$ there is a 10% probability of multiple scattering. By $T_r = 0.5$ that probability has risen to almost 70%.

If multiple scattering is suspected, two strategies for mitigating its effects are to thin the sample and/or use shorter wavelengths and then re-measure and look for differences.

## 6.11 Radiation Damage

An important consideration during SAXS, particularly synchrotron-SAXS, experiments is that the X-ray beam can be extremely destructive to some samples if mitigating strategies are not deployed, such as the use of flow-through cells or cryogenic cooling. This is because the energy of the X-ray photons [for example, 8047 eV at the copper (Cu) (Kα) wavelength of 0.154 nm] is far greater than the strength of the bonds holding the sample together (for example, the C–C bond energy is just 4 eV), and hydrogen bonds are weaker still. Even visible photons from a modest blue laser can cause sample degradation if left to irradiate the sample for long enough. Conversely, the energy of neutrons of equivalent wavelengths are orders of magnitude lower (for example, a mere 0.034 eV at a wavelength of 0.154 nm), meaning neutrons are a truly non-destructive probe, especially of sensitive biological material.

## 6.12 Sample Deuteration

Although selective deuteration is a valuable experimental tool for extracting maximum information from a SANS experiment, it is unfortunately not without complications. The slightly larger atomic volume of deuterium, differences in the polarizability of C–H bonds and C–D bonds, and differences in the strength of O…H and O…D hydrogen bonds all contribute to subtle modifications of the underlying thermodynamics in the sample which can, and do, affect the phase behavior. For example, cloud points, critical micelle concentration, micelle aggregation numbers, and theta temperatures have all been known to change (sometimes in opposite directions depending on whether the solute or the solvent is deuterated). These challenges should be considered when designing a SANS experiment.

# 7. DATA PROCESSING

SAS data processing has two steps:

1. Data reduction: The transformation of the as-measured instrument-dependent data into portable instrument-independent data (ideally in absolute units on an absolute scale)
2. Data analysis: The interpretation of the instrument-independent data to extract physical information characterizing the sample

## 7.1 Data Reduction

Data reduction procedures, and the factors for which they are corrected, will vary from one SAS instrument to the next. In the case of SANS, these procedures can even vary between reactor-SANS instruments and TOF-SANS instruments. However, every SAS instrument manufacturer or large facility will provide the necessary software to correctly reduce the data collected from their instrument(s). This software is typically graphical user interface (GUI)-driven (although scripts are still used) and will offer both “novice” and “expert” modes of use, for either “manual”/“single” or “automated”/“batch” data reductions.

In general, the data reduction process will include most or all of the following steps:

1. Correct for the detector efficiency, dark current, dead time, and spatial linearity (flatness)
2. Calculate the $q$ values of each detector pixel
3. Integrate the recorded counts at a given $q$ within some region of interest (azimuthal angle, sector, etc.)
4. Normalize for the incident flux or quantum efficiency of the source
5. Correct for the sample transmission (absorbance correction)

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6. Correct for the volume of sample illuminated (beam area × sample thickness)
7. Apply an intensity calibration factor
8. Convert the intensity scale into absolute units

This process is repeated for every sample measured, and for any backgrounds measured. The appropriate background is then subtracted from a sample to yield the final, fully reduced SAS data set.

## 7.2. Data Analysis

SAS data analysis for a single sample may use one or more of the following methodologies:

- Model-fitting/curve-fitting methods:
  - Involve optimized fitting of analytical functions for $P(q)$ and/or $S(q)$ to the experimental $I(q)$ data, for example, to determine generic shape information (sphere, cylinder, coil, etc.), size (radius, radius-of-gyration, cross-sectional radius, etc.), and interaction potentials (hard sphere, Percus–Yervick, etc.) (see *Figure 7*)
  - Applicable to all sample types
  - Computationally straightforward

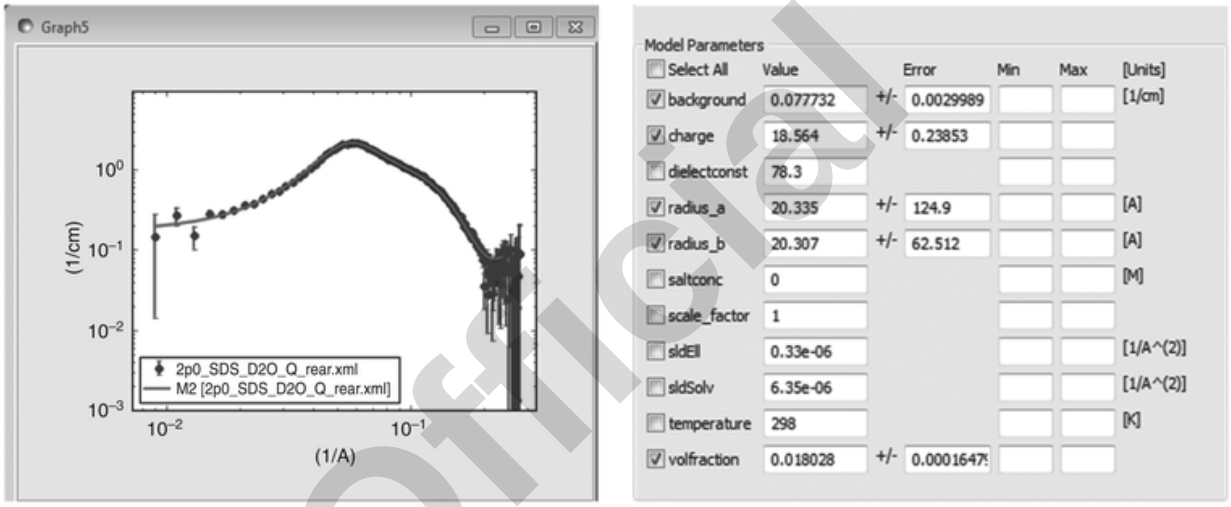


Figure 7. Left: Fit of a model calculation (line) to the SANS from a 2% (w/w) solution of surfactant sodium dodecyl sulfate (SDS) in $D_2O$ (shaded circles). At this concentration, above the critical micelle concentration (CMC), the system comprises electrostatically interacting, near-spherical, charged micelles. Right: The model parameter set, illustrating the level of sophistication that this approach can bring. Data and figures courtesy of Doucet et al., 2017 (*13*).

- Real-space methods:
  - Involve mathematical transformation of the experimental $I(q)$ data from reciprocal-space to real-space, for example, to obtain density correlation functions ($\Gamma(r)$ ), distance distribution functions [pair-distance distribution function (PDDF) or $P(r)$], or scattering length/segment density profiles [$\rho(r)$] (see *Figure 8*)
  - Applicable to all sample types
  - Computationally straightforward

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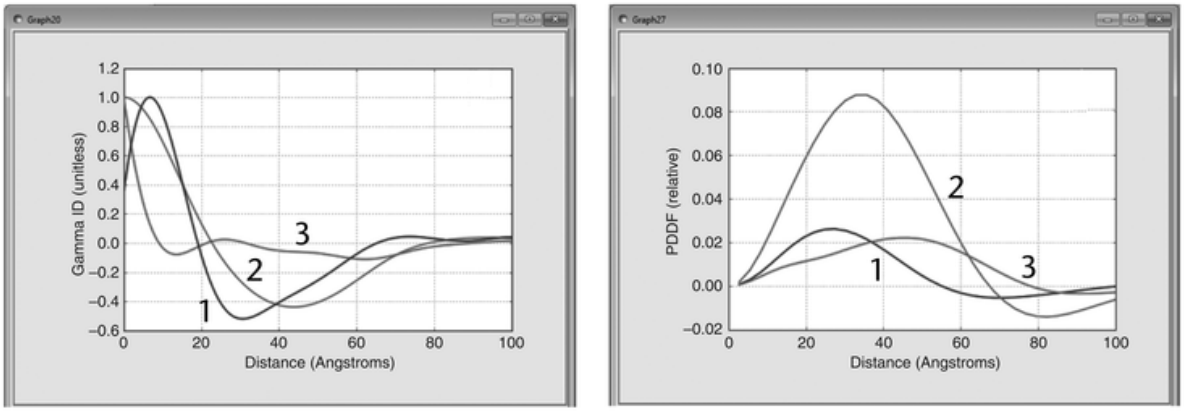


Figure 8. Two representations of the structure of a surfactant-stabilized oil-in-water emulsion system obtained from the SANS at three different scattering contrasts: "core" (line 1: where the scattering arises from the oil droplets only); "drop" (line 2: where the scattering arises from the oil cores and the surfactant layers); and "shell" (line 3: where the scattering arises from the surfactant layers only). For this system, the model-fitting approach suggests relatively monodisperse spherical objects of 25 Å radius with a 6 Å thick surfactant shell. Left: The one-dimensional density correlation functions. Right: The pair-distance distribution functions. Data and figures courtesy of Doucet et al., 2017 (*13*).

- $\Gamma(r)$ depicts the average separation of regions of similar scattering-length density in the sample. The minima and maxima in the function represent structural boundaries, for example, the edge of the object:

$$\Gamma(r) = \frac{1}{Q^*}\int_0^\infty I(q)q^2\cos(qr)dq$$

where the "scattering invariant" (also called the "total scattering") is given by:

$$Q^* = \int_0^\infty I(q)q^2dq$$

- The PDDF depicts the relative number of instances that two points in each scattering object are separated by a given distance, *r*. It is a function of distance only and is not a three-dimensional representation of the structure. The more symmetric the curve, the more rotationally symmetric the shape of the scattering object. Basic shapes like spheres, rods, and disks produce distinct distributions.

$$PDDF = \frac{1}{2\pi^2}\int_0^\infty I(q)qr\sin(qr)dq$$

- Ab-initio methods:
  - Involve the iterative creation of complex-shape envelopes, for example, from assemblies of homogeneous spheres ordered on a lattice or using spherical harmonic functions, the computed scattering of which is then compared with the experimental $I(q)$ data (*14*,*15*)
  - Are particularly useful for approximating the tertiary structure of biological molecules, but can also be applied to self-assembled soft matter systems like micelles
  - Do not take into account the chemical physics of the sample (e.g., bond lengths, bond angles, packing fractions, electrostatics)
  - Are computationally modest
- Simulation methods:
  - Involve the iterative manipulation of atomistic structures, for example, as derived from X-ray or high-resolution NMR crystal structures, by Boltzman-weighted Monte Carlo/Molecular Dynamics methods, the computed scattering of which is then compared with the experimental $I(q)$ data (*16*,*17*)
  - Are particularly useful for determining the tertiary structure of biological molecules
  - Take into account the chemical physics of the sample
  - Are computationally expensive, but can be used with coarse-graining methods

As SAS data analysis procedures become ever more sophisticated, so too does the degree of detail about the sample that they can return. However, one must never take this information in isolation; always look to cross-validate it with information derived from alternative techniques or the scientific literature. For example, model-fitting a large number of parameters without a priori information can result in "local" rather than "global" solutions. And PDDF, ab initio, and simulation methods work best when they can be constrained (e.g., to a known radius-of-gyration).

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## 8. ADDITIONAL SOURCES OF INFORMATION

- For further information about SAS, visit http://smallangle.org.
- For further information about synchrotron X-ray sources, visit http://lightsources.org.
- For further information about neutron sources, visit http://neutronsources.org.
- The interests of the world-wide SAS community are represented by a subcommission of the International Union of Crystallography (IUCr) (http://www.iucr.org/resources/commissions/small-angle-scattering) augmented by the community network CanSAS (http://www.cansas.org/). Together, these bodies advocate for and foster best practice, standardization, and data interchange, particularly where SAXS and SANS are concerned.
- The IUCr also operates a moderated SAS-specific email discussion list. To join it, visit http://mailman.iucr.org/cgi-bin/mailman/listinfo/sa_scat.

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# ⟨1430.6⟩ ANALYTICAL METHODOLOGIES BASED ON SCATTERING PHENOMENA—PARTICLE COUNTING VIA LIGHT SCATTERING

1. INTRODUCTION
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3. INSTRUMENTATION
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    3.2 Airborne Counters
    3.3 Light Scattering Aerosol Spectrometers
4. FACTORS THAT AFFECT THE TESTING
5. QUALIFICATION OF LIGHT SCATTERING PARTICLE COUNTING INSTRUMENTS
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## 1. INTRODUCTION

Light-scattering particle counter instrumentation can monitor or evaluate levels of particles with an equivalent diameter below 2 μm in both liquids and gases. The monitoring and/or evaluation of these particles may be needed for certain applications in the pharmaceutical industry.

A light scattering liquid-borne particle counter (LSLPC) is used for monitoring particle contamination in liquids for sizes lower than the typical sizes measured by light extinction particle counters that are specified in *Particulate Matter in Injections* ⟨788⟩ and ▲*Methods for the Determination of Subvisible Particulate Matter* ⟨1788⟩▲ (CN 1-May-2021).

Monitoring the size, number concentration, and number size distribution of particles suspended in a gas may also be needed in the pharmaceutical industry. Depending on the application, measuring particles suspended in a gas is performed using a light scattering aerosol spectrometer (LSAS) or a light scattering airborne particle counter (LSAPC).

LSLPCs are used for the evaluation of the cleanliness of pure water and chemicals, as well as the measurement of number and size distribution of particles in various liquids. The measured particle size using the LSLPC depends on the refractive index of particles and medium; therefore, the measured particle size is defined as being equivalent to the size of calibration particles in pure water.

LSASs can be used to measure particle number concentrations and particle size distribution of particles with an equivalent diameter of approximately 0.06–45 μm in several pharmaceutical applications including but not limited to: the characterization of metered dose inhalers (MDI), dry powder inhalers (DPI), and nebulizers in pharmacy; process control of active pharmaceutical ingredient production; and the fractional separation efficiency determination of filters.

Alternatively, an LSAPC can be used in the pharmaceutical industry to measure the size distribution and number concentration of particles suspended in air. The method is used to measure particles with a typical size range of 0.05–10 μm. LSAPCs are used for the classification of air cleanliness in cleanrooms and other controlled environment rooms as well as the measurement of number and size distribution of particles in various aerosols.

## 2. THEORY

The light scattering particle counter operation is based on the principles of light scattering phenomena. Particles may be sized using a light scattering technique based on the amount and direction of light that is scattered by a particle passing through the detection area of the particle counter. The measurement principle of a particle counter or sizer considers the particles as passing individually through a light beam, i.e., there is always only one particle in the scattering volume. The resulting signal is processed before the next measurement, and the cumulative resulting scattered light is then recorded. Simultaneously, the intensity of the scattered light is correlated with the particle size by using preselected scattering models depending on the type and size of the targeted sample.

Different models cover different scattering regimes, e.g., the Rayleigh regime, Mie regime, or Fraunhofer regime (see *Analytical Methodologies Based on Scattering Phenomena—General* ⟨1430⟩ and *Analytical Methodologies Based on Scattering Phenomena—Light Diffraction Measurements of Particle Size* ⟨1430.2⟩ for general principles of scattering and scattering regime models).

Depending on the choice of model, the height of the scattered light pulses would depend, in addition to the particle size, on the particle material (usually represented by the refractive index) and the particle shape. Hence, the final measurement output is the equivalent diameter of a sphere that scatters light as defined by the calibration with spherical latex particles.

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## 3. INSTRUMENTATION

Instrumentation for both LSLPC and LSAPC consists of a laser light source, an optical system, a scattered light detector(s) [photodetector(s)] to measure the scattered light from the particles, and appropriate hardware and software to conduct the necessary computations. A sample handler is used to introduce either liquid or gas at a known flow rate into the light scattering instrument. The laser light source is usually a laser diode or helium–neon laser. The optical system components include laser collimators, mirrors, and beam splitters as appropriate for a given optical arrangement. The scattered light detectors are in general photomultipliers or photodiodes. Commercial instruments use both low-angle and right-angle scattering optical configurations. A generic example of a right-angle light scattering particle counter is shown in *Figure 1*. The laser light is scattered by passing particles. The optics (collimators and/or mirrors) collect and focus the scattered light onto the photodetector, which converts it into a voltage signal pulse.

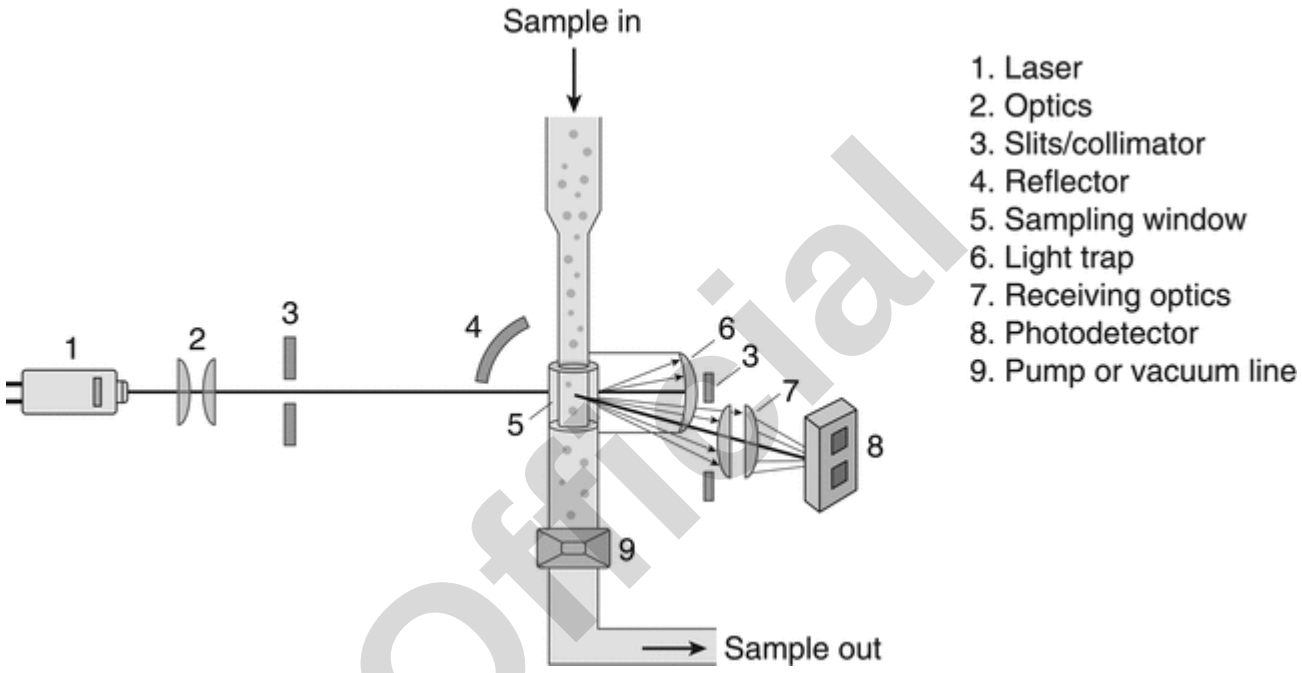


Figure 1. Schematic of a right-angle scattering particle counter.

Further descriptions of common instrumentation configurations are shown in ISO 21501. The instrumentation for light scattering particle counters is similar to but not identical to the instrumentation used for light obscuration particle counters. The primary difference between light scattering and light obscuration particle counters is the use of light scattering detectors to detect smaller-sized particles that are below the size range of particles detected by light obscuration.

### 3.1 Liquid Counters

Liquid particle counters are used to count and size the number of particles in a volume of liquid that flows at a set flow rate for a predefined time. The choice of an appropriate scattering model is very important for the liquid counters. The models assume that the refractive index of particles and the medium is the same as the calibration particles; hence, the measured particle size is the light scattering equivalent size of the calibration particles in the same medium. The actual size of the particle may be different from this reported size.

### 3.2 Airborne Counters

Aerosol particle counters are used to count and size the number of particles in a volume of air. The instrumentation and the principle of measurement are similar to the liquid counters. The preferred optic alignment for airborne counters is the right-angle scattering configuration, i.e., the airflow, laser, and collection optics are all at right angles to one another. The airborne counters differ from the liquid counters in the measuring cell and associated accessories assembly and the mode of accomplishing the fluid movement through the cell. The liquid counters have a syringe or pump at the cell's outlet that draws the sample through the cell, whereas the airborne counters have a vacuum line attached to the cell assembly outlet that draws air through the cell.

### 3.3 Light Scattering Aerosol Spectrometers

Light scattering aerosol spectrometers are a specialized type of aerosol particle counter that may be used in several pharmaceutical applications evaluating various types of aerosolized formulations and determining filter retention characteristics. The instrumentation and the principle of measurement of the light scattering aerosol spectrometers are similar to the airborne

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counters, but light scattering aerosol spectrometers generally have the capability of collecting data in more size channels than the conventional airborne counter to allow for a more complete assessment of the particle size distribution.

***Change to read:***

## 4. FACTORS THAT AFFECT THE TESTING

As light scattering aerosol and liquid particle counters are typically calibrated using polystyrene latex spherical particles of known size and concentration with a preset flow rate, differences in the physical properties of the actual particles present in an unknown sample, including changes in the refractive index and/or shape of the particles, or deviations from the calibrated flow conditions when measurements are performed may affect the observed particle size and count. The presence of an immiscible fluid, such as silicone oil in aqueous solutions or gas bubbles in an unknown sample, may also affect the observed particle count and size measured from a liquid counter as these may artificially increase the observed particle count. The presence of dispersed water droplets in sampled air may also affect the observed particle count when using an airborne ▲▲ (ERR 1-May-2021) counter. In these cases, the observed particle counts and sizes from an unknown sample may require additional examination, including isolation using an appropriate filtration technique and further characterization by an orthogonal technique such as scanning electron microscopy to confirm the values reported by the light scattering particle counter.

Elevated particle concentrations in the unknown samples may also affect the observed particle size and count when the concentration of particles in the sample is above the coincidence limit of the detector. When the coincidence limit of the detector is exceeded, the concentration of particles is high enough that multiple smaller particles may be counted as fewer larger-sized particles, thus preventing accurate sizing and counting of the particulates. This can be managed by performing serial dilutions of the unknown sample with a particle-free diluent to lower the particle concentration and verifying that the particle counts match the applied dilution factors.

## 5. QUALIFICATION OF LIGHT SCATTERING PARTICLE COUNTING INSTRUMENTS

The following are the key system qualification parameters needed to ensure accuracy and precision of the measurements performed by the LSLPC, LSAS, or LSAPC:

- Size calibration
- Verification of size setting
- Counting efficiency
- Size resolution
- Sampling flow rate
- Sampling volume
- Calibration interval

The above characteristics are similar to the counterpart characteristics used in the light obscuration particle counters (see ⟨788⟩ and ⟨1788⟩). Hence, the information in these chapters may be useful to serve as references for completing performance qualification and for testing of samples when using the various light scattering particle counters and sizers.

### 5.1 Size Calibration

It is recommended to calibrate an optical instrument experimentally by means of test particles of known size and refractive index. The output of such a calibrated instrument will then be related to light scattering equivalent particle diameter.

When calibrating a light scattering particle counter with calibration particles of known size, the particles are dispersed in a fluid or gas and introduced to the counter. The particles will produce a distribution of voltages where the median voltage [or internal pulse height analyzer (PHA) channel] corresponding to the particle size (see *Figure 2*) is selected for each size. Other-sized particles are then analyzed in the same manner to generate a calibration curve.

A monotonic, steadily increasing calibration curve is important for high size resolution and classification accuracy. Due to the influence of optical properties of the particle material, particle shape, and the optical setup (light source and detection angle), experimentally measured calibration curves may differ from theoretical predictions.

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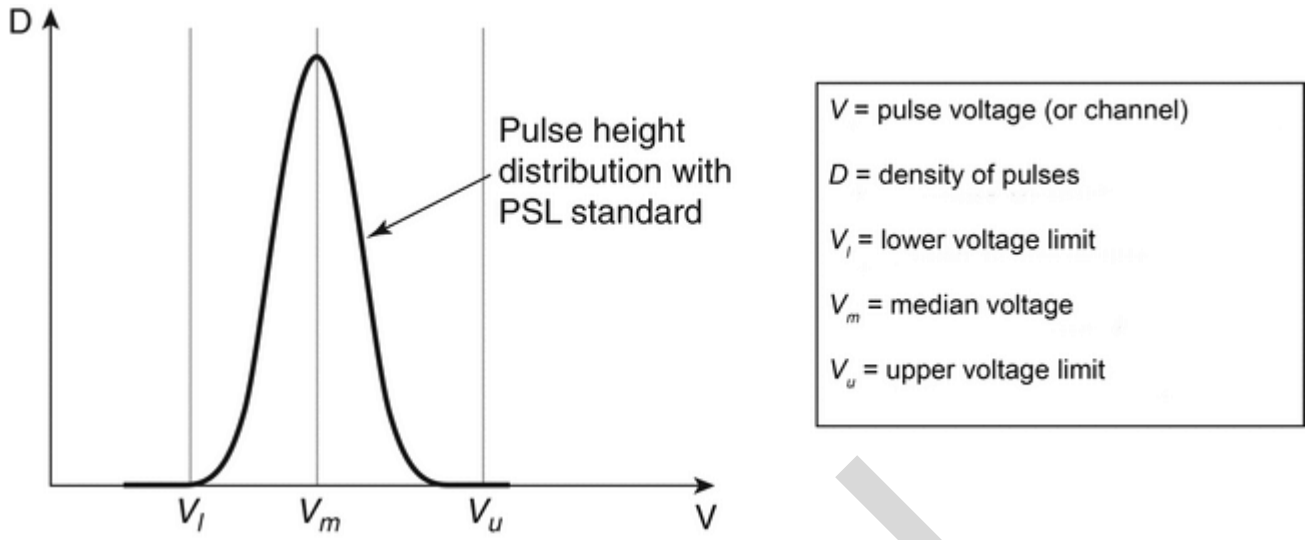


Figure 2. Pulse height distribution of polystyrene latex (PSL) particle signals.

The voltages of channels corresponding to particle size should be determined in accordance with the calibration curve provided by the manufacturer (see *Figure 3*). In general, a cubic spline fit may be necessary to generate the calibration curve.

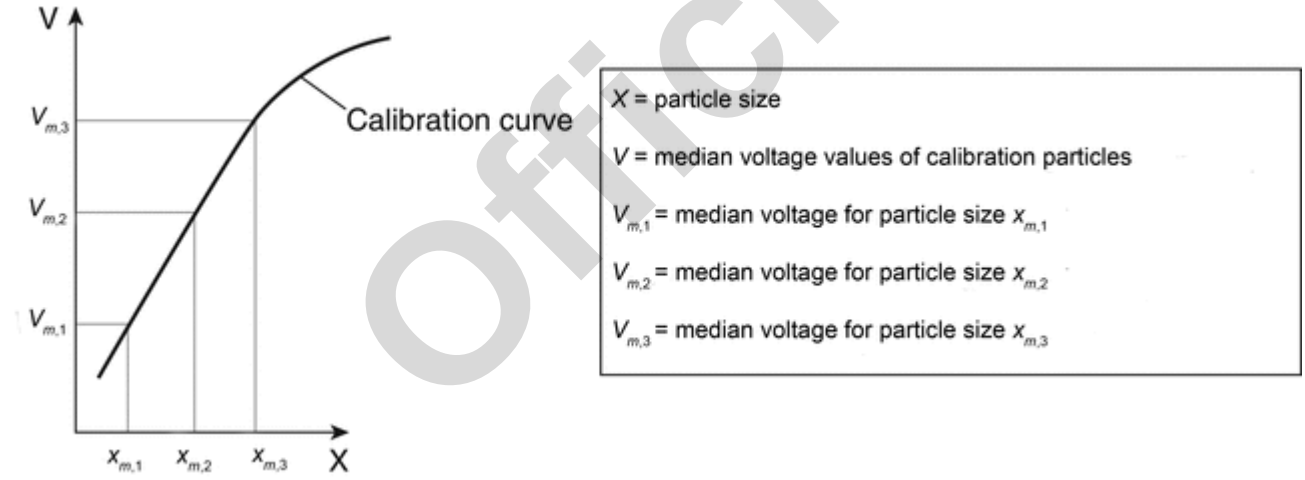


Figure 3. Calibration curve.

## 5.2 Verification of Size Setting

After calibration is completed, the calibration curve settings can be verified by obtaining response voltages (or internal PHA channel) using at least 3 sizes of calibration particles that span most of the reported size range, $x_r$, of the counter LSLPC.

Calculate the corresponding particle size, $x_s$, from the voltage setting (or internal PHA channel) of the LSLPC using the calibration curve. Obtain the percentage size-setting error, ε(%), by means of *Equation 1*. The recommended size-setting error is NMT 15%.

$$\varepsilon(\%) = \frac{x_s - x_r}{x_r} \times 100 \quad (1)$$

$x_s$ = calculated particle size (µm)
$x_r$ = reported size range (µm)

## 5.3 Counting Accuracy

To test the counting accuracy of the LSLPC, use 2 sizes of calibration particles—one that is close to the minimum detectable reported size range and another that is 1.5–2 times larger than the minimum detectable size.

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Measure the particle number concentration of both particles with the LSLPC under test and either a microscopic method or a calibrated LSLPC as a reference instrument.

The counting efficiency is the ratio of the particle number concentration measured by the LSLPC under test and the particle number concentration measured by the reference instrument.

## 5.4 Sensor Resolution

The width of a measured size distribution includes contributions from both the actual range of particle sizes in the sample and the inherent variability of the system (mainly the sensor resolution). The resolution of the particle sizer can be determined by using a single-size particle standard with a known size and standard deviation and comparing the theoretical standard deviation to the experimentally measured standard deviation determined with the instrument. The procedures for determining sensor resolution are similar to those detailed in the sensor resolution section of ⟨1788⟩. The sensor resolution determination is illustrated in *Figure 4*, in which the graph represents the voltage (pulse height) density distribution of a monodispersed particle standard, where the *D*-axis represents the density and the *V*-axis represents either the raw sensor voltage (or PHA channel).

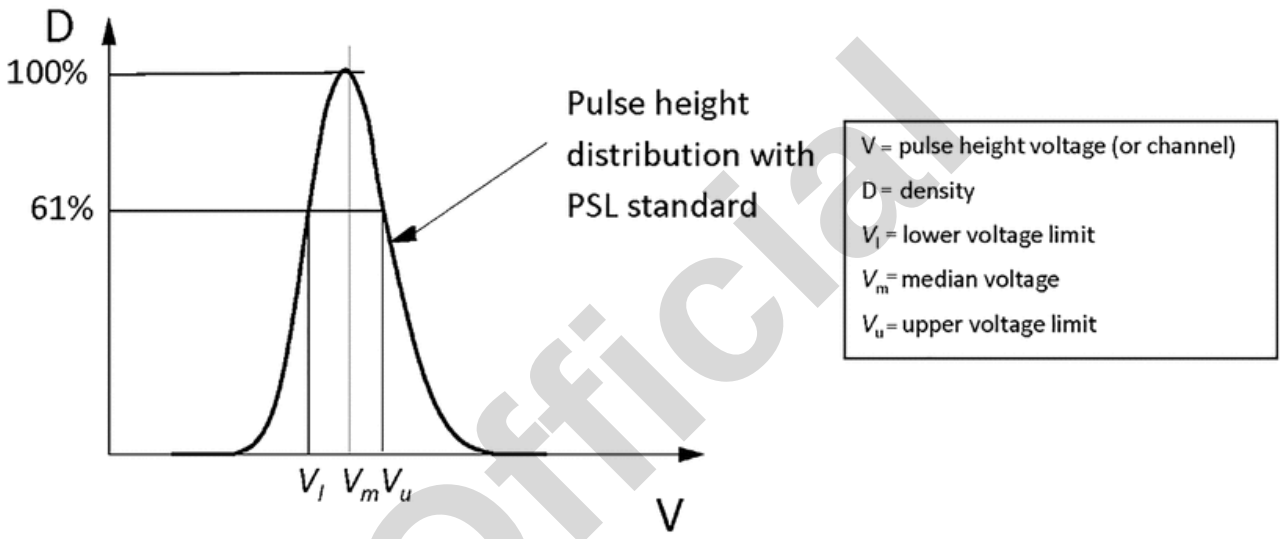


Figure 4. Sensor resolution.

The median voltage (or internal PHA channel), $V_m$, is determined. The voltages (or channels) corresponding to 61% density of the median voltage ($V_m$) are defined as the lower voltage limit ($V_l$) and the upper voltage limit ($V_u$). The particle sizes corresponding to $V_l$ and $V_u$ are determined from the calibration curve. The absolute differences in size of the nominal particle size from these sizes represent the upper and lower standard deviation. The greater of these represents the experimentally determined standard deviation, σ. The percentage size resolution, *R*(%), of the LSLPC is obtained using *Equation 2*. The recommended size resolution is NMT 15%.

$$R(\%) = \frac{\sqrt{\sigma^2 - \sigma_p^2}}{x_p} \times 100 \quad (2)$$

σ = experimentally determined standard deviation of LSLPC (µm)
$\sigma_p$ = supplier-reported standard deviation of calibration particles (µm)
$x_p$ = size of the calibration particle (µm)

## 5.5 Sampling Volume (For Liquid Counters Only)

An accurate sampling volume is critical for an accurate concentration determination. The procedure is the same as in ⟨1788⟩. The acceptable error of the sampling volume shall be NMT 5% of the preset value.

## 5.6 Sampling Flow Rate

Flow rate is important for the measurement, especially for the airborne counters, and should match the flow rate of the calibration that was performed. The acceptable error in the sampling flow rate shall be specified by the manufacturer, and the user should verify that the sampling flow rate is within the range specified by the manufacturer at the time of use.

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### 5.7 Calibration Interval

The recommended calibration interval of an LSLPC, LSAPC, or LSAS is one year or less or as described by the manufacturer.

***Change to read:***

## 6. ADDITIONAL SOURCES OF INFORMATION


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- ISO 9276-1, Representation of results of particle size analysis—Part 1: Graphical representation.
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***Add the following:***

# ▲⟨1430.7⟩ ANALYTICAL METHODOLOGIES BASED ON SCATTERING PHENOMENA—NEPHELOMETRY AND TURBIDIMETRY

1. INTRODUCTION
2. THEORY
    2.1 Nephelometry and Turbidimetry
    2.2 Rate Nephelometry
    2.3 Rate Turbidimetry
3. INSTRUMENTATION
    3.1 Rate Nephelometry Instrumental Parameter Settings
    3.2 Calibration of Rate Nephelometric Instruments
4. APPLICATIONS
5. METHOD DEVELOPMENT
    5.1 Method Design
    5.2 Sample Considerations
    5.3 Reagents
    5.4 Method Specific Reference Standards
    5.5 Controls
    5.6 Robustness
6. METHOD VALIDATION
7. DATA ANALYSIS
8. ADDITIONAL SOURCES OF INFORMATION

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## 1. INTRODUCTION

Nephelometry and turbidimetry are based on the direct (nephelometry) or indirect (turbidimetry) measurement of the scattered light intensity of an incident light beam from a monochromatic light source. In the rate nephelometry technique, the change of the scattered light intensity is measured as a function of time. Turbidimetry or nephelometry may be useful for the measurement of precipitates formed by the interaction of highly dilute solutions of reagents or other particulate matter such as suspensions of bacterial cells. All variables must be carefully controlled to achieve consistent results. Where such control is possible, extremely dilute suspensions may be measured.

## 2. THEORY

### 2.1 Nephelometry and Turbidimetry

For all relevant terms and definitions see *Nephelometry and Turbidimetry* ⟨855⟩. Both techniques are governed by the same equation, which describes the (time-averaged) intensity of the light beam as it interacts with matter along its path, i.e., when the incident light detected at an angle of zero decreases exponentially with the thickness, $x$, of the layer of material. See *Equation 1*. (For more information, see *Analytical Methodologies Based on Scattering Phenomena—General* ⟨1430⟩ and ⟨855⟩.)

$$I = I_0 e^{-\tau x} \quad (1)$$

### 2.2 Rate Nephelometry

As discussed above, the scattered light intensity when a sample is submitted to a constant incident monochromatic light beam depends on the number and size of the particles that scatter the light. If either or both of these factors change over time as a result of a chemical or physical process, the cumulative scattered light intensity at a given direction (usually 90° to the incident light direction) will change accordingly. Thus, rate nephelometry measures the change, or rate of change, in the intensity of light scattered during the formation of complexes (e.g., antigen-antibody) in solution. A correlation between the physicochemical changes and the scattered light intensity can be established and used to evaluate the property changes in the samples under test.

The extent of formation of light scattering complexes depends on concentrations of complex formation entities, e.g., antibodies and antigen molecules for immunonephelometry. The maximum rate response occurs when the antibody and antigen are in optimal proportions. Generally a fixed amount of antibody is used. Standard applications typically use 670-nm lasers with the detector aligned at 90° to the incident light direction, and the scattered signal is measured at 5-s intervals. System software calculates the rate of change at the end of reaction. The rate of change is converted to concentration by the system software.

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## 2.3 Rate Turbidimetry

Rate turbidimetric methods measure the decrease in intensity of light as it passes through a solution while complexes form. The light source is a light emitting diode (LED) at 940 nm. Turbidimetric measurements are made at 0° from the incident beam. The intensity decrease is converted to an increasing scatter signal by *Equation 2*.

$$\text{Scatter signal} = -\log_{10}\left|\frac{\text{light intensity}}{\text{initial light intensity}}\right| \quad (2)$$

# 3. INSTRUMENTATION

A schematic diagram and principles of operation of a nephelometric (turbidimetric) instrument are shown in *Nephelometry and Turbidimetry* ⟨855⟩, *Figure 1*. A typical instrumental design dedicated for rate nephelometric measurement is shown below in *Figure 1*. The main components are: a light source that is usually a laser (635 nm) with a highly collimated beam to avoid stray light, a sample cell, and a light detector. The laser beam passes through the sample well into a scattered light detector assembly. Typically an integrating sphere (Ulbricht sphere), which helps improve the signal-to-noise ratio and enhance the signal for very low concentrations, is attached to a photodiode and used as the detector in rate nephelometry instruments. The unscattered light (light not deflected by particles) passes straight through the sphere and no signal is generated. Light scattered by particles is reflected around the interior of the sphere and then detected by the photodiode detector. The angle of detection of the scattered light is usually 90°, which is, in general, suitable for smaller aggregates ($d \leq \lambda/20$). A light trap is attached to the Ulbricht sphere at the opposite side of the light beam entrance window to absorb the unscattered light. The amount of scattered light is proportional to the concentration of particles. Some instruments can perform both rate nephelometry and rate turbidimetry measurements. These instruments are equipped with a laser source at 670 nm with 90° scatter detection for nephelometric measurements and an LED source at 940 nm with 0° scatter detection for turbidimetric measurements.

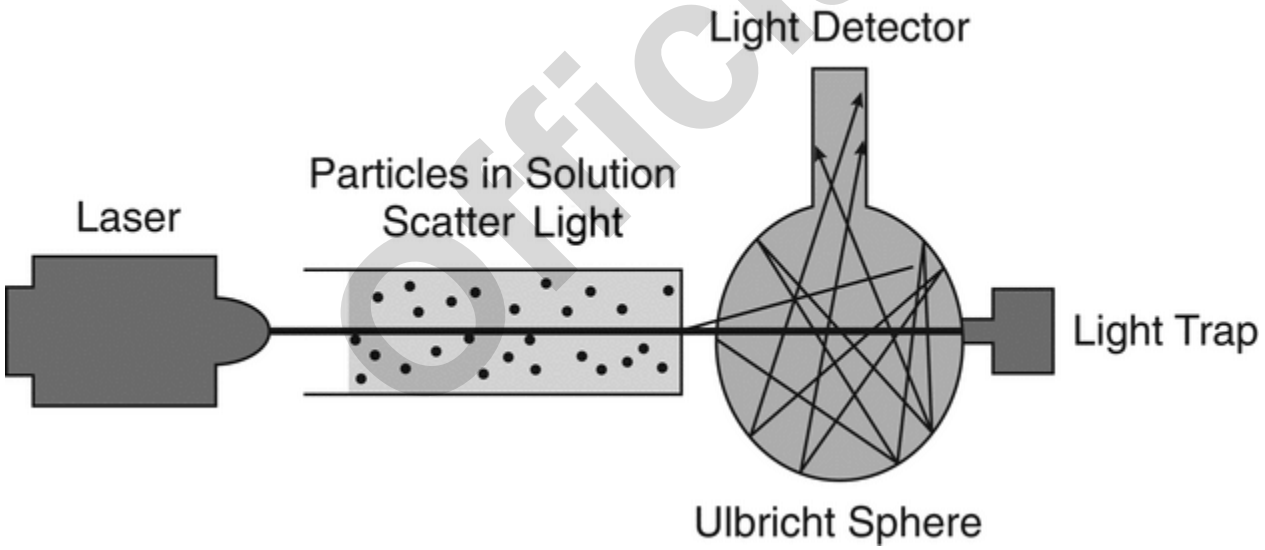


Figure 1. Schematic of a typical nephelometer.

## 3.1 Rate Nephelometry Instrumental Parameter Settings

The most important rate nephelometric system parameter settings are:

- **Acquisition time:** Reaction cuvettes, housed on a reaction wheel, are scanned in cycles of 5 s, for total reaction times ranging from 1.5 to 10 min. Acquisition time commences upon completion of a 5-min preset baseline acquisition period, also in 5-s cycles. Most reactions in nephelometric applications achieve peak rates within 2 min, whereas those used in turbidimetric applications require longer reaction times (3 min or longer).
- **Gain settings:** These settings are related to signal amplification. Generally, decreasing analyte concentration requires a concomitant increase in gain to maintain sensitivity.
- **Onboard compartment temperatures:** Reaction wheel temperature is maintained at 37° to enhance aggregate formation. The reagent compartment is cooled to extend stability of antibody reagents, whereas the sample compartment temperature is ambient.
- **Laser beam width and intensity adjustment:** If applicable, the laser beam width and intensity are adjustable in some instruments and can be optimized to obtain the best scattered signal response.

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### 3.2 Calibration of Rate Nephelometric Instruments

Calibration of the rate nephelometer is typically a multitier process consisting of preprogrammed and user-defined components. The preprogrammed component is executed with each instrument run and is comprised of measurements of an onboard reference cuvette of known scatter values. The user-defined component relies on protein controls. Certified protein controls are routinely used for initial instrument calibration and may be utilized for periodic instrument performance qualification. For method calibration and performance monitoring, specially formulated product-relevant reference materials are used.

Data assessment is accomplished by comparison against the control manufacturer's published range, peer data, and/or internally established acceptance criteria. Each analytical run typically includes a calibration curve of reference material and, where applicable, a single concentration of control, each selected to represent the product of interest. The validity of an instrument run is dependent on satisfaction of acceptance criteria established through method development and statistical analysis of temporal method performance.

## 4. APPLICATIONS

⟨855⟩ gives a general overview of the applications of turbidimetric and nephelometric techniques including the rate nephelometry and rate turbidimetry (see *Nephelometry and Turbidimetry* ⟨855⟩, *3. Applications*). The following discussions represent additional and more detailed information on the applications of rate nephelometry and rate turbidimetry in biopharmaceutical industry and clinical laboratories, which are summarized in *Table 1*.

In bacterial polysaccharide-containing vaccine analysis applications, the reaction of excess of (bivalent, polyvalent) monoclonal or polyclonal antibodies with high molecular weight bacterial polysaccharides—such as those from *Haemophilus influenzae* type b (Hib vaccine), *Neisseria meningitidis* (meningococcal vaccine) or *Streptococcus pneumoniae* (pneumococcal vaccine)—causes crosslinking and the formation of high molecular weight aggregates that scatter light. Therefore, the rate at which aggregates develop allows the polysaccharides, free or conjugated, to be quantified. Endpoint nephelometry is an alternative application in which the endpoint of the reaction is determined in the analysis.

In the case of multivalent vaccines, the specificity of the antibody used allows the selective detection and quantification of a specific polysaccharide, and the use of different antibodies with different specificities allows multiple components to be quantified.

Immunonephelometry for biologics analysis is based on antibody-antigen immune complex formation (immunoprecipitation). Formation of the immunoprecipitation is dependent on the presence of the antigen and antibody molecules in optimal proportions. Quantitative methods are based on the principle that when the amount of antibody is held at a constant excess, the degree of immunoprecipitation will increase with increasing antigen content up to a maximal amount. A standard curve can be generated by maintaining a constant level of antibody and measuring the light scatter with increased concentrations of antigen. Due to the reliance upon antibody and antigenic epitope recognition, the above applications are employed for characterization purposes, monitoring of functional group content, stability, and assessment of lot-to-lot consistency, i.e., for quantification of the antigenic saccharide content in components and/or products of vaccine manufacture. Such components and/or products include: (a) bulk monovalent glycoconjugate immunogens; (b) bulk polyvalent glycoconjugate immunogen blends; (c) bulk polyvalent polysaccharide blends; (d) glycoconjugate vaccine final fills; and (e) polysaccharide vaccine final fills. Rate nephelometric analysis of the multicomponent product is generally employed in conjunction with direct saccharide quantitation of the individual components by orthogonal methods.

Turbidimetry is widely used for measurement of activity in recombinant coagulation factor and other hemophilia replacement therapies, as well as for patient monitoring in clinical hemostasis laboratories. The turbidimeter monitors the transmittance of light at 670 nm as fibrin converts into fibrinogen during clot formation. Calibrators in the form of reference plasmas, generally traceable to World Health Organization standards, serve for instrument calibration and may be used for relative activity determination for the analyzed product.

**Table 1. Examples of Analytical Applications**

| Turbidimetry | Nephelometry |
|---|---|
| Immunoagglutination assay: detection of Chagas disease | Polysaccharide quantitation: pneumococcal vaccine |
| Multiple latex immunoagglutination assays | Polysaccharide quantitation: meningococcal vaccine |
| Fibrinogen assays | Relative molecular size (nephelometry coupled with high pressure size exclusion chromatography) |

## 5. METHOD DEVELOPMENT

Thorough method development encompasses designing the method to fit the application through assessing a complex set of conditions including choice of reagents, standards, and controls; sample handling; and instrument settings. The following areas should be considered during method development in order to determine operating ranges and limits.

### 5.1 Method Design

Competitive versus noncompetitive assay modes are available. The option chosen will be dictated by the sample molecular weight/size and concentration of analyte to be analyzed; where noncompetitive immunoprecipitation mode is preferred for

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molecular weight (MW) > $10^4$ Da. Sample concentration directs the selection of instrument gain, where lower concentrations require higher gain levels.

For each method, the optimal acquisition time, sequence of administration of components, and gain level must be determined.

Sample volumes will dictate the type and size of sample containers.

A precision study should be performed during development to determine the replication testing scheme for samples in order to obtain the desired method precision (see *Analytical Data—Interpretation and Treatment* ⟨1010⟩).

A response curve between the analyte concentration and measured light scattering unit (LSU) values (or antigenicities calculated from LSU values) is prepared from standard solutions at concentrations encompassing the anticipated concentration of the test solution. The standard curve is then evaluated using appropriate statistical methods, such as least-squares regression.

## 5.2 Sample Considerations

Care must be taken to ensure samples, as with all other components, are essentially free from particulates and air bubbles in order to minimize nonspecific contributions to light scattering and liquid level sensing errors, respectively. Analytes such as monovalent polysaccharide-protein conjugates are diluted in several steps to a single point targeting the midpoint of an established standard curve.

Samples that contain adjuvants require solubilization of the adjuvant, carefully controlled and replicated with the reference material. Select applications, such as the specific analysis of adjuvant-bound antigenicity or the separation of conjugated antigenicity from free antigenicity, may require additional manipulations such as pH adjustment, preadsorption with Excel adjuvant, or temperature treatment prior to nephelometric analysis. If adjuvant solubilization is used for the sample, then the respective reference material must be subjected to the same treatment. Solubilization should be tightly controlled to minimize variability.

Specific pretreatments may need to be developed depending on the type of antigen. For example, polysaccharide-carrier protein conjugates may be analyzed for bound, conjugated, free, or total polysaccharide content. Example pretreatments include pH adjustment, adsorption with excess adjuvant, temperature treatment, and physical separation through centrifugation.

Cross-reactivity between component analytes may also need to be addressed during reagent selection (see the antibody discussion under *5.3 Reagents*).

## 5.3 Reagents

The choice of reaction buffer is dictated by compatibility with the analyte formulation buffer. Presence of aggregation enhancers or inhibitors should be considered. Interactions with containers such as glass test tubes may need to be considered. Detergent may be required for certain analytes, and the type and concentration must be determined experimentally.

Polyclonal antibodies are preferred for rate nephelometry as they are typically optimal for appropriate lattice formation; although monoclonal antibodies are frequently required for detection of specific functional groups. All antibodies must be sufficiently specific for the analysis of individual product components and of appropriate titer and avidity.

## 5.4 Method Specific Reference Standards

Reference materials are carefully selected from process representative batches, formulated, and packaged to optimally mimic the analyte of interest. Reference samples and standards must be treated equally.

## 5.5 Controls

Both positive and negative controls are likewise selected as product representative materials that may be formulated for extended stability via freezing or lyophilization.

## 5.6 Robustness

Method robustness assessments should include the following:

- Instrument parameters, e.g., gain (signal amplification), reaction time (interval between addition of last reagent and measurement)
- Concentration optimums
  - Antigen
  - Antibody
- Mix times
- Selectivity (lack of cross-reactivity) of antibodies
- Reagent use/hold times
- Type of ions, ionic strength of solution

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## 6. METHOD VALIDATION

In addition to generally applicable validation characteristics as described in *Validation of Compendial Procedures* ⟨1225⟩, the following aspects are recommended for consideration in the context of nephelometry and/or turbidimetry:

- **Accuracy:** Quantitation of polysaccharides determined by assessment of known values generated with an orthogonal validated assay.
- **Linearity:** Should be assessed in both reference standards and analyte samples.
  - Standard curve required for each type of polysaccharide being assessed.
  - Standard polysaccharide concentration determined by alternative validated method (e.g., meningococcal serogroup C polysaccharide concentration determined by the sialic acid colorimetric method of Svennerholm.)
- **Lifecycle considerations:** Appropriate performance parameters are trended over time and utilized for bridging of new reference standards and for assessment of period of use of antibodies/reference standards/controls.
- **System suitability:** Positive control is assessed at the validated limits of adjustable instrument parameters. Results for the positive control must be within its defined range.

## 7. DATA ANALYSIS

A calibration curve is generated from the duplicate readings for the reference standard versus the standard concentration (from a validated orthogonal assay, e.g., nephelometric polysaccharide quantitation standard values from suitable sugar-specific colorimetric methods, i.e., hexose, uronic acid, sialic acid, or PABA assays.)

- Percent difference between duplicate readings for standards, controls, and samples is calculated.
- Antigenicity is calculated, in μg/mL, using sample LSU values and the slope and the intercept of the standard curve as shown in *Equation 3*:
  Antigenicity = $(\bar{A}_v - y_{int})/S$ (3)

$\bar{A}_v$ = average sample values (LSU)
$y_{int}$ = *y*-intercept (LSU)
$S$ = slope$\left(\frac{\text{LSU} \cdot \text{mL}}{\mu\text{g}}\right)$

Acceptance criteria for replicate measurements of each standard solution and/or sample (in LSU) should be developed such that valid results meet the desired accuracy and precision of the method, e.g., linearity, slope, and intercept may be monitored and assessed against acceptance criteria during routine laboratory use. Acceptance criteria shall be established for slope and linearity, satisfactory precision of replicates, minimum and maximum response for matching (positive control), and baseline (negative control).

## 8. ADDITIONAL SOURCES OF INFORMATION


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▲ (USP 1-Dec-2020)

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# ⟨1430⟩ ANALYTICAL METHODOLOGIES BASED ON SCATTERING PHENOMENA—GENERAL

1. OVERVIEW: GENERAL CHAPTERS BASED ON SCATTERING PHENOMENA
2. INTRODUCTION
3. THEORY (GENERAL PRINCIPLES OF SCATTERING)

***Change to read:***

## 1. OVERVIEW: GENERAL CHAPTERS BASED ON SCATTERING PHENOMENA

This chapter provides a general overview of the scientific principles and analytical procedures used in scattering techniques and their applications. *Figure 1* shows a taxonomy (tree diagram) of the techniques that are covered in this chapter. The first branch classifies the techniques according to the wavelength of the incident electromagnetic (EM) radiation: visible light (700–400 nm), X-rays (0.33–0.06 nm), as well as neutrons (2.5–0.15 nm). The next branch classifies techniques into those based on the measured intensities (i.e., scattering), where the radiation exiting the sample is measured at an angle relative to the incident beam, or transmission, where this angle is equal to zero. Scattering techniques can be further classified based on how the exiting radiation is quantified, in either a time-averaged (static) or time-dependent (dynamic) mode. Finally, because scattering techniques are often coupled with separation techniques, such as chromatography or field flow fractionation, these are included as well.

Two of the most important parameters of the EM radiation–matter interaction are the wavelength of the incident EM radiation ($\lambda = \lambda_0/m_0$, where $\lambda_0$ is the wavelength in vacuo and $m_0$ is the refractive index of the medium) and the size of the particle, often expressed as an equivalent spherical radius ($r$). A dimensionless parameter ($\alpha = 2\pi r/\lambda$) can be used to describe different scattering regimes. For $\alpha \ll 1$, the scattering is considered as being in the Rayleigh regime and the Rayleigh scattering theory applies (named after Lord Rayleigh, who developed the theory in 1871); for $\alpha \sim 1$, the scattering is considered as being in the Mie regime and the Mie scattering theory applies (named after Gustav Mie, who developed the theory in 1908); and for $\alpha \gg 1$, geometric optics apply. These theories are further addressed in the respective chapters where they are applied. *Table 1* lists all chapters whose fundamental physical principles are addressed in this overarching chapter. These chapters deal with elastic light scattering in heterogeneous systems and its applications in the pharmaceutical industry.

**Table 1. List of ⟨1430.X⟩ General Chapters Family**

| Chapter Number | Chapter Title | Measured Property | Primary Purpose |
|---|---|---|---|
| ⟨1430⟩ | *Analytical Methodologies Based on Scattering Phenomena—General* | Overarching chapter | General overview; N/A |
| ⟨1430.1⟩ | *Analytical Methodologies Based on Scattering Phenomena—Static Light Scattering* | Scattered light intensity as a function of detector's angle | Molecular weight, size, shape; molecular interactions |
| ⟨1430.2⟩ | *Analytical Methodologies Based on Scattering Phenomena—Light Diffraction Measurements of Particle Size* | Diffracted light intensity at multiple angles | Particle size distributions in the approximate range of 0.01–3000 µm |
| ⟨1430.3⟩ | *Analytical Methodologies Based on Scattering Phenomena—Dynamic Light Scattering* | Fluctuations of the scattered light intensity | Average hydrodynamic diameter and polydispersity index |
| ⟨1430.4⟩ | *Analytical Methodologies Based on Scattering Phenomena—Electrophoretic Light Scattering (Determination of Zeta Potential)* | Doppler shift of scattered light frequency | Stability of suspensions and emulsions |
| ⟨1430.5⟩ | *Analytical Methodologies Based on Scattering Phenomena—Small-Angle X-Ray Scattering and Small-Angle Neutron Scattering* | Scattered intensity of a beam of X-rays or neutrons | Direct probing of the size, shape, ordering, and interactions of individual molecules, their assemblies in the approximate length scales from 0.1–2500 nm for SAXS and 0.2–1000+ nm for SANS. Also properties of condensated phases (e.g., porosity and crystallinity) can be determined. |
| ⟨1430.6⟩▲▲ (ERR 1-Oct-2021) | *Analytical Methodologies Based on Scattering Phenomena—Particle Counting via Light Scattering* | Light scattered from individual particles passing through a light beam | Quantitation of particles in liquids and gases from 0.1–2 µm |
| ⟨1430.7⟩▲▲ (ERR 1-Oct-2021) | *Analytical Methodologies Based on Scattering Phenomena—Nephelometry and Turbidimetry* | Direct or indirect measurement of the scattered light intensities | Suspended particles in liquid or gas samples; nephelometry for lower aggregate sizes and analyte concentrations; turbidimetry for higher aggregate sizes and analyte concentrations |

Because these methods are based on the same underlying physics, the general principles are given only once in this overarching chapter. The relationships between these methods are shown in *Figure 1*.

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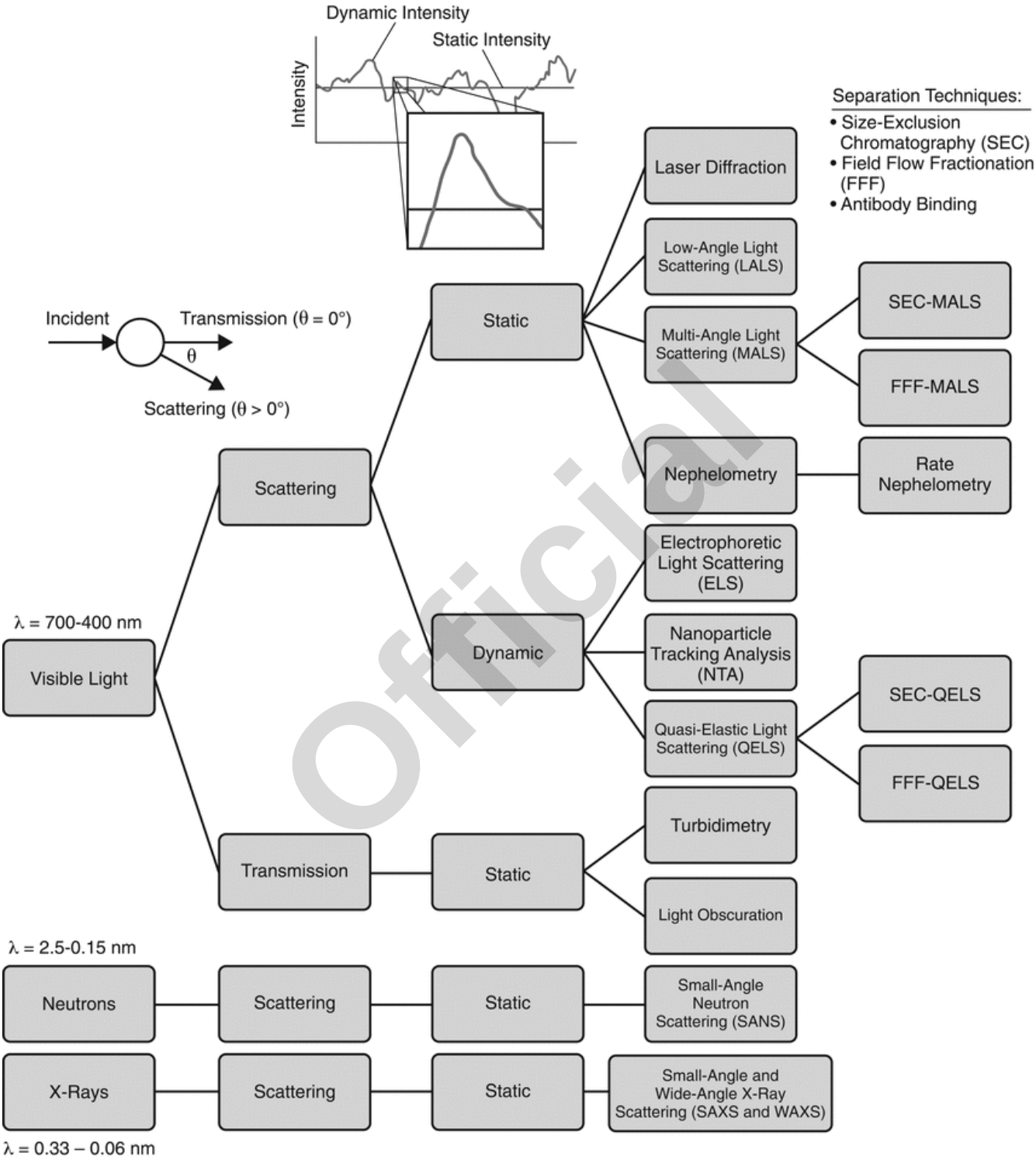


Figure 1. Taxonomy of scattering methods.

***Change to read:***

## 2. INTRODUCTION

When an EM wave strikes/interacts with a small object (a particle or a molecule) and thereby changes its direction, the phenomenon is called scattering. If the scattered EM radiation has exactly the same energy (wavelength) as the incident one, it is called elastic scattering. When the energy (wavelength) of the scattered EM radiation is different from that of the incident

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EM radiation, the scattering process is termed "inelastic". Inelastic scattering is exploited, as an example, by Raman spectroscopy (refer to *Raman Spectroscopy—Theory and Practice* ⟨1858⟩). Scattered radiation may be detected and measured either directly: as a function of the angle between the incident beam direction and the detector, or indirectly: when the actual measurement is that of the transmitted light. The latter is the case for both turbidity and light obscuration methods (see the following chapters on turbidity and light obscuration: *Nephelometry, Turbidimetry, and Visual Comparison* ⟨855⟩, *Subvisible Particulate Matter in Therapeutic Protein Injections* ⟨787⟩, *Particulate Matter in Injections* ⟨788⟩, and *Methods for the Determination of Subvisible Particulate Matter* ⟨1788⟩). Both methodologies exploit time averaged signals.

In static light scattering (SLS), the inevitable short-term temporal fluctuations in scattering intensity due to Brownian motion are averaged over a range of times from tens to hundreds of milliseconds. SLS therefore measures the "time average" intensity of scattered light from particles in a (suitably prepared) sample. Depending on the specific technique, SLS provides information about molecular weight, particle size, particle shape, and molecular interactions (see *Analytical Methodologies Based on Scattering Phenomena—Static Light Scattering* ⟨1430.1⟩. Light scattered in the near-forward direction by particles is analogous to diffraction of light through an aperture. This is exploited by (laser) diffraction techniques, which are optimized to afford the derivation of a full size distribution, with moderate to high resolution, rather than a single characteristic size as in low-angle light scattering (LALS)/multi-angle light scattering (MALS).

Small-angle X-ray scattering (SAXS) and small-angle neutron scattering (SANS) are also static techniques in that they make use of time averaged signals. These differ from LALS (and MALS) in that they are based on scattering of X-rays (SAXS) or neutrons (SANS) rather than visible light. Like SLS, these techniques also measure size, shape, and interactions but on much shorter length scales, typically ranging from around 1 nm to several hundred nanometers. Very/ultra small-angle (VSAXS/VSANS, USAXS/USANS) and wide-angle (WAXS/WANS) analogues extend these length scales to the micrometer and sub-nanometer regimes, respectively. These applications are discussed in *Analytical Methodologies Based on Scattering Phenomena—Small-Angle X-Ray Scattering and Small-Angle Neutron Scattering* ⟨1430.5⟩.

Dynamic light scattering (DLS), which is addressed in *Particle Size Analysis by Dynamic Light Scattering* ⟨430⟩[1] and *Analytical Methodologies Based on Scattering Phenomena—Dynamic Light Scattering* ⟨1430.3⟩, differs from SLS in that DLS measures the fluctuation of the scattered light intensity over very short time intervals (e.g., approximately 200, 400 ns).

Electrophoretic light scattering (determination of zeta potential), which is addressed in more detail in *Determination of Zeta Potential by Electrophoretic Light Scattering* ⟨432⟩ ▲▲ (ERR 1-Oct-2021) and *Analytical Methodologies Based on Scattering Phenomena—Electrophoretic Light Scattering (Determination of Zeta Potential)* ⟨1430.4⟩, measures the Doppler shift of the frequency of scattered light as the result of particle movements from the cumulative effect of electrophoresis and electroosmosis.

## 3. THEORY (GENERAL PRINCIPLES OF SCATTERING)

Light/EM scattering is the result of a complex interaction between incident light/EM waves and matter. An EM wave that is redirected, i.e. changes direction, when it encounters obstacles such as a molecule or molecular aggregates, or particles, is said to have been scattered. As the EM radiation, such as light or X-rays, interacts with a discrete particle, the electron orbits within the particle's constituent atoms or molecules are perturbed periodically with the same frequency ($\nu_0$) as the electric field of the incident wave. The oscillation or perturbation of the electron cloud results in a periodic separation of charge within the molecule, which is called an induced dipole moment. The oscillating induced dipole moment manifests itself as a source of EM radiation (see *Figure 2*). Neutrons, on the other hand, are always scattered by a very short-range nuclear interaction with the nuclei in the atoms.

[1] This chapter ▲appeared in issue 46(3) of the▲ (ERR 1-Oct-2021) *Pharmacopeial Forum* (*PF*). ▲▲ (ERR 1-Oct-2021)

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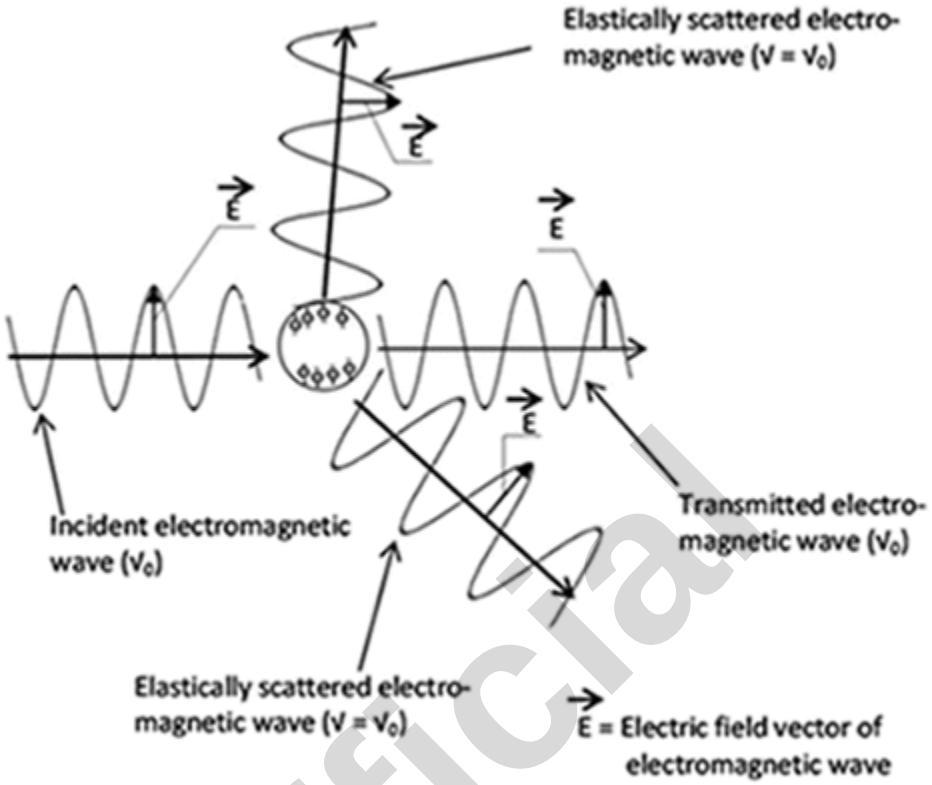


Figure 2. Scattering of an incident EM wave by an induced dipole moment.

The (time averaged) intensity ($I$) of the light beam as it interacts with matter along its path (i.e., when the incident light is detected at an angle of zero) decreases exponentially with the thickness, $x$, of the layer of material as follows in *Equation 1*:

$$I = I_0 e^{-\tau x} \qquad (1)$$

where $\tau$ is the turbidity and $x$ is the distance that the incident light travels through the sample.

This equation is the basis for turbidimetry and nephelometry (see ⟨855⟩).

In SLS, the theoretical description of the relationship between the (time averaged) intensities of incident and scattered light, although quite complex, originates from the following simple relationship (*Equation 2*).[2]

$$I = I_0 \frac{1}{r^2} \sigma_{scat} \qquad (2)$$

The scattering coefficient, $\sigma_{scat}$, and with it the intensity of the scattered light depend on factors determined by the interaction of the incident light with the material/particles itself as well as those stemming from the equipment used for generating and detecting the signal (e.g., $r$, the distance of the detector from the sample; $I_0$, the intensity of the incident light; and, $\Theta$, the angle of observation).

On the other hand, when studying the fluctuations in scattered intensity (DLS), the way to extract useful, quantitative information is by calculating the so-called "autocorrelation function", denoted $g_2(\tau)$ based on the measured intensities (*Equation 3*):

$$g_{2,\text{theory}}(\tau) = (\Sigma A_i e^{B_i \tau})^2 + 1 \qquad (3)$$

where in this case, $\tau$ denotes a correlation time (not to be confused with the $\tau$ in *Equation 1* above), and the use of the index, $i$, accounts for different particle types (e.g., different sizes) present. A computer program is used to find the parameters $A_i$, $B_i$ (= $A_1$, $B_1$, $A_2$, $B_2$,…) that produce the best agreement between the measured and theoretical autocorrelation function.

Depending on the methodology, the equations above are transformed to obtain a measure that relates the measured intensities, $I$ and $I_0$, respectively, and the autocorrelation function $g_2(\tau)$ to the material itself, independent of equipment characteristics. These are further discussed in the specific chapters in the *USP–NF* dedicated to the corresponding techniques (see *Table 1*).

[2] Hahn DW. *Light Scattering Theory*. University of Florida; 2009. http://plaza.ufl.edu/dwhahn/Rayleigh%20and%20Mie%20Light%20Scattering.pdf.

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# ⟨1467⟩ RESIDUAL SOLVENTS—VERIFICATION OF COMPENDIAL PROCEDURES AND VALIDATION OF ALTERNATIVE PROCEDURES

The procedures defined in *Residual Solvents* ⟨467⟩ have been validated for Class 1 and 2 solvents over the range of 50%–150% of the concentrations described in the standard preparation for procedures A and B in *Residual Solvents* ⟨467⟩*, 8. Analytical Procedures for Class 1 and Class 2 Residual Solvents*.

The suitability of these residual solvent procedures must be verified under actual conditions of use. When using the procedures for testing residual solvents described in ⟨467⟩, verification can be accomplished using the recommendations below. There are separate recommendations for limit and quantitative procedures in *Residual Solvents* ⟨467⟩*, 8. Analytical Procedures for Class 1 and Class 2 Residual Solvents*.

Alternative procedures include different analytical procedures from those described in ⟨467⟩, as well as modifications to the procedures described therein that go beyond the stated validation of the method (e.g., different concentrations or other analytes or variations to chromatographic conditions beyond those permitted by *Chromatography* ⟨621⟩). Alternative procedures are permitted (see *General Notices, 6.30 Alternative and Harmonized Methods and Procedures*), provided that they are properly validated. A risk-based approach may be appropriate to determine the degree and extent of the verification or validation process to assure the fitness for purpose of the procedure. Recommendations for validation of alternative procedures (limit and quantitative tests) are described in *Validation of Alternative Procedures*. A summary of the requirements is shown in *Table 1*.

**Table 1. Summary of Verification and Validation Requirements**

| | Verification of Compendial Procedures | | Validation of Alternative Procedures | |
|---|---|---|---|---|
| **Validation/Verification Characteristics** | **Limit Test Methods** | **Quantitative Methods** | **Limit Test Methods** | **Quantitative Methods** |
| Specificity | Yes | Yes | Yes | Yes |
| Detection limit | Yes[a] | No | Yes | No |
| Quantitation limit[b] | No | Yes[a] | No | Yes |
| Accuracy | No | Yes | No | Yes |
| Precision/repeatability | No | Yes | No | Yes |
| Linearity | No | No | No | Yes |
| Range | No | Demonstrated by accuracy and precision | No | Demonstrated by accuracy and precision |
| Intermediate precision | No | No | No | Yes |
| Solution stability[c] | Yes | Yes | Yes | Yes |
| Robustness[d] | No | No | Yes[e] | Yes[e] |

[a] System suitability may be used instead to demonstrate sensitivity.
[b] In quantitative tests, quantitation limit may be demonstrated by accuracy and precision determination.
[c] Solution stability should be determined for the timeline of the test.
[d] Evaluation of robustness should be considered during the development phase and depends on the type of procedure under study.
[e] The "Yes" quotation here is intended to emphasize the importance of assessing the robustness of the procedure before the implementation.

***Change to read:***

## VERIFICATION OF COMPENDIAL PROCEDURES

### Limit Procedures: Procedure A and Procedure B

The analytical characteristics to be verified include specificity, detectability, and solution stability.

VERIFICATION WHEN SOLVENTS LIKELY TO BE PRESENT (LTBP) ARE NOT KNOWN

**Samples**

Prepare a reagent blank.

Prepare *Standard solutions* as described in *Residual Solvents* ⟨467⟩*, 8. Analytical Procedures for Class 1 and Class 2 Residual Solvents, 8.2 Screening of Water-Soluble Articles* or *8.3 Screening of Water-Insoluble Articles*.

Prepare the *Spiked sample solution* as described in *Class 1 System suitability solution* in *Residual Solvents* ⟨467⟩*, 8. Analytical Procedures for Class 1 and Class 2 Residual Solvents, 8.2 Screening of Water-Soluble Articles* or *8.3 Screening of Water-Insoluble Articles*.

**Specificity**

*Recommended acceptance criteria:* An appropriate blank should be injected to assure the lack of a significant interference. A significant interference is one producing a deviation in the fitness for purpose of the procedure that affects precision and/or

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accuracy. The procedure must be able to separate acetonitrile and methylene chloride (*Procedure A* in *Residual Solvents* ⟨467⟩, *8. Analytical Procedures for Class 1 and Class 2 Residual Solvents, 8.2 Screening of Water-Soluble Articles* or *8.3 Screening of Water-Insoluble Articles*), or ▲methylisobutylketone▲ (ERR 1-Dec-2020) and *cis*-dichloroethene (*Procedure B* in *8. Analytical Procedures for Class 1 and Class 2 Residual Solvents, 8.2 Screening of Water-Soluble Articles* or *8.3 Screening of Water-Insoluble Articles*) with a resolution of NLT 1.0.

**Detection Limit (see** *Table 1, Footnote a***)**

*Recommended acceptance criteria:* The mean signal-to-noise ratio for each solvent in the *Standard solution* and *Spiked sample solution* [after correction for native (original) solvent content] from at least three determinations from a single preparation is NLT 3.

**Solution Stability**

*Recommended acceptance criteria:* Detection limit should meet the requirements throughout the testing period.

VERIFICATION WHEN SOLVENTS LTBP ARE KNOWN

**Samples**

Prepare a reagent blank.

Prepare *Standard solution(s)* and *Sample solution(s)* as described in *Residual Solvents* ⟨467⟩, *8. Analytical Procedures for Class 1 and Class 2 Residual Solvents, 8.2 Screening of Water-Soluble Articles* or *8.3 Screening of Water-Insoluble Articles* depending on the specific sample solubility using only those solvents likely to be present (LTBP).

Prepare *Spiked sample solution(s)* as described in *Quantitative Procedures: Procedure C* for solvents LTBP [corrected for native (original) solvent content].

**Specificity**

*Recommended acceptance criteria:* The reagent blank does not produce any significant interference with any of the peaks from solvents LTBP.

The procedure must be able to separate each of the solvents in the *Standard solution(s)* from each other and from other peaks in the *Spiked sample solution* with a resolution of NLT 1.0. If the resolution between any pair of peaks is less than 1.5, then verification must demonstrate that the method is suitable for its intended use.

If the solvents present in the *Standard solution(s)* are not separated with a resolution of NLT 1.0 when using the *Chromatographic System* of *Procedure A*, then *Procedure B* in *Residual Solvents* ⟨467⟩, *8. Analytical Procedures for Class 1 and Class 2 Residual Solvents, 8.2 Screening of Water-Soluble Articles* or *8.3 Screening of Water-Insoluble Articles* should be used as confirmatory.

**Detection Limit**

*Recommended acceptance criteria:* The mean signal-to-noise ratio for each solvent in the *Standard solution* and the *Spiked sample solution* [after correction for native (original) solvent content] from at least three determinations from a single preparation is NLT 3.

**Solution Stability**

*Recommended acceptance criteria (not required if only running fresh solutions):* Detection limit for limit tests and quantitation limit for quantitative tests should be met throughout the testing period.

## Quantitative Procedures: Procedure C

[NOTE—When performing *Residual Solvents* ⟨467⟩, *8. Analytical Procedures for Class 1 and Class 2 Residual Solvents, 8.5 Quantitative Tests—Procedure C*, solvents LTBP are typically known, either based on results from *Procedure A* or *Procedure B* in *Residual Solvents* ⟨467⟩, *8. Analytical Procedures for Class 1 and Class 2 Residual Solvents, 8.2 Screening of Water-Soluble Articles* or *8.3 Screening of Water-Insoluble Articles*, or based on available knowledge. The analytical characteristics to be verified include specificity, accuracy (which addresses quantitation limit), range, repeatability, and solution stability.]

**Samples** Prepare a reagent blank.

**Standard stock solution:** Prepare a solution containing each solvent LTBP or each peak identified and verified by *Procedure A* and *Procedure B* in *Residual Solvents* ⟨467⟩, *8. Analytical Procedures for Class 1 and Class 2 Residual Solvents, 8.2 Screening of Water-Soluble Articles* or *8.3 Screening of Water-Insoluble Articles*, with a concentration as described in *Residual Solvents* ⟨467⟩, *8. Analytical Procedures for Class 1 and Class 2 Residual Solvents, 8.5 Quantitative Tests—Procedure C* for water-soluble articles or water-insoluble articles, as appropriate.

**Sample stock solution:** Prepare as described in *Residual Solvents* ⟨467⟩, *8. Analytical Procedures for Class 1 and Class 2 Residual Solvents, 8.2 Screening of Water-Soluble Articles* or *8.3 Screening of Water-Insoluble Articles*.

**Spiked sample solution:** Prepare as described in *Residual Solvents* ⟨467⟩, *8. Analytical Procedures for Class 1 and Class 2 Residual Solvents, 8.5 Quantitative Tests—Procedure C*, using the *Standard stock solution*.

**Spiked sample solutions A, B, C, etc.:** Prepare *Spiked sample solutions* with the sample matrix and spiked with each solvent LTBP or identified and verified by *Procedure A* and *Procedure B* in *Residual Solvents* ⟨467⟩, *8. Analytical Procedures for Class 1 and Class 2 Residual Solvents, 8.2 Screening of Water-Soluble Articles* or *8.3 Screening of Water-Insoluble Articles*, in triplicate, at NLT 3 levels covering the range of interest or at least 50%–150% of the corresponding concentration of the *Standard solution* as described in *Procedure A* in *8. Analytical Procedures for Class 1 and Class 2 Residual Solvents, 8.2 Screening of Water-Soluble Articles* or *8.3 Screening of Water-Insoluble Articles*.

[NOTE—The *Spiked sample solution* may be used as one of the solutions.]

**Specificity**

**Recommended acceptance criteria:** The procedure must be able to separate each of the solvents in the *Standard solution(s)* from each other and from other peaks in the *Spiked sample solution* with a resolution of NLT 1.0. If the resolution between any pair of peaks is less than 1.5, then verification must demonstrate that the method is suitable for its intended use.

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If the solvents present in the *Standard solution(s)* are not separated with a resolution of NLT 1.0, then chromatography described in *Procedure B* in *Residual Solvents* ⟨467⟩, *8. Analytical Procedures for Class 1 and Class 2 Residual Solvents, 8.2 Screening of Water-Soluble Articles* or *8.3 Screening of Water-Insoluble Articles* should be used as confirmatory.

**Quantitation Limit**

**Recommended acceptance criteria:** The mean signal-to-noise ratio for each solvent in the *Standard solution* and the *Spiked sample solution* [after correction for native (original) solvent content] from at least three determinations is NLT 10, or the *Quantitation Limit* may be demonstrated by *Accuracy* and *Precision*.

**Accuracy**

**Recommended acceptance criteria:** The mean recovery for *Spiked sample solutions A, B,* and *C, etc.*, when calculated relative to the *Spiked sample solution*, is 80%–120% of the expected theoretical amount.
[NOTE—Recoveries should be corrected for native (original) content of any solvent under test.]

**Precision/Repeatability** Use at least six independent *Spiked sample solution* preparations from the same lot, prepared as described in the *Spiked sample solution* in the *Accuracy* test above, or use nine independent preparations as described in *Spiked sample solutions A, B, C, etc.*

**Recommended acceptance criteria:** Relative standard deviation is NMT 20% for each solvent present.

**Solution Stability:** Demonstrate acceptable solution stability for the period of time to run the test.

**Recommended acceptance criteria:** NMT 20% change in solvent(s) content compared to the initial time point

## VALIDATION OF ALTERNATIVE PROCEDURES

Chromatographic alternative procedures should meet the acceptance criteria for the analytical performance characteristics shown below. When non-chromatographic alternative procedures are validated, the analytical performance characteristics listed should be addressed, although, it may be appropriate to apply other analytical performance characteristics. For more information, refer to *Validation of Compendial Procedures* ⟨1225⟩.

### Limit Procedures

Analytical characteristics to be validated are specificity, detection limit, and solution stability. The same criteria used for *Verification when solvents LTBP are known*, as described above, may be used.

### Quantitative Procedures

Analytical characteristics to be validated are specificity, linearity and range, quantitation limit, accuracy, repeatability, intermediate precision, and solution stability.

**Samples** Prepare a reagent blank.

**Spiked sample solutions P, Q, R, etc.:** Prepare *Spiked sample solutions* with the sample matrix and spiked with each solvent LTBP at NLT 5 levels covering the range of interest. [NOTE—Results should be corrected for native (original) content of any solvent under test.]

**Specificity**

**Recommended acceptance criteria:** The analytical procedure must have the ability to assess unequivocally the analytes of interest in the presence of the components expected to be present.

**Linearity and Range**

**Recommended acceptance criteria:** Perform linear regression analysis on the results for *Spiked sample solutions P, Q, R, etc.* The coefficient of determination, $r^2$, is NLT 0.90.

**Quantitation Limit**

**Recommended acceptance criteria:** The mean signal-to-noise ratio for each solvent in the standard solution and the *Spiked sample solution* [after correction for native (original) solvent content] from at least three determinations is NLT 10. The *Quantitation Limit* may also be demonstrated by *Accuracy* and *Precision*.

**Accuracy**

**Recommended acceptance criteria:** The mean recovery for each *Spiked sample solution* should be 80%–120%.
[NOTE—Recoveries should be corrected for native (original) content of any solvent under test.]

**Precision/Repeatability:** Prepare at least six independent *Spiked sample solution* preparations from the same lot.

**Recommended acceptance criteria:** Relative standard deviation is NMT 20% for each solvent present.

**Precision/Intermediate Precision:** Perform the *Repeatability* test over at least two independent events, e.g., on different days, and/or using different instruments and/or analysts.

**Recommended acceptance criteria:** Testing for intermediate precision should demonstrate that the method is suitable for its intended use.

**Solution Stability:** Standard solutions and *Spiked sample solutions* should be stable throughout the testing period.

**Recommended acceptance criteria:** NMT 20% variation

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***Add the following:***

# ▲⟨1469⟩ NITROSAMINE IMPURITIES

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4. NITROSAMINE RISK ASSESSMENTS—DEVELOPMENT OF A CONTROL STRATEGY
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## 1. INTRODUCTION

The presence of nitrosamine impurities has been detected recently in several drug substances and drug products. In 2018, *N*-nitrosodimethylamine (NDMA) and *N*-nitrosodiethylamine (NDEA) were detected in some valsartan drug substances and the drug products manufactured from drug substances using specific synthetic routes. This observation triggered extensive synthetic route assessments and development of analytical procedures to quantify these two nitrosamine impurities. As additional pharmaceuticals were evaluated and, in some cases tested, other nitrosamines beyond NDMA and NDEA were added as impurities of concern. Given the potentially broad implications of the presence of carcinogenic members of this class of chemicals, this chapter has been developed to provide a science- and risk-based approach for the control of nitrosamine impurities to ensure that the potential presence of nitrosamines in drug substances and drug products is identified, assessed, and controlled.

Recommendations are provided regarding: a) the establishment of controls of nitrosamine levels in order to ensure their elimination or reduction; and b) analytical procedure performance characteristics for procedures used to monitor nitrosamine levels.

## 2. NITROSAMINE IMPURITIES

Nitrosamines addressed in this general chapter are listed in *Table 1* by their common names and chemical names. This list is a compilation of the information shared by multiple global health authorities. As additional nitrosamines are identified as potential concerns, the principles described herein should be applied for the assessment of these nitrosamines. If a manufacturer finds a nitrosamine not listed in *Table 1*, the appropriate regulatory authority should be contacted for determining appropriate AI limits. The potential presence of any one or more of these impurities is dependent on the reaction chemistries and processes. The list of nitrosamines is not intended to be exhaustive but represents those that have been observed and communicated by regulators and manufacturers as being potentially present or observed.

*N*-nitroso compounds are among the structural groups of high potency mutagenic carcinogens in several animal species, and some are classified as probable or possible human carcinogens referred to as the “cohort of concern" in ICH M7: *Assessment and Control of DNA Reactive (Mutagenic) Impurities in Pharmaceuticals to Limit Potential Carcinogenic Risk* (*1*), a designation that carries with it a recommendation to control the impurities at or below the acceptable cancer risk. As a result of the potential toxicity associated with these impurities, it is recommended to take steps to control and limit their presence in pharmaceutical materials.

**Table 1. Nitrosamines Found as Contaminants in Drug Substances and Drug Products**

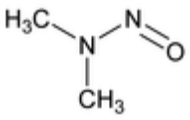
| Common Name and Chemical Name | Acronym | CAS # | Structure | Chemical Formula | Molecular Weight |
|---|---|---|---|---|---|
| Nitrosodimethylamine<br>*N*-Methyl-*N*-nitrosomethanamine | NDMA | 62-75-9 |  | $C_2H_6N_2O$ | 74.08 |

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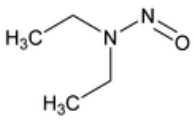
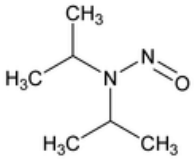
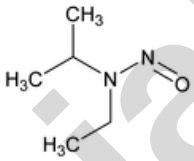
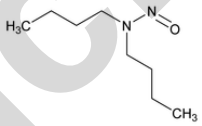
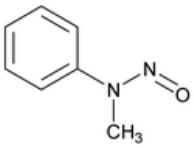
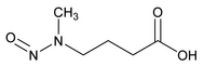
**Table 1. Nitrosamines Found as Contaminants in Drug Substances and Drug Products** *(continued)*

| Common Name and Chemical Name | Acronym | CAS # | Structure | Chemical Formula | Molecular Weight |
|---|---|---|---|---|---|
| Nitrosodiethylamine<br>*N*-Ethyl-*N*-nitrosoethanamine | NDEA | 55-18-5 |  | $C_4H_{10}N_2O$ | 102.14 |
| Nitrosodiisopropylamine<br>*N*-Isopropyl-*N*-nitrosoisopropylamine | NDIPA | 601-77-4 |  | $C_6H_{14}N_2O$ | 130.19 |
| Nitrosoethylisopropylamine<br>*N*-Ethyl-*N*-nitroso-2-propanamine | NEIPA | 16339-04-1 |  | $C_5H_{12}N_2O$ | 116.16 |
| Nitrosodibutylamine<br>*N*-Butyl-*N*-nitroso-1-butanamine | NDBA | 924-16-3 |  | $C_8H_{18}N_2O$ | 158.25 |
| Nitrosomethylphenylamine<br>*N*-Methyl-*N*-nitrosophenylamine | NMPA | 614-00-6 |  | $C_7H_8N_2O$ | 136.15 |
| Nitrosomethylaminobutyric acid<br>4-[Methyl(nitroso)amino] butanoic acid | NMBA | 61445-55-4 |  | $C_5H_{10}N_2O_3$ | 146.15 |

## 3. SOURCES OF NITROSAMINES

There are a number of pathways by which nitrosamines can be introduced into or generated as impurities in pharmaceutical drug products. Specifically, nitrosamines are formed by chemical reaction of secondary or tertiary amines with nitrites (the latter via intermediate degradation) under acidic conditions (see *3.1. Nitrosamine Formation Reaction*). Some examples of the reported sources or pathways leading to the generation of nitrosamines identified empirically or reported in the literature (*2–3*) include (but are not limited to) the following:

- Drug substance processing under specific conditions and in the presence of certain reagents, solvents, raw materials, and processing aids. There is evidence that despite processing and purification steps, reactive species, whether intentionally added to or formed during the process/reaction sequence (e.g., nitrites and secondary amines in the presence of acidic conditions), can carry over to subsequent steps (see *3.1 Nitrosamine Formation Reaction*). Special attention should be given to the formation of nitrogen-containing heterocycles by employing azide followed by quenching with nitrous acid to remove excess azide.
- The drug substance itself, which may degrade under some conditions resulting in the formation of nitrosamines (e.g., ranitidine).
- Degradation of solvents (e.g., dimethylformamide [DMF]) leading to the formation of dialkyl amines.
- Impurities in raw materials, solvents (including recycled solvents), reagents, or catalysts.
- Impurities in materials and intermediates, reagents, and solvents used to prepare the starting materials or intermediates.
- Impurities in water, excipients, or processing aids used in the production of the finished drug product.
- During drug product manufacture under certain reaction conditions and in the presence of requisite precursors necessary for the formation of nitrosamines.

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- Impurities in the container–closure system for the finished drug product, which may include impurities capable of forming nitrosamines, especially if associated with materials containing amines and potential sources of a nitrosating agent (e.g., nitrite, nitrocellulose).

A risk assessment should be conducted to determine the materials that contribute to the potential for inclusion of nitrosamines in the drug product. All potential sources for the introduction of nitrosamines should be considered in the risk assessment including, for example, the drug substance, excipients, water, solvents, the manufacturing process, packaging components, and formation on stability. See *Figure 1* for a diagram of some potential sources to be considered.

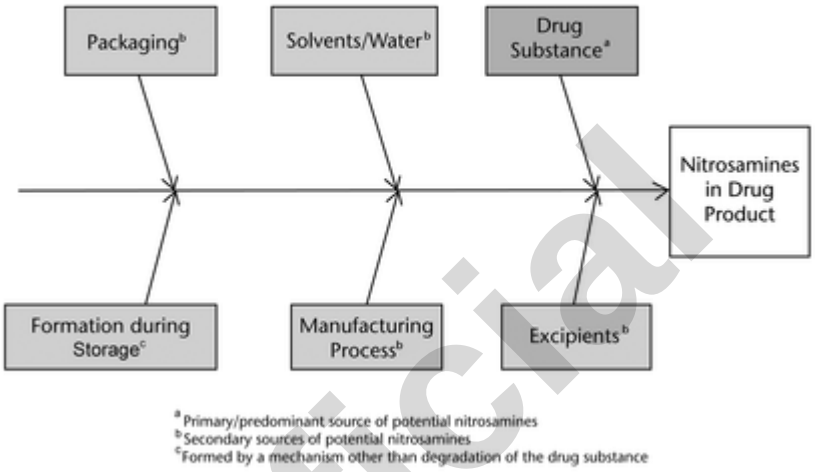


Figure 1. Potential sources of nitrosamine impurities in drug product.

Ongoing assessments and evaluations have identified risks associated with several of the potential sources of nitrosamines. Some of the examples identified are summarized in *Table 2*.

**Table 2**

| Potential Source of Nitrosamines | Observed Risk[a] |
|---|---|
| Solvents | • Presence of residual dialkyl amines or tri-substituted amines that can degrade to form intermediates that can further react with nitrosating agents<br>• Presence of nitrites or other nitrosating agents<br>• Presence of acid<br>• Limited controls/specification limits for recycled solvents<br>• Poor quality solvents |
| Water | • Presence of residual dialkyl amines or impurities that can degrade to form dialkyl amines<br>• Presence of acid and nitrosating agents |
| Excipients | • Presence of nitrites or other nitrosating agents and/or nitrosamine impurities (if applicable) |
| Drug substance | • Use of sodium azide in the synthesis followed by use of nitrites in acidic medium (nitrous acid) for quenching excess azides<br>• Use of di- or tri-alkylamines and amides (e.g., dimethylformamide [DMF], dimethylamine [DMA], triethylamine [TEA], *N*-methylpyrrolidone [NMP]) in the presence of nitrites and acid media<br>• Use of recycled solvents that may contain nitrosamines or their precursors<br>• Use of sanitized water (e.g., chloramines)<br>• Insufficient purification<br>• Degradation of drug substances containing functional groups that can then participate in nitrosation reactions |
| Manufacturing process | • Contamination<br>• Use of poor quality or recycled solvents that may contain nitrosamines or their precursors<br>• Presence of nitrous oxides in air used to dry the drug substance or drug product<br>• Carryover of relevant reactive species into subsequent steps |

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**Table 2** *(continued)*

| Potential Source of Nitrosamines | Observed Risk[a] |
|---|---|
| Drug product (including stability) | • Secondary, tertiary, or quaternary amine group in molecule of drug substance<br>• Presence of nitrate counter ions (potentially containing nitrite as an impurity)<br>• Potential reactions within the formulation matrix during stability/shelf life (e.g., presence or generation of acidic conditions, moisture, and heat) |
| Container–Closures | • Packaging materials containing vulnerable amines that might react with nitrosating agents present in the packaging material itself (e.g., amines in inks reacting with nitrocellulose print base) |

[a] General chemical reactions leading to formation of nitrosamines can be found in *3.1 Nitrosamine Formation Reaction*.

### 3.1 Nitrosamine Formation Reaction

The general schematic representation of the chemical reaction responsible for the formation of nitrosamines from secondary amines is described in *Figure 2*. Examples of representative reactions are described in the scientific literature (*2*–*3*).

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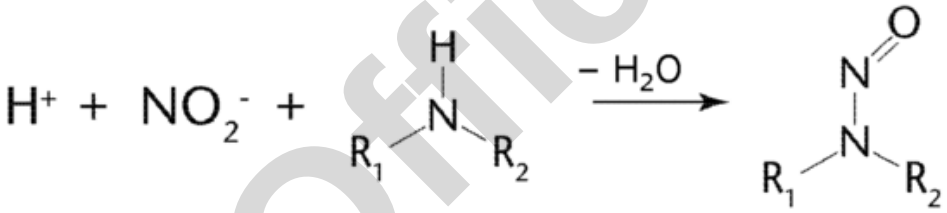


Figure 2. General example of formation of nitrosamines, where $R_1$ and $R_2$ are alkyl or functionalized alkyl groups (and only one of them could be an aryl or functionalized aryl group).

If the potential for the presence of nitrosamines is identified, where appropriate, a control strategy should be developed. If nitrosamines are identified as impurities in ingredients, they may be controlled as appropriate in the ingredients (e.g., manufacture of the drug substance or controls placed on the drug substance). If nitrosamines are identified as degradation products (i.e., being formed during manufacturing of the drug product or formed during product storage), they should be controlled as appropriate in the drug product. In some cases, changes to the manufacturing process(es) or ingredients may be required to achieve acceptable levels or the elimination of nitrosamine impurities in the drug product.

## 4. NITROSAMINE RISK ASSESSMENTS—DEVELOPMENT OF A CONTROL STRATEGY

In order to determine the level of control, if any, which may be required for ensuring that levels of nitrosamines are at or below the acceptable intake (AI) if their presence cannot be avoided, the components of drug products should be assessed by the drug product manufacturer for the potential to form nitrosamines or to be contaminated with nitrosamines. Although one of the sources with the highest potential for nitrosamines is the drug substance synthetic route, the drug substance manufacturing process, drug product manufacturing process, and excipients and raw materials should also be included in a risk assessment to establish if controls or additional controls are needed. An example of high-level process flow for evaluating materials is shown in *Figure 3*.

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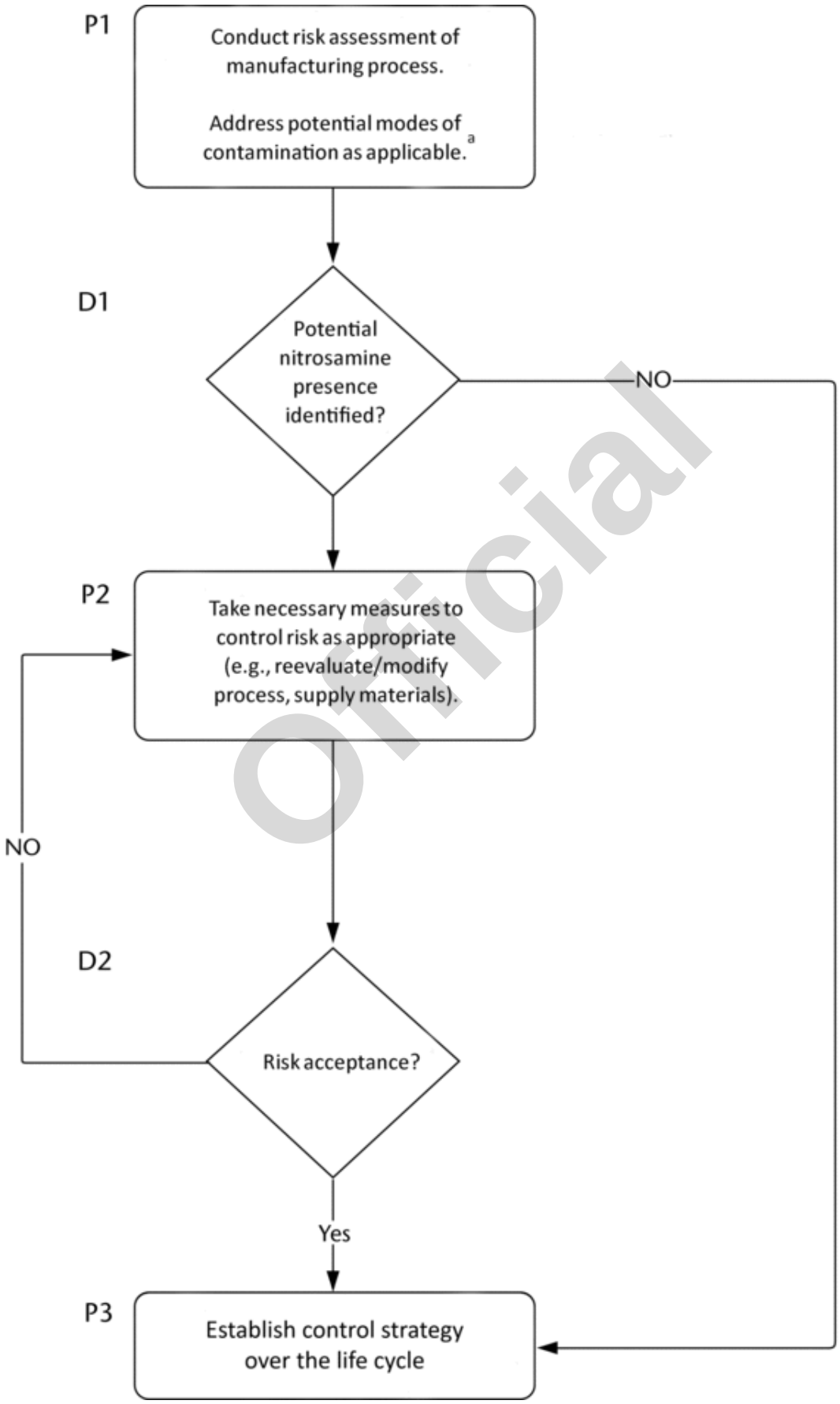


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Figure 3. High level process for development of a nitrosamine impurity control strategy. ([a] Refer to *Table 2*; P1, P2, P3 = Process

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1, 2, 3; D1, D2 = Decision 1, 2)

In all cases, if nitrosamines are predicted by the risk assessment or confirmed to be present through testing of the drug substance, drug product, or other materials, a control strategy should define an approach to ensure that the nitrosamine levels comply with the established AIs. The control strategy should be aligned with the current regulatory requirements in place.

## 5. LIMITS OF NITROSAMINES

Nitrosamine impurities identified in this chapter have potential and established toxicity with no therapeutic value. Because nitrosamines are among the structural groups of high potency mutagenic carcinogens of the "cohort of concern" in ICH M7 (*1*), the threshold of toxicological concern (TTC) does not apply. Instead, the available safety data should be used to establish a material-specific AI on a case-by-case basis. The AI is defined as an intake level that poses a negligible health risk.

### 5.1 Derivation of AI Limits

There are a number of methodologies that toxicologists have applied in establishing AIs. In this case, the median tumorigenic dose ($TD_{50}$) of NDMA, NDEA, and other nitrosamines was used as representative data in a linear extrapolation to establish an acceptable risk level. The limits have been published in the FDA Guidance for Industry: *Control of Nitrosamine Impurities in Human Drugs* (*4*).

### 5.2 Example Calculations of Nitrosamine Limits

The AIs in nanograms per day and the maximum daily dose (MDD) of the drug substance (DS) from the drug label in milligrams per day can be used to calculate the maximum nitrosamine concentration limits, in ppm, for individual drug products using the following equation:

$$\text{Concentration} = \text{AI}/\text{DS}_{\text{dose}}$$

Since the exposure to nitrosamines is related to the MDD of the DS, different concentrations of nitrosamines (ng/g) may be acceptable for each material evaluated. The acceptable concentration in the material can be calculated using the following equation:

$$\text{Acceptable nitrosamine content} = \text{AI}/\text{MDD}$$

AI = acceptable intake of the nitrosamine (µg/day)
MDD = maximum daily dose of the drug substance (g/day)

**Table 3. Example Using an AI of 96 ng/day for the Target Nitrosamine**

| Name | Acceptable Concentration (µg/g) | | | |
|---|---|---|---|---|
| | 0.050 (50-mg dose) | 0.100 (100-mg dose) | 0.250 (250-mg dose) | 1.00 (1000-mg dose) |
| Nitrosamine 1 | 1.920 | 0.960 | 0.384 | 0.096 |

[NOTE—If multiple nitrosamines are identified in the material and the total exceeds 26.5 ng/day, the appropriate regulatory authority should be consulted to determine an acceptable approach.]

## 6. TESTING FOR THE PRESENCE OF NITROSAMINES

Upon completion of the risk assessment, exploratory testing may need to be performed to confirm the conclusions of the risk assessment and proposed control strategy. On the basis of the controls identified (e.g., incoming material testing or specification limit; drug substance or drug product specification limit), it may be necessary to implement routine testing for nitrosamines. If testing is applied to ensure that the nitrosamine(s) concentration(s) do not exceed the AI, methods should be established following the recommendations detailed in this chapter. Example analytical procedures can be found in *8. Analytical Procedures*.

### 6.1 Presence of Two or More Nitrosamines

The current published AIs reflect limits for the presence of a single nitrosamine. If multiple nitrosamines are possible and are determined analytically to be present at levels exceeding the maximum amount permitted by the regulatory authority, the relevant health authority should be consulted to determine a specific path forward. Manufacturers should contact FDA for determining the AI limits if multiple nitrosamine impurities are detected in a drug substance or drug product in which the total nitrosamine level exceeds 26.5 ng/day based on MDD.

## 7. TEST METHOD PERFORMANCE CHARACTERISTICS OF NITROSAMINE METHODS

The AIs associated with nitrosamines require the application of sensitive analytical procedures. In many cases, the most reliable procedures take advantage of the sensitivity and selectivity of chromatographic separation techniques coupled with quantitation by mass spectrometry (e.g., HPLC–MS/MS, GC–MS/MS). For additional guidance on validation of alternative methods for nitrosamines, see *Validation of Compendial Procedures* ⟨1225⟩.

### 7.1 Considerations for Sample Preparation

Appropriate sample preparation is a critical step in trace impurity analyses such as those required to evaluate the levels of nitrosamines in drug substances and drug products. This is particularly critical to prevent the loss or generation of nitrosamines as artifacts of the analytical procedure itself, as in the following circumstances.

- The presence of dialkyl amine (dimethylamine) as a process impurity or counter ion of the salt form of the active ingredient in the presence of nitrite and acid can lead to in situ formation of nitrosamines as an artifact, especially in GC analyses.
- Total solubilization versus selective extraction: If the active ingredient contains a dimethylamino group, total dissolution of the drug substance should be avoided when applying GC techniques. High concentration of the active ingredient, when injected in the GC instrument can generate nitrosamines in the injection port if a

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nitrosating agent is present. In these situations, sample extractions should be modified to prevent the solubilization of the active ingredient while maintaining the extraction efficiency for nitrosamines present in the material.

The recommended method performance characteristics that need to be evaluated for quantitative analysis of nitrosamines include range, accuracy, repeatability, intermediate precision, and limit of quantitation. If a limit test is intended for use, the recommended method performance characteristics to be assessed include specificity, recovery, detectability, and solution stability. The performance criteria for these parameters should be properly set and confirmed through validation to ensure that the method is suitable for its intended use based on the specific analytes, matrices, and required precision and accuracy of the analytical procedures. Precision and recovery depend highly on concentration and matrix complexity, and the final proposed acceptance criteria need to be justified in the procedure validation documentation. Higher variability may be tolerated or acceptable at concentrations approaching the limit of quantitation of the procedure while lower variability (higher precision) would be expected at higher concentrations. Examples of quantitative analytical procedures are included in *8. Analytical Procedures*. Additional example methods may be provided as they become available for these additional nitrosamines.

## 8. ANALYTICAL PROCEDURES

The following procedures have been established as suitable for their intended (specified) purpose. Users should validate these methods while considering the effect of sample solubility and extraction efficiency on the test results for other materials for which they are intended to be applied (see ⟨1225⟩ and *Verification of Compendial Procedures* ⟨1226⟩).

### 8.1 Quantitative Procedures

**Procedure 1: Quantitation of NDMA, NDEA, NDIPA, NEIPA, NMBA, NMPA, and NDBA in selected sartans (valsartan, irbesartan, and losartan potassium) by HPLC–HRMS**

**Diluent:** Methanol

**Solution A:** 0.1% formic acid in water

**Solution B:** 0.1% formic acid in methanol

**Mobile phase:** See *Table 4*.

**Table 4**

| Time (min) | Solution A (%) | Solution B (%) |
|---|---|---|
| 0 | 90 | 10 |
| 1.5 | 90 | 10 |
| 7.0 | 45 | 55 |
| 17.0 | 45 | 55 |
| 17.1 | 10 | 90 |
| 21.0 | 10 | 90 |
| 21.1 | 90 | 10 |
| 25.0 | 90 | 10 |

**Sensitivity solution:** 1.0 ng/mL each of USP *N*-Nitrosodimethylamine RS, USP *N*-Nitrosodiethylamine RS, USP *N*-Nitrosoethylisopropylamine RS, USP *N*-Nitrosodiisopropylamine RS, USP *N*-Nitrosodibutylamine RS, USP *N*-Nitrosomethylphenylamine RS, and USP *N*-Nitrosomethylaminobutyric Acid RS in *Diluent*

**Standard solution:** 6.0 ng/mL each of USP *N*-Nitrosodimethylamine RS, USP *N*-Nitrosodiethylamine RS, USP *N*-Nitrosoethylisopropylamine RS, USP *N*-Nitrosodiisopropylamine RS, USP *N*-Nitrosodibutylamine RS, USP *N*-Nitrosomethylphenylamine RS, and USP *N*-Nitrosomethylaminobutyric Acid RS in *Diluent*

**Sample solution:** 20 mg/mL of drug substance prepared as follows. Transfer 100 mg of drug substance into a suitable container. Add 5.0 mL of *Diluent* and vortex until fully dispersed or dissolved. Pass the solution through a suitable filter of 0.2-µm pore size. Use the filtrate for analysis.

**Chromatographic system**

(See *Chromatography* ⟨621⟩, *System Suitability*.)

**Mode:** LC

**Detector:** High resolution mass spectrometer

**MS conditions**

**Ionization:** Electrospray ionization (ESI)

**Scan settings:** See *Table 5*.

**Table 5**

| Nitrosamine Impurity | NDMA | NMBA | NDEA | NEIPA | NDIPA | NMPA | NDBA |
|---|---|---|---|---|---|---|---|
| Scan type | SIM[a] | SIM | PRM[b] | SIM | SIM | SIM | PRM |
| Polarity | positive | negative | positive | positive | positive | positive | positive |
| Scan start–end (min) | 1.0–3.5 | 3.5–5.5 | 5.5–7.0 | 7.0–8.5 | 8.5–10.0 | 8.5–10.0 | 13.0–15.5 |

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**Table 5** *(continued)*

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **m/z[c] isolated for PRM** | N/A | N/A | 103.0866 | N/A | N/A | N/A | 159.1492 |
| **NCE[d]** | N/A | N/A | 25 | N/A | N/A | N/A | 20 |
| **Scan range (m/z)** | 74.3–75.8 | 144.3–145.8 | 50.0–114.0 | 116.4–117.9 | 130.4–131.9 | 136.3-137.8 | 50.0–170.0 |
| **Microscans** | 3 | 3 | 3 | 3 | 3 (1)[e] | 3 (1)[e] | 3 |
| **Resolution** | 30,000 | 60,000 | 30,000 | 60,000 | 60,000 | 60,000 | 30,000 |
| **AGC target value (%)[f]** | 250 | 250 | 250 | 250 | 250 | 250 | 250 |

[a] SIM = selected ion monitoring.
[b] PRM = parallel reaction monitoring.
[c] m/z = mass to charge ratio.
[d] NCE = normalized collision energy.
[e] 1 microscan must be used only if both NDIPA and NMPA are present.
[f] AGC = automatic gain control.

[NOTE—Divert the drug substance from the MS source during the elution.]

**Data processing:** Peak areas in the extracted ion chromatograms (EIC) with an m/z extraction window of ±15 ppm are used for quantitation. The m/z values extracted are listed in *Table 6*.

**Table 6**

| **Nitrosamine Impurity** | NDMA | NMBA | NDEA | NEIPA | NDIPA | NMPA | NDBA |
|---|---|---|---|---|---|---|---|
| **m/z to be extracted** | 75.0553 | 145.0619 | 75.0553, 103.0866 | 117.1022 | 131.1179 | 137.0709 | 57.0704, 103.0872, 159.1492 |

**Column:** 4.6-mm × 10-cm; 2.6-µm packing L43
**Temperatures**
**Autosampler:** 4°
**Column:** 40°
**Flow rate:** 0.6 mL/min
**Flow rate to ion source:** 0.6 mL/min
**Injection volume:** 3 µL

**System suitability**
**Samples:** *Sensitivity solution* and *Standard solution*
[NOTE—The relative retention times for NDMA, NMBA, NDEA, NEIPA, NDIPA, NMPA, and NDBA are 0.20, 0.31, 0.46, 0.57, 0.66, 0.67, and 1.00, respectively.]
[NOTE—NMBA and NEIPA exist as syn- and anti-conformers due to the restricted rotation of the N–N bond. These conformers are partially separated by the method's chromatographic conditions. The NMBA peak is observed as a doublet. Integrate both of the NMBA peaks and use the combined peak areas for calculation of the NMBA concentration. The NEIPA peak may appear as a doublet or a single peak with a tailing shoulder. For NEIPA, if the conformers are resolved, integrate both peaks and combine the peak areas for the calculation of the NEIPA concentration. If the NEIPA conformers are not fully resolved (e.g., evidence of a shoulder is present), integrate the main peak and shoulder as a single peak and use the combined peak area to calculate the NEIPA concentration.]
**Suitability requirements**
**Relative standard deviation:** NMT 20.0% from 6 replicate injections, *Standard solution*
**Signal-to-noise ratio:** NLT 10, *Sensitivity solution*

**Analysis**
**Samples:** *Standard solution* and *Sample solution*
Calculate the concentration, in ppm, of each specified nitrosamine impurity in the portion of drug substance taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 10^6$$

$r_U$ = peak response of the individual specified nitrosamine impurity from the *Sample solution*
$r_S$ = peak response of the corresponding nitrosamine impurity from the *Standard solution*
$C_S$ = concentration of USP *N*-Nitrosodimethylamine RS, USP *N*-Nitrosodiethylamine RS, USP *N*-Nitrosoethylisopropylamine RS, USP *N*-Nitrosodiisopropylamine RS, USP *N*-Nitrosodibutylamine RS, USP *N*-Nitrosomethylphenylamine RS, or USP *N*-Nitrosomethylaminobutyric Acid RS in the *Standard solution* (µg/mL)
$C_U$ = concentration of the drug substance in the *Sample solution* (µg/mL)

Report the nitrosamine impurity concentration in the drug substance in ppm (µg/g).

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**Procedure 2: Quantitation of NDMA, NDEA, NDIPA, and NEIPA in selected sartans (valsartan, irbesartan, losartan potassium, olmesartan medoxomil, candesartan cilexetil, and telmisartan) by headspace GC–MS**

**Diluent:** Methanol

**Internal standard stock solution:** 0.4 µg/mL of NDMA-d6 in *Diluent*

**Internal standard solution:** 0.016 µg/mL of NDMA-d6 prepared as follows. Transfer 2.0 mL of *Internal standard stock solution* into a 50-mL volumetric flask, and dilute with *Diluent* to volume.

**Nitrosamine RS stock solution:** 0.4 µg/mL each of USP *N*-Nitrosodimethylamine RS, USP *N*-Nitrosodiethylamine RS, USP *N*-Nitrosoethylisopropylamine RS, and USP *N*-Nitrosodiisopropylamine RS prepared as follows. Transfer an appropriate amount of USP *N*-Nitrosodimethylamine RS, USP *N*-Nitrosodiethylamine RS, USP *N*-Nitrosoethylisopropylamine RS, and USP *N*-Nitrosodiisopropylamine RS into a suitable volumetric flask, and dilute with *Diluent* to the volume.

**Standard stock solution:** 0.016 µg/mL each of USP *N*-Nitrosodimethylamine RS, USP *N*-Nitrosodiethylamine RS, USP *N*-Nitrosoethylisopropylamine RS, and USP *N*-Nitrosodiisopropylamine RS prepared as follows. Transfer 2.0 mL of *Nitrosamine RS stock solution* and 2.0 mL of *Internal standard stock solution* into a 50-mL volumetric flask, and dilute with *Diluent* to volume.

**Standard solution:** Transfer 1 mL of *Standard stock solution* to an appropriate headspace vial containing about 100 mg of imidazole and 1.0 mL of acetonitrile. Apply the stopper, cap, and crimp tightly.

**Sensitivity stock solution:** 0.004 µg/mL each of USP *N*-Nitrosodimethylamine RS, USP *N*-Nitrosodiethylamine RS, USP *N*-Nitrosoethylisopropylamine RS, and USP *N*-Nitrosodiisopropylamine RS in *Diluent* prepared as follows. Transfer 0.5 mL of the *Nitrosamine RS stock solution* and 2.0 mL of *Internal standard stock solution* into a 50-mL volumetric flask and dilute with *Diluent* to volume.

**Sensitivity solution:** Transfer 1 mL of *Sensitivity stock solution* to an appropriate headspace vial containing about 100 mg of imidazole and 1.0 mL of acetonitrile. Apply the stopper, cap, and crimp tightly.

**Sample solution:** Transfer 200 ± 10 mg of drug substance and about 100 mg of imidazole into a headspace vial, and then add 1.0 mL of *Internal standard solution* and 1.0 mL of acetonitrile. Apply the stopper, cap, and crimp tightly.

**Blank:** Transfer about 100 mg of imidazole into a headspace vial, and then add 1.0 mL of *Internal standard solution* and 1.0 mL of acetonitrile. Apply the stopper, cap, and crimp tightly.

**Chromatographic system**

(See *Chromatography* ⟨621⟩, *System Suitability*.)

**Mode:** GC

**Injector:** Headspace (see *Table 7* for parameters)

**Table 7**

| | |
|---|---|
| **Equilibration temperature** | 95°–110° |
| **Loop temperature** | 150° |
| **Rate 1** | 10°/min |
| **Transfer line temperature** | 160° |
| **Pressurizing gas pressure** | 20.00 psi |
| **Equilibration time** | 10.00 min |
| **Pressurizing time** | 2.00 min |
| **Load time** | 2.00 min |
| **Injection time** | 1.00 min |
| **Vial size** | 20 mL |

**Injection type:** (Split, Split ratio 1:1 or 1:3)

[NOTE—Split ratio can be modified to optimize sensitivity.]

**Detector:** Mass spectrometer

**Column:** 0.32-mm × 30-m fused-silica, coated with a 1.0-µm layer of phase G16

**Column temperature:** See *Table 8*.

**Table 8**

| Initial Temperature (°) | Temperature Ramp (°/min) | Final Temperature (°) | Hold Time at Final Temperature (min) |
|---|---|---|---|
| 45 | 0 | 45 | 3 |
| 45 | 10 | 130 | 3 |
| 130 | 15 | 190 | — |
| 190 | 40 | 240 | 10 |

**Carrier gas:** Helium

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**Flow rates**
**Gas:** Constant flow at 1.8 mL/min (adjustment and verification are necessary for other carrier gases)
**Purge:** 3.0 mL/min or default value
**MS conditions:** See *Table 9*.

**Table 9**

| | |
|---|---|
| **Ionization mode** | Electron impact (EI) |
| **Polarity** | positive |
| **Event 1** | |
| **Name** | NDMA |
| **Start time** | 10.0 min |
| **End time** | 12.0 min |
| **Acquisition mode** | multiple reaction mode (MRM) |
| **Ch 1 m/z** | 74.00 > 44.00 |
| **CH 1 collision energy** | 4.00 V |
| **Ch 2 m/z** | 74.00 > 42.00 |
| **CH 2 collision energy** | 15.00 V |
| **Event 2** | |
| **Name** | NDMA-d6 |
| **Start time** | 10.0 min |
| **End time** | 12.0 min |
| **Acquisition mode** | MRM |
| **Ch 1 m/z** | 80.00 > 50.00 |
| **CH 1 collision energy** | 5.00 V |
| **Event 3** | |
| **Name** | NDEA |
| **Start time** | 12.00 min |
| **End time** | 12.75 min |
| **Acquisition mode** | MRM |
| **Ch 1 m/z** | 102.00 > 85.1 |
| **CH 1 collision energy** | 6.00 V |
| **Ch 2 m/z** | 102.00 > 56.1 |
| **CH 2 collision energy** | 15.00 V |
| **Event 4** | |
| **Name** | NEIPA |
| **Start time** | 12.75 min |
| **End time** | 13.35 min |
| **Acquisition mode** | MRM |
| **Ch 1 m/z** | 116.00 > 99.10 |
| **CH 1 collision energy** | 6.00 V |
| **Ch 2 m/z** | 99.00 > 44.10 |
| **CH 2 collision energy** | 9.00 V |
| **Event 5** | |
| **Name** | NDIPA |
| **Start time** | 13.35 min |
| **End time** | 14.00 min |
| **Acquisition mode** | MRM |
| **Ch 1 m/z** | 130.00 > 42.00 |
| **CH 1 collision energy** | 10.00 V |

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**Table 9** *(continued)*

| | |
|---|---|
| **Ch 2 m/z** | 130.00 > 43.10 |
| **CH 2 collision energy** | 18.00 V |

[NOTE—Ch 1 m/z in multiple reaction mode (MRM) is used for quantitation.]

**System suitability**

**Samples:** *Standard solution*, *Sensitivity solution*, and *Blank*

[NOTE—The relative retention times for NDMA, NDMA-d6, NDEA, NEIPA, and NDIPA are 0.80, 0.80, 0.90, 0.96, and 1.00, respectively.]

**Suitability requirements**

**Relative standard deviation:** NMT 20.0% for the ratio of the impurity standard peak response to the internal standard peak response from 6 replicate injections, *Standard solution*

**Signal-to-noise ratio:** NLT 10 for the impurity peak, *Sensitivity solution*

**Interference:** There should be no interfering peak in the *Blank*.

**Analysis**

**Samples:** *Standard solution* and *Sample solution*

Calculate the concentration, in ppm, of each specified nitrosamine impurity in the portion of drug substance taken:

$$\text{Result} = 1/W \times (R_U/R_S) \times C_S$$

$W$ = weight of the drug substance in the *Sample solution* (g)
$R_U$ = peak response ratio of the specified nitrosamine impurity to that of the internal standard from the *Sample solution*
$R_S$ = peak response ratio of the specified nitrosamine impurity to that of the internal standard from the *Standard solution*
$C_S$ = concentration of USP *N*-Nitrosodimethylamine RS, USP *N*-Nitrosodiethylamine RS, USP *N*-Nitrosoethylisopropylamine RS, and USP *N*-Nitrosodiisopropylamine RS in the *Standard stock solution* (µg/mL)

Report the nitrosamine impurity concentration in the drug substance in ppm (µg/g).

**Procedure 3: Quantitation of NDMA, NDEA, NDIPA, NEIPA, NMBA, and NDBA in selected sartans (valsartan, losartan potassium, olmesartan medoxomil, candesartan cilexetil, and telmisartan) by HPLC–MS/MS**

**Diluent:** 1% formic acid in water

**Solution A:** 0.1% formic acid in water

**Solution B:** 0.1% formic acid in methanol

**Mobile phase:** See *Table 10*.

**Table 10**

| Time (min) | Solution A (%) | Solution B (%) |
|---|---|---|
| 0 | 97 | 3 |
| 1.5 | 97 | 3 |
| 4.0 | 50 | 50 |
| 7.0 | 25 | 75 |
| 8.1 | 15 | 85 |
| 9.2 | 5 | 95 |
| 12.0 | 5 | 95 |
| 12.1 | 97 | 3 |

**Internal standard solution:** 10 µg/mL each of NDMA-d6 and NMBA-d3, 1 µg/mL each of NDEA-d10 and NDBA-d18 in water

**Nitrosamine standards stock solution mixture:** Prepare a mixture of 200 ng/mL each of *N*-nitrosodimethylamine, *N*-nitrosoethylisopropylamine, *N*-nitrosodiisopropylamine, *N*-nitrosodibutylamine, and *N*-nitrosomethylaminobutyric acid by mixing appropriate volumes of the respective USP Reference Standards and dilute with water.

[**CAUTION**—Prepare *Nitrosamine standards stock solution mixture* in amber vials and store at –18° to –20°).]

**NDEA standard stock solution:** Prepare a solution of 132 ng/mL of *N*-nitrosodiethylamine by diluting USP *N*-Nitrosodiethylamine RS with water.

**Standard solutions:** Depending on the targeted nitrosamine concentration in the sample, prepare a set of 5 consecutive linearity solutions from *Table 11* from the *Nitrosamine standards stock solution mixture* and *NDEA standard stock solution* by mixing specified volumes of each solution as indicated. [NOTE—*Table 11* represents an example for preparing solutions for constructing the calibration curve. Other dilution schemes may be used for preparing the set of 5 linearity solutions covering the range of interest. L1 is used only for NDEA when applicable. For others, linearity starts with L2.]

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**Table 11**

| Linearity Solution # | Concentration Level | Concentration of NDMA, NMBA, NDBA, NEIPA, NDIPA (ng/mL)/ NDEA (ng/mL) | Content of NDMA, NMBA, NDBA, NEIPA, NDIPA (ppb)/NDEA (ppb) | Volume of Nitrosamine Standard Stock Solution Mixture (µL) | Volume of NDEA Standard Stock Solution (µL) | Volume of Water (µL) | Volume of Internal Standard (µL) | Total Volume (µL) |
|---|---|---|---|---|---|---|---|---|
| 1 | L1 | 1.33/0.66 | 19.95/10 | 8 | 6 | 1174 | 12 | 1200 |
| 2 | L2 | 2/0.88 | 30/13.5 | 12 | 8 | 1168 | 12 | 1200 |
| 3 | L3 | 5/3.3 | 75/49.5 | 30 | 30 | 1128 | 12 | 1200 |
| 4 | L4 | 7.5/4.95 | 112.5/74.25 | 45 | 45 | 1098 | 12 | 1200 |
| 5 | L5 | 10/6.6 | 150/99 | 60 | 60 | 1068 | 12 | 1200 |
| 6 | L6 | 15/9.9 | 225/148.5 | 90 | 90 | 1008 | 12 | 1200 |
| 7 | L7 | 30/19.8 | 450/297 | 180 | 180 | 828 | 12 | 1200 |
| 8 | L8 | 60/39.6 | 900/594 | 360 | 360 | 468 | 12 | 1200 |
| 9 | L9 | 90/59.4 | 1350/891 | 540 | 540 | 108 | 12 | 1200 |

**Sample solution:** Transfer about 80 mg of the drug substance into a 2-mL lidded centrifuge tube. Add 1188 µL of *Diluent* and 12 µL of the *Internal standard solution*. Vortex at 2500 rpm for 20 min (except for losartan potassium, which should be vortexed NMT 5 min). Centrifuge at about 10,000 rpm for 10 min, and filter into a vial using a hydrophilic polytetrafluoroethylene (PTFE) filter of 0.45-µm pore size.

**Chromatographic system**

(See *Chromatography* ⟨621⟩, *System Suitability*.)

**Mode:** LC

**Detector:** MS/MS (triple quadrupole mass spectrometer)

**MS conditions**

**Ionization:** Atmospheric pressure chemical ionization (APCI)

**Scan settings:** See *Table 12*.

**Table 12**

| Nitrosamine Impurity | Acquisition Mode | Polarity | MRM Transitions (m/z) | |
|---|---|---|---|---|
| | | | MRM-1[a] | MRM-2 |
| NDMA | MRM | Positive | +75.0 → +43.0 | +75.0 → +44.1 |
| NDMA-d6 | MRM | Positive | +81.2 → +46.0 | +81.2 → +64.1 |
| NDEA | MRM | Positive | +103.1 → +75.1 | +103.1 → +47.1 |
| NDEA-d10 | MRM | Positive | +113.2 → +34.2 | +113.2 → +49.1 |
| NMBA | MRM | Positive | +147.1 → +44.1 | +147.1 → +117.1 |
| NMBA-d3 | MRM | Positive | +150.1 → +47.1 | +150.1 → +120.2 |
| NDBA | MRM | Positive | +159.2 → +41.1 | +159.2 → +29.1 |
| NDBA-d18 | MRM | Positive | +177.3 → +66.2 | +177.3 → +46.2 |
| NEIPA[b] | MRM | Positive | +117.1 → +75.1 | +117.1 → +47.2 |
| NDIPA[b] | MRM | Positive | +131.2 → +89.1 | +131.1 → +47.1 |

[a] MRM-1 is used for quantitation.
[b] NDEA-d10 is used as internal standard for NEIPA and NDIPA.

**Column:** 3.0-mm × 15-cm; 2.7-µm packing L1

**Temperatures**

**Autosampler:** 18°

**Column:** 60°

**Flow rate:** 0.5 mL/min

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**Flow rate to ion source:** 0.5 mL/min
**Injection volume:** 20 µL

**System suitability**
**Samples:** *Standard solutions*
Generate the peak response ratio of the specified impurity to that of the internal standard versus the concentration standard curve for each nitrosamine impurity under test using the corresponding selected *Standard solutions* and perform the linear regression analysis.
[NOTE—The relative retention times for NDMA, NMBA, NDEA, NEIPA, NDIPA, and NDBA are 0.20, 0.31, 0.46, 0.57, 0.66, and 1.00, respectively.]

**Suitability requirements**
**Correlation coefficient:** NLT 0.99
***y*-Intercept:** NMT 25% of the response of the medium concentration solution used in standard curve generation

**Analysis**
**Samples:** *Standard solutions* and *Sample solution*
Calculate the concentration, in ppm, of each specified nitrosamine impurity in the *Sample solution* using the corresponding calibration curve:

$$\text{Result} = [(R_U - y_{int})/a] \times (1/C_U) \times 10^3$$

$R_U$ = peak response ratio of the specified nitrosamine impurity to that of the internal standard from the *Sample solution*
$y_{int}$ = *y*-intercept of the calibration curve for the specified nitrosamine impurity from the *Standard solutions*
$a$ = slope of the calibration curve for the specified nitrosamine impurity from the *Standard solutions* [(µg/mL)$^{-1}$]
$C_U$ = concentration of the drug substance in the *Sample solution* (mg/mL)

Report the nitrosamine impurity concentration in the drug substance in ppm (µg/g).

**Procedure 4: Quantitation of NDMA, NDEA, NDIPA, NEIPA, NMPA, and NDBA in selected sartans (valsartan, losartan potassium, and candesartan cilexetil) by GC–MS/MS (triple-quad)**
**Internal standard solution:** 50 ng/mL of NDMA:C13-d6 in methylene chloride
**Standard solution:** Prepare a mixture of 0.1 µg/mL each of *N*-nitrosodimethylamine, *N*-nitrosodiethylamine, *N*-nitrosoethylisopropylamine, *N*-nitrosodiisopropylamine, *N*-nitrosomethylphenylamine and *N*-nitrosodibutylamine by mixing appropriate volumes of respective USP Reference Standards and diluting with *Internal standard solution*.
**Calibration solutions:** Depending on the targeted nitrosamine concentration in the sample, prepare a set of 5 consecutive solutions from *Table 13* that are used for generating the calibration curve by following the preparation scheme shown in the table. Volumes may be adjusted to prepare larger quantities of the calibration solutions as needed, maintaining final concentrations of the nitrosamines. For each calibration solution, transfer the designated aliquot of *Standard solution* to the designated volumetric flask, and adjust the volume with the *Internal standard solution*.

**Table 13**

| Calibration Solution ID | Standard Solution Aliquot (µL) | Final Volume (mL) | Final Nitrosamine Concentration (µg/mL) | Equivalent Nitrosamine Concentration (µg/g) |
|---|---|---|---|---|
| Cal 1 | 50 | 10 | 0.0005 | 0.005 |
| Cal 2 | 100 | 10 | 0.001 | 0.010 |
| Cal 3 | 200 | 10 | 0.002 | 0.020 |
| Cal 4 | 300 | 10 | 0.003 | 0.030 |
| Cal 5 | 400 | 10 | 0.004 | 0.040 |
| Cal 6 | 500 | 10 | 0.005 | 0.050 |
| Cal 7 | 1000 | 10 | 0.010 | 0.100 |
| Cal 8 | 1500 | 10 | 0.015 | 0.150 |

**Sample solution:** Transfer 500 mg of the drug substance into a disposable 10- to 15-mL glass centrifuge tube. Add 5.0 mL of the *Internal standard solution*. Cap the tube. Vortex the sample for 1 min, and then place in the centrifuge. Centrifuge the sample at 4000 rpm for 2.5 min. Transfer 2 mL of the bottom methylene chloride layer to a 5-mL syringe fitted with a 0.45-µm nylon filter. Filter 1 mL of sample extract into a 2-mL GC autosampler vial and cap.

**Chromatographic system**
(See *Chromatography* ⟨621⟩, *System Suitability*.)
**Mode:** GC
**Injector:** Split/splitless
**Injection type:** Splitless with purge
**Purge time:** 0.5 min
**Detector:** MS/MS (triple quadrupole mass spectrometer)
**MS conditions**
**Ionization:** Electron impact

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**Scan settings:** See *Table 14*.

**Table 14**

| Nitrosamine Impurity | Acquisition Mode | Polarity | MRM Transitions (m/z) | |
|---|---|---|---|---|
| | | | MRM-1[a] | MRM-2 |
| NDMA | MRM | Positive | 74 → 44 | 74 → 42 |
| NDMA:c13-d6 | MRM | Positive | 82 → 48 | – |
| NDEA | MRM | Positive | 102 → 85 | 102 → 56 |
| NEIPA | MRM | Positive | 116 → 99 | 71 → 56 |
| NDIPA | MRM | Positive | 130 → 88 | 130 → 42 |
| NMPA | MRM | Positive | 106 → 77 | 77 → 51 |
| NDBA | MRM | Positive | 158 → 99 | 84 → 56 |

[a] MRM-1 is used for quantitation.

**MS1 and MS2 resolution:** Q1: normal; Q3: wide (1.5)
**Minimum window:** 1 min
**Emission current:** 50 µA
**Column:** 0.25-mm × 30-m; fused-silica coated with a 1.0-µm layer of phase G16
**Temperatures**
**Injector:** 250°
**Transfer line to MS detector:** 250°
**Ionization source:** 250°
**Column:** See *Table 15*.

**Table 15**

| Initial Temperature (°) | Temperature Ramp (°/min) | Final Temperature (°) | Hold Time at Final Temperature (min) |
|---|---|---|---|
| 40 | 0 | 40 | 0.5 |
| 40 | 20 | 200 | 0 |
| 200 | 60 | 250 | 3 |

**Carrier gas:** Helium
**Flow rate:** Constant flow at 1.0 mL/min (adjustment and verification are necessary for other carrier gases)
**Injection volume:** 2 µL

**System suitability**
**Samples:** *Calibration solutions*
[NOTE—The relative retention times for NDMA, NDEA, NEIPA, NDIPA, NDBA, and NMPA are 0.73, 0.78, 0.81, 0.82, 0.99, and 1.00, respectively.]
Generate the peak response ratio of the specified impurity to that of the internal standard versus concentration standard curve for each nitrosamine impurity under test using the corresponding *Calibration solutions* and perform the linear regression analysis.

**Suitability requirements**
**Correlation coefficient:** NLT 0.98
**Signal-to-noise:** NLT 10 for the impurity peak of the lowest concentration *Calibration solutions* used in the calibration curve

**Analysis**
**Samples:** *Calibration solutions* and *Sample solution*
Calculate the concentration, in ppm, of each specified nitrosamine impurity in the *Sample solution* using the corresponding calibration curve:

$$\text{Result} = 5 \times (1/W) \times [(R_U - y_{int})/a]$$

$W$ = weight of the drug substance in the *Sample solution* (g)
$R_U$ = peak response ratio of the specified nitrosamine impurity to that of the internal standard from the *Sample solution*
$y_{int}$ = *y*-intercept of the calibration curve for the specified nitrosamine impurity from the corresponding *Calibration solutions*

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$a$ = slope from the calibration curve for the specified nitrosamine impurity from the corresponding *Calibration solutions* [$(\mu g/mL)^{-1}$]

Report the nitrosamine impurity concentration in the drug substance in ppm (µg/g).

**8.2. Limit Test Procedures**

While a limit test analytical procedure for nitrosamines content is not currently available, recommended sample preparation procedures are shown in *Table 16*.

**Table 16**

| Solutions | Solution Preparation |
|---|---|
| Internal standard solution | Prepare a suitable *Internal standard solution* that, when added to the *Sample solution*, will have the resultant peak response at the highest appropriate target limit of the nitrosamines of interest in the sample. |
| Sample solution | Prepare a solution of the article to be examined, spiked with the internal standard, and prepared as described in the sample preparation. The amount of substance to be examined is chosen in such a way that the amount, in ppm, of a target *N*-nitrosamine, if present at its limit concentration for that substance, would be equal to the contribution of the respective spiking solution. |
| Spiking solution | A solution of target *N*-nitrosamine(s) of a concentration that, if added to the amount of article used for the preparation of the *Sample solution*, would result in the acceptance limit. |
| Spiked sample solution | Prepare a solution of the article to be examined spiked with a) appropriate *Spiking solution*(s) and b) *Internal standard solution* prepared as described in the *Sample solution*. |

**Analysis**

**Samples:** *Spiked sample solutions* and *Sample solution*

Determine the peak response ratio of the respective target *N*-nitrosamine to the internal standard from the *Sample solution*: $R_{U(i)}$

Determine the peak response ratio of the respective target *N*-nitrosamine to the internal standard from the *Spiked sample solution*: $R_{ST(i)}$

**Acceptance criteria:** $R_{U(i)}/R_{ST(i)}$ is NMT 0.5

## 9. ADDITIONAL SOURCES OF INFORMATION

Several test procedures have been developed for the specific testing of nitrosamines in sartans and/or other official articles based on different scientific principles and are publicly available from many regulatory agencies. The hyperlinks in this section direct the user to the respective regulatory agencies' procedures. These can be used as alternative procedures and must be validated under actual use for the respective performance characteristics recommended in *7. Test Method Performance Characteristics of Nitrosamine Methods*.

1. FDA-published testing methods to provide options for regulators and industry to detect NDMA and NDEA impurities: https://www.fda.gov/drugs/drug-safety-and-availability/fda-updates-and-press-announcements-angiotensin-ii-receptor-blocker-arb-recalls-valsartan-losartan/?utm_campaign=UPDATE on angiotensin II receptor blocker (ARB) recalls - FDA publishes LC-HRMS and RapidFire-MS/MS&utm_medium=email&utm_source=Eloqua#testingmethods.
2. *Ph. Eur.* 2.5.42 *N*-Nitrosamines in active substances: https://www.edqm.eu/sites/default/files/medias/fichiers/European_Pharmacopoeia/News/european_pharmacopoeia_n-nitrosamines_in_active_substances.pdf.
3. EDQM projects on sampling strategies and testing methods with the Official Medicines Control Laboratory (OMCL) Network: https://www.edqm.eu/en/ad-hoc-projects-omcl-network.

## 10. *USP REFERENCE STANDARDS* ⟨11⟩

USP *N*-Nitrosodibutylamine RS
USP *N*-Nitrosodiethylamine RS
USP *N*-Nitrosodiisopropylamine RS
USP *N*-Nitrosodimethylamine RS
USP *N*-Nitrosoethylisopropylamine RS
USP *N*-Nitrosomethylaminobutyric Acid RS
USP *N*-Nitrosomethylphenylamine RS

## REFERENCES

1. International Council for Harmonisation of Technical Requirements of Pharmaceuticals for Human Use. ICH M7: *Assessment and Control of DNA Reactive (Mutagenic) Impurities in Pharmaceuticals to Limit Potential Carcinogenic Risk*, 2017. https://www.ich.org/page/multidisciplinary-guidelines.
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4. Food and Drug Administration. *Control of Nitrosamine Impurities in Human Drugs-Guidance for Industry*, 2021. https://www.fda.gov/media/141720/download. ▲ (USP 1-Dec-2021)

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***Add the following:***

# ▲⟨1503⟩ QUALITY ATTRIBUTES OF SYNTHETIC PEPTIDE DRUG SUBSTANCES

INTRODUCTION
PEPTIDE DEFINITION
MANUFACTURING METHODS
RAW MATERIALS
PEPTIDE GENERAL CHARACTERISTICS AND SPECIFICATION
- Color and Appearance
- Identification
- Bioassays
- Assay and Peptide Content
- Impurities
- Microbiological Contamination
- Bacterial Endotoxins
- Specific Tests

## INTRODUCTION

The pharmaceutical industry's interest in peptides as drug candidates—based on their potency, enormous functional diversity, high degree of specificity, and general lack of toxicity—has existed since their general structure was first elucidated more than 100 years ago. The early development of synthetic peptides for therapeutic use was, however, challenged by the high costs of production, their inherently short in-vivo half-lives, the lack of sensitive and discriminating analytical methods for intermediates, and the relatively complex manufacturing methods, which are now referred to as "solution peptide synthesis" or "liquid-phase peptide synthesis" (LPPS).

The introduction of solid-phase peptide synthesis (SPPS) was a key factor in facilitating the expansion of peptide research and made the production of peptides with sequences longer than 20 amino acids feasible. Also, in recent years, new formulations and conjugation strategies have resulted in alternative routes of administration and longer half-lives for peptide therapeutics, which have facilitated patient compliance. As a result, more peptide drugs have been approved and are in clinical development. In spite of these successes—or perhaps because of them—some challenges in peptide manufacturing remain, especially with the increasingly complex molecules entering development.

Because synthetic peptides are not easily classified as small molecules or biological products, they are specifically excluded from many guidance documents. Hence, this chapter provides an overview of the current status and the quality attributes of synthetic therapeutic peptides, with particular emphasis on drug substance impurities and related contributing factors, including 1) manufacturing methods, 2) raw materials, 3) peptide general characteristics and specifications, 4) assay and peptide content, 5) impurities, 6) microbiological contamination, 7) bacterial endotoxins, and 8) specific tests.

Drug product quality attributes and recombinant DNA technologies, which can also be employed for manufacturing peptides for pharmaceutical use, are not included in this chapter.

## PEPTIDE DEFINITION

Peptides are short chains of two or more amino acids covalently linked by amide bonds. They can be derived from rDNA or from a biological source or made by chemical synthesis. In the absence of clear scientific consensus on the criteria that distinguish proteins from peptides, including the exact size at which a chain or chains of amino acids become a protein, the FDA, in its final rule on the *Definition of the Term "Biological Product"* (*Federal Register* 85: 10057; February 21, 2020), distinguishes proteins from peptides based on size (i.e., number of amino acids).

FDA regulations state that a "protein is any alpha amino acid polymer with a specific defined sequence that is greater than 40 amino acids in size. When two or more amino acid chains in an amino acid polymer are associated with each other in a manner that occurs in nature, the size of the amino acid polymer for purposes of this interpretation will be based on the total number of amino acids in those chains, and will not be limited to the number of amino acids in a contiguous sequence" (21 CFR §600.3(h)(6)). FDA considers any polymer composed of 40 or fewer amino acids to be a peptide. Unless a peptide meets the statutory definition of a "biological product" (e.g., a peptide vaccine), it would not be considered a "biological product", and it would be regulated as a drug product under the Federal Food, Drug, and Cosmetic Act (FD&C Act). The concepts and approaches described in this chapter generally are applicable to chemically synthesized peptides.

## MANUFACTURING METHODS

A number of chemistries and methods have been developed for building peptide chains. The strategies selected are largely determined by the size and characteristics of the peptide needed. The classical approach to peptide synthesis, LPPS, is often

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chosen for manufacturing small peptides; however, SPPS has become the preferred option for manufacturing peptides of larger sizes. A hybrid SPPS/LPPS technique can also be used for manufacturing large peptides and peptide conjugates.

Differences in the LPPS and SPPS processes are particularly important, as they lead to differences in the impurity profiles of the final drug substance. In LPPS, potential impurities may be predicted based on the reaction scheme and usually can be identified and controlled individually for each reaction step. Typically, peptide-related impurities are easier to control in LPPS because they are less similar to the target peptide and their accumulation can be minimized by intermediate purification.

In SPPS processes, the peptide chain is assembled on a solid support, enabling excess reagents and amino acid derivatives to be washed off the resin-bound peptide on a filter screen at each step. Hence, non-peptide process-related impurities usually are not a major concern. However, all peptide-related impurities that are incorporated into the resin-bound peptide accumulate throughout the coupling and deprotection steps, leading to a complex profile of impurities very similar to that of the target peptide.

Regardless of the synthetic strategy, manufacturing highly purified peptides under good manufacturing practices (GMP) is a complex process and requires robust control strategies. Quality should be built into every step of the process, and a quality-by-design (QbD) strategy is often beneficial for a successful outcome.

## RAW MATERIALS

Starting material (SM) selection, impurity characterization, control strategies, supplier qualifications, and regulatory acceptance are integral to the strategy for the development of any synthetic peptide manufacturing process. The term "raw materials" is used to describe the SMs, solvents, and reagents that are used in the production of the peptide drug substance.

A "starting material" is a raw material, intermediate, or an active pharmaceutical ingredient that is used in the production of—and incorporated as a significant structural fragment into—the drug substance, as described in the following International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use guidelines: ICH Q7—*Good Manufacturing Practice Guide for Active Pharmaceutical Ingredients*; ICH Q11—*Development and Manufacture of Drug Substances (Chemical Entities and Biotechnological/Biological Entities)*; and the ICH Implementation Working Group's Q11 *Questions and Answers* document.

Typically, the most relevant quality attributes to be controlled for SMs are identity and purity, including related impurities and stereoisomeric impurities content, due to their impact on the quality of the drug substance. The determination of adequate acceptance criteria for the SM quality attributes is based on development data, validation of the manufacturing process, and the corresponding risk assessment. Historical batch data may also be used to set SM specifications. Guidance is provided for the management of SMs and their suppliers in ICH Q7 Sections 7 and 13; ICH Q10—*Pharmaceutical Quality System*, Section 2.7; ICH Q11; and ICH Q11—*Questions and Answers*. It is recommended that SMs be manufactured under a quality system that assures the consistency of batches and the ability to trace the operations carried out to reach the desired material. SMs coming from different qualified suppliers should comply with the overall control strategy for the drug substance. Thus, the selection of SMs should be based on knowledge of the intended commercial process and should take into account the relationship of the SM to the impurity profile of the drug substance.

The ICH Q11 guideline emphasizes that appropriate controls should be in place for the impurities present in the SMs, and that the impact of the impurities on the drug substance quality should be assessed. ICH Q11 also makes an important distinction between commercially available chemicals and custom synthesized chemicals. ICH Q11 states that while the use of a commercially available chemical as an SM generally does not need to be justified, chemicals produced by custom synthesis are not considered to be commercially available. Thus, their proposed use as an SM should be justified.

In some cases, amino acids, protected amino acid derivatives, or any other compound such as dipeptide derivatives may not have a pre-existing, non-pharmaceutical use to meet the ICH Q11 definition of commercially available chemicals; but provided they are simple enough in structure, they may be acceptable as SMs with the appropriate justification. Per ICH Q11—*Questions and Answers*, Section 5.6, a rationale should be provided explaining why the SM is considered appropriate and why the proposed strategy is suitable for controlling impurities in the drug substance. This usually includes, but is not limited to, justification of the proposed SM specifications, including the acceptance criteria for amino acid impurities and their enantiomers [and diastereomers for isoleucine (Ile) and threonine (Thr)], as well as data to support that the proposed control strategy is adequate for manufacturing a peptide drug substance of acceptable purity. Likewise, fragments proposed as SMs in the synthesis of peptides are considered custom synthesized chemicals. Therefore, manufacturers should consider all of ICH Q11 Section 5.1, *General Principles*, and ICH Q11—*Questions and Answers*, clarifications for the selection and justification of such fragments as SMs.

The origin of the SMs should be known in order to evaluate their potential risk of contamination with adventitious agents, including agents that cause transmissible spongiform encephalopathies (TSE). When possible, use of materials from "non-TSE-relevant animal species" or non-animal origin is preferred. If animal sources are necessary, efforts should be made to minimize the risk of transmission of TSE, including not obtaining materials from specific geographic regions.

The most common impurities found in the SMs used for the synthesis of peptides are listed in *Table 1*.

**Table 1. Potential Impurities in Starting Materials**

| Impurities | Origin |
|---|---|
| Incorrect enantiomers (e.g., D-amino acid instead of L-amino acid or L-amino acid present in D-amino acid) | Poor chiral purity of the SM |
| Dipeptides in fluorenylmethyloxycarbonyl protecting group (Fmoc) amino acid derivative (e.g., Fmoc-Leu-Leu-OH [Leu=leucine] instead of Fmoc-Leu-OH) | Synthetic impurities |
| β-Alanine-containing impurities (e.g., Fmoc-β-Ala-OH [Ala=alanine] and Fmoc-β-Ala-AA-OH [AA=amino acid or amino acid derivative]) | Generated during Fmoc-protection of the amino acid when using 9-fluorenylmethyl *N*-succinimidyl carbonate |
| Free amino acids (not amine protected); partially protected amino acids | Loss of N-terminal α-amino-protecting group or incomplete protection |

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**Table 1. Potential Impurities in Starting Materials** *(continued)*

| Impurities | Origin |
|---|---|
| Isomeric contaminants (e.g., residual isoleucine [Ile] in Leu derivatives) | Poor SM purity |
| Residual acetic acid (e.g., ethyl acetate) | From residual solvent used during synthesis of amino acid derivatives |

Solvents, reagents, and resins (with the exception of resins preloaded with the first amino acid or amino acid derivative) are not considered SMs, but the impact of their quality on the final drug substance quality should be evaluated through a formal risk assessment. For solvents and reagents, it is recommended to use the highest quality material available. The use of lower grade raw materials is appropriate if their use has been justified. High quality grade water (i.e., at least purified water) should also be used for the purification of peptides.

## PEPTIDE GENERAL CHARACTERISTICS AND SPECIFICATIONS

The specification for a drug substance needs to reflect the critical quality attributes proposed and justified by the manufacturer. The drug substance specification also needs to be approved by the regulatory authorities, as recommended by ICH Q6A—*Specifications: Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Chemical Substances* and by the International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH) guideline GL-39—*Specifications: Test Procedures and Acceptance Criteria for New Veterinary Drug Substances and New Medicinal Products: Chemical Substances*.

The process of setting a specification ensures that the quality of a drug substance is controlled through general characteristics, identity, assay, impurities determination, and specific tests. For reference, the *USP* general chapters that describe the test methods most frequently used for characterization and quality control of peptide drug substances are listed in *Table 2*.

**Table 2. Summary of Tests for Characterization and Quality Control of the Drug Substance**

| Test | Method | Comments |
|---|---|---|
| **Characteristic** | | |
| Appearance[a] | Visual inspection | — |
| **Identification** | | |
| High-performance liquid chromatography (HPLC) coelution with reference standard[a] | ⟨621⟩[b] | The method used for the identification of the active pharmaceutical ingredient (API) may be the same as the one used for the detection of related substances or for determination of the assay based on comparison with a reference standard |
| Mass spectrometry (MS)[a] | ⟨736⟩[c] | Monoisotopic mass |
| Amino acid analysis (AAA) | ⟨1052⟩[d] and ⟨507⟩[e] | Hydrolysis and derivatization protocols should be specified |
| Tandem mass spectrometry (MS–MS) sequencing | ⟨736⟩[c] | May be complicated for longer sequences |
| Nuclear magnetic resonance (NMR) spectroscopy (i.e., $^{1}H$, $^{13}C$ and $^{15}N$) | ⟨761⟩[f] | Complex interpretation for longer sequences |
| Peptide mapping by chemical or enzymatic cleavage methods | ⟨1055⟩[g] | Used for longer sequences (e.g., >20 amino acids); equivalent to MS–MS |
| Enantiomeric purity | ⟨621⟩[b] and ⟨736⟩[c] | AAA in combination with chiral chromatography and MS detection |
| N-Terminal sequence analysis | Edman degradation | Complicated analysis, especially for sequences that are N-terminally blocked; largely superseded by LC with MS–MS (LC–MS/MS) |
| Infrared spectroscopy | ⟨197⟩[h] and ⟨854⟩[i] | Limited use for peptides |
| Higher order structures | Circular dichroism, NMR, and Fourier-transform infrared spectroscopy (FTIR) | Used for investigation of secondary and tertiary structures in aqueous solutions |
| Bioidentity | ⟨1032⟩[j], ⟨1033⟩,[k] and ⟨1034⟩[l] | No longer a routine test for most peptides; may be required for large peptides or those with complex sequences |
| **Assay** | | |
| Assay by HPLC[a] | ⟨621⟩[b] | Method is based on a comparison with a reference standard and may be the same method used to measure related substances and for identification |
| **Peptide Content** | | |
| Peptide content by AAA | ⟨1052⟩[d] and ⟨507⟩[e] | Hydrolysis protocols must be validated; only well-recovered amino acids should be included in the calculation of mean peptide content |

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**Table 2. Summary of Tests for Characterization and Quality Control of the Drug Substance** *(continued)*

| Test | Method | Comments |
|---|---|---|
| Peptide content by nitrogen analysis | ⟨461⟩[m] | Direct analysis by elemental analysis, or Kjeldahl, or using HPLC with chemiluminescence nitrogen detector (CLND) |
| UV Spectroscopy | ⟨197⟩,[h] ⟨857⟩,[n] and ⟨507⟩[e] | Only useful for drug substances containing amino acids with suitable chromophores |
| Quantitative NMR (qNMR) | ⟨761⟩[f] and ⟨1761⟩[o] | A suitable internal standard is required |
| **Impurities** | | |
| Peptide-related substances[a] | ⟨621⟩[b], LC–MS | Method specific for drug substances; must be validated for both process-related impurities and degradation products; limits for total and individual impurities should be specified, LC–MS is a commonly used method for characterization |
| Residual solvents[a] | ⟨467⟩[p] | If justified, may be limited to solvent used in the final steps of the manufacturing process |
| Elemental impurities[a] | ⟨232⟩,[q] ⟨233⟩,[r] and ⟨1065⟩[s] | Required if metal catalysts are used in the manufacturing process; elemental impurities may be required based on the risk assessment |
| Residual trifluoroacetic acid[a] | ⟨503.1⟩[t] | Required if TFA is used during the manufacturing process |
| Residual fluoride[a] | ⟨1065⟩[s] or ion-selective electrodes | Required if hydrofluoric acid (HF) is used during the manufacturing process (e.g., due to *tert*-butyloxycarbonyl protecting group or *tert*-butoxycarbonyl protecting group [Boc]-chemistry) |
| Other small-molecule impurities | Impurity dependent | Non-peptide impurity limits are recommended to follow ICH Q3A(R2), *Impurities in New Drug Substances*; potentially mutagenic impurities require additional evaluation |
| **Specific Tests** | | |
| Counter-ion content[a] | For acetate, ⟨503⟩[u], for others, ⟨1065⟩[s] | Titration with silver nitrate may be used to determine chloride |
| Water content[a] | ⟨921⟩[v] | ⟨921⟩, *Method I, Method Ic (Coulometric Titration)*[v] preferred |
| Optical rotation | ⟨781⟩[w] | For characterization only |
| Thiol groups | Ellman's test | Only required if the reduced form(s) of a peptide containing disulfide bonds cannot be determined using the HPLC methods for related substances |
| Bioburden[a] | ⟨61⟩[x] and ⟨62⟩[y] | Often required for the drug substances used in the manufacture of parenteral drug products |
| Bacterial endotoxins[a] | ⟨85⟩[z] | Required for the drug substances used in the manufacture of parenteral drug products |

[a] Recommended test to be performed as part of the release.
[b] *Chromatography* ⟨621⟩.
[c] *Mass Spectrometry* ⟨736⟩.
[d] *Biotechnology-Derived Articles—Amino Acid Analysis* ⟨1052⟩.
[e] *Protein Determination Procedures* ⟨507⟩.
[f] *Nuclear Magnetic Resonance Spectroscopy* ⟨761⟩.
[g] *Biotechnology-Derived Articles—Peptide Mapping* ⟨1055⟩.
[h] *Spectroscopic Identification Tests* ⟨197⟩.
[i] *Mid-Infrared Spectroscopy* ⟨854⟩.
[j] *Design and Development of Biological Assays* ⟨1032⟩.
[k] *Biological Assay Validation* ⟨1033⟩.
[l] *Analysis of Biological Assays* ⟨1034⟩.
[m] *Nitrogen Determination* ⟨461⟩.
[n] *Ultraviolet-Visible Spectroscopy* ⟨857⟩.
[o] *Applications of Nuclear Magnetic Resonance Spectroscopy* ⟨1761⟩.
[p] *Residual Solvents* ⟨467⟩.
[q] *Elemental Impurities—Limits* ⟨232⟩.
[r] *Elemental Impurities—Procedures* ⟨233⟩.
[s] *Ion Chromatography* ⟨1065⟩.
[t] *Trifluoroacetic Acid (TFA) in Peptides* ⟨503.1⟩.
[u] *Acetic Acid in Peptides* ⟨503⟩.
[v] *Water Determination* ⟨921⟩.
[w] *Optical Rotation* ⟨781⟩.
[x] *Microbial Enumeration Tests* ⟨61⟩.

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[y] *Tests for Specified Microorganisms* ⟨62⟩.
[z] *Bacterial Endotoxins Test* ⟨85⟩.

## Color and Appearance

The color and appearance test by visual inspection is included in the specification of a drug substance because it gives a qualitative description of the color and physical state. The test also ensures a consistent appearance from batch to batch. However, because the processes adopted by different manufacturers can result in subtle variations in the color and appearance, tests by visual inspection are not included in *USP* monographs. In some cases, the description is available in *Reagents and Reference Tables, Reference Tables, Description and Relative Solubility*.

## Identification

Identification tests are specific for a peptide drug substance and, as stated by ICH Q6A, "should optimally be able to discriminate between compounds of closely related structure which are likely to be present". Identity is normally verified by multiple orthogonal techniques, generally including at least one specific test. The most commonly used identification tests for synthetic peptides are reverse-phase high-performance liquid chromatography (RP-HPLC) (see *Chromatography* ⟨621⟩) and mass spectrometry (MS) (see *Mass Spectrometry* ⟨736⟩). Other identification tests such as amino acid analysis (AAA), peptide mapping, or nuclear magnetic resonance (NMR) spectroscopy may be used.

HPLC identification tests rely on the comparison of the retention times of the peptide drug substance's main peak from a reference standard and the test sample, when injected separately, or by coelution of a reference standard and the test sample when co-injected as an equal mixture. Co-injection of an equal mixture of a reference standard and the sample is the recommended best practice. If the HPLC method is capable of distinguishing between the peptide and its closely related impurities, the same method can be used for the identification and impurity measurements.

The monoisotopic mass can be determined by MS, an orthogonal identification test. For peptides larger than 2 kDa, the use of high-resolution instruments may be necessary. In addition, tandem mass spectrometry (MS–MS) can be used for peptide mapping and sequencing to further characterize the synthetic peptide drug substance and to confirm the primary sequence of the peptide. However, peptide mapping and sequencing by MS–MS are normally not part of routine release testing of peptides.

Although AAA was previously a commonly used technique for identification, it has been gradually replaced by techniques such as HPLC and MS. Despite providing the amino acid composition of a peptide, AAA is unable to differentiate aspartic acid (Asp) from asparagine (Asn) and glutamic acid (Glu) from glutamine (Gln) or pyroglutamic acid (Pyr). However, AAA is still a useful tool when discrimination between leucine (Leu) and isoleucine (Ile) is required.

Infrared spectroscopy (IR) is of limited use for identification of a peptide drug substance due to the complexity of peptide molecules, but it is sometimes still used as an orthogonal identification test.

## Bioassays

Bioassays are laboratory tests that mimic the drug substance's mechanism of action towards a therapeutic target. In the past, many of these methods used animal models. Today, depending on the complexity of the biologic response, cell-based, or ligand- or receptor-based binding methods are used instead. During drug discovery and development, bioassays are commonly used to discover new chemical or biologic entities that may be agonists or antagonists of a specific therapeutic target. In later stages, for small molecules and many simple, small peptides, bioassay methods are not used for release once the more precise physicochemical methods are validated and justified. ICH Q6A does not mention bioassay use.

For more complex or long peptides, regulatory authorities may require that a bioassay remain as a release test to measure potency or to confirm that the functional activity of the peptide drug is maintained throughout the manufacturing process. Bioassays may be needed because a very small chemical change may cause a loss of functional activity. A "bioidentity" test, sometimes found in *USP* monographs, is a subset of the bioassay class and is used as an orthogonal identity test and to confirm the functionality of a drug substance or drug product. In the case of peptide monographs that include an HPLC-based assay and a bioidentity test, the number of replicates may be reduced because the acceptance criterion is often a limit (e.g., "activity not less than").

ICH Q6B—*Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products*, which applies to proteins, allows bioassays to be replaced by physicochemical tests when a well-established manufacturing history exists and when "sufficient physicochemical information about the drug, including higher-order structure, can be thoroughly established by such physicochemical methods, and relevant correlation to biologic activity demonstrated".

Manufacturers are encouraged to contact regulators early in development for control strategy related to bioassays, e.g., the need for a bioassay, its purpose, etc. Additional guidance for development, validation, and analysis of bioassays can be found in *Biological Assay Chapters—Overview and Glossary* ⟨1030⟩, *Design and Development of Biological Assays* ⟨1032⟩, *Biological Assay Validation* ⟨1033⟩, and *Analysis of Biological Assays* ⟨1034⟩.

## Assay and Peptide Content

The peptide drug substance assay is normally defined on an anhydrous, counter-ion free basis. Routine assay testing is normally performed by a specific chromatographic method. Assay by HPLC, based on the use of an established, quantitative standard, e.g., a pharmacopeial reference standard or an in-house developed peptide standard, is a relative method that is often the method of choice for peptides. If the HPLC method separates all peptide-related impurities, no correction for impurities is generally required. However, a correction for levels of additional impurities determined from a separate method may be applied. The specific method(s) used and the basis for the calculation should be defined and justified for each peptide drug substance. It is important to note that significant variability of assay results must be considered when establishing acceptable

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ranges. The main causes for assay variability are difficulty of sample preparation due to the hygroscopicity of peptides and batch-to-batch variability of reference standards. Peptide content (sometimes referred to as net peptide content) is the percentage of total peptides (i.e., drug substance plus peptide-related impurities) relative to everything else present in the sample, such as water, counter ions, residual solvents, and salts. Peptide content is usually determined by an absolute method such as quantitative AAA or nitrogen content determination. Peptide content may be helpful in the following cases:

- Drug substance testing, if no quantitative reference standard is available
- Qualification of a quantitative reference standard
- Investigations of an assay out-of-specification result, especially of the reference standard used

Peptides normally contain residual water, a counter ion, peptide-related impurities, and residual solvents. While all of these components can be determined individually, there are tests that will determine combinations of them. While primary or absolute methods do not require a reference standard, relative methods are based on a comparison to a reference standard. The quantitative qualification of a primary reference standard requires absolute methods. There are several absolute methods available to determine the peptide content.

It is important to consider that most absolute methods are non-specific and will only determine the peptide content, not discriminating between the drug substance and peptide-related impurities. If a specific result for the peptide drug substance assay is needed to substitute for an HPLC assay result, the peptide content may be corrected for the total related impurities, which are usually determined by HPLC. Where relevant, other impurities, such as oligomers measured by size-exclusion chromatography (SEC) or peptide impurities measured by ion-exchange chromatography, should also be included in the correction.

The easiest approach to determine the net peptide content is a simple mass balance calculation that consists of deducting the percentage of water, of counter ion, and, if relevant, of total other non-peptide impurities from 100%.

Peptide content can also be determined by elemental nitrogen analysis, Kjeldahl analysis, or chromatographic methods with nitrogen chemiluminescence detection (NCD) (see *Biotechnology-Derived Articles—Total Protein Assay* ⟨1057⟩ and *Nitrogen Determination* ⟨461⟩).

Quantitative AAA (see *Biotechnology-Derived Articles—Amino Acid Analysis* ⟨1052⟩ or *Protein Determination Procedures* ⟨507⟩, *Procedure, Method V. Amino Acid Analysis*) has traditionally been used for the determination of peptide content; however, because the method involves a relatively complex sequence of operations, results may vary. Peptide content by quantitative AAA compares the peak areas of the amino acids in a hydrolyzed sample with the peak areas of the same amino acids in an external standard. The peptide hydrolysis procedure should be validated to ensure good recovery, and only well-recovered amino acids should be used in the calculation of mean peptide content. Some peptide sequences containing multiple, adjacent hydrophobic residues, such as isoleucine (Ile), phenylalanine (Phe), leucine (Leu), and valine (Val), or sterically hindered amino acids, such as valine (Val) and isoleucine (Ile), may give a lower recovery than expected.

Quantitative NMR (qNMR) is also a useful tool for peptide content determination, although it requires a suitable internal standard with signals that fully resolve from the signals associated with the peptide. The versatility of qNMR for quantitative analysis of peptides that do not contain an appropriate UV chromophore makes it well-suited for the determination of peptide concentration in aggregation studies.

UV at 280 nm is used during characterization to determine the content of peptides containing tryptophan (Trp) or tyrosine (Tyr). The molar absorption coefficient required for the measurements at UV 280 nm can be calculated theoretically based on knowledge of the sequence; however, the method should be independently validated using orthogonal techniques. Peptide content by UV absorption may be applied as a fast and simple method for frequent or on-line, in-process determination of peptide concentrations.

## Impurities

A peptide impurity profile may differ significantly depending on the technology used for manufacturing. Hence, it is difficult to generalize the impurities and their control strategies. In addition, because the manufacturing technologies for the production of peptides are diverse and complex compared to small molecules, the impurity guidelines applicable to small molecules usually cannot be applied to peptides.

### CLASSIFICATION

*Impurities in Drug Substances and Drug Products* ⟨1086⟩ classifies impurities into three categories: organic impurities, inorganic impurities, and residual solvents. However, from a practical perspective, impurities can be classified as either peptide related or non-peptide.

**Peptide-related impurities:** Peptide-related impurities, also referred to as "related substances" in this chapter, comprise all impurities that are related to the structure of the target molecule. These are by-products derived from SM impurities, side-reactions during synthesis, or degradations during storage. Thus, peptide-related impurities are either process related or drug related. Stereoisomers are also considered process-related impurities or degradation products. *Table 3* summarizes the categories and types of peptide-related impurities, their origin and/or relevance, and the recommended analytical techniques for their identification. The information provided focuses on the most-common examples and should not be considered exhaustive.

**Non-peptide impurities:** Non-peptide impurities are impurities that are introduced by the manufacturing process and are not related to the structure of the target molecule. These comprise residues of raw materials, reagents, and auxiliary materials such as catalysts and solvents. Such residues also include reaction by-products and contaminants carried over by these materials, as shown in *Table 4*. Peptide drug substances are often defined with counter ions such as acetate or chloride. These counter ions are not considered impurities but concomitant components according to *Ordinary Impurities* ⟨466⟩. Nevertheless, excess bases or acids should be limited.

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## IMPURITIES IN SYNTHETIC PEPTIDES

The purity of a peptide is usually determined by HPLC. The method should be capable of separating SM, process-related, and degradation impurities. When using multiple methods, orthogonal separation techniques are recommended. One or more analytical procedures may be required for comprehensive quantitation of all relevant peptide-related impurities. For peptides containing hydrophobic residues, RP-HPLC methods may be appropriate, although alternatives, such as ion-exchange or mixed-mode methods could be considered. For hydrophilic peptides, hydrophilic interaction chromatography stationary phases may achieve the necessary resolution. Anion- and cation-exchange chromatography may be also useful. Liquid chromatography–mass spectroscopy (LC–MS) compatible methods are also advantageous for the identification of impurities. At least one of the methods should be a stability-indicating method.

In order to set acceptance criteria for peptide-related impurities, it is helpful to further classify impurities as specified or unspecified. Specified impurities can be identified or nonidentified. Impurities are classified as identified when their structure has been elucidated. Impurities are classified as qualified when their limits have been justified by toxicological studies, clinical trials, or by a structure-based scientific rationale. If present in the drug substance, qualified identified and unidentified impurities are usually included in the final release specification as specified impurities. Non-qualified impurities should be limited by a general acceptance criterion for “any unspecified impurity”. While ICH Q3A(R2), *Impurities in New Drug Substances*, limits each unspecified impurity to 0.10% or 0.05%, peptides are excluded from this guideline. The higher threshold (0.5% for identification) adopted by the *European Pharmacopoeia* (*Ph. Eur.*) is recognized by authorities of *Ph. Eur.* member states. Other authorities, e.g., the FDA may consider the limits for unspecified impurities for new peptides on a case-by-case basis.

In order to define appropriate targets for process and analytical development, regulators encourage case-specific meetings [e.g., pre-investigational new drug (pre-IND)]. As appropriate, the specification parameters should include each specified identified impurity, each specified unidentified impurity, any unspecified impurity, and the total impurities as the sum of all impurities.

**Peptide-related impurities:** A control strategy for peptide-related impurities, built on process characterization, should be implemented. The control strategy should account for the impurities introduced with the SMs and the impurities generated during the peptide synthesis and purification processes. Appropriately validated in-process control analytical procedures may be used to ensure the quality of the final drug substance, especially when an impurity cannot sufficiently be controlled by the release method.

The purity of a very complex peptide (i.e., very large or conjugated peptides) may be difficult to monitor due to broad signals and poor resolution in HPLC. The control strategy should then comprise the control of related substances for appropriate key intermediates, e.g., the peptide prior to conjugation. To determine possible peptide polymerization, a test for the presence of multimers is needed. A specific methodology such as SEC can be used to quantitate multimers.

HPLC with UV detection is often chosen for the determination of impurities. Because of the frequent similarities between the peptide-related impurities and the drug substance, and because of their potential coelution, smaller particle sizes allow for superior resolution of related compounds on C18, C8, and biphenyl columns. Incomplete separation may be observed with conjugated peptides or as the peptide chain length increases. UV detection is not specific for peptide-related impurities and cannot unequivocally identify these impurities by retention time and UV absorbance, or quantify impurities that coelute. Understanding the impurity profile is important to ensure drug quality and safety, such as any adverse events associated with immunogenicity. Hence, more sensitive and specific LC-HRMS method is recommended for characterizing peptide-related impurities.

**Table 3. Peptide-Related Impurities: Origin and Commonly Used Analytical Techniques**

| Type | Description | Origin | Analytical Techniques |
|---|---|---|---|
| Deletion (clipped forms, fragments, truncations) | Loss of one or more amino acids | Synthesis (incomplete coupling or deprotection) or storage (hydrolysis of N- or C-terminal amino acids or fragments) | LC–MS or LC–MS/MS |
| Insertion | Presence of one or more additional amino acids | Raw materials (SMs containing the respective protected dipeptide) or synthesis (loss of the N-protecting group during coupling, presence of unprotected amino acids in the starting material) | LC–MS or LC–MS/MS |
| Substitution | Presence of one or more different amino acids | SM contaminants or insufficient washes during peptide-resin synthesis | HPLC/UHPLC spiking with synthesized analogs or isolation/AAA/LC–MS or LC–MS/MS |
| Truncation | Sequences containing N-terminal deletions | Raw materials (materials containing residual acetic acid), synthesis (steric hindrance, deliberate acetylation of incomplete coupling reactions), or storage | LC–MS or LC–MS/MS |
| Stereoisomers (diastereomers) | Sequences containing epimerized amino acids | SMs, synthesis, or degradation | HPLC/UHPLC spiking with synthesized diastereomeric analogs or chiral analysis |

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**Table 3. Peptide-Related Impurities: Origin and Commonly Used Analytical Techniques** *(continued)*

| Type | Description | Origin | Analytical Techniques |
|---|---|---|---|
| Asp/Asn-related impurities | Aspartimide/succinimide-containing sequences | Cyclization to the backbone via loss of water or ammonia, respectively, from the sidechain of Asp or Asn | HPLC, LC–MS, LC–MS/MS |
| | β-Asp-containing sequences | Hydrolytic ring-opening of the aspartimide/succinimide intermediate during processing or storage | HPLC/UHPLC spiking with synthesized analogs or isolation |
| | Asp stereoisomers (diastereomers) | Epimerization of the aspartimide/succinimide intermediate followed by ring-opening during processing or storage | HPLC/UHPLC spiking with synthesized analogs or isolation |
| | Chain cleavage products | Total hydrolysis of the aspartimide/succinimide intermediate during processing or storage | HPLC/UHPLC spiking with synthesized analogs or isolation |
| β-Alanine insertion | Sequences containing β-alanine | SM contaminants | LC–MS or LC–MS/MS |
| Pyroglutamic acid | Sequence containing N-terminal glutamine or glutamic acid | Synthesis | HPLC or LC–MS |
| Oligomers | Polymers or aggregates | Conformational changes or peptide polymerization | SEC or SEC–multi-angle light scattering (SEC–MALS), sedimentation velocity analytical ultracentrifugation (SV–AUC), dynamic light scattering (DLS), field-flow fractionation (FFF), or ion mobility spectrometry (IMS) |
| Disulfide reduction | Sequences containing disulfides | Synthesis or storage | LC–MS or LC–MS/MS |
| Others | Deamidation of glutamine, asparagine, or C-terminus | Synthesis or storage | LC–MS or LC–MS/MS |
| | Acetylation of amino functional group | Synthesis or storage | LC–MS or LC–MS/MS |
| | Oxidation of certain residues, e.g., aromatic or sulfur-containing side-chains | Synthesis, storage, or analytical sample preparation | LC–MS or LC–MS/MS |
| | Alkylation, acylation of amino acids | Deprotection (cleavage) | LC–MS or LC–MS/MS |

*Stereoisomers:* Non-routine determination of stereoisomeric purity is possible using chiral AAA. The method is based on acid hydrolysis of the peptide, suitable derivatization of the resulting amino acids, and determination of the optical isomers of the constituent amino acids, e.g., by chiral gas chromatography with mass spectrometry (GC–MS) detection. HPLC-based methods of chiral AAA involve pre- or post-column derivatization of amino acid hydrolysates, e.g., using Marfey's reagent or *o*-phthalaldehyde (OPA), respectively. The method should be validated to account for potential epimerization during hydrolysis or subsequent derivatization. The use of deuterated reagents, e.g., deuterium chloride (DCl) and deuterium oxide ($D_2O$), results in substitution of the α-carbon hydrogen with deuterium during the epimerization process, permitting correction for the epimerization during hydrolysis and derivatization when using MS-based detection methods. It is recommended that stereoisomeric purity data are established for at least three representative batches to demonstrate that the manufacturing process does not lead to unacceptable levels of diastereomers. If there are indications of significant epimerization, the respective diastereomers should be identified and routinely controlled.

**Non-peptide impurities:** Reagents used in the manufacturing process should be included in the drug substance specification if there is a risk of residues of such reagents in the drug substance. One example is trifluoroacetic acid (TFA) used in the cleavage and deprotection steps when fluorenylmethyloxycarbonyl protecting group (Fmoc) chemistry is utilized. The drug substance specification should include tests for the determination of residual solvents "likely to be present" in accordance with ICH Q3C(R7)—*Impurities: Guideline for Residual Solvents*; acceptance criteria are to be set in accordance with the same guideline.

Other examples of potential process residues include elemental impurities. As described in ICH Q3D(R1)—*Guideline for Elemental Impurities*, *Elemental Impurities—Limits* ⟨232⟩, and *Elemental Impurities—Procedures* ⟨233⟩, elemental impurities may arise from several sources: they may be residual catalysts that were added intentionally during synthesis, they may be present as impurities (e.g., through interactions with processing equipment or container–closure systems), or they may be derived from the raw materials.

The acceptance criteria for residual elemental impurities are established based on permitted daily exposure (PDE) for each element of toxicological concern. Guidance for elemental impurity limits is provided in ICH Q3D(R1). If the risk assessment fails to demonstrate that an elemental impurity level is consistently below the control threshold, additional control should be implemented to ensure that the elemental impurity level in the drug product does not exceed the PDE. In some cases, lower levels of elemental impurities may be warranted when levels below toxicity thresholds have been shown to have an impact on other quality attributes of the drug product (e.g., element-catalyzed degradation of drug substances).

For many process-related impurities, it is possible to rely on the manufacturing process to remove them. HPLC purification methods may also be able to remove residual process materials. For analysis of residual solvents, elemental impurities, and residual anions and cations, methods such as GC, inductively coupled plasma mass spectrometry (ICP–MS), and ion chromatography (IC) should be used, respectively. NMR is particularly useful during process characterization because diffusion experiments allow for rapid differentiation between small-molecule organic impurities and peptide-bound modifications.

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**Table 4. Non-Peptide Impurities: Origin and Commonly Used Analytical Techniques and Reference Guidance**

| Type | Origin | Analytical Techniques and Reference Guidance |
|---|---|---|
| SMs, intermediates, reagents, and their respective reaction or degradation products | SMs or reagents | ⟨621⟩[a] (e.g., HPLC or GC) |
| Residual solvents | Solvents used in late stages of manufacturing (e.g., acetonitrile used as eluant for the preparative HPLC purification) | ⟨467⟩[b] and ICH Q3C(R7) |
| Inorganic impurities, residual anions of organic acids, and residual cations of bases | Residual anions or cations present as counter ions or buffer components (e.g., TFA or triethylammonium phosphate (TEAP), or fluoride originating from hydrogen fluoride used with Boc-SPPS) | ⟨503⟩,[c] ⟨503.1⟩,[d] and ICH Q3C(R7) (e.g., for acetic acid and triethylamine) |
| Elemental impurities | SMs, catalysts during synthesis; equipment | ⟨232⟩,[e] ⟨233⟩,[f] and ICH Q3D(R1) |
| Bioburden | Aqueous solutions or buffers at neutral pH | ⟨61⟩,[g] ⟨62⟩,[h] and ⟨1111⟩[i] |
| Bacterial endotoxins | Materials used in late stages of manufacturing | ⟨85⟩[j] |

[a] *Chromatography* ⟨621⟩.
[b] *Residual Solvents* ⟨467⟩.
[c] *Acetic Acid in Peptides* ⟨503⟩.
[d] *Trifluoroacetic Acid (TFA) in Peptides* ⟨503.1⟩.
[e] *Elemental Impurities—Limits* ⟨232⟩.
[f] *Elemental Impurities—Procedures* ⟨233⟩.
[g] *Microbial Enumeration Tests* ⟨61⟩.
[h] *Tests for Specified Microorganisms* ⟨62⟩.
[i] *Microbiological Examination of Nonsterile Products: Acceptance Criteria for Pharmaceutical Preparations and Substances for Pharmaceutical Use* ⟨1111⟩.
[j] *Bacterial Endotoxins Test* ⟨85⟩.

## Microbiological Contamination

In contrast to biotechnological processes, the manufacturing of peptides by chemical synthesis normally involves organic solvents and conditions that do not support microbiological growth. Unless pure aqueous solutions or buffers at neutral pH are involved, microbiological growth can easily be prevented. Bioburden should be controlled according to *Microbial Enumeration Tests* ⟨61⟩ and *Tests for Specified Microorganisms* ⟨62⟩. Appropriate acceptance criteria are recommended in *Microbiological Examination of Nonsterile Products: Acceptance Criteria for Pharmaceutical Preparations and Substances for Pharmaceutical Use* ⟨1111⟩.

## Bacterial Endotoxins

In most cases, when used in injection products, peptides should be controlled for bacterial endotoxins according to *Bacterial Endotoxins Test* ⟨85⟩. The final endotoxin content of a synthetic peptide is mainly dependent on the endotoxin content of materials introduced in late manufacturing stages, as synthesis conditions will normally not favor microbiological growth. Consequently, the endotoxin contribution of these materials needs to be controlled.

## Specific Tests

### WATER

Water is often a constituent of the peptide drug substance when it is isolated from aqueous solutions by, for example, lyophilization. As peptides are typically hygroscopic, the water content can significantly affect the peptide content and stability of the drug substance and should therefore be controlled. The acceptance criterion for water content is specific to each drug substance and is usually set based on batch history. This specification parameter may contribute to the peptide stability. It is usually included in the stability study of the peptide drug substance because it is required for the calculation of the assay by HPLC. As per ICH Q6A, the acceptance criterion may be justified with data on the effects of hydration or moisture absorption.

Typically, *Water Determination* ⟨921⟩ is used to monitor the water content. The more specific Karl Fischer titration in ⟨921⟩, *Method I, Method Ic* is preferred. If volatile components in addition to water are also expected, then *Loss on Drying* ⟨731⟩ is a suitable alternative. In addition, if residual solvents are expected, they should be quantitated independently (see *Residual Solvents* ⟨467⟩).

### COUNTER ION

Most peptide drug substances contain a counter ion, commonly acetate or chloride. The counter ion acceptance criterion is specific to each drug substance and is usually set based on batch data history. Counter-ion determination usually is not included in stability studies of peptide drug substances. A common method for acetate determination is described in *Acetic Acid in Peptides* ⟨503⟩. Alternatively, *Ion Chromatography* ⟨1065⟩ provides additional information on common techniques used for this purpose.▲ (USP 1-Aug-2021)

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***Add the following:***

# ▲⟨1504⟩ QUALITY ATTRIBUTES OF STARTING MATERIALS FOR THE CHEMICAL SYNTHESIS OF THERAPEUTIC PEPTIDES

## SCOPE

This general chapter is intended to provide recommendations on the minimum quality attributes for starting materials used in the manufacture of synthetic therapeutic peptides. Because it is not possible to cover the wide variety of available starting materials that may be used, including amino acids, protected amino acid derivatives, and peptide fragments, the discussion in this chapter will be limited to the most commonly used protected amino acid derivatives (AAD), i.e., 9-fluorenylmethyloxycarbonyl (Fmoc) amino acid derivatives (including resin-bound AAD). However, it is intended that the general concepts and guidance provided for this class of starting materials should be applied to all peptide starting materials, where applicable. This chapter is to be used in conjunction with *Quality Attributes of Synthetic Peptide Drug Substances* ⟨1503⟩.

## INTRODUCTION

The increased interest in peptides as drug candidates during the past few decades can be attributed to a number of factors, including advances in proteomics and high-throughput screening techniques, which have accelerated the development of new potential therapeutic candidates. Furthermore, the development of efficient synthetic chemical strategies, combined with robust purification processes and more powerful and discriminating analytical methodologies, have facilitated the manufacture of peptides with high-quality standards. The evolution of these synthetic strategies started with the manufacture of relatively short peptides but today, the manufacture of longer peptides, which traditionally have been manufactured by biological processes, is feasible using synthetic chemical techniques.

Because synthetic peptides are not classified as small molecules or biological products [final rule on the *Definition of the Term "Biological Product"* (85 FR 10057, February 21, 2020)], they are excluded from many guidance documents. ⟨1503⟩ provides an overview of the current status regarding the quality attributes of synthetic peptides used as drug substances.

In the manufacture of any active pharmaceutical ingredient (API; this term is used interchangeably with "drug substance"), the term "raw material" is used independently for describing starting materials (SMs), solvents, and reagents. From a quality point of view, the "most critical" raw materials are those that are incorporated as significant structural fragments into the structure of the API. From a regulatory perspective, these critical raw materials are known as SMs because their introduction into the manufacturing process establishes the point at which the production of the API begins.

Special attention must be paid to the quality attributes of these SMs because they have the potential to directly impact the quality of the drug substance. For the manufacture of peptides by chemical synthesis, the starting materials are amino acids, protected amino acid derivatives, and fragments. Hence, this chapter will focus on the quality criteria for the starting materials used in the manufacturing process with a particular emphasis on impurities.

## SUPPLIER QUALIFICATION AND EVALUATION OF SYNTHETIC ROUTE

The scope of good manufacturing practices for APIs as described in the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines ICH Q7—*Good Manufacturing Practice Guide for Active Pharmaceutical Ingredients* and ICH Q11—*Development and Manufacture of Drug Substances (Chemical Entities and Biotechnological/Biological Entities) Questions and Answers (Regarding the Selection and Justification of Starting Materials) Section 5.13* excludes the steps prior to the introduction of the "API starting material" into the manufacturing process. This lack of specific guidance for the manufacture of starting materials does not exclude the responsibility of peptide manufacturers to establish a qualification program for their suppliers and the starting materials they manufacture that is aligned with their process design and their process risk assessment.

It is recommended that the suppliers of starting materials manufacture their products under a quality system that ensures the consistency of batches manufactured and the traceability of the operations carried out to reach the product. The level of the requirements of this quality system should be higher in the case of fragments used as starting materials than for amino acids or protected amino acid derivatives. For more discussion of this topic, refer to ⟨1503⟩.

The implementation of technical agreements or quality agreements for the suppliers of starting materials is strongly recommended. These agreements must consider the obligation for the supplier to inform the customer about changes in the synthetic route used for the protected amino acid derivatives. Peptide manufacturers must assess the impact of these changes on the quality of their final drug substances. Starting material manufacturers are required to provide sufficient information to support regulatory submissions. Publicly available, published synthetic routes for the manufacture of starting materials can be used as supportive information. Implementation of confidentiality agreements between peptide manufacturers and their suppliers of critical raw materials will be helpful in performing such impact assessments.

Manufacturers of drug substances must assess any changes of starting material supplier or significant changes in the starting material manufacturing process for their impact on the quality attributes of the drug substance. The level of requirements for this assessment must take into consideration whether the starting material is an amino acid, a protected AAD, or a fragment. The existence of a supplier qualification program and a common control strategy at the reception of the starting material (common quality requirements aligned with the assessment of their impact on the quality of the final product) should be adequate. For fragments, as they may be customized, a change of manufacturer should be appropriately justified.

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The synthetic route for the amino acid derivative used for the manufacture of synthetic peptides potentially could result in different impurities. Impurities can be classified into three main categories:

1. AAD-related impurities originating from the amino acids
2. AAD-related impurities originating from the AAD manufacturing process
3. Non-AAD-related impurities

The first two categories are considered to be critical because they can increase the level of related impurities in the drug substance. The third category refers to process impurities (for example, solvents, elemental impurities, and nitrosamines) that do not generally interfere with the manufacture of peptides especially for solid-phase synthesis; however, their levels must be assessed to determine whether their presence poses a risk to the safety of the product and the health of the patients.

## AAD-RELATED IMPURITIES ORIGINATING FROM AMINO ACIDS

One potential cause for critical impurities in the protected amino acid derivatives is related to contaminants of the unprotected amino acids used to manufacture those AADs. Amino acid-related impurities, such as stereoisomers and foreign amino acids, especially, may become critical AAD-related impurities after the introduction of the protecting groups. This will be discussed in this section.

For the sake of simplicity within this chapter, the term “amino acid” shall be limited in its meaning to the 20 proteinogenic (“natural”) α-amino acids and their stereoisomers.

### Amino Acid Enantiomers

Of the 20 proteinogenic α-amino acids, 19 have an asymmetrical center at their α-carbon. The only achiral exception is glycine. The chiral proteinogenic amino acids have the L-configuration in the Fisher projection that is equivalent to the *S*-configuration in International Union of Pure and Applied Chemistry (IUPAC) nomenclature according to Cahn–Ingold–Prelog sequence rules.

Most chiral amino acid isomers exist in two forms that are enantiomers to each other, namely the *S*- and *R*-enantiomers (or L- and D-, respectively). Exceptions are isoleucine and threonine as discussed below.

Due to their abundance in nature, the *S*-amino acids are the ones that are most frequently required for peptide synthesis. They are commercially available in excellent optical purity (<0.1%–0.5% D-content) as *R*-amino acids as their most likely isomeric contaminants are rare in natural sources. For the same reason, *R*-amino acids are much more difficult to obtain in high optical purity when needed for peptide synthesis.

### Amino Acid Diastereomers

Isoleucine and threonine have two asymmetrical centers: one at the α-carbon and one at the β-carbon. Hence, four isomeric configurations exist and lead to the two enantiomeric pairs *SS*/*RR* and *SR*/*RS*, respectively. These two pairs of enantiomers are diastereomers of each other. Proteinogenic isoleucine and threonine have the *SS*-configuration; whereas their enantiomers have the *RR*-configuration. The *SR*/*RS*-diastereomers of isoleucine and threonine are called *allo*-isoleucine and *allo*-threonine, respectively. The diastereomers are also the most likely isomeric impurities of isoleucine and threonine due to inversion at the α-carbon.

### Foreign Amino Acids

Amino acids are normally manufactured from natural protein sources and must be separated from all other amino acids present. Hence, amino acids may be contaminated with residual foreign amino acids that could carry over into the AAD and finally be incorporated into the target peptide. Therefore, foreign amino acids are critical impurities of amino acids and must be controlled and limited if relevant. In particular, contamination with isomeric impurities such as isoleucine in leucine is especially challenging.

### Control of the Critical Impurities

The critical amino acid-related impurities may be controlled either directly in the amino acids or in the subsequently manufactured AAD. In most cases, separating foreign or isomeric amino acids is easier at the unprotected stage than at the protected stage as AAD properties are mainly determined by the bulky protecting groups, i.e., the Fmoc group in Fmoc chemistry. Therefore, it is recommended that AAD manufacturers should control these critical impurities directly in the amino acids used for manufacturing the AADs. Peptide manufacturers who purchase the AADs should ensure appropriate quality by:

1. Verifying that their AAD manufacturers appropriately control the critical impurities in their starting amino acids
2. Including the resulting critical impurity derivatives into their AAD specification and incoming material testing

The stereoisomers of both amino acids and their protected derivatives can be quantitated by chiral chromatographic methods. Chiral gas chromatography (GC) analysis with pre-column derivatization and flame ionization detector (FID) detection is recommended for unprotected amino acids, whereas HPLC on chiral reverse-phase (RP)-columns is the recommended technique for Fmoc amino acid derivatives.

Chiral GC analysis is also frequently used for AADs. In this case, the protecting groups must first be removed by hydrolysis. Because the hydrolysis may lead to epimerization, it must be performed in deuterated solvents (deuterium chloride/deuterium oxide), in which case molecules that epimerize during hydrolysis will have the α-hydrogen replaced by deuterium. By using the chiral GC method with mass detection, the isomers that epimerized during hydrolysis will be disregarded. However, the accuracy

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of this method is generally limited to a standard deviation (SD) of 0.1%. For the determination of very low isomer levels, chiral RP-HPLC is the more accurate technique.

Foreign amino acids may also be determined together with the stereoisomers by the methods described above. Of course, the usual methods for amino acid analysis may also be applied to screen for foreign amino acids.

## AAD-RELATED IMPURITIES ORIGINATING FROM THE AAD MANUFACTURING PROCESS

Several types of impurities related to the AAD production process may be found in protected amino acids used for peptide synthesis. They can be divided into several groups based on their origin and potential impact on the quality of the peptide drug substance.

### Partially Protected and Unprotected Amino Acids

The first group is the unprotected and partially protected amino acids. These impurities can be formed during the AAD production process or by degradation on storage or transportation. In general, the incomplete protection of functional groups causes a significant change in polarity compared to the fully protected AAD. Therefore, partially protected AADs can normally be removed easily from the target AAD but, nevertheless, these impurities should be controlled through the specification. Partially protected AADs can be divided into three subgroups.

#### PARTIAL LOSS OF THE SIDE CHAIN PROTECTING GROUP

In peptide synthesis, fully protected amino acids are used to avoid formation of impurities due to various possible side reactions of the unprotected functional groups. However, the presence of a small amount of side chain unprotected amino acid derivatives will not usually result in significant impact on the purity of the peptide (depending on the amino acid, the target peptide, and the synthesis conditions). Therefore, the acceptance criteria for such impurities should be set based on careful evaluation of the particular production process during development. Because amino acid derivatives are frequently protected by an Fmoc group at the N-terminus, these impurities can be easily controlled by regular HPLC methods and identified by liquid chromatography-mass spectrometry (LC-MS).

#### PARTIAL LOSS OF THE FMOC GROUP

These impurities can have a critical impact on the quality of the drug substance since their presence will lead to formation of double insertion impurities due to double coupling of the amino acid. Moreover, depending on the coupling conditions, the free amino groups may cause further Fmoc cleavages in the coupling mixture thus increasing the impurity concentration autocatalytically. If the amino acid also contains suitable side chain protection, they can be controlled by RP-HPLC method and identified by LC-MS.

#### UNPROTECTED AMINO ACIDS

Similar to the previous type of impurities without the Fmoc group, these impurities are also critical for the quality of the drug substance, since they can lead to double insertion due to double coupling and also side chain derivatization. Most of the amino acids are very polar and don't have a chromophore to enable sensitive detection by UV; consequently, they elute at the beginning of the run on an RP-HPLC column and are difficult to detect. Therefore, in most cases regular HPLC methods are not adequate for their determination and the development of specific methods is required, usually based on pre-column derivatization. Thin-layer chromatographic (TLC) methods may also be appropriate for this purpose if a limit test is sufficient. In general, free amino acids should not be present in AADs since they can be removed readily from the respective Fmoc derivative.

### β-Alanine (Ala) Impurities

The second group of impurities originating from the manufacturing process are derived from the side reaction that takes place during the introduction of the Fmoc-protecting group. If this is accomplished using Fmoc-succinimide (Fmoc-OSu) as the reagent, Fmoc-β-alanine (Ala) and Fmoc-β-Ala-amino acid (AA) impurities may be formed. Because these impurities can be formed in all Fmoc-protected amino acid derivatives, without strict control of the starting material, many potential impurities derived from the addition of β-alanine can be formed at different positions during the synthesis of the peptide sequence. Some of these impurities are very difficult to separate from the target peptide. Control of the presence of these impurities in the AAD and limiting them to acceptable levels is critical for the reduction of β-alanine contamination to acceptable levels during the production of peptide drug substances using Fmoc chemistry. Levels of Fmoc-β-Ala and Fmoc-β-Ala-AA can be controlled by RP-HPLC techniques through use of the appropriate Reference Standard with parallel identification by LC-MS methods, if required.

### Other Process-Related Impurities

Additional impurities may be found in the AAD, which are related to the manufacturing process. Such impurities can be dipeptides (for example, Fmoc-glycine-glycine) or derivatives of the AAD formed due to side reaction during the process, especially if Fmoc-chloride is used to introduce the Fmoc protection group. In most cases, such impurities can be controlled by

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HPLC methods and identified by LC-MS. The acceptable limit of each impurity should be defined based on its ability to form potential impurities in the drug substance and the relative ease of removal of the impurities during purification.

## NON-AAD IMPURITIES

Control of non-AAD impurities in starting materials is very important to assure the safety, quality, and efficacy of the resulting peptide drug substances. The major potential non-AAD related impurities in the starting materials are:

- Residual solvents and reagents used in the preparation of starting materials
- Elemental impurities
- Residual allergens and melamine
- Bovine spongiform encephalopathy-transmissible spongiform encephalopathy (BSE-TSE) prions
- Nitrosamines and other genotoxic impurities

### Residual Solvents and Reagents Used in the Preparation of Starting Materials

The most commonly used solvents in amino acid and Fmoc amino acid manufacturing processes are Class 2 and Class 3 solvents such as acetonitrile, acetic acid, triethylamine, dioxane, dichloromethane, chloroform, tetrahydrofuran, methanol, etc. The quality of these solvents must be controlled to eliminate the presence of any Class 1 solvent contaminants. For this reason, the manufacturers of all solvents should limit the content of benzene unless they can certify that solvents are benzene-free.

Fmoc amino acids may contain traces of acetic acid, ethylacetate (a potential source of acetic acid), or other potentially reactive solvents such as aldehydes, which can cause chain termination during synthesis. For this reason, it is recommended that very low limits for acetic acid content, as well as the content of other potentially reactive solvents, should be established for all Fmoc amino acids.

### Elemental Impurities in Starting Materials

Elemental impurities are important quality attributes for the safety of peptide drug substances. Whereas the processes used for solid-phase synthesis of peptides are likely to eliminate elemental impurities in starting materials, it is important to consider these impurities as part of the risk assessment during development in order to ensure that the drug substance complies with the limits specified in ICH Q3D, *Elemental Impurities* and *USP* chapters *Elemental Impurities—Limits* ⟨232⟩ and *Elemental Impurities—Procedures* ⟨233⟩.

### Residual Allergens and Melamine

Sources of amino acids in starting materials are either synthetic or of plant origin and may be derived from potential allergens such as soybeans, wheat, rye, oat, barley, corn, maize, spelt, kamut, sugar, and alcohol. Because amino acids that are produced from proteins undergo harsh hydrolysis conditions and are further purified, it is extremely unlikely that allergens would pass through the hydrolysis and purification steps. Allergen- and melamine-free statements should be obtained from the starting material vendor and may also be ensured by the quality supply agreement.

### BSE/TSE-Free Starting Materials

Only synthetic or plant-derived starting materials should be used in peptide synthesis. Amino acids that originate from or come into contact with animal materials (waste from slaughterhouses, bones, cartilage, gelatin, etc.) should be avoided. The origin of the amino acids should be known and manufacturers of starting materials should provide adequate certification of TSE compliance. Preferably, human- and animal-derived amino acids should be avoided. If use of amino acids of animal origin cannot be avoided, the respective material should meet the requirements of the *European Pharmacopoeia* general chapter *5.2.8. Minimising the Risk of Transmitting Animal Spongiform Encephalopathy Agents Via Human and Veterinary Medicinal Products*. The requirement for BSE/TSE-free statements should be part of the quality supply agreement.

### Nitrosamines and Other Genotoxic Impurities

Chemically synthesized peptide manufacturers are required to assess the risk for the presence of *N*-nitrosamines impurities in their products. Nitrosamines are chemical compounds with potential carcinogenic risk. It is commonly accepted that the formation of *N*-nitrosamine impurities can occur due to the presence of a nitrosating agent together with a secondary or tertiary amine (named as the nitrosatable substance) usually under acidic reaction conditions. Although the most common source of nitrosating agents from a synthetic point of view are nitrite salts, all potential nitrosating agents should be considered (nitrate salts, nitrous acid, nitrous acidum ion, nitryl chloride, nitrosothiol, nitrosyl halides, and alkyl nitrites, among others). Amino acid derivatives used for the manufacturing of chemically synthesized peptides are considered a potential source of *N*-nitrosamines that could be transferred to the drug substance. It is recommended to avoid those manufacturing processes that could lead to the formation of *N*-nitrosamines. The presence of other genotoxic impurities should be carefully considered and evaluated during development, taking into consideration the requirement of ICH M7—*Assessment and Control of DNA Reactive (Mutagenic) Impurities in Pharmaceuticals to Limit Potential Carcinogenic Risk*.

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## CONCLUSION AND RECOMMENDATIONS FOR AAD SPECIFICATIONS

Based on the considerations outlined above, the most common quality attributes to be used for protected amino acid derivative starting materials are:

- Appearance
- Identification
- Related impurities
  - Total
  - Specified
  - Foreign amino acids
  - Optical isomers (diastereomers and enantiomers)
  - Unspecified
- Other impurities (not related)
  - E.g., residual reagents, solvents, elements
- Assay (e.g., by titration)
- Other components as necessary

Limits for specified impurities (e.g., unprotected amino acids, other amino acid derivatives, foreign amino acids, and chiral isomers) are set and justified based on prior batch history and an understanding and risk assessment of the API manufacturing process. The correlation between the content of a specified impurity in the starting material and the content of the related impurity in the API must be considered. For example, will the manufacturing process produce a related impurity in the API and to what extent is the process able to reduce a related impurity that has been formed? For unspecified impurities, batch history and the API specification for unspecified impurities are used as a starting point for the specification in the starting material. Assay by base titration is recommended for verification that the amino acid derivative is supplied as the carboxylic acid form suitable for activation and coupling and that no salts of the amino acid derivative are present. Other impurities (not related) and potential components are included in the specification based on their potential effect on the API manufacturing process and the quality of the final API.▲ (USP 1-Dec-2023)

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# ▲⟨1567⟩ PYRROLIZIDINE ALKALOIDS AS CONTAMINANTS

## 1. INTRODUCTION

This informational general chapter is intended to provide an overview of pyrrolizidine alkaloids (PAs), heterocyclic organic compounds that are synthesized by many plant species (*1–2*) and can contaminate or be present in many plant-related products. The most potent PAs exhibit hepatotoxic, genotoxic, carcinogenic, or mutagenic activities. PAs can be found as contaminants in botanical drugs, herbal medicines, botanical dietary ingredients, and botanical dietary supplements, thereby posing a potentially serious threat to human health. PAs are derived from ornithine and are characterized by bicyclic pyrrolizidine moieties that are referred to as "necines" (*3–4*). PAs occur in two forms: pyrrolizidine alkaloids *N*-oxide (PANO) and tertiary bases (*4–5*). More than 660 PAs have been identified in approximately 6000 plant species (*2*) or in 3% of the world's flowering plants (*6–7*). PAs are reported to be present mainly in the Boraginaceae (all genera), Asteraceae or Compositae (specifically in the tribes Senecioneae and Eupatorieae), and Leguminosae or Fabaceae (genus *Crotalaria*) families (*5,8–9*). PAs are mainly concentrated in the seeds and flowering parts of plants and, in lesser amounts, the leaves, stems, and roots (*5*). PAs of concern have been detected in dietary supplements (*6*); teas and herbal infusions (*2*); honey and pollen (*4,10*), predominantly due to the presence of PA-containing plants growing near plants of interest and being accidentally coharvested with plants used in these products. Because of the wide distribution of PAs in many plant species, dietary exposures to potent PAs are a safety concern (*6*). Published literature reports suggest that tea and herbal infusions were the main contributors to total unsaturated PA exposure. Therefore, efforts should be made to obtain information specifically on the sources of PA contamination in tea, such as the adventitious PA-containing plants responsible for the contamination, and to use measures to control infestation and coharvest (*11*).

## 2. PA CHEMISTRY

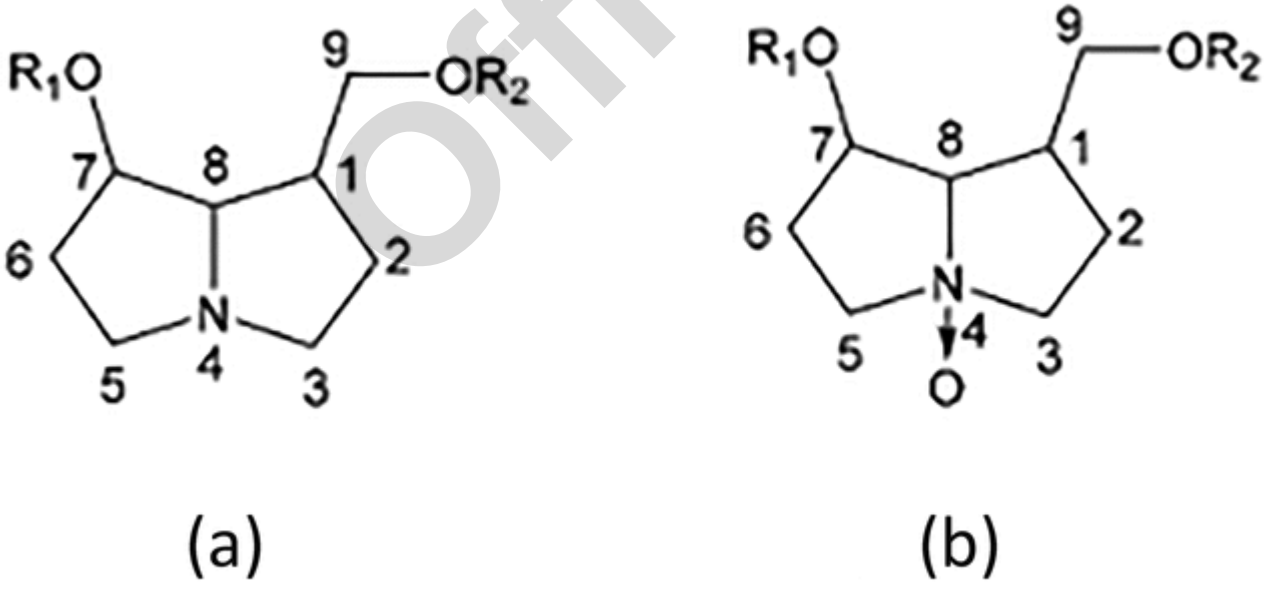


Figure 1. General structure of a) PA molecule and b) PANO molecule. $R_1$ and $R_2$ are hydrogens or necic acids.

PAs are typically found as esters composed of a necine base and one or more necic acids (*Figure 1*).The necine base typically includes pyrrolizidine, a bicyclic aliphatic hydrocarbon comprised of two fused 5-membered rings with a nitrogen atom at position 4. The necine base usually has a hydroxymethyl group at C-1 and a hydroxyl group at C-7 (*12*) (when $R_1$ and $R_2$ are hydrogens in *Figure 1*[a]). Esterification with necic acids occurs at these hydroxyl groups forming monoester (*Figure 2*), open-chain diester (*Figure 3*), or macrocyclic diester alkaloids (*Figure 4*) (*13*). Necic acids are either mono- or dicarboxylic acids with branched carbon chains bearing hydroxy, epoxy, carboxy, acetoxy, methoxy, or other alkoxy groups as substituents (*14*). The high diversity in the structures of PAs is due in part to combinations of various necine bases and necic acids and the formation of monoesters at different positions (*5*). The diversity is further enhanced with their presence in plants as both tertiary bases and as *N*-oxides (PANO) *Figure 1*(b).

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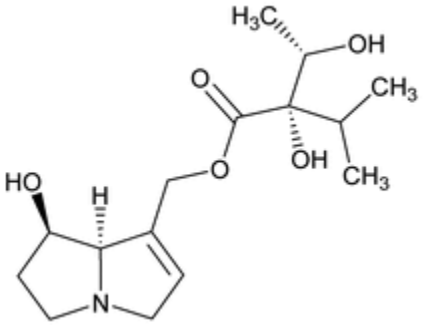


Figure 2. Chemical structure of lycopsamine, an ester of monocarboxylic acid.

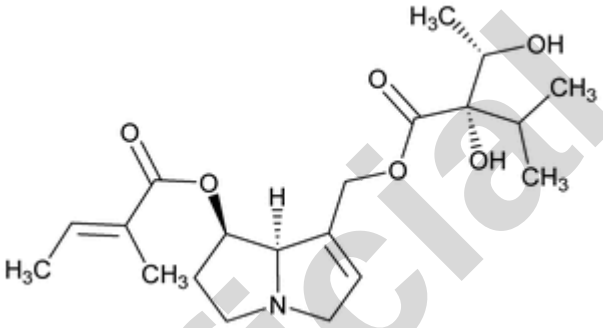


Figure 3. Chemical structure of symphytine, a diester of monocarboxylic acid.

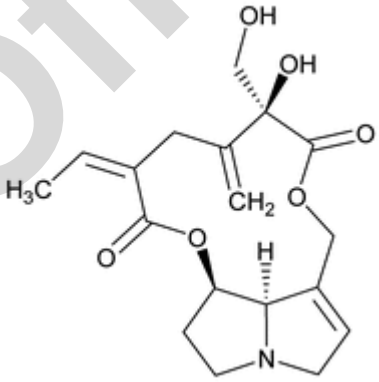


Figure 4. Chemical structure of riddelliine, a macrocyclic diester.

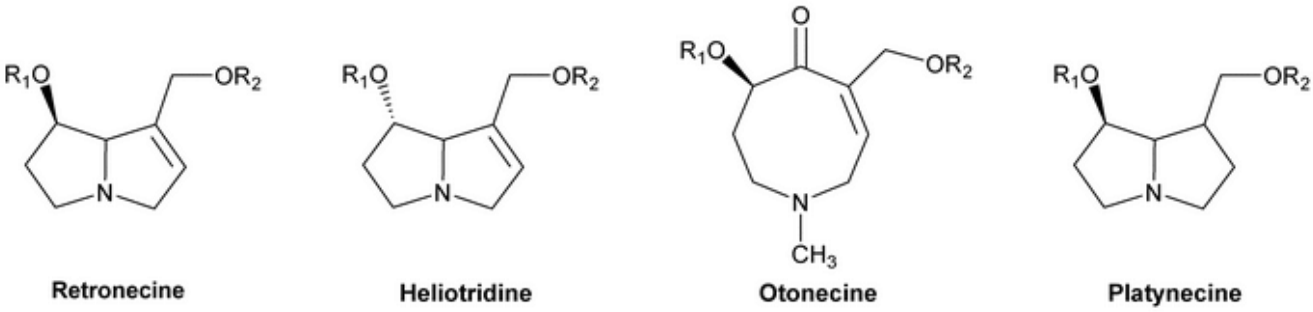


Figure 5. Classification of PAs based on the structure of the necine base.

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Based on the structure of necine base, PAs are further categorized into four groups: retronecine, heliotridine, otonecine, and platynecine types (*Figure 5*) (*15*). Retronecine, heliotridine and otonecine are unsaturated bases, i.e., they contain a double bond in the 1,2-position, whereas platynecine is a saturated base (*16–17*). Structurally, otonecine stands as the most distinct among all types because it is oxidized at C-8 and contains a monocyclic ring, thus deviating from other types that contain a bicyclic ring (*18–19*). Some esters of 1,2-unsaturated necine bases are toxic while esters with platynecine type are considered nontoxic PAs (*14,20*).

### 2.1 Structural Requirements for Toxicity

Not all PAs are toxic to humans at the levels found in herbal products. The toxic potential of a PA varies considerably with chemical structure (*21*). The structural requirements for PAs to exhibit toxicity are outlined below:

- The presence of a 1,2-double bond in the necine base (*22*). Cytochrome P-450 mediated metabolism of 1,2-unsaturated PAs forms reactive pyrroles, which can then form protein and DNA adducts (*17,23*). In contrast, the saturated structure of the platynecine-type PAs forms nonreactive polar compounds that are water soluble and readily excreted.
- The presence of one or two hydroxyl groups in the necine base.
- The esterification of one or two hydroxyl groups in the necine base and branching in at least one of the carboxylic acids (*24*). Due to this, cyclic diesters exert the highest toxicity, followed by open-chain diesters (intermediary), with monoesters having the lowest toxicity (*25*).

For the remainder of this paper, the term "PAs" will refer to more-potent and most-potent pyrrolizidine alkaloid molecules with the chemical characteristics described above, not all PAs.

### 2.2 Stability of PAs

It has been suggested that PAs, particularly their *N*-oxides, are heat sensitive, and thus, heat treatment could be utilized to control PA levels (*26*). Although high concentrations of some 1,2-unsaturated PAs and their *N*-oxides may occur in certain plants, much lower and variable concentrations of these substances are present in finished products due to processing (*26*). Drying plant materials is reported to cause significant decomposition of 1,2-unsaturated PAs, especially their *N*-oxides (*27*).

## 3. DIETARY EXPOSURE TO PAS

Seeds of PA-containing plants can contaminate grains, and prolonged consumption of such grains or grain products have resulted in rare but serious large-scale outbreaks of PA poisoning (pyrrolizidine alkaloidosis). One such example is a serious outbreak in 1974 that occurred in Afghanistan due to the consumption of bread made from wheat contaminated with seeds of *Heliotropium* sp. plants (*28*).

Lately there has been a growing interest in the use of botanical ingredients, leading to concern about the potential for PA poisoning primarily due to contamination from PA-containing plants in widely distributed botanical and herbal products. Recent literature reports have revealed the presence of PAs in herbal teas, predominantly senecionine-type, followed by lycopsamine- and heliotrine-types (*29*). Cross-contamination with plants containing PAs during the harvesting of herbal ingredients is responsible for the occurrence of PAs in ingredients of botanical drugs and botanical dietary supplements such as *Matricaria chamomilla* and *Mentha* × *piperita* L, which are not known to contain PAs (*30–31*).

Apart from exposure to PAs through herbal products, secondary contamination in foods of animal origin such as milk, eggs, honey, and pollen products was also observed.

Honey and supplement products containing bee pollen frequently contain PAs (*5,11*) such as senecionine, echimidine, lycopsamine and their *N*-oxides (*32–33*).

### 3.1 Measures to Avoid PA Contamination

Total eradication of PA-containing plants is not possible. It is necessary to identify potential risks associated with every step involved in the process chain, such as cultivation, harvesting, inspection of incoming goods, and drug processing, until the release of the botanical drug, botanical dietary ingredient, or botanical dietary supplement. For example, the risk of coharvesting PA-containing weeds during the harvest of the cultivated plant material can be controlled by optimization of timing, technology, cutting height, and other such factors associated with harvesting technology. Apart from coharvesting of PA-containing weeds, an alternate path of contamination that involves horizontal transfer and exchange of PA and/or PANO between living plants via the soil was recently discovered (*34*). To avoid PA contamination through this process, alkaloidal weeds have to be extracted completely from the soil and entirely removed from the fields rather than just leaving them to rot (*34*).

## 4. TOXICOLOGY

PAs are absorbed in the gastrointestinal tract and approximately 80% of PAs are excreted in the urine, feces, and breast milk (*4*). Because PAs are highly lipophilic, some are able to cross the placenta (*4*). The toxicity of potent PAs is mainly associated with their metabolic byproducts (*2,4*). PAs require metabolic activation to form highly reactive electrophiles (*4,35*). The liver is the main target organ of toxicity because the metabolic activation of PAs primarily occurs in the liver (*4*). Metabolic activation occurs through the oxidation of PAs, mostly catalyzed by the cytochrome P450 enzymes CYP3A and CYP2B, to form reactive pyrrolic esters. The hydrolysis of pyrrolic esters produces dihydropyrrolizines (DHP) (*4,5*). Liver toxicity results from the electrophilic pyrrolic esters and DHP, which further binds or forms adducts with vital cellular macromolecules such as proteins and DNA (*5,36*). The lungs are also a target organ of PA exposure as a result of reactive metabolites reaching the lungs through

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the systemic circulation (*4–5,37*). Experimental animal studies on potent PAs have reported hepatotoxicity, developmental toxicity, genotoxicity, and carcinogenicity (*37*). Both acute and chronic toxicity of PAs have been described and are characterized by different symptoms (*2,4*) as outlined below.

### 4.1 Acute and Subchronic Toxicity

Acute liver toxicity in humans and animals due to PA exposure is characterized by hepatic veno-occlusive disease (HVOD), which is considered a marker of PA toxicity (*2,5,37–38*). It is estimated that in 20% of acute PA poisoning cases humans die, 50% recover clinically, and 20% may develop chronic HVOD and cirrhosis years later (*39*). Acute PA poisoning symptoms include hemorrhagic necrosis, hepatomegaly, and ascites, while subacute liver effects present as hepatic sinusoidal obstruction syndrome (HSOS) due to blockage of the hepatic veins, (*4*) which is characterized by portal hypertension and liver dysfunction (*40*). Acute PA toxicity may also target the lungs (*5,37,39*) causing damage similar to HVOD, resulting in pulmonary hypertension and congestive heart failure (*4,38–39*). The lowest exposures of PAs associated with acute or short-term toxicity range from 0.8–3 mg/kg per day (*5*). However, based on available human and experimental data, even one-time exposure to unsaturated PAs may lead to liver cirrhosis; the level of exposure was not provided (*41*). Importantly, long-term exposure to much lower levels of PAs (15 µg/kg per day for 6 months) may cause chronic liver diseases including HVOD (*5,35*).

### 4.2 Chronic Toxicity

Animal studies have shown that long-term exposure to some PAs is associated with carcinogenicity, primarily in the liver (*5*), although other organs have also been rarely implicated. The occurrence of PA-associated tumors has also been reported in the lungs, bladder, skin, brain, spinal cord, pancreas, and gastrointestinal tract (*4*). Tumorigenic PAs belong to heliotridine-, retronecine- and otonecine-type structural classes. The DHPs formed by metabolism of these PAs form DNA adducts, which are thought to be the main mechanism responsible for tumorigenesis (*4–5*). Lasiocarpine, monocrotaline, and riddelliine are classified as possibly carcinogenic to humans (Category 2B) based on carcinogenic effects observed in rodent studies. Riddelliine has been shown to form DNA adducts in human liver microsomes similar to the DNA adducts observed in rodents, suggesting that PAs could be genotoxic and carcinogenic in humans (*4*).

## 5. PAS RECOMMENDED FOR MONITORING

Twenty-eight PAs, shown in *Table 1*, are selected for monitoring in botanical drugs, botanical dietary ingredients, and botanical supplements.

**Table 1. List of PAs Selected for Monitoring**

| PA | CAS number | PA | CAS number |
|---|---|---|---|
| Echimidine | 520-68-3 | Lycopsamine | 10285-07-1 |
| Echimidine-*N*-oxide | 41093-89-4 | Lycopsamine-*N*-oxide | 95462-15-0 |
| Erucifoline | 40158-95-0 | Monocrotaline | 315-22-0 |
| Erucifoline-*N*-oxide | 123864-94-8 | Monocrotaline-*N*-oxide | 35337-98-5 |
| Europine | 570-19-4 | Retrorsine | 480-54-6 |
| Europine-*N*-oxide | 65582-53-8 | Retrorsine-*N*-oxide | 15503-86-3 |
| Heliotrine | 303-33-3 | Seneciphylline | 480-81-9 |
| Heliotrine-*N*-oxide | 6209-65-0 | Seneciphylline-*N*-oxide | 38710-26-8 |
| Intermedine | 10285-06-0 | Senecionine | 130-01-8 |
| Intermedine-*N*-oxide | 95462-14-9 | Senecionine-*N*-oxide | 13268-67-2 |
| Jacobine | 6870-67-3 | Senecivernine | 72755-25-0 |
| Jacobine-*N*-oxide | 38710-25-7 | Senecivernine-*N*-oxide | 101687-28-9 |
| Lasiocarpine | 303-34-3 | Senkirkine | 2318-18-5 |
| Lasiocarpine-*N*-oxide | 127-30-0 | Trichodesmine | 548-90-3 |

## 6. REGULATORY AND PROPOSED TOLERABLE DAILY INTAKE LEVELS

The US current good manufacturing practice (cGMP) regulations for dietary supplements (21 CFR §111) require manufacturers to take certain measures to prevent contamination in the manufacturing process. The cGMP regulations do not specifically require testing for PAs, but manufacturers are required to set limits on the types of contamination that may adulterate or may lead to adulteration of the product within the meaning of the Federal Food, Drug and Cosmetic Act (FFDCA). Manufacturers also generally have a responsibility to ensure that their dietary supplement products do not present a significant or unreasonable risk of illness or injury that would cause the FDA to deem the product adulterated under §402(f)(1)(A) of the FFDCA. Additionally, under §402(a)(1) of the FFDCA, certain foods, including dietary supplements, are deemed adulterated if they contain poisonous or deleterious substances. Currently, there are no required FDA limits for PAs. In the absence of required

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FDA limits, the Division of Risk and Decision Analysis evaluates the safety of contaminants in food on a case-by-case basis using a safety assessment (SA) approach. The SA considers exposure and toxicity of the contaminant in the food product. Exposure is estimated using the contaminant level reported in the food and consumption or recommended intake amounts for specific subpopulations.

Regulations governing levels of PAs in food and food supplements have been established in several countries based on the potential exposure to contaminants from the increased use of herbal medicines leading to PA intoxication as a public health concern (*4,42*). The range of tolerable daily intake levels for PAs provided in *Table 2* may provide some guidance to manufacturers on the highly potent PAs (e.g., cyclic diesters).

**Table 2. Proposed Tolerable Daily Intake Levels for PAs**

| Regulatory Authority | Tolerable Daily Intake | Reference |
|---|---|---|
| German Federal Department of Health *Bundesanzeiger* (1992) | ≤1 µg/day (maximum 6 weeks use per year) ≤0.1 µg/day (for prolonged use) | (*4,42,43*) |
| US FDA (2001) | Ban on PA-containing comfrey supplements; no tolerable intake level established | (*42,45*) |
| Netherlands (2001) | ≤1 µg/kg or ≤1 µg/L PA concentration in final product (herbal preparation or plant extract) | (*4,42,46*) |
| Food Standards Australia New Zealand (FSANZ) (2001) | ≤1 µg/kg/day (provisional) based on veno-occlusive disease (cancer risk considered not proven in humans) | (*41,47*) |
| EFSA (2011) | MOE of 10,000 or higher than BMDL10 of 70 µg/kg/day considered a "low concern from a public health point of view" (based on NTP carcinogenicity data) | (*5*) |
| BfR (2011) | 0.007 µg/kg/day | (*48*) |
| JECFA (2015) | MOE of 10,000 or higher than BMDL10 of 182 µg/kg/day (based on NTP carcinogenicity data) | (*49*) |
| EFSA (2017) | MOE of 10,000 or higher than BMDL10 of 237 µg/kg/day considered a "low concern from a public health point of view" (based on updated BMD modeling approach and NTP carcinogenicity data) | (*37*) |
| European Commission | 400 µg/kg in food supplements and 500 µg/kg in pollen-based food supplements | (*50*) |
| EMA (2020) | Accepted limit of 1.0 µg/day with no temporary restrictions | (*44*) |

## 7. RELATIVE POTENCIES OF PAS

The risk-based decisions for tolerable daily intake levels presented above are based on approaches that assume all PAs are equally potent, i.e., as potent as the highly toxic PAs such as lasiocarpine (*5*) or riddelliine (*49*). There is another risk-based approach based on relative risk factors (RRF) that may be appropriate. The RRF approach may be applicable because large structure-dependent differences have been documented between subgroups of unsaturated PA congeners with respect to their genotoxicity and cytotoxicity (*51*). The concept of relative potency (REP) was developed to address cases where a group of compounds share the same major mode of action, but individual members exhibit extraordinarily different potencies sometimes spanning several orders of magnitude. REP factors represent the potency of the congener relative to the most-toxic congener(s). The latter is assigned an REP factor of 1.0, and then all other congeners have REP factors of ≤1.0. For a given sample (e.g., a mixture of PAs) the sum potency can be derived by multiplying the amount of the specific congeners and their respective REP factors, which are then summed up for all congeners present in the sample. This REP concept has been successfully applied in evaluating the toxicology of polychlorinated dioxins, furans, and PCBs (*52*), and has also been suggested for phototoxic furanocoumarins (*53*). In an attempt to factor in the varied PA potencies in risk assessment, efforts have been made to derive PA potency factors relative to riddelliine. REP factors for PAs can be derived based on an approach that correlated fundamental structural differences between PAs and acute toxicity data from rodents, cytotoxicity data in mammalian cell culture, and genotoxicity data in *Drosophila*. Cyclic PA diesters are markedly more toxic than open-chain diesters followed by monoesters, consistent with experimental data. Generally, PA monoesters and open diesters showed lower toxicity than cyclic diesters. However, some PAs such as lasiocarpine and lasiocarpine *N*-oxide (PA monoesters) had the same REP factors as monocrotaline, riddelliine, and retrorsine (cyclic diesters, most potent) (*54*).

Manufacturers of dietary supplements containing PAs should aim at minimizing the presence of PAs in their products.

## 8. ANALYTICAL METHODS AND CHALLENGES

A wide range of analytical techniques to quantitate PAs and PANOs—such as chromatography, nuclear magnetic resonance (NMR), enzyme-linked immunosorbent assay (ELISA), capillary electrophoresis (CE), droplet countercurrent chromatography (DCCC), and miscellaneous electrokinetic chromatography (MEKC) methods—have been reported in the literature (*55–57*). However, selective and sensitive analytical methods are needed to meet the limits set by different regulatory bodies as the methods cited above should be validated for the different matrices at the analytical range required to establish the level of PA

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contaminants. Chromatographic separation methods, particularly high- or ultra-high performance liquid chromatography (HPLC, UHPLC) and gas chromatography (GC), are preferred to quantitate PAs. Detection methods including ultraviolet (UV), flame ionization (FI), evaporative light scattering (ELS), and refractive index (RI) could not achieve the required limit of detection (LoD) and limit of quantitation (LoQ). Mass spectrometric techniques offer improved detection limits and provide the advantage of mass selectivity via detectors such as quadrupole, triple quadrupole, time of flight (ToF), and orbital ion-trap along with various ionization techniques. It is not feasible to quantitate PANOs using GC without prior derivatization or reduction due to their instability at high temperatures. Further, the extraction and clean-up procedures associated with GC methods are time consuming and require very large volumes of solvent. As a result, high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) has become the method of choice because of the extraction methods, which are more appropriate for the matrix, and the capability to simultaneously analyze PANOs along with PAs.

The most preferred analytical technique is LC–MS/MS, but this also has limitations. A baseline chromatographic separation is not always achieved for a few PA isomers such as intermedine/lycopsamine or senecionine/senecivernine, as well as their respective *N*-oxides (*37*). Also, because these isomers have the same molecular weight, it is not possible to distinguish them by mass spectrometry. An accurate quantification of individual PAs is not always possible for the above reasons. This necessitates the use of multiple methods for the accurate quantification of isomeric PAs. Furthermore, coelution of indicine could happen with the intermedine/lycopsamine pair (*58*). Similarly, coelution of indicine *N*-oxide has been observed with *N*-oxides of the intermedine/lycopsamine pair.

There is a need for highly sensitive and selective analytical methods to provide the level of quantification required to control contaminant PAs in botanical drugs, botanical dietary ingredients, and botanical dietary supplements. Apart from the above-mentioned challenges associated with analytical methods for the quantitation of PAs, there are challenges related to the reference standards: most of them are not commercially available, are very expensive to procure, are difficult to synthesize or isolate, and are not stable.

## CONCLUSIONS

PAs are naturally occurring heterocyclic phytotoxins that have been reported to be hepatotoxic, mutagenic, and carcinogenic. PAs have been detected in various dietary sources such as grains, herbal teas, honey, milk, and eggs. We included a list of PAs to be monitored in *Table 1*. Manufacturers should evaluate the levels of PAs in their product considering their potential presence arising from the plant itself, from contamination with external PAs (e.g., due to coharvesting, absorbed from soil), and/or from supply chain vulnerabilities that may increase the risk of contamination of ingredients. Manufacturers should use a risk-based approach for determining appropriate limits and testing requirements, which should take into consideration that select PAs are more toxic and their levels should be minimized. The range of tolerable daily intake levels for PAs provided in *Table 2* may provide some guidance to manufacturers for the highly potent PAs (e.g., cyclic diesters). Extraction and analytical quantitation of PAs is associated with several challenges and would benefit from evolving strategies for both efficient extraction and advanced selective analytical quantification techniques. Future developments will hopefully lead to a greater availability of reference standards and method validation as regulatory bodies create requirements for accurate quantitation of PAs.

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# ⟨1601⟩ PRODUCTS FOR NEBULIZATION—CHARACTERIZATION TESTS

Products used for nebulization and intended for pulmonary delivery are characterized using the following tests:

- *Drug Substance Delivery Rate and Total Drug Substance Delivered*;
- *Aerodynamic Assessment of Nebulized Aerosols*.

These tests standardize the approach to the assessment of the dose that would be delivered to a patient but are not intended to provide assessment of the nebulizer device itself.[1] The mass rather than the number-weighted size distribution is more appropriate to evaluate product performance. Drug substance mass as a function of aerodynamic diameter is more indicative of therapeutic effect within the respiratory tract.

## DRUG SUBSTANCE DELIVERY RATE AND TOTAL DRUG SUBSTANCE DELIVERED

These tests are performed to assess the rate of delivery to the patient and the total drug substance delivered to a patient using standardized conditions of volumetric flow rate. Breath-enhanced and breath-actuated nebulizers should be evaluated by a breathing simulator because the output of these types of device is highly dependent on inhalation flow rate. The methodology below describes the use of a standard breathing pattern defined for adults. Should a particular product for nebulization be indicated only for pediatric, i.e., neonate, infant, or child use, then pediatric breathing pattern(s) must be used.[2] Breathing patterns are used, rather than continuous flow rates, to provide a more appropriate measure of the mass of drug substance that would be delivered to patients.

Drug substance delivery rate and total drug substance delivered are appropriate characteristics because they allow the mass delivered to be characterized in a standard way regardless of the nebulizer used. Accordingly, the test methodology described below (a) measures the mass of drug substance delivered in the first period (typically 1 min) consequently giving an assessment of drug substance delivery rate and (b) captures the total drug substance mass delivered.

### Apparatus

#### BREATHING SIMULATOR

A commercially available breathing simulator that is able to generate the breathing profiles specified in *Table 1* is used for the test. The breathing profile indicated for adults is used unless the medicinal product is specifically intended for use in pediatrics, when alternate patterns should be used, as indicated in *Table 1*.

**Table 1. Breathing Simulator Specification**

| Item | Specification | | | |
|---|---|---|---|---|
| | Adult | Neonate | Infant | Child |
| Total volume | 500 mL | 25 mL | 50 mL | 155 mL |
| Frequency | 15 cycles/min | 40 cycles/min | 30 cycles/min | 25 cycles/min |
| Waveform | sinusoidal | sinusoidal | sinusoidal | sinusoidal |
| Inhalation:exhalation ratio | 1:1 | 1:3 | 1:3 | 1:2 |

#### FILTER SYSTEM

A suitably validated low-resistance filter, capable of quantitatively collecting the aerosol and enabling recovery of the drug substance with an appropriate solvent, is used for the test. The dead volume of the filter casing does not exceed 10% of the tidal volume used in the breath simulation.

### Procedure

Attach the filter (contained in the filter holder) (A) to the breath simulator (B) according to *Figure 1*. Fill the nebulizer (C) with the volume of the drug product as specified in the patient instructions. Attach the mouthpiece of the nebulizer to the inhalation filter using a mouthpiece adapter if required, ensuring that connections are airtight. Position the nebulizer in the same orientation as intended for use. This may require tilting the breathing simulator and filter holder. Set the breathing simulator to generate the specified breathing pattern.

[1] European Standard 13544-1:2001. *Respiratory Therapy Equipment*. Part 1: Nebulizing systems and their components. European Committee for Standardization. Brussels, Belgium. 2001.

[2] Suitable breathing patterns for pediatric use may be found, for example, in Canadian Standard CAN/CSA/Z264.1-02:2002, *Spacers and Holding Chambers for Use with Metered Dose Inhalers*. Canadian Standards Association, Mississauga, Canada 2002.

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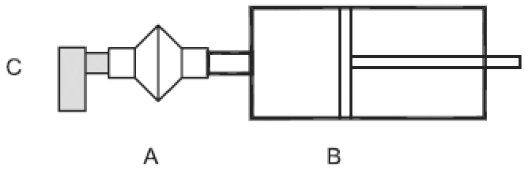


A. inhalation filter and filter holder B. breathing simulator C. nebulizer

Figure 1. Experimental Set-Up for Breathing Simulator Testing.

Start the breathing simulator and at the beginning of an inhalation cycle, start the nebulizer. Operate the nebulizer for a defined initial time period. The length of the time interval ensures that sufficient drug substance is deposited on the inhalation filter for quantitative analysis. A time of 60 ± 1 s typically enables direct determination of the drug substance delivery rate. The time chosen, usually 60 ± 1 s, must allow sufficient drug substance deposition on the inhalation filter to allow quantitative analysis. If the quantity of drug substance deposited on the inhalation filter in 60 s is insufficient for this analysis, the length of the time interval for aerosol collection can be increased. If the filter is soaked with the product, this time can be decreased. At the end of this initial period, stop the nebulizer.

Place a fresh filter and filter holder in position and continue until nebulization ceases. Interrupt nebulization and exchange filters, if necessary, to avoid filter saturation.

## Results

Using a suitable method of analysis, determine the mass of drug substance collected on the filters and filter holders during each time interval. Determine the drug substance delivery rate by dividing the mass of drug substance collected on the first inhalation filter by the time interval used for collection. Determine the total mass of drug substance delivered by summing the mass of drug substance collected on all inhalation filters.

***Change to read:***

## AERODYNAMIC ASSESSMENT OF NEBULIZED AEROSOLS

Nebulized products need to be size-characterized at flow rates lower than the range that is typically used for powder inhalers and metered-dose inhalers. The CEN standard recommends a flow rate of 15 L/min because this value represents a good approximation to the mid-inhalation flow rate achievable by a healthy adult breathing at 500 mL tidal volume.

Although low-angle laser light-scattering instruments (laser diffractometers) can provide rapid size-distribution measurements of nebulizer-generated aerosols, these techniques do not detect the drug substance. Rather, they measure the size distribution of the droplets irrespective of their content. This may not be a problem with homogeneous solutions, but it can result in significant error if the product to be nebulized is a suspension, or if droplet evaporation is significant, as can be the case with certain nebulizer types. Cascade impactors enable the aerosol to be characterized unambiguously in terms of the mass of drug substance as a function of aerodynamic diameter. Laser diffraction may be used if validated against a cascade impaction method.

▲The Next Generation Impactor without pre-separator used with inhalation aerosols, inhalation sprays, and nasal aerosols is described in▲ (ERR 1-Aug-2021) *Inhalation and Nasal Drug Products: Aerosols, Sprays, and Powders—Performance Quality Tests* ⟨601⟩. ▲An archival version of this cascade impactor▲ (ERR 1-Aug-2021) has been calibrated at 15 L/min specifically to meet the recommendation of the CEN Standard and ▲this configuration▲ (ERR 1-Aug-2021) is therefore used for this test.[3] Determining mass balance in the same way as for powder inhalers and metered-dose inhalers is not straightforward because the dose is being captured as a continuous output and hence is not included. Recovery experiments must be performed as part of method development and validation. Control of evaporation of droplets produced by nebulizers may be critical to avoid bias in the droplet size assessment process. Evaporation can be minimized by cooling the impactor to a temperature of about 5°, typically achieved by cooling the impactor in a refrigerator for about 90 min.

Typically, at least after each day of use, the apparatus must be fully cleaned, including the inter-stage passageways, because of the greater risk of corrosion caused by the condensation/accumulation of saline-containing droplets on inter-stage metalwork associated with cooling the impactor. All surfaces of the apparatus should be dried after each test, e.g, with compressed air. [NOTE—The micro-orifice collector (MOC) should not be dried with compressed air.]

[3] Marple VA, Olson BA, Santhanakrishnan K, *et al.* Next generation pharmaceutical impactor: A new impactor for pharmaceutical inhaler testing. Part III: Extension of archival calibration to 15 L/min. *J Aerosol Med* 2004; 17(4):335–343.

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## Apparatus

▲*Inhalation and Nasal Drug Products: Aerosols, Sprays, and Powders—Performance Quality Tests* ⟨601⟩*, C.4 Next Generation Impactor without Pre-separator for Inhalation Aerosols, Inhalation Sprays, and Nasal Aerosols*▲ (ERR 1-Aug-2021) includes details of critical dimensions and the qualification process for the impactor (stage mensuration).

A back-up filter in addition to the MOC must be used to ensure quantitative recovery of drug substance from the nebulized aerosol at the specified flow rate of 15 L/min. The filter is located below the MOC (internal filter option), or a filter in holder external to the impactor is used to capture any fine droplets that pass beyond the last size fractionating stage. A pre-separator is not used for testing nebulizer-generated aerosols.

## Method Validation

### IMPACTOR STAGE OVERLOADING

During method development and validation, confirm that the volume of liquid sampled from the nebulizer does not overload the impactor. Visual inspection of the collection surfaces on stages collecting most of the droplets may reveal streaking if overloading has occurred. This phenomenon is usually also associated with an increase in mass of drug substance collected on the final stage and back-up filter. Reducing the sampling period ($T_o$) is the most effective way to avoid overloading in any given system, balancing overloading with analytical sensitivity.

### RE-ENTRAINMENT

Droplet bounce and re-entrainment are less likely with nebulizer-produced droplets than with solid particles from inhalers, thus coating would not normally be required.

## Procedure

Pre-cool the assembled impactor and induction port in a refrigerator (set at about 5°) for not less than 90 min, and start the determination within about 5 min of impactor removal from the refrigerator. Other methods that maintain the impactor at a constant temperature (e.g, use of a cooling cabinet) can also be employed when validated.

Set up the nebulizer with a supply of driving gas (usually air or oxygen), or use a compressor at the pressure and flow rate specified by the manufacturer of the nebulizer. Ensure that the gas supply line does not become detached from the nebulizer when under pressure. Fill the nebulizer with the volume of the medicinal product as specified in the patient instructions.

Remove the impactor from the refrigerator. Attach the induction port to the impactor, and connect the outlet of the impactor/external filter to a vacuum source that is capable of withdrawing air through the system at 15 L/min, as specified in *Figure 2*. Turn on the flow through the impactor.

▲

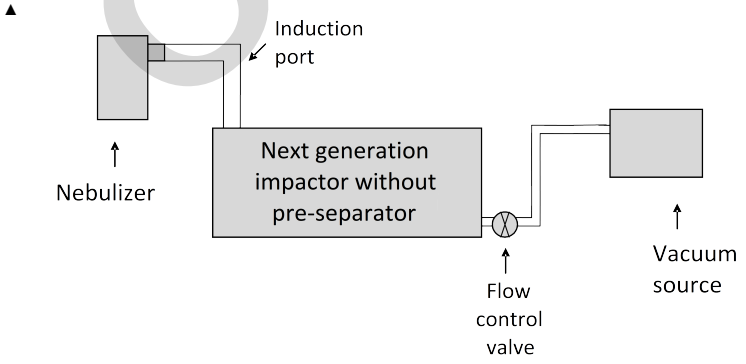


Figure 2. ▲Next Generation Impactor without Pre-separator▲ (ERR 1-Aug-2021) for Measuring the Size Distribution of Products for Nebulization.

Connect a flow meter, calibrated for the volumetric flow leaving the meter, to the induction port. Adjust the flow control valve located between the impactor and the vacuum source to achieve a steady flow through the system at 15 L/min (±5%). Remove the flow meter.

Position the nebulizer in the same orientation as intended for use, then attach the mouthpiece of the nebulizer to the induction port, using a mouthpiece adapter if required. Switch on the flow/compressor for the nebulizer. Sample for a predetermined time ($T_o$). Once determined, this time ($T_o$) must be defined and used in the analytical method for a particular drug product to ensure that mass fraction data can be compared. At the end of the sampling period, switch off the driving gas flow/compressor to the nebulizer, remove the nebulizer from the induction port, and switch off the flow from the vacuum source to the impactor. Dismantle the impactor and, using a suitable method of analysis, determine the mass of drug substance collected in the induction port on each stage and on the back-up filter as described ▲in *Inhalation and Nasal Drug Products: Aerosols, Sprays, and Powders—Performance Quality Tests* ⟨601⟩*, C.4 Next Generation Impactor without Pre-separator for Inhalation*

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*Aerosols, Inhalation Sprays, and Nasal Aerosols*.▲ (ERR 1-Aug-2021) Add the mass of drug substance collected in the MOC to that deposited on the back-up filter/external filter and treat as a single sample for the purpose of subsequent calculations.

Calculate the fine particle mass of the drug substance based on the predetermined time ($T_o$). Calculate the mass fraction ($F_{m,comp}$) of the drug substance deposited on each component of the impactor, commencing with the induction port and proceeding in order through the impactor, using the following expression:

$$F_{m,comp} = m_{comp}/M$$

$m_{comp}$ = mass associated with the component under evaluation;

M = total mass collected by the system.

Present $F_{m,comp}$ in order of location within the measurement equipment, beginning at the induction port and ending with the back-up filter of the impactor (see *Figure 3*). $F_{m,comp}$ values for adjacent stages of the impactor may be combined in order to report the mass fraction collected on a group of stages as a single value.

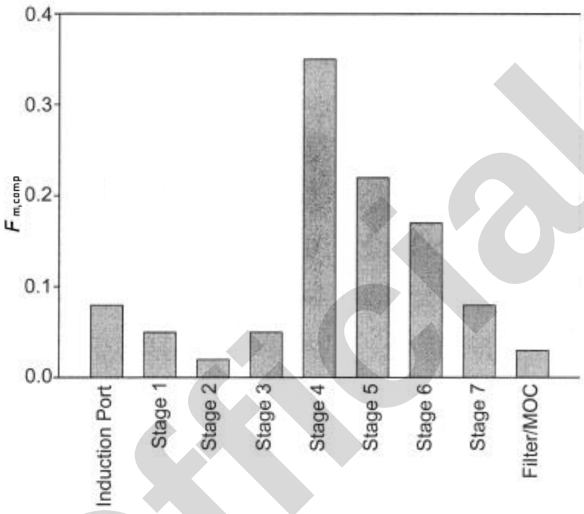


Figure 3. Example of Mass Fraction of Droplets Presented in Terms of Location within the Sampling System.

Determine the cumulative mass-weighted particle-size distribution of the aerosol size-fractionated by the impactor in accordance with the procedure given in ▲*Inhalation and Nasal Drug Products: Aerosols, Sprays, and Powders—Performance Quality Tests* ⟨601⟩*, C.4 Next Generation Impactor without Pre-separator for Inhalation Aerosols, Inhalation Sprays, and Nasal Aerosols*▲ (ERR 1-Aug-2021). Starting at the filter, derive a cumulative mass versus effective cut-off diameter of the respective stages (see *Table 2* for the appropriate cut-off diameters at 15 L/min). Plot the cumulative fraction of drug substance versus cut-off diameter in a suitable format, e.g, logarithmic or log-probability format. Where appropriate, use this plot to determine by interpolation the fraction either less than a given size or between an upper and lower size limit.

**Table 2. Cut-off Sizes for ▲Next Generation Impactor without Pre-separator▲ (ERR 1-Aug-2021) at 15 L/min**

| Stage | Cut-off Diameter (µm) |
|---|---|
| 1 | 14.1 |
| 2 | 8.61 |
| 3 | 5.39 |
| 4 | 3.30 |
| 5 | 2.08 |
| 6 | 1.36 |
| 7 | 0.98 |

If necessary, and as appropriate, use this plot to determine values for the mass median aerodynamic diameter (MMAD) and the geometric standard deviation (GSD).

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# ⟨1602⟩ SPACERS AND VALVED HOLDING CHAMBERS USED WITH INHALATION AEROSOLS—CHARACTERIZATION TESTS

1. INTRODUCTION
- 1.1 Background
- 1.2 Purpose
- 1.3 Rationale
- 1.4 Recommendations
- 1.5 Definitions of Key Terms Relating to This Chapter
- 1.6 Choice of Drug Product with which to Test

2. TEST METHOD SELECTION
- 2.1 Spacer/VHC Configurations
- 2.2 Comments on Test Methods

3. MEASUREMENT OF APSD
- 3.1 No Delay between the MDI Actuation and Sampling Onset
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4. MASS OF DRUG DELIVERED FROM A SPACER/VHC WHILE SIMULATING PATIENT TIDAL BREATHING
- 4.1 Without Facemask
- 4.2 With Facemask

***Change to read:***

## 1. INTRODUCTION

### 1.1 Background

Spacers and valved holding chambers (VHCs) are widely used in conjunction with inhalation aerosols [commonly known as pressurized metered-dose inhalers (MDIs)]. When used correctly, VHCs assist with the administration of inhalation aerosols to patients who have poor coordination of MDI actuation and inhalation, and VHCs reduce oro-pharyngeal deposition of the drug. Patients using these spacers/VHCs in general breathe tidally when inhaling their medication, regardless of any delay, and this is particularly true for young and elderly users. Also, spacers and VHCs often come with a facemask instead of the mouthpiece normally supplied for inhalation aerosols. These add-on devices (spacers/VHCs) interface with the actuator/mouthpiece of the MDI and provide additional volume for the aerosol plume to develop. In this chapter, these aspects are addressed by providing patient-use appropriate scenarios that have been evaluated extensively.

Although existing methods for evaluating inhalation aerosols rely on sampling at a constant flow rate, spacers and VHCs may be used by patients who have poor inhalation technique (i.e., they delay inhalation after actuating the MDI). This chapter describes tests that are intended to provide information on how the spacer/VHC modifies the aerosol emitted by the MDI.

### 1.2 Purpose

The purpose of this chapter is to define potential standardized methods for characterizing the in vitro performance of a given MDI drug product with a specific spacer and VHC. It is recognized that spacer/VHC configurations will deliver a modified particle-size distribution and dose compared with the dose indicated on the label by the manufacturer of the MDI product, chiefly by collecting almost all of the portion of the dose contained in particles too large to be available for inhalation by the patient and potentially reach the lungs. This chapter does not advise on the outcome of such changes, rather the interpretation of data obtained using the methods described should be developed by the user after discussion with the appropriate receiving organization.

### 1.3 Rationale

This chapter includes separate testing by a multi-stage cascade impactor to determine aerosol aerodynamic particle size distribution (APSD) and tests mimicking fully coordinated and fully uncoordinated use, with tidal breathing simulated to establish emitted mass (EM) with the MDI-spacer/VHC coupled to a breathing simulator. ▲▲ (USP 1-Dec-2019) It is now possible to combine both APSD and EM measurements by means of a mixing inlet. ▲▲ (USP 1-Dec-2019) However, the more conservative approach adopted herein reflects the absence of method validation data for multiple marketed drug products delivered by MDI. Spacers and VHCs modify the APSD substantially from what is emitted by the MDI when used alone. In particular, almost all of the mass fraction of drug associated with particles having ballistic trajectories, by virtue of propellant expansion upon actuation, is contained within the add-on device. The user should refer to the Canadian Group standard for further interpretation of changes in APSD brought about by the presence of a spacer or VHC.

▲▲ (USP 1-Dec-2019)

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## 1.4 Recommendations

The use of the induction port identified in *Inhalation and Nasal Drug Products: Aerosols, Sprays, and Powders—Performance Quality Tests* ⟨601⟩ is recommended for compliance with the pharmacopeial standard. However, it is acknowledged that alternative designs of the induction port, as yet to be included as pharmacopeial standards, offer unique testing opportunities. These are either anatomically correct or idealized to have aerosol transport properties similar to an anatomically correct induction port. Given the widespread use of spacers/VHCs for infants and small children, it is important to note that many of these anatomically appropriate induction ports are scaled in terms of such potential users (e.g., the "Alberta" idealized throats, ▲▲ (USP 1-Dec-2019) developed at the University of Edmonton, Canada). The user is free to adopt such an induction port but should specify the induction port design and age group with which the induction port will be used.

The evaluation of facemask performance requires a model face of the appropriate age range specified in the labeling for the add-on device (i.e., infant, small child, or adult). This arrangement is necessary because there is no other way to accurately simulate the magnitude of the dead space or test for the possibility of leak pathways between facemask and face, both of which are known to influence efficiency of the drug delivery. Some face models are available commercially.▲▲ (USP 1-Dec-2019) Therefore, because the use of a face model is an appropriate way to test these add-on devices, the user will need to either acquire a model from a source where age-appropriate face models have been developed and validated, or develop and validate his or her own design. Whichever pathway is chosen, a description of the model, in particular the dimensions of the face where the facemask comes into contact, should be provided in the data report to the recipient.

## 1.5 Definitions of Key Terms Relating to This Chapter

**Spacers:** Open tubes that do not have a valve at the exit nearest the patient interface (either the mouthpiece or facemask) to retain the aerosol before the user can inhale (see *Figure 1*). Spacers simply increase the distance between the MDI mouthpiece and the mouth of the patient. If the patient exhales instead of inhaling, the medication will be blown out of the spacer and lost.

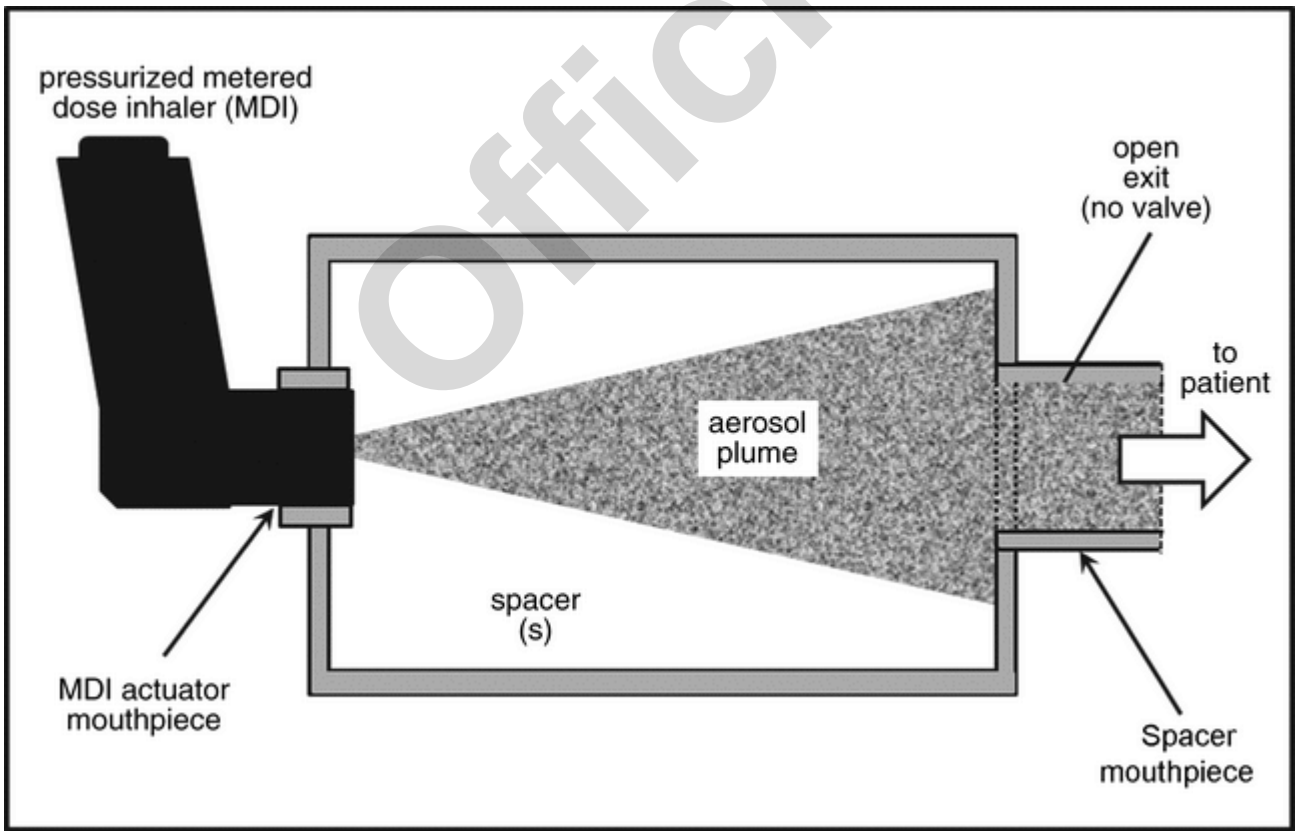


Figure 1. Open-tube spacer with no mechanism for aerosol conservation during exhalation.

**VHCs:** Contain at least one valve that opens to allow the patient to inhale aerosol on inspiration. This inhalation valve remains closed at other times during each breathing cycle (see *Figure 2*). Some VHCs may also contain an exhalation valve that is open only during exhalation to direct the exhaled flow away from the inhalation valve.

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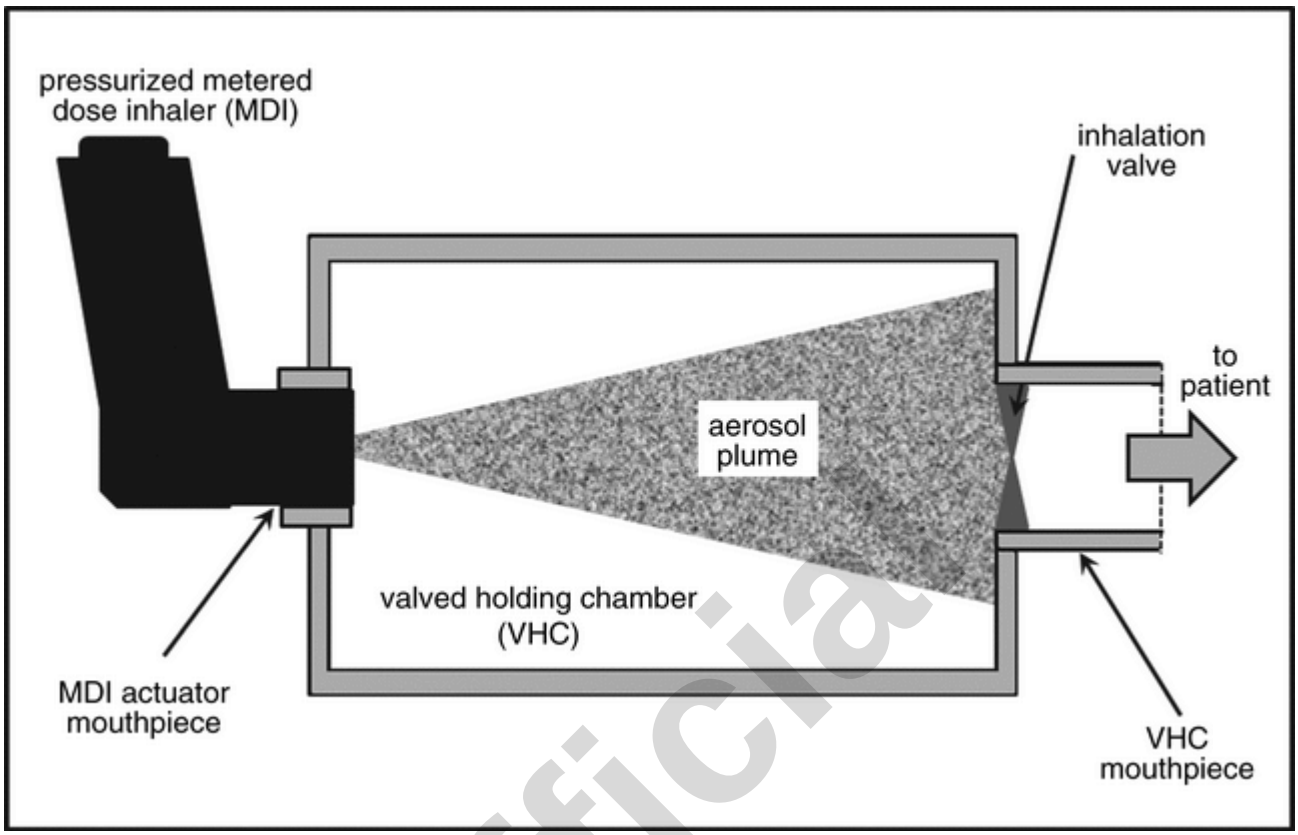


Figure 2. Conventional VHC with valve opening upon inhalation, accepting MDI with forward-firing actuator mouthpiece.

**Integral actuator:** A companion piece with some spacers and VHCs that accepts a MDI canister removed from its actuator-mouthpiece. A "reverse firing" feature may enable the design to be modified such that the built-in actuator faces away from rather than toward the user. In the example illustrated in *Figure 3*, the spacer operates in this way. It has some of the characteristics of a VHC, in that an air dam is created by closure of the valve located distally from the user on exhalation into the chamber. This type of spacer can therefore be evaluated as if it were a VHC, because the aerosol released on MDI actuation is conserved during exhalation.

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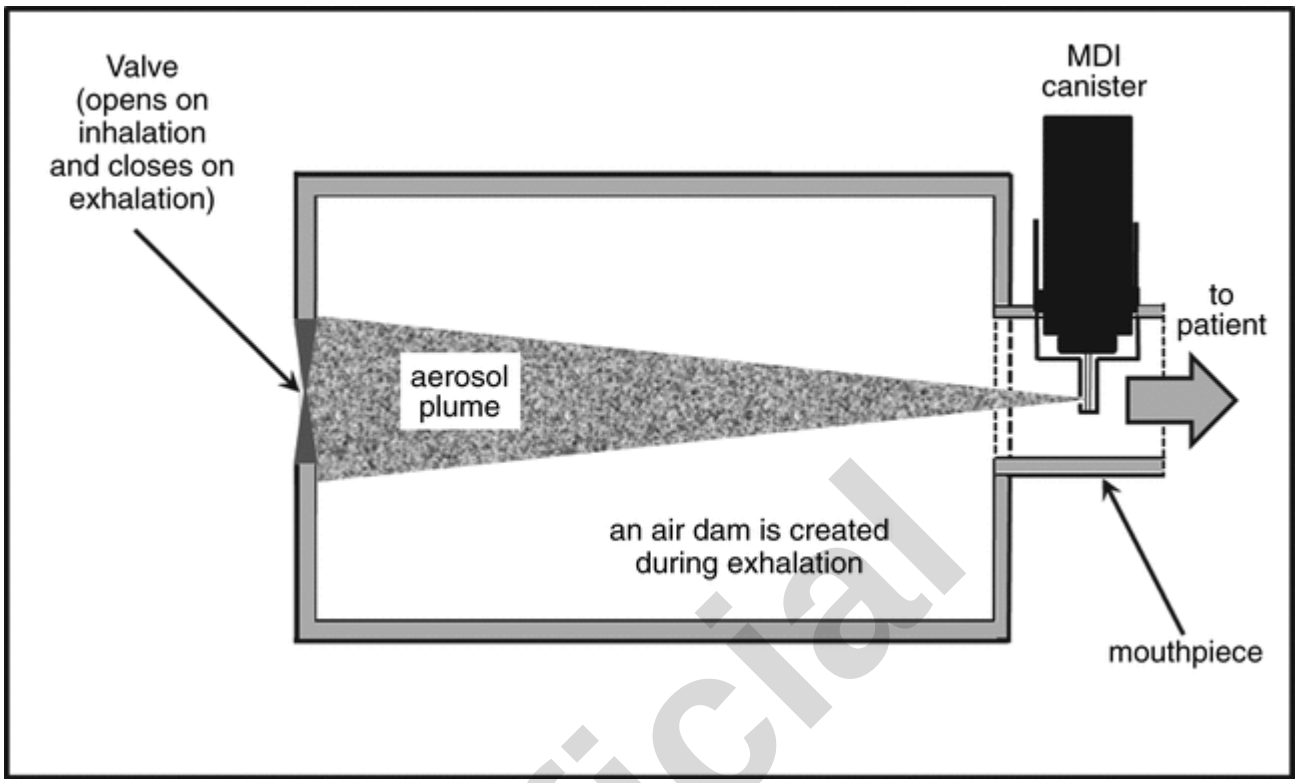


Figure 3. Holding chamber with reverse-firing integral actuator for MDI canister.

### 1.6 Choice of Drug Product with which to Test

It is understood that testing will take place with each single active component within the drug product or with each strength of multi-strength drug products as required by the recipient of the data.

***Change to read:***

## 2. TEST METHOD SELECTION

### 2.1 Spacer/VHC Configurations

Four spacer/VHC configurations have been identified that require different test methods to complete the evaluation process (see *Table 1*).

**Table 1. Identification of Test Configurations of the Spacer/VHC**

| Configuration | Spacer | VHC | Mouthpiece | Facemask |
|---|---|---|---|---|
| A | +[a] | –[b] | + | – |
| B | + | – | – | + |
| C | – | + | + | – |
| D | – | + | – | + |

[a] + denotes that this item was part of the condition.
[b] – denotes that this item was not part of the condition.

In *Configuration A*, the device being tested is a spacer with a mouthpiece. Likewise, in *Configuration B*, the device under test is a spacer with a facemask. In *Configuration C*, the device is a VHC with a mouthpiece, and in *Configuration D* the device is a VHC with a facemask. The evaluation of devices without patient interface (i.e., those intended for use by patients on mechanical ventilation) is outside the scope of this chapter.

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## TESTS FOR CONFIGURATION A

*Configuration A*, spacer with mouthpiece (see *Figure 4*), comprises two tests: 1) APSD measurement with no delay (see *Part 1A*); and 2) EM by breathing simulation—fully coordinated (see *Part 2A*). Testing with a delay would be inappropriate for this configuration because the medication cannot be retained within the spacer because it has no valving mechanism.

## TESTS FOR CONFIGURATION B

*Configuration B*, spacer with facemask (see *Figure 4*), comprises one test with the facemask in place: 1) EM by breathing simulation—fully coordinated (see *Part 3A*). ▲ [NOTE—The facemask should NOT be removed for this test, because it is important to be able to evaluate the effect of dead space between facemask and face, as well as the correct function of an exhalation valve, if provided.]▲ (USP 1-Dec-2019) It is recommended that this class of device be evaluated for APSD measurement with no delay (see *Part 1A*) by removing the facemask and coupling the spacer on-axis to the induction port entry with a suitable connector. Testing with delay would be inappropriate for this configuration, because the medication cannot be retained within the spacer because it has no valving mechanism. ▲ [NOTE—Removal of the facemask is acceptable for APSD determination, because the entire bolus of medication leaving the spacer is sampled in the continuous vacuum that is required to operate the cascade impactor.]▲ (USP 1-Dec-2019)

## TESTS FOR CONFIGURATION C

*Configuration C*, VHC with mouthpiece (see *Figure 4*), comprises four tests: 1) APSD measurement with no delay (see *Part 1A*); 2) APSD measurement with delay (see *Part 1B*); 3) EM by breathing simulation—fully coordinated (see *Part 2A*); and 4) EM by breathing simulation—fully uncoordinated (see *Part 2B*).

## TESTS FOR CONFIGURATION D

*Configuration D*, VHC with facemask (see *Figure 4*), comprises two tests with the facemask in place: 1) EM by breathing simulation—fully coordinated (see *Part 3A*); and 2) EM by breathing simulation—fully uncoordinated (see *Part 3B*). ▲ [NOTE—The facemask should NOT be removed for this test, because it is important to be able to evaluate the effect of dead space between facemask and face, as well as the correct function of an exhalation valve, if provided.]▲ (USP 1-Dec-2019) It is recommended that this class of device be evaluated for APSD measurement with and without delay (see *Parts 1A* and *1B*) by removing the facemask and coupling the VHC on-axis to the induction port entry with a suitable connector. ▲ [NOTE—Removal of the facemask is acceptable for APSD determination, because the entire bolus of medication leaving the spacer is sampled in the continuous vacuum that is required to operate the cascade impactor.]▲ (USP 1-Dec-2019)

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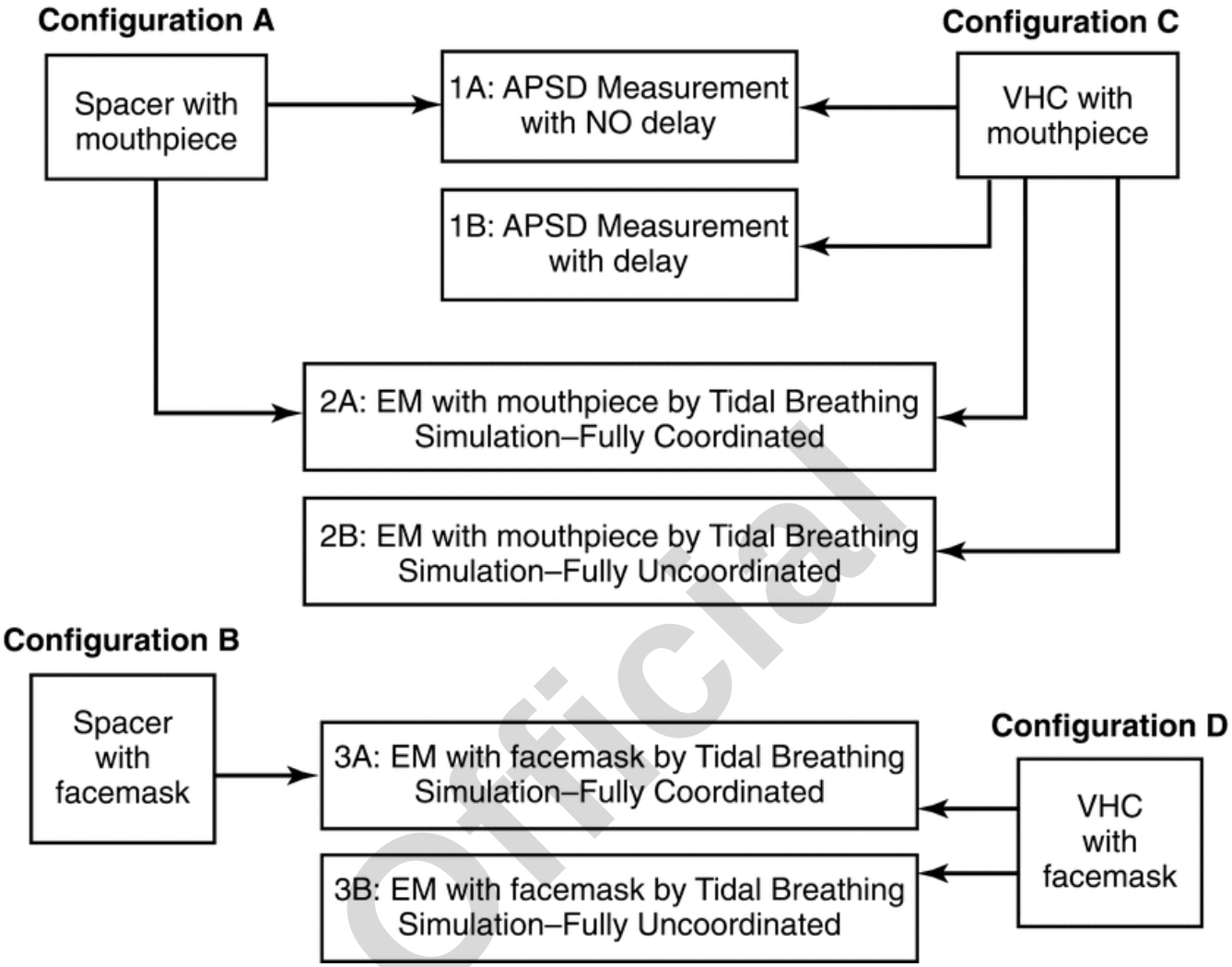


Figure 4. Decision tree for test selection by add-on device type.

## 2.2 Comments on Test Methods

The tests in *Parts 1A* and *1B* (see *Table 2*) are an extension of the procedures described in ⟨601⟩. However, sampling at a constant flow rate via a cascade impactor does not do more than enable the assessment of the APSD of the emitted aerosol. In the later parts of *Products for Nebulization—Characterization Tests* ⟨1601⟩, tests are described in which tidal breathing is simulated to assess spacer/VHC performance in terms of the delivered dose (equivalent to EM) in situations that are more representative of patient use. In *Part 1A* (see *Table 2*), the purpose is to enable the determination of metrics for which a direct comparison can be made with and without the add-on device. This methodology is the same as that described using either ▲the Andersen Cascade Impactor without pre-separator or the Next Generation Impactor without pre-separator (see ⟨601⟩)▲ (USP 1-Dec-2019) and is therefore not repeated here. In *Part 1B* (see *Table 2*), delayed sampling is introduced because add-on devices, especially VHCs, are widely prescribed for individuals who, for whatever reason, are unable to coordinate MDI actuation with the onset of inhalation as described for the MDI alone in "patient information" and "instructions for use". All of the tests in *Part 1* are undertaken with the facemask removed from the spacer/VHC equipped with this type of patient interface. This modification is allowed so that the device can be readily interfaced by fitting the facemask adapter directly to the entry of the induction port without the complication of a direct facemask-to-induction port connection, where internal dead space would likely be both ill-defined and unrepresentative of the "in-use" condition.

**Table 2. In Vitro Tests for Spacers and VHCs**

| Test | Delay at Constant Flow Rate | Breathing Simulation | Suitability | Relevance to Patient Use Scenarios |
|---|---|---|---|---|
| Part 1A: Measurement of APSD with 4-L sample volume | None | Not applicable | Spacers and VHCs | Baseline performance compared with MDI alone |

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**Table 2. In Vitro Tests for Spacers and VHCs** *(continued)*

| Test | Delay at Constant Flow Rate | Breathing Simulation | Suitability | Relevance to Patient Use Scenarios |
|---|---|---|---|---|
| Part 1B: Measurement of APSD with 4-L sample volume | For example, 2, 5, or 10 s[a] | Not applicable | VHCs only | Simulation of a delay by a poorly coordinated user |
| Part 2▲A:▲ (USP 1-Dec-2019) Measurement of EM with sufficient number of inhalations from the VHC following guidance in the patient instructions to ensure that the drug is delivered from the VHC as intended[b] | Not applicable | Filter collection of aerosol simulating tidal breathing in coordinated ▲▲ (USP 1-Dec-2019) use | Spacers and VHCs | Simulation of optimum use (fully coordinated) with ▲pMDI▲ (USP 1-Dec-2019) actuation ▲▲ (USP 1-Dec-2019) |
| ▲Part 2B: Measurement of EM with sufficient number of inhalations from the VHC following guidance in the patient instructions to ensure that the drug is delivered from the VHC as intended[b] | Not applicable | Filter collection of aerosol simulating tidal breathing in uncoordinated use | VHCs only | Simulation of fully uncoordinated use with pMDI actuation▲ (USP 1-Dec-2019) |
| Part 3▲A:▲ (USP 1-Dec-2019) Measurement of EM delivered by facemask with sufficient number of inhalations from the VHC following guidance in the patient instructions to ensure that the drug is delivered from the VHC as intended[b] | Not applicable | Filter collection of aerosol behind lips or at nares of model, simulating tidal breathing in coordinated ▲▲ (USP 1-Dec-2019) use | Spacers and VHCs | Simulation of optimum use (fully coordinated) with ▲pMDI▲ (USP 1-Dec-2019) actuation. ▲▲ (USP 1-Dec-2019) Simulation of use with facemask applied with a 1.6-kg force[c] |
| ▲Part 3B: Measurement of EM delivered by facemask with sufficient number of inhalations from the VHC following guidance in the patient instructions to ensure that the drug is delivered from the VHC as intended[b] | Not applicable | Filter collection of aerosol behind lips or at nares of model, simulating tidal breathing in uncoordinated use | VHCs only | Simulation of fully uncoordinated use with pMDI actuation. Simulation of use with facemask applied with a 1.6-kg force[c]▲ (USP 1-Dec-2019) |

[a] Other delay intervals may also be used as required, if justified.
[b] Representative breathing patterns for different patient ages are listed in *Table 3*; other patterns may be used as required, if justified.
[c] 1.6-kg applied force has been shown to be clinically appropriate, but the force used may differ from this value if justified.

The tests in *Parts 2* and *3*, depending on whether a mouthpiece or facemask is present as the patient interface, respectively (see *Table 2*), evaluate the performance of the inhalation valve (and exhalation valve, if equipped) of the add-on device. This evaluation is accomplished by simulating tidal breathing, with parameters that are appropriate for the intended user group (e.g., infant, small child, or adult). This testing approach creates the continually varying flow conditions that are expected in use. Note that in the determination of EM, a sufficient number of inhalations should be taken in accordance with the instructions for use of the VHC, to ensure that the intended amount of drug is delivered.

Spacers/VHCs using a facemask as the patient interface should be evaluated ideally with the facemask in place (see *1.3 Rationale*). The desirable approach is such that in vitro testing makes use of a model face that is representative of the age range for which the spacer/VHC is intended. There are currently limited commercially available model faces; however, the peer-reviewed literature contains several articles providing the necessary technical information to enable suitable age-appropriate model(s) to be created for the purpose of testing spacer/VHCs. It is also important that the surfaces of the face coming into contact with the facemask have mechanical characteristics (e.g., deformability) that are representative of the skin and underlying soft tissues when the facemask is applied to the face with an appropriate force. In *Part 3* (see *Table 2*), the spacer/VHC is evaluated by a breathing simulation with the facemask fitted, mimicking similar fully coordinated use as defined in *Part 2A* and also fully uncoordinated use as defined in *Part 2B* (see *Table 2*). By comparing the EM of the drug with and without the use of a facemask, it is possible to assess the influence of the facemask.

[NOTE—Not all of the tests described in this chapter are applicable to open-tube spacers because such devices require fully coordinated use; otherwise, drug delivery will be significantly reduced. *Figure 4* shows the decision tree to be followed so that the tests chosen are appropriate to the type of add-on device being evaluated.]

*Table 2* further defines the test, method, applicable device, and relevance to patient use scenarios of the procedures that are described in this chapter. Example delay intervals of 2, 5, and 10 s are noted in the table, although other delay intervals may be substituted or added as required and justified. For the testing in *Parts 3▲A* and *B*▲ (USP 1-Dec-2019) (see *Figure 4*), it is recognized that currently there are limited commercially available face models representing the most widely recognized age categories, which may be required (i.e., infant, small child, and adult). The user is advised to select an appropriate commercial model or develop his or her own, providing justification for the selection as required. Additionally, *Table 3* shows representative tidal-breathing patterns for the various patient age categories from neonate to adult.

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**Table 3. Representative Tidal Breathing Patterns[a]**

| Parameter | Pediatric | | | Adult | |
|---|---|---|---|---|---|
| | Neonate | Infant | Small Child | Normal 1[b] | Normal 2[b] |
| Tidal volume (mL) | 25 | 50 | 155 | 770 | 500 |
| Frequency ($min^{-1}$) | 40 | 30 | 25 | 12 | 13 |
| I/E ratio[c] | 1:3 | 1:3 | 1:2 | 1:2 | 1:2 |
| Minute volume (mL) | 1000 | 1500 | 3875 | 9240 | 6500 |

[a] With the permission of the Canadian Standards Association (operating as the CSA Group), material is reproduced from CSA Group standard CAN/CSA-Z264.1-02 (R2011), "Spacers and Holding Chambers for Use with Metered-Dose Inhalers", which is copyrighted by CSA Group, 5060 Spectrum Way, Suite 100, Mississauga ON, L4W 5N6. This material is not the complete and official position of the CSA Group on the referenced subject, which is represented solely by the standard in its entirety. Although use of the material has been authorized, CSA is not responsible for the manner in which the data are presented, nor for any interpretations thereof. For more information or to purchase standards from the CSA Group, please visit shop.csa.ca/ or call 1-800-463-6727.
[b] *Normal 1* represents a resting adult of large build; *Normal 2* represents an active adult of normal build.
[c] *I/E ratio* = inspiratory time/expiratory time.

▲Testing is undertaken (see *3.1 No Delay between the MDI Actuation and Sampling Onset*) to ensure that there are no leakages of ambient air into the measurement apparatus. The purpose is not to evaluate leakages associated with the spacer/VHC. Such testing would be impractical because of the variety of adapters that would be needed to interface the flow meter with the different designs associated with these add-on devices.▲ (USP 1-Dec-2019)

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# 3. MEASUREMENT OF APSD

## 3.1 No Delay between the MDI Actuation and Sampling Onset

### BACKGROUND

In *Part 1A* (see *Figure 4*), the measurement of APSD from the spacer or VHC with no delay following actuation of the inhaler is defined as an important test of the optimum performance of the device. This test also provides information with which to compare the in vitro performance of the MDI with and without the add-on device present.

### TEST PROTOCOLS

If the spacer/VHC is intended for adult use, and comparison is being made with the MDI alone, follow ▲one of▲ (USP 1-Dec-2019) the procedures given for ▲inhalation aerosols and sprays,▲ (USP 1-Dec-2019) as required. Alternatively, ▲the pediatric version of▲ (USP 1-Dec-2019) the Marple-Miller Cascade Impactor ▲(Model 150P)▲ (USP 1-Dec-2019) may be used.

If the spacer/VHC is intended for neonates, infants, or small children, it may be appropriate to use an alternative apparatus that operates at the reduced flow rate(s) more appropriate for these classes of patients. Examples are ▲the Next Generation Impactor (without pre-separator)▲ (USP 1-Dec-2019) operated at 15.0 L/min or the pediatric version of ▲▲ (USP 1-Dec-2019) the Marple-Miller Cascade Impactor (Model 150P) that functions at either 4.9 or 12.0 L/min.

▲Set up the cascade impactor as described in the manufacturer's literature. To ensure efficient particle capture, coat appropriately the particle collection surface of each stage with glycerol, silicone oil, or other suitable liquid unless this has been demonstrated to be unnecessary. Connect an induction port with internal dimensions as defined in ⟨601⟩ to the impactor inlet. Connect the impactor to a vacuum pump via a suitable flow control valve.

Attach a flowmeter capable of providing volumetric flow rate, within the range of operation leaving the meter, to the induction port, ensuring an airtight seal. Verify that the flow rate for the test is within ±5% of the specified flow rate. This is achieved by applying a vacuum to the test apparatus by means of the flow control valve located between the cascade impactor and vacuum source. Turn off the source of vacuum to this apparatus after this check has been completed and remove the flow meter.

Attach a suitable coupling for the spacer/VHC to the induction port.▲ (USP 1-Dec-2019) Conduct the ▲remainder of the▲ (USP 1-Dec-2019) evaluation with the mouthpiece of the spacer or VHC, if so equipped, connected to the test apparatus. If the spacer or VHC is equipped with a facemask, remove the facemask and perform the evaluation with the add-on device connected to the test apparatus by means of the facemask adapter.

▲Prepare the spacer/VHC▲ (USP 1-Dec-2019) for the test by washing in accordance with the manufacturer's instructions, if so indicated. If no instructions for preparation are provided, test the device out of the package without prewashing, and note this information in the test report.

Connect the spacer or VHC via the mouthpiece or facemask adapter to the entry to the induction port described in ⟨601⟩. This connection can be made either with a short piece of flexible tubing or with a purpose-built coupling that ensures on-axis alignment with the entry to the induction port. If flexible tubing is used for this connection, ensure that the add-on device is supported in such a way that its long axis aligns with the axis of the induction port entry. Care should be taken to ensure that the coupling, however constructed, does not result in a significant loss of the drug and/or change in measured APSD.

▲▲ (USP 1-Dec-2019)

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The practice of actuating the MDI with the spacer or VHC initially disconnected from the induction port and then rapidly connecting the add-on device after MDI actuation is not recommended, because there is always a small delay interval and medication will be lost in an uncontrolled manner from an open-tube spacer if this procedure is followed.

Follow the directions in the patient information and instructions for use for the MDI to prime it before inserting into the MDI adapter of the spacer/VHC. In the case of an add-on device equipped with an integral actuator, after priming the MDI canister in its mouthpiece adapter as supplied, remove the canister from the adapter and clean the external valve stem with a suitable wipe. Insert the canister valve stem into the receptacle following the instructions for use for the particular add-on device, taking care to avoid premature actuation.

In cases where more than one actuation of the MDI is needed to collect sufficient mass of drug product, follow the directions in the patient information and instructions for use; if no directions are provided, allow a minimum of 30 s between actuations. Turn on the vacuum to the cascade impactor before delivering any actuations. It is important to deliver the minimum number of actuations to obtain an adequate sample of the size-fractionated drug mass for measurements to be made to the same degree of precision as would be the case if the MDI was evaluated alone.

After the MDI actuation and aerosol sampling are completed, recover the drug from each component of the cascade impactor, including the induction port, and also from within the spacer/VHC by using a validated method appropriate to the product being measured. Carefully remove the backup filter of the cascade impactor, whose purpose is to capture the drug mass passing the last impaction stage, from its location and place it in a suitable container. For each sample, add a predetermined volume of a suitable solvent to the container and agitate to dissolve the collected drug. Transfer a sample to a cuvette or vial for drug assay using a syringe equipped with an in-line filter to retain any suspended material. Care must be taken with the recovery of a drug retained by the add-on device, because a greater volume of solvent may be needed, compared with volumes needed for recovery from each impaction plate/cup of the impactor. Perform separate assays to measure the mass of drug recovered from each component, using a validated procedure.

Repeat the measurement with the required number of devices and replicates per device. Typically, five separate spacers or VHCs are each tested once, but other testing protocols may be adopted as required, if justified. These protocols may include replicate measurements per device to obtain information about intra- as well as inter-device performance.

MEASUREMENTS

**Total mass of drug collected:** This procedure is not a test of the product or add-on device but serves as a system suitability test to ensure that the results are valid. The total mass of drug collected comprises the sum of the drug mass in all of the components, including the MDI mouthpiece and from within the spacer/VHC [material balance (MB)] divided by the number of actuations of the MDI. Note that typically up to 5% of the target-delivered label claim may be retained by the cascade impaction apparatus as inter-stage wall losses.

**Total mass of drug emitted:** The total mass of drug emitted from the spacer/VHC with zero seconds delay (delivered dose, equivalent to EM) is determined from the sum of the values for the mass of drug collected in all of the components of the test apparatus divided by the number of actuations of the MDI. Spacers and VHCs are intended to reduce the mass of coarse particles inhaled by the patient; therefore, an important part of data interpretation of the APSD should involve separate assessments of the pertinent subfractions relevant to the inhalation aerosol product. The precise size limits for these subfractions will agree with the recipient of the test data. However, to provide initial guidance on this matter, it is suggested that the following be considered as a minimum:

- Determination of the mass of drug retained by the spacer/VHC together with the mass recovered from the induction port
- Stage-by-stage profile of the remaining EM

Further interpretation of these data is at the discretion of the user. The performance of the spacer/VHC is compared with that of the MDI alone, following the procedure described in ⟨601⟩.

## 3.2 Delay between the MDI Actuation and Sampling Onset

[NOTE—This test is inappropriate for spacers.]

BACKGROUND

This test requires the adaptation of ▲the Andersen Cascade Impactor without pre-separator and the Next Generation Impactor without pre-separator (see ⟨601⟩)▲ (USP 1-Dec-2019) to accept a means by which the delay interval can be realized. The actuation of the MDI into the VHC on test with its mouthpiece or facemask adapter disconnected from the entry to the induction port, followed by connection to the port with the vacuum applied to the apparatus immediately after the delay interval has elapsed, is difficult to perform accurately and may result in leakage or an inaccurate realization of the delay interval. For these reasons, an apparatus of the type described in *Figure 5* is recommended, because it enables the VHC mouthpiece/facemask adapter to remain in position throughout the measurement. The methodology for delay testing is based on the apparatus illustrated, but other equipment offering similar capability may be used if justified.

TEST PROTOCOLS

The vertically mounted shutter plate comprises a circular opening in its upper half and is mounted at setup such that the gap between the VHC mouthpiece/facemask adapter and induction port entry is closed, as illustrated (see *Figure 5*). The induction port and VHC adapter fit tightly into the mounting block. In this position, when vacuum is applied to the test apparatus, air is sampled by the apparatus at the desired flow rate via a bypass channel on the side of the adapter facing the induction port. This arrangement avoids the need to start the flow through the apparatus after the delay interval has elapsed, so that the cascade impactor is always operated at a constant flow rate.

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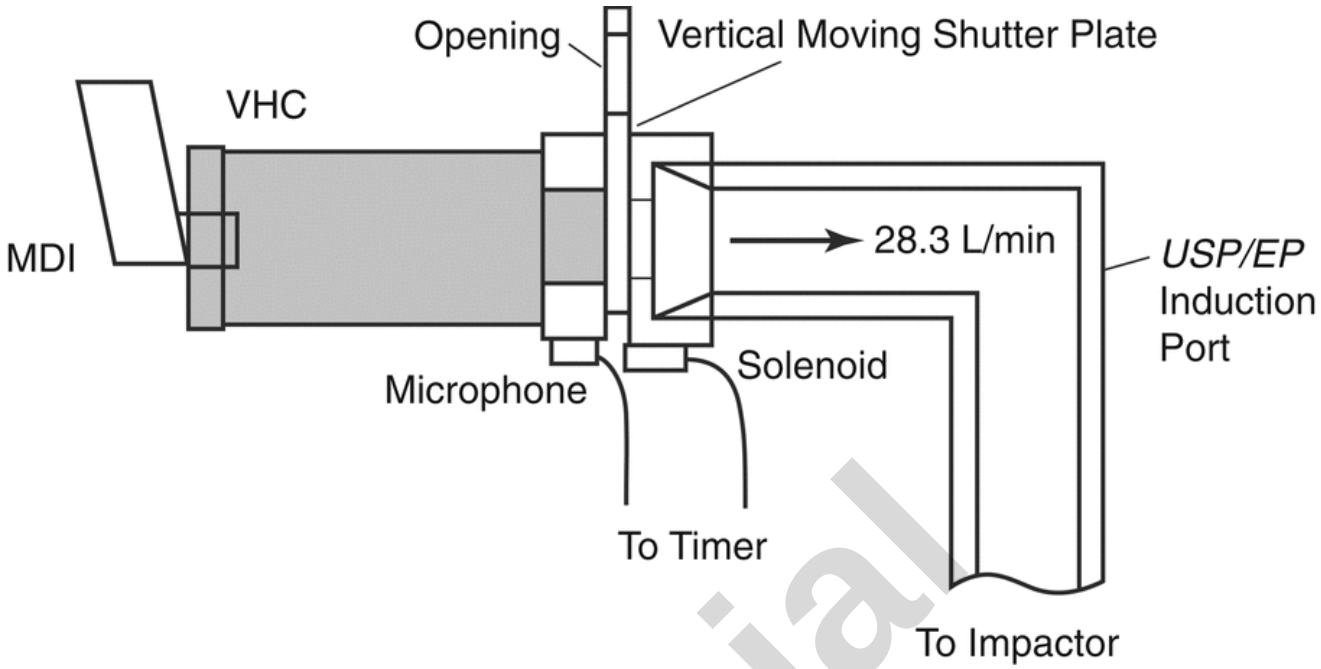


Figure 5. A configuration for an apparatus to assess the effect of a delay between the MDI actuation and sampling onset.

In the configuration described in *Figure 5*, when the MDI is actuated, a microphone located on the adapter block detects the sound emitted at actuation of the MDI, starting a timer that operates a solenoid valve that retracts a pin immediately after the preset delay interval has expired. This process permits gravity to operate on the shutter, which drops to the lower position in which the aperture is aligned with the VHC mouthpiece/facemask adapter and induction port entrance. The aerosol retained within the VHC is sampled as soon as the shutter moves to the "open" position. This procedure avoids the risk of capturing any "blow-by" aerosol that might escape the VHC as the propellant expands immediately following MDI actuation but would not be inhaled by a user. If blow-by is observed, it should be noted in the test report. In the configuration shown in *Figure 5*, the adapter introduces <5 mL of additional volume to the aerosol pathway from the VHC to the filter, and the minimum delay interval achievable is 1 s.

Example delay intervals of 2, 5, and 10 s have been noted earlier in this chapter, although other delay interval(s) may be substituted or added if required and justified.

▲Connect the impactor to a vacuum pump via a suitable flow control valve.

The delay apparatus, if constructed in accordance with *Figure 5* will introduce leakage into the apparatus, once attached to the induction port because the bypass flow passage is open in order that in-flow comes in via this passage rather than through the VHC before the shutter drops after the prescribed delay period. The flow rate verification test is therefore conducted before the delay apparatus is attached. Connect an induction port with internal dimensions as defined in ⟨601⟩ to the impactor inlet. Attach a flowmeter capable of providing volumetric flow rate, within the range of operation leaving the meter, to the coupling, ensuring an airtight seal. Verify that the flow rate for the test is within ±5% of the specified flow rate. This is achieved by applying a vacuum to the test apparatus by means of the flow control valve located between the cascade impactor and vacuum source. Turn off the source of vacuum to this apparatus after this check has been completed and remove the flow meter.▲ (USP 1-Dec-2019)

Securely attach the exit port from the delay apparatus to the entry of the induction port. ▲Conduct the remainder of the evaluation with the mouthpiece of the spacer or VHC, if so equipped, connected to the test apparatus. If the spacer or VHC is equipped with a facemask, remove the facemask and perform the evaluation with the add-on device connected to the test apparatus by means of the facemask adapter. If this is the first testing of the spacer/VHC or the intention is to evaluate the add-on as new for each delay condition, prepare it for the test by washing in accordance with the manufacturer's instructions, if so indicated. If no instructions for preparation are provided, test the device out of the package without prewashing, and note this information in the test report.▲ (USP 1-Dec-2019) Connect the VHC via the patient interface (mouthpiece or facemask adapter) to the entry port of the delay apparatus. ▲▲ (USP 1-Dec-2019)

Before actuating the MDI for the first time into the VHC being tested, ensure that the delay apparatus is set such that the shutter is in the "up" or "closed" position. Select the desired delay interval for the timer.

Follow the directions in the patient information and instructions for use before inserting the actuator/mouthpiece of the MDI into the MDI adapter of the VHC before the first actuation into the test apparatus. If the spacer/VHC is designed with an integral actuator, after priming the MDI canister in its mouthpiece adapter as supplied, remove the canister from the adapter and clean the external valve stem with a suitable wipe. Insert the canister valve stem into the receptacle following the instructions for the particular add-on device, taking care to avoid premature actuation.

In cases where more than one actuation of the MDI is needed to collect sufficient mass of drug product, follow the directions in the patient instructions, or if no direction is provided, allow a minimum of 30 s between actuations. Deliver the minimum number of actuations needed to obtain an adequate sample of the size-fractionated drug mass so that measurements can be made to the same degree of precision as if the MDI was evaluated alone. Reposition the shutter of the delay apparatus in the

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up or closed position, and check that the timer is set for the desired delay after each MDI actuation. Maintain the vacuum through the entire sequence until all actuations are completed.

After the sampling part of the measurement is completed, recover the drug from each component of the cascade impactor, including the induction port, and from within the spacer/VHC by using a validated method appropriate to the specific product. In the configuration shown in *Figure 5*, the surface area of the shutter that is exposed to aerosol particles is minimal. Therefore, recovery of drug from this component needs to be done only for the most accurate work; in which case, the inner surfaces of the aperture in the shutter plate and the exit port are washed with recovery solvent. Care should be taken with the recovery of drug retained by the VHC, because a greater volume of solvent than is needed for recovery from each impaction plate/cup of the impactor may be needed to be sure that drug is quantitatively recovered. Assay for the mass of drug recovered from each component separately by using a validated procedure.

Repeat the measurement with the required number of devices and replicates per device. Typically, five separate spacers or VHCs are each tested once, but other designs, including replicate measurements per device, may be adopted as required.

MEASUREMENTS

**Total mass of drug collected:** The total mass of drug collected in all of the components, including the MDI mouthpiece and from within the spacer/VHC (MB) is divided by the number of actuations of the MDI. The component of the dose that may escape past the inhalation valve of the VHC on MDI actuation (due to momentary pressurization of the interior of the chamber) is termed "blow-by", and it may not be captured for the assay. If this phenomenon is observed, it must be noted.

[NOTE—Quantification of blow-by is not a practical proposition because the act of collecting the aerosol will inevitably apply back-pressure to the valve, reducing or eliminating the phenomenon altogether.]

The performance of the spacer/VHC tested with delay (see *3.2 Delay between the MDI Actuation and Sampling Onset*) is compared with that of the spacer/VHC tested with no delay (see *3.1 No Delay between the MDI Actuation and Sampling Onset*).

VHCs are intended to reduce the mass of coarse particles inhaled by the patient, so an important part of data interpretation of the APSD should involve separate assessments of the pertinent subfractions relevant to the inhalation aerosol product. The precise size limits for these subfractions will agree with the recipient of the test data. However, to provide initial guidance on this matter, it is suggested that the following be considered as a minimum:

- Determination of the mass of drug retained by the spacer/VHC together with the mass recovered from the induction port
- Stage-by-stage profile of the remaining EM

Further interpretation of these data is at the discretion of the user.

***Change to read:***

## 4. MASS OF DRUG DELIVERED FROM A SPACER/VHC WHILE SIMULATING PATIENT TIDAL BREATHING

### 4.1 Without Facemask

Cascade impactors used in the assessment of APSD are designed to operate at fixed flow rates. However well the add-on device may perform during an in vitro test of function using such an apparatus, the operation of critical moving components (i.e., inhalation and exhalation valves of VHCs) is not evaluated in the way that these components would perform when the VHC is used by the patient. An additional test that simulates tidal breathing is therefore included, because this type of respiratory pattern is most commonly encountered with patients using an add-on device, particularly neonates, infants, and children who are incapable of a forced inhalation maneuver. Several representative, age-related breathing patterns are listed in *Table 3*.

There is no breathing simulator recognized in ⟨601⟩. However, ⟨1601⟩ provides for the use of a commercially available breathing simulator that is able to generate the same breathing profiles as those specified in *Table 3*. The methodology provided assumes that equipment meeting this standard is available. *Figure 6* illustrates schematically a setup that has been effective for these measurements, but other configurations may be used as required.

The filter system used to collect the aerosol at the mouthpiece/facemask adapter of the spacer/VHC should be a suitably validated, low-resistance filter capable of quantitatively collecting the aerosol and enabling recovery of the drug substance with use of an appropriate solvent. If the filter is contained in its own housing, the dead volume of the filter casing must not exceed 10% of the tidal volume used in the breath simulation. This restriction will likely make it necessary to use filters without a separate housing when performing tests that simulate neonatal or infant use.

The first part of the test is suitable for both spacers and VHCs, because it simulates the delivery of medication mimicking a fully coordinated user actuating the MDI at the onset of inhalation. However, the second part of the test is only suitable for VHCs because the MDI is actuated at the onset of exhalation to simulate a fully uncoordinated user.

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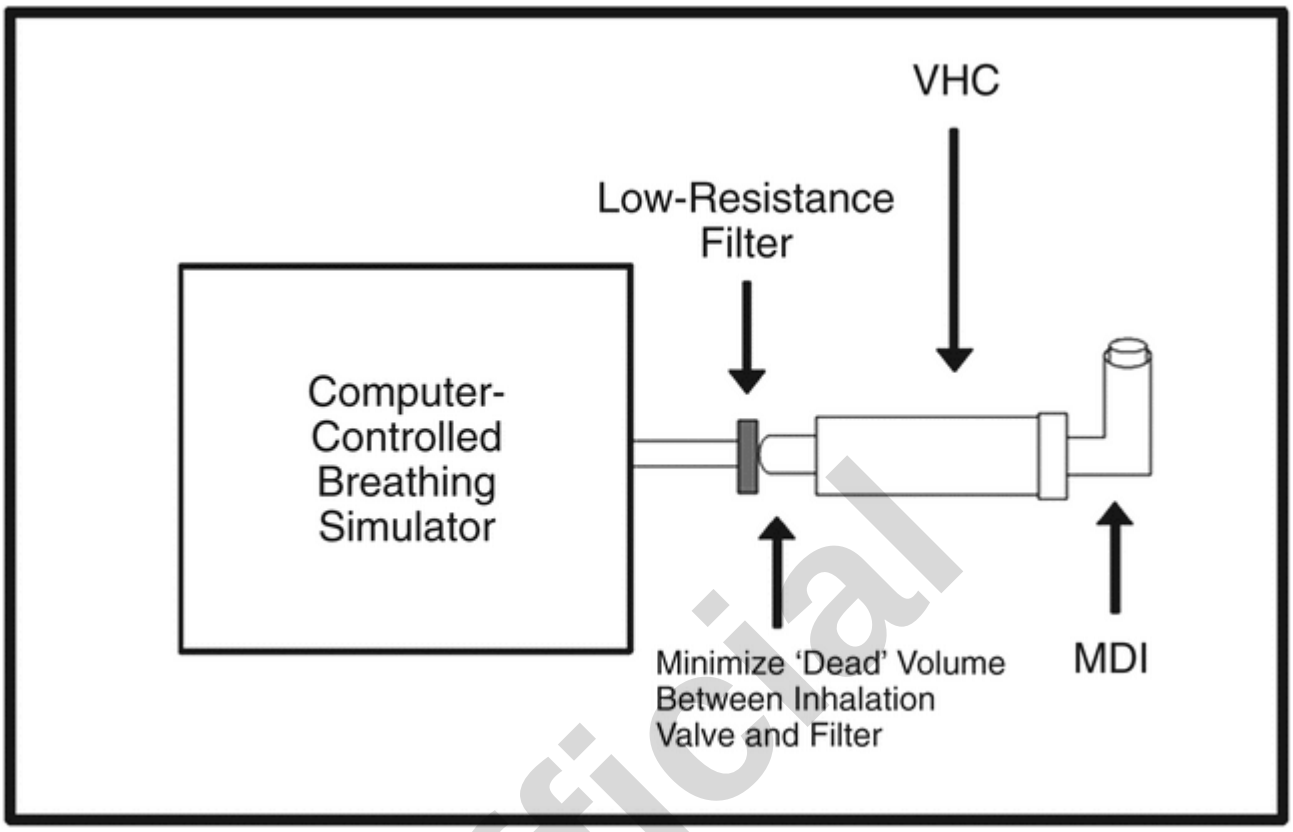


Figure 6. VHC evaluation by breathing simulator.

TEST, PART 1

Set the breathing simulator to the required breathing pattern (identified from *Table 3*) in accordance with the manufacturer's instructions. Alternative breathing patterns may be used if required. Ensure that the simulator has been calibrated before use so that the actual volumes and frequency are within ±5% of the indicated value during each breathing cycle. Calibration syringes are available from various suppliers of mechanical ventilation equipment to verify volumes. To verify frequency, the method varies among breathing simulator manufacturers, and therefore the advice of the particular supplier/manufacturer should be sought if the method is not explicitly stated in the operating instructions for the apparatus.

Ensure that the selected flow waveform from the computer-controlled breathing simulator (*Figure 6*) is stable before proceeding. Prepare each device in the group of spacers or VHCs to be tested by washing in accordance with the manufacturer's instructions, if indicated. If no instructions for preparation are provided, test the devices out of package without prewashing and note this fact in the test report.

Connect the mouthpiece or facemask adapter of the spacer/VHC to the breathing simulator by means of a short length of flexible hose. Locate the aerosol filter as close as possible to the mouthpiece of the spacer/VHC using an adapter or other means that will enable a leak-tight seal to be formed with the minimum amount of space (dead volume) between the device and the filter. Ensure that the adapter does not restrict the path of the aerosol.

Filtrete is a suitable electret filter medium ▲▲ (USP 1-Dec-2019) that readily releases collected drug quantitatively upon addition of a suitable solvent during the assay procedure. However, other suitable filter media may be used provided that the tester is satisfied that the filter is capable of collecting the entire emitted dose and that drug recovery for assay is quantitative.

Follow the directions in the patient information and instructions for use for the MDI to prime it before inserting into the MDI adapter of the spacer/VHC before the first actuation into the test apparatus. In the case of an add-on device equipped with an integral actuator, after priming the MDI canister in its mouthpiece adapter as supplied, remove the canister from the adapter and clean the external valve stem with a suitable wipe. Insert the canister valve stem into the receptacle by following the instructions for use for the particular add-on device, taking care to avoid premature actuation. This test typically should require only one actuation of the MDI into the spacer/VHC per determination. However, for certain highly potent products delivering low unit mass of drug per actuation, more than one actuation of the MDI may be needed to collect a sufficient mass of drug product. Under such circumstances, follow the directions in the patient information and instructions for use for the MDI, or allow a minimum of 30 s between actuations if no direction is provided. It is important to deliver the minimum number of actuations to obtain an adequate sample of the drug mass for measurements to be made with acceptable precision.

For the first part of the test, perform a single actuation timed to coincide with the *beginning* of an inhalation. Allow sampling to occur for five additional breathing cycles for the actuation. If additional actuations are required to improve analytical sensitivity, ensure that the sampling time is of sufficient length for the spacer/VHC to be emptied of remaining aerosol before the next actuation is delivered, and do not disconnect the spacer/VHC from the breathing simulator between actuations.

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Remove the filter carefully from its location and place it in a suitable container. Add a predetermined volume of a suitable solvent to the container and agitate to dissolve the collected drug. Transfer a sample to a cuvette or vial for drug assay using a syringe equipped with an in-line filter to retain any suspended material. Recover the mass of drug from the filter by using a validated procedure appropriate to the specific product, and assay for the mass of drug collected by using a validated procedure. Calculate the total mass of drug per actuation; this is the emitted mass in the fully coordinated condition ($EM_c$). Repeat the measurement with the required number of devices and replicates per device.

TEST, PART 2

[NOTE—This part of the test is inappropriate for spacers.] For the second part of the test, either clean the VHC between measurements or evaluate a new VHC out of its packaging, as required and justified. Report whether cleaning or replacement of the VHC was carried out. Repeat the procedure above in *Test, Part 1* with the same VHC, this time actuating the MDI timed to coincide with the onset of exhalation. Calculate the total mass of drug per actuation; this is the EM in the fully uncoordinated condition ($EM_{uc}$). Repeat the measurement with the required number of devices and replicates per device.

Calculate the ratio of $EM_c$ with the $EM_{uc}$.

## 4.2 With Facemask

TEST, PART 1

The purpose of this test is to compare the EM from a spacer/VHC equipped with a facemask to that obtained in the fully coordinated simulation with the facemask removed. Facemasks are widely prescribed for infants, small children, and adults who lack coordination to use a mouthpiece-based product. The evaluation of spacers/VHCs supplied with a facemask, rather than a mouthpiece, requires additional attention beyond the methods described previously, because the facemask itself has a major part to play in the transport of the drug-containing aerosol particles from the add-on device to the patient (*Figure 7*). It is therefore important that the facemask be tested in situ as part of the add-on device, rather than separately. A critical component of the test apparatus is the representation of a human face appropriate to the age range for which the add-on device is intended (e.g., neonate, infant, small child, or adult). There are few models that can be defined as an apparatus in a way that is similar to what is done for cascade impactors in the aerodynamic particle size analysis of MDI-generated aerosols, although some face models are currently available commercially. Ideally, standardized, age-appropriate models would be preferred. ▲▲ (USP 1-Dec-2019)

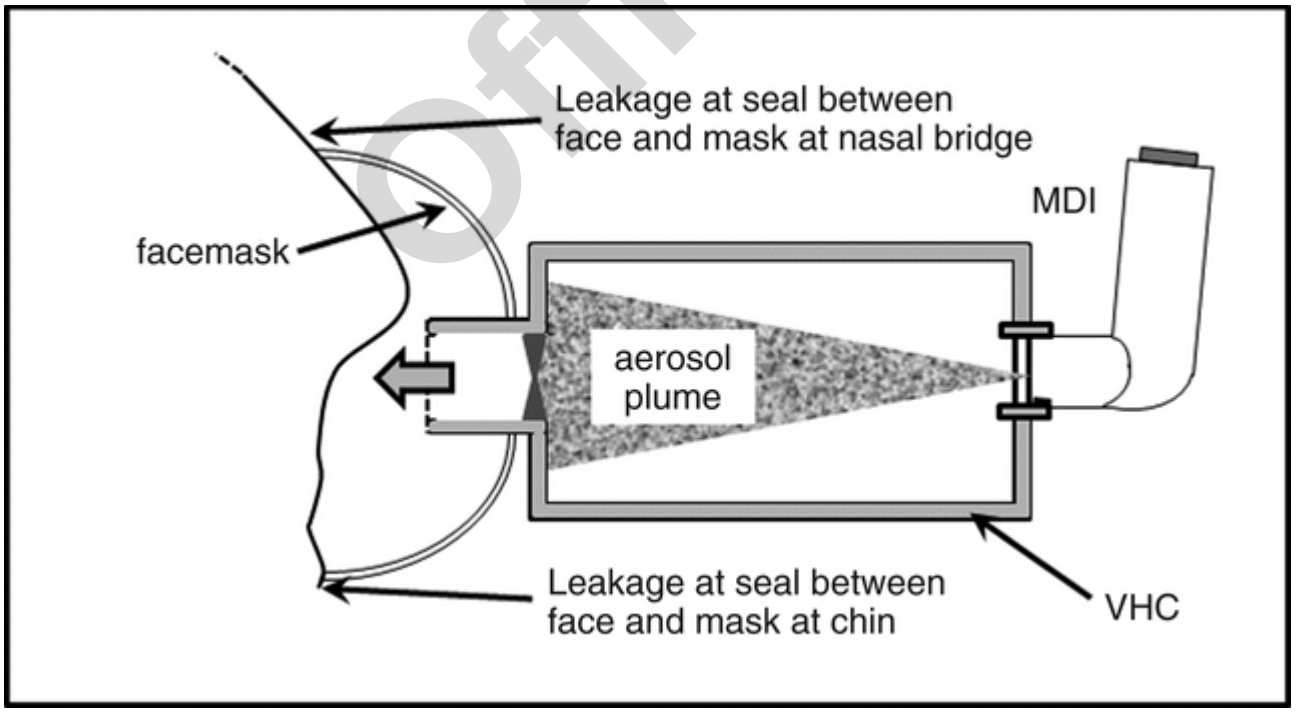


Figure 7. VHC with facemask applied to face.

Ideal attributes for face models are the following:

- Appropriate facial dimensions for the intended user age range
- Ability to apply the facemask with the predicted amount of dead space when it is applied with a clinically appropriate force to the model
- Physiologically accurate soft facial tissue modeling around the chin, cheeks, and nose where the facemask makes contact

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- Means of correctly mounting the spacer/VHC so that the facemask is oriented with the correct alignment to the face, as would occur when in use by a patient

[NOTE—Some models may include anatomically accurate realization of the upper airway (naso- or oropharynx), with the aerosol collection filter located at the distal port of the model representing the entry to the lungs.]

For the sake of simplicity and to realize a measure of spacer/VHC performance comparable to the measure obtained with facemask removed in *Parts 2*▲*A* and *B*▲ (USP 1-Dec-2019) (see *Figure 4*), the default assumption is that the model face simulates open-mouth breathing without an anatomically accurate upper airway, and that the aerosol collection filter is located in a cavity immediately behind the lips of the model. Alternatively, if an obligate nasal breathing infant model is being used, then the filter can be placed behind the nares. The mass of drug is reported as the emitted mass ($EM_{facemask}$).

Select a face model appropriate to the intended age range for the spacer/VHC facemask, and mount the face model in an appropriate fixture. The fixture should enable the facemask to be located at an appropriate angle to the face model, such that an effective seal between the facemask and face model is created with a clinically appropriate force. This is typically a loading force of 1.6 kg but may differ from this value if justified by the design of the facemask. The arrangement shown schematically in *Figure 8* is one way of achieving the desired result, but other approaches may be adopted as required, if justified.

Locate and secure the filter media in the cavity behind the lips of the face model. Filtrete is a suitable electret filter medium ▲▲ (USP 1-Dec-2019) that readily releases collected drug quantitatively upon addition of a suitable solvent during the assay procedure. However, another suitable filter medium may be used, provided that the tester is satisfied that it is capable of collecting the entire emitted dose, and that drug recovery for the assay is quantitative.

Verify that a seal has been obtained between the facemask and face model. This is conveniently done by connecting the outlet (distal) port of the face model, using a short length of flexible hose, to an in-line calibrated gas flow meter whose distal port is in turn connected to the vacuum source via a regulating valve capable of setting the flow rate to a suitable value, typically 30 L/min. Connect a similar flow meter to the port for the MDI (MDI adapter) on the spacer/VHC, and ensure that the mask is positioned in order to maximize flow rate. ▲▲ (USP 1-Dec-2019)

[NOTE—Leakage from the facemask to face seal, as detected by lower flow rate, is known to reduce drug delivery.]

Turn off the vacuum source after this check has been completed, and disconnect the flexible hose from the in-line flow meter. Remove the upstream flow meter from the MDI adapter of the spacer/VHC.

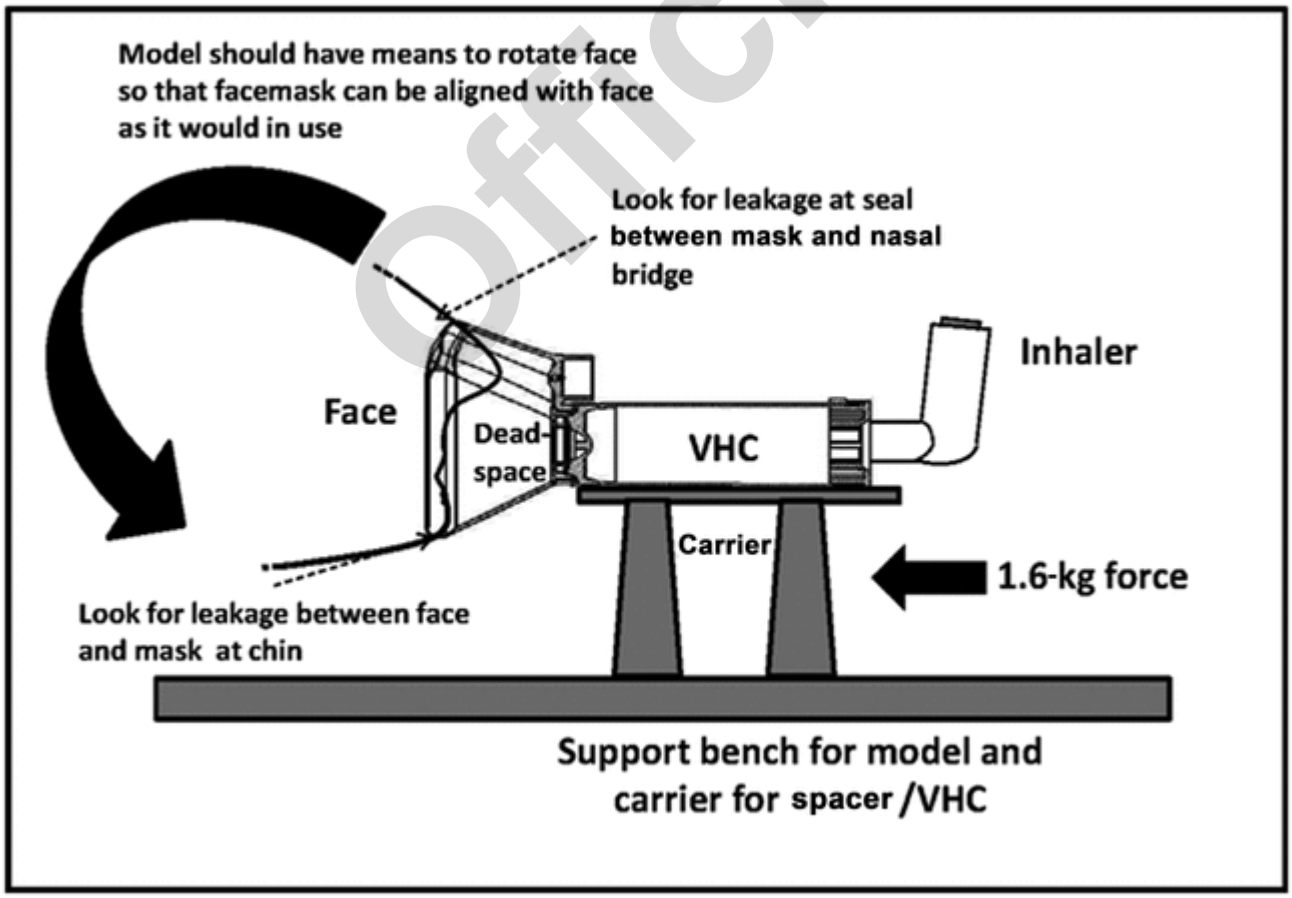


Figure 8. Mount for face model with alignment fixture including carriage for spacer/VHC; a fixed (i.e., 1.6-kg) weight acts on the pulley below the VHC and draws the carriage toward the face with the required force.

Connect the outlet (distal) port of the face model to the appropriate port of the breathing simulator using a short length of flexible hose. Set the breathing simulator to the required breathing pattern (identified from *Table 3*) in accordance with the manufacturer's instructions. Alternative breathing patterns may be used if required. Ensure that the simulator has been

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calibrated before use so that the actual volumes and frequency are within ±5% of the indicated value during each breathing cycle. Calibration syringes are available from various suppliers of mechanical ventilation equipment to verify volumes. To verify frequency, the method varies among breathing simulator manufacturers, and therefore the advice of the particular supplier/manufacturer should be sought if the method is not explicitly stated in the operating instructions for the apparatus. Ensure that the selected flow waveform from the breathing simulator is stable before proceeding.

Follow the directions in the patient information and instructions for use for the MDI to prime it before inserting it into the MDI adapter of the spacer/VHC before the first actuation into the test apparatus. In the case of an add-on device equipped with an integral actuator, after priming the MDI canister in its mouthpiece adapter as supplied, remove the canister from the adapter, and clean the external valve stem with a suitable wipe. Insert the canister valve stem into the receptacle according to the instructions for use for the particular add-on device, taking care to avoid premature actuation. Typically, it should require only one actuation of the MDI into the spacer/VHC per determination. However, for certain highly potent products delivering a low-unit mass of drug per actuation, more than one actuation of the MDI may be needed to collect a sufficient mass of drug product. Under such circumstances, follow the directions in the patient instructions or allow a minimum of 30 s between actuations if no direction is provided. Deliver the minimum number of actuations needed to obtain an adequate sample of the drug so that mass measurements can be made with acceptable precision.

Perform a single actuation in the spacer/VHC, timed to coincide with the beginning of an inhalation (see *Figure 4*, *Part 3A*). Allow sampling to occur for five additional breathing cycles for the actuation. If additional actuations are required to improve analytical sensitivity, ensure that the sampling time is of sufficient duration for the spacer/VHC to be emptied of remaining aerosol before the next actuation is delivered, and do not disconnect the spacer/VHC from the breathing simulator between actuations.

Following the tests, remove the filter carefully from its location and place it in a suitable container. Add a predetermined volume of a suitable solvent to the container and agitate to dissolve the collected drug. Transfer a sample to a cuvette or vial for drug assay using a syringe equipped with an in-line filter to retain any suspended material. Recover the mass of drug from the filter by using a validated method appropriate to the specific product, and assay for the mass of drug collected by using a validated procedure. Calculate the total mass of drug per actuation; this is the emitted mass in the fully coordinated condition with facemask fitted to the spacer/VHC ($EM_{c–fm}$). Repeat the measurement with the required number of devices and replicates per device.

The values of $EM_c$ from the test undertaken in *Part 2*▲*A*▲ (USP 1-Dec-2019) (see *Figure 4*) in which the facemask adapter was removed for comparison of EM, simulating fully coordinated use by a breathing simulator can be compared to the $EM_{c–fm}$ in order to understand the impact of the facemask on drug delivery.

## TEST, PART 2

For a VHC only, the same process can be repeated, but with the MDI actuation(s) timed to coincide with the beginning of exhalation (rather than inhalation) (see *Figure 4*, *Part 3B*). In this instance, the mass of drug collected per actuation is the emitted mass in the uncoordinated condition ($EM_{uc–fm}$). The ratio of $EM_{c–fm}$ from *Part 1* ▲of this test▲ (USP 1-Dec-2019) and the $EM_{uc–fm}$ can now be calculated.

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# ⟨1603⟩ GOOD CASCADE IMPACTOR PRACTICES

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## 1. INTRODUCTION

### 1.1 Background and Rationale

The multi-stage cascade impactor is the mainstay methodology for the determination of aerodynamic particle size distribution (APSD) of aerosols emitted by Inhalation Aerosols and Sprays, and Inhalation Powders, as described in *Inhalation and Nasal Drug Products: Aerosols, Sprays, and Powders—Performance Quality Tests* ⟨601⟩. This equipment is also recommended for APSD assessment associated with the use of drug products dispersed by nebulizing systems in *Products for Nebulization—Characterization Tests* ⟨1601⟩ as well as for the assessment of add-on devices for use with inhalation aerosols in *Spacers and Valved Holding Chambers Used With Inhalation Aerosols—Characterization Tests* ⟨1602⟩. Both chapters provide instructions on how to determine APSD, but due to necessity are focused on the measurement process itself, and are therefore limited in the advice given to assist in obtaining consistent measurements by these apparatuses. The cascade impactor is a complex apparatus to set-up, use, and maintain correctly. This chapter provides guidance to optimize the accuracy and precision of the methods described in ⟨601⟩, ⟨1601⟩, and ⟨1602⟩ for the determination of APSD.

Regulatory activity in the mid-1990s that led to the 1998 publication of a draft US FDA Guidance for Industry on metered dose inhaler (MDI) and dry powder inhaler (DPI) drug products chemistry, manufacturing, and controls documentation obviated the need for an informational chapter covering good cascade impactor practice (GCIP). This draft Guidance (Metered Dose Inhaler (MDI) and Dry Powder Inhaler (DPI) Products—Quality Considerations) was updated in 2018. Following the release of this document, the International Pharmaceutical Aerosol Consortium on Regulation and Science (IPAC-RS) and later the Product Quality Research Institute (PQRI), recognized that there was a need to define a method of addressing a failure to achieve a mass balance in the quality testing of orally inhaled products (OIPs). After the PQRI working group was concluded, the Cascade Impactor Working Group of the IPAC-RS continued to address reports from stakeholders that the cascade impactor method was complex, resulting in measurement failures that were not attributable to product quality. Later, the European Pharmaceutical Aerosol Group (EPAG) established the acceptability of stage mensuration as a means for a regular check on aerodynamic size-separating performance of the component stages and exploring methods of collection surface coating, eliminating leakage into the apparatus and setting the volumetric flow rate correctly.

The purpose of GCIP is therefore:

1. To ensure appropriate size-fractionation of the inhaled product;
2. To establish the APSD profile of the delivered dose reliably, minimizing sources of potential bias and optimizing measurement precision;
3. To obtain adequate resolution of the fine particle mass component of the delivered dose;

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4. To establish the precision of manufacture of impactor apparatuses and accessories with consideration for allowed tolerances and material of construction; including information on calibration, verification, and mensuration-related issues of the apparatus upon receipt and after use.

The rationale for the chapter is to guide both new and existing cascade impactor equipment users as to industry best practices in connection with set-up, use, maintenance, and storage between uses. The ultimate goal is to establish and maintain the capability to determine the APSD and quality consistency of drug product batches.

## 1.2 Purpose and Scope

The purpose of this chapter is to define a series of measures that should be taken to improve the likelihood of reliably making valid measurements of APSD from a product for inhalation by any of the cascade impactor apparatuses described in ⟨601⟩. Its focus is on the measurement apparatus itself.

The scope is in four main parts:

1. Generic description of the operating principle of the cascade impactor;
2. Apparatus maintenance, including ancillary equipment such as the pre-separator (PS), used for some APSD measurements and the induction port entry to the measurement system;
3. Method development for inhaler APSD determinations, providing guidance on how to structure a series of measurements to obtain information regarding the variability associated with the determination, as well as advice regarding the mitigation of common causes of measurement bias;
4. In-use aspects, in particular the step-by-step checks that should be made before each measurement, addressing cleaning and storage of the apparatus components, maintaining the integrity of individual apparatus sets where more-than-one cascade impactor and the associated equipment is in service, and lastly advising on checks that should be made to provide assurance of component interchangeability following appropriate component validation procedure(s) (for example, the use of a common PS with several different impactors or the use of the same collection plate or cup in several different impactors of the same type).

This chapter is not intended to provide a line-by-line guide to APSD measurement, as some of this information is provided in ⟨601⟩, ⟨1601⟩, and ⟨1602⟩ for the various inhaler product types. Neither does it provide detailed procedures, because in many cases, such information will be product-specific. Rather, the information presented is meant to summarize current thinking as to the implementation of best practices in cascade impactor maintenance and use in terms of robust protocols for the laboratory making these measurements. When developing such protocols, users are also advised to consult with current regulatory guidance documents that address inhaler APSD measurement, for example, those published from time to time by the FDA.

## 1.3 Specific Information Regarding Impactor Stage Mensuration

In *4.1 Stage Mensuration, Measurement Traceability, and Mensuration Interval*, information regarding the stage optical mensuration of nozzles associated with the sizing stages of cascade impactors is given to aid the user in identifying the critical aspects of the process and how mensuration provides traceability of the measures of size ultimately to the international length standard via intermediate calibrated objects, such as optical reticles or orifices of known diameter, having a circular profile. However, since specialized optical inspection equipment is required to acquire the mensuration data, it is assumed likely that the user will employ a specialized service in order to undertake the task, so detailed methodology is not provided.

# 2. DEFINITIONS OF KEY TERMS RELATING TO THIS CHAPTER

**Aerodynamic diameter ($D_{ae}$):** The aerodynamic diameter of an irregular particle is defined as the diameter of the spherical particle with a density of water [1000 kg/m$^3$ ($\rho_0$)] and having the same settling velocity under the influence of gravity as the irregular particle. $D_{ae}$ is related to the microscopy-measured physical diameter (volume equivalent diameter, $D_p$) in accordance with:

$$D_{ae}C_{ae} = \left[\frac{\rho_\rho}{\rho_0 \chi}\right]^{1/2} D_\rho C_\rho \quad [1]$$

$C_{ae}$ and $C_p$ = slip correction factors corresponding to $D_{ae}$ and $D_p$, respectively, both of which can be regarded as being close to unity for the sizes of aerosol particles from inhalers for which APSD is being determined
$\rho_p$ = particle density
$\chi$ = dynamic shape factor[1] (1.00 for spherical solid particles or liquid droplets, >1.00 for non-spherical particles)

**Effective diameter ($W_{eff}$):** The representative diameter for a collection of nozzles forming a multi-nozzle stage of a multi-stage cascade impactor. Effective diameter is defined analytically as the product of the area mean diameter ($W^*$) raised to the power of 2/3 and the area median diameter ($W_{median}$) raised to the power of 1/3:

$$W_{eff} = (W^*)^{2/3}(W_{median})^{1/3} \quad [2]$$

$W^*$ = area mean diameter
$W_{median}$ = area median diameter

[1] Hinds W.C. 1999 "Properties, Behavior and Measurement of Airborne Particles." Wiley Interscience, NY 1999.

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For a single-nozzle stage, $W_{eff}$ defaults to the mensurated diameter of that nozzle.

**Stage cut-point size ($d_{50}$):** The size on the aerodynamic diameter scale at which a given impactor stage collects incoming particles with 50% efficiency. The relationship between collection efficiency and $D_{ae}$ is described further in *3. Cascade Impactor Operating Principles*.

**Stokes number (*St*):** The dimensionless term characterizing the behavior of solid or liquid particles suspended in a fluid flow, such as air. A particle with a low Stokes number follows the air streamlines. In contrast, a particle possessing a large Stokes number is dominated by its inertia and so that it continues along its initial trajectory when the flow passes an obstruction, for example, as it diverges upstream of the collection surface of an impactor stage (see *Figure 1*). $St_{50}$ is the Stokes number corresponding to the cut-point size at which the impactor stage is 50% efficient at collecting the incoming particles.

**Stage mensuration:** The process of establishing the representative diameter at the exit of each nozzle of a stage forming part of a multi-stage impactor. Although pin or go/no gauges can be used to mechanically measure nozzles having diameters in excess of about 2 mm, it is more common currently to make the determination non-invasively by using automated optical microscopy combined with image analysis.

## 3. CASCADE IMPACTOR OPERATING PRINCIPLES

A cascade impactor size-fractionates incoming aerosol particles into several sub-fractions on the basis of their differing inertia. A typical design, such as the apparatuses described in ⟨601⟩, comprises several consecutive stages, each of which functions as an inertial size-separator or fractionator of the incoming aerosol in a gas stream moving at constant velocity (flow rate). The basic component is a single stage that comprises a nozzle plate containing one or more circular orifices located a fixed distance from an impaction plate/cup surface that is usually horizontal (see *Figure 1*). The following characteristic dimensions describe the operating property of the stage:

$W_{eff}$: effective nozzle diameter—most stages will contain *n* nozzles machined to be as close to identical as possible
*T*: thickness of the nozzle plate
*S*: separation distance between exit plane of nozzle plate and the collection surface

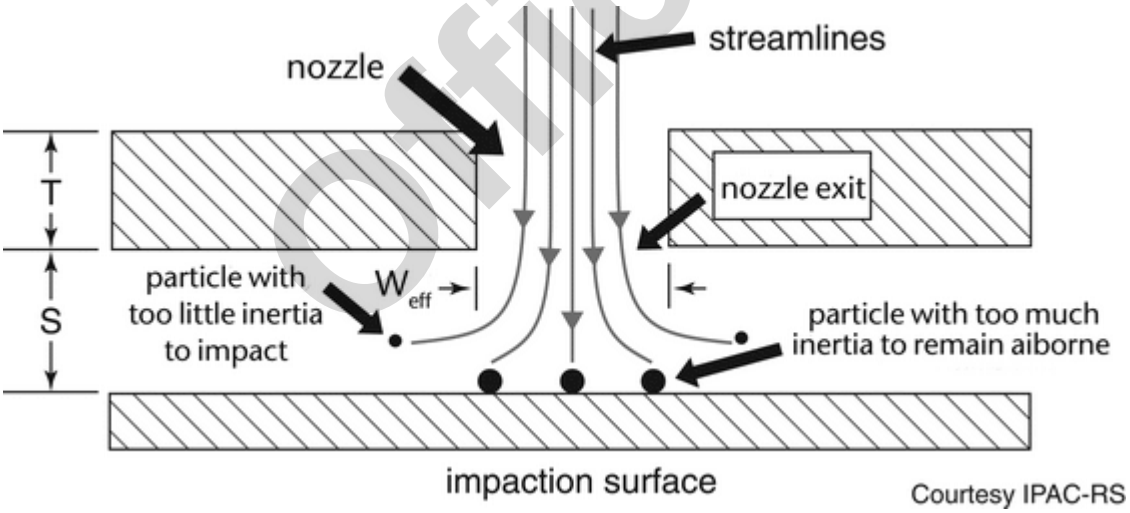


Figure 1. Generic single-stage impactor showing nozzle plate and collection surface located immediately below the plane of the nozzle exit. Several consecutive stages connected together with nozzle sizes that progressively get smaller constitutes a cascade impactor.

The stage functions by fractionating incoming particles of various sizes on the basis of their differing inertia, the magnitude of which reflects the resistance to a change in direction of the laminar flow streamlines passing through each nozzle. As the incoming flow passes through the nozzle plate, the streamlines diverge on approach to the collection surface, whereas the finite inertia of the particles causes them to cross the streamlines. Depending upon how far a given particle diverges from the streamlines, its trajectory may cause it to collide (impact) with the collection surface located at a distance, *S*, close to the exit plane of the nozzle set.

The magnitude of *T* is set by the manufacturer during design of the impactor stage, and in general, does not change measurably in use. In contrast, the size of *S* can be reduced by coating of the collection surface to mitigate particle bounce (see *5.4 Mitigation of Particle Bounce and Re-Entrainment*), as well as by the accumulation of deposited particles on the collection surface. The ratio, $S/W_{eff}$, can vary widely in the range 1–10 for effective size fractionation to occur, and therefore changes in this ratio are unlikely to influence stage performance, given typical mass loadings associated with inhaler APSD measurements, even on the stages where most of the particulate deposits.

The dimensionless Stokes number (*St*), which is the ratio of the stopping distance of a particle to a characteristic dimension, in this case the nozzle diameter, $W_{eff}$, describes the size fractionation process by defining a critical particle size that will reach

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the collection surface for a particular stage geometry. *St* is defined in terms of the stage effective diameter by the following relationship:

$$St = \frac{\rho_\rho C_{\text{ae}} d_{ae}^2 u}{9\mu W_{eff}} \text{ [3]}$$

$C_{ae}$ = designated as unity from this point onwards
$d_{ae}$ = particle aerodynamic diameter
$u$ = air velocity through the nozzle plate
$\mu$ = air viscosity

In a cascade impactor, several stages are connected together such that $u$ progressively increases through the cascade by reducing $W_{eff}$ (see *Figure 2*).

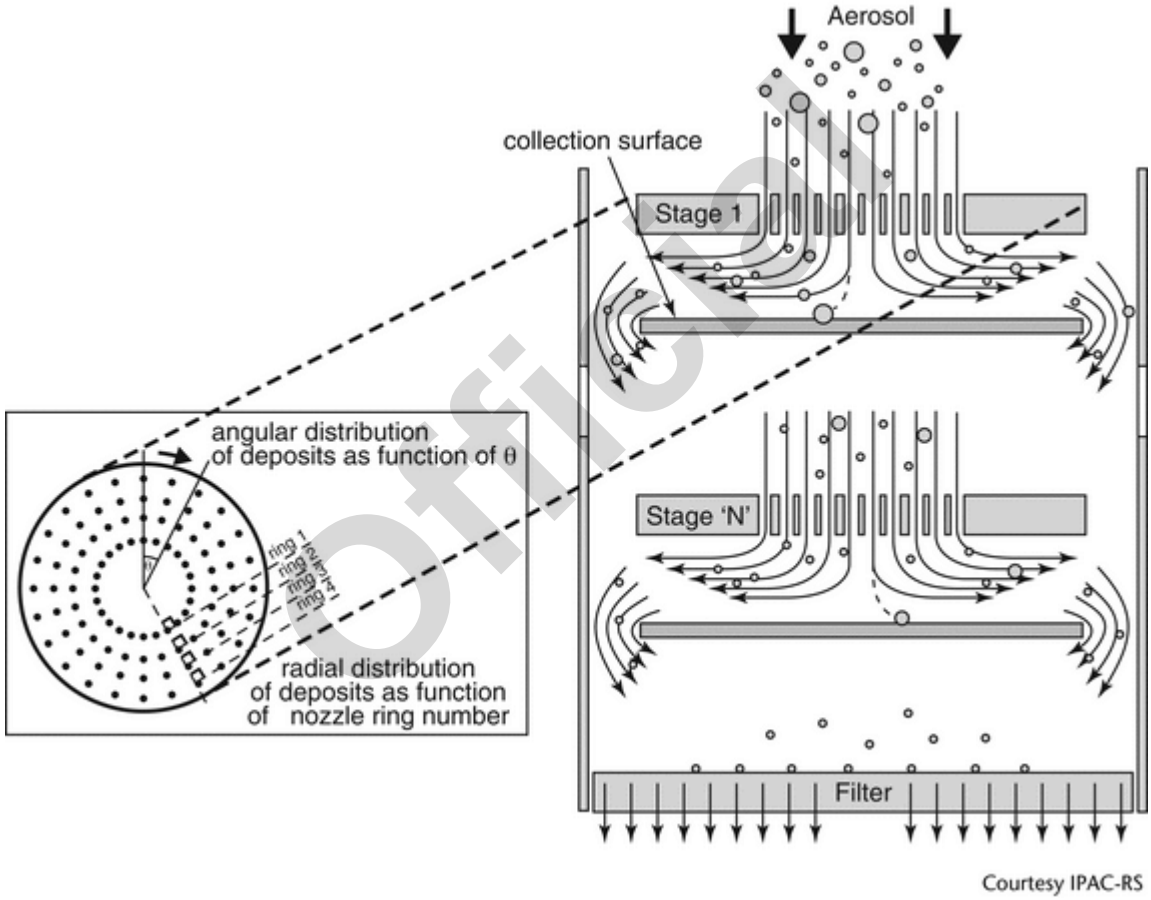


Figure 2. Conceptual multi-stage cascade impactor—Typically there are 7 or 8 stages from the entry to the back-up filter or micro-orifice collector (MOC) in the case of the Next Generation Impactor; the function of the filter/MOC is to capture any particles that have penetrated beyond the lowermost stage 'N'.

*Equation 4* predicts that the particle collection efficiency of an ideal impactor stage, $E_{stage}$, expressed as a percentage, will increase in a step-wise manner between limits of 0%–100%, as $d_{ae}$ increases. In practice, for a well-designed stage, $E_{stage}$ is a monotonic sigmoidal function of $d_{ae}$ that increases steeply from $E \approx 0\%$ to >95%, reaching its maximum steepness when $E_{stage}$ is 50% (see *Figure 3*). At this location, defined as the stage cut-off diameter ($d_{50}$), this equation also defines the relationship between $d_{50}$, $St_{50}$, and volumetric flow rate ($Q$) for a multi-orifice stage comprising $n$ circular nozzles:

$$d_{50} = \left[\frac{9\pi\mu n W_{eff}}{4\rho_0 Q}\right]^{1/2} \sqrt{St_{50}} \text{ [4]}$$

$\sqrt{St_{50}}$ is close to 0.49 at $d_{50}$ for all stages of each cascade impactor type described in ⟨601⟩.

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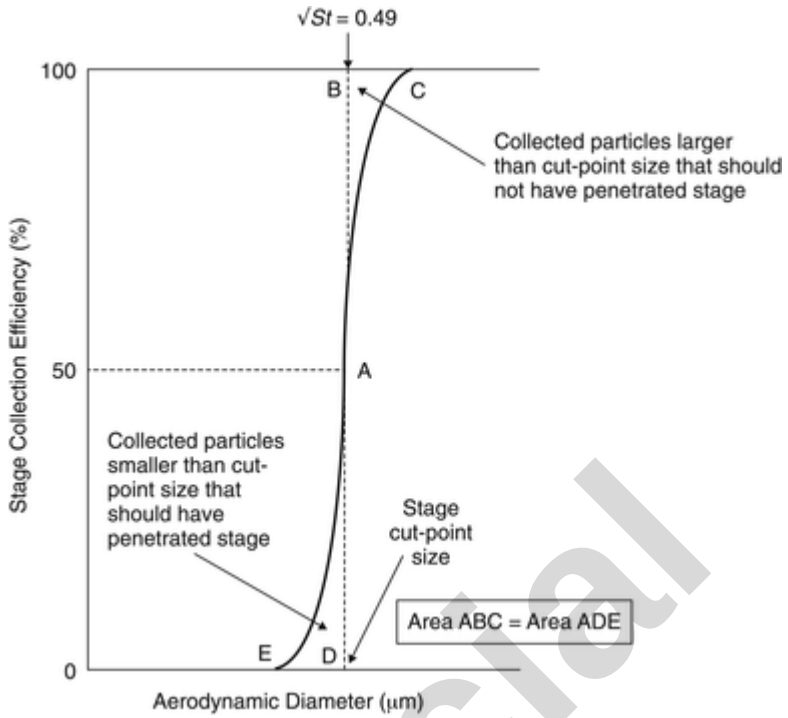


Figure 3. Generic impactor stage collection efficiency curve—The ideal step function at the size corresponding to the cut-off diameter is shown together with the sigmoidal curve representing normal behavior.

In a well-designed stage, the area ABC of *Figure 3*, representing the portion of the incoming aerosol contained in particles larger than the cut-point size that should have been collected, but instead bypassed the collection plate/cup, equals the corresponding area ADE that represents the portion of the aerosol contained in particles smaller than the cut-point size that should have penetrated the stage, but instead were collected there. The result is that $d_{50}$ for a given stage can be used to define its size-fractionating performance as if it was ideal in this respect.

An important outcome arising from *Equation 4* is that the cut-off diameters of all the stages in a given cascade impactor are constant only when $Q$ is also constant. It follows that for the testing of dry powder inhalers in accordance with the methodology in ⟨601⟩, the stage cut-points will not be at their final steady state values until the volumetric flow rate is constant after initiation of sampling from no flow by opening the two-way solenoid valve located between the exit from the impactor and the vacuum source. This process can take up to 400 ms for impactors having large internal volume, in particular, the Next Generation Impactor (NGI) with its PS. In practice, this finite start-up time is ignored in such assessments on the basis that it represents a small fraction of the total time during which the 4.0-L volume is sampled for inhalation powders. More importantly, however, any leakage of ambient air into the impactor at locations other than via the induction port, will increase the local value of $Q$ after the leakage point, and in consequence result in an uncontrolled decrease in $d_{50}$ for stages downstream. Bias arising from leakage into the impactor is therefore discussed further in *6.5 Mitigation of Air Leakage into the Apparatus*.

## 4. APPARATUS MAINTENANCE

### 4.1 Stage Mensuration, Measurement Traceability, and Mensuration Interval

The performance of a specific stage of a cascade impactor depends primarily upon the physical integrity of the nozzles. It follows that the precision of manufacture of these critical components is a factor that may contribute to variability of APSD results when measurements of the same inhaled product are made with more than one impactor. Furthermore, use-related changes to the nozzle dimensions of a given impactor may result in the development of APSD measurement bias during time in service. Mensuration is therefore appropriate both for new impactors and at intervals during their lifetime in service.

Nozzles can become partially plugged by deposited particles or through corrosion of the metal from which the stage nozzle plate is manufactured. Alternatively, they can become widened with time, either through corrosive action or by mechanical damage, for instance during cleaning if a sharp object is used to penetrate the nozzles. In the past, stage performance was determined by calibrating the stage with uniform-sized (monodisperse) particles of known aerodynamic diameter. However, the process is time-consuming as many different sizes of calibration particles are required to define the collection efficiency curve to the required precision. Since the advent of automated microscopy-image analysis, the procedure of stage mensuration has replaced particle calibration. In stage mensuration, the diameter of each nozzle exit is directly measured to a high degree of accuracy and precision, and the effective diameter ($W_{eff}$) is calculated to represent the entire group of nozzles where more than a single nozzle is present. In the latter case, the single nozzle diameter is identical with $W_{eff}$. The calculated value of $W_{eff}$ is compared with the tolerance range established for that stage for either the Andersen Cascade Impactor or the NGI in ⟨601⟩ to establish if the stage is within specification. The mensuration equipment is in turn calibrated with reference objects that are

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traceable ultimately to the international length standard. It is anticipated that the cascade impactor user will have the procedure undertaken by an external provider, who will typically be the supplier of the impactor, so the focus here is on establishing a regimen for maintaining assurance that any given stage of any cascade impactor remains within the manufacturer's specification as defined in ⟨601⟩.

The scheme shown in *Figure 4* illustrates the components of a mensuration protocol for initial receipt and subsequent assessment of any of the impactors described in ⟨601⟩. The following checks are recommended:

1. Installation qualification (IQ): At receipt of a new impactor, ensure, as part of IQ, that each stage is compliant with the manufacturer's dimensional specifications. These initial measurements are intended to establish baseline data for each stage of each individual cascade impactor. Undertake mensuration for each and every nozzle of each stage and determine its $W_{eff}$. Compare this measurement against the critical dimensions specified for that stage/cascade impactor in ⟨601⟩. Reject any stage whose $W_{eff}$ is outside the specification. For the ACI, it may be appropriate to substitute the equivalent rejected stage(s) with alternative stages of the same number(s), whose $W_{eff}$ value(s) are within specification. However, in the case of the NGI, the entire unit will need to be returned to the supplier for remedial action to provide stage(s) whose $W_{eff}$ values are within specification. Once values of $W_{eff}$ for all stages are ascertained to be within specification, the entire cascade impactor can be accepted for use.

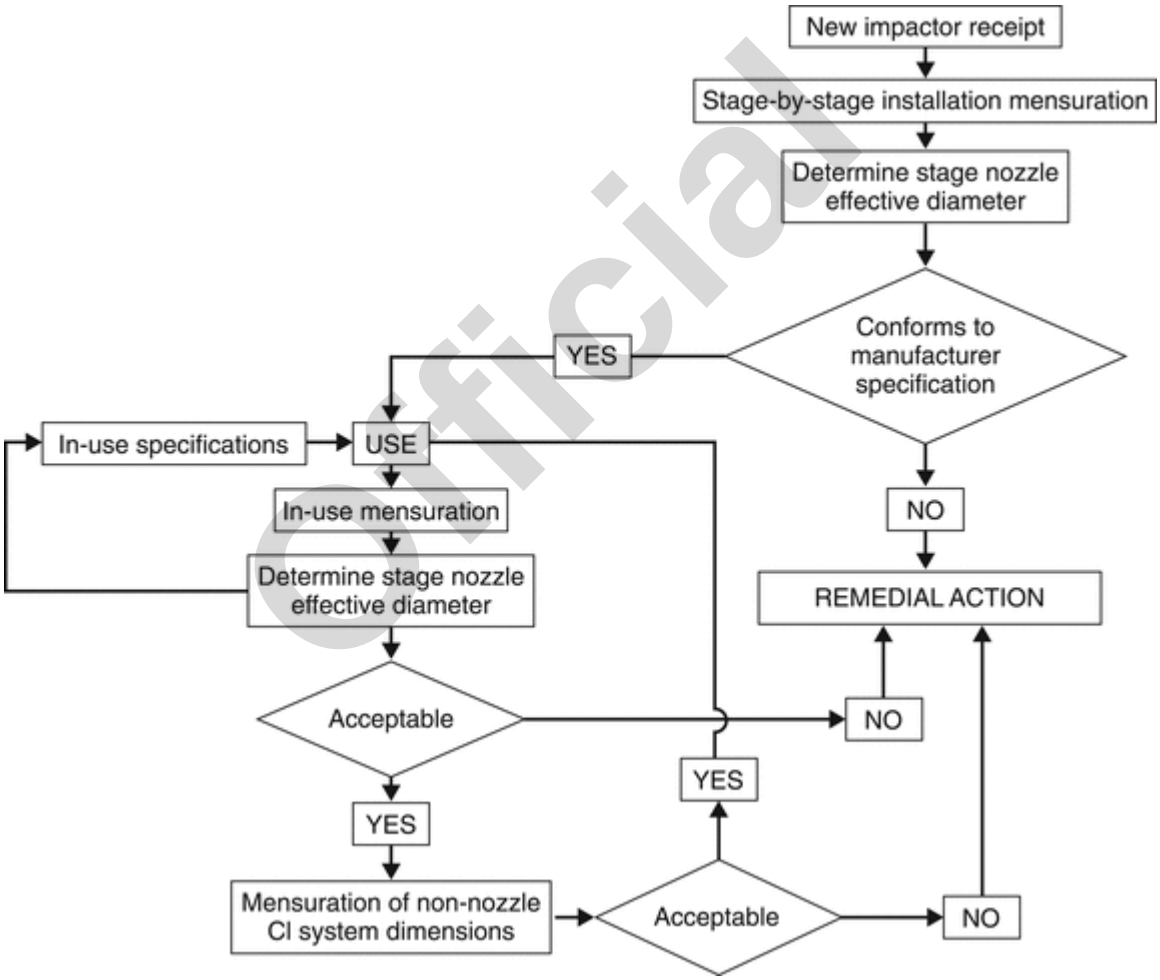


Figure 4. Scheme for the application of stage mensuration to confirm suitability for use of a cascade impactor at initial receipt and at subsequent performance assessment.

[NOTE—The frequency of the "In-use mensuration" check is determined by the amount of impactor use, the material from which the impactor is constructed, the product ingredients, and recovery solvent characteristics.]

2. For stages that are deemed acceptable in terms of the specifications in ⟨601⟩, establish the in-use stage mensuration regimen, whose frequency is determined by the amount of impactor use and the product/active pharmaceutical ingredient solvent chemical characteristics. Each in-use mensuration will always include measuring the individual stage nozzle diameters, determining $W_{eff}$ as the performance metric. The specifications established at initial acceptance will normally be used as benchmarks to judge whether the stage(s) may continue in service or require remediation or replacement. Note that remediation could be as simple as careful cleaning to remove deposits that may be plugging nozzles. Acquisition of data from several subsequent mensuration exercises after exposure to the product(s) being evaluated in the typical testing environment may also provide the opportunity to develop meaningful in-service "at

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mensuration" limits for $W_{eff}$ for a given stage that will likely be chosen to be tighter than those used to begin with. These internal limits can subsequently be used to indicate the need for remediation before the stage $W_{eff}$ breaches either the upper or lower boundary of the tolerance range provided in ⟨601⟩.

3. Following mensuration failure of an impactor, undertake the following:
   A. In the case of the ACI, remove those stages from service that are not acceptable in terms of the manufacturer's specifications, and either recondition them or replace the affected stages altogether.
   B. In the case of an NGI, the entire unit will need to be returned to the supplier for remedial action, if $W_{eff}$ for one or more stages is/are found to be out of specification.
4. Performance qualification (PQ): Establish the frequency between mensuration events. Although annual evaluation will be sufficient for most users, the duration between mensuration exercises should ideally be governed by the frequency of cascade impactor use, the type of compounds being collected, and the solvents used to recover the collected active pharmaceutical ingredient(s) particulate matter as well as the end user established cleaning procedures.
5. Ensure that other non-nozzle dimensional data for the cascade impactor apparatus are checked and confirmed as being acceptable through a mensuration or appropriate visual inspection process. Guidance on the important dimensions for ancillary equipment, such as the induction port and PS (if used), has been provided by Nichols, et al.[2] In the unlikely event that any of the critical dimensions (e.g., entry and exit diameters for the induction port, or nozzle diameters for the PS) are deemed to be unacceptable, remedial action may be as simple as cleaning the affected component. Under such circumstances, discussion with the manufacturer to establish the most appropriate procedure beforehand is strongly recommended. However, if remedial action does not resolve the problem, then the individual component should be replaced before the ancillary apparatus is placed back into service.
6. If any impactor stage or item of ancillary equipment fails specification, establish an appropriate remediation strategy, most likely involving the supplier/manufacturer of the affected equipment.

## 4.2 Internal Losses

Internal losses of particles on surfaces of the cascade impactor, other than the intended collection plates or cups, as well as losses associated with ancillary equipment, in particular the induction port and the PS (if used), are likely to result in a divergence in the recovered mass balance from the ideal label claim value. The magnitude of so-called wall losses, or inter-stage losses, if located within the impactor, is related to the issue of particle bounce covered in *5.4 Mitigation of Particle Bounce and Re-Entrainment*, since particles having adhesive surfaces that have trajectories which graze internal surfaces of the apparatus are more susceptible to be retained there by virtue of their increased ability to adhere once initial contact is made. Furthermore, electrostatic charge associated with the aerosol/dry powder particles being sampled may also result in increased internal losses. Chapter ⟨601⟩ therefore sets an upper limit of 5% of the total delivered drug mass per actuation from the inhaler as a validation requirement to limit the impact of such internal losses on measurement accuracy.

The magnitude of internal losses is also likely to be dependent on the physicochemical properties of the particles being sampled (e.g., surface roughness) that are in turn formulation dependent. Likewise, these losses are dependent upon the internal geometry of the passageways between stages of the impactor and are therefore different from one impactor design to another. In the initial development of a method for a new drug product or when a new design of cascade impactor is placed in service, it is recommended that internal surfaces of the induction port, PS (if used) and cascade impactor (entrance cone and nozzle plates for the ACI or inter-stage passageways for the NGI) be washed from both each other and from the collection surfaces separately with the established minimum necessary volume of recovery solvent to determine the magnitude of the associated internal losses as well as their location. In the product development stage, if the overall losses in APSD determination exceed 5% of the mass balance, and steps to mitigate particle bounce in the cascade impactor (see *5.4 Mitigation of Particle Bounce and Re-Entrainment*) and electrostatic charge (see *5.5 Mitigation of Electrostatic Charge Accumulation*) prove ineffective at reducing such losses, the user may wish to consider an alternative cascade impactor (e.g., replace the ACI with the NGI) to investigate if losses can be reduced below 5%. If this outcome is not possible, the inhaler aerosol APSD measurement procedure can be performed including the total internal wall losses in the assessment of mass balance to confirm that the mass recovery is within the recommended range of label claim for mass of active pharmaceutical ingredient delivered per actuation. In defining the ASPD profile, it is important to note that it is not possible to apportion wall losses from stage to stage within the cascade impactor in an attempt to assign specific size ranges to these losses. The wall loss deposition processes lack the requisite size selectivity associated with each impaction stage.

## 4.3 Inspection of Damaged/Deformed Collection Surfaces

Cascade impactors are typically manufactured from durable materials that are less prone than their predecessor materials (often aluminum) to chemical attack by solvents used in recovery of collected deposits. Nevertheless, the possibility remains that accidental damage may take place in use, especially to the more vulnerable components such as the collection plates (ACI) or collection cups/MOC (NGI). In the case of accidental damage, such as dropping during transit from one location to another, immediate physical inspection for superficial damage should be undertaken. The component should be taken out of use and replaced if damage/deformation is apparent, and is likely to affect performance.

[2] Nichols SC, Mitchell JP, Shelton CM, Roberts DL. Good cascade impactor practice (GCIP) and considerations for "in-use" specifications. *AAPS PharmSciTech*. 2013;14(1):375–390.

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# 5. CASCADE IMPACTOR METHOD DEVELOPMENT

## 5.1 Initial Considerations

The development of a cascade impactor-based method for evaluating the APSD of a specific inhaler product is typically undertaken in consultation with the appropriate regulatory authority. These authorities from time-to-time publish guidance to industry relating to the determination of APSD for inhaled products. The information provided in this part of this chapter is therefore intended to provide underlying information associated with good practice that is intended to assist in the process of establishing an acceptable methodology.

## 5.2 Number of Inhaler Actuations per Determination

Ideally, only one actuation from the inhaler product would be necessary for the APSD determination, and this situation may be possible for products having a high drug mass unit-dose delivery. In practice, more than one actuation may be needed to achieve the required deposition profile throughout the stages of the cascade impactor to enable acceptable measurement precision to be achieved by the drug recovery and assay procedure. However, repeated actuations may disguise any underlying variability in APSD that occur from one actuation of the inhaler to the next, and therefore it is recommended that determination for each inhaler should be performed with the minimum number of actuations justified by the sensitivity of the analytical procedure used to quantitate the deposited drug. The amount of drug deposited on the critical stages of the cascade impactor should be sufficient for reliable assay, but not so excessive as to bias the results by masking individual actuation variability. Here, critical stages are those that collect the smallest particle mass per actuation, where the sensitivity of the recovery method and subsequent assay for the active pharmaceutical ingredient are often limiting factors.

## 5.3 Number of Replicate APSD Determinations

Although the cascade impactor-based method for determining inhaler APSD performance is both a labor-intensive and exacting procedure, automated or semi-automated systems are available to assist in the process. It is common to undertake at least five replicate measurements at each condition being assessed. However, this number will typically be decided by consultation with the appropriate regulatory agency, involving considerations such as prior knowledge of the variability in delivery associated with the product and the number of actuations required through the delivery life of the inhaler.

## 5.4 Mitigation of Particle Bounce and Re-Entrainment

In normal operation, particles collected by a particular stage of the cascade impactor should be retained on the collection surface until the drug is recovered for assay. For certain inhalation aerosols containing surfactant as an excipient as well as products for nebulization that are generated as aqueous droplets, it is likely that the collection surfaces will not need coating with a viscous substrate to mitigate bounce of incoming particles and their subsequent re-entrainment in the flow passing further into the impactor. However, for inhalation powders, coating of collection surfaces other than the filter/MOC will almost certainly be necessary. Such pre-coating is normally used for inhalation powders where the incoming drug particles are accompanied by larger carrier particles.

Particle bounce and associated re-entrainment is easily diagnosed by the presence of a larger than expected mass of drug recovered from the back-up filter/MOC, because once re-entrained, the particles have sufficient velocity to bounce off the collection surfaces of subsequent stages. In method development, it is therefore recommended that evaluation of such behavior be undertaken in a validation study with and without collection surface coating present.

There are numerous methods for collection surface coating, all of which involve the deposition of a thin layer of a viscous substance, such as glycerol, silicone oil, or other suitable substance, such as the surfactant, polyoxyethylene lauryl ether.[3] The coating substance is typically prepared as a solution in a volatile solvent which subsequently evaporates, leaving behind a uniform deposit. The choice of coating material will depend upon the physicochemical properties of the drug product being sampled as well as the subsequent recovery and analytical procedure. Whatever method is chosen for applying the coating substance, each collection surface should be visually inspected for non-uniformities in the coating, and the procedure repeated after cleaning the collection surface, if imperfections are found.

The interior surfaces of the induction port are not normally coated in the procedures described in ⟨601⟩. However, an appropriate pre-defined volume of an appropriate solvent may be dispensed when the PS is used (up to 10 mL to coat the PS interior used with the ACI or up to 15 mL to the central cup of the PS used with the NGI). Alternatively, it may be appropriate to coat the particle collection surfaces with a viscous substance as described for the stage collection surfaces in order to prevent re-entrainment of impacted particles therein.

## 5.5 Mitigation of Electrostatic Charge Accumulation

Acquisition of electrostatic charge can affect the measured APSD by modifying the particle size properties of the aerosol delivered from the device (intrinsic aerosol electrostatic charge) or by changing the deposition of the aerosolized particles within the cascade impactor test assembly (delivery device/apparatus-acquired charging). Both processes result in an increase of variability of APSD measurements, especially when seasonal changes in relative humidity are not controlled, and such behavior is more likely with inhalation powders rather than inhalation aerosols or products for nebulization.

[3] Polyoxyethylene-23-lauryl ether.

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Appropriate setup and handling of the inhaler as well as the entire measurement apparatus can significantly reduce variability in test results caused by electrostatics, and practical steps include grounding the measurement equipment as well as the operator (typically via use of a wrist strap and an antistatic laboratory coat). Controlling the local environmental conditions such that testing is undertaken at values of relative humidity in the range 40%–60% is also recommended to avoid variability from electrostatic effects.

***Change to read:***

# 6. IN-USE ASPECTS

## 6.1 Initial Considerations

There are several precautions that should be taken for each and every cascade impactor measurement. The purpose of this section is to provide an ordered sequence of verifications [Operational Qualification (OQ)] for the user to follow to avoid bias associated with errors associated with apparatus set-up (i.e., incorrect impactor assembly) and also in the establishment of the correct operating conditions (i.e., flow rate setting). From a regulatory science perspective, the mass of active pharmaceutical ingredient deposited on a stage-by-stage basis is initially reported to establish the APSD profile of the drug product. At the appropriate time, stage groupings may be proposed based on agreement with the regulatory agency.

## 6.2 Inspection of Cascade Impactor Components Susceptible to Deterioration

Regardless of incidents involving accidental damage, a regimen should be established for periodic visual inspection of all cascade impactor apparatus components, including the induction port and PS (if used), connection tubing, and related flow control equipment. This physical examination is in addition to the stage mensuration of the nozzles of individual stages described in *4.1 Stage Mensuration, Measurement Traceability, and Mensuration Interval*. The frequency of such inspections will depend upon the amount of use the components receive, as well as experience gained with time in service; however, as a start an annual inspection is recommended. Inspected components that are deemed damaged or marginally functional should be identified as such and removed from service. Visual inspection of the seal body and inter-stage passageways of the NGI can be difficult, and may therefore best be undertaken by the supplier/manufacturer. The component should be replaced or repaired to the manufacturer's specification, if any damage/deformation is apparent.

The external 'O'-ring seal associated with each stage of the ACI is particularly susceptible to crack formation in normal use. Each seal should therefore be visually inspected for cracking, scratch marks, and gross deformation on each occasion of use and replaced if damaged. The corresponding elastomer rings located in the seal body of the NGI should likewise be visually inspected before use and replaced if damaged.

## 6.3 Assurance of Stage Collection Surface Coating

Coating of the stage collection plates (ACI) or cups (NGI) may be necessary to mitigate bias in the APSD measurement caused by particle bounce and re-entrainment (see *5.4 Mitigation of Particle Bounce and Re-Entrainment*). Since the coating procedure can be highly operator dependent, even if semi-automated, once a suitable coating method has been established and validated, each coated collection plate or cup should be visually inspected before assembly into the impactor to be sure that the applied coating is uniform and that no locations are apparent that remain uncoated.

## 6.4 Assertion of Correct Assembly

### 6.4.1 ANDERSEN CASCADE IMPACTOR

The individual stages are separated when the impactor is disassembled fully between determinations. The manufacturer may identify each nozzle plate with its stage number. However, if there is more than a single impactor in service, nozzle plates should be further identified as belonging to a particular impactor assembly, either by embossing the impactor serial number on the underside of each plate (so as not to affect the particle deposition surface), or by an acceptable means of identification, in order to preserve the integrity of the stage mensuration process by linking it to a specific cascade impactor assembly (see *4.1 Stage Mensuration, Measurement Traceability, and Mensuration Interval*).

If an inhalation aerosol or spray is being tested, the stage order is as illustrated for configuration A in *Table 1*, and the cascade impactor is operated at 28.3 L/min for evaluating an inhalation aerosol or spray (see also ⟨601⟩, *Table 4* for critical nozzle dimensions). If an inhalation powder is under evaluation, there are three configurations A, B, and C of *Table 1* for stage ordering, depending upon the target flow rate of 28.3 L/min, 60 L/min, or 90 L/min, respectively (see also ⟨601⟩, *Table 3a* for stage cut-point sizes).

The annular collection plates for the uppermost two stages are interchangeable, as are the solid surfaced plates for the following 6 stages so there is no need to identify each collection plate to belong to a specific impactor. However, during assembly it is important not to mix the order in which the two types of collection plate are assigned to each stage.

Following completion of the collection plate coating procedure (see *5.4 Mitigation of Particle Bounce and Re-Entrainment*), set out the individual stages, each with their collection plate in the order in which they will be assembled (see *Table 1*) on the bench-top. Starting with the base-plate, insert a suitable filter disk into the recess of the filter stage and secure with the 'O'-ring provided. Secure the filter stage on top of the base-plate. Locate the collection plate for the bottom-most size-fractionating stage on the three supports at the periphery of the filter stage, then secure the bottom-most stage nozzle plate to the filter stage. Continue assembling the stack vertically, first positioning the collection plate for the next stage, followed by securing

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the corresponding nozzle plate. Attach the inlet cone to the uppermost nozzle plate if an inhalation aerosol or spray is being tested, or the PS, assembled in accordance with the instructions in ⟨601⟩, if the impactor is being used to assess an inhalation powder, unless justified by data that PS is not necessary. Finally, secure the induction port to the entry of the inlet cone (inhalation aerosol or spray assessment) or PS (inhalation powder assessment) to complete the assembly of the impactor stack.

**Table 1. The Three Configurations of the Andersen Cascade Impactor—Configuration A is Used at 28.3 L/min for the Evaluation of Inhalation Aerosols and Sprays; Configurations A, B, or C are Used for Evaluation of Inhalation Powders Depending Upon the Target Flow Rate (28.3, 60, or 90 L/min, Respectively)**

| Stage Configuration | | |
|---|---|---|
| **A** | **B** | **C** |
| 28.3 L/min | 60 L/min | 90 L/min |
| 0 | –1 | –2 |
| 1 | –0 | –1 |
| 2 | 1 | –0 |
| 3 | 2 | 1 |
| 4 | 3 | 2 |
| 5 | 4 | 3 |
| 6 | 5 | 4 |
| 7 | 6 | 5 |
| Filter | Filter | Filter |

[NOTE—Stage '0' is modified to accept stage '–1' for operation at 60 L/min or stages '–1' and '–2' together for operation at 90 L/min. In either case, the inlet cone is replaced by the modified stage '0' and used above either stage '–1' or stage '–2' depending upon the chosen flow rate.]

### 6.4.2 NEXT GENERATION IMPACTOR

There is only a single configuration of the NGI that can be operated at 15 L/min for the assessment of products for nebulization (see ⟨1601⟩, *Table 2*), at 30 L/min for inhalation aerosols and sprays or at flow rates between 30 and 100 L/min for the assessment of inhalation powders (see ⟨601⟩, ▲*Table 3b*▲ (CN 1-May-2021) for stage cut-point sizes).

There are two sizes of collection cups; the two larger cups are located beneath the collection plate for stage 1 and under the MOC (or internal filter, if used), the six smaller cups are located beneath stages 2–7. Cups of a given size are interchangeable. Although this impactor can be operated without the cup tray, it is normal practice to insert the cups into the tray before each determination, since their order with respect to the stage number is easily preserved after each measurement by removing the tray and cups as a single unit from the bottom frame for transfer to the station where drug recovery takes place. Alternatively, multiple cup-sets may be used with a single cup tray or each cup set can be assigned to a particular cup tray, depending on the number of APSD determinations that need to be made per day, as well as the operating regimen in force within the laboratory. For instance, assigning cup sets to a particular tray is useful if separate operators are responsible for the APSD determination and subsequent drug recovery/assay processes. Nevertheless, it is advisable to mark the base of each cup so they are identified as a single set.

In typical use, following completion of the collection cup coating procedure (see *5.4 Mitigation of Particle Bounce and Re-Entrainment*) and inspection of the seals in the seal body, each cup is located in the appropriate order in the cup tray. The MOC is normally present to capture any particles that penetrate beyond stage 7. However, for some inhaled products (i.e., inhalation aerosols dispersing a plume in which a significant portion of the particles have sizes finer than about 1.0 μm in aerodynamic diameter), the MOC is replaced by either an internal filter and/or a separate external filter assembly which can be inserted between the impactor body and the vacuum source. Next, insert the cup tray into the impactor, such that the cup bases fit into the corresponding spaces of the bottom frame. Close and secure the seal body containing the stages nozzle plates over the cup tray using the external lever located at the front of the impactor. Insert the tapered fitting of the induction port to the entry port located on the uppermost surface of the seal body if an inhalation aerosol or spray is being tested. Alternatively, insert the tapered fitting of the PS, assembled in accordance with the instructions in ⟨601⟩, if the impactor is being used to assess an inhalation powder, unless justified by data that PS is not necessary, and secure the induction port to the entry of the PS to complete the assembly of the impactor.

## 6.5 Mitigation of Air Leakage into the Apparatus

*Equation 4* in *3. Cascade Impactor Operating Principles* illustrates the relationship between impactor stage cut-point sizes and volumetric flow rate of the air through the cascade impactor. Since the source of the air flow through the measurement apparatus is by the creation of a vacuum downstream of the impactor exit and the flow rate is set at the entry to the induction port (see *6.6 Setting of Flow Rate*), it follows that any leakage of ambient air into the sampling train at the time of setting the flow rate for the determination, other than via the intended entry of the induction port will result in an unintended increase in flow rate through stages downstream of the leak. In consequence, such leakage will result in bias of part or all of the APSD towards coarser sizes. Avoidance of air leakage is therefore a paramount requirement that should be verified before every APSD measurement.

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Leakage testing is most conveniently undertaken immediately following assembly (see *6.4 Assertion of Correct Assembly*) by connecting the set-up, including the induction port, PS (if used), and external filter (if used with the NGI), to the vacuum source. This procedure also serves to check the leakage integrity of the entire set-up including tubing and flow control equipment before use. The entry of the induction port is temporarily plugged with an elastomeric bung for the duration of the test. *Figure 5* illustrates the configuration for the ACI without PS, but the external components, if equipped with its PS or for the NGI with/without PS, are similar. A side-connection via a gate valve (G2) is made to a sensitive differential pressure transducer (0–50 kPa and capable of determining a change of ±0.5 kPa at 20.0 kPa) that is located immediately after the exit port from the cascade impactor or the external filter assembly if the NGI is used with this additional equipment. Another gate valve (G1) is located between the side-connection and the variable flow control valve that, in turn, is connected to the vacuum source/pump.

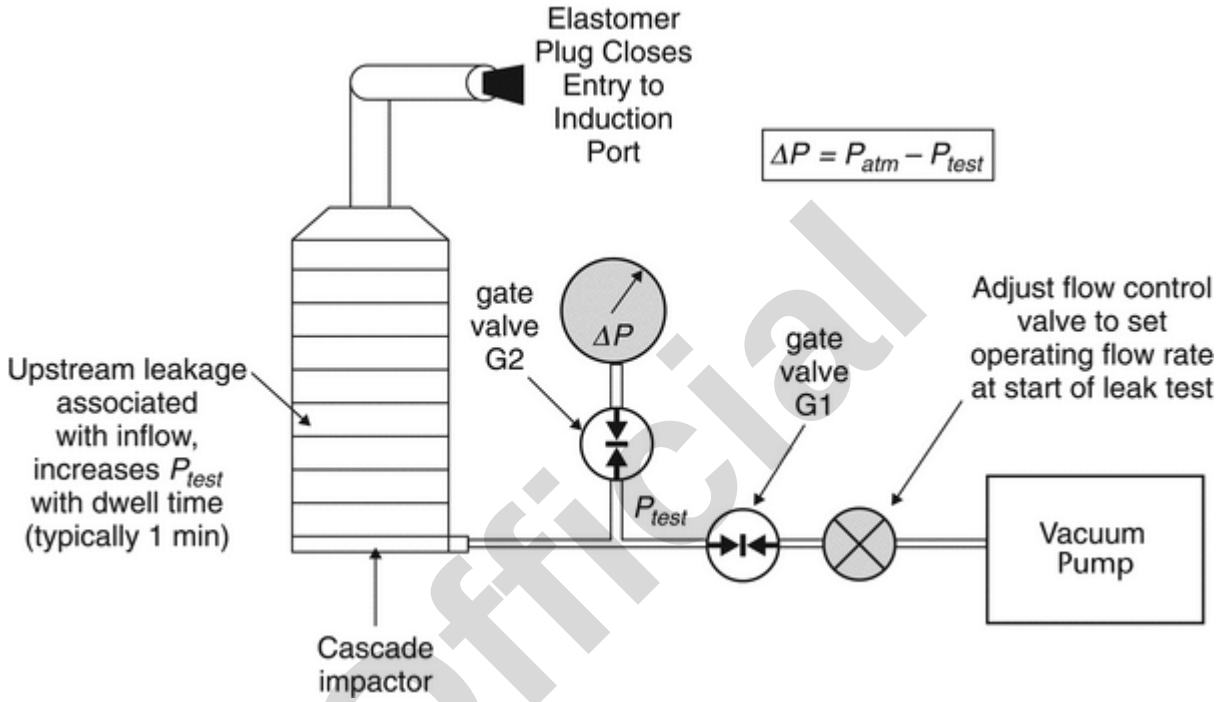


Figure 5. Set-up for leak testing impactor and associated equipment before use—the Andersen Cascade Impactor is shown without its PS, but the set-up is similar if the PS is present or the Next Generation Impactor is substituted.

[NOTE—The system is shown for inhalation aerosols, but the same procedure will work by substituting the appropriate flow control equipment for testing inhalation powders in place of the flow control valve.]

The following procedure is then followed:

1. With the flow control valve closed, the gate valves, G1 and G2, are opened, and the vacuum source is activated;
2. A partial vacuum is applied to the test system by adjusting the flow control valve to set $P_0$ to 20.0 ± 0.5 kPa observing the reading of the output from the differential pressure meter. The differential pressure is measured by connecting the differential pressure transducer at its negative pressure port to the side-connection from the test system;
3. After the required vacuum is displayed on the meter of the pressure gauge, the gate valve, G1, is fully closed to isolate the test apparatus from the vacuum source, and a timer is started;
4. The initial differential pressure ($P_0$) is documented;
5. Allow at least 1 min to elapse, then observe and record the displayed pressure ($P_1$);
6. Carefully remove the rubber bung from the inlet, ensuring not to disturb the apparatus, close the gate valve, G2, and open the gate valve, G1, to isolate the differential pressure meter and to bring the entire test system back to atmospheric pressure conditions, ready for next use;
7. The vacuum source can be deactivated at this stage;
8. The leak rate ($L$) is calculated in accordance with *Equation 5*:

$$L = \frac{P_0 - P_1}{t} \quad [5]$$

$P_0$ = initial differential pressure
$P_1$ = displayed pressure
$t$ = time (min)

Use the leak test specification from the manufacturer. If this is unavailable, then $L$ should be ≤10 kPa/min.

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If *L* exceeds this limit, examine the components of the assembly for leakage(s). Begin by inspecting the seals associated with the stages, then the connections from the induction port to PS (if used) or to cascade impactor, and finally from the impactor to the vacuum source. Replace defective components as necessary and reassemble. Repeat the leak test above until *L* is lower than the specified limit.

## 6.6 Setting of Flow Rate

In *3. Cascade Impactor Operating Principles*, *Equation 4* links the stage cut-point sizes of the cascade impactor to the air volumetric flow rate through the apparatus. It follows that the accuracy of a measured APSD is critically dependent upon the flow rate setting. The flowmeters supplied by some impactor manufacturers that are located between the impactor exit and the vacuum source should be treated as flow indicators and NOT used as a means of setting flow rate.

The volumetric flow rate is set at the entry of the induction port, as illustrated in *Figure 6*.

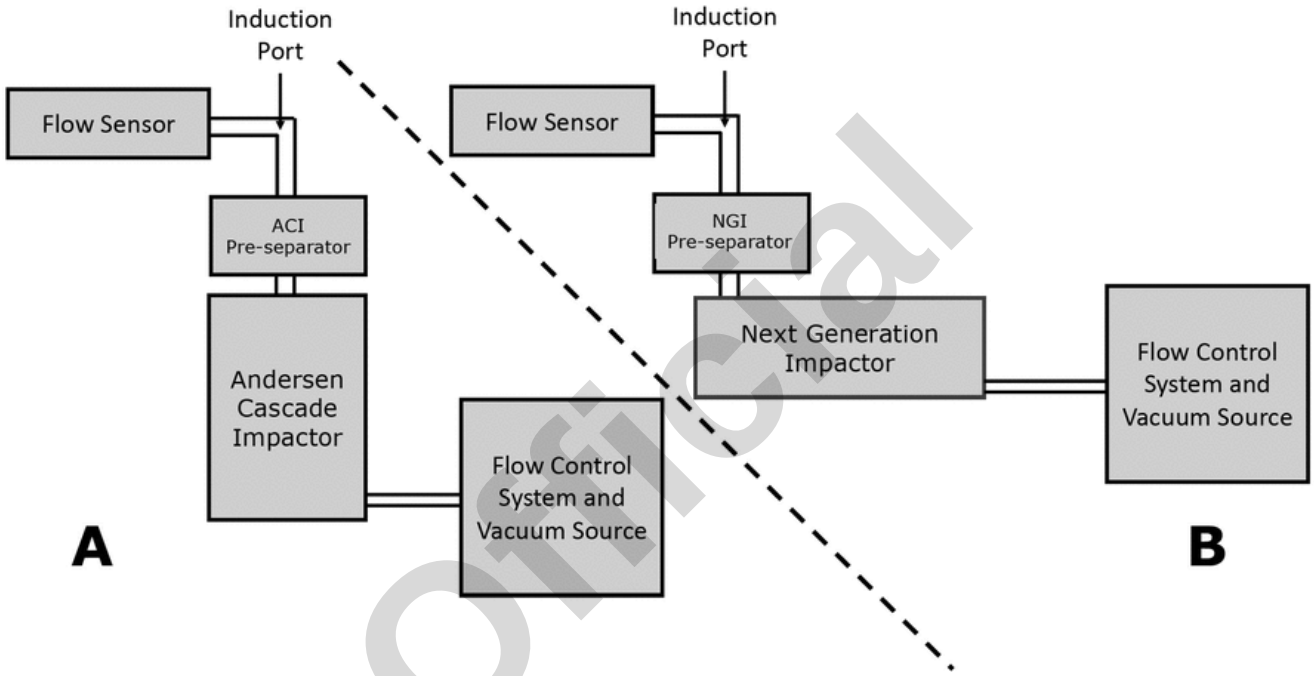


Figure 6. Flow rate setting for (A) Andersen Cascade Impactor with PS, (B) Next Generation Impactor with PS; the flow sensor location at the entry to the induction port remains the same if the PS is not used.

Set the volumetric flow rate to the target value for the inhaler being tested immediately before making the APSD determination. Note that a low flow resistance mass flowmeter may be used to make this measurement, as the air pressure at the point of flow measurement is close to room ambient.

## 6.7 Cleaning and Storage Between Uses

Although cascade impactors and their related equipment are constructed from durable materials, careful storage between uses will prolong the life of vulnerable components, such as seal rings and collection plates/cups. A regimen should be established that ensures that all components are cleaned periodically, especially the nozzle plates (ACI) and the inter-stage passageways of the seal body (NGI). The frequency of cleaning will depend upon the chemical properties of the formulations being assessed as well as the drug recovery solvent(s).

If cascade impactor equipment is not in continuous use, it should be stored at room ambient temperature, in a dry environment, and protected from airborne contaminants. The collection plates/cups should be stored separately from the impactors and also protected from airborne contaminants.

## 6.8 Assertion that Individual Cascade Impactor Assemblies of the Same Type (i.e., Andersen Cascade Impactor or Next Generation Impactor) are Interchangeable

In many laboratories, there are likely to be many cascade impactor assembles of the same type in service. There may therefore be a desire to interchange different impactors when making APSD performance measurements on the same inhaler product in order to speed up the process. Provided that stage mensuration (see *4.1 Stage Mensuration, Measurement Traceability, and Mensuration Interval*) has demonstrated that $W_{eff}$, determined as the representative measure of the diameter of the nozzles of each stage is within the manufacturer's specification, as provided in ⟨601⟩, *Table 4* (ACI) and ⟨601⟩, ▲*Table 6*▲ (CN 1-May-2021) (NGI), and checks have been made to ensure that seals are intact (see *6.2 Inspection of Cascade Impactor Components Susceptible to Deterioration*), and other considerations as discussed in *4. Apparatus Maintenance*. The impactors may be interchangeable.

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However, it is advisable to perform comparative testing to validate equivalence. Nevertheless, it is recommended that the stages together with their collection surfaces are uniquely identified to a particular cascade impactor assembly for the purpose of traceability as to the source for each APSD determination.

A similar consideration applies to the PS used with the NGI, because there is a specification for the nozzle diameters in ⟨601⟩, *Table 6*. The situation is less clear for the PS for the ACI, although a manufacturer-supplied specification for the diameter of the three exit tubes, where available, would fulfill the same purpose as the PS specification for the NGI PS.

The specifications for the critical dimensions (entry and exit tube internal diameters) of the induction port are provided in ⟨601⟩, ▲*Figure 6b*▲ (CN 1-May-2021) and should be used to check for the interchangeability of this component, where multiple induction ports are in service.

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# ▲⟨1604⟩ PRESENTATION OF AERODYNAMIC PARTICLE SIZE DISTRIBUTION (APSD) MEASUREMENT DATA FOR ORALLY INHALED PRODUCTS

## INTRODUCTION

Aerodynamic particle size distribution (APSD) is a critical quality attribute of all orally inhaled drug products (OIP). Characterization of the delivered mass of drug from the inhaler is important to assess product safety and efficacy. This mass includes both (and is the sum of) the sized and non-sized fraction emitted from the patient interface (e.g., the inhaler mouthpiece) (see *Figure 1*).

The delivered mass excludes drug product deposited on the mouthpiece and is consistent with the target delivered dose as specified in *Inhalation and Nasal Drug Products: Aerosols, Sprays, and Powders—Performance Quality Tests* ⟨601⟩.

Although nasal drug products can produce particles whose size can be measured by multistage cascade impactors (CIs), almost all the emitted particulates of a well-designed nasal spray drug product are larger than the normal operating size ranges for CIs (see ⟨601⟩).

For determination of aerodynamic particle size distribution, the number of actuations should be minimized but sufficient to allow quantification of drug deposited on the stage with lowest deposition without overloading the stage with highest deposition. For details on performing determination of aerodynamic particle size distribution, see ⟨601⟩.

[NOTE—All figures in this chapter are for illustrative purposes.]

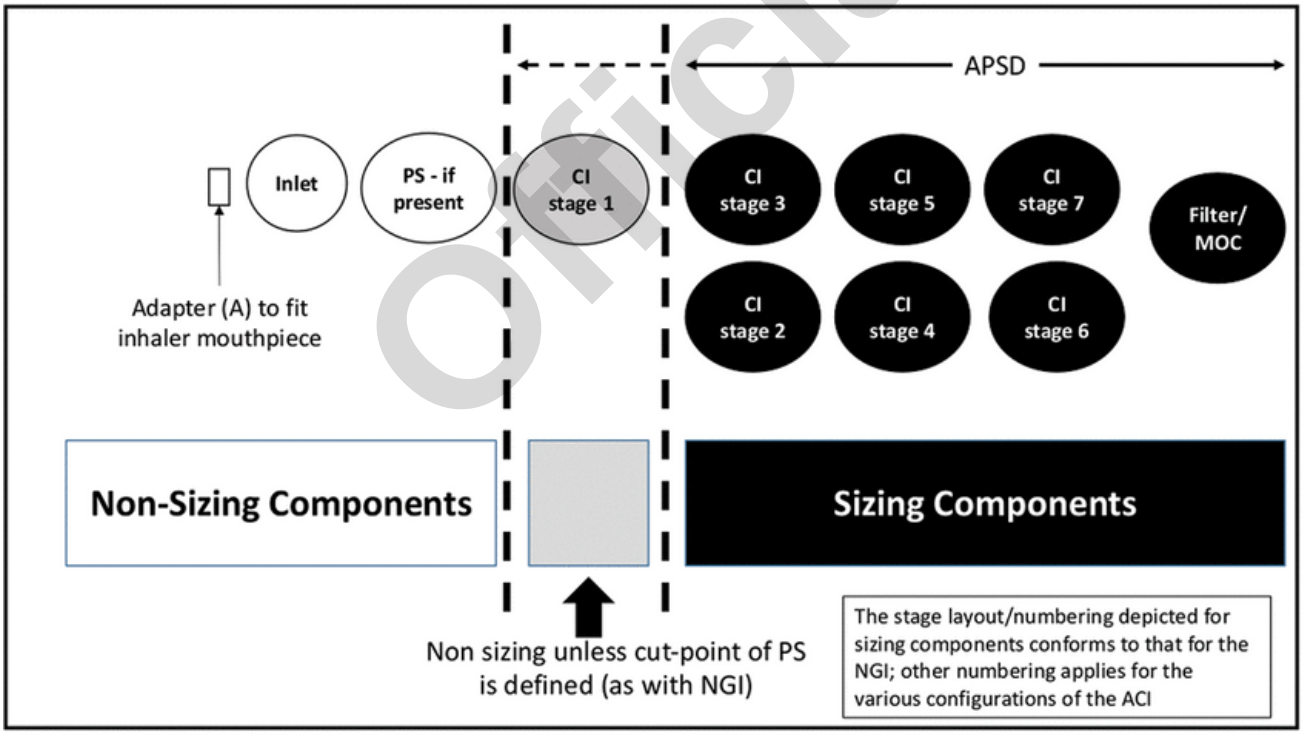


Figure 1. CI sizing and non-sizing components of the sampling apparatus used for determination of APSD for OIPs.

[NOTE—A filter may replace the micro-orifice collector (MOC), which is used with the Next Generation Impactor (NGI), or may be used in addition to the MOC.]

It is not the intention of this chapter to provide guidance on the assessment of comparability of APSDs.

This chapter presents two pharmacopeial approaches that may be used to evaluate CI analysis data, collected as per ⟨601⟩ (i.e., at a single flow rate):

- Representation of the deposition profile for the entire delivered mass of drug product per actuation, sampled as an aerosol from the inhaler mouthpiece (combination of sized and non-sized particles)
- Assessment of the deposition profile of the delivered mass of drug product per actuation from the inhaler mouthpiece by grouping CI stages

Optional uses for APSD data, which are developmental in nature, include:

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- Representation of the cumulative APSD for the portion of the emitted aerosol sized aerodynamically and determination of associated moments [e.g., mass median aerodynamic diameter (MMAD) and, where appropriate, geometric standard deviation (GSD)] (*1*)
- Assessment of the APSD in terms of pertinent sub-fractions (e.g., when comparing APSD data collected at different flow rates)

The approach used depends on the question being addressed. It might be appropriate to use more than one approach.

### Choice of CI and Sampling Flow Rate for the APSD Measurement

The effective cutoff diameters that define the aerodynamic diameters associated with the sizing components at the CI operational flow rate differ between impactor types [e.g., NGI versus the Andersen Cascade Impactor (ACI)]. The same impactor must be used for stage grouping comparisons; comparison of data for different impactors of the same type can be established through a cumulative distribution curve or sized fractions. Furthermore, comparative measurements should normally be undertaken at the same flow rate unless the intent is to investigate flow rate-dependent changes to the APSD. However, when inhalers with different flow resistances are compared, measurements should be made at the same pressure differential, which will require operating at different flow rates. For some passive inhalation systems [e.g., breath-actuated dry powder inhalers (DPIs)], it is difficult to make meaningful comparisons of aerosol metrics such as fine particle dose (FPD), fine particle fraction (FPF), or MMAD between measurements collected on an NGI versus those collected using an ACI due to the differences in the two impactors' internal volumes. The difference in internal volume alters the initial acceleration and aerosolization of particles emitted from the inhaler early in the profile before the peak flow rate is achieved. Additionally, the NGI and ACI differ because of different stage effective cutoff diameters (ECDs), further prohibiting a direct comparison between the two impactors. [See sections on the ACI or NGI with pre-separator (PS) in ⟨601⟩, *C. Aerodynamic Particle/Droplet Size Distribution—Inhalation Aerosols, Inhalation Sprays, Inhalation Powders, Nasal Aerosols, and Nasal Powders* and sections on Apparatus B in ⟨601⟩, *A. Delivered Dose Uniformity*.]

## APPROACHES FOR PHARMACOPEIAL APSD DATA PRESENTATION

### System Suitability

As part of the CI system suitability verifications during standardized quality control testing, it is required that the mass balance (delivered amount from the inhaler through the impactor as a percentage of the target delivered label claim) for each determination is within 85%–115% (see ⟨601⟩). However, where losses due to internal non-sizing components (also commonly referred to as *wall losses*) are known to be ≤5% of the total delivered drug mass into the impactor, the procedure may be simplified by assaying only drug on the collection plates. [NOTE—For testing nonstandard flow rates, the method must be validated.]

### Deposition Profile

From the analysis of the solutions recovered from the entire apparatus including the patient interface, induction port, pre-separator if used, and all stage collection surfaces of the CI including the back-up filter or MOC (NGI only), calculate the mass of drug substance per actuation deposited on each of the components of the apparatus. Do not include the mass of the drug substance recovered from the interior walls of the CI, as the aerodynamic particle size of such deposits is undefined. The mass of drug substance recovered from the internal walls may, however, be used in calculations to estimate the total mass of drug substance recovered from the apparatus (mass balance; see *System Suitability*).

Construct a graph with the mass of drug substance per actuation as the ordinate (*y*) and the collection site within the sampling train as the abscissa (*x*) (see *Figure 2*).

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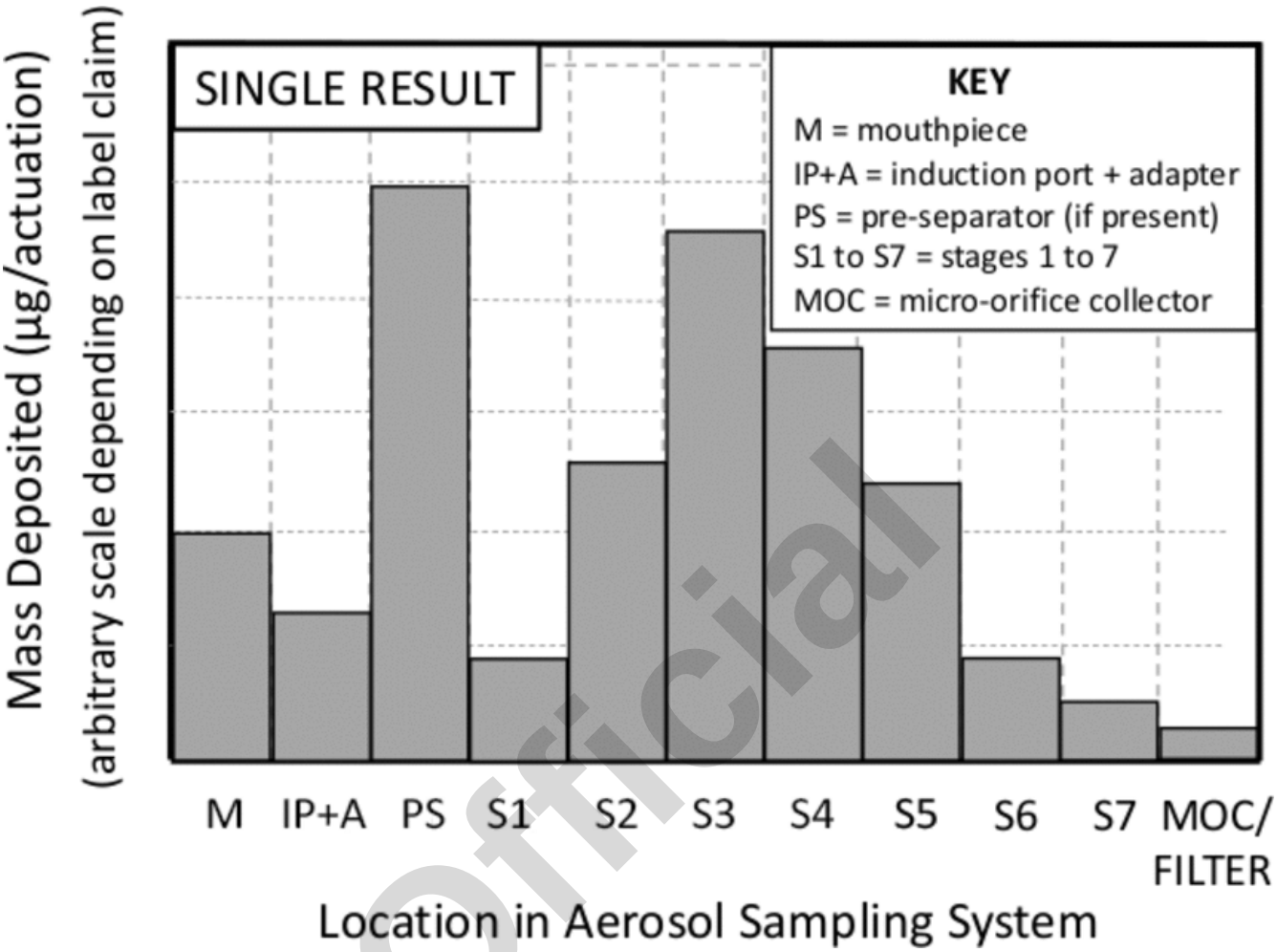


Figure 2. CI deposition profile (NGI with PS as example) for a single measurement.

## MULTIPLE DETERMINATIONS

In certain situations, it may be appropriate to compare the mean of several CI determinations. To do so repeat the data analysis procedure for the number of multiple determinations (i.e., *X*-replicate measurements), by calculating the mean mass of drug substance per actuation for each component together with its standard deviation (SD). Construct a graph with the average mass of drug substance per actuation as the ordinate and the collection site within the sampling apparatus as the abscissa; moreover, error bars may be added, typically representing ±1 SD (see *Figure 3*). It is also recommended that when presenting data based on multiple determinations that the sample size be noted (i.e., N = *X*, where *X* is the number of samples). Additionally, for each component, individual data can be grouped as shown in *Figure 4*.

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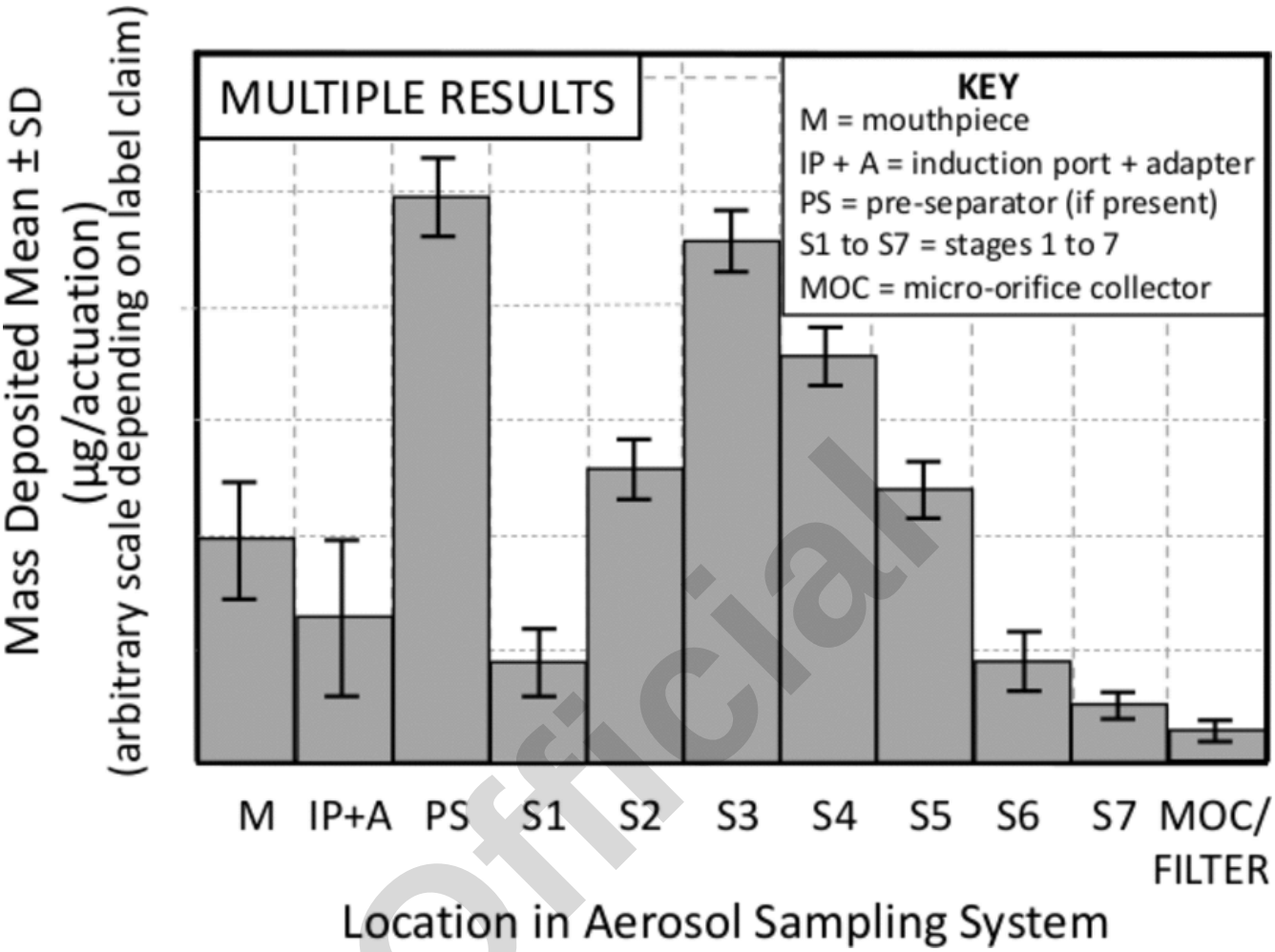


Figure 3. CI deposition profile (NGI with PS) for multiple determinations based on a sample size of *X*. Error bars represent +/ −1 SD.

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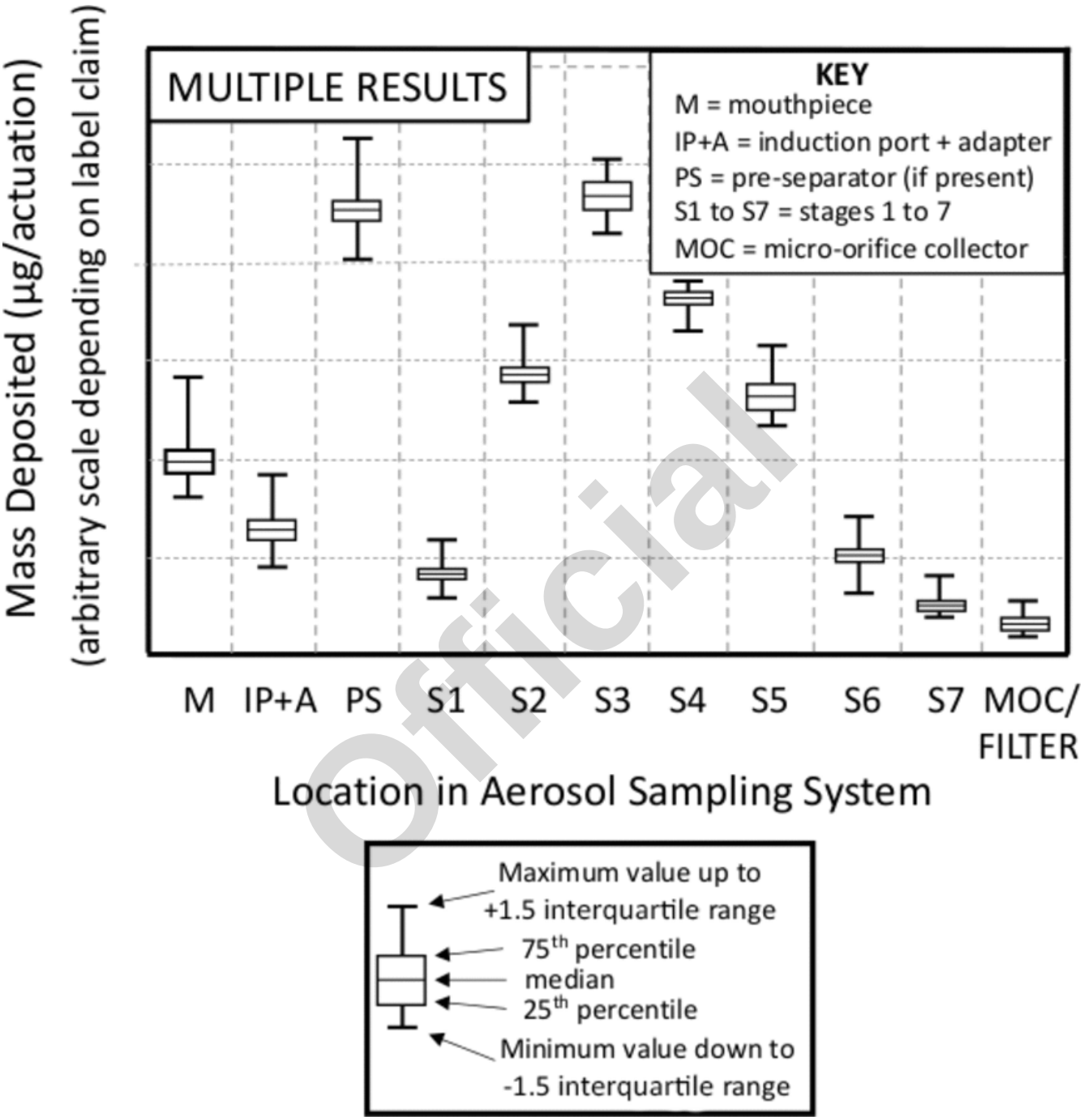


Figure 4. CI deposition profile (NGI with PS) for multiple determinations based on a sample size of *X*.

See ⟨601⟩ for more details regarding multiple determinations.

## The APSD: Sized Deposition Profile

The sized deposition profile (SDP) is a representation of the mass of drug substance per actuation versus particle size, expressed in terms of aerodynamic diameter (*Figure 5*). [NOTE—Some distributions may not conform to the illustration in *Figure 5*, which is specific to an NGI.] The SD associated with each measurement may also be presented. Only the mass collected on each of the sizing components of the apparatus contributes. Hence, the mass recovered from the initial stage of either the ACI or the NGI when a PS is not present does not contribute to the SDP because there is no maximum size associated with either component. In contrast, when the PS is used with the NGI, mass on the initial stage 1 (S1) does contribute to the SDP because the effective cutoff diameter of the PS is defined through the archival calibration of this apparatus.

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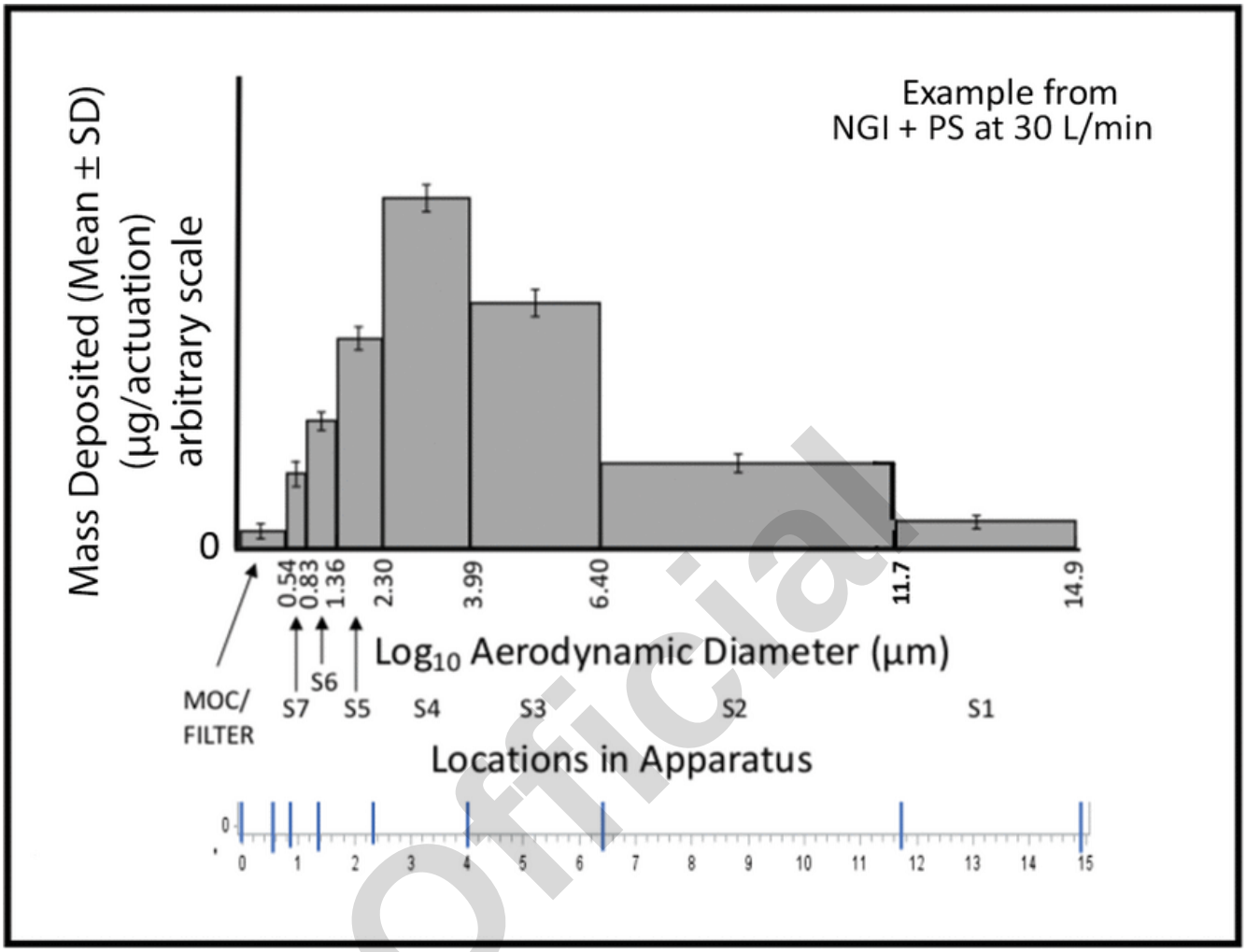


Figure 5. Differential mass-weighted APSD profile (NGI with PS at 30 L/min) based on a sample size of N = *X*.

## Stage Grouping of the Deposition Data

For quality control purposes, the mass deposition data from both non-sizing and sizing components of the sampling apparatus can be consolidated into nonoverlapping groups to summarize the distribution of the mass of drug substance emitted from the inhaler. An illustration of this concept is presented in *Figure 6*. Other groupings are possible and the user should therefore seek guidance on this matter from the appropriate regulatory agency. In the illustration provided, the groups have been defined in terms of four relative categories:

- Group 1: Mass of drug substance recovered from non-sizing components
- Group 2: Mass of drug substance recovered from stages that might be taken as representing the “coarse” particle fraction of the mass
- Group 3: Mass of drug substance recovered from stages that might be taken as representing the “fine” particle fraction of the mass
- Group 4: Mass of drug substance recovered from stages that might be taken as representing the “extra-fine” particle fraction of the mass

The words "coarse", "fine", and "extra-fine" are intended to illustrate sizes only for discussion in this chapter.

Discussion and agreement with the relevant regulatory authority regarding the definition of the boundary sizes of each of the groupings and the related specification setting is strongly encouraged.

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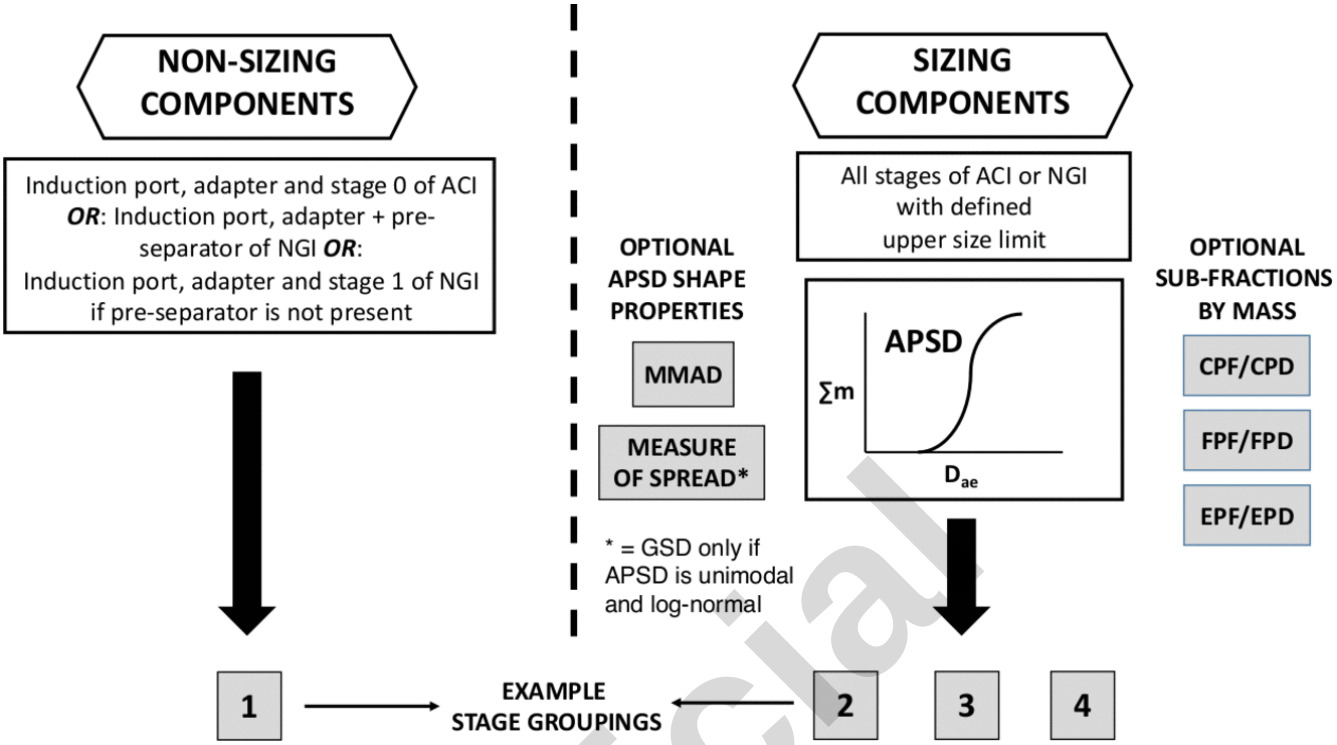


Figure 6. The left side shows the contribution from the non-sizing components of the sampling train to example stage group 1 together with the contributions from the sizing components to example stage groups 2, 3, and 4; the cumulative mass distribution and derived metrics are shown on the right side. CPF: "coarse" particle fraction; CPD: "coarse" particle dose (mass); FPF: "fine" particle fraction; FPD: "fine" particle dose (mass); EPF: "extra-fine" particle fraction; EPD: "extra-fine" particle dose (mass). If the size distribution is unimodal and log-normal, it is optionally permissible to calculate GSD as a measure of spread in addition to MMAD.

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## FURTHER TREATMENTS OF APSD DATA

### The APSD: Cumulative Distribution Profile

The cumulative distribution profile (CDP) is a representation of the cumulative mass of the drug substance less than or equal to the stage cutoff diameter versus the stage cutoff diameter.

[NOTE—For comparative purposes, the use of percent CDP alone would be misleading in absence of comparable impactor sized mass (ISM).]

The CDP is commonly expressed as a percentage of the ISM. The sum of the mass collected starting from the first stage, where the upper bound size is defined by virtue of the cutoff diameter of the stage immediately preceding, or of the PS in the case of the NGI, and all stages including the back-up filter or MOC. Starting at the final collection site (filter or MOC), derive a table of cumulative mass versus cutoff diameter of the respective stage. *Figure 7* shows how such data can be plotted.

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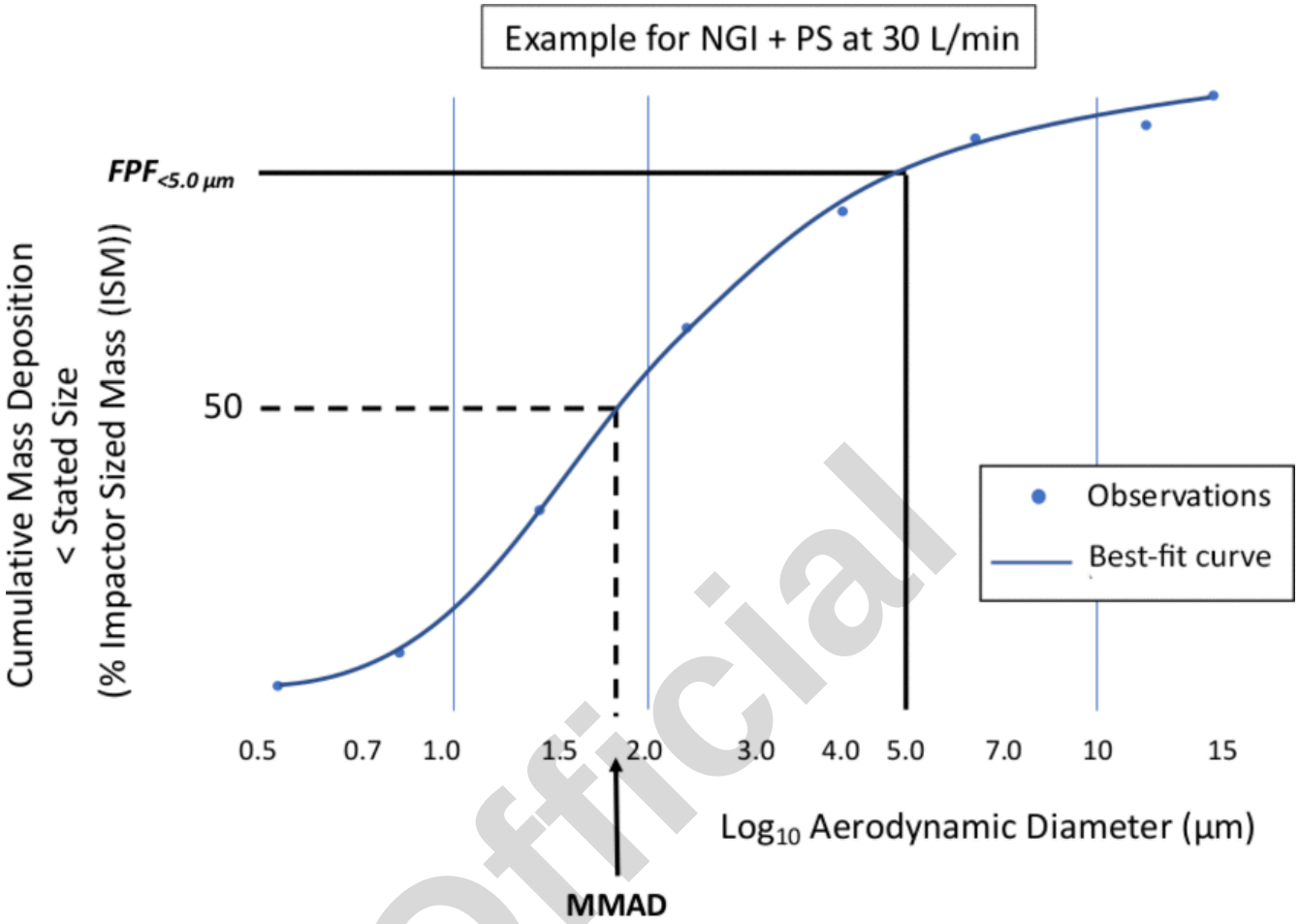


Figure 7. Curve fitting of cumulative mass-weighted deposition data to generate APSD as a CDP.

[**CAUTION**—Investigation of the APSD in isolation from the non-sized mass fraction of the delivered dose per actuation from the inhaler would likely not provide adequate information on the quality and consistency of a drug product or its batches.]

[NOTE—For further guidance, refer to *Table 1A*, *Table 1B*, and *Table 1C*, representing the configurations of the ACI used at 28.3, 60, and 90 L/min, respectively (see ⟨601⟩); or *Table 2A* (without PS) and *Table 2B* (with PS), representing the configuration of the NGI used at flow rate (*Q*), which can be any flow rate between 30 and 100 L/min for the evaluation of inhalation aerosols, sprays, and powders, or *Table 2C* for a flow rate of 15 L/min; or *Table 2A* (without PS) and *Table 2B* (with PS), representing the configuration of the NGI used at flow rate (*Q*), which can be 15 L/min or any flow rate between 30 and 100 L/min.]

**Table 1A. Calculations for the ACI Apparatus When Used at a Flow Rate of 28.3 L/min**

| Cutoff Diameter (µm) | Mass of Drug Substance Deposited per Discharge | Cumulative Mass of Drug Substance Deposited per Discharge | Cumulative Fraction of Drug Substance (%) |
|---|---|---|---|
| $d_7 = 0.4$ | mass from back-up filter, $m_f$ | $c_7 = m_f$ | $f_7 = (c_7/c_0) \times 100$ |
| $d_6 = 0.7$ | mass from stage 7, $m_7$ | $c_6 = c_7 + m_7$ | $f_6 = (c_6/c_0) \times 100$ |
| $d_5 = 1.1$ | mass from stage 6, $m_6$ | $c_5 = c_6 + m_6$ | $f_5 = (c_5/c_0) \times 100$ |
| $d_4 = 2.1$ | mass from stage 5, $m_5$ | $c_4 = c_5 + m_5$ | $f_4 = (c_4/c_0) \times 100$ |
| $d_3 = 3.3$ | mass from stage 4, $m_4$ | $c_3 = c_4 + m_4$ | $f_3 = (c_3/c_0) \times 100$ |
| $d_2 = 4.7$ | mass from stage 3, $m_3$ | $c_2 = c_3 + m_3$ | $f_2 = (c_2/c_0) \times 100$ |
| $d_1 = 5.8$ | mass from stage 2, $m_2$ | $c_1 = c_2 + m_2$ | $f_1 = (c_1/c_0) \times 100$ |
| $d_0 = 9.0$ | mass from stage 1, $m_1$ | $c_0 = c_1 + m_1$ | $f_0 = (c_0/c_0) = 100$ |
| Undefined[a] | mass from stage 0, $m_0$ | *assign to non-sized mass* | — |

[a] There is no component to set the upper size limit. The 100th percentile in the last column represents the total mass that enters the impactor. While this size range is not defined, it is a fundamental component for the calculations of the APSD.

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**Table 1B. Calculations for the ACI Apparatus When Used at a Flow Rate of 60 L/min**

| Cutoff Diameter (µm) | Mass of Drug Substance Deposited per Discharge | Cumulative Mass of Drug Substance Deposited per Discharge | Cumulative Fraction of Drug Substance (%) |
|---|---|---|---|
| $d_6 = 0.26$ | mass from back-up filter, $m_f$ | $c_6 = m_f$ | $f_6 = (c_6/c_{-1}) \times 100$ |
| $d_5 = 0.55$ | mass from stage 6, $m_6$ | $c_5 = c_6 + m_6$ | $f_5 = (c_5/c_{-1}) \times 100$ |
| $d_4 = 1.2$ | mass from stage 5, $m_5$ | $c_4 = c_5 + m_5$ | $f_4 = (c_4/c_{-1}) \times 100$ |
| $d_3 = 1.9$ | mass from stage 4, $m_4$ | $c_3 = c_4 + m_4$ | $f_3 = (c_3/c_{-1}) \times 100$ |
| $d_2 = 3.2$ | mass from stage 3, $m_3$ | $c_2 = c_3 + m_3$ | $f_2 = (c_2/c_{-1}) \times 100$ |
| $d_1 = 4.4$ | mass from stage 2, $m_2$ | $c_1 = c_2 + m_2$ | $f_1 = (c_1/c_{-1}) \times 100$ |
| $d_{-0} = 6.5$ | mass from stage 1, $m_1$ | $c_0 = c_1 + m_1$ | $f_0 = (c_0/c_{-1}) \times 100$ |
| $d_{-1} = 8.6$ | mass from stage –0, $m_{-0}$ | $c_{-1} = c_0 + m_0$ | $f_{-1} = (c_{-1}/c_{-1}) = 100$ |
| Undefined[a] | mass from stage –1, $m_{-1}$ | *assign to non-sized mass* | — |

[a] There is no calibrated component located before the first impaction stage to set the upper size limit. The 100th percentile in the last column represents the total mass that enters the impactor. While this size range is not defined, it is a fundamental component for the calculations of the APSD.

**Table 1C. Calculations for the ACI Apparatus When Used at a Flow Rate of 90 L/min**

| Cutoff Diameter (µm) | Mass of Drug Substance Deposited per Discharge | Cumulative Mass of Drug Substance Deposited per Discharge | Cumulative Fraction of Drug Substance (%) |
|---|---|---|---|
| $d_5 = 0.22$ | mass from back-up filter, $m_f$ | $c_5 = m_f$ | $f_5 = (c_5/c_{-2}) \times 100$ |
| $d_4 = 1.0$ | mass from stage 5, $m_5$ | $c_4 = c_5 + m_5$ | $f_4 = (c_4/c_{-2}) \times 100$ |
| $d_3 = 1.7$ | mass from stage 4, $m_4$ | $c_3 = c_4 + m_4$ | $f_3 = (c_3/c_{-2}) \times 100$ |
| $d_2 = 2.6$ | mass from stage 3, $m_3$ | $c_2 = c_3 + m_3$ | $f_2 = (c_2/c_{-2}) \times 100$ |
| $d_1 = 3.5$ | mass from stage 2, $m_2$ | $c_1 = c_2 + m_2$ | $f_1 = (c_1/c_{-2}) \times 100$ |
| $d_{-0} = 5.2$ | mass from stage 1, $m_1$ | $c_{-0} = c_1 + m_1$ | $f_{-0} = (c_{-0}/c_{-2}) \times 100$ |
| $d_{-1} = 6.5$ | mass from stage –0, $m_{-0}$ | $c_{-1} = c_{-0} + m_{-0}$ | $f_{-1} = (c_{-1}/c_{-2}) \times 100$ |
| $d_{-2} = 8.0$ | mass from stage –1, $m_{-1}$ | $c_{-2} = c_{-1} + m_{-1}$ | $f_{-2} = (c_{-2}/c_{-2}) = 100$ |
| Undefined[a] | mass from stage –2, $m_{-2}$ | *assign to non-sized mass* | — |

[a] There is no calibrated component located before the first impaction stage to set the upper size limit. The 100th percentile in the last column represents the total mass that enters the impactor. While this size range is not defined, it is a fundamental component for the calculations of the APSD.

**Table 2A. Calculations for NGI Apparatus without Pre-Separator When Operated at Flow Rates from 30–100 L/min[a]**

| Cutoff Diameter (µm) | $x$ | Mass of Drug Substance Deposited per Discharge | Cumulative Mass of Drug Substance Deposited per Discharge | Cumulative Fraction of Drug Substance (%) |
|---|---|---|---|---|
| $d_7 = 0.34 \times q$ | 0.67 | mass from MOC or terminal filter, $m_8$ | $c_7 = m_8$ | $f_7 = (c_7/c_1) \times 100$ |
| $d_6 = 0.55 \times q$ | 0.60 | mass from stage 7, $m_7$ | $c_6 = c_7 + m_7$ | $f_6 = (c_6/c_1) \times 100$ |
| $d_5 = 0.94 \times q$ | 0.53 | mass from stage 6, $m_6$ | $c_5 = c_6 + m_6$ | $f_5 = (c_5/c_1) \times 100$ |
| $d_4 = 1.66 \times q$ | 0.47 | mass from stage 5, $m_5$ | $c_4 = c_5 + m_5$ | $f_4 = (c_4/c_1) \times 100$ |
| $d_3 = 2.82 \times q$ | 0.50 | mass from stage 4, $m_4$ | $c_3 = c_4 + m_4$ | $f_3 = (c_3/c_1) \times 100$ |
| $d_2 = 4.46 \times q$ | 0.52 | mass from stage 3, $m_3$ | $c_2 = c_3 + m_3$ | $f_2 = (c_2/c_1) \times 100$ |
| $d_1 = 8.06 \times q$ | 0.54 | mass from stage 2, $m_2$ | $c_1 = c_2 + m_2$ | $f_1 = (c_1/c_1) = 100$ |
| Undefined[b] | — | mass from stage 1, $m_1$ | *assign to non-sized mass* | — |

[a] Use $q = (60/Q)^x$, where $Q$ is the test flow rate in L/min, and $x$ is listed in the table.
[b] There is no calibrated component before the first impaction stage to set the upper size limit. The 100th percentile in the last column represents the total mass that enters the impactor. While this size range is not defined, it is a fundamental component for the calculations of the APSD.

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**Table 2B. Calculations for NGI Apparatus with Pre-Separator When Operated at Flow Rates From 30–100 L/min[a]**

| Cutoff Diameter (μm) | $x$ | Mass of Drug Substance Deposited per Discharge | Cumulative Mass of Drug Substance Deposited per Discharge | Cumulative Fraction of Drug Substance (%) |
|---|---|---|---|---|
| $d_7 = 0.34 \times q$ | 0.67 | mass from MOC or terminal filter, $m_8$ | $c_8 = m_8$ | $f_8 = (c_8/c_1) \times 100$ |
| $d_6 = 0.55 \times q$ | 0.60 | mass from stage 7, $m_7$ | $c_7 = c_8 + m_7$ | $f_7 = (c_7/c_1) \times 100$ |
| $d_5 = 0.94 \times q$ | 0.53 | mass from stage 6, $m_6$ | $c_6 = c_7 + m_6$ | $f_6 = (c_6/c_1) \times 100$ |
| $d_4 = 1.66 \times q$ | 0.47 | mass from stage 5, $m_5$ | $c_5 = c_6 + m_5$ | $f_5 = (c_5/c_1) \times 100$ |
| $d_3 = 2.82 \times q$ | 0.50 | mass from stage 4, $m_4$ | $c_4 = c_5 + m_4$ | $f_4 = (c_4/c_1) \times 100$ |
| $d_2 = 4.46 \times q$ | 0.52 | mass from stage 3, $m_3$ | $c_3 = c_4 + m_3$ | $f_3 = (c_3/c_1) \times 100$ |
| $d_1 = 8.06 \times q$ | 0.54 | mass from stage 2, $m_2$ | $c_2 = c_3 + m_2$ | $f_2 = (c_2/c_1) \times 100$ |
| $d_0 = 12.8–0.07 \times (Q - 60)$ | — | mass from stage 1, $m_1$ | $c_1 = c_2 + m_1$ | $f_1 = (c_1/c_1) = 100$ |
| Undefined[b] | — | mass from pre-separator, $m_p$ | *assign to non-sized mass* | — |

[a] Use $q = (60/Q)^x$, where $Q$ is the test flow rate in L/min, and $x$ is listed in the table.
[b] There is no calibrated component before the pre-separator to set the upper size limit. The 100th percentile in the last column represents the total mass that enters the pre-separator and impactor. While this size range is not defined, it is a fundamental component for the calculations of the APSD.

**Table 2C. Calculations for NGI Apparatus without Pre-Separator When Operated at a Test Flow Rate of 15 L/min**

| Cutoff Diameter (μm) | Mass of Drug Substance Deposited per Discharge | Cumulative Mass of Drug Substance Deposited per Discharge | Cumulative Fraction of Drug Substance (%) |
|---|---|---|---|
| $d_7 = 0.98$ | mass from terminal filter, $m_8$ | $c_7 = m_8$ | $f_7 = (c_7/c_1) \times 100$ |
| $d_6 = 1.36$ | mass from stage 7, $m_7$ | $c_6 = c_7 + m_7$ | $f_6 = (c_6/c_1) \times 100$ |
| $d_5 = 2.08$ | mass from stage 6, $m_6$ | $c_5 = c_6 + m_6$ | $f_5 = (c_5/c_1) \times 100$ |
| $d_4 = 3.30$ | mass from stage 5, $m_5$ | $c_4 = c_5 + m_5$ | $f_4 = (c_4/c_1) \times 100$ |
| $d_3 = 5.39$ | mass from stage 4, $m_4$ | $c_3 = c_4 + m_4$ | $f_3 = (c_3/c_1) \times 100$ |
| $d_2 = 8.61$ | mass from stage 3, $m_3$ | $c_2 = c_3 + m_3$ | $f_2 = (c_2/c_1) \times 100$ |
| $d_1 = 14.1$ | mass from stage 2, $m_2$ | $c_1 = c_2 + m_2$ | $f_1 = (c_1/c_1) = 100$ |
| Undefined[a] | mass from stage 1, $m_1$ | *assign to non-sized mass* | — |

[a] There is no calibrated component before the first impaction stage to set the upper size limit. The 100th percentile in the last column represents the total mass that enters the impactor. While this size range is not defined, it is a fundamental component for the calculation of the APSD.

## Sized Fractions

The total mass of particles in a particular size range is sometimes of interest, for example, when investigating flow dependence or when comparing inhalation powders (DPIs) with different flow resistances. This amount can be expressed in micrograms or milligrams depending on the product (e.g., FPD) and in percentage (e.g., FPF). The normalizing factor can either be labeled delivered dose (*or* target delivered dose) by the CI or ISM, depending on purpose. For example, FPD may be considered to be <5 μm aerodynamic diameter and, similarly, extra fine particle dose (EPD) may be considered to be <2 μm aerodynamic diameter because these parameters may be used for quality control purposes in regions outside the United States and may also be believed to correlate to clinical responses.

The $FPD_{<X\mu m}$, where $X$ is an aerodynamic diameter within the range of the cutoff diameters for the impactor in use at the relevant flow rate, can be estimated from the raw distribution in several ways. The most straight-forward approach is to use linear interpolation of the CDP between stages with cutoff diameters adjacent to $X$. Consider, for example, the estimation of FPD for data from an NGI (with pre-separator) with a test flow of 60 L/min (see *Table 2B*). $FPD_{<5\mu m}$ is then calculated as:

$$\frac{\text{FPD} < 5\mu m - F_3}{F_2 - F_3} = \frac{5 - 4.46}{8.06 - 4.46}$$

where $F_2$ and $F_3$ are the values of the cumulative dose up to stage 2 and 3, respectively, which simplifies to:

$$FPD_{<5\mu m} = F_3 + \left(F_2 - F_3\right)\frac{0.54}{3.60} = 0.15F_2 + 0.85F_3$$

Other sized fractions, also at other flow rates, can be determined in the corresponding manner. For best results, the CDP should be approximately linear in the region for the estimation. If this is not the case, transformation of the CDP and/or the cutoff diameters can be considered. An alternate approach is to fit a growth curve (*F*) to the CDP (microgram scale), so FPD =

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*F*(*X*). The advantage with this approach is that all data, not only the data for the stages with cutoff diameters adjacent to *X*, are directly influencing the estimation.

Relevant to, and associated with, the sized fractions is a measure of the central point of the APSD: the MMAD. The USP growth curve method for calculating MMAD (*2*) assumes that the distribution of the CDP raw data has the same form as a log-normally distributed random variable. The pharmacopeial method does not provide an alternative approach when the distribution fails to meet this criterion. If there are significant deviations from log-normality, performing a linear regression over the entire data set could result in considerable bias depending on where and by how much such deviations from log normality occur.

Published data show that for many, if not most, OIPs the CDP does not follow a log-normal distribution; however, several other growth functions, which are not based on an underlying log-normal distribution have been shown suitable for fitting the CDP of OIPs (*2*). As an example, *Figure 7* shows a Morgan-Mercer-Flodin growth curve fit to the CDP in which the median (i.e., 50$^{th}$ percentile) particle size was estimated to be approximately 1.9 μm in aerodynamic diameter. Other percentiles could also be considered. In general, modeling practices should follow the principles outlined in *Analysis of Biological Assays* ⟨1034⟩.

## GLOSSARY

**A:** Adapter
**ACI:** Andersen cascade impactor
**APSD:** Aerodynamic particle size distribution
**CDP:** Cumulative distribution profile
**CI:** Cascade impactor
**CPD:** Coarse particle dose
**CPF:** Coarse particle fraction
**DPI** Dry powder inhaler
**ECD:** Effective cutoff diameter
**EPD:** Extra-fine particle dose
**EPF:** Extra-fine particle fraction
**FPD:** Fine particle dose
**FPF:** Fine particle fraction
**GSD:** Geometric standard deviation
**IP:** Induction port
**IQR:** Interquartile range
**ISM:** Impactor sized mass
**M:** Mouthpiece
**MMAD:** Mass median aerodynamic diameter
**MOC:** Micro-orifice collector
**NGI:** Next Generation Impactor
**OIP:** Orally inhaled product
**PS:** Pre-separator
**Q:** Test flow rate
**S:** Stage
**SD:** Standard deviation
**SDP:** Sized deposition profile
***X*:** Sample size

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# ⟨1644⟩ THEORY AND PRACTICE OF ELECTRICAL CONDUCTIVITY MEASUREMENTS OF SOLUTIONS

This general chapter provides information in support of instrumental methods for procedures that measure electrical conductivity. Pharmaceutical applications include: chemical dosing, cleaning in place, fermentation control, and liquid mixing verification, among others. Although the general chapter focuses on aqueous systems, conductivity measurements can be extended to organic fluids. The general chapter also focuses on contacting conductivity measurements and does not cover applications which may use noncontacting inductive conductivity. After an introduction, the general chapter covers the following major topics: theory of operation, operational considerations, calibration, and operation for at-line, in-line, and off-line measurement procedures.

## INTRODUCTION

Conductivity is the measurement of the ability of a fluid to conduct electricity via its chemical ions. The ability of any ion to electrically conduct is directly related to its ion mobility. Some of the common applications of conductivity measurements include water treatment and purification, clean-in-place process fluid management, fermentation process monitoring, dosing applications, nutrient media preparation, buffer production (e.g., distribution and dilution for dialysis and chromatography applications), chromatography detection of gradient and eluent, active pharmaceutical ingredient chemical synthesis, and concentration determination of basic chemicals. Fluids should be measured in a single homogenous phase—i.e., conductivity should not be applied to mixed immiscible fluids unless they are separated. Electrical conductivity measurements cannot be applied to solids or gases, but they can be applied to the condensate of gases.

Besides its use to monitor ionic concentrations of process fluids, conductivity is also useful for the detection of ionic impurities in compendial waters (see *Water Conductivity* ⟨645⟩) and for the detection of ionic impurities in organic matrices.

The measurement is non–ion-specific, and all ions respond with different efficiency or equivalent conductance, λ. Despite the lack of ionic specificity, conductivity is a valuable laboratory and process tool for measurement and control of total ionic content because it is proportional to the sum of the concentration of each ionic species (anions and cations) as described in *Equation 1*:

$$\kappa = 1000 \sum_{i}^{all\ ions} C_i \lambda_i$$

where κ is the conductivity (S/cm), $C_i$ is the concentration of chemical ion *i* (mole/L), and $\lambda_i$ is the specific conductance of ion *i* ($S \cdot cm^2$/mole). Though S/m is the appropriate SI unit for conductivity (i.e., the base SI units are the ampere and the meter) units of S/cm historically have been selected as the accepted unit of expression.

At low ion concentrations (typically $<10^{-3}$ mole/L), the conductivity–concentration relationship is linear and valid because λ is constant for each ion, but there are three notable exceptions to this strict linearity and proportionality. First, at higher concentrations (approximately $10^{-3}$ to 1 mole/L) small negative deviations from linearity (<5% per decade) arise because of the decrease in λ for each ion, and the negative deviations vary from ion to ion. Second, at higher concentrations for weak acids and bases the extent of dissociation into ions decreases depending on their dissociation constants. As the concentration of a weak acid/base increases, the conjugate cation/anion concentration increases as the square root of the acid/base concentration. Third, at high concentrations (>20%) of certain strong acids such as $HNO_3$ and $H_2SO_4$, the negative deviations persist, and, in some cases, the conductivity decreases with increasing concentration. The conductivity of high-concentration acid systems is well documented.

Another variable that influences conductivity measurements is the fluid temperature. A stricter expression of *Equation 1* is shown below as *Equation 2*:

$$\kappa\left(T\right) = 1000 \sum_{i}^{all\ ions} C_i\left(T\right)\lambda_i\left(T\right)$$

where the conductivity measurement, ion concentrations, and specific ion conductances are temperature (*T*) dependent. As fluid temperature increases, the ions become more efficient electrical conduits, making this physicochemical phenomenon the predominant reason for the temperature-compensation requirement when testing conductive fluids. The specific ion conductance of all ions increases with increasing temperature. In addition, the concentration of ions can also change as a function of temperature. For example, the auto-dissociation constant of water, $K_w$, increases with temperature from 0° to 100°, resulting in the increased production of $H^+$ and $OH^-$ .

Strictly speaking, it is challenging to temperature-compensate perfectly the conductivity measurement to a reference temperature unless the ionic species are well known. In many applications the ionic species are well known, and in most other cases simple assumptions make this issue less demanding. Temperature compensation is discussed in further detail in *Temperature Compensation*, below.

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# THEORY OF OPERATION

## Alternating Current Measurement Method

Conductivity is measured by applying a voltage (or current) between two conducting electrodes and measuring the resistance of the fluid using Ohm's Law. Various methods are used to apply the voltage/current, but all have the property of using an alternating voltage/current (AC) in order to minimize polarization (or collection of ions) at the electrodes or any electrolytic reaction. If a direct voltage/current (DC) is used, then the positive ions will collect at the negative electrode, and negative ions will collect at the positive electrode. The collection of ions at the electrode prevents the flow of current and adversely affects the accuracy and stability of the conductivity measurement. The measuring frequency of the AC signal depends on the technology and can range from as low as 30 Hz in low-conducting fluids and up to 4 kHz in highly conductive fluids. The specific frequencies are not relevant to operation of the system because the drive frequency is embedded in the instrument's measurement systems and is integrally linked to the supplier's measurement technology. This chapter does not seek to evaluate different measurement technologies because they are usually microprocessor-controlled systems and are proprietary.

The two-electrode AC measurement technique is valid for use with all concentrations of ionic species ranging from acids and bases (high conductivity) to *Water for Injection* and *Purified Water* (low conductivity) and even to organic, weakly ionic species such as alcohols and glycols. The measurement can be sensitive to ion concentrations as low as 0.05 µg/L. For high ion concentrations, analysts can use an alternative 4-electrode measurement method in which the current is applied between two of the electrodes and the voltage is measured between the two other electrodes. Ions are driven to the current electrodes while the voltage electrodes make the measurement with limited polarization effects.

## Units of Expression

There is no difference in the physical measurement of conductivity and resistivity—they are multiplicative inverse measurements of each other. Therefore, if one measurement is known, then the other value is readily calculated by taking the reciprocal of the numerical value and the units. There is also no difference in the instrumentation or the sensors. The only difference is how the measured value is reported or displayed for the convenience of the analyst. For example, 18.2 MΩ · cm = 0.0550 µS/cm and 5.23 kΩ · cm = 0.191 mS/cm = 191 µS/cm. Although the proper SI units are Ω · m or S/m, the traditionally used units are Ω · cm or S/cm.

*Figure 1* shows relationships among conductivity, resistivity, and some example process fluids of various grades of purity. The conductivity of fluids in pharmaceutical systems varies over approximately 8 orders of magnitude. In high-purity water systems, the quantity of ions present in *Purified Water* or *Water for Injection* is very low, resulting in a conductivity <5 µS/cm and often approaching 0.055 µS/cm or less. [NOTE—The conductivity of the purest waters at temperatures less than 25° is less than 0.055 µS/cm.] In drinking waters, the conductivity may vary from 30 to 2000 µS/cm. In chromatographic separations, the conductivity of the eluent may vary from 0.1 to 100 mS/cm. For hot concentrated acids, the conductivity may be as high as 1 S/cm.

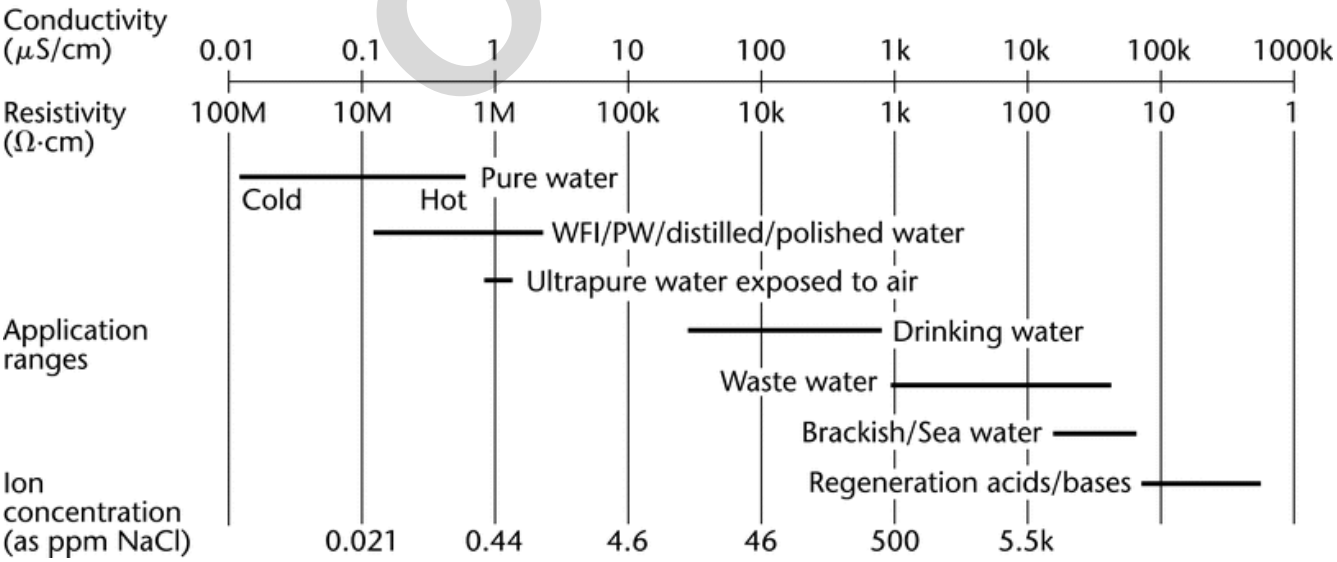


Figure 1. Relationships among conductivity, resistivity, and some example process fluids of various grades of purity.

## Cell Constant Determination

The purpose of the sensor's cell constant is to normalize the conductance/resistance measurement for the geometrical construction of two electrodes. When two electrodes are placed in a conducting fluid and a voltage is applied to them, there is a conductance (resistance) between the electrodes. If the electrodes are placed farther apart, the conductance decreases (resistance increases). If the area of the electrodes increases, then the conductance increases (resistance decreases). In both

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cases, the ion concentrations between the electrodes do not change, but the geometrical construction of the sensor (cell constant) alters the measured conductance (resistance). The conductivity of a solution ($\kappa$, S/cm) is related to the conductance, $G$ (siemens) according to *Equation 3:*

$$\kappa = G \times \left(\frac{d}{A}\right) = G \times \theta$$

where $A$ is the area of the conducting electrodes ($cm^2$) and $d$ is the distance between the electrodes (cm). The other common unit of expression is the reciprocal of conductivity, or resistivity, $\rho$ ($\Omega \cdot cm$), as described in *Equation 4*:

$$\rho = \frac{1}{\kappa} = \frac{1}{G \times \theta} = \frac{R}{\theta}$$

where $R$ is the resistance of the fluid between the electrodes ($1\ \Omega = 1\ S^{-1}$). The geometrical ratio, $d/A$ (or $\theta$), is known as the cell constant ($cm^{-1}$) of the sensor.

Determination of the cell constant by the direct measurement of $d$ and $A$ is impractical because of variations in the geometrical configurations and the nonuniformity of the electric field between the electrodes. Practically, the cell constant is determined by the measurement of aqueous solutions of known conductivity. See *Calibration* below.

### Temperature Compensation

Temperature compensation is a typical requirement for most conductivity measurements, although there are exceptions such as those contained in *Water Conductivity* ⟨645⟩. As noted previously, the conductivity of a fluid is related to its temperature. As the temperature increases, ions become more mobile and the conductivity increases. The effect of temperature depends on the type and concentration of the ion, but for most solutions >10 µS/cm the impact of temperature is in the range of 1.9%/° to 2.2%/°. For strong acids, this may be as low as 1.5%/°. For high-purity compendial waters, the temperature coefficient varies from 2.0%/° to 7.5%/° depending on the temperature and purity of the water. For each case described here, some knowledge of the type of impurity is needed in order to ensure adequate temperature compensation. If the conductivity vs. temperature function is linear and the temperature coefficient is constant, the equation that relates the compensated conductivity to the non–temperature-compensated conductivity is described in *Equation 5*:

$$\kappa_{25} = \frac{\kappa_T}{[1 + \alpha(T - 25)]}$$

where $T$ is the measured temperature, $\kappa_{25}$ is the conductivity compensated to 25°, $\kappa_T$ is the conductivity at $T$, and $\alpha$ is 0.02 for a temperature coefficient of 2%/°.

Most conductivity measurement systems measure the uncompensated conductivity/resistivity and the temperature, and the temperature-compensated conductivity is determined via mathematical algorithms (e.g., by application of *Equation 5*) in the microprocessor of the transmitter. Depending on the application and knowledge of the content of the fluid, different compensation algorithms may be available. For most process control applications, temperature compensation is recommended because when the uncompensated conductivity changes it is impractical to distinguish whether this change is caused by temperature fluctuation or a change in ionic content. Temperature compensation allows the analyst to distinguish between changes in temperature and ionic content. Compensation to a reference temperature of 25° is standard practice, but some methods specify temperature compensation to 20°.

Most conductivity sensors have temperature devices such as a platinum RTD (resistance temperature device) or NTC (negative temperature coefficient) thermistor embedded inside the sensor, although external temperature measurement is possible.

## OPERATIONAL CONSIDERATIONS

### System Components

The usual components of a conductivity measurement system are the sensor, the transmitter, and the cable that connects the sensor to the transmitter. The sensor is the device that is in direct contact with the fluid. The sensor consists of the electrodes, usually an embedded temperature device, and a process connection (e.g., a tri-clamp if the sensor is intended for in-process sanitary applications). Traditionally, sensors are passive electromechanical devices and do not contain measurement circuitry.The transmitter is the device that measures the resistance between the sensor's electrodes, measures the resistance of the temperature-measuring device, converts the resistance measurement to a conductivity (or resistivity), and performs temperature corrections to compensate the signal to a reference temperature. The cable connects the sensor to the transmitter. In process control or monitoring systems, the cable allows the sensor to be attached to a process tank or pipe, and the transmitter can be located at a control panel or other remote location, if necessary. The distance between the sensor and the transmitter may affect measurement accuracy caused by the added cable resistance and the susceptibility of the cable to external noise. Therefore, this distance should be considered during selection of the measurement system. In some conductivity measurement systems, the measurement circuitry is directly attached to the conductivity sensor, allowing digital transfer of the measurement results to a remote display at greater distances than traditional measuring systems with wire transmission of analog signals.

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### Materials of Construction

The sensor's materials of construction are critical when the sensor may be in contact directly or indirectly with the product. For most laboratory applications, materials of construction are less critical. All sensors contain measuring electrodes and an insulating material between the electrodes. Technically, the only requirements are for electrodes to be electrically conducting and to be able to withstand the physical and chemical environment. Electrodes can be made of various grades of stainless steel, titanium, graphite, and many other metals. The insulating materials must isolate the electrodes from each other so that only the fluid conductivity is measured. Insulating materials are usually made of an inert polymer, epoxy, or ceramic.

If the sensor is connected directly to a process vessel or the piping system, then the sensor's materials of construction must meet the thermal and hydraulic (pressure) requirements of the process system. The sensor must have a proper process connection to the vessel/piping. Depending on the application, the sensor also may be required to meet biological compatibility, material, or hygienic design requirements. The sensor must not degrade during installation and operation. The sensor must be able to withstand any other processes that the system may encounter such as clean-in-place or steam-in-place processes. Otherwise analysts may need to remove the sensor from the process. When immersion of the sensor into the process places the process at risk, the sensor should be installed in a housing that is attached to the sidestream. In this case, a fraction of process fluid flows to the housing and sensor and then to drain. This allows indirect measurement of the process fluid without harm to the process or product.

If the conductivity measurement is performed off-line, e.g., in a laboratory environment, then the operating considerations are reduced because the thermal, hydraulic, and other product-contact concerns are reduced or eliminated.

## CALIBRATION

The process of calibrating a complete conductivity measurement system generally consists of three parts. First, the transmitter's electronic circuitry is calibrated. Second, the temperature sensor device is calibrated. Third, the cell constant of the conductivity sensor is determined. In each case, verification may precede any calibration to determine if an adjustment is necessary. In most cases for microprocessor-controlled instrumentation, there is no formal mechanical or electronic adjustment of these subsystems. Instead, these adjustments are usually made in software-controlled calibrations that are computed automatically by the transmitter.

A calibrated transmitter should be used in order to calibrate the temperature sensor and cell constant. The transmitter used to calibrate the temperature sensor and cell constant can be the transmitter used in normal operation, or another transmitter can be used instead. Because of differences in a supplier's wiring and electronic compatibility, it may be necessary to use the same type of transmitter.

As is the case for all instrument-based methods, calibration frequency depends on many factors. Depending on the type of conductivity sensor and transmitter, calibration cycles vary from weekly to annually based on manufacturers' recommendations, historical performance of the instrumentation, internal requirements, and the criticality of the application. For robust process instrumentation, typical calibration cycles for the electronics and the sensor take place approximately every 12 months. When the sensor's cell constant can be altered by the fluid or the process conditions, more frequent calibration may be needed.

### Instrument Calibration

The transmitter's electronic circuitry is calibrated by disconnecting the sensor from the transmitter, connecting precision resistors (resistors of known value) to the transmitter, and comparing the traceable resistance value to the measured resistance value. The resistance values should be traceable to a competent national authority. The resistance values should be selected so that they are in the range of (1) the measurement capability of the transmitter, and (2) the resistance that will be measured during operation. The transmitter may have multiple circuits internally, so verification of the appropriate circuit (or all circuits) and measurement range is necessary. Comparison of the measured resistance to the actual resistance verifies if the transmitter is properly calibrated. The resulting difference must be within a predetermined $x_1$% of the actual resistance, where $x_1$ indicates the desired conductivity circuit electronics performance. Typical conductivity circuit electronics performance is usually in the range of 2% or less of the target value. Otherwise, adjustment of the resistance measurement circuit is recommended.

An example of the transmitter's measurement electronics calibration process follows: A typical operating range for a clean-in-place process may be in the 50 to 75 mS/cm range. A sensor with a cell constant of 5.0 $cm^{-1}$ is used in this example. Based on *Equation 4*, this requires a measuring resistance of 67 to 100 Ω. To verify the conductivity measuring circuit during calibration, analysts should use a resistor(s) with a traceable value in or near this range. When compendial waters are used, typical measurements are in the range of 1 to 20 MΩ · cm and use sensors with a cell constant of 0.1 $cm^{-1}$. This computes to a resistance of 0.1 to 2 MΩ. Resistors with a traceable value in this range should be used. If the recorded values are within the range of the pre-established acceptance criteria ($x_1$%), then adjustment is not required.

### Temperature Measurement and Sensor Calibration

If a temperature measurement circuit is integrated into the transmitter and is used as part of the measurement system, then verification and/or calibration of this circuit is required. Depending on the type of temperature device in the sensor, an appropriate signal source (e.g., resistance) should be input to the transmitter. Comparison of the measured temperature and the simulated temperature verifies if the temperature measurement circuit is properly calibrated. The resulting difference must be within $x_2$° of the simulated temperature where $x_2$ indicates the desired temperature circuit electronics performance. Typical temperature circuit electronics performance usually is in the range of ±1° or less. If the difference meets the pre-established acceptance criteria, no further action is required. Otherwise, adjustment of the temperature measurement circuit is recommended using the protocol in the transmitter's calibration function.

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If a temperature sensor is integrated into the conductivity sensor, the temperature sensor can be calibrated by comparison of the sensor's temperature measurement to a reference system. This is accomplished by immersing the process and reference sensors in the same fluid. The reference system can be another traceable temperature measurement device or a fluid system of known temperature such as boiling water (corrected for elevation, if this degree of accuracy is necessary) or an ice-water bath.

The accuracy of the calibration is related to the accuracy of the reference sensor, the thermal homogeneity of the fluid, and the elimination or reduction of any artifacts that can negatively influence accuracy. For example, if the sensor is not fully immersed in the fluid, thermal conduction from the ambient environment can alter the temperature measurement. Careful insulation of the sensor from the ambient environment may improve the accuracy of temperature measurements, particularly if the reference temperature is substantially different than the ambient temperature.

Comparison of the measured temperature and the reference temperature verifies if the temperature sensor is properly calibrated. The resulting difference must be within $x_3$° of the reference temperature where $x_3$ indicates the desired temperature accuracy that is required for the process. Temperature sensor accuracy is usually in the range of ±2° or less. If the difference meets the pre-established acceptance criteria, no further action is required. Otherwise, adjustment of the temperature sensor calibration factors is recommended using the protocol in the sensor's calibration function.

### Cell Constant Calibration

The cell constant of the sensor is determined by comparing the conductivity measurement of the system to that from a reference conductivity system and adjusting the cell constant. Place the sensor in a reference solution of known conductivity whose value is traceable to a competent authority. The reference solution can be a solution of known conductivity in one of three ways:

1. The solution can be produced according to a standard method that is traceable to a competent authority. One method is to prepare one of the solutions listed in ASTM D1125. Another method is to use ultra-pure deionized water with no exposure to air whose conductivity is known.
2. The solution can be procured from a third-party supplier with traceability to an acceptable authority. Cell constant calibration should be performed in the recommended temperature range of the reference solution.
3. The solution can be any fluid whose conductivity is known by measurement from an alternative and traceable reference conductivity measurement system.

In all three cases, regardless of the type of calibration methodology, there are some fundamental requirements. The sensor's cell constant should be calibrated at a conductivity that is in the measurement range of the measuring system. For example, if the system is designed to measure in the 0–100 µS/cm range, then use of a 1000 µS/cm reference solution is not advised.

Because the cell constant is a geometrical property of the sensor and is a constant, the determination and/or calibration of the cell constant does not need to be in the operational range of the pharmaceutical process as long as the conductivity is in the operational range of the measurement system. Also, because the cell constant is a geometrical property of the sensor, a single-point calibration typically is sufficient. In some applications, a 2-point calibration can provide improved accuracy at very high conductivity. Depending on the reference solution, cell constant calibration can be performed with or without temperature compensation. For reference solutions exceeding 10 µS/cm, if temperature compensation is needed a temperature coefficient ($\alpha$) of 2%/° is sufficient over the range of 25 ± 10° unless otherwise specified.

Adjustment of the cell constant is recommended if the difference between the measured and reference conductivity exceeds $x_4$% of the reference conductivity where $x_4$ indicates the conductivity accuracy that is required for the process. Typical conductivity accuracy is 5% or less. If the accuracy measurement meets the pre-established acceptance criteria, no further action is required. Otherwise, adjustment of the sensor's cell constant is recommended using the protocol within the sensor's calibration function.

## OPERATION

### In-line, At-line, and Off-line Measurements

Depending on the application, in-line, at-line, and off-line measurements have specific universal requirements. Each has advantages and disadvantages depending on the application, but the fundamental technologies (AC measurement) supporting each measurement type are constant. The primary differences between the in-line/at-line and the off-line systems are sensor features related to process robustness and transmitter features related to output functions, external communication capabilities, and system installation costs. In-line and at-line systems have added benefits of real-time measurements and continuous data acquisition. They also have added one-time electrical and plumbing installation costs, and the instrumentation is fixed in a specific location for a single process and purpose. Also, sample is diverted to drain, resulting in product loss. Note that the fluid flow velocity should be high enough that the velocity does not affect the measurement. Off-line (or laboratory-based) systems have the benefit of measuring many sample types under controlled conditions. Off-line batch testing has added costs associated with cleaning containers and collecting samples as well as risks of sample contamination.

In-line conductivity measurements are suitable when the following conditions are met: (1) there is a need for or value of real-time, continuous data; (2) the sensor and the application/fluid are compatible and cause no harm to each other; and (3) atmospheric contamination must be avoided. In these cases, continuous process control, decisions, and intervention are available.

At-line (or sidestream) conductivity measurements are used when the following conditions are met: (1) a need exists for or value of real-time, continuous data; (2) the sensor and the application are compatible with each other; (3) the sensor can cause harm to the application and/or fluid; and (4) atmospheric contamination must be avoided. In such cases the measurement is made by delivering the sample from the tank/piping using hydraulic pressure (flow) to the conductivity sensor, and the fluid passes through the sensor and goes to drain. In these cases, continuous process control, decisions, and intervention are available

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and have no affect on product or application. The only requirement is positive pressure upstream from the sensor to prevent backstreaming of the sample fluid back into the process.

Off-line conductivity measurements are used when the following conditions are met: (1) no need exists for or value of real-time or continuous data; (2) the sensor and the application are compatible with each other, but the sensor can cause harm to the application and/or fluid; and (3) sampling needs are infrequent or are needed on a limited basis.

## Sample Preparation and Off-line Measurement

When performing off-line measurements, analysts must ensure that the container used to collect the fluid sample(s) is sufficiently clean so that the container does not alter the result. Collect an amount of fluid needed for rinsing and measuring. For samples such as *Purified Water* where the analysis can be affected by gasses, containers should be filled completely to reduce headspace. The volume of fluid needed to make a measurement depends on the design of the sensor and can range from <10 mL to 1 L. Transfer a portion of the sample fluid to the clean measuring container to rinse the container walls. Discard the fluid. The sample container also can be used as the measuring vessel. The sensor should be rinsed with a suitable quality of water or other appropriate fluid before use so the measurement is not affected and then rinsed at least once with the solution to be measured, which then is discarded. This rinse with sample solution removes any residue or fluids with which the conductivity sensor previously was in contact. The exact procedure varies depending on the solution tested. For example, when testing very low conductivity samples such as *Purified Water*, analysts must do more rinsing to remove any remaining residue from the sensor. Maximum holding time, temperature, and container type must be controlled for some low-conductivity samples, e.g., high-purity waters, because of the potential impact of ionic leachables from certain containers.

Transfer the fluid to the measuring container, immerse the sensor into the fluid, and ensure acceptable clearance around the sensor's measurement area. Stir or agitate the sample to prevent bubbles from attaching to the electrodes and disturbing the measurement. If a temperature-compensated measurement is used, then select the appropriate temperature-compensation algorithm in the transmitter, and adjust the sample temperature to the recommended range, if necessary, using an appropriate temperature bath. Otherwise, disable the temperature compensation. Verify that the temperature is sufficiently stable (<0.25° change per min), and record the conductivity reading and the temperature, if necessary.

## In-line Measurement

For in-line testing, the sensor is installed into the process piping or vessel. The orientation of the sensor relative to the flow of the fluid is critical to ensure that (1) particles and sediment do not collect between the measuring electrodes, and (2) no air pockets are trapped between the measuring electrodes. Both factors can adversely affect the measurement accuracy. Depending on the electrode design, manufacturers usually provide recommendations. Ensure the sensor is clean before installation.

After the sensor is immersed in the closed process, such as a piping system or tank, the sensor does not require removal and cleaning before a measurement. If a temperature-compensated measurement is used, then select the appropriate temperature-compensation algorithm. Otherwise, disable the temperature compensation. Although temperature stability is desirable, it may not be possible depending on the temperature control of the process. Record the conductivity reading and the temperature, if necessary.

## At-line Measurement

For at-line testing, a piece of tubing is used to connect the process vessel/piping to the sensor and its housing. The tubing should be cleaned or flushed with an appropriate cleaning agent to remove any impurities that could alter the conductivity measurement. The tubing can be made of metal or plastic depending on the application and chemical compatibility. Installation of the sensor into a housing permits fluid to be directed into the sensor and then to drain. Installation considerations are similar to those for in-line installations. Ensure the sensor and housing are clean before installation.

After the sensor and housing are connected to the sidestream, the sensor does not require removal and cleaning before a measurement. If a temperature-compensated measurement is used, then select the appropriate temperature-compensation algorithm. Otherwise, disable the temperature compensation. Although temperature stability is desirable, it may not be possible depending on the temperature control of the process. Record the conductivity reading and the temperature, if necessary.

## Other Considerations

In all cases mentioned above, the use of abrasive materials to clean the sensor typically is discouraged. Two reasons to avoid abrasive cleaning materials are that (1) the passive layer of stainless steel sensors (when used) can be destroyed, and (2) the surface of the measurement area can affect the measurement accuracy. For these reasons, the use of appropriate chemically compatible fluids is preferred instead of mechanical methods for cleaning.

Unlike the requirements for many electrochemical measurements, the need for flowing, circulating, or agitating fluid is not a fundamental requirement for a conductivity measurement. In general, there is no difference in the conductivity measurement of a static or a flowing sample, but two conditions must be met for static samples: First, bubbles cannot be allowed to collect on the measuring area of the electrodes because they can interfere with the flow of current conductivity measurement. Second, if sample homogeneity is affected by the lack of agitation or circulation, then the conductivity measurement may not represent the conductivity of the bulk fluid.

In all cases, the installation of the sensor should take into consideration wall effects from the vessel, piping, or laboratory container. If the proximity of the wall interferes with the electromagnetic field for the conductivity measurement, then the measurement could be positively or negatively altered. Some 2-electrode sensor designs, such as coaxial concentric electrodes, are not affected by nearby objects. The sensor's installation instructions may indicate if this must be considered.

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If unstable conductivity readings are observed, some common causes could be inadequate grounding of the water system, electronic noise from pumps and other high-frequency generators, or internal leakage of the sensor. Various diagnostic approaches are available and can help identify the cause.

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# ⟨1660⟩ EVALUATION OF THE INNER SURFACE DURABILITY OF GLASS CONTAINERS

## PURPOSE

This general information chapter provides information about factors that affect the durability of the inner surface of glass containers. Recommended approaches are provided to evaluate the potential of a drug product to cause formation of glass particles and delamination of the inner surface. Screening methods are provided to detect glass particles and delamination, allowing a comparison to be made of glass durability on a lot-to-lot basis or between different glass manufacturers.

## SCOPE

This chapter addresses bottles and vials manufactured by molding and ampuls, cartridges, vials, and prefillable syringes manufactured from tubing glass. Glass for pharmaceutical packaging is classified as Type I borosilicate glass, Type II treated soda-lime-silica glass, or Type III soda-lime-silica glass on the basis of the hydrolytic resistance of the glass, as defined in *Containers—Glass* ⟨660⟩. Type I glass containers are suitable for most products for parenteral and nonparenteral use. Type II glass containers are suitable for most acidic and neutral aqueous products for parenteral and nonparenteral uses, and can be used for alkaline parenteral products when stability data demonstrate their suitability. Type III glass containers usually are not used for parenteral products or for powders for parenteral use, except when suitable stability test data indicate that Type III glass is satisfactory. This chapter focuses primarily on Type I glass, because it is the most widely used in the pharmaceutical and biopharmaceutical industry for parenteral products although the guidance can be equally applied to Type II and Type III glass used for parenteral products.

The chapter should be useful for the following:

- Molded and tubular glass container manufacturers and converters
- Pharmaceutical and biopharmaceutical companies
- Contract manufacturing and filling organizations

Glass delamination may be described as the appearance of thin flexible flakes of glass (or lamellae) that can range in size from <50 µm to 200 µm in a drug product solution. This is a serious quality issue and can result in a product recall. The appearance of glass lamellae is a lagging indicator of a strong interaction between the drug product and the inner surface of the glass. Although delamination is the most obvious visual indicator, it represents the final stage of a complex glass corrosion reaction, and can be observed only at a point where prevention is no longer an option. Adding further complexity to detection, mechanical energy from shaking or vial-to-vial contact during transportation may be required to dislodge the lamellae from the internal surface of a filled vial and facilitate observation.

Tests for delamination combine the visual examination of the solution, an examination of the vial's internal surface and analysis of an aggressive test solution to assess the propensity of the internal glass surface of vials to delaminate. These examinations and the use of an aggressive test solution are intended to be conducted by the pharmaceutical manufacturer, not the glass manufacturer or converter.

## GLASS TYPES

Glass in its pure form consists of silicon dioxide with a melting point in excess of 1700°. However, this is rarely used commercially because of the cost of working at these elevated temperatures. Added network modifiers, such as sodium, potassium, or boron oxide, lower the melting point and lower the chemical durability, whereas added network stabilizers, such as calcium and aluminum oxides, improve the durability of the glass. Colored glass (e.g., amber glass) is produced by transition metal oxides such as iron oxides. All additives to pure silicon dioxide, as well as silicon itself, can be viewed as potential extractables from glass containers.

Glass compositions do not exist as stoichiometric chemical compounds but rather are expressed by a range of compositions. Thus, there is allowable variation within a glass type, and glass types may vary slightly among glass producers. Soda-lime-silica glass consists of silicon dioxide (60–75 wt%), sodium and potassium oxides (12–18 wt%), and smaller amounts of calcium, magnesium, and aluminum oxides (5–12 wt%). This glass has a relatively high coefficient expansion (COE) of 80–90 × $10^{-7}$ per degree and is susceptible to breakage by thermic shock. Borosilicate glass consists of silica (65–80 wt%), boric oxide (7–13 wt %), and smaller amounts of sodium, potassium, and aluminum oxides. The presence of boron provides greater resistance to thermal shock through a reduction in COE and to hydrolytic attack by increasing the connectivity of the glass network. Type I glass is available in multiple formulations: tubular glass is available with a low COE, described as 32–33 expansion glass and with a relatively low COE (range, 48–56 expansion), for example 51 expansion glass, in reference to their individual COEs of 32.5 × $10^{-7}$ per degree and 51.0 × $10^{-7}$ per degree, respectively. Molded glass has a higher COE in the region of 60–63 expansion.

## FORMATION OF MOLDED AND TUBULAR GLASS CONTAINERS

Formation of molded and tubular glass containers requires a number of steps. The quality of the container used in packaging depends on the conditions and the quality control of each step. Both molded and tubular containers originate from a glass furnace, and different furnaces are dedicated to borosilicate or soda-lime-silica glass. The refractory bricks lining the furnace

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deteriorate with time and must be replaced. Worn bricks can contribute to cosmetic defects such as stones (inclusions in the glass) that become incorporated into the molded glass containers or glass tubing.

Molded glass vials and bottles are manufactured in a one-step process whereby a stream of molten glass is cut into a gob, which then enters a mold where air or tooling is used to shape the container to the mold. Formation of containers from tubing glass is a two-step process. Glass tubes of a specific diameter are formed from a stream of molten glass that exits the furnace, is cooled, and is sectioned into standard lengths. These tubes are subsequently converted into glass containers (ampuls, cartridges, syringes, or vials) by either the tubing glass manufacturer or by independent converters. It is technically difficult to form glass tubing with a diameter sufficient to make bottles containing 100 mL or more, so these containers are produced by molding.

Gas flames are used to soften tubing glass to form the neck, to melt the glass to form the base of ampuls or vials, and to separate the container from the glass tube. In the case of cartridges and prefillable syringes, the glass tube is cut to length, and the ends are softened to form the nozzle and flange of the syringe and the neck and rear of the cartridge. Heating rate, maximum glass temperature, and production speed are critical parameters that can be adjusted for individual forming machines. After formation, both tubular and molded containers pass through an annealing oven (lehr) that heats the containers to 20° to 30° above the transformation temperature ($T_g$) of the individual glass formulation ($T_g$ for borosilicate glass is approximately 570°) and then gradually cools them in order to remove stresses in the container due to the manufacturing process. This too is a critical process because poorly annealed containers show reduced chemical and mechanical durability.

The process of forming tubular vials and ampuls has an effect on the local surface composition of the glass. During formation of the neck and particularly the base, the temperature of the inner surface of the containers can exceed the evaporation point of some of the glass components such as alkali borates. Under certain time–temperature conditions, the glass can phase separate during forming, creating nonhomogenous surface chemistry on the interior of the container. Both scenarios are undesirable for the storage of aggressive liquids from a surface chemical durability perspective. Evidence of this can be obtained by appropriately etching the glass with acid, after which an opaque ring will appear above the heel of the container, indicating a negative change in the inner surface chemistry. The same phenomenon can be observed at the shoulder of the container as well, but in many instances this area does not experience prolonged contact with a liquid.

## PROCESSING OF MOLDED AND TUBULAR GLASS CONTAINERS

At times, the inner surfaces of glass ampuls, vials, and bottles undergo additional treatments. As an example, heating glass propagates sodium oxide toward the inner surface of the container, but washing with water does not remove sodium oxide because of the latter's limited solubility. When glass is exposed to an aqueous solution, sodium ions diffuse into the solution from the glass surface to produce hydroxide ions, resulting in an elevated pH in unbuffered solutions. One common treatment is the use of ammonium sulfate which converts the sodium oxide on the inner surface to a depth of approximately 10–100 nm into highly soluble sodium sulfate that then can be removed by washing. Although removal of sodium ions from the surface does reduce the propensity for pH shift, the treatment does remove structural elements, leaving a thin silica-rich inner surface layer. The process originally was designed to raise the surface hydrolytic resistance of Type III soda-lime-silica glass to that of Type II glass in order to mimic the hydrolytic resistance of Type I glass. This process also can be applied to Type I glass.

In summary, the key factors that influence glass surface durability of containers manufactured from Type I glass are primarily the manufacturing conditions, such as the forming temperature, the time of exposure to heat, and the annealing conditions. The temperatures used for subsequent steps are lower than those used for forming and annealing (see *Table 1*), and do not pose an additional risk to the chemical durability of the glass from phase separation or volatilization. Post-manufacturing operations such as storage in humid conditions and processing, such as depyrogenation in the presence of water vapor and terminal sterilization via autoclaving, can also impact glass surface chemical durability.

**Table 1. Temperatures Encountered During Formation and Processing of Type I Tubular Glass Containers**

| Key Operations | Typical Temperatures (°) |
|---|---|
| Furnace | 1500–1650 |
| Sectioning of tube and base formation | 1300–1500 |
| Working range | 1000–1250 |
| Softening | 750–850 |
| Annealing | 550–600 |
| Depyrogenation range | 250–350 |
| Terminal sterilization | 110–130 |

## GLASS CONTAINER SOURCING

A pharmaceutical manufacturer has a range of choices when selecting a glass container for a drug product. These include the type of glass (I, II, or III), the production method (tubular or molded), surface treatments, as well as the size and neck finish of the container. It is important that the pharmaceutical manufacturers provide sufficient information on their requirements, such as the drug product formulation and the manufacturing and filling process, to allow the glass vendors to make informed judgments as to what containers to recommend.

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Pharmaceutical manufacturers should consider the upstream provenance of the containers they purchase in that they should have sufficient knowledge of the glass manufacturing process and glass composition. This is essential to qualify a particular glass container type from a glass manufacturer for a particular drug product. The following knowledge is useful in this regard:

- Glass formulation
- COE for Type I tubular glass (32–33 or 48–56 expansion)
- Whether the glass converter makes its own glass feedstock or sources the glass feedstock from a third party
- The manufacturing site for the glass containers. If multiple sites manufacture glass containers for a given product, information to determine if the glass containers made at the different sites will perform comparably
- Whether the glass surface has been modified through chemical treatment such as ammonium sulfate by the glass manufacturer or converter.

The maker and user of a glass container should collaborate to assure that glass quality is monitored and maintained throughout the extent of the glass supply relationship. Glass quality should also be monitored and inferred by the user through observations made during storage throughout the product's use-by-date. The glass manufacturer and glass user quality management programs should include the following:

- Quality audits of glass supplier (glass manufacturer and/or converter) by the glass user
- Establishing mutually agreed upon acceptable quality levels for lots of glass containers
- Monitoring and trending of the quality of glass batches, including but not necessarily limited to, monitoring the values obtained by the *Surface Glass Test* in ⟨660⟩
- Monitoring and trending of glass quality and glass manufacturing process performance by the glass container manufacturer, including the effectiveness of methods used during the manufacturing process of the glass container to measure geometric tolerances and identify cosmetic defects
- Assurance that differences among different glass manufacturing sites do not significantly affect quality of a specified glass container sourced from multiple sites
- Presence of a system to monitor and qualify changes made to the glass manufacturing process and to inform customers of such changes.

## GLASS SURFACE CHEMISTRY

After manufacturers are assured of the quality and consistency of the glass containers they purchase, they can use the complex aqueous chemistry of surface glass to decide on potential drug product formulation and treatment steps that could increase glass stability. The first reaction between the glass surface and an aqueous phase (water or water vapor) involves ion exchange between hydrogen ions (or hydronium ions $H_3O^+$) from the aqueous phase and alkaline ions in the glass (*Equation 1*). This ion-exchange occurs in a short reaction time in acidic or neutral solutions. In basic solutions, the reaction occurs at the glass/water interface and dissolves the silica network. Further reaction of the silica releases silicic acid into the solution, thereby lowering the pH (*Equation 2*). These reactions result in hydration of the glass surface and an alkali-depleted, silica-rich layer.

$$H^+ + Na^+SiO^-(glass) \Rightarrow SiOH + Na^+ [1]$$

$$H_2O + Na^+SiO^-(glass) \Rightarrow SiOH + Na^+ + OH^- [2]$$

The presence of water in the leachate promotes hydrolysis of the Si–O bond forming a silica-gel layer (*Equation 3*).

$$H_2O + Si–O–Si \Leftrightarrow 2\ SiOH\ [3]$$

The mechanical properties of the surface gel that forms are different from those of bulk glass. Repeated hydration and dehydration of the layer leads to the cracking of the gel layer and eventual generation of particles. This process is worsened as the gel layer increases in thickness. This phenomenon is well known in glass exposed to ambient moisture (known as weathering). At higher pH values, the mechanism of glass degradation changes from the leaching of alkali elements to the dissolution of the silicate network as shown in *Equations 4* and *5*.

$$SiO_2 + 2H_2O \Leftrightarrow H_4SiO_4 [4]$$

$$H_4SiO_4 + OH^- \Leftrightarrow H_3SiO_4^- + H_2O\ [5]$$

Reaction (*Equation 5*) increases the solubility of the silicic acid in solution, driving the reaction forward. At some point the limit of solubility is exceeded, and particles are formed via precipitation. If the solution is not buffered, a decrease in the solution pH will take place. These reactions and scenarios apply only to the reactions of glass with water; the presence of drug product formulations can complicate the situation considerably.

## FACTORS THAT INFLUENCE INNER SURFACE DURABILITY

A number of factors have the potential to negatively influence the chemical durability of the inner surface of glass containers. These factors include glass composition, the conditions under which the containers were formed, subsequent handling and

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treatments, and the drug product in the container (*Table 2*). Not only can an aggressive drug substance corrode the inner surface, but excipients such as buffers, chelating agents and organic acids and high pH can also have a deleterious effect. For example, neutral solutions of sodium citrate attack glass with a severity similar to that of substantially alkaline solutions. Organic acids, such as gluconic and malonic acids, also corrode glass through a proposed mechanism of an ion exchange reaction in which metal ions on the glass surface are replaced by hydrogen ions from the acid. Not all listed factors negatively influence surface durability to the same degree, and can contribute to delamination either acting alone or in combination. Because of the range of variables, end users should examine all relevant variables for an individual drug product and assess the degree of risk for delamination and formation of subvisible and visible glass particles. In some situations, the accumulation of risk factors may indicate that the selection of a glass container for a particular formulation should be done following a predictive screening study to establish more stringent glass quality requirements or may indicate that a glass container should not be used for a formulation.

**Table 2. Factors That Influence the Inner Surface Durability of Glass**

| Container Manufacture | Container Processing and Storage | Drug Product: Formulation, Processing, and Storage |
|---|---|---|
| • Glass composition<br>• Molded or tubular container<br>• Tubular manufacturing process:<br>— Converting speed<br>— Converting temperature | • Post-formation treatments:<br>— Ammonium sulfate<br>—Washing<br>—Depyrogenation<br>• Storage conditions:<br>— High humidity | • Drug substance<br>• Formulation:<br>— Acetate, citrate, phosphate buffers<br>— Sodium salts of organic acids, e.g., gluconate, malate, succinate, tartrate<br>— High ionic strength, e.g., >0.1 M of alkaline salts<br>— Complexing agents, e.g., EDTA<br>— High pH, e.g., >8.0<br>• Terminal sterilization<br>• Labeled storage conditions (refrigerated or controlled room temperature)<br>• Shelf life |

## EVALUATION OF THE INNER SURFACE DURABILITY

Each lot of Type I, II, or III glass containers received by a pharmaceutical manufacturer must comply with the *Surface Glass Test* in chapter ⟨660⟩. This test provides an indication of inner surface chemical durability but does not appear to provide a clear direct correlation with the propensity to form glass particles or to delaminate. The alkalinity value represents the sum of all the internal surfaces of the container, and although this is representative for molded containers, tubular glass vials can have different degrees of surface chemical durability, depending on the location (e.g., just above the heel versus the side wall). A low surface alkalinity value can be obtained from containers treated with ammonium sulfate but the treatment itself may reduce the inner surface chemical durability, dependent upon the drug product formulation used to fill the vial. The most important variable that affects the surface durability is the drug product itself, and because it uses water as the extracting medium, the *Surface Glass Test* does not take this into consideration. Therefore, the *Surface Glass Test* represents only a first step in quality control of surface chemical durability, and additional screening methods should be used to demonstrate the suitability of vials for a formulation from a particular source before formal stability studies begin.

### Predictive Screening Methods

Screening methods help evaluate glass containers from different vendors (molded or tubular), glass formulations (e.g., 32–33 or 48–56 expansion for tubular glass), and post-formation treatments. Screening also establishes lot-to-lot variation from individual vendors during the drug development process, as well as lot-to-lot variations for products that have been shown to have a particular propensity to form glass particles or to delaminate. Screening methods can use a number of different technologies to examine three key parameters: visual examination and chemical profile of the inner surface layer, the amount and identity of extracted elements in solution, and the number of subvisible and visible particles in solution. Taken together, these elements are assessed by predictive tests for formation of glass particles and delamination, processes that reflect reduced durability. Predictive tests should look for precursors that lead to delamination rather than looking only for glass lamellae, and should be able to quickly provide predictive indication of surface durability. This makes the tests useful not just for vendor selection but also for evaluation of individual lots if necessary. Some of the more commonly used analytical techniques for evaluating the three key parameters are shown in *Table 3*.

**Table 3. Analytical Techniques for Screening Studies**

| Parameter | Test Parameter | Instrumentation |
|---|---|---|
| Glass inner surface | • Degree of surface pitting<br>• Chemical composition as a function of depth | • DIC Microscopy[a] or EM[b]<br>• SIMS[c] |
| Extracted elements in solution | • Conductivity/pH<br>• $SiO_2$ concentration | • Conductivity/pH meter<br>• IC–MS[d] or ICP–OES[e] |
| Lamellae and visible and subvisible glass particles | • Presence of lamellae and visible particles<br>• Lamellae or particle number and size<br>• Lamellae or particle morphology and composition | • Visual inspection<br>• Particle size analyzer<br>• SEM–EDX[f] |

[a] Differential interference contrast microscopy.
[b] Electron microscopy.
[c] Secondary ion mass spectrometry.
[d] Inductively coupled plasma–mass spectrometry.

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[e] Inductively coupled plasma–optical emission spectrometry.
[f] Scanning electron microscopy–energy-dispersive X-ray spectroscopy.

## Aggressive Screening Conditions

In selecting an appropriate primary glass container for pharmaceutical liquids, analysts should consider two approaches. The first is a series of accelerated temperature exposures using aggressive conditions that establish, in rank order, the chemical durability of the container without any specific reference to a given compound. Such testing can be helpful when selecting a packaging system for which the most chemically durable glass is desired. This testing also can be helpful in determining if changes in glass quality have occurred or in assessing processing changes that have been made by the primary container manufacturer. *Table 4* provides three examples of model systems that could be used for this assessment. Other model systems may be developed by the end users.

**Table 4. Formulations and Conditions Used to Accelerate Delamination**

| Formulation | 0.9% KCl<br>pH 8.0 | 3% Sodium Citrate<br>pH 8.0 | 20 mM<br>Glycine<br>pH 10.0 |
|---|---|---|---|
| Conditions | 1 h at 121°<br>1 or 2 cycles | 24 h at 80° | 24 h at 50° |

## Screening Strategy for Drug Products

Indicators include the appearance of a pitted, fractured inner surface particularly around the heel of the vial instead of a smooth surface, as well as a number of changes in the test solution, especially increases in $SiO_2$ concentration, the number of subvisible particulates in the solution, and a change in pH.

If the purpose of the glass screening is to determine the suitability of a given glass container for a specific product, the testing proposed in *Table 4* is insufficient. The exposure conditions are too harsh and do not provide a direct link to the product itself. In these instances, accelerated conditions are still relevant, but they must link to the relevant conditions for the given product. For example, if a product will be stored at 5° and the appropriate accelerated conditions are 30°, then testing should occur at 30°. Many products or formulations cannot withstand the elevated temperatures or high pH shown in *Table 4*. In addition, false positive testing results could be obtained because the unusually high temperatures shown in *Table 4* could cause signs of delamination, but moderate exposure at 30° would produce no evidence of glass incompatibility.

Because lower temperatures are required for actual product testing, the duration of testing must be longer, ranging from weeks to months. A larger number of vials also is appropriate for this scenario because the goal of the testing is to ensure the results are representative of the quality of the glass that will be used for the drug product. *Table 5* shows some of the conditions that could be used for testing with a specific product.

**Table 5. Screening Strategy for Glass Vials**

| Stress Test | Water Control | Drug Product Control |
|---|---|---|
| • Vials: washed, depyrogenated<br>• Filled with Stress Test solution<br>• Accelerated time and temperature treatment conditions | • Vials: washed, depyrogenated<br>• Filled with Water for Injection<br>• Autoclave if applicable to Drug Product<br>• Accelerated Drug Product stability storage conditions | • Vials: washed, depyrogenated<br>• Filled with Drug Product<br>• Autoclave if applicable<br>• Accelerated Drug Product stability storage conditions |

# CONCLUSIONS

Evaluation of the internal surface of glass containers begins with the *Surface Glass Test*, which uses water as the extracting medium. A low value is not always an indicator of a durable inner surface if the results are obtained using surface treatments (e.g., ammonium sulfate). Such treatments can lead to a silica-rich inner surface layer that represents a weakened glass structure, and risk of delamination increases when the vial is filled with formulations that contain aggressive agents such as organic acids, EDTA, or solutions that have high ionic strength or high pH. The screening methods and strategies described in this chapter can assist in the evaluation of glass containers from different suppliers or on a lot-to-lot basis and can provide an indication of the propensity of the selected formulation to cause delamination over time. Selection of glass vials intended to contain a drug product with one or more of the formulation risk factors identified in *Table 2* should undergo particular scrutiny.

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# ⟨1661⟩ EVALUATION OF PLASTIC PACKAGING SYSTEMS FOR PHARMACEUTICAL USE AND THEIR MATERIALS OF CONSTRUCTION

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3. GENERAL PRINCIPLES—THE OVERALL ASSESSMENT PROCESS
4. MATERIALS ASSESSMENT: CHARACTERIZATION, SCREENING, AND SELECTION, *USP* ⟨661.1⟩
5. PACKAGING SYSTEM ASSESSMENT AND QUALIFICATION, *USP* ⟨661.2⟩
    5.1 Extractables and Leachables
6. APPLICABILITY AND APPLICATION OF ⟨661.1⟩
    6.1 Applicability
    6.2 Application
    6.3 Description of Plastics Contained in ⟨661.1⟩
7. APPLICABILITY AND APPLICATION OF ⟨661.2⟩
    7.1 Applicability
    7.2 Application

## 1. INTRODUCTION

Drug products can chemically interact with their associated packaging systems and/or the system's plastic materials of construction while the drug product is being manufactured, shipped, stored, and administered. The magnitude of these interactions should not adversely affect the suitability of the packaged drug product, which includes both quality aspects and performance aspects such as efficacy, stability, purity, and compendial compliance.

Suitability for use, as determined by the impact of the interaction between a drug product and its packaging system, is assessed and established via the appropriate testing of the materials of construction, components, and packaging systems. *Plastic Packaging Systems and Their Materials of Construction* ⟨661⟩ establishes the tests and acceptance criteria that are necessary and appropriate for ensuring that such systems are suitable.

## 2. SCOPE

The purpose of this chapter is to communicate the key concepts behind ⟨661⟩ and its related sub-chapters, *Plastic Materials of Construction* ⟨661.1⟩ and *Plastic Packaging Systems for Pharmaceutical Use* ⟨661.2⟩, and to provide additional information and guidance regarding the application and applicability of these chapters.

## 3. GENERAL PRINCIPLES—THE OVERALL ASSESSMENT PROCESS

The objective of *USP* plastic packaging standards is to establish the tests and acceptance criteria to ensure packaging systems do not materially impact the effectiveness of the drug product. Given the complex nature of packaging systems and their development and manufacturing processes, multiple testing procedures are needed to establish their suitability with a specific drug product. The logical development and manufacturing process for packaged drug products, starts with the packaging system's materials of construction, continues with the packaging system itself, and ends with the packaged drug product. This progression forms the basis of a three-stage approach to packaging systems qualification, as illustrated in *Figure 1*.

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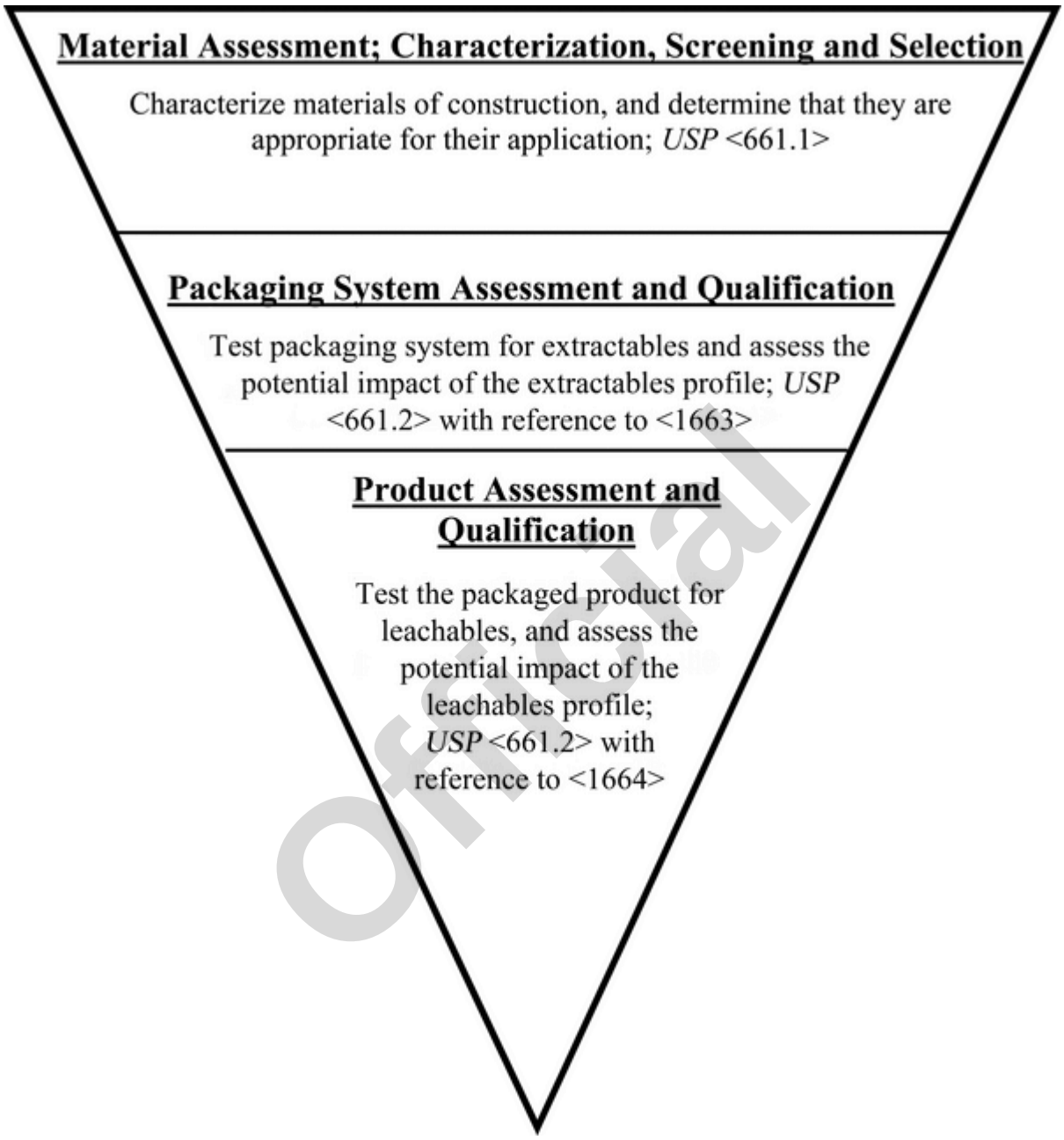


Figure 1. The three-stage process for the characterization and qualification of packaging systems and their materials of construction.

The process for establishing a packaging system's suitability includes: characterization of its materials of construction (ingredients); testing and assessment of the system itself (extractables); and testing and assessment of the packaged drug product (leachables). The initial step of this process involves chemically characterizing candidate materials of construction so the selection of materials can be rationally made and scientifically justified. The intermediate step of system assessment is useful and necessary because it bridges the risk assessment gap between testing starting materials and testing the finished drug product, while providing a means for optimizing drug product testing. This intermediate test is necessary because materials of construction undergo considerable stress, such as exposure to high temperatures, as they are being converted into either components of the packaging system or the packaging system itself. Processing aids and additional additives may be introduced during the manufacturing process for a packaging system, so the extractables profile is likely to be different from, and potentially more complex than, the extractables profiles of its materials of construction. Therefore, the initial hazard identification performed during material selection is appropriately revisited by testing and qualification of the overall packaging system.

Ultimately, the effect the packaging system may have on the drug product end-user is mediated by packaging system-derived substances that are present in the drug product. The third stage of the process includes product assessment, specifically leachables testing of the packaged product and impact assessment, which considers the user's exposure to the leachables.

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## 4. MATERIALS ASSESSMENT: CHARACTERIZATION, SCREENING, AND SELECTION, *USP* ⟨661.1⟩

Testing and characterizing materials of construction for attributes relevant to their suitability provides a rational basis for material selection in designing a packaging system and minimizes the risk that a system made from those materials will be unsuitable. Therefore, the characterization of materials of construction is the first step in the process of developing and qualifying suitable packaging materials. Additionally, chemical characterization data may also provide the basis for effective and appropriate change control.

The intent of ⟨661.1⟩ is to establish whether potential material candidates could adversely affect the quality of packaged drug products. The basic tenet of materials assessment is that knowing the general composition and certain general characteristics of a material of construction allows one to:

- Rationally assess the potential suitability of materials with a degree of certainty that is appropriate for early product development and/or manufacturing.
- Forecast with some degree of accuracy the identity of extractables from that material of construction and from systems that use that material of construction.
- Use the assessment and forecast to establish and justify the use (or rejection) of a particular material in a particular packaging system.

To this end, ⟨661.1⟩ defines a well-characterized plastic material of construction as one whose:

- Identity has been definitively established.
- Biological reactivity has been established.
- General physicochemical properties have been established.
- Extractable elements (when necessary).
- Additives have been quantified.

Chapter ⟨661.1⟩ testing is not a guarantee that packaging systems constructed from plastic materials meeting ⟨661.1⟩ requirements will be suitable for their intended use. Characterization of a material using ⟨661.1⟩ establishes the composition or characteristics of the material, which can be used to determine if the material is an appropriate candidate for use in a packaging system. Nevertheless, ⟨661.1⟩ testing leverages the logical connection between material additives, material extractables, and system extractables, and thus provides a useful indication of the probable suitability issues for materials and systems. The actual qualification of the material occurs when the entire system is qualified for use in a particular application via ⟨661.2⟩ testing.

## 5. PACKAGING SYSTEM ASSESSMENT AND QUALIFICATION, *USP* ⟨661.2⟩

The impact of packaging systems on the chemical composition of packaged drug product can be established in two ways: 1) the packaging system itself can be characterized with respect to substances that can extract from it (extractables); or 2) the packaged drug product can be tested for packaging-derived substances that have leached into the drug product (leachables). In the case of extractables assessment, the impact is predicted based on a relationship that is established (or inferred) between extractables and leachables. In the case of leachables assessment, the impact is specifically measurable, assuming that all the relevant leachables can be discovered, identified, and quantified in the packaged drug product. In either case, ⟨661.2⟩ establishes the tests and acceptance criteria for the packaging system, while referring users to relevant informational chapters (e.g., *Assessment of Extractables Associated with Pharmaceutical Packaging/Delivery Systems* ⟨1663⟩ for extractables and *Assessment of Drug Product Leachables Associated with Pharmaceutical Packaging/Delivery Systems* ⟨1664⟩ for leachables) for insights on how to design and execute relevant studies.

Considering the packaging system as the test article, the intent of ⟨661.2⟩ is to define and delineate the testing needed to produce the data required for establishing the packaging system's suitability. Chapter ⟨661.2⟩ refers to this process of establishing the suitability of packaging systems as chemical suitability for use assessment and notes that a packaging system is suited for its intended use if:

- The packaging system is constructed from well-characterized materials, as established by testing according to ⟨661.1⟩.
- The packaging system's general physicochemical properties have been established.
- The packaging system's biological reactivity has been established.
- The packaging system has been established to be suitable by means of the appropriate chemical suitability for use assessment.
- The packaging system is chemically compatible with the packaged product, as established by appropriate compatibility assessments (e.g., stability studies).

### 5.1 Extractables and Leachables

For high risk dosage forms, in addition to being the basis for toxicological assessments, information about a packaging system's extractables can be used in several ways to optimize finished product testing for leachables. The potential quality or impact of extractables may facilitate identification of leachables that might adversely affect product quality. Such leachables of potential concern would necessarily be among the targeted analytes in testing of a final drug product within its packaging system. The targeting of specific leachables, as opposed to the screening of drug products for unspecified leachables, has significant analytical benefits, including the ability to develop, validate, and utilize test procedures that are appropriately sensitive, specific, and accurate. Extractables (and their accumulation levels in extracts) can be used to forecast the levels of leachables in the finished product, depending on how well the extraction conditions mimic the drug product's composition and actual conditions of clinical use. If the extraction conditions are such that they accelerate and modestly exaggerate the product's clinical use conditions, then the extractables and their levels in the extracts can be extrapolated to estimate the

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maximum levels of leachables in the finished product. If such extractables are assessed for their quality impact, the results of that assessment can also be extrapolated to, and considered to be relevant for, the drug product. Finally, if no adverse impact is found based on the extractables data, then no adverse impact can be inferred for the leachables in the packaged drug product.

***Change to read:***

## 6. APPLICABILITY AND APPLICATION OF ⟨661.1⟩

### 6.1 Applicability

1. The holder of the drug product application and drug product manufacturer [in the case of many over-the-counter products (OTCs), where there is no application] bear primary responsibility and accountability for ensuring that the requirements of the chapters are met. The means by which the holder of the drug product application and drug product manufacturer obtain information to meet the requirement is at the discretion of the holder.
2. The testing required and acceptance criteria for materials of construction contained within ⟨661.1⟩ are relevant to and applicable for all dosage forms, because it is the universal expectation that packaging materials be constructed from well-characterized materials, regardless of the potential interaction between a dosage form. It has been established that the concepts and principles of risk management has a strategic role in designing, implementing, and interpreting effective and efficient material suitability testing and assessments. It is well-established that risk-management tools and principles can be used to define the nature and magnitude of assessment (including testing), where low-risk situations require reduced or alternate assessment (testing) versus high-risk situations noted in *Plastic Materials of Construction* ⟨661.1⟩, *Table 1*, which establishes biological reactivity and chemical tests that differ for low-risk dosage forms (such as oral and topical) versus high-risk dosage forms (such as inhalation and injections).
3. Application of ⟨661.1⟩ to materials of construction used for systems other than packaging systems for finished drug product is beyond the scope of this chapter at the present time, but the concepts and principles of the chapter may be applicable and relevant to other systems and their materials for construction (e.g., manufacturing systems for pharmaceutical products, and packaging/storage systems for drug substances).
4. The scope of ⟨661.1⟩ is materials of construction for packaging systems for finished drug products and ⟨661.2⟩ is packaging systems for finished drug products. A third type of test article is components and if component testing is considered to be necessary, the relevant testing and acceptance criteria for the component are contained within ⟨661.2⟩.

### 6.2 Application

1. There are two means of demonstrating that a plastic material of construction has met the requirements of ⟨661.1⟩. The first is to perform the testing contained within ⟨661.1⟩ and meet the acceptance criteria. The second is the use of the material in a packaging system that meets the requirements of ⟨661.2⟩.
2. The outcome of ⟨661.1⟩ testing is that the tested material of construction has been well-characterized. Characterization data generated during ⟨661.1⟩ testing can be used to support decisions on the proper use of the tested material. The characterization data do not specifically or universally qualify the material for use in packaging systems because the material's use can vary depending on the packaging applications.
3. It is the responsibility of the user of the tested material to decide if the material is appropriate for their intended application. It is the user's expert review of ⟨661.1⟩ test results, coupled with additional information as necessary and appropriate, that establishes whether a well-characterized material is suitable for a specific application.

#### 6.2.1 IDENTIFICATION TESTS

The identification tests required in ⟨661.1⟩ serve the purpose of categorizing a material so that it is properly tested and evaluated against the appropriate acceptance criteria. The acceptance criteria for identification are based on a comparison of the test result obtained for the test material versus the relevant Reference Standard. This comparison is based on the concept of substantial equivalence as opposed to an exact quantitative acceptance criteria.

Establishing substantial equivalence requires that the test results and test material versus Reference Standard be considered to be equivalent. For example, although the infrared (IR) identification acceptance criteria may include wavenumber targets, these targets are not acceptance criteria but rather serve the purpose of establishing the expected general characteristics of the IR spectra. An identification test is considered successfully completed if the analytical results obtained for the test article and the appropriate Reference Standard are substantially equivalent, and where all differences between the test results for the article and the Standard are explained by the nature, processing, and/or composition of the test article.

#### 6.2.2 CHEMICAL TESTING

The chemical testing prescribed in ⟨661.1⟩ is orthogonal: physicochemical tests provide a general overview of extracted substances whereas plastic additives tests address potential organic extractables. It is also the case that chemical testing alone may not demonstrate all potential impacting attributes. Thus, chemical testing is augmented by the orthogonal approach of establishing biological reactivity.

#### 6.2.3 EXTRACTABLE ELEMENTS

Knowledge of the potential extractable elements present in the materials of construction for plastic packaging components is important in establishing that the material is well-characterized. Knowledge of the elements that are likely to be present and

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the concentrations at which they may be observed provides information to determine potential drug product quality. Materials of construction can vary widely in terms of their intentionally and unintentionally added elements, the levels of the elements, and the chemical form of the elements. Because of this variety in composition, it is challenging to provide universally effective and efficient test methodologies for extracted elements (including extraction conditions), lists of elements to target, and reporting requirements. Therefore, it is the material user's responsibility to evaluate the need for extractable elements testing and, if such testing is necessary, to establish and justify the means by which testing is accomplished, taking into account extraction conditions, target elements, extract analysis, and reporting requirements.

In general, however, the following points are relevant:

- Extraction conditions should be such that elements that could be leached into pharmaceutical drug products are fully extracted from the test material. Thus, the combination of extraction solvent(s), extraction temperature, extraction duration, extraction process, and extracted surface area to extraction solution volume should be carefully considered. It is important to remember that the objective is to perform an extraction, not to digest or dissolve the test material. Furthermore, the extraction process should produce an extract that is analytically expedient, meaning that the extract is amenable to analysis by the chosen test methodology with the necessary levels of sensitivity, accuracy, and precision.
- Target elements should minimally reflect those elements that have been intentionally added (e.g., catalyst and processing aids) to the material, those elements that could be unintentionally added to the material (e.g., processing contaminants, ingredient impurities), and those elements of potential toxicological concern, whether they are intentionally or unintentionally added.
- Analytical test methods should be compatible with the extract and should have the specified sensitivity, accuracy, and precision.
- Reporting requirements must reflect the analytical capabilities of the method and the levels at which an extracted element could have an adverse effect on drug product quality. The desired situation is that the test method is capable of producing reliable information at levels near those at which an element could have such an adverse effect. In this case, the reporting requirement is established considering the juxtaposition of analytical capability and potential effect.

### 6.2.4 PLASTIC ADDITIVES

The sole purpose of the tests for plastic additives is to establish which additives are present and to ensure that the levels of these additives are known. This information is relevant because additives are typically a source of extractables and leachables.

In ⟨661.1⟩, the chapter requires that materials be tested for all relevant analytes. Clearly, an analyte will be present in a material if it is intentionally or knowingly added to the material during its production or if testing of the material has revealed the presence of the analyte. Although test methods included in ⟨661.1⟩ may be of sufficiently broad scope to detect all relevant additives, this is not always the case and one cannot rely on these methods to reveal all relevant analytes. It may be the case that the material's vendor has knowledge that may be unavailable to the material's user, which is germane to establishing relevant analytes. It is reasonable to expect that material vendors and users work together to produce a complete and robust list of relevant analytes.

It is reasonable to anticipate that there may be some information that the vendor is not in a position to share with a material's user. Nevertheless, it is in the interest of both the vendor and the user that a material be well characterized and that the characterization include all relevant analytes. Thus, it is strongly recommended that the vendor and the user find a means of establishing all relevant analytes.

### 6.2.5 UNADDRESSED MATERIALS

For the materials that are not currently listed in ⟨661.1⟩ (unaddressed plastics), it is the responsibility of the user to: 1) develop those test methods (e.g., ▲physicochemical▲ (ERR 1-Nov-2020), biological reactivity, and plastic additives) and acceptance criteria; 2) justify those test methods and acceptance criteria, specifically considering their consistency with test methods and acceptance criteria that exist for materials that are listed in ⟨661.1⟩; and 3) possess the test results obtained when the material is tested.

### 6.2.6 CONCEPT OF NON-INTERACTION OF MATERIALS

Testing of materials of construction via ⟨661.1⟩ is predicated on the circumstance that the material will most likely interact with the packaged drug product when the material is used in a packaging system. It is not necessary for a material used in a packaging system to be well-characterized if there is little or no chance of the material and the packaged drug product interacting. Under these conditions the materials of construction would be considered non-interacting and would be exempt from ⟨661.1⟩ and ⟨661.2⟩ testing.

It is relevant to differentiate between "no direct contact" and "non-interacting", where the term "no direct contact" means that the material and the packaged drug product do not come into direct physical contact under the clinical conditions of use.

In certain applications a "no direct contact" material of construction is also a "non-interacting" material of construction, this does not ensure no interaction, especially when the conditions of contact include long durations and/or substantially elevated temperatures. To explain the concepts of "no direct contact" and "non-interacting", consider the following example. An aqueous drug product is packaged in a flexible plastic container, which is placed in a foil overpouch and the combination is terminally sterilized. An adhesive label is then applied to the outside of the foil overpouch.

Here, both the foil overpouch and the label are examples of "no direct contact", because there is at least one physical barrier (the primary container) between the packaged drug product and these two items. However, if the flexible plastic primary container is permeable, the foil overpouch can be considered a "potentially interacting" component, as substances from the overpouch could migrate through the primary packaging, especially under the high-temperature conditions of terminal sterilization. The label is a "non-interacting" component because 1) the foil overpouch is impermeable and 2) the label is applied after the thermal stress associated with terminal sterilization.

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The difference between a “potentially interacting” and “non-interacting” “no direct contact” component is the permeability of the barrier that separates the “no direct contact” components from the drug product. If the barrier is incomplete, then the component (and its materials of construction) is “potentially interacting” and the materials should be tested per ⟨661.1⟩. If the barrier is complete, then the component (and its materials of construction) is “non-interacting” and the materials need not be tested per ⟨661.1⟩.

## 6.3 Description of Plastics Contained in ⟨661.1⟩

### 6.3.1 CYCLIC OLEFINS

Cyclic olefin copolymers are manufactured by the copolymerization of cyclic monomers, such as tetracyclododecene or norbornene, with an olefin such as ethylene or propylene; or by ring-opening metathesis polymerization (ROMP) of cyclic monomers, followed by hydrogenation facilitated by Ziegler-Natta catalysts to produce cyclic olefin polymers. Cyclic olefins are commonly supplied in pellet form and are suited for standard polymer processing techniques such as extrusion, injection molding, injection blow molding, compression molding, thermoforming, and others. As they are amorphous, and given their high purity, moisture barrier, clarity, and sterilization compatibility, cyclic olefins are an excellent alternative to glass in a wide range of medical products, including packaging. Cyclic olefins exhibit good chemical resistance and are generally considered to be of high purity with low levels of extractables. Nevertheless, cyclic olefins may contain residual processing aids, colorants, and antioxidants.

### 6.3.2 POLYAMIDE 6

Polyamide 6 is synthesized by ring-opening polymerization of caprolactam. Hydrolytic or catalytic ring-opening polymerization of caprolactam produces epsilon-aminocaproic acid, which readily condenses to polyamide 6 at high temperatures and under a vacuum. The high strength, flexibility, and chemical resistance of crystalline polyamide 6 make it well suited for pharmaceutical applications ranging from soft and flexible tubing to catheters and containers to stiff components for surgical and dental instruments. Entities present in commercial polyamide 6 include residual monomers, residual reaction intermediates, residual catalysts (copper and chromium oxides) and activators (acetyl lactams, oxazolines, ethylenebisamides, and isocyanates) and certain additives including stabilizers (mixtures of metal and alkali metal halides), processing aids (nucleating agents and lubricants), and modifiers (chain extenders, plasticizers, and impact modifiers).

### 6.3.3 POLYCARBONATES

Polycarbonates are a group of thermoplastic polymers containing carbonate groups in their chemical structures. In interfacial polymerization, the polycarbonate material is produced by the reaction of bisphenol A (BPA) and phosgene. This process is being replaced by an alternative, termed “melt polymerization”, which entails transesterification from BPA and diphenyl carbonate, thus avoiding the use of phosgene. Entities present in commercial polycarbonates include residual monomers, solvents or catalysts (triethylamine or lithium halides, and hydroxides or aluminum hydrides), processing aids (e.g., mold release agents), UV stabilizers, impact modifiers, flame retardants, colorants, and sterilization stabilizers (free radical scavengers including propylene glycol, aromatic bromate, or disulfide compounds).

### 6.3.4 POLYETHYLENES

High- and low-density polyethylenes are long-chain ethylene-based polymers synthesized under controlled conditions of heat and pressure with the aid of catalysts from NLT 85.0% ethylene and NLT 95.0% total olefins. Other olefin ingredients that are most frequently used are butene, hexene, and propylene. Low-density polyethylene (LDPE) contains many long-chain branches along the polymer backbone, preventing the alignment and packing of the chains, thus forming a low-density material. Linear low-density polyethylene (LLDPE) contains several short chains along the polymer backbone that prevent the alignment and packing of the polymer chains, thus creating a poor crystalline material. High-density polyethylene (HDPE) contains relatively few side chains, allowing the polymer backbone to align and pack together, thus forming a crystalline, high-density plastic. High-, low-, and linear low-density polyethylene all have an IR absorption spectrum that is distinctive for polyethylene, and each possesses characteristic thermal properties. High-density polyethylene has a density between 0.941 and 0.965 g/cm$^3$. Low-density polyethylene has a density between 0.850 and 0.940 g/cm$^3$. Additives are added to the polymer in order to optimize its chemical, physical, and mechanical properties, thereby rendering it suitable for its intended use. These additives may include nucleating agents, clarifying agents, antioxidants, colorants, lubricants, antiblocking agents, and others. These additives typically are present individually in the polyethylene at levels of 0.01–0.3 weight %, and the total levels of the antioxidants typically are < 0.3%. Other additives, specifically amides and stearates, typically are present in polyethylenes individually at levels of NMT 0.5 weight %. Polyethylene materials that provide light protection can contain as much as 4% by weight titanium oxide.

### 6.3.5 POLYETHYLENE TEREPHTHALATE AND POLYETHYLENE TEREPHTHALATE G

Polyethylene terephthalate (PET) polymers are long-chain crystalline [e.g., polyethylene terephthalate glycol-modified (PETG)] polymers prepared by the condensation of ethylene glycol with dimethyl terephthalate or terephthalic acid. PET copolymer resins are prepared in a similar way except that they may also contain a small amount of either isophthalic acid (NMT 3 mole %) or 1,4-cyclohexanedimethanol (NMT 5 mole %). Polymerization is conducted with the aid of catalysts and stabilizers. PET polymers may contain silica or silicates (NMT 0.5% by weight) and may contain colorants.

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#### 6.3.6 POLY(ETHYLENE-VINYL ACETATE)

Poly(ethylene-vinyl acetate) polymers are typically obtained by copolymerization of mixtures of ethylene and vinyl acetate. Poly(ethylene-vinyl acetate) used in containers has a defined quantity of vinyl acetate of NMT 25%. Poly(ethylene-vinyl acetate) used in tubing has a defined quantity of vinyl acetate of NMT 30%. A certain number of additives are present in the polymer to optimize its chemical, physical, and mechanical properties, thereby rendering it suitable for its intended use. These additives may include antioxidants, amides, stearic acid salts, a source of base (calcium carbonate or potassium hydroxide), and inorganic fillers. Poly(ethylene-vinyl acetate) can contain NMT 3 antioxidants with individual levels of NMT 0.2 weight %. This material may also contain: 1) oleamide and/or erucamide at individual levels of 0.5 weight %; 2) calcium stearate, zinc stearate, or both at levels NMT 0.5 weight %; 3) sources of base at levels NMT 0.5 weight %; and 4) colloidal silica at 0.2 weight %.

#### 6.3.7 POLYPROPYLENE

Propylene polymers are long-chain polymers synthesized from propylene or other olefins, for example, ethylene or butene, under controlled conditions of heat and pressure with the aid of catalysts. A certain number of additives are added to the polymer in order to optimize its chemical, physical, and mechanical properties, thereby rendering it suitable for its intended use. These additives may include nucleating agents, clarifying agents, antioxidants, colorants, lubricants, antiblocking agents, and others. These additives typically are present individually in the polypropylene at levels of 0.01–0.3 weight %, and the total levels of the antioxidants typically are less than 0.3%. Polypropylene that provides light protection can contain as much as 4% by weight titanium dioxide.

#### 6.3.8 POLYVINYL CHLORIDE

Polyvinyl chloride (PVC) is produced by polymerization of the vinyl chloride monomer in a process that uses initiators that break down to start the radical chain reaction. The polymerization reactions used to produce PVC are designed to produce levels of residual vinyl chloride monomer of <1 ppm on a weight basis. Polyvinyl chloride may contain NMT 15% by weight of: 1) copolymers based on acrylic and/or methacrylic acids and/or their esters, 2) styrene, and/or 3) butadiene.

To obtain the required mechanical and stability characteristics, materials based on PVC may contain: 1) epoxidized oils at levels up to 8 weight %; 2) calcium and/or zinc salts of long chain fatty acids at levels NMT 1.5 weight %; 3) liquid paraffin, waxes, and/or hydrogenated oils at individual levels NMT 2 weight %; 4) nonylphenyl phosphite-type compounds at levels NMT 1 weight %; 5) sorbitol and macrogel esters, NMT 1.5 weight % each; 6) stabilizers at levels of either 0.25 weight % (for tin-based stabilizers) or 1 weight % (for other stabilizers); and 7) colorants, pigments, or opacifiers. The additives used and their allowed levels are dictated by the application of the polyvinyl chloride.

#### 6.3.9 POLYVINYL CHLORIDE, PLASTICIZED

PVC polymers are long-chain vinyl chloride polymers synthesized from vinyl chloride monomers via free radical polymerization. Various additives are compounded into PVC to provide the materials with properties that render it suitable for its intended use. These additives may include heat stabilizers, primary and secondary plasticizers, stabilizers, impact modifiers, lubricants, pigments, and others. These additives typically are present individually in the PVC at levels ranging from 0.1 to 45 weight %. The additives used and their allowed levels are dictated by the application of the plasticized PVC.

## 7. APPLICABILITY AND APPLICATION OF ⟨661.2⟩

### 7.1 Applicability

1. The holder of the drug product application and drug product manufacturer [in the case of many OTCs, where there is no application] bear primary responsibility and accountability for ensuring the requirements of the chapter are met. The means by which the holder of the drug product application and drug product manufacturer obtain information to meet the requirement are at the discretion of the holder.
2. The scope of ⟨661.2⟩ is packaging systems for finished drug products. If component testing is considered to be necessary, the relevant testing and acceptance criteria for components are contained within ⟨661.2⟩.
3. Application of ⟨661.2⟩ to systems other than packaging systems for finished drug products is beyond the scope of these chapters at the present time, but the concepts and principles of these chapters may be applicable and relevant to other systems.

### 7.2 Application

1. Chapter ⟨661.2⟩ requires that all packaging systems be demonstrated to be suitable by performing a chemical suitability for use assessment but does not specify the details of the chemical suitability for use assessment process acceptance criteria. Rather, for the chemical suitability for use assessment, ⟨661.2⟩ discusses the use of a risk-based approach in determining the level of testing that is deemed necessary for a packaging system. It is currently a regulatory expectation that extractables and leachables testing be performed for high risk dosage forms. Thus, ⟨661.2⟩ references chapters ⟨1663⟩ for extractables and ⟨1664⟩ for leachables, thereby providing users with a means for designing and implementing an effective, efficient, risk-based, and more or less customized extractables or leachables assessments that comply with regulatory requirements.

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2. Chapter ⟨661.2⟩ provides holders of packaging system or drug product applications and/or packaged drug products manufacturers with the flexibility to operate within the context of their own specific situation and their own specific risk-management philosophy. The trade-off for having such flexibility is that it is the responsibility of the holders and manufacturers to justify their test methods and acceptance criteria. It is proper and appropriate that the justification exists and that it be judged (and approved) on the basis of its individual scientific and risk-management merits.

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# ⟨1663⟩ ASSESSMENT OF EXTRACTABLES ASSOCIATED WITH PHARMACEUTICAL PACKAGING/DELIVERY SYSTEMS

## PURPOSE

This general information chapter presents a framework for the design, justification, and execution of an extractables assessment for pharmaceutical packaging and delivery systems. The chapter establishes critical dimensions of an extractables assessment and discusses practical and technical aspects of each dimension. Although intended to be helpful and generally applicable, the chapter is for informational purposes and does not establish specific extraction conditions, analytical procedures, or mandatory extractables specifications and acceptance criteria for particular packaging and delivery systems or dosage forms; nor does it delineate every situation in which an extractables assessment is required. It is not possible for a general discussion of extractables to anticipate and cover all situations where an extractables assessment might be required. Designing an individual extractables assessment is a process that balances sound science, prudent resource allocation, and effective risk management. Achieving this balance is the responsibility and obligation of the drug product manufacturer and assumes due consideration of all applicable legal and regulatory requirements. The principles and best demonstrated practices outlined in this general chapter represent a consensus interpretation of sound science and can therefore be applied to any situation in which an extractables assessment is required for pharmaceutical application.

## KEY TERMS

This general chapter uses the following key terms listed below (*1*, *2*; also see *Packaging and Storage Requirements* ⟨659⟩). Note that the terms *Packaging System*, *Packaging Component*, *Primary Packaging Component*, *Secondary Packaging Component*, and *Materials of Construction* are also defined in ⟨659⟩, and the definitions below are intended for clarification purposes within the context of this chapter and are not intended to supersede those provided in ⟨659⟩.

*Packaging System (also referred to as a container–closure system):* The sum of packaging components and materials that together contain and protect the product. This includes primary, secondary, and tertiary packaging components.

*Delivery System:* The sum of components and materials that are used to transport a drug product from its packaging to the point of administration into the patient. For example, an administration set is a delivery system that is used to transfer liquid drug products from their plastic packaging system to the site of administration to the patient. It is noted that in some cases, the packaging system itself may perform the delivery function.

*Container:* A receptacle that holds an intermediate compound, active pharmaceutical ingredient, excipient, or dosage form and is in direct contact with the product.

*Closure:* A material that seals an otherwise open space on a container and provides protection for the contents. It also provides access to the contents of the container.

*Packaging Component* is any single part of the package or container–closure system including the container (e.g., ampuls, prefilled syringes, vials, bottles); closures (e.g., screw caps, stoppers); ferrules and overseals; closure liners; inner seals; administration ports; overwraps; administration accessories; labels; cardboard boxes; and shrink wrap.

*Primary Packaging Component* is a packaging component that is in direct contact or may become in direct contact with the product (e.g., IV bag).

*Secondary Packaging Component* is a packaging component that is in direct contact with a primary packaging component and may provide additional protection for the product (e.g., overpouch or dustcover for an IV bag).

*Tertiary Packaging* is a packaging component that is in direct contact with a secondary packaging component and may provide additional protection of the product during transportation and/or storage (e.g., shipping carton for an overpouched IV bag).

*Ancillary Component* is a component or entity that may come into contact with a tertiary packaging component during the distribution, storage, and transportation of the packaged product (e.g., pallets, skids, shrink wrap).

*Packaging Materials of Construction* are substances used to manufacture packaging components. These are also referred to as *Raw Materials*.

*Extractables* are organic and inorganic chemical entities that are released from a pharmaceutical packaging/delivery system, packaging component, or packaging material of construction and into an extraction solvent under laboratory conditions. Depending on the specific purpose of the extraction study (discussed below), these laboratory conditions (e.g., solvent, temperature, stoichiometry, etc.) may accelerate or exaggerate the normal conditions of storage and use for a packaged dosage form. Extractables themselves, or substances derived from extractables, have the potential to leach into a drug product under normal conditions of storage and use and thus become leachables.

*Leachables* are foreign organic and inorganic chemical entities that are present in a packaged drug product because they have leached into the packaged drug product from a packaging/delivery system, packaging component, or packaging material of construction under normal conditions of storage and use or during accelerated drug product stability studies. Because leachables are derived from the packaging or delivery system, they are not related to either the drug product itself or its vehicle and ingredients. Leachables are present in a packaged drug product because of the direct action of the drug product on the source of the leachable. Thus leachables are typically derived from primary and secondary packaging, as the primary and secondary packaging can serve as a barrier between the packaged drug product and other potential sources of foreign chemical entities (such as tertiary packaging and ancillary components). In certain circumstances, packaging may directly contact the patient under typical clinical conditions of use (for example, the mouthpiece of a metered dose inhaler). As a result of this contact, patients may be exposed to leachables from the packaging without the action of the drug product. Leachables are typically a subset of extractables or are derived from extractables.

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*Migrants* are also foreign organic and inorganic chemical entities that are present in a packaged drug product because they have leached into the packaged drug product from a packaging/delivery system, packaging component, or packaging material of construction under normal conditions of storage and use or during accelerated drug product stability studies. However, migrants are differentiated from leachables by the circumstance that migrants accumulate in the packaged drug product after the migrant has crossed a physical barrier, such as that provided by primary and secondary packaging. Because migrants cross a physical barrier, they are not present in the packaged drug product due to direct action of the drug product on the source of the migrant because the barrier prevents such direct action. Thus migrants are derived from secondary and tertiary packaging and ancillary components. Regardless of whether a substance is a leachable or a migrant, it is still a foreign substance in the packaged drug product and thus it must be impact assessed in the same manner. However, as the means by which a leachable and a migrant become entrained in a packaged drug product may be different, extractables studies meant to address leachables may be designed and implemented differently than extractables studies meant to address migrants.

*Extraction Studies* are the overall laboratory processes required in order to create extractables profile(s) of particular pharmaceutical packaging/delivery systems, packaging components, or materials of construction. Extraction studies are also referred to as *Controlled Extraction Studies*.

*Characterization* is the discovery, identification, and quantitation of each individual organic and inorganic chemical entity present in an extract above a specified level or threshold. Such thresholds can be based on patient safety considerations, materials considerations, the capabilities of analytical technology, etc.

*Scouting* is the process of acquiring general chemical information that provides insight into the nature and magnitude of extractables.

*Discovery* is the process of searching for, and ultimately finding, individual organic and inorganic chemical entities present in an extract.

*Identification* is the process of assigning a molecular structure to an organic extractable, or assigning constituent elements in the case of an inorganic extractable.

*Quantitation* is the process of measuring the level, or concentration, of an individual organic or inorganic chemical entity contained in an extract.

*Extractables Profiles* are qualitative and/or quantitative analytical representations of the extractables content of a particular extracting medium and set of laboratory extraction conditions.

*Leachables–Extractables Correlations* are established when observed drug product leachables are linked both *qualitatively* and *quantitatively* to extractables from associated packaging/delivery systems, packaging components, or materials of construction.

*Safety Concern Threshold (SCT)* is the threshold below which a leachable has a dose so low that it presents negligible safety concerns from carcinogenic and noncarcinogenic toxic effects.

*Analytical Evaluation Threshold (AET)* is the threshold at or above which a leachable should be characterized and reported for toxicological assessment. The AET can be mathematically derived from the SCT (or other threshold concepts) based on factors that include the dosing parameters of the drug product. When an extraction study is performed for the purpose of estimating the accumulation levels of leachables, the AET may be applicable to extractables as well as leachables. The concept of the AET is discussed in greater detail in *Assessment of Drug Product Leachables Associated with Pharmaceutical Packaging/Delivery Systems* ⟨1664⟩.

As noted, additional terminology and associated definitions are available (*1*, *2*, and ⟨659⟩).

## SCOPE

The scientific principles and best-practices described in this general chapter are intended to apply to any extractables assessment or extraction study of pharmaceutical packaging/delivery systems, packaging components, or materials of construction; the results of which are intended for establishing extractables profiles. Extractables profiles can be used in a variety of pharmaceutical development and manufacturing applications, including the characterization, selection, and qualification of materials of construction, packaging components or packaging/delivery systems; establishing leachables–extractables correlations; and/or simulating worst-case drug product leachables profiles. When appropriate, extractables profiles can also be used in establishing leachables–extractables correlations as described in *Assessment of Drug Product Leachables Associated with Pharmaceutical Packaging/Delivery Systems* ⟨1664⟩. These scientific principles and best practices can also be applied to materials of construction and functional components of equipment used to manufacture drug substances and drug products; e.g., filters, tubing, and tanks. In addition, these principles and best practices can be applied to materials of construction for the medical device component of a combination product (*2*), with appropriate consideration of the guidances and regulations that apply specifically to medical devices. These scientific principles and best practices apply to all organizations and individuals involved in the manufacture of drug substances, drug products, and in their stability studies, including but not limited to:

- Manufacturers of drug substances and drug products for human and veterinary use where manufacturing may involve operations at the applicant holder's facilities (i.e., facilities that belong to the holder of an approved New Drug Application or Abbreviated New Drug Application) or at those of a contractor for the applicant holder
- Manufacturers of combination products
- Packaging operations by the manufacturer or a designated contractor for the application holder
- Repackaging operations in which the drug product may be owned by an organization other than the primary manufacturer

Manufacturers and fabricators of pharmaceutical packaging/delivery systems, packaging components, and materials of construction may also apply these scientific principles and best-practices as appropriate.

## BACKGROUND INFORMATION

During the course of manufacturing, packaging, storage, distribution, and administration; dosage forms and their constituents can contact components and materials of construction of manufacturing and packaging equipment, and primary

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and secondary packaging components and systems. Such contact may result in interactions between the dosage form and these components and materials. One such interaction is the migration or leaching of substances from any of these components and materials into the dosage form with subsequent delivery to the patient during drug administration. Patients also can be directly exposed to substances via direct contact with the packaging/delivery system during drug administration. Leachables, which can include both organic and inorganic chemical entities with wide chemical diversity, are of concern due to their potential safety risk to patients and potential compatibility risks for the drug product (e.g., drug substance interaction/degradation, pH change, appearance change, particle formation, protein aggregation/structure change, etc.). In order to assess these risks and manage the potential issues posed by leachables, it is necessary to know the identities and the levels to which leachables will accumulate in the finished drug product over its shelf-life. These two pieces of information can be used to establish the magnitude of patient exposure (dose) and therefore the safety risk posed by an individual leachable, as well as the likelihood of any compatibility issues involving the drug product.

Regulatory guidelines and various best-practice recommendations state that assessment of the potential impact of contact between a component or material and a final dosage form involves evaluating the final dosage form with respect to leachables. This assessment can include a migration or leachables study whose purpose is to discover, identify, and quantitate leachables that have migrated from the contacted system, components, or materials and accumulated in the dosage form. Alternatively, this assessment may involve performing a simulation extraction study, when use of such a study in lieu of a migration study can be justified. There are many science-based and practical reasons why such a leachables assessment typically does not stand alone as the single means of assessment. Since the pharmaceutical packaging/delivery system is the primary source of potential leachables, it is generally appropriate that any leachables assessment be preceded by an extractables assessment performed on the packaging/delivery system, its primary and certain critical secondary packaging components that are noncontacting but potentially interacting, and/or packaging and delivery system materials of construction; consistent with regulatory guidelines and best-practice recommendations. Such an extractables assessment can also be performed on particular components and/or materials of construction of manufacturing and packaging equipment, as well as certain tertiary packaging components, that are deemed of high leaching potential or have been implicated in an identified leachables problem with a particular drug product.

Extractables assessments can be used to:

- Characterize packaging/delivery systems, packaging components, combination product medical device components, manufacturing components, and their various materials of construction
- Facilitate the timely development of safe and effective dosage form packaging/delivery systems, manufacturing systems and processes by assisting in the selection of components and materials of construction
- Understand the effects of various manufacturing processes (e.g., sterilization) on packaging components and their potential leachables
- Establish the worst-case potential leachables profile in a manner which facilitates leachables studies, the development of leachables specifications and acceptance criteria (should these be required), and the safety evaluation/qualification of potential and actual leachables
- Establish the worst-case potential leachables profile in a manner which facilitates the safety evaluation/qualification of probable leachables when it is not scientifically possible to determine actual leachables
- Facilitate the assessment of patient exposure to chemical entities resulting from direct contact between a patient's body tissue(s) (e.g., mouth, nasal mucosa) and a packaging or combination product medical device component (e.g., a metered dose inhaler's plastic actuator/mouthpiece)
- Facilitate the establishment of qualitative and quantitative leachables–extractables correlations
- Facilitate the development of extractables specifications and acceptance criteria (if these are required) for packaging components, combination product medical device components, and materials of construction
- Facilitate investigations into the origin(s) of identified leachables whose presence causes quality and/or safety issues (such as out-of-specification results) for a marketed product

In these ways, extractables assessments can support Quality by Design (QbD) principles for the development and manufacture of pharmaceutical packaging/delivery systems and drug products. Note that although characterization of packaging/delivery systems and materials is a goal of many extractables assessments, regulatory guidances and best-practice recommendations clearly stress that extractables assessments also serve as investigations into potential leachables (*1*–*5*).

As stated previously, it is not a goal of this chapter to identify each case in which an extractables assessment is required for packaging/delivery systems, individual packaging components, or materials of construction for any particular type of dosage form. This is the responsibility of the Holder of the NDA (applicant holder), and assumes appropriate consideration of applicable regulatory guidance documents. Rather, this chapter addresses the question "If an extractables assessment is required, what are the scientific principles and best demonstrated practices under which it should be accomplished?"

As performing an extractables assessment involves the processes of discovery and identification, an extractables assessment can be facilitated by knowledge about the test article, especially its composition. Thus it is strongly recommended that the test article assessor and the test article vendor collaborate in such a way that the test article assessor has access to critical information which will aid in the design and implementation of an effective and efficient extraction study. Additionally, it is noted that characterization of the test article per *Plastic Materials of Construction* ⟨661.1⟩ will provide information that is useful in the design and implementation of extraction studies.

Achieving the objectives of an extractables assessment requires performance of an extraction study in order to create extractables profiles. An extraction study has two critical dimensions: laboratory generation of the extract (extraction) and testing the extract (characterization).

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# GENERATING THE EXTRACT

## General Concepts and Critical Experimental Design Parameters

Extractables are derived from a variety of sources and exhibit extensive chemical diversity. Primary sources of extractables include:

- Chemical additives in individual elastomeric/polymeric packaging components and raw materials, including impurities in these additives
- Chemical entities and additives that are present in packaging components composed of glass and metals
- Entities related to the dissolution of the packaging component itself (e.g., iron extracted from a stainless steel material, silicon extracted from glass)
- Monomers and higher molecular weight oligomers derived from incomplete polymerization
- Migrants from secondary and tertiary packaging components, such as inks, label adhesives, and volatiles from cardboard shipping containers, plastic storage bags, and other shipping aids such as wooden pallets
- Surface residues, such as heavy oils and degreasing agents on metal canisters and containers
- Chemical substances on the surfaces of component fabrication machinery or other drug product manufacturing systems, such as mold release agents, and antistatic and antislip agents
- Chemical additives, monomers/oligomers, impurities, etc., in various parts of component fabrication machinery or other drug product manufacturing systems

As noted above, the chemical diversity of extractables is significant. For example, the chemical additive category *antioxidants* includes hindered phenols, secondary aromatic amines, hindered amines, organosulfur compounds, organophosphorus compounds, and other chemical classes.

Extraction is a process of treating a material with a solvent to remove soluble substances. Extraction is a complex process influenced by time, temperature, surface area to volume ratio (i.e., stoichiometry), extraction medium, and the phase equilibrium of the material (*3*). There are two reasons why an extraction study is the necessary and appropriate means of accomplishing the various objectives of an extractables assessment. First, there is no other viable analytical alternative. Characterizing a material for potential leachables in its natural solid state is a goal of modern analytical chemistry rather than an accomplishment. Second, even if a direct characterization could be accomplished, it would at best only establish the identities and levels of chemical entities present in the material, and not assess the leaching characteristics of these chemical entities. A compositional assessment does not take into account any chemical reactions that can alter the molecular structures of potential leachables over the dosage form's lifecycle. For example, in the case of phenolic antioxidants that are leached by an aqueous drug product, hydrolysis and oxidation products can accumulate in the drug product. The only viable means for producing data related to leaching is to use a process such as laboratory extraction that is mechanistically similar to leaching.

The design of an extraction study is dictated by the purpose of the extractables assessment and the question(s) being asked, as well as the available information regarding the chemical composition of the test article(s) to be extracted. Extraction studies can be designed to answer questions such as:

- What are the chemical additives in a particular packaging component or material of construction?
- What are the maximum accumulations of chemical additives from a particular packaging component into the dosage form?
- What are the likely contents of an end-of-shelf-life drug product leachables profile?

Addressing each of these questions requires a particular set of parameters, such as extraction time, extraction temperature, extracting solvent, extraction technique, sample surface area to extracting solvent volume ratio, etc. Clearly, the intent of the extractables assessment must be established before the study design is finalized. For example, if the purpose of the extraction study is to simulate a worst-case leachables profile, then the study can be termed a simulation study that should produce an extract that:

- Contains all the substances (extractables) that could leach into the final product at levels considered potentially significant
- Contains these extractables at concentrations that are greater than or equal to the maximum concentration that these chemical entities will accumulate in the drug product as leachables at any time during the shelf-life
- Is generated more efficiently and in less time than that required for a drug product leachables study
- Is amenable to chemical analysis

The concept of a simulation study is addressed in greater detail in *Assessment of Drug Product Leachables Associated with Pharmaceutical Packaging/Delivery Systems* ⟨1664⟩. The means by which an extraction process is accomplished are reflected in the juxtaposition of several experimental parameters including:

- The chemical nature of the extracting media
- The time duration of the extraction process
- The temperature and pressure at which the extraction is performed
- The stoichiometry of the extraction process (extracted surface area per unit volume of extracting solution)
- The mechanism or process by which the extraction is accomplished

Extraction processes have been described as "accelerated", "aggressive", "exhaustive", "vigorous", "harsh", and so on, and for medical device studies certain of these terms have been defined. In general, extraction processes should allow completion in a reasonable time frame but should not be so aggressive that they alter the qualitative and/or quantitative nature of the resulting extractables profile. The most aggressive extraction conditions are reserved for the quantitative determination of chemical additive contents in components and materials. Because such studies are intended to quantitate specific known chemical additives and not to simulate a drug product leachables profile, it is acceptable to use extraction conditions which

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disrupt or dissolve the component or material being extracted, and thus to alter the resulting extractables profile, while recovering the target additive(s) without loss or chemical decomposition.

## Chemical Nature of the Extracting Medium

Of all the parameters involved in generating the extract, the extracting medium is the most critical because it is the extracting medium that accomplishes the extraction, and all other parameters merely facilitate the extraction. Establishing and justifying the extracting medium (or media) is both straightforward *strategically* and complex *tactically*. *Strategically*, if the purpose of a particular extraction study is, for example, to simulate a worst-case leachables profile, then the ideal situation is for the extracting solvent to have a similar or greater propensity to extract substances as the formulation, thus obtaining a similar qualitative and quantitative extractables profile. This is clearly stated in regulatory guidances and best practice recommendations (*1*, *4*, *5*). Therefore, the most logical *tactic* for this simulation study is to use the formulation itself as the extracting medium and in the absence of complicating factors, such an approach is recommended. However, in certain cases the use of the formulation as an extracting medium complicates extract characterization to such an extent that it is impractical. The various guidances and recommendations suggest that if the use of the drug product as the extracting solvent is not feasible, then the drug product vehicle, or placebo, could be used as an effective extracting medium. This recommendation is derived from the fact that the drug substance itself does not typically create the "leaching power" of a drug product but rather that it is the formulation's ingredients (drug product vehicle) that establish the drug product's ability to leach substances from a contacted material.

When circumstances require that an extraction study must be accomplished with a simulating solvent(s), it is necessary to establish and justify the composition of these solvents. In order to accomplish this objective, one must consider all the physicochemical characteristics of a formulation and/or simulating solvent that influence its "extracting power". In certain circumstances, the formulation is sufficiently simple that the critical characteristics can be readily delineated and simulated. For example, the extracting power of polar aqueous drug products consisting of soluble ingredients (such as an injectable with a drug substance, buffers, and diluent) is, for organic extractables, driven primarily by drug product pH. In such a circumstance, simulating the drug product pH with an analytically viable buffer system for the extraction study may be appropriate and justifiable. For inorganic extractables, utilization of a simulating solvent having similar metal-chelating properties as the drug product vehicle may also be appropriate and justifiable. It may also be the case that largely non-polar drug products can be readily simulated with analytically expedient organic solvents. For example, chlorofluorocarbon and hydrofluoroalkane propellants used in metered dose inhalers (MDIs) can be simulated with dichloromethane as an extracting solvent and isopropanol can be used to simulate ethanol, a common co-solvent in MDI formulations.

Many drug products are compositionally intermediate between the polar and nonpolar examples just discussed. Examples of such products include "aqueous" drug products that contain stabilizers, solubilizing agents, chelating agents and buffers, lipid-containing products, and biotechnology products containing proteins, peptides, and blood-derived products. Such products have a characteristic polarity which establishes their "extracting power". Thus, an appropriate simulating solvent will have a polarity that matches that of the drug product. Binary mixtures of miscible solvents (such as alcohol/water) have been utilized as simulating solvents for these types of drugs product.

It may be that a single simulating solvent cannot be established and justified for a specific drug product, or that the focus of the extractables assessment is a material or system that will be characterized for use with multiple, compositionally diverse drug products. In such circumstances, the drug product's ability to leach chemical entities from a packaging system can be established based on the use of multiple extracting solvents, each of which addresses one (or more) of the extracting "mechanisms" that are relevant to the drug product (or drug products) under investigation. The use of multiple solvents is consistent with industry-driven best-practice recommendations for drug products that have a relatively high risk of dosage form interaction with the packaging system and a relatively high safety risk relative to the route of administration (e.g., inhalation aerosols and solutions, injectables and injectable suspensions) (*1*). Therefore, the use of multiple solvents (or extracting media) with different polarities, pH, ionic strength, or extracting powers, is recommended for high risk dosage form packaging system components and materials requiring extraction studies in order to simulate a drug product leachables profiles (see *Table 1*). If the goal of an extractables assessment is materials characterization, then simulating a drug product vehicle is both unnecessary and undesirable since this goal requires qualitatively and quantitatively efficient extractions. Such extractions generally are only achieved with relatively powerful organic solvent systems capable of softening, swelling, or dissolving the material's polymer matrix and releasing quantitative levels of additives and other chemical entities.

**Table 1. Possible Extracting Media Relative to Particular Packaging Components**

| Packaging Component | Possible Extracting Media[a] |
|---|---|
| MDI valve elastomer seal (MDI formulation contains 1,1,1,2-tetrafluoroethane and ethanol) | Nonaqueous solvents (e.g., Dichloromethane Isopropanol Hexane)[b] |
| Dry powder inhaler mouthpiece | Water (unbuffered)<br>Isopropanol[c] |
| Small-volume parenteral vial rubber stopper (aqueous formulation buffered at pH 6.5) | Water (pH 5.2)<br>Water (pH 9.5)<br>Isopropanol:water (50:50)[d] |
| Large-volume parenteral plastic bag (aqueous formulation buffered at pH 7.2) | Water (pH 5.2)<br>Water (pH 9.5)<br>Isopropanol:water (50:50)[d] |

[a] The possibilities listed in *Table 1* are provided for example only and should not be interpreted as standard practice recommendations.
[b] These extraction media reflect the varying polarities of the organic solvents used in MDI formulations.
[c] These extraction media reflect both the hydrophilic and lipophilic character of human saliva and allow materials characterization.

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[d] These extraction media reflect the chemical nature of the formulation. Using media whose pH range encompasses, and slightly exceeds, the pH limits of the product addresses the potential effect of pH on the extractables profile. The use of an aqueous mixture containing an organic solvent takes into account the possible presence of formulation additives such as solubilizing agents that can influence the leaching power of the formulation. The specific organic solvent used and its proportion in the extracting medium depends on the specific chemical nature of the formulation and on practical issues associated with testing the extract.

If the goal of an extractables assessment is materials characterization, then simulating a drug product vehicle is both unnecessary and undesirable since these goals require qualitatively and quantitatively efficient extractions. Such extractions generally are only achieved with relatively powerful organic solvent systems capable of softening, swelling, or dissolving the material's polymer matrix and releasing quantitative levels of additives and other chemical entities.

## Extraction Time and Temperature

Extraction time and temperature are critical factors in the extraction process. Although the nature of the extraction solvent establishes the magnitude of the extraction (i.e., the amount of substances that can be extracted from a material at equilibrium), the combination of extraction time and temperature establishes the magnitude of the driving force and the degree to which equilibrium is actually achieved. In a simulating extraction study the purpose of elevated temperature is to increase the extraction rate, so that a short experimental time may simulate longer leaching times (*1*, *5*, and *6*).

Because extraction is a diffusion process, the relationship between the diffusion rate and temperature can be expressed empirically by the Arrhenius equation. The mathematics involved in a process that is driven by Arrhenius kinetics have been established in ASTM F1980-07 (2011) Standard Guide for Accelerated Aging of Sterile Barrier Systems for Medical Devices (*7*), which may be a useful guide for establishing accelerated contact conditions. As with all such models, the proper use of this model requires an understanding of the model's basis and essential principles, assumptions, and limitations (*2*).

Extractables profiles obtained with a given extracting medium and extraction technique can and should be monitored for equilibrium or the attainment of asymptotic levels of extractables (see *Figure 1*).

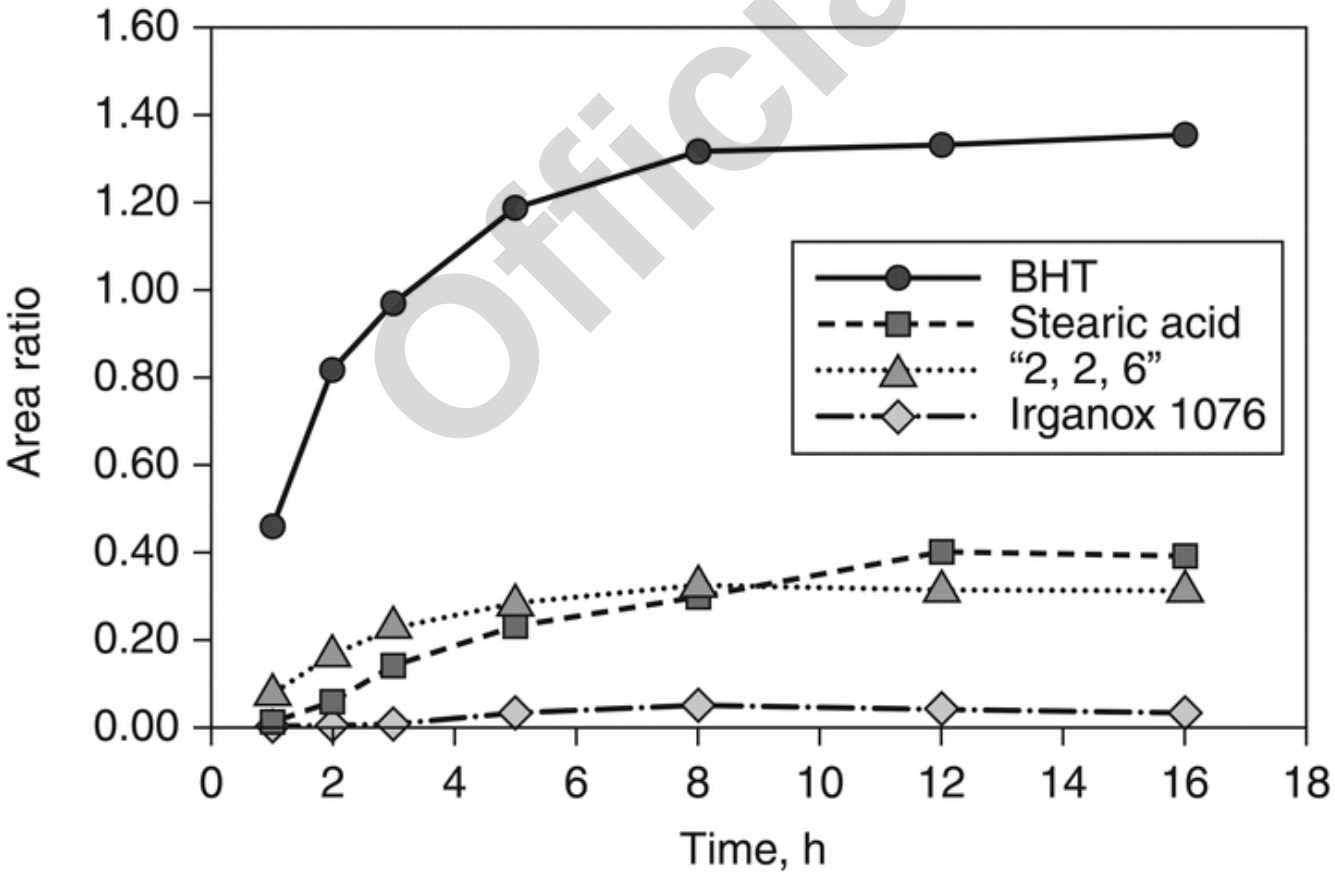


Figure 1. A graphical representation of an extraction that has attained equilibrium as indicated by the achievement of asymptotic levels of target individual extractables as a function of extraction time (i.e., GC/MS peak area ratios of target extractables relative to an internal standard plotted versus extraction time).

## Extraction Stoichiometry

Extraction stoichiometry considers the physical mass and/or surface area of the test article relative to the volume of the extracting medium, and the actual physical state of the material when it is extracted. Extraction stoichiometry can be manipulated to facilitate production of a more concentrated extract. For example, consider the case of a rubber stopper for a

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vial that contains 5 mL of a liquid drug product. A more concentrated extract than the drug product (i.e., an extract that contains higher levels of extractables than the leachables level in the drug product) could be produced by extracting 20 stoppers in 200 mL of extracting solvent. Another aspect of extraction stoichiometry is the physical state of the test article. It is not uncommon that components or materials are cut, opened, ground, or otherwise altered in size or configuration prior to being extracted. For inhomogeneous or layered materials such as film laminates, the process of cutting or grinding prior to extraction may alter the extractables profile as it may provide a means for the extracting solvent to come into contact with (and thus more effectively extract) materials (layers) that are shielded from contact with the solution under normal conditions of use. One can argue that the use of such sized material further facilitates the extraction process, however it is possible for sizing to reveal extractables that might not appear as leachables. Nevertheless, some sizing of components or materials before extraction can be useful in certain situations and for certain purposes, including: (a) reducing sample-to-sample variability by the consistent preparation of ground homogeneous polymeric material; (b) reducing the sizes of large packaging components to allow use of standard laboratory glassware for extraction studies; (c) increasing the surface area of a packaging component or material test article (e.g., via extruding, pressing, or grinding) in order to increase extraction efficiency. In any event, careful consideration should be given to the effect of physical sizing of test articles on the extractables profile before such sizing methods are employed in extraction studies.

For extractables assessments involving components or materials whose chemical ingredients are known based on information from the supplier or fabricator, analysts can manipulate extraction stoichiometry based on the known levels of chemical additives and the known sensitivity of the analytical technique(s) that will be used to characterize the extract. For example, consider the formulation for a peroxide-cured ethylene-propylene-diene-monomer (i.e., EPDM) gasket from an MDI valve shown in *Table 2*.

**Table 2. Ingredients in a Peroxide Cured Rubber Gasket Test Article that are Used in an MDI**

| Elastomer Ingredient | Amount (Nominal) |
|---|---|
| EPDM polymer | 64.0% |
| Mineral fillers (may include stearic acid) | 34.4% |
| Antioxidant 1: (butylated hydroxytoluene) | 0.3% |
| Antioxidant 2: (2,2′-methylene-bis-[6-(1,1-dimethylethyl)-4-methyl] phenol) | 0.3% |
| Peroxide curing agent | 1.0% |

Such information, when available from component and material suppliers, can be useful in designing an extraction study.

Analysts can also base the extraction stoichiometry on established safety thresholds for leachables. For example, an exposure of 0.15 µg/day total daily intake for an individual organic leachable has been proposed as an SCT for inhalation drug products, also termed orally inhaled and nasal drug products. Leachables present at or above the SCT, in an MDI for example, should be analytically and toxicologically evaluated, suggesting that extractables assessment also should be guided with the SCT in mind. The application of thresholds such as the SCT and AET to leachables assessments is discussed in greater detail in *Assessment of Drug Product Leachables Associated with Pharmaceutical Packaging/Delivery Systems* ⟨1664⟩.

In summary, extraction stoichiometry (and thus the "sensitivity" of an extraction study) can be based on:

- The known chemical ingredients in a component or material
- Safety-based thresholds for drug product leachables
- The known or determined sensitivities of analytical instrumentation used for extract characterization

## Mechanism of Extraction—Extraction Technique

An extraction can be accomplished in a variety of ways. It is necessary that the means of performing the extraction match the objectives of the extractables assessment. Common laboratory extraction techniques include:

- *Maceration (solvent soaking)*—in which the test article is allowed to soak for a period of time in an organic or aqueous extracting solvent at temperatures below the solvent's boiling point. Analysts can also fill packaging system units with extracting solvent and store them at relevant temperatures.
- *Reflux*—in which the test article is immersed in boiling solvent for a period of time.
- *Soxhlet*—in which the test article is placed in the "thimble" of a Soxhlet extraction apparatus that is slowly filled with redistilled solvent from a boiling flask/condenser system; and periodically, the extracting solvent (containing extractables) is siphoned back into the boiling flask and the process begins again (for as many times as required to attain equilibrium).
- *Sealed vessel*—in which the test article and extracting solvent are sealed inside a container capable of withstanding elevated temperatures and pressures, placed into a laboratory autoclave and heated with steam for a period of time.
- *Instrument-based solvent extraction*—in which the test article is placed inside a sealed apparatus and extracted in an automated cycle; examples include pressurized fluid extraction, microwave-assisted extraction, and supercritical fluid extraction.
- *Sonication*—in which the test article and extracting solvent are placed into a glass container and partly immersed in water inside an ultrasonic bath.

Each of these extraction mechanisms/techniques has its own unique advantages and limitations. For example, reflux extraction is very efficient, but may be too harsh for certain applications and can lead to thermal decomposition of certain organic extractables; the extracting power of sonication can be difficult to control; and because of its relatively high boiling point, water performs poorly in reflux and Soxhlet but well in a sealed vessel.

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If the goal of the extractables assessment is identification and quantitation of the chemical additive content of a component or material, it is typical to use extraction techniques and processes that soften, swell, or dissolve (or in the case of inorganic extractables, *digest*) the component or material, thereby releasing quantitative amounts of chemical additives for analysis.

### Extractions That are Not Solvent Mediated

Not all drug product or material-contact situations are solution mediated and not all issues related to leaching of material-derived entities involve a solution phase. For example, doses of inhalation powder contained in a capsule or blister pack for use in a dry powder inhaler may have volatiles leached from the capsule or blister material, or by specialty surface additives such as mold-release agents; a solid oral dosage form could contain volatile leachables derived from the adhesive of a paper label affixed to the plastic bottle that contains the dosage form; and inhalation solutions packaged in low-density polyethylene containers could contain volatile migrants from tertiary packaging or auxiliary components such as wooden shipping pallets. In the latter two cases, chemical entities can migrate through the plastic containers and volatilize into the airspace, subsequently accumulating as migrants in the dosage forms.

Extraction techniques specifically designed for application to volatile organic compounds are usually directly coupled to analytical instruments. These extraction techniques include headspace analysis (as headspace gas chromatography; HD/GC), direct thermal desorption (usually coupled to gas chromatography; TD/GC), and thermogravimetric analysis (TGA/GC).

## CHARACTERIZING THE EXTRACT

### Objectives and Challenges

Once an extract has been generated, the next objective is to perform a thorough chemical characterization of the extract. Setting a threshold (as described above), which is a specified level of an individual extracted chemical entity which requires characterization, can be based on safety considerations such as the SCT; functional considerations including nominal levels of known chemical additives in the formulation of an extracted component or material; or technological considerations such as the known or determined sensitivity of an analytical technology, instrument, or method. The extract characterization phase of the extraction study must enable the realization of the overall goals of the extractables assessment.

The ultimate objective of thorough extract characterization as defined above cannot be realized in all cases, even when state-of-the-art analytical chemistry is practiced with best available skill and diligence. It is a reality that there is no analytical technique or combination of analytical techniques that is capable of the discovery, identification, and quantitation of any and all organic and inorganic extractable chemical entities known to science. In some cases, authentic reference compounds for organic extractables may not be available for confirmation of identifications, or for quantitative instrument calibration. Thus, the practical objective of extract characterization must therefore be an exercise of due diligence in the discovery, identification, and quantitation to a reasonable degree of scientific certainty of all individual extractable chemical entities present in an extract above a specified level or threshold.

### Processes Involved in Extract Characterization

#### 1. SCOUTING

The most useful analytical techniques in a scouting exercise are not compound specific, as they do not provide chemical information specific to the molecular structure of any particular extractable or chemical class of extractables. These analytical techniques provide information regarding bulk chemical properties of organic and/or inorganic chemical entities present in an extract, which can be used to guide extractables discovery, identification, and quantitation. Scouting analysis is not capable in and of itself of realizing the practical objective of extract characterization, regardless of which scouting technique or combination of techniques is applied.

Analytical techniques which can be employed for scouting are listed in *Table 3*, along with the particular bulk chemical property (and potential utility of this property) available from each technique. Some examples of the utility of scouting include the following:

- Significant levels of nonvolatile residue determined by gravimetric analysis could suggest the presence of significant levels of inorganic chemical entities in the extract. This suggestion would be reinforced if significant mass remained after ashing the extracted nonvolatile residue (residue on ignition).
- Significant UV absorbance of an extract suggests that organic chemical entities are present which contain UV chromophores within their molecular structure, such as phenolic antioxidants.
- Characteristic features in an infrared spectrum of this extract could provide more detailed insights into the chemical classes of organic extractables present. These insights could be used to develop and apply analytical methods for discovery and identification that would detect the chemical classes of extractables suggested by the scouting process.
- For aqueous extracts, total organic carbon provides a measure of the total amount of organic extractables present.

The scouting process and scouting analyses are optional for extract characterization. The utility of scouting is in the guidance it potentially provides for discovery, identification, and quantitation.

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**Table 3. Survey of Analytical Methods for Extract Analysis**

| Analytical Technique | Analytical Method | Application | | | | Information/Utility |
|---|---|---|---|---|---|---|
| | | Scouting | Discovery | Identification | Quantitation | |
| Spectroscopy | UV | X | | X | | Bulk property of UV absorbing organic extractables; semi-quantitative with limited identification ability |
| | FTIR[a] | X | | X | | Bulk property of IR absorbing organic extractables, moderate identification ability |
| Wet Chemical | NVR[b], ROI[c] | X | | | | Bulk property reflecting total amount of nonvolatile organic and/or inorganic extractables |
| | pH | X | | | | Bulk property of acidic or basic extractables |
| | TOC[d] | X | | | | Quantitative measure of organic extractables |
| Gas Chromatography | FID[e] | | X | X | X | Discovery and quantitative assessment of individual organic extractables; note that qualitative identification is possible |
| | MS | | X | X | X | Discovery, identification, and quantitation of individual organic extractables; note that identification can be either qualitative or structural |
| | FTIR[a] | | X | X | | Discovery and identification of individual organic extractables; note that FTIR has limitations relative to structural analysis (however identification via qualitative analysis is possible) |
| Liquid Chromatography | UV, CAD[f], and ELSD[g] | | X | | X | Discovery and quantitative assessment of individual organic extractables; note that identification via qualitative analysis is possible and that Diode Array UV detectors can assist with structural analysis |
| | MS | | X | X | X | Discovery, identification, and quantitation of individual organic extractables; note that identification can be by either qualitative or structural and that ionization sources with different selectivities are available |
| | FTIR[a] | | X | X | | Discovery and identification of individual organic extractables; note that FTIR has limitations relative to structural analysis (however identification via qualitative analysis is possible) |
| | NMR[h] | | X | X | | Identification of individual organic extractables; note that identification can be by either qualitative or structural |
| Ion Chromatography | Conductivity | | X | | X | Discovery and quantitation typically of individual ionic species |
| | MS | | X | X | X | Discovery, identification, and quantitation of individual ionic extractables; note that identification can be by either qualitative or structural and that ionization sources with different selectivities are available |
| Spectrometry | MS | | | X | | Identification of individual organic extractables |
| | NMR[h] | | | X | | Identification of individual organic extractables |
| | IMS[i] | | X | X | X | Discovery and quantitative assessment of individual organic extractables; note that various ionization sources are available and that qualitative identification is possible |
| Atomic Spectroscopy | AAS[j] | | X | X | X | Discovery, identification, and quantitation of individual extracted elements (trace elements, metals); note that AAS can be applied to only one element at a time. Identification of the chemical form or speciation of the extracted element may require additional testing |
| | ICP-AES[k] | | X | X | X | |
| | ICP/MS[l] | | X | X | X | |

[a] FTIR = Fourier Transform Infrared spectroscopy.
[b] NVR = Nonvolatile Residue.
[c] ROI = Residue on Ignition.
[d] TOC = Total Organic Carbon.
[e] FID = Flame Ionization Detection. Additional GC detectors, such as Thermal Energy Analysis Detector (TEA), may provide greater sensitivity for specific compound classes.
[f] CAD = Charged Aerosol Detector.
[g] ELSD = Evaporative Light Scattering Detector.
[h] NMR = Nuclear Magnetic Resonance spectroscopy.
[i] IMS = Ion Mobility Spectrometry.
[j] AAS = Atomic Absorption Spectroscopy.
[k] ICP-AES = Inductively Coupled Plasma Atomic Emission Spectroscopy.

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[I] ICP/MS = Inductively Coupled Plasma Mass Spectrometry.

## 2. DISCOVERY

The process of discovery involves testing an extract and thereby producing one or more analytical results that are attributable to individual extractables. The process of discovery is accomplished by detecting instrumental responses from the individual organic and inorganic extractables that are proportional to the levels of these individual extractables within the extract. It is in the discovery process that analytical techniques typically associated with trace organic and inorganic analysis are first required for extract characterization. Trace organic analysis typically involves the use of chromatographic techniques, particularly gas chromatography (GC) and high-performance liquid chromatography (HPLC). GC has enormous separating capability for volatile and semi-volatile organic compounds while HPLC is most applicable to semi-volatile and relatively nonvolatile organic compounds, making the two separation techniques complementary and orthogonal for application to the significant chemical diversity of extractables. A discussion of the principles of both gas and liquid chromatography is available in *Chromatography* ⟨621⟩.

The chemical diversity of extractables with respect to polarity and volatility can require alternative sample introduction techniques or sample modification, particularly for GC. Relatively volatile extractables like methanol are most amenable to headspace sampling of aqueous-based extracts into a GC. Organic acids and bases can often be analyzed more effectively by GC after chemical derivatization, such as methylation or silylation for organic acids. Both GC and HPLC can employ detection systems with different specificities (*Table 3*) which take advantage of unique structural properties of various chemical classes of extractables.

The analytical techniques useful for organic extractables discovery can also be applied to identification as well as quantitation. Analytical techniques such as gas chromatography/mass spectrometry (GC/MS), that are most often applied to identification, can also be used for both discovery and quantitation (*Table 3*). Inorganic extractables such as trace elements and metals are typically discovered, identified, and quantitated by the same suite of analytical techniques, such as atomic emission spectroscopy. Analytical techniques designed to study inorganic speciation, particularly in aqueous extracts, are considered beyond the scope of this chapter.

It is important to state that the overall goals of an extraction study always require the identities and quantitative amounts of individual organic and inorganic extractables, and so the mere discovery of extractables does not achieve the ultimate objectives of an extraction study.

## 3. IDENTIFICATION

Identification of an extractable can be accomplished either by structural analysis or qualitative analysis. *Structural analysis* is the process by which the molecular structure of an unknown analyte is elucidated from compound-specific data, and therefore requires compound-specific detection of the unknown analyte. A compound-specific detector is one that provides information specific to the molecular structure of the individual unknown analyte (not just its chemical class). *Qualitative analysis* is the process by which an unknown analyte is matched with an authentic reference compound via one or more analytical techniques. The analytical techniques used for qualitative analysis can, but do not need to be, compound specific.

The analytical techniques most applicable to structural analysis, and to trace organic analysis problems in general, involve the combination of chromatography with mass spectrometry. These are the so-called “hyphenated” techniques of GC/MS and high-performance liquid chromatography/mass spectrometry (LC/MS). A discussion of the principles of mass spectrometry (including both GC/MS and LC/MS) is available in *Mass Spectrometry* ⟨736⟩.

Both GC/MS and LC/MS are capable of generating extractables profiles in the form of chromatograms. However, since LC/MS includes a relatively high chemical background of HPLC mobile phase ions, it is typical to include a non-destructive UV detector in series with the mass spectrometer to assist in locating peaks of individual extractables. The compound-specific data available from mass spectrometry include:

- The monoisotopic molecular weight of the extractable based on confirmation of the molecular ion from one or more ionization processes
- The molecular formula of the extractable based on accurate mass measurements, and/or accurate isotope ratio measurements, of the molecular ion
- The fragmentation behavior of the extractable based on in-source fragmentation or tandem mass spectrometry

GC/MS interfaced with electron ionization produces mass spectra which can be searched through computerized databases, or libraries, of mass spectra from known compounds. Note that searchable mass spectra are generally unavailable for LC/MS ionization processes because of the variable nature of such spectra over time and between various instruments and laboratories. Both GC/MS and LC/MS also include the retention time (or retention index) of the unknown extractable which can be compared with that of authentic reference compounds.

Given the number and chemical diversity of organic extractables, it is unreasonable to expect that authentic reference compounds will be available (or can be made available) to confirm every identification. It is therefore necessary that levels of identification confidence be established and appropriately utilized. Data typically available from GC/MS and LC/MS analyses (see items a through e below) are used to designate individual extractables identifications in the categories of *Confirmed*, *Confident*, or *Tentative* (*2* ):

- Mass spectrometric fragmentation behavior/expert mass spectrum interpretation
- Confirmation of molecular weight
- Confirmation of elemental composition
- Mass spectrum matches automated library or literature spectrum
- Mass spectrum and chromatographic retention index match authentic reference compound
- Supporting spectral information from an orthogonal method (e.g., NMR)

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- A *Tentative* identification means that data have been obtained that are consistent with a class of molecule only. This is typically the case when only information such as a or d is available.
- A *Confident* identification means that the tentative identification has been bolstered by additional and sufficient confirmatory information to preclude all but the most closely related structures. This would be the case, for example, if the tentative information (a and/or d) were augmented by b, c, or f. The more confirmatory information obtained, the greater the level of confidence.
- A *Confirmed* identification means that the preponderance of evidence confirms that the entity in question can only be the identification that is provided. Although it is possible that a highly confident identification may meet the standard implied by the preponderance of evidence (for example, having a, b, c, e, and f), the only means of providing a confirmed identification is via mass spectral and retention time match with an authentic reference compound (item e).

Although these identification categories are based on mass spectrometry, it is possible to use data from other analytical techniques to assist in extractables identification. Such techniques include GC/FTIR (Fourier Transform Infrared Spectroscopy) and LC/NMR (Nuclear Magnetic Resonance Spectroscopy). These and potentially other analytical techniques are capable of producing compound-specific data which are complementary to mass spectrometry.

The level of identification required for any individual extractable depends on the intended use of that identification. It is up to the organization responsible for the extraction study to determine this after appropriate consideration of applicable regulatory guidances.

Since the list of potential inorganic extractables, such as trace elements and metals, is finite compared with the population of organic extractables, identification and quantitative analysis for inorganic extractables are achieved simultaneously. While elemental analysis is relatively straightforward, it is not without its challenges. The issue of false positive responses and spectral or mass interference must be addressed in order for identifications based on atomic spectroscopy to be rigorous and accurate. It is also noted that elemental analysis provides element-specific identifications and quantitations, and not the chemical speciation of the extractable. Thus, interpretation of the impact of the elemental results may require further studies, such as detailed chemical speciation of elements deemed of significance. For example, while finding sulfur in an extract by atomic spectroscopy is an important outcome, the safety impact of this finding cannot be ascertained until the speciation of the sulfur is established. This is the case as the safety impact of sulfur as elemental sulfur may be different than that of sulfur as sulfate.

## 4. QUANTITATION

Quantitation is typically based on the instrumental response of an individual extractable relative to an authentic reference compound, and therefore requires that individual extractables be separated (either directly with chromatography or indirectly with selective detection) and produce detector responses that are directly proportional to the level (or concentration) of the extractable in a given extract. Calibration of an analytical system is accomplished by analysis of authentic reference compounds (external standards). One or more internal standards can also be included in both the extract and reference calibration solutions to increase accuracy and precision. The levels of extractables for which authentic reference compounds are not available can be estimated using their responses (or response factors) relative to internal standards, or other surrogate reference compounds of similar molecular structure. While such an analytical process can provide reliably accurate concentration estimates, diligence must be exercised in terms of establishing and justifying the choice and use of internal standards. Criteria for the selection of appropriate internal standards have been described (*2*).

## Preparation of Extracts for Analysis

Extracts can often be analyzed directly without significant preparation or concentration. Many organic solvent extracts (e.g., dichloromethane, ethyl acetate, hexane) can be directly injected into a gas chromatograph, while others (e.g., methanol, ethanol, isopropanol) are either too reactive in the heated GC injection port or too high boiling. Organic solvent extracts with inappropriate physical/chemical properties for direct analysis by GC can be switched to more appropriate solvents. Certain extractables, such as fatty acids (e.g., palmitic acid, stearic acid) perform better in gas chromatographic analysis when they are derivatized to either methyl esters or trimethylsilyl esters.

It is usually considered inappropriate to directly analyze aqueous extracts by gas chromatography, due to the reactivity and high boiling point of water. In addition, pH-buffered aqueous extracts contain nonvolatile salts which are not suitable for GC injection. Aqueous extracts are typically back extracted with an organic solvent to remove organic extractables from the water, with the resulting organic extract being injected into the GC. Unlike GC, liquid chromatography (HPLC, LC/MS) is perfectly suited to the direct analysis of aqueous extracts, since most HPLC methods include water and water-miscible mobile phases. Water-immiscible organic solvents (e.g., hexane) cannot be injected onto these reversed-phase HPLC systems, so these must be dried and the resulting extractable residue taken up in a solvent suitable for HPLC (e.g., acetonitrile, methanol, or mixtures of these with water).

Organic or aqueous extracts with insufficient levels of extractables for analysis can be concentrated by various techniques. Many organic solvents can be dried down under inert gas, a rotary evaporator, or a Kuderna-Danish concentrator. Aqueous extracts can be lyophilized, concentrated under vacuum, or back extracted into an organic solvent which is then further concentrated.

The final concentration at which an extract is analyzed depends on the goal(s) of the extractables assessment and the inherent sensitivities of the analytical techniques applied. A good “rule of thumb” is that in order to accomplish a complete structural analysis of an unknown extractable, a GC/MS requires approximately 5 ng injected into the instrument. This suggests a concentration in the injected extract of 5 ng/µL or 5 µg/mL. In a 200-mL dichloromethane extract, this converts to a total of 1 mg of this particular extractable recovered from the extracted test article. If this analyte concentration is insufficient to meet the goal of the extractables assessment, then the following parameters can be optimized:

- Extraction stoichiometry (i.e., extract more material or use more extracting solvent)

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- Extraction conditions (i.e., use higher temperatures, longer times, solvents with greater extraction power, more aggressive extraction technique, etc.)
- Extract processing (i.e., concentration of the extract)

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## SUMMARY

### Assessing the Completeness of an Extractables Assessment

The completeness of an extractables assessment can only be judged against the overall goals of the assessment. For example, an extractables assessment accomplished solely for materials characterization might include one extracting solvent, one extraction technique, and one set of extraction conditions; along with a materials-based threshold (e.g., 10 ppm w/w). Such an extractables assessment might be considered complete if all extractables above the defined threshold were identified to the confident level (defined above) and quantitated. For an extractables assessment designed to establish a rigorous leachables–extractables correlation for a high risk drug product, where a challenging safety threshold might apply (e.g., 0.15 µg/day), good scientific practice and due diligence requires the following:

- Generation of extracts should be accomplished with
  - Multiple solvents or extracting media with varying extracting power based on the known extracting power of the drug product vehicle;
  - Multiple and complementary extraction techniques, including those with the capability for volatiles analysis;
  - Extraction conditions that allow equilibrium to be achieved.
- Characterization of extracts should use
  - Multiple and complementary analytical techniques;
  - Careful sample preparation, keeping the analytical technique(s) in mind;
  - A systematic process for identification and quantitation of extractables.

In this case, the extractables assessment might be considered complete if all extractables above the defined threshold were identified to at least the confident level, quantitated, and correlated both qualitatively and quantitatively with drug product leachables data (if available) and the known ingredients in the packaging system, packaging component(s), or material(s) of construction.

It should be noted that limited extractables assessments with relatively narrow goals can be accomplished to required completeness with a relatively focused effort. For example, extraction studies designed to quantitate the levels of specific chemical additives in specific packaging components/materials can be done with specified extraction parameters and analytical methods (see *Plastic Packaging Systems and Their Materials of Construction* ⟨661⟩, *Plastic Materials of Construction* ⟨661.1⟩, and *Plastic Packaging Systems for Pharmaceutical Use* ⟨661.2⟩). The reader is also referred to various sources which describe extractables assessments and extraction studies for pharmaceutical applications (*2*, *5*), as well as other general sources which refer to extractables assessments for medical devices and food contact (*8*).

Reference is also made to compendial chapters in this Pharmacopeia which include extraction studies with specific goals and purposes:

1. *Biological Reactivity Tests, In Vitro* ⟨87⟩
2. *Biological Reactivity Tests, In Vivo* ⟨88⟩
3. ▲*Elastomeric Components Used in Injectable Pharmaceutical Packaging/Delivery Systems* ⟨381⟩▲ (CN 1-Dec-2020)
4. *Plastic Packaging Systems and Their Materials of Construction* ⟨661⟩
5. *Plastic Materials of Construction* ⟨661.1⟩
6. *Plastic Packaging Systems for Pharmaceutical Use* ⟨661.2⟩

### Example Extractables Profiles—Materials Characterization

As stated previously, extraction studies are usually designed to produce extractables profiles, which are qualitative and/or quantitative analytical representations of the extractables content of a particular extracting medium. To illustrate the concept of an extractables profile, the following is an example of an extractables study performed for the purpose of material characterization. Extractables profiles are commonly produced by analysis of laboratory extracts by instrumental chromatographic techniques. *Figure 2* and *Figure 3* show example extractables profiles (GC/MS and HPLC/UV, respectively) from a hexane Soxhlet extract of a cyclic olefin copolymer (COC) material of construction. COC materials are used in the fabrication of pre-filled syringes, vials for small-volume parenterals, and bags for large-volume parenterals. The extracts were generated by subjecting approximately 5 g of suitably sized material to 16 hours of Soxhlet extraction with 125 mL of hexane. The resulting extract was spiked with internal standards (details not relevant to this discussion) and was analyzed directly by GC/MS (see *Table 4*). For HPLC/UV analysis, an aliquot of the hexane extract was reduced in volume and diluted with methanol prior to analysis (see *Table 5*). Since the purpose of this extractables assessment was materials characterization, extraction study parameters were adjusted relative to an extractables identification threshold of 10 ppm (µg/g). It is clear from these chromatograms that the techniques used are both complementary and orthogonal, illustrating the concept that multiple analytical methods are required to typically elucidate the complete extractable profile.

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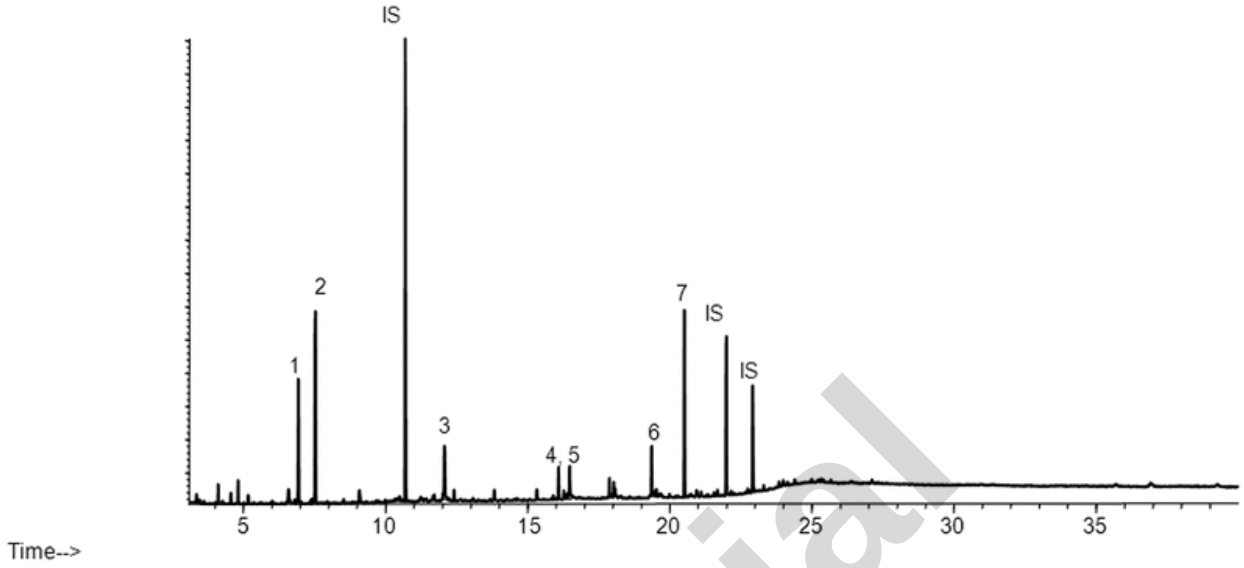


Figure 2. GC/MS chromatogram (extractables profile) for a hexane Soxhlet extract of a cyclic olefin copolymer. Internal standards (IS) producing peaks in this chromatogram include: 2-Fluorobiphenyl at 10.7 min, Irganox 415 at 22.0 min, and Bisphenol M at 23.0 min. Numbered peaks represent identified extractables above the materials-based threshold.

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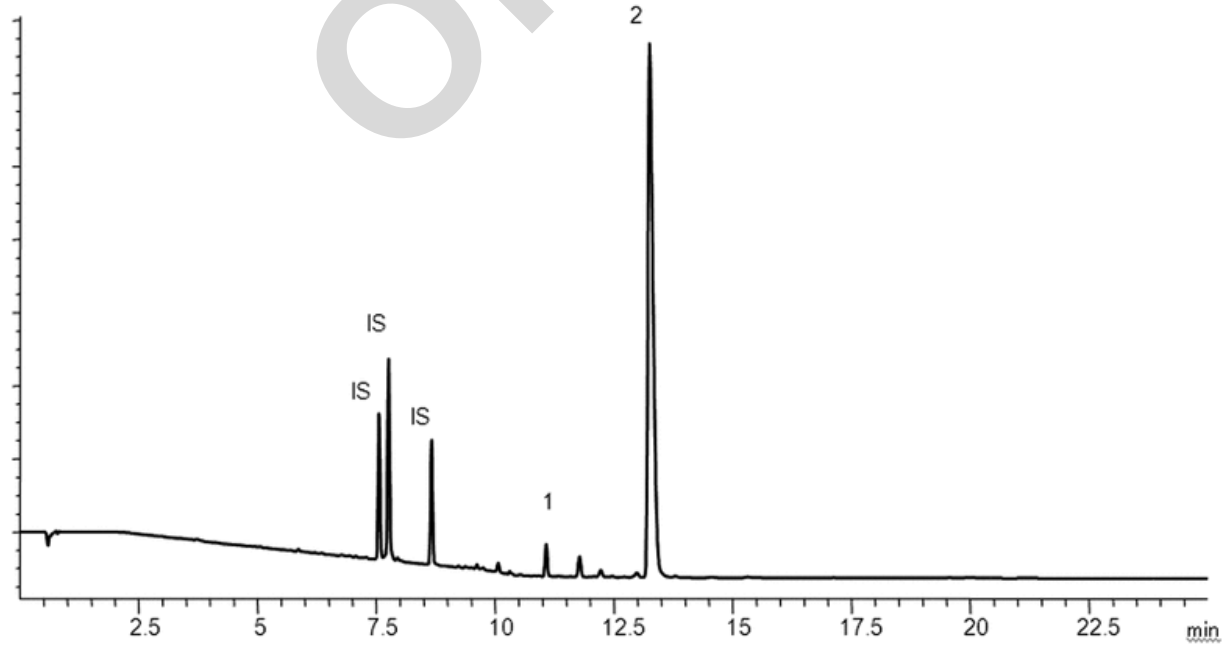


Figure 3. HPLC/UV Chromatograms (λ = 220 nm; extractables profile) for a hexane Soxhlet extract of a cyclic olefin copolymer. Internal standards (IS) producing peaks in this chromatogram include: Bisphenol M at 7.6 min, 2-Fluorobiphenyl at 7.8 min, and Irganox 415 at 8.7 min. The major peak in this extractables profile above the materials-based threshold is the known additive (antioxidant) Irganox 1010 (Peak 2).

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**Table 4. Operating Parameters, GC/MS Analysis of the Hexane COC Soxhlet Extract**

| Operating Parameter | Operating Value |
|---|---|
| Column | J&W DB-5HT, 30-m × 0.25-mm, 0.25 µm film thickness |
| Oven Program | Start at 50°, hold for 1 min; ramp at 12°/min to 315°, hold for 16 min |
| Carrier Gas | He at 1.2 mL/min |
| Injection | Split (1:5); 1 µL |
| Injector Temperature | 300° |
| FID Detector Temperature | N/A |
| MS Transfer Line Temperature | 180° |
| MS Detection Details | 70 eV (+) EI (electron ionization), mass range of 33–650 amu (3.0-min solvent delay) |

**Table 5. Operating Parameters, HPLC/UV Analysis of the Hexane COC Soxhlet Extract**

| Operating Parameter | Operating Value | |
|---|---|---|
| Column | Agilent Zorbax Eclipse Plus $C_{18}$, 100- × 3.0-mm, 3.5-µm particles | |
| Column Temperature | 40°–50° | |
| Mobile Phase Components | A = 10 mM ammonium acetate, B = acetonitrile | |
| Mobile Phase Gradient | Time | %B |
| | 0.0 | 5.0 |
| | 8.4 | 100.0 |
| | 35.0 | 100.0 |
| | 36.0 | 5.0 |
| | 39.0 | 5.0 |
| Mobile Phase Flow Rate | 0.8 mL/min | |
| Sample Size | 10–50 µL | |
| Detection, UV | 205–300 nm; spectra recorded at λ = 210, 220, 230, 250, and 270 nm | |

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# ⟨1664.1⟩ ORALLY INHALED AND NASAL DRUG PRODUCTS

## INTRODUCTION

This section addresses specific considerations for leachables in orally inhaled and nasal drug products (OINDP), including metered dose inhalers (MDIs); nasal sprays; inhalation solutions, suspensions, and sprays; and dry powder inhalers (DPIs). Although OINDP can be a combination of products that are comprised of drug and device constituent parts, the primary mode of action is typically through the drug. For this reason OINDP are treated as drugs from a regulatory perspective. Regulatory guidance documents and detailed best practice recommendations specific to OINDP are available (*1*–*4*). Note that the following discussion is primarily devoted to organic leachables. For consideration of inorganic (i.e., elemental) leachables, see *Assessment of Drug Product Leachables Associated with Pharmaceutical Packaging/Delivery Systems* ⟨1664⟩.

## KEY TERMS

In addition to the key terms listed in ⟨1664⟩, some additional key terms more specific to OINDP are the following:

- *Critical components* are packaging components that contact either the drug product formulation or the patient, or that affect the mechanics of the overall performance of the packaging and delivery system, including any necessary secondary packaging. The identification of critical components for a particular OINDP dosage form is the responsibility of the applicant in consultation with appropriate regulatory authorities.
- *Special case compounds* are individual (or classes of) compounds that have special safety or historical concerns as drug product leachables in OINDP, and therefore must be evaluated and controlled as leachables (and extractables) by specific analytical techniques and technology-defined thresholds.

Additional terminology and associated definitions specific to OINDP are available in the cited references (*1*–*4*).

## LEACHABLES ASSESSMENT RATIONALE FOR ORALLY INHALED AND NASAL DRUG PRODUCTS

OINDP are generally categorized as high-risk dosage forms due to safety considerations related to the route of administration and high probability of packaging component interaction with the formulation (see *Table 1* in ⟨1664⟩). The packaging systems used in these drug products incorporate components of various types, including components composed of polymeric (plastic or elastomeric) raw materials with complex chemical compositions and therefore a variety of potential leachables. Chemical entities may migrate (i.e., leach) into the formulation when there is direct contact with the primary packaging and delivery components for extended periods of time. In certain cases, there is also the potential for leaching from secondary and tertiary packaging. In addition, for OINDP, contact of the delivery device with mucosal tissue (mouth or nasal) is generally expected. Leachables studies for some OINDP may be considered separately for packaging components that are in continuous contact with the formulation (e.g., vials, bottles, blisters, metering valve components) versus those that are only in transient contact (e.g., DPI mouthpiece, MDI mouthpiece).

OINDP typically require:

- A leachables stability study for drug product registration that supports intended storage and use conditions throughout the proposed shelf-life (see *Table 1*), ideally on primary drug product stability batches manufactured with the same lots of packaging components used in extraction studies (in order to facilitate a leachables–extractables correlation)
- Sensitive, selective, and fully validated leachables analytical methods
- Leachables assessments based on safety thresholds [Safety Concern Threshold (SCT): 0.15 µg/day, and Qualification Threshold (QT): 5 µg/day total daily intake (TDI) for an individual organic leachable; however, for exceptions see *Special Case Compounds*]
- Complete qualitative and quantitative leachables–extractables correlations (which require that extractables assessments be accomplished on all critical packaging components; see *Assessment of Extractables Associated with Pharmaceutical Packaging/Delivery Systems* ⟨1663⟩)
- Leachables specifications including acceptance criteria (assumes a complete extractables assessment for each critical packaging component). (Note that in many cases routine extractables testing for release of critical components can be used to control drug product leachables in lieu of routine drug product leachables testing, providing that a comprehensive leachables–extractables correlation is established.)

For OINDP dosage forms based on formulations with relatively lower leaching potential for organic compounds (e.g., aqueous formulations, dry powder formulations), the above requirements should be considered and evaluated on a case-by-case basis, including consultations with the appropriate regulatory authorities.

**Table 1. Example Stability Storage Conditions and Testing Time Points for an OINDP Registration Leachables Study (*4*)**

| Condition (temperature/relative humidity) | Time Points (months) |
|---|---|
| 25 ± 2°C/60 ± 5%RH | 3, 6, 12, 18, 24, 36 (to end of shelf-life) |
| 30 ± 2°C/65 ± 5%RH | 3, 6, 12, 18, 24, 36 (to end of shelf-life) |
| 40 ± 2°C/75 ± 5%RH | 3, 6 |

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# ORALLY INHALED AND NASAL DRUG PRODUCTS DOSAGE FORM TYPES

## Metered Dose Inhaler

MDIs or pressurized MDIs (pMDIs) are defined as "drug products that contain active ingredient(s) dissolved or suspended in a propellant, a mixture of propellants, or a mixture of solvent(s), propellant(s), and/or other excipients in compact pressurized aerosol dispensers" (*2,4*). Typical MDIs include a metal canister (stainless steel or aluminum; coated or uncoated), a fixed-volume metering valve (with plastic or elastomeric components), elastomeric seals, and a plastic actuator or mouthpiece (see *Inhalation and Nasal Drug Products: Aerosols, Sprays, and Powders—Performance Quality Tests* ⟨601⟩). MDIs are multidose drug container closure and delivery systems that can contain sufficient formulation for up to several hundred actuations (label claim) per container. Because many of the critical packaging components are in continuous contact with an organic solvent-based formulation, the MDI has the highest risk for formulation-packaging component interaction, and therefore the highest risk for leachables, of all OINDP dosage forms (or any other dosage form). Because of the leaching potential of their organic solvent-based formulations, MDIs would typically be expected to show complete qualitative and quantitative leachables–extractables correlations. Leachables in MDIs should be characterized (i.e., identified and quantitated) at levels above a calculated analytical evaluation threshold (AET). An AET can be calculated for any OINDP dosage form with consideration of the SCT for OINDP (i.e., 0.15 µg/day for an individual organic leachable). An example AET calculation for an MDI follows.

Given an MDI drug product with 200 labeled actuations per canister, a maximum recommended patient exposure of 12 actuations per day, and a critical valve component mass per valve of 200 mg, for an individual organic leachable derived from this valve component, the following AET can be estimated:

$$\text{Estimated AET} = \left(\frac{0.15\ \mu\text{g/day}}{12\ \text{actuations/day}}\right) \times (200\ \textit{labeled}\ \text{actuations/canister})$$

$$\text{Leachables Estimated AET} = 2.5\ \mu\text{g/canister}$$

To convert to an estimated AET, which would be a useful guide for characterizing potential leachables via extraction studies of this particular valve component (see ⟨1663⟩):

$$\text{Extractables Estimated AET} = \left(2.5\ \mu\text{g/Canister}\right) \times \left(\frac{1\ \text{canister/value}}{0.2\ \text{g elastomer/value}}\right)$$

$$\text{Extractables Estimated AET} = 12.5\ \mu\text{g/g}$$

The AET calculation should not be modified to account for variables, such as manufacturing overfill in the canister to compensate for leak rate or fill variability, unless such modification can be scientifically justified.

Analytical methods for leachables testing of MDI drug products can be based on processes such as "cold filtration" of suspension formulations to remove active ingredient and excipient particles (*5*) or careful venting of the volatile organic propellant, which retains leachables in a residue within the canister (*6*). Because sample preparation procedures for MDI formulations can be complex and typically require the volatile propellant to be reduced to dryness at some point, creating the possibility for loss of leachables before sample analysis, it is particularly important to demonstrate adequate recoveries of leachables through the use of spiked MDI samples.

Although it is unlikely to contribute leachables to the emitted drug product aerosol plume, potential patient exposure to chemical entities from the MDI plastic actuator or mouthpiece should be assessed at a threshold of 20 µg/g (see ⟨1663⟩). Additional studies and references required to assess patient exposure to actuator- or mouthpiece-derived chemicals include reference to indirect food additive regulations and the application of *Biological Reactivity Tests, In Vitro* ⟨87⟩ and *Biological Reactivity Tests, In Vivo* ⟨88⟩ (*2*). Note that "spacers" and other devices designed for use with MDIs should also be characterized if a particular device is specified on the drug product label.

When constructed from materials acceptable for food contact, MDI actuators and mouthpieces, "spacers", and other components and devices specified in the drug product labeling generally only require appropriate characterization (i.e., extraction studies and routine extractables testing) in order to assure continued consistent composition of the component or device.

In addition, based on applicable regulatory guidance (*2*), drug product applicants should consider the following (see ⟨1663⟩):

- Development and validation of surface organic residue release tests for incoming uncoated metal canisters, with appropriate acceptance criteria
- Development and validation of extractables release tests for the inner surfaces of incoming coated canisters, with appropriate acceptance criteria
- Development and validation of extractables release tests for incoming metering valve critical components, with appropriate acceptance criteria
- Development and validation of extractables profile release tests for incoming actuators or mouthpieces, with appropriate qualitative and quantitative acceptance criteria.

## Nasal Sprays

Nasal sprays are defined as "drug products that contain active ingredients dissolved or suspended in a formulation, typically aqueous-based, which can contain other excipients and are intended for use by nasal inhalation" (*1,4*). Nasal sprays include a plastic container and components (usually plastic) that are responsible for formulation metering, atomization, and delivery to

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the patient (see ⟨601⟩). Critical components include those that are in constant contact with the formulation (e.g., the container, dip tube) and components that are in the liquid pathway during actuation of the device and that do not permit quick evaporation of residual surface liquid (*3*). Because nasal sprays are typically aqueous-based formulations, and the vast majority of potential organic leachables are relatively lipophilic, the risk for formulation-packaging component interaction is lower relative to the organic propellant-based MDIs, and the risk for organic leachables is lower. Leachables in nasal sprays should be characterized (i.e., identified and quantitated) at levels above a calculated AET. An AET can be calculated for any OINDP dosage form with consideration of the SCT for OINDP (i.e., 0.15 µg/day for an individual organic leachable). An example AET calculation for a nasal spray follows.

Given a nasal spray drug product with 120 labeled actuations per container, a maximum recommended patient exposure of 4 actuations per day, and a critical component (plastic dip tube) mass of 250 mg, for an individual organic leachable derived from this component, the following AET can be estimated:

$$\text{Estimated AET} = \left(\frac{0.15\ \mu\text{g/day}}{4\ \text{actuations/day}}\right) \times (120\ \textit{labeled}\ \text{actuations/container})$$

$$\text{Leachables Estimated AET} = 4.5\ \mu\text{g/container}$$

Given a total fill volume of 10 mL:

$$\text{Estimated AET} = (4.5\ \mu\text{g/container})/(10\ \text{mL/container})$$

$$\text{Estimated AET} = 0.45\ \mu\text{g/mL}$$

To convert to an estimated AET, which would be a useful guide for characterizing potential leachables via extraction studies of this particular plastic dip tube (see ⟨1663⟩):

$$\text{Extractables Estimated AET} = (4.5\ \mu\text{g/container}) \times \left(\frac{1\ \text{container}}{0.25\ \text{g material/tube}}\right)$$

$$\text{Extractables Estimated AET} = 18\ \mu\text{g/g}$$

The AET calculation should not be modified to account for variables, such as manufacturing overfill, unless such modification can be scientifically justified.

All nasal spray packaging system critical components should be subjected to extractables assessments (see ⟨1663⟩). Potential patient exposure to chemical entities from nasal spray critical components not in continuous contact with the drug product formulation should be assessed at a threshold of 20 µg/g (see ⟨1663⟩). Additional studies and references required to assess patient exposure to nonformulation contact critical component derived chemicals include reference to indirect food additive regulations and the application of ⟨87⟩ and ⟨88⟩ (*1*).

When constructed from materials acceptable for food contact, nasal spray critical components not in continuous contact with the drug product formulation generally only need to be appropriately characterized (i.e., extraction studies and routine extractables testing) in order to assure continued consistent composition of the component.

In addition, based on applicable regulatory guidance (*1*), drug product applicants should consider the following (see ⟨1663⟩):

- Development and validation of extractables release tests for incoming container closure and pump critical components, with appropriate qualitative and quantitative acceptance criteria.

## Inhalation Solutions, Suspensions, and Sprays

Inhalation solutions, suspensions, and sprays are defined as “drug products that contain active ingredients dissolved or suspended in a formulation, typically aqueous-based, which can contain other excipients and are intended for use by oral inhalation” (*1,4*). Inhalation solutions and suspensions are intended for use with a nebulizer (*1,4*). Inhalation sprays, like MDIs and nasal sprays, are combination products where the components responsible for the metering, atomization, and delivery of the formulation to the patient are a part of the container closure system (*1,4*). Critical components include components that are in constant contact with the formulation and components that are in the liquid pathway during actuation of the device and that do not permit quick evaporation of residual surface liquid. Leachables in inhalation sprays should be characterized (i.e., identified and quantitated) at levels above a calculated AET. An AET can be calculated for any OINDP dosage form with consideration of the SCT for OINDP (i.e., 0.15 µg/day for an individual organic leachable). An example AET calculation for an inhalation spray follows.

Given an inhalation spray drug product with 120 labeled actuations per container, a maximum recommended patient exposure of 4 actuations per day, and a critical component (plastic dip tube) mass of 400 mg, for an individual organic leachable derived from this component, the following AET can be estimated:

$$\text{Estimated AET} = \left(\frac{0.15\ \mu\text{g/day}}{4\ \text{actuations/day}}\right) \times (120\ \textit{labeled}\ \text{actuations/container})$$

$$\text{Leachables Estimated AET} = 4.5\ \mu\text{g/container}$$

Given a total fill volume of 4.5 mL:

$$\text{Estimated AET} = (4.5\ \mu\text{g/container})/(4.5\ \text{mL/container})$$

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$$\text{Estimated AET} = 1\ \mu g/mL$$

To convert to an estimated AET, which would be a useful guide for characterizing potential leachables via extraction studies of this particular plastic dip tube (see ⟨1663⟩):

$$\text{Estimated AET} = \left(\frac{0.15\ \mu g/\text{day}}{4\ \text{actuations/day}}\right) \times (120\ \textit{labeled}\ \text{actuations/container})$$

$$\text{Extractables Estimated AET} = 11.3\ \mu g/g$$

The AET calculation should not be modified to account for variables, such as manufacturing overfill, unless such modification can be scientifically justified.

Because inhalation solutions and suspensions are similar to nasal spray and inhalation spray drug products in that they are typically aqueous-based formulations, and the vast majority of potential organic leachables are relatively lipophilic, the risk for formulation-packaging component interaction is lower relative to the organic propellant-based MDIs, and the risk for organic leachables is lower. However, unlike MDIs, nasal and inhalation spray drug products, inhalation solutions, and suspensions are typically packaged in plastic unit dose containers (i.e., nebules). Leaching can potentially occur from the unit dose container [e.g., low-density polyethylene (LDPE)], which is in long-term continuous contact with the drug product formulation. It is also possible that organic chemical entities associated with paper labels, adhesives, inks, etc. in direct contact with the permeable unit dose container can migrate through the container and into the formulation. Leachables from tertiary packaging systems (e.g., cardboard shipping containers) are also possible. Leachables in inhalation solutions and suspensions should be characterized (i.e., identified and quantitated) at levels above a calculated AET. An AET can be calculated for any OINDP dosage form with consideration of the SCT for OINDP (i.e., 0.15 µg/day for an individual organic leachable). An example AET calculation for an inhalation solution follows.

Given an inhalation solution with 3 mL of drug product contained in a LDPE unit dose vial (1 g total weight of LDPE), with a maximum recommended patient exposure of three vials per day, for an individual organic leachable derived from this component, the following AET can be estimated:

$$\text{Estimated AET} = \left(\frac{0.15\ \mu g/\text{day}}{3\ \text{doses/day}}\right) \times (1\ \textit{labeled}\ \text{dose/container})$$

$$\text{Leachables Estimated AET} = 0.05\ \mu g/\text{container}$$

$$\text{Estimated AET} = (0.05\ \mu g/\text{container})/(3\ mL/\text{container})$$

$$\text{Estimated AET} = 0.017\ \mu g/mL$$

To convert to an estimated AET, which would be a useful guide for characterizing potential leachables via extraction studies of this particular plastic unit dose vial (see ⟨1663⟩):

$$\text{Extractables Estimated AET} = (0.05\ \mu g/\text{container}) \times \left(\frac{1\ \text{container}}{1\ \text{g material/container}}\right)$$

$$\text{Extractables Estimated AET} = 0.05\ \mu g/g$$

The challenge of characterizing drug product leachables at levels of 17 ng/mL in an aqueous drug product is considerable, even given the capabilities of modern analytical chemistry. For this particular inhalation solution example, it might be appropriate to implement a simulation study (see ⟨1663⟩ and ⟨1664⟩) to facilitate the discovery and identification of probable leachables, with actual drug product leachables being quantitated (if required) with high-sensitivity target compound analytical techniques and methods.

All inhalation solution, suspension, and spray packaging system critical components should be subjected to extractables assessments (see ⟨1663⟩). Potential patient exposure to chemical entities from inhalation solution, suspension, and spray critical components not in continuous contact with the drug product formulation should be assessed at a threshold of 20 µg/g (see ⟨1663⟩). Additional studies and references required to assess patient exposure to nonformulation contact critical component-derived chemicals include reference to indirect food additive regulations and application of ⟨87⟩ and ⟨88⟩ (*1*). When constructed from materials acceptable for food contact, inhalation solution, suspension, and spray critical components not in continuous contact with the drug product formulation generally only need be appropriately characterized (i.e., extraction studies and routine extractables testing) in order to assure continued consistent composition of the component. Critical components of nebulizers and other devices designed for use with inhalation solutions and suspensions should also be characterized with respect to extractables and leachables if a particular device is specified in the drug product labeling.

Based on applicable regulatory guidance for inhalation solutions, suspensions and sprays (*1*), drug product applicants should consider the following (see ⟨1663⟩):

- Development and validation of extractables release tests for incoming container closure and pump critical components, with appropriate qualitative and quantitative acceptance criteria
- Consideration of validated tests for probable leachables from labels, inks and adhesives, etc., with appropriate acceptance criteria (should these be appropriate and applicable).

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## Dry Powder Inhalers and Inhalation Powders

DPIs are defined as "drug products designed to dispense powders for inhalation" (*2,4*). The drug substance in an inhalation powder has a particle size distribution in the respirable range, and may be a physical mixture of active pharmaceutical ingredient(s) with carrier particles or a formulated combination of active ingredient and excipients (see ⟨601⟩). The powder may be contained in a unit dose packaging system (e.g., capsule, blister), or reside in bulk in a reservoir inside the delivery device itself. In the latter case, the dose is metered by the device. The delivery device may actively disperse the powder from the container or rely on patient inspiration to supply the energy necessary to disperse the particles. The components and the design of the device are integral to the aerosol characteristics (i.e., mass and particle size distribution) of the formulation delivered to the patient. There is a wide diversity of DPI designs and characteristics (*2*).

Of all OINDP, the DPI has the lowest risk of exposing a patient to leachables at significant levels. The reasons for this are:

1. The DPI drug product formulation is a dry powder, and contains no solvent, either organic or aqueous, which can promote leaching of organic (or inorganic) chemical entities.
2. In a unit dose DPI, the drug product formulation is contained in a separate packaging system, and is usually only in transient contact with critical components of the device itself.

The most likely source of leachables in a unit dose DPI would be the material composing the unit dose container, such as a foil laminate blister or capsule material, or the material composing the drug product reservoir in a multidose DPI (including antistatic surface additives). Leaching would have to occur either via direct contact of the drug product powder with the packaging material, via volatilization of organic chemical entities from the container closure material with deposition on the dry powder, or via migration of organic chemical entities through the primary packaging material with deposition on the dry powder. The possibility of observing leachables from the DPI unit dose container is best evaluated with detailed extraction studies on the container material to identify potential leachables, which could possibly migrate to the dry powder by either solid-solid contact or volatilization and have potential safety concerns.

The device and packaging materials are typically evaluated for potential leachables by extraction and simulation studies (see ⟨1663⟩) to determine whether there are chemical entities at levels that would pose a safety concern. The evaluation of materials that contain the inhalation powder must consider the inks and any other processing aids used in the manufacture of the container so that all potential leachables are characterized. The types of compounds of greatest concern for inhalation powders are those that may migrate from the primary packaging (i.e., unit dose container or multidose reservoir) into the formulation. Extraction and simulation studies should consider all possible mechanisms of leaching, including volatilization. Actual and potential leachables in inhalation powders derived from critical components of the packaging system or device that may have continuous long-term contact with the drug product formulation should be characterized (i.e., identified and quantitated) at levels above a calculated AET. An AET can be calculated for any OINDP dosage form with consideration of the SCT for OINDP (i.e., 0.15 µg/day for an individual organic leachable). An example AET calculation for an inhalation powder follows.

Given a DPI containing 13 mg of inhalation powder in a unit dose blister with 50 mg of blister material either in direct contact with the formulation or capable of volatilizing leachables into the headspace above the formulation, with a maximum recommended daily exposure of 2 actuations per day, for an individual organic leachable derived from this material, the following AET can be estimated:

$$\text{Estimated AET} = \left(\frac{0.15\ \mu g/day}{2\ \text{doses/day}}\right) \times (1\ \textit{labeled}\ \text{dose/blister})$$

$$\text{Leachables Estimated AET} = 0.075\ \mu g/\text{blister}$$

To convert relative to the total mass of drug product in a blister:

$$\text{Estimated AET} = (0.075\ \mu g/\text{blister})/(0.013\ \mu g\ \text{drug product/blister})$$

$$\text{Estimated AET} = 5.8\ \mu g/g\ \text{drug product}$$

To convert to an estimated AET, which would be a useful guide for characterizing potential leachables via extraction studies of this particular blister material (see ⟨1663⟩):

$$\text{Extractables Estimated AET} = (0.075\ \mu g/\text{blister}) \times \left(\frac{1\ \text{blister}}{0.05\ g\ \text{material/blister}}\right)$$

$$\text{Extractables Estimated AET} = 1.5\ \mu g/g$$

The challenge of characterizing drug product leachables at levels of 5.8 µg/g in an inhalation powder is considerable, even given the capabilities of modern analytical chemistry. For this particular DPI example, it might be appropriate to implement a simulation study (see ⟨1663⟩ and ⟨1664⟩) to facilitate the discovery and identification of probable leachables from the blister material, with actual drug product leachables being quantitated (if required) with high-sensitivity target compound analytical techniques and methods.

All inhalation powder packaging system and DPI device critical components should be subjected to extractables assessments (see ⟨1663⟩). Potential patient exposure to chemical entities from inhalation powder packaging system and DPI device critical components not in continuous contact with the drug product formulation should be assessed at a threshold of 20 µg/g (see ⟨1663⟩). Additional studies might be required to assess patient exposure to nonformulation contact critical component derived chemicals, including reference to food additive regulations and application of ⟨87⟩ and ⟨88⟩ (*2*).

When constructed from materials acceptable for food contact, inhalation powder packaging system and DPI device critical components not in continuous contact with the drug product formulation generally only need be appropriately characterized (i.e., extraction studies and routine extractables testing) to assure continued consistent composition of the component.

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In addition, for DPIs and inhalation powders, and based on applicable regulatory guidance (*2*), drug product applicants should consider the following (see ⟨1663⟩):

- Development and validation of extractables release tests for incoming inhalation powder packaging system and DPI device critical components, with appropriate qualitative and quantitative acceptance criteria.

# ADDITIONAL CONSIDERATIONS

## Analytical Uncertainty

An AET is that concentration above which unknown leachables should be characterized and reported for toxicological assessment. Target leachables (previously characterized as potential or probable leachables from extractables or simulation studies) will have known safety profiles and previously established leachables thresholds. In addition, reference compounds for previously characterized potential leachables will allow for accurate and precise quantitation of those target leachables as actual drug product leachables. Characterization of unknown leachables requires consideration of analytical uncertainty, as the location of an AET in a given leachables profile (e.g., a gas chromatography/mass spectrometry [GC/MS] chromatogram) must be accomplished relative to an internal standard(s) within the leachables profile. Analytical uncertainty for a particular analytical technique or method can be estimated based on the analysis of a series of reference compounds to create a response factor database. The reference compounds included in this database should represent all known potential leachables (i.e., as determined from extractables assessments). For OINDP, it is recommended (*4*) that the estimated AET be lowered by a factor defined as 1% relative standard deviation in an appropriately constituted response factor database, or a factor of 50% of the estimated AET, whichever is greater. Detailed examples of response factor databases and AET determinations are available (*4*).

## Special Case Compounds

Polycyclic Aromatic Hydrocarbons (PAHs) or Polynuclear Aromatics (PNAs), *N*-nitrosamines, and 2-mercaptobenzothiozole (2-MBT) are considered to be "special case" compounds (i.e., compounds with special safety and historical concerns), requiring special characterization studies using specific analytical techniques and methods (*1,2,4*). Thresholds for characterization of these compounds as extractables or leachables in OINDP are typically based on the limits of these specific analytical techniques and methods. *Table 2* lists the PNAs and *N*-nitrosamines that, along with 2-MBT, are typically investigated as extractables and leachables in OINDP.

**Table 2. PAHs, PNAs, and *N*-Nitrosamines Typically Investigated as Extractables and Leachables for OINDP**

| Target PAHs/PNAs | Target *N*-nitrosamines |
|---|---|
| Naphthalene | *N*-Nitrosodimethylamine |
| Acenaphthylene | *N*-Nitrosodiethylamine |
| Acenaphthene | *N*-Nitrosodi-*n*-butylamine |
| Fluorene | *N*-Nitrosomorpholine |
| Phenanthrene | *N*-Nitrosopiperidine |
| Anthracene | *N*-Nitrosopyrrolidine |
| Fluoranthene | — |
| Pyrene | — |
| Benzo(*a*)anthracene | — |
| Chrysene | — |
| Benzo(*b*)fluoranthene | — |
| Benzo(*k*)fluoranthene | — |
| Benzo(*e*)pyrene | — |
| Benzo(*a*)pyrene | — |
| Indeno(123-*cd*)pyrene | — |
| Dibenzo(*ah*)anthracene | — |
| Benzo(*ghi*)perylene | — |

PNAs have been associated with carbon black filler used in many types of elastomer. Analysis of PNAs, either as elastomer extractables or as drug product leachables, usually involves quantitative extraction followed by highly specific and sensitive analysis of resulting extracts. GC/MS with selected-ion-monitoring has been reported for analysis of target PNAs as leachables in MDI drug products, for example (*5*). *N*-Nitrosamines are reaction products between specific organic precursor molecules, secondary amines ($R_2NH$), and a "nitrosating agent". In the compounding of rubber, secondary amines are likely formed from certain vulcanization accelerators such as thiurams and dithiocarbamates. Potential nitrosating agents include $NO^+$, $N_2O_3$, $N_2O_4$, etc., certain of which can be formed from commonly used chemicals such as sodium nitrite ($NaNO_2$), which has many industrial uses. Analysis of *N*-nitrosamines in rubber as potential leachables involves quantitative

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extraction followed by analysis of extracts with gas chromatography/thermal energy analysis detection (GC/TEA) (*7*). Analysis of *N*-nitrosamines as leachables in MDI drug products using GC/TEA has been reported (*6*). The 2-MBT is a vulcanization accelerator, which is used in certain sulfur-cured elastomers, and can be analyzed by extraction followed by LC/MS (*4*).

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# ⟨1664⟩ ASSESSMENT OF DRUG PRODUCT LEACHABLES ASSOCIATED WITH PHARMACEUTICAL PACKAGING/DELIVERY SYSTEMS

## PURPOSE

This general chapter presents a framework for the design, justification, and implementation of assessments for drug product leachables derived from pharmaceutical packaging and delivery systems. A scientifically sound leachables assessment is important to manufacturers and their various suppliers primarily as a means of establishing the suitability for use of pharmaceutical packaging/delivery systems, as leachables can potentially affect drug product efficacy, safety, and quality. Additionally, such a leachables assessment could provide an understanding of the sources of leachables and how to evaluate and manage leachables during the drug development and manufacturing processes. The chapter establishes critical dimensions of a leachables assessment and discusses practical and technical aspects of each dimension. The chapter does not establish specific analytical methods or leachables specifications and acceptance criteria for any particular dosage form or packaging system or drug product combination; nor does it delineate every situation in which a leachables assessment is required. It is not possible for a general discussion of drug product leachables to anticipate and cover all situations which can occur in the pharmaceutical industry where a leachables assessment might be required. Designing an individual leachables assessment is a process that strikes a balance between sound science, prudent resource allocation, and effective risk management with an emphasis on patient safety and product quality. Achieving this balance is the responsibility and obligation of the drug product manufacturer, and assumes due consideration of applicable legal and regulatory requirements. The principles and best demonstrated practices outlined in this general chapter represent a consensus interpretation of sound science and can therefore be extrapolated and applied to any situation in which a leachables assessment is required for pharmaceutical application.

In many cases, drug product leachables assessments are based on or facilitated by knowledge from extractables assessments accomplished on drug product packaging systems, packaging components, and packaging materials of construction (see *Assessment of Extractables Associated with Pharmaceutical Packaging/Delivery Systems* ⟨1663⟩).

## KEY TERMS

This general chapter uses the following key terms (*1*,*2*; also see *Packaging and Storage Requirements* ⟨659⟩). Note that the terms *Packaging System*, *Packaging Component*, *Primary Packaging Component*, *Secondary Packaging Component*, and *Materials of Construction* are also defined in ⟨659⟩, and the definitions below are intended for clarification purposes within the context of this chapter and are not intended to supersede those provided in ⟨659⟩.

*Packaging Systems* are the sum of packaging components that together contain and protect the dosage form. Packaging systems are also referred to as *Container Closure Systems* and may include primary, secondary, and tertiary packaging.

A *Container* is a receptacle that holds an intermediate compound, active pharmaceutical ingredient, excipient, or dosage form and is in direct contact with the product.

A *Closure* is a material that seals an otherwise open space on a container and provides protection for the contents. It also provides access to the contents of the container.

A *Packaging Component* is any single part of the package or container–closure system including the container (e.g., ampuls, prefilled syringes, vials, bottles), closures (e.g., screw caps, stoppers), ferrules and overseals, closure liners, inner seals, administration ports, overwraps, adminstration accessories, labels, cardboard boxes, and shrink wrap.

A *Primary Packaging Component* is in direct contact or may come into direct contact with the product (e.g., IV bag).

A *Secondary Packaging Component* is in direct contact with a primary packaging component and may provide additional protection of the product (e.g., overpouch or dustcover for an IV bag).

A *Tertiary Packaging Component* is in direct contact with a secondary packaging component and may provide additional protection of the product during transportation and/or storage (e.g., shipping carton for an overpouched IV bag).

An *Ancillary Component* is a component or entity that may come into contact with a tertiary packaging component during the distribution, storage, and transportation of the packaged product (e.g., pallets, skids, shrink wrap, active containers).

*Packaging Materials of Construction* are substances used to manufacture packaging components. These are also referred to as *Raw Materials*.

A *Delivery System* is the sum of components and materials that are used to transport a drug product from its packaging to the point of administration into the patient. For example, an administration set is a delivery system that is used to transfer liquid drug products from their plastic packaging system to the site of administration to the patient.

*Extractables* are organic and inorganic chemical entities that can be released from a pharmaceutical packaging/delivery system, packaging component, or packaging material of construction and into an extraction solvent under laboratory conditions. Depending on the specific purpose of the extraction study (discussed below), these laboratory conditions (e.g., solvent, temperature, stoichiometry, etc.) may accelerate or exaggerate the normal conditions of storage and use for a packaged dosage form. Extractables themselves, or substances derived from extractables, have the potential to leach into a drug product under normal conditions of storage and use and become leachables. Thus extractables are potential leachables.

*Leachables* are foreign organic and inorganic chemical entities that are present in a packaged drug product because they have leached into the packaged drug product from a packaging/delivery system, packaging component, or packaging material of construction under normal conditions of storage and use or during accelerated drug product stability studies. Because leachables are derived from the packaging or delivery system, they are not related to either the drug product itself or its vehicle and ingredients. Leachables are present in a packaged drug product because of the direct action of the drug product on the source of the leachable. Thus leachables are typically derived from primary and secondary packaging, because the primary and secondary packaging can serve as a barrier between the packaged drug product and other potential sources of foreign chemical

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entities (e.g., tertiary packaging and ancillary components). In certain circumstances, packaging may directly contact the patient under typical clinical conditions of use (e.g., the mouthpiece of a metered dose inhaler). As a result of this contact, patients may be exposed to leachables from the packaging without the action of the drug product. Leachables are typically a subset of extractables or are derived from extractables. Note that chemical entities can also migrate from packaging/delivery systems to patients via direct contact.

Migrants are also foreign organic and inorganic chemical entities that are present in a packaged drug product because they have leached into the packaged drug product from a packaging/delivery system, packaging component, or packaging material of construction under normal conditions of storage and use or during accelerated drug product stability studies. However, migrants are differentiated from leachables by the circumstance that migrants accumulate in the packaged drug product after the migrant has crossed a physical barrier, such as that provided by primary and secondary packaging. Because migrants cross a physical barrier, they are not present in the packaged drug product due to direct action of the drug product on the source of the migrant because the barrier prevents such direct action. Thus migrants are derived from secondary and tertiary packaging and ancillary components.

Regardless of whether a substance is a leachable or migrant, it is still a foreign substance in the packaged drug product, and thus its impact must be assessed in the same manner. However, as the means by which a leachable and a migrant become entrained in a packaged drug product may be different, extractables studies meant to address leachables may be designed and implemented differently than extractables studies meant to address migrants.

*Leachables Studies* are laboratory investigations into the qualitative and quantitative nature of a particular leachables profile(s) over the proposed shelf-life of a particular drug product.

*Characterization* is the discovery, identification, and quantitation of each individual organic and inorganic leachable present in a drug product formulation above a predetermined level or threshold. Such thresholds should be based mainly on patient safety considerations, with consideration also given to the capabilities of analytical technology, and other related issues.

*Identification* is the process of assigning a molecular structure to an organic leachable, or assigning constituent elements in the case of an inorganic leachable.

*Quantitation* is the process of measuring the level, or concentration, of an individual organic or inorganic leachable contained in a drug product formulation.

*Leachables Profiles* are qualitative and/or quantitative analytical representations of the leachables content of a particular drug product formulation.

*Leachables–Extractables Correlations* are established when observed drug product leachables are linked both *qualitatively* and *quantitatively* to extractables from associated packaging/delivery systems, packaging components, or materials of construction.

*Threshold of Toxicological Concern* (TTC) is a level of exposure for all chemicals, whether or not there is specific toxicity data, below which there would be no appreciable risk to human health (*6*). The TTC approach is a form of risk characterization in which uncertainties arising from the use of data on other compounds are balanced against the low level of exposure.

*Safety Concern Threshold* (SCT) is the threshold below which a leachable would have a dose so low as to present negligible safety concerns from carcinogenic and noncarcinogenic toxic effects.

*Qualification Threshold* (QT) is the threshold below which a given noncarcinogenic leachable is not considered for safety qualification (toxicological assessments) unless the leachable presents structure-activity-relationship (SAR) concerns.

*Analytical Evaluation Threshold* (AET) is the threshold at or above which a leachable should be characterized and reported for toxicological assessment. The AET can be mathematically derived from the SCT (or other threshold concepts) based on factors that include the dosing parameters of the drug product.

As noted, additional terminology and associated definitions are available (*1,2*; see also ⟨659⟩).

## BACKGROUND

Management of leachables is important to pharmaceutical and biotechnology/biologic product manufacturers and regulatory authorities because certain leachables above specific concentrations can present safety concerns for patients and/or compatibility issues for drug product formulations. During the 1980s, the U.S. Food and Drug Administration (FDA) began to formally and comprehensively address leachables in drug products after findings of patient sensitivity induced by leachables and other potential safety concerns related to leachables (*2–4*). Since then, management of both extractables and leachables for packaging systems and final drug products has become an important part of pharmaceutical development and regulatory submissions for many dosage form types, particularly for those deemed of relatively high risk for dosage form interaction with the packaging system, along with a relatively high safety risk relative to the route of administration (see *Table 1*). Note that *Table 1* is a version of the original concept that appears in the FDA guidance *Container Closure Systems for Packaging Human Drugs and Biologics* (*1*), in which certain dosage forms in the above guidance have been downgraded to having lower potential for interaction with packaging components. Remaining relatively high-risk dosage forms include: inhalation aerosols and solutions, injectables and injectable suspensions, ophthalmics, and transdermal ointments and patches. It is important to note, however, that even low-risk dosage forms present some risk and that appropriately rigorous leachables assessments can be important to particular drug products in lower risk dosage form categories (e.g., topicals and oral dosage forms, etc.).

**Table 1. Modified FDA/CDER/CBER Risk-Based Approach to Consideration of Leachables[a] (*1*)**

| Examples of Packaging Concerns for Common Classes of Drug Products | | | |
|---|---|---|---|
| **Degree of Concern Associated with the Route of Administration** | **Likelihood of Packaging Component-Dosage Form Interaction** | | |
| | **High** | **Medium** | **Low** |
| Highest | Inhalation Aerosols and Sprays | Injections and Injectable Suspensions; Inhalation Solutions | Sterile Powders and Powders for Injection; Inhalation Powders |

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**Table 1. Modified FDA/CDER/CBER Risk-Based Approach to Consideration of Leachables[a] (*1*) *(continued)***

| Examples of Packaging Concerns for Common Classes of Drug Products | | | |
|---|---|---|---|
| **Degree of Concern Associated with the Route of Administration** | **Likelihood of Packaging Component-Dosage Form Interaction** | | |
| | **High** | **Medium** | **Low** |
| High | Transdermal Ointments and Patches | Ophthalmic Solutions and Suspensions; Nasal Aerosols and Sprays | — |
| Low | Topical Solutions and Suspensions; Topical and Lingual Aerosols; Oral Solutions and Suspensions | — | Oral Tablets and Oral (Hard and Soft Gelatin) Capsules; Topical Powders; Oral Powders |

[a] While this table provides a convenient overview of the general level of regulatory concern with various dosage forms regarding leachables, it should not be inferred that "low-risk" dosage forms (e.g., oral tablets) by that definition carry no risk for leachables issues.

This chapter will describe scientific principles and best practices for the assessment of drug product leachables, and will cover various important concepts, including: 1) the requirement for leachables studies; 2) fundamental concepts for leachables studies; 3) the basis of thresholds for leachables and general guidance about application of these thresholds; 4) design and implementation of leachables studies; 5) leachables method development and validation; 6) correlation of results from extractables assessments and routine extractables testing with leachables studies; and 7) establishment of leachables specifications including acceptance criteria.

These scientific principles and best practices apply to all organizations and individuals involved in the manufacture, marketing, and qualification of drug products and in their stability studies, including but not limited to:

- Manufacturers of drug products for human and veterinary use where manufacturing may involve operations at the applicant holder's facilities (i.e., facilities that belong to the holder of an approved New Drug Application or Abbreviated New Drug Application) or at those of a contractor for the applicant holder
- Manufacturers of combination drug products
- Packaging operations by the manufacturer or a designated contractor for the applicant holder
- Repackaging operations in which the drug product may be owned by an organization other than the primary manufacturer.

Although it is ultimately the drug product applicant's responsibility to ensure that appropriate leachables assessments are completed, manufacturers and fabricators of pharmaceutical packaging/delivery systems, packaging components, and materials of construction should also apply these scientific principles and best practices as appropriate, and applicants are encouraged to work with component manufacturers and fabricators to this end.

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# CONCEPTS

## General Concepts for Leachables Assessment

During the course of manufacturing, packaging, storage, distribution, and administration, dosage forms and/or their formulation constituents contact components and materials of construction of manufacturing and packaging equipment, and primary and secondary packaging components and systems. Such contact may result in interactions between the dosage form and these components and materials. One such interaction is the migration, or leaching, of substances from any of these components and materials into the dosage form. Leachables, which can include both organic and inorganic (i.e., elemental) chemical entities with wide chemical diversity, are of concern due to their potential safety risk to patients and potential compatibility risks for the drug product. In order to assess these risks and manage the potential issues posed by leachables, it is necessary to know their identities and the levels to which they will accumulate in the finished drug product over its shelf-life. These two pieces of information can be used to establish the magnitude of patient exposure (dose) and therefore the safety risk posed by an individual leachable, as well as the likelihood of any drug product compatibility issues.

Regulatory guidelines, requirements, and various best practice recommendations all state that the definitive assessment of the potential impact of contact between a packaging/delivery system and a final dosage form involves testing the final drug product for leachables. In its most essential form, this impact assessment involves performing a migration, or leachables, study whose purpose is to discover, identify, and quantitate leachables that have migrated from the contacted system, components, or materials and accumulated in the finished dosage form under the product's actual manufacturing, storage and clinical use conditions. A leachables study is a laboratory investigation into the qualitative and quantitative nature of a particular leachables profile(s) over the proposed shelf-life of a particular drug product. The purpose of a leachables study is to systematically and rationally identify and quantify (i.e., characterize) drug product leachables to the extent practicable, and within certain defined analytical threshold parameters. The results of leachables studies are used in the overall leachables assessment to understand the impact of leachables on patient safety and drug product quality and stability.

Leachables studies can be used within the context of an overall leachables assessment to:

- Facilitate the timely development of safe and effective dosage form packaging/delivery systems, manufacturing systems, and processes by assisting in the selection of components and materials of construction
- Facilitate the establishment of qualitative and quantitative leachables–extractables correlations in drug products, when coupled with an appropriate extractables assessment(s)
- Establish the worst-case drug product leachables profile in a manner that facilitates the development of drug product leachables specifications and acceptance criteria (should these be required), and the safety evaluation/qualification of leachables

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- Identify trends in drug product leachables accumulation levels over the shelf-life of a particular drug product
- Facilitate change-control processes for drug product packaging/delivery systems (as appropriate), packaging components, materials of construction, formulation constituents, etc.
- Facilitate investigations into the origin(s) of identified leachables whose presence causes out-of-specification (OOS) results for a marketed drug product.

In these ways, leachables studies and assessments can support Quality by Design (QbD) principles for the development and manufacture of pharmaceutical packaging/delivery systems and drug products.

A complete leachables assessment includes understanding the safety impact of individual leachables, safety qualification of individual leachables, and developing an understanding of the impact of individual leachables on drug product stability (i.e., compatibility) and stability. Although safety qualification is presented in general terms, the details of attaining this goal are beyond the scope of this chapter, which is limited to general scientific principles and best practices for the conduct of leachables studies and the other stated uses of the results of leachables studies within an overall leachables assessment. The reader is directed to authoritative manuscripts on this topic (*2, 8*).

Note that certain packaging and combination product medical device components are (or can be) in direct contact with a patient's mouth, nasal mucosa, or other body tissue(s) during normal use of the drug product. Such packaging components include metered dose inhaler and dry powder inhaler mouthpieces, transdermal patches, etc. Patients are potentially exposed to chemical entities by direct contact from such components. Assessment of patient exposure in such direct contact scenarios is best accomplished with appropriate extractables assessments and extraction studies, and the reader is referred to ⟨1663⟩.

## Safety Thresholds

Although leachables represent a particular class of drug product impurity, current regulatory guidance for drug product impurities specifically considers leachables to be out of scope (*5*). Thresholds that have been specifically proposed for drug product leachables are based on either patient safety considerations or the current capabilities of analytical technology. Safety thresholds are particularly important in a leachables assessment because current analytical technology allows detection of trace organic and inorganic chemical entities at extremely low levels (i.e., ng/mL; ng/g). Identification and risk assessment (or qualification) of every individual chemical entity in a typical leachables profile at the limits of current analytical technology is neither necessary from a toxicological perspective nor feasible in a typical drug product. Safety thresholds allow for a science- and risk-based determination of acceptable levels of leachables and can be based on established toxicological information, as well as additional safety risk factors that consider, e.g., route of administration, daily exposure, and treatment duration. Because safety thresholds are derived from exposure data they are considered in terms of units of exposure, such as Total Daily Intake (TDI). Thus, any safety threshold must be converted into units of concentration (e.g., µg/mL) so that it can be applied as an analytical threshold in the laboratory. The analytical threshold is a guide as to which chemical entities in the leachables profile should be considered for chemical characterization (i.e., confirmed identification) and safety evaluation and qualification.

An example of a safety threshold concept that has been practically applied in pharmaceutical development is the Threshold of Toxicological Concern (TTC) approach (*6*). The TTC concept was adopted by the European Medicines Agency (EMA) to evaluate genotoxic impurities, using an excess cancer risk factor of $10^{-5}$ (1 in 100,000) (*7*). The EMA's proposed safety threshold for genotoxic impurities using the TTC approach is 1.5 µg/day TDI. Other examples of safety thresholds include the Product Quality Research Institute (PQRI) Safety Concern Threshold (SCT) and Qualification Threshold (QT), derived and proposed for individual organic leachables in Orally Inhaled Nasal Drug Products (OINDP) (*2, 8*). The SCT is 0.15 µg/day TDI, and the QT is proposed at 5 µg/day TDI for an individual organic leachable. The development of the TTC approach provided a foundation, precedent, and guide for derivation of the PQRI SCT, which incorporates a $10^{-6}$ (1 in 1,000,000) risk factor rather than the $10^{-5}$ value used for the EMA threshold. This lower threshold was considered appropriate for leachables in OINDP because of considerations regarding the direct delivery of some of these dosage forms to diseased organs of a sensitive patient population, and assuming lifetime exposure. In addition, leachables are typically industrial chemicals with no direct structural relationship to any active ingredient or other formulation constituent. Below the SCT, identification and safety evaluation of leachables generally would not be required. Below the QT, leachables without structure alerts for carcinogenicity or irritation would not require compound-specific safety risk assessment. Note that neither the SCT nor the QT is a control threshold or safety-driven limit. Rather, they are leachables evaluation thresholds. The SCT in particular is designed to establish a threshold for characterization of unknown drug product leachables. Individual levels of safety concern, different from the SCT value, could be determined for known leachables and potential leachables (i.e., extractables).

For OINDP there are certain "special case" compounds and compound classes, that due to particular safety concerns (e.g., carcinogenic) were deemed to require lower thresholds based on the capabilities of specific analytical technologies and methods. These special case compounds for OINDP include: polyaromatic hydrocarbons or polynuclear aromatics (PAHs or PNAs), *N*-nitrosamines, and the individual chemical entity 2-mercaptobenzothiozole (see *Orally Inhaled and Nasal Drug Products* ⟨1664.1⟩).

## Information Sharing

To successfully manage leachables throughout the drug product lifecycle, it is critical to establish close and regular communication among those stakeholders throughout the development and drug product lifecycle responsible for the quality of the drug product: chemists, toxicologists, packaging engineers, manufacturing operations, procurement, etc. With respect to leachables, communication between the analytical chemist and toxicologist is critical. For example, if a leachable is found to be above an accepted limit, or a new leachable is found, a safety evaluation will need to be performed. The chemist will need to provide the toxicologist with information that will help to qualify the leachable, including the identity of the leachable, which may include compound class or more specific information, such as chemical formula and structure; and the amount and concentration of the leachable in the drug product.

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Information sharing between packaging component manufacturers/suppliers and drug product developers/manufacturers is also important in order to guide packaging component and materials of construction selection, provide knowledge of potential extractables and leachables, and facilitate leachables–extractables correlations via knowledge of packaging component chemical compositions, etc. (also see ⟨1663⟩).

## LEACHABLES STUDY DESIGN

Although leachables studies may be accomplished at any time during the drug product development/manufacturing lifecycle, leachables studies are especially relevant during late stage product development or during formal product stability assessment. Ideally, leachables assessment is conducted as follows:

- The assessment is performed on the actual drug product and not simulations thereof (however, see *Simulation Studies*).
- The assessment is performed with the actual packaging and delivery system in the form it will be commercialized, not with a prototype or on system components.
- The related extractables assessments are accomplished on the same lots of packaging components used to manufacture the drug product lots on which the leachables assessments are performed.
- The assessment is performed on a product that is manufactured under conditions that reflect the actual commercial processes of production of the drug product and the packaging/delivery system, filling of the drug product into the packaging/delivery system, post-filling treatment of the filled packaging (e.g., terminal sterilization), distribution, storage, and clinical use of the drug product. Although leachables studies may include accelerated storage conditions, they cannot be limited to accelerated conditions and must include real-time assessment.

Leachables studies can also be performed early in the drug product development process (e.g., preclinical stage) in order to facilitate the selection of packaging components and their materials of construction. Such leachables studies are particularly useful for certain "high-risk" dosage forms (see *Table 1*) where selecting appropriate packaging components and materials of construction is critical. A variety of packaging components and materials of construction can be evaluated at the same time and drug product leachables profiles determined and evaluated for each configuration. For primary packaging systems or combination drug/device products this can be accomplished by using either the drug product formulation or a placebo formulation in contact with the proposed packaging system. In the latter case, the placebo formulation can be considered as a simulating solvent to characterize extractables as probable leachables (see *Simulation Studies*). In either case, the leachables study conditions (i.e., time, temperature, etc.) should be based on conditions that are relevant for either the use-life or shelf-life of the drug product. Preclinical development stage leachables studies can be designed in a systematic way in order to support QbD processes and principles. It is important to also note that during early stage drug product development for high-risk dosage forms, leachables characterization is recommended for any drug product batches that are used as test articles in any definitive toxicology or clinical studies. For "low-risk" dosage forms (e.g., solid orals, topical powders) leachables studies conducted throughout development might be appropriate in order to assess, and thereby avoid, problems with packaging systems that might appear either in later stage development or marketed product.

During later stage development of high-risk dosage forms in support of product registration, when the final market form of the packaged drug product is available, leachables studies may be accomplished on definitive registration batches of drug product during the course of overall product stability studies. The results of these leachables stability studies can be used to establish leachables–extractables correlations, identify trends in leachables accumulation levels, evaluate individual leachables and qualify them on a safety basis, and develop leachables specifications with acceptance criteria (should these be required). For inhalation aerosols and other OINDP, leachables testing should be an integral part of the larger ICH registration stability program (*2*), and storage conditions and stability time points should be planned accordingly. For cases where a packaging/delivery component is in direct contact with the patient (e.g., a metered dose inhaler or dry powder inhaler actuator mouthpiece), chemical entities that a patient might be exposed to can be evaluated as extractables (i.e., potential leachables) using appropriate simulating fluids under time/temperature exposure conditions relevant to the intended use (see ⟨1663⟩).

Additionally leachables assessments may be appropriate on certain occasions post-market. For example, drug product leachables studies may also be appropriate in many cases where necessary or desired changes in a marketed drug product are made. Such leachables studies are normally required to support change-control processes for many high-risk dosage forms, particularly those with in-place leachables specifications and acceptance criteria, and could also be appropriate for other dosage form types, drug/device combination products, etc. Changes may include but are not limited to: composition of the drug formulation; manufacturing processes for the drug product; primary and secondary packaging components or their materials of construction; manufacturing or assembly processes for primary and secondary packaging components or their materials of construction; and delivery system(s) that are part of the drug product labeling. Any change that results in the patient being exposed to a different leachables profile than the one approved during registration will require leachables studies as part of any change-control process unless adequate scientific justification is provided to the contrary.

Although low-risk dosage forms (e.g., solid orals, topical powders) typically do not rigorously require leachables studies as part of the drug product registration process (*Table 1*), it is possible that leachables could appear in drug product impurity profiles either during registration stability studies or in marketed products. For example, it has been documented that chemical additives in label adhesives can migrate through plastic primary packaging and appear in impurity profiles of solid oral dosage forms packaged within these containers. Thus, it is appropriate to consider performing leachables studies on "low-risk" dosage forms in certain cases. If leachables assessment is not performed proactively, such an event could lead to an OOS result for a development or marketed product and require an "emergency" leachables study as part of an investigation process. The design of this type of leachables study depends on the particular situation; however, in general it would be necessary to identify and quantify the leachable(s), evaluate safety and possibly qualify the leachable(s), and correlate the leachable(s) with packaging component extractables. It is also possible that leachables could result from contact with manufacturing equipment and tertiary packaging systems (e.g., shipping materials).

The design of a particular leachables study depends on the purpose and goals of the overall leachables assessment. Although the leachables studies described above have different purposes and overall goals, they require similar types of information for

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their proper design. First, it is important to have information as to the identities and maximum possible accumulation levels of all potential leachables. The packaging component manufacturers may provide chemical composition details for the packaging/delivery system and various materials of construction, as well as details regarding the manufacturing processes for these components and materials. Such information may be in the form of material safety data sheets, technical data sheets, test reports, or confidential communications, and can be used to infer potential leachables. An extractables assessment (including an extraction study) can also be accomplished on packaging components and/or their materials of construction to directly assess potential leachables (see ⟨1663⟩). Regardless of how the chemical information is obtained, it is important to ensure that all possible sources of potential leachables from the finished packaging system are considered. These may include chemical entities from any of the primary and secondary packaging components and their materials of construction, coatings, cleaning, lubricating, cutting, sterilization, assembly, or other processes associated with the manufacture of the final packaging/delivery system as used in the drug product. The chemical information on the packaging and delivery system is used to create a list of potential leachables and their possible accumulation levels.

Potential leachables have a significant chemical diversity, and therefore a diversity of physical and chemical properties, including polarity, volatility, solubility, etc. Whereas relatively volatile compounds can more readily migrate into any type of formulation through indirect contact, nonvolatiles generally require direct contact. Two aspects of formulation contact should be considered: the nature of the formulation contact (i.e., direct or indirect) and the time of contact (transient or continuous). If the formulation is not in direct contact with the packaging component (e.g., inhalation powder in a capsule packaged in a blister) then it is less likely that any relatively nonvolatile compounds would migrate into the formulation from the packaging system; however, volatile compounds might. If the formulation is only briefly contacting the packaging component (e.g., an inhaler mouthpiece) it is less likely that any migration of chemicals from the component would occur on this transient timescale. However, if the formulation is in continuous contact with the packaging component (e.g., parenteral in a bag delivered through an administration set) then all types of compounds could potentially migrate into the formulation.

A rigorous leachables assessment considers leachables from sources other than primary packaging, such as necessary secondary packaging and, in certain situations, tertiary packaging. If the primary packaging consists of a semipermeable polymer (e.g., a low density polyethylene container), then potential leachables from labels, inks, adhesives, etc. that are used in the secondary packaging must be evaluated. Similarly, volatile compounds that are present in tertiary packaging (e.g., wooden pallets, cardboard boxes, plastic overwraps, etc.) could migrate into a formulation contained in such a plastic bottle. These migrants that are derived from tertiary packaging should be considered in the event that an unknown impurity is detected or suspected in the drug product.

Various characteristics of the drug product formulation must also be considered in designing any leachables study. For example, formulations are typically either solids or liquids, and it is well documented that physical state affects the leaching process. In the event that a formulation has a change in state during the course of production (e.g., lyophilization; liquid to solid) then the leachables study should be designed taking into consideration the time periods that the formulation is expected to be in each physical state. In the event that a formulation has a change in state during the course of use (e.g., nebulization of a liquid to vapor) then consideration should be given to both the leachables acquired during storage from the container of the liquid and those acquired during use of the prescribed delivery device. In addition, typically only the final packaged product is evaluated for leachables; however, there may be cases in which an intermediate (e.g., bulk capsule for an inhalation powder) is stored for long periods of time in different primary packaging (e.g., foil pouch) from which compounds may leach. If these compounds that migrate from bulk packaging persist through the drug product's manufacturing process and are entrained in the finished drug product, then they are properly treated as leachables.

The nature of any contact that the packaging and delivery system has with the patient must also be considered. If the contact is only surface contact, then the likelihood of direct chemical migration to the patient is much less than if the contact is with mucosa, tissue, bone, or dentin. The various contact categories are described in *The Biocompatibility of Materials Used in Drug Containers, Medical Devices, and Implants* ⟨1031⟩ or ISO 10993 (*9*).

## LEACHABLES CHARACTERIZATION

The primary goal of any leachables study is leachables characterization; i.e., the discovery, identification, and quantitation of leachables present in a particular drug product. Analytical methods for leachables characterization are developed based on the nature of the drug product matrix, the identities and possible accumulation levels of potential leachables, and the required sensitivity based on an adopted leachables evaluation threshold and the capabilities of the analytical methods employed. Unlike a typical drug product impurity method where target analytes are related to the drug substance, leachables have a wide chemical diversity and can come from various sources in the packaging/delivery system. Leachables also have a wide range of possible accumulation levels in a drug product. Taken in total, these factors present a significant challenge for trace analysis, especially in the case of organic leachables identification. Under certain circumstances, this challenge can be mitigated by performing the process of potential leachables identification outside of the leachables assessment, for example via extractables assessment in simulated extractables studies (see *Simulation Studies*).

Before describing the processes, analytical techniques, and methods involved in leachables characterization, it is appropriate to state that the ultimate objective of thorough leachables characterization as defined above cannot be realized in all cases, even when state-of-the-art analytical chemistry is practiced with best available skill and diligence. It is a reality that there is no analytical technique or combination of analytical techniques that is capable of the discovery, identification, and quantitation of any and all organic and inorganic leachables. For example, authentic reference compounds for organic leachables may not be available in all cases for confirmation of identifications or for quantitative instrument calibration. Given these circumstances, the practical objective of leachables characterization must therefore be the discovery, identification, and quantitation of individual leachables present in a drug product above a predetermined level, or "threshold", to a reasonable degree of scientific certainty and exercised with appropriate due diligence.

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## Analytical Thresholds

The starting action in leachables method development is to establish the level at which the method must perform at to accomplish the appropriate leachables characterization functions. This level is known as the analytical threshold. Minimally, an appropriate method must function at all levels greater than or equal to the analytical threshold. As discussed previously, such an analytical threshold can be based on various criteria, including safety considerations. An example of a safety-based threshold is the SCT as established for OINDP. In order to define the SCT in terms that facilitate laboratory analysis, it must be converted from units of exposure (i.e., µg/day) to units of concentration (e.g., µg/mL, µg/g, µg/canister, µg/vial, etc.). This is accomplished by considering the dose parameters for a given drug product per the drug product's label claim. The resulting analytically useful threshold is termed the Analytical Evaluation Threshold (AET) (*2*). Previously characterized target leachables will have known safety profiles and previously established leachables thresholds. In any event, thresholds can be used for the basis for analytical method development unless other product considerations, such as compatibility, dictate a lower level is necessary.

A general formula for converting the SCT (0.15 µg/day) to an AET is as follows:

$$\mathrm{AET}\left(\frac{\mu g}{\text{container}}\right) = \left(\frac{0.15 \mu g/\text{day}}{\text{doses/day}}\right) \times \left(\frac{\text{labeled doses}}{\text{container}}\right)$$

Further, for liquid dosage forms:

$$\mathrm{AET}\left(\frac{\mu g}{mL}\right) = \frac{\mu g}{\text{container}} \div \frac{mL}{\text{container}}$$

Further, for solid dosage forms:

$$\mathrm{AET}\left(\frac{\mu g}{g}\right) = \frac{\mu g}{\text{container}} \div \frac{g}{\text{container}}$$

This AET establishes the level at which unknown leachables should be identified and quantified in a particular drug product, and can therefore be used as a basis for analytical method development.

## Analytical Method Requirements

Analytical method requirements for leachables characterization are based on the determined AET (or an alternative valid threshold concept), and information on potential leachables obtained from extractables assessments of packaging components and materials, including information from component/material suppliers. Since leachables are typically a subset of extractables or chemically linked to extractables, it may be the case that analytical methods used for leachables characterization can be based on those used for extractables characterization (see ⟨1663⟩). Any analytical method for leachables that is used for drug product stability studies in support of product registration, establishing leachables–extractables correlations for high-risk dosage forms, or the development of leachables specifications and acceptance criteria must be subject to complete validation using industry-accepted validation practices.

## Preparing the Drug Product for Analysis—Sample Preparation

Sample preparation for leachables characterization is a function of the chemical nature of the potential leachables, the chemical nature of the drug product sample matrix, and the analytical technique(s) to be applied. The drug product matrix can present a significant challenge for organic leachables characterization. Drug product matrices contain the active pharmaceutical ingredient and excipients, which are typically present at high levels relative to leachables (except in certain high potency drug products). Analytical methods for organic leachables usually incorporate sample preparation procedures to separate leachables from the drug product matrix and concentrate them for analysis. The exact details of sample preparation procedures are unique to the individual drug product and while it is impossible to anticipate every scenario, the following general statements can be made:

- *Aqueous dosage form* (e.g., inhalation solutions, small and large volume parenterals, ophthalmic solutions, etc.) leachables can be recovered using liquid-liquid extraction with water immiscible organic solvents, such as dichloromethane, hexane or petroleum ether, etc. The pH of the aqueous sample can be manipulated (i.e., raised or lowered) in order to enhance extraction of weakly acidic or basic leachables, or reduce extraction problems caused by the relatively high concentration of active pharmaceutical ingredient and excipients. The resulting organic extract can be dried if required (e.g., with magnesium sulfate drying agent) and concentrated if required by techniques that remove the solvent, such as evaporation with a gentle stream of dry nitrogen, rotary evaporation, or a Kuderna-Danish concentrator, etc. Concentrated organic extracts can be analyzed directly by GC-based methods; however, for HPLC-based methods using aqueous mobile phases the organic extract can be reduced to dryness (or near dryness) and the resulting residue of leachables taken up in a water miscible solvent (e.g., acetonitrile, methanol, etc.). Volatile leachables (e.g., solvents) can be analyzed directly from aqueous drug product samples with GC combined with headspace sampling. Note that recoveries of certain leachables can be affected by extraction and extract concentration procedures.
- *Solid dosage form* (e.g., solid orals, inhalation powders, lyophilized powders, etc.) leachables can be recovered (for example) by dissolving the drug product with an aqueous solution and applying liquid-liquid extraction and extract concentration, as above. Headspace sampling and GC analysis of volatile leachables can be accomplished on the aqueous samples or, in some cases, directly on the solid dosage form. It is also possible to dissolve the drug product sample in another appropriate and analytically expedient medium (e.g., an organic solvent) for direct analysis by GC; however it is possible that matrix effects and interferences from the active ingredients and excipients could result.

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- *Oral liquid dosage form* leachables can be recovered by diluting the drug product sample in aqueous solution and applying liquid-liquid extraction and extract concentration, as above. Headspace sampling and GC analysis of volatile leachables can be accomplished on the aqueous samples or, in some cases, directly on the oral liquid dosage form. It is also possible to dissolve the drug product sample in another appropriate and analytically expedient medium (e.g., an organic solvent) for direct analysis by GC; however, it is possible that matrix effects and interferences from the active ingredients and excipients could result.
- *Cream and ointment dosage form* leachables can be recovered by dissolving the drug product sample in an aqueous solution, filtering, and applying liquid-liquid extraction and extract concentration, as above. Headspace sampling and GC analysis of volatile leachables can be accomplished on the aqueous samples. It is also possible to dissolve the drug product sample in another appropriate and analytically expedient medium (e.g., an organic solvent) for direct analysis by GC; however, it is possible that matrix effects and interferences from the active ingredients and excipients could result.
- Dosage forms with nonaqueous drug product vehicles (e.g., metered dose inhalers with organic solvent propellants) require special sample preparation procedures, which are discussed in ⟨1664.1⟩.

Development of sample preparation methods for leachables characterization can be accomplished through the use of appropriate test samples, such as accelerated drug product samples that have been aged under accelerated conditions (e.g., 40°/75%RH on 3-month storage), drug product or placebo samples spiked with known potential leachables, and/or a simulated drug product matrix spiked with known potential leachables. Recoveries of spiked potential leachables should be assessed and optimized during method development. Internal standards can be included to improve quantitative accuracy and precision.

Note that the sample preparation for leachables characterization must create a test sample in a form amenable to the analytical technique to be applied, and appropriately concentrated so that individual leachables can be characterized relative to the selected threshold.

## Analytical Techniques

Analytical techniques applied to leachables characterization are the same as those applied to extractables characterization, which are summarized and discussed in ⟨1663⟩. Scouting analyses in general are not applied to leachables characterization, as the drug formulation may interfere with the scouting methods (see ⟨1663⟩). The most useful analytical techniques for discovery, identification (either by qualitative or structural analysis), and quantitation of organic leachables are those that combine GC and HPLC with mass spectrometry [i.e., GC/MS and HPLC (or LC)/MS]. Headspace sampling can also be interfaced with GC/MS to address volatile compounds. Other detection systems for both GC and HPLC that are not compound specific (e.g., FID, UV, etc.) are potentially useful for leachables discovery and quantitation, but not in general for identification. The combination of GC and HPLC techniques has the sensitivity and specificity required to characterize the diversity of chemical compounds found in many leachables samples. Analytical methods for leachables should be capable of characterizing target as well as new (or unspecified) leachables (e.g., scanning GC/MS or LC/MS); however, when additional sensitivity is required due to the use of analytically challenging thresholds, dedicated target compound methods (e.g., GC/MS with selected ion monitoring) can be used. It is also possible, with appropriate validation, to use methods based on techniques that are not compound specific (i.e., GC/FID, HPLC/UV, etc.).

Structural analysis of leachables should be accomplished with a systematic process identical to that described in ⟨1663⟩ for extractables, and to a level of confidence sufficient for safety assessment. A discussion of the principles of both gas and liquid chromatography is available in *Chromatography* ⟨621⟩. A discussion of the principles of mass spectrometry (including both GC/MS and LC/MS) is available in *Mass Spectrometry* ⟨736⟩. Although chromatographic-based hybrid analytical techniques are most commonly applied to leachables characterization, other analytical techniques with compound-specific detection capability (e.g., nuclear magnetic resonance spectroscopy) can be employed.

## Quantitative Methods—Validation Considerations

Validation of quantitative leachables methods should be accomplished according to industry accepted practices, criteria, and standards, such as *Validation of Compendial Procedures* ⟨1225⟩ and (*10*). The extent of validation required depends on the goals of the leachables study in which the analytical method is being utilized. Validation parameters may include: accuracy, precision (repeatability, intermediate precision), specificity, limit-of-detection, limit-of-quantitation, linearity and range, and robustness. System suitability tests and criteria should also be developed for each leachables method. Special considerations for individual validation parameters relative to leachables methods are as follows:

- *Accuracy and precision*—The validation parameters of accuracy and precision (repeatability and intermediate precision) are typically evaluated using drug product samples spiked with known amounts of target leachables. The choice of drug product spiking matrix used for these evaluations should be one that has had little-to-no contact with the packaging materials used in the final drug product, and therefore little-to-no measurable levels of endogenous leachables. Suitable spiking matrices can include freshly manufactured drug product and simulated drug product vehicles. Spiking levels should be determined based on results from accelerated stability studies or estimated from the known amounts of potential target leachables determined from extraction studies. Accuracy is typically performed at three spiking levels, which can also be determined based on results from accelerated stability studies or estimated from the known amounts of potential target leachables determined from extraction studies.
- *Linearity and range*—Since potential leachables are present in packaging components at widely varying levels, actual drug product leachables can likewise appear at widely varying levels. The best accuracy and precision are achieved when the validated linear range considers the potential maximum accumulation levels of each target leachable or chemical class of leachables.
- *Limit of detection/Limit of quantitation*—To detect and quantitate unknown leachables, the limit of quantitation should be at or below the designated analytical threshold (e.g., AET).

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- *Specificity*—Evaluation of method specificity can be accomplished by evaluating chromatographic peak purity in spiked and nonspiked drug product samples. For GC-based quantitative methods, this can be accomplished by GC/MS. For HPLC-based methods, either LC/MS or LC/DAD (diode array detection) can be used. Specificity can be qualitatively demonstrated if there are no observable method interferences related to the chemical entities present in the drug product.
- *Robustness*—A design-of-experiments statistical approach with consideration of critical analytical method parameters (e.g., HPLC flow-rate, HPLC column, mobile phase gradient, etc.) should be used to create robustness evaluation protocols. Other approaches, such as serial change of critical parameters, can also be applied.
- *System suitability*—Chromatography-based analytical methods, such as those described in ⟨621⟩, should include appropriate system suitability criteria for routine method evaluation, including tests for method linearity, precision, sensitivity, and specificity as appropriate. These parameters should be evaluated with an appropriately constituted test mixture(s) each time the quantitative leachables method is used, and should include appropriate system suitability acceptance criteria based on the method validation results. For example, sensitivity may be confirmed by analysis of standards prepared at the analytical threshold.

Several examples of validated leachables methods from the scientific literature have been documented in the chemical literature (*11*–*16*).

## ESTABLISHING A LEACHABLES–EXTRACTABLES CORRELATION

A leachables–extractables correlation is established when actual drug product leachables can be linked either qualitatively or quantitatively with extractables from corresponding extractables assessments of individual materials of construction, packaging components, or packing systems. Leachables–extractables correlations are important for several reasons, including justifying the use of routine extractables release tests of packaging components as an alternative to leachables testing during stability studies for high-risk drug products, establishing the source of a leachable producing an OOS result for a low-risk drug product, change control, and ongoing quality control, etc.

A *qualitative correlation* is demonstrated when a leachable is linked either *directly* or *indirectly* to an extractable (i.e., potential leachable). For example, hexadecanoic acid observed in a leachables profile can be directly linked with hexadecanoic acid observed in the extractables profiles of one or more primary packaging components. The ethyl ester of hexadecanoic acid observed in the same leachables profile can be indirectly linked with hexadecanoic acid observed in one or more extractables profiles, if ethanol is a known drug product formulation constituent and it is shown that an esterification reaction can occur in the drug product during storage. For an appropriate quantitative leachables–extractables correlation to exist, the quantity of any individual leachable over the shelf-life of a drug product must be mathematically related to the quantity of the corresponding extractable in its source. One of the more simple mathematical relationships between an extractable and a leachable is that the quantity of the leachable in the drug product should be less than or equal to the quantity of the corresponding extractable. For example, the concentration of butylatedhydroxytoluene (BHT) present in a drug product formulation was determined to be 5 µg/mL. BHT was extracted from a primary packaging system component at a level of 300 µg/component. If the drug product packaging system incorporates one of these components per dosage form and the packaged formulation has a volume of 50 mL, then a quantitative leachables–extractables correlation is established, as BHT was extracted in the amount of 300 µg (300 µg/component × 1 component) and was leached in the amount of 250 µg (5 µg/mL × 50 mL). As a result, it can be concluded that on the average 50 µg of BHT is unaccounted for (300 µg extracted – 250 µg leached), and that this quantity was either not leached from the packaging component into the formulation (more likely) or lost by some other process (less likely).

For high-risk drug products, leachables–extractables correlations may be established over multiple batches of drug product (accelerated or at end of shelf-life) and multiple batches of packaging components. Extractables studies should ideally be conducted on the same lots of components that were used to manufacture the drug product batches used in primary stability studies (and therefore on the drug product batches on which leachables testing was conducted to establish leachables–extractables correlations).

If the maximum level of any specific leachable in the formulation during stability studies was substantially greater than the calculated maximum potential accumulation levels of that same leachable as established by the extraction study, and the extraction studies were conducted on the same lots of components used to make the primary drug product stability batches, it can be concluded that the extraction study was incomplete and therefore a leachables–extractables correlation for that specific leachable cannot be established. In this case, either the extraction study can be augmented with experiments that produce an extractable level exceeding the maximum level of the leachable, or the leachable can be controlled via the drug product specifications for shelf-life stability testing, and release testing as an extractable at the component level is inadequate to control this leachable.

If a leachable–extractables correlation cannot be established, possible explanations include: inadequate extractables assessments of packaging components (see ⟨1663⟩); unreported changes in packaging component composition or manufacturing processes; unreported changes in the identity of packaging components.

## CONSIDERATIONS IN DEVELOPING LEACHABLES SPECIFICATIONS AND ACCEPTANCE CRITERIA

The validated analytical methods and information obtained from those methods in a drug product leachables study can be used to develop drug product leachables specifications and acceptance criteria (i.e., limits). In certain circumstances, most commonly encountered with high-risk dosage forms (such as OINDP), it may be meaningful, useful, and at times required to routinely monitor finished drug products for leachables. Under such circumstances, leachables specifications and acceptance criteria must be established. One means by which such specifications and acceptance criteria could be developed includes testing a minimum of three drug product batches to determine their leachables levels. After thorough chemical and safety

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evaluation, the test data from the three or more batches can be used to establish acceptance criteria for targeted leachables, consistent with 1) the qualitative and/or quantitative results of leachables studies, 2) a consideration of the capabilities of the drug product's manufacturing process, and 3) a consideration of the potential safety, compatibility, and/or drug product quality impact of the leachables. It is important to note that leachables specifications should be applicable to a product during all stages of its shelf-life, including release and at end of shelf-life. This is the case since leachables accumulate over the entire shelf-life of a drug product.

When a change occurs in a product for which leachables specifications and acceptance criteria have been established, it is important to review the analytical method and re-evaluate the acceptance criteria and make adjustments to the specifications and acceptance criteria as appropriate and scientifically justified. A change in components that results in an increase in leachables concentrations beyond the levels qualified will necessitate the toxicological evaluation of the proposed levels, as would be the case for any impurity.

Acceptance criteria can be both qualitative and quantitative for both known and unspecified leachables. For example, a typical leachables specification could include:

- Quantitative end of shelf-life limits for target leachables, which apply over the shelf-life of the drug product
- A quantitative limit for "unspecified" (i.e., previously unidentified and uncorrelated) leachables (identification of unspecified leachables is required for accurate quantitation and toxicological evaluation).

## ADDITIONAL CONSIDERATIONS

### Simulation Studies

Occasions may arise in which it is not analytically feasible (due to challenging thresholds, for example) to successfully discover and identify all actual leachables in a drug product leachables study. This circumstance can be managed if the activities of discovery and identification of probable leachables are accomplished in an extraction study, where samples and analyte concentrations are more easily manipulated to achieve the necessary analytical performance. In such a circumstance, the actual drug product leachables assessment is simplified to a high-sensitivity quantitation of targeted leachables that have been discovered and identified as part of this extraction study.

In order to facilitate the discovery and identification of probable leachables, the extraction study must be similar in design to a drug product leachables study. Such an extraction study seeks to simulate the circumstances experienced by the drug product but should produce a test sample that is easier to characterize than the drug product itself. For such a study to be relevant in establishing appropriate target leachables, the solvent(s) used to generate the test sample must have nearly the identical propensity to leach as the drug product formulation. Such a study should be accelerated versus the leachables study so that the extractables, reflecting potential target leachables, can be discovered and identified in a timely fashion. Differences in the study design between this simulation study and the drug product leachables study are: 1) that the drug product formulation has been replaced with a simulating solvent that mimics the formulation; 2) that the conditions of contact have been accelerated, so as to increase both the concentrations of probable leachables and the rates of migration of probable leachables into the simulating solvent; and 3) that the test article can be either the complete packaging and delivery system or separate components of that system. Factors to consider in designing and justifying the simulating solvent(s), along with recommendations on the analytical approach used to characterize the simulating extract for extractables as potential target leachables are discussed in ⟨1663⟩. Given the intent of the simulation study, which is the discovery and identification of extractables as target leachables, simulation studies must also be driven by relevant thresholds.

It is possible that in cases of very low thresholds (e.g., AETs), quantitation of drug product leachables might still not be analytically feasible, even with high sensitivity target compound analytical methods. In such cases, the results of the simulation study (probable leachables identities and concentrations) may be sufficient to establish patient safety and the quality impact of the actual drug product leachables. **To the extent that the simulation study mimics the drug product leachables study**, the potential safety or quality impact of a compound as an extractable is an estimate of the potential safety or quality impact of the compound as an actual leachable. If it can be established that a compound quantitated as an extractable under these conditions has an acceptably small impact on safety and quality, then it follows that the same compound as a leachable in the drug product formulation may be assumed to have a similarly low impact on safety and quality as a leachable in the drug product formulation. The acceptability of this approach for any particular drug product needs to be scientifically justified by the drug product applicant.

If a compound is measured as an extractable in a simulation study and targeted as a leachable in a drug product leachables study, the extractables and leachables data for that compound become the basis upon which a leachables–extractables correlation can be made. For such a correlation to be considered to be valid, it is necessary that each leachable concentration in the drug product be less than or equal to the corresponding extractable concentration in the simulated extract, accounting for the uncertainty in the analytical measurements and any justifiable "exaggeration factors" that may have been utilized in the simulation study. Note that in certain justifiable circumstances drug product placebo batches may be used as test articles in drug product leachables studies (stability studies). However, there are also circumstances when placebo batches are not acceptable, such as when there is significant reason to believe that leachables might have an adverse effect on an active pharmaceutical ingredient (e.g., therapeutic proteins).

### Inorganic (Elemental) Leachables

The topic of leachables as elemental impurities in pharmaceuticals can be addressed within the overall context of elemental impurities in drug products (e.g., *Elemental Impurities—Limits* ⟨232⟩. Elemental impurities leached from packaging or delivery systems represent only one source of elemental impurities in a drug product, and thus testing a drug product for elemental impurities does not establish that the impurities are leachables.

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Testing of the plastic packaging systems and their materials of construction will establish those extractable elemental impurities that are relevant to a particular packaging system, and it may be appropriate to quantify such elemental impurities as leachables in the drug product. Therefore, the results of testing plastic packaging systems should be used to establish those elemental impurities that should be monitored as targeted elemental leachables in the drug product.

In general, guidelines and recommendations about elemental impurities in drug products address safety concerns associated with the elemental impurities. However, it is proper to consider elemental leachables from the broader perspective of the overall quality of the drug product. Thus the process of evaluating elemental leachables may include both the aspects of user safety and product quality.

Note that one of the differences between the testing for organic leachables (and extractables) and for elemental impurities is the nature of the information generated. The testing for organic leachables is based on having established the identity of the chemical compound that is the leachable. Alternatively, the test methods most commonly employed to address elemental impurities (atomic spectroscopy) do not establish the compound, or form, that the detected element is present in. For example, sulfur might be present in the form of elemental sulfur ($S_8$), as the sulfate ($SO_4^{-2}$), or as a sulfur-containing organic compound (e.g., 2-mercaptobenzothiazole). Also, silicon might be present either as silicone oil or as silicon dioxide ($SiO_2$). As the form of the elemental impurity may have a marked effect on the impurity's impact on product quality or safety, it may be the case that testing beyond elemental impurity profiling may be necessary to establish the exact chemical form of the elemental impurity and thus ascertain its potential safety or product impact.

An important issue to consider in testing drug products for elemental leachables is establishing which elements to measure as leachables. Considering this, it is noted that testing of the plastic packaging systems and their materials of construction will establish those extractable elemental impurities that are relevant to a specific packaging system. Therefore, the results of testing plastic packaging systems should be used as one means of establishing those elemental impurities that should be monitored as targeted elemental leachables.

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## SUMMARY

The requirement for, and completeness of, a leachables assessment for any particular drug product can only be determined by the drug product applicant with reference to appropriate regulatory guidance documents. Detailed recommendations for OINDP are presented in ⟨1664.1⟩.

Reference is also made to other compendial chapters in this Pharmacopeia that describe related extraction studies:

1. *Biological Reactivity Tests, In Vitro* ⟨87⟩
2. *Biological Reactivity Tests, In Vivo* ⟨88⟩
3. ▲*Elastomeric Components Used in Injectable Pharmaceutical Packaging/Delivery Systems* ⟨381⟩▲ (CN 1-Dec-2020)
4. *Plastic Packaging Systems and Their Materials of Construction* ⟨661⟩
5. *Plastic Materials of Construction* ⟨661.1⟩
6. *Plastic Packaging Systems for Pharmaceutical Use* ⟨661.2⟩

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***Add the following:***

# ▲⟨1665⟩ CHARACTERIZATION AND QUALIFICATION OF PLASTIC COMPONENTS AND SYSTEMS USED TO MANUFACTURE PHARMACEUTICAL DRUG PRODUCTS AND BIOPHARMACEUTICAL DRUG SUBSTANCES AND PRODUCTS

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## 1. INTRODUCTION

A manufacturing process is the sum of the steps required to convert starting raw materials into a manufactured biopharmaceutical drug substance (DS) or a pharmaceutical or biopharmaceutical drug product (DP). Manufacturing processes are performed utilizing manufacturing systems which are the sum of the components that together comprise the equipment that converts starting raw materials into biopharmaceutical DSs or pharmaceutical or biopharmaceutical DPs.

Manufacturing systems can be partially or completely constructed from plastic materials. Plastics used in manufacturing systems possess a range of molecular weights and contain plastic additives such as antioxidants, stabilizers, lubricants, plasticizers, and colorants. Several factors dictate the types of plastics that are used in manufacturing components, the types and amounts of additives and polymeric resins that are used in plastic materials and the processes used to convert the plastic materials into components or systems.

The process stream, production intermediates, DS, or the DP itself could directly come in contact, and potentially interact, with one or more plastic components of the manufacturing system at some point during the manufacturing process. During contact, substances could leach from a manufacturing component and become incorporated into the process stream. If these substances persist in the process stream through subsequent process operations, they could accumulate in either a DS or DP as process equipment-related leachables (PERLs). If they are present in the DP, PERLs have the potential to alter critical quality attributes of the DP, such as safety, efficacy, and stability.

*Plastic Components and Systems Used to Manufacture Pharmaceutical Drug Products and Biopharmaceutical Drug Substances and Products* ⟨665⟩ addresses these interactions by providing a risk-based means for chemically characterizing and qualifying plastic components used to manufacture biopharmaceutical DSs and pharmaceutical and biopharmaceutical DPs. That chapter provides guidance on qualification procedures applicable to manufacturing components and systems that are within its scope. Chapter ⟨1665⟩ communicates the key concepts behind and provides additional information and guidance regarding the applicability and the application of ⟨665⟩.

## 2. SCOPE

In general, components of pharmaceutical and biopharmaceutical manufacturing systems can be grouped based on the function or operation that the component performs, including:

- Fluid transfer and transport (e.g., tubing and connectors)
- Mixing, reacting, and fermenting (e.g., tank liners, mixer bags, bioreactors, impellers, tubing, and container ports)

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- Storage (e.g., containers [and associated ports and/or tubing] for raw materials, production reagents, and process intermediates)
- Processing (e.g., filters and chromatography columns)
- Filling devices (e.g., filling needles)
- Ancillary components (e.g., o-rings, gaskets, check valves, septa, diaphragms, polymer pump surfaces, and sensors)

This chapter is applicable to all manufactured DPs, including pharmaceuticals ("traditional" or "small molecule" products) and biopharmaceuticals (biologics), and is also applicable to manufactured biopharmaceutical DSs. This chapter is also applicable to substances that are manufactured (collected, isolated, purified, etc.) with plastic components and systems and used in gene and cell therapies or as antibody–drug conjugates (ADCs). Active pharmaceutical ingredients (APIs) that are the precursors to non-biologic and non-biopharmaceutical drug products and are typically manufactured via chemical, as opposed to biological, processes are referred to as "traditional" or "small molecule" drug substances and are out of scope with regard to ⟨665⟩ and this chapter as they are well-characterized substances that have sufficiently low levels of PERLs that they do not require characterization. Thus, manufacturing components used in the production of APIs are considered to be suitable for use without the chemical testing required in ⟨665⟩. However, APIs formulated in upstream processes, which include reactive process streams (i.e., containing organic solvents in large proportion, exceptionally high or low pH), high contact temperatures, long contact durations and/or multiple plastic components followed with limited purification processes, are considered to be at a higher risk. Applying ⟨665⟩ to the qualification of plastic components for such processes is determined on a case-by-case basis using good scientific judgment such as the concepts and processes described in ⟨665⟩.

This chapter is applicable to plastic manufacturing components that are used once and discarded (single-use systems) and components that are used once, rendered suitable for re-use (e.g., cleaning, sterilization) and then re-used in multiple-use systems.

Manufacturing components such as o-rings and gaskets may be constructed of rubber-based elastomers, which could be a source of PERLs and thus must be qualified. However, rubber-based components are not within the scope of ⟨665⟩ and thus qualification tests and specifications must be found elsewhere. Although rubber components used in manufacturing are outside the scope of *Elastomeric Components in Injectable Pharmaceutical Product Packaging/Delivery Systems* ⟨381⟩, that chapter contains tests and specifications that might be relevant to rubber-based elastomeric manufacturing components.

This chapter and ⟨665⟩ exclude items that are referred to as auxiliary items. While assorted plastic auxiliary items (e.g., scoops, funnels, pipettes, graduated cylinders, weighing dishes, and beakers) could be used in manufacturing operations for the dispensing and transferring of ingredients into a process stream or solution, their conditions of use are such that they are unlikely to contribute PERLs to the process stream. For example, such auxiliary items contact manufacturing ingredients for relatively short periods of time. Furthermore, many auxiliary items are used to transfer solid ingredients, which present a low risk of PERL transfer. The use of auxiliary items poses little risk in terms of the transfer of PERLs to the process stream. Thus, such auxiliary items are outside the scope of ⟨665⟩ and such items are considered to be qualified for use without ⟨665⟩ testing.

The applicant who secures and is responsible for the regulatory approval of a manufacturing system or the manufactured pharmaceutical or biopharmaceutical drug product derived from that system is accountable for establishing that the product's manufacturing system meets these expectations and is suited for its intended use.

This is accomplished by ensuring that the manufacturing system itself and/or the manufactured pharmaceutical DP or biopharmaceutical DS or DP has been appropriately tested and that the test results have been appropriately evaluated. The avenues through, and means by, which the applicant obtains test results and secures test result evaluations are left to the discretion of the applicant.

## 3. GENERAL PRINCIPLES

### 3.1 Discussion

The most effective means of ensuring that a manufacturing system is suitable for its intended use is to use well-characterized and intentionally selected components.

While the selection process for components increases the likelihood that a component and a system composed of components will be suitable for use, it does not necessarily establish that the component is actually suitable to use. Qualification testing of a component and/or system is required to establish the component's (or system's) ability to produce an acceptable DS or DP. When appropriate, system qualification may be augmented by DS or DP testing to directly establish what effect the manufacturing system had on the DS's or DP's key quality attributes.

This chapter and ⟨665⟩ both deal with the qualification of manufacturing components, where ⟨665⟩ specifically establishes the testing requirements for the qualification of plastic components.

Given the nature of manufacturing processes and systems, certain components may be isolated from the DS or DP. Although such components are essential aspects of the production system, they do not add PERLs to the DS or DP because they are isolated. If the isolation of a process component can be established (e.g., waste bag), then characterization of the component per ⟨665⟩ is not required.

### 3.2 Material Characterization

The most effective means of ensuring that a manufacturing component is suitable for its intended use is to use well-characterized and intentionally selected materials of construction. Thus, a component manufacturer is advised to collect chemical characterization data on candidate materials for the component, as such information may guide material selection. Guidance on how to chemically characterize materials of construction can be found in the following *USP* chapters:

- ⟨381⟩
- *Plastic Materials of Construction* ⟨661.1⟩

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Although chemical characterization of materials may enable appropriate material selection, chemical characterization of materials is not a prerequisite for the qualification of manufacturing components or systems.

### 3.3 Component Characterization

Components shall be characterized to the extent that is consistent with the risk that the component could add PERLs into a process stream and that the PERLs could persist through the manufacturing process and adversely affect the suitability for use of the process output. Matching the risk with the required level of characterization is achieved by a two-stage component assessment approach consisting of an initial assessment followed by a risk assessment.

## 4. ASSESSMENT PROCESS

### 4.1 Initial Assessment

The initial assessment as illustrated in ⟨665⟩, *Figure 1* addresses three aspects of the component and its use:

1. Is the component with a process stream, a biopharmaceutical DS, or a pharmaceutical or biopharmaceutical DP during use?
2. Is the process stream, DS, or DP that contacts a manufacturing component either a liquid or semisolid?
3. Does a potential comparator component or system exist, and, if so, can equivalence be established between the comparator and the component or system under consideration?

Aspect 1 addresses whether the component contacts a process stream, biopharmaceutical DS, or pharmaceutical or biopharmaceutical DP directly. If the component contacts an auxiliary stream, then it is isolated from the process stream. It is unlikely that such a component's PERLs will become entrained into the biopharmaceutical DS, or pharmaceutical or biopharmaceutical DP. In this circumstance, the component is deemed to be qualified for use with no further assessment and is out of scope for ⟨665⟩.

Aspect 2 addresses the physical state of the process stream and is based on the generalization that interactions between a process stream and a component are likely to be more extensive if the process stream is a liquid or semisolid (as opposed to a gas or solid). Moreover, liquid and semisolid process streams are more common in pharmaceutical manufacturing than are solid or gaseous process streams. Thus, when components are contacted by either solid or gaseous process streams, the risk associated with PERLs is very low, no characterization is required, and the component is deemed to be qualified for use with no further assessment.

An example of such a component is one that is used during the transfer of drug product powders into their final packaging. As another example, it is noted that a DS may be stored frozen in a container at some point in the manufacturing process, raising the question as to whether the container is actually within the scope of ⟨665⟩ (a frozen DS is a solid). As such storage typically includes periods in which the DS is thawed in the container; this situation is within the scope of ⟨665⟩ and requires risk assessment and appropriate testing. As the PERL's risk during frozen storage is very low, such an assessment focuses solely on the conditions during which the DS exists in a liquid or semisolid form.

A component that does not contact a liquid or semisolid process stream is out of scope for ⟨665⟩. If the component or system is contacted by a liquid or semisolid process stream, process intermediate, biopharmaceutical DS, or biopharmaceutical or pharmaceutical DP, then Aspect 3 is addressed.

Aspect 3 addresses the concept of a comparator component. When a manufactured pharmaceutical or biopharmaceutical product has been approved for commercial marketing, then by direct association, the process and system used to manufacture that product has been established to be suitable for their intended purpose. Once a manufacturing system has been established to be suitable to manufacture a biopharmaceutical DS, or pharmaceutical or biopharmaceutical DP by the relevant regulatory authority, then every component used in that manufacturing system has been established to be acceptable. These manufacturing components or systems may then be used as comparators and may provide the means of establishing whether a component or system under consideration for use in another manufacturing process and/or to produce a different biopharmaceutical DS, or pharmaceutical or biopharmaceutical DP is suitable.

If equivalence can be established between a component under consideration and a comparator component, then this justification is adequate to establish that the component under consideration is suited for its intended use without testing the component per ⟨665⟩. In this case, a justification should be provided and the qualification process is complete.

Equivalence can be established if both the component under consideration and the comparator are:

1. Constructed from the same materials of construction
2. Manufactured via the same process and the same processing conditions
3. Equivalent in terms of the function they perform
4. Equivalent in terms of how they have been processed (e.g., sterilized) and/ or reprocessed, if appropriate, prior to their use, either by the component's vendor or the component's user. For multiuse components/systems, equivalence with respect to how they have been processed post use.
5. Equivalent in terms of their conditions of use in the manufacturing process
6. Prepared for use (e.g., flushed, sterilized) in the same manner
7. Used to produce the same type of output (biopharmaceutical DS or pharmaceutical or biopharmaceutical DP), and the outputs are administered in the same clinical manner (route of administration and dose)

Although it is highly desirable that the equivalence in all seven aspects be exact, it may be the case that essential equivalence can be established based on strong similarities between the component under consideration and the comparator. The concept of strong similarities implies that the differences in any of the above-listed seven items of comparison are minor. Here "minor" means the differences are sufficiently small that the PERLs derived from the component in question:

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- Will be the same in identity and amount to the PERLs from the comparator, or
- Will have the same process or patient effect as the PERLs derived from the comparator

Any minor differences between the component under consideration and a largely representative comparator component may be addressed by risk assessing the minor differences in a manner consistent with ⟨*665*⟩ and this chapter. Alternatively, minor differences between the component of interest and a largely representative comparator component may be addressed by an alternate risk assessment process. The purpose of this risk assessment is to ensure that a minor difference will have a minor impact on the suitability of a component for its intended use. If it is established that the minor difference will likely have a minor and insignificant impact on a component's suitability, then the comparator approach is an appropriate means of qualifying the component. However, if it is possible that the minor difference could have a significant effect on the component's suitability, then the comparator approach is not an appropriate means of qualifying the component and the component must be qualified by testing as established by risk assessment.

Ultimately, it is the responsibility of the component's user to establish and justify equivalence, or essential equivalence, to a comparator.

## 4.2 Risk Assessment of Components

Components that have direct contact with a liquid or semisolid process stream, starting material, reagent, process intermediate, biopharmaceutical DS, or pharmaceutical or biopharmaceutical DP, and that do not have comparators, must be characterized to the extent dictated by their risk profiles whereby a greater risk is associated with a more extensive characterization. The risk profile of a component or system, used in a particular circumstance, is established by the application of a risk evaluation matrix constructed so that it:

- Establishes the appropriate contributors to, or dimensions of, risk
- Provides a means of quantifying the risk, in each of its dimensions (e.g., see *Table A-1*)
- Provides a means of quantifying the total risk as a combination of the individual risks established for each dimension
- Links the quantified total risk to appropriate characterization strategies (e.g., see *Table A-3*)

### 4.2.1 DEVELOPMENT AND APPLICATION OF THE RISK EVALUATION MATRIX

The risk evaluation matrix establishes the relative risk that a plastic component or system will be leached with sufficient tenacity that a process stream contacting the plastic component will likely contain potentially impactful PERLs and that the PERLs will likely persist in the process stream to be incorporated in the production output. The matrix then links that risk to a prescribed level of chemical characterization.

If the contact conditions between a process stream and a manufacturing component are mild, then it is unlikely that the leaching of PERLs will occur. On the other hand, if the contact conditions are harsh, then it is very likely that PERL leaching will occur.

Secondly, a PERL generally is likely to have an adverse effect on a key drug product quality attribute only if it persists through the manufacturing process and is incorporated into the finished DP. If the PERL is cleared from the process stream by subsequent manufacturing steps, then it will not persist to the end of the manufacturing process. As a result, the PERL will not be incorporated into the DP, and it will not have an effect on any of the DP's key quality attributes. Conversely, if the PERL is not removed from the process stream, then it will likely be entrained into the DP. [NOTE—For biologics, key quality attributes and process performance parameters can also be potentially impacted by a PERL during drug substance manufacturing prior to the finished DP.]

These two actions, leaching and persistence, can be used as dimensions in a risk evaluation diagram (see *Figure 1*), where the risk that is being evaluated is whether the finished DP will contain PERLs at high enough levels that they could have an adverse effect on a key quality attribute of the DP. The level of risk is associated with the nature and amount of testing that is required for components per ⟨*665*⟩.

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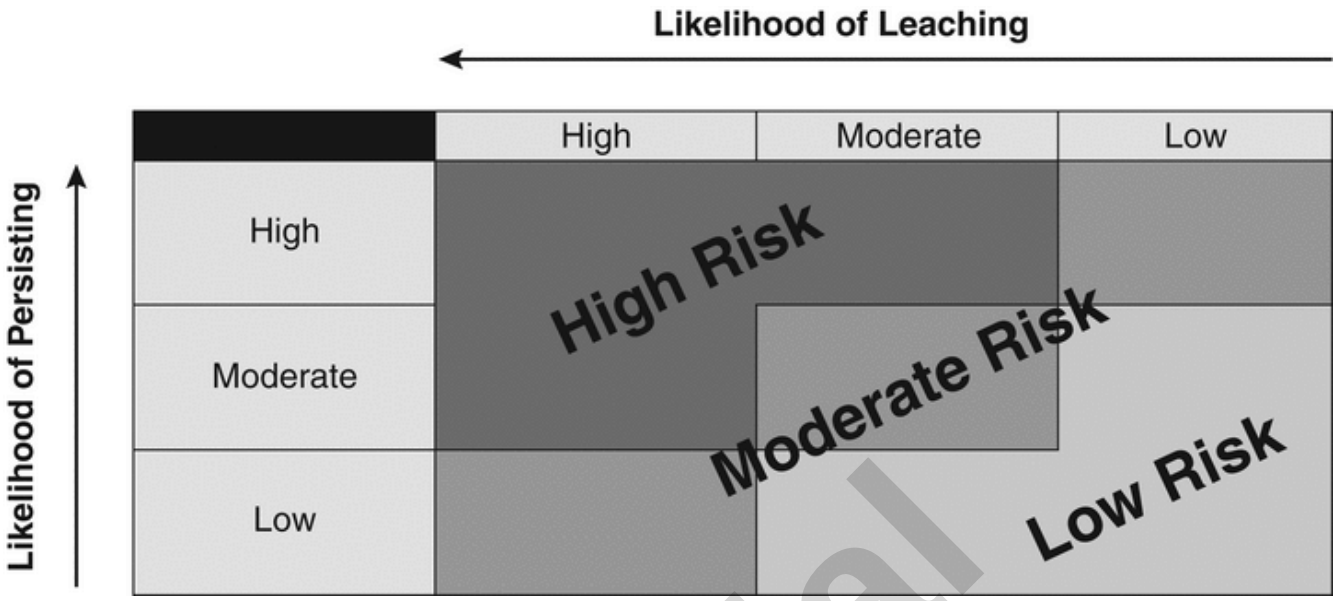


Figure 1. Risk evaluation diagram.

An individual manufacturing circumstance (e.g., component type or process conditions under which the component is contacted by the process stream) is positioned in this risk evaluation diagram to establish the component's required level of testing per ⟨665⟩. Although each individual drug product sponsor must establish the manufacturing circumstance's position in the risk evaluation diagram, the means by which this is accomplished is not specified in ⟨665⟩, and it is the responsibility of the sponsor to establish and justify these means. In establishing and justifying the means, the sponsor must consider the multitude of factors that impact the likelihood of leaching and the likelihood of PERLs to persist. Although general comments on how to fit a specific circumstance into the risk evaluation diagram can be found in this chapter, the finished drug product's sponsor has the ultimate responsibility for performing and justifying the risk evaluation.

While the individual details of individual sponsors' risk evaluation processes may differ, it is proper and reasonable to expect that the processes would be similar in terms of the general concepts applied. To properly address the issue of PERLs, the risk evaluation process should address the following points:

1. The chemical and physical nature of the contacted component, establishing the component's "propensity to be leached"
2. The chemical nature of the contacting process stream, establishing the process stream's "leaching power"
3. The conditions of contact, addressing the "driving force" for leaching (e.g., temperature, duration)
4. The ability of purification process operations to eliminate the PERL from the process stream or to dilute the PERL to such an extent that an adverse effect is unlikely
5. The inherent risk associated with the manufactured drug product, considering such factors as the nature of the manufactured dosage form, e.g., inhalation solution (higher risk) versus parenteral solutions (moderate risk) versus aqueous-based oral and topical (lower risk); the clinical dosing of the DP (e.g., daily dose volume); and the duration of the clinical therapy (acute versus chronic)

Regardless of the justified means by which the risk assessment is carried out, the outcome of the risk assessment must be that the risk is defined as low, moderate, or high, consistent with the risk levels illustrated in *Figure 1*. However, it is recognized that the final risk output from some drug product sponsors may differ from the three levels of risk evaluation matrix as shown in *Figure 1*. Other risk models outputs may yield two or more risk evaluation levels. In these cases, it is the responsibility of the drug product sponsor to align their risk outputs with the three risk levels required in ⟨665⟩. In addition, at least one of the sponsor's risk evaluation levels must be aligned with the ⟨665⟩ high risk level.

An example of a risk evaluation matrix can be found in the *Appendix*. As the purpose of this example is education and illustration, it is not required that the matrix in the *Appendix* be adopted in order for a sponsor to claim that their assessment complies with ⟨665⟩.

### 4.2.2 LINKING RISK TO CHARACTERIZATION METHODOLOGIES

The various adjusted characterization levels established previously are linked to the following characterization processes:

- Low Risk = general chemistry testing, "worst-case" extraction solution
- Moderate Risk = organic extractables profiling, "worst-case" extraction solution
- High Risk = organic extractables profiling, multiple extraction solutions reflecting the effects of pH and polarity on extractions, and extracted elements as deemed appropriate by the component's user

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## 4.3 Establishing the Level of Component Chemical Characterization

### 4.3.1 LOW RISK ASSESSMENT

Low risk chemical characterization data are generated by subjecting the component of interest to an extraction involving the ethanol–water solvent *Solution C1* from the standard extraction protocol described in ⟨665⟩, *4.2 Test Methods*. The ethanol–water extraction solution is chosen as it is generally recognized as producing the most substantial organic extractables profile of all three extraction solutions specified in the standard extraction protocol, where substantial means the largest number of extractables at their highest levels. This extraction solution is profiled, in general, for organic extractables by measuring its level of nonvolatile residue (NVR) and UV absorbance, where NVR provides a level of total extracted nonvolatile substances and UV absorbance provides an indication of the amount and type of UV-absorbing organic extractables. NVR and UV absorbance are chosen because they are tests that are compatible with the extraction solution. Although pH and total organic carbon (TOC) are useful general chemistry tests, they are not compatible with the extraction solution.

Although these general chemical properties of the extracts may not serve as the only basis for a risk assessment, they are important and relevant indicators that the component in question may have levels or types of extractables that could present a higher risk than was indicated by the risk assessment. For example, a large value for NVR would suggest that large amounts of non-volatile extractables were present in the extract. In such a circumstance, even though the component has been judged to be low risk, the judgment may have underestimated the risk. The conclusion that the risk has been underestimated would mean that the risk level should be elevated to moderate risk, triggering the generation of full extractables profiling in *Solution C1*. It is reasonable to expect that the extractables profile would establish those extractables responsible for the general test result that triggered the extractables profiling effort.

### 4.3.2 MODERATE RISK ASSESSMENT

Consistent with the greater risk, moderate risk components require a more rigorous chemical characterization whereby extractables are addressed by full organic extractables profiling of extraction *Solution C1*. As noted under *Low Risk Assessment*, *Solution C1* is chosen as the extraction solution as it is generally the case that, of the three extraction solutions specified in ⟨665⟩, the most complex organic extractables profile is obtained with this extraction solution, where "most complex" means the greatest number of individual extractables at their individually highest concentration in the extract.

### 4.3.3 HIGH RISK ASSESSMENT

High risk components must be characterized to the extent that a full organic extractables profile has been established, addressing potentially extreme process conditions such as process solutions with a high "leaching power" or conditions of contact with a high "driving force" for leaching (e.g., higher temperatures or longer duration periods of contact). Such a rigorous and complete characterization is obtained by implementing the standard extraction protocol, which adds two more extraction solutions (low pH and high pH aqueous *Solution C2* and *Solution C3*) to the ethanol–water solution, whereas *Solution C1* is required in the low and moderate risk cases. This extraction protocol is discussed in detail in *5.1 Standard Extraction Protocol*.

# 5. TESTING OF PLASTIC COMPONENTS AND SYSTEMS

## 5.1 Standard Extraction Protocol

Components and systems are used in many different ways and perform many different functions in manufacturing systems. Because it is reasonable to anticipate that the conditions of contact between the process stream and a manufacturing component may be strongly influenced by the function of the component (and the chemical nature of the process solutions at the relevant stage of manufacturing), component extraction procedures vary across the various component functions.

Given the diversity of components used in manufacturing systems and the widely varying conditions of contact in manufacturing operations, it is not possible to establish a single extraction procedure that is a perfect match for every manufacturing circumstance. On the other hand, it is unreasonable and impractical to impose a large set of extraction conditions because it would inevitably lead to testing and test results that are irrelevant. The compromise between universal applicability and practicality is to establish a standard extraction protocol, based on a minimum set of extraction conditions, which provides information that is useful in a majority of circumstances and is more completely relevant in the more commonly encountered circumstances.

The standard extraction protocol was designed to produce extracts whose analytical characterization would generate extractables profiles that can be used to drive component qualification in commonly encountered situations. As such, it represents a compromise between completeness and practicality.

### 5.1.1 EXTRACTION SOLUTIONS

From a practical perspective, it is desirable to limit the number of extraction solutions to a minimal number of solutions with a sufficiently varied chemical composition that effectively addresses the diversity in the composition of process streams. Furthermore, the solvents themselves should be analytically expedient, facilitating the chemical analysis of the extracts.

Three extraction solutions are specified in ⟨665⟩: a 50% by volume ethanol–water solution to address process streams that contain organic solvents, organic solubilizing or stabilizing agents, lipids (up to 15% of the process stream's composition by weight), proteins, and blood or blood-derived components (*Solution C1*); a pH 3 salt solution to address low pH (*Solution C2*); and a pH 10 buffer to address high pH (*Solution C3*). The pH values or ethanol content of the extraction solutions encompass many of the process streams encountered in typical manufacturing operations. The differing nature of the solvents could

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produce three separate extractables profiles, the combination of which would be relevant to the most commonly encountered manufacturing situations.

Other solvents may be used in addition to the three solvents specified in the standard extraction protocol at the discretion of the assessor. For example, there may be process situations where the pH of the process solutions falls outside the range encompassed by *Solution C2* and *Solution C3*, and in those situations it may be appropriate to consider alternative or additional extraction solutions.

Considering aqueous solvents, the pH range specified in the standard extraction protocol was chosen to 1) reflect a reasonable range of pH values for process solutions commonly encountered in pharmaceutical manufacturing processes and 2) encompass the pH range over which pH materially impacts the "extracting power" of the solvent.

For extraction at a low pH specifically, studies that included the pH 3 extraction solution (*Solution C2*) specified in ⟨665⟩ and a more acidic 0.1 M phosphoric acid extraction solution have demonstrated that the extractables profiles produced are sufficiently similar that these solvents can be considered interchangeable. Thus, an extraction performed with 0.1 M phosphoric acid as the extraction solution meets the extraction requirements in ⟨665⟩ for *Solution C2*. Furthermore, extraction solutions that achieve pH 3 by various means (e.g., the use of different acids to achieve the pH) are considered to be appropriate surrogates for the pH 3 solution specified in the standard extraction protocol, so long as the surrogate solvent is analytically viable.

For extraction at a high pH, studies that included the pH 10 extraction buffer *Solution C3* specified in ⟨665⟩ and more caustic extraction solutions (e.g., 0.5 N sodium hydroxide) have demonstrated that the extractables profiles produced may be sufficiently dissimilar that these solvents cannot be considered interchangeable. This circumstance is noteworthy as high pH caustic solutions such as 0.5 N sodium hydroxide are used in certain commonly encountered process situations. For example, it is fairly commonplace in biopharmaceutical manufacturing for manufacturing components to be cleaned and/or sanitized prior to use by contact with caustic solutions. Although extractables may be released from the components and become entrained in the caustic solution, such situations are not of concern with respect to ⟨665⟩, as the caustic solutions are directed to waste and contact between the component and the caustic solution is usually followed by rinsing, with the rinsate also being directed to waste. Thus, the released extractables do not enter the process stream and cannot become PERLs.

On the other hand, caustic solutions are frequently used in biopharmaceutical manufacturing to adjust the pH of process solutions (e.g., media fills in bioreactors). Such caustic solutions may be stored in plastic containers, clarified by plastic filters, or delivered to the process stream by plastic tubing and connectors. In these situations, extractables from the plastic components that become entrained in the caustic solution enter the process stream and become PERLs, making this situation relevant to ⟨665⟩. However, the manufacturing process is such that the caustic solutions are greatly diluted by the process streams. The levels of these PERLs in the actual process stream are small and likely to be inconsequential.

These circumstances notwithstanding, if all of the following criteria are met, then the pH 10 extraction solution specified in ⟨665⟩ can be replaced with the contact solution or the proposed simulating solvent. Alternatively, if an extraction study has already been performed with the contact solution or the proposed simulating solvent, the extractables profile obtained from that study can be used in place of the organic extractables data obtained with the pH 10 extraction solution.

1. The pH of the contacting solution is >10
2. There is a reasonable expectation that extractables resulting from the action of the high pH contacting solution will remain in the process stream (or else the component is isolated from the process stream and extraction is not required per ⟨665⟩, *3.1 Initial Assessment)*
3. Assessment of the risk associated with this contact situation produces a high risk characterization (as other risk characterizations do not require a high pH extraction)
4. The contact solution itself, or a proposed simulating solvent, are analytically expedient (meaning that the analytical process of extractables discovery, identification, and quantitation can be accomplished at the necessary concentration levels via the expert application of generally practiced, state-of-the-art analytical practices and technology)

A component that is tested with the replacement extraction solution and meets the requirements of ⟨665⟩ is considered to be compliant with ⟨665⟩.

Substitution of a high pH contact solution or simulating solvent for the pH 10 extraction solution (*Solution C3*) in circumstances where all four of these criteria are not met is an inappropriate application of ⟨665⟩.

An alternative solvent should not be used in place of *Solution C1, Solution C2*, or *Solution C3* if the "extraction (or leaching) power" of the process stream used in the manufacturing process is less than that of the corresponding solution (*Solution C1, Solution C2* or *Solution C3*).

The options available for extractions solvents are summarized in *Figure 2*.

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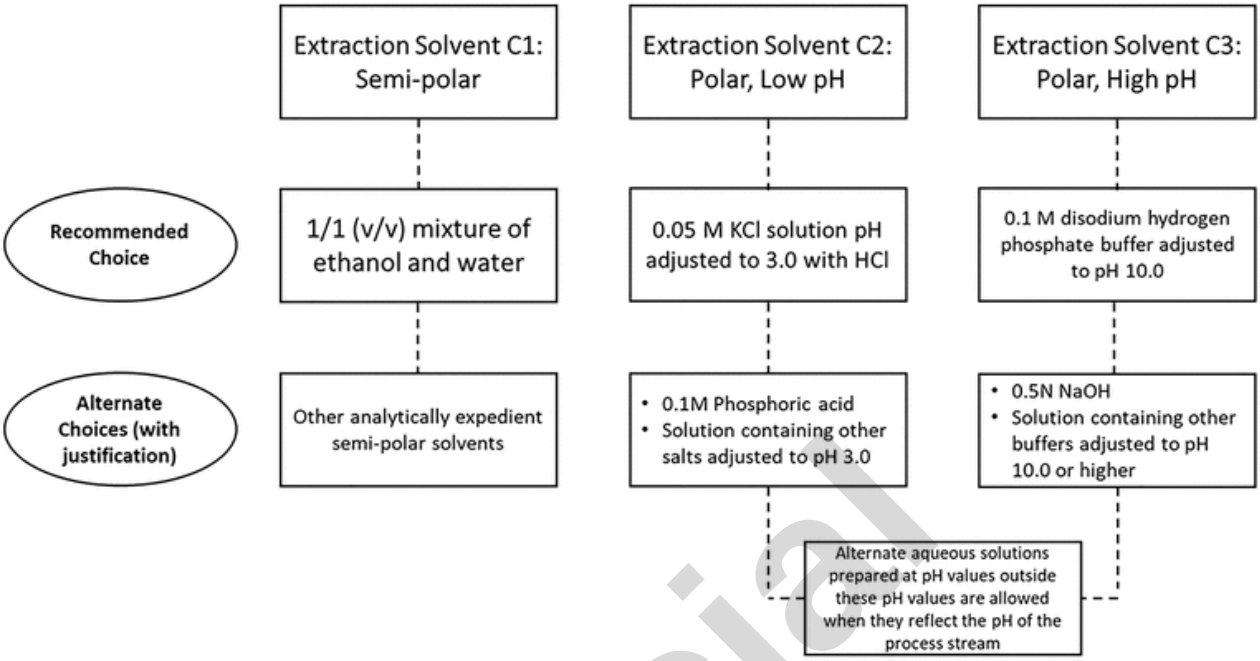


Figure 2. Extraction solution selection for manufacturing components.

The required extraction solutions do not include water, as the extractables profile obtained in water will be intermediate between the combined extractables profiles obtained from the pH extreme aqueous solvents. The extraction solutions do not include a high inorganic salt solution (e.g., 5 M sodium chloride), as the high salt will reduce leaching. The extraction solutions do not include solvents containing surfactants as the "leaching power" because the ethanol–water solvent generally has the same or greater "leaching power" as a surfactant-containing process stream. Additionally, extraction solutions containing surfactants are more difficult to analyze than an ethanol–water solutions.

### 5.1.2 EXTRACTION TEMPERATURE

The choice of the extraction temperature (40°) was dictated by the desire to accelerate, but not alter, the extraction process. A 40° extraction temperature provides an appropriate acceleration factor. Still, it is not so much higher than the temperature of typical manufacturing operations that the nature of the components at the extraction temperature would differ greatly from the nature of the component at the manufacturing temperature.

### 5.1.3 EXTRACTION DURATION

Durations of extraction were established specifically for individual components, consistent with the duration of contact between the component and a process stream during typical manufacturing operations. In some cases, such as bioreactor bags, the duration of contact specified in the standard extraction protocol is a value intermediate between the shortest and longest possible contact time during manufacturing operations. Profiles generated by testing extracts obtained at such intermediate durations will generally apply to manufacturing conditions at both contact duration extremes and will be sufficient to facilitate and justify component qualification.

Extraction durations longer than contact time during typical use are used for manufacturing situations where the contact between the component and the process stream is short, such as manufacturing operations involving a flowing process stream. The shortest extraction time was established as 1 day.

Extraction durations shorter than contact time during typical use are used for manufacturing situations where the contact between the component and the process stream is long, such as long-term storage of process intermediates. Nevertheless, the longest extraction time established, 21 days, still reflects approximately 2.5 months of contact at ambient temperature and 8 months storage under refrigerated conditions, estimated as follows.

The accelerated aging techniques noted in ASTM F1980-16 (*1*) are based on the hypothesis that the chemical reactions being considered follow the Arrhenius reaction rate function and can be applied generally to temperatures between 2° (non-frozen) and 70° (depending on the glass transition temperature of the plastic being considered).

The technique is based on an accelerated aging factor (AAF), which is expressed mathematically in *Equation 1*.

$$\text{AAF} = Q_{10}^{\left[\left(T_{AA} - T_{RT}\right)/10\right]} \quad \text{Equation 1}$$

$Q_{10}$ = aging factor, which has a conventionally accepted value of 2.0 for a first-order chemical reaction
$T_{AA}$ = elevated temperature to be used for extraction

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$T_{RT}$ = temperature at which the manufacturing operation is performed

For example:

If $T_{AA}$ = 40° and $T_{RT}$ = 22° (ambient temperature), then the AAF = 3.48.

If $T_{AA}$ = 40° and $T_{RT}$ = 5° (refrigerated temperature), then the AAF = 11.3.

The AAF value is used to calculate the accelerated aging time (AAT) as expressed in *Equation 2*.

$$t_{RT} = \text{AAT} \times \text{AAF} \quad \text{Equation 2}$$

$t_{RT}$ = contact time at $T_{RT}$

Using the example of the values of AAF obtained above and a 21 days extraction period, the calculated AATs for process steps that occur at ambient or refrigerated temperatures become 73 and 237 days, respectively. Even if these values are not exactly correct due to the assumptions inherent in the calculation, they establish the extent to which the duration specified in the standard extraction protocol reflect actual manufacturing conditions.

For extrapolation of 21 days of extraction at 40° to the equivalent storage duration at ambient room temperature (22°), *Equation 2* becomes:

$t_{RT}$ = AAT × AAF

$t_{RT}$ = 21 days × 3.48

$t_{RT}$ = 73.1 days

For extrapolation of 21 days of extraction at 40° to the equivalent storage duration at nominal refrigerated temperature (5°), *Equation 2* becomes:

$t_{RT}$ = AAT × AAF

$t_{RT}$ = 21 days × 11.3

$t_{RT}$ = 237.3 days

### 5.1.4 DYNAMIC EXTRACTION

Many manufacturing operations involve flowing or agitated process streams. Additionally, extractions may be facilitated by movement of extraction solutions by either agitation or recirculation. Thus, dynamic extractions are performed on components via either agitation or recirculation. Specific dynamic requirements are detailed for individual components in ⟨665⟩.

### 5.1.5 EXTRACTED SURFACE AREA TO EXTRACT SOLUTION VOLUME RATIO (SA/V)

It is generally the case that extractions described in relevant regulatory guidelines and relevant standards are defined in terms of the ratio of a test article's contact surface area and the volume of the extraction solution. To be consistent with these precedents, extractions in ⟨665⟩ are defined in terms of SA/V. Additionally, since components used in manufacturing are available in multiple sizes and configurations, specifying extractions based on SA/V allows for a consistent basis of comparison. The target SA/V of 6 $cm^2$/1 mL was adopted from relevant regulatory guidelines and standards. In all cases, the SA/V used in an extraction must be specified. In cases where components are extracted via immersion, reporting the mass/extraction volume or the number of components/extraction volume ratios may also facilitate the assessment of the extractables data.

### 5.1.6 NON-STANDARD EXTRACTIONS

Extractions that replace those specified in the standard extraction protocol may be necessary and appropriate in certain circumstances. It is a fundamental aspect of extractables assessment that the item being extracted is compatible with the conditions of extraction; that is, the item should not be altered chemically or physically by the extraction process. Although the extreme circumstance of complete dissolution of the test item is an obvious indication of an incompatibility (as it is surely the case that a manufacturing component is not dissolved by a process stream during use), less extreme indicators of incompatibility exist (e.g., deformation or discoloration of the test item or generation of a precipitate-laden extract).

If an extraction solution specified in the standard extraction protocol is incompatible with the component being tested (i.e., the component is rendered nonfunctional by the extraction), then an alternate extraction solution should be established, and the alternate solution should replace the specified solution. The alternate extraction solution must be suitable for determining either the NVR and UV absorbance or the organic extractables profile and, as appropriate, extractable elements, meaning that the solvents must be analytically compatible with the analytical techniques used for organic and inorganic extractables and profiling.

Considering the required surface area to solution volume ratio of 6 $cm^2$/1 mL specifically, when such an extraction ratio cannot be readily achieved, then an alternate extraction should be designed so that it is based on the highest possible surface area to solution volume ratio and a justification for the alternate extraction ratio must be provided.

Additionally, the standard extraction protocol was established to be applicable to a majority of the most commonly encountered manufacturing circumstances. However, one can encounter extreme manufacturing circumstances where the contact conditions between the process stream and a manufacturing component are more aggressive, in terms of extraction potential, than the extraction conditions in the standard extraction protocol. An example of such a circumstance is the situation where the pH of the process stream exceeds the pH of the basic extraction *Solution C3*. In this specific circumstance, the use of an extraction solution with a pH greater than *Solution C3* (pH 10) as a substitute for *Solution C3* may be appropriate (see *5.1.1 Extraction Solutions*).

As another example, higher temperature or longer extraction duration may be appropriate based upon the worst-case conditions of use in the manufacturing process (e.g., maximum component use time including holding/pause and restart). In

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this case, the higher temperature and/or longer duration is used in place of the temperature or duration established in the standard extraction protocol.

In other circumstances of extreme manufacturing conditions, an alternate extraction process that is as aggressive as the manufacturing conditions must be designed and justified. This alternate extraction process is then used in place of the process established in the standard extraction protocol.

On the other hand, it is possible that the extraction conditions of the standard extraction protocol are more aggressive than the manufacturing conditions of contact. The substitution of a less aggressive extraction for the extraction specified in the standard extraction protocol is not appropriate, as it is the intent of the standard extraction protocol to produce an extractables profile in the laboratory that is at least as complex as the PERL profile under actual process conditions, where complex refers to the number of compounds and their concentrations.

The generation and testing of extracts produced by alternate extractions will generate extractables profiles that could replace those profiles generated by the implementation of the standard extraction protocol at the discretion of the component's assessor. However, the generation and testing of such additional extracts cannot be used to replace or substitute for the extractables profiles generated by implementation of the standard extraction protocol unless:

1. The substitution is driven by incompatibilities, or
2. The substitution can be justified by a rigorous technical discussion of the "extracting power" of the proposed substitution solvent and the solvent (*Solution C1*, *Solution C2*, or *Solution C3*) it is replacing, or
3. The alternative testing condition represents the worst-case scenario of the extraction power of the process stream during manufacturing.

An example of such a justification would be the discussion about the interchangeability of the pH 3 extraction *Solution C2* and an extraction solution consisting of 0.1 M phosphoric acid.

It is noted that any alternate extraction process can only replace the extraction conditions that it directly addresses. For example, the use of a high pH process stream in manufacturing means that while *Solution C3* can be replaced with an alternate extraction solution, it does not mean that testing with *Solution C1* and *Solution C2* can be eliminated; testing of these solutions must still be performed.

The circumstances just discussed address the substitution of an alternate extraction process for an extraction process specified in the standard extraction protocol. Extractions performed in addition to those specified in the standard extraction protocol may be performed at the discretion of the component's assessor.

#### 5.1.7 ACCOUNTING FOR CONDITIONING AND RELATED STEPS EMPLOYED IN MANUFACTURING

Certain components used in manufacturing suites may be subjected to processes during manufacturing that have the potential to affect their extractables profiles. For example, tubing may be cleaned, rinsed, or sterilized by autoclaving or irradiation prior to, or between, manufacturing events. In addition to cleaning, rinsing, and sterilization, filters and chromatography columns may be conditioned prior to or after use.

Two aspects of such process steps are potentially important. The first aspect is whether manufacturing components must be tested by extracting them with solutions and under conditions that mimic such process steps. Since it is likely that the solutions used in these processing steps are isolated from the process stream, and thus that the finished DP is not exposed to extractables leached during such steps, the components do not have to be extracted with the cleaning, rinsing, or conditioning solutions. Nevertheless, it still may be necessary to establish that the components are compatible with these manufacturing processes and solutions.

The second aspect is that such process steps could alter the extractables profile of the affected components. Especially when such process steps are repeated numerous times over the component's useful lifetime (e.g., if the component is reused after processing), it is necessary to establish a component's extractables profile at various points during its useful lifetime, unless the assessor can provide evidence that the extractables profile does not change with each successive processing and reuse.

### 5.2 Testing the Extracts and Generating the Extractables Profile

#### 5.2.1 ORGANIC EXTRACTABLES

For moderate and high risk components, the extracts shall be analytically tested to establish the identities of the organic extractables and to estimate their concentration in the extracts using appropriate and orthogonal analytical methods, consistent with *Assessment of Extractables Associated with Pharmaceutical Packaging/Delivery Systems* ⟨1663⟩. Thus, chromatographic methods with appropriate sampling and detection processes are used to screen extracts for organic extractables.

The reporting of organic extractables must be consistent with the application of relevant and appropriate reporting thresholds. An example of such a reporting threshold is the analytical evaluation threshold (AET), as defined in ⟨1663⟩. The reporting threshold should be clearly communicated and justified in conjunction with the extractables profile.

Chromatographic methods used to address organic extractables should be qualified as being suited for this intended use.

#### 5.2.2 EXTRACTED ELEMENTS (POTENTIAL ELEMENTAL PERLS)

Techniques such as atomic spectroscopy (e.g., inductively coupled plasma–optical emission spectroscopy [ICP–OES]—see *Elemental Impurities—Procedures* ⟨233⟩) can be used to screen extracts for extracted elements.

Considering the reporting of extracted elements, the relevance of extractable elements testing should be considered by the component's potential user. Should such testing be deemed necessary, it is the user's responsibility to establish and justify the means by which testing is accomplished, taking into account extraction conditions, target elements, and reporting requirements. Furthermore, the component's user may wish to consult ⟨233⟩ when establishing its manufacturing components'

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extracted elements strategy, recognizing that ⟨233⟩ is specifically applicable to finished drug products and elements listed in the chapter do not reflect all potentially impactful elements but rather those elements for which permissible daily exposures (PDEs) could be derived.

### 5.3 Evaluation of the Extractables Profile Established by Implementing the Standard Extraction Protocol

Although the generation of a component's extractables profile is a necessary step in component qualification, the mere existence of an extractables profile does not establish whether a component is appropriate for use. Rather, the extractables profile must be interpreted in terms of the probable impact that the extractables would have on key performance and/or key quality attributes of either the process itself or the process output if the extractables were to become PERLs.

It is beyond the scope of this document to specify the exact means by which the probable impact of a component's extractables (as potential PERLs), as revealed by application of the standard extraction protocol, is established. However, *Section 6* discusses certain aspects of such a qualification process.

## 6. QUALIFICATION OF MANUFACTURING COMPONENTS AND SYSTEMS

### 6.1 General

A manufacturing system is chemically suited and qualified for its intended use if:

1. The manufacturing system is constructed from well-characterized components that have been intentionally chosen for use as established by testing.
2. The manufacturing system has been established to be safe by means of the appropriate chemical testing of components, such as extractables profiling, or DPs, leachables testing as necessary and appropriate, and toxicological assessment of the test data. In this context, a manufacturing system is considered to be safe if the DS or DP it produces contains PERLs at levels sufficiently low that they pose a negligible risk to patient safety. This combination of chemical testing and toxicological assessment is termed "chemical suitability for use assessment".

In certain circumstances and at the drug product sponsor's discretion, the suitability for use assessment could consider the possibility that the efficiency and/or effectiveness of the manufacturing process would be adversely impacted by PERLs that are derived from manufacturing components and present in the process stream(s).

This section of this chapter deals solely with manufacturing components and systems and should not be applied to materials from which manufacturing systems are constructed. It is also noted that it is relatively rare that the entire manufacturing system is qualified by performing extraction studies on the entire system, given the practical difficulties in so doing, and that, most commonly, a manufacturing system is qualified by performing extraction studies on the components that make up the system and then using the individual components' extractables profiles to establish the components' cumulative potential effect as a system. The means used to establish either the cumulative extractables profile and/or its cumulative potential effect must be justified.

In certain circumstances, individual manufacturing components may be connected to produce a manufacturing assembly and it may be desired that the assembly be tested per ⟨665⟩. Such a desire potentially complicates the testing of an assembly versus the component particularly when the assembly is comprised of components that individually would be tested in different manners, for example, duration of extraction.

It is beyond the scope of ⟨665⟩ to envision all possible assemblies that could be used in manufacturing and to provide extraction conditions for such assemblies. Ultimately, it the assembly user who is responsible for establishing and justifying the extraction conditions used to ascertain the assembly's compliance with ⟨665⟩. If appropriate, the justification must specifically address the situation where the extraction conditions used for the assembly are less rigorous (e.g., of shorter duration) than the most rigorous extraction conditions specified in ⟨665⟩ for the assembly's individual components.

### 6.2 Chemical Qualification

A manufacturing system is qualified as being suitable for use based on relevant and appropriate chemical testing of the manufacturing system, its components of construction, or the system's output. Generally, an appropriate and rigorous chemical assessment would include extractables testing of the manufacturing system (and/or its components) and/or PERL testing of the manufactured biopharmaceutical DS or pharmaceutical or biopharmaceutical DP. It is expected that the design of the extractables study would be based on sound and justifiable scientific principles and that the studies themselves would be consistent with the:

- Composition of the components
- Conditions of use of the components
- Chemical properties of the process stream
- Clinical use of the process output
- Perceived risk associated with the manufacturing system and the process output

No manufacturing system or process output is excluded from extractables or leachables testing; however, the nature and degree of testing depends on factors listed above and the risk that PERLs from the manufacturing component could affect the effectiveness of the manufacturing process or the quality of the manufactured DS or DP. Thus ⟨665⟩ has established a process for qualifying components that establishes three levels of risk and utilizes three levels of testing.

Considering a component that has been classified as low risk, such a component is deemed to be qualified for use, consistent with ⟨665⟩ if:

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1. The tests specified for low risk components have been performed.
2. The test results have been reviewed in the context of the validity of the risk classification.

As an example of the second point, consider one of the required tests for a low-risk component, NVR. If the component assessor establishes that the potential process- or patient-impact of the measured NVR is likely to be low, then the risk classification is verified, and the component is qualified for use. On the other hand, if the component assessor establishes that the impact of the measured NVR could be significant, then the risk classification of "low" is refuted, and the component can only be qualified by performing the required moderate- or high-risk testing, as established by the assessor.

The concept of an NVR result being “high” is subjective and can only be established on a case by case basis by the assessor based on the exact manufacturing process being considered, the exact DS or DP being assessed, and the component’s conditions of use.

Considering a component that has been classified as either moderate or high risk, such a component is deemed to be qualified for use, consistent with ⟨665⟩, if:

1. The test(s) specified for moderate or high risk components have been performed.
2. The resulting extractables profiles have been assessed for possible process impact and to establish the suitability of the manufactured DS or DP for use.
3. The outcome of the assessment is that the impact of the extractables (as PERLs) is such that process efficiency and/or product suitability for use is not adversely affected by the PERLs.

The means by which a component assessor establishes that the extractables (as PERLs) would have no adverse impact on the efficiency or effectiveness of the manufacturing process must be established by the assessor. Similarly, the means by which the component assessor establishes that the extractables (as PERLs) would not adversely affect the output’s (DS or DP) suitability for use must be established by the assessor. Most typically, interpretation of the chemical data includes the toxicological assessment of extractables and/or PERL data, supported, as appropriate, by other relevant testing to establish the potential patient safety impact of PERLs in the DS or DP. In this circumstance, the toxicological assessment should be performed for each individual relevant extractable (or each relevant PERL as appropriate) to demonstrate that patient safety is not adversely affected by each individual PERL (or extractable as worst-case PERLs). The term “relevant extractable” or “relevant PERL” refers to those extractables that are present in a manufacturing system and those PERLs that are present in a DS or DP at levels sufficiently high that they could affect a key quality attribute of the drug product such as patient safety (e.g., extractables or PERLs whose levels are greater than the AET).

Considering the impact of PERLs on the suitability for use of a DP or DS, the previously mentioned approach of interpreting extractables data (as potential PERLS) is one means of establishing that a component is qualified. A second means would be the actual characterization of the manufactured DS and DP for PERLs. If it is established that the actual levels of PERLs in the DS and DP are acceptable (meaning that assessment of the PERLs has concluded that the PERLs do not compromise the DS’s or DP’s suitability for use), then the manufacturing system, and each of the individual manufacturing components, have been qualified.

### 6.3 Alternate Approaches for Qualification

Chapter ⟨665⟩ establishes qualification procedures and qualification acceptance criteria applicable to manufacturing components that are within its scope. Alternative chemical qualification procedures and acceptance criteria may be appropriate in justified circumstances, subject to agreement by an appropriate regulatory authority. When considering such alternate approaches, the sponsor is advised to consult with the regulatory authority during the development of such alternate procedures so that regulatory concurrence with the outcome of the alternate qualification is more likely.

Chapters ⟨1663⟩ and *Assessment of Drug Product Leachables Associated with Pharmaceutical Packaging/Delivery Systems* ⟨1664⟩, are applicable to pharmaceutical packaging/delivery systems but may be helpful resources for designing and justifying rigorous and appropriate studies by establishing general essential principles and demonstrated best-practice recommendations for extractables and leachable studies and assessments.

## GLOSSARY

Additional terms are defined in *Packaging and Storage Requirements* ⟨659⟩.

**Auxiliary stream:** A solution or semisolid that is used during manufacturing but is decoupled from the process stream and thus the manufactured DP. For example, filling lines may be rinsed with cleaning solutions in preparation for product manufacturing. Additionally, filters and chromatography columns may be rinsed and conditioned prior to use. As these rinsing and conditioning solutions are directed to waste, they do not become part of the process stream and they are designated as auxiliary streams. As substances extracted from components, or systems by auxiliary streams cannot be entrained into the manufactured DP as leachables, auxiliary streams are not tested for process equipment-related leachables and components or systems contacted solely by auxiliary streams do not require rigorous chemical assessment supported by testing.

**Characterization:** The process of establishing the characteristics or properties of a test article. In the context of this chapter, characterization is defined as the process of establishing physicochemical and chemical characteristics of components used in manufacturing operations. Characterization is accomplished by extraction testing of components as specified in ⟨665⟩.

**Comparator component:** A component that can be established as equivalent (or nearly equivalent) to another component in terms of its composition, design, processing, performance, and conditions of use in the manufacturing process. Generally, the comparator component is (or has been) used in a regulatory-approved manufacturing process or is used to produce a regulatory-approved and marketed biopharmaceutical DS, or pharmaceutical or biopharmaceutical DP. Linking a component under consideration to a comparator component is the sole necessary justification that is required to approve and qualify the component under consideration for use.

**Component:** An item consisting of one or more materials that performs a single function in a manufacturing system or process. Silicone tubing is an example of a component that consists of a single material. A filter is an example of a component

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that likely consists of multiple materials such as its membrane (a single material), its housing (a single material), and its various gaskets (each of which may be a single material).

**Extractable:** An organic or inorganic chemical entity that is released from a manufacturing component or system into an extraction solution under laboratory conditions. Depending on the specific purpose of the extraction study, these laboratory conditions (e.g., solvent, temperature, stoichiometry, and others) may accelerate or exaggerate the normal conditions of use for a manufacturing component or system.

**Extractables profile:** A list of all substances that are extracted from a test article including the substance's identity and concentration (or amount) in the extract. The extractables profile typically includes only those substances whose level in the extract is above a chosen and justified reporting threshold.

**Extraction study:** A study, performed under laboratory conditions, whose purpose is to establish what substances are extracted from a test article and at what levels the substances are extracted under the conditions of the study. Extraction studies are also referred to as "controlled extraction studies".

**Isolated component or system:** A component or system that is chemically isolated, in terms of process equipment related leachables, from the manufactured DP due to the nature of its use in the manufacturing process. For example, while a drain or waste bag contacts an auxiliary solution, its purpose is to collect waste solutions after their use. The waste solutions collected in the drain bag are meant to be discarded and thus are not part of the process stream.

**Leachable:** A foreign organic or inorganic chemical entity that is present in a manufactured biopharmaceutical DS, or pharmaceutical or biopharmaceutical DP because it has leached from a component used in the manufacturing system and has persisted through the entire manufacturing process.

**Manufacturing system:** The sum total of all components that together are used to convert inputs (starting raw materials) into an output (manufactured DS or DP).

**Process:** A series of steps involving the use of systems or components that start with a DP's ingredients, converts those ingredients into a process stream, and then converts the process streams into the manufactured DP.

**Process equipment-related leachable (PERL):** A foreign organic or inorganic chemical entity that is present in a process stream because it has leached from a component used in the manufacturing system. A PERL may be present in a manufactured biopharmaceutical DS or pharmaceutical or biopharmaceutical DP as a leachable if it persists through the entire manufacturing process.

**Process stream:** Those solutions encountered in manufacturing that can be directly linked throughout the manufacturing process to the manufactured DP. Process streams either carry process intermediates through the manufacturing process or are process intermediates themselves. In essence, the manufacturing process converts the various process streams into the manufactured DP.

**Qualification:** The process of establishing that a test article is suited for its intended use in terms of a key performance attribute. This process typically includes obtaining and documenting evidence that addresses suitability for use.

**System:** An entity, consisting of multiple components, that accomplishes the intended purpose of a manufacturing process, producing either a DS or a DP.

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# APPENDIX

## An Example of a Risk Evaluation Matrix

As noted in *4.2 Risk Assessment of Components* and in ⟨665⟩, components are risk assessed with respect to their ability to contribute PERLs to DPs and the outcome of the risk assessment establishes the level of chemical characterization that must be performed. Although the DP sponsor is responsible for developing and justifying the risk evaluation matrix used to accomplish the risk assessment, ⟨665⟩ provides requirements for the risk dimensions that must be addressed in the matrix. This appendix provides one example of a risk evaluation matrix that meets these requirements.

The risk evaluation matrix evaluates those dimensions that address the risk that a plastic component will be leached by a process stream to such an extent that its extractables may be impactful. These dimensions include the:

1. Duration of contact
2. Temperature of contact
3. Chemical composition of the process stream
4. Nature of the component's materials of construction

The matrix then evaluates each dimension separately and assigns a level of risk based on certain measures relevant to each dimension (see *Table A-1*).

In most cases, the individual risk dimensions are considered to be equal in terms of their impact and in terms of their use in the risk evaluation matrix. However, in certain cases, especially those at the boundaries between the characterization levels, the logical application of the risk evaluation matrix is facilitated if a distinction is made between the dimensions in terms of their effect on the accumulation of PERLs. When it is necessary and appropriate to do so, the dimensions of temperature, solvent, and duration are weighted more heavily than the material dimension as it is logical that these dimensions could have a greater effect on leaching than does the material dimension.

**Table A-1. Dimensions Relevant to Risk Level**

| Risk Dimension | Duration of Contact | Temperature of Contact[a] | Chemical Composition of the Process Stream | Chemical Composition of the Component |
|---|---|---|---|---|
| Level 1 | <24 h | Refrigerated (2°–8°) | Aqueous (≤5% organic v/v; pH ≥3 and pH ≤9) | Low risk |

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**Table A-1. Dimensions Relevant to Risk Level** *(continued)*

| Risk Dimension | Duration of Contact | Temperature of Contact[a] | Chemical Composition of the Process Stream | Chemical Composition of the Component |
|---|---|---|---|---|
| Level 2 | 1–7 days | Ambient (15°–25°) | Somewhat organic (>5% and ≤40% v/v) | Intermediate risk |
| Level 3 | >7 days | Elevated (>30°) | Highly organic (>40% v/v) or aqueous, extreme pH (pH <3 or pH >9) | High risk |

[a] The gaps in the temperature ranges reflect temperature ranges that are rarely experienced in manufacturing processes.

## DURATION OF CONTACT

It is observed that as the duration increases, the magnitude of leaching increases. Durations of contact between components or systems and manufacturing process streams were established and the duration of contact dimension was divided into three levels: short term (<24 h, Level 1), intermediate term (1–7 days, Level 2), and long term (>7 days, Level 3). When determining the duration of contact, any holding and pausing times should be included so that the worst-case scenario for the manufacturing process is represented.

## TEMPERATURE OF CONTACT

It is observed that as the temperature increases, the magnitude of leaching increases. Temperatures relevant to components or systems used in manufacturing systems were established and the temperature dimension was divided into three levels: low (refrigerated, Level 1), intermediate (ambient, Level 2), and high (elevated, Level 3).

## CHEMICAL COMPOSITION OF THE PROCESS STREAM

The terms “aqueous”, “somewhat organic”, and “highly organic” are defined as shown in *Table A-2*:

**Table A-2. Chemical Composition of the Process Stream**

| Process Streams Containing... | Definition of Process Stream | | |
|---|---|---|---|
| | Aqueous | Somewhat Organic | Highly Organic |
| Organic solvents | <5% by volume | >5% and ≤40% by volume | >40% by volume |
| Surfactants, (e.g., polysorbate 80) | ≤0.1% by weight | >0.1% and ≤0.5% by weight | >0.5% by weight |
| Blood or blood-derived substances, (e.g., albumin) | <1% by weight | ≥1% and <25% by weight | ≥25% by weight |
| Lipids and proteins | <1% by weight | ≥1% and <5% by weight | ≥5% by weight |

Some process streams could possess multiple solubilizers (e.g., protein solutions that also contain surfactants). In such circumstances, the process stream is risk assessed as the compounded risk associated with the individual agents. For example, a process stream containing 2% protein would be classified as “somewhat organic” and a process stream containing 0.2% surfactants would also be classified as “somewhat organic”. A process stream that contains both 2% protein and 0.2% surfactants would be classified as “highly organic”, reflecting the compounding of the classifications of the individual agents.

Solubilizers could also include agents such as cyclodextrins, chelators, and niacinamide. As the effect of such agents on leaching has not been extensively studied and quantified, distinctions such as those applied to surfactants cannot be proposed for these additional agents, and it is therefore the risk assessor’s responsibility to properly account for these agents in the risk assessment.

## CHEMICAL COMPOSITION OF THE COMPONENT

Certain materials are more likely to contribute extractables to solutions they contact, based on their composition and physical properties. For example, the greater the number and level of additives in a plastic material, the greater the total pool of potential extractables and the greater the risk that extractables will migrate into a contacting solution. Additionally, high energy processing of materials such as sterilization may cause degradation of the plastic and its plastic additives, further increasing the number and pool of potential extractables. Lastly, in-use processing of materials and components, such as rinsing, could reduce extractables levels.

The term “chemical composition of the component” is used to assess the component’s likely overall risk of extractables. It is based on the logic that the greater the number of additives (or the greater the level of the additives), the greater risk of extractables, as additives are logical sources of extractables. The terms “low risk”, “intermediate risk”, and “high risk” are applied to the material’s contribution and are defined as follows:

- If the total level of plastic additives in the component is ≤0.1% by weight, then the component is considered to be “low risk”
- If the total level of plastic additives in the component is > 0.1% and ≤ 1% by weight, then the component is considered to be “intermediate risk”
- If the total level of plastic additives in the component is >1% by weight, then the component is considered to be “high risk”.

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If the component is irradiated or heated at high temperature (e.g., autoclaved) prior to its use during manufacturing, then the component is considered to be "high risk".

If the assembly of the component requires the use of bonding solvents, adhesives, or other chemical means of connecting a component's materials or subassemblies, then the component must be considered either "intermediate risk" or "high risk", depending on the amount of bonding agents used and documented evidence of their loss from the component prior to its use.

If the component is flushed or rinsed during use and the flush/rinse solution is directed towards waste and thus is not part of the process stream, then the flushing and rinsing has the potential to reduce the number or amount of extractables. If the ability of flushing/rinsing to eliminate or reduce extractables has been established, then such flushing/rinsing can be used to reduce the material reactivity term's risk level by one level; for example, a rinsed "high risk" component becomes an "intermediate risk" component.

This assessment of component composition focuses on the concept that the greater the number and quantity of plastic additives, the greater the likelihood that the plastic additives and their related substances will become extractables. Furthermore, when components are stressed as part of their processing, plastic additives degrade, producing additional extractables that may be more soluble. The assessment notes that certain processes used to prepare a component for use can either reduce the levels of extractables available to the process stream (such as rinsing) or increase the number of potential extractables (such as solvent bonding).

Other factors, such as the diffusion of compounds through a component, may affect the compound's ability to be extracted from the component. As diffusivity through a component is impacted by the nature of the component, this is a reasonable factor to consider in assessing a component's propensity to be leached.

Additional factors may also be relevant. For example, the propensity of a component to be leached could be assessed by considering information other than merely the levels of additives and the component's treatment prior to use. The use of additives levels discussed previously is one approach to assess risk and its inclusion in this chapter is not meant to imply that all risk assessors be limited to using this approach.

Component evaluators should consider taking these additional factors and information into account in assessing the component's propensity to be leached. Such assessments must be science-based in that they are supported by information or data that justify the ranking of the component in terms of its propensity to be leached.

## USING THE RISK EVALUATION MATRIX

The risk evaluation matrix uses a 3-step process:

**Step 1—Establish values for each risk dimension:** A component being assessed for risk is "rated" with respect to these four dimensions shown in *Table A-1*, and the resulting rating results in a level assignment of 1, 2, or 3 in each of the four dimensions. A numerical risk sequence can be generated based on these assignments. For example, a component or system that is rated as highest risk in all four dimensions has a generated numerical risk sequence of 3333. The numerical order of the risk sequence values is largely inconsequential, and the proper use of the numerical risk sequence requires that the sequence be given in order of decreasing digit values. Although the numerical risk sequences 3212 and 3221 reflect different risk profiles, both sequences are linked to the same level of characterization. Both risk sequences can be expressed as 3221 for ease of use in establishing the proper level of characterization.

**Step 2—Link the numerical risk sequence with a level of characterization:** On the basis of a consideration of all possible outcomes of the application of the risk evaluation matrix, links between the numerical risk sequence and the characterization level have been established (see *Table A-3*).

**Step 3—Use mitigating factors to adjust the characterization level:** Mitigating factors take into account the fact that the leaching of a component alone does not necessarily mean that the leached substance will have an adverse effect on the production process output. For example, extractables from a component that are removed (or cleared) from a process stream by a process step that comes after the leaching cannot affect the safety of a manufactured DP because the extractables will not be present in the DP as leachables. Furthermore, the clinical use of the manufactured DS or DP may be such that any adverse effect of the PERL would be moderate. The potential adverse patient safety impact of a leachable in a low-risk dosage form (e.g., liquid oral or topical) used in acute therapy is much less than the potential adverse patient safety impact of the same leachable in a high-risk dosage form (liquid injectable) used in a chronic therapy. Therefore, both clearance and clinical mitigating factors must be taken into account when establishing the component's risk and characterization level.

**Clearance:** Is there a post-contact (downstream) processing step that clears extracted substances?

- If yes, then use the factor (mitigating factor = 1)
- If no, then do not use the factor

To a certain extent, dilution can be considered similar to clearance in terms of its ability to mitigate the effect of extractables. While some process steps may not eliminate extractables from the process stream, they can reduce the concentration of extractables by diluting them via the process stream, for example, the addition of a concentrated acid or base used for pH adjustment. In such circumstances, extractables associated with the small volume reagent are diluted by the process stream, mitigating their potential hazard. In such cases, dilution can be the basis of assigning a value of 1 to the clearance mitigating factor if the effect of dilution can be quantified and the use of the factor can be justified.

**Clinical use:** What is the risk of leachables given the clinical use of the process output (DS or DP)? Factors to consider include dosage form, duration of clinical use, and daily dose volume (if applicable).

- **a.** If the dosage form is a solid oral, liquid oral (not containing surfactants, co-solvents [such as alcohols] or other solubilizing agents) or topical (semisolid or solid), then use the factor (mitigating factor = 1)
- **b.** If the duration of clinical use is <7 days, then use the factor (mitigating factor = 1)
- **c.** If the daily dose volume is <10 mL, then use the factor (mitigating factor = 1)
- **d.** Otherwise, do not use the factor

[NOTE—A mitigating factor is used only in the first instance that requirements *a–c* are met. Mitigating factors obtained by considering requirements *a–c* are not additive; so the highest value that the mitigating factor can have due to clinical use is 1.]

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The mitigating factors are used as follows. Add the clearance mitigating factor and the mitigating factor due to clinical use. Possible values of this sum are 0, 1, and 2.

- If the sum = 0, then there is no adjustment of the characterization level
- If the sum = 1, then the characterization level established by the other dimensions is reduced by one level of testing (e.g., Level B testing is reduced to Level A testing or Level C testing is reduced to Level B testing)
- If the sum = 2, then characterization Level A is applicable in all circumstances.

**Table A-3. Linking the Numerical Risk Sequence with a Level of Characterization**

| If... | And... | Then the Characterization Level is... |
|---|---|---|
| Four of the dimension scores are Level 3 | There is no additional qualifier (3333) | Level C (High Risk) |
| Three of the dimension scores are Level 3 | The other dimension score is Level 2 (3332) | Level C |
| | The other dimension score is Level 1 (3331) | Level C |
| Two of the dimension scores are Level 3 | The other two dimension scores are both Level 2 (3322) | Level C |
| | One dimension score is Level 2 (3321) | Level B (Moderate Risk) or C[a, b] |
| | The other two dimension scores are Level 1 (3311) | Level A (Low Risk) or B[b, c] |
| One of the dimension scores is Level 3 | All of the other dimension scores are Level 2 (3222) | Level B |
| | One of the other dimension scores is Level 1 (3221) | Level B |
| | Two of the other dimension scores are Level 1 (3211) | Level A or B[b, c] |
| | All of the other dimension scores are Level 1 (3111) | Level A |
| None of the dimension scores is Level 3 | All of the dimension scores are Level 2 (2222) | Level B |
| | Not all of the dimension scores are Level 2 | Level A |

[a] If the Level 2 score is in temperature, solvent, or duration dimensions, then Level C; otherwise, Level B.
[b] In these cases the temperature, solvent, or duration dimensions have a greater influence on risk than do component composition.
[c] If one of the Level 1 scores is in the component composition dimension, then Level A; otherwise, Level B.

## REFERENCES

1. ASTM F1980. *Standard Guide for Accelerated Aging of Sterile Barrier Systems for Medical Devices*, ASTM International, 100 Barr Harbor Drive, P.O. Box C700, West Conshohocken, PA 19428-2959.

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# ▲⟨1671⟩ THE APPLICATION OF MOISTURE VAPOR TRANSMISSION RATES FOR SOLID ORAL DOSAGE FORMS IN PLASTIC PACKAGING SYSTEMS

INTRODUCTION
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## INTRODUCTION

A common cause for failure to meet specifications of packaged solid oral dosage forms (SODFs) is their instability as a result of moisture uptake during storage. Failures are usually related to out-of-specification or out-of-range values in any of the following quality attributes (alone or in combination with other physical properties): assay, related substances, degradation by-products, and dissolution rate. Therefore, the moisture barrier properties of the packaging system must be characterized properly to demonstrate the relative moisture vapor transmission rate (MVTR) performance required to protect SODFs from moisture uptake.

Common packaging systems used for SODFs are plastic bottles that include a plastic closure with a foil seal over the bottle opening, or formed plastic and aluminum blisters, with a foil seal over the blister cavity. Plastic packaging systems are considered to be permeable, and therefore the moisture barrier properties must be characterized.

*Containers—Performance Testing* ⟨671⟩ includes desiccant-based MVTR methods for SODF packaging systems that align with the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) accelerated storage conditions for stability testing [40°/75% relative humidity (RH)] and a water-based method that aligns with the ICH storage condition of 40°/25% RH. These methods have the accuracy to measure the performance of high-barrier packaging systems, which has been traditionally difficult to measure. They provide specific MVTR values for packaging systems with sufficient sensitivity and precision to allow clear differentiation across packaging systems. This chapter provides guidance on the application of these methods to determine MVTR and the use of MVTR data to support demonstrating the equivalence of packaging systems when packaging SODFs.

### Scope

For packaging systems containing more than one unit of product, the concept of normalizing the MVTR of the packaging system to the number of units contained in the system (MVTR/unit) allows for a relative comparison of barrier performance. This ratio can be used to demonstrate equivalent moisture protection, when comparing a variety of packaging systems that contain different numbers of units. Thus, to complement—and in some cases, reduce—product stability testing for SODFs, the use of MVTR/unit can provide evidence that a proposed packaging system may provide adequate moisture protection to maintain product stability. However, annual stability may never be replaced by MVTR studies and changes based upon MVTR studies are made with some risk that stability failures may occur.

This chapter is intended to:

- Describe the methodology of providing evidence for the moisture barrier equivalency of two similar packaging systems (e.g., bottle A to bottle B, blister 1 to blister 2), based on the MVTR measurements described in *Containers—Performance Testing* ⟨671⟩*, Moisture Vapor Transmission for Plastic Packaging Systems*
- Define the upper limit of the MVTR for an SODF
- Compare the desiccant-based and water-based MVTR methods
- Explain how to leverage MVTR data to complement and, possibly, reduce stability studies when a new packaging system, a new packaging configuration for a marketed drug product, or a change to an existing packaging system is introduced

The scope of this chapter is restricted to:

- SODFs

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- MVTR based on *Method 1*, *Method 2*, and *Method 3* (desiccant-based) described in *Containers—Performance Testing* ⟨671⟩*, Moisture Vapor Transmission for Plastic Packaging Systems*
- MVTR based on *Method 4* (water-based) described in *Containers—Performance Testing* ⟨671⟩*, Moisture Vapor Transmission for Plastic Packaging Systems*
- Repackaging of SODFs

This chapter is applicable to:

- Pharmaceutical manufacturers
- Packagers
- Repackagers

## PACKAGING SYSTEMS

Pharmaceutical products are manufactured in different dosage forms and are packaged in a packaging system for storage and distribution. A packaging system performs multiple functions, among which protection is the most fundamental. Such protection ensures that the quality of the packaged drug product is maintained and not affected by physical hazards during the shipping and distribution process or by environmental hazards such as moisture, light, and oxygen throughout the shelf life of the drug product.

For SODFs, the most commonly used packaging systems are plastic bottles and blisters. A number of different plastic materials are used to manufacture SODF packaging, including cyclic olefins, polyethylene (PE), polyethylene terephthalate (PET), polyethylene terephthalate G (PETG), polypropylene (PP), and polyvinyl chloride (PVC). A general chemical description of these plastics is contained in *Evaluation of Plastic Packaging Systems and Their Materials of Construction with Respect to Their User Safety Impact* ⟨1661⟩, and the specifications for use in oral dosage packaging systems are defined in *Plastic Materials of Construction* ⟨661.1⟩. Two other plastic materials used to form blisters are polyvinylidene chloride (PVDC) and polychlorotrifluoroethylene (PCTFE).

Chapter ⟨671⟩ classifies these packaging systems into three main categories: multiple-unit containers, single-unit containers, and unit-dose containers as defined in *Packaging and Storage Requirements* ⟨659⟩. In the context of this chapter, packaging systems for SODFs are categorized as:

1. Multiple-unit container: a sealed plastic bottle that contains more than one unit of drug product. An example of a multiple-unit container is a high-density polyethylene (HDPE) bottle containing 30 tablets. *Method 1* or *Method 4* in *Containers—Performance Testing* ⟨671⟩*, Moisture Vapor Transmission for Plastic Packaging Systems* should be used to determine the MVTR of the multiple-unit container.
2. Single-unit or unit-dose container: A sealed blister card that contains 1 or more blister cavities and 1 unit of drug product per blister cavity. An example of such a container is a sealed PVDC blister card containing 7 cavities. *Method 2* or *Method 4* in *Containers—Performance Testing* ⟨671⟩*, Moisture Vapor Transmission for Plastic Packaging Systems* should be used to determine the MVTR of the container unless the container is a low-barrier blister (see *Glossary*), in which case *Method 3* or *Method 4* in *Containers—Performance Testing* ⟨671⟩*, Moisture Vapor Transmission for Plastic Packaging Systems* should be followed.

## DESICCANT VERSUS WATER MVTR METHOD

Traditionally, the MVTR of container–closure systems for solid oral drug products has been determined by using desiccant-filled test containers stored in an environmental chamber to create a difference in RH or vapor pressure between the inside and outside of the test containers. The use of desiccants can be problematic because an inadequate amount of desiccant or incompletely dried desiccant may result in variability in the vapor-pressure differences between the inside and outside of the container during the study. This potential variability in the vapor-pressure difference can increase variability in MVTR determinations.

### Use of Desiccants for MVTR Determination

According to MVTR *Method 1*, *Method 2*, and *Method 3* described in ⟨671⟩, test containers are filled with sufficient anhydrous desiccant to maintain an inside RH close to 0%. This RH is maintained if the prescribed desiccant preparations are handled properly. However, mishandling of the desiccant during shipping to the filling site or during filling of the blisters will compromise the dryness of the desiccant. Desiccant-filled test containers are held in an environmental chamber under controlled environmental conditions, and weight determinations are made over time. The inside RH remains constant during testing if adequate anhydrous desiccant is present, which becomes more challenging to achieve with a low-barrier blister (*Method 3*) because the desiccant can become saturated before the completion of testing. The weight gain of the desiccant is determined during this steady-state condition and is translated into a permeation rate MVTR (i.e., water vapor) for the test packaging system.

Percent relative humidity (%RH) is used to express the environmental chamber conditions and the headspace inside the test containers. To obtain reliable MVTR determinations, humidity must remain constant inside and outside the test container throughout the study. Results from desiccant-filled test containers do not account for potential variability introduced if the internal RH of the test containers neither starts at 0% nor remains at 0% during the study. The desiccant methods are designed to maintain the internal RH below 10% throughout the study. However, this internal RH can range between 0% and 10%, which can translate to the variability of the internal RH. In test containers filled with water, the RH is maintained at a constant (100%) from the beginning to the end of the MVTR study, thereby reducing potential variability.

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## Use of Water as an Alternative Fill Medium

*Method 4*, as described in ⟨671⟩, is an alternative approach for the determination of MVTR for packaging systems for SODFs. While desiccant-filled test containers stored in a 40°/75% RH chamber achieve a vapor-pressure difference of 65%–75% if the internal RH created by the desiccant is between 0% and 10%, water-filled test containers stored in an environmental chamber at 40°/25% RH achieve and maintain a constant vapor-pressure difference of 75%.

There are potentially multiple benefits of the MVTR method using water as the test medium. With water, the variability of the fill amount does not result in variability of internal RH, and no interaction with container walls is known to exist for the common blister plastic and lidding materials. The stability of the internal RH afforded by water facilitates holding the samples or reusing them at a later time, or in multiple studies (e.g., different storage conditions using the same samples). The concerns about shipping desiccant-filled samples and delays in testing are mitigated by the use of water. Additional measurements or extensions of studies are possible without concern about depleting the water.

The use of water eliminates special handling requirements to protect desiccant-filled blister samples from excessive exposure to moisture before testing. Also, samples filled with aqueous dye can be used for leak testing before or after MVTR testing, which can enhance visual detection of gross leaks during the study.

Sample preparation techniques are important for filling blisters with water. The best practice for delivering water into the cavities on the packaging line is to use a manual syringe filled with water; this method allows the delivery of a few drops of water to each cavity without causing sealing problems. However, there are concerns about introducing an inadequate amount of water. Chapter ⟨671⟩ describes how to estimate the amount of water to avoid the total loss of water before the completion of the test. In contrast to the desiccant method in which the saturation of desiccant is a concern, the water-filled method allows low-barrier blisters to be tested over an extended period in the same manner as high-barrier blisters.

*Table 1* summarizes recommended test methods to measure the MVTR of different packaging systems. *Method 3* in ⟨671⟩ is not recommended for measuring the MVTR of low-barrier blisters due to the rationales discussed earlier in this chapter.

**Table 1. Recommended MVTR Test Methods for Different Packaging Systems**

| Packaging System | Test Method (recommended) |
|---|---|
| Low-barrier blister | ⟨671⟩, *Method 4* |
| High-barrier blister | ⟨671⟩, *Method 2* or *Method 4* |
| Ultra-high barrier blister | ⟨671⟩, *Method 2* or *Method 4* |
| Bottle containers (e.g., multiple-unit containers) | ⟨671⟩, *Method 1* or *Method 4* |

## COMPARISON OF MVTR RESULTS FOR WATER AND DESICCANT METHOD

Several sets of data are provided in *Table 2* (*1*). The user should use an appropriate statistical tool to analyze the data and prove the equivalency from the statistical perspective. The raw data for each set are shown graphically in *Figure 1*.

**Table 2. Summary of MVTR Studies Comparing Desiccant-Filled and Water-Filled Samples**

| Sample Description | Fill Medium | Chamber Conditions | Number of Test Containers | Average MVTR |
|---|---|---|---|---|
| 7.5 mil PVC | Molecular sieves | 40°/75% RH | 10 | 1.46 mg/(day × cavity) |
| 7.5 mil PVC | Water | 40°/25% RH | 5 | 1.39 mg/(day × cavity) |
| 0.6 mil PCTFE | Calcium chloride | 40°/75% RH | 10 | 0.161 mg/(day × cavity) |
| 0.6 mil PCTFE | Water | 40°/25% RH | 6 | 0.162 mg/(day × cavity) |
| 2 mil PCTFE | Molecular sieves | 40°/75% RH | 10 | 0.104 mg/(day × cavity) |
| 2 mil PCTFE | Water | 40°/25% RH | 10 | 0.105 mg/(day × cavity) |
| 325-mL HIS[a] HDPE bottle | Calcium chloride | 40°/75% RH | 10 | 2.97 mg/(day × package) |
| 325-mL HIS[a] HDPE bottle | Water | 40°/25% RH | 5 | 3.20 mg/(day × package) |

[a] Heat induction sealed.

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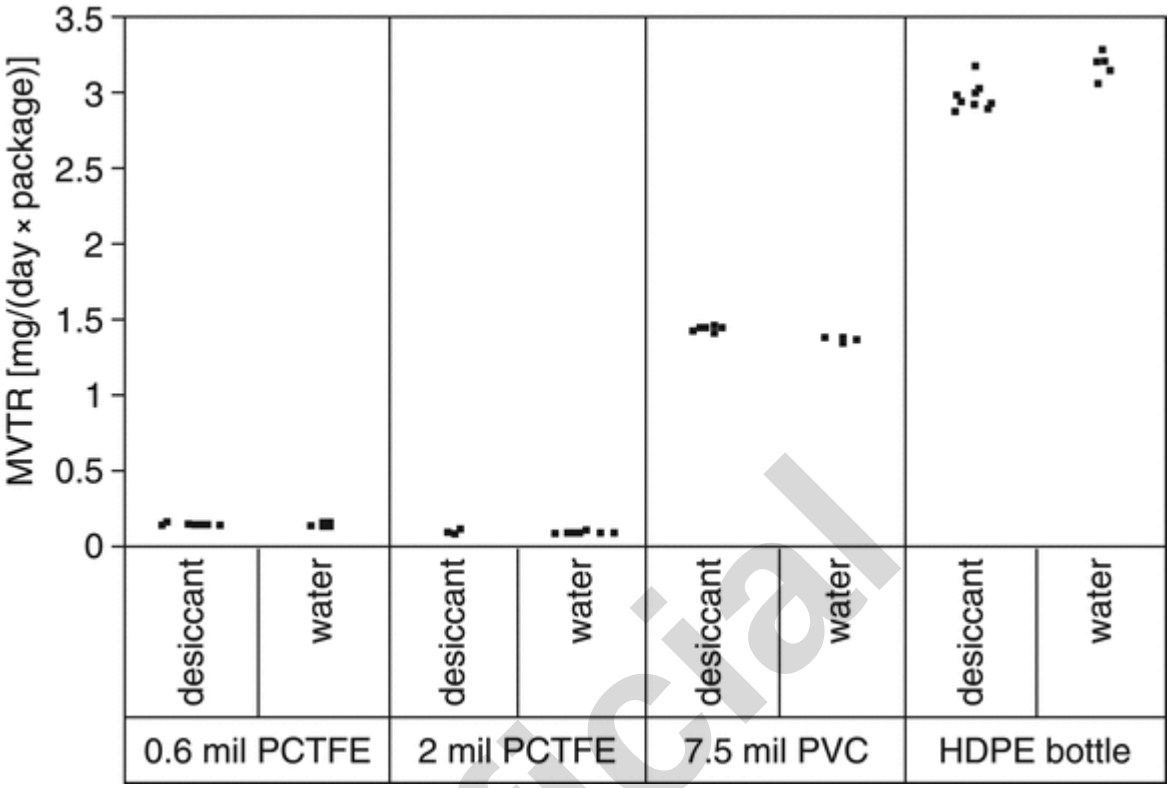


Figure 1. Graphic representation of raw data for *Table 2*.

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## EQUIVALENCY AND APPLICATION OF MVTR DETERMINATION METHOD

### Description

For SODF packaging systems containing more than one unit of product, the concept of normalizing the MVTR of the packaging system to the number of SODF units contained in the system allows for the comparison of the barrier performance and the demonstration of equivalent moisture protection for each SODF unit when comparing various packaging systems containing varying numbers of SODF units.

### Rationale

The moisture permeability of a packaging system can be a product Critical Quality Attribute (CQA) for SODFs. Once the stability performance has been assessed and suitability demonstrated in one or more packaging systems, a maximum demonstrated level of moisture permeability of the packaging systems, defined by MVTR normalized to the number of dosage units in the container (MVTR/unit), can be established. The MVTR/unit links the performance of the packaging system to the quality of the product in the container. Having established the maximum demonstrated level of MVTR/unit for a given SODF, and having demonstrated the suitability of one or more packaging systems through stability testing and/or other means, the MVTR/unit may be used as a measure of moisture protection to support changes to the packaging systems.

Use of a proper moisture permeation test method and a product-centric measurement of MVTR/unit for blisters and bottles permits the pooling of performance data from both packaging systems to establish a maximum demonstrated level for moisture permeability as a product CQA and accompanies stability study data to establish drug product quality attributes. For the pharmaceutical manufacturer, the product-centric model enables and facilitates a scientifically justified determination of the maximum demonstrated level of MVTR/unit unique to the product. This presents the ability to demonstrate that the new packaging system provides the equivalent or better MVTR/unit for a specific product, with the potential of not having to wait for the results of stability testing, providing that suitability of the bottle or blister packaging system is demonstrated through other means [such as compliance with the appropriate Code of Federal Regulations (CFR) and *USP* tests] and the packaging systems have previously been used in a regulatory-approved SODF.

### Application

By utilizing MVTR/unit and product-specific stability studies, scientifically justified limits for packaging moisture permeability can be defined. The moisture permeation test results and the maximum demonstrated level derived from those tests should be

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discussed in the Pharmaceutical Development section of the regulatory submission to support the selection of the packaging systems and materials of construction. It is also important to indicate the control strategy and the steps that will be taken to ensure that the packaging system attributes, and specifically the MVTR, will be met in day-to-day production. Moisture permeation through a material is affected by the properties of the material and its thickness. The MVTR can be controlled through acceptance specifications to confirm material identity and the weight or thickness of the material, and in-process control checks to confirm seal quality.

A maximum demonstrated level for moisture permeability of packaging systems can be ascertained based on the MVTR/unit for each package configuration and the acceptable product stability performance. Consider the following hypothetical example for SODF product X, in which MVTR and stability testing were performed on unit-dose blisters, using two different blister films, and an HDPE packaging system with a fill count of 30 tablets. In this example, the dissolution rate is a drug product CQA, and a decrease in the dissolution rate caused by moisture ingress was observed on stability.

**Table 3. MVTR for Different Packaging Systems (tested according to ⟨671⟩)**

| Packaging System | MVTR/Container (mg/day) | MVTR/Unit (mg/day) | Drug Product Dissolution |
|---|---|---|---|
| 250-µm PVC unit-dose blister | 3.1 | 3.1 | Fail |
| 250-µm PVC/60-gm² PVDC unit-dose blister | 0.8 | 0.8 | Pass |
| 60-cc HDPE bottle, 30-count | 1.2 | 0.04 | Pass |

In *Table 3* (*2*), PVC blisters with an MVTR of 3.1 mg/day/tablet were unable to provide adequate protection against moisture ingress, and the product failed to meet the specification for dissolution rate. However, the use of a higher-barrier PVDC-coated PVC blister packaging or a 30-count 60-cc HDPE bottle, with MVTR values of 0.8 mg/day/tablet and 0.04 mg/day/tablet, respectively (*Table 3*) (*2*), did provide adequate protection. A maximum demonstrated moisture permeability of ≤0.8 ▲mg▲ (ERR 1-Dec-2020)/day/tablet was established.

The maximum demonstrated level of MVTR may be leveraged in order to introduce new packaging presentations without the need of specific drug product stability testing prior to implementation. MVTR is one of many factors in assessing packaging system change including introducing new packaging configuration (e.g., HDPE bottle with a new count), the composition of the container–closure system, product requirements, and stability. However, MVTR may be useful in a product development setting, to decide on the packaging components before packaging exhibit batches, or in a pharmacy setting where the product is being repackaged.

For example, after launch, a commercial need may arise for a bottle containing a weekly dose of 7 tablets or a bottle containing 90 tablets. These two presentations may be considered based on the data shown in *Table 3* (*2*) and *Table 4* (*2*).

**Table 4. MVTR for Different Bottle Sizes and Counts (Tested According to ⟨671⟩)**

| Packaging System | MVTR/Container (mg/day) | MVTR/Unit (mg/day) | Maximum Demonstrated MVTR/Unit (mg/day) |
|---|---|---|---|
| 40-cc HDPE bottle, 7-count | 0.95 | 0.14 | 0.8 |
| 100-cc HDPE bottle, 90-count | 1.7 | 0.019 | 0.8 |

# REPACKAGING OF SODFs

## Repackaging an SODF into a Unit-Dose Container

Multiple-unit packages of SODFs (e.g., unit-of-use or bulk packaging systems) may be reprocessed or repackaged as instructed by the manufacturer. A blister package may not be reprocessed or repackaged after it has been deblistered from a unit-dose container. If the manufacturer's labeling includes "Protect from Moisture", the repackager must use a high-barrier or an ultra-high barrier film. If light protection is required for the SODF, the repackager should follow the requirements for spectral transmission established in ⟨671⟩. This testing should be conducted on the formed blister because the light-protective properties of the film are compromised once the film is thinned during the forming process. It is recommended that these tests, in conjunction with any guidance provided by the manufacturer, be considered for any packaging system used to repackage an SODF. The packaging system used to repackage an SODF must be at least as protective or provide better protection than the packaging system used by the original manufacturer of SODFs, taking into consideration the planned shelf life of the repackaged drug.

In the event that a product should not be repackaged, the manufacturer may state this in the labeling.

## Determination of Equivalence for Packaging Systems for SODFs

The suitability of a repackaging system for an SODF may be characterized by establishing equivalency compared to the original packaging system. Equivalence is established when the repackaging system is at least as protective as the original packaging system in terms of MVTR, oxygen transmission, light transmission, and compatibility of the materials of construction. Packaging system equivalency extends to any special protective materials, such as those used for light protection; seals; auxiliary packaging components, such as fillers (see *Auxiliary Packaging Components* ⟨670⟩); and desiccants and their canister or sachet,

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which are associated with the original packaging system (see *Packaging and Storage Requirements* ⟨659⟩*, General Definitions, Packaging Definitions, Equivalent container–closure system*).

For moisture, the determination of MVTR should be made using a method provided in ⟨671⟩ (*Method 1*, *Method 2*, or *Method 4*) for both the original packaging system and the packaging system used to repackage the SODF to provide evidence of equivalence.

## GLOSSARY

**Blister:** Formed, lidded, and sealed plastic or foil dome that contains the capsule or tablet (usually a single-unit or unit-dose).

**Blister card:** A contiguous group of blisters formed and sealed with lid in place. The number of blisters per card commonly ranges from 1 to 10 but may be more. The blister card may sometimes be referred to as a packaging system.

**High-barrier blister:** A blister whose moisture vapor transmission rate, when tested at 40° and an RH difference of 75% between inside and outside the sealed blister, is less than 1.0 mg/cavity-day, but greater than 0.01 mg/cavity-day. An example of a high-barrier blister is a thermoformed PCTFE blister sealed with foil.

**Low-barrier blister:** A blister whose moisture vapor transmission rate, when tested at 40° and an RH difference of 75% between inside and outside the sealed blister, is greater than 1.0 mg/cavity-day. An example of a low barrier blister is a thermoformed PVC blister sealed with foil.

**Ultra-high barrier blister:** A blister whose moisture vapor transmission rate, when tested at 40° and an RH difference of 75% between inside and outside the sealed blister, is less than 0.01 mg/cavity-day. An example of an ultra-high barrier blister is a cold-formed foil blister sealed with foil.

**Cavity:** Formed, lidded, and sealed plastic or foil dome (see *Blister*).

**Equivalency:** A claim that two container–closure packaging systems for a particular dose of an SODF are suitable for maintaining the stability of the drug in accordance with its specifications. In this chapter, equivalency refers to equivalent packaging systems from a moisture barrier perspective.

**Equivalent container–closure system:** See *Packaging and Storage Requirements* ⟨659⟩*, General Definitions, Packaging Definitions, Equivalent container–closure system*.

**Moisture vapor transmission rate:** The steady-state moisture vapor transmission (in unit time) through a packaging system, under specific conditions of temperature and humidity. These test methods use gravimetric measurement to determine the rate of weight gain as a result of moisture vapor transmission into the packaging system and subsequent uptake by a desiccant enclosed within the packaging system or the rate of weight loss as a result of moisture vapor transmission out of the packaging system and subsequent loss of water enclosed within the packaging system.

**Test specimen (or specimen):** For multiple-unit containers, the bottle is the test specimen; and for single-unit or unit-dose containers, the blister card containing multiple blister cavities is the test specimen. For blisters, more than one card may be bundled to form a test specimen.

**Test unit:** For multiple-unit containers, the bottle is the test unit as well as being the test specimen; and for single-unit or unit-dose containers, the test unit is a group of test specimens (blister cards) processed together for temperature and humidity exposure and for weighing at each time point. The purpose of the test unit for single-unit or unit-dose containers is to gain the advantage of additive weight gain resulting from more blister cavities than are present on a single card. The test unit, when applied to bottles, is used to maintain congruence of naming among the three test methods.

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***Add the following:***

# ▲⟨1711⟩ ORAL DOSAGE FORMS—PERFORMANCE TESTS

## INTRODUCTION

This general information chapter provides approaches for the development of performance tests, mainly dissolution and disintegration procedures for oral dosage forms intended for human and animal applications, which are not covered by *Disintegration* ⟨701⟩, *Dissolution* ⟨711⟩, *Drug Release* ⟨724⟩, *The Dissolution Procedure: Development and Validation* ⟨1092⟩, *Capsules—Dissolution Testing and Related Quality Attributes* ⟨1094⟩, and *Disintegration and Dissolution of Dietary Supplements* ⟨2040⟩.

The oral dosage forms referred to, but not restricted to, in this chapter are: effervescent tablets, chewable tablets, sublingual tablets, orally disintegrating tablets, gastroretentive tablets, delayed-release dosage forms, granules or pellets that are administered with food or beverages, suspensions, powder for suspension, granules for suspensions, tablets for suspension, lozenges, oral pastes, oral gels, chewable gels, Type A medicated articles, and Type B and Type C medicated feeds that deliver animal drugs and drug products in animal feeds.

## PRODUCT PERFORMANCE PROCEDURE DEVELOPMENT

The dissolution test should be, in most cases, discriminative for the critical quality attributes of the product. The FDA Guidance for Industry *Dissolution Testing and Acceptance Criteria for Immediate-Release Solid Oral Dosage Form Drug Products Containing High Solubility Drug Substances* (*1*) discusses the cases in which the dissolution test does not need to be discriminative. The test conditions (media, apparatus, tolerances/acceptance criteria) are typically defined in a case-by-case approach, supported and justified by data obtained with samples from the formulation(s) under evaluation. Generally, the compendial apparatus and procedures that can be used are described in ⟨711⟩, ⟨724⟩, and ⟨2040⟩. Any deviations from or modifications to the conditions stated in these chapters are acceptable with appropriate justification. For additional information see also ⟨1092⟩ and *In Vitro and In Vivo Evaluation of Dosage Forms* ⟨1088⟩. Other useful documents are FDA Guidance for Industry *Dissolution Testing of Immediate-Release Solid Oral Dosage Forms* (*2*) and FDA Guidance for Industry *Extended Release Oral Dosage Forms: Development, Evaluation, and Application of In Vitro/In Vivo Correlations* (*3*).

The dissolution media to be evaluated should have pH values within the physiological range, e.g. from 1.2–6.8. Dissolution media with pH values outside this range can be used with appropriate justification. The dissolution media for animal applications may be animal species specific.

Examples of dissolution tests can be found at the FDA Dissolution Methods database (*4*) and at the USP Dissolution Methods database (*5*).

### Solid Oral Dosage Forms

The general descriptions of and definitions for solid oral dosage forms can be found in *Pharmaceutical Dosage Forms* ⟨1151⟩.

#### TABLETS

**Effervescent tablets:** Effervescent tablets that result in a solution when handled according to the instructions to the patient do not require a dissolution test. In this case, a disintegration test is to be conducted.

For effervescent tablets that result in a suspension after being handled according to the instructions to the patient, a dissolution test should be developed. Depending on the size of the tablet, it may be necessary to position the paddles of USP *Apparatus 2* described in ⟨711⟩ at a distance higher than 2.5 cm, to avoid having the dosage form being hit by the paddle.

**Chewable tablets:** The dissolution test should be conducted using intact tablets. Since these types of tablets may be harder or more elastic than the regular immediate-release tablets, the dissolution procedure may require more aggressive conditions such as higher agitation speed and longer test time.

In the case of chewable tablets for use in animals, when developing a disintegration test, it is important to consider their size as they may be too big to fit in the equipment described in ⟨701⟩. An alternative is *Apparatus B* described in ⟨2040⟩. When developing a dissolution test, depending on the size of the tablet, it may be necessary to position the paddles of USP *Apparatus 2* described in ⟨711⟩ at a distance higher than 2.5 cm, to avoid having the dosage form being hit by the paddle.

In addition to dissolution testing, a disintegration test should be conducted using intact tablets in an appropriate medium using the appropriate equipment described in ⟨701⟩ or ⟨2040⟩ [see FDA Guidance for Industry *Quality Attribute Considerations for Chewable Tablets* (*6*)].

**Sublingual tablets:** Typically, sublingual tablets are immediate-release formulations which disintegrate very rapidly and contain a drug substance that has a good solubility at the pH value representing the pH of the buccal cavity. A dissolution test should be developed for this type of dosage form. However, the dissolution test may be replaced by a disintegration test, when justified. It may be advantageous to use a dissolution medium with a pH close to the saliva pH. Normal, healthy, human saliva in the oral cavity has, typically, a pH between 6.7 and 7.4. Other media may be used with appropriate justification.

**Orally disintegrating tablets:** The technological concept and manufacturing process have a great influence on the disintegration behavior of orally disintegrating tablets. The tablets manufactured by lyophilization will have a very short disintegration time, typically 1–3 s, when compared to orally disintegrating tablets manufactured by conventional means that have a disintegration time of around, or more than, 30 s.

Products labeled as orally disintegrating tablets require disintegration and dissolution tests [see FDA Guidance for Industry *Orally Disintegrating Tablets* (*7*)].

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The evaluation of the amount of drug dissolved at early time points (less than 5 min) may be useful.

**Gastroretentive tablets:** Gastroretentive tablets are tablets intended to be retained in the stomach for a sufficient time while withstanding gastric mobility, and releasing the drug substance in a controlled manner. The advantages of this type of dosage form are the enhanced bioavailability of drugs having a narrow absorption window at the upper part of the gastrointestinal tract or drugs showing a higher solubility, stability, or absorption at an acidic pH. They can also be advantageous for drugs which are locally active in the gastric mucosa.

Different strategies have been proposed to prolong the gastric residence time, including floating, expanding, high density and bioadhesive systems. Floating formulations should float on the gastric contents preventing its passage through the pylorus. Floating hydrodynamically balanced systems or effervescent matrix systems are the most common approaches for floating dosage forms. Depending on the formulation, a lag time may precede the dosage form floating. Gas-generating coated floating tablets, which form balloon-like floating devices upon hydration, can have a shorter floating lag time independent from surrounding pH (*8*).

The dissolution medium should be hydrochloric acid in the concentration of 0.1 N or 0.01 N. Other dissolution media can be used with appropriate justification.

Besides the dissolution test, floating tablets may also require a buoyancy or a floating strength test.

## Delayed-Release Dosage Forms

Delayed-release dosage forms are, typically, formulated with acid-resistant, or enteric or gastro-resistant coatings or matrices to protect acid-labile drug substances from the gastric environment, to avoid absorption in the proximal segment of the gastrointestinal tract, to prevent local adverse effects such as irritation, or to target drug release at the site of action or where the absorption is desired (i.e., in the colon region).

If the release of the drug substance is intended to occur in the proximal portion of the intestine, the procedures described in ⟨711⟩, with one acid stage and one buffer stage, should be followed. If the release of the drug substance is intended for the distal portion of the intestine (colon), the dissolution test consists of up to three stages—one acid stage and one or two buffer stages.

For non-pH-dependent delayed-release dosage forms the onset of dissolution is not intended to occur in a specific segment of the gastrointestinal tract but at a defined time point. The release mechanism is usually not pH dependent but driven by the propagation rate of a moisture front within the dosage form. The medium pH switch from acid to base may not be applicable. Instead, the dissolution procedure is similar to that used for immediate-release dosage forms, with the use of only one dissolution medium. It may require multivariate specifications for the time of the onset of the dissolution and the amount dissolved. The sampling time points have to be defined to include the pivotal time point of the onset of drug release.

## Granules, Powders, or Pellets That Are Administered with Food or Beverages

These granules, powders, or pellets are sprinkled, according to the instructions to the patient, into a small amount of soft food (e.g., applesauce, fruit- or vegetable-based baby food, or yogurt) or into a small amount of liquid (e.g., infant formula, apple juice, or pediatric electrolyte solution).

Granules or pellets that result in a solution when used according to the instructions to patients do not require a dissolution test. In this case, a disintegration test is to be conducted.

For granules or pellets that result in a suspension when used according to the instructions to the patient, a dissolution test should be developed. In this case, the amount of sample to be transferred to the dissolution equipment should be equivalent to 1-unit dose. If the product has different doses according to body weight or age, the amount of sample to be transferred to the dissolution equipment should correspond to the highest unit dose that can be administered. If labeled for single use, each sample should come from a different container/packet.

Sometimes, such granules or pellets are filled in capsules, which are then opened prior to being administered by the patient. In this case, the dissolution test needs to be conducted only with the capsule contents.

Food and beverages may influence the bioavailability of the drug substance(s) incorporated into the granules or pellets. However, adding food or beverage to the dissolution media is typically not required. See FDA Draft Guidance for Industry *Use of Liquids and/or Soft Foods as Vehicles for Drug Administration: General Considerations for Selection and In Vitro Methods for Product Quality Assessments* (*9*).

## Suspensions

The product needs to be re-suspended according to the instructions to the patient before the test. The sample agitation procedure should be defined in order to ascertain a homogeneous and representative sample and to avoid introducing too many bubbles into the sample to ensure the precision of dosing. The sample preparation procedure should be well described and standardized in the final version of the method.

The amount of sample to be transferred to the dissolution equipment should be equivalent to 1-unit dose. If the product has different doses according to body weight or age, the amount of sample to be transferred to the dissolution equipment should correspond to the highest unit dose that can be administered at one time. If labeled for single use, each sample should come from a different container/packet.

The sample can be transferred to the dissolution apparatus by volume or by weight. Other alternative transferring procedures can be used.

The sample should be introduced into the dissolution medium in a way to be rapidly dispersed. The dispensing point/place and the dispensing time should be evaluated and defined in a case-by-case approach during method development. The evaluation of the amount of drug dissolved at early time points (5–10 min) may be useful. Lower paddle speeds may be appropriate for this type of dosage form.

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## Powders for Oral Suspension, Granules for Oral Suspension, or Tablets for Oral Suspension

Powders, granules, or tablets for oral suspension are prepared according to the instructions to the patient to constitute a suspension, and the resulting suspension is placed into the dissolution equipment.

For additional information regarding dissolution test conditions for these type of dosage forms, see *Suspensions*.

## Lozenges

Lozenges can be, basically, manufactured in two ways: 1) molded, where the drug substance(s) is/are dissolved in a hot mixture of sugars, syrups, and other ingredients, the final mixture is transferred to molds and cooled down; and 2) compressed, where all the formulation components, in the powder form, are compressed together.

In case of molded lozenges, a dissolution or disintegration test may not be required. In the case of compressed lozenges, the development of the dissolution or disintegration test should follow the same steps as any other immediate-release solid oral dosage form.

## Oral Pastes and Oral Gels

In addition to the equipment described in ⟨711⟩ and ⟨724⟩, it may be useful to consider the equipment and procedures described in *Semisolid Drug Products—Performance Tests* ⟨1724⟩.

## Chewable Gels

The development of the dissolution test for chewable gels should follow the same steps as for any other immediate-release solid oral dosage form.

## Type A Medicated Articles and Type B and Type C Medicated Feeds

For definitions and general descriptions of animal drugs and drug products delivered in animal feeds, see *Animal Drugs for Use in Animal Feeds* ⟨1152⟩.

Usually, dissolution testing is not required for Type A medicated articles and Type B and Type C medicated feeds that deliver animal drugs and drug products in animal feeds, but it may be required to support certain formulation changes for approved products or bioequivalence determinations for generic products.

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# ⟨1724⟩ SEMISOLID DRUG PRODUCTS—PERFORMANCE TESTS

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## ▲SCOPE

This chapter provides general information about developing in vitro performance tests to evaluate drug release or skin permeation for topical and transdermal semisolid and liquid-based dosage forms, including but not limited to, creams, gels, ointments, pastes, suspensions, lotions, and foams. For information related to in vitro performance tests that evaluate drug release for transdermal delivery systems (TDS), refer to *Drug Release* ⟨724⟩.

In this chapter, the term "drug" is utilized to refer more generally to active ingredients, potentially including active ingredients that may not be considered drugs (e.g., sunscreen active ingredients).

For information related to product quality tests for topical and transdermal dosage forms, refer to *Topical and Transdermal Drug Products—Product Quality Tests* ⟨3⟩.

## INTRODUCTION

This chapter provides general information for developing in vitro release test (IVRT) methods to assess the rate of drug release from topical and transdermal semisolid and liquid-based dosage forms, including but not limited to, creams, gels, ointments, pastes, suspensions, lotions, and foams applied on the skin and other mucosal membranes. Definitions and descriptions of these dosage forms can be found in *Pharmaceutical Dosage Forms* ⟨1151⟩. This chapter also provides general information for developing in vitro permeation tests (IVPT) to assess the rate and extent of drug permeation into and through the skin from semisolid and liquid-based drug products. The same equipment and similar methodological principles/procedures may be relevant for IVPT methods with other epithelial membranes (for information on appropriate mucosal membranes, see *Biological Membrane* below).

### Drug Product Quality and Performance Tests

Drug product tests are divided into two categories: 1) those that assess general quality attributes i.e., product quality tests, and 2) those that assess product performance, e.g., using an IVRT/IVPT method. Product quality tests characterize the physicochemical and/or structural attributes (e.g., pH, particle size/morphology etc.) of the formulation. By contrast, product performance tests assess how a drug product functions under specified conditions, which may provide information relevant to its in vivo performance.

Product quality tests are generally useful to characterize and/or compare product quality attributes that can control the performance of the product, including, but not limited to, identity, strength, purity, uniformity, pH, particle size/morphology, and apparent viscosity. Details about these product quality tests can be found in ⟨3⟩. The performance tests that are the focus of this general chapter are specifically useful to characterize and/or compare the linear (steady state) drug release rate (using an IVRT) or the dynamic rate and extent to which a drug permeates into and through the skin (using an IVPT) from semisolid and/or liquid-based dosage forms, under the conditions of the test.

Potential contexts for use of IVRT and IVPT methods are summarized below.

#### IVRT

- Characterizes the steady-state drug release rate for a product batch. When the performance (release rate) of a topical product has been characterized by a validated IVRT method using the same batch of product that supported a demonstration of the safety and/or efficacy of the product, that performance (release rate) may have the potential to serve as a basis of reference for the product in the future
- Characterizes the influence of processes, formulations, and/or manufacturing differences on a drug product by comparing the steady-state drug release rate for the postchange (test) and prechange (reference) products, typically in the context of scale-up or post–approval changes for an approved drug product, which can support a demonstration of equivalence in certain situations
- Characterizes the influence of processes, formulations, and/or manufacturing differences on a drug product by comparing the steady-state drug release rate for a prospective generic (test) product and an approved (reference standard) product, which can support a demonstration of bioequivalence in certain situations

#### IVPT

- Characterizes the rate and extent to which a drug permeates into and through the skin for a product batch
- Characterizes the influence of process, formulation, and/or manufacturing differences on a drug product by comparing the rate and extent to which a drug permeates into and through the skin. This may occur in the context of comparing prototype formulations of a topical product, or potentially in the context of comparing postchange (test) and prechange (reference) products
- Characterizes the influence of process, formulation, and/or manufacturing differences on a drug product by comparing the rate and extent to which a drug permeates into and through the skin for a prospective generic (test) product and an approved (reference standard) product, which can support a demonstration of bioequivalence in certain situations

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(See the FDA *Guidance for Industry, Nonsterile Semisolid Dosage Forms; Scale-Up and Postapproval Changes: Chemistry, Manufacturing, and Controls; In Vitro Release Testing and In Vivo Bioequivalence Documentation*; available at www.fda.gov/media/71141/download.)

## IN VITRO RELEASE TEST (IVRT)

An IVRT is intended to characterize a steady-state drug release rate from a semisolid formulation under a specific set of conditions (i.e., a given set of method parameters). IVRT methods are typically precise and reproducible. However, if the parameters of the IVRT method change, then the release rate measured may also change. Notably, when the IVRT method is consistent, then changes in the release rate between a test and reference formulation are reflective of a difference in the physicochemical properties and/or the structural arrangement of matter in the formulations. Thus, IVRT methods can provide a sensitive and discriminating way to monitor for differences in the physicochemical or structural properties of a test versus the reference formulation.

The steady-state drug release kinetics are not representative of the finite dose (non-steady-state) kinetics of drug permeation through the skin. The drug release rate measured by an IVRT method should not be misconstrued to represent a “true” drug release rate for that formulation, or even a biorelevant release rate. For these reasons, IVRT methods are most appropriate to assess (compare) the "sameness" of formulations (e.g., before and after certain manufacturing or formulation changes).

If the drug release rate is found to be different between a test and reference formulation of comparable components and composition, it typically indicates that there is a difference in the physicochemical and/or structural attributes between the formulations. The IVRT methods discussed in this chapter are not expected to predict whether an observed difference in measured release rate may impact in vivo bioavailability, nor can they identify the exact nature of the difference. Instead, the utility of IVRT studies is that they can efficiently assess whether test and reference products perform in a manner that appears to be the same. When the drug release rates are equivalent for two formulations with similar components and compositions, it suggests that there may be a high degree of sameness in physicochemical and structural attributes between the formulations, thereby providing evidence that mitigates the risk of a potential difference in therapeutic performance. Consequently, IVRT studies may provide evidence to support a demonstration of equivalence between prechange and postchange batches of a product, or to support a demonstration of bioequivalence in certain situations.

### Theory

After a short lag period, release of drug from the semisolid dosage form is kinetically described by diffusion of a chemical out of a semi-infinite medium into a sink, by the following equation:

$$m = \sqrt{2 \times Q \times D_m \times C_s \times t}$$

Where $m$ is the amount of drug released, $Q$ is total amount of the drug in solution and suspended in the matrix, $D_m$ is the drug diffusion coefficient in the semisolid matrix, $C_s$ is the drug solubility, and $t$ is time.

A plot of $m$ versus $\sqrt{t}$ will be linear with a slope of:

$$m = \sqrt{2 \times Q \times D_m \times C_s}$$

### IVRT Method Development, Validation, and Transfer

IVRT method development should encompass key aspects that influence the IVRT results, including the IVRT receptor solution sample analysis method. IVRT method validation is outside of the scope of this chapter. An IVRT method transfer between laboratories should include a demonstration that the IVRT method and the associated IVRT receptor solution sample analysis method produces comparable, valid results when compared between the laboratory where the methods were validated and the laboratory to which the methods are transferred.

### Synthetic Membrane

Filter membranes (e.g., 0.45-µm pore size) comprised of synthetic materials (e.g., mixed cellulose esters, nylon, polysulfone, polyethersulfone) are frequently suitable for IVRT methods as they are typically resistant to binding most drugs, and they permit drugs to diffuse through the membrane. Filter membranes comprised of a synthetic material, sometimes with different pore sizes, are commonly evaluated during IVRT method development. Other types of simple, monolayer synthetic membranes, like dialysis membranes, may also be suitable for an IVRT method. The use of biological membranes or synthetic membranes developed to emulate a biological membrane are not appropriate for an IVRT method because such membranes may inappropriately influence the apparent rate of drug release.

### Diffusion Cells

Suitable equipment for the IVRT method includes various models of vertical diffusion cell (VDC) and immersion cells. The most commonly used equipment for IVRT methods are VDC (see *Figures 1–5*), although immersion cells (*Figure 6* and *Figure 7*) also have been used successfully. Equipment features (e.g., bubble-free cells or cells with a vent hole) other than the ones depicted below may be suitable once qualified. The total receptor compartment volume of VDC typically ranges from 5–15 mL, while the total vessel volume for the immersion cell typically varies between 50 and 200 mL; values outside of those typical

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ranges may be available depending on the manufacturer of the equipment. VDCs may offer advantages over immersion cells; for example, in the assessment of lower strength dosage forms and/or dosage forms with low amounts of drug release, VDCs provide less drug dilution in the receptor solution, minimizing potential analytical challenges regarding drug quantification. Also, many VDC models allow the user to adjust the amount of the dosage form utilized, which can allow users to increase the amount of the dosage form in the donor compartment, thereby reducing the percent dose depletion and sustaining the pseudo-infinite dose conditions that support steady-state drug release kinetics. See *Equipment* for further details on the equipment used in IVRT.

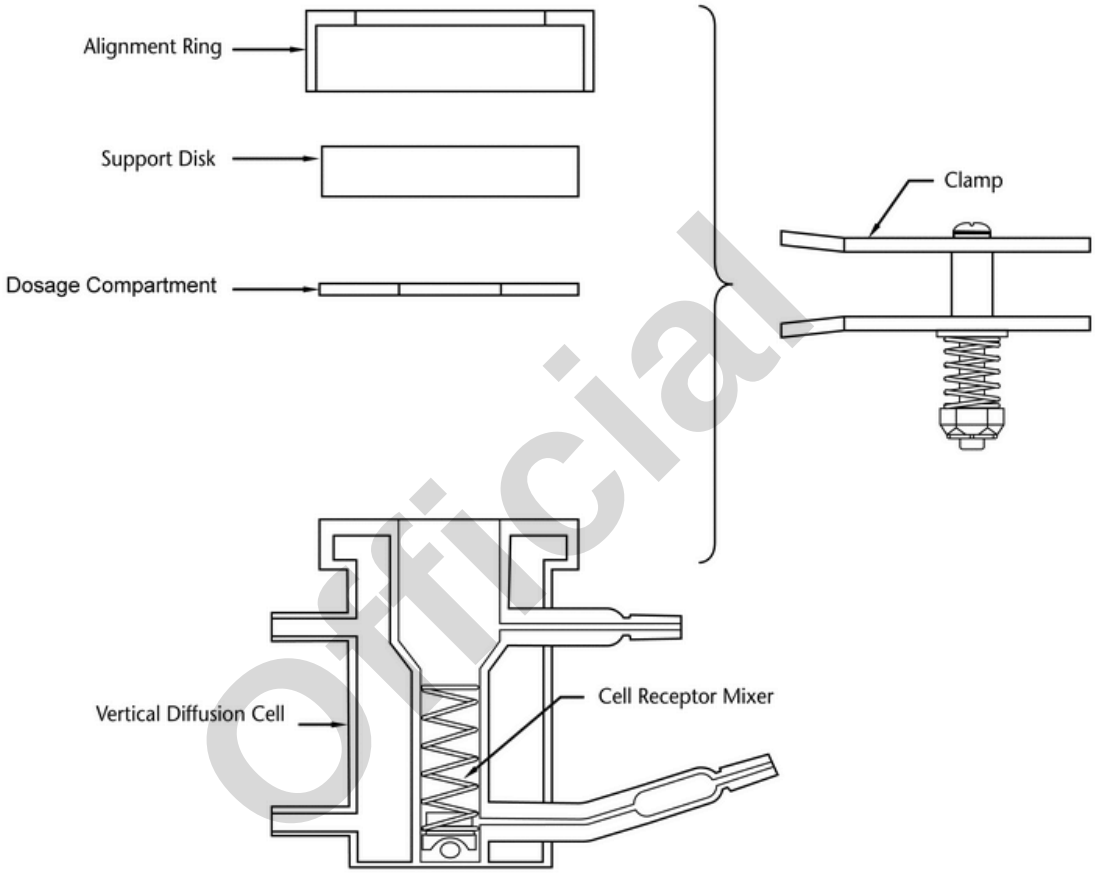


Figure 1. Example of possible design for VDC—Model A.

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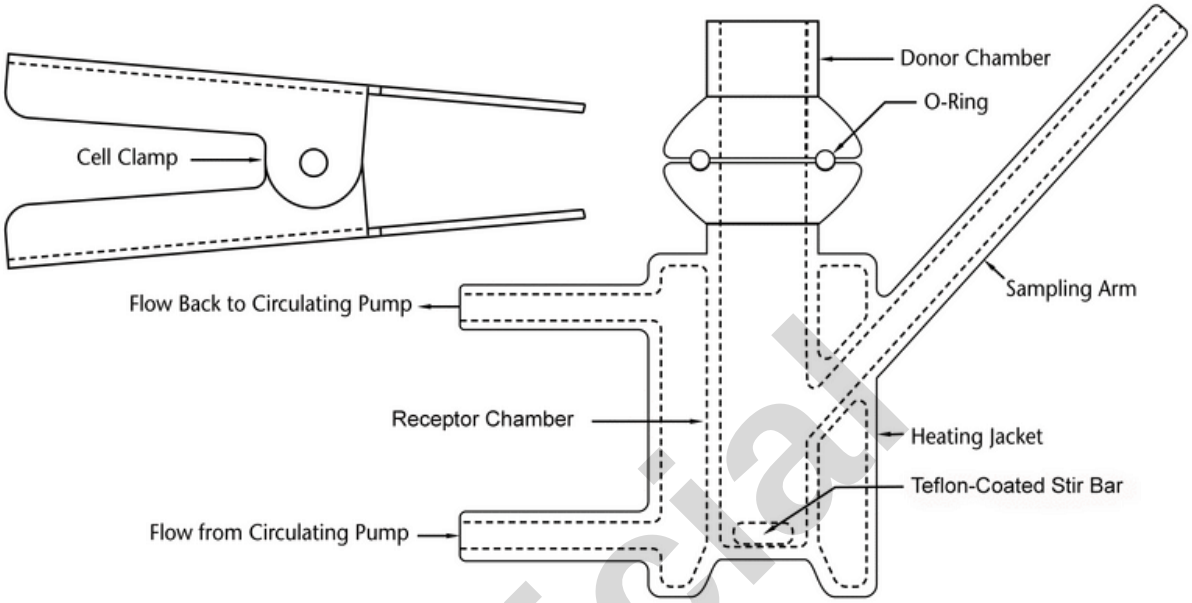


Figure 2. Example of possible design for VDC—Model B.

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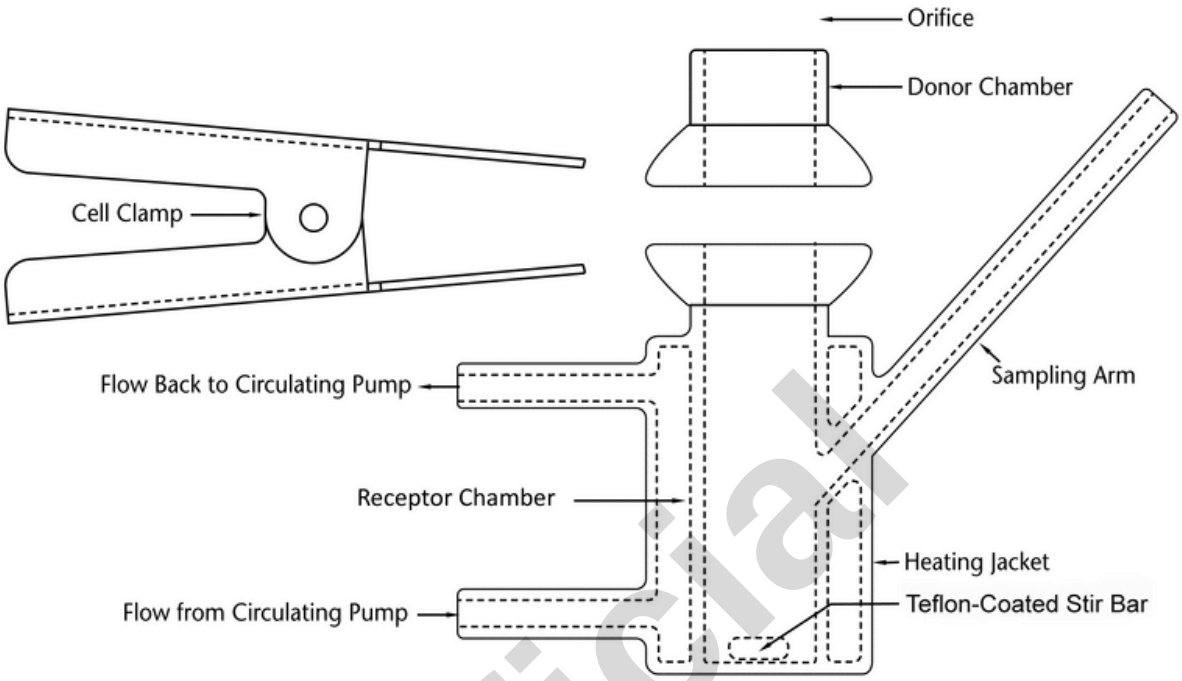


Figure 3. Example of possible design for VDC—Model C.

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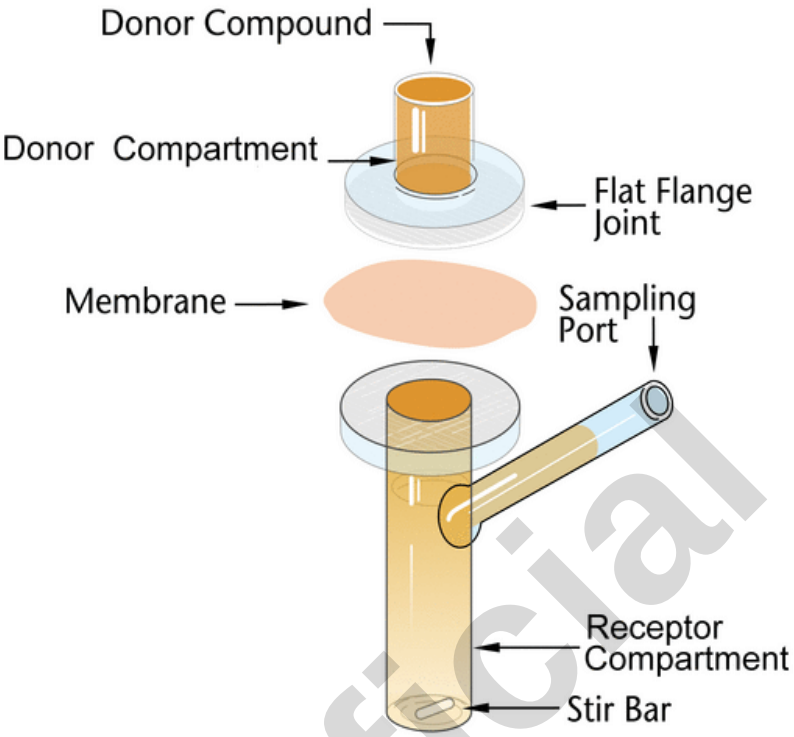


Figure 4. Schematic diagram of a generic design of a VDC.

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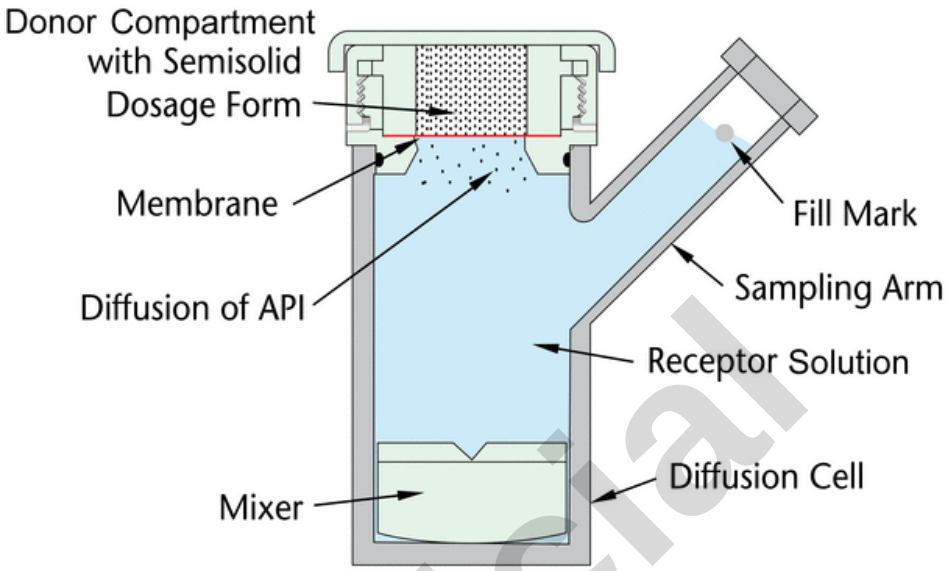


Figure 5. Schematic diagram of a generic design of a VDC.

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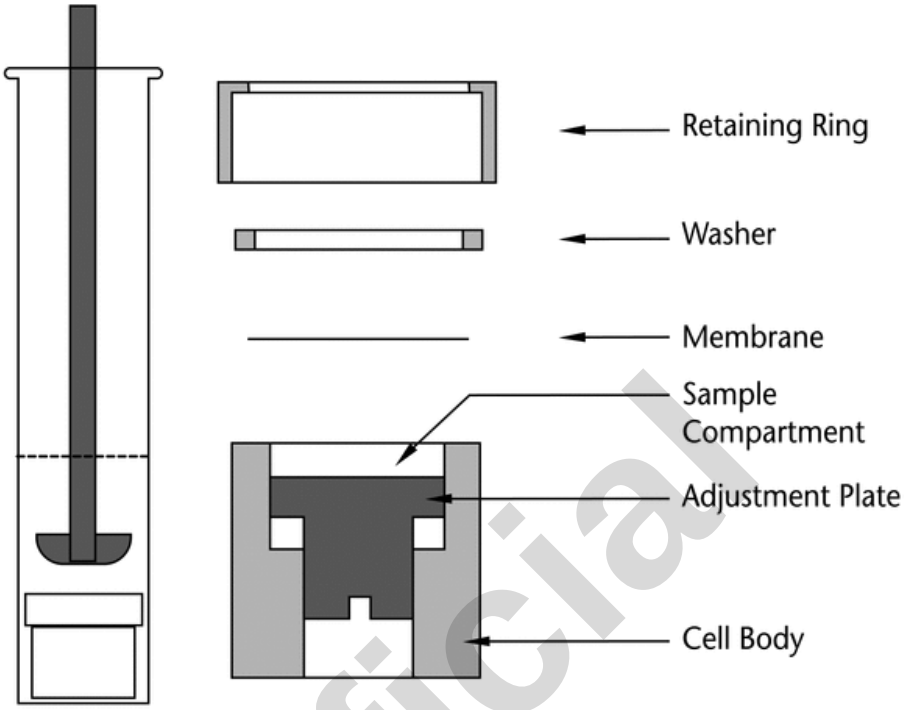


Figure 6. Example of possible design for an immersion cell.

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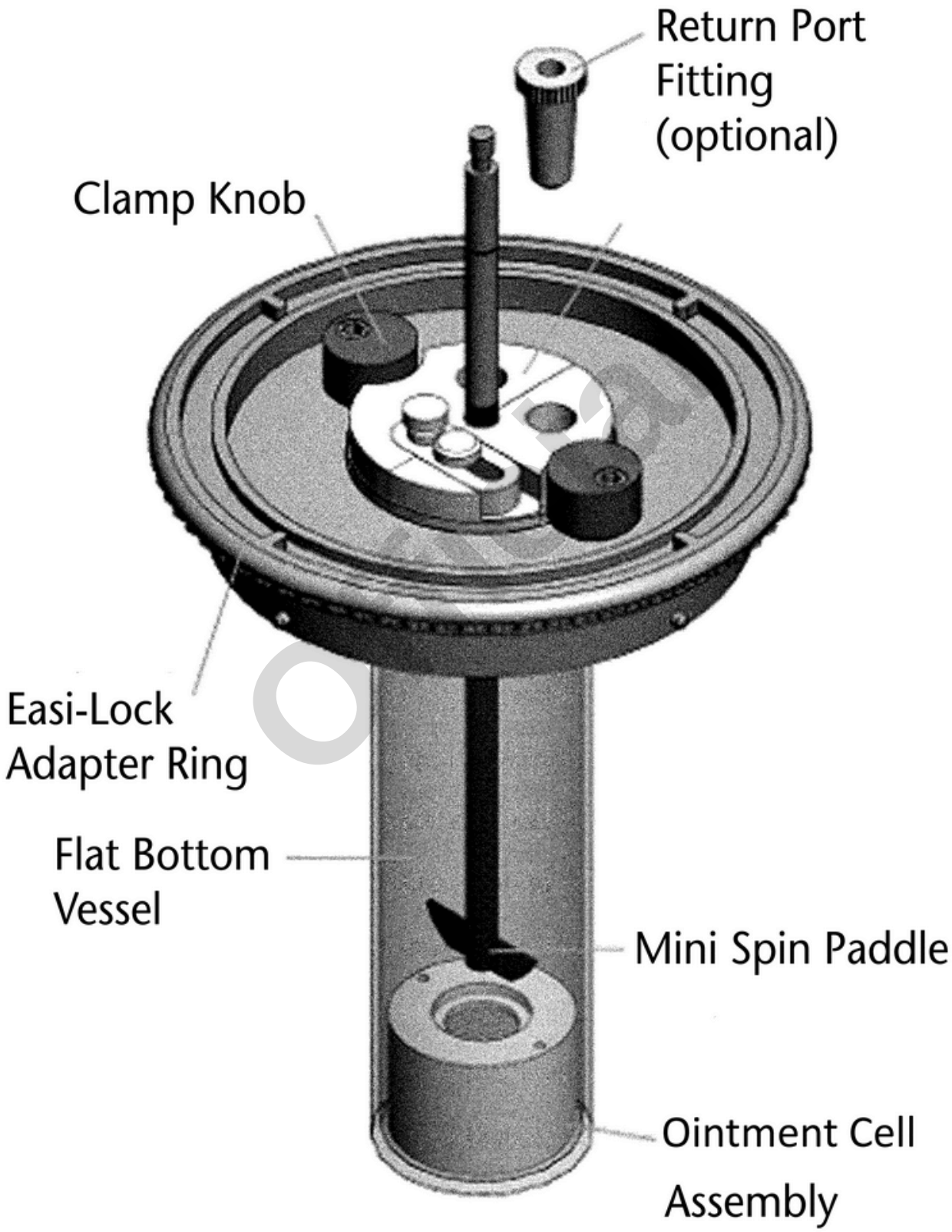


Figure 7. Example of possible design for an immersion cell.

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## Analytical Method

The analytical method should be precise, accurate, and specific for the drug in the receptor solution. The use of validated analytical methods with multipoint calibration curves is encouraged. The validation of an analytical method for IVRT sample analysis is done separately from the validation of an IVRT method, which follows specific procedures not described in this chapter.

## Experimental Design

### DOSE

When utilizing an appropriately selected receptor solution, membrane, equipment, dose, and sampling duration (as well as other potential method parameters) the release rate of the drug should be linear (when plotted as the amount of drug released versus the square root of time) and reproducible. The amount of drug product in the donor compartment should ensure that dose depletion does not occur to an extent that alters the linear (steady state) release kinetics during the duration of the test (i.e., a pseudo-infinite dose amount should be utilized). Excessive dose depletion is characterized by a nonlinear region in the plotted cumulative amount of drug released curve (when plotted as the amount of drug released versus the square root of time).

The dose application can be done by different methods, such as, but not limited to, dispensing directly on the membrane from a tube, transferring and spreading on the membrane with a spatula, or transferring and dispensing with a positive-displacement pipette or a disposable harvester (which minimizes shear stress during dosing). The shear stress imposed upon the dosage form during the dispensing (e.g., from a product tube) and dosing (e.g., using a positive displacement pipette) should be considered, and the dosing method should preferably minimize and routinely control the shear stress in a consistent manner for all diffusion cells. It is also important that a consistent amount of the dosage form is applied uniformly upon the membrane, without air pockets, making a compact cylinder of the formulation. The diffusional surface area of the product on the membrane needs to be consistent and well controlled as it is a factor in the calculation of the release rate. The donor compartment should be occluded to avoid evaporation of volatile components (including water) from the dosage form that is in contact with the membrane, because that evaporative metamorphosis may lead to changes in the composition of the drug product and may alter its linear (steady state) drug release kinetics.

### RECEPTOR SOLUTION

The selection of the receptor solution is guided by the solubility of the drug substance(s) in potential solvents/solvent combinations. Most commonly, combinations of aqueous and alcoholic/organic solvents are used, and the solubility of the drug in the receptor solution should exceed the highest sample concentration in the IVRT study, ideally by an order of magnitude, but demonstrably sufficient to facilitate a linear (steady state) release rate for the duration of the study (even when evaluating a relatively higher release rate of a formulation that is 150% of the nominal reference strength).

The receptor solution should be adequately mixed, typically by stirring at a constant and well-controlled speed throughout the experiment. The stirring speed or the flow rate should also be part of system qualification and is further described under *Equipment*.

Another factor to consider is the stability of the drug in the receptor solution at the temperature of the test during the IVRT experiment.

### SYSTEM EQUILIBRATION

The system should be appropriately equilibrated under the predefined experimental conditions prior to the beginning of the IVRT run. No air bubbles should be present in the receptor solution (under the membrane) immediately prior to, or during the experiment; if appropriate, the receptor solution can be degassed. The system temperature (including the membrane surface temperature and the circulating water bath temperature, if applicable) should be stable and well controlled. IVRT membranes can be presoaked with receptor solution prior to assembling in the diffusion cell or equilibrated with the receptor solution in situ within the diffusion cell prior to dosing (e.g., for 30 min at a membrane temperature of 32°) depending upon the method.

### EXPERIMENT LENGTH AND SAMPLING

The test duration should be suitable to characterize the linear (steady state) drug release kinetics of the product formulation. This is typically demonstrated based upon a sustained steady-state release between 4-6 h. The data obtained during the first hour may not represent the steady-state release kinetics, and for many IVRT methods steady-state release kinetics are calculated based upon timepoints from 1 h onward (e.g., at 1, 2, 3, 4, and 5 h, or at 2, 3, 4, 5, and 6 h). At least 5 sampling time points should be planned to obtain a well-characterized release rate. Sampling durations of at least 4 h are suitable to assess steady-state release rates, whereas shortened (e.g., 2 h) sampling durations may not be representative of the steady-state release kinetics. The specific sampling times may be varied depending on the formulation (e.g., every 30 min, or every hour); however, caution should be taken regarding the precise timing of each sample collection to ensure that sample collections happen within the predefined tolerance range. Samples should be withdrawn within a tolerance of ±15 min or ±2% of the nominal time, selecting the tolerance that results in the narrowest time interval.

Sampling is dependent upon the equipment used and should follow instructions provided by the manufacturer, when appropriate. The receptor solution used to replace the volume removed during sampling may be pre-warmed to the temperature of the receptor solution in the receptor compartment, and it is important to ensure that air bubbles are not present beneath the membrane after sampling and refilling the receptor solution at each time point.

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### NUMBER OF REPLICATES

The test is often conducted with a group of 6 or 12 cells per test run. The results from 6 diffusion cells dosed with a specific product are routinely sufficient to characterize the performance (release rate) of that product using that specific IVRT method. In instances when two products are compared, the results from 6 cells of a test product are often compared with the results from 6 cells of a reference product during the first stage of data analysis. See *Data Reporting* for more details on the use of additional replicates in instances when a second stage of testing is performed in a comparative IVRT study.

### MEMBRANE TEMPERATURE

The test is conducted at 32 ± 1° for products applied to the skin (measured at the surface of the membrane, when possible, or inferred based upon the temperature of the receptor solution for immersion cells or VDC Model A). The test is conducted at 37 ± 1° for products intended for internal application (e.g., rectal and vaginal products).

During the entire test, the nominal system conditions, like the temperature of the receptor solution, should be maintained so that the temperature at the membrane remains within specified parameters (e.g., 32 ± 1°) for the duration of the IVRT. The temperature control qualification is described under *Equipment*.

### DATA REPORTING

For each cell, the amount of drug released (typically in µg/cm$^2$) at each sampling time ($t_1$, $t_2$, etc.) is determined, and the cumulative amount released is plotted versus $\sqrt{t}$. The slope of the resulting line is a measure of the rate of drug release.

For each cell, the individual amount of drug released is plotted versus the square root of time. The slope of the resulting line is the rate of drug release.

IVRT is a useful tool during semisolid product development to assess whether there may be differences in the arrangement of matter within the semisolid product matrix of compositionally identical (or similar) test and reference products, which may alter their rates of drug release, and that may have the potential to alter product performance in clinical use. When test and reference products exhibit equivalent drug release rates based upon a validated IVRT method, it can mitigate the risk of potential differences in product performance. Therefore, evidence from an IVRT can support a demonstration of bioequivalence, along with other evidence that collectively mitigates the risk of a difference in product performance between a reference product and a prospective generic (test) product.

IVRT is commonly used to assess the sameness of a drug product after postapproval changes. Because common testing artifacts, such as air bubbles and membrane defects, yield measurements that are not normally distributed, a nonparametric statistical technique is used to evaluate the test results. The Mann-Whitney *U* test is used to calculate the 90% confidence interval for the ratio of the slopes between the test and the reference batches. This is illustrated by the following example in which the initial drug product batch is referred to as the reference batch (*R*) and the changed or subsequent batch is referred to as the test batch (*T*). The individual amounts of drug released from *R* are plotted versus the square root of time, and the resulting slopes are determined. Those are the reference slopes. The process is repeated for the test batch (*T*).

The *T*/*R* slope ratios are calculated for each test-to-reference combination of all pairs of *T*/*R* slopes. This procedure is facilitated with a table where the values for the slopes for *T* are listed down the left side of the table and the slopes for *R* are listed across the top of the table. The *T*/*R* slope ratios are then determined. See *Table 1*.

**Table 1. Comparison of Test Slope (TS) and Reference Slope (RS) Ratios**

| | RS1 | RS2 | RS3 | RS4 | RS5 | RS6 |
|---|---|---|---|---|---|---|
| TS1 | TS1/RS1 | TS1/RS2 | TS1/RS3 | TS1/RS4 | TS1/RS5 | TS1/RS6 |
| TS2 | TS2/RS1 | TS2/RS2 | TS2/RS3 | TS2/RS4 | TS2/RS5 | TS2/RS6 |
| TS3 | TS3/RS1 | TS3/RS2 | TS3/RS3 | TS3/RS4 | TS3/RS5 | TS3/RS6 |
| TS4 | TS4/RS1 | TS4/RS2 | TS4/RS3 | TS4/RS4 | TS4/RS5 | TS4/RS6 |
| TS5 | TS5/RS1 | TS5/RS2 | TS5/RS3 | TS5/RS4 | TS5/RS5 | TS5/RS6 |
| TS6 | TS6/RS1 | TS6/RS2 | TS6/RS3 | TS6/RS4 | TS6/RS5 | TS6/RS6 |

After the *T*/*R* ratios have been calculated, they are ordered from the lowest to the highest. The 8th and 29th *T*/*R* ratios are identified and converted to percent (multiplied by 100). These values represent the 90% confidence interval for the ratio of test to reference release rates. To pass first stage testing, those ratios must be within the range of 75%–133.33%.

If the results do not meet this criterion, 4 additional tests (2 reference and 2 test) of 6 cells each should be performed, resulting in 12 additional slope determinations for each product tested. The *T*/*R* slope ratios for all 18 slopes for each product tested are determined. All 324 individual *T*/*R* slope ratios are ordered from the lowest to the highest. To pass this second stage testing, the 110th and 215th slope ratios, representing the 90% confidence interval, must be within the range of 75%–133.33%.

## IN VITRO PERMEATION TEST (IVPT)

The in vitro permeation test (IVPT) is intended to characterize the rate and extent to which a drug applied on the surface of a biological membrane permeates into and through it, using method parameters aimed to simulate in vivo conditions. IVPT methods can utilize a variety of biological membranes, including excised human skin, which can exhibit natural variations in permeability that are reflective of the variability observed in vivo. This variability can be substantial; it is not uncommon to observe a 10-fold difference in skin permeability for a given compound (in the same formulation) between individuals in the

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population or between different anatomical regions on the same individual. Experimental variability may also be a result of the physicochemical properties of a molecule. For all products or treatments compared in an experiment, the replicate skin sections used should be sourced from the same donor (or the same set of donors), the same anatomical site (e.g., abdomen, back, etc.), the same source (e.g., elective surgery or cadavers) and manner of preparation (e.g., dermatoming, freezing, etc.) to minimize variability.

An IVPT study can be sensitive and discriminating to differences in the rate and extent to which compounds applied on the skin from different formulations become available in and through it. Notably, unlike an IVRT study, differences in permeation that are observed in an IVPT study comparing a test versus reference product, if any, may correlate with and/or be predictive of differences in bioavailability in vivo. The IVPT is routinely carried out to guide semi-solid topical formulation development.

## IVPT Method Development and Validation

IVPT method development should encompass key aspects that influence the IVPT results (some of these aspects are discussed below), including the bioanalytical methodology used to quantify the amount of the drug in the IVPT study samples. The scope of the IVPT method development is dependent upon the purpose of the study. For example, a bioequivalence study is likely to require a larger number of skin donors in order to adequately power a statistical analysis, compared to a study intended to qualitatively evaluate different formulation prototypes. Further, whereas IVPT studies designed to support a demonstration of bioequivalence generally rely upon assessments of the rate and extent to which drugs permeate the skin based upon receptor solution results, the selection of new chemical entities or formulation prototypes may also employ assessments of tissue distributions. A comprehensive discussion on IVPT method validation is outside of the scope of this chapter.

### Biological Membrane

Dermatomed human skin (split-thickness, typically around 250–500 µm), sourced from cadavers or elective surgery (typically abdominoplasties or breast reductions), is often the most appropriate membrane of choice due to its intrinsic relevance to a human drug product development, as well its ease of handling during experimental procedures. The thickness of the dermatomed skin should be confirmed prior to assembling the membrane in the diffusion cell, as significant variability in thickness may increase the variability of IVPT results. Epidermal preparations (sheets of epidermis that have been separated from the dermis at the dermal-epidermal junction) require technical experience to process, have less structural integrity compared to dermatomed skin, and may tear or lose barrier integrity more easily. However, epidermal preparations are often more consistent in thickness than dermatomed skin, and the magnitude of permeation for some compounds may be greater through epidermal preparations than through dermatomed skin. Therefore, while dermatomed skin is a good choice to evaluate in most cases, in some situations (e.g., when the drug of interest is not feasible to quantify permeating through dermatomed skin) epidermal preparations may be useful to evaluate during IVPT method development.

Dermatomed porcine skin has a relatively similar morphology to human skin and can be used as a secondary option (as a surrogate for human skin) for research and development studies, unless regulatory guidance recommendations specify otherwise. The use of rodent skin (as a surrogate for human skin) is discouraged due to significant dissimilarity to human skin in terms of morphology and stratum corneum structure and thickness, which can make the results difficult to interpret in terms of their relevance to human skin permeation. The use of cultured skin constructs (reconstructed human epidermis) is not ideal as a surrogate for natural excised human skin because the permeability properties of such tissues is currently not representative of excised human skin. The use of any synthetic membrane (including the types developed to emulate a biological membrane) is not appropriate for an IVPT method; the results of tests with any synthetic membrane may not reflect the rate and extent to which a drug permeates into and through skin and may be misleading.

The use of human or porcine full-thickness (non-dermatomed) skin can pose experimental challenges and is generally not recommended. Other membranes such as rectal, vaginal, or corneal epithelial tissue may also be employed, depending on the intended route of administration for the drug product under evaluation. In such circumstances, several parameters of the test method may differ from those used for an IVPT with skin due to specific considerations for modeling the relevant in vivo conditions. For example, the appropriate temperature at which to maintain the membrane may be different.

### Diffusion Cells

Two types of diffusion cells are commonly used for IVPT studies: the vertical diffusion cell (VDC) (see *Figures 1–5*) and the flow-through diffusion cell (FDC) (see *Figures 8–10*). Equipment features (e.g., bubble-free cells or cells with a vent hole) other than the ones depicted below may be suitable once qualified. VDCs are well characterized and can be utilized for both, IVRT and IVPT studies. FDCs are used exclusively for IVPT studies (not IVRT studies) and have the advantage that the sampling is more easily automated, which can make it easier to acquire samples at times when investigators are typically not in the laboratory (e.g., the middle of the night). VDCs and FDCs can both produce excellent results when the IVPT method is appropriately developed, and each type of cell has its advantages.

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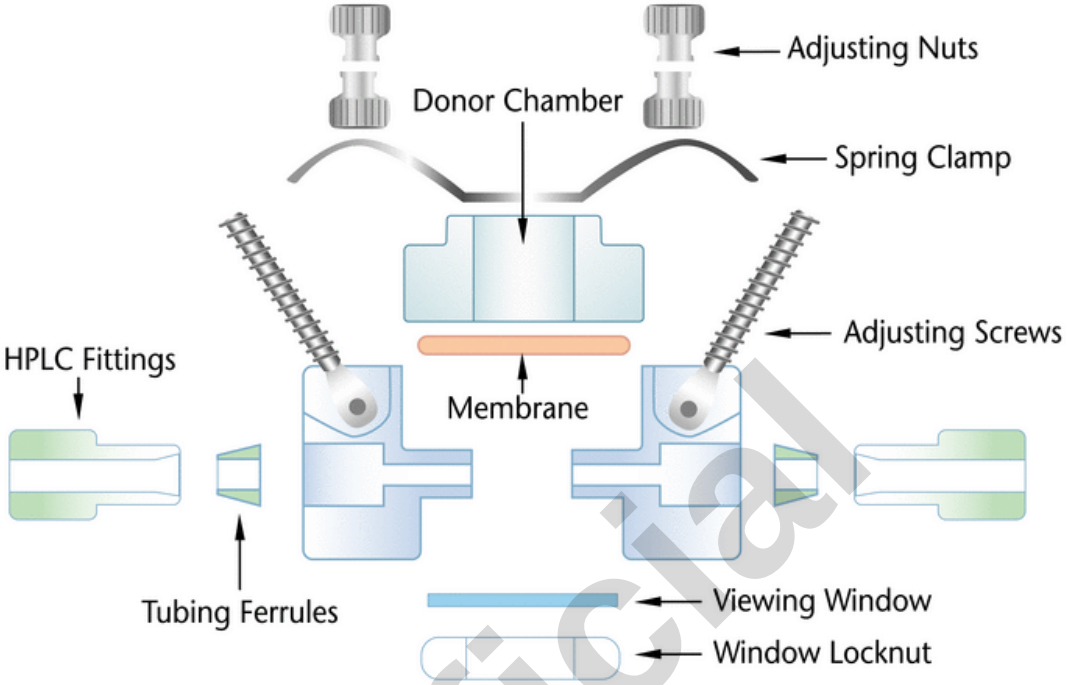


Figure 8. Schematic diagram detailing individual parts of a horizontal flow-through cell.

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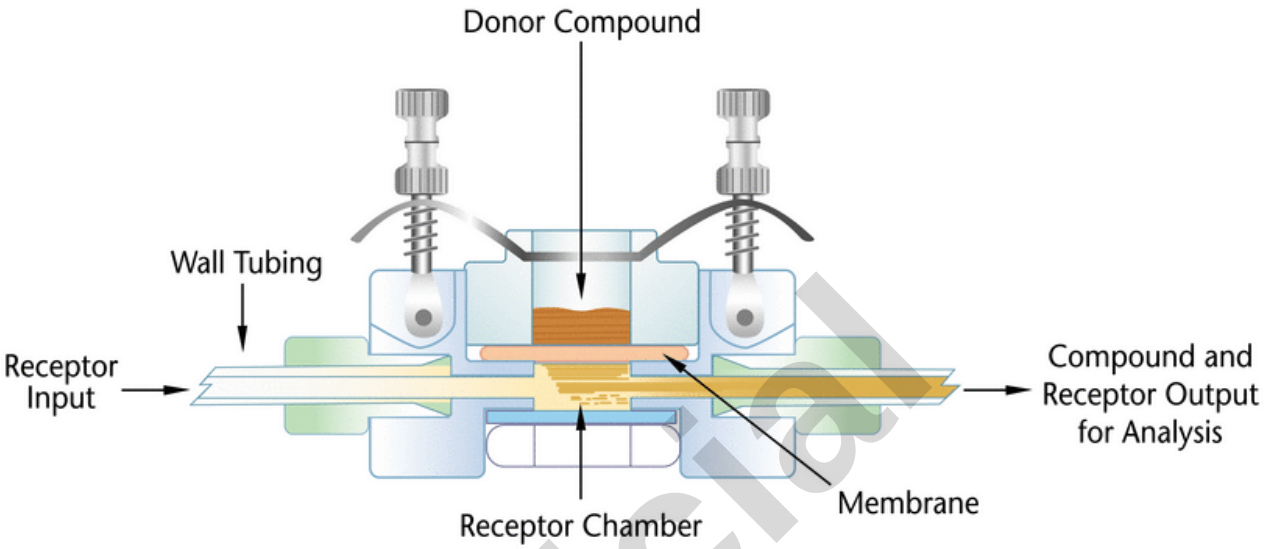


Figure 9. Schematic diagram detailing an assembled horizontal flow-through cell.

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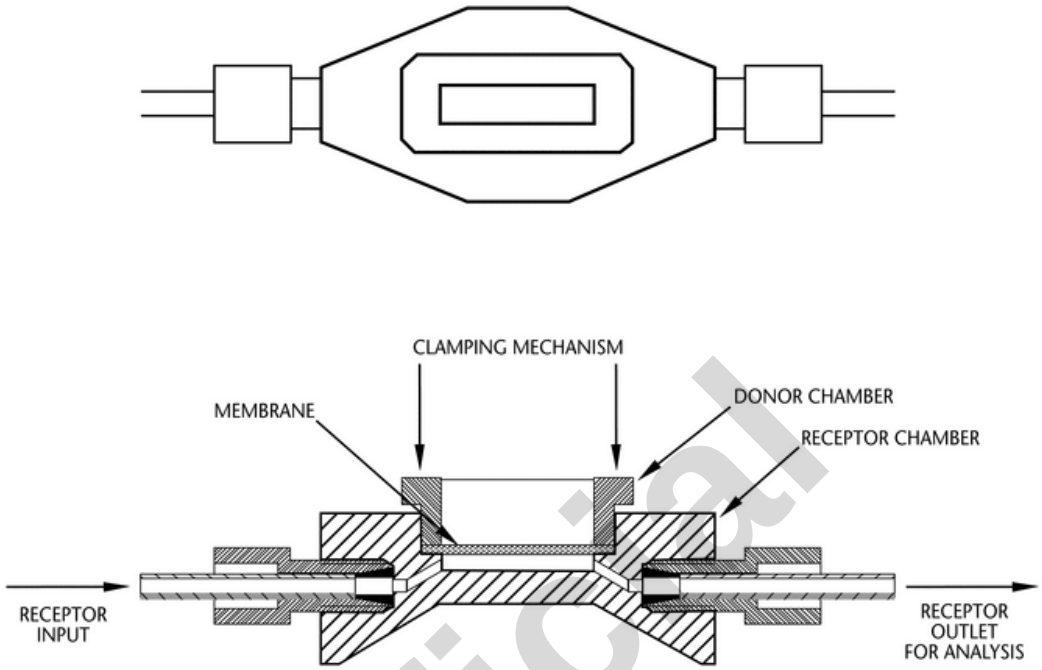


Figure 10. Schematic diagrams of top view and lateral view of an assembled horizontal flow-through cell.

## Bioanalytical Method

The choice of analytical method should consider the physicochemical properties of the analyte(s) present in the formulation and the actual formulation composition; these factors combined will directly affect the ability of a drug to cross the stratum corneum, therefore impacting drug concentrations in each skin layer and in the receptor solution. Liquid chromatography (LC) coupled with a mass spectrometer (MS) or a tandem mass spectrometer (MS/MS) detector provides excellent sensitivity and selectivity, albeit at the expense of higher complexity in terms of method development/validation, troubleshooting, and higher maintenance costs. LC coupled with an ultraviolet (UV) detector, diode array detector (DAD), or fluorescence detector (FLD) offers lesser technical complexity but is generally less sensitive and can pose selectivity challenges for analytes present in biological samples.

The use of validated bioanalytical methods with multipoint calibration curves is encouraged. The validation of an analytical method for IVPT sample analysis is done separately from the validation of an IVPT method, each of which follow specific procedures not described in this chapter.

## Experimental Design

### DOSE

The thermodynamic activity of the drug(s) present in a formulation will affect the rate and extent of skin permeation. It is prudent to understand the drug(s) solubility in the solvents/excipients of interest, as well as in the formulation prototypes under evaluation.

The finite dose typically ranges from 2–15 mg of formulation per square centimeter of dosing area, with a dose of 5–10 mg/cm$^2$ often used as a starting dose for method development. Ensuring consistency in the dispensing of the formulation (e.g., from the product tube) and uniform dose administration (spreading/coverage) on the skin section placed in the diffusion cell can help minimize experimental variability. The use of positive-displacement pipettes is recommended to ensure dosing precision. For semisolid dosage forms such as ointments, creams, and certain gels that do not flow like a solution, a glass rod or the bottom of a glass vial can be used to evenly spread the formulation within the dosing area.

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## RECEPTOR SOLUTION

The receptor solution should ensure sufficient solubility of the drug(s) of interest throughout the length of the study, thus not limiting the rate of diffusion/partitioning of the drug(s) from the skin to the receptor solution. The solubility of the drug in the receptor solution should exceed the highest sample concentration in the IVPT study, ideally by an order of magnitude, if possible. The inclusion of 0.1% (w/v) polyoxyethylene (20) oleyl ether is ideal to enhance the solubility of physiological buffer based (aqueous) receptor solutions for hydrophobic drugs. If additional solubility is needed, small increases in the concentration of polyoxyethylene (20) oleyl ether [e.g., from 0.1% (w/v) to 0.2% (w/v)] is typically adequate for most hydrophobic drugs. Higher concentrations of polyoxyethylene (20) oleyl ether may be evaluated, as needed, but should not exceed 6% because higher concentrations may alter the skin barrier. Other solubility modifiers may also be effective but may not be suitable for studies assessed by a regulatory agency unless the impact of that solubility modifier on the permeability of the skin barrier has been adequately characterized. Other strategies to improve the solubility of the drug in the receptor solution that may have the potential to alter the permeability of the skin (e.g., inclusion of organic solvents and alcohols in the receptor solution) are not prudent and may invalidate the IVPT method. Additionally, labile compounds may require modifications in the receptor solution, including the addition of antioxidants and/or chelating agents (e.g., ethylene diamine tetra acetic acid; EDTA) or the use of acidified water (e.g., pH 4.0) instead of phosphate-buffered saline (PBS). The addition of antibacterial/antimycotic agents [e.g., 0.1% (w/v) sodium azide; 0.01% (w/v) gentamicin sulfate] in the receptor solution is also important to prevent microorganism growth and membrane tissue degradation.

## SYSTEM EQUILIBRATION

The system should be appropriately equilibrated under the predefined experimental conditions prior to the beginning of the IVPT run. No air bubbles should be present in the receptor solution (under the membrane) immediately prior and during the experiment; if appropriate, the receptor solution can be degassed. The system temperature (including the membrane surface temperature and the circulating water bath temperature, if applicable) should be stable and well controlled. Frozen biological membranes should be thawed at ambient temperature prior to assembling on the diffusion cells. Once mounted, the skin sections should be allowed to equilibrate to a physiologically relevant state of hydration and temperature (e.g., for 30 min at a membrane surface temperature of 32°).

## EXPERIMENT LENGTH AND SAMPLING

The test duration should be sufficient to characterize a suitable permeation profile. For IVPT studies that are intended to support a demonstration of bioequivalence, the selected sampling schedule and study duration should be sufficient to characterize the cutaneous pharmacokinetics of the drug, which ideally includes a sufficiently complete flux profile to identify the maximum (peak) flux and a decline in the flux thereafter across multiple subsequent time points. IVPT studies typically range from 12–72 h in length; IVPT bioequivalence studies may require longer periods of time to observe a sufficient decline in the skin flux to adequately characterize the cutaneous pharmacokinetics.

The IVPT method development should support the selection of an appropriate sampling schedule, intended to provide suitable resolution for the flux profile. A study with fewer than 8 nonzero sampling time points may not provide adequate characterization of the flux profile.

## NUMBER OF DONORS AND REPLICATES

Depending on the stage of method development, the number of donors may vary. A study with fewer than 4 donors and 4 replicates per donor per treatment may be difficult to interpret. IVPT bioequivalence studies may require a larger number of donors and replicates to adequately power a statistical analysis. The inclusion of a nondosed control (no formulation) is recommended and may help ensure the skin source(s) and receptor solution are absent of contaminants that may influence the results.

## MEMBRANE TEMPERATURE

The surface of each skin section should be kept constant at 32 ± 1° throughout the length of the study. Temperature fluctuations of the skin surface will affect drug diffusion and may increase experimental variability. The use of a thermoregulated diffusion cell is encouraged to control the temperature of the membrane surface.

## MEMBRANE INTEGRITY

The skin barrier integrity in each diffusion cell should be confirmed prior to dosing by using techniques such as transepidermal water loss (TEWL), electrical impedance/conductance or tritiated water permeation; only skin sections with acceptable barrier integrity results should be dosed. TEWL is often a preferred method because, unlike the other methods, it measures the flux of water through the skin barrier while it is dry and in contact with the air, as it is in the normal in vivo state, and TEWL is relatively rapid and convenient.

## DRUG TISSUE DISTRIBUTION AND MASS BALANCE

Most IVPT experiments report skin flux and the total cumulative amount of drug that has permeated into the receptor solution (or as a percentage of drug from the total applied dose). Depending on the purpose of the study, an evaluation of drug tissue distribution (amounts in the stratum corneum, viable epidermis, and dermis) and total mass balance may provide additional insights.

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Full mass balance requires more intensive bioanalytical method development to ensure that the drug(s) of interest are efficiently extracted from each sample matrix type, such as cotton swabs used to remove the excess of formulation present on the skin surface at the end of the experiment, tape strips used to remove stratum corneum, and homogenized viable epidermis and dermis.

Epidermis and dermis heat splitting can be accomplished by placing the skin sections (harvested at the end of the IVPT study) on a sheet of aluminum foil and incubating it (dry heat) at 60° for 2–3 min. After this step, the epidermis can be separated (by scraping) from the dermis by using forceps. Because the drug levels in the epidermis are generally higher than the dermis, the splitting should be done carefully to avoid cross contamination, and clean forceps should be used in between samples.

### DATA REPORTING

Depending on the purpose of the study (bioequivalence, formulation development, prototype screening, etc.), data may be reported and assessed using different parameters (endpoints). For example: a bioequivalence study will focus on the cutaneous pharmacokinetic endpoints of maximum flux ($J_{max}$), and total cumulative amount (AMT) of drug permeated. Studies conducted in early stages of drug development, such as those intended to select a new chemical entity (NCE), or to rank different formulation prototypes, may report tissue (epidermis and dermis) concentrations in addition to the parameters (endpoints) above, and may include full mass balance data.

The flux (rate of drug permeation) should be calculated for each time point in units of mass/area/time (e.g., ng/cm$^2$/h) on the *y*-axis versus time in the *x*-axis. The extent of drug permeation should also be reported in units of mass/area (e.g., ng/cm$^2$) in the *y*-axis versus time in the *x*-axis—the slope of this curve can help determine the overall drug flux through the skin at different points in time. Drug levels in the viable epidermis and dermis can be reported as absolute mass amount (e.g., nanograms or micrograms) or concentration (e.g., nanograms or micrograms of drug per milligram of tissue [ng/mg or µg/mg]).

Full mass balance results are generally reported as percentage of drug(s) in each compartment (e.g., stratum corneum, viable epidermis, dermis and receptor solution) relative to the total amount applied at the beginning of the experiment.

## IVRT Versus IVPT

The selection between IVRT or IVPT should be based on the intended objective(s) of the formulation assessment, as described in *Table 2*. Intrinsic experimental differences between the two methodologies are further described in *Table 3*.

**Table 2. Guide for Selecting an Appropriate Test Method (IVRT or IVPT) Depending Upon the Goal**

| Evaluation/Goal | Test |
|---|---|
| Assessment of in vitro/in vivo correlation | IVPT |
| Assessment of the ability of a drug in a formulation to cross the stratum corneum and of the distribution of the drug in the epidermis, dermis, and/or receptor solution | IVPT |
| Selection of a new chemical entity (NCE) for further development as a semisolid formulation | IVPT |
| Comparison of the permeation kinetics of different topical semisolid formulations (different composition of different dosage forms, such as solutions, gels, creams, ointments, etc.) | IVPT |
| Comparison of the rate and extent to which a topically administered drug permeates through the skin and becomes available at or near a site of action from a reference and a test product formulation | IVPT |
| Evaluation of the effect of inactive ingredients on the rate of drug release from the formulation matrix | IVRT |
| Assessment of sameness in the formulation matrix between formulations with the same amount of an active ingredient and the same or similar composition of inactive ingredients | IVRT |
| Comparison of the effect of critical manufacturing/process steps on the microstructure of semisolid formulation | IVRT |
| Compare batch-to-batch variability in drug release rate at the time of manufacture, and during stability | IVRT |

**Table 3. Key Differences Between IVRT and IVPT Methodologies**

| Parameter | IVRT | IVPT |
|---|---|---|
| Membrane | Synthetic (e.g., mixed cellulose esters, nylon, polysulfone, polyethersulfone) | Biological (e.g., ex vivo human epithelial tissue such as skin, rectal or vaginal tissue, corneal tissue, nails, other mucous membranes) |
| Receptor solution | Aqueous and aqueous–organic combination, may include high percentages (≥30%) of alcohols and/or organic solvents (e.g., ethanol, isopropyl alcohol, acetonitrile) | Aqueous, typically phosphate buffered saline; low levels (typically ≤5%) of additives may be added to enhance solubility of hydrophobic compounds (see the IVPT *Receptor Solution* section) |

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**Table 3. Key Differences Between IVRT and IVPT Methodologies** *(continued)*

| Parameter | IVRT | IVPT |
|---|---|---|
| Sampling | Aliquot sampling of a portion of the receptor solution at designated sample time points | Removal of the entire volume of the receptor solution, or sampling of relatively large volume aliquots of the receptor solution for VDCs; FDCs offer a different sampling methodology due to the continuous flow of receptor solution into the collection vials |
| Apparatus | VDC or immersion cell | VDC or FDC |
| Dose | Pseudo-infinite, occluded | Finite (typically ≤15 mg/cm$^2$), unoccluded |
| Experimental duration | Typically ≤6 h | Typically ≥24 h |
| Receptor solution drug levels | Microgram to milligram range | Picogram to nanogram range |
| Analytical technique | HPLC/UPLC with detection by UV, DAD, or FLD | HPLC/UPLC with MS or MS/MS detection |
| Key data obtained | Release rate (slope) | Flux profile including peak flux ($J_{max}$) and cumulative amount (AMT) permeated; drug distribution in the different layers of the skin may also be assessed in certain study designs |

# EQUIPMENT

## General Recommendations for Equipment Qualification and Requalification

The VDC, immersion cells, and FDC should match the general descriptions provided below and may have design variations of the types shown among specific examples illustrated in *Figures 1–5* for VDCs, *Figures 6* and *7* for immersion cells, or *Figures 8–10* for FDCs. The VDC, immersion cell, and FDC components should be manufactured with inert materials that do not adsorb, absorb, bind, or react with the analyte. The diffusion cell and its components should not alter the amount of diffusing drug that is measured, either by adsorbing, absorbing, binding, or reacting with the drug, or by releasing drug that was adsorbed, absorbed, bound, or reacted with in a previous experiment.

The operating principles and specific test procedures differ among the various equipment; relevant procedures from the manufacturer may be used for installation, operational, and performance qualifications, if available.

However, regardless of what qualification information is provided by a diffusion cell manufacturer, the laboratory performing the test should perform an initial qualification of each diffusion cell. Qualified diffusion cells can be used in numerous experiments, and do not need to be qualified again (i.e., requalified) each time. The condition of the orifices and diffusion cells should be ascertained prior to each test. Certain diffusion cell components such as O-rings or tubing may require re-evaluation and occasional replacement. Automated systems are too varied to be covered in any detail within this chapter; however, they should be requalified routinely (e.g., every 6-12 months) based on the manufacturers' recommendations.

The initial qualification of each diffusion cell should, at minimum, include:

1. Measurements of the diffusional area of the orifices of the donor and receptor compartments between which the membrane is mounted
2. Empirically measured volume of the receptor solution compartment/vessel for each VDC or immersion cell, or the empirically measured outflow tube length for each FDC
3. Stability of the temperature measured at the membrane surface (e.g., 32 ± 1°) across a relevant duration (e.g., 6 h for IVRT or 48 h for IVPT)
4. Rate of stirring or agitation for VDCs or immersion cells, or the flow rate for FDCs, as applicable

In addition to the quantitative assessments described above, qualitative assessments should verify that diffusion cells do not leak, and that the membrane can be mounted securely in the diffusion cell so that there is no bulk flow of a dosage form from the donor compartment to the receptor compartment that circumvents the membrane. Several other considerations relevant to a well-controlled test may be included in the qualification.

1. **Qualification of Orifice Diameter:** The orifice diameter for the donor and receptor compartments can be determined using vernier calipers. The orifice should be inspected for any chips or other damage which may alter the surface area.
2. **Qualification of Volume of Receptor Solution Compartment/Vessel:** In the case of a VDC, the volume of the receptor solution should be empirically determined for each cell, individually, and recorded in a manner that can be uniquely associated with each diffusion cell thereafter, for use in calculations. The volume should be filled in a consistent manner for all diffusion cells (some cells may have a fill line marked on the cell which can be utilized) and the procedure used should consider whether the displacement by a stir bar (or other mechanism) may impact the volume measured such that the measured volume would not be relevant to that diffusion cell if a different size stir bar was used during the test. The nominal volume of the diffusion cells provided by the manufacturer should be used only as a guide.
   For immersion cells, the volume of receptor solution to be filled in the dissolution vessel should be accurately measured with the appropriate volumetric glassware and transferred to the dissolution vessel.
3. **Qualification of Temperature Control:** The temperature of each cell should be equilibrated to provide the target temperature at the membrane, and the initial qualification of the equipment should verify that the temperature can be maintained for the duration of the test when using the relevant equipment and method parameters. Depending on the VDC or FDC equipment design, a measurement of the membrane temperature can often be made conveniently using an infrared thermometer, or using a thermocouple mounted with the membrane; for immersion cells, the temperature at the membrane may be assumed to be the same as the bulk volume of the receptor solution in the dissolution vessel,

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once equilibrated and stabilized. The temperature of each cell should typically remain within ±1° of the target temperature (typically 32° or 37°) during the test.

4. **Qualification of Stirring/Agitation/Flow Rate:** The stirring rate should be verified with a suitable device, such as a photo tachometer. For VDC, stirring rates in the range of 400–600 rpm are common. The stirring rate measured is typically applicable to the specific location where the stirring impeller is (e.g., a specific position in a rack that holds multiple VDC, or a specific stir plate). Stirring rate can be measured using a surrogate stir bar if the stir bar used in the diffusion cell is too small to obtain an accurate measurement. The paddle stirring rate for immersion cells of 50 or 100 rpm is common and should be verified and maintained during the test.
Stirring rates are relevant for VDC, but not necessarily for FDC or immersion cells. In case of FDC, the flow rate of the receptor solution should be set at a specific value and maintained throughout the test. The flow rate (typically expressed in units of milliliters per minute) can be determined by setting the desired flow rate and collecting a sample into a tared vial for a specific duration. A suitable flow rate allows for a sample of sufficient size to ensure an accurate weighing and should not be substantially impacted by normal variability that may arise due to a drop that fails to fall from the tip of the dispensing tube. It is essential that the lengths of all the dispensing tubes are exactly the same, because differences in dispensing tube lengths can alter the void volume and lag time for samples to be dispensed into collection tubes.

## Vertical Diffusion Cell (VDC)

A VDC consists of two compartments (a donor compartment and a receptor compartment with a sampling arm) separated by a membrane and held together by a clamp, screw top, or other means (see *Figures 1–5*). The VDC body (i.e., donor and receptor compartment with associated cell structures like the sampling arm and water jacket, if present) is usually made from borosilicate glass, although different materials may be used to manufacture the body and other parts of the VDC assembly. Other diffusion cells that are similar in design principles and features to those depicted in *Figures 1–3* can generally be used.

The diameters of the orifices of the donor compartment and receptor compartment, which influence the diffusional area for the test, should be sized within ±5% of the specified diameter. The diameter of the donor and receptor compartment orifices may vary for different diffusion cell models. The receptor compartment orifice should be the same size as the donor compartment orifice. The design of the VDC should facilitate proper alignment of the orifices in the donor and receptor compartments. The membrane should be held in a horizontal plane between the donor and receptor compartments. Also, the membrane should be mounted so that it is flat, with no folds or wrinkles. The receptor compartment in each unit of a given model of VDC should be manufactured consistently, with a uniform height and geometry. All the cells (units) of the same VDC model should have the same nominal volume, and the actual volume should be measured and recorded for each individual cell. Care should be taken to minimize the intercell volume variability (e.g., not more than ±5%).

When conducting IVRT or IVPT studies, the VDC units are typically positioned in a stirrer rack (not depicted) that holds multiple VDC units (e.g., in sets of 6 or 7) in the correct orientation, providing magnetic stirring at a calibrated rate.

A magnetic nonstick (e.g., Teflon-coated), inert stir bar or similar stirring/agitation device in the receptor compartment is used as the internal stirring mechanism. The stirring bar or device should provide enough circulation to ensure contents of the receptor compartment are uniform. Samples of the receptor solution are removed at intervals throughout the test, and regardless of whether the entire receptor compartment volume or only an aliquot is sampled, an equivalent volume of stock receptor solution is replaced at each sampling event. The aliquots should be removed from the well-mixed center of the receptor compartment, unless sufficient proof exists that the entire receptor solution volume, including the portion in the sampling arm, are homogeneous and at the same concentration. For most VDC models, it is ideal for the volume of receptor solution in the sampling arm to be sampled and dispensed (repeatedly, a few times) into the well-mixed center of the receptor compartment, to ensure that the entire volume of the receptor compartment is homogeneous.

## General IVRT VDC Equipment Set Up

Observational checks should be performed prior to testing. The donor and receptor compartment orifices should be evaluated for any chips or other damage which may affect the orifice area. Chipped or damaged cells should not be used.

### MEMBRANE MOUNTING AND DOSING

Dosing procedures may be dictated by the requirements of the test method and/or the design of the diffusion cell. In general, two approaches are used: 1) the membrane is mounted and equilibrated in the diffusion cell prior to dosing the cell, or 2) the membrane is mounted and dosed during the assembly of the diffusion cell (e.g., VDC Model A in *Figure 1*), before the membrane comes into contact with the receptor solution. The dosing of the membrane prior to mounting on the diffusion cell in VDC Model A is acceptable because VDC Model A incorporates unique design elements to control the consistency of the dose and its alignment with the diffusion cell components. For all other VDC models, the membrane should be mounted and equilibrated in the diffusion cell prior to dosing the cell.

### DOSING VDC WITH OPEN DONOR COMPARTMENTS

It is conventional to install the membrane and then fill the receptor compartment with receptor solution. Any bubbles present underneath the membrane should be removed by tipping the cell and allowing the bubble to rise through the arm.

The system should be equilibrated, and the temperature should be measured at the membrane. The membrane should be within ±1° of the target temperature (typically 32° or 37°) prior to applying the dosage form. The stirrers should be turned on during the equilibration period.

For specific dosing techniques to apply the product to the membrane, refer to *In Vitro Release Test (IVRT), Experimental Design, Dose*.

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### DOSING VDC MODEL A DIFFUSION CELLS

Model A VDC are intended to have the membrane prepared and the dosing performed as part of the assembly of the cell top, which is separate from the cell bottom of the Model A VDC. In this model, the unique design and operation of the Model A VDC, as defined by the manufacturer, should be taken into consideration along with requirements of the test method. This method should not be used with conventional VDC designs like VDC models B and C.

During the preparation and dosing of the membrane in the Model A VDC cell top assembly, the membrane should be placed below the Model A VDC donor compartment's dosage wafer on a flat and firm surface. The dosage form is then applied to the membrane within the dosage wafer. The Model A VDC donor compartment (dosage wafer) is filled to capacity; a small spatula can be used to dispense and spread the dosage form evenly within the dosage wafer and to remove any excess formulation.

The receptor compartment of the Model A VDC should be filled with receptor solution that is equilibrated to the target temperature (typically 32° or 37°) during the preparation and dosing of the membrane in the cell top assembly. The stirrers can be run during this period to speed up temperature stabilization. The receptor solution temperature should be measured and ensured to be within ±1° of the target temperature prior to mounting the cell top assembly. The stirrers can be run to help speed up temperature stabilization.

To start the test, the donor compartment cell top assembly is mounted on the cell bottom assembly of the Model A VDC so that the membrane is facing the receptor compartment, and the membrane separates the dosage form from the receptor solution. After assembly of the Model A cell top and bottom, the fully assembled cell should be checked to ensure that no bubbles are present underneath the membrane, and that the membrane has not slipped out of position whereby the dosage form might flow directly into the receptor compartment, circumventing the membrane.

### SAMPLING VDC (GENERAL PROCEDURE FOR ALL MODELS OF VDC)

VDC are typically sampled one of two ways for IVRT, either withdrawal and replacement, or displacement. Cells which use withdrawal and replacement will typically only have one arm leading to the receptor compartment. Cells which are sampled by displacement will have two arms leading to the receptor compartment. For cells sampled by different methods it is recommended to refer to the manufacturer's documentation.

### SAMPLING BY WITHDRAWAL AND REPLACEMENT

The receptor solution volume in the arm of many VDC may not mix adequately with the volume of receptor solution in the stirred receptor compartment. To ensure that the entire volume of the receptor compartment is homogeneous, it is ideal for the volume of receptor solution in the sampling arm to be sampled (drawn out) from the arm and re-dispensed into the well-mixed center of the receptor compartment multiple times prior to collecting a sample of the receptor solution for analysis. A precise volume of the sample should be withdrawn from the well-mixed center of the receptor compartment, typically using a precise syringe or pipette. Typical sample amounts are 200 to 500 µL. The same amount of receptor solution is then replaced through the same arm. Care should be taken to ensure that a bubble is not introduced during the sampling withdrawal and replacement process. If a bubble is present, it should be removed by tipping the cell and guiding the bubble up the arm. Depending upon the test method, the stirring may continue throughout the sampling procedure, or prior to the sample time point, the stirring may be stopped and then resumed shortly after the volume of receptor solution is replaced.

### DISPLACEMENT SAMPLING

Cells with two sampling arms are often sampled by displacement. Prior to the sample time point, all stirring is stopped. The replacement receptor solution is slowly added to the receptor compartment to prevent mixing. The receptor solution displaces the sample out the other sampling arm for collection. Once the sample is collected the stirring is resumed. Typically, a rinse volume is required in the process for displacement sampling to ensure that the current sample does not contain remnants of a previous sample. This is especially important for automated systems.

## General IVPT VDC Equipment Set Up

Observational checks should be performed prior to testing. The donor and receptor compartment orifices should be evaluated for any chips or other damage which may affect the orifice area. Chipped or damaged cells should not be used.

Receptor solution can either be preheated or allowed to equilibrate to temperature in the receptor compartments of the diffusion cells.

Skin should be cut to the appropriate size for the diffusion cell (larger than the diffusional area of the orifice) to ensure that the orifice is completely covered.

Ideally, the skin should be gently stretched to ensure that it is flat (with no folds or wrinkles) when mounted upon the diffusion cell with the stratum corneum of the skin facing toward the air. An inert support membrane (i.e., a synthetic membrane validated for use with an IVRT method for the same drug product) may be used beneath the skin, as appropriate, as long as it does not impede the diffusion of the drug from the skin to the receptor solution. The donor compartment should then be installed on top of the skin ensuring that the skin is mounted securely in the diffusion cell. The receptor compartment may be filled with receptor solution either before or after mounting of the skin. Any bubbles underneath the skin should be removed by tipping the cell and guiding the bubble up the sampling arm. The thermoregulatory mechanism of the VDC system should then be utilized to equilibrate the skin to the target temperature.

Once the hydration and temperature of the skin has equilibrated (i.e., after 30 min) the skin surface temperature should be measured. The skin surface temperature should be within ±1° of the target temperature (32°) prior to applying the dosage form. The temperature of the receptor solution, or the circulating water bath, or the set point of a dry heat system, or any other component of the system should not be assumed to be the same as the skin surface temperature. Instead, the skin surface

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temperature should be directly measured (with an infrared thermometer or a thermocouple), and the thermoregulatory mechanism of the VDC system should be adjusted as needed to produce the target temperature of 32° at the skin surface. The stirrers can be run during the duration of the equilibration to speed up temperature stabilization.

For specific dosing techniques to apply the product to the membrane, refer to *In Vitro Permeation Test, Experimental Design, Dose*.

One approach to sampling involves draining the receptor compartment of all the receptor solution and refilling it completely with fresh receptor solution. Removal of the entire receptor solution volume and full volume replacement of the receptor solution at each time point may provide optimal solubility sink conditions. Although the entire receptor solution volume is sampled, an aliquot is typically used for analysis. Another approach to sampling involves the removal of only a partial amount (i.e., an aliquot) of the entire receptor solution volume, leaving behind a partial volume of the receptor solution at each sampling. This approach can allow the concentration of a compound to increase above a quantification limit during time periods of low flux. However, the sampling of relatively small aliquots of the receptor solution for an IVPT study may introduce anomalous measurements of apparently negative flux in certain regions of the IVPT study and produce flux profiles that are difficult to interpret. Therefore, when sampling partial volumes, it is prudent to maximize the volume of the sample aliquot, in order to minimize the potential for apparent negative flux results.

## VDC Qualification

VDC systems should generally match the description of the equipment described in this chapter. Other designs can be used with sufficient scientific justification so long as they consist of a donor compartment separated from a receptor compartment by a membrane.

VDCs should be individually qualified and uniquely identifiable either by a manufacturer issued serial number or other means. The orifice area for the donor and receptor compartments can be determined using vernier calipers. Receptor compartment volume should be determined with all components (e.g., stir bars) in place. The receptor compartment volume should be determined (gravimetrically or volumetrically) to a precision of 0.01 mL. The nominal receptor compartment volume (e.g., 7 mL) should never be used in place of an accurately determined VDC volume (e.g., 6.89 mL). Using the nominal volume fails to account for variations of the receptor compartment volume due to manufacturing which could affect the accuracy and precision of test results.

## VDC Preventative Maintenance Procedure and Requalification

All VDC systems should be properly maintained and requalified on a regular basis (as described previously). The relevant VDC systems (heating/thermoregulation, water bath and circulation tubes/manifolds, magnetic impeller/stirring, mechanical sampling, etc.) and other components of the VDC system should be kept clean and their operation should be evaluated to ensure that each diffusion cell can maintain the skin surface temperature within the target range (32 ± 1°), maintain the stirring speed within the specified range, and support the performance of the test within the specifications of the test method.

Any stirring device that is not stirring properly with the receptor solution present should be repaired or replaced. Since direct measurement of the stirring device used for testing is not always feasible, the stirring system (e.g., the rotating magnetic impeller) can be evaluated with a surrogate. For example, if stirring within a VDC is done with a 5 mm stir bar, the VDC can be temporarily removed, and a 25 mm stir bar can be placed in the cell block which can be measured to determine the stirring speed.

Sampling devices, such as pipettes, syringes, or automated systems should be evaluated to ensure that they are withdrawing and replacing sample volumes of the receptor solution with sufficient accuracy and precision.

Requalification of automated systems should be performed (e.g., every 6–12 months) based on considerations including the extent of equipment usage, the risk tolerance of the laboratory performing the test, and manufacturer's recommendations. Requalification should also be performed if the system is relocated or undergoes a major repair. In situations where a new VDC unit is incorporated into a previously qualified VDC system (with a set of specific VDC units), the elements of VDC qualification relevant to the new VDC unit should be performed, and the elements of VDC qualification relevant to the (previously qualified) VDC system should be requalified, as relevant (e.g., this may include a requalification of the skin surface temperature control for the new/replacement VDC introduced into the previously qualified VDC system).

## Immersion Cells

For immersion cells, USP *Apparatus 2* should be qualified according to the procedure described in *Dissolution* ⟨711⟩.

The immersion cell system consists of the following components:

- immersion cell which is an inert housing that holds a membrane and donor compartment.
- a smaller version of USP *Apparatus 2* (see ⟨711⟩) with vessel volumes that vary from 50–200 mL; however, 150- or 200-mL vessels are typically used.

An example immersion cell configuration can be seen in *Figure 6* and *Figure 7*.

The components of the immersion cell are listed in *Figure 6*. Formulation is placed in the sample compartment and sealed to prevent leaks by the membrane, washer, and retaining ring. The diameters of the orifices of the donor compartment, which define the dosage delivery surface area for the test, should be sized within ±5% of the specified diameter. The membrane should be held horizontal and faced up toward the paddle. Also, the membrane should be mounted so that it is flat, not wrinkled from overtightening the retaining ring. Donor compartments range from holding 300 mg to 4 g of the formulation; some models are adjustable.

The immersion cell can be used with a smaller version of USP *Apparatus 2* (see ⟨711⟩) with vessel volumes that vary from 50–200 mL; however, the 150- or 200-mL vessels are more commonly used. A flat-bottom variation of the 150- or 200-mL vessel

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should be used to avoid the issue of dead space under the cell and standardize the orientation of the immersion cell in the vessel. Flat bottom vessels with a small peak at the base, to keep the immersion cell centered, may also be used.

Use of the 150- or 200-mL vessel with USP *Apparatus 2* requires the following modifications:

- holders for the small-volume vessels (e.g., 200 mL vessels instead of standard 900-mL vessels)
- adapter plate to position the small-volume vessel in the center of the spindle
- smaller size shaft/paddle [2/3 cm (1/4 in)] to fit in the small-volume vessels

## General IVRT Immersion Cell Equipment Set Up

Immersion cell sample compartment volume should be set to a constant volume (compartment volume can be adjusted using an adjustment tool). Alignment of the retaining ring over the sample compartment is ensured using an alignment tool for the specific surface area of the immersion cell.

Observational checks should be performed prior to testing. The donor and receptor compartment orifices should be evaluated for any damage which may affect the orifice area. Damaged cells should not be used.

Before loading the cells and placing the receptor solution in the vessel, set the paddle height, which is 10 ± 2 mm above the surface of the membrane. All other operational parameters, such as level, vibration, wobble, etc., should be set at the same conditions defined for USP *Apparatus 2*. The small-volume condition is qualified by first using the standard *Apparatus 2* setup and *Performance Verification Test, Apparatus 1 and 2* (see ⟨711⟩).

Fill the reservoir dosage area with the sample under test. Ensure that the reservoir is filled to the top to minimize the possibility of air bubble formation between the surface of the sample and the membrane. A uniform surface can be obtained with the aid of a spatula. The typical quantity of sample is between 300 mg and 4 g, depending on the type of immersion cell used. Using forceps or tweezers, remove the membrane from the soaking receptor solution and place it over the top of the sample compartment by rolling it onto the surface to avoid entrapment of air. Ensure that the membrane is free of wrinkles. Assemble the immersion cell components as specified by the device manufacturer. Carefully place the completed assembly into the bottom of the dissolution vessel with the membrane facing up. The appropriate preheated receptor solution may be preloaded in the vessel or can be added after immersion of the immersion cell to start the test.

At the end of the test period, dismantle the cell and examine the contents for anything unusual that could explain any anomalous data (e.g., leaks, bubbles, etc.).

## Immersion Cell Qualification

Immersion cells should match the description of one of the instruments listed in this chapter. Other designs can be used with sufficient scientific justification so long as they consist of a donor compartment separated from a receptor compartment by a membrane.

At a minimum, the orifice area and donor compartments volume must be certified. Additional dimensions should also be certified when possible. Due to the uniformity in manufacturing immersion cells, individual identification of the cells and components is recommended, but not required.

Orifice area for the donor and receptor compartments can be determined using vernier calipers. Volume of the donor compartment can be calculated using the donor compartment diameter and height.

A known volume of receptor solution should be transferred to the vessel when testing by suitably accurate method or device.

Immersion cells should only be used on suitably qualified dissolution apparatus.

Requalification should be performed every 6–12 months, based on equipment usage, risk tolerance, and manufacturers recommendation. Requalification should also be performed if the system is relocated or undergoes a major repair.

## Flow-Through Diffusion Cell (FDC) for IVPT

The components of the FDC are displayed in *Figures 8–10*. The skin is placed above the receptor compartment and kept in place by the donor compartment and a clamping mechanism. The skin should be held in a horizontal plane between the donor and receptor compartments. The donor and receptor compartments should be made out of an inert material. The surface area of the orifices of the donor compartment, which define the dosage delivery surface area for the test, should be sized within ±5% of the nominal value.

The FDC is connected to a pump which provides a constant or pulsation flow of receptor solution that passes through the receptor compartment, into a sample line then collected by a sample vial. Typically, the receptor compartment within the cell holds relatively small volumes (≤0.5 mL) as sink conditions are controlled, in part, by the flow rate of the pump. Flow rates in the range of 2–200 µL/min are common, however some methods may have flow rates in the thousands-of-microliters-per-minute range (i.e., in the milliliter-per-minute range), and the appropriate flow rate for each IVPT method may need to be optimized during method development depending upon the solubility of the drug in the receptor solution, the permeation rates during relevant sampling durations, and the limit of quantification of the receptor sample solution analysis method. The cells are typically placed on the arm of a fraction collector which will align the sample lines with vials or test tubes for each sample point. The FDC is heated to allow the membrane to be maintained at appropriate temperature.

## General IVPT FDC Equipment Set Up

Observational checks should be performed prior to testing. The donor and receptor compartment orifices should be evaluated for any damage which may affect the orifice area. Damaged cells should not be used.

Ideally, the skin should be gently stretched to ensure that it is flat (with no folds or wrinkles) when mounted upon the diffusion cell with the stratum corneum facing the air. An inert support membrane (e.g., a synthetic membrane validated for use with

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an IVRT method for the same drug product) may be used beneath the skin, as appropriate, as long as it does not impede the diffusion of the drug from the skin to the receptor solution. The donor compartment should then be installed on top of the skin ensuring that the skin is mounted securely in the diffusion cell. The receptor compartment may be filled with receptor solution either before or after mounting of the skin. Any bubbles underneath the skin should be removed by tipping the cell and/or facilitating the removal of the bubble via the outflowing receptor solution. The thermoregulatory mechanism of the FDC system should then be utilized to equilibrate the skin to the target skin surface temperature of 32°.

The FDC is connected to a pump and inflow as well as outflow tubing. When all the FDC for testing are connected to pumps, air bubbles may need to be purged from the receptor compartments. A typical method is the cells are tipped upward, with the outflow sample line raised, while the pump is run at a high speed which facilitates the removal of the air bubble from under the skin via the outflowing receptor solution. The pump can be stopped after all air has been purged from the receptor compartment. The cell should be lowered to the testing position and allowed to equilibrate.

Once the hydration and temperature of the skin has equilibrated (e.g., after 30 min) the skin surface temperature should be measured. The skin surface temperature should be within ±1° of the target temperature (32°) prior to applying the dosage form. The temperature of the receptor solution, or the circulating water bath, or the set point of a dry heat system, or any other component of the system should not be assumed to be the same as the skin surface temperature. Instead, the skin surface temperature should be directly measured (with an infrared thermometer or a thermocouple), and the thermoregulatory mechanism of the FDC system should be adjusted as needed to produce the target temperature of 32° at the skin surface.

Collection vials or test tubes should be weighed when empty to determine the exact volume of sample present after collection has completed.

For specific dosing techniques to apply the product to the skin, refer to the *In Vitro Permeation Test, Experimental Design, Dose*.

Set the pump to the flow rate to be used for testing and align the outflow sample tubes (which should all be the same length) with the first set of vials or test tubes.

After samples have been collected, the vials or test tubes should be weighed to determine the exact volume of receptor solution collected. This volume should be used when determining the total amount of drug present in each sample.

## FDC Qualification

FDC systems should generally match the description of the equipment described in this chapter. Other designs can be used with sufficient scientific justification so long as they consist of a donor compartment separated from a receptor compartment by the skin.

FDCs should be individually qualified and uniquely identifiable either by a manufacturer issued serial number or other means. The orifice area for the donor and receptor compartments can be determined using vernier calipers. It may be appropriate to qualify the lengths of (inert) tubing for each of the flow-through diffusion cells, and their associated dead volumes, to accurately calculate the lag time before a sample elutes through the tubing and is collected.

The flow rate of the pump should be qualified for the rate used during testing. For flow rates in the microliters-per-minute range, it is recommended to extend the sampling time to reduce the effects of droplets adhering to the sample line. If an automated system is used it should be ensured that the sample lines are properly aligned over the vials.

Skin surface temperature should be measured using an IR thermometer. The temperature of the heating block must be set higher than the desired membrane temperature (e.g., 33° for a 32° skin surface temperature).

## FDC Preventative Maintenance Procedure and Requalification

All FDC systems should be properly maintained and requalified on a regular basis (as described previously). The relevant FDC systems (heating and thermoregulation, pumps, tubes, mechanical sampling systems, etc.) and other components of the FDC system should be kept clean and their operation should be evaluated to ensure that each diffusion cell can maintain the skin surface temperature within the target range of 32 ± 1°, maintain the flow rate within the specified range, and support the performance of the test within the specifications of the test method.

The pump should be evaluated to ensure it is working properly. If a peristaltic pump is used, the tubing should be unclamped once the test has completed to prevent unnecessary wear on the tubing, which may cause variation in the flow rate. Peristaltic pump tubing should be replaced at regular intervals to ensure the flow rate remains consistent across all the cells. For automated sampling systems, sample tube alignment should also be checked to ensure that all samples are entirely collected into the vials or test tubes, without leakage, failure to capture samples, or sample loss for other reasons.

Requalification of automated systems should be performed (e.g., every 6–12 months) based on considerations including the extent of equipment usage, the risk tolerance of the laboratory performing the test, and manufacturer's recommendations. Requalification should also be performed if the system is relocated or undergoes a major repair. In situations where a new FDC unit is incorporated into a previously qualified FDC system (with a set of specific FDC units), the elements of FDC qualification relevant to the new FDC unit should be performed, and the elements of FDC qualification relevant to the (previously qualified) FDC system should be requalified, as relevant (e.g., this may include a requalification of the skin surface temperature control for the new/replacement FDC introduced into the previously qualified FDC system).▲ (USP 1-Dec-2023)

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# ⟨1730⟩ PLASMA SPECTROCHEMISTRY—THEORY AND PRACTICE

## OUTLINE

1. *Introduction*
2. *Sample Preparation*
3. *Sample Introduction*
4. *Standard Preparation*
5. *Inductively Coupled Plasma (ICP)*
6. *Inductively Coupled Plasma Optical Emission Spectroscopy (ICP–OES)*
    - *6.1 Calibration*
    - *6.2 Standardization*
    - *6.3 Procedure*
7. *Inductively Coupled Plasma Mass Spectrometry (ICP–MS)*
    - *7.1 Calibration*
    - *7.2 Standardization*
    - *7.3 Procedure*
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9. *References*

## 1. INTRODUCTION

The purpose of this general chapter is to provide a general overview of fundamental principles, instrumentation and application of inductively coupled plasma optical emission spectroscopy (ICP–OES) and inductively coupled plasma mass spectrometry (ICP–MS). It is the companion chapter to *Plasma Spectrochemistry* ⟨730⟩. A glossary of terms is located at the end of this general chapter.

## 2. SAMPLE PREPARATION

Sample preparation is critical to the success of plasma-based analysis and is the first step in performing any analysis via ICP–OES or ICP–MS. Plasma-based techniques are heavily dependent on sample transport into the plasma, and because ICP–OES and ICP–MS share the same sample introduction system, the means by which samples are prepared may be applicable to either technique. The most conventional means by which samples are introduced into the plasma is via solution nebulization. If solution nebulization is employed, solid samples must be dissolved in order to be presented into the plasma for analysis. These samples may be dissolved in any appropriate solvent. There is a strong preference for the use of aqueous or dilute nitric acid solutions, because there are minimal interferences with these solvents compared to other solvent choices. Hydrogen peroxide, hydrochloric acid, sulfuric acid, perchloric acid, combinations of acids, or various concentrations of acids can all be used to dissolve the sample for analysis. Dilute hydrofluoric acid can be used, but great care must be taken when using this acid to ensure the safety of the analyst, and to protect the components of the sample introduction system, specifically: peristaltic pump tubing, the nebulizer, spray chamber, and inner torch tube should be manufactured from hydrofluoric acid-tolerant materials. Proper safety procedures must be followed to protect the analyst, as well. Additionally, alternative means of dissolving the sample can be employed. These include, but are not limited to: the use of dilute bases, straight or diluted organic solvents, combinations of acids or bases, and combinations of organic solvents, or any solvent that is compatible with the instrumentation.

When samples are introduced into the plasma via solution nebulization, it is important to consider the potential matrix effects and interferences that might arise from the solvent. The use of an appropriate internal standard and/or matching the standard matrix with samples should be applied for ICP–OES and ICP–MS analyses in cases where accuracy and precision are not adequate. The use of an internal standard should be considered the rule, rather than the exception, in the case of ICP–MS analyses. In either event, the selection of an appropriate internal standard should consider the analyte in question, ionization energy, wavelengths or masses, and the nature of the sample matrix.

Where a sample is found not to be soluble in any acceptable solvent, a variety of digestion techniques can be employed. These include hot-plate digestion and microwave-assisted digestions, including open-vessel and closed-vessel approaches. The decision regarding the type of digestion technique to use depends on the nature of the sample being digested, as well as on the analytes of interest.

Use acids, bases, and hydrogen peroxide of ultra-high purity, especially when ICP–MS is employed. Deionized water must be at least 18 MΩ. Check diluents for interferences before they are used in an analysis. Because it is not always possible to obtain organic solvents that are free of metals, use organic solvents of the highest quality possible with regard to metal contaminants. Open-vessel digestion is generally not recommended for the analysis of volatile metals, e.g., selenium and mercury. The suitability of a digestion technique, whether open-vessel or closed-vessel, should be supported by spike recovery experiments in order to verify that, within an acceptable tolerance, volatile metals have not been lost during sample preparation. Additionally, it may be necessary to extract the analyte(s) of interest, should a sample not completely dissolve. In such an instance, the validity of the extract must be demonstrated by means of spike and recovery studies.

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It is important to consider the selection of the type, material of construction, pretreatment, and cleaning of analytical lab ware used in ICP–OES and ICP–MS analyses. The material must be inert and, depending on the specific application, resistant to caustics, acids, and/or organic solvents. For some analyses, diligence must be exercised to prevent the absorption of analytes onto the surface of a vessel, particularly in ultra-trace analyses. Contamination of the sample solutions from metal and ions present in the container can lead to inaccurate results.

The use of lab ware that is not certified to meet Class A tolerances for volumetric flasks is acceptable if the linearity, accuracy, and precision of the method have been experimentally demonstrated to be suitable for the purpose at hand.

## 3. SAMPLE INTRODUCTION

There are two ways to introduce the sample into the nebulizer: by means of a peristaltic pump, and by self-aspiration. The peristaltic pump is preferred and serves to ensure that the flow rate of sample and standard solution to the nebulizer is the same irrespective of sample viscosity. The speed setting of the peristaltic pump should remain constant throughout an analysis during the time period when readings are being taken by the instrument. In some cases, where a peristaltic pump is not required, self-aspiration can be used.

The purpose of a nebulizer is to generate very small droplets of the sample, with the goal of generating a fine aerosol mist. A wide variety of nebulizer types is available, including pneumatic (concentric and cross-flow), grid, and ultrasonic nebulizers. Micronebulizers, high-efficiency nebulizers, direct-injection high-efficiency nebulizers, and flow-injection nebulizers are also available. The selection of the nebulizer for a given analysis should consider the sample matrix, analyte, and desired sensitivity. Some nebulizers are better suited for use with viscous solutions or those containing a high concentration of dissolved solids, whereas others are better suited for use with organic solutions.

Note that the self-aspiration of a fluid is due to the Bernoulli, or Venturi, effect. Not all types of nebulizers will support self-aspiration. The use of a concentric nebulizer, for example, is required for self-aspiration of a solution.

Once a sample leaves the nebulizer as an aerosol, it enters the spray chamber, which is designed to permit only the smallest droplets of sample solution into the plasma; as a result, typically only 1%–2% of the sample aerosol reaches the ICP, although some special-purpose nebulizers have been designed that permit virtually all of the sample aerosol to enter the ICP.

As with nebulizers, there is more than one type of spray chamber available for use with ICP–OES or ICP–MS. Examples include the Scott double-pass spray chamber, as well as cyclonic spray chambers of various configurations. The spray chamber must be compatible with the sample and solvent and must equilibrate and wash out in as short a time as possible. When a spray chamber is selected, the nature of the sample matrix, the nebulizer, the desired sensitivity, and the analyte should all be considered. Gas and liquid chromatography systems can be interfaced with ICP–OES and ICP–MS for molecular speciation, ionic speciation, or other modes of separation chemistry, based on elemental emission or mass spectrometry.

Ultimately, the selection of sample introduction hardware should be demonstrated experimentally to provide sufficient specificity, sensitivity, linearity, accuracy, and precision for the analysis at hand.

In addition to solution nebulization, it is possible to analyze solid samples directly via laser ablation (LA). In such instances, the sample enters the torch as a solid aerosol. LA–ICP–OES and LA–ICP–MS are better suited for qualitative analyses of pharmaceutical compounds because of the difficulty in obtaining appropriate standards. Nonetheless, quantitative analyses can be performed if it can be demonstrated, through appropriate method validation, that the available standards are adequate (*1*).

## 4. STANDARD PREPARATION

Single- or multi-element standard solutions, whose concentrations are traceable to primary reference standards, such as those of the National Institute of Standards and Technology (NIST), can be purchased for use in the preparation of working standard solutions. Alternatively, standard solutions of elements can be accurately prepared from standard materials, as appropriate, and their concentrations can be determined independently. Working standard solutions, especially those used for ultra-trace analyses, may have limited shelf life, depending on the analyte in question, the type of storage container, the solution's concentration, and the storage conditions. As a general rule, working standard solutions with concentrations less than 10 ppm (w/v) should be retained for NMT 24 h unless stability is demonstrated experimentally. The selection of the standard matrix is of fundamental importance in the preparation of element standard solutions. Spike recovery experiments should be conducted with specific sample matrices in order to determine the accuracy of the method. If sample matrix effects cause excessive inaccuracies, standards, blanks, and sample solutions should be matrix matched, if possible, in order to minimize matrix interferences.

In cases where matrix matching is not possible, an appropriate internal standard or the method of standard additions should be used for ICP–OES or ICP–MS. The method of standard additions may be necessary, even with the use of matrix-matched solutions and internal standards. In any event, the selection of an appropriate internal standard should consider the analytes in question, their ionization and excitation energies, their chemical behavior, their wavelengths or masses, and the nature of the sample matrix. Ultimately, the selection of an internal standard should be demonstrated experimentally to provide sufficient specificity, sensitivity, linearity, accuracy, and precision of the analysis at hand.

The method of standard additions involves adding a known concentration of the analyte element to the sample at no fewer than two concentration levels plus an unspiked sample preparation. The instrument response is plotted against the concentration of the added analyte element, and a linear regression line is drawn through the data points. The absolute value of the *x*-intercept multiplied by any dilution factor is the concentration of the analyte in the sample.

Optimization of the ICP–OES or ICP–MS method is also highly dependent on the plasma parameters and means of sample introduction. Forward power, gas flow rates, and torch position may all be optimized to provide the best signal. Selection of wavelengths or isotopes must be carefully considered. The presence of dissolved carbon at concentrations of a small percentage in aqueous solutions enhances ionization of selenium and arsenic in an inductively coupled argon plasma. This phenomenon frequently results in a positive bias for ICP–OES and ICP–MS selenium and arsenic quantification measurements, which can be

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remedied by using the method of standard additions or by adding a small percentage of carbon, such as analytically pure glacial acetic acid, to the linearity standards.

## 5. INDUCTIVELY COUPLED PLASMA (ICP)

The components that make up the ICP excitation source include the argon gas supply, torch, radio frequency (RF) induction coil, impedance-matching unit, and RF generator. Argon gas is almost universally used in the ICP. The plasma torch consists of three concentric tubes designated as the inner, the intermediate, and the outer tube. The intermediate and outer tubes are almost universally made of quartz. The inner tube can be made of quartz or alumina if the analysis is conducted with solutions containing hydrofluoric acid. The nebulizer gas flow carries the aerosol of the sample solution into and through the inner tube of the torch and into the plasma. The intermediate tube carries the intermediate (sometimes referred to as the auxiliary) gas. The intermediate gas flow helps to lift the plasma off the inner and intermediate tubes to prevent their melting and the deposition of carbon and salts on the inner tube. The outer tube carries the outer (sometimes referred to as the plasma or coolant) gas, which is used to form and sustain the toroidal plasma. The tangential flow of the coolant gas through the torch constricts the plasma and prevents the ICP from expanding to fill the outer tube, keeping the torch from melting.

An RF induction coil, also called the load coil, surrounds the torch and produces an oscillating magnetic field, which in turn sets up an oscillating current in the ions and electrons produced from the argon. The impedance-matching unit serves to efficiently couple the RF energy from the generator to the load coil. The unit can be of either the active or the passive type. An active matching unit adjusts the impedance of the RF power by means of a capacitive network, whereas the passive type adjusts the impedance directly through the generator circuitry. Within the load coil of the RF generator, the energy transfer between the coil and the argon creates a self-sustaining plasma. Collisions of the ions and electrons liberated from the argon ionize and excite the analyte atoms in the high-temperature plasma. The plasma operates at temperatures of 6,000–10,000 K, so most covalent bonds and analyte-to-analyte interactions are eliminated.

## 6. INDUCTIVELY COUPLED PLASMA OPTICAL EMISSION SPECTROSCOPY (ICP–OES)

The ICP can use either an optical or a mass spectral detection system. In the former case, ICP–OES, analyte detection is achieved at an emission wavelength of the analyte in question. Because of differences in technology, a wide variety of ICP–OES systems are available, each with different capabilities as well as different advantages and disadvantages. Simultaneous-detection systems are capable of analyzing multiple elements at the same time, thereby shortening analysis time and improving background detection and correction. Sequential systems move from one wavelength to the next (sometimes referred to as slewing) to perform analyses, and often provide a larger number of analytical lines from which to choose.

Modern instruments typically use array detectors as detection devices. Array detectors, including charge-coupled devices and charge-injection devices, have detectors assembled on a chip, making it possible to combine the advantages of both simultaneous and sequential systems. These types of detection devices are used in the most powerful spectrometers, providing rapid analysis and a wide selection of analytical lines. Some instruments may use photomultiplier tubes (PMT's) for detection. PMT's are best-suited for simultaneous analyses; however, the use of PMT's is quickly waning, and array detectors are more commonly found.

The ICP can be viewed in either axial or radial (also called lateral) mode. The torch is usually positioned horizontally in axially viewed plasmas and is viewed end on, whereas it is positioned vertically in radially viewed plasmas and is viewed from the side. Axial viewing of the plasma can provide higher signal-to-noise ratios (better detection limits and precision); however, it also incurs greater matrix and spectral interferences. Axial plasmas normally require the use of a shear gas, which effectively removes the coldest part of the plasma to help reduce self-absorption. Methods validated on an instrument with a radial configuration may not be completely transferable to an instrument with an axial configuration, and vice versa.

Additionally, dual-view instrument systems are available, making it possible for the analyst to take advantage of either torch configuration. The selection of the optimal torch configuration will depend on the sample matrix, the analyte in question, the analytical wavelength(s) used, the cost of instrumentation, the required sensitivity, and the type of instrumentation available in a given laboratory.

Regardless of torch configuration or detector technology, ICP–OES is a technique that provides a qualitative and/or quantitative measurement of the optical emission from excited atoms or ions at specific wavelengths. These measurements are then used to determine the analyte concentration in a given sample. Upon excitation, an atom or atomic ion emits an array of different frequencies of light that are characteristic of the distinct energy transition allowed for that element. The intensity of the light is generally proportional to the analyte concentration. It is necessary to correct for the background emission from the plasma. Sample concentration measurements are usually determined from a working curve of known standards over the concentration range of interest. It is, however, possible to perform a single-point calibration under certain circumstances, such as limit tests, if the method has been validated for sufficient specificity, sensitivity, linearity, accuracy, precision, ruggedness, and robustness.

Because there are distinct transitions between atomic energy levels, and because the atoms in the ICP are rather dilute, emission lines have narrow bandwidths. However, because the emission spectra from the ICP contain many lines, and because "wings" of these lines overlap to produce a nearly continuous background on top of the continuum that arises from the recombination of argon ions with electrons, a high-resolution spectrometer is required in ICP–OES.

The decision regarding which spectral line to measure should include an evaluation of potential spectral interferences. All atoms in a sample are excited simultaneously; however, the presence of multiple elements in some samples can lead to spectral overlap. Spectral interference can also be caused by background emission from the sample or plasma. Modern ICPs usually have background correction available, and a number of background correction techniques can be applied. Simple background correction typically involves measuring the background emission intensity at a baseline level away from the main peak and subtracting this value from the total signal being measured. Mathematical modeling to subtract the interfering signal as a

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background correction can also be performed with certain types of ICP–OES spectrometers. One simple way to avoid spectral interferences is to select an analytical line that is free of interferences, if possible.

The selection of the analytical spectral line is critical to the success of the ICP–OES analysis, regardless of torch configuration or detector type. Though some wavelengths are preferred, the final choice must be made in the context of the sample matrix, the type of instrument being used or the sensitivity required. Analysts might choose to start with the wavelengths recommended by the manufacturer of their particular instrument and select alternative wavelengths based on manufacturer recommendations or published wavelength tables (*2, 3, 4, 5, 6*). Ultimately, the selection of analytical wavelengths should be demonstrated experimentally to provide sufficient specificity, sensitivity, linearity, accuracy, and precision of the analysis at hand.

Forward power, gas flow rates, viewing height, and torch position can all be optimized to provide the best signal. However, it must also be kept in mind that these same variables can influence matrix and spectral interferences.

The analysis of the Group I elements can pose some challenges. When atomic ions are formed from elements in this group, they assume a noble gas electron configuration, with correspondingly high excitation energy. Because the first excited state of these ions is extremely high, few are excited, so emission intensity is correspondingly low. This situation can be improved by reducing the fractional ionization that can in turn be achieved by using lower forward power settings in combination with adjusted viewing height or nebulizer gas flow, or by adding an ionization suppression agent to the samples and standards.

When organic solvents are used, it is often necessary to use a higher forward power setting, higher intermediate and outer gas flows, and a lower nebulizer gas flow than would be employed for aqueous solutions, as well as a reduction in the nebulizer gas flow. It could be necessary to reduce the peristaltic pump speed, and alter the selection of the spray chamber. When using organic solvents, it could be necessary to bleed small amounts of oxygen into the torch to prevent carbon buildup in the torch.

### 6.1 Calibration

The wavelength accuracy for ICP–OES detection must comply with the manufacturer's applicable operating procedures. Because of the inherent differences among the types of instruments available, there is no general system suitability procedure that can be employed. Calibration routines recommended by the instrument manufacturer for a given ICP–OES instrument should be followed.

### 6.2 Standardization

The instrument must be standardized for quantitation at time of use. Because ICP–OES is a technique generally considered to be linear over a range of 6–8 orders of magnitude, it is not always necessary to continually demonstrate linearity by the use of a standard curve composed of multiple standards. Once a method has been developed and is in routine use, it is possible to calibrate with a blank and a single standard. One-point standardizations are suitable for conducting limit tests, as well as other analyses, on production materials and final products if the method has been rigorously validated for sufficient specificity, sensitivity, linearity, accuracy, precision, ruggedness, and robustness. The use of single-point standardization is also acceptable for qualitative ICP–OES analyses, where the purpose of the experiment is to confirm the presence or absence of elements without the requirement of an accurate quantitation.

An appropriate blank solution and standards that bracket the expected range of the sample concentrations should be assayed and the detector response plotted as a function of analyte concentration, as in the case where the concentration of a known component is being determined within a specified tolerance. The plot of the analyte concentration against the known concentrations of components is usually performed automatically by the instrument.

It is not always possible to employ a bracketing standard when an analysis is performed at or near the detection limit. This lack of a bracketing standard is acceptable for analyses conducted to demonstrate the absence or removal of elements below a specified limit. The number and concentrations of standard solutions used should be based on the purpose of the quantitation, the analyte in question, the desired sensitivity, and the sample matrix. Regression analysis of the standard plot should be employed to evaluate the linearity of detector response, and individual monographs may establish other criteria.

### 6.3 Procedure

It is important to follow the procedure for the instrument parameters, as directed in the individual monograph. The specification of definitive parameters in a monograph does not preclude the use of other suitable operating conditions, and adjustments of operating conditions may be necessary. Because of differences in manufacturers' equipment configurations, the manufacturer's suggested default conditions could be used and modified as needed. Alternative conditions must be supported by suitable validation data, and the conditions in the monograph will take precedence for official purposes. Data collected from a single sample introduction are treated as a single result. This result might be the average of data collected from replicate sequential readings from a single solution introduction of the appropriate standard or sample solution. Sample concentrations are calculated versus the working curve generated by plotting the detector response versus the concentration of the analyte in the standard solutions, which is often calculation directly by the instrument.

## 7. INDUCTIVELY COUPLED PLASMA MASS SPECTROMETRY (ICP–MS)

When using the ICP–MS, analytes are detected directly at their atomic masses. Because these masses must be charged to be detected in ICP–MS, the method relies on the ability of the plasma source to both atomize and ionize sample constituents. As is the case with ICP–OES, a wide variety of ICP–MS instrumentation systems are available.

The systems most commonly in use are quadrupole-based systems. Additionally, high-resolution sector field instruments and time of flight-based instruments are available, as are multiple quadrupole systems. Regardless of instrument design or configuration, ICP–MS provides both a qualitative and a quantitative measurement of the components of the sample.

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Ions are generated from the analyte atoms by the plasma, and ions are then extracted from the atmospheric-pressure plasma through a sampling cone into a lower-pressure zone, ordinarily held at a pressure near 1 Torr. In this extraction process, the sampled plasma gases, including the analyte species, form a supersonic beam, which dictates many of the properties of the resulting analyte ions. A skimmer cone, located behind the sampling cone, "skims" the supersonic beam of ions as they emerge from the sampling cone. Behind the skimmer cone is a lower-pressure zone, often held in milliTorr ranges. Lastly, the skimmed ions pass a third-stage orifice to enter a zone held near microTorr pressures, where they encounter ion optics and are passed into the mass spectrometer. The pressure differences aid in moving the ions along and into the mass spectrometer, which separates the ions according to their mass-to-charge (m/z) ratios. The ICP–MS has a mass range up to 240 atomic mass units.

Depending on the equipment configuration, analyte adducts can form with diluents, with argon, or with their decomposition products. Also formed are oxides and multiply-charged analyte ions, which can increase the complexity of the resulting mass spectra. Interferences can be minimized by appropriate optimization of operational parameters, including gas flows (central, intermediate, and outer gas flow rates), sample-solution flow, RF power, extraction-lens voltage, etc., or by the use of collision or reaction cells, or cool plasma operation, if available on a given instrument. Unless a laboratory is generating or examining isotopes that do not naturally occur, a list of naturally occurring isotopes will provide the analyst with acceptable isotopes for analytical purposes. Isotopic patterns also serve as an aid to element identification and confirmation. Additionally, tables of commonly found interferences and polyatomic isobaric interferences and correction factors can be used, and are often pre-programmed into an instrument.

ICP–MS generally offers considerably better detection limits than ICP–OES, largely because of the extremely low background noise that it generates. This ability is a major advantage of ICP–MS for determination of very low analyte concentrations or when elimination of matrix interferences is required. In the latter case, some interferences can be avoided simply by additional dilution of the sample solution. In some applications, analytes can be detected below the parts per trillion (ppt) level using ICP–MS. As a general rule, ICP–MS as a technique requires that samples contain significantly less total dissolved solids than does ICP–OES.

The selection of the analytical mass to use is critical to the success of the ICP–MS analysis, regardless of instrument design. Though some masses are often considered to be the primary ones, because of their high natural abundance, an alternative mass for a given element is often used to avoid spectral overlaps (isobaric interferences). Selection of an analytical mass must always be considered in the context of the sample matrix, the type of instrument being used, and the concentrations to be measured. Analysts could choose to start with masses recommended by the manufacturer of their particular instrument and select alternate masses based on manufacturer's recommendations or published tables of naturally occurring isotopes (*7*).

Optimization of the ICP–MS method is also highly dependent on the plasma parameters and means of sample introduction. Forward power, gas flow rates, and torch position may all be optimized to provide the best signal. When organic solvents are used, it is often necessary to use a higher forward power setting and a lower nebulizer flow rate than would be used for aqueous solutions. Additionally, when organic solvents are used, it could be necessary to introduce small amounts of oxygen into the central or intermediate gas to prevent carbon buildup in the torch or on the sampler cone orifice. The use of a platinum-tipped sampling or skimmer cone may also be required in order to reduce cone degradation with some organic solvents.

## 7.1 Calibration

The mass spectral accuracy for ICP–MS detection must be in accordance with the applicable operating procedures. Because of the inherent differences between the types of instruments available, there is no general system suitability procedure that can be employed. Analysts should refer to the tests recommended by the instrument manufacturer for a given ICP–MS instrument.

## 7.2 Standardization

The instrument must be standardized for quantification at the time of use. Because the response (signal vs. concentration) of ICP–MS is generally considered to be linear over a range of 6–8 orders of magnitude, it is not always necessary to continually demonstrate linearity by the use of a working curve. Once a method has been developed and is in routine use, it is common practice to calibrate with a blank and a single standard. One-point standardizations are suitable for conducting limit tests on production materials and final products, provided that the method has been rigorously validated for sufficient specificity, sensitivity, linearity, accuracy, precision, ruggedness, and robustness. An appropriate blank solution and standards that bracket the expected range of the sample concentrations should be assayed and the detector response plotted as a function of analyte concentration, which are normally performed by the instrument. The number and concentration of standard solutions used should be based on the analyte in question, the expected concentrations, and the sample matrix, and should be left to the discretion of the analyst.

The method of standard additions should be employed in situations where matrix interferences are expected or suspected. This method involves adding a known concentration of the analyte element to the sample solution at no fewer than two concentration levels. The instrument response is plotted against the concentration of the added analyte element, and a linear regression line is drawn through the data points. The absolute value of the *x*-intercept multiplied by any dilution factor is the concentration of the analyte in the sample. In many instances, the instrument will perform this calculation automatically after being programmed to use the method of standard additions.

## 7.3 Procedure

Follow the procedure for the detection mode and instrument parameters for ICP–MS, as directed in the individual monograph. The specification of definitive parameters in a monograph does not preclude the use of other suitable operating conditions, and adjustments of operating conditions may be necessary. Alternative conditions must be supported by suitable validation data, and the conditions in the monograph will take precedence for official purposes. Because of differences in manufacturers' equipment configurations, the analyst could begin with the manufacturer's suggested default conditions and

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modify them as needed. Data collected from a single sample introduction are treated as a single result. Data collected from replicate sequential readings from a single introduction of the appropriate standard or sample solutions are averaged as a single result. Sample concentrations are calculated versus the working curve generated by plotting the detector response versus the concentration of the analyte in the standard solutions. With modern instruments, this calculation is often performed by the instrument.

## 8. GLOSSARY

**Auxiliary gas:** See *Intermediate (or auxiliary) gas.*

**Axial viewing:** A configuration of the plasma for AES in which the plasma is directed toward the spectrometer optical path, also called "end-on viewing."

**Central (or nebulizer) gas:** One of three argon gas flows in an ICP torch. The central gas is used to help create a fine mist of the sample solution when solution nebulization is employed. This fine mist is then directed through the central tube of the torch and into the plasma.

**Collision cell:** A design feature of some ICP–MS instruments. Collision cells are used to reduce interferences from argon species or polyatomic ions and facilitate the analysis of elements that might be affected by those interferences.

**Cool plasma:** Plasma conditions used for ICP–MS that result in a plasma that is cooler than that normally used for an analysis. This condition is achieved by using a lower forward power setting and higher central-gas flow rate, and is used to help reduce isotopic interferences caused by argon and some polyatomic ions.

**Coolant gas:** See *Outer (or coolant or plasma) gas.*

**Forward power:** The number of watts used to ignite and sustain the plasma during an analysis. Forward power requirements may vary, depending on sample matrix and analyte.

**Intermediate (or auxiliary) gas:** Gas used to "lift" the plasma off the surface of the torch, thereby preventing melting of the intermediate tube and the formation of carbon and salt deposits on the inner tube.

**Internal standard:** An element added to or present in the same concentration in blanks, standards, and samples to act as an intensity reference for the analysis. An internal standard should be used for ICP–AES work and must always be used for quantitative ICP–MS analyses.

**Lateral viewing:** See *Radial viewing.*

***m*:** The ion mass of interest.

**Multiply-charged ions:** Atoms that, when subjected to the high-ionization temperature of the ICP, can form doubly or triply charged ions ($X^{++}$, $X^{+++}$, etc.). When detected by MS, the apparent mass of these ions will be ½ or ⅓ that of the atomic mass.

**Nebulizer:** Used to form a consistent sample aerosol that mixes with the argon gas, which is subsequently sent into the ICP.

**Outer (or coolant or plasma) gas:** The main gas supply for the plasma.

**Plasma gas:** See *Outer (or coolant or plasma) gas.*

**Radial viewing:** A configuration of the plasma for AES in which the plasma is viewed orthogonal to the spectrometer optic path. Also called "side-on viewing." See also *Lateral viewing.*

**Reaction cell:** Similar to *Collision cell,* but operating on a different principle. Designed to reduce or eliminate spectral interferences. Used in ICP–MS.

**Sampling cone:** A metal cone (usually nickel-, aluminum-, or platinum-tipped) with a small opening, through which ionized sample material flows after leaving the plasma in ICP–MS.

**Sequential:** A type of detector configuration for AES or MS in which discrete emission lines or isotopic peaks are observed by scanning or hopping across the spectral range by means of a monochromator or scanning mass spectrometer.

**Simultaneous:** A type of detector configuration for AES or MS in which all selected emission lines or isotopic peaks are observed at the same time by using a polychromator or simultaneous mass spectrometer, offering increased analysis speed for analyses of multi-element samples.

**Skimmer cone:** A metal cone through which ionized sample flows after leaving the sampling cone and before entering the high-vacuum region of an ICP–MS.

**Standard additions:** A method used to determine the actual analyte concentration in a sample when viscosity or matrix effects might cause erroneous results.

**Torch:** A series of three concentric tubes, usually manufactured from quartz, in which the ICP is formed.

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# ⟨1735⟩ X-RAY FLUORESCENCE SPECTROMETRY—THEORY AND PRACTICE

## OUTLINE

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6. *Qualitative XRF Analysis*
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## 1. INTRODUCTION

This general chapter provides information regarding the theory and acceptable practices for the consistent analysis and interpretation of X-ray fluorescence spectroscopic data. X-ray fluorescence (XRF) spectrometry is an instrumental technique based on the measurement of characteristic X-ray photons caused by the excitation of atomic inner-shell electrons by a primary X-ray source. The XRF technique can be used for both qualitative and quantitative analysis of liquids, powders, and solid materials. Although some vendors supply radioactive isotope-based source instruments, nearly all modern instruments use an X-ray tube as the source.

## 2. PRINCIPLES OF X-RAY FLUORESCENCE SPECTROMETRY

The X-rays produced by an X-ray tube include characteristic lines corresponding to the anode material, and a continuum also known as Bremsstrahlung radiation. Both types of X-rays can be used to excite atoms in a specimen and thus induce X-rays. The intensity of both of these types can be adjusted by the voltage and current settings of the X-ray generator. These parameters can be adjusted to optimize the flux of X-ray photons for each element of interest. Further adjustments, such as the addition of filters in the primary beam, can be used to remove undesirable and potentially interfering tube spectral lines. Secondary targets may be used to produce an excitation X-ray beam that differs from the primary X-ray tube spectrum, with the aim of achieving optimum excitation conditions. Numerous instrument designs with different geometrical configurations, for example, secondary filters and polarization, are used to optimize X-ray detection and reduce background contributions.

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Although many variations exist, XRF instrumentation can be divided into two categories: wavelength-dispersive XRF (WDXRF) and energy-dispersive XRF (EDXRF) instruments. The main distinguishing factor between these two technologies is the method used to separate the spectrum emitted by all atoms in the sample according to X-ray photon energy.

The *energy* (or the *wavelength*) of the X-ray photon is characteristic of a given electron transition in an atom, and is therefore *qualitative* in nature. The *intensity* of the emitted radiation is indicative of the number of atoms in the sample, and therefore constitutes the *quantitative* nature of the technique.

# 3. PHYSICS OF X-RAY EXCITATION AND EMISSION

## 3.1 Ionization and the Photo-Electric Effect

The emission of characteristic X-ray radiation results from an electron transition between two inner shells of an atom, after ionization. After, for example, the ejection of an electron from the K-shell, the atom is ionized and the ion is left in a high-energy state, $E_1$, with $E_1$ being equal to the energy required to remove the K-electron from the shell to a situation where it is at rest (no remaining kinetic energy) at infinite distance from the nucleus. This interaction between electromagnetic radiation (a photon) and an atom resulting in an electron being excited is called the *photo-electric effect*. It is typically the largest contribution to absorption of X-rays. $E_1$ thus corresponds to the binding energy of the electron, which is identical to the energy of the atomic level. The excited state has a very limited life span, and will decay rapidly by a transition of an electron from an outer shell to the vacancy in the inner shell. The atom is still ionized, with an electron vacancy in the other shell. Let the energy state after this transition be represented by $E_2$, corresponding to the binding energy of the electron prior to the transition. The energy difference, $\Delta E$, is represented by

$$\Delta E = E_1 - E_2 \text{ [1]}$$

and can be released in two competing processes: the Auger effect, or the emission of a photon. For XRF, obviously the latter is of interest. The energy of the photon emitted is thus equal to $\Delta E$. The binding energies of the inner electrons are not affected by the chemical state of the atom concerned. The atomic energy levels are unique (characteristic) to the element, thus the energy difference between two given levels is also a characteristic. Therefore, the resulting X-ray photon is called a *characteristic* photon. The energy of the characteristic photons is varying in a systematic way with the atomic number $Z$ of the elements, a fact that can be represented using Moseley's law [*1*]:

$$E_{photon} = k(Z - s)^2 \text{ [2]}$$

where $k$ is a constant for a given series (as defined by the shell with the initial electron vacancy) and $s$ is the screening constant. A fairly complete tabulation of the energies of characteristic radiation can be found in the work by Bearden [*2*]. Assume that the initial vacancy was created in the K-shell. After an electron transition from, for example, the L3-shell, the atom still has an electron vacancy, but in the L3-shell, while a photon with an energy $E$ corresponding to

$$E_{photon} = E_k - E_{L3} \text{ [3]}$$

has escaped the atom. The vacancy in the L3-shell can subsequently be filled by an electron from the M5-shell (assuming that this is a high-atomic-number element). This results in a vacancy in the M5-shell that can then decay further. At the end of this cascade, the ion is back in the neutral state.

## 3.2 Scattering

The most important interaction between X-rays and matter is the photo-electric effect, described above. Two other mechanisms are coherent and incoherent scatter.

### 3.2.1 COHERENT SCATTER

Coherent scatter is elastic scattering of electromagnetic radiation by a free, charged particle. It is also known as Thomson scattering. The electric field of the incident photon accelerates the charged particle. This particle will subsequently emit radiation with the same energy as the incident photon, but travelling in a different direction.

### 3.2.2 INCOHERENT SCATTER

Incoherent scatter of a photon is usually called Compton scatter. Compton scatter is inelastic in nature. Both momentum and energy are conserved in the process. After Compton scattering, the electron has acquired considerable momentum (and is excited from the atom), and a photon with a longer wavelength than the incident photon is emitted (see *Figure 1*). The conservation of energy and of momentum leads to a simple relationship between the scattering angle $\Phi$ and the wavelength difference $\Delta\lambda$ between the incident and the Compton scattered photon:

$$\Delta\lambda = 0.00243 \cdot (1 - \cos\Phi) \text{ [4]}$$

*Equation [4]* calculates the wavelength shift in nm. The peaks of Compton scattered radiation are typically broader than those corresponding to characteristic radiation or coherently scattered radiation. The reason is that most X-ray fluorescence

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spectrometers do not have a collimated incident beam. The incident angle on the specimen can thus vary significantly. This contributes to wide variation in total scattering angles $\Phi$, leading to variation in $\Delta\lambda$.

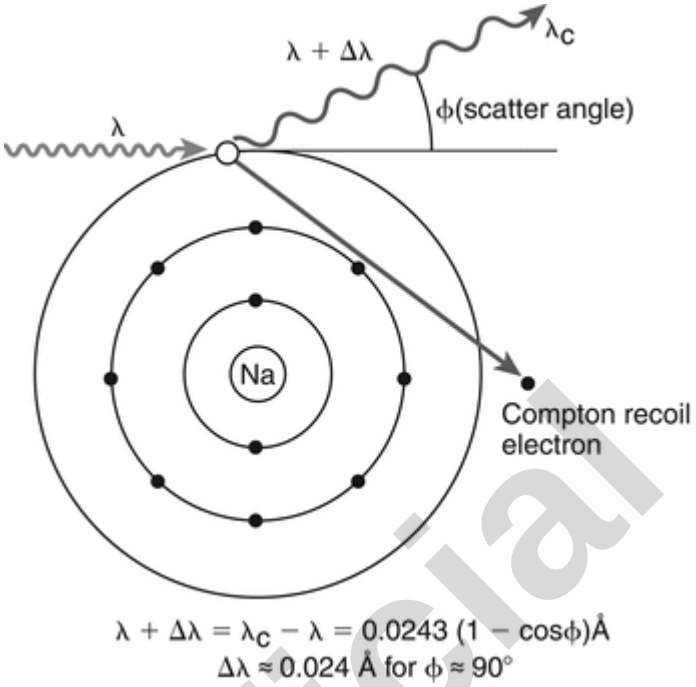


Figure 1. Compton scattering of an X-ray photon; $\Phi$ is the scattering angle (from Willis and Duncan [*3*] with permission).

## 3.3 Conversion between Energy and Wavelength

Photons exhibit a wave-particle duality [*4*]. The energy, *E,* of the "particle" and the wavelength $\lambda$ of the "wave" are related through Planck's constant, *h*:

$$E = \frac{hc}{\lambda} \text{ [5]}$$

where *E* is the photon energy; *h* is Planck's constant; and *c* is the speed of light in vacuum. By substituting these values in *Equation [5]*, and expressing photon energy *E* in keV and wavelength $\lambda$ in nm, the following is obtained:

$$E = \frac{1.24}{\lambda} \text{ [6]}$$

This allows quick and easy conversion between energy and wavelength scales. Note that *shorter* wavelengths correspond to *higher* energies.

Commonly, textbooks designed around WDXRF will use wavelength units for the characteristic radiation. On the other hand, when dealing with EDXRF, the scale of choice is an energy scale with keV as the unit. Photon energy can be converted to photon wavelength and vice versa using *Equation [6]*. The *frequency* of the electromagnetic radiation (which can be calculated using $c/\lambda$ or from $E/h$) is not used when relating to XRF analysis.

## 3.4 The Electromagnetic Spectrum

The X-ray range is typically the spectral range between 0.01 and 10 nm, and it spans 4 orders of magnitude. In practice, however, the range of X-rays commonly analyzed varies from about 0.04 to 1 nm.

The X-ray region of the electromagnetic spectrum can also be expressed in terms of the characteristic radiation of the elements. Most wavelength-dispersive X-ray spectrometers can be equipped to analyze characteristic lines between about 0.04 and 4.4 nm. This allows analysis of all elements in the periodic table from carbon upwards ($Z = 6$). With dedicated analyzers, the elemental range can be extended to include beryllium, although issues with limited specificity and sensitivity severely limit the practical applications for the quantitative analysis of beryllium.

## 3.5 Selection Rules

The transitions in the process described above are governed by quantum-mechanical selection rules. XRF radiation is observed with reasonable probability only from those transitions where

$$\Delta j = -1, 0, +1$$

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and

$$\Delta l = -1 \text{ or } +1$$

where *j* and *l* are the usual quantum numbers. The correspondence between shell designations (K, L, M…) and the quantum numbers *n*, *l*, and *m* is given in *Table 1*. This means that in transitions such as K ← L1 and L1 ← M1, both shells with *l* = 0 for initial and final state are forbidden radiative transitions. These transitions, however, can be accompanied by the emission of an Auger electron.

**Table 1. Correspondence between Shell Designations (K, L, M, N) and Quantum Numbers (*n*, *l*, *m*)**

| Shell | Quantum Numbers | | | Number of Orbitals | Subshell Designation | Number of Electrons to Fill Subshell | Total Number of Electrons in Shell |
|---|---|---|---|---|---|---|---|
| | *n* | *l* | *m* | | | | |
| K | 1 | 0 | 0 | 1 | 1s | 2 | 2 |
| L | 2 | 0 | 0 | 1 | 2s | 2 | 8 |
| | 2 | 1 | –1,0,1 | 3 | 2p | 6 | |
| M | 3 | 0 | 0 | 1 | 3s | 2 | 18 |
| | 3 | 1 | 1,0,–1 | 3 | 3p | 6 | |
| | 3 | 2 | –2,–1,0,1,2 | 5 | 3d | 10 | |
| N | 4 | 0 | 0 | 1 | 4s | 2 | 32 |
| | 4 | 1 | 1,0,–1 | 3 | 4p | 6 | |
| | 4 | 2 | –2,–1,0,1,2 | 5 | 4d | 10 | |
| | 4 | 3 | –3,–2,–1,0,1,2,3 | 7 | 4f | 14 | |

After the initial vacancy has been created, the ion is in a highly excited state. After an electron transition, for example, K ← L3, an energy equal to the difference in the binding energies (see *Equation [3]*) between the two shells involved can be released. In the case on hand, this gives rise to the emission of a Kα photon. Photon emission is, however, only one of two competing processes. The other is the emission of an Auger electron. This is the process in which the energy is transferred to another electron, which escapes the ion with a certain kinetic energy $E_{Kin}$. The kinetic energy can be calculated in much the same fashion as the photon energy, using the energy levels from the shells involved. If the energy released from the K ← L3 transition is transferred to an L1 electron, the kinetic energy can be calculated from

$$E_{Kin} = E_{Auger} = E_K - E_{L1} - E_{L3} \text{ [7]}$$

The kinetic energy of the Auger electron is characteristic because it is made up of characteristic quantities. The vacancies created after the emission of an Auger electron can subsequently decay, either by other Auger electrons or by photons.

## 3.6 Fluorescence Yield

The fluorescence yield (symbol $\omega$) is defined as the probability that a vacancy is filled through a radiative transition. For the K-shell this is simply

$$\omega_K = \frac{l_K}{n_K} \text{ [8]}$$

where $I_k$ is the total number of K-shell X-ray photons and $n_k$ is the number of vacancies created in the K-shell. For the L-shell and M-shell, a similar definition applies for each of the subshells, but the number of primary vacancies needs to be corrected for the cascade effects and the occurrence of Coster-Kronig transitions [*5*]. These are nonradiative transitions between subshells having the same principal quantum number. Data regarding values for fluorescence yields can be found in the work by Bambynek et al. [*5*] and Hubbell et al. [*6*].

## 3.7 Counting Statistical Error

The *counting statistical error* (CSE) is the uncertainty in the measurement of the number of photons, which is subject to a Poisson distribution. The distribution of the number of photons can be approximated by a normal distribution, because the number of photons counted is usually sufficiently large. The standard deviation $\sigma_N$ of an intensity measurement of *N* counts is given by the square root of the number of counts:

$$\sigma_N = \sqrt{N} \text{ [9]}$$

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Most measurements in XRF are now expressed as an intensity, *I*, which is simply the ratio of the number of photons counted divided by the measuring time, *t*. The counting statistical error on a measured intensity can be calculated using

$$\sigma_I = \sqrt{\frac{I}{t}} \text{ [10]}$$

This indicates that the counting statistical error on an intensity measurement becomes smaller with longer measurement times. Measuring longer on an XRF instrument (under otherwise constant conditions of measurement) reduces the relative uncertainty on the measurement. This is generally applicable until the resulting CSE becomes comparable to the *overall instrument error*. For high-end instrumentation, the order of magnitude of this overall instrument error is about 0.1% or better. Performing measurements with a CSE significantly lower than the overall instrumental error will not lead to more precise results. The CSE thus imposes a theoretical lower limit to the precision of an X-ray measurement. The contribution of the instrumental errors is added to the CSE to obtain the total error on the measurement.

The *intensity* of a photon beam is calculated from the number of photons collected divided by the time taken for the measurement, expressed in counts per second (cps) or kilocounts per second (kcps).

## 3.8 Detection Limit

The lower limit of detection (LLD) is defined as the concentration that will yield a positive intensity above background intensity with a given confidence level. For analysis near the detection limit, the intensity of the peak will be comparable to the intensity of the background. For example, a net signal that is 3 times larger than the CSE of the background will satisfy this criterion with 99.7% confidence. This intensity can then be converted to a concentration using the sensitivity, *S*, of the spectrometer for the analyte considered:

$$LLD = \frac{k \times CSE}{S} \text{ [11]}$$

where *k* is a factor depending upon the confidence level chosen, *S* is the sensitivity (expressed as net intensity per unit concentration), and *CSE* is the counting statistical error of the determination. Substituting *Equation [10]* where $\sigma_I$ is the CSE of the intensity of the background yields

$$LLD = \frac{k}{S}\sqrt{\frac{I_B}{t}} \text{ [12]}$$

where $I_B$ is the intensity of the background radiation at the analytical line considered. The LLD can be improved by the following:

- Increasing the sensitivity, *S*, of the spectrometer
- Decreasing the intensity of the background, $I_B$
- Increasing the measurement time, *t*.

The third method, increasing the measurement time, seems to be an especially easy way to improve detection limits, but it is limited because long counting times can make the method impractical. Hence, for a given spectrometer configuration, any increase in the sensitivity results in a similar increase of the background intensity. Quadrupling the sensitivity also leads to a 4-fold higher background intensity and an improvement of the LLD by a factor of 2.

## 3.9 Nomenclature of X-Ray Emission Lines

There are different nomenclatures in use involving the designation of the X-ray emission lines. The International Union of Pure and Applied Chemistry (IUPAC) published a systematic notation for X-ray emission lines and absorption edges, based on the energy-level designation. In practice and in many publications, Siegbahn's notation is still dominant. For the most important characteristic lines, the correspondence between Siegbahn and the IUPAC notation is given in *Table 2*.

**Table 2. Correspondence between Siegbahn and IUPAC Notation for the Most Important Characteristic Lines**

| K-series | | M-series | | L-series | |
|---|---|---|---|---|---|
| **Siegbahn** | **IUPAC** | **Siegbahn** | **IUPAC** | **Siegbahn** | **IUPAC** |
| $K\alpha_{1,2}$ or Kα | $K–L_{2,3}$ | $L\alpha_1$ | $L_3–M_5$ | $M\alpha_{1,2}$ | $M_5–N_{6,7}$ |
| $K\alpha_1$ | $K–L_3$ | $L\alpha_2$ | $L_3–M_4$ | Mβ | $M_4–N_6$ |
| $K\alpha_2$ | $K–L_2$ | $L\beta_1$ | $L_2–M_4$ | | |
| $K\beta_1$ | $K–M_3$ | $L\beta_2$ | $L_3–N_5$ | | |
| $K\beta_{1,3}$ | $K–M_{2,3}$ | $L\gamma_1$ | $L_2–N_4$ | | |
| $K\beta_2$ | $K–N_{2,3}$ | Lη | $L_2–M_1$ | | |
| | | Lι | $L_3–M_1$ | | |

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# 4. SAMPLE PREPARATION

With XRF it is possible to analyze most materials, irrespective of their physical state—whether they are liquids, powders, or solids—with little or no additional sample preparation. A significant advantage is the sample-size capacity: the XRF technique can accommodate large sample masses (usually in the gram weight range), thereby minimizing analytical sampling errors. This enhances the degree to which the sample presented to the spectrometer is representative of the bulk material submitted for analysis. A primary concern is that the final sample surface is flat when it is placed into the measuring position.

Measurements can be performed in a variety of environmental conditions including air, nitrogen, helium, and vacuum. Most X-ray spectrometers typically use vacuum as the medium of analysis, with the exception of some bench-top instruments. Liquid samples are incompatible with vacuum environments, so helium, air, or nitrogen at near-ambient pressure is used. Air and nitrogen readily absorb low-energy X-rays; therefore, their use is generally limited to the analysis of high-energy X-rays. Liquid and powder samples are commonly analyzed in helium, as this significantly lowers the absorption of the characteristic radiation from the specimen, compared with the effects of air or nitrogen.

## 4.1 Liquid Samples

Liquid samples need to be placed into disposable sample cups before being introduced to the spectrometer. The sample cup should be constructed with an appropriate supporting film known to be free of contaminant elements. Suitable films, such as polyester or polypropylene, may have a thickness as low as 1.5 µm. It is also important that the disposable cup is an appropriate size so that it is not viewed by the spectrometer; this ensures that the signals measured are coming from the sample only, and not from the sample cup.

Furthermore, it is important to understand the relationships among the sample matrix, elements of interest, and concept of infinite thickness. In general, when analyzing samples composed of low-atomic-number elements (e.g., organic matrices), it is important to use a thicker sample than when analyzing for the same elements in a heavier (e.g., metallic) matrix. This ensures that the intensity of the X-rays produced (and detected) is only dependent upon the specimen's composition and not also on the quantity of mass analyzed. The thickness at which the intensities are no longer dependent upon the thickness of the specimen presented to the spectrometer is called the *critical thickness* (also known as *infinite thickness*). The critical thickness depends upon the energies of the emission lines considered, the sample matrix, and—to a lesser degree—also on the excitation conditions. In many cases, it may not be possible to obtain infinitely thick samples because of lack of sufficient material or because the critical thickness may exceed sample cup depth; the latter can happen when analyzing liquids that are mainly composed of water or organic compounds. In these cases, correction procedures are applicable. In many cases, a simple modification to the analytical procedure—ensuring that all samples analyzed are of *constant* mass—is sufficient to avoid problems relating to non-infinite thickness.

An additional consideration is variation of the density between samples and standards. The analysis of samples that do not satisfy the requirement of critical thickness will be further complicated by limitations imposed by the geometry of the instrument. The X-rays incident on the sample and the X-rays emitted from the sample form a complex shape in three dimensions that is essentially a cone. The geometry is referred to as a *wedge*. This geometry limits the usefulness of traditional matrix correction because these conventional methods assume a uniform X-ray sampling volume, as opposed to a cone. In most cases, the difference between a cone-shaped geometry and the simpler geometry model is insignificant. This effect is more pronounced when measuring heavy elements in light matrix samples (such as organics). In these cases, it can have severe effects on the calculated concentrations if it is not considered. However, it is possible to include a geometric model of the wedge with a matrix correction technique referred to as the *wedge effect correction* [*7*].

An alternative solution is to have an infinitely thin layer of sample, thereby removing any possible sample interference (matrix effects), as well as the geometrical effect described above. This is not always practical, and in practice it can be difficult to prepare reproducible samples and standards.

## 4.2 Powder Samples

To ensure infinite sample thickness, loose powder samples may be simply weighed and placed directly into a disposable sample cup as described above. For reasons described below, it is often advantageous to lightly pack the powder, using constant pressure and a clean tool. Variations in sample compaction due to manual pressing can be compensated for by using the Compton corrections, which can account for small variations in sample thickness and/or density. These powders can also be ground to a finer particle size (if required) and then pressed to produce a solid pellet.

Preferably, all non-homogenous solid samples, such as coated tablets, should be ground using a grinding device. Similarly, any homogenous and flat sample that has too small an area to cover the instrument optical aperture should also be ground. For softer materials, a pestle and mortar may be sufficient, and for other materials swing mills, ball mills, and/or cryogenic grinding mills may be required. The analyst should ensure that the grinding means do not contribute traces of the analyte elements; this can be ascertained easily by grinding/milling pure compounds and comparing the spectra. By convention, the maximum powder (particle) size should be 50 µm or less. It is, however, more important to have a consistent particle size for both standards and samples. This ensures the homogeneity of the sample and provides an accurate representation of the entire sample, not just the near-surface layer. It is then possible to load a sample into a disposable sample cup, or to press the sample into a pellet. The disadvantage of pressing a sample into a pellet is that it may be necessary to blend a binder (e.g., wax or cellulose) into the sample. The use of such a material must be considered as a possible source of contamination. A number of different binders are available to improve the mechanical properties of the pellet. An advantage of pressing a sample is that it removes air voids, which can absorb X-rays, and thus after pressing, the measurements typically show enhanced light element reproducibility.

Furthermore, it is possible to directly measure a pressed pellet without a supporting film. It is important to note that supporting films can result in signal attenuation, depending upon the element of interest. This effect is commonly seen with

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lighter elements such as sodium (Na), magnesium (Mg), and aluminum (Al). The other key advantage is that the pellet ensures a flat surface that is available for reproducible excitation from all angles. In addition, pelletized samples can be measured in vacuum conditions, and this reduces any photon absorption by air or any other gas such as helium (He). Some materials such as lactose and cellulose are self-binding, thus there are no deleterious considerations when preparing a pellet except for the additional time spent on sample preparation. It is important to note that standards and samples must be treated the same from sample preparation through analysis.

Because the majority of pharmaceutical materials have an organic matrix, it is recommended to use a quantity of material corresponding to a thickness of about 1.5 cm. This can be used for both liquids and loose powder samples (provided all samples and standards have a similar matrix composition). Furthermore, any minor sample thickness variations can be compensated for by Compton ratio corrections.

## 5. INSTRUMENTATION

All spectrometers include a source (e.g., X-ray tube), some kind of source filtration mechanism, a sample chamber, and a detector. In addition, wavelength-dispersive systems have a set of collimators and an analyzing crystal. These components, and a few optional items, are discussed below and are further divided according to type of instrument. A diagram of a wavelength-dispersive spectrometer is shown in *Figure 2*. An energy-dispersive spectrometer is simpler in that the collimators and the crystal shown in *Figure 2* are not present, and the detector is pointed directly at the sample.

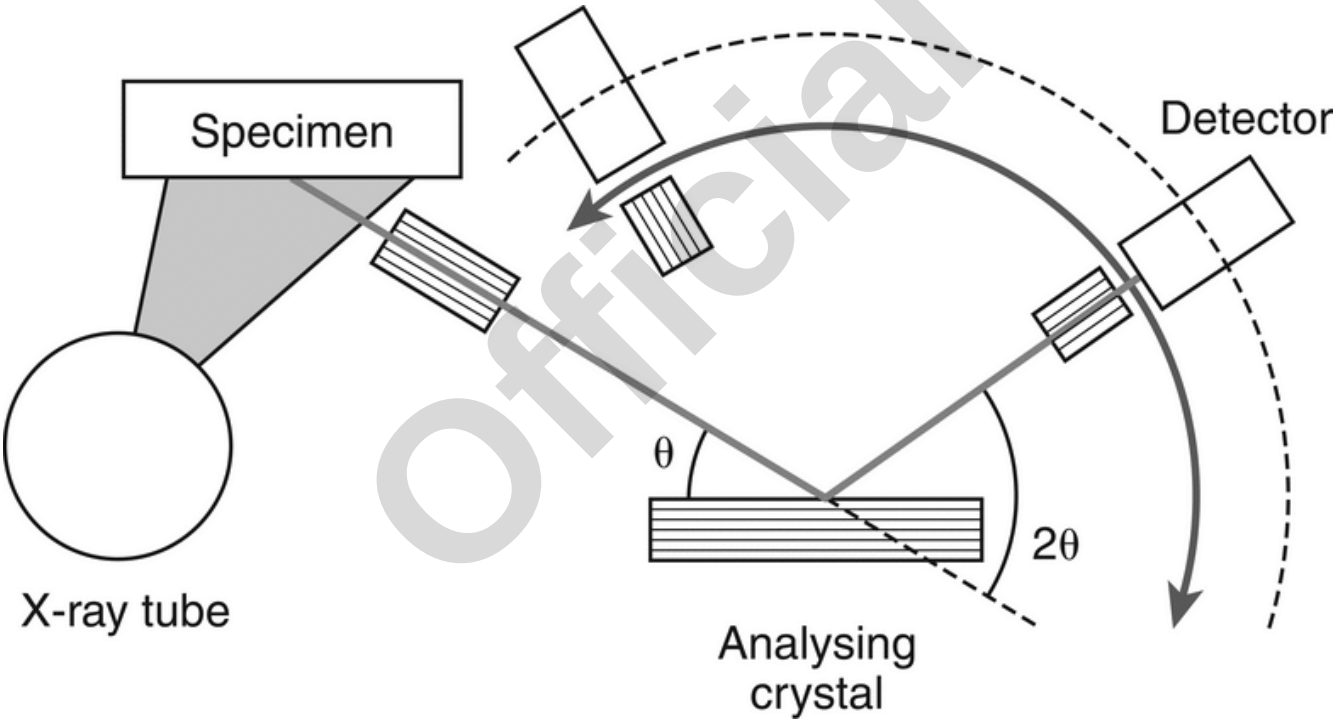


Figure 2. Schematic diagram of a wavelength-dispersive XRF spectrometer, indicating the main components.

### 5.1 X-Ray Tube

The two most important components of an X-ray tube are the filament (used as a cathode) and the anode. The filament is the source of electrons, which are accelerated to the anode material by a strong electric field between filament and anode. Upon impact with the anode, the electrons can gradually lose their kinetic energy, generating heat and a continuous spectrum of X-ray radiation in the process. Alternatively, when the electrons ionize atoms from the anode, the resulting vacancy can decay by emitting characteristic X-ray radiation. The total spectrum of an X-ray tube thus consists of the characteristic lines of the anode element (which is discrete in nature) superimposed on a continuum. Note that the continuum radiation, scattered by the specimen, is the main source of the background observed in XRF analysis.

### 5.2 Primary Beam Filter

Most X-ray spectrometers are equipped with one or more programmable beam filters. These filters are thin metal foils (typically between 50 and 1,000 microns in thickness) that are mounted on a mechanical device that can move a particular filter in the path of the primary beam. The main purpose of the primary beam is to eliminate the characteristic lines of the X-ray tube anode. If for example a rhodium anode tube is used, a significant part of the incident rhodium X-rays will be scattered by the sample. The fraction that is coherently scattered cannot be distinguished from rhodium lines that are created by atoms in the sample. In such a case, to allow for analysis of rhodium in the sample, the primary beam filter is used to prevent

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characteristic lines from the X-ray tube from reaching the sample. Another application of beam filters is the reduction of background in certain energy ranges. The lower limit of detection can be improved in cases where the background intensity is significant. However, the use of primary beam filters also leads to a degradation of sensitivity, and care must be taken to achieve optimum setup conditions.

## 5.3 Wavelength-Dispersive X-Ray Fluorescence Spectrometry

### 5.3.1 PRINCIPLE OF OPERATION—BRAGG'S LAW

As the name implies, the wavelength-dispersive spectrometers employ an X-ray *monochromatizing* strategy, using *Bragg's Law of Diffraction*, to split the X-ray spectrum into its individual components (or rather, narrow energy bands):

$$n\lambda = 2d\sin\theta \text{ [13]}$$

where $n$ is the order of reflection; $\lambda$ is the wavelength of the photons considered; $d$ is the spacing of the crystallographic planes, and $\theta$ is the angle of incidence (and reflection) with respect to the crystal's plane. In the vast majority of applications, the first order ($n = 1$) is used. For diffraction of photons with wavelength $\lambda$ to occur, the angle $\theta$ must be selected such that the product of $2d\sin\theta$ equals $\lambda$. Bragg's Law can be viewed as the requirement for positive interference of X-ray photons. Consider a monochromatic and parallel beam of X-rays of a specific wavelength $\lambda$ with electrical vectors of equal amplitude in phase along any point of the direction of propagation. This beam is incident on a crystal at an angle $\theta$. The beam is scattered in all directions. However, constructive interference only occurs in those directions for which the phase relationship is conserved. This happens at an angle $\theta$ for incident rays 1 and 2 (see *Figure 3*), for which the path difference $AB + BC$ is equal to an integer number of wavelengths $\lambda$. The integer $n$ refers to the order of the diffraction. The most intense diffraction peaks—and thus the highest measured intensity—can be obtained when $n = 1$ (first order). Higher-order diffractions with $n = 2$, 3, or 4 do occur, but the efficiency is low.

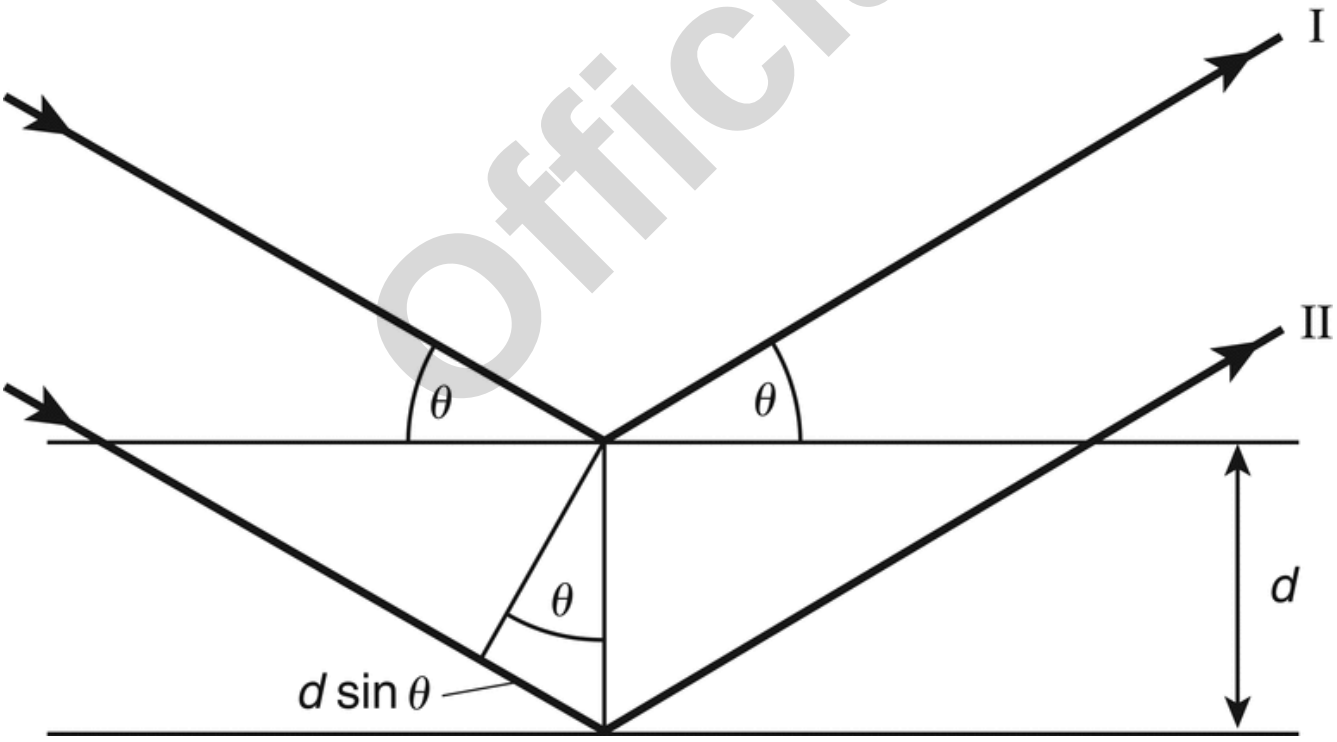


Figure 3. Geometry involving Bragg's law.

### 5.3.2 DISPERSION

The *dispersion* of a WDXRF instrument can be calculated by differentiating *Equation [13]* as follows:

$$\frac{d\theta}{d\lambda} = \frac{n}{2d\cos\theta} \text{ [14]}$$

Dispersion is a measure of the angular separation between peaks corresponding to two different wavelengths. A larger value for the dispersion indicates a larger angular separation, thus a smaller (potential) overlap between photons of slightly different wavelengths. Decreasing the $2d$ spacing is an obvious way to improve on angular dispersion; the $2d$ spacing is, however, determined by the crystal used. For most of the wavelength range, there are at least two crystals available; the one with the

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smaller spacing offers the better angular separation, but typically this involves a loss of intensity. Well-defined crystals with known *2d* spacing are used as monochromators. A list of commonly used crystals appears in *Table 3*.

**Table 3. Commonly Used Monochromators in WDXRF**

| Crystal | Name | Reflection Plane | *2d* Spacing (nm) | Element Range |
|---|---|---|---|---|
| LiF(220) | Lithium fluoride | (220) | 0.2848 | V-U |
| LiF(200) | Lithium fluoride | (200) | 0.4028 | K-U |
| Ge | Germanium | (111) | 0.6532 | P, S, Cl |
| PE or PET | Pentaerythritol | (002) | 0.8742 | Cl-Al |
| TlAP | Thallium acid phthalate | (1010) | 2.575 | Mg-O |
| | Layered synthetic microstructure | | 3–12 | Mg-Be |

*Dispersion* only describes the *angular separation* between two peaks; it does not describe the width of the peak, which is typically referred to as resolution (in other forms of spectrometry) and is calculated from the full width of the peak at half height. The actual resolution from the detector system has no discernible influence on peak separation.

The choice of crystal and angle of the detector relative to the crystal determine which elemental X-ray lines may enter the detector. The main task of the detectors in WDXRF is reduced to counting photons with a known energy, as the use of the monochromator crystal thus separates the selection of the wavelength of the photons from the actual counting.

### 5.3.3 DETECTORS FOR WDXRF

**Gas-filled detectors:** Gas-filled detectors consist of a cylindrical volume filled with a noble gas such as argon (Ar) or krypton (Kr), and a wire running through the center of the detector (see *Figure 4*).

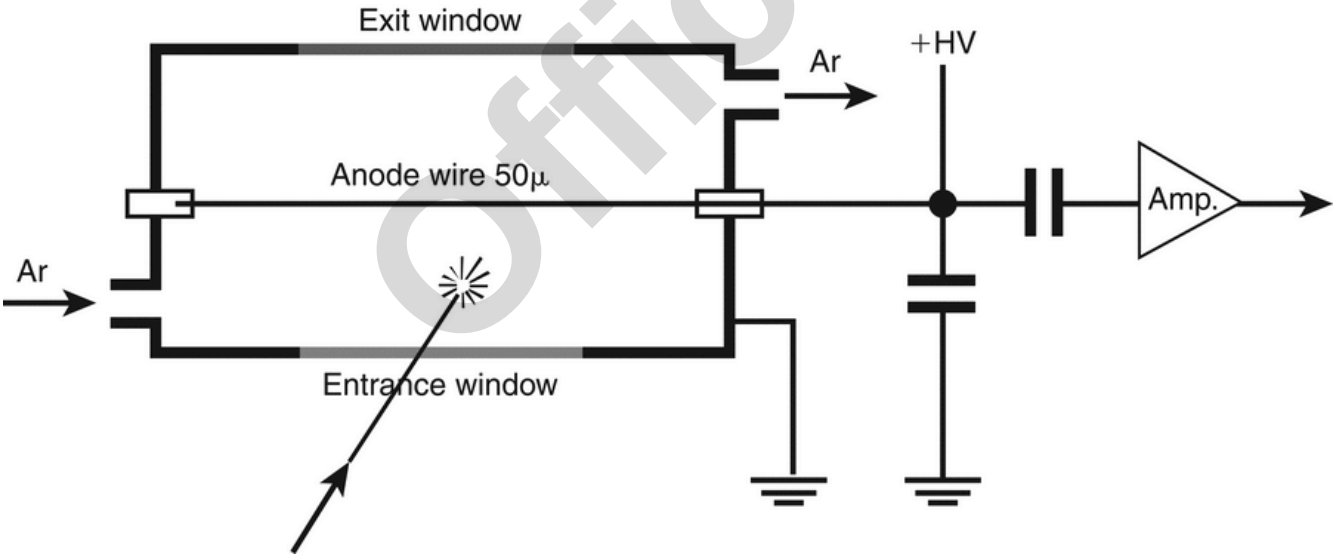


Figure 4. Schematic diagram of a flow counter.

An incoming photon (represented by its energy $h\nu$) can remove an outer (valence) electron of one of the inert gas atoms. For an argon-filled detector:

$$Ar \xrightarrow{h\nu} Ar^{+} + e^{-} \text{ [15]}$$

The energy required for the ionization, $e_i$, depends on the element; for argon (Ar), about 26 eV is required to create an ion-electron pair. The total number of such primary ionizations, $n_p$, caused per incident photon with energy, $E$, can then be calculated using

$$n_p = \frac{E}{e_i} \text{ [16]}$$

The electric field between the wire and the outer body will cause the electrons to accelerate toward the anode wire. The positive ions, on the other hand, will migrate toward the housing. The resulting electric field gives rise to a cascade of ionization events. The result of this cascade effect is as follows: for each of the individual electrons originally created by the incident photon,

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many more electrons are finally collected at the anode wire (this is called the *gas amplification factor* and is typically around $10^4$ to $10^6$), creating a voltage drop that is then processed by the counting electronics.

The entrance window should absorb as few of the incoming photons as possible, because any photons absorbed into the window are lost from the counting circuit, thus reducing the sensitivity. Large windows with low attenuation for long wavelengths can be made from stretched polymers, such as polyethylene or polyester, coated with a conductive material at the interior of the counter to provide for a homogeneous electric field. Such thin windows need a mechanical support to withstand the pressure difference caused by the vacuum in the spectrometer chamber. The collimator is usually preferred as a support because it will also reduce the amount of radiation that is scattered from the crystal or the multilayer.

On the other hand, the polymer windows have small pinholes. These pinholes allow the counting gas to gradually leak away. Such detectors would have only a very short lifetime before the counting gas leaked away or became so contaminated that it would effectively render the detector useless. To compensate for the leaks, a constant flow of counting gas is used, the excess of which is vented away out of the spectrometer chamber; such detectors are therefore called *flow proportional counters* (or gas flow detectors). Only a fraction of this flow is leaking into the spectrometer chamber, whereas the remainder is leaving the detector through a tube and is vented into the atmosphere. It is essential for the overall stability of the spectrometer that the pressure and temperature of the gas are kept constant. Hence, most instruments are equipped with a gas-density stabilizer.

Gas flow proportional counters are the preferred counters for the detection of X-rays with very long wavelengths in wavelength-dispersive spectrometers. The counting efficiency of the gas-filled detectors is determined by the absorption in the entrance window and the capture efficiency (absorption of the incident radiation) of the counting gas for the wavelengths of interest. The capture efficiency depends on the path length available within the detector, the composition of the counting gas, and its pressure. The absorption properties of the window determine the efficiency of the detector at the long wavelengths, whereas the absorption properties of the gas determine the efficiency at the shorter wavelengths. Considering the typical dimensions, the nature of the gas, and the pressure, the absorption of the gas as function of the wavelength of the incident photon can be calculated. Around wavelengths of 0.2–0.3 nm, about half of the incident photons are not absorbed, reflecting a low efficiency. This is the main reason why sequential WDXRF instrumentation is equipped with more than one detector.

**Scintillation counter:** WDXRF instruments are generally fitted with a scintillation counter for the detection of shorter wavelengths for which the gas proportional counter is less efficient. The operation of the scintillation counter is based on two distinct stages: a scintillation crystal and a photo-multiplier tube (see *Figure 5*).

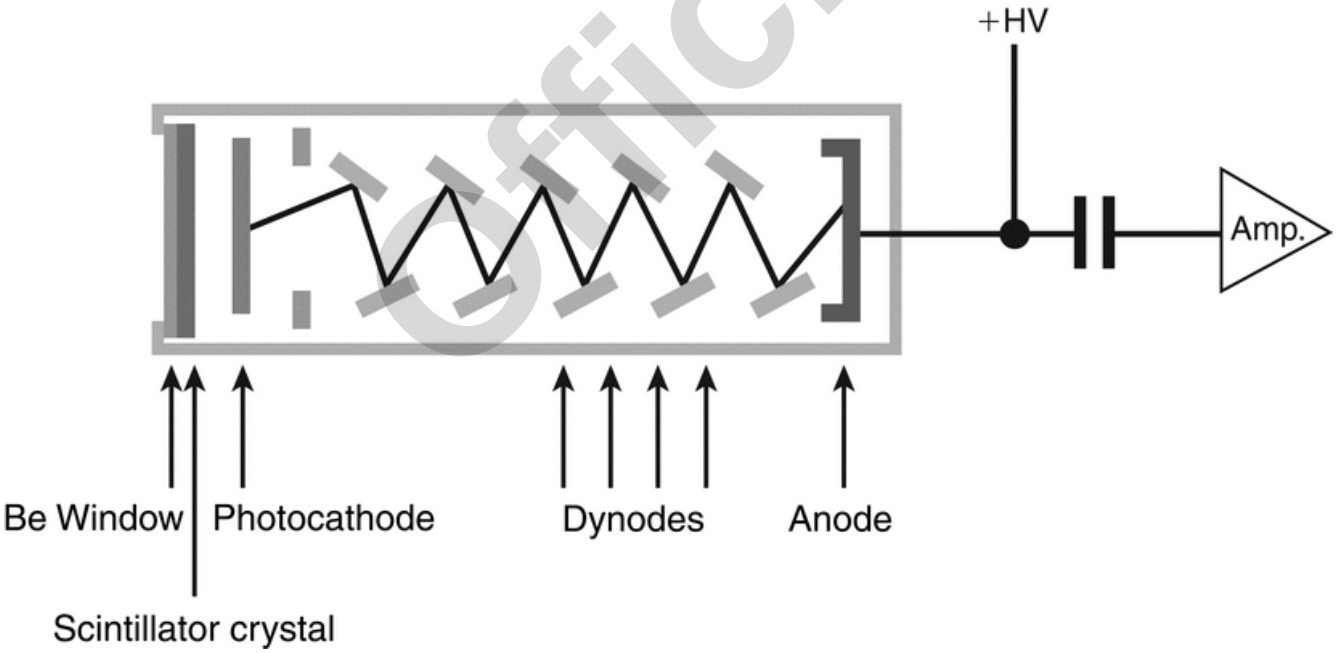


Figure 5. Schematic diagram of a scintillation counter.

In the first stage, the incoming photon is absorbed by a “scintillator crystal”. A scintillator is a material that emits light when it is excited by ionizing radiation, such as X-rays. Many different scintillator materials exist, with different properties regarding radiation hardness, operating temperature range, decay times, optical properties, and other properties. The most commonly used scintillator material in WDXRF instruments is a sodium iodide (NaI) crystal, doped with thallium (Tl). The scintillating crystal is optically coupled to a “photo-multiplier”, which consists of a photo-cathode and a series of dynodes. In the second stage, upon impact by a photon, the photo-cathode releases free electrons. These are then accelerated toward a series of dynodes. At each dynode, the impact of the electrons generates more electrons. If 10 dynodes are used, the total amplification provided is about a factor of $10^6$.

The counting efficiency of the scintillation counter is largely determined by the thickness of the sodium iodide thallium [NaI(Tl)] crystal. The counting efficiency for a 3-mm-thick crystal is better than 99% for wavelengths of 0.05 nm and longer. This allows the detection of high-energy photons such as Sn Kα with high efficiency.

The production of light photons is higher in the scintillation detector than the number of electron-ion pairs created in a flow counter; this should lead to a better resolution. However, this advantage is cancelled out by the low yield of photo-electrons

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by the photo-cathode, where less than 1 electron is freed for every 10 incident light photons. Compared to the gas-filled counters, the resolution of a scintillation counter is worse.

The use of the WDXRF technique (typically with power in the kW range) for organic matrix sample analysis is limited because heat generated by the X-rays may be sufficient to induce significant specimen alteration, such as browning or burning of the sample surface. Furthermore, even if the sample is measured for a very short time by WDXRF, the heat imparted to the sample surface may induce the loss of volatile elements [e.g., mercury (Hg) and selenium (Se)]. The WDXRF technique is, however, perfectly suited to measure inorganic materials that can withstand elevated temperatures. Examples of common inorganic materials that are suitable for WDXRF measurement are calcium carbonate ($CaCO_3$) and sodium chloride (NaCl).

## 5.4 Energy-Dispersive X-Ray Fluorescence Spectrometry

### 5.4.1 PRINCIPLE OF OPERATION

The energy-dispersive XRF technique typically involves simultaneous detection of photons of different energies that are emitted by the atoms in the sample. The detector determines both the photon's energy and the intensity, which is the number of photons per unit of time. To provide adequate resolution for distinguishing between characteristic lines and overlapping lines from other elements, most EDXRF instruments are equipped with a high-resolution detector and a multi-channel analyzer (MCA). The MCA essentially provides a histogram representation of the spectrum. High-end instruments use sophisticated calculation (deconvolution) algorithms to identify the peaks (i.e., to establish the presence of certain elements) and to obtain intensity data from the histogram. EDXRF is theoretically a simultaneous technique because it measures the complete spectrum in a single counting phase. However, significant improvements in performance can be achieved by measuring the sample using two or more different excitation conditions, each optimized for a certain energy range.

Compared to WDXRF, the design of an EDXRF spectrometer is much simpler because it lacks a high-precision goniometer and the associated collimators. The positioning of the detector relative to the sample is very simple; the most obvious requirement is that there should not be a direct path between the anode of the X-ray tube through the entrance window of the detector onto the active body. Distances between the X-ray tube window and specimen, and between the specimen and detector entrance window, can be small, in the order of 1–2 cm. Furthermore, as only limited collimation is required, the angle of acceptance of the photons from the specimen can be quite large. Low-power X-ray tubes with ratings up to 50 W are used. Because of the inherent simplicity of their design, wide variation of systems with varying capabilities exists. A typical spectrum on an energy-dispersive spectrometer is shown in *Figure 6*. The energy on the horizontal axis increases from left to right. The peak shape is nearly perfect Gaussian.

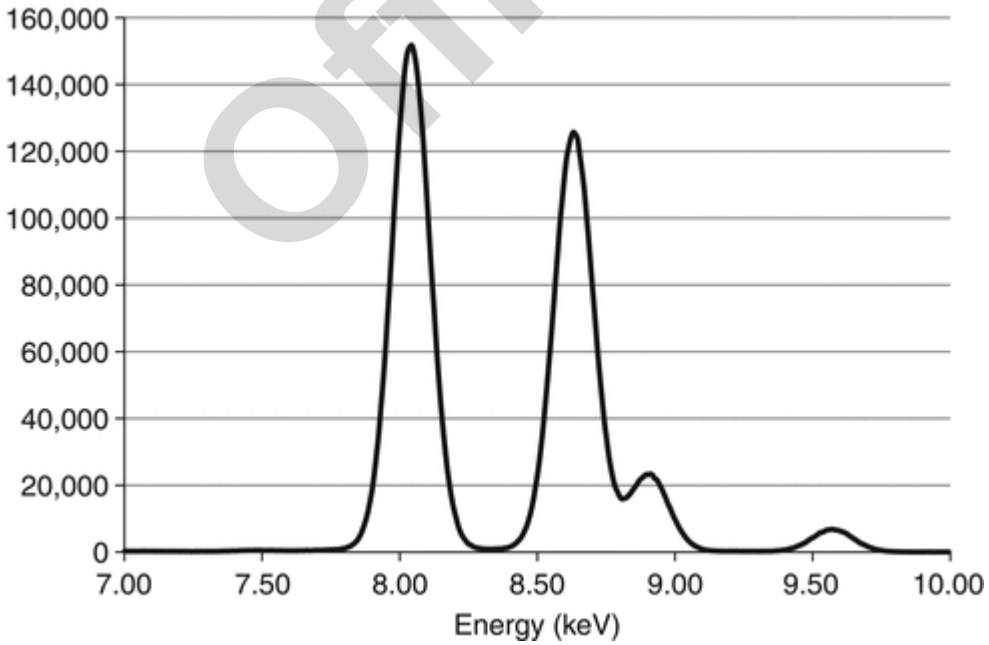


Figure 6. Energy-dispersive spectrum of a brass specimen. From left, the peaks are due to Cu Kα, Zn Kα, Cu Kβ, and Zn Kβ. The horizontal axis is energy and the vertical axis is in counts per channel. This spectrum was recorded at 75,000 cps with 50 kV and an Ag primary filter to reduce the background. The full width at half maximum (FWHM) is about 165 eV at the Cu Kα peak.

### 5.4.2 DETECTORS

The resolution of the detector system determines both the peak width (expressed as FWHM) and the peak separation. Systems may be equipped with gas-filled, solid-state, or semi-conductor detectors. The three most common solid-state detectors used are the following:

- Liquid nitrogen-cooled silicon-lithium drifted Si(Li)

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- Liquid nitrogen-cooled germanium detectors, such as the LeGe and HPGe
- Detectors based on intrinsic silicon, such as the PIN (positive, intrinsic, and negative) detector and the SDD (silicon drift detector).

The first two types of detectors allow for high photon-capture efficiency but require relatively bulky, well-insulated containers (Dewar) for the liquid nitrogen. These detectors are generally rather large, which improves sensitivity, and thick, which improves capture efficiency for high-energy photons. They are typically found on floor-standing equipment. Bench-top systems are equipped with detectors that are based on intrinsic silicon. These types of detectors do not require cooling to temperatures as low as the other two categories; temperatures between –25° and –15° are acceptable. Peltier-cooling or electro-thermal cooling is sufficient to reach those temperatures. The detectors are smaller and thinner than their bulk siblings. Older equipment might still be using so-called PIN detectors, but all of the newer equipment tends to be equipped with an SDD. The SDDs have much better resolution and can be used at much higher intensities than the PIN detectors, which are typically limited to about 220 eV at Mn Kα. The SDDs combined with state-of-the-art electronics have a resolution (expressed as FWHM) of better than 145 eV at Mn Kα. This is comparable to liquid nitrogen-cooled semi-conductor detectors. A word of caution is required as the resolution worsens with increasing count rate or intensity. Most manufacturers therefore specify resolution at an impractical, low count rate of 1,000 or 2,000 cps.

## 5.5 Polarized X-Ray Spectrometry

### 5.5.1 PRINCIPLE OF OPERATION

The most elaborate EDXRF spectrometers use polarization or a secondary target to modify the spectral distribution of the X-ray source; the aim is to reduce, or ideally eliminate, the intensity of the continuum background. This effort is a logical consequence of *Equation [3]*. Due to the fact that the major component of the background is scattered tube radiation, one method of preventing radiation from the X-ray tube from reaching the detector system is the use of polarized radiation. These systems have applications in the analysis of, for example, residues of palladium (Pd) catalyst in some pharmaceutical ingredients.

### 5.5.2 POLARIZED RADIATION AND POLARIZING TARGET

The orientation of the electric field vector, *E*, of the photons generated in an X-ray tube is random but always perpendicular to the direction of propagation. Using a Cartesian coordinate system, with the *z*-direction coinciding with the direction of propagation, the two components of the electric field vector in the *x*-direction (denoted by $E_x$) and *y*-direction ($E_y$) are identical and obey the following relationship:

$$E_x^2 = E_y^2 = \frac{1}{2}E^2 \quad [17]$$

The component along the axis of propagation, $E_z$ is zero; this is because electromagnetic radiation is a transverse wave. After *coherent scattering* in the *yz* plane, the field vector of photons traveling in the *y* direction, $E_y$, is also reduced to zero. This beam of photons, all with their electric field vector parallel to the *x*-direction, is directed at the sample and interacts in the usual way. The photons cause ionizations and can be scattered, but they cannot be scattered in the direction along the *x*-axis, because the electric field vector cannot have a component parallel to the direction of propagation. If the detector is now placed somewhere along the *x*-direction (still in the same frame of reference), the background is significantly reduced because the main contribution (i.e., scatter from the incident radiation) is absent. The process is inefficient because there is no active polarization; it is entirely achieved by suppressing one component of the electromagnetic wave, thereby significantly reducing its intensity. Therefore, high-power X-ray tubes are used.

### 5.5.3 SECONDARY TARGETS

The use of secondary targets in energy-dispersive spectrometry has been widespread for high-power instrumentation. The aim is to reduce the background, thus improving the lower limit of detection. When *electrons* decelerate, they generate Bremsstrahlung of a continuous nature; however, photons do not generate Bremsstrahlung. The principle behind an instrument equipped with secondary targets is to use a classic X-ray tube to provide a beam of X-rays (including the troublesome continuum) and direct it at a secondary target, which is typically a metal. The radiation emanating from the secondary target will consist of the very intense characteristic radiation corresponding to the nature of the target and scattered continuum. The intensity of the scattered background is relatively low, especially when the target material is a metal of medium-to-high atomic number. There is no additional continuum created by the incident photons, but a tiny contribution to background radiation is created by the high-energy photo-electrons. The radiation from the secondary target is directed at the sample for excitation purposes. The spectrum from the sample is composed of the characteristic lines from the elements present, the scattered characteristic radiation from the secondary target, and the concomitantly scattered continuum from the tube. The latter contribution is very weak because it has been subjected to two scattering events, one at the secondary target and another one at the sample. If the instrument is equipped with a selection of secondary targets, then significant improvements in the lower limit of detection can be obtained. The two-step process requires a higher-power X-ray tube to get the same flux at the sample when compared to direct excitation, so it is generally not incorporated into bench-top instrumentation. Currently, secondary targets are often used in combination with polarization targets. This combination is straightforward, because both types of targets can use the same geometrical arrangement.

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### 5.6 Total Reflection X-Ray Fluorescence Spectrometry

Another variety, *total reflection X-ray fluorescence* (TXRF, also sometimes abbreviated to TRXRF), specializes in the analysis of infinitely thin and extremely homogenous samples derived from liquids. Typically, TXRF instrumentation employs an X-ray source, which is directed at the sample under a very small angle with respect to the surface. The detector is typically placed above the sample at a 90° angle. The TXRF technique commonly uses internal standards (mixed into the unknowns) and geometrical relationships to determine elemental concentrations.

## 6. QUALITATIVE XRF ANALYSIS

This section applies to both WDXRF and EDXRF.

### 6.1 X-Ray Spectra

Qualitative analysis by means of X-ray fluorescence is relatively simple because the characteristic line spectra of the elements are simple and are not affected by chemical bonding; i.e., these spectra are truly elemental. For qualitative (and quantitative) analysis, the main lines are those from the K- and L-series. The K-lines result from electron transitions after one of the K-electrons has been expelled. Due to quantum mechanical selection rules, not all transitions are allowed. Transitions from the L-shell to the K-shell are limited to transitions involving L2 and L3, as radiative transitions between the L1-shell and K-shell are forbidden. These transitions are the most probable, leading to the most dominant line in the spectra. For most elements, the energy difference between the L2-shell and L3-shell is rather small (in the order of a few eV), so the emitted lines are typically not separated and simply considered a doublet. Radiative transitions from M- and N-shells to the K-shell are also occurring. These give rise to a more complex part of the K-spectrum. In practice, however, only the $K\beta_{1,3}$ doublet (resulting from transitions between M3 and K and M2 and K) is of importance. The relative intensity between the Kα and the Kβ varies from 1 in 1,000 for sodium (Na) to 1 in 6 for plutonium (Pu), with the Kα always being the most intense line of the series.

The Kα-line is generated by an electron transition from an electron in the L3 shell, following the removal of a K-shell electron. The Kα-line is the most dominant line in an element's series. The K electrons of an element, however, have the highest binding energy of all electrons in the atom, and thus are only excited by high-energy incident photons. This is generally not a problem for the low- and middle-atomic number elements, but excitation of the K-lines of elements with atomic numbers above 50, for example, might not be possible, as the excitation potential is higher than the maximum operational voltage on most X-ray tubes.

Whereas the K-line spectra are typically limited to two lines only, the situation for the L-series spectra is more complicated. For lead (Pb), a total of 46 L-lines are listed in Bearden [*2*]. Many of these are weak, or are doublets. For qualitative analysis, not all of these lines are to be considered. In all cases, it is important to realize that if the Kβ is visible, then the Kα must be visible too and must have a higher intensity.

### 6.2 Qualitative Analysis

Qualitative analysis typically consists of a few simple steps. After the spectra have been collected, the background is subtracted. This background subtraction can be done in a separate step, or it can be done in conjunction with a peak search algorithm. A fraction of these photons propagate in the direction of the analyzing crystal (WDXRF) or directly to the detector (EDXRF). Next, a peak search is performed, and finally, these peaks are identified, i.e., each is attributed to a particular element. Efficient algorithms for qualitative analysis look for the presence of each element, rather than trying to identify each peak. This is different from X-ray diffraction, where each peak is checked for a match with a phase. In this method, the peak search is performed first. Then, for each element, the presence of the $K\alpha_{1,2}$-doublet is verified. If this line is found in the spectrum, then the weaker lines of the same series ($K\beta_{1,3}$ and $K\beta_2$) are investigated. Note that all characteristic lines in the same series should appear, except in cases where the intensity of a weaker line is similar to the intensity of the background. For elements with higher atomic numbers where the K-lines are not excited (or not detected due to limitations in detection hardware) the process starts with the L-line spectra. The intensity ratios of, for example, Kα to Kβ are listed in several databases [*8*,*9*]. The tabulated ratios are for the "isolated" atom; they reflect the intensities as obtained from an atom without any matrix effects. These ratios can be corrected quite easily for matrix effects, even in the case of overlapping lines. Only elements that are present at trace levels (with intensities comparable to the standard deviation of the background intensity) are usually deemed "not present". Note that, in principle, line interference between elements is not much of a problem in qualitative analysis; this is due to the fact that in qualitative analysis, many more data points that cover alternative lines are available. Hence, establishing the presence of elements is not impacted as much as one would suspect. Also, in energy-dispersive spectrometry, where the line overlap is most severe, adequate corrections can be performed because the peak shape is rather well known.

## 7. QUANTITATIVE XRF ANALYSIS

### 7.1 Selection of Analytical Line

The characteristic emission spectra of most elements contain several lines. The choice of an analytical line for a given element is largely dependent on three factors: the concentration of the element in the sample, the potentially interfering lines, and the excitation capabilities of the spectrometer. In most cases, the Kα emission line of an element is preferred. When the excitation of the K-lines is not possible due to limitations of the tube and/or generator, the use of L-lines, with their lower energy, is necessary. Use of the Lα-line (when the Kα-line is not excited) does not involve much of a detriment because for those elements,

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the intensity of the L-lines is quite high. Also, the detection efficiency of the L-line photons is then much higher than that of the high-energy K-lines of the same element. For elements with high atomic numbers (i.e., Z larger than about 72), the Lβ offers a very good alternative to the Lα and these lines have comparable sensitivities.

In general, on a typical WDXRF instrument, all elements with atomic numbers up to 56 are measured on their Kα-line. For elements with higher atomic numbers, the Lα-line is used. For EDXRF spectrometers equipped with SDD-using generators (and tubes) with a maximum voltage of at least 30 kV, K-lines are available to measure for elements up through about Z = 47; the L-line spectra are used for all elements with higher atomic numbers. The exact point at which the switch over occurs between the K-line and the L-line spectra depends on factors such as the application and the anode material of the X-ray tube used.

Quantification procedures are carried out using routine spectroscopic techniques involving the measurement of blanks and standards of varying elemental concentrations. The measured intensities are then corrected for background and for line overlap, if required. In a number of cases, a straightforward linear relationship between the intensity of the analyte and its concentration can be established. This is especially the case with low concentrations in a constant matrix. In the more general case, however, corrections for matrix effects are required. Matrix effects are not the limiting factor in the precision of the final analysis, but they must be accounted for in order to obtain an accurate measurement.

## 7.2 Matrix Correction Techniques

### 7.2.1 ORIGIN OF MATRIX EFFECTS

XRF is typically applied to condensed samples, without resorting to dissolution of the sample in an abundance of acids or other solvents. Thus, the samples remain concentrated, in contrast to many other techniques. Because of this, the XRF technique is prone to matrix effects, in that the relationship between the intensity of a particular analyte and its concentration is generally not as simple and straightforward as with diluted specimens. With the latter, this relationship between concentration and intensity is often expressed as a straight line. However, the presence of other elements at high concentrations can cause deviations of this straight-line relationship. The physics of X-ray emission and detection is well understood and relatively simple for bulk materials, and this provides a solid theoretical basis for a mathematical description of matrix effects. Matrix effects can thus be described accurately and correctly using a variety of mathematical techniques, and several methods have been developed to address them. Two main matrix effects can be distinguished and are discussed below—absorption and enhancement.

**Absorption:** Absorption as a matrix effect occurs when the matrix elements and the analyte element have different absorption properties for the characteristic radiation of the analyte. If the mass attenuation coefficient of one or more of the matrix elements is larger than the mass attenuation coefficient of the analyte, the matrix will absorb more of the analyte radiation than expected and the measured intensity of the analyte will be lower. This results in a concave calibration curve. If, on the other hand, the absorption of the matrix elements is lower, a higher intensity of the analyte will be measured and a convex calibration curve will be observed. Absorption as a matrix effect can be large, reducing the intensity by as much as 100% (and more) or "increasing" it by as much as 10%, depending upon the analyte and matrix elements. The magnitude of the absorption effect depends on the wavelength of the analytical line considered and the composition of the specimen. In a given specimen, the absorption effect can be significant for the characteristic line of one analyte and rather insignificant for another. The absorption effect can also differ significantly for the same analyte when measured on two different characteristic lines.

The three processes that cause absorption are the photo-electric effect, the coherent scatter, and the incoherent scatter. In general terms, photons with a given energy will be absorbed more with increasing atomic number of the absorbing element. Also, the absorption by a given element decreases in a steady, smooth manner when the photon energy increases. However, when the photon energy exceeds the binding energy of electrons in an inner shell, the photo-electric effect (see *3.1 Ionization and the Photo-Electric Effect*) causes an increase in absorption. This leads to discontinuities in the absorption curve at energies corresponding to the binding energy of the electron levels. These discontinuities are called absorption edges; the energy at which the absorption edge occurs is equal to the binding energy of the electron shell concerned.

**Enhancement:** *Enhancement* as a matrix effect is the process whereby the characteristic radiation of matrix elements is absorbed by atoms of the analyte, subsequently causing characteristic radiation of the analyte. The intensity of the analyte is thus enhanced, which leads to concave calibration curves. For a given analyte, all characteristic lines from the same series are enhanced to the same degree. This is because the root cause of the enhancement is the photo-electric absorption of radiation from matrix elements. The effect of enhancement is typically smaller than that of absorption. It is most pronounced in alloys of the first-row transition elements, where it can reach 25%. In many other applications, the enhancement effect is smaller, but is still important enough to warrant consideration and correction.

### 7.2.2 FUNDAMENTAL PARAMETER METHODS

The physical processes involved in absorption and excitation in XRF are well understood and easy to model mathematically. All entities required are listed in a variety of tables and databases. The calculations were previously tedious and time consuming, but with the current computing power available, the calculation effort is no longer an obstacle. Fundamental parameter methods are based on the underlying physical processes governing X-ray fluorescence, including the specifics of the instrument's geometry, the anode material of the X-ray tube, the voltage on the tube, and the composition of the specimen. The derivation of the equations is beyond the scope of this chapter, but is addressed by Criss and Birks [*10*] and Shiraiwa and Fujino [*11*].

### 7.2.3 COMPTON CORRECTION

The use of Compton scattered radiation as a means to correct for matrix effects (as well as for variations in sample preparation) has been described by Reynolds [*12*] and Taylor and Andermann [*13*]. The Compton correction is based on the fact that the intensity of incoherent (or Compton) scatter is inversely related to the mass attenuation coefficient of the sample. In practice, a

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high-energy characteristic line from the tube anode is used. Measuring the intensity of the Compton scattered tube line thus reveals information about the absorption properties at the wavelength of the Compton scattered line. At first glance this seems to be of limited use, but in the field of X-rays, values of the mass attenuation coefficients for the same sample at different energies/wavelengths are proportional. This relationship, and the fact that absorption is the major source of matrix effects, explains why the Compton correction is a powerful correction method in a variety of matrices.

It is, however, not generally applicable and will fail in cases where there are significant absorption edges between the wavelengths of the Compton scattered radiation, at the wavelength of the analytical line used. The correction is based on the relationship between Compton scatter and the sample's absorption properties, and it does not correct for enhancement.

#### 7.2.4 INFLUENCE COEFFICIENT ALGORITHMS

Matrix correction methods based on influence coefficients have the general format:

$$C_i = K_i l_i \left[ 1 + \sum_{\substack{j=1 \\ j \neq i}}^{n} \alpha_{ij} C_j \right] \text{[18]}$$

(based on Lachance and Traill [*14*]) where $C_i$ and $C_j$ are the concentrations of the analyte *i* and the interfering element *j*, respectively, and $\alpha_{ij}$ is the influence coefficient, expressing the influence of interfering element *j* on the analyte *i*. All concentrations are expressed as weight fraction. The summation in *Equation [18]* has $n - 1$ terms, for a sample consisting of n elements or compounds; this is a simple yet essential feature of the algorithm. The same set of equations with *n* terms in the summation would lead to a homogeneous set of simultaneous equations, from which only *ratios* rather than absolute values could be derived.

Because the sum of the concentrations of all elements (whether they are measured or not) in a specimen always totals 1, one element can be eliminated. Most influence coefficient algorithms in software are based on this equation, however they may differ in *how the values of the coefficients are calculated*.

#### 7.2.5 EMPIRICAL INFLUENCE COEFFICIENTS

Lachance and Traill [*14*] indicate that the value of the coefficients should be calculated from theory, but they only provide a simple and limited method that fails to consider enhancement or the polychromatic nature of the incident beam. Therefore, in many cases the coefficients were determined using multiple regression methods. Many early variations on influence coefficient algorithms were based on regression techniques that were used to calculate the value of the coefficients. However, the use of regression methods should be discouraged as they often lead to overly optimistic indications of precision and then often fail during validation. This is especially problematic if not enough certified reference materials are used in the calibration. The minimum number of standards used should be at least 3 times the number of parameters. Each of the influence coefficients whose value is determined by regression consumes one degree of freedom, as does the determination of slope and intercept.

The total number of parameters to be determined can grow rapidly for a single analyte. For example, assume that there are three interfering elements, and thus with slope and intercept, a total of five parameters need to be determined. This requires a minimum of 15 *independent* and *uncorrelated* standard samples. Note that replicates of the same standard sample do not count as individual samples, but instead count as a single one; this is a common source of error. Also, care must be taken to ensure that the standards are uncorrelated. This implies that a series of dilutions from a given standard cannot be used when empirical influence coefficients are to be determined. In a dilution series, two (or more) components *A* and *B* increase (or decrease) in the same direction. Thus the effect of component *A* on the intensity of the analyte cannot be distinguished from component *B*'s effects, leading to erroneous values for the influence coefficients.

#### 7.2.6 THEORETICALLY CALCULATED INFLUENCE COEFFICIENTS

De Jongh [*15*] was the first to publish a method detailing the calculation of influence coefficients, using theory only to eliminate a compound other than the analyte. The algorithm is still in common use today. The eliminated compound can be the non-fluorescing matrix, such as cellulose, or the matrix when its concentration is not certified. Note that the concentration range over which the algorithm delivers precise results is rather limited when the matrix effects are severe. This is a direct consequence of the fact that the values of the influence coefficient are calculated for a particular composition of the sample. For routine analysis involving relatively limited concentration ranges, the algorithm delivers excellent results. In contrast to the situation where the values of influence coefficients are determined by regression (see *7.2.5 Empirical Influence Coefficients*) it is feasible to calibrate using dilution series.

### 7.3 Measurement Method Development and Calibration

#### 7.3.1 OVERVIEW

Once the elements of interest have been identified, the spectrometer measurement parameters must be set appropriately. This involves determining the "ideal" measurement conditions for each element. The list of parameters to be selected for each analyte can include, for example, the voltage and current on the X-ray tube, the crystal and the collimator (WDXRF), the type of primary beam filter (if used), and other parameters. For elements that are similar in atomic number, the settings will be quite similar. The precision of XRF measurements is theoretically limited by the CSE. It has been shown that the CSE of a measurement of *N* counts (photons) is given by the square root of *N*. It is a generally accepted practice to determine the measurement times that obtain an acceptable CSE for each element of interest. Sample preparation errors and the CSE are the

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largest contributors to the total analytical precision. Once the measurement time has been established, it is important to ensure that the background positions and methods for peak fitting are also appropriate.

After the net intensities of the elements of interest have been obtained from a suitable number of reference standards, a calibration can be performed. For low concentrations of analytes in an otherwise constant matrix, linear regression may be appropriate. When evaluating specimens with higher concentrations of analytes, the calibration also involves correction for matrix effects. The fact that the EDXRF technique is not capable of measuring the organic constituents of an actual pharmaceutical composition is of little concern. This is certainly true if the composition of the matrix is constant, and if the total content of measurable elements is low. But if these two conditions are not satisfied, the difference in scattering properties can result in calculation errors if not corrected. Fortunately, there are numerous strategies that accommodate these differences, such as the fundamental parameters method or the Compton correction method. The nature of matrix effects, as well as methods to correct for them, have been discussed above. The application of these methods to calibration and analysis of unknowns will be discussed in the sections below.

### 7.3.2 CALIBRATION USING INFLUENCE COEFFICIENT ALGORITHMS

Calibration using influence coefficients is rather straightforward. Once the values for the coefficients have been calculated for each of the standard samples, the summation

$$M_i = \left[1 + \sum_{\substack{j=1 \\ j \neq e}}^{n} \alpha_{ij} C_j\right] \text{[19]}$$

can be calculated where for standard samples, the concentrations, $C_j$, are known. The value of $M_i$ for one standard sample will be different from the value for another standard sample, and it does not matter whether the difference is significant or slight (large or small). If adequate corrections for background have been made, calibration is then represented by

$$C_i = K_i I_i M_i \text{ [20]}$$

where the product of the measured intensity, $I_i$, and the corresponding value for the matrix effect ,$M_i$, are regressed against the given, known concentration, $C_i$, to determine the slope, $K_i$. This equation assumes that the intensity, $I_i$, is properly corrected for the background. In those cases where the intensity of the background is constant between samples and standards, a slightly modified equation can be used:

$$C_i = B_i + K_i I_i M_i \text{ [21]}$$

where $B_i$ is the intensity of the background. In this case, both $B_i$ and $K_i$ are determined by regression analysis. In situations where the background intensity varies significantly between samples, or between samples and standards, proper background correction must be applied to the measured intensities, and the corrected intensities must be used for the calibration.

### 7.3.3 CALIBRATION USING COMPTON CORRECTION

In this case, both the intensity of the analyte, $I_i$, and the intensity of the Compton scattered line, $I_C$, are measured. The intensity of the Compton is inversely proportional to the mass attenuation coefficient [*12*], or

$$M_i = \frac{1}{I_C} \text{ [22]}$$

Combining this with *Equation [20]* leads to

$$C_i = K_i I_i \frac{1}{I_C} \text{ [23]}$$

The intensity of the analyte and the Compton line are measured on a suite of standard samples and regressed against the analyte concentration to determine the value of the slope of the calibration. A background factor, $B_i$, can be determined as well.

### 7.3.4 CALIBRATION USING FUNDAMENTAL PARAMETER METHODS

The physical processes involved in absorption and excitation during XRF are well understood, and it is possible to calculate the value of the matrix effect based on several factors: the composition of the sample, the type and settings of the tube, the geometry of the spectrometer, and the fundamental parameters:

$$M_i = f(C_i, C_j, \ldots, C_n, \textit{tube}, \textit{geometry}, \textit{fp}) \text{ [24]}$$

This value of $M_i$ is calculated for each standard and for each analyte of interest, and then applied in the usual manner.

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#### 7.3.5 INTERNAL STANDARD METHOD

An alternative to the methods described above is the internal standard method. A fixed quantity of an element *s* (other than the analyte *i*) is added to all specimens. The element *s* is chosen such that it is not present in the specimens, thus ensuring that its concentration is the same for all specimens. In the absence of matrix effects, the measured intensity of its characteristic radiation would also be constant. Any variation in its intensity (other than the variation resulting from the counting statistical error) is attributed to the presence of matrix effects. The idea is to choose the internal standard element *s* in such a way that the intensity of its characteristic radiation is subject to the same matrix effects as the intensity of the analyte element under consideration. Both intensities—of the analyte and of the added element—increase (or decrease) in proportion to one another depending upon the matrix effect. *Equation [22]* can be modified for the internal standard element *s*:

$$C_s = K_s I_s M_s \text{ [25]}$$

In this equation, $C_s$ is a constant. Taking the ratio of *Equation [20]* and *Equation [25]* and solving for the only unknown, $C_i$, yields

$$C_i = \frac{K_i M_i C_s}{K_s M_s} \frac{I_i}{I_s} \text{ [26]}$$

It is important to note that the terms of the first fraction in *Equation [26]* are either all constants ($K_i$, $K_s$, and $C_s$) or are proportional to one another ($M_i/M_s$). *Equation [26]* can thus be reduced to

$$C_i = K_i^* \frac{I_i}{I_s} \text{ [27]}$$

where all of these constants are collated in a single variable $K_i^*$.

The main challenge in applying this method is the selection of the element to be used as the internal standard. This element must not be present in the specimens (or at most, present at very low levels that will not interfere with the analysis). Also, its characteristic radiation must be subject to the same matrix effects as the analyte(s) of interest, and its emission spectrum should not cause interference. In particular, care must be taken to ensure that the analyte element and the internal standard are both subject to (or both *not* subject to) enhancement by a matrix element. In the same fashion, when the analyte is in the presence of matrix elements that strongly absorb the characteristic radiation of the analyte, the same should apply to the characteristic intensity of the internal standard element.

When the samples are organic matrices typical of pharmaceuticals, it is easy to satisfy the essential requirement that the characteristic radiation of both the analyte(s) and the internal standard are subject to the same matrix effects. These organic matrices do not exhibit characteristic radiation of their constituent elements, which makes it easier to find a suitable internal standard element. A simple guideline is to choose an element that is not present in the samples and that has an emission line close to the one of the analyte's (with regard to either wavelength or energy). The characteristic lines themselves do not have to be the same or even from the same series, and the method also works when the analytical line is a Kα and the line of the internal standard is, for example, an Lβ. It must be noted that there is no requirement for the internal standard element to be a pure element. Stable compounds that are easily soluble in a solvent can be used. The solvent is, in many cases, the same as the base material of the specimens and the calibration standards. If more than one internal standard element is to be used, it is recommended that both elements/compounds are added in a single internal standard solution, to minimize errors.

### 7.4 System Suitability Criteria

Performance characteristics that demonstrate the suitability of an XRF method are similar to those required for any analytical procedure. A discussion of the applicable general principles is found in *Validation of Compendial Procedures* ⟨1225⟩.

#### 7.4.1 LIMIT OF DETECTION AND LIMIT OF QUANTITATION

The lower limit of detection may be expressed as:

$$LLD = \frac{3.3 \times CSE}{S} = \frac{3.3 \times C}{I_{net}} \sqrt{\frac{I_b}{t_b}} \text{ [28]}$$

with $I_{net}$ representing the net count rate at the concentration, *C*; $I_b$ as the count rate of the background; and $t_b$ as the background measurement time. It is noteworthy that the detection limit will improve with the square root of the measuring time.

The limit of quantitation can be estimated by calculating the standard deviation of NLT six replicate measurements of a blank and multiplying by 10. Other suitable approaches may be used (see ⟨1225⟩).

#### 7.4.2 LONG-TERM STABILITY AND DRIFT CORRECTION

Under standard operating conditions, instrumental drift of X-ray spectrometers is less than 1% relative and can be as small as 0.1% relative for high-end instrumentation in well-maintained laboratories over the same time period. To monitor instrument drift, one or more stable sample(s) with reasonable intensities (to minimize the CSE) for the analytes of interest can be measured on a regular basis. It is not necessary to use *in-type* drift correction samples. It is common to use glassy samples and alloys. Most instruments have drift correction routines within the software, and because XRF is a very robust technique, it may only be

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necessary to utilize a drift-monitor once per month. However, it is recommended that most laboratories practice drift monitoring more frequently.

### 7.4.3 ANALYSIS

Once a method has been established through the calibration procedure, a sample analysis may be performed. It is essential to ensure that samples and standards are treated in exactly the same manner. This includes specimen preparation and specimen presentation to the instrument.

### 7.4.4 CALCULATIONS AND REPORTING

In the event that the free-pressed-pellet sample method is used (i.e., pressed without binder), the spectrometer results represent final sample concentrations, and no further calculations are required. Similarly, both loose powders (i.e., pure, fine materials) and undiluted liquids measured in disposable sample containers require no further calculation and the spectrometer results represent final sample concentrations. Any material that has been diluted, such as a liquid or pressed pellet, will require additional calculation. Most modern XRF instrumentation comes with software packages that include calculation functions that accommodate dilution factors and automatically back-calculate sample concentrations. For diluted, pressed-pellet samples, the reported units may be weight % or ppm. The actual units for the concentrations are of little importance, as long as care has been taken to work with the proper conversion factors. If a calibration for liquid samples is setup to deliver results in µg/mL, the final concentration of a given element can be calculated in µg/g from the solution element concentration in µg/mL.

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# ⟨1736⟩ APPLICATIONS OF MASS SPECTROMETRY

## 1. INTRODUCTION

Mass spectrometry (MS) is an integral part of modern pharmaceutical research and development in academic, industrial, and clinical laboratories. Trace analytical measurement (both qualitative and quantitative), specifically the demand for trace-mixture analysis, has increased the need for this powerful tool. In many cases, the analytical demands of trace-mixture sample analysis has made MS the method of choice for qualitative and quantitative assays for target analytes such as proteins, peptides, drug substances, metabolites, impurities, and degradation products. Because of its analytical capabilities, MS has found widespread application in the pharmaceutical industry. Specifically within the compendial context, MS has been, or has the potential to be, applied to both qualitative (identification tests) and quantitative measurements (assays).

For qualitative tests, MS-based methods can provide molecular mass information via the detection of the molecular ion or ions related to the molecular mass of the analyte as a first level or step of identification. A variety of instrumental methods permit molecular mass determinations for a wide range of materials, up to and including large biomolecules (e.g., proteins) and polymers. Along with the molecular mass information, MS also can provide unique structural information via the generation of fragment ions. The diversity of fragmentation approaches available in modern MS allows structurally significant fragmentation approaches for a wide variety of compendially relevant materials (e.g., small molecules and peptides).

Molecular mass thus can become a surrogate for confirmation or can even be used for the identification of a targeted compound, particularly when used in conjunction with an authentic standard or a chromatographic method. Advanced studies that involve one or two more dimensions of mass analysis also can be used to obtain specific structural detail (fragment ions that correspond to structurally unique portions of the target molecule) or more selectivity to enable powerful approaches for quantitation. Moreover, higher resolution methods that feature mass spectrometry and chromatography can routinely provide a benefit to the scientific community.

Modern analytical MS includes a diverse range of available instrumentation and experimental approaches. The specific mass spectrometer, and, of course, specific chemistries (i.e., sample preparation, chromatography, and ionization) define the final analytical procedure.

## 2. MASS SPECTROMETERS

The following sections describe the principles of MS, including the general layout of modern MS instrumentation used in the pharmaceutical industry and the specific components (sample introduction, ionization, and mass analyzer). This section also includes a brief description of the operational modes of a mass spectrometer, focusing on fragmentation production and analysis.

### 2.1 Overview

Analytical measurements that use MS generally involve the following processes: sample preparation, chromatography or sample introduction, ionization, and mass analysis and detection. The resulting output from the mass spectrometer is depicted in a mass spectrum, a survey of ions made in the ion source, and is represented in a graphic representation of mass-to-charge ratio ($m/z$) versus intensity.

Ions are separated by a property of mass such as $m/z$, where $m$ is the mass of the ion and $z$ is the number of charges on the ion. The method by which ions are separated typically defines the mass spectrometer type. Regardless of type, the mass analyzer separates ions according to the $m/z$. The mass analyzer continuously acquires data across a predefined range of masses to generate the resulting mass spectra.

### 2.2 General Layout

A general layout of a mass spectrometer platform for pharmaceutical analysis is shown in *Figure 1*. Samples are prepared via a procedure defined for the specific sample and analyte. The resulting sample then is injected into the mass spectrometer via a chromatographic system such as high-performance liquid chromatography (HPLC) or gas chromatography (GC) or via direct injection. From the inlet, the discrete analytes are ionized for subsequent analysis by the mass spectrometer. All mass spectrometers require four components:

1. Sample introduction technique
2. An ionization source to charge the analyte
3. A mass analyzer to separate the analytes on a mass/charge ($m/z$) scale
4. A detector to measure the ions.

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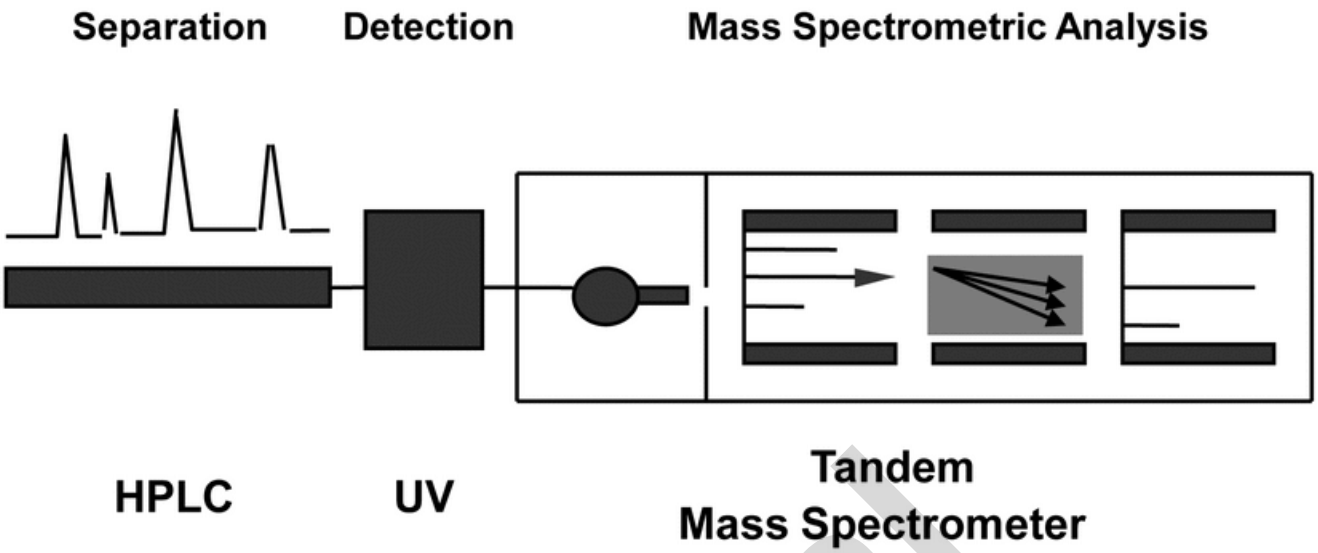


Figure 1. General MS system. HPLC = high-performance liquid chromatography; UV = ultraviolet detector. (Courtesy of Milestone Development Services, Newtown, PA)

MS can be categorized by the dimensions of mass analysis: single-stage mass spectrometers, hybrid or tandem mass spectrometers (MS/MS), and multiple stages of mass analysis ($MS^n$). These mass spectrometer formats are described in this section.

### 2.2.1 SINGLE-STAGE MS

A single-stage mass spectrometer provides one dimension of mass analysis. As shown in the schematic in *Figure 2*, a single mass spectrometer can be viewed simply as providing a survey of all ions generated in the ion source. For example, if a 10-component mixture is injected into a chromatograph and each component is separated with adequate resolution, then the mass spectrometer would provide discrete measurement of mass-to-charge of each of the 10 components. The resulting mass spectrum of each component is generated for subsequent interpretation and assignment of molecular mass.

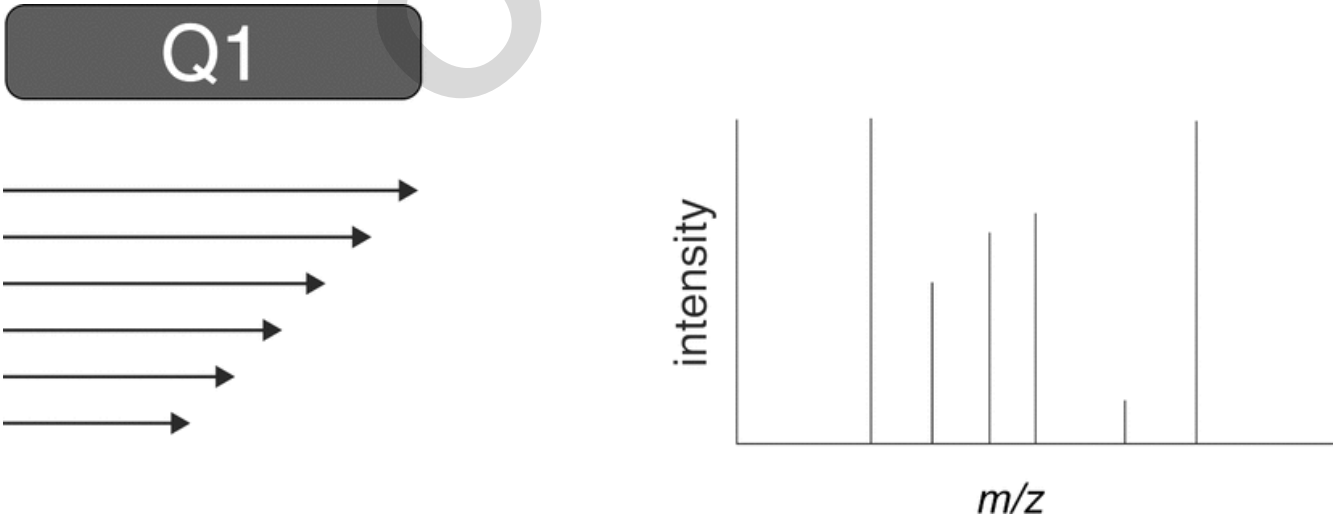


Figure 2. One-dimensional MS scan. Q1 = single-stage mass spectrometer or a single stage of analysis. (Courtesy of Milestone Development Services, Newtown, PA)

### 2.2.2 TANDEM MASS SPECTROMETRY (MS/MS)

A system capable of carrying out two sequential *m/z* analysis events (i.e., a tandem mass spectrometer) provides increased selectivity for detailed structure elucidation or quantitative analysis.

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### 2.2.3 MULTIPLE STAGES ($MS^n$)

A unique feature of some MS formats is the capability to perform multiple stages of MS and to generate further fragment information. These mass spectrometers are capable of isolating an ion of interest, inducing fragmentation, isolating a specific product ion and then repeating this process on the resulting or selected product ion.

## 2.3 Sample Introduction

Several approaches can be used to introduce the sample into the mass spectrometer. The direct, infusion, and chromatographic procedures are described in this section.

### 2.3.1 DIRECT INTRODUCTION

For some sample types and applications, a direct insertion probe is used to introduce the sample into the mass spectrometer. The sample, usually a pure compound or relatively pure compound, is dissolved in an appropriate solvent. A small amount of the sample (typically 1 µL or less) is deposited onto the probe. Typically, the probe consists of a metal filament, such as platinum, located at its tip. The metal filament is heated, and the sample is desorbed into the ion source of the mass spectrometer. The probe then is removed from the mass spectrometer and is prepared for the next sample.

### 2.3.2 INFUSION INTRODUCTION

Sample introduction via infusion typically is done to provide a relatively long analysis time or perhaps to conduct a quick survey of a sample. Infusion introduction also can be done when analysts optimize the instrumental conditions (source and mass spectrometer operational parameters) for a specific analyte as well as to obtain greater numbers of spectra. This sample introduction method may require more sample than conventional flow rates. However, infusion sample introduction is widely practiced for nanospray ionization applications for the analysis of proteins and peptides as well as during specialized nanospray applications with small molecules in drug metabolism to determine structure or equimolar response ratios.

### 2.3.3 CHROMATOGRAPHIC INTRODUCTION

The use of chromatography methods for sample introduction into the mass spectrometer is perhaps the most common approach.

**2.3.3.1 Gas chromatography:** GC procedures are preferred for nonpolar, volatile analytes (see *Chromatography* ⟨621⟩. Samples contained in solvent are injected onto the injection port of the GC. The sample is volatilized, and a nonreactive, inert gas such as helium carries the sample through the GC column, which is contained in a temperature-controlled oven. The combination of carrier gas and heat moves the analyte through the column. Capillary GC columns are commonly used in pharmaceutical applications. Molecules separate within the capillary column and are introduced directly into the ion source of the mass spectrometer.

**2.3.3.2 High-performance liquid chromatography:** A popular approach for sample introduction into the mass spectrometer involves HPLC (see ⟨621⟩). HPLC procedures are preferred for nonvolatile and thermally labile analytes, but are suitable for use with any analyte that is readily ionizable in a solution environment with the appropriate chemical modifiers. Samples are prepared in solution and then injected onto the HPLC column. Analytes are separated based on the partitioning between the mobile phase and stationary phase and are introduced into the ion source of the mass spectrometer. The effluent is volatilized in the ion source and ionization occurs. The subsequent ions then are introduced into the mass spectrometer for analysis.

**2.3.3.3 Capillary electrophoresis:** Capillary electrophoresis (CE), also called capillary zone electrophoresis (CZE), is a separation method that exploits subtle differences in the ionic composition of analytes to separate them based on electrophoretic mobility in a conductive liquid. Details regarding the principles and use of this methodology can be found in *Capillary Electrophoresis* ⟨1053⟩. During the past 15 years this separation method periodically has been used with MS as an alternative to LC–MS for certain classes of compounds. However, with the widespread use of HPLC in analytical and MS laboratories, CE-coupled MS methods often are superseded by LC–MS methods.

## 2.4 Ion Polarity

Ionization involves the process of converting the analyte into the gas phase and depositing a charge onto the molecule. The final charge of the analyte determines the ion polarity to be used in the mass analysis. Generally, selection of the ionization mode depends on the ability of the desired analyte to accept or lose a proton during the ionization process. MS can be performed in the positive or negative ion polarity mode.

### 2.4.1 POSITIVE ION MODE

Many MS pharmaceutical analyses use the positive ion mode. For example, compounds that contain a basic functional group such as an amine (e.g., proteins and peptides) are excellent candidates for MS analyses in the positive ion mode because under acidic conditions these compounds readily accept a proton to form positively charged ions.

### 2.4.2 NEGATIVE ION MODE

The negative ion mode is well suited for compounds that can easily lose a proton. Compounds that contain a carboxylic acid, phosphate, or a sugar, for example, are good candidates for negative ion MS.

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## 2.5 Ionization Procedures

A variety of ionization procedures can be used with a mass spectrometer for pharmaceutical analysis. The various ionization procedures are described in this section.

### 2.5.1 ELECTRON IONIZATION

Electron ionization (EI) is used mostly with GC applications when the analytes of interest are nonpolar and are easily volatilized. Mass spectra produced by EI procedures are characterized by extensive fragmentation. Because EI typically produces extensive fragmentation, EI is considered a hard-ionization mode. Mass spectra are obtained when a 70 eV electron beam enters the source and impacts the analyte molecules present in the gas phase:

$$M + e^- \rightarrow M^{+\cdot} + 2e^-$$

Nearly all EI applications are conducted in the positive ion mode. These spectra are highly reproducible. Thus, EI spectral libraries can be used to determine the structure and to confirm the identity of unknown compounds. Additionally, fragmentation patterns have been extensively studied for electron ionization and can help determine structure of unknown compounds.

### 2.5.2 CHEMICAL IONIZATION

Chemical ionization (CI) procedures rely on electron ionization of reagent ions such as methane, ammonia, or isobutene. The reagent ions react with the analyte molecules (ion–molecule reaction) in the source of the mass spectrometer. In the positive ion mode, analyte molecules are ionized via proton transfer and/or adduct formation, producing even electron molecular ion species or adduct ions. Depending on the analyte, both positive and negative ion spectra can be obtained with CI procedures. CI is considered a softer ionization (less fragmentation of the molecular ions) mode than EI although some approaches can induce fragmentation depending on the CI gas chosen and the structure of the analyte molecule. CI is very useful for reactive and unstable compounds where a molecular mass determination is desired. Representative CI ionization reactions for both GC–MS and LC–MS methods follow.

Protonated molecule formation (GC–MS example):

$$CH_4 + e^- \rightarrow CH_4^+ + 2e^-$$
$$CH_4^+ + CH_4 \rightarrow CH_5^+ + CH_3$$
$$CH_3^+ + CH_4 \rightarrow C_2H_5^+ + H_2$$
$$M + C_2H_5^+ \rightarrow [M + H]^+ + C_2H_4$$

Protonated molecule formation (LC–MS example):

$$M + NH_4^+ \rightarrow NH_3 + [M + H]^+$$

Adduct ion formation (LC–MS example):

$$M + NH_4^+ \rightarrow [M + NH_4]^+$$

### 2.5.3 ATMOSPHERIC PRESSURE IONIZATION

Atmospheric pressure ionization (API) procedures allow the direct introduction of samples into the mass spectrometer from a liquid interface such as an HPLC. The two most common forms of API used in pharmaceutical analyses are electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI).

**2.5.3.1 Electrospray ionization:** ESI is a method that produces fine charged droplets of a liquid phase that carries the analyte of interest. The liquid phase typically is a volatile combination of water and organic solvent (e.g., acetonitrile or methanol). A small percentage of a reagent (e.g., 0.1% formic acid) also is included to increase the conductivity of the solution. ESI typically is used with HPLC and involves the nebulization of the sample delivered at flow rates that range from nL/min to mL/min to produce a fine spray of droplets (radius = 0.5–1.0 µm). Solvent evaporation results in an increased charge concentration at the droplet surface until ions are liberated directly from the droplet. Ions are transported or focused directly into the mass spectrometer, and the resulting spectra contain ions that are typically indicative of the molecular mass of the analyte.

ESI often is referred to as a soft-ionization procedure because typically it does not result in fragmentation of the analyte during the ionization process. The development of this ionization technology was crucial for the analysis of large biopolymers (e.g., proteins) because it allows the addition of multiple charges to a single molecule, thus bringing the $m/z$ of the analyte into a suitable $m/z$ range for many types of mass analyzers. For example, *Figure 3* shows the ESI charge state distribution for equine heart apomyoglobin as analyzed on a simple single quadrupole instrument with an upper scan limit of $m/z$ 2000. The series of multiply charged ions allows the spectrum to be collected within the instrument's working $m/z$ range. The ability to calculate the intact mass of the protein from a combination of all the charge states allows a more accurate average mass (see inset in *Figure 3*) to be determined (within 0.1% on a simple quadrupole instrument). Thus ESI methods are critically important for the analysis of large biomolecules.

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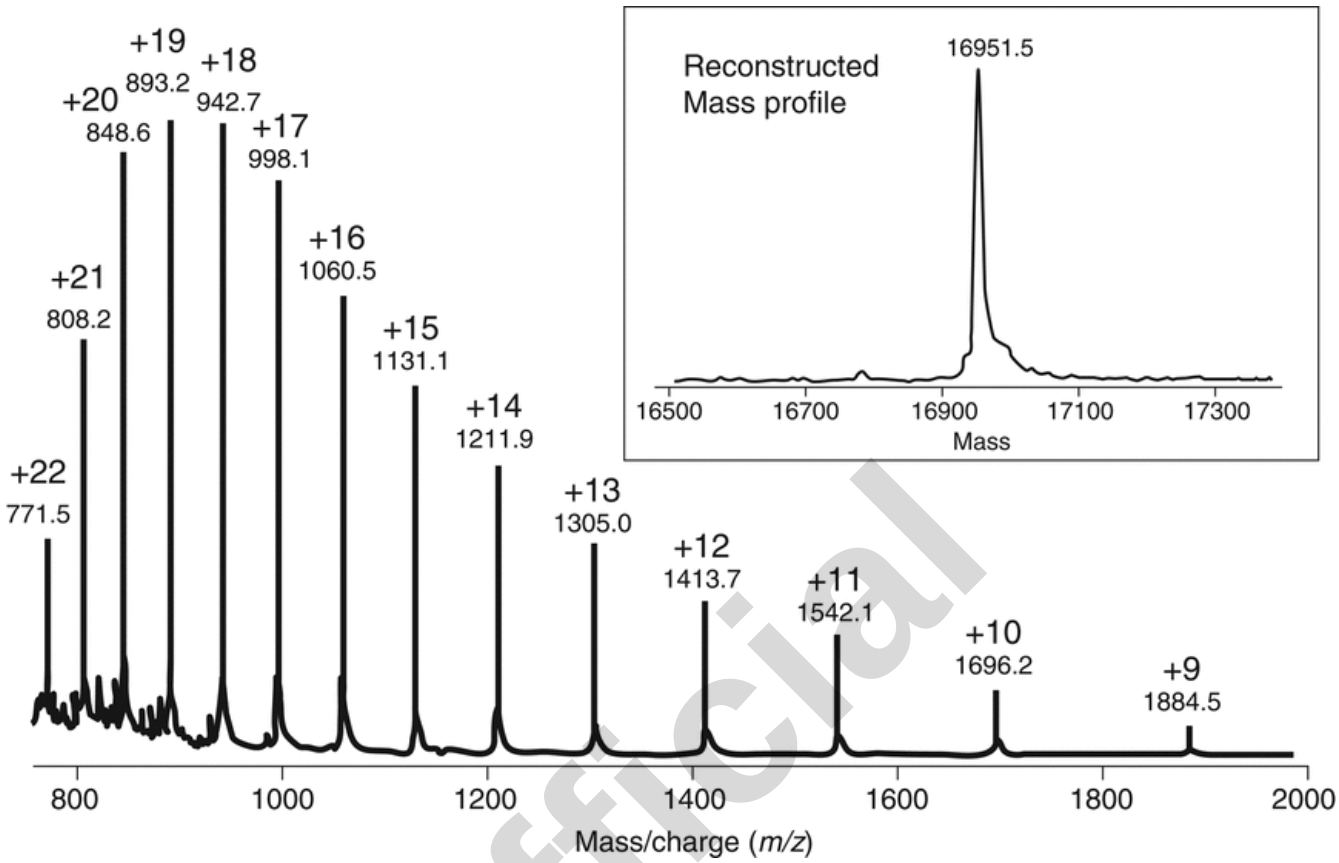


Figure 3. Electrospray ionization mass spectrum for equine heart apomyoglobin.

**2.5.3.2 Atmospheric pressure chemical ionization:** APCI procedures are used with HPLC and involve the nebulization and heating of the sample to liberate neutral molecules. A corona discharge produces reagent ions (e.g., $H_3O^+$ or $NH_4^+$) from the mobile phase. The reagent ions react with analyte molecules via proton transfer or adduct formation. The resulting positive ion spectrum typically contains $[M + H]^+$ ions that are indicative of molecular mass and fragment ions that correspond to unique substructure(s) of the analyte molecule. Negative ion spectra are generated when reagent ions (e.g., $OH^-$ or $CH_3COO^-$) react with the analyte molecule to produce $[M – H]^-$ ions.

## 2.5.4 MATRIX-ASSISTED LASER DESORPTION IONIZATION

Matrix-assisted laser desorption ionization (MALDI) is a soft-ionization procedure used primarily for biomolecules that relies on the addition of a chemical matrix dried with the analyte of interest. This matrix compound can absorb laser energy at a particular wavelength during the laser ablation process. By a mechanism that has not been fully elucidated, some of the ions generated in the matrix can transfer protons to the analyte, and the resulting gas-phase ions are focused into the mass spectrometer. Specific matrices have been empirically developed for various classes of analytes, including 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) and α-cyano-4-hydroxycinnamic acid for proteins and peptides and picolinic acid for oligonucleotides. Unlike ESI, MALDI typically produces lower charge-state ions, with singly charged ions being the most favorable.

## 2.5.5 AMBIENT IONIZATION PROCEDURES

Ambient ionization generally refers to a collection of MS procedures that permit direct sampling and interrogation of analytes from sample matrices or surfaces under ambient conditions with little or no pretreatment. In recent years, the number and type of ambient ionization procedures have rapidly expanded. This is evident from the proliferation in recent literature of acronyms that represent various ambient ionization procedures. Ambient ionization has enjoyed broad application in fields that include forensics; food safety; monitoring of environmental contaminants; polymers; fuels; detection of explosives and drugs of abuse; molecular imaging of surfaces and tissues; profiling and characterization of metabolites, proteins, and biomolecules; as well as monitoring of chemical reactions and processes.

Ambient ionization procedures typically produce analyte ions directly from sample surfaces or require initial production of analytes that subsequently are ionized by any of several processes. Therefore, ambient ionization procedures can be broadly categorized as representing either direct or multistage ionization mechanisms. Direct ionization procedures generate analyte ions from a sample solution or droplet in an electric field, or desorb analyte ions by impinging a sample surface with charged droplets or solvent ions, photons, or metastable atoms. Multistage ionization procedures initially produce analyte particle or droplets with a liquid or gas stream, thermal desorption, irradiation or ablation with a laser, or nebulization. Analytes

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subsequently react with a charged species or with metastable atoms generated by ESI, APCI, or photoionization to produce analyte ions.

*Table 1* is a representative, but not comprehensive, listing of ambient ionization procedures and illustrates the diversity and similarity among various approaches.

**Table 1. Ambient Ionization Procedures**

| Acronym | Description | Mechanisms |
|---|---|---|
| APGDDI | Atmospheric pressure glow discharge desorption ionization | Thermal desorption, gas discharge ionization |
| APPeI[a] | Atmospheric pressure Penning ionization | Similar to APCI |
| AP–TD/ESI[a] | Atmospheric pressure thermal desorption/electrospray ionization | Thermal desorption, ESI |
| ASAP[a] | Atmospheric pressure solids analysis probe | Thermal desorption |
| DAPCI | Desorption atmospheric pressure chemical ionization | Thermal desorption, APCI |
| DAPPI[a] | Desorption atmospheric pressure photoionization | Thermal desorption, photoionization |
| DART[a] | Direct analysis in real time | Gas discharge ionization |
| DBDI | Dielectric barrier discharge ionization | Thermal desorption, gas discharge ionization |
| DCBI | Desorption corona beam ionization | Thermal desorption, APCI |
| DESI | Desorption electrospray ionization | Similar to ESI |
| EADESI | Electrode-assisted desorption electrospray ionization | Similar to ESI |
| EASI | Easy ambient sonic spray ionization | Supersonic spray ionization |
| EESI[a] | Extractive electrospray ionization | Similar to ESI |
| ELDI[a] | Electrospray laser desorption ionization | Laser desorption or ablation, ESI |
| FD–ESI[a] | Fused droplet electrospray ionization | Similar to ESI |
| IR–LADESI[a] | Infrared laser-assisted desorption electrospray ionization | Laser desorption or ablation, ESI |
| LAESI[a] | Laser ablation electrospray ionization | Laser desorption or ablation, ESI |
| LD–APCI[a] | Laser desorption atmospheric pressure chemical ionization | Laser desorption, APCI |
| LD–ESI[a] | Laser desorption electrospray ionization | Laser desorption, ESI |
| LDSPI[a] | Laser desorption spray postionization | Laser desorption or ablation |
| LDTD[a] | Laser diode thermal desorption | Thermal desorption |
| LEMS[a] | Laser electrospray mass spectrometry | Laser desorption or ablation |
| LESA[a] | Liquid extraction surface analysis | Similar to ESI, surface extraction with solvent droplet |
| LIAD–ESI[a] | Laser-induced acoustic desorption electrospray ionization | Laser desorption or ablation, similar to ESI |
| LPI–MS[a] | Liquid surface Penning ionization mass spectrometry | Similar to APCI |
| LSI | Laser spray ionization | Laser desorption or ablation |
| MALDESI[a] | Matrix-assisted laser desorption electrospray ionization | Laser desorption or ablation |
| PADI | Plasma-assisted desorption ionization | Thermal desorption, gas discharge ionization |
| SESI[a] | Secondary electrospray ionization | Similar to ESI |
| TDAMS[a] | Thermal desorption based ambient mass spectrometry | Thermal desorption |
| TD/APCI[a] | Thermal desorption/ atmospheric pressure chemical ionization | Thermal desorption, similar to APCI |

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[a] Multistage ionization procedure.

## 3. MASS ANALYSIS

The method by which ions are separated typically defines the mass spectrometer type. Essentially, the mass analyzer is responsible for filtering the ions generated during the ionization process. The various mass analyzers that typically are used in the pharmaceutical industry are described in this section.

### 3.1 Quadrupole

A quadrupole mass spectrometer consists of a set of four parallel rods. When a combination of constant (DC) and alternating (AC) voltage are applied to the opposing rods respectively, the resulting electric fields allow ions of a specific $m/z$ to stably transit the quadruple and to pass through to the detector. Quadrupole mass spectrometers are relatively low-cost instruments and provide good qualitative and quantitative analytical capabilities. Generally, quadrupole mass spectrometers are limited to production of low-resolution mass spectra.

### 3.2 Magnetic Sector

A magnetic sector mass spectrometer filters ions by the means of the application of a magnetic field. The magnetic field is varied, and ions are deflected to follow a curved path so that ions with different $m/z$ ratios are separated.

### 3.3 Ion Traps and Ion Cyclotron Resonance

Most commercial mass spectrometers fall broadly into two categories: those that rely on continuous beams of ions being sent to a detector for sequential $m/z$ detection (like cars on a highway driving from point A to point B), and those devices that "trap" ions in discrete, repeating orbits (like cars on a race track). The latter devices are broadly referred to as *ion traps*. The orbital paths are complex and unique to each device. The ions can be trapped by static electric fields (DC voltage), dynamic quadrupole electric fields [radio frequency (RF) AC voltage], magnetic fields, or some combination of the types. Detection of the ions may take place by sequential ion ejection (after trapping, by $m/z$) or by detection of all ions simultaneously [while trapped, i.e., by image current detection and subsequent Fourier transformation (FT) of the data]. Examples include the popular linear and 3D ion trap MS systems (RF AC voltage only with ion ejection for detection), the FT Orbital Trapping MS (which uses DC voltage and a quadrupole field for trapping, with image current detection), and the less common FT Ion Cyclotron Resonance MS (which uses a superconducting magnet to provide the primary trapping field). The FT devices generally provide very high mass resolution (e.g., $10^5$–$10^6$ at $m/z$ 400) at reasonable scan speeds (1 Hz), and the RF-based devices generally offer fast scan rates (0.1 Hz) but lower resolution ($10^3$). Lastly, the most popular FT equipment is now hybridized with a quadrupole RF device (especially the linear trap) to yield instrumentation with multiplexed experiment capabilities (multiple sequential MS–MS experiments with accurate $m/z$ determinations for structure elucidation).

#### 3.3.1 TIME-OF-FLIGHT

A time-of-flight (TOF) mass spectrometer uses differences in transit time through a field-free drift region to separate ions. Ions generated in the ion source are pulsed into the field-free drift region (flight tube) by an electric field. Lighter ions have a higher velocity and reach the detector sooner. The TOF mass spectrometer has benefited significantly from the use of fast electronics and fast computers to provide systems characterized by high speed, high sensitivity, and high resolution.

## 4. MS/MS AND $MS^N$ SPECTROMETRY INSTRUMENTATION

A wide variety of tandem MS instruments are available to the pharmaceutical research and development community. Each of the tandem instruments has some unique features that make it particularly suited for some specific applications, but this does not preclude the use of other tandem instruments to meet a specific analytical need. The appropriate selection of a tandem instrument that is suitable to meet a specific need depends on the type of analysis required (e.g., quantitation or structural elucidation), a variety of sample-specific factors (e.g., analyte concentration or the complexity of the matrix), and whether sample fractionation is incorporated before the introduction in the mass spectrometer (e.g., by LC–MS). A brief summary of some of the common types of tandem instruments follows, highlighting some of the typical applications.

### 4.1 MS/MS and $MS^n$ Spectrometers

#### 4.1.1 TRIPLE QUADRUPOLE

The triple quadrupole mass spectrometer is used for both qualitative and quantitative analysis. A triple quadrupole mass spectrometer features a first stage of mass analysis (Q1) for the selection of a precursor ion, followed by an RF-only collision quadrupole region (Q2). A second stage of mass analysis (Q3) is used for product ion analysis. Most quantitative LC–MS-based analyses feature the use of triple quadrupole mass spectrometers. Furthermore, the triple quadrupole mass spectrometer is capable of providing all the most common qualitative scan modes: full scan, product ion, precursor ion, and neutral loss (see *Figure 4* and *4.2 MS/MS and $MS^n$ Spectrometry Operational Modes*).

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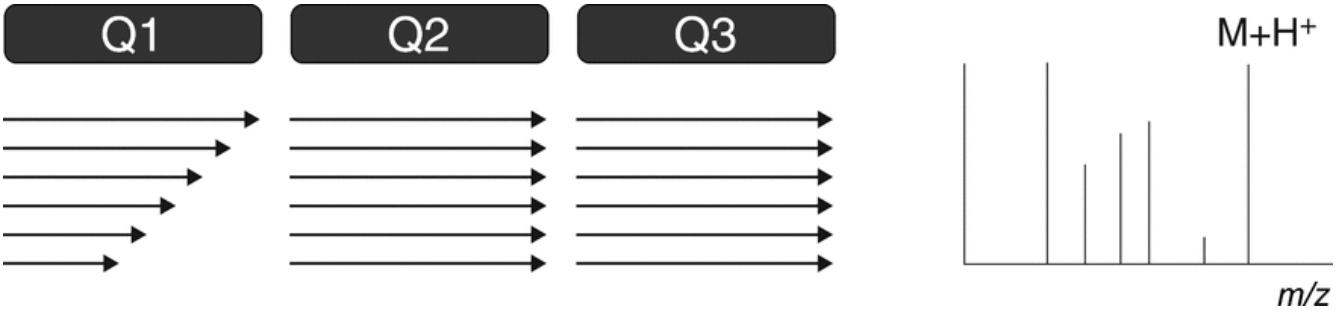
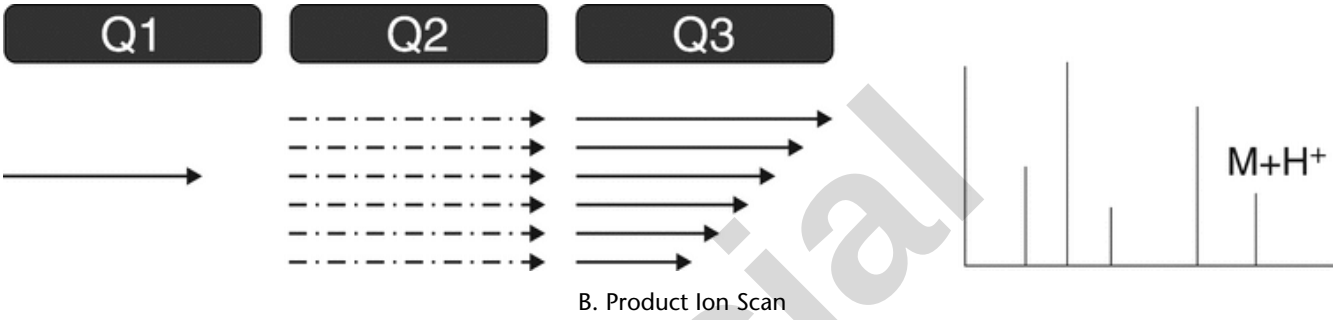
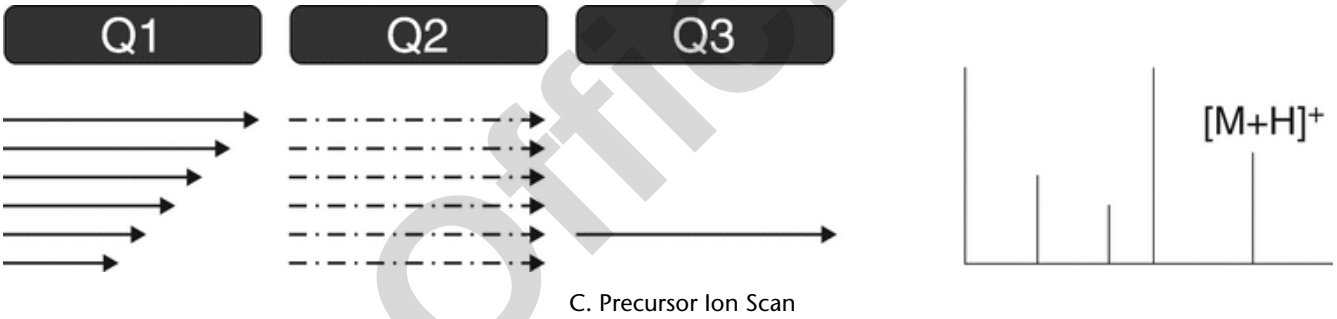


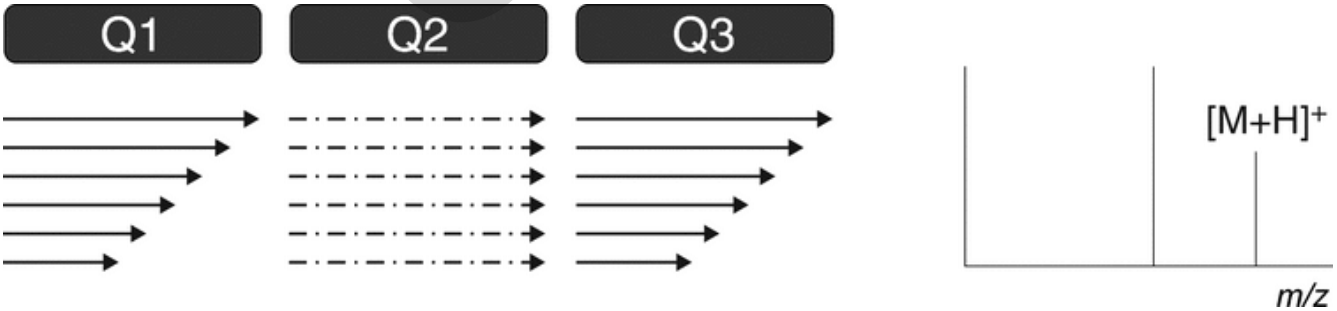

A. Full Scan




B. Product Ion Scan




C. Precursor Ion Scan




D. Neutral Loss Scan Figure 4. Triple quadrupole MS. (A) *Full scan*—Q1 scans the ions produced in the ion source. This scan also can be conducted with Q3. (B) *Product ion scan*—Q1 is set to select a specific molecular ion produced in the ion source. Fragmentation of the ion(s) selected in Q1 occurs in the collision cell (Q2). Q3 scans the resulting fragments. The product ion spectrum contains all the fragments of the selected precursor ion(s). (C) *Precursor ion scan*—Q1 scans the ions produced in the ion source. Ions fragment in the collision cell (Q2). Q3 is set to select a specific product ion (derived from the product ion spectrum). The scan results in a spectrum that contains all precursor ions that generate the specific product ion. (D) *Neutral loss scan*—Q1 scans the ions produced in the ion source, and ions fragment in the collision cell (Q2). Q3 also scans, but at a difference in mass equal to a selected neutral loss (as derived from the product ion spectrum). The scan results in a spectrum that contains all molecular ions that generate a specific neutral loss fragment. (Courtesy of Milestone Development Services, Newtown, PA)

### 4.1.2 TOF–TOF SYSTEM

A tandem configuration for TOF systems typically is used for the characterization and sequencing of peptides and other biomolecules from fairly simple mixtures (e.g., proteolytic digestion of a single-protein therapeutic). These systems often are

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configured for ionization by MALDI, which allows peptide sequence confirmation without the need for an initial separation. The relative ease of use of such instruments and the robust fragmentation data collected for confirmation of peptides may allow TOF–TOF instruments to be used for monograph identification testing in the future.

#### 4.1.3 MAGNETIC SECTOR

Tandem configurations for magnetic sector instruments traditionally have been used for analyses that require high resolution and mass accuracy, such as structural elucidation of unknowns. However, because of greater resolution and mass accuracy becoming available in other tandem MS platforms (e.g., ion traps and quad-TOF devices), magnetic sector instruments are now less commonly used for these purposes.

#### 4.1.4 ION TRAP

The tandem configuration for ion trap systems allows the isolation and fragmentation of ions in a time-dependent manner, unlike other tandem instruments that have spatially distinct mass analyzers in the path of the ion beam. One advantage of the trap-isolate-fragment-detect mechanism in an ion-trap instrument is the ability to re-isolate product ions from the initial precursor ions for further fragmentation. This sequential fragmentation allows more detailed structural determination to assist in elucidating structural unknowns. For protein and peptide applications, ion-trap instruments have been widely used to identify and sequence peptides and to characterize protein modifications (e.g., phosphorylation and glycosylation).

#### 4.1.5 FOURIER-TRANSFORM ION CYCLOTRON RESONANCE

Fourier-transform ion cyclotron resonance (FT–ICR) mass spectrometers, like ion traps, facilitate a time-dependent isolation of ions. FT–ICR mass analyzers offer high resolution (typically $10^5$–$10^6$) and can achieve sub-ppm mass accuracy, which permits determination of accurate mass and elemental composition for unknown compounds with a high degree of certainty. Ions can be selectively excited or ejected from the ICR cell by generation of a waveform that contains resonant frequencies of the $m/z$ values of the ions of interest. Several fragmentation procedures are available for achieving the structural characterization of small and large molecules with FT–ICR MS, including collision-induced dissociation (CID), electron capture dissociation (ECD), and infrared multiphoton dissociation (IRMPD). Some commercial FT–ICR MS systems use a tandem ion trap to isolate and fragment precursor ions, the product ions of which are subsequently transferred to the ICR cell for detection or additional fragmentation by other procedures. The high resolution and mass accuracy of FT–ICR MS make this instrument particularly well suited for the structural characterization of components that comprise highly complex mixtures of small and large organic molecules. FT–ICR MS also has been widely used in the characterization of lipids and carbohydrates and in the characterization and sequencing of peptides and proteins, including examination of unique features such as post-translational modifications.

#### 4.1.6 HYBRIDS

A variety of hybrid tandem mass spectrometers have emerged to either enhance specific applications or to broaden the overall utility of the instrument in a variety of applications. Currently the most common hybrid instrument used for pharmaceutical characterization is the quadrupole TOF (Quad-TOF) MS, which combines the scanning speed of a quadrupole mass filter with the resolving power and mass accuracy of the TOF mass analyzer. This combination is well suited for structural elucidation because the improved resolution and mass accuracy of the TOF (by comparison with a quadrupole) allow a greater degree of certainty in determining the probable elemental makeup of both the precursor and product ions. In addition, the use of a quadrupole mass filter in this tandem configuration allows scanning functions like those outlined for a triple-quadrupole instrument (*Figure 4*), although additional postprocessing of the data is required. An increasing number of hybrid configurations have been marketed to take advantage of the unique feature of the combinations for resolution, mass accuracy, and quantitation. Some of these configurations include Quadrupole–Trap, Trap–TOF, Trap–IRC, and orbital trapping mass spectrometers.

### 4.2 MS/MS and $MS^n$ Spectrometry Operational Modes

#### 4.2.1 FRAGMENTATION PRODUCTION MODES

Collision-Induced Dissociation (CID) or Collisionally Activated Dissociation (CAD) is the process by which a selected precursor ion undergoes collisions with neutral gas molecules in a collision region to yield product ions. Fragmentation occurs at relatively low energies (1–100 eV) primarily at the site(s) of ionization to yield a fragment ion and a neutral molecule. Nitrogen, argon, or helium typically is used as the collision gas with collision energies in the 10–50 eV range.

Electron Capture Dissociation (ECD) and Electron Transfer Dissociation (ETD) are two distinct but related fragmentation methods used primarily to fragment proteins and peptides to generate *c*- and *z*-type ions that are useful for determining protein sequence and characterization of protein modifications. The fragmentation in each case relies on the introduction of either a free electron (ECD) or a radical anion (ETD) into the gas phase of positively charged molecules to induce chemical fragmentation. The advantage of these methods for proteins and peptides versus CID is that the chemical fragmentation occurs almost exclusively along the peptide backbone, thereby preserving the position and context of protein modification that often are lost during CID.

**4.2.2 fragmentation analysis modes**

The most common MS/MS scan modes are described in this section.

*4.2.2.1 Product ion:* The product ion scan mode is illustrated in *Figure 4B* for a triple-quadropole (QQQ) instrument. Application of the product ion scan mode is performed when the first mass analyzer (Q1) is held at a specific $m/z$. This ion, typically the protonated molecule, $[M + H]^+$, is fragmented in the collision cell (Q2). The second mass analyzer (Q3) scans the

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resulting product ions. For a trapping device such as an ion trap mass spectrometer, ions of a specific $m/z$ are isolated and are collisionally activated, followed by scanning of the product ions. In either case, the resulting product ions' MS–MS spectrum contains the diagnostic fragment ions indicative of the characteristic substructures of the selected analyte. This scan mode requires that a full scan mass spectrum be obtained before the product ion experiment to determine the appropriate selection of the molecular ion (typically $[M + H]^+$). The product ion scan mode is available in all mass spectrometers capable of MS–MS operation.

*4.2.2.2 Precursor ion:* The precursor ion scan mode is illustrated in *Figure 4C*. Application of the precursor ion scan mode is performed when the first mass analyzer (Q1) is scanned and allows all ions created in the ion source to pass into the collision cell (Q2). The second mass analyzer (Q3) is held at a specific $m/z$ ratio that corresponds to a diagnostic or unique fragment ion of the molecule or class of molecules. The resulting precursor ion MS–MS spectrum contains all molecular ions that contain a diagnostic fragment ion. The precursor ion scan mode requires that the product ion experiment be performed to confirm the diagnostic fragment ion(s). This scan mode can be used to screen for molecules that contain a specific substructural feature such as a peptide residue. The precursor ion scan mode is available on triple-quadrupole, magnetic sector, and some hybrid instrumentation.

*4.2.2.3 Neutral loss:* The neutral loss scan mode is illustrated in *Figure 4D*. Application of the neutral loss scan mode is performed when both mass analyzers (Q1 and Q3) are scanned at the same rate. The second mass analyzer (Q3) is offset (lower) from the first by a constant $m/z$ difference. Molecular ions enter Q1 and fragment in the collision cell (Q2), and the resulting fragment ions are detected after passage through Q3. Because of the offset scan function described above, the resulting spectrum presents all molecular ions that have undergone the selected neutral loss. This scan mode also requires that the product ion scan be performed to confirm the diagnostic neutral loss(es). Like the precursor ion scan mode, this scan mode is used to screen for molecules with a diagnostic structural feature such as phosphate or glucuronide conjugation. The neutral loss ion scan mode is available on triple-quadrupole, magnetic sector, and some hybrid instrumentation.

*4.2.2.4 $MS^n$:* The $MS^n$ acquisition mode is essentially a sequential application of the product ion scan discussed in *4.2.2.1 Product ion*. That is, it is used to further fragment product ions from a previous product ion experiment. Thus it can be utilized to obtain additional structural information for identification of analytes. This acquisition mode typically is available only on ion trap or FT–ICR instruments.

## 5. QUALITATIVE ANALYSIS

MS is a powerful method and important tool for structure identification because of its ability to provide information about the mass, elemental composition, and structural features of known and unknown molecular entities. In its simplest terms, an MS separates and mass-measures ions related to the sample. A mass spectrometer separates, detects, and records the presence of ions, not neutral molecules. For the purposes of small-molecule organic MS, the charge state ($z$) typically is 1.

A wide range of mass spectrometers and ionization interfaces are available for specific types of MS measurements. Thus a diverse range of experimental approaches can be employed for any given MS measurement needed. Some of the more common and applicable approaches are presented in this section. The appropriateness of a given MS experimental approach is demonstrated by the respective validation data provided in the submission supporting a new monograph or monograph procedure.

### 5.1 The Mass Spectrum

The mass spectrum typically is displayed as a plot of $m/z$ on the abscissa versus ion intensity as the ordinate and frequently is normalized to the most intense ion in the spectrum. Some characteristic features in a typical full-scan mass spectrum include (depending on ionization procedure) the molecular ion (e.g., $M^+$), protonated or deprotonated molecules ($[M + H]^+$ or $[M - H]^-$), or adduct ions (e.g., $[M + NH_4]^+$), all of which are indicative of the molecular mass of the analyte. Mass spectra may be more or less complex, depending on the specific analyte(s) and ions detected. EI mass spectra typically are rich in fragment ions, but API and soft-ionization procedures may produce spectra that are sparse in fragment ions but often feature more prominent or base peak ions that are indicative of MW. Therefore, the interpretation of mass spectra may be more or less straightforward. Typically, the availability of any supporting information about the sample (e.g., origin, history, preparation, stability, solubility) can greatly assist with interpretation. Two primary considerations merit initial discussion because of their importance in the interpretation of mass spectra: resolution and mass accuracy.

#### 5.1.1 MASS RESOLUTION

In MS, resolution is the extent to which two ions of adjacent $m/z$ can be distinguished from each other, and has been expressed in different ways. Two adjacent peaks in a spectrum at values $m$ and $m - \Delta m$ may be separated by a valley, which at its lowest point is a specified maximum amount of overlap between the peaks. The resolution would be expressed as $m/\Delta m$. For example, the term "10% valley" describes a 10% amount of overlap between two peaks and is appropriate when the peaks are of equal height and shape. In practice, however, adjacent peaks of equal height and shape are rarely observed. Therefore, resolution also can be calculated by using the apparent width of a single peak at a given point in mass range as $\Delta m$, and it often is expressed as the measured width of a peak at half-height (full-width half-height maximum, or FWHM). Resolution is an important factor in establishing mass accuracy, evaluating isotope patterns and abundance, determining charge state of multiply charged ions, and distinguishing nominally isobaric interferences.

Nominal mass spectrometers instruments typically provide mass resolution on the order of 1000, whereas for high-resolution mass spectrometry (HRMS) this can range from 5000 to 1,000,000. Improved resolution includes the ability to separate and distinguish isobaric interferences (analytes with the same nominal mass but with different empirical formulae) and to mine complex data sets more efficiently (using increased specificity).

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**5.1.1.1 Nominal resolution:** A number of different types of mass spectrometers are capable of generating nominal resolution mass spectra. Probably the most common mass spectrometer employed for nominal mass resolution work is the quadrupole mass spectrometer. In a quadrupole mass spectrometer, ions pass through a set of four rods such that opposing rods ramp through RF or DC, effectively scanning through the desired $m/z$ range and allowing only a single $m/z$ to pass at any given instant. The net effect is to obtain nominal mass resolution (e.g., $m/z$ 400 can be reliably distinguished from $m/z$ 399 and $m/z$ 401) and to obtain a spectrum that can define monoisotopic mass and associated isotopic cluster (e.g., A + 1 and A + 2). With this type of mass spectrometer (nominal resolution), the primary information obtained is an indication of the molecular mass of the analyte and, depending on the experimental approach used (e.g., EI ionization), ion fragments related to the structure of the analyte.

**5.1.1.2 High resolution:** HRMS has important advantages for the identification of analytes. The various forms of this technology allow determination of the mass of small molecules to the third or fourth decimal place (i.e., approximately 0.1 to 10 ppm mass error). This provides an added degree of specificity (certainty) that, when combined with isotope ratio information and chemical sense, results in elucidation of the analyte's empirical formula as a starting point for further analysis. Thus HRMS offers advantages compared to nominal MS, for which nominal mass is the starting point. HRMS can be performed with a number of different technologies, including TOF–MS, as well as orbital-trapping MS and ion cyclotron resonance MS, where the latter two approaches utilize Fourier transformation to process the raw data. These systems have different advantages, including improved sensitivity for full spectrum data collection (versus scanning instruments like quadrupoles), as well as resolution, mass accuracy, and calculation of the isotopic ratio intensities.

### 5.1.2 MASS ACCURACY

Mass accuracy is the comparison of an experimentally determined $m/z$ to the true $m/z$ ratio. Mass accuracy is influenced by a number of factors, including mass resolution, signal abundance, calibration type and algorithms, choice and number of calibrants used, interferences such as chemical noise, and linear dynamic range inherent with a particular type of mass spectrometer. Mass accuracy may be expressed as millimass unit (0.001 μ) differential or ppm. Mass accuracy, as ppm, is given by the following:

$$\Delta m_{acc} = m_{true} - m_{meas}$$

$$ppm = (\Delta m_{acc}/m_{true}) \times 10^6$$

$m_{meas}$ = measured value

$m_{true}$ = calculated or true mass

Although resolution and accuracy may not be directly correlated, HRMS typically allows improved assignment of exact mass. In practice, high resolution and high mass accuracy are both necessary to minimize error introduced into the mass measurement by isobaric interferences. Sufficiently high resolution and mass accuracy together can facilitate assignment of elemental composition to ions in a mass spectrum, assisting with confirmation or elucidation of structure, respectively, for known or unknown compounds.

## 5.2 Interpretation of Mass Spectra

When interpreting the mass spectra of unknown compounds, analysts typically can differentiate unknowns into two broad categories.

The first category, representing the situation more commonly encountered, can be described as known unknowns or compounds for which supportive information may be available or can be determined by comparison with standards, related substances, metabolites, synthetic impurities, or intermediates. In this case, the objective is verification of a compound's identity by demonstrating its consistency with a standard material by using predetermined criteria. This objective often is achieved by the direct comparison of mass spectral features of an unknown with either a concurrently analyzed standard or an archived library standard mass spectrum generated with similar instrumentation and conditions. For high-resolution accurate mass spectra, confirmation of identity may include the verification of elemental composition. Characteristic fragmentation patterns obtained under consistent conditions also can be used for the confirmation of identity by providing a fingerprint that indicates functional groups, substituents, and structural differences or modifications. The importance of chromatographic separation (i.e., demonstration of retention characteristics that are similar to a standard) also should be considered as part of an overall identification confirmation. The importance of chromatographic separation is particularly evident with the identification of various structural isomers or enantiomers for which MS alone may not provide sufficient distinction.

The second category comprises true unknowns or compounds for which no or very limited information about structure or background is available and presents the most complex cases for spectral interpretation. True unknowns are encountered less frequently and often necessitate the combination of multiple spectral procedures in order to achieve a complete structural elucidation. For true unknowns, any relevant information (e.g., origin, preparation, matrix, solubility, other compounds if the sample is a mixture) can aid interpretation.

The first step in the interpretation of mass spectra is the determination of monoisotopic mass. This peak is highly dependent on the ionization procedure employed, analytical conditions, and characteristics of the molecules from which the ions are formed. There are prominent differences between EI and API mass spectra, which, for purpose of this discussion, generally result from hard- and soft-ionization procedures, respectively. EI forms primarily an odd-electron radical molecular cation, $M^{+\cdot}$, and API and other soft-ionization procedures typically produce characteristic even-electron protonated $[M + H]^+$ or deprotonated $[M - H]^-$ molecules.

The Nitrogen Rule can be useful in identifying the molecular ion peak: it states that an odd-electron ion containing either no nitrogen or an even number of nitrogen atoms will be observed at even nominal mass and that an odd-electron ion with an odd number of nitrogen atoms will appear at odd nominal mass because nitrogen has an even integer mass (14) and an

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odd valence (3). This rule holds for all compounds that contain carbon, hydrogen, oxygen, nitrogen, sulfur, and the halogens, as well as phosphorous, boron, silicon, sulfur, and alkaline earth metals. Applied to even-electron ions (e.g., $[M + H]^+$ or $[M - H]^-$), the Nitrogen Rule states that an even-electron ion containing either no nitrogen or an even number of nitrogen atoms will be observed at odd nominal mass and that an even-electron ion with an odd number of nitrogen atoms will appear at even nominal mass.

The formation of molecular adducts can assist with determining and verifying mass in both positive and negative ionization modes. Some common adducts observed in positive ion mass spectra are $[M + Na]^+$, $[M + K]^+$, and $[M + NH_4]^+$ adducts. Adducts with other inorganic or organic cations, such as Li, Ag, Cs, $H_2O$, acetonitrile, methanol, isopropanol, and small organic protonated or quaternary amines, also can be generated. Adducts present in negative ion mass spectra include those formed with Cl, Br, and formic or triflouoroacetic acid. Multiple adducts are not uncommon in either positive or negative ionization mode mass spectra from API and other soft-ionization procedures and can include combinations of protonated or deprotonated molecules accompanied by additional protons, multiple inorganic or organic cations, or anions. For some applications, it may be desirable to deliberately produce adducts by the modification of the sample solution, matrix, or mobile phase to induce characteristic fragmentation or to denote components in a mixture with common or unique structural features. Dimeric or polymeric analyte ions $[2M + H]^+$ or $[2M - H]^-$ may be observed respectively in positive or negative ion API mass spectra, often with high analyte concentrations. Analogous dimeric adducts with Na, K, and other inorganic cations also can be formed and observed and can provide verification of the MW. Overall, adduct formation and ultimately the presence of adduct ion peaks in mass spectra are highly dependent on ionization procedure, ionization source conditions, type of mass spectrometer or analyzer, sample matrix, mobile phase composition, and the analyte itself.

The relative intensity of mass spectral peaks can provide information about molecular and ion structure. The most intense peak in a mass spectrum is referred to as the base peak and represents 100% relative abundance in normalized mass spectra. In EI mass spectra, the base peak may or may not represent the molecular ion. However, with API and softer ionization procedures, $[M + H]^+$ or $[M - H]^-$ often appear as a prominent peak in the mass spectrum. The relative intensities of peaks in EI mass spectra, and to some extent in API and other mass spectra, can indicate ion stability.

## 5.3 Elemental Composition and Structure

Each molecule has a specific elemental formula or composition that by itself does not suggest anything about the structure of the molecule, because completely different molecules can have identical empirical composition. The mass spectrum of an analyte indicates its monoisotopic mass, but the larger the mass, the greater the number of possible empirical formulae or combinations of atoms that could produce that same nominal *m/z* signal.

## 5.4 Isotope Patterns

The relative intensity and position of isotope cluster peaks in a mass spectrum provide orthogonal information to the *m/z* data. Isotopes represent variants of a given element with the same number of protons and a different number of neutrons. The number and relative abundance of isotopes are unique to each element and together comprise the isotope pattern for a particular molecule in a mass spectrum. The relative abundance of each isotope also is a function of the number of each element in a molecule. Natural isotopic abundances depend on the source or provenance of a material and are influenced by variations of the isotopic abundances of its constituent elements. Because a unique elemental formula is consistently indicated by each peak coinciding with the *m/z* and relative abundance of each isotope in a molecular formula, the resulting isotopic fingerprint can be a powerful tool for the prediction of elemental composition.

Chlorine and bromine are examples of elements that produce prominent and easily recognizable isotope patterns in a mass spectrum. Chlorine atoms are characterized by the isotopes $^{35}Cl$:$^{37}Cl$ (approximately 3:1) and bromine atoms by unmistakable doublets ($^{79}Br$:$^{81}Br$ = approximately 1:1) that enable easy identification of ions containing these atoms in a mass spectrum. Combinations of multiple Cl or Br atoms in a molecule yield characteristic isotope peaks that differ by two units and have relative abundances that can be calculated. For some types of HRMS (TOF in particular), the relative intensities of the isotopic cluster can be carefully defined. Because the relative intensity of the isotope cluster is exactly a function of the elemental composition of the molecule (that is easily calculated), isotope fitting of experimental data to theoretical intensity can help provide the correct formula.

*Figure 5* presents the isotopic pattern in the molecular ion region for *n*-butyl benzenesulfonamide collected using ESI with different mass spectrometers, each having different mass resolution. With nominal mass (i.e., quadrupole mass spectrometer) resolution, one can clearly see the A + 1 (*m/z* 215) and A + 2 (*m/z* 216) peaks of the $[M + H]^+$ ion at *m/z* 214. The major contributor to the A + 1 peak results from the occurrence of versions of the molecule containing $^{13}C$, and the major contributor to the A + 2 peak, is naturally occurring $^{34}S$. With a higher-resolution mass spectrum, the more accurately calculated relative abundances of the A + 1 and A + 2 peaks could be used to estimate the number of carbon and sulfur atoms in the molecule, respectively. An examination of the FT–ICR mass spectrum illustrates the additional isotopic information that can be obtained under even higher-resolution conditions. This spectrum clearly shows that the A + 1 peak, seen as one peak with the other two mass spectrometers, also has contributions from other isotopes. At resolutions afforded by ICR or orbital-trapping mass spectrometers, the various contributors to the A + 1 peak can be observed, and one would get a more accurate estimate of the atoms present.

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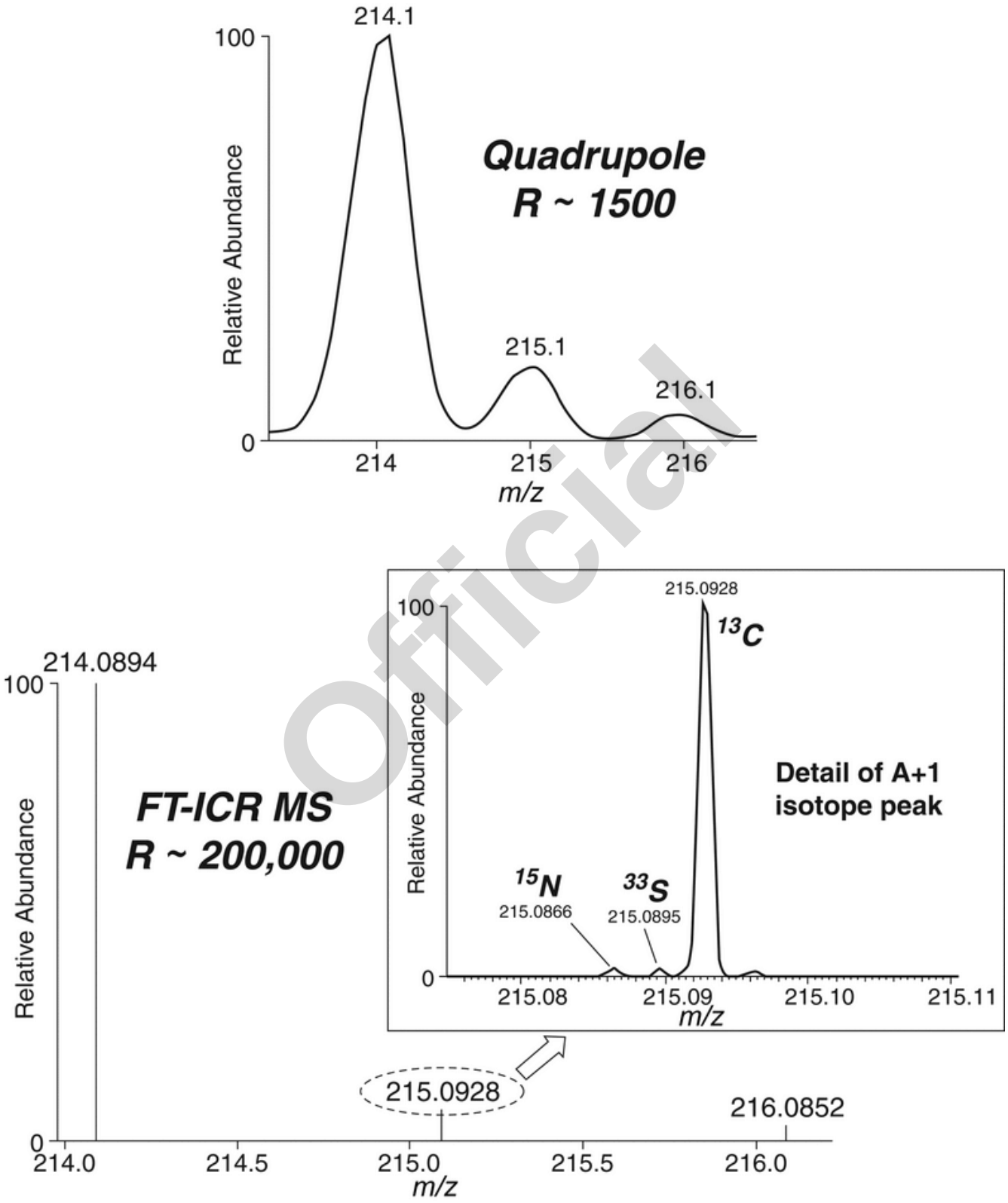


Figure 5. Partial ESI mass spectrum of *n*-butylbenzenesulfonamide under different mass resolution conditions.

Examination of isotope peaks also can reveal information regarding the charge state of an ion. For example, the difference between the A and the A + 1 isotope peaks is 1 *m*/*z* for a singly charged ion, a mass difference of 0.5 *m*/*z* denotes a doubly charged ion, and a mass difference of 0.3 *m*/*z* denotes a triply-charged ion. Thus, the correct charge state can be determined when only one peak or one set of isotope peaks is present, which supports the determination of MW and elemental composition.

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Because accurate mass and isotope fitting are orthogonal data that each separately can greatly reduce the number of possible empirical formulae for a given nominal mass, the information used together can reduce experimental data to a single or limited set of possible choices.

## 5.5 The Monoisotopic Ion

The monoisotopic ion is an important feature of a mass spectrum, and recognizing this ion is valuable in determining the elemental composition of a molecule or ion, particularly with accurate mass measurements. The monoisotopic mass of an ion or molecule is the exact mass calculated using the mass of the most abundant naturally occurring isotope of each constituent element. Therefore, the monoisotopic ion is represented by the isotopic peak composed of the most abundant isotopes of its elements. Importantly, MS observes the monoisotopic mass of the analyte, not its molecular weight (i.e., the average including contributions from heavy isotopes). Recognizing the monoisotopic ion peak can be straightforward, and some singly charged ions and elements present simple or easily recognizable isotope patterns. However, the combination of multiply charged ions and multiple elements that collectively contribute to complex isotope patterns occasionally can make determination of the monoisotopic ion peak quite challenging, particularly with polymers and biomolecules. In such cases the benefit of instrumentation that offers higher resolving power is readily apparent.

## 5.6 Fragmentation

Fragmentation of an organic molecule in a mass spectrometer and examination of the ions produced in the fragmentation process can provide detailed information about unique structural features such as functional groups, substituents, and connectivity. Fragmentation generally is the dissociation of an activated analyte ion. Observed fragmentations are quite diverse and can range from simple events such as loss of water or ammonia to highly complex pathways that involve adduction, multiple rearrangements, and atom migration. In MS, fragmentation is highly dependent on the type of instrumentation and operating conditions. Primary considerations include ionization source, collision zone conditions, and type of mass analyzer. To some extent, controlling these factors permits the selection and optimization of conditions that are conducive to generating desired or structurally diagnostic fragmentation.

EI fragmentation results entirely from unimolecular dissociation that occurs in high vacuum. Formation of a radical molecular ion ($M^{+\cdot}$) in an electron beam (typically 70 eV) is accompanied by redistribution and accumulation of energy into different vibrational modes, and as a result the molecular ion may undergo fragmentation. Fragmentation typically occurs by bond cleavage and produces an even-electron cation and a neutral odd-electron radical. The converse also can occur, albeit less frequently: an odd-electron radical cation is observed with an even-electron neutral fragment loss. Fragment ions can further dissociate to generate additional fragments. Generally, fragmentation of odd-electron ions may produce either odd-electron or even-electron ions, but even-electron ions fragment to other even-electron ions. The probability of cleavage of a particular bond in a molecule can be correlated with bond strength and the relative stability of the resulting fragment ions. Some general principles apply with regard to the presence of fragment ion peaks observed in EI mass spectra and can assist with interpretation. These principles have been thoroughly discussed in numerous texts on the topic and are not discussed here.

API and soft-ionization procedures predominantly produce ions such as $[M + H]^+$ or $[M - H]^-$ that exhibit limited fragmentation. API and other softer ionization procedures achieve fragmentation by activating ions in a variety of ways, depending on the type of instrumentation.

## 5.7 Biomacromolecules

Structural elucidation of biomacromolecules using MS offers a number of challenges that are not common among traditional drug substances described previously in the chapter. To start with, biomacromolecular therapeutics encompass many compounds with very different chemical properties that range from proteins and peptides to a variety of glycoconjugates, lipids, and even DNA and RNA complexes. To further complicate the matter, the molecular mass of biomacromolecules can range from under 1000 in the case of some bioactive peptides and lipids to well over 100,000 for vaccines, antibodies, and heparin complexes. Furthermore, many of these biologically derived substances exhibit considerable heterogeneity and thus may not be characterized as a single drug substance but instead as a mixture that is monitored for consistency from batch to batch using a variety of analytical methods.

This section provides guidance about MS characterization of biomacromolecules. Because of the complexity of this broad class of compounds, this section focuses on a few types of compounds to illustrate the benefits of MS, then comments briefly about how MS can be implemented for other biomacromolecular compounds.

### 5.7.1 PEPTIDES AND PROTEINS

Details of the process for characterizing proteins and peptides are provided in *Biotechnology-Derived Articles—Peptide Mapping* ⟨1055⟩ and are only summarized in this section to provide context.

**5.7.1.1 Spectral interpretation:** Mass spectral evaluation of proteins and peptides typically encompasses MS data collected on the intact compound(s) to establish the overall mass of the protein followed by tandem MS to generate information about the primary amino acid sequence of the protein or to characterize sites of protein modification. Interpretation of fragmentation spectra to generate the sequence information is a critical component of this approach, and thus some guidelines for the spectral interpretation of peptide fragments are provided in *5.7.1.2 Database searching*. Primary and secondary MS-based approaches have emerged as the standards for sequencing proteins by fragmentation and are known as bottom-up and top-down sequencing.

*5.7.1.1.1 Bottom-up*—This approach encompasses fractionation of individual peptides generated by proteolytic digestion and sequencing of the peptides by tandem MS. The complete protein sequence then is recapitulated by piecing together the

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individual sequences. To get complete coverage of the protein, it is necessary to do separate digestions with multiple proteases with different cleavage specificities to get overlapping sequence coverage to rebuild the overall sequence. This approach is currently the most common MS-based approach used to characterize protein therapeutics.

*5.7.1.1.2 Top-down*—Advances in MS-based technologies and particularly ECD and ETD fragmentation offer another approach for sequencing proteins, whereby the intact protein is fragmented to generate sequence information without prior need to digest the protein into smaller peptides. Current challenges with this approach include the need for purified proteins; difficulty in getting complete sequence coverage, particularly for larger proteins; the need for considerably larger quantities of proteins than for the bottom-up approach; and a requirement for a high-resolution instrument with ECD or ETD capabilities. However, during the development of proteins therapeutics the availability of large quantities of purified protein is a likely scenario, and with the continued advancement of MS technologies, top-down proteomics may offer a direct option for characterization of some proteins therapeutics in the future.

**5.7.1.2 Database searching:** Because of progress in whole-genome sequencing and the computing power of predictive algorithms for the annotation of all possible genes, mRNAs, and proteins from genomic information, the characterization of unknown protein and peptide therapeutics rarely requires complete de novo sequence evaluation. Instead, the compound can be initially identified by digestion and sequencing by peptide fragmentation. This is possible because various fragmentation strategies (e.g., CID and ETD as described previously) produce gas-phase fragment ions from protonated peptides that represent consistent cleavage positions along the peptide backbone. The Roepstorff nomenclature for general peptide fragmentation along the peptide backbone is shown in *Figure 6A*. Peptide fragments that maintain a charge on the C-terminus are the *x*, *y*, or *z* ions, and those that maintain a charge on the N-terminus are known as *a*, *b*, or *c* ions. By optimizing the fragmentation energies and the collision gas to target only a single fragmentation event per protonated peptide molecule, analysts can use the collective fragmentation spectra from a given peptide to produce a set of fragment ions from the same series (i.e., *b* ions or *y* ions) that vary by the residue mass of each amino acid. In this manner they can derive the sequence. A schematic of a peptide fragmentation profile with a complete set of *y* ions is shown in *Figure 6B*. The complete set of *y* ions allows the sequence to be determined from the mass difference between the ions that correspond to residue masses for each amino acid. With the development of complete databases of many genes, transcripts, and proteins, a variety of commercially available computer algorithms have been developed to match the fragmentation masses with protein sequences in databases, along with false discovery rates and confidence probabilities. As a result, confirmation of protein sequences for known proteins can be fully automated for data collection and evaluation.

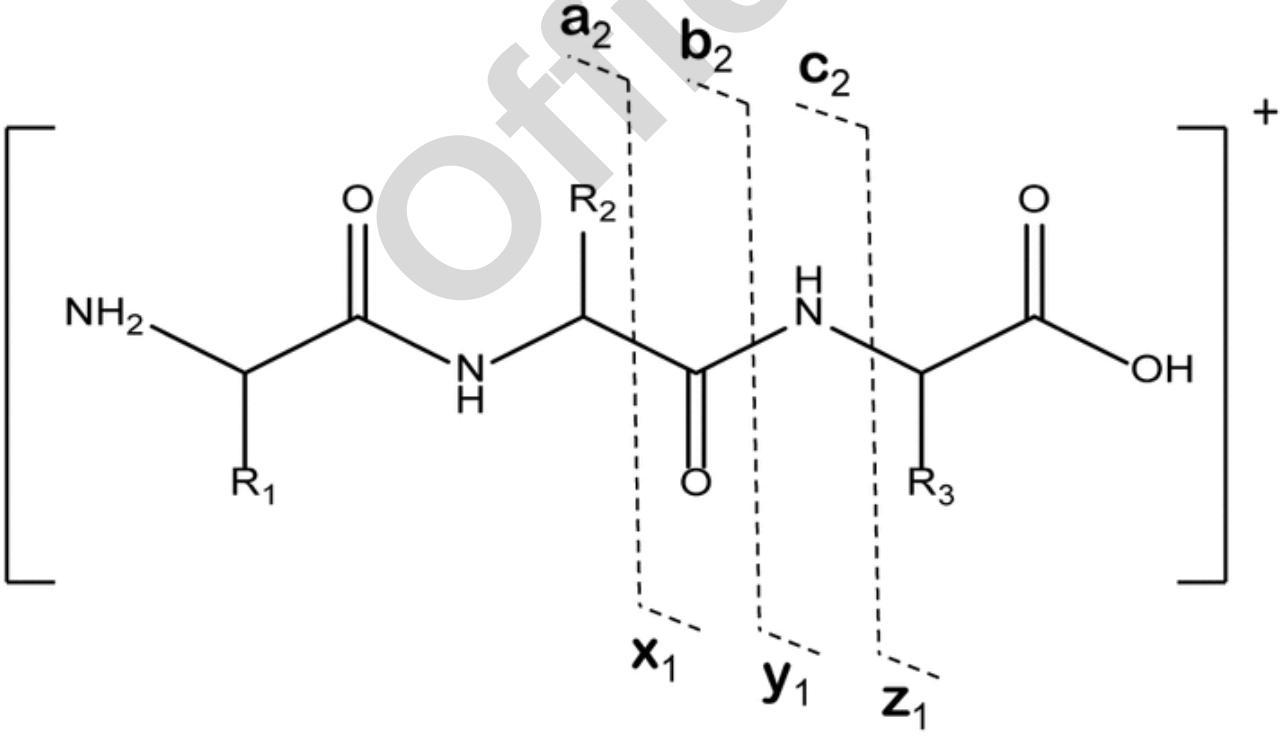


Figure 6A. Peptide fragmentation nomenclature.

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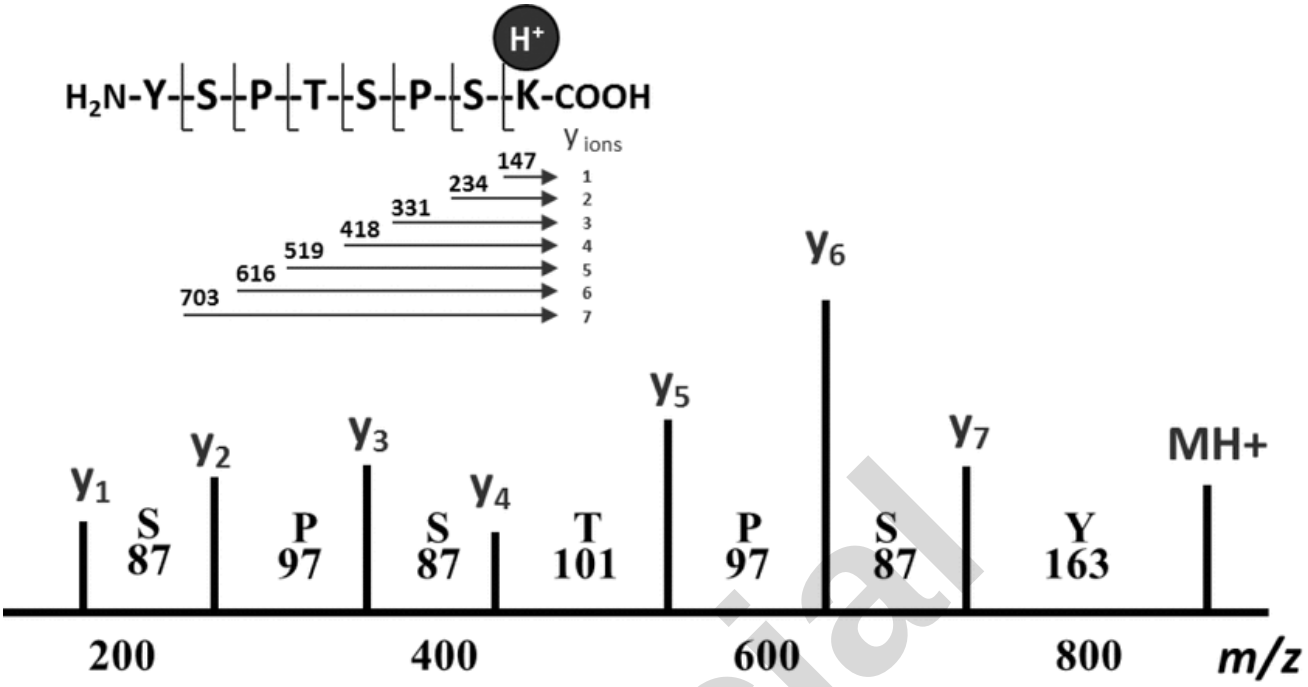


Figure 6B. Peptide fragmentation sequence.

**5.7.1.3 De novo sequencing approaches:** When a protein is completely unknown, MS-based fragmentation can be used to generate sequence information as described. The primary challenge is the inability to always get complete, unambiguous sequence information for some peptides by MS fragmentation because of the inability to assign all amino acids from a given fragmentation spectrum without a template of known sequences. In cases such as these, a combination of chemical sequencing using Edman degradation and MS may be necessary. Alternatively it may be necessary to digest and sequence the protein with multiple proteases in order to generate overlaying sequences to ensure the reliability of the overall sequence assignments.

### 5.7.2 GLYCOCONJUGATES

The analysis of drug substances in this class is discussed in *Glycoprotein and Glycan Analysis—General Considerations* ⟨1084⟩. Thus this section addresses only the application of MS to the characterization of protein glycosylation.

Many biopharmaceuticals are glycoproteins for which glycosylation plays critical roles in maintaining function, stability, and solubility. The most common glycosylations are *N*-linked and *O*-linked glycosylation in addition to other glycosylation modifications such as GPI anchors. *N*-glycosylation refers to glycan modification on the asparagine side chain, and *O*-glycosylation occurs to the side chains of serine and threonine. MS analysis of protein glycosylation can be performed on three different levels: intact proteins, peptides, and glycans. This section provides a brief overview of glycoprotein analysis.

**5.7.2.1 Intact protein analysis:** For proteins with small size or relatively simple glycosylation, direct MS analysis of intact protein provides a mass profile that reflects the protein's primary sequence and major modifications, including glycosylation. This type of experiment usually is performed using TOF analyzers or analyzers with higher mass resolution equipped with ESI or MALDI ionization. MALDI forms mainly singly charged ions and requires MS analyzers with a wide *m*/*z* range for measurement of intact protein mass (e.g.,TOF). ESI–MS is commonly used, because it leads to the formation of multiply charged protein ions in the *m*/*z* range of 700–5000, resulting in better mass accuracy in protein mass measurement (<100 ppm when coupled with a high-resolution mass sepctrometer). MS analysis in many cases can resolve protein peaks by their glycoform distribution because of the mass differences (e.g., hexose 162 Da, HexNAc 203 Da, and *N*-acetyl neuraminic acid 291 Da). The mass information can lead to identification of the monosaccharide composition of the glycoforms. The intensity of the intact protein peaks can be used to roughly estimate the glycoform distribution on the protein. For monoclonal antibodies modified mainly by neutral *N*-glycans, quantification of glycosylation distribution by intact protein analysis appears to be comparable with measurement using glycan separation methods. Antibodies containing multiple glycosylation sites can be enzymatically cleaved into major domains before MS analysis to gain more specific information about the position of the glycosylation on the intact protein.

**5.7.2.2 Peptide analysis:** For proteins with complicated glycoforms and multiple glycosylation sites, site-specific glycosylation information usually is desirable and is achieved by using a bottom-up proteomics approach via analysis of peptides generated by enzymatic digestion. The peptides are separated by reversed-phase LC followed by ESI–MS/MS analysis. MS–MS fragmentation can be performed using CID or ETD. In CID–MS/MS, the peptides can be identified to be glycosylated peptides by the detection of oxonium ions in the peptide MS–MS spectra [e.g., *m*/*z* 163 (hexose), 204 (HexNAc), 292 (sialic acid), and 366 (hexose-HexNAc)]. However, CID–MS/MS spectra of glycosylated peptides usually are dominated with fragmentation from the glycan with minimal fragmentation between the amino acid residues of the peptide backbone, thus hampering the ability to identify the protein sequence. ETD–MS–MS spectra of glycosylated peptides contain mainly fragmentation between the amino acids (e.g., *c*- and *z*-type peptide backbone cleavages), while the attached glycans remain intact. ETD–MS–MS provides

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information about peptide sequence, glycosylation site, and the glycan mass of the glycosylated peptides. CID– and ETD–MS–MS may be used alternatively in the same LC–MS analysis of complicated protein digests, which may provide the information necessary for identification of sequence, glycosylation sites, glycan mass, and glycan branching. *O*-glycosylated peptides also can be analyzed by using similar approaches. Peptide glycosylation can be identified by observation of oxonium ion such as *m*/*z* 204 in CID–MS–MS, and the peptide sequence is identified by ETD–MS–MS when the *O*-glycan moieties remain attached to the peptide.

**5.7.2.3 Glycan analysis:** *N*- and *O*-glycans can be enzymatically or chemically released from proteins followed by MALDI–MS or ESI–MS analysis either directly or after derivatization with chemical tags for enhancement of their ionization.

The direct MALDI–MS analysis of released glycans can be performed in both positive and negative mode for the detection of neutral and acidic glycans, respectively. The commonly used matrices include 2,5-dihydroxybenzoic acid (DHB) and 2′,4′,6′-trihydroxyacetophenone. The neutral glycans usually are detected as $[M + Na]^+$ in positive ion MALDI–MS. ESI–MS analysis of glycans suffers from poor ionization of the glycans and thus are used only following chemical derivatization. For example, derivatization of released glycans from glycoproteins can be performed on the reducing end of glycans by reductive amination using fluorescent tags such as 2-aminobenzamide. The labeled glycans are analyzed using hydrophilic interaction chromatography followed by ESI–MS analysis in positive ionization mode. The molecular mass of the glycans can be determined, and subsequent MS–MS analysis can provide structure information about the glycans. Permethylation is another important glycan derivatization method that neutralizes acidic glycans and allows both neutral and acidic glycans to be detected by MS in positive ion mode. MS/MS spectra of glycans contain ions from two major types of cleavages, as illustrated in *Figure 7*. Glycosidic cleavage occurs at the bonds between two sugar rings and results in *B*, *C*, *Y*, and *Z* ions, which provides important information regarding monosaccharide sequence and glycan branching. Cross-ring cleavages (shown in *Figure 7* by dashed lines) involve rupture of two bonds on the same sugar ring and lead to *A* and *X* ions that assist in identification of the type of sugar linkages in the glycans. With increasing understanding of glycan fragmentation, the identification of glycan from its MS–MS spectrum now is possible via database searches using recently developed computational algorithms.

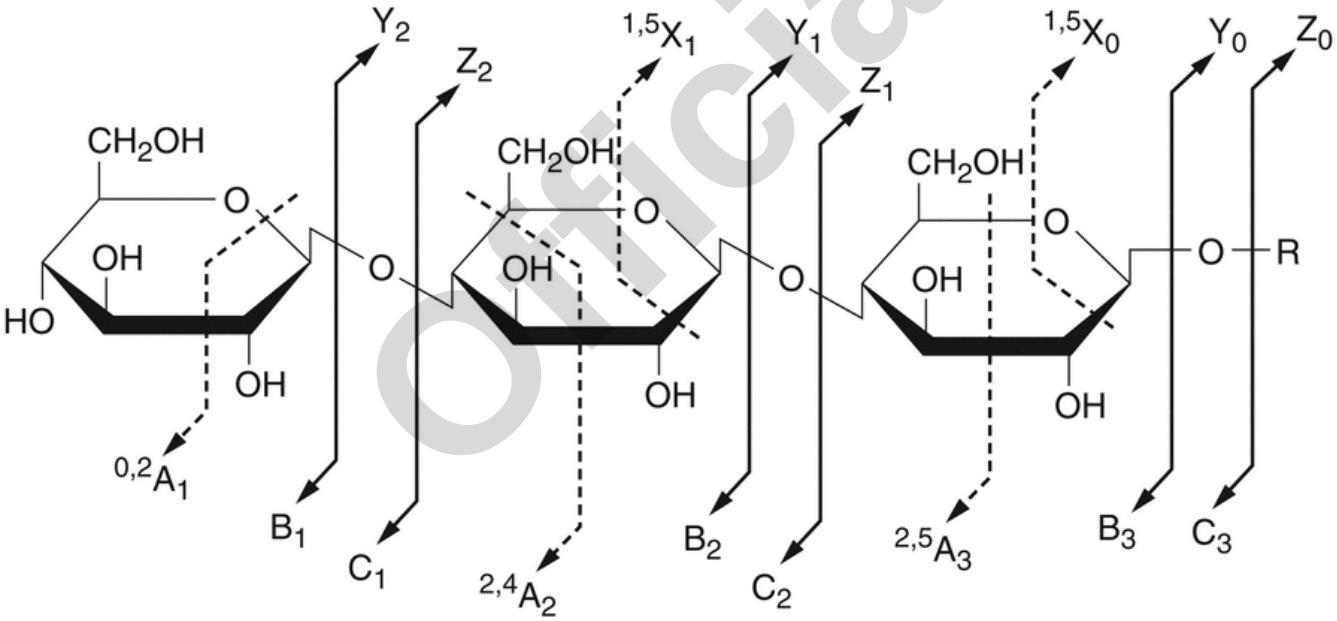


Figure 7. Nomenclature for carbohydrate fragmentation. Source: Adapted from Domon B and Costello CE (1988). *Glycoconj. J. 5*, 397–409.

## 5.7.3 VACCINES

Vaccines present a unique challenge because often they include complex mixtures of proteins, polysaccharides, and lipids in the form of intact bacteria or viruses, outer membrane vesicle preparations, or combinations of recombinant proteins. General information regarding bacterial vaccines is available in *Vaccines for Human Use—Bacterial Vaccines* ⟨1238⟩. Quality control of such mixtures typically relies on characterization by SDS–PAGE, SEC, amino acid or monosaccharide compositional analyses, RP–HPLC, and other analytical methods. However, to date MS has not been used as a primary QC method even though it is frequently used for confirmation of identity and characterization of impurities. Because of the many advantages of MS described in this chapter and its widespread use in the characterization of biotherapeutics and vaccines that contain complex protein mixtures, MS and MS–MS offer the ability to identify product and impurity simultaneously, and likely will be adapted in routine QC environments in the future. Additionally, when a complication occurs (e.g., discrepancy between assays, activity changing after formulation, or posttranslational modification), the quantitation of the recombinant protein (within a complex proteome) or key antigen in a complex protein vaccine via a highly specific procedure such as MS may be desirable. The use of isotope dilution MS–MS in combination with multiple reaction monitoring scans can provide highly specific and accurate quantitation of protein in complex samples.

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### 5.8 Monograph Methods for Qualitative Identification

The discussions in this chapter and in *Mass Spectrometry* ⟨736⟩ covering the qualitative capabilities of MS provide strong technical justification for its use as part of a compendial identification strategy for a wide range of molecular classes. The structural information content (e.g., molecular mass, empirical formula, structurally significant fragmentation, and characterization of biomolecular modifications such as glycosylation) has been and will be an integral part of identification strategies. One or more specific aspects of mass spectral information can be combined to increase the specificity and reliability of the proposed test. Development of the MS portion of the compendial identification test should strive to provide unique and complimentary information for use with other identification tests intended in the monograph. Method development also should consider the availability of a reference standard for successful mass spectrometric identification.

## 6. QUANTITATIVE ANALYSIS

MS is useful for the quantitative determination of actives or impurities in a drug substance or a drug product because of its selectivity and sensitivity. The former typically comes into play when target compounds reside in complex matrices (e.g., natural or formulated), and the latter is a benefit when low-level impurities must be measured. Selectivity is achieved in MS-based methods by the combination of well-characterized attributes of the target compound, including molecular mass, intrinsic ionization characteristics, and fragmentation with additional data such as chromatographic retention time. In addition to chromatographic selectivity, use of a chromatographic inlet helps isolate the target compound from other chemical entities within the sample that could create problematic artifacts (e.g., ionization suppression) if they were introduced concomitantly.

This general approach to sample analysis (i.e., chromatography coupled with MS) typically introduces the target compound into the mass spectrometer as a well-defined peak (ideally, Gaussian) that can be reproducibly integrated for optimal quantification. While other forms of sample introduction (e.g., flow injection and direct-insertion procedures such as MALDI) potentially can be employed and may be necessary in some applications, chromatographic introduction systems are more common and are preferred for MS quantification.

Although various mass spectrometer types can be used, the quadrupole and triple quadrupole, in particular, are most commonly employed for quantitative analysis. These instruments provide a combination of specificity, sensitivity, stability, and linear response, which is essential for accurate measurement of target compounds, particularly within complex formulations. For these reasons, the balance of this quantification discussion emphasizes the use of chromatographic separations in concert with quadrupole MS detection.

### 6.1 MS Quantitation Overview

Employing a chromatographic inlet, analysts introduce a sample solution via injection onto an appropriate chromatographic column, and a flowing carrier stream (gas or liquid mobile phase) advances the target compound(s) and other matrix components through the column at various rates that are determined by strength of the carrier (gas characteristics for GC; organic, along with mobile-phase modifiers, for HPLC), temperature, and affinity for a stationary phase (see ⟨621⟩). Pressurized or near-atmospheric-pressure chromatographic effluent is passed through an appropriate interface into the vacuum environment of the mass spectrometer. In this manner, chemical components within the sample enter the mass spectrometer as chromatographic peaks that are subject to ionization as defined by the chosen ionization mode and according to the propensity of each chemical to become ionized under those experimental conditions.

In the case of single-quadrupole MS detection, the mass analyzer is set to pass to the detector an ion with an $m/z$ value that is characteristic of the target compound. In this selected ion monitoring (SIM) mode, a chromatographic profile for a single $m/z$ value is created. This overall process yields a chromatographic peak at the retention time expected for the target compound. When integrated, the area under this peak leads to a measure of target compound concentration in the original sample. Accuracy in an MS-based quantitative assay is achieved by the proper use of a well-characterized reference standard and, ideally, an appropriate internal standard, as described in *6.2.1 Internal Standards*.

For many analyses, the combination of chromatographic separation and SIM MS analysis will provide sufficient selectivity to yield an analysis, which is sufficiently free of chemical interference to meet the measurement need. However, in some situations, it may be necessary to utilize the additional selectivity provided by tandem MS detection that commonly is provided by a triple-quadrupole instrument. The need can be particularly acute in Category II analytical procedures for which a trace impurity must be measured within a complex formulation.

The relative benefit of MS/MS detection is illustrated in the extreme example presented in *Figure 8*. The antitussive dextromethorphan (Dex) is present in a plasma extract at 100 pg/mL. This extract is first analyzed by LC–MS using ESI–SIM at $m/z$ 272 (protonated molecule). In this experiment the SIM chromatographic peak corresponding to Dex is barely discernible above the relatively high background noise. Alternatively, a triple quadrupole can be employed to set up a selected reaction monitoring (SRM) detection scheme for added selectivity. SRM is a special case of product ion MS–MS in which the second mass analyzer is tuned to pass only a selected fragment ion corresponding to Dex ($m/z$ 147 in this example). A marked enhancement in selectivity, and therefore signal-to-noise ratio, is evident and typically leads to improved performance for quantitative analytical methods.

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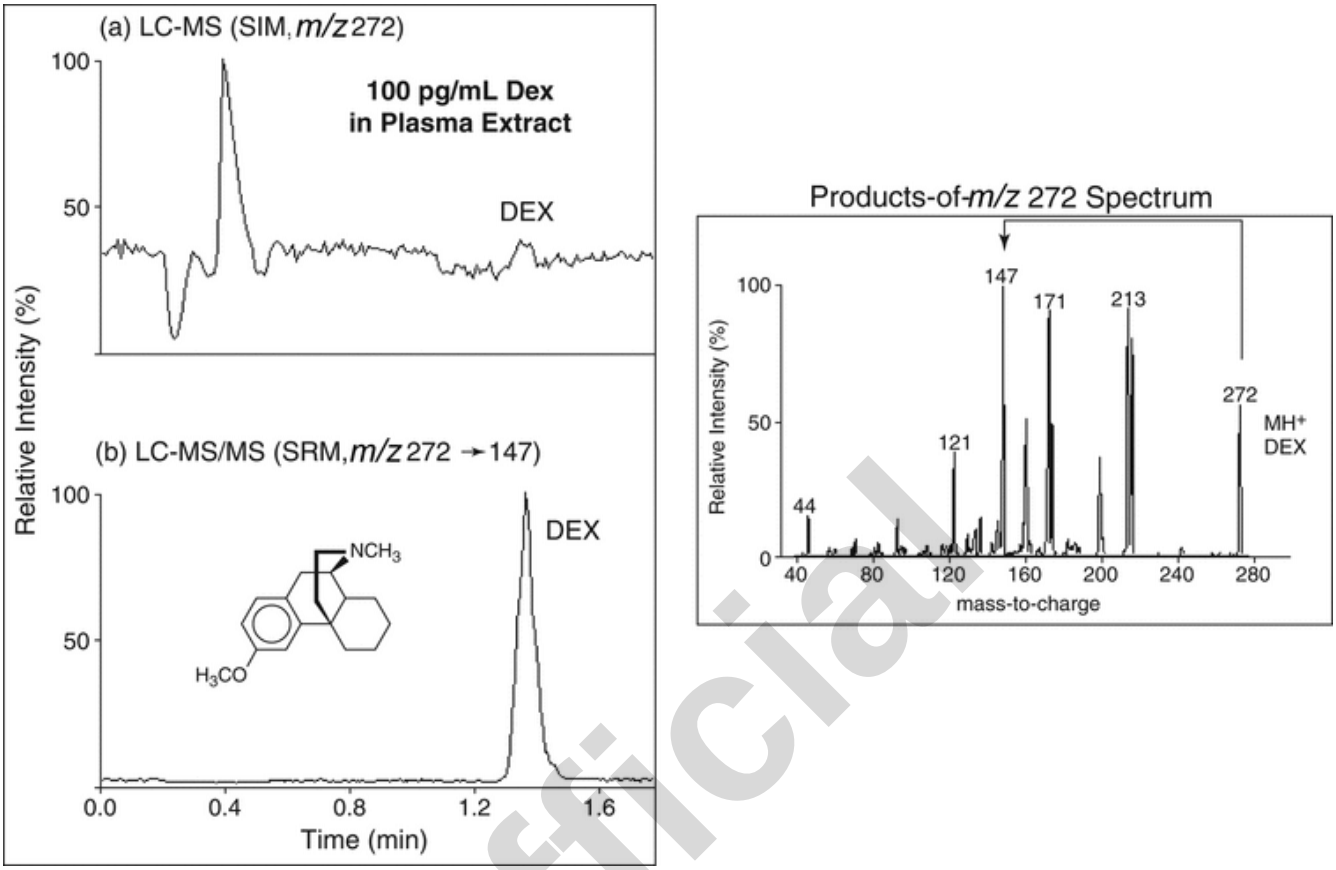


Figure 8. MS (SIM) versus MS–MS (SRM) for quantitative methods.

## 6.2 Quantification Considerations and Procedures

Methods suitable for Category I and Category II applications require the use of a reference standard for each target compound. Quantification of a target compound in an appropriately prepared test sample is achieved by relating its MS response (e.g., SRM-derived chromatographic peak area) to that of similarly prepared reference standards spanning the required concentration range (see *Mass Spectrometry* ⟨736⟩*, Validation and Verification of Mass Spectrometry Analytical Procedures*).

### 6.2.1 INTERNAL STANDARDS

For optimal accuracy and precision, internal standards should be employed at a consistent concentration in both the test samples and reference standards. Internal standards compensate for small sample-to-sample differences in dilution volume or loss of material during sample processing. They also compensate for analysis-to-analysis fluctuations in MS response or sensitivity drift over the course of batch analyses, both of which occur in all MS-based methods. In practice, selective detection schemes are set up to monitor both the target compound (analyte) and its corresponding internal standard. The key instrumental measure for each standard and test sample is, therefore, a ratio of the analyte-to-internal standard chromatographic peak areas. Internal standards can be either structural or stable-isotope-labeled analogs of the target compound. The former may be adequate and often has the advantage of lower cost and availability. The isotopically labeled (e.g., $^{2}H$, $^{13}C$, or $^{15}N$) alternative offers superior method performance, because it is essentially identical to the analyte in chemical and physical properties, with the exception of greater molecular mass. The only requirements are that the isotopic labels are not subject to chemical exchange during storage or sample preparation, are retained in the MS ionization process, and provide a sufficient molecular mass shift (typically 3+ Da using multiple labels) versus the analyte to ensure negligible cross-talk (interference) between analyte and internal standard signals (because of natural isotopes and variable *m*/*z* resolution across different mass analyzers). In addition, care must be taken to ensure that the internal standard contains inconsequential levels of unlabeled target compound impurities that potentially could bias results. Suitability of the internal standard is confirmed after successful completion of the analytical procedure validation.

### 6.2.2 DATA ACQUISITION

For accurate integration of analyte and internal standard chromatographic peaks, the data acquisition parameters (e.g., sampling rate, scan range, or masses monitored) must provide a sufficient number of intensity samples across the peak width. The number of samples may vary depending on method performance requirements (see *Table 1* in general chapter ⟨736⟩) and practical considerations related to chromatography conditions, mass spectrometer type, and the number of analytes and

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internal standards monitored. For example, for single-analyte assays on quadrupole-based instruments, analyte and internal standard SIM or SRM detection schemes are alternately sampled throughout the analytical run. With modern quadrupole instruments, dwell times of 100 ms or less per sampling point are readily achieved and should result in at least eight samples across each peak (this is the suggested minimum for good quantitative representation of the peak).

#### 6.2.3 CALIBRATION

A calibration curve is created by plotting analyte-to-internal standard peak area ratios versus concentration for the reference standards analyzed. For the somewhat narrow calibration range required for Category I and Category II measurements, a simple (nonweighted) linear fit is sufficient to define this response versus concentration function. However, $1/x$ or $1/x^2$ weighting also can be used, as well as nonlinear (e.g., quadratic) functions, as supported by appropriate and provided validation data.

The calibration concentration range is selected based on instrument sensitivity and the required method performance attributes. In general, the lowest concentration should yield sufficient signal for reproducibility, and the highest concentration ideally should be within the linear response range of the MS system. In extreme cases, the upper end of the linear response range may result from limitations of the mass analyzer or detector (e.g., as with TOF and ion trap MS instruments, but much less so with quadrupole mass analyzers). More often, loss of linearity results from diminishing ionization efficiency at higher concentrations. This is a common consideration with, for example, electrospray ionization where high analyte concentrations may increasingly deplete available ionizing adduct ions, leading to a less-than-proportional increase in detected signal with increasing concentration. This so-called self-suppression phenomenon can be somewhat compensated by the use of a stable-isotope-labeled internal standard that co-elutes with the analyte and, therefore, experiences similar proportional suppression. However, best-method performance is achieved when calibration falls within the linear range.

#### 6.2.4 TEST SAMPLE PREPARATION

The scope of acceptable sample preparation procedures for quantitative MS analysis is broad and depends on many factors, including the analyte chemical class, the composition and complexity of the matrix, the target analyte concentration, and the type of analytical instrumentation available. If appropriate, the preferred approach is simple dissolution followed by internal standard addition and then dilution to a target concentration within the calibration range. This simplicity minimizes the potential for analyte recovery issues and contamination or other procedural errors that may compromise the robustness of more complex methods. Provided sufficient instrument sensitivity, optimal method performance typically is achieved with greater dilution factors (lower analyte concentrations in the prepared sample). This approach not only avoids self-suppression issues but also minimizes the potential for ionization suppression due to co-eluting matrix components.

More complex sample preparation procedures may be required to address issues related to the chemical class or concentration of the analyte, as well as the nature of the matrix. In such cases, the use of an internal standard (stable-isotope-labeled standards in particular) is almost essential to ensure adequate method performance. For best results, the addition of the internal standard should take place at the earliest practical stage of sample preparation (e.g., immediately following test sample dissolution).

For example, procedures such as liquid–liquid and solid-phase extraction may be required to enrich (concentrate) target compound(s) for greater assay sensitivity or for isolation from high levels of ionization-suppressing components within the matrix (e.g., salts or other excipients). Chemical derivatization of certain functional groups of a target compound may be desirable to stabilize the sample (e.g., for GC–MS analysis), improve method chromatographic performance, or enhance intrinsic sensitivity for detection (e.g., for ESI). Although such procedures add complexity, they are acceptable provided the overall analytical procedure can be properly validated.

A special case of complex sample preparation that may be required for quantification of macromolecular compounds such as vaccines and biotherapeutics involves the intentional degradation of the target molecule(s) into smaller entities that are more amenable to quantitative MS analysis. For example, partial degradation of a protein therapeutic may be achieved by hydrolytic (chemical) or enzyme-based (e.g., tryptic) digestion to yield peptide fragments that are characteristic of the protein. These smaller molecules can be analyzed by using principles described in this section (including the use of stable-isotope-labeled internal standards of each targeted peptide), and the results can be correlated to determine the concentration of the original target protein. Further, by measuring multiple peptides derived from the protein of interest, analysts can achieve qualitative (identity) verification of the protein by means of the same method. Of course, this indirect approach usually requires some degree of isolation and qualitative identification of the macromolecule to ensure that the subsequent quantitative measure of the representative peptides is relevant.

## 7. EMERGING MS APPLICATIONS

### 7.1 Drug Product Authentication and Contamination Detection

Counterfeit and contaminated or adulterated pharmaceutical materials are a threat to patient safety, consumer confidence, and product security and are therefore of concern to pharmaceutical manufacturers and regulatory agencies. Because increases in the incidence of counterfeited and contaminated pharmaceutical materials have elevated concerns about public health safety and product security, numerous measures have been employed to detect counterfeit and contaminated drug products and to ensure supply chain integrity. Because of its sensitivity and specificity, MS is an effective analytical tool for broad application in the determination of authenticity, source, or contamination of drug products. Approaches include verifying the identity of the active ingredient(s), determining and comparing impurity profiles, and isotopic characterization.

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### 7.1.1 IDENTIFICATION AND/OR VERIFICATION OF THE ACTIVE INGREDIENT

Counterfeit drug products may contain multiple or different active ingredients or no active ingredient at all. GC–MS, LC–MS, or direct-infusion MS procedures can provide a verification of the presence or absence of an active ingredient or the identity of other active ingredients that may be present. Counterfeit drug products containing multiple active ingredients or very high levels of a single active ingredient (superpotent samples) typically are of greater concern to human safety than those that contain little or none. In cases such as these, MS can provide positive, unequivocal proof of identity, as well as quantitative information sufficient to pursue those responsible for producing the counterfeit products.

### 7.1.2 IMPURITY PROFILING

Impurity profiles can indicate contamination of a drug product or can distinguish an authentic product from a counterfeit. Impurity profiles serve as fingerprints for a drug substance or formulated product from a particular source. Hyphenated techniques such as LC–MS and GC–MS can be used to resolve and identify process-specific impurities that are characteristic of a particular synthetic route, which can be useful in determining the origin and distribution of counterfeit materials. Variations in the impurity profile of the authentic material and changes in the profile with time and process conditions must be considered when analysts use impurity profiles for identification or as indicators of potential contamination. With suspect counterfeit products, similarity of impurity profiles may not necessarily indicate a suspect sample is authentic. In such cases, additional investigation using orthogonal procedures may be necessary.

### 7.1.3 ISOTOPIC CHARACTERIZATION

Stable isotopes of elements such as carbon, nitrogen, hydrogen, and oxygen occur naturally in pharmaceutical products and raw materials. Isotopic ratios principally depend on starting materials and processes used to produce drug substances and drug products and can be highly specific for a given batch. Therefore, isotope ratios can define an isotopic fingerprint for a given material, and observed differences in specific isotope ratios between batches can be used to authenticate materials or indicate potential contamination. Isotope ratio mass spectrometry (IRMS) is a powerful tool for pharmaceutical authentication or detection of potential contamination in drug products and raw materials. IRMS affords a high level of specificity, accuracy, and precision in the determination of certain elemental (e.g., carbon, nitrogen, oxygen, and hydrogen) isotope ratios in drug substances or products. Because specific isotope ratios can be influenced by multiple factors such as location, climate, process differences, and source and quality of raw materials, IRMS is able to provide information that is not only useful in authenticating a drug product but also in indicating the origin of a drug product or material. This is particularly important in determining whether distributed counterfeit drug products obtained at discrete locations originate from the same manufacturing process or when linking a diverted product with its original manufacturing site. *Figure 9* illustrates subtle but notable differences between authentic drug product samples from three global manufacturing sites and overt differences between authentic and three counterfeit drug products. Additionally, IRMS approaches authentication from a postproduction perspective and does not involve marking or altering the drug product, as may be required by other pharmaceutical authentication technologies.

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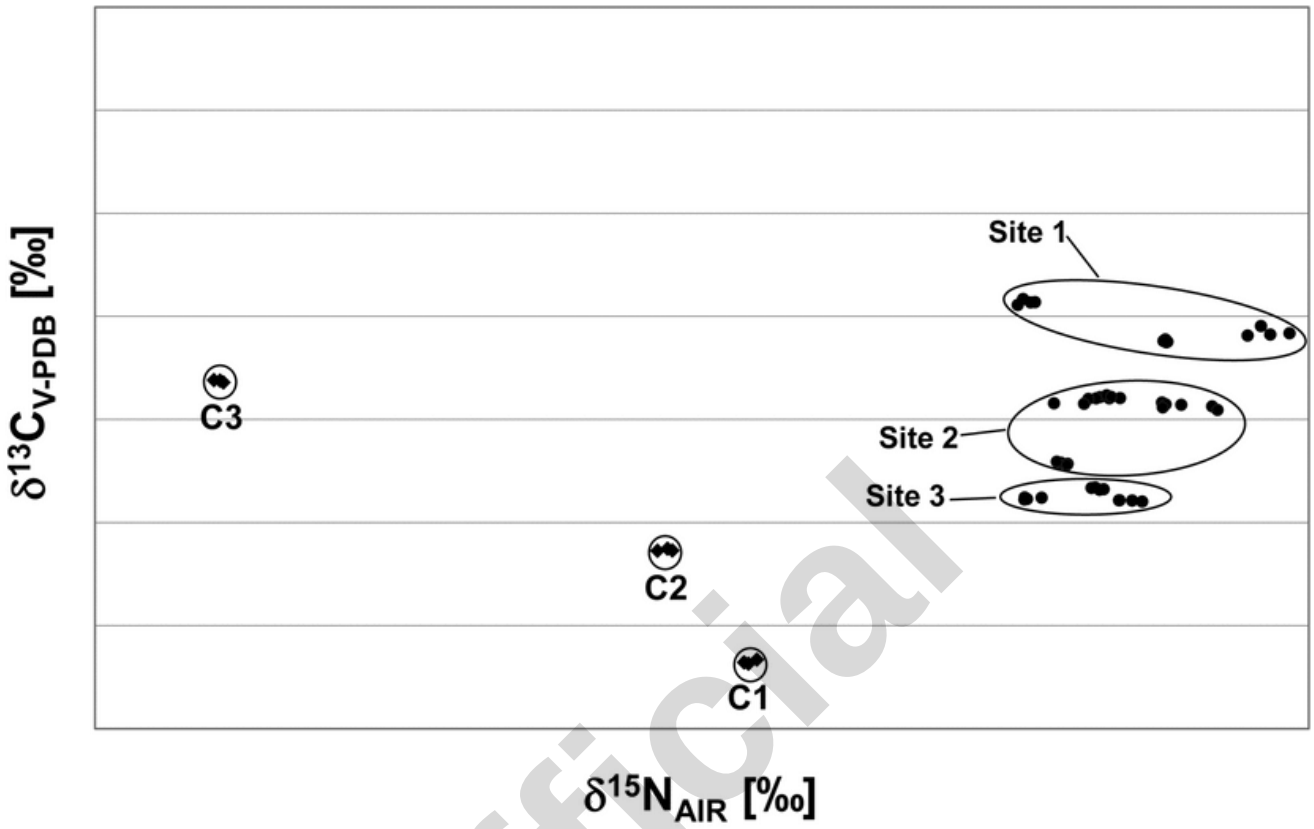


Figure 9. Bivariate plot of stable isotopic composition ($\delta^{13}C$ versus $\delta^{15}N$) of authentic drug product from three global manufacturing sites (Sites 1–3) and three counterfeit drug products (C1–C3).

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# ⟨1761⟩ APPLICATIONS OF NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

## PRINCIPLES OF NMR

Nuclear magnetic resonance (NMR) spectroscopy is an analytical technique based on the magnetic properties of certain atomic nuclei. NMR is similar to other types of spectroscopy in that absorption of electromagnetic energy at characteristic frequencies provides analytical information. NMR differs from other types of spectroscopy because the discrete energy levels between which the transitions take place are created by placing the nuclei in a magnetic field of strength $H_0$. Although the initial field strength of the applied field is $H_0$, when the sample is inserted into the magnet, the field strength throughout the sample becomes $B_0$, defined as follows:

$$B_0 = \mu_S H_0 \text{ [1]}$$

in which $\mu_S$ is the magnetic susceptibility of the sample.

Atomic nuclei behave as if they were spinning on the nuclear axis. The angular momentum, $\rho_0$, of the nucleus is characterized by a spin quantum number ($I$). The maximum observable component of the angular vector, $\rho$, is

$$\rho = I\text{h}/2\pi = I\,\overline{\text{h}} \text{ [2]}$$

in which h is Planck's constant and $\overline{\text{h}}$ is modified Planck's constant.

*Table 1* shows the values of $I$ as a function of the mass number and the atomic number.

**Table 1. Nuclear Spin Values as a Function of Mass and Atomic Numbers**

| Mass Number | Atomic Number | Nuclear Spin ($I$) |
|---|---|---|
| Odd | Even or odd | 1/2, 3/2, 5/2 ... |
| Even | Even | 0 |
| Even | Odd | 1, 2, 3 ... |

The angular momentum creates a magnetic moment, $\mu$, which is parallel to and directly proportional to $\rho$.

$$\mu = \gamma\rho = \gamma I\overline{\text{h}} \text{ [3]}$$

where $\gamma$ is the magnetogyric ratio and is a constant for all nuclei of a given isotope, regardless of their position in a molecule.

Nuclei that have a spin quantum number $I \neq 0$, when placed in an external uniform static magnetic field, align with respect to the field in $(2I + 1)$ possible orientations. Thus, for nuclei with $I = 1/2$, which includes most isotopes of analytical significance (*Table 2*), there are two possible orientations, corresponding to two different energy states. The energies of these two states are $\pm \mu B_0$, and their separation is

$$E = \mu B_0 - (-\mu B_0) = 2\mu B_0 \text{ [4]}$$

with more nuclei populating the lower energy state ($-\mu B_0$) than the higher energy state ($+\mu B_0$). The populations are in accordance with the Boltzmann distribution:

$$N_+/N_- = \exp(-E/kT) \text{ [5]}$$

where $N_+$ and $N_-$ are the populations of the high and low energy states, respectively; $k$ is the Boltzmann constant; and $T$ is the temperature in K.

A nuclear resonance is the transition between these states, and upward as well as downward transitions are possible. In a static magnetic field the nuclear magnetic axis precesses (Larmor precession) about the $B_0$ axis. The precessional angular velocity is often referred to as the Larmor frequency, $\omega_0$, and is related to $B_0$:

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$$E = h\nu = 2\mu B_0$$
$$= 2\gamma I \bar{h} B_0$$
$$= \gamma \bar{h} B_0$$
$$\nu = \gamma B_0 / 2\pi$$
$$\omega_0 = \gamma B_0 \quad [6]$$

If energy from an oscillating radio-frequency (rf) field is introduced, then resonance is achieved when the rf frequency is the same as the precessional angular velocity. Although the probability of an upward transition is equal to that of a downward transition, more upward transitions take place than downward transitions because $N_-$ is greater than $N_+$. Hence, an overall absorption of energy takes place. As shown in *Table 2*, the resonance frequency of a nucleus increases in direct proportion with the increase of the magnetic field strength.

**Table 2. Properties of Some Nuclei Amenable to NMR Study**

| | | | | Resonance Frequency (MHz) at | | |
|---|---|---|---|---|---|---|
| **Nucleus** | ***I*** | **Natural Abundance (%)** | **Sensitivity** | **1.4093 T[a]** | **7.0463 T** | **11.7440 T** |
| $^{1}H$ | 1/2 | 99.98 | 1.00 | 60.000 | 300.000 | 500.000 |
| $^{13}C$ | 1/2 | 1.108 | 0.0159 | 15.087 | 75.432 | 125.721 |
| $^{19}F$ | 1/2 | 100 | 0.83 | 56.446 | 282.231 | 470.385 |
| $^{31}P$ | 1/2 | 100 | 0.0663 | 24.289 | 121.442 | 202.404 |
| $^{11}B$ | (3/2) | 80.42 | 0.17 | 19.250 | 96.251 | 160.419 |

[a] T = tesla: 1.4093 T = 14.093 kilogauss.

NMR is a technique of high specificity but relatively low sensitivity. The basic reason for the low sensitivity is the comparatively small difference in energy between the upper and lower energy states (0.08 Joules at 1.5 to 2.0 T field strength), which results in a population difference between the two levels of only a few parts per million. Another important aspect of the NMR phenomenon, with negative effects on sensitivity, is the long lifetime of most nuclei in the excited state. Long lifetimes affect the design of the NMR analytical test, especially in repetitive pulsed experiments. Simultaneous acquisition of the entire range of resonant frequencies instead of frequency-swept spectra can give increased sensitivity per unit time.

All characteristics of the signal–chemical shift, multiplicity, linewidth, coupling constants, relative intensity, and relaxation time contribute analytical information. The analytical usefulness of NMR arises from the observation that the same types of nuclei, when located in different molecular environments, exhibit different resonance frequencies. The reason for this difference is that the effective field associated with a particular nucleus is a composite of the external field provided by the instrument and the field generated by the circulation of the surrounding electrons. The latter field is generally opposed to the external field and lowers the overall field strength at the nuclear site. The phenomenon is termed *shielding*. Hence, the more shielded nuclei have lower Larmor frequencies.

It is not convenient to accurately measure the absolute values of transition frequencies, as is done with other spectroscopic procedures. However, it is convenient to accurately measure the difference in frequencies between two resonance signals. The position of a signal in an NMR spectrum is described by its separation from another resonance signal arbitrarily taken as standard. This separation is called *chemical shift*, which may be expressed in units of magnetic field or in frequency units that are readily interconverted by the equation for the resonance condition, *Equation 6*. This equation shows that when the separation is expressed in frequency units, it is directly proportional to the field strength. It is more convenient, therefore, to express the chemical shift in terms of the dimensional unit δ, which is independent of the field strength, and is defined by

$$\delta = (\nu_{SS} - \nu_{RS})/\nu_0 \quad [7]$$

where $\nu_{SS}$ is the test substance resonance frequency, in Hz; $\nu_{RS}$ is the reference resonance frequency in Hz; and $\nu_0$ is the instrument frequency, in MHz. When $\nu_0$ is expressed in units of MHz, then *Equation 7* is expressed in units as parts per million

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(ppm). Hence, it is common to use the unit ppm to express the chemical shift difference between the resonance peak of the test substance and that of the reference.

By using this equation, one can use (with appropriate caution) the chemical shift of any known species (such as the residual $^{1}$H-containing species in a deuterated solvent) as a chemical shift reference. This equation, now in common use, is applicable to nearly all nuclei.

Tetramethylsilane (TMS) is the most widely used chemical shift reference for proton and carbon spectra. It is chemically inert, exhibits only one peak (which is more shielded than most signals), and is volatile, which allows ready specimen recovery. Either 2,2,3,3-$d_4$ sodium 3-(trimethylsilyl)propionate (TMSP) or sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) is used as an NMR reference for aqueous solutions. The resonance frequency of the TMSP or DSS methyl groups closely approximates that of the TMS signal. Where the use of an internal NMR reference material is not desirable, an external reference may be used, such as a reference standard in a separate NMR tube.

Conventional NMR spectra are shown with shielding increasing and chemical shift decreasing from left to right because less shielded nuclei have higher Larmor frequencies than do more shielded nuclei. Resonances on the left are said to be deshielded (i.e., they show lower electron density). Resonance peaks appearing at the right are termed more shielded (i.e., they show greater electron density) than those appearing at the left. Resonances from the more shielded and the less shielded nuclei often are inappropriately called the high-field or upfield peaks and the low-field or downfield peaks, respectively, as a result of the outdated method of acquiring data by sweeping the magnetic field. Today, the overwhelming majority of spectra are acquired on a pulsed Fourier transform (FT) spectrometer, which sweeps neither the magnetic field nor the transmitter frequency. Therefore, it is more appropriate to refer to resonances at the left side of the spectrum as high-frequency or deshielded resonances and those on the right as low-frequency or shielded resonances.

The coupling between two nuclei can be described in terms of the spin-spin coupling constant, *J*, which is the separation (in Hertz) between the individual peaks of the multiplet. When two nuclei interact and cause reciprocal splitting, the measured coupling constants in the two resulting mutiplets are equal. Furthermore, *J* is independent of magnetic field strength.

Coupled spin systems are usually referred to as weak or strong. These terms depend on the separation of the Larmor frequencies of the coupled nuclei compared to the coupling constant between them. Both of these values are easily measured from the spectrum. For a weakly coupled system, the separation expressed in Hz ($\Delta\nu$) is large compared to *J*, which is always expressed in Hz. Thus, the ratio of the two is dimensionless. For a weakly coupled system, the ratio is large. Typically, spectroscopists consider a ratio above 10 to be weak. Only weakly coupled spin systems produce first-order spectra, which are comparatively easy to analyze. The number of individual peaks that are expected to be present in a multiplet and the relative peak intensities are predictable. The number of peaks is determined by $2nI + 1$, where $n$ is the number of identical nuclei on adjacent groups that are active in splitting and $I$ is the spin of those nuclei causing the splitting. For protons this becomes $(n + 1)$ peaks. In general, the relative intensity of each peak in the multiplet follows the coefficient of the binomial expansion $(a + b)^n$. These coefficients can conveniently be found by use of Pascal's triangle, which produces the following relative areas for the specified multiplets: doublet, 1:1; triplet, 1:2:1; quartet, 1:3:3:1; quintet, 1:4:6:4:1; sextet, 1:5:10:10:5:1; and septet, 1:6:15:20:15:6:1. Two examples of first-order spectra arising from weak coupling are shown in *Figure 1*.

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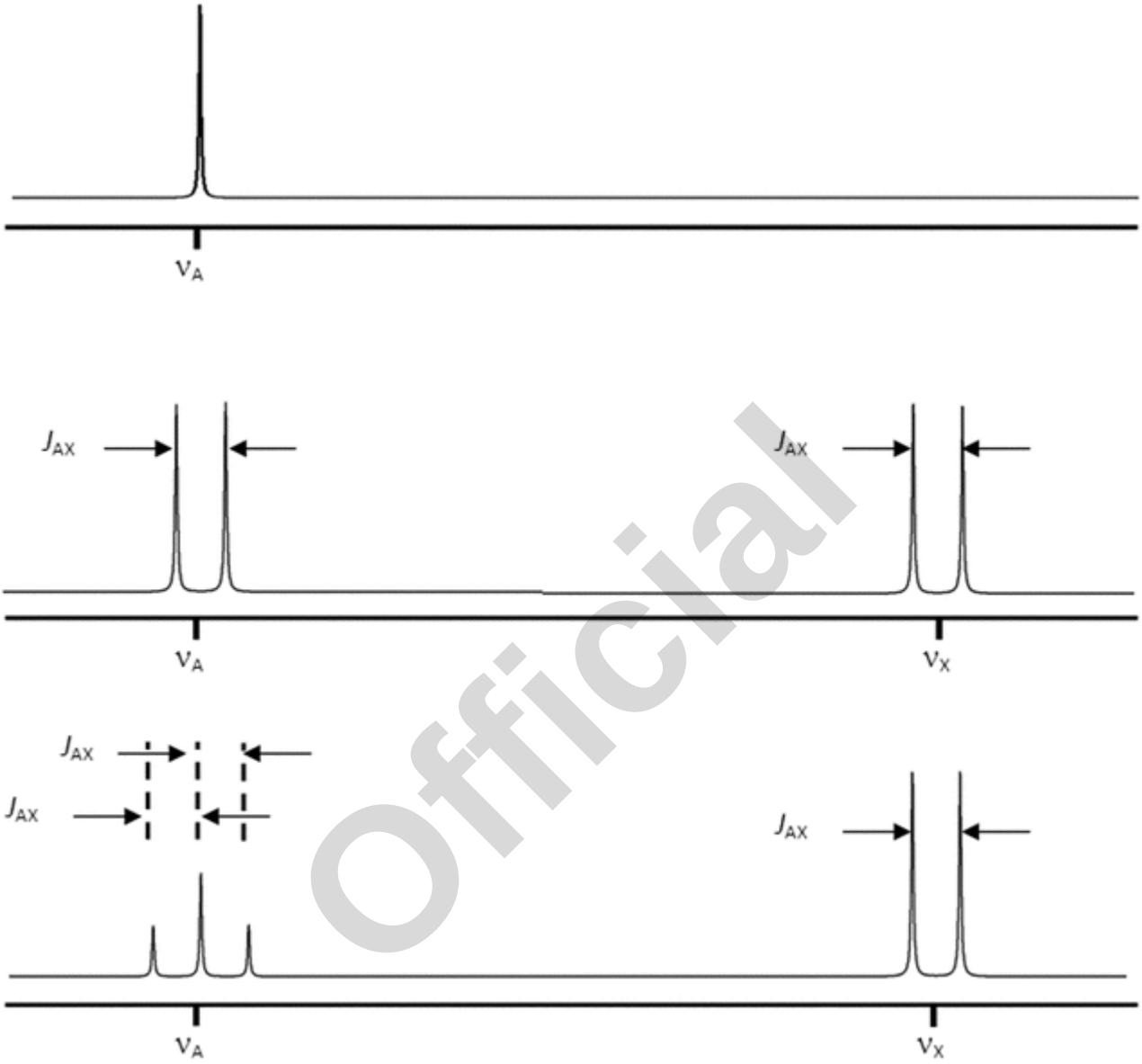


Figure 1. Diagrammatic representation of simple spectra resulting from weakly coupled spin systems.

Coupling may occur between $^{1}H$ and other nuclei, such as $^{19}F$, $^{13}C$, and $^{31}P$. This type of coupling can frequently be observed between nuclei separated by 1–5 bonds.

Magnetically active nuclei with $I \geq 1$, such as $^{14}N$, possess a nuclear quadrupole moment, which produces line broadening of the signals from neighboring nuclei.

Another characteristic of an NMR signal is its relative intensity, which has wide analytical applications. In carefully designed experiments, the area or intensity of a signal is directly proportional to the number of protons that give rise to the signal. As a result, NMR can be used for quantitation (see *Quantitative Analysis* in this chapter and in *Nuclear Magnetic Resonance Spectroscopy* ⟨761⟩, *Qualitative and Quantitative NMR Analysis*. NMR spectra may contain spinning side bands, peaks that appear symmetrically located around each signal. These signals are due to the failure to optimize the off-axis (*x* and *y*) shims. The homogeneity of modern superconducting magnets, coupled with computer shimming techniques, reduce the need for sample spinning and completely eliminate these sidebands.

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# NMR SPECTROMETERS

## Introduction

NMR spectrometers have evolved since the first commercial instrument, a Varian HR-30 that operated at 30 MHz, was produced in 1952. Initially NMR spectrometers used a data acquisition technique known as continuous wave (CW), which was based on sweeping the magnetic field. The limitations of a CW spectrometer include low sensitivity and long analysis time.

Today's spectrometers operate at frequencies up to 1 GHz and apply an rf pulse to the sample to produce a time-domain signal known as a free induction decay (FID), which is then Fourier transformed into a frequency-domain signal. This technique is known as FT NMR spectroscopy. Current NMR spectrometers are composed of several key components: the magnet, the probe, the console, and the computer.

The instruments are described by the approximate resonance frequency of the $^{1}H$ resonance, e.g., 600 MHz, or by their field strength, e.g., 14.1 T.

## The Magnet

Until the early 1970s, NMR magnets were either ferromagnetic-core electromagnets or permanent magnets that operated at field strengths of 1.41–2.35 T, corresponding to $^{1}H$ resonance frequencies of 60, 80, 90, and 100 MHz. The first NMR magnets based on superconducting magnets were introduced in the 1960s and allowed access to much higher field strengths that currently are as high as 23.5 T (1 GHz).

The superconducting magnet is the most expensive component of an NMR spectrometer and can cost up to several million dollars. These magnets consist of miles of $Nb_3Sn$ or NbTi wire. When these materials are wound into a solenoid that is immersed in liquid helium at 4.2 K, they become superconducting. That is, an electrical current can be induced in them by an energizing power supply, and that current will persist for many years even after the power supply is removed. This essentially constant electrical current is used to generate high static magnetic fields that can be several times higher than those obtained with ferromagnetic-core magnets. Ensuring that the superconducting coil is immersed in liquid helium at all times is the only maintenance needed to sustain these high fields.

*Figure 2* contains a diagram of a typical superconducting magnet. The superconducting solenoid is immersed in a liquid-helium Dewar at 4.2 K. This unit is itself contained in a liquid-nitrogen Dewar at 77.4 K. Each Dewar is surrounded by a vacuumed space and reflective film coating to prevent outside heat of the laboratory from entering the helium Dewar. The central core or room-temperature bore provides room for the stack that contains the shim coils for room-temperature shimming. Finally the probe is inserted into the stack. Samples are injected or ejected from the probe by means of a jet of filtered and dried air or nitrogen.

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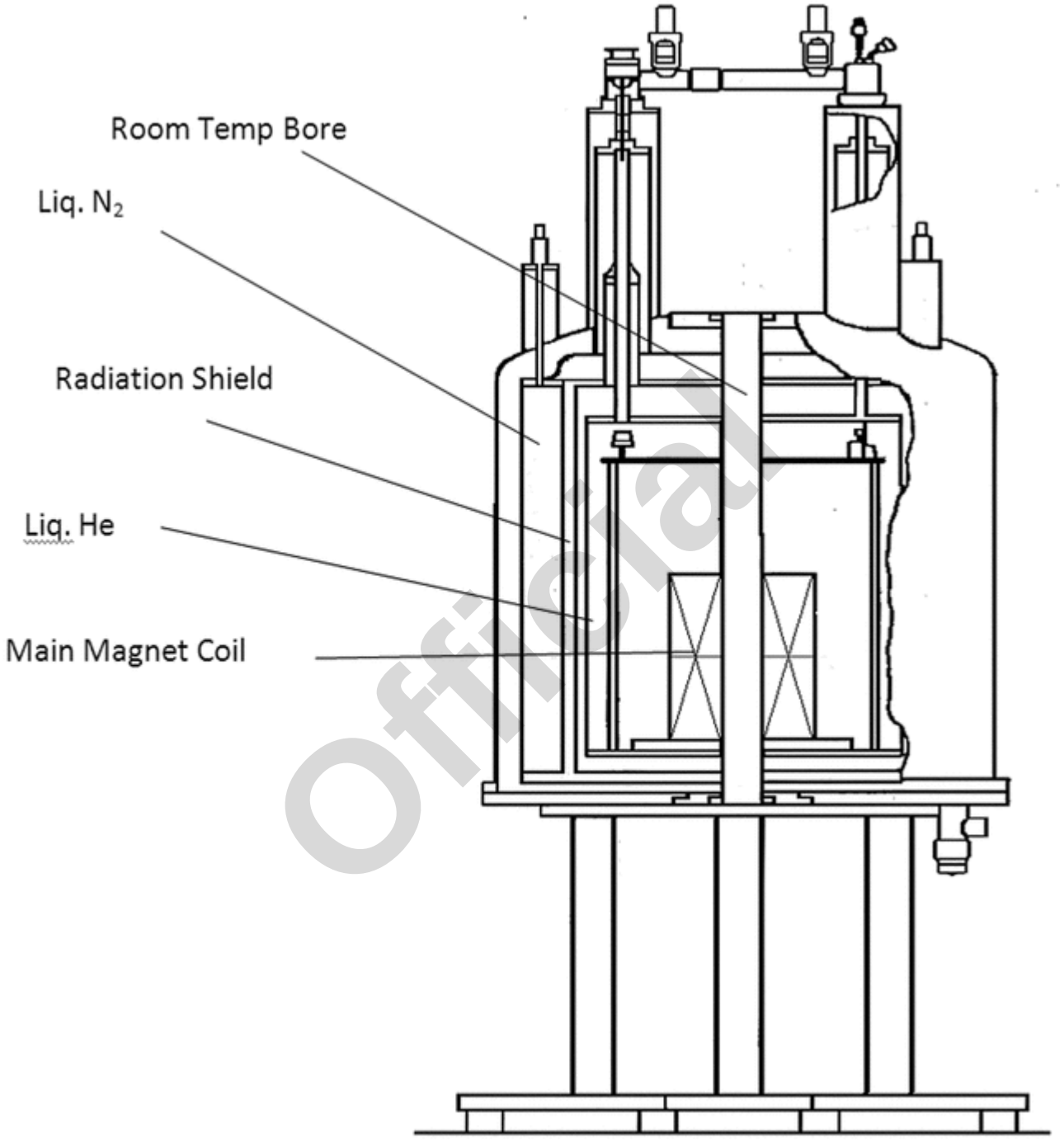


Figure 2. Schematic representation of a superconducting magnet.

An important recent advance in magnet technology has been the construction of shielded magnets with stray fields that can extend only one or two meters from the center of the magnet in three dimensions, thereby making magnet siting a far easier task than it was with unshielded magnets.

In addition to the main solenoid, the helium Dewar also contains several other superconducting coils that are used to shim the main magnetic field as a first step in attaining very high field homogeneity. Further shimming is accomplished by 20–30 shim coils in the room-temperature shim stack that is inserted in the bore of the magnet. These coils operate at ambient temperature and are used to generate small magnetic fields that oppose and cancel inhomogeneities caused by the surroundings, the probe, or the sample itself. Computer software has taken over a large amount of the tedious job of shimming the magnet homogeneity, a critical task for obtaining good NMR data. Using the lock signal from the sample, spectroscopists can generate a field map for each of the shim coils. Using this map, the computer then calculates the amount of current that should be applied to each of the shim coils to maximize the magnet homogeneity. Typically this operation takes less than a

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minute for shimming the on-axis ($z_1$ to $z_6$) shim coils. The off-axis ($x/y$) inhomogeneities can be compensated for in a similar manner but usually in a longer period of time (15–20 minutes) because of the larger number of off-axis coils.

## The Probe

The NMR probe may be the most important part of the instrument. The probe consists of one or two rf coils. Each coil, which is inductive (L), is in a circuit that contains several tunable capacitive (C) elements. These elements are adjusted to enable the probe to transmit and receive at the Larmor frequency, $\nu_0$. For a given nucleus, the probe tuning is determined by [$\nu = 1/(2\pi(LC)^{1/2})$]. A pulse of rf at the Larmor frequency results in an applied magnetic field ($B_1$). To induce a transition, $B_1$ must be applied perpendicular to the static field generated by the magnet superconducting coil. The rf coil not only transmits the excitation pulse but is also electronically switched to receive the rf signal from the sample.

The most common NMR sample tube is the 5-mm (od) NMR tube. However, probes have been designed in many forms. Some probes can accommodate 10- or 20-mm tubes for samples that are not in limited supply, such as petroleum and polymers. For limited amounts of precious samples, probes have been made to accept tubes as small as 1 mm, which can accommodate solutions as small as 5 µL. Also, flow probes are available to obtain data directly from a liquid chromatography effluent.

Probes are available in a large number of coil configurations. The most common probe usually contains a broadband observe coil that is tunable over a wide range of frequencies ($^{31}$P to $^{109}$Ag), a decoupler ($^1$H) coil, and a coil tuned to deuterium ($^2$H) for field-frequency lock. Usually, the decoupler coil is double-tuned for both $^1$H and $^2$H. Probe configurations can include as many as four channels for multidimensional work with biological macromolecules.

The probe can come in a format where the X-nucleus observe-coil (e.g., $^{13}$C) is wound closest to the sample for highest sensitivity, and the decoupler coil ($^1$H) is farther away. The inverse configuration is also available to provide the maximum signal-to-noise (S/N) ratio for $^1$H, the detected nucleus in two-dimensional (2-D) indirect heteronuclear experiments, such as heteronuclear single-quantum coherence spectroscopy (HSQC) and heteronuclear multiple-bond correlation (HMBC) where the X-nucleus is indirectly detected via the $^1$H frequency. Recent advances in probe technology have resulted in a single probe combining the sensitivity of the direct and inverse detection probes described above. Another advancement in probe design is the cryogenically cooled probe wherein the rf coils and their preamplifiers are held at close to liquid helium temperatures (20 K). Because rf electronics generate lower noise levels at colder temperatures, S/N ratios can be increased by at least a factor of four in these probes. Because the S/N ratio of a spectrum is given by $n^{1/2}$, where $n$ is the number of acquisitions, an enhancement by a factor of four to the initial S/N ratio translates to a savings of 16 in time or to a four-fold reduction in sample size.

Probes can also be equipped with gradient coils that can apply a magnetic field gradient in the $z$ only or $x$, $y$, and $z$ directions. These gradients are used to study diffusion or, more commonly, to be integral parts of pulse sequences because they provide an efficient means for selecting specific coherences in 2-D experiments.

In addition to the electronic coils, probes usually come equipped with a heater coil that enables variable temperature work from –100° to +150°. Probes also have gas outlets to allow sample insertion and ejection from the probe as well as sample spinning.

For solid-state samples the probes come with cylindrical rotors that are filled with the sample and capped. The entire rotor can then be oriented at an angle of 54.74° (known as the magic angle) relative to the magnetic field direction and spun at rates up to 70 kHz. Rapid rotation at this angle helps remove the chemical shift anisotropy and dipolar coupling so that the very broad resonances observed in the solid state can be reduced considerably.

## The Console and Computer

The NMR console has the primary function of generating the various Larmor frequencies required for a given experiment, amplifying and transmitting these frequencies to the probe, and detecting the resulting signals that are transmitted from the probe so that they can be used to create an NMR spectrum. In addition to these primary functions, the console performs many more operations, all of which are computer-controlled. *Figure 3* contains a schematic diagram illustrating the various components of the current majority of spectrometer consoles.

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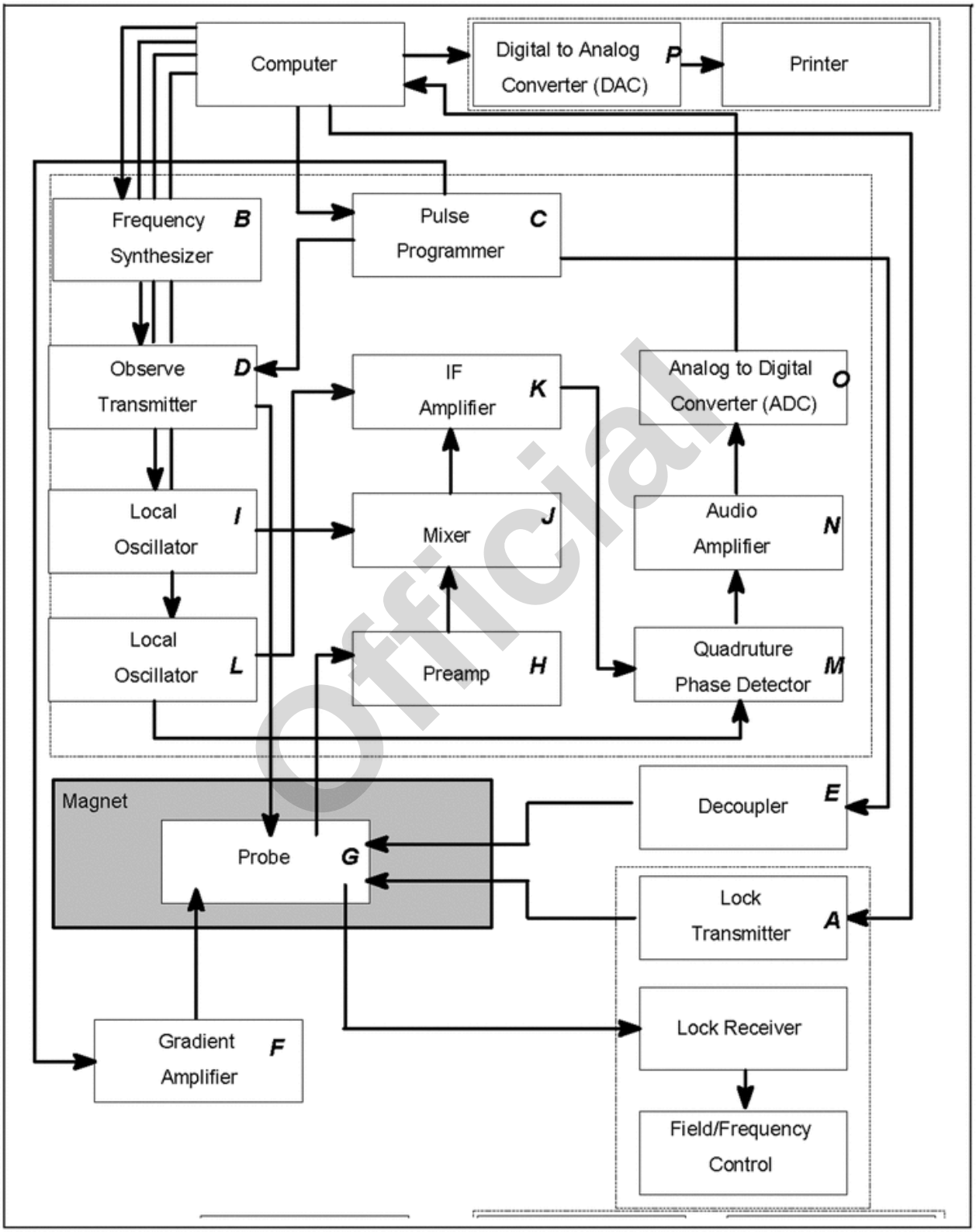


Figure 3. Schematic diagram of an NMR spectrometer.

The computer continually controls the signal to the lock transmitter (***A***) so the resonance of the lock material can be detected in the lock receiver to maintain field/frequency control. The computer also controls the frequency synthesizer (***B***) that generates

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the various frequencies that are close to the Larmor frequencies of the observed or perturbed nuclei. For a specific experiment, the computer triggers the pulse programmer (***C***), which, in turn, sends timing signals (pulse width, phase, and shape) to the observer transmitter (***D***), the decoupler (***E***), and the gradient amplifier (***F***), depending on which of these units are needed for that experiment. Once the observed nucleus in the probe (***G***) is excited by a pulse from the observed transmitter, the resultant rf signal is amplified in the preamplifier (***H***). Then the signal is mixed (***J***) with a local oscillator (***I***) to generate a lower rf frequency, called the intermediate frequency (IF) that is further amplified (***K***). A second mixing stage, this time with a different local oscillator (***L***), results in an audio signal that is then detected in quadrature (***M***) before being amplified (***N***), converted (***O***) from an analog to a digital signal, and stored in the computer. After the signal is processed to create a spectrum, the digital data can be converted back to an analog signal in a digital-to-analog converter (***P***) and printed.

The output from the phase-sensitive detector (***M***) is the free induction decay (FID), which is a time-domain signal, $f(t)$.

When two detectors are used (***M***) with their reference frequencies shifted from each other by 90°, frequencies that are positive with respect to the reference can be distinguished from those that are negative. This system is referred to as quadrature phase-sensitive detection (QPD). Each detector produces an FID, but they will always be 90° out of phase with respect to each other. One FID is called the real FID, and the other is called the imaginary FID. The Fourier transform that is performed in this case is called a complex Fourier transform. The combination of the real and imaginary FIDs yields the frequency spectrum, $F(\omega)$.

$$F(\omega) = \int_{-\infty}^{+\infty} f(t)\exp^{-i\omega t} dt \qquad [8]$$

In addition to the Fourier transform process, the computer is also used for post-acquisition processing of the data. The frequency-domain spectrum that results from the complex Fourier transform can then be phased, baseline corrected to remove distortions, integrated to obtain peak areas, and peak picked to provide chemical shift information. The computer is also capable of providing spectra calculated from chemical shift and coupling values, curve-fitting resonances, and deconvoluting complex overlapped peaks. Finally, the digital data can be converted to their analog form by a DAC (***O***) and printed.

## RELAXATION

NMR includes two types of relaxation: *Spin-Spin Relaxation*, sometimes referred to as *Transverse Relaxation*, or $T_2$ Relaxation, and *Spin-Lattice Relaxation*, sometimes referred to as *Longitudinal Relaxation*, or $T_1$ Relaxation. At least two mechanisms contribute to *Transverse Relaxation*: loss of signal due to $B_0$ inhomogeneity and the natural relaxation that would take place even in a perfectly homogeneous field. The combined effects of these two mechanisms produce a new time constant for the relaxation, which is referred to as $T_2$*.

### Spin-Spin Relaxation (Transverse Relaxation)

After an rf pulse, the component of $M_0$ in the $(x,y)$ plane, $M_{xy}$, will gradually decay toward zero. The process is first order, and as it is in other types of first-order processes, the instantaneous rate of decay of $M_{xy}$ is directly proportional to its displacement from equilibrium. The further $M_{xy}$ is displaced from zero, the faster it decays, and as it approaches zero it decays more and more slowly. Hence, *Equation 9* applies.

$$dM_{xy}/dt \propto (-M_{xy}) \quad [9]$$

The process is analogous to the decay of a radioactive element. However, spectroscopists do not speak of the rate of decay of an FID in terms of its half-life. Instead, they speak of its 1/e-life, that is, how long it takes for the FID to decay to a value that equals 1/e of its original value at time zero. Standard mathematical manipulations of *Equation 9* yield

$$M_{xy} = M_0\exp(-t/T_2^*) \quad [10]$$

where $M_0$ is the equilibrium distribution given by *Equation 9*, and $T_2$* is the rate constant for the decay. $T_2$* is a *measure* of how fast the signal decays, not how long it takes to decay. The rate of decay is fastest immediately after the pulse because then it is furthest from its equilibrium position, zero. If the rate remained constant at this initial rate, then the signal would be completely decayed after one $T_2$*.

*Table 3* was prepared from *Equation 10*. It shows the percentage of $M_{xy}$ remaining as a function of time after a 90° pulse. In *Table 3* time is given as the number of $T_2$*s.

**Table 3. Percent of $M_{xy}$ Remaining as a Function of Time, in Units of $T_2$***

| Time/$T_2$* | 0.0 | 0.5 | 1.0 | 1.5 | 2.0 | 3.0 | 4.0 | 5.0 |
|---|---|---|---|---|---|---|---|---|
| % Remaining | 100.0 | 60.7 | 36.8 | 22.3 | 13.5 | 5.0 | 1.8 | 0.7 |

$M_{xy}$ asymptotically approaches zero, and it would take infinitely long for complete decay, but the decay is normally considered to be complete when the time has reached 3 to 5 times $T_2$*. Hence, these times are commonly used as acquisition

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times. If acquisition times that are shorter than 3 times $T_2$* are used, the FID is truncated and subsequent Fourier transform leads to visible baseline artifacts.

The decay of the FID produces the peak width in the final spectrum. The faster the decay, the broader is the line. The mathematical relationship is:

$$\Delta\nu_{1/2} = 1/(\pi T_2^*) \text{ [11]}$$

where $\Delta\nu_{1/2}$ is the width of the peak at its half-height.

## Spin-Lattice Relaxation (Longitudinal Relaxation)

After an rf pulse, nuclei are excited from the low-energy state into the high-energy state. The nuclei will eventually relax back to establish the Boltzmann distribution (see *Equation 5*), and this process is called spin-lattice relaxation. The recovery process is first-order, and as it is in other types of first-order processes, the instantaneous rate of growth of $M_z$ is directly proportional to its displacement from equilibrium. The farther $M_z$ is displaced from $M_0$, the faster it grows back, and as it approaches $M_0$ it grows back more and more slowly. Hence, *Equation 12* applies.

$$dM_z/dt \propto (M_0 - M_z) \text{ [12]}$$

Standard mathematical manipulations yield

$$M_z = M_0(1 - \exp(-t/T_1)) \text{ [13]}$$

Note that $T_1$ is a measure of how fast $M_z$ grows back to $M_0$—it is not how long it takes to grow back. *Table 4* was prepared from *Equation 13* and shows the percentage of recovery of $M_z$ as a function of time after a 90° pulse. In this table, time is given as the number of $T_1$*s*.

**Table 4. Percent Recovery of $M_z$ as a Function of Time, in Units of $T_1$**

| Time/$T_1$ | 0.0 | 0.5 | 1.0 | 1.5 | 2.0 | 3.0 | 4.0 | 5.0 |
|---|---|---|---|---|---|---|---|---|
| % Recovery | 0.0 | 39.3 | 63.2 | 77.7 | 86.5 | 95 | 98.2 | 99.3 |

As $M_z$ asymptotically approaches $M_0$, it takes infinitely long for 100% recovery. However, recovery is normally considered to be complete when it has reached 99%. Hence, relaxation delays of $5T_1$ are commonly used in pulse sequences. The rate of return to $M_0$ is fastest immediately after the pulse because that is when $M_0$ is farthest from its equilibrium position. If the rate remained constant at this initial rate, then full recovery would be achieved after one $T_1$.

# TIP ANGLE

During an rf pulse, a magnetic field ($B_1$) is applied to the sample. The magnetization vector *M* precesses about $B_1$ according to

$$\omega_1 = \gamma B_1 \text{ [14]}$$

where $\omega_1$ is the precessional frequency and $B_1$ is the strength of the magnetic field applied to the sample. During the time that the pulse is applied, *M* precesses to an angle ($\alpha$) given by the precessional rate multiplied by the width of the pulse, in time, (*PW*). Then,

$$PW \times \omega_1 = \alpha = PW \times \gamma B_1 \text{ [15]}$$

Tip angles typically are expressed in units of degrees or in radians. For example, a 90° pulse is sometimes referred to as a $\pi/2$ pulse.

## Optimum Tip Angle or Ernst Angle

Time averaging to improve the S/N ratio is accomplished by a delay-pulse-acquire sequence that is repeated as necessary. Suppose that during the first pulse, $M_0$ precesses 30° about $B_1$ before the pulse is turned off. At this time the magnitude of $M_z$ is $M_0\cos 30°$. At the end of the pulse, $M_z$ will begin to grow back toward its equilibrium value, $M_0$. Typically, the second pulse is applied before $M_z$ reaches $M_0$, driving $M_z$ down even farther away from $M_0$. Consequently, $M_z$ begins to grow back faster because it is even farther away from its equilibrium value. After 6–10 pulses, $M_z$ will grow back an amount that is equal to the incremental displacement caused by each succeeding pulse and will reach a new equilibrium or steady state. Each succeeding pulse continues to tip this new steady-state value of $M_z$ by 30°, resulting in signal intensity for each pulse.

The position of the steady-state equilibrium is determined by three factors: the $T_1$, the tip angle, and the time between pulses. On the one hand, for a given $T_1$ and time between pulses, if the tip angle is too large then the steady-state value of $M_z$ will be close to the origin, affording only a small signal. On the other hand, if the tip angle is too small, e.g., 5°, then the steady-state value will be large, but with a small angle the value of $M_z\sin 5°$ will also be small, again affording a small signal. The optimum angle is frequently called the Ernst angle, which is given by:

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$$\cos \alpha_{opt} = \exp(-PR/T_1) \quad [16]$$

$$\alpha_{opt} = \text{arc cos}[\exp(-PR/T_1)] \quad [17]$$

where *PR* is the time between pulses, or pulse-repetition time. This time is the sum of the acquisition time used to collect the FID plus any relaxation delay used. This angle provides a reasonably large steady-state value combined with a reasonably large angle and will produce the best S/N ratio per unit time. The value of $T_1$ to be used in *Equation 17* should be for the longest relaxing nucleus in the molecule.

## RELAXATION DELAY

Surprisingly, the optimum S/N ratio per unit time is obtained when no relaxation delay is used for a given $T_1$. That is when *PR* equals to *AT*, the acquisition time. However, because the nuclei in most molecules do not have the same $T_1$ values, the relative intensity relationship will be lost.

For typical quantitation experiments, a relaxation delay is used and should be at least five times the longest $T_1$ expected for any of the nuclei in the molecule; and the pulse width should be set to 90°. Further details are provided in the section *Quantitative Applications*.

## RESOLUTION

In NMR spectroscopy the typical definition of resolution is the ability to distinguish between two closely spaced resonance peaks in a spectrum. The industry standard for measuring resolution is to measure the width of a single peak, in units of Hz, at the half-height of the peak.

The uncertainty principle determines the best resolution that can be achieved in an NMR spectrum. The maximum resolution possible, or the minimum separation that can be observed between two frequencies, $\Delta\nu$, in a spectrum is equal to the reciprocal of the acquisition time, *AT*, of the FID.

$$\Delta\nu = 1/\Delta t = 1/AT \quad [18]$$

The time set by the spectrometer operator should not exceed the required *AT*, because this would result in the collection of only noise after the signal has decayed to near zero. Collecting this noise does not improve the resolution.

## POSTACQUISITION DATA PROCESSING

The final appearance of the spectrum can usually be improved by applying a variety of mathematical procedures to the FID before the Fourier transform is performed. The two most common procedures are multiplying the FID by a mathematical function, generally known as a *window* function; or appending zeros to the end of the FID, generally known as *zero filling*.

### Window Functions

Two types are generally used: one for increasing the resolution and another for increasing the S/N ratio.

INCREASING THE RESOLUTION

The decay of the signal produces a peak width in the spectrum, and if this decay can be removed, then the resonance peak would consist of a single point, i.e., an infinitely narrow peak. The decay of the signal can be represented by $\exp(-t/T_2^*)$. Hence the full equation representing the decaying signal is

$$A(t) = A_0\exp(-t/T_2^*)\cos(\omega t + \theta) \quad [19]$$

If the FID is multiplied by an increasing function that exactly cancels the decay, then the peak width will be removed. This can be achieved by multiplying the FID by $\exp(t/T_2^*)$. Then *Equation 19* becomes

$$A(t) = A_0\exp(0)\cos(\omega t + \theta) = A_0\cos(\omega t + \theta) \quad [20]$$

Unfortunately, the application of this function as described above will also disproportionately increase the noise power at the tail of the FID. The S/N ratio in the final spectrum is so poor that this function is not used without modifications. Typically, the beginning of the FID, where the S/N ratio is better, is multiplied up but then the tail of the FID, where S/N ratio is poorer, is multiplied down. Two commonly used functions that accomplish this are the Gaussian function and the transform of reversed-added FIDs (TRAF) function for resolution. The latter is sometimes given the name TRAFR by instrument manufacturers. It affords the same resolution enhancement as the Gaussian function but with much less degradation in overall S/N ratio. Numerous other window functions have been proposed though not always widely used. It should also be noted that in the quantitative experiments increasing the resolution should be used with caution because it may change the accuracy of signal integration in the spectrum.

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INCREASING THE S/N RATIO

The overall S/N ratio in the spectrum can be increased by weighting the points at the beginning of the FID more highly than at the tail. This is because the S/N ratio is the highest in the beginning and the lowest at the tail. The weighting is often accomplished by multiplying the raw FID by a function that decreases with time. A popular function that gives the greatest increase in the S/N ratio is called the *matched filter*. It weights each point in the FID by an amount proportional to the S/N ratio at that point. A function that accomplishes this must match the decay. Hence, the FID is multiplied by $\exp(-t/T_2^*)$.

The penalty for the use of the matched filter is a loss of resolution that equals a doubling of the peak width. When the original decay is multiplied by the matched filter, then the new decay is given by the following:

$$\exp(-t/T_2^*) \times \exp(-t/T_2^*) = \exp(-2t/T2^*) = \exp(-t/0.5T_2^*) \quad [21]$$

The FID then appears to have decayed with a $T_2^*$ equal to one-half of the original, and according to *Equation 11* and *18* the peak width will double. Multiplying the FID by a steeper decay in an attempt to weight the beginning points eore results only in less of an increase in S/N ratio and a greater increase in peak width.

Another function that accomplishes the same increase in S/N ratio, but without any change in resolution, is the TRAF function for sensitivity, which is sometimes given the name TRAFS by instrument manufacturers.

## Zero Filling

Spectroscopists can improve the overall appearance of the spectrum considerably by appending zeros to the end of the FID before the Fourier transform is performed. This process results in placing more points on every resonance peak in the spectrum. The most common procedure is to append a number of zeros equal to the number used to collect the FID. Adding more zeros will result in only a very slight further improvement.

Although all of the peaks are better defined with zero filling, the resolution is not increased. For example, in a case where the separation between two lines is closer than the peak widths, only a single broadened line will result. Zero filling will not resolve these peaks—it will only place more points on an already broadened line. Only an increase in the acquisition time of the signal or the use of a window function could resolve the peaks.

Zero filling has a beneficial effect on quantitation. Integration of a digital spectrum is accomplished by taking the intensity at a given point and adding to it the intensity at successive points. If the number of data points is insufficient to depict the actual peak shape, the resultant integral of that peak will not be accurately determined. Therefore, zero filling until each peak is represented by at least 7–10 data points results in a more accurate integration. To obtain reliable peak representation and quantitative peak integration there should be at least 4–5 data points above the full width at half height of a peak.

# GENERAL PROCEDURE FOR STRUCTURE IDENTIFICATION

NMR spectroscopy is a powerful technique for structure identification because of its specificity of detecting certain nuclei such as $^1H$, $^{13}C$, $^{31}P$, and $^{19}F$. Typically, a routine identification test can be performed by $^1H$ NMR spectroscopy in a short period of time for simple molecules. The basis for identification is provided by a comparison of the signals from the test sample with the expected signals from a qualified reference standard. A positive identification can be concluded when the chemical shifts, multiplicities, and coupling constants of the spectrum of the test sample match those of the reference standard or, in the case of a *USP* monograph, the values listed in the monograph.

Data may be made unacceptable for analysis if incorrect sample preparation or poor adjustment of spectrometer parameters leads to poor resolution, decreased sensitivity, and spectral artifacts. It is preferable that the operator be familiar with the basic theory of NMR and operation of the spectrometer. Frequent checks of instrument performance are essential.

The procedures discussed here refer specifically to $^1H$ and $^{13}C$ NMR, but they are applicable, with modification, to other nuclei. The discussion assumes that the NMR spectra are obtained from solutions in suitable solvents.

## Selection of Solvent

Deuterated solvents are usually used to prepare solutions for NMR analysis because they are readily available, have greatly reduced $^1H$ signals from solvents in $^1H$ spectra, and have the added advantage of providing a lock signal. Select a solvent whose residual $^1H$ signals will not interfere with signals of the analyte. If a residual $^1H$ solvent peak might interfere with any signals from the sample solution and another solvent is not possible, then the $^2H$ isotopic purity of the solvent should be as high as possible. Some solvents (e.g., $D_2O$ or $CD_3OD$) have labile protons that can enter into fast exchange reactions with the labile protons in the analyte. This may eliminate resonance signals from –COOH, –OH, and $–NH_2$ structural groups. The most commonly used solvents for proton and carbon NMR are listed in *Table 5* along with their residual $^1H$ and $^{13}C$ chemical shifts as well as the multiplicities of these resonances caused by coupling to deuterium.

**Table 5. Solvents Commonly Used for $^1H/^{13}C$ NMR Chemical Shifts**

| Solvent | Residual $^1H/^{13}C$ Signal ($\delta^{a,b}$) and Multiplicity | |
|---|---|---|
| | $^1H$ | $^{13}C$ |
| $CDCl_3$ | 7.27 | 77.23 (3) |
| $CD_3OD$ | 3.35, 4.78 | 49.15 (7) |

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**Table 5. Solvents Commonly Used for $^{1}H/^{13}C$ NMR Chemical Shifts** *(continued)*

| Solvent | Residual $^{1}H/^{13}C$ Signal ($\delta^{a,b}$) and Multiplicity | |
|---|---|---|
| $(CD_3)_2CO$ | 2.05 | 206.68 (1)<br>29.92 (7) |
| $D_2O$ | 4.7[c] | — |
| $(CD_3)_2SO$ | 2.50 | 39.51 (7) |
| $C_6D_6$ | 7.20 | 128.39 (3) |
| Dioxane-$d_8$ | 3.55 | 66.66 (5) |
| $CD_3CO_2D$ | 2.05, 11.65[c] | 178.99 (1)<br>20.0 (7) |
| $(CD_3)_2NCDO$ | 2.77, 2.93, 8.05 | 163.15 (3)<br>34.89 (7)<br>29.76 (7) |

[a] Chemical shifts were measured at 295 K>.
[b] δ in ppm relative to TMS at 0 ppm.
[c] Labile hydrogen.

## Sample Preparation

For *USP* procedures, directions are usually given in individual monographs. The solute concentration depends on the objective of the experiment. Typically, NMR sample solutions are prepared so that they contain from a few to 50 mg/mL. Detection of minor contaminants may require higher concentrations. In some cases such as polymers, even higher concentrations can be used. The solutions are prepared in separate vials and are transferred to the NMR tube. The volume required depends on the size of the NMR tube and on the geometry of the probe. The level of the solution in the tube must be high enough to extend beyond the coils when the tube is inserted in the instrument probe.

The NMR tubes must meet narrow tolerance specifications in diameter, wall thickness, concentricity, and camber. The most widely used tubes have a 5- or 10-mm outside diameter (OD) and a length between 15 and 20 cm, but 1- and 3-mm (OD) NMR tubes are becoming more common, and tubes as large as 20 mm (OD) have been used.

## Procedure

The NMR tube is placed in a probe located in the magnetic field. Although samples traditionally have been spun to average the nonradial field gradients, the quality of the shim coils no longer makes spinning a requirement, and, in the case of many 2-D experiments, the sample should not be spun. The magnetic field's homogeneity is optimized by shimming, a function that is largely being taken over by the computer in most modern spectrometers. Probe tuning is optimized for the frequency being observed and is matched to the impedance of the spectrometer.

The computer serves to control all operations of the spectrometer from running the pulse program to storing and processing the data. The experimental setup involves selecting values for a large number of variables, including the spectral width to be examined, the duration of the excitation pulse (*PW*), the time interval over which data will be acquired (*AT*), the number of transients to be accumulated, and the delay between one acquisition and the next (relaxation delay). The acquisition time for one transient is on the order of seconds. The number of transients is a function of the specimen concentration, the type of nucleus, and the objective of the experiment and can vary from a few for most $^{1}H$ experiments to several thousand for $^{13}C$ spectra. At the end of the experiment, the signal (FID) is stored in digitized form in the computer memory and may be displayed on the monitor. The signal can be processed mathematically to enhance either the resolution or the sensitivity, and it can be Fourier-transformed into a frequency-domain spectrum, which can be further analyzed to obtain peak positions (chemical shifts) and intensities.

## Structure Elucidation by NMR

The simplest case of using NMR spectroscopy to elucidate an unknown structure is to obtain a match with a spectrum from a reference standard or from a database. The informational content of an NMR spectrum is sufficient for deducing structures of organic molecules even when qualified reference standards or spectra are not available. Relatively simple structures can be identified using chemical shifts, coupling patterns, and intensities obtained from one-dimensional (1-D) $^{1}H$ and $^{13}C$ spectra. For more complex structures, spectroscopists may have to obtain two-dimensional (2-D) spectra from experiments that have been developed to determine homo- or heteronuclear connectivities.

Two-dimensional spectra are characterized by two frequency axes. The intensity, which is mathematically another dimension, is not considered to be a dimension in 2-D NMR because it is not an axis that spreads out the chemical shift. All modern 2-D experiments consist of at least two pulses separated by a time period, labeled $t_1$, the evolution period, and a period of time used for collecting the signals, labeled $t_2$, the detection period. A COrrelation SpectroscopY (COSY) sequence is the simplest of all and is shown in *Figure 4*.

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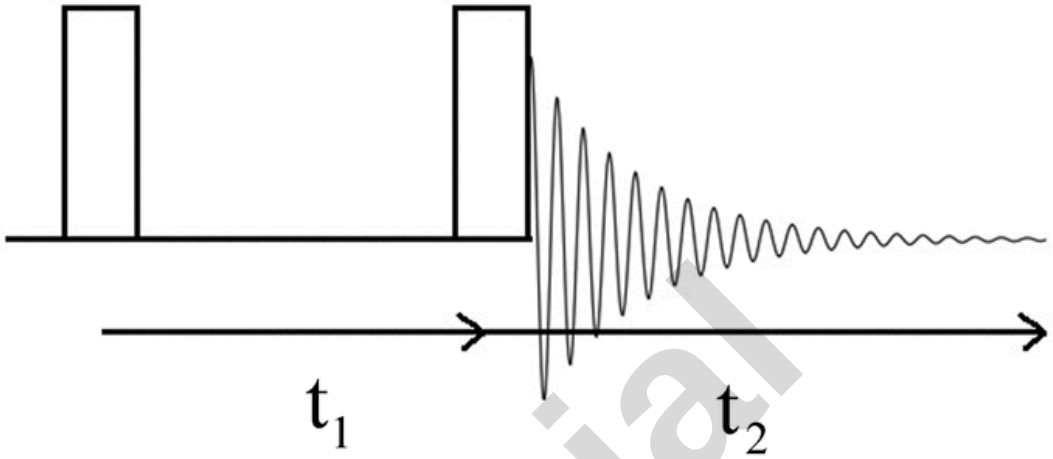


Figure 4. Pulse sequence for a COSY experiment.

A series of such sequences is performed using an incremental increase in the evolution period. Fourier transforms of the FIDs produced during each of the $t_2$ detection periods are stacked, a second Fourier transform is performed along this $t_1$ new axis, resulting in a plot of amplitude along two frequency axes, $F_2$ and $F_1$.

The next simplest addition to the COSY sequence is another pulse. The Nuclear Overhauser Effect SpectroscopY (NOESY) sequence is an example and is shown in *Figure 5*.

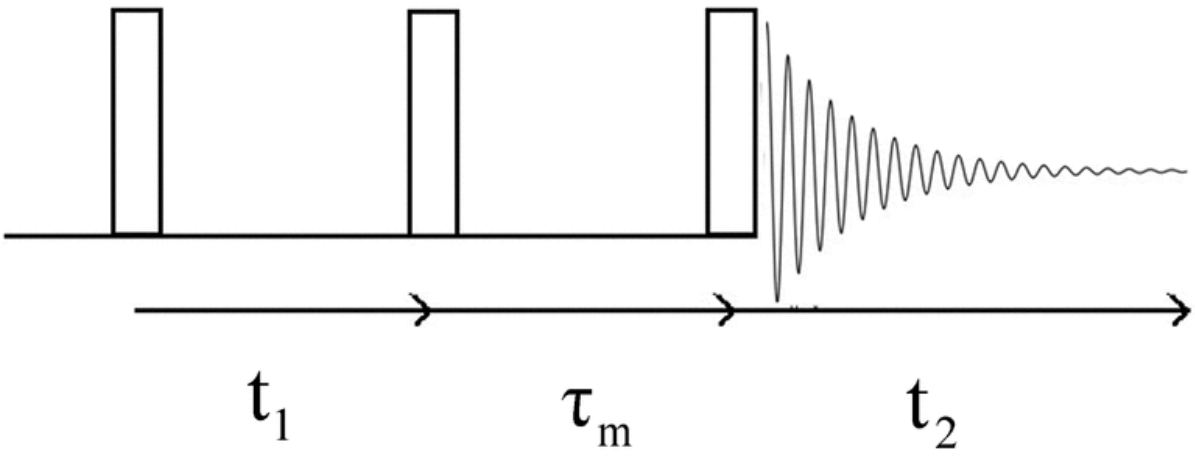


Figure 5. Pulse sequence for a NOESY experiment.

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The evolution period is again the time between the first two pulses and is incremented as in the COSY experiment. The time between the second and third pulses is fixed and is not incremented. This period of time is labeled $\tau_m$ and is called the mixing time. Cross-polarization occurs during this period.

The COSY and NOESY sequences described above are the two simplest 2-D experiments to perform. Many other experiments have been developed and contain a very complicated series of pulses with different pulse widths, time delays, gated decoupling, and pulsed field gradients. Some of these experiments are described below.

## Strategies for Establishment of Homonuclear Connectivities

Assigning signals based on chemical shifts, spin multiplicity, and coupling constants serves as a starting point for structural elucidation. Structure elucidation is simplified if one can establish molecular connectivity between homonuclear spins. This can be done using correlations via bonds (scalar coupling, sometimes referred to as *J* coupling) or via spatial (dipolar coupling) interactions. This section describes NMR techniques that can be used for the study of homonuclear connectivity. Popular experiments in this category are COSY, total correlated spectroscopy (TOCSY), NOESY, and rotating frame Overhauser effect spectroscopy (ROESY).

### COSY

COSY has become a routine 2-D $^1$H NMR experiment that can quickly provide the proton–proton connectivity for spin systems connected by two or three chemical bonds. In a COSY spectrum, the contour plot typically shows diagonal and off-diagonal cross-peaks. The diagonal peaks correspond to the places in 2-D space where chemical shifts of the same nucleus in the 1-D $^1$H NMR spectrum intersect. The off-diagonal cross-peaks occur in 2-D space where the chemical shift of one nucleus intersects the chemical shift of a different nucleus to which it is coupled. The COSY spectrum enables the identification of scalar-coupled spins that are in geminal or vicinal positions. Generally, one starts the assignment process by selecting a resonance in the COSY spectrum that has already been identified in the 1-D $^1$H NMR spectrum. Then, the off-diagonal peaks between this resonance and any others determine the resonances of the neighboring protons to which it is coupled. The neighboring protons identified in this way then serve as the next points to examine for cross-peaks to other protons, and so forth. The process continues until all coupled spin systems are identified.

Thus, a COSY experiment can provide useful information about proton–proton connectivity for various fragments of the molecules under examination. The relationship between these fragments may be difficult to establish because of an interruption of the coupling between the two fragments. For instance, if two segments are connected through quaternary carbons or heteronuclei, the very small four-bond coupling between two protons on the two separate fragments usually cannot be detected by COSY. Basic COSY experiments are usually processed using magnitude mode, which results in broad bases to the peaks. The diagonal peaks may be so broad that spin systems with close chemical shifts may not be observable because the broadness of the diagonal peaks will cause them to overlap the off-diagonal peaks. An example of a COSY experiment, specifically a gradient, or gCOSY, is shown in *Figure 6*.

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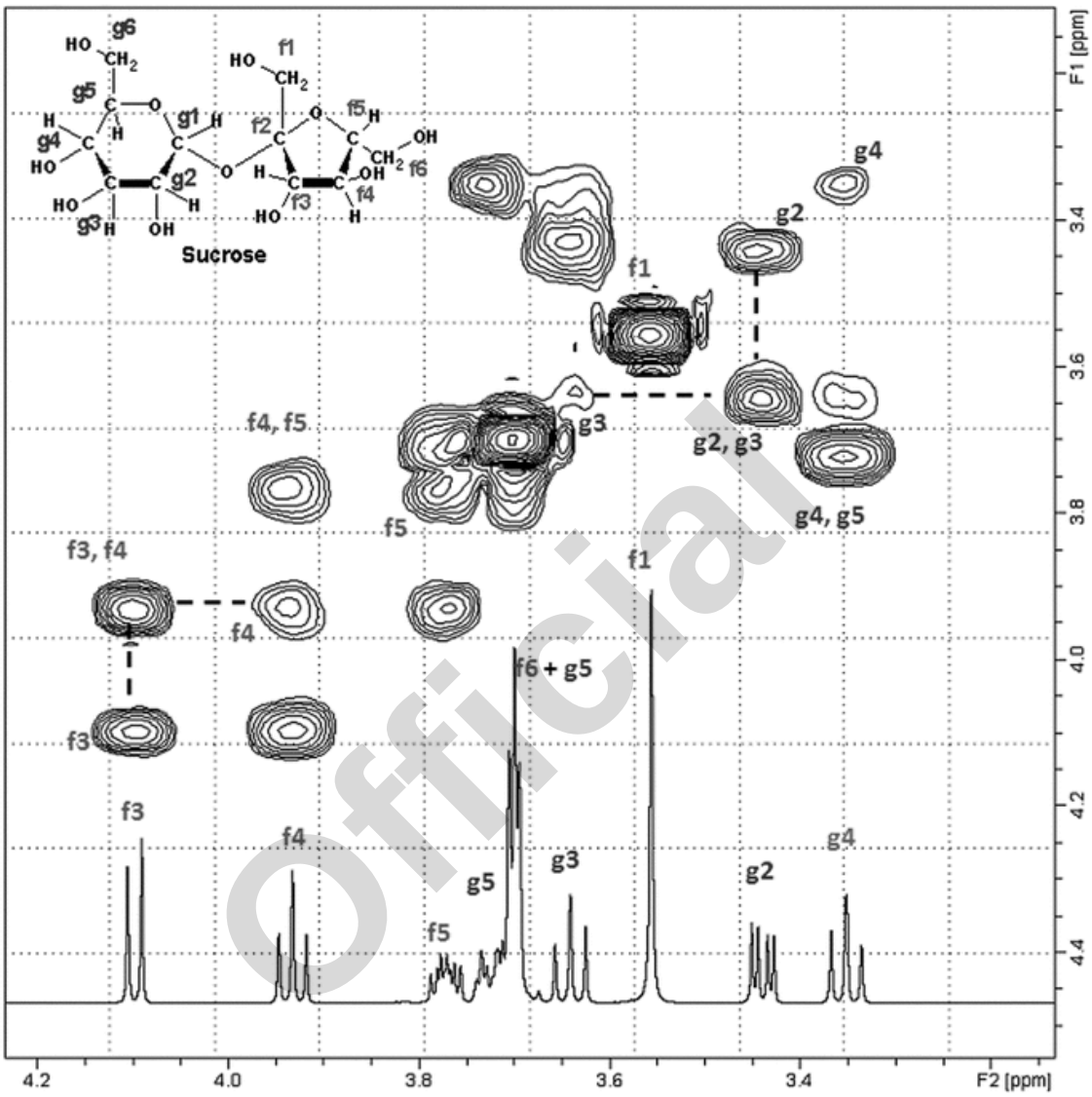


Figure 6. Partial COSY spectrum of sucrose. Dotted lines show how two off-diagonal (f3, f4 and g2, g3) contours are used to determine which nuclei are coupled to each other.

A variety of COSY experiments have been devised to improve upon the original experiment. Perhaps the most important change has been to use gradients to acquire a gCOSY spectrum. The classic COSY experiment uses a 90° pulse to generate transverse magnetization and relies on elaborate phase cycling to cancel unwanted signals over many scans, resulting in long experiment times. In a gCOSY experiment, which requires a probe capable of a pulsed field gradient (PFG), a magnetic field gradient pulse dephases any coherent magnetization in the *xy* plane. If a second gradient pulse of the proper strength is applied in the opposite direction, it will cause any dephased double-quantum magnetization to refocus. Hence, only those signals will be received. The strengths of the gradients can be used to select single-, double- or triple-quantum coherences.

The gradient pulses in a gCOSY experiment can be used to prevent the refocusing of magnetizations that cause the artifacts in the classic COSY experiment. Hence, instead of requiring a minimum of eight phase-cycled acquisitions for each data increment in the second dimension, a gCOSY spectrum requires only a single acquisition per increment. For samples that are sufficiently concentrated to produce an acceptable S/N in only one acquisition, this greatly shortens the experimental time.

Phase-sensitive COSY experiments have been developed to overcome the problem of overlap with closely spaced chemical shifts. A phase-sensitive COSY results in pure absorptive peaks with narrower peaks than are generated by the classic-magnitude COSY. These narrower peaks allow better resolution of resonances that are close to diagonal peaks.

Double-quantum filtered COSY (DQF-COSY) was developed to overcome the problem caused by intense signals from functional groups such as methyls. The singlets from these groups do not provide useful connectivity information, but their intensity often limits the dynamic range of the experiment, making it difficult to observe other weaker signals. The pulse sequences of DQF-COSY detect the spin systems that have only double-quantum transitions. Isolated singlets are not selected

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and thus are filtered out of the final 2-D spectrum. In addition, a reduction in the overall intensity of the diagonal signals is achieved with an increase in the intensities of off-diagonal signals.

## TOCSY (OR HOHAHA—HOMONUCLEAR HARTMANN HAHN)

The $^{1}H$–$^{1}H$ TOCSY experiment is closely related to COSY but differs because it yields correlations for every spin in a coupled network. This is especially useful when multiplets overlap or there is extensive strong coupling. For example, consider the network –CHa–CHb–CHc–CHd–CHe–, where each CHn stands for a spin that is coupled through three bonds to the adjacent spin. A COSY spectrum would show correlations for each adjacent pair of hydrogens. On the one hand, the Hb resonance would show connectivities to Ha and Hc but not to Hd. A partial correlation is revealed for each CHn. On the other hand, a TOCSY spectrum would show all off-diagonal contours for every spin in this network. That is, for every peak in this coupling network there would be off-diagonal contours corresponding to CHa, CHb, CHc, CHd, and CHe. Thus, a TOSCY spectrum, such as the one shown in *Figure 7*, affirmatively identifies all of the spins within the same coupling network. This pattern is easily recognized, especially when there is extensive overlap with other coupled networks. However, a TOSCY experiment cannot establish connectivity between separate networks that are interrupted by heteronuclear atoms, quaternary carbons, or a carbon bearing only exchangeable protons. A TOCSY experiment is useful for the study of large molecules with many separated coupling networks such as peptides, proteins, oligosaccharides, and polysaccharides.

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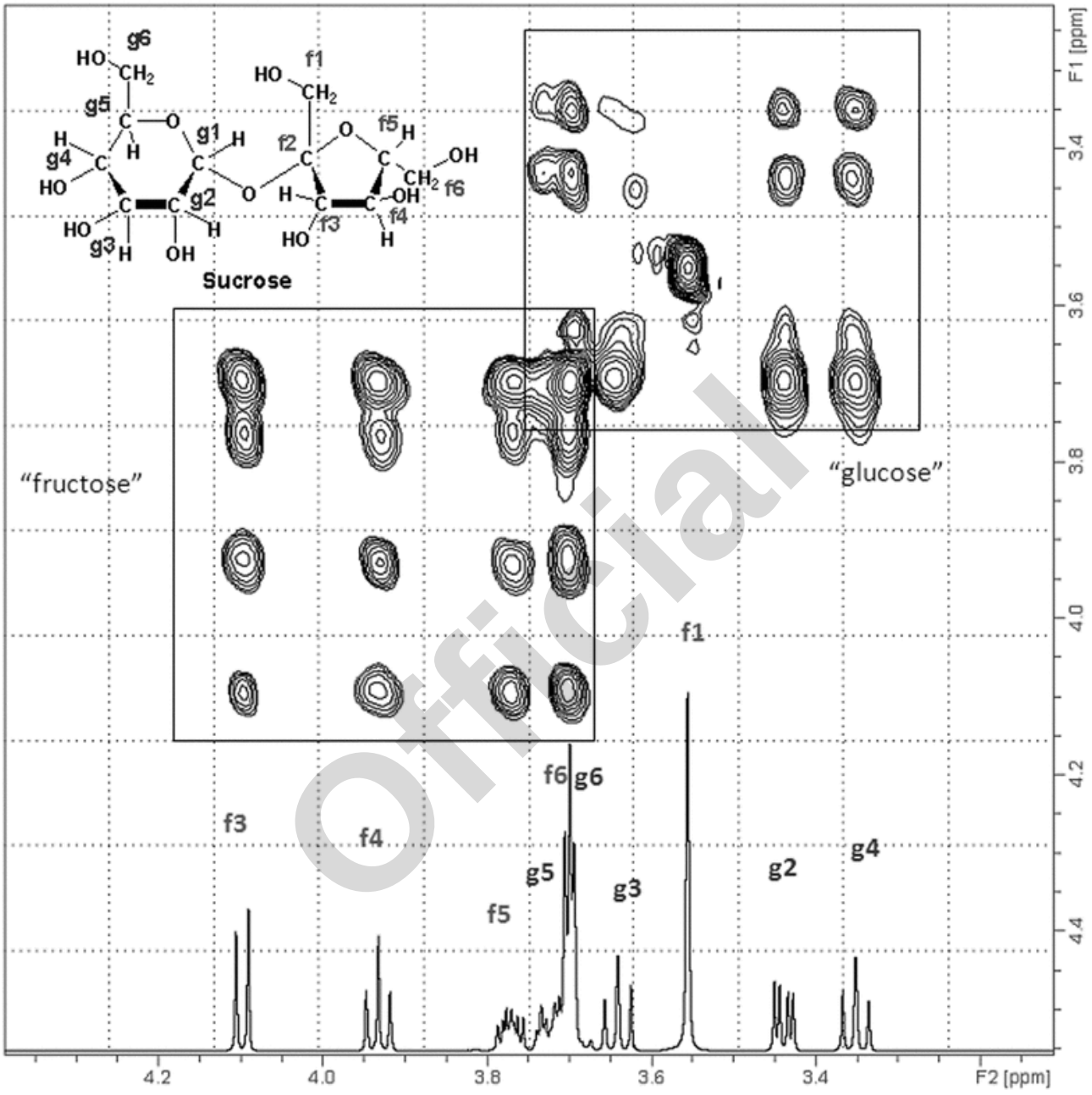


Figure 7. Partial TOCSY spectrum of sucrose showing two sets of contours corresponding to all the coupled nuclei in the glucose and fructose rings in sucrose. The contour for the f1 resonance falls within the glucose spin system and has no coupling.

## NOESY

The NOESY experiment gives correlations between protons that are close to each other in space even though they may not be connected by bonds. These through-space correlations are made via spin-lattice relaxation. Dipole interactions between protons close in space generate NOE transfers, and the magnetization is aligned along the $z$ axis ($B_0$), producing positive or negative intensity changes that yield cross-peaks that are not normally observable in a COSY spectrum. The sign of the NOESY peaks depends on the size and mobility of the molecule under study. Used in combination with other techniques, a NOESY experiment can establish spatial relations for particular spins and can provide critical information about ring structures and conformations.

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### $^{1}H–^{1}H$ ROESY

The ROESY experiment is similar to a NOESY experiment insofar as it also provides correlations between protons that are close to each other in space, whether or not they are connected via bonds. A ROESY spectrum yields through-space correlations via spin-spin relaxation in the rotating frame. The ROESY experiment utilizes a spin–lock sequence as a mixing time during which NOE transfer occurs among all components of the spins locked in the *xy* plane. In contrast to a NOESY experiment, in which NOE transfer occurs while magnetizations are aligned along the *z* axis ($B_0$) producing positive or negative intensity changes, the ROESY experiment depends on NOE transfers occurring in the rotating frame under the influence of a $B_1$ magnetic field. This always results in positive signals no matter how large the molecule or whether its motion is fast or slow. Therefore, a ROESY experiment frequently will provide through-space correlations when the same correlations in a NOESY experiment cannot be detected because of molecule mobility.

### INCREDIBLE NATURAL ABUNDANCE DOUBLE QUANTUM TRANSFER EXPERIMENT

The Incredible Natural Abundance Double Quantum Transfer Experiment (INADEQUATE) uses a double quantum coherence to provide information about $^{13}C$ nuclei directly coupled to other $^{13}C$ nuclei. Thus it provides the same sort of information that is available using a COSY experiment for proton couplings. Because of the low probability of two $^{13}C$ nuclei being attached to each other (only 1 in 10,000 molecules), this technique is usually one of last resort. The ever-increasing sensitivity of cryogenically cooled probes used in modern instrumentation makes this experiment practical in some cases.

## Strategies for Establishment of Heteronuclear Connectivities

Although homonuclear $^{1}H–^{1}H$ connectivity is one important aspect of structure elucidation of organic molecules, the establishment of heteronuclear connectivities is equally important, although somewhat more difficult to obtain given the lower abundance of most heteroatoms. If one has partial assignments in either the $^{1}H$ or $^{13}C$ spectrum, the knowledge of this connectivity leads to a much fuller assignment of both spectra. Heteronuclear 2-D spectra do not exhibit a diagonal as is seen in homonuclear correlations. Rather, cross-peaks occur at the point of intersection of the $^{1}H$ and $^{13}C$ chemical shifts in the 2-D space as shown in *Figure 8*.

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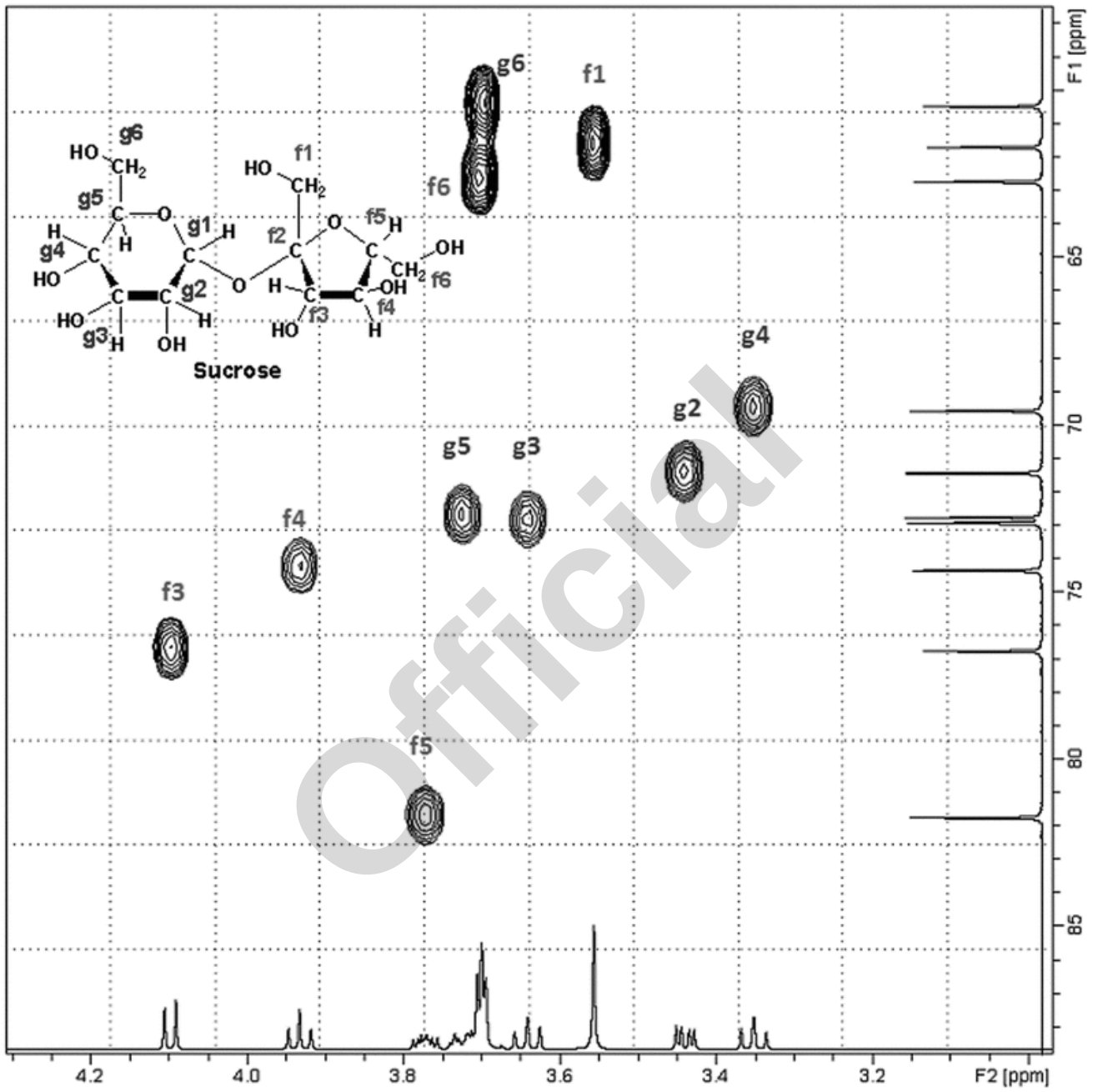


Figure 8. Partial $^{1}H/^{13}C$ HSQC spectrum of sucrose showing contours that indicate the one-bond correlation between a given hydrogen and the carbon to which it is attached.

Heteronuclear 2-D spectra are designed so that $^{1}H$ is the detected nucleus and are usually acquired using inverse- detection probes because the $^{1}H$ coil is wound closer to the sample than the broadband coil. This results in a better filling factor and a greater sensitivity for the $^{1}H$ coil. It should be noted that a newer configuration of coils has been developed that provides the same sensitivity but does not utilize the inverse-detection coil arrangement. Typical heteronuclear 2-D experiments include Heteronuclear Single Quantum Coherence (HSQC), Heteronuclear Multiple Quantum Coherence (HMQC), and Heteronuclear Multiple Bond Correlation (HMBC) experiments.

HMQC

The 2-D HMQC experiment provides information about correlation between protons and their attached heteronuclei via the heteronuclear scalar coupling. The sequence selects double quantum coherence transfer between scalar-coupled spins ($^{13}C–^{1}H$).

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HSQC

HSQC spectroscopy is also an inverse chemical shift correlation experiment that yields the same information as HMQC, i.e., the identification of directly bonded hydrogen–carbon interactions. The correlation between heteronuclei is detected via the selection of single quantum coherence transfer using the insensitive nuclear enhancement by polarization transfer (INEPT) sequences. The main advantage of using this sequence instead of the HMQC sequence is that the $F_1$ domain does not contain any proton–proton couplings. Hence, the resolution is improved.

An interesting modification of this sequence is an edited HSQC experiment. This is a phase-sensitive experiment that not only gives one-bond correlations between hydrogen and carbon but also gives methyl and methine correlation peaks that are 180° out of phase with methylene resonances.

HMBC

HMBC spectroscopy is a modified version of HSQC and is suitable for determining long-range (> 1-bond) $^{1}H$–$^{13}C$ connectivity. Long-range heteronuclear correlation spectroscopy can yield signals for those nuclei that are separated by 2–4 bonds. This experiment, in conjunction with the other 2-D experiments discussed above, allows one to define the structure of a molecule in great detail.

## QUANTITATIVE APPLICATIONS

NMR is one of the most useful techniques for quantitative analysis in chemistry. If appropriate experimental conditions are chosen, the relative intensities of resonances are proportional to the population of the nuclei causing those resonances. NMR experiments can be designed for relative or absolute quantitation, either with an internal standard or without one.

### Experimental Design for Quantitative NMR

Design for quantitation involves the elimination or precise measurement of differences in intensities due to spin-lattice relaxation and NOE. The spin-lattice relaxation time ($T_1$) for all resonances used in the procedure can be measured with an inversion recovery pulse sequence. If a 90° pulse is used for excitation, quantitation at the level of 99.3% may be achieved with a recycle time, $T_r$ (the sum of the relaxation delay and aquisition time), of $5 \times T_1$, and improved to even higher levels by using longer recycle times or shorter pulses. The general equation for the degree of quantitation, $Q$, of a resonance as a function of the pulse angle, $\alpha$, and $T_r$, and $T_1$ is given by:

$$Q = \frac{1 - e^{-Tr/T1}}{1 - [e^{-Tr/T1} \cdot \cos(\alpha)]} \qquad [22]$$

If a 45° excitation pulse is used, with a $T_r = 5T_1$, $Q = 0.998$. However, the accuracy of quantitation in the final spectrum depends not only on the $Q$ values of the resonances but also on the accuracy of the integration method and on the S/N ratio in the spectrum. Hence, $Q$ values somewhat less than unity may be warranted and other angles and recycle times should be used.

The minimization of systematic quantitation bias should be sufficient for the intended use of the procedure. Alternatively, quantitative procedures may be developed using conditions for which $Q$ is not unity for some or all resonances, provided the value of $Q$ is precisely known and corrected for.

For quantitative methods using heteronuclei, the possibility of differential NOEs should be avoided by using a $T_r$ at least 5 times the longest $T_1$ value and by using inverse gated decoupling (decoupler gated on only during the acquisition time). Preferably, 90° pulses should be used. For quantitative methods, relaxation agents are often used for shortening the $T_1$ values of heteronuclei.

The reproducibility of an NMR method depends on a variety of acquisition and processing parameters, all of which should be described in the procedure. These include pulse angle, acquisition time, relaxation delay, spectral width, number of points in the FID, number of acquisitions, number of points used (if any) for zero filling, line broadening, baseline correction, integral breaks, and temperature. For best reproducibility, integral breaks should be specified to 0.01 ppm for $^{1}H$ NMR methods and to 0.1 ppm for $^{13}C$ methods.

Quantitative analysis, as well as detection of trace impurities, has markedly improved with modern instrumentation. Stronger magnetic fields and improved probe technology have enhanced the sensitivity of NMR procedures in recent years.

## SOLID-STATE NMR

The analytical usefulness of solid-state NMR spectroscopy for studying solid materials lies in the fact that the same types of nuclei in different *solid-state* environments exhibit different resonance frequencies. Applications of this technique in pharmaceutical analysis range from solid form (polymorph, solvate) identification and quantitation in bulk drug substances to physical/chemical profiling of dosage forms. The technique has the unique ability to probe electronic environments of specific nuclei in the solid state over a large timescale without the requirement of single-crystal substrates or even homogeneous

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samples. Methods and procedures presented herein are directed at observing $^{13}C$, the most popular NMR nucleus for solids. The concepts may be equally applied to other relevant spin-1/2 nuclei such as low-natural-abundance $^{15}N$ as well as high-natural-abundance $^{31}P$.

## Cross-Polarization Magic Angle Spinning (CPMAS) NMR Technique

The basic principles of NMR are the same for solution and solid-state measurements, but conventional solution-phase 1-D NMR data acquisition techniques do not normally produce detectable spectra for solid samples because of low sensitivity and extensive line broadening. The sensitivity of the solid-state experiment is low for $^{13}C$ based on its 1.1% natural abundance and long spin-lattice ($T_1$) relaxation times. Line broadening arises primarily from dipole–dipole interactions and chemical shift anisotropy (CSA), which are not averaged to zero because of the fixed orientation of molecules in a packed solid sample vs the rapid molecular tumbling of the molecules when they are in solution. If not averaged, CSA results in the simultaneous observation of all different orientations of molecules with respect to the applied magnetic field. CSA patterns may span the width of an entire liquid spectrum. Three modifications to standard solution methods—cross-polarization (CP), magic angle spinning (MAS), and high-power $^{1}H$ decoupling—are routinely used in combination to obtain high-resolution solid-state NMR spectra.

### CROSS-POLARIZATION (CP)

CP addresses the low sensitivity associated with collecting NMR spectra of dilute spin-1/2 nuclei such as $^{13}C$. CP is a double-resonance procedure wherein abundant $^{1}H$ and rare $^{13}C$ spins are brought into resonance by simultaneously applying two spin–locking rf fields ($B_{1H}$ and $B_{1C}$), the magnitude of which will satisfy the Hartmann–Hahn matching condition,

$$\gamma_H B_{1H} = \gamma_C B_{1C} \text{ [23]}$$

During this contact time, polarization transfer occurs allowing the rare $^{13}C$ spins to take on the magnetization and relaxation behavior of the abundant $^{1}H$ spins, leading to a sensitivity enhancement (up to four-fold based on the ratio of the $^{1}H$ and $^{13}C$ magnetogyric ratios) and a reduction in the pulse repetition time. Reducing the pulse repetition time allows a greater number of aquisitions to be accumulated per unit time, which yields a better S/N ratio. In instances where it may be difficult or impossible to record CP spectra because of weak $^{13}C$–$^{1}H$ coupling or short spin-lattice relaxation times in the rotating frame ($T_{1\rho H}$), direct polarization (Bloch decay) may be the only approach to recording solid-state NMR spectra.

### MAGIC ANGLE SPINNING (MAS)

Line broadening in solid-state NMR is eliminated or averaged by both MAS and high-power $^{1}H$ decoupling. MAS involves mechanically rotating the sample at an angle of 54.7° (the magic angle) relative to the static magnetic field in order to simulate rapid molecular tumbling in solution. Rotating a solid sample at the magic angle to minimize line broadening requires that the sample be spun faster (in Hz) than the width of the CSA. High spinning rates are possible with current MAS probe technology, but complications can arise with CP and may require techniques such as ramped-amplitude CP (RAMP-CP) and variable-amplitude CP (VACP) to improve the efficiency of magnetization transfer from $^{1}H$. Additionally, MAS can raise the sample temperature significantly if it is not controlled, and pressures at the periphery of the rotor may be thousands of times the ambient pressure. These stresses may induce phase transformations, loss of solvent, and other effects.

#### SIDEBAND SUPPRESSION

As shown in *Figure 9*, slower spinning rates can be used to avoid compromising the solid sample, but when CSA is incompletely averaged, spinning sidebands will appear in solid-state NMR spectra. These artifacts are separated from the centerbands by integer multiples of the spinning rate (in Hz) and can be readily identified as the peaks that shift in spectra acquired at different spinning speeds. Spinning sideband manifolds contain useful information but can interfere with the signals of interest and may be particularly problematic when one uses higher field instruments. The total suppression of spinning sidebands (TOSS) procedure is commonly used to eliminate spinning sidebands from solid-state NMR spectra.

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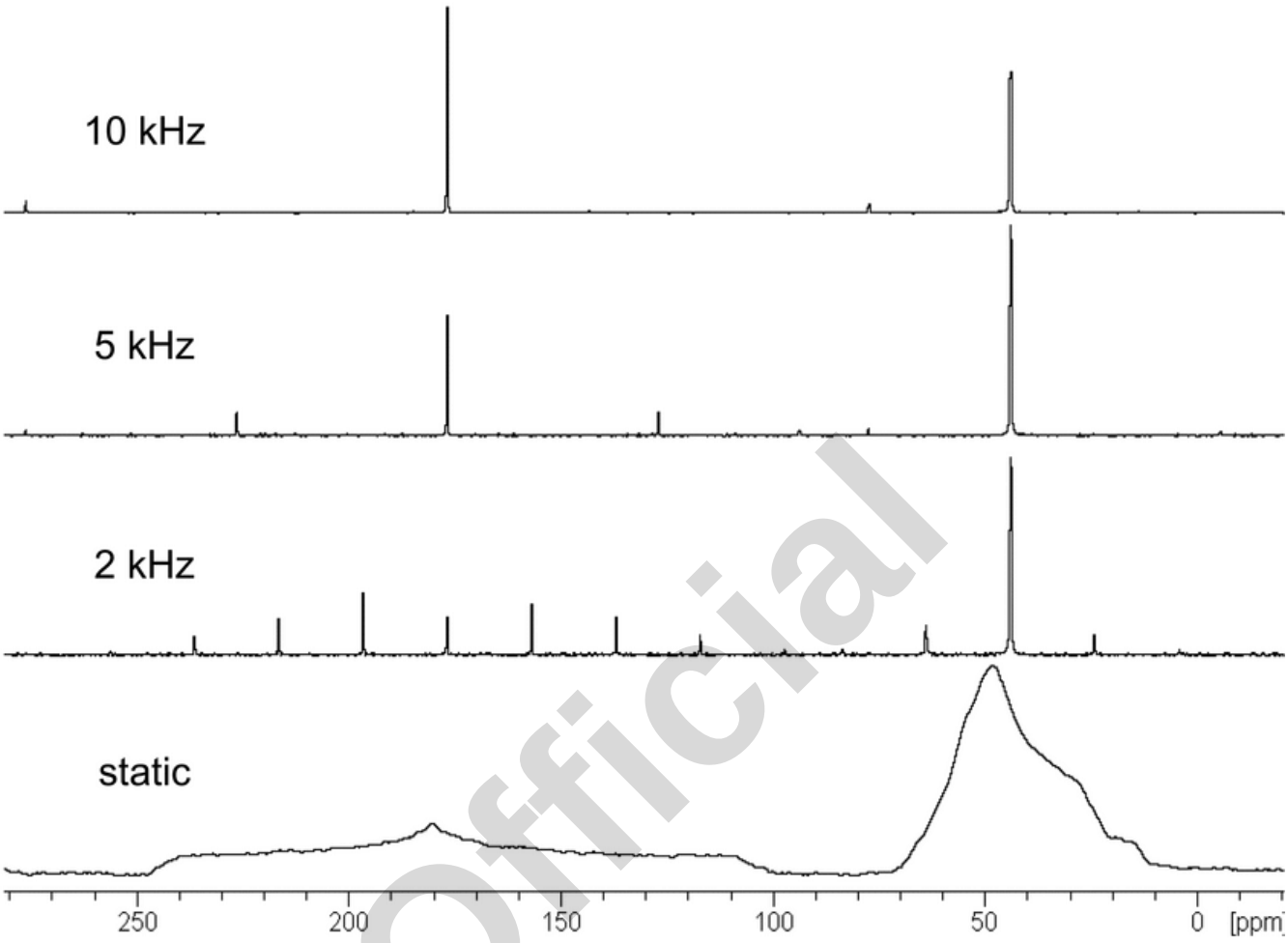


Figure 9. $^{13}C$ CPMAS NMR spectra of polycrystalline α-glycine collected at different sample spinning (MAS) rates. Spinning sidebands appear in solid-state NMR spectra when the sample spinning frequency is less than the width of the CSA pattern.

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## HIGH-POWER $^{1}H$ DECOUPLING

High-power $^{1}H$ decoupling is used to further reduce the line broadening from dipolar coupling to $^{1}H$ spins in the solid state. Specialized hardware is required to deliver the rf power needed for $^{1}H$ decoupling in solids, an rf power that is two orders of magnitude greater than that required to remove scalar coupling in liquids. CW decoupling is commonly used in solid-state NMR, although two-pulse phase-modulated and small phase incremental alteration (SPINAL-64) decoupling are increasingly used to improve the sensitivity and resolution of dilute spin spectra.

Typically only $^{1}H$–$^{13}C$ heteronuclear dipolar interactions are significant when one acquires solid-state $^{13}C$ NMR spectra of organic materials. Homonuclear $^{13}C$–$^{13}C$ dipole–dipole and scalar coupling interactions are negligible. Because of their low natural abundance, the probability that two $^{13}C$ nuclei are in close proximity is very small. However, homonuclear $^{13}C$–$^{13}C$ dipolar coupling can be a concern for $^{13}C$-labeled substrates.

## EXPERIMENTAL SETUP

The basic setup for a CPMAS experiment will necessarily include magic angle setting, shimming, pulse calibration, Hartmann–Hahn matching, and spectral referencing, each of which is typically conducted on standard samples. Accurate measurement of the pulse lengths and associated rf power levels is essential for solid-state NMR experiments. Setting up the magic angle, shimming a CPMAS probe, and measuring its sensitivity for different tuning ranges are parts of probe setup and performance assessment. Shimming a solid-state NMR probe for MAS is more complicated than that for a solution probe because shim gradients are designed for the vertical orientation of solution NMR tubes. Solid-state NMR probes have no $^{2}H$ lock channel, so shimming must be performed manually. Because of the intrinsically broad peaks, shimming is not nearly as critical in solid-state NMR as it is in solution NMR.

Standard compounds used for setting the magic angle and optimizing pulse lengths and the associated rf power levels for CP are listed in *Table 6*. KBr is commonly used for magic angle adjustments, observing the $^{79}Br$ resonance and adjusting the sample spinning angle to maximize (to 10 ms and beyond) the duration of the rotational echo train in the FID. Liquid samples can be used to shim solid-state NMR probes, although solid adamantane is commonly used for this purpose. Adamantane,

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glycine, and hexamethylbenzene (HMB) are commonly used for Hartmann–Hahn matching and testing sensitivity. Typically, the reference sample for testing sensitivity is permanently packed into a rotor to ensure that the same amount of sample is used.

**Table 6. Standard Samples Commonly Used for Setting Up CPMAS Experiments**

| Setup Procedure | Nucleus | Standard Sample(s) |
|---|---|---|
| Setting the magic angle | $^{79}$Br | KBr |
| Shimming | $^{13}$C | adamantane |
| Pulse calibration | $^{1}$H, $^{13}$C | adamantane, HMB |
| Hartmann-Hahn matching | $^{1}$H/$^{13}$C | adamantane, HMB |
| Sensitivity | $^{13}$C | HMB, adamantane, α-glycine |

Solid-state NMR spectrometers are generally used without field/frequency locking, so the resulting chemical shifts are less accurate than those for solutions. Calibration of the chemical shift can be done using either a primary or secondary standard. Spectral referencing is typically performed by sample replacement (external referencing). Standard compounds commonly used for spectral referencing in solid-state NMR are listed in *Table 7*. Note that glycine is polymorphic, so its crystal form should be ensured before its use in referencing spectra.

**Table 7. Reference Compounds Commonly Used for Solid-State NMR**

| Nucleus | Primary Standard | Secondary Standard(s) | Chemical Shift in ppm from Primary Standard |
|---|---|---|---|
| $^{13}$C | TMS | HMB<br>adamantane<br>α-glycine | 17.35 ($CH_3$)<br>38.48 ($CH_2$)<br>176.45 (carboxyl) |
| $^{15}$N | nitromethane | $NH_4{}^{15}NO_3$<br>$^{15}NH_4Cl$<br>α-glycine-$^{15}$N | –5.1 ($NO_3$)<br>–338.1<br>–349.5 |
| $^{31}$P | 85% $H_3PO_4$ | $CaHPO_4 \cdot 2H_2O$ (brushite) | 1.4 |
| $^{29}$Si | TMS | tetratrimethylsilylmethane | –1.4 |
| $^{19}$F | $CF_3Cl$ | perfluorobenzene | –166.4 |

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## General Test Procedure

Spectrometer performance should be demonstrated first for a reference sample as described in *Cross-Polarization Magic Angle Spinning (CPMAS) NMR Technique, Experimental Setup*. The magic angle, pulse lengths, and associated rf power levels for CP that are established using the reference compounds are sample independent. To obtain a CP spectrum of the sample, only the recycle delay needs to be chosen, followed by the contact time. When quantitative signal intensities are not required, an optimum recycle delay, i.e., one that affords the best S/N ratio, is $1.2T_{1H}$, and the contact time is generally that which provides the optimum S/N ratio or that which best shows the features of most interest. For quantitative CP, a recycle delay of at least five times the longest $T_{1H}$ of a heterogeneous mixture is suggested to ensure full relaxation, and a full analysis of the CP signal as a function of contact time must be conducted. See the subsection *Quantitative Analysis.*

### SAMPLE PREPARATION

Sample-handling procedures used in solid-state NMR are substantially different from those used in liquid NMR. Solid samples are packed in ceramic rotors that are capped with fluted drive tips specifically designed for MAS. Fine powders are typically tamped into MAS rotors, although solid plugs, e.g., whole tablets, can be cut to fit the exact inner dimensions of the rotor and can be inserted directly into the rotor. Crushing or grinding may be used to reduce the sample to a fine powder, but caution should be used in order not to induce phase transformations. Depending on the compressibility of the powder and the rotor volume, 40–400 mg of material is typically required to fill a sample rotor.

## Physical Characterization

Specific components in heterogeneous systems can be searched based on unique nuclei or different NMR relaxation properties. The identification of crystalline and amorphous materials can be accomplished by comparison of the solid-state NMR spectrum of the sample preparation with that of a reference standard. Chemical shifts and relative peak intensities can be used in the comparison. Amorphous materials generally give good MAS spectra with broader peaks than those seen for crystalline materials. Highly crystalline samples typically give $^{13}$C linewidths of the order of a few tens of Hz. The shape of the signals generally is between Lorentzian and Gaussian, a fact that should be recognized in deconvoluting overlapped spectra.

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## Relaxation

Relaxation parameters of interest in solids include spin-lattice relaxation ($T_1$), spin-lattice relaxation in the rotating frame ($T_{1\rho}$), spin-spin relaxation ($T_2$), and cross-relaxation ($T_{CP}$). In organic solids, $^1$H spin diffusion is generally efficient so that pure compounds normally give single values for each of the relaxation times, $T_1$ and $T_{1\rho}$. For CP experiments, $T_{1H}$ is used to establish the recycle delay between acquisitions. $T_{1H}$ relaxation times can be measured using either progressive saturation or inversion recovery pulse sequences. In CP experiments, $T_{CP}$ and $T_{1\rho H}$ characterize the magnetization build-up and decay, respectively. $T_{1\rho H}$ is measured via the $^{13}$C signal using a delayed contact CP pulse sequence that has a variable delay time before the CP contact.

## Quantitative Analysis

To quantitatively assess solid-state NMR spectra under CP conditions, extra measures must be taken. In addition to ensuring that the sample spinning axis is precisely set to the magic angle (54.7°) to minimize CSA broadening, the temperature and spin rate must be carefully controlled and the MAS probe properly tuned. Suggested recycle delays of $5T_{1H}$ are allotted between successive pulses to ensure that the magnetization has returned to its full equilibrium value. Both $T_{CP}$ and $T_{1\rho H}$ relaxation must then be accounted for in the selection of the contact time. Typically, the CP contact time chosen is that which provides maximum sensitivity for the signals of interest. Quantitative analysis can be performed using either internal or external referencing methods. The use of internal standards can compensate for variability in sample volume and $B_1$ inhomogeneity throughout the sample.

By properly setting data acquisition parameters (recycle time, pulse widths, contact time, Hartmann–Hahn match, and decoupling power for each chemical system), signals can be obtained that are proportional to the number of nuclei producing them. For quantitative analysis, integrated signal intensities should be used rather than peak heights because linewidths in solid-state spectra often vary. When spinning sidebands are not eliminated by MAS, the intensity of the spinning sideband manifold must be added to the centerband intensity.

## Spectral Editing

A key step in the analysis of any NMR spectrum is the assignment of individual resonances to unique phases and, in some cases, to specific atoms in the molecule. Special pulse sequences are available and may assist in simplifying CPMAS spectra and assigning signals. Dipolar dephasing, also known as nonquaternary suppression or interrupted decoupling, yields spectra that typically contain signals only from quaternary and methyl carbons. Spectral subtraction of dipolar dephasing spectra from normal CP spectra or short contact time CP may be used to produce subspectra that contain signals from methylene and methine carbons only. Polarization-inversion techniques can be used to identify methylene and methine carbons.

# LOW-FIELD NMR

Low field NMR (LF-NMR), sometimes referred to as time domain NMR (TD-NMR), experiments are performed by measuring relaxation, relaxometry, or diffusion. Instrumentation for these applications is based on low-field permanent magnet technologies that operate in the 2–25 MHz frequency range. Inexpensive stationary bench-top and portable TD-NMR spectrometers are commercially available. Typical bore sizes are 10–50 mm in diameter. A recent development in spectrometer design uses a mobile mouse probe as an alternative to a stationary magnet. This design allows analysis of samples of unrestricted size.

Most TD-NMR applications are based on simple pulsing sequences, including FID, Hahn–echo, Carr–Purcell– Meiboom–Gill, and solid–echo acquisition. The choice of pulse sequence depends on the physical and chemical properties of the sample as well as the information that is desired from the experiment. These systems can be used to measure longitudinal (spin-lattice, $T_1$) and transverse (spin-spin, $T_2$) relaxation times. Diffusion properties of compounds can be exploited using pulsed field gradient (PFG-NMR) experiments.

The classical application of relaxometry is for the determination of food product components based on differences in longitudinal and transverse relaxation times of water, fats, and proteins.

## Relaxivity

The magnitude of a substance's capacity to enhance the relaxation rate of a nucleus is referred to as relaxivity, expressed in units of $sec^{-1}mM^{-1}$. An example of such a substance commonly used in NMR spectroscopy is paramagnetic chromium acetylacetonate. Paramagnetic species are used in the medical industry as contrasting agents for magnetic resonance imaging. The relaxivity of a substance is determined experimentally by measuring the spin-lattice relaxation time ($T_1$) of a test substance and plotting $1/T_1$ against the concentration in units of mM (mmol/L). The slope of the curve is the numerical relaxivity.

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# ⟨1771⟩ OPHTHALMIC PRODUCTS—PERFORMANCE TESTS

## INTRODUCTION

This chapter provides information on performance tests to assess drug release from finished ophthalmic products. These tests are applicable to products that have an extended-release mechanism (beyond 1 day); the dissolution/drug release rate is rate limiting for absorption and is expected to provide a controlled therapeutic response. Examples of such products include intraocular matrix-type and polymer-based bioerodible implants, non-bioerodible intraocular matrix and reservoir implants, intraocular injectable suspensions/colloidal systems, intraocular in situ–forming depots and gels, punctal plug-based delivery systems, non-biodegradable drug release devices (e.g., drug-coated stents, drug-coated contact lenses), biodegradable and non-degradable ocular inserts/bioadhesives (for cul-de-sac or conjunctival-sac applications), and other such dosage forms. For products having a localized and immediate response when applied to the eye (e.g., topically applied dosage forms, including dispersed systems, having very short residence time for absorption), a dissolution/drug release test may have no practical value.

Application of a dissolution/drug release test to assess performance as a surrogate for in vivo testing should be considered only with appropriately validated in vivo–in vitro correlations (see *In Vitro and In Vivo Evaluation of Dosage Forms* ⟨1088⟩). Dissolution/drug release tests are used as a quality control tool for specific product attributes (e.g., burst release from matrix-type, biodegradable, polymeric systems). These tests may have the ability to distinguish between different lots of a drug product having one or more formulation and/or process changes.

Changes may be related to the drug substance(s) or excipients present in the formulation, physical and/or chemical attributes of the finished formulation, critical manufacturing variables, shipping and storage effects, aging effects, or other formulation and/or process factors critical to the performance of the ophthalmic product. Such changes may affect the performance characteristics of the dosage form when applied to the eye. Consideration should be given to whether processing or components are intended to provide for prolonged exposure to any part of the eye and whether a dissolution/drug release test could be used to demonstrate this effect.

## PERFORMANCE TESTS (DISSOLUTION/DRUG RELEASE)

Depending on the design and release mechanism of the dosage form, the dissolution/drug release test can be developed using any apparatus described in *Dissolution* ⟨711⟩ or *Drug Release* ⟨724⟩. Novel dosage forms may require the use of noncompendial equipment and/or conditions. Any dissolution/drug release test should be discriminative for the intended critical quality attributes of the product and should be properly validated (see *The Dissolution Procedure: Development and Validation* ⟨1092⟩). The test conditions should reasonably mimic the method of administration of the product and in vivo conditions to establish, if possible, an in vivo–in vitro correlation that can be used to predict in vivo performance of the product.

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***Add the following:***

# ▲⟨1776⟩ IMAGE ANALYSIS OF PHARMACEUTICAL SYSTEMS

1. INTRODUCTION AND SCOPE
    1.1 Image
    1.2 Image Analysis
2. APPLICATIONS OF IMAGE ANALYSIS OF PARTICLES
    2.1 Characterization of Raw Materials in Development
    2.2 Image Analysis through Life Cycle/Late Stage Manufacturing
    2.3 Assessing the Contribution of Particle Size and Shape to Bulk Powder Properties Such as Flow and Behavior
    2.4 Particle Quantitation and Identification of “Foreign Particulate Matter”
    2.5 Assessing Crystal Growth, Milling, and Dissolution
    2.6 Studying Particle Properties as a Function of Environmental Conditions
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## 1. INTRODUCTION AND SCOPE

Image analysis is the qualitative or quantitative characterization of two- or three-dimensional digital images. In its simplest form, image analysis is the manual measurement of an image using a scaled ruler. This is a time-intensive way to generate data associated with an image, and it is not in the scope of this chapter (see *Optical Microscopy* ⟨776⟩). Image analysis is primarily a size and shape analysis and yields data that are complementary to many other techniques such as laser diffraction and sieve analysis.

Digital image processing and analysis started in the late 1970s, and technology and automation have advanced to enable more routine use of image analysis. Therefore, its applications and use in the pharmaceutical industry are expanding rapidly. Currently, the use of automated image analysis has been expanded to be a valuable research and quality-control tool. For example, where monitoring of particle-size-related parameters was predominant historically, image analysis is increasingly being adopted to complement size with numerous shape measurements and images that offer additional insight into the materials in question.

The purpose of this chapter is to provide general considerations regarding the use of images for particle and material characterization (size and shape analysis, identity, etc.) as well as a standardized approach for the use of the technique in pharmaceutical applications. The chapter also describes the principles of the science that supports the measurement tool.

### 1.1 Image

An image is an array of pixels where the pixel position represents the spatial arrangement of the imaged objects within the field of view. Pixel values represent a measured property such as intensity (gray scale), color (intensities for multiple colors), and spectra (absorbance versus wavelength). This chapter concentrates on two-dimensional images, however, it should be noted that the third dimension may also be employed in tomographic methods [nanotomography, atomic force microscopy (AFM), microtomography, etc.] and 3D shape characterization, for example.

Image resolution is important and is defined by the number of pixels per unit area. More pixels improve shape definition and detection of smaller features. This is illustrated in *Figure 1*, where we can see better resolution for sizing accuracy and shape discrimination. However, system resolution is also impacted by other optical components such as optics and lighting.

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Figure 1. An example of pixel arrays with increasing resolution.

Images may be obtained in several ways:

- Macroscopic imaging (e.g., photography)
- Microscopy [optical microscopy, scanning electron microscopy (SEM), confocal scanning microscopy (CSM), transmission electron microscopy (TEM), and AFM]
- Electromagnetic reflection, illumination, and detection [terahertz, infrared, visible, ultraviolet, X-ray, micro-computed tomography (micro-CT), neutron]

Images may also be generated in different environmental conditions (e.g., different temperatures) or during processing, enabling the analysis of transformations.

## 1.2 Image Analysis

Image analysis is the derivation of information from the numerical processing of the pixel array. Images are processed using algorithms to differentiate the component parts (often particles) from the background. The data are then analyzed to provide quantitative information such as:

- Size (dimensions, separations, etc.)
- Shape (curvature, aspect ratio, etc.)
- Surface texture and/or roughness
- Number density of features with particular characteristics
- Chemical nature (*Mid-Infrared Spectroscopy—Theory and Practice* ⟨1854⟩, *Near-Infrared Spectroscopy—Theory and Practice* ⟨1856⟩, and *Raman Spectroscopy—Theory and Practice* ⟨1858⟩)
- Classification of different types of features within a sample; this could be done to quantify components of the same species with different morphological profiles (in its simplest form, those that are larger/smaller) or it could be to differentiate between components of the image

Image analysis is employed in many fields with applications as diverse as astronomy, medicine/biology, meteorology, satellite imaging, facial recognition, geology, agriculture, materials science, pharmaceuticals, and other industrial applications. The content of this chapter is limited to pharmaceutically relevant applications of image analysis, including the following:

- Characterization of particle size, shape, and texture
- Mixture composition and uniformity

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- Orthogonal technique complementary to other physical analyses
- Bulk materials characteristics and processability
- Foreign particulate matter detection, quantification, and/or identification in the visible and subvisible ranges

Within pharmaceutical, medical, and biological applications there are other examples not covered in this chapter, including the following:

- Defect detection and identification (e.g., vials, tablets, packaging, printing) using machine vision systems
- Fill level detection and verification
- Biological tissue characterization such as microscopic and macroscopic imaging in pathology and radiology [e.g., X-ray, CT, magnetic resonance imaging (MRI)]
- Clinical applications such as pathology and diagnostics

## 2. APPLICATIONS OF IMAGE ANALYSIS OF PARTICLES

### 2.1 Characterization of Raw Materials in Development

Image analysis is a useful tool in characterization of the active pharmaceutical ingredients (APIs) throughout the development process. One of the advantages at the early development stage is that a multiparameter, full description of particles is possible from a small quantity of material (e.g., milligrams). At the early stages, physical characteristics may vary between different lots of raw materials. Aspects of particle morphology can be monitored and optimized for drug delivery, formulation, and processing. This also enables the optimization of the API synthesis process. Examples include assessing particle roughness for respiratory formulations, particle size and shape distribution for solid dosage formulations, and particle morphology for suspension formulations. Use of early phase image analysis can support formulation and process development in the later phases.

In the later stages of development, a broader understanding of the particle morphology of the API and its impact on the formulation process can be obtained by image analysis than by the more traditional techniques (e.g., laser diffraction, optical and scanning microscopy, and specific surface area). Image analysis may also be a key technique in characterization of excipients, intermediates, and the formulated drug product. For example, image analysis may show that a well-defined range of size distribution of raw materials is needed to avoid segregation during powder-blending steps or that the granule size distribution can be optimized for compression.

### 2.2 Image Analysis through Life Cycle/Late Stage Manufacturing

Image analysis is also employed throughout the product life cycle in such areas as the investigation of process changes and quality by design (QbD). This enables predictive trending for raw material changes (API and excipients) and tracking of crystallization/crystal growth, thereby allowing the definition of in-process specifications.

Quality control is another area where image analysis can serve as a useful tool. Raw materials can be tested for size as well as for aspects of shape, such as smoothness, which impact both flowability and mixing (*1*). Ensuring consistency in overall size and shape as well as identifying an overpopulation of fine particles or larger agglomerates is an advantage of image analysis over size-only methods. In API manufacturing, image analysis systems also allow for number-based assessment of crystal growth and concentration as well as crystal milling to ensure proper size and shape of API material.

Raw materials and finished product may also be assessed for the presence and quantity of intrinsic or foreign particles.

### 2.3 Assessing the Contribution of Particle Size and Shape to Bulk Powder Properties Such as Flow and Behavior

Particle morphology may have a significant impact on a powder's suitability for different pharmaceutical applications (*2*). Even relatively minor variations in these physical properties can affect interparticulate forces, which in turn influence powder flow, packing, and in-process/application behavior. Image analysis enables quantification of particle size and shape properties of the bulk powder. In addition, as it is fundamentally a number-based technique (see *4.2.3 Data Reporting*), it provides insight into the presence of low quantities of particles with a specific morphology compared to the bulk, such as the presence of fine particles, information that may not be obvious when measuring by volume-based techniques alone.

Clear relationships have been observed between particle size, shape, and flow properties under a range of different process conditions (*3*) such as:

- The presence of fine particles may cause sticking
- Particle shape may determine the extent of particle interlocking, entrained air, and bed permeability
- Fine particles and/or irregular particle shape can lead to bridging and blockages
- Morphology may influence particle–particle friction, determining the ease with which particles shear against each other whether as APIs, excipients, or blends
- Particle shape may influence particle interactions with equipment surfaces and other media

Process operations where particle parameters are important for considering powder behavior include:

- Flow out of hoppers and intermediate bulk containers (IBCs) and flow into tablet dies (*4*)
- Use of dry powder inhalers (DPIs), as particles entrained in an air flow during conveying or dispersion will experience different drag forces and therefore different terminal velocities

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- Variation in aerodynamic behavior contributes to the complex interactions between the carrier and drug particles within an assembly during aerosolization and affects impact energies during collisions, which are recognized as important factors contributing to DPI efficacy (*5*)

## 2.4 Particle Quantitation and Identification of "Foreign Particulate Matter"

It is possible to generate information on the quantity, size, and shape of particles including contaminants. Contaminants may be identified through a combination of size, shape, opacity, and/or color differentiation or identified from the particle images themselves. In some cases, staining the sample may help identify and separate contaminants. If a suitable combination technology is used, such as electron microscopy coupled with energy dispersive X-ray (EDX) or optical microscopy coupled with Raman spectroscopy, the chemical composition of contaminants can also be determined.

The maximum number of particles that are permissible in a specific mass or volume of sample is addressed in *Particulate Matter in Injections* ⟨788⟩, *Visible Particulates in Injections* ⟨790⟩, *Methods for the Determination of Subvisible Particulate Matter* ⟨1788⟩, and *Visual Inspection of Injections* ⟨1790⟩.

## 2.5 Assessing Crystal Growth, Milling, and Dissolution

Crystallization is a key step in almost every process in the manufacturing of pharmaceutical compounds. Crystallization is often used as a purification step. Image analysis of crystal growth can be used to detect the starting point (when nucleation begins) and then used to record event data. As the crystallization process advances, image analysis systems can capture in real time the size, shape, and concentration data as well as thumbnail images that serve as objective evidence of the process. This is all captured in a series of sequential analyses and compared over time. These data can then be used to study the rate of crystallization and how the crystal shapes form over time under different conditions. In some cases, instrumentation can be used to notify the end user when select shape and size conditions are met. The different shapes can help identify how these crystals can impact bioavailability, solubility, and dissolution rates.

The images in *Figure 2* were taken from a crystallization process where sequential analyses were taken. Each analysis captures the size, shape, concentration, and thumbnails of the crystal particles. As the subsequent runs are taken, the crystal particles are growing in size, changing in shape, and increasing in concentration. Thumbnail images are used as objective evidence to backup the large amount of particle data saved for each analysis.

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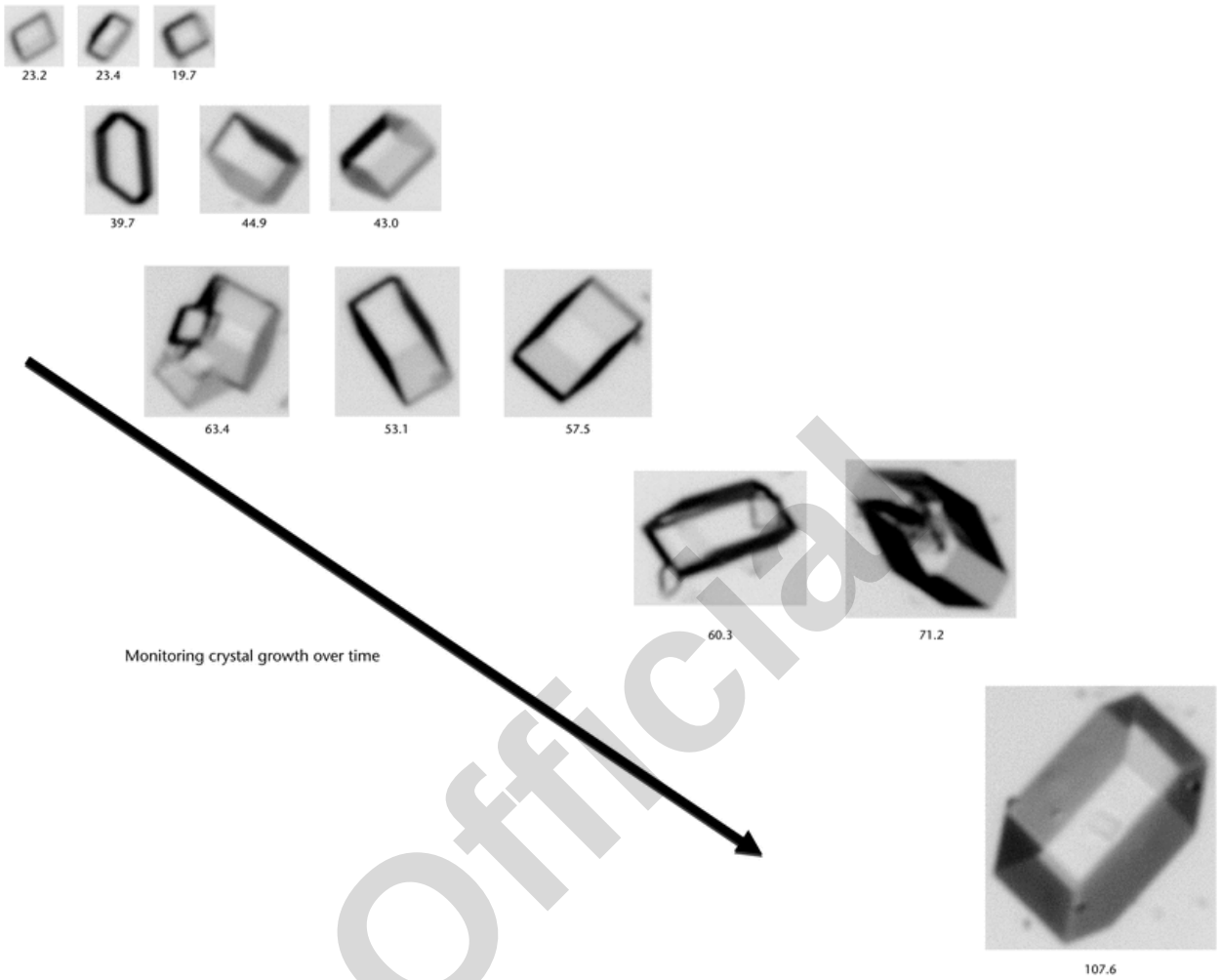


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Figure 2. Monitoring crystal growth using image analysis. The measuring abilities of image analysis make it possible to capture quantitative information. The stored images themselves enable qualitative review of the samples. Monitoring the dissolution of the particles exhibits the opposite trend. Note that different magnifications were used in these images; measurement values are in micrometers.

## 2.6 Studying Particle Properties as a Function of Environmental Conditions

Another example of using image analysis as a tool to study particles while environmental conditions are applied is dissolution testing. This method is the reverse of the process described above. Image analysis can be used to sample small aliquots of the representative sample from a starting point to an end point. Because image analysis can classify particles based on shape, it is possible to monitor the size and concentration of multiple components being released by therapeutics over time, with images captured to serve as objective evidence.

Time-lapse image analysis can be used to monitor particle size and shape as a result of being subjected to changes in process parameters and environmental conditions (e.g., heat, humidity, physical stress, and time), typically achieved by using an environmentally controlled chamber. An image analysis system can capture images of the samples as the conditions are adjusted, or it can record images at set intervals. The added value that image analysis provides over size-only systems for monitoring particle properties over time is the ability to classify particles based on shape, to quantify particles per unit volume (concentration of the particles of interest), and to provide objective evidence of the particles as they change over time with thumbnail images. Analysis intervals can be established using the starting point of interest and the point at which the ending condition occurs based on the environmental condition applied. Particle data results can then be compared in a sequence over time to study how the environmental conditions to which the particles were subjected caused physical changes in shape and concentration.

Another example is hot-stage microscopy (HSM), which can be utilized to analyze particle properties as a function of temperature. This microscopy technique monitors both particle size and shape as materials are heated on a "hot stage" from which the technique gets its name (*6*). In pharmaceutical applications, this technique is typically used to investigate API crystal

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growth and polymorphism. A lack of understanding and control over the different polymorphs formed by an API can lead to significant changes in particle morphology, influencing tableting performance and dissolution rates.

## 2.7 In-Process Monitoring of Particle Properties

In-process monitoring of particle properties can have many advantages over the more conventional off-line analyses. For example, process downtime can be minimized as it is not necessary for aliquots to be removed from process equipment for off-line analysis and results. In addition, materials are measured under their processing conditions, and the data is likely to be representative of a greater proportion of processed material.

The increased use of in-process techniques for particle imaging is consistent with guidance from the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) and the US Food and Drug Administration (FDA) for better process understanding and monitoring (*7*).

## 2.8 Evaluating the Chemical Composition of Materials

Image analysis combined with spectral information may provide chemical insight into materials. The area of chemical imaging is growing with an ever-expanding range of combination techniques, and instrumentation. Such techniques may use vibrational, fluorescence, physical, or other responses to probe the chemical composition of a species. These techniques include: SEM with EDX, optical image analysis with Fourier-transform infrared (FTIR) or Raman microscopy (*8*), confocal fluorescence microscopy, atomic force microscopy, nanotomography, and MRI. With X-ray microcomputed tomography (XMT) (*9*), it may even be possible to image the packed product, for example within a blister or a tube.

These techniques may be applied to particles, granules, and certain formulated products, which are DPIs (*10*), oral solid drug products, nasal sprays (*11*), and semisolid formulations, to gain insights into the chemical distribution of materials within a sample (*12*). These techniques may also be useful for understanding the chemical changes in a formulation over time, and in investigations of possible contamination.

# 3. THEORY AND INSTRUMENTATION

The following discussion is limited to optical systems as these cover the majority of applications.

## 3.1 Basics of Image Acquisition

Image acquisition systems consist of several instruments: an illumination source, a sample presentation unit, an optical system that magnifies the sample, an image capture device, and data processing software to perform the image analysis that provides the particle data.

Image analysis systems typically come in two broad types, static and dynamic, which differ on the basis of sample presentation method (*Figure 3*). With static systems, the particles (dispersed as dry powder or as wet suspension, or captured on a filter) remain static on a presentation cell within an automated stage that moves under the optics. With dynamic systems, particles dispersed as a suspension (either dry in air or wet in a liquid medium) travel through a flow cell and images are taken as the particles are in motion.

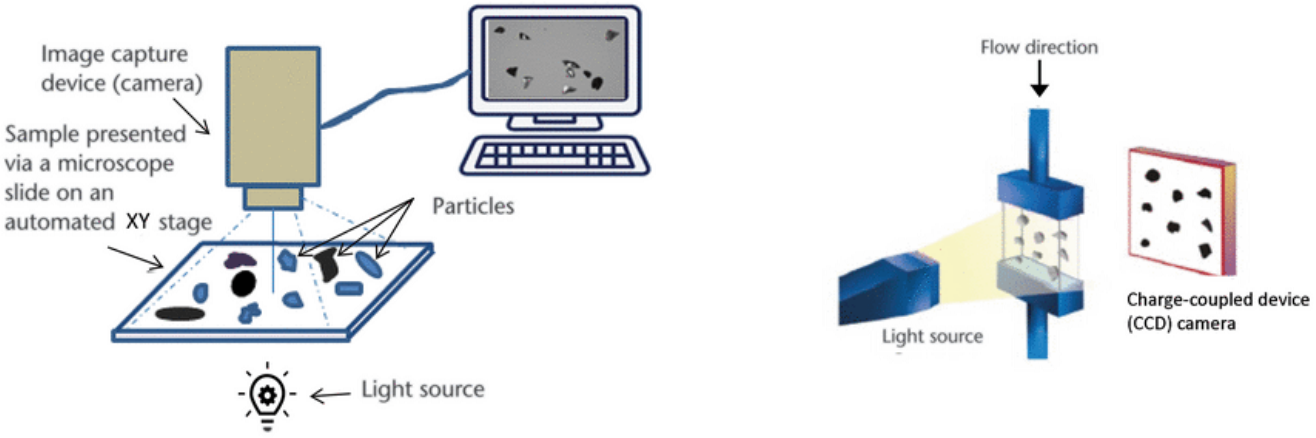


Figure 3. Example diagram of static and dynamic image analyses.

### 3.1.1 ILLUMINATION

Illumination techniques are either stroboscopic or fixed, and the goal is to obtain a clear image with as much edge definition as possible to enable suitable detection and measurement. Depending on the system configuration, illumination options may

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include diascopic (transmitted) or episcopic (reflected) and brightfield or darkfield (*Figure 4*). In addition to proper illumination, optical filters and polarizers may be used to further improve the edge definition of particles being analyzed.

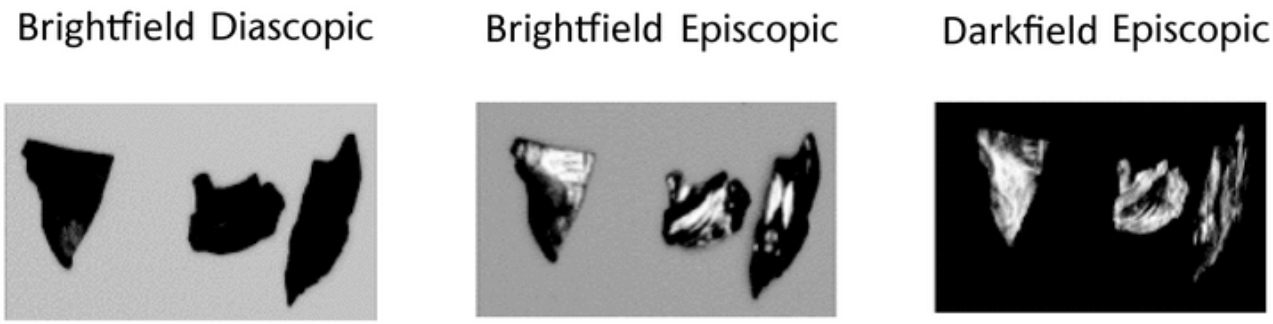


Figure 4. Examples of different types of illumination.

### 3.1.2 SAMPLE PRESENTATION

For successful image analysis, it is critical that the sample presentation be appropriate for the measurement technique. When measuring bulk properties, specific components, or foreign particulate matter, it is important to ensure that the aliquot measured is representative of the bulk material (see *Bulk Powder Sampling Procedures* ⟨1097⟩).

Factors that affect results include the sample volume or concentration and the conditions used to disperse the sample. Adequate particle separation is required without particle breakage. Particles should be appropriately dispersed to ensure that there are clear boundaries between distinct particles and agglomerates, thereby avoiding coincidence or unwanted agglomeration/flocculation, as these could be misrepresented as larger particles. Equally important, particle damage can result if dispersion aids such as pressure or ultrasound are used, which would also misrepresent the sample results. For wet or dry dispersions, it is important to optimize the appropriate dispersion methods and conditions.

Specific applications discussed above may have different sample preparation considerations.

Dynamic image analysis systems may include wet and/or dry dispersion units. Wet dispersion is usually achieved by controlled stirring, and the use of ultrasound may also be an option. Dry dispersion is usually achieved via gravity fall or with the aid of differential air pressure. Many static image analysis systems utilize dry powder dispersion units, which control differential air pressure for dispersing the sample. Wet samples are presented in specific cells or are filtered. The latter is typical for low-concentration foreign particulate matter applications. Some sample types such as topicals and viscous suspensions (e.g., nasal sprays) are prepared using classic microscope techniques with the sample sandwiched between a slide and coverslip.

### 3.1.3 OPTICAL SYSTEM

Once the sample is properly prepared to ensure statistical accuracy, the next step is to magnify the images to fit on the optical field of view. As larger magnifications are used, the field of view becomes smaller (see *Figure 5*).

When zooming into an image, the detection zone becomes smaller (see *Figure 5*, far below). It is important to take this into consideration to ensure that proper sampling is performed. Use of excessive magnification may underrepresent larger particles because it becomes less likely that they will appear fully in the field of view. Statistically, this would skew the results toward the smaller sample population. Conversely, as magnification is decreased to see the larger particles properly, smaller particles may fall outside the detection ability of the instrument configuration, skewing the results toward the larger size particles. Due to inherent limitations for a given magnification, a compromise between these two factors may be needed, with consideration of the purpose of the application.

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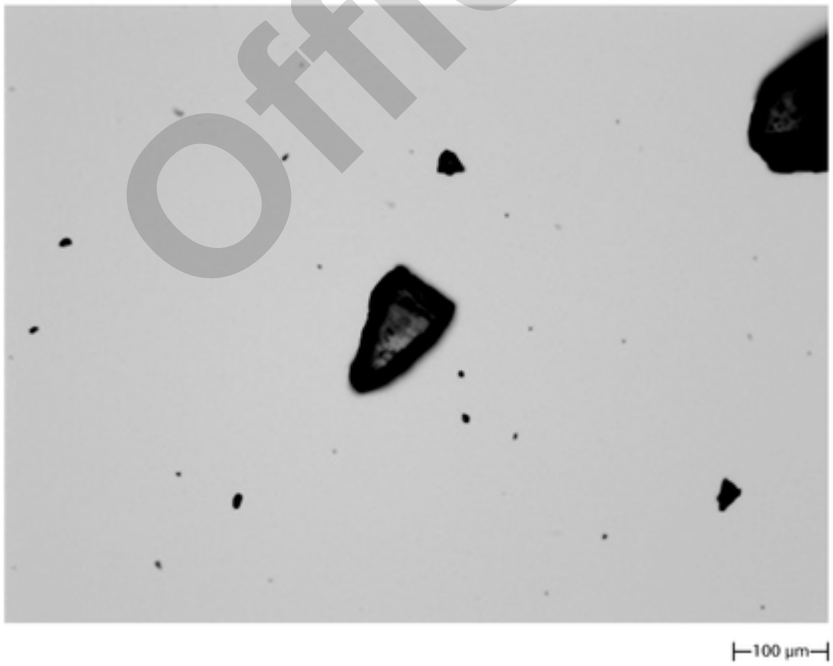


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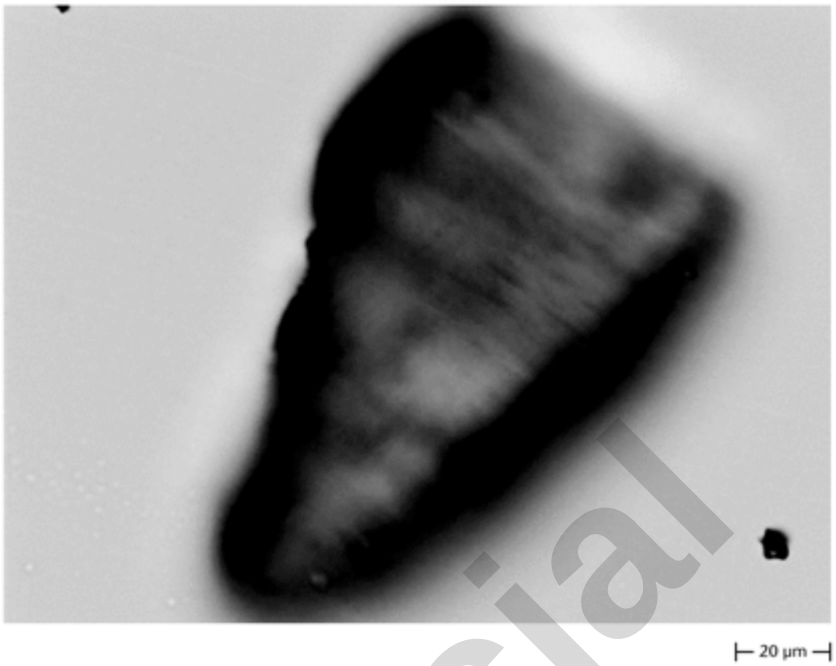


Figure 5. Images of the same particles taken at 2.5×, 10×, and 50×. As the magnification is increased the field of view is reduced and the resolution is increased. The third image shows that the depth of field of the magnification is not sufficient for the object. This could be overcome using *z*-stacking algorithms, but this would also increase the measurement time. For an image analysis measurement, a compromise may be needed; for this example the 10× magnification may be optimal.

*Figure 6* shows the field of view and depth of field of flow cell in a dynamic image analysis system.

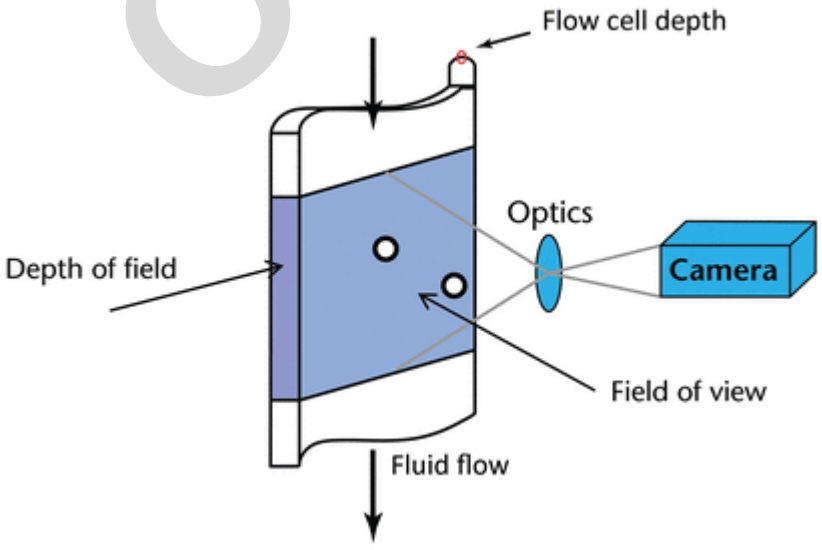


Figure 6. A diagram (shaded area) of the field of view and depth of field of an image.

Depth of field (DoF) will also be a factor; it is the distance between the nearest and farthest objects (particles) that are in an acceptably sharp focus within an image (see *Figure 7*). DoF is generally controlled by the optical components of the imaging system, which includes the aperture or f-stop. Generally, as the aperture is smaller, the DoF is larger. The degree to which a user has DoF control will depend on the instrumentation; many manufacturers provide the largest DoF possible in order to minimize out-of-focus particles for analysis.

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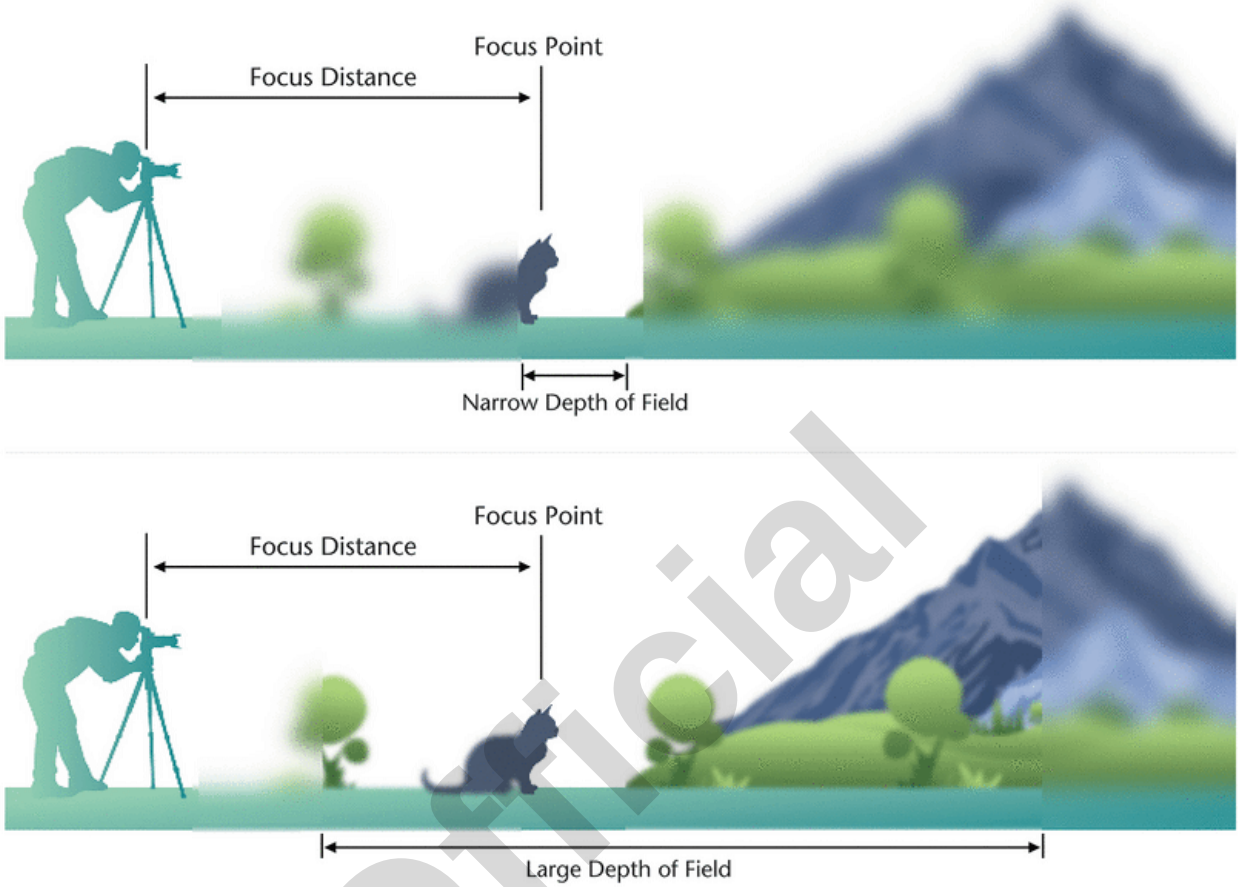


Figure 7. Images captured at different depths of field.

## 4. DATA PROCESSING, INTERPRETATION, AND REPORTING

### 4.1 Data Processing

Software processing is the next step after image capture. Once images have been captured and are available for processing on a computer, image analysis systems perform a series of functions to identify particles on every image.

Operations may be applied to the gray level images to enhance further processing, for example adjustments to brightness and contrast.

For the scope of this chapter, the majority of applications being considered involve measurements of individual particles, often a large number of them, within a given image. It is necessary to identify and define the boundaries of the individual particles. This is often done by defining a threshold gray level value and defining the desired objects as above or below that level (light, dark, color, or other appropriate value). However alternative edge detection and combined algorithms are used by some systems.

Once the desired objects have been identified, further processing is necessary for accurate measurements (e.g., separation of closely spaced or touching objects). The required criterion for an analyzable discrete object will be a minimum number of associated pixels, which will depend on the application (see *Figure 1*).

The gray-scale images are converted to a binary format for the purposes of size and shape calculations. The size and the number of pixels are used to accurately characterize images and calculate the particle size and shape parameters. Some systems report the binarized particle images, whereas others retain the gray-scale images, which may provide additional information about the samples, such as texture. This is important if qualitative information about the objects is required.

### 4.2 Data Interpretation and Reporting

#### 4.2.1 SIZE PARAMETERS

Image analysis calculates a variety of particle size parameters derived from the size of the pixels, which relates to the magnification used and the number of pixels per unit area. Size parameters that are typically reported are described in *Table 1*.

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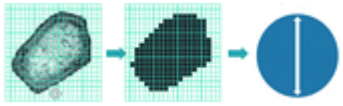
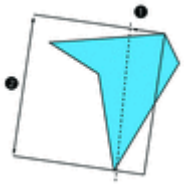
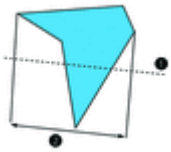
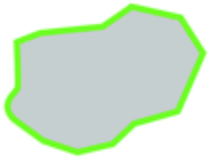
There are a number of aspects used to describe the dimensions of a particle, such as various diameters or chords. Common measures include the Feret and Martin diameters or the dimensions of bounding circles, rectangles, or other polygons. Depending on the measure used, the definition of descriptors such as length or width may differ. One must be aware of the definitions used by specific instrument systems and software.

**Table 1. Most Common Size Parameters Used in Image Analysis**

| | | |
|---|---|---|
| **Area** | The area (1) is the visual projected area of the particle and is usually reported in both pixels and microns. | |
| **Circular equivalent diameter (CED)** | CED is the diameter of a circle with the same 2D image (area) as the particle. It can be calculated from either the area of the particle or the perimeter of the particle. [NOTE—CED is a very important parameter as it is often used to compare with other particle sizing techniques. Most other particle-size-only techniques perform indirect measurements. As a result, these size-only techniques report particle size under the assumption that particles are spherical in nature.] |  |
| **Length** | Length can be described in a number of ways. Lines from all points on the perimeter are projected onto the major axis (1) of the particle as shown. The longest distance between the points where two of these projections meet the axis is the length (2) of the particle. |  |
| **Width** | Lines from all points on the perimeter are projected onto the minor axis (1) of the particle as shown. The longest distance between the points where two of these projections meet the axis is the width (2) of the particle. |  |
| **Perimeter** | The length of the total distance around the shape under analysis, e.g., length of the green line in the image to the right. |  |

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### 4.2.2 SHAPE PARAMETERS

Shape measures are essentially combinations of the basic size measurements. Those typically reported are described in *Table 2*.

**Table 2. Most Common Shape Parameters Used in Image Analysis**

| | | |
|---|---|---|
| **Aspect ratio and elongation** | These parameters are used to describe the overall form of an object.<br>Aspect ratio = width/length<br>Elongation = 1 – (width/length)<br>[NOTE—Although the convention is to define aspect ratio such that the values range from 0–1, the inverse (length/width) is also commonly used.] | |
| **Convexity and solidity** | These parameters are edge roughness of an object.<br>Convexity = convex hull perimeter (in green)/actual perimeter (in blue)<br>Solidity = area (in green)/convex hull area (in blue) | |

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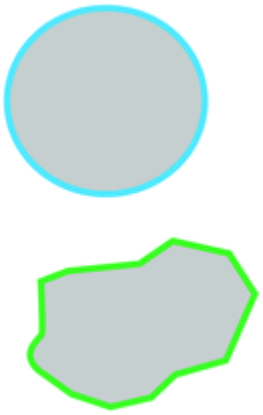
**Table 2. Most Common Shape Parameters Used in Image Analysis** *(continued)*

| | | |
|---|---|---|
| **Circularity** | Circularity is a measure of how close the object's shape is to a perfect circle. There are two definitions that are often used:<br>1. Circularity is the ratio of the circumference of a circle equal to the object's projected area to the perimeter of the object.<br>$$\text{Circularity} = \frac{2 \times \sqrt{\pi \times \text{area}}}{\text{perimeter}}$$<br>$$\text{Circularity} = \frac{\text{circular equivalent perimeter (in blue)}}{\text{perimeter (in green)}}$$<br>2. High-sensitivity (HS) circularity is equivalent to squaring the numerator and denominator of the circularity calculations to obtain a more sensitive measure; it is sometimes called compactness.<br>$$\text{HS Circularity} = \frac{4 \times \pi \times \text{area}}{\text{perimeter}^2}$$ |  |

Some applications may make use of gray level measurements. These could include gray level averages or gray level distributions in the image or specific regions of interest, determination of a gray level profile along a defined line, or other measurements. One common application for gray level processing is the differentiation of particles based on opacity where two particles can be identical in size and shape but one may be darker than the other when viewed as an image (see *Figure 8*).

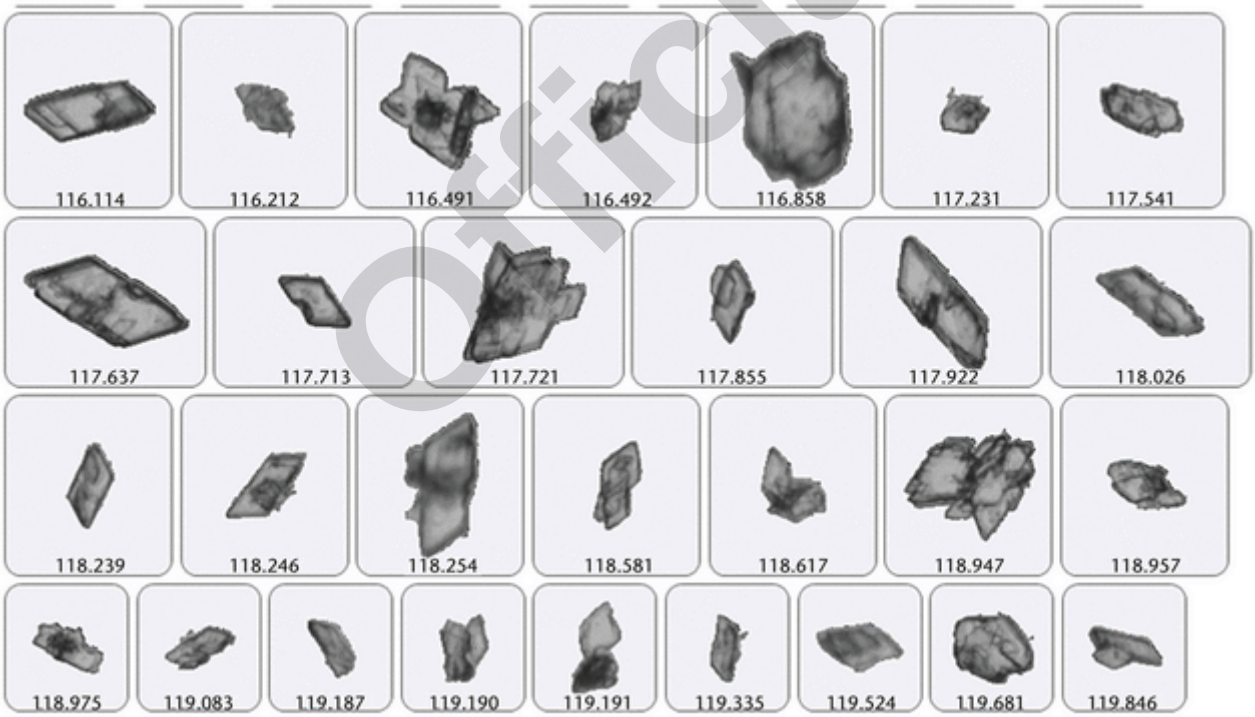



Figure 8. Differentiation of particles based on the opacity.

### 4.2.3 DATA REPORTING

Detailed individual particle data are typically reported along with the particle images providing key qualitative information as well as whole sample distributions and statistics.

For every parameter measured, statistical histograms are created (see *Figure 9*). The full distributions can be compared across samples, which may enable the detection of differences among samples when statistically relevant data are used to differentiate populations of particles from one another.

Imaging is a number-based technique but mathematical conversion to volume results is typically available to compare results with those of other techniques (*13*). However, care must be taken with such comparison because different techniques calculate sizes on the basis of different factors.

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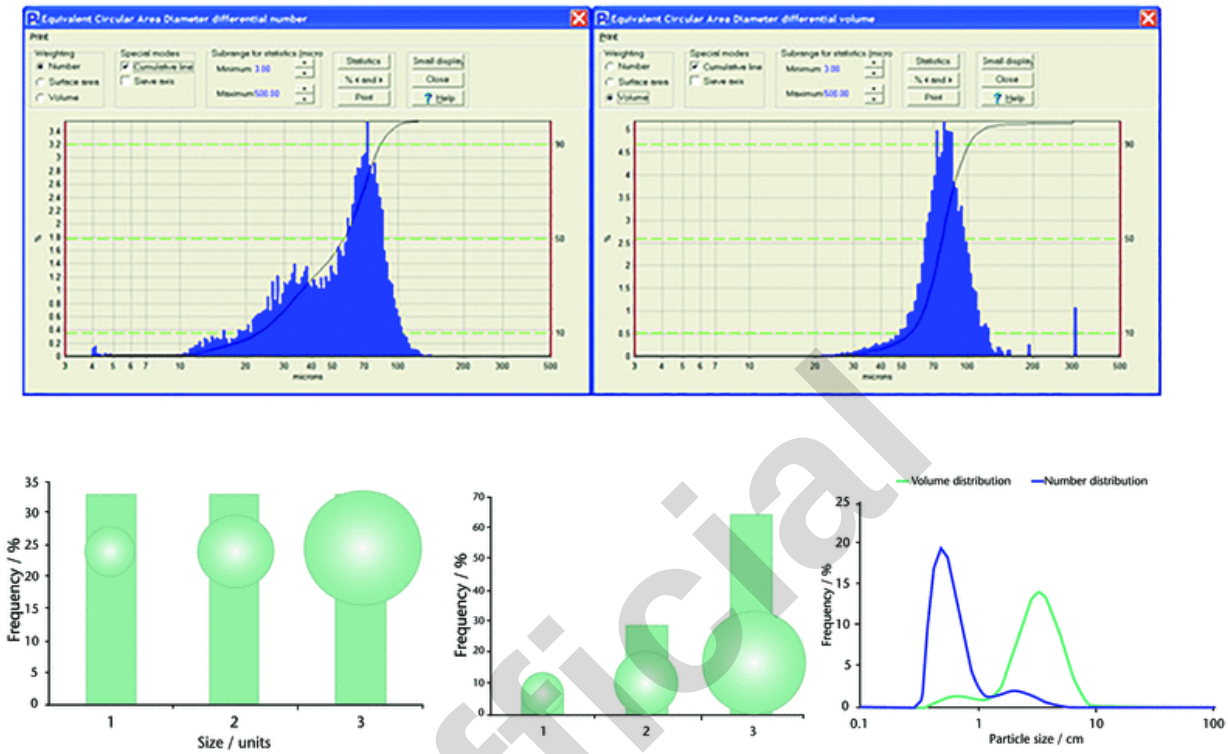


Figure 9. Illustration of number-weighted versus volume-weighted distributions. The first illustration shows a number-weighted distribution for three objects where each object contributes equally. The second illustration shows the volume-weighted distribution for the same three objects. The third illustration shows the number- and volume-weighted distributions for the same sample. This illustrates that the small number of particles that are bigger than 1 μm in size contain most of the volume in the sample. It is important to note that both of these distributions are correct; they are just different ways of representing the same particles.

The two histograms in *Figure 9* may look very different, but they represent the same sample. The histogram on the left is a number-weighted distribution, and the one on the right is a volume-weighted distribution. Because it is a number-based method, image analysis has the ability to identify and quantify fine particles, as is well shown with the number-weighted distribution. This enables end users to identify the presence of fine particles in their sample regardless of the amount. As discussed in this and other *USP* chapters, the presence of fine particles can significantly impact the powder flowability of the sample. This can also be used as a tool to assess the performance of milling processes. Volume-weighted distributions are also very useful for identifying and quantifying large, rare events such as agglomerates, bubbles, or contamination. It is important to keep in mind that with a number-based system, end users are able to view single particle events that appear on histograms as objective evidence.

Many systems also offer the ability to classify particles according to particle size and/or shape combinations, providing further insight into the samples. Systems combined with spectroscopic techniques allow further classification by elemental or chemical nature.

## 5. MEASUREMENT AND EXPERIMENTAL CONSIDERATIONS

### 5.1 Size Calibration

For accurate size measurements, the magnification of the system must be calibrated. A size standard of suitable quality must be used. For optical microscopy-based systems, this is most often a stage micrometer or reticle. The image of the standard should span as large a portion of the image field as possible to maximize the number of pixels used. This method is most suitable for static systems. For dynamic systems, although calibration with a reticle or micrometer may be possible, it is most commonly accomplished using well-characterized standard particles of known size. Once calibrated, a given optical arrangement (e.g., a

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set of lenses) will normally be maintained. However, it is good practice to check the sizing accuracy periodically, or prior to critical measurements. Shape factors are scale independent and should not be affected by the sizing accuracy.

## 5.2 Factors That Affect the Measurement

### 5.2.1 SPATIAL ACCURACY

In most applications, it is presumed that the pixel array accurately represents the geometry of the imaged object. However, there are potential sources of inaccuracy and asymmetry in the image.

Asymmetry may arise from both the optics used to acquire the image and the detector elements. To assess the potential for inherent asymmetry in the experimental arrangement, spatial calibration should be checked at multiple angles, both within a given image and in the arrangement of the dimensional standard.

Another source of geometric inaccuracy within an image is due to the DoF and focus. If objects are not in the same physical focal plane, their relative perceived sizes could be inaccurate due to perspective. Likewise, with a limited DoF, objects at different distances from the collecting optics will differ in focus. The measured size of objects not sharply focused will be dependent on the thresholding values used to discriminate boundaries (see *3.1.3 Optical System*).

### 5.2.2 GRAY LEVEL ACCURACY

For many applications, especially those dealing with the size and shape of individual particles, the accuracy of gray level values is not needed. When extracting quantitative information from gray levels, such as a concentration, it is necessary to consider factors affecting the accuracy of gray level values. Detector response to intensity may not be linear or may be affected by illumination wavelength. Software factors such as adjustments to brightness and contrast or the application of other image enhancement filters, such a gamma distribution, will affect the apparent response. Users should be aware of default software settings for such factors to avoid unintended effects.

# 6. METHOD DEVELOPMENT AND VALIDATION

The major consideration for method development is the purpose of the measurement (i.e., is the primary goal to measure size or shape?). Among the factors to be considered are:

- Sample characteristics (e.g., size range of the particles)
- Level of assurance needed for accuracy and precision
- Sample, equipment, or time limitations

Optimization of the conditions will apply to all phases of the image analysis process:

- Sampling and sample preparation
  - The sample must be representative
  - The sample preparation must disperse the particles as intended
  - There must be adequate numbers of particles in the image (i.e., images of a statistically representative number of particles are captured for analysis)
- Appropriate image acquisition parameters
  - Illumination should be uniform and suitable to aid discrimination of particles from background
  - Magnification must be appropriate for the purpose
    - Fine shape features will often require higher magnification
    - Magnification must balance accuracy of sizing of small particles while avoiding exclusion of larger particles
- Application of appropriate data processing and statistical analysis
- Sample orientation (fixed versus random orientation)

As with all analytical methods, consistency is key to ensuring accurate, representative results. Analysts must be familiar with the equipment and potential sources of error. Detailed method conditions will be equipment specific. For method validation, the normal considerations apply, namely precision, accuracy, and ruggedness.

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## ADDITIONAL SOURCES OF INFORMATION

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- ASTM Designation: E2651–19: Standard Guide for Powder Particle Size Analysis.▲ (USP 1-Aug-2022)

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# ⟨1782⟩ VIBRATIONAL CIRCULAR DICHROISM SPECTROSCOPY—THEORY AND PRACTICE

1. INTRODUCTION
2. DEFINITION OF VIBRATIONAL CIRCULAR DICHROISM
3. VCD INSTRUMENTATION
4. MEASUREMENT OF VCD SPECTRA
5. QUALITATIVE AND QUANTITATIVE ANALYSIS
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    5.2 Determination of the Noise Level at Each Point in the Spectrum
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    5.4 VCD Baseline Absorption Artifacts and Single-Enantiomer Measurement Capability
    5.5 VCD Measurement Stability and Noise Level Reduction
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8. COMPARISON OF MEASURED AND CALCULATED SPECTRA
    8.1 Degree of Confidence of Correct Assignment
9. DETERMINATION OF ENANTIOMERIC EXCESS
10. CONCURRENT USE OF VCD FOR ABSOLUTE CONFIGURATION AND EE
    10.1 Chiral Raw Material Identification
    10.2 Chiral Quality Control

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## 1. INTRODUCTION

Chirality is a ubiquitous aspect of the world of three spatial dimensions. Molecules that possess sufficient structural complexity so that their mirror-image structures are non-superimposable are termed chiral. For chiral pharmaceutical molecules, two important structural measures critical to their physical characterization are absolute configuration (AC) and enantiomeric excess (EE), also termed enantiomeric purity. The AC of a chiral molecule specifies its three-dimensional structure in space and distinguishes it from its mirror-image structure. Structures related by mirror symmetry are known as "enantiomers". The AC of a particular enantiomer is critical to its action as a pharmaceutical agent because mirror-image structures, i.e., enantiomers, have different therapeutic effects, both desired and undesired. The EE of a sample specifies, usually as an excess percentage, the relative amounts of enantiomers. An EE of 100% is a pure enantiomer (100% enantiomeric purity), 0% is a racemic mixture with equal amounts of enantiomers and no excess, and –100% specifies a pure sample of the opposite enantiomer with respect to the EE definition.

The principal chapter that addresses molecular chirality in the *USP–NF* is *Optical Rotation* ⟨781⟩. The optical rotation (OR) of a sample is the measure of the angle of rotation of a plane of polarized light by a chiral sample at a particular wavelength, typically in the visible or near-UV region. The AC and EE of any sample are determined by a measure of its OR if the measurement has been previously calibrated using a sample of known AC and EE. The sign of the OR determines the larger enantiomer amount, and the magnitude of the OR determines the EE, where 100% EE corresponds to the maximum OR possible for the chiral molecule under consideration. Although OR is a simple, well-established method for determining AC and EE relative to their known calibrations, not all molecules have measurable OR, particularly with limited sample quantities in which the presence of chiral impurities cannot be determined by an OR measurement. The OR is simply a number with no structural information about the sample molecule.

In the past several decades, the AC of an unknown chiral molecule has been determined using the Bijvoet method of single-crystal X-ray crystallography. The Bijvoet method requires a single high-purity crystal of the molecule with a single chiral phase. Analysis by this method is not always possible, for example, for noncrystallizable liquids or insoluble solids, and in many cases analysis using the Bijvoet method requires lengthy efforts and time to achieve crystallization.

This chapter presents a technique for the determination of AC that relies on vibrational circular dichroism (VCD), a procedure that is now widely used throughout the pharmaceutical industry for chiral molecules for which the AC is unknown. The technique involves comparing the measured VCD spectrum for a chiral molecule to the quantum chemistry calculation for the same molecule. If the measured and calculated VCD spectra show agreement on the principal features and their signs, this means that the AC of the physical sample is the same as the AC of the structure used in the calculation. If the signs are opposite, the AC of the sample is the opposite of that used in the calculation.

As described below, statistical methods have been developed for assessing the degree of confidence that the AC has been correctly determined by the VCD method. Usually the AC of a chiral molecule is specified by connecting its structural chirality, labeled with *R* or *S* for chiral centers or *P* or *M* for chiral axes, to the sign of its measured OR as (+) or (–). Once the AC of a molecule has been determined by VCD, for example, (S)-(–)-α-pinene, its VCD spectrum can become a reference standard of the AC of this molecule, and the AC of any subsequent sample of this molecule can be determined by comparison to its VCD reference standard. An important advantage of determining AC by VCD is the spectral richness of a VCD spectrum that supports the simultaneous determination of the structural identity of the molecule and its absolute chirality. In contrast to OR, every chiral molecule has a VCD spectrum, because all molecules have infrared (IR) absorption bands, each one of which acts as a chromophore for a VCD spectrum. VCD also is sensitive to molecular conformations. As demonstrated below, information about the conformation of a chiral molecule is obtained as a bonus from the VCD determination of AC, but AC is the principal

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informational content of chiral pharmaceutical molecules. An extensive body of literature describing the AC of biological molecules has been created during the past 30 years and can be used as a basis for determining secondary and higher-order structural states of biological drug substances. Stereospecific methods using VCD can be developed to characterize the production, formulation, and stability of biopharmaceutical products.

This chapter also presents a method for the determination of EE using VCD. EE determination with VCD takes advantage of the fact that the magnitude of a VCD spectrum, measured as the circular polarization absorbance difference for a constant parent IR absorbance spectrum, is directly proportional to EE with no offset. Thus, the maximum relative size of a VCD spectrum is obtained for an EE of 100%, is zero for an EE of 0% (racemic mixture), and is maximum with opposite signs (all positive VCD bands are negative and all negative VCD bands are positive) for an EE of –100%. The advantage of VCD EE determination is that the EE of any molecular sample can be determined by a single VCD measurement once the relative size of VCD for 100% EE (or any %EE value) is known for that molecule. Separation of enantiomers with chiral chromatography is not needed.

## 2. DEFINITION OF VIBRATIONAL CIRCULAR DICHROISM

VCD is defined as:

$$\Delta A = A_L - A_R$$

$\Delta A$ = the difference in the IR absorbance
$A_L$ = sample absorbance $A$ for left circularly polarized (LCP) radiation
$A_R$ = sample absorbance $A$ for right circularly polarized (RCP) radiation

Unpolarized IR absorbance intensity is defined as the average of LCP and RCP intensities:

$$A = (A_L + A_R)/2$$

The IR intensities of enantiomers are identical, whereas enantiomers have equal and opposite-signed VCD intensities.

## 3. VCD INSTRUMENTATION

The measurement of a VCD spectrum and its parent mid-IR spectrum is based on Fourier transform-IR (FT-IR) absorption spectroscopy. The optical–electronic layout for the measurement of VCD using an FT-VCD spectrometer is illustrated in *Figure 1*. The output beam of an FT-IR spectrometer is optically filtered and then linearly polarized before passage through a photoelastic modulator (PEM). The zinc selenide (ZnSe) PEM, typically used for VCD measurement, changes the polarization state of the beam between LCP and RCP at a frequency of 37 kHz. A sample solution in a standard IR cell is placed in the beam, and a liquid nitrogen-cooled detector of mercury–cadmium telluride (MCT) detects the transmitted beam. The detector produces signals in two frequency ranges: 1) $I_{DC}$, in the range of 1–2 kHz, represents the FT-IR interferogram; and 2) $I_{AC}$, centered at 37 kHz, represents the FT-VCD interferogram. The FT-IR interferogram may be directly Fourier-transformed to yield the IR spectrum. After synchronous demodulation (using a lock-in amplifier or purely numerical processing) with a reference at 37 kHz, the VCD interferogram can be Fourier-transformed to yield the VCD spectrum. Both the IR and VCD spectra are presented in dimensionless absorbance units, $A$, and hence the ratio of the VCD to the IR intensity for any band in the spectrum yields the dimensionless anisotropy ratio for that vibrational transition. *Figure 1* illustrates the minimum setup for VCD measurement, but technically advanced instruments use two PEMs to increase baseline stability and two sources to increase signal quality or reduce sample measurement time. Measurement of VCD in other spectral regions, for example the hydrogen-stretching or near-IR, can be carried out with appropriate changes in sources, polarizers, filters, PEM, cells, and detectors.

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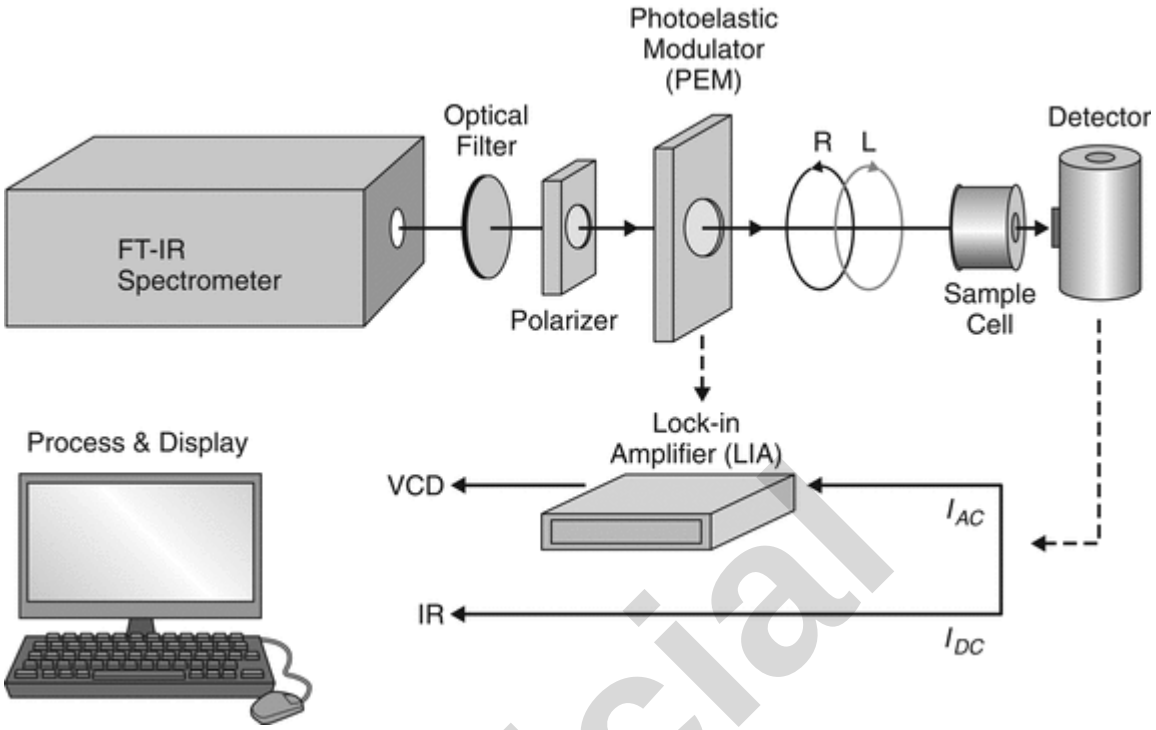


Figure 1. Diagram illustrating the typical features of an FT-VCD spectrometer for the measurement of the IR and VCD spectra of a chiral sample.

## 4. MEASUREMENT OF VCD SPECTRA

*Figure 2* shows the IR and VCD spectra for (+)-*R*- and (–)-*S*-α-pinene as a neat liquid measured at 4 $cm^{-1}$ spectral resolution for 1 h. Also shown are the stereostructures of the two opposite enantiomers (+)-*R*- and (–)-*S*-α-pinene. This figure shows that the IR spectra are nearly identical (superimposed dash and solid traces) for these enantiomers and that IR is therefore blind to chirality. By contrast, the corresponding VCD spectra have intensities that are equal in magnitude with opposite signs for each band in the spectrum. The two stereostructures for α-pinene are presented in a form that makes clear their mirror symmetry, and it follows that mirror-symmetric pairs of chiral molecules have mirror-symmetric VCD spectra about the zero baseline. The VCD baseline for these spectra is offset slightly above zero and can be baseline corrected as discussed below. Inspection of the intensity scales for the IR and VCD spectra reveals that the VCD intensities are approximately four orders of magnitude smaller than the corresponding IR intensities. Finally, each band in the IR spectrum has a corresponding VCD band. The richness of the resolved spectral bands in the VCD spectrum, each one representing what has traditionally been called a chromophore in electronic circular dichroism (ECD), gives VCD its exceptional combination of structural richness and stereochemical specificity.

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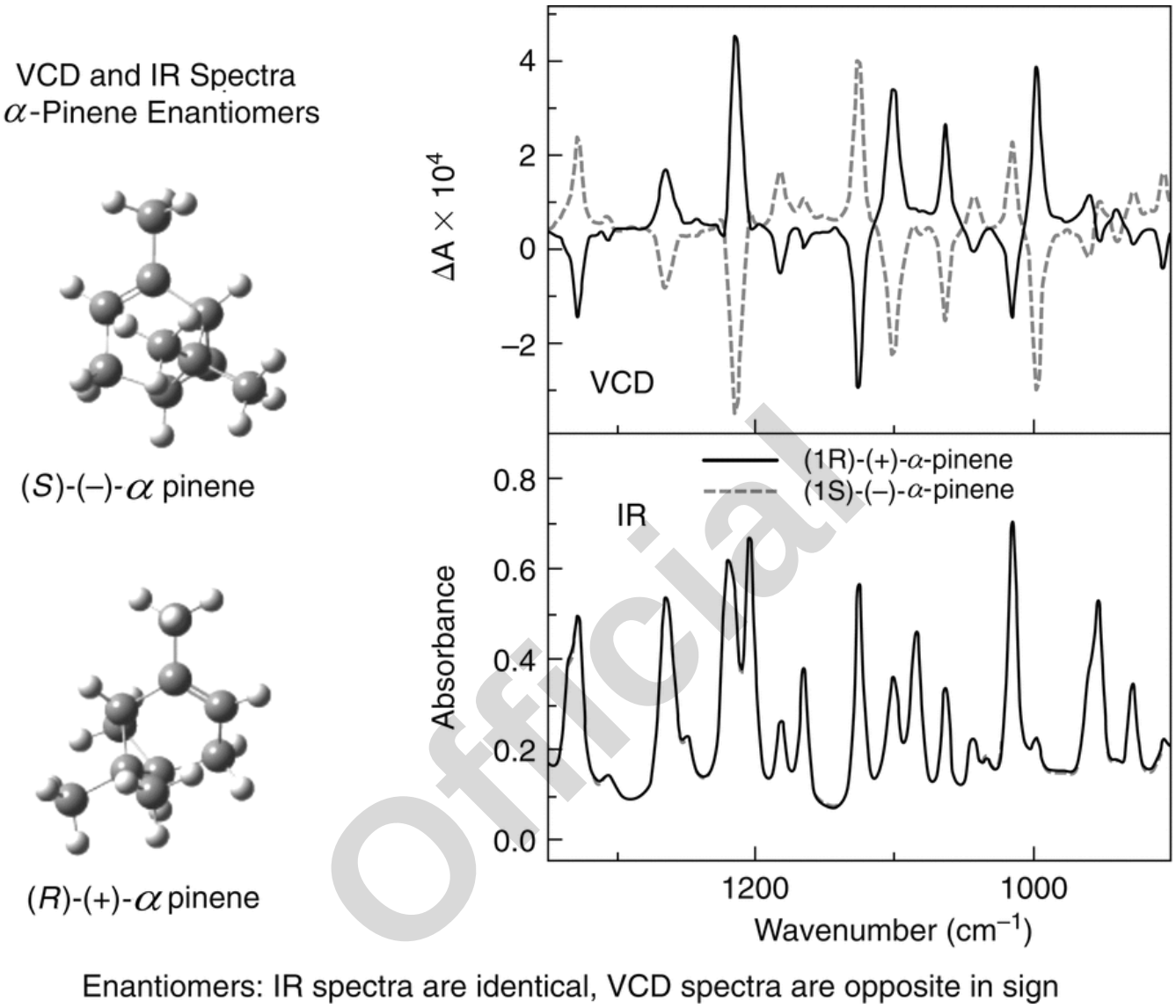


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Figure 2. IR (lower) and VCD (upper) of (1*S*)-(–)-α-pinene (dash) and (1*R*)-(+)-α-pinene (solid). To the left are presented the quantum chemistry-optimized stereostructures of (1*S*)-(–)-α-pinene (upper) and (1*R*)-(+)-α-pinene (lower). The IR and VCD spectra were measured at 4 $cm^{-1}$ spectral resolution, and the VCD spectra were averaged for 1 h.

Sampling requirements for VCD are similar to those needed to obtain a good IR spectrum. Ideally, as shown in *Figure 2*, analysts choose a combination of path length and sample concentration to obtain an average IR absorbance value *A* in the range between 0.2 and 0.8 and concentrations between 0.1 and 1.0 M, depending on the molecular weight of the sample. Typical path lengths for organic solvents are in the range of 50–100 µm, and sample quantities needed are typically 5–10 mg, although amounts as small as 2 mg are possible. Typical solvents for VCD measurement of organic molecules are deuterated solvents that have reduced solvent absorption in the mid-IR region. Besides hydrogen-free solvents, such as carbon tetrachloride ($CCl_4$), other commonly used solvents for VCD are deuterated chloroform ($CDCl_3$) and deuterated dimethyl sulfoxide (DMSO-$d_6$).

IR and VCD spectra can be plotted either in absorbance units, as *A* and $\Delta A$, that reflect the sampling conditions, or in molar absorption coefficients, ε and $\Delta\varepsilon$, that remove the concentration and path-length dependence of the intensity to give a molecular-level property that can be compared quantitatively to calculated IR and VCD intensities. The conversion between these two sets of quantities is given by:

$$A = \varepsilon C \times l$$

*A* = IR absorbance
ε = molar absorption coefficient
*C* = concentration (mol/L)
*l* = path length (cm)

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and

$$\Delta A = \Delta\varepsilon C \times l$$

$\Delta A$ = difference in the IR absorbance
$\Delta\varepsilon$ = difference in molar absorption coefficient

A useful dimensionless measure of the intrinsic strength of a VCD band is the anisotropy ratio, defined as $g = \Delta A/A = \Delta\varepsilon/\varepsilon$. Including the spectral frequency dependence of IR and VCD, the measured spectra are expressed respectively either as $A(\nu)$ and $\Delta A(\nu)$ or as $\varepsilon(\nu)$ and $\Delta\varepsilon(\nu)$.

VCD spectra require baseline corrections for instrumentation and solvent intensity. Deviations from a perfectly flat baseline with zero offset must be removed before a final calibrated VCD spectrum is complete. Solvent baseline correction can be achieved for VCD spectra by subtraction of the VCD spectrum of the solvent in the same sample cell. If the opposite enantiomer or racemic mixture of the sample is available, baseline correction can be achieved as one-half the difference between these two equal and opposite VCD spectra in the case of enantiomers or as simple subtraction in the case of the racemic mixture. Baseline deviations are caused by the unavoidable presence of linear birefringence in the optical elements of the instrument and the sample cell that can become important for all but the largest VCD intensities. An example of a baseline-corrected VCD spectrum is presented in *Figure 3*, along with the corresponding IR and VCD noise spectrum. The spectrum of this sample of neat (–)-(*S*)-α-pinene was collected for a period of 1 h at 4 $cm^{-1}$ spectral resolution in barium fluoride ($BaF_2$) with a path length of 75 µm. The noise spectrum is less than $1 \times 10^{-5}$ absorbance (*A*) units across the spectrum. The IR, VCD, and noise spectra presented in *Figure 3* may be taken as a validation standard for the performance of a mid-IR FT-VCD instrument, as described in *Vibrational Circular Dichroism Spectroscopy* ⟨782⟩.

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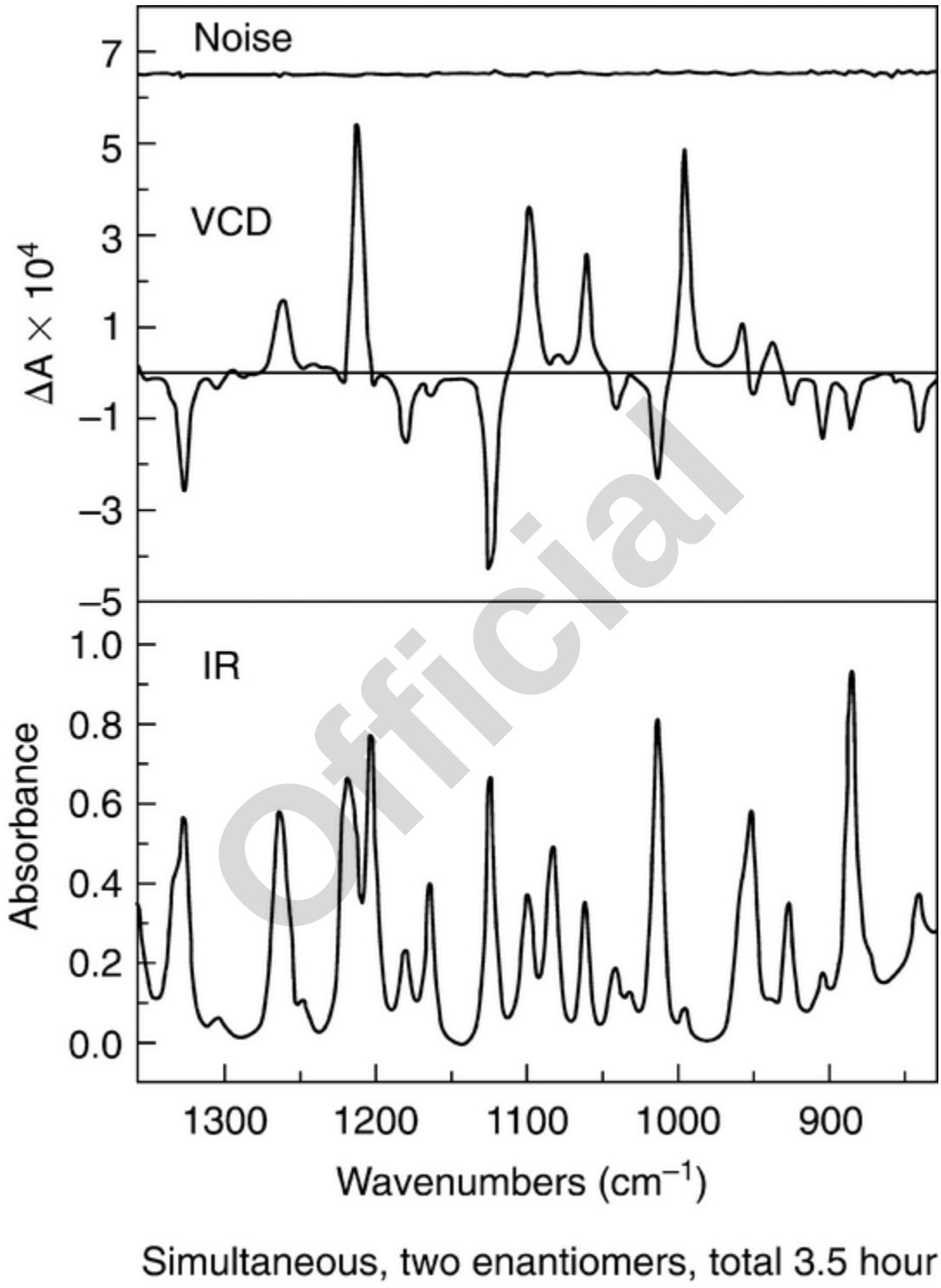


Figure 3. IR absorbance A (bottom), baseline-corrected VCD (middle), and VCD noise spectrum (top) for (–)-(1*S*)-α-pinene as a neat liquid averaged for 1 h at 4 $cm^{-1}$ spectral resolution and in a 75-µm path-length barium fluoride cell.

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# 5. QUALITATIVE AND QUANTITATIVE ANALYSIS

## 5.1 Ensure That Signs and Intensities Are Correct

Qualitative analysis can be carried out with VCD by comparing the VCD spectrum of an unknown to a reference spectrum of that molecule to identify common features and to confirm that the VCD spectrum of the sample is a close match to the reference spectrum.

Quantitative analysis using VCD involves measuring the VCD intensity of the sample molecule and comparing it to a standard reference spectrum for that molecule. In this way, the concentration and the %EE of the sample can be determined as follows. The intensity of the IR spectrum relative to the IR standard of the sample determines the concentration for measurements with the same path length. After normalizing the IR intensity of the unknown sample to the standard, the relative intensities of the VCD determine the %EE of the sample. Such comparisons need to be carried out with all sampling conditions being the same, including the solvent and instrument spectral resolution.

To determine that the VCD intensities are correct, the uncalibrated VCD intensities must be calibrated. This is accomplished by placing a multiple-wave plate in the sample position, followed by a polarizer that is either parallel or perpendicular to the instrument polarizer in *Figure 1*. Measurement of the VCD spectrum of this multiple-wave plate set-up for two settings of the wave plate and polarizer that differ by 90° produces two sets of curves that have crossing points. The curve connecting the crossing points follows the shape of a first-order Bessel function and represents unit VCD intensity. Dividing this curve into the uncalibrated VCD spectrum produces a VCD spectrum with the correct calibrated intensities. The two calibration curves and their crossing-point curve are illustrated in *Figure 4*.

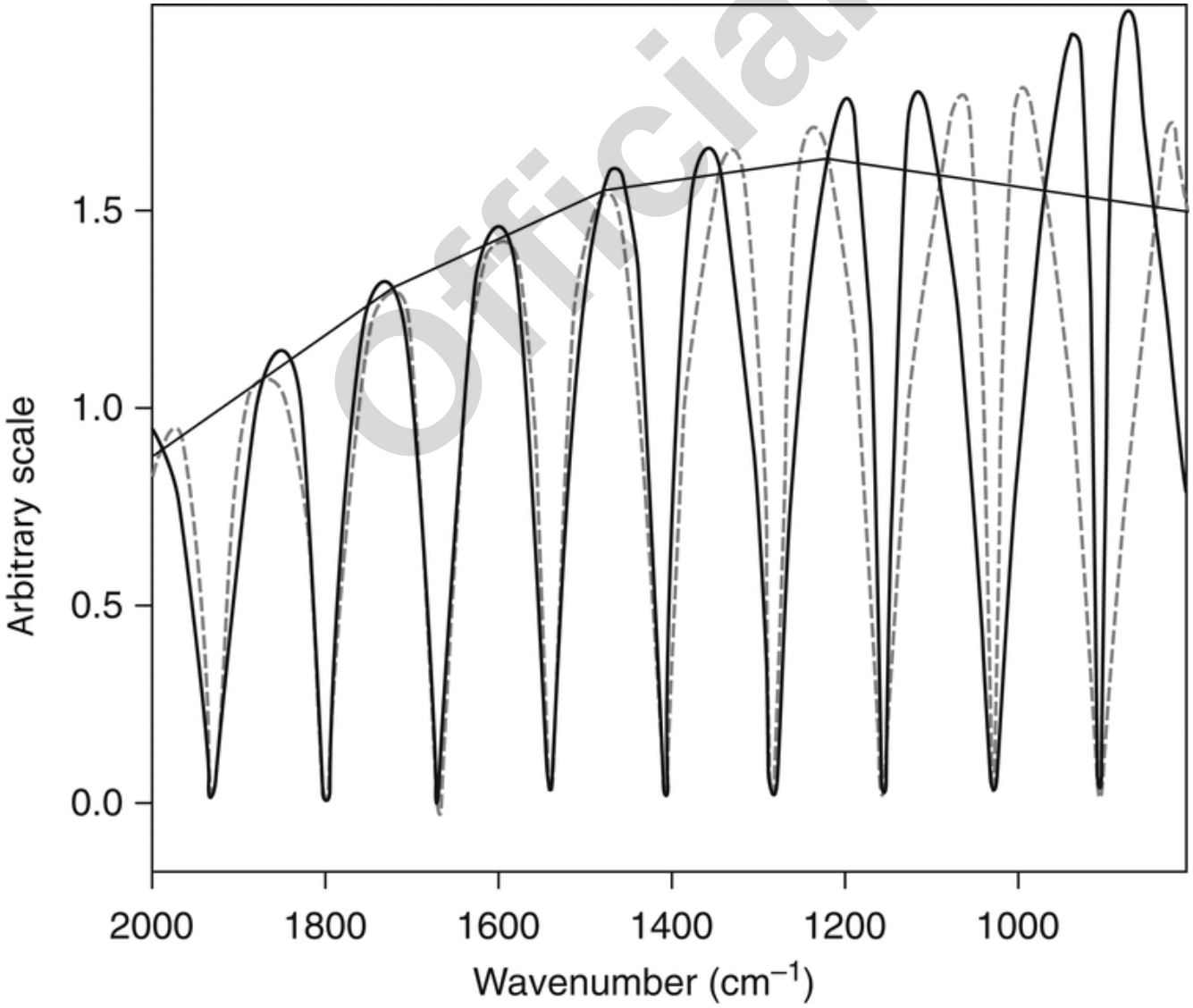


Figure 4. Calibration curves for VCD intensity generated by placing a multiple-wave plate and a polarizer in the IR beam and connecting the crossing points with a curve representing unit VCD intensity for maximum VCD intensity at 1200 cm$^{-1}$.

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## 5.2 Determination of the Noise Level at Each Point in the Spectrum

A critical factor in quantitative analysis using VCD is the degree of accuracy of the spectrum as given by the VCD noise level and baseline stability of the measurement. The noise level depends on a number of factors such as the D* value of the detector, the amount of radiation passing through the sample from the source, and the amount of absorption of the sample. The noise level is obtained by dividing the VCD measurement into two halves. Addition of these halves gives the VCD spectrum, including noise, whereas subtraction eliminates the VCD features and leaves the equivalent noise spectrum. An example of a VCD noise spectrum is given in *Figure 3*, which shows, from bottom to top, the IR, VCD, and VCD noise spectra for a measurement of (–)-*S*-α-pinene. The VCD noise level can be reduced by increasing the measurement time through co-addition of the spectra, as desired.

## 5.3 VCD Baseline Accuracy

A second critical factor in the measurement of a VCD spectrum is the characteristics of the VCD baseline, namely its location, straightness, and stability. The VCD zero intensity line between positive and negative VCD intensities needs to lie as close as possible to the electronic zero of the measurement (zero of spectral display). Due to the sensitivity of the VCD intensity scale, some offset of the baseline from electronic zero almost always occurs, as discussed above in reference to the need for baseline correction of the VCD spectra of α-pinene in *Figure 2* and as carried out for *Figure 3*. Use of α-pinene as a validation standard is advantageous, because α-pinene has an unusually large intensity and thus, its spectrum is relatively easy to measure on a regular basis. α-Pinene can also be used as a measure of the baseline of a VCD measurement relative to the electronic zero of the measurement. This is illustrated in *Figure 5*, where the VCD spectra of (–)-α-pinene (*dash*) and (+)-α-pinene (*black*) are presented along with the VCD spectrum of a racemic mixture [equal quantities of (–)-α-pinene and (+)-α-pinene, hence cancelling the VCD intensity] of α-pinene (*gray*). The VCD spectrum of the racemic mixture of α-pinene is the VCD baseline for this measurement. A typical standard for baseline quality is deviations from electronic zero of <10% of the maximum separation of positive and negative VCD intensity of the neat α-pinene spectrum, or approximately $1 \times 10^{-4}$. The baseline illustrated in *Figure 5* satisfies this standard to within 5%. The baseline at 1350 $cm^{-1}$ is slightly above electronic zero and has maximum excursions away from electronic zero of <0.00005, or $5 \times 10^{-5}$ at 1170 $cm^{-1}$, which can be compared to the maximum positive and negative intensity values of (–)-α-pinene of $+5 \times 10^{-4}$ at 1220 $cm^{-1}$ and a negative peak value of $-4 \times 10^{-4}$ at 1130 $cm^{-1}$.

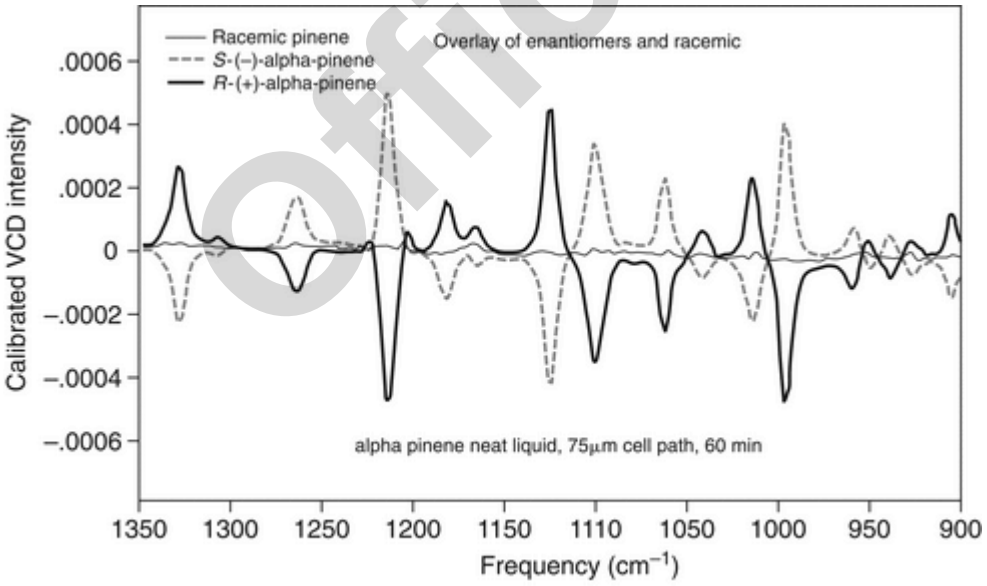


Figure 5. VCD spectrum of *R*-(+)-α-pinene (black), *S*-(–)-α-pinene (dash), and racemic (gray) α-pinene as a neat liquid for a 60-min measurement in a 75-µm path-length barium fluoride cell.

## 5.4 VCD Baseline Absorption Artifacts and Single-Enantiomer Measurement Capability

For tests of baseline accuracy and stability, the sample camphor in carbon tetrachloride solution is used as a qualification standard and provides a more stringent test than α-pinene, because the VCD spectrum of camphor is approximately one-fifth the magnitude, relative to the IR absorbance spectrum, of that of neat α-pinene. The VCD spectra of both enantiomers, as well as two measures of the VCD baseline, are provided in *Figure 6*. It is clear, as in *Figure 2* and *Figure 5*, that the VCD spectra of enantiomers, in this case (+)-camphor and (–)-camphor, are equal in intensity and opposite in sign relative to the VCD baseline to within the noise level of the measurement. The two VCD baselines provided are both zero VCD measurements. The VCD spectrum of racemic camphor [equal mixture of (+)-camphor and (–)-camphor] is the true VCD baseline for the individual (+)-camphor and (–)-camphor spectra, even if there are offsets (artifacts) due to absorption bands in the IR spectrum of camphor. The absence of such artifacts to within the noise level of the measurement is demonstrated by the congruence of the VCD spectrum of the racemic mixture and that of the solvent, carbon tetrachloride. Because carbon tetrachloride has no

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significant level of absorbance in the region of the spectrum displayed, the agreement of these two baselines within the noise level demonstrates the absence of baseline artifacts in the VCD spectrum of racemic camphor, and therefore, for the VCD measurement of either of the two enantiomers of camphor.

Achieving a VCD baseline that is free of absorption artifacts is usually done by optical alignment of the VCD instrument such that the baseline is as close as possible to the true electronic zero of the instrument of the measurement. In *Figure 6*, the VCD baseline can be seen to wander above and below zero across the spectrum, but in this case, the deviations from the electronic zero are small (approximately $2 \times 10^{-5}$) and <20% of the positive and negative maximum VCD intensities of the spectrum (approximately $1 \times 10^{-4}$). This value of deviation is consistent with the maximum baseline magnitudes discussed above for α-pinene and displayed in *Figure 5*, because α-pinene has a much larger (approximately 5×) VCD spectrum, relative to its IR spectrum, than camphor. The baselines for camphor in *Figure 6* are noisier than that of α-pinene in *Figure 5*, because the VCD spectrum scale is smaller and the measurement time is 20 min instead of 1 h. As in *Figure 3*, the VCD baseline can be corrected by subtraction of an appropriate baseline spectrum, racemic or solvent. In *Figure 7*, the baseline-corrected VCD spectra for both enantiomers and the racemic mixture have been obtained by subtraction of the VCD spectrum of the solvent carbon tetrachloride. As a result the VCD zero baseline and the instrument electronic zero are the same to within the noise level, and the mirror symmetric properties of these spectra are even more apparent. This VCD test verifies that by subtracting the VCD spectrum of the solvent, an accurate VCD spectrum of camphor can be obtained using only one enantiomer, either the (+)- or (–)-enantiomer, and hence, the instrument possesses a single-enantiomer VCD measurement capability.

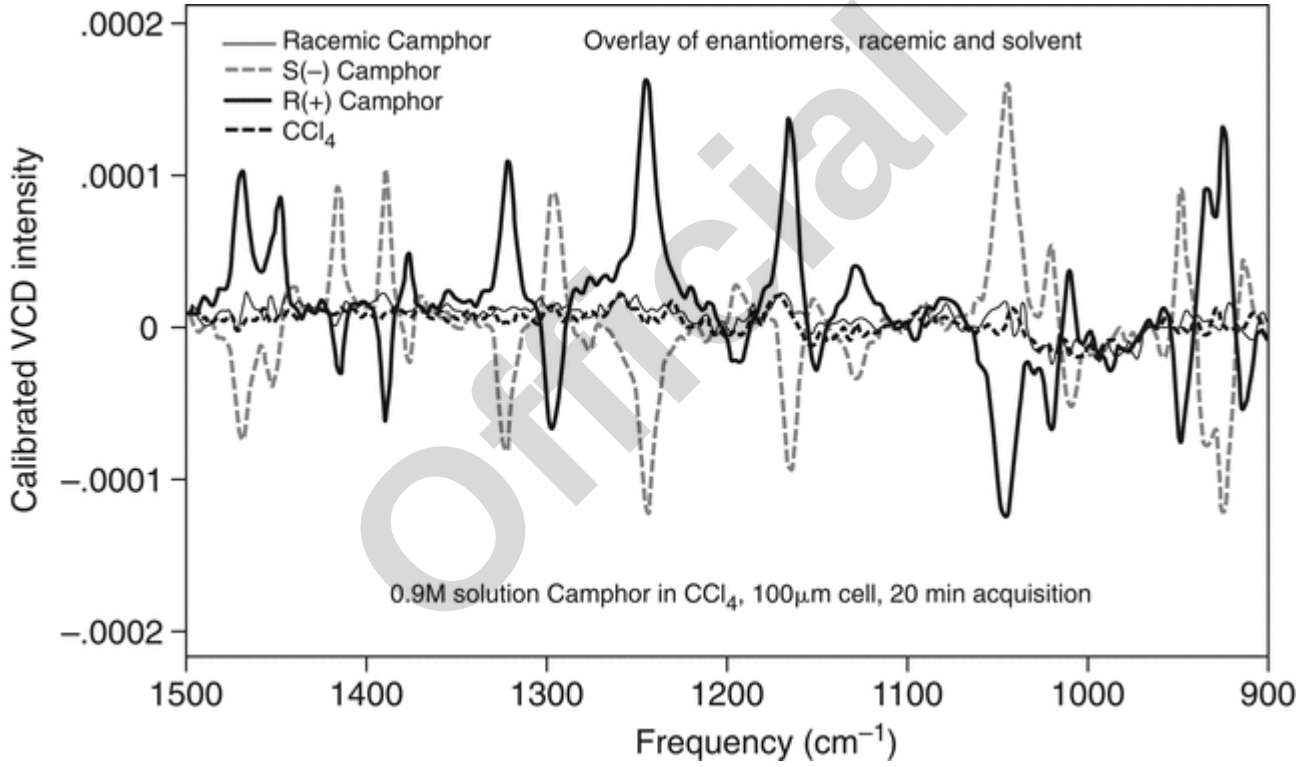


Figure 6. VCD spectrum of *R*-(+)-camphor (black), *S*-(–)-camphor (gray dash), and racemic camphor mixture (gray) as a 0.9 M solution in carbon tetrachloride. Also provided with the same cell and path length is the VCD spectrum of pure carbon tetrachloride (black dash). The sampling conditions were a spectral collection time of 20 min with a 100-μm path-length barium fluoride cell.

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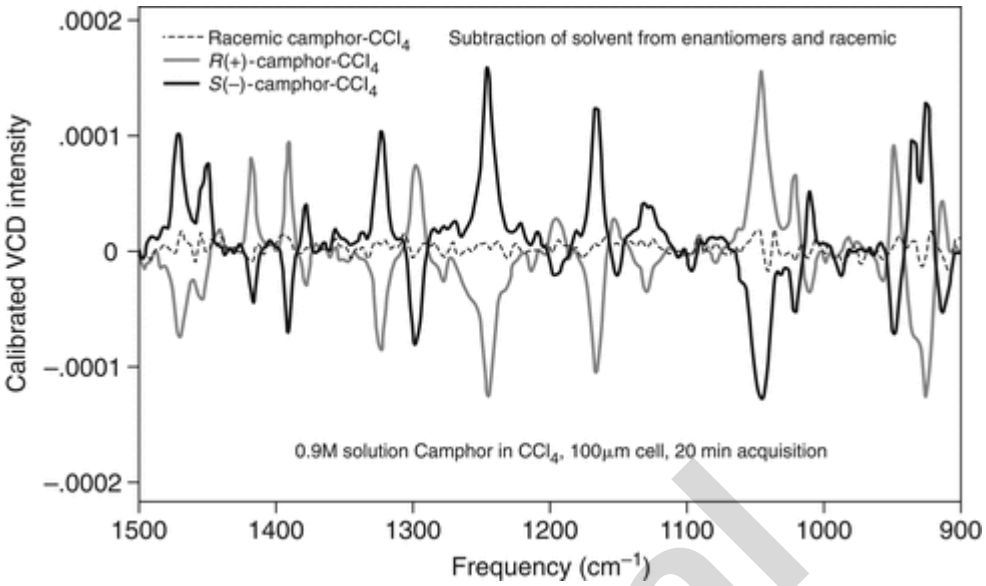


Figure 7. Solvent baseline-corrected VCD spectrum of *R*-(+)-camphor-carbon tetrachloride (gray), *S*-(–)-camphor-carbon tetrachloride (black), and racemic camphor-carbon tetrachloride mixture (dash) of the VCD spectra shown in *Figure 6*.

## 5.5 VCD Measurement Stability and Noise Level Reduction

Comparison of VCD measurements of camphor for 20 min to that measured over a period of 4 h in blocks of 20 min permits evaluation of instrument measurement stability as well as the reduction of noise level over time. In *Figure 8*, 12 such 20-min VCD spectra measured in 4 h are presented to show the stability of a VCD spectrum over time. It is clear that there are no deviations of VCD spectra outside the noise level of any one 20-min spectrum, and hence, there is no long-term drift in the VCD baseline over this time period.

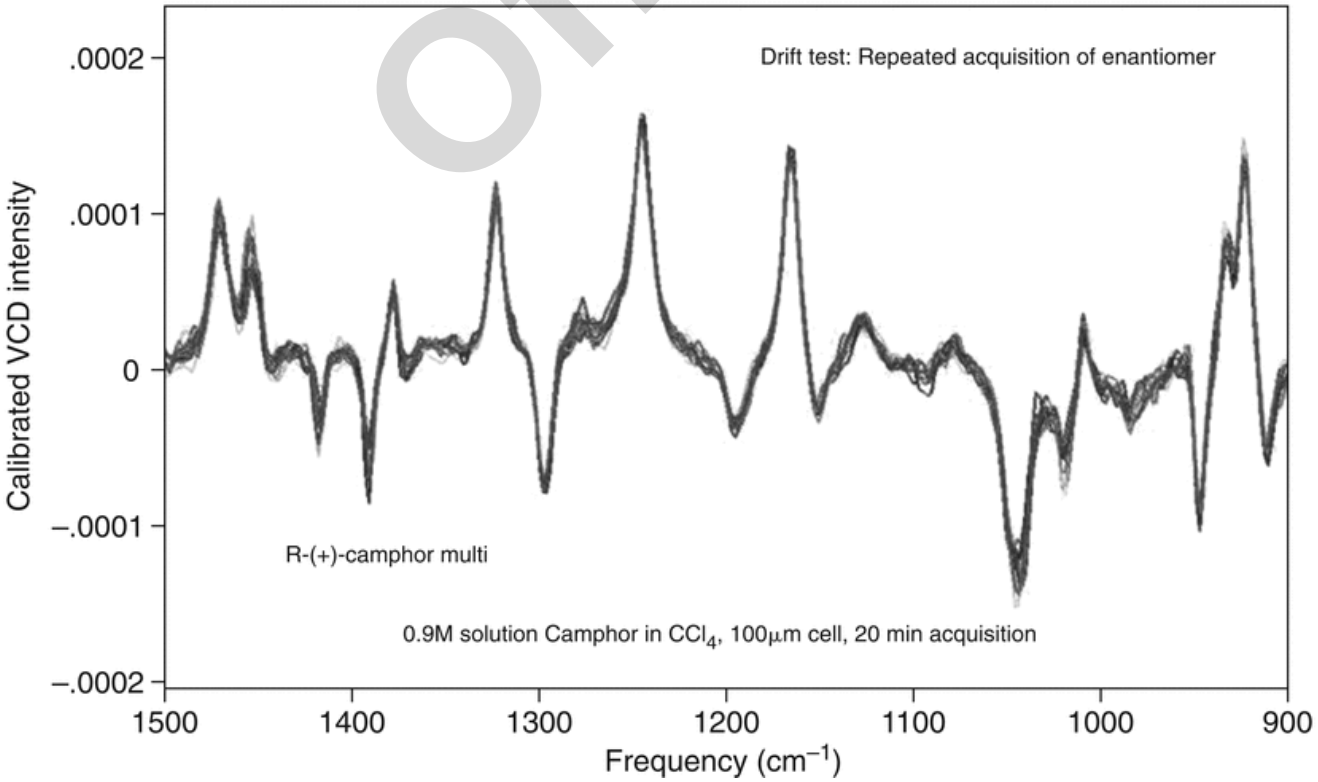


Figure 8. Superposition of 12 20-min VCD spectra of *R*-(+)-camphor under the conditions of *Figure 6* and *Figure 7*.

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If the 12 blocks of 20-min VCD spectra in *Figure 8* are averaged, the resulting 4-h VCD spectrum can be compared to a 20-min VCD spectrum of the same sample to demonstrate the noise reduction that is achieved upon signal averaging, provided there are no other systematic noise sources in the VCD spectrometer. In this case, the noise reduction should be a factor of the square root of 12, or approximately 3.5. Thus, the signal-to-noise ratio should improve by a factor of 3.5. Such a comparison is provided in *Figure 9*.

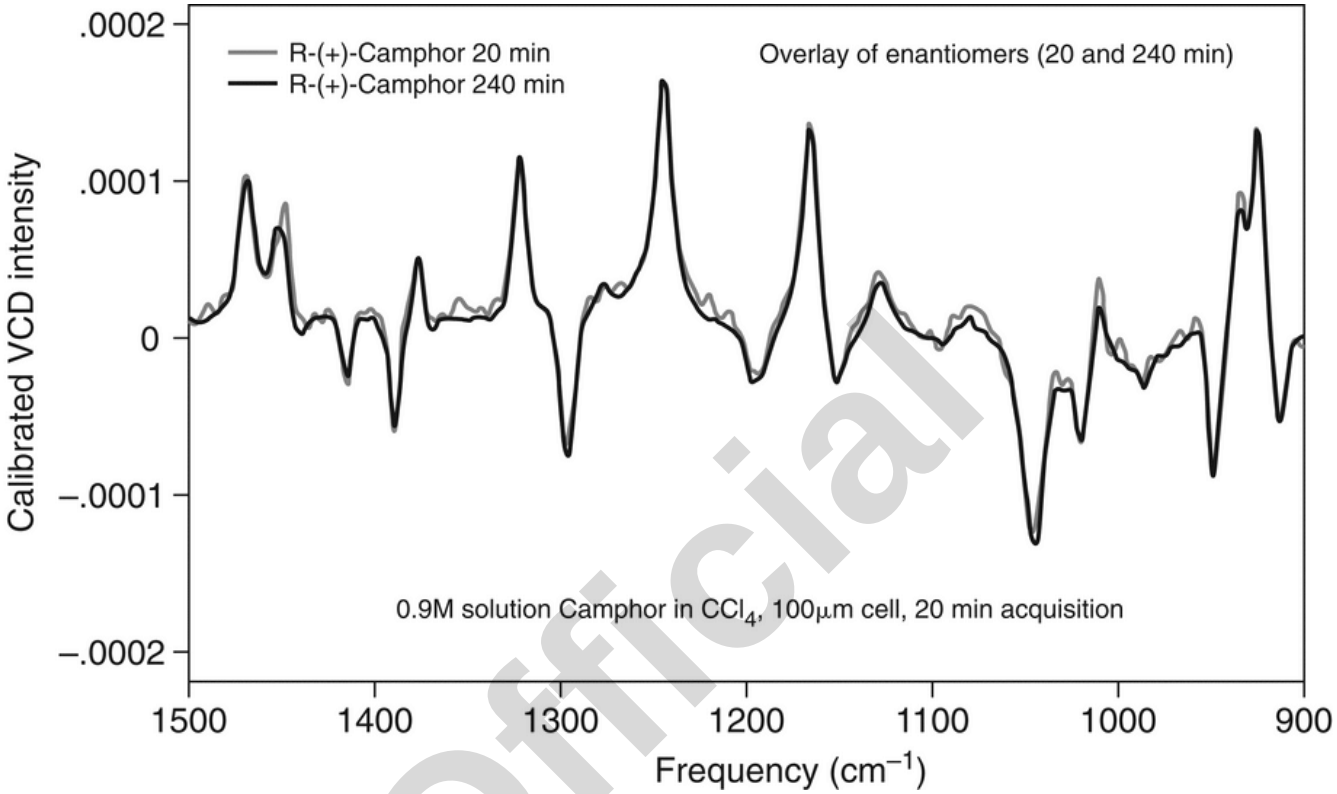


Figure 9. Comparison of the 4-h average of 12 20-min VCD spectra [*R*-(+)-camphor 240 min (gray)] to one 20-min measurement of camphor [*R*-(+)-camphor 20 min (black)], under the conditions of *Figures 6*, *7*, and *8*, showing improvement in signal-to-noise ratio with increased collection time.

The noise level can be visualized directly on a 5× more sensitive scale to better evaluate the noise levels by comparing the noise spectrums, as illustrated and described in *Figure 3*, for the 4-h and 20-min VCD measurements. This is presented in *Figure 10*.

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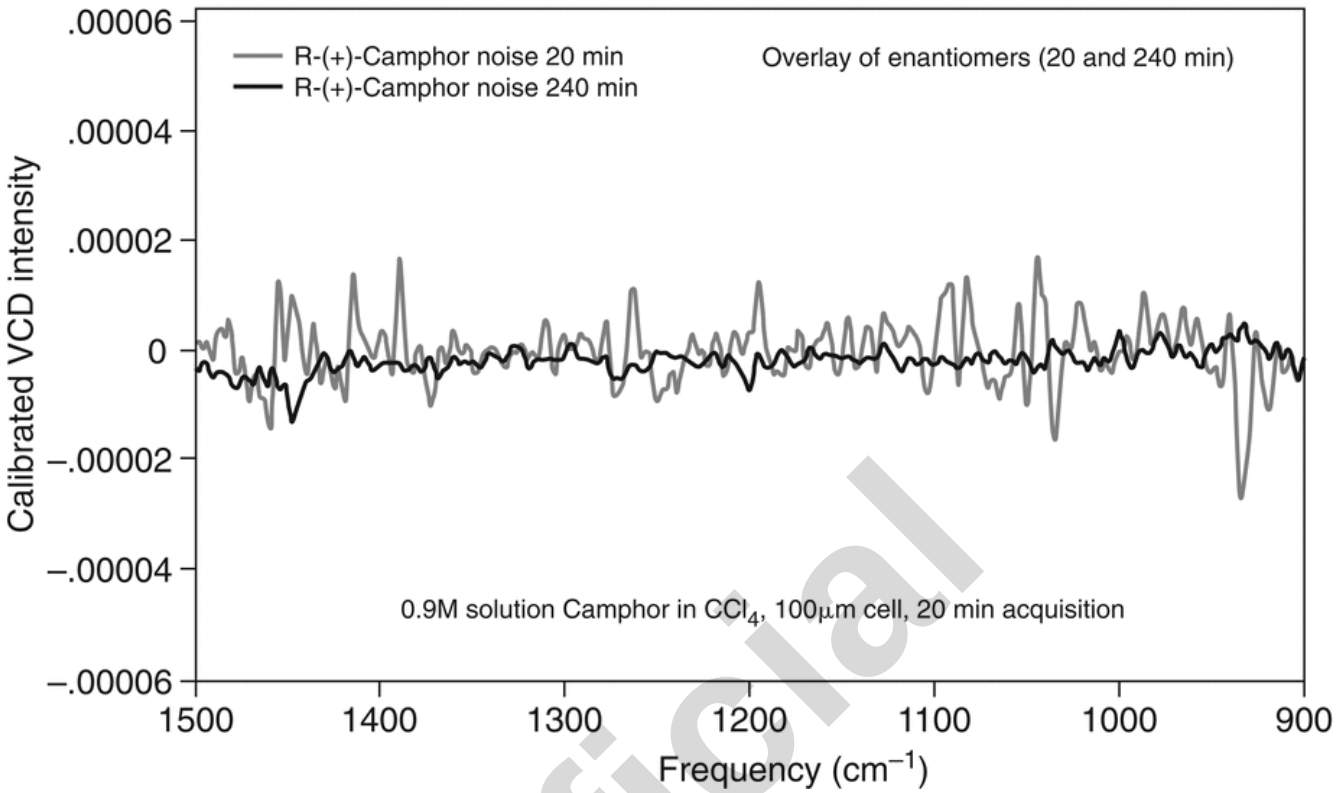


Figure 10. Comparison of the VCD noise spectra of *R*-(+)-camphor for one 20-min measurement [*R*-(+)-camphor noise (gray)] versus a 4-h measurement [*R*-(+)-camphor noise (black)] under the conditions of *Figures 6*, *7*, *8*, and *9*, where the intensity scale has been expanded to accommodate noise spectra that are 5× enlarged for better viewing from the noise levels in the previous four figures.

## 6. DETERMINATION OF ABSOLUTE CONFIGURATION

The IR and VCD spectra of a sample are measured in solution in a matter of minutes to hours without the requirement of prior crystallization or high sample optical purity. The AC of an unknown sample is determined by comparison of its measured VCD spectrum to a VCD quantum chemistry calculation of the same molecule, where the AC used in the calculated VCD spectrum is chosen arbitrarily. If the signs of the bands in the measured VCD spectrum match those in the calculated VCD spectrum, the AC of the sample is identical to that used in the calculation. If the signs are opposite, then the sample has the opposite AC compared with that calculated. An example of the determination of the AC of a pharmaceutical molecule is shown in *Figure 11*. The AC of mirtazapine, which is sold in racemic form, is determined by comparison of the measured IR and VCD spectra of the (–)-enantiomer to the calculated IR and VCD spectra of the *R*-enantiomer. The close match in VCD signs—vibrational frequencies, intensities of the observed and calculated spectra, and IR frequencies and intensities—demonstrates clearly that the AC of mirtazapine is (–)-*R*. Also shown in *Figure 11* is the stereospecific quantum chemistry calculated structure of *R*-mirtazapine.

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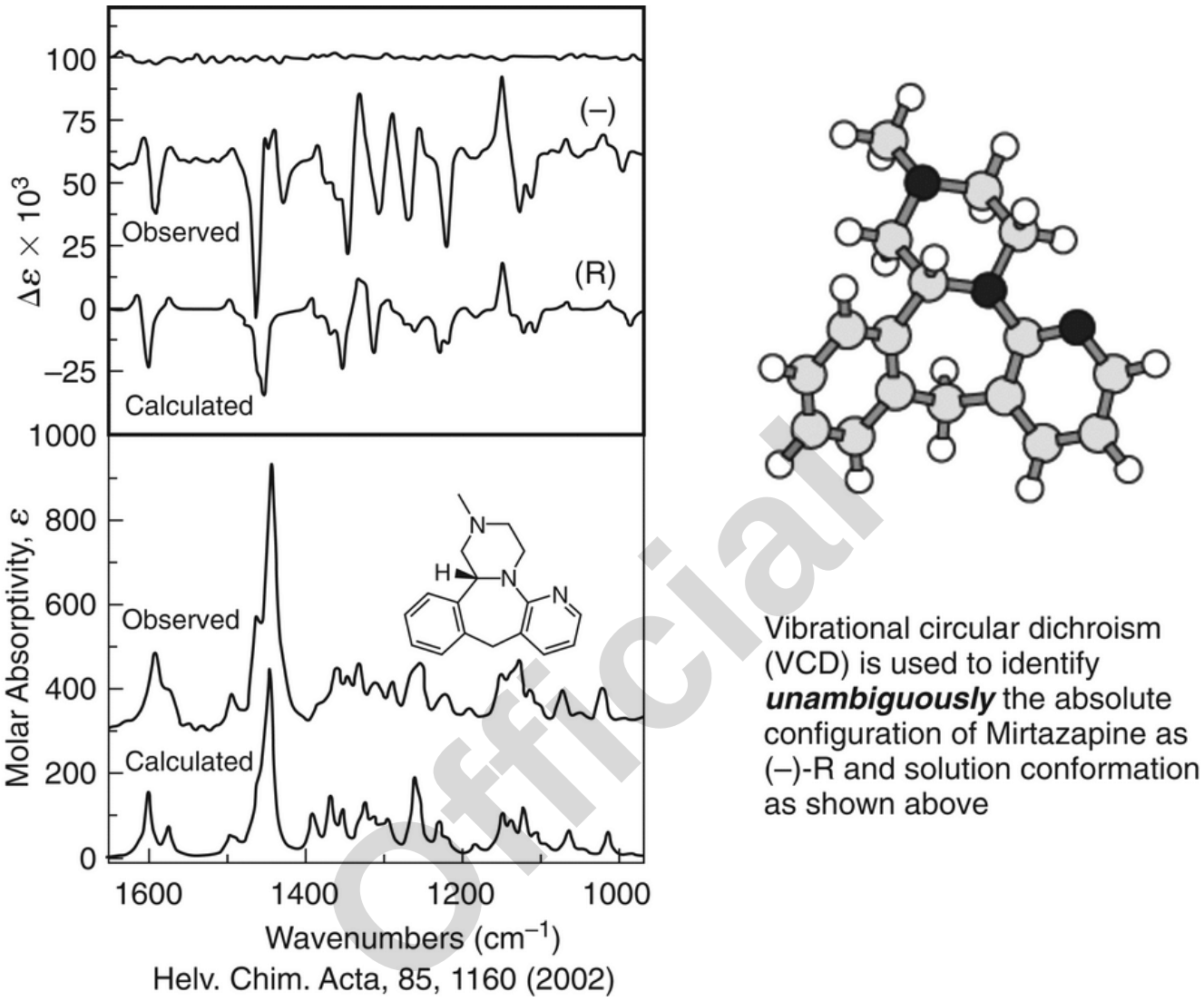


Figure 11. Comparison of the measured (observed) and calculated IR and VCD spectra of mirtazapine, allowing assignment of its AC to (–)-*R*. Reproduced in part with permission from John Wiley & Sons.

If the AC of a molecule has previously been established, for example, by X-ray crystallography, a VCD calculation of the molecule is not necessary (but could be performed as a check of the X-ray assignment), and the measured VCD spectrum of the molecule may be taken as a reference standard for the AC of the molecule, supported, for example, by USP. For a molecule with unknown structural chirality, the determination of its AC by VCD requires a comparison of the measured and calculated IR and VCD spectra, $\varepsilon(\nu)$ and $\Delta\varepsilon(\nu)$, as shown in *Figure 11*. The key point of the comparison is correlating the major VCD bands to determine whether they have the same or opposite signs. The IR spectrum is a guide in the process. Seeing a close correspondence between the calculated and measured IR spectra provides confidence that a good vibrational force field has been calculated and the most important vibrational bands are correlated. If a corresponding correlation of the signs of the major VCD bands can be made, as in *Figure 11*, then the AC has been determined.

When the measured and calculated VCD spectra are compared, the magnitudes of the corresponding intensities are not critical. What is important is the relative intensities of the bands in the two spectra, and in fact, that the comparison of measured and calculated spectra can be made using different sets of intensities for $\varepsilon(\nu)$ and $\Delta\varepsilon(\nu)$ for the calculated spectra and $A(\nu)$ and $\Delta A(\nu)$ for the measured spectra. In general, an exact match of the measured and calculated band frequencies is not expected because of several limitations of the calculations. Examples of these limitations include the assumption of the harmonic approximation when some anharmonicity is present in the measured spectra, the need to ignore the effects of solvent interactions with the chiral solute molecule, limitations in the size of the basis set used for the quantum calculation, and the choice of density functional required for the density functional theory (DFT) calculation.

## 7. CALCULATION OF VCD SPECTRA

To determine the AC of a molecule with unknown chirality using the VCD method as an alternative to, or supplement to, X-ray crystallography, analysts conduct a quantum chemistry calculation of the IR and VCD intensities of the molecule. Since

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the late 1990s, commercial DFT software has been available and can be used effectively by a trained user because it does not require special expertise or extensive experience in quantum chemistry calculations. For example, VCD calculations are now part of some undergraduate chemistry curricula. The following steps are required to perform a DFT calculation of IR and VCD:

1. The stereospecific structure of the molecule is entered through a visual graphics interface.
2. The quantum chemistry program then automatically performs the following steps:
    A. The geometry is optimized to a minimum energy conformation.
    B. A force field is calculated that determines the vibrational modes of the molecule, with 3N-6 modes for a molecule with N atoms.
    C. Atomic polar tensors that yield the IR spectrum are calculated.
    D. Atomic axial tensors that yield the VCD spectrum are calculated.
3. The output of the calculation consists of a table where for each mode *i* there is a frequency, $\nu_i$, IR intensity (dipole strength), $D_i$, and VCD intensity (rotational strength), $R_i$.
4. Commercially available programs calculate the IR and VCD spectra for the molecule using the following steps:
    A. A line shape, $f(\nu)$, is applied to each dipole and rotational strength value, $D_i$ and $R_i$, centered at each vibrational frequency, $\nu_i$.
    B. Summation of these normal mode line-shaped intensities automatically produces the final calculated IR and VCD spectra $\varepsilon(\nu)$ and $\Delta\varepsilon(\nu)$ that can be compared directly to the corresponding measured IR and VCD spectra.

Another important factor that often is encountered in the assignment of the AC of pharmaceutical molecules and natural products (because of the structural complexity of these types of molecules) is the presence of more than one important conformer under the measurement conditions. The fractional populations of different conformers can be predicted by using their relative calculated energies in the Boltzmann distribution. The lowest-energy conformer has the highest fractional population and so on, to increasingly less-populated conformers with higher relative energies. To calculate the VCD, one must calculate the IR and VCD of each important conformer and then add these spectra together, weighted by the fractional Boltzmann population. Usually, one can ignore the spectral contributions of conformers that account for less than a few percent of the total population.

Programs for calculating VCD and IR spectra to the level of accuracy sufficient for comparison to measured IR and VCD are available commercially from a variety of sources. First, one needs a program for evaluating and finding the geometry of all the lowest-energy conformations of a chiral molecule. A variety of programs using molecular mechanics are available for this purpose. Second, one needs a full quantum chemistry software program for calculating the ab initio force fields, vibrational frequencies, and VCD and IR intensities for each of the lowest-energy conformers. A common chemical model of sufficient accuracy for AC determination is DFT, using a basis set minimum of 6-31G(d), and a choice of hybrid functionals, such as B3LYP or B3PW91. Higher-level basis sets and alternative choices of hybrid functionals may be made. Finally, one needs a software method for comparing measured and calculated VCD and IR spectra to assess the degree of spectral agreement between the measured and calculated spectra, and hence the level of confidence that the correct assignment of AC has been predicted. Additional details are described below.

In *Figure 12*, the AC of the bioactive enantiomer of the analgesic (*S*)-(+)-ibuprofen is determined by comparing the measured and calculated IR and VCD spectra. The calculated IR and VCD are the sums of the IR and VCD conformer spectra, one for each conformer, weighted by the fractional populations 0.37, 0.36, 0.12, and 0.07, plus smaller contributions from another eight conformers that have a total contribution of 0.08. Clearly, there are two important conformers that are nearly equally populated, and the basic features of the final IR and VCD spectra are determined by these spectra. As a result of this analysis, not only has VCD been able to confirm the AC of (+)-ibuprofen as *S*, but two dominant solution-state conformers have been identified, along with two additional less-populated conformers.

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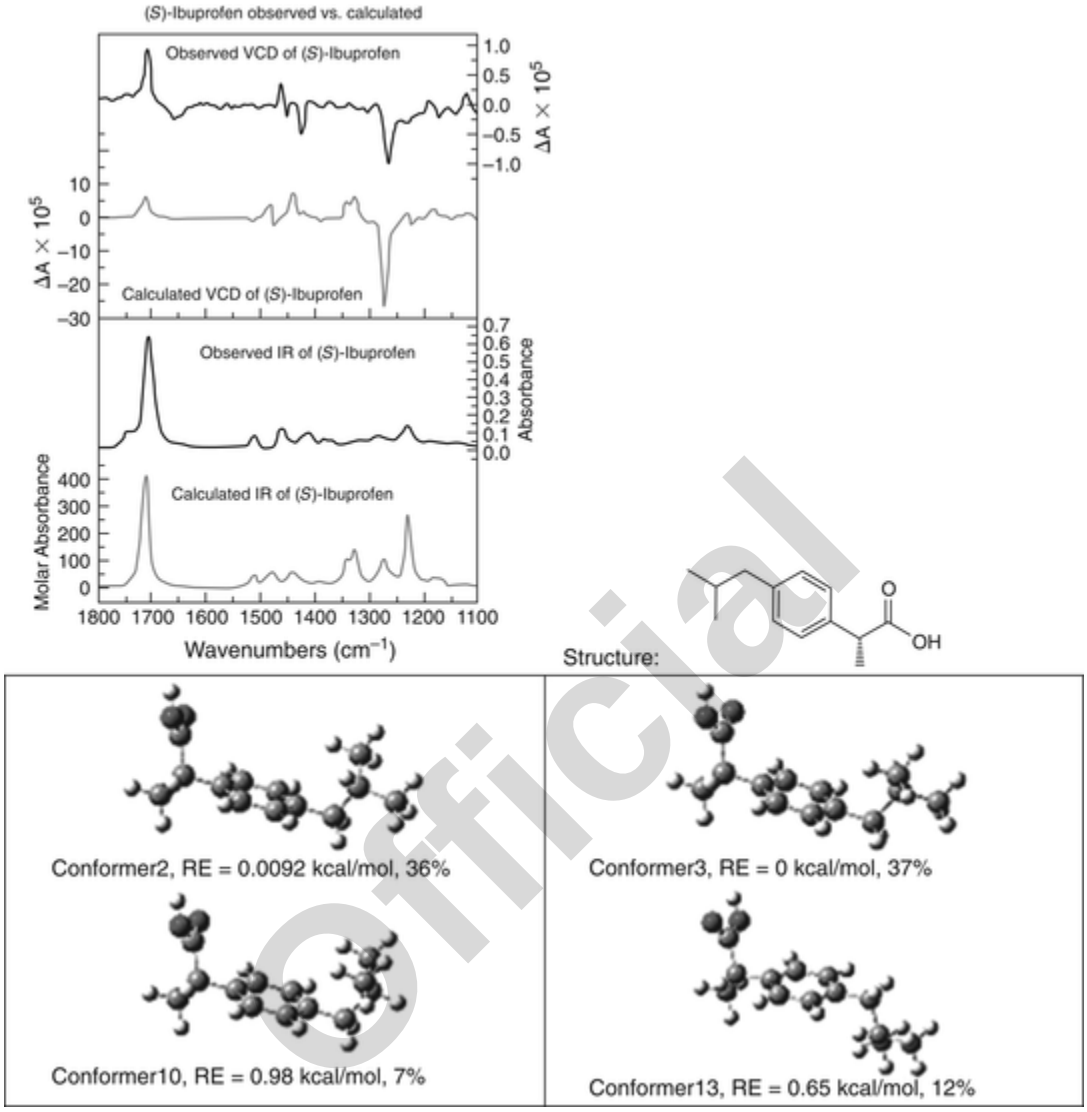


Figure 12. On the top are displayed the measured (observed; +) and calculated (*S*) IR and VCD spectra of (+)-ibuprofen (above) and the stereostructure of (*S*)-ibuprofen (below). On the bottom are displayed the stereostructures, relative energy (RE), and percentage Boltzmann population (BP) for the four most important solution-state conformers.

## 8. COMPARISON OF MEASURED AND CALCULATED SPECTRA

To provide an unbiased statistical measure of the degree of similarity between measured and calculated IR and VCD spectra, an analytical method based on a convolution algorithm has been developed. The degree of similarity, or congruence, between a measured and a calculated spectrum can be calculated and used to determine a degree of confidence that the AC determined by visual inspection is correct.

### 8.1 Degree of Confidence of Correct Assignment

The results of the use of such statistical measures are shown in *Figure 13*. The degree of similarity (the total neighborhood similarity, TNS) of the measured and calculated IR is 92.5, where 100 is a perfect match. The same TNS measure for the VCD is 82.2. Because VCD bands can be either positive or negative, a more refined analysis is carried out for VCD, and only regions of sign agreement are compared for each enantiomer. In this case, the value of the signed neighborhood similarity (SNS) for the *S*-enantiomer is 92.8, and for the *R*-enantiomer it is only 12.1. Clearly, the analysis confirms the visual agreement of the measured VCD spectrum (*upper solid line*) and the calculated VCD spectra (*lower solid line*) for the *S*-enantiomer versus the *R*-enantiomer (*dashed line*). An additional numerical comparison is calculated as the enantiomeric similarity index (ESI), which equals SNS(*S*)– SNS(*R*). The result (*black dot*) is then plotted against a database of 89 prior correct VCD assignments, where the axes are SNS (*vertical*) and ESI (*horizontal*). The closer a statistical point is to the *upper right-hand corner* of the plot, the higher the similarity of the comparison and the higher the degree of confidence. The upper right-hand corner of the plot is defined as 100% spectral similarity. The degree of confidence is a statistical measure that is set to be 100% confidence for points

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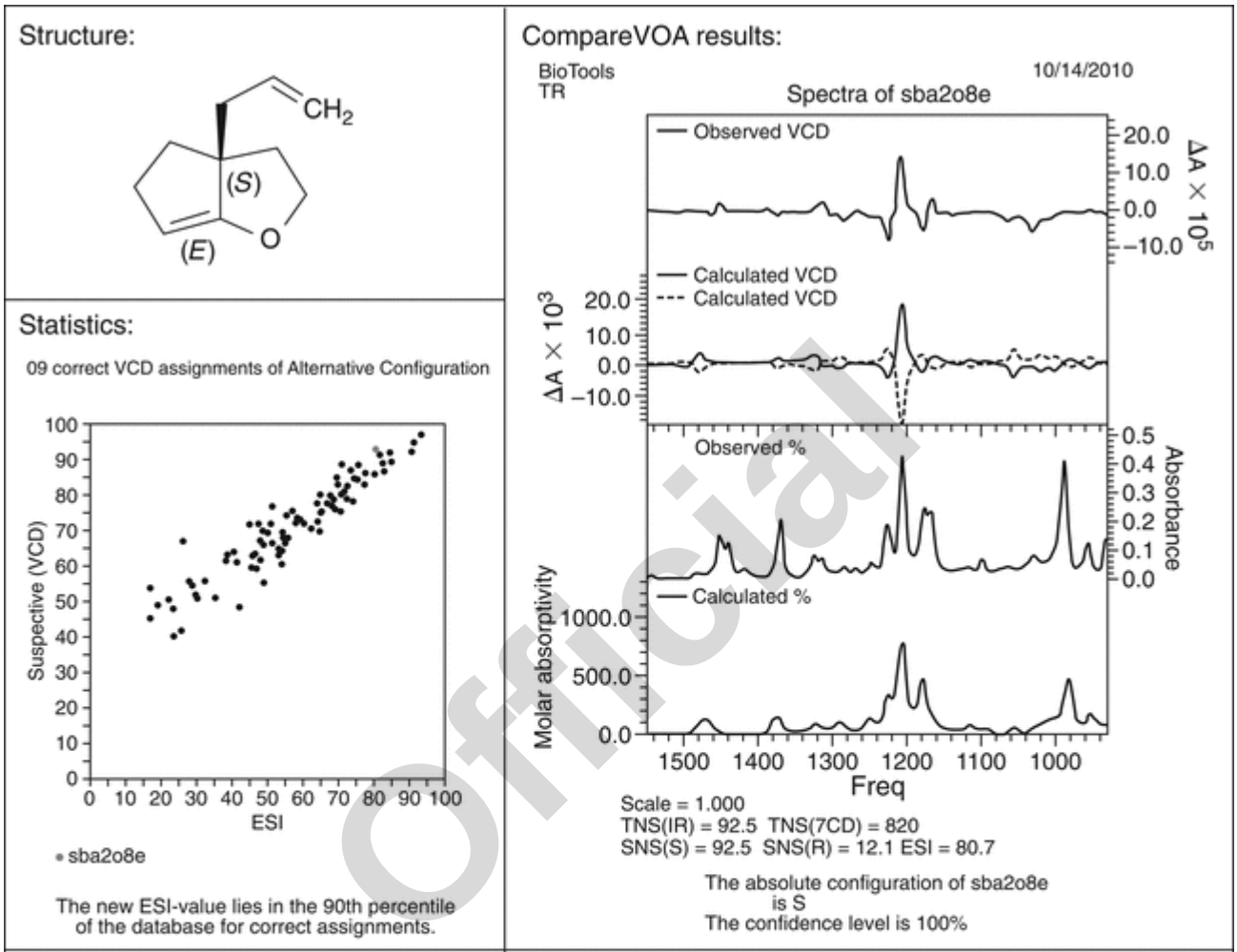
clustered in the *upper right-hand region* of the plot. Statistical measures such as these eliminate the reliance on only visual judgment for the comparison of measured and calculated VCD spectra and provide a statistical basis for assigning a degree of similarity between the measured and calculated spectra.




| Experimental Details | | Calculation Details | |
|---|---|---|---|
| Instrument | Chiral R2X with DualPEM | Gaussian Version | Gaussian 09 |
| | | DFT-level of theory | B3LYP/6-31G* |
| Solvent | CDCl3 | Configuration calculated | As shown above |
| Acquisition time | 10 h | Total calculated conformers | 6 |
| Concentration | 7.5 mg/0.15 mL | Total low-energy conformer used for Boltzmann sum | 4 |
| Pathlength | 100 μm | | |
| Assigned Absolute Configuration: (*S*)- | | Confidence level: 100% | |

Figure 13. Output of a commercial program for comparison of measured (observed) VCD and IR spectra with calculated VCD spectra of both enantiomers and the calculated IR spectrum. Statistical data are explained in the text.

## 9. DETERMINATION OF ENANTIOMERIC EXCESS

After AC, the next most important property of a chiral sample is the EE, as described in *1. Introduction*. For enantiomers labeled *R* and *S*, the EE for the *R* enantiomer is defined as EE = $(N_R - N_S)/(N_R + N_S)$ and %EE = EE × 100%. Here, $N_R$ represents the number of moles of the *R* enantiomer present in the sample; for a solution, $N_R$ can represent the concentration of the *R* enantiomer. Thus, %EE for the *R* enantiomer can vary from +100% to –100%.

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VCD spectra also can be used to determine the EE of a sample once the sample has been calibrated by a single IR and VCD measurement of a sample with a known EE. VCD scales linearly with EE with a maximum VCD intensity for a given IR intensity at 100% EE, one-half VCD intensity for the same IR intensity at 50% EE, and zero VCD for the racemic mixture of 0% EE. These points are illustrated in *Figure 14* for 11 measurements of *R*-(+)-α-pinene for which the %EE decreases from 100% to 6.7%. The IR spectra are identical, but the VCD spectra grow smaller linearly as EE is reduced by the addition of measured amounts of the opposite enantiomer to the sample cell. The degree of accuracy of this determination of %EE was slightly >1%, as indicated in the plot of actual prepared EE versus VCD predicted EE, using a partial least-squares chemometric analysis as presented in *Figure 15*.

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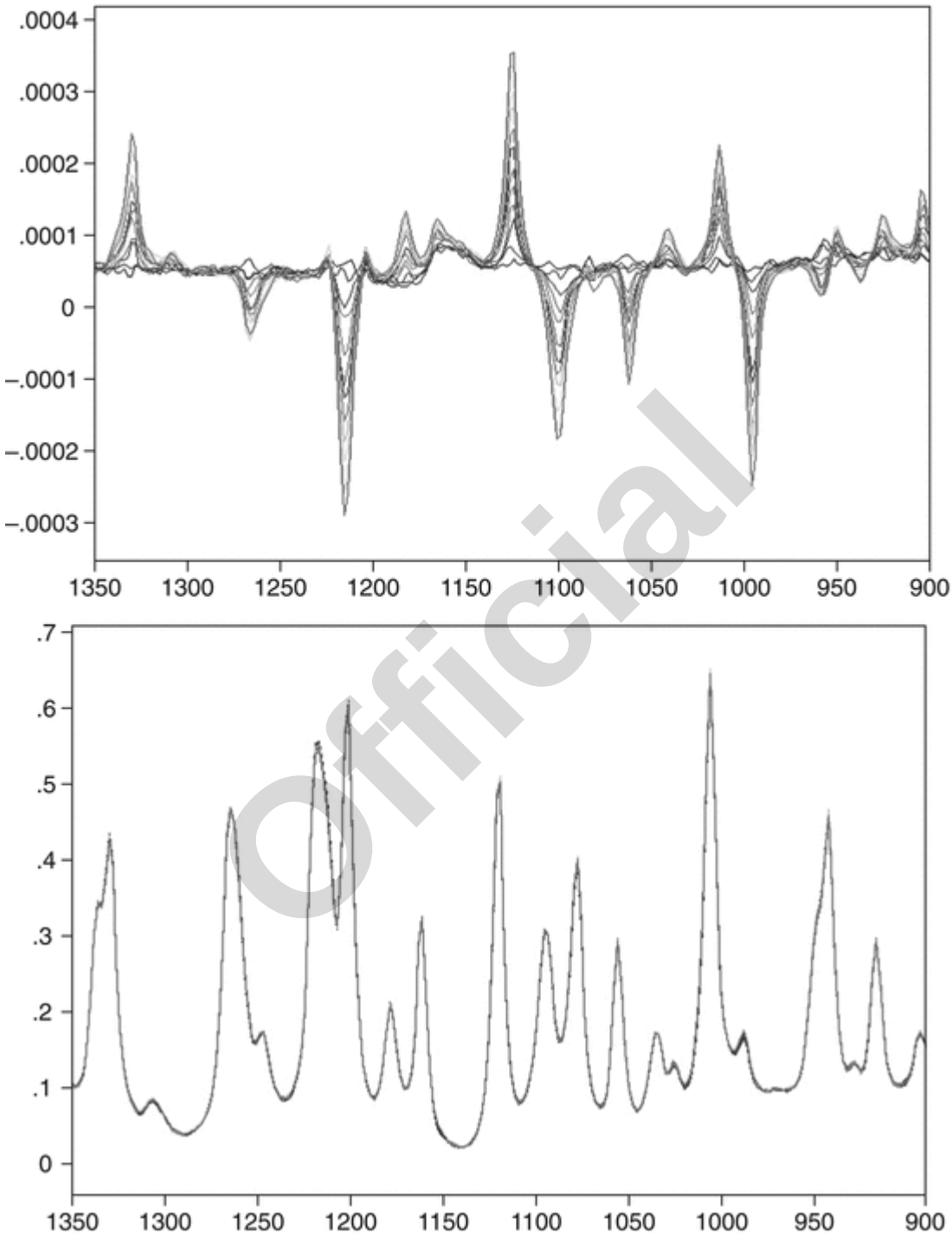


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Figure 14. Superposition of 11 VCD (upper) and IR (lower) spectra of a 3.1 M solution of (+)-(*R*)-α-pinene in carbon tetrachloride for samples with %EE values of 100.0%, 88.2%, 77.8%, 68.4%, 60.2%, 52.4%, 45.5%, 33.3%, 23.1%, 14.3%, and 6.7%. Reproduced in part with permission from John Wiley & Sons.

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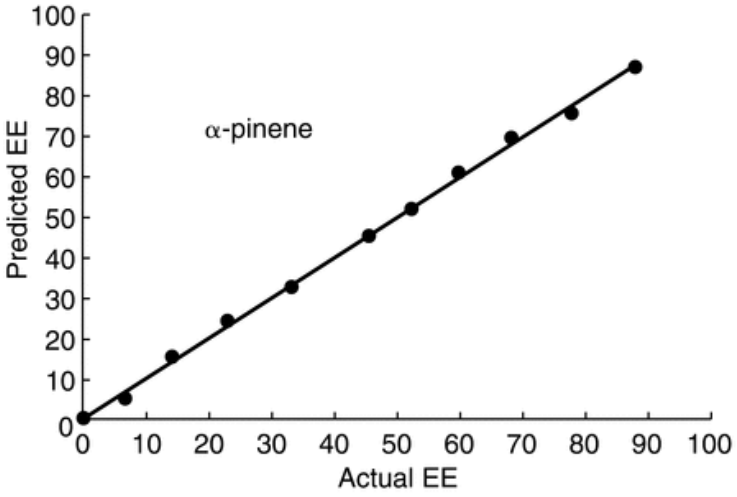


| Actual EE% | Predicted EE% | Variance (EE%) |
|---|---|---|
| | | |
| 88.2 | 87.2 | 1.0 |
| 77.8 | 75.9 | 1.9 |
| 68.4 | 69.7 | –1.3 |
| 60 | 61.3 | –1.3 |
| 52.4 | 52.2 | 0.2 |
| 45.5 | 45.3 | 0.2 |
| 33.3 | 32.8 | 0.5 |
| 23.1 | 24.4 | –1.3 |
| 14.3 | 15.4 | –1.1 |
| 6.7 | 4.9 | 1.8 |
| 0 | 0.3 | –0.3 |
| RMSD | 1.15 | |
| STDEV | 1.21 | |

Figure 15. Plot and corresponding table of actual versus VCD predicted values of %EE using partial least-squares (PLS) chemometric analysis that achieves a root-mean-square deviation (RMSD) of 1.15 and a standard deviation error of cross-validation (STDEV) of 1.21.

***Change to read:***

## 10. CONCURRENT USE OF VCD FOR ABSOLUTE CONFIGURATION AND EE

### 10.1 Chiral Raw Material Identification

Currently, there is no routine, real-time chiral measurement for material identification (ID). Chiral drug substances must comply with the *USP–NF* monograph tests that typically rely upon nonchiral test procedures such as mid-IR spectroscopy (*Mid-Infrared Spectroscopy* ⟨854⟩ and *Mid-Infrared Spectroscopy—Theory and Practice* ⟨1854⟩). In practice, near-IR (for additional information, see ▲*Near-Infrared Spectroscopy—Theory and Practice* ⟨1856⟩▲ (CN 1-May-2020)) is used in many manufacturing facilities. For near-IR analysis of solids, analysts commonly use fiber-optic probes and then confirm the raw material by comparison of the measured spectrum against that of the USP Reference Standard. To determine the AC and EE of a chiral material, analysts must make a separate optical rotation measurement, which requires large amounts of sample in a 10-cm path-length cell, where uncertainty errors are possible for materials with small OR values.

A single measurement of the VCD spectrum and its associated IR spectrum in the mid-IR or near-IR region of a raw material, either as a crystalline solid or in solution, simultaneously contains information for the three critical measures: ID, AC, and EE. By comparison to a USP Reference Standard, the simultaneous measurement of IR and VCD spectra of a test material identifies the sample and the presence of impurities and separates impurities into achiral (IR only) or chiral (IR and VCD). The signs of the VCD identify the AC of the dominant enantiomer, and the ratio of the VCD to the IR gives the EE, as described above. Typical VCD accuracy for EE determinations is in the range of 0.1%–1%.

### 10.2 Chiral Quality Control

VCD can be used as a chiral measure for characterization of raw materials for process analytical technology (PAT) during development, synthesis, formulation, and final production of drug substances and drug products. VCD also can be used to test the interaction between formulated chiral drug substances and excipients. Currently, there are no protocols for monitoring EE as a quality control measure in the pharmaceutical industry. Because of the importance of chirality as a critical measure of sample integrity, there is a need for the incorporation of a new technology, such as VCD, to ensure the desired level of chiral quality of pharmaceutical products from discovery through to final formulation.

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# ⟨1787⟩ MEASUREMENT OF SUBVISIBLE PARTICULATE MATTER IN THERAPEUTIC PROTEIN INJECTIONS

## INTRODUCTION

Parenteral products are designed and manufactured to minimize particulate matter, which is differentiated into two broad categories: visible and subvisible. The absolute limit of visibility, or detectability, depends on the test conditions and the nature of the particulate matter. This general information chapter covers subvisible particles in the range of 2–100 µm. Particles larger than 100 µm are generally considered visible particles (see *Visible Particulates in Injections* ⟨790⟩).

Foreign and particulate matter are undesirable in the final product. These undesired particles arise from two sources: extrinsic or foreign matter and intrinsic or product-related matter. Extrinsic matter cannot be associated with product or process. Intrinsic particles may be a byproduct of the assembly of the product or result from change over time. A third category, inherent matter, describes a physical state or particles that are an expected attribute of the product. The aim of this chapter is to provide additional information to support *Subvisible Particulate Matter in Therapeutic Protein Injections* ⟨787⟩, which has limits for ≤10-µm and ≤25-µm particle content. Because the monitoring of the sub-10-µm population may be an important product quality parameter, collection of data in the 2–10-µm range (e.g. 2–5 µm and 5–10 µm) is recommended. This chapter will focus on enumeration, characterization, and when possible, identification of inherent particles, distinguishing them from extrinsic and intrinsic particles. The chapter does not cover formulations that are suspensions or emulsions, or those that contain adjuvants or similar intended particle components. However, foreign and particulate matter should be minimized in the final drug product, and the strategy and methods utilized are ultimately determined by the manufacturer.

### Extrinsic Particles

Materials that are not part of the formulation, package, or assembly process, but rather are foreign and unexpected. Examples of extrinsic particles include materials from biological sources (e.g., fibers, insect parts, pollens, and vegetative matter), from process and building materials (e.g., cellulose, lint, minerals, glass, plastics, rubber, metal, and paint), and from personnel (e.g., epithelial cells, clothing fragments, and hairs).

### Intrinsic Particles

Materials occurring in the final product that arise from sources within the formulation ingredients, assembly process, or packaging. Their association with the production process and/or package-related sources can be indicative of systemic problems. For example, silicone oil is an important manufacturing and product component that may affect particle counts and, in excess, is considered intrinsic. The incidence of silicone oil-related particles may correlate with processing sequences or formulation component sources. Detection, identification, and measurement of silicone oil-related particles are discussed further in another section of this chapter. Intrinsic particles can be similar in some ways to extrinsic particles such as rubber, metal, plastic, and glass, but may arise because of insufficient cleaning of processing equipment. Intrinsic particles can also arise from changes in the product over time. These changes may be related to ionic or organic extracts, instability of the active pharmaceutical ingredient (API), formulation degradation, or product–package interaction. Intrinsic particles may promote protein, and one should be aware of this possibility.

### Inherent Particles

Materials that are expected from the drug substance and other formulation components, and thus represent a potentially acceptable characteristic of the product. In the context of therapeutic protein injections, the primary source of inherent particles is proteinaceous aggregates formed either solely by interactions of the protein with itself or in combination with other formulation ingredients. The presence of proteinaceous particles should be assessed for process consistency and product quality. Identification and characterization of the aggregates are critical for distinguishing them from extrinsic and intrinsic particles. The tools and techniques discussed in this chapter are applicable to all sources but are especially useful for the characterization of protein aggregates.

Heterogeneous particles consist of more than one chemical entity. They are classified as either extrinsic or intrinsic based on the nonproteinaceous component that presents the greatest risk.

Historically, test methods contained in *Particulate Matter in Injections* ⟨788⟩ were developed for detection and control of extrinsic and intrinsic particle content and rely on light obscuration (LO) and/or microscopic determination of particles that are between 10- and 25-µm thresholds. These sizes were selected to allow consistent measurement of subvisible particle content and allow determination of product acceptability from the perspective of patient safety and process consistency. The membrane microscopic method was developed first and was used only for large-volume (>100 mL) products. Later, improvements in the microscopic method and development of an automated light extinction (obscuration) method provided an efficient and robust means of tracking particulate content in a variety of products with different fill volumes. Evolution of analytical application is typical of the compendial standard-setting process, where methods are updated to reflect technological advances, patient safety considerations, and regulatory requirements.

Since ⟨788⟩ became official, the varieties of dosage forms and the numbers of therapeutic protein products have increased markedly. The LO method described in ⟨788⟩ has some technical limitations when used for analyzing certain particle types (e.g., those that have low contrast in the product medium and/or may change shape/size during analysis, as is typical for inherent particles in therapeutic protein injections). Other ⟨788⟩ limitations for therapeutic protein products relate to sample handling

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and low product volumes. These considerations have led to the development of *Subvisible Particulate Matter in Therapeutic Protein Injections* ⟨787⟩.

## OBJECTIVE

This chapter was written to describe strategies for identifying and characterizing extrinsic and intrinsic particle populations, compared with inherent proteinaceous particle population(s), in therapeutic protein injections. Information about specific methods that can be used for these purposes, as well as their advantages and limitations, is discussed; the information applies to therapeutic protein injection products and their dilution or infusion solutions. The overarching goal is to provide comprehensive guidance on use of a broad array of analytical methods, one or more of which may be used beyond the methods described in ⟨787⟩ and ⟨788⟩. This is expected to achieve enhanced or orthogonal characterization of therapeutic protein products during development and support, root cause analysis for nonconformance investigations, stability studies, and others. This includes methods that allow assessment of a variety of characteristics of the inherent protein aggregates including morphology, conformation, reversibility/dissociation, and covalent modification. Guidance on sample handling and preparation is discussed in general terms.

## BACKGROUND

Extrinsic and intrinsic particles should be minimized in all parenteral products. However, protein-related particles may be inherent to therapeutic protein products. Inherent particles should be understood and controlled. These inherent particles are known and expected, arising from the association of protein molecules that can be present in a continuum of sizes, ranging from nanometers (dimers) to hundreds of micrometers (multimers and visible particles). The distribution of particle size is affected by amino acid sequence, solution conditions, sample handling history, and other factors. The specific phenomenon of protein association that gives rise to particles is called aggregation. The terms “proteinaceous particles” and “aggregates” are used interchangeably in this chapter.

Because multiple potential sources of particles exist, it is important to identify the particles and determine whether they are extrinsic, intrinsic, or inherent. Once this has been accomplished, it is possible to develop and apply appropriate control strategies. If deviations occur, particle identity will guide the root cause analysis, risk assessment, corrective actions, and control strategy.

Protein aggregates inherent in therapeutic protein products may consist of a heterogeneous population, and therefore size alone does not adequately describe them. When aggregate populations are being compared across products, labs, etc., it is important to consider other characteristics such as morphology, the ability to dissociate, the protein conformations (or higher-order structures) within the aggregates, and the chemical modifications present (*1*). These are summarized in *Table 1* [adapted from (*1*)]. It is important to realize that the results obtained depend on the technique and methodology used, and this information must be included in any description of protein aggregate characterization. The tools available for aggregate characterization are described later in this chapter. The tools used and the characteristics analyzed should be thoughtfully chosen based on the protein being studied. A more detailed description of attributes that should be considered during aggregate characterization follows.

Size is the particle attribute that has been monitored historically and remains a primary descriptor. When measurements are reported, they should include the size range measured and technique used. Thus, subvisible particles could also be described as aggregates/particles between 1 and 100 µm based on: the equivalent circular diameter, determined using LO; the longest chord, using light microscopy; the equivalent spherical volume, using electrical zone sensing; or other relevant dimensions obtained using other available technologies. The sample preparation, determination method, and algorithms applied are all important factors to include when presenting the results.

The ability of any aggregate species to dissociate (i.e., revert to the monomeric state) and the conditions required to accomplish this are also important characteristics to consider. Sample preparation could affect the results obtained if the aggregates are dissociable; therefore, understanding this aspect of the aggregates is critical when selecting techniques for counting, measuring, and characterizing them. The dissociability of protein aggregates can be assessed by diluting the aggregates into the formulation buffer or other solutions and then re-analyzing them with the original technique. This can also include simply returning the sample to the original conditions and assessing how much of the aggregate has dissociated. Ability to dissociate is thus a characteristic of protein aggregates that can inform sample handling and preparation (e.g., dilution conditions) for particle counting. The ability to dissociate may also apply to intrinsic and, to a lesser extent, extrinsic particles species.

The structure and conformation of protein molecules in the aggregates can be investigated by using several biophysical techniques. The findings may help in investigations and troubleshooting. It is also useful to know about the presence of chemical modifications such as oxidation, deamidation, cross-links, or fragments in the aggregate. Conformation of protein molecules within protein aggregates, as well as their state of covalent modification, may help explain the potential biological impact of the inherent particle.

Morphology of the particles can serve as another descriptor and also help identify their source. More specifically, characterization of morphology may allow one to distinguish between particles that are inherent to the therapeutic protein product and those that are extrinsic or intrinsic. Proteinaceous as well as many intrinsic and extrinsic particles are generally of irregular shape, whereas silicone oil and air bubbles tend to be spherical. The morphology of protein aggregates also constitutes important data that can facilitate comparisons across studies and over time.

*Table 1* provides an overview of protein aggregate characteristics to consider, which can be used to classify them.

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**Table 1. Description of Categories of Protein Aggregates (*1*)**

| Category | Classification |
|---|---|
| Size | <100 nm (nanometer)<br>100–1000 nm (submicrometer)<br>1–100 µm (subvisible)<br>>100 µm (visible) |
| Dissociability | Reversible (revert to initial state when returned to original conditions)<br>Irreversible (do not dissociate under conditions tested)<br>Dissociable (dissociate under specific conditions tested, e.g., dilution, changes in buffer, etc.)<br>Dissociable under physiological conditions |
| Reversibility | Reversible<br>Irreversible<br>Dissociable<br>Dissociable under physiological conditions<br>Dissociable under defined (list) conditions |
| Secondary/tertiary structure | Native<br>Partially unfolded<br>Unfolded<br>Inherently disordered<br>Ordered (e.g., amyloid) |
| Covalent modification | Cross-linked (reducible and nonreducible)<br>Intramolecular modification<br>Oxidation<br>Deamidation<br>Fragmentation<br>No modification |
| Morphology | Number of monomeric subunits<br>Aspect ratio<br>Surface roughness<br>Internal morphology<br>Optical properties<br>Heterogeneous (e.g., protein–silicone oil) |

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### Silicone Oil

A standard lubricant in pharmaceutical products and packaging. In excess, it may contribute to elevated particle counts in products. Assessing the effect of silicone oil on particle counts in therapeutic protein injections, where relevant, is critical to the overall particle control strategy. In parenteral manufacturing, silicone oil is used primarily to facilitate packaging component handling, alleviate adherence of rubber parts, allow free stopper flow in the bowl/hopper, and promote tracking onto the waiting, filled container. Silicone oil is also used as a lubricant to decrease glide force and allow plunger stopper movement in glass, pre-filled syringe presentations. For parenteral container preparation, controlled stages of washing, rinsing, siliconization, and sterilization are routine. The tumbling of closures (e.g., vial stoppers) with a measured amount of silicone has been a routine process for uniformly coating surfaces before use. Application of a known amount of an accepted lubricant, rendered sterile, is common.

Even when applied judiciously, silicone oil can migrate from the product-contact surface to the fill-over time during product transport, storage, and use. Silicone oil droplets can contribute significantly to the subvisible particle counts of the product, specifically, particles in the <10-µm range in the case of therapeutic protein injections. The particle count depends on the amount of silicone oil, and in some cases small amounts of silicone oil can have a significant effect on particle counts (*2*).

Within therapeutic protein products, protein may become adsorbed to silicone oil droplets. Agitation resuspends and rearranges the oily matrix into different particle forms and sizes. The size range of the silicone oil droplets in protein formulations may be similar to inherent protein particles, but the morphology and optical properties of the two substances differ. Silicone oil in aqueous solution is typically spherical, with a higher refractive index than that of protein aggregates and a regular decrease in contrast from the outside to the center of the particle. This is unlike protein aggregates, which are typically amorphous and translucent and have a refractive index very similar to the protein monomer background of the drug formulation. These distinctions can be used to develop algorithms for differentiating silicone oil particles from protein particles. It is also possible to monitor changes in one population and distinguish them from changes in the other.

Because silicone oil serves a function in the product, particle counts arising from the oil are considered intrinsic and generally will vary over time. However, poorly controlled application processes can lead to excessive amounts of silicone oil. Careful design, control, and application of the silicone oil are recommended to obtain proper functionality with the minimum amount needed for the shelf life of the product.

## PARTICLE STANDARDS

Analysis of particle size distribution depends on the instrument used and its method of calibration. Some available particle standards (count and size) are polystyrene latex beads, mono-disperse silica or polymethylmethacrylate (PMMA) beads, and polydisperse glass beads. All of these standards are spheres, with a refractive index and density that are distinctly different from those of therapeutic protein products. Because typical protein particles have irregular morphology and low optical contrast, there are currently no standards available to consistently mimic the morphology and parameters of protein particles.

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Protein particles have a refractive index that is quite close to that of the matrix solution. This difference in refractive index is a critical parameter for detection of protein particles by methods that rely on light interaction to measure size distribution. It is important to establish standard reference materials that mimic the properties of protein particles (*3*). The development of standards that better resemble protein particles is currently an active area of research. These standards should serve as surrogates for the actual protein particles in that the surrogate's properties mimic those of actual proteins for the detection method of choice. This approach is necessary because standards using proteins are limited by the following factors: (a) storage and transport should be at temperatures close to –80° to achieve acceptable stability; (b) any non-ambient technique may affect the standard; and (c) protein particles are themselves quite variable, making it difficult to have a single protein that would match all applications.

## SUBVISIBLE PARTICLE MEASUREMENT AND CHARACTERIZATION TECHNOLOGIES

The following sections review the advantages, limitations, and appropriate uses of each methodology (see *Table 2*) that allow assessment of a variety of characteristics of all particles, especially for the inherent protein aggregates, including size and distribution, size and morphology, and characterization. The methodology selected will affect reported particle size because of instrumental measurement principles, mode of measurement, particle characteristics, preparation, and handling. It can also be affected by the algorithms applied to estimate size, volume, and count. Thus with any size measurement, the method used should be specified. Also, the units should be clearly stated, because size can be provided using various parameters, usually hydrodynamic diameter, equivalent circular diameter (ECD), longest chord, equivalent spherical diameter (ESC), Feret's diameter, or molecular weight. The list in *Table 2* is not meant to be all inclusive.

**Table 2. Methodologies Useful in Measuring Properties of Subvisible Particles**

| Section I: Size and Distribution | | |
|---|---|---|
| **Technique** | **Principle of Operation** | **Range** |
| Light obscuration | The size of the particle in the product fluid is determined by the amount of light that it blocks when passing between the source and the detector. | 1–300 µm |
| Electrical sensing zone (Coulter) | The size of the particle in the product fluid or selected electrolyte is measured in terms of the change in resistance as the particle passes through a microchannel (orifice). | 0.4–1600 µm |
| Laser diffraction | The size of the particles in product fluid or dilution is determined by measuring the angle of the scattered light. | 0.1–3500 µm |
| | | |
| **Section II: Size and Morphology** | | |
| **Technique** | **Principle of Operation** | **Range** |
| Light microscopy | Photon imaging of substances directly in product fluids or mounts, or of isolated specimens on substrates | 0.3 µm to 1 mm |
| Flow imaging analysis | Digital image capture of the particles' magnified image in streaming product fluid, revealing size, shape, and optical properties | 0.7–100 µm for size distribution; 4–100 µm for morphology |
| Electron microscopy (EM): Scanning EM, scanning transmission EM, and transmission EM | Electron imaging of specimen isolates on substrates. High vacuum or near-ambient pressures is required. | Å to mm |
| | | |
| **Section III: Characterization** | | |
| **Technique** | **Principle of Operation** | **Range** |
| Fourier Transform Infrared (FTIR) microspectroscopy | Photon imaging of isolated specimens on substrates | 10 µm to 1 mm |
| Dispersive-Raman microspectroscopy | Photon imaging of isolated specimens on substrates, or in product fluids or fluid mounts | 0.5 µm to 1 mm |
| Electron microscopy (EM) with energy-dispersive X-ray spectrometry (EDS) | X-ray photon emission from specimens energized by a focused electron beam | Å to mm for imaging; 1 µm to 1 mm for elemental composition |
| Electron microscopy (EM) with electron energy loss spectroscopy (EELS) | Inelastic scattering from specimens energized by a focused e-beam; e-loss is characteristic of the source element; complementary to EDS | Å to mm for imaging; 0.5 µm to 1 mm for elemental composition |
| Time-of-Flight Secondary Ion Mass Spectrometer (TOF-SIMS) | Identification of particles according to their mass spectra profile | µm to near mm |
| Staining assay | Visible staining to gain qualitative confirmation of unknown materials | 0.3 µm to 1 mm |

The accuracy of equipment used to determine particle size distribution is verified by calibration with reference particles of known concentration and size traceable to the International System of Units.

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The concept of three-dimensional particle size is critical to understanding how particle standards interact with the various types of measurement equipment and how to compare the results across methodologies. Different techniques use different measurements and algorithms to determine size (e.g., volume equivalent diameter, surface equivalent diameter, and drag or Stokes diameter).

***Change to read:***

## SIZE AND COUNT DISTRIBUTION

### Light Obscuration (working range 1–300 µm)

PRINCIPLE OF OPERATION

The sample is passed between a light source and a sensor. Counts are generated when individual particles pass between the two, disrupting the light, which yields a voltage spike. The height of each voltage spike depends on the size of the particle causing it. The particle size to be recorded is generated from a size–voltage response calibration curve constructed using monosphere, certified reference size standards (typically polystyrene beads). LO tabulates particle size as the diameter of a circle having an equivalent cross-section. The product may be sampled directly from the container, pooled from several containers, or prepared as a dilution (see general chapters *Globule Size Distribution in Lipid Injectable Emulsions* ⟨729⟩, ⟨788⟩, *Particulate Matter in Ophthalmic Solutions* ⟨789⟩, and ▲*Methods for the Determination of Subvisible Particulate Matter* ⟨1788⟩▲ (CN 1-May-2021)).

ADVANTAGES

- Primary *USP–NF* method with large archive of historical data
- Particles directly observed in solution
- Measurements are straightforward and quick
- Capable of unit–unit sampling; analyzes almost entire sample
- Easily implemented
- Medium-to-high sample throughput.

LIMITATIONS

- One sensor head cannot cover the full working range
- Tabulates particle size as the diameter of a circle having an equivalent cross-section
- Dilution of the sample may be required and may change sample properties
- Upper limits for particle load that can be measured depend on sensor used and potential for coincidence counting
- May underestimate subvisible protein particles formed in formulation because of low contrast
- Medium should be transparent for laser light
- Sensitive to air bubbles, and some degassing techniques can change sample properties
- Destructive, i.e., sample cannot be reused.

### Electrical Sensing Zone (Coulter Principle) (working range 0.4–1600 µm)

PRINCIPLE OF OPERATION

The sample is diluted into an electrolyte solution and drawn through a small aperture, passing between active electrodes, interrupting an electric field. Response is based on the displacement volume of the electrolyte, and thus size is reported as the equivalent spherical diameter (the diameter of a calibration standard sphere of the same volume as the particle). The response is unaffected by particle type (e.g., variations in color, hardness, opacity, refractive index).

ADVANTAGES

- Determines particle size in terms of equivalent spherical volume
- Not affected by optical properties of the solution
- Orthogonal technique to light-based methods, i.e., LO
- Medium-to-high sample throughput for a single orifice.

LIMITATIONS

- Low particle concentration required
- Each orifice has limited measuring range (2%–60% of orifice diameter)
- Near the lower end of the working range, electrical noise may be hard to distinguish from true particle counts
- For broad particle size distribution, more than one orifice may be necessary, which will involve reduced sample throughput
- Sample must be diluted in electrolyte solution if the formulation buffer does not have sufficient conductivity; the dilution may alter sample properties

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- Smaller orifices require higher conductivity of medium and can require significantly increased buffer concentration
- Destructive, i.e., sample cannot be reused.

### Laser Diffraction (working range 0.1–3500 µm)

PRINCIPLE OF OPERATION

Laser diffraction detects particles passing through a laser beam as they scatter light at an angular distribution of intensity that depends on the size of the particles. Therefore, it is possible to calculate particle size distributions if the intensity of light scattered from a sample is measured as a function of angle. This angular information needs to be compared with a scattering model (Mie theory) to calculate the size distribution.

ADVANTAGES

- Brief analysis time (up to 30 s)
- Analytical samples can be retrieved
- Limited sample preparation
- Scattering pattern allows molecular mass calculation
- High sample throughput
- Large dynamic range.

LIMITATIONS

- Intensity is directly proportional to size, which can lead to misinterpretation of the population
- Analysis relies on a theoretical fit that requires refractive index and other optical factors
- Medium should be transparent for laser light
- Assumes particles are spherical and not interacting with each other
- Low concentration of particles required; dilution may be necessary and may change sample properties
- Complications can occur when comparing data from one instrument vendor to another
- Particle–particle interactions and occasionally multiple scattering may yield apparent size results that differ significantly from actual particle size
- Poor results for polydispersions when small and large particles are presented simultaneously
- For exact molecular weight calculation, monodisperse particles are required.

## SIZE AND MORPHOLOGY

### Light Microscopy (LM) (working range 0.3 µm to 1 mm)

PRINCIPLE OF OPERATION

Optical or light microscopy involves passing light through a series of lenses after it is transmitted through or reflected from the prepared sample. The light is detected directly by the eye, imaged on a photographic plate, or captured digitally. Samples can be analyzed directly or after filter capture. Light microscopy can be combined with static imaging analysis, thereby allowing digital images to be captured and further deconvolved using the software systems in image analysis. When using image analysis, one may select certain parameters to screen out unwanted artifacts or particle populations (circularity, aspect ratio, and others) and then begin studying the selected population set (see *Optical Microscopy* ⟨776⟩, ⟨788⟩, ⟨789⟩, and ⟨1788⟩).

ADVANTAGES

- Direct observation of particles in product fluid and filter capture
- Visualization, counting, sizing, and morphological characterization of particles are readily obtained
- Direct observation of particle may provide immediate recognition and identification
- Can be coupled with spectroscopic analyses (infrared, Raman) to identify chemical composition
- Particle identification can be enhanced via image database search and comparison
- Only small sample volume is needed for direct observation
- Membrane filtration is secondary to the ⟨788⟩ LO method.

LIMITATIONS

- Time consuming
- Low sample throughput
- Limited depth of field at high magnification can affect sizing accuracy
- Only a small sample volume is analyzed, so sampling must be done carefully
- For filter-captured samples, protein particles can pass through membranes and may present as amorphous background

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- For filter-captured samples, potential exists for sample preparation artifacts
- Difficult to visualize particles with low optical contrast; must use phase, differential interference contrast microscopy, polarized light microscopy, and other methods
- Destructive, i.e., sample cannot be reused for filter-captured samples.

## Flow Imaging Analysis [working range 0.7–100 µm (size) and 4–100 µm (morphology)]

### PRINCIPLE OF OPERATION

This method captures digital images of materials within a flowing sample stream and uses post-collection analyses of the stored images. As in optical microscopy, which is essentially a static image analysis system, dynamic image analysis or flow microscopy captures multiple separate images, in this case using a dynamic, flowing system. Dynamic image analysis uses the components of illumination, objective lens, and focusing lens of a microscope, and adds a closed fluid pathway within a defined dimensional flow cell, plus camera and processor for acquisition and analysis. Thus, the method provides in situ conditions with realistic views of the particles. Visualization of the particles of gas, liquid, semisolids, or solids with this technique aids the interpretation of other in-suspension measurements that cannot visualize the particles being studied. The particles are moving and are likely tumbling, and this must be accounted for in the system design or data consideration. Increasing the system magnification and pixel resolution and reducing the flow cell depth will augment the quality of the captured image. An additional advantage of dynamic flow microscopy is the ability to apply size and feature analyses to the particle image. Also, any number of additional algorithms may be applied to study the collected image set as necessary. Particle image clarity, contrast, and shape may indicate possible source or type of particle, and with sufficient comparator data, mathematical filters can be applied to analyze and quantify different populations in a sample. This approach has been used to help distinguish silicone oil from protein aggregates.

### ADVANTAGES

- Direct observation of particles in product fluid (in situ)
- Analysis of captured digital images allows visualization, counting, sizing, and characterization of particles
- Retained images may be further analyzed by different algorithms
- Actual particle images facilitate understanding of contamination sources and product nature
- Compared to LO or membrane microscopy (MM), increased sample volume and reduced analysis time
- Morphology and relative refractive index of particles apparent, easily calibrated
- Technique can be used with image databases to identify some particles
- High repeatability, high resolution, and good selectivity
- Medium-to-high sample throughput.

### LIMITATIONS

- Minimum particle size necessary
- Results dependent on algorithm used to select, classify, and size particles
- Difficult to visualize and appropriately quantify number and size of particles with low optical contrast, such as translucent proteins
- Dilution of sample may be required and may change sample properties
- Destructive, i.e., sample cannot be reused.

## Electron Microscopy (working range Angstroms to mm)

### PRINCIPLE OF OPERATION

In EM, a beam of electrons is used to illuminate the samples and produce a magnified image. The practical spatial resolution limit of EM for biological samples is approximately 1 nm, which greatly exceeds resolution requirements for typical >0.1-µm particles in protein solutions. For EM analysis, subvisible particles should be either individually isolated or captured on a suitable filter and then mounted onto special grids or stabs for observation in the microscope. Traditional EM operates in high vacuum (about $10^{-6}$ Torr). Sample drying and coating is a required preparation step. Newer technologies allow examination of uncoated, as-is samples (environmental or wet EM). Detection techniques are divided as follows: (a) scanning electron microscopy (SEM), which may use secondary electron or back-scattering signals; (b) transmission electron microscopy (TEM), which visualizes electrons penetrated through sample; and (c) scanning tunneling electron microscopy (STEM), which combine SEM and TEM principles (see also *Scanning Electron Microscopy* ⟨1181⟩).

### ADVANTAGES

- Visualization, counting, sizing, and morphological characterization of particles are readily obtained
- Direct observation of particle may provide immediate recognition and identification
- High repeatability, high resolution, and good selectivity
- Information about chemical composition of object can be obtained using energy-dispersive spectroscopy (EDS) or electron energy loss spectroscopy (EELS) (see below)

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- Enhanced depth of field compared with photon methods
- Image analysis and building permits quasi-3D imaging of particles.

LIMITATIONS

- Time-consuming preparation and analysis
- Low sample throughput
- Possible changes in structure and size may arise from sample preparation and high-vacuum operation
- Specialized sample preparation requirements may exist, depending on the nature of the sample
- Contrast staining with heavy metals salts may be required for enhancing protein samples
- Protein particles are flexible and can pass through membranes
- Destructive, i.e., sample cannot be reused.

## CHARACTERIZATION

Characterization of the protein active ingredient includes not only size distribution assessment but also determination of the chemical nature of the primary protein as well as conformation, aggregation, and self-associated species (see *Table 1*). The size of the fundamental particle–aggregate is important; however, the nature of the solids and the association with other species are similarly important.

### Fourier-Transform Infrared (FTIR) Microspectroscopy (working range 10 µm to 1 mm or greater)

PRINCIPLE OF OPERATION

When light interacts with a substance, the light can be reflected, absorbed, or scattered. Infrared spectroscopy determines the vibrational properties of matter, typically in the 200–4000 $cm^{-1}$ range, by analyzing the interaction with light while detecting the absorption of photons. In addition, the use of microscopy-based instruments, thus microspectroscopy, allows selection of individual particles or domains of interest, with the benefit of filters and polarizers for microscopes and the ability to capture an image, along with vibrational or other imaging data. Vibrational spectroscopy techniques are particularly useful for analysis of particles in protein solutions.

Particles can be isolated individually or captured on a membrane filter and then analyzed using FTIR microscopy. Because the infrared signal from water interferes strongly with protein bands, these samples usually require thorough drying before analysis. Typically, FTIR microscopes can operate in either image mode or spot mode. In image mode, the infrared signals are collected from individual pixels in the pre-defined area. However, spot mode usually provides higher signal-to-noise ratio, because the combined infrared signal is collected from the area defined by the aperture size. Many organic compounds have unique infrared signatures. Spectra collected for an individual particle can be analyzed by performing an infrared spectra library search or by comparing the particle's spectra results with those of suspected materials.

ADVANTAGES

- Microspectroscopy has the same sample advantages as LM
- FT instruments excel compared with dispersive spectrometers (grating monochromator) for particle analysis
- Provides rapid chemical identification and classification of particles, manually or automatically, in comparison with spectral databases
- Identification, structure, and composition can be derived from spectral data
- Provides some information on protein structure
- Nondestructive; samples can be reused.

LIMITATIONS

- May have the same limitations as LM
- FTIR provides strong absorption for common materials, such as silicones, water, and glass, which may interfere with or hinder sample analysis
- Identification depends on availability of information on possible components in spectral libraries
- Particle size limit is dependent on instrument resolution (~10 µm for FTIR)
- Process of particle identification becomes time consuming, if not available in a database
- FTIR is not sensitive to many inorganic materials or other molecules that do not possess a dipole moment
- Certain detectors are not sensitive to inorganic materials.

### Dispersive-Raman Microspectroscopy (working range 0.5 µm and greater)

PRINCIPLE OF OPERATION

As with infrared spectroscopy, Raman spectroscopy is the study of how light interacts with a substance. A defined, monochromatic photon source (laser) is focused on the sample, thereby producing reflectance, absorption, and scatter, probing

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the same vibrational states probed by infrared microspectroscopy. Specifically, Raman spectroscopy is the study of the inelastically scattered photons (Raman scattering). Photons are also scattered elastically (Rayleigh scattered) with no change in wavelength, but are of use only to mark the laser excitation energy. A virtual excitation state is attained, which then relaxes to a base vibrational or rotational state, emitting energy characteristic of a functional group such as molecular and related atoms. Lower (Stokes) and higher (anti-Stokes) shifts are recorded due to interaction with the electron cloud of the functional group bonds. Lasers are used because only a very small proportion of Raman-scattered photons (only about 1 in $10^6$–$10^8$) exhibit wavelength shift. Raman microspectroscopy combines a light microscope with a coincident laser and white light pathway. The microscope, with the benefit of filters and polarizers, aids in selection of individual particles or domains of interest to allow capturing of an image, along with vibrational spectra.

Fourier-Transform Raman is generally complementary to dispersive Raman, and in using longer wavelength excitation yields little sample fluorescence. One important advantage of Raman microscopy compared with infrared is the ability to analyze aqueous samples directly, often in glass sample holders. Individual particles can be analyzed in situ, and in some cases directly in the container, if it does not interfere with the Raman signal. Similar to the infrared analysis, unknown spectra can be analyzed either by a spectral library search or by matching the spectra of individual reference compounds.

Raman spectroscopy theory and techniques are further described in *Raman Spectroscopy—Theory and Practice* ⟨1858⟩.

#### ADVANTAGES

- Same sample advantages as LM
- Dispersive instruments excel over Fourier-Transform spectrometers for particle analysis
- Rapid chemical identification and classification of particles, manually or automatically by comparison with spectral databases
- Identification, structure, and composition can be derived from spectral data
- Provides some information on protein structure
- Good lower size limit; instrument resolution is ~0.5 µm for dispersive-Raman technique
- Raman analysis viable for many organic and most inorganic compounds
- Identification uses Raman shift data, known for molecular categories and functional groups
- Nondestructive; samples can be reused
- Glass and plastic packaging have weak Raman spectra, thus interference from packaging is minimized.

#### LIMITATIONS

- Same limitations as with LM
- Specimen fluorescence may hinder data collection
- With sensitive samples, there can be laser-induced changes (heat, light)
- Many organic species yield no Raman shift; that is, there is no molecular polarization
- Identification may depend on the information available in proprietary and public spectral libraries.

### Electron Microscopy (EM) with Energy-Dispersive X-Ray Spectrometry (EDS or EDX) (working range for qualitative analysis, 0.1- to 3-µm particles; for semiquantitative analysis, >3-µm particles)

#### PRINCIPLE OF OPERATION

Energy-dispersive X-ray spectrometry (EDS or EDX) is based on characteristic X-ray photon emission generated from a specimen through interaction with a high-energy source, such as an electron beam. Typically, an elemental intensity is plotted against X-ray emission energy, which correlates with the content of atomic valence shell transition energies. Current instrument technology uses thin-window and windowless detectors positioned near the sample in EM systems, which improves detection and signal-to-noise ratio for all elements, especially lighter elements, e.g., $Z < 11$ (sodium). Immediate, qualitative elemental composition is attained for solid specimens (see also ⟨1181⟩ for general EM and related EDS background information).

#### ADVANTAGES

- Complementary to EM and available for most commercial electron microscopes
- Determines elemental composition of the sample
  - Rapid qualitative collection for small particles
  - Semiquantitative analysis (1% LDL and ±20% RSD) possible in controlled experiments
- Most useful during survey investigation of particle origin
- Involves manual elemental screening and/or search of databases
- Elemental mapping can assist in protein particle visualization.

#### LIMITATIONS

- Not all instruments are equipped for determination of light elements (e.g., $Z < 11$)

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- Quantitative analysis entails measuring line intensities for each element in the sample and for the same elements in calibration standards of known composition
- Strong background may be contributed by filter membrane material and sample coating.

## Electron Microscopy (EM) with Electron Energy Loss Spectroscopy (EELS) (working range 0.5 µm to 1 mm)

OPERATION PRINCIPLE

Electron energy loss spectroscopy (EELS) measures the vibrational motion of atoms and molecules on and near the surface by analyzing the energy spectrum of low-energy electrons back-scattered from it.

ADVANTAGES

- Used in conjunction with transmission electron microscopy
- Determines elemental composition of the sample, especially light elements (e.g., $Z < 11$)
- Can provide information about chemical bonding
- Qualitative and quantitative analyses are possible
- Has lower detection limits than in EDS.

LIMITATIONS

- Difficult to interpret
- Strong background
- Quantitative analysis entails measuring line intensities for each element in the sample and for the same elements in calibration standards of known composition.

## Time-of-Flight Secondary Ion Mass Spectrometer (TOF-SIMS) (working range µm to near mm)

OPERATION PRINCIPLE

Time-of-Flight Secondary Ion Mass Spectrometer (TOF-SIMS) is a surface-sensitive analytical method that uses a pulsed ion beam [microfocused cesium (Cs) or gallium (Ga)] to remove molecules from the very outermost surface of the sample. The particles are removed from atomic monolayers on the surface (secondary ions). These particles are then accelerated into a "flight tube", and their mass is determined by measuring the exact time at which they reach the detector (i.e., time-of-flight).

ADVANTAGES

- Surveys of all masses on material surfaces; these may include single ions (positive or negative), individual isotopes, and molecular compounds
- Elemental and chemical mapping on a submicron scale
- High mass resolution, to distinguish species of similar nominal mass
- High sensitivity for trace elements or compounds, on the order of ppm to ppb for most species
- Surface analysis of insulating and conducting samples
- Depth profiling (in the near surface environment, on the order of individual atomic layers to 10 s of nanometers)
- Nondestructive analysis
- Retrospective analysis, for post-data acquisition analysis and interpretation of stored images and spectra

LIMITATIONS

- Generally does not produce quantitative analyses (semi-quantitative at best)
- Optical capabilities typically limited, making it difficult to find grains or specific regions of interest for analysis
- Charging may be a problem in some samples, although charge compensation routines are generally sufficient to overcome these problems
- There is commonly an image shift when changing from positive to negative ion data collection mode, making it difficult to collect positive and negative ion data on exactly the same spot.

## Staining Assay (working range 0.3 µm to 1 mm)

OPERATION PRINCIPLE

Staining methods utilize reagents to provide specific reactions, such as visible staining or fluorescence, which provide qualitative confirmation of an unknown materials category or identity. Generally conducted via microscopical methods; microscopy advantages and limitations apply.

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ADVANTAGES

- Direct observation of staining effects upon particles in product fluid and by filter capture
- Visualization, counting, sizing, and morphological characterization of particles are readily obtained
- Direct observation of particle may provide immediate recognition and identification
- Can be coupled with spectroscopic analyses (infrared, Raman) to identify chemical composition
- Surface analysis of insulating and conducting samples
- Particle identification can be enhanced via image database search and comparison
- Only small sample volume is needed for direct observation

LIMITATIONS

- Low sample throughput
- Limited depth of field at high magnification
- Only a small sample volume is analyzed, so sampling must be done carefully
- For filter-captured samples, protein particles can pass through membranes and may present as amorphous background
- For filter-captured samples, potential exists for sample preparation artifacts
- Difficult to visualize particles with low optical contrast; must use phase, differential interference contract microscopy, polarized light microscopy, and other methods
- Destructive; that is, sample cannot be reused for filter-captured samples

## STRATEGY

Particles in therapeutic protein products generally have continuous size distributions with exponentially higher numbers of the smaller (<10 µm) particles. Information generated from analysis of inherent protein particles can be used for guiding development efforts and to facilitate communication and understanding of this important quality attribute of therapeutic protein products. A rational strategy should be developed for characterizing protein aggregates or particles. The categories in *Table 1* provide a guideline for differences observed between types of protein aggregates, recognizing that size distribution analysis of these species is important, but it is not the only informative characteristic. Describing the characteristics of the protein aggregates in the manner shown in *Table 1* can help define the population and/or characteristics that may be the most important for assessment of the effect on product quality.

To obtain a comprehensive characterization of the particle distribution, a variety of analytical techniques must be applied. There are a number of techniques available to measure and characterize particles, each with its advantages and limitations. Multiple methods are needed because they differ in their ability to analyze specific attributes of the particle. By using orthogonal methods, one can better ascertain the nature of the particles (extrinsic, intrinsic, or inherent), the size distribution, and other aspects that may allow differentiation between particles. Information about the particle characteristics can be used to perform a risk assessment around the presence of aggregates and to help identify potential mechanisms of formation. This knowledge about the particles' characteristics can also be used during formulation and process development to inform choices of the formulation and process steps, which can help in reducing the overall particle content of the drug product.

A comprehensive strategy for assessing subvisible particles involves multiple phases from development through commercialization and during marketing. Particle measurement/characterization may begin as early as candidate selection, with the use of high-throughput techniques to predict particulate-forming tendencies of the molecules under consideration. This involves using small amounts of material and providing a relative ranking of aggregation propensity.

### Early Development

At this stage, the focus is on understanding the typical particle profile for the product, including the size distribution and the characteristics of the aggregates most commonly seen in the drug product. The particle size and count in the 1- to 100-µm range should be monitored at the time of manufacture and during stability.

### Late Development

At this stage, the focus is on understanding the drug product and comparability between lots, as well as the linkage between particle size analysis and formulation, manufacturing, and use. Particle size and count in the 1- to 100-µm range should be monitored in clinical batches. Enhanced characterization of aggregates and particles, including pivotal product batches, should be performed. Quantitative and qualitative data about the subvisible particles formed under storage, use, and stress conditions should be collected, and risk mitigation strategies created. When the final method for monitoring the product's particle size and count is chosen, control strategies for inherent and intrinsic particles should be established. The overall control strategy developed may include action limits beyond which an investigation may be warranted.

### Marketing

At this stage, one should collect data over commercial batches to align with the overall control strategy, which may include the sub-10-µm (1- to 10-µm) range. Use quantitative as well as qualitative analysis during the management changes for the

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post-marketing life cycle. When an investigation occurs, the knowledge obtained during development can be used to design mitigation strategies. Stability studies are also part of life cycle management, and are addressed later in the *Application* section.

### Overall Perspective

In-depth characterization of aggregates during development, coupled with correlation of results from these tests to those used for product release (such as LO), may provide the basis for a rational, overall control strategy. Aggregates are usually present in a continuum of size from oligomers to particles that are hundreds of micrometers. If a correlation (or mathematical relationship) can be demonstrated in the counts of particles from oligomers through the >25-µm sizes assessed as part of the compendial method, it might be possible to rely on the LO determination of the >10- and >25-µm particle content to reveal the effect of changes and trends in manufacturing on 1- to 10-µm particles. In this situation, the overall control strategy would be used to establish action limits for particles >10 µm, which when exceeded would trigger an investigation, including the characterization methods discussed in this chapter.

## SAMPLE CONSIDERATIONS

Certain aspects of sample handling and volume require special consideration. Handling and degassing procedures need to be developed and applied to avoid artifacts such as false positives attributable to bubbles, or aggregates generated during the sample preparation steps. Under certain circumstances, dilution of samples may be necessary to obtain reliable results. Aspects of sample handling are described in ⟨787⟩.

## APPLICATION

A key focus during development of a protein drug product is to gain knowledge about the nature, source, and number of inherent particles/aggregates present in the therapeutic formula, as well as their stability and overall effects on product quality, safety, and efficacy.

Protein aggregates in the 1- to 10-µm size range may also carry a risk of potentiating immunogenicity against the therapeutic activity (*4,5*). From a product-development perspective, changes in the concentrations of proteinaceous subvisible particles, particularly in the 1- to 10-µm range, can be an early and sensitive indicator of potential issues with product stability (*6,7*). Apart from stability, particles in these size ranges should also be measured as part of the risk assessment exercise to improve understanding of how various stressors and conditions of use affect the product. These conditions could include thermal, freeze/thaw, light, transport, dilution/admixture, and use characteristics. Particle counts in the 1- to 10-µm range tend to be significantly (orders of magnitude) greater than those in the ≥10-µm range. This makes the 1- to 10-µm range useful for the purpose described above, because changes can be easier to observe. However, the counts also tend to be quite variable, and thus the ability to discern trends requires proper controls, adequate sampling, and good technique.

Detailed characterization of subvisible particles, especially inherent particles, provides information that can support the formulation and product development program, leading to a robust process and commercial control strategy. On the other hand, although characterization alone may not reveal possible correlations between an immune response and aggregates/particles, the use of multiple methods applied to multiple drug product lots helps inform the definition of product quality, which can be linked to safety and efficacy data obtained from clinical trials. This knowledge is also useful in life cycle management, such as movement of product between manufacturing sites, changes in presentation (liquid to lyophilized; vial to prefilled syringe), and changes in strength.

To illustrate these points, two examples are presented below; they show ways that complementary techniques can be used to gain product knowledge.

### Example 1: Comparison of Drug Product Configurations

Not all drug products are formulated as liquids. In one case of a lyophilized drug product filled in glass vials, a liquid formulation in a prefilled syringe configuration was developed to facilitate patient administration. A comparison of subvisible particle counts by LO was expected to show that the particle count of the reconstituted lyophilized formulation was higher than in the liquid formulation, but the opposite results were observed, with the liquid formulation containing significantly higher levels of particles >2 µm [~130,000 particles per container (ppc) compared to ~30,000 ppc]. These samples were then assessed by the MM method, and the results were more consistent with the expected outcome: for particle sizes >5 µm, the lyophilized formulation contained ~70 ppc compared to ~14 ppc for the liquid formulation. Analysis was then performed using flow microscopy to understand the discrepancy in results between the MM and LO methods. Flow microscopy supported the LO results, with the liquid formulation showing higher numbers of particles >2 µm (~600,000 ppc) compared to the lyophilized formulation (~150,000 ppc). Examination of the structure of the particles revealed a different morphology in the liquid formulation, with a predominant number of the particles exhibiting a spherical shape, consistent with the presence of silicone oil droplets from the prefilled syringe container. These results were confirmed by testing a sample of the liquid formulation that had not been exposed to the prefilled syringe (the source of the silicone oil), which showed <20,000 ppc. Once the silicone oil droplets were accounted for, the number of remaining particles in the liquid formulation in the prefilled syringe was lower than in the lyophilized formulation, consistent with the MM results.

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## Example 2: Selection of Filling Technology

Filling processes can affect product quality by introducing physical stresses such as adsorption, shear, friction, and cavitation, which may lead to protein aggregation. Certain drug product filling pumps may shed extrinsic particles that can lead to heterogeneous nucleation-induced aggregation.

The effect of using a stainless steel displacement piston pump on the subvisible particulate content of solutions was investigated using multiple techniques to analyze the particulates in solution (*8*). Coulter counter analysis of a buffer solution passed through the pump revealed that the pump shed particles in solution, with the pumped solution containing >13,000 particles per mL in the 1.5- to 6-µm range. Elemental analysis confirmed that the particles were stainless steel. Analysis of the particle size distribution in this solution, performed using a laser diffraction particle sizer, showed that most of the particles were between 0.25 and 0.95 µm. Incubation of stainless steel nanoparticles with a solution of MAb-Y led to increases in both the number of particles and the particle size distribution over time. Characterization of the particles by FTIR spectroscopy showed that the protein adsorbed onto the metal particles contained a slightly perturbed secondary structure compared to the protein in solution. It was concluded that the metal particles served as nucleation sites for protein aggregation. In this case, selection of a different type of filler may be warranted to mitigate protein aggregation.

A comparison of the effect of different pump types on subvisible particulate formation in a solution of MAb-Z was performed (*9*). Flow microscopy showed that piston pumps, made of either stainless steel or ceramic, produced particles in the size range of 1–100 µm at levels approximately 100-fold higher than the control samples. Examination of the microscopic images of these particles revealed that the particles were translucent, suggesting they were protein based. Incubation of the protein solution with shed particles from the piston pumps did not lead to a significant increase in either the particle size distribution or particle counts for these solutions, indicating that extrinsic particles did not serve as nucleation sites for the protein. In this case, the formation of particles in the solution was considered to be from shear stress caused during the pumping operation. These results were supported by examination of other filler types (peristaltic, time-pressure, and rolling diaphragm), which produced solutions with significantly lower particle load.

## SUMMARY

This chapter provides strategies for describing, characterizing, and identifying the particle content in therapeutic protein injections, including extrinsic and intrinsic particle population. Information regarding specific methods that can be used for these purposes, as well as their advantages and limitations, is discussed. A rational, comprehensive control strategy can be developed on the basis of an in-depth characterization of aggregates during development, coupled with correlation of results from these tests to those used for release. Aggregates are usually present in a continuum of sizes, from oligomers to particles that are hundreds of micrometers. Correlation in the counts of particles from oligomers through the >25-µm size, assessed as part of the compendial method, may make it possible to rely on the LO determination alone to reveal changes and trends in manufacturing. In this situation, the overall control strategy would be used to establish action limits for particles >10 µm, which when exceeded would trigger an investigation, including the characterization methods discussed in this chapter.

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# ⟨1788.1⟩ LIGHT OBSCURATION METHOD FOR THE DETERMINATION OF SUBVISIBLE PARTICULATE MATTER

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## 1. INTRODUCTION

Light obscuration (LO) is useful for an approximate particle range from 1 to 300 μm, depending on the instrument and configuration (see *Measurement of Subvisible Particulate Matter in Therapeutic Protein Injections* ⟨1787⟩ and *Methods for the Determination of Subvisible Particulate Matter* ⟨1788⟩). Certain solution formulations may not be easily analyzed by LO. The LO method may encounter problems with products that do not have the clarity and viscosity approximating that of water. Further, formulation characteristics such as color and high viscosity, or inherent formulation properties such as shear-induced changes, may generate erroneous LO data. Similarly, products that produce air or gas bubbles when drawn into the LO sensor, such as bicarbonate-buffered formulations, may generate erroneous data. For these product types, the membrane microscope (MM) method may be more appropriate.

LO is based on light blockage/light extinction on a photodiode detector induced by particles passing a flow cell illuminated by a laser beam. The degree of obscuration is converted into electronic pulses, which can be related back to the equivalent circular diameter of the particles, based on a calibration curve generated with polystyrene standard beads. Because of the nature of the detection and counting system, air bubbles and immiscible liquids may block sufficient light to be recorded along with the target suspended particles. These artifacts must be diminished through proper preparation techniques. Solutions with excessive immiscible liquids may not be amenable to LO analysis. A variety of suitable devices of this type are commercially available. It is the responsibility of those performing the test to ensure that the operating parameters of the instrumentation are appropriate for the required accuracy and precision of the test results, and that the artifacts and interferences inherent in certain products and with certain methods of preparation are eliminated or accommodated.

## 2. PRINCIPLES OF OPERATION

The apparatus is a liquid-borne particle counting system that uses a LO sensor with a suitable sample-feeding device to deliver controlled aliquots of sample for analysis. Suspended particles in the sample fluid flow between a light source and sensor to obscure the transmitted light by either scattering or absorbing light. The corresponding signal reduction correlates to the area of each particle. In other words, larger particles cause a larger reduction in transmitted light, and the extent of the reduction represents the projected area of the particle passing through. LO instruments report the equivalent circular diameter of a polystyrene (PS) microsphere that attenuates the light beam by the same magnitude as the particle in the test sample. Because of the nature of the detection and counting system, air bubbles and immiscible liquids may diffract or block sufficient light and be recorded as particles. These artifacts must be diminished through proper sample preparation. Solutions with excessive immiscible liquids may not be amenable to LO analysis.

LO instruments detect, size, and count particles that are opaque or have a refractive index that is significantly different from the surrounding liquid. This includes not only ordinary (extrinsic) dirt particles but also gels, immiscible liquid droplets such as silicone oil droplets, air bubbles, and refractive index anomalies such as Schlieren effects. Semitransparent particles allow some laser light to pass through the particle, potentially resulting in the assignment of a smaller than actual size. All of the considerations for quantification, calibration, etc., discussed in ⟨1788⟩, *Verification of Compendial Procedures* ⟨1226⟩, *Particulate Matter in Injections* ⟨788⟩, *Subvisible Particulate Matter in Therapeutic Protein Injections* ⟨787⟩, and ⟨1787⟩ apply here. When performing the LO test on new drug products that have not been tested using this method, orthogonal methods should be

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considered as well. Ideally, subvisible particle analysis should be included during product development such that product history can be used to inform analysis and data interpretation or the product's release and commercial use.

A variety of suitable LO devices are commercially available. It is the responsibility of those performing the test to ensure that 1) the operating parameters of the instrumentation are appropriate to the required accuracy and precision of the test result and 2) the artifacts and interferences inherent in certain products and with certain methods of preparation are eliminated or accommodated. Because LO does not distinguish between air bubbles and particles, sample handling is critical (see ⟨1788⟩ and ⟨1787⟩). Adequate training must be provided for individuals responsible for the technical performance of the test.

It is important to note that for pharmacopeial applications, the ultimate goal is that the particle counter can reproducibly size and count particles present in the material under investigation. The instruments available range from those systems where calibration and other components of standardization must be carried out by manual procedures to more sophisticated systems that incorporate hardware- and software-based functions for the standardization procedures. Thus, it is not possible to specify exact methods to be followed for standardization of the instrument, and it is necessary to emphasize the required result of a standardization procedure rather than a specific method for obtaining this result. This section is intended to emphasize the criteria that must be met by a system rather than specific methods to be used. It is the responsibility of the user to apply the various methods of standardization applicable to a specific instrument. Critical operational criteria include the following two considerations.

### 2.1 Sensor Concentration Limits

Use an instrument that has a concentration limit (the maximum number of particles per milliliter) identified by the manufacturer that is greater than the concentration of particles in the test specimen to be counted. The vendor-certified concentration limit for a sensor is specified as the count level at which the coincidence error caused by the simultaneous presence of two or more particles in the sensor view volume comprises less than 10% of the counts collected for 10-µm particles. The coincidence error at sizes below 10 µm also should be known or evaluated.

### 2.2 Sensor Dynamic Range

The dynamic range of the instrument used (range of sizes of particles that can be accurately sized and counted) must include the smallest particle size to be enumerated in the products.

## 3. INSTRUMENT STANDARDIZATION TESTS

Because different brands of instruments may be used in the test, the user is responsible for ensuring that the counter used is always operated according to the manufacturer's specific instructions. For LO, the instrument standardization tests described in this section are consistent with installation qualification (IQ) and operational qualification (OQ) principles described in *Analytical Instrument Qualification* ⟨1058⟩.

The following discussion of instrument standardization emphasizes performance criteria rather than specific methods for calibrating or standardizing a given instrument system. A wide variety of approaches can be taken to achieve this, and the underlying principle and final results should be the focus. Appropriate instrument standardization (calibration and routine qualification) is essential to performance of the test according to requirements.

The following information for instrument calibration helps ensure that the sample volume accuracy, sample flow rate, particle size response curve, sensor resolution, and count accuracy are appropriate to the performance of the test. Routine performance qualification should be carried out prior to use of the instrument or with sufficient frequency to verify that the instrument provides accurate and consistent results within the manufacturer's specifications and for the intended use. The following sections focus on the instruments most commonly in use. For those older instruments without built-in routines to define particle size directly or those that do not incorporate computer-based software routines, see *Appendix*.

### 3.1 Sample Volume Accuracy

Because the particle count from a sample aliquot varies directly with the volume of fluid sampled, it is important that the sampling accuracy is known to be within a certain range. To accomplish this, transfer a volume of particle-free water that is greater than the sample volume to a container and weigh the container. Using the sample feeding device, withdraw a volume that is appropriate for the specific sampler and equal to the volume to be run during testing, and again weigh the container. Determine the sample volume by subtracting the tare volume from the combined sample plus tare volumes. Verify that the value obtained for the sample volume is within 5% of the appropriate sample volume for the test. Alternatively, the sample volume may be determined using a suitable Class A graduated cylinder (see *Volumetric Apparatus* ⟨31⟩). [NOTE—Instruments of this type require a variable tare volume. This is the amount of sample withdrawn before counting. This volume may be determined for syringe-operated samplers by setting the sample volume to zero and initiating sampling so that the only volume of solution drawn is the tare. Subtract the tare volume from the total volume of solution drawn in the sampling cycle to determine the sample volume.]

The choice of syringe has a significant effect on both the volume accuracy required to provide small volumes, as noted in ⟨787⟩, and the associated flow rate required.

### 3.2 Sample Flow Rate

Verify that the flow rate is within the manufacturer's specifications for the sensor used. This can be accomplished by using a calibrated stopwatch to measure the time required for the instrument to withdraw and count a specific sample volume (i.e.,

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the time between beginning and end of the count cycle as denoted by instrument indicator lights or other means). Sensors can be operated accurately over a range of flow rates.

## 3.3 Diameter Calibration

Refer to ⟨1788⟩ for general guidance on diameter calibration. Laser LO instruments should be calibrated using spherical PS particles with an approximate refractive index of 1.59. Diameter readings are sensitive to the particle refractive index in two cases: 1) particle diameters below approximately 5 µm and 2) particles of all diameters with a refractive index within approximately 0.2 of the refractive index of the liquid. In these cases, instrument calibration ensures that the LO instrument is functioning properly and gives reproducible results, but it does not ensure that the reported diameters match the actual physical size of the test particles. Prior to conducting the calibration procedure, it is recommended that a system suitability test be performed to determine the "as found" performance of the instrument.

Two options for conducting diameter calibration are the automated method and the electronic method. With the automated method, the calibration (size response) curve can be determined for the instrument–sensor system using validated software routines offered by instrument vendors. These routines may be included as part of the instrument software or may be used in conjunction with a microcomputer interfaced with the counter. The use of these automated methods is appropriate if the vendor supplies written certification that the software provides a response curve equivalent to that attained by the manual half-count method or the electronic method and if the automated calibration is validated as necessary by the user.

The electronic method uses a multichannel peak height analyzer to determine the center channel of the particle counter pulse response for each standard suspension. This peak voltage setting becomes the threshold used for calculation of the voltage response curve for the instrument. The standard suspensions used for the calibration are run in order, and median pulse voltages for each are determined. These thresholds are used to generate the size response curve manually or via software routines. The thresholds determined from the multichannel analyzer data are then transferred to the counter to complete the calibration. Instrument indications other than a direct voltage reading may be used to construct the response curve.

## 3.4 Sensor Size Resolution

The particle size resolution test determines the amount of peak broadening due to instrument response. Determine the resolution of the particle counter for 10-µm particles using the 10-µm calibrator spheres. The relative standard deviation of the size distribution of the standard particles used, as reported by the manufacturer, must be NMT 5%. Acceptable methods of determining particle size resolution are 1) an electronic method of measuring and sorting particle sensor voltage output with a multichannel analyzer or direct reporting of the particle size distribution and 2) automated methods. Refer to *Appendix* for details on the electronic method.

### AUTOMATED METHODS

Software that allows for the automated determination of sensor resolution is available for some counters. This software may be included in the instrument or used in conjunction with a microcomputer interfaced with the counter. The use of these automated methods is appropriate if the vendor supplies written certification that the software provides a resolution determination equivalent to the electronic method and if the automated resolution determination is validated as necessary by the user. LO resolution can degrade due to contaminated flow-cell walls; routine checks of resolution are useful since the flow-cell wall cannot be inspected otherwise.

Perform a system suitability test upon completion of the instrument calibration to ensure that diameters are accurately reported and that the count accuracy is verified using any of the three methods described in *7. Particle Counting Accuracy (System Suitability)*.

## 3.5 Qualification Considerations

### DIAMETER BIASED

For particles of nonspherical shape and low refractive index, the reported diameter may differ substantially from the actual diameter (*1*).

The reported diameter of a particle is affected most by the following:

- The refractive index of the particle
- The orientation of nonspherical particles
- The degree of particle elongation, especially for long fibers (*2*)

If the diameter is reported incorrectly, the particle size distribution will be affected and the particle concentration for affected size channels (bins) will be incorrect. Concentration errors are most prevalent for particles that have a low refractive index (e.g., protein aggregates).

### OPALESCENCE

Particle detection may be hindered in samples that are highly opalescent. To assess the visibility of particles, spike microspheres of known diameter and concentration into a test sample, measure the microsphere diameter and concentration, and then compare to the diameter and concentration expected. The microsphere chosen for this analysis should have a refractive index similar to that of the particles of interest in the test sample (e.g., silica microspheres could be used to mimic lower-contrast protein aggregates; polystyrene could be used to mimic higher-contrast particles).

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VISCOSITY

For some samples, especially highly concentrated biologics such as antibody therapeutics, the drug product can be highly viscous. This can complicate the measurement by affecting instrument performance as well as the amount of material actually sampled and measured by the instrument. This measurement process should be tested during method development. It might be necessary to dilute the sample in order to get reliable measurements. If so, the effect of dilution on the particle distribution obtained should be determined, and appropriate procedures should be used to minimize differences due to this sample handling.

## 4. SYSTEM SUITABILITY

First, measure the particle concentration of nominally particle-free water. The measured particle concentrations obtained must in all cases be sufficiently small as to be insignificant relative to any acceptance limits for the tests to be run. The actual background level obtained will be dependent on the size range, the instrument used, and the type of samples routinely run in the instrument. Records of past blank runs are useful to document the level of performance expected of particular instruments and to identify deviations from acceptable performance (or unacceptable levels of particles in the water used).

### 4.1 Mixing

For Reference Standards, such as the USP Particle Count Set RS (used for particle count accuracy, recovery studies, or calibration), homogenization of the mixture may be attained by agitating the preparation for 20 s and sonicating for NMT 30 s. Mixing of most product samples will require different handling than that for the standard. Please note that this sample handling is not appropriate for many samples themselves, especially for the biologics.

The instrument should be checked for the correct size and concentration readings using at least one size of PS microspheres; bead sizes from 5 to 15 µm are typical for this test. Size and concentration should agree within the combined uncertainty of the manufacturer's specifications and the tolerances listed on the size and concentration standard. Bead concentration standards should have a concentration three times lower than the manufacturer's stated upper concentration limit for 10% coincidence error. Size and concentration tests may be conducted with a single bead suspension, or size and concentration may be tested independently.

If reduced particle count is observed, instrument fouling is suspected. Back-flushing the sensor to remove the clog may be helpful, followed by reconfirming the count accuracy using larger PS microspheres (e.g., 25 µm). When flushing sensors to change samples or to clean the sensor, alternate liquid and air in the fluid line to achieve the best flush of the sensor.

Refer to ⟨1788⟩, *11.1 Guidelines for the Use of Calibrator Spheres* if the instrument fails a system suitability test. If failure of the standard is ruled out, then follow the manufacturer's directions for thoroughly cleaning all fluid lines and the sensor head. The instrument is deemed suitable for use if it passes the system suitability tests after cleaning. Flush the system to remove traces of the particle standard prior to routine use.

## 5. TEST ENVIRONMENT

Refer to ⟨1788⟩, *12. Test Environment and Preparation* for general guidance.

## 6. BLANK AND SAMPLE HANDLING

Refer to ⟨1788⟩, *12. Test Environment and Preparation* for general guidance.

## 7. PARTICLE COUNTING ACCURACY (SYSTEM SUITABILITY)

Determine the particle counting accuracy of the instrument using *Method 1* [for sensors requiring the moving window half-count (MWHC) method for calibration], *Method 2* (for multichannel sensors), or *Method 3* for any instrument (manual comparison to MM method). Prepare the standard as described in *6. Blank and Sample Handling*, including continuous stirring throughout the analysis. Perform five counts on 5-mL volumes of the suspension using the particle counter's 10-µm size threshold. Obtain the mean cumulative particle count per milliliter.

Note that the three methods in this section verify particle counts. Only *Method 1* verifies particle size as well as the count for MWHC instruments. *Method 2* and *Method 3* verify counts (>10 µm) but not size for instruments with fewer than 15 size channels, which means the instrument size accuracy can be off by more than 10% and still provide the correct counts (>10 µm) when using these two methods. Automated instruments with 512 or more size-channels (bins) and validated software routines provide accurate mean size and concentration for each standard peak as well as other statistical parameters for each peak. The user needs to understand the limits and capabilities of the instrument being used and proceed accordingly.

### 7.1 Method 1 (Manual Method for MWHC Instruments)

See *Appendix*.

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## 7.2 Method 2 (Multichannel Instruments)

PROCEDURE

Use one of three standards: 1) USP Particle Count Set RS (a dilution may be used if deemed necessary); 2) a commercial preparation of standard calibrator spheres with a nominal diameter of 10–30 µm, certified by the manufacturer; or 3) a laboratory-prepared suspension of standard calibrator spheres with a nominal diameter of 10–30 µm. The use of non-*USP* standards for options 2 or 3 is acceptable when they are compliant with *USP* standardization criteria: five successive counts are NMT ±10% of the stated count.

Stir continuously throughout the analysis and perform five counts on 5-mL volumes of the suspension using the particle counter's 10-µm size threshold. Stirring should be sufficiently thorough that the set of five counts does not show an obvious trend. Obtain the mean cumulative particle count per milliliter. Note that multichannel instruments provide a mean size and other statistical information as well as the particle counts within the size range selected by the user, i.e., the region that contains the peak.

INTERPRETATION

The instrument meets the requirements for *7. Particle Counting Accuracy (System Suitability)* if the count obtained at ≥10 µm conforms to the values obtained for the USP Particle Count Set RS, or the expected particle number if other acceptable standards were used. If the instrument does not meet the requirements, repeat the procedure. If the results of the second test are within the limits given above, the instrument meets the requirements of the test for *7. Particle Counting Accuracy (System Suitability)*. If the system does not meet the requirements of the test on the second attempt, identify and correct the source of the failures and retest the instrument.

## 7.3 Method 3 (Alternate Manual Method)

PROCEDURE

Prepare a suspension of standard calibrator spheres with a nominal diameter of 10–30 µm that contains 50–200 particles/mL, and determine the particle concentration as described in *Membrane Microscope Method for the Determination of Subvisible Particulate Matter* ⟨1788.2⟩. Stir continuously throughout the analysis and perform five counts on 5-mL volumes of the suspension using the particle counter's 10-µm size threshold. Stirring should be sufficiently thorough that the set of five counts does not show an obvious trend. Obtain the mean cumulative particle count per milliliter. This mean count should be within 20% of the count per milliliter for the suspension.

***Change to read:***

# APPENDIX

## Instrument Standardization Tests

These sections describe the development and use of methods to accommodate the few remaining older LO instruments still in use and for instrument manufacturers to refer to for development and verification of automated methods. In modern instruments, these methods or their equivalents have been incorporated into instrument software, and users will generally not need to execute the tests as described below.

## Diameter Calibration Methods

MANUAL METHOD FOR DEVELOPING THE CALIBRATION CURVE

In this method, the diameter indications of an LO instrument are obtained by finding the LO instrument voltage corresponding to the midpoint of the particle size distribution (PSD) for nominally monodisperse PS spheres of known size. Refer to *3.3 Diameter Calibration*, which describes the requirements for standards to be utilized in the calibration. When using the manual method, ensure that the total number of spheres counted is within the sensor's concentration limit. Prepare suspensions of the calibrator spheres in water at a concentration of 1000–5000 particles/mL, and determine the channel setting that corresponds to the highest count setting for the sphere distribution. This is determined by using the setting for the highest count threshold to split the distribution into two bins containing equal numbers of particles, with the instrument set in the differential count mode (MWHC). Use only the central portion of the distribution in this calculation to avoid including asymmetrical portions of the peak.

The portion of the distribution that must be divided equally is the count window. The window is bound by threshold settings that will define a threshold voltage window of ±20% around the mean diameter of the test spheres. The window is intended to include all single spheres, taking into account the standard deviation of the spheres and the sensor resolution while excluding noise and aggregates of spheres. The value of 20% was chosen on the basis of the worst-case sensor resolution of 10% and the worst-case standard deviation of the spheres of 10%. Because the thresholds are proportional to the cross-sectional area of the spheres (and all particles) rather than the diameter, the lower and upper voltage settings are determined by the following equations:

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$$V_L = 0.64\ V_S$$

in which $V_L$ is the lower voltage setting and $V_S$ is the voltage at the peak center, and

$$V_U = 1.44\ V_S$$

in which $V_U$ is the upper voltage setting. Once the center peak thresholds are determined, use these thresholds for the standards to create a regression of log voltage versus log particle size from which the instrument settings for the 10- and 25-µm sizes can be determined.

### SENSOR SIZE-RESOLUTION METHODS

The test for sensor size resolution calculates the standard deviation of reported sizes from the measured PSD for monodisperse PS spheres. The instrument resolution is obtained by correcting the observed standard deviation of the reported sizes for the standard deviation of the PS sphere size standard, as reported by the manufacturer.

**Manual method:** The manual method applies to instruments with size bins that have adjustable voltage thresholds. When using the manual method, adjust the particle counter to operate in the cumulative mode or total count mode. Refer to the calibration curve obtained earlier, and determine the threshold voltage for the 10-µm spheres. Adjust three channels of the counter to be used in the calibration procedure as follows:

- *Channel 1*—set for 90% of the threshold voltage
- *Channel 2*—set for 100% of the threshold voltage
- *Channel 3*—set for 110% of the threshold voltage

Draw a sample through the sensor, observing the count in *Channel 2*. When the particle count in that channel has reached approximately 1000, stop counting and observe the counts in *Channels 1* and *3*. Check to see if the *Channel 1* count and *Channel 3* count are 1.68 ± 10% and 0.32 ± 10%, respectively, of the count in *Channel 2*. If not, adjust the *Channel 1* and *Channel 3* thresholds to meet these criteria. When these criteria have been satisfied, draw a sample of suspension through the counter until the counts in *Channel 2* have reached approximately 10,000, or until an appropriate volume (e.g., 10 mL) of the sphere suspension has been counted. Verify that the *Channel 1* and *Channel 3* counts are 1.68 ± 3% and 0.32 ± 3%, respectively, of the count in *Channel 2*. Record the particle size for the thresholds just determined for *Channels 1, 2,* and *3*. Subtract the particle size for *Channel 2* from the size for *Channel 3*. Subtract the particle size for *Channel 1* from the size for *Channel 2*. The values so determined are the observed standard deviations on the positive side and negative side of the mean count for the 10-µm standard. One commonly used method for calculating the percentage resolution of the sensor is the following:

$$\% \text{ Resolution} = (100/d) \times [(s_{observed})^2 - (s_{standard})^2]^{1/2}$$

in which $d$ is the diameter of the spheres, in µm, as specified by the supplier; $s_{observed}$ is the highest observed standard deviation determined for the sphere standard; $s_{standard}$ is the supplier's reported standard deviation for the spheres. The resolution is NMT 10%.

**Electronic method:** Either record the voltage output distribution of the particle sensor using a multichannel analyzer while sampling a suspension of the 10-µm particle size standard or record the reported particle size distribution of an LO system that reports counts within adjustable diameter bins directly. (Other sizes ranging from 5 to 25 µm may be used provided the resolution is initially compared to the results for 10-µm microspheres and the subsequent measurements all use the same-size microsphere.) Particle size bins must each have a diameter range ±0.5 of the standard deviation of the main singlet bead peak, and the number of bins used for determination of the standard deviation must cover a minimum size range from ($d_{observed} - 2s_{observed}$) to ($d_{observed} + 2s_{observed}$). From the recorded particle size distribution, first identify the range of size bins that bracket the main singlet bead peak. From these identified bins, calculate the standard deviation ($s_{observed}$) of the readings:

$$s_{observed}^2 = \frac{1}{N-1} \sum_{i=1}^{M} n_i \left(d_i - d_{average}\right)^2$$

where $N$ is the total number of particulates; $M$ is the number of bins; $n_i$ is the number of particles in the $i$th bin; $d_i$ is the mean diameter of the $i$th bin; and $d_{average}$ is the average diameter of all the particles in the bins calculated as follows:

$$d_{average} = \frac{1}{N-1} \sum_{i=1}^{M} n_i d_i$$

The resolution is obtained using the formula given above for the manual method.

## Particle Count Accuracy Manual Method Test for MWHC Instruments

### OVERVIEW

The USP Particle Count Set RS contains PS spheres of approximately 15 µm in diameter. Counting with a size threshold ≥10 µm gives a measure of all the PS spheres. On setting the threshold to ≥15 µm, however, only approximately half the spheres will be counted. Comparison of the counts at these two size thresholds with values on the USP certificate provides a test of count accuracy. The test is also sensitive to size accuracy at 15 µm, since small errors in size will translate into large errors in count at the midpoint of the ▲particle size▲ (ERR 1-Oct-2021) distribution.

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## PROCEDURE

Prepare the suspension and blank using the USP Particle Count Set RS. With the instrument set to count in the cumulative (total) mode, collect counts at settings of ≥10 µm and ≥15 µm. Withdraw three consecutive volumes directly from the container. Historically, these have been volumes of NLT 5 mL each, due to instrument limitations and the desire to maximize sample volume. However, where desired, volumes that meet the standardization criteria and address the sensitivities of the formulation may be utilized. Obtain the particle counts and discard the data from the first portion. Complete the procedure within 5 min. Repeat the procedure using the suspension in place of the blank. From the averages of the counts resulting from the analysis of the two portions of the suspension at ≥10 µm and from the analysis of the two portions of the blank at ≥10 µm, calculate the number of particles in each milliliter:

$$(P_S - P_B)/V$$

in which $P_S$ is the average particle count obtained from the suspension; $P_B$ is the average particle count obtained from the blank; and $V$ is the average volume, in mL, of the four portions tested. Repeat the calculations using the results obtained at the setting of NLT 15 µm.

## INTERPRETATION

The MWHC instrument meets the requirements for *7. Particle Counting Accuracy (System Suitability)* if both the count obtained at ≥10 µm and the ratio of the counts obtained at ≥10 µm to those obtained at ≥15 µm conform to the values that accompany the USP Particle Count Set RS. If the instrument does not meet the requirements for *7. Particle Counting Accuracy (System Suitability)*, and adequate test volumes remain, repeat the procedure; if insufficient volumes remain, prepare new a suspension and blank and then repeat the procedure. If the results of the second test are within the limits given above, the instrument meets the requirements of the test for *7. Particle Counting Accuracy (System Suitability)*. On the second attempt, if the system does not meet the requirements of the test, determine and correct the source of the failures and retest the instrument. When using this ratio method, it is recommended to check the instrument size accuracy at both 10 µm and 15 µm prior to measurement of the count ratio, since a size error at 15 µm can translate into a large count error.

## REFERENCES

1. Ripple DC, Hu Z. Correcting the relative bias of light obscuration and flow imaging particle counters. *Pharm Res*. 2016;33:653–72.
2. Cavicchi RE, Carrier MJ, Cohen JB, Boger S, Mongomery CB, Hu z, et al. Particle shape effects on subvisible particle sizing measurements. *J. Pharm. Sci.* 2015;104:971–987.

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***Add the following:***

# ▲⟨1788.2⟩ MEMBRANE MICROSCOPE METHOD FOR THE DETERMINATION OF SUBVISIBLE PARTICULATE MATTER

1. INTRODUCTION
2. TEST APPARATUS
    2.1 Principles of Operation
    2.2 Basic Configuration
    2.3 Instrument Dynamic Range
3. BLANK AND SAMPLE HANDLING
4. TEST ENVIRONMENT
    4.1 Microscope Preparation
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    4.3 Particle Counting Accuracy (System Suitability)
5. TEST PROCEDURE
    5.1 Sample Preparation
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6. AUTOMATED APPROACHES
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## 1. INTRODUCTION

The membrane microscope method (MM) may be applied to samples of both large-volume and small-volume parenteral injections, as well as ophthalmic solutions. This test enumerates solid[1] particulate matter that is ≥10 µm after collecting, rinsing, and drying on a microporous membrane filter. Semisolid substances may also be isolated, although all liquid and sub-micrometer material will pass through the membrane. Because a wide range of test aliquots may be utilized, particle counts may be determined on a per-volume or a per-container basis without dilution or extrapolation.

Contrary to the light obscuration (LO) or flow imaging (FI) methods, particles are not measured using the MM method in their in-situ state. Particles are isolated from the formulation and examined on an air-dried substrate, and thus they may have changed from their wetted state in the final product. MM is not a reliable method for analyzing particles <10 µm; however, these particles can be analyzed using LO and FI. Because of their deformable shape and low contrast, protein particles can be difficult to detect or pass through the filter. For these reasons, this technology is best-suited for the analysis of foreign particles, which is useful in root cause analysis.

To perform the MM assay, the analyst estimates the size of retained materials viewed at 100× magnification, tabulating them into two (or more) size categories. In this process, materials on the membrane surface that do not appear solid may be encountered, showing little or no surface relief like a "stain" on the membrane. *Particulate Matter in Injections* ⟨788⟩ advises not to size or enumerate such semisolid particles due to comments in the past from large-volume injectable manufacturers that encountered brown stain-like residues after heat sterilization of dextrose solutions using terminal sterilization. However, if a carbohydrate solution or similar formulation is not being tested, recognizing the presence of such material may help to determine changes that would increase formulation robustness. Consistent evidence of such materials may be an indication that further product development is necessary to understand their content. The nature of these materials and the subsequent decision to count or investigate must be based upon product formulation experience. Interpretation of microscopical enumeration may be aided by testing a sample by the LO method or a validated alternative method and characterizing by Fourier transform infrared spectroscopy (FTIR) or another spectral microscopy method (see *Measurement of Subvisible Particulate Matter in Therapeutic Protein Injections* ⟨1787⟩). Information is provided in this chapter to aid the analyst in using the MM method.

## 2. TEST APPARATUS

### 2.1 Principles of Operation

Sample packages are mixed and opened, and product fluid is drained or transferred into a clean filtration barrel. Free particles from the product are captured on a membrane surface. The membrane-retained material is rinsed and dried for subsequent microscopic examination. Sizing and counting of particles is conducted at 100× using two size thresholds: ≥10 µm and ≥25 µm. Particle content is reported as the total count in each category.

[1] Soft particles and semisolid substances may also be retained.

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## 2.2 Basic Configuration

Elements of the filtration and microscopic equipment are described in ⟨788⟩. Additional considerations are discussed here.

### FILTRATION APPARATUS

Use a filter funnel suitable for the volume to be tested, such as a 25-mm 50 mL or 47-mm 300 mL barrel. Larger barrels are also available. However, the barrel inner diameter will determine the final counting surface; for example, an inner diameter of about 16 mm for 25-mm membranes or about 37 mm for 47-mm membranes. The funnel may be made of plastic, glass, or stainless steel. Use a filter support made of stainless steel or sintered glass as the filtration base. The goal is a flat, dry, even-colored, and unmarked membrane background.

Cleaning and rinsing the apparatus is facilitated with a pressurized solvent dispenser capable of delivering fluids passed through a terminal membrane filter at a range of pressures from 10 to 80 psi.

### MEMBRANES

Chapter ⟨788⟩ recommends a black or dark gray membrane with a pore size of <1 µm; however, finer pore size membranes will have smoother surfaces, enhancing the resolution of particles in the microscope. Smaller pore sizes may impede the filtration of more viscous sample fluids during the assay. The black or dark gray membrane is recommended to provide good contrast for the array of particle colors, shapes, and transparencies. Other colored membrane types may be suitable depending on the product.

Alternative membrane choices may also be used to improve or alleviate incompatibility during product filtration. Product compatibility is an issue for mixed cellulose ester membranes or for the isolated materials used for further compositional analysis. Gold-coated or metallic silver filters may be used for these applications, especially spectroscopic analysis or particle type identification.

The test apparatus is described in ⟨788⟩. Additional comments are the following:

- A compound binocular microscope that corrects for changes in interpupillary distance by maintaining a constant tube length must be used.
- The objective must be of 10× nominal magnification with flatness correction and color correction, such as a planar achromat or better in quality, with a minimum 0.25 numerical aperture (NA).
- The objective must be compatible with an episcopic illuminator attachment.
- The eyepieces must be matched. In addition, one eyepiece must be designed to accept and focus an eyepiece graticule.
- The microscope must have a mechanical stage capable of holding and traversing the entire filtration area of a 25-mm or 47-mm membrane filter in order to completely scan the isolate surface.
- One episcopic and one oblique illuminator are required. Both illuminators must be of sufficient output to provide a bright and even source of illumination and may be equipped with blue daylight filters to decrease operator fatigue during use.
- The *USP* graticule as described in ⟨788⟩ is used.

### STAGE MICROMETER

Used for calibrating the microscope configuration and graduated in 10-µm increments, the stage micrometer is preferably utilized each day of use. For initial calibration, utilize a stage micrometer that is traceable to the National Institute of Standards and Technology (NIST) to verify the *USP* graticule dimension features. A commercial stage micrometer graduated in 10-µm increments to verify proper setup may be used for daily calibration/verification.

## 2.3 Instrument Dynamic Range

The following considerations apply to both human operator and machine vision. Although the instrument has a wide dynamic range and this technique may be used for visible particles at the upper end, the focus in this section is at the lower end, a subvisible range.

### SIZE ACCURACY

The microscopical assay uses 100× observation by reflected illumination in an air mount that produces optical diffraction effects, e.g., blurriness. Thus, the practical resolution at the specified size thresholds is about 1 µm. At a nominal 100× magnification, the smallest particles (10 µm) are easily discriminated from the background. Particles <10 µm can be observed via the MM method, but differentiation of particles close to the thresholds is important in the sizing process.

Because there is a diffraction limit of about 1 µm, it may be difficult to discriminate 9 µm from 10 µm or 24 µm from 25 µm due to suboptimal illumination, interfering background, particle features, et cetera. As a best practice, if there is doubt in determining whether an object is a particle, it should be counted. The operator should note the predominant particle morphologies descriptively and may perform further analysis if appropriate.

### PARTICLE CONCENTRATION

The filtration operation will capture any solid material that is suspended within the sample fluid. For a blank (e.g., filtered water), there may be several particles ≥10 µm. The particle load is typically higher for samples.

Considering the ⟨788⟩ small-volume injection limit of 3000 particles that are ≥10 µm per container, several thousand particles may be isolated from a 10-container pool. That particle load is atypical, but when encountered, must be processed. Full counting

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of a high particle load membrane is possible but time consuming and fatiguing when thousands of particles are present. Partial or statistical counting is recommended and is described later in this chapter in *Partial Count Procedure*. The partial counting approach extrapolates the count of a high particle load membrane from a defined portion of the filtration area. A practical upper limit for counting is around ≥3000 particles per membrane. The ≥3000 particle load typically takes a trained analyst an hour or more to count. Consider reducing the sample volume (e.g., fewer product samples) to reduce the total load of isolated particles. Finally, when a high particle load sample cannot be adequately processed, pursue an alternative analytical strategy.

THRESHOLDING/SENSITIVITY

Training is essential because the assay requires the operator's judgment of particle size by comparison to the calibrated reticle. Calibration of the microscope after optimization of the illumination allows the operator to make the best judgment on whether a particle should or should not be included in the particle count.

For automated systems, the same rules for image analysis apply. Because the 10-µm and 25-µm thresholds are used, the sensitivity at these sizes is essential. The *General Notices* require that accuracy be within 10%; therefore the system must be able to recognize 10 ± 1 µm. Thresholding implies the definition of actual particle boundaries versus background, which can be difficult for the human operator and even more so for the instrumental logic applied for feature recognition.

## 3. BLANK AND SAMPLE HANDLING

A blank determination is necessary at the beginning of each test sequence to verify minimal contribution from the background, equipment, or personnel. Test sequence can be defined as per shift, product family, series of filtrations (manifold), or sample. The suitability of the selected test sequence is dependent upon the operational needs of the laboratory system; the ability to clean glassware between samples, the number of different products, and the volume of sample tested. However, the blank should be considered as a system suitability check. Failure of the blank would suggest that all samples that were run after the previous blank are questionable.

To determine the blank count, duplicate the sample preparation process regarding the apparatus and membrane types. Assemble a clean filtration apparatus with a fresh membrane, rinse the interior with filtered water to drain, and then deliver 50 mL or more volume of filtered water to the filtration funnel while applying vacuum, drawing the entire volume of water through the membrane filter. Remove the membrane from the filter funnel base and place it onto a holding device, typically, on a strip of double-sided tape on a microscope slide or in a commercial membrane holder or Petri dish. After allowing the membrane to dry (it must be counted dry), examine the entire filtration area microscopically at 100× magnification. If NMT 20 particles ≥10 µm and NMT 5 particles ≥25 µm or larger are present within the filtration area, the background particle level is sufficiently low to meet the criteria in ⟨788⟩, *Method 2 Microscope Particle Count Test*. If particle load exceeds these limits, repeat the procedure.

There is value in further limiting the background counts for both ⟨788⟩ and *Particulate Matter in Ophthalmic Solutions* ⟨789⟩ testing in regard to good laboratory practice, and more specifically in regard to the ≥25-µm and ≥50-µm particle limits in ⟨789⟩, which may be more restrictive than injectable limits considering the total particle content allowed for the (usually) small unit volumes.

## 4. TEST ENVIRONMENT

The recommended operational detail to enhance the successful performance of the MM assay is given in this section. It is ideal to use two unidirectional airflow hoods (UAFH) or other unidirectional airflow (UAF) enclosures, one for "wet" sample preparations and the other an enclosure for the microscope counting phase. The UAFH should have a capacity sufficient to envelop the area in which the analysis is prepared. The UAFH provides high-efficiency particulate air (HEPA) filtered air that meets the International Organization for Standardization ISO 14644-1 standard (*1*).

Throughout the operational procedure (in the HEPA environment), it is preferable to use powder-free gloves and low-shedding clothing. Before conducting the test, clean the work surfaces of the UAF enclosure with an appropriate filtered solvent. Glassware and equipment should be rinsed successively with a warm, residue-free solution of detergent, hot water, and filtered distilled or deionized water. [NOTE—Prior to use, pass the distilled or deionized water through membrane filters of at least 0.2-µm nominal pore size.] If an alternate solvent must be used, use proper safety precautions. Perform the rinse in the UAF enclosure. Allow the glassware and filtration apparatus to dry in the enclosure, upstream of all other operations. The enclosure is preferably located in a separate room that is supplied with filtered cool, conditioned air and maintained under positive pressure with respect to the surrounding areas.

### 4.1 Microscope Preparation

The microscope optical alignment and illumination are critical for the success of this method. Although it is not difficult to differentiate a 10-µm particle from a 25-µm particle at 100× with reflected light, the decision regarding the boundary at each size category is difficult with inadequate equipment, maintenance, or optical alignment. Also, operator fatigue is caused by poor microscope alignment. The operator will have to determine if a particle is above or below the limit (e.g., the particle size is 9 µm or 11 µm). Optimized system resolution (i.e., the ability to discern discrete points of minimal separation) relies upon good and well-aligned optical systems. Factors including instrument cleanliness, resolution (e.g., objective NA[2]), and focus of both eyepieces, and the graticule will all play significant roles in attaining the best images. In consideration of optimizing the use of the binocular compound microscope, it is best to utilize operators familiar with the instrument and comfortable with its

[2] NA is an indicator of optical light-gathering capability and, thus, resolution. High NA correlates to high resolution.

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alignment. The operator conducting the method should align the optics and illumination for his/her use with supervisory/trainer approval.

It is recommended to start with the alignment of the microscope for a typical transmitted illumination observation using a known sample. Any specimen familiar to the operator will suffice; however, a common particle count reference standard suspension, such as the USP Particle Count Set RS, is a good selection because it is also utilized in method system suitability evaluation. A drop of USP Particle Count Set RS is placed between a glass microscope slide and coverslip and viewed microscopically[3] With appropriate interpupillary distance and a comfortable sitting position at the microscope, the operator examines the fields of suspended spheres. One should observe (with ease) the small standard spheres, crisp on a combined field for both eyes. One attains crisp focus and ease of view after the focal adjustment of each eyepiece on a single point on the specimen.

Rotate the graticule in the right microscope eyepiece so that the linear scale is located at the bottom of the field of view, bringing the graticule into sharp focus by adjusting the right eyepiece diopter ring while viewing an out-of-focus specimen. Focus the microscope on a specimen, looking through the right eyepiece only. Then, looking through the left eyepiece, adjust the left eyepiece diopter to bring the specimen into sharp focus.

When the operator is not comfortable using the microscope or does not attain an equivalent crisp focus for each eye in a merged field of view, counting will become a difficult experience and will result in fatigue and flawed size comparison.

Nothing is better for preparing the operator for counting particles than to examine a test membrane as a positive control. Seasoned microscopists may not require this step, but for new operators or individuals conducting many different types of methods in the modern laboratory, familiarization is a prudent exercise. A filter membrane of the type being used for the method —such as a 25-mm, color-contrast, plain, membrane filter of 0.45-µm nominal pore size, containing particles—is a good choice. This may be a sample from a previous method that contains a variety of particle types or one prepared for familiarization. This positive test control will contain natural particles (e.g., flakes, threads, equant particles, and particles of various colors/opacity in a range of sizes) to effectively refresh the operator's sensitivity and facilitate microscope and illumination alignment for optimal viewing.

To examine the membrane preparation, locate a typical array of particles and first bring the illumination into good alignment:

1. Adjust the external, incident illumination at an oblique angle (10°–20° inclined to the stage) so that an even ellipse of reflected light is visible on the membrane and even illumination is evident through the eyepiece field of view (even across the full field). Shadows will be evident from larger particles, such as those with *z*-axis dimension >5 µm (*z*-axis is the microscope's vertical axis).
2. Now adjust the internal episcopic brightfield illuminator to yield an even illumination at a high setting on the transformer control. More importantly, when dialing down the illumination, one observes the evident shadow from the larger particles. In this manner, the high reflectivity of flat, glassy particles and the distinct shadows of more equant (*x*:*y*:*z* ~ 1:1:1) particles are evident.

## 4.2 Using the Circular Diameter Graticule

In order to properly focus the ocular lenses and attain a balanced single-field view, each operator must bring the *USP* graticule lines into sharp focus by adjusting the eyepiece diopter ring. Next, focus the microscope on a specimen through this same eyepiece, and then, looking only through the other eyepiece, adjust its diopter ring to bring the specimen into sharp focus. The *USP* graticule and specimen particles are now in focus on a well-balanced illumination field.

The *USP* graticule is specifically fabricated for each microscope. The relative error of the graticule used must be ±2% and is initially measured with an NIST traceable stage micrometer. To accomplish this, align the graticule micrometer scale with the stage micrometer so that they are parallel. Compare the scales, using as large a number of graduations on each as possible. Read the number of graticule scale divisions (*GSD*), compared to stage micrometer divisions (*SMD*). Calculate the relative error:

$$\% \text{ Relative error} = 100[(GSD - SMD)/SMD]$$

A relative error of ±2% is acceptable and verifies good alignment, focus, and proper magnification. Thereafter, a day-of-use verification by the microscope operator with the NIST traceable stage micrometer is sufficient to demonstrate proper setup.

The basic technique of measurement, applied with the use of the circular diameter graticule, is to count all particles 10 µm and larger, further categorizing in sizes ranging from 10 to 24 µm and 25 µm or greater. The circular zone or graticule field of view (GFOV) is a useful zone for active sizing and counting. Particles are compared to the linear scale and/or circles to determine their size against the circular diameter design. This is conducted by mentally transforming the image of each particle onto the 10- and 25-µm graticule reference circles. The sizing process is carried out without having to physically superimpose the particle on the reference circles; particles are not moved from their locations within the GFOV (the large circle) for comparison to the reference circles. Compare the longest chord of the particle being sized to the diameter of the black or transparent circles. The circles allow comparison of the particles regardless of their orientation. The linear scale may also be used for size comparison. Use the clear graticule reference circles to size white or transparent particles. Use the black reference circles to size dark particles.

The intent of comparing particles to an equivalent circular diameter is correlation to the LO particle sizing methodology, for which many manufacturers have extensive databases. In practice, particles with nearly circular areas will correlate well with the graticule circle diameters. For particles with one long axis, such as rods and needles, the conversion to circular area will produce more significant bias to smaller estimated sizes. It is simpler, and most conservative, to count particles in longest chord. To use an extreme example, the total count of monodispersions of fine needle crystals would vary greatly dependent upon the size determination utilized.

[3] The microscope objective requires a defined cover slip thickness, nominally 170 µm, or No. 1½.

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### 4.3 Particle Counting Accuracy (System Suitability)

- It is valuable to use a reference count preparation for verifying illumination and as a means to discuss counting and sizing criteria.
- Calibration of the graticule/linear scale should be done as needed to verify proper setup (ocular, objective, and intermediate lenses are easily removed and changed).
- Particle counting accuracy is an important training operation and a reasonable annual check. A monodisperse sphere standard, such as a membrane deposit of the USP Particle Count Set RS, is useful for comparative counts between the trainer and the operators.
- Blank samples are an effective means of confirming the cleaning preparations for the method.

## 5. TEST PROCEDURE

### 5.1 Sample Preparation

Preparation of the filtration apparatus and test preparations are discussed in ⟨788⟩ and *Methods for the Determination of Subvisible Particulate Matter* ⟨1788⟩.

Filter large-volume injection (LVI) units individually. Small-volume injection (SVI) units having a volume of 25 mL or more may be filtered individually.

Transfer to the filtration funnel the total volume of a solution pool or of a single unit, and apply vacuum. If the volume of solution to be filtered exceeds the volume of the filtration funnel, add a portion of the solution, stepwise, until the entire volume is filtered. It is prudent to maintain more than half of the funnel volume of liquid in the filtration funnel between refills, especially if the partial count procedure is to be used (see *Partial Count Procedure*). [NOTE—This step is necessary in order to ensure even distribution of particles on the analytical membrane.] After the last addition of solution, begin rinsing the walls of the funnel by directing a low-pressure stream of filtered water in a circular pattern along the walls of the funnel, and stop rinsing the funnel before the volume falls below about one-fourth of the fill level. Maintain the vacuum until all the liquid in the funnel is gone.

Remove the filtration funnel from the filtration base while maintaining vacuum, then turn the vacuum off, and remove the filter membrane with nonserrated forceps. Place the filter in the prepared holder and label with sample identification. Allow the filter to air-dry in the UAF enclosure with the cover ajar.

### 5.2. Particle Enumeration

The MM test described in this section is flexible in that typical artifacts such as air and immiscible liquids do not interfere with the final count. The method has a broad size-detection and counting range if applying the partial count procedure. This method may be used where all particles on an analysis membrane surface are counted or where only those particles on some fractional area of a membrane surface are counted.

TOTAL COUNT PROCEDURE

The microscope method is a slower process than the LO or FI methods, but they all have their value. Because the results rely upon the training and experience of human operators (e.g., visual inspection), insufficient training and lack of diligence can result in imprecision (e.g., poor agreement within and among labs) and inaccuracy (e.g., particle size estimates that are inaccurate for nonspherical particle shapes). Operator fatigue can be a problem and is promoted by poor ergonomic fit (chair height), poor or imbalanced ocular focus, and inattention to detail due to interruptions and lack of mental focus. Restricting the eye movement to a field-defining graticule, such as the *USP* counting graticule, restricts eye movement to the central one-third of the field of view. This significantly limits eye movement and may induce fatigue.

Sample size is an important consideration in counting precision. Care must be taken to sample many containers within a batch for a good representation of the particle distribution. Accordingly, the portion of the individual package sampled is critical. Particles may float or settle. Sampling only the first 25 mL of a large volume parenteral or sampling without adequate and recent mixing will lead to serious undercounting. Sampling whole, well-mixed containers with the particles in suspension is the best approach.

Counting the isolated particles is an important parameter. Counting all of the particles retained on the membrane is certainly the best approach, and then the simple problem is determining the correct size for placement into the threshold bins, 10 µm and 25 µm. This will be increasingly important for methods utilizing additional bins for population determination, such as 5-, 50-, and 100-µm. Note that the particle limits for ⟨788⟩ and ⟨789⟩ must be reported as all particles ≥10 µm and all particles ≥25 µm (and all particles ≥50 µm). If the lab method has been configured to count in several bins, such as 10–24 µm, 25–49 µm, and ≥50 µm, et cetera, all bins ≥10 µm must be added to yield the total ≥10-µm count; all bins ≥25 µm need to be added to yield the total ≥25-µm count. Using a number of narrow size bins may be beneficial in product improvement efforts to separate particle groups.

In performing a total count, the graticule GFOV is defined by the large circle of the graticule, and the vertical crosshair is used as a counting target. Count and size particles as they pass across the full diameter line of the GFOV, keeping mental track of the path over the membrane. Scan the membrane in paths that cover the effective filtration area (EFA) adjoining but not overlapping previous scan paths. Repeat this procedure; tabulate particle counts minimally in the 10–24-µm and ≥25-µm thresholds, moving across the membrane until all particles on the membrane within the EFA are counted. Record the total number of particles that are 10–24 µm and the number that are ≥25 µm.

For large-volume products, calculate the particle count, in particles per milliliter, for each unit tested:

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$$P_{\Sigma\geq10}/V$$
$$P_{\Sigma\geq25}/V$$
$$P_{\Sigma\geq50}/V$$

in which $P_{\Sigma\geq10}$ is the total particle count obtained from all portions ≥10 µm analyzed; $P_{\Sigma\geq25}$ is the total particle count obtained from all portions ≥25 µm analyzed; $P_{\Sigma\geq50}$ is the total particle count obtained from all portions ≥50 µm analyzed; and $V$ is the volume, in milliliters, of the solution tested.

For example, the analyst has counted the test samples in four bins: a) 10–24 µm, b) 25–49 µm, c) 50–99 µm, and d) ≥100 µm. This would then be calculated as:

$$P_{\Sigma\geq10} = P_{10–24\ \mu m} + P_{25–49\ \mu m} + P_{50–99\ \mu m} + P_{\geq100\ \mu m}$$

For small-volume products, calculate the particle count, in particles per container:

$$P_{\Sigma\geq10}/\eta$$
$$P_{\Sigma\geq25}/\eta$$
$$P_{\Sigma\geq50}/\eta$$

in which $P_{\Sigma\geq10}$ is the total particle count obtained from all portions ≥10 µm analyzed; $P_{\Sigma\geq25}$ is the total particle count obtained from all portions ≥25 µm analyzed; $P_{\Sigma\geq50}$ is the total particle count obtained from all portions ≥50 µm analyzed; and $n$ is the number of units pooled (1 in the case of an individual unit).

## PARTIAL COUNT PROCEDURE

When encountering a membrane full of particles, the task of adequately counting all of them is daunting. Consider that an small volume parenteral with an at-limit content of small particles, sampled in a 10-vial pool would have 30,000 10-µm particles on the membrane. Partial or statistical counting of the membrane's EFA may be the only means to attain consistent results. Partial counting should be used not to reduce count times but to estimate the total load on a high-count isolate. Field-defining devices, such as grids on the membrane surface or an ocular GFOV, have been used reliably. An ocular graticule provides a sharp boundary for area definition. Gridded membrane lines are rather broad and have ink-spatter that may be mistaken for particulate matter.

For 25-mm membranes, the EFA is 16-mm in diameter using typical commercial filtration funnels; therefore the EFA ($\pi \times r^2$) = 201 mm$^2$. Based upon earlier proposals from the Health Industry Manufacturers Association (HIMA) and discussion by Draftz (*2*), acceptable confidence intervals (Poisson distribution, two standard deviations) dictate that for samples with <1000 particles, the imprecision of statistical counting is objectionable. Full count is recommended for such samples. For samples with >1000 particles on a 25-mm isolate membrane, a reasonable estimate of particle population is attained using 20 GFOVs. If a smaller confidence interval for the result is desired, a larger number of fields and particles may be counted.

For 47-mm membranes the EFA has a 37-mm diameter. These larger-diameter membranes may be selected for formulations needing more membrane surface area (i.e., formulations that have slow flow characteristics through 25-mm membranes); in which case, the EFA ($\pi \times r^2$) is ($\pi \times 18.5$ mm$^2$) = 1075 mm$^2$. Thus, for 47-mm membrane EFAs, many more GFOVs must be counted to attain similar confidence levels. Using 100 GFOVs for partial counting of 47-mm membranes provides similar statistical confidence to the 20-GFOV/25-mm approach. Accordingly, when a particle load of 1000 or less is present, a full count is recommended.

When a partial count of particles on a membrane is to be performed, the analyst must first ensure that an even distribution of particles is present on the membrane. This is assessed by rapid scanning at 50× to qualitatively scan for clumps of particles. If heterogeneity is observed, one should perform a full count on the membrane. Next, count the 10-µm or larger particles in one GFOV at the edge of the filtration area as well as one GFOV in the center of the membrane. The number of 10-µm or larger particles in the GFOV with the highest total particle count must not be more than twice that of the GFOV with the lowest total particle count. If this criterion is not met, perform a full count of the membrane.

To perform a partial count of the particles on a membrane, include all particles 10–24 µm and ≥25 µm within the GFOV and those that are in contact with the right side of the GFOV circle. Do not count particles outside of the GFOV. Ignore those that touch the left side of the GFOV circle. The dividing line between right and left sides of the GFOV circle is the vertical crosshair and is a useful counting line.

[NOTE—Make the best possible judgment on particle size without changing the membrane position, microscope magnification, or illumination.]

Start at the center edge of the filtration area and begin counting adjacent GFOVs. When the other edge of the filtration area is reached, move one GFOV toward the top of the filter and continue counting GFOVs by moving in the opposite direction. This can be accomplished by one of two methods: define a landmark (particle or surface irregularity in the filter) and move over one GFOV in relation to the landmark, or use the Vernier scale on the microscope method to move 1 mm between GFOVs. To facilitate the latter, adjust the microscope *x*- and *y*-method positioning controls to a whole number at the starting position at the center right edge of the filtration area so each GFOV will be one whole division of movement of the *x*-method positioning control. If the top of the filtration area is reached before the desired number of GFOVs is reached, begin again at the right center edge of the filtration area one GFOV lower than the first time. This time, move downward on the membrane when the end of a row of GFOVs is reached. Continue as before until the number of GFOVs is complete.

For large-volume products, extrapolate the total count of particles per milliliter:

$$P_{\Sigma\geq10}\,A_T/A_P V$$
$$P_{\Sigma\geq25}\,A_T/A_P V$$

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in which $P_{\Sigma \geq 10}$ is the total particle count obtained from all fields of view and all size thresholds; $A_T$ is the filtration area of the membrane in millimeters$^2$ (inner filtration barrel diameter); $A_P$ is the partial area counted in millimeters$^2$, based on the number of graticule fields counted (GFOV area × number of GFOV counted); $V$ is the volume of solution filtered in milliliters; and $P_{\Sigma \geq 25}$ is the total particle count obtained from all fields of view and all size thresholds ≥25 µm.

For a solution pool (for small-volume product units containing less than 25 mL) or for a single unit of a small-volume product, extrapolate the total count of particles per unit:

$$P_{\Sigma \geq 10} A_T / A_P n$$
$$P_{\Sigma \geq 25} A_T / A_P n$$

in which $P_{\Sigma \geq 10}$ is the total particle count obtained from all fields of view and all size thresholds; $P_{\Sigma \geq 25}$ is the total particle count obtained from all fields of view and all size thresholds ≥25 µm; and $n$ is the number of units counted (one, in the case of an individual unit). For all types of product, if the tested material has been diluted to decrease viscosity, the dilution factor must be accounted for in the calculation of the final test result.

# 6. AUTOMATED APPROACHES

## 6.1 Background

The original MM method was designed as an operator-intensive operation, from preparation to filtration to counting. The judgment of the operator regarding the presence and "countability" of isolates is paramount to effective detection, sizing, and counting of the retained particles. There may be alternative approaches. Static image analysis systems offer automated tracking and detection of particles based on contrast against the background. There is increased potential for objective accuracy and precision versus subjective human decision and tabulation. Primary considerations for the qualification of image systems are: recognition of thin or indistinct particles, "acceptance" of background features, discrimination of particles from background, and counting of artifacts. Use of these systems is supported with proper qualification using typical particle types as challenge sets.

To provide a more comprehensive guidance for human or machine vision, this chapter includes qualification recommendations for operators running the membrane assay for demonstrating the applicability of an automated method.

Qualification is a quality or accomplishment that makes someone suitable for a particular job or activity. Discussion points to address this simple goal are as follows:

- Microscope selection, preparation, and use are critical in detecting, sizing, and counting particles in the collected isolate. See *2. Test Apparatus* for background on the type of microscope and illumination used in daily counting.
- Familiarization of the operator is essential in particle recognition and size/count decision making. The following are elements of a proper training program:
  - It is imperative to fully scan the EFA for countable particles:
    - The membrane is tracked under the 10× objective with an *x-y* stage control
    - The lateral tracks must be parallel and not underpassed (gaps) or overpassed (recounted)
    - The edge of the EFA must be recognized and taken as the limit for tracking
  - Real particles ≥10 µm must be tabulated and discerned from the background
  - Blank samples familiarize the operator with the characteristics of the membrane texture, color, and reflectivity
  - Differentiation of 10–24 µm and ≥25 µm (additionally ≥50 µm for ⟨789⟩) particles is imperative

## 6.2 Qualification

A bimodal suspension of standard spheres is a reasonable means to verify operator recognition of ≥10 µm and ≥25 µm standards to satisfy the above criteria. Use two sphere standards just above the two size thresholds, such as 12–15 µm and 30–35 µm. Prepare a suspension of equal portions of the standard spheres in particle-free water, limiting the total content to ≤1000 in the volume tested. Training operators are charged with preparing and counting the preparations to verify acceptability.

The trainer counts the preparation, once acceptable, three times within a workday. This is the reference count. To promote stability, the membrane standard preparation may be sprayed with a fixative, such as a clear photoprotectant aerosol spray or a hand-pumped clear hairspray to fix the particles to the membrane surface. These applications may be used for months but should always be verified by trainers.

Trainees count the preparation three times in the same workday. The relative standard deviation (RSD)[4] for each threshold for one operator should be small, such as ≤5%. The RSD for each threshold between operator and trainer must be ≤10%.

In addition to manual microscopy, advances in technology have provided for automated testing of membrane-filtered samples. These techniques replace the human eye with a charge-coupled device (CCD) camera for image collection and the human decision-making process with advanced image processing software. While these techniques may seem to be simple, human-error free substitutes, careful consideration of the experimental variables, much like those for manual microscopy, is required for proper method qualification.

System particle size accuracy and count may be determined using traceable etched slides available from national standards organizations. These suffice to provide a calibrated measure of particle size, typically using chromed circular spots on a rigidly controlled sample. This approach also offers the user a self-contained system check with limited overhead once method qualification is complete (i.e., internal verification of system performance).

[4] The relative standard deviation (RSD), also known as the coefficient of variation (CV), is a standardized measure of dispersion of a probability distribution or frequency distribution. It is often expressed as a percentage and is defined as the ratio of the standard deviation to the mean (or its absolute value).

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Beyond the initial system verification, it is necessary to directly compare the results of operator-conducted MM to automated methods. Although the mixed-sphere samples suggested for operator familiarization/training are excellent for comparing data collection methods, they represent optimal samples that may not highlight differences between manual and automated approaches. Instead, it is imperative that manual and automated methods be compared in a vein similar to that expressed for manual operator training.

- Using a minimum of three real samples, have the manual operator conduct the assessment of particulates ≥10, ≥25, and ≥50 µm
- Using the automated method for the same samples, have the system conduct three replicates of each sample for assessment of particulates ≥10 and ≥25 µm
- The RSD for the automated method replicates should be ≤3%
- The RSD between the manual and automated methods should be ≤10%
- The manual operator should review automated results to ensure that the automated system accurately captures particle populations observed manually

In general, repeatability of automated methods should be reasonably high (≤1%), and that of a single human observer slightly lower (≤5%). However, differences in method variables between manual and automated methods can lead to large changes in the apparent count and/or size results when comparing techniques. This variance can be far outside that expected for a human operator versus an automated method, which typically points to a shortcoming of the automated method. Such a situation warrants an understanding of why the method results diverge and a question of whether automation of the method is reasonable.

These considerations are usually related to either the microscope illumination or the thresholding mechanism defined in the automated method. Illumination varies among automated systems and can include diascopic (transmitted), episcopic (axial reflected), or oblique lighting. Manual methods have evolved to utilize oblique lighting exclusively, while automated methods may utilize alternate illumination, depending on the substrate selection. This can lead to automated methods with considerably different contrast quality from corresponding manual methods, so care should be taken to closely match the lighting conditions when comparing size/count results. Single-value thresholding schemes, which are quite common for automated systems, may produce fragmented images or miss particles entirely, whereas a human operator can clearly resolve the particle projection and accurately size/count the particles. It is critical to ensure that the qualification of an automated method includes a review of detected particles.

A final topic of consideration involves the selection of membrane type. Not all product forms are amenable to using cellulosic membranes, so alternatives to the standard cellulose-based membrane substrate may be appropriate. Novel membranes are now available that feature superior homogeneity, compatibility with organic solvents, and spectroscopic compatibility. These membrane choices may be used to develop manual or automated methods that meet ⟨788⟩ requirements, with an appropriate qualification of the method.

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***Add the following:***

# ▲⟨1788.3⟩ FLOW IMAGING METHOD FOR THE DETERMINATION OF SUBVISIBLE PARTICULATE MATTER

1. INTRODUCTION
2. TEST APPARATUS
    2.1 Principles of Operation
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## 1. INTRODUCTION

Flow imaging (FI) is useful for an approximate particle range from 2 to 100 µm, depending on the instrument and its configuration, covering particle sizes from the accepted subvisible size range to hundreds of micrometers (see *Measurement of Subvisible Particulate Matter in Therapeutic Protein Injections* ⟨1787⟩ and *Methods for the Determination of Subvisible Particulate Matter* ⟨1788⟩). Specialized instruments or configurations can cover expanded size ranges. FI instruments provides both individual microscopic images of observed particles and reports of particle size distribution (PSD) and particle concentration at selected size thresholds. Values of morphological parameters and sample images corresponding to the stated size bins or thresholds may be added to the report. Particle size can be reported as an equivalent circular diameter [similar to light obscuration (LO)] or based on Feret's diameter, which captures some information about the shape. Algorithms categorizing particles are usually based on optical density and morphological parameters such as circularity, allowing them to differentiate to some degree between elongated fibers, dense symmetrical air bubbles, circular silicone oil droplets, and amorphous protein particles, etc.

This is an essential characterization method that is often used as an orthogonal method to the compendial LO and membrane microscope (MM) methods, especially during development. This method is described, along with other orthogonal techniques, in ⟨1787⟩. Because of its increasing use and importance as a technique to size, count, and bin by morphology, more details on how to optimize the application of this technology are provided here.

## 2. TEST APPARATUS

### 2.1 Principles of Operation

The principles of operation and the strengths and weaknesses of FI are discussed in ⟨1787⟩. Briefly, the apparatus is a liquid-borne particle counting and imaging system using a sample-feeding device to deliver controlled aliquots of liquid for analysis. The samples flow through an optical cell positioned between a light source and camera, allowing the capture of a digital stream of particle images present in the sample.

Image analysis of the captured image frames can provide necessary information. Fundamentally, information may be obtained about particle size and number within a size range from about 2 to 100 µm (exact size range and larger size ranges will depend on the instrument, cell size, and magnification). Other parameters such as shape, contrast, and transparency of the particles allow for the evaluation of the particle population.

FI combines the benefits of in situ observation, as in LO, with the ability to qualitatively evaluate some characteristics of the particles, as in MM. Air bubbles and immiscible liquids are detected because imaging is performed on flowing liquid samples. With software algorithms, these common artifacts may be discriminated from "real" particles. The effectiveness of particle discrimination is a function of magnification, resolution, software algorithms, and particle properties.

Therefore, product fluids with air bubbles and immiscible liquid droplets (e.g., silicone oil), which are not discriminated from other types of particles when using LO analysis, are imaged by FI and can be discriminated through software processing. It is the responsibility of those performing the test to ensure that the operating parameters of the instrumentation meet the required

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accuracy and precision, that artifacts and interferences observed in certain products and preparation methods are eliminated or accommodated, and that the algorithm is assigning particle type correctly.

High-resolution optics have a small depth of field relative to optical configurations with lower resolution. Thus, for typical instruments where the particle suspension travels through a flow cell of approximately 100-µm thickness, there is a tradeoff between ensuring that all particles remain in focus versus obtaining high-resolution images. It may be appropriate to obtain high-quality images with different hardware settings than typically required to obtain an accurate particle count.

FI instruments vary in the flexibility and range of adjustment of instrument hardware. Some instruments will have user-selectable flow cells, objectives, and illumination. Other instruments will have predominantly fixed settings. It is the user's responsibility to determine if the optical configuration of a particular instrument, whether fixed or variable, serves the stated purpose of the measurement.

It is important to note that for compendial applications, the ultimate goal is that the imaging instrument accurately size and count the particles present. FI enables classification of particles according to particle image properties (e.g., aspect ratio, brightness, transparency, and circularity), which can assist the user in understanding the nature of particle subpopulations and in diagnosing possible particle count interferences (e.g., as air bubbles). Establishing an image library will aid in the categorization of particulate unknowns and could be part of risk assessment. The instruments that are available range from manual calibration and standardization systems to sophisticated hardware- and software-based standardization systems. Thus, it is not possible to specify the exact methods to be followed for instrument standardization, and it is necessary to emphasize the required end result of a standardization procedure rather than a specific method for obtaining this result. This chapter is intended to emphasize the criteria that must be met by a system rather than specific methods to be used in their determination. It is the responsibility of the user to apply the various methods of standardization applicable to a specific instrument. Indeed, no single method will apply to all experiments. Factors such as the desired range of detection, formulation characteristics, available sample volumes, and nature of the particulate matter are typical considerations for the method used. Validation concepts for a limits test in *Validation of Compendial Procedures* ⟨1225⟩ provide sufficient guidance for these purposes.

## 3. INSTRUMENTATION STANDARDIZATION TEST

### 3.1 Concentration Calibration

The particle concentration reported by a FI instrument is based on the total number of particles measured, within a specified range of diameters, divided by the total volume of fluid that has been imaged. Each camera image records particles within a volume equal to the imaged area multiplied by the interior thickness of the flow cell. Consequently, the imaged sample volume requires accurate knowledge of the flow cell thickness but does not depend directly on the liquid flow rate through the cell. Because direct measurement of flow cell inner dimension is difficult for some types of flow cell, the thickness is often inferred by measuring the particle concentration of a suspension of microspheres of certified concentration. Calibration procedures are given in the *Appendix*.

### 3.2 Sample Flow Rate

The flow rate through the cell should be consistent with the manufacturer's recommendations in order to ensure that the instrument is functioning properly. This may be accomplished by weighing the amount of liquid passed through the system at a specified rate, using a calibrated stopwatch to measure the elapsed time. Instruments may be operated accurately over a range of flow rates. Perform *7. Test Procedure* at the same flow rate as that selected for calibration of the instrument.

### 3.3 Diameter Calibration

Refer to ⟨1788⟩ for general guidance on diameter calibration. A microscopic particle image depicts the projected area of a particle. From this area, a variety of effective diameters may be obtained, depending on the instrument model and software settings. Instruments should be calibrated so that the reported effective diameter for polystyrene (PS) microspheres equals the actual microsphere diameter. Calibration procedures are given in the *Appendix*. Calibration of an instrument with PS microspheres may result in incorrect reported diameters for particles with low optical contrast. Refer to *Diameter Bias* for guidance in this circumstance.

### 3.4 Sensor Performance or Resolution

The particle size resolution of the instrumental particle counter is dependent upon the instrument model, flow cell, and hardware and software settings. Poor particle size resolution will lead to broadening of the PSD, which can lead to errors in reported particle concentrations. A procedure for determining instrument resolution is given in the *Appendix*.

### 3.5 Qualification Considerations

Qualification of the instrument should be based on frequency of use, maintenance schedule and accuracy requirements. Specific qualification considerations are similar to those for LO. Critical operational criteria consist of the following:

- *Integrated microscopy and fluid handling*—The fluidics system, microscope, and instrument control should be designed to provide repeatable imaging of particles, in a known fluid volume for each frame, at a controlled sample flow rate.
- *Instrument dynamic range*—The dynamic range of the instrument used (i.e., the range of particle sizes that can be accurately sized and counted) must include the smallest particle size to be enumerated in the product.

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- *Automated particle identification*—Particles should be identified with image threshold settings suitable for the detection of protein aggregates or other species of interest. The system should support classification of particles by size, morphology, and image intensity parameters. Agreement with visual analysis of the images should be verified by a skilled operator.

Reliable and accurate measurements require attention to several stages in the measurement process. Key quality activities include:

- Initial instrument qualification
- Method development
- Periodic operational checks
- Routine running of blanks and inspection of data
- Instrument maintenance

## INITIAL QUALIFICATION

All methods should be qualified for performance, considering the guidance in ⟨1225⟩. Specifically, volume accuracy, standards for particle size, particle count accuracy, and a determination of image resolution should be included in qualification.

## METHOD DEVELOPMENT

**Adsorption:** Compared to PS microspheres and other inert particles, some drug substances (e.g., proteins) are much more likely to adsorb onto the walls of the fluidic system. The degree of adsorption can vary with the nature of the formulation, the nature of the particulate matter, and the active pharmaceutical ingredient (API). To assess adsorption, run different prime or rinse volumes and check for variations in particle concentration. Run buffer blanks after the completion of a run and observe the particle counts in the blanks as a function of blank volume.

**Diameter bias:** Estimates of diameter bias may be corrected by adding an appropriate correction (which may be positive or negative) to the measured diameter of each particle, then recalculating the PSD. Diameter bias may be assessed by measuring monodisperse particles of a known size or polydisperse particles of known PSD provided that the chosen particles have optical properties similar to those in the test sample. Refer to ⟨1788⟩, *11.3 Validation Considerations for All Techniques* for further guidance. Consult manufacturer's recommendations for additional information and specific guidance.

The apparent diameter of a particle is affected by the following:

- The choice of threshold
- The degree to which the particle is out of focus
- Diffraction effects
- The ability of the instrument software to identify adjacent, low-contrast regions as a single particle
- Alignment of elongated or platelike particles in the flowing fluid

If the incorrect diameter is reported, PSD and particle concentration for affected size bins will be inaccurate. Concentration errors are most prevalent for small particles near the lower limit of the instrument's resolution and those that are difficult for the instrument to resolve (e.g., glass lamellae or protein aggregates in high-concentration formulations).

**Opalescence:** Particles may be obscured in samples that are highly opalescent. To assess the visibility of particles, spike microspheres of known diameter and concentration into a test sample, measure the microsphere diameter and concentration, and compare to the diameter and concentration expected. The choice of microsphere for this analysis should have a similar refractive index as the particles of interest in the test sample (e.g., silica microspheres could be used to mimic lower-contrast protein aggregates; PS could be used to mimic higher-contrast particles).

**Viscosity:** The low volumetric flow rate of FI instruments makes them generally insensitive to typical fluid viscosities. Confirm that no bubbles are introduced on priming or running at standard speed. For systems that use a peristaltic pump, confirm that the delivered volume is correct if the sample viscosity differs substantially from the viscosity of water. Reduce volumetric flow rates if necessary.

**Stuck particles:** Stuck particles may be observed during the actual data collection or in post-collection analysis. If particles or air bubbles become temporarily affixed in the detection zone of the sample cell, the data around the affixed particle should be eliminated using software recognition to avoid overcounting particles; if this is not possible, the entire data set should be discarded and the sample cell should be changed.

**Schlieren effects:** Inhomogeneities in solvent refractive index due to mixing (typically when water or buffer is mixed with a high-concentration protein suspension or cold solvent) lead to localized variance in the background intensity, which can impact analysis. Schlieren effects are often more pronounced when the sample viscosity is high relative to buffer or water. These inhomogeneities should be removed from the flow channel before the system background is recorded or data is collected. Priming with a sufficient volume of the sample to be measured, or with an equivalent solution, to reach the equivalent refractive index and viscosity of the test samples prior to its injection can remove such artifacts.

**Particle classification:** After data has been acquired, many FI systems enable the sorting of images by size, image intensity attributes, and morphological image attributes. This type of classification can be very useful in distinguishing silicone oil or air bubbles from other types of particles. However, care must be taken to ensure that the classification algorithms work as intended. Studies of different types of particles can be used to generate reference libraries, and additional measurements combining known populations of particles can be used to confirm the performance of the algorithms. As mentioned above, the "binning" should be verified by a subject matter expert to ensure the algorithm differentiates populations as expected.

**Software settings:** Instruments will differ in the degree to which software settings can be altered. The manufacturer should be consulted for recommended instrument settings for the type of samples being investigated. Common settings may include:

- Prime and sample volumes
- Flow rate through the cell
- Illumination intensity

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- Particle identification threshold
- Image processing settings (e.g., closing holes in the binary image)

**Hardware setup:** Instruments will differ in the degree to which hardware settings can be altered. The manufacturer should be consulted for recommended instrument settings for the type of samples being investigated. Common settings may include:

- Magnification
- Collimating lens
- Cell inner dimensions
- Sample delivery and mixing

There is a tradeoff between high optical resolution (obtained using high-magnification objectives with a high-numerical aperture) and large depth of field (obtained using objectives with low-numerical aperture). Large depth of field is necessary for accurate particle counts, but high resolution may be necessary for detection of low-contrast particles and accurate classification. Care must be taken that the choice of magnification and cell is compatible with the desired purpose of the test. For an instrument where sheath flow is used, throughput is less, but all of the particles are within the depth of field.

**Fouling:** Test samples with high particle loads or with particle sizes approaching the flow cell thickness may cause fouling of the cell entrance. Often, the flow path will only be partially clogged such that the liquid will continue to flow through the cell and small-size particles will still be observed. Evidence of partial fouling is typically a reduction in the observed concentration of larger size particles, a variation in count rate during a run, or visual observation of contaminants near the entrance of the flow cell. If fouling is observed, it may be helpful to backflush the flow cell to remove the clog, and then reconfirm the count accuracy using larger-size PS microspheres (e.g., 25 µm). For the best result, alternate liquid and air in the fluid line when flushing to change samples or to clean the flow cell.

### SYSTEM SUITABILITY

**Instrument configuration:** Verify appropriate instrument hardware and software settings and record them as appropriate (see *Analytical Instrument Qualification* ⟨1058⟩).

**Blanks:** Run filtered water as needed throughout the day to ensure cleanliness of the system. The measured concentration for a well-cleaned system is typically <60 particles ≥5 µm per milliliter. Some debris inside the instrument may be loosened and introduced into the count when exposed to surfactant solutions. Additional runs with filtered buffer or filtered test sample may be useful to confirm that carryover from the previous sample is acceptably low.

Prior to testing blanks, degas according to the guidance in ⟨1788⟩.

**Size and concentration standards:** It is prudent to verify system performance often according to the guidance in ⟨1788⟩. Failure to reproduce the size and/or count reference values can often be traced to a clogged or dirty flow cell, compromised tubing or pump, or other hardware problems. Errors in measured concentration may be due to partial clogs of the flow cell, especially for particles near the upper size limit. Refer to *Fouling* in the previous section for guidance. If counts are below the certified value or deviate statistically from prior readings, backflush, clean the cell, and repeat the concentration measurement.

### PRACTICAL MAINTENANCE

**Routine flushing:** Maintain system cleanliness by flushing with water or filtered formulation buffer between samples. Users may wish to introduce small slugs of air to assist in removing any trapped particulates from the flow channel between samples. Samples with particles that adhere preferentially to the flow cell may require rinsing with 70% isopropanol and/or surfactant solution to remove. The system should be flushed between samples to ensure background levels do not significantly affect the counts.

**Short- and long-term storage:** For short-term storage, the system should be cleaned as recommended by the manufacturer, rinsed with copious amounts of purified water, and kept filled with water to limit the accumulation of material or growth of organisms between routine uses. Long-term storage may necessitate the removal of all solvent from the FI system, possibly with the use of dry nitrogen to drive all water from the flow path.

## 4. TEST ENVIRONMENT

Specimens, sampling and specimen-preparation equipment (e.g., pipet tips, vials), and the FI instrument itself must be cleaned to the extent that the level of particles added by testing has a negligible effect on the outcome of the test. For FI instruments, the predominant sources of contaminating particles are often the fluidics components that come into direct contact with the test specimen. When cleaning these components, it may be necessary to disconnect tubing lines, etc., to permit cleaning of tubing joints or other crevices that can harbor particles.

See ⟨1788⟩, *12. Test Environment and Preparation*.

## 5. PARTICLE COUNTING ACCURACY (SYSTEM SUITABILITY)

The particle counting accuracy (system suitability) should follow the process described in ⟨1788⟩, *13. Instrument Standardization Tests*, using one of three standards: 1) USP Particle Count Set RS (a dilution may be used if deemed necessary); 2) commercial preparation of standard calibrator spheres of nominal diameter 5–30 µm, certified by the manufacturer; or 3) a laboratory-prepared suspension of standard calibrator spheres having a nominal diameter of 5–30 µm. (Refer to *3.1 Concentration Calibration* for additional information.) Spheres with diameters <5 µm are harder to distinguish from environmental or sample-bottle debris. Spheres with diameters >30 µm are more prone to measurement errors due to sedimentation.

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Stir continuously throughout the analysis, if possible. In systems without continuous stirring, the use of standards with a nominal diameter of 5–10 µm is recommended. Perform five counts on samples of the suspension. Obtain the mean cumulative particle count per milliliter for the main singlet particle peak, excluding the first run. The lower and upper diameter limits for delineation of the peak may be set by consulting the FI instrument vendor and any available information from the standard manufacturer.

Thorough mixing is imperative. Analysis must be conducted in a timely manner to avoid count bias due to the settling of dense species but in a careful manner to avoid generating new particles or breaking existing ones.

### 5.1 Interpretation

The instrument meets the requirements for *5. Particle Counting Accuracy (System Suitability)* if the count obtained within the specified diameter limits conforms to the values that accompany the USP Particle Count Set RS or other certificate of particle concentration. If the instrument does not meet the requirements for *5. Particle Counting Accuracy (System Suitability)*, repeat the procedure. If the results of the second test are within the limits given above, the instrument meets the requirements of the test. If on the second attempt the system does not meet the requirements of the test, determine and correct the source of the failures, and retest the instrument.

## 6. BLANK TEST

Refer to ⟨1788⟩ for guidance on blank handling.

Conduct a blank test (perform after the *5. Particle Counting Accuracy (System Suitability)* test to ensure that any microspheres have been cleared from the system). The blank test for the FI method ensures that the instrument, diluent, glassware, and environment are suitable. Failure of a blank consisting of nominally particle-free water is most often a reflection of contamination in the flow cell or in the fluidics upstream of the flow cell. The acceptance criteria of the blank may be set using the desired statistical precision based on the expected sample counts and the historical background levels observed with clean instruments. Obtain the mean cumulative particle concentration. The concentration obtained should be below a predetermined cutoff specified by the method developed in *3. Instrumentation Standardization Test*.

## 7. TEST PROCEDURE

### 7.1 Product Determination

Because the volumes for FI are small, the nominal volume of the product is not important. The analysis will reveal the nature of the particles present in the product and will be used to support the counts from the other techniques.

Deliver the aliquot into the sampler and count particles according to the developed method. Commercial FI instruments will generate reports of the PSD or of the number of particles above stated diameters, as determined by software settings and the specified method. On the basis of the volume of fluid imaged, the software will normalize the particle counts so that results are reported as particle concentrations as well as particle counts.

## 8. FLOW IMAGING CALCULATIONS

FI instruments typically report particle load as a number concentration of particles per milliliter. For determination of the particles per container, multiply the average particle concentration (in units of particles per milliliter) by the liquid volume of the full container (in milliliters):

Particles per container = average particles/mL × nominal product volume (mL).

## 9. CONSIDERATIONS OF CONCENTRATION LIMITS BY FLOW IMAGING

For properly conducted measurements, particle concentrations as obtained by FI will be more accurate than those obtained by LO for particles with low optical contrast (such as aggregated proteins). However, LO remains an important tool for assessment of particle load because:

- LO instruments are highly reliable and repeatable
- Regulatory requirements for non-US markets may require the use of LO
- There is a long history and significant accumulated experience with LO testing of pharmaceutical products, and the historical values of measured particle concentration are readily compared to contemporary LO measurements

The correlation between FI and LO results will be repeatable if the measured particles have relatively high optical contrast (i.e., a refractive index difference between the particle and the matrix liquid >0.15), or if the nature of the particles in the test samples is similar to that of the samples used to establish the correlation in the drug-characterization phase of development. PS particle standards give equivalent PSD profiles with FI and LO. However, very different particle counts may be obtained with natural particle populations, especially those with protein or other semisolid particles.

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# APPENDIX

## Methods for FI Instrument Verification

### CONCENTRATION CALIBRATION

The particle concentration reported by an FI instrument may be checked by measurement of commercial PS concentration standards. The particle suspensions should have a nominal microsphere concentration between 3,000 and 10,000 $mL^{-1}$, with a stated uncertainty of the certified value of 10% or better in an aqueous media. The PSD should be largely monodisperse with a mean diameter of either 5 or 10 µm. Microsphere suspensions should be thoroughly mixed prior to use by following the manufacturer's instructions. Perform a preliminary measurement of the PSD to identify the measured diameter of the main peak. Adjust the measurement protocol to establish diameter limits corresponding to the lower and upper bounds of the main peak. Manufacturer's guidance on the diameter limits corresponding to the certified value may be useful for this purpose.

Instrument hardware should be adjusted so that each run records at least 1100 counts in the main peak (to obtain a relative standard deviation from Poisson count variance of ≤3%). If the instrument cannot be so adjusted to achieve this count, the results of multiple runs may be combined to obtain sufficient counts.

### DIAMETER CALIBRATION

**Background:** Calibration and adjustment of FI instruments to give correct diameter readings are typically performed at the manufacturer's factory or by field technicians, using proprietary methods. The tests below give recommendations for instrument verification suitable for implementation by manufacturers or field technicians.

Users, in general, will implement the less complex system suitability tests discussed in *5. Particle Counting Accuracy*, which suffice to demonstrate continued reliability of instrument performance.

**Manual method:** Calibrate the instrument with a minimum of three calibrators, such as near-monosize PS microspheres having diameters of approximately 2, 5, 10, 15, and 25 µm, in an aqueous, particle-free vehicle.[1] The calibrator spheres must have a mean diameter within 5% of the nominal diameters and be standardized against materials traceable to National Institute of Standards and Technology (NIST) standard reference materials. The microsphere suspensions should have a nominal microsphere concentration between 3,000 and 10,000 $mL^{-1}$, in an aqueous media. The PSD should be largely monodisperse. Microsphere suspensions should be thoroughly mixed prior to use by following the manufacturer's instructions. Perform a preliminary measurement of the PSD to identify the measured diameter of the main peak. Adjust the measurement protocol to establish diameter limits corresponding to the lower and upper bounds of the main microsphere peak. Manufacturer's guidance on the diameter limits corresponding to the certified value may be useful for this purpose. The range between lower and upper bounds is intended to include all single spheres, taking into account the standard deviation of the spheres and the instrument resolution while excluding noise and aggregates of spheres. Determine the mean of the measured particle diameters, and compare it to the stated mean diameter of the calibrator spheres.

**Automated method:** The calibration (size response) curve may be determined for the instrument by the use of validated software routines offered by instrument vendors; these may be included as part of the instrument software or used in conjunction with a microcomputer interfaced with the counter. The use of these automated methods is appropriate if the vendor supplies written certification that the software provides an appropriate response curve and if the automated calibration is validated as necessary (see also ⟨1058⟩.

### METHOD FOR DETERMINING INSTRUMENT RESOLUTION

Determine the resolution of the particle counter for 10-µm particles, using the 10-µm calibrator spheres. Other particle diameters can be used when justified. The relative standard deviation of the size distribution of the standard particles used should be NMT 5%. Run the microsphere suspension through the system to obtain at least 1000 counts in the main microsphere peak. Filter the data to remove any particles with circularity <0.85. the data to obtain the PSD with bin sizes NMT 0.25 µm. Identify a range of particle bins that are symmetric about the bin that has the maximum count and a sufficiently broad range of diameters such that the lowest- and highest-diameter bin have <1% of the count of the bin with the maximum count. Compute the standard deviation of the diameters of the filtered data corresponding to the range identified by data binning. The instrument resolution may be calculated from the observed deviation using the formula for percent resolution given in *Light Obscuration Method for the Determination of Subvisible Particulate Matter* ⟨1788.1⟩, *Appendix.*▲ (USP 1-May-2021)

[1] ASTM standard F658-00a provides useful discussions pertaining to calibration procedures applying near-monosize latex spheres.

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***Change to read:***

# ▲⟨1788⟩ METHODS FOR THE DETERMINATION OF SUBVISIBLE PARTICULATE MATTER▲ (USP 1-May-2021)

***Add the following:***

▲

1. INTRODUCTION
2. SCOPE
3. BACKGROUND
    3.1 Testing Considerations
    3.2 The Nature of Particulate Matter
4. DETECTION AND ENUMERATION OF PARTICLES IN DIFFERENT SIZE RANGES
5. APPLICABILITY OF METHODS
6. PARTICULATE MATERIAL EVALUATION
    6.1 Final Filter Exemption
7. USP SUBVISIBLE PARTICULATE MATTER LIMITS
8. CONTROL OF PARTICULATE MATTER
    8.1 Development
    8.2 Statistical Sampling Plan
    8.3 Risk-Based Approach
9. SILICONE DROPLETS AND AIR BUBBLES VERSUS NON-SILICONE PARTICLES
10. CHALLENGING SAMPLES
11. FACTORS AFFECTING ALL METHODS
    11.1 Guidelines for the Use of Calibrator Spheres
    11.2 Elements of Measurement Quality Control
    11.3 Validation Considerations for All Techniques
12. TEST ENVIRONMENT AND PREPARATION
    12.1 Sample Handling
    12.2 Sampling Practices
13. INSTRUMENT STANDARDIZATION TESTS
    13.1 Diameter Calibration
    13.2 System Suitability Tests
14. CALCULATIONS
    14.1 Pooled Samples
    14.2 Individual Samples
    14.3 Individual Unit Samples
GLOSSARY
APPENDICES
REFERENCES

▲ (USP 1-May-2021)

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## ▲1. INTRODUCTION

It is the intent of *USP* general test chapters *Injections and Implanted Drug Products* ⟨1⟩ and *Particulate Matter in Injections* ⟨788⟩ that systems, packaging, and formulation be selected and developed with regard to commercial product cleanliness and stability. Monitoring and limiting particulate levels are an important effort in ensuring product quality and controlling the risk of adverse effects or loss of drug efficacy.

A related chapter, *Measurement of Subvisible Particulate Matter in Therapeutic Protein Injections* ⟨1787⟩, categorizes particulate matter as intrinsic, extrinsic, or inherent. Process streams, equipment, and other factors related to the manufacturing process may contribute intrinsic particulate matter. Formula components and container/closure systems may contribute to immediate and even long-term generation of intrinsic particles. Poor selection or cleaning of components and systems can exacerbate these problems. The production environment, including personnel gowning and behavior, may contribute extrinsic particulate matter to the filled product. Finally, some products may have a small level of unintended particulate matter that is inherent to the product, even after careful formulation. All of these contributors to particulate matter and final product quality may be evaluated by the methods in ⟨788⟩ or variations thereof.

Historically, the first USP requirements for determination of subvisible particulate matter used the microscopic membrane (MM) method. Membrane filtration has the advantage of collecting a wide size range of particles and retaining these for later analysis, but MM is labor intensive and requires special facilities and operator training. Because it is automated and robust, the

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light obscuration (LO) method was introduced in USP standards in 1985. By 1995, both methods had been optimized and applied in ⟨788⟩ to both large and small volume products. *Appendix 1* contains further information on the historical development of ⟨788⟩.

Several *USP* chapters are related to subvisible particles, including three compendial chapters, *Subvisible Particulate Matter in Therapeutic Protein Injections* ⟨787⟩, ⟨788⟩, *Particulate Matter in Ophthalmic Solutions* ⟨789⟩, and the aforementioned informational chapter ⟨1787⟩. This informational chapter is meant to complement the others and focuses on the description of the LO, MM, and flow imaging (FI) methods. All methods should address the specific analytical life-cycle requirements for the individual methodologies (*1*).

Chapters ⟨788⟩ (a harmonized chapter) and ⟨789⟩ provide an LO test (*Method 1*) for sizing and counting of subvisible particles (generally 2–100 µm) for nearly transparent, low viscosity ("waterlike") product samples, and an MM particle count test (*Method 2*) for manual particle sizing and counting as an alternate method. ⟨787⟩ also includes LO and is especially pertinent to protein therapeutics. FI is recommended as a third method to complement LO and MM, especially for particle characterization, and during product development. Although FI is not included in ⟨788⟩, FI can provide useful information on the types of particles in a test sample, aiding interpretation of LO and MM results, particularly for protein therapeutics.

In the LO method, a laser beam shines through the liquid sample as it is drawn through an optical flow cell. Any particles, air bubbles, or liquid droplets will scatter or absorb light to some degree, briefly reducing the total transmitted light. Each transient drop in detected light is counted as one particle. The MM method consists of filtering the liquid sample, inspecting the dried filter through a microscope, and manually counting particles above stated size thresholds. Soft objects, such as liquid droplets or aggregated proteins, can pass through or absorb onto the filter and are not reliably counted by this method For the FI method, the sample is drawn through a flow cell and a sequence of photographic images obtained of particles in the sample. Subsequent automated analysis gives image parameters such as size, morphology, and image intensity, which are useful to categorize particle images and provide improved understanding of the different particle types observed.

Product packaging, formulation, administration, and sourcing have become much more diverse since the 1980s. Improvements in manufacturing and filling arena design and control and the evolving quality of packaging components have steadily reduced the total particle load from adventitious contamination. This particle reduction has come through particle remediation efforts and improvements in product process control. (See *Visual Inspection of Injections* ⟨1790⟩*, 4. Inspection Life-Cycle*.)

The complexity of products has expanded to include smaller delivery volumes, specialized dosing and devices, the advent of therapeutic proteins and other biologics, and issues of product and package physical instability. This has led to increased scrutiny of particle load, with an emphasis on particles resulting from formulation-related changes (intrinsic) and product characteristics (inherent), especially for the subvisible particles (defined here as particles between 1 and 100 µm).

## 2. SCOPE

This chapter is meant to expand upon the information in ⟨787⟩, ⟨788⟩, and *Particulate Matter in Ophthalmic Solutions* ⟨789⟩ by including FI techniques and by providing additional guidance on the recommended use of LO and MM.

The use of the FI method for particle sizing and enumeration in this chapter constitutes a substantial update to past approaches. FI has become a valuable orthogonal method that provides morphology data. These data contribute to a better understanding of the particle population, help resolve discrepancies between other methods, and provide insight into further definition of the sources of the particle load, supporting risk assessment and evaluation of acceptability. This technique is commonly used for characterization of product and process. FI is not currently planned for inclusion in ⟨788⟩ because: 1) ⟨788⟩ is a harmonized chapter, and addition of a new technique would require new discussions for acceptance; 2) this is an emerging technique and there is both a lack of historical data from products that have patient exposure and an undetermined relationship of the particle size distribution obtained with FI relevant to that determined by LO or MM, making the establishment of specifications difficult; and 3) specifications (if/when set in the future) should be set by the stakeholder/company and should be product specific, based on prior knowledge and risk assessment.

This chapter directly supports ⟨788⟩ and ⟨789⟩; however, the chapter is also useful for ⟨787⟩, which addresses particulate matter testing for therapeutic protein products. Therapeutic protein products require special care in terms of particle measurement, and ⟨787⟩ provides modifications to ⟨788⟩ methods that are suitable for protein and other large-molecule formulations. This chapter incorporates those improvements because these are applicable to small molecule formulations as well.

The informational chapter ⟨1787⟩ describes additional tools and instrumentation for analyzing subvisible particles and remains a solid resource for forensic methods. The recommendations in ⟨1787⟩ are applicable to ⟨788⟩ and ⟨789⟩ as well. The techniques included in ⟨1787⟩ are all commercially available and can be used during product and process characterization. However, more experience and development is often necessary before methods can be validated and qualified to the extent necessary for lot release, for use in quality control laboratories, and for application in all product forms.

Tracking and identifying particles allow identification of the sources of the particle load and provide a means to evaluate product acceptability and drive product improvement. USP subvisible particle concentration limits apply to particle sizes ≥10 µm. Note that ⟨788⟩ carries limits for 10- and 25-µm particles, and ⟨789⟩ carries limits for 10-, 25-, and 50-µm particles. These limits were based on historical clinical exposure, not necessarily the demonstrated impact on patient safety (*2*). The use of the LO and FI methods at smaller diameters can provide valuable insights into product quality, especially in the development of biotherapeutics, as described in ⟨1787⟩. The impetus for evaluation of the <10 µm particle population for protein formulas is addressed by Carpenter et al. (*3*), Singh et al. (*4*), and Cao et al. (*5–6*).

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# 3. BACKGROUND

## 3.1 Testing Considerations

LO is the preferred first-pass method for ⟨787⟩, ⟨788⟩, and ⟨789⟩, although certain solution formulations may not be easily analyzed using this technique. The LO method may encounter problems with a product that does not have clarity or viscosity approximating that of water. Further, formulation characteristics (e.g., color or viscosity) or inherent formulation properties (e.g., shear-induced changes) may generate erroneous LO data. Similarly, products that produce air or gas bubbles when drawn through the LO sensor, such as bicarbonate-buffered formulations, may generate erroneous high-bias data. LO has an inherent bias for sizing larger or fibrous particles (Feret's diameter ≥50 µm). For fibers that are longer than the illumination zone, LO will report an equivalent circular diameter that is closer to the fiber width than the equivalent circular diameter based on the projected area of the fiber (*7*).

The LO method can also be applied to particle sizes considerably smaller than the ⟨788⟩ lower limit of 10 µm with good reproducibility (*5*) and to particles sizes much larger than the upper limit of 25 µm, with proper sensor selection. Thus, valuable information on the product for visible and subvisible particle content is enhanced by including a few more size categories in the subvisible methods such as ≥2 µm and ≥5 µm, the compendial thresholds ≥10 µm and ≥25 µm, and perhaps ≥50 µm and ≥100 µm. Chapter ⟨1787⟩ provides guidance and advice for many orthogonal approaches that can also cover expanded size ranges.

FI is also useful for long-axial-dimension particles, yet it may encounter flow restriction biases. During calibration, size thresholding for LO is determined against spherical calibration standards, thus the results are reported in terms of equivalent circular diameter. MM and FI utilize direct comparison to linear standards. For particles of low refractive index close to that of the solution (such as protein aggregates), the diameter reported by LO will be smaller than the physical diameter of the particle, especially at sizes ≤5 µm (*8*). Any particles of entrained air or immiscible liquids (primarily silicone oil) commonly encountered in liquid samples are counted and added to the total particle population. Immiscible liquids and air are not detected by MM and are observed directly as high-contrast images by FI.

The MM method is useful for testing formulations that are not amenable to LO, such as those with color and viscosity affecting optical detection and those that are emulsions. MM isolates particles as a filter retentate, which a trained and qualified operator then uses to determine the count and size population. The isolate may be used for characterization of the individual particles and forensic microscopic categorization. The filter isolate in the membrane assay can retain particles ranging in size from the nominal pore size to millimeters. The dried, retained solid may differ from its nature in the product liquid. While the operator counts particles on the membrane surface that meet counting criteria (≥10 µm and <25 µm or ≥25 µm), there is also a concurrent, streaming view of particle appearance that provides an impression of the particle types or categories that are present. MM methods will not be useful for evaluation of silicone oil droplets or protein aggregates, because these flexible, amorphous particles are difficult to detect on the membrane and can pass through the pores as well. However, MM often can more accurately measure the size of particles with unusual aspect ratio or physical/optical properties.

The FI method has the advantage that all particles, liquid droplets, and air bubbles are captured in microscopic images. The images can be classified by morphological and image-intensity parameters, as well as by particle size. The classification can be useful in determining the relative proportions of different particle types, such as silicone oil droplets and protein aggregates. The reliability of the classification must be carefully qualified, especially for sizes <5 µm. FI has the additional advantage that particles of low refractive-index difference from the matrix formulation are more accurately sized than in LO, although these particles can still be problematic. Selection of proper flow cells and optics configurations in FI can provide particle detection <10 µm or >50 µm. Chapter ⟨1787⟩ provides more detail on alternate techniques for protein particle characterization. See also ASTM E3060-2016 for additional guidance on FI methods (*9*).

With past development of ⟨787⟩, ⟨788⟩, and ⟨789⟩, the focus was always on extraneous or foreign matter present in the finished drug product. Concerns about safety related to capillary occlusion and sterility, as well as pharmaceutical elegance, primarily drove this focus. Safety concerns about large numbers of subvisible extraneous particles were expressed by Langille (*2*) and Bukofzer et al. (*10*). However, there is an increased understanding that additional particles may result from the manufacturing process, from product or container degradation, or from properties of the drug product such as self-association. The presence of particles from any of these sources is an important product quality factor. Accurate particle concentration measurements are critical for understanding the content and source of the particulate matter and for ensuring long-term product stability. A more comprehensive explanation of particle categories is provided in *3.2 The Nature of Particulate Matter*.

In the case of protein-based pharmaceutics, there is an additional source of particles and associated product safety concerns. Proteins self-associate based on the inherent molecular properties of the specific molecule and can form aggregates in a broad size range, including 1–100 µm and larger. Proteinaceous particles in this size range can be quantified using LO and FI. Aggregated proteins have the potential to be immunogenic, adding another level of complexity to the monitoring and control of subvisible particles in protein therapeutics. On the other hand, protein-based vaccines may consist of aggregates or organized complexes such as viruslike particles, as inherent components of the vaccine product. In vaccines, immunogenicity is a desirable attribute, and subvisible particle monitoring may be performed to monitor product quality and batch consistency. The strategy and practices described in this chapter provide approaches for the safety and quality evaluation of the drug product. Safety perspectives for proteinaceous subvisible particles were introduced (as counterpoints) by Carpenter et al. (*3*) and Singh et al. (*4*) and more recently addressed in commentaries by Corvari et al. (*11*) and Narhi et al. (*12*). This has resulted in a substantial body of work exploring the potential immunogenicity of protein particles, which was reviewed by Moussa et al. (*13*), among others.

## 3.2 The Nature of Particulate Matter

Particles arise from many different sources. Over time, with growing experience in manufacturing and administration of therapeutics, it has become apparent that not all particles are equivalent in terms of their effects on safety or indications for

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product stability. One way to capture this difference in the risk associated with particles is to classify them into three broad categories: extrinsic, intrinsic, and inherent (See ⟨1787⟩). However, within these categories, different particle types can pose different patient safety risks. Since particle counting instruments have limited ability to differentiate among particle types, no single technique may be adequate to delineate the categories and track the particulates of most concern for product quality.

*Ophthalmic Products—Quality Tests* ⟨771⟩, *Injections and Implanted Drug Products* ⟨1⟩, ⟨787⟩, and ⟨1787⟩ have all adopted a unified definition for particulate matter. Two categories, extrinsic and intrinsic, describe the traditionally held concept of particles as foreign matter. A third category, inherent, describes particles that represent the character of the drug formulation and typify acceptable product. For further details, refer to ⟨1787⟩. The *European Pharmacopoeia* and *Japanese Pharmacopoeia* use two categories, extrinsic, which is from outside the manufacturing process, and intrinsic, which includes anything from the manufacturing process or the active ingredient and formulation components (the latter two described as inherent in the *USP*).

Heterogeneous particle populations are common (e.g., more than one chemical entity with many variations of density, refractive index, color, and shape). Heterogeneous particles are classified as either extrinsic or intrinsic based on the non-proteinaceous component that presents the greatest risk. Orthogonal techniques must be used to fully understand this particle type. As with all quality attributes, the ultimate decision on what levels are allowed should be tied to risk assessment and the clinical history of the product.

## 4. DETECTION AND ENUMERATION OF PARTICLES IN DIFFERENT SIZE RANGES

Particle formation often occurs across a size continuum with the lower end of the visible range near the upper region of the detection methods described herein (see *Figure 1* and ⟨1790⟩). The size range detected depends on the particle type and method used, and care should be taken to choose the appropriate method for the population being tested.

The techniques reviewed in this chapter are applicable for the determination of unwanted particle content, such as contamination, solids from instability, and insufficient package cleaning, as well as particles that arise from lubricants, product contact, formulation components, and the active pharmaceutical ingredient (API) itself. These particles vary in size from tens of nanometers to hundreds of micrometers, and many particle types do not have a defined size. Instead, they occur as a broad distribution of sizes, often with many more particles in the smaller size portion of the distribution.

Pharmaceutical products come in various forms with large differences in particle size distributions and in concentrations of particle types. Drug forms may include nanoparticulate suspensions, solid drug suspensions, and emulsions. Extremes of drug concentration and factors such as color and transmission variability, along with a wide array of active ingredient sources that have small to very large molecular weights, may all play a role in particulate formation. Packaging varies in terms of fill volumes and materials, which include soft polymers, glass, and hard polymers. Medical devices, combination devices, and unique drug delivery systems are also on the market. Sampling many of these systems presents a challenge to existing conventional procedures.

For example, products formulated as non-solutions are inherently particulate in nature and can thus complicate the analysis of the unwanted subvisible particles within the overall product. For this reason, it may be necessary to monitor subvisible product at non-adjuvanted/intermediate product stages, where justified. One can also evaluate the particle load of a placebo or media fill on the same line to demonstrate quality. Whatever their nature, monitoring of subvisible particles is important to ensure consistency from lot to lot and between clinical and commercial products.

Evaluation, test method development, and control for particles in the formulation in the <10-µm range can be important for some of these products. For biologics and vaccines, detection below 10 µm is important to capture protein aggregate contents (see ⟨787⟩ and ⟨1787⟩). Ophthalmic solutions require particle determination at an additional 50-µm threshold, as specified by ⟨789⟩. Correlation of subvisible content with the visual inspection program (see ⟨1790⟩) promotes the use of higher-end instruments to probe the lower end of the visible size range. Certainly, there are other applications that may affect the system design and use. Calibration is described in ⟨788⟩ as "….apparatus is calibrated using dispersions of spherical particles of known sizes between 10 and 25 µm. These standard particles are dispersed in particle-free water." When detecting particles <10 µm and those ≥50 µm, as discussed in ⟨789⟩, calibration may be necessary far outside the conventional range set for in ⟨788⟩ in order to address the above considerations.

The sizes reported and the populations interrogated are quite dependent upon the technique, so it may be necessary to run orthogonal techniques plus qualitative particle identification to assess the particle population with confidence. Chapter ⟨1787⟩ provides an overview on the practical use of commercial instrumentation for the pharmaceutical subvisible range (2–100 µm). The difficult <2 µm domain, which is of interest for quantifying inherent protein-particle content of biotherapeutics, will require multiple techniques for full understanding (*14*). Note that ⟨787⟩ and ⟨1787⟩ focus on the inherent protein (or other formulation) particles, rather than contamination analysis.

No single method should be used during development, but rather orthogonal testing should be employed for full characterization and understanding of the expected particle population for that particular product. During production, if the LO lot release method fails, these methods can be deployed to determine root cause and other factors.

All three methods produce appropriate counts in size thresholds when used properly and applied to appropriate sample formulations and particle populations. But each can be misused, and each has inherent drawbacks to consider. Calibration and operator training are imperative. Instrument standardization and count accuracy demonstrate appropriate configuration. See *Table 1*.

The comparison in *Table 1* considers visible to subvisible techniques recommended for all particle sizes. It underscores that an orthogonal approach for particle evaluation and tabulation is important to obtain comprehensive information about the particle population and thus product fill integrity and product stability. The ⟨788⟩ guidance methods are used as a baseline and as prospective release methodology. The use of additional non-compendial techniques may be necessary to develop a full understanding of the particle population. An example of these techniques has been reviewed in ⟨1787⟩ and in a 2016 *Stimuli* article (*15*).

Final product quality regarding particulate matter must include both the visible and subvisible size ranges, which may be related for certain incidents involving contamination or product instability. Inherent particles resulting from protein

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self-association often span a continuum from sub-micrometer to visible particles as well. Chapter ⟨1790⟩ provides a comprehensive discussion of visible particle detection systems and product development considerations.

## 5. APPLICABILITY OF METHODS

All sterile solutions for injection or parenteral infusion are included within the scope of these test regimens. However, some solutions are amenable to the methods while others are not. All will require qualification of the methods for specific product forms and formulation families.

Clear, aqueous-solution formulations with low to moderate concentrations of the active pharmaceutical ingredient (API), and simple vehicles, will be the most suitable (with minimal method development or changes) and amenable to all ⟨1787⟩ and ⟨1788⟩ methods. Of greatest concern for all techniques are formulations that have competing solids, suspensions (either as-is or after resuspension), emulsions, and nanodispersions. Many of these issues may be addressed by additional sample treatment. Informational chapter ⟨1790⟩ and *PDA Technical Report No. 79: Particulate Matter Control in Difficult to Inspect Parenterals* (*16*) discuss options for analyzing the visible particles in these products, which are also applicable to subvisible assays.

Certain products may exist as suspensions, i.e., containing insoluble components in the formulation. Examples include cellular therapeutics and adjuvanted vaccines. Such products may not be tested directly unless a phase separation of the product is feasible, allowing subsequent analysis. Other techniques described in ⟨1787⟩ may be useful.

**Table 1. Comparison of Visual Inspection, LO, MM, and FI**

| Technique | *USP* General Chapter(s) | Sample Volume | Compendial Method-Based Detection Ranges | Method Artifacts | Method Limitations |
|---|---|---|---|---|---|
| Visual | *Visible Particulates in Injections* ⟨790⟩, ⟨1790⟩ | Container volume in a defined cylinder of view | Probabilistic, nominally single particles >100 µm in reflected light; color, opacity, and refractive index (RI) difference aid detection | Subjective decisions, container seams and irregularities, air bubbles | Particulate matter (PM) of small size, transparency, or low contrast, dwell time and depth of field or container/formula limitations on detection |
| LO | ⟨787⟩, ⟨788⟩, ⟨789⟩, ⟨1787⟩, ⟨1788⟩ | 0.2–5 mL per draw of product or test fluid | PM >2 µm up to sensor limit; shadow on the detector from particles in confined cell between illuminator and sensor; upper limit depends on the sensor used; opacity, RI difference aid detection | Air, immiscible liquids, Schlieren effects, cell soiling, high PM concentration | Low contrast and large PM, >500 µm; floaters, sinkers, very thin flakes; size range determined by sensor choice |
| MM | ⟨788⟩, ⟨789⟩, ⟨1788⟩ | 0.1 to hundreds of milliliters per draw of product or test fluid | PM >5 µm, virtual optical image by reflection and shadowing on membrane substrate; particles are retained on the membrane for subsequent analysis, if desired | Membrane features, PM in rinse or glassware, deposition/drying effects on membrane | Air, immiscible liquids, soft or gelatinous semi-solids that may sink into surface; sample must be chemically compatible with membrane and also visually discernible |
| FI | ⟨1788⟩ | 0.5–1 mL per draw of product or test fluid | PM generally >2 µm to an upper limit depending on the flow cell, digital camera images captured; image and dimension may be evaluated post-collection | Particles adhering to cell walls, transient air gaps; cell contamination is effectively removed by background subtraction; changes in cell contamination are a bigger issue | Low-contrast PM, floaters, fibers; size range determined by flow cell |

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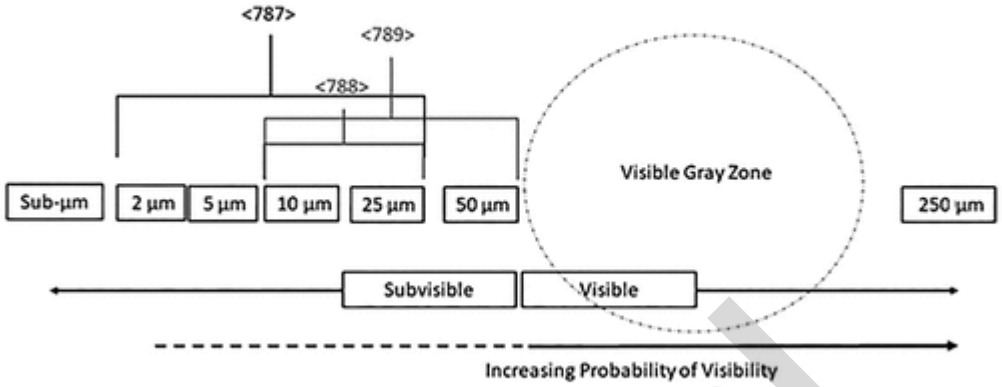


Figure 1. Continuum of particle sizes from subvisible through visible, indicating the scope of ⟨787⟩, ⟨788⟩, and ⟨789⟩.

*Figure 1* provides a schematic overview of the size ranges within the scope of ⟨787⟩, ⟨788⟩, and ⟨789⟩. Brackets indicate the range of lower size limits requiring particle concentration determinations for each of these chapters. The "Visible Gray Zone" refers to the size range where particle detection by visible inspection is probabilistic, with the probability of detection falling to zero near 50 µm.

## 6. PARTICULATE MATERIAL EVALUATION

For all particle determination efforts, and ultimately to reach a comprehensive control strategy, it is necessary to understand the category, if not the identity, of the particle population. As described in this chapter, extrinsic and intrinsic particles may be quantified (using LO), quantified and described (using MM and FI), and isolated (using MM) for further analysis.

For extrinsic and intrinsic particle varieties, it is recommended to use a database consisting of an internal particle library that provides particle references. The database is a key component of particle classification, risk assessment, and the ultimate identification. Some public domain and vendor databases are available, but the best database comes from compilation of particle species from your own operations.

Evaluation of the inherent particle-population baseline will utilize ⟨788⟩ techniques but also those described in ⟨1787⟩. For protein therapeutic drugs, establishment of the inherent baseline of proteinaceous particles is necessarily important and has been discussed in detail (*17*).

LO provides only particle concentration for specified size bins. Although these data are valuable for tracking product quality and manufacturing consistency, LO does not differentiate among particles based on particle types. Both MM and FI provide additional information on particle attributes other than size. FI reveals the images of all particles streaming by the camera system and thus allows classification according to morphology and image intensity. MM not only tabulates counts but also reveals the particle image and provides an isolate for analysis after counting.

However, much more can be done to fully characterize and possibly identify particulate matter with an integrated approach using light microscopy, vibrational spectroscopy, elemental analysis, and electron microscopy (*18–20*). These references describe a forensic pathway of identification, and ⟨1787⟩ provides a review of useful techniques with their advantages and disadvantages.

Applicability of any of these tests for specific products should be evaluated using appropriate qualification steps. Qualification of the instrument should be based on frequency of use, maintenance schedule, and accuracy requirements. General guidance on performing qualification is provided in *Validation of Compendial Procedures* ⟨1225⟩ and *Verification of Compendial Procedures* ⟨1226⟩.

### 6.1 Final Filter Exemption

General chapter ⟨788⟩ provides the following: "Parenteral products for which the labeling specifies use of a final filter prior to administration are exempt from the requirements of ⟨788⟩, provided that scientific data are available to justify this exemption." The purpose of this exemption is to offer a mechanism to reduce the subvisible particulate burden inherent in the final drug product when the amount would exceed acceptable limits and cannot otherwise be reduced through formulation adjustments or the manufacturing process prior to use. The utilization of an in-line filter should be driven by risk benefit evaluation of the potential clinical implication of subvisible particulates for the intended user population, but not be recommended to mitigate a Current Good Manufacturing Practices (cGMP)-related product quality issue (e.g., the presence

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of extrinsic particles). The application of this exemption should be supported by scientific evidence sufficient to justify its adoption and generally include the composition and mechanism of formation of the particles in addition to demonstrating that process-optimization strategies failed to reduce the particle load. Labeled recommendations of filter type and size should also be supported by appropriate drug product compatibility assessments, consistency in particle removal, and clinical data as necessary. Demonstration that drug potency is not significantly affected is essential. An associated risk assessment is strongly encouraged to evaluate the potential clinical implications to the intended user populations if a final in-line filter is not utilized.

## 7. USP SUBVISIBLE PARTICLE LIMITS

Current limits for ⟨787⟩ and harmonized ⟨788⟩ methods are set at maximum allowable particles per container or particles per unit volume at size thresholds ≥10 and ≥25 µm. These limits are historical and have changed over time based on method development and species of concern. Chapter ⟨789⟩ applies the 10- and 25-µm thresholds as well, plus a 50-µm threshold. In ⟨789⟩, the limits are provided only on a per milliliter basis. Chapter ⟨771⟩ provides the direction for the testing of ophthalmic products, using ⟨787⟩, ⟨788⟩ or ⟨789⟩ limits, based upon product application.

The calculation of allowable particle content is based on product volume (as determined by the manufacturer) for the SVI market, and on a particles-per-milliliter basis for the LVI market. However, these limits are generous, and actual particle loads are often an order of magnitude below them. All efforts to reduce particle load and maintain a consistent low-particle contribution are encouraged.

**Table 2.** ⟨788⟩ **Particle Content Limits**

| | Technique | | | |
|---|---|---|---|---|
| | LO | | MM | |
| Technique range | 2-500 µm | | 5 µm to several millimeters | |
| Particle size | ≥ 10 µm | ≥ 25 µm | ≥ 10 µm | ≥ 25 µm |
| SVI ≤100 mL | 6000/container | 600/container | 3000/container | 300/container |
| LVI >100 mL | 25/mL | 3/mL | 12/mL | 2/mL |

[NOTE—Chapter ⟨787⟩ limits the total content of particles in LVI products to 6000 particles ≥10 µm and 600 particles ≥25 µm.]
[NOTE—For ophthalmic products, please consult ⟨771⟩]

*Table 2* gives the particles per container or particles per volume requirements from ⟨788⟩. No limits have been established for the use of FI or for the collection of data with size thresholds <10 µm for ⟨787⟩, ⟨788⟩, and ⟨789⟩. As *Table 2* shows, the registration limits vary according to technique and injection product volume. Furthermore, as discussed previously, the technique capabilities are certainly not equivalent across a broad range of sizes.

Particle concentration should be monitored even for samples that give results below the *Table 2* limits. Although technically within USP specifications, particle content in a single container near the limit may be a concern and may indicate problems in product quality. Results that are out of trend with what is normal for a product, even if well below the specification limits, would be of concern as well and should also be investigated. The use of additional analytical methods and the imparting of controls specific to the medication and dose are important extensions of this guidance.

The *Table 2* particle concentration limits are often significantly higher than the particle concentrations observed with many commercial products. The limits apply both at time of release and at the end of shelf life, and they apply to a range of product types, from the simplest (e.g., sterile water in an ampule) to the most complex formulas with stability issues. As mentioned in *1. Introduction*, this is a limit based on historical experience; internal target choices, discussed in *8.1 Development*, allow for consideration of product histories, product types, therapeutic uses, and patient populations.

The intent of these tests is to provide a control strategy framework for designing the most stable formula, selecting the most stable and inert package, and employing an elegant fill/finish in a high-quality, state-of-the-market factory. The limits are imposed as a minimal standard of control, with the intent that the innovator, contract manufacturing operation, or generic producer will meet and exceed this standard across all injectable dosage forms, as appropriate to ensure high product quality. A strategy to achieve these goals is discussed in *8. Control of Particulate Matter*.

## 8. CONTROL OF PARTICULATE MATTER

It is the intent of ⟨1⟩ that in the design of formulations, selection of packages, and final assembly of packages, a stable final product is attained. Using the methods and limits in ⟨788⟩ and ⟨789⟩, high-quality commercial product cleanliness and stability are verified. This state can be described as "clean" (few particles) and "stable" (avoiding changes that promote particle growth). Process streams, equipment, filling lines, pumps, staging tanks, personnel gowning, packaging cleanliness, and environmental quality all may contribute particulate matter to the product. Insufficient cleaning of product components and inappropriate selection of both container–closure systems and formula components may contribute to immediate and long-term generation of particles. Intrinsic sources of detectable particulate matter may be present but not yet evident until particles form over time, sometimes long after lot release. This should be assessed by performing drug stability studies, and subsequently controlled in the commercial product.

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## 8.1 Development

Due diligence using particle detection methods endorsed by USP or alternate method strategies (new methods, not in *USP–NF*) for the best product design is recommended during product and process development and may be continued in selected evaluations during commercial production. The use of many methods early in development is an important consideration for limiting the number of particles in the product and for full understanding and characterization of the particle population.

To accomplish this, start early and conduct analyses often, using methods appropriate to the product development phase (*11*). Analysis using only lot release methods will give a limited understanding of the particle population. As the development cycle progresses, the use of orthogonal techniques provides greater confidence in particle assessment. It is important to explore the incidence of change in the number of particles during early phases of development to provide the optimal selection of formulation, manufacturing process, and packaging. Also, prior to commercialization, the character of inherent solids must be fully explored to establish the range of its acceptability.

Particles may form during manufacturing or over time due to any one or more of the following factors:

- Formulation instability
- Package cleanliness
- Package selection (quality/stability)
  - Inorganic extracts
  - Organic extracts
  - Product-package interaction
- Process contributions
  - Stresses throughout the process (e.g., pH swings, surface exposures)
  - Inorganic extracts
  - Organic extracts
- Silicone content

When evaluating particle data, consider the basis for size and number generation as well as the types and sizes of particles that each technique can or cannot reliably detect. Also consider when a change of particulate amount and/or distribution is or is not significant. Likely it depends upon the basis of the technique and its demonstrated precision for quantification of the particle type of interest. Important considerations are matrix and particle character such as opacity, density, color, refractive index, shape/morphology, and true nature (e.g., liquid, matrix-aggregate, agglomerate). Results should be qualified by known standards matching the particle type(s) of interest to the extent feasible. In the case of interfering particle populations (e.g., silicone droplets interfering with counts of protein aggregates), qualification may include measurement of two single-component populations and mixtures of these.

The chosen target specifications unique to a product are an important extension of the USP limits in *Table 2*. In the implementation of a target-specification strategy, several tenets apply:

- Understand and minimize inherent particle load when appropriate
- Minimize intrinsic particle load
- Identify and characterize particles during development, and establish a particle database
- Correlate particle load to visible particle rejects
- Track the particle population within product families and across historical batches to reveal anomalous trends and initiate remediation
- Establish targets for particle content relevant to product family, including considerations of manufacturing arena, packaging, and formulation complexity

## 8.2 Statistical Sampling Plan

Chapter ⟨788⟩ provides the sampling plan according to product volume, while ⟨787⟩ emphasizes a statistically relevant sample plan independent of volume. For all products, regardless of volume, comprehensive experience regarding the integrity and consistency of the batch is gained throughout development by analyzing a number of samples throughout each batch. Experience obtained during product development indicates the proper sampling plans to be applied in commercial production to ensure that sample selection is representative of batch quality. All batches must have sampling plans that accommodate desired statistical measures of batch quality and facilitate process control. Earlier stages of drug development will require less statistical precision than late-stage development or release. The sampling plan should consider the statistical precision of the obtained counts, the likely heterogeneity within a vial or other vessel, the likely heterogeneity between vials or other vessels, and variations in particle concentration with time or other variables.

A combination of individual and pooled counting data should be considered in statistical evaluations to provide confidence in commercial release testing. For example, during development, primary consideration is given to formulation stability, because production is usually in smaller batches and not in the final at-volume commercial arena. Ideally, all containers contain the same low level of particulate matter; however, there are conditions that promote heterogeneity or, more specifically, produce containers with higher particle load than average. These conditions include the following:

1. Insufficient or inconsistent container washing
2. Particle load on the stoppers
3. Variations in silicone coater performance
4. Particles contributed by the filling equipment (particle load may vary from pump to pump)
5. Particles contributed by the drying/sterilization system
6. Failure of aseptic filling conditions

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7. Self-association of active ingredient or formulation components
8. Particles generated over time, due to formulation instability or product-package interaction. Inherent particles have been observed to have highly non-repeatable growth kinetics such that one vial may have a single large particle while other vials of the same lot have multiple small particles.

Chapter ⟨788⟩ provides a sampling plan according to product volume. This plan is based solely upon the limitations of traditional optical counting instruments for adequate, multiple-sample aliquot draws. Originally, the sample volume for the LO method was set at 25 mL and maintained for years, primarily due to the limited capability of the then-primary instrument, the high-accuracy particle counter (HIAC), to draw 5-mL sample volumes. Collecting sample aliquots for averaging provided a single value for the batch. Accordingly, the same sample volume was maintained for MM, but this volume was not due to a method restriction. Due to the current capability of MM to process any liquid aliquot regardless of volume, a wide range of sampling volumes can now be accommodated.

In the current analytical instrument market, much smaller sample-volume draws are accurate and precise for optical particle counters and other techniques. Additionally, due to the expansion of dosage forms to include very small volumes of a highly concentrated drug, and the cost of certain pharmaceutical drug products, there is compelling stakeholder interest in allowing much smaller sample aliquots such as those described in ⟨787⟩.

Unit variability is an important quality parameter 1) during development of the formulation; 2) after process changes, such as container or stopper washing or fill-arena changes; and 3) when changing package vendors. Evaluation of the non-pooled vials can give a better measure of inter-vial variability and also can eliminate the introduction of particles in the pooling process. However, use of smaller sampling volumes must be balanced by batch sampling, which will provide adequate statistical power for evaluation of acceptability.

For all products, regardless of volume, comprehensive experience regarding the integrity and consistency of the batch is gained throughout development. This allows the proper batch sampling plans to be applied for commercial production to ensure that sample selection is representative of batch quality. All batches must have sampling plans that accommodate desired statistical measures of batch quality and facilitate process control, regardless of volume tested or method used (e.g., ⟨787⟩, ⟨788⟩, or ⟨789⟩). The FDA provides sampling plan guidance (*21–22*), and guidance is also available from ISO (*23*) and ANSI (*24*).

Final unit variability should be minimal. While 100% visual inspection is a comprehensive check for the entire batch, it is limited to fill color, fill haze, and larger particles in the fill. Subvisible particles are only visible when large numbers produce light blockage and scatter. There is low probability of visibly detecting particles <100 µm.

The above factors account for heterogeneity of the sampled particle load. Lack of repeatability in the particle measurement process introduces additional variability. Differences in sample preparation by different analysts can cause substantial variation in count, especially for samples sensitive to handling. Repeatability studies using samples similar to the samples of interest, rather than polystyrene microspheres, will give a more accurate assessment of measurement repeatability.

### 8.3 Risk-Based Approach

The USP limits in *Table 2* mirror the historical approach of obtaining particle concentrations above given size thresholds and treating all particles as equivalently deleterious to product quality. However, the increased variety of particle types in modern pharmaceuticals, each with different risk factors, suggests that a better approach would be to identify the particle concentrations of the component subtypes of the total particle load and to then assess the risk to product quality posed by each particle type.

For example, the presence of extrinsic particles could be evidence of a breach in sterility, with a high product risk. Increases in intrinsic particle load may be associated with the production process and/or package-related sources, which can be indicative of systemic problems. Conversely, the presence of air bubbles is not a concern.

Risk for elevated final product particulate levels will be reduced when there is deep product knowledge and when systems are utilized to control the process stream in the reduction of particle load (type). Consideration should also be given to the following:

- Package selection with regard to formulation (stability)
- Package source and preparation quality (removal and addition)
- Product-contact surfaces (particle load and extractable substances)
- Filling arena cleanliness and practices (addition)
- Formulation design (stability)

Successful risk analysis may require considerable particle characterization, beyond what can be achieved by LO, MM, and FI, and utilization of prior knowledge (*25*).

## 9. SILICONE DROPLETS AND AIR BUBBLES VERSUS NON-SILICONE PARTICLES

With the historical use of a single optical particle counting approach (LO), there was high reliability, reproducibility, and throughput for the quality control lab analysis of particle load. However, the technique does not provide differentiation between solid, liquid, and gaseous matter initiating the counting pulse. Both FI and MM can be useful adjuncts to LO in distinguishing air bubbles and silicone oil droplets from other particle types.

Certainly, the content of gas bubbles identified as counts by a particle counter may be considered an artifact of the formulation and/or sample preparation and may be alleviated or negated by sampling or preparation methodology or data manipulation. The counts by optical methods of immiscible liquids, which are largely composed of the ubiquitous silicone lubricants in pharmaceutical articles, must be considered. For some dosage forms such as cartridges, syringes, and some devices, lubrication (usually by silicone oil) is necessary.

There is a difference of opinion regarding the relative risk posed by silicone oil droplets. These opinions range from "no risk"—based on the droplet sterility and on the long history of medical use and patient exposure to silicone oil—to "potential concerns" about the increased immunogenicity of biologic drugs. Immunogenicity would arise from possible interactions between the

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droplets and the protein, resulting in an increase in protein aggregation or the silicone oil acting as an adjuvant. This is an active area of research. Regardless of the acceptability of the presence of high levels of silicone oil, an analytical strategy to accommodate and explain the presence of silicone droplets must be achieved by selection of the instrumental technique and methods applied during counting.

Historically, MM was used as a "referee" method to resolve the occurrence of elevated LO particle counts by verifying whether the counts were due to solid particles rather than gases and liquids. With the addition of FI, it is now possible in many cases to begin to resolve and differentiate gases and liquids counted as particles from the semisolid to solid particle fraction. Even in those cases where the differentiation of air bubbles and silicone oil from other particles is imperfect, the partial differentiation obtained with FI can provide useful guidance for optimization of sample handling and assessment of comparability of the protein particle population.

Air and immiscible liquids do not affect the membrane count. Membrane surface artifacts and semisolid particles can, however, complicate this analysis, while many protein samples spread out on the surface such that they are not detectable. This procedure is also time consuming and difficult to reproduce, requiring extensive operator training. It is usually only applied to biologics when identification of foreign particles is performed during development of nonconformance investigations.

## 10. CHALLENGING SAMPLES

Alternate methods may be necessary for dosage forms for which ⟨788⟩ cannot be directly applied. This includes some of the sample types described in *5. Applicability of Methods* (e.g., process streams, vehicles, or components). Alternate methods and/or evaluation should be considered for dosage forms and process samples for which optical detection is not practical.

For example, some products have inherent properties that affect or preclude particle counting so ⟨788⟩ methods cannot be used as written. For other products, development activity may be needed (see ⟨1787⟩). Direct evaluation of container–closure rinses and product vehicles may be used for these suspension systems by the ⟨788⟩ methods. When the product is not a solution or is viscous or will otherwise confound the ⟨788⟩ methods, it must be altered or accommodated to facilitate the assay. Examples of alternate methodologies are:

- Particle determination of product after treatment with solvent to dissolve all product solids
- Use of centrifugation to separate product solids from the vehicle
- Use of selected screens or sieves to separate product solids from the product vehicle to minimize interference
- Evaluation of the product filling system by using an alternate, clear fluid such as sterile media or saline

Use the methods that are best adapted to the particle type and volume of sample. Selected sampling requires careful statistical evaluation of the batch population when using small sample volumes to evaluate product acceptability; this applies to all methods. Regardless of the sample size, the number of samples tested should be determined statistically based on acceptable risk and development history.

## 11. FACTORS AFFECTING ALL METHODS

### 11.1 Guidelines for the Use of Calibrator Spheres

#### 11.1.1 GENERAL

Polystyrene spheres of stated diameter, or stated diameter and concentration, are commercially available for calibrating particle counting instruments. All such standards are only valid if the spheres are predominantly unaggregated. When measuring particle mean diameter, particle concentration, or instrument resolution, the user should inspect the particle size distribution to confirm the absence of microsphere aggregates. Aberrant calibration results may be due to instrument or method problems or calibrant quality. If there is evidence of aggregation, the bottle of calibrator spheres used should be discarded. Any results obtained from a vial that has demonstrated aggregation of spheres should be disregarded, as long as a satisfactory result with a new source of spheres is obtained. If the instrument is running properly but the calibration results deviate from the manufacturer's specifications, and the calibrator spheres show no indication of aggregation, the manufacturer of the instrument should be consulted.

#### 11.1.2 SPECIFIC STANDARDS

Chapter ⟨788⟩ specifies the use of mono-sized dispersions of traceable spherical particles with known diameters that include the 10- and 25-µm required sizes for particulate-matter testing. This must also include an expanded range of sizes to cover the calibrated range between 2 and 100 µm. The calibration curve should include additional sizes such as 2, 5, 10, 15, 25, and 50 µm, especially when particle load data are expected outside the traditional ⟨788⟩ thresholds. All particles are suspended in particle-free water (do not use *Water for Injection*), with small amounts of surfactant and preservative allowed.

Each calibrator sphere size should have a mean diameter within 5% of the nominal diameter and should be traceable to National Institute of Standards and Technology (NIST) standard reference materials or certified reference materials from other national metrology institutes.

The concentration of calibrator microspheres may be certified by the manufacturer or by the user via MM or other methods (*26*). For an MM determination of particle concentration, pipet a volume of the suspension containing 250–500 particles into a filter funnel. After drying the membrane, count the total number of standard spheres collected on the membrane filter. The size calibration should be done with the same standards. It is very important to calibrate for both size and number of particles, as the accuracy of one can influence the other.

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## 11.2 Elements of Measurement Quality Control

Quality control of measurements is ensured by a hierarchy of activities. See *11.3 Validation Considerations for All Techniques*. This topic can be generally summarized as the following:

- Method validation ensures that the chosen method will reliably measure the quantity of interest at known levels of accuracy, precision, and specificity (see ⟨1225⟩)
- Instrument qualification verifies that a particular instrument is fit for purpose (see *Analytical Instrument Qualification* ⟨1058⟩)
- Instrument standardization tests (ISTs) are the historical parameters of control for LO and are described in this chapter
- System suitability tests (SSTs) are routine and usually single-pass measurements performed to demonstrate a high degree of confidence that the instrument is performing correctly in use. The SST contained in this chapter for LO is a series of tests that demonstrates that each parameter complies with the manufacturer requirements.

Elements of method validation will be discussed for each technique; however, each method should include an assessment of the following:

- Recovery
- Accuracy
- Precision
- Specificity
- Lower detection limit
- Upper detection limit
- Sampling volume
- Linearity/range

For all the methods, there are general laboratory and sample considerations. First, remember that the main objective is to determine the level of unwanted particulate matter in a final parenteral product. The handling of the article during setup, selection, preparation, and testing should not significantly affect the results. For articles with a higher resident particle load, the impact of handling and area cleanliness is not as pronounced; however, the same general, clean, systematic approach must apply for all samples. See *Appendix 2* for further details on cleaning procedures.

As described in ⟨788⟩, determine the particulate matter in 5 samples of filtered water, each with a volume of 5 mL (or a blank volume that is equivalent to the sample volume for your specific method). For the 5-mL blank volume, if the number of particles that are ≥10 µm in size exceeds 25 for the combined 25 mL (NMT 1/mL), then this indicates that the precautions taken for the tests were not sufficient. Achieving a low number in the blank verifies that the equipment, operations, and sample residuals are not contributing significant counts. This is most important for ⟨789⟩ operations, which have strict limits. This guidance provides a practical limit for all methods, as it demonstrates insignificant impact of the operations for article preparation, glassware, sample handling, and instrument/apparatus cleanliness. Having a low-traffic, designated area for particle counting operations is recommended but may not be necessary depending on the method and the sample condition. Finally, when utilizing any technique for ⟨789⟩, the blank test should be considered carefully, as the limits are more restrictive than for ⟨787⟩ and ⟨788⟩, and there is an additional size threshold, ≥50 µm, to consider. Blank counts for ⟨789⟩ should be consistently very low to zero within a properly configured lab and using clean equipment and glassware.

## 11.3 Validation Considerations for All Techniques

Although the ⟨788⟩ methods are deemed robust for most product forms, deviation from clear, noncolored, and waterlike formulations may need further study. At the very least, an alternative particle counting strategy and a recovery study with known particle size standards will demonstrate the performance of the method for a specific medium. As described in the rest of this chapter, orthogonal methods may be necessary to fully understand the properties of the product. The following guidance is useful for comprehensive evaluation.

Chapter ⟨1225⟩ and ICH Q2(R1) (*27*) provide comprehensive guidance for validation of analytical procedures, as do the IST routines endorsed by the vendors. The aspects of assay validation in these guidelines are as follows:

- Chapter ⟨1225⟩: Accuracy, Precision, Specificity, Detection limit, Quantitation limit, Linearity, Range, Robustness
- ICH Q2(R1): 1. Specificity, 2. Linearity, 3. Range, 4. Accuracy, 5. Precision, 6. Detection limit, 7. Quantitation limit, 8. Robustness, 9. System Suitability Testing
- IST for LO: *Light Obscuration Method for the Determination of Subvisible Particulate Matter* ⟨1788.1⟩*, 3. Instrument Standardization Tests, 3.1 Sample Volume Accuracy, 3.2 Sample Flow Rate, 3.3 Diameter Calibration, 3.4 Sensor Size Resolution*, and *3.5 Qualification Considerations*

Validation requirements are discussed in ⟨1225⟩. However, for a limits test such as the testing described in ⟨788⟩, it has been common and acceptable in the US to use the following for evaluation of performance:

- IST as described in *13. Instrument Standardization Tests*
- Particle count reference standard compliance for counts conducted daily, weekly, monthly, or quarterly
- Spiked recovery studies to verify optimal sample preparation, mixing, and presentation

Both LO and MM methods provide a robust and reproducible particle content determination. Many companies have collected these data over an extended history, and the data are appropriate for most product formulas as written. Clear, low-concentration formulations that mix easily often require no further method modifications for validation. However, verifying acceptability for specific product families is an important step. Interference of test-sample attributes with various particle counting methods is discussed in *4. Detection and Enumeration of Particles in Different Size Ranges*. Method development as discussed in this section is a critical aspect of method validation. The fewer samples that deviate from non-labile particles in waterlike formulations, the greater the need for careful method validation.

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For those formulations that are highly concentrated, colored, or viscous, or that have specific gravity significantly different from water, the effects of the formulation character will require further consideration for method acceptability. Particle-recovery and -counting linearity should be standardized and verified for test methods that require sample mixing and pooling. When this is required, the test method should use the same procedure. MM samples the entire (drained) product fill and thus has no similar sampling issues.

Chapter ⟨1225⟩ indicates that specificity and detection limit are necessary elements for limit tests and, depending on the nature of the test, accuracy and range are as well.

For all techniques:

### 11.3.1 SPECIFICITY

As discussed previously, LO instruments will count air bubbles, immiscible liquid droplets, and semisolid and solid particles. Thus, LO has low specificity, and the specificity for detecting the particles of interest should be qualified by other methods. MM is insensitive to air bubbles and liquid droplets. FI will capture images of all bubbles, droplets, and particles. The specificity of FI can be assessed by measurement of mixtures of known particle concentrations followed by analysis with the algorithm that will also be used for the specific product.

### 11.3.2 RECOVERY

The use of microspheres of lower refractive index than polystyrene (such as polymethylmethacrylate or silica) or other particles of low refractive index may be appropriate to model particles of low refractive index difference for both LO and FI. In all cases, the ability of the method to accurately report the number of particles within the assigned sizing bins should be determined.

### 11.3.3 LINEARITY AND RANGE

Linearity and range can be assessed by the measurement of microsphere mixtures at different dilutions followed by comparison of the measurement result with the particle concentration expected from the known dilution factor. All three methods are subject to coincidence errors in which two particles appear as a single particle. LO method has typical coincidence errors of 10% at particle concentrations ranging from several thousand to a hundred thousand particles per milliliter, depending on the sensor choice. Typical FI instruments have much lower coincidence errors than MM or LO. For all methods, coincidence errors will depend on the nature of the particle size distribution, with larger errors for a size distribution with larger particles. The range extends from the level of background counts observed to the highest concentration with acceptable coincidence error.

### 11.3.4 ACCURACY

For particle counters, there are two types of accuracy that must be assessed: accuracy of reported diameter and accuracy of reported particle count. Both may be assessed at a basic instrumental level by measuring microsphere suspensions of known diameter and/or concentration. However, LO, FI, and MM will all report different diameters, even if the instruments have been properly calibrated to give accurate diameters with microspheres, because of the different dependencies of the instruments on particle morphology and refractive index. In every case, 2-dimensional images are being used to describe a 3-dimensional object.

While there is no consensus method for adjusting the reported diameter to a corrected diameter that addresses these effects, there are some methods that can be helpful in understanding them.

Silica and polymethylmethacrylate microspheres have lower refractive index than commonly used polystyrene microspheres.

Suspensions of these microspheres in glycerol/water solutions, or in other liquid mixtures of tunable refractive index, can be used to study the change in reported diameter with change in the refractive index difference between the particle and the matrix fluid.

Polydisperse suspensions of particles that optically mimic protein aggregates are in development. Use of these suspensions will be helpful for understanding the response of instruments to irregular particles of low optical contrast. Typical protein aggregates, for example, have an average refractive difference from the matrix fluid of only approximately 0.02 for sizes ≥2 µm.

Currently, there are two fundamental limitations on correcting errors of reported diameter. First, the refractive index of certain common particles such as protein aggregates is not yet established. Second, there is no accepted method for correcting diameter errors for heterogeneous mixtures of particles (e.g., silicone oil droplets and protein aggregates in a single test sample).

### 11.3.5 PRECISION

Precision may be assessed by considering the repeatability of multiple assays. Assays performed with microspheres or microsphere mixtures may be useful for assessing the fundamental precision limit of a method. Assays performed with samples representative of actual particles will provide a more accurate assessment of the precision that is possible in regular use.

### 11.3.6 DETECTION LIMIT

The detection limit is usually defined as the particle concentration corresponding to a 3:1 signal-to-noise (S/N) ratio. Particle counters may be theoretically capable of counting single particles without ambiguity, but noise is introduced in the form of background counts seen with blank or filtered buffer samples. The detection limit will vary with the size of the particle.

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#### 11.3.7 ROBUSTNESS

Assay results for particle counts can be sensitive to sample preparation, sample variability, and instrument cleanliness for all methods. MM is especially dependent on operator judgment.

## 12. TEST ENVIRONMENT AND PREPARATION

The nature of the sample preparation and the analytical method used can substantially alter the environmental requirements. Especially for MM, the pooling of aliquots, and other procedures requiring sample manipulation or samples exposed to the environment, will require more attention to cleanliness than procedures that only require drawing samples out of a vial as for LO. In all cases, perform the test in an environment that minimizes background particulate matter. Glassware must be cleaned to the extent that the level of particles added by testing has a negligible effect on the outcome of the test. Methods are acceptable as long as blank samples or other negative controls provide good results.

### 12.1 Sample Handling

Best practices for minimizing particle contamination from packaging or the testing environment should be followed at every step. Remove tertiary and secondary packaging outside of the test area and ensure that the exterior of the product containers has been cleaned. Rinse the exterior of containers with filtered water after removing all retention hardware (e.g., ferrules, clips, bands) and as directed in *12.2 Sampling Practices* for each technique. Protect the containers from environmental contamination until analyzed.

#### 12.1.1 LIQUID PREPARATIONS

Prepare the container(s) as directed in ⟨788⟩*, Method 1 Light Obscuration Particle Count Test*, either pooling units to get the 25-mL test aliquot when using ⟨788⟩ or testing individual units if using ⟨787⟩. When using ⟨788⟩ for containers of 25 mL or more, simply open the container, insert the counter probe in the middle of the solution and measure. For all liquid samples, gently mix the samples by hand swirling, gentle inversion, or mechanical means. Care should be taken to avoid introduction of air bubbles or contaminants and to avoid disruption of particles in all cases.

#### 12.1.2 DRY OR LYOPHILIZED PREPARATIONS

Prepare the containers as directed under ⟨788⟩*, Method 1 Light Obscuration Particle Count Test*. Open each container, taking care not to contaminate the opening or cover. Reconstitute as directed by the product insert instructions, allowing time for the sample to stand if so directed. Alternatively, during product development, prior to the availability of the package insert, use conditions as close as possible to those expected at the point of administration or use.

Replace the closure and manually agitate the container sufficiently to ensure dissolution of the drug, being careful not to agitate too vigorously and cause particle formation. For some dry or lyophilized products, it may be necessary to let the containers stand for a suitable interval and then agitate again to achieve complete dissolution. After the drug in the constituted sample is completely dissolved, prepare the units as directed in *12.1.1 Liquid Preparations*. Gently stir the contents of the unit by hand swirling or by mechanical means to mix and suspend any particulate matter. Proceed as directed for the appropriate unit volume under the applicable *USP* chapter. When sampling, ensure that the sipping probe is inserted into the middle of the solution.

#### 12.1.3 PRODUCTS PACKAGED WITH DUAL COMPARTMENTS TO HOLD THE DRUG PRODUCT AND A SOLVENT IN SEPARATE COMPARTMENTS

Prepare the units to be tested as directed under ⟨788⟩*, Method 1 Light Obscuration Particle Count Test* and according to product insert directions. Mix each unit as directed in the labeling, activating and agitating it to ensure thorough mixing of the separate components and to ensure drug dissolution. Prepare as described in ⟨1788.1⟩*, 6. Blank and Sample Handling*, and proceed as appropriate.

#### 12.1.4 PRODUCTS LABELED AS "PHARMACY BULK PACKAGE NOT FOR DIRECT INFUSION"

Proceed as directed for *Liquid Preparations* where the volume is 25 mL or more. Calculate the test result on a portion that is equivalent to the maximum dose given in the labeling. For example, if the total bulk package volume is 100 mL and the maximum dose volume is 10 mL, then the average LO particle count per milliliter would be multiplied by 10 to obtain the test result based on the 10-mL maximum dose. [NOTE—For the calculations of test results, consider this maximum dose portion to be the equivalent of the contents of one full container.]

### 12.2 Sampling Practices

After proper mixing, open the container(s) and pour or withdraw by syringe the contents under test to transfer in a manner least likely to generate particles. The number of product samples is determined by the fill category (small volume or large volume) or the intent of the determination, such as container variation or batch matrix experiment (BME) or design of experiment (DOE).

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- *Direct sampling*—Contents of containers with removable stoppers may be poured out directly after removing the closures for MM. Products packaged in flexible plastic containers may be sampled by cutting the medication or administration port tube or a corner from the unit with a suitably cleaned razor blade or scissors. Sampling devices that have a needle to penetrate the unit closure may also be used for all three methods. With an appropriate instrument "sipper", the contents of the product package may be directly drawn for LO or FI analysis.
- *Pooling*—If there is insufficient volume for the test method, the contents of multiple containers may be pooled from freshly opened product into clean glassware (cleanliness verified by testing).
- *Reconstitution*—Dry or lyophilized products must be constituted using either their insert diluent or particle-free water (as desired). The closure is removed to add the fluid, which is injected via hypodermic syringe. If test specimens are to be pooled, allow each to constitute fully before sampling directly or pooling.
- *Dilution*—Some products will require dilution for LO or FI analysis due to insufficient volume or due to suboptimal fluid properties, such as color or viscosity. All MM procedures utilize particle-free water as a receiving and rinsing fluid, thus they are diluted by design. The diluent must be particle-free water (or filtered product vehicle) and dilution vessels must be scrupulously cleaned and verified. The impact of dilution must be evaluated for acceptability through feasibility studies. Feasibility is verified when it is demonstrated that the dilution does not dissolve resident particles and, more importantly, does not induce formulation change.

#### 12.2.1 MIXING

For reference standards, such as the USP Particle Count Set RS (used for system suitability particle count accuracy, recovery studies, or calibration), homogenization of the mixture may be attained by agitating the preparation for 20 s and sonicating for up to 30 s. Mixing of most product samples will require different handling than that for the standard.

Gently mix the contents of the sample just prior to analysis by hand-swirling, tipping of the vial, or mechanical means. Take care not to introduce air bubbles, or if open, contamination. Mixing of samples is necessary to resuspend settled particles; however, mixing must be done carefully, because agitation may alter fragile or shear-labile particles. Swirling is gentler than tipping the vial but may not resuspend all particles. To the extent feasible, confirm on the sample of interest that the mixing method produces an acceptably homogeneous sample and that multiple stirring operations do not introduce unacceptable shifts in the particle size distribution.

#### 12.2.2 DEGASSING

The handling of microspheres and some blanks is different from the handling of some test samples. Microspheres will not degrade under sonication; however, sonication will strongly affect some particle types and is never acceptable for protein-containing solutions.

Use a consistent degassing procedure appropriate for the samples. For protein-containing solutions, sonication must be avoided. Recommended degassing procedures include 1) sonication at 20–80 watts for about 30 s, 2) standing and equilibrating at room temperature for 10 min, or 3) applying a mild vacuum, e.g., 75 torr, for 10 min. The standing of the sample can result in particle stratification and/or settling; thus, a sufficient and gentle agitation should be applied before analysis to bring all particles into the sampling zone. For cases where procedure 2 is ineffective, such as viscous samples or gas-particle associations, gentle vacuum may remove these entrained bubbles without disrupting the inherent particle population.

There are caveats to sonication and standing. With sonication, much energy is dissipated as heat, resulting in denaturation of the biologics; this could also affect certain particles in the sample. Allowing the sample to stand to dissipate gas is effective, but stratification and settling of particulate matter occurs during standing. For this reason, use of the standing approach must be accompanied by sufficient gentle agitation to bring all particles into the sampling zone. Allowing the sample to stand also has the benefit of bringing the solution to room temperature, mitigating any thermal effects encountered when introducing the sample to the counting system. Sometimes, allowing the sample to stand is not sufficient to remove microbubbles (e.g., if the solution is viscous or if the air bubbles associate with the particles). Gentle vacuum can often remove these entrained bubbles without disrupting the inherent particle population.

#### 12.2.3 BLANK SAMPLES

For the ⟨788⟩ method, an environment blank verifies system cleanliness. To collect blank counts, use a cleaned vessel of the type and volume to be used in the test. Place a defined volume of filtered water consistent with the volume of sample to be counted in the vessel and agitate the water sample in the cleaned glassware by inversion or swirling. Using the same method as for product samples is recommended.

Best practice includes running additional blank samples, especially between product families. Measurement of additional blanks consisting of filtered formulation buffer may be useful for assessing the introduction of air bubbles or desorption of particles from wetted surfaces in surfactant-containing formulations.

## 13. INSTRUMENT STANDARDIZATION TESTS

ISTs are used to verify that the instrument performs in accordance with the manufacturer's specifications upon calibration and continues to provide accurate and consistent results between calibrations. ISTs, as described in *13. Instrument Standardization Tests*, are equivalent to the performance qualification (PQ) in ⟨1058⟩ and are intended to verify the acceptable performance of the instrument for its intended use. See ⟨1058⟩ for an in-depth description of PQ, operational qualification (OQ), and system suitability. These tests are also applicable for MM but in a much-simplified approach (see *Membrane Microscope Method for the Determination of Subvisible Particulate Matter* ⟨1788.2⟩).

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ISTs for LO and FI include several tiers. Instrument calibration is the most comprehensive set of tests, and PQ tests provide assurance that the instrument meets all key performance requirements. Other verification tests provide daily assurance that the instrument performance is acceptable.

## 13.1 Diameter Calibration

Perform instrument calibration according to the instrument manufacturer's instructions. The count accuracy of an instrument can be significantly affected by the size accuracy of the instrumental particle counter. Count accuracy is also dependent upon the sensor used and may vary with individual sensors of the same model, as described previously.

Perform a system suitability test upon completion of the instrument calibration to ensure that the particle sizes of interest for the application (i.e., 10 and 25 µm) and other sizes (e.g., 5 and 15 µm), are accurately reported and that the count accuracy is verified. Count accuracy should be performed during or after the diameter accuracy is confirmed. Particle count accuracy may be assessed using commercial microsphere count standards. For LO and FI, corrections are incorporated into instrument software to give accurate particle diameters of the calibration microspheres.

## 13.2 System Suitability Tests

The key attributes of a particle counting system are that it counts particles accurately and that the level of background counts is acceptably low. Count accuracy may be assessed by routine measurement of microsphere count standards. Background counts may be assessed by routine measurement of blanks or filtered formulation. These two types of tests are the core system suitability tests.

A few factors influence how often a system suitability test should be run:

- Do the test samples tend to clog flow cells in the LO and FI methods?
- Do the particles in the test sample tend to adhere to wetted surfaces, thereby causing increased counts in subsequent blanks or test sample runs?
- What are the consequences if assay failure is detected at a date later than when the assay was run?

### 13.2.1 SYSTEM SUITABILITY FAILURE

Instruments that fail system suitability tests can often be remediated by thorough cleaning or by evaluating the particle standard used (these can aggregate over time). If failure of the standard is ruled out, the manufacturer's directions should be followed for thoroughly cleaning all fluid lines and the sensor head. Back-flushing to remove partial clogs may also be beneficial. The instrument is deemed to be suitable for use if it passes the system suitability tests after cleaning. Flush the system to remove traces of the particle standard prior to routine use.

Depending upon the dosage form being tested, proceed as directed under the appropriate category in *14. Calculations*.

# 14. CALCULATIONS

## 14.1 Pooled Samples

Average the counts from the aliquot portions analyzed. Calculate the number of particles in each container:

$$P_{\Sigma\geq 10} V_T / V_A n$$
$$P_{\Sigma\geq 25} V_T / V_A n$$
$$P_{\Sigma\geq 50} V_T / V_A n$$

in which $P_{\Sigma\geq 10}$ is the average particle count per threshold obtained from all portions analyzed and $P_{\Sigma\geq 25}$ is the average particle count per threshold obtained from all portions ≥25 µm analyzed. [NOTE—$P_{\Sigma\geq 50}$ is additionally used for ⟨789⟩. $V_T$ is the volume, in milliliters, of pooled sample; $V_A$ is the volume, in milliliters, of each portion analyzed; and $n$ is the number of containers pooled.] .

## 14.2 Individual Samples

Average the counts obtained for the aliquot portions from each separate unit analyzed and calculate the number of particles in each container:

$$P_{\Sigma\geq 10} V / V_A$$
$$P_{\Sigma\geq 25} V / V_A$$
$$P_{\Sigma\geq 50} V / V_A$$

in which $P_{\Sigma\geq 10}$ is the average particle count per threshold obtained from all portions analyzed and $P_{\Sigma\geq 25}$ is the average particle count per threshold obtained from all portions 25 µm analyzed. For ⟨789⟩, $P_{\Sigma\geq 50}$ is also reported and is the average particle count per threshold obtained from all portions ≥50 µm. $V$ is the volume, in mL, of the tested unit and $V_A$ is the volume, in mL, of each portion analyzed. For individual unit samples measured directly, the results are reported as $P_{\Sigma\geq 10}$ or $P_{\Sigma\geq 25}$ per unit volume, such as counts per milliliter.

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## 14.3 Individual Unit Samples

Average the counts obtained for the aliquot portions taken from the solution unit. Calculate the number of particles in each milliliter of product solution taken:

$$P_{\Sigma\geq 10}/V$$
$$P_{\Sigma\geq 25}/V$$
$$P_{\Sigma\geq 50}/V$$

in which $P_{\Sigma\geq 10}$ is the average particle count per threshold obtained from all portions analyzed and $P_{\Sigma\geq 25}$ is the average particle count per threshold obtained from all portions 25 µm analyzed. For ⟨789⟩, $P_{\Sigma\geq 50}$ is also reported and is the average particle count per threshold obtained from all portions ≥50 µm. $V$ is the volume, in milliliters, of the portion taken.

For all types of product, if the tested material has been diluted to decrease the viscosity, the dilution factor must be accounted for in the calculation of the final test result.

[NOTE—For all methods, when reporting particle load the particle counts must be reported in cumulative mode. If the instrument has been configured to count in differential bins (e.g., ≥10–24 µm, >25–50 µm, >50 µm), all bins ≥10 µm must be added to yield a total ≥10-µm count and all bins ≥25 µm need to be added to yield a total 25-µm count. Reports should clearly state the diameter limits, the units of particle counts (e.g., counts per container or counts per milliliter), and that the particle loads are for all particles greater than or equal to the stated diameter limits.]

## Glossary

**Diameter bias:** Deviation of a derived particle diameter from the actual diameter due to technique parameters and limitations.

**Diascopic illumination:** Transmitted light illumination for transparent to translucent samples.

**Episcopic illumination:** An internal illumination path coincident with the optical path, providing top-reflected light for the detection of thin, flat particles and films.

**Equivalent circular diameter:** The diameter of a standard sphere that produces an equivalent electronic pulse or other signal to an analyte particle.

**Feret's diameter:** The dimension of an object along a specified direction, also known as caliper diameter.

**Flow imaging:** An instrumental system configured to collect digital images of solids in sample fluid flowing between a light source and a camera.

**Fouling:** Plugging or coating of a particle counting system sensor or flow cell; may be a full blockage, partial blockage, or an extraneous substance affecting the analysis.

**Image analysis, dynamic:** A software program used to collect images from a flowing display, store the images, and retrospectively analyze the collected data.

**Image analysis, static:** A software application used to collect images and discriminate the images from a live, unchanging image.

**Light obscuration:** An optical particle-sensing technique that records impulses on a photodetector from obscuration of a source illumination by analyte particles. Particle size correlates with the response curve of a known spherical standard.

**Longest chord:** The longest dimension that can be measured across the perimeter of an object.

**Membrane microscopy:** A particle determination method that isolates solids from product samples by filtration and then examines them with a light microscope to count the collected material in size thresholds.

**Operational qualification (OQ)/Performance qualification (PQ):** Essential elements of method validation: OQ specifies the principal operational elements of the device in both hardware and method controls; PQ verifies the acceptable performance characteristics for a specified instrument OQ.

**Optical particle counter:** An instrument that provides detection, sizing, and enumeration of substances in a fluid medium using visible spectrum illumination and an electronic detector.

**Particle concentration:** Number of particles (count) per unit volume.

**Particle count accuracy:** An evaluation of the capability of an instrumental system to collect particle counts for evaluation against a standard.

**Particle size:** *Visible*: In the context of pharmaceutical products, the size at which one can visualize a single particle within a final product package. USP has set a conservative threshold at 100 µm, which must be considered the size at which the optimal methods will reliably detect particles. The caveat for setting a size threshold is that detection by manual (human) methods is a probabilistic process. *Subvisible*: 2–100 µm. *Nanoparticles*: <2 µm. [NOTE—The <2 µm threshold is a cut-off based on the limitation of typical instrument capabilities.]

**Refractive index (RI):** The numerical ratio of the speed of light through a vacuum over the speed of light through a substance. The RI of water is 1.33, with fluid oils ranging to 1.50 and solids spanning a wide range upwards from 1.34. RI is wavelength specific.

**Relative standard deviation/Coefficient of variation:** A measure of dispersion of a probability distribution. It is the ratio of the standard deviation to the mean and is expressed as percentage.

**Schlieren effects:** These are regions of varying refraction in a transparent medium that are often caused by differences in temperature or pressure and are detectable by optical means, generally by the passage of a beam of light through the medium.

**Sheath flow:** A technique using a "wall" of higher-viscosity fluid encasing a lower-viscosity analyte sample flowing through it, thereby producing a limited focal plane due to hydrodynamic channels.

**System suitability:** A measurement of the system output with a known standard that demonstrates acceptable performance of the system, often the result of several operational parameters.

**Validation:** Validation of an analytical procedure is the process by which it is established, by laboratory studies, that the performance characteristics of the procedure meet the requirements for the intended analytical applications. This typically

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includes measurement of method accuracy, precision, specificity, detection limit, linearity, range, and robustness. Described in ⟨1225⟩.

# APPENDICES

## Appendix 1: Historical Development of *USP* Chapters on Subvisible Particulate Matter

The determination of subvisible particulate matter in injections was initiated in 1975 as an MM method for large-volume injections (LVI) in *USP XV*. This one method was quite effective at revealing the content of unwanted solid particles ≥10 µm in the product, because filtration could accommodate a wide range of product volumes and the microscopist could evaluate the retained solids. However, the microscope method was much slower per sample than corollary instrumental techniques and required high-efficiency particulate air (HEPA) environmental hoods for isolation operations, with special microscope count training for the analysts.

Higher throughput with a robust electronic method was sought. Historically, the *British Pharmacopoeia* had utilized electrical sensing zone (Coulter counter) counting for all particles ≥2 µm; however, the method required dispersion of the product in an electrolyte solution, which did not work for certain products and formulations and could require extensive particle sampling prior to analysis.

The current USP subvisible particle determination methods were established in 1986 for determination of particulate matter in sterile injections, and in 1995, an improved version of this methodology was adopted (*28*) following extensive development by an expert volunteer group (*29*). The MM method was improved, and the combination of LO and MM was proposed for use as a two-stage test for both LVI and small-volume injection (SVI) products. In ⟨788⟩ and ⟨789⟩, "small-volume parenteral" is synonymous with SVI, and "large-volume parenteral" is synonymous with LVI. SVI and LVI are used throughout this chapter. The improved method for MM became official in 1995, and the first- and second-stage methods could be used for any sterile product amenable to testing by either method. Chapters ⟨788⟩ (a harmonized chapter) and ⟨789⟩ provide two methods: an LO test (*Method 1*) for sizing and counting of subvisible particles (generally 2–100 µm) for nearly transparent, low viscosity ("waterlike") product samples, and an MM particle count test (*Method 2*) for manual particle sizing and counting as an alternate method. The MM method also retains particles for further evaluation. Barber (*30*) has a good review of the limits history and instrument choices.

## Appendix 2: Cleaning Procedures to Limit Particle Contamination

Cleanse glassware, closures, and other required equipment, preferably by immersing and cleaning the items using warm, nonionic detergent solution. Rinse in flowing tap water, and then rinse again in flowing filtered water. Organic solvents may also be used to facilitate cleaning. [NOTE—These steps describe one way to clean equipment; alternatively, particle-free equipment may be obtained from a suitable vendor.] Preferably, the test specimen, glassware, closures, and other required equipment are then given a final rinse with filtered water using a hand-held pressure nozzle with final filter or other appropriate filtered water source within an environment protected by HEPA cabinets. While conducting the assay, non-shedding garments and powder-free gloves are worn within the HEPA environment.

For example, prepare the test specimens in the following sequence. Outside of the unidirectional airflow cabinet (UAFC) or HEPA filters to be used for the test, remove outer hardware or sealing bands. Labels typically don't need to be removed; however, if necessary, they may be removed or taped over before the next cleaning step. Rinse the exteriors of the containers with filtered water. Protect the containers from environmental contamination until analyzed.

Within the UAFC, open the containers and withdraw, pour, or otherwise sample the contents of the containers for testing in a manner least likely to generate particles that could enter the test specimen. Contents of containers with removable stoppers may be withdrawn directly by removing the closures. A sampling device with a needle to penetrate the unit closure may also be used. Products packaged in flexible plastic containers may be sampled by cutting the medication or administration port tube or a corner from the unit with a suitably cleaned razor blade or scissors.

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# ⟨1790⟩ VISUAL INSPECTION OF INJECTIONS

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## 1. SCOPE

### 1.1 Introduction

This chapter provides guidance on the inspection of injections for visible particles. The terms "particle," "particulates," and "particulate matter" are equivalent and do not have different meanings when used in this chapter. Particulate matter is defined in *Particulate Matter in Injections* ⟨788⟩ as "extraneous mobile undissolved particles, other than gas bubbles, unintentionally present in the solutions." Visual inspection is a probabilistic process, and the specific detection probability observed for a given product for visible particles will vary with differences in dosage form, particle characteristics (such as size, shape, color, and density), and container design. Some products and packages limit the ability to inspect for particles when compared to clear solutions in transparent containers. Additional guidance when inspecting these difficult-to-inspect products (DIP) are provided later within this chapter.

The requirement for injections to be "true solutions" appeared in *USP IX* in 1915, and the first appearance of "solution clarity" for parenteral products occurred in 1936 in *NF IV*. Since then, there have been numerous modifications to the compendia in this regard. A comprehensive history of compendial inspection standards is available in the *Pharmacopeial Forum* (*1*).

The practices discussed in this chapter are also applicable to the detection of other visible defects that are not the subject of *Visible Particulates in Injections* ⟨790⟩ but are critical to a qualified, comprehensive inspection process. These include, but are not limited to, container–closure integrity defects such as cracks, misplaced closures, or incomplete seals, any of which may compromise the sterility of the product. Additional container system defects (*2–3*), as well as other product characteristics such

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as fill level, discoloration, or clarity, may also be detected during visual inspection, and nonconforming units should be rejected using the methods described in this chapter. Inspection for these container–closure quality attributes often occurs at the same time as the inspection for particles using a specifically designed and qualified inspection sequence.

The reference method described in this chapter and in ⟨*790*⟩ is a manual inspection of a single container for particulate matter. However, multiple-container manual inspection, semi-automated, and automated inspection methods are also discussed and permitted by the Pharmacopeia. These alternate inspection methods must be qualified to demonstrate equivalent or better defect detection when compared to the reference manual inspection described in ⟨*790*⟩.

### 1.2 Related Chapters

*Injections and Implanted Drug Products* ⟨1⟩ provides an overview of injectable dosage forms and the quality tests associated with them. Another chapter, ⟨*790*⟩, has been added to the *USP–NF* to provide a clear definition of routine inspection procedures for injectable products; the goal is to comply with the expectation that products be essentially free of visible particulate matter. Additionally, information on the detection of subvisible particulates and particulates in specific dosage forms is provided in *Ophthalmic Products—Quality Tests* ⟨771⟩, *Subvisible Particulate Matter in Therapeutic Protein Injections* ⟨787⟩, ⟨788⟩, and *Particulate Matter in Ophthalmic Solutions* ⟨789⟩. *Measurement of Subvisible Particulate Matter in Therapeutic Protein Injections* ⟨1787⟩ and *Methods for the Determination of Subvisible Particulate Matter* ⟨1788⟩ provide additional supporting information on measurement methods for subvisible particles.

### 1.3 Defect Prevention

Although this chapter focuses on the detection and removal of product units that show evidence of visible particles, the need for preventing such contamination should not be overlooked. No inspection process, manual or automated, can guarantee complete removal of all visible particulate matter or other visible defects; thus, prevention of such defects is an important consideration. Good process and product design, along with environmental control, are necessary to ensure the reliable production of products with a low particle burden. To ensure the control of defects throughout the process, manufacturers should consider an inspection life-cycle approach (*4*). This approach begins with developing quality attributes based on incoming component specifications, followed by component-level acceptance testing. It extends to component preparation and product-filling procedures, followed by 100% in-process inspection of filled product, and concluding with final acceptance sampling and testing of the finished product. The life-cycle approach must encompass purchased, ready-to-use (RTU) components such as primary containers or closures, where there is no opportunity for further particle removal before use. Single-use systems (SUS) and consumable cleanroom materials such as wipes, should also be considered as potential sources of particulates. Stability and retention sample inspection, customer complaint evaluation, and in-house investigative procedures support this integrated approach. The inspection life cycle is composed of and supported by sub-cycles involving defect detection method development, qualification of methods and personnel, maintenance, and personnel training. Categorization and identification of product-contact particles found during inspection are useful to support source identification. Forensic analytical methods and the use of libraries containing visual standards (e.g., photographs and/or drawings) and characterized defect samples further support these objectives. The final element of the life cycle is a feedback loop of trending and data review from each of these process areas, resulting in a quality system that supports continuous process improvement.

## 2. BACKGROUND

### 2.1 Inspection Process Capability

Visual inspection of injections is necessary to ensure articles for administration consistently exhibit a high-quality level and minimize the introduction of unintended particles to patients during the delivery of injectable medications. Such inspection also offers the opportunity to reject containers whose integrity has been compromised, such as those with cracks or incomplete seals, which pose a risk to the sterility of the product. The desire to detect these defects, despite their very low frequency and the randomness of their occurrence, has resulted in the longstanding expectation that each finished unit will be inspected (100% inspection). Although having zero defects is the goal and this should drive continuous process improvement, zero defects is not a feasible specification for visible particles given current packaging components, processing capability, and the probabilistic nature of the inspection process.

The visible particulate detection process is probabilistic: the likelihood of detection is a cumulative function of visible attributes such as particle quantity, size, shape, color, density, and reflectivity as well as optical characteristics of the surrounding product and package. Understanding human performance is therefore critical to establishing visual inspection criteria. Analysis of inspection results pooled from several studies (*5*–*7*) conducted with standards prepared with single spherical particles shows that the probability of detection (PoD) for a seeded sample with a single 50-µm particle in a clear solution contained in a clear 10-mL vial utilizing diffuse illumination between 2000 and 3000 lux is only slightly greater than 0%. The detection probability increases to approximately 40%-60% for a seeded standard with a 100-µm particle, and the threshold for routine, reliable detection (≥70% PoD) of individual visible particles is often near 150 µm in diameter. Particles 200 µm and larger are often necessary to achieve greater than 95% PoD (*5*). The PoD for fibers is often less than that discussed for similarly sized spherical particles above, with reliable detection often commencing at or above 500 µm (*7*). Thus, in a qualified visual inspection system, the vast majority of nonfibrous particles that might go undetected and be introduced into the pharmaceutical supply chain will be smaller than 200 µm. Some injectable products are more difficult to inspect due to the nature of the primary container (*8*) (e.g., increasing size, color, and opacity), as well as dosage form characteristics (e.g., fill level; solution color, clarity, or opalescence; and suspensions, lyophilized powders, emulsions, and implants). Particle characteristics beyond size (e.g., color, shape, and density) will also affect the PoD that can be achieved for a specific product and container system (*7*).

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### 2.2 Patient Risk

A complete review of the medical literature is beyond the scope of this chapter, but the effect of particles on the patient must be considered. Several reviews on this subject are available (*9–15*). The clinical implications of particulate matter in injections are determined by many factors, including the size and number of particles, the composition of the material, the potential for microbiological contamination, the route of administration, the intended patient population, and the clinical condition of the patient. For example, an otherwise healthy individual receiving a subcutaneous or intramuscular injection containing sterile, inert particulates would likely experience no adverse effects, or at worst would develop a small granuloma. On the other hand, a critically ill premature infant receiving a particle-laden infusion directly through an umbilical catheter might suffer considerable pathophysiologic injury (*16–17*).

Numerous animal studies have been conducted to determine the fate of intravenous particles with different sizes and compositions (*18–21*). Most studies have focused on subvisible particles with a diameter <50 µm. In these studies, a massive infusion of particles has been accompanied by histologic evidence of injury to pulmonary capillary endothelial cells (*22*), microscopic thrombi in the pulmonary capillaries (*23*), pulmonary microscopic granulomata (*24*), and hepatic inflammatory effects (*25*). Although useful for understanding the pathophysiologic response to particulate matter, the large number of particles used in these studies (e.g., $10^9$ particles/kg per injection) provides little insight into the risk to humans posed by small numbers of visible particles.

Garvin and Gunner were among the first to report a concern about the effects of particles in human patients (*26–27*). For obvious ethical reasons, there is a lack of controlled clinical studies on the effects of particles purposely infused into human patients. Some anecdotal information about human patient safety may be obtained by examining case reports of intravenous drug abusers (*28–30*). In these cases, solid oral dosages are often ground up and injected as a slurry; pulmonary foreign-body emboli and granulomas were observed in these patients (*31*). Unfortunately, the clinical risks to human patients posed by small numbers of particles are difficult to infer from these observations due to the extreme number of insoluble particles and the uncontrolled conditions in which they were administered.

Arterial embolization using materials such as polyvinyl alcohol (PVA), collagen-coated acrylic microspheres, and gelatin spheres also provides some insight into the potential human pathophysiologic implications of non-target embolization of extraneous-particle intravenous infusions. In these cases, massive particle loads moving from the arterial injection site into the venous circulation were also reported (*32–36*). In addition, case reports have documented injury associated with the infusion of significant quantities of precipitated admixtures or therapeutic use of particles for embolization (*16–17,37*). Finally, recent studies evaluating the utility of in-line filtration (0.2- and 1.2-µm-rated filters) of intravenous fluids administered in a pediatric intensive care unit setting suggest that reducing the particulate burden associated with high volume infusions and multiple infused medications through bedside filtration may provide a reduction in morbidity in select patient populations (*38*).

Ultimately, the safety considerations related to particulate matter in injections must be assessed for each drug product, intended patient population, and method of administration. No single set of inspection criteria can adequately anticipate all potential risks to the patient. Despite the administration of an estimated 15 billion doses of injectable medicines each year (*39*), no reports of adverse events associated with the injection of individual visible particles have been found. The methods outlined in ⟨*790*⟩ should serve as essential requirements when assessing the adequacy of the visual inspection procedure, but alternative acceptance criteria (e.g., the use of tightened sampling plans) should be implemented when the patient population and intended use of the product warrant these additional measures.

## 3. TYPICAL INSPECTION PROCESS FLOW

### 3.1 100% Inspection

Chapter ⟨*790*⟩ establishes the expectation that each finished unit of injectable product will be inspected as part of the routine manufacturing process. This inspection should take place at a point when and where defects are most easily detected, for example, prior to labeling or insertion into a device or combination product. Each unit should be examined with the unaided eye under conditions appropriate for the container system (manual inspection) or by using a conveyor to transport and present the containers to a human inspector (semi-automated inspection), or by means of light obscuration or electronic image analysis (automated inspection). Manual and semi-automated inspection should be performed only by trained, qualified inspectors. Inspection may be conducted by means of a device that holds more than a single unit at one time for examination. This inspection may be performed at-line or in-line with filling or packaging or in a separate, off-line inspection. The intent of this inspection is the detection and removal of any observed defective unit. When in doubt, units should be removed. *Figure 1* shows a simplified process flow where solid boxes indicate required process operations, and those with dotted lines (supplemental testing) may be required for DIPs as discussed in *5.2 Unique Product and Container Considerations*. Alternative strategies, such as reinspection or two-stage inspection, may be required and are discussed in *3.3 Remediation and Alternative Practices*.

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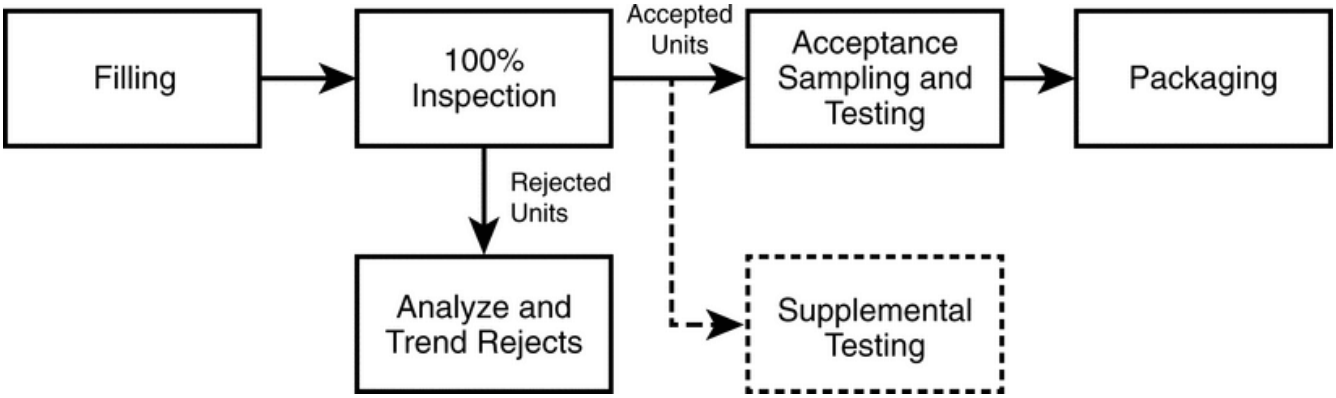


Figure 1. Typical process flow chart.

[NOTE—100% inspection refers to the complete non-destructive inspection of the container–closure system and its contents. Inspection may be accomplished in a single operation with specifically designed sequences for detecting visible particulate and/or container system defects or in multiple steps using a combination of technologies. See additional discussion in *3.3 Remediation and Alternative Practices* and *6. Inspection Methods and Technologies*.]

[NOTE—Supplemental destructive testing is required when the nature of the product or container limits visual inspection of the contents (e.g., with a lyophilized cake or powder, or with an amber glass or opaque container). See additional discussion in *5.2 Unique Product and Container Considerations*. Representative samples should be taken throughout the batch for supplemental testing from any point in the process after 100% inspection.]

During 100% inspection, limits on typical rejection rates should be established to identify atypical lots (*40*). These limits may be established for categories of defects (e.g., critical, major, and minor) or for specific types of defects (e.g., particles). A review of historical performance is useful in establishing these limits, and the review may include grouping products similar in appearance and manufacture. Periodic reassessment of these limits is recommended to account for expected process improvements and/or normal fluctuations in the process baseline (*41*). If a limit is exceeded, it should trigger an investigation. The investigation may include an additional inspection, or it may determine whether additional inspection is necessary.

## 3.2 Acceptance Sampling and Testing

After 100% inspection, a statistically valid sample is taken from the units accepted by the inspection process. These sampled units should be manually inspected under controlled conditions by trained inspectors. Chapter ⟨790⟩ provides reference inspection conditions for this purpose. The sample may be a random or a representative sample (e.g., at fixed time intervals or a fixed number per tray). Defects may not be distributed equally over the lot, and therefore a sampling process that represents the whole lot is required. Typical sampling plans used for this purpose can be found in ANSI/ASQ Z1.4 (*42*). Equivalent plans may also be found in ISO 2859 (*43*) or JIS Z9015 (*44*). These plans were historically developed for larger lot sizes and thus oversampled smaller batches (*45*). Alternative sampling plans may be justified for smaller batch sizes resulting in a smaller sample size. For batch release, the sampling plans listed as Normal II are typically used. Tightened sampling plans may be appropriate when an atypical result is observed, or reinspection is performed. All sampling plans specify a sample size for a range of batch sizes and require selection of an acceptable quality limit (AQL) to establish the sensitivity of the plan. The AQL is the defect rate at which 95% of the lots examined will be accepted and is a measure of falsely rejecting good batches. Critical defects (those that pose the greatest risk to the patient) should be assigned an AQL with a very low value. Often, the *accept number* (the number of defective units allowed in the sample) for a critical defect is zero. Major and minor defects, which pose less risk to the patient, will have increasing (less stringent) AQL values and accept numbers greater than zero. Further definition and discussion on defect categories are found in *5.1 Defect Classification*. *Table 1* shows the range of AQL values typically used for visual inspection processes (*46*).

**Table 1. Typical AQL Values for Visual Inspection Processes.**

| Defect Category | AQL Range (%) |
|---|---|
| Critical | 0.010–0.10 |
| Major | 0.10–0.65 |
| Minor | 1.0–4.0 |
| [NOTE—When selecting a sampling plan for AQL testing after 100% inspection using ANSI/ASQ Z1.4, ISO 2859, or JIS Z9015, choose the sample size to satisfy the AQL value for the most critical category (e.g., critical) of defects being evaluated. Then use the accept numbers for this sample size for the AQL values chosen for the other defect categories (e.g., major and minor). This assures that the sample size will produce a statistically valid result for all defect categories examined. The defect categories shown here represent a common basic approach to grouping defects by risk; however, additional categories may be added to these for more detailed analysis.] | |

While the standards referenced in the paragraph above are indexed by AQL, it is important to also know the unacceptable quality limit (UQL) for the sampling plan(s) used. These can be found in the operational characteristic (OC) curve data supplied for each plan found in these standards or calculated independently using qualified software. The UQL is the defect rate at which

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90% of the lots examined will be rejected and is a better measure of the customer or patient risk. The protection afforded by any sampling plan is represented by its OC curve. This is a plot of the probability of lot acceptance versus the defect rate in the lot. The AQL and UQL are two points on this curve. Sampled units should be manually inspected under controlled conditions by trained inspectors. Inspection conditions should be aligned with the 100% inspection process.

Manual acceptance sampling should be performed after any type of 100% inspection process, including manual, semi-automated, and automated inspection processes. It provides a measure of the performance of the overall inspection process and the quality of a specific lot, compared with predefined acceptance criteria. Although automated systems are validated before use and are routinely challenged to ensure acceptable performance, the use of manual acceptance sampling inspection detects unexpected defects that were not included in the development and training of the automated system by the manual inspection process.

Acceptance criteria are comprised of the product specifications and acceptance/rejection criteria, such as the AQL and UQL values, with an associated sampling plan that is necessary for deciding to accept or reject a lot or batch (or any other convenient subgroups of manufactured units) as described in 21 CFR 210.3 (*47*). If the acceptance criteria of the sampling plan are not met, an investigation should be conducted. Depending on the nature of the failure, this investigation should include forensic classification/identification of the particle(s), and examinations of the manufacturing process, the raw materials, and the packaging materials, as well as the inspection process. If, after investigation, the inspection process is deemed capable of detecting the defect(s) in question, the batch may be reinspected. An alternative inspection process better suited to detection of a specific defect may also be chosen for reinspection.

After reinspection (performing a second 100% inspection of the batch), a new statistically valid sample of the accepted units is taken and compared against established acceptance criteria. It is a good practice to use a tightened sampling plan and acceptance criteria under these circumstances because of the atypical nature of this process step.

Typical sampling plans for supplemental testing can be found in the special sampling plans S-3 and S-4 in ANSI/ASQ Z1.4 (*42*). These higher sensitivity S-plans offer a practical compromise between sample size and statistical power. For batch sizes between 200 and 100,000 they suggest a sample size of 20 with an accept number of 0 (based on an AQL of 0.65%). Sample sizes larger than 20, as found in these sampling plans, may be appropriate for larger batch sizes or when additional sensitivity to support reduced AQL values or a more conservative defect classification is desired. Refer to *Table 2*.

**Table 2. Typical 0.65% AQL sampling schemes for normal, single-pass inspection using S-3 and S-4 levels, dependent upon lot size.**

| Lot Size | (Sampling Letter Code) Accept Number, Reject Number/Sample Size | |
|---|---|---|
| | Inspection Level S-3 | Inspection Level S-4 |
| 200–10,000 | (F) 0,1/20 | (G) 0,1/20 |
| 10,001–500,000 | (G) 0,1/20 | (J) 1,2/80 |
| >500,000 | (H) 1,2/80 | (K) 2,3/125 |

Alternative plans are acceptable, but care should be taken to examine the UQL of such plans to assess their sensitivity.

## 3.3 Remediation and Alternative Practices

### REINSPECTION

As discussed in the preceding section, reinspection (repeating the 100% inspection followed by acceptance sampling inspection) may be appropriate if the initial 100% inspection is not successful in meeting in-process control levels or the initial acceptance sampling inspection criteria. This includes instances when the established 100% inspection failure rate(s) and/or the accept/reject number(s) associated with the chosen AQL values have been exceeded. Reinspection should only be conducted using a procedure that has been approved by the quality organization and addresses key parameters such as the inspection conditions (e.g., same as primary inspection or modified to enhance detection of a specific defect type), the number of times reinspection may be performed (this should be limited and justified), and the acceptance criteria (e.g., same as primary inspection or tightened). If reinspection is required often, consideration should be given to improving the sensitivity of the primary inspection process or of the manufacturing controls to prevent defects in the upstream process as determined by root cause analysis.

### TWO-STAGE INSPECTION

In cases where an assignable cause, such as the formation of air bubbles or specific container or closure variation, results in a high false-rejection rate (rejection of acceptable units), the use of a second inspection step may be considered. *Figure 2* shows a typical process flow for a two-stage inspection. Such an inspection strategy is more common with automated inspection systems, where there is less ability to tolerate normal variation in the product or container.

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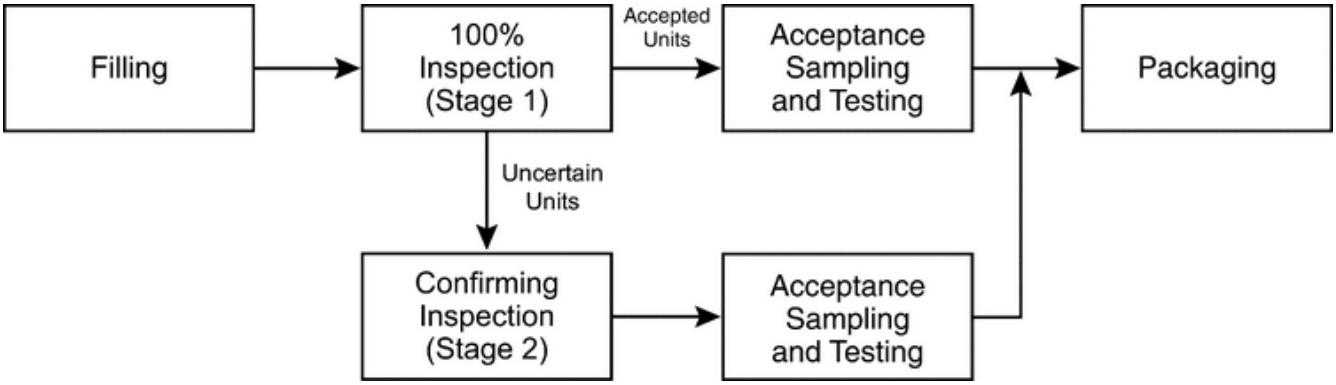


Figure 2. Two-stage inspection process flow chart.

Under these circumstances, the inspection system is adjusted to ensure acceptance of good units. Those not accepted during the primary inspection are considered of uncertain disposition until inspected by another means (e.g., enhanced manual inspection) to provide confirmation of acceptance or rejection. Inspection conditions may be adjusted to provide greater sensitivity in this second inspection step (e.g., additional inspection time) to ensure a higher probability that true defective units will be rejected. The limitations of the first inspection and the reason for conducting a second stage of inspection should be clearly defined and documented. The second inspection of these units by the same method and conditions (e.g., automated inspection with the same parameters after an initial automated inspection) is generally not recommended because the same limitation in inspection method is present for both inspections unless enhanced manual or more sensitive automated inspection parameters are employed for the second stage inspection. However, it may be suitable when the root cause is specifically related to air bubbles in the solution, and a study has been performed to establish an appropriate holding time to allow the bubbles to dissipate before performing the second inspection. It is recommended that each inspection stream (those accepted by the first stage and those accepted by the second stage) be sampled separately and evaluated against the sampling plan acceptance criteria before they are confirmed as accepted and recombined into a single batch. If a two-stage inspection strategy is used, it must be validated as intended for use. Defective containers with less than a 100% PoD will have the PoD reduced further with each stage of inspection; thus, the PoD should be determined after inspection through both stages to ensure that acceptable sensitivity is maintained.

## 4. INSPECTION LIFE CYCLE

### 4.1 Extrinsic, Intrinsic, or Inherent Particles

Particles may originate from many sources. These are discussed here, as well as in other chapters in the *USP* (e.g., ⟨1787⟩). Those that are foreign to the manufacturing process are exogenous or "extrinsic" in origin; these include hair, non-process-related fibers, starch, minerals, insect parts, and similar inorganic and organic materials. Extrinsic material is generally a one-time occurrence and should result in the rejection of the affected container in which it is seen; however, elevated levels in the lot may implicate a broader contribution from the same source. These particles may carry an increased risk of microbiological or extractable contamination because less is known about their path prior to deposition in the product container or their interaction with the product.

A second category of undesired particles is considered "intrinsic" or from within the process. The determination of whether the particulate is extrinsic or intrinsic to the process is based upon appropriate characterization of the particle's physicochemical properties. Process-related intrinsic particles may come from production equipment or primary packaging materials that were either added during processing or not removed during container or closure cleaning and preparation. These primary product-contact materials may include stainless steel, elastomers from seals and gaskets, container glass or plastic, fluid transport tubing, and silicone lubricant. Such particles still pose the risk of a foreign body, but they generally come from sterile or sanitized materials, and more is known about their interactions when in contact with the product. Any process-related intrinsic particles should have controls established based on the use of a life-cycle approach, as outlined in *1.3 Defect Prevention*. Another group of particles considered intrinsic is interrelated with the stability of the product. These product-stability-indicating particles arise from container or closure interaction (glass delamination, component erosion, and elemental extraction), changes to the drug preparation (insoluble degradation products), recrystallization, or temperature sensitivity over time. Stability-indicating intrinsic particles should be identified and addressed as early in the product development process as possible.

A third category is that of an inherent particle type that varies with dosage form and includes solutions, suspensions, emulsions, and other drug delivery systems that are designed as particle assemblies (agglomerates, aggregates). Such dosage-form-related particulates should be studied in the development phase and in samples placed on stability to determine the normal characteristics and time-based changes that can occur. Measurement of particle size distribution or characterization by image analysis in the subvisible (for particle sizes ≥2 µm) and visible ranges (≥100 µm) may be required to fully characterize inherent dosage-form-related particles. In biologics, protein particles are generally considered inherent and may be accepted when their presence is measured, characterized, and determined to be part of the clinical profile. Aggregation or agglomeration of proteins associated with a change in preparation constituents or other causative agents like excessive siliconization should be minimized. The manufacturer may allow inherent particles if the product appearance specification allows their presence or if the product is an emulsion, suspension, or implant.

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An evaluation of the potential impact of particles identified from any of these sources may be enhanced by incorporating a clinical risk assessment (*15*). This assessment may include factors such as the intended patient population, route of administration, source and type of particles, and implications for product sterility. For intrinsic or inherent particulate matter sources, a risk assessment may be useful in developing product-specific control strategies. Given the probabilistic nature of particle detection, it is important to assess the possible implications of particles identified through the product life cycle to better ensure the product's safe use.

## 4.2 Prevention of Particulates

The manufacturing process is designed to keep the final container and its contents clean within the control parameters established for process-related particulates. Once the container is filled, the stability of the product needs to be maintained throughout its shelf life. Changes that occur as the product ages during its normal shelf life must be characterized. Avoidance of intrinsic particle sources that may affect final product stability depends on careful consideration of the entire product and packaging system. If these intrinsically sourced changes occur, and they affect stability, particles ranging from subvisible to visible may develop. Typically, these particles result from change mechanisms that slowly affect the on-shelf product.

### ROBUST DESIGN DURING DEVELOPMENT

To anticipate potential sources of instability that yield intrinsic particles, the product design should be evaluated from many perspectives, beginning with a literature review of similar preparations and packages. Points to consider include the reported sensitivities of the active ingredient, the dosage form type, and the final container–closure system needed for delivery. Knowledge of how glass containers are fabricated, controlled, sterilized, and tested is important as this may affect the tendency to form glass lamellae as discussed in *Evaluation of the Inner Surface Durability of Glass Containers* ⟨1660⟩ and by the FDA (*48*).

Obtaining further information on possible extracts and leachates, metals, or solubility limits is important as these factors may promote formation of solid material in the aging solution. Several additional factors for successful product design include the product concentration, solution pH, associated buffers, critical micelle concentration, oligomerization content/potential, package effects (large surface area, product volume, headspace, light/oxygen transmission), and compatibility of the specific preparation with the package. Furthermore, shock/vibration sensitivity at the air/liquid interface can be a significant contributor to particle formation that requires investigation during product development. Some key dosage form design factors include the formula components chosen and their purity, the solubilities of the active ingredient(s) and excipients, and consideration of potential salt forms. Finally, to maximize product stability, consider the final product preparation for delivery, product dilutions, and shelf stability of the commercial product or its therapeutic preparations.

To examine the appropriateness of the product design for maintaining product stability, there are two levels of evaluation. Both levels examine retained containers for visible changes using methods described in this chapter, but neither level dwells on low percentage defects.

For the first level of stability study, visual inspection and testing of trial containers will show general compatibility of the chosen components over time with regard to clarity, color, and both subvisible and visible particle formation. Careful product assembly in clean containers, with consideration of the container type, headspace, and sealing, will yield a beneficial first-pass trial of stability over several months' time. The nature of particles detected, and the investigation of type and frequency, is essential to differentiate additive types from instability or package interaction. Pursuit of extrinsic particles at this stage of development is generally not significant, as the particles do not reflect on the suitability of the specific preparation or expected manufacturing process under development but rather the way these study samples were prepared.

The second, more refined level of stability study involves conducting visual inspections of the injection in defined, International Council for Harmonisation (ICH)-relevant trials (*49*). This may include periodic inspection of the same containers over time if the product does not require reconstitution or is not affected by frequent temperature changes. Detection of minor or subtle differences in these containers is not the goal at this stage of development. Catastrophic change and the occurrence of intrinsic product-related visible particles should be the focus. Typically, a set of containers is carefully prepared to exclude extrinsic particles and is then inspected to cull out any units with visible defects. Next, a numbered set of containers appropriate for the batch size is placed on stability and visually inspected periodically for changes due to degradation or any sign of instability. It is important to be able to analyze the particulate matter or condition (change in color, turbidity, opalescence) to identify the cause of events due to instability. A typical sample size is 80–100 units. This quantity should be sufficient to assess package and preparation interaction. Additional sets of containers stored at selected extremes of ICH temperatures can be followed to aid discovery of solubility-edge phenomena. When unwanted changes are detected—such as particle formation, solution color change, solution haze, and package changes—the process of isolation, characterization, and identification can commence. Identification of the material comprising these changes aids in the determination of the cause of formation as well as development of improvements for future use.

### COMMON SOURCES OF PARTICULATES

Process-related intrinsic particles originating from product-contact materials tend to be stable and unchanging (e.g., glass, elastomers, or metal). In contrast, there may also be particles resulting from product-stability-related change mechanisms within the final product. It is very important to understand that these changes only need be slight in certain cases, far below the detection limit of most release or stability assays, to result in visible changes to the product. The threshold levels for the formation of visible change for certain substances may be as low as 10–100 ppm (0.001%–0.01%) based on the ability to detect a single 100-µm particle or many sub-10-µm particles creating a hazy or cloudy appearance. However, if all this insoluble material were contained in a single particle, it likely would not cause rejection of the container.

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DOSAGE FORM COMPONENTS

The active ingredient may also contribute to the presence of stability-indicating intrinsic particles. For example, significant haze and particles have been observed in aqueous dosage forms due to extraction of plasticizers from filtration media during bulk drug production (*6*). Metal content in the active ingredient has contributed to organometallic salt formation and has also been observed as precipitated inorganic salts, blooming long after product release. The active ingredient and related degradation products may also be relatively insoluble and may grow to form visible particles. Isolated particulate material must be analyzed to determine its chemical nature and possible source.

Monomers or single molecules may join through chemical processes to form dimers, trimers, and oligomers (a limited assemblage of monomers, short of polymerization). Such changes are not unexpected (*50*). In high-concentration and/or saturated preparations, and especially for micellar drug associations, the solubility of related forms is significant when the aging preparations contain progressively higher concentrations of these substances. Larger molecules may have a greater effect on solution integrity due to their inherent insolubility, especially if the active drug is in a micellar preparation.

Polymorphs are unique crystalline forms of identical chemical entities. Although uncommon in solutions that have been mixed homogeneously and filtered, small seed crystals of a relatively stable polymorph may form over time, especially at nucleation sites such as container-surface defects. More common than formation of polymorphs is the formation of a modified crystal lattice containing an integral liquid, typically water or a solvent. The lattice may form slowly, promoted by evaporation, nucleation, and temperature extremes (*51*–*52*).

PACKAGING COMPONENTS

"Extractables" and "leachables" are terms commonly used to describe the potential for primary packaging materials to contribute unwanted agents to the product. Extractables represent all the materials that could be contributed, and leachables represent the practical contribution upon contact between packaging components and drug preparation as discussed in *Assessment of Extractables Associated with Pharmaceutical Packaging/Delivery Systems* ⟨1663⟩ and *Assessment of Drug Product Leachables Associated with Pharmaceutical Packaging/Delivery Systems* ⟨1664⟩ as well as in other references (*53*). These substances can also contribute to the formation of subvisible and visible particles. All product contact materials should be evaluated for extractables and leachables or product reactivity.

Product preparation attack of the container is a dramatic change and most often occurs in glass container systems. Glass containers undergo corrosion that is 25 times greater at pH 8 than at pH 4 (*54*, ⟨1660⟩). A preparation with a pH above 7, especially with high-ionic strength solutions, promotes attack of the inner glass surface, resulting in glass delamination and/or siliceous particle generation.

Silicone oil is added to stoppers and prefilled syringe systems to enhance lubricity for stopper or plunger insertion and its movement within the syringe barrel. Silicone may also come from tubing used for fluid transfer and a variety of polymeric fittings and seals that are used in the processing equipment. All these components must be compatible with the preparation to minimize leachates. Although silicones are processed to be sterile and are widely used, their use must still be strictly controlled, in other words, minimized. Silicone can cause container sidewall droplets and a variety of visible semisolid forms. No more than the minimum quantity should be used during processing to achieve acceptable machineability. Silicone and other hydrophobic substances have the capacity to coalesce and agglomerate with proteinaceous drug substances (*55*) or other particles, eventually reaching a visible size over time.

Residual tungsten from the manufacture of syringes has been known to increase protein aggregation (*56*).

## 4.3 Recommendations for Specific Components and Processes

GLASS CONTAINERS

Each step of the glass-container washing and rinsing process performed internally or by an external supplier, should be evaluated for particle-reduction capability. Incoming glassware may have significant particle load. The washer validation studies should demonstrate a reduction in naturally occurring particles (particle burden) or should use seeded containers to demonstrate such reduction capability. The use of statistical sampling plans with light obscuration (LO) for subvisible particle levels and membrane-microscope particle-counting method for the enumeration of visible particles can provide a means to demonstrate reduction of both subvisible and visible particles during washing cycle development and validation. The membrane filtration microscopic method is superior to the LO method for capturing and characterizing larger foreign particles in the visible range (>100 µm) during validation or monitoring activities. During process development, validation, and routine use, container-washing procedures should include periodic visual operational checks. This routine verification ensures that effective draining of all containers is occurring during all washing and rinsing steps. Review the wash-water recirculating filter maintenance procedures to ensure that particle overloading or breakthrough is being prevented.

Glass breakage that occurs during the component washing process could affect surrounding containers, and the washing cycle should be evaluated for possible glass particle generation and distribution. Written procedures describing container-clearance following glass breakage in the washer, in the depyrogenation tunnel, or on the filling line should specify the number of containers to be removed from the affected portion of the line. Removing units around the immediate area where the breakage occurred aids in minimizing particle transfer to the downstream process.

Processes that use racks or trays for transporting and holding samples, as are typically used in batch ovens, should be monitored for metal particle generation. The racks or trays should have a formal maintenance program associated with their routine use. Trays should be inspected for wear and scoring, which can be sources of particulates. Periodic cleaning, polishing, and/or resurfacing may be warranted to control particles effectively. Tunnels used for depyrogenation should also have a routine maintenance program for periodic cleaning, inspection, and replacement of parts that may wear and generate particles. Glass-to-glass and glass-to-metal contact should be minimized where possible to reduce weakening of the glass surface resulting

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in increased risk of subsequent fracture. The use of polymeric facing on guides can help to reduce such damage; however, coatings on metal surfaces must be monitored for degradation or particle generation by frictional wearing over time.

### ELASTOMERIC CLOSURES

Each step of the elastomeric-component washing and rinsing process should be evaluated for particle-reduction opportunities. Utilize statistically valid sampling plans to collect meaningful test units. The use of LO (for subvisible particles) or other automated particle counting or membrane-microscopic particle-counting methods may be used to demonstrate reduction of both subvisible and visible particles during washing validation. As stated above for glass containers, the membrane filtration microscopic method is superior to LO methods for capturing and characterizing larger foreign particles in the visible range (>100 µm) during validation or monitoring activities with elastomeric closures. During process development and validation and in routine use, container-washing procedures should include visual checks to ensure that stoppers are not routinely sticking together. Such sticking surfaces reduce cleaning efficacy and entrap particles or cause reduced sterilization and depyrogenation effectiveness. Periodic assessment of component cleanliness and supplier washing capabilities should be included as part of the supplier qualification program when using ready-to-sterilize (RTS) or RTU components.

Evaluate siliconization processes, whether in-house or by the supplier, to minimize excess silicone levels while maintaining machinability of the stoppers. LO or other automated particle-counting methods may be used to compare overall particle level reduction (subvisible background silicone oil droplets) during process development or validation. The level of residual silicone oil will affect the particulate quality of the final filled product, observed as dispersed droplets and particle-forming matrices.

### EQUIPMENT PREPARATION

It is important to minimize redeposition of particles on product contact surfaces after cleaning. Cleaned and sterilized equipment should be protected by HEPA-filtered, unidirectional airflow until transferred to, and installed on, the filling line. For cleaned equipment that needs to be wrapped or bagged prior to sterilization, utilize low-shedding, noncellulosic (synthetic) wrapping materials. Cellulose fibers are one of the most common particles found in the injections-manufacturing environment, and injectable products and their source will be a prime concern (*46*).

Personnel are a concern for the introduction of extrinsic particle types such as facial hair and skin cells. The equipment preparation staff should be adequately gowned with hair covers, facial hair covers, and goggles to prevent contaminating cleaned process equipment. During validation and monitoring activities of clean- and/or steam-in-place (CIP-SIP) systems (vessels, filters, tubing, and other product contact equipment), foreign particle burden should be evaluated after cleaning. Membrane filtration/microscopic-particle-capture and -counting methods provide a useful tool to assess and document performance.

### FILLING LINE

The transfer of open containers should be evaluated and reviewed to mitigate particle contamination. For example, for aseptically filled products, the transfer should be conducted in Grade A (ISO 5, Class 100), unidirectional airflow to minimize particle contamination. The air in critical zones should be monitored continuously during operation to confirm compliance. Routine checks to detect particles and potential particle-generation locations should be explained in the procedures. Effective, written container-clearance procedures to be used after glass breakage should specify the number of containers to remove from the affected portion of the line. Note that improper setup and adjustment of the filler can lead to “needle strikes,” where the filling needles contact the container being filled. This can generate either stainless steel or glass particles.

Filling pump design and the pump’s compatibility with the product are important considerations. Metal-on-metal piston pumps have the potential for generating metal particles. Pump maintenance is essential and includes a requirement to resurface the cylinders and pistons periodically. Peristaltic-action pumps must be monitored for generation of silicone tubing particles, especially with aggressive, near-saturated solutions or suspensions. Friction in the peristaltic roller area can break down the tubing, resulting in the generation of particles (spallation). Diaphragm pumps must have specific maintenance cycles to replace the pump lining.

Stopper bowl surfaces should have a formal maintenance program, and stopper handling or replenishment by operators should be specifically designed to minimize particle transfer to the stoppers. Proper operator positioning and avoidance of open containers is important in good aseptic-filling practices to avoid microbial contamination. These same principles help reduce particle transfer to the open containers and exposed elastomeric closures.

Careful selection of cleaning and gowning materials will help reduce contamination from extrinsic particles and fibers. These clean-room materials (typically polyester or Tyvek) should be selected for their superior non-shedding and low-particle properties.

## 4.4 Trending

Data obtained from the in-process 100% inspection followed by AQL inspection are used for batch release. Both 100% and AQL inspection data should also be analyzed for adverse trends on a periodic basis, typically at least annually. Data from the 100% inspection provides the best source of typical defect types and rates during normal production. High-volume products may generate enough data to allow quarterly analysis, whereas a longer period may be necessary to accumulate data for products that are produced infrequently. Data from component inspection, production 100% inspection, and the AQL inspections should be evaluated based upon sound statistical principles to determine whether the current action levels accurately reflect current process capability. Alert and/or action levels may be established and/or adjusted if the statistical analyses indicate that lower defect levels are being observed consistently. When establishing new action or alert levels, a preliminary target value may be used until enough production experience is obtained. Consideration should be given to planned improvements in the

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manufacturing and inspection processes. If significant improvements are planned, the reduction of the action/alert level should not be instituted until the impact of the improvement is measured over enough time to establish the validity of the new value.

# 5. INTERPRETATION OF INSPECTION RESULTS

## 5.1 Defect Classification

Defects are commonly grouped into classifications based on patient and compliance risk (*2*). The most common system uses three groups: critical, major, and minor. Critical defects are those that may cause serious adverse reactions or death of the patient if the product is used. This classification includes any nonconformity that compromises the integrity of the container and thereby risks microbiological contamination of the sterile product. It may also include anomalous extrinsic particle types such as insects or other filth or adulteration. Major defects carry the risk of temporary impairment or medically reversible reactions or involve a remote probability of a serious adverse reaction. Visible intrinsic particulate matter typically falls into the major defect category. This classification is also assigned to any defect that impairs or makes the product unusable. Minor defects do not impact product performance or compliance; they are often cosmetic in nature, affecting only product appearance or pharmaceutical elegance.

Upon 100% inspection, visible extrinsic and intrinsic particles should be reliably removed. Particle motion aids in detection, and stationary particles (due to neutral buoyancy or fill viscosity) are difficult to detect, exhibiting a significantly reduced PoD. The test method allows inherent particles to be accepted if the product appearance specification allows inherent particle types. PoD for all particles is dependent on the container characteristics (e.g., size, fill level, shape, and transparency), inspection conditions (lighting and duration), preparation characteristics (color and clarity), and particle characteristics (size, shape, color, and density). The PoD at 70% or greater is known as the "reject zone" described in Knapp's methodology (*57–58*), which is used worldwide as a common industry practice for rejecting particle defects. Test sets characterized by repeated inspections, as described in *7.4 Rejection Probability Determination*, are used to "calibrate" the inspection method's PoD, inspector performance, or automated inspection systems, and to demonstrate the sensitivity to threshold particle size at the reject zone of >70% PoD. The limitation of the reject zone at 70% detection is that threshold particles at this size may routinely be missed or go undetected up to 30% of the time. These undetected units may contain some level of threshold-sized particles or subvisible particles at a lower PoD. Therefore, it is important to characterize and size any particles recovered from AQL testing, retention sample inspection, and product returned from distribution to understand how they could have gone undetected during the initial 100% in-process inspection.

## 5.2 Unique Product and Container Considerations

### DIFFICULT TO INSPECT PRODUCTS (DOSAGE FORMS, CONTAINERS, AND DELIVERY SYSTEMS)

As discussed throughout this chapter, 100% visual inspection is required as a final control mechanism for particulate and other container and closure defects in injectable products. This unit-level inspection allows for the final removal of any visually detectable and undesirable nonconforming units to ensure that batch-quality acceptance criteria are met. There are dosage forms for which the sensitivity of direct examination is significantly reduced. Often referred to as DIPs, the products hinder or block the inspectors' ability to reliably detect visible particulate matter. This is attributable to interference from the characteristics of the dosage form and/or the container or delivery system. A DIP, unlike a clear solution in a transparent container, is not as amenable to defect reduction even by an optimized 100% inspection process. Thus, there is a higher probability after the 100% inspection of such products, that undetected visible particulates may remain in the batch. Supplemental testing, as briefly described in *3.1 100% Inspection*, is required to better assess residual defects in the DIP. Because fewer particles can be detected and removed during 100% inspection, there is an increased risk that the statistical sampling for the supplemental destructive testing may not reliably meet the acceptance criteria stated in ⟨790⟩. Increased preventative measures can help to compensate for the reduced detectability in DIPs. Such measures should be part of a life-cycle approach to trend and identify sources of particulate matter and use this information to reduce or eliminate their entry into finished product.

Examples of DIPs include those having opaque or deeply colored solutions, lyophilized cakes, powders, concentrated suspensions, emulsions, and ointments. Examples of DIP container types include translucent and opaque plastic; blow-fill-seal and flexible bags; foiled containers; plastic syringes; and specialty containers such as cartridges and combination products. The following sections provide additional recommendations for visual inspection and supplemental testing of DIP examples. Test methods for DIP products are discussed in *5.3 Alternate Inspection Strategies for Supplemental Testing*. Expanded information on these and additional test methods for DIP products have been published separately (*8*).

### LYOPHILIZED PRODUCTS

Lyophilized products should receive 100% inspection after the freeze-drying and sealing of each unit. However, the solid, lyophilized cake can mask the presence of visible particles because they cannot be seen within the solid matrix. The cake surfaces (top, bottom, side) are visible during inspection but account for only a small fraction of the cake volume. Because of these challenges in evaluating acceptability, a small sample of units is reconstituted and inspected for visible particles in addition to the 100% inspection of the cakes for visible particles. When reconstituted, the sample has a particle detection probability like that of solutions. Care must be taken during reconstitution to avoid contamination that can lead to false-positive results. Sample preparation should be done in a clean environment with appropriate particle-control measures. Reconstituted samples should be inspected using the same qualified methods as those for routine detection of visible particles. The destructive nature of this test limits the size of the test sample.

Once inspection of these reconstituted samples has been performed, they may be used for other required testing, such as that for subvisible particles, potency, impurities, or other specified tests. If particles are detected in this relatively small sample,

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additional units may be reconstituted as part of an investigation to assess the compliance of the entire batch. The size of the additional sample should be based on the total combined sample size (initial plus additional sample) required to have an accept number greater than 0 for a sampling plan with an AQL of 0.65% or less. This will be based on the batch size. The results from the initial and second sample must be combined, rather than resampling and repeating the test and basing the accept decision on the results of the second sample only. The following is an example of a two-stage test plan as described above: Using an S-3 plan for a batch size of 100,000 units, the initial sample size is 20, with accept on 0 and reject on 1. If 1 unit is found with one or more visible particles, the S-4 (stricter) plan may be selected, and the new sample size of 80 may be used. An additional 60 samples are then tested and, if no further evidence of visible particles is detected, the batch meets the acceptance criterion.

Alternatives to reconstitution testing can be considered, such as collection of liquid samples from the filling line after stopper insertion and prior to the lyophilization process. Such samples represent the majority of particle exposure risk. This is a typical evaluation during development activity to provide a system check for unwanted particulate matter. These risks should be assessed and documented to justify this approach. Additional process information may be supported by inspection of media fill or water fill trials for visible particle types and frequencies.

## POWDER PRODUCTS

Sterile powders are difficult to inspect for particles due to powder flow and the occlusion of white or light-colored particles by the drug product itself. Sterile powders should be reconstituted and inspected for visible foreign particles using an approach like that for lyophilized products, as discussed above.

## EMULSION AND SUSPENSION PRODUCTS

The manufacturer may not be able to detect all extrinsic and intrinsic particle types due to the inherent characteristics of an emulsion or suspension. A test dissolving the suspension or disrupting the emulsion to provide for extrinsic and intrinsic particle detection, either by visual inspection or by filtration/microscopic exam, is also recommended as part of destructive supplemental testing of a small sample, as described above for lyophilized products.

## CELL AND GENE THERAPY PRODUCTS

Cell and gene therapies present unique challenges to the normal inspection strategies employed for particulate control and assessment. Particles may be introduced from the external environment, during the manufacturing process or because of cellular or protein degradation. As cell therapies do not undergo final filtration steps, particulates must be controlled and assessed during production with visual inspection at release and prior to administration. Because the cells are the intended final product, there is an inherent limitation to the product's purification, limiting particulate removal from the ultimate formulated material. Testing is often further complicated by the small batch sizes, thus making more extensive destructive testing undesirable. Establishing acceptance limits should be based on process and inspection capability along with clinical risk assessment following similar exposure in pivotal clinical trial materials.

## AMBER CONTAINERS

Inspecting amber containers is challenging because selected glass component elements have been added to mask UV light penetration into the Type I glass container. Light transmission is blocked below 500 nm, thus increased light intensity (e.g., 8000–10,000 lux or higher) may be required to observe visible particles during inspection. Directional lighting from below or behind the container may also be beneficial. At the extreme, solutions filled in practically opaque containers may be audited via sampling and transferred to clear, clean containers. The membrane filtration microscopic method is also suitable for capturing and characterizing larger foreign particles in the visible range (>100 µm).

## PLASTIC CONTAINERS

Transparent plastic containers (e.g., vials, syringes, and larger containers) should be inspected by procedures similar to those for products in clear glass, as described throughout this chapter. The only differences may be specific consideration for the unique properties of plastic molding and physical design.

Translucent plastic containers (e.g., plastic syringes, blow-fill-seal [BFS]) are chosen for break resistance or other properties that glass cannot offer, such as the ability to be injection molded into shapes that minimize hold-up volume or that can be used in a combination product. Plastic containers may have optical properties that require significantly increased light (e.g., 8000–10,000 lux) to illuminate any visible particles against black and white backgrounds. Directional lighting from behind the container or from the bottom (Tyndall light) may also be beneficial.

Opaque plastic containers are utilized for break resistance or other properties such as metered dispensing commonly used in topical ophthalmic container systems. The expectation for 100% inspection in process for particulates during manufacture is not feasible and necessitates alternate inspection strategies (*8*). The acceptance limits for visible particles should be based on process capability as established during product development. The expectation is that this capability is judged according to industry standards and product benchmarking. The risk profile for topical ophthalmic products is reduced relative to parenteral injectable products; therefore, application of an AQL value of <0.65% for particulate matter may not be appropriate for these topical dosage forms. The recommendation is to study and trend the particle burden of the product solution and the container–closure contribution, develop a reasonable life-cycle control strategy, and establish limits based on risk assessment.

## LARGE-VOLUME CONTAINERS

Large-volume containers (>100 mL) may require additional time for a thorough inspection to be completed. For flexible containers, in addition to solution opacity, container material choices may range from clear to semitransparent with potential

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changes to container clarity following sterilization. Opportunities to inspect the empty container prior to printing or labeling, or the filled container prior to pouching or overwrapping, may be preferred to finished-package inspection. The point of inspection may be determined based upon the last opportunity for the introduction of the defect of concern and where the probability of detection is greatest. The use of higher illumination intensities along with directional lighting combinations (e.g., from behind the container), as discussed above for translucent plastic containers, should be considered to enhance the visibility of particles. The manipulation sequence for inspection should be established and may differ from traditional small-volume containers. Increased inspection time may be considered for larger fill-volume container systems.

#### COMBINATION PRODUCTS

When inspecting the unlabeled primary drug container for a combination product, the inspection considerations should be the same as those specified for a conventional drug product in a conventional package such as a vial or syringe. Sensitivity may be improved by performing inspection before assembly into the device. Where there are critical attributes that are only visible after assembly (such as alignment with a fill-level window), a second inspection after assembly may also be required.

### 5.3 Alternate Inspection Strategies for Supplemental Testing

#### TRANSFER

When the container limits thorough inspection, transfer to a clear and readily inspectable container is recommended. Using verified clean and clear containers, the sample is opened, transferred to the receiving container, stoppered, and then visually inspected.

#### FILTRATION

Membrane filtration methods, such as in ⟨788⟩, *Method 2 Microscopic Particle Count Test* focusing on particles >100 um, collect all solid particles from the filled unit(s) onto a membrane. Samples may be individual or pooled for analysis. This method will reveal all solid particles (visible and subvisible), which may be sized microscopically, and permits qualitative categorization of these retained solids.

#### CLARIFICATION

In the case of suspensions, there is a wide range of active ingredient particle sizes, from submicron (<1 µm) to tens of micrometers. In many cases, the solids may be clarified/dissolved in the original container with an appropriate filtered solvent to allow subsequent visual inspection or membrane filtration/microscopic exam. Dissolution solvent compatibility with the formula or package and filtration membrane, if used, must be demonstrated.

#### SIEVING

As above, if the solid particle suspension is small enough to allow selective sieving, this may be used as an alternative to membrane filtration. The very small solids pass through the sieve, and larger particles (>100 µm) are retained, counted, and categorized.

## 6. INSPECTION METHODS AND TECHNOLOGIES

### 6.1 Manual Visual Inspection

Manual visual inspection (MVI) is the reference inspection method described in all major pharmacopeias (*59*–*60*). It consists of viewing filled and sealed containers under controlled conditions. This process may be aided using a tool to allow consistent examination of more than one container at a time.

The quality decision, to either accept or reject the container, is made by a trained person. Inspection is a probabilistic process, and detection rates less than 100% are to be expected, especially for smaller or low-contrast defects.

#### CRITICAL PROCESS PARAMETERS IN MVI

**Light intensity:** The results of the manual inspection process are influenced by the intensity of the light in the inspection zone. In general, increasing the intensity of the light that illuminates the container being inspected will improve inspection performance; chapter ⟨790⟩ recommends that the minimum light levels be not less than 2000–3750 lux at the point of inspection for routine inspection of clear glass containers. This range was chosen to harmonize with current *European Pharmacopoeia* requirements (*59*) and is further supported by recent studies performed in Japan (*61*) as part of a review of the current *Japanese Pharmacopoeia* requirements (*60*). Special attention should be given to ensure that inspection is not performed below the lower limit of 2000 lux. Increased light levels are recommended for translucent plastic containers or those made from amber glass. Under these circumstances, light levels as high as 10,000 lux may prove beneficial. Care should be taken to avoid glare and direct viewing of the light source at these high intensities, as this may result in eye strain and fatigue. The final qualified inspection conditions of minimum light level will depend on measured performance using visual standards to ensure appropriate particle size detection sensitivity.

Light should be diffuse and even across the inspection zone, and it is a good practice to clearly identify this zone within the inspection station where the intensity meets the required levels. Fluorescent lamps have often been used as the light source for

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inspection. When fluorescent lamps are used, high-frequency ballasts are recommended to reduce visible flicker (and associated inspector fatigue). Incandescent lamps have also been used successfully for this purpose, but they generate significant heat during use. Light-emitting diodes (LED) offer an energy-efficient, stable source of light without the added heat of incandescent lamps.

Light intensity in each inspection station should be measured periodically to ensure continued compliance within the specified range. The frequency of monitoring should be based on historical experience with the type of light source in use. A lower light-intensity action limit should be established to trigger corrective action before inspection is performed below the lower limit of the range.

**Background and contrast:** Contrast between the defect of interest and the surrounding background is required for detection. Increased contrast improves detection. The use of both black and white backgrounds is described in ⟨*790*⟩, as well as other global pharmacopeias. Matte/nonglossy backgrounds are recommended to avoid interference from reflection. The use of both backgrounds provides good contrast for a wide range of particulate and container defects, which can be light or dark in appearance.

**Inspection rate:** Enough time must be provided to allow for thorough inspection of each container; ⟨*790*⟩ specifies a reference time of 10 s/container (5 s each against both black and white backgrounds). Larger or more complex containers may require additional time for inspecting all attributes. Increased time may facilitate detection of defects near the threshold of detection, but studies by Wolfe et al. (*62–63*) suggest that there are diminishing or limited gains with increasing inspection time. Time spent per container is typically a function of the inspector training or may be controlled using an inspector-triggered pacing device (proximity switch) using an indicator light or tone. Timing devices that automatically repeat the timing cycle can be distracting to other inspectors; these devices may be used during training, much as a musician uses a metronome during practice to learn the tempo of a musical piece for later performance. Recording the time spent inspecting each batch and then calculating a nominal inspection rate is a good way to confirm that the rate of inspection was within established limits. Correction can be made for non-inspection activities performed by the inspectors during this time to better document the nominal inspection rate.

**Container handling and movement:** When observing objects, the human eye is very sensitive to movement. Good techniques for manual inspection include a careful swirl or inversion of the liquid product within the container. This displaces any particles from the upper inner surfaces of the container and the closure and puts them into motion. A technique that minimizes the introduction of air bubbles is important, as air bubbles can appear as particles and interfere with detection of offending particles. If multiple containers are qualified to be equivalent to the single-container inspection method per ⟨*790*⟩, they may be held during the particle detection sequence using a tool that holds these containers for consistent presentation. Holding many containers by hand at once should be avoided, as it is difficult to obtain a complete view of all container surfaces and contents. Full rotation (360°) of the container during the container–closure defect inspection sequence is recommended for identifying small container defects such as cracks or chips.

**Magnification:** Some inspection processes use a large magnifier to increase image size and thus increase the probability of detecting and rejecting containers with defects near the threshold of detection. Although magnification can be useful for critical examination of a portion of the container, it does not often lead to increased overall detection rates for defects of interest. This may be due, in part, to the added eye strain that often results from the use of magnification. As such, it is not recommended as part of the reference inspection method described in ⟨*790*⟩ or in other global pharmacopeias (*59–60*). Although not recommended for use during routine inspections, magnification can be helpful for critical examination of a small number of units, as may be needed during an investigation.

### INSPECTOR FATIGUE AND ERGONOMIC CONSIDERATIONS

Inspecting for extended periods of time can cause inspector fatigue and a decrease in inspection performance. On the basis of industry experience (*46*), it is recommended that inspectors be given a documented break from performing inspection at least every hour. This break should allow time to rest the eyes and mind and may be achieved with a short rest (e.g., 5 min) or a longer meal break. This need for regular breaks may also be met through rotation to a non-inspection function, such as material handling or documentation.

Inspection stations should be designed and operated in a manner that minimizes the inspector's risk of repetitive-motion injury. Adjustable chairs and careful positioning of light sources as well as incoming and inspected product can reduce the risk of such injury. These adjustments can also reduce inspector fatigue and discomfort, both of which can be distracting and thus can decrease performance.

The inspection room environment should also be considered. Temperature and humidity should be controlled for inspector comfort. Reduced ambient lighting is recommended to focus the inspection process and to reduce distraction from extraneous reflections. Special care should be given to inspection rooms with exterior windows that allow daylight into the room, thereby changing ambient lighting throughout the day and with the seasons.

## 6.2 Semi-Automated Visual Inspection

Semi-automated visual inspection combines automated material handling of the containers to be inspected with human vision and judgment to make the decision to accept or reject. These systems often use a conveyor equipped with rollers to transport the containers in front of the inspector inside an inspection booth or station. For inspection of liquids, the booth can be equipped with a high-speed spin station to set particles in motion. The rollers can also be used to slowly rotate the containers in front of the inspector as they traverse the inspection zone. These systems offer a means to control the presentation of the vials and can offer additional lighting options, such as Tyndall lighting, which may enhance the appearance of some defects such as cracks or small particles. Mirrors may also be used to provide a clear view of the top and bottom of each container. Rejected units may be removed from the rollers by hand, whereas some systems are equipped with a remote rejection system that can be triggered by the inspector. Care should be taken in the qualification and operation of these systems to ensure full rotation of vials in the inspection zone to assure examination of all surfaces. In addition, studies should be conducted to ensure

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the detection of heavy particles, which may not be lifted from the bottom of the container, and to ensure that the rate of inspection produces an acceptable detection rate for defects of interest.

With semi-automated visual inspection, performance should be demonstrated to be equivalent or better than that of MVI. Some increase in throughput may be achieved because the inspector spends all the available time viewing the containers rather than splitting the time between inspection and material handling.

#### CRITICAL PROCESS PARAMETERS FOR SEMI-AUTOMATED INSPECTION

Light intensity must be controlled, as with MVI. The rate of inspection is controlled by the speed of the roller/conveyor or some equipment that allows the inspector to call for a group of containers each time. Spin speed for liquid products and rotation rate for all containers should be established during validation/qualification and maintained within the validated range for routine inspection. The background color is controlled by the color of the rollers selected and the color of the background seen through the spaces between the rollers. Qualification of inspectors and validation of the inspection equipment should be based on comparison with the compendial single-container manual-inspection process with an expectation that alternative methods such as semi-automated inspection demonstrate equivalent or better performance.

### 6.3 Automated Visual Inspection

Automated visual inspection (AVI) combines automated material handling of the containers with electronic sensing of product appearance. Containers that do not meet preprogrammed acceptance criteria are automatically rejected by the machine. Early machines performed inspection for particles and fill level, but manual or semi-automated inspection was still required for the container and closure system. Newer models have the capability to inspect all attributes of the containers, along with the contents. As with MVI, machines often spin the containers to set particles in motion and make them easier to detect. Multiple cameras are used to image various regions on the container in detail. Each camera is coupled with unique lighting to highlight specific defects in the region of interest. Light- and dark-field lighting techniques offer the same benefits as white and black backgrounds as discussed above, offering contrast for a full range of light- and dark-colored defects. A defect found by any camera is tracked through the machine to allow accurate ejection by the reject system. These machines also offer detailed reporting of defects observed in a specific production lot.

AVI offers advantages in the areas of throughput and consistency, compared with MVI (*5*). AVI may also offer enhanced sensitivity for some defects, compared with MVI, but may suffer from higher false rejection rates due to the inability to tolerate normal variation in containers or product. This is especially true for molded glass containers and flexible bags.

Validation of the automated inspection equipment should be based on comparison with the compendial manual inspection process with an expectation that alternative inspection methods demonstrate equivalent or better performance. Significant effort is required to program these systems and to test their performance against a range of known defects, as well as acceptable containers. Recent experimentation with artificial intelligence (AI) or deep learning (DL) has shown promise in improving the discrimination ability of the AVI system (*64*). Early studies show both reduced false reject rates and improved detection performance (*65*). The use of these tools may also reduce the time to develop a recipe for a new product or container.

#### LIGHT-OBSCURATION METHODS

Some systems use an optical sensor to detect the shadow of particles in solution products. This LO technique has been used successfully for detection of both subvisible and visible particles. For the detection of visible particles, it requires particles to be in motion, typically using a high-speed spin and rapid braking of the container to achieve this motion. Spin conditions must be optimized to provide sensitivity for heavier particles while minimizing false rejections due to bubbles. Some biological products experience shear-induced agglomeration, so care should be taken to avoid excessive agitation and flow rates with these products.

LO methods are optimized for sensitivity to moving particles and can thus be made less sensitive to minor container imperfections. These methods can be used with both tubing and molded containers. Results are generally robust in detecting single particles that are 100 µm in diameter and larger, though detection of smaller particles at or below the visible threshold is improved when multiple particles are present.

These systems can also detect fill height by detecting the shadow of the solution meniscus. Generally, this process is not sensitive enough to ensure compliance with dose- or fill-weight specifications, but it can provide a secondary check of gross fill. Sensitivity is a function of the container shape, with greater sensitivity achieved in small-diameter containers.

#### IMAGING METHODS

Continuing advances in camera technology now allow the rapid capture of high-resolution images for inspection. When coupled with high-speed processors that have ever-increasing computational capability, a powerful inspection tool can result. Images are divided into inspection windows, and an array of tools such as image subtraction, pixel counting, intensity analysis, and others are used to assess the images against programmed quality attributes.

Imaging systems can detect particles and fill level, as well as other container and closure attributes. Inspection in this manner can provide a comprehensive inspection of visual attributes. These systems can offer high sensitivity but may also have high false-rejection rates if container and product attributes are not tightly controlled.

X-ray imaging has also been explored to detect particles within freeze-dried cakes, powders, or suspensions (*66*).

These technologies may be used alone or in combination with other inspection methods (e.g., leak detection or container integrity) to provide a comprehensive assessment of product quality before labeling and packaging.

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# 7. QUALIFICATION AND VALIDATION OF INSPECTION PROCESSES

## 7.1 Standards

The use of standards for visual inspection has been described by Melchore and Berdovich (*67*). Development of inspection standards begins with a description of the defect types that will be represented in the test set(s). This information typically comes from observations made during product development and the manufacturing area, where naturally occurring defective units can be identified from rejected product. The defects are categorized as critical, major, or minor. These defects must be further characterized to allow for the selection of naturally occurring particulates and physical or cosmetic production rejects removed from the product lots. Characterization information on defects should include, where appropriate, the range of sizes typically observed and the specific location on the container. If feasible, a photograph of the defect should be included. All information that could support consistent re-creation of the defect standards should be included in the characterization description.

## 7.2 Preparing Defect Standards

Visual-inspection particulate standards should include particles identified during product development, acquired from known production rejects, or created manually with well-characterized particulate material. A single particle seeded into each container should be used when fabricating sets to determine detection thresholds. The focus should be to demonstrate single-particle detection sensitivity, especially at the edge of the reject and "gray" zones. The use of multiple particles in a container is not recommended in order to avoid skewing the data by increasing the PoD.

## 7.3 Particle Types

The primary packaging materials that directly contact the product and the potential environmental contaminants can be divided into specific particle groups such as glass, stainless steel, elastomeric closure, plastic, and fibers (synthetic or natural). Naturally occurring visible particles from production rejects containing these materials may be used for training and to assess inspection performance. Physically prepared particles can be sieved initially to target a specific size, and then the individual particles are measured using optical microscopy prior to seeding into a prepared container. The use of characterized production defects or physically prepared standards is preferred over standard spherical particles for inspector training and qualification, as well as machine validation as they better represent actual inspection challenges and performance.

Spherical standard particles may be utilized as surrogates for naturally occurring particulates; however, these are best used for routine machine calibration rather than validation or inspector qualification, as they do not move or look like actual production defects.

## 7.4 Rejection Probability Determination

Once a well-defined defect standard is available, it is assigned a detection frequency or PoD by conducting a documented, human-inspection qualification that is accomplished by repeated manual inspection. This repeated inspection is the basis for qualifying the defect standard. This approach has been described by Knapp and Kushner (*57–58*). The Knapp methodology recognizes that the detection of particles is probabilistic, and repeated inspections with strict controls on lighting and inspection pacing/sequencing generate the statistical confidence to assign a reject probability to each standard unit. A manual, visual inspection PoD of 0.7, or 70% or greater is required to assign the container to the reject zone for subsequent calculation of the reject zone efficiency (RZE). The RZE is simply a measure of the probability or percentage of containers assigned to the reject zone that are detected during inspection. Additional discussion on the calculation and use of RZE is provided in *7.8 Inspector Qualification Requirements*. Secure probabilistic data for particulate standards can be achieved with 30–50 inspections of each container. This is best achieved with multiple inspectors. Inspection reject probability is calculated for the defect as follows:

$$\text{PoD} = (\text{Number of times rejected})/(\text{Number of times inspected})$$

## 7.5 Test Sets

These qualified defect standard units are then assembled into test sets, which may be used to specifically challenge the particle detection technique of human inspectors, used as part of a defect test set (including container–closure defects) for human qualification, or used for comparison of PoD results during automated equipment qualification and validation. Acceptance criteria should reflect the different risk categories (i.e., critical, major, and minor) of these defects when combined into a single test set, and a single acceptance criterion should not be used with a set with multiple defect risk categories. When possible, the test set should be prepared with duplicate product units per particle type and size to ensure that backup units are available if a standard container is broken, or the particle is trapped or lost within the container. Particle standard units of the same type or size may not have identical performance due to subtle differences in morphology leading to different inspection (PoD) results. Therefore, each unit (including duplicates) must be calibrated to determine its average PoD by manual inspection, see *7.4 Rejection Probability Determination*. When using test sets, it is a good practice to verify the presence of particles before and after use, as particles may become lodged between the container and the closure. A stereomicroscope with top and bottom illumination is very helpful for this. When a freely moving particle cannot be verified in liquid preparations, the unit should not be used, and the data should be excluded from subsequent calculations. When this happens, it may be possible to free the particle with the use of an ultrasonic bath. If this is not possible, the unit should be replaced. For dry solid preparations, such as powders or lyophilized cakes, the appearance of the standards may degrade with repeated handling, requiring frequent

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replacement and adding to the difficulty in performing these types of studies. The number of defective units in each test set should be limited to approximately 10% to prevent rejection bias (*62*). The accept containers will be identified as having a predetermined manual visual inspection PoD of <0.3 or 30%. The accept zone containers are used as blank units or may be used to study the false reject rate of the inspection method. Any particle standards found to fall within the acceptable gray zone, indicating a manual inspection rejection probability ≥30% and <70%, may be included as an "acceptable unit" in a test set, if desired. The true utility of the gray zone units is to study the units from ≥50% and <70% PoD and demonstrate manual detection frequency of subvisible particles just below the reject zone threshold. These data can be used for illustrating the subtle differences between the manual inspection and alternate inspection methods (semi-automatic or fully automatic) during comparison studies. The human efficiency in detecting gray zone containers should be used for information only; formal acceptance criteria is not typically applied to gray zone containers (*68*).

It is important to prepare a written procedure for the creation and maintenance of standards. This procedure should define the qualification criteria, appropriate storage conditions, periodic examination and requalification, expiration, and sample custody during use. Test sets should be approved by the quality unit. The container in which the specific particle set is stored must be clearly labeled with the test set identification information.

## 7.6 Types of Test Sets

The particle detection threshold can be determined for a specific inspection method and product/package combination. For example, it can be displayed as a standard curve of detection probabilities at various particle types and sizes in an approximate range of 100–500 µm (with recommended increments of 100 µm). Fibers are typically observed in sizes greater than 500 µm. The typical size range of particles used in threshold studies incorporates a variety of particle types and densities that are typically found in the manufacturing environment.

Threshold studies are conducted to determine the sensitivity of manual inspection methods, using a range of particle sizes, in a blinded study that yields the particle-size detection capabilities of a defined group or of an individual inspector. The threshold studies indicate that the method of inspection is valid and has appropriate detection sensitivity. For example, for clear solutions in 10-mL tubing glass vials, past threshold studies indicate that particles within the range of 150–250 µm (500–2000 µm for fibers) can be detected with a PoD of 70% or greater. Results can differ due to differences in dosage forms as well as container type and size. Particulate threshold studies are also useful as an assessment tool when evaluating or qualifying visual inspection staff on a specific method with fixed testing parameters. Container and closure defects should also be evaluated for detection performance (PoD) with prepared test sets using the assigned risk categories (e.g., critical, major, and minor) in a manner similar to that for particles. Detection threshold studies are typically the first step in evaluating the performance of any new inspection method.

Depending on the product and/or presentation, rejects in the test set should represent all defect types anticipated for a given container type or product family. For particles, use a bracketed range of types (densities) and sizes, from near the lower limit of the visible range (>100 µm in clear solutions) to the largest sizes routinely observed or expected in the pool of rejects. Separate test sets should be designed to represent that the unique visual characteristics of DIPs (*8*), such as lyophilized powders, suspensions, or emulsions or containers such as those made from amber glass, are inspected. Threshold studies should be used to determine the size and type of particles that can be routinely detected (≥70% PoD) in this DIP. These data can be used to support risk assessment for an optimized inspection program and help identify when and where additional upstream controls may be needed.

For an individual manual test set, it is important that all containers and closures are of the same type, and the samples are blinded. UV ink (invisible to the inspectors) may be used to mark containers. Alternatively, bar codes or other coded labels may be used. Manual test sets can be used initially to qualify, or periodically to requalify, human inspectors. These test sets may also be used for direct comparison of manual inspection to semi-automated or automated inspection methods during initial qualification or for periodic requalification.

A bracketing approach may be used to represent a range of different container sizes when other visual characteristics are similar. Other specialized test set types may be prepared as needed to be used for daily challenge or suitability checks for automated inspection systems.

## 7.7 Training and Qualification of Human Inspectors

Before training, potential inspectors should be tested for visual acuity (*69*) and color perception. Near-vision performance should be the equivalent of 6/6 m (20/20 ft), but not conducted at this distance, and with no impairment of color vision. Both the Snellen and Jaeger charts are useful for verifying visual acuity; they test far and near vision, respectively. The use of corrective lenses to achieve the desired visual acuity is permitted. Training should include a phased approach with a specified number of training hours expected for each segment. Initially, train the potential inspectors with defect photographs or a video library and clear, written descriptions. Utilize subject matter experts to mentor and provide hands-on training with defect standards for the specified method. Reinforce mental or silent counting and follow the paced sequence to achieve consistent inspection timing. Stress the importance of strict adherence to the inspection process (procedure, sequence, and timing). Inspector fatigue may be addressed in the qualification process by testing under worst-case conditions (e.g., at the end of a typical inspection shift).

Train all inspectors (QC, QA, and production) with common procedures used for 100% inspection and AQL inspection. All inspection practices should be standardized and consistently executed by all inspection groups.

Qualification should be performed for each product type and package that the inspector will encounter. A bracketed or matrix approach can be used to simplify qualification of products with similar physical or visual characteristics such as container type and size, dosage form, product viscosity, color, and others (product families or groups). It is common to initially train and qualify personnel on clear solutions in clear containers (if produced at the facility) and then expand their expertise to the inspection of more difficult dosage forms or presentations.

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## 7.8 Inspector Qualification Requirements

The qualification of all inspection personnel utilizes a manual test set to be inspected under normal operating conditions and inspection critical parameters, including inspection timing and sequence, physical environment, and inspection duration. Three successful inspections of the test set are recommended to demonstrate consistent performance for initial qualification of new inspectors. Acceptance criteria for each defect class should be based on the PoD, or RZE observed during test set qualification for the package.

There are generally two basic approaches to establishing qualification acceptance criteria for the particulate matter detection sequence. Other qualification criteria may be used with sound rationale.

PERCENT RECOVERY QUALIFICATION METHOD: A test set is prepared with bracketing of each particle type with a one-seeded unit being near-threshold (just above 70% PoD) and the other larger with a higher PoD. Typically, not less than 80% or 90% of the units containing visible particles are expected to be detected from the qualification test set.

RZE QUALIFICATION METHOD: The previously determined test set RZE is used to assess the results obtained by the inspector to be qualified. Each inspector to be qualified would then inspect the blinded set, and the RZE (the average PoD for all units containing particles >70% PoD) is calculated for each trial. The acceptance criterion is to achieve an RZE during the qualification that is equal to or better than the RZE originally determined during the threshold study.

$$\%PoD = (\text{Number of times rejected})/(\text{Number of times inspected}) \times 100$$

RZE = the average of all PoD values for all units in the test set with an individual %PoD >70%

When assessing inspector performance, a limit is also needed for false rejection. A target of <5% falsely rejected good units in the test set is recommended.

## 7.9 Requalification

Inspectors should be requalified at least annually. Requalification includes a test of visual acuity and testing with at least one product/test set configuration. A single successful inspection of the test set is sufficient for requalification. Requalification may also be necessary if poor performance is observed during routine inspection or if the inspector has been away from the inspection operation for an extended period (e.g., more than 3 months).

If an inspector fails the requalification test, a retraining process should be initiated to identify the root cause and allow the inspector to receive additional instruction. After this process has been completed, the inspector may attempt to meet the acceptance criteria one additional time. If the inspector fails, he or she may attempt to qualify again after a specified time period.

# 8. PRODUCTS IN DISTRIBUTION

Chapter ⟨790⟩ states, "If it becomes necessary to evaluate product that has been shipped to customers (e.g., because of a complaint or regulatory concern), sample and inspect 20 units. If no particles are observed in the sample, the batch is considered essentially free of visible particulates. If available, additional units may be inspected to gain further information on the risk of particulates in the batch."

For products in distribution, questions regarding batch quality will occasionally arise from customer complaints, observations in the field, customer use questions, and from the use of nonstandard (sensitive) conditions of inspection. As discussed in this chapter, the detection process is probabilistic, and the likelihood of detection is a cumulative function of the particle's visible attributes, drug product and container characteristics, and the inspection method used. In an appropriately qualified manufacturing process, the batch is presumed to have been prepared according to robust processes and all containers with package defects and visible particles (nonconforming units) removed prior to labeling. In that regard, the evaluation outlined in ⟨790⟩, *Inspection Procedure, Sampling at Batch Release (After 100% Manufacturing Inspection)* and *Product in Distribution* is only permissible if both sampling at batch release and a 100% manufacturing inspection has been successfully completed.

The particle detection threshold should be determined for a specific inspection method and product/package combination incorporating a variety of particle types and densities that are typically found in the manufacturing environment. For example, the detection threshold for routine, reliable detection (≥70% probability) of a single spherical particle in a clear solution contained in a 10-mL vial utilizing diffuse illumination between 2000 and 3000 lux is often near 150 µm in diameter (*5*). Units returned from distribution may be false positive, may contain particle(s) larger than the acceptance threshold that were missed, may contain particle(s) in the gray zone (i.e., less than the detection threshold), or may have suffered a physicochemical change that resulted in a visible change. Ideally, there will be no visible particles in the containers released to market; however, there is always a low probability that this may occur.

Upon receipt, suspect containers should be subjected to the same inspection conditions and methodology used in the release inspection. Particle(s) verified in the returned or re-evaluated supply must be carefully characterized by an analytical forensic process to determine their source and likely cause. Single particles of typical product-contact materials are unlikely to present a concern. Multiple particles, large particle sizes, and any particles indicative of physical or chemical change are significant events and should be subject to further investigation. Rare instances of particulate material falling into the gray zone should be expected given the probabilistic nature of the inspection process and should not routinely trigger further evaluation of retention samples. Chapter ⟨790⟩ provides that zero particles found in the sampling and inspection of 20 units signify that the batch is "essentially free of visible particulates." If multiple suspect containers from the same batch are detected, additional units should be inspected, and an appropriate rationale provided to support the batch's conformance to the registered specifications.

Overall batch quality using internal systems to control particulate matter and the means to investigate these occurrences is key to the life cycle approach for modern pharmaceutical production. Evaluation of retention and stability samples provides insight to batch quality, as do the field-use effects for any medication. While the presence of particles or product or container

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defects discovered in retained or returned product do not necessarily incriminate the quality of the batch, a careful investigation should be conducted to exclude systemic risks.

## 9. CONCLUSIONS AND RECOMMENDATIONS

Visual inspection for particles and other visible defects continues to be an important part of the manufacturing process for injections. Chapter ⟨790⟩ provides a useful reference method and acceptance criteria for visible particulates in injections. Successful execution of visual inspection requires an understanding of the inspection process and careful control of inspection conditions. Both visual inspection procedures and investigative processes for elevated rejects consist of comprehensive and well-documented systems relevant for the products being manufactured. Inspectors must be trained to ensure consistent, high-quality performance. Alternative inspection methods, either semi-automated or fully automated, may be used in place of manual inspection methods. Where machine methods are used, the equipment must be validated to demonstrate equivalent or better performance when compared to manual inspection. The use of test sets that contain standard defects is an important element in inspector training and qualification as well as machine validation. Good product development will lead to a stable product with a lower risk of particle formation. Identification of the type or types of particles found during product development and routine manufacturing is critical to reducing their occurrence. Inspection results should be trended to further aid in continuous process improvement with the ultimate goal of defect prevention.

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# ⟨1821⟩ RADIOACTIVITY—THEORY AND PRACTICE

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## 1. INTRODUCTION

Radioactive drugs and devices require specialized techniques in their production, testing, handling, dispensing, and administration to ensure optimal effectiveness and maintain safety for workers, patients, and the public. All operations involving these articles should be carried out by or under the supervision of personnel who have been appropriately trained in the handling of radioactive materials.

The facilities for the production, storage, and use of radioactive drugs and devices are generally subject to licensing by the U.S. Nuclear Regulatory Commission, an appropriate state agency, or similar governmental agencies outside of the United States. Most radioactive drugs and devices, although not identified as hazardous drugs, are classified as hazardous materials and are therefore subject to other regulations relating to transportation, environmental release, and workplace safety.

The purpose of this chapter is to provide information regarding radioactivity—including definitions, types of decay, and general considerations relating to radioactive decay, counting, radionuclide production, purity, and labeling—as well as instrumentation for detection and measurement of radioactive emissions.

Specific information on standards for radionuclide identification and assay, including instrument qualification, performance checks, identification of radionuclides and radionuclidic impurities, and assay of radionuclides are provided in *Radioactivity* ⟨821⟩.

## 2. TYPES OF DECAY

"Radioactive decay" is the process by which an unstable nuclide transitions to a lower energy configuration. Depending on the particular starting radionuclide, the result of the transition may be either a stable nuclide or a different radionuclide. Typically, these transitions are accompanied by the emission of radiation from the nucleus, which is broadly classified as either particulate or nonparticulate. Some radionuclides emit multiple types of radiation in this process, whereas others emit only a single type. The main types of particulate radiation commonly seen in nuclear medicine are alpha, beta, and positron. Nonparticulate types of radiation include gamma rays and X-rays. Nuclear medicine imaging is accomplished through detection and localization of nonparticulate radiation, whereas therapeutic effects arise from the energy deposited in the target organ by particulate radiation.

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### 2.1 Alpha Decay

"Alpha decay" is radioactive decay with the emission of alpha particles, or helium nuclei, and is generally limited to elements with an atomic number >83. In some cases, beta particles and gamma rays may also be emitted during alpha decay. An example of a radionuclide that decays by alpha decay is:

Radium-226 → Radon-222

### 2.2 Beta Decay

"Beta decay" is radioactive decay with the emission of an electron. This type of decay typically occurs in neutron-excessive radionuclides wherein a neutron is transformed into a proton. In some cases, the emission of a positively charged electron, or positron, may occur. This type of decay typically occurs in neutron-deficient radionuclides with a lower atomic number wherein a proton is transformed into a neutron. In some cases, gamma rays may also be emitted during beta decay. An example of beta decay through emission of an electron is:

Iodine-131 → Xenon-131

An example of beta decay through emission of a positron ($\beta^+$) is:

Fluorine-18 → Oxygen-18

Because a positron is an anti-electron, when it interacts with an electron, the two particles annihilate, and their combined mass is transformed into energy in the form of two 511 kiloelectron volt (keV) gamma rays. These gamma rays are produced simultaneously and travel away from the point of interaction in nearly opposite directions. These two characteristics form the basis for positron emission tomography (PET) imaging techniques.

### 2.3 Electron Capture Decay

"Electron capture" is radioactive decay that involves nuclear capture of an inner orbital electron, nuclear transformation of a proton into a neutron, and emission of one or more gamma rays. Electron capture generally occurs in higher atomic number radionuclides that are neutron deficient. An example of a radionuclide that decays by electron capture decay is:

Iodine-123 → Tellurium-123

### 2.4 Isomeric Transition

An "isomeric transition" is radioactive decay that involves a transition between nuclear isomers with the emission of one or more gamma rays. In contrast to other types of decay, the number of protons and neutrons remains the same in an isomeric transition. Isomeric transition generally occurs in radionuclides that are metastable. An example of a radionuclide that decays by isomeric transition is:

Technetium-99m → Technetium-99

## 3. GENERAL CONSIDERATIONS

### 3.1 Radioactivity

Radioactive decay is a first-order process (i.e., a fraction of atoms decay per unit time). The rate of decay for each radionuclide is a unique and constant value, which gives rise to the term "decay constant".

Each radionuclide's rate of decay is a unique and constant value (its decay constant) and can be described by the following equation:

$$A = \lambda N$$

$A$ = amount of radioactivity in a source at a given time
$\lambda$ = rate of decay of the radionuclide
$N$ = number of radioactive atoms

The traditional unit for radioactivity is the curie (Ci), which is equal to $3.7 \times 10^{10}$ atoms undergoing radioactive decay, or disintegrations per second (dps). Commonly used prefixes associated with the Ci include the millicurie (mCi) and the microcurie (μCi). The SI unit for radioactivity is the becquerel (Bq), which is equal to 1 dps. Commonly, prefixes associated with the Bq include the megabecquerel (MBq) and the gigabecquerel (GBq). Hence, 1 Ci = 37 GBq.

### 3.2 Fundamental Decay Law

The decay of a radioactive source is described by the equation:

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$$N_t = N_0 e^{-\lambda t}$$

$N_t$ = number of radioactive atoms remaining at elapsed time $t$
$t$ = time elapsed (time unit, such as seconds, minutes, or hours)
$N_0$ = number of radioactive atoms when $t = 0$
$\lambda$ = decay constant of the specific radionuclide

The above equation can be rewritten in terms of radioactivity:

$$A_t = A_0 e^{-\lambda t}$$

$A_t$ = amount of radioactivity at elapsed time ($t$)
$t$ = time elapsed (time unit, such as seconds, minutes, or hours)
$A_0$ = amount of radioactivity when $t = 0$
$\lambda$ = decay constant of the specific radionuclide

"Decay tables" that provide radionuclide-specific decay factors (i.e., fraction remaining) calculated from $A_0 e^{-\lambda t}$ at various elapsed times ($t$) are commonly available.

The half-life is defined as the time interval required for a quantity of radioactivity to decay to one-half of its initial value and is related to the decay constant $\lambda$ by the equation:

$$T_{1/2} = 0.69315/\lambda$$

$T_{1/2}$ = half-life of the radionuclide
$\lambda$ = decay constant of the specific radionuclide

The activity of a pure radioactive substance as a function of time can be obtained from the exponential equation, from decay tables, or by graphical means based on the half-life (*Figure 1*).

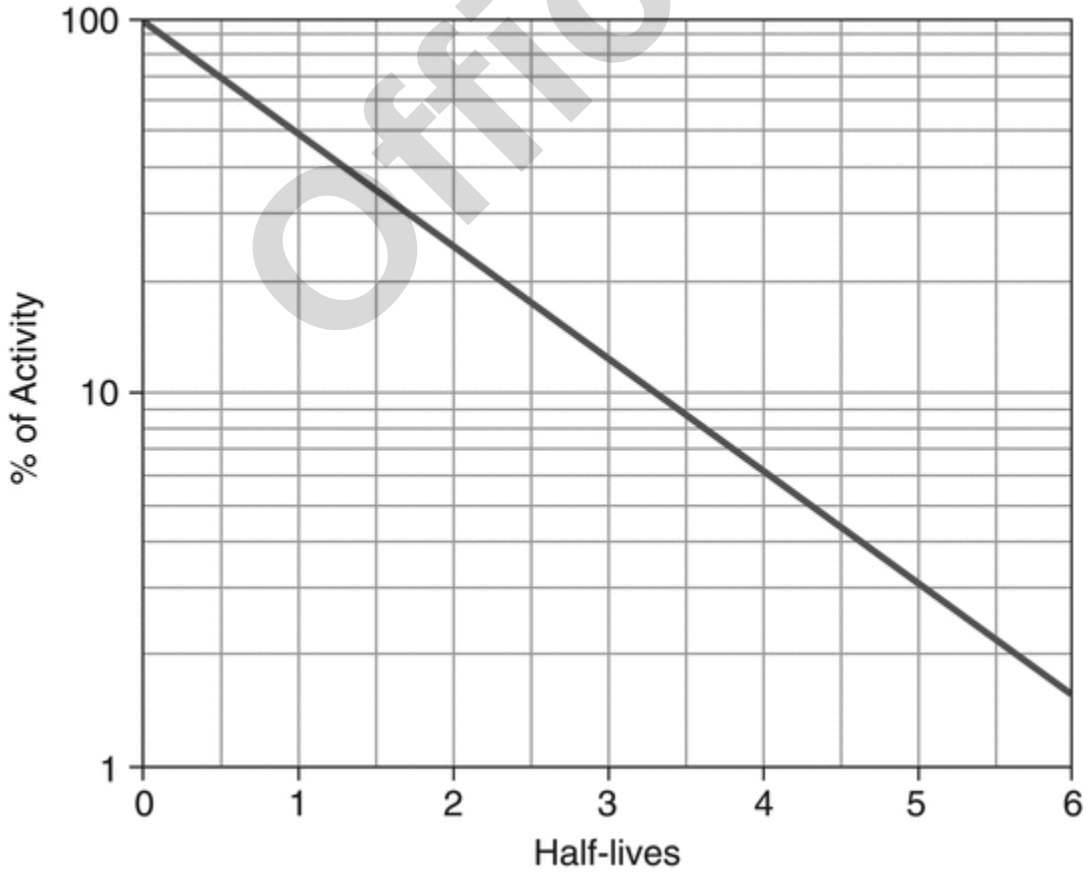


Figure 1. Normalized decay chart.

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## 3.3 Counting Efficiency

The validity of any radionuclide measurement is dependent upon the reproducibility of the relationship between the source, the detector, and its surroundings. Appropriate allowances should be made for source configuration. Measurements of radioactivity require calculation of a calibration factor, or efficiency, and are dependent on the type of detector, the container in which the radioactivity is placed, and the source–detector geometry. The basic efficiency equation for detectors designed to count discrete decay events over a period of time is given as:

$$\text{disintegrations/s} = (\text{counts/s})/\varepsilon$$

$\varepsilon$ = efficiency or calibration factor

Because 1 dps is defined as 1 Bq, the above equation gives radioactivity in units of Bq. Through the application of the appropriate unit conversion, Bq can be transformed into µCi or any other unit of radioactivity. Detectors should be calibrated with a source of known radioactivity and in a fixed geometry.

## 3.4 Background

Cosmic rays, radioactivity present in the detector or shielding materials, and radiation from improperly shielded sources contribute to the background radiation. All radioactivity measurements should be corrected by subtracting the background count rate from the gross count rate in the test sample.

## 3.5 Statistics of Counting

Modern radiation detection systems often incorporate statistical analysis into their software. The user should understand the use and limitations of these programs to ensure accurate results.

Because the process of radioactive decay is a random phenomenon, the events being counted form a random sequence in time. Therefore, counting for any finite time can yield only an estimate of the true counting rate. The precision of this estimate, being subject to statistical fluctuations, is dependent upon the number of counts accumulated in a given measurement and can be expressed as:

$$\sigma = \sqrt{n}$$

$\sigma$ = standard deviation
$n$ = number of counts accumulated in a given measurement

The probability of a single measurement falling within $\pm 100/\sqrt{n}\%$ of the mean of a great many measurements is 0.68, which means that if each count were to lie within $\pm 100/\sqrt{n}\%$ of the mean for approximately two-thirds of the observations, then approximately one-third of the observations would lie outside of this interval.

Because of the statistical nature of radioactive decay, repeated counting of an undisturbed source will yield count-rate values in accordance with the frequency of a normal distribution. Any deviations in these values from the normal distribution conform to the chi-square ($\chi^2$) statistical test. For this reason, the $\chi^2$ test is frequently applied to determine the performance and correct operation of a counting assembly. The term "figure of merit" of a radioactive counting instrument is expressed as:

$$\text{Figure of merit} = \varepsilon^2/B$$

$\varepsilon$ = counter efficiency
$B$ = background count rate (cps)

In the selection of instruments and conditions for assay of radioactive sources, the figure of merit should be maximized.

## 3.6 Minimum Detectable Activity

In situations where only very small quantities of radioactivity are to be measured, the lower limit of the ability of the instrument to detect that particular radionuclide should be known. The "minimum sensitivity", also referred to as "limit of detection", is defined as the net count rate above background that must be exceeded before a sample is deemed to contain detectable radioactivity with a specified level of confidence. The minimum sensitivity is generally considered to be 3 standard deviations above the mean background count rate and is calculated as:

$$\text{Minimum sensitivity} = (3 \times \sqrt{B})/t$$

$B$ = background count rate (cps)
$t$ = count time

The minimum detectable activity is defined as the smallest quantity of radioactivity that can be measured under the specific conditions of minimum sensitivity and counting efficiency of the instrument. It is calculated as:

$$\text{Minimum detectable activity} = (\text{Minimum sensitivity})/(\varepsilon \times F)$$

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$\varepsilon$ = counting efficiency
$F$ = unit conversion factor

For example, if minimum sensitivity is in units of dpm and minimum detectable activity is desired to be in units of Bq, then $F$ = 60 dpm/Bq. If minimum sensitivity is in units of cpm and minimum detectable activity is desired to be in units of µCi, then $F = 2.2 \times 10^6$ cpm/µCi.

## 3.7 Limit of Quantification

The limit of quantification is the smallest quantity of radioactivity that can be quantitatively determined with suitable precision and accuracy. The limit of quantification is used particularly for the determination of impurities and degradation products. In practical terms, the limit of quantification is usually considered to be 10 standard deviations above the mean background count rate.

## 3.8 Counting Losses

The minimum time interval that is required for the counter to resolve two consecutive signal pulses is known as the dead time. The dead time is typically on the order of microseconds for proportional and scintillation counters and to hundreds of microseconds for Geiger counters. Nuclear events occurring within the dead time of the counter will not be registered. The corrected count rate, $R$, can be calculated using the formula:

$$R = r/(1 - r \times \tau)$$

$r$ = observed count rate
$\tau$ = dead time

The correction formula assumes a nonextendable dead time. The observed count rate, $r$, refers to the gross sample count rate and is not to be corrected for background before use in this equation. For general validity, the value of $r \times \tau$ should not exceed 0.1. Most modern counting systems have the ability to account for dead time, but dead time may still be a factor to consider in some circumstances.

## 3.9 Linearity and Range

When a radiation detection instrument is used in a quantitative measurement, the instrument should be suitable for the type(s) of radiation to be measured, and the instrument response should be linear over the range of measurements or a correction factor should be applied. Normally, a minimum of five different quantities of radioactivity are used to establish linearity. These quantities should bracket the range of radioactivity levels that are routinely measured in a particular application.

## 3.10 Calibration Standards

All radioactivity assays should be performed using measurement systems that have been calibrated with appropriately certified radioactivity standards. Such calibration standards may be purchased either directly from an appropriate National Metrology Institute (NMI) or from other sources that have established traceability to the NMI, through participation in a program of inter-comparative measurements. Where such calibration standards are unavailable, nuclear decay data required for calibration can be obtained from the Evaluated Nuclear Structure Data File maintained at the Brookhaven National Laboratory.[1]

## 3.11 Production of Radionuclides

The various radionuclides found in nature generally have undesirable properties for nuclear medicine applications, including very long half-lives (e.g., thousands or millions of years), decay with emissions of alpha or beta particles, and low isotopic purity because of the presence of other isotopes of that element. Because of these properties, naturally occurring radionuclides are rarely used for radiopharmaceuticals, except for some alpha-emitting members of the actinide decay series, which are used for certain therapeutic radiopharmaceuticals (e.g., radium-223).

There are four common production routes for artificially produced radionuclides: fission, neutron activation, charged-particle-induced reactions (cyclotron), and radionuclide generators.

"Fission byproducts" refer to radionuclides that are obtained as byproducts of the fission of uranium (uranium-235). These radionuclides, whether directly produced fission fragments or subsequent members in a decay chain originating from a fission fragment, can be chemically separated into individual forms from the mixture of fission products. Desirable properties of fission byproducts include high isotopic purity and moderate cost. Undesirable properties may include beta-particle emissions, low specific activity of certain radionuclides, and the limited selection of radionuclides produced. Examples of fission byproducts used in nuclear medicine applications include iodine-131 and xenon-133. Currently, the most widely used fission byproduct is molybdenum-99, which is used in technetium-99m generators.

"Neutron activation" refers to the production of radionuclides in a nuclear reactor by bombarding target atoms with thermal neutrons. Nuclear transformations, induced by neutron capture, result in isotopes possessing one additional neutron, and thus an atomic mass increased by one. The excess energy of the newly formed isotope is emitted as a gamma ray. These reactions

[1] http://www.nndc.bnl.gov/ensdf.

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are often termed ($n,\gamma$) reactions. Desirable properties of neutron-activated radionuclides include a wide variety of isotopes that can be produced and the moderate cost to produce them. Undesirable properties may include decay with beta-particle emissions and relatively low isotopic purity (i.e., unreacted stable target atoms are mixed with their radioisotope products). Because of their beta emissions, however, several of these radionuclides have been used in therapeutic radiopharmaceuticals. Examples of neutron-activated radionuclides used in therapeutic radiopharmaceuticals include strontium-89, yttrium-90, iodine-131, samarium-153, and lutetium-177.

Cyclotron production of radionuclides occurs by bombarding stable atoms with charged particles (e.g., protons or deuterons) that have been accelerated in the cyclotron's oscillating electromagnetic field. Nuclear transformations induced by particle capture usually result in a radioisotope of a different element with the emission of one or more neutrons or protons. For example, if a proton is captured and a neutron is emitted, the reaction is often termed a ($p,n$) reaction. Desirable properties of cyclotron-produced radionuclides include the wide variety of isotopes that can be produced, the availability of alternate production schemes, radionuclide decay by electron capture or positron decay rather than by beta decay, and high isotopic purity. Undesirable properties may include contaminating radioisotopes from side reactions and the relatively high cost of radionuclide product. Examples of cyclotron-produced radionuclides used in nuclear medicine applications include carbon-11, fluorine-18, gallium-67, indium-111, iodine-123, and thallium-201.

Generators refer to a special method of radionuclide production whereby a short-lived radionuclide results or is generated from the decay of a longer-lived radionuclide. The longer-lived parent radionuclide is generally bound to a column, and the short-lived radionuclide daughter product is then extracted (eluted) from the column. After elution, subsequent decay of the long-lived parent radionuclide generates more of the short-lived radionuclide daughter product, which can then be extracted. A generator provides a specific radionuclide in sequential elutions over a prolonged period of time. Desirable properties of generator-produced radionuclides include ready availability, portability, low-to-moderate cost, variety of radionuclides and type of decay, and relatively high isotopic purity. Undesirable properties may include the limited number of parent-daughter pairs and the potential for generator breakthrough of the parent radionuclide in the eluate. Examples of generator-produced radionuclides used in nuclear medicine applications include technetium-99m (daughter of molybdenum-99), rubidium-82 (daughter of strontium-82), and gallium-68 (daughter of germanium-68). The characteristics of all four production methods are summarized in *Table 1*.

**Table 1. Production Methods of Radionuclides**

| Production Method | Nuclear Reactor (fission byproduct) | Nuclear Reactor (neutron activation) | Cyclotron | Radionuclide Generator |
|---|---|---|---|---|
| Bombarding particle | Neutron | Neutron | Proton, deuteron, triton, alpha | Production by decay of parent |
| Product | Neutron excess | Neutron excess | Neutron poor | Neutron poor or excess |
| Typical decay pathway | $\beta^-$ | $\beta^-$ | Positron emission, electron capture | Several modes |
| Typically carrier free | Yes | No | Yes | Yes |
| High specific activity | Yes | No | Yes | Yes |
| Relative cost | Moderate | Moderate | High | Low to moderate |
| Radionuclides for nuclear medicine applications | Molybdenum-99, iodine-131, xenon-133 | Iodine-131, strontium-89 yttrium-90, samarium-153, lutetium-177 | Thallium-201, iodine-123, gallium-67, indium-111, fluorine-18, carbon-11 | Technetium-99m, krypton-81m, gallium-68, rubidium-82 |

Other methods of radionuclide production have been developed but currently are not used to produce radionuclides used in radiopharmaceuticals.

## 3.12 Carrier

The total mass of radioactive atoms or molecules in a radiopharmaceutical is directly proportional to the amount of radioactivity and is usually too small to be measured by ordinary chemical or physical methods. For example, 37 MBq (1 mCi) of iodine-131 has a mass of $8 \times 10^{-9}$ g. Because such small quantities behave in an anomalous manner, such as nonspecific adsorption to container walls, a carrier may be added during processing to permit ready handling. Amounts of the carrier, however, should be sufficiently small so that undesirable physiological, pharmacological, or toxicological effects are not produced. Also, because the carrier is chemically identical to the radionuclide, the amount of carrier should be limited to avoid competitive interference with the desired chemical reactions and overall radiochemical yield.

The term "carrier free" refers only to radioactive preparations in which other isotopes of the radionuclide are absent (i.e., free from the presence of carrier). In practice, a true carrier-free state may be difficult or impossible to achieve because of the ubiquity of certain elements or molecules. Hence, the term "no carrier added" may more appropriately describe a preparation that may contain a trivial amount of carrier but for which additional carrier has not been purposefully added. Radionuclides produced by neutron activation reactions generally contain substantial amounts of nonradioactive isotope remaining from unreacted target material and thus cannot be considered carrier free. However, there are select cases in which this is not the case, such as ($n,p$) reactions.

The radioactivity per unit volume of a medium or vehicle containing a radionuclide is referred to as the "radioactivity concentration", "specific concentration", or "strength" and is expressed in units such as Bq/mL or Ci/mL. The radioactivity of a radionuclide per unit mass is referred to as "specific activity" and is expressed in units such as Bq/g, Ci/g, or Bq/mol. The

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maximum specific activity of a radioactive preparation exists when it is in a carrier-free state; the addition of a carrier results in lowered specific activity.

## 3.13 Radiochemical Identity and Purity

"Radiochemical identity" may be defined as the molecular structure of the compound that contains the radionuclide. Because it is nearly impossible to analyze the structure of radiolabeled compounds with the traditional tools used for organic structure determination, the radiochemical identity of a radiopharmaceutical is often determined indirectly. This process begins with the preparation and characterization of a nonradioactive analog, which is commonly referred to as the "cold compound". The radiolabeled compound is often chromatographically analyzed simultaneously with the cold compound. The identical response of the two compounds demonstrates the structural identity of the radiolabeled compound.

The radiochemical purity of a radiopharmaceutical preparation refers to the fraction of the stated radionuclide present in the stated chemical form. Radiochemical purity is important for radiopharmaceuticals because radiochemical impurities may affect the biodistribution and interfere with image interpretation (diagnostic accuracy). In addition, radiochemical impurities may alter radiation absorbed doses to various organs. When using therapeutic radiopharmaceuticals, radiochemical purity is very important because altered biodistribution associated with radiochemical impurities may result in insufficient irradiation of the target tissue (suboptimal treatment response) or excessive irradiation of other tissues (undesired radiation damage).

Radiochemical impurities in radiopharmaceuticals may result from byproducts of the preparative method or from decomposition. Radiation causes decomposition of water, a main component of aqueous radiopharmaceutical preparations, leading to the production of reactive hydrogen atoms and hydroxyl radicals, hydrated electrons, hydrogen, hydrogen ions, and hydrogen peroxide. Hydrogen peroxide is formed in the presence of oxygen radicals, originating from the radiolytic decomposition of dissolved oxygen. Many radiopharmaceuticals show improved stability if oxygen is excluded or restricted. Radiation may also affect the radiopharmaceutical itself, giving rise to ions, radicals, and excited states. These species may combine with one another and/or with the active species formed from water. Radiation decomposition may be minimized by the use of chemical agents that act as electron or radical scavengers. Radiochemical purity must meet compendial standards throughout the time of use until the stated expiration of the radiopharmaceutical.

Determination of radiochemical purity is typically a two-stage process: 1) the different chemical species are separated by paper, thin-layer, or column chromatography or other suitable analytical separation technique; and 2) the radioactivity content in each of the separated chemical species is measured with a suitable radiation detector and counting device. The ultimate confirmation of acceptable radiochemical purity of a radiopharmaceutical is its intended biodistribution after administration.

## 3.14 Radionuclidic Identity and Purity

"Radionuclidic identity" is a critical parameter in radiopharmacy because the radionuclide used determines the radiation dose to the patient, the biodistribution of the radiopharmaceutical, the quality of any images obtained, or the efficacy of any therapeutic preparation. Radionuclide identity can be established either by measuring the half-life or the energy of the radiations emitted by the sample.

The radionuclidic purity of a radiopharmaceutical preparation refers to the fraction of radioactivity attributable to the desired radionuclide in the total radioactivity measured. Hence, a radionuclidic impurity is the presence of an unwanted radionuclide. Radionuclidic purity is important for radiopharmaceuticals, because unwanted radionuclides may cause several undesired consequences:

- Radionuclidic impurities may cause the radioactive assay of the radiopharmaceutical to deviate from the prescribed amount.
- Radionuclidic impurities may deliver higher-than-desired, radiation-absorbed doses to various organs and tissues.
- In some situations, radionuclidic impurities may interfere with image interpretation (diagnostic accuracy).
- It should be remembered that radionuclidic purity will change with time and is generally specified as a percentage of the desired radionuclide's activity at the time of calibration or at the time of administration (e.g., molybdenum-99 in technetium-99m).

The radionuclidic purity must meet compendial standards throughout the useful life of the radiopharmaceutical. In addition, the impurity(ies) themselves will decay. Consideration must be given to the acceptable minimum and maximum allowable times between expiration of the product and analysis for impurities.

Radionuclidic impurities commonly arise during radionuclide production relating to impurities in target materials, differences in the values of various competing production cross-sections, and different excitation functions of competing reactions at the energy of the bombarding particles. In the case of generator-produced radionuclides, some generator breakthrough of parent radionuclide typically occurs and represents a radionuclide impurity in the eluate of the daughter radionuclide.

Determination of radionuclidic purity is typically based on evaluation of radioactive emissions, the principal analysis of the gamma spectrum obtained from a sample of the product. For short-lived isotopes, the half-life measurement could be an appropriate approach to assess radionuclidic purity. In cases involving radionuclidic impurities that have long half-lives relative to the desired radionuclide, measurement of the radionuclidic impurities can be performed after a sufficient time delay to allow the desired radionuclide to fully decay. In cases involving radionuclidic impurities that have substantially higher energy gamma emissions relative to the desired radionuclide, measurement of the radionuclidic impurities can be performed after placing the product inside a properly calibrated radiation shield that affords differential attenuation of the gamma rays emitted from the desired radionuclide versus those emitted from the radionuclidic impurities. Positron-emitting radionuclides typically cannot be differentiated, because their emitted energy (511 keV) is the same for each radionuclide; thus, gamma-ray spectrometry is not a recommended prerelease test for radionuclidic identity for PET radionuclides. Some positron-emitting radionuclides have characteristic gamma-ray emissions in addition to 511 keV, which may be used for purposes of identification (e.g., germanium-69 and sodium-22). In any case, the appropriate instrument should be chosen to detect potential impurities and should be properly calibrated to accurately quantify any identified impurities.

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### 3.15 Chemical Purity

The chemical purity refers to the fraction of the total chemical species present in the product as the specified chemical component(s). Hence, a chemical impurity is the presence of an unwanted nonradioactive chemical. Chemical purity is important for radiopharmaceuticals, because chemical impurities may cause undesirable consequences such as chemical interactions (e.g., precipitation) and toxic biologic effects.

Chemical impurities are typically associated with production procedures and may include contaminants from raw materials, synthetic byproducts, solvents, excipients, equipment, preparative or purification columns, and containers. For certain radiopharmaceuticals, chemical impurities may also be associated with generator breakthrough of resin material from the generator column (e.g., alumina) in the eluate solution.

Determination of chemical purity is generally not performed and reported as a single attribute. Rather, determinations of individual chemical impurities are performed and compared to specifications (limits) for the respective individual chemical impurities. Such determinations of chemical impurities use analytical techniques as appropriate and described in the individual radiopharmaceutical monograph.

### 3.16 Labeling

Individual radiopharmaceutical monographs indicate that the labeling is to include the date and time of calibration, the amount of radioactivity associated with the radiopharmaceutical expressed as total MBq (μCi or mCi) and concentration as MBq (μCi or mCi)/mL at the time of calibration, the expiration date (and time, if appropriate), and the statement, "Caution—Radioactive Material". The labeling indicates that in making dosage calculations, a correction is to be made for radioactive decay and also indicates the radioactive half-life of the radionuclide. Other labeling requirements may apply to biologics or articles intended for injection. Beyond-use dates of compounded preparations should be included as appropriate. Additional labeling requirements may be required by various regulatory agencies.

### 3.17 Naming Conventions for Isotopes

Various naming conventions exist for isotopes that are associated with radiopharmaceuticals and radioactive devices. For example, the name of an isotope may or may not use a superscripted value for the mass number. Superscripted values should precede the elemental symbol for the isotope, and non-superscripted values should follow the elemental symbol, preferably with a hyphen between the symbol and the mass number. Examples include $^{68}$Ga and O-18, respectively. Square brackets should be used to denote a specific isotope when necessary within a chemical name, for example, 2-[$^{18}$F]fluoro-2-deoxyglucose. Individual radiopharmaceutical monographs use nonproprietary names assigned by the U.S. Adopted Names (USAN) Council, which use the elemental symbol followed by the mass number separated by a space, for example, *Thallous Chloride Tl 201 Injection*. Although exceptions to these conventions undoubtedly exist, efforts should be made to adopt standardized conventions for radiopharmaceuticals and radioactive devices that fall within the scope of this general chapter.

## 4. INSTRUMENTATION FOR DETECTION AND MEASUREMENT OF RADIOACTIVE EMISSIONS AND APPLICATIONS

### 4.1 Ionization Chambers

Radioactive materials are not readily detected by ordinary chemical or physical methods. Instead, detection methods for radioactive materials rely on the ionization of matter that results from the emitted radiation. The charge separation created during this process forms the basis of radiation detection systems, which may be based on the ionization properties of gaseous, liquid, and solid materials.

An ionization chamber is an instrument that directly measures ions produced in a gas as the result of the interaction of radiation with the gas. The most common usage in nuclear medicine applications is as the detector used in a dose calibrator. The dose calibrator is an instrument used to measure the quantity of radioactivity in a radiopharmaceutical. The key component of a dose calibrator is an argon-filled chamber with an applied electrical potential that allows the detection of ions produced by the passage of gamma rays through the chamber. Calibration of the system may involve one or more radionuclides with gamma energies and quantities that span the range of typical analyses. The calibration of the ionization chamber should be performed, when possible, with suitable NMI-traceable radionuclide standards. Routine system suitability testing should include checks for these parameters. Frequency of testing should occur as appropriate. Please refer to ⟨821⟩ for additional details on typical instrument requirements.

The position of the radioactive sample in the dose calibrator is ideal when it simulates 4π geometry. The geometric goal is placement of the sample at a point in the center of the cylindrical detector. Reproducibility of placement within the chamber is critical, because the response typically drops off at the top and bottom of the cylinder because of a combination of geometry and electronic effects. The value of the ionization current per unit of radioactivity, known as the "calibration factor", is characteristic of each gamma-ray-emitting radionuclide. The current produced in a dose calibrator is related to the mean energy of the emitted radiation and is proportional to the intensity of the radiation. The calibration of the dose calibrator for a specific radionuclide is ideally performed with a radioactive calibration source of the same radionuclide. Alternatively, it may be performed by measuring radioactive calibration sources with gamma energies above and below the gamma energy for the radionuclide to be measured and interpolating these values, also correcting for differences in gamma abundance, to establish the calibration factor for that radionuclide.

The upper limit of the dose calibrator is normally specified by the manufacturer. If not, testing is required to ascertain this upper limit. With a deep reentrant well-type chamber, reproducibility within approximately 5% or less can be readily obtained

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in a few seconds for quantities of radioactivity in the MBq (mCi) range and within about 30 s for quantities in the kBq (µCi) range.

The calibration of a dose calibrator should be maintained by relating the measured response of a standard to that of a long-lived reference standard, such as radium-226 in equilibrium with its daughters, cesium-137 in equilibrium with its daughters, barium-133, cobalt-60, or cobalt-57. The instrument should be checked on each day of use with the reference standard source to ascertain the stability over a long period of time. This check should include reference standard readings at all radionuclide settings used. Any necessary corrections for radioactive decay of the reference standard source should first be applied. It is also recommended that the reproducibility and/or stability of multi-range instruments be checked with the use of standards with appropriate activities for all ranges.

The size, shape, and location of a radioactive sample within the well will affect the response of a dose calibrator. This is usually referred to as "geometry". The shape, composition, and dimensions of the container holding the radioactive material can affect the result. Effects relating to container properties are generally more pronounced with radionuclides that emit beta particles (because of differences in Bremsstrahlung production) or emit low energy gamma or X-rays (because of differences in photon attenuation). It is important that geometric correction factors, if needed, be determined for each combination of radionuclide and configuration (i.e., size, shape, location with the well chamber, volume within the container, and container properties). The manufacturer's calibration factor for each radionuclide is determined using a specific geometry and container, which may not match the geometry or container used operationally.

## 4.2 Liquid Scintillation Counters

The liquid scintillation counter (LSC) detection method uses liquid scintillation cocktails to transform emitted radiation into detectable light photons. Alpha- and beta-emitting radionuclides may be assayed with the use of a liquid-scintillation detector system. In the liquid scintillator, the emitted radiation is converted into light quanta that are usually detected by two multiplier phototubes arranged to detect only coincidence radiation. The liquid scintillator is a solution consisting of a solvent, primary and secondary solutes, and additives. As the emitted particle dissipates energy in the solvent, a fraction of this energy is converted into fluorescent light by the primary solute. The function of the secondary solute is to absorb the primary fluorescence and re-emit the light at a longer wavelength that is more efficiently detected by the multiplier phototubes. Traditionally used solvents (cocktails) are toluene and *p*-xylene; primary solutes are 2,5-diphenyloxazole (PPO) and 2-(4′-*tert*-butylphenyl)-5-(4-biphenylyl)-1,3,4-oxadiazole (butyl-PBD); and secondary solutes are 2,2′-*p*-phenylenebis[4-methyl-5-phenyloxazole] (dimethyl-POPOP) and *p*-bis(*o*-methylstrylyl)benzene (bis-MSB). Many aqueous scintillating solution cocktails that are less hazardous are available. Aqueous solutions tend to have a shorter shelf-life; therefore, it is important to ensure that they have not expired before use. As a means of attaining compatibility and miscibility with aqueous samples to be assayed, additives, such as surfactants and solubilizing agents, are also incorporated into the scintillator. For an accurate determination of radioactivity of the sample, care should be exercised to prepare a sample that is truly homogeneous. Quenching is a major concern for liquid scintillation and refers to any mechanism that causes a reduction of emitted light by the source. Quenching can result from multiple factors, including oxygen and dilution effects; therefore, it is critical to perform quenching corrections by counting comparative measurements of standard samples using the same conditions of volume, additives, and solvent to accurately account for these effects.

Alternatively, an external source, typically barium-133 or europium-152, is placed in close proximity to the sample vial to release Compton electrons. The shape of the resulting spectrum is analyzed to compute a quench-indication parameter. This parameter can then be related to the counting efficiency by measuring sources of known radioactivity at a determined level of quenching agent. The resulting quench curve allows the determination of the radioactivity of an unknown sample knowing the count rate and value of the quenching parameter. The scintillation fluid may require special handling for disposal, in addition to any residual radioactivity. Static electricity on the vials may also cause spurious counts in the system, especially in the case where low-energy beta emitters are being assayed. This problem is often greater in a low-humidity environment.

The disintegration rate of a beta-emitting source may be determined by a procedure in which the integral count rate of the sample is measured as a function of the pulse-height discriminator bias, and the emission rate is then obtained by extrapolation to zero bias. Energetic alpha-emitters may be similarly measured by this method.

## 4.3 Nuclear Spectroscopy Systems

### GAMMA-RAY SPECTROMETRY

Each gamma-emitting radionuclide has a unique spectrum of mono-energetic photons emitted that allows the identification and quantification of radioactive materials in a sample by comparing the energy(ies) of the photon(s) detected and the intensity at each energy. This gamma spectrum allows for both the quantitative determination of purity as well as identity of the radionuclide. Gamma-spectrum analysis can be performed by using either a scintillation crystal, typically sodium iodide activated with thallium [NaI(Tl)], or by using a semiconductor detector consisting of a germanium–lithium (Ge–Li) crystal or a high-purity germanium detector (HPGe). Semiconductor detectors have a much higher energy resolution than NaI(Tl) detectors, with the ability to resolve gamma rays differing in energy by only a few keV, as opposed to the 20–80 keV required for a NaI(Tl) detector. Because of their increased resolution, semiconductor detectors are the preferred analytical method for gamma spectral analysis. A lanthanum–bromide detector is also available that has a significantly better resolution (10–12 keV) than the NaI(Tl) detector, without the necessary requirement of liquid nitrogen cooling for a HPGe detector. The use of spectroscopy software to automate the analysis is acceptable; however, the operator should have an understanding of the parameters chosen to ensure that the performance of the system is adequate to meet test requirements.

Semiconductor detectors are, in essence, solid-state ionization chambers, but the energy required to create an electron-hole pair or to promote an electron from the valence band to the conduction band in the semiconductor is about one-tenth the energy required for creation of an ion-pair in a gas-filled ionization chamber or proportional counter. This energy threshold is

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also far less than the energy needed to produce a photon in a NaI(Tl) scintillation crystal. The energy resolution is a measure of the ability to distinguish the presence of two gamma rays closely spaced in energy and is defined by convention as the full width of the photopeak at its half maximum (FWHM), expressed as a percentage of the photopeak energy. For example, with 1.33 MeV gamma rays from cobalt-60, a HPGe detector has an energy resolution of about 0.3% FWHM, whereas a 3-in × 3-in NaI(Tl) crystal has a value of about 6%.

Gamma-ray spectra exhibit one or more sharp, characteristic photopeaks, or full-energy peaks, as a result of total absorption in the detector of the full energy of incident gamma radiations. These photopeaks are useful for identification purposes. Other secondary peaks are observed as a consequence of backscatter, annihilation radiation, coincidence summing, fluorescent X-rays, and other factors, accompanied by a broad band known as the "Compton continuum", which arises from the scattering of photons in the detector and surrounding materials. Because the photopeak response varies with gamma-ray energy, calibration of the gamma-ray spectrometer should be achieved with radionuclide standards having well-known gamma-ray energies and emission rates from an NMI. The shape of the gamma-ray spectrum is dependent upon the shape and size of the detector, the types of shielding materials used, and the electronic processing characteristics of the instrument.

One of the most useful applications of gamma-ray spectrometry is the identification of radionuclides and the determination of radionuclidic impurities. When confirming the identity of a radionuclide by gamma-ray spectrometry and/or quantifying the radioactivity, it is necessary to ensure that the detector has been accurately calibrated using a known source, as described above, and in the same geometry as the unknown sample. Where the radionuclides emit coincident gamma or X-radiations, the character of the pulse-height distribution often changes quite dramatically because of the summing effect of these coincident radiations in the detector as the efficiency of detection is increased (e.g., by bringing the source closer to the detector); this is referred to as "cascade summing". Such an effect is particularly evident in the case of iodine-125. Most commercially available software packages include an ability to correct this source of error.

When identification of a radionuclide by means of a calibrated spectrometry system is not possible, the identity of the radionuclide may instead be established by measuring two or more of the following nuclear decay scheme parameters: (1) half-life; (2) energy of each gamma ray or X-ray emitted; (3) the abundance of each emission; and (4) $E_{max}$, the maximum energy of emitted beta particles, for those radionuclides that decay with beta-particle emissions. Such measurements should be performed as directed in ⟨821⟩. Agreement of two or more of the measured parameters within 10% of the corresponding published nuclear decay scheme data confirms the identity of the radionuclide.

As with other types of detectors, the background should be determined and subtracted from the measurement. In addition, the background should be stable, especially in situations where long counting times are required. This can be achieved by running a background spectrum before analysis and comparing it to a previously obtained background spectrum. Generation of a background spectrum will also allow the calculation of a minimum detectable activity for each possible impurity.

## BETA PARTICLE COUNTING SYSTEMS

Beta (β) particles are emitted with a distribution of energies ranging from zero to a definite maximum value. The maximum energy of the electrons is characteristic of a particular radionuclide and is normally the $E_{max}$ shown in nuclear data tables. The determination of the maximum beta energy may aid in the identification of the beta-emitting radioisotope, and careful measurements can routinely quantify activities. Emitted beta particles rarely possess the maximum energy. On average, emitted beta particles possess one-third of the maximum energy. Beta particles can be difficult to detect because they only penetrate small thicknesses of solid materials. [NOTE—Radioisotopes that emit gamma radiation in addition to beta radiation are more easily quantified and identified by using gamma-ray spectroscopy. In some cases, gamma-ray detection is the preferred method for measuring these radioisotopes and is usually the best means of identification of a beta/gamma-emitting radionuclide.]

Several detectors can be used for the detection and measurement of beta particles. These can be ionization chambers, proportional counters, and scintillation counters with their associated electronics. Self-absorption and backscattering can be an issue in beta-particle analysis and can result in a lower or higher number. Ionization chambers and proportional counters can be used for the quantitation of beta particles but are less suited for identification, because they cannot measure the maximum beta energy. Scintillation counters can be used for both the quantitation and identification of beta particles. When all or part of the energy of beta radiation is dissipated within scintillators, photons of an intensity proportional to the amount of dissipated energy are produced. These pulses are detected by an electron multiplier phototube and converted to electrical pulses, which are subsequently analyzed with a pulse-height analyzer to yield a pulse-height spectrum related to the energy spectrum of the incident radiation. In general, a beta-particle, scintillation pulse-height spectrum approximates the true beta-energy spectrum, provided that the beta-particle source is prepared in such a manner that self-absorption is minimized. Beta-particle energy spectra may be obtained by using calcium fluoride or anthracene as the scintillator. The spectra of charged particles may also be obtained using silicon semiconductor detectors.

The penetration power of beta particles is significantly larger than for alpha particles; a few millimeters of aluminum will stop beta particles. Care should be taken for the Bremsstrahlung X-rays that are created during the deceleration of the beta particles, because they may influence the measurement and can be a radiation safety concern.

Beta emitters can be quantitatively measured with ionization chambers and proportional counters that measure electrical current generated by the ionization of a select gas in a high-voltage field. Depending on the energy of the beta particle, the composition of the sample and container, and the design of the detector, the measurement may be based on Bremsstrahlung. For a radionuclide in solution, most of the beta energy is absorbed by the solvent, leaving only Bremsstrahlung exiting the sample. In the case of ionization chambers (e.g., dose calibrators), the walls housing the ionizable gas effectively convert all beta energy to secondary photons. As is the case for all radiation measurements, quantitative determinations with an ion chamber or a proportional counter depend on rigorous calibration and standardization of the sample type and geometry.

Depending on the detection setup, alpha particles can interfere with measurement of the beta particles. This can be easily prevented by positioning an absorber for alpha particles between the source and the measuring equipment. However, because low-energy beta particles (<200 keV) may also be absorbed, the count rate should be corrected for the absorption of these low-energy beta particles.

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An LSC can also be used for the quantification of beta particles and aid in radionuclide detection. Because of the high efficiency of the method, LSC is particularly useful in measuring very low levels of radioactivity and is commonly used for complex biological samples. The sample is normally dissolved in a solution containing a phosphor that converts the beta emission to light pulses, which are detected by a very sensitive system of photomultiplier tubes. At energies greater than ~100 keV, the energy conversion and therefore the counting efficiency, is essentially 100%. The method is somewhat compromised by quenching effects, but these can be easily overcome with careful calibration.

When high energy (>800 keV) beta particles are measured, it is possible to count without scintillation cocktails, because the beta particles create Cherenkov radiation, which can be directly detected with a photomultiplier. [NOTE—Although alpha particles can interfere with beta counting, this problem can be corrected for by use of thin absorbers or by instrumental energy discrimination.]

The identification of pure beta-emitting radioisotopes without accompanying gammas is best done by a combined measurement of the half-life of the radioisotope and the maximum energy of the beta radiation emitted by the radioisotope.

The approximate maximum energy of a beta particle can be determined by two procedures: (1) by measuring the radioactivity as a function of absorber thickness; and (2) by plotting the logarithm of the count rate versus the thickness of the absorber in $mg/m^2$; then, an absorption curve can be made. The absorption curve can be compared with standardized absorption curves, which aids in the identification of the radioisotope.

Using LSC, by measuring the pulse height over a calibrated energy range, a beta-energy spectrum can be generated from which the maximum energy can be estimated. LSC instruments typically automate the calibration factor, but care should be taken in correcting for quenching effects.

#### ALPHA PARTICLE COUNTING SYSTEMS

Several detectors can be used for the detection and measurement of alpha particles. These can be ionization chambers, proportional counters, silicon semiconductor detectors, and scintillation counters with their associated electronics. For the identification and quantification of alpha particles, ionization chamber and proportional counters are not suitable.

Special precautions should be taken in the measurements of alpha particles because of their high energy but limited penetration power (about 40 µm in human skin).

For the identification and assay of alpha-particle emitters, spectrometry using liquid scintillation is mostly used. For the identification and determination of radionuclidic purity of alpha-particle emitters, spectrometry using a silicon-diode semiconductor detector can be used.

In a scintillation counter, the energy of the alpha particle is transformed to a light pulse, which can be detected by a photomultiplier tube. The intensity of the pulse is a measure of the energy of the detected alpha particle. Solid scintillation counters or phosphor detectors can be used as well as liquid scintillation.

When solid-state detectors are used, the sample is usually electroplated on a planchet disc, and the detector is brought close to or immediately on the sample. The counting efficiency of solid-state detectors tends to be low. In liquid scintillation counting, when the sample is dissolved in the appropriate medium, the efficiency can very high. Each method of counting has its own advantages and disadvantages. For solid detectors, advantages are lower background noise and a better differentiation between alpha and beta particles. Disadvantages are that no volatile samples can be measured, and self-absorption by the sample material (related to thickness of the sample layer) can lower the detected count rate, giving a falsely low outcome of the assay.

For liquid scintillation, advantages are the ease of sample preparation and no self-absorption, because the sample is mixed with the scintillation fluid. Disadvantages are higher background noise levels and poor separation between alpha and beta particles.

## 4.4 Detector Systems for Chromatographic Applications

Chromatographic applications, wherein the radioactive components of a mixture are separated based on their distribution between a stationary phase and a mobile phase, represent a unique usage of radiation detection systems. The most common chromatography applications include thin-layer chromatography (TLC), gas chromatography (GC), and liquid chromatography (high-pressure liquid chromatography and high-performance liquid chromatography, typically denoted as HPLC). Depending on the chromatographic application and the type of emitted radiation, various detector systems may be used in the analysis of separated radioactive components.

Gamma-ray detector systems can be used to dynamically measure separated radioactive components in GC and HPLC applications. In these applications, the eluate from the chromatography column is directed over or through the scintillating detector. Typically, these detector systems are not used for radionuclide identification; therefore, NaI(Tl) crystals tend to be the detector of choice in these applications. Depending on the required sensitivity of the technique, the geometry of detector may consist of a well that surrounds the eluate tubing, or a flow cell that passes the eluate across the surface of the detector. In either case, the design of the system should provide a reproducible geometric relationship between the tubing and the detector. In addition, the detector should be shielded sufficiently to prevent spurious peaks or baseline drift due to background radiation. For this application, the output pulses of the photomultiplier tube may be converted into an analog signal whose voltage is proportional to the number of pulses (i.e., the amount of radioactivity). In this manner, the resulting electric signal is used by a data acquisition and chromatography system to provide a chromatogram in a similar fashion as more traditional chromatography detection systems. The flow rate of the eluate and the amount of radioactivity in the eluate should be controlled to provide a count rate that is within the linear range of the detector system.

LSC methods can also be used in HPLC separations of beta-emitting components. Several techniques may be used in this application. In the first, the eluate from the chromatographic column is collected in discrete fractions. Each fraction is then mixed with the liquid scintillation cocktail before analysis in the LSC. Additional processing steps may be necessary before the addition of the cocktail to minimize quenching. In the second technique, the scintillation cocktail may be mixed with the eluate from the column before passage of the resulting mixture through an online flow cell surrounded by the photomultiplier tubes. Finally, inline solid scintillators may be used with in situ mixing of the eluate with a liquid scintillation cocktail.

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Beta-particle detector systems can be used to measure separated radioactive components in TLC applications. In this application, windowless gas ionization detectors are most commonly used, and the detector is automatically scanned over the TLC plate, yielding a two-dimensional plot of the radioactivity. Because of the nature of the sample, self-adsorption is negligible, and the beta radiation is efficiently counted. If all of the components on the TLC plate contain the same beta emitter, the output signal is proportional to the fraction of radioactivity in the separated components. If the separated species contain different radionuclides, the detector should be calibrated with each radionuclide to correct for different signal responses. Alternatively, if such a detector is not available, the TLC plate can be cut into multiple strips and counted by an appropriate detector. The individual strips should be counted using the same geometry. For the highest sensitivity, the strips may be extracted with solvent and then counted by LSC.

## GLOSSARY

**Alpha particles** ($\alpha$)**:** Positively charged particles that are emitted from nuclei during radioactive decay. Alpha particles are Helium-4 nuclei, consisting of two protons and two neutrons but no electrons.

**Beta particles** ($\beta^-$)**:** Negatively charged particles that are emitted from nuclei during radioactive decay. Beta particles are electrons.

**Bremsstrahlung:** Electromagnetic radiation produced by the acceleration or especially the deceleration of a charged particle after passing through the electric and magnetic fields of a nucleus.

**Calibration factor:** The coefficient used to convert the measured ionization chamber current to a nominal radioactivity. This term is often referred to as the "calibration coefficient".

**Calibration time:** An arbitrary time at which the specified amount of radioactivity is present on a specific date.

**Carrier free:** A preparation free from stable isotopes of the same element as the radionuclide.

**Counting assembly:** An instrument that consists of a sensing unit and an electronic scaling device. The sensing unit may be a Geiger-Müller tube, a proportional counter, a scintillation detector in which a photomultiplier tube is used in conjunction with a scintillator, or a solid-state semi-conductor.

**Dose calibrator** (also referred to as radionuclide calibrator)**:** A well-type ionization chamber commonly used to assay radiopharmaceuticals. Display units are typically in curies (μCi or mCi or Ci) or becquerels (kBq or MBq or GBq).

**Gamma rays** ($\gamma$-rays)**:** Electromagnetic radiation emitted from nuclei during radioactive decay. Gamma rays have a wide range of energies. The gamma rays emitted from a given radionuclide are always at the same energy(ies), providing a unique signature that enables the identification of a gamma-emitting radionuclide.

**Geiger-Müller counter** (often referred to as a G-M counter or Geiger counter)**:** An instrument in which a high-voltage potential is applied across a volume of inert gas for the purpose of collecting ions produced by a radiation field. The negative electrons are internally multiplied to produce a readily detectable current pulse. Display units are typically counts per minute (cpm) or milliroentgen per hour (mR/h).

**Geometry:** The characteristics of a radioactive source (i.e., container type, container wall thickness, volume and position of the container in the well chamber) that along with the physical characteristics of the ionization chamber affect the magnitude of the calibration coefficient for a specific radionuclide. The principal geometry considerations that may affect the accuracy of a source measurement in a dose calibrator are container configuration, source volume, position of the source in the chamber well, and the radionuclide itself. [NOTE—It is customary to compare a standardized preparation and radiopharmaceutical drug or preparation using identical geometry conditions for assay, identification, and other parameters. The validity of the result is critically dependent upon the reproducibility of the spatial relationships of the source to the detector and its surroundings and upon the accuracy of the standardized preparation.]

**Ionization chamber:** An instrument in which an electric field is applied across a volume of inert gas for the purpose of collecting ions produced by a radiation field. The positive ions and negative electrons drift along the lines of force of the electric field and are collected on electrodes, producing an ionization current. The most commonly used form of ionization chambers for measurement of the activities of radiopharmaceuticals is a well-type ionization chamber known as a dose calibrator.

**Isobars:** Nuclides with the same mass number (protons + neutrons).

**Isomers:** Atoms with the same number of protons and neutrons, but a different nuclear energy configuration. Short-lived radioactive isomers are generally referred to as metastable. Different isomers are different nuclides based on their nuclear energy configurations.

**Isotones:** Nuclides with the same number of neutrons and a different number of protons. Isotones are different elements with different atomic masses.

**Isotopes:** Nuclides with the same number of protons and a different number of neutrons. Isotopes are the same element with a different atomic mass.

**Isotopic carrier** (also referred to as carrier)**:** A stable isotope of the element concerned either present in or added to the radioactive preparation in the same chemical form of the radionuclide.

**Liquid scintillation counter** (LSC)**:** An instrument that detects scintillation light from the absorption of radiation energy in a scintillation liquid. This instrument is used primarily for beta-emitting radionuclides that do not also emit gamma photons. For best results, the radioactive sample must be able to be dissolved in the scintillation liquid.

**Minimum detectable activity:** The smallest quantity of radioactivity that can be detected above the background with a specified level of confidence.

**National Metrology Institute** (also known as NMI)**:** A measurement standards body that is a laboratory of metrology that establishes standards for a country or organization. For example, the National Institute of Standards and Technology (NIST) is the NMI for the United States.

**No carrier added:** A preparation where no stable isotopes of the same element as the radionuclide being tested are intentionally added in the stated chemical form or at the position of the radionuclide in the molecule being tested.

**Nuclide:** An atom with a specific number of protons and neutrons in a given nuclear energy state.

**Positrons** ($\beta^+$)**:** Positively charged particles emitted from a nucleus during radioactive decay. Positrons are anti-electrons.

**Radioactivity:** (1) The spontaneous transformation of nuclei characterized by the emission of particles or photons. Radioactivity is typically described as atoms undergoing radioactive decay per unit time (or disintegrations per unit time). (2)

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The quantity of radioactive material, as measured in units of curies (U.S. units) or becquerels (SI units). The quantity of radioactive material may also be referred to as activity.

**Radiochemical identity:** The molecular structure of the intended active radioactive drug ingredient that is present in the radiopharmaceutical preparation.

**Radiochemical purity:** The ratio, expressed as a percentage, of the radioactivity of the intended active radiopharmaceutical ingredient to the total radioactivity of all radioactive ingredients present in the radiopharmaceutical preparation.

**Radioisotope:** A radioactive atom, generally used in the context of an isotope of an element.

**Radionuclide:** An unstable nuclide that undergoes radioactive decay; a radioactive nucleus. The terms radionuclide and radioisotope are commonly used interchangeably.

**Radionuclidic identity:** The intended radionuclide in the radiopharmaceutical preparation.

**Radionuclidic purity:** The ratio, expressed as a percentage, of the radioactivity of the intended radionuclide to the total radioactivity of all radionuclides in the radiopharmaceutical preparation.

**Radiopharmaceutical** (radiopharmaceutical preparation/radioactive drug)**:** A finished dosage form that contains a radioactive substance in association with one or more other ingredients and that is intended to diagnose, stage a disease, monitor treatment, or provide therapy. A radiopharmaceutical includes any nonradioactive reagent kit or radionuclide generator that is intended to be used in the preparation of any such substance. The terms radiopharmaceutical and radioactive drug are commonly used interchangeably.

**Scintillation crystal counter:** An instrument consisting of a crystal scintillator, such as NaI(Tl), with an attached photomultiplier tube and associated electronics. Scintillation light produced from the absorption of gamma and X-rays in the crystal is converted to electrons and amplified in the photomultiplier tube. The resultant current pulse may be further analyzed with regard to photon energy. A commonly used form of this instrument that has a hole in the crystal of sufficient size to allow placement of a test tube or similar container is known as a well counter.

**Semiconductor detector:** An instrument consisting of a semiconductor material, such as silicon or germanium crystals, that detects ionizing radiation through generation of charge carriers (passage of electrons through holes). The current pulse produced by migration of these charge carriers, under the influence of a voltage potential across the material, can be further amplified and analyzed to determine the quantity and energy of the incident radiation.

**Solid-state detector:** A crystal-based detector, in contrast to a gas-based detector; often is used as a synonym for a semiconductor detector.

**Specific activity:** The radioactivity of a radionuclide per unit mass of the element or compound. The unit of specific activity is radioactivity per mass expressed on a gram or mole basis [e.g., mCi/µg (MBq/µg); Ci/mmol (GBq/mmol)].

**Strength:** The radioactivity concentration of the radiopharmaceutical at the calibration time. The unit of strength is the amount of radioactivity on a volume basis (e.g., mCi/mL or MBq/mL).

**Total radioactivity:** The radioactivity of the radionuclide, expressed per unit (e.g., vial, capsule, ampule, generator, and others) at the calibration time.

**Validation:** Establishment of documented evidence that a method, process, or system meets its intended requirements.

**Verification:** Confirmation that an established method, process, or system meets predetermined acceptance criteria.

**X-rays:** A type of electromagnetic radiation emitted from the electron orbitals. Although they do not arise from the nucleus, they are often present immediately after a decay event if there are interactions between the emitted radiation and the orbital electrons.

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# ⟨1823⟩ POSITRON EMISSION TOMOGRAPHY DRUGS—INFORMATION

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## 1. INTRODUCTION

Positron emission tomography (PET) drugs contain radionuclides that undergo nuclear transformation, or radioactive decay, predominantly by the emission of a positron. Positrons undergo annihilation upon interaction with electrons to produce two photons that are emitted in nearly opposite directions to each other. Each photon possesses an energy of 511 keV, which lies in the gamma ray portion of the electromagnetic spectrum. These radionuclides are used in a wide range of PET imaging studies, including research, investigational, and clinical applications.

The radionuclides used in PET imaging studies typically possess short physical half-lives (denoted as $T_{1/2}$). Some common examples of PET radionuclides and their associated half-lives are included in *Table 1*. Note that *Table 1* includes radionuclides currently in predominant use and is not intended to illustrate all positron-emitting radionuclides used in PET.

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**Table 1**

| PET Radionuclide | Half-Life, $T_{1/2}$ |
|---|---|
| Fluorine-18 | 109.8 min |
| Carbon-11 | 20.4 min |
| Nitrogen-13 | 10.0 min |
| Oxygen-15 | 2.0 min |
| Copper-64 | 12.7 h |
| Gallium-68 | 68 min |
| Rubidium-82 | 75 s |

Because certain of these radionuclides such as *Carbon-11* and *Nitrogen-13* when found in biological systems have the same chemical and physical properties as their stable counterparts found in biological systems, PET offers a unique platform for *in vivo* imaging studies of complex biochemical pathways. As a result, PET radionuclides have found widespread use in cardiology, oncology, and neurology applications. PET drugs have also attracted interest as potential tools to accelerate and reduce the cost of therapeutic drug discovery efforts.

Most PET radionuclides are produced at the point of use by a particle accelerator (e.g., a cyclotron) or a radionuclide generator. A cyclotron accelerates charged particles such as protons or deuterons to velocities sufficient to induce a nuclear transformation of the target nucleus into a different element. High-energy particles and/or radiation are emitted from the target nucleus during the transformation process. An example of a transformation is the bombardment of stable Oxygen-18 nuclei with accelerated protons to produce *Fluorine-18* nuclei along with the concomitant emission of a neutron. This process may be summarized according to the following shorthand notation:

$$^{18}O(p,n)^{18}F$$

The chemical form and physical state of the bombarded nuclei vary, depending on the target and the subsequent usage of the PET radionuclide. This allows the production of PET radionuclides in gaseous, solid, and solution phases.

Radionuclide generators offer an alternative method for point-of-use access to some PET radionuclides. A radionuclide generator contains an immobilized parent radionuclide that undergoes radioactive decay to a daughter radionuclide, which may be eluted from the generator. An example of a PET radionuclide generator is the Germanium-68/Gallium-68 generator. The parent Germanium-68 ($T_{1/2}$ = 271 days) is adsorbed onto the column packing material. The Germanium-68 undergoes radioactive decay by electron capture into Gallium-68, which is not adsorbed onto the column packing material and may be eluted from the generator.

Regardless of the production method, other radionuclides may be present in addition to the desired PET radionuclide. In the case of cyclotron-produced PET radionuclides, other radionuclides may result from the bombardment of the target holder and/or other nuclei present in the target material. For generator-produced PET radionuclides, break-through of the parent radionuclide may occur. Safeguards are readily available to avoid unnecessary contamination with radionuclidic impurities of the final PET drug.

Once the PET radionuclide has been produced, it should be processed into a suitable form of a PET drug. The simplest form of processing involves the purification and/or formulation of the PET radionuclide. An example is the purification of the [$^{18}$F]fluoride ion. After production of the [$^{18}$F]fluoride ion by cyclotron bombardment, ion exchange procedures may be used to remove impurities and yield a formulation suitable for PET imaging studies. Another example is the purification of gaseous [$^{15}$O]oxygen through various solid supports before administration of the finished PET drug by inhalation.

Simple forms of processing are rare, because PET drug products are typically synthesized from the appropriate radionuclide through multiple synthetic processing steps. The exact process depends on the desired PET drug. The synthesis of 2-[$^{18}$F]fluoro-2-deoxyglucose ([$^{18}$F]FDG) provides an illustration. The first step in this synthesis is the preparation of the anhydrous [$^{18}$F]fluoride ion from cyclotron-produced Fluorine-18. The [$^{18}$F]fluoride ion is activated with a phase transfer catalyst such as tetraethylammonium or [2.2.2]-cryptand to enhance its reactivity toward nucleophilic substitution. The resulting complex reacts with mannose triflate to yield a fluorinated $^{18}$F intermediate, which is deprotected and purified to yield [$^{18}$F]FDG. This process is summarized in the following set of equations:

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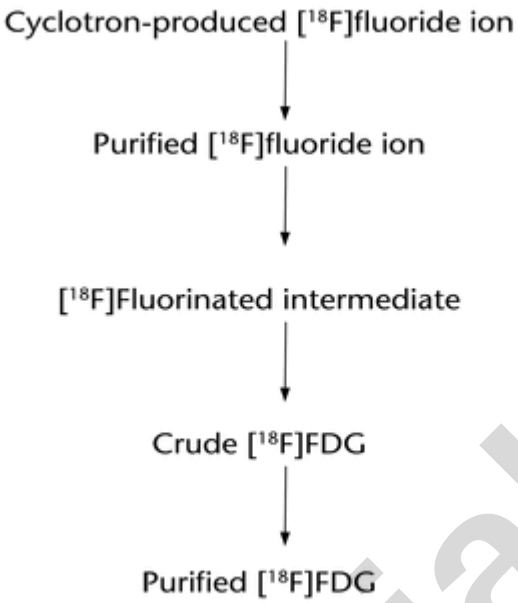


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In addition to the chemical processing and/or purification steps used to generate the desired radiochemical of interest, other steps are typically required to formulate the PET drug product. For injectable PET drug products, formulation steps may include dilution, addition of a stabilizer, pH adjustment, and other steps. The final formulation of the PET drug product should take place in a manner that minimizes the presence of bacterial endotoxins and bioburden. Finally, the product should be sterilized (e.g., by passage through a membrane sterilizing filter) to provide a solution suitable for intravenous administration. In the above example, [$^{18}$F]FDG becomes the drug product *Fludeoxyglucose F 18 Injection*.

Before use, the finished PET drug product should be tested to ensure that the product meets suitable standards of identity, strength, quality, and purity. Because of the short half-life of PET radionuclides, testing should be completed in a timely fashion; however, it is not possible to complete certain quality control (QC) tests within a suitable timeframe. In some cases, it may be necessary to adopt a "sub-batch" approach for QC testing, wherein a sub-batch of the PET drug product is prepared solely for purposes of QC testing. Examples of PET drug products with such short half-lives include [$^{15}$O]water and [$^{13}$N]ammonia.

Ultimately, the short half-lives of PET radionuclides create unique constraints for the production and testing of PET drug products and define how these products are used in research, investigational, and clinical settings. PET drugs are a unique class of products defined by the following characteristics:

- The mass of the radioactive ingredient in a PET drug product usually ranges from nanogram to microgram quantities. This affects pharmacological and toxicological considerations for PET drugs, usually by creating large safety margins due to low mass of the active ingredient in the administered dose.
- An entire batch of a PET drug product may be contained in a single vial. Samples withdrawn for QC testing are representative of the entire batch. PET drug products produced in this fashion undergo 100% QC testing.
- PET drug products are produced and handled in environments with overlapping areas of regulatory authority. For example, a site may be subject to requirements of any combination of the following authorities: Nuclear Regulatory Commission or Agreement State Agency, Food and Drug Administration, Environmental Protection Agency, occupational safety entities, etc. These agencies may have different and conflicting requirements of the manufacturing facility, some through regulation, some through guidelines published by the authorities, and some through license commitments made by the facility.
- PET drug products are generally produced at or near their point of use in small-scale facilities with limited personnel and resources. This requires:
  - Allowance for multiple operations in one area with adequate controls
  - Allowance for making and testing multiple PET drug products using shared equipment with appropriate cleaning between batches
  - Appropriate requirements for aseptic operations
  - Appropriate requirements for system suitability and other day-of-use activities
  - Appropriate QC requirements for components, materials, and supplies
  - Self-verification of significant steps in radionuclide production, PET drug production, compounding, and testing
  - Single-person oversight of production or compounding, testing, review of batch records, and release authorization
- PET drug products do not enter a traditional distribution chain. Instead, PET drug products require just-in-time deliveries typically performed by dedicated carriers with experience in handling radioactive materials.
- It is not possible to complete sterility testing for PET drug products before their use. Therefore, considerations are made to provide for the assurance of sterility for injectable PET drugs intended for human use.

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- Procedures should be in place to notify the responsible individuals in a timely manner if a PET drug product is found to be in noncompliance after release for human use.

## 2. TECHNIQUES FOR PRODUCTION AND QUALITY CONTROL

The unique characteristics of PET drug products play a large role in the choice of instrumentation and techniques used in production and QC testing. Production techniques and analytical methods should be efficient and rapid. The selection of techniques and methods is also strongly influenced by the development stage of the PET drug. For example, early development efforts focus on radiolabeling, purification, QC methods development, and others. These efforts are designed to support the usage of the PET drug for *in vitro* studies, animal studies, and may even include first-in-human studies. At this stage, relatively small quantities of the PET drug are required at a limited number of institutions or geographic areas. Manual production techniques or semi-automated equipment may provide sufficient quantities to meet this demand. Analytical methods should also be suitable for the usage of the PET drug at the early stages of development. Typically, method development efforts focus on accuracy, precision, and linearity. Before the use of injectable PET drugs in human studies, provisions are made to ensure that bacterial endotoxins are controlled at suitable levels and that the product is sterile.

In later development stages, larger quantities of the PET drug may be required in more geographic areas. At this point, the synthesis, purification, and testing of the PET drug product should be well defined to support clinical trials. Development efforts at this stage typically focus on optimization and reliability of the production process. Manual production techniques are rarely used. Instead, semi-automated or fully automated techniques are typically required to provide sufficient quantities of the PET drug at multiple geographic locations. This approach simultaneously assures consistent quality attributes of the PET drug at multiple production facilities and minimizes radiation exposure of operators. Method development activities at this stage focus on specificity, ruggedness, robustness, and other characteristics required to support QC testing of the PET drug at multiple geographic locations.

Finally, at the commercial stage of production, semi-automated or fully automated techniques and equipment may be required to provide sufficient quantities of the PET drug in numerous geographic areas. Production techniques and analytical methods for commercial PET drug products should be well defined and adequately described in the appropriate marketing authorization.

Not all PET drugs are intended to advance through the various development stages from *in vitro* studies, to animal studies, to clinical trials, to commercial production. For example, a PET drug product may be produced at several institutions for research purposes without the intention of commercialization. In other instances, a PET drug product may be Food and Drug Administration (FDA) approved but is only available in a single geographic area. These factors often result in different strategies for production techniques, analytical methodologies, and the underlying studies and documentation to support these strategies.

## 3. QUALITY ASSURANCE

The goal of quality assurance (QA) is to ensure that the techniques and equipment used in production and testing result in a product that meets established standards for that specific PET drug product. The QA program should be sufficient to establish the reliability and suitability of the techniques and equipment as appropriate to the development stage of the PET drug product. The following topics should be considered in a QA program.

### 3.1 Reagents and Materials

Reagents and materials used in the synthesis and testing of a PET drug product should conform to established acceptance criteria. Procedures for procuring, receiving, testing, storage, and use of reagents and materials should also be considered. An audit trail may be established for the traceability of specific lots of reagents and materials to specific batches of product. These acceptance criteria, procedures, and the audit trail should be appropriate for the development stage of the PET drug product.

### 3.2 Change Control

Changes in the synthesis and test methods should be evaluated for their potential for altering the product quality and be approved in advance through established procedures. If the resultant PET drug product does not meet the criteria appropriate to the development stage, the process change is unacceptable. Acceptable changes should be appropriately documented.

### 3.3 Validation

Production and analytical test methods should be validated at a level that is appropriate for the development stage of the PET drug product. Validation of the production process should result in a process that is reliable and consistent. Analytical method validation demonstrates that a method can quantitatively measure a PET drug product reliably and reproducibly. It is not necessary to validate analytical methods that are described in USP compendia. The emphasis on validation typically increases as the PET drug product moves through the development stages toward commercialization. Validation of the computer software used to control automated production equipment should also be considered as appropriate.

#### QUALIFICATION

Equipment used in the synthesis and testing of PET drug products should be qualified as appropriate for the development stage of the PET drug product. This may include qualification procedures (installation, operational, and performance), as well

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as procedures for periodic maintenance and equipment calibration. For commercially available equipment, the equipment vendor may be able to support qualification requirements.

STABILITY STUDIES

Stability studies should be performed on PET drug products to establish suitable storage conditions and the expiration time and date. Quality attributes such as radiochemical purity, appearance, pH, stabilizer, preservative effectiveness, and chemical purity should be evaluated. The frequency and extent of these studies should be appropriate for the development stage of the PET drug product. Significant process changes require stability studies to be repeated.

# 4. PRODUCTION

## 4.1 Equipment for Manual Synthesis

Equipment intended for the manual synthesis of PET drug products may be based on apparatus found in a typical chemistry laboratory such as glass reaction vessels, heating blocks, tubing, and other items. These items should be suitable for their intended use and selected according to the needs of the process. Items that are reused and come into contact with the reactants and/or final drug product of the synthesis should be cleaned and depyrogenated as appropriate for the use of the final product.

Written procedures for the manual syntheses of PET drug products should contain sufficiently detailed steps to ensure that a reproducible process is followed for each batch by the operator. Observations of critical process parameters should be documented as appropriate in the batch record.

## 4.2 Equipment for Automated Synthesis

Preparation of *Fludeoxyglucose F 18 Injection* and other common PET drug products can be adapted readily to automated synthesis. The use of programmable controllers and/or computers with switches, solenoids, and sensors allows the operator to control the sequence of steps in an automated fashion. Thus, an automated synthesis requires minimal operator intervention, and batch-to-batch consistency is maintained with each step executed in a reproducible manner. Software may provide feedback to the operator such as alarm conditions, status reports, or an end-of-synthesis report. Modules for automated synthesis may be commercially available. These modules may use individual reagents or cassettes required for the appropriate PET drug product synthesis. The cassettes provide preassembled collections of reagents, reaction vessels, tubing, filters, and other items necessary to produce a batch of a PET drug product.

Laboratory robots may be appropriate in situations where complex manipulations are required for the procedure, such as the physical movement of a reaction vessel from one station to another, or the dispensing of doses from a vial into individual syringes. Customized equipment may also be fabricated to perform a specific function in the PET drug product synthesis.

# 5. QUALITY CONTROL

QC tests should be designed and executed in a manner that is appropriate for PET drugs. The short half-life of PET radionuclides significantly limits the timeframe for the performance of QC tests, and it may not be possible to complete all QC tests before the use of the PET drug product.

## 5.1 Quality Control Sampling

In most cases, the entire batch of a PET drug is contained within a single vial. The relatively small batch volume of PET drugs limits the amount of material available for QC testing. This is different from traditional manufacturing practices, where a batch may consist of numerous vials and not all vials are sampled for purposes of QC testing. QC sampling of PET drug products use a larger proportion of the drug volume, and thereby offers a greater degree of assurance that quality-related problems will be identified in the QC testing process.

## 5.2 Reference Standards

Reference standards are necessary to identify the API and impurities in the PET drug product. Reference standards may be commercially available or prepared in-house. Commercial reference standards should include documentation from the supplier to ensure they are properly characterized. The characterization and qualification of reference standards should be appropriate for the development stage of the PET drug product.

## 5.3 Conditional Release

When a required QC test for a PET drug product cannot be completed because of a malfunction of test equipment, it may be appropriate to conditionally release the batch. It is not appropriate in the absence of critical QC tests to conditionally release batches (e.g., radiochemical identity and purity). Procedures should be developed to describe the conditional release process, including the role of historical data, other QC test results, sample retention, and other procedures.

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### 5.4 Out-of-Specification Results

When the results of a QC test do not meet product specifications, an investigation should be conducted to determine if the result is due to an analytical error (false positive) or if the result is due to a product failure. Because of the short half-life of PET radionuclides, it may not be possible to conduct investigations in the same manner as in traditional drug testing laboratories.

## 6. ANALYTICAL METHODOLOGIES

Various analytical methodologies and chromatographic techniques may be used for the testing of PET drug products. The most commonly used methodologies are discussed in this section for purposes of illustration. Similar principles should apply to other methodologies not included in this section.

### 6.1 Thin-Layer Chromatography

Thin-layer chromatography (TLC) involves separation, identification, and quantification of analytes using a stationary phase on a support or plate and a developing solvent that may also be known as mobile phase. The stationary phase may be silica or alumina spread in a uniform layer onto a plate of glass, metal, or plastic. A small sample of the PET drug product is applied (spotted) onto the stationary phase, and the plate is developed in a chromatographic tank. The mobile phase moves through the stationary phase by capillary action, and the analytes are separated based on partition, ion exchange, and/or adsorption. The retardation factor ($R_f$) and radiochemical purity of the PET drug on the developed plate can be determined by either counting the developed plate on a radio–TLC scanner, or cutting the plate into pieces and counting each piece in a radiation detector. The $R_f$ of an analyte, under specific TLC conditions, is considered an identifying characteristic of the analyte.

### 6.2 Gas Chromatography

Gas chromatography (GC) involves separation, identification, and quantification of volatile analytes using a stationary phase and a gaseous mobile phase. A small sample of the solution is introduced into the GC instrument, which vaporizes the sample for passage over the stationary phase that is immobilized within a GC column. As the gaseous mobile phase passes over the stationary phase, the analytes are separated based on their partition between the gas and stationary phases. A detector located at the exit of the column provides an electronic signal to produce the gas chromatogram.

A GC instrument consists of a gas source, injection port, column, detector, and a data collection device. The injection port, column, and detector are temperature controlled and may be varied as part of the analysis. The gas source depends on the column and detector in use. The type of detector depends on the nature of the compounds analyzed. Typical detectors include flame ionization and thermal conductivity. In addition, radiation detectors may be used in GC analysis. Detector output is recorded over time, and the instrument response, measured as peak area or peak height, is a function of the amount present. The retention time of an analyte under specific GC conditions is considered an identifying characteristic. GC analysis is most typically used to quantify residual solvents in PET drug products but may also be used to determine radiochemical and chemical purity of the PET drug product formulation.

### 6.3 High-Performance Liquid Chromatography

High-performance liquid chromatography (HPLC) is an analytical technique used to separate, identify, and quantify the components of a solution. HPLC separations are based on the interaction of the analytes between the stationary and mobile phases, which in turn leads to the retention of the analytes. The mechanism of interaction between the analytes and stationary phase may be partition, size exclusion, adsorption, and/or ion exchange chromatography. The retention time of an analyte under specific HPLC conditions is considered an identifying characteristic.

HPLC instruments consist of a reservoir containing the mobile phase, a pump to force the mobile phase through the system at high pressure, an injector to introduce the sample into the mobile phase, a chromatographic column, a detector, and a data collection device. The type of detector used depends on the nature of the compounds analyzed. In addition to mass detectors such as refractive index, ultraviolet, and conductivity, radiation detectors are also used in HPLC analysis. An HPLC system with a radiation and mass detector allows the simultaneous determination of radiochemical and chemical purity, retention time, and the subsequent identity of the PET drug product. This configuration also allows the determination of specific activity. Elution methods include gradient or isocratic methods with aqueous and/or organic buffers.

The calibration of a GC or HPLC instrument may be achieved by different means. One approach involves the creation of a calibration curve from a range of standards with known concentrations. The calibration curve may be used over a specified period of time for product testing. A second approach for calibration involves the creation of a single-point calibration at the beginning of each testing cycle. The results may be averaged and used to provide a calibration factor for product testing. Regardless of the approach for calibration, the tailing factor and chromatographic resolution (or column efficiency as appropriate) should be determined routinely as a part of system suitability. Routine system suitability testing should include checks for these parameters.

### 6.4 Radiation Detectors Used in Chromatographic Techniques

Radiation detectors used in TLC, GC, and HPLC for quantitative measurements should be calibrated when possible with suitable NMI-traceable radionuclide standards at appropriate frequency. Routine system suitability testing should be performed on the radiation detection system to ensure proper operation. Because of the nature of TLC, system suitability for a radio–TLC

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scanner should address uniformity, positional accuracy, detector linearity, and resolution. The calibration of radiation detectors should be repeated as appropriate.

### 6.5 Multichannel Analyzer

A multichannel analyzer (MCA) is an instrument used to obtain a spectrum of gamma rays emitted by a PET radionuclide. The key component of a MCA is an energy-sensitive detector. On the basis of the gamma spectrum of a sample, radionuclides present in the sample may be identified and quantified. Calibration of the system is typically performed with certified standards and may involve one or more radionuclides with gamma energies and quantities that span the range of typical analyses at appropriate frequency.

### 6.6 Ionization Chamber

An ionization chamber, often referred to as a dose calibrator, is an instrument used to measure the quantity of radioactivity in a PET drug product. The key component of an ionization chamber is an argon-filled chamber with an applied electrical potential that allows the detection of ions produced by the passage of gamma rays through the chamber. Calibration of the system is typically performed with certified standards at appropriate frequencies and may involve one or more radionuclides with gamma energies and quantities that span the range of typical analyses.

## 7. QUALITY ATTRIBUTES

Quality attributes should be defined and assessed for each PET drug. These attributes should be suitable for the intended use of the PET drug and should also reflect the dosage form. Because most PET drug products are administered by intravenous injection, this section focuses on quality attributes associated with injectable PET drug products. Different attributes may be appropriate for other dosage forms. In addition, the definition and assessment of quality attributes may vary with the development phase of the PET drug product in comparison to the final drug phase.

### 7.1 Appearance

Injectable PET drug products should be free of visible particulate matter. Because of the radioactive nature of PET drug products, the test for appearance should be a visual inspection that meets radiation safety requirements. The use of a visual standard should be considered to ensure the accuracy of appearance determinations.

### 7.2 pH

Injectable PET drug products should be in the pH range suitable for intravenous administration. Because of the limited volume of a PET drug product, pH measurement is typically performed using narrow-range paper strips. The use of pH standards should be considered to ensure the accuracy of pH determinations.

### 7.3 Total Radioactivity and Strength

The total radioactivity of a PET drug product may be determined by an ionization chamber and should be stated at a given date and time. From the total radioactivity and the volume of the PET drug product, the strength may be determined. The strength of the PET drug product should be stated, along with the date and time of the determination of total radioactivity.

### 7.4 Radionuclidic Identity

Half-life (also referred to as approximate half-life) is a characteristic of the radionuclide that may be used for its identification. To adequately confirm the identity of a PET radionuclide, the half-life should be measured in a suitable counting device over a period of time appropriate to the half-life of the radionuclide.

### 7.5 Radionuclidic Purity

By definition, all positron-emitting radionuclides emit 511 keV gamma rays but may also emit gamma rays with different energies. Therefore, it is generally not possible to determine the radionuclidic purity of PET drug products with a MCA. One suitable solution to this problem is the use of validation studies to ensure the removal and/or decay of accelerator-produced radionuclidic impurities during the production process. Other approaches may be appropriate depending on the characteristics and source of the radionuclidic impurities. In addition, periodic analysis of decayed samples in routine production with a MCA may be used to quantify radionuclidic impurities developed.

### 7.6 Radiochemical Identity and Purity

Depending on the physical and chemical properties of the PET drug product, the radiochemical identity and purity may be determined by TLC, HPLC, or GC. The identity of the active pharmaceutical ingredient (API), and possibly other analytes, should be based on the known retention time of the analyte. The simultaneous use of reference standards during sample analysis should

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be considered for purposes of radiochemical identification. The radiochemical purity of the PET drug product should be determined based on the sum of all chemical forms of the radionuclide of interest. As appropriate, it should be established during validation that all radiochemical analytes are eluting from the chromatographic system and that the radioactive detector is operating within its linear range.

### 7.7 Chemical Purity

The chemical purity of the PET drug product should be assessed for the presence of volatile impurities, residual reagents and/or precursors, and by-products. For example, residual solvents, such as acetonitrile and ethyl alcohol, may assessed by GC. [2.2.2]-Cryptand, a common reagent used in the preparation of PET drug products, may be assessed by colorimetric techniques.

### 7.8 Total Mass of the Active Pharmaceutical Ingredient and Specific Activity

Potential toxicity issues and pharmacological effects may be associated with the API in a PET drug product (e.g., the mass-dependent localization of neurotransmitters or other more generalized forms of toxicity). In these instances, the total mass of the API should be determined before use of the PET drug product. The total mass of the API contained in a patient dose should be defined for PET drug products where mass-related localization or toxicity concerns require such assessment, which may be determined by HPLC or GC. On the basis of the mass of the API, the specific activity may be calculated. To determine the appropriate quality of the injected drug product in the patient dose, any two of three measured parameters (total mass, total activity, or specific activity) are sufficient. Specific activity should be stated along with the date and time of determination.

### 7.9 Bacterial Endotoxin

The quantity of bacterial endotoxin in an injectable PET drug product should comply with USP standards (<175 USP Endotoxin Units/patient dose). The test for bacterial endotoxin uses a solution of limulus amebocyte lysate (LAL). Various methods exist for this test, including gel-clot, chromogenic, and others. Test samples should be obtained and handled in a manner that minimizes contamination before testing. In addition, control measures should be used to ensure accurate test results without interferences from the test solution, such as certain formulations that enhance or inhibit the interaction of LAL with bacterial endotoxin. This effect may be corrected by dilution of the PET drug product before testing. The necessity of dilution in this test is determined in the validation of the specific formulation of the PET drug product.

### 7.10 Sterility

Injectable PET drug products should be sterile. For reasons described in *1. Introduction*, it is not possible to complete the sterility test before the use of PET drug products. The test for sterility should consist of inoculating a test sample into media capable of supporting the growth of aerobic and anaerobic microbes, which may be established with an appropriate certificate of analysis. The test samples should be obtained and handled in a manner that minimizes contamination before testing. To reduce radiation exposure to operators, the test samples may be allowed to decay before inoculation. The inoculation should use techniques that minimize the potential for false positives during the sterility test. In addition, control measures should be used to ensure accurate test results without interferences from the test solution. After inoculation, the sterility test samples should be incubated for the appropriate period of time at the prescribed temperature. If visible growth is detected during the incubation period, the investigator or physician should be informed in a timely manner, and an investigation should be initiated to determine whether the growth was due to accidental contamination during the sample handling process or to a true product failure. The investigation should include identification of the microbe(s) in the sterility test sample to determine the source of the contamination. This aspect of the investigation may be limited if production and sterility test sampling occur in the same laboratory.

## 8. STERILITY ASSURANCE

A suitable sterility assurance program should be established for PET drug products that are intended for intravenous injection. This section describes appropriate measures that assure the routine production of a sterile product suitable for injection.

### 8.1 Sterile Membrane Filtration

Injectable PET drug products are typically sterilized by passage of the solution through a sterile membrane filter into a presterilized vial. To provide the greatest assurance of sterility, the PET drug product should pass through an appropriate sterilizing filter into a presterilized vial that has been assembled using aseptic techniques.

### 8.2 Aseptic Techniques

Critical steps that affect the sterility of the PET drug product should be identified and, where appropriate, aseptic techniques should be used to complete these steps. Aseptic techniques should include the use of a suitable gowning, proper handling of components, environmental controls, and others. Aseptic techniques should be described in written procedures.

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## 8.3 Presterilized Components

PET drug production typically employs various components and containers that are presterilized and pyrogen free. Appropriate sterile components are generally assembled aseptically in a controlled environment before use in production. Presterilized vials and sterile diluents such as sterile water for injection or sodium chloride solution are typically used. Other sterile components are sometimes utilized in nonsterile applications in production where a commercially available product may be useful. An example is use of a sterile transfer line for fluid transfer prior to a sterilizing filter, where the diameter or length of a commercially available sterile line is appropriate to the application.

## 8.4 Environmental Controls

The environment where aseptic techniques are executed should be controlled to ensure appropriate aseptic conditions. These controls may include temperature and humidity, ventilation and air filtration, cleaning and disinfection, equipment maintenance, proper garb, and microbiological monitoring. Air filtration standards should conform to local and/or national standards relevant to environmental standards, for example, International Organization for Standardization (ISO) standards.

## 8.5 Media Fills

A media fill, also known as a “process simulation”, is the performance of an aseptic procedure using a sterile microbiological growth medium in place of the PET drug product. The goal of a media fill is to test whether the aseptic procedure is adequate to prevent microbiological contamination during the actual process. Media fills may be used to evaluate aseptic techniques used in the assembly of presterilized components and to qualify operators for aseptic techniques. A media fill should be designed to ensure that the simulation is representative of the aseptic manipulations performed during the actual process, including personnel, components, gowning, locations, batch size, number of replicates, and other factors. After completing the media fill, components filled with media should be incubated appropriately to permit the growth of microbes. The use of negative controls should be considered during the media fill procedure.

## 8.6 Suitability of Media

Sterility test media should be tested before use to ensure that the media adequately support the growth of microbes. These tests are typically referred to as growth-promotion tests and may be conducted in-house, by the supplier, or by a contract laboratory. Media should be used within the manufacturer’s expiration date.

## 8.7 Suitability of the Sterility Test Method

The sterility test should be tested to ensure that the PET drug product is not bacteriostatic or fungistatic. The suitability of the sterility test may be conducted in-house or by a contract laboratory.

## 8.8 Membrane Filter Integrity Test

To ensure the proper sterilization of the PET drug product by passage through a membrane sterilization filter, the bubble point of the filter should be determined after completing the filtration process. The bubble point of the filter, measured in pounds per square inch (psi), is the pressure required to force air through the pores of the wetted membrane in the device. Other suitably validated procedures may be used.

## 8.9 Operator Training and Qualification

Operators involved in aseptic techniques should be trained in proper gowning, environmental controls, handling sterile components, and other techniques. Operators should be qualified through successful completion of media fills. Media fills should be periodically repeated to ensure ongoing competency of the technique.

# 9. LABELING

The labeling associated with PET drug products may evolve as the drug progresses through the various development stages. For example, the labeling for PET drug products used for *in vitro* and animal studies may be very simple and designed to avoid mix-ups in the routine use of the PET drug product. In later development stages, the labeling may include information required for the investigational use of the PET drug product in humans. Finally, at the commercial stage of production, the labeling should include ingredients, warnings, approved indications, and other elements required by the appropriate regulatory agencies (e.g., FDA, Nuclear Regulatory Commission, and others).

# GLOSSARY

The following definitions apply to words and phrases as they are used in this chapter:

**Accuracy:** The closeness of test results obtained by that method to the true value established across the range of the method.

**Active pharmaceutical ingredient (API):** A radioactive substance that exhibits spontaneous disintegration of unstable nuclei by the emission of positrons and is incorporated into a PET drug product to furnish a direct effect in the diagnosis or

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monitoring of a disease or a manifestation of a disease in humans, or monitoring treatment of disease or therapeutic procedures (e.g., tumor therapy). Both radioactive and nonradioactive forms of the PET drug are included in the API.

**Batch:** A quantity of PET drug product that is intended to have uniform character and quality, within specified limits, and that is made in a single, defined operational cycle.

**Chemical purity:** The purity of a PET drug product based on the nonradioactive components of the formulation, for example, residual solvents and/or volatile impurities, reagents, and or precursors used in the synthesis and purification, stabilizers, excipients, or by-products produced in the synthesis.

**Compounding:** The process of synthesis or formulation of a PET drug for use under the practice of pharmacy and medicine.

**Conditional final release:** A final release for patient administration before completion of a required test because of a breakdown of analytical equipment.

**Limit of detection:** The lowest amount of analyte in a sample that can be detected but not necessarily quantified under the stated method conditions.

**Linearity:** The ability to elicit test results that are, directly or by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range.

**Lot:** A quantity of materials (e.g., reagents, solvents, gases, purification columns, and other auxiliary materials) that have uniform character and quality within specified limits and are used to make a PET drug product.

**Lower limit of quantification:** The lowest amount of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated method conditions.

**National Metrology Institute (NMI):** A measurement standards body that is a laboratory of metrology that establishes standards for a country or organization. The National Institute of Standards and Technology (NIST) is the NMI for the United States.

**PET drug:** A radioactive substance (active pharmaceutical ingredient) that exhibits spontaneous disintegration of unstable nuclei by the emission of positrons and is incorporated into a PET drug product to furnish direct effect in the diagnosis or monitoring of a disease or a manifestation of a disease in humans, or monitoring treatment of disease or therapeutic procedures (e.g., tumor therapy).

**PET drug product:** A finished dosage form that contains a PET drug, whether or not in association with one or more other ingredients.

**Precision (as repeatability):** The degree of agreement among individual test results when the method is applied repeatedly to multiple samplings of a homogenous sample within a lab over a short period of time using the same analyst with the same equipment.

**Quality assurance (QA):** A planned and systematic program to ensure that a PET drug product possesses the quality required for its intended purpose.

**Quality control (QC):** A system for testing the quality of components, materials, supplies, and PET drug products by procedures, tests, analytical methods, and acceptance criteria.

**Radiochemical identity:** The molecular structure of the intended active radiopharmaceutical ingredient that is present in the radiopharmaceutical preparation.

**Radiochemical purity:** The ratio, expressed as a percentage, of the radioactivity of the intended active radiopharmaceutical ingredient to the total radioactivity of all radioactive ingredients present in the radiopharmaceutical preparation.

**Radionuclidic identity:** The intended radionuclide in the radiopharmaceutical preparation.

**Radionuclidic purity:** The ratio, expressed as a percentage, of the radioactivity of the intended radionuclide to the total radioactivity of all radionuclides in the radiopharmaceutical preparation.

**Range:** The interval between the upper and lower levels of a quality attribute that can be determined with a suitable level of precision and accuracy.

**Reagents and materials:** A reagent is a chemical used in the synthesis and/or testing of a PET drug, whereas a material is an ancillary object, such as tubing, glassware, vials, and others.

**Retardation factor (TLC):** The ratio of the distance the analyte moved from the origin line divided by the distance the solvent moved from the origin line denoted by the variable $R_f$.

**Retention time (HPLC or GC):** The time required, after the injection, for the analyte to move through the column and reach the detector.

**Robustness:** The measure of the capacity of an analytical method to remain unaffected by small variations in method parameters.

**Ruggedness (as reproducibility):** The degree of reproducibility of test results obtained by the analysis of the same samples under a variety of conditions, such as different labs, different analysts, different instruments, different lots of reagents, different assays, and different days.

**Specific activity:** The radioactivity of a radionuclide per unit mass of the element or compound. The unit of specific activity is the amount of radioactivity on a mass basis [e.g., mCi/μg (MBq/μg) or Ci/mmol (GBq/mmol)].

**Specificity:** The ability to assess unequivocally the analyte in the presence of components that may be expected to be present such as impurities, degradation products, and matrix components.

**Strength:** The radioactivity concentration of the active pharmaceutical ingredient in a PET drug product at a given date and time. The unit of strength is the amount of radioactivity on a volume basis [e.g., mCi/mL (MBq/mL)].

**Sub-batch:** A quantity of PET drug product having uniform character and quality, within specified limits, that is produced during one succession of multiple irradiations using a given synthesis or purification operation. A group of sub-batches collectively forms a batch that is intended to have uniform character and quality, within specified limits. Sub-batches may be required for PET drug products with very short-lived radionuclides (e.g., $^{13}N$ and $^{15}O$), because QC tests cannot be completed before use.

**System suitability:** Requirements used to verify that the system performs according to established criteria.

**Tailing factor (symmetry factor):** A measure indicating the non-ideality of a chromatographic peak resulting from the distribution and the migration of the analyte through the chromatographic column.

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**Validation:** Establishment of documented evidence that a method, process, or system meets its intended requirements.
**Verification:** Confirmation that an established method, process, or system meets predetermined acceptance criteria.

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***Add the following:***

# ▲⟨1850⟩ EVALUATION OF SCREENING TECHNOLOGIES FOR ASSESSING MEDICINE QUALITY

1. INTRODUCTION
2. APPLICATION OF THE TECHNOLOGY
3. GENERAL INFORMATION ABOUT THE TECHNOLOGY
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4. PERFORMANCE EVALUATION
    4.1 Technology Applications and Analytical Performance Characteristics to Evaluate
5. FIELD EVALUATION
    5.1 Access, Handling, Maintenance, and Repair
    5.2 Durability and Use
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## 1. INTRODUCTION

The proliferation and spread of substandard and falsified (SF) medical products has been and continues to be a growing global concern. Examples have been reported on a range of SF medicinal products, including but not limited to essential medicines. These products threaten global public health by jeopardizing patient safety, adding to the cost of care, diminishing confidence in health workers and systems, increasing the risk of treatment failure, wasting valuable resources, and contributing to the development of drug resistance.

Fortunately, many analytical technologies and tools exist to evaluate product quality within the laboratory. In general, these technologies are well understood and characterized. Over the last decade, many of these tools have been miniaturized for portability to help combat the growing proliferation of SF medical products. One of the benefits of the field-deployable platform of these technologies is that it facilitates the screening of samples as part of a risk-based testing approach.

The capabilities of the portable screening technologies complement, but do not obviate the need for, laboratory-based technologies and their confirmatory power. Portable tools may help conserve the limited resources of these laboratories and drive a sustainable "work smart" approach to ensuring medicine quality by utilizing complementary methodologies of progressively increasing complexity to rapidly and reliably analyze large numbers of samples. The following examples describe where and how these technologies are currently used or may be used in the future:

- Manufacturing controls
- Supply chain screening
- Border control
- Customs inspection
- Post-market quality surveillance or regulatory monitoring
- Point-of-care screening

However, the potential and actual capabilities and limitations of many of these portable screening tools, and especially their performance in field settings, have not been established. To address this need, this chapter provides the structure and suggested requirements for performing an appropriate and pragmatic review of a given technology. Such a review would typically comprise general information, a performance evaluation, and a field evaluation. The information garnered from the review can then be used to inform selection, procurement, and deployment of the technology of interest. Within this chapter, the term technology refers to screening instruments, solutions, technologies, or tools that provide basic information about the authenticity or quality of a drug substance or product.

## 2. APPLICATION OF THE TECHNOLOGY

The first and most important aspect of an evaluation is to identify the target application of the technology. A clear understanding of the intended application of any technology will help identify the parameters that need to be evaluated and inform the decision as to whether multiple screening technologies might be needed in a particular setting. For example, some screening technologies evaluate non-chemical features of a product, such as appearance, packaging, labeling, and origin. The technology will need to correctly identify a falsified medicine, but it will not need to determine the level of chemical impurities within the drug product.

Furthermore, the intended application of the technology comprises that of both the original equipment manufacturer (OEM) and the user. If these applications align, then the initial part of an evaluation should involve reviewing the technology against the manufacturer's claims. However, if these applications do not align, further steps should be taken to evaluate the technology against the user's requirements and intended application. Intended applications may vary based on factors such as:

- Medicine types
- Anticipated users

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- Geographic locations
- Points within the supply chain
- The public health consequences related to poor-quality medicines within supply chains

## 3. GENERAL INFORMATION ABOUT THE TECHNOLOGY

The first step of a review, prior to implementing the performance and field evaluations, is to acquire general information about the technology of interest. This should include, but is not limited to, answering the questions listed below.

### 3.1 Specifications, Relative Cost, and Data

SPECIFICATIONS

Questions regarding specifications include:

- Which model or version of the technology is being reviewed?
- What technique (e.g., Raman spectroscopy, infrared spectroscopy, chromatography) does the technology employ?
- What are the dimensions (i.e., size, weight) of the technology?
- What type of power source (i.e., electricity, battery, solar) is needed to run the technology?
- What additional power requirements (i.e., converters, power conditioners, uninterrupted power supply) are involved?
- Are there any safety precautions that should be taken into consideration before using the technology (i.e., lasers, heat generating)?
- Is waste generated when using the technology? If so, what kind of waste, and how is it safely handled?
- How easily can the technology's equipment be cleaned?
- What additional resources (i.e., chemicals, water, electricity) are required to clean the technology?

RELATIVE COST

Questions regarding relative cost include:

- What is the up-front cost of the technology?
- What are the recurring costs and how much are they (e.g., consumables, maintenance costs)?
- What is the cost of performing a test?
- Will the technology and intended methods be utilized with enough frequency to offset the cost?
- What warranties are available when purchasing the technology, and how comprehensive are they?

DATA

Questions regarding data include:

- What alternative languages are available for the hardware/software?
- Does the technology have a readily accessible user manual?
- Does the technology need or have internet connectivity capabilities?
- What format are data files in?
- Can data be transferred between devices?
- Can data be transferred to external sources?
- How can the data be displayed?
- Can the data be easily summarized into a report that supports decision-making?
- Can the software capabilities maintain data integrity?
- What are the levels of software permissions (i.e., administrator, user, guest), and what level of access do they provide?
- Does the instrument have a usable bar or Quick Response (QR) code reader?

It is important to note that some of these questions complement and supplement those listed in *5. Field Evaluation*. Generally speaking, the questions listed in that section can be answered through the review of publicly available information on the technology of interest or discussion with the OEM.

## 4. PERFORMANCE EVALUATION

The analytical performance portion of an evaluation may include parameters discussed in various guidances and standards on method development and validation. These include *Validation of Compendial Procedures* ⟨1225⟩ (*1*); *Analytical Instrument Qualification* ⟨1058⟩ (*2*); the Food and Drug Administration (FDA) Guidance for Industry, *Analytical procedures and methods validation for drugs and biologics* (*3*); and the International Council for Harmonisation (ICH) (harmonised tripartite guideline), *Validation of analytical procedures: text and methodology Q2(R1)* (*4*). However, except for ⟨1058⟩, these references are geared toward the validation of a method; their application in the context of this chapter should therefore focus on the performance qualification of a specific instrument rather than a procedure.

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## 4.1 Technology Applications and Analytical Performance Characteristics to Evaluate

This section provides potential reviewers with the performance characteristics to evaluate, depending on the intended applications of a given technology. Ideally, this analytical performance evaluation will take place in a laboratory where variables can be controlled and a confident assessment can be made regarding the technology's analytical capabilities and whether they align with the reviewer's needs. With the exception of *Application I*, below, the performance evaluation should also encompass a comparison of the analytical results of the screening technology with those of a well-accepted confirmatory or compendial procedure (e.g., high-performance liquid chromatography) using reference material or standards. This will help inform the application and implementation of the technology within a broader quality assurance and quality control system.

*Table 1*, *Table 2*, and the *Applications* below, have been adapted from ⟨1225⟩ and tailored to the context of this chapter. However, they provide a broad guide and should be adapted to fit the needs of the user and the capabilities of the technology of interest. Definitions of the listed characteristics are included in the *Glossary*.

QUALITATIVE APPLICATIONS

**Application I:** Verification of packaging, labeling, origin, and appearance of the sample or drug product
**Application II:** Identification of bulk drug substances or active pharmaceutical ingredients (APIs) in finished pharmaceutical products
**Application III:** Identification of contaminants or impurities in bulk drug substances or finished pharmaceutical products

**Table 1. Qualitative Analytical Characteristics to Evaluate**

| Analytical Characteristic | Application I | Application II | Application III |
|---|---|---|---|
| Specificity | Yes | Yes | Yes |
| Detection limit | No | No | Yes |

QUANTITATIVE APPLICATIONS

**Application IV:** Quantification of major components of bulk drug substances or APIs in finished pharmaceutical products
**Application V:** Quantification of contaminants, impurities, or adulterants in bulk drug substances or finished pharmaceutical products
**Application VI:** Determination of product performance characteristics (e.g., disintegration, dissolution, drug release) in finished pharmaceutical products.

**Table 2. Quantitative Analytical Characteristics to Evaluate**

| Analytical Characteristic | Application IV | Application V | Application VI |
|---|---|---|---|
| Accuracy | Yes | Yes | Yes |
| Precision | Yes | Yes | Yes |
| Specificity | Yes | Yes | Yes |
| Detection Limit | No | No | No |
| Quantitation Limit | No | Yes | No |
| Linearity | Yes | Yes | Yes |
| Range | Yes | Yes | Yes |

# 5. FIELD EVALUATION

A field fitness evaluation of a screening technology should be conducted as a follow-up to a performance evaluation. This part of the review assesses the ability of a technology to operate effectively and efficiently in field settings, which may include, but is not limited to, resource-limited areas. Certain parameters should be assessed and certain questions answered. Although some of these parameters can be evaluated through online searches and discussion with the instrument manufacturers, other parameters will need to be evaluated in the field and this should be taken into consideration when planning a review. The parameters and questions listed in the section below are not exhaustive and some may not be applicable, depending on the technology. However, they provide baseline information necessary for making an informed decision about the field suitability of a given technology within a given country.

Determine whether the results provided by the technology when analyzing a medical product in the field correspond to results obtained from the performance evaluation in the laboratory. This does not mean that the exhaustive analytical testing done in the laboratory needs to be replicated in the field, but a smaller, pragmatic subset of samples and tests should be run to give the reviewer confidence that the technology does, indeed, function in a field setting.

## 5.1 Access, Handling, Maintenance, and Repair

Some questions to address include:

- Is the technology commercially available in the country?

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- In countries where the technology is commercially available but needs to be imported, what are the marketing authorization requirements for importing the technology?
- What are the control requirements for exporting this technology out of its country of origin or distribution?
- Are there local service providers or authorized distributors in the country?
- Can the technology be maintained and repaired by trained users or is a local service provider required?
- If there is a local service provider, then where is it located and how can it be reached (i.e., in country office, via email, or by phone)?
- Are there other shipping requirements if there is a need to send the technology for calibration or repair?
- Are there additional resources required to calibrate, recalibrate, or repair the technology (i.e., internet access, reagents, standards, spare parts)?
- How accessible are these additional resources in terms of availability and affordability?
- How often are recalibration and performance maintenance of the technology required?

## 5.2 Durability and Use

DURABILITY

Some considerations regarding durability of the technology include:

- What is the operating temperature range of the technology?
- How is the technology affected by humidity?
- How are results affected by changes in temperature or humidity?
- How tolerant is the technology of various changes in the operational environment?
  - Temperature
  - Humidity
  - Dust
  - Vibration
  - Electromagnetic interference
  - Light
  - Water
  - Electrical variability (e.g., voltage, surge, frequency)
- How tolerant is the technology of rough handling by users?
- Is the technology intrinsically safe and suitable for the intended environment (e.g., temperature, humidity)?
- Can the technology withstand a drop test [i.e., according to U.S. Military Standard [MIL-STD]-810G *(5)*, International Electrotechnical Commission [IEC] 60068 *(6)*, or ASTM D5276-98 *(7)*]?

USE

Some considerations regarding use of the technology include:

- Is the technology suitable to use for the intended application?
- What level of training (e.g., remote vs. in-person, time, complexity) is required to use the tool?
- What are the sample preparation requirements for analysis and is the testing destructive or non-destructive?
- What additional accessories, consumables, reagents, and standards are required for sample analysis?
- How long does it take to analyze a sample and how easily can the results be interpreted?
- How many samples can be run simultaneously?
- What offline data analysis is required to interpret the results?
- What offline data analysis is available for experienced users?
- What requirements are there for fleet management of multiple instruments?
- What types of products (e.g., tablets, injections) can the technology analyze?
- Can models and/or methods developed on another instrument be remotely transferred to field units as needed?

## 5.3 Protocol and Statistics

PROTOCOL

When evaluating a screening technology for suitability, the applicable sections of this chapter should be used to develop a robust, practical, and ideally, standardized protocol that specifically outlines the work to be done. After selection of the applicable sections, additional variables need to be taken into account to ensure that the evaluation generates evidence-based data that can inform decision-making. These variables include but are not limited to the following:

- Number of units of the screening technology needed to determine variability between instruments (any evaluation should include at least two units of the technology in question)
- Types of dosage forms (e.g., solid oral dosage forms, injectables) to use for the evaluation
- Types of APIs to use for the evaluation

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- Number of batches per product to use for the evaluation
- Number of units per sample to use for the evaluation

STATISTICS

The prudent use of statistics as part of an evaluation is a critical component for ensuring the integrity and reliability of any data generated. When possible, the specific statistical approaches and methodologies to be used should be identified, and a professional statistician should be consulted to devise an appropriate protocol. Concomitantly, it is valuable to collect available information from the literature and specific OEMs about the statistics associated with various applications of the technology. This approach provides a strong baseline to expand upon.

The following statistical guidances, references, and standards can be used to help with planning statistical approaches and methodologies:

- *Analytical Data—Interpretation and Treatment* ⟨1010⟩ (*8*)
- *Statistical Tools for Procedure Validation* ⟨1210⟩ (*9*)
- Annals of Internal Medicine Research and Reporting Methods—QUADAS-2: a revised tool for the quality assessment of diagnostic accuracy studies (*10*)
- ASTM E2586-16 Standard practice for calculating and using basic statistics (*11*)
- BMJ Research Methods & Reporting—STARD 2015: an updated list of essential items for reporting diagnostic accuracy studies (*12*)
- *Guidance for Industry and FDA Staff—Statistical guidance on reporting results from studies evaluating diagnostic tests* (*13*)
- International Organization for Standardization (ISO) 3534-2, *Statistics—vocabulary and symbols—part 2: applied statistics* (*14*)
- ISO 3534-3 *Statistics—vocabulary and symbols—part 3: design of experiments* (*15*)
- ISO 5725-1 *Accuracy (trueness and precision) of measurement methods and results—Part 1: general principles and definitions* (*16*)

## GLOSSARY

For reference purposes, some definitions are based on ⟨1225⟩.

**Accuracy:** The accuracy of an analytical procedure is the closeness of the test results obtained by that procedure to the true value. The accuracy of an analytical procedure should be established across its range. [NOTE—The definitions of accuracy in ⟨1225⟩ and the ICH harmonized tripartite guideline, *Validation of analytical procedures: text and methodology* Q2 (R1) (*4*) correspond to unbiasedness only. In the *International Vocabulary of Metrology* and documents of ISO, "accuracy" has a different meaning. In ISO 5725, accuracy combines the concept of unbiasedness (termed "trueness") and precision, and is defined as the closeness of agreement between a test result and the accepted reference value.]

**Adulterant:** Any substance that has been (1) mixed or packed with a drug so as to reduce its quality or strength or (2) substituted wholly or in part for the drug.

**Detection limit:** The detection limit is a characteristic of limit tests. It is the lowest amount of analyte in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions. Thus, limit tests merely substantiate that the amount of analyte is above or below a certain level. The detection limit is usually expressed as the concentration of analyte (e.g., percentage, parts per billion) in the sample.

**Linearity:** ICH documents define linearity as the ability to obtain test results that are proportional to the concentration of analyte in the sample across a given range.

**Precision:** The precision of an analytical procedure is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of a homogeneous sample. The precision of an analytical procedure is usually expressed as the standard deviation or relative standard deviation (coefficient of variation) of a series of measurements. Precision may be a measure of either the degree of reproducibility or of repeatability of the analytical procedure under normal operating conditions. In this context, reproducibility refers to the use of the analytical procedure in different laboratories, as in a collaborative study. Intermediate precision (also known as ruggedness) expresses within-laboratory variation, as on different days or with different analysts or equipment within the same laboratory. Repeatability refers to the use of the analytical procedure within a laboratory over a short period of time using the same analyst with the same equipment.

**Quantitation limit:** The quantitation limit is a characteristic of quantitative assays for low levels of compounds in sample matrices, such as impurities in bulk drug substances and degradation products in finished pharmaceuticals. It is the lowest amount of analyte in a sample that can be determined with acceptable *Precision* and *Accuracy* under the stated experimental conditions. The quantitation limit is expressed as the concentration of analyte (e.g., percentage, parts per billion) in the sample.

**Range:** The range of an analytical procedure is the interval between the upper and lower levels of analyte (including these levels) that have been demonstrated to be determined with a suitable level of *Precision*, *Accuracy*, and *Linearity* using the procedure as written. The range is normally expressed in the same units as the test results (e.g., percent, parts per million) obtained by the analytical procedure.

**Robustness:** The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in procedural parameters listed in the procedure documentation. Robustness provides an indication of the procedure's suitability during normal usage. Robustness may be determined during development of the analytical procedure.

**Screening:** The process by which a sample undergoes an initial assessment of quality to determine acceptability or the need for additional testing.

**Specificity:** ICH documents define specificity as the ability to identify the analyte in the presence of components that may be expected to be present, such as impurities, degradation products, and matrix components (*4*). [NOTE—Other reputable international authorities (e.g., International Union of Pure and Applied Chemistry, AOAC-International) have preferred the term

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"selectivity," reserving "specificity" for those procedures that are completely selective.] The definition of specificity, above, has the following implication for the tests discussed below:

1. Identification tests ensure the identity of the analyte.
2. Purity tests ensure that all the analytical procedures performed allow an accurate statement of the content of impurities of an analyte (e.g., heavy metals, organic volatile impurities).
3. Assays provide an exact result, which allows an accurate statement of the content or potency of the analyte in a sample.

**Supply chain:** The system of manufacture, distribution, and dispensing of pharmaceutical products.

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# ⟨1852⟩ ATOMIC ABSORPTION SPECTROSCOPY—THEORY AND PRACTICE

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## THEORY

The instrumental technique of atomic absorption spectrometry (AAS▲ (USP 1-Aug-2022)) uses the Beer–Lambert Law (Beer's Law), which relates the concentration of an analyte in a sample to the sample's absorption of electromagnetic radiation. Beer's Law states that the optical absorbance of a chromophore in a transparent solvent is linearly proportional to the chromophore's concentration and also to the sample cell path length. Beer's Law is applicable only if the spectral bandwidth of the light is narrow compared to the spectral line widths in the spectrum and is expressed as follows:

$$A = \varepsilon bc$$

$A$ = absorbance
$\varepsilon$ = molar absorptivity [L/(mol · cm)]
$b$ = path length of the sample cell (cm)
$c$ = concentration (mol/L)

In addition:

$$A = \log_{10}(P_0/P) = \log_{10}(1/T) = \log_{10}(100/\%T) = 2 - \log_{10}\%T$$

$P_0$ = intensity of light passing through a sample
$P$ = intensity of light leaving the sample
$T$ = transmittance
$\%T$ = $100 \times T$

AAS methods fall into two categories: flame (FAAS) and flameless. Flameless methods include graphite furnace AAS (GFAAS), also known as electrothermal vaporization AAS (ETVAAS), for ultra-trace analyses. Other flameless methods are cold vapor AAS (CVAAS), intended specifically for the analysis of mercury, and hydride generation AAS (HGAAS), intended specifically for the analysis of arsenic, bismuth, germanium, lead, antimony, selenium, tin, and tellurium.

A basic AAS consists of a radiation source, a sample introduction device, a means to atomize the sample, a monochromator or a polychromator, a detector, and some means of acquiring data (usually a computer). Most instruments also have some sort of background correction system, which will be discussed later in this chapter.

In the case of FAAS, the flame through which the sample passes is considered to be the sample cell. In the case of electrothermal vaporization (ETV), the graphite tube into which the sample is deposited is considered to be the sample cell. In the case of CVAAS, a quartz absorption cell is mounted on top of the burner head, and the space above the burner in the optical path is considered to be the sample cell. In the case of hydride generation, hydrides are swept into a heated cell.

## INSTRUMENTATION

Figure 1. Basic components of an atomic absorption spectrophotometer.

All atomic absorption spectrophotometers share fundamental components (*Figure 1*). A wide variety of AAS is available commercially and is based on one of two designs: a single-beam or a double-beam light path.

*Figure 2* illustrates the light path for a single-beam spectrophotometer. The dotted line represents the modulated light signal from the external light source (line source), and the solid line represents the direct current signal from the atomizer, represented by a flame. The ovals represent lenses.

In this example, the beam from the line source is electrically modulated, and an amplifier placed after the detector is tuned to this modulation frequency. The beam from the line source becomes coded in this manner. The result is that the noise from the radiation emitted at all frequencies, except for the resonance frequency, is rejected, and the signal-to-noise ratio is improved.

Single-beam spectrophotometers rely on a very stable line source to maintain accuracy. It is also necessary to run a separate blank solution in order to provide a correct 100% $T$ adjustment.

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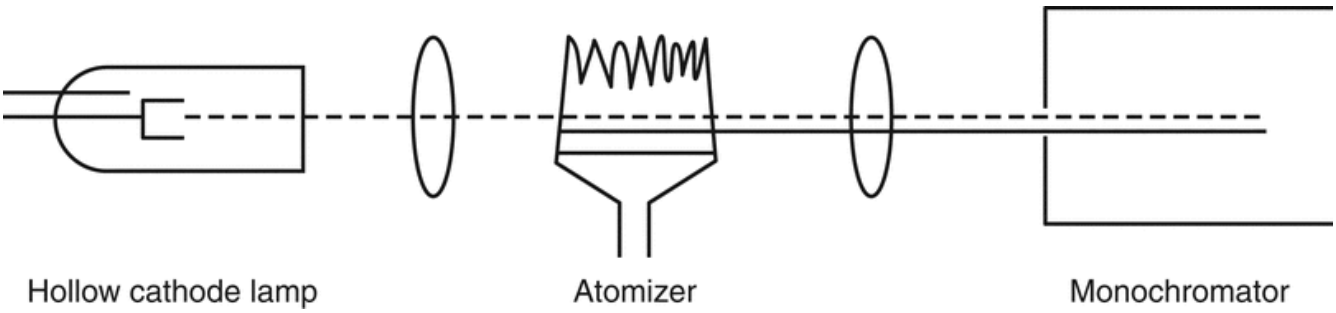


Figure 2. Schematic of a single-beam atomic absorption spectrophotometer. Reproduced with permission of the Royal Society of Chemistry.

*Figure 3* illustrates the light path for a double-beam spectrophotometer. The radiation beam from the line source is divided by a mirrored chopper. One half is directed through the atomizer (sample beam), and the other half is directed around it (reference beam). Note that the beam from the line source is mechanically modulated by the chopper. The two beams are then recombined by a half-silvered mirror and are directed into a monochromator where the photons at the characteristic wavelength are measured by the detector. The ratio between the sample signal and the reference signal is amplified and processed as the absorbance reading. Because the sample and reference beams are generated from a common line source, are separated into their characteristic wavelengths by the same monochromator, and are processed by the same electronics, any variations in the radiation source, detector properties, or electronics are canceled out. In principle, the stability of a double-beam spectrophotometer is superior to that of a single-beam spectrophotometer.

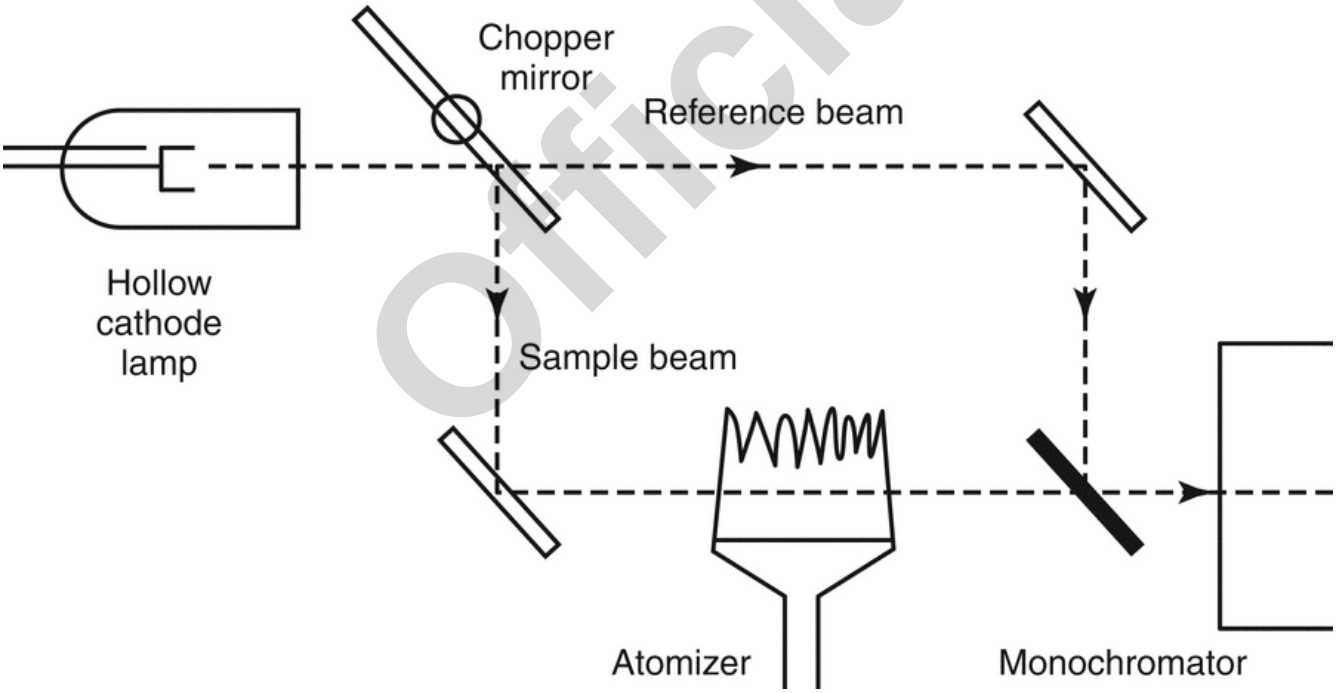


Figure 3. Schematic of a double-beam atomic absorption spectrophotometer. Reproduced with permission of the Royal Society of Chemistry.

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## SAMPLE CELL DESIGNS

### Flame Atomic Absorption Spectrometry

In FAAS, a liquid sample is aspirated into a flame via a nebulizer. Via the nebulizer, the sample is converted to a mist that is composed of uniform droplets that are easily introduced into the flame. The flame desolvates and atomizes the sample providing a source of neutral atoms or molecules for analysis in the spectrophotometer.

Although other flame types have been documented, the most commonly used flame is an air–acetylene flame. Because the temperature of the air–acetylene flame is not sufficient to destroy oxides that might form or are present, a nitrous oxide–

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acetylene flame often is used, depending on the analyte and nature of the sample. The air–acetylene flame burns within a temperature range of 2125°–2400°, but the nitrous oxide–acetylene flame burns within a temperature range of 2650°–2800°.

Flames can be optimized for a particular analysis by either increasing or decreasing the fuel to oxidant ratio. The fuel to oxidant ratio can be adjusted to be either lean or rich—oxidizing or reducing—depending on the analyte of interest. Most instrument manufacturers provide guidance regarding the type of flame to use for a specific element, and analysts should refer to the manual provided by the instrument manufacturer for optimal flame conditions for a given analyte.

Some AAS can also be used in the flame emission mode. In flame emission, the atoms and molecules achieve an excited electronic state following thermal collisions within the flame, and upon their return to a lower or ground electronic state they emit light at characteristic wavelengths for each analyte. Although instrumentation may be capable of operation in the flame emission mode, this will not be discussed further in this chapter.

## Electrothermal Vaporization and Graphite Furnace Atomic Absorption Spectrometry

In ETVAAS or GFAAS, a liquid sample is deposited through a small opening into a graphite tube known as a mini-Massmann furnace. Inside the furnace, the sample is heated at increasing temperatures until the solvent is evaporated, solid residue is ashed or pyrolyzed, and neutral atoms are atomized in their ground states. The atoms are then excited by absorption of radiation at characteristic wavelengths. Samples can be deposited either directly onto the wall of the graphite furnace or onto a small graphite platform, known as a L'vov platform, which sits inside of the graphite furnace. With ETVAAS, a series of heating steps is usually employed, including drying, charring or ashing, atomizing, and clean-out. Other intermediate heating steps can be used, depending on the nature of the sample. Throughout the heating process, the graphite furnace is purged with an inert gas, usually nitrogen or argon. At the atomization step, the furnace is quickly heated to a high temperature (usually to incandescence). The purge gas flow is stopped temporarily as a transient absorption signal produced by the atomized analyte is measured. As in FAAS, Beer's Law can be used to relate the concentration of the analyte and the absorption signal.

▲A figure of merit commonly used in GFAAS analyses is the characteristic mass. The characteristic mass is defined as the mass of an element required to produce an absorbance of 1% (99% transmittance, *T*) or 0.0044 absorbance units (AU). The characteristic mass, in picograms, is calculated as follows:

$$M = (0.0044 \times C \times V)/A$$

*M* = characteristic mass (pg)
*C* = concentration of the sample (pg/µL)
*V* = volume of the sample (µL)
*A* = measured absorbance (AU)

The optimum characteristic mass for each element is often provided by the instrument manufacturer or publication authors and can be useful for method development of difficult matrices.▲ (USP 1-Aug-2022)

## Cold Vapor and Hydride Generation Atomic Absorption Spectrometry

Cold vapor and hydride generation techniques are often used for the determination of mercury or for some hydride-forming elements such as tin, arsenic, selenium, antimony, and bismuth.

In the case of mercury, a chemical reduction generates atoms, and a stream of inert gas sweeps the cold vapor into a cold quartz cell in the optical path of the instrument. The technique is very sensitive and has detection limits that range from parts per billion (ppb) to parts per trillion (ppt) depending on the sample and the laboratory environment.

In the case of hydride-forming elements, a reaction with sodium borohydride and hydrochloric acid generates the hydride of the analyte of interest. The resulting gas is swept into an inert quartz cell that is positioned on top of the burner. The cell can be externally heated, or it can be heated by an air–acetylene flame. The heat of the flame breaks down the hydride and creates the elemental form of the analyte. This is known as the direct-transfer mode of hydride generation. Commercial direct-transfer hydride generators are available in two configurations, continuous flow and flow injection. As with the cold vapor detection of mercury, hydride generation also can be very sensitive and has detection limits in the ppb or ppt range.

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# LINE SOURCES

An external light source (line source) is used to emit spectral lines corresponding to the energy required to elicit the electronic transition from the ground state to an excited state in the sample. The external light sources most commonly used are hollow cathode lamps (HCL) or continuum electrodeless discharge lamps (EDL). Absorption of radiation from the external light source is proportional to the population of the analyte species in the ground state, which is proportional to the concentration of the analyte that is aspirated into the flame, thereby making it possible to use Beer's Law to determine the concentration of an analyte in the sample. The absorption is measured by the difference in transmitted signal in the presence and absence of the analyte.

A suitable line source for AAS must:

- Produce lines of sufficiently narrow bandwidths specific to a particular atomic absorption peak
- Produce a beam of radiation of sufficient intensity to allow high signal-to-noise absorption measurements
- Produce a beam of radiation that is stable for extended periods of time
- Be easy to start and have a short warm-up time and an extended shelf life.

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The gas contained within the HCL tube is ionized when an electrical potential is applied across the electrodes. Gaseous cations then acquire sufficient kinetic energy to dislodge some of the metal atoms from the anode surface, a process known as sputtering. A portion of the resulting cloud of metal ions is excited. Upon relaxation to the ground state, the ions emit photons at the characteristic wavelengths for that metal. HCLs are available in a variety of configurations and can be specific for a single element or multiple elements.

For certain elements, EDLs produce much more intense radiation beams than do HCLs. They are available for the analysis of antimony, arsenic, bismuth, cadmium, cesium, germanium, lead, mercury, phosphorus, selenium, tellurium, thallium, tin, and zinc. They are similar to HCLs because they rely on the sputtering of a metal by accelerated ions, but they ionize the inert gas by means of an intense radio-frequency field instead of hard-wired electrodes.

The requirement for an individual line source for each metal limits AA to a single-element technique. Some modern systems allow multi-element analyses by automatic lamp switching or by positioning multiple lamps in an array. High-resolution continuum source AAS combines a high-resolution echelle monochromator with a Xenon short-arc lamp to obtain multi-element analytical capabilities (see ▲the first entry in *Additional Sources of Information*▲ (USP 1-Aug-2022) for additional details).

## WAVELENGTH SELECTORS

Because atomic resonance lines are narrow, spectrometers are frequently equipped with monochromator gratings of moderate resolution, such as Ebert and Czerny–Turner monochromator systems.

Although the application is not common, high-resolution echelle polychromators have been used in AAS designed for simultaneous measurements of multiple elements. Spectrophotometers of this type usually mate a high-resolution polychromator with either a Xenon arc continuum source or several individual line sources. They typically are equipped with a solid-state detector system.

## DETECTION SYSTEMS

Detection systems convert radiant energy, photons, into a concentration-proportionate electronic signal. This signal is amplified and processed into an absorbance reading or a concentration, as illustrated in *Figure 1*. Photomultiplier tubes (PMTs) are widely used in AAS to convert photons passed through the monochromator into voltages. Some spectrophotometers are designed so that the applied PMT currents are under operator control. As mentioned above, some modern AAS are equipped with solid-state detection systems. There are two types: a charge-injection device or a charge-coupled device. Advantages and disadvantages of each design depend on specific applications. In some applications, a solid-state detector can produce a superior signal-to-noise ratio, provide a flatter response across the UV/Vis spectrum, and/or have improved background correction capabilities when compared to a PMT.

## BACKGROUND CORRECTION

Nonspecific absorption can compromise the accuracy of AAS measurements. This is particularly the case for ultra-trace analyses using GFAAS. The type of background correction used for GFAAS can have a large impact on the success of the analysis. Three types of background correction designs are available for modern AAS: continuum source, Smith–Hieftje or variable giant pulse, and Zeeman effect.

### Continuum Source

A continuum source, frequently a deuterium lamp, is configured in the spectrophotometer optics so that radiation from the continuum source and the line source is alternately passed through the sample cell by a beam chopper (*Figure 4*). The width of the continuum source bandpass is equal to the slit width.

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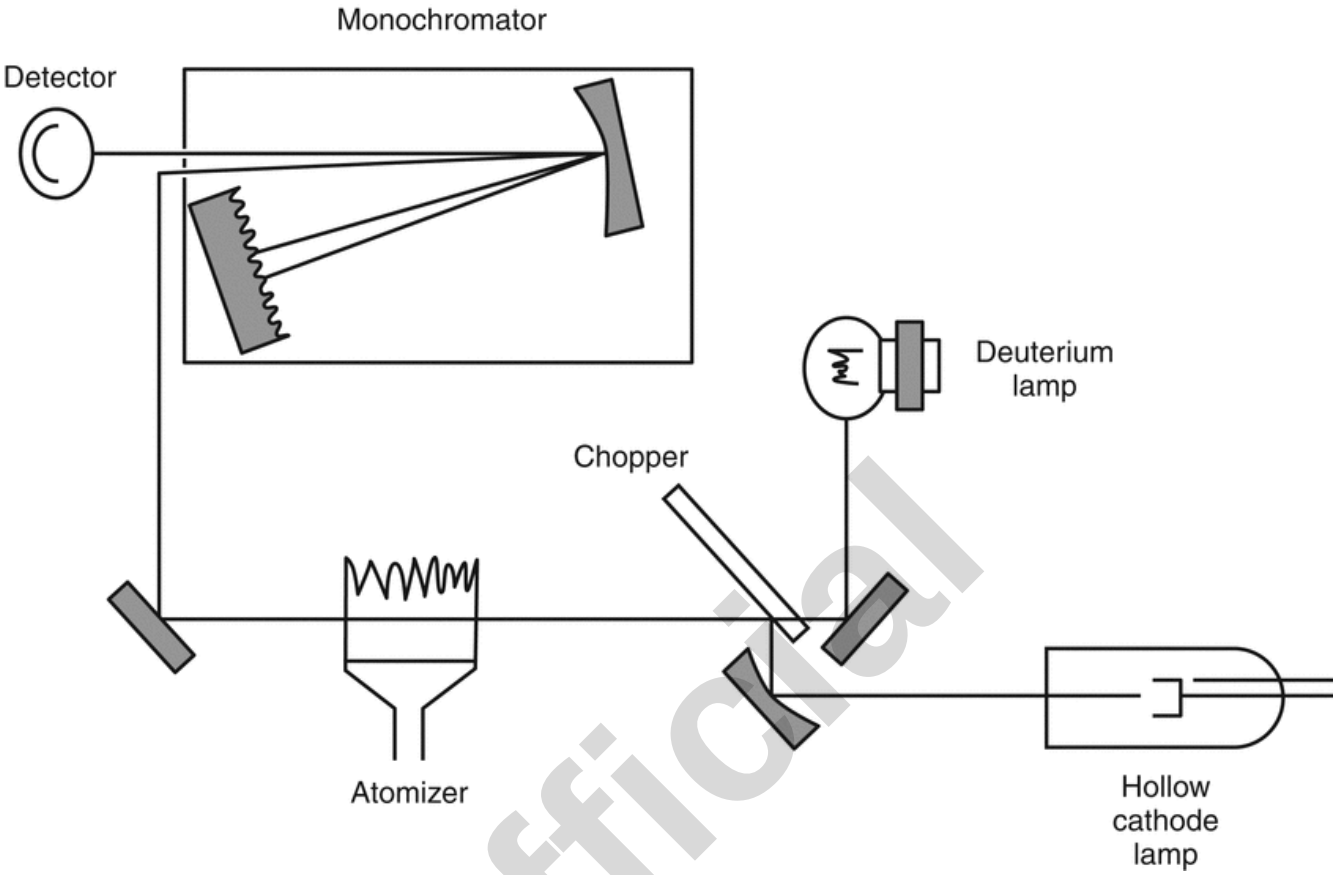


Figure 4. Schematic of an atomic absorption spectrophotometer with a continuum source background corrector. Reproduced with permission of the Royal Society of Chemistry.

The signal intensity from both sources can then be measured, and the ratio can be calculated.

*Figure 5* displays the operation of a continuum-source background-correction system. Note that before the analysis, the intensity of the deuterium lamp and the HCL are equalized at the analyte's resonance wavelength. Also note that the bandwidth of the continuum source is equal to the width of the monochromator's exit slit, usually 0.2–0.7 nm, compared to the width of the resonance line of approximately 0.002 nm. If a solution containing the analyte of interest is introduced, the signals from both sources are attenuated because of atomic absorption (*Figure 5*, Panels A and B). If a solution containing the analyte of interest plus components that lead to nonspecific broad-band absorbance is introduced, the intensity of atomic absorption of the line source (HCL) is correctly subtracted from the intensity of the continuum source (*Figure 5*, Panels C and D).

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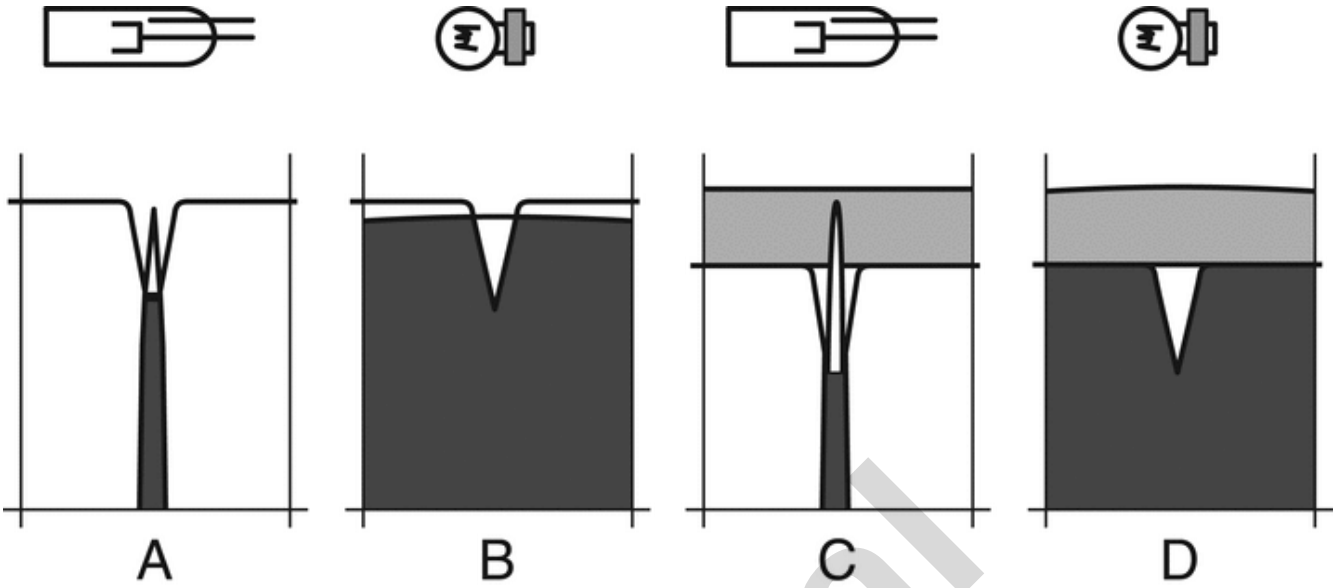


Figure 5. Panels A and B: atomic absorption only. Panels C and D: atomic absorption with background absorption. Reproduced with permission of the Royal Society of Chemistry.

Although continuum-source background correction is a useful design, it has some limitations. Basically, the introduction of an additional lamp and chopper causes a degradation of the overall signal-to-noise ratio of measurements. In addition, if the line source and the continuum source are not in perfect alignment, an erroneous correction will result when the individual beams are passed through an inhomogeneous gaseous sample, as this frequently occurs in the sample cell. This is particularly the case for highly structured backgrounds. The radiant intensity of the deuterium source in the visible region is too low to use for analytical wavelengths above 350 nm. Some instrument manufacturers equip their spectrophotometers with alternative continuum sources, such as tungsten halogen lamps, to remedy this issue.

## Smith–Hieftje

When high currents are applied to an HCL, the emission-line profile is altered: the line is broadened and a dip appears in the center because of self-absorption. Self-absorption occurs when the photons emitted by excited atoms are absorbed by gaseous ground-state atoms contained within the quartz tube of the HCL. At currents used for normal data acquisition, the HCL is used to measure the sum absorbance of the element of interest and the nonspecific background. At high lamp currents, the measured absorbance is predominantly caused by the background. The absorbance caused by the element of interest is the difference between the intensities of analyte and nonspecific background absorbances. *Figure 6* illustrates the Smith–Hieftje background correction process.

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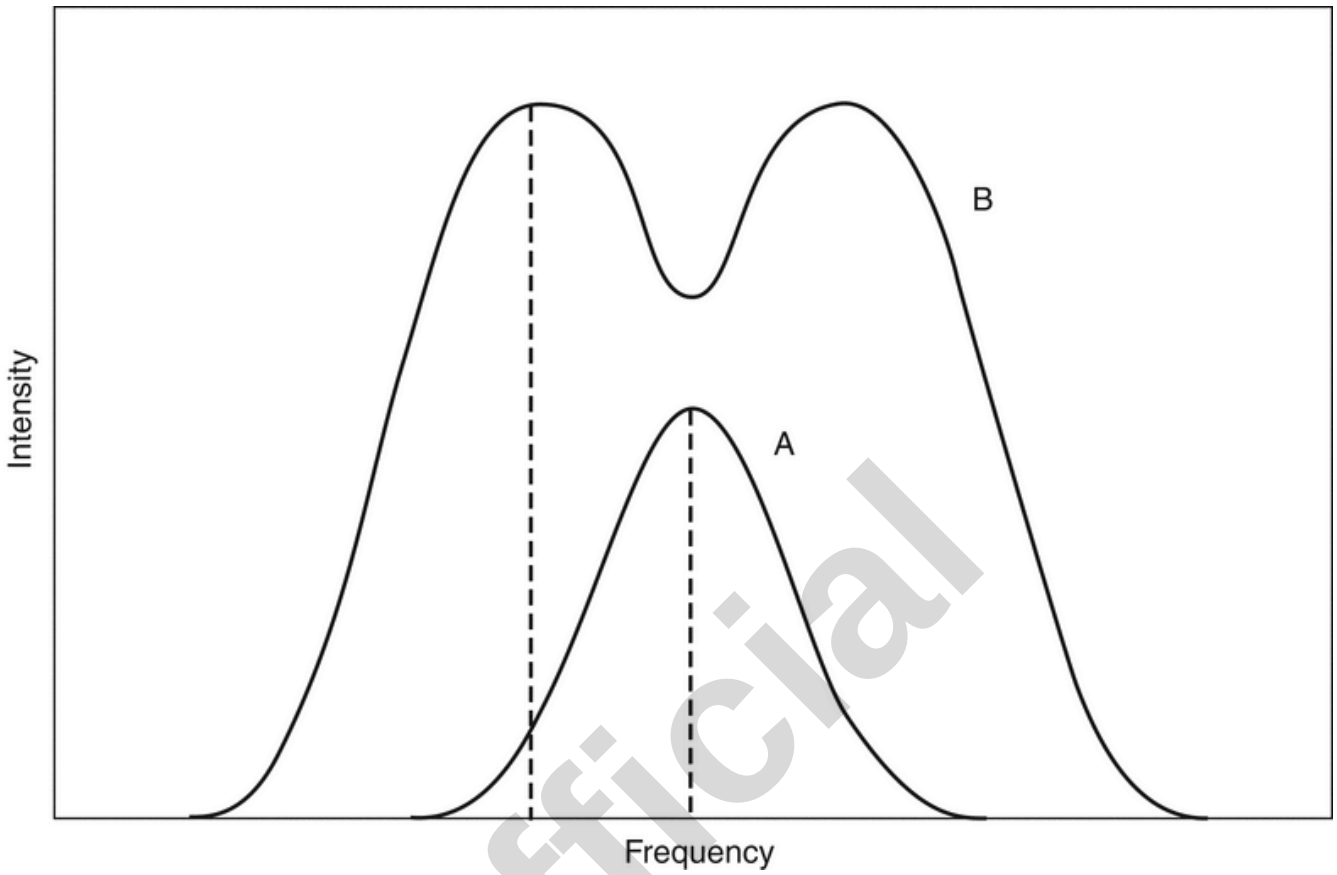


Figure 6. Emission profile of an HCL at (A) low current and (B) high current. Low current absorbance is caused by background plus analyte. High current is background absorbance only. Adapted from S.B. Smith, Jr. and G.M. Hieftje, *Applied Spectroscopy*, 1983, 37, 419.

The advantages of Smith–Hieftje background correction are that the HCL serves as the single source for any analytical wavelength, obviating any alignment issues arising from alternative continuum source. This system allows correction of structured backgrounds very near the resonance wavelength. Disadvantages are that the lifetimes of the HCLs are notably shortened. Compared with continuum-source background correction, the Smith–Hieftje procedure may result in a shorter dynamic range and/or may require higher sample dilutions to reduce background absorbances.

## Zeeman Effect

The Zeeman effect background correction is based on the principle that in the presence of a magnetic field, the absorption line of an element is split into three optically polarized components. The π component is positioned at the center of the resonance line. Positioned at equal distances on either side of the π component are the σ+ and σ– components. The σ components are linearly polarized perpendicular to the magnetic field. The distance of the separation of the σ+ and σ– components from the π component increases as the magnetic field strength increases. Commercial instruments use magnets with field strengths of approximately 1 Tesla, which corresponds to a σ component separation of approximately 0.01 nm on each side of the π component.

Absorbance lines from different elements have normal or anomalous Zeeman patterns. Normal Zeeman patterns are composed of a single π component and two σ components. A normal Zeeman pattern occurs for absorbance wavelengths that correspond to a singlet electron transition. An anomalous Zeeman pattern has further splitting of the π and σ components and occurs for wavelengths that correspond to more complex electron transitions.

Three basic designs are available for commercial AAS equipped with Zeeman background correction: transverse DC Zeeman AAS, transverse AC Zeeman AAS, and longitudinal AC Zeeman AAS.

### TRANSVERSE DC ZEEMAN ATOMIC ABSORPTION SPECTROMETRY

This design uses a DC magnet positioned around the atomizer. The term *transverse* refers to the parallel alignment of the magnetic field with the optical axis. The Zeeman effect is permanently applied in this design. A rotating polarizer is positioned in the light path between the line source and the atomizer. This splits the incident radiation from the line source into the characteristic π and σ components. See *Figure 7* for a schematic of the configuration and an illustration of the operation of DC Zeeman AAS.

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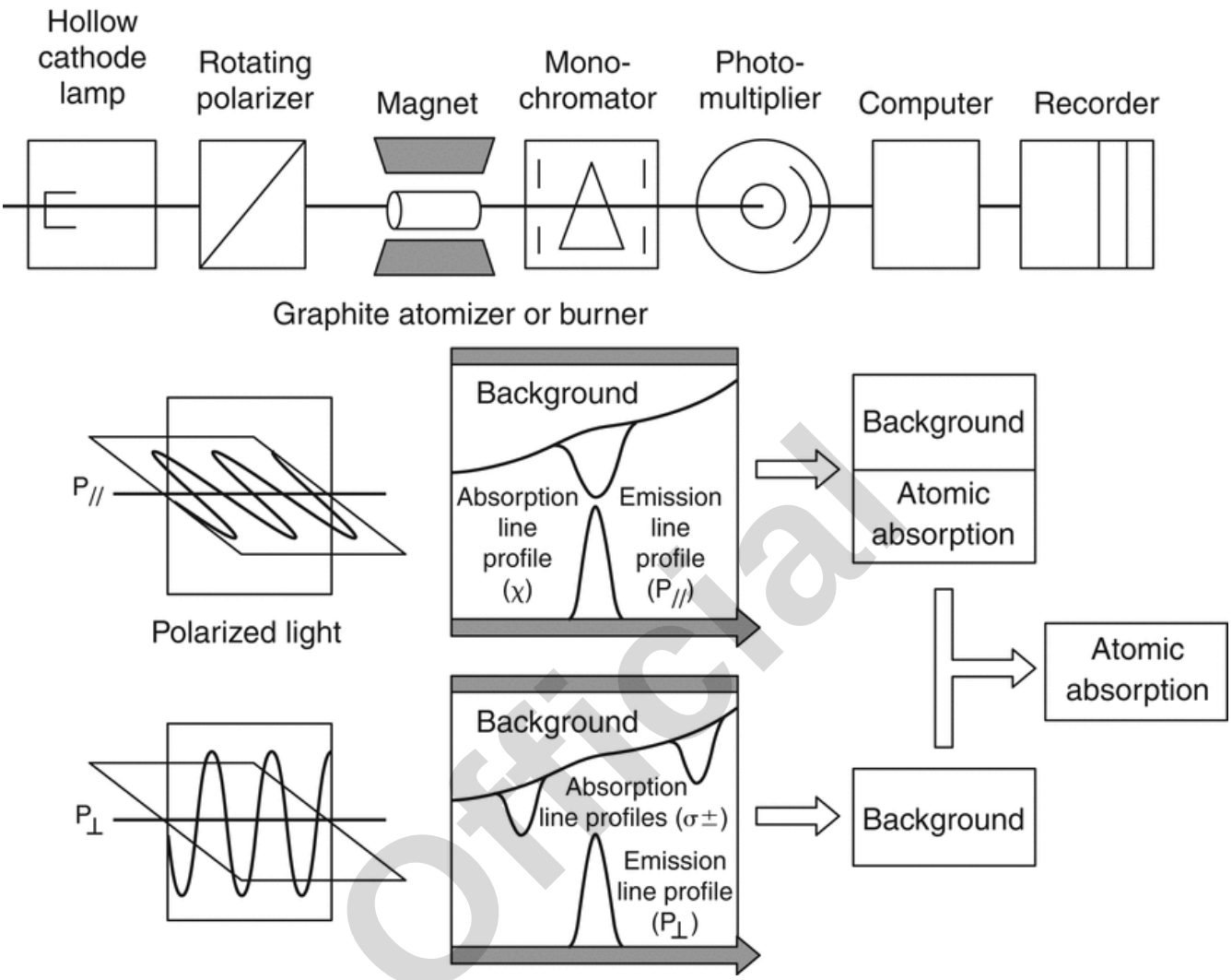


Figure 7. Configuration and operation of a transverse DC Zeeman atomic absorption spectrophotometer. Adapted from H. Koizumi and K. Yasuda, *Spectrochimica Acta*, 1976, 31B, 523.

## TRANSVERSE AC ZEEMAN ATOMIC ABSORPTION SPECTROMETRY

This design uses an AC magnet positioned around the atomizer. The magnetic field is in parallel alignment with the optical axis. The AC magnet is switched on and off at one-half the modulation frequency of the line source (50 or 60 Hz, typically). A fixed polarizer is positioned between the atomizer and the monochromator. See *Figure 8* and *Figure 9* for illustrations of the operation of transverse AC Zeeman AAS.

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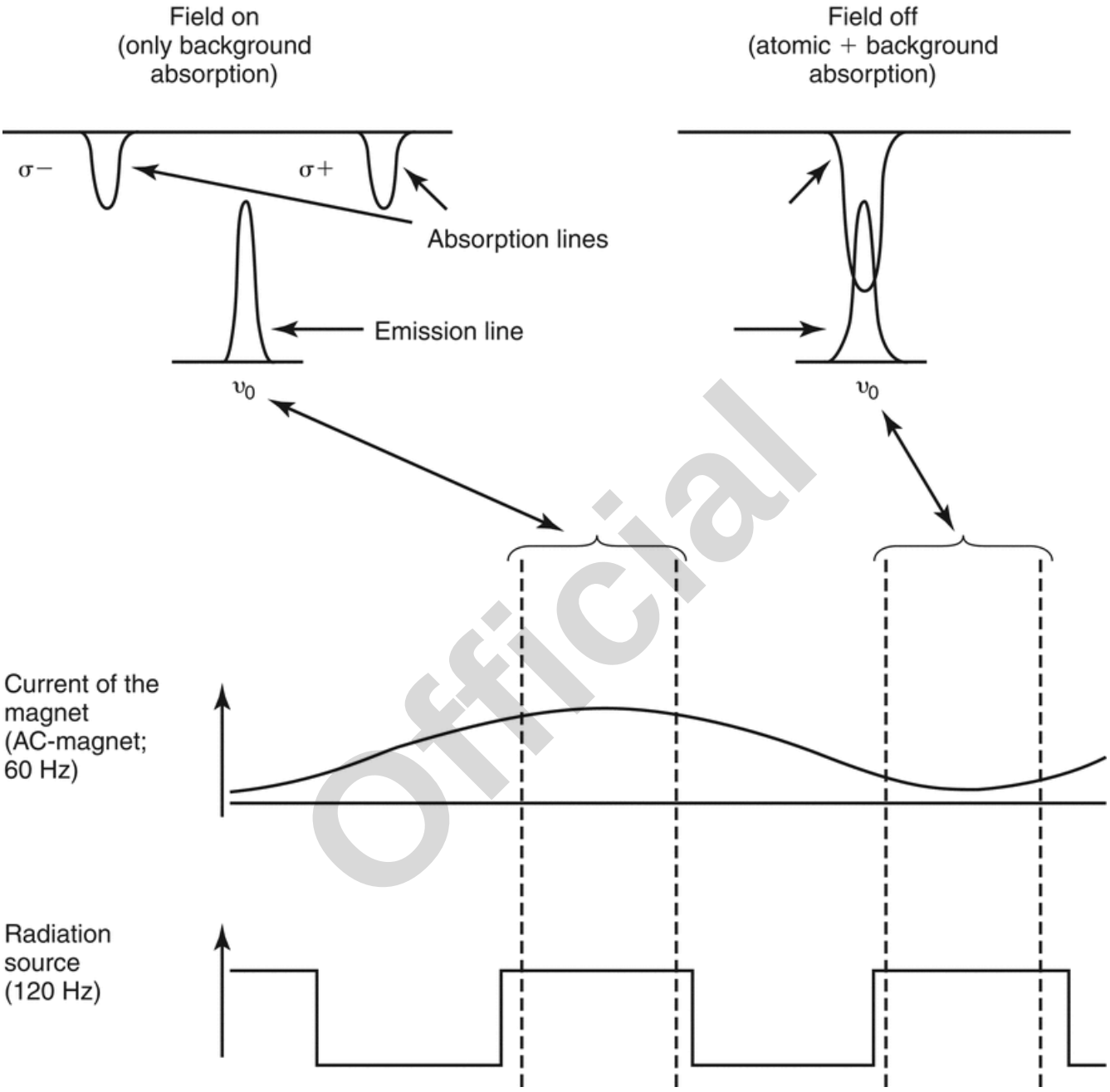


Figure 8. Operational principle of a transverse AC Zeeman atomic absorption spectrophotometer. Printed with permission of PerkinElmer, Inc. All rights reserved.

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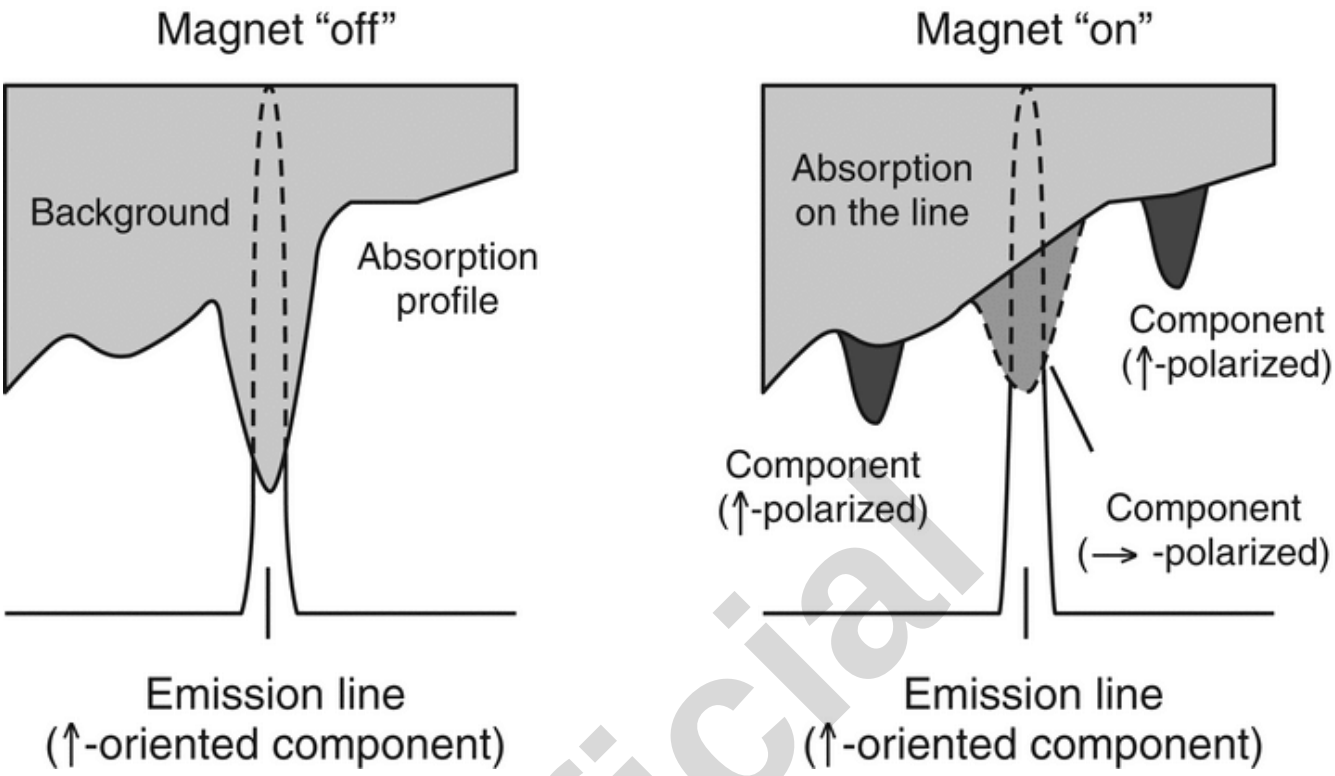


Figure 9. Illustration of transverse AC Zeeman atomic absorption spectrophotometer operation. Printed with permission of PerkinElmer, Inc. All rights reserved.

## LONGITUDINAL AC ZEEMAN ATOMIC ABSORPTION SPECTROMETRY

In this design, the magnetic field is in perpendicular alignment with the optical axis. With this configuration the π component is absent and the σ components are circularly polarized, so a polarizer is not required. See *Figure 10* for comparison between transverse and longitudinal AC Zeeman AAS.

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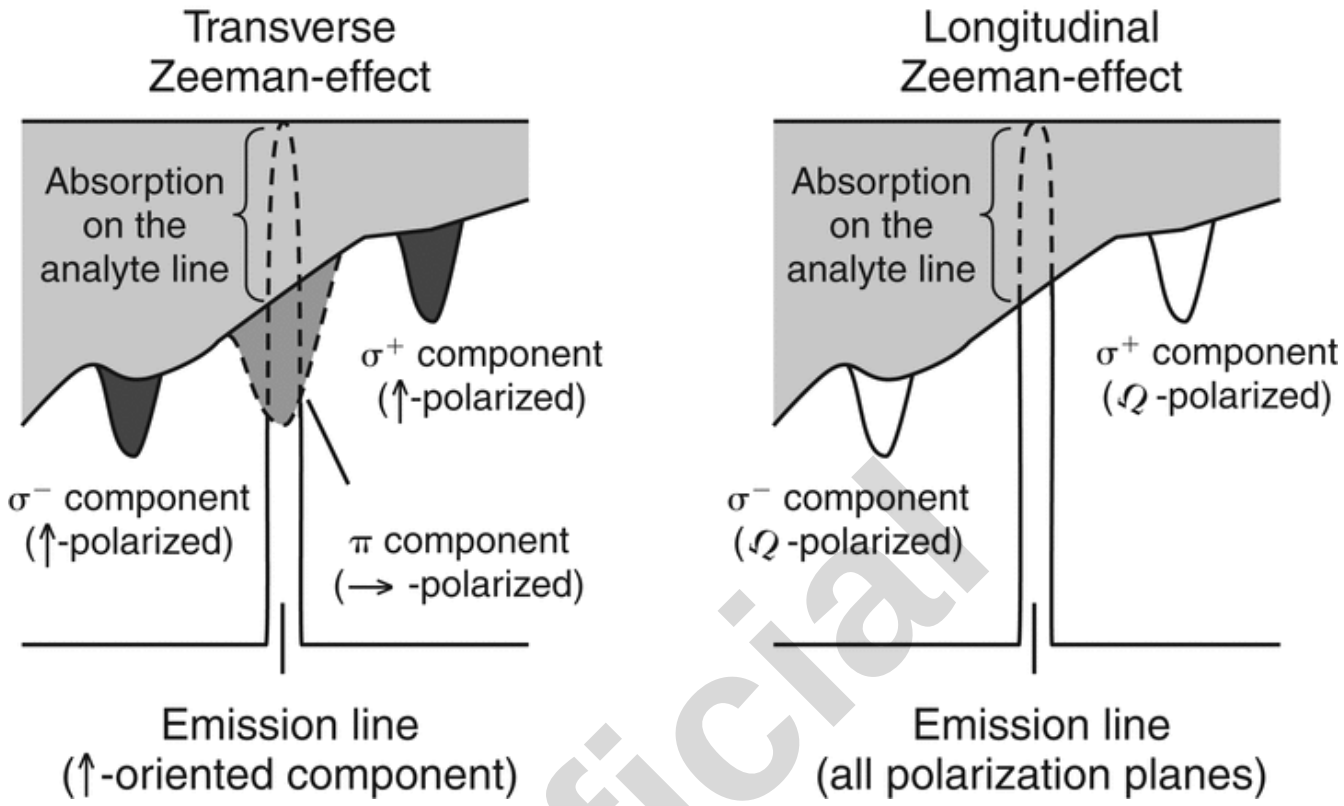


Figure 10. Comparison of transverse and longitudinal AC Zeeman atomic absorption spectrophotometer. Printed with permission of PerkinElmer, Inc. All rights reserved.

Zeeman effect background correction offers the advantage of background correction at the resonance wavelength and correction of relatively high nonspecific absorbances. Compared to continuum source background correction, Zeeman effect background correction can result in a reduction of the linear dynamic range in some applications and may require additional dilution of test solutions. Depending on the specific analyte and application, Zeeman effect background correction offers advantages and disadvantages that must be considered for each application.

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## ANALYTICAL CONSIDERATIONS

### Sample Preparation

FAAS requires the introduction of a liquid sample into the nebulizer. ETVAAS/GFAAS is normally performed using liquid samples, but analyses can be performed using slurries and solid samples. Because the analysis of liquid slurries is far more common, the analysis of solid samples is not discussed in this chapter.

Samples can be prepared in a variety of ways to dissolve or solubilize them. In some cases, direct dilution of a solid sample can be performed using deionized water, dilute acids, or organic solvents. When a sample does not dissolve, however, some form of acid digestion is required. Acid digestion is also required if the sample is first ashed. Options for acid digestion include hot-plate digestions, open-vessel microwave digestion, and closed-vessel microwave digestion. Procedures for acid digestion are specific for a given sample matrix and analyte. When using microwave digestion systems, analysts should refer to the microwave manufacturer's guidelines for use and programming of the instrument.

### Interferences

AAS is subject to several types of interferences. The most common are:

- Spectral interferences: Spectral interferences can arise when there is an overlapping signal from another element that is a component of the sample or sample matrix.
- Ionization of the analyte: Some elements such as sodium, potassium, calcium, and cesium are easily ionized, and ionization of the analyte reduces the analytical signal.
- Matrix effects: Interferences can arise from differences between sample, standard, and blank viscosities or can be introduced by surface tension.

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- Spectral line broadening: Spectral line broadening can occur as a result of several factors, including self-absorption, Lorentz effect, Doppler effect, or quenching.
- Compounds that do not dissociate in the flame.

## Matrix Modification, Releasing Agents, and Ionization Suppressants

To counteract potential interferences or to enhance their ability to monitor an analyte, analysts sometimes use a matrix modifier, a releasing agent, or an ionization suppressant.

Matrix modifiers are added to samples, standards, and blanks with GFAAS with the chief goals of changing the nature of the sample or analyte in the sample by:

- Increasing the volatility of the sample matrix so that matrix components are removed during the ashing or pyrolysis step
- Reducing the volatility of the analyte, which helps to eliminate loss of the analyte during the ashing or pyrolysis step
- Reducing background absorption by eliminating matrix components so that they do not interfere with the analyte signal during atomization.

It may be necessary to use more than one matrix modifier during an analysis. Commonly used matrix modifiers include magnesium nitrate, nickel nitrate, palladium, and lanthanum. Manufacturers of graphite furnace instrumentation provide detailed information regarding the use of matrix modifiers, and analysts should consult that information when they perform an analysis. In some instances, GFAAS analyses require applying the method of standard additions in order to overcome matrix induced result biases. Many modern GFAAS systems are equipped with autosamplers that automatically spike sample solutions at multiple levels specified by the analyst, perform the linear regression, and report the final concentration result based upon the intercept.

Releasing agents and ionization suppressants are used in FAAS to eliminate certain potential interferences. Releasing agents are added in excess to samples, standards, and blanks to prevent the formation of refractory compounds by combining with a potential interferent. Ionization suppressants are added in excess to samples, standards, and blanks to help control ionization of the analyte. By adding an ionization suppressant that has an ionization potential lower than that of the analyte, analysts create an excess of electrons in the flame, and the ionization of the analyte is suppressed. Commonly used releasing agents include lanthanum and strontium. Commonly used ionization suppressants include sodium, potassium, cesium, and lanthanum. Manufacturers of flame atomic absorption (AA) instrumentation provide detailed information regarding the use of releasing agents and ionization suppressants, and analysts should consult that information when performing an analysis.

Because of the separation of the analyte from the matrix in cold-vapor and hydride-generation AA, spectral interferences are notably reduced when compared to the other AA methods. Nonselective background interferences can occur, however, when a sufficient amount of an absorbing species is transported to the cell. Typically this occurs when an excess of another hydride-forming species is introduced from the sample matrix or in the case of hydride formation when nonaqueous media are used. Transport interferences take place during transport of a hydride from the sample solution to the sample cell. This either causes a delay in hydride formation, known as transport kinetics interference, or loss of the hydride altogether, known as transport efficiency interference. Volatile compounds transported to the cell with the hydride can also interfere nonselectively. These interferences can be remedied either by using the method of standard additions or by further diluting the sample.

## ▲Method of Standard Additions

For the validation of procedures that use the method of standard additions, it is important to consider the appropriate standard addition concentrations applied to the linear regression and the impact on the associated intercept that defines the test solution concentration. It is important to consider not only how well correlated the linear regression is determined to be via the correlation coefficient or the coefficient of determination calculations but also the error of each measurement at each standard addition concentration. If, for example, standard addition concentrations are significantly higher than the targeted sample concentration, the regression line is likely to be very highly correlated but will carry a large bias error at the intercept. It is for this reason that the accuracy validation parameter for a method using the method of standard additions applies to the recovery of a spiked sample determined by the intersection of the concentration axis by the standard addition regression line. The same practice also applies to the system suitability standard measurement. This process is simplified by manufacturers of modern AA spectrometers, which provide for an automated method of standard addition analyses.▲ (USP 1-Aug-2022)

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## ▲APPENDIX

### Acronyms

A: absorption
AA: atomic absorption
AAS: atomic absorption spectrometry
CVAAS: cold vapor AAS
EDL: electrodeless discharge lamp
ETV: electrothermal vaporization
FAAS: flame AAS
GFAAS: graphite furnace AAS
HCL: hollow cathode lamp

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HGAAS: hydride generation AAS
PMT: photomultiplier tube
T: transmittance▲ (USP 1-Aug-2022)

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## ▲ADDITIONAL SOURCES OF INFORMATION▲ (USP 1-AUG-2022)

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# ⟨1853⟩ FLUORESCENCE SPECTROSCOPY—THEORY AND PRACTICE

## THEORY

Fluorescence is a two-step process that requires an initial absorption of light followed by emission. [NOTE—Many terms and variables used in this general chapter are explained in the *Glossary*.] Fluorescence spectroscopy is an electronic spectroscopic method related to ultraviolet–visible–near infrared (UV–Vis–NIR) absorption spectroscopy. It is also a background-free method that involves light emitted from the sample in all directions, as is the case with Raman spectroscopy. The initial absorption of a photon by a molecule in the sample promotes an electron to an excited state. The excited electron returns to the ground electronic state by emitting a photon. If the emission arises from an "allowed" transition that typically has a short lifetime between 1 ns and 10 ns, then it is called *fluorescence*. If the emission arises from a "forbidden" transition that typically has a long lifetime between 1 ms and 1 s, then it is called *phosphorescence*. Under similar conditions phosphorescence usually is less intense than fluorescence. This general chapter discusses fluorescence spectroscopy, but many points raised here also apply to phosphorescence. The basic concepts behind fluorescence spectroscopy have been well established, but its applications and standardization are still expanding and progressing, making it a developing rather than a mature method.

The most common type of fluorescent sample is a dilute, transparent solution that absorbs light following the Beer–Lambert Law and that emits a corresponding fluorescence intensity that is directly proportional to the concentration, the absorptivity, and the fluorescence quantum yield of the fluorescent species or fluorophore. A conventional fluorescence spectrometer has both excitation and emission wavelength selectors. It collects a spectrum by fixing the wavelength of one of the selectors and scanning the other wavelength selector over a range. When the excitation wavelength is fixed and the emission wavelength is scanned, the resulting spectrum is termed an *emission spectrum*. When the emission wavelength is fixed and the excitation wavelength is scanned, the resulting spectrum is termed an *excitation spectrum* (*Figure 1*). The fluorescence spectrum is plotted as relative intensity or counted photons of fluorescence vs. wavelength. The appearance of a fluorescence spectrum is much like a UV–Vis–NIR absorption spectrum. In fact, the shape or contour of an excitation spectrum often is identical to that of the corresponding absorption spectrum for an organic dye in solution over the same wavelength range.

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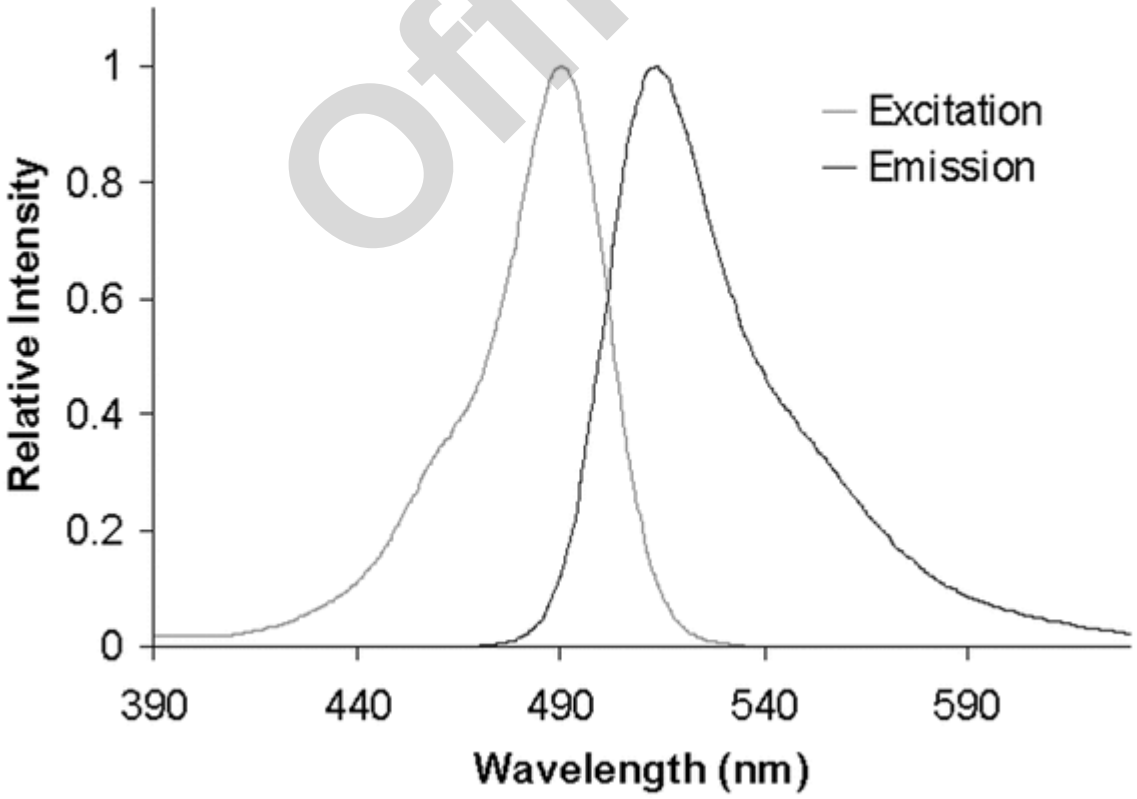


Figure 1. Fluorescence excitation and emission spectra for fluorescein in borate buffer. The wavelength axis shows excitation and emission wavelengths.

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Polyatomic fluorophores in condensed media (e.g., solutions, thin films, and solids at room temperature) exist in ground or excited electronic states in a broad distribution of vibrational energy levels and cause homogeneous broadening of excitation or emission spectra, respectively. A microenvironment or shell also surrounds each fluorophore in condensed media, and differences in the structures of these shells among individual fluorophores cause inhomogeneous broadening. These two types of broadening cause fluorescence spectra to be broader than some other types of spectra (e.g., mid-infrared or Raman spectra). The typical width of a fluorescence band is between 10 nm and 100 nm. Once it is electronically excited, a polyatomic fluorophore experiences vibrational relaxation before emitting a photon, causing a red shift or Stokes shift of the fluorescence spectrum relative to the wavelength at which it was excited.

Few naturally occurring biological compounds fluoresce strongly. However, the development of a large array of fluorescent indicator dyes, used mainly to bind to ions or indicate pH or polarity, has led to increased interest in the use of both direct and secondary fluorescence techniques, e.g., fluorescence resonance energy transfer (FRET). While many of these probes inherently do not have very high quantum yields, their fluorescence changes greatly upon binding and upon the associated solution chemistry. For instance, the pH of the sample solution is an important factor to control when considering not only its impact on the fluorescence of the probe, but also on the detection of other possible fluorescing of fluorescent dye binding species. Some fluorescent probes, such as fluorescein (pH dependent) and rhodamine and its derivatives, have very large absorptivities and quantum yields approaching one—i.e., they fluoresce nearly as many photons as they absorb. Fluorescence methods are also termed *background-free* because very little excitation light reaches the detector. These advantages make fluorescence detection highly sensitive, down to single-molecule detection in some cases. Specificity and sensitivity are two of the more important strengths of fluorescence methods. Fluorescence spectroscopy also typically is not destructive to the sample, and measurements can be made quickly (on the order of seconds to minutes).

A right-angle or 0°/90° geometry often is used to measure dilute solutions and other transparent samples. In such cases, the excitation beam is normal to the sample, and fluorescence is detected at a 90° angle relative to the beam (*Figure 2a*). A front-face geometry is used to measure optically dense samples where the excitation beam is incident on the sample at <90° and the fluorescence is collected at an angle ≤90° (*Figure 2b*). The epifluorescence geometry is a special case of the front-face geometry that often is used in fluorescence microscopy and optical fiber–based fluorometers. In epifluorescence geometry the excitation beam and collected fluorescence are both normal to and are on the same side of the sample, i.e., a 0°/0° geometry. A 0°/180° transmitting geometry often is used in microscopy (*Figure 2c*).

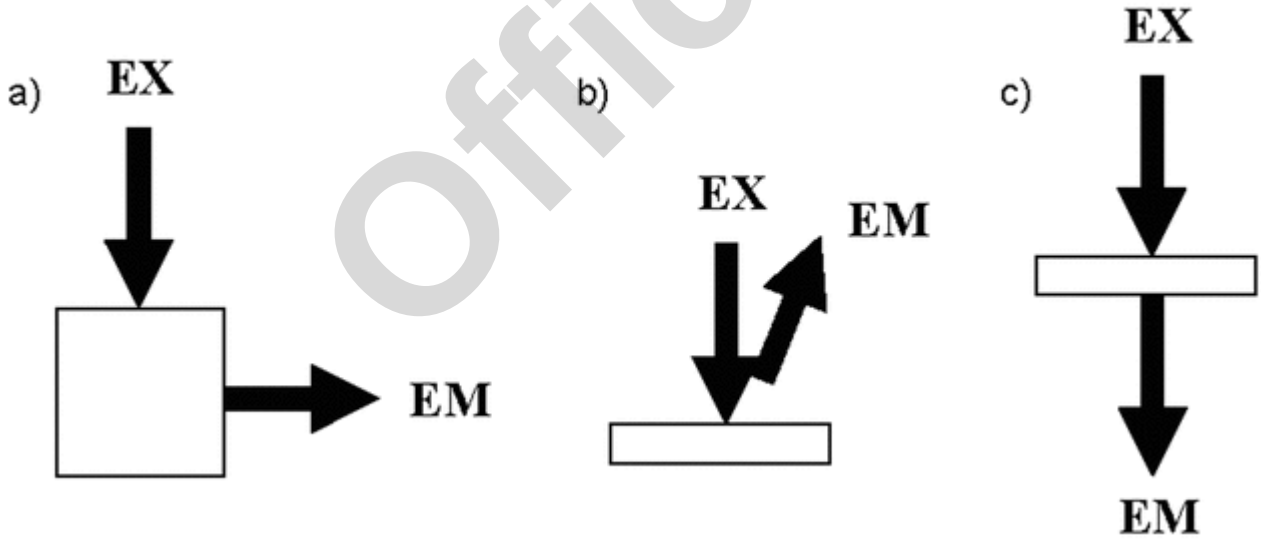


Figure 2. Schematic of the excitation beam (EX) and detected emission (EM) orientations for (a) 0°/90° right-angle transmitting, (b) front-face, and (c) 0°/180° transmitting geometries.

The number of chemical assays and screening methods using fluorescence detection continues to increase rapidly and has resulted in a corresponding increase in the need for standardization of fluorescence measurements. Only a few standard methods and reference materials have been well established and are readily available at present for the characterization of fluorescence measuring systems. National metrology institutes and international standards organizations are working to provide new fluorescence standard materials and methods in the near future. This general chapter briefly discusses the major issues that should be considered by users of fluorescence instruments who aim to achieve high-quality measurements. Standard methods and materials are also described where appropriate. A few guidelines and recommendations have appeared, but this general chapter aims to be most useful to nonexpert users of fluorescence spectrometers.

## INSTRUMENTATION

All modern fluorescence measurements involve irradiating the sample with the beam from a suitable light source, selecting the excitation wavelength, collecting the resulting fluorescence, rejecting the Rayleigh-scattered light, selecting the emission

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wavelength, and detecting the fluorescence signal. The following functions will be discussed individually, along with the equipment used to achieve these functions in commercial instruments:

1. excitation light source
2. excitation wavelength selector
3. sampling device
4. emission wavelength selector
5. detector.

### Excitation Light Source

A variety of lamps, lasers, and light-emitting diodes (LEDs) are used as excitation sources. Continuous and pulsed versions of these sources are used for steady-state and time-resolved instruments, respectively. Xenon lamps are the most commonly used because of their relatively high intensity and broad wavelength range (UV to NIR). Lasers are the highest-intensity sources and are used in applications where short collection times and small amounts of sample are required, e.g., for flow cytometry or microarrays.

### Excitation Wavelength Selector

The intensity of scattered light at the excitation wavelength (i.e., Rayleigh scattering) can be comparable to or greater than that of the fluorescence at the sample. Therefore, the excitation wavelength profile should not overlap the emission wavelength region being detected. This is achieved for lamps by using an excitation wavelength selector (e.g., a filter or a monochromator with a known peak transmission wavelength and bandwidth) between the lamp and sample. The inherent bandwidth of the radiation from a laser or an LED often is narrow enough that an excitation wavelength selector is not necessary. This selector also enables fluorescence excitation spectra to be resolved.

### Sampling Device

The sampling device includes all optics and other equipment needed to deliver the excitation beam to the sample, collect the emission from the sample, and hold the sample in place. Sample formats include cuvettes, microwell plates, microarrays, microscope slides, and flow systems and may be accompanied by a variety of optical delivery and collection systems, including conventional transmitting, front-face, and epifluorescence systems and fiber optic–based probes.

### Emission Wavelength Selector

As with the selector for excitation, the emission wavelength selector helps to ensure that the emission wavelength region being detected does not overlap with the excitation wavelength profile. This approach enables individual fluorescence bands to be detected when multiple bands are present and allows fluorescence emission spectra to be resolved. Emission wavelength selectors also are important for the rejection of stray light. Filters, monochromators, and grating polychromators often are used for emission wavelength selection.

### Detector

For the detection of emitted light, a photomultiplier tube (PMT) or a charge-coupled device (CCD) array is placed after the emission wavelength selector. The detection of the excitation beam in order to monitor its intensity commonly is done by a quantum counter detector or a photodiode placed before the sample and to which a small fraction of the excitation beam is split off from the rest.

## FACTORS THAT AFFECT QUANTITATION

### Instrument-Based Factors

Measurements on a fluorescence instrument require that instrument parameters such as wavelengths, bandwidths, and detector gain be set. All of these parameters can be set with varying degrees of repeatability and accuracy, depending on the instrument used. These factors can introduce measurement uncertainty or bias that is particularly significant when measured values are compared between instruments. For instance, the measured peak positions of the emission bands of two analytes may differ between instruments because of a wavelength bias. A corresponding bias between instruments could be introduced in the results of an assay that depends on the ratio of the fluorescence intensities at the two specified emission wavelengths.

The intensity of the excitation beam can change significantly with excitation wavelength or with time because of the wavelength dependence of the intensity of the light source and the transmittance of the excitation wavelength selector or the time dependence of the light source intensity. Thus analysts should monitor the excitation beam intensity and correct the measured fluorescence intensity for these fluctuations. This monitoring can be particularly important when excitation spectra are collected because the excitation intensity often has sharp peaks and valleys when lamp sources such as a xenon (Xe) lamp are used.

The responsivity of a detection system is not linear with intensity at all intensities, so analysts should know the linear intensity range of the detection system used. The linear range for most detection systems ranges from its limit of detection up to a

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threshold intensity above which the responsivity becomes increasingly nonlinear with increasing intensity. Analysts should establish the linear range of the fluorescence detection system before they attempt to calibrate the responsivity of the detection system.

The responsivity of the detection system also is a result of the wavelength dependence of the transmittance of the emission wavelength selector and the responsivity of the detector. These factors can affect the shape of a measured emission spectrum.

The diffraction efficiency of gratings and the responsivity of detectors often depend on polarization. Changes to instrumental polarization settings can result in changes in the observed excitation intensity and the responsivity of the detection system. Even when polarizers are not used within the instrument, the excitation beam may be polarized by the optical system itself and may affect the responsivity of the detection system, and is instrument dependent. In addition, emission polarization effects not only can cause intensity differences but also can change spectral correction factors.

The passing of multiple wavelengths by a diffraction grating can introduce unexpected sharp peaks into a fluorescence spectrum. So that incident light is diffracted at a desired wavelength, a grating equation is used to set the angle of the grating with respect to incident light:

$$m\lambda = d(\sin\alpha + \sin\beta),\ m = 0, 1, 2, \ldots$$

$m$ = diffraction order
$d$ = groove spacing on the grating
$\alpha$ = angle of the incident wavefront relative to the grating normal
$\beta$ = angle of the diffracted wavefront relative to the grating normal

The value of $m\lambda$, not $\lambda$, is fixed, where $m$ is an integer termed the *diffraction order*. Therefore, the grating equation can be satisfied by more than one wavelength for a single grating position. For instance, if a grating in an emission monochromator is set to pass 500-nm light at first order, it also will pass 250-nm light at second order. As a result, the scattered light from a 250-nm excitation beam will be detected as a peak at an emission wavelength of 500 nm unless a suitable optical filter is inserted in the beam.

## Sample-Based Factors

The fluorescence intensity of optically dense samples (e.g., absorbance $A$ >0.05 at a path length of 1 cm) does not increase linearly with concentration because of significant absorption of the excitation beam and/or emission (reabsorption) by the sample. These inner filter effects also can greatly reduce the amount of fluorescence that reaches the detector, especially when a right-angle transmitting geometry is used. The fluorescence intensity can become strongly dependent on sample position and optical geometry. At even higher concentrations, aggregation of fluorophores often occurs, causing the shape of the fluorescence spectrum to be different from that of a dilute sample and also causing nonlinear concentration behavior.

The fluorescence intensity of a sample may decrease with time of exposure to light because of photobleaching and photodegradation. This is particularly true of most organic dyes, which are the most widely used fluorescent probes. Analysts should limit the time that such samples are exposed to light in order to obtain reproducible fluorescence intensities and in some cases even reproducible spectral shapes.

The fluorescence intensity of fluorophores is temperature dependent. Typically, the rates of fluorescence quenching processes, such as collisional quenching in solutions, increase with temperature and cause a decrease in fluorescence intensity. Temperature coefficients for fluorescence intensity for particular fluorophores can be used to correct for this temperature dependence.

The absorbance and consequently the intensity of fluorescence from a sample depend on the orientation of the sample's absorption transition dipole with respect to the polarization of the excitation light. The polarization of fluorescence is parallel to the direction of polarization of the fluorescent species' emission transition dipole. Fluorescence polarization is parallel to the orientation of the fluorescent species' emission transition dipole. Fluorescence anisotropy ($r$) is used to describe the extent of polarization of emission and is defined by:

$$r = (I_{\parallel} - I_{\perp})/(I_{\parallel} + 2I_{\perp})$$

$I_{\parallel}$ = observed fluorescence intensity when the fluorometer's emission polarizer is oriented parallel to the direction of the polarized excitation
$I_{\perp}$ = observed fluorescence intensity when the fluorometer's emission polarizer is oriented perpendicular to the direction of the polarized excitation

A sample whose fluorophores are oriented nonrandomly and have a rotational period that is long compared to their fluorescence lifetime will emit anisotropic fluorescence. The spectral shape and intensity of such fluorescence depend on the viewing angle and the instrument's polarization factors.

A fluorophore's fluorescence intensity and peak position, and sometimes even its spectral shape, often depend on the environment, including changes caused by the solvent used, the solution's pH, or the species to which the fluorophore is bound. For the above reasons care should be taken during the experimental design of a new methodology to consider and evaluate these effects, and matrix match the reference and unknown as appropriate.

A Raman signal can introduce peaks into the fluorescence spectrum. The Raman peaks of the sample's solvent or matrix are those most commonly encountered. For instance, the Raman peak of water, which is found red-shifted 3382 $cm^{-1}$ from the chosen excitation wavelength, typically is observed in the fluorescence spectrum of any aqueous solution excited by UV or blue light.

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# CALIBRATION OF FLUORESCENCE INSTRUMENTS

Two types of fluorescence instrument calibrations are used. The first and most commonly used is analyte specific and determines the relationship between the response of the instrument (fluorescence intensity) and the concentration or amount of a specific analyte. The second is analyte independent and is intended for spectral instruments. In this case, the wavelength accuracy for emission and excitation and the spectral responsivity of the detection system are calibrated across the entire or a continuous part of the wavelength range of the instrument.

## Analyte Concentration—Calibration Curves

Calibration curves of instrument response (fluorescence intensity) vs. analyte concentration are determined using reference materials that contain the analyte of interest. For instance, the fluorescence intensities of a set of solutions at different, known analyte concentrations that cover a desirable concentration range can be measured and plotted vs. concentration. The plot then is fitted to a polynomial, typically a straight line. The resulting calibration curve is both analyte and instrument specific and can be used to determine analyte concentrations of unknown samples. This type of calibration may not be accurate when the microenvironment surrounding the fluorophore is different in the reference and unknown samples. In addition, users must ensure that the fluorescence intensities of samples are reproducible and do not decrease over the time when they are being excited and measured because the organic dyes typically used may be prone to photobleaching.

In some cases, calibration samples at known concentrations and prepared from appropriate reference materials may not be available. Firstly, organic dyes that are used as fluorescent probes often are not commercially available at a known high purity that enables production of reference solutions. Secondly, in complex systems where fluorophores are bound to large molecules, cells, or microbeads, the concentration of bound fluorophores in a solution or suspension may be difficult to determine. In the latter case, the molecules of equivalent soluble fluorophore (MESF) scale has been proposed as an alternative way to use calibration curves to quantify fluorescence intensity for a particular analyte.

## Emission Wavelength and Spectral Slit Width

A variety of reference standards have been proposed for use in the determination of emission wavelength accuracy, including atomic lamps and inorganic and organic fluorophores. The most widely used and best characterized of these are low-pressure atomic lamps, commonly termed *pen lamps.* In this case, the type of pen lamp (e.g., Hg, Xe, etc.) is chosen so that its radiated atomic lines are within the desired wavelength range. The lamp is placed at the sample position so that its light is centered in the optical path of the detection system of the instrument. The accuracy of this method may decrease if the pen lamp is not properly aligned. The emission wavelength selector–detector then measures the signal over the wavelength range of interest. The measured wavelength positions of the resulting sharp peaks then are compared with the known positions to determine wavelength accuracy.

The spectral slit width accuracy of the emission wavelength selector can be confirmed by measuring the spectral bandwidth, taken to be the full width at half the peak maximum, of a single line of a pen lamp. For fluorescence spectrometers with both excitation and emission monochromators, an alternative method can be used when one monochromator is scanned over the position of the other.

## Excitation Wavelength and Spectral Slit Width

Many of the reference samples that are used for determining emission wavelength accuracy also can be used for excitation wavelength accuracy. For instance, a pen lamp can be placed at the excitation light source position so that the resulting spectrum is detected after the excitation wavelength selector using the instrument's reference detector. However, in this case, a relatively weak signal may limit the number of useful atomic lines, and therefore alignment of the pen lamp is more critical in this instance than for the emission wavelength accuracy determination.

Once the accuracy of the emission wavelength selector has been determined, use a diffuse scatterer, e.g., a scattering solution or a diffuse reflector, at the sample to scatter a fraction of the excitation beam into the detection system to determine excitation wavelength accuracy. One wavelength selector is set at a fixed wavelength and the other is tuned over the same wavelength to obtain a spectrum. The wavelength bias between the two wavelength selectors is equal to the difference between the set wavelength position and the observed peak position of the collected spectrum. This method can be used at any wavelength, unlike many other methods that depend on a limited number of set excitation wavelengths determined by the reference material chosen. Methods similar to those used for spectral slit width accuracy of the emission wavelength selector also can be used to determine the spectral slit width accuracy of the excitation wavelength selector.

## Linearity of the Detection System

Several approaches are available to determine the detection system's linear intensity range. They can be separated into three types, based on the tools used to vary the intensity of light that reaches the detector: (1) double aperture, (2) optical filters and/or polarizers, and (3) fluorophore concentrations. The double-aperture method is the best established and probably is the most accurate when done correctly, but it also is the most difficult to perform. A variety of methods using optical filters, polarizers, or a combination of the two have been reported. These methods require high-quality, often costly, components and some user expertise. The third method is the most popular and is easiest. It uses a set of solutions obtained by serial dilution of a fluorescent stock solution that is similar to one used for obtaining calibration curves for analyte concentration, as described earlier. In this case, analysts use solutions with low concentration ($A$ <0.05 at 1-cm path length), but fluorophore adsorption to cuvette walls may affect measurements at very low concentrations. Users must ensure that the fluorescence intensities of

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samples are reproducible and do not decrease over the time that they are being excited and measured because the organic dyes typically used may be prone to photobleaching.

## Signal Level (Relative Emission)

Calibration of the relative responsivity of the emission detection system with emission wavelength, also referred to as spectral correction of emission, is necessary for successful quantification when intensity ratios at different emission wavelengths are compared or when the true shape or peak maximum position of an emission spectrum must be known. Such a calibration is required because the relative spectral responsivity of a detection system can change significantly over its wavelength range (*Figure 3*). Analysts should know the degree of photometric precision required for successful quantitation. The linear range of the detection system is determined before this calibration is performed, so that appropriate steps are taken (e.g., the use of attenuators) to ensure that all intensities measured during this calibration are within the linear range. When one uses an emission polarizer, the spectral correction for emission depends on the polarizer setting.

Two methods are preferred for calibrating photometric responsivity: one (*Method A*) uses light from a calibrated source (CS), and the other (*Method B*) uses certified reference materials (CRMs). Both give results that are traceable to national metrology institutes. A calibrated tungsten white light source is used most commonly for *Method A* and covers the wavelength range from about 350 nm into the NIR. Standard reference materials from the U.S. National Institute of Standards and Technology and CRMs from the German Federal Institute for Materials Research and Testing currently are available for use in *Method B*. Corrected emission spectra of some commonly used dyes also have been reported in the literature. *Method A* is more difficult to implement than *Method B* and requires periodic recertification of the CS. A third method, *Method C*, uses a calibrated detector and a calibrated diffuse reflector. This method typically has larger uncertainties than *Method A* and *Method B* but is recommended in UV and NIR wavelength regions that are not covered by the other two methods.

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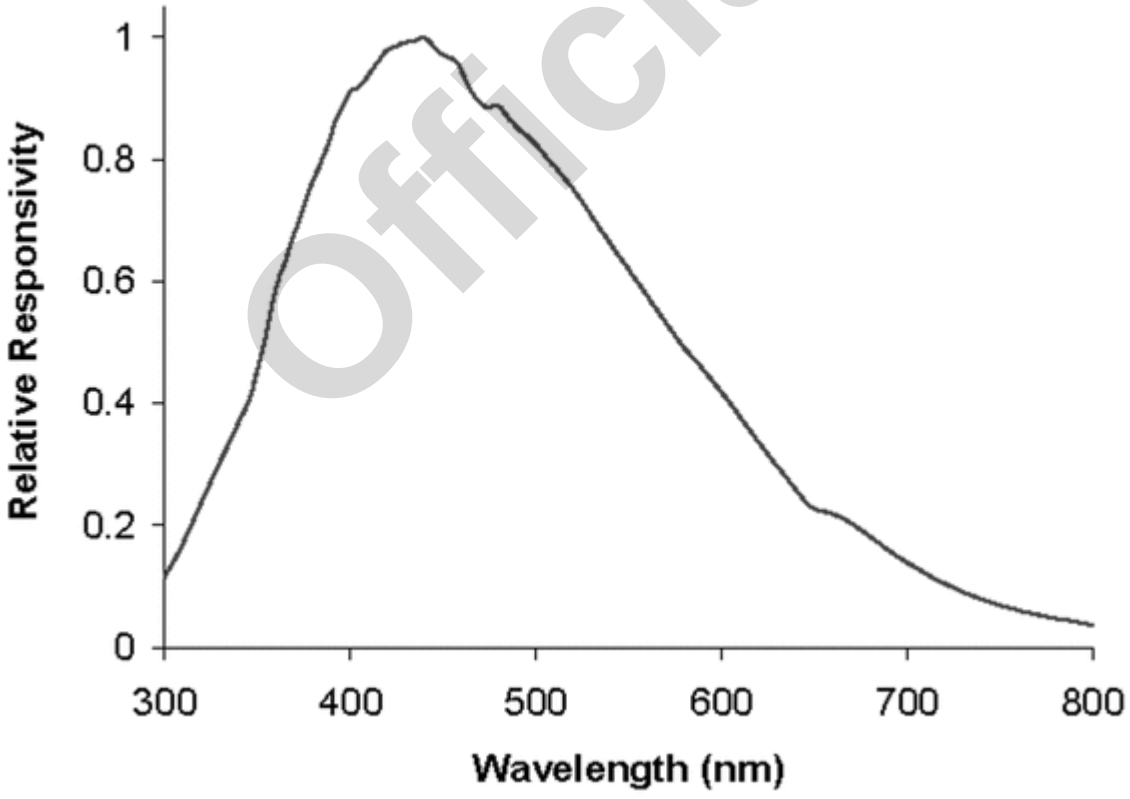


Figure 3. Example of the relative spectral responsivity of an emission detection system (PMT-based grating monochromator) for which a correction must be applied to a measured emission spectrum to obtain the true spectral shape (relative intensities).

### METHOD A

In *Method A*, analysts direct CS light into the emission detection system by placing the CS at the sample position. If the CS is too large to be placed at the sample position, analysts can place a calibrated diffuse reflector (CR) at the sample position to reflect the light from the CS into the emission detection system. The emission wavelength selector is scanned over the emission region of interest using the same instrument settings as used with the sample, and the signal channel output ($S'$) is collected.

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The known radiance of the CS incident on the detection system ($L$) can be used to calculate the relative correction factor ($C_{CS}$) so that:

$$C_{CS} = L/S''$$

$C_{CS}$ = relative correction factor
$L$ = radiance of the CS incident on the detection system
$S''$ = signal channel output

The corrected emission intensity is equal to the product of the signal output of the sample and $C_{CS}$.

METHOD B

In *Method B*, analysts place the fluorescence standard at the sample position. Its spectrum is collected and is compared to the certified spectrum according to the instructions given on the accompanying certificate, which yields spectral correction factors for the instrument.

METHOD C

*Method C* involves two steps: Step 1 uses a calibrated detector (CD) at the sample position to measure the flux of the excitation beam as a function of excitation wavelength. Step 2 uses a CR to reflect a known fraction of the flux of the excitation beam into the detection system. This is done by placing the CD at the sample position at a 45° angle, assuming a 0°/90° instrument geometry, and synchronously scanning both the excitation and emission wavelength selectors over the emission region of interest while collecting both the signal output and the reference output. This method allows analysts to calculate the relative correction factor. This method has larger uncertainties than those for *Method A* or *Method B* and typically is more difficult to implement.

## Reference Signal Level (Relative Excitation)

Calibration of the excitation intensity with excitation wavelength is necessary for successful quantitation when analysts compare intensity ratios at different excitation wavelengths or when analysts must know the true shape or peak maximum position of an excitation spectrum. Such a calibration is necessary because the relative spectral flux of an excitation beam at the sample can change extensively over its wavelength range (see *Figure 4*). The neglect of excitation intensity correction factors can cause even greater errors than neglect of emission correction factors. Fortunately, many fluorescence instruments have a built-in reference detection system to monitor the intensity of the excitation beam. This monitoring usually is done using a photodiode, PMT, CCD, or a quantum counter detector to measure a fraction of the excitation beam that is split off from the rest of the beam. The collected reference signal can be used to correct the fluorescence signal for fluctuations caused by changes in the excitation beam's intensity. Reference detectors often are not calibrated with excitation wavelength, which introduces errors that can be particularly large over longer excitation wavelength ranges (e.g., >50 nm) or in a wavelength region where the excitation intensity changes rapidly with excitation wavelength (e.g., the UV range). When an excitation polarizer is used, the spectral correction for excitation intensity depends on the polarizer setting.

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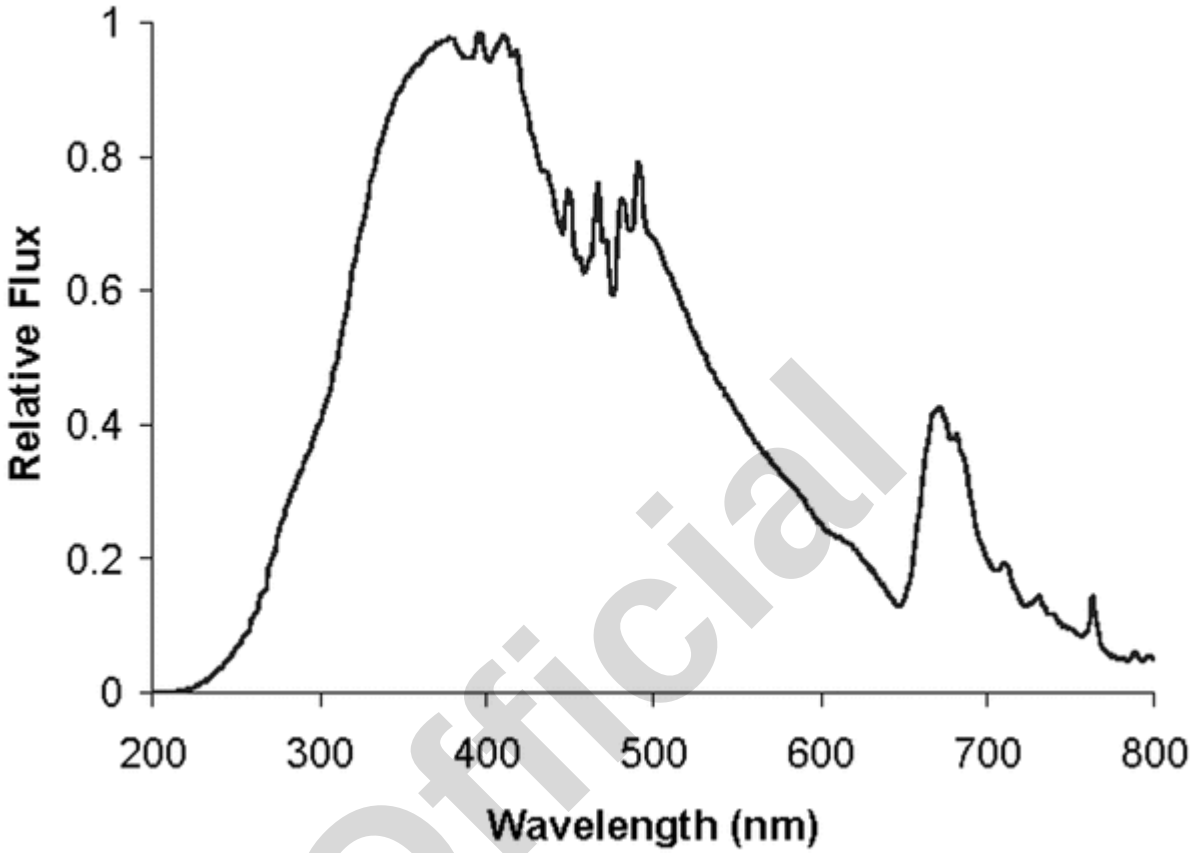


Figure 4. Example of the relative flux of an excitation beam (Xe lamp grating monochromator) for which a correction must be applied to a measured excitation spectrum in order to obtain its true spectral shape.

When a reference detector is not built into an instrument, a spectral correction for the reference channel or an independent spectral correction of excitation intensity is required. Three methods can be used to determine the spectral correction of excitation intensity: a CD (Method 1); a calibrated diffuse reflector (Method 2); or a quantum counter (Method 3). The latter two methods use the instrument's fluorescence detection system as a detector.

For Methods 1 and 2, the detector and diffuse reflector are calibrated for responsivity and reflectance, respectively, as a function of wavelength. For Method 2, excitation and emission wavelength selectors are scanned synchronously, and the spectral correction for the emission channel [see *Signal Level* (*Relative Emission*)] must be applied to the measured intensities. Method 3 should be used only in the quantum counter's effective wavelength range where a wavelength-independent response can be achieved. Method 1 using a CD has fewer caveats than do the other two methods. A CD is placed in the sample position, and the output is measured as a function of emission wavelength by scanning the excitation wavelength selector over the excitation region of interest using the same instrument settings as those used with the sample. The known responsivity of the CD is used to calculate the flux of the excitation beam. If the instrument's reference detector is used to measure the intensity of the excitation beam simultaneously with the CD, then the correction factor for the responsivity of the reference detector also can be calculated.

## Intensity and Sensitivity

The absolute value of the fluorescence signal measured by the detection system depends not only on the sample itself but also on the excitation intensity of the sample and the optical geometry of the instrument. Therefore, determination of instrument-independent fluorescence intensity of any sample or the absolute responsivity of any detection system in terms of the intensity of the sample or measured by the detector, respectively, relative to the excitation intensity can be difficult.

The most accurate way to calibrate an instrument for absolute intensity is to use conventional standards-based methods such as those that employ a calibrated light source or a calibrated detector in combination with a calibrated reflector. These methods require user skill and knowledge. Also, the (typically annual) certification and recertification are expensive. In addition, these standards tend to be bulky and are not compatible with many instruments. Thus, most researchers use simpler alternative standards and methods.

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One approach is to correlate fluorescence signals to analyte concentrations using calibration curves or MESF units (see *Analyte Concentration—Calibration Curves*). Another approach is to measure the intensity of a standard sample that can be expected to always give the same fluorescence intensity under the same conditions.

Organic dyes, such as those used as fluorescent probes, generally are not good choices for intensity standards because of issues with photobleaching, stability, and reproducible concentration. If organic dyes are used, then those with known high purity and known shelf life, such as those produced by national metrology institutes, are recommended for single use (i.e., analysts should use a fresh solution for every measurement).

A better alternative is to use fluorescent samples that are stable over time even when exposed to light. For example, fluorescent, inorganic glasses with well-characterized photostability and spectral properties and long shelf lives are commercially available. Such materials can be used for determining a quasi-absolute intensity scale by measuring fluorescent signal at fixed wavelength values within their recommended range using specified experimental parameters such as bandwidths, excitation intensities, and temperatures.

The sensitivity of a fluorescence instrument is determined by measuring the signal-to-noise ratio of the fluorescence signal of intensity standards. The Raman line of water often is used to measure sensitivity in a similar way, but the Raman signal typically is strong enough only to be useful in the UV region. Analysts can use organic dye solutions to measure instrument sensitivity or limits of detection with caveats that are identical to those that apply when the solutions are used as intensity standards.

The methods outlined here yield a quasi-absolute intensity scale that should be instrument independent for instruments with similar optical geometries, designs, and settings. Results of these measurements enable comparison of the sensitivity of different fluorescence instruments, but these comparisons should be approached with caution because of the relatively large and difficult-to-quantify uncertainties involved.

## PROCEDURE VALIDATION

Validation of an analytical procedure using fluorescence demonstrates that the result is valid within a specified, acceptable uncertainty budget. Instrument qualification, which also may involve instrument calibration, usually is part of the process, and analysts also must consider sample-related errors (see *Sample-based Factors*). These can arise from concentration, anisotropy, photostability, and shape of the sample, in combination with effects of the instrument's optical geometry. All suspected errors should be quantified and combined to give a total estimated error that must be less than the method-specific, acceptable limit.

## GLOSSARY

**Absorptivity (a):** A measure of the absorption of radiation from an incident beam as it traverses a sample, which is equal to the quotient of:

$$A/bc$$

$A$ = absorbance
$b$ = path length (cm)
$c$ = concentration (mg/mL)

Also referred to as *specific absorption coefficient* by the International Union of Pure and Applied Chemistry.

**Absorption coefficient (α):** A measure of absorption of radiation from an incident beam as it traverses a sample according to Bouguer's Law:

$$I/I_0 = e^{-ab}$$

$I$ = transmitted intensity
$I_0$ = incident intensity
$e$ = base of natural logarithm
$a$ = absorptivity
$b$ = path length of the beam through the sample

Note that transmittance $T = I/I_0$ and absorbance $A = -\log T$.

**Beer–Lambert law (or Beer's law or Beer–Lambert–Bouguer law):** In the absence of any other physical or chemical factors, $A_\lambda$ is proportional to path length, $b$, through which the radiation passes and to the concentration, $c$, of the substance in solution in accordance with:

$$A_\lambda = \varepsilon_\lambda cb$$

$\varepsilon_\lambda$ = molar absorptivity
$c$ = solute concentration (M/L)
$b$ = path length (cm)

**Calibrated detector (CD):** A light detector whose responsivity as a function of wavelength has been determined along with corresponding uncertainties.

**Calibrated light source (CS):** A light source whose radiance as a function of wavelength has been determined along with corresponding uncertainties.

**Calibrated diffuse reflector (CR):** A Lambertian reflector whose reflectance as a function of wavelength has been determined along with corresponding uncertainties.

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**Certified reference material (CRM):** A material with properties of interest, the values and corresponding uncertainties of which have been certified by a standardizing group or organization. A "reference material, accompanied by documentation issued by an authoritative body and providing one or more specified property values with associated uncertainties and traceabilities, using valid procedures" [International Vocabulary of Metrology (VIM) 5.14].

**Diffuse scatterer:** A material that scatters light in multiple directions. This includes diffuse reflectors, which often are Lambertian, and scattering solutions, which are not Lambertian.

**Fluorescence anisotropy (*r*):** A measure of the degree of polarization of fluorescence:

$$r = (I_{\parallel} - I_{\perp})/(I_{\parallel} + 2I_{\perp})$$

$I_{\parallel}$ = observed fluorescence intensity when the fluorometer's emission polarizer is oriented parallel to the direction of the polarized excitation

$I_{\perp}$ = observed fluorescence intensity when the fluorometer's emission polarizer is oriented perpendicular to the direction of the polarized excitation

**Fluorescence band:** A region of a fluorescence spectrum where the intensity passes through a maximum, usually corresponding to a discrete electron transition.

**Fluorescence lifetime:**[1] A parameter describing the time decay of the fluorescence intensity of a sample component. If a sample decays by first-order kinetics, this is the time required for its fluorescence intensity and corresponding excited-state population to decrease to $1/e$ of its initial value.

**Fluorescence quantum efficiency:** The ratio of the number of fluorescence photons leaving an emitter vs. the number of photons absorbed.

**Fluorescence quantum yield (Φ):** The probability that a molecule or species will fluoresce once it has absorbed a photon. This quantity is an innate property of the species and typically is calculated for a sample as the ratio of the number of molecules that fluoresce vs. the number of molecules that absorb. Fluorescence quantum yield values range from 0 (i.e., no molecules fluoresce) to 1 (theoretical maximum in which all molecules that had absorbed radiation fluoresce).

**Flux (or radiant flux):** The rate of propagation of radiant energy, typically expressed in watts. *Spectral flux* is the flux per unit spectral bandwidth, typically expressed in watts per nanometer.

**Grating equation:** It describes the relationship between the angle of diffraction and the wavelength of radiation that is incident on a grating:

$$m\lambda = d(\sin\alpha + \sin\beta)$$

$m$ = diffraction order

$d$ = groove spacing on the grating

$\alpha$ = angle of the incident wavefront relative to the grating normal

$\beta$ = angle of the diffracted wavefront relative to the grating normal

**Inner filter effects:** A decrease in the measured quantum efficiency of a sample caused by extensive absorption of the excitation beam or reabsorption of the emission by the sample itself. This causes the measured quantum efficiency to depend on the absorbance, concentration, and excitation and emission path lengths of the sample.

**Intensity:** A measure of the amount of electromagnetic energy present. This general definition is synonymous with or directly proportional to the signal output of a photodetector or the flux of a sample or light source. A more specific definition often used in radiometry is: the radiant flux per unit solid angle from a point source, which typically is expressed as watts per steradian ($W/sr$). [NOTE—Steradian corresponds to the SI unit of solid angle.]

**Lambertian reflector:** A surface that reflects light according to Lambert's law, i.e., the light is unpolarized and has a radiance that is isotropic or independent of viewing angle.

**Limit of detection (LOD):** An estimate of the lowest concentration of an analyte that can be measured with a given procedure, often taken to be the analyte concentration with a measured signal-to-noise ratio of 3.

**Noise level:** The peak-to-peak noise of a blank.

**Photobleaching:** A loss of emission or absorption intensity by a sample caused by exposure to light. This loss can be reversible or irreversible, and the latter typically is referred to as *photodegradation* or *photodecomposition.*

**Quantum counter:** A photoluminescent emitter with a quantum efficiency that is independent of excitation wavelength over a defined spectral range. When a quantum counter is combined with a detector to give a response proportional to the number of incident photons, the pair is called a *quantum counter detector*.

**Quasi-absolute fluorescence intensity scale:** A fluorescence intensity scale that has been normalized to the intensity of a fluorescent reference sample or artifact under a fixed set of instrumental and experimental conditions. This artifact should demonstrably yield a fluorescence intensity that is reproducible with time and between instruments under a fixed set of conditions.

**Raman scattering:** The inelastic scattering of radiation (the wavelengths of the scattered and incident radiation are not equal) by a sample that occurs because of changes in the polarizability of the relevant bonds of a sample during a molecular vibration. Unlike fluorescence, the radiation being scattered is not required to be in resonance with electronic transitions in the sample.

**Rayleigh scattering:** The elastic scattering of radiation by a sample; i.e., the scattered radiation has the same energy (same wavelength) as the incident radiation.

**Responsivity (spectral):** The ratio of the photocurrent output and the radiant power collected by a light-detection system. *Spectral responsivity* is the responsivity per unit spectral bandwidth.

**Sensitivity:** A measure of an instrument's ability to detect an analyte under a particular set of conditions.

[1] Boens N, Qin W, Basaric N, et al., Fluorescence lifetime standards for time and frequency domain fluorescence spectroscopy. *Anal Chem.* 2007;79(5): 2137–2149.

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**Spectral bandwidth (or spectral bandpass or resolution):** A measure of the capability of a spectrometer to separate radiation or resolve spectral peaks of similar wavelengths. Usually observed as the triangular dispersion of an emission line, this parameter is taken to be the full width at half the peak maximum (FWHM).

**Spectral slit width:** The mechanical width of the exit slit of a spectrometer divided by the linear dispersion in the exit slit plane. In practice, observed as the triangular dispersion of an emission line, this width includes all the transmitted wavelengths for a given slit setting.

**Transition dipole moment:** An oscillating dipole moment induced in a molecular species by an electromagnetic wave that is resonant with an energy transition of the species, e.g., an electronic transition. Its direction defines the transition polarization, and its square determines the intensity of the transition.

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# ⟨1854⟩ MID-INFRARED SPECTROSCOPY—THEORY AND PRACTICE

## PRINCIPLES OF MID-INFRARED SPECTROSCOPY

Mid-infrared (mid-IR) spectroscopy involves measurement of the absorption of electromagnetic radiation over the wavenumber range of 4000–400 $cm^{-1}$ (which corresponds to the wavelength range of 2.5–25 μm) caused by the promotion of molecules from the ground state of their vibrational modes to an excited vibrational state. The most commonly used parameter to denote the energy of the transitions is the wavenumber, i.e., the number of waves per centimeter. The wavelength, λ (μm), and wavenumber, $\tilde{\nu}$ ($cm^{-1}$), of radiation are related by the expression:

$$\tilde{\nu} = 10^4/\lambda \quad (1)$$

The mid-IR spectrum extends from 4000 $cm^{-1}$ (2.5 μm) to 400 $cm^{-1}$ (25 μm). Molecules can move in a certain number of vibrational modes. The energy of mode, *i*, is given by:

$$E_i = hc\tilde{\nu}_i\left(v_i + 1/2\right) + hc\tilde{\nu}_i \times x_i\left(v_i + 1/2\right)^2 \quad (2)$$

$h$ = Planck's constant
$c$ = velocity of light
$\tilde{\nu}_i$ = fundamental vibrational frequency of mode *i* ($cm^{-1}$)
$v_i$ = vibrational quantum number of this mode
$x_i$ = so-called anharmonicity constant

The strongest bands in the mid-IR spectrum are caused by fundamental transitions from the ground state of a given mode ($v_i = 0$) to its first excited vibrational state ($v_i = 1$), although weaker overtone and combination bands also are observed in the spectrum. Overtone bands are caused by the promotion of molecules from their ground state to their second and higher vibrational states ($v_i = 2$, 3, etc.). Overtones are observed only for those modes for which $x_i$ is non-zero. Combination bands are caused by the simultaneous promotion of molecules to two excited vibrational states.

Vibrational modes involve the motion of all atoms of the molecule. Many modes involve only large-amplitude vibrations of the atoms in localized regions of the molecule, and the remaining atoms are largely unaffected. When molecules contain a certain functional group, the transitions often occur in narrow spectral ranges. In this case, the wavenumbers at which these transitions occur are known as *group frequencies*. When a vibrational mode involves atomic motions of more than just a few atoms, the frequencies occur over wider spectral ranges and are not characteristic of a particular functional group. Instead, they are more characteristic of the molecules as a whole. Such bands are known as *fingerprint bands*. All strong bands that absorb at wavenumbers above 1500 $cm^{-1}$ are group frequencies. Strong bands that absorb below 1500 $cm^{-1}$ can either be group frequencies or fingerprint bands. Thus, not all strong bands in the IR spectrum of a given molecule can be attributed to the presence of a particular functional group.

The motion of atoms during a particular vibrational mode, *i*, is characterized by the normal coordinate, $Q_i$. The intensity of fundamental bands is governed by the square of the change in dipole moment, μ, during the vibrational cycle $(\partial\mu/\partial Q_i)^2$. Thus vibrational transitions of modes involving polar groups, such as C–O, C=O, O–H, N–H, and C–F, typically give rise to strong bands in the spectrum. Where $(\partial\mu/\partial Q_i)$ is small, transitions are weak. When the symmetry of a molecule leads to the condition that $(\partial\mu/\partial Q_i) = 0$ for a certain mode, the band corresponding to this mode does not appear in the IR spectrum. Overtone and combination bands are always weaker than the fundamental modes from which they are derived.

For functional groups that have the form $XY_2$ (such as $–CH_2$, $–NH_2$, $–NO_2$, and $–SO_2$) and $XY_3$ (such as $–CH_3$ and $–NH_3^+$), first-order coupling can occur so that the mode is split into a symmetrical mode (where both the Y atoms move to and from the X atom with the same phase) and an antisymmetrical mode (where one Y atom moves 180° out of phase with the other). The amount by which the two bands are split depends on the Y–X–Y angle. The more closely this angle approaches 180°, the greater the splitting. Thus, for example, for the symmetric and antisymmetric stretching modes of ketenes and isocyanates where the angle is approximately 180°, the splitting can approach 1000 $cm^{-1}$, whereas for $CH_2$ and $CH_3$ groups where the angle is approximately 108°, the splitting is on the order of 100 $cm^{-1}$.

Second-order coupling, sometimes known as Fermi resonance, occurs when an overtone or combination band happens to occur at the same (or near coincident) wavenumber as a fundamental mode of the molecule that involves motion of the same atoms. In this case, the overtone or combination borrows intensity from the fundamental band, and the two bands split apart by as much as 40 $cm^{-1}$. The closer the bands that interact in this manner, the greater the splitting and the closer the intensity for the resulting bands.

## SAMPLING PROCEDURES

Mid-IR spectra can be measured by transmission, external reflection, internal reflection [often called attenuated total reflection (ATR)], diffuse reflection, and photoacoustic spectroscopy. Each of the major approaches is presented below.

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## Transmission Measurements

The alkali halide disk and mulling procedures are the traditional mid-IR transmission sample presentation methods for materials that are in the form of a finely divided powder, as is the case for many drug substances and excipients. During preparation of a sample suitable for IR spectroscopy, the powdered material is uniformly dispersed throughout either the alkali halide or mulling agent, which acts as a support matrix for the analyte. Other procedures by which transmission spectra can be acquired include the use of solutions and compression cells. Neat compounds can be examined in a compression cell, as a self-supporting film (for polymers), as a capillary film between the IR-transparent cell windows (for liquids and semisolids), and as a gas.

The ratio of the single-beam spectrum of the sample and an appropriate background spectrum at a given wavenumber, $\tilde{\nu}$, is known as the transmittance, $T$-($\tilde{\nu}$). A transmittance spectrum is the direct output of most prism or grating spectrophotometers. For spectra measured on a Fourier transform-IR (FT-IR) spectrophotometer, the two single-beam spectra are measured at different times and are ratioed subsequently. An appropriate background spectrum should be measured. For alkali halide disks and mineral oil mulls where the diameter of the sample is usually greater than that of the focused beam, the background is measured with nothing in the sample compartment. If the sample diameter is smaller than that of the beam, the empty sample holder should be in place when the background spectrum is being measured. Similarly, the background for measurements made with an IR microscope should be measured with the same aperture that is used to measure the sample spectrum. For the measurement of ATR spectra, the background spectrum should be the clean internal reflection element.

Because transmission spectra of nonscattering samples obey the Beer–Lambert Law (usually abbreviated as Beer's Law), the transmittance commonly is converted to absorbance, $A$ ($\tilde{\nu}$), i.e., $\log_{10} 1/T$ ($\tilde{\nu}$). Beer's Law states that the absorbance of component $i$ at wavenumber, $A_i$ ($\tilde{\nu}$), is the product of the absorptivity of $i$ at that wavenumber, $a_i$ ($\tilde{\nu}$), the path length of the sample, $b$, and the concentration of $i$, $c_i$. The measured absorbance of a mixture at each wavenumber is the sum of the absorbances of each component of the mixture. Certain powdered alkali halides, such as potassium bromide, potassium chloride, and caesium iodide, coalesce under high pressure and can be formed into self-supporting disks that are transparent to mid-IR radiation.

## Potassium Bromide (KBr) Disks

The alkali halide most commonly used is powdered, dry, highly pure potassium bromide, which is transparent to mid-IR radiation to approximately 400 $cm^{-1}$. From this point, alkali halide disks will be referred to as *KBr disks* even though they can be made with other alkali halides such as potassium chloride and caesium iodide. IR spectroscopic-grade potassium bromide with a particle size of 100–200 mesh (about 100 µm in diameter) can be purchased commercially. Nonspectroscopic-grade material may contain impurities with absorption bands in the mid-IR region. One of the more common impurities is potassium nitrate, which has a sharp absorption band at approximately 1378 $cm^{-1}$. Powdered potassium bromide has a tendency to adsorb molecules from the air over a long period of time, so it must be stored properly. If the potassium bromide is not dry, its spectrum exhibits a broad absorption band caused by adsorbed water at approximately 3400 $cm^{-1}$, along with a weaker band near 1640 $cm^{-1}$.

Commercial presses and dies in a range of diameters are available for the preparation of alkali halide and similar disks. The most common diameter of KBr disks is 13 mm, but mini-disks with a diameter as small as 0.5 mm can be prepared using commercially available presses. Follow the manufacturer's recommended procedures for operating the disk-making accessory and the press.

Typically, the weight ratio of sample to alkali halide is on the order of 1 part of the sample to 100–400 parts of potassium bromide. An optimal procedure for preparing a 13-mm diameter disk is to pregrind the sample to a fine particle size in an agate mortar. A vibrating agate or steel ball mill also can be used. Then 1–2 mg of the ground sample is weighed and transferred to a clean mortar (or vial). A weighed amount of dry ground potassium bromide powder (300 mg) is then added and mixed gently with the analyte to form a homogeneous mixture. The optimum way of achieving homogeneity is to add about 10 mg of ground potassium bromide to the sample in a mortar and then to gently mix with the pestle. This is followed by successively adding doubled quantities of potassium bromide (i.e., approximately 20, 40, 80, and 160 mg) with mixing after each addition. The applied pestle action should induce good mixing with minimal grinding because further reduction of the potassium bromide particle size will lead to increased water absorption. (If the mixing is done in a vial, then the mixing time is shortened.) The mixture of potassium bromide and analyte is then transferred completely to a clean 13-mm die, which is filled and assembled according to the manufacturer's instructions. When the die is connected to a rotary vacuum pump, evacuate for about 2 min. The die (still under vacuum) is placed in a hydraulic press, and a pressure sufficient to form a disk that shows uniform transparency is applied. Discard any disk that visually shows lack of uniform transparency or exhibits poor transmittance at about 2000 $cm^{-1}$. Faulty, unsatisfactory, or poor-quality disks may be a consequence of inadequate or excessive grinding, moisture/humidity, or impurities in the dispersion medium.

## Mulls

To prepare a mull, homogeneously distribute the finely divided powder sample in a thin layer of a viscous liquid that is semi-transparent to mid-IR radiation and has a refractive index closely matched to that of the sample. The prepared mull is sandwiched between a pair of mid-IR–transparent windows, and a transmission spectrum of the mull preparation is recorded. The mull, which should have the consistency of a paste, is formed in such a manner as to minimize radiation scattering effects (radiation scatter from particles is worse when there is greater mismatch between the refractive index of the dispersant and surrounding medium). The sandwich can be clamped together in a mull cell. Commercial mull cells are available for both macro- and micro-preparations.

The most widely used mulling agent for the mid-IR region is a saturated hydrocarbon mineral oil (liquid paraffin, Nujol). This material has strong absorption bands of 3000–2800 $cm^{-1}$ and of 1500–1340 $cm^{-1}$, and a weaker band at 720 $cm^{-1}$ that may

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obscure the absorption bands of the sample in these regions. In this case, a mull may require preparation in a chemically different oil. This can be achieved by the use of a perhalogenated oil mulling agent such as chlorofluoro substituted polymers. Combining the spectra of the sample prepared in the two mulling oils and ignoring the regions where one of them has strong absorption bands enables observation of the full mid-IR absorption spectrum with minimal interference.

For the preparation of a mineral oil mull, the particle size of the sample must be reduced to below that of the shortest wavelength of the interrogating radiation (2.5 µm) in order to minimize light-scattering effects that decrease spectral contrast and cause band distortions. The spectrum from a coarse powder or one that is poorly ground will show a high degree of scatter that is manifested as a sloping baseline that decreases toward shorter wavelengths (higher wavenumbers). Also, a coarser powder will increase the Christiansen effect, which is caused by reflection from the interface between materials of different refractive index. The refractive index of materials with strong absorption bands varies in a way that is similar to the first derivative of the profile of the absorption band—a phenomenon that is referred to as *anomalous dispersion*. The Christiansen effect is manifested as a transmission increase on the short-wavelength (high-wavenumber) side of an absorption band with a concomitant decrease on the longer wavelength side. Furthermore, a coarse, poorly dispersed powder can lead to a severely distorted mid-IR spectrum in which the relative intensity of the weaker bands is enhanced and the intensity of the more intense bands in the spectrum appears weaker and distorted. These effects are a consequence of radiation that has reached the detector but has not been transmitted through a representative sample of the analyte. Similar effects can be seen in the spectra of poorly prepared KBr disks.

During the preparation of mulls and KBr disks, some work is done on the analyte, either in the form of grinding, mixing, or pressing, and consequently there is the potential to induce solid-state form transformations. Although laboratory mechanical mills can produce powders with a small particle size, hand grinding using a mortar and pestle usually achieves better control and less aggressive processing for organic pharmaceutical materials. Practitioners generally accept that of the two procedures, the mull procedure is the less aggressive and is less prone to induce solid-state form changes such as changes in the crystallinity (polymorphism) or changes in the hydration or solvation state (pseudopolymorphism). The KBr disk procedure does, however, have advantages over the mull presentation method because potassium bromide exhibits no absorption bands above 400 $cm^{-1}$ (neglecting any adsorbed water or impurities) and is better adapted to micro-sample preparations. When the sample is a salt, as is frequently the case for active pharmaceutical ingredients (APIs), ion exchange can occur between the analyte and alkali halide, and the sample is better prepared as a mull.

## Compression Cells

The use of a compression cell has become a popular sampling procedure for recording a mid-IR transmission spectrum of a small or limited-quantity solid sample such as a single particle of an API or excipient, a contaminant such as a short length of fiber, or a small fragment from a packaging material. This is particularly the case for investigations using an IR microscope system. Type IIa diamonds are quite transparent over much of the mid-IR region, although they exhibit fairly strong absorption between approximately 2000 and 2400 $cm^{-1}$. Because of the high strength of diamond, it is commonly used as the window material of compression cells. The sample is placed between the diamond windows of the cell, the cell is then tightened, and the sample thickness is reduced to an optimum for a transmission measurement. The compressed sample can be examined while it is contained within the compression cell.

## Self-Supported Polymer Films

The mid-IR transmission spectrum of many polymers used as packaging materials can be recorded from samples prepared as thin self-supporting films. Films of appropriate thickness can be prepared by, for example, hot compression moulding a sample or microtoming a thin section from a sample. Soft and low-melting solids that do not crystallize when cooled can be prepared either as a thin layer sandwiched between two mid-IR–transparent windows by gently warming the sample or from the melt. Thin films from some materials can be cast from solution onto an IR-transparent window.

## Capillary Films

Nonvolatile liquids can be examined neat in the form of a thin layer sandwiched between two matching windows that are transparent to IR radiation. The liquid layer must be free of bubbles and must completely cover the diameter of the IR beam focused onto the sample.

## Liquids and Solutions in Transmission Cells

For the examination of liquid and solution samples, transmission cell assemblies that comprise a pair of windows constructed of mid-IR–transparent materials such as potassium bromide spacers, filling ports, and a holder are available commercially in both macro- and micro-sample configurations. They can be sealed, semi-permanent, or flow-through. A wide range of standard thickness spacers and window materials is available. For laboratory applications, spacers are typically formed from lead, poly(tetrafluoroethylene), or poly(ethylene terephthalate) and can be supplied, depending on spacer materials, in standard thickness path lengths from approximately 6 µm to 1 mm or larger.

The optimum path length required for examining a particular liquid or solution usually must be determined empirically and depends on its absorption characteristics and whether the application is qualitative or quantitative. In some instances for qualitative work, transmission cells can be replaced with disposable porous media such as polyethylene or poly(tetrafluoroethylene) mounted on a suitable backing.

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## Gases

Mid-IR transmission cells for static or flow-through gas and vapor sampling are available in a wide range of materials to suit the application, from laboratory to process scale. In the laboratory, the traditional gas cell has been a 10-cm long cylinder made from borosilicate glass or stainless steel with an aperture of about 40 mm at each end. Each open end is covered with an end cap that contains one of a pair of mid-IR–transparent windows constructed from, for example, potassium bromide, zinc selenide, or calcium fluoride. The cell body is fitted with appropriate inlet and outlet ports. Different gases may present different sealing requirements that should be taken into account. For the detection of gases at the ppm level, long-path length cells constructed from borosilicate glass or metal are used. These cells can be of fixed path length of up to about 2 m or of variable path lengths of 10–200 m. Gas cells can be jacketed and operated at temperatures of 250° or greater, and their pressure ratings can range from vacuum to more than 50 atmospheres. Small differences in temperature and pressure have significant effects on the spectrum, and care must be taken to ensure that the calibration and analysis are performed under similar conditions such that the calibration remains valid.

## Attenuated Total Reflection Spectroscopy

Attenuated total reflection (ATR) spectroscopy, alternatively known as internal reflection spectroscopy or evanescent wave spectroscopy, has become a widely used procedure. This is largely a consequence of a new generation of simple-to-use single-reflection accessories.

ATR spectroscopy relies on the optical property that radiation passing through a medium of high refractive index, $n_2$ [the *optically dense* medium, also known as the internal reflection element (IRE)], at an angle of incidence greater than the critical angle will be totally internally reflected at a boundary in contact with a material of lower refractive index, the sample, $n_1$ (the *optically rare* medium). The critical angle, $\theta_C$, is given by $n_1/n_2$. The electric field of the radiation penetrates a short distance into the optically rare medium. The intensity of this electric field, which is known as the *evanescent wave*, is confined within the vicinity of the surface of the denser medium. Its intensity decreases exponentially with distance, normal to the surface, into the optically rare medium. It can, therefore, be envisaged as penetrating the surface layer of the rarer medium. The depth of penetration, $d_p$, is a convenient comparative term for different experimental arrangements. It is the distance from the surface of the IRE at which the amplitude of electric field amplitude falls to 37% ($1/e$) of its value at the surface:

$$d_p = \frac{\lambda_0}{2\Pi n_2 \sqrt{\left(\sin^2(\theta) - {n_{12}}^2\right)}} \quad (3)$$

(3)

$\lambda_0$ = wavelength of the radiation in vacuum
$\theta$ = angle at which the beam strikes the internal surface of the IRE
$n_{12} = n_2/n_1$

Note that, to achieve total internal reflection, $\theta$ must be greater than $\theta_C$, so that $n_2$ is usually greater than 2.3. Some of the more commonly used IRE materials are the following: zinc selenide ($n_2$ ~ 2.4), Type IIa diamond ($n_2$ ~ 2.4), silicon ($n_2$ ~ 3.4), and germanium ($n_2$ ~ 4.0). Furthermore, $d_p$ decreases with increasing angle of incidence and increases with increasing wavelength (decreasing wavenumber). As a consequence of the increase in $d_p$ with increasing wavelength, the band intensities within an ATR spectrum appear—by comparison with a conventional transmission spectrum—to be relatively increasingly enhanced with decreasing wavenumber.

Furthermore, the refractive index of all molecules is not constant and varies across absorption bands (anomalous dispersion). For strong absorption bands, $n_1$ can vary between approximately 1 and 2. This effect causes a shift in the measured wavenumber of a band with respect to transmission spectra. Particularly when equipment is operating at high angles of incidence or with an IRE with a low refractive index such as zinc selenide or diamond, strong bands may also be accompanied by the appearance of an underlying first-derivative-like shape (see above for a discussion of the Christiansen effect in KBr disks).

Many different geometrical shapes and sizes are used for IREs. A trapezoidal IRE is commonly incorporated into so-called horizontal ATR (H-ATR) units. Hemispherical IREs are the core of some micro-sampling ATR accessories. Rod or rod-like multiple internal reflection IREs are often used for on-line monitoring of liquid processes.

Multiple internal reflection (MIR) elements allow the internally reflected radiation in the IRE to interact several or many times with the surface layer of the sample with which it is in contact, thereby increasing the intensity (effective path length) of the recorded sample spectrum. Still, the spectrum recorded is characteristic only of the depth probed by a single reflection. The number of internal reflections depends on the length and thickness of the IRE. MIR elements may be several centimeters in length. A typical configuration for a vertically mounted 45° angle of incidence IRE may allow 25 internal reflections. Today the most commonly used MIR systems in the pharmaceutical laboratory are those in which the IRE is mounted horizontally. These are often referred to as H-ATR accessories. MIR systems based on cylindrical rods with cone-shaped ends or similar geometries are often incorporated as liquid-sampling devices in flow-through solution cells or are used for on-line process monitoring. The trapezoidal-shaped MIR elements incorporated into H-ATR accessories enable or facilitate study of a wide range of sample forms, including liquids, solutions, dispersions, creams, pastes, waxes, semi-solids and soft powders, continuous flat surface solids, solutions, films cast from solution, and many more. Micro– and macro–H-ATR accessories are commercially available with 1, 3, 9, or more sample-interaction reflections.

Single-reflection, simple prism, and novel design IREs are also used in commercial H-ATR units. They provide an effective and convenient means of analyzing and studying samples in a diverse range of physical forms. In particular, small contact area, single-reflection, fixed angle of incidence H-ATR accessories have become popular no-preparation sampling devices within the pharmaceutical industry because they provide a ready means to record, in a simple, quick manner, a mid-IR spectrum from a

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limited quantity of almost any condensed-phase material. Hemispherical IREs act as focusing lenses so that the area sampled is generally smaller than that sampled with prismatic IREs. Even though the IRE may be opaque to visible light, many accessories listed above allow some form of viewing capability so that the sample under test can be inspected.

Single-reflection micro-ATR units have the advantage of virtually no requirement for a solid sample to have a uniformly flat surface. The test sample is placed in contact with the IRE sampling area and, if it is a solid, a clamp is used to compress and secure the sample against the IRE. Hemispherical ATR elements of zinc selenide, germanium, and silicon also form the sensing elements for ATR objectives that can be fitted to mid-IR FT-IR microscopes. Several of the accessories are capable of operation at controlled elevated temperatures that permit, for instance, studies relating to thermally induced, solid-state form transformations.

The hardness, scratch resistance, chemical inertness, and mid-IR transparency over a wide wavenumber range make the Type IIa diamond a unique material for ATR measurements. Even though it does have a broad absorption feature between approximately 2400 and 2000 $cm^{-1}$, for most pharmaceutical applications, this is not prohibitive because this is the region in which only characteristic stretching bands occur for triple and cumulated double bonds. Because of cost, the use of Type IIa diamond as an IRE material is usually restricted to micro-ATR accessories or when the IRE is used as the sensing element in an ATR immersion probe for process monitoring. Diamond has a refractive index that is closely matched to that of zinc selenide, so composite IREs can be constructed. Lower-cost focusing or support optics made from zinc selenide can be optically interfaced with a Type IIa diamond ATR sensing element, thereby minimizing the overall cost of the IRE while still benefiting from the properties of the diamond. On the basis of this technology, 3- and 9-reflection MIR configurations have been designed for both laboratory systems and process probes.

## External Reflection Spectroscopy

Several types of external reflection IR spectra can be measured. Among them are Fresnel reflection, transflection, reflection–absorption spectroscopy, and photoacoustic spectroscopy, but with the exception of diffuse reflection they are not widely used in pharmaceutical applications.

## Diffuse Reflection

Spectra recorded from powders or fairly fine granular samples are known as *diffuse reflection* (DR) spectra. Most of the spectrum originates from radiation that has penetrated through the surface of the sample and has been transmitted through multiple particles. A relatively small fraction of the DR spectrum originates from radiation that has been reflected from the front surface of the samples and therefore has the shape of a Fresnel reflection spectrum. Because the shapes of bands in mid-IR Fresnel reflection spectra are asymmetrical, the fraction of Fresnel reflection that contributes to a DR spectrum should be reduced to be as small as possible. This may be achieved in a number of ways, the most important and commonly used of which is to dilute the sample by mixing it with 90%–99% of a nonabsorbing diluent such as finely powdered potassium bromide or potassium chloride. The sample dilution has the added benefit of reducing absorption band intensities to an appropriate level.

DR spectra largely result from photons that have been transmitted through tens to hundreds of particles and, therefore, have an appearance similar to that of transmission spectra. However, DR spectra do not obey Beer's Law. Instead, the DR spectrum measured at infinite depth, $R_\infty$ (i.e., band intensities do not change if the thickness of sample is increased), is converted by the Kubelka–Munk function:

$$f\left(R_\infty\right) = \frac{\left(1 - R_\infty\right)^2}{2R_\infty} \quad (4)$$

The function $f(R_\infty)$ is equal to the ratio of the absorption coefficient to the scattering coefficient of the sample. DR is usually calculated by taking the ratio of the single-beam spectrum of the diluted sample to the single-beam spectrum of the neat diluent. Ideally, the sample is ground to the point that the average particle diameter is <5 µm. The sample–diluent mixture is thick enough that any increase in its thickness does not lead to a change in the spectrum. Samples measured in this way are said to be measured at *infinite depth*, and the reflectance is given the symbol, $R_\infty$. For mid-IR DR spectrometry, the infinite depth criterion is usually obeyed when the thickness is at least 100 µm, but to be conservative the depth of most sampling cups for mid-IR DR spectrometry is at least 1 mm.

A common way of preparing samples for DR spectrometry is to overfill the cup and to level the sample with a spatula or razor blade. However, this way of preparing the sample can lead to a difference between the scattering coefficient near the surface and in the bulk of the sample. Because the intensity of DR spectra depends on the scattering coefficient, a better sample preparation procedure is to slightly overfill the cup and to tap the base of the cup on a bench until the top surface is level with the rim of the cup.

Several types of accessories are used for the measurement of DR spectra. On-axis, or bright-field, DR accessories are similar to very efficient specular reflection accessories. They are the most efficient devices for the measurement of DR spectra but generally give the least rejection of Fresnel reflection. Off-axis, or dark-field, DR accessories are less efficient than on-axis devices but reject specular reflection more efficiently. Compound parabolic concentrators also have been adapted for DR measurements and appear to be intermediate in efficiency and Fresnel reflection between on-axis and off-axis accessories. Finally, integrating spheres have been used for DR spectrometry. These devices are the most accurate photometrically but are the least efficient.

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# MICROSPECTROSCOPY AND IMAGING

## Transmission Microscopy

In any microscope, the area of a sample under study is defined by one or more remote masking apertures mounted at focused image planes conjugate to the sample focus plane. If the sample is mounted on a computer-controlled *x*–*y* stage, then successive neighboring or specified regions can be measured sequentially. These single-point spectra can be used to generate absorbance-intensity contour maps or false-color images that highlight differences or inhomogeneities across the area mapped. Such maps can also be generated by moving the sample manually, but this procedure can be very time consuming. The linear dimension of the smallest sample that can be studied by mid-IR FT-IR microscopy is approximately equal to the wavelength, i.e., approximately 10 µm. In practice, one can usually record a single-point spectrum of acceptable signal-to-noise ratio (SNR) and spectral resolution within a reasonable time-scale from a masked sample area of 10-µm diameter in about 1 min. For mapping over a relatively large sample area, where hundreds or even thousands of spectra are needed, a much more time-efficient process is to use an FT-IR microscope equipped with an array detector. Variable-temperature studies with thermomicroscopy observations and variable-temperature studies undertaken on an FT-IR microscope can be particularly useful for studying solid-state form and thermally induced transitions in situ.

As with macroscopic sampling, micro-samples presented for transmission FT-IR microscopy measurements should be flat. For a full mid-IR fingerprint spectrum, they should be of an appropriate thickness which, in the case of many APIs, may require a sample of approximately 10-µm thickness or less. Compression cells are particularly useful for thinning a sample. A thinned sample can be examined under compression or (less preferably) with the top window of the cell removed and the thinned sample examined while supported on the bottom window. A good practical means of providing an appropriate sample from which to record a single-beam background spectrum is to mount a small particle of potassium bromide alongside the sample and thin both by compression under the same conditions. The single-beam spectra of the sample and reference materials then can be measured under the same conditions simply by moving the compression cell using the *x*–*y* stage of the microscope.

Many continuous solids such as polymers used in packing or fibers found as contaminants can be prepared to an appropriate thickness and examined in a compression cell by FT-IR microscopy. Rolling with a tool specifically designed for this purpose can also sometimes decrease the thickness of soft samples. Laminated samples can be sectioned using a microtome and can be examined either free-standing or supported on an IR-transparent window. Analysts commonly use a microtome to get a cross-section from a multi-layer polymer laminate film so that its layer structure can be analyzed by FT-IR microscopy.

## Reflection Microscopy

All major types of reflection spectroscopy discussed above can be implemented on microscopes. Most microscopes have optical configurations that enable them to be switched from transmission to reflection measurements if the operator switches a simple flip mirror. The angle of incidence for these measurements is usually between 30° and 45° so that both Fresnel reflection and transflection measurements can be carried out easily. In contrast, special microscope objectives are required for ATR or reflection–absorption spectrophotometry.

For ATR microspectroscopy, the sample under investigation is supported on the microscope stage, and the ATR objective is lowered until the IRE is in optical contact with the uppermost surface of the sample. Reproducible contact pressure can be achieved by the use of a pressure gauge. A disadvantage of using a germanium IRE is that, unlike zinc selenide, it does not transmit visible light, which precludes in situ visual inspection of the sample. ATR mapping using a computer-controlled mapping stage in a manner analogous to that of transmission mapping is also possible, but this is usually restricted to soft materials.

## Hyperspectral Imaging

The term *hyperspectral imaging* came into use to describe the process whereby a focal plane array (FPA) detector is used to record simultaneously an array of spectra from a stationary sample. Each pixel of the array records a spectrum of a different region of the sample. This approach is a much more time-efficient process than single-point mapping if many spectra are measured from a large sample area. Typical array sizes for mid-IR applications are 256 × 256, 128 × 128, and 64 × 64. The individual mercury cadmium telluride (MCT) detector elements (pixels) of most FPAs are 6.25 µm × 6.25 µm. The detectors that are incorporated in the FPA are photovoltaic (PV) detectors, unlike the single-element photoconductive (PC) MCT detectors that are used for many FT-IR measurements (see below). Whereas PC MCT detectors operate at least to 750 $cm^{-1}$, PV detectors that are installed in FPAs typically have a low wavenumber cut-off in the region of approximately 900 $cm^{-1}$. Advances in digital electronics and FPA design enable 64 × 64 MCT FPAs to be used with an FT-IR spectrophotometer operating in the more conventional continuous-scan mode. The three-dimensional array of data sets, two spatial and one spectral, recorded in such an imaging measurement has become known as a *hypercube* or data cube.

An alternative way of hyperspectral imaging that is now commercially available is a hybrid approach in which a linear array of small PC MCT detectors is used in combination with a computer-controlled mapping stage. Adjacent regions are rapidly repositioned under the array detector until the full spatial region of interest has been covered. The full field image is built up as a mosaic of the individual area images recorded. In one system, which incorporates a 16-detector linear array, the lower wavenumber cut-off is approximately 700 $cm^{-1}$.

# INSTRUMENTATION

The majority of mid-IR spectra are measured with an FT-IR spectrophotometer. These instruments generally incorporate an incandescent silicon carbide (Globar®-type) source. The radiation emitted by the source is collimated and passed into a

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continuous scanning two-beam interferometer. The rate of change of optical path difference in the interferometer (usually known as the *optical velocity*) is typically on the order of 0.2–5.0 cm · $s^{-1}$. The actual value depends on the detector and the analog-to-digital converter (ADC). The beam emerging from the interferometer is then focused at the center of the sample compartment of the spectrophotometer by an off-axis paraboloidal mirror. After being transmitted through, or reflected from, the sample, the beam is focused onto the detector. This signal is called an *interferogram*. The interferogram is a record of the variation of the AC component of the energy incident on the detector as a function of the optical path difference (retardation) of the interferometer. The Fourier transform of the interferogram is the single-beam spectrum.

The measurement usually involves passing a laser beam through the center of the interferometer, with the resulting sinusoidal interferogram measured by a detector of visible radiation at the same time the IR interferogram is measured by the IR detector. The usual laser used for this purpose is a helium–neon (HeNe) laser with a wavelength of 632.8 nm (15,802 $cm^{-1}$). This laser interferogram allows the exact position of the moving optical element in the interferometer to be determined. In FT-IR spectrophotometers equipped with an externally triggerable ADC, the IR signal is digitized at each wavelength of the laser interferogram, typically at the zero crossings. If the IR interferogram is sampled once per wavelength of a HeNe laser, the spectral range is restricted to 0–7901 $cm^{-1}$, i.e., one-half the wavenumber of the laser. In some contemporary FT-IR spectrophotometers, a sigma-delta ADC is used. These ADCs sample the interferogram at constant time intervals rather than at constant intervals of optical path difference, but they have a greater dynamic range than do externally triggered ADCs. In the latter case, the instrument's software calculates what the value of the interferogram would have been at the laser zero crossings.

In obtaining the spectrum of a sample, the single-beam spectrum of the sample and an appropriate reference are measured, and the ratio of these two single-beam spectra is calculated. If the spectrum is obtained in the transmission mode, the ratio is known as the transmittance spectrum, $T(\tilde{\nu})$. For many measurements, the negative logarithm of $T(\tilde{\nu})$ is calculated to give the absorbance, $A(\tilde{\nu})$. If the sample is interrogated in the reflection mode, the ratio is the reflectance spectrum, $R(\tilde{\nu})$. For ATR, transflection and reflection–absorption measurements, $R(\tilde{\nu})$, are usually converted to absorbance in the same way as for transmission spectroscopy. Conversion to $A(\tilde{\nu})$ is essential for quantitative measurements. For Fresnel reflection measurements, $R(\tilde{\nu})$ is often subjected to a Kramers–Kronig transformation to yield the absorption index spectra, $k(\tilde{\nu})$, and the refractive index spectra, $n(\tilde{\nu})$. For mid-IR diffuse reflection measurements, $R(\tilde{\nu})$ is usually converted by the Kubelka–Munk function (see *Equation 4*).

The standard detector used in FT-IR spectrophotometers is a room-temperature pyroelectric bolometer, most commonly deuterated triglycine sulfate (DTGS) or deuterated L-alanine-doped triglycine sulfate. These detectors respond to IR radiation of all wavelengths, and their low wavenumber cut-off is determined by the window behind which they are mounted. The window is typically selected to match the material on which the beamsplitter is deposited and is usually potassium bromide, so that the range is restricted to 400 $cm^{-1}$.

When the sensitivity of pyroelectric bolometers is inadequate, e.g., for measurements made through a microscope or with a gas chromatography interface, the more sensitive MCT detector is used. MCT detectors are generally operated in the photoconductive mode and are usually cooled to 77 K with liquid nitrogen ($LN_2$). Thermoelectrically cooled MCT detectors are available, but their low wavenumber cut-off is usually well above 1000 $cm^{-1}$, and they are not as sensitive as $LN_2$-cooled MCT detectors. Liquid nitrogen-cooled indium antimonide (InSb) detectors can also be used. These detectors are more sensitive than MCT but have a cut-off at 1800 $cm^{-1}$.

Two problems with MCT detectors should be noted. First, because they are more sensitive than DTGS detectors, they should be used only when the sample or sampling accessory attenuates the beam by at least a factor of 10. Otherwise, the ADC will be overloaded and the photometric accuracy of the spectrophotometer will be seriously affected. If the instrument is equipped only with an MCT detector (a rare circumstance), a neutral-density filter can be mounted in the spectrophotometer beam to reduce the energy of the beam at the detector to an appropriate level. Second, even when the ADC is not saturated, the response of MCT detectors is often nonlinear at high signal levels near the centerburst of the interferogram. The effect of this nonlinearity is to cause the baseline of the calculated single-beam spectrum to be displaced from zero. This effect can be seen readily when one plots the single-beam spectrum between 0 and 4000 $cm^{-1}$. If the average energy in the single-beam spectrum between, for instance, 400 and 300 $cm^{-1}$ is above or below zero, the detector is responding in a nonlinear manner in the region of the centerburst, and the photometric accuracy of the measurement is degraded concomitantly. Some vendors supply software to correct for this effect, but in general the signal should be reduced by inserting a neutral-density filter, not an aperture stop, in the beam.

## FACTORS THAT IMPACT MEASUREMENT PERFORMANCE

### Spectral Resolution

The main factor that affects the resolution of an FT-IR spectrophotometer is the maximum optical path difference of the interferogram. The nominal resolution, $\Delta\tilde{\nu}$, is the reciprocal of the maximum optical path difference.

The divergence angle of the beam passing through the interferometer may also degrade the resolution. The effective collimation of the beam that passes through the interferometer is determined by the limiting aperture of the optical system. In instruments designed for high resolution ($\Delta\tilde{\nu} > 0.5$ $cm^{-1}$), an adjustable aperture that serves the same purpose as the entrance aperture of a monochromator is installed at a focus between the source and the interferometer. As the desired resolution is increased (i.e., $\Delta\tilde{\nu}$ is made numerically smaller), the diameter of this aperture, which is known as the *Jacquinot stop* or J-stop, is decreased, making the divergence angle of the beam in the interferometer smaller; however, this procedure may increase the noise in the spectrum. In lower resolution instruments (typically those with a maximum resolution of 1 or 2 $cm^{-1}$), the detector serves the purpose of the Jacquinot stop.

If the true full-width at half-height of the bands or lines in the spectrum is less than $\Delta\tilde{\nu}$, side lobes are seen on each narrow spectral feature. These side lobes can be eliminated by apodization, i.e., multiplying the interferogram by a function that is equal to 1 at the centerburst and decays monotonically with optical path difference. Besides reducing the amplitude of the side

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lobes, apodization also has the effect of degrading the resolution (broadening the bands). When an interferogram is not weighted, it is often (incorrectly) said to be apodized with a boxcar apodization function, although a better term would be a boxcar truncation function. The selection of apodization function should be made on the basis of the purpose of the experiment, but it is usually limited to a few choices.

The Norton–Beer weak, medium, and strong apodization functions give the optimum combination of side-lobe amplitude for a loss in resolution of 20%, 40%, and 60%, respectively. The Norton–Beer medium apodization function is generally appropriate for many measurements of condensed-phase samples. The Happ–Genzel apodization function is also a nearly optimal function that degrades the resolution by about 50% (i.e., it is midway between the Norton–Beer medium and strong functions). A commonly available function is the triangular apodization function. This function has the effect of causing significant deviations from Beer's Law and is not recommended.

## Wavenumber Accuracy

The main factors that affect wavenumber accuracy are the alignment of the laser and IR beams in the interferometer and the divergence of the beam passing through the interferometer as observed with the IR detector. One might think that because the wavenumber of the laser beam is known very accurately, the wavenumber scale of a spectrum measured on an FT-IR spectrophotometer should be known to equal accuracy. However, the laser beam is highly collimated, whereas the beam from the IR source is not (a collimated beam can be obtained only from a point source). Because both the source and detector have a finite size, equal accuracy (of the wavenumber of the laser beam and the wavenumber scale of the spectrum measured) is never the case. As the diameter of the Jacquinot stop is decreased, a small wavenumber shift (always less than 0.25 $\Delta\tilde{\nu}$) will be observed. When the diameter of a sample is less than the diameter of the beam focus in the sample compartment or the sampling accessory, the effect is also to vignette the beam (i.e., to stop the beam down), and a small wavelength shift will be observed.

## Photometric Accuracy

The main factor that affects the photometric accuracy of FT-IR spectrophotometers is the linearity of the detector response. As noted above, the response of pyroelectric bolometers usually varies linearly with the energy on the detector, but this is not the case for MCT detectors. With MCT detectors, the best way of detecting photometric error is measurement of the nonphysical energy in the single-beam spectrum below the detector cut-off.

## Sensitivity

The sensitivity of the instrument can be determined by measuring two single-beam spectra under exactly the same conditions and calculating their ratio to produce what is commonly known as a 100% line. The noise level in different spectral regions can be estimated either as the peak-to-peak noise, i.e., the difference between the maximum and minimum values of the percent transmission in the selected spectral region(s), or the root-mean-square (RMS) noise, i.e., the standard deviation of the spectrum in that region. The RMS noise level is the preferred metric because this calculation involves all the data in the selected region rather than just the two most deviant points. An example of suitable measurement conditions to test the sensitivity of an FT-IR spectrophotometer equipped with a DTGS detector are 16 co-added scans, a resolution of 2 $cm^{-1}$, and Norton–Beer medium apodization. The most commonly used spectral region is 2200–2000 $cm^{-1}$ because (a) this is where the performance of most mid-IR spectrophotometers is highest, and (b) no common atmospheric interferent such as water or carbon dioxide absorbs strongly in this region. However, other regions should be tested close to the ends of the spectrum, such as 650–450 $cm^{-1}$ and 4000–3800 $cm^{-1}$. The SNR of the spectrophotometer operating with certain parameters in a given spectral region is estimated as 100/(RMS noise level in percent transmission).

## Beer's Law Linearity

For quantitative measurements, the spectrum is measured at a resolution that is at least twice as narrow as the narrowest band in the spectrum. The use of either the Norton–Beer medium or Happ–Genzel apodization function is recommended. For optimal photometric accuracy, the maximum absorbance of the analytical bands is no greater than 1.0 absorbance unit. Higher absorbance values can be tolerated with certain combinations of resolution and apodization functions. The effect of band width and peak absorbance on Beer's Law linearity for strong bands depends on the resolution and apodization function, and should be validated on a case-by-case basis.

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# ▲⟨1856⟩ NEAR-INFRARED SPECTROSCOPY—THEORY AND PRACTICE

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## 1. THEORY

Near-infrared (NIR) spectroscopy is a technique with broad and varied applications in pharmaceutical analysis. The NIR spectral region lies between the ultraviolet (UV)-visible and infrared regions, and at one time was considered the forgotten region of the electromagnetic spectrum. It is a branch of vibrational spectroscopy that shares many of the principles that apply to other spectroscopic techniques. The NIR spectral region comprises two sub-ranges (see *Figure 1*) associated with detectors used in instrumentation. The short-wavelength (Herschel) region extends from approximately 780–1100 nm (12,821–9000 $cm^{-1}$), and the longer wavelengths, which fall between 1100 and 2500 nm (9000–4000 $cm^{-1}$), compose the traditional NIR spectral region. It is common to express the wavelength ($\lambda$) in nanometers (nm) and the frequency ($\nu$) in reciprocal centimeters (wavenumber) as acquired by the instrument. Usually, Fourier-transform (FT) spectrometers report the *x*-axis in wavenumber ($cm^{-1}$), whereas a dispersive, monochromator-based instrument will show the *x*-axis in wavelength (nm).

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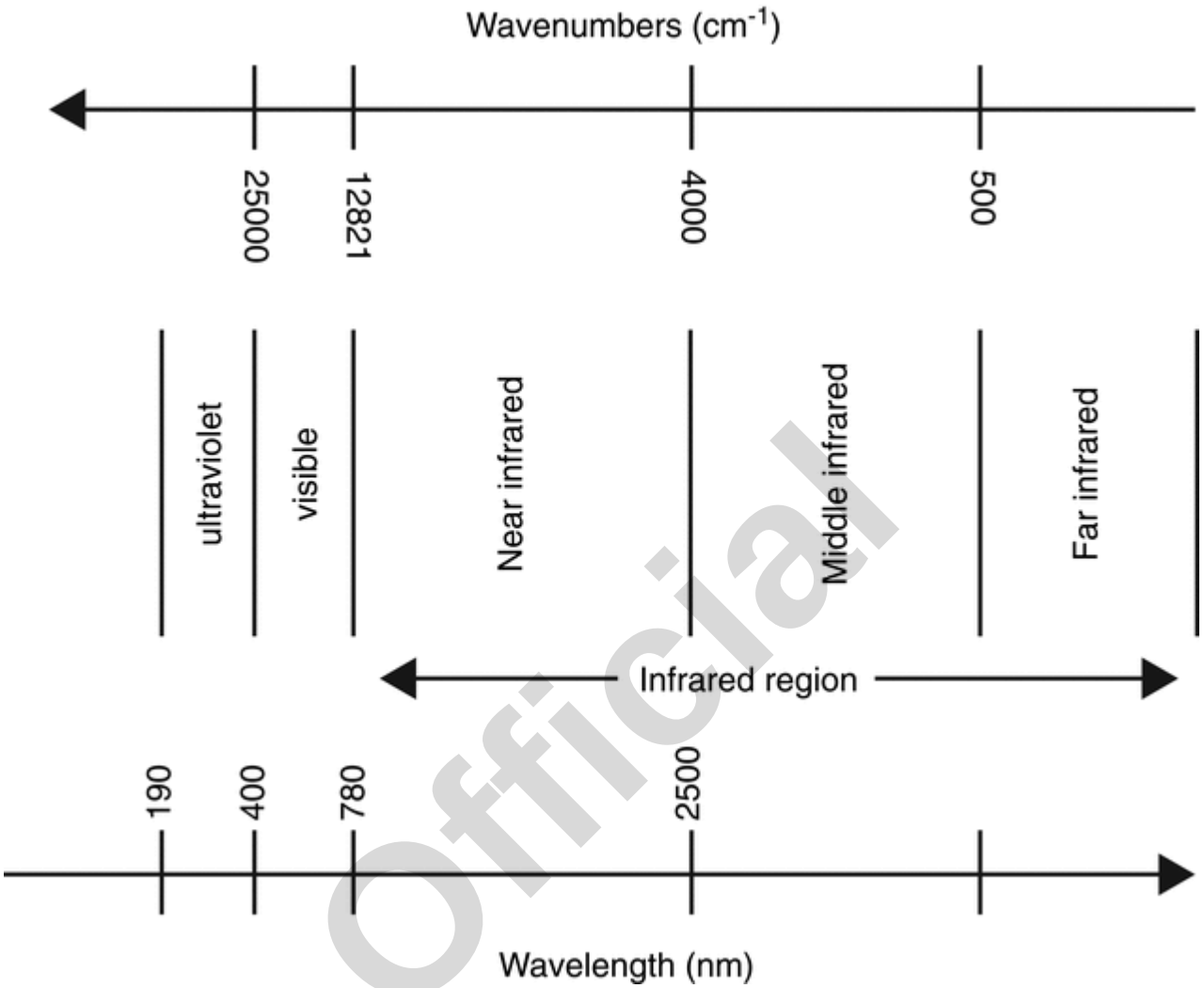


Figure 1. NIR region of the electromagnetic radiation spectrum.

NIR spectra are dominated by C-H, N-H, O-H, and S-H overtone resonances and combinations of fundamental mid-infrared vibrational modes from which they originate. Because molar absorptivities in the NIR range are low, radiation can penetrate several millimeters into materials, including solids where it can be absorbed when the wavelength of the radiation corresponds to a transition between the ground vibrational state of the analyte and either a harmonic of a given vibrational mode (an overtone) or the sum of two or more different modes (a combination band). Fiber-optic technology is readily implemented in the NIR range, which allows real-time, or near real-time, monitoring of processes in environments that might otherwise be inaccessible. As is the case with other spectroscopy measurements, interactions between NIR radiation and matter provide information that can be useful for both qualitative and quantitative assessment of the chemical composition of samples. In addition, qualitative and quantitative characterization of a sample's physical properties can be made because of the sample's influence on NIR spectra.

Measurements can be made directly on samples in situ in addition to applications during standard sampling and testing procedures. Applications of qualitative analysis include identification of raw material, in-process control testing, and finished-product release testing. NIR measurements can be performed off-line, but also at-line, in-line, and on-line for process analytical technology. These applications often involve comparing an NIR spectrum from a sample to a reference spectrum and assessing similarities against acceptance criteria developed and validated for a specific application. In contrast, applications of quantitative analysis involve the development of a predictive relationship between NIR spectral attributes and sample properties. Because of the highly covariant nature of the NIR signal, these applications typically use chemometrics models to quantitatively predict chemical and/or physical properties of the sample on the basis of its spectral attributes.

## 2. TRANSMISSION, REFLECTION, AND TRANSFLECTION MODES

The most common measurements performed in the NIR spectral range are transmission and reflection spectroscopy. Incident NIR radiation is absorbed or scattered by the sample and is measured as transmittance or reflectance, respectively.

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## 2.1 Transmission Mode

Transmittance ($T$) is the intensity ratio of the transmitted radiation ($I$) to the incident radiation ($I_0$), which represents the decrease in intensity at given wavelengths when radiation is passed through the sample. The sample is placed in the optical beam between the source and the detector. The arrangement is analogous to that in many conventional spectrophotometers, and the result can be presented directly in terms of transmittance and/or absorbance ($A$). NIR spectra are usually measured in absorbance ($A$).

$$T = \frac{I}{I_0} \text{ or } T = 10^{-A}$$

$I$ = intensity of transmitted radiation
$I_0$ = intensity of incident radiation

The measurement of transmittance is dependent on a background transmittance spectrum for its calculation. Examples of background references include air, a polymeric disc, an empty cell, a solvent blank, or in special cases, a reference sample. The method generally applies to gases, liquids (diluted or undiluted), dispersions, solutions, and solids, including tablets and capsules. For transmittance measurements of solids, a suitable sample accessory is to be used. Tablets and capsules can be analyzed using suitably engineered holders, thereby ensuring reproducible measurements. Liquid samples are examined in a cell of suitable path length (typically 0.5–4 mm) that is transparent to NIR radiation, or alternatively by immersion of a fiber-optic probe of a suitable configuration. Gases are usually measured using dedicated long path-length, multiple reflection cells.

## 2.2 Reflection Modes

### 2.2.1 REFLECTANCE

Reflectance ($R$) is the ratio of the intensity of light reflected from the sample, $I$, to that reflected from a background or reference reflective surface, $I_R$.

It is composed of both the specular (mirror) and the diffuse components (see *Figure 2*).

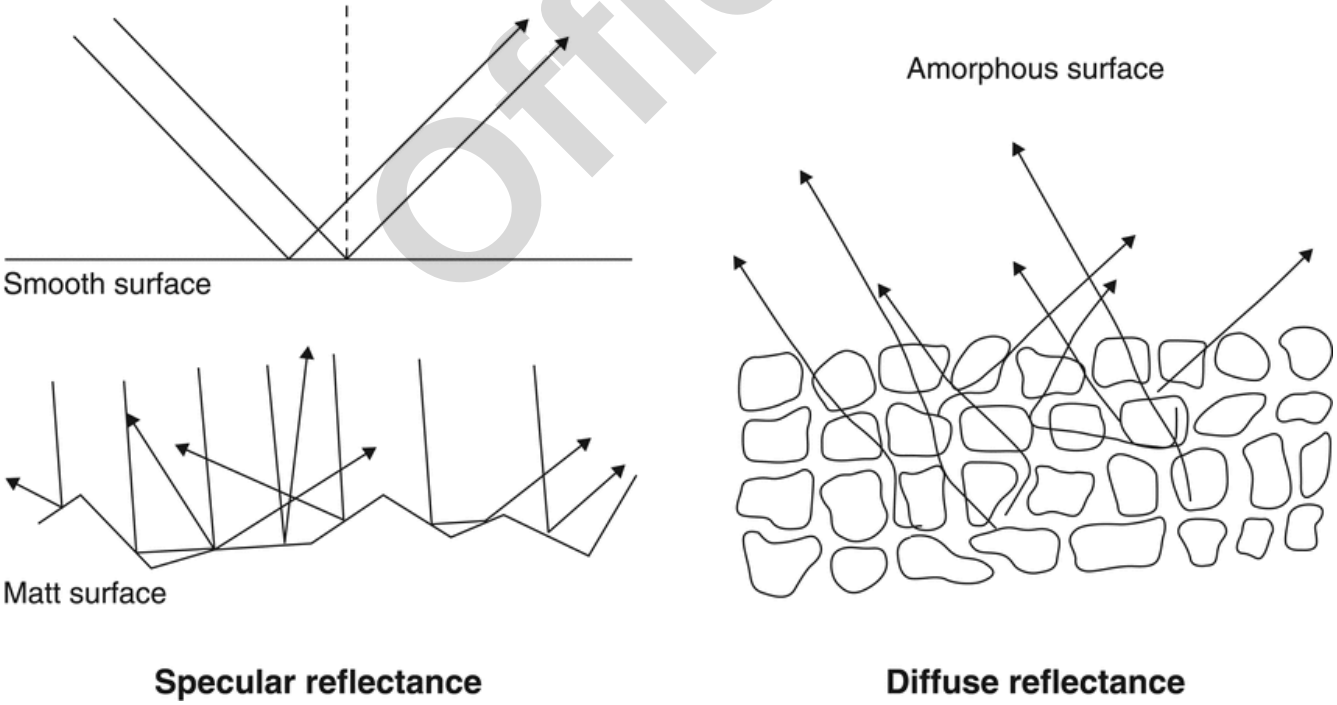


Figure 2. Specular and diffuse reflectances.

The determination of reflectance requires the measurement of a reference reflection spectrum to determine the attenuation of reflected light relative to the unattenuated incident beam. The reflectance spectrum is calculated as the ratio of the single-beam spectrum of the sample to that of the reference material.

$$R = \frac{I}{I_R}$$

$I$ = intensity of light diffusively reflected from the sample

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$I_R$ = intensity of light reflected from the background or reference reflective surface

Most reflection measurements in the NIR spectral region are made of scattering samples such as powders and slurries. For such materials, NIR radiation can penetrate a substantial distance into the sample, where it can be absorbed when the wavelength of the radiation corresponds to a transition between the ground vibrational state of the analyte and either a harmonic of a given vibrational mode (an overtone) or the sum of two or more different modes (a combination band). Nonabsorbed radiation is scattered back from the sample to the detector. NIR reflectance spectra typically are accessed by calculating and plotting log (1/*R*) versus wavelength. Other abscissa data transformations can also be used. This logarithmic form is the pseudo-absorbance of the material and is commonly called absorbance.

#### 2.2.2 Diffuse Reflection

The diffuse reflection mode gives a measure of reflectance (*R*) (penetrating the sample surface, interacting with the sample, and passing back through the sample's surface), which is the ratio of the intensity of light reflected from the sample (*I*) to that reflected from a background or reference reflective surface ($I_r$), where by using careful instrumental design, the specular component is often excluded. NIR radiation can penetrate a substantial distance into the sample, where it can be absorbed by vibrational combinations and overtone resonances of the analyte species present in the sample. Nonabsorbed radiation is partially reflected back from the sample to the detector.

$$R = \frac{I}{I_r}$$

$I$ = intensity of light diffusively reflected from the sample
$I_r$ = intensity of light reflected from the background or reference reflective surface

This mode generally applies to solids. The sample is examined in a suitable device. For process monitoring, material can be analyzed through a polished glass (e.g., sapphire) window interface, or using an in-line probe. Care must be taken to ensure that the measuring conditions are as reproducible as possible from one sample to another. The reflected radiation of a background reference is scanned to obtain the baseline, and then the reflectance of one or more analytical samples is measured. Common reflectance references include ceramic tiles, thermoplastic resins, and gold. Other suitable materials may be used. In some process-analysis situations, it may be impossible to remove a probe for reference background data collection; in these cases, consider various options including internal referencing; measurement of a background reference using a second detector; and others. Only spectra measured against a background possessing the same optical properties can be compared directly with one another.

### 2.3 Transflection Mode

The term transflection (*T**) is used to describe any double-pass transmission technique. Transflection spectrometry is a hybrid of transmission and reflection wherein a reflector is placed behind the sample so that the optical path through the sample and back to the detector is doubled, compared with a transmission measurement of a sample of the same thickness. Nonabsorbed radiation is reflected back from the sample to the detector. The light may be reflected from a diffuse or specular (mirror) reflector placed behind the sample.

$$T^* = \frac{I}{I_{T^*}}$$

$I$ = intensity of transflected radiation measured with the sample
$I_{T^*}$ = intensity of transflected radiation of the reference material as background

This mode generally applies to liquids and clear plastic materials. This configuration can be adapted to share the same instrument geometry with reflectance and fiber-optic probe systems where the source and the detector are on the same side of the sample. The sample is examined through a cell with a mirror or a suitable diffusive reflector made of either metal or an inert substance (e.g., dried titanium dioxide) not absorbing in the NIR region. Liquids can also be measured using in-line transflectance probes to increase intensity levels for more sensitive applications.

## 3. FACTORS THAT AFFECT NIR SPECTRA

NIR spectroscopy is advantageous because, as a result of lower molar absorptivities in this region of the electromagnetic spectrum, accurate measurements can often be made quickly without destroying the sample, and with minimal or no sample preparation; this applies whether the samples are solid, semi-solid, liquid, or gas. The NIR spectrum contains information on overtone resonances and combination of fundamental vibrational modes of the sample that can yield both sample and process understanding. Samples may be analyzed directly through packaging or directly in media that is transparent to wavelengths in the NIR spectral region. In addition, by definition NIR spectra are produced using the corresponding NIR radiation, and therefore standard glass/quartz and fiber optics may be used.

The following discussion is not exhaustive, but it includes many of the major factors that affect NIR spectra.

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## 3.1 Environmental Factors

The environmental temperature and humidity must be considered before carrying out measurements. Ideally, ambient conditions will be controlled to meet the operating specifications of the instrument manufacturer. However, tighter controls may not always be practical to the degree necessary for the application, and as such, another justifiable means of accounting for the influence of the environment (e.g., spectral preprocessing) should be utilized, with frequent verification. This is particularly relevant when considering the effect of temperature when using fiber-optic probes.

## 3.2 Sampling Factors

Depending on the measurement mode, sample preparation and presentation can vary. The following factors should be considered for all sampling techniques:

- Find the best suitable measurement mode for the intended application (transmission, diffuse reflection, or transflection)
- Find the best suitable accessory (e.g., transmission or immersion probes)
- Optimize path length in transmission and transflection modes
- Find a suitable spectroscopic background reference sample
- Show that the background reference sample is reliable over time and that the measurement of the background is reproducible and stable over time
- When measuring moving materials or samples (for process-related measurements), it is important to obtain a representative spectrum (e.g., by adjusting the measuring time or number of scans, by co-adding individual spectra, or by increasing the beam size)
- Ensure that there is not fouling of the sensor, for example, with build-up of material or contamination
- When measuring through packaging material, consider the variability of content and thickness
- Where multiple crystalline forms are present, care must be taken to ensure that the model calibration samples have a distribution of forms relevant to the intended application

### 3.2.1 SAMPLE PRESENTATION AREA

The sample presentation area or probe end must be clean and free of residue prior to the measurement. Similarly, the in-line or on-line interface to the sample should not have significant product or contamination build-up, which would interfere with the desired measurement.

### 3.2.2 SAMPLE TEMPERATURE

Sample temperature influences spectra obtained from aqueous solutions and other hydrogen-bonded liquids, and a difference of a few degrees may result in significant spectral changes. Temperature may also affect spectra obtained from less polar liquids, as well as solids that contain solvents and/or water.

### 3.2.3 MOISTURE AND SOLVENT

Moisture and solvent that are present in the sample material and analytical system may change the spectrum of the sample. Both absorption by moisture and solvent, and their influence on hydrogen bonding of the active pharmaceutical ingredients and excipients, can change the NIR spectrum.

### 3.2.4 SAMPLE THICKNESS

Sample thickness is a known source of spectral variability and must be understood and/or controlled, particularly for tablet and capsule analysis. The sample thickness in transmission mode is typically controlled by using a fixed optical path length for the sample. In diffuse reflection mode, the sample thickness may be controlled by using samples that are “infinitely thick” relative to the detectable penetration depth of NIR light into a solid material. Here, the term “infinite thickness” implies that the reflection spectrum does not change if the thickness of the sample is increased. For the measurement of compressed powders, an infinite thickness is typically reached after 5 mm of sample depth (e.g., in a filled vial).

### 3.2.5 SAMPLE OPTICAL PROPERTIES

With solids, both surface and bulk scattering properties of model calibration standards and analytical samples must be taken into account. Surface morphology and refractive index properties affect the scattering properties of solid materials. For powdered materials, particle size and bulk density influence scattering properties and the NIR spectrum. The spectra of physically, chemically, or optically heterogeneous samples may require sample averaging, examination of multiple samples, or spinning the sample to obtain a representative spectrum of the sample. Certain factors, such as differing degree of compaction or particle size in powdered materials and surface finish, can cause significant spectral differences.

### 3.2.6 SOLID-STATE FORMS

The variations in solid-state forms (polymorphs, hydrates, solvates, and amorphous forms) influence vibrational spectra. Hence, different crystalline forms as well as the amorphous form of a solid may be distinguished from one another on the basis of their NIR spectra. Where multiple crystalline forms are present, care must be taken to ensure that the calibration samples have a distribution of forms relevant to the intended application.

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#### 3.2.7 AGE OF SAMPLES

Samples may exhibit changes in their chemical, physical, or optical properties over time. Depending on the storage conditions, solid samples may either absorb or desorb water/solvent, and portions of amorphous materials may crystallize. Materials used for NIR model calibration are representative of future samples and their matrix variables. Hence, care must be taken to ensure that samples for NIR analysis are representative.

## 4. PRETREATMENT OF NIR SPECTRAL DATA

NIR spectral data to be used in qualitative or quantitative applications often need preprocessing to attenuate environmental or sampling factors, enhance a certain signal, or for other purposes that may include transformation, normalization, or other mathematical treatment. Preprocessing of samples (rows of data) may include mean or median centering, scaling, and other procedures. NIR responses (columns of data) also can be transformed, centered, or scaled. Because of the highly covariant nature of the NIR signal, it is common to remove linear or polynomial trends or apply filtering or smoothing techniques such as derivatives, wavelets, or a Savitzky–Golay filter. Spectral pretreatment techniques may include the numerical calculation of the first- or second-order derivative of the spectrum. Higher-order derivatives are not recommended because of increased spectral noise. For more detail, see *Chemometrics* ⟨1039⟩.

## 5. INSTRUMENTATION

### 5.1 Apparatus

All NIR measurements result from exposing material to incident NIR light radiation and measuring the attenuation of the emerging (transmitted, scattered, or reflected) light. Spectrophotometers for measurement in the NIR region consist of a suitable light source, such as a highly stable quartz–tungsten lamp, a monochromator or interferometer, and a detector. Common monochromators are acousto-optical tunable filters, gratings, or prisms. Traditionally, many NIR instruments have had a single-beam design, although some process instruments use internal referencing and can therefore be dual beam (for example, in diode array instruments). Silicon, lead sulfide, and indium gallium arsenide are examples of detector materials. Examples of sampling devices include conventional cuvette sample holders, fiber-optic probes, transmission dip cells, neutral borosilicate vials, and spinning or traversing sample holders. The choice is made on the basis of the intended application, with particular attention paid to the suitability of the sampling system for the type of sample to be analyzed. Suitable data processing and evaluation units (e.g., software and computer) are usually part of the system.

### 5.2 Imaging Techniques

NIR imaging is a combination of NIR spectroscopy with digital image processing. A NIR imaging system is basically composed of an illumination source, an imaging optic, a spectral encoder selecting the wavelengths, and a focal plane array. NIR imaging in particular has a huge potential for gaining rapid information about the chemical structure and related physical or biopharmaceutical properties of all types of pharmaceutical dosage forms, thus improving product quality and enhancing production speed.

Within a given material, the distribution of the various compounds can be characterized three-dimensionally by using imaging. Use of NIR imaging allows for the collection of detailed chemical information. NIR microscopy techniques have the following advantages:

- No preparation of the sample required
- Good spatial resolution
- Clear image quality
- Excellent chemical differentiation

It is possible to collect a signal from the entire sample (e.g., a tablet), disperse it into a spectrum using a spectrometer, and detect the spectrum using a multi-channel detector such as a charge couple detector or photo-diode array. In this case, instead of obtaining trivial cumulative information about the spot signal intensity, one can obtain a signal spectrum which can be transformed into detailed information about the chemical composition of the given spot on a sample. The three-dimensional array of data sets, two spatial and one spectral, recorded in such an imaging measurement has become known as a hypercube or data cube. These microscopy systems are capable of determining a spatial resolution down to approximately 1–5 µm.

NIR imaging systems are capable of characterizing the structure and distribution of the active components, and excipients within formulations, dosage forms, and delivery devices, to name a few. For example, chemical mapping for homogeneity testing of solid oral dosage forms, creams, and ointments may be useful in select applications.

### 5.3 Instrument Calibration Considerations

NIR instrument calibration involves three components: wavelength accuracy and uncertainty (*x*-axis); photometric linearity and response stability (*y*-axis); and photometric noise. *Table 1* provides an overview of performance verification criteria for a wide range of NIR systems.

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### 5.3.1 PHOTOMETRIC NOISE

For any given method, the validation should include an estimation of the photometric noise of the system, as a change in this parameter may affect any future model calibration.

### 5.3.2 WAVELENGTH ACCURACY AND UNCERTAINTY (*X*-AXIS)

In the case of FT-NIR instruments, primary instrument wavelength-axis calibration is maintained, at least to a first approximation, with an internal helium–neon laser. NIR spectra collected by the conventional grating based spectra from sample and/or reference standard materials can be used to demonstrate an instrument's suitable wavelength-dispersion performance against target specifications. Suitable materials for demonstrating wavelength-accuracy performance include methylene chloride, talc *R*, spectral calibration lamps, polystyrene, mixtures of rare earth oxides, and absorption by water vapor. Certified traceable standards are available from the National Institute of Standards and Technology (NIST) for transmittance measurements (SRM 2035a) and reflectance (SRM 2036) and can be used for wavelength verification. Other suitable standards may also be used. Instrument manufacturers may use polystyrene films (thickness of 0.75–2 mm) as an internal standard for wavelength qualification and control. However, because of the weak asymmetric signals emanating from polystyrene at short wavelengths, this material may not be suitable as a reference standard across the whole NIR range. When choosing an appropriate reference standard, it is important to confirm that the operating parameters (e.g., resolution, band width, and others) are within the limits of the certified material.

The effect of temperature on assignment of wavelength must be taken into account. Typically, standards are measured in environments near 25°C, and the temperature at which certified values were taken will be indicated. The effect of resolution can also have an impact on the measured values. Resolution for FT-NIR spectrometers is typically 8 $cm^{-1}$, but modern spectrometers are capable of higher resolutions (2 $cm^{-1}$ or 1 $cm^{-1}$).

Standard certificates will indicate resolution and possibly even peak assessment algorithms. The choice of reference standard for a performance test should always match the resolution and environmental conditions expected for the execution of the test. Additional standards may be necessary, depending on the range of wavelengths or reference standards meeting these conditions.[1] Refer to *Table 1* and *Table 2* for additional information.

### 5.3.3 PHOTOMETRIC LINEARITY AND RESPONSE STABILITY (*Y*-AXIS)

Calibration of the photometric axis can be critical for successful quantification. Both FT-NIR and dispersive NIR spectrometers should undergo similar instrument calibration procedures. The tolerance of photometric precision acceptable for a given measurement should be assessed during the method development and validation stages.

NIR spectra from samples and/or reference standard materials with known relative transmittance or reflectance can be used to demonstrate a suitable relationship between NIR light attenuation (resulting from absorption) and instrument response. Verification of photometric linearity and verification of photometric noise are not required for instruments using methods to perform simple identifications that do not use the photometric absorbance as part of the model strategy (e.g., simple correlation with absorbing wavelengths). The USP Near IR System Suitability RS is recommended, but other certified traceable standards exist in the market and may be used. For reflectance measurements, commercially available reflectance standards with known reflectance properties are often used.

Spectra obtained from reflection standards are subject to variability because the experimental conditions under which they were factory calibrated differ from those under which they are subsequently put to use. Hence, the reflectance values supplied with a set of calibration standards may not be useful in the attempt to establish an "absolute" calibration for a given instrument. The reproducibility of the photometric scale will be established over the range of standards provided that 1) the standards do not change chemically or physically, 2) the same reference background is also used to obtain the standard values, and 3) the instrument measures each standard under identical conditions (including precise sample positioning). Subsequent measurements on the identical set of standards provide information on long-term stability. Refer to *Table 1* and *Table 2* for additional information.

Photometric linearity is demonstrated by using a set of transmission or reflection standards with known values for percentage transmittance or reflectance. For reflectance measurements, carbon-doped polymer standards are available. It is important to ensure that the absorbance of the materials used is relevant to the intended linear working range of the method. Nonlinear calibration models, which are acceptable, may be used, as long as the user can demonstrate adequate justification for this approach.

[1] Burgess C, Hammond J. Wavelength standards for the near-infrared spectral region. *Spectroscopy*. 2007;22(4):40–48.

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**Table 1. Control of Bench/Mobile Instrument Performance**

| Measurement Mode | Reflection | Transflection | Transmission |
|---|---|---|---|
| | Measure talc via a suitable medium or by fiber-optic probe. Talc has suitable-for-calibration, characteristic peaks at 948, 1391, 2077, and 2312 nm. Alternatively, other suitable standards may also be used that ensure wavelength accuracy in the region of working methodology. For example, measure an internal polystyrene standard if built in, or measure an NIST standard or other traceable material (e.g., USP Near IR System Suitability RS) and assess 3 peaks across the wavelength range for calibration. | A suspension of 1.2 g of dry titanium dioxide in about 4 mL of methylene chloride is used directly through the cell or using a probe. Titanium dioxide has no absorption in the NIR range. Spectra are recorded with a maximum nominal instrument bandwidth of 10 nm at 2500 nm (16 $cm^{-1}$ at 4000 $cm^{-1}$). Methylene chloride has characteristic sharp bands at 1155, 1366, 1417, 1690, 1838, 1894, 2068, and 2245 nm. Choose 3 peaks across the wavelength range for calibration. Other suitable standards may also be used, such as TS5 liquid mixed with titanium dioxide or some other reflective medium. | Methylene chloride may be used and has characteristic sharp bands at 1155, 1366, 1417, 1690, 1838, 1894, 2068, and 2245 nm. Choose 3 peaks across the wavelength range for calibration. Other suitable standards may also be used, such as NIST SRM 2065, Polystyrene 65 μm, and TS5 liquid. |
| Verification of wavelength scale (except for filter apparatus) | For FT instruments, the calibration of the wavenumber scale may be performed using a narrow, isolated water-vapor line (for example, the line at 7306.74, 7299.45, or 7299.81 $cm^{-1}$). | | |
| Verification of wavelength repeatability (except for filter apparatus) | The standard deviation of the wavelength is consistent with the specifications of the instrument manufacturer, or otherwise scientifically justified. Verify the wavelength repeatability using a suitable external or internal standard. | | |
| Verification of photometric linearity and response stability[a] | Measure 4 photometric standards across the working method absorbance range. Analyze 4 reference standards, for example, in the range of 10%–90%, including 10%, 20%, 40%, and 80% with respective absorbance values of 1.0, 0.7, 0.4, and 0.1. Evaluate the observed absorbance values against the reference absorbance values (for example, perform a linear regression). Acceptable tolerances are 1.00 ± 0.05 for the slope and 0.00 ± 0.05 for the intercept for the first verification of photometric linearity of an instrument. Subsequent verifications of photometric linearity can use the initial observed absorbance values as the reference values. | | |
| Verification of photometric noise | Determine the photometric noise using a suitable reflectance or transmittance standard (for example, white reflective ceramic tiles or reflective thermoplastic resins [i.e., polytetrafluoroethylene (PTFE)]). Follow the manufacturer's methodology and specifications. Scan the reflectance standard over a suitable wavelength range in accordance with the manufacturer's recommendation and calculate the photometric noise as peak-to-peak noise. | | |

[a] Burgess C, Hammond J. Wavelength standards for the near-infrared spectral region. *Spectroscopy*. 2007;22(4):40–48.

**Table 2. Control of Process Instrument Performance**

| Measurement Mode | Reflection | Transflection | Transmission |
|---|---|---|---|
| Verification of wavelength scale (except for filter apparatus) | If it is not practically possible to measure a traceable standard material at the point of sample measurement, use internal material such as polystyrene, fiberglass, or solvent and/or water vapor. Alternatively, adopt a second external fiber/probe. For FT instruments, the calibration of the wavenumber scale may be performed using a narrow, isolated water-vapor line (for example, the line at 7306.74, 7299.45, or 7299.81 $cm^{-1}$) or a narrow line from a certified reference material. | | |
| Verification of wavelength repeatability (except for filter apparatus) | The standard deviation of the wavelength is consistent with the specifications of the instrument manufacturer, or otherwise scientifically justified. Verify the wavelength repeatability using a suitable external or internal standard. | | |
| Verification of photometric linearity and response stability[a] | Measure 4 photometric standards across the working method absorbance range. | | |
| | If photometric reflectance standards cannot be measured at the point of sample measurement, use the manufacturer's internal photometric standards. Process instruments can use internal photometric standards for photometric linearity. Follow the manufacturer's verified tolerances in such cases. | Analyze 4 reference standards to cover the absorbance values over the working absorbance range of the modelled data. Evaluate the observed absorbance values against the reference absorbance values (for example, perform a linear regression). Acceptable tolerances are 1.00 ± 0.05 for the slope and 0.00 ± 0.05 for the intercept for the first verification of photometric linearity of an instrument. Subsequent verifications of photometric linearity can use the initial observed absorbance values as the reference values. | |
| Verification of photometric noise | Determine the photometric noise using a suitable reflectance or transmittance standard [for example, white reflective ceramic tiles or reflective thermoplastic resins (i.e., PTFE)]. Follow the manufacturer's methodology and specifications. | | |
| | As above, or if not practically possible, use the manufacturer's internal standard for noise testing and specifications. | Scan the transmittance high-flux standard over a suitable wavelength/wavenumber range in accordance with the manufacturer's recommendation and calculate the photometric noise as peak-to-peak noise. | |

[a] Burgess C, Hammond J. Wavelength standards for the near-infrared spectral region. *Spectroscopy*. 2007;22(4):40–48.

### 5.3.4 EXTERNAL INSTRUMENT CALIBRATION

Detailed functional validation employing external reference standards is recommended to demonstrate instrumental suitability of laboratory instruments, even for instruments that possess an internal calibration approach. The use of external reference standards does not obviate the need for internal quality-control procedures; rather, it provides independent documentation of the fitness of the instrument for the specific analysis or purpose. For instruments installed in a process location or in a reactor where positioning of an external standard routinely is not possible, including those instruments that employ an

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internal calibration approach, the relative performance of an internal versus an external calibration approach should be evaluated periodically. The purpose of this test is to check for changes or drifts in components (e.g., the process lens or fiber-optic probe) that might not be included in the internal calibration method; this could, for example, affect the photometric calibration of the optical system.

#### 5.3.5 MULTI-INSTRUMENT CALIBRATION

There are many approaches for ensuring a transferable calibration across multiple instruments of the same type (same make and model) and different types. This is an area of growing interest because of the globalization of manufacturing and regulatory controls. Today, reference standards have been developed to cover this region, but many deficiencies still exist regarding wavelength uncertainty and photometric response curves. Therefore, verification of compendial procedures, method transfers, and spectrometer platform changes is compromised by the bias involved in differences between instruments. Understanding the risk associated with these variations should be a part of any quality-by-design initiative to use NIR spectroscopic procedures for product control.

The use of appropriate certified reference materials can be helpful in establishing instrumental variance and/or bias. As stated above, the value assignments included on the certification of such materials will contain significant contributions to the overall expanded uncertainty budget from the instrumental factors used in generating these values. For example, the optical geometry, polarization, and other aspects of a reflectance measurement will significantly affect the measured value, and therefore wherever possible these characteristics should be matched to the required measurement configuration. However, by definition, a reference material should have the essential characteristics of appropriate stability and homogeneity to at least allow comparative measurements to be made, albeit they may be biased by known or unknown factors.

## 6. APPLICATIONS

NIR spectroscopy has a wide variety of applications for chemical, physical, and process analysis.

For chemical analysis, the applications include:

- Identification of active substances, excipients, dosage forms, manufacturing intermediates, chemical absorbance materials, and packaging materials
- Qualification of ingredients, intermediates, and drug products, including batch-to-batch spectral comparison and supplier change assessment
- Quantification of active substances and excipients; determination of chemical values such as hydroxyl value; determination of absolute water content; determination of degree of hydroxylation; and control of solvent content

For physical analysis, the applications include:

- Crystalline form and crystallinity, polymorphism, solvates, and particle size
- Analysis of intact pharmaceutical dosage forms: tablets, capsules, lyophilized products, and implants (e.g., polymeric and microspheres)
- Dissolution behavior, disintegration pattern, and hardness
- Examination of film properties

For process monitoring and process control analysis, the applications include:

- Monitoring of unit operations such as synthesis, crystallization, blending (e.g., powder), pelletization, tableting, capsule filling, drying, granulation, coating (e.g., film), and packaging for the purpose of process control

Measurements in the NIR spectral region are influenced by many chemical and physical factors. The reproducibility and relevance of the results depend on the control of these factors. Usually, measurements are only valid for a defined system.

### 6.1 Qualitative Analysis: Identification and Characterization

#### 6.1.1 ESTABLISHMENT OF A SPECTRAL REFERENCE LIBRARY

To establish a spectral reference library, record the spectra of a suitable number of representative samples of the substance; the samples should have known, traceable identities. These representative samples should exhibit the variation that is typical for the substance to be analyzed (e.g., variation in solid-state form and particle size). The set of spectra obtained represents the information that can be used for chemical and/or physical identification of the sample to be analyzed.

The collection of spectra in the library may be represented in different ways, which are defined by the mathematical technique used for identification. These may be:

- All individual spectra representing the substance
- A mean spectrum of the measured batches for each chemical substance or physical form
- If necessary, a description of the variability within the substance spectra

The number of substances to be included in the library depends on the specific application. All spectra in the library will have the same spectral range and number of data points; technique of measurement; and data pretreatment. If subgroups (libraries) are created, the above criteria should be applied independently to each group. Origin spectral data for the preparation of the spectral library must be archived. Caution must be exercised when performing any mathematical transformation, as artifacts can be introduced or essential information can be lost. The suitability of the algorithm chosen should be demonstrated by successful method validation, and in all cases, the rationale for the use of transformation must be documented.

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#### 6.1.2 NIR REFERENCE SPECTRA

NIR references provide known, stable measurements to which other measurements can be compared; thus, they are used to minimize instrumental and environmental variations that would affect the measurement. Direct comparison of representative spectra of the substance to be examined and a reference substance for qualitative chemical or physical identification purposes may not require use of a reference spectral library, where specificity permits.

#### 6.1.3 DATA EVALUATION

Direct comparison is made between the representative spectrum of the substance to be examined and the individual or mean reference spectra of all substances in the database on the basis of their mathematical correlation or other suitable algorithms. A set of known reference mean spectra and the variability around this mean can be used with an algorithm for classification; alternatively, this can be achieved visually by overlaying spectral data if specificity is inherent. Different calibration techniques are available, such as principal component analysis, cluster analysis, etc.

#### 6.1.4 VALIDATION OF THE MODEL

Chemical or physical identification methods using direct spectral comparison must be validated in accordance with identification method validation procedures. The validation parameters for qualitative methods are robustness and specificity.

#### 6.1.5 RELATIVE COMPARISON OF SPECTRA

A model calibration is not required when comparing a set of spectra for limit analysis purposes, such as determining the maximum or minimum absorbance at which an analyte absorbs. Also, in-process control of a drying operation may use a qualitative approach around a specific absorbing wavelength. Appropriate spectral ranges and pretreatments (if used) must be shown to be fit for purpose.

#### 6.1.6 SPECIFICITY

The relative discriminatory power and selectivity of a limit test must be demonstrated. The extent of specificity testing is dependent on the application and the risks being controlled. Variations in matrix concentrations within the operating range of the method must not affect the measurement.

### 6.2 Trend Analysis

#### 6.2.1 RELATIVE COMPARISON OF SPECTRA

NIR-based models can also be used to assess process trajectories. An example application is the use of NIR-based models to determine the endpoint of blending operation. Typically, a reference trajectory is defined based on a pool of batches that are deemed to present nominal variability; then each new batch trajectory gets compared with the reference trajectory and associated tolerances.

A model calibration is not necessarily required when comparing a set of spectra for trend analysis purposes, for example, using the moving-block approach to estimate statistical parameters such as mean, median, and standard deviation. For example, blend-uniformity monitoring using NIR spectroscopy has adopted such data analysis approaches. Appropriate spectral ranges and algorithms must be used for trend analyses.

#### 6.2.2 SPECIFICITY

The relative discriminatory power and selectivity for trend analysis must be demonstrated. The extent of specificity testing is dependent on the application and the risks being controlled. Variations in matrix concentrations within the operating range of the method must not affect the trend analysis.

### 6.3 Quantitative Analysis

#### 6.3.1 SPECTRAL REFERENCE LIBRARY FOR A CALIBRATION MODEL

Calibration is the process of constructing a mathematical model to relate the response from an analytical instrument to the properties of the samples. Any calibration model that can be defined clearly in a mathematical expression and gives suitable results can be used. Record the spectra of a suitable number of representative samples with known or future-established values of the attribute of interest (for example, content of water) throughout the range to be measured. The number of samples for calibration will depend on the complexity of the sample matrix and interferences (e.g., temperature, particle size, and others). It is encouraged to take a risk management approach to identify critical interferences that may need to be assessed. All samples must give quantitative results within a calibration interval as defined by the intended purpose of the method. Multiple linear regression, principal component regression (PCR), and partial least squares regression (PLS) are commonly used. For PLS or PCR calibrations, the regression coefficients and/or the loadings should be plotted, and the regions of large coefficients or loadings should be compared with the spectrum of the analyte. Predicted residual error sum of squares plots or similar plots are useful for facilitating the optimization of the number of PCR or PLS factors. For more information, see ISO 12099.

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### 6.3.2 PRETREATMENT OF DATA

Wavelength selection, or exclusion of certain wavelength ranges, may enhance the efficiency of calibration models. Wavelength compression (wavelength averaging) techniques, multiplicative signal correction (MSC), standard normal variate transformation (SNV), and derivatives may be applied to the data.

### 6.3.3 MODEL VALIDATION PARAMETERS

Analytical performance characteristics to be considered for demonstrating the validation of NIR methods are similar to those required for any analytical procedure. Specific acceptance criteria for each validation parameter must be consistent with the intended use of the method. Validation parameters for quantitative methods are accuracy, linearity over the operational range, precision (repeatability and intermediate precision), robustness, and specificity.

### 6.3.4 ONGOING MODEL EVALUATION

NIR models validated for use are subjected to ongoing performance evaluation and monitoring of validation parameters. If discrepancies are found, corrective action is necessary.

## 7. PROCEDURE VALIDATION

Validation of NIR methods will follow the same protocols described in *Validation of Compendial Procedures* ⟨1225⟩ in terms of accuracy, precision, and other suitable parameters. It may be necessary to determine QL for methods of detection and quantification of an impurity or polymorphic form.

Detector linearity must be confirmed over the range of possible signal levels. Method precision must also encompass sample position. The sample presentation is a critical factor for both solids and liquids, and must be either tightly controlled or accounted for in the calibration model. Sample-position sensitivity can often be minimized by appropriate sample preparation or sample holder geometry, but will vary from instrument to instrument on the basis of excitation and optical configuration. In addition, many suitable chemometric algorithms for data pretreatment and calibration are available. Selection of an algorithm should be based on sound scientific judgment and suitability for the intended application.

## 7.1 Ongoing Method Evaluation

Validated NIR methods should be subject to ongoing performance evaluation, which may include monitoring accuracy, precision, and other suitable method parameters. If performance is unacceptable, corrective action is necessary. This involves conducting an investigation to identify the cause of change in method performance, and may indicate that the NIR method is not suitable for continued use. Improving the NIR method to meet measurement suitability criteria may require additional method development and documentation of validation experiments demonstrating that the improved method is suitable for the intended application. The extent of revalidation that is required depends on the cause of change in method performance and the nature of corrective action needed to establish suitable method performance. Appropriate change controls should be implemented to document ongoing method improvement activities.

Revalidation of a qualitative model may be necessary as a result of the following:

- Changes in instrument hardware
- Addition of a new material to the spectral reference library
- Changes in the physical properties of the material
- Changes in the source of material supply
- Identification of previously unknown critical attribute(s) of material(s)

Revalidation of a quantitative model may be necessary as a result of the following:

- Changes in the composition of the test sample or finished product
- Changes in the manufacturing process
- Changes in the sources or grades of raw materials
- Changes in the reference analytical method
- Major changes in instrument hardware

## 7.2 Method Transfer

Controls and measures for demonstrating the suitability of NIR method performance following method transfer are similar to those required for any analytical procedure. Any exceptions to general principles for conducting method transfer for NIR methods should be justified on a case-by-case basis. The transfer of an NIR method is often performed by using an NIR calibration model on a second instrument that is similar to the primary instrument used to develop and validate the method. When a calibration model is transferred to another instrument, procedures and criteria must be applied to demonstrate that the calibration model meets suitable measurement criteria on the second instrument. The selection of an appropriate calibration-model transfer procedure should be based on sound scientific judgment.

## USP Reference Standards ⟨11⟩

USP Near IR System Suitability RS

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## GLOSSARY

[NOTE—This section refers to the technique of NIR in general. Please see *Analytical Instrument Qualification* ⟨1058⟩ and ⟨1039⟩ for additional information.]

**Absorbance:** Absorbance (*A*) is represented by the equation:

$$A = -\log T = \log (1/T)$$

where *T* is the transmittance of the sample. Absorbance is also frequently given as:

$$A = \log (1/R)$$

where *R* is the reflectance of the sample.

**Background spectrum:** This is used for generating a sample spectrum with minimal contributions from instrument response; it is also referred to as a "reference spectrum" or "background reference". The ratio of the sample spectrum to the background spectrum produces a transmittance or reflectance spectrum dominated by NIR spectral response associated with the sample. In reflection measurements, a highly reflective, diffuse standard reference material is used for the measurement of the background spectrum. For transmission measurement, the background spectrum may be measured with no sample present in the spectrometer or using a cell with the solvent blank or a cell filled with appropriate reference material.

**Diffuse reflectance:** Ratio of the spectrum of radiated light (penetrating the sample surface, interacting with the sample, and passing back through the sample's surface) reaching the detector to the background spectrum. This is the component of the overall reflectance that produces the absorption spectrum of the sample.

**Fiber-optic probe:** This consists of two components: optical fibers that may vary in length and number, and a terminus, which contains specially designed optics for examination of the sample matrix.

**Installation qualification:** The documented collection of activities necessary to establish that an instrument is delivered as designed and specified and is properly installed in the selected environment, which is suitable for the instrument's intended purpose.

**Instrument bandwidth or resolution:** A measure of the ability of a spectrometer to separate radiation of similar wavelengths.

**Operational qualification:** The process of demonstrating and documenting that an instrument performs according to specifications and that it can perform the intended task. This process is required following any significant change, such as instrument installation, relocation, or major repair.

**Overall reflectance:** The sum of diffuse and specular reflectance.

**Performance qualification:** The process of using one or more well-characterized and stable reference materials to verify consistent instrument performance. Performance qualification may employ the same or different standards for different performance characteristics.

**Photometric linearity:** Also referred to as "photometric verification", it is the process of verifying the response of the photometric scale of an instrument.

**Pseudo-absorbance:** *A*, is represented by the equation:

$$A = -\log R = \log (1/R)$$

where *R* is the diffuse reflectance of the sample.

**Reference spectrum:** See *Background spectrum*.

**Reflectance:** Is described by the equation:

$$R = \frac{I}{I_R}$$

in which *I* is the intensity of radiation reflected from the surface of the sample and $I_R$ is the intensity of radiation reflected from a background reference material and its incorporated losses due to solvent absorption, refraction, and scattering.

**Root-mean-square (RMS):** Calculated using the equation:

$$\text{RMS} = \sqrt{\sum_{i=1}^{N} \frac{\left(\hat{A} - A_i\right)^2}{N}}$$

where $\hat{A}$ is the mean absorbance over the spectral segment; $A_i$ is the absorbance for each data point; and *N* is the number of points per segment.

**Specular (surface) reflectance:** The reflectance from the front surface of the sample, where the angle of reflection matches the angle of incidence (mirror effect).

**Standard error of calibration (SEC):** A measure of the capability of a model to fit reference data. SEC is the standard deviation of the residuals obtained from comparing the known values for each of the calibration samples to the values that are calculated from the calibration. SEC should not be used as an assessment tool for the expected method accuracy (trueness and precision of prediction) of the predicted value of future samples. The method accuracy should generally be verified by calculating the *Standard error of prediction (SEP)* using an independent validation set of samples. An accepted method is to mark a part of the calibration set as the validation set. This set is not fully independent but can be used as an alternative for the determination of the accuracy.

**Standard error of the laboratory (SEL):** A calculation based on repeated readings of one or more samples to estimate the precision and/or accuracy of the reference laboratory method, depending on how the data were collected.

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**Standard error of prediction (SEP):** A measure of model accuracy of an analytical method based on applying a given calibration model to the spectral data from a set of samples different from, but similar to, those used to calculate the calibration model. SEP is the standard deviation of the residuals obtained from comparing the values from the reference laboratory to those from the method under test for the specified samples. SEP provides a measure of the model accuracy to be expected when one measures future samples.

**Surface reflectance:** Also known as "specular reflection", it is that portion of the radiation not interacting with the sample but simply reflecting back from the sample surface layer (i.e., the sample–air interface).

**Transflection:** A transmittance measurement technique in which the radiation traverses the sample twice. The second time occurs after the radiation is reflected from a surface behind the sample.

**Transmittance:** Represented by the equation:

$$T = \frac{I}{I_0} \text{ or } T = 10^{-A}$$

where $I$ is the intensity of the radiation transmitted through the sample; $I_0$ is the intensity of the radiant energy incident on the sample ; and $A$ is the absorbance.▲ (USP 1-May-2020)

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# ⟨1857⟩ ULTRAVIOLET-VISIBLE SPECTROSCOPY—THEORY AND PRACTICE

***Change to read:***

## THEORY

Ultraviolet-visible (UV-Vis) spectroscopy is an electronic transition spectroscopic technique in which the interaction between incident radiation and electrons results in the promotion of one or more of the outer or the bonding electrons from a ground state into a higher-energy state. This quantum effect results in a specific absorption of radiation, the frequency and wavelength of which are governed by the equation:

$$E = h\nu = \left(\frac{hc}{\lambda}\right) \times 10^9$$

where:

$E$ = energy
$h$ = Planck's constant ($6.63 \times 10^{-34} J \cdot s$)
$\nu$ = frequency (Hz), related to the energy change $\Delta E$, induced when electromagnetic radiation is absorbed ($\Delta E = h\nu$ per photon)
$c$ = velocity of light ($2.998 \times 10^8$ m/s)
λ = wavelength (nm)

▲All absorption of ultraviolet (UV) and visible (Vis) radiation in organic molecules is restricted to certain functional groups (chromophores) that contain valence electrons of low excitation energy. The spectrum of a molecule containing these chromophores is complex. This is because the superposition of rotational and vibrational transitions on the electronic transitions gives a combination of overlapping lines. This appears as a continuous absorption band, in the form of a bell-shaped peak. As a general rule, small molecules absorb somewhere in the ultraviolet region, and the greater the extent to which the p electrons are delocalized over a ring or a larger complex multiple-ring or branched structure, the longer the wavelength of the first absorption band (i.e., the band of lowest energy and longest wavelength). This delocalization over multiple-ring structures can shift the wavelength into the visible region and is responsible for the observed color of the substance.▲ (USP 1-Dec-2022)

***Change to read:***

## ▲UV-VIS SPECTROMETERS▲ (USP 1-DEC-2022)

All modern UV-Vis measurements involve detecting and measuring the intensity ratio of the radiation at a certain wavelength in the presence or absence of the absorbing sample ▲(transmitted radiation and incident radiation). *Figure 1* shows schematics of four typically encountered optical designs: single-beam, single split-beam (where the reference detector is located within the internal optics of the system), double-beam, and reverse optic array spectrometers.▲ (USP 1-Dec-2022) Dispersion of light to achieve the desired resolution can occur before ▲(as in the first three configurations)▲ (USP 1-Dec-2022) or after introduction of the sample, but all commercial UV-Vis instruments share the following features to perform these functions:

- Continuum source
- Monochromator or polychromator
- Sampling area
- Detector

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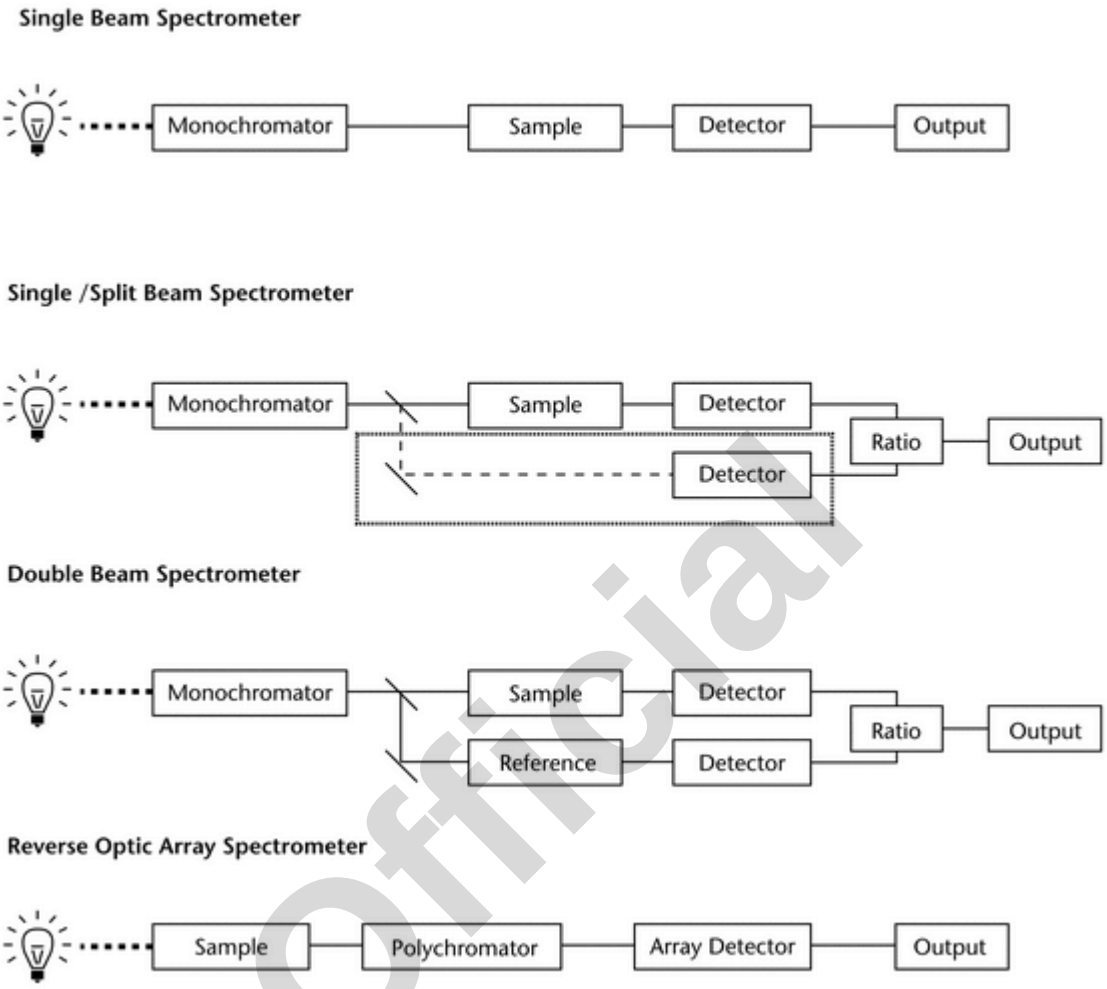


Figure 1. Typical spectrometer designs.

▲ (USP 1-Dec-2022)

Care must be taken to ensure that the sample is not degraded by the incident light beam and that any heating effects are minimized. This is particularly important when ▲photodiode array▲ (USP 1-Dec-2022) instruments are used because the sample is irradiated at all wavelengths.

## Continuum Source

Two major types of continuum source are currently in use: continuous and pulsed. Continuous sources include tungsten halogen for visible, deuterium arc for UV, and xenon arc for both. The source for pulsed radiation is the xenon flash lamp. Many UV-Vis instrument systems use a combination of deuterium and tungsten halogen to effectively cover the UV and visible regions, respectively. By necessity, source selection is achieved either by the use of a mirror ▲system to select the appropriate source, positioned manually or by using a hardware and software combination.▲ (USP 1-Dec-2022) This change usually is performed in the region of 320–350 nm, and thus qualification of the system must be performed using both source and mirror positions. Systems based on xenon lamps have the benefit of a single source and a higher energy output, but they are more expensive.

## Monochromator

The wavelength scale can be encoded by either a scanning monochromator or a grating polychromator (see *Figure 2*), as is the case for ▲spectrometers▲ (USP 1-Dec-2022) equipped with linear or two-dimensional array detectors. A discussion of the specific benefits and drawbacks of each of the dispersive designs is beyond the scope of this general chapter. Any properly qualified instrument should be suitable for qualitative measurements. Care must be taken when selecting an instrument for quantitative measurements because dispersion, response linearity, and stray light may not be uniform across the full spectral range. ▲Appropriate qualification procedures can be found in *Ultraviolet-Visible Spectroscopy* ⟨857⟩.▲ (USP 1-Dec-2022)

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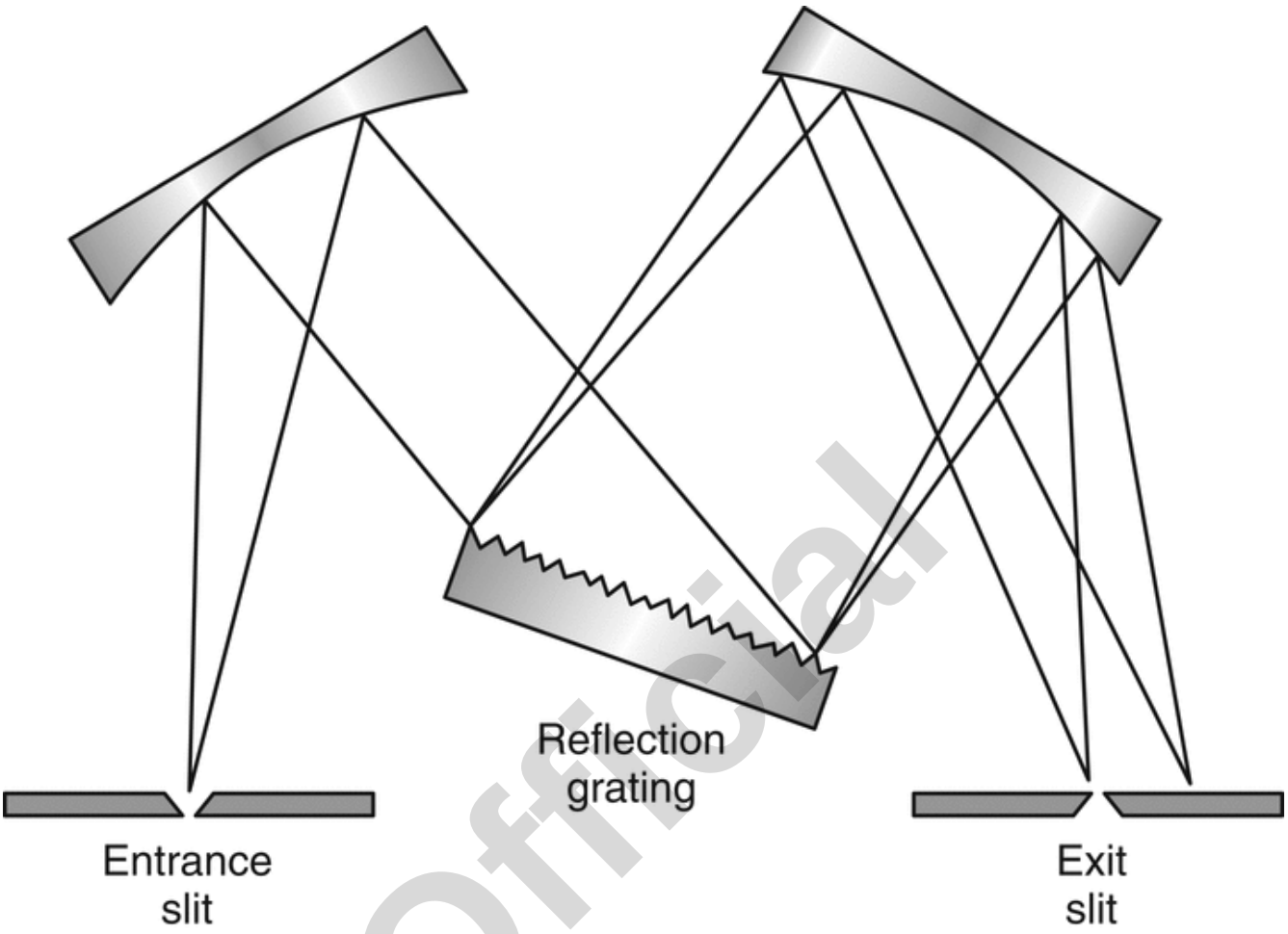


Figure 2. Grating monochromator.

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## Sampling Area

Numerous sampling arrangements are available in addition to the ▲cuvette▲ (USP 1-Dec-2022) holders that are designed to accommodate various path-length configurations based on conventional rectangular ▲cuvettes.▲ (USP 1-Dec-2022) These include flow ▲cuvettes,▲ (USP 1-Dec-2022) fiber-optic-based immersion probes, ▲multi-channel▲ (USP 1-Dec-2022) plate configurations, and automated sample changers, among others. Considerations such as sampling volume, speed of measurement, and reproducibility of sample presentation should be evaluated to optimize the sampling device for specific applications.

## Detector

Photoelectric detectors, which are the most common form of UV-Vis detectors, generate an electric current that is directly proportional to the intensity of the radiant energy incident upon them. They may take the form of photosensitive semiconductor devices, either discrete detectors, linear or two-dimensional arrays, or photomultipliers. Photosensitive semiconductor devices include solid-state photodiodes, charge-coupled device arrays (CCDs), and phototransistors. The most common semiconductor material is silicon, which is sensitive to wavelengths of 400–800 nm, but some silicon devices have extended sensitivity from as short a wavelength as 190 nm to as long a wavelength as 1100 nm. The dynamic response of these detectors typically is four orders of magnitude. Most ▲photodiode▲ (USP 1-Dec-2022) array detectors are made of silicon and hence have a similar wavelength response. Other semiconductor materials can provide wavelength response to several micrometers.

In contrast, photomultipliers are vacuum detectors that have a photocathode in which photon energy releases electrons that are directed by the field applied to electron-sensitive plates. By a cascade effect, these dynodes amplify the electrons released initially by the absorption of the incident radiation. Photomultipliers have typical wavelength responses of 160–900 nm, although some photocathode materials can provide response to higher wavelengths. The dynamic response of these detectors typically is six orders of magnitude or higher.

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## ALTERNATIVE UV-VIS ▲SPECTROMETER▲ (USP 1-DEC-2022) CONFIGURATIONS

### ▲Photodiode Array Spectrometers

With photodiode array detection,▲ (USP 1-Dec-2022) the optical configuration is reversed from that in a conventional ▲spectrometer,▲ (USP 1-Dec-2022) and the light beam passes through the sample before being dispersed by the polychromator. This gives the benefit of fast, full spectral data with no moving parts that can wear out. The perception is, therefore, that ▲photodiode▲ (USP 1-Dec-2022) arrays are more reliable than other detectors. This is true, but the reverse-optics design requires that both the sample and optics before the dispersing element (usually a grating) are subjected to the full-spectrum radiation also encountered by these components in a conventional ▲spectrometer.▲ (USP 1-Dec-2022) In a conventional ▲spectrometer,▲ (USP 1-Dec-2022) the sample and optics are outside the monochromator, and optical beam deflection and/or scattering simply reduces the intensity of the light that enters the monochromator. In an array-based ▲spectrometer,▲ (USP 1-Dec-2022) deflection occurs within the ▲polychromator,▲ (USP 1-Dec-2022) causing scatter within the confined environment ▲of these devices, resulting in▲ (USP 1-Dec-2022) an associated increase in stray light. ▲As detailed in *Stray Light*, that leads to a reduction in optimum photometric range as shown in *Figure 3*.▲ (USP 1-Dec-2022)

### HPLC ▲UV-Vis▲ (USP 1-Dec-2022) Detectors

The design of a UV-Vis ▲spectrometer▲ (USP 1-Dec-2022) is always the result of a number of considered compromises. For example, optical performance (absolute wavelength resolution) is governed by the focal length of the monochromator, which in turn dictates the physical size of the instrument. In the case of HPLC ▲UV-Vis▲ (USP 1-Dec-2022) detectors ▲used for peak homogeneity assessment and identification procedures using the spectrum at the peak apex,▲ (USP 1-Dec-2022) the need to provide a high-stability, low S/N ratio output at high transmittance levels through a small-aperture flow ▲cuvette▲ (USP 1-Dec-2022) requires that by design these systems may not have the dynamic range or wavelength accuracy of their conventional spectroscopic counterparts.

### Fiber-Optic-Based Modular Systems

In recent years there has been a rapid increase in the availability and use of systems built around the ability of fiber optics to channel and multiplex optical systems. Although these systems have the advantages of flexibility and ease of use and they allow measurements to be performed on ▲multi-channel plate readers, modular user-designed and -built systems, etc.,▲ (USP 1-Dec-2022) analysts must consider the following disadvantages:

- Some analysts assume that these array-based fiber-optic systems are immune to room light interference at the sample interface, which may or may not be true. This assumption is easily tested by using a black photographic film-changing bag or a simple black cloth. Use of a masking technique should not change the measured value if the assumption is true.
- Custom-built systems ▲may▲ (USP 1-Dec-2022) not have additional shuttering, stray light filtering, and other capabilities that are found in commercially designed ▲spectrometers.▲ (USP 1-Dec-2022) Light levels transmitted directly down fibers from high-intensity sources such as Xenon flash lamps may cause photodegradation ▲of the sample being measured by the system. In these▲ (USP 1-Dec-2022) systems, the source may be coupled by fiber directly to the sample interface, and the only control is the source on/off switch.
- ▲Therefore, by design, the performance characteristics, i.e., the optimum photometric range, may have to be significantly reduced, if possible, to allow the system to be qualified according to ⟨857⟩.▲ (USP 1-Dec-2022)

***Add the following:***

## ▲UV-VIS INSTRUMENT PERFORMANCE INDICATORS

### Spectral Bandwidth—Effect on Resolution and Signal-to-Noise Ratio

Appropriate spectral bandwidth (SBW) is important to ensure adequate peak resolution. The selection of the SBW always requires balancing adequate resolution against maximization of the signal-to-noise (S/N) ratio. During method development, analysts should optimize the resolution by selecting the narrowest slit width that provides an adequate S/N ratio because optimum resolution is achieved when the S/N ratio is maximized. By design, the entrance and exit slits of the monochromator will be mechanically matched together to achieve the same physical separation and associated dispersion (usually triangular) to produce the required SBW. If access to a variable-bandwidth instrument is available, then the optimum setting can be defined as the narrowest slit width at which no significant reduction in peak intensity is observed. If accurate absorbance measurements must be made on benzenoid compounds or other compounds with sharp absorption bands (natural half-bandwidths of <15 nm), the SBW of the spectrometer used should not be greater than $1/8^{th}$ of the natural half-bandwidth of the compound's absorption; i.e., this equates to a spectrometer with a SBW of 2 nm or less. In practical terms, for molecules of pharmaceutical interest in solution, this bandwidth of 2 nm is considered adequate in also providing the required balance of high resolution while maintaining good S/N ratio.

However, it should be noted that nicotinic acid, which is shown to be acceptable for use as a photometric accuracy reference, does have transmittance characteristics that are highly correlated to the SBW, and therefore it is always necessary to match the SBW of the certified absorbance values to those of the instrument system being qualified.

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## Stray Light

Stray radiation, commonly referred to as stray light, can be defined as radiant energy at wavelengths other than those indicated by the monochromator setting and all radiant energy that reaches the detector without having passed through the sample or reference solutions (see *Figure 3*). It may be caused by any scattered radiation from imperfections in the dispersing medium, which commonly is a grating. The use of a holographic grating substantially reduces the levels of this source of stray radiation. Higher-quality ruled gratings yield a low level of scattered radiation. In higher-performance instruments, stray radiation can be reduced by the use of double monochromators or double-pass monochromators. Stray radiation, or apparent stray radiation, also may be caused by light leaks in the system, incorrect wavelength calibration, incorrect optical alignment, reduced source output, or reduced detector response.

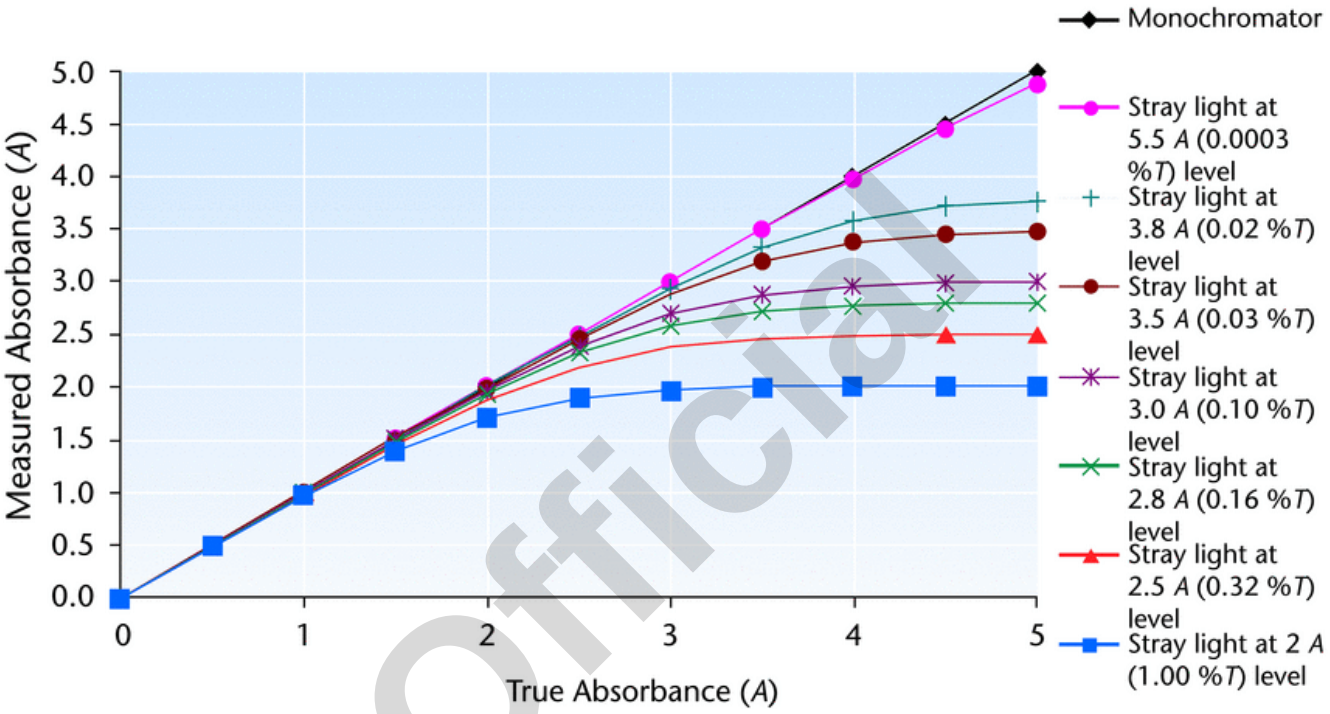


Figure 3. Effect of stray light on measured absorbance.

The usual way of assessing stray light is to measure, at the desired analytical wavelength, a sample that totally absorbs the radiation at that wavelength but transmits at all other wavelengths. Any light detected by the instrument is then stray light.

In 1982, Mielenz et al. at the National Institute of Standards and Technology (NIST) defined stray light as spurious radiant energy that has departed from its regular path in a spectrometer and then re-enters the path so that it is sensed by the detector and causes false readings of transmittance and absorbance. Mielenz also offered a refinement on the estimation method in current use at the time. This method has since been incorporated into ⟨857⟩, *Qualification of UV-Vis Spectrometers, Estimation of the Limit of Stray Light (Stray Radiant Energy), Procedure A*. In this method, the reference beam is attenuated by a 5-mm path length cuvette containing the same edge filter solution as used in a 10-mm path length cuvette placed in the sample beam. A maximum peak differential absorbance is observed ($\Delta A$) while scanning through the edge absorbance, which is related empirically to the stray light level ($s_\lambda$) by:

$$s_\lambda = 0.25 \times 10^{-2\Delta A}$$

*Table 1* provides a list of differential absorbance values, the "Mielenz measured absorbance", and their corresponding stray light levels.

**Table 1. Value Comparison Table and Critical "Mielenz" Measured (Differential) Absorbance Values**

| "Mielenz" Measured Absorbance ($\Delta A$) | Stray light level, $s_\lambda$, expressed as... | | |
|---|---|---|---|
| | Transmittance (*T*) | % Transmittance (%) | Absorbance (*AU*) |
| 0.3 | 0.063 | 6.3 | 1.2 |
| 0.5 | 0.025 | 2.5 | 1.6 |
| 0.7 | 0.01 | 1.0 | 2.0 |
| 1.0 | 0.0025 | 0.25 | 2.6 |

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**Table 1. Value Comparison Table and Critical "Mielenz" Measured (Differential) Absorbance Values** *(continued)*

| "Mielenz" Measured Absorbance (Δ*A*) | Stray light level, $s_\lambda$, expressed as... | | |
|---|---|---|---|
| | Transmittance (*T*) | % Transmittance (%) | Absorbance (*AU*) |
| 1.5 | 0.00025 | 0.025 | 3.6 |
| 2.0 | 0.000025 | 0.0025 | 4.6 |
| 25 | 0.0000025 | 0.00025 | 5.6 |

This method therefore avoids the potentially difficult procedure (see ⟨857⟩, *Qualification of UV-Vis Spectrometers, Estimation of the Limit of Stray Light (Stray Radiant Energy), Procedure B*) to find appropriate attenuation filters in the UV region required to "back-off" the system, especially if using a modern double-monochromator spectrometer, and also avoids the measurement performed at the limit of the instrumental range and analog-to-digital electronic resolution. *Figure 4* shows spectral profiles acquired of a potassium chloride solution using both ⟨857⟩, *Procedure A* and ⟨857⟩, *Procedure B*.

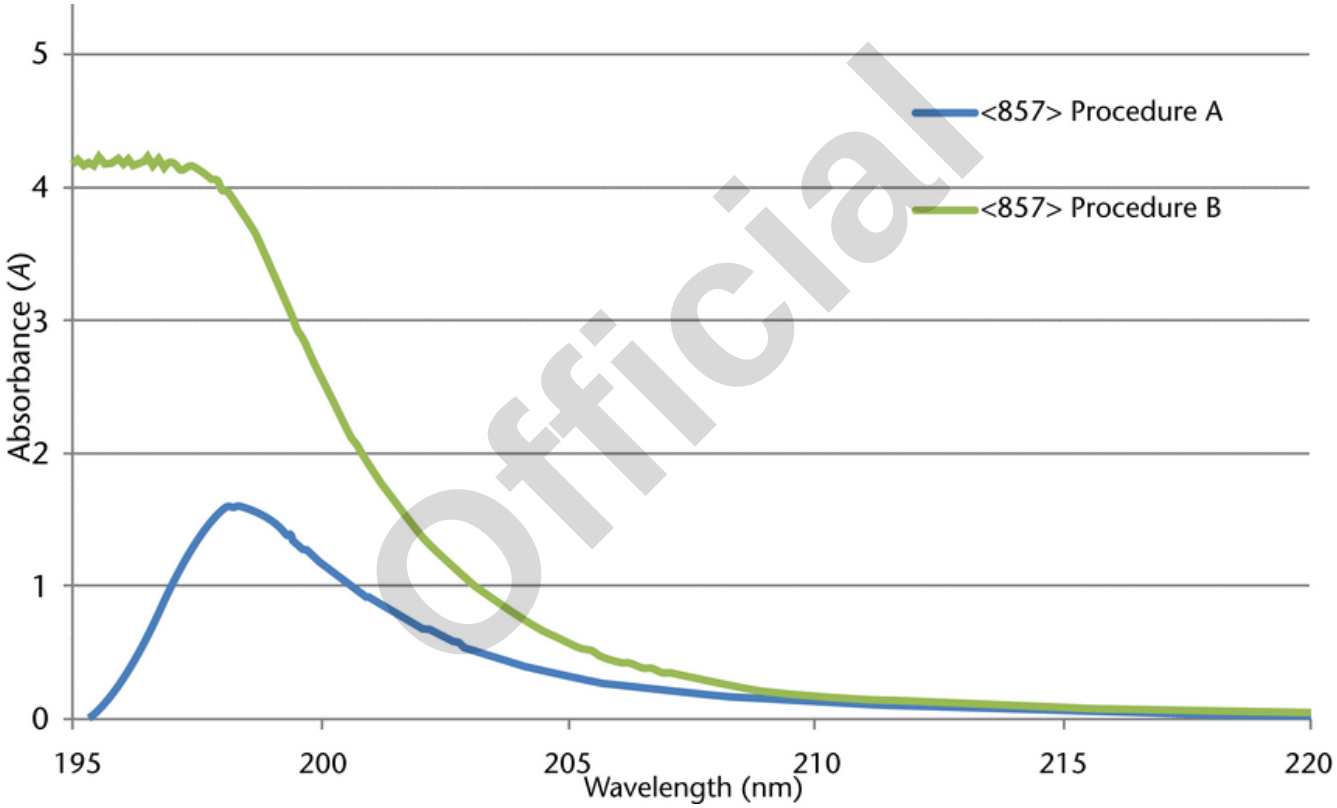


Figure 4. Typical spectrum of 1.2% aqueous potassium chloride vs. water as reference, 10-mm path length (*Procedure B*). Differential absorbance spectrum of 1.2% aqueous potassium chloride, 10-mm path length, vs. the same solution as reference, 5-mm path length (*Procedure A*).

## Optimum Working Photometric Range

Determination of the optimum photometric range is fundamental to establishing the capability of a given instrument before and during method validation. The importance of this procedure can be shown by the fact that in 1945 Vandenbelt et al. attempted to establish the optimum absorbance range on the then-new Beckman DU spectrometer (*1*). They suggested a simple approach in which the molar absorptivities, $A^{1\%}_{1\ \mathrm{cm}}$, of various compounds are measured at different concentrations. This approach is shown theoretically in *Figure 5*, where the center plateau is used to define the photometric range.

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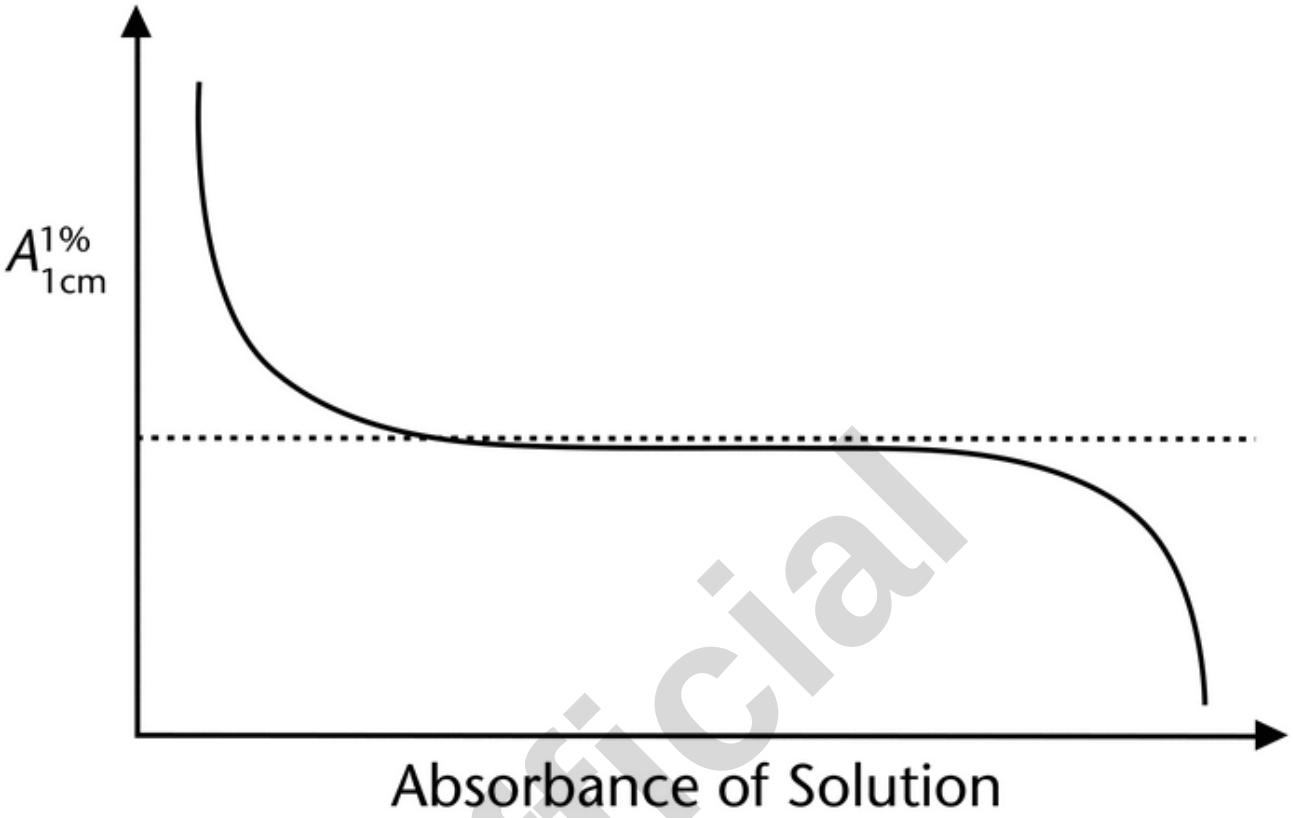


Figure 5. Theoretical photometric response curve.

In practice, data are more variable. *Figure 6* shows three typical curves.

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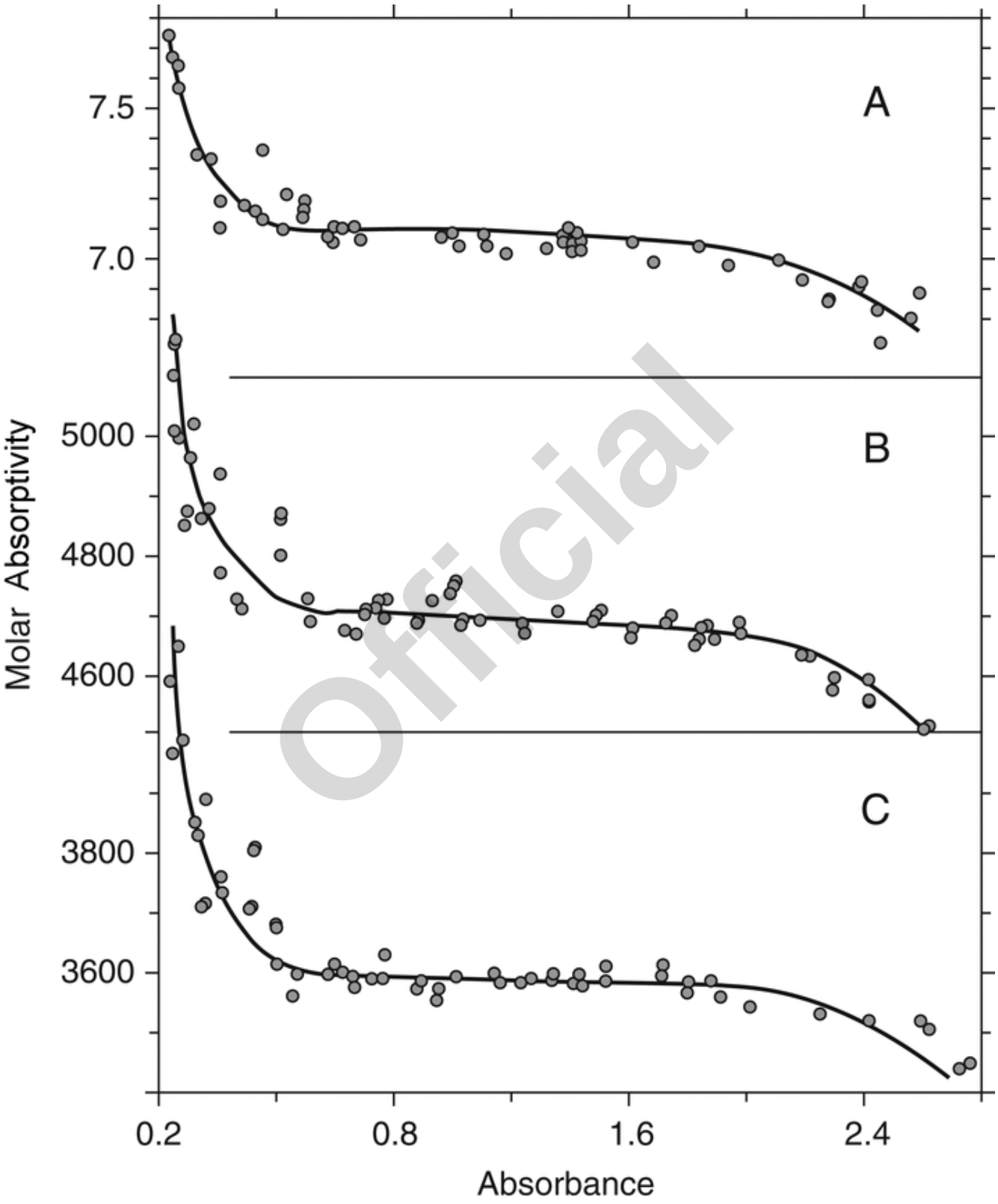


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Figure 6. Molar absorptivity vs. absorbance for three solutions: A) aqueous potassium nitrate measured at 301 nm; B) potassium chromate in 50 mM potassium hydroxide at 373 nm; C) potassium chromate in 50 mM potassium hydroxide at 273 nm (*2*).

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A more rigorous method for estimating an instrument's region of maximum precision is given by Youmans and Brown (*3*). The error in the measurement of the concentration of a solution of transmission $T$ can be expressed as:

$$\Delta c = \frac{1}{\epsilon b}\log\frac{T_{\text{av}}}{T_{\text{min}}}$$

where $\Delta c$ is the change in concentration, $\varepsilon$ is the molar absorptivity of the compound, $b$ is the path length, and $T_{av}$ and $T_{min}$ are the mean and minimum values, respectively, from a series of $n$ measurements on the sample.

The statistical error is (see *Figure 7*):

$$\frac{\Delta c}{c} = \frac{1}{-\log\left(T_{\text{av}}\right)}\log\frac{T_{\text{av}}}{T_{\text{min}}}$$

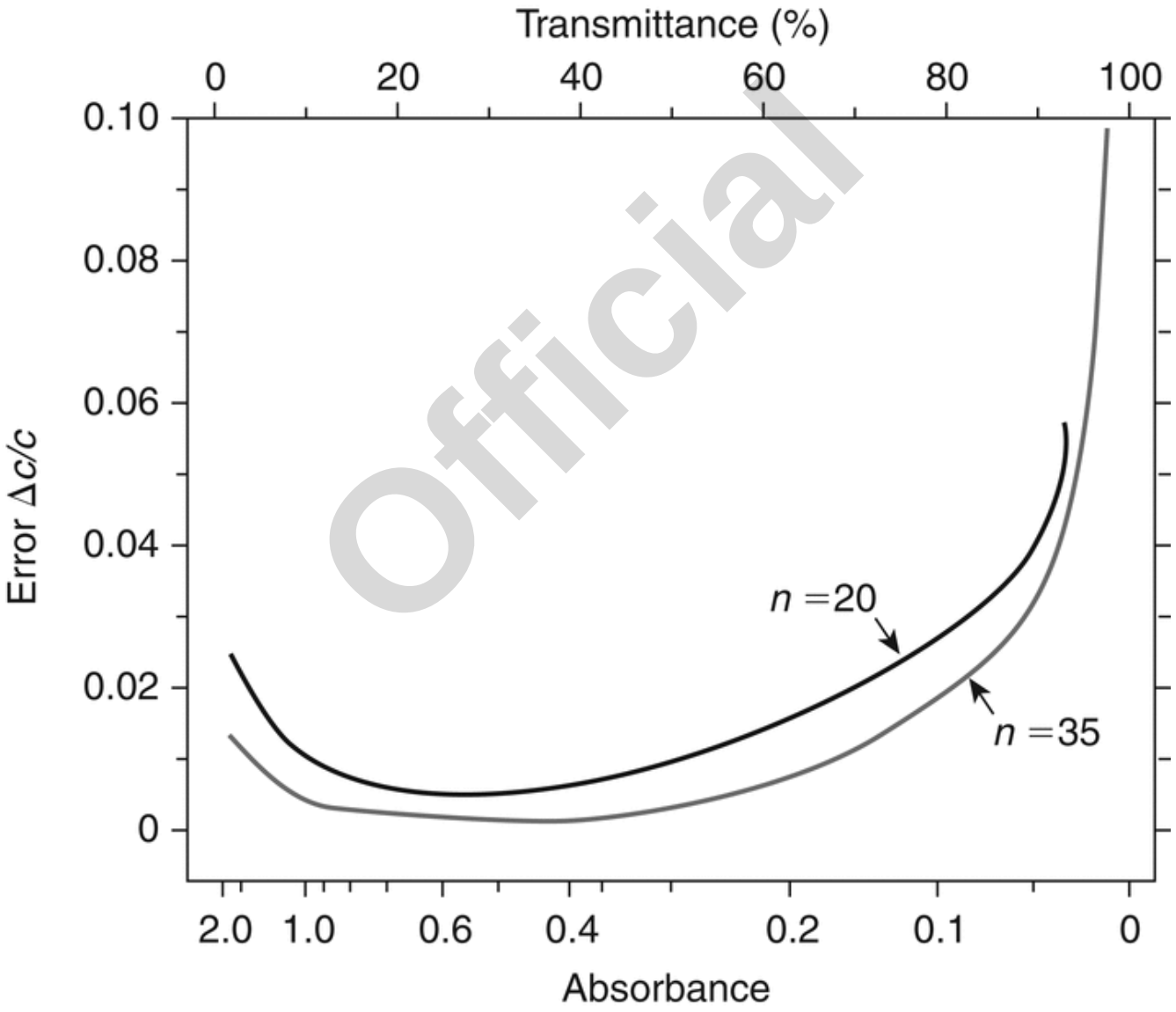


Figure 7. Youmans and Brown plot (*2*).

The noise levels, and hence the precision of measurement, depend primarily on detector noise type, as shown in *Figure 8*.

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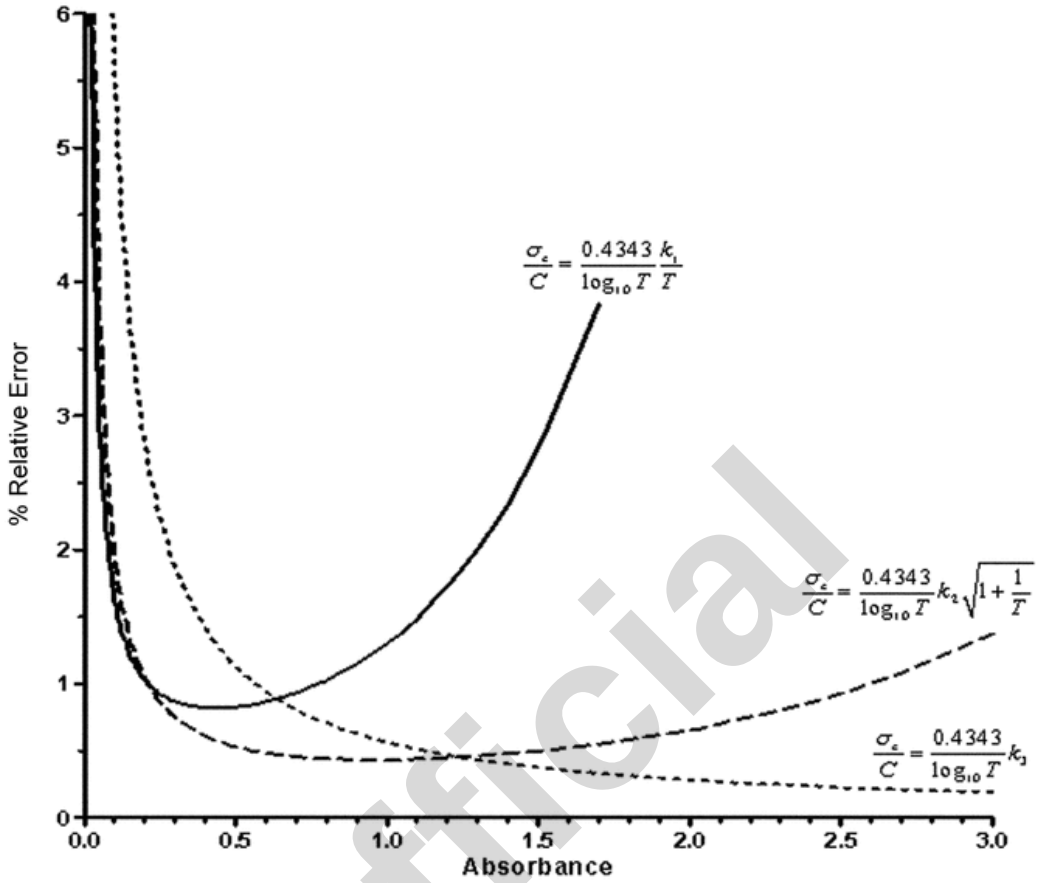


| Standard deviation of a measurement $\sigma_T$ | Source of variability | Relative error function |
|---|---|---|
| $\sigma_T = k_1$ | Thermal detector, amplifier, and dark current noise. [Independent of $T$] | $\frac{\sigma_c}{C} = \frac{0.4343}{\log_{10} T}\frac{k_1}{T}$ |
| $\sigma_T = k_2\sqrt{T^2 + T}$ | Shot noise from the detector | $\frac{\sigma_c}{C} = \frac{0.4343}{\log_{10} T}k_2\sqrt{1+\frac{1}{T}}$ |
| $\sigma_T = k_3 T$ | Cell positioning, non-parallelism errors and incident beam intensity fluctuations | $\frac{\sigma_c}{C} = \frac{0.4343}{\log_{10} T}k_3$ |

Figure 8. Measurement precision as a function of noise type (*4*).

Both approaches clearly show that in addition to the expected increasing error at higher absorbance levels caused by stray light and other factors, there is a similar, if not larger possibility for increasing error at low absorbance levels caused by instrumental variances in the form of noise from the detector, source, etc. This variance often is overlooked in the desire for economy of scale, when analysts use smaller samples and shorter path lengths and therefore lower measured absorbance values.

Good practice is to optimize the measured absorbance within 0.2–2.0 absorbance units (AU), where possible. In addition, the use of shorter path length cuvettes should be the preferred option to reduce a measured solution absorbance rather than the corresponding ratio dilution with solvent. Invariably, the path length tolerances on a modern manufactured cuvette are significantly smaller than those associated with volumetric glassware; and not diluting the sample avoids the risk of solution chemistry changes caused by dilution.▲ (USP 1-Dec-2022)

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## CALIBRATION

UV-Vis ▲spectrometer▲ (USP 1-Dec-2022) calibration involves two components: primary wavelength (*x*-axis) and ▲photometric scale▲ (USP 1-Dec-2022)(*y*-axis), and often is performed when the instrument is initialized. Most dispersive UV-Vis

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▲spectrometers▲ (USP 1-Dec-2022) use atomic emission lines from either the source or a secondary lamp for primary wavelength (*x*-axis) calibration, in addition to the zero-order position in the monochromator. ▲How (and to what level of "user") these fundamental calibration procedures are made available will depend on the vendor's configuration of the instrument. Calibration of both the wavelength (*x*-axis) and the photometric scale (*y*-axis) is critical for successful quantitation and method transfer between instruments. In many cases, the effect of wavelength error in the *x*-axis can be minimized by the use of careful method selection, e.g., wavelength at a spectral peak.▲ (USP 1-Dec-2022) Although the fundamental measurement relies on a simple ratio measurement, modern instruments may use several gain settings or amplification factors to ensure a linear detector response, particularly at low intensity levels and high-power settings. These gain settings often are initially set by the manufacturer, but the settings may be recalibrated as the optics age or as other changes take place.

Detailed functional validation based on certified reference materials ▲as detailed in ⟨857⟩▲ (USP 1-Dec-2022) is recommended to demonstrate the suitability of laboratory instruments, even for instruments that possess an internal calibration capability. The use of external reference materials does not obviate the need for internal quality control procedures. Rather, it provides independent documentation of the fitness of the instrument to perform the specific analysis.

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## ANALYTICAL CONSIDERATIONS

### ▲Temperature and Coefficient of Expansion Effects

Temperature impacts absorption measurements and, thereby, the photometric accuracy in several significant ways. Changes in temperature can induce expansion or contraction of the solvent, leading to lower or higher concentrations and absorbances. While this is a particular issue with some organic solvents, careful temperature control, i.e., within ±2° may be required where high-accuracy absorbance values are required for aqueous solutions, for example, to verify compliance with photometric value assignments stated for a reference material and/or calculation of a molar extinction coefficient. In ⟨857⟩, this accurate control of temperature is particularly important when liquid photometric accuracy references are being used in the qualification process, i.e., nicotinic acid and acidic potassium dichromate solutions. In such cases, additional temperature monitoring and/or control of the sample measurement process may be required. Also, shifts in equilibria between the chemical forms of an analyte may cause the nature of the absorbing species to be changed.

### Solvent Selection Effects

Solvents play an important role in UV spectra. In a spectrum, the compound peak can be obscured by the solvent peak. So, a most suitable solvent is one that does not itself absorb and is transparent in the region under investigation. As a general rule, it should be possible to measure a solvent at the wavelength of interest at a transmittance value of >75%.

A dilute solution of sample is always prepared for analysis. See *Table 2* for a list of most commonly used solvents. In this table, approximate wavelengths below which the solvent absorbance may be unacceptable are specified. For quantitative applications, the cut-off may be set at a wavelength ($L_0$) where the absorbance for the 10-mm path length of the solvent exceeds 0.05 AU (relative to water), i.e., $A_{1\ cm} > 0.05$ AU. For qualitative work, it may still be feasible to operate at significantly lower wavelengths; most analysts accept a cut-off based on the wavelength ($L_1$) for $A_{1\ cm} > 1.0$ AU.

**Table 2. Solvent Cut-Off of Commonly Used Solvents**

| Solvent (Common Name) | $L_0$ (nm) | $L_1$ (nm) |
|---|---|---|
| Acetone | 340 | 330 |
| Acetonitrile | 200 | 190 |
| Isobutanol | 250 | 200 |
| *n*-Butanol | 245 | 215 |
| *n*-Butyl acetate | 275 | 255 |
| Cyclopentane | 220 | 195 |
| Decalin | 250 | 230 |
| Dichloromethane | 245 | 230 |
| Diethyl ether | 255 | 220 |
| *N,N*-Dimethylformamide | 300 | 270 |
| Dimethylsulfoxide | 330 | 285 |
| Ethanol | 240 | 205 |
| Ethyl acetate | 280 | 260 |
| Heptane | 230 | 200 |
| Hexane | 225 | 195 |
| Methanol | 240 | 205 |

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**Table 2. Solvent Cut-Off of Commonly Used Solvents** *(continued)*

| Solvent (Common Name) | $L_0$ (nm) | $L_1$ (nm) |
|---|---|---|
| Isopropanol | 240 | 205 |
| *n*-Propanol | 250 | 210 |
| Tetrachloroethylene | 320 | 290 |
| Tetrahydrofuran | 280 | 220 |
| Water | 190 | 185 |

In addition, there are three other solvent related effects. First, dissolved gases may cause absorption at wavelengths above the pure solvent cut-off. For example, dissolved atmospheric gases may cause absorption to occur at 190–205 nm in an aqueous system. Second, organic solvents may contain other stabilizing chemicals that will absorb at higher wavelengths. Last, changes in physical solvent properties, e.g., polarity or degree of hydrogen bonding, may cause chromatographic shifts in the band spectrum.▲ (USP 1-Dec-2022)

## Sample-Based Factors

The most important sample-based factors that negatively affect quantitative UV-Vis ▲spectrometry▲ (USP 1-Dec-2022) are fluorescence and light scattering. If the sample matrix includes fluorescent compounds, the measured signal usually will contain a contribution from fluorescence. The wavelength range and intensity of the fluorescence depend on the chemical composition of the fluorescent material. Suspended particles scatter light by the Tyndall effect, causing a decrease in the measured intensity that increases as the wavelength decreases. Unless there is no alternative, absorbance should not be determined on turbid samples. Procedures for removing turbidity include filtering, centrifuging, or flocculating the sample and are performed before any additional procedures that generate a chromophore, provided that they do not affect the concentration of the analyte or the chromophore in the test solution. Any measurements performed on a turbid solution are highly instrument specific and can be used only for comparative purposes in the same system. A further complication can arise at higher analyte concentrations with respect to the coordination chemistry and system matrix. Ionic association, complex formation, and similar factors can cause deviation from the expected linear response.

## Sampling Factors

### ▲SOLUTION PREPARATION

As previously stated, all solutions used for measurement should be particle free and prepared as accurately as possible. The errors associated with any solution preparation can be minimized by ensuring that all gravimetric processes are used within the center median of the given working range of the balance(s) and that a gravimetric process, in preference to a volumetric process, is used for all subsequent required dilutions. Again, the use of reduced path length cuvettes should be chosen in preference to an additional dilution step.

### CUVETTES▲ (USP 1-Dec-2022)

Quantitative absorbance measurements usually are made on solutions of the substance in liquid-holding ▲cuvettes.▲ (USP 1-Dec-2022) The most common ▲cuvette▲ (USP 1-Dec-2022) path length is 10 mm, although path lengths from 0.01–100 mm are commercially available (see *Figure 9*). For samples with low absorbance, improved sensitivity generally can be obtained by increasing the ▲cuvette▲ (USP 1-Dec-2022) path length. For example, the theoretical absorbance of a solution in a 50-mm ▲cuvette▲ (USP 1-Dec-2022) is greater by a factor of 5 compared to the same solution in a 10-mm ▲cuvette.▲ (USP 1-Dec-2022) Errors in absorption readings arising from ▲cuvettes▲ (USP 1-Dec-2022) almost always are caused by dirty windows that can absorb a significant proportion of the incident light beam. Less frequent causes of error are an incorrect choice of ▲cuvette▲ (USP 1-Dec-2022) material for the wavelength required, e.g., use of glass ▲cuvettes▲ (USP 1-Dec-2022) less than 320 nm, nonrepeatability of ▲cuvette▲ (USP 1-Dec-2022) positioning, differences in ▲cuvette▲ (USP 1-Dec-2022) window thickness, nonparallel optical windows, or impurities in ▲cuvette▲ (USP 1-Dec-2022) window materials. A 10-mm ▲cuvette▲ (USP 1-Dec-2022) manufactured with a ±0.05 mm tolerance will add a 0.005 AU contribution to the overall uncertainty, when measuring absorbance values at the 1 AU level. There are 10-mm ▲cuvettes▲ (USP 1-Dec-2022) with a ±0.01 mm tolerance commercially available. As a general guide, the ▲cuvette▲ (USP 1-Dec-2022) chosen should have a transmittance value of >75% when filled with the appropriate solvent▲, as previously discussed,▲ (USP 1-Dec-2022) and measured at the wavelength of interest.

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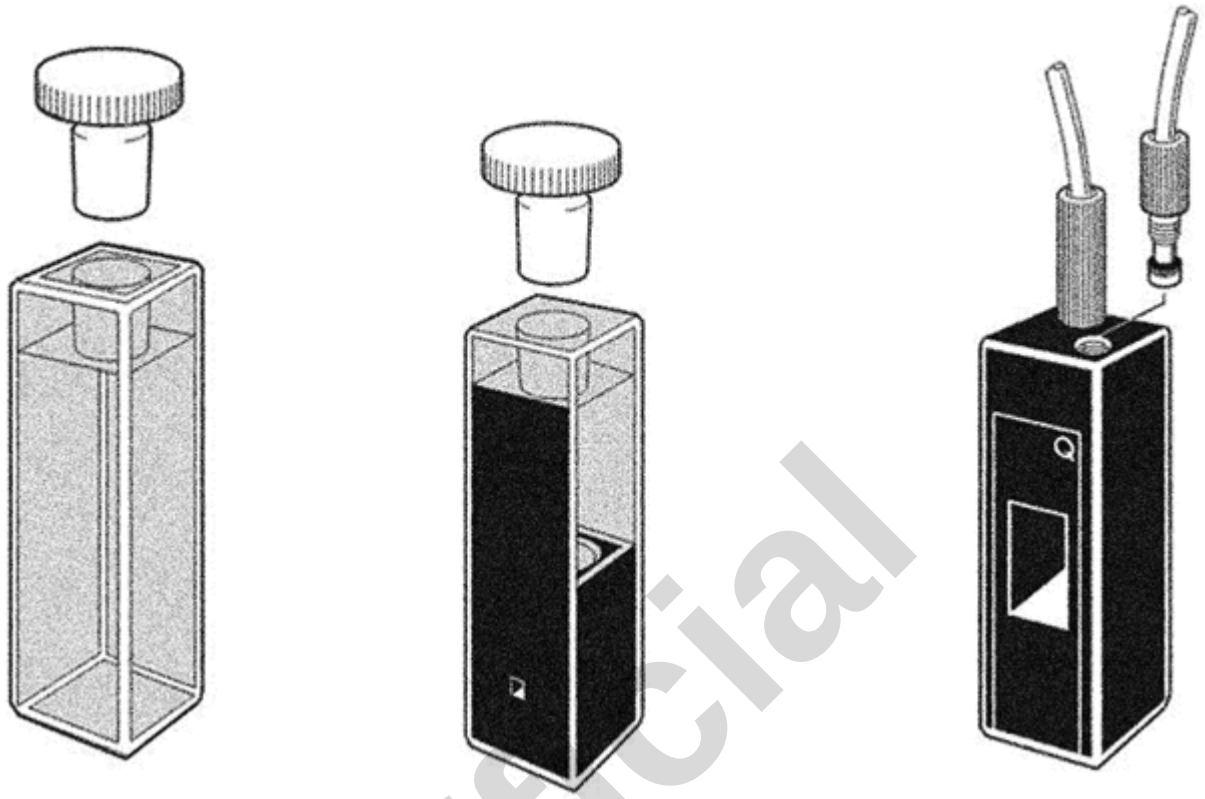

Figure 9. Commonly used ▲cuvette▲ (USP 1-Dec-2022) types: 10-mm regular, low-volume, and flow ▲cuvettes▲ (USP 1-Dec-2022) (from left to right).

Analysts can mitigate the variability introduced by ▲cuvettes▲ (USP 1-Dec-2022) by adhering to the following set of best practices. Carefully clean ▲cuvettes▲ (USP 1-Dec-2022) before use, and store them in a manner that avoids contamination when they are not in use. ▲Cuvettes▲ (USP 1-Dec-2022) in frequent and repeated use, e.g., flow ▲cuvettes,▲ (USP 1-Dec-2022) can be stored wet in high-purity water or 1% (v/v) nitric acid. Do not use cracked or scratched ▲cuvettes.▲ (USP 1-Dec-2022) Wipe ▲cuvettes▲ (USP 1-Dec-2022) carefully with a soft, clean, lint-free cloth before they are placed in the ▲spectrometer.▲ (USP 1-Dec-2022) Do not use lens-cleaning paper or any other form of paper cleaning tissue because these will either scratch or allow contamination of the optical face.

It is preferable to use a single ▲cuvette▲ (USP 1-Dec-2022) for all measurements of both the reference and samples▲, and where possible, never move the cuvette if you can move the solution. Liquid transfer should be made using plastic-tipped devices to avoid scratching the optical surfaces. This approach, which eliminates interreflection errors and nonparallelism effects, should always be used if work of the highest accuracy is needed. The use of a flow cuvette with peristaltic aspiration is recommended.▲ (USP 1-Dec-2022) When several sample ▲cuvettes▲ (USP 1-Dec-2022) must be tested against one reference ▲cuvette,▲ (USP 1-Dec-2022) they all should be closely matched in their characteristics. Whenever possible, measure standards and samples at the same time and under the same conditions to minimize the potential for bias. The reference ▲cuvette▲ (USP 1-Dec-2022) should contain the same solvent as the sample ▲cuvette▲ (USP 1-Dec-2022) and should be checked against the sample ▲cuvette▲ (USP 1-Dec-2022) at all wavelengths for which measurements are required. Analysts can apply corrections for differences between ▲cuvettes▲ (USP 1-Dec-2022) to absorbance readings obtained from the solutions under examination. The maximum allowable correction is ±0.01 AU. Best practices include using the same ▲cuvette▲ (USP 1-Dec-2022) in the reference beam with the same face incident to the light beam, and flushing the sample ▲cuvette▲ (USP 1-Dec-2022) three times with the sample solution before the final filling before measurement. ▲When solutions using volatile solvents are to be measured, it is advisable to use polytetrafluoroethylene (PTFE) stoppered-cuvettes, ideally one of the many screw-capped variants available.▲ (USP 1-Dec-2022)The use of flow ▲cuvettes▲ (USP 1-Dec-2022) effectively addresses many of these ▲handling and filling▲ (USP 1-Dec-2022) issues. The contents in both the sample ▲cuvette▲ (USP 1-Dec-2022) and the reference ▲cuvette▲ (USP 1-Dec-2022) must be free from gas bubbles and particles. If flow ▲cuvettes▲ (USP 1-Dec-2022) are used, analysts may be required to degas the solution(s) before measurement in order to avoid the formation of air bubbles in the ▲cuvette.▲ (USP 1-Dec-2022)

A sequence of activities is used to monitor the quality of the measurement process and to ensure that the contents are representative of the sample under measurement. Drift of the ▲spectrometric▲ (USP 1-Dec-2022) baseline can be detected by measuring the blank at the start and end of the sample sequence▲and also at interim points if the time scale requires them.▲ (USP 1-Dec-2022) Adequate flushing of the ▲cuvette▲ (USP 1-Dec-2022) can be confirmed by alternating between measurement of the standard (high concentration) and the blank (zero concentration) at the start of the measurement sequence. The latter check is particularly important for automated systems that can generate reproducible but inaccurate results because of inadequate flushing.

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### CARE OF ▲CUVETTES▲ (USP 1-Dec-2022)

Contaminated ▲cuvettes▲ (USP 1-Dec-2022) are the greatest single source of error in ▲UV-Vis spectrometry. Cuvettes▲ (USP 1-Dec-2022) should never be handled by the optical polished faces, and analysts should rinse off residual or spilled solution. If ▲cuvettes▲ (USP 1-Dec-2022) are properly cared for, rigorous cleaning methods rarely will be needed, especially if the ▲cuvettes▲ (USP 1-Dec-2022) are cleaned immediately after use. Although acid solutions will simply contaminate surfaces, alkaline solutions can etch all types of polished glass surfaces, and the degree of attack depends on the pH and contact time. When cleaning ▲cuvettes,▲ (USP 1-Dec-2022) analysts should ensure that the optical faces are not scratched or chipped. Commercial cleansing solutions are available and can be used, provided that they are diluted before use in accordance with the manufacturers' recommendations. After they are cleaned with such agents, the ▲cuvettes▲ (USP 1-Dec-2022) should be carefully and thoroughly rinsed with high-purity water. If this fails to clean the ▲cuvettes,▲ (USP 1-Dec-2022) then soaking them in cold, concentrated nitric or hydrochloric acid is acceptable. Flow ▲cuvettes▲ (USP 1-Dec-2022) are best cleaned in situ, provided that the solvent does not interact with the connecting tubing. If the ▲cuvettes▲ (USP 1-Dec-2022) are stored for an extended period, then they should be dried quickly after cleaning by blowing dry with a compressed gas stream in a clean, dust-free environment. ▲Many laboratory "compressed air gas lines" may contain lubricating oil from the machinery and thereby deposit a film of oil on the optical face.▲ (USP 1-Dec-2022)

### ALIGNMENT AND FILLING OF ▲CUVETTES▲ (USP 1-Dec-2022)

Analysts should align the ▲cuvettes▲ (USP 1-Dec-2022) so that the same optical face is always incident to the light beam. Most ▲cuvettes▲ (USP 1-Dec-2022) have type identification engraved on one face; this can be used to ensure consistent orientation. If there are no markings, a small mark can be made on one face outside the area of the light beam. The use of ▲cuvettes▲ (USP 1-Dec-2022) with no type markings is not recommended, but, if ▲cuvettes with type markings▲ (USP 1-Dec-2022) are used, analysts should confirm their suitability for the intended application. If ▲cuvettes▲ (USP 1-Dec-2022) are provided with the instrument, individual ▲cuvette▲ (USP 1-Dec-2022) holders should always be used in the same beam as identified, e.g., by use of appropriate markings.

### ▲CUVETTE▲ (USP 1-Dec-2022) CORRECTIONS

If multiple ▲cuvettes▲ (USP 1-Dec-2022) are used, differences in the optical transparency of individual ▲cuvettes▲ (USP 1-Dec-2022) can introduce a systematic bias unless the latter is accounted for by a ▲cuvette▲ (USP 1-Dec-2022) correction. ▲Depending on where the zero point is set, this correction can be either positive or negative; however,▲ (USP 1-Dec-2022)do not use corrections in excess of 0.01 ▲AU.▲ (USP 1-Dec-2022) To determine if ▲cuvette▲ (USP 1-Dec-2022) corrections are necessary, analysts can fill all ▲cuvettes▲ (USP 1-Dec-2022) with the appropriate solvent and measure the differences in absorbance at the required analytical wavelengths. These measurements are repeated after cleaning to ensure that differences in absorbance are related to the ▲cuvettes▲ (USP 1-Dec-2022) and are not the result of contamination. Analysts should fill and check the ▲cuvettes▲ (USP 1-Dec-2022) repeatedly until the results are consistent, and they should investigate any appreciable change in ▲cuvette▲ (USP 1-Dec-2022) correction because it indicates contamination, damage to the ▲cuvettes,▲ (USP 1-Dec-2022) or incorrect adjustment of the instrument.

## APPENDIX

## Other Sources of Information

1. Vandenbelt J M, Forsyth J, and Garrett A. *Anal. Chem. (Industrial and Engineering Chemistry, Analytical Edition)*. 1945;17(4): 235.
2. Burgess C, Frost T (Eds.). *Standards and Best Practice in Absorption Spectrometry*. Blackwell Science; 1999. ISBN 0-632-05313-5.
3. Youmans HL, Brown WD. *Anal. Chem.* 1976;48:1152.
4. Skoog DA, West DM, Holler FJ. *Fundamentals of Analytical Chemistry*. 7th ed.; 1996: Table 24-4.

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# ▲⟨1858⟩ RAMAN SPECTROSCOPY—THEORY AND PRACTICE

THEORY
APPLICATIONS
QUALITATIVE AND QUANTITATIVE RAMAN MEASUREMENTS
- Qualitative Raman Measurements
- Quantitative Raman Measurements

SAMPLING FACTORS
APPARATUS
- Components
- Excitation Source (Laser)
- Sampling Device
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SPECIALIZED TECHNIQUES
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CALIBRATION
- Primary Wavelength
- Laser Wavelength
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SAMPLE-BASED FACTORS THAT AFFECT MEASUREMENT PERFORMANCE
PROCEDURE VALIDATION
- Ongoing Procedure Evaluation
- Method Transfer

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## THEORY

Raman is a vibrational spectroscopic technique; it is complementary to mid-infrared (IR) and near-infrared (NIR) spectroscopy where there is center of symmetry, because both techniques rely on interaction with the molecular structure of the material. The Raman effect itself arises as a result of a change in the polarizability of molecular bonds during a given vibrational mode and is measured as inelastically scattered radiation, whereas absorption intensity at IR or NIR wavelengths occurs due to differences in dipole moments. Raman spectroscopy is particularly sensitive to non-polar bonds (e.g., C–C, single or multiple bonds), and thus, strong dipole oscillators do not show Raman scattering because the molecular geometry does not give rise to polarizability. The appearance of a Raman spectrum is much like an IR absorbance spectrum. The intensities, or the number of Raman photons counted, are plotted against the shifted energies. The *x*-axis is generally labeled "Raman shift/$cm^{-1}$" or "wavenumber/$cm^{-1}$". The Raman shift is usually expressed in terms of wavenumber and represents the energy difference between the incident photon energy and the inelastically scattered photon energy. In addition, water, which has a strong IR absorption spectrum, only exhibits a weak Raman signature and is a useful solvent for this technique. The spectral features and intensities of Raman and IR/NIR are therefore different for most organic molecules, and thus the spectra of the different techniques may be used to complement specificity and characterization.

A Raman spectrum is generated by exciting the sample of interest to an excited state with an appropriate light source. Light elastically scattered (no change in wavelength) is known as Rayleigh scatter and is not of interest in Raman spectroscopy, except for marking the laser wavelength. However, if the sample relaxes to a vibrational energy level that differs from the initial state, either up or down, the scattered radiation is shifted in energy. This shift is commensurate with the energy difference between the initial and final vibrational states. This "inelastically scattered" light is referred to as Raman scatter. Only about 1 of $10^6$–$10^8$ photons incident on the sample undergoes Raman scattering. Lasers can provide high photon densities and are used in Raman spectrometers to compensate for this inherently low Raman scattering efficiency; also, the Rayleigh scatter can be filtered easily to reduce stray light interferences. Lasers can also be tuned to avoid fluorescence from the sample or matrix or to excite to resonant modes to enhance the signal. If the Raman-scattered photon is of lower energy than the excitation source, it is referred to as Stokes scattering, and if it is of higher energy, it is referred to as anti-Stokes scattering. In practice, nearly all analytically useful Raman measurements make use of Stokes-shifted Raman scatter.

## APPLICATIONS

Raman spectroscopy can be used for a variety of applications ranging from verification of raw materials to process monitoring of drug production to quality control of products. These applications include optimizing polymorph (or crystalline structure)

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analysis; synthesis of new drugs; gaining process understanding through real-time monitoring of reactors, crystallizers, and blenders; verifying raw materials; and ensuring product quality. Once a potential new drug is identified, the method for synthesizing the drug is developed, and Raman spectroscopy is ideal for monitoring reactant, intermediate, and product concentrations, as well as determining pathways, kinetics, mechanisms, end-points, and yields for a variety of reaction types. Quality-by-design control of the product requires assurance that its final form (e.g., gel capsules or tablets) contains the correct amount of active pharmaceutical ingredient (API), its polymorph (if appropriate), excipient, and other additives such as dyes. Raman spectroscopy has been used to monitor mixing in blenders, as well as to inspect individual products before shipment, and may be a useful alternative to HPLC in stability studies. Analysis of the data using chemometrics tools is common for many applications. Proper design of experiment, calibration standard, instrument selection, and maintenance are important considerations for any chemometric model, and it is these requirements that will dictate the specification of the instrumentation required to ensure that it is fit for purpose. For further discussion of the theory and applications of chemometrics, see *Chemometrics* ⟨1039⟩.

## QUALITATIVE AND QUANTITATIVE RAMAN MEASUREMENTS

There are two general classes of measurements that are commonly performed using Raman spectroscopy: qualitative and quantitative.

### Qualitative Raman Measurements

Qualitative Raman measurements yield spectral information about the molecular structure of the sample. Because the Raman spectrum is specific for a given compound, qualitative Raman measurements can be used as a compendial identification test. See *Spectroscopic Identification Tests* ⟨197⟩.

### Quantitative Raman Measurements

For instruments equipped with a detector that measures optical power, such as Fourier transform (FT)–Raman spectrometers, quantitative Raman measurements use the following relationship:[1, 2]

$$I(\omega_s) = I(\omega_L) N \frac{d\sigma(\omega_S)}{d\Omega} Z$$

$I(\omega_s)$ = Raman scattering intensity at wavelength $\omega_s$
$I(\omega_L)$ = incident laser intensity at wavelength $\omega_L$
$N$ = number density of scatters
$d\sigma(\omega_s)/d\Omega$ = Raman cross-section at $\omega_s$
$Z$ = geometric factor that takes into account the spectrometer depth of field

## SAMPLING FACTORS

Raman spectroscopy is a zero-background technique in that the signal at the detector is expected to be zero in the absence of a sample. This situation can be contrasted with absorption spectroscopy, where the intensity at the detector is at a maximum in the absence of a sample. Zero-background techniques are inherently sensitive because small changes in sample concentration lead to proportionate changes in the signal level. The instrument is sensitive to other sources of light that can cause sample-to-sample variations in the measured signal level. In addition, a large background signal can be caused by fluorescence. Thus, it may be very difficult to use the absolute Raman signal intensities for direct determination of an analyte. Other potential sources of variation are changes in the sample opacity and heterogeneity, changes in the laser power at the sample, and changes in optical collection geometry or sample position. These effects can be minimized by careful method design related to preparation and presentation of the sample, as well as instrument factors.

Use of an internal reference standard is the most common and robust method for eliminating variations caused by absolute intensity fluctuations. There are several options for this approach. An internal standard can be added deliberately, and isolated peak(s) from this standard can be used. Alternatively, the analyst can use a band due to a moiety such as an aromatic ring, the Raman intensity of which does not change with the way the sample is prepared. For solution spectra, an isolated solvent band can be used because the solvent will remain relatively unchanged from sample to sample. In a formulation, an excipient peak can possibly be used if it is present in a substantial excess, when compared to the analyte, in a homogeneous matrix.

A second, important sampling-based factor to consider is spectral contamination. Raman scattering is a weak effect that can be masked by a number of external sources. Common sources of contamination include sample-holder artifacts (container or substrate) and ambient light. Typically, these issues can be identified and resolved by careful experimentation.

[1] Biggs KB, Camden JP, Anker JN, Van Duyne RP. *J Phys Chem A.* 2009;113(16):4581–4586.
[2] McCreery RL. Raman Spectroscopy for Chemical Analysis. New York, NY: John Wiley and Sons, Inc.; 2000.

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# APPARATUS

## Components

Regarding the instrumentation, modern systems are easy to use, provide fast analysis times (milliseconds to several minutes), and are reliable. Conventional Raman systems are usually benchtop instruments. However, handheld devices based on solid-state electronics and detection systems are widely available and mainly used for the identification of raw materials. Raman spectra are ordinarily excited with visible or NIR radiation, and standard glass/quartz and fiber optics may be used. The signal is typically in the visible or NIR optical range, allowing samples to be analyzed directly through packaging or directly in media that are transparent at these wavelengths. In addition, process Raman analyzers used as process analytical technology (PAT) are also available. Acceptance criteria for fitness for purpose over their operational range are the same for both benchtop and handheld devices.

All commercial Raman instruments have the following components:

- Excitation source (laser)
- Sampling device
- Device to filter/reject light scattered at the laser wavelength
- Wavelength processing unit
- Detector and electronics

## Excitation Source (Laser)

*Table 1* identifies several common lasers used for pharmaceutical applications or Raman spectroscopy.

The danger of using high-powered lasers must be recognized, especially when their wavelengths are outside of the visible range, i.e., in either the UV or NIR regions. Fiber optic probes should be used with caution.

**Table 1. Lasers Used in Pharmaceutical Applications**

| Nominal Laser λ (nm) | Type | Typical Power at Laser | Wavelength Range (nm) (Stokes Region, 100–3000 $cm^{-1}$ shift) | Comments |
|---|---|---|---|---|
| **NIR Lasers** | | | | |
| 1064 | Solid state [neodymium-doped:yttrium–aluminum–garnet (Nd:YAG)] | Up to 3 W | 1075–1563 | Commonly used in FT instruments |
| 830 | Diode | Up to 650 mW | 836–1105 | Typically limited to 2000 $cm^{-1}$ Raman shift because of charge-coupled device (CCD) spectral response; less common than the other lasers |
| 785 | Diode | Up to 500 mW | 791–1027 | Most widely used dispersive Raman laser |
| **Visible Lasers** | | | | |
| 632.8 | Helium–Neon (He–Ne) | Up to 500 mW | 637–781 | Relatively small fluorescence risk |
| 532 | Doubled Nd:YAG | Up to 1 W | 535–633 | High fluorescence risk |
| 514.5 | Argon (Ar)-Ion | Up to 1 W | 517–608 | High fluorescence risk |
| 488.0–514.5 | Ar-Ion | Up to 1 W | 490–572 | High fluorescence risk |
| **UV Lasers** | | | | |
| 257.3 | Doubled Ar-Ion | Up to 200 mW | 258–279 | — |
| 248.3 | Neon–Copper (Ne–Cu) | 20 mW to 1.8 W peak | 249–268 | — |
| 244.0 | Doubled Ar-Ion | Up to 100 mW | 245–263 | — |
| 229.0 | Doubled Ar-Ion | Up to 10 mW | 230–246 | — |
| 224.3 | Helium–Silver (He–Ag) | 20 mW to 1.8 W peak | 225–241 | — |

## Sampling Device

Several sampling arrangements are possible, including direct optical interfaces, microscopes, fiber optic-based probes (either non-contact or immersion optics), and sample chambers, including specialty sample holders and automated sample changers. The sampling optics can also be designed to obtain polarization-dependent Raman spectra. The choice of sampling device is often dictated by the analyte and sample. However, considerations such as sampling volume, speed of the measurement, laser safety, and reproducibility of sample presentation should be evaluated to optimize the sampling device for any given application.

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### Filtering Device

The intensity of scattered light at the laser wavelength (Rayleigh scatter) is many orders of magnitude more than the Raman signal and must be rejected before it reaches the detector. Notch filters are almost universally used for this purpose and provide excellent rejection and stability combined with small size. The traditional use of multi-stage monochromators for this purpose, although still viable, is now rare. In addition, various filters or physical barriers to shield the sample from external radiation sources (e.g., room lights or laser plasma lines) may be required, depending on the collection geometry of the instrument.

### Wavelength Processing Unit

The wavelength scale may be encoded by either a scanning monochromator, a grating polychromator [in charge-coupled device (CCD)-Raman spectrometers], or an interferometer (in FT-Raman spectrometers). A discussion of the specific benefits and drawbacks of each of the dispersive designs compared to the FT instrument is beyond the scope of this chapter. Any properly qualified instrument should be suitable for qualitative measurements. However, care must be taken when selecting an instrument for quantitative measurements because dispersion and response linearity might not be uniform across the full spectral range.

### Detector

The silicon-based CCD array is the most common detector for dispersive instruments. The cooled array detector allows measurements over the spectral range from 4500 to 100 $cm^{-1}$ Raman shift with low noise when most visible lasers, such as frequency-doubled neodymium-doped:yttrium–aluminum–garnet (Nd:YAG; 532 nm) or helium–neon (He–Ne; 632.8 nm) lasers, are used. When a 785-nm diode laser is used, the wavelength range is reduced to about 3100 to 100 $cm^{-1}$. The most commonly used CCD has its peak wavelength responsivity when matched to the commonly used 632.8-nm helium–neon (He–Ne) gas laser or 785-nm diode laser. FT instruments typically use single-channel germanium or indium–gallium–arsenide (InGaAs) detectors responsive in the NIR to match the 1064-nm excitation of an Nd:YAG laser.

## SPECIALIZED TECHNIQUES

In addition to “normal” Raman spectroscopy, there are several more specialized Raman-based techniques. These include Raman microscopy and confocal Raman microscopy (CRM), spatially offset Raman spectroscopy (SORS), resonance Raman (RR), deep UV resonance Raman spectroscopy (DUVRRS), surface-enhanced Raman spectroscopy (SERS), Raman optical activity (ROA), coherent anti-Stokes Raman spectroscopy (CARS), Raman gain or loss spectroscopy, and hyper-Raman spectroscopy. Some of these techniques are currently in use, and these are described below. Those that have very specialized uses in pharmaceutical laboratories are not addressed in this chapter.

### Confocal Microscopy/Imaging

The distribution of the various compounds within a given material can be characterized either on the surface by microscopy or inside the sample, three dimensionally, using confocal imaging. The use of Raman scattering allows for the collection of detailed chemical information. Raman microscopy techniques have the following advantages:

- Preparation of the sample may not be required
- Excellent spatial resolution
- Clear image quality
- Outstanding chemical differentiation

It is possible to collect a signal from a single point on a sample, disperse it into a spectrum using a spectrometer, and detect the spectrum using a multi-channel detector such as a CCD or photo diode array (PDA). In this case, instead of obtaining trivial cumulative information about the spot signal intensity, we can obtain a signal spectrum, which can be transformed into detailed information about the chemical composition of the given spot on the sample. The three-dimensional array of data sets, two spatial and one spectral, which is recorded in such an imaging measurement, has become known as a “hypercube” or data cube. These microscopy systems are capable of determining a spatial resolution down to approximately 250 nm but will be influenced by the sample matrix.

The pharmaceutical industry has specific challenges for confocal Raman microscopy in characterizing the structure and distribution of the active components within surface coatings of formulations, dosage forms, and delivery devices, to name a few. For example, high-resolution chemical mapping for homogeneity testing of solid oral dosage forms, creams, and ointments may be useful in select applications.

### Spatially Offset Raman Spectroscopy

SORS provides highly accurate chemical analysis of objects beneath obscuring surfaces (e.g., coatings or non-interfering packaging materials). Examples of uses include, but are not limited to, the identification of tablets inside plastic bottles or screening for counterfeit tablets inside blister packs.

A SORS measurement will make at least two Raman measurements: one at the surface and one at an offset position, typically a few millimeters away. The two spectra can be subtracted using a scalar to produce two spectra representing the surface and subsurface spectra. For a simple, two-layer system, such as powder in a plastic bottle, the powder spectrum can be measured without knowing the bottle material or its relative signal contribution. To do this without using an offset measurement would

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involve severe restriction by the interfering signals of the packaging and any fluorescence signals originating from the surface layer. Scaled subtraction works well for two-layer systems, but multi-variate analysis may be required for more complicated situations, such as where the overlying material contains components included in the sublayer (living tissue, for example).

### Deep UV Resonance Raman Spectroscopy

DUVRRS usually uses excitation wavelengths in the 190–260 nm range. There are four major advantages of DUVRRS procedures over their near-UV, visible, or NIR counterparts. These advantages relate to:

- Sensitivity or signal enhancement
- Fluorescence background elimination
- Spectral complexity
- Ambient solar or artificial light background elimination

Current DUVRRS systems come in a large variety of configurations. These variations are primarily related to the light source (laser) and detector systems.

## CALIBRATION

Raman instrument calibration involves three components: primary wavelength (*x*-axis), laser wavelength, and intensity (*y*-axis).

### Primary Wavelength

In the case of FT-Raman instruments, primary wavelength (*x*-axis) calibration is maintained, at least to a first approximation, with an internal helium–neon (He–Ne) laser. Most dispersive instruments use atomic emission lamps for primary wavelength (*x*-axis) calibration. In all instruments suitable for analytical Raman measurements, the vendor offers a procedure for *x*-axis calibration that can be performed by the user. For dispersive Raman instruments, a calibration based on multiple atomic emission lines is preferred. The validity of this calibration approach can be verified after laser wavelength calibration by using a suitable Raman shift standard. For scanning dispersive instruments, calibration might need to be performed more frequently, and precision in both a scanning mode and a static operation mode may need to be verified.[3]

### Laser Wavelength

Laser wavelength variation can affect both the wavelength precision and the photometric (signal) precision of a given instrument. Even the most stable current lasers can vary slightly in their measured wavelength output. The Raman shift wavelength(s) for a given material must therefore be confirmed to ensure that the wavelength scale is accurate for both FT-Raman and dispersive Raman instruments. A reference Raman shift standard material such as those outlined in American Society for Testing and Materials (ASTM) E1840[3] or other suitably verified materials, where the observed peak position(s) have been verified, can be used for this purpose. [NOTE—Reliable Raman shift standard values for frequently used liquid and solid reagents required for wavenumber calibration of Raman spectrometers are provided in the ASTM Standard Guide cited. These values can be used in addition to the highly accurate and precise low-pressure arc lamp emission lines that are also available for use in Raman instrument calibration.] Spectrometric-grade material can be purchased from appropriate suppliers for this use. Certain instruments may use an internal Raman standard that is separate from the primary optical path. External calibration devices reproduce exactly the optical path taken by the scattered radiation. [NOTE—When chemical standards are used, care must be taken to avoid contamination and to confirm standard stability.]

Unless the instrument is of a continuous-calibration type, the primary wavelength (*x*-axis) calibration should be performed, as per vendor procedures, just before measuring the laser wavelength. For external calibration, the Raman shift standard should be placed at the sample location and measured using appropriate acquisition parameters. The peak center of a strong, well-resolved band in the spectral region of interest should be evaluated. The position can be assessed manually or with a suitable, valid peak-picking algorithm. The software provided by the vendor might measure the laser wavelength and adjust the laser wavelength appropriately so that this peak is at the proper position. If the vendor does not provide this functionality, the laser wavelength should be adjusted manually. Depending on the type of laser, the laser wavelength can vary with temperature, current, and voltage. Wavelength tolerances can vary depending on the specific application.

### Intensity

Calibration of the photometric *y*-axis can be critical for successful quantification using certain analytical methods and for method transfer between instruments. Both FT-Raman and dispersive Raman spectrometers should undergo similar calibration procedures. The photometric precision acceptable for a given measurement should be assessed during the procedure development stage.

To calibrate the photometric response of a Raman instrument, a broadband emission source should be used. Most manufacturers will provide appropriate calibration sources and software. If the manufacturer does not provide a procedure or method, the user can accomplish the task using a source obtained from National Institute of Standards and Technology (NIST)

[3] ASTM International. ASTM E1840. Standard Guide for Raman Shift Standards for Spectrometer Calibration. West Conshohocken, PA: ASTM International.

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and appropriate software. If a manufacturer's method is used, attention must be paid to the calibration procedure and source validity. The user should obtain appropriate documentation from the manufacturer to ensure a qualified approach.

There are two accepted methods for calibration; however, other appropriate procedures may be used. *Method A* uses a tungsten white light source.[4] The output power of such sources is traceable to the National Metrology Institute. In the United Kingdom, the National Physical Laboratory also provides calibrated light sources. Several other vendors provide NIST-traceable irradiance calibration standards. *Method A* is applicable to all common laser excitation wavelengths listed in *Table 1*. In *Method B*, NIST standard reference materials (SRMs) are used.[5, 6] Several doped-glass fluorescence standards are currently available.

METHOD A

The source should be placed at the sample location with the laser off and the response of the detector measured (using parameters appropriate for the instrument). The output of the source used for calibration should be known. The ratio of the measured response to the true response should be determined, and a correction file should be generated. This correction should be applied to all spectra acquired with the instrument.

METHOD B

The fluorescence standard should be placed at the sample location. With the laser on, a spectrum of the SRM should be obtained (using parameters appropriate for the instrument). The output of the standard used for calibration should be known. The ratio of the measured response to the true response should be determined, and a correction file should be generated. This correction should be applied to all spectra acquired with the instrument. [NOTE—*Method B* is currently appropriate for systems with 785-nm (SRM 2241), 532-nm (SRM 2242), 514.5-nm and 488-nm (SRM 2243), 1064-nm (SRM 2244), 632.8-nm (SRM 2245), and 830-nm (SRM 2246) excitation.]

## External Calibration

Detailed functional validation using external reference standards is recommended to demonstrate instrumental suitability of laboratory instruments, even for instruments that possess an internal calibration approach. The use of external reference standards does not obviate the need for internal quality control procedures; rather, it provides independent documentation of the fitness of the instrument to perform the specific analysis or purpose. For instruments installed in a process location or in a reactor where routine positioning of an external standard is not possible, including those instruments that use an internal calibration approach, the relative performance of an internal versus an external calibration approach should be checked periodically. The purpose of this test is to detect changes in components that might not be included in the internal calibration method (process lens, fiber optic probe, and others).

# SAMPLE-BASED FACTORS THAT AFFECT MEASUREMENT PERFORMANCE

The most important sample-based factor that deleteriously affects quantitative Raman spectroscopy is fluorescence. Other factors, such as local sample heating by the light source, photobleaching, absorption by the matrix or the sample itself, and the effect of polarization, can also be problematic and must be addressed during procedure development. If the sample matrix includes fluorescent compounds, the measured signal will usually contain a contribution from fluorescence. Fluorescence will be observed only if the laser excitation wavelength overlaps with an absorption band of a fluorescent compound. Fluorescence is typically observed as a broad, sloping background underlying the Raman spectrum. Fluorescence can cause both a baseline offset and a reduced signal-to-noise ratio. The wavelength range and intensity of the fluorescence depend on the chemical composition of the fluorescent material. Because fluorescence is generally a much more efficient process than Raman scattering, even minor amounts of fluorescent impurities can lead to significant degradation of the Raman signal. Fluorescence can be reduced by using longer wavelength excitation sources such as 785 or 1064 nm. As the intensity of Raman scattering is proportional to the fourth power of the absolute wavenumber of scattered light, a significant improvement in Raman scattering efficiency can be expected when higher exciting wavenumbers are used. However, the advantage of using a long-wavelength excitation laser to minimize fluorescence is at least partially offset by the reduced strength of the Raman signal. The greatest signal-to-noise ratio will be obtained by balancing fluorescence rejection, signal strength, and detector response.

Fluorescence in solids can sometimes be mitigated by photobleaching, where the sample is exposed to the laser radiation for a period of time before measurement, and operates by degrading the highly absorbing species. Although this is typically a factor to avoid, photobleaching may be used in exceptional circumstances to mitigate the effect of fluorescence, if no other preprocessing of the sample is possible. Photobleaching is less effective in liquids, where the sample is mobile, or if the amount of fluorescent material is more than a trace.

Sample heating by the laser source can cause a variety of effects, such as physical form change (melting), polymorph conversion, or sample burning. The likelihood of sample heating is greatest when the spot size at the sample is the smallest, i.e., when a microprobe or microscope is being used. This is usually an issue for colored, highly absorbing species, or very small particles that have low heat transfer. The effects of sample heating are usually observable, either as changes in the Raman spectrum over time or by visual inspection of the sample. Besides decreasing the laser flux, a variety of methods can be used

[4] NIST traceable tungsten white light source statement: While the calibration of the Raman frequency (or Raman shift, $cm^{-1}$) axis using pure materials and an existing ASTM standard is well accepted, techniques for calibration of the Raman intensity axis are not. Intensity calibrations of Raman spectra can be accomplished with certified white light sources.

[5] National Institute of Standards and Technology. NIST SRM 2241.

[6] Ray KG, McCreery RL. *Appl Spectrosc.* 1997;51:108–116. Raman intensity correction standard for systems operating with 785-nm excitation.

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to diminish laser-induced heating, such as moving the sample or laser during the measurement or improving the heat transfer from the sample with thermal contact or liquid immersion.

Absorption of the Raman signal by the matrix or the sample itself can also occur. This problem is more prevalent with long-wavelength FT-Raman systems where the Raman signal can overlap with an NIR overtone absorption. This effect will be dependent on the optics of the system, as well as on the sample presentation. Associated with this effect is variability from scattering in solids as a result of packing and particle-size differences. The magnitude of all of these effects is typically smaller than in NIR spectroscopy because of the limited depth of penetration and the relatively narrower wavelength region sampled in Raman spectroscopy.

Finally, it should be recognized that laser radiation is polarized, and the Raman spectra of crystalline materials and other oriented samples can differ significantly, depending on the way that the sample is mounted. If the Raman spectrometer is capable of producing linearly polarized radiation at the sample, then a polarization scrambler is recommended for routine sample analysis.

## PROCEDURE VALIDATION

Validation of Raman procedures will follow the protocols described in *Validation of Compendial Procedures* ⟨1225⟩. Several of these criteria are affected by variables specific to Raman spectroscopy. Fluorescence is the primary variable that can affect the suitability of a procedure. The presence of fluorescent impurities in samples can be quite variable and can have little effect on the acceptability of a material. The procedure must be flexible enough to accommodate different sampling regimens that may be necessary to minimize the effects of these impurities.

Detector linearity must be confirmed over the range of possible signal levels. Fluorescence might drive both the signal baseline and the noise higher than the levels used in the validation, in which case the fluorescence must be decreased, or the procedure must be modified to accommodate the higher fluorescence levels. This is also true for the precision, limit of detection, and limit of quantification of the procedure, as increased baseline noise will adversely affect all of these values. Because fluorescence can also affect quantification caused by baseline shifts, acceptable quantification at different levels of photobleaching, when used, should be confirmed.

The effect of the laser on the sample must be determined. Visual inspection of the sample and qualitative inspection of the Raman spectrum will confirm that the sample is not being altered (other than by photobleaching). Specific variables to confirm in the spectrum are shifts in peak position, changes in peak height and band width, and unexpected changes in background intensity.

Precision must also encompass sample position. The sample presentation is a critical factor for both solids and liquids and must be either tightly controlled or accounted for in the calibration model. Sample-position sensitivity can often be minimized by appropriate sample preparation or sample-holder geometry but will vary from instrument to instrument, based on excitation and collection optical configuration.

In addition, many suitable chemometric algorithms for data pretreatment and calibration exist. The selection of these algorithms should be based on sound scientific judgment and suitability for the intended application.

### Ongoing Procedure Evaluation

Validated Raman procedures should be subject to ongoing performance evaluation, which may include monitoring of accuracy, precision, and other suitable procedure parameters. If the performance is unacceptable, corrective action is necessary. This involves conducting an investigation to identify the cause of the change in the performance of the procedure and may indicate that the Raman procedure is not suitable for continued use. Improving the Raman procedure so that it meets measurement suitability criteria may require additional procedure development as well as validation experiments, with documentation, demonstrating that the improved procedure is suitable for the intended application. The extent of the revalidation required depends on the cause of the change in performance of the procedure and the nature of corrective action required to establish suitable performance. Appropriate change controls should be implemented to document ongoing continuous-improvement activities.

Revalidation of a qualitative model may be necessary as a result of the following:

- Addition of a new material to the spectral reference library
- Changes in the source of material supply
- Identification of previously unknown critical attribute(s) of material(s)

Revalidation of a quantitative model may be necessary as a result of the following:

- Changes in the composition of the test sample or finished product
- Changes in the manufacturing process
- Changes in the sources or grades of raw materials
- Changes in the reference analytical procedure
- Major changes in instrument hardware

#### OUTLIERS

Sample spectra that produce a Raman response that differs from the qualitative or quantitative calibration model may produce an outlier. This does not necessarily indicate an out-of-specification result, but rather an outlier indicates that further testing of the sample may be required. If subsequent testing of the sample by an appropriate procedure indicates that the property of interest is within specifications, then the sample meets its specifications. Outlier samples may be incorporated into an updated calibration model after execution and documentation of suitable validation studies. For further discussion on model lifecycle, see ⟨1039⟩.

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## Method Transfer

Controls and measures for demonstrating the suitability of performance after method transfer are similar to those required for any analytical procedure. Exceptions to general principles for conducting method transfer for Raman procedures should be justified on a case-by-case basis. The transfer of a Raman procedure is often performed by using a Raman calibration model on a second instrument that is similar to the primary instrument used to develop and validate the procedure. When a calibration model is transferred to another instrument, procedures and criteria must be applied to demonstrate that the calibration model meets suitable measurement criteria on the second instrument. The selection of an appropriate calibration model transfer procedure should be based on sound scientific judgment.▲ (USP 1-Aug-2020)

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***Add the following:***

# ▲⟨1901⟩ THEORY AND PRACTICE OF ASBESTOS DETECTION IN PHARMACEUTICAL TALC

To view the Notice from the Expert Committee that posted in conjunction with this chapter, please click https://www.uspnf.com/notices/talc-official-dates-20230601.

## INTRODUCTION

This chapter provides background information to support *Detection of Asbestos in Pharmaceutical Talc* ⟨901⟩. It describes analytical procedures for X-ray diffraction (XRD)—also termed X-ray powder diffraction (XRPD)—and polarized light microscopy (PLM), both of which can be effective for testing of asbestos in talc. Some of the principles applied in this method were first described in the J4-1 method developed by the Cosmetics, Toiletries, and Fragrance Association (CTFA)(*1*) and applied to the analysis of talc for asbestos, which was implemented in 1976, although the method applied only to the analysis of amphibole asbestos. The original asbestos test, which was added to the *Talc* monograph in 2005, expanded on this protocol, applying the principles to chrysotile and amphibole asbestos. For a full description of the current update to this procedure, see the related USP publication (*2*).

XRD in combination with PLM and/or an electron microscopy technique, such as transmission electron microscopy (TEM) or scanning electron microscopy (SEM), can ensure adequate specificity of the asbestos test because definitive microscopic identification and characterization of asbestos and mineral fibers are critical to detect the presence or confirm the absence of asbestos. Specificity encompasses a determination of a particle's mineral identity and whether or not the mineral is asbestiform. This can only be accomplished definitively by microscopy.

As with all of the techniques discussed for asbestos analysis, XRD should not be used alone for asbestos determination, as XRD cannot distinguish morphology and therefore cannot be used exclusively to determine whether amphibole or serpentine are asbestiform or nonasbestiform. However, XRD is critical for the identification of phases present in a mineral mixture and can also determine if foreign materials are present.

Both XRD and PLM allow for testing of larger sample amounts than electron microscopic methods, leading to more representative analyses in general. The largest amount of material can be analyzed in the XRD test, followed by PLM, SEM, and TEM.

The asbestos testing methods for pharmaceutical talc—including XRD, PLM, SEM, and TEM—are compared in *Table 1*.

**Table 1. Methods for Asbestos Detection and Quantification in a Talc Matrix**

| Method | Description | Advantages | Disadvantages |
|---|---|---|---|
| X-Ray diffraction | The presence of amphiboles is detected by a diffraction peak at 10.5 ± 0.1° 2θ, and the presence of serpentines is detected by diffraction peaks at 24.3 ± 0.1° 2θ to 12.1 ± 0.1° 2θ. | • Important in fully characterizing mineral assemblage<br>• Gives information about bulk purity<br>• Can give information about the origin of the talc deposit and associated risk<br>• Can indicate if problematic levels of any phase are present | • Cannot distinguish asbestos from nonasbestos forms of the same mineral<br>• Limit of detection may be too high for public health and regulatory purposes<br>• Detection limit of serpentine is severely affected by the presence of chlorite<br>• May give a false negative if used as a screening method |
| Polarized light microscopy | The presence of asbestos is confirmed by the occurrence of particles that have asbestos morphology and are identified as an asbestos mineral by optical properties and dispersion staining. | • Identification is based on morphology and phase determination; can be conclusive<br>• Particles characterized by PLM are in the size range easily distinguished as asbestos vs. nonasbestos<br>• Good for larger-size products typical of personal care talc products | • Normal quantitation limit may be too high for public health and regulatory purposes if concentration techniques are not used<br>• Particles of milled material (<5 µm) may be below the resolution limit |
| Scanning electron microscopy | The presence of asbestos is confirmed by the occurrence of particles that have asbestos morphology and are identified as an asbestos mineral by energy dispersive x-ray spectroscopy (EDS) elemental analysis. | • A larger sample size (microgram range) is analyzed relative to TEM<br>• Identification is based on morphology and elemental analysis<br>• Resolution is better than PLM<br>• Capable of disclosing surface morphology | • Fibrils of chrysotile may be below the resolution limit of older microscopes<br>• Because it is a presumed ID based on chemistry and morphology alone, it may give a false positive<br>• Structural information methods are currently in development<br>• Interferences: talc vs. anthophyllite, etc. |

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**Table 1. Methods for Asbestos Detection and Quantification in a Talc Matrix** *(continued)*

| Method | Description | Advantages | Disadvantages |
|---|---|---|---|
| Transmission electron microscopy | The presence of asbestos is confirmed by the occurrence of particles that have asbestos morphology and are identified as an asbestos mineral by EDS elemental analysis and electron diffraction. | • Identification is based on morphology, elemental analysis, and electron diffraction (structural information)<br>• May be the only method with the resolution high enough to routinely detect fibrils of chrysotile | • May be prohibitive for quality control due to protracted preparation and analysis time, high cost, irreproducibility, and small sample size (nanogram range)<br>• Because it may miss the larger fibers associated with amphibole asbestos, it may give a false negative |

XRD in combination with PLM can be effective in the characterization of asbestos particles in the >5-µm size range, and PLM has, as its strength, the ability to distinguish asbestiform from nonasbestiform particles. While electron microscopy techniques such as TEM and SEM have the ability to analyze particle sizes that are below the resolution limit of optical microscopy, optical microscopy has sufficient resolution to cover the majority of the size range in coarser pharmaceutical products (e.g., 200 mesh) and a significant portion of the finest products (i.e., <325 mesh). Furthermore, due to the high tensile strength associated with asbestos and the higher hardness of serpentine and amphibole minerals relative to talc—the softest mineral known—asbestos, if present, is not likely to occur solely as individual particles in the finest size range of a milled talc–asbestos mixture, but rather as larger particles and bundles, due to resistance to grinding. This was demonstrated during round robin testing, where spiked samples were prepared using aggressive grinding to simulate production of ground product. Even though the samples were ground, asbestos bundles were still present and identifiable by PLM (*3*).

## PROCEDURE 1: X-RAY DIFFRACTION

XRD is used to determine whether amphibole and/or serpentine are present in a talc powder matrix, and when applicable, to estimate the percentage present. It is intended to be used in combination with one or more microscopy techniques capable of determining whether asbestos is present. XRD is the initial analysis in the monograph asbestos testing and is an important tool for mineral or material characterization.

XRD can be used to determine overall purity of a talc product with respect to accessory minerals and is important in determining the suitability of talc for pharmaceutical use. Depending on a talc's formation conditions, accessory minerals include, but are not limited to, dolomite, magnesite, calcite, and quartz, as well as a variety of micas, chlorites, feldspars, and sometimes serpentines and amphiboles. Chlorite group, feldspars, and dolomite minerals can interfere with the detection of serpentine group minerals during analysis. Refer to *Table 2* in *Appendix 1*, which includes XRD peak positions for talc and minerals that can be associated with talc as well as critical interferences. An example of an XRD qualitative scan of talc (used as the base material for reference material spikes) is included in *Appendix 2*.

This test method uses XRD to detect and identify the presence of total amphiboles and total serpentine in a powdered talc product. [NOTE—XRD cannot distinguish between asbestiform and nonasbestiform varieties of amphibole or serpentine and therefore on its own cannot detect if asbestos is present. XRD can, however, detect whether serpentine or amphibole minerals are present and provide information about their upper limits of occurrence.] XRD can also indicate the presence of foreign substances.

For monoclinic amphiboles (e.g., tremolite, actinolite), the primary diagnostic peak is at approximately 10.5° 2-theta (2θ), and for orthorhombic amphiboles (specifically anthophyllite), the primary diagnostic peak is at approximately 10.7° 2θ with copper Kα radiation. Refer to *Appendix 2* for examples of quantitative XRD scans of talc spiked with 1% tremolite, 1% anthophyllite, and 1% chrysotile showing peak positions for these minerals. For serpentine group minerals, partial or full overlap of peaks from chlorite group minerals can occur (i.e., serpentine diagnostic peaks at 12.1° 2θ and 24.4° 2θ can overlap with chlorite diagnostic peaks at 12.5° 2θ and 25.1° 2θ, respectively). For this method, an amphibole reporting limit of detection has been empirically determined to be 0.2%. However, a lower limit of detection for amphiboles can be attained at 0.1%, as verified by an individual laboratory. For this method, a serpentine limit of detection has been determined to be 0.2% with no chlorite interference and 1% with up to 15% chlorite interference. *Appendix 2* includes examples of quantitative XRD scans of talc spiked with 1% serpentine and 1% chlorite to illustrate the potential interference of these two minerals.

[NOTE—XRD evaluations of amphibole and serpentine minerals may have compositional variation or inherent crystallinity variation or combinations thereof that can be observed through slight shifts in *d*-values and associated peak profile variations associated with full width half maximums (FWHM).]

XRD findings on potential total amphibole or total serpentine content should be consistent with the findings from PLM and any additional microscopic techniques. For example, PLM would not be expected to exhibit percentages of amphibole or serpentine if XRD did not detect them above the detection limit.

When used under quantitative scan conditions specific to this test method, XRD can also be used to determine a relative detection limit of total amphibole and total serpentine minerals in a pharmaceutical talc product. XRD analysis, with respect to asbestos screening of bulk materials, is also discussed in the Environmental Protection Agency's (EPA/600/R-93/116) and the International Organization for Standardization's (ISO 22262-3)(*4*) methods.

For background information about talc, amphibole, and serpentine group minerals, see the relevant USP publication (*2*).

### Detection Limit

An XRD round robin study using samples prepared with known amphibole and serpentine amounts was completed to determine a relative limit of detection for amphiboles (tremolite and anthophyllite), serpentine with no chlorite interference, and serpentine with trace levels of chlorite interference (*2*). Samples included the following:

- 1% tremolite nonasbestiform

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- 1% anthophyllite asbestiform (A-181; as received)
- 1% serpentine—chrysotile (#1422 WCD, as received)
- 0.25% tremolite nonasbestiform
- 0.25% anthophyllite asbestiform (A-181; as received)
- 0.25% serpentine—chrysotile (#1422 WCD, as received)
- 1% serpentine—chrysotile (#1422 WCD, as received)
- 1% chlorite (highly crystalline spike, fine powder)
- Baseline talc (no chlorite, no carbonates, macro-crystalline talc)

Based on these round robin evaluations, the method provided the following:

- Acceptable resolution and detection of tremolite and anthophyllite at a concentration of 0.2%
- Easily resolved and detected tremolite and anthophyllite at a concentration of 1%
- Acceptable detection of serpentine at a concentration of 0.2% without chlorite interference
- Easily detected serpentine at a concentration of 1% without chlorite interference
- Acceptable resolution and detection of serpentine at a concentration of 1% with a chlorite level of up to 15%–20%

Individual laboratories should confirm that these detection limits (or lower) can be achieved by using similar (or lower concentration) spiked standards.

The instrument, equipment, apparatus, and supplies—as well as radiation safety—are described in *Characterization of Crystalline and Partially Crystalline Solids by X-Ray Powder Diffraction (XRPD)* ⟨941⟩.

## Lab Testing Considerations

The lab should be capable of implementing a cGMP-based quality program as described in ⟨941⟩. The use of one or more of the recommended National Institute of Standards and Technology (NIST) XRD reference materials (e.g., NIST SRM 640c–Si) noted in *Appendix 4* should be used to check the alignment of the instrument and sample as follows:

- *Instrument alignment:* A condition where the geometry of the goniometer and sample stage are ideal, and analysis will generate an accurate *d*-value
- *Sample alignment:* A condition where the specimen surface lies on the sample stage within the geometric focal plane of the goniometer and in conjunction with the instrument alignment, providing the most-precise and accurate *d*-values

It is helpful to run talc standards to become familiar with peak positions. Certified reference materials (or other noncertified talc samples) can be obtained for this purpose (see *Appendix 4*.) A list of peak positions for talc and common materials associated with talc is included in *Appendix 2*.

## Further Information

For more information on XRD procedures, laboratories should have access to either *Elements of X-Ray Diffraction* (*5*)—which covers fundamentals, experimental methods, and applications—or *X-ray Diffraction Procedures: for Polycrystalline and Amorphous Materials* (*6*), which provides an effective guide to the science and practical techniques of geometrical crystallography and XRD of crystals as well as more complete coverage of space lattices, point and space groups, production and measurement of X-rays, powder techniques, other related topics, and additional references.

# PROCEDURE 2: POLARIZED LIGHT MICROSCOPY

PLM is a transmitted light microscopic technique where optical properties of minerals can be measured and definitively identified with respect to mineral phase. Estimates of content can also be determined. PLM is described in detail in several publications (*7*–*9*).

Using standard immersion methods with PLM has three methodological strengths. First, the refractive indices (RIs) of the asbestos minerals of concern (e.g., chrysotile, anthophyllite-asbestos, tremolite-asbestos, or actinolite-asbestos) differ from talc such that individual particle identification is possible. This is especially true for the distinction of anthophyllite versus talc, which by other methods may be problematic. Second, tens of thousands of particles can be observed for selected optical properties in a relatively short time. Third, the morphology of each particle can also be determined.

Another technique, dispersion staining, is used to screen for particles of interest based on their color in dispersion staining mode. Dispersion staining is a specific configuration of the microscope, not a physical staining technique of the particles. When the RIs of the mineral and liquid are similar, the RI of the mineral can be estimated using this technique. Alternatively, additional preparations using more RI liquids can be used to determine a more precise value. A heating and cooling stage (optional for this method and discussed below) can also be used to vary the RI liquid values to determine matching indices of the phases analyzed more precisely.

Compared to *Optical Microscopy* ⟨776⟩, the addition of polarized light techniques described in this chapter, along with RI screening by dispersion staining, facilitates particle phase identification.

The PLM technique described below and in ⟨901⟩ involves a wet sieving preparation procedure to evaluate the coarser particles in the mineral powder matrix. This technique takes advantage of the tendency of talc to have a much finer particle size than harder mineral particles in a ground mineral mixture because talc is softer than all other minerals (Moh's hardness of 1 on a 1–10 scale). This causes minerals that are more difficult to grind to concentrate in the coarser size fraction of mixtures containing talc. This is further enhanced by the fact that asbestos is characteristically difficult to grind due to its inherent tensile strength, especially relative to talc. This concept is readily confirmed by attempting to grind asbestos standards using a mortar and pestle. Therefore, enhanced separation of asbestos particles from talc is achieved with wet sieving by removing talc (the

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main component) from the matrix. Enhanced separation of nonasbestos particles of serpentine and amphibole is also achieved due to differences in their hardness (Moh's hardness of 5–6 versus 1, respectively), allowing for a more complete evaluation of morphology of the remaining phases. An added benefit of wet sieving is the removal of fine particles that are too small to be characterized by optical methods and act to dilute the sample.

The dispersion staining method applied to the analysis of chrysotile in talc involves immersing the particles in an RI liquid of 1.550. In the dispersion staining configuration of the PLM with the 1.550 RI liquid, talc plates appear yellow during the entire rotation of the stage. Edge-view talc appears yellow when the particle is oriented with its direction of elongation parallel to the microscope polarizer (*n* parallel) and blue when the particle is oriented with its direction of elongation perpendicular to the microscope polarizer (*n* perpendicular). In contrast, chrysotile is easily distinguished from talc, appearing magenta in the *n*-parallel orientation and blue in the *n*-perpendicular orientation. See *Appendix 3* for an example image of chrysotile with talc in a 1.550 RI liquid.

The dispersion staining method applied to the analysis of amphibole in talc involves immersing the particles in an RI liquid of 1.605. In the dispersion staining configuration of the PLM with the 1.605 RI liquid, talc plates and edge-view talc appear pale blue to white during the entire rotation of the stage. In contrast, amphibole is easily distinguished from talc, appearing yellow when the particle is oriented with its direction of elongation parallel to the microscope polarizer (*n*-parallel) and a deep blue, magenta, or yellow when the particle is oriented with its direction of elongation perpendicular to the microscope polarizer (*n*-perpendicular). Refer to *Appendix 3* for an example image of tremolite with talc in a 1.605 RI liquid.

The 1.605 RI liquid is used to screen for the presence of amphibole in talc. It will be incapable of measuring the gamma prime ($\gamma'$) RI of any amphibole and thus alone cannot be used to identify the mineral species; in some cases, the RIs and thus the colors observed are common across multiple amphiboles.

Use the appropriate matching RI liquid(s) specified in *Table 2* of ⟨901⟩, for suspected amphiboles that may not be conclusive in the dispersion staining screening step (e.g., amphiboles with both RIs significantly higher than 1.605).

The wavelength of match can be determined using dispersion staining charts in Annex C of ISO 22262-1 (*10*) or Becke lines, as shown in Figure 2 of Gunter (*11*). RIs can be determined from the wavelength of match using references (*7*,*12*–*13*). Ranges of RIs for the amphibole minerals can be found elsewhere (*9*,*14*–*15*). A table of typical RI ranges for the regulated asbestos types can be found in EPA/600/R-93/116, Table 2-2 Optical Properties of Asbestos Fibers (*16*).

Optionally, a heating and cooling stage can be used to determine RI more precisely. In this method, the heating and cooling stage can be used with 1.595 RI liquid in plane-polarized light (PPL), cross-polarized light (XPL), and cross-polarized light with the first-order red plate (RPL), starting at room temperature. Scan the entire slide using medium to high magnification. The advantage of using this stage is that one RI liquid can achieve a range of RIs at different temperatures (e.g., when using 1.595 RI liquid, the RI at 125° = 1.551, 50° = 1.584, and 0° = 1.606). For better identification, this procedure can be used to match the RIs of the mineral more precisely.

## Detection Limit

It has been determined empirically that the working detection limit of this method is 0.01%, or 100 ppm by weight (µg/g). This was established in an interlaboratory test involving five laboratories and standards with known concentrations (*3*). In some cases, a concentration of 0.001%, or 10 ppm (µg/g), was detected, although this was not achieved consistently using the average amount of material analyzed at each laboratory. Individual laboratories can establish alternative detection limits if they are validated using standards of known composition.

## Quantitation

Although quantification of results is not required in ⟨901⟩, the following is an optional quantification method using the dimensions of particles documented during analysis:

Calculate the mass of asbestos, *m*, in grams:

$$\text{Result} = l \times w^2 \times f \times \rho \times 10^{-12}$$

$l$ = length (µm)
$w$ = width (µm)
$f$ = shape factor (0.7854 for chrysotile; 0.5 for amphibole) [refer to ISO 22262-2 (*17*)]
$\rho$ = density (mg/m$^3$)

Calculate the total asbestos mass observed for all slides by summing the mass of each asbestos particle, $m_T$, in grams.

Calculate the total mass of the sample analyzed by summing the mass of sample placed on each slide, $m_A$, in grams.

Calculate the total fiber mass as a percentage:

$$\text{Result} = m_T/m_A \times 100$$

[NOTE—When quantitating the portion retained on a sieve (following wet sieve preparation), the above calculation applies only to the size fraction analyzed.]

## Lab Testing Considerations

It is helpful to ensure that the microscope is set up and aligned properly by obtaining dispersion staining colors on prepared standards of amphibole and chrysotile asbestos before analyzing unknown material.

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An analyst performing this method should have training specific to PLM and the various modes of PLM operation and must have a rigorous working knowledge of all terms described.

An example of a quality control program relating to PLM analysis can be found in EPA/600/R-93/116, Section 3.0 (*16*).

## Standards and Calibration

- NIST or NIST-traceable standards should be used for all types of asbestos, both common and uncommon asbestos types (the six regulated species), as well as interfering minerals and talc itself. [NOTE—Other characterized standards may be used as well, such as those provided by the National Institute of Environmental Health Sciences (NIEHS) or the Union for International Cancer Control (UICC).]

  Mounting media calibration (RI liquids):
  - Calibrate upon opening a new bottle from the manufacturer and quarterly thereafter for RI liquids that are used on a regular basis (e.g., 1.550, 1.605)
  - Calibrate infrequently used liquids before each use
  - Perform calibration using either NIST-traceable reference particles (e.g., Cargille precision optical glass) or an Abbe refractometer calibrated with a similar NIST-traceable standard

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# APPENDICES

## Appendix 1. X-Ray Diffraction Peak Positions

**Table 2. X-Ray Diffraction Peak Positions for Talc and Associated Minerals[a]**

| 2-Theta (°) | *d*-Spacing | Mineral | Example ICDD[b] File Number(s) | Intensity ($I/I_{100}$)[c] | Comments/Potential Interferences |
|---|---|---|---|---|---|
| 8.8–8.9 | 9.9–10.0 | Mica—biotite, muscovite | 42-603, 7-42 | 100 | — |
| 9.5 | 9.3 | Talc | 13-558, 19-770 | 100 | — |

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**Table 2. X-Ray Diffraction Peak Positions for Talc and Associated Minerals[a]** *(continued)*

| 2-Theta (°) | *d*-Spacing | Mineral | Example ICDD[b] File Number(s) | Intensity ($I/I_{100}$)[c] | Comments/Potential Interferences |
|---|---|---|---|---|---|
| 10.5 | 8.4 | Amphibole—actinolite | 41-1366 | 75 | — |
| 10.5 | 8.4 | Amphibole—magnesio-hornblende | 20-481 | 100 | — |
| 10.5 | 8.4 | Amphibole—riebeckite | 16-1061, 60-343 | 100 | — |
| 10.5 | 8.4 | Amphibole—tremolite | 13-437, 44-1402 | 100 | — |
| 10.6 | 8.3 | Amphibole—grunerite | 44-1401 | 100 | — |
| 10.7 | 8.3 | Amphibole—anthophyllite | 9-455, 45-1343 | 57 | — |
| 11.7 | 7.5 | Sepiolite | 26-1226 | 50 | — |
| **12.1** | **7.3** | **Serpentine—lizardite** | **9-444, 50-1606** | **100** | **Interference between serpentine phases, chlorite, and sepiolite** |
| **12.1** | **7.3** | **Serpentine—chrysotile** | **21-543, 52-1562** | **100** | |
| **12.1** | **7.3** | **Sepiolite** | **13-595, 26-1226** | **100** | |
| **12.1** | **7.3** | **Serpentine—antigorite** | **21-963** | **100** | |
| **12.4** | **7.1** | **Chlorite—clinochlore** | **24-506, 12-242** | **100** | |
| 20.9 | 4.3 | Quartz | 33-1161, 46-1045 | 22 | — |
| **24.3** | **3.7** | **Serpentine—lizardite** | **9-444, 50-1606** | **100** | **Interference between serpentine phases, chlorite, and sepiolite** |
| **24.4** | **3.7** | **Serpentine—chrysotile** | **21-543, 52-1562** | **70** | |
| **24.6** | **3.6** | **Serpentine—antigorite** | **21-963** | **80** | |
| **25.0** | **3.6** | **Chlorite—clinochlore** | **24-506, 12-242** | **53** | |
| **25.1** | **3.5** | **Sepiolite** | **26-1226** | **19** | |
| 26.5 | 3.4 | Sepiolite | 13-595, 26-1226 | 30 | — |
| 26.7–26.8 | 3.3 | Mica—biotite, muscovite | 42-603, 6-263 | 80–100 | — |
| 26.7 | 3.3 | Quartz | 33-1161, 46-1045 | 100 | — |
| 27.5 | 3.2 | Feldspar—microcline | 19-932 | 100 | — |
| 27.4 | 3.2 | Rutile | 21-1276 | 100 | — |
| 27.9 | 3.2 | Feldspar—albite | 9-466 | 100 | — |
| 27.9 | 3.2 | Feldspar—anorthite | 12-301 | 100 | — |
| **28.6** | **3.1** | **Amphibole—tremolite** | **13-437, 44-1402** | **100** | **Interference between amphibole and talc (use lower 2θ region)** |
| **28.6** | **3.1** | **Amphibole—riebeckite** | **19-1061, 60-343** | **55** | |
| **28.6** | **3.1** | **Amphibole—actinolite** | **41-1366** | **100** | |
| **28.6** | **3.1** | **Talc** | **13-558, 19-770** | **70** | |
| **29.4** | **3.0** | **Calcite** | **5-586** | **100** | **Interference between calcite and anthophyllite** |
| **29.3** | **3.1** | **Anthophyllite** | **9-455, 45-1343** | **100** | |
| 30.9 | 2.9 | Dolomite | 36-426 | 100 | — |
| 31.9 | 2.8 | Fluorapatite | 15-876, 60-667 | 100 | — |
| 32.4 | 2.8 | Amphibole—grunerite | 44-1401 | 38 | — |
| 32.6 | 2.7 | Magnesite | 8-479 | 100 | — |
| 33.1 | 2.7 | Fluorapatite | 15-876, 60-667 | 60 | — |
| 39.4 | 2.3 | Calcite | 5-586 | 18 | — |
| 41.1 | 2.2 | Dolomite | 36-426 | 19 | — |
| 43.0 | 2.1 | Magnesite | 8-479 | 45 | — |
| 54.3 | 1.7 | Rutile | 21-1276 | 60 | — |

[a] Potential interferences are noted (2θ based on copper Kα radiation).
[b] International Centre for Diffraction Data (*18*).
[c] $I$ = peak intensity; $I_{100}$ = intensity of the most intense peak.

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## Appendix 2. Representative XRD Diffractograms

The figures in this appendix show example scans of baseline talc and talc spiked with tremolite, anthophyllite, chrysotile, and chlorite. (Baseline talc has been characterized and found to contain no detectable asbestos or amphibole.) Peak positions in these scans can serve as references for these minerals (also refer to the peak position list in *Appendix 1*).

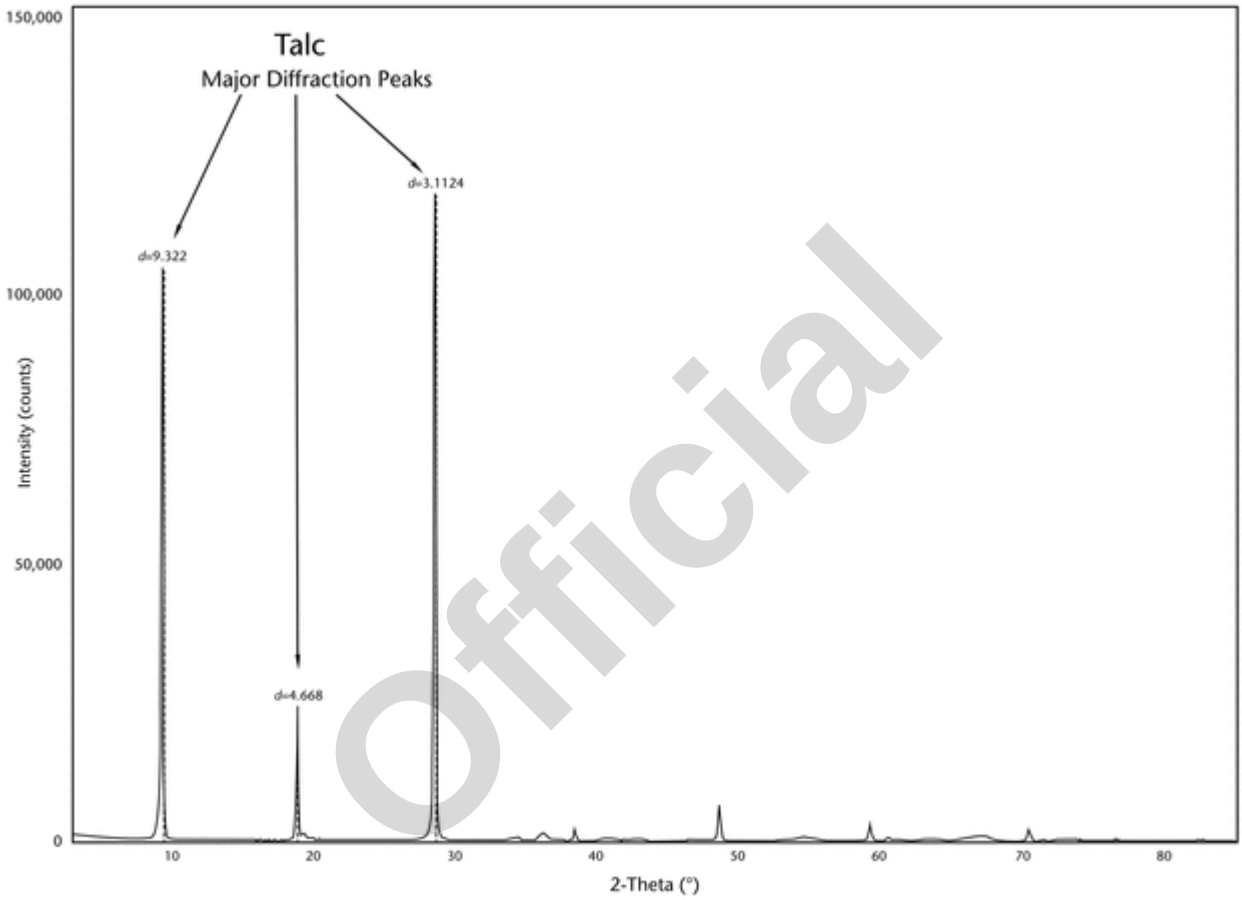


Figure 1. Typical talc scan. Qualitative analysis conditions using copper Kα radiation are 3°–85° 2θ; talc major diffraction peaks with *d*-values and spacings noted.

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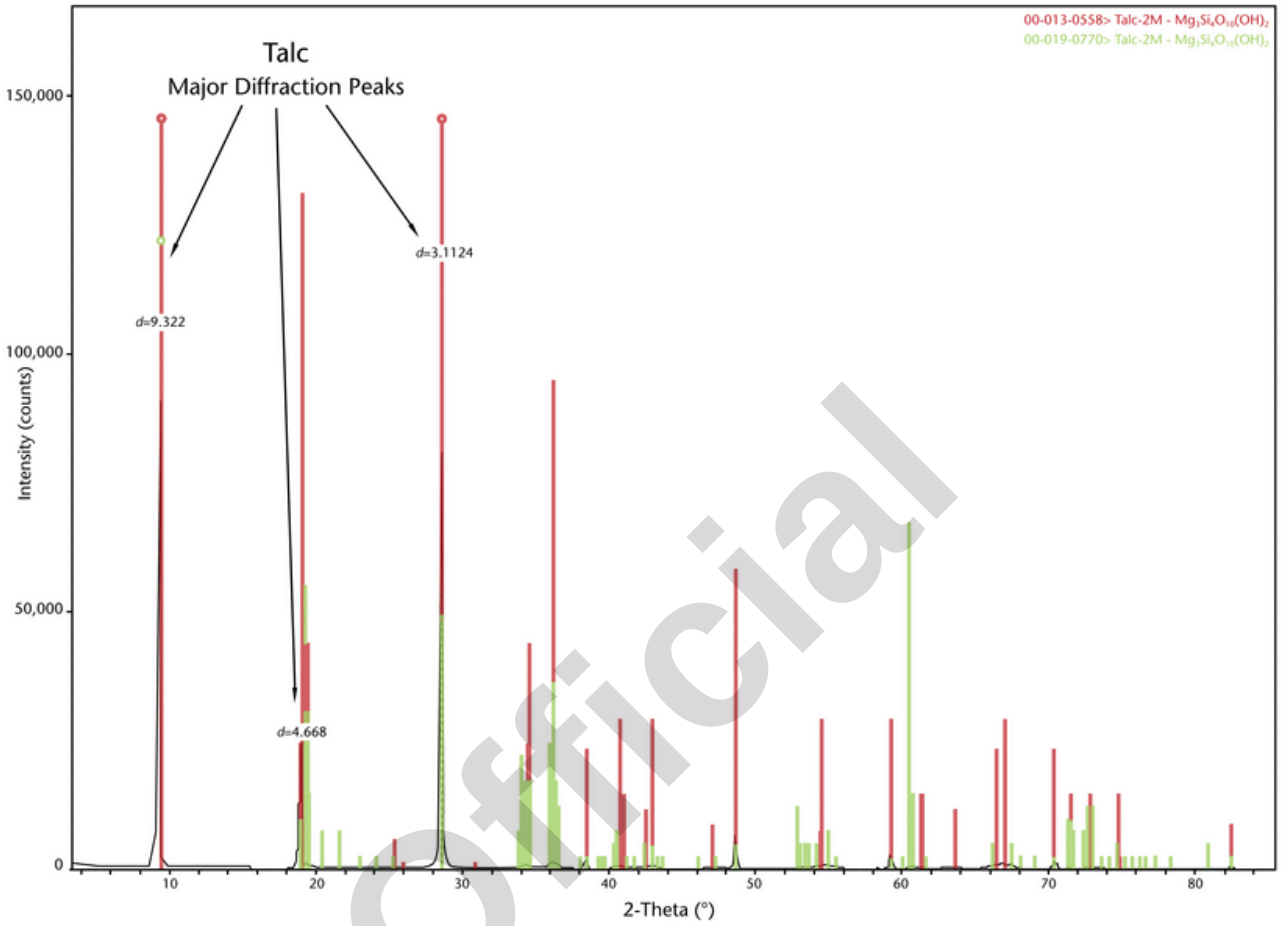


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Figure 2. Typical talc scan. Qualitative analysis conditions using copper Kα radiation are 3°–85° 2θ; talc major diffraction peaks with *d*-values and spacings noted. Overlay of ICDD talc data files 13-558 and 19-770 are shown (*18*).

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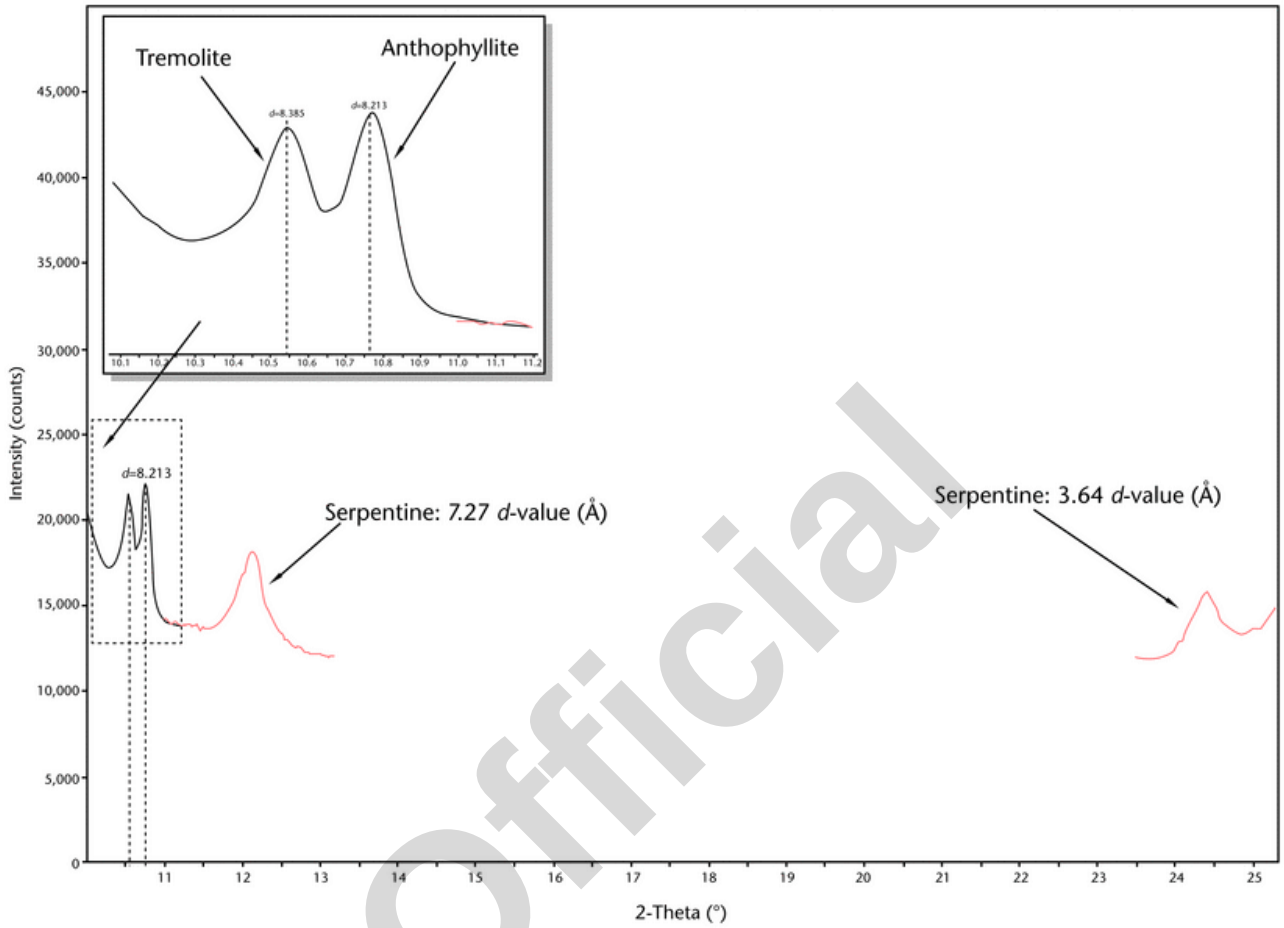


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Figure 3. Talc spiked with 1% tremolite (amphibole—monoclinic), 1% anthophyllite (amphibole—orthorhombic), and 1% serpentine. Quantitative analysis conditions using copper Kα radiation for amphiboles are 9.8°–11.2° 2θ and for serpentines are 11.0°–13.2° 2θ and 23.5°–25.6° 2θ.

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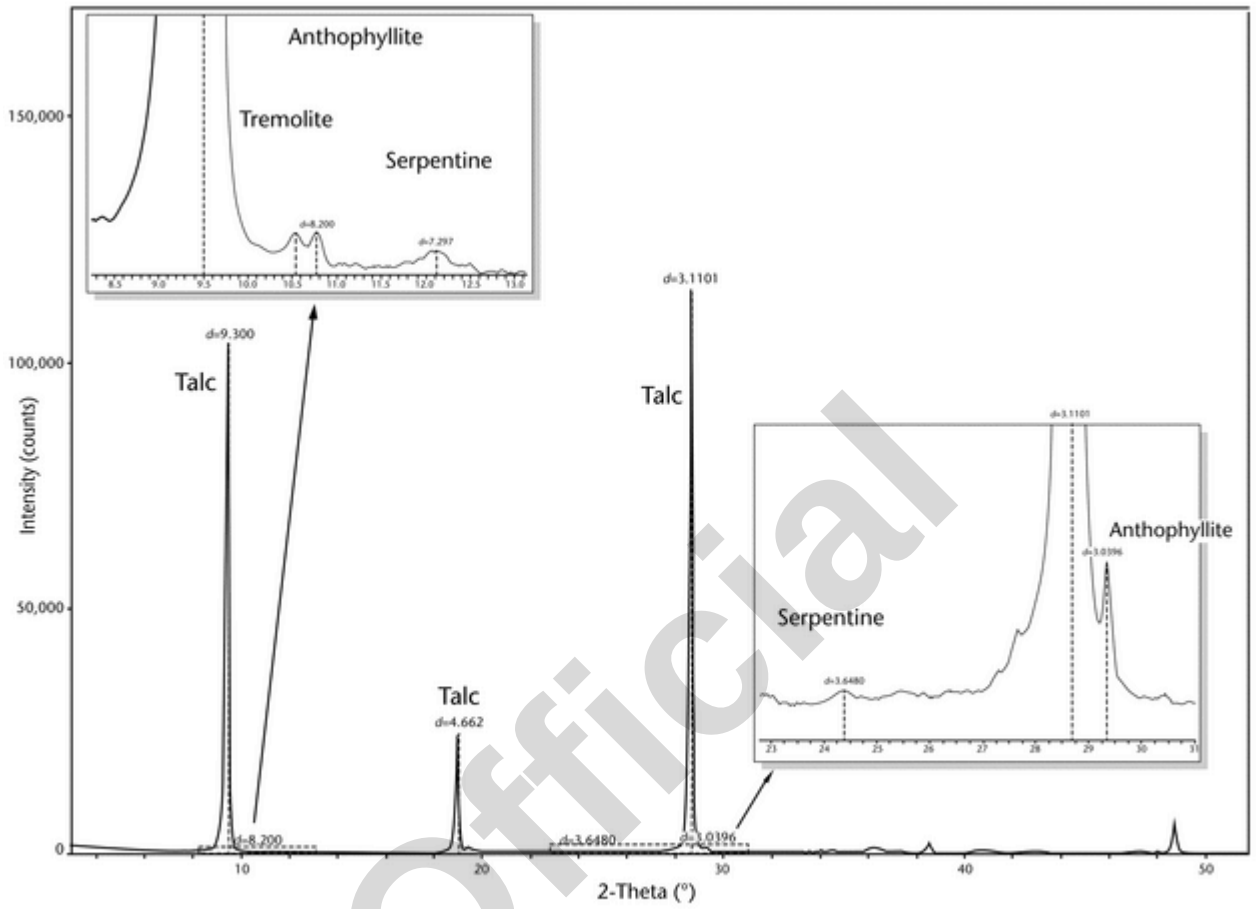


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Figure 4. Talc spiked with 1% tremolite nonasbestiform (amphibole—monoclinic), 1% anthophyllite (amphibole—orthorhombic), and 1% serpentine. Qualitative analysis conditions using copper Kα radiation are 3°–52° 2θ; Expanded intensity scale for the qualitative scans are shown for amphiboles and serpentines.

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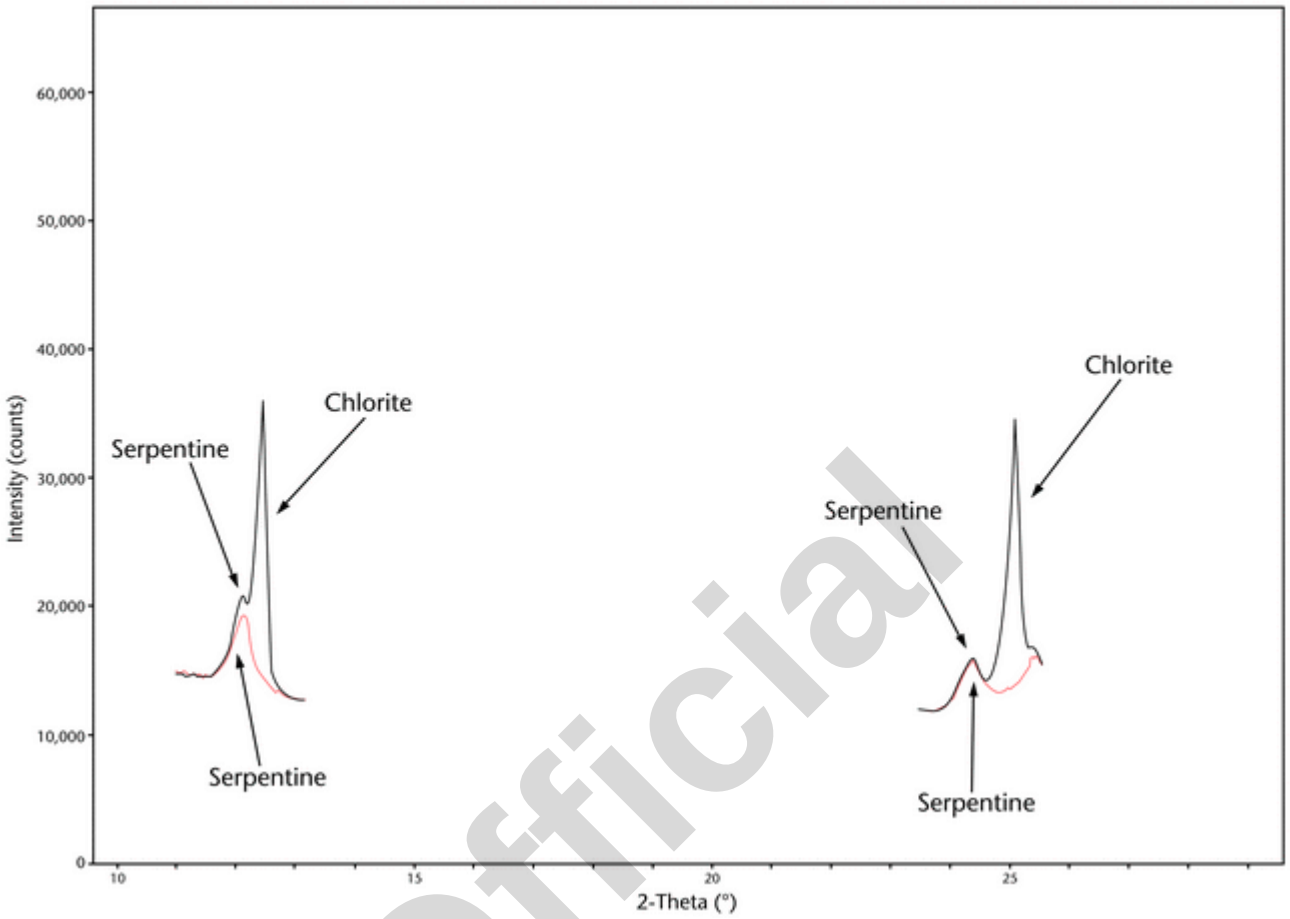


Figure 5. Talc spiked with 1% serpentine with and without chlorite interference (1% serpentine with no chlorite $n_D$<0.1% shown in red; 1% serpentine with 1% chlorite shown in black). Quantitative analysis conditions using copper Kα radiation for serpentines are 11.0°–13.2° 2θ and 23.5°–25.6° 2θ.

## Appendix 3. Representative PLM Images

The following are PLM image examples of chrysotile and amphibole asbestos spiked into talc. Additional images are contained in *Appendix 5*.

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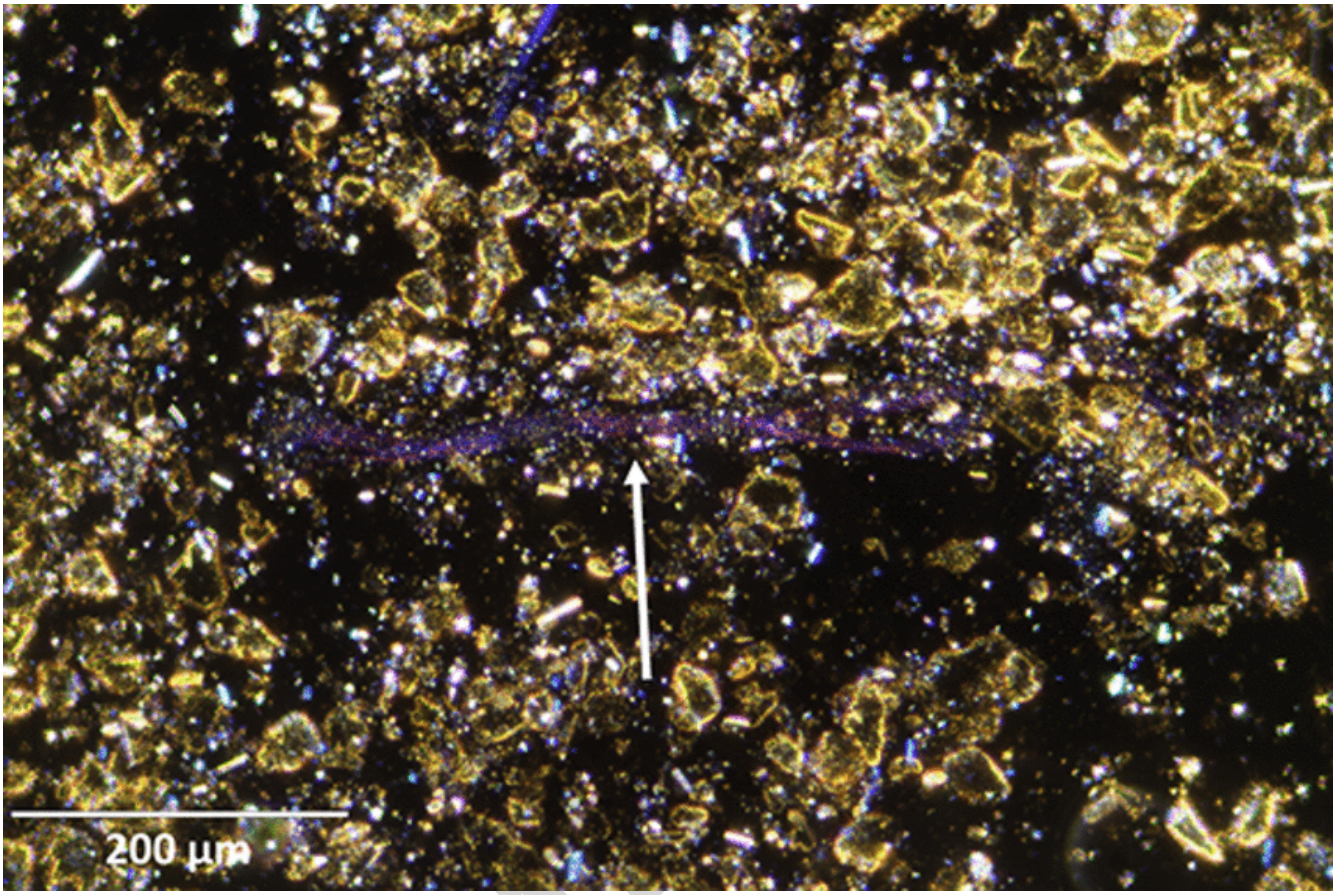


Figure 6. Chrysotile (magenta) compared to talc particles (yellow) in 1.550 RI liquid (magenta particle is oriented parallel to the lower polarizer). Spiked material was retained on a 400-mesh sieve.

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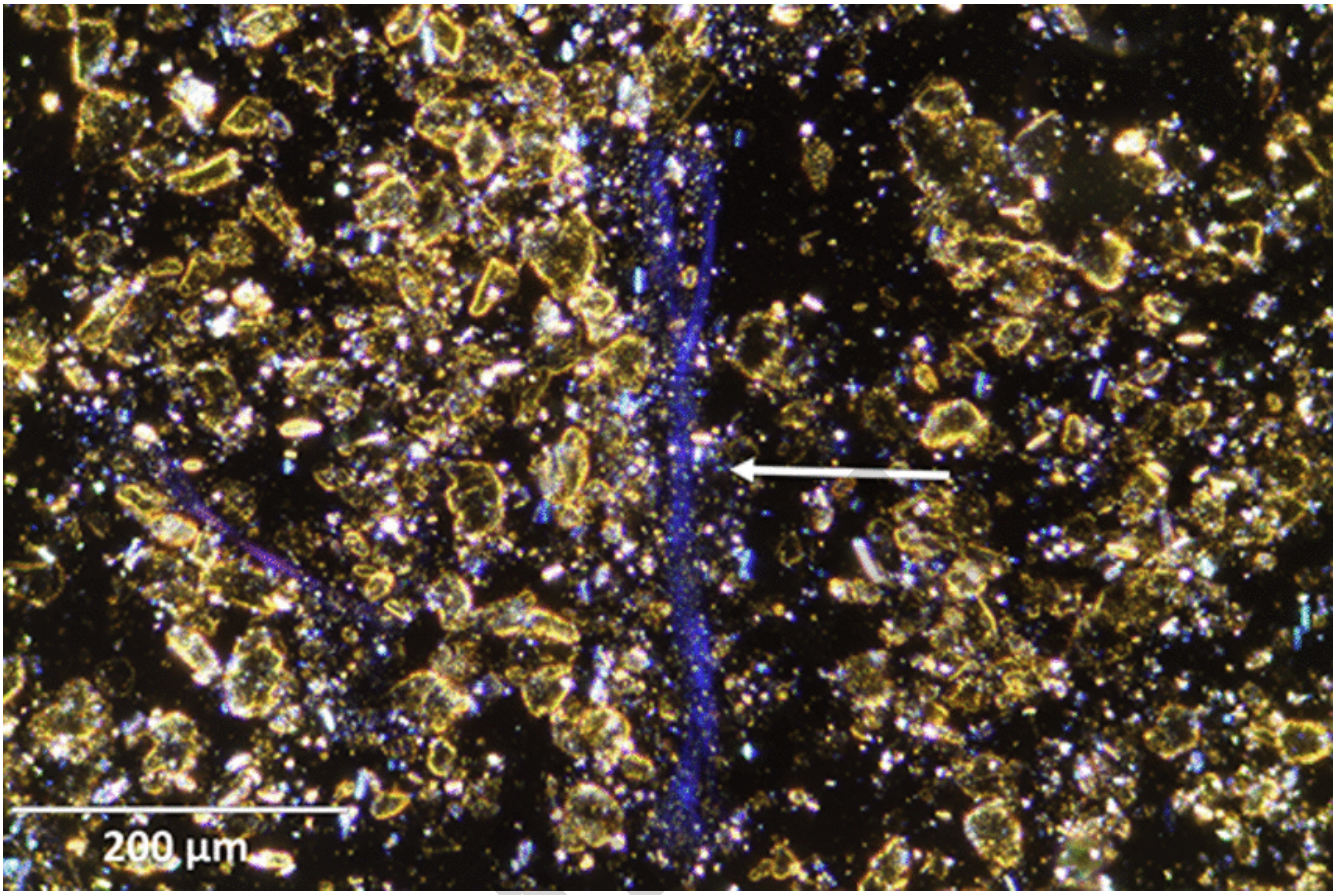


Figure 7. Chrysotile (blue) compared to talc particles (yellow) in 1.550 RI liquid (blue particle is oriented perpendicular to the lower polarizer). Spiked material was retained on a 400-mesh sieve.

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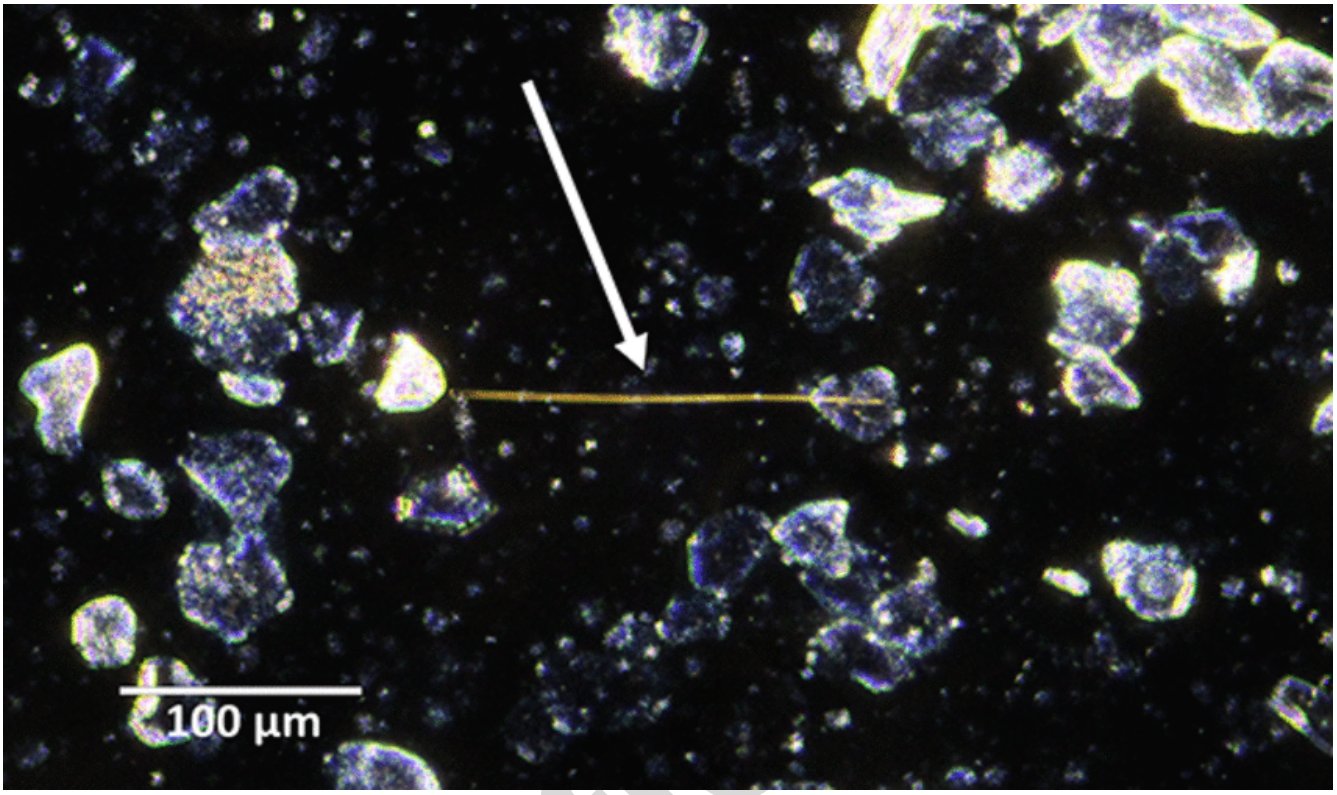


Figure 8. Tremolite (yellow) compared to talc particles (pale blue) in 1.605 RI liquid (yellow particle is oriented parallel to the lower polarizer). Spiked material was retained on a 400-mesh sieve.

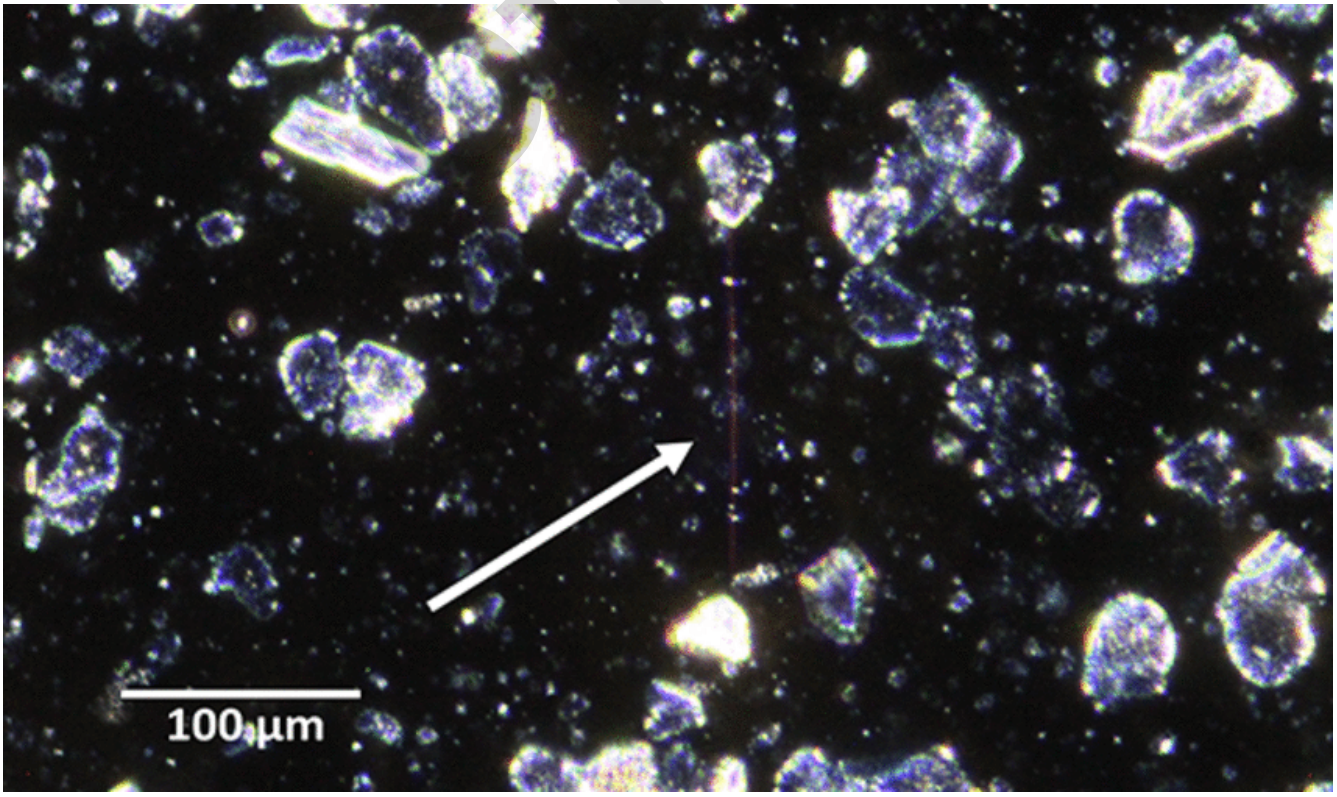


Figure 9. Tremolite (magenta) compared to talc particles (pale blue) in 1.605 RI liquid (magenta particle is oriented perpendicular to the lower polarizer). Spiked material was retained on a 400-mesh sieve.

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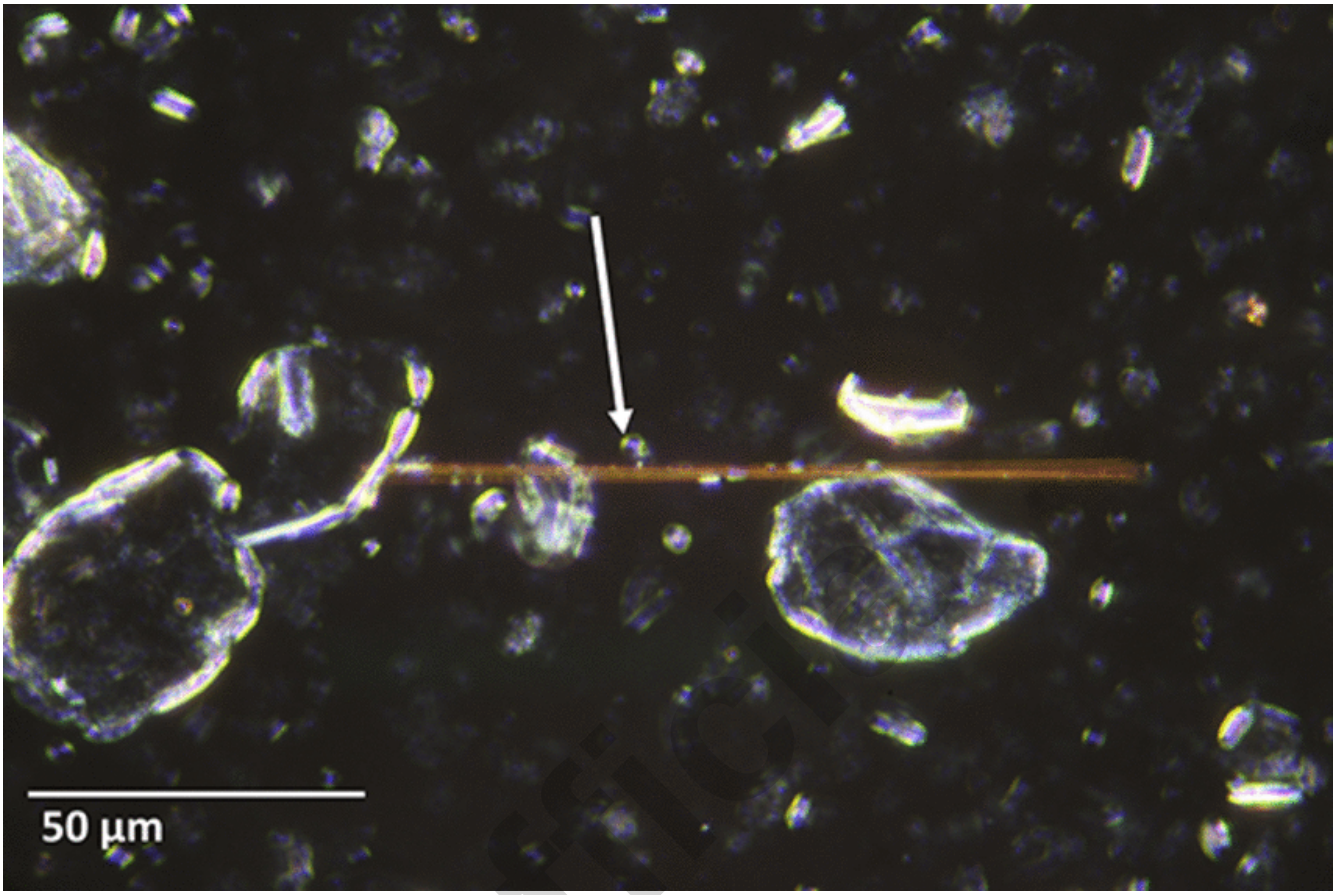


Figure 10. Tremolite (yellow) compared to talc particles (pale blue) in 1.620 RI liquid (yellow particle is oriented parallel to the lower polarizer). Spiked material was retained on a 400-mesh sieve.

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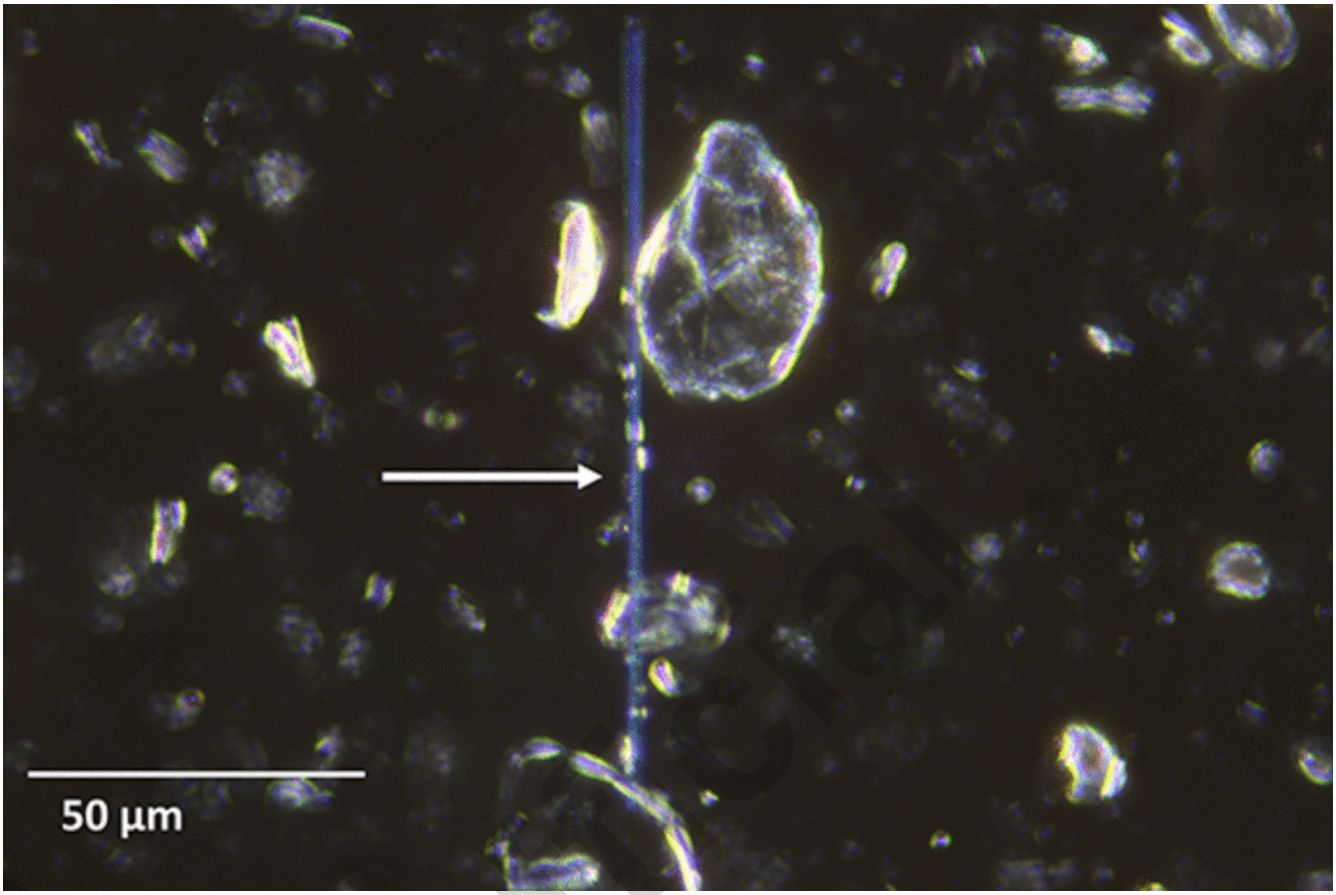


Figure 11. Tremolite (blue) compared to talc particles (pale blue) in 1.620 RI liquid (blue particle is oriented perpendicular to the lower polarizer). Spiked material was retained on a 400-mesh sieve.

## Appendix 4. Example Materials

[NOTE—Asbestos and nonasbestos standards are becoming difficult to obtain from national laboratories or institutes. The use of in-house or noncertified reference materials may be needed. It is recommended that permanent mounts for PLM be made or purchased to be used for lab reference. Available example materials are listed below.]

- XRD available certified reference materials
  - NIST SRM 640b or above silicon powder, *d*-spacing standard
  - NIST SRM 674b or above x-ray powder diffraction intensity set
  - NIST SRM 660a or above LaB6, line position and line shape standard
  - NIST SRM 675 or above fluorophlogopite, low angle (large *d*-spacing) standard
- Talc available certified reference materials
  - Brammer primary certified reference material; JCRM R901, R902, R903 (set of three)
  - Brammer certified reference material, BCS 203a RM asbestos and nonasbestos reference materials
- Amphibole
  - NIST 1867a, uncommon commercial asbestos—tremolite, anthophyllite, actinolite (set of three)
  - Lone Pine tremolite (as provided by Research Triangle Institute)
  - Anthophyllite and talc (Fisher A-181, Gooch filter)
- Serpentine
  - Whittaker, Clark, and Daniels #1422 asbestos (1971)
  - NIST SRM 1866a commercial asbestos—chrysotile

For XRD, homogeneity concerns must be considered when using dry preparations of reference standards, regardless of preparation size. Therefore, it is recommended that the laboratory analyst use ethanol, isopropanol, or methanol for all reference standard preparations.

Mortar and pestle preparation can be used for small preparations (<10 g) but should include multiple wet grinding or blending and drying cycles (minimum of three). For large reference standard preparations (>10 g), the laboratory analyst should use ethanol, isopropanol, or methanol in conjunction with milling or grinding using puck or puck and ring mills under shear and impact conditioning or by cylindrical media-mill grinding with impact and planar shearing.

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## Appendix 5. PLM Digital Image Library

The following images represent the different minerals immersed in various RI liquids and can be used for reference.

- Milled chrysotile in talc
  - *Milled chrysotile in talc matrix—1.550 RI liquid (not sieved)*
  - *Milled chrysotile in talc matrix (0.1%)—1.550 RI liquid (material retained on 400-mesh sieve)*
  - *Milled chrysotile in talc matrix (0.01%)—1.550 RI liquid (material retained on 400-mesh sieve)*
  - *Milled chrysotile in talc matrix (0.001%)—1.550 RI liquid (material retained on 400-mesh sieve)*
- Milled tremolite asbestos in talc
  - *Milled tremolite asbestos in talc matrix—1.605 RI liquid (not sieved)*
  - *Milled tremolite asbestos in talc matrix—1.615 RI liquid (not sieved)*
  - *Milled tremolite asbestos in talc matrix (0.1%)—1.620 RI liquid (material retained on 400-mesh sieve)*
  - *Milled tremolite asbestos in talc matrix (0.01%)—1.605 RI liquid (material retained on 400-mesh sieve)*
  - *Milled tremolite asbestos in talc matrix (0.01%)—1.620 RI liquid (material retained on 400-mesh sieve)*
  - *Milled tremolite asbestos in talc matrix (0.001%)—1.605 RI liquid (material retained on 400-mesh sieve)*
- Milled tremolite (not tremolite asbestos) in talc
  - *Milled tremolite (not tremolite asbestos) in talc matrix—1.605 RI liquid (not sieved)*
  - *Milled tremolite (not tremolite asbestos) in talc matrix—1.615 RI liquid (not sieved)*
  - *Milled tremolite (not tremolite asbestos) in talc matrix (0.1%)—1.620 RI liquid (material retained on 400-mesh sieve)*
- Milled talc
  - *Milled talc in 1.550 RI liquid (not sieved)*

MILLED CHRYSOTILE IN TALC

**Milled chrysotile in talc matrix—1.550 RI liquid (not sieved)**

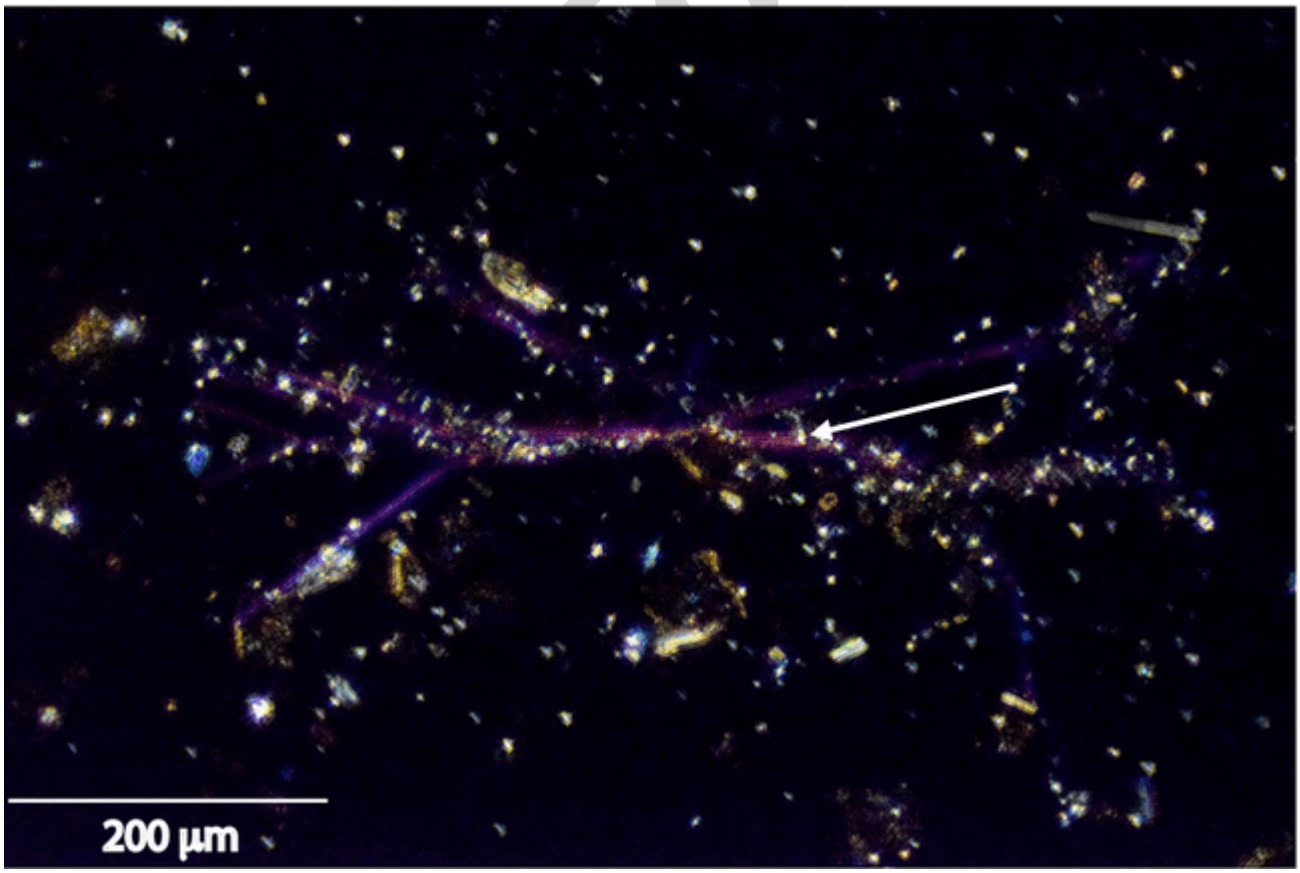


Figure A1. Milled chrysotile in talc matrix immersed in Cargille $n_D$ 1.550 HD liquid at 25° oriented so that $\gamma'$ (and fiber length) is parallel with the direction of the lower polarizer (East–West [E–W]) in central stop dispersion staining.

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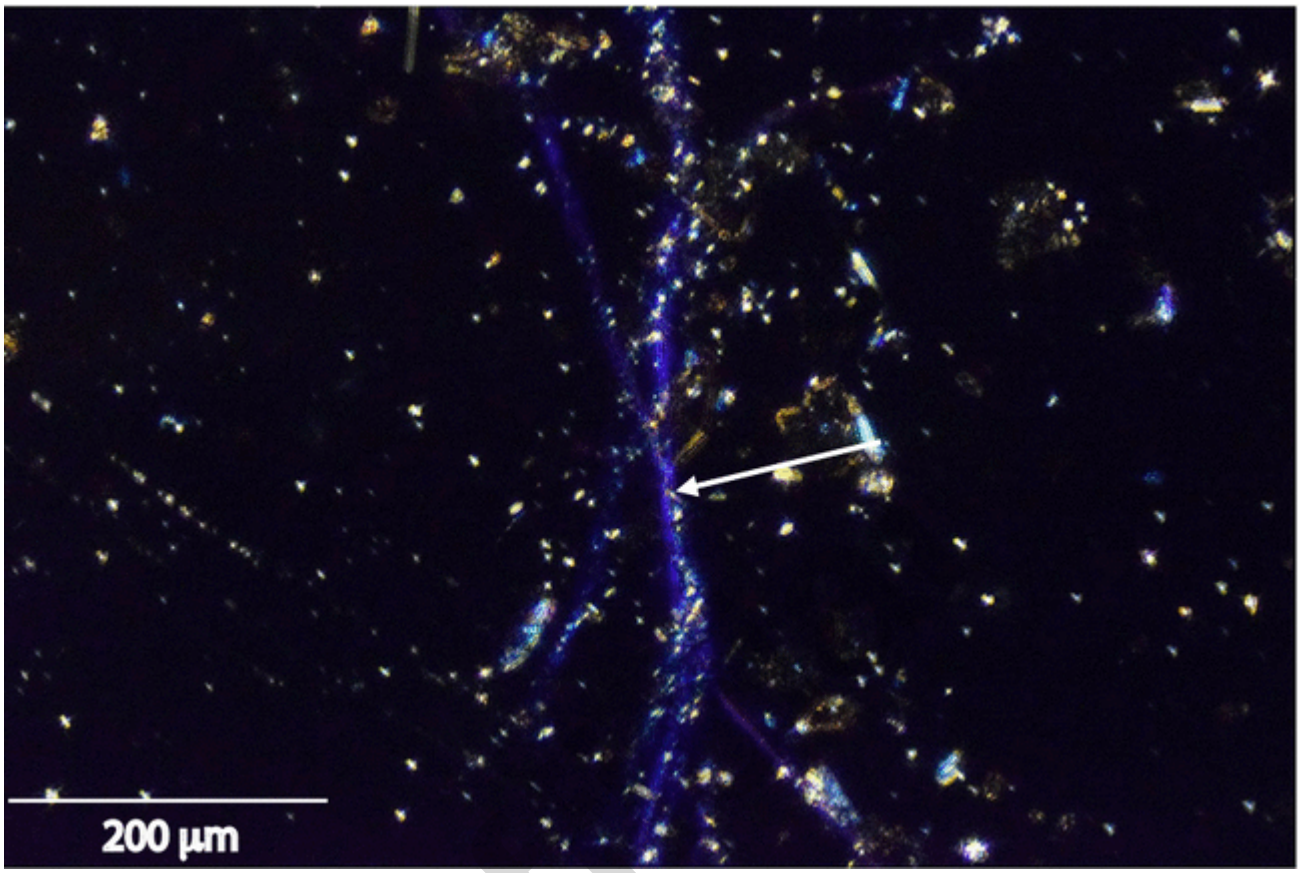


Figure A2. Milled chrysotile in talc matrix immersed in Cargille $n_D$ 1.550 HD liquid at 25° oriented so that α′ is parallel with the direction of the lower polarizer (E–W) in central stop dispersion staining (fiber length is perpendicular to the lower polarizer).

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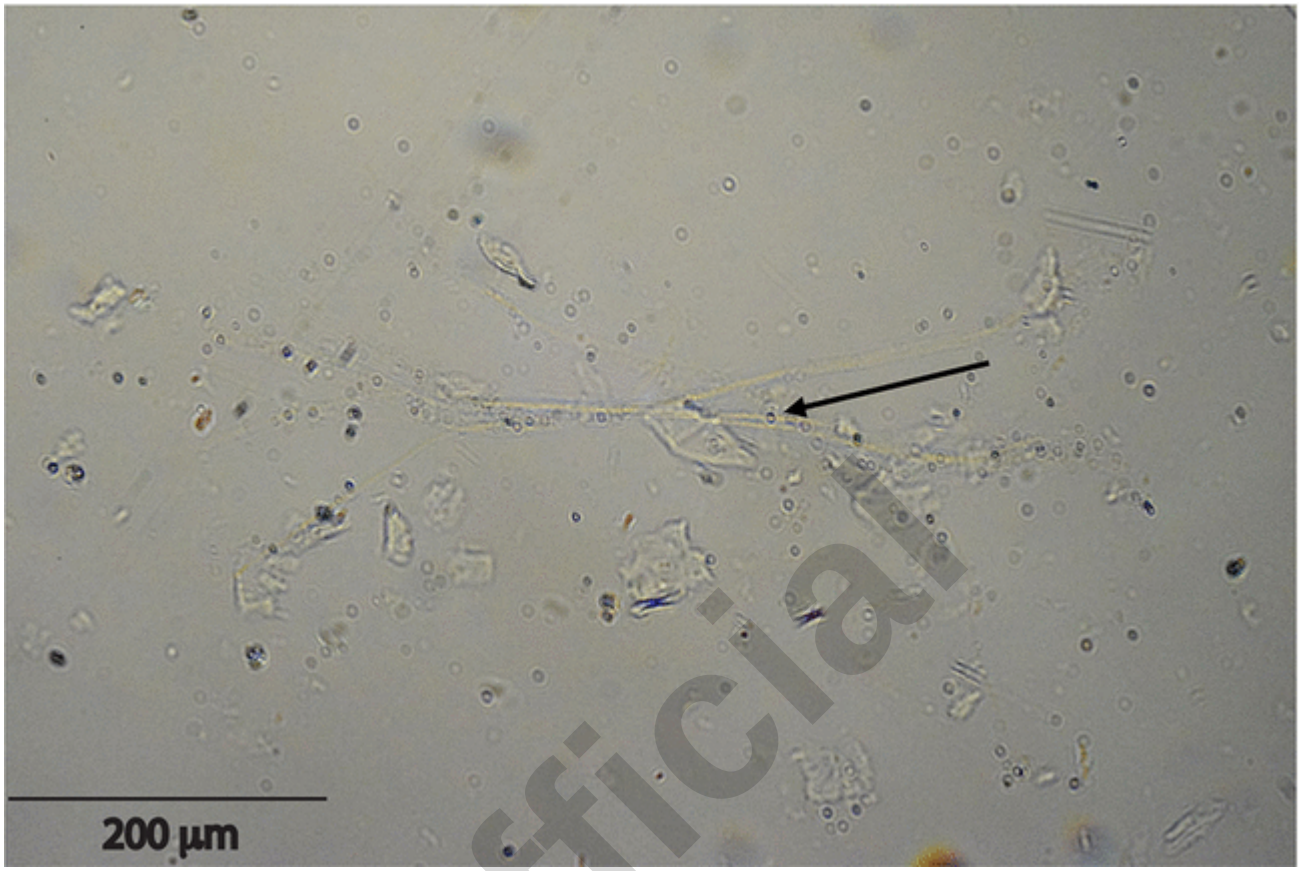


Figure A3. Milled chrysotile in talc matrix immersed in Cargille $n_D$ 1.550 HD liquid at 25° oriented so that $\gamma'$ (and fiber length) is parallel with the direction of the lower polarizer (E–W) in plane-polarized light slightly defocused to observe the Becke line.

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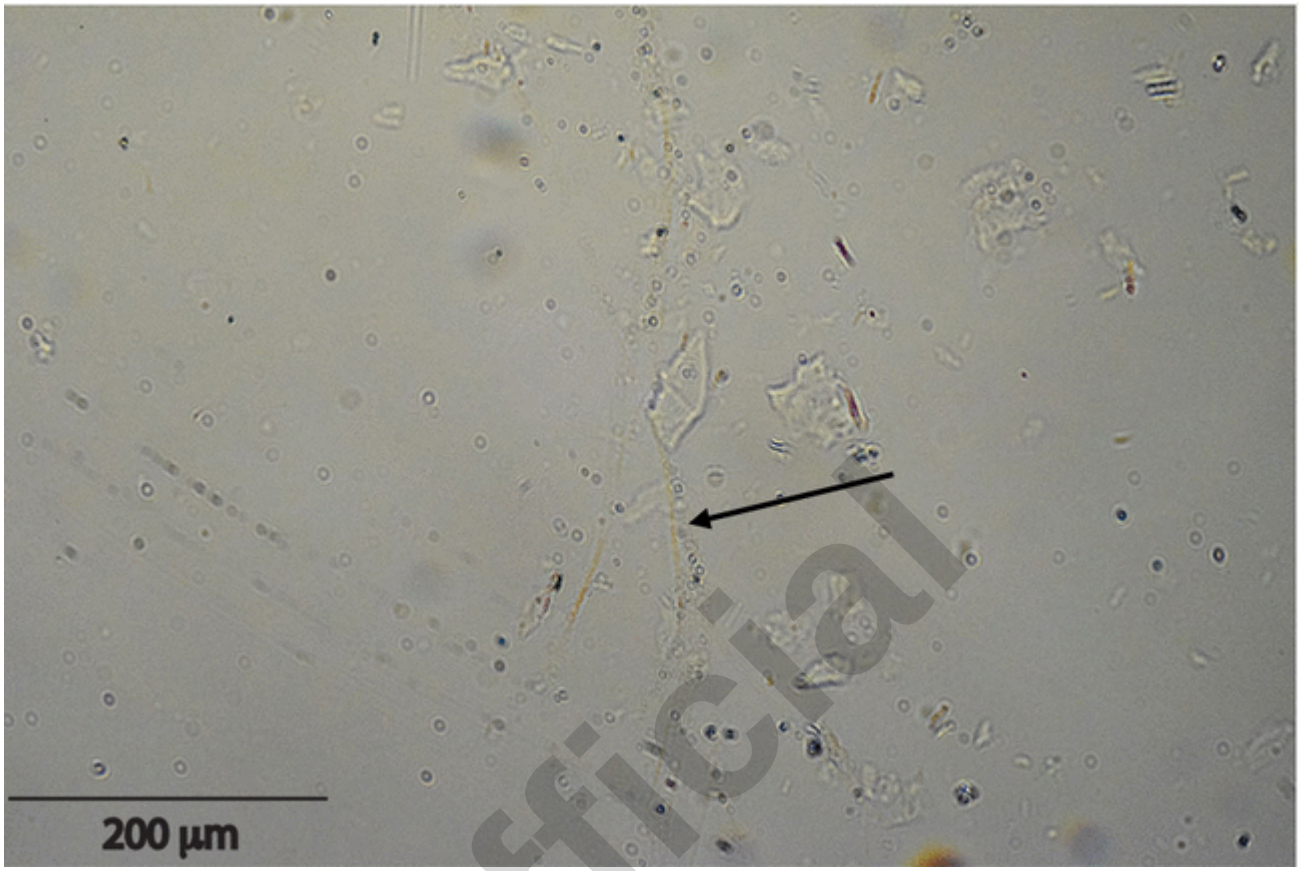


Figure A4. Milled chrysotile in talc matrix immersed in Cargille $n_D$ 1.550 HD liquid at 25° oriented so that α′ is parallel with the direction of the lower polarizer (E–W) in plane-polarized light slightly defocused to observe the Becke line (fiber length is perpendicular to the lower polarizer).

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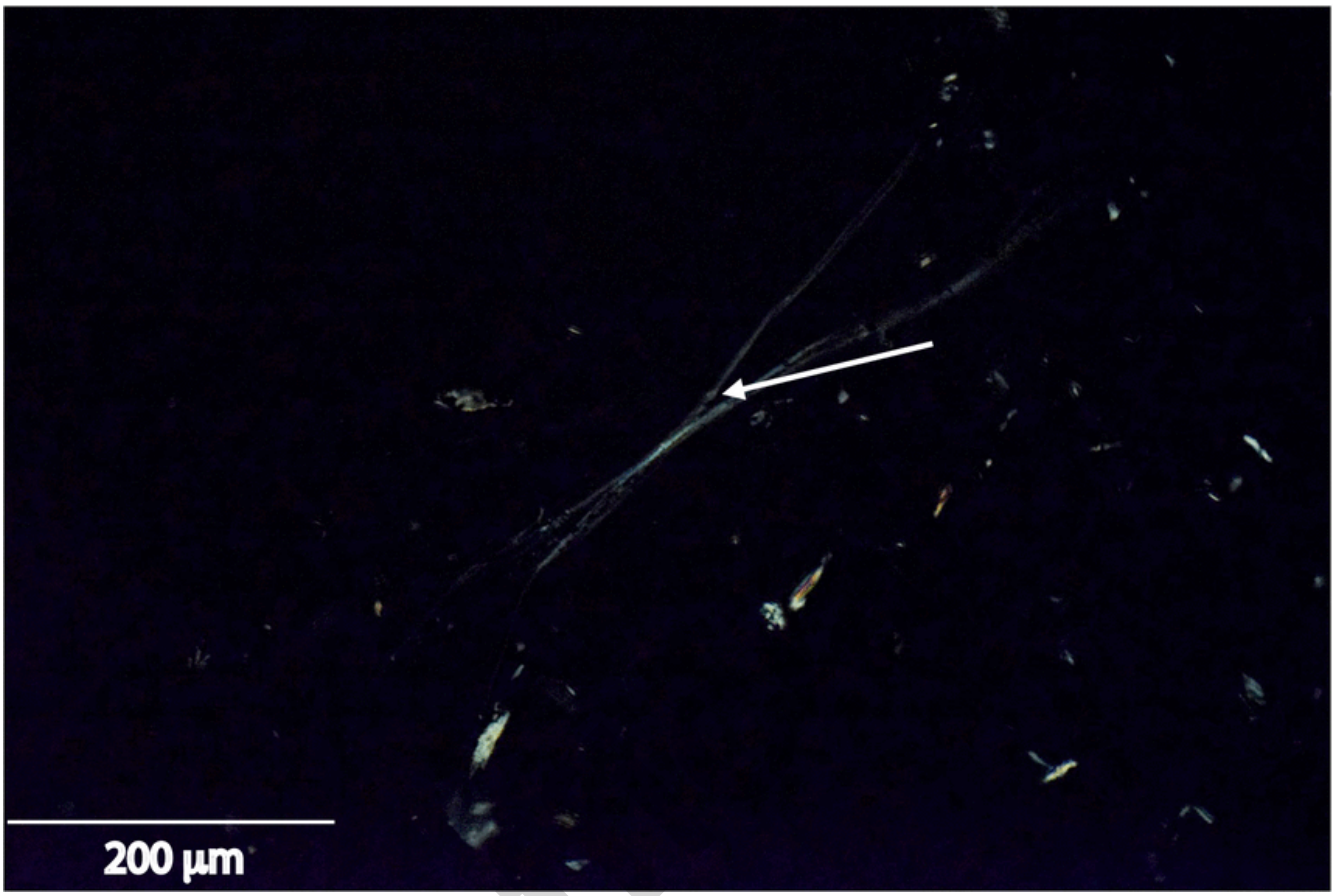


Figure A5. Milled chrysotile in talc matrix immersed in Cargille $n_D$ 1.550 HD liquid at 25° oriented so that $\gamma'$ is 45° counterclockwise up from the lower polarizer (E–W) and parallel with N (the larger of the privileged directions of the accessory plate) in cross-polarized light.

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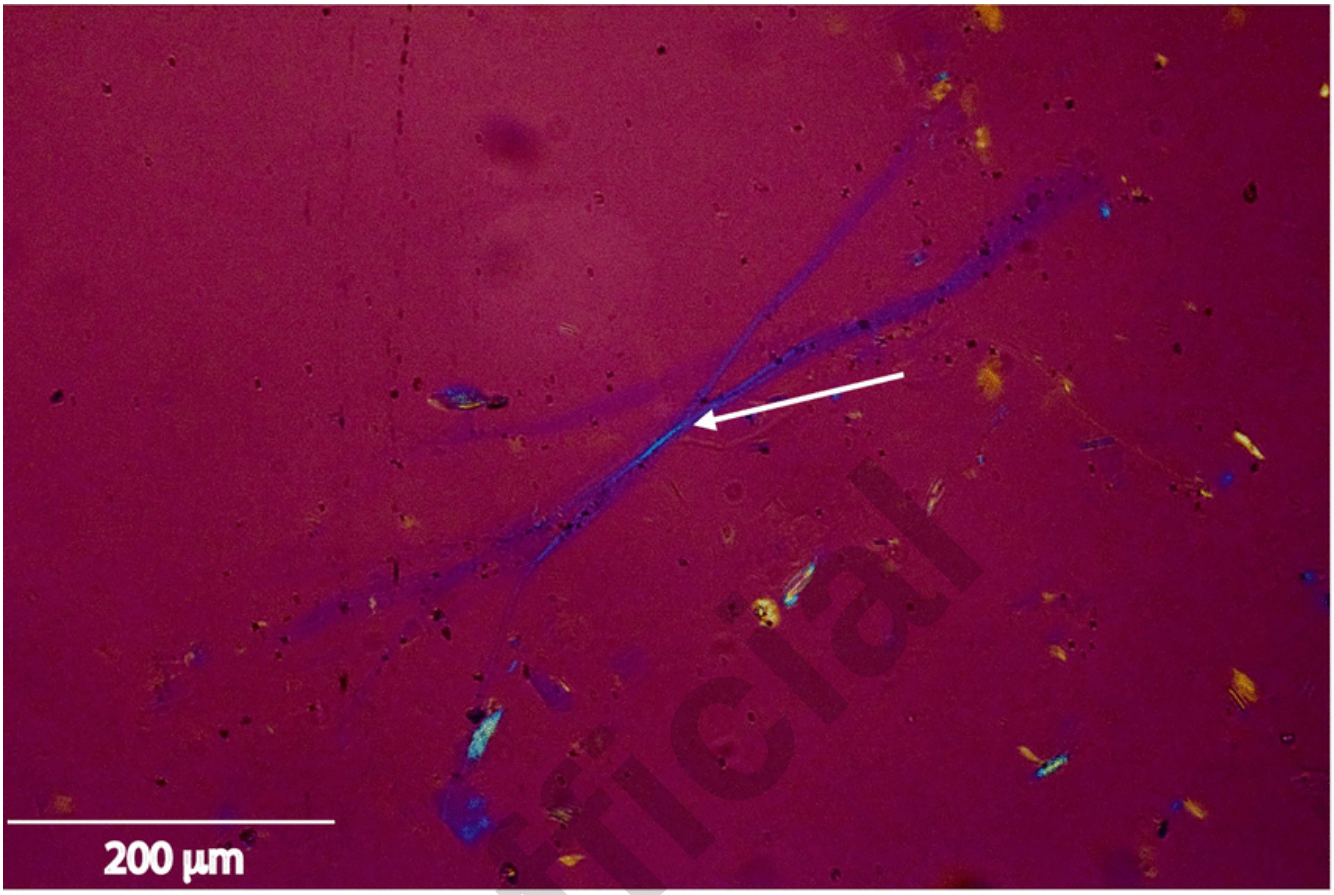


Figure A6. Milled chrysotile in talc matrix immersed in Cargille $n_D$ 1.550 HD liquid at 25° oriented so that $\gamma'$ (and fiber length) is 45° from the direction of the lower polarizer (E–W) and parallel with N of the inserted full wave accessory plate in cross-polarized light.

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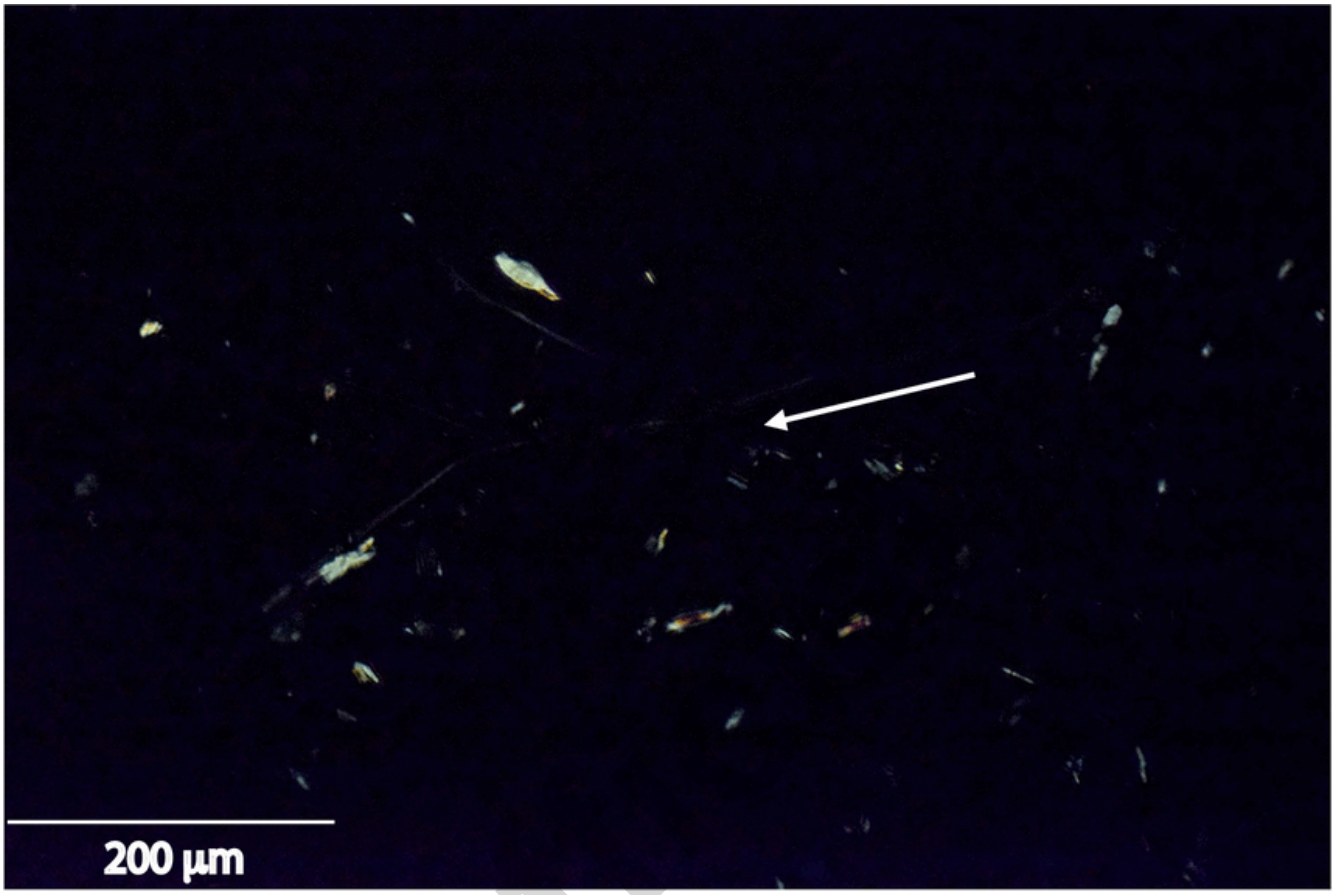


Figure A7. Milled chrysotile in talc matrix immersed in Cargille $n_D$ 1.550 HD liquid at 25° oriented so that $\gamma'$ (and fiber length) is parallel with the direction of the lower polarizer (E–W) with the full wave accessory plate inserted in cross-polarized light. The chrysotile is at or near extinction.

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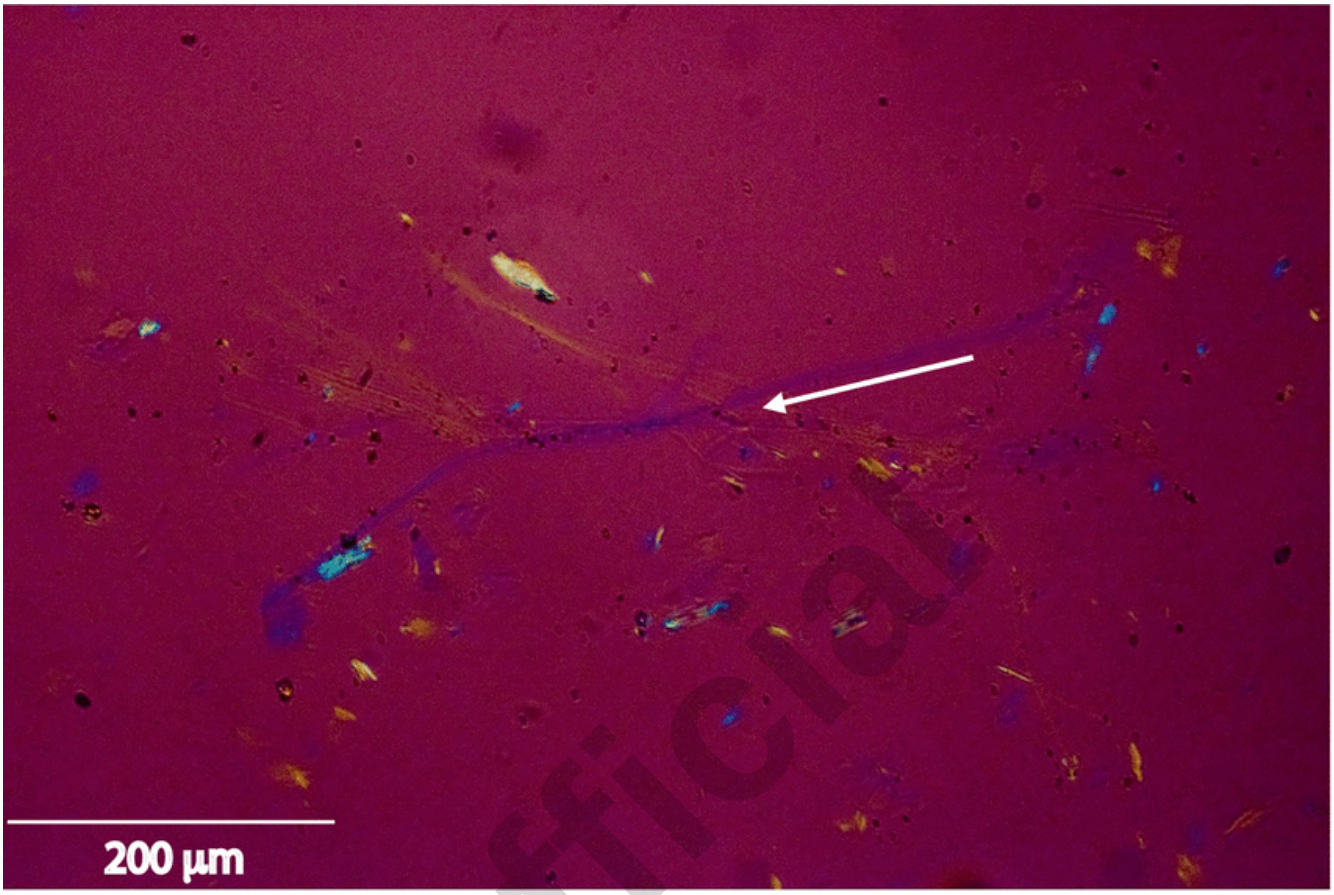


Figure A8. Milled chrysotile in talc matrix immersed in Cargille $n_D$ 1.550 HD liquid at 25° oriented so that $\gamma'$ (and fiber length) is parallel with the direction of the lower polarizer (E–W) with the full wave accessory plate inserted in cross-polarized light. The chrysotile is at or near extinction.

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**Milled chrysotile in talc matrix (0.1%)—1.550 RI liquid (material retained on 400-mesh sieve)**

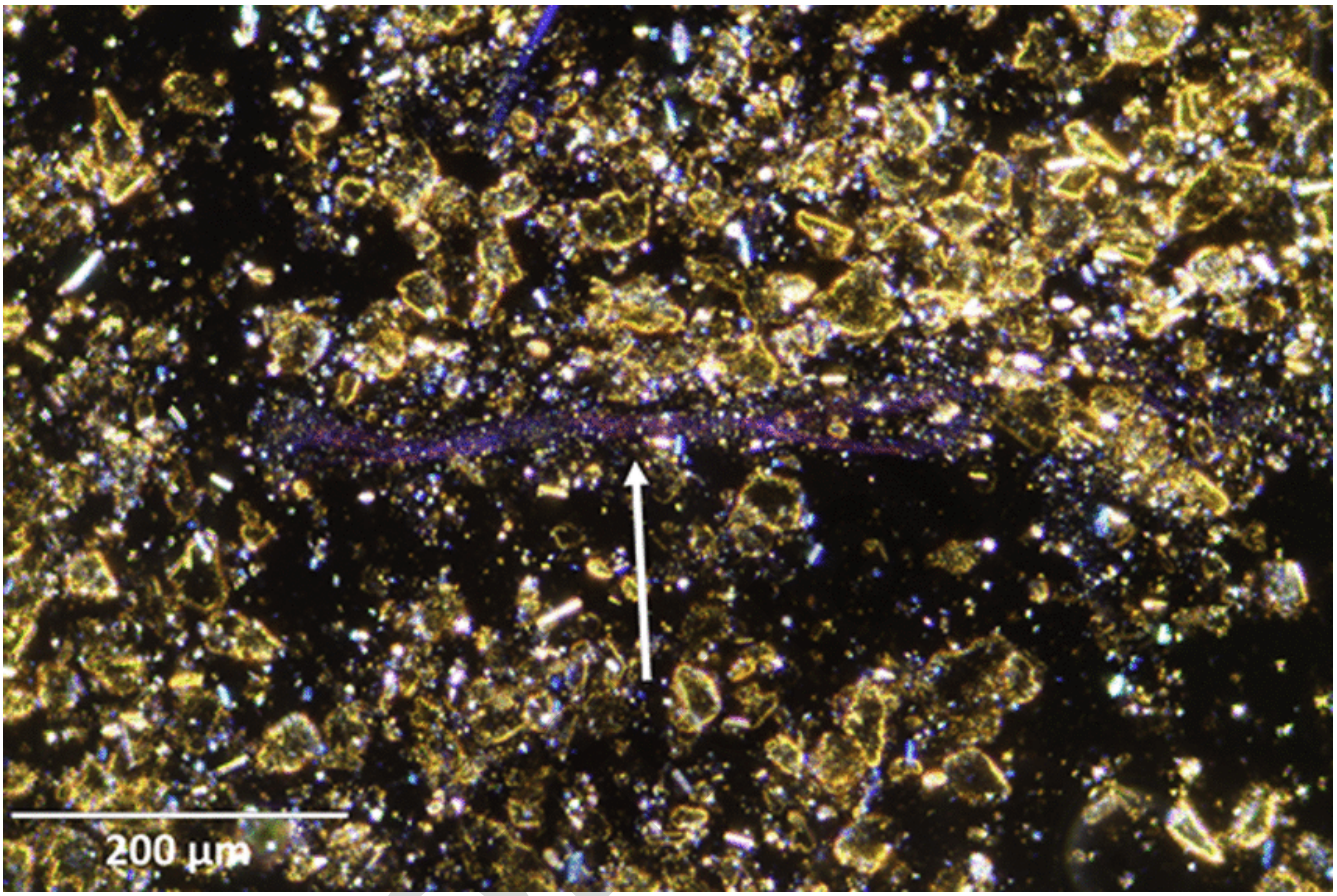


Figure B1. Milled chrysotile in talc matrix (0.1%). Material retained on a 400-mesh sieve was immersed in Cargille $n_D$ 1.550 HD liquid at 25° and oriented so that $\gamma'$ (and fiber length) is parallel with the direction of the lower polarizer (E–W) in central stop dispersion staining.

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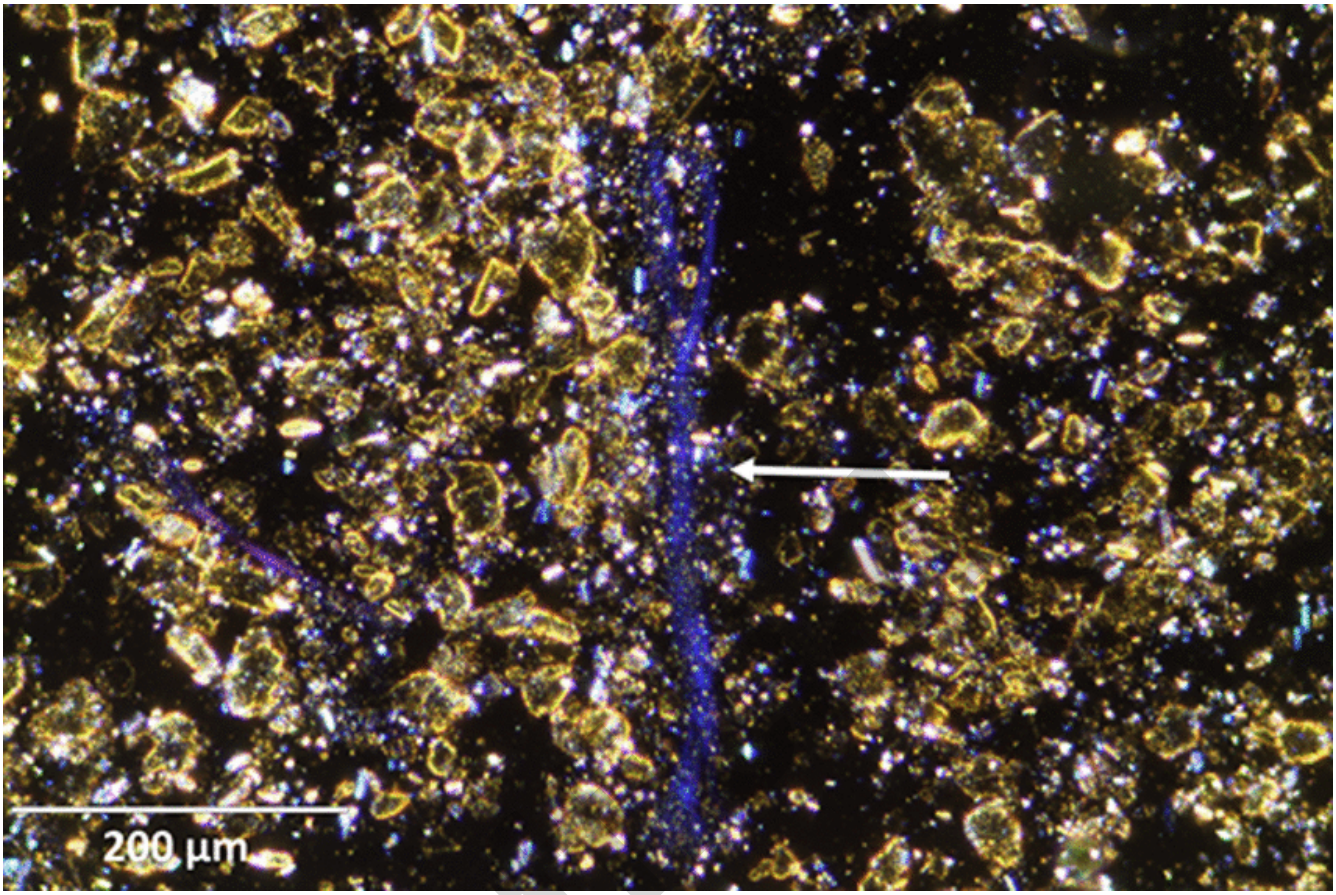


Figure B2. Milled chrysotile in talc matrix (0.1%). Material retained on a 400-mesh sieve was immersed in Cargille $n_D$ 1.550 HD liquid at 25° and oriented so that α′ is parallel with the direction of the lower polarizer (E–W) in central stop dispersion staining (fiber length is perpendicular to the lower polarizer).

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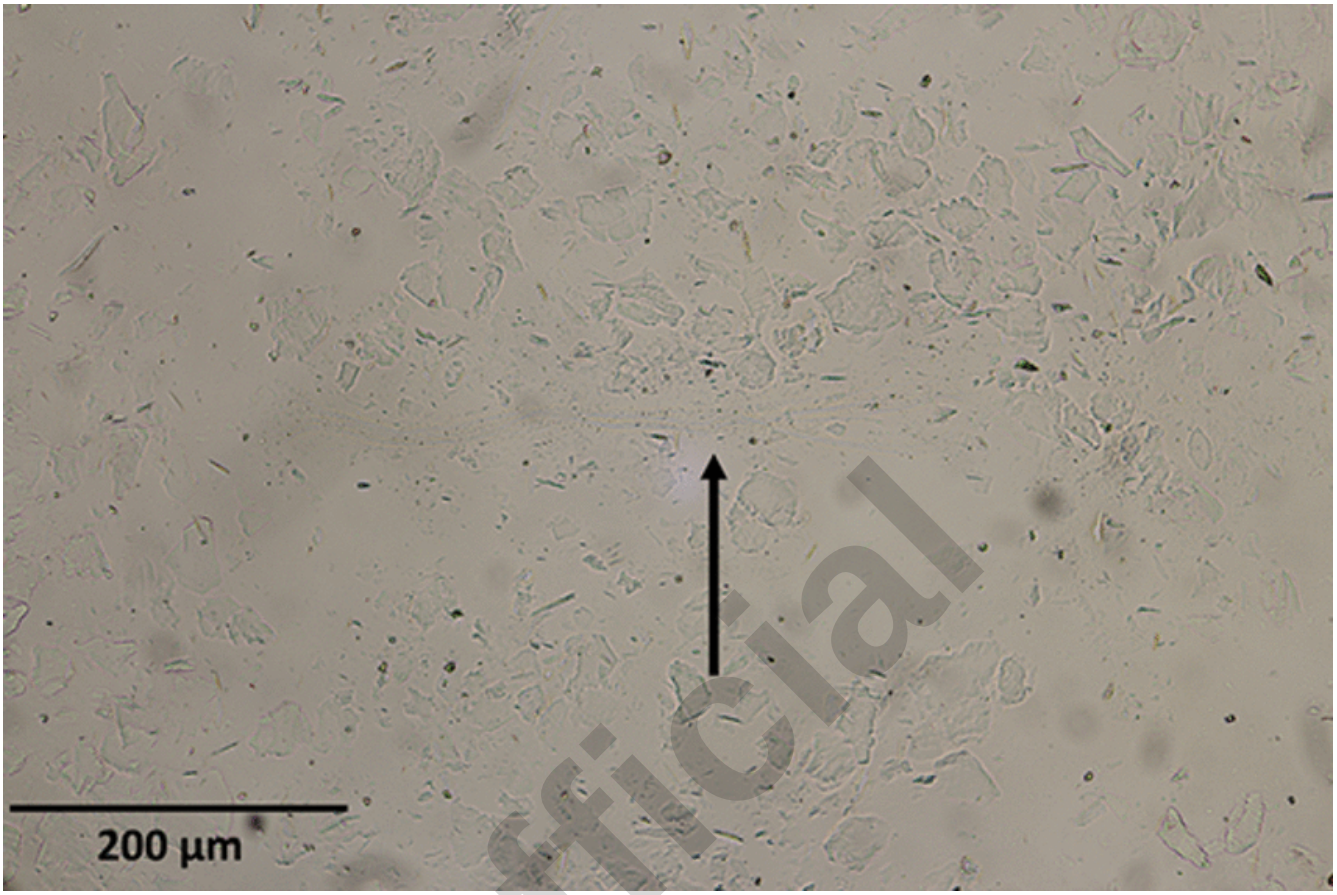


Figure B3. Milled chrysotile in talc matrix (0.1%). Material retained on a 400-mesh sieve was immersed in Cargille $n_D$ 1.550 HD liquid at 25° and oriented so that γ′ (and fiber length) is parallel with the direction of the lower polarizer (E–W) in plane-polarized light.

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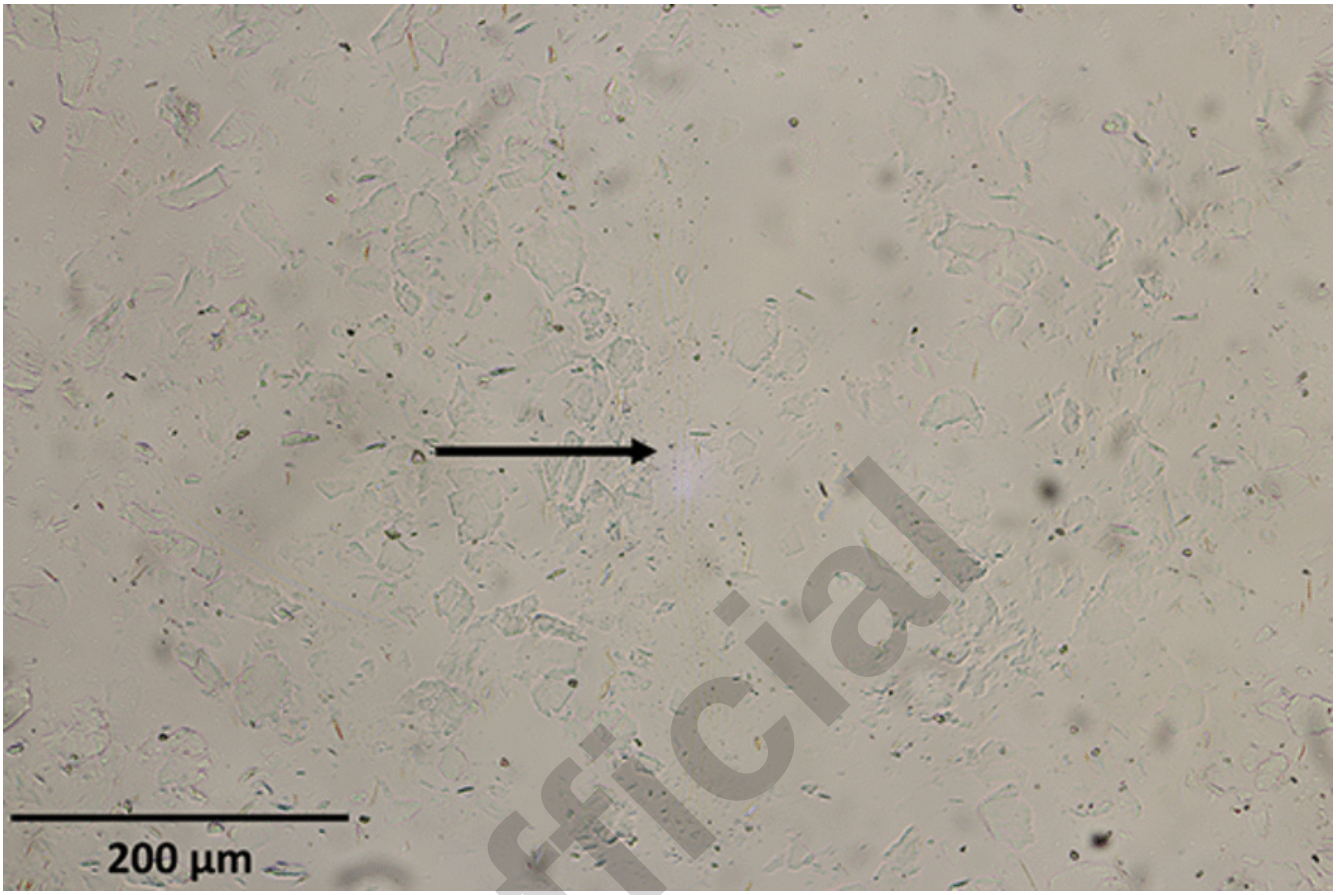


Figure B4. Milled chrysotile in talc matrix (0.1%). Material retained on a 400-mesh sieve was immersed in Cargille $n_D$ 1.550 HD liquid at 25° and oriented so that α′ is parallel with the direction of the lower polarizer (E–W) in plane-polarized light (fiber length is perpendicular to the lower polarizer).

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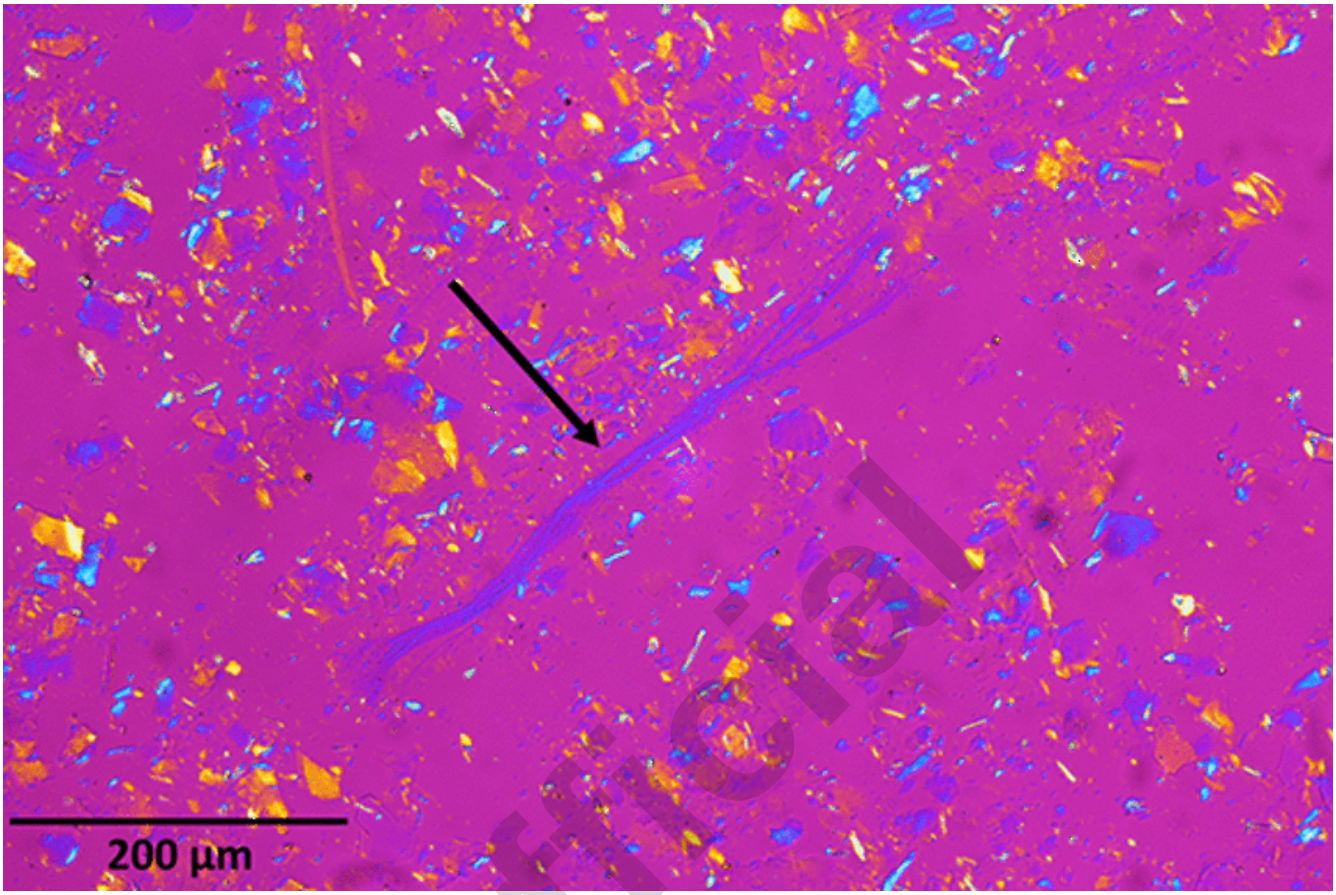


Figure B5. Milled chrysotile in talc matrix (0.1%). Material retained on a 400-mesh sieve was immersed in Cargille $n_D$ 1.550 HD liquid at 25° and oriented so that $\gamma'$ (and fiber length) is 45° counterclockwise from the direction of the lower polarizer (E–W) and parallel with N of the inserted full wave accessory plate in cross-polarized light.

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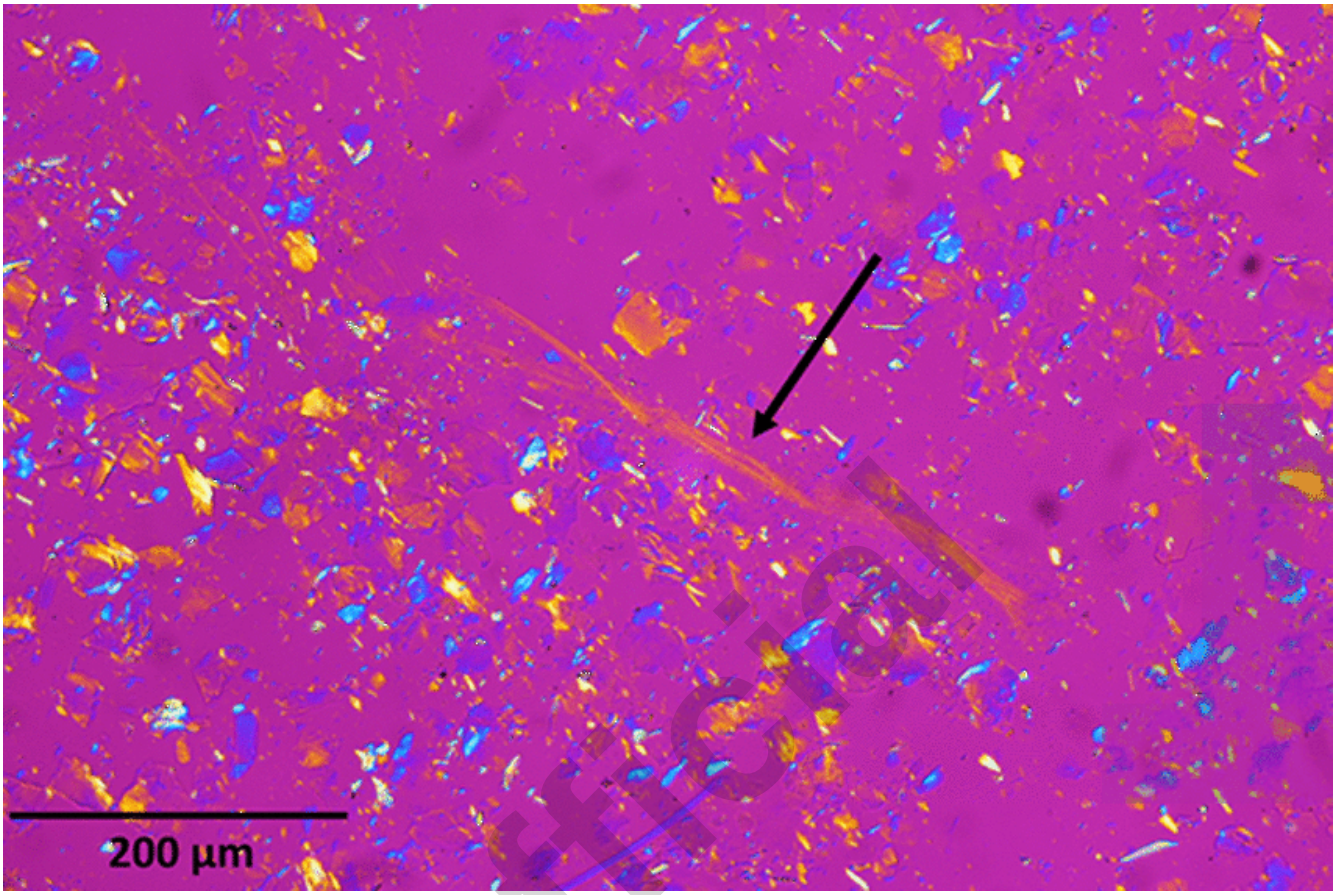


Figure B6. Milled chrysotile in talc matrix (0.1%). Material retained on a 400-mesh sieve was immersed in Cargille $n_D$ 1.550 HD liquid at 25° and oriented so that $\gamma'$ (and fiber length) is 45° clockwise from the direction of the lower polarizer (E–W) and perpendicular with N of the inserted full wave accessory plate in cross-polarized light.

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**Milled chrysotile in talc matrix (0.01%)—1.550 RI liquid (material retained on 400-mesh sieve)**

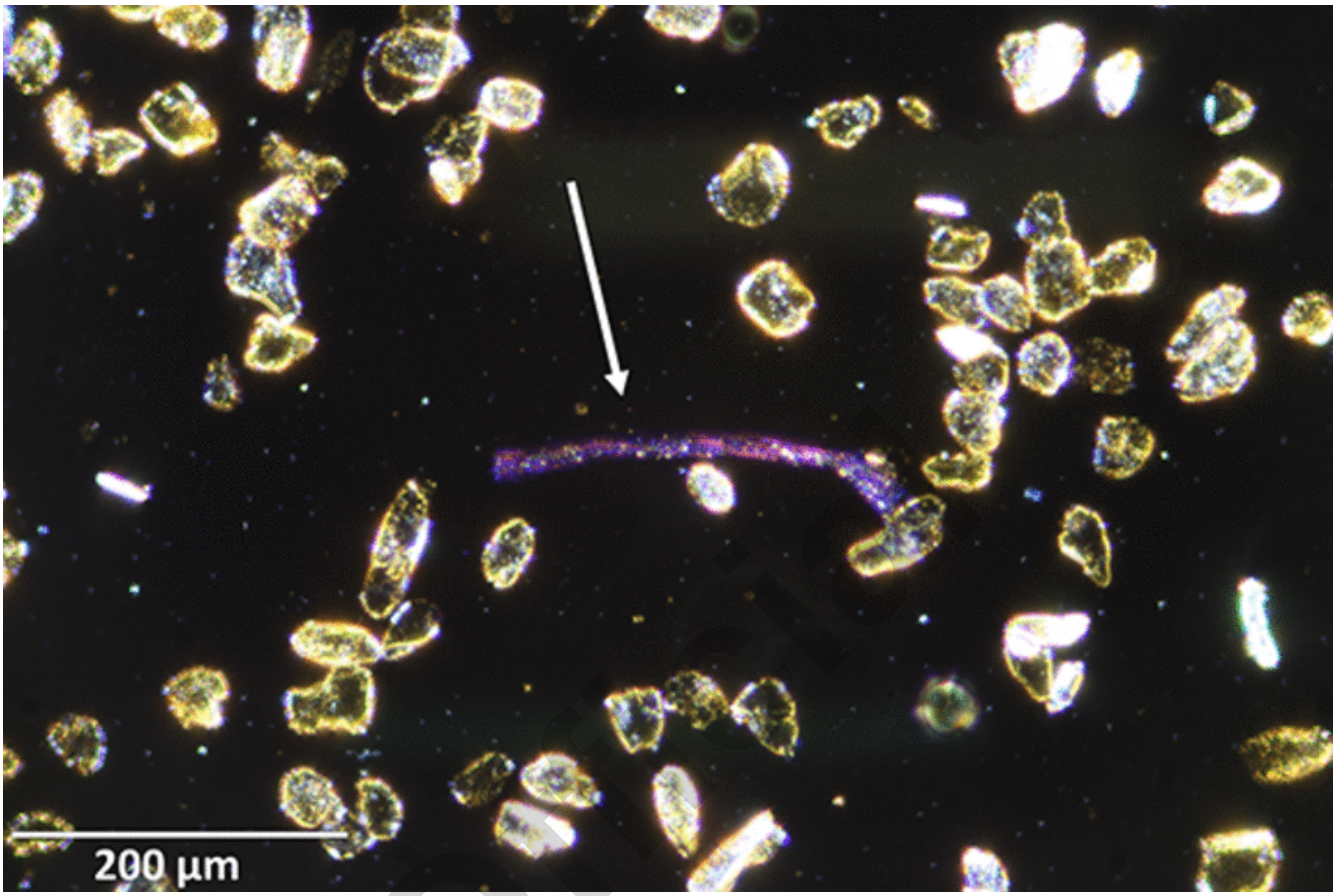


Figure C1. Milled chrysotile in talc matrix (0.01%). Material retained on a 400-mesh sieve was immersed in Cargille $n_D$ 1.550 HD liquid at 25° and oriented so that γ′ (and fiber length) is parallel with the direction of the lower polarizer (E–W) in central stop dispersion staining.

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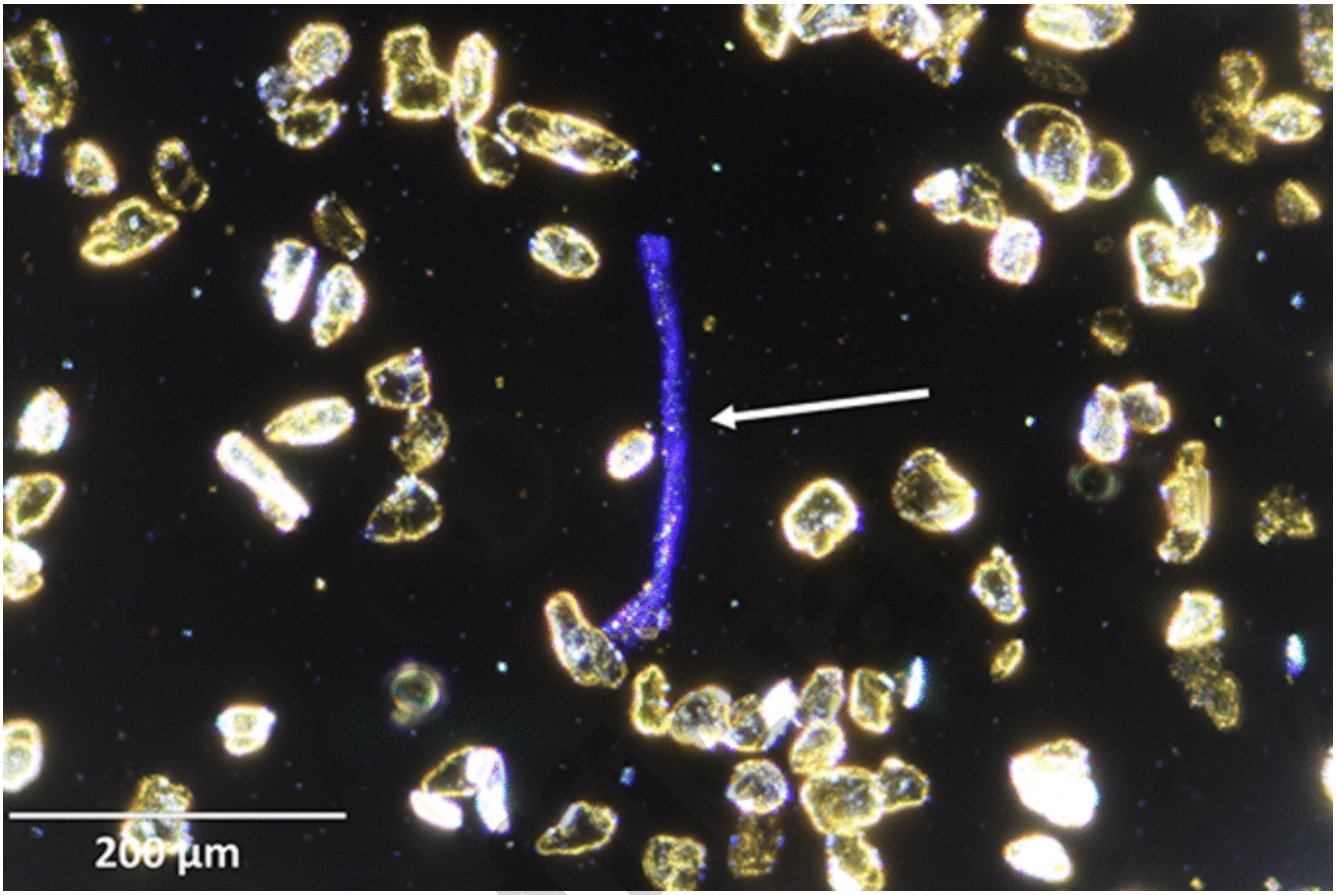


Figure C2. Milled chrysotile in talc matrix (0.01%). Material retained on a 400-mesh sieve was immersed in Cargille $n_D$ 1.550 HD liquid at 25° and oriented so that α′ is parallel with the direction of the lower polarizer (E–W) in central stop dispersion staining (fiber length is perpendicular to the lower polarizer).

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Figure C3. Milled chrysotile in talc matrix (0.01%). Material retained on a 400-mesh sieve was immersed in Cargille $n_D$ 1.550 HD liquid at 25° and oriented so that γ′ (and fiber length) is parallel with the direction of the lower polarizer (E–W) in plane-polarized light.

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Figure C4. Milled chrysotile in talc matrix (0.01%). Material retained on a 400-mesh sieve was immersed in Cargille $n_D$ 1.550 HD liquid at 25° and oriented so that α′ is parallel with the direction of the lower polarizer (E–W) in plane-polarized light (fiber length is perpendicular to the lower polarizer).

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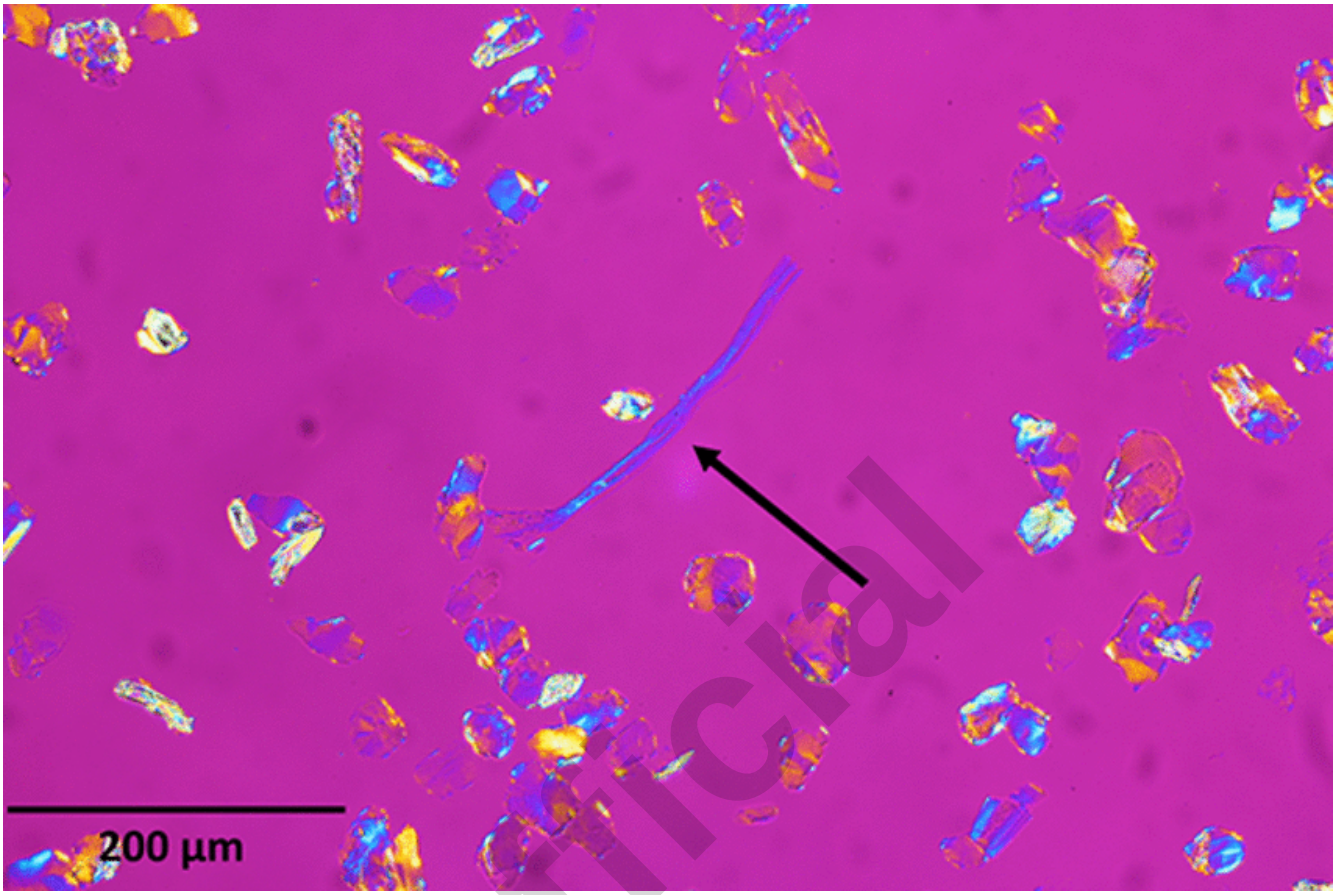


Figure C5. Milled chrysotile in talc matrix (0.01%). Material retained on a 400-mesh sieve was immersed in Cargille $n_D$ 1.550 HD liquid at 25° and oriented so that γ′ (and fiber length) is 45° counterclockwise from the direction of the lower polarizer (E–W) and parallel with N of the inserted full wave accessory plate in cross-polarized light.

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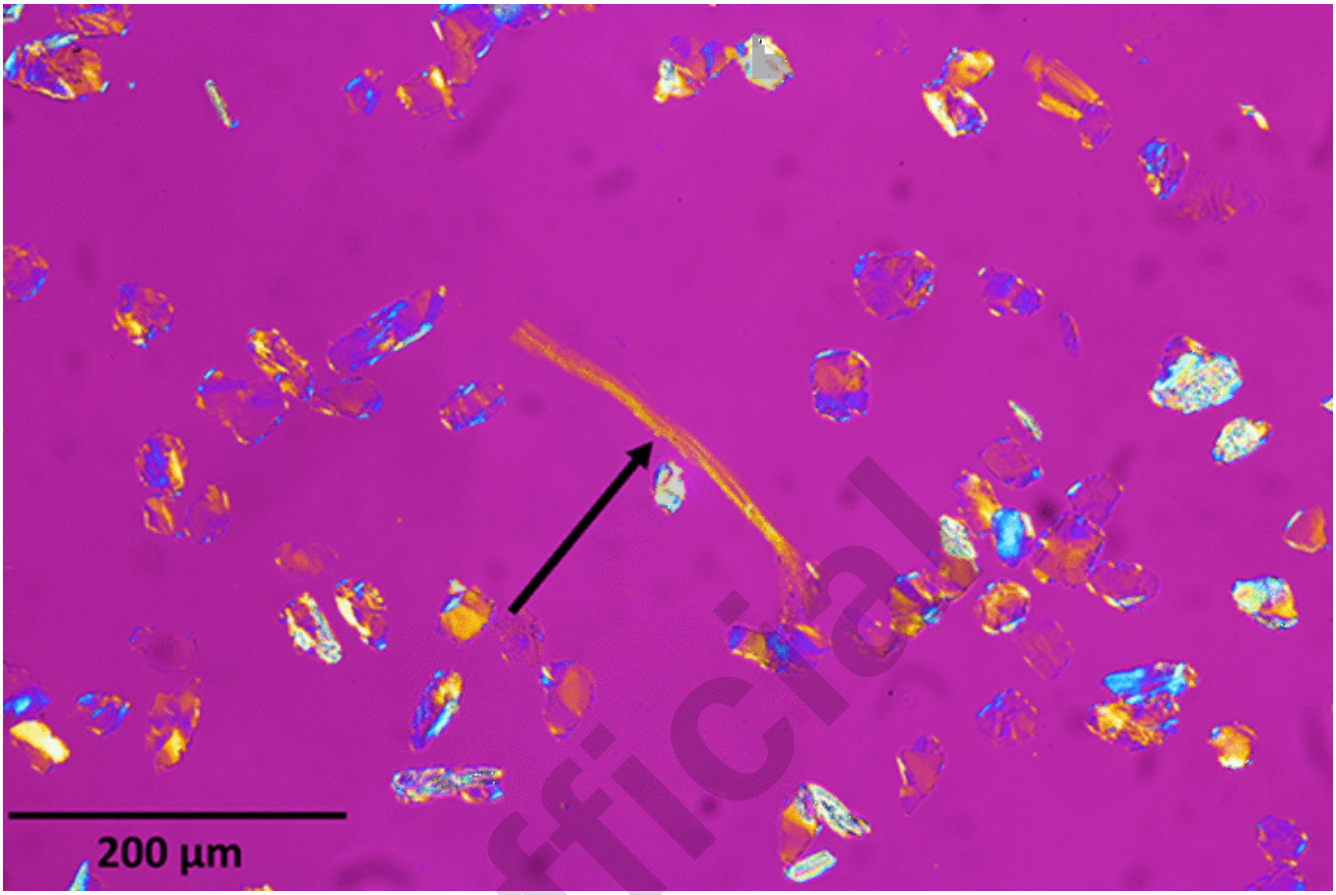


Figure C6. Milled chrysotile in talc matrix (0.01%). Material retained on a 400-mesh sieve was immersed in Cargille $n_D$ 1.550 HD liquid at 25° and oriented so that $\gamma'$ (and fiber length) is 45° clockwise from the direction of the lower polarizer (E–W) and perpendicular with N of the inserted full wave accessory plate in cross-polarized light.

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**Milled chrysotile in talc matrix (0.001%)—1.550 RI liquid (material retained on 400-mesh sieve)**

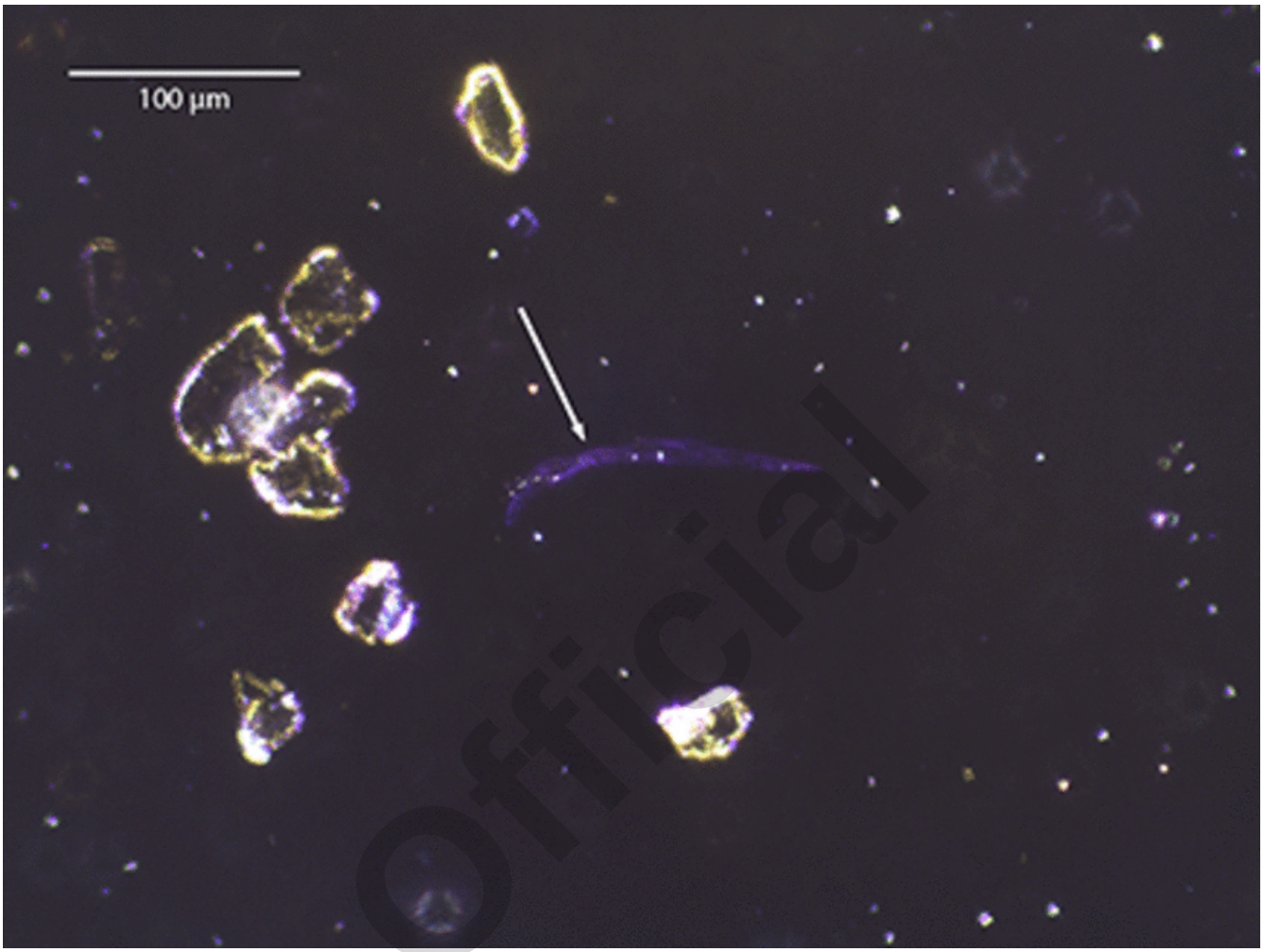


Figure D1. Milled chrysotile in talc matrix (0.001%). Material retained on a 400-mesh sieve was immersed in Cargille $n_D$ 1.550 HD liquid at 25° and oriented so that γ′ (and fiber length) is parallel with the direction of the lower polarizer (E–W) in central stop dispersion staining.

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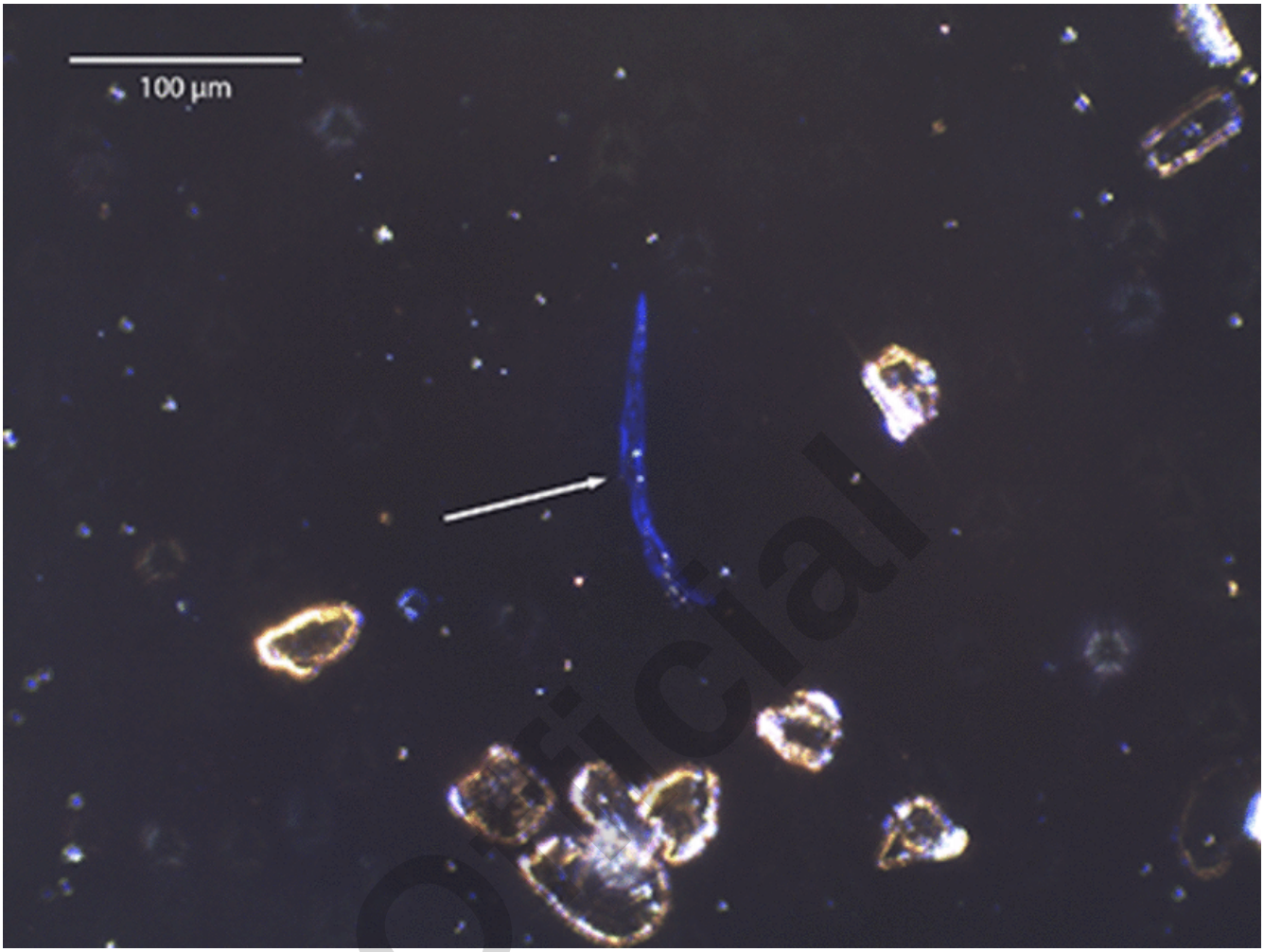


Figure D2. Milled chrysotile in talc matrix (0.001%). Material retained on a 400-mesh sieve was immersed in Cargille $n_D$ 1.550 HD liquid at 25° and oriented so that α′ is parallel with the direction of the lower polarizer (E–W) in central stop dispersion staining (fiber length is perpendicular to the lower polarizer).

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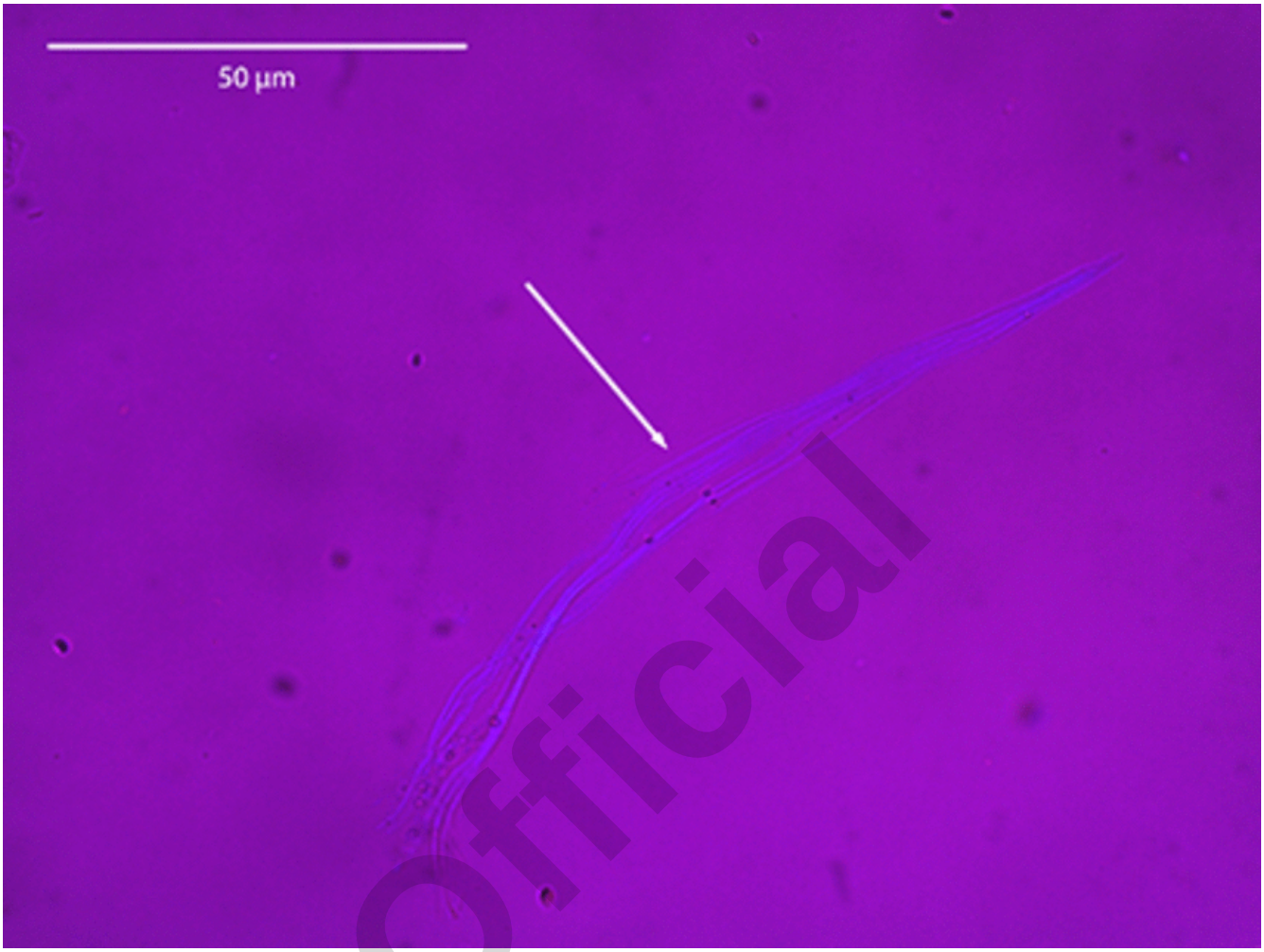


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Figure D3. Milled chrysotile in talc matrix (0.001%). Material retained on a 400-mesh sieve was immersed in Cargille $n_D$ 1.550 HD liquid at 25° and oriented so that γ′ (and fiber length) is 45° counterclockwise from the direction of the lower polarizer (E–W) and parallel with N of the inserted full wave accessory plate in cross-polarized light.

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Figure D4. Milled chrysotile in talc matrix (0.001%). Material retained on a 400-mesh sieve was immersed in Cargille $n_D$ 1.550 HD liquid at 25° and oriented so that γ′ (and fiber length) is 45° clockwise from the direction of the lower polarizer (E–W) and perpendicular with N of the inserted full wave accessory plate in cross-polarized light.

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**Milled tremolite asbestos in talc matrix—1.605 RI liquid (not sieved)**

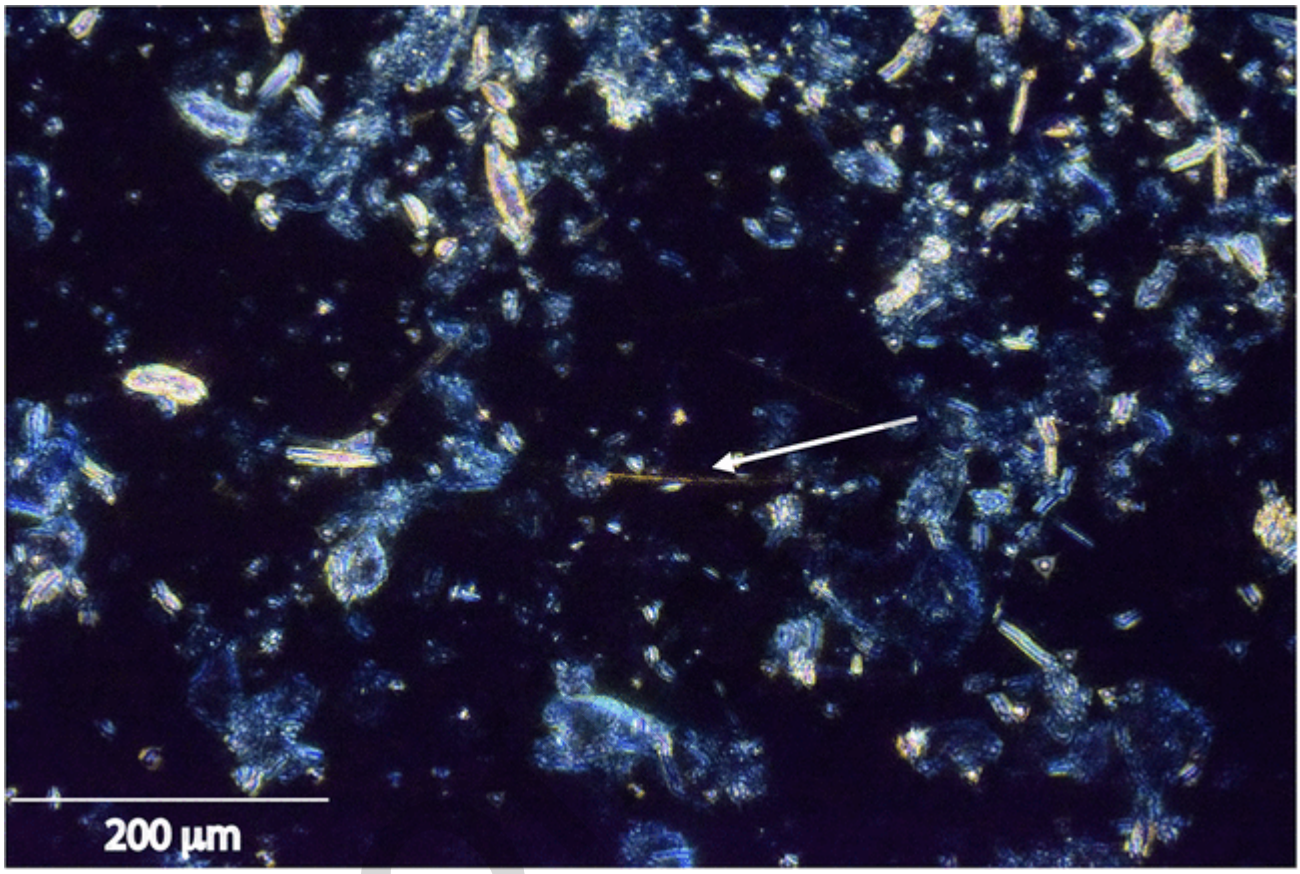


Figure E1. Milled tremolite asbestos in talc matrix immersed in Cargille $n_D$ 1.605 HD liquid at 25° oriented so that $\gamma'$ (and fiber length) is parallel with the direction of the lower polarizer (E–W) in central stop dispersion staining.

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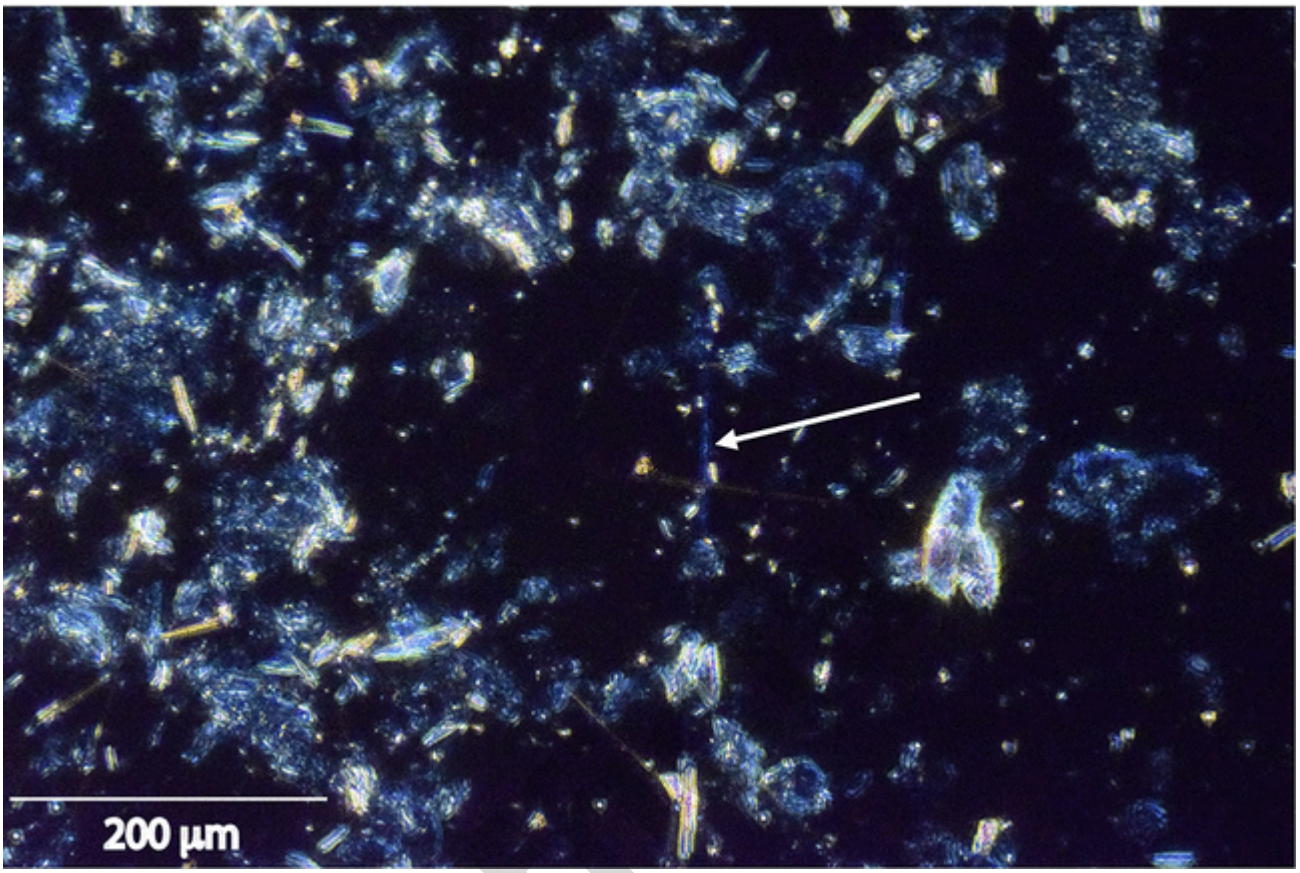


Figure E2. Milled tremolite asbestos in talc matrix immersed in Cargille $n_D$ 1.605 HD liquid at 25° oriented so that α′ is parallel with the direction of the lower polarizer (E–W) in central stop dispersion staining (fiber length is perpendicular to the lower polarizer).

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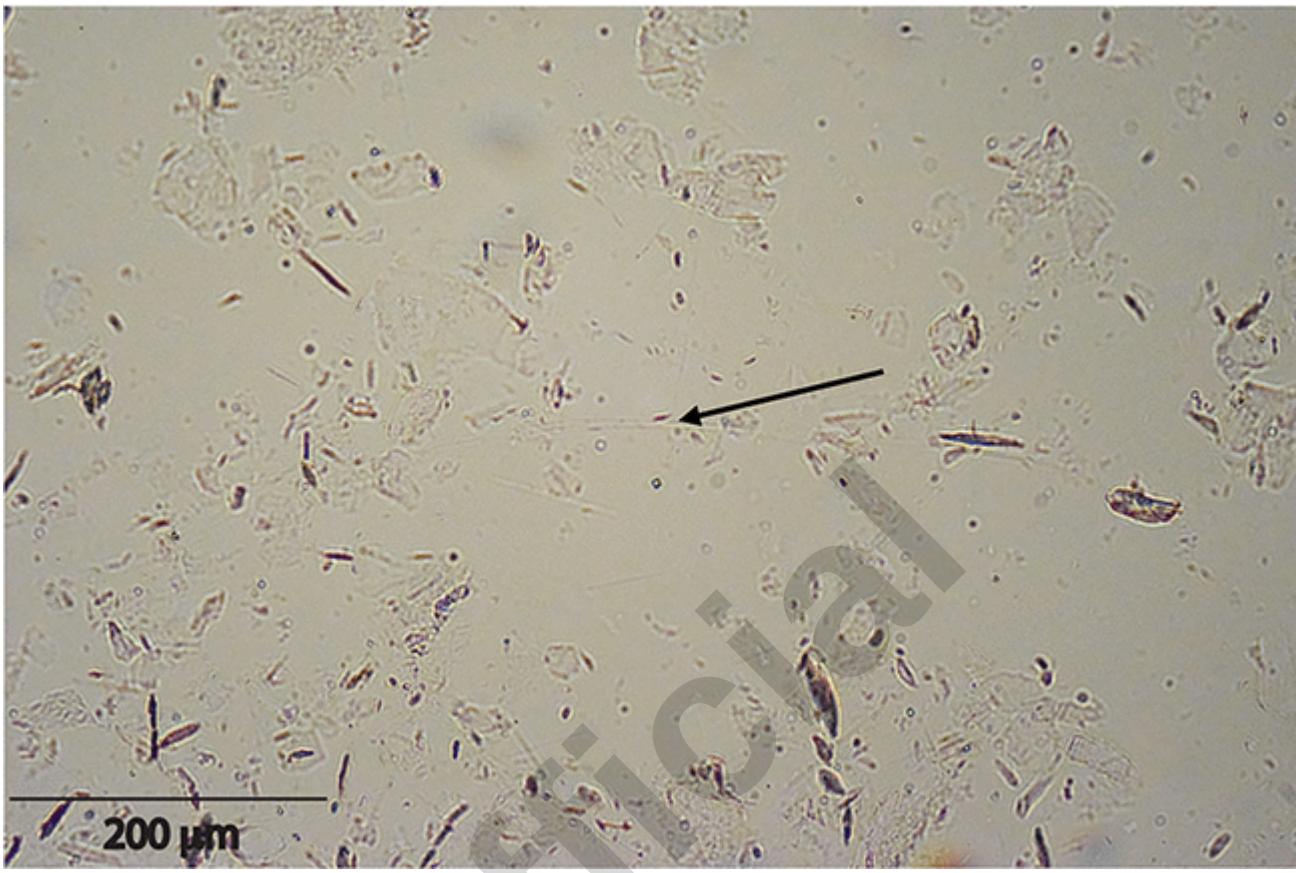


Figure E3. Milled tremolite asbestos in talc matrix immersed in Cargille $n_D$ 1.605 HD liquid at 25° oriented so that γ′ (and fiber length) is parallel with the direction of the lower polarizer (E–W) in plane-polarized light slightly defocused to observe the Becke line.

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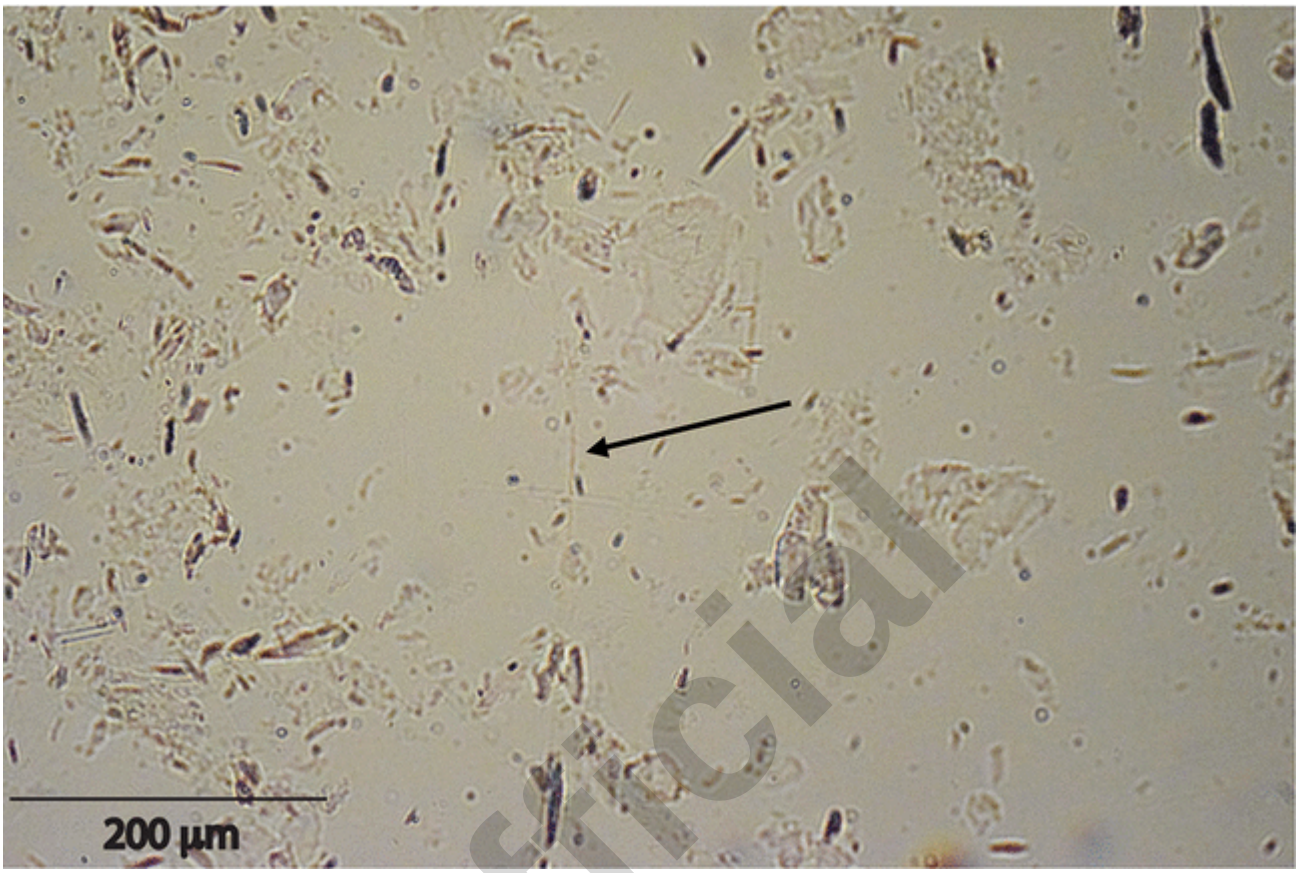


Figure E4. Milled tremolite asbestos in talc matrix immersed in Cargille $n_D$ 1.605 HD liquid at 25° oriented so that α′ is parallel with the direction of the lower polarizer (E–W) in plane-polarized light slightly defocused to observe the Becke line (fiber length is perpendicular to the lower polarizer).

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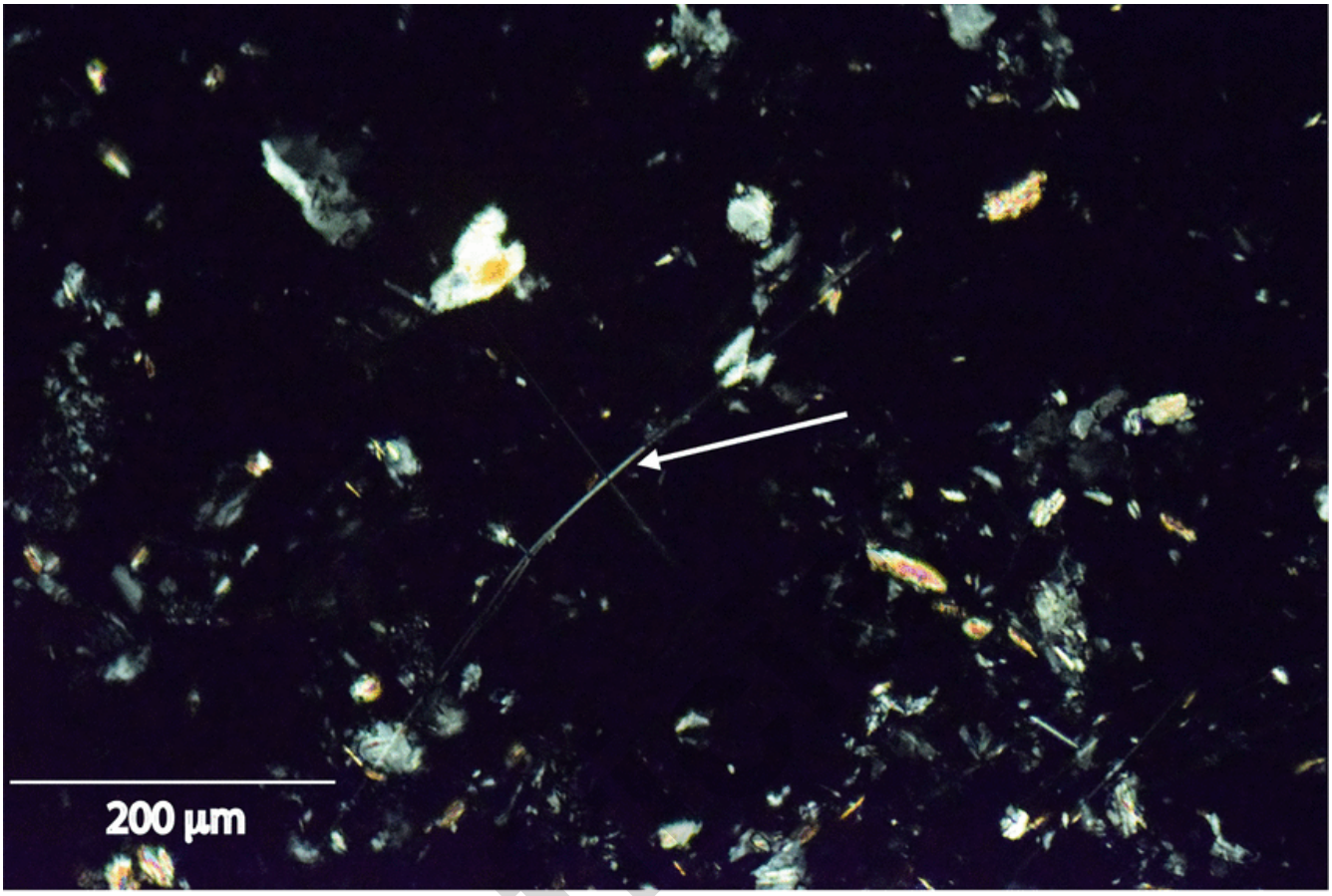


Figure E5. Milled tremolite asbestos in talc matrix immersed in Cargille $n_D$ 1.605 HD liquid at 25° oriented so that $\gamma'$ (and fiber length) is 45° counterclockwise from the lower polarizer (E–W) in cross-polarized light.

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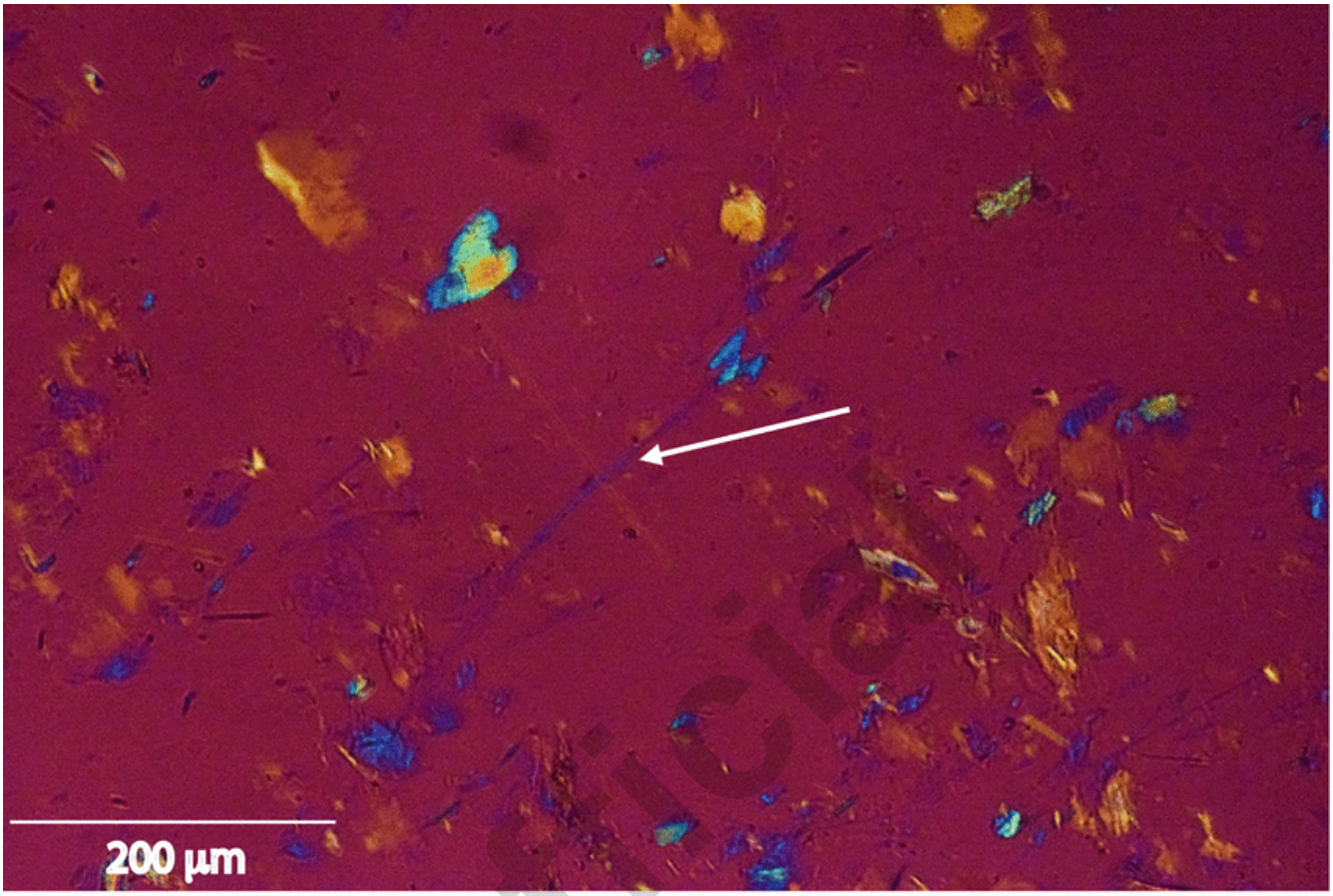


Figure E6. Milled tremolite asbestos in talc matrix immersed in Cargille $n_D$ 1.605 HD liquid at 25° oriented so that $\gamma'$ (and fiber length) is 45° from the direction of the lower polarizer (E–W) and parallel with N of the inserted full wave accessory plate in cross-polarized light.

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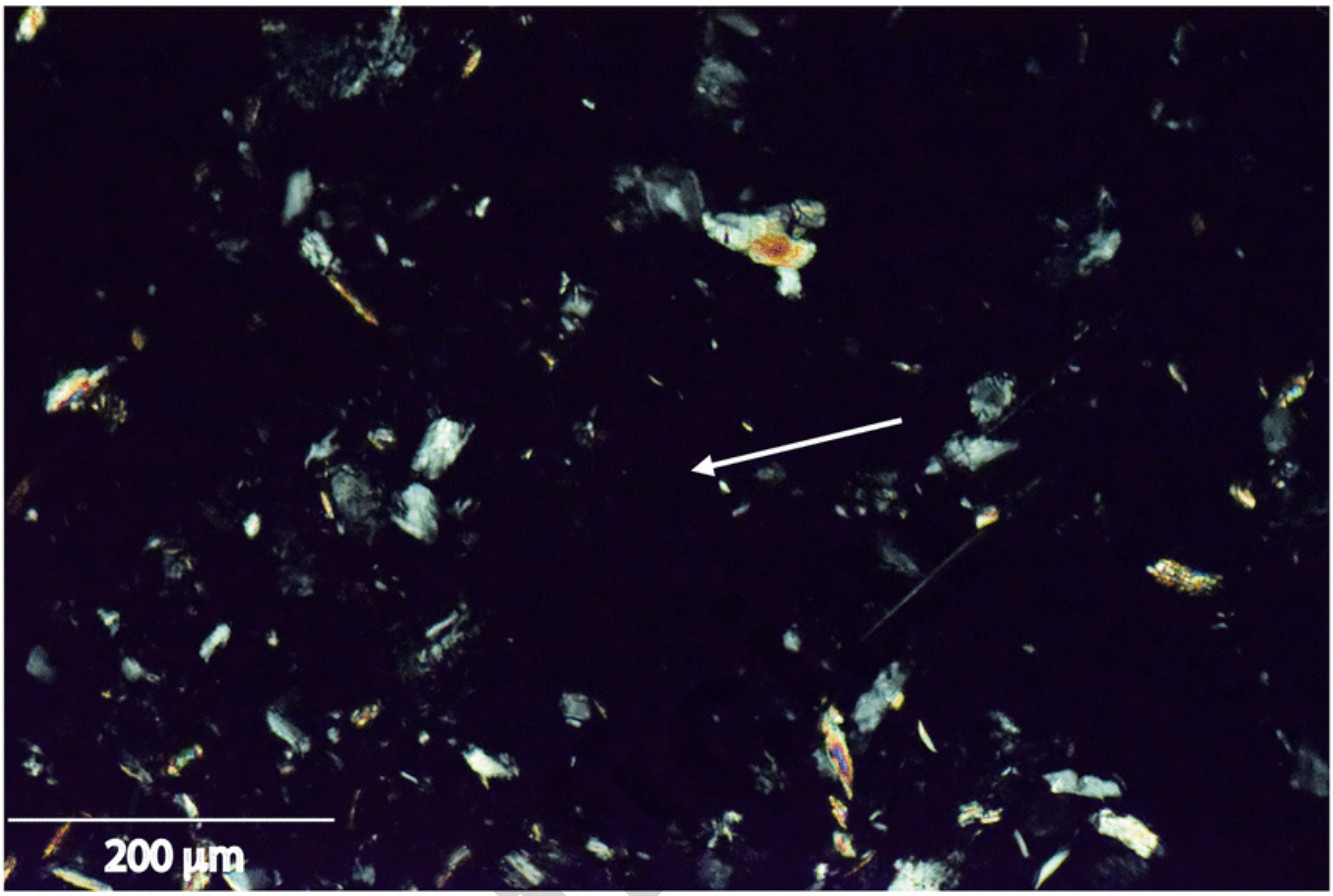


Figure E7. Milled tremolite asbestos in talc matrix immersed in Cargille $n_D$ 1.605 HD liquid at 25° oriented so that γ′ (and fiber length) is parallel with the direction of the lower polarizer (E–W) in cross-polarized light. The tremolite asbestos is at or near extinction.

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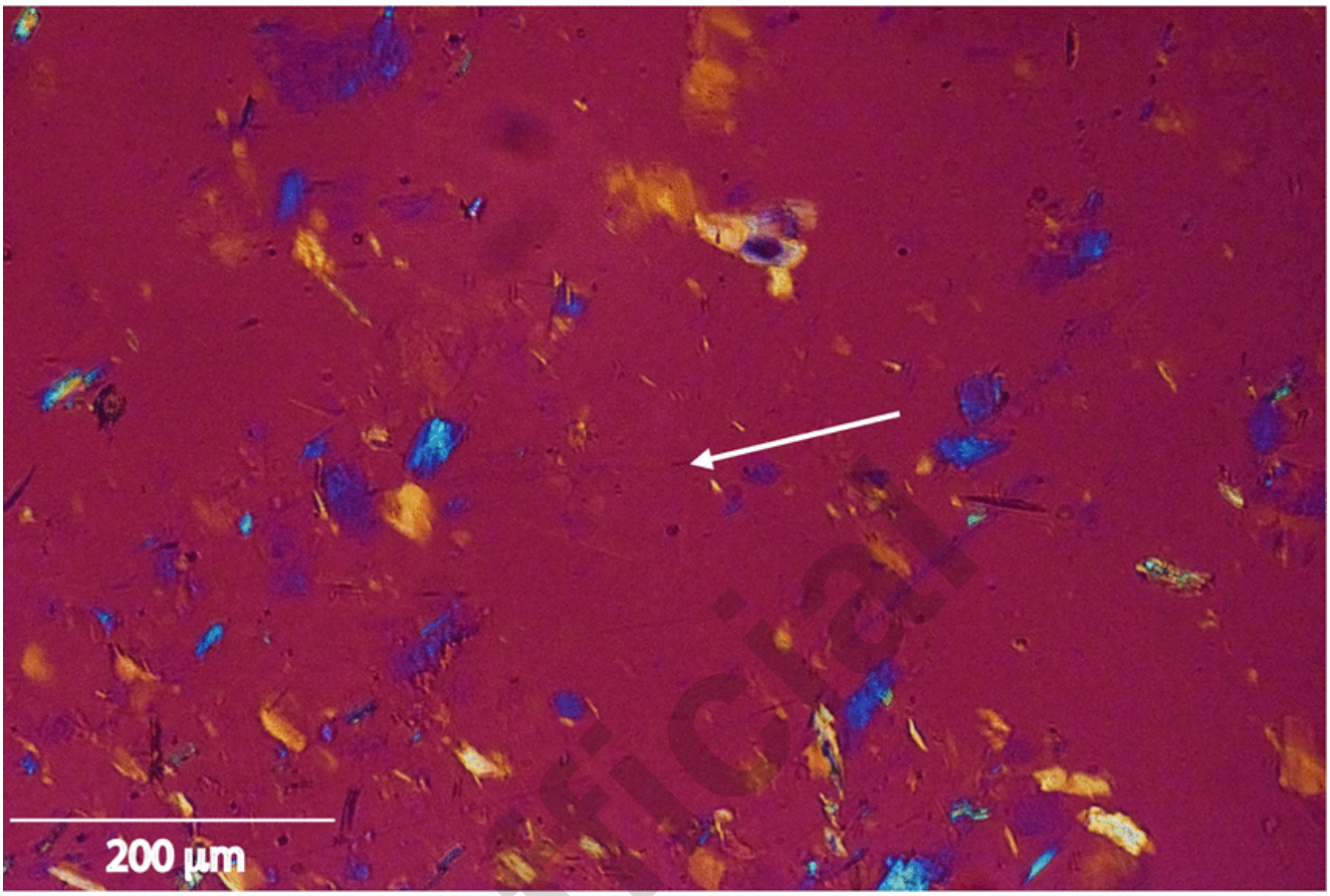


Figure E8. Milled tremolite asbestos in talc matrix immersed in Cargille $n_D$ 1.605 HD liquid at 25° oriented so that $\gamma'$ (and fiber length) is parallel with the direction of the lower polarizer (E–W) with the full wave accessory plate inserted in cross-polarized light. The tremolite asbestos is at or near extinction.

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**Milled tremolite asbestos in talc matrix—1.615 RI liquid (not sieved)**

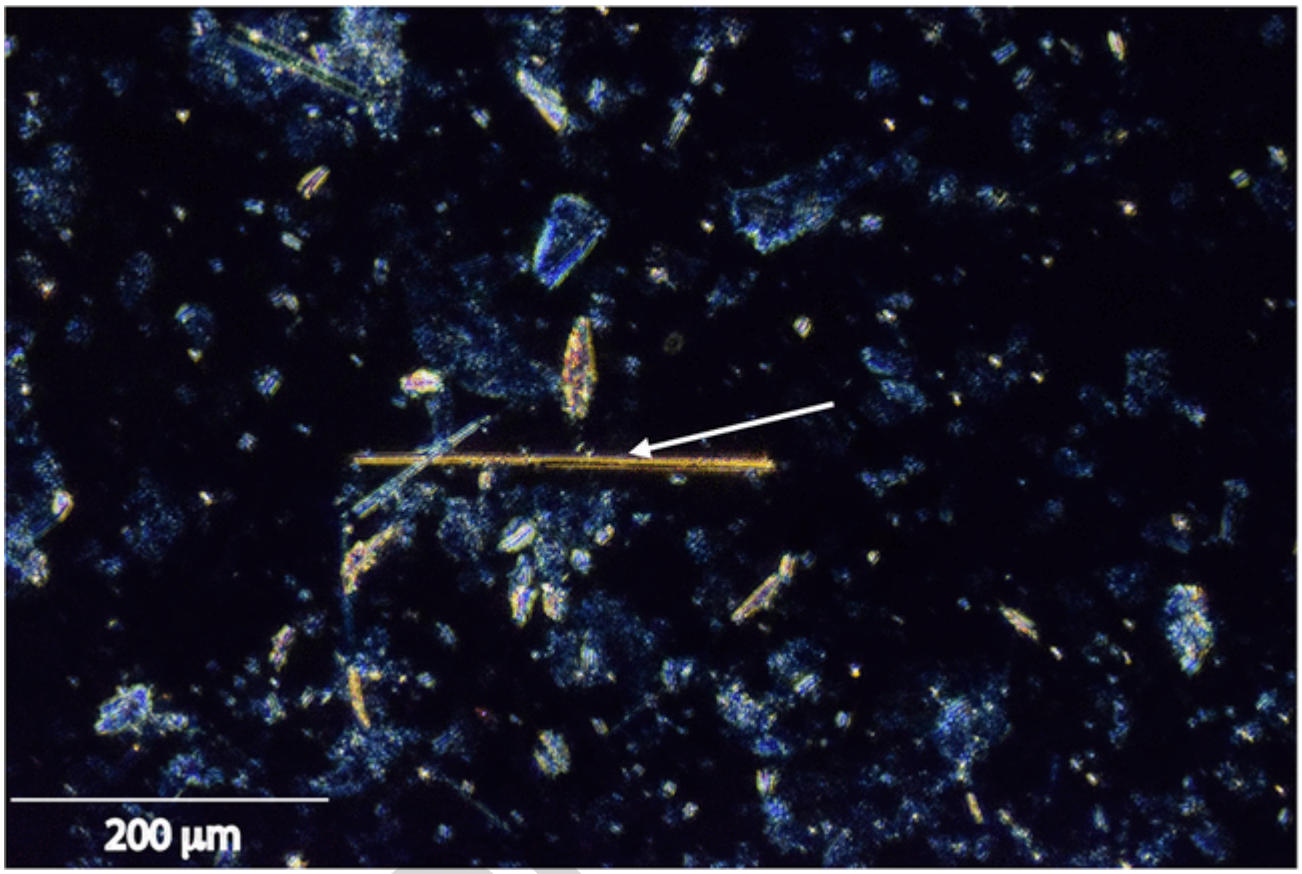


Figure F1. Milled tremolite asbestos in talc matrix immersed in Cargille $n_D$ 1.615 HD liquid at 25° oriented so that $\gamma'$ (and fiber length) is parallel with the direction of the lower polarizer (E–W) in central stop dispersion staining.

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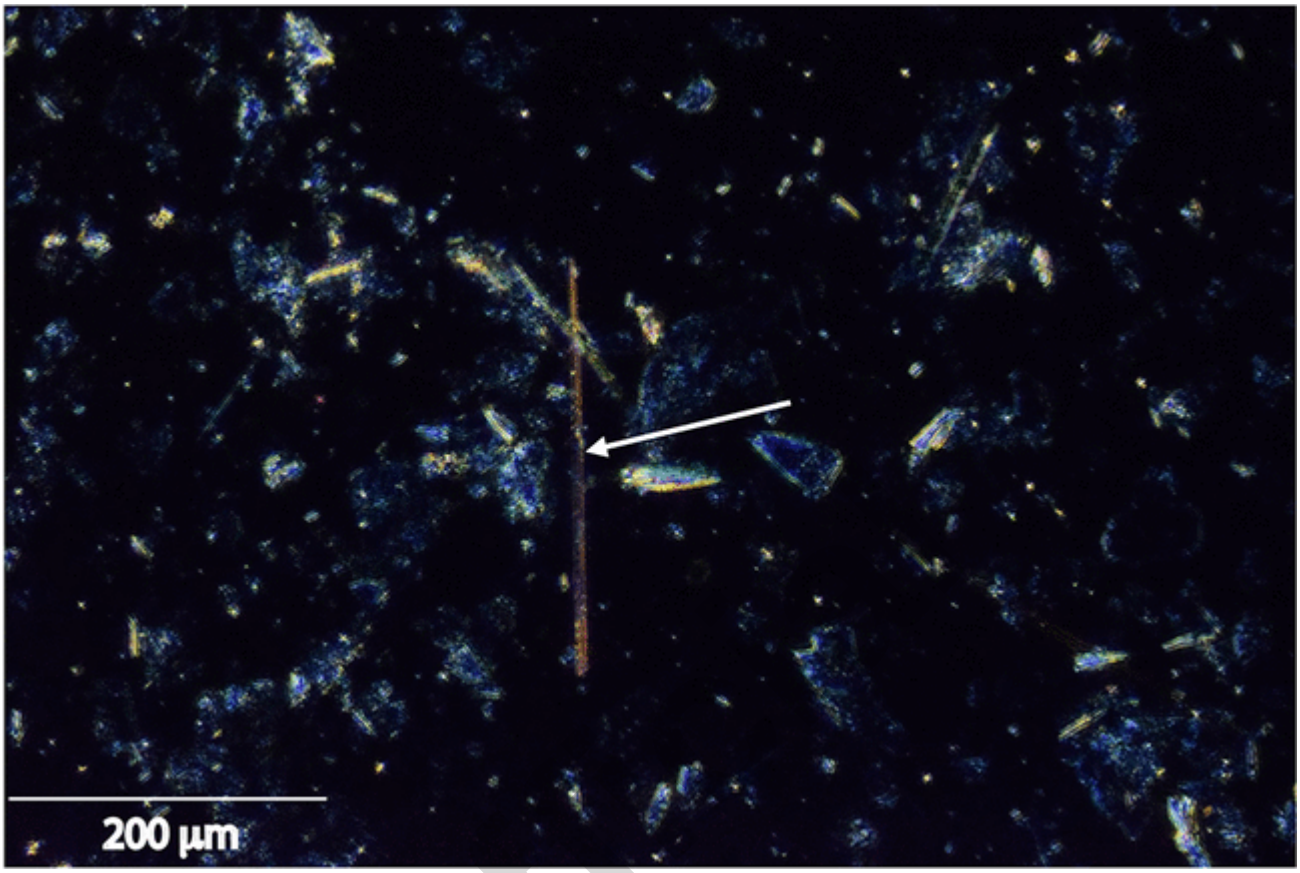


Figure F2. Milled tremolite asbestos in talc matrix immersed in Cargille $n_D$ 1.615 HD liquid at 25° oriented so that α′ is parallel with the direction of the lower polarizer (E–W) in central stop dispersion staining (fiber length is perpendicular to the lower polarizer)

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Figure F3. Milled tremolite asbestos in talc matrix immersed in Cargille $n_D$ 1.615 HD liquid at 25° oriented so that γ′ (and fiber length) is parallel with the direction of the lower polarizer (E–W) in plane-polarized light slightly defocused to observe the Becke line.

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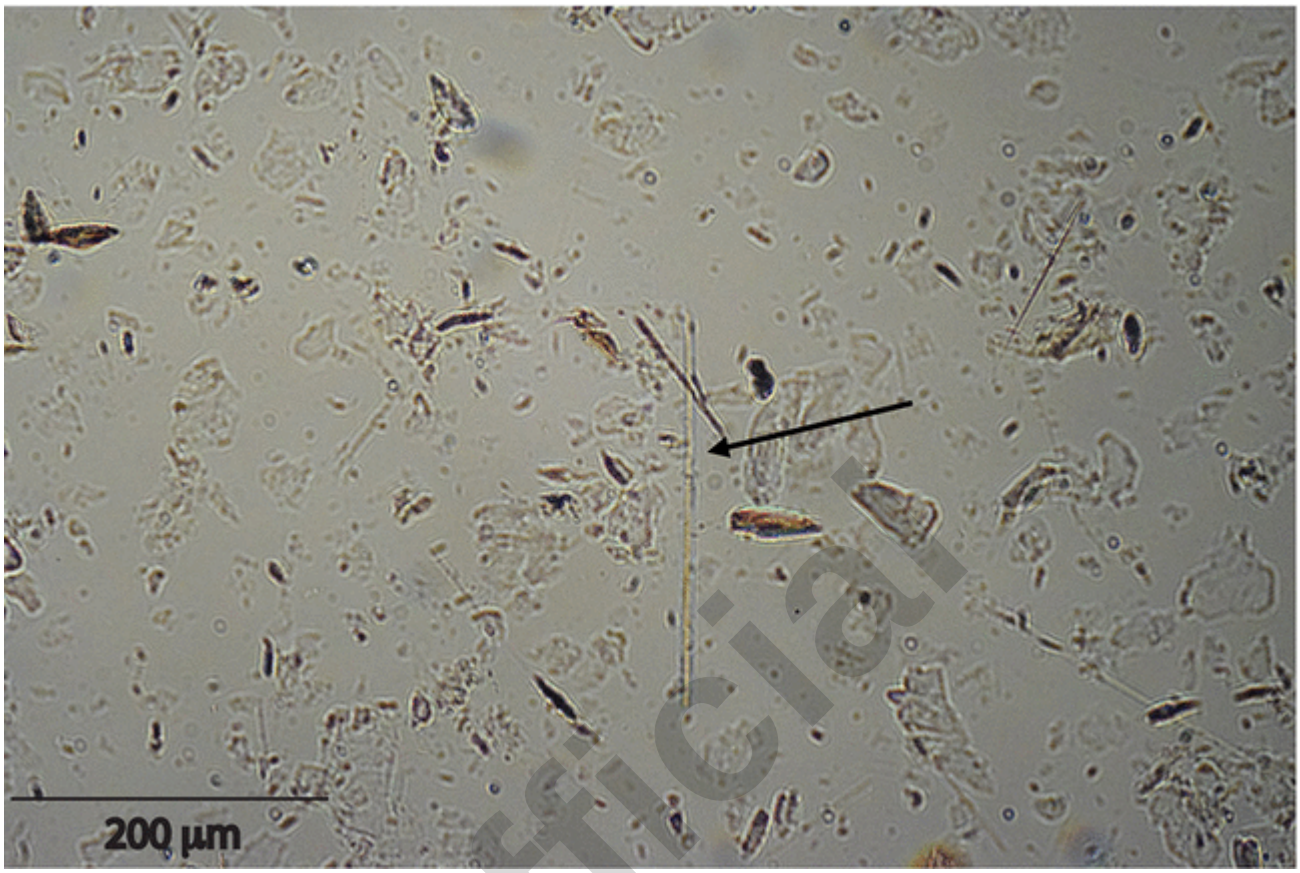


Figure F4. Milled tremolite asbestos in talc matrix immersed in Cargille $n_D$ 1.615 HD liquid at 25° oriented so that α′ is parallel with the direction of the lower polarizer (E–W) in plane-polarized light slightly defocused to observe the Becke line (fiber length is perpendicular to the lower polarizer).

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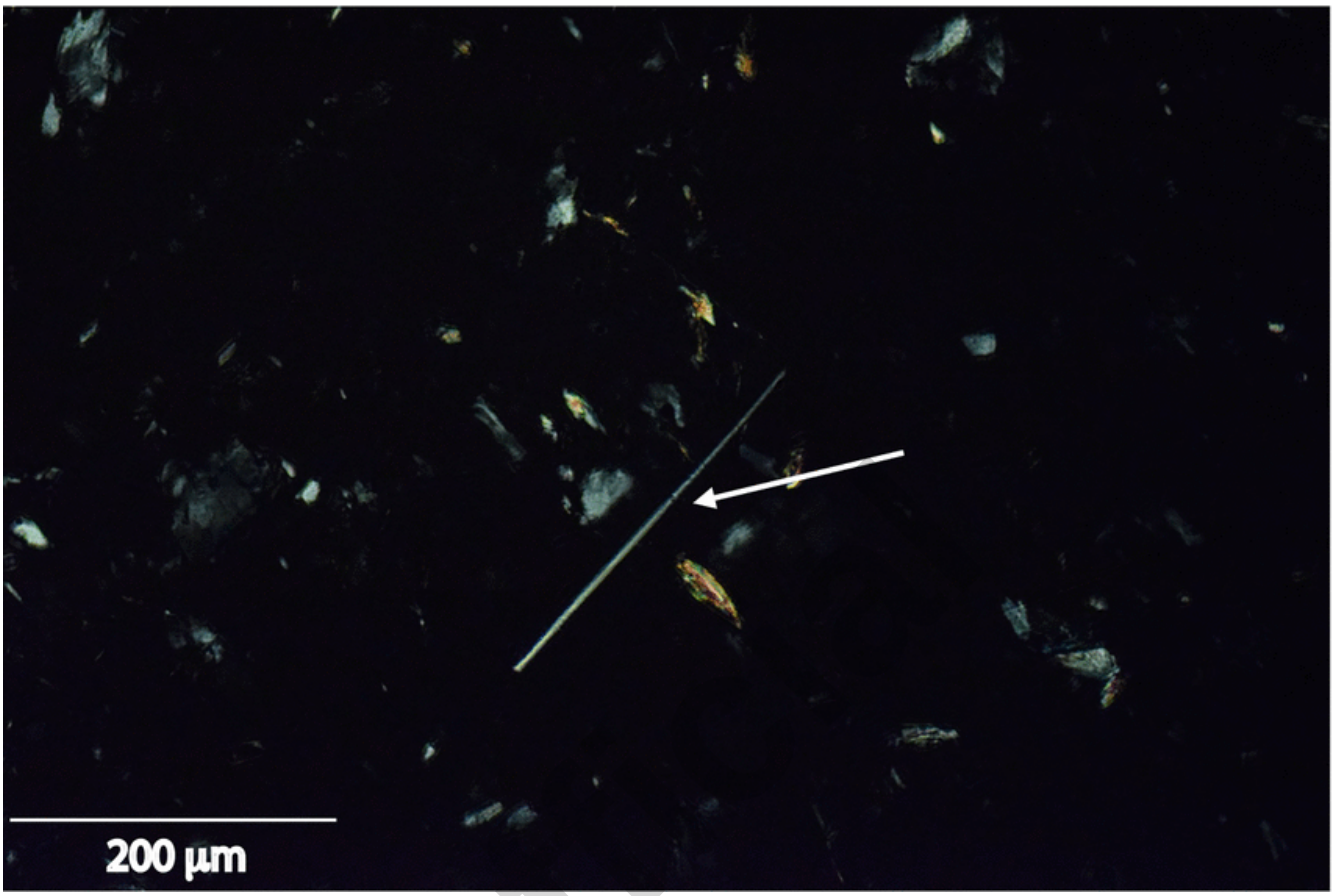


Figure F5. Milled tremolite asbestos in talc matrix immersed in Cargille $n_D$ 1.615 HD liquid at 25° oriented so that γ′ (and fiber length) is 45° counterclockwise up from the lower polarizer (E–W) and parallel with N of the accessory plate in cross-polarized light.

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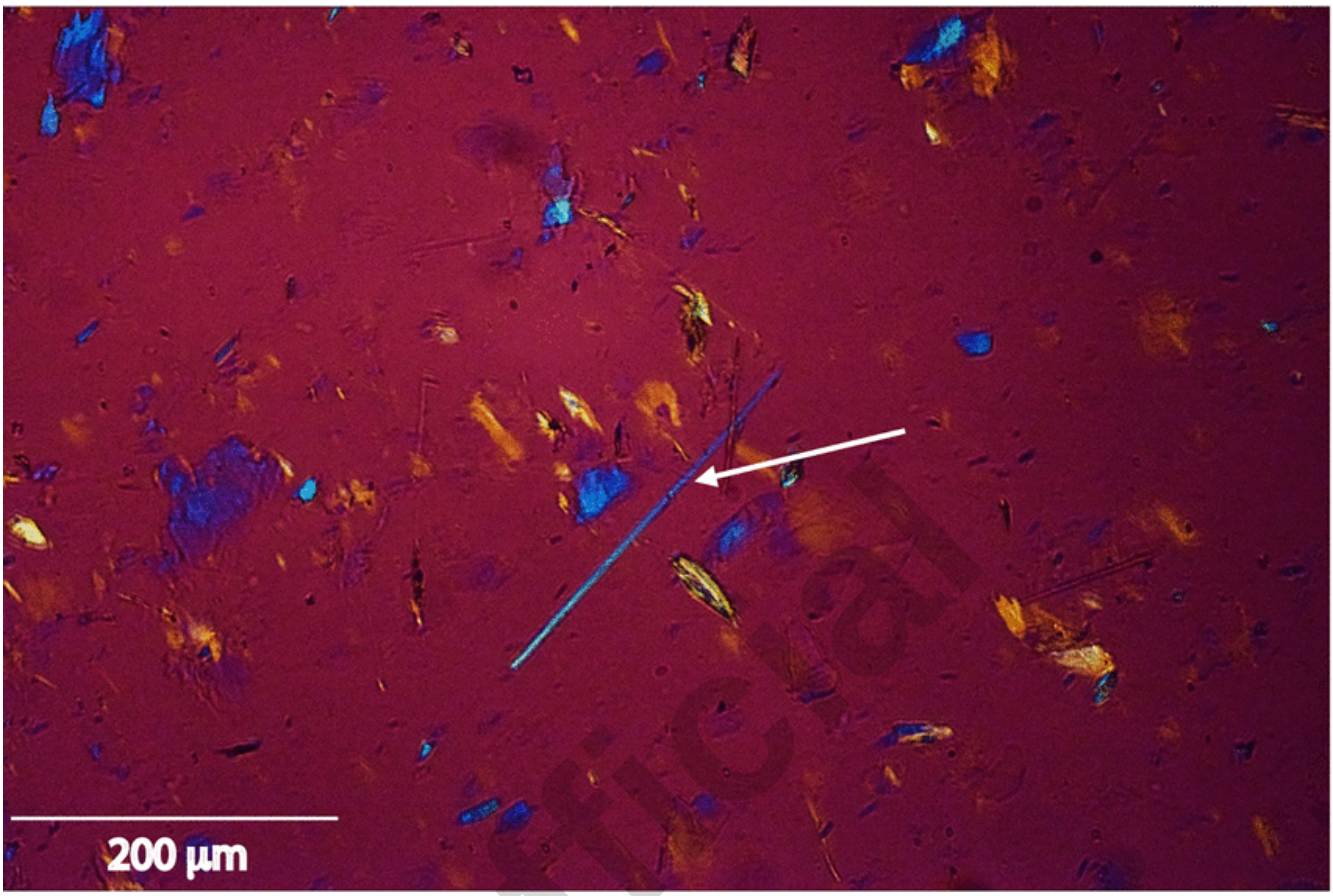


Figure F6. Milled tremolite asbestos in talc matrix immersed in Cargille $n_D$ 1.615 HD liquid at 25° oriented so that γ′ (and fiber length) is 45° from the direction of the lower polarizer (E–W) and parallel with N of the inserted full wave accessory plate in cross-polarized light.

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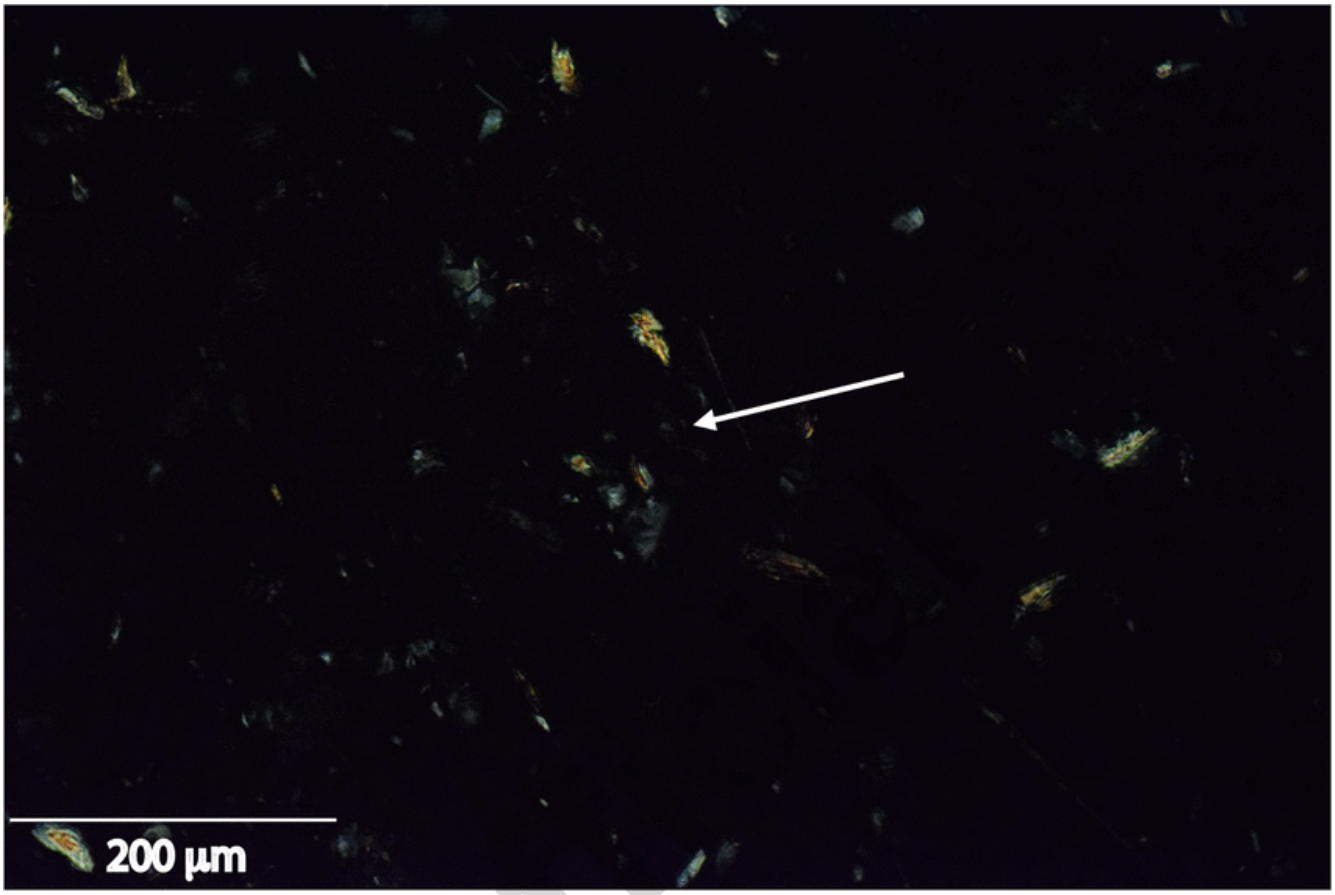


Figure F7. Milled tremolite asbestos in talc matrix immersed in Cargille $n_D$ 1.615 HD liquid at 25° oriented so that γ′ (and fiber length) is parallel with the direction of the lower polarizer (E–W) in cross-polarized light. The tremolite asbestos is at or near extinction.

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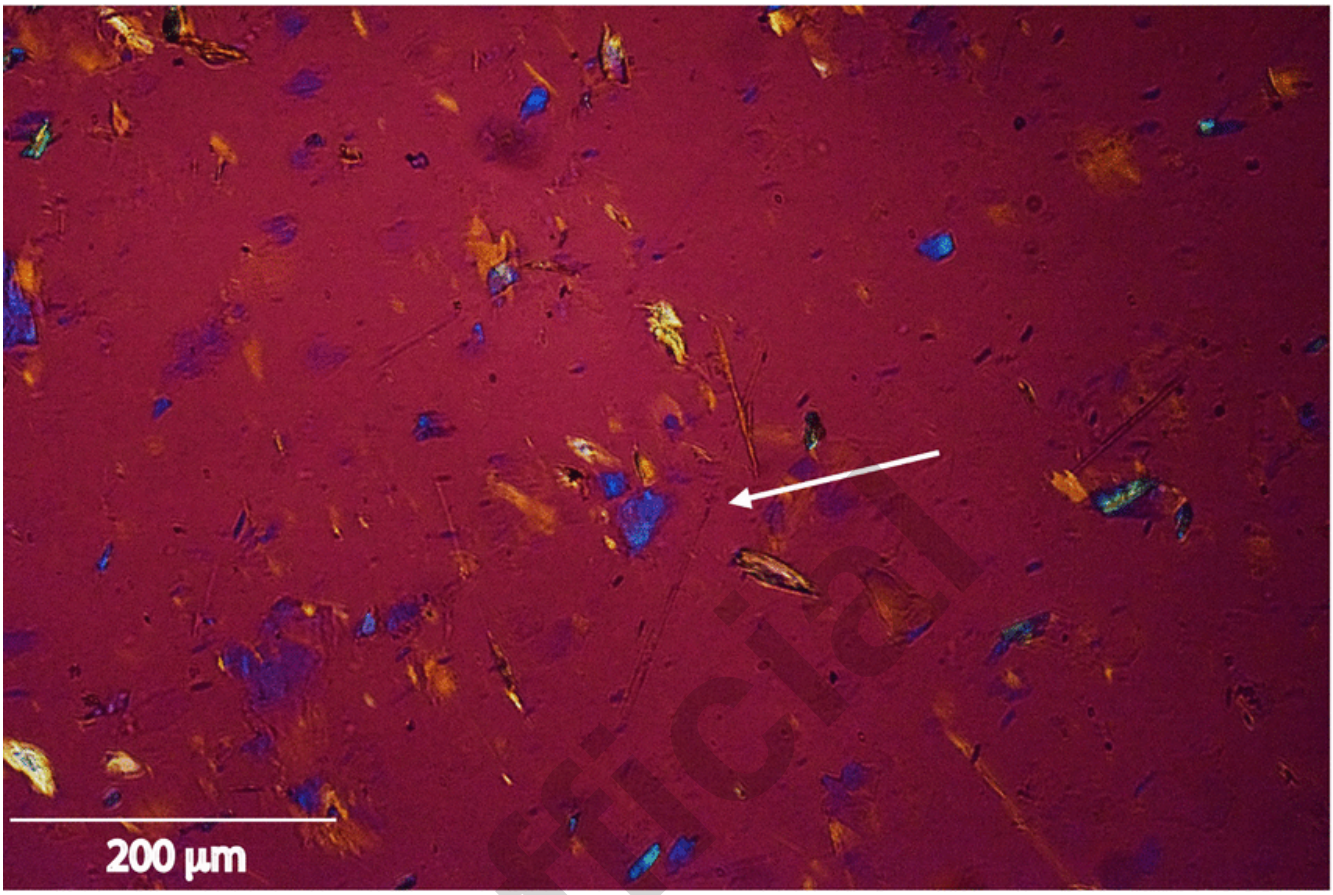


Figure F8. Milled tremolite asbestos in talc matrix immersed in Cargille $n_D$ 1.615 HD liquid at 25° oriented so that γ′ (and fiber length) is parallel with the direction of the lower polarizer (E–W) with the full wave accessory plate inserted in cross-polarized light. The tremolite asbestos is at or near extinction.

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**Milled tremolite asbestos in talc matrix (0.1%)—1.620 RI liquid (material retained on 400-mesh sieve)**

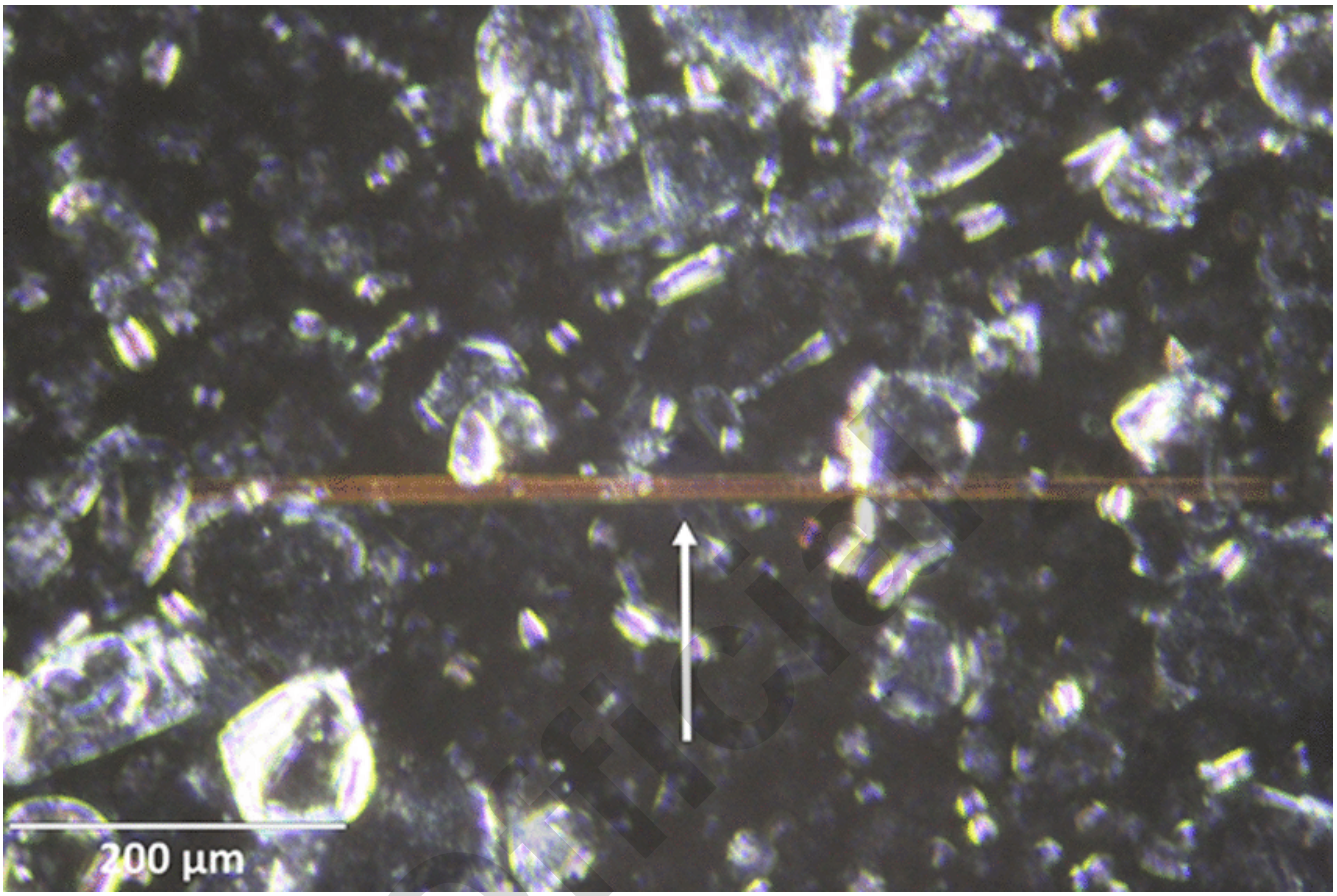


Figure G1. Milled tremolite asbestos in talc matrix (0.1%). Material retained on a 400-mesh sieve was immersed in Cargille $n_D$ 1.620 HD liquid at 25° and oriented so that $\gamma'$ (and fiber length) is parallel with the direction of the lower polarizer (E–W) in central stop dispersion staining.

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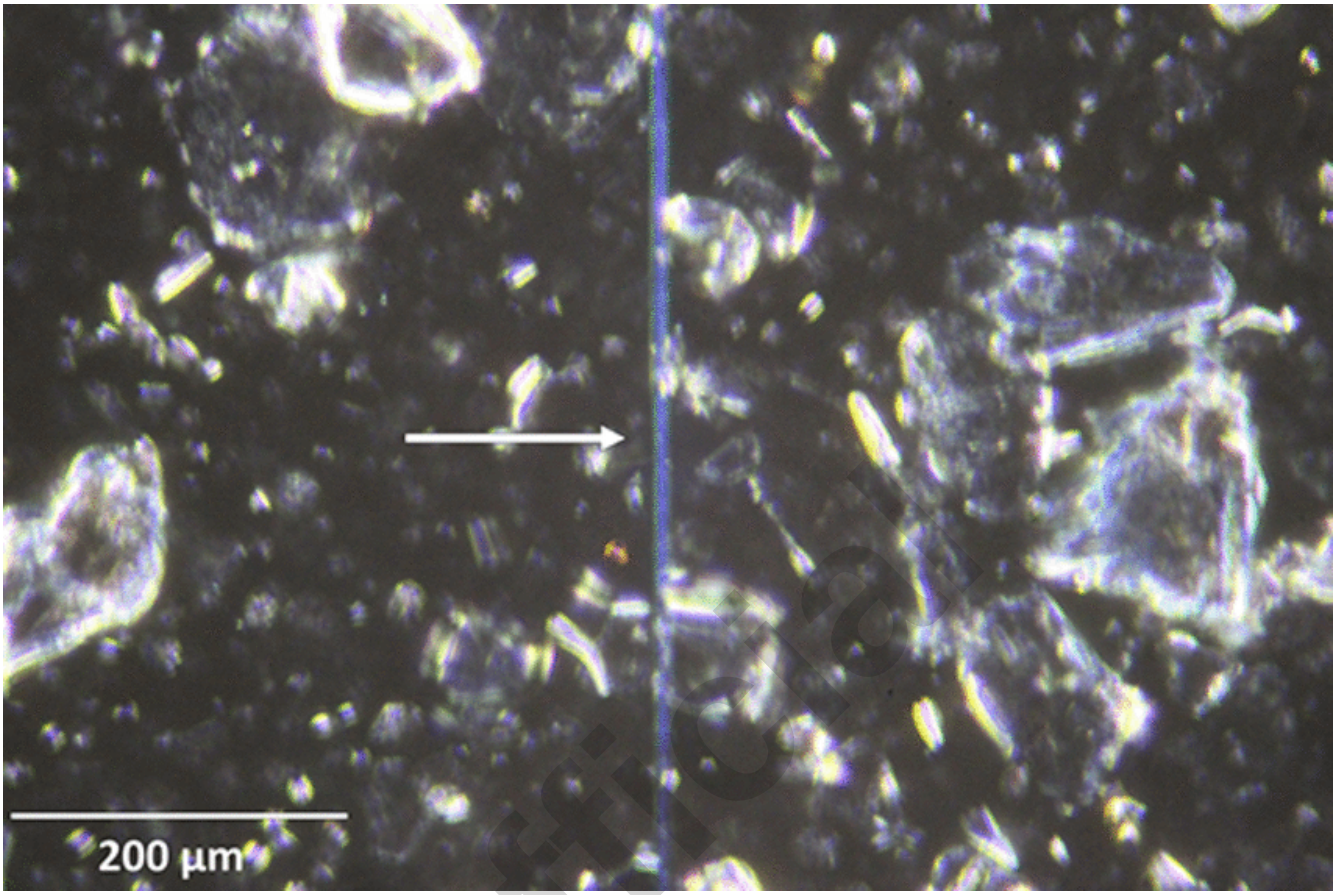


Figure G2. Milled tremolite asbestos in talc matrix (0.1%). Material retained on a 400-mesh sieve was immersed in Cargille $n_D$ 1.620 HD liquid at 25° and oriented so that α′ is parallel with the direction of the lower polarizer (E–W) in central stop dispersion staining (fiber length is perpendicular to the lower polarizer).

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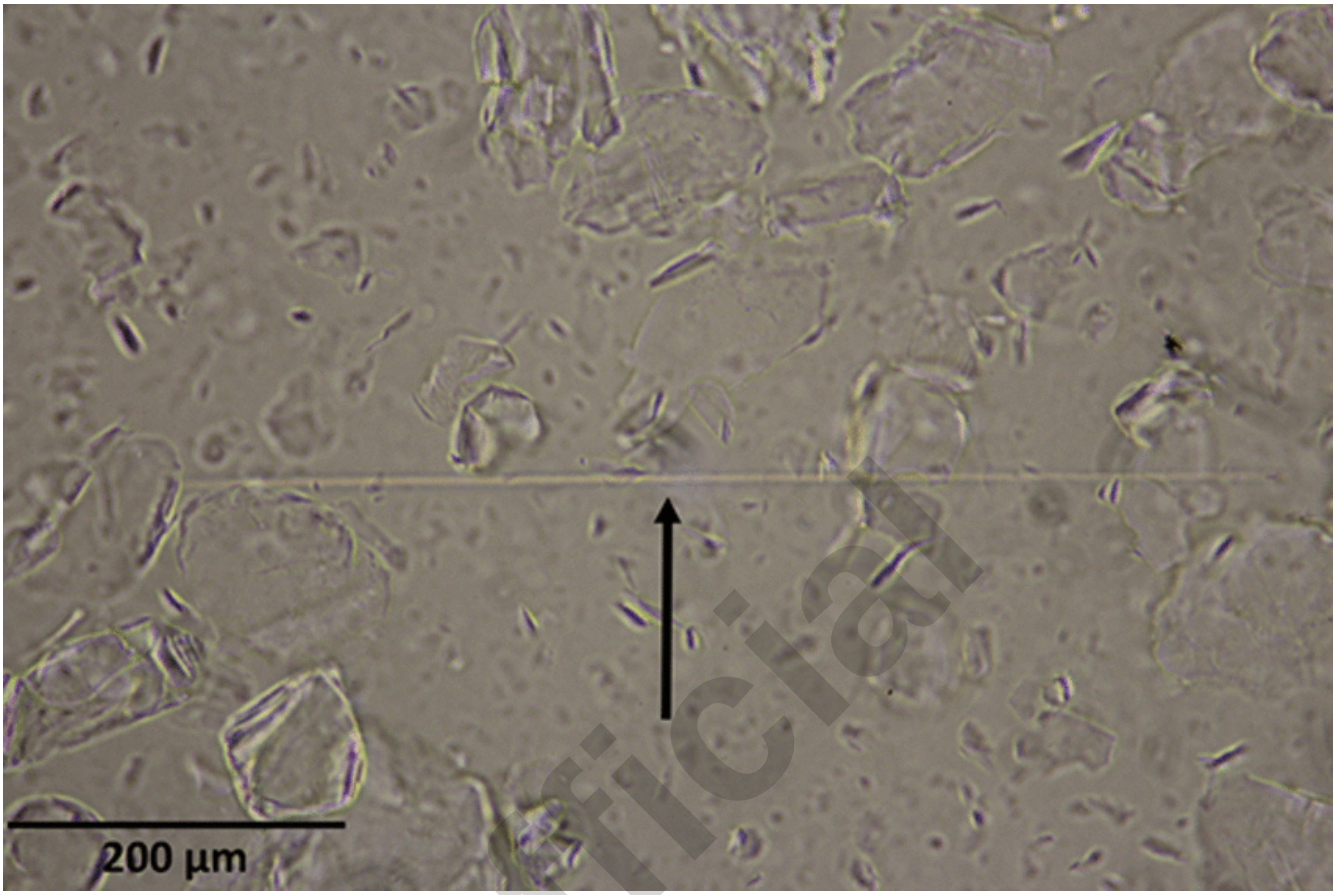


Figure G3. Milled tremolite asbestos in talc matrix (0.1%). Material retained on a 400-mesh sieve was immersed in Cargille $n_D$ 1.620 HD liquid at 25° and oriented so that $\gamma'$ (and fiber length) is parallel with the direction of the lower polarizer (E–W) in plane-polarized light.

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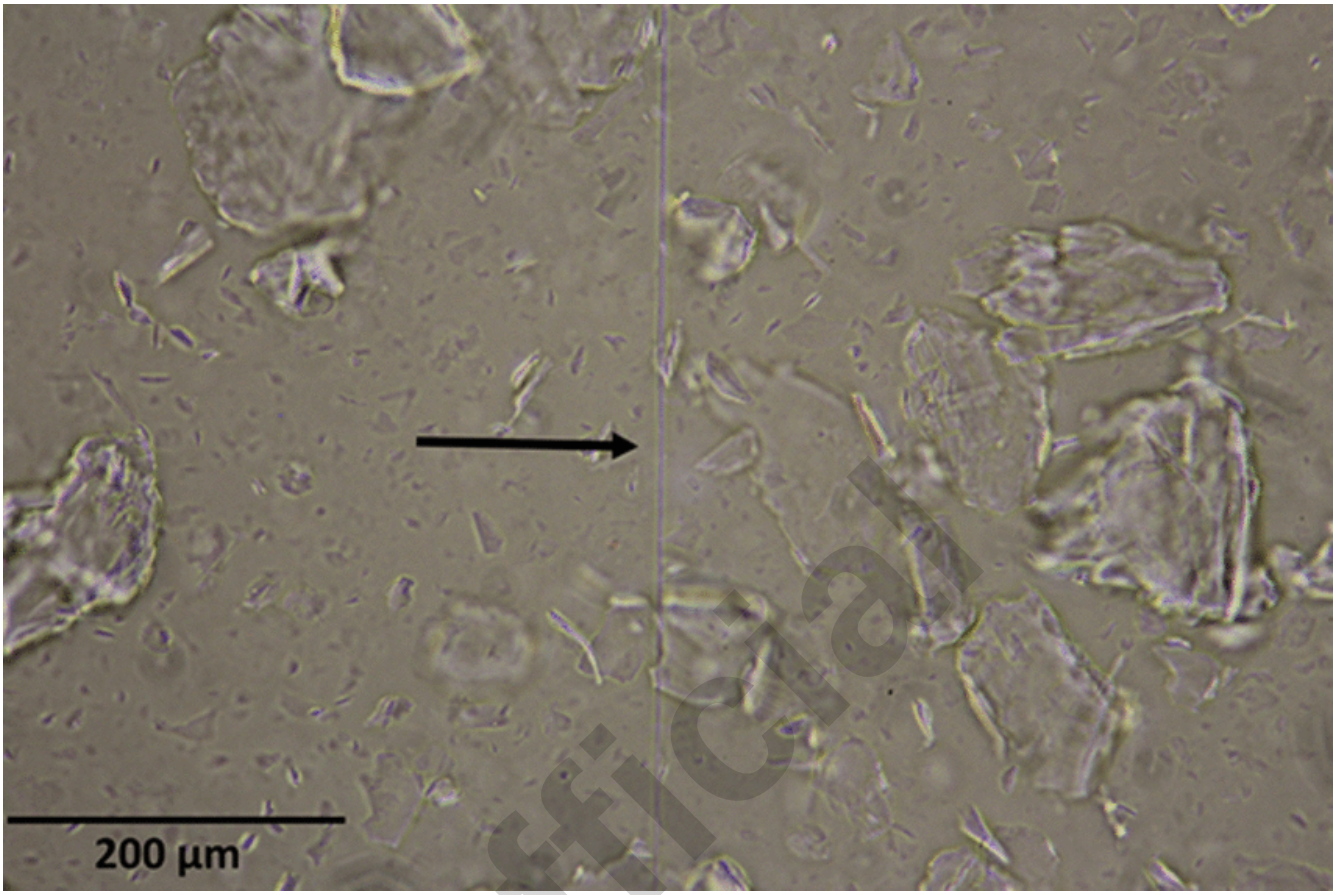


Figure G4. Milled tremolite asbestos in talc matrix (0.1%). Material retained on a 400-mesh sieve was immersed in Cargille $n_D$ 1.620 HD liquid at 25° and oriented so that α′ is parallel with the direction of the lower polarizer (E–W) in plane-polarized light (fiber length is perpendicular to the lower polarizer).

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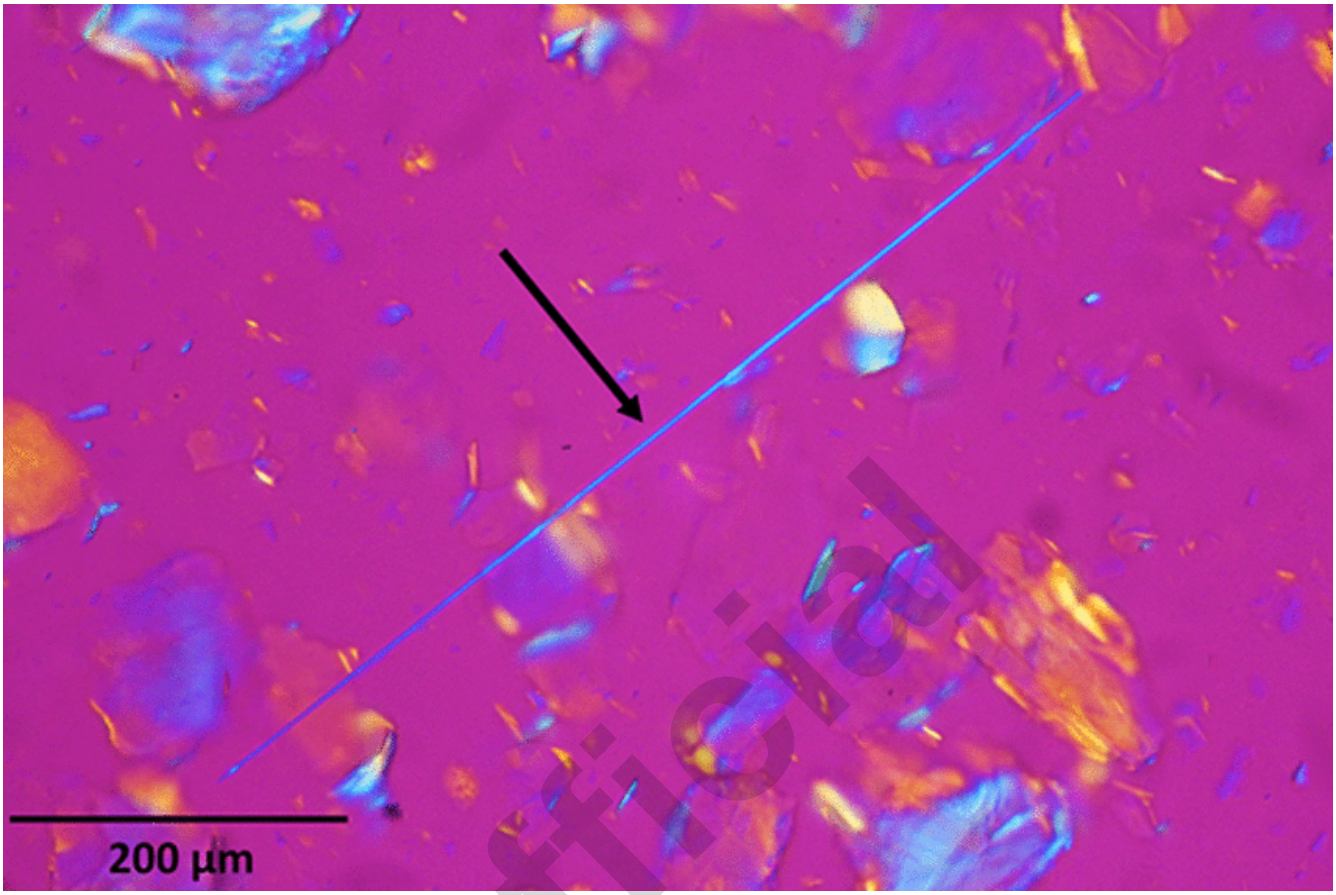


Figure G5. Milled tremolite asbestos in talc matrix (0.1%). Material retained on a 400-mesh sieve was immersed in Cargille $n_D$ 1.620 HD liquid at 25° and oriented so that γ′ (and fiber length) is 45° counterclockwise from the direction of the lower polarizer (E–W) and parallel with N of the inserted full wave accessory plate in cross-polarized light.

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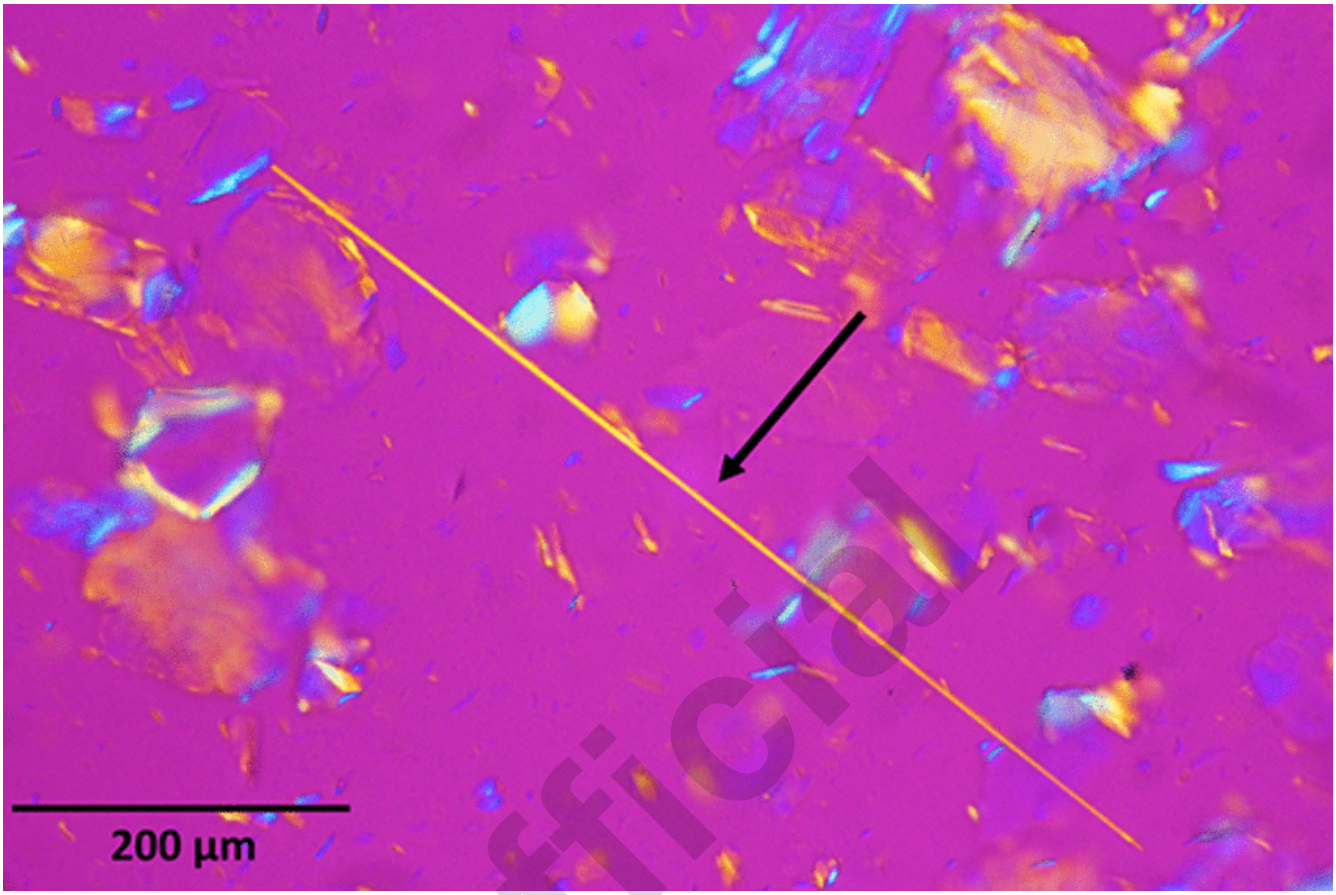


Figure G6. Milled tremolite asbestos in talc matrix (0.1%). Material retained on a 400-mesh sieve was immersed in Cargille $n_D$ 1.620 HD liquid at 25° and oriented so that $\gamma'$ (and fiber length) is 45° clockwise from the direction of the lower polarizer (E–W) and perpendicular with N of the inserted full wave accessory plate in cross-polarized light.

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**Milled tremolite asbestos in talc matrix (0.01%)—1.605 RI liquid (material retained on 400-mesh sieve)**

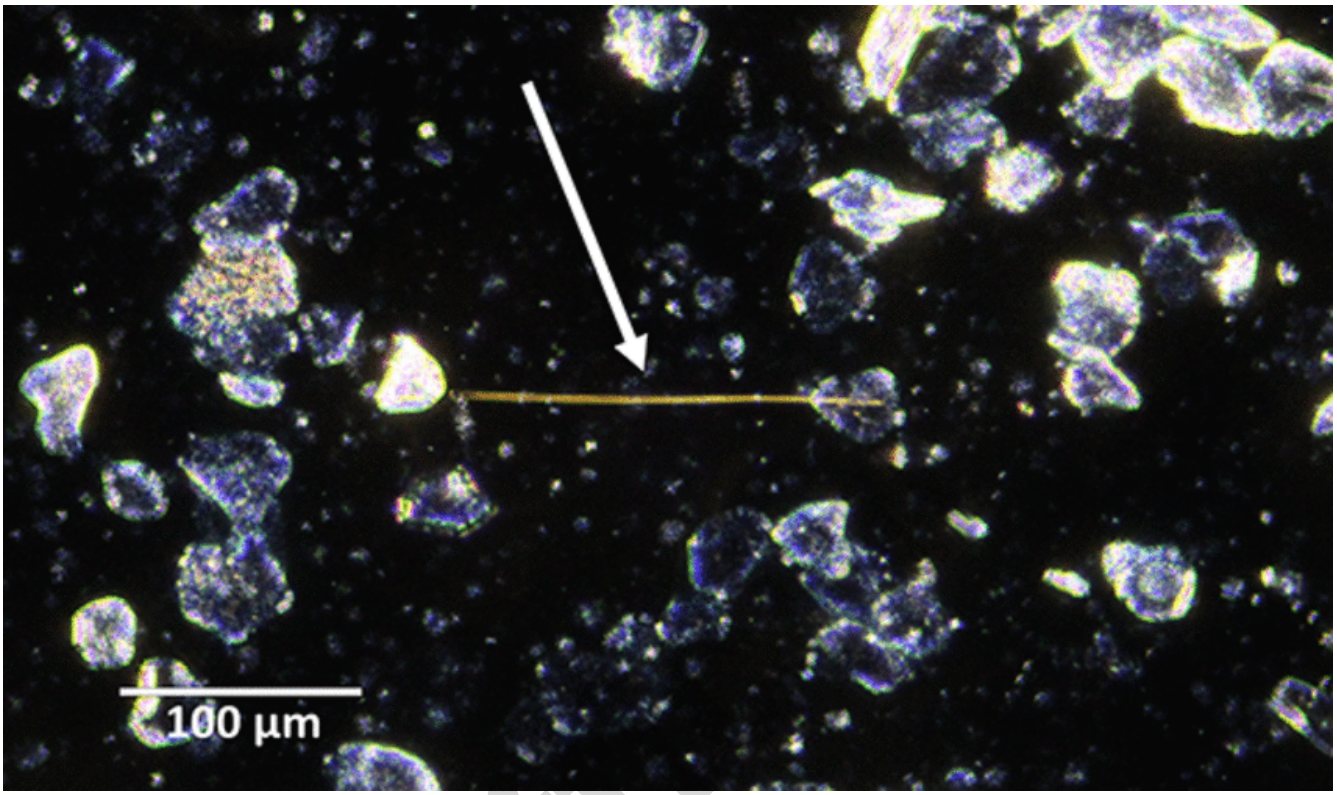


Figure H1. Milled tremolite asbestos in talc matrix (0.01%). Material retained on a 400-mesh sieve was immersed in Cargille $n_D$ 1.605 HD liquid at 25° and oriented so that $\gamma'$ (and fiber length) is parallel with the direction of the lower polarizer (E–W) in central stop dispersion staining.

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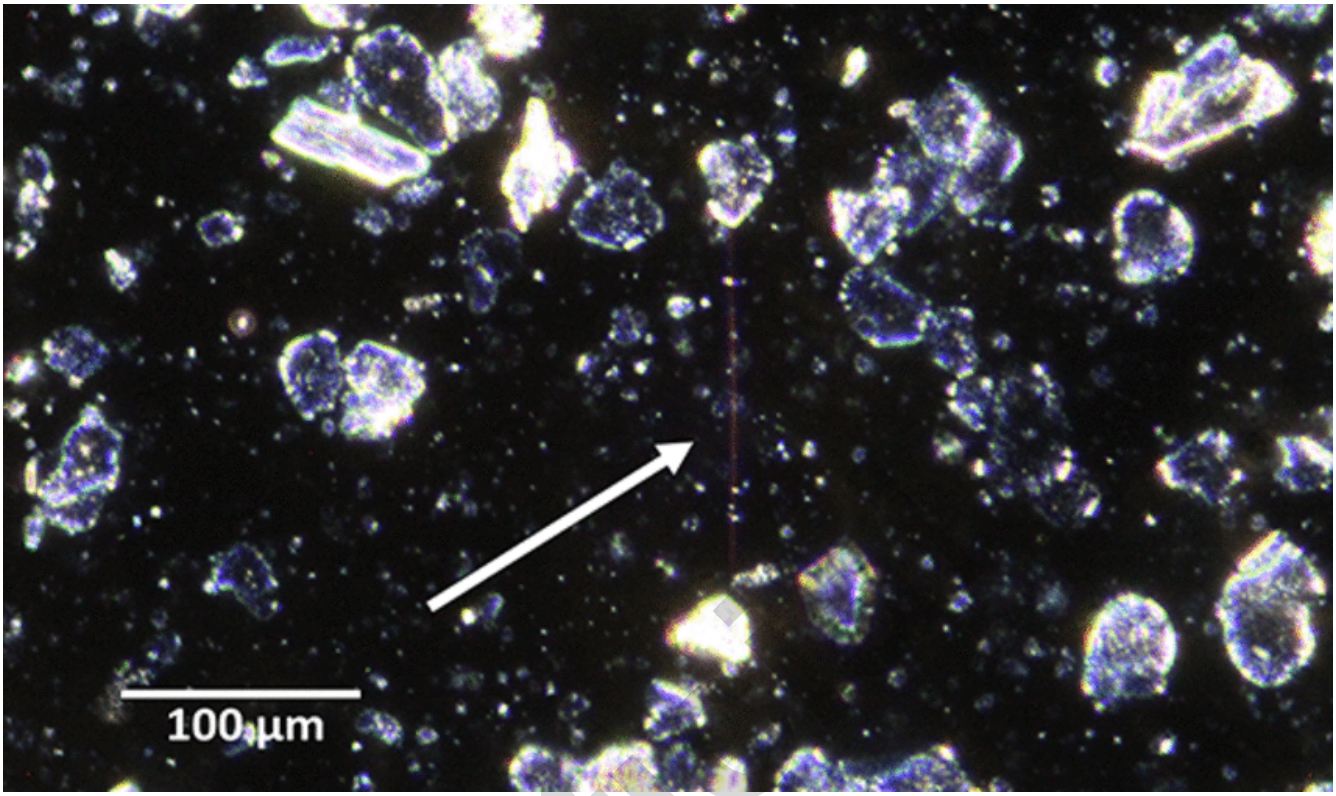


Figure H2. Milled tremolite asbestos in talc matrix (0.01%). Material retained on a 400-mesh sieve was immersed in Cargille $n_D$ 1.605 HD liquid at 25° and oriented so that α′ is parallel with the direction of the lower polarizer (E–W) in central stop dispersion staining (fiber length is perpendicular to the lower polarizer).

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**Milled tremolite asbestos in talc matrix (0.01%)—1.620 RI liquid (material retained on 400-mesh sieve)**

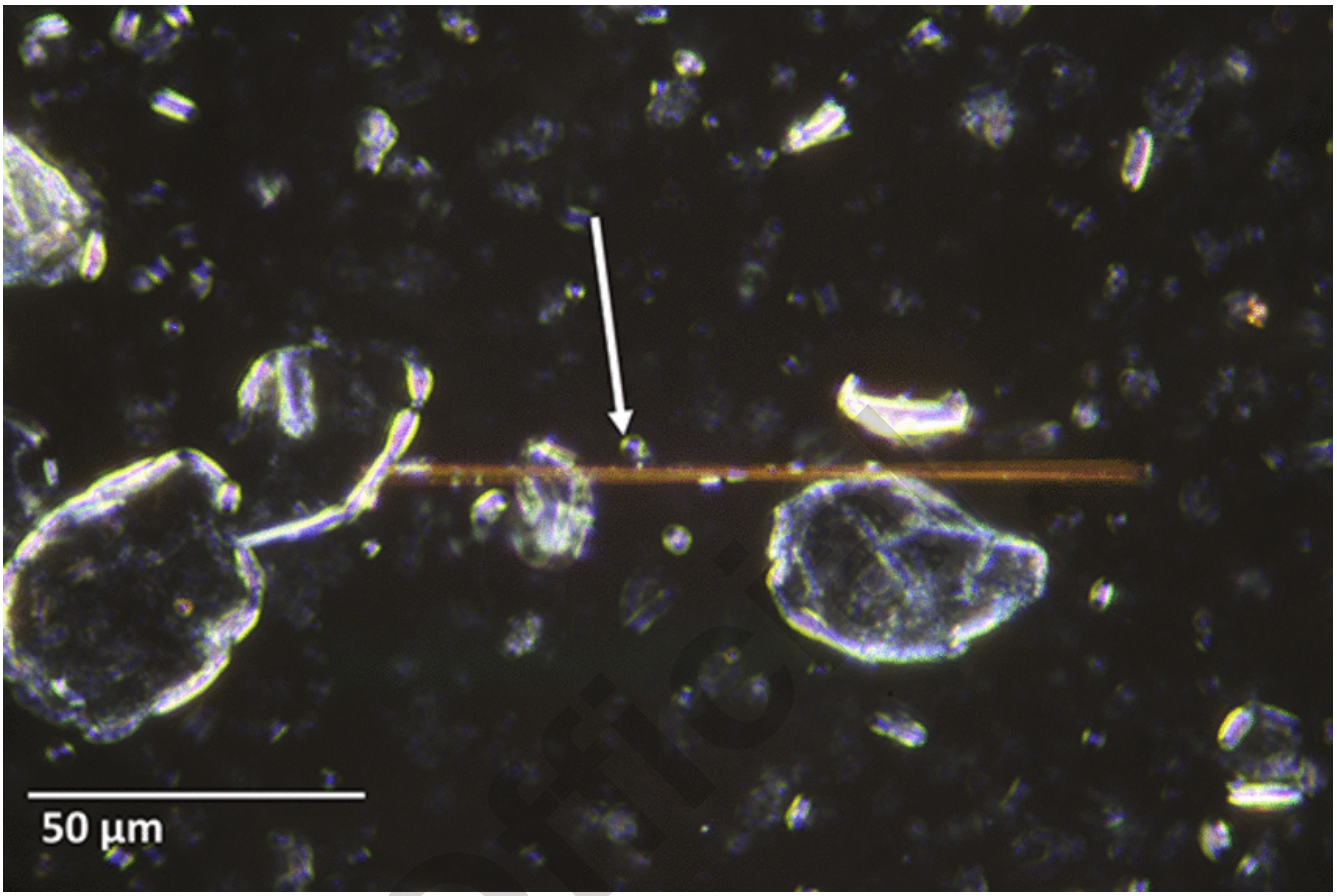


Figure I1. Milled tremolite asbestos in talc matrix (0.01%). Material retained on a 400-mesh sieve was immersed in Cargille $n_D$ 1.620 HD liquid at 25° and oriented so that $\gamma'$ (and fiber length) is parallel with the direction of the lower polarizer (E–W) in central stop dispersion staining.

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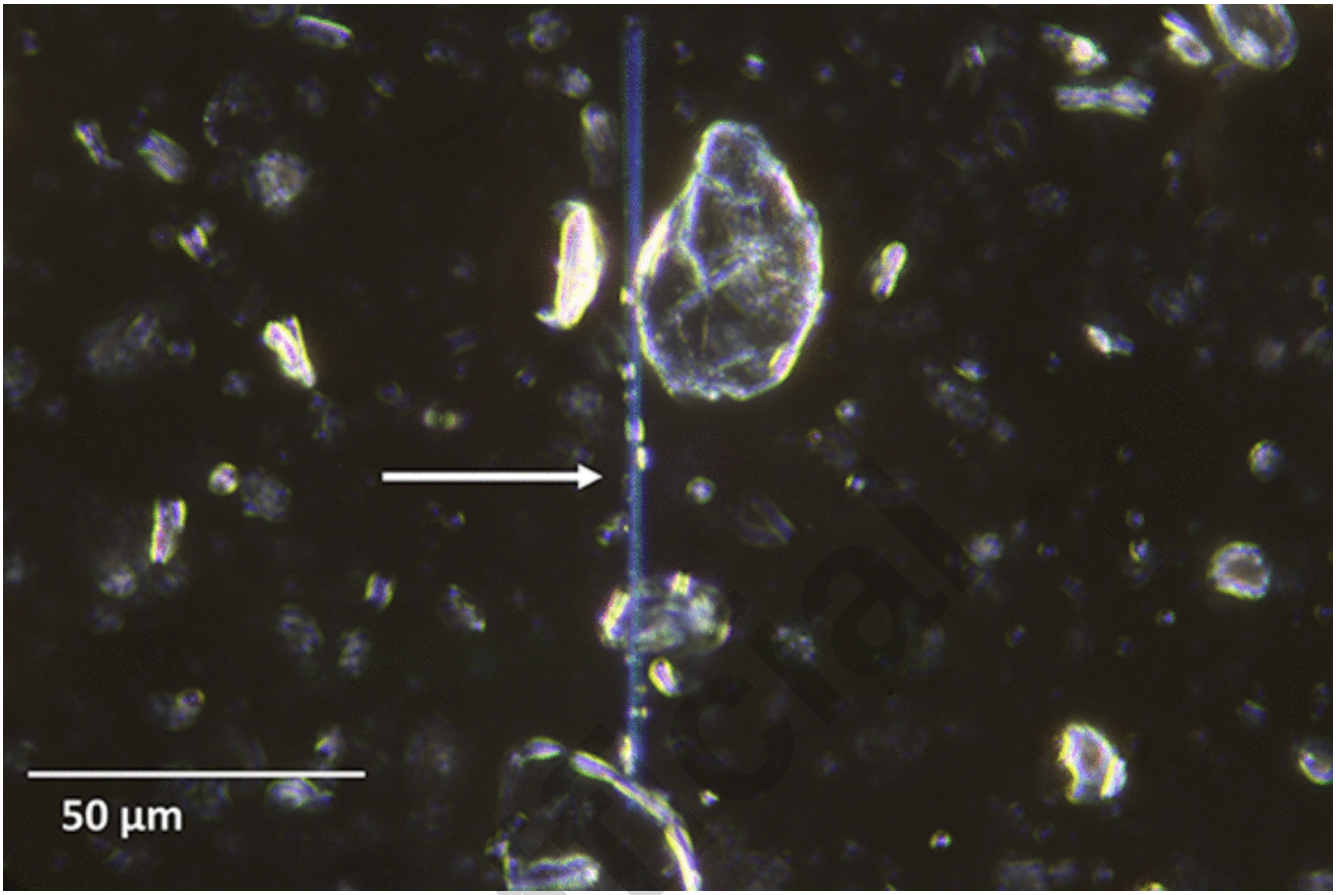


Figure I2. Milled tremolite asbestos in talc matrix (0.01%). Material retained on a 400-mesh sieve was immersed in Cargille $n_D$ 1.620 HD liquid at 25° and oriented so that α′ is parallel with the direction of the lower polarizer (E–W) in central stop dispersion staining (fiber length is perpendicular to the lower polarizer).

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Figure I3. Milled tremolite asbestos in talc matrix (0.01%). Material retained on a 400-mesh sieve was immersed in Cargille $n_D$ 1.620 HD liquid at 25° and oriented so that $\gamma'$ (and fiber length) is parallel with the direction of the lower polarizer (E–W) in plane-polarized light.

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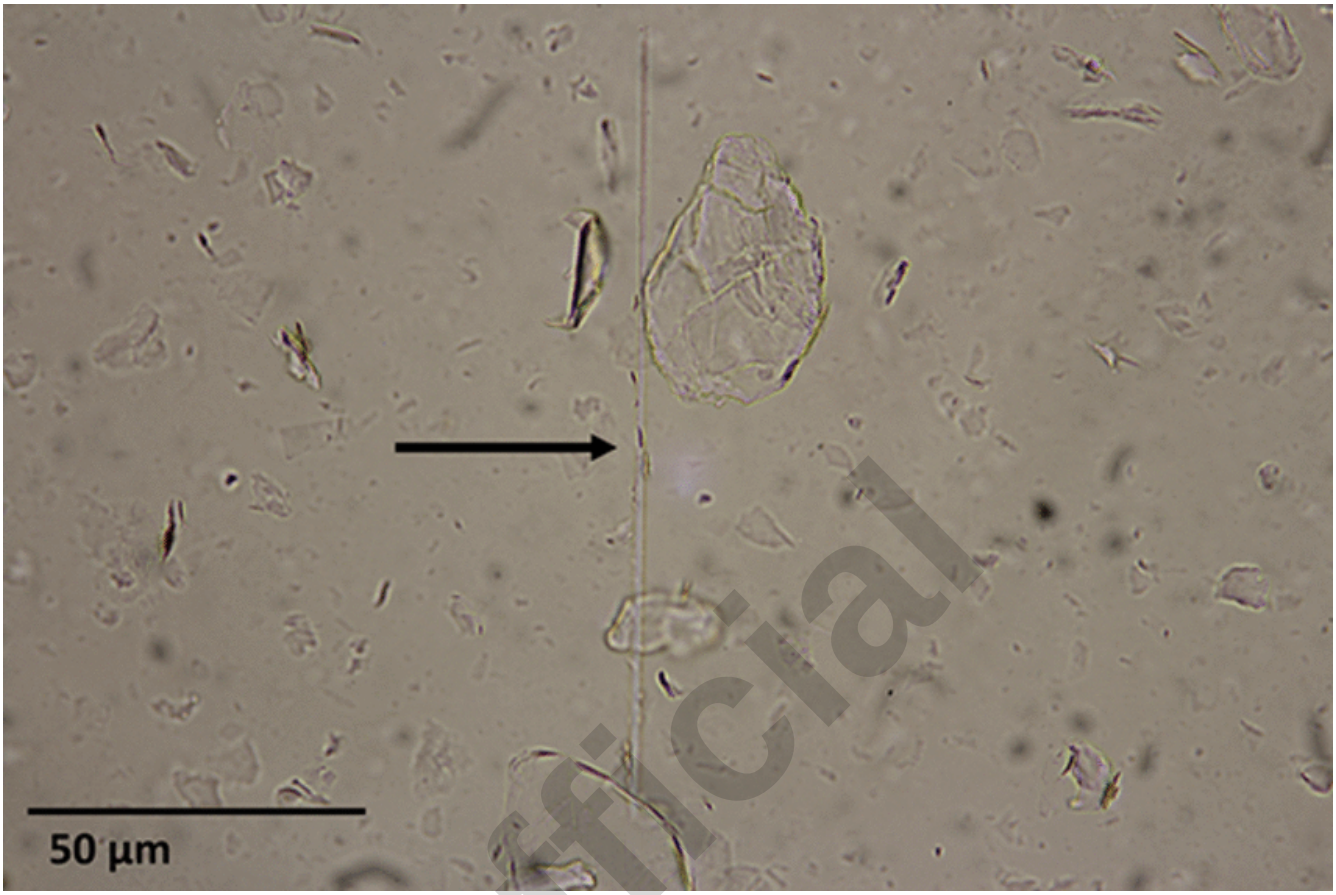


Figure I4. Milled tremolite asbestos in talc matrix (0.01%). Material retained on a 400-mesh sieve was immersed in Cargille $n_D$ 1.620 HD liquid at 25° and oriented so that α′ is parallel with the direction of the lower polarizer (E–W) in plane-polarized light (fiber length is perpendicular to the lower polarizer).

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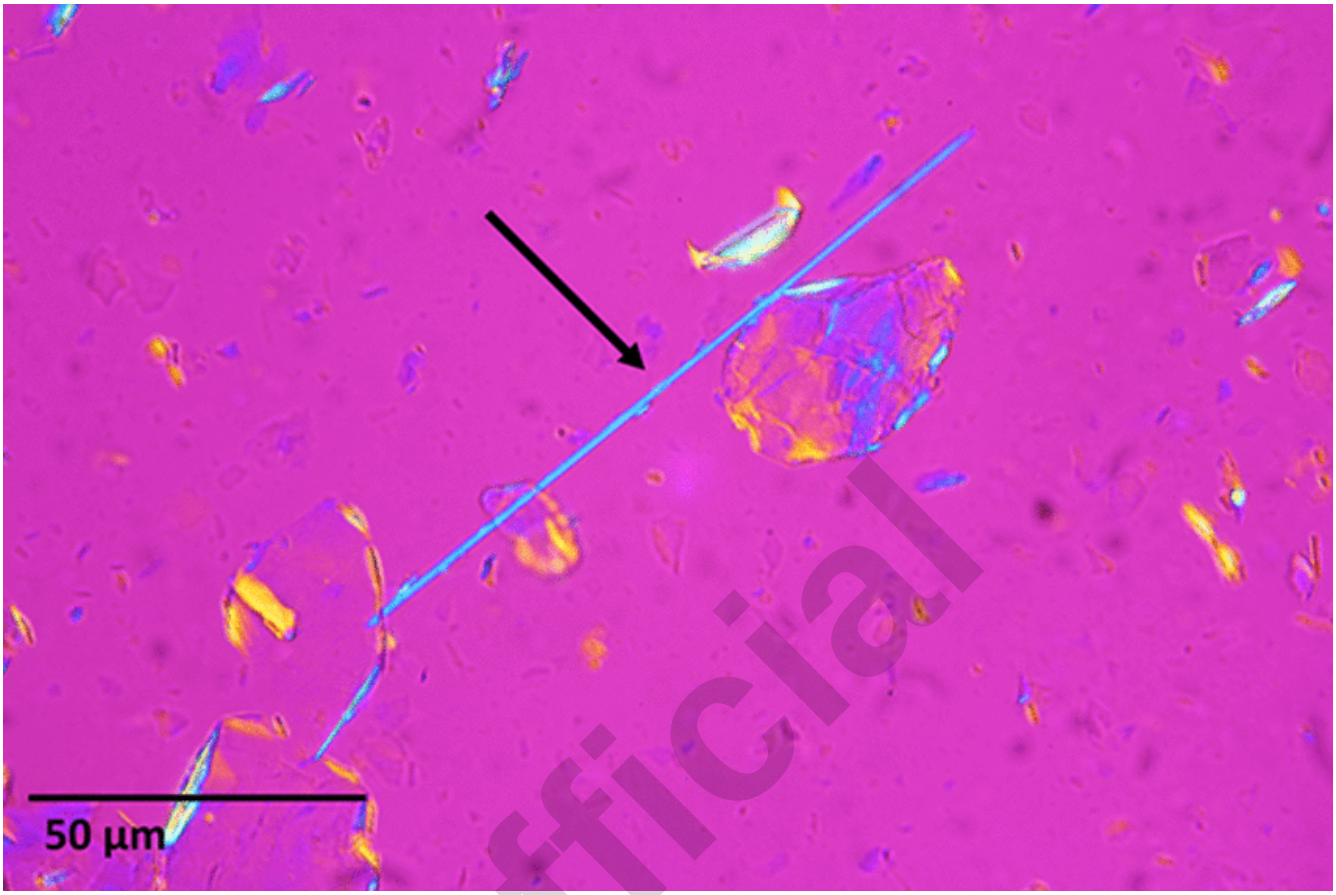


Figure I5. Milled tremolite asbestos in talc matrix (0.01%). Material retained on a 400-mesh sieve was immersed in Cargille $n_D$ 1.620 HD liquid at 25° and oriented so that γ′ (and fiber length) is 45° counterclockwise from the direction of the lower polarizer (E–W) and parallel with N of the inserted full wave accessory plate in cross-polarized light.

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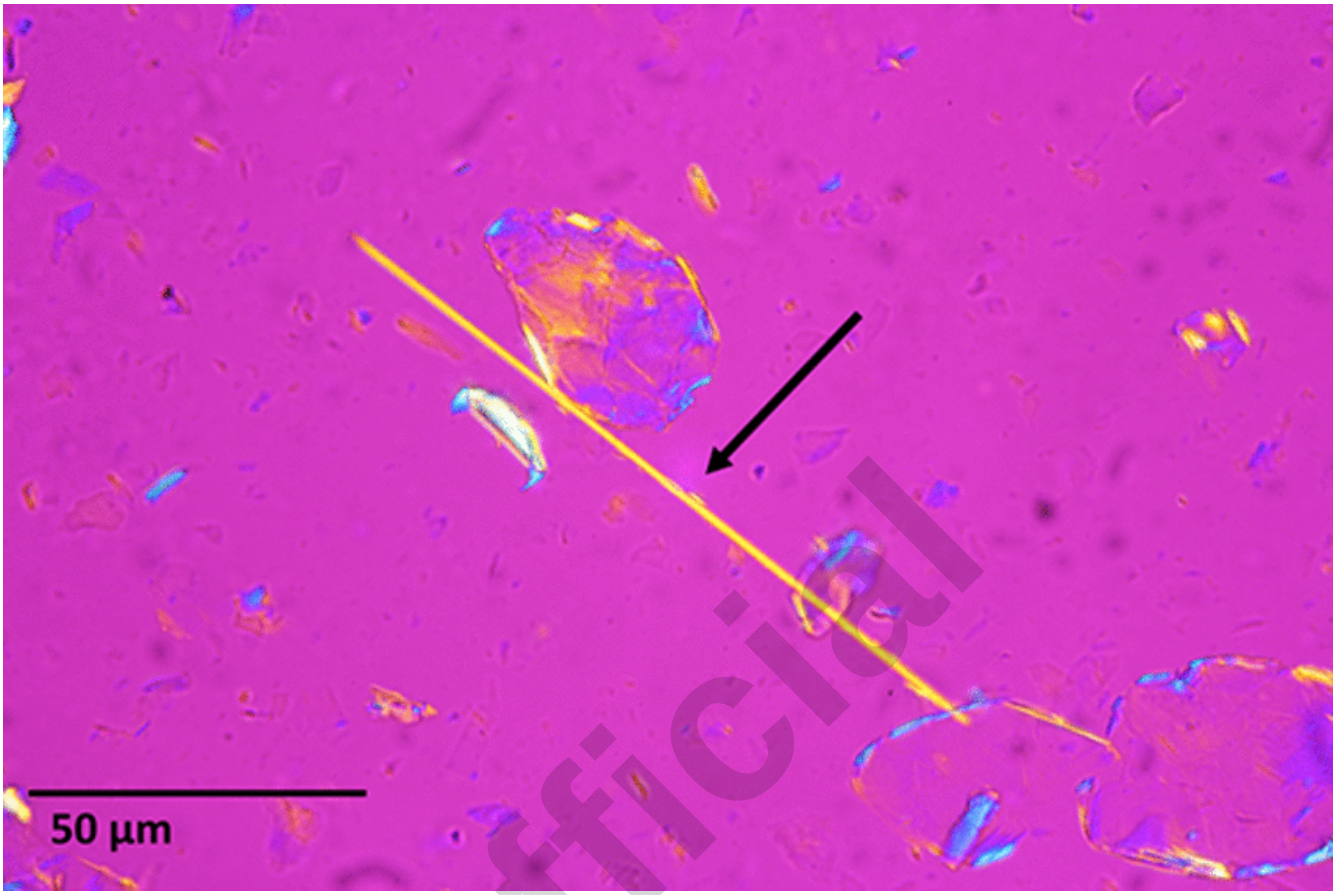


Figure I6. Milled tremolite asbestos in talc matrix (0.01%). Material retained on a 400-mesh sieve was immersed in Cargille $n_D$ 1.620 HD liquid at 25° and oriented so that $\gamma'$ (and fiber length) is 45° clockwise from the direction of the lower polarizer (E–W) and perpendicular with N of the inserted full wave accessory plate in cross-polarized light.

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**Milled tremolite asbestos in talc matrix (0.001%)—1.605 RI liquid (material retained on 400-mesh sieve)**

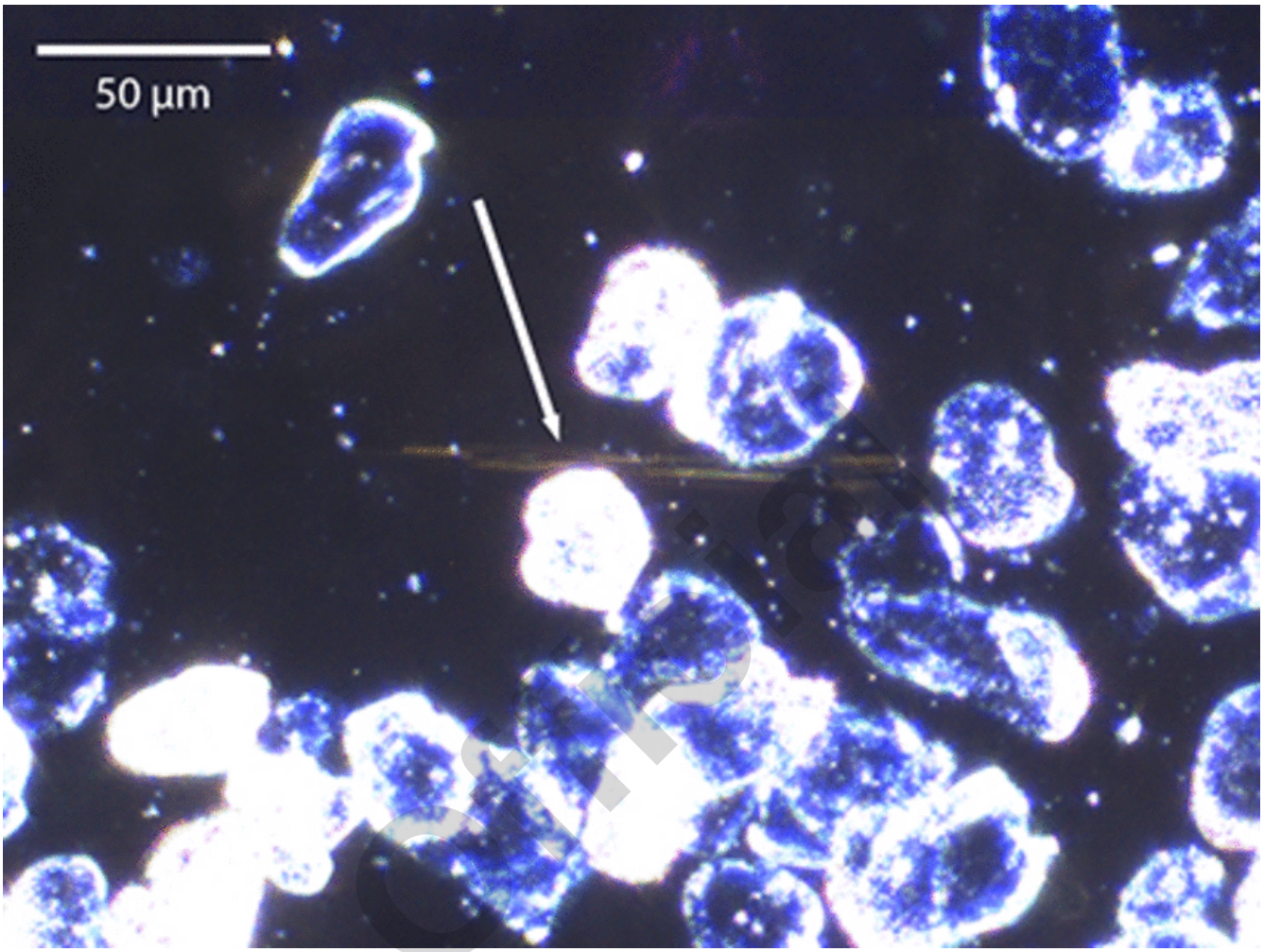


Figure J1. Milled tremolite asbestos in talc matrix (0.001%). Material retained on a 400-mesh sieve was immersed in Cargille $n_D$ 1.605 HD liquid at 25° and oriented so that $\gamma'$ (and fiber length) is parallel with the direction of the lower polarizer (E–W) in central stop dispersion staining.

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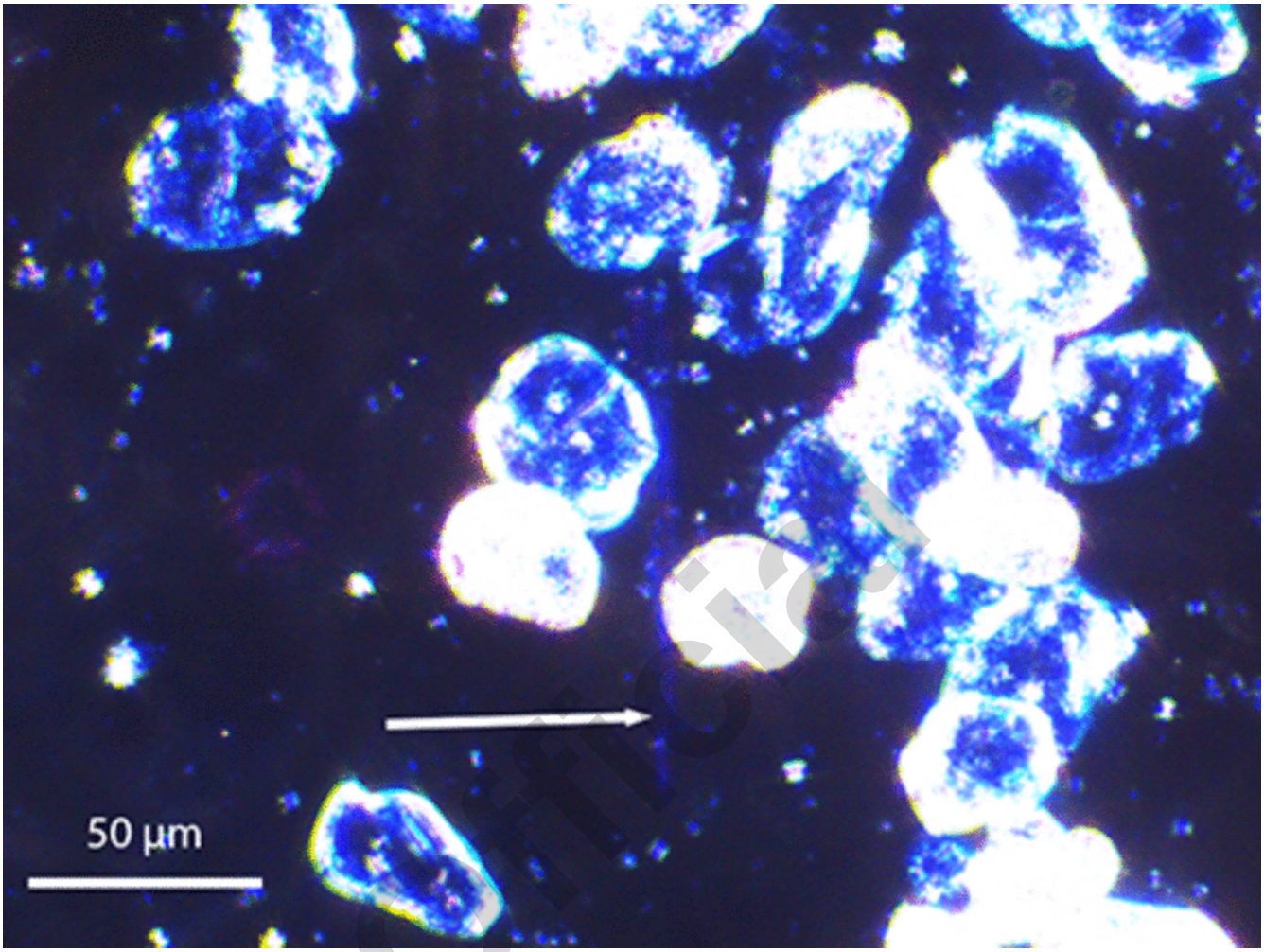


Figure J2. Milled tremolite asbestos in talc matrix (0.001%). Material retained on a 400-mesh sieve was immersed in Cargille $n_D$ 1.605 HD liquid at 25° and oriented so that α′ is parallel with the direction of the lower polarizer (E–W) in central stop dispersion staining (fiber length is perpendicular to the lower polarizer).

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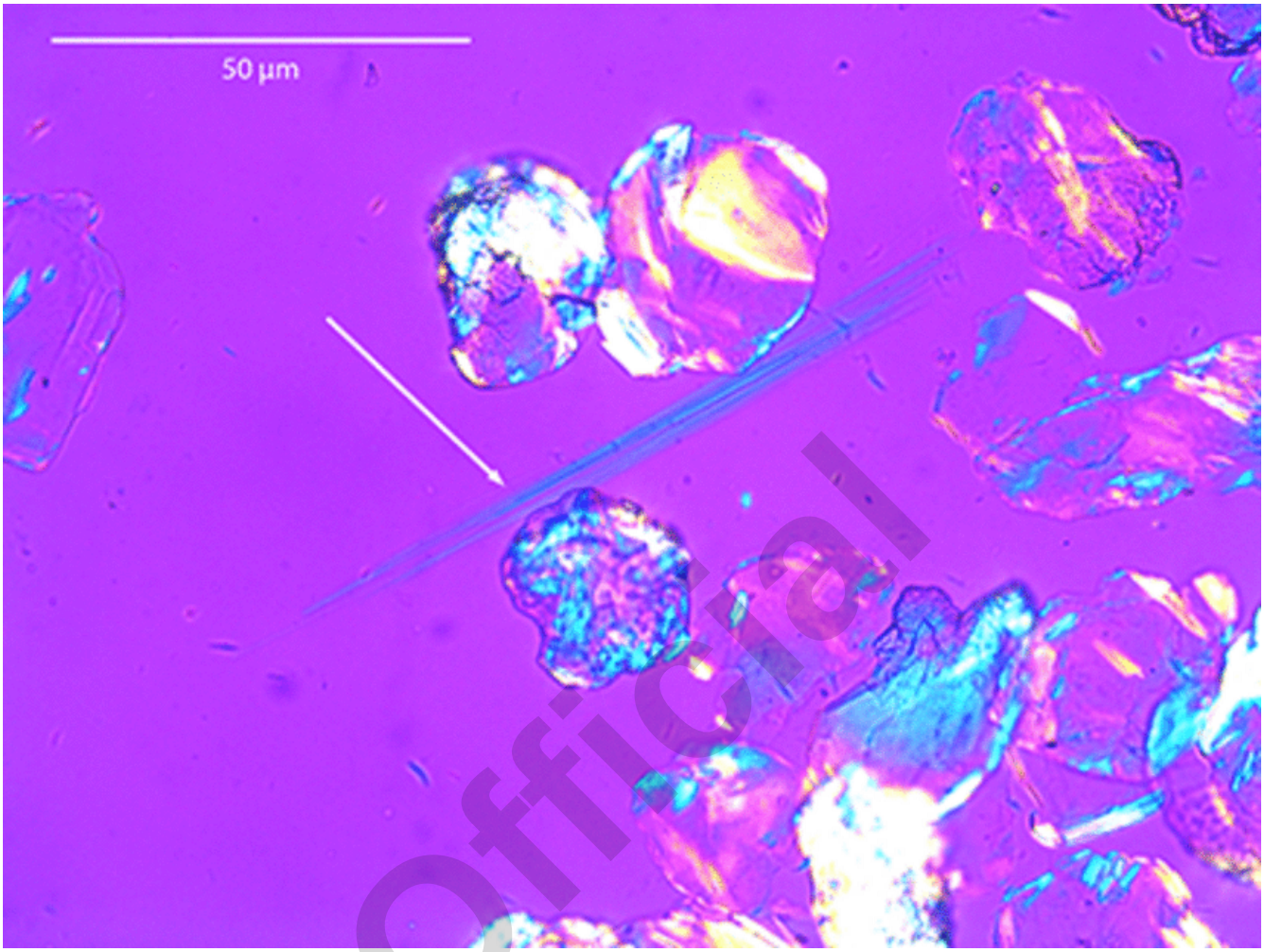


Figure J3. Milled tremolite asbestos in talc matrix (0.001%). Material retained on a 400-mesh sieve was immersed in Cargille $n_D$ 1.605 HD liquid at 25° and oriented so that $\gamma'$ (and fiber length) is 45° counterclockwise from the direction of the lower polarizer (E–W) and parallel with N of the inserted full wave accessory plate in cross-polarized light.

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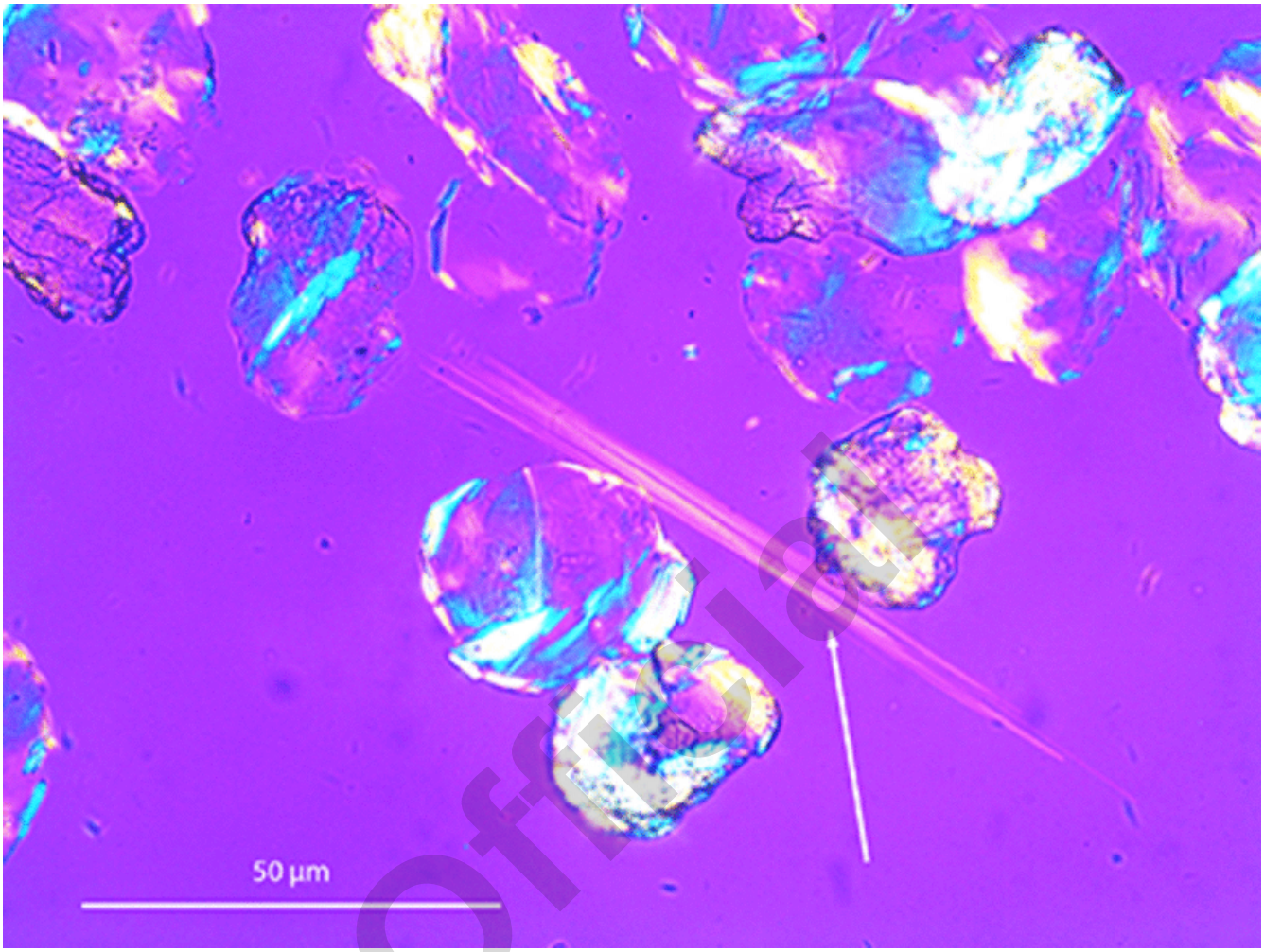


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Figure J4. Milled tremolite asbestos in talc matrix (0.001%). Material retained on a 400-mesh sieve was immersed in Cargille $n_D$ 1.605 HD liquid at 25° and oriented so that $\gamma'$ (and fiber length) is 45° clockwise from the direction of the lower polarizer (E–W) and perpendicular with N of the inserted full wave accessory plate in cross-polarized light.

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## MILLED TREMOLITE (NOT TREMOLITE ASBESTOS) IN TALC

**Milled tremolite (not tremolite asbestos) in talc matrix—1.605 RI liquid (not sieved)**

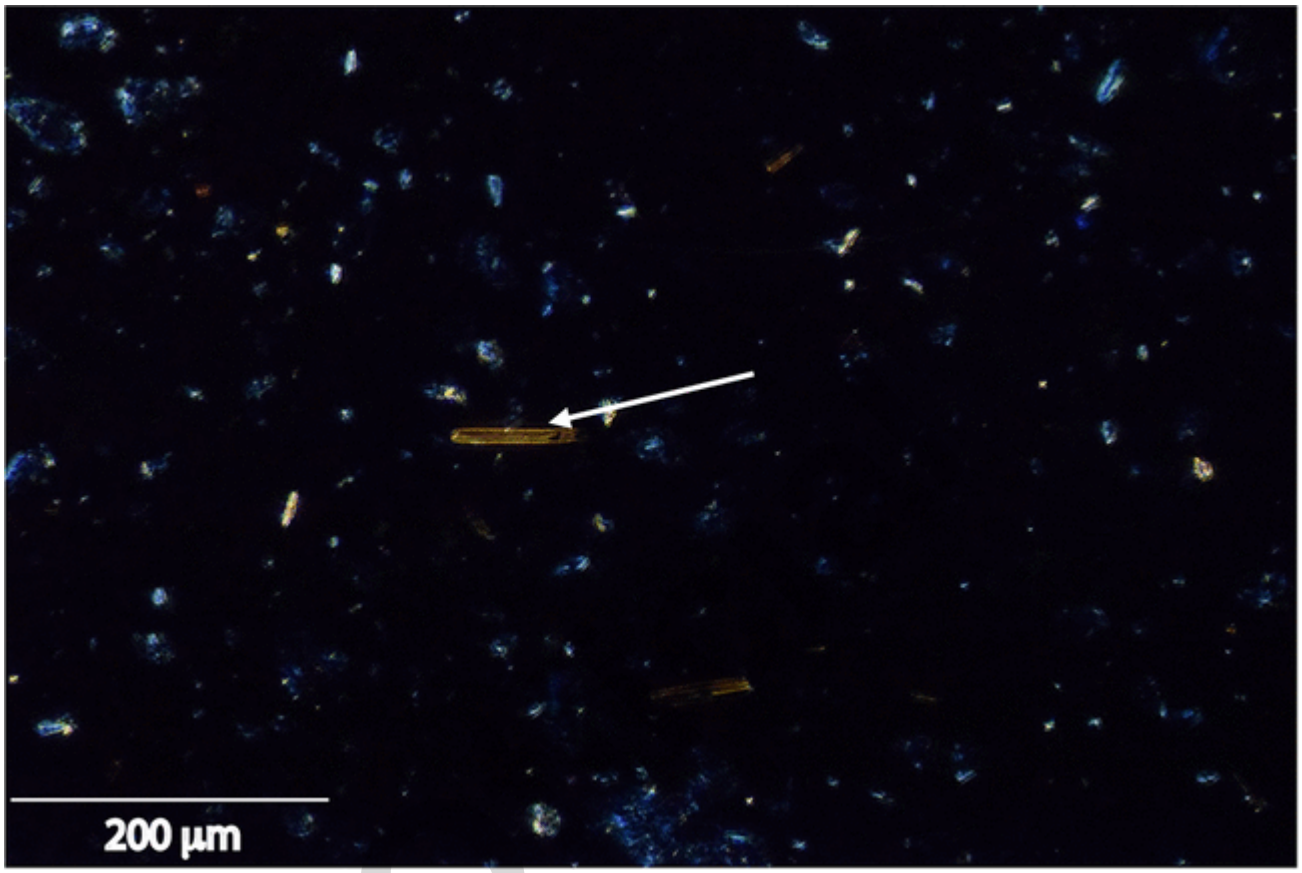


Figure K1. Milled tremolite (not tremolite asbestos) in talc matrix immersed in Cargille $n_D$ 1.605 HD liquid at 25° oriented so that $\gamma'$ (and particle elongation) is parallel with the direction of the lower polarizer (E–W) in central stop dispersion staining.

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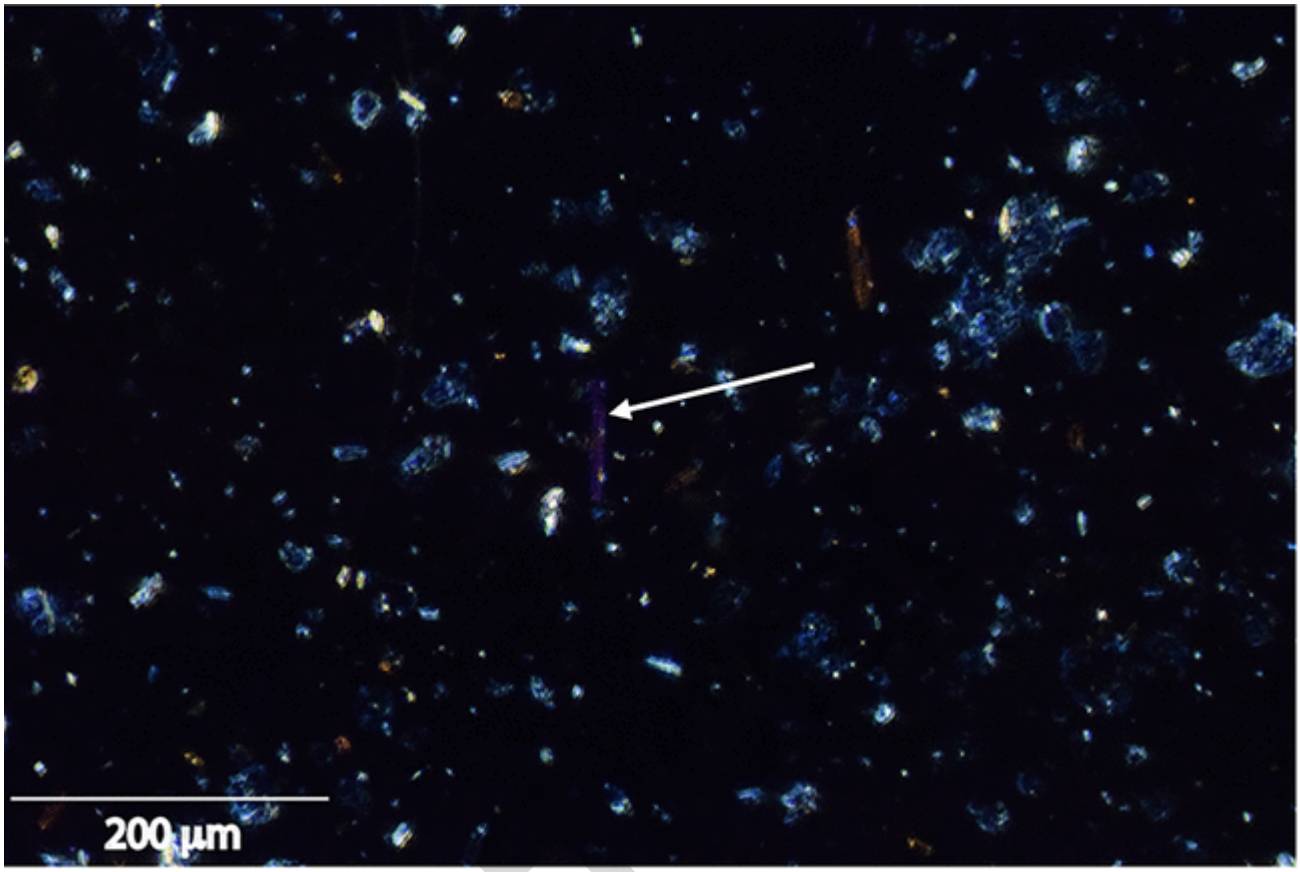


Figure K2. Milled tremolite (not tremolite asbestos) in talc matrix immersed in Cargille $n_D$ 1.605 HD liquid at 25° oriented so that α′ is parallel with the direction of the lower polarizer (E–W) in central stop dispersion staining (particle elongation is perpendicular to the lower polarizer).

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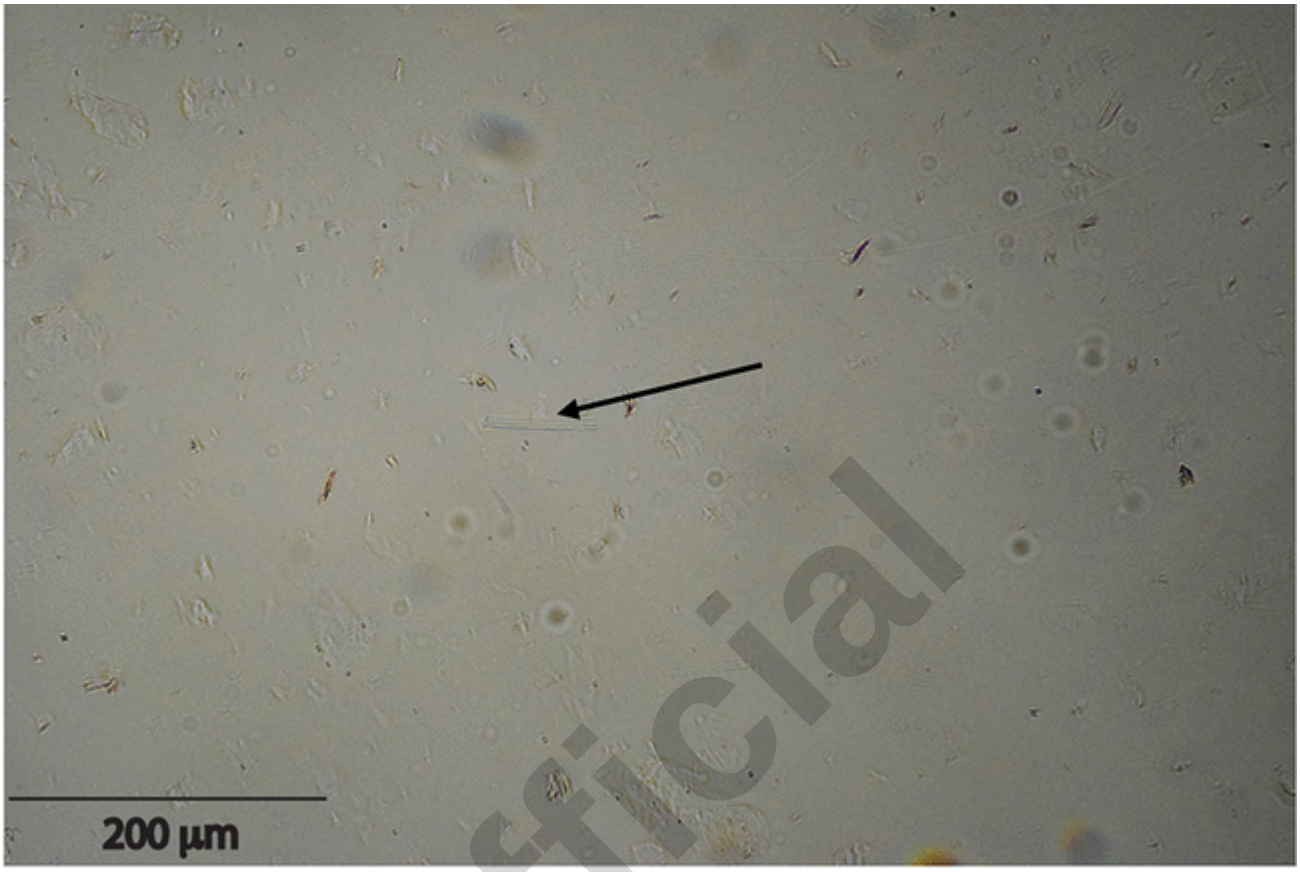


Figure K3. Milled tremolite (not tremolite asbestos) in talc matrix immersed in Cargille $n_D$ 1.605 HD liquid at 25° oriented so that γ′ (and particle elongation) is parallel with the direction of the lower polarizer (E–W) in plane-polarized light slightly defocused to observe the Becke line.

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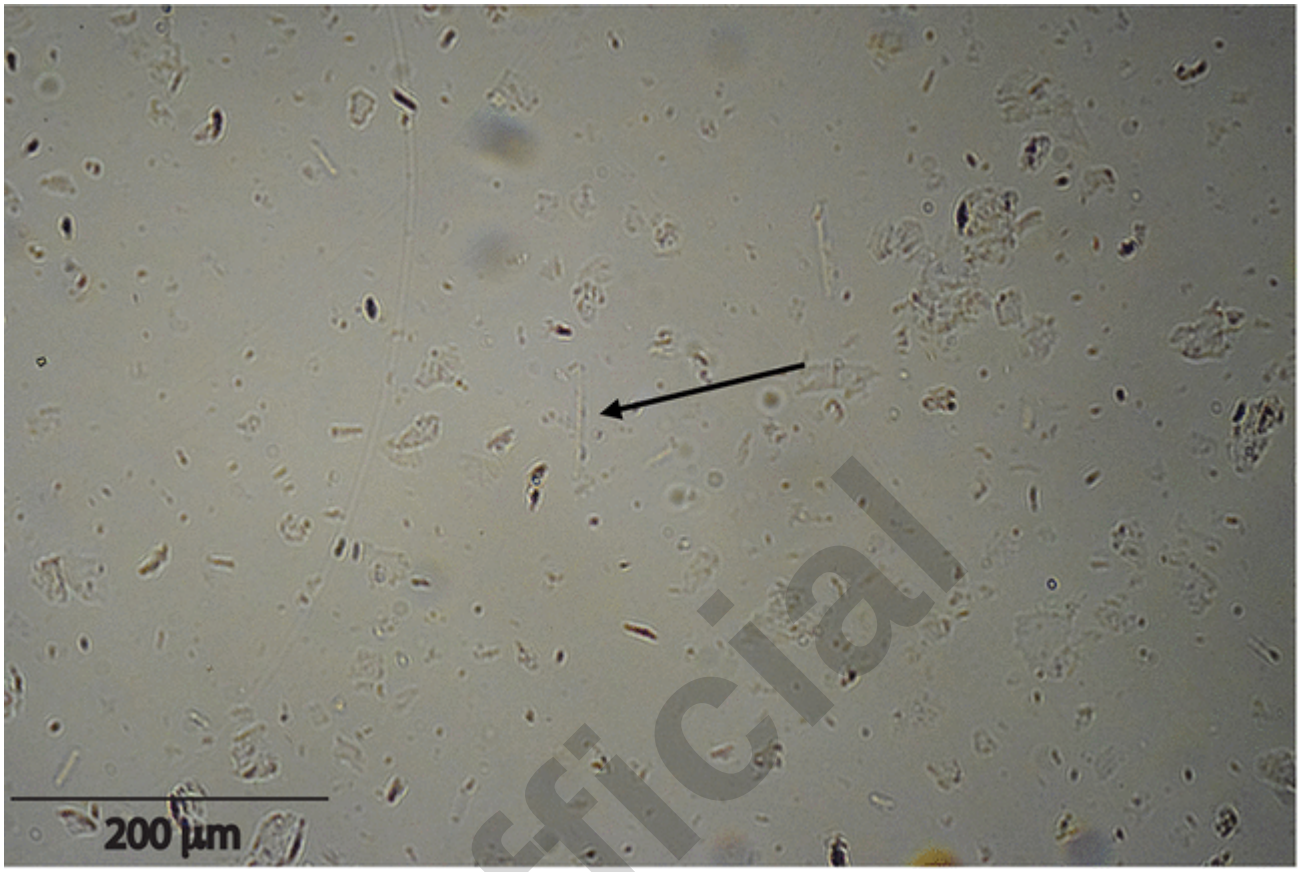


Figure K4. Milled tremolite (not tremolite asbestos) in talc matrix immersed in Cargille $n_D$ 1.605 HD liquid at 25° oriented so that α′ is parallel with the direction of the lower polarizer (E–W) in plane-polarized light slightly defocused to observe the Becke line (particle elongation is perpendicular to the lower polarizer).

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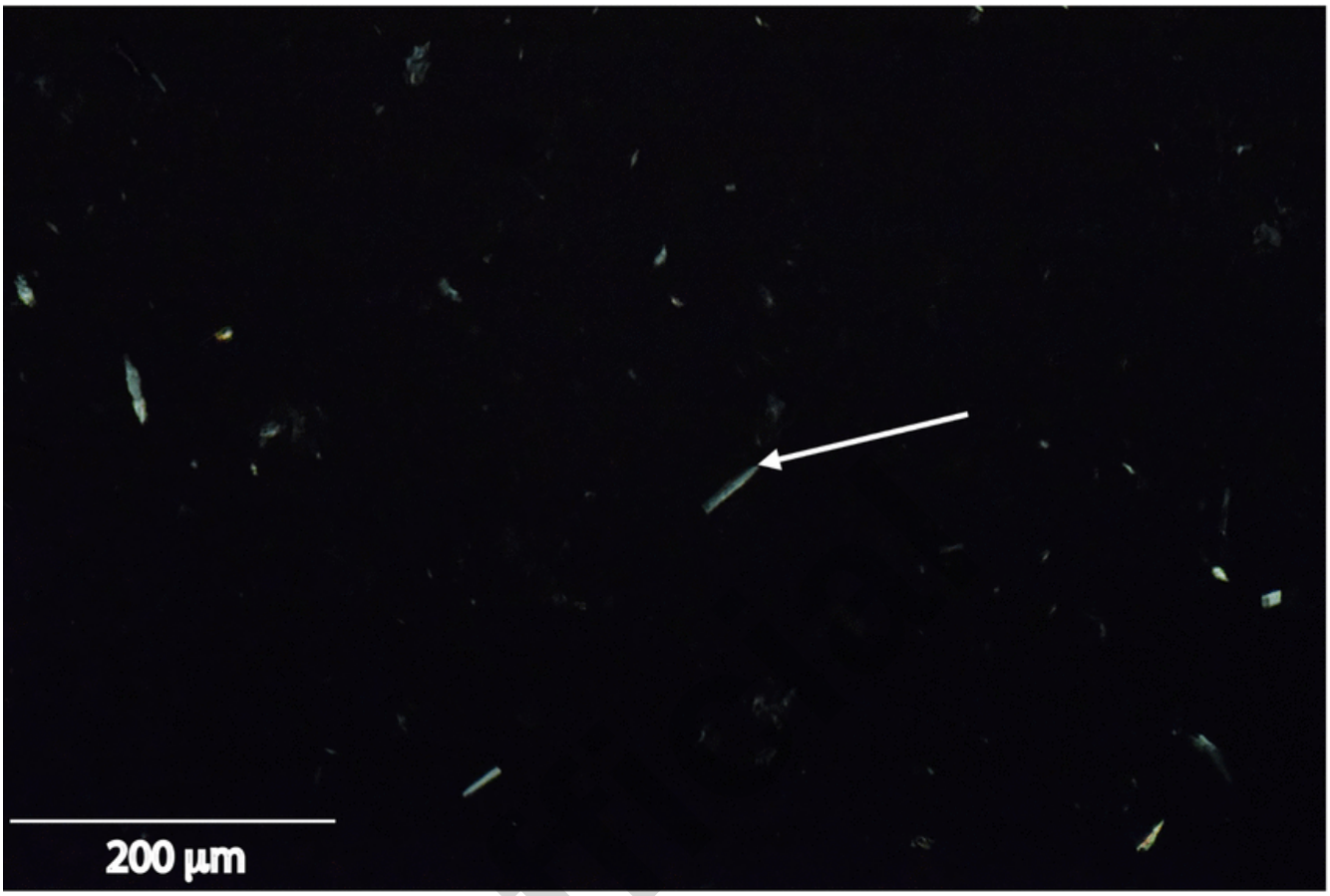


Figure K5. Milled tremolite (not tremolite asbestos) in talc matrix immersed in Cargille $n_D$ 1.605 HD liquid at 25° oriented so that $\gamma'$ (and particle elongation) is 45° counterclockwise from the lower polarizer (E–W) and parallel with N of the inserted full wave accessory plate in cross-polarized light.

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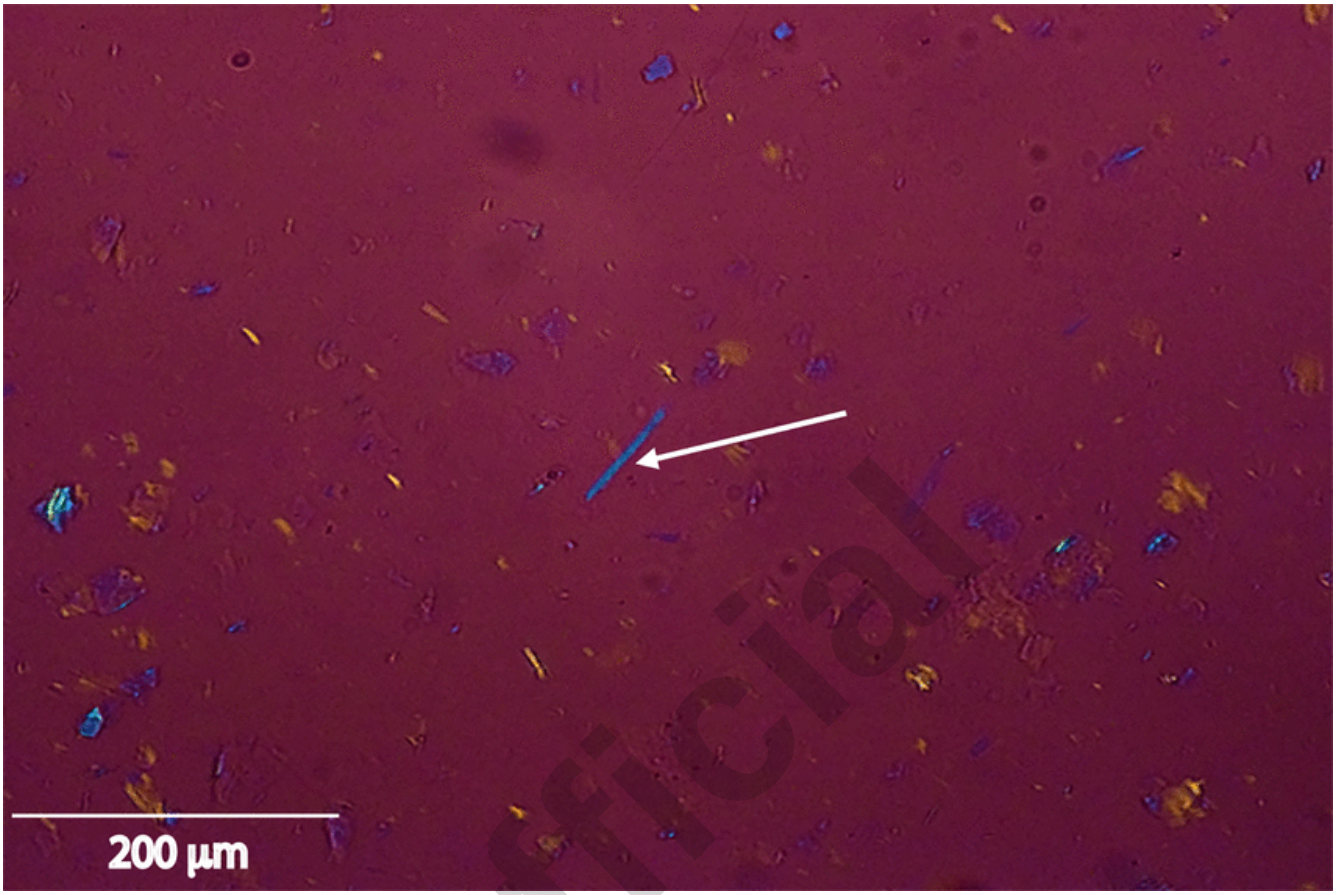


Figure K6. Milled tremolite (not tremolite asbestos) in talc matrix immersed in Cargille $n_D$ 1.605 HD liquid at 25° oriented so that γ′ (and particle elongation) is 45° from the direction of the lower polarizer (E–W) and parallel with N of the inserted full wave accessory plate in cross-polarized light.

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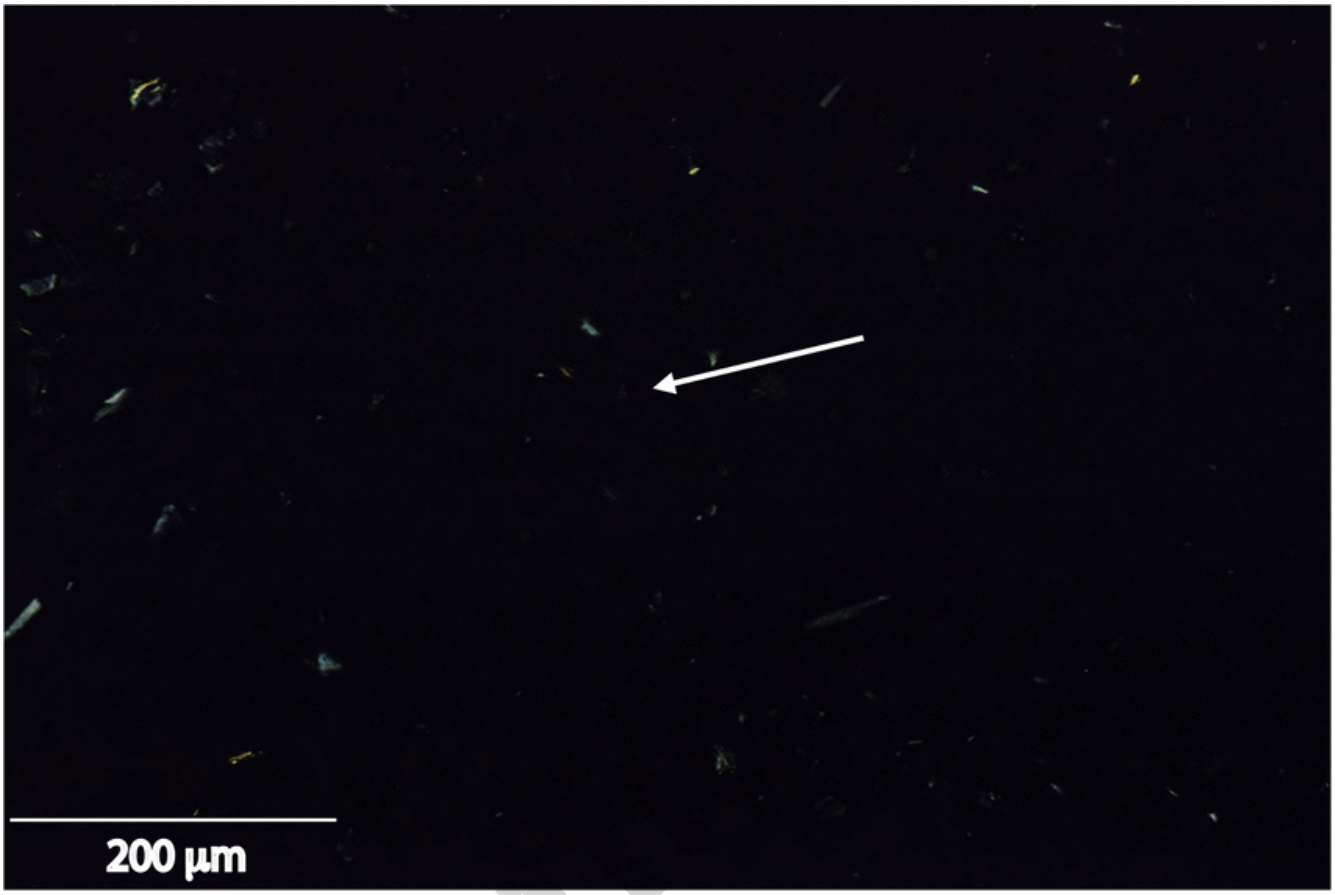


Figure K7. Milled tremolite (not tremolite asbestos) in talc matrix immersed in Cargille $n_D$ 1.605 HD liquid at 25° oriented so that γ′ (and particle elongation) is parallel with the direction of the lower polarizer (E–W) in cross-polarized light. The tremolite is at or near extinction.

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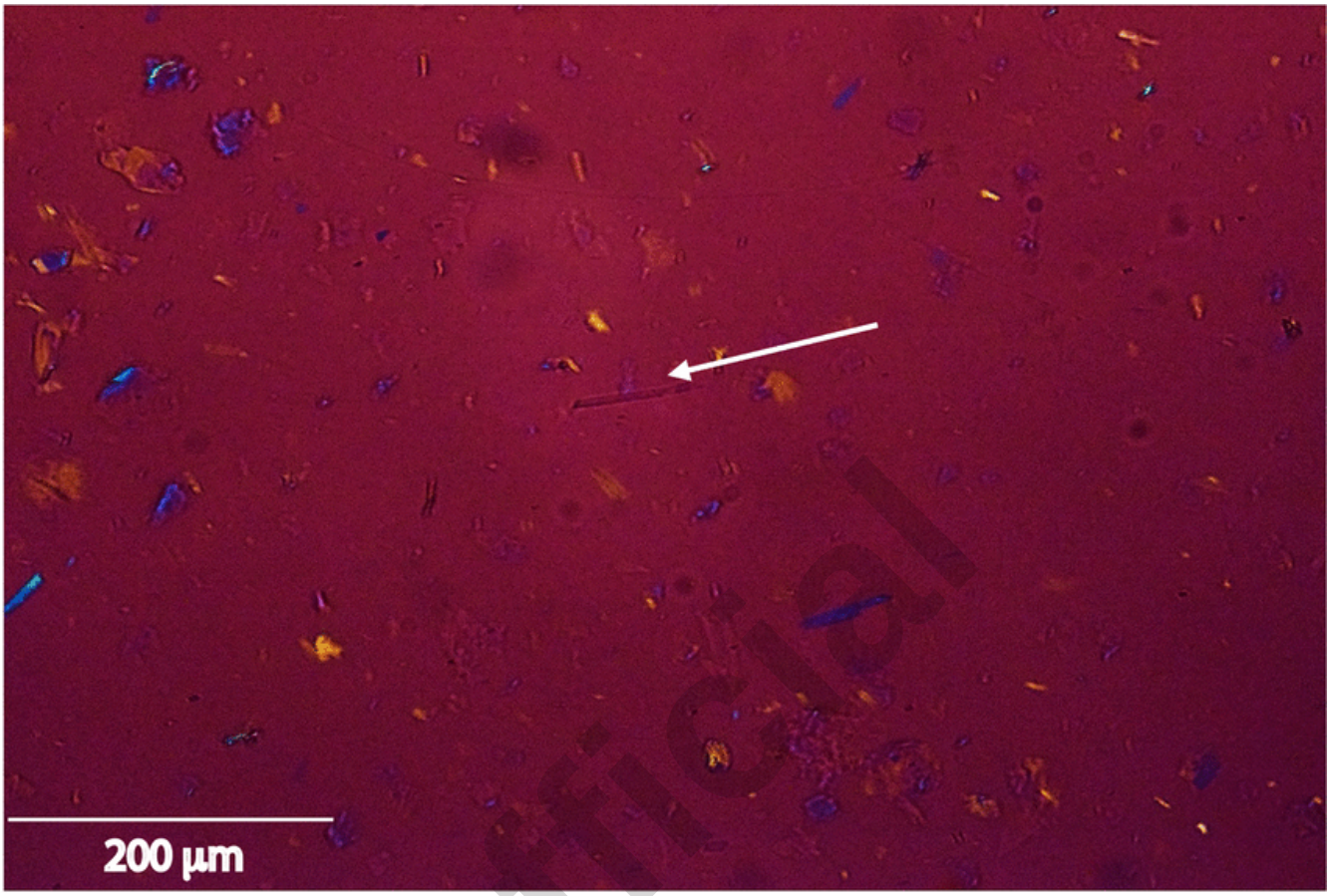


Figure K8. Milled tremolite (not tremolite asbestos) in talc matrix immersed in Cargille $n_D$ 1.605 HD liquid at 25° oriented so that γ′ (and particle elongation) is parallel with the direction of the lower polarizer (E–W) with the full wave accessory plate inserted in cross-polarized light. The tremolite is at or near extinction.

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**Milled tremolite (not tremolite asbestos) in talc matrix—1.615 RI liquid (not sieved)**

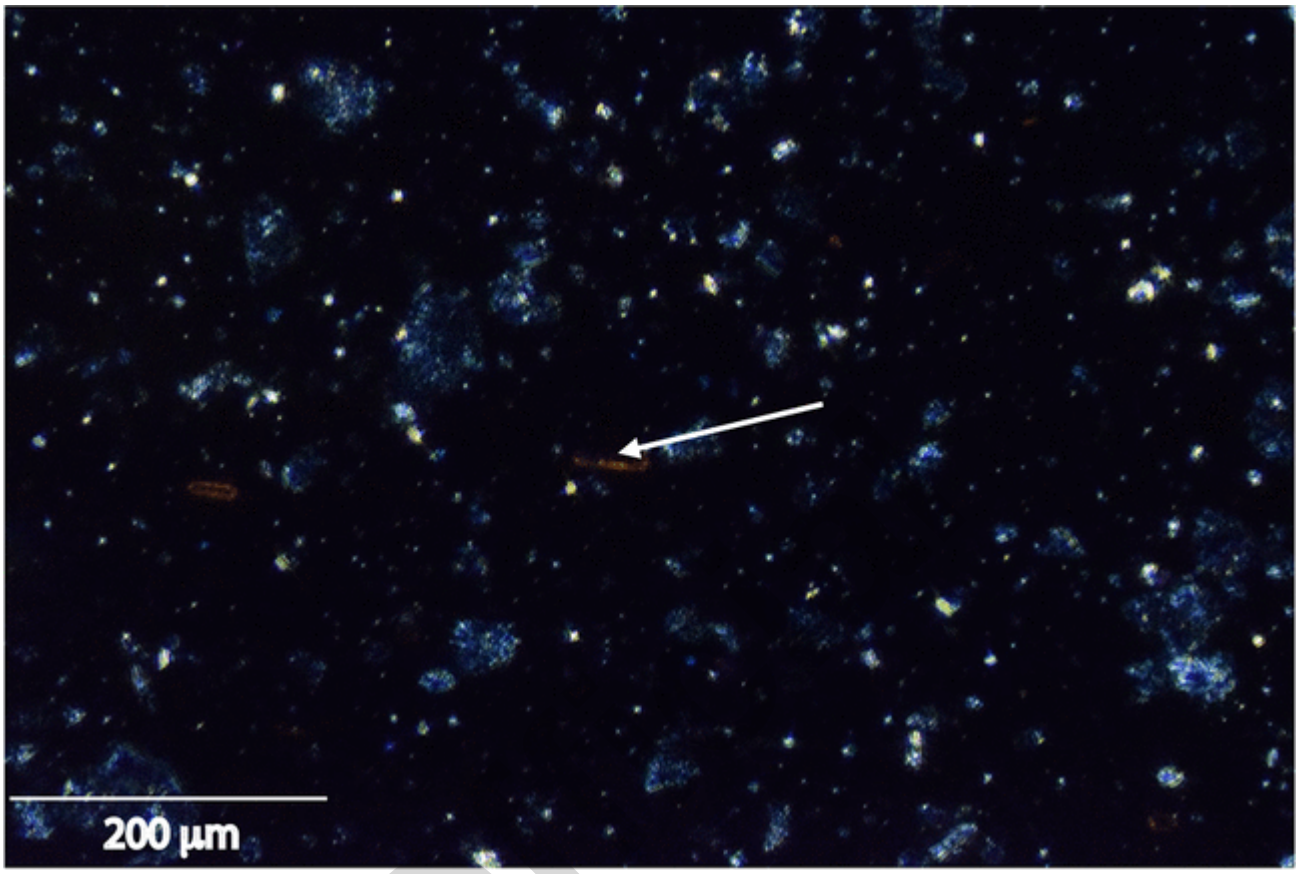


Figure L1. Milled tremolite (not tremolite asbestos) in talc matrix immersed in Cargille $n_D$ 1.615 HD liquid at 25° oriented so that γ′ (and particle elongation) is parallel with the direction of the lower polarizer (E–W) in central stop dispersion staining.

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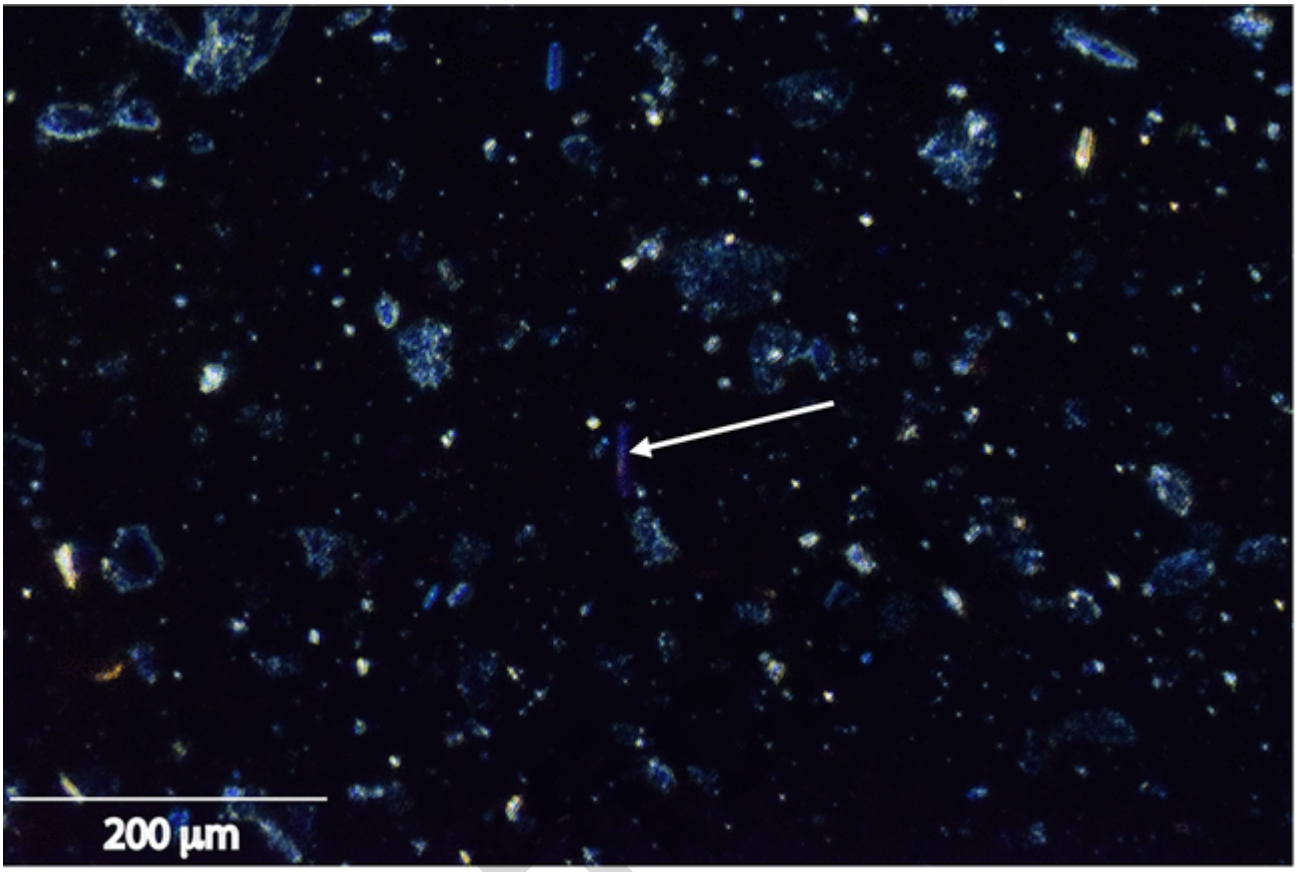


Figure L2. Milled tremolite (not tremolite asbestos) in talc matrix immersed in Cargille $n_D$ 1.615 HD liquid at 25° oriented so that α′ is parallel with the direction of the lower polarizer (E–W) in central stop dispersion staining (particle elongation is perpendicular to the lower polarizer).

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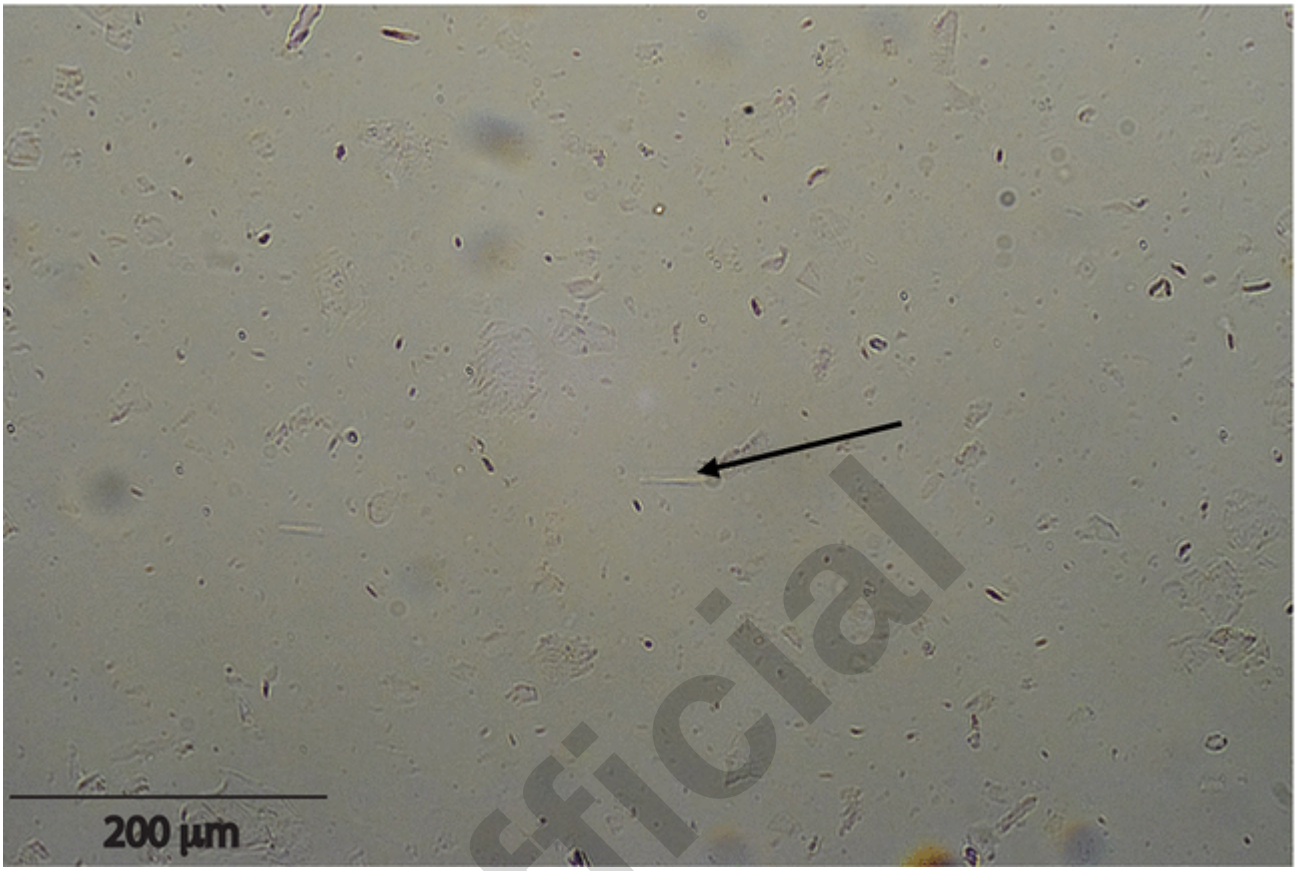


Figure L3. Milled tremolite (not tremolite asbestos) in talc matrix immersed in Cargille $n_D$ 1.615 HD liquid at 25° oriented so that γ′ (and particle elongation) is parallel with the direction of the lower polarizer (E–W) in plane-polarized light slightly defocused to observe the Becke line.

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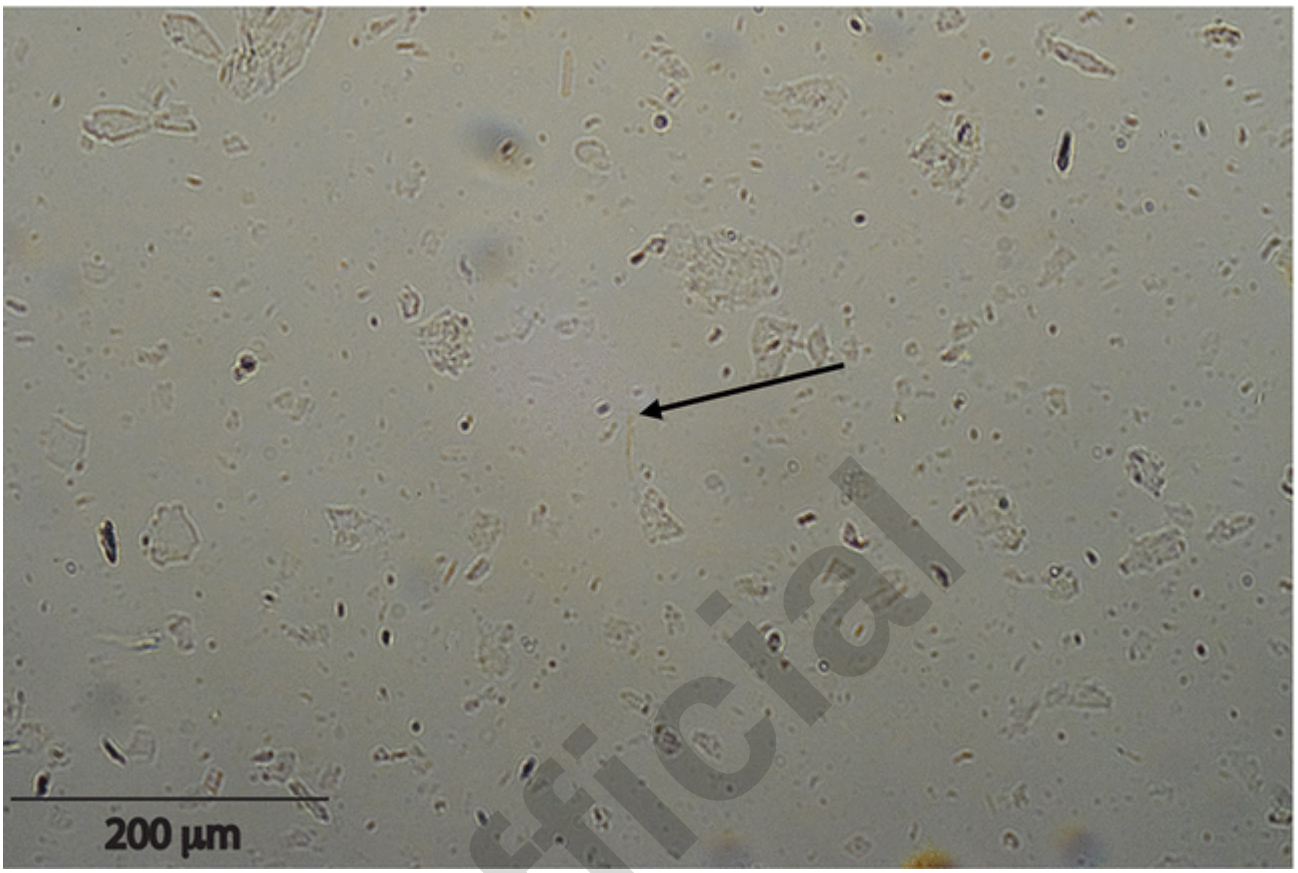


Figure L4. Milled tremolite (not tremolite asbestos) in talc matrix immersed in Cargille $n_D$ 1.615 HD liquid at 25° oriented so that α′ is parallel with the direction of the lower polarizer (E–W) in plane-polarized light slightly defocused to observe the Becke line (particle elongation is perpendicular to the lower polarizer).

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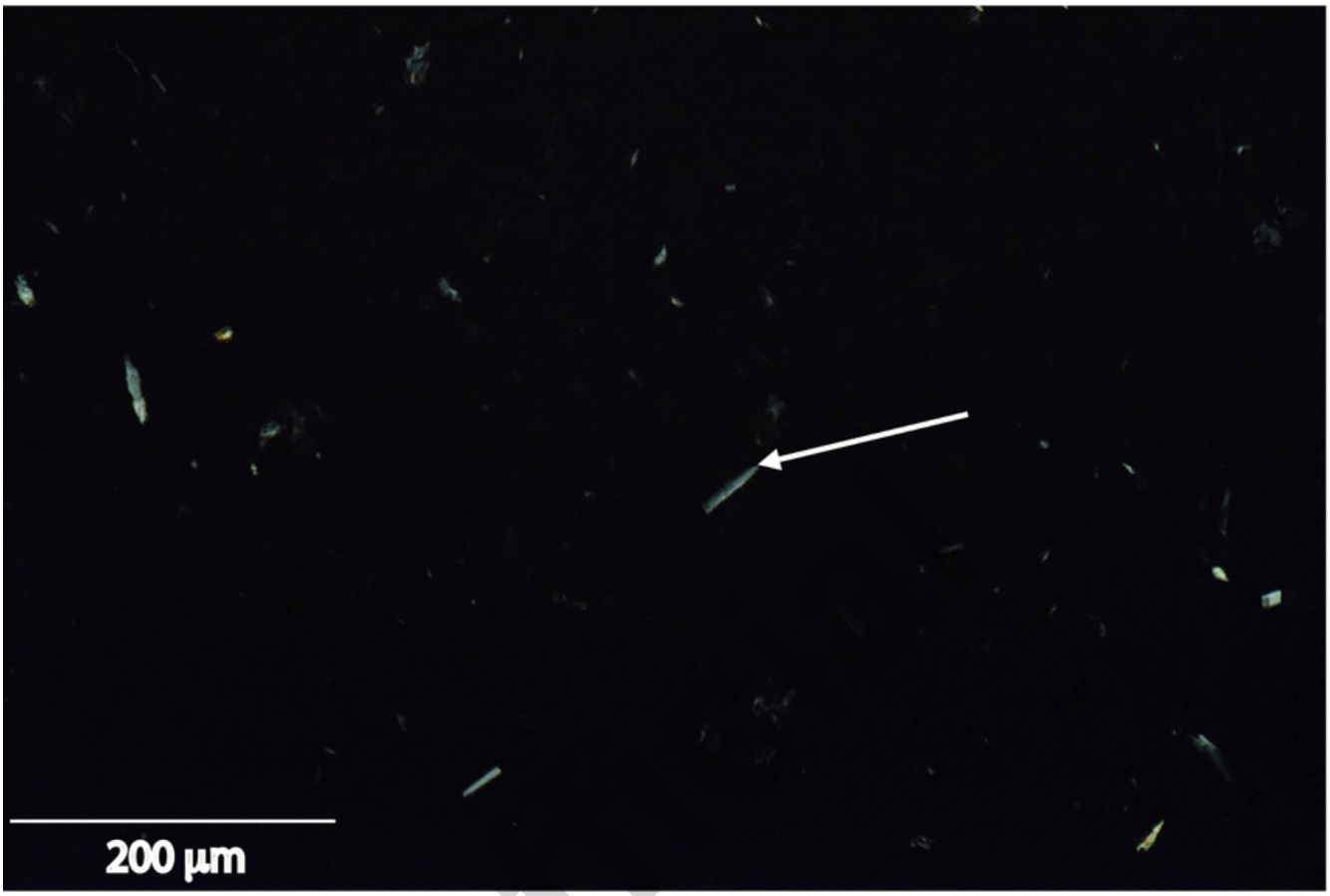


Figure L5. Milled tremolite (not tremolite asbestos) in talc matrix immersed in Cargille $n_D$ 1.615 HD liquid at 25° oriented so that γ′ (and particle elongation) is 45° counterclockwise from the lower polarizer (E–W) and parallel with N of the accessory plate in cross-polarized light.

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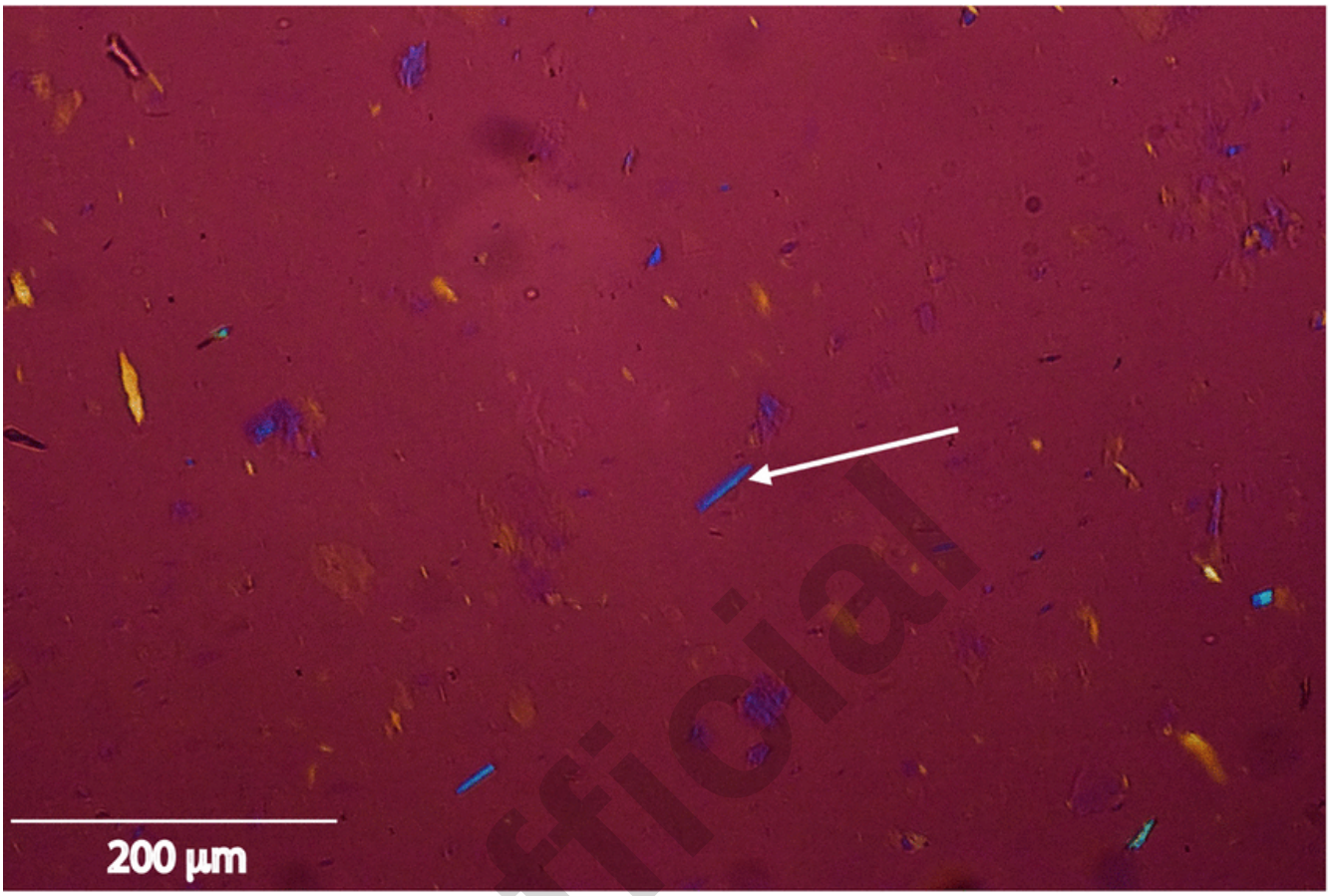


Figure L6. Milled tremolite (not tremolite asbestos) in talc matrix immersed in Cargille $n_D$ 1.615 HD liquid at 25° oriented so that γ′ (and particle elongation) is 45° from the direction of the lower polarizer (E–W) and parallel with N of the inserted full wave accessory plate in cross-polarized light.

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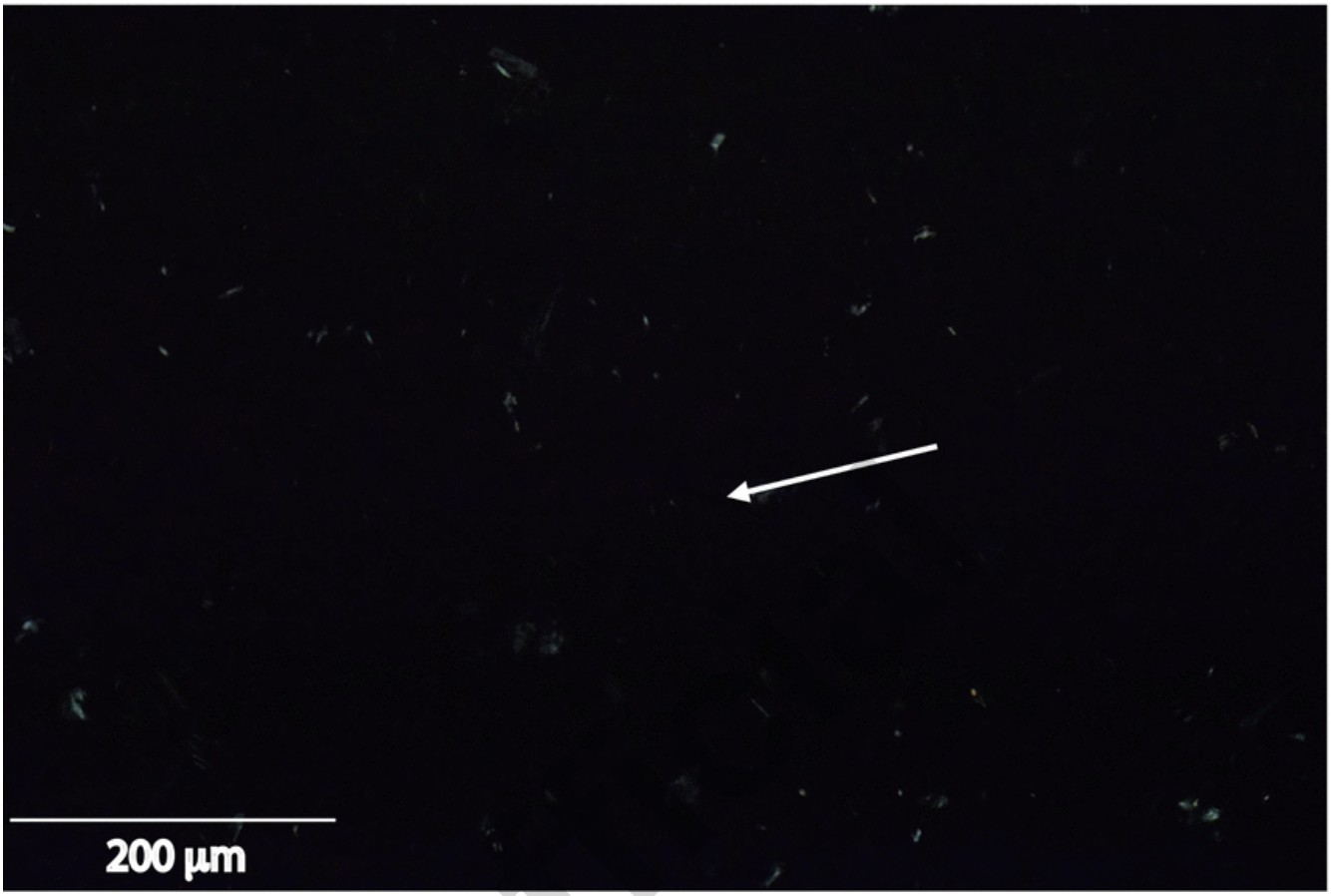


Figure L7. Milled tremolite (not tremolite asbestos) in talc matrix immersed in Cargille $n_D$ 1.615 HD liquid at 25° oriented so that γ′ (and particle elongation) is parallel with the direction of the lower polarizer (E–W) in cross-polarized light. The tremolite is at or near extinction.

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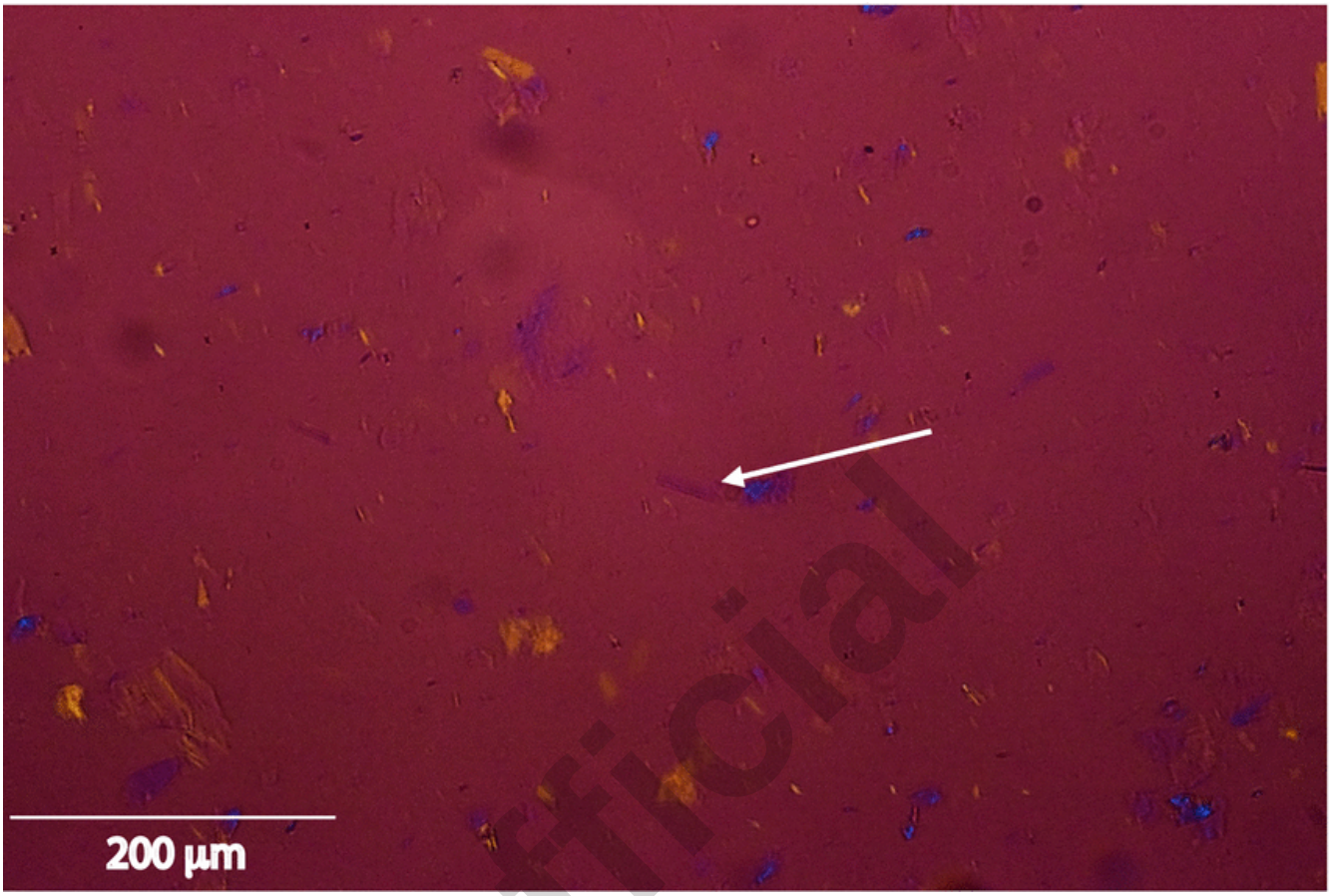


Figure L8. Milled tremolite (not tremolite asbestos) in talc matrix immersed in Cargille $n_D$ 1.615 HD liquid at 25° oriented so that γ′ (and particle elongation) is parallel with the direction of the lower polarizer (E–W) with the full wave accessory plate inserted in cross-polarized light. The tremolite is at or near extinction.

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**Milled tremolite (not tremolite asbestos) in talc matrix (0.1%)—1.620 RI liquid (material retained on 400-mesh sieve)**

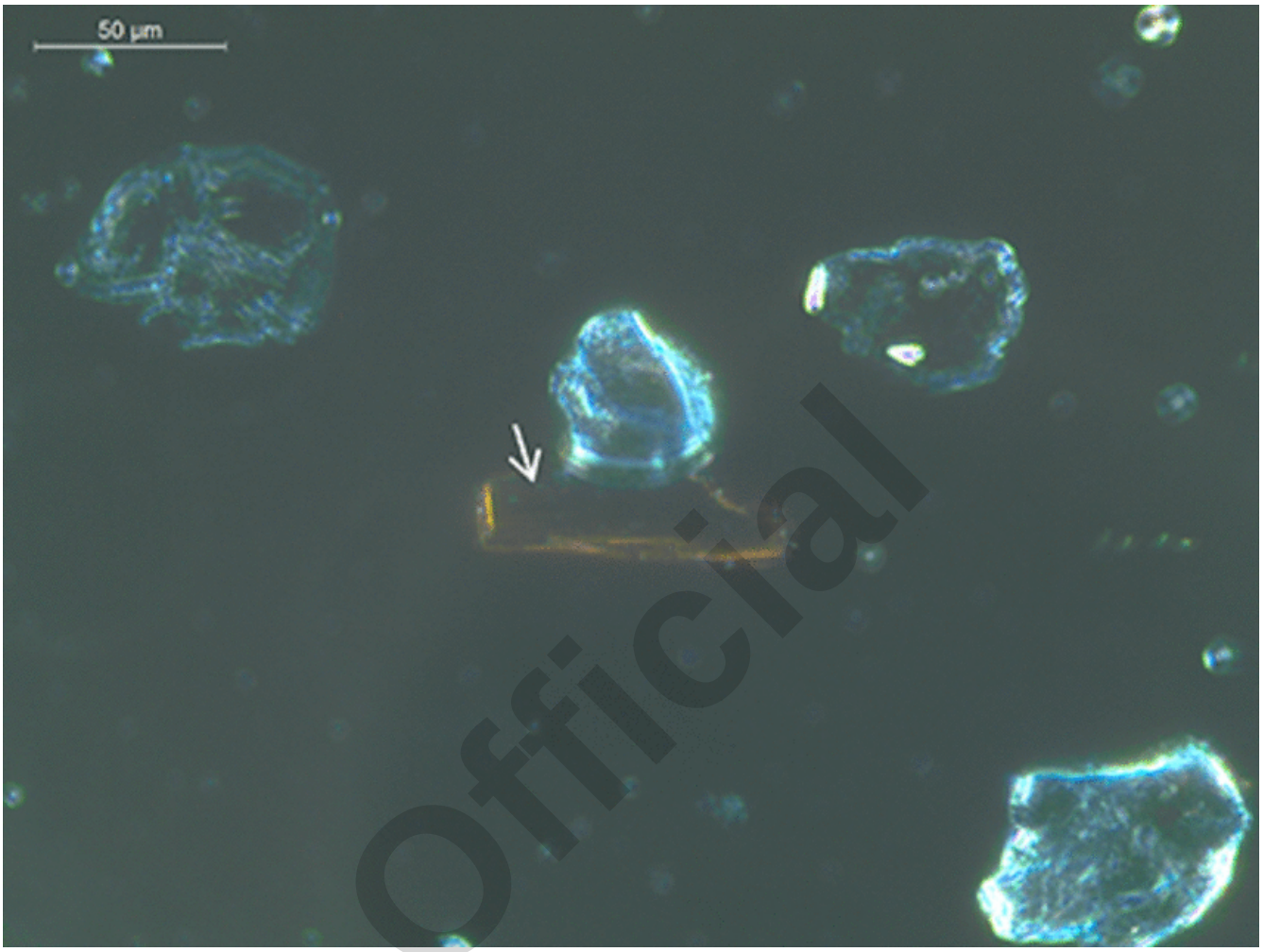


Figure M1. Milled tremolite (not tremolite asbestos) in talc matrix (0.1%). Material retained on a 400-mesh sieve was immersed in Cargille $n_D$ 1.620 HD liquid at 25° and oriented so that $\gamma'$ (and fiber length) is parallel with the direction of the lower polarizer (E–W) in central stop dispersion staining.

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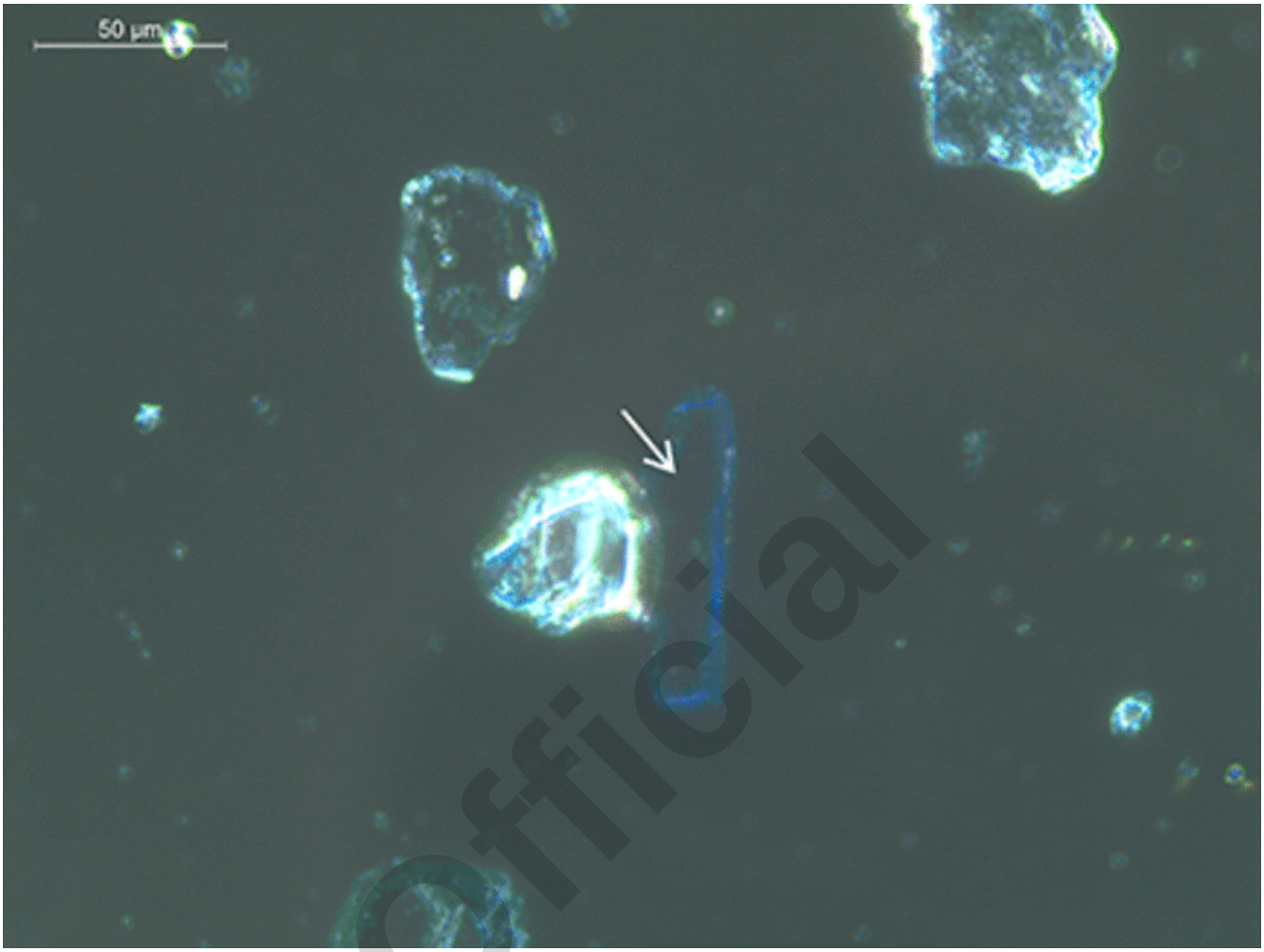


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Figure M2. Milled tremolite (not tremolite asbestos) in talc matrix (0.1%). Material retained on a 400-mesh sieve was immersed in Cargille $n_D$ 1.620 HD liquid at 25° and oriented so that α′ is parallel with the direction of the lower polarizer (E–W) in central stop dispersion staining (fiber length is perpendicular to the lower polarizer).

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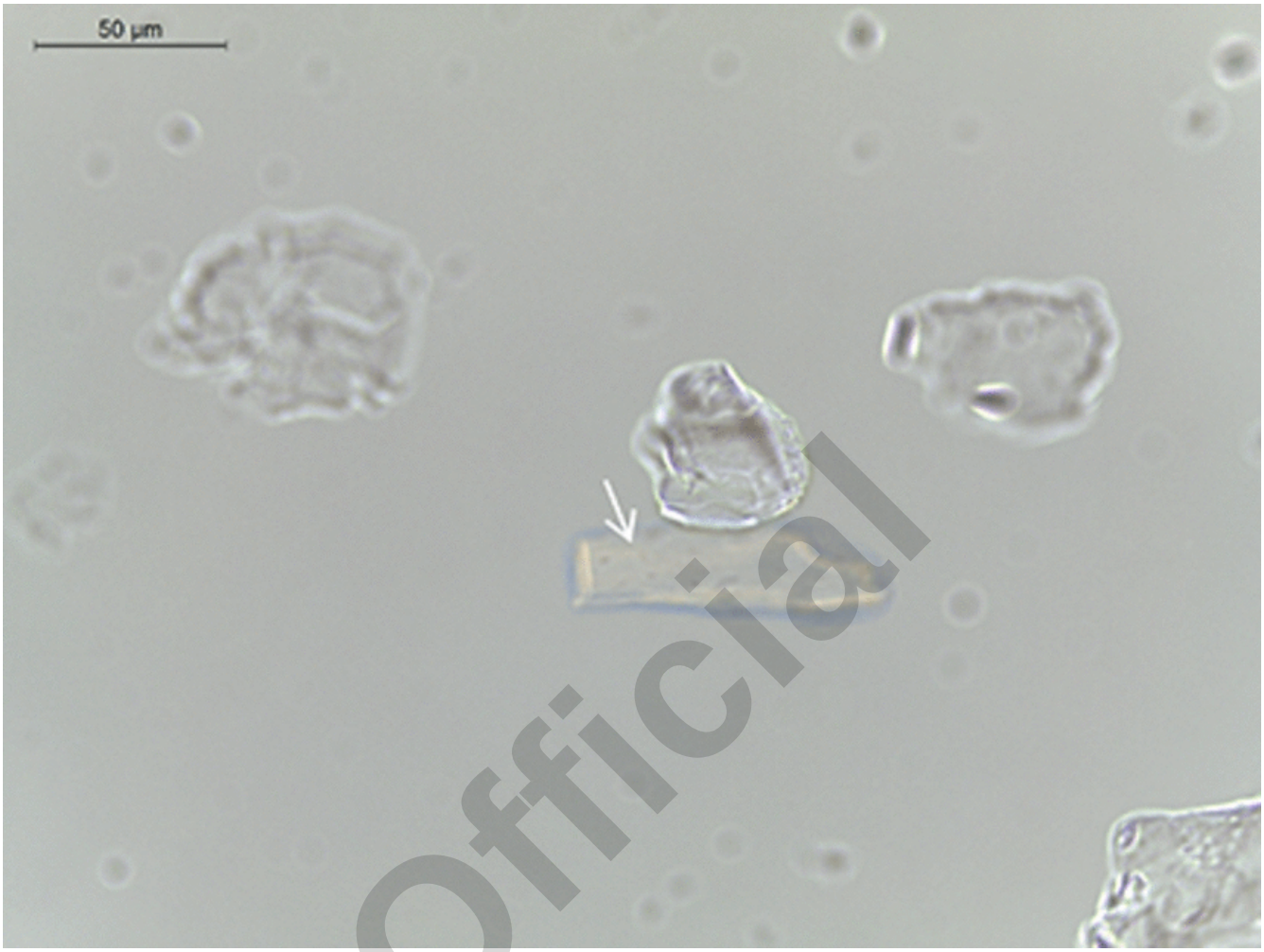


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Figure M3. Milled tremolite (not tremolite asbestos) in talc matrix (0.1%). Material retained on a 400-mesh sieve was immersed in Cargille $n_D$ 1.620 HD liquid at 25° and oriented so that $\gamma'$ (and fiber length) is parallel with the direction of the lower polarizer (E–W) in plane-polarized light slightly defocused to observe the Becke line.

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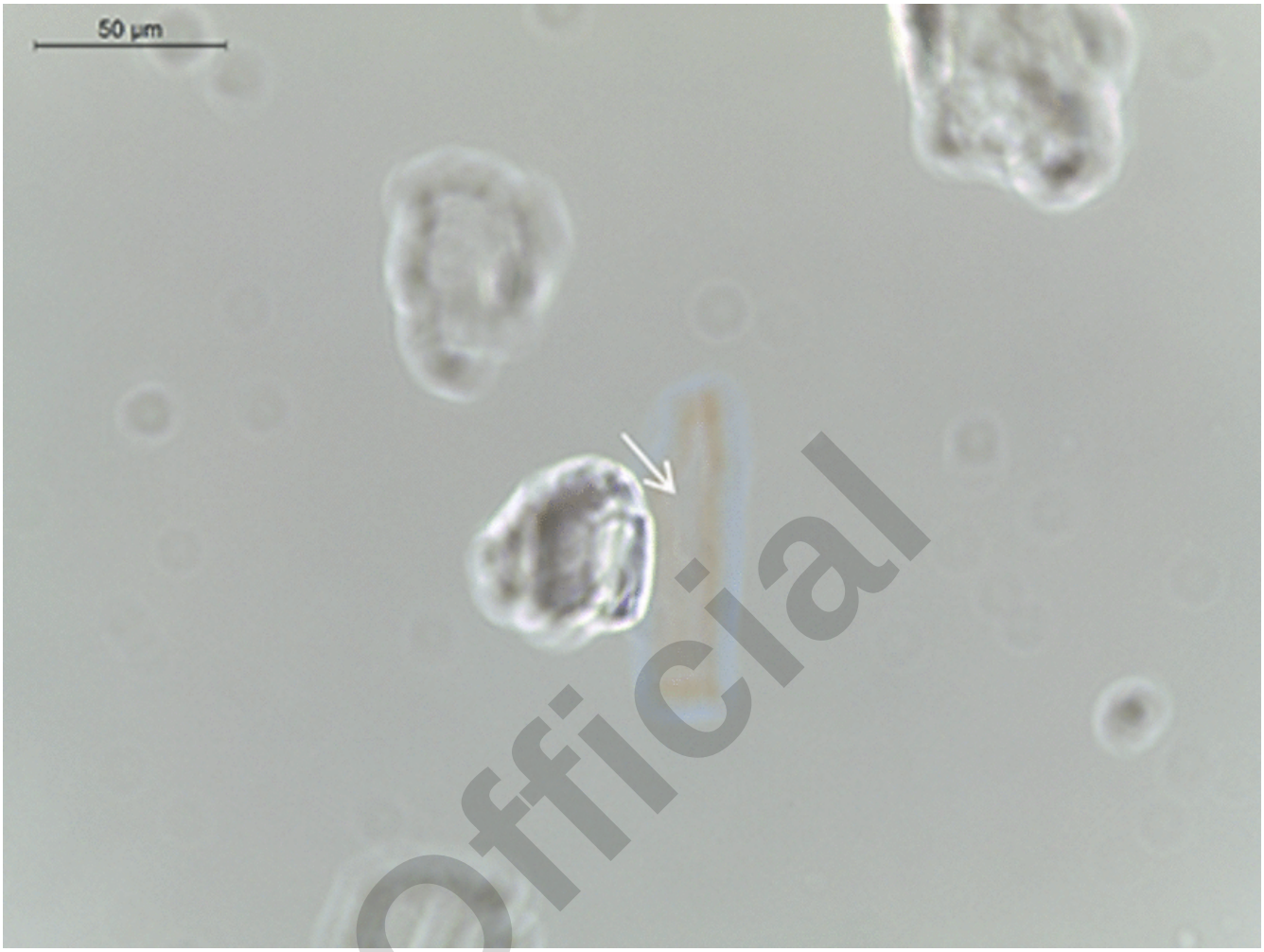


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Figure M4. Milled tremolite (not tremolite asbestos) in talc matrix (0.1%). Material retained on a 400-mesh sieve was immersed in Cargille $n_D$ 1.620 HD liquid at 25° and oriented so that α′ is parallel with the direction of the lower polarizer (E–W) in plane-polarized light slightly defocused to observe the Becke line (fiber length is perpendicular to the lower polarizer).

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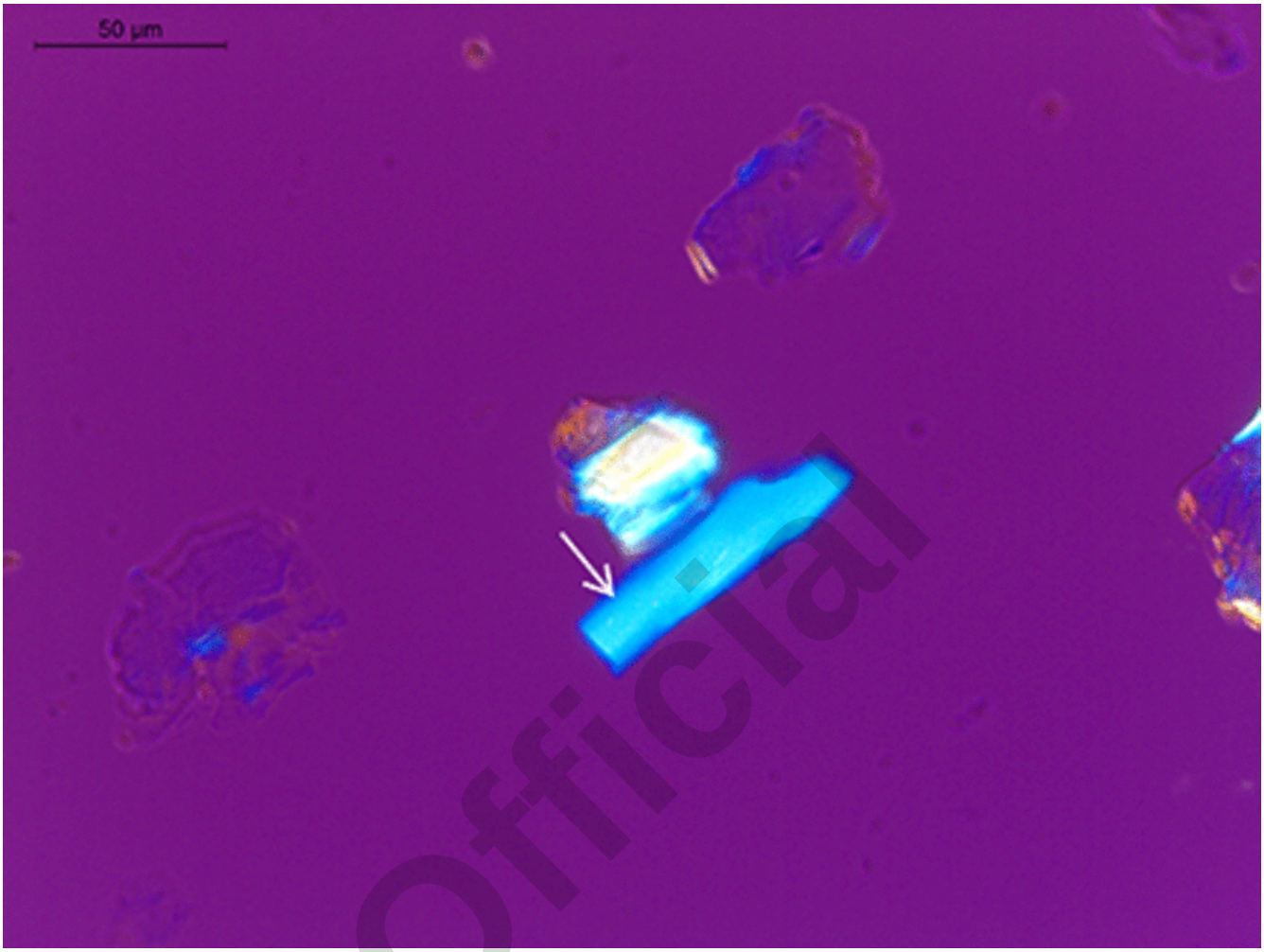


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Figure M5. Milled tremolite (not tremolite asbestos) in talc matrix (0.1%). Material retained on a 400-mesh sieve was immersed in Cargille $n_D$ 1.620 HD liquid at 25° and oriented so that γ′ (and fiber length) is 45° counterclockwise from the direction of the lower polarizer (E–W) and parallel with N of the inserted full wave accessory plate in cross-polarized light.

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MILLED TALC

**Milled talc in 1.550 RI liquid (not sieved)**

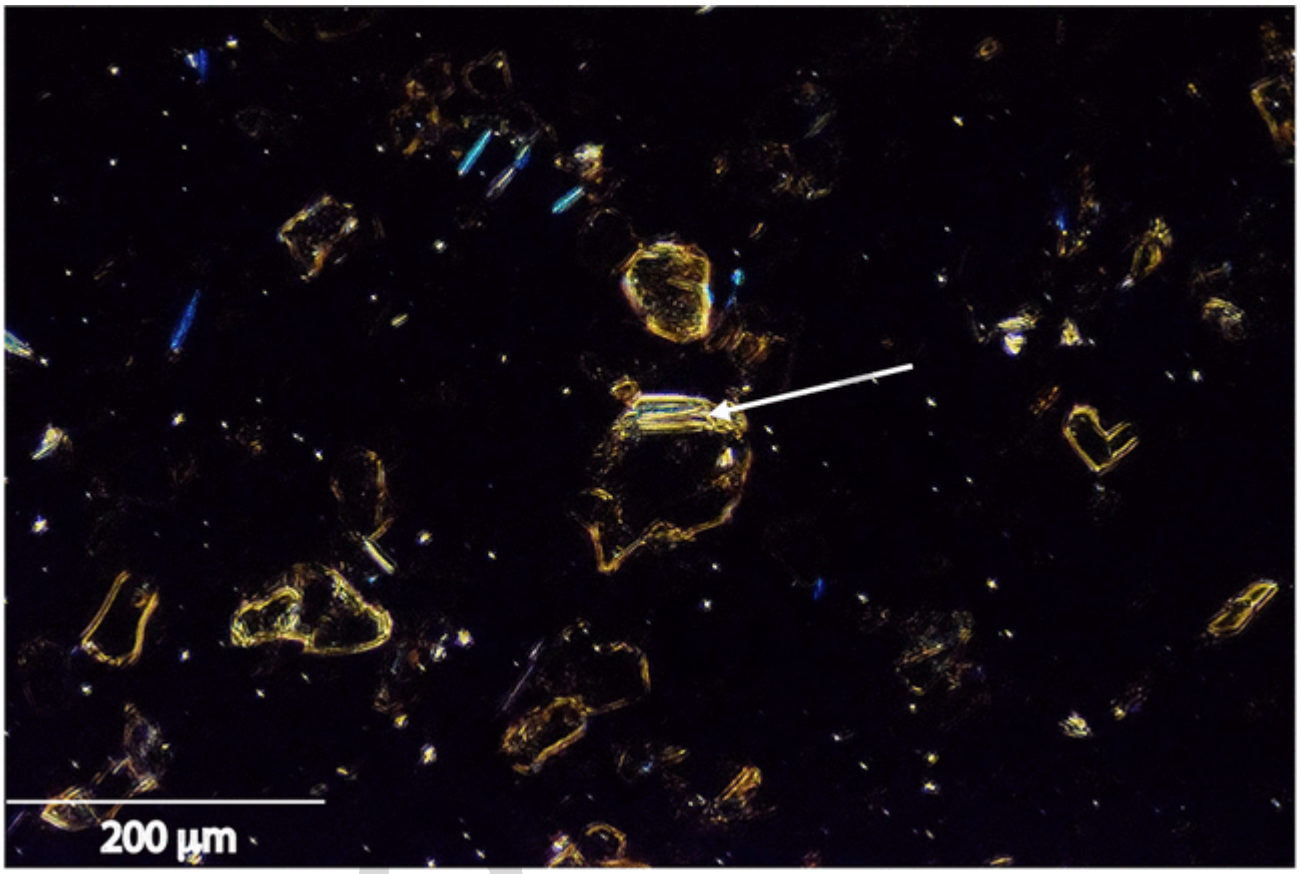


Figure N1. Milled talc immersed in Cargille $n_D$ 1.550 HD liquid at 25° oriented so that the upturned edge of the plate or $\gamma'$ is parallel with the direction of the lower polarizer (E–W) in central stop dispersion staining.

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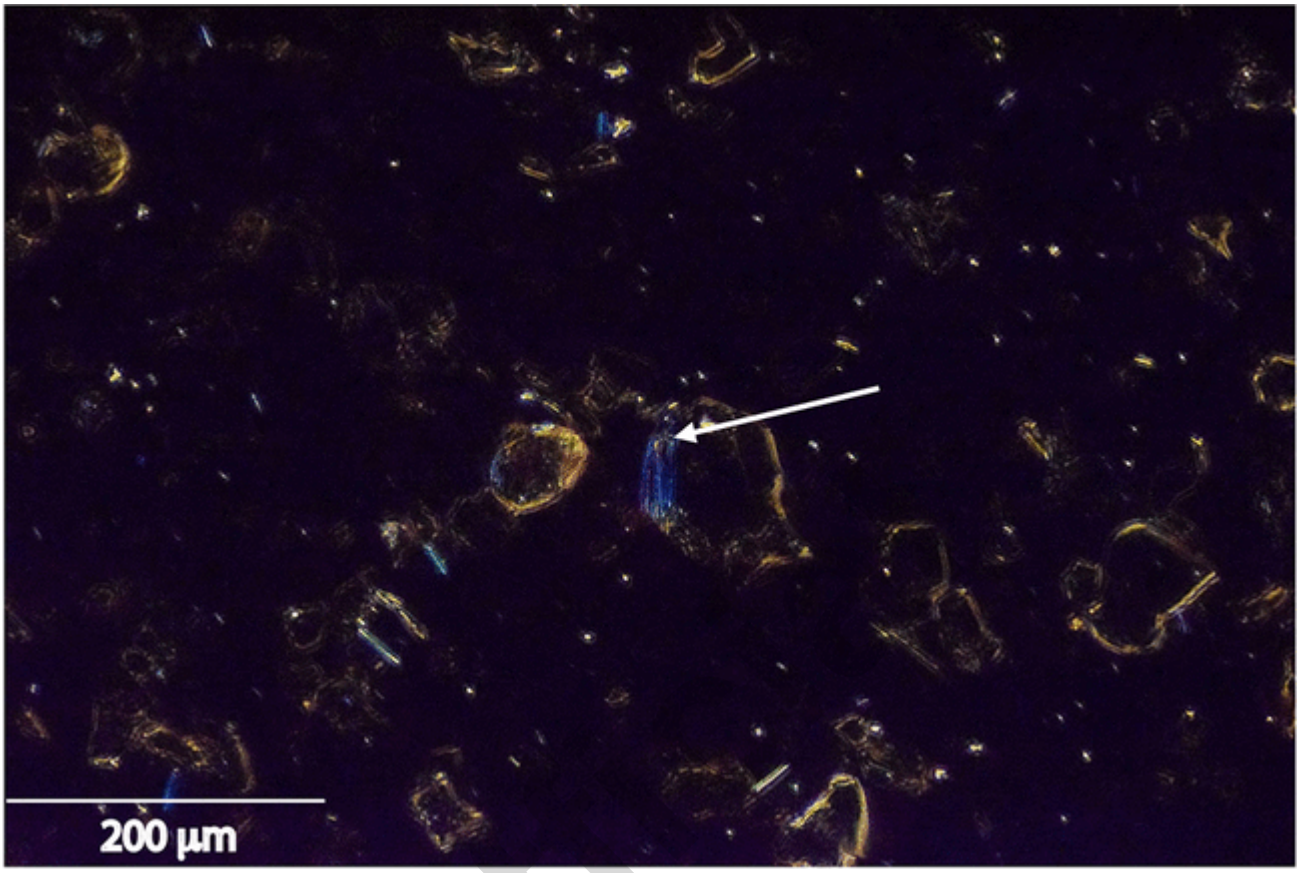


Figure N2. Milled talc immersed in Cargille $n_D$ 1.550 HD liquid at 25° oriented so that the upturned edge of the plate or α′ is parallel with the direction of the lower polarizer (E–W) in central stop dispersion staining.

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Figure N3. Milled talc matrix immersed in Cargille $n_D$ 1.550 HD liquid at 25° oriented so that the upturned edge of the plate or γ′ is parallel with the direction of the lower polarizer (E–W) in plane-polarized light slightly defocused to observe the Becke line.

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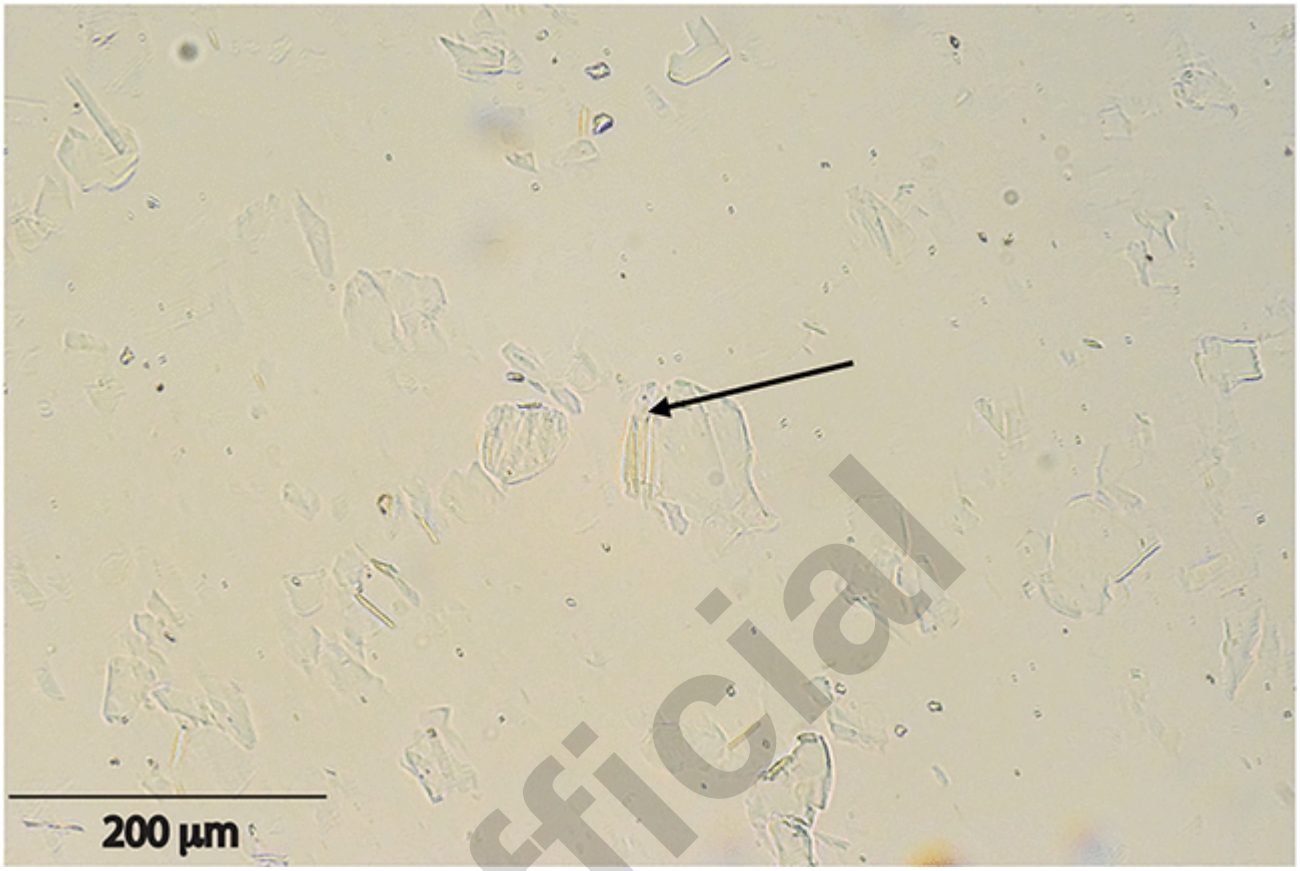


Figure N4. Milled talc immersed in Cargille $n_D$ 1.550 HD liquid at 25° oriented so that the upturned edge of the plate or α′ is parallel with the direction of the lower polarizer (E–W) in plane-polarized light slightly defocused to observe the Becke line.

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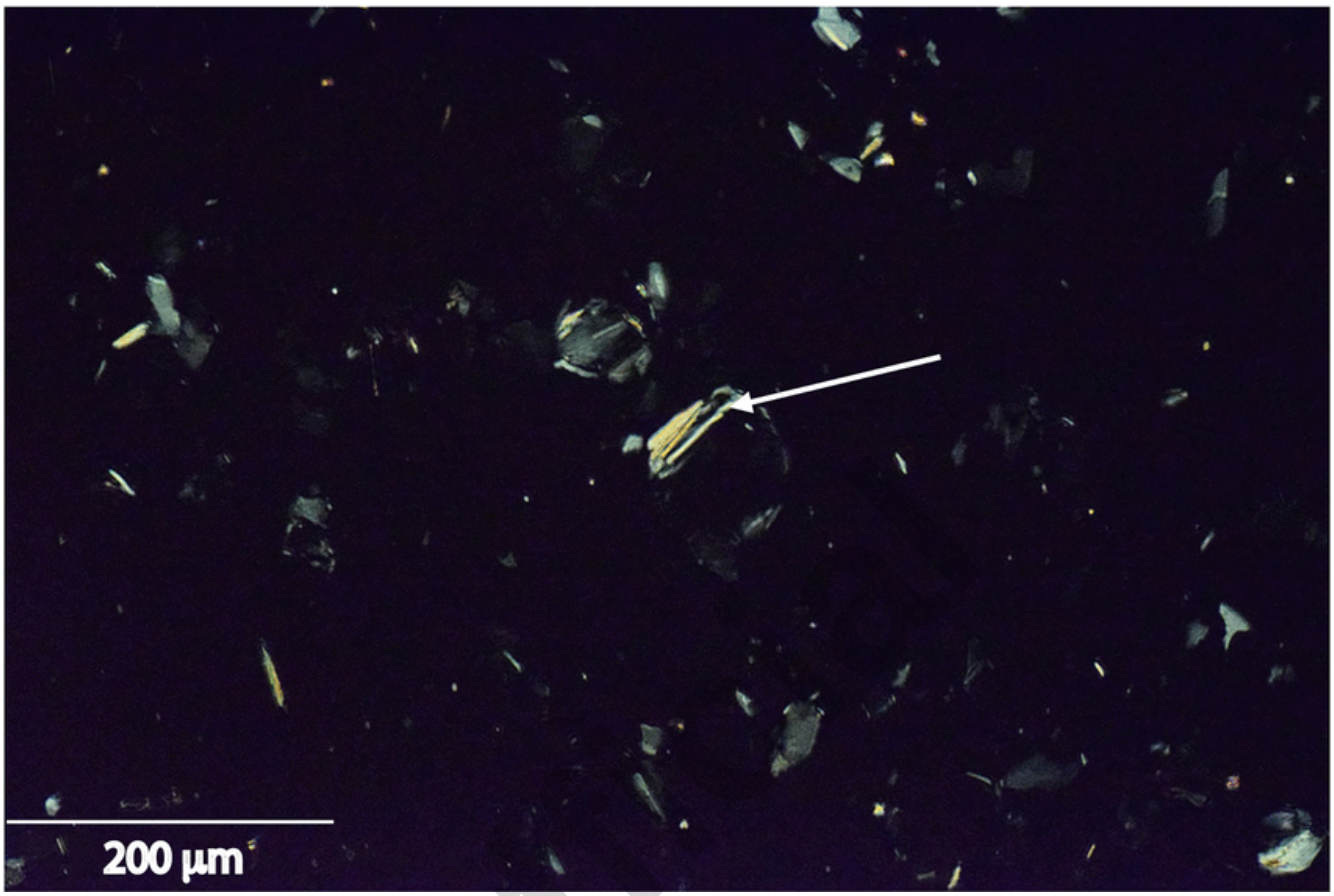


Figure N5. Milled talc immersed in Cargille $n_D$ 1.550 HD liquid at 25° oriented so that the upturned edge of the plate or $\gamma'$ is 45° counterclockwise from the lower polarizer (E–W) in cross-polarized light.

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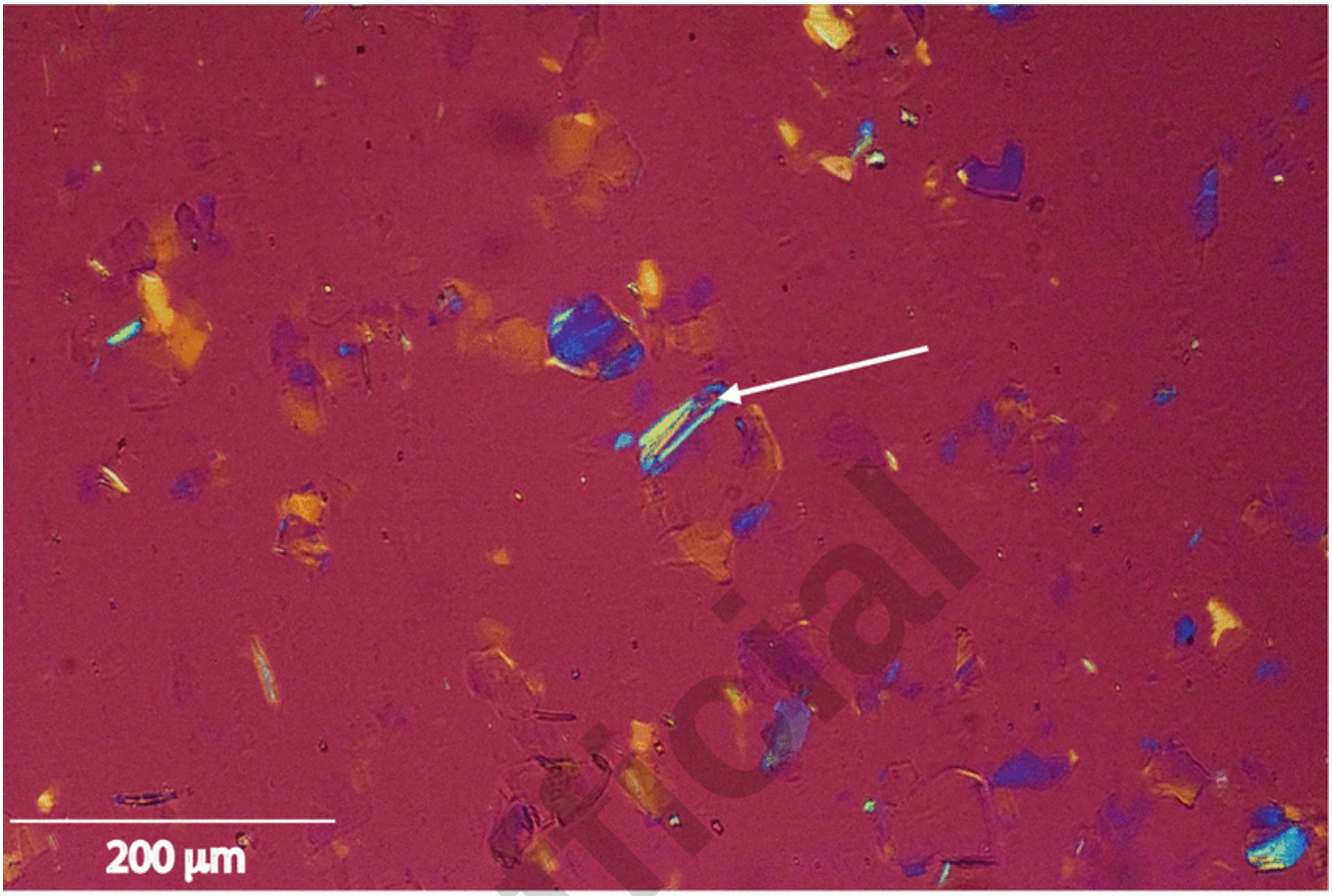


Figure N6. Milled talc immersed in Cargille $n_D$ 1.550 HD liquid at 25° oriented so that the upturned edge of the plate or $\gamma'$ is 45° from the direction of the lower polarizer (E–W) and parallel with N of the inserted full wave accessory plate in cross-polarized light.

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# ⟨1911⟩ RHEOMETRY

- **Newtonian Viscosity**

If an external stress is exerted on a fluid, the latter will flow to a degree that is determined by internal friction forces derived from internal molecular interactions and by the magnitude of the external stress applied. The measure of resistance to flow is defined as the viscosity of the fluid. The coefficient of viscosity, $\eta$, historically termed the absolute viscosity, is defined by Newton's law of viscous flow:

$$\sigma = \eta \cdot \dot{\gamma} \quad (1)$$

$\sigma$ = shear stress, defined as the applied stress that causes successive parallel layers of a material body to move in their own planes relative to each other

$\dot{\gamma}$ = shear rate, defined as the rate of change of shear strain with time

It should be noted that although *stress* and *strain* are often used interchangeably, the terms are not used interchangeably in rheology: *stress* is synonymous with force per unit area or a system of forces per unit area, and *strain* refers to deformation (i.e., a change in shape or size). A fluid is said to exhibit Newtonian flow if the viscosity is a constant that is independent of the shear rate or applied shear stress. Because viscosity depends on temperature, the temperature of the substance being measured should be controlled to within ±0.1°.

In general, Newton's law is obeyed by liquids of low molecular weight and by solutions of low molecular weight solutes in low molecular weight solvents. However, liquids of high molecular weight, solutions that contain high molecular weight solutes and colloidal dispersions (e.g., suspensions and emulsions) typically do not obey Newton's law of viscous flow and are referred to as non-Newtonian fluids.

In the International System of Units (SI), the unit of viscosity is the pascal-second (Pa · s), which is equivalent to 1 kg · $m^{-1}$ · $s^{-1}$ or 10 Poise in the centimeter-gram-second (cgs) system. The viscosities of most Newtonian fluids are measured in millipascal-seconds (1 Pa · s = 1000 mPa · s; 1 mPa · s = 1 centipoise). Kinematic viscosity, $\nu$, relates the viscous force to the inertial force by relating the Newtonian viscosity of a fluid to its density, $\rho$, at the same temperature:

$$\nu = \frac{\eta}{\rho} \quad (2)$$

The units of kinematic viscosity in SI units are $m^2 \cdot s^{-1}$ or, more commonly, $mm^2 \cdot s^{-1}$. [Note—1 $mm^2 \cdot s^{-1}$ = 1 centistoke (cS or cSt). Stokes or centistokes are the units in the cgs system for kinematic viscosity.]

**Measurement of Newtonian Viscosity Using a Capillary Viscometer**

The viscosity of a Newtonian fluid is measured by the use of glass capillary viscometers, examples of which are shown in *Figure 1* (simple U-tube capillary viscometer, also referred to as an Ostwald-type capillary viscometer) and *Figure 2* (constant or suspended-level viscometer, also referred to as an Ubbelohde-type capillary viscometer). [Note—When the term viscosity is used herein, it refers to absolute or Newtonian viscosity.] The constant- or suspended-level viscometer is a more robust design that is less influenced by temperature variations.

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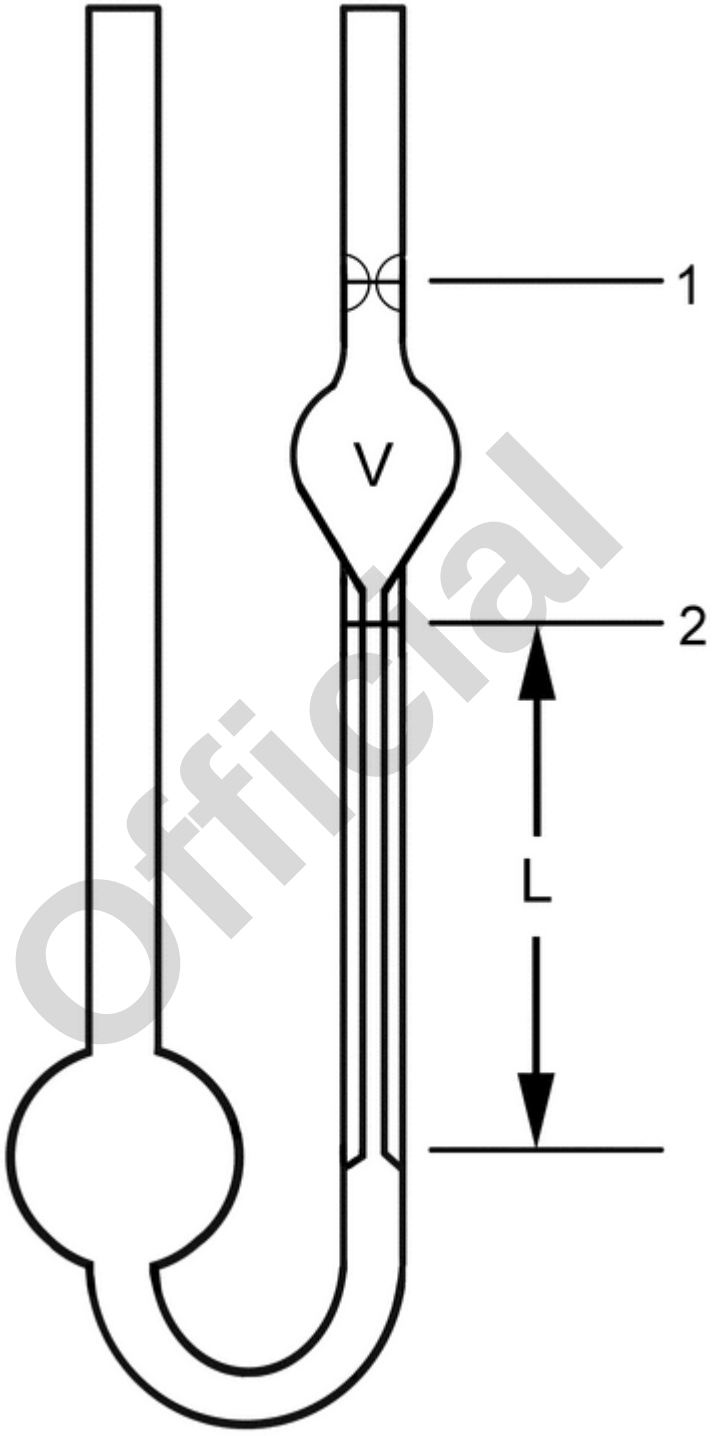


Figure 1. Simple U-tube (or Ostwald-type) capillary viscometer. [NOTE—Variables are defined in the text.]

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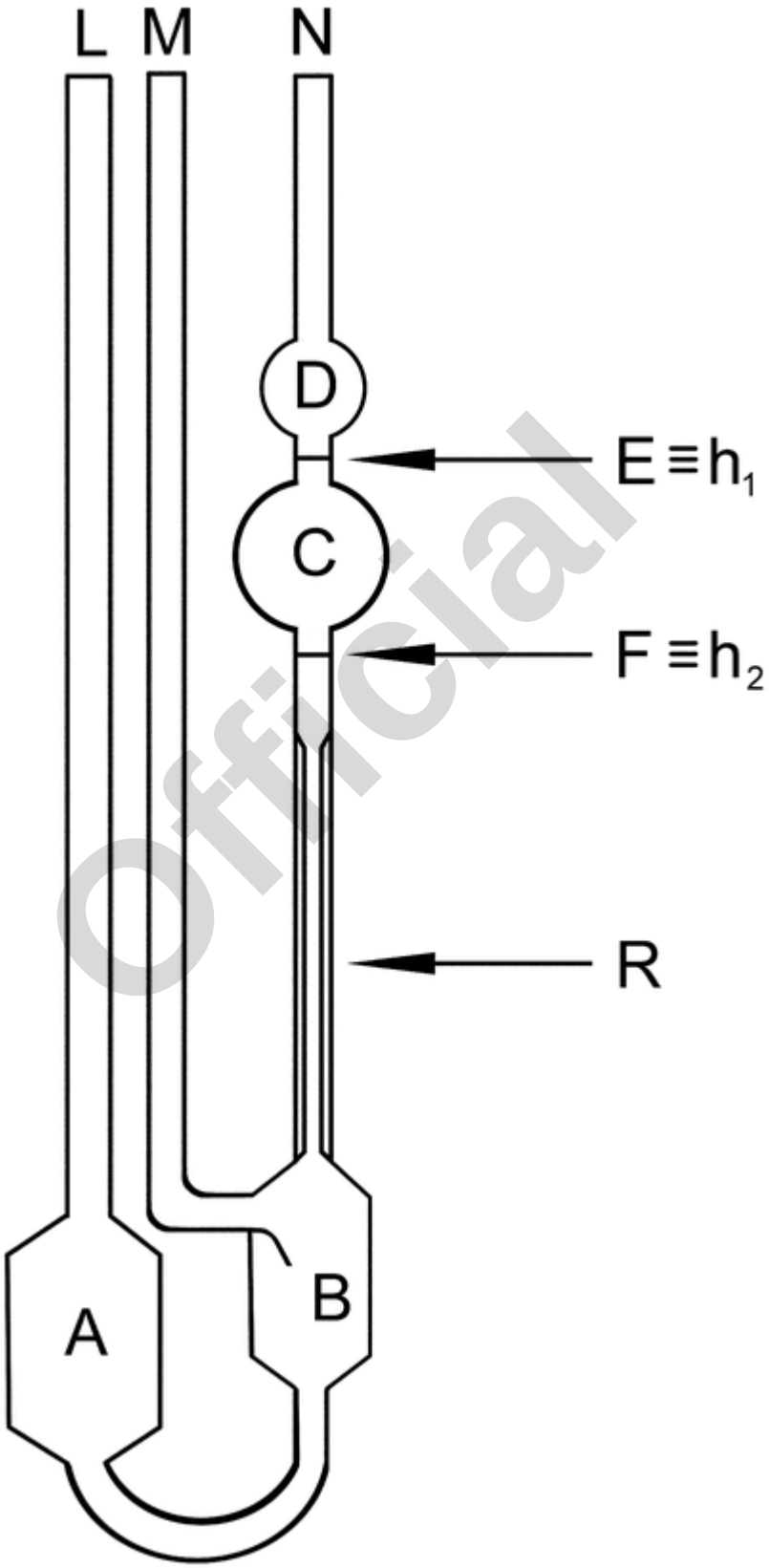


Figure 2. Suspended-level (or Ubbelohde-type) capillary viscometer. [NOTE—Variables are defined in Viscosity—Capillary Methods ⟨911⟩.]

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The following example is based on *Figure 1*, and the same principle is applicable to *Figure 2*. With the apparatus in *Figure 1*, the flow time, *t*, is measured for a given volume of fluid, *V*, as defined between lines 1 and 2 of *Figure 1*, to flow through a capillary tube having a length equal to *L* and a radius equal to *r*. As long as the density of the fluid, $\rho$, is known, the viscosity can be calculated using the Poiseuille equation:

$$\eta = \frac{\pi r^4 t \Delta P}{8LV} \quad (3)$$

$P$ = pressure

The applied pressure differential, $\Delta P$, is defined as:

$$\Delta P = (h_1 - h_2) \times g \times \rho \quad (4)$$

$h_1$ = height of the fluid in the tube at line 1 measured from the bottom position
$h_2$ = height of the fluid in the tube at line 2 measured from the bottom position
$g$ = gravitational acceleration (standard value = 9.80665 $m \cdot s^{-2}$)

Glass capillary viscometers generally are categorized as low shear stress instruments because $\Delta P$ is relatively small. The typical ranges for shear stress, $\sigma$, and shear rate, $\dot{\gamma}$ (at the wall), are 0.1–15 Pa and 100–20,000 $s^{-1}$, respectively.

The absolute dimensions of a capillary tube usually are too difficult to measure conveniently. As a result, an empirical calibration is employed for each viscometer tube. Collecting all the constants in *Equations 3* and *4* into a single viscometer constant, *k*, yields the approximate equation for kinematic viscosity:

$$\nu = k \times t \quad (5)$$

To determine *k*, analysts measure the flow time for a liquid of known viscosity at a given temperature. This result is used to calculate the viscometer constant for that particular temperature.

In work requiring a high degree of accuracy, analysts can use the following equation:

$$\nu = k \times t - (E/t^2) \quad (6)$$

$E$ = kinetic energy factor, in $mm^2 \cdot s$

The viscometer constant, *k*, and kinetic factor, *E*, are determined from flow times measured for a set of stable, clean, Newtonian fluids of known kinematic viscosity (viscosity standard reference materials).

Once the viscometer constant(s) have been determined for a given temperature by the use of appropriate viscosity standards, the values can be used until the viscometer is subjected to thermal or mechanical stress that alters the dimensions of the capillary.

Generally, calibration, operation, and cleaning of viscometers should be performed according to instrument manufacturer's recommendations. Capillary viscometers must be scrupulously clean and should display no residual adsorbed or particulate organic or inorganic contamination. Surface contaminants may alter the dimensions of the capillary and the interfacial tension.

The procedures detailed in *Viscosity—Capillary Methods* ⟨911⟩ are intended for use with fluids that exhibit Newtonian behavior when subjected to shear stress. These procedures are not suitable for the characterization of non-Newtonian fluids because they may give erroneous and variable results if used with such fluids.

Capillary viscometers described in ⟨911⟩ typically measure rheological properties at only one shear rate during one pass of the sample through the capillary. Because of the nonlinearity of the shear rate–shear stress relationship for non-Newtonian fluids, this measurement is inadequate unless provisions are made for flow measurements at multiple rates of shear. Even more problematic for non-Newtonian fluids are the decrease in shear rate from the capillary wall to the center of the capillary and the variation in shear stress with time as the liquid level in the capillary viscometer decreases. Thus, capillary viscometers are inherently unsuitable for use in characterizing non-Newtonian fluids.

Because of the small $\Delta P$ attained with glass capillary viscometers, their use in measurements of high-viscosity fluids is impractical. Capillary-extrusion viscometers that use an external pressurized gas reservoir or other source of constant pressure are the only practical capillary instrumental alternative in these instances. In addition, capillary-extrusion viscometers may allow the operator to vary the shear rate or stress, thus enabling the characterization of non-Newtonian fluids. Chapter ⟨911⟩ does not address such devices.

**Measurement of Newtonian Viscosity Using a Rotational Viscometer**

The principle of the method is to measure the force (torque) acting on a rotor when it rotates at a constant angular velocity (rotational speed) in a liquid. Rotational viscometers are used for measuring the viscosity of Newtonian fluids or the apparent viscosity of non-Newtonian fluids. Detailed methods and procedures for rotational instruments are provided in *Viscosity—Rotational Methods* ⟨912⟩. Measurement of Newtonian viscosity can be performed on rotational viscometers/rheometers following the methods and procedures proposed in *Viscosity—Rotational Methods* ⟨912⟩. The calculated viscosity of Newtonian fluids should be the same (within experimental error), regardless of the rate of shear (or rotational speed).

- **NON-NEWTONIAN RHEOLOGY**

Newton's law of viscous deformation or flow, described in the section on Newtonian viscosity, describes the relationship between an applied stress, $\sigma$, and the resultant flow with a rate of shear, or velocity gradient, of $\dot{\gamma}$. Disperse systems (i.e., suspensions or emulsions) or fluids that contain macromolecular components usually do not obey Newton's law. These exceptions to Newtonian behavior are the subject of this section. The categorization of such exceptions into different flow

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behaviors such as shear-thinning (pseudoplastic), shear-thickening (dilatant), Bingham, etc., is not rigid. Non-Newtonian fluids may exhibit different rheological behavior depending on shear rate, shear stress, and temperature. Time-dependent rheological behavior, such as rheopexy or thixotropy, is not considered in this chapter.

The rheological behavior of a non-Newtonian fluid is evident when shear stress is plotted as a function of shear rate. The shear stress–shear rate relationship for Newtonian compositions is linear and passes through the origin, whereas the shear stress–shear rate relationship for non-Newtonian fluids is nonlinear and may not pass through the origin, or it is linear but does not pass through the origin (Bingham body). Rheograms, or flow curves, of some typical non-Newtonian fluids are contrasted with those of Newtonian fluids in *Figure 3*.

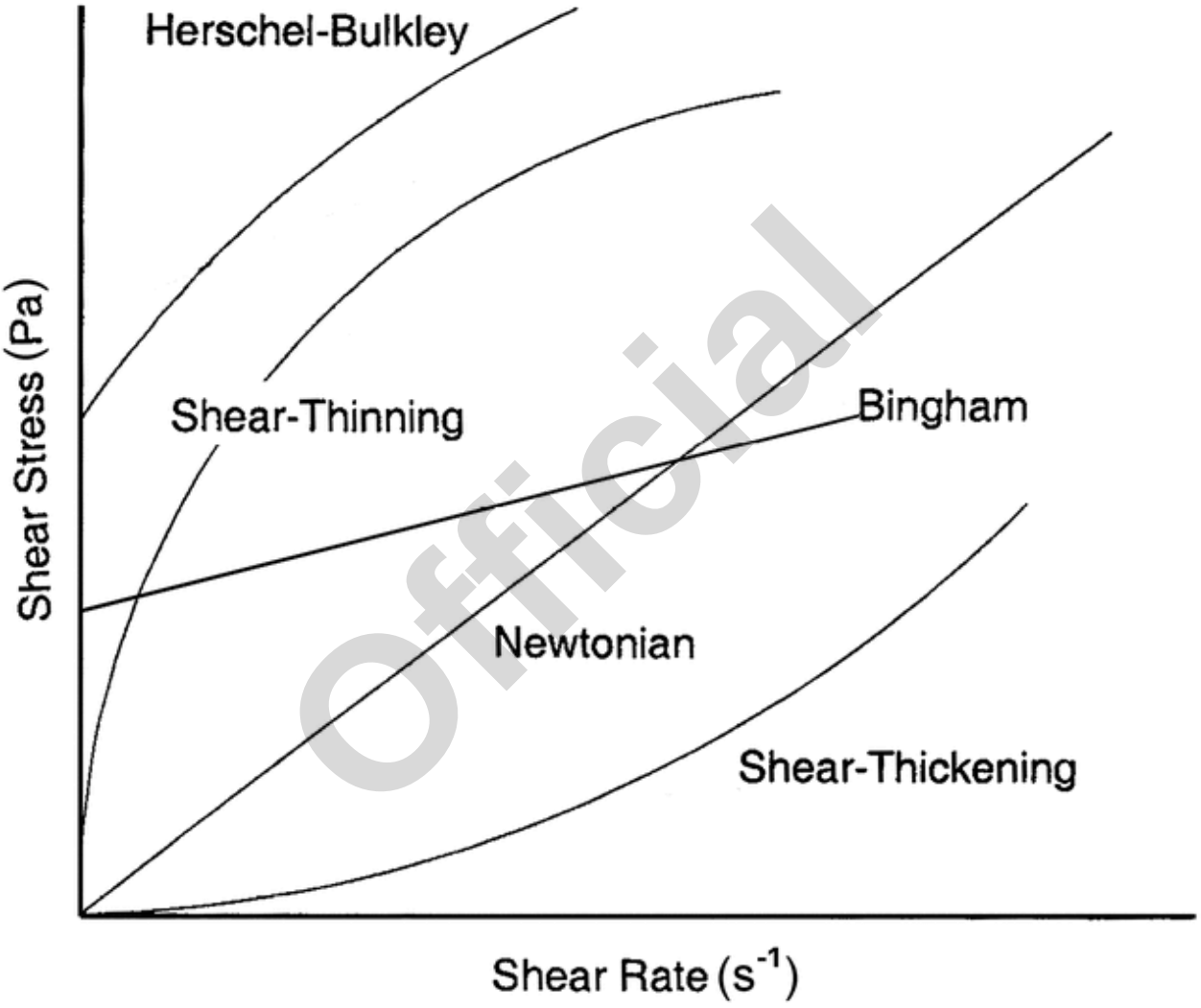


Figure 3. Rheograms of Newtonian and typical time-independent non-Newtonian fluids.

All the rheograms in *Figure 3* can be described by the Herschel–Bulkley equation:

$$\sigma = K \cdot \dot{\gamma}^{n} + \sigma_0 \quad (7)$$

$\sigma$ = shear stress, in Pa
$K$ = consistency coefficient
$\dot{\gamma}$ = shear rate, in $s^{-1}$
$n$ = the flow behavior index
$\sigma_0$ = yield stress, in Pa

For Newtonian fluids and Bingham plastics, $K$ is designated as the viscosity ($\eta$) and plastic viscosity ($\eta_{pl}$), respectively. Because the viscosity of Newtonian fluids and the plastic viscosity of Bingham plastics are independent of the shear stress or shear rate, the flow behavior index, $n$, is equal to 1 for Newtonian fluids and Bingham plastics. For shear-thinning fluids, $0 < n < 1$, and for shear-thickening fluids, $1 < n < \infty$. Shear thinning can be observed in certain complex solutions such as aqueous

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hydrocolloid solutions. Dilatant behavior is generally exhibited by concentrated suspensions of nonaggregating monodisperse solid particles such as mixtures of corn starch and water. At very high viscosities or very low shear stresses, Bingham plastics exhibit a yield stress, $\sigma_0$, a shear stress below which no apparent deformation occurs (*Figure 3*). Other non-Newtonian fluids do not appear to exhibit a yield stress, $\sigma_0$, because flow occurs even at very low shear stresses. Thus, for non-Bingham bodies, in the absence of $\sigma_0$ the Herschel–Bulkley equation reduces to the power law, i.e., the Ostwald–de Waele equation:

$$\sigma = K \cdot \dot{\gamma}^{n} \quad (8)$$

Many pharmaceutical macromolecular materials exhibit non-Newtonian behavior in solution. Although the viscosity of Newtonian fluids is a constant, i.e., $\eta = \sigma/\dot{\gamma}$ so that $\eta$ is independent of shear rate or shear stress, there is no singular value of viscosity for non-Newtonian fluids because the viscosity of non-Newtonian fluids varies with shear rate or shear stress. Accordingly, applying the Newtonian concept of viscosity to non-Newtonian fluids is inappropriate. The viscosity value observed for a non-Newtonian fluid is an apparent viscosity, $\eta_{app}$, and is a function of the conditions of measurement. As the shear rate or shear stress changes, the apparent viscosity is likely to change as well. *Figure 4* depicts the dependence of $\eta_{app}$ on the shear rate for an aqueous solution of a typical hydrocolloid.

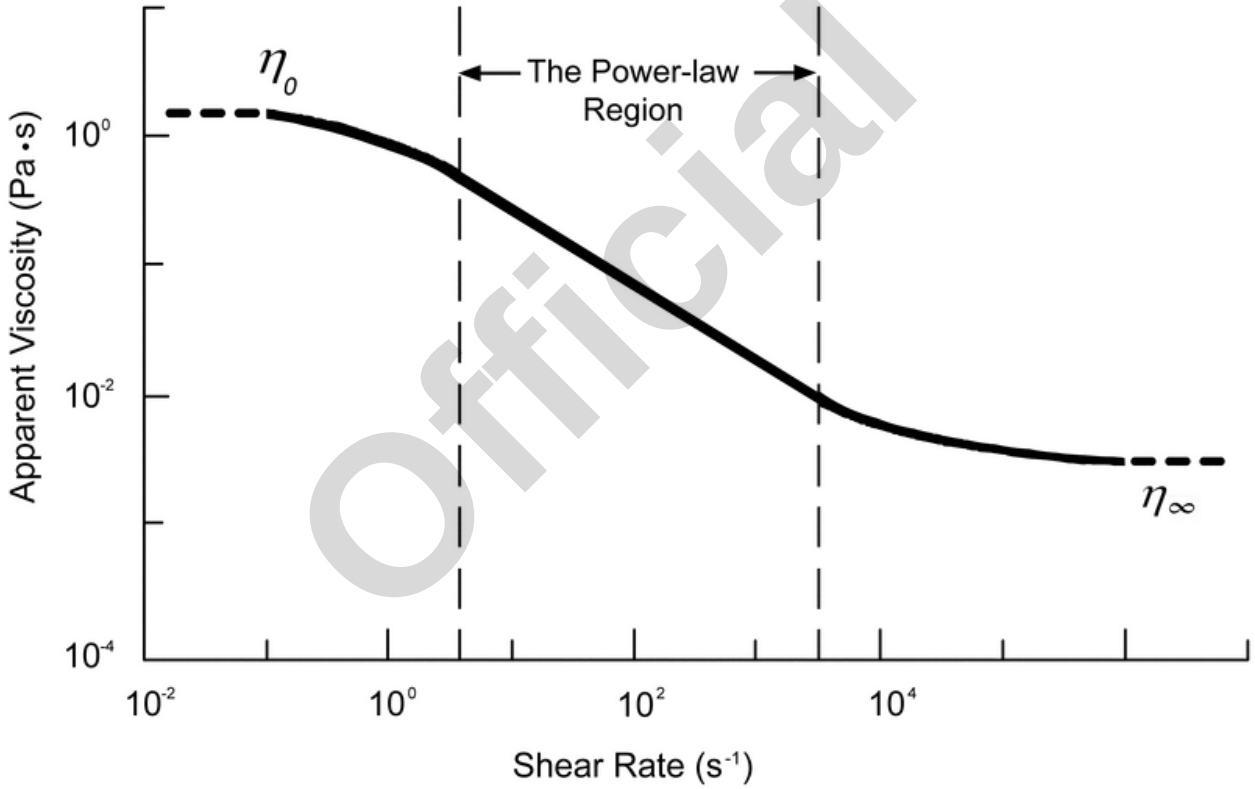


Figure 4. Log–log plot of the dependence of $\eta_{app}$ on the shear rate for an aqueous solution of a typical hydrocolloid.

*Figure 4* depicts apparent viscosities, $\eta_{app}$, ranging from high values at very low rates of shear to low values at very high rates of shear. The curve's asymptotes—corresponding to the zero-shear viscosity ($\eta_0$) and the infinite-shear viscosity ($\eta_\infty$)—together with the linear log–log region at intermediate rates of shear, are characteristic of the shear-thinning behavior of these materials.

During the course of manufacturing, in-process materials that exhibit non-Newtonian behavior may be subjected to wide ranges of shear rates and shear stresses. Fluid dynamic conditions during drug product storage and administration and release of the active constituent(s) could broaden those ranges even further. For materials that exhibit non-Newtonian behavior in a solution or dispersion, the rheological profile (rheogram or flow curve), as described in *Figure 3*, gives a better functional characterization than a single viscosity measurement of the apparent viscosity at a single shear rate.

Determination of the rheological profiles of non-Newtonian solutions or dispersions requires the use of a rheometer or a viscometer that is capable of measuring rheological behavior over the range of shear rates and/or shear stresses encompassing the conditions that the excipient or product is likely to encounter. The exact mathematical characterization of deformation and flow of a test substance requires the precise delineation of sample dimensions, the forces exerted, and the resulting deformation or flow. Instruments that enable a thorough, geometrically accurate analysis of deformation and flow are termed *absolute* rheometers. Such absolute rheometers are preferred for measurements of non-Newtonian fluids so that results can be compared from instrument to instrument for corresponding ranges of shear stresses and shear

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rates. When flow or deformation is not well defined or is indeterminate, the resulting measurements are likely to correspond, at best, to approximations of the true rheological behavior of the sample. Rheometers that can provide useful information about material behavior, but do not enable the true rheological character of the material to be determined, are known as *relative* rheometers. Such instruments are subject to geometry-dependent limitations. Results obtained with relative rheometers generally are not comparable to those obtained with absolute rheometers or other differently configured relative rheometers. Relative rheometers often are called viscometers. However, the term *viscometer* should be reserved for a subset of rheometers that measure only steady shear flow and the corresponding Newtonian viscosity.

Temperature control during the course of measurement is critical to the rheological evaluation of non-Newtonian fluids. An Arrhenius-type equation has often been invoked to characterize Newtonian and non-Newtonian flow properties as a function of temperature:

$$\frac{\eta}{\eta_{ref}} = A \cdot e^{\left(\frac{E_0}{R}\left(\frac{1}{T} - \frac{1}{T_{ref}}\right)\right)} \quad (9)$$

$\eta$ = Newtonian or non-Newtonian viscosity at some absolute temperature $T$
$\eta_{ref}$ = Newtonian or non-Newtonian viscosity at some reference temperature $T_{ref}$
$A$ = constant
$E_0$ = energy for viscous flow
$R$ = universal gas constant

Energies for viscous flow and the constant $A$ are determined from experimental data. Energy dissipation by viscous flow is a function of measuring system geometry, temperature control, shear rate, and shear stress, but increased variability is observed in particular at high shear rates and for fluids that exhibit high apparent viscosity. Sample temperatures of non-Newtonian fluids should be controlled to ±0.1°, as is the case for Newtonian viscosity measurements.

**Measurement of Viscosity Using a Rotational Rheometer**

Rotational rheometers represent a more useful alternative to most capillary viscometers for measuring the rheological characteristics of non-Newtonian fluids as long as variations in shear rate, sample temperature, and edge or end effects can be minimized. The most common absolute rotational rheometer designs include concentric (coaxial) cylinder, cone-and-plate, and parallel-plate instruments. All of these are available in models that can provide absolute rheological data—that is, data that do not require substantial corrections for end or edge effects, viscous heating, or variations in shear rate or shear stress in the sample being evaluated. Rotating spindle rheometers—often described as viscometers—are relative rheometers that typically are used to assess apparent viscosity.

An additional factor with all rheometers/viscometers that must be taken into consideration, particularly with multiphase materials, is the potential formation of a thin, less-viscous layer of fluid at the wall of the instrument. When wall effects, or wall slip, occur, instrument measurements may be misleading because of the actual rheological behavior of the sample. Identification of wall effects is instrument specific. One useful method entails the comparison of results with a roughened vs. a smooth rotor. Instrument manufacturers generally provide recommendations for identification of and correction for wall slip.

Generally, calibration, operation, and cleaning of rheometers should be performed according to the instrument manufacturer's recommendations.

General chapter *Viscosity—Rotational Methods* ⟨912⟩ describes the procedures for the most common types of rheometers: spindle rheometers (viscometers) and concentric (coaxial) cylinder, cone-and-plate, and parallel-plate (parallel disk) instruments.

**Calculation of shear rate, shear stress, and viscosity using a concentric (coaxial) cylinder rheometer:**

For non-Newtonian liquids, it is essential to specify the shear stress, $\sigma$, or the shear rate, $\dot{\gamma}$, at which the viscosity is measured. Under narrow gap conditions (conditions satisfied in absolute rheometers), the shear rate $\dot{\gamma}$ in $s^{-1}$, and the shear stress $\sigma$, in Pa ($N \cdot m^{-2}$ or $kg \cdot m^{-1} \cdot s^{-2}$), are calculated using the following equations:

$$\dot{\gamma} = \left(\frac{R_o^2 + R_I^2}{R_o^2 - R_I^2}\right)\omega \quad (10)$$

$$\sigma = \left(\frac{1}{4\pi h R_o^2} + \frac{1}{4\pi h R_I^2}\right)M \quad (11)$$

$R_o$ = radius of the outer cylinder (m)
$R_I$ = radius of the inner cylinder (m)
$\omega$ = angular velocity (radian/s)
$M$ = torque acting on the cylinder surface ($N \cdot m$)
$h$ = height of immersion of the inner cylinder in the liquid medium (m)

Generally, the angular viscosity can be calculated using the equation:

$$\omega = \left(\frac{2\pi}{60}\right)n \quad (12)$$

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$n$ = rotational speed, in revolutions per min (rpm)

For laminar flow, the viscosity $\eta$ (or apparent viscosity $\eta_{App}$), in Pa · s, is given by the following equation: [NOTE—1 Pa · s = 1000 mPa · s.]

$$\eta \text{ or } \eta_{\text{App}} = \frac{1}{4\pi h}\left(\frac{1}{R_I^2} - \frac{1}{R_o^2}\right)\frac{M}{\omega} = k\frac{M}{\omega} \quad (13)$$

$k$ = constant of the apparatus (radians/m³)

**Calculation of shear rate, shear stress, and viscosity using a cone-and-plate rheometer:**
The shear rate $\dot{\gamma}$ in $s^{-1}$, and the shear stress $\sigma$, in Pa, are calculated by the following equations:

$$\dot{\gamma} = \left(\frac{1}{\alpha}\right)\omega \quad (14)$$

$$\sigma = \left(\frac{1}{\frac{2}{3}\pi R^3}\right)M \quad (15)$$

$\alpha$ = angle between the flat plate and the cone (radians)
$R$ = radius of the cone (m)
$\omega$ = angular velocity (radian/s)
$M$ = torque acting on the cylinder surface (N · m)

For laminar flow, the viscosity $\eta$ (or apparent viscosity $\eta_{App}$), in Pa · s, is given by the following equation:

$$\eta \text{ or } \eta_{\text{App}} = \left(\frac{3\alpha}{2\pi R^3}\right)\left(\frac{M}{\omega}\right) = k\frac{M}{\omega} \quad (16)$$

$k$ = constant of the apparatus (radians/m³)

**Measurement of Rheological Properties Using a Nonrotational Rheometer**

Alternative nonrotational rheometer procedures for measuring rheological properties of non-Newtonian fluids include slit rheometers, small- and large-amplitude oscillatory shear-based rheometers, and capillary-breakup elongational rheometers. Equipment manufacturers generally provide detailed procedures, and these instruments and methods may be appropriate for use after users complete characterization and validation studies for both the equipment and the material under test. These types of rheometers are not described in *Viscosity—Rotational Methods* ⟨912⟩, as these are not rotational rheometers.

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# ⟨1912⟩ MEASUREMENT OF ▲YIELD STRESS▲ (USP 1-Dec-2023) OF SEMISOLIDS

## ▲BACKGROUND

Semisolids are viscoelastic materials that exhibit a yield stress. The yield stress for a raw material or dosage form[1] is the applied stress at which a change in the viscoelastic properties of a semisolid is observed: below the yield stress the material response is dominated by completely reversible elastic deformation, whereas above the yield stress the material response is changing to irreversible elastic behavior (as the material remains a viscoelastic semisolid) or is dominated by viscous flow behavior. Often, the yield stress is identified as the applied stress below which a material appears to not show any motion or flow (i.e., where the shear rate is near-zero).

This observation can be dependent on the sample preparation, methods of testing and analysis, the time scale of the measurement, the scan rate used to locate the change in the behavior, and the direction from which the transition is approached. Most rotational measurement techniques identify the yield stress by locating the onset of flow; however, penetrometry, which is another rheological technique, looks for the yield stress where the semisolid stops yielding. Although the yield stress for various materials is expected to correlate when measured with different techniques, the values should not be considered to be independent of the method of testing and analysis. Yield stress is reported with units of shear stress [Pascals (Pa)].

Characterizing and monitoring the viscoelastic properties of semisolids is not straightforward because the properties can be dominated by either solid- or liquid-like behavior depending on how much deformation or stress is applied to the material. If the quality of a raw material or dosage form is primarily dependent on the behavior under high-shear conditions, then viscosity may be an appropriate parameter to monitor. In this case, efforts should be made to avoid wall slip and make measurements where the applied shear stress is much greater than the yield stress. However, if the properties of the raw material or dosage form are critical to quality at low shear or at rest (e.g., uniformity and stability of an ointment suspension or residence time at site of application), then measurement of the yield stress using one or more of the methods described below may be required.

For temperature-sensitive materials, viscoelastic properties at both ambient conditions (i.e., storage conditions) and physiological conditions [i.e., skin surface temperature (32°) or body temperature (37°)] are useful. For example, an anesthetic topical cream may require characterization at 32°, whereas a vaginal cream may require characterization at 37° (*1*–*2*).

A brief summary of the mathematical models used to quantify the viscoelastic properties of semisolids is presented below, followed by a summary of the most common experimental methods for assessing the viscoelastic properties and determining the yield stress for semisolids. [NOTE—Additional models for viscosity versus shear rate are also discussed in *Rheometry* ⟨1911⟩, see *Figure 3*).]

### Hooke's Elasticity Law and Newton's Viscosity Law

For elastic materials, Hooke's Elasticity Law applies,

$$\sigma = G\gamma \quad \text{Equation 1}$$

where $\sigma$ is the shear stress (= force/surface area, in units of pascals, Pa), $G$ is the shear modulus that represents the rigidity of a material, and $\gamma$ is the shear strain or shear deformation (= displacement/distance or shear gap, dimensionless).

For viscous materials, Newton's Viscosity Law applies,

$$\sigma = \eta\dot{\gamma} \quad \text{Equation 2}$$

where $\sigma$ is the shear stress, $\eta$ is the shear viscosity that represents the resistance of the material to flow, and $\dot{\gamma}$ is the shear rate (or strain rate = velocity/distance or shear gap, in units of $s^{-1}$).

Semisolid raw materials and dosage forms will have both viscous and elastic properties and, hence, are categorized as viscoelastic materials.

### Herschel–Bulkley Equation

The Herschel–Bulkley equation is suitable for parameterizing the observed shear stress versus shear rate information for a wide range of viscoelastic materials including semisolids exhibiting a yield stress. The Herschel–Bulkley equation is:

$$\sigma = \kappa\dot{\gamma}^{\eta} + \sigma_{\gamma} \quad \text{Equation 3}$$

where, $\sigma$ is the shear stress, $\kappa$ is the proportionality constant (formerly also referred to as consistency), $\dot{\gamma}$ is the shear rate, $\eta$ is the exponent, and $\sigma_{\gamma}$ is the yield stress according to Herschel–Bulkley.

The exponent, $n$, is less than 1 for shear-thinning fluids and is greater than 1 for shear-thickening fluids.

When $n = 1$, the Herschel–Bulkley equation simplifies to the Bingham equation with $\kappa$ referred to as the Bingham viscosity. When $n = 0.5$, the Herschel–Bulkley equation simplifies to the Casson equation with $\kappa$ typically referred to as the Casson viscosity.

[1] [NOTE—Throughout this chapter, the use of the term "raw material or dosage form" refers to its semisolid state.]

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### Oscillatory Measurements of Viscoelastic Properties

If a sinusoidal oscillating shear strain (shear deformation) is applied to a material,

$$\gamma(t) = \gamma_0 \sin(\omega t) \quad \text{Equation 4}$$

where $\gamma(t)$ is the amplitude at time ($t$), $\gamma_0$ is the amplitude at time zero, and $\omega$ is the angular frequency of the shear strain, the corresponding shear rate is calculated as follows.

$$\dot{\gamma}(t) = \gamma_0 \omega \cos(\omega t) = \dot{\gamma_0} \cos(\omega t) \quad \text{Equation 5}$$

According to Hooke's Elasticity Law, the ideal elastic response to this applied shear strain will be sinusoidal:

$$\sigma(t) = \gamma_0 G \sin(\omega t) \quad \text{Equation 6}$$

which indicates that the ideal elastic response is in-phase with the strain ($G$ is the shear modulus).

According to Newton's Viscosity Law, the ideal viscous response will also be sinusoidal:

$$\sigma(t) = \eta \gamma_0 \omega \cos(\omega t) \quad \text{Equation 7}$$

which indicates that the ideal viscous response is out-of-phase (shifted by 90°, showing the phase shift angle of $\delta = 90°$) relative to the shear strain.

For a viscoelastic material, the response will be sinusoidal with a phase shift angle, $\delta$ (also referred to as the loss angle) of $0° \leq \delta \leq 90°$.

$$\sigma(t) = \sigma_0 \sin(\omega t + \delta) \quad \text{Equation 8}$$

The response of a viscoelastic material to an applied sinusoidal shear strain is used to determine the values of the loss angle, $\delta$, and the amplitude of the response, $\sigma_0$. This response can be separated into a viscous and elastic component according to *Equation 9* and *Equation 10*,

$$G' = \frac{\sigma_0}{\gamma_0} \cos\delta \quad \text{Equation 9}$$

$$G'' = \frac{\sigma_0}{\gamma_0} \sin\delta \quad \text{Equation 10}$$

where $G'$ is called the elastic (storage) modulus and $G''$ is called the viscous (loss) modulus. The dimensionless loss factor (or damping factor) as the ratio of the viscous and the elastic portions is defined as: $\tan\delta = G''/G'$ (unit: 1).

Therefore, the sinusoidal response of a viscoelastic material to an oscillatory shear strain (or shear stress) can be used to separate the viscous and elastic responses for a semisolid material in the range of low strains and low stresses without requiring the material to actually flow.

When the elastic response of a material is dominant ($G' > G''$), the material is referred to as a viscoelastic solid or a gel. When the viscous response of a material is dominant ($G' < G''$), the material is referred to as viscoelastic liquid or a sol. The point where $G' = G''$ ($\tan\delta = 1$) is referred to as the sol–gel transition.

## EXPERIMENTAL METHODS

### Yield Stress via the Viscosity Maximum

A viscosity maximum is performed by presetting a constant, very low ("near-zero") shear rate (e.g., of 0.01 or 1 $s^{-1}$) or a very low rotational speed (e.g., of 0.01 or 1 rpm). The relationship between rotational speed $n$ (in rpm) and angular velocity $\omega$ (in rad/s or 1/s) is: $\omega = (2\pi/60) \times n$. As a result, the yield stress corresponds to the maximum viscosity (or shear stress or torque), which can be displayed in a diagram showing one of these parameters on the $y$-axis and time (or shear strain) on the $x$-axis. The following applies according to Newton's Viscosity Law (see *Equation 2*): Because this experiment is run at a constant shear rate (or speed), the shear viscosity and the shear stress are proportional. The shape of the plots of the shear viscosity and shear stress (or torque) are identical. The resulting viscosity (or shear stress or torque) values will begin to increase with time (transient viscosity function, i.e., time-dependent). When approaching the yield stress, the increase in the values will slow, and thus, the curve will become flatter. There are two possible results:

- The plot will clearly show a maximum (or peak). The yield point (in Pa) is read off as the shear stress value appearing at the maximum point of the curve (see *Figure 1A*).
- The plot will steadily approach to a (constant) plateau value. The yield point (in Pa) is read off as the shear stress value when first reaching the plateau value (see *Figure 1B*).

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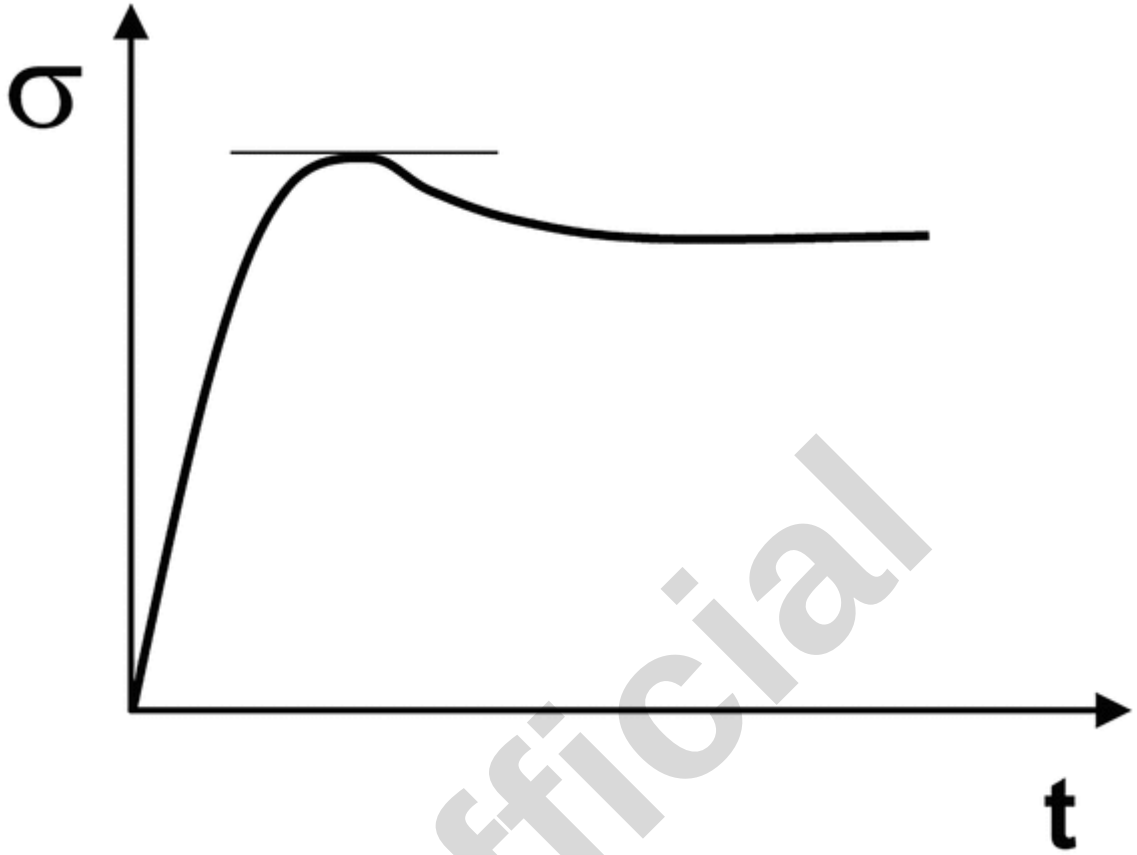


Figure 1A. Yield stress determination via the shear stress maximum (alternative 1): After presetting a constant low rotational speed (or shear rate), the yield stress is read off as the shear stress value that is obtained at the peak of the shear stress curve.

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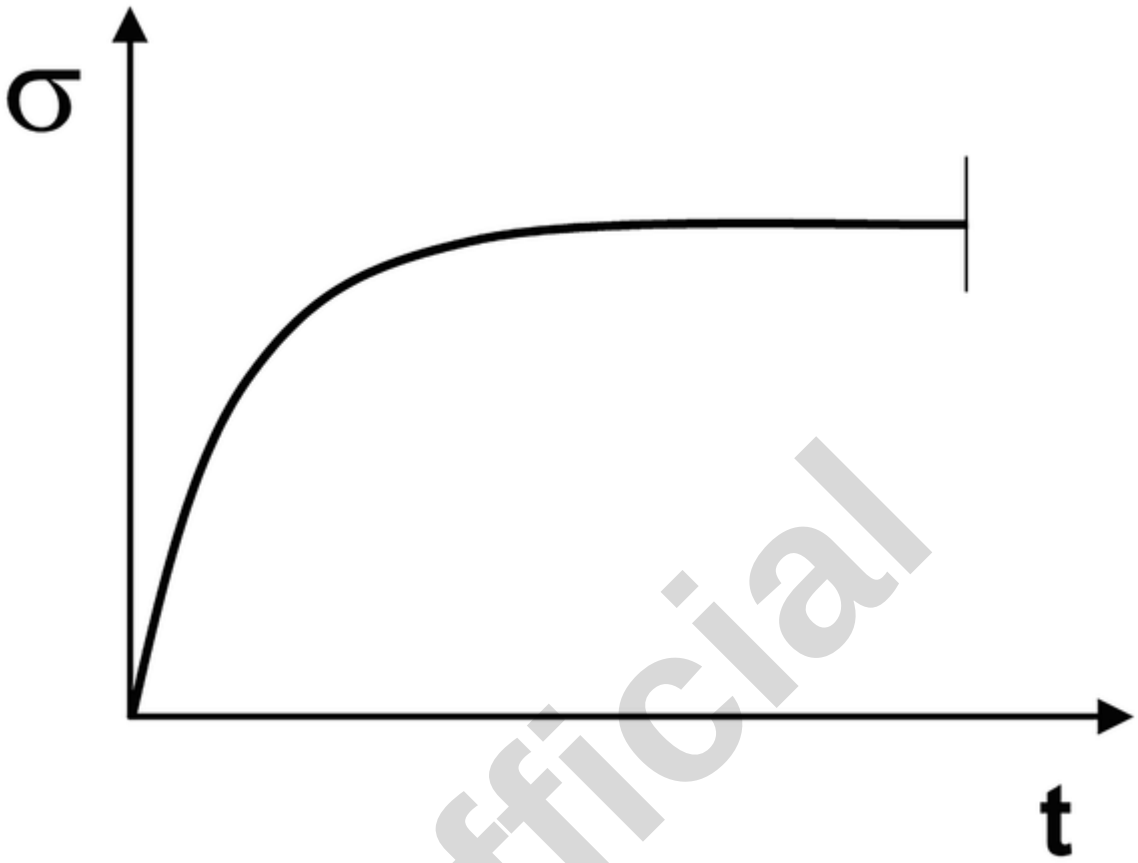


Figure 1B. Yield stress determination via the shear stress maximum (alternative 2): After presetting a constant low rotational speed (or shear rate), the yield stress is read off as the shear stress value that is obtained at the point when the shear stress curve finally reaches a constant value.

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The experimental parameters that influence this measurement method include the sample loading technique, the placement of the measurement geometry (often a vaned rotor) in the sample cup, and the sample history. Because air pockets may be of concern, ideally, the sample should be loaded gently, without significant shearing and without introducing any air, and the sample cup should be large enough that the vane or cylinder can be placed at least $2H$ above the bottom of the sample cup (where $H$ is the height of the vane or cylinder) and at least a distance $D$ away from the side of the sample cup (where $D$ is the diameter of the vane or cylinder). To avoid end effects at the top of the vaned rotor or cylinder, either the top should be at least $H/2$ below the air interface, or the geometry should be positioned so that the top is at or above the air interface. After carefully immersing the geometry into the sample, an equilibration time may be required to relieve any stresses developed during the loading of the sample. The yield stress may be affected by the shear rate (or speed) used. When comparing different raw materials or dosage forms, it is important and recommended to use the same measurement speed.

## Yield Stress via Shear Rate Ramps or Shear Stress Ramps (flow curves)

The yield stress of a material can be estimated in two ways:

- Using a non-linear regression approach based on the Herschel–Bulkley equation (*Equation 3*), see *Figure 2*.
- Reading the plateau value at low shear rate. As shown in *Figure 3*, a material with a yield stress will exhibit a plateau on this log–log plot and the yield stress will correspond to this asymptotic value at low shear rate. If the plateau is not apparent, then fitting to the Herschel–Bulkley equation will provide a better estimate of the yield stress.

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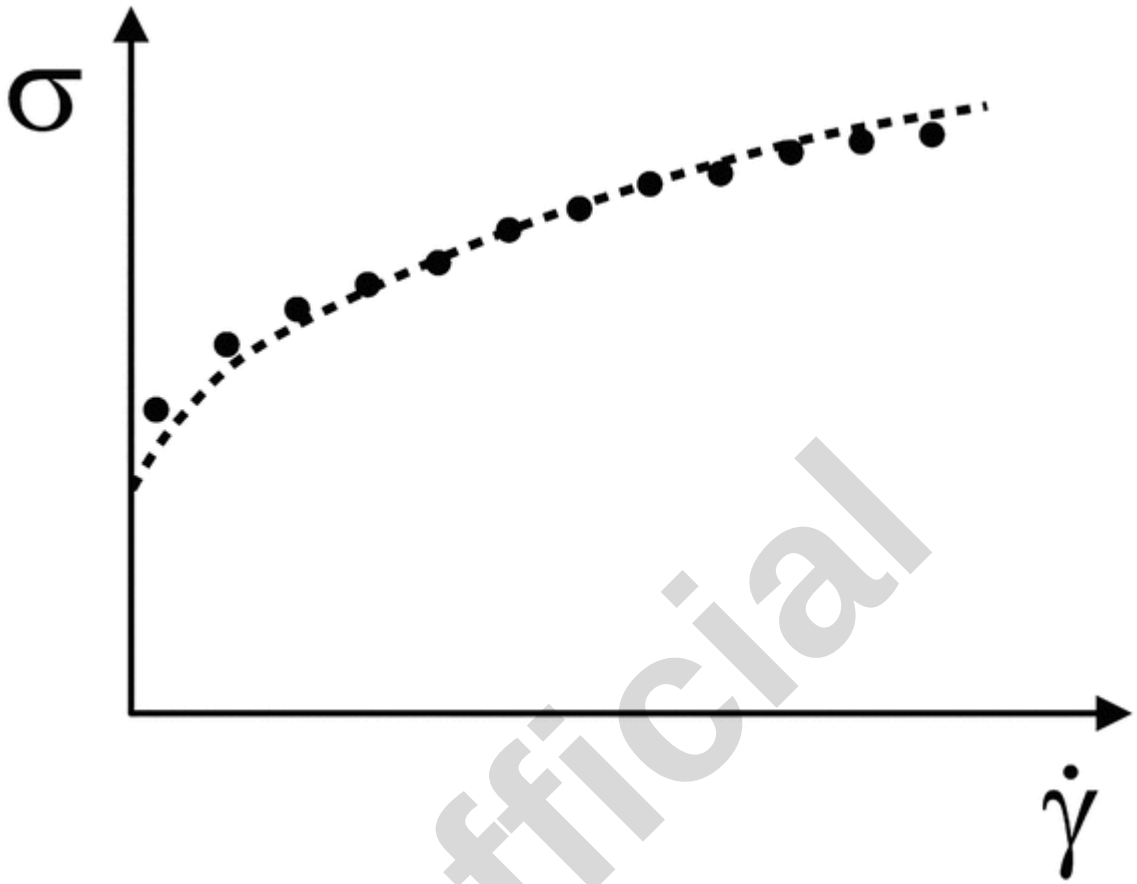


Figure 2. Yield stress determination using a curve-fitting function (e.g., according to the model of Herschel–Bulkley). Here, the individual measuring points of a flow curve measurement are shown (on a linear scale for both axes) as dots and the fitting curve as dotted line. The yield stress is read off at the intersection of the fitting curve and the σ-axis, or is calculated for the shear rate at the value zero, respectively.

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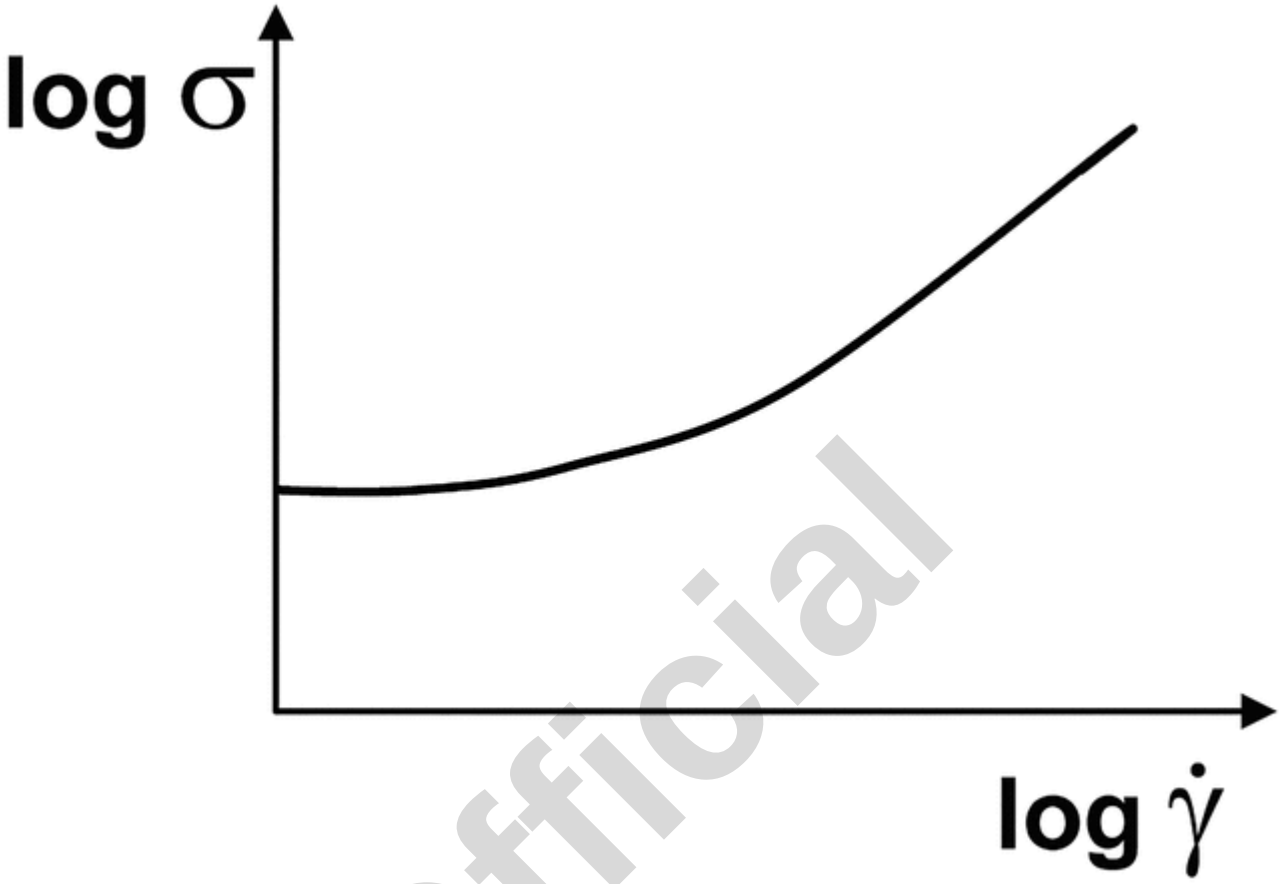


Figure 3. Flow curve diagram on a logarithmic scale for both axes. The yield stress is read off as the plateau value of the shear stress curve in the low-shear range.

Modern rotational rheometers can perform flow curve measurements using two modes of operation: 1) controlled shear rate (CSR) and 2) controlled shear stress (CSS). For CSR, typical examples for testing include: a) on a linear scale, preset of 20 measuring points from shear rate 0 to 1000 $s^{-1}$; and b) on a logarithmic scale, preset of 10 measuring points per decade between 0.01 $s^{-1}$ (or 1 $s^{-1}$) and 1000 $s^{-1}$.

For these experiments, wall slip at low shear rates will significantly affect the results. It is highly recommended that either a vaned rotor or a roughened (e.g., cross-hatched) parallel plate (PP) measurement geometry be used. In extreme cases of wall slip, a material with a yield stress may appear to switch to a state of flow and may lead to the incorrect conclusion that the material does not exhibit any yield stress at all. One benefit of dynamic oscillation stress or strain sweep tests is that it provides a lower probability of wall slippage.

## Yield Stress by Amplitude Sweep

For oscillatory measurements in the form of amplitude sweeps, the amplitude of the shear strain (or shear stress) is increased from low to high on a logarithmic scale (e.g., shear strain from 0.01%–100% or shear stress from 1 to 1000 Pa, preset on a log scale, with 10 points per decade). Users often perform amplitude sweeps at $\omega$ = 10 rad/s (or, alternatively, at frequency ($f$) = 1 Hz). This test may be used to evaluate the viscoelastic behavior and the stiffness (rigidity) of a gel in the linear viscoelastic range (LVR) . The LVR is the low shear range where the response of a sample behaves predominantly according to Hooke's Elasticity Law, the shear strain (shear deformation) is reversible, and the resulting parameters ($G'$ and $G''$) are constant, both showing plateau values with $G' > G''$ within this region. The LVR limit typically is determined in terms of the shear strain or shear stress value when the $G'$ curve deviates from the plateau value by 5% (see ISO 3219-3 for more information), usually downward.

The yield stress value is correlated with the storage modulus drop that occurs beyond the limit of the LVR. The limit of the LVR may be considered the onset of yielding or, alternatively, the onset point for yielding may be located as the intersection of an initial tangent line (from the LVR) with a final tangent line (where the $G'$ curve or complex viscosity drops rapidly). Additionally, the $G'$ and $G''$ crossover point may be considered a transition from elastic to viscous behavior. A yield point or yield stress may be identified from these plots in multiple ways.

Because the oscillatory tests do not require the measuring sample to flow, wall slip may not be a significant concern for these experiments, although vaned rotor and roughened measurement geometries may still be used.

Each amplitude sweep is performed at a single frequency. [For the conversion between the angular frequency $\omega$ (in rad/s) and the frequency (in Hz): $\omega = 2\pi \times f$.] The frequency selected for the amplitude sweep will affect the value of $G'$ in the LVR and

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may affect the location of the crossover point of the curves of *G′* and *G″*.Frequency sweeps may be performed to determine the significance of the frequency (or time) dependence of the results.

*Figure 4* shows an example of an amplitude sweep of a typical semisolid. In the LVR, the *G′* value of the material is constant and may be used as a measure of the stiffness of the material. At the limit of the LVR, the value of *G′* begins to depart from the plateau, typically by sloping downward, and at even higher strain (or stress) amplitudes, the material exhibits the crossover point of *G′* and *G″*. Both the limit of the LVR and the crossover point of *G′* and *G″* represent significant changes in the viscoelastic properties of the material that sometimes are selected as a yield stress value. This can be interpreted as follows:

- The limiting shear stress, or $\sigma_L$, is the shear stress at the limit of the LVR. At this limit, the semisolid begins to yield but *G′* still dominates *G″*, and thus the elastic (solid) behavior over the viscous (liquid) one.
- The shear stress at the crossover point (also known as the flow point), or $\sigma_{co}$, is the point where *G″* dominates *G′*, and thus, the viscous (liquid) behavior over the elastic (solid) one. In other words, at this point the material is predominantly in a flowing state. Many practical users prefer the crossover point for two reasons: It can be located more precisely as it occurs clearly as a single point in the diagram, and it has been found to correlate particularly with the yield stress values resulting from rotational tests. [NOTE—However, for amplitude sweeps, the values of the crossover point (*G′* = *G″*) in terms of the shear strain and shear stress are clearly measured outside of the LVR.]

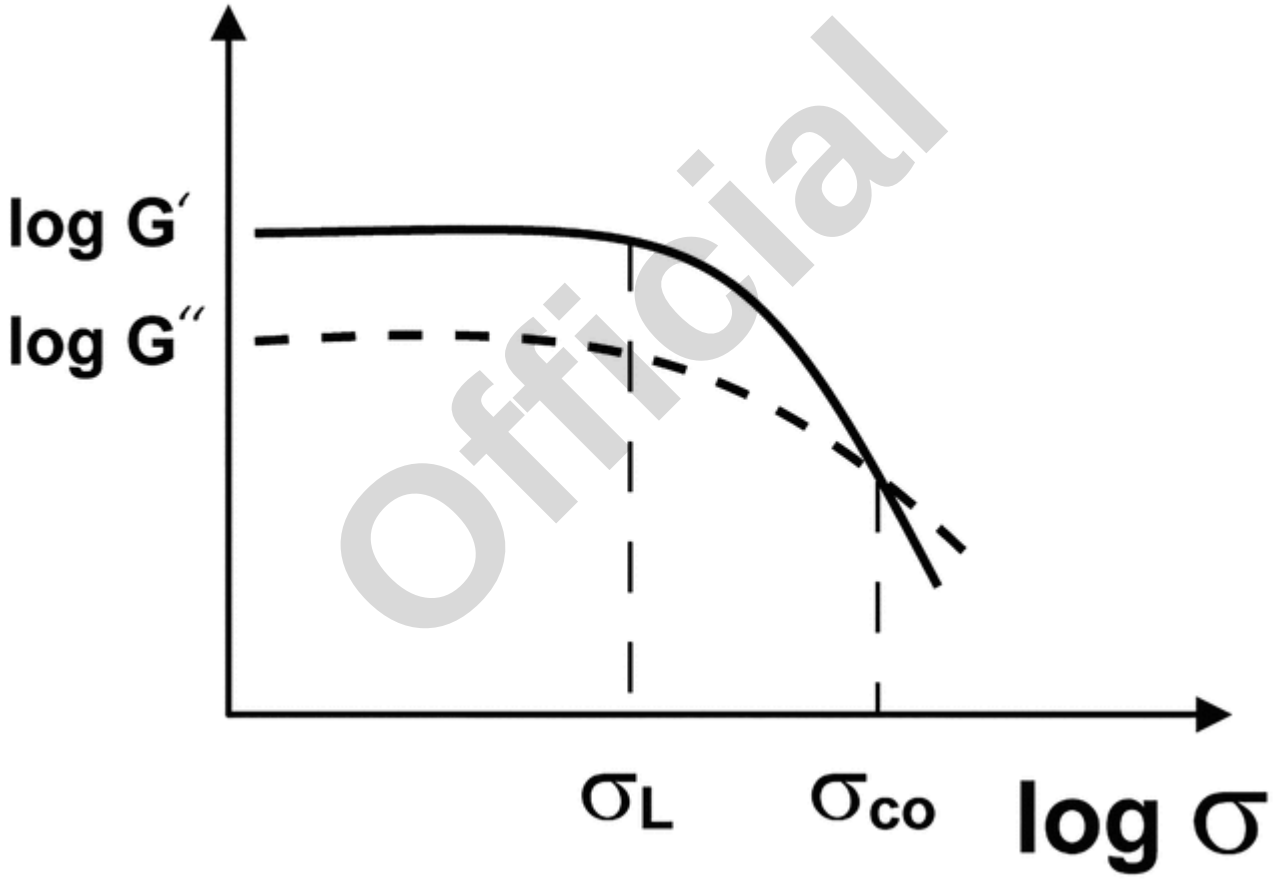


Figure 4. Amplitude sweep: in the LVR, the curves of *G′* and *G″* show plateau values. The LVR ends at the limiting value of the shear stress ($\sigma_L$), which is often also used for the yield stress. The shear stress value ($\sigma_{co}$) at the crossover point of the curves of *G′* and *G″* is the point where the viscoelastic behavior of the measuring sample changes from predominantly elastic (or solid) to predominantly viscous (or liquid); therefore, this point is often referred to as the flow stress (or flow point).

## Penetrometry Measurements

In the penetrometry experiment, a cone with an angle of 2α is driven into the semisolid by gravity. The most widely used penetrometer is a gravity-driven instrument (*Figure 5*), which is typically used to perform a penetrometry experiment according to ASTM D217, ASTM D937, the *European Pharmacopoeia*, 2.9.9 *Measurement of Consistency by Penetrometry*, or *Measurement of Structural Strength of Semisolids by Penetrometry* ⟨915⟩. These methods require measurement of the sample at 23.5 ± 2.0°.

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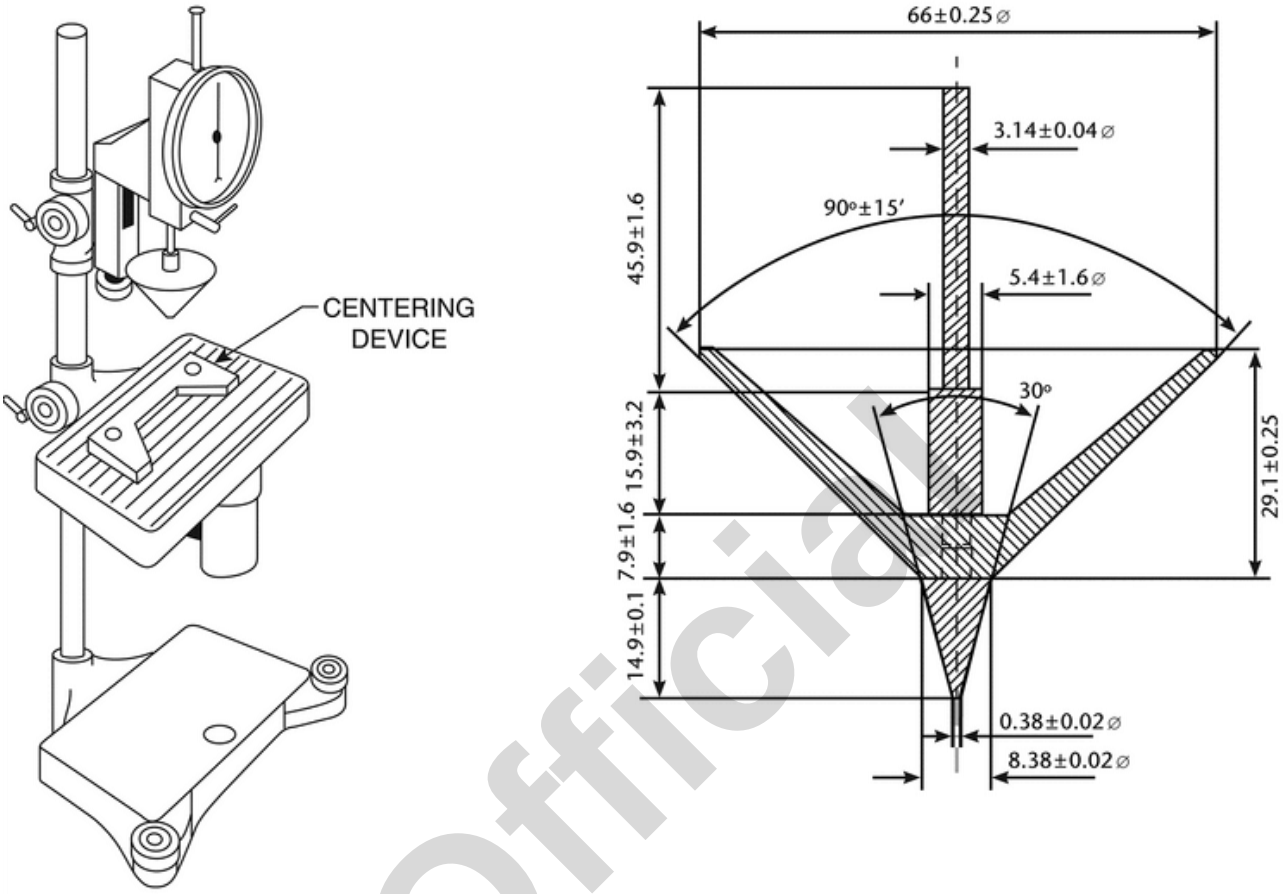


Figure 5. Typical penetrometer and cone used in hardness measurements. (Adapted with permission from *Pharmeuropa*, Vol. 9, No. 8, July 2019. ©Council of Europe, 2019.)

Briefly, the penetrometer cone positioned just above the surface of the semisolid is released and allowed to drop freely into the sample for 5 s. The penetration depth is recorded and reported in units of 0.1 mm. The penetration unit is decimillimeter (dmm). Three or more determinations are made, and the results are averaged to give the reported result. These penetrometry methods require the use of a two-piece cone (a small 30° cone attached to a larger 90° cone) that has a total effective mass of 150.0 ± 0.1 g. For this cone, gravity drives the cone into the semisolid with 1471 millinewtons (mN) of force.[2] The effective penetration force (the total force minus the buoyancy force) results in the application of a shear stress to the semisolid at the surface of the penetrating cone. In general, the semisolid will respond to this applied shear stress according to the Herschel–Bulkley equation (*Equation 3*).

The cone will continue to penetrate until the applied shear stress is equal to the yield stress of the semisolid. At this point, the cone will stop penetrating and the shear rate will go to zero. It can be shown that, at this point, the yield stress will be a function of the penetration depth, *h*, according to *Equation 11* below:

$$\sigma_0 = \left(\frac{\cos^2\alpha}{\pi \tan\alpha}\right)\frac{1}{h^2}\left(W - \frac{\rho_f g \pi h^3 \tan^2\alpha}{3}\right) \quad \text{Equation 11}$$

where $\alpha$ is the half-angle of the cone, $W$ is the weight of the penetrating cone, $\rho_f$ is the density of the semisolid, and $g$ is the acceleration from gravity. If the buoyancy correction is negligible, this equation simplifies as shown in *Equation 12*, indicating that the yield stress is essentially equal to the weight of the cone over the square of the penetration depth multiplied by a cone constant.

$$\sigma_0 = \left(\frac{\cos^2\alpha}{\pi\tan\alpha}\right)\frac{W}{h^2} \quad \text{Equation 12}$$

[2] Force (mN) = 1000 × m × g = 0.15 kg × 9.80665 m $s^{-2}$ = 1471 mN.

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The term "hardness" (*H*) has been defined by several authors as shown in *Equation 13*,

$$H = C\frac{W}{h^n}$$ Equation 13

where *C* is a constant dependent on the cone geometry, *W* is the weight of the penetrating cone, *h* is the penetration depth, and *n* is an exponent. If *n* = 2, then *H* will be equivalent to the yield stress and have the same units (Pa).

The monographs for petrolatum and white petrolatum each require the result of the penetrometry measurement to be in the 100–300-dmm range (NLT 10.0 mm and NMT 30.0 mm). *Figure 6* shows the calculated yield stress for the standard 150-g, two-piece cone as a function of penetration depth.

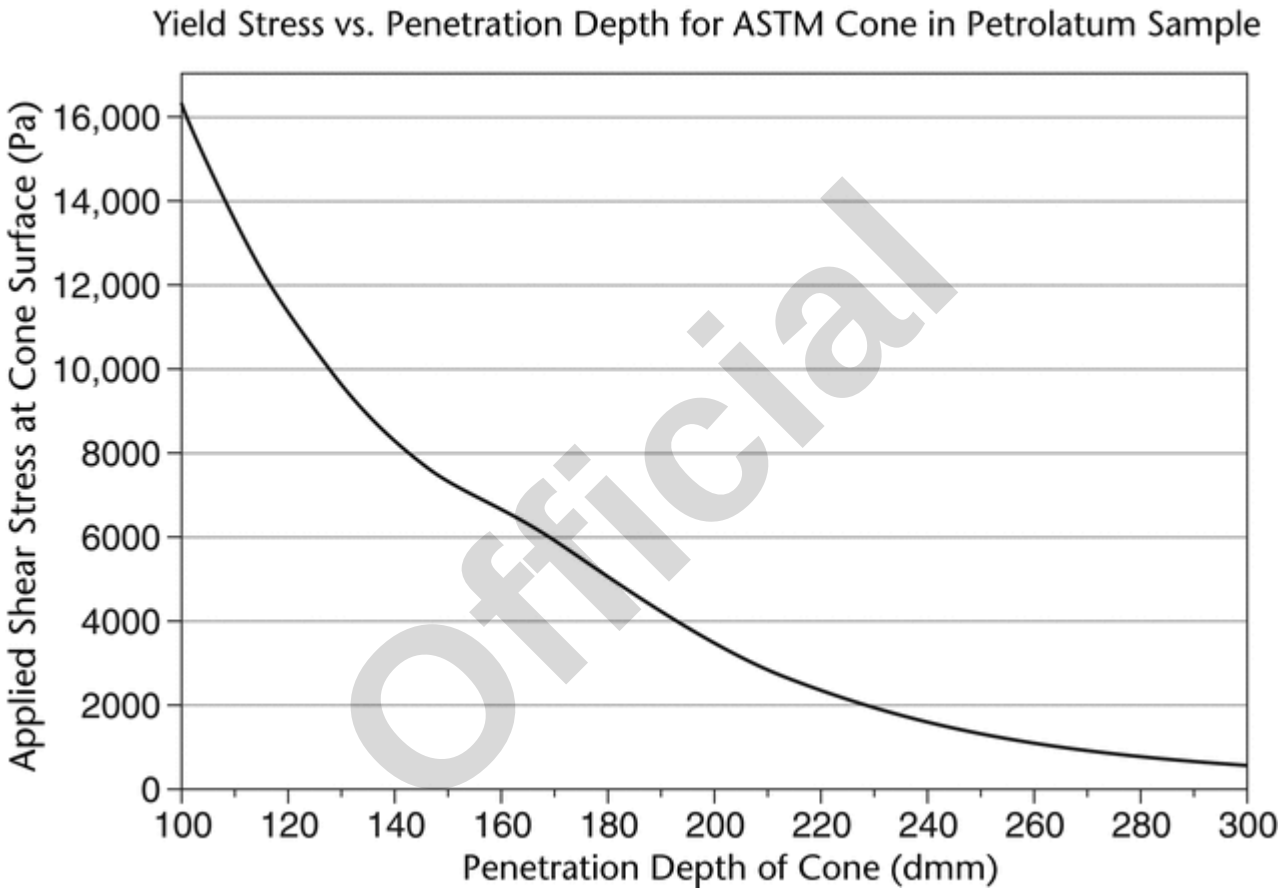


Figure 6. Yield stress (hardness) as a function of penetration depth for the two-piece, 150-g standard cone (from ⟨915⟩, *Procedure, Determination of Penetration, Method I*). This calculation uses *Equation 3* for penetration into sample with a density of 0.85 g/cm$^3$ and includes the buoyancy correction. Based on the ideal geometry, the smaller cone is truncated at the tip (by 7 dmm) and the two cones are joined at 150 dmm where they have the same diameters. This penetration depth corresponds to yield stresses in the 200–16,000 Pa range. The plot shows that there is a slight inflection point near 150 dmm where the two cones are joined.

## GLOSSARY

Note that the following definitions are provided to clarify the use of these terms in the context of this chapter. These definitions are not intended to supersede or contradict definitions found elsewhere in *USP–NF*.

**Consistency:** See Structural strength.

**Gel and Sol:** A gel is defined as a material for which $G' > G''$. A sol is defined as a material for which $G' < G''$. The behavior of a gel is dominated by its elastic response to strain (shear deformation). The behavior of a sol is dominated by its viscous response to strain. The point where $G' = G''$ is called the sol–gel transition. Rheology is used to characterize the continuous phase of a dosage form to classify it as either a gel or sol based on which behavior dominates the viscoelastic properties.

**Hardness:** Hardness is a term used synonymously with tensile stress, and yield stress is a type of tensile stress. Hardness is proportional with yield stress—a harder semisolid also exhibits a larger yield stress. In penetrometry, hardness has been more specifically defined as $H = C \times W/p^n$, where *C* is a constant dependent on the cone geometry, *W* is the weight of the penetrating cone, *p* is the depth of penetration, and *n* is an exponent. When *n* = 2, hardness has the same units as yield stress (Pa).

**Penetrometry:** A method for quantifying the yield stress of semisolid materials in which a metal cone, with standardized dimensions and weight, penetrates into a semisolid until the buoyancy of the cone and the yield stress of the semisolid exactly balances the gravity-applied force driving the penetrating object into the semisolid. See ⟨915⟩ for more details.

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**Semisolids:** Materials that exhibit viscoelastic properties that are classified as more solid-like at rest and at room temperature. Typically, these materials will transition to more fluid-like behavior under applied stress or as a result of temperature changes. This transition from a sol to a gel is identified as a yield stress. When used as dosage forms semisolids may be further classified as gels, ointments, or creams.

**Strain:** The shear deformation of a material. If a cubic volume of material is deformed by moving the top plane of the cube relative to the bottom plane of the cube, the strain is equal to the linear displacement of the top plane divided by the height of the cube (distance between the planes). This strain that results from the shear deformation of the viscoelastic material is unitless as it has units of distance (displacement) over distance. It is sometimes multiplied by 100 and reported as a percentage strain.

**Structural strength (Consistency):** Consistency is sometimes used synonymously with "viscosity", albeit incorrectly. Viscosity represents the proportionality of the shear stress to the shear rate for a Newtonian fluid, whereas consistency is the term for this proportionality for semisolids that exhibit non-Newtonian rheological behavior. Because the term consistency may be confused with uniformity or homogeneity, the term structural strength is now the preferred term.

**Surface area:** The surface area of the sheared fluid volume on which the shear force is acting or the cross-sectional area of material with area parallel to the applied force vector.

**Wall slip:** Term describing the shear-thinning of a semisolid dosage form at the wall of a measurement system. When shear stress is applied to a dosage form, the material at the wall of the measurement system will yield and shear thin before the remaining bulk material. This slipping of the material at the wall of the measurement system results in incomplete transfer of the shear stress into the bulk. Wall slip will result in erroneously low viscosity results for shear thinning and yield stress fluids and will result in erroneously high shear rate for a given applied shear stress. Wall slip is most significant for low shear measurements and can be reduced by using plate-plate measurement systems with roughened surfaces or by using a vaned rotor rather than a cylinder.

**Yield stress:** The applied stress at which a change in the viscoelastic properties of a semisolid is observed. Below the yield stress the material response is dominated by completely reversible elastic deformation, whereas above the yield stress, the material response is changing to irreversible elastic behavior (as the material remains a viscoelastic semisolid) or is dominated by viscous flow behavior.

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# ⟨2021⟩ MICROBIAL ENUMERATION TESTS—NUTRITIONAL AND DIETARY SUPPLEMENTS

## INTRODUCTION

This chapter provides tests for the estimation of the number of viable aerobic microorganisms present in nutritional supplements of all kinds, from raw materials to the finished forms. Alternative methods may be substituted for the tests, provided that they have been properly validated as giving equivalent or better results. In preparing for and in applying the tests, observe aseptic precautions in handling the specimens. The term "growth" is used in a special sense herein, i.e., to designate the presence and presumed proliferation of viable microorganisms.

## PREPARATORY TESTING

The validity of the results of the tests set forth in this chapter rests largely upon the adequacy of a demonstration that the test specimens to which they are applied do not, of themselves, inhibit the multiplication, under the test conditions, of microorganisms that may be present. Therefore, preparatory to conducting the tests on a regular basis and as circumstances require subsequently, inoculate diluted specimens of the material to be tested with separate viable cultures of the challenge microorganisms.

For the *Soybean–Casein Digest Agar Medium* used for *Total Aerobic Microbial Count*, inoculate duplicate plates with 25–250 cfu of *Staphylococcus aureus* (ATCC[1] No. 6538), *Escherichia coli* (ATCC No. 8739), and *Bacillus subtilis* (ATCC No. 6633) to demonstrate a greater than 70% bioburden recovery in comparison to a control medium. For the *Sabouraud Dextrose Agar Medium* used for *Total Combined Molds and Yeasts Count*, inoculate duplicate plates with 25–250 cfu of *Candida albicans* (ATCC No. 10231) and *Aspergillus brasiliensis* (ATCC No. 16404) to demonstrate a greater than 70% bioburden recovery in comparison to a control medium. For *Enterobacterial Count (Bile-Tolerant Gram-Negative Bacteria)*, appropriate dilutions of *Escherichia coli* (ATCC No. 8739) and *Salmonella typhimurium* (ATCC No. 13311) are used. Failure of the organism(s) to grow in the relevant medium invalidates that portion of the examination and necessitates a modification of the procedure by (1) an increase in the volume of diluent, the quantity of test material remaining the same, or by (2) the incorporation of a sufficient quantity of suitable inactivating agent(s) in the diluents, or by (3) an appropriate combination of modifications to (1) and (2) so as to permit growth of the inoculum.

The following are examples of ingredients and their concentrations that may be added to the culture medium to neutralize inhibitory substances present in the sample: soy lecithin, 0.5%; and polysorbate 20, 4.0%. Alternatively, repeat the test as described in the preceding paragraph, using *Fluid Casein Digest–Soy Lecithin–Polysorbate 20 Medium* to demonstrate neutralization of preservatives or other antimicrobial agents in the test material. Where inhibitory substances are contained in the product and the latter is soluble, a suitable, validated adaptation of a procedure set forth under *Procedure* using the *Membrane Filtration Method* may be used.

If, in spite of the incorporation of suitable inactivating agents and a substantial increase in the volume of diluent, it is still not possible to recover the viable cultures described above, and where the article is not suitable for the employment of membrane filtration, it can be assumed that the failure to isolate the inoculated organism is attributable to the bactericidal or bacteriostatic activity of such magnitude that treatments are not able to remove the activity. This information serves to indicate that the article is not likely to allow proliferation or contamination with the given species of microorganism. Monitoring should be continued in order to determine the inhibitory range and bactericidal activity of the article.

## BUFFER SOLUTION AND MEDIA

Culture media may be prepared as follows, or dehydrated culture media may be used provided that, when reconstituted as directed by the manufacturer or distributor, they have similar ingredients and/or yield media comparable to those obtained from the formulas given herein.

In preparing media by the formulas set forth herein, dissolve the soluble solids in the water, using heat if necessary to effect complete solution, and add solutions of hydrochloric acid or sodium hydroxide in quantities sufficient to yield the desired pH in the medium when it is ready for use. Determine the pH at 25 ± 2°.

Where agar is called for in a formula, use agar that has a moisture content of NMT 15%. Where water is called for in a formula, use *Purified Water*.

### pH 7.2 Phosphate Buffer

Prepare a stock solution by dissolving 34 g of monobasic potassium phosphate in about 500 mL of water contained in a 1000-mL volumetric flask. Adjust to a pH of 7.2 ± 0.1 by the addition of sodium hydroxide TS (about 175 mL), add water to volume, and mix. Dispense and sterilize. Store under refrigeration. For use, dilute the stock solution with water in the ratio of 1–800, dispense as desired, and sterilize.

[1] Available from ATCC, 10801 University Boulevard, Manassas, VA 20110-2209. Equivalent microorganisms, provided that they are from a national collection repository, can be used in lieu of ATCC strains. However, the viable microorganisms used in the test must not be more than five passages removed from the original ATCC or national collection culture.

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## Media

Prepare media for the tests as described below. Alternatively, dehydrated formulations may be used provided that, when reconstituted as directed by the manufacturer or distributor, they meet the requirements of *Growth Promotion Testing*. Unless otherwise indicated elsewhere in this chapter, media are sterilized in autoclaves using a validated process. The exposure time within the autoclave at 121° will depend on the volume of media to be sterilized. Thus, for example, a 500-mL volume would need to be autoclaved using a temperature and time relationship that will ensure that the medium has attained at least an $F_0$ of 12–15 in the sterilization process. However, the appropriate time and temperature duration for sterilizing prepared media at any given volume should be confirmed by a thermal penetration study using a thermocouple or thermoprobe placed within the liquid medium.

### FLUID CASEIN DIGEST–SOY LECITHIN–POLYSORBATE 20 MEDIUM

| | |
|---|---|
| Pancreatic Digest of Casein | 20 g |
| Soy Lecithin | 5 g |
| Polysorbate 20 | 40 mL |
| Water | 960 mL |

Dissolve *Pancreatic Digest of Casein* and *Soy Lecithin* in 960 mL of water, heating in a water bath at 48°–50° for about 30 min to effect solution. Add 40 mL of *Polysorbate 20*. Mix, dispense as desired, and sterilize.

### SOYBEAN–CASEIN DIGEST–AGAR MEDIUM

| | |
|---|---|
| Pancreatic Digest of Casein | 15.0 g |
| Papaic Digest of Soybean Meal | 5.0 g |
| Sodium Chloride | 5.0 g |
| Agar | 15.0 g |
| Water | 1000 mL |

pH after sterilization: 7.3 ± 0.2.

### FLUID SOYBEAN–CASEIN DIGEST MEDIUM

| | |
|---|---|
| Pancreatic Digest of Casein | 17.0 g |
| Papaic Digest of Soybean Meal | 3.0 g |
| Sodium Chloride | 5.0 g |
| Dibasic Potassium Phosphate | 2.5 g |
| Dextrose | 2.5 g |
| Purified Water | 1000 mL |

Dissolve the solids in the water, heating slightly to effect a solution. Cool the solution to room temperature, and adjust the pH with 1 N sodium hydroxide so that after sterilization it will have a pH of 7.3 ± 0.2. Filter, if necessary, and dispense into suitable containers. Sterilize at a temperature and time relationship that will ensure that the medium has attained at least an $F_0$ of 12–15 in the sterilization process, or by a validated filtration process.

### SABOURAUD DEXTROSE–AGAR MEDIUM

| | |
|---|---|
| Dextrose | 40.0 g |
| Mixture of Peptic Digest of Animal Tissue and Pancreatic Digest of Casein (1:1) | 10.0 g |
| Agar | 15.0 g |
| Water | 1000 mL |

Mix, and boil to effect solution.
pH after sterilization: 5.6 ± 0.2.

### VIOLET-RED BILE AGAR WITH GLUCOSE AND LACTOSE

| | |
|---|---|
| Yeast Extract | 3.0 g |

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| | |
|---|---|
| Pancreatic Digest of Gelatin | 7.0 g |
| Bile Salts | 1.5 g |
| Lactose | 10.0 g |
| Sodium Chloride | 5.0 g |
| D-Glucose Monohydrate | 10.0 g |
| Agar | 15.0 g |
| Neutral Red | 30 mg |
| Crystal Violet | 2 mg |
| Water | 1000 mL |

Adjust the pH so that it is 7.4 ± 0.2 after heating. Heat to boiling, but do not heat in an autoclave. Pour onto plates.

MOSSEL–ENTEROBACTERIACEAE ENRICHMENT BROTH

| | |
|---|---|
| Pancreatic Digest of Gelatin | 10.0 g |
| D-Glucose Monohydrate | 5.0 g |
| Dehydrated Ox Bile | 20.0 g |
| Monobasic Potassium Phosphate | 2.0 g |
| Dibasic Potassium Phosphate | 8.0 g |
| Brilliant Green | 15 mg |
| Water | 1000 mL |

Suspend the solids in water, and heat to boiling for 1–2 min. Transfer 120-mL portions to 250-mL volumetric flasks or 9-mL portions to test tubes, all being capped with cotton plugs or loose-fitting caps. Heat on a steam bath for 30 min. Adjust the pH so that it is 7.2 ± 0.2 after heating.

GROWTH PROMOTION TESTING

Each lot of dehydrated medium bearing the manufacturer's identifying number or each lot of medium prepared from basic ingredients must be tested for its growth-promoting qualities. Cultures of *Staphylococcus aureus* (ATCC No. 6538), *Escherichia coli* (ATCC No. 8739), *Bacillus subtilis* (ATCC No. 6633), *Candida albicans* (ATCC No. 10231), and *Aspergillus brasiliensis* (ATCC No. 16404) are used. A $10^{-3}$ dilution of a 24-hour broth culture of the microorganism to the first dilution (in *pH 7.2 Phosphate Buffer* or *Fluid Soybean–Casein Digest Medium*) may be used as the inocula. Serially streak plates of the media with the appropriate inocula to obtain isolated colonies to demonstrate the growth-promotion qualities of the *Soybean–Casein Digest Agar* and *Sabouraud Dextrose Agar* media. Inoculate the *Fluid Soybean–Casein Digest Medium* and *Mossel–Enterobacteriaceae Enrichment Broth* with 10–100 cfu of the appropriate challenge organisms to demonstrate their growth-promotion qualities.

## SAMPLING

Provide 10-mL or 10-g specimens for the tests called for in the individual monograph.

## PROCEDURE

Prepare the specimen to be tested by a treatment that is appropriate to its physical characteristics and that does not alter the number and kind of microorganisms originally present, in order to obtain a solution or suspension of all or part of it in a form suitable for the test procedure(s) to be carried out.

For a solid that dissolves to an appreciable extent but not completely, reduce the substance to a moderately fine powder, suspend it in the vehicle specified, and proceed as directed under *Total Aerobic Microbial Count*.

For a fluid specimen that consists of a true solution, or a suspension in water or a hydroalcoholic vehicle containing less than 30% of alcohol, and for a solid that dissolves readily and practically completely in 90 mL of *pH 7.2 Phosphate Buffer* or the media specified, proceed as directed under *Total Aerobic Microbial Count*.

For water-immiscible products, prepare a suspension with the aid of a minimal quantity of a suitable, sterile emulsifying agent (such as one of the polysorbates), using a mechanical blender and warming to a temperature not exceeding 45°, if necessary, and proceed with the suspension as directed under *Total Aerobic Microbial Count*.

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## Total Aerobic Microbial Count

For specimens that are freely soluble, use the *Membrane Filtration Method* or *Plate Method*. For specimens that are sufficiently soluble or translucent to permit use of the *Plate Method*, use that method; otherwise, use the *Multiple-Tube Method*. With either method, first dissolve or suspend 10.0 g of the specimen if it is a solid, or 10 mL, accurately measured, if the specimen is a liquid, in *pH 7.2 Phosphate Buffer, Fluid Soybean–Casein Digest Medium*, or *Fluid Casein Digest–Soy Lecithin–Polysorbate 20 Medium* to make 100 mL. For viscous specimens that cannot be pipeted at this initial 1:10 dilution, dilute the specimen until a suspension is obtained, i.e., 1:50 or 1:100, etc., that can be pipeted. Perform the test for absence of inhibitory (antimicrobial) properties as described under *Preparatory Testing* before the determination of *Total Aerobic Microbial Count*. Add the specimen to the medium NMT 1 h after preparing the appropriate dilutions for inoculation.

### MEMBRANE FILTRATION METHOD

Dilute the fluid further, if necessary, so that 1 mL will be expected to yield 30–300 colonies. Pipet 1 mL of the final dilution into 5–10 mL of *pH 7.2 Phosphate Buffer, Fluid Soybean–Casein Digest Medium*, or *Fluid Casein Digest–Soy Lecithin–Polysorbate 20 Medium*. Wash each membrane with an appropriate amount of one of the above diluents. Transfer each membrane to a Petri dish containing *Soybean–Casein Digest–Agar Medium*, previously solidified at room temperature. Incubate the plates at a temperature 30°–35° for 48–72 h. Following incubation, examine the plates for growth, count the number of colonies, and express the average for the two plates in terms of the number of microorganisms per g or per mL of specimen. If no microbial colonies are recovered from the dishes representing the initial 1:10 dilution of the specimen, express the results as “less than 10 microorganisms per g or per mL of specimen”.

### PLATE METHOD

Dilute the fluid further, if necessary, so that 1 mL will be expected to yield 30–300 colonies. Pipet 1 mL of the final dilution onto each of two sterile Petri dishes. Promptly add to each dish 15–20 mL of *Soybean–Casein Digest–Agar Medium*, previously melted and cooled to about 45°. Cover the Petri dishes, mix the sample with agar by gently tilting or rotating the dishes, and allow the contents to solidify at room temperature. Invert the Petri dishes and incubate for 48–72 h. Following incubation, examine the plates for growth, count the number of colonies, and express the average for the two plates in terms of the number of microorganisms per g or per mL of specimen. If no microbial colonies are recovered from the dishes representing the initial 1:10 dilution of the specimen, express the results as “less than 10 microorganisms per g or per mL of specimen”.

### MULTIPLE-TUBE METHOD

Into each of 14 test tubes of similar size, place 9.0 mL of sterile *Fluid Soybean–Casein Digest Medium*. Arrange 12 of the tubes in four sets of three tubes each. Put aside one set of three tubes to serve as the controls. Into each of three tubes of one set (“100”) and into a fourth tube (A) pipet 1 mL of the solution or suspension of the specimen, and mix. Pipet 1 mL from tube A into the one remaining tube (B), not included in a set, and mix. These two tubes contain 100 mg or 100 µL and 10 mg or 10 µL of the specimen, respectively. Into each of the second set (“10”) of three tubes pipet 1 mL from tube A, and into each tube of the third set (“1”) pipet 1 mL from tube B. Discard the unused contents of tubes A and B. Close well, and incubate all of the tubes. Following incubation, examine the tubes for growth: the three control tubes remain clear, and the observations in the tubes containing the specimen, when interpreted by reference to *Table 1*, indicate the most probable number of microorganisms per g or per mL.

**Table 1. Most Probable Count by Multiple-Tube Method**

| Observed Combinations of Numbers of Tubes Showing Growth in Each Set | | | Most Probable Number of Microorganisms per g or per mL |
|---|---|---|---|
| Number of mg or µL of specimen per tube | | | |
| 100 | 10 | 1 | |
| 3 | 3 | 3 | More than 1100 |
| 3 | 3 | 2 | 1100 |
| 3 | 3 | 1 | 500 |
| 3 | 3 | 0 | 200 |
| 3 | 2 | 3 | 290 |
| 3 | 2 | 2 | 210 |
| 3 | 2 | 1 | 150 |
| 3 | 2 | 0 | 90 |
| 3 | 1 | 3 | 160 |
| 3 | 1 | 2 | 120 |
| 3 | 1 | 1 | 70 |
| 3 | 1 | 0 | 40 |
| 3 | 0 | 3 | 95 |

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**Table 1. Most Probable Count by Multiple-Tube Method** *(continued)*

| Observed Combinations of Numbers of Tubes Showing Growth in Each Set | | | Most Probable Number of Microorganisms per g or per mL |
|---|---|---|---|
| Number of mg or µL of specimen per tube | | | |
| 100 | 10 | 1 | |
| 3 | 0 | 2 | 60 |
| 3 | 0 | 1 | 40 |
| 3 | 0 | 0 | 23 |
| 2 | 2 | 0 | 21 |
| 2 | 1 | 1 | 20 |
| 2 | 1 | 0 | 15 |
| 2 | 0 | 1 | 14 |
| 2 | 0 | 0 | 9 |
| 1 | 2 | 0 | 11 |
| 1 | 1 | 0 | 7 |
| 1 | 0 | 0 | 4 |
| 0 | 1 | 0 | 3 |
| 0 | 0 | 0 | <3 |

## Total Combined Molds and Yeasts Count

PROCEDURE

Proceed as directed for *Membrane Filtration Method* or *Plate Method* under *Total Aerobic Microbial Count*, except to use the same amount of *Sabouraud Dextrose–Agar Medium* instead of *Soybean–Casein Digest–Agar Medium* and to incubate the plates for 5–7 days at 20°–25°.

RETEST

For the purpose of confirming a doubtful result by any of the procedures outlined in the foregoing tests following their application to a 10-g specimen, a retest on an additional 10-g specimen from the original sample and a 10-g specimen from the new sample of the nutritional supplement may be conducted. Proceed as directed under *Procedure*.

## Enterobacterial Count (Bile-Tolerant Gram-Negative Bacteria)

Dissolve or suspend the sample in a sufficient volume of *pH 7.2 Phosphate Buffer* or *Fluid Soybean–Casein Digest Medium* and dilute with *Fluid Soybean–Casein Digest Medium* to 100 mL. Pre-incubate for 2–5 h at 20°–25° in soybean–casein digest broth diluent; inoculate suitable quantities of *Mossel–Enterobacteriaceae Enrichment Broth* to contain 0.1, 0.01, or 0.001 g or mL of the product. Incubate at 30°–35° for 24–48 h. Subculture onto a plate of *Violet-Red Bile Agar with Glucose and Lactose*, and incubate at 30°–35° for 18–24 h. Growth of well developed, generally red or reddish, colonies of Gram-negative bacteria reveal the presence of enterobacteria. Determine the most probable number of microorganisms per g or per mL by reference to *Table 2*.

**Table 2. Most Probable Enterobacterial Count**

| Observed Presence of Enterobacteria | | | Most Probable Number of Enterobacteria per g or per mL |
|---|---|---|---|
| Number of g or mL of specimen per tube | | | |
| 0.1 | 0.01 | 0.001 | |
| + | + | + | More than $10^3$ |
| + | + | – | Fewer than $10^3$ but more than $10^2$ |
| + | – | – | Fewer than $10^2$ but more than $10^1$ |
| – | – | – | Fewer than $10^1$ |

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# ⟨2022⟩ MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS—NUTRITIONAL AND DIETARY SUPPLEMENTS

## INTRODUCTION

Good manufacturing practices require that objectionable organisms be absent from nonsterile nutritional and dietary products. A microorganism can be considered objectionable if it represents a potential health hazard to the user who is using the product as directed, or if it is capable of growing in the product. Objectionable microorganisms are defined as contaminants that, depending on the microbial species, number of organisms, dosage form, intended use, and patient population, would adversely affect product safety. Additionally, microorganisms may be deemed objectionable if they adversely affect product stability or if they may damage the integrity of the container closure system.

This chapter describes the testing of nutritional and dietary articles for specified microorganisms, which are specified in the individual monographs or whose absence is recommended by the guidance under *Microbiological Attributes of Nonsterile Nutritional and Dietary Supplements* ⟨2023⟩. When objectionable microorganisms are not specified in the individual monograph, it is the manufacturers' responsibility to determine which microorganisms in their products are objectionable. It is not intended that all nonsterile nutritional and dietary articles be tested for the absence of all of the microorganisms mentioned in this chapter, nor is the testing of relevant microorganisms restricted to those presented in this chapter.

Alternative microbiological, physicochemical, and biotechnological methods, including automated methods, may be substituted for these tests, provided they have been validated as being equivalent in their suitability for determining compliance.

***Change to read:***

## BUFFER AND MEDIA

### General Considerations

See *Buffer Solution and Media* under *Microbial Enumeration Tests—Nutritional and Dietary Supplements* ⟨2021⟩. The appropriateness of each medium for the intended purpose is to be assessed. Control sets of *Fluid Soybean–Casein Digest Medium* for *Preparatory Testing* are also used to assess the appropriateness of these media in the growth promotion of the specified microorganisms. For other media, streak agar plates to obtain isolated colonies of appropriate microorganisms, and inoculate the fluid media with the appropriate microorganisms at a final concentration of less than 100 cfu per mL. Observe the growth to establish the appropriateness of the media.

### Buffer

#### BUFFER STOCK SOLUTION and pH 7.2 PHOSPHATE BUFFER

Proceed as directed under *Microbial Enumeration Tests—Nutritional and Dietary Supplements* ⟨2021⟩.

### Media

#### FLUID SOYBEAN–CASEIN DIGEST MEDIUM

Prepare as directed under *Microbial Enumeration Tests—Nutritional and Dietary Supplements* ⟨2021⟩.

#### MANNITOL–SALT–AGAR MEDIUM

| | |
|---|---|
| Pancreatic Digest of Casein | 5.0 g |
| Peptic Digest of Animal Tissue | 5.0 g |
| Beef Extract | 1.0 g |
| D-Mannitol | 10.0 g |
| Sodium Chloride | 75.0 g |
| Agar | 15.0 g |
| Phenol Red | 0.025 g |
| Water | 1000 mL |

Mix, then heat with frequent agitation, and boil for 1 minute to effect solution.
pH after sterilization: 7.4 ± 0.2.

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## FLUID TETRATHIONATE MEDIUM

| | |
|---|---|
| Pancreatic Digest of Casein | 2.5 g |
| Peptic Digest of Animal Fat | 2.5 g |
| Bile Salts | 1.0 g |
| Calcium Carbonate | 10.0 g |
| Sodium Thiosulfate | 30.0 g |
| Water | 1000 mL |

Heat to boiling. Do not autoclave; use the same day. Immediately before use, add a solution prepared by dissolving 5 g of potassium iodide and 6 g of iodine in 20 mL of water. Then add 10 mL of a solution of brilliant green (1 in 1000), and mix. Do not heat after adding the brilliant green solution.

## BRILLIANT GREEN–AGAR MEDIUM

| | |
|---|---|
| Yeast Extract | 3.0 g |
| Peptic Digest of Animal Tissue | 5.0 g |
| Pancreatic Digest of Casein | 5.0 g |
| Lactose | 10.0 g |
| Sodium Chloride | 5.0 g |
| Sucrose | 10.0 g |
| Phenol Red | 80.0 g |
| Agar | 20.0 g |
| Brilliant Green | 12.5 mg |
| Water | 1000 mL |

Boil for 1 minute. Sterilize just prior to use, melt, pour into Petri dishes, and allow to cool.
pH after sterilization: 6.9 ± 0.2

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## XYLOSE–LYSINE–DESOXYCHOLATE–AGAR MEDIUM

| | |
|---|---|
| Xylose | 3.5 g |
| L-Lysine | 5.0 g |
| Lactose | 7.5 g |
| Sucrose | 7.5 g |
| Sodium Chloride | 5.0 g |
| Yeast Extract | 3.0 g |
| Phenol Red | 80 mg |
| Agar | 13.5 g |
| Sodium Desoxycholate (as Bile Salts) | 2.5 g |
| Sodium Thiosulfate | 6.8 g |
| Ferric Ammonium Citrate | 800 mg |
| Water | 1000 mL |

Heat, with swirling, just to the boiling point. Do not overheat or sterilize. Transfer at once to a water bath maintained at about 50°, and pour into Petri plates as soon as the *Medium* has cooled.
Final pH: 7.4 ± 0.2.

## HEKTOEN ENTERIC AGAR MEDIUM

| | |
|---|---|
| Protease Peptone | 12.0 g |
| Yeast Extract | 3.0 g |
| Lactose | 12.0 g |

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| | |
|---|---|
| Sucrose | 2.0 g |
| Salicin | 9.0 g |
| Bile Salts No. 3 | 9.0 g |
| Sodium Chloride | 5.0 g |
| Sodium Thiosulfate | 5.0 g |
| Ferric Ammonium Citrate | 1.5 g |
| Acid Fuchsin | 0.1 g |
| Bromothymol Blue | 65 mg |
| Agar | 14.0 g |
| Water | 1000 mL |

Mix, and allow to stand for 10 minutes. Heat gently, and allow to boil for a few seconds to dissolve the agar. Do not sterilize. Cool to 60°, and pour into Petri dishes.
Final pH: 7.5 ± 0.2.

### TRIPLE SUGAR–IRON–AGAR MEDIUM

| | |
|---|---|
| Pancreatic Digest of Casein | 10.0 g |
| Pancreatic Digest of Animal Tissue | 10.0 g |
| Lactose | 10.0 g |
| Sucrose | 10.0 g |
| Dextrose | 1.0 g |
| Ferrous Ammonium Sulfate | 200 mg |
| Sodium Chloride | 5.0 g |
| Sodium Thiosulfate | 200 mg |
| Agar | 13.0 g |
| Phenol Red | 25 mg |
| Water | 1000 mL |

pH after sterilization: 7.3 ± 0.2.

### MACCONKEY AGAR MEDIUM

| | |
|---|---|
| Pancreatic Digest of Gelatin | 17.0 g |
| Pancreatic Digest of Casein | 1.5 g |
| Peptic Digest of Animal Tissue | 1.5 g |
| Lactose | 10.0 g |
| Bile Salts Mixture | 1.5 g |
| Sodium Salts Mixture | 5.0 g |
| Agar | 13.5 g |
| Neutral Red | 30 mg |
| Crystal Violet | 1.0 mg |
| Water | 1000 mL |

Boil for 1 minute to effect solution.
pH after sterilization: 7.1 ± 0.2.

### LEVINE EOSIN–METHYLENE BLUE–AGAR MEDIUM

| | |
|---|---|
| Pancreatic Digest of Gelatin | 10.0 g |
| Dibasic Potassium Phosphate | 2.0 g |
| Agar | 15.0 g |

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| | |
|---|---|
| Lactose | 10.0 g |
| Eosin Y | 400 mg |
| Methylene Blue | 65 mg |
| Water | 1000 mL |

Dissolve pancreatic digest of gelatin, dibasic potassium phosphate, and agar in water, with warming, and allow to cool. Just prior to use, liquefy the gelled agar solution, and add the remaining ingredients, as solutions, in the following amounts: for each 100 mL of the liquefied agar solution, add 5 mL of lactose solution (1 in 5), 2 mL of the eosin Y solution (1 in 50), and 2 mL of methylene blue solution (1 in 300). Mix. The finished *Medium* may not be clear.

pH after sterilization: 7.1 ± 0.2.

### BAIRD–PARKER AGAR MEDIUM

| | |
|---|---|
| Pancreatic Digest of Casein | 10.0 g |
| Beef Extract | 5.0 g |
| Yeast Extract | 1.0 g |
| Lithium Chloride | 5.0 g |
| Agar | 20.0 g |
| Glycine | 12.0 g |
| Sodium Pyruvate | 10.0 g |
| Water | 950 mL |

Heat with frequent agitation, and boil for 1 minute. Sterilize, cool to between 45° and 50°, and add 10 mL of sterile potassium tellurite solution (1 in 100) and 50 mL of egg yolk emulsion prepared as follows. Disinfect the surface of whole-shell eggs, aseptically crack the eggs, transfer intact yolks to a sterile graduated cylinder, add sterile saline TS to obtain a 3 to 7 ratio of egg yolk to saline, add to a sterile blender cup, and mix at high speed for 5 seconds. Mix all ingredients well but gently, and pour into plates.

pH after sterilization: 6.8 ± 0.2.

### VOGEL–JOHNSON AGAR MEDIUM

| | |
|---|---|
| Pancreatic Digest of Casein | 10.0 g |
| Yeast Extract | 5.0 g |
| Mannitol | 10.0 g |
| Dibasic Potassium Phosphate | 5.0 g |
| Lithium Chloride | 5.0 g |
| Glycine | 10.0 g |
| Agar | 16.0 g |
| Phenol Red | 25.0 mg |
| Water | 1000 mL |

Boil for 1 minute. Sterilize, cool to between 45° and 50°, and add 20 mL of sterile potassium tellurite solution (1 in 100).

pH after sterilization: 7.2 ± 0.2.

### FLUID SELENITE–CYSTINE MEDIUM

| | |
|---|---|
| Pancreatic Digest of Casein | 5.0 g |
| Lactose | 4.0 g |
| Sodium Phosphate | 10.0 g |
| Sodium Acid Selenite | 4.0 g |
| L-Cystine | 10.0 ▲mg▲ (ERR 1-Nov-2019) |
| Water | 1000 mL |

Mix, and heat to effect solution. Then heat in flowing stream for 15 minutes. Do not sterilize.

Final pH: 7.0 ± 0.2.

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REINFORCED MEDIUM FOR CLOSTRIDIA

| | |
|---|---|
| Beef Extract | 10.0 g |
| Peptone | 10.0 g |
| Yeast Extract | 3.0 g |
| Soluble Starch | 1.0 g |
| Glucose Monohydrate | 5.0 g |
| Cysteine Hydrochloride | 0.5 g |
| Sodium Chloride | 5.0 g |
| Sodium Acetate | 3.0 g |
| Agar | 0.5 g |
| Water | 1000 mL |

Dissolve agar in water by heating to boiling, while stirring continuously. Adjust the pH if necessary, and sterilize.
pH after sterilization: 6.8 ± 0.2.

COLUMBIA AGAR

| | |
|---|---|
| Pancreatic Digest of Casein | 10.0 g |
| Meat Peptic Digest | 5.0 g |
| Heart Pancreatic Digest | 3.0 g |
| Yeast Extract | 5.0 g |
| Cornstarch | 1.0 g |
| Sodium Chloride | 5.0 g |
| Agar | 15.0 g |
| Water | 1000 mL |

Dissolve agar in water by heating to boiling and with continuous stirring. If necessary, adjust the pH. Sterilize, and allow to cool to 45° to 50°. Add, when necessary, gentamicin sulfate, equivalent to about 20 mg of gentamicin base, and pour into Petri dishes.
Pre-reduction of the medium is recommended.
pH after sterilization: 7.3 ± 0.2.

RAPPAPORT VASSILIADIS SALMONELLA ENRICHMENT BROTH

| | |
|---|---|
| Soya Peptone | 4.5 g |
| Magnesium Chloride Hexahydrate | 29.0 g |
| Sodium Chloride | 8.0 g |
| Dipotassium Phosphate | 0.4 g |
| Potassium Dihydrogen Phosphate | 0.6 g |
| Malachite Green | 0.036 g |
| Purified Water | 1000 mL |

Dissolve, warming slightly. Sterilize in an autoclave using a validated cycle, at a temperature not exceeding 115°.
The pH is 5.2 ± 0.2 at 25° after heating and autoclaving.

## PREPARATORY TESTING

Proceed as directed for *Preparatory Testing* under *Microbial Enumeration Tests—Nutritional and Dietary Supplements* ⟨2021⟩.
For enrichment broth, selective media, and differential media use an inoculating loop to transfer the inoculum of each test organism to the plated or liquid media being tested. If a plated medium is being tested, streak the surface of plate with the loop in four directions to obtain a pattern of isolated colonies. Incubate the media, and examine the plated or liquid media for the characteristic growth of the inocula (See *Tables 1*, *2*, *3*, and *4*).

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## SAMPLING

Proceed as directed for *Sampling* under *Microbial Enumeration Tests—Nutritional and Dietary Supplements* ⟨2021⟩.

## TEST PROCEDURES

**Test Preparation—**Prepare as directed for *Sampling.* Transfer to a suitable container with 100 mL of *Fluid Soybean–Casein Digest Medium (FSCD).* Mix by shaking gently. [NOTE—On the basis of results for *Preparatory Testing,* modify the *Test Preparation* as appropriate.]

### Test for Absence of *Staphylococcus aureus*

Incubate at 30° to 35° for 18 to 24 hours. Streak a loopful from *FSCD* onto the surface of one or more of the following media: *Vogel–Johnson Agar Medium (VJ Agar), Mannitol–Salt–Agar Medium (MS-Agar),* and *Baird-Parker Agar Medium (BP Agar).* Cover the Petri plates, invert them, and incubate at 30° to 35° for 24 to 48 hours.

Examine the plates of *VJ Agar, MS-Agar,* and/or *BP Agar*, and interpret the results with reference to *Table 1:* if no plate contains colonies having the characteristics described, the test specimen meets the requirement for the absence of *Staphylococcus aureus.* If characteristic colonies are present, perform coagulase test as follows. Transfer representative colonies to separate tubes containing 0.5 mL of rabbit plasma, horse plasma, or any other mammalian plasma. Incubate in a water bath at 37°. Examine for coagulation after 3 hours of incubation and at suitable intervals up to 24 hours. Comparing with positive and negative controls, the absence of a coagulase reaction indicates the absence of *Staphylococcus aureus* in the tested article.

**Table 1. Characteristics of *Staphylococcus aureus* on Specified Agar Media**

| Agar Medium | Colonial Morphology | Gram Stain |
|---|---|---|
| *Vogel–Johnson* | Black surrounded by yellow zone | (+), cocci |
| *Mannitol–Salt* | Yellow colonies with yellow zone | (+), cocci |
| *Baird–Parker* | Black, shiny surrounded by 2–5-mm clear zones | (+), cocci |

### Test for Absence of *Salmonella* Species

Incubate at 30° to 35° for 18 to 24 hours. From *FSCD,* pipet a 1-mL aliquot into 10 mL of *Rappaport Vassiliadis Salmonella Enrichment Broth,* mix, and incubate at 30° to 35° for 18 to 24 hours. Streak a loopful from both incubated media onto individual surfaces of one or more of following media: *Brilliant Green Agar Medium (BG-Agar), Xylose–Lysine–Desoxycholate–Agar Medium (XLDC-Agar),* and *Hektoen Enteric Agar Medium (HE Agar).* Cover, invert the plates, and incubate at 30° to 35° for 24 to 48 hours. Examine the inoculated plates of *BG-Agar, XLDC-Agar,* and/or *HE Agar,* and interpret the results with reference to *Table 2*: if no colonies having the characteristics described are observed, the test specimen meets the requirement for the absence of *Salmonella* species. If colonies with characteristics described in *Table 2* are present, the suspect colonies are transferred to a slant of *Triple Sugar–Iron–Agar Medium (TSI)* using an inoculating wire, by first streaking the surface of the slant, and then stabbing the wire well beneath the surface. Incubate at 30° to 35° for 24 to 48 hours. If the tubes do not have red alkaline slants and yellow acid butts, with or without concomitant blackening of the butts from hydrogen sulfide production, the test specimen meets the requirement for the absence of *Salmonella* species.

**Table 2. Characteristics of *Salmonella* Species on Specified Agar Media**

| Agar Medium | Colonial Morphology | Gram Stain |
|---|---|---|
| *Brilliant Green* | Small, transparent and colorless; or opaque, pink or white (often surrounded by pink to red zone) | (–), rods |
| *Xylose–Lysine–Desoxycholate* | Red, with or without black centers | (–), rods |
| *Hektoen Enteric* | Blue-green, with or without black centers | (–), rods |

### Test for Absence of *Escherichia coli*

Incubate at 30° to 35° for 24 to 48 hours. From *FSCD,* pipet a 1-mL aliquot into a container containing 10 mL of *MacConkey Broth,* mix, and incubate at 42° to 44° for 24 to 48 hours. Streak a loopful from both incubated media onto individual surfaces of *MacConkey Agar Medium (MC Agar),* and incubate at 30° to 35° for 18 to 24 hours. Examine the inoculated *MC Agar* plate, and interpret the results with reference to *Table 3*: if no colonies having the characteristics described are observed, the test specimen meets the requirement for the absence of *Escherichia coli.* Suspect colonies showing the characteristics described in *Table 3* are transferred individually, using an inoculating loop, to the surface of a plate with *Levine Eosin–Methylene Blue–Agar Medium (LEMB-Agar).* If a large number of suspect colonies are to be transferred, divide the surface of each plate into quadrants, each quadrant being inoculated with a different colony. Cover the plates, invert, and incubate at 30° to 35° for 24 to 48 hours. If none of the colonies exhibit a characteristic metallic sheen under reflected light, and if none exhibit a blue-black appearance under transmitted light, the test specimen meets the requirement for the absence of *Escherichia coli.*

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**Table 3. Characteristics of *Escherichia coli* on *MacConkey Agar Medium***

| Colonial Morphology | Gram Stain |
|---|---|
| Brick red, may have surrounding zone of precipitated bile | (–), rods |

## Test for Absence of *Clostridium* Species

### Test Preparation

Prepare as directed for *Sampling.* [Note—On the basis of results for *Preparatory Testing,* modify the *Test Preparation* as appropriate.]

Procedure—Take two equal portions of the *Test Preparation,* heat one to 80° for 10 minutes, and cool rapidly. Transfer 10 mL of each portion to separate containers, each containing 100 mL of *Reinforced Medium for Clostridia,* and incubate under anaerobic conditions at 35° to 37° for 48 hours. After incubation, subculture each specimen on *Columbia Agar Medium* to which gentamicin has been added, and incubate under anaerobic conditions at 35° to 37° for 48 hours. Examine the plates, and interpret with reference to *Table 4*: if no growth of microorganisms is detected, the test specimen meets the requirement for the absence of *Clostridium* species.

**Table 4. Characteristics of *Clostridium* Species on Specified Media**

| Medium | Gram Stain | Catalase |
|---|---|---|
| *Reinforced Medium for Clostridia* | (+), rods | Negative |
| *Columbia Agar* | (+), rods | |

If growth occurs, subculture each distinct colony on *Columbia Agar Medium,* and separately incubate in aerobic and in anaerobic conditions at 35° to 37° for 48 hours. The occurrence of only anaerobic growth of gram-positive bacilli, giving a negative catalase reaction, indicates the presence of *Clostridium sporogenes.* To perform the catalase test, transfer discrete colonies to glass slides, and apply a drop of dilute hydrogen peroxide solution: the reaction is negative if no gas bubbles evolve. If the test specimen exhibits none of these characteristics, it meets the requirement for the absence of *Clostridium* species.

### Retest

For the purpose of confirming a doubtful result by any of the procedures outlined in the foregoing tests following their application to a 10 g specimen, a retest on a 25 g specimen of the nutritional or dietary supplement may be conducted. Proceed as directed under *Procedure,* but make allowances for the larger specimen size.

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# ⟨2023⟩ MICROBIOLOGICAL ATTRIBUTES OF NONSTERILE NUTRITIONAL AND DIETARY SUPPLEMENTS

## INTRODUCTION

The raw materials, pharmaceutical ingredients, and active ingredients used in the manufacture of nutritional and dietary articles may range from chemically synthesized vitamins to plant extracts and animal byproducts, and these ingredients are typically not sterile. Considerable experience has accrued with these highly refined plant- and animal-derived pharmaceutical ingredients, such as microcrystalline cellulose, modified starch, lactose, and magnesium stearate, and their microbiological attributes are well established. Botanicals may be microbiologically contaminated at any point during cultivation, harvesting, processing, packing, and distribution. Major sources of microbial contamination are associated with human or animal feces used as plant manure; contaminated irrigation water and/or process water; and poor worker hygiene and sanitation practices during harvesting, sorting, processing, packaging, and transportation. Furthermore, it is essential that microbiological contamination be minimized during the manufacture of nonsterile dietary supplements. To achieve this, Good Manufacturing Practices are employed and adequate microbiological specifications are established.

Microbiological process control, control of the bioburden of raw materials, and control of the manufacturing process to minimize cross-contamination are necessary to guarantee acceptable microbial quality in the final dosage forms. Because nonaqueous or dry dosage forms do not support microbial growth because of low water activity, the microbial quality of such articles is a function of the microorganisms introduced through ingredients or during processing. In addition to considering the intended use of the product, the frequency of microbial testing for the finished nonsterile dietary supplement would be a function of the historical microbial testing database of that product, knowledge of the manufacturing processes, the susceptibility of the formulation to microbial proliferation, and the demonstrated effectiveness of programs controlling the raw materials.

## FORMULATION AND PROCESS DESIGN

From a microbiological perspective, the development of the formulation of nutritional or dietary supplements includes an evaluation of raw materials and their suppliers and the contribution made to the products by each ingredient and the manufacturing processes. Characterization of these elements allows the adequacy of the manufacturing process to be demonstrated. For example, if a product is formulated with an ingredient of botanical or animal origin known to possess a high, variable, or unpredictable level of microbiological contamination, it is necessary to ensure that the microbiological monitoring identifies ingredients that have an inappropriate bioburden level and that a premanufacturing process such as drying, extraction, heat treatment, irradiation, or gaseous sterilization treatment will inactivate or remove any objectionable contaminant possibly present.

However, the selected treatment technique should not have any adverse effects. The treatment of raw materials by irradiation and ethylene oxide may cause unwanted changes affecting the safety and efficacy of the raw material. For instance, when treated by ethylene oxide, crude extracts containing alkaloids have shown reduced contents of alkaloids. Dry heat treatment has been used for inactivation as well, but requires further evaluation because it may adversely affect stability and degradation of the raw material. With regard to the design of the manufacturing process, appropriate consideration should be given to the microbiological effect of wet granulation manufacturing processes. Wetting of a dry powder can result in increased levels of microorganisms if the granulation is stored prior to drying. However, it is recognized that the pressure and temperature associated with compression of tablets will decrease microbial counts. Antimicrobial activity is also achieved, especially with aqueous preparations, by the addition of chemicals that have known antimicrobial properties and that are compatible with the formulation.

However, antimicrobial preservation is not a substitute for Good Manufacturing Practices. A process has to be designed to minimize the microbiological population. Operating procedures, temperatures, and time limits, including holding times, are established to protect the product from microbiological contamination and growth. All processes have to be validated for their intended purposes. Moreover, in-process manufacturing and testing controls necessary for microbiological quality should be identified and implemented.

## FACILITIES, EQUIPMENT, WATER, AND SANITIZATION

### Facilities

The facilities, including the building and the heating, ventilation, and air-conditioning (HVAC) systems, should be designed to minimize microbiological contamination. The design of facilities used for the manufacture of supplements and their operating parameters should be documented, and the documentation should include, when appropriate, HVAC filter types, space pressure differentials, temperature, and relative humidity and air changes. Dry products processed in a dry environment do not possess a high potential for increased microbial levels. However, some control is warranted to minimize microbiological and chemical contamination. Potentially problematic areas are those that utilize *Purified Water* for wet granulation, batching liquid products, and film-coating tablets, because water encourages microbial growth.

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## Equipment

Equipment used for the processing of semisolid and dry supplements should be designed to promote sanitary conditions, to be self-drying, and to be easy to clean. Dryers, ovens, wet granulation equipment, bulk tanks, and equipment for preparation of coating solutions are periodically evaluated to ensure that cleaning procedures are adequate.

## Water

As one of the major components in nutritional and dietary supplement manufacturing processes, water deserves a special consideration in the microbiological control of these articles. It is a growth medium for a variety of microorganisms that present a threat to product quality, safety, preservation, and stability. Water may even act as a carrier of objectionable microorganisms. In view of this, water used in manufacturing is *Purified Water*. For the manufacture of raw materials, process water that meets specific microbiological objectives and U.S. Environmental Protection Agency National Drinking Water standards or equivalent European and Japanese standards may be used.

## Cleaning and Sanitization

Detailed and specific cleaning and sanitization procedures should be evaluated, developed, and validated, with special attention given to product contact surfaces. Personnel should possess sufficient knowledge of these procedures.

# SUPPLEMENT COMPONENTS

Raw materials, excipients, and active substances as components of nutritional and dietary supplements can be a primary source of microbiological contamination. Specifications should be developed and sampling plans and test procedures should be employed to guarantee the desired microbiological attributes of these materials. The nature and extent of microbiological testing should be based upon a knowledge of the material's origin, its manufacturing process, use, historical data, and experience. For instance, materials of animal or botanical origin that are not highly refined might require special, more frequent testing than synthetic products.

Since members of the family Enterobacteriaceae are a major component of the normal epiphytic and endophytic microflora (e.g., members of genera *Klebsiella, Enterobacter*, and *Erwinia*) and have been associated with the seeds, pods, roots, leaves, and stems of plants of economic importance, coliform or Enterobacteriaceae counts will not be an appropriate general microbiological criterion for botanicals. However, when it is considered advantageous, coliform or Enterobacteriaceae counts may be included in the individual monographs. Typically on new leaves, bacteria predominate in the microflora, while yeast and filamentous fungi succeed bacteria and become dominant late in the growing season. With dried botanicals, the bacterial population will tend to change from Gram-negative bacteria to Gram-positive spore formers and fungi. Refinement of botanicals from chopped or powdered plant material to powdered extracts using alcoholic, alkaline, acid hydro-alcoholic, or aqueous extracting materials will reduce the likelihood of vegetative microorganisms within the botanical material. The classification of botanical materials is contained in *Table 1*.

**Table 1. Definitions of a Range of Botanical Materials**

| Botanical Preparation | Definition |
|---|---|
| Chopped or Powdered Botanicals | Hand-picked portions of the botanical (e.g., leaves, flowers, roots, tubers, etc.) that are air dried, chopped, flaked, sectioned, ground, or pulverized to the consistency of a powder. |
| Botanical Extracts | Extracts are solids or semisolid preparations of a botanical that are prepared by percolation, filtration, and concentration by evaporation of the percolate. The extracting material may be alcoholic, alkaline, acid hydro-alcoholic, or aqueous in nature. Typically, an extract is 4–10 times as strong as the original botanical. The extracts may be semisolids or dry powders termed powdered extracts. |
| Tinctures | Tinctures are solutions of botanical substances in alcohol obtained by extraction of the powdered, flaked, or sectioned botanical. |
| Infusions | Infusions are solutions of botanical principles obtained by soaking the powdered botanical in hot or cold water for a specified time and straining. Typically, infusions are 5% in strength. |
| Decoctions | Decoctions are solutions of botanicals prepared by boiling the material in water for at least 15 min and straining. Typically, decoctions are 5% in strength. |
| Fluidextracts | A fluidextract is an alcoholic liquid extract made by percolation of a botanical so that 1 mL of the fluidextract represents 1 g of the botanical. |
| Botanicals to be treated with boiling water before use | Dried botanicals to which boiling water is added immediately prior to consumption. |

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# MICROBIOLOGICAL TESTING

## Frequency of Sampling and Testing

Microbiological attribute sampling and testing plans vary widely. In some cases, no sampling or testing is necessary; in other cases, periodic monitoring is warranted; and yet for some articles, each batch requires sampling and testing. The design of the sampling and testing plans and the kind of attributes examined depend on the application and the type of the product, the potential for contamination from components and processing, the growth promotion or inhibition properties of the formulation, and the target population for the supplement. For example, a powdered botanical may have highly variable microbiological attributes so that an incoming batch would be sampled and composite testing would not be advised, while a highly refined botanical extract may not require routine microbial testing. Similarly, products with a low water activity will not be susceptible to microbial growth during their shelf life provided they are protected from elevated humidity by their containers.

## Microbial Enumeration Tests

See the *Introduction* under *Microbial Enumeration Tests—Nutritional and Dietary Supplements* ⟨2021⟩. These tests provide meaningful information regarding the microbiological acceptability of excipients, active substances, and nonsterile supplement formulations. If the individual monograph does not specify microbial enumeration limits, the guidance provided in this chapter is used. Acceptable general limits of microbial levels for raw materials, excipients, and botanical products are shown in *Table 2*; and those for raw materials, excipients, active ingredients, and other nonsterile finished articles that are nutritional supplements, but do not contain botanicals, are shown in *Table 3*.

**Table 2. Recommended Microbial Limits for Botanical Ingredients and Products**

| Material | Recommended Microbial Limit Requirements (cfu/g or mL) |
|---|---|
| Dried or Powdered Botanicals | Total aerobic microbial count NMT $10^5$ |
| | Total combined yeasts and molds count NMT $10^3$ |
| | Bile-tolerant Gram-negative bacteria NMT $10^3$ |
| | Absence of *Salmonella* spp. and *E. coli* in 10 g |
| Powdered Botanical Extracts | Total aerobic microbial count NMT $10^4$ |
| | Total combined yeasts and molds count NMT $10^3$ |
| | Absence of *Salmonella* spp. and *E. coli* in 10 g |
| Tinctures | Total aerobic microbial count NMT $10^4$ |
| | Total combined yeasts and molds count NMT $10^3$ |
| Fluidextracts | Total aerobic microbial count NMT $10^4$ |
| | Total combined yeasts and molds count NMT $10^3$ |
| Infusions/Decoctions | Total aerobic microbial count NMT $10^2$ |
| | Total combined yeasts and molds count NMT 10 |
| Nutritional Supplements with Botanicals | Total aerobic microbial count NMT $10^4$ |
| | Total combined yeasts and molds count NMT $10^3$ |
| | Absence of *Salmonella* spp. and *E. coli* in 10 g |
| Botanicals to be treated with boiling water before use | Total aerobic microbial count NMT $10^6$ |
| | Total combined yeasts and molds count NMT $10^4$ |
| | Bile-tolerant Gram-negative bacteria NMT $10^2$ |
| | Absence of *E. coli* and *Salmonella* spp. in 10 g |

**Table 3. Recommended Microbial Limits for Dietary Supplement Ingredients and Products**

| Material | Recommended Microbial Limit Requirements (cfu/g or mL) |
|---|---|
| Other raw materials and dietary supplement ingredients | Total aerobic microbial count NMT $10^3$ |
| | Total combined yeasts and molds count NMT $10^2$ |
| | Absence of *E. coli* in 10 g |
| Nutritional supplements with synthetic or highly refined ingredients | Total aerobic microbial count NMT $10^3$ |
| | Total combined yeasts and molds count NMT $10^2$ |
| | Absence of *E. coli* in 10 g |

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## Absence of Objectionable Microorganisms

See *Introduction* under *Microbiological Procedures for Absence of Specified Microorganisms—Nutritional and Dietary Supplements* ⟨2022⟩. Absence of one or more species of objectionable microorganisms is required in some individual monographs.

## Test for Aflatoxins

Dietary and nutritional articles containing botanical products with a history of mycotoxin contamination are also typically tested for aflatoxins, especially if the material is obtained from roots or rhizomes. See *Articles of Botanical Origin* ⟨561⟩ for the details of a test for aflatoxins. Where necessary, this test is included in the individual monograph.

## Solid Oral Dosage Forms

Among all dosage forms, solid oral dosage forms present the lowest microbiological risk because of their method of manufacture, low water activity, and route of administration. When justified, reduced microbiological testing may be appropriate.

## Other Concerns

The presence of some microorganisms in articles can be an indicator of processes that are not under microbiological control. For example, *Purified Water* used at some stage of the manufacture of these products might contain a typical flora of Gram-negative microorganisms. As with pharmaceutical products, inadequate processing of water and poor maintenance of water systems may result in the contamination of processed formulations by Gram-negative microorganisms.

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# ⟨2030⟩ SUPPLEMENTAL INFORMATION FOR ARTICLES OF BOTANICAL ORIGIN

This general chapter provides information about several aspects of botanical articles not covered in *USP* standards monographs. Although the standards in the monographs address the quality issues associated with botanical plant materials, extracts, and preparations of Pharmacopeial articles, there is a need to develop appropriate information to optimize the pre-harvest conditions for appropriate growth and the post-harvest handling to achieve consistent quality with minimum variations in the composition of chemical constituents.

## PROTOCOL CONTENTS

**Black Cohosh** (*Actaea racemosa* L.)
**Ginger** (*Zingiber officinale* Roscoe)
**Valerian** (*Valeriana officinalis* L.)
**Elm** (*Ulmus rubra* Muhlenberg)

## GENERAL GUIDANCES

It is recommended that, at a minimum, growers and others involved in the handling and distribution of botanical products should become familiar with and follow the WHO Guidelines on Good Agricultural and Collection Practices (GACP) for Medicinal Plants (found at http://apps.who.int/medicinedocs/pdf/s4928e/s4928e.pdf).

Commercial trade in natural products occurs in a global market. Material of domestic origin must be produced in compliance with all federal laws of the United States. Material of foreign origin, imported into the U.S., must be produced and transported in compliance with the laws of the U.S., the country of origin, and relevant international treaties. These include, but may not be limited to, the following:

1. The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) is an international agreement between governments. Its aim is to ensure that international trade in specimens of wild animals and plants does not threaten their survival. Information about CITES is available at http://www.cites.org.
2. The Convention on Biological Diversity (CBD) establishes three main goals: the conservation of biological diversity, the sustainable use of its components, and the fair and equitable sharing of the benefits from the use of genetic resources. Each country that has ratified and is a party to the Convention is responsible for implementation by means of national enabling legislation that can differ from country to country.
3. The Endangered Species Act (ESA) was originally adopted in 1973. The ESA is a law that aims to protect species of fish, wildlife, and plants believed to be threatened with extinction. The ESA is administered primarily by the U.S. Fish and Wildlife Service. Full text of the act is available at: http://epw.senate.gov/esa73.pdf.

Provided below is additional information not covered in the compendial specifications: compendial history; sources; collection and cultivation, including common adulterants; and drying, storing, and shipping. This information is provided to complement the standards for quality control in the monographs for botanical articles.

**Compendial History—**The focus in this section is on historical compendial use that has strong validity, with only brief reference to anecdotal use. This is important information because traditional use is one of the elements taken into consideration to support the safety and the presumptions of benefits of botanical dietary supplements.

**Sources—**Included here is the point of origin of the botanical; it also encompasses cultivation (defined as agricultural growing) and wildcrafting (defined as collected in the wild), along with a listing of the primary geographical (native) areas of production.

**Collection and Cultivation—**This section discusses wildcrafting, the conservation of restricted and rare species, and the trend to cultivation as an ecological alternative; such optimal harvesting and collection practices serve to preserve the integrity of species and botanical products. It is divided into three subsections:

1. Collection (conservation and ecology)
2. Cultivation Practices
3. Optimal Times for Harvest

**Post-Harvest Handling (Optimal Handling and Processing Practices, Drying, Storage, and Shipping)—**Important factors regarding storage of herbal products and how they should be maintained include the following.

1. *Light:* Protection from light is important for botanical articles. Light accelerates numerous chemical processes that may lead to degradation or changes in the constituents of the articles.
2. *Temperature:* Storage temperatures in this *Pharmacopeia* are defined in *Packaging and Storage Requirements* ⟨659⟩*, Storage Conditions*. Excessive heat may affect the content of volatile constituents (essential oils) and accelerate degradation processes. However, heat treatments are sometimes useful in the maintenance of the article's quality and can be used in drying, reducing microbial load, and inhibiting certain enzymes. Heat application during these processes must be carefully controlled to achieve the desired balance between degradation and quality conservation.
3. *Humidity:* Moisture in the articles may allow certain enzymes such as glycosidases to become active, hence degrading constituents. High humidity also increases the danger of microbial proliferation. As a rule, it is advisable to store botanical

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articles below 60% relative humidity. Although controlled humidity and temperature warehouses are now required in many good manufacturing practices for natural products, much of the world still lacks access to these facilities.

4. *Degree of Comminution:* The degree of comminution plays a role in determining the stability of the botanical articles during storage. The increased surface area in fine powders allows oxidation and other degradation processes to occur more extensively and rapidly than in the case of a whole article. Plants containing tannins, bitter substances, and essential oils are particularly sensitive to the degree of comminution. In general, dried crude botanicals should be stored in a minimally processed form.
5. *Containers:* Appropriate containers are defined in this *Pharmacopeia* in *Packaging and Storage Requirements* ⟨659⟩*, General Definitions*.

**Constituents—**Where known, the substances mainly responsible for the activity of the product are listed, along with other compounds contained in the plant.

## SUPPLEMENTAL INFORMATION AND GENERAL GUIDANCE PROTOCOLS

### **Black Cohosh** *Actaea racemosa* L.[*Cimicifuga racemosa* (L.) Nutt.] (Fam. Ranunculaceae)

#### BOTANICAL IDENTIFICATION

*Actaea racemosa* L. Herbaceous perennial from rhizome.
**Stem:** Erect, solitary, to 2.5 m tall, glabrous.
**Leaves:** Basal and cauline, alternate, 2–4-ternately compound, petioles 15 to 60 cm long, bases clasping stem; leaflets 20 to 70; terminal leaflet of central division 3-lobed, 6 to 15 cm long, 6 to 16.5 cm wide, with 3 prominent veins arising from base; subterminal leaflets with blades ovate-lanceolate to obovate, 4 to 12 cm long and 3 to 8 cm wide; margins toothed to deeply incised; green above, paler below; glabrous or rarely pubescent along veins of undersurface.
**Inflorescence:** Terminal panicle of 4 to 9 slender branches, each 7 to 60 cm long, pubescent; 1 bract subtending each pedicel.
**Flowers:** Perfect, radially symmetric; sepals 4, greenish-white, caducous; petals 0; staminodes (1–) 4 (–8), petaloid, cream-colored, 2 to 3 mm long, clawed, apex bifid; stamens 55 to 110; pistils 1 (–3), glabrous to pubescent, ovary superior, style short, stigma 0.5 mm wide.
**Fruit:** Many-seeded follicle, 5 to 10 mm long, ovoid, laterally compressed with curved, stout beak (peristent style), pubescent; seeds hemispheric, brown, scales lacking.
**Chromosome number:** n = 8.

There are currently two varieties of *A. racemosa* recognized on the basis of differences in leaf morphology: var. *racemosa* and var. *dissecta*. The former variety has triternate-pinnate leaves with serrate margins, while the latter has quadriternate-pinnate leaves that are deeply incised with serrate lobes. Variety *dissecta* is only known from very few herbarium specimens, all of which were collected well over 100 years ago, making this taxon of uncertain taxonomic significance.

#### COMPENDIAL HISTORY

Black cohosh appeared on the secondary list of substances in the first *United States Pharmacopoeia (USP)* of 1820, where it was listed as an anti-inflammatory and antispasmodic. It soon rose to the primary list in 1830, a position it held until the 10th decennial revision of 1920. Black cohosh appeared in the first edition of the *United States Dispensatory (USD)* in 1833 and remained through 1955 for a total of 122 years. Carrying forward the traditional Native American use of black cohosh for women's ailments and Barton's use for throat complaints, current therapeutics finds the plant used in a number of preparations for coughs and for gynecological disorders. In 2001, both the rhizome and the dry rhizome extract of black cohosh were proposed once again for inclusion in the *United States Pharmacopeia–National Formulary (USP–NF).* (See revised proposal on page 1455 of *PF* 28(5) [Sept.–Oct. 2002].) The monograph became official in the *Second Supplement* to *USP 30–NF 25*.

#### CONSTITUENTS

Major constituents of black cohosh are triterpene glycosides principally as beta-xylopyranosides and alpha-arabinopyranosides. The aglycones are mostly derived from acteol and cimigenol. The nomenclature of these compounds is quite confusing in the literature, with different names often given to the same compounds. A cyclopropane ring is a common feature of these compounds, which are structurally related to cycloartenol. The isoflavone formononetin has been reported in some publications; however, recent evidence indicates its absence in the roots and rhizomes of *Actaea racemosa.* Other constituents include tannins, resin, fatty acids, starch, sugars, and aromatic acids including ferulic acid, isoferulic acid, caffeic acid, and salicylic acid.

#### SOURCES AND DISTRIBUTION

**Sources**—Black cohosh can be found in moist deciduous forests, ravines, moist meadows, creek margins, and mountainous terrain. Black cohosh flowers from June to September and is native to eastern North America from Ontario south to Georgia and west to Missouri. The entire supply of black cohosh comes from the United States. The major producers of black cohosh are Kentucky and Tennessee, with additional supplies coming from Georgia, Michigan, North Carolina, Ohio, South Carolina, Virginia, West Virginia, and Wisconsin. Although there are reports of black cohosh being grown in China and India for export, the true identity of the cultivated material has not been verified and may well be an Asian species of *Actaea* such as *A. cimicifuga* (syn. *Cimicifuga foetida*). The vast majority of the commercial black cohosh is wild harvested. Concern over the conservation of black cohosh due to increasing demand makes this species a good candidate for cultivation.

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**Distribution**—North America (Ontario; Georgia, Kentucky, Michigan, Missouri, North Carolina, Ohio, South Carolina, Tennessee, Virginia, West Virginia, and Wisconsin); China; and India.

## COLLECTION AND CULTIVATION

**Collection (Conservation and Ecology)**—Traditionally, black cohosh has been harvested after plants become reproductive, which occurs anywhere from 2 to 8 years of age in cultivated plants, depending on growing techniques (see *Cultivation Practices*). A portion of the rhizome with a visible bud on it should be left in the ground to resprout the following year. There is no published information on the relationship between the constituent profile of the rhizome and its age, growing conditions, or place of origin, although such studies are underway. The impact of harvest on wild populations of black cohosh is currently unknown, and sources differ in their opinion about it. Whereas some maintain that current levels of harvest threaten the viability of wild populations, others feel that sustainable harvesting is possible at current levels of demand. A study of sustainable harvest limits is currently underway. The regulatory status regarding the trade of black cohosh is under review by CITES. Refraining from harvesting plants until after they have set seed and leaving a portion of the rhizome in the ground to resprout are key components to sustainable harvesting.
**Cultivation Practices**—Black cohosh is grown from rhizome cuttings or seeds and requires some shading, depending on altitude and other environmental conditions. If grown from rhizome cuttings, a plant takes 2 to 3 years to become reproductive; grown from seed sown in the greenhouse and then planted, takes 4 to 6 years; direct-seeded may take from 6 to 8 years. Preliminary work indicates that black cohosh can be propagated successfully using in vitro techniques.
**Optimal Times for Harvest**—Rhizomes and roots should be harvested in autumn when the plant is dormant. At that time the underground portions of the plant have lower moisture content than in other seasons. Fall harvesting also allows plants to produce mature seeds before being uprooted.

## POST-HARVEST HANDLING

**Optimal Handling and Processing Practices**—Rhizomes with roots may be processed fresh or dried. They should be thoroughly washed directly after harvest and then laid out to dry. Freshly harvested roots should be solid but not woody.
**Drying**—Rhizomes with roots are cut and air-dried at 35° to 45°. They are fully dried when they are brittle and snap easily and when no moisture is evident in cross section, either visibly or to the touch.
**Storage**—Follow general guidelines for storage by packing in airtight containers protected from light, heat, moisture, and insect infestation.

## ADULTERANTS AND CONTAMINANTS

Other species of *Actaea,* especially yellow cohosh (*A. podocarpa* syn. *Cimicifuga americana*), have commonly been mixed with *A. racemosa* because of similarity in aboveground appearance and common growing habitat between species. The two species can be distinguished by differences in their freshly harvested underground parts: the fresh rhizome of *A. podocarpa* has a distinct yellowish hue, whereas that of *A. racemosa* is black. The rhizomes of both species are far more difficult to tell apart when dry because *A. podocarpa* darkens upon drying. The underground portions of baneberry *(Actaea pachypoda* and *A. rubra)* occur as occasional adulterants of black cohosh supplies. Fruiting plants of baneberry may be distinguished from black cohosh by their fleshy white or red poisonous berries, which contrast with the dry follicles of black cohosh. No information was available on how to distinguish the underground portions of black cohosh and baneberry from each other. According to one herb dealer, the roots of baneberry are smaller than those of black cohosh, and therefore are not often harvested by wildcrafters. In the Pacific Northwest, *Actaea elata* (syn. *Cimicifuga elata*) is collected for medicinal use.

# **Ginger** *Zingiber officinale* Roscoe (Fam. Zingiberaceae)

## BOTANICAL IDENTIFICATION

*Zingiber officinale* Roscoe. Herbaceous perennial from tuberous rhizome, aromatic because of the presence of volatile oils.
**Stem:** Erect, unbranched pseudostem formed by the tight overlap of sheathing leaf bases; 0.9 to 1.5 m tall.
**Leaf:** Simple, alternate and two-ranked, sessile or petioles short with bases sheathing the stem and a ligule where the leaf base meets the stem; blade linear to narrowly lanceolate, 15 to 25 cm long, 1.5 to 3 cm wide; margin entire; glabrous to pubescent.
**Inflorescence:** Terminal spike, 3.5 to 8 cm long, 1.5 to 2 cm wide, with conspicuous spirally arranged primary bracts; usually borne on specialized leafless stems.
**Flower:** Perfect, bilaterally symmetric; calyx tubular with 3 lobes; corolla tube 2 to 2.5 cm long with lanceolate apical lobes, 1.5 to 2 cm long, 2 to 3.5 mm wide, greenish yellow; stamen 1, anther cream-colored with dark purple, elongated connective grasping upper part of style; staminodes 4, petaloid, 2 fused into an erect, ovate-oblong lip that is dull purple with cream mottling; ovary inferior; style 1, slender, exerted beyond connective.
**Fruit:** Loculicidal capsule; seeds shiny black with a white aril.
**Chromosome number:** n = 11.
There are several different varieties and forms of ginger. The varying morphological characteristics of these are displayed in *Table 1*.

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**Table 1. Morphological and Key Characteristics of Ginger from Different Areas of Production**

| Source | Form | Aroma | Color (External) |
|---|---|---|---|
| **Africa** | Flat surfaces, mostly peeled, starchy and fibrous; 9 cm long, 1.5 cm wide | Poor quality is recognized by its camphoraceous aroma | Uncut surface dark grayish-brown; cut surface brownish-black |
| **Australia** | | Citrus-like | Buff |
| **Bengal** | Flat surfaces, scraped | | Gray-brown |
| **China** | Short stumpy lobes, unscraped, mostly sliced | Strong, floral to citrus | Pale brown |
| **Cochin** | Lateral surfaces lacking cork | Strong, floral to citrus | Cream color with numerous black resin dots |
| **Jamaica (unbleached)** | Up to 12 cm long, 1 cm wide; surfaces completely peeled; starchy and fibrous thin cortex | Delicate, citrus-like | All surfaces yellow-brown |
| **Japan** | Up to 7 cm long, 12 mm wide; flat surfaces usually completely peeled; starchy and fibrous thick cortex | Bergamot-like | Externally gray-white to light grayish-brown, often with white powder from being coated with lime |
| **Malabar (Cochin and Calcutta)** | Cork layer completely removed, mostly treated with chalk | Citrus-like | Almost white |
| **Nigeria** | Smaller in size than other varieties, rather less deeply scraped | Delicate | Somewhat darker than other varieties |

## COMPENDIAL HISTORY

Ginger was official in the *United States Pharmacopoeia* from the first edition of 1820 through the fourteenth revision of 1950, often appearing in multiple preparations. It also appeared in all editions of the *United States Dispensatory* from 1833 through the final edition of 1973, where it was described as "a stimulant and carminative that has been used for treatment of dispepsia and flatulent colic".

## CONSTITUENTS

The essential oils and the pungent principles make up some of the major components of the rhizome of ginger: 4.0% to 10.0% of the rhizome consists of an oleoresin composed of nonvolatile, pungent principles (phenols such as gingerols and their related dehydration products, shogaols); nonpungent fats and their waxes. The essential oil (1% to 3%) contains sesquiterpenes and monoterpenes, mainly geranial and nerals. Generally, but not always, sesquiterpenes predominate (30% to 70%), such as zingiberene, sesquiphellandrene, and beta-bisabolene, which decompose on drying and storage. The nonvolatile pungent principles include the phenylalkanones, the gingerols, and the phenylalkanonols, shogaols with varying chain lengths.

## SOURCES AND DISTRIBUTION

**Sources**—Ginger is cultivated in most tropical and subtropical countries to greater or lesser degrees. The world production is estimated to be 100,000 tons. China and India are reported to be the primary areas of production. Approximately 5000 tons of ginger are imported into the United States. An estimated 80% of this comes from China. In China, Sichuan and Guizhou provinces reportedly produce the largest quantities and highest quality. It is also produced in Guangdong, Hubei, Shandong, Shanxi, and Zhejian provinces. Most of the dried ginger from China available in the United States has had the cortex scraped or rubbed off before it is dried. This gives it a whitish appearance. The freshly dug root is soaked overnight in water, scraped with a knife to remove the outer cortex, and then sun-dried. It has been reported that high arsenic levels in the soil of Changning County of Hunan Province, China, has negatively affected ginger yields.

In India, ginger is grown on a large scale in the warm, moist regions of Madras and Cochin, and to a lesser extent in Bengal and the Punjab. Varieties grown in Bengal are reportedly the highest quality material in India. Other areas of production include Africa (Nigeria and Sierra Leone), Australia, the East Indies, Fiji, Hawaii, and Jamaica. The morphological characteristics of ginger cultivated in these different areas are outlined in *Table 1*.

In older literature, Jamaican ginger is reported to be the highest quality and the most aromatic, though supplies are limited.
**Distribution**—Most tropical and subtropical countries, such as Australia, China (Guangdong, Guizhou, Hubei, Shandong, Shanxi, Sichuan, and Zhejian provinces), India (Bengal, Cochin, Malabar), the East Indies, Fiji, Jamaica, Japan, Nigeria, and Sierra Leone. Hawaii in the United States.

## COLLECTION AND CULTIVATION

**Collection (Conservation and Ecology)**—When the stems wither and are white, the rhizomes are ready for collection. Usually ginger is harvested after 6 months of growth at the earliest, and sometimes not until as late as 20 months; or to obtain larger roots, it is harvested in January or February of the second year of growth. In tropical and subtropical areas, roots are harvested

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as early as 4 months of growth, because they tend to become fibrous and tough as they get older. As ginger matures, it becomes more fibrous and stronger in flavor. Ginger harvest can be described in three stages:

1. Ginger that has been harvested early is known as green ginger and is traded as fresh ginger. It is succulent and tender, mellow, and mildly aromatic with a floral or lemony aroma and mild flavor.
2. Ginger harvested a few months later is more fibrous and drier and is collected for drying and may be sold as a full-flavored, pungent dried whole ginger.
3. The last harvest is usually around 9 months and yields the strongest ginger, which is quite dry and also richest in pungent components. This ginger is dried and then ground into powder.

**Cultivation Practices**—Ginger is a perennial herb that grows well at subtropical temperatures where the rainfall is at least 1.98 meters per year. The plant is sterile and is grown by vegetative means. Selected pieces of rhizome ("seed pieces" or "setts"), each bearing a bud, are planted in holes or trenches. Ideally the soil should be well-drained, rich clay loam. The growing conditions resemble those of potato cultivation. Mulching or manuring is necessary because the plant rapidly exhausts the soil of nutrients.

Ginger is susceptible to waterlogging and root rot. Preventive methods include using only the cleanest ginger for planting and washing it with fungicide before planting. A study growing ginger hydroponically yielded up to 125 tons per hectare in 6 to 7 months compared to 35 tons per hectare when grown in soil.

**Optimal Times for Harvest:** typically in December or January.

### POST-HARVEST HANDLING

**Optimal Handling and Processing Practices**—After harvesting, the rhizome is cleaned and stripped of its stems and roots. Each area processes its ginger differently after harvest. This results in the different quality and commercial grades available on the market. Green ginger consists of the rhizomes sent to market without drying. Unscraped or partially scraped varieties are traded as coated or black ginger. These roots have been scalded with boiling water and dried quickly. When dry, black ginger breaks with a horny, blackish, somewhat diaphanous fracture, due to the pasty condition of the starch. White ginger is bleached, usually by rubbing with chalk or lime, to lighten its color and to prevent insect infestation. Preserved ginger consists of soft, yellowish-brown pieces obtained by steeping the fresh ginger in hot syrup and carefully bottling. It is soft, brown-yellow and translucent. When baked, ginger loses its pungency and acquires a bitter taste.

**Drying**—In general, after harvest, the fresh roots are washed, and the whole dark outer skin, consisting of cork and a little underlying parenchyma, is scraped away. Scraping speeds up the drying time of the crude drug. However, excessive scraping can result in lower concentrations of essential oil that is lost with the discarded epidermal tissue. After scraping, the rhizomes are then laid out on clean floors and dried in the sun for 7 to 10 days. During this time they are occasionally turned and are piled up every night. If the fresh rhizomes are too fleshy or moist, drying will take longer and the product will end up looking shriveled. To obtain a whiter product, the ginger is moistened after 5 or 6 days and dried for another 2 days, at which time it is ready for export. Dried ginger is more pungent and stronger in taste than fresh ginger.

**Storage**—Store in a tightly closed container, protected from light and moisture, in a cool area. A study was done on ginger harvested after 8, 9.5, 11, or 12 months. Samples were stored at 10° to 15° and 45% to 55% relative humidity or 25° to 30° and 75% relative humidity for 0, 4, or 8 weeks. Oil and oleoresin yields increased with the age of the ginger. Room temperature storage had adverse effects, but refrigerated storage for up to 4 weeks had no effect on quality. When stored for extended periods of time, ground ginger loses its pungency.

### ADULTERANTS

Because ginger is so characteristic, unintentional adulterants are rare. However, in East Asia sometimes the much larger *Zedoary cassumer* and *Zedoary zerumbet*, along with *Alpinia allughas*, are used and found in European commerce. They are easy to distinguish because of their characteristic aromas. Occasionally, Chinese sugar-candied "ginger" is prepared from *Alpinia galangal*.

In older literature, other herbs have reportedly been used as adulterants. These include various species of *Curcuma, Capsicum,* and Grains of Paradise *(Amomum melegueta)* added to exhausted material in order to enhance color and pungency.

Ginger powder is sometimes adulterated with plant starches such as those from wheat middlings, potatoes, corn, barley, rice, legumes, acorns, flaxseed meal, mannihot, oil cakes from linseed, rapeseed, mustard, almond meal, palm kernel or olives, hazelnut shells, and mineral additives. These may be easily identified microscopically. The extent of this type of adulteration in trade is unknown.

Exhausted material should be considered an adulterant.

## **Valerian** *Valeriana officinalis* L. (Fam. Valerianaceae)

### BOTANICAL IDENTIFICATION

*Valeriana officinalis* L. Herbacious perennial, rhizomatous.

**Stem:** Solitary, hollow, 15 to 150 cm.

**Leaf:** Basal and cauline, opposite, oddly once pinnately lobed, lobes 11 to 21 lanceolate, entire or dentate, basal leaves petiolate, cauline leaves subsessile to clasping.

**Inflorescence:** Compound cyme, terminal or axillary, many pale pink to white, strongly scented flowers.

**Flower:** Calyx 5-lobed, lobes inconspicuous in flower, becoming elongate and pappus-like in fruit, corolla funnel-form, slightly saccate at the base, 5-lobed, tube 4 mm, lobes 1 mm, stamens 3, filaments attached to corolla tube alternate to corolla lobes, ovary inferior, triloculate, uniovulate, only 1 locule fertile, stigma tripartite.

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**Fruit:** Achene crowned by persistent calyx, lanceolate-oblong, 4.5 to 5 mm, hairy or glabrous. Populations of *V. officinalis* range in ploidy level from diploid to tetraploid or octaploid. British *V. officinalis* is usually octaploid, and central European supplies are tetraploid.

There are three subspecies of *V. officinalis*: ssp. *officinalis,* ssp. *collina* (Wallr.) Nyman, and ssp. *sambucifolia* (Mikan fil.) Celak. All three of these subspecies, as well as the other European species of valerian, *V. repens* Host, have been considered acceptable source material for medicinal preparations.

**Macroscopic Identification**—Various chemotypes will have slightly different characteristics. When dried, the whole rhizome is up to 50 mm long and up to 30 mm in diameter, obconical to cylindrical, with an elongated or compressed base. It has a yellowish-brown to dark brown exterior with a circular stem and leaf scars. The rhizome contains numerous thick, light to dark brown rootlets that are located around a thin ligneous cord. The root is longitudinally wrinkled, approximately 100 mm long and 1 to 3 mm in diameter, almost cylindrical, and almost the same color as the rhizome. In longitudinal section, the pith exhibits a central cavity transversed by septa. The stolons are 20 to 50 mm long, pale yellowish grey with prominent nodes separated by longitudinally striated internodes. It is commonly sliced in half for ease of cleaning. The rootlets, which contain the majority of the essential oil, are brittle and break in short, horny fractures and are whitish or yellowish internally. Aroma: when dried properly, *V. officinalis* L., s.l. has only a very faint characteristic, valeric acid–like aroma that becomes stronger as it ages. Improperly dried or old material possesses a strong and characteristic odor due to the enzymatic hydrolysis of esters of the valepotriates (isovaleric acid and hydroxyvaleric acid). Taste: mildly sweet and camphoraceous with a slightly bitter and spicy aftertaste.

## COMPENDIAL HISTORY

Valerian was official in the *United States Pharmacopoeia* from the first edition of 1820 through the eleventh revison of 1930, often appearing in multiple preparations. At its peak from 1850 through 1880 it appeared six to seven times in different preparations. Valerian is among the top 30 most listed botanicals in the history of the *USP*. The root of valerian has been used as a sedative and spasmolytic in Europe since the 16th century.

## CONSTITUENTS

Major constituents of valerian have been identified as sesquiterpenes of volatile oils and iridoids (epoxy-triesters) known as valepotriates. The total content of volatile oil varies widely within a single species and between different species. European *Valeriana officinalis* L. usually contains 0.1% to 2.8% volatile oil. The oil consists of mixtures of monoterpene and sesquiterpene derivatives. The amount of valepotriates present also varies widely between species and genera and even within a species, generally ranging from 0.5% to 1.2%. Valepotriates are particularly unstable; they decompose easily under the effect of moisture, temperatures above 40°, or acidity (pH <3).

Valerian also contains small amounts of aliphatic acids, alkaloids, amino acids, phenolic acids, flavonoids, free fatty acids, sugars, and salts. Valerian constituents that have possible sedative effects include acetoxyvalerenic acid, 1-acevaltrate, baldrinal, didrovaltrate, hydroxyvalerenic acid, kessane derivatives, valeranone, valerenal, valerenic acid, and valtrate.

## SOURCES AND DISTRIBUTION

**Sources**—Valerian is found in damp or dry meadows, scrub, or woods in most of Europe, although rare in the south, and it is cultivated and naturalized in North America. Valerian is cultivated in Belgium, Britain, Eastern Europe, France, Germany, Japan, the Netherlands, North America, and Russia. The majority of standardized extract products and crude cut and sifted material on the domestic market are prepared from European supplies. A large number of liquid extracts are prepared from domestically cultivated material. Many species other than *V. officinalis* are reported to be traded as medicinal valerian. These include *V. edulis* Nutt. ex Torr. & A. Gray, *V. corneana* Briq. k, *V. stubendorfi* Kreyer ex Kom., *V. amurensis* P. Smirn. ex Kom., *V. hardwickii* Wall., *V. exaltata* Mikan, and *V. wallichi* DC. syn. *V. jatamansi* Jones.* The most frequently used North American species include *V. sitchensis* Bong and *V. edulis* Nutt.* = *V. edulis* Nutt. ex Torr. & Gray ssp. *procera.* Other species reported to be used locally include *V. arizonica* Gray, *V. capitata* Pall ex Link., *V. diocia* L., and *V. scouleri* Rydb. Detailed chemical analyses of most American species are lacking. A limited number of assays of material cultivated in the Pacific Northwest show varying levels of essential oil ranging from 0.4% to 1.3%. Valerenic acid and valepotriates have been found to be present in fresh and dry samples of *V. sitchensis* Bong. *V. sitchensis* Bong exhibits a strong pungency when fresh. High quality material is reported to contain from 1.0% to 1.5% essential oil, ≥30% extractable matter, and ≥0.5% valerenic acid.

**Distribution**—Europe (Belgium, Britain, Eastern Europe, France, Germany, the Netherlands), Japan, North America, and Russia.

## COLLECTION AND CULTIVATION

**Collection (Conservation and Ecology)**—The majority of valerian in trade comes from cultivated material. Harvest times will vary geographically. The composition of the essential oil varies greatly among different populations of the same subspecies and even between the same population of plants from year to year. Essential oil content also varies with genotypes, harvest times, growing conditions, age of root, drying techniques, and method of analysis. It has been reported that valerian harvested in higher elevations, grown in dryer regions, or cultivated in phosphate-rich soil yields relatively high levels of essential oil.

Older literature reports that valerian should be harvested in the fall, between August and September, preferably in the second year of growth. Analyses of material cultivated in the Netherlands report that the majority of constituents, including the essential oil and valerenic acid, were highest in roots harvested in the first year of growth, with essential oil being highest in September and November (1.2% to 2.1%). The next highest level of essential oil was reported for material harvested in March (0.9% to 1.6%). Valerenic acid and its derivatives were found to be highest in February and March (0.7% to 0.9%), followed by material

* *V. wallichi* DC. and *V. edulis* Nutt. reportedly are lacking in valerenic acid and its derivatives.

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harvested in September (0.5% to 0.7%) and then in January (0.3% to 0.4%). From a commercial standpoint, it is more cost effective to harvest the roots in the same year the plants are sown than in the second year.
**Cultivation Practices**—Sowing seeds has been reported to be preferred over planting of seedlings. Best results were achieved by flat field planting at row spacings of 50 cm and a seed rate of 3 kg per hectare. Cutting off the flowering tops before the plant has set seed causes the rhizome to develop more fully.
**Optimal Times for Harvest**—Wagner reports that harvest should take place in the morning during relatively cool weather, a general recommendation for roots rich in essential oils.

### POST-HARVEST HANDLING

**Optimal Handling and Processing Practices**—The essential oil is located in the hypodermis of the rhizome in large thin-walled cells. Therefore, care must be taken not to damage these cells during handling. Excess washing of the roots can result in a significant reduction of extractive matter. Because of the sensitivity of volatile oils to heat, it is necessary to minimize the amount of time generated in the grinding or powdering process by doing small lots at a time, with frequent interruptions in run times, or by using a cryogenic grinder.
**Drying**—For maximum preservation of the essential oils, valerian should be dried at 40° with a flow rate of 0.05 kg per sec per $m^2$. Alternatively, drying at 20° for approximately 10 days, shade drying at approximately 45°, low temperature vacuum-drying, and freeze-drying are also reported to be appropriate drying techniques.

Careless or prolonged drying produces a darker color in the roots and results in the hydrolysis of the isovalerianic esters and the liberation of isovaleric and hydroxyisovaleric acid. This produces the characteristic valerianic aroma. Properly dried valerian will produce this same aroma over time.
**Storage**—Store in closed containers protected from light, air, and moisture. Hydroxyvalerenic acid, a decomposition product of acetoxyvalerenic acid, is formed when the herb is stored at too high humidity.

Improper storage conditions can cause significant deterioration of the material. Although the essential oil is relatively stable, it can evaporate with excessive exposure to air. The essential oil can degrade quickly in powdered material. In powdered root, the essential oil content can decrease by 50% within 6 months.

Valepotriates are sensitive to humidity, temperatures above 40°, and acid media (pH <3) and are generally not detected in commercial products after 60 days.

### ADULTERANTS

Other species of valerian: An unidentified *Apiaceae* species may be found in valerian trade. Adulteration of valerian in the American market is not common. Many species other than *V. officinalis* are reported to be traded as medicinal valerian. These include *V. edulis* Nutt. ex Torr. & A. Gray, *V. coreana* Briq. k, *V. stubendorfi* Kreyer ex Kom., *V. amurensis* P. Smirn. ex Kom., *V. hardwickii* Wall, *V. exaltata* Mikan, and *V. wallichi* DC. syn. *V. jatamansi* Jones.

## **Elm** *Ulmus rubra* Muhlenberg [*Ulmus fulva* Michaux] (Fam. Ulmaceae)

### BOTANICAL IDENTIFICATION

*Ulmus rubra* Muhlenberg; tree to 35 m high, with spreading branches and open flat crown; preparations derived from inner bark. *U. rubra* appears to be more closely related to the introduced Asian species *U. pumila* L. than to other native American species of *Ulmus*; where the two co-occur, interbreeding is common.
**Trunk:** 18 to 35 m high, to 1 m in diameter; the trunk rises free of branches until about 5 to 6 m.
**Branches:** Erect, spreading; young twigs are scabrous-pubescent.
**Bark:** Dark brown to reddish-brown, deeply furrowed. Inner bark is whitish (outer surface yellow-orange; inner surface pale yellow), fragrant (upon powdering, a distinctive fenugreek-like odor) and very mucilaginous upon chewing or moistening.
**Leaves:** Alternate; simple; petiolate with petiole (3–)5–7(–9) mm long; 7–18(–23) cm long, 5–10(–15) cm broad; elliptical to ovate, oblong, or obovate with oblique base and acuminate apex; margins serrate toward base, elsewhere doubly serrate; upper surface scabrous, rough; lower surface tomentose; secondary veins parallel, slightly curved, running to tips of marginal teeth.
**Inflorescence:** Axillary fascicles, roughly hemispherical, to 1.5(–2.5) cm in diameter.
**Flowers:** Small, perfect; pedicels 1–2(–3) mm long; calyx campanulate, 5–9-lobed at apex, about 2.6–3.5 mm in diameter, reddish-pubescent; petals absent; stamens 5–9, exserted at flowering; styles 2. Flowers occur before the leaves from March through early May.
**Fruit:** Winged samara, yellowish, irregularly suborbicular or occasionally broadly elliptical or obovate, 10–20 mm in diameter, reddish-pubescent over seed; wing papery-textured.

### COMPENDIAL HISTORY

Slippery elm (Ulmus) inner bark appeared in the list of materia medica in the first *United States Pharmacopoeia (USP)* of 1820 and remained official until it was removed from *USP XI* (1936). The *USP 1820* included instructions for the preparation of *Infusion of Slippery Elm: "Take of Slippery elm, sliced, one ounce. Boiling water, one pint. Infuse for twelve hours in a covered vessel, near the fire with frequent agitation, and strain."* Immediately following its removal from *USP XI* (official: June 1, 1936), *Slippery Elm Bark* became an official monograph in the sixth edition of the *National Formulary* (*NF*; official: June 1, 1936) until its elimination from the 11th edition (official: October 1, 1961). It became official again, as *Elm,* on November 15, 1995, in the *USP* section of the *Third Supplement* to the *United States Pharmacopeia–National Formulary* (*USP 23–NF 18*). A revision was published in the *Seventh Supplement* on November 15, 1997.

In 1982, Elm Bark appeared in the Food and Drug Administration (FDA) Advance Notice of Proposed Rulemaking (ANPR) for the establishment of a therapeutic monograph for oral health care drug products for over-the-counter (OTC) human use.

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In the ANPR (1982) as well as in the subsequent tentative final monograph of 1988 and in the amendment to the monograph of 1991, Elm bark was classified as a Category I (Generally Recognized as Safe and Effective (GRASE)) OTC oral demulcent active ingredient, and appropriate standards were urged to be developed in the official compendia.

Aside from *USP–NF*, the monograph of *Elm* had already appeared in the second edition of *The Dispensatory of the United States of America* (1834), and its last appearance was in the 25th edition, 1960.

## CONSTITUENTS

Constituents of relevance for conformance to *Identification A* under *Elm* are mucilaginous substances. Elm inner bark mucilage is readily extractable by water and consists principally of a polysaccharide which on hydrolysis yields D-galactose, D-methyl galactose, L-rhamnose, and glucose. Borohydride reduction of the periodate-oxidized polysaccharide affords, on partial hydrolysis with hot acid, three oligosaccharides:
*O*-(3-*O*-methyl-β-D-galactopyranosyl)-(1→4)-*O*-(3-*O*-methyl-β-D-galactopyranosyl)-(1→4)-L-rhamnose,
*O*-(3-*O*-methyl-β-D-galactopyranosyl)-(1→4)-L-rhamnose, and *O*-(3-*O*-methyl-β-D-galactopyranosyl)-(1→4)-3-*O*-methyl-D-galactose.

Other Elm constituents are traces of tannins, including proanthocyanidins, some starch, traces of oxalate salts, beta-sitosterol, and minerals.

## SOURCES AND DISTRIBUTION

**Sources**—Slippery elm bark is harvested from wild populations in eastern Canada and the United States, from southern Quebec west to North Dakota, south to south-central Texas, and Florida. It is common throughout eastern, southern, and midwestern U.S., and it grows in more than 25 states. An increasing amount of the commercial supply is being collected according to sustainable wild resource management plans as a condition of organic certification for wild crops. Conversely, Dutch elm disease has had a significant negative impact on elm populations, from 1930 when it was first found in the United States affecting over 50% of elm trees in the northern states.

**Distribution**—Canada (New Brunswick, Ontario, Quebec), the United States (Alabama, Arkansas, Connecticut, Delaware, the District of Columbia, Florida, Georgia, Illinois, Indiana, Iowa, Kansas, Kentucky, Louisiana, Maine, Maryland, Massachusetts, Michigan, Minnesota, Mississippi, Missouri, New Hampshire, New Jersey, New York, North Carolina, North Dakota, Ohio, Oklahoma, Pennsylvania, Rhode Island, South Carolina, South Dakota, Tennessee, Texas, Virginia, Vermont, Wisconsin, and West Virginia).

## COLLECTION AND CULTIVATION

**Collection (Conservation and Ecology)**—"Natural" (both inner and outer bark) and "rossed" (inner bark only) are harvested, but only the rossed bark will conform to the standards of the *USP* monograph. Harvesting should occur on dry, preferably warm days with no chance of precipitation, and it usually commences in late morning after the morning dew and humidity pass. This is because post-harvest processing (rossing) generally takes place at the collection site outdoors and humidity can damage the quality of the inner bark. Because the inner bark contains polysaccharide mucilage, when it comes in contact with moisture, it begins to gel.

The bark, from trees of a minimum age of 10 years (some harvesters recommend selecting at least 12- to 15-year-old trees) is obtained mainly by pruning or trimming the lower limbs and branches, but can also be obtained from the bole (trunk) and, very rarely, even from the roots in cases where the entire tree is felled. While the inner bark of the root reportedly contains more mucilage than that of the trunk or branches, sustainable wild resource management dictates harvesting only the branches of mature trees. In practice, commercial bark collectors are more likely to select branches from trees that are at least 30 to 50 years old in order to obtain a high enough yield. After pruning, the tree will heal over where harvested and continue to grow, but the trunk and/or branches should never be girdled, because this will kill the tree. Girdling is the stripping away of bark from the trunk or a branch all the way around. In practice, the most sustainable method of harvest from mature healthy trees is to prune off entire lower branches in a way that will not harm the tree. If sawn properly so that rainwater will not drop directly into the exposed cut area, the tree will grow over the cut area within a couple of years. The proper cut, which is made just outside the branch collar and the branch bark ridge, does the least amount of damage to the trees.

Although elm bark should not be gathered from already dead trees, selective collection from dying trees—for example, those affected with Dutch elm disease—is feasible. The fungus that causes Dutch elm disease, *Ophiostoma ulmi* and *Ophiostoma novo-ulmi* (syn: *Graphium ulmi* or *Ceratocystis ulmi*), is carried from tree to tree by the European elm bark beetle (*Scolytus multistriatus*), which arrived in North America on a ship carrying logs from Europe around 1930, and to a much lesser extent by the native elm bark beetle (*Hylurgopinus rufipes*). Elms appear to succumb to the disease at about 10 years of age and then die off in a two-year period. Once a tree is diagnosed with Dutch elm disease, healthy bark can still be harvested for about two years, up until the tree is near death. To monitor how long harvesting can continue, one can use a drawknife to take samples. Once the inner bark is showing increasingly noticeable black streaking, and as more and more limbs die and debarking is evident, the tree is near death. The near-dead tree can be dropped, and the remaining healthy bark can be stripped from the entire tree. Discolored (black-streaked) inner bark should be separated out and discarded. Pruning lower limbs from healthy trees and/or selectively harvesting entire older trees that are near death can both be acceptable methods as part of a sustainable resource management plan for a specific area of forest under organic supervision.

Following organic production system rules (e.g., for harvest site selection, absence of prohibited substances, testing of soil and water, disallowed inputs, periodic residue testing, documentation control, independent inspection) will more likely result in botanical raw material that meets the general requirements for pesticide residue and heavy metals limits, among other potential contaminants. Certified organic production of wild elm bark requires producers to promote ecological balance and conserve biodiversity. Wild collected elm bark that is to be certified organic must be harvested from a designated area that has had no prohibited substance applied to it for a period of 3 years immediately preceding the harvest, and must be harvested

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in a manner that ensures that such harvesting or gathering will not be destructive to the environment and will sustain the growth and production of the wild crop. Wild crop producers must comply with the same organic system plan requirements and conditions, as applicable to their operation, as their counterparts who produce cultivated crops. The producer of organic wild harvested elm bark must initiate practices to support biodiversity and avoid, to the extent practicable, any activities that would diminish it. Production practices must maintain or improve the natural resources of the operation, including soil, water, wetlands, woodlands, and wildlife. This is accomplished, in part, by developing and executing a resource management plan that requires wild harvest from stable populations, minimizing disruption of priority species/sensitive habitats, avoiding erosion, allowing reestablishment, and monitoring wild crop sustainability.

**Cultivation Practices**—Even though the commercial supply is harvested from wild populations, slippery elm trees can be propagated by cuttings or by seed. For propagation by seed, the ripe seeds are collected from April to June from healthy and successful (dominant) trees from an area similar to the proposed planting site. A ripeness indicator is when the samaras (fruits) are green. It is best to collect seed from trees within 160 km north or south of the planting site, as potential for success is optimal within this range from the parents. Twenty-five seeds per square foot can be scattered, 0.6 cm deep. Slippery elm may be sown as in its normal cycle in the spring in a raised peat moss soil and sand bed. The seedbeds may need a wire top to protect young seedlings. Germination rate is 10% to 25%, with light germination in summer and increased germination the following spring. The young trees can be transplanted into tree tubes within the first month of germination and field planted after one or two years, depending on the size of the tree tube. The tree saplings must be watered during times of drought and routinely checked for insect predation and indications of fertilization needs.

**Optimal Times for Harvest**—Harvest should preferably occur in the spring (March to May), but can also take place in the autumn. In the spring, bark is harvested from mature trees (minimum 10 years) when the sap begins to rise.

## POST-HARVEST HANDLING

**Optimal Handling and Processing Practices**—To produce pharmacopeial quality elm inner bark, the outer corky layer of bark must be removed, exposing the inner bark. If post-harvest processing occurs at the wild collection site, the pruned limbs and branches should be placed onto clean tarps and not directly on the ground. The very small branches with leaves are stripped off the pruned limbs by hand and discarded. To optimize conformance to standards for composition, identity, purity, and quality (e.g., NMT 2% of adhering outer bark, NMT 2% foreign organic matter, NMT 10% total ash, and NMT 0.65% acid-insoluble ash), a clean bark rosser (hand tool with handle and knife blade) should be used to shave off the outer bark. The rough, scaly matter on the surface of the bark is called *ross*, and to ross bark is to scrape or shave the outer bark from the limb. An experienced rosser can visually discern that at least 98% of the outer bark has been shaved off. The inner bark is white in color (in the spring; reddish later in the season) in obvious visible contrast to the brown outer bark layer. After most of the outer bark is rossed off, greater care must be exercised to very carefully slice off the remaining thin layer of outer bark so as not to waste any of the inner bark in the process. After removal of the outer bark, the inner bark can then be removed in strips, squares, or chips. An incision is made with a clean knife down the center of the limb. Then a clean crowbar is slipped underneath the incision in order to lift and peel the inner bark off from the cambium. The strips of inner bark are stacked on a clean tarp and later bundled for transport to the drying facility.

**Drying**—*Elm USP* requires a loss on drying limit of NMT 12%. So long as rain is not expected, fresh elm inner bark can be sun-cured within a temperature range of 32° to 60°. Drying can also be carried out in a warm room with airflow or in a greenhouse. Greenhouse drying takes about 3 to 4 days. Drying indoors can take 5 to 7 days, depending on the heat source. Drying at commercial scale, however, is done typically in enclosed drying chambers, in which time and temperature can be better controlled. The strips of elm bark are placed onto a clean screen floor and dried over about 2 days' time at about 50° with fan-forced heat through the floor. Because of additional phytosanitary requirements for export of tree barks to Europe, higher heat exposure is necessary, usually at least 65° but up to 93° for up to two days. Post-drying, the strips of inner bark can be cut or sawn into pieces of equal length and bound into bundles with wire. The bundles usually consist of flat, oblong pieces, about 30 cm in length and from 10 to 15 cm in width. The bark strips can be stored this way until further processing (e.g., cutting or powdering) is scheduled.

**Storage**—To maintain pharmacopeial purity and quality (e.g., to prevent accumulation of excess moisture), dried elm inner bark should be preserved in well-closed containers, and stored in a cool, dry place.

## ADULTERANTS AND CONTAMINANTS

Common contaminants that could cause a material not to conform with the identification tests in the *Elm* monograph in *USP* would include other plant parts: for example, greater than 2% outer bark, which lacks mucilage. Insufficient shaving or rossing of outer bark could cause the material to exceed the monograph limit of NMT 2% of adhering outer bark. Other possible contaminants would include visible discolored inner bark, although no maximum limit has been established (for example, inner bark with visible black streaking obtained from a diseased tree). Powdered bark can also be adulterated with cornmeal, rice flour, starch, or other starchy substances. Consequences of contamination with outer bark or adulteration with flour or starch are lower mucilage content, lower swelling index value, and correspondingly less of a therapeutic demulcent effect that is mucilage-dependent. Excess outer bark could also cause the material to fail the quantitative standard of NMT 10% total ash. Methods to determine the presence of adulterants include microscopic examination in order to determine the presence of excess outer bark or any other adulterant and the concentration of mucilage cells. The *Elm* mucilage test (*Identification A*) as well as a modified swelling volume test (based on the test in the *USP* monograph *Psyllium Husk*) may also be useful to investigate if adulteration is suspected.

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# ⟨2040⟩ DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS

## INTRODUCTION

This general chapter is provided to determine compliance with the disintegration and dissolution standards for dietary supplements where stated in the individual monographs.

For the purposes of this chapter, dietary supplement dosage forms have been divided into three categories: *Vitamin–Mineral Dosage Forms*, *Botanical Dosage Forms*, and *Dietary Supplements Other Than Vitamin–Mineral and Botanical Dosage Forms*. *Vitamin–Mineral Dosage Forms* include articles prepared with vitamins, minerals, or combinations of these dietary ingredients, as described in *Table 1*. *Botanical Dosage Forms* comprise formulations containing ingredients of botanical origin, including plant materials and extracts. *Dietary Supplements Other Than Vitamin–Mineral and Botanical Dosage Forms* encompass dietary supplements formulated with lawfully recognized dietary ingredients that are different from those pertaining to the two foregoing categories (e.g., amino acids, chondroitin, and glucosamine).

Where a dietary supplement represents a combination of the categories mentioned above, and there is a difference between the requirements for the individual categories, the more stringent requirement applies. [NOTE—"More stringent requirement" means stricter acceptance criteria and/or milder operational conditions.]

Disintegration and dissolution tests as described in this chapter are quality-control tools to assess performance characteristics of dietary supplement finished dosage forms. These performance standards are intended to detect problems that may arise due to use or misuse, or changes in coatings, lubricants, disintegrants, and other components. These performance tests are also intended to detect manufacturing process issues, such as overcompression and overdrying, that would affect the release characteristics of the final dosage forms. These tests are not intended to be used as a demonstration or as a surrogate for in vivo absorption, bioavailability, or effectiveness, unless an in vitro–in vivo correlation (IVIVC) has been established.

### DISINTEGRATION

This test is provided to determine whether dietary supplement capsules or tablets disintegrate within the prescribed time when placed in a liquid medium at the experimental conditions presented below. Compliance with the limits on *Disintegration* stated in the individual monographs for dietary supplements is required, except where the label states that the products are intended for use as troches, are to be chewed, or are designed as extended-release dosage forms. Dietary supplements claiming to be extended-release dosage forms must comply with standards other than disintegration to verify that the release of the dietary ingredients from the dosage form is for a defined period of time. Dietary supplements claiming to be extended-release dosage forms must not be labeled as in compliance with USP unless a *USP* monograph exists for such product. Determine the type of dosage form under test from the labeling and from observation, and apply the appropriate procedure to 6 or more units.

For purposes of this test, disintegration does not imply complete solution of the unit or even of its active constituent. Complete disintegration is defined as that state in which any residue of the unit, except fragments of insoluble coating or capsule shell, remaining on the screen of the test apparatus or adhering to the lower surface of the disk (if used) is a soft mass having no palpably firm core.

- **APPARATUS**

**Apparatus A:** Use the *Apparatus* described in *Disintegration* ⟨701⟩*, Apparatus* for capsules or tablets that are NMT 18 mm long. For larger capsules or tablets, use *Apparatus B*.

**Apparatus B:** The apparatus consists of a basket-rack assembly, a 1000-mL low-form beaker for the immersion fluid, a thermostatic arrangement for heating the fluid between 35° and 39°, and a device for raising and lowering the basket in the immersion fluid at a constant frequency rate between 29 and 32 cycles/min through a distance of 53–57 mm. The volume of the fluid in the vessel is such that at the highest point of the upward stroke, the wire mesh remains at least 15 mm below the surface of the fluid and descends to NLT 25 mm from the bottom of the vessel on the downward stroke. At no time should the top of the basket-rack assembly become submerged. The time required for the upward stroke is equal to the time required for the downward stroke, and the change in stroke direction is a smooth transition rather than an abrupt reversal of motion. The basket-rack assembly moves vertically along its axis. There is no appreciable horizontal motion or movement of the axis from the vertical.

**Basket-rack assembly:** The basket-rack assembly (see *Figure 1*) consists of three open-ended transparent tubes, each 77.5 ± 2.5 mm long and having an inside diameter of 32.0–34.6 mm and a wall 2.0–3.0 mm in thickness; the tubes are held in a vertical position by two plastic plates, each 97 ± 2 mm in diameter and 7.5–10.5 mm in thickness, with three holes, 36.0–40.6 mm in diameter, equidistant from the center of the plate and equally spaced from one another. Attached to the undersurface of the lower plate is 10-mesh No. 23 (0.025-inch) W- and M-gauge woven stainless-steel wire cloth having a plain square weave. The parts of the apparatus are assembled and rigidly held by means of three bolts passing through the two plastic plates. A suitable means is provided to suspend the basket-rack assembly from the raising and lowering device, using a point on its axis. The design of the basket-rack assembly may be varied somewhat, provided that the specifications for the glass tubes and the screen mesh size are maintained.

**Beaker:** Low form, 1000 mL; the difference between the diameter of the plastic plates, which hold the tubes in a vertical position, and the inside diameter of the beaker should be NMT 6 mm.[1]

**Disks:** Each tube is provided with a perforated cylindrical disk 15.3 ± 0.15 mm in thickness and 31.4 ± 0.13 mm in diameter. The disk is made of a suitable, transparent plastic material having a specific gravity of between 1.18 and 1.20. Seven holes 3.15 ± 0.1 mm in diameter extend between the ends of the cylinder, one of the holes being in

[1] 1000-mL low-form beakers, designed in compliance with the current ASTM E 960 Type I or Type II or ISO 3819 specifications, meet the size requirements.

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the center and the other six parallel to it and spaced equally tangent to a circle with a radius of 4.2 mm from the center of the disk. All surfaces of the disk are smooth.[2]

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[2] The use of automatic detection using modified disks is permitted where the use of disks is specified or allowed. Such disks must comply with the requirements for density and dimensions given in this chapter.

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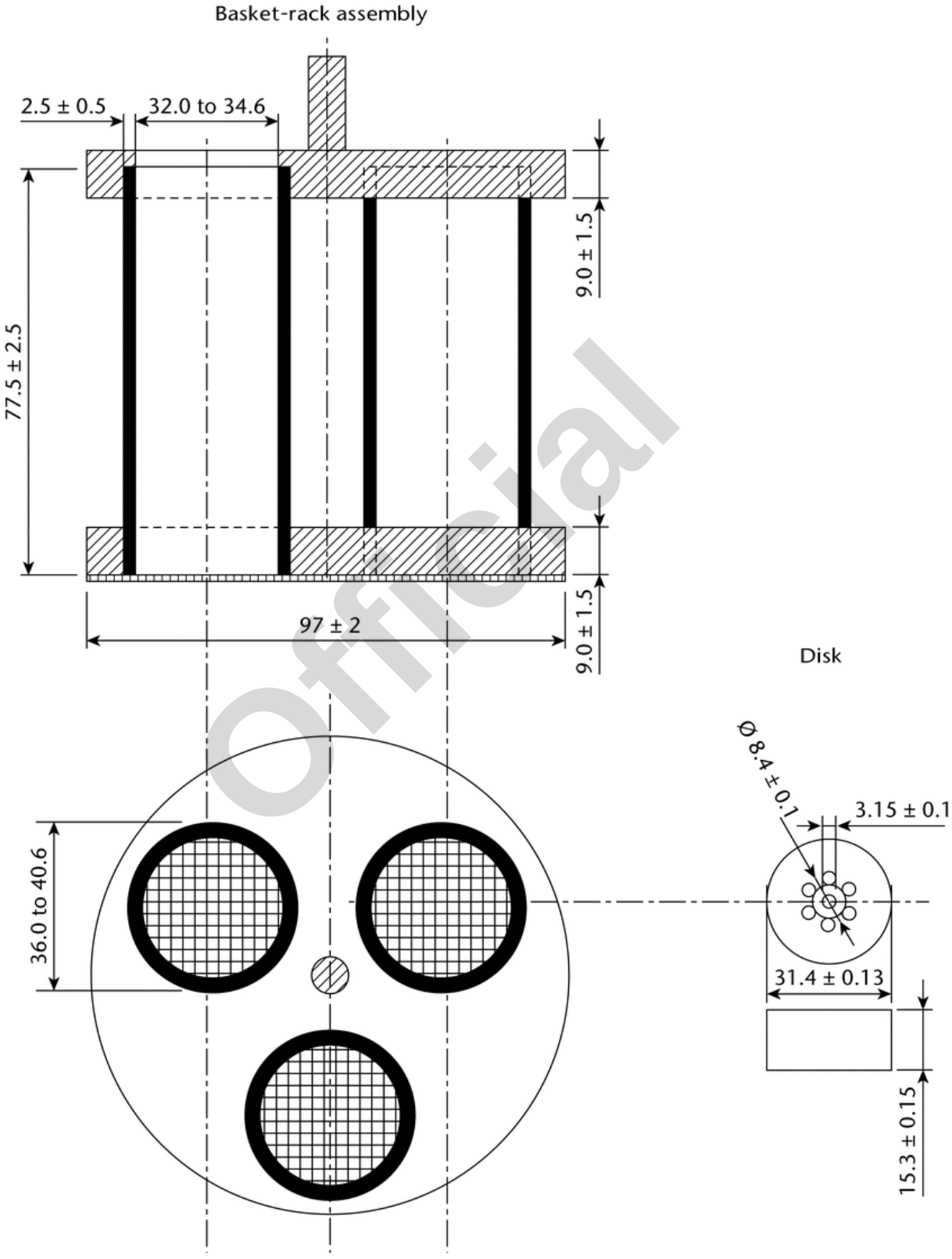


Figure 1. Basket-rack assembly, *Disintegration, Apparatus B* (dimensions in mm).

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- **PROCEDURE:** Test 6 dosage units as described below for each type of dosage form. [NOTE—Two basket arrangements for a total of six tubes are necessary for *Apparatus B*.] If 1 or 2 dosage units fail to disintegrate completely, repeat the test on 12 additional dosage units.

  **Uncoated tablets:** Place 1 tablet in each of the tubes of the basket and, if prescribed, add a disk to each tube. Operate the apparatus, using water or the specified medium as the immersion fluid, maintained at 37 ± 2°. At the end of 30 min, lift the basket from the fluid and observe the tablets.

  **Plain-coated tablets:** Place 1 tablet in each of the tubes of the basket and, if the tablet has a soluble external sugar coating, immerse the basket in water at room temperature for 5 min. Then, if prescribed, add a disk to each tube and operate the apparatus, using water or the specified medium as the immersion fluid, maintained at 37 ± 2°. At the end of 30 min, lift the basket from the fluid and observe the tablets.

  **Delayed-release (enteric-coated) tablets:** Omit the use of a disk. Place 1 tablet in each of the six tubes of the basket, and if the tablet has a soluble external sugar coating, immerse the basket in water at room temperature for 5 min. Then operate the apparatus using simulated gastric fluid TS, maintained at 37 ± 2°, as the immersion fluid. After 1 h of operation in simulated gastric fluid TS, lift the basket from the fluid and observe the tablets: the tablets show no evidence of disintegration, cracking, or softening. Operate the apparatus using simulated intestinal fluid TS, maintained at 37 ± 2°, as the immersion fluid for the time specified in the monograph. Lift the basket from the fluid and observe the tablets.

  **Delayed-release (enteric-coated) soft-shell capsules:** Place 1 softgel capsule in each of the six tubes of the basket. Omit the use of a disk. Operate the apparatus using simulated gastric fluid TS, maintained at 37 ± 2°, as the immersion fluid. After 1 h of operation in simulated gastric fluid TS, lift the basket from the fluid and observe the softgels: the softgels show no evidence of disintegration or rupture that would permit the escape of the contents. Operate the apparatus with disks using simulated intestinal fluid TS, maintained at 37 ± 2°, as the immersion fluid for NMT 60 min. Lift the basket from the fluid and observe the capsules.

  **Hard-shell capsules:** Apply the test for *Uncoated tablets* using, as the immersion fluid, maintained at 37 ± 2°, a 0.05 M acetate buffer prepared by mixing 2.99 g of sodium acetate trihydrate and 1.66 mL of glacial acetic acid with water to obtain a 1000-mL solution with a pH of 4.50 ± 0.05. Attach a removable wire cloth, as described in *Basket-rack assembly*, to the surface of the upper plate of the basket-rack assembly. At the end of 30 min, lift the basket from the fluid and observe the capsules.

  **Soft-shell capsules:** Proceed as directed in the *Rupture Test for Soft-Shell Capsules*.

- **USE OF DISKS**

  **Vitamin–mineral dosage forms:** Add a disk to each tube unless otherwise specified in the *Procedure* above or in the individual monograph.

  **Botanical dosage forms:** Omit the use of disks unless otherwise specified in the *Procedure* above or in the individual monograph.

  **Dietary supplements other than vitamin–mineral and botanical dosage forms:** Omit the use of disks unless otherwise specified above or in the individual monograph.

- **TOLERANCES:** All of the 6 dosage units initially tested or NLT 16 of a total of 18 dosage units tested disintegrate completely.

**RUPTURE TEST FOR SOFT-SHELL CAPSULES**

**Medium:** Water; 500 mL

**Apparatus:** Use *Apparatus 2* as described in *Dissolution* ⟨711⟩, *Apparatus*, operating at 50 rpm.

**Time:** 15 min

- **PROCEDURE:** Place 1 capsule in each vessel, and allow the capsule to sink to the bottom of the vessel before starting rotation of the blade. Use sinkers if the capsules float. Observe the capsules throughout the test and at the end of the test. The capsule shell is considered ruptured if breached, exposing or allowing the fill contents to escape.
- **TOLERANCES:** The requirements are met if all of the capsules tested rupture in NMT 15 min. If 1 or 2 of the capsules rupture in >15 min but NMT 30 min, repeat the test on 12 additional capsules: NMT 2 of the total of 18 capsules tested rupture in >15 min but NMT 30 min. For soft gelatin capsules that do not conform to the above rupture test acceptance criteria, repeat the test with the addition of papain to the *Medium* in the amount that results in an activity of NMT 550,000 units/L of *Medium* or with the addition of bromelain in the amount that results in an activity of NMT 30 gelatin-digesting units (GDU)/L of *Medium*. [NOTE—Determine papain activity using the *Assay* in the monograph for *Papain* and bromelain activity using the procedure in bromelain, in the *Reagent Specifications* section.]

**DISSOLUTION**

This test is provided to determine compliance with the *Dissolution* requirements where stated in the individual monographs for dietary supplements. The operative assumption inherent in this test is that if the index vitamin or mineral or marker compound(s) for a botanical is dissolved within the time frame and under conditions specified, the dosage form does not suffer from formulation- or manufacturing-related problems affecting the adequate release of the active ingredients.

- **FOR DOSAGE FORMS CONTAINING OR COATED WITH GELATIN**

For hard or soft gelatin capsules and gelatin-coated tablets that do not conform to the dissolution specification because of the presence of cross-linking, the dissolution procedure should be repeated with the addition of enzymes to the medium, as described below.

**Dissolution medium with pH ≤4.0**

**Enzyme:** Pepsin, activity determined by the procedure in pepsin, in the *Reagent Specifications* section

**Amount:** A quantity of pepsin that results in an activity of NMT 750,000 units/L of dissolution medium

**Dissolution medium with pH >4.0 and <6.8**

**Enzyme:** Papain, activity determined by the *Assay* in the monograph for *Papain*; or bromelain, activity determined by the procedure in bromelain, in the *Reagent Specifications* section

**Amount:** A quantity of papain that results in an activity of NMT 550,000 units/L of dissolution medium, or a quantity of bromelain that results in an activity of NMT 30 GDU/L of dissolution medium

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**Dissolution medium with pH ≥6.8**

**Enzyme:** Pancreatin, protease activity determined by the procedure in *Assay for protease activity* (*Casein digestive powder*) in the monograph for *Pancreatin*

**Amount:** A quantity of pancreatin that results in a protease activity of NMT 2,000 units/L of dissolution medium

**Dissolution medium containing surfactants or other components known to denature the enzyme:** If the dissolution medium contains surfactants or other components known to denature the enzyme to be used, a pretreatment step should be applied. The pretreatment step is performed under the same dissolution conditions (apparatus, rotation, and flow rate), except to use a medium with the corresponding amount of enzyme as directed in the preceding section and without the surfactant or component known to denature the enzyme. To achieve the final specified volume of medium, the pretreatment step may be conducted with a smaller volume of medium without the surfactant or component in such a manner that the final specified volume is achieved after the addition of the surfactant or component at the end of the pretreatment step. Perform the pretreatment step until capsule rupture, but for NMT one-half of the total dissolution time specified in the procedure. The pretreatment time is included in the total dissolution time specified in the procedure.

- **Apparatus:** See ⟨711⟩ for a description of the apparatus used, apparatus suitability test, and other related information. Where the procedure specifies the use of a stationary basket, use the quadrangular basket of stainless steel wire gauze as shown in *Figure 2a* and *Figure 2b*.

The capsule is placed in a basket, soldered in one of its upper, narrow sides to the end of a steel rod (see *Figure 2a*). The capsule cover is placed in the horizontal diagonal of the basket. The rod assembly is inserted vertically through the cover of the dissolution vessel, and fixed by means of two teflon nuts, 3.2 cm from the center of the vessel, or by any other appropriate means. The lower edge of the bottom of the basket is adjusted to about 1 cm above the top of the paddle blade (see *Figure 2b*).

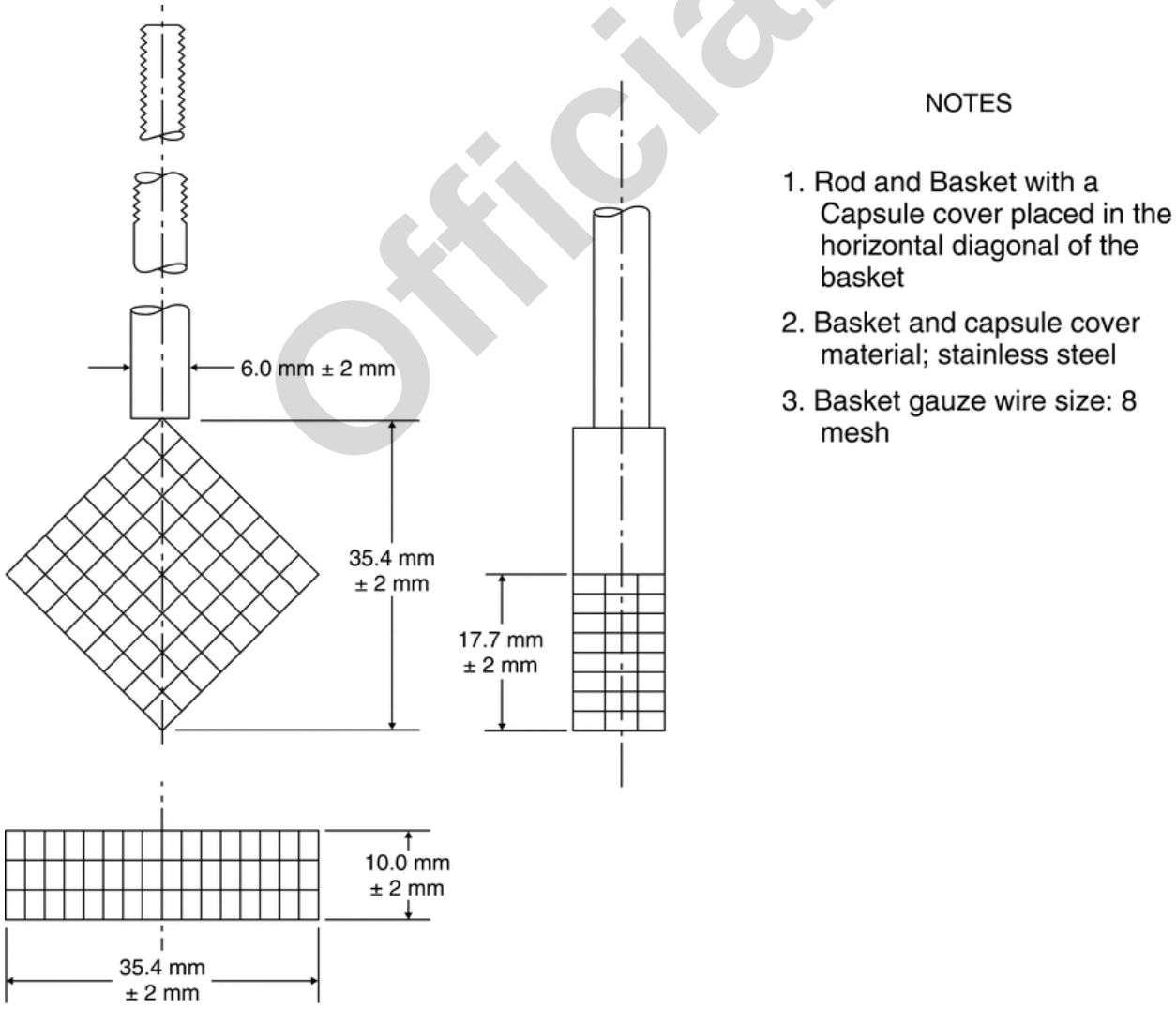


Figure 2a. Stationary Basket

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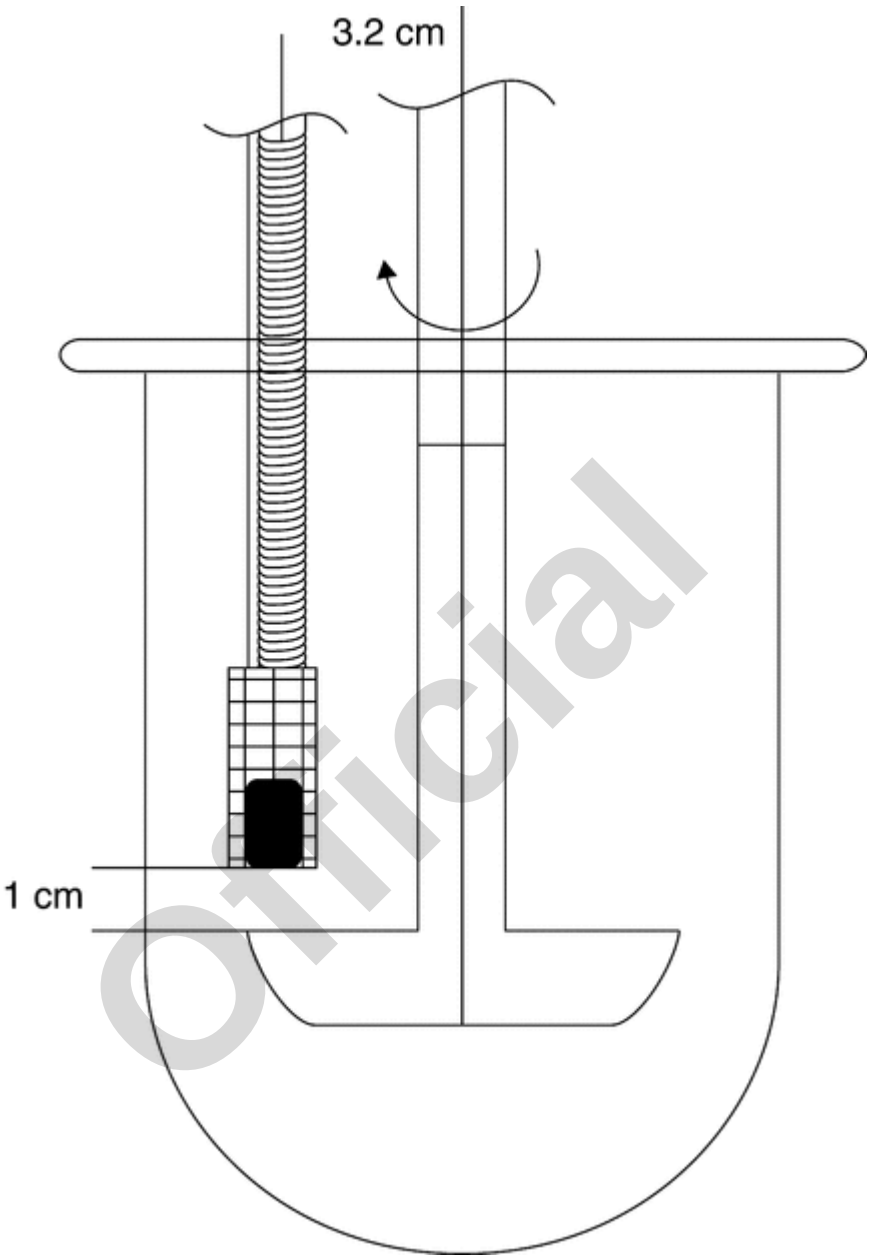


Figure 2b. Stationary Basket Configuration Diagram

Of the types of apparatus described in ⟨711⟩, use the one specified in the individual monograph.

- **Vitamin–Mineral Dosage Forms**

All dietary supplement capsules, tablets, or chewable gels containing folic acid are subject to the dissolution test and criteria for folic acid described in this chapter. This test is required because of the importance of the relationship between folate deficiency and the risk of neural tube defects. Dietary supplement capsules, tablets, or chewable gels containing water-soluble vitamins, minerals, or their combination are subject to the dissolution test and criteria for index vitamins, index minerals, or both, described in this chapter. Dietary supplement tablets, chewable gels, and hard-shell capsules with solid content dosage forms containing vitamin A are subject to the dissolution test and criteria for vitamin A described in this chapter. Dissolution standards were not established and therefore are not applicable to vitamin A in dietary supplement soft-shell capsules filled with liquids. *Table 1* summarizes the dissolution requirements for the assigned USP classes of dietary supplements. Vitamin–mineral combinations that do not belong to any of the USP classes listed in *Table 1* are subject to the *Dissolution* test and criteria specified in the individual monographs.

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**Table 1. Dietary Supplements—Vitamin–Mineral Dosage Forms**

| USP Class | Ingredients | Dissolution Requirements for Tablets, Chewable Gels, and Hard-Shell Capsules with Solid Contents | Dissolution Requirements for Soft-Shell Capsules Filled with Liquids |
|---|---|---|---|
| I | Oil-soluble vitamins | Vitamin A (if present) | Not applicable |
| II | Water-soluble vitamins | One index water-soluble vitamin and folic acid (if present) | One index water-soluble vitamin and folic acid (if present) |
| III | Water-soluble vitamins with minerals | One index water-soluble vitamin, one index element, and folic acid (if present) | One index water-soluble vitamin, one index element, and folic acid (if present) |
| IV | Oil- and water-soluble vitamins | Vitamin A (if present), one index water-soluble vitamin, and folic acid (if present) | One index water-soluble vitamin and folic acid (if present) |
| V | Oil- and water-soluble vitamins with minerals | Vitamin A (if present), one index water-soluble vitamin, one index element, and folic acid (if present) | One index water-soluble vitamin, one index element, and folic acid (if present) |
| VI | Minerals | One index element | One index element |
| VII | Oil-soluble vitamins with minerals | Vitamin A (if present) and one index element | One index element |

**Selection of index water-soluble vitamins and index elements:** Compliance with the dissolution requirements for dietary supplements representing combinations of water-soluble vitamins and combinations of oil- and water-soluble vitamins is determined by measuring the dissolution of a single index vitamin from the water-soluble vitamins present. Riboflavin is the index vitamin when present in the formulation. For formulations that do not contain riboflavin, pyridoxine is the index vitamin. If neither riboflavin nor pyridoxine is present in the formulation, the index vitamin is niacinamide (or niacin), and in the absence of niacinamide (or niacin), the index vitamin is thiamine. If none of these four water-soluble vitamins are present in the formulation, the index vitamin is ascorbic acid.

Compliance with the dissolution requirements for dietary supplements representing combinations of minerals is determined by measuring the dissolution of only one index element. Iron is the index element when present in the formulation. For formulations that do not contain iron, the index element is calcium. If neither iron nor calcium is present, the index element is zinc. In the absence of all three of these elements, magnesium is the index element.

Compliance with the dissolution requirements for dietary supplements representing combinations of water-soluble vitamins and minerals and combinations of oil- and water-soluble vitamins and minerals is determined by measuring the dissolution of one index water-soluble vitamin and one index element, designated according to the respective hierarchies described above.

**Dissolution conditions for vitamin A:** [NOTE—Perform this test under light conditions that minimize photodegradation.]
**Medium:** 1% (w/v) sodium ascorbate and 1% (w/v) octoxynol 9 in 0.05 M phosphate buffer, pH 6.8; 900 mL
**Apparatus 2:** 75 rpm
**Time:** 45 min

**Dissolution conditions for folic acid:** [NOTE—Perform this test under light conditions that minimize photodegradation.]
**Test 1**
**Medium:** Water; 900 mL
**Apparatus 1:** 100 rpm, for capsules
**Apparatus 2:** 75 rpm, for tablets
**Time:** 1 h
If the units tested do not meet the requirements for dissolution in water, use the following conditions:
**Buffer:** Mix 95 mL of 0.1 M citric acid monohydrate and 405 mL of 0.1 M sodium citrate dihydrate, dilute with water to 1000 mL, mix, and adjust to a pH of 6.0 by using either 0.1 M hydrochloric acid or 0.1 M sodium hydroxide solution.
**Medium:** Buffer; 900 mL
**Apparatus 1:** 100 rpm, for capsules
**Apparatus 2:** 75 rpm, for tablets
**Time:** 1 h
**Test 2** (for lipid-filled soft-shell capsules): Proceed as directed for *Test 2* under *Dissolution conditions for index water-soluble vitamins and index minerals*. If the article complies with this test, the labeling indicates that it meets USP *Dissolution Test 2*.
**Test 3** (for lipid-filled soft-shell capsules): Proceed as directed for *Test 3* under *Dissolution conditions for index water-soluble vitamins and index minerals*. If the article complies with this test, the labeling indicates that it meets USP *Dissolution Test 3*.
[NOTE—Compliance with the dissolution requirements for folic acid does not exempt the article from compliance with the dissolution requirements of the pertinent index vitamin or the corresponding index mineral.]

**Dissolution conditions for index water-soluble vitamins and index minerals**
**Test 1**
**Medium:** 0.1 N hydrochloric acid; 900 mL
**Apparatus 1:** 100 rpm, for capsules
**Apparatus 2:** 75 rpm, for tablets
**Time:** 1 h

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For formulations containing 25 mg or more of the index vitamin, riboflavin, use the following conditions:
**Medium:** 0.1 N hydrochloric acid; 1800 mL
**Apparatus 1:** 100 rpm, for capsules
**Apparatus 2:** 75 rpm, for tablets
**Time:** 1 h

**Test 2** (for lipid-filled soft-shell capsules)**:** If the article complies with this test, the labeling indicates that it meets USP *Dissolution Test 2*.
**Medium:** 0.25% (w/v) octoxynol 9, 0.02% (w/v) ascorbic acid, and 0.04% (w/v) simethicone in simulated gastric fluid TS; 250 mL
**Apparatus 3:** 15 dpm
**Screen** (top and bottom)**:** 20-mesh
**Time:** 1 h

**Test 3** (for lipid-filled soft-shell capsules)**:** If the article complies with this test, the labeling indicates that it meets USP *Dissolution Test 3*.
**Medium:** 0.25% (w/v) octoxynol 9 and 0.02% (w/v) ascorbic acid in simulated gastric fluid TS; 500 mL
**Apparatus 2:** 125 rpm; dosage unit placed in stationary basket (*Figure 2a* and *Figure 2b*)
**Time:** 1 h

[NOTE—Compliance with dissolution requirements for the pertinent index vitamin or index mineral does not exempt the article from compliance with the dissolution requirements for folic acid, if present.]

**Procedures:** In the following procedures, combine equal volumes of the filtered solutions of the six individual specimens withdrawn, and use the pooled sample as the test specimen. Determine the average amount of vitamin A, folic acid, or the index vitamin or element dissolved in the pooled sample. Make any necessary modifications, including concentration of the analyte in the volume of *Sample solution* taken. Use the *Medium* for preparation of the *Standard solution* and dilution, if necessary, of the *Sample solution*.

**Vitamin A:** Determine the percentage of retinyl acetate or retinyl palmitate dissolved by using the following procedure.
**Standard solution:** Dissolve a suitable amount of USP Retinyl Acetate RS or USP Retinyl Palmitate RS in isopropyl alcohol, and dilute with *Medium* to obtain a concentration similar to that expected in the *Sample solution*. [NOTE—The amount of isopropyl alcohol should be 5%–10%.]
**Sample solution:** Withdraw a portion of the solution under test, pass through a suitable filter of 0.45-µm pore size, and use the pooled sample as the test specimen.
**Solution A:** Methanol and water (90:10)
**Solution B:** Methanol and isopropyl alcohol (55:45)
**Mobile phase:** See *Table 2*.

**Table 2**

| Time (min) | Solution A (%) | Solution B (%) |
|---|---|---|
| 0 | 100 | 0 |
| 8 | 0 | 100 |
| 13 | 0 | 100 |
| 13.1 | 100 | 0 |
| 15 | 100 | 0 |

**Chromatographic system**
(See *Chromatography* ⟨621⟩*, System Suitability*.)
**Mode:** LC
**Detector:** UV 325 nm
**Column:** 4.6-mm × 10-cm; 3-µm packing L1
**Flow rate:** 1.0 mL/min
**Injection volume:** 50 µL

**System suitability**
**Sample:** *Standard solution*
**Suitability requirements**
**Tailing factor:** NMT 1.5 for retinyl acetate; NMT 2.0 for retinyl palmitate
**Relative standard deviation:** NMT 2.0%

**Analysis**
**Samples:** Appropriate *Standard solution* and *Sample solution*

$$\text{Result} = (r_U/r_S) \times (C_S \times V/L) \times 100$$

$r_U$ = peak area of the all-*trans*-retinyl ester from the *Sample solution*
$r_S$ = peak area of the all-*trans*-retinyl ester from the appropriate *Standard solution*
$C_S$ = concentration of retinol in the appropriate *Standard solution* (µg/mL)
$V$ = volume of *Medium*, 900 mL
$L$ = label claim of vitamin A, as retinol (µg/tablet)

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**Folic acid:** Determine the amount of folic acid ($C_{19}H_{19}N_7O_6$) dissolved by using the procedure set forth in the assay for folic acid in the individual monograph. Make any necessary modifications.

**Niacin or niacinamide, pyridoxine, riboflavin,** and **thiamine:** Determine the amount of the designated index vitamin dissolved by using the procedure set forth in the assay for niacin or niacinamide, pyridoxine hydrochloride, riboflavin, and thiamine in the individual monographs. Make any necessary modifications.

**Ascorbic acid:** Determine the amount of ascorbic acid ($C_6H_8O_6$) dissolved by using the procedure set forth in the assay for ascorbic acid in the individual monograph. Make any necessary modifications.

**Iron, calcium, magnesium,** and **zinc:** Determine the amount of the designated index element dissolved by using the procedure set forth in the appropriate assay in the individual monographs. Make any necessary modifications.

**Tolerances:** The requirements are met if NLT 75% of the labeled content of vitamin A, NLT 75% of the labeled content of folic acid, and NLT 75% of the labeled content of the index vitamin or the index element from the units tested is dissolved.

- **Botanical Dosage Forms**

Compliance with dissolution requirements necessitates the testing of 6 dosage units individually, or testing 2 or more dosage units in each of the six vessels of the dissolution apparatus, and measuring the dissolution of one or more index/marker compound(s) or the extract specified in the individual monograph.

**Procedures:** Combine equal volumes of the filtered solutions of the six or more individual specimens withdrawn, and use the pooled sample as the *Sample solution*. Determine the average amount of index or marker compound(s) or the extract dissolved in the pooled sample by the procedure specified in the individual monograph. Make any necessary modifications, including concentration of the analyte in the volume of the *Sample solution* taken. Use the *Medium* for preparation of the *Standard solution* and dilution, if necessary, of the *Sample solution*.

**Tolerances:** Unless otherwise specified in the individual monograph, the requirements are met if NLT 75% of the labeled content of the index or marker compound(s) or the extract from the units tested is dissolved in 1 h.

- **Dietary Supplements Other Than Vitamin–Mineral and Botanical Dosage Forms**

Unless otherwise stated in the individual monographs for dietary supplement dosage forms in this category, compliance requires the testing of 6 individual units, measuring the dissolution of the dietary ingredient as the average of the 6 units tested.

**Procedures:** Combine equal volumes of the filtered solutions of the six specimens withdrawn, and use the pooled sample as the *Sample solution*. Determine the average amount of the dietary ingredient dissolved in the pooled sample by the procedure specified in the individual monograph. Make any necessary modifications, including concentration of the analyte in the volume of the *Sample solution* taken. Use the *Medium* for preparation of the *Standard solution* and for dilution, if necessary, of the *Sample solution*.

**Tolerances:** Because of the diversity of chemical characteristics and solubilities of dietary ingredients pertaining to this category, general tolerances cannot be established. See individual monographs for *Tolerances*.

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# ⟨2091⟩ WEIGHT VARIATION OF DIETARY SUPPLEMENTS

***Change to read:***

## INTRODUCTION

The following tests provide limits for the permissible variations in the weights of individual tablets, capsules, ▲or chewable gels▲ (USP 1-Dec-2019) expressed in terms of the allowable deviation from the average weight of a sample. Separate procedures and limits are described herein for capsules, uncoated tablets, coated tablets, ▲and chewable gels▲ (USP 1-Dec-2019) that are intended for use as dietary supplements.

## CAPSULES

Capsules meet the requirements of the following test with respect to variation in weight of contents.

### Hard Shell Capsules

Weigh 20 intact capsules individually, and determine the average weight. The requirements are met if each of the individual weights is within the limits of 90% and 110% of the average weight.

If not all of the capsules fall within the aforementioned limits, weigh the 20 capsules individually, taking care to preserve the identity of each capsule, and remove the contents of each capsule with the aid of a small brush or pledget of cotton. Weigh the emptied shells individually, and calculate for each capsule the net weight of its contents by subtracting the weight of the shell from the respective gross weight. Determine the average net content from the sum of the individual net weights. Then determine the difference between each individual net content and the average net content: the requirements are met if (a) NMT 2 of the differences are greater than 10% of the average net content and (b) in no case is the difference greater than 25%.

If more than 2 but NMT 6 capsules deviate from the average between 10% and 25%, determine the net contents of an additional 40 capsules, and determine the average content of the entire 60 capsules. Determine the 60 deviations from the new average: the requirements are met if (a) NMT 6 of the 60 capsules have differences that are greater than 10% of the average net content and (b) in no case does the difference exceed 25%.

### Soft Shell Capsules

Proceed as directed under *Hard Shell Capsules*, but determine the net weight of the contents of individual capsules as follows. Weigh the intact capsules individually to obtain their gross weights, taking care to preserve the identity of each capsule. Then cut open the capsules by means of a suitable clean, dry cutting instrument, such as scissors or a sharp open blade, and remove the contents by washing with a suitable solvent. Allow the occluded solvent to evaporate from the shells at room temperature over a period of about 30 min, taking precautions to avoid uptake or loss of moisture. Weigh the individual shells, and calculate the net contents. The requirements are as stated under *Hard Shell Capsules*.

## TABLETS

Tablets conform to the criteria given in *Table 1*.

### Uncoated Tablets and Film-Coated Tablets

Weigh 20 whole tablets individually, and calculate the average weight. The requirements are met if the weights of NMT 2 of the tablets differ from the average weight by more than the percentage listed in *Table 1*, and no tablet differs in weight by more than double that percentage.

### Coated Tablets (Other Than Film-Coated Tablets)

Weigh 20 whole tablets individually, and calculate the average weight. If the coated tablets do not conform to the criteria in *Table 1*, place 20 tablets in a beaker of water at 37° and swirl gently for NMT 5 min. Examine the cores for evidence of disintegration and repeat the procedure for a shorter time if disintegration has begun. Dry the cores at 50° for 30 min. Accurately weigh 20 individual tablet cores, and calculate the average weight.

The requirements are met if the weights of NMT 2 of the tablets differ from the average weight by more than the percentage listed in *Table 1* and no tablet differs in weight by more than double that percentage.

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## Acceptance Criteria

**Table 1. Weight Variation Tolerances for Uncoated Tablets, Film-Coated Tablets, and Coated Tablets (Other Than Film-Coated Tablets)**

| Average Weight of Tablet (mg) | Difference (%) |
|---|---|
| 130 or less | 10 |
| From 130 through 324 | 7.5 |
| More than 324 | 5 |

***Add the following:***

## ▲CHEWABLE GELS

Chewable gels meet the requirements of the following test with respect to weight variation. Individually weigh an equal number of units of each color and shape to obtain a total of NLT 20 and NMT 30 individual weights, and calculate the average weight.

The requirements are met if no individual weight deviates from the average weight by more than 7.5%. If more than 1 unit exceeds the specified limit, the test fails.

If 1 unit falls outside of the limits, repeat the procedure with an additional set of NLT 20 and NMT 30 individual chewable gels. The requirements are met if none of the units tested in the second set differ from their average weight by more than 10%.▲ (USP 1-Dec-2019)

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# ⟨2232⟩ ELEMENTAL CONTAMINANTS IN DIETARY SUPPLEMENTS

The objective of this general chapter is to limit the amounts of elemental contaminants in finished dietary supplement dosage forms labeled as conforming to *USP* or *NF* standards. This general chapter is not intended to set limits for dietary ingredients. Those limits are set in the corresponding individual monographs.

The focus of this general chapter is on the four major elements of toxicological concern: arsenic, cadmium, lead, and mercury. The extent of testing can be determined using a risk-based approach that takes into account the likelihood of contamination. Manufacturers should consider the presence of unexpected elemental contaminants to determine compliance.

## LIMITS OF ELEMENTAL CONTAMINANTS

The levels of elemental contaminants should be restricted as shown in *Table 1* unless otherwise stated in the individual monograph. Specific monographs may provide different limits for articles that need to be consumed in large quantities.

**Table 1**

| Element | PDE (µg/day)[a] |
|---|---|
| Arsenic (inorganic) | 15 |
| Cadmium | 5 |
| Lead | 5 |
| Mercury (total) | 15 |
| Methylmercury (as Hg) | 2 |

[a] Permitted Daily Exposure (PDE) is derived from the Provisional Tolerable Weekly Intake (PTWI) that is recommended by the Food and Agriculture Organization of the United Nations and World Health Organization (FAO/WHO) by subtracting the daily exposure (µg/day) to each elemental contaminant from air, food, and drinking water. A body weight of 50 kg and a safety factor are used to calculate the PDE. Other regulations (e.g., Proposition 65 in California) may require different limits; manufacturers are responsible for compliance with applicable local requirements differing from these PDE values.

Arsenic may be measured using a nonspeciation procedure under the assumption that all arsenic contained in the supplement is in the inorganic form. Where the limit is exceeded using a nonspeciation procedure, compliance with the limit for inorganic arsenic shall be demonstrated on the basis of a speciation procedure. Methylmercury determination is not necessary when the content for total mercury is less than the limit for methylmercury.

## OPTIONS FOR COMPLIANCE WITH THE LIMITS OF ELEMENTAL CONTAMINANTS

In order for a dietary supplement finished dosage form to comply with the limits for elemental contaminants as described in this chapter, the level of elemental contaminant in the finished dietary supplement should be NMT the PDE. The following three options are available for determining compliance with the limits for elemental contaminants in dietary supplements.

- **Dietary Supplement Analysis Option**

  This option is generally applicable. In this option the finished dietary supplement dosage form is analyzed according to the procedures in the general chapter *Elemental Impurities—Procedures* ⟨233⟩ or the speciation procedures given in this chapter. The results obtained from the analysis of a typical serving size, scaled to a maximum daily intake, are compared to the PDE, as stated in *Table 1*.

  **Analysis:** Proceed as directed below in this chapter.

  Calculate the measured amount of each elemental contaminant, in µg/daily intake, as:

  $$\text{Result} = MVSS \times N$$

  $MVSS$ = measured amount of each elemental contaminant (µg/serving size)

  $N$ = maximum daily intake of the supplement recommended in the labeling (servings/day)

  **Acceptance criteria:** The measured amount/daily intake is NMT the PDE value given in *Table 1*.

- **Individual Component Option**

  This option is applicable to finished dietary supplement dosage forms with a maximum daily intake of NMT 10 g of the dietary supplement finished product.

  **Analysis:** Unless otherwise specified in the individual monograph, proceed with the individual ingredient as directed below in this chapter.

  **Acceptance criteria:** The product meets the requirements when each component used to prepare the finished dietary supplement meets the limits given in *Table 2*.

**Table 2**

| Element | Individual Component Limits (µg/g)[a] |
|---|---|
| Arsenic (inorganic)[b] | 1.5 |
| Cadmium | 0.5 |
| Lead | 0.5 |
| Mercury (total) | 1.5 |
| Methylmercury (as Hg)[c] | 0.2 |

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[a] The limits for individual components are based on a maximum daily intake of 10 g of a dietary supplement and are intended for use only with *Options for Compliance with the Limits of Elemental Contaminants*, *Individual Component Option*.
[b] Arsenic may be measured using a nonspeciation procedure under the assumption that all arsenic contained in the supplement is in the inorganic form. Where the limit is exceeded using a nonspeciation procedure, compliance with the limit for inorganic arsenic shall be demonstrated on the basis of a speciation procedure.
[c] Methylmercury determination is not necessary when the content for total mercury is less than the limit for methylmercury.

[NOTE—If all components in a formulation meet the limits given for the *Individual Component Limits*, these components can be used in any proportion. No further calculation is necessary.]

- **SUMMATION OPTION**

This option can be used for finished dietary supplement dosage forms that are consumed in quantities greater than 10 g/day or where the acceptance limit for any contaminant in any component of the dietary supplement exceeds the applicable *Individual Component Limits*.

**Analysis:** Unless otherwise specified in the individual monograph, proceed with the individual ingredient as directed below in this chapter.

Calculate the amount of each elemental contaminant, in µg/daily intake, present in the finished dietary supplement dosage form:

$$\text{Result} = \Sigma(C_i \times W_i) \times N$$

$C_i$ = elemental contaminant concentration in the individual component (µg/g)

$W_i$ = weight of each individual component per serving of the dietary supplement (g/serving)

$N$ = maximum daily intake of the supplement recommended in the labeling (servings/day)

**Acceptance criteria:** The calculated amount of each elemental contaminant/daily intake is NMT the PDE value given in *Table 1*.

***Change to read:***

## ANALYTICAL PROCEDURES FOR TOTAL ELEMENTAL CONTAMINANTS

Performance-based methodology for analysis of total elemental contaminants in general chapter *Elemental Impurities—Procedures* ⟨233⟩ is applicable for dietary supplements. The validation necessity will vary depending on the situation. In all three options described in the section *Options for Compliance with the Limits of Elemental Contaminants*, the use of *Validation of Limit Procedures* (see *Elemental Impurities—Procedures* ⟨233⟩) may be appropriate. However, for the *Summation Option*, acceptable levels of validation must be determined on a case-by-case basis. Validation of a procedure using the *Validation of Quantitative Procedures* (see *Elemental Impurities—Procedures* ⟨233⟩) is acceptable for all options under all circumstances and is generally preferred. The determination of the level of validation necessity is at the discretion of the manufacturer and the competent regulatory authority.

- **ANALYTICAL PROCEDURE FOR INORGANIC ARSENIC**

Where the level of total arsenic exceeds the limit recommended in this chapter, speciation may be used to determine the amount of inorganic arsenic present. The following procedure is suggested for determination of inorganic arsenic, but any validated procedure shown to give equivalent or better results can be used.

**Apparatus:** See *Figure 1*.

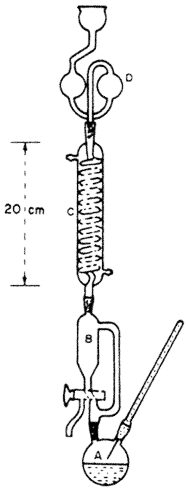


Figure 1. Special apparatus for the determination of inorganic arsenic (A, 250-mL distillation flask; B, receiver chamber, approximately 50-mL capacity; C, reflux condenser; D, splash head or security funnel).

**Reagents**

**Distillation-reducing solution:** 30.0 g of potassium iodide in 100 mL of water. [NOTE—Prepare fresh on the day of use.]

**Control:** 6.0 µg of arsenic (As) (6.0 mL of *Standard Arsenic Solution*). [NOTE—Use this amount rather than the 3.0 mL specified for *Standard Preparation* in the general chapter ▲*Arsenic* ⟨211⟩, *Procedures, Procedure 1*▲ (CN 1-Jun-2023).]

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**Sample solution:** Transfer a 2.00-g sample that has previously been ground to pass through a 60-mesh screen to a distillation flask (A). To the flask add 35 mL of hydrochloric acid 6.6 N and 15 mL of *Distillation-reducing solution*, let stand for 5 min to ensure reduction of arsenic [As(V)], connect the flask to the receiver chamber (B), complete the assembly of the apparatus, and begin circulating tap water through the condenser (C). Half-fill the lower two bulbs of the splash head (D) with water. Maneuver the stopcock to cause the contents of the receiver chamber to drain into the distillation flask, heat the flask until the temperature above the solution reaches 100°–110°, and continue refluxing at this temperature for 15 min. Close the stopcock, continue heating at 108°–110°, and collect 30–33 mL of distillate in the receiver chamber. Remove the heating source, and allow the temperature to drop to about 80°.

Close the stopcock, and add a second 35-mL portion of 6.6 N hydrochloride (HCl) and 15 mL of the *Distillation-reducing solution* through the thermometer opening to the distillation flask. Replace the thermometer, increase the temperature to 100°–110°, and collect a second 30- to 33-mL portion of distillate in the receiver chamber. Drain the second distillate into the beaker containing the first portion, and cool the combined distillate to room temperature. Remove the splash head, and wash its contents into the beaker. Also, wash down the inside of the condenser and receiver chamber with water, collecting the washings into the beaker. Transfer to a 100-mL volumetric flask, and complete with water to volume.

**Analysis:** Determine the arsenic content by the ICP-MS procedure in *Elemental Impurities—Procedures* ⟨233⟩. Alternatively, add 2 mL of potassium iodide TS and 0.5 mL of stronger acid stannous chloride TS to the *Sample solution* contained in the Erlenmeyer flask, and proceed as directed in ▲*Arsenic* ⟨211⟩*, Procedures, Procedure 1*▲ (CN 1-Jun-2023)*, Procedure*, beginning with "Allow to stand at room temperature for 30 minutes."

- **Analytical Procedure for Methylmercury**

Where methylmercury determination is required, the following procedure is suggested. However, any validated procedure shown to give equivalent or better results can be used.

**Procedure 1**

This procedure uses an aqueous extraction of the mercury species with an L-cysteine solution, HPLC separation of the derivatized mercury species with a mobile phase also containing L-cysteine, and ICP-MS detection.

**Cysteine solution:** 1% L-cysteine hydrochloride monohydrate in water

**Mobile phase:** 0.1% L-cysteine hydrochloride monohydrate and 0.1% L-cysteine free base in water

**Standard stock solution:** 1 µg/mL of mercury (Hg) as methylmercury chloride, and 1 µg of mercury (Hg) as mercury chloride in 5% hydrochloric acid containing 0.2 mg/mL of L-cysteine hydrochloride monohydrate

**Standard solution:** 2 ng/mL of mercury as methylmercury from *Standard stock solution* in *Cysteine solution*

**Sample solutions**

**For supplements in tablet form:** Weigh, and finely powder a counted number of tablets. Transfer an accurately weighed portion of the powder equivalent to 0.5 times the daily recommended intake to a tared vial. Add 50.0 mL of *Cysteine solution* accurately weighed, cap the vial, and shake vigorously. Place the vial in a water bath at 60° for 60 min. Shake the vial again, and return to the water bath for another period of 60 min. Shake the vial again, and allow to cool at room temperature for about 20 min. Filter through a 0.45-µm polyethylene membrane. [NOTE—Prepare fresh on the day of use.]

**For supplements in capsule form:** Weigh accurately NLT 20 capsules, and determine the average weight. Place a number of capsules equivalent to about 5 times the daily recommended intake in a blender, add 500.0 mL of *Cysteine solution* and blend to obtain a homogenous analytical suspension. Transfer 50.0 mL of this analytical suspension to a vial, cap the vial, and shake vigorously. Place the vial in a water bath at 60° for 60 min. Shake the vial again, and return to the water bath for another period of 60 min. Shake the vial again, and allow to cool at room temperature for about 20 min. Filter through a 0.45-µm polyethylene membrane.

[NOTE—Prepare fresh on the day of use.]

**For supplements in liquid form:** Weigh accurately an amount of the liquid equivalent to 0.5 times the daily recommended intake into a vial, and add 50.0 mL of *Cysteine solution*. Cap the vial, and shake vigorously. Place the vial in a water bath at 60° for 60 min. Shake the vial again, and return to the water bath for another period of 60 min. Shake the vial again, and allow to cool at room temperature for about 20 min. Filter through a 0.45-µm polyethylene membrane.

[NOTE—Prepare fresh on the day of use.]

**Chromatographic system**

(See *Chromatography* ⟨621⟩*, System Suitability*.)

**Mode:** LC

**Detector:** ICP-MS at a mass-to-charge ratio of 202

**Column:** 4.6-mm × 15-cm; 4-µm packing L1

**Flow rate:** 1 mL/min

**Injection volume:** 50 µL

**System suitability**

**Sample:** *Standard solution*

**Suitability requirements**

**Resolution:** NLT 3 between the peak representing mercury ($Hg^{2+}$) species at a relative retention time of about 0.56 and the peak representing methylmercury ($MeHg^{+}$) species at a relative retention time of 1.0

**Relative standard deviation:** NMT 10.0%, *Standard solution*

**Analysis**

**Samples:** *Standard solution* and the appropriate *Sample solution*

Determine the peak area of the peak representative of methylmercury in the chromatograms of the *Sample solution* and *Standard solution*.

**Acceptance criteria:** 0.2 µg/daily intake: The peak area for methylmercury in the chromatogram of the *Sample solution* is NMT the peak area for methylmercury in the chromatogram of the *Standard solution*.

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# ⟨2250⟩ DETECTION OF IRRADIATED DIETARY SUPPLEMENTS

## INTRODUCTION

Federal regulations do not permit irradiation of dietary ingredients or dietary supplements for sanitation purposes. Under section 201(s) of the Federal Food, Drug, and Cosmetic Act [21 USC 321(s)], irradiation is considered an additive, and as such it requires FDA approval. Foods that are irradiated should be adequately labeled according to international and national guidelines with statements such as "Treated with radiation" or "Treated by irradiation" in addition to information required by other regulations, including the irradiation logo, the Radura [21 USC 321(s)]. Overexposure to irradiation may have negative effects on product quality. Currently, several independent methods are used to identify irradiated foodstuffs, including dietary supplements. These methods have been validated and are recognized worldwide.

Procedures based on luminescence are widely applied and include screening by photostimulated luminescence (PSL), which is a rapid, simple preliminary screening method to detect irradiation, and a subsequent thermoluminescence (TL) procedure to confirm that the sample has been irradiated. PSL is less time consuming than TL because the inorganic/silicate mineral source of luminescence does not need to be isolated from any organic components present. Both PSL and TL signal intensities are affected by irradiation dose as well as by the nature and amount of inorganic material. TL analysis is one of the detection methods used for confirming the presence of irradiated foods, herbs, spices, vegetables, and fruits, although it has certain limitations: for example, samples must contain sufficient amounts of silicates that can be successfully separated from the samples. The lengthy preparation and need for irradiation in all cases limits TL to a small number of laboratories.

The procedures described in this chapter can be used both by regulatory authorities and by producers and suppliers of foods, including dietary supplements, to detect undeclared irradiated products in the market for purposes of determining compliance with regulations.

## PRINCIPLES OF PHOTOSTIMULATED LUMINESCENCE AND THERMOLUMINESCENCE

Most of the natural dietary ingredients that are either cultivated or wild contain silicate minerals, calcite, or hydroxyapatite. Exposure to ionizing radiation from gamma rays ($^{60}Co$ or $^{137}Cs$), electron beams (up to 10 MeV), or x-rays (up to 5 MeV) stimulates electrons in those types of crystals, resulting in energized electrons being stored in the crystal lattice. Trapped high-energy electrons can be released by stimulation with light or controlled heat, leaving electron holes in the crystal lattice. The energy thus released is detected as luminescence. PSL occurs if light is applied to the system, and TL occurs when heat is applied to the system. The recorded luminescence intensity is proportional to the initial radiation dose absorbed.

Samples for both PSL and TL measurements must be taken from a light-protected position of the bulk samples, and exposure to high temperature and light must be avoided after the samples are taken from the bulk. Sample preparation and subsequent PSL or TL measurement must be conducted under subdued lighting conditions when possible.

TL has been widely used for detection of irradiation in food and dietary ingredients from which silicate minerals can be isolated. In this chapter, PSL and TL are described as the most appropriate detection methods for the materials typically used as dietary ingredients. The two methods are both radiation-specific phenomena, based on the observation that photon counts of irradiated samples usually are higher than those of nonirradiated samples. However, all available methods have some limitations in terms of their specific application range, product-to-product variation, complexity of or interference from the food matrix, and low concentration of radiation-induced markers.

## GENERAL PROCEDURE FOR DETECTION OF IRRADIATION IN DIETARY INGREDIENTS AND DIETARY SUPPLEMENTS

### Screening Using Photostimulated Luminescence

PSL, also known as optically stimulated luminescence, is based on the emission of light in the 300–600 nm range from irradiated samples when illuminated at longer wavelengths in the near-IR region. PSL analysis allows multiple measurements to be performed without sample pretreatment in a short period of time. PSL is also a nondestructive method that does not require separation of inorganic minerals and organic compounds. PSL is based on optical stimulation of mineral debris, typically silicates, and bioinorganic materials such as calcite, feldspar, or hydroxyapatite, and its sensitivity depends on the quantities and types of minerals present in the sample. Before the measurement of PSL, two thresholds are set: the lower typically is set at 700 counts/min (T1), and the upper typically is set at 5000 counts/min (T2). These two thresholds serve to classify the samples. After initial screening of PSL intensity from the samples is performed, the results are classified into negative (counts/ min less than T1), intermediate (counts/min NLT T1 and NMT T2), or positive (counts/min more than T2). A second measurement after a known irradiation dose, known as calibrated PSL (CalPSL), is applied in cases of poorly defined sample matrices whose luminescence sensitivities are not well established. By comparing the screening PSL measurement against its CalPSL response, analysts can take into account variation in detection sensitivity due to variable amounts and different types of minerals present in a given sample. CalPSL measurements are recommended to rule out false negative results due to low mineral content.

#### INSTRUMENTATION

The PSL system consists of pulsed IR sources for photostimulation, a single-photon counting system for highly sensitive detection of luminescence, a sample chamber, and a computer for data analysis.

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The instrumental setup procedure includes checks of irradiated and nonirradiated materials, as well as establishing measurement parameters (cycle time, thresholds, and data-recording conditions). Measurement of initial background counts and periodic measurement of counts in the empty chamber should be conducted in subdued lighting to confirm lack of instrument contamination. The PSL signals (photon counts) emitted from the sample/second are automatically accumulated in the computer and are reported as counts/min. [NOTE—All experiments should be conducted under subdued lighting. In order to minimize the risk of cross-contamination, all preparations are performed in a laminar-flow cabinet.]

### PHOTOSTIMULATED LUMINESCENCE MEASUREMENT

**Procedure**

*PSL screening*—Dispense and weigh two portions of the sample (5–10 g, depending on the density of the product) into two separate 50-mm disposable Petri dishes to cover the Petri base in a thin layer. Dispense samples in subdued lighting to minimize bleaching and under a laminar-flow cabinet to minimize cross-contamination. Measure the PSL of the sample for 1 min on the duplicate aliquots, and calculate the mean.

CALIBRATED PSL (CALPSL)— [NOTE—CalPSL testing may be done only once as part of the validation of the procedure for each material.]

Dispense and weigh two portions of the sample (5–10 g, depending on the density of the product) into 50-mm disposable Petri dishes to cover the Petri base in a thin layer. Measure the PSL of the sample for 1 min on duplicate aliquots, and for each sample calculate the mean. Irradiate the sample to a known dose of 1 kGy after the initial screening. Repeat the PSL measurement (CalPSL).

*Evaluation*—Classify results in the following manner:

<T1: negative, no evidence of PSL

>T1 and <T2: intermediate, weak PSL signal

>T2: positive, stronger signal.

ACCEPTANCE CRITERIA—Nonirradiated samples produce negative results in the PSL screening and are known to produce positive results in the CalPSL. [NOTE—Negative signals in the PSL screening are generally associated with nonirradiated material but can result from low-sensitivity irradiated materials. To evaluate whether a sample is a low-sensitivity material, assessment of the signals using the CalPSL option is necessary. Positive signals in the PSL screening are associated with irradiated material. Samples that are classified as intermediate require further investigation by the TL method to determine their irradiation status.]

## Confirmatory Analysis Using Thermoluminescence

The TL analysis is based on physical changes in silicate minerals that are present in many food samples and dietary ingredients. The silicate minerals are able to store the absorbed radiation energy. A TL reader measures the amount of light that is emitted during controlled heating. The TL of the sample ($TL_1$) is compared to that of the same sample following irradiation at 1 kGy ($TL_2$). If the TL ratio ($TL_1/TL_2$) is greater than 0.1, the sample is considered to be irradiated. In order to use the TL method for the detection of irradiated foods or dietary ingredients, silicate minerals must be isolated from the samples.

### INSTRUMENTATION

TL measurements can be performed with a TL detector that meets the specifications in *Table 1*.

**Table 1. Thermoluminesence Detector Specifications[a]**

| | |
|---|---|
| Radiation[b] | Photon: energies >5 keV<br>Neutron: thermal to 100 MeV<br>Electron/beta: energies >70 keV |
| Measurement ranges | 10 µGy to 1 Gy (1 mrad to 100 rad) linear<br>1 Gy to 20 Gy (100 rad to 2000 rad) supralinear |
| Repeatability | For 1 mGy (100 mrad) $^{137}$Cs doses, <2% standard deviation of 10 sequential measurements |
| Heating plate | 50° to approximately 500°,<br>approximately 6°/s |

[a] Harshaw TLD 3500 (Thermo Fisher Scientific, Waltham, MA) and Nanogray TL2000 (Nanogray, Osaka, Japan) are suitable.
[b] Gamma rays from $^{60}$Co or 10-MeV electron beams are suitable radiation sources.

### PROCEDURE FOR SAMPLE PREPARATION

**Sodium polytungstate solution:** Prepare a solution of sodium polytungstate in water with a final density of 2 g/mL.
**Mineral extraction:** Suspend 3–20 g of sample with 50–100 mL of pure water, and sonicate for about 5 min. Sieve through a 250-µm nylon mesh, rinsing the mesh with water each time by using a strong jet from a wash bottle into a larger beaker (500–1000 mL). Allow the minerals to settle for 5 min and decant most of the water, leaving the minerals in <50 mL of water. Transfer the mineral fraction to a centrifuge tube, and centrifuge for 1 min at 1000 × g. Discard the water layer.
**Preconcentration density separation:** Add 5 mL of the *Sodium polytungstate solution* to the minerals in the centrifuge tube. Shake or mix on a vortex mixer, then sonicate for 5–15 min. Centrifuge for 2 min at 1000 × g. Silicate minerals (density 2.5–2.7 g/mL) precipitate, whereas organic components float. Carefully overlay a layer of water on the *Sodium polytungstate solution*, and discard the water layer and the organic materials by decantation or vacuum suction, leaving the minerals behind in the polytungstate layer. Carefully remove the *Sodium polytungstate solution* layer, and wash the minerals twice with water

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(centrifuge at 1000 × g briefly). Add 1–2 mL of 1 M hydrochloric acid, shake, and leave for 10 min in the dark to dissolve carbonates adhering to the silicate materials. Neutralize the acid with 1 M ammonium hydroxide. Fill the tube with water. Allow the minerals to settle, and centrifuge. Discard the water, and wash the minerals twice with water.

**Fixing the minerals on disc for TL measurement:** Add 3 mL of acetone to the preconcentrated minerals, and shake to displace residual water. Use a stainless steel disc suitable for the TL reader in use. [NOTE—Discs typically are 9–10 mm in diameter and 0.25–0.50 mm in thickness] . Carefully clean the disc by rinsing in water, wash two to three times with acetone, and dry in an oven. Store under dust-free conditions. Record the weight of the clean disc immediately before use. Transfer the minerals (in acetone), and dry the disc at 50° overnight (lab oven). Weigh the disc, and calculate the mass of minerals. Repeat the extraction, if necessary, to meet the system suitability requirements. [NOTE—The mineral sample amount is typically between 0.1 mg and 5 mg] . The deposited minerals can be fixed on the disc by using silicone spray (or by layering with 0.2% carboxymethylcellulose) and drying at 50° overnight.

**Blank discs:** Use clean discs.

## TL MEASUREMENT

TL measurements are performed using a TL reader. Register the TL emission as a function of temperature (glow curves).

**Minimum detectable integrated TL intensity level (MDL):** Integrate the TL intensity of the first glow of the blank discs, and calculate the standard deviation. The MDL is three times the standard deviation of the integrated TL intensity of the blank discs.

**System suitability:** The integrated TL intensity of the irradiated sample ($TL_2$) should be at least 10 times the MDL.

**Measurement of $TL_1$**

*First-glow $TL_1$ measurement*—Set the instrument to an initial temperature of 70°, a heating rate of 6°/s, and a final temperature of 350°. Flush the chamber with nitrogen. Place the sample disc on the heating plate of the TL reader, and measure the glow curve. Determine $TL_1$ as the integrated TL signal between 150° and 250°. Measure the background glow for the sample ($BG_1$) after cooling the sample to 50°. After measuring $TL_1$ and $BG_1$, measure the weight of the sample ($BW_1$).

*Irradiation*—Irradiate the disc with the minerals, with a defined radiation dose of about 1 kGy. [NOTE—A source of $^{60}Co$ gamma rays is suitable.] Store at 50° overnight.

**Measurement of $TL_2$**

*Second-glow $TL_2$ measurement*—Measure the TL for the irradiated sample ($TL_2$) as described for $TL_1$. Measure the background glow for the irradiated sample ($BG_2$) after cooling the sample to 50°, and record the weight of the sample plate with the sample ($BW_2$). Measure the blank levels for process control (without the sample), following the procedures at each stage. Calculate the MDL as the integrated TL intensity of the blank plus three standard deviations.

**TL glow ratio:** The TL glow ratio per sample weight is calculated as described below:

$$\text{TL glow ratio} = [(TL_1 - BG_1)/BW_1]/[(TL_2 - BG_2)/BW_2]$$

$TL_1$ = irradiated sample for the first-glow measurement
$BG_1$ = background glow for the irradiated first-glow sample
$BW_1$ = weight of the irradiated first-glow sample
$TL_2$ = irradiated sample for the second-glow measurement
$BG_2$ = background flow for the irradiated second-glow sample
$BW_2$ = weight of the irradiated second-glow sample

**Evaluation:** TL glow ratios from irradiated samples are typically greater than 0.1. The ratios from nonirradiated samples are below 0.1.

**Acceptance criteria:** The temperature that gives the maximum glow from $TL_1$ measurement must be equal to or higher than the one from a nonirradiated sample. The TL glow ratio must be NMT 0.1 for nonirradiated materials.

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# ⟨2251⟩ SCREENING FOR UNDECLARED DRUGS AND DRUG ANALOGUES

## INTRODUCTION

The illegal addition of undeclared synthetic compounds to products marketed as dietary supplements[1] (DS) is an issue of universal concern. This fraud is practiced to impart therapeutic effects that cannot be achieved by the dietary ingredients alone. Increasingly, synthetic intermediates and structural analogues of the pharmaceuticals and drugs that have been discontinued or withdrawn from the market are being used as adulterants. Multiple adulterating compounds may be added to a single product, frequently in erratic amounts.

The proposed test methodologies facilitate screening for synthetic adulterants. No individual technique is capable of addressing all potential analytes; thus, a combination of orthogonal approaches adds certainty to the analytical outcome. Mass spectrometric techniques provide strong substantiation of the analytical findings. In some cases, e.g., with hormonal drugs, the amounts of physiologically relevant adulterants may be so low that GC-MS or LC-MS may be the only fitting analytical options.

The express purpose of assembling the procedures recommended herein is their suitability for screening. The level of evidence achievable by application of one or several of the recommended procedures is ultimately dictated by the specific requirements of the end-user. It should be noted that structure elucidation and quantitative assessment are beyond the scope of this chapter.

This chapter is meant to be updated regularly, as new concealment methodologies for the adulterants are introduced, or improvements to the methods of analysis are realized.

## ADULTERATION CATEGORIES

The following major categories of adulterated products are recognized:

- **Sexual Enhancement:** This category is also referred to as the Erectile Dysfunction (ED) category. It encompasses a functionally coherent group of adulterants, including several approved drugs, their numerous approved and unapproved analogues, and synthetic intermediates. Their functionality is manifested by inhibition of phosphodiesterase type 5 enzyme (PDE5), which hydrolyzes cyclic guanosine 3′,5′-monophosphate (cGMP); this group of compounds is frequently identified as PDE5 inhibitors. Screening methods for products adulterated with ED compounds are presented in *Appendix A*.
- **Weight Loss (WL):** This category comprises a functionally and chemically diverse collection of compounds that include stimulants, laxatives, diuretics, anorexiants, and psychoactive drugs. Although stimulants constitute an important segment of WL adulterants, the oral anorexiant sibutramine dominates this category, frequently in combination with phenolphthalein, a laxative. Methods for analysis of products adulterated with WL compounds will be addressed in *Appendix B* (to come).
- **Sports Performance Enhancement (SPE):** These compounds constitute the third major category of adulteration. Professional and amateur athletes are targeted with designer anabolic steroids and stimulants, which are systematically banned by the World Anti-Doping Agency. Functional and structural diversity, synthetic proclivity of the adulterators, and the generally small amounts of the infringing substances required to elicit a therapeutic effect make this category especially challenging to address. These supplements are customarily formulated in protein- and fat-rich matrices, thereby further complicating detection. For these reasons, GC- and LC-$MS^n$ techniques constitute primary analytical methodologies within this category. Analysis of products adulterated with SPE compounds will be addressed in *Appendix C* (to come).

## BULK INGREDIENTS AND DOSAGE FORMS

Adulteration may occur either at the level of bulk ingredients or at any subsequent stage of the finished product manufacturing. Analysts should be mindful of the possibility of adulterants physically associated with the finished dosage matrix or excipients, as well as components. In the latter, synthetic compounds have been found embedded into the capsule shell body. This underscores the need for deliberate adjustment to the laboratory procedures that typically focus on the capsule contents alone. Appropriate sampling practices for powders and finished dosage forms should be exercised, particularly when only a limited amount of sample is available.

## RECOMMENDED ANALYTICAL METHODOLOGIES

Adulteration analysis may be broadly categorized into targeted and nontargeted methods. The distinction between these types may be subtle, and a minor adjustment to the methodology will transform a nontargeted method into a targeted method.

### Targeted

These techniques are warranted when the analytes are known. An example of a targeted approach would be monitoring a chromatographic run at a particular wavelength (or mass), and quantifying the analyte that appears within a predefined retention time window. Targeted analysis is conceptually straightforward, because it relies on pre-existing knowledge of the analyte and allows optimization of test methodology for its reliable detection. The targeted approach also is a rarity in the adulterated products analysis, where the nature of the analyte may be anticipated only tentatively, and variable amounts of multiple adulterants belonging to several functional categories are commonplace.

[1] In the United States, dietary supplements are defined as substances that are ingested, in agreement with 21 U.S. Code §321(ff)(2)(A)(i). Definitions of dietary supplements, nutritional supplements, functional foods, and bioactive food additives may vary extensively, depending on local or national legislation. In the marketplace, there is a trend toward expanding the mode of delivery of the adulterating compounds to routes not covered by the regulatory definition for dietary supplements, i.e., topical oils, creams, lotions, e-cigarettes, chewing gums, sprays, and others. Such novel delivery systems present unique challenges, particularly from the standpoint of sample preparation, and are not considered for the purposes of this chapter to be dietary supplements. However, recognizing the emerging threat, USP chooses to highlight the existence of these products. In no way should mention of these products be interpreted as a comment on their legal status or be perceived as an expansion of the definition of DS.

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**Nontargeted**

These methods are better suited to a broad-spectrum detection requirement presented by adulterated products. Nontargeted screening trades precise knowledge of the analyte identity, along with specificity and accuracy, for a wider detection scope. Examples of nontargeted chromatographic screening include acquisition of photodiode array data and full mass-spectral scanning following a chromatographic separation. The procedures in this chapter are written with an eye toward applying all techniques in a nontargeted mode, even the ones considered to be inherently targeted, thereby facilitating detection of a suspect adulterant even in the absence of a matching reference compound.

It is generally recommended to apply a broadly nontargeted methodology first, followed by a targeted procedure. It is crucial to clearly define the end-purpose of analysis, and only then decide on the appropriate instrumentation and assemble a logical testing strategy from the procedures provided. Thus, application of a nontargeted screening method may satisfy the requirements of a manufacturer for the purposes of monitoring bulk raw materials. Conversely, a laboratory requiring a higher level of evidence to enact an enforcement action may opt for a two-step procedure: a preliminary screen, followed by confirmatory analysis of suspect samples.

USP Reference Standards recommended for screening are listed at the end of each relevant *Appendix*. However, considering the rate of propagation of structural analogues and proliferation of newly developed "designer" molecules, establishing and maintaining an all-inclusive catalog of reference materials is both challenging and impractical. Several commercial sources of the compounds of interest exist.[2] Please note that mention of the external reference materials suppliers does not in any way constitute their endorsement, as neither does the listing of reagents, supplies, and instrumentation.

## APPENDIX

- **Appendix A. Screening Methodologies for PDE5 Inhibitors**

### 1. HPLC with Photodiode Array Detection

**Solution A:** 0.1% Formic acid in water
**Solution B:** 0.1% Formic acid in acetonitrile
**Mobile phase:** See *Table 1*.

**Table 1**

| Time (min) | Solution A (%) | Solution B (%) |
|---|---|---|
| 0 | 95 | 5 |
| 15 | 5 | 95 |
| 23 | 5 | 95 |
| 24 | 95 | 5 |
| 31 | 95 | 5 |

**Diluent:** Acetonitrile and water (50:50)
**Standard solution:** 100 µg/mL each of USP Sildenafil Citrate RS, USP Tadalafil RS, and USP Vardenafil Hydrochloride RS in *Diluent*
**Sample solution:** Combine one-fifth of the dosage unit, 10–20 mg of bulk material, or a small fragment of the capsule shell (3 mm × 3 mm) with 10 mL of *Diluent*, sonicate for 30 min, and pass through a 0.2-µm PTFE syringe filter.
**Chromatographic system**
(See *Chromatography* ⟨621⟩, *System Suitability*.)
**Mode:** LC
**Detector:** Photodiode array, 200–400 nm
**Analytical wavelength:** 290 nm
**Column:** 2.1-mm × 15-cm; 5-µm packing L1[3]
**Column temperature:** 40°
**Flow rate:** 0.2 mL/min
**Injection volume:** 1 µL
**System suitability**
**Sample:** *Standard solution*
**Suitability requirements**
**Column efficiency:** NLT 3000 theoretical plates
**Tailing factor:** NMT 1.5
**Analysis**
**Sample:** *Sample solution*
Examine the UV spectra of the prominent peaks for similarity to those in the *Standard solution* or other known PDE5 inhibitor compounds (*Figure 1* and *Table 5*). Typical retention times of several PDE5 inhibitors are provided in *Table 5*. However, neither retention time match nor the absorbance spectrum similarity should be construed as sufficient confirmation of the chemical identity of an adulterant.

[2] CacheSyn (http://www.cachesyn.com/); Santa Cruz Biotechnology, Inc. (http://www.scbt.com); TLC Pharmachem (http://www.tlcpharmachem.com/); and Toronto Research Chemicals (http://www.trc-canada.com/) are some of the potential sources of rare and hard-to-find adulterant reference materials.
[3] The procedure was developed on the Agilent Technologies Zorbax SB-C18 column.

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**2. HPLC with Mass-Spectrometric Detection**

Preferably, a mass-spectrometric detector is connected in sequence to the UV-Vis detector. The settings below apply to an ion-trap mass spectrometer. Other MS detectors are suitable; however, it is advisable to use spectrometers that possess MS/MS capability.

**Solution A:** 0.1% Formic acid in water

**Solution B:** 0.1% Formic acid in acetonitrile

**Mobile phase:** See *Table 2*.

**Table 2**

| Time (min) | Solution A (%) | Solution B (%) |
|---|---|---|
| 0 | 95 | 5 |
| 15 | 5 | 95 |
| 23 | 5 | 95 |
| 24 | 95 | 5 |
| 31 | 95 | 5 |

**Diluent:** Acetonitrile and water (50:50)

**Standard solution:** 5 µg/mL each of USP Sildenafil Citrate RS, USP Tadalafil RS, and USP Vardenafil Hydrochloride RS in *Diluent*

**Sample solution:** Combine one-fifth portion of the dosage unit, 10–20 mg of bulk material, or a small fragment of the capsule shell (3 mm × 3 mm) with 10 mL of *Diluent*, sonicate for 30 min, and pass through the 0.2-µm PTFE syringe filter. Dilute the filtrate 20-fold with *Diluent* before injection.

**Chromatographic system**

(See *Chromatography* ⟨621⟩, *System Suitability*.)

**Mode:** LC

**Detector:** UV 290 nm

**Column:** 2.1-mm × 15-cm; 5-µm packing L1[4]

**Column temperature:** 40°

**Flow rate:** 0.2 mL/min

**Injection volume:** 1 µL

**Mass spectrometric system**[5]

(See *Mass Spectrometry* ⟨736⟩.)

**Ionization:** ESI

**Polarity:** Positive or negative

**Sheath gas:** 35 mL/min

**Sweep gas:** 5 mL/min

**Capillary temperature:** 300°

**Source voltage:** 5 kV

**Collision:** 45 meV

**Scanning:** *m/z* 90–1050 and dependent scan on the most intense ion

**System suitability**

**Sample:** *Standard solution*

**Suitability requirements**

**Resolution:** NLT 2.0 between vardenafil and sildenafil peaks

**Tailing factor:** NMT 1.5

**Analysis**

**Sample:** *Sample solution*

Compare mass-to-charge ratios of the molecular ions $[M+H]^+$ or $[M-H]^-$ and fragments to those of the *Standard solution* or other known analytes listed in *Table 4*. Typical retention times of several common PDE5 inhibitors are provided in *Table 5*.

**3. High Performance Thin-Layer Chromatography (HPTLC) with Visual, UV, and/or MS Detection**

**Standard solution:** A composite of 0.2-mg/mL each of USP Sildenafil Citrate RS, USP Tadalafil RS, and USP Vardenafil Hydrochloride RS in methanol, with sonication if necessary. Additional reference materials may be available commercially.

**Sample solution:** Comminute 1 dosage unit, including the capsule shell and tablet coating, or about 500 mg of raw material; combine with 10 mL of methanol, and subject to ultrasonication for 30 min. Centrifuge or filter the solution, and use the supernatant. [NOTE—Upon development, if the chromatographic bands appear too saturated and UV densitometric spectra are distorted, dilute the *Sample solution* 10-fold with methanol.]

**Developing solvent system:** *tert*-Butyl methyl ether, methanol, and 28.0% (w/w) ammonium hydroxide (20:2:1). [NOTE—Strength of ammonium hydroxide was found to be crucial for adequate method performance. It is therefore

[4] The procedure was developed on the Agilent Technologies Zorbax SB-C18 column.

[5] The settings were found appropriate for ThermoElectron LTQ XL Linear Ion Trap Mass Spectrometer. Users will need to optimize their respective instrumentation according to the manufacturer's recommendations.

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advisable to establish the titer of higher-concentration ammonia[6] and to adjust the latter to exactly 28.0% immediately before the experiment.]

**Chromatographic system**

(See *HPTLC for Articles of Botanical Origin* ⟨203⟩.)

**Adsorbent:** Chromatographic silica gel with an average particle size of 5 µm

**Application volume:** 3 µL, as 8-mm bands

**Relative humidity:** Condition the plate to a relative humidity of 47% using a suitable device in the presence of a saturated solution of potassium isothiocyanate.

**Temperature:** Ambient

**Saturation:** 20 min, with paper

**Developing distance:** 6 cm

**Derivatization reagent:** None

**Drying:** 5 min in a current of cold air

**Detection 1:** Visual, under illumination with 254- and 365-nm UV light

**Detection 2:** UV-Vis spectrometry (scanning densitometer), 190–550 nm

**Detection 3:** Mass spectrometry, *m/z* 90–1050

**Mass spectrometric system**[7]

(See *Mass Spectrometry* ⟨736⟩.)

**Ionization:** ESI

**Polarity:** Positive, negative, or rapid switching

**Desolvation gas ($N_2$):** 300 L/h

**Cone gas ($N_2$):** 80 L/h

**Temperatures**

**ESI probe:** 105°

**Desolvation:** 150°

**Capillary voltage:** 3.0 kV

**Cone voltage:** 50 V

**Scanning:** *m/z* 90–1050

**System suitability**

**Sample:** *Standard solution*

**Suitability requirements:** Under UV light at 254 nm, sildenafil, tadalafil, and vardenafil appear as dark bands against the fluorescent background. Under UV light at 365 nm, sildenafil, tadalafil, and vardenafil appear as blue fluorescent bands.

**Analysis:** Inspect the plate under short-wave (254 nm) and long-wave (365 nm) UV light. PDE5 inhibitors appear as dark bands against the fluorescent background at 254 nm and typically exhibit different shades of blue fluorescence under 365 nm. Note the similarities in $R_F$ values between the bands in the *Standard solution* and *Sample solution*; these may be informative, however they do not constitute sufficient proof of identity. Relative intensities of the bands permit approximation of the amounts. Using scanning densitometry, obtain UV spectra of the prominent bands in the *Sample solution*, and compare them to those of the PDE5 inhibitors in the *Standard solution* and those provided in *Table 5* and *Figure 1*. Mass-spectrometric interface, if available, may facilitate more definitive assignment of the analyte bands: compare mass-to-charge ratios of the molecular ions $[M+H]^+$ or $[M-H]^-$ and fragments to those of the common adulterants listed in *Table 4*.

**4. Ambient Ionization Mass Spectrometry**

**Diluent:** Acetonitrile and water (50:50), with 0.1% formic acid

**Standard solution:** A composite solution containing 20 µg/mL each of USP Sildenafil Citrate RS, USP Tadalafil RS, and USP Vardenafil Hydrochloride RS in *Diluent*. Additional reference materials may be available commercially.

**Sample solution:** Grind the entire dosage form, including the capsule shell and tablet coating, to a fine powder. Weigh about 50 mg of the resulting powder, or about 50 mg of bulk material, and combine with 5 mL of *Diluent*. Cap tightly, subject to ultrasonication for 2 min, and vortex thoroughly. Centrifuge or filter the resulting solution, and dilute an aliquot of the supernatant or filtrate 100-fold with *Diluent*.

**Mass spectrometric system**

(See *Applications of Mass Spectrometry* ⟨1736⟩*, Mass Spectrometers, Ionization Procedures, Ambient Ionization Procedures*.)

**Ionization:** Ambient with thermal desorption

**Mode:** Thermal profile

**Polarity:** Positive, negative, or rapid switching

**Gas temperature:** 150°, 250°, 350°, and 450°

**Scanning:** *m/z* 90–1050

**System suitability:** Deposit 3-µL aliquots of the *Standard solution* onto the disposable sample cards. Set the compatible mass spectrometer to a 30-s acquisition of 90–1050 Da. Using one sample card for each temperature setting, acquire mass spectra at each of the following desorption gas temperatures: 150°, 250°, 350°, and 450° in positive ionization mode. Switch polarity, and re-acquire spectra at the same four temperatures in the negative ionization mode. [NOTE—If the mass spectrometer permits rapid polarity switching, both positive and negative spectra may be acquired

[6] 32% Ammonia solution is available from EMD Millipore.

[7] Procedure was developed using Expression CMS mass spectrometer from Advion, equipped with a TLC-MS interface available from CAMAG. If other mass spectrometers are used, relevant settings will have to be optimized. The bands were directly eluted with a mixture of water and acetonitrile (80:20) containing 0.1% formic acid.

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simultaneously using a single sample.] Confirm that the $[M+H]^+$ or $[M-H]^-$ ions of sildenafil, tadalafil, and vardenafil are observed as listed in *Table 4*.

**Analysis:** Deposit 3-µL aliquots of the *Sample solution* onto the disposable sample cards, and follow the procedure outlined above for the *Standard solution*. In the event that a single ion dominates the mass spectrum at every temperature setting, dilute the *Sample solutions* 10-fold with *Diluent* and re-analyze. Compare mass-to-charge ratios of the molecular ions $[M+H]^+$ or $[M-H]^-$ and fragments to those of the known analytes listed in *Table 4*.

**5. NMR Spectroscopy–Low-Field and High-Field**

(See *Nuclear Magnetic Resonance Spectroscopy* ⟨761⟩*, Qualitative and Quantitative NMR Analysis*.) [NOTE—Deuterated acetonitrile ($CD_3CN$) should be NLT 99.8 atom % D, and should contain 0.05% tetramethylsilane (TMS) as a chemical shift reference. Use of solvents in sealed ampules is recommended. NMR tubes should be suitable for use at the selected magnetic field strength.]

**Standard solutions:** Dissolve 10 mg of USP Sildenafil Citrate RS, USP Tadalafil RS, or USP Vardenafil Hydrochloride RS in separate 1-mL aliquots of $CD_3CN$, and transfer 700-µL aliquots of the resulting solutions into individual NMR tubes. Additional reference materials are available commercially.

**Sample solution:** Grind the entire dosage form, including the capsule shell and tablet coating, to a fine powder. Transfer 100–200 mg of the ground material, or an equivalent amount of bulk raw material powder, into a 5-mL sealable glass vial. Add 1 mL of $CD_3CN$, vortex thoroughly, and allow the solids to settle. Transfer about 700 µL of the supernatant to an NMR tube, taking care to minimize transfer of solids.

**Instrument performance qualification**

(See *Nuclear Magnetic Resonance Spectroscopy* ⟨761⟩.)

**Magnetic field strength:** NLT 42.5 MHz ($^1H$ operating frequency)

**Data collection:** Use the parameters specified in *Table 3*; perform 90° pulse width calibration before the analysis according to the recommendations of the equipment manufacturer.

**Table 3**

| Parameter | $^1H$-NMR Qualitative Measurement |
|---|---|
| Pulse program | Single pulse $^1H$ |
| Spectral width | 14 ppm (–1 to 13 ppm) |
| Transmitter offset | Center of spectral width |
| Relaxation delay | 5–10 s |
| Acquisition time | 2–5 s |
| Number of data points per FID[a] | NLT 16,000 |
| Temperature | 25° |

[a] Free induction decay.

**System suitability:** Acquire a $^1H$ spectrum of the *Standard solution* using the settings outlined in *Data collection*. Record a sufficient number of scans to ensure that signal-to-noise ratio of the TMS signal is NLT 10.

**Analysis:** Acquire a $^1H$ spectrum of the *Sample solution* under the conditions outlined in *Data collection*. Record a sufficient number of scans to ensure that the signal-to-noise ratio of the TMS signal is NLT 10. Reference all acquired spectra to the $^1H$ signal of TMS (0 ppm). Measure and record the chemical shift and multiplicity of the NMR signals in the spectra of the *Standard solutions* and *Sample solution*. Compare the $^1H$ NMR spectrum of the *Sample solution* to those of the *Standard solutions*, paying particular attention to the aromatic region (5–9 ppm). Determine whether the chemical shift and multiplicity of the NMR signals in the *Sample solution* exhibit sufficient similarity to those found in the *Standard solutions*.

**6. Bioassay**[8]

**PDE5 enzyme**[9] **stock solution:** Prepare a concentration of approximately 3000 Units/µL. If necessary, dilute with 40 mM tris(hydroxymethyl)aminomethane hydrochloride (tris-HCl), pH 8.0, 110 mM sodium chloride (NaCl), 2.2 mM potassium chloride (KCl), 3 mM dithiothreitol, and 20% glycerin. Vortex gently to mix.

**PDE5 working solution** (100 Units per 6.5 µL)**:** Combine 400 µL of PDE-Glo™ Reaction Buffer 5X, 10 µL of *PDE5 enzyme stock solution*, and 1590 µL of water. Vortex gently to mix.

**cGMP solution:** Combine 400 µL of PDE-Glo™ Reaction Buffer 5X, 40 µL of 1-mM cGMP stock solution, and 1560 µL of water. Mix thoroughly by vortexing.

**100-mM IBMX stock solution in DMSO:** Prepare a 22.2-mg/mL solution of 3-isobutyl-1-methylxanthine (IBMX) in dimethylsulfoxide (DMSO), e.g., dissolve 100 mg of IBMX in 4.5 mL of DMSO. Mix thoroughly by vortexing.

**Reaction buffer:** Combine 400 µL of PDE-Glo™ Reaction Buffer 5X and 1600 µL of water. Mix thoroughly by vortexing.

**Reaction buffer with 4% DMSO:** Combine 400 µL of PDE-Glo™ Reaction Buffer 5X, 80 µL of DMSO and 1520 µL of water. Mix thoroughly by vortexing.

[8] The procedures were developed using commercial Promega PDE-Glo™ Phosphodiesterase Assay Kit, Catalog # V1361. It includes the following reagents: PDE-Glo™ Reaction Buffer 5X (Catalog # V133A); PDE-Glo™ Detection Buffer 5X (Catalog # V134A); Protein Kinase A Solution (Catalog # V135A); PDE-Glo™ Termination Buffer 5X (Catalog # V136A); cGMP Stock Solution, 1 mM (Catalog # V641A); cAMP, 1 mM (Catalog # V642B); Kinase-Glo™ Substrate (Catalog # V672A); and Kinase-Glo™ Buffer (Catalog # V673A). Kits from alternative suppliers may also be used, e.g., BPS Science, Catalog # 60350, although re-optimization of test procedures will be required.

[9] The procedures were developed using human phosphodiesterase 5A from BPS Bioscience Catalog # 60050. The enzyme is available from numerous suppliers, e.g., Sigma-Aldrich Catalog # E9034.

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**Termination buffer:** Combine 400 µL of PDE-Glo™ Termination Buffer 5X, 40 µL of *100-mM IBMX stock solution in DMSO*, and 1560 µL of water. Mix thoroughly by vortexing.

**Detection buffer:** Combine 400 µL of PDE-Glo™ Detection Buffer 5X, 16 µL of Protein Kinase A Solution, and 1584 µL of water. Vortex gently to mix.

**Kinase-Glo™ reagent:** Add 10 mL of Kinase-Glo™ Buffer to the vial of Kinase-Glo™ Substrate, and vortex gently.

**Standard solution** (400 nM)**:** Dissolve 5 mg of USP Sildenafil Citrate RS in 3.0 mL of DMSO to obtain a 2.5-mM stock solution. Combine a 10-µL aliquot of the resulting solution with 240 µL of DMSO, and mix thoroughly (100 µM). Combine a 10-µL aliquot of the resulting solution with 90 µL of DMSO, and mix thoroughly (10 µM). Combine a 10-µL aliquot of the resulting solution with 240 µL of *Reaction buffer*, and mix thoroughly (400 nM); the last dilution is the *Standard solution*.

**Control solution:** Combine 10 µL of DMSO with 240 µL of *Reaction buffer*, and mix thoroughly by vortexing.

**Sample solution:** Grind the entire dosage form, including the capsule shell and tablet coating, to a fine powder. Transfer 100 mg of the ground material into a 5-mL polypropylene vial. Add 3.0 mL of DMSO, and vortex for 60 s. Allow solids to settle, combine 50 µL of the clear supernatant with 200 µL of DMSO, and mix thoroughly by vortexing. Combine a 10-µL aliquot of the resulting solution with 90 µL of DMSO, and mix thoroughly by vortexing. Combine a 10-µL aliquot of the resulting solution with 240 µL of *Reaction buffer*, and mix thoroughly by vortexing; the last dilution is the *Sample solution*.

**Analysis**

1. Dispense 12.5-µL aliquots of the *Standard solution*, *Control solution*, and *Sample solution* into microplate wells, in triplicate. Use a white, flat-bottom, opaque polystyrene, nontreated, 96-well microtiter plate.[10] [NOTE—Do not use treated plates, black plates, or clear plates.]
2. Add 6.5 µL of *PDE5 working solution* to each well. Incubate for 5 min.
3. Add 6.0 µL of *cGMP solution* to effect a 5-µM cGMP concentration in a 25-µL volume. Incubate for 30 min.
4. Add 12.5 µL of *Termination buffer*. Incubate for 5 min.
5. Add 12.5 µL of *Detection buffer*. Incubate for 20 min.
6. Add 50 µL of *Kinase-Glo™ reagent*. Incubate for 10 min.
7. Record luminescence at 560 nm with a microplate luminometer at 0.5 s/well.

[NOTE—Incubate the plate at room temperature, preferably using a plate shaker.]

Calculate average luminescence values for the replicate preparations. Assess the degree of PDE5 inhibition in the *Sample solution* relative to that observed in the *Standard solution* and *Control solution*. Inhibition of PDE5 is manifested as reduction of luminescence: the samples that exhibit suppression of luminescent output comparable to or in excess of that observed with the *Standard solution* are likely adulterated with synthetic PDE5 inhibitors.

- **USP REFERENCE STANDARDS** ⟨11⟩

USP Sildenafil Citrate RS
USP Tadalafil RS
USP Vardenafil Hydrochloride RS

**Table 4. Mass Spectral Data for Select PDE5 Inhibitors[a]**

| # | Name | CAS Number | Chemical Formula | Exact Mass | $[M+H]^+$ | $[M-H]^-$ | Fragments |
|---|---|---|---|---|---|---|---|
| 1 | Acetaminotadalafil | 1446144-71-3 | $C_{23}H_{20}N_4O_5$ | 432.1434 | 433 | — | 455 $[M+Na]^+$, 391, 311, 269, 250 |
| 2 | Acetil acid | 147676-78-6 | $C_{18}H_{20}N_4O_4$ | 356.1485 | 357 | — | 329, 300, 285, 268, 256, 242, 166, 131 |
| 3 | Acetildenafil (Hongdenafil) | 831217-01-7 | $C_{25}H_{34}N_6O_3$ | 466.26924 | 467.28 | — | 449, 439, 420, 404, 396, 381, 355, 353, 339, 325, 324, 311, 297, 285, 166, 127, 111, 99, 97 |
| 4 | Acetylvardenafil | 1261351-28-3 | $C_{25}H_{34}N_6O_3$ | 466.2692 | 467 | — | 439, 396, 341, 317, 270 |
| 5 | Aildenafil (Dimethylsildenafil, Methisosildenafil) | 496835-35-9 | $C_{23}H_{32}N_6O_4S$ | 488.22057 | 489.23<br>— | —<br>487.40 | 432, 377, 313, 311, 283, 113, 99<br>460, 310, 282 |
| 6 | Aminotadalafil | 385769-84-6 | $C_{21}H_{18}N_4O_4$ | 390.1328 | 391.14<br>— | —<br>389.1248 | 269, 262, 241, 239, 224, 197, 169<br>362, 298, 262, 234, 233, 232 |
| 7 | (*S,R*)-Aminotadalafil [(+)-*trans*-Aminotadalafil] | 1093940-70-5 | $C_{21}H_{18}N_4O_4$ | 390.1328 | 391 | — | No data |
| 8 | Avanafil | 330784-47-9 | $C_{23}H_{26}N_7O_3Cl$ | 483.1786 | 484.186 | — | 375, 349, 221 |

[10] Conforming plates are available from Corning (Costar 3912 or Costar 3963), Thermo Scientific (Nunc™ Catalog #236105), and other vendors.

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**Table 4. Mass Spectral Data for Select PDE5 Inhibitors[a]** *(continued)*

| # | Name | CAS Number | Chemical Formula | Exact Mass | $[M+H]^+$ | $[M-H]^-$ | Fragments |
|---|---|---|---|---|---|---|---|
| 9 | Benzylsildenafil | 1446089-82-2 | $C_{28}H_{34}N_6O_4S$ | 550.2362 | 551 | — | 377, 283 |
| 10 | *N*-Butylnortadalafil | 171596-31-9 | $C_{25}H_{25}N_3O_4$ | 431.18451 | 432.25 | — | **310**,[b] *282,*[c] *197, 169* |
| 11 | Carbodenafil (Fondenafil) | 944241-52-5 | $C_{24}H_{32}N_6O_3$ | 452.2536 | 453 | — | 283 |
| 12 | Chlorodenafil | 1058653-74-9 | $C_{19}H_{21}ClN_4O_3$ | 388.8485 | 390 | — | 360, 311, 291, 254, 183, 136 |
| 13 | Chloropretadalafil | 171489-59-1 | $C_{22}H_{19}ClN_2O_5$ | 426.0982 | 427 | — | 429, 395, 349, 334, 302, 287, 262, 229, 159, 135 |
| 14 | Cinnamyldenafil | 1446089-83-3 | $C_{32}H_{38}N_6O_3$ | 554.3005 | 554 | — | 488, 354, 297, 283, 215, 166, 117, 91 |
| 15 | Cyclopentynafil | 1173706-34-7 | $C_{26}H_{36}N_6O_4S$ | 528.2519 | 529 | — | 461, 153 |
| 16 | Depiperazinothiosildenafil | 1353018-10-6 | $C_{17}H_{20}N_4O_4S_2$ | 408.0926 | 409<br>— | —<br>407.0845 | 381, 351, 327, 299, 285, 272<br>378, 314 |
| 17 | Descarbonsildenafil | 1393816-99-3 | $C_{21}H_{30}N_6O_4S$ | 462.2049 | 463 | — | 418, 377, 360, 311, 299, 283, 255, 151, 87 |
| 18 | *N*-Desethylvardenafil | 448184-46-1 | $C_{21}H_{28}N_6O_4S$ | 460.18927 | 461.20 | — | 392, 377, 376, 329, 313, 312, 299, 284, 283, 151 |
| 19 | Desmethylcarbodenafil | 147676-79-7 | $C_{23}H_{31}N_6O_3$ | 438.523 | 439.2451 | — | 339, 311 |
| 20 | *N*-Desmethylsildenafil | 139755-82-1 | $C_{21}H_{28}N_6O_4S$ | 460.18927 | 461.19 | — | 377, 313, 311, 299, 283 |
| 21 | Dimethylacetildenafil | 1417999-76-8 | $C_{25}H_{34}O_3N_6$ | 466.2771 | 467 | — | 279, 149, 177 |
| 22 | Dioxo-acetildenafil | 1609405-33-5 | $C_{25}H_{30}N_6O_5$ | 494.2278 | 495 | — | No data |
| 23 | Dithio-desmethylcarbodenafil | 1333233-46-7 | $C_{23}H_{30}N_6OS_2$ | 470.6572 | 471.1991 | — | 371, 343 |
| 24 | Gendenafil | 147676-66-2 | $C_{19}H_{22}N_4O_3$ | 354.16919 | 355.31 | — | 327, 298, **285**, 283, *256, 242* |
| 25 | Gisadenafil | 334826-98-1 | $C_{23}H_{33}N_7O_5S$ | 519.22639 | 520 | — | No data |
| 26 | Homosildenafil | 642928-07-2 | $C_{23}H_{32}N_6O_4S$ | 488.22057 | 489.23 | — | 467, 461, 377, 313, 311, 283, 127, 111, 113, 99, 97 |
| 27 | Hydroxyacetildenafil (Hydroxyhongdenafil) | 147676-56-0 | $C_{25}H_{34}N_6O_4$ | 482.26415 | 483.27 | — | 465, 447, 439, 396, 353, 339, 325, 311, 297 |
| 28 | Hydroxychlorodenafil | 1391054-00-4 | $C_{19}H_{23}ClN_4O_3$ | 390.1459 | 391 | — | No data |
| 29 | Hydroxyhomosildenafil | 139755-85-4 | $C_{23}H_{32}N_6O_5S$ | 504.21549 | 505.22<br>— | —<br>503.31 | 487, 461, 423, 377, 312, 311, 283, 284, 225, 166, 129, 112, 99, 97<br>475, 310, 282 |
| 30 | 2-Hydroxypropylnortadalafil | 1353020-85-5 | $C_{24}H_{23}N_3O_5$ | 433.16377 | 434.15 | — | **312**, *284, 197, 169* |
| 31 | Hydroxythiohomosildenafil (Hydroxyhomosildenafil thione, Sulfohydroxyhomosildenafil) | 479073-82-0 | $C_{23}H_{32}N_6O_4S_2$ | 520.19264 | 521.20 | — | 503, 477, 461, 419, 393, 355, 354, 327, 325, 291 |
| 32 | Hydroxythiovardenafil | 912576-30-8 | $C_{23}H_{32}N_6O_4S_2$ | 520.1926 | 521 | — | No data |
| 33 | Hydroxyvardenafil | 224785-98-2 | $C_{23}H_{32}N_6O_5S$ | 504.2155 | 505.26<br>— | —<br>503 | 344, 312, 253, 169, 99<br>475, 310, 282 |
| 34 | Imidazosagatriazinone (Desulfovardenafil) | 139756-21-1 | $C_{17}H_{20}N_4O_2$ | 312.1586 | 313 | — | 284, 256, 169, 151 |
| 35 | Isopiperazinonafil | 1335201-06-3 | $C_{25}H_{34}N_6O_4$ | 482.2642 | 483<br>— | —<br>481 | —<br>453, 422, 379, 336, 325, 311, 309 |

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**Table 4. Mass Spectral Data for Select PDE5 Inhibitors[a]** *(continued)*

| # | Name | CAS Number | Chemical Formula | Exact Mass | $[M+H]^+$ | $[M-H]^-$ | Fragments |
|---|---|---|---|---|---|---|---|
| 36 | Lodenafil carbonate | 398507-55-6 | $C_{47}H_{62}N_{12}O_{11}S_2$ | 1034.4102 | 1035 | — | 518, **487**, *377, 311* |
| 37 | Mirodenafil | 862189-95-5 | $C_{26}H_{37}N_5O_5S$ | 531.6698 | 532 | — | 488, 404, 362, 296, 268 |
| 38 | Mutaprodenafil | 1387577-30-1 | $C_{27}H_{35}N_9O_5S_2$ | 629.7635 | 630.2279 | — | 489, 377, 142, 113 |
| 39 | Nitrodenafil | 147676-99-1 | $C_{17}H_{19}N_5O_4$ | 357.3647 | 358 | — | 307, 289, 261, 217, 176, 154, 136, 107, 89 |
| 40 | Nitroso-prodenafil | 1266755-08-1 | $C_{27}H_{35}N_9O_5S_2$ | 629.2203 | 630 | — | 142 |
| 41 | Noracetildenafil (Demethylhongdenafil) | 949091-38-7 | $C_{24}H_{32}N_6O_3$ | 452.25359 | 453.26 | — | 425, 406, 396, 380, 367, 355, 353, 339, 325, 324, 313, 297, 296, 253 |
| 42 | Norneosildenafil (Piperidino sildenafil) | 371959-09-0 | $C_{22}H_{29}N_5O_4S$ | 459.19403 | 460.20 | — | 432, 377, 329, 311, 299, 283 |
| 43 | Norneovardenafil | 358390-39-3 | $C_{18}H_{20}N_4O_4$ | 356.1485 | 357 | — | 329, 307, 289, 176, 154, 136, 107, 99 |
| 44 | Nortadalafil (Demethyltadalafil) | 171596-36-4 | $C_{21}H_{17}N_3O_4$ | 375.1219 | 376 | 374.1138 | 262, 234, 233, 232 |
| 45 | *N*-Octylnortadalafil (Octylnortadalafil) | 1173706-35-8 | $C_{29}H_{33}N_3O_4$ | 487.2471 | 488 | — | 366, 227 |
| 46 | Oxohongdenafil | 1446144-70-2 | $C_{25}H_{32}N_6O_4$ | 480.2485 | 481 | — | 451, 396, 354, 339, 312, 297, 289 |
| 47 | Piperazinonafil (Piperazonifil, Dihydroacetildenafil) | 1335201-04-1 | $C_{25}H_{34}N_6O_4$ | 482.2642 | 483<br>— | —<br>481 | —<br>453, 435, 348, 336, 321, 311, 309, 282, 267 |
| 48 | Piperidino acetildenafil (Piperiacetildenafil) | 147676-50-4 | $C_{24}H_{31}N_5O_3$ | 437.2427 | 438 | — | 410, 408, 355, 353, 341, 325, 297, 288 |
| 49 | Piperidinovardenafil (Piperidenafil, Pseudovardenafil) | 224788-34-5 | $C_{22}H_{29}N_5O_4S$ | 459.19403 | 460.20 | — | 432, 403, 391, 377, 349, 329, 312, 311, 301, 299, 284, 283, 270, 256, 169, 151 |
| 50 | Propoxyphenyl aildenafil | 1391053-82-9 | $C_{24}H_{34}N_6O_4S$ | 502.2362 | 503 | — | 252 |
| 51 | Propoxyphenyl hydroxyhomosildenafil (Methylhydroxyhomosildenafil) | 139755-87-6 | $C_{24}H_{34}N_6O_5S$ | 518.2311 | 519 | — | 501, 475, 391, 331, 325, 299, 283, 129, 112, 99 |
| 52 | Propoxyphenyl sildenafil | 877777-10-1 | $C_{23}H_{32}N_6O_4S$ | 488.2205 | 489.2272 | — | 447, 391, 325, 299, 283, 100 |
| 53 | Propoxyphenyl thioaildenafil (Propoxyphenyl thiomethisosildenafil) | 856190-49-3 | $C_{24}H_{34}N_6O_3S_2$ | 518.2133 | 519 | — | 260 |
| 54 | Propoxyphenyl thiohydroxyhomosildenafil | 479073-90-0 | $C_{24}H_{34}N_6O_4S_2$ | 534.2083 | 535.2150 | — | 517, 359, 341, 315, 299, 271, 129, 112, 99 |
| 55 | Sildenafil | 139755-83-2 | $C_{22}H_{30}N_6O_4S$ | 474.20492 | 475.21<br>— | —<br>473.45 | 447, 418, 391, 377, 374, 346, 329, 311, 297, 283, 255, 163, 160, 100<br>445, 310, 282 |
| 56 | Tadalafil (Tildenafil) | 171596-29-5 | $C_{22}H_{19}N_3O_4$ | 389.13756 | 390<br>— | —<br>388.1288 | 302, 268, 262, 250, 240, 197, 169, 135<br>262, 234, 233, 232 |
| 57 | (–)-*trans*-Tadalafil (ent-Tadalafil) | 629652-72-8 | $C_{22}H_{19}N_3O_4$ | 389.1376 | 390 | — | 779 $[2M+H]^+$, 262, 250, 135 |
| 58 | Thioaildenafil (Sulfoaildenafil, Thiomethisosildenafil, Sulfodimethyl sildenafil, Dimethylthiosildenafil) | 856190-47-1 | $C_{23}H_{32}N_6O_3S_2$ | 504.19773 | 505.21 | — | 448, 393, 327, 299, 113, 99 |

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**Table 4. Mass Spectral Data for Select PDE5 Inhibitors[a]** *(continued)*

| # | Name | CAS Number | Chemical Formula | Exact Mass | $[M+H]^+$ | $[M-H]^-$ | Fragments |
|---|---|---|---|---|---|---|---|
| 59 | Thiohomosildenafil (Sulfohomosildenafil, Homosildenafil thione) | 479073-80-8 | $C_{23}H_{32}N_6O_3S_2$ | 504.19773 | 505.21 | — | 477, 421, 393, 357, 355, 343, 327, 315, 299, 271, 113, 99 |
| 60 | Thioquinapiperifil (KF31327) | 220060-39-9 | $C_{24}H_{28}N_6OS$ | 448.2045 | 449 | — | 363, 246, 225, 204, 121 |
| 61 | Thiosildenafil (Sulfosildenafil, Sildenafil thione) | 479073-79-5 | $C_{22}H_{30}N_6O_3S_2$ | 490.18208 | 491.19 | — | 407, 393, 343, 341, 327, 315, 313, 299, 283, 271, 163, 99 |
| 62 | Udenafil | 268203-93-6 | $C_{25}H_{36}N_6O_4S$ | 516.2519 | 517.260 | — | 474, 418, 347, 325, *299*, 283 |
| 63 | Vardenafil | 224785-90-4 | $C_{23}H_{32}N_6O_4S$ | 488.22057 | 489.2274<br>— | —<br>487.33 | 461, 420, 377, 376, 375, 346, 339, 329, 312, 299, 284, 283, 169, 151, 123, 99<br>459, 310, 282 |
| 64 | Xanthoanthrafil (Benzamidenafil) | 1020251-53-9 | $C_{19}H_{23}N_3O_6$ | 389.15869 | 390.31 | — | 344, 252, 223, **151**, *107, 91* |

[a] Compiled from peer-reviewed literature, and communications with USP collaborators. See corresponding chemical structures in *Figure 2*.
[b] **Bold**, fragment subjected to $MS^3$ fragmentation.
[c] *Italics*, $MS^3$ fragments derived from the parent fragment (**bold**).

**Table 5. UV Absorbance Maxima and Retention Time Data for Select PDE5 Inhibitors[a]**

| # | Name | CAS Number | Chemical Formula | UV Absorbance Maxima (nm) | UV Absorbance Spectrum Type (*Figure 1*) | Retention Time (min)[b] | Relative Retention Time with Respect to Sildenafil |
|---|---|---|---|---|---|---|---|
| 1 | Acetaminotadalafil | 1446144-71-3 | $C_{23}H_{20}N_4O_5$ | 202, 222, 282 | b | 14.3 | 1.1 |
| 2 | Acetil acid | 147676-78-6 | $C_{18}H_{20}N_4O_4$ | 230, 260, 285 | — | — | — |
| 3 | Acetildenafil (Hongdenafil) | 831217-01-7 | $C_{25}H_{34}N_6O_3$ | 234, 282 | e | 12.5 | 1.0 |
| 4 | Acetylvardenafil | 1261351-28-3 | $C_{25}H_{34}N_6O_3$ | 218, 246, 268(s)[c] | d | 11.4 | 0.9 |
| 5 | Aildenafil (Dimethylsildenafil, Methisosildenafil) | 496835-35-9 | $C_{23}H_{32}N_6O_4S$ | 226, 294 | a | 13.3 | 1.0 |
| 6 | Aminotadalafil | 385769-84-6 | $C_{21}H_{18}N_4O_4$ | 200, 220, 284, 290(s) | b | 14.2 | 1.1 |
| 7 | (*S,R*)-Aminotadalafil [(+)-*trans*-Aminotadalafil] | 1093940-70-5 | $C_{21}H_{18}N_4O_4$ | 225, 283 | — | — | — |
| 8 | Avanafil | 330784-47-9 | $C_{23}H_{26}N_7O_3Cl$ | 198, 244 | f | 13.0 | 1.0 |
| 9 | Benzylsildenafil | 1446089-82-2 | $C_{28}H_{34}N_6O_4S$ | 291 | — | — | — |
| 10 | *N*-Butylnortadalafil | 171596-31-9 | $C_{25}H_{25}N_3O_4$ | 222, 284 | — | — | — |
| 11 | Carbodenafil (Fondenafil) | 944241-52-5 | $C_{24}H_{32}N_6O_3$ | 295 | — | — | — |
| 12 | Chlorodenafil | 1058653-74-9 | $C_{19}H_{21}ClN_4O_3$ | 211, 235, 279 | — | — | — |
| 13 | Chloropretadalafil | 171489-59-1 | $C_{22}H_{19}ClN_2O_5$ | 204, 222, 284 | b | 17.4 | 1.3 |
| 14 | Cinnamyldenafil | 1446089-83-3 | $C_{32}H_{38}N_6O_3$ | 239 | — | — | — |
| 15 | Cyclopentynafil | 1173706-34-7 | $C_{26}H_{36}N_6O_4S$ | 218, 290 | — | — | — |
| 16 | Depiperazinothiosildenafil | 1353018-10-6 | $C_{17}H_{20}N_4O_4S_2$ | 295, 354 | — | — | — |
| 17 | Descarbonsildenafil | 1393816-99-3 | $C_{21}H_{30}N_6O_4S$ | 225, 295 | — | — | — |
| 18 | *N*-Desethylvardenafil | 448184-46-1 | $C_{21}H_{28}N_6O_4S$ | 226, 246(s) | d | 11.9 | 0.9 |
| 19 | Desmethylcarbodenafil | 147676-79-7 | $C_{23}H_{31}N_6O_3$ | 226,296 | a | 11.9 | 0.9 |
| 20 | *N*-Desmethylsildenafil | 139755-82-1 | $C_{21}H_{28}N_6O_4S$ | 224, 294 | a | 12.9 | 1.0 |
| 21 | Dimethylacetildenafil | 1417999-76-8 | $C_{25}H_{34}O_3N_6$ | 233, 276 | — | — | — |
| 22 | Dioxo-acetildenafil | 1609405-33-5 | $C_{25}H_{30}N_6O_5$ | No data | — | — | — |

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**Table 5. UV Absorbance Maxima and Retention Time Data for Select PDE5 Inhibitors[a]** *(continued)*

| # | Name | CAS Number | Chemical Formula | UV Absorbance Maxima (nm) | UV Absorbance Spectrum Type (*Figure 1*) | Retention Time (min)[b] | Relative Retention Time with Respect to Sildenafil |
|---|---|---|---|---|---|---|---|
| 23 | Dithio-desmethylcarbodenafil | 1333233-46-7 | $C_{23}H_{30}N_6OS_2$ | 258, 285, 356 | — | — | — |
| 24 | Gendenafil | 147676-66-2 | $C_{19}H_{22}N_4O_3$ | 232, 274 | f | 16.8 | 1.3 |
| 25 | Gisadenafil | 334826-98-1 | $C_{23}H_{33}N_7O_5S$ | No data | — | — | — |
| 26 | Homosildenafil | 642928-07-2 | $C_{23}H_{32}N_6O_4S$ | 226, 292 | a | 13.3 | 1.0 |
| 27 | Hydroxyacetildenafil (Hydroxyhongdenafil) | 147676-56-0 | $C_{25}H_{34}N_6O_4$ | 234, 280 | e | 12.2 | 0.9 |
| 28 | Hydroxychlorodenafil | 1391054-00-4 | $C_{19}H_{23}ClN_4O_3$ | 212, 303 | — | — | — |
| 29 | Hydroxyhomosildenafil | 139755-85-4 | $C_{23}H_{32}N_6O_5S$ | 226, 296 | a | 12.9 | 1.0 |
| 30 | 2-Hydroxypropylnortadalafil | 1353020-85-5 | $C_{24}H_{23}N_3O_5$ | 222, 284 | — | — | — |
| 31 | Hydroxythiohomosildenafil (Hydroxyhomosildenafil thione, Sulfohydroxyhomosildenafil) | 479073-82-0 | $C_{23}H_{32}N_6O_4S_2$ | 228, 296, 352 | c | 15.0 | 1.2 |
| 32 | Hydroxythiovardenafil | 912576-30-8 | $C_{23}H_{32}N_6O_4S_2$ | 203, 235, 316 | — | — | — |
| 33 | Hydroxyvardenafil | 224785-98-2 | $C_{23}H_{32}N_6O_5S$ | 216 | — | — | — |
| 34 | Imidazosagatriazinone (Desulfovardenafil) | 139756-21-1 | $C_{17}H_{20}N_4O_2$ | 212, 253 | — | — | — |
| 35 | Isopiperazinonafil | 1335201-06-3 | $C_{25}H_{34}N_6O_4$ | 221, 290 | — | — | — |
| 36 | Lodenafil carbonate | 398507-55-6 | $C_{47}H_{62}N_{12}O_{11}S_2$ | 226, 296 | a | 15.4 | 1.2 |
| 37 | Mirodenafil | 862189-95-5 | $C_{26}H_{37}N_5O_5S$ | 216, 248 | f | 14.5 | 1.1 |
| 38 | Mutaprodenafil | 1387577-30-1 | $C_{27}H_{35}N_9O_5S_2$ | 218, 240, 283, 297, 335 | — | — | — |
| 39 | Nitrodenafil | 147676-99-1 | $C_{17}H_{19}N_5O_4$ | 212, 298 | — | — | — |
| 40 | Nitroso-prodenafil | 1266755-08-1 | $C_{27}H_{35}N_9O_5S_2$ | 241, 301 | — | — | — |
| 41 | Noracetildenafil (Demethylhongdenafil) | 949091-38-7 | $C_{24}H_{32}N_6O_3$ | 234, 280 | e | 12.3 | 0.9 |
| 42 | Norneosildenafil (Piperidino sildenafil) | 371959-09-0 | $C_{22}H_{29}N_5O_4S$ | 226, 300 | a | 18.7 | 1.4 |
| 43 | Norneovardenafil | 358390-39-3 | $C_{18}H_{20}N_4O_4$ | 215, 241 | — | — | — |
| 44 | Nortadalafil (Demethyltadalafil) | 171596-36-4 | $C_{21}H_{17}N_3O_4$ | No data | — | — | — |
| 45 | *N*-Octylnortadalafil (Octylnortadalafil) | 1173706-35-8 | $C_{29}H_{33}N_3O_4$ | 281 | — | — | — |
| 46 | Oxohongdenafil | 1446144-70-2 | $C_{25}H_{32}N_6O_4$ | 481 | — | — | — |
| 47 | Piperazinonafil (Piperazonifil, Dihydroacetildenafil) | 1335201-04-1 | $C_{25}H_{34}N_6O_4$ | 221, 290 | — | — | — |
| 48 | Piperidino acetildenafil (Piperiacetildenafil) | 147676-50-4 | $C_{24}H_{31}N_5O_3$ | 234, 284 | e | 13.1 | 1.0 |
| 49 | Piperidinovardenafil (Piperidenafil, Pseudovardenafil) | 224788-34-5 | $C_{22}H_{29}N_5O_4S$ | 224, 246(s) | d | 16.5 | 1.3 |
| 50 | Propoxyphenyl aildenafil | 1391053-82-9 | $C_{24}H_{34}N_6O_4S$ | 215, 225, 295 | — | — | — |
| 51 | Propoxyphenyl hydroxyhomosildenafil (Methylhydroxyhomosildenafil) | 139755-87-6 | $C_{24}H_{34}N_6O_5S$ | 226, 294 | a | 13.4 | 1.0 |
| 52 | Propoxyphenyl sildenafil | 877777-10-1 | $C_{23}H_{32}N_6O_4S$ | 226, 292 | a | 13.6 | 1.0 |
| 53 | Propoxyphenyl thioaildenafil (Propoxyphenyl thiomethisosildenafil) | 856190-49-3 | $C_{24}H_{34}N_6O_3S_2$ | 227, 295, 355 | — | — | — |
| 54 | Propoxyphenyl thiohydroxyhomosildenafil | 479073-90-0 | $C_{24}H_{34}N_6O_4S_2$ | 227, 295, 353 | — | — | — |

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**Table 5. UV Absorbance Maxima and Retention Time Data for Select PDE5 Inhibitors[a]** *(continued)*

| # | Name | CAS Number | Chemical Formula | UV Absorbance Maxima (nm) | UV Absorbance Spectrum Type (*Figure 1*) | Retention Time (min)[b] | Relative Retention Time with Respect to Sildenafil |
|---|---|---|---|---|---|---|---|
| 55 | Sildenafil | 139755-83-2 | $C_{22}H_{30}N_6O_4S$ | 224, 294 | a | 13.0 | 1.0 |
| 56 | Tadalafil (Tildenafil) | 171596-29-5 | $C_{22}H_{19}N_3O_4$ | 200, 222, 284, 292(s) | b | 15.0 | 1.2 |
| 57 | (–)-*trans*-Tadalafil (ent-Tadalafil) | 629652-72-8 | $C_{22}H_{19}N_3O_4$ | 231, 282, 289 | — | — | — |
| 58 | Thioaildenafil (Sulfoaildenafil, Thiomethisosildenafil, Sulfodimethyl sildenafil, Dimethylthiosildenafil) | 856190-47-1 | $C_{23}H_{32}N_6O_3S_2$ | 228, 250(s), 296, 352, 366(s) | c | 15.5 | 1.2 |
| 59 | Thiohomosildenafil (Sulfohomosildenafil, Homosildenafil thione) | 479073-80-8 | $C_{23}H_{32}N_6O_3S_2$ | 228, 248(s), 296, 354, 370(s) | c | 14.4 | 1.1 |
| 60 | Thioquinapiperifil (KF31327) | 220060-39-9 | $C_{24}H_{28}N_6OS$ | 211, 268, 363 | — | — | — |
| 61 | Thiosildenafil (Sulfosildenafil, Sildenafil thione) | 479073-79-5 | $C_{22}H_{30}N_6O_3S_2$ | 228, 250(s), 296, 356, 368(s) | c | 15.2 | 1.2 |
| 62 | Udenafil | 268203-93-6 | $C_{25}H_{36}N_6O_4S$ | 228, 298 | f | 13.6 | 1.0 |
| 63 | Vardenafil | 224785-90-4 | $C_{23}H_{32}N_6O_4S$ | 226, 252(s) | d | 12.1 | 0.9 |
| 64 | Xanthoanthrafil (Benzamidenafil) | 1020251-53-9 | $C_{19}H_{23}N_3O_6$ | 202, 228, 278, 390 | f | 15.3 | 1.2 |

[a] Compiled from peer-reviewed literature, and contributed by the USP collaborators. See corresponding chemical structures in *Figure 2*.
[b] Retention times derived from the experiments conducted as described in *HPLC with Photodiode Array Detection* and in *HPLC with Mass-Spectrometric Detection* methods.
[c] (s) denotes a shoulder.

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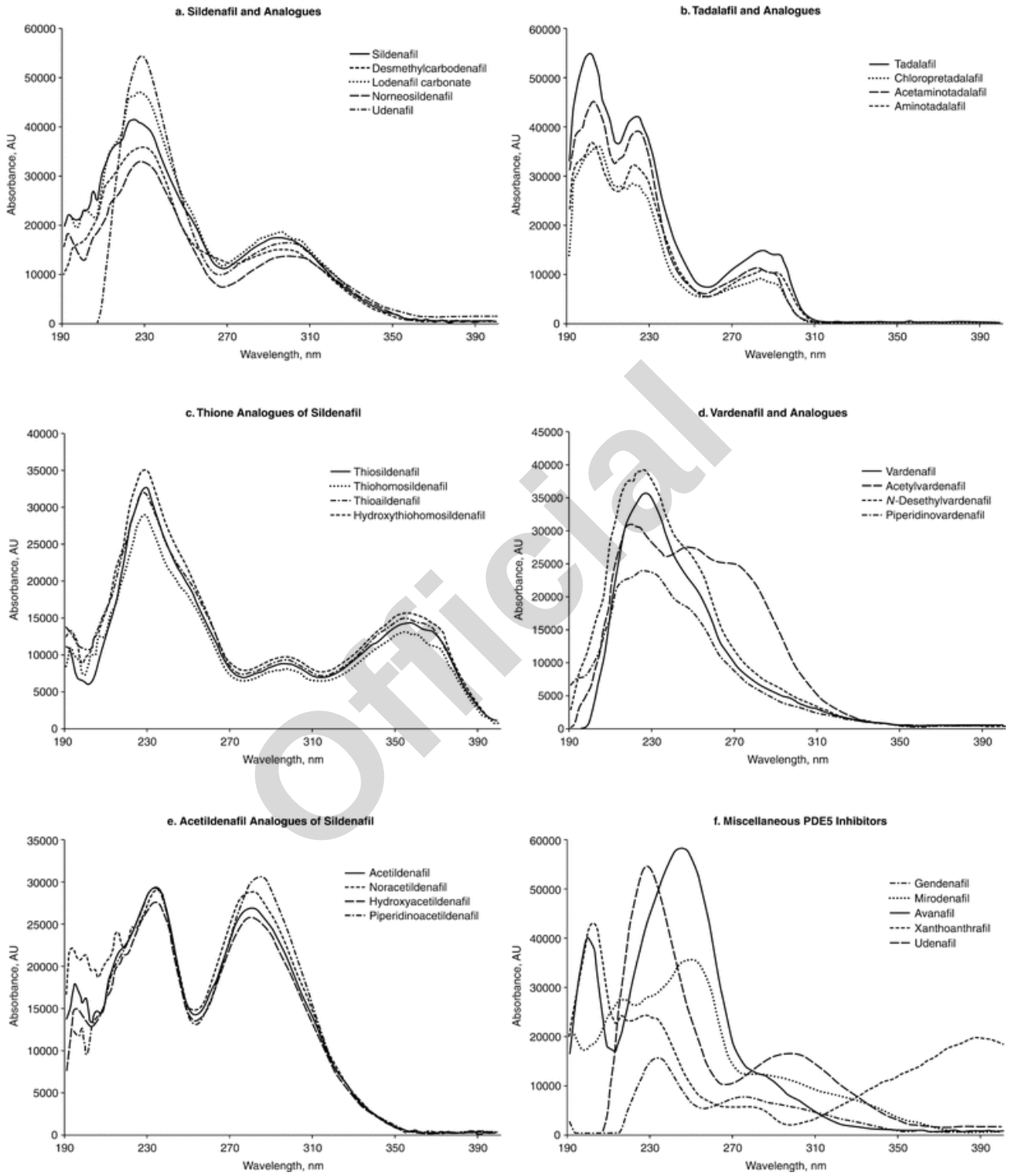


Figure 1. UV absorbance spectra of select PDE5 inhibitors.[11]

[11] Data contributed by USP collaborators.

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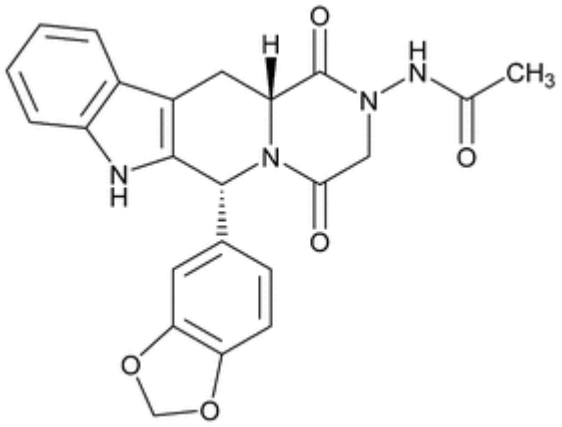
Figure 2. Chemical structures of select PDE5 inhibitors.[12]



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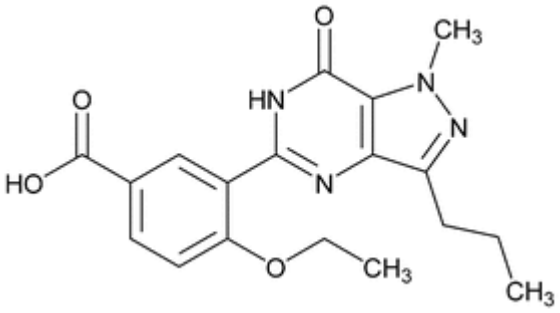

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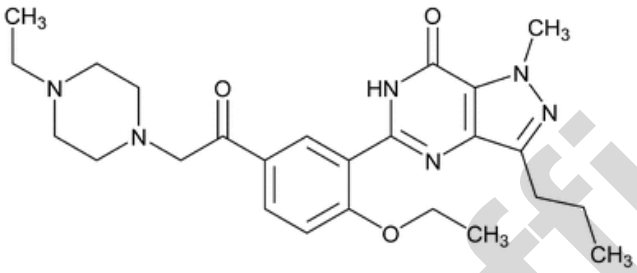
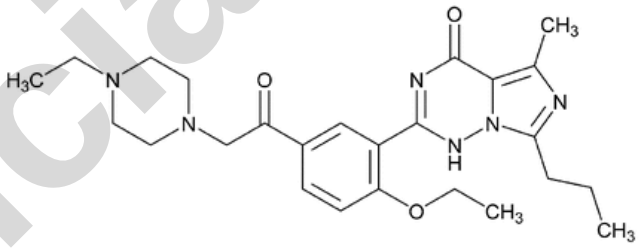
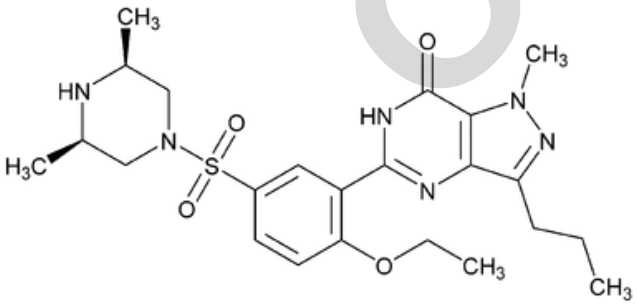


Acetildenafil



Acetylvardenafil



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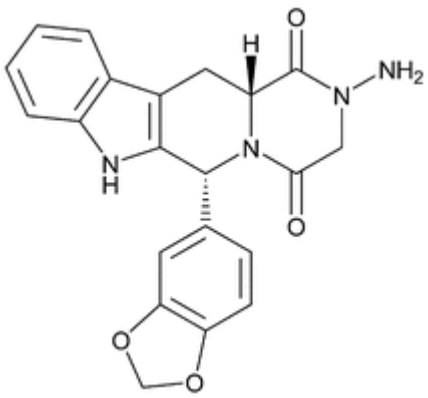

Aminotadalafil

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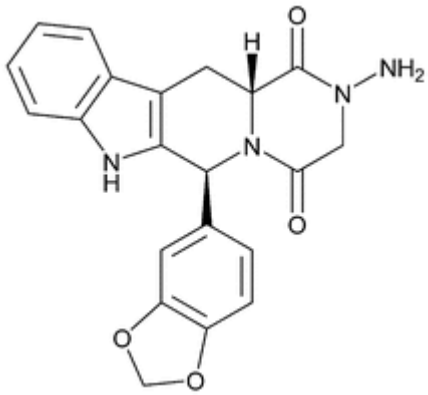
Figure 2. Chemical structures of select PDE5 inhibitors.[12] *(continued)*



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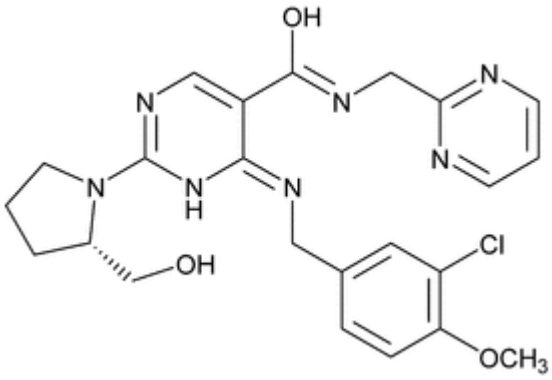

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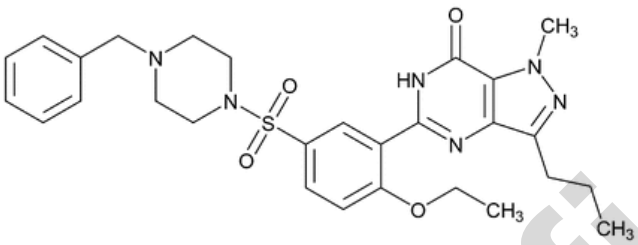

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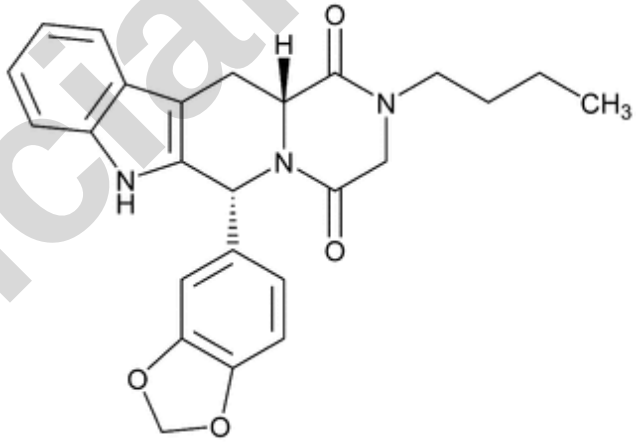
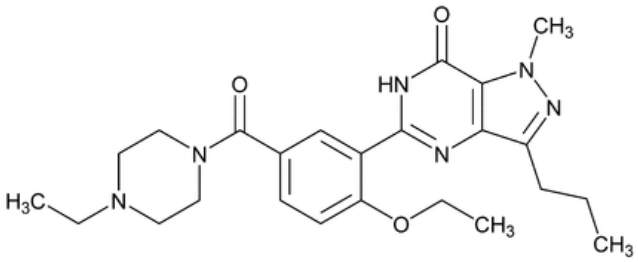


*N*-Butylnortadalafil



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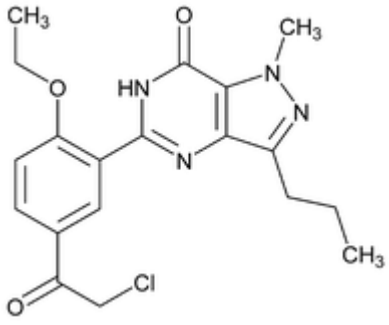

Chlorodenafil

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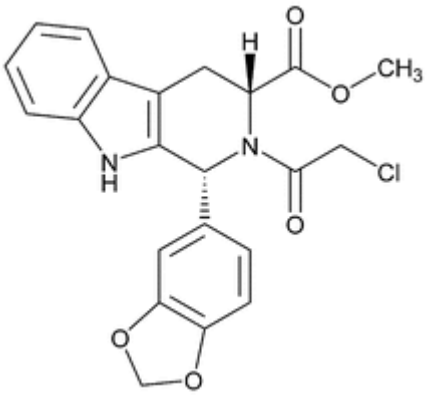
Figure 2. Chemical structures of select PDE5 inhibitors.[12] *(continued)*



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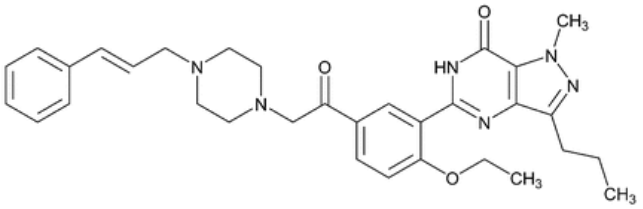

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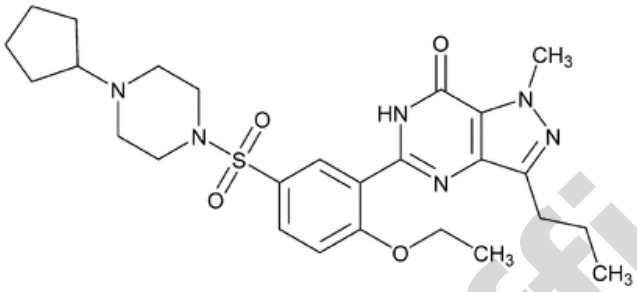

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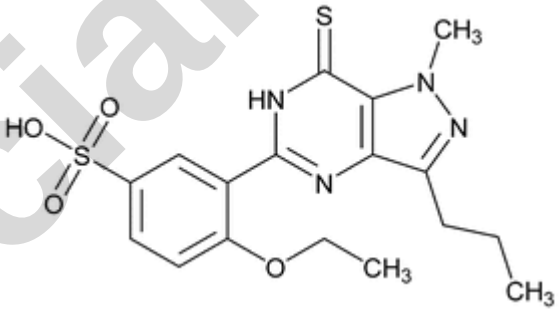
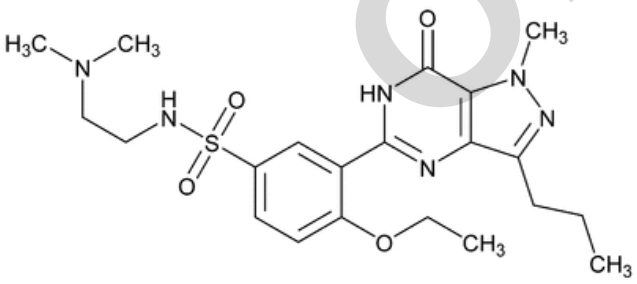


Depiperazinothiosildenafil



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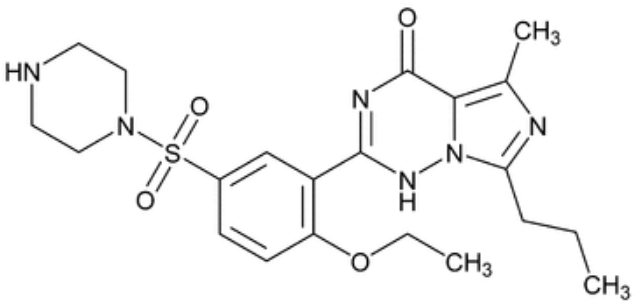

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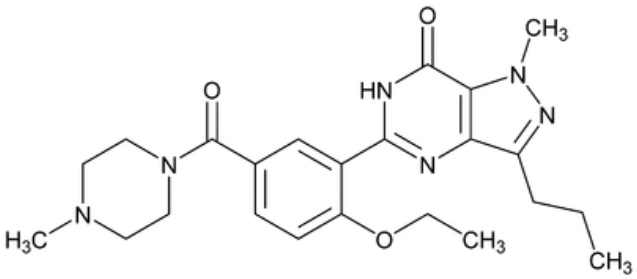

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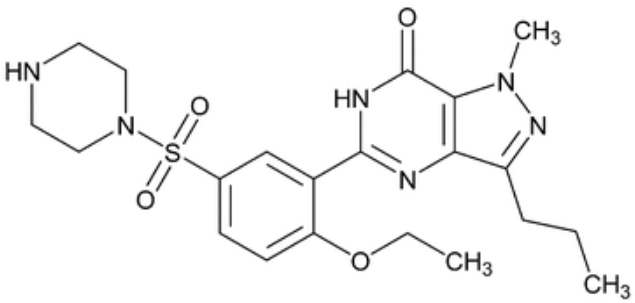

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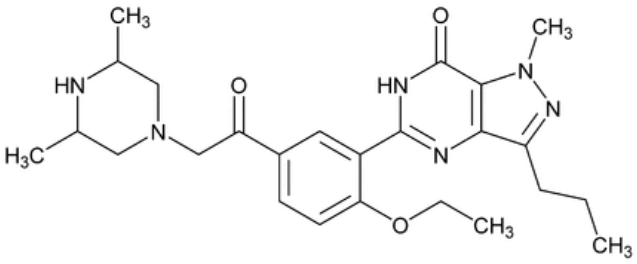
Figure 2. Chemical structures of select PDE5 inhibitors.[12] *(continued)*



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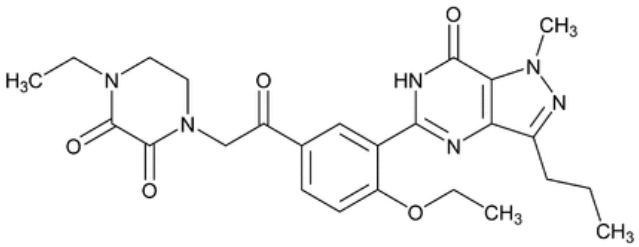

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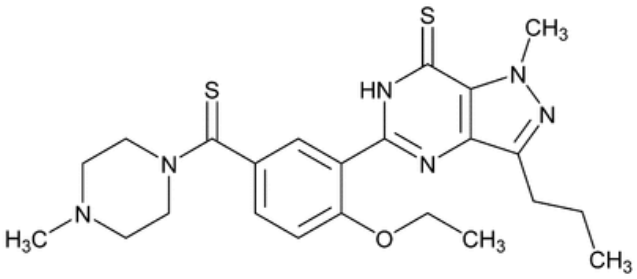

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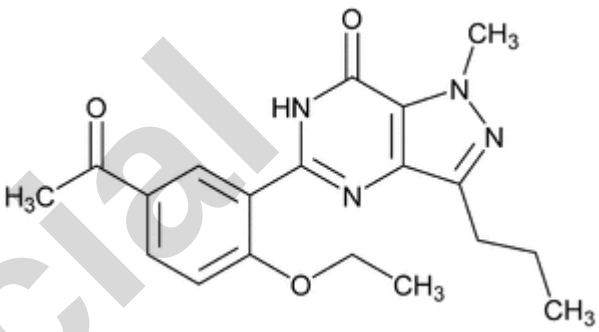

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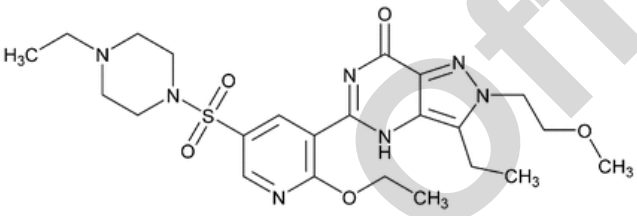

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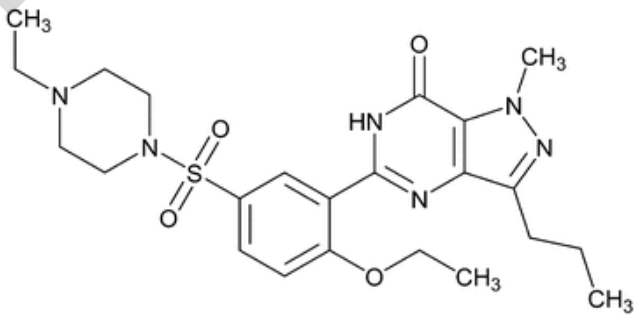

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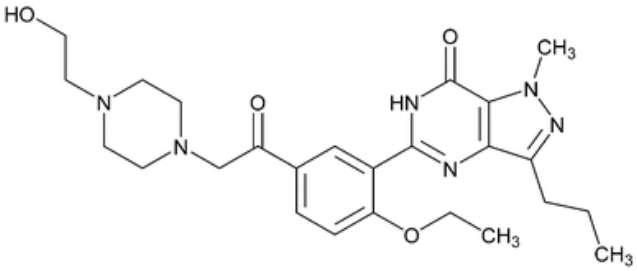

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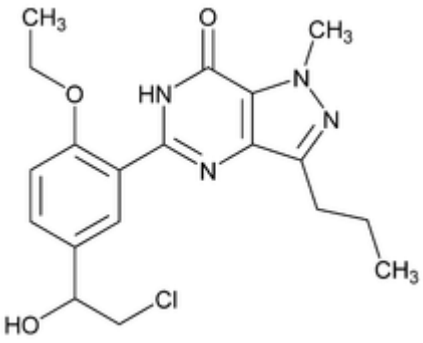

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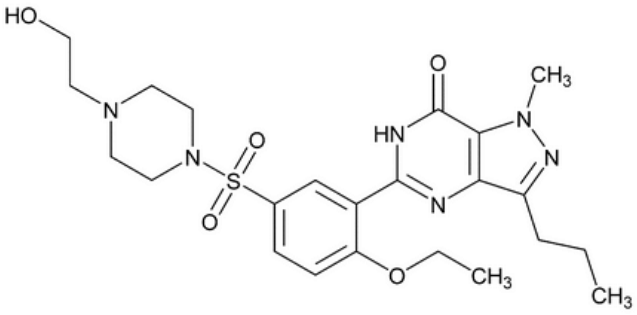
Figure 2. Chemical structures of select PDE5 inhibitors.[12] *(continued)*



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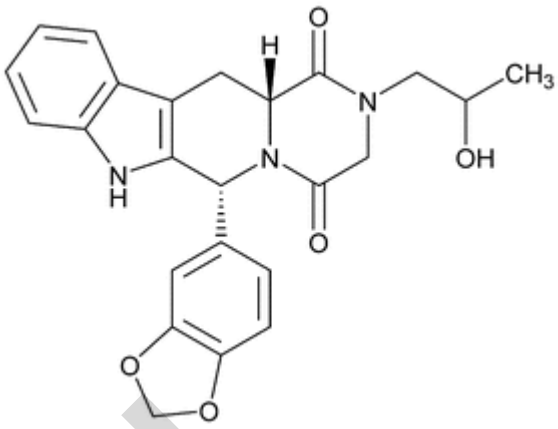

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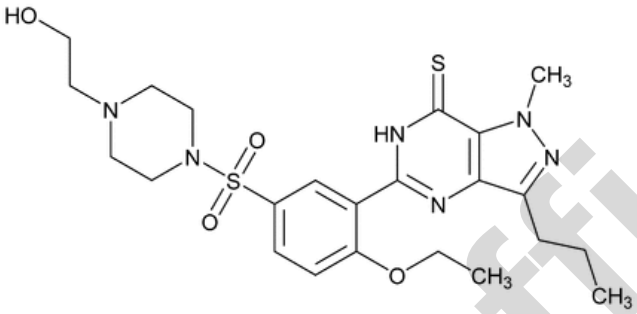

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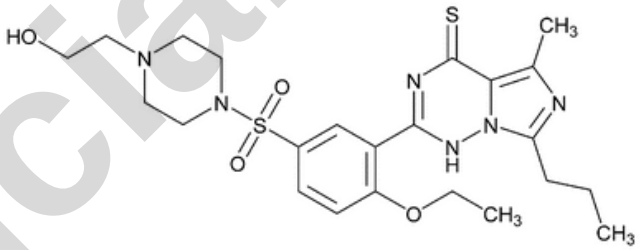

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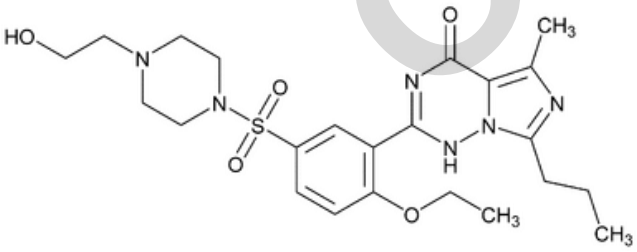

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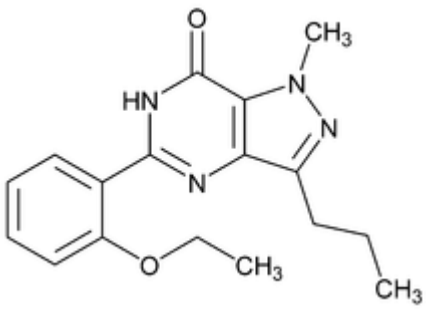

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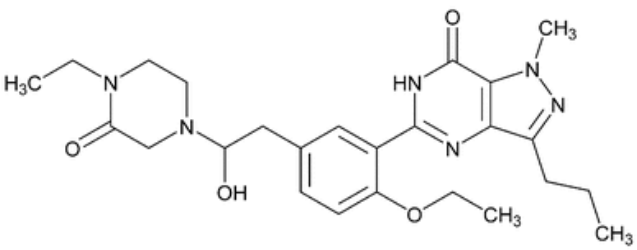

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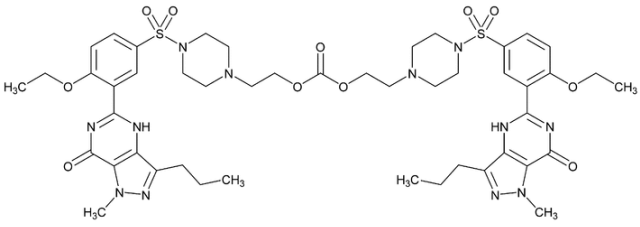

Lodenafil carbonate

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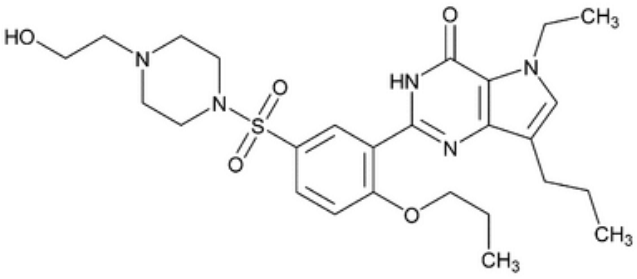

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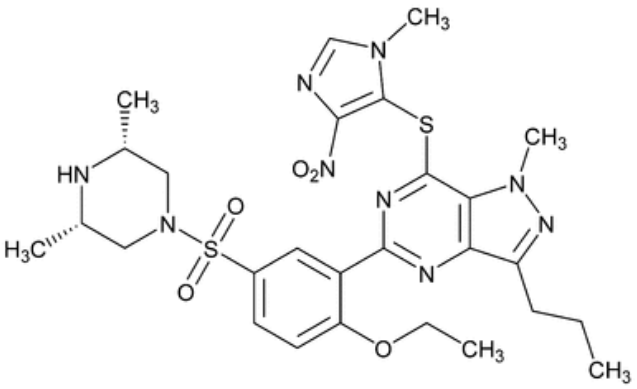

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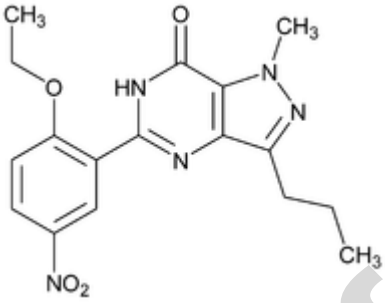

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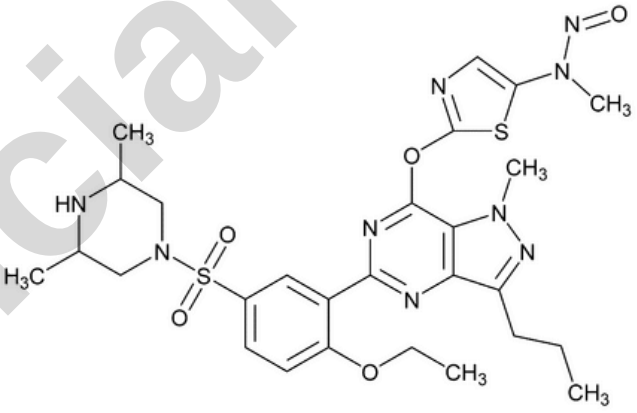
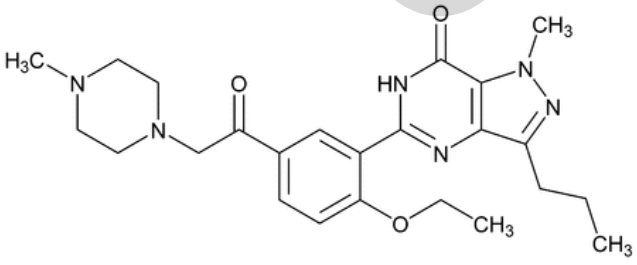


Nitroso-prodenafil



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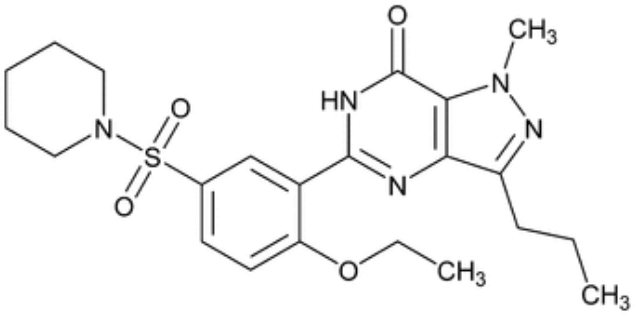

Norneosildenafil

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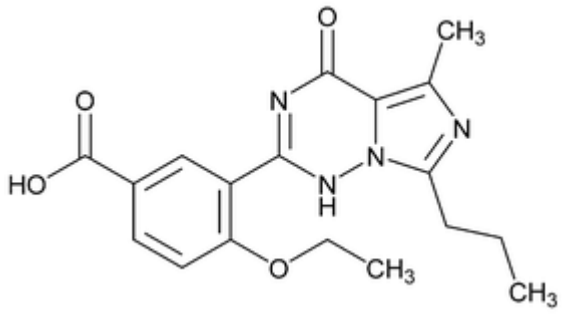

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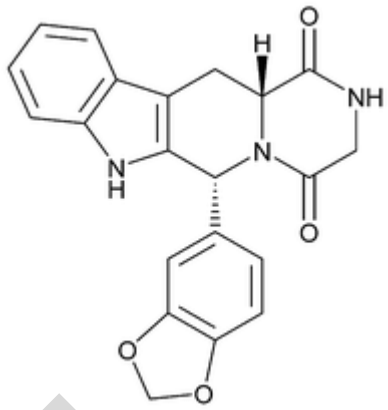

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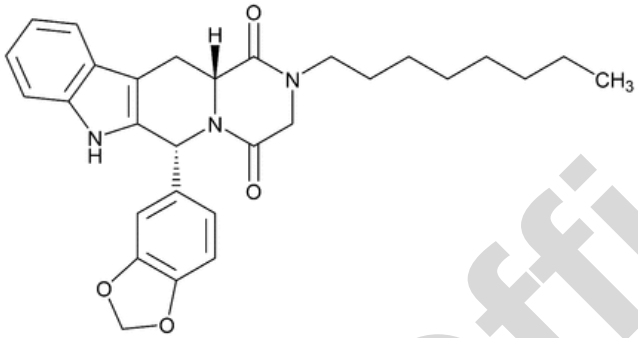

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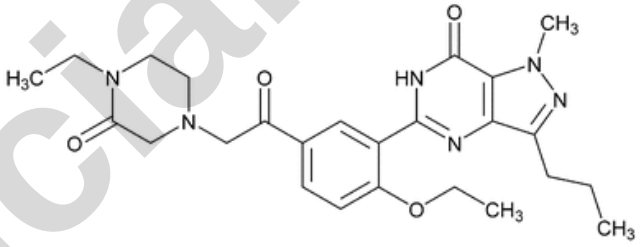

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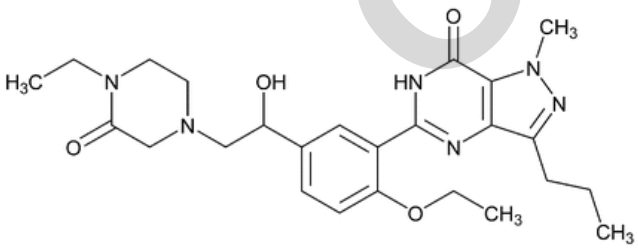

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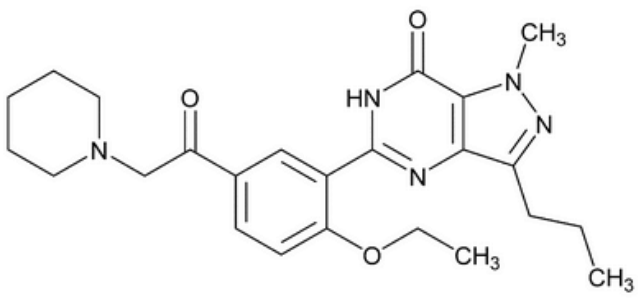

Piperidino acetildenafil

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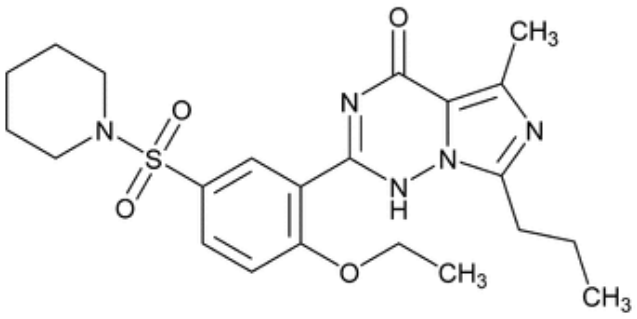

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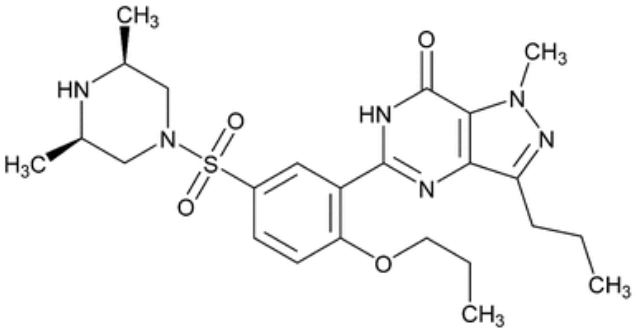

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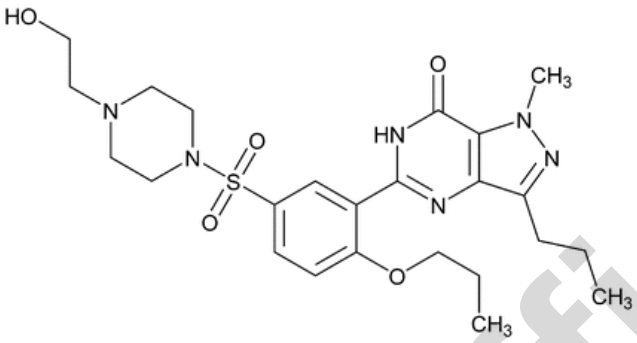

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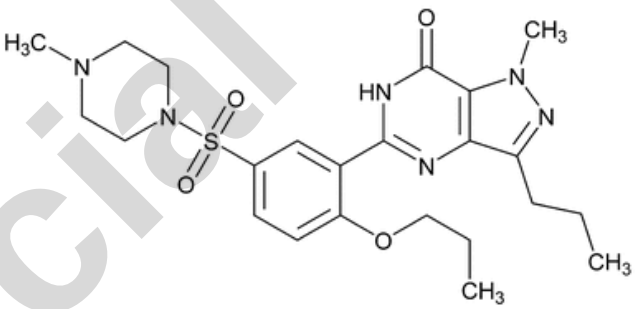

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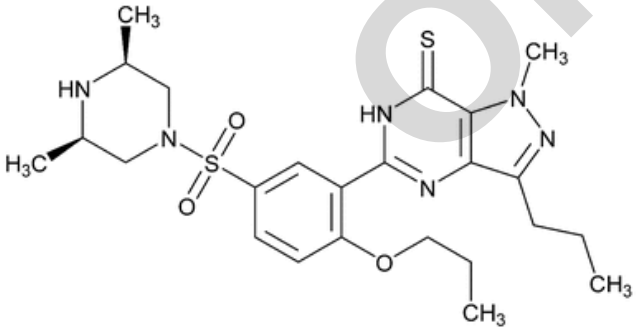

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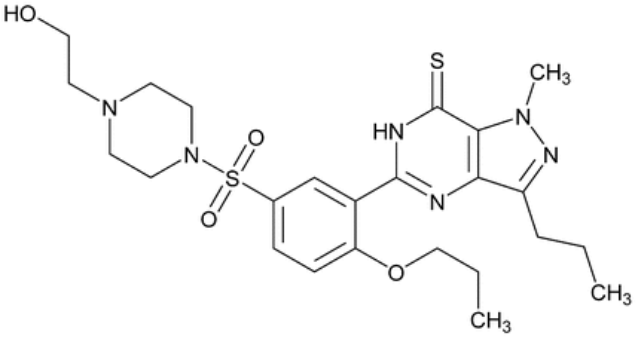

Propoxyphenyl thiohydroxyhomosildenafil

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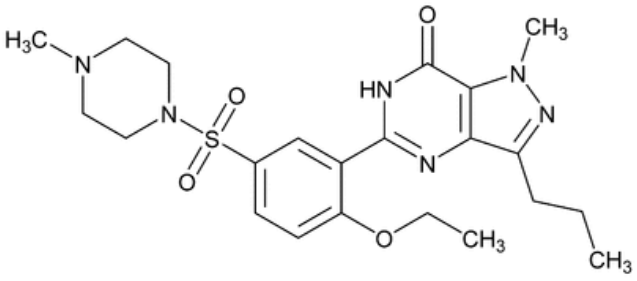

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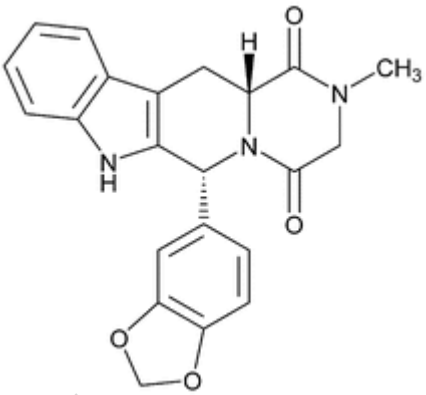

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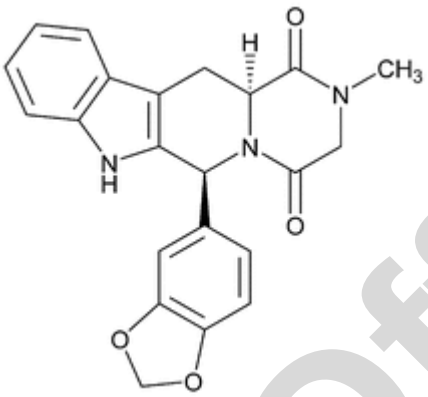

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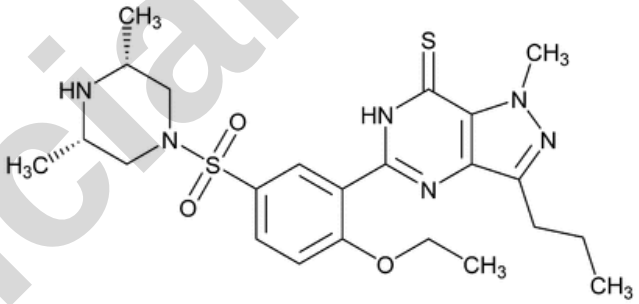

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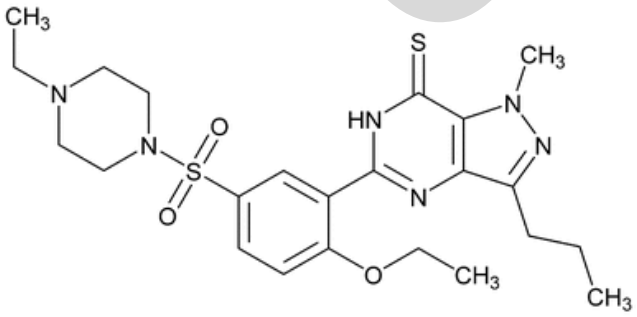

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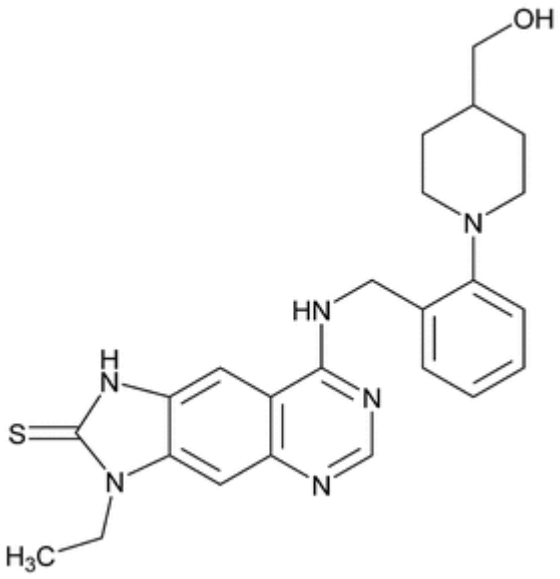

Thioquinapiperifil

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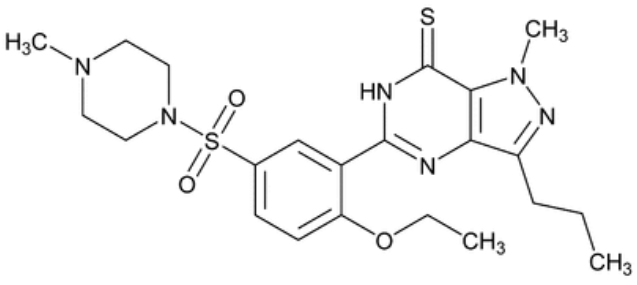

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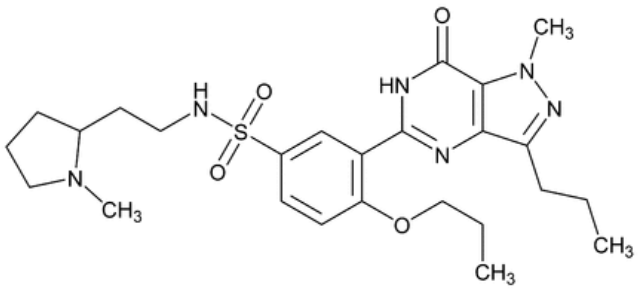

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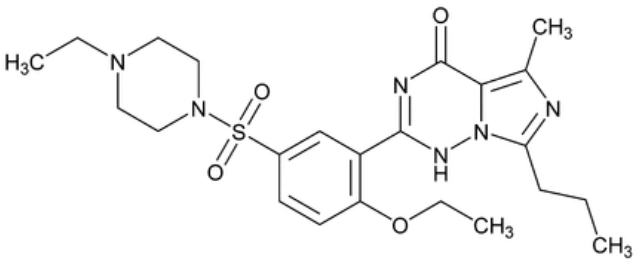

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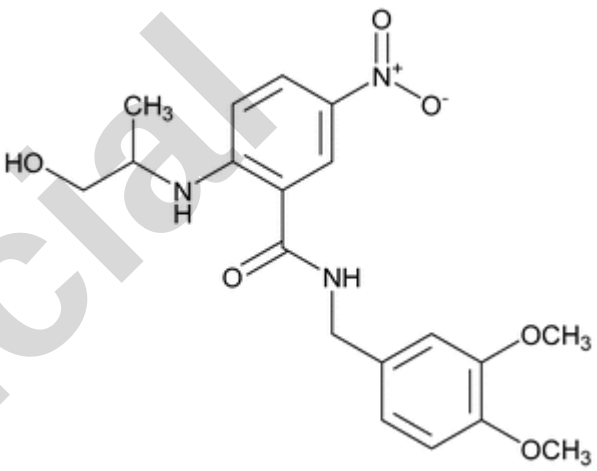

Xanthoanthrafil

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[12] Data compiled from published sources.

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***Add the following:***

# ▲⟨2740⟩ MANUFACTURING PRACTICES FOR DIETARY INGREDIENTS

1. GENERAL PROVISIONS
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    2.3 Documentation and Records
    2.4 Change Control
    2.5 Deviations and Material Reviews
    2.6 Corrective and Preventative Actions
    2.7 Hazard Analysis and Risk-Based Preventive Controls (HARPC)
    2.8 Contract Manufacturers and Contract Laboratories
    2.9 Complaints
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    3.3 Physical Plant Sanitation and Facilities
    3.4 Equipment, Instruments, and Utensils
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4. MATERIALS MANAGEMENT OPERATIONS AND CONTROLS
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    4.6 Materials Management Operations and Control Documentation
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    7.4 Test Procedures
    7.5 Transfer of Analytical Test Procedures
    7.6 Reduced Testing
    7.7 Out-of-Specification (OOS) Investigations
    7.8 Stability Testing
    7.9 Laboratory Testing Records
    7.10 Laboratory Operations and Controls Documentation
GLOSSARY
APPENDIX

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# 1. GENERAL PROVISIONS

This general information chapter provides recommended minimum good manufacturing practices for the methods to be used in and the facilities and manufacturing controls to be used for the manufacture, holding, packaging, labeling, and distribution of dietary ingredients. These principles are set forth to assist manufacturers as well as auditors in establishing whether the facilities and controls used for the manufacture of dietary ingredients are adequate and whether the dietary ingredients possess the identity, strength, and meet the quality and purity characteristics that they purport to possess.

The requirements pertaining to dietary ingredients apply to determining whether or not a dietary ingredient is adulterated within the meaning of §402(a)(3) of the US Federal Food, Drug, and Cosmetic Act (FD&C Act) in that the dietary ingredient has been manufactured under such conditions that is unfit for food; or §402(a)(4) of the FD&C Act in that the food has been prepared, packed, or held under insanitary conditions whereby it may have become contaminated with filth, or whereby it may have been rendered injurious to health; and in violation of §361 of the Public Health Service Act (42 USC §264). The operation of a facility that manufactures, processes, packs, or holds food for sale in the United States if the owner, operator, or agent in charge of such facility is required to comply with, and is not in compliance with, §418 of the FD&C Act or Subpart C, D, E, or F of this part is a prohibited act under §301(UU) of the FD&C Act.

A production and in-process control system must be designed and implemented to cover all stages of manufacturing, packaging, labeling, holding, and distribution of the dietary ingredient and that is designed to ensure quality as specified in the batch production, packaging control record, and dietary ingredient specification.

An overall scheme of quality systems adopted in the chapter consists of the following:

1. *Quality management system*—This system assures overall compliance with current good manufacturing practices (cGMPs) and internal procedures and specifications. This system includes the quality control (QC) unit and all of its review and approval duties (e.g., change control, deviations and material reviews, batch release, product quality review, validation protocols, and reports, etc.). It also includes Hazard Analysis and Risk-Based Preventive Controls (HARPC) or the Hazard Analysis Critical Control Points (HACCP) which details the food safety plan (FSP), food allergen controls, sanitation controls, supply-chain program, and HARPC implementation records. The quality management system must define the quality unit's obligations and authorities to ensure that all material use and disposition decisions are independent of any conflict of interest.
2. *Physical plant and equipment system*—This system includes the measures and activities that provide an appropriate physical environment and resources used in the manufacture of dietary ingredients. It includes buildings and facilities along with maintenance, equipment qualifications, calibration and preventative maintenance, computerized systems, and utilities that are not intended to be incorporated into the product such as water systems.
3. *Materials management system*—This system includes measures and activities to control dietary ingredients including specifications and determining compliance with specifications. It also includes the procedures and process for receiving and release of materials and collecting representative and reserve samples along with storage, distribution controls, and records. The materials management system also includes electronic systems that are used to quarantine and control the movement of physical inventory to ensure that only approved and released materials are allocated to a production order.
4. *Production operations and controls system*—This system includes measures and activities to control the manufacture of dietary ingredients, including batch and continuous production and control records, production and process controls, and operation and control documentation. This system also includes an evaluation of automatic electronic weighing and control systems that are designed to minimize the potential for errors in production.
5. *Packaging and labeling operations and controls system*—This system includes measures and activities that control the packaging and labeling of dietary ingredients. It includes written procedures, label examination and usage, on-site label printing, label storage and issuance, packaging and labeling operations controls, and validation of these operations.
6. *Laboratory control system*—This system includes measures and activities related to laboratory procedures, testing, analytical methods development and validation or verification, and the stability program.

# 2. QUALITY MANAGEMENT

## 2.1 General Principles

Quality unit operations must be implemented in manufacturing, packaging, labeling, laboratory, and holding operations for producing the dietary ingredients to ensure the quality of the dietary ingredient and that the dietary ingredient is packaged and labeled as specified in the master manufacturing and packaging control record.

Quality should be the responsibility of all persons involved in manufacturing, testing, packaging, labeling, and holding dietary ingredients. An effective system for managing quality that involves the active participation of management and appropriate manufacturing personnel must be established, documented, and followed. Management oversight of quality should encompass the organizational structure, procedures, processes, resources, and activities necessary to ensure that the dietary ingredient will meet its intended specifications for quality. All quality-related activities must be defined and documented.

There must be a quality unit(s) that is independent of production and that fulfills both quality assurance (QA) and QC responsibilities. This can be in the form of separate QA and QC units or a single individual or group, depending on the size and structure of the organization.

All quality- and manufacturing-related activities must be recorded and reviewed contemporaneously, and then signed and dated.

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## 2.2 Organization and Personnel

### 2.2.1 GENERAL

The owner, operator, or facility in charge and the management of the establishment must take reasonable measures and precautions to ensure all individuals who manufacture, process, pack, or hold dietary ingredients are qualified to perform their assigned duties.

Written procedures must be established and followed for creating and maintaining job position descriptions, listing job responsibilities and required qualifications for education, training and experience. There must be an organizational chart clearly delineating the structure of the organization and the relationships and relative ranks of its parts and job positions.

### 2.2.2 RESPONSIBILITIES OF THE QUALITY UNIT

A quality unit must be established that is involved in all quality-related matters and that must review and approve all appropriate quality-related documents. Quality unit personnel must ensure that manufacturing, packaging, labeling, and holding operations ensure the quality of the dietary ingredient and that the dietary ingredient is packaged and labeled as specified in the master production and control record.

The primary responsibilities of the independent quality unit(s) should not be delegated and these responsibilities must be described in writing. The quality unit(s) must have the primary responsibility and authority for the following:

- Establishing the HARPC and FSP
- Approving or rejecting (i.e., making a disposition decision for) the release of all dietary ingredients for distribution or of in-process materials for use outside the control of the manufacturer
- Establishing a system to approve or reject the release of raw materials, other ingredients, in-process materials, packaging materials, labeling materials, and the finished dietary ingredient
- Reviewing and approving or rejecting the release of products manufactured, packaged, labeled, or held under contract by another company
- Reviewing and approving all specifications and master production, packaging, and labeling records
- Reviewing and approving all procedures affecting the quality of in-process materials and dietary ingredients
- Reviewing completed production records to ensure that no errors have occurred or, if errors have occurred, that they have been investigated and resolved
- Ensuring that all deviations are properly documented and that deviations are investigated and resolved
- Ensuring that corrective and preventive actions as a result of investigations (deviations or complaints) and internal audits are implemented in a timely manner
- Ensuring that internal audits (self-inspections) are performed
- Establishing a supply chain control system to evaluate and approve suppliers of raw materials, other ingredients, packaging materials and labeling, and suppliers of manufacturing and laboratory services (i.e., contract manufacturers and contract laboratories)
- Establishing a system to evaluate and approve changes that potentially impact the quality of dietary ingredients
- Reviewing and approving test method validation protocols and reports
- Establishing a system to handle complaints and ensure that quality-related complaints are investigated and resolved
- Establishing effective systems for maintaining and calibrating equipment and instruments
- Ensuring that materials are properly tested and the results are reported
- Ensuring that there is stability data to support the retest or expiry date and labeled storage conditions of dietary ingredients
- Performing product quality reviews and trend analysis, as needed

The QC unit (i.e., QC laboratory) must have the responsibility for approving or rejecting all laboratory processes, procedures, specifications, controls, tests, examinations, and deviations from or modifications to them that may affect the identity, strength, quality, and purity of the dietary ingredient. All responsibilities and procedures applicable to the QC unit must be in writing.

The quality unit personnel must be identified and qualified to perform QA and/or QC operations and have responsibilities related to performing quality unit operations distinct and separate from responsibilities that person otherwise has when not performing such operations.

The designated QC personnel who conducts a material review and makes the disposition decision must, at the time of performance, document any material review and disposition decisions.

### 2.2.3 RESPONSIBILITIES OF THE PREVENTIVE CONTROLS-QUALIFIED INDIVIDUAL AND QUALIFIED AUDITOR

One or more preventive controls-qualified individuals must do or oversee the following:

- Preparing the HARPC and FSP
- Validating the preventive controls
- Writing justification for validation to be performed in a time frame that exceeds the first 90 calendar days of production of the applicable dietary ingredient
- Determining that validation is not required with written justification
- Reviewing records
- Writing justification for review of records of monitoring and corrective actions within a time frame that exceeds 7 working days
- Re-analyzing the FSP

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- Determining that re-analysis can be completed, and additional preventive controls validated as appropriate to the nature of the preventive control and its role in the facility's food safety system, in a time frame that exceeds the first 90 calendar days of production of the applicable dietary ingredient

To be a preventive controls-qualified individual, the individual must have successfully completed training in the development and application of risk-based preventive controls at least equivalent to that received under a standardized curriculum recognized as adequate by the FDA or be otherwise qualified through job experience to develop and apply a food safety system. Job experience may qualify an individual to perform these functions if that experience has provided knowledge at least equivalent to that provided through the standardized curriculum. The preventative controls-qualified individual may be, but is not required to be, an employee of the facility.

A qualified auditor must conduct an on-site audit. To be a qualified auditor, a qualified individual must have technical expertise obtained through education, training, or experience (or a combination thereof) necessary to perform the auditing function.

All applicable training in the development and application of risk-based preventive controls must be documented in records, including the date of the training, the type of training, and the person(s) trained.

### 2.2.4 RESPONSIBILITIES FOR PRODUCTION OPERATIONS

The responsibilities for production operations must be documented in writing and should include the following:

- Preparing, reviewing, approving and distributing the instructions for the production of in-process materials and dietary ingredients according to written procedures
- Reviewing all production batch records and ensuring that they are completed and signed
- Ensuring that production facilities are clean and sanitized, if needed
- Ensuring that HVAC, dust collection, and other systems are appropriately designed to minimize the potential for cross-contamination
- Ensuring that physical and spatial separation are used in combination to minimize the potential for cross-contamination and to prevent mix-ups, when appropriate
- Ensuring that the premises and equipment are maintained and records are kept
- Ensuring that manufacturing process validation protocols and reports are reviewed and approved
- Evaluating proposed changes in product, process, or equipment
- Ensuring that new and any modified facilities and equipment are qualified through an appropriate documented change control system

### 2.2.5 PERSONNEL QUALIFICATIONS

Each person engaged in the manufacture of dietary ingredients (including temporary and seasonal personnel) or in the supervision thereof must be a qualified individual having the proper education, training, and experience (or any combination thereof) needed to perform the assigned functions.

Responsibility for ensuring compliance by individuals with the requirements of this general information chapter must be clearly assigned to supervisory personnel. Each person responsible for supervising the manufacture of dietary ingredients must have the proper education, training, and experience (or any combination thereof) to perform assigned functions in such a manner as to provide assurance that the dietary ingredient has the safety, identity, strength, quality, and purity that it is represented to possess.

An adequate number of qualified personnel to perform and supervise the manufacture of dietary ingredients should be provided.

Personnel qualifications apply equally to contractors, consultants, and temporary employees. Records should be maintained stating the name, address, qualifications, and type of service provided by contractors and consultants.

### 2.2.6 TRAINING

Training should be regularly conducted by qualified persons and should cover, at minimum, the particular operation(s) that the employee performs and cGMPs as it relates to the employee's functions. Training must also cover the principles of food hygiene and food safety, including the importance of employee health and personal hygiene, and, as appropriate to the dietary ingredient, the facility and the employee's assigned duties.

Training requirements should be specified (e.g., read and understand documents, instruction with trainer, on-the-job training). Training effectiveness should be periodically assessed. Training programs including on the job training should be re-assessed. Appropriate documentation of any training must be retained by the company, including the date of the training, the type of training, the name of trainer if applicable, the person trained, and any assessment results (e.g., test results).

### 2.2.7 PERSONNEL RESPONSIBILITIES

The company management must take all reasonable measures and precautions to ensure the prevention of microbial contamination and to maintain hygienic practices.

**2.2.7.1 Preventing microbial contamination:** Any person who, by medical examination, by the person's acknowledgment, or by supervisory observation, is shown to have, or appears to have, an illness, infection, or open lesion, or has been exposed to communicable disease or any other abnormal source of microbial contamination that could result in microbial contamination of raw materials, other ingredients, in-process materials, dietary ingredients, or contact surfaces, must be excluded from working in any operations that may result in such contamination until the health concern no longer exists or unless conditions such as

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open lesions, boils, and infected wounds are adequately covered (e.g., by an impermeable cover). Personnel must be instructed to report such health conditions to their supervisors.

**2.2.7.2 Hygienic practices:** All persons working in an operation in direct contact with raw materials, other ingredients, in-process materials, dietary ingredients, or food-contact surfaces must conform to hygienic practices to the extent necessary to protect against allergen cross-contact, microbial contamination, and other types of contamination of the dietary ingredient. These practices include the following:

- Wearing outer garments in a manner that protects against the contamination of raw materials, other ingredients, in-process materials, or dietary ingredients, or any food-contact surfaces
- Maintaining adequate personal cleanliness
- Removing cosmetics from parts of the body that may contact raw materials, other ingredients, in-process materials, dietary ingredients, or food-contact surfaces during performances of their duties
- Washing hands thoroughly (and sanitizing, if necessary, to protect against contamination with undesirable microorganisms) in an adequate hand-washing facility before starting work, as well as at any other time when the hands may have become soiled or contaminated
- Removing all unsecured jewelry and other objects that might fall into raw materials, other ingredients, in-process materials, dietary ingredients, equipment, or packaging materials, and removing hand jewelry that cannot be adequately sanitized during periods in which raw materials, other ingredients, in-process materials, or dietary ingredients are manipulated by hand. If hand jewelry and cosmetics cannot be removed, they must be covered by material that is maintained in an intact, clean, and sanitary condition, and that effectively protects against contamination of raw materials, other ingredients, in-process materials, dietary ingredients, or food-contact surfaces
- Maintaining gloves used in handling raw materials, other ingredients, in-process materials, or dietary ingredients in an intact, clean, and sanitary condition; the gloves must be of impermeable material if bare hands or gloves have the potential to come in contact with these materials during the performances of a person's duties
- Wearing hair nets, caps, beard covers, or other hair restraints when appropriate, such as when the raw material, in-process material, or ingredient is exposed, and in an effective manner
- Storing clothing or other personal belongings in areas separate from where raw materials, other ingredients, in-process materials, dietary ingredients, or any food-contact surface are exposed or where food-contact surfaces are washed
- Not eating food, chewing gum, drinking beverages, taking personal medication, or using tobacco products in areas where raw materials, other ingredients, in-process materials, dietary ingredients, or food-contact surfaces are exposed or where food-contact surfaces are washed
- Taking any other precautions necessary to protect against allergen cross-contact and contamination of raw materials, other ingredients, in-process materials, dietary ingredients, or food-contact surfaces with microorganisms, allergens, filth, foreign substances, or other extraneous materials, including perspiration, hair, cosmetics, tobacco, chemicals, and medicines applied to the skin. These precautions may include restricting individuals working in unit operations with known allergens from moving to non-allergen production areas, and/or color-coding of in-process allergen containing materials to differentiate them from non-allergen containing materials

## 2.3 Documentation and Records

### 2.3.1 GENERAL

Documentation can be viewed as the foundation of all quality systems, because clear, complete, and accurate records are essential to all operations and procedures. A guiding cGMP principle is that if it is not written down, it is not done. *Good Documentation Guidelines* ⟨1029⟩, which provides guidelines on good documentation practices for cGMP-regulated industries, should be followed.

All documents related to the manufacture of dietary ingredients must be initiated, prepared, reviewed, approved, distributed, and archived according to written procedures. Such documents can be in paper or electronic form.

When entries are made in records, these must be made legibly and indelibly in spaces provided for such entries, directly after performing the activities. Entries should identify the person making the entry, as well as the day and time when the entry was made. All dates must include the day, month, and year in a consistent and unambiguous format such as establishing a date format (e.g., DD-MM-YYYY) or spelling out the month. Numerical results must have appropriate numerical units, and calculations must be made with appropriate rounding and significant figures. Corrections to entries must be dated and signed and leave the original entry still legible. If the reason for the correction is not obvious (e.g., date transcription error), then a statement should be made to provide clarity for the correction.

### 2.3.2 CONTROL OF DOCUMENTATION

The issuance, revision, superseding, and withdrawal of all documents must be controlled by the maintenance of revision histories. A procedure should be established for retaining all appropriate documentation (e.g., procedures and records). There should be proper control over the following:

- Forms: content, revision, review
- Spreadsheets: spreadsheet for specific project (accuracy checked by reviewer), spreadsheet for routine use (validated, access/revision controlled)
- External documents: proprietary documents, guidance documents issued by outside agency
- Uncontrolled documents
- "For reference only" documents

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- Employee notes and memos

A formal documentation filing system (paper and electronic) should be established with appropriate security, and documentation should be readily retrievable.

#### 2.3.3 RECORDS RETENTION

The retention periods for these documents must be specified. Written records must be kept, at minimum, for 1 year past the retest or expiry date of the dietary ingredient associated with those records. Records must be kept as original records, as accurately reproduced copies (e.g., photocopies, microfilm, compact disc), or as electronic copies. All electronic records must comply with 21 CFR Part 11 (*Electronic Records; Electronic Signatures*).

All records or copies of records required by this general information chapter must be readily available during the retention period for inspection by the FDA when requested. If reduction techniques such as microfilming are used, suitable reader and photocopying equipment must be made readily available to the FDA.

## 2.4 Change Control

A formal change control system must be established to evaluate all changes that may affect the production and quality of dietary ingredients. Written procedures should provide for the identification, documentation, appropriate review, and approval of changes to raw materials, other ingredients, packaging materials, labeling material specifications, analytical methods, facilities, utility systems, equipment, computer systems, production operations, and packaging and labeling operations.

Any proposals for cGMP-relevant changes should be drafted, reviewed, and approved by the appropriate manufacturing units, as well as reviewed and approved by the quality unit(s).

The potential impact of the proposed change on the quality of the dietary ingredient should be evaluated. A classification procedure may help in determining the level of testing, validation, and documentation needed to justify changes to a validated process. Changes can be classified (e.g., as minor or major) depending on the nature and extent of the changes, and the effects these changes may impart on the process. Scientific judgment should determine what additional testing and validation studies are appropriate to justify a change in a validated process. When implementing approved changes, measures should be taken to ensure that all documents affected by the change are revised.

The potential for critical changes to affect established retest or expiry dates (e.g., changes to product manufacturing process or formulation) should be evaluated. If necessary, the dietary ingredient produced by the change should be placed on an accelerated stability program and/or added to the stability monitoring program.

Dietary ingredient manufactures should require their raw material suppliers or packagers to notify them of changes from established production process controls that can affect the quality of the dietary ingredient.

## 2.5 Deviations and Material Reviews

#### 2.5.1 DEVIATIONS

Written procedures must be established and followed for documenting all deviations and for investigating deviations. Any deviation from established procedures and standards should be documented and explained. Deviations should be investigated, and the investigation and its conclusions should be documented. The investigation should extend to any and all batches of material that may have been associated with the specific failure or deviation. The quality unit must ensure that deviations are investigated and resolved.

#### 2.5.2 MATERIAL REVIEW

Written procedures must be established and followed for conducting material reviews and making disposition decisions, and for the quality unit to approve or reject any treatment or in-process adjustment of a raw material, other ingredient, in-process material that is rejected, or reprocessing of a dietary ingredient that is rejected.

Quality unit personnel must conduct all required material reviews and make all required disposition decisions. Quality unit personnel must conduct a material review and make a disposition decision if one of the following occurs:

- A specification is not met for a raw material, other ingredient, in-process material, packaging, labeling, dietary ingredient, a dietary ingredient received for packaging and labeling, or finished packaged and labeled dietary ingredient
- A batch deviates from the master production and control record, including when any step is not completed and any deviation from specifications occurs
- Any unanticipated occurrence during manufacturing operations adulterates or could lead to adulteration of the raw material, other ingredient, in-process material, dietary ingredient, or packaging, or could lead to the use of a label not specified in the master production and control record
- Calibration of an instrument or control suggests a problem may have resulted in a failure to ensure the quality of a batch or batches of a dietary ingredient
- A dietary ingredient is returned

Quality unit personnel must reject the raw material, other ingredient, in-process material, dietary ingredient, packaging, or labeling when an established specification is not met or when a deviation or unanticipated occurrence during the batch production and in-process control system results in or could lead to adulteration of the product. However, the quality unit can approve a treatment, an in-process adjustment, or a reprocessing to correct the deviation or occurrence, ensuring that the quality of the final dietary ingredient and that its packaging and labeling meets the specifications of the master production and control record.

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The quality unit personnel and each qualified individual who provides information relevant to the material review and disposition decision and subsequent approval or rejection of the treatment, in-process adjustment, or reprocessing of the subject material must document performance of the review by signing and dating in the deviation investigation and material review report at the time of performance.

Deviation investigation and material review reports must include the following:

- Description of the specific deviation or unanticipated occurrence
- Description of the root cause investigation
- Evaluation of whether the deviation or unanticipated occurrence has resulted in or could lead to a failure to ensure the quality of the dietary ingredient or failure to package and label the dietary ingredient as specified in the master production and control record
- Identification of corrective action(s) and preventive action(s) taken with timelines
- Description of any material reviews and a scientifically valid reason for any treatment, in-process adjustment, or reprocessing so as to correct the applicable deviation or unanticipated occurrence, along with the quality unit personnel's approval signature and date when performed
- Conclusion(s), including the explanation of the final disposition decision (approval or rejection) made for the subject article
- Signatures of individual(s) who are designated to perform quality unit operations and who conducted the material review and made the disposition decision, and of each qualified individual who provides information relevant to that material review and disposition decision

Documentation of any material review and disposition decision or reference to the deviation investigation and material review report must be included in the appropriate batch production and control record.

## 2.6 Corrective and Preventative Actions

Corrective Actions and Preventative Actions (CAPA) programs are important to continuous improvement of the quality management system. The CAPA operating system is an essential governance document by which personnel plan and implement control to mitigate and control failures within the quality management systems. CAPAs may rise from many sources, including deviations, customer complaints, investigations, internal audit findings, and external audit findings.

The quality unit must establish, monitor, and maintain a CAPA program. The CAPA program should include a tracking system for deviations and nonconformities, including out-of-specification (OOS) laboratory results, and corrective and/or preventive action(s). All persons must identify deviations and nonconformities and notify management and/or the quality unit. Supervisors must determine root causes and develop and implement appropriate remedial, corrective, and/or preventive action(s). The quality unit must monitor CAPAs and manage the CAPA program to assist with adequate development, implementation, and closure of CAPAs. See *2.7.4.2 Corrective actions and correction procedures* for further details regarding corrective actions and correction procedures as they apply to the FSP.

A *Glossary* that includes CAPA terms (i.e., corrective action, preventive action, remedial (short-term) action, nonconformity, deviation, root cause) is presented at the end of the chapter.

## 2.7 Hazard Analysis and Risk-Based Preventive Controls (HARPC)

### 2.7.1 FOOD SAFETY PLAN (FSP)

The HARPC include the requirement to prepare and implement a written FSP that is overseen by one or more preventive controls-qualified individuals. The scope of HARPC/FSP includes evaluating hazards that could affect food safety; implementing preventive steps or controls to minimize or prevent hazards; monitoring the controls to ensure that they work; maintaining records of monitoring; and specifying what actions will be taken to correct problems that arise, including a recall plan for the dietary ingredient. The FDA's draft guidance *Hazard Analysis and Risk-Based Preventive Controls for Human Food: Guidance for Industry* should be consulted.

The contents of the written FSP must include the following:

- Hazard analysis
- Preventive controls
- Supply chain controls
- Recall plan
- Monitoring implementation of preventive controls
- Corrective actions and correction procedures
- Verification and validation procedures
- Associated records

### 2.7.2 HAZARD ANALYSIS

A hazard analysis must be conducted to identify and evaluate known or reasonably foreseeable hazards for each type of dietary ingredient manufactured, processed, packed, or held at the facility to determine whether there are any hazards requiring preventive control. This hazard analysis is based on experience, illness data, scientific reports, and other information.

Hazard identification must include known or reasonably foreseeable hazards, such as the following:

- Biological hazards, including microbiological hazards such as parasites, environmental pathogens, and other pathogens
- Chemical hazards, including radiological hazards, substances such as pesticides and drug residues, natural toxins, decomposition, unapproved or color additives, and food allergens

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- Physical hazards, such as stones, glass, and metal fragments
- Hazards that may be present in the dietary ingredient for any of the following reasons:
  - The hazard occurs naturally
  - The hazard may be introduced unintentionally
  - The hazard may be introduced intentionally for the purpose of economic gain

The identified hazards must be evaluated to assess the severity of the illness or injury if the hazards were to occur and the probability that the hazard will occur in the absence of preventive controls. Hazard evaluation must include environmental pathogens whenever a dietary ingredient is exposed to the environment prior to packaging and the packaged dietary ingredient does not receive a treatment or another control measure, such as formulations with antimicrobial properties that would significantly minimize or reduce the likelihood for the presence of pathogenic microorganisms. The hazard evaluation analysis also must consider the effect of the following factors on the safety of the finished dietary ingredient for the intended consumer:

- Physical and chemical properties of the dietary ingredient (e.g., water activity, pH)
- Formulation of the dietary ingredient
- Condition, function, and design of facility and equipment
- Raw materials and other ingredients
- Transportation practices
- Manufacturing/processing procedures
- Packaging activities and labeling activities
- Storage and distribution
- Intended or reasonably foreseeable use
- Sanitation, including employee hygiene
- Any other relevant factors, such as the temporal (e.g., weather-related) nature of some hazards (e.g., levels of some natural toxins)

### 2.7.3 PREVENTIVE CONTROLS

Preventive controls must be identified and implemented to provide assurances that any hazards requiring a preventive control will be significantly minimized or prevented and the dietary ingredient manufactured, processed, packed, or held by the facility will not be adulterated or misbranded. Preventive controls must be written. Preventive controls include controls at any critical control points (CCPs) and other controls appropriate for food safety.

Preventive controls include the following, as appropriate to the facility:

- Process controls
- Food allergen controls
- Sanitation controls
- Supply-chain controls
- Recall plan

**2.7.3.1 Process controls:** These include procedures, practices, and processes to ensure the control of parameters during operations such as heat processing, acidifying, irradiating, and refrigerating foods. As appropriate to the nature of the applicable control and its role in the facility's food safety system, process controls must include parameters associated with the control of the hazard and the maximum or minimum value, or combination of values, to which any biological, chemical, or physical parameter must be controlled to significantly minimize or prevent a hazard. Where practical, dietary ingredient manufacturers should employ process analytical technologies such as electronic metal detection to mitigate the risk of hazards.

**2.7.3.2 Food allergen controls:** These include procedures, practices, and processes to control food allergens. As appropriate, food allergen controls must include those procedures, practices, and processes employed for ensuring the protection of food from allergen cross-contact, including during storage, handling, and use; and labeling the finished food, including ensuring that the finished food is not misbranded.

**2.7.3.3 Sanitation controls:** These include procedures, practices, and processes to ensure that the facility is maintained in a sanitary condition adequate to significantly minimize or prevent hazards such as environmental pathogens, biological hazards due to employee handling, and food allergen hazards. As appropriate to the facility and the dietary ingredient, sanitation controls must include procedures, practices, and processes for the cleanliness of food-contact surfaces, including food-contact surfaces of utensils and equipment, and prevention of allergen cross-contact and cross-contamination from insanitary objects, from personnel to food, food packaging material, and other food-contact surfaces, and from raw product to processed product.

**2.7.3.4 Supply-chain controls:** When dietary ingredient manufacturers who receive the raw materials and other ingredients identify a hazard that requires a supply-chain-applied control, a written risk-based supply-chain control program must be established and implemented for those raw materials and other ingredients. The supply-chain program must provide assurance that a hazard requiring a supply-chain-applied control has been significantly minimized or prevented.

If the receiving facility is an importer that is in compliance with the foreign supplier verification program requirements in 21 CFR Part 1, Subpart L, and that has documentation of verification activities conducted in 21 CFR 1.506(e) (which provides assurance that the hazards requiring a supply-chain-applied control for the raw material or other ingredient have been significantly minimized or prevented), it does not need to conduct supplier verification activities for that raw material or other ingredient.

When a supply-chain-applied control is applied by an entity other than the dietary ingredient manufacturer's supplier (e.g., a raw material or other ingredient manufacturer applies controls under different management than the supplier), the dietary ingredient manufacturer must verify the supply-chain control or obtain documentation of appropriate verification activity from the entity, review and assess the entity's applicable documentation, and document that review and assessment. For the purposes of this section, "verification" means the application of methods, procedures, tests, and other evaluations, in addition to

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monitoring, to determine whether a control measure or combination of control measures is or has been operating as intended and to establish the validity of the FSP.

The supply-chain control program must include the following:

- Using approved suppliers and documenting that approval before receiving raw materials and other ingredients from those suppliers
  - Written procedures for receiving raw materials and other ingredients must be established and followed and must ensure that raw materials and other ingredients are received only from approved suppliers (or, when necessary and appropriate, on a temporary basis from unapproved suppliers whose raw materials and other ingredients are subjected to adequate verification activities before acceptance for use)
  - Use of the written procedures for receiving raw materials and other ingredients must be documented
- Determining appropriate supplier verification activities, including determining the frequency of conducting the activity by considering the following:
  - The hazard analysis of the raw material or other ingredient, including the nature of the hazard controlled before receipt of the raw material or other ingredient
  - The entity or entities that will be applying controls for the hazards requiring a supply-chain-applied control
  - Supplier performance, including the following:
    - The supplier's procedures, processes, and practices inherent to the hazards associated with the raw material and other ingredients
    - Applicable FDA food safety regulations and information relevant to the supplier's compliance with those regulations, including an FDA warning letter or import alert relating to the safety of the raw material and other ingredients, and other FDA compliance actions related to food safety (or, when applicable, relevant laws and regulations of a country whose food safety system FDA has officially recognized as comparable or has determined to be equivalent to that of the United States, and information relevant to the supplier's compliance with those laws and regulations)
    - The supplier's food safety history relevant to the raw materials and other ingredients that the manufacturer receives from the supplier, including available information about results from testing raw materials and other ingredients for hazards, audit results relating to the safety of the raw material and other ingredients, and responsiveness of the supplier in correcting problems
  - Any other factors as appropriate and necessary, such as storage and transportation practices
- Conducting one or more supplier verification activities as determined to be appropriate for raw materials and other ingredients, and approving each supplier before using the raw materials and other ingredients
  - Risk-based supplier verification activities may include the following:
    - On-site audits
    - Sampling and testing of raw materials and other ingredients to verify the accuracy of information on the supplier certificate of analysis
    - Review of supplier's relevant food safety records
    - Other appropriate supplier verification activities based on supplier performance and the risk associated with the raw materials and other ingredients
  - When a hazard in a raw material or other ingredient will be controlled by the supplier and is one for which there is a reasonable probability that exposure will result in serious adverse health consequences or death to humans, the appropriate supplier verification activity is an on-site audit of the supplier, which must be conducted annually unless there is a written determination that other verification activities and/or less frequent on-site auditing of the supplier provide adequate assurance that the hazards are controlled
  - There must not be any financial conflicts of interests that influence the results of the verification activities, and payment must not be related to the results of the activity
- Taking and documenting prompt corrective measures to ensure that raw materials and other ingredients from the supplier do not cause the dietary ingredient to be adulterated or misbranded, if the manufacturer has identified through auditing, verification testing, document review, relevant consumers, and customer or other complaints that the supplier is not controlling hazards requiring a supply-chain-applied control
- Documenting supplier qualification activities

*2.7.3.4.1 On-site audit:* An on-site audit of a supplier must be performed by a qualified auditor. If the raw material or other ingredient at the supplier is subject to one or more FDA food safety regulations, an on-site audit must consider such regulations and include a review of the supplier's written plan (e.g., HACCP plan or other FSP), if any, and its implementation for the hazard being controlled (or, when applicable, an on-site audit may consider relevant laws and regulations of a country whose food safety system the FDA has officially recognized as comparable or has determined to be equivalent to that of the United States).

One of the following may be substituted for an on-site audit, provided that the inspection was conducted within 1 year of the date that the on-site audit would have been required to be conducted:

- The written results of an appropriate inspection of the supplier for compliance with applicable FDA food safety regulations conducted and reported by the FDA, by representatives of other federal agencies (such as the US Department of Agriculture), or by representatives of state, local, tribal, or territorial agencies
- For a foreign supplier, the written results of an inspection by the FDA or the food safety authority of a country whose food safety system the FDA has officially recognized as comparable or has determined to be equivalent to that of the Unites States

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- For inspections conducted by the food safety authority of a country, the raw material or other ingredient that is the subject of the on-site audit must be within the scope of the official recognition or equivalence determination, and the foreign supplier must be in and under the regulatory oversight of that country.

An on-site audit is not subject to the requirements in 21 CFR Part 1, Subpart M, if it is solely conducted to meet the requirements of the manufacturer's supply-chain control program by an audit agent of a certification body accredited in accordance with those regulations.

*2.7.3.4.2 Responsibilities of the receiving facility:* The receiving facility must approve suppliers. The receiving facility must determine and conduct appropriate supplier verification activities and satisfy all supply-chain-program documentation requirements.

An entity other than the receiving facility may do any of the following provided that the receiving facility reviews and assesses the entity's applicable documentation and documents that review and assessment:

- Establish written procedures for receiving raw materials and other ingredients by the entity
- Document that written procedures for receiving raw materials and other ingredients are being followed by the entity
- Determine and/or conduct the appropriate supplier verification activities with appropriate documentation

As a supplier verification activity for a particular lot of product, the supplier may conduct and document sampling and testing of raw materials and other ingredients for the hazard controlled by the supplier and provide such documentation to the receiving facility, provided that the receiving facility reviews and assesses that documentation and maintains a record of that review and assessment.

A receiving facility may not accept any of the following as a supplier verification activity:

- A determination by its supplier of the appropriate supplier verification activities for that supplier
- An audit conducted by its supplier
- A supplier-conducted review of that supplier's own relevant food safety records

The requirements of this section do not prohibit a receiving facility from relying on an audit provided by its supplier when the audit was conducted by a third-party qualified auditor.

*2.7.3.4.3 Supply-chain program documentation and records:* The manufacturer receiving raw materials and other ingredients must document and review the following records as applicable to its supply-chain program:

- The written supply-chain program
- Documentation that a receiving facility that is an importer is in compliance with the foreign supplier verification program requirements in 21 CFR part 1, subpart L, including documentation of verification activities in 21 CFR 1.506(e)
- Documentation of the approval of a supplier
- Written procedures for receiving raw materials and other ingredients
- Documentation demonstrating use of the written procedures for receiving raw materials and other ingredients
- Documentation of the determination of the appropriate supplier verification activities for raw materials and other ingredients
- Documentation of the conduct of an on-site audit, which must include:
  - The name of the supplier subject to the on-site audit
  - Documentation of audit procedures
  - The dates the audit was conducted
  - The conclusions of the audit
  - Corrective actions taken in response to significant deficiencies identified during the audit
  - Documentation that the audit was conducted by a qualified auditor
- Documentation of sampling and testing conducted as a supplier verification activity, which must include:
  - Identification of the raw material or other ingredient tested (including lot number) and the number of samples tested
  - Identification of the test(s) conducted, including the analytical method(s) used
  - The date(s) on which the test(s) were conducted and the date of the report
  - The results of the testing
  - Corrective actions taken in response to detection of hazards
  - Information identifying the laboratory conducting the testing
- Documentation of the review of the supplier's relevant food safety records, which must include:
  - The name of the supplier whose records were reviewed
  - The date(s) of the review
  - The general nature of the records reviewed
  - The conclusions of the review
  - Corrective actions taken in response to significant deficiencies identified during the review
- Documentation of other appropriate supplier verification activities based on the supplier performance and the risk associated with the raw material or other ingredient
- Documentation of any determination that verification activities other than an on-site audit and/or less frequent on-site auditing of a supplier provide adequate assurance that the hazards are controlled when the supplier controls a hazard in a raw material or other ingredient and that hazard is one for which exposure could probably result in serious adverse health consequences or death to humans
- The written results of an appropriate inspection of the supplier for compliance with applicable FDA food safety regulations by the FDA, by representatives of other federal agencies (such as the US Department of Agriculture), or by representatives

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from state, local, tribal, or territorial agencies, or the food safety authority of another country when the results of such an inspection is substituted for an on-site audit
- Documentation of actions taken with respect to supplier non-conformance
- Documentation of verification of a supply-chain-applied control applied by an entity other than the receiving facility's supplier
- When applicable, documentation of the receiving facility's review assessment, which could include:
  - Applicable documentation from an entity other than the receiving facility:
    - Of written procedures for receiving raw materials and other ingredients being followed
    - Of the determination of the appropriate supplier verification activities for raw materials and other ingredients
    - Of conducting the appropriate supplier verification activities for raw materials and other ingredients
    - Of the verification activities when a supply-chain-applied control is applied by an entity other than the receiving facility's supplier
  - Applicable documentation from its supplier:
    - Of the results of sampling and testing conducted by the supplier
    - Of the results of an audit conducted by a third-party qualified auditor

Supplier verification activities must be documented. Formal reports should be generated for each raw material or other ingredient from each supplier summarizing initial supplier verification activities and analyses, and plans and schedule for continuous monitoring. An approved list of raw materials, other ingredients, and suppliers should be readily available to staff. A unique material item code should be assigned for each material from each supplier; however, the manufacturer may identify equivalencies between the same raw materials and other ingredients from different suppliers as long as they meet the same critical specifications for identity, strength, purity, composition, and limits on contaminants.

Quality unit personnel must review and approve the documentation setting forth the basis for the qualification of any supplier.

**2.7.3.5 Recall plan:** For dietary ingredients with a hazard requiring a preventive control, the manufacturer must establish a written recall plan for the dietary ingredient. The written recall plan must include procedures that describe the steps to be taken and assign responsibility for taking those steps and to perform the following actions as appropriate to the facility:
- Directly notify the direct consignees of the dietary ingredient being recalled, including how to return or dispose of the affected dietary ingredient
- Notify the public about any hazard presented by the dietary ingredient when appropriate to protect public health
- Conduct effectiveness checks to verify that the recall is carried out
- Appropriately dispose of the recalled dietary ingredient (e.g., through reprocessing, reworking, diverting to a use that does not present a safety concern, or destroying the dietary ingredient)

A written procedure should define the circumstances under which recall of a dietary ingredient should be considered. The recall procedure should designate who should be involved in evaluating the information, how a recall should be initiated, who should be informed about the recall, and how the recalled dietary ingredient should be treated. If the event of a health hazard is not self-evident, then an independent health hazard assessment by a qualified individual or organization should be conducted to determine the depth and scope of a recall. In the event of a serious or potentially life-threatening situation, local, national, and/or international regulatory authorities should be informed, and their advice sought to ensure regulations are properly followed and executed for disposition of the recalled article.

Mock recalls should be performed annually.

**2.7.3.6 Other controls:** Other controls include any other procedure, practice, and process necessary to ensure that food will not be adulterated or misbranded; examples of other controls include hygiene training and other good cGMPs.

### 2.7.4 PREVENTIVE CONTROL MANAGEMENT

Preventive controls are subject to the following preventive control management components as appropriate to ensure the effectiveness of the controls while considering their nature and their role in the facility's food safety system:
- Monitoring
- Corrective actions and corrections
- Verification

The supply-chain program is subject to the following preventive control management component as appropriate to ensure the effectiveness of the supply-chain program while considering the nature of the hazard controlled before receipt of the raw material or other ingredient:
- Corrective actions and corrections that consider the nature of any supplier non-conformance:
  - Review of records
  - Re-analysis

**2.7.4.1 Monitoring:** As appropriate to the nature of the preventive controls and their role in the facility's food safety system, written procedures must be established and implemented and include the frequency with which they are to be performed for monitoring the preventive controls. Preventive controls must be monitored with adequate frequency to provide assurance that they are consistently performed. The monitoring of preventive controls must be documented in records that are subject to verification and records review.

**2.7.4.2 Corrective actions and correction procedures:** As appropriate to the nature of the hazard and the nature of the preventive controls, the manufacturer must establish and implement written corrective action procedures that must be taken if preventive controls are not properly implemented, including procedures to address, for the presence of the following hazards:
- The presence of a pathogen or appropriate indicator organism detected as a result of product testing

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- The presence of an environmental pathogen or appropriate indicator organism detected through environmental monitoring

The corrective action procedures must describe the steps to be taken to ensure that:

- Appropriate action is taken to identify and correct a problem that has occurred with implementation of a preventive control
- Appropriate action is taken when necessary to reduce the likelihood that the problem will reoccur
- All affected dietary ingredients are evaluated for safety
- All affected dietary ingredients are prevented from entering commerce, if the manufacturer cannot ensure that the affected dietary ingredient is not adulterated or misbranded

The manufacturer must follow these steps and, if appropriate, re-analyze the FSP to determine whether modification of the FSP is required if any of the following circumstances occur:

- A preventive control is not properly implemented and a corrective action procedure has not been established
- A preventive control, combination of preventive controls, or the FSP is found to be ineffective
- A review of records finds that the records are not complete, the activities conducted did not occur in accordance with the FSP, or appropriate decisions were not made about corrective actions

All corrective actions (and corrections, when appropriate) taken must be documented in records that are subject to verification and records review.

**2.7.4.3 Verification activities:** Verification activities must include the following preventive control management components as appropriate to the nature of the preventive controls and their role in the facility's food safety system:

- Validation
- Verification that monitoring is being conducted as required in *2.7.4.1 Monitoring*
- Verification that appropriate decisions about corrective actions are being made as required in *2.7.4.2 Corrective actions and correction procedures*
- Verification of implementation and effectiveness
- Re-analysis

All verification activities must be documented in records and are subject to review.

*2.7.4.3.1 Validation:* The manufacturer must validate that the preventive controls identified and implemented are adequate to control the hazard as appropriate to the nature of the controls and their role in the facility's food safety system. The validation of the preventive controls must be performed (or overseen) by a preventive controls-qualified individual at the following times:

- Prior to implementation of the FSP
- When necessary to demonstrate the control measures can be implemented as designed (within 90 calendar days, or within a reasonable justifiable time frame after production of the applicable dietary ingredient first begins)
- Whenever a change to a control measure or combination of control measures could affect whether the control measure or combination of control measures, when properly implemented, will effectively control the hazards
- Whenever a re-analysis of the FSP reveals the need to do so

The validation of the preventive controls must include obtaining and evaluating scientific and technical evidence (or, when such evidence is not available or is inadequate, conducting studies) to determine whether the preventive controls, when properly implemented, will effectively control the hazards.

The manufacturer does not need to validate the food allergen controls, the sanitation controls, the recall plan, the supply-chain controls, and other preventive controls if the preventive controls-qualified individual prepares (or oversees the preparation of) a written justification that validation is not applicable based on factors such as the nature of the hazard and the nature of the preventive control and its role in the facility's food safety system.

*2.7.4.3.2 Verification of implementation and effectiveness:* The manufacturer must verify that the preventive controls are consistently implemented and are effectively and significantly minimizing or preventing the hazards by conducting the following activities as appropriate to the facility, the dietary ingredient, and the nature of the preventive controls and their role in the facility's food safety system:

- Calibration of process monitoring instruments and verification instruments (or checking them for accuracy)
- Product testing for a pathogen (or appropriate indicator organism) or another hazard
- Environmental monitoring for an environmental pathogen or for an appropriate indicator organism
- Other activities appropriate for verification of implementation and effectiveness
- Review of the following records within the specified time frames by (or under the oversight of) a preventive controls-qualified individual to ensure that the records are complete, the activities reflected in the records occurred in accordance with the FSP, the preventive controls are effective, and appropriate decisions were made about corrective actions:
    - Records of monitoring and corrective action records within 7 working days after the records are created or within a reasonable time frame, provided that the preventive controls-qualified individual prepares (or oversees the preparation of) a written justification for a time frame that exceeds 7 working days. All records should be reviewed and approved by a qualified individual before the products are released in the market
    - Records of calibration, testing (e.g., product testing, environmental monitoring), supplier and supply-chain verification activities, and other verification activities within a reasonable time after the records are created

As appropriate to the facility, the food, the nature of the preventive control, and the role of the preventive control in the facility's food safety system, the manufacturer must establish and implement written procedures for the following activities:

- The method and frequency of calibrating process monitoring instruments and verification instruments (or checking them for accuracy)
- Product testing procedures that must:

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- Be scientifically valid
- Identify the test microorganism(s) or other analytes
- Specify the procedures for identifying samples, including their relationship to specific lots of product
- Include the procedures for sampling, including the number of samples and the sampling frequency
- Identify the test(s) conducted, including the analytical method(s) used
- Identify the laboratory conducting the testing
- Include the corrective action procedures

- Environmental monitoring procedures that must:
  - Be scientifically valid and based on risk management procedures
  - Identify the test microorganism(s)
  - Identify the locations from which samples will be collected and the number of sites to be tested during routine environmental monitoring (the number and location of sampling sites must be adequate to determine whether preventive controls are effective)
  - Identify the timing and frequency for collecting and testing samples (the timing and frequency for collecting and testing samples must be adequate to determine whether preventive controls are effective)
  - Identify the test(s) conducted, including the analytical method(s) used
  - Identify the laboratory conducting the testing
  - Include the corrective action procedures

*2.7.4.3.3 Re-analysis:* The manufacturer must conduct a re-analysis of the FSP in its entirety once every 3 years. In addition, the manufacturer must conduct a re-analysis either in its entirety or of the affected portion of the FSP when one of the following occurs:

- Whenever a significant change in the activities conducted at the facility creates a reasonable potential for a new hazard or creates a significant increase in a previously identified hazard
- Whenever the manufacturer becomes aware of new information about potential hazards associated with the dietary ingredient
- Whenever appropriate after an unanticipated food safety problem
- Whenever the manufacturer finds that a preventive control, combination of preventative controls, or the FSP in its entirety is ineffective
- Whenever the FDA determines it is necessary to respond to new hazards and developments in scientific understanding

The manufacturer must complete the re-analysis and validate as appropriate to the nature of the preventive controls and their role in the facility's food safety system any additional preventive controls needed to address the hazard identified in one of the following time frames:

- Before any change in activities (including any change in preventive control) at the facility is operative
- When necessary to demonstrate the control measures can be implemented as designed within one of the following:
  - 90 calendar days after production of the applicable dietary ingredient
  - A reasonable time frame, provided that the preventive controls-qualified individual prepares (or oversees the preparation of) a written justification for a time frame that exceeds 90 calendar days after production of the applicable dietary ingredient first begins

The manufacturer must either revise the written FSP if a significant change in the activities conducted at the facility creates a reasonable potential for a new hazard or a significant increase in a previously identified hazard, or document the basis for the conclusion that no revisions are needed. A preventive controls-qualified individual must perform (or oversee) the re-analysis and revisions to the FSP.

### 2.7.5 HARP IMPLEMENTATION RECORDS

The manufacturer must establish and maintain the following records documenting implementation of the FSP:

- Records that document the monitoring of preventive controls
- Records that document corrective actions
- Records that document verification, including, as applicable, those related to:
  - Validation
  - Verification of monitoring
  - Verification of corrective actions
  - Calibration of process monitoring and verification instruments
  - Product testing
  - Environmental monitoring
  - Records review
  - Re-analysis
- Records that document the supply-chain program
- Records that document applicable training for the preventive controls-qualified individual and the qualified auditor

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## 2.8 Contract Manufacturers and Contract Laboratories

All contract manufacturers and contract laboratories should comply with the requirements of this chapter. Special consideration should be given to the prevention of allergen cross-contact and cross-contamination and to maintaining traceability.

Contract manufacturers and contract laboratories should be evaluated by the contract giver to ensure cGMP compliance of the specific operations occurring at the contract sites.

A written and approved contract or quality agreement should define in detail the cGMP responsibilities, including the quality measures, of each party. The contract acceptor should permit the contract giver to audit the contract acceptor's facilities for compliance with cGMPs. When subcontracting is allowed, the contract acceptor should use a third party for any of the work entrusted to it under the contract without the contract giver's prior evaluation and approval of the arrangements.

Manufacturing and laboratory records should be kept at the site where the activity occurs and be readily available. Changes in the process, equipment, test methods, specifications, or other contractual requirements should not be made unless the contract giver is informed and approves the changes.

## 2.9 Complaints

All quality-related complaints, whether received orally or in writing, should be recorded and investigated according to written procedures.

Complaint records should include the following:

- Name, address, and phone number of complainant
- Name of dietary ingredient, item code, and lot number
- Nature of the complaint, including how the product was used if known
- Date complaint was received
- Investigation undertaken and initial findings, including dates and identity of person(s) conducting the investigation
- Action initially taken, including dates and identity of person(s) taking the action
- Any follow-up action taken
- Response provided to the complainant, including the date the response was sent
- Final findings and decision on product lot
- Quality unit personnel review and approval signature

A qualified person must review and investigate all product complaints to determine whether the product complaint involves a possible failure of a dietary ingredient to meet any of its specifications or any other requirements of this general chapter, including those specifications and other requirements that, if not met, may result in risk of illness or injury. Quality unit personnel must review and approve decisions about whether to investigate a product complaint and review and approve the findings and follow-up action of any investigation performed. The review of the product complaint and any investigation performed must extend to all relevant batches and records.

Records of complaints should be retained in order to evaluate trends, product-related frequencies, and severity with a view of taking additional and, if appropriate, immediate corrective action.

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## 2.10 Internal (Self-Inspection) Audits

To verify compliance with the requirements of this chapter, regular internal audits should be performed according to an approved schedule. Audit findings and corrective actions should be documented and brought to the attention of responsible management. Acceptable corrective action should be completed in a timely and effective manner. Internal audits also should verify that computer systems with an audit trail feature have it enabled.

## 2.11 Product Quality Reviews

Regular quality reviews of dietary ingredients should be conducted annually or by using a matrix and bracketing approach on a rotation cycle over a 3-year period with the objective of verifying the consistency of the manufacturing process.

Such review should normally include a review of the following:

- Critical dietary ingredient test results
- All batches that failed to meet established specification(s)
- Deviations or nonconformities and related investigations
- Adequacy of corrective actions
- Changes to the manufacturing process and analytical test methods
- Results of the stability monitoring program
- Quality-related returns, complaints, and recalls

The results of this review should be evaluated, and an assessment made of whether any additional corrective action or any revalidation should be undertaken. Reasons for such corrective actions should be documented. Acceptable corrective actions should be completed in a timely and effective manner.

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## 2.12 Quality Management Documentation

The following documentation must be made and kept for fulfilling the requirements of the quality management system (see *1. General Provisions*).

### 2.12.1 WRITTEN PROCEDURES

Written procedures must be established and followed for the following:

- Creating and maintaining job position descriptions and listing job responsibilities and required qualifications for education, training, and experience
- Implementing a training program
- Implementing a documentation and records control management system
- Implementing a change control program
- Implementing a deviation management program
- Conducting material reviews
- Implementing a corrective action and preventive action management program
- Implementing an FSP
- Implementing a supplier-chain control program
- Handling recalls
- Implementing a contract (manufacturing and laboratory) services program
- Implementing a complaint handling program
- Conducting internal audits
- Conducting product quality reviews

### 2.12.2 RECORDS

Records must meet the following standards:

- Be kept as original records, true copies (e.g., photocopies, pictures, scanned copies, microfilm, microfiche, or other accurate reproductions of the original records), or electronic records including associated metadata
- Contain the actual values and observations obtained during monitoring and, as appropriate, during verification activities
- Be accurate, indelible, and legible
- Be created concurrently with performance of the activity documented
- Be as detailed as necessary to provide history of work performed
- Include the following information:
  - Information adequate to identify the plant or facility (e.g., the name and when necessary the location of the plant or facility)
  - The date and, when appropriate, the time of the activity documented
  - The signature or initials of the person performing the activity
  - When appropriate, the identity of the product and the lot code

Records must be made and kept for the following:

- Organizational chart, job position descriptions, and curriculum vitae for all key management staff in manufacturing and quality operations
- Training, including the date of the training, the type of training, name of trainer if applicable, the person(s) trained, content of the training or reference to a document that describes the content, and any assessment results (e.g., test results)
- List and copies of current standard operating procedures, manufacturing instructions, and standard test methods
- List and reports of changes that affect the production and quality of dietary ingredients
- List and reports of unanticipated occurrences and deviations from approved instructions or established standards
- List and reports of material reviews
- List and reports of corrective actions and preventative actions taken
- List of approved suppliers of raw materials, other ingredients, packaging materials and labeling, and corresponding supplier qualification evaluation reports
- List of approved suppliers of manufacturing and laboratory services, including any provided service such as pest control and metrology, and of corresponding evaluation reports and related quality agreements
- List of complaints and corresponding investigation reports
- Recall and mock recall reports
- Internal (self-inspection) audit schedule and reports
- Product quality review reports

The owner, operator, or agent in charge of the facility must sign and date the FSP upon initial completion and upon any modification.

All records must be retained at the plant or facility for at least 2 years after the date they were prepared. Records that relate to the general adequacy of the equipment or processes being used by a facility, including the results of scientific studies and evaluations, must be retained by the facility for at least 2 years after their use is discontinued (e.g., because the facility has updated the written FSP or records that document validation of the written FSP).

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Except for the FSP, off-site storage of records is permitted if such records can be retrieved and provided on-site within 24 h of request for official review. The FSP must remain on-site, except when the plant or facility is closed for a prolonged period, in which case the FSP may be transferred to some other reasonably accessible location but must be returned to the plant or facility within 24 h for official review upon request. Electronic records are on-site if they are accessible from an on-site location.

Existing records (e.g., records kept to comply with other federal, state, or local regulations, or for any other reason) do not need to be duplicated if they contain all of the required information and satisfy applicable requirements. Existing records may be supplemented as necessary to include all required information and satisfy the applicable requirements. The information required does not need to be kept in one set of records. If existing records contain some of the required information, any new information required may be kept either separately or combined with the existing records.

# 3. PHYSICAL PLANT AND EQUIPMENT

## 3.1 Grounds Maintenance

The grounds around the physical plant must be kept in a condition that protects against the contamination of raw materials, other ingredients, in-process material, dietary ingredients, and food-contact surfaces. The methods for adequate ground maintenance include the following:

- Properly storing equipment, removing litter and waste, and cutting weeds or grass within the immediate vicinity of the physical plant so that it does not constitute an attractant, breeding place, or harborage for pests
- Maintaining roads, yards, and parking lots so that they do not become a source of contamination in areas where raw materials, other ingredients, in-process material, dietary ingredients, or food-contact surfaces are exposed
- Adequately draining areas that may contribute to the contamination of raw materials, other ingredients, in-process materials, dietary ingredients, or food-contact surfaces by seepage, filth, or any other extraneous materials, or by providing a breeding place for pests
- Adequately operating systems for waste treatment and disposal so that they do not constitute a source of contamination in areas where raw materials, other ingredients, in-process materials, dietary ingredients, or food-contact surfaces are exposed
- Exercising care if the physical plant grounds are bordered by grounds not under the operator's control and not maintained in the aforementioned manner, using inspection, extermination, or other means to exclude pests, dirt, filth, or any other extraneous materials that may be a source of contamination

## 3.2 Physical Plant Design

Any physical plant used in the manufacture of dietary ingredients must be of suitable size, construction, and design to facilitate maintenance, cleaning, and sanitizing operations. The physical plant must have adequate space for the orderly placement of equipment and holding of materials as is necessary for maintenance, cleaning, and sanitizing operations and to prevent contamination and mix-ups of materials.

The physical plant must take adequate precautions to reduce the potential for allergen cross-contact and for contamination of materials and food-contact surfaces with microorganisms, chemicals, filth, and other extraneous materials. The potential for allergen cross-contact and for contamination may be reduced by adequate food safety controls and operating practices or effective design, including the separation of operations in which allergen cross-contact and contamination are likely to occur, by one or more of the following means: location, time, partition, air flow systems, dust control systems, enclosed systems, or other effective means.

The flow of materials and personnel throughout the physical plant should be designed to prevent contamination and mix-ups of materials.

### 3.2.1 SEPARATE AND DEFINED AREAS

The physical plant must have and the manufacturer must use separate, defined areas of adequate size or other control systems, such as computerized inventory controls or automated systems of separation, physical and spatial to prevent contamination or allergen cross-contact and mix-ups of raw materials, other ingredients, in-process materials, dietary ingredients, and packaging materials and labels, during the following operations:

- Receiving, identifying, sampling, holding, and withholding from use materials to be used in manufacturing
- Separating materials to be used in manufacturing from materials awaiting material review and disposition (i.e., in quarantine)
- Performing production, packaging, labeling, and holding operations, as appropriate
- Physically separating allergen-containing materials from non-allergen-containing materials
- Holding rejected materials before further disposition (e.g., return, reprocessing, or destruction)
- Performing and separating the manufacturing, packaging, labeling, and holding of different product types, including different types of dietary ingredients and other foods, cosmetics, and pharmaceutical products
- Cleaning and sanitizing food-contact surfaces as necessary
- Performing and separating chemical and microbiological laboratory analyses and holding of laboratory samples and supplies

Laboratory areas/operations normally should be separated from production areas. Some laboratory areas, particularly those used for in-process controls, can be located in production areas, provided the operations of the production process do not

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adversely affect the accuracy of the laboratory measurements and the laboratory and its operations do not adversely affect the production process.

Dedicated production areas, which can include facilities, air-handling equipment, and/or process equipment should be considered when materials of an allergenic nature are involved, unless validated cleaning procedures are established and maintained.

#### 3.2.2 DESIGN AND CONSTRUCTION

The physical plant must be designed and constructed in a manner that prevents contamination of materials and contact surfaces by having the following:

- Floors, walls, and ceilings that can be adequately cleaned and kept in good repair
- Fixtures, ducts, and pipes that do not contaminate materials and contact surfaces by dripping or other leakage or by condensation
- Adequate ventilation or environmental control equipment such as airflow systems, including filters, fans, and other air-blowing equipment that minimize odors and vapors (including steam and noxious fumes) in areas where they may cause allergen cross-contact or contamination of materials or food-contact surfaces, and that are located and operated in a manner that minimizes the potential for allergens, microorganisms, and particulate matter to contaminate materials or food-contact surfaces
- Equipment that controls air pressure, temperature, and humidity as needed to ensure the quality of the dietary ingredient; attention should be given to areas where raw materials, other ingredients, in-process materials, and dietary ingredients are exposed to the environment
- Aisles or working spaces between equipment and walls that are adequately unobstructed and of adequate width to permit staff to perform their duties and to protect against contamination of materials or food-contact surfaces with clothing or personal contact
- Adequate lighting in all areas where material is examined, processed, or held, or where food-contact surfaces are cleaned, and in hand-washing areas, dressing and locker rooms, and bathrooms
- Safety-type light bulbs, fixtures, skylights, or other glass or glass-like materials when suspended over exposed materials in any step of manufacture
- Adequate screening or other protection against pests and insects, where necessary
- Adequate protection of materials in bulk fermentation vessels by using, for example, protective coverings; placement in areas where harborages for pests can be eliminated over and around the vessels and where staff can check regularly for pests, pest infestation, filth, or any other extraneous materials; or use of skimming equipment

### 3.3 Physical Plant Sanitation and Facilities

The physical plant must be kept clean and sanitary and maintained in repair adequate to prevent materials and contact surfaces from becoming contaminated. Cleaning and sanitizing of utensils and equipment must be conducted in a manner that protects against allergen cross-contact and against contamination of food, food-contact surfaces, or food-packaging materials.

Overall sanitation of the physical plant must be under the supervision of one or more competent individuals assigned responsibility for this function.

Written procedures should be established assigning responsibility for sanitation and describing the cleaning schedules, methods, equipment, and materials to be used in cleaning buildings and facilities.

The physical plant must be equipped with adequate sanitary facilities and accommodations including water supply, plumbing, sewage disposal, toilet facilities, hand-washing facilities, and rubbish and offal disposal.

Utilities that could affect dietary ingredient quality (e.g., steam, gases, compressed air, and heating, ventilation, and air conditioning) should be qualified and appropriately monitored and action should be taken when acceptance criteria are not met. Drawings for these utility systems should be made and available.

#### 3.3.1 CLEANING COMPOUNDS, SANITIZING AGENTS, PESTICIDES, AND OTHER TOXIC MATERIALS

Cleaning compounds and sanitizing agents must be free from undesirable microorganisms and must be safe and adequate under the conditions of use. All toxic materials should be purchased with a safety data sheet and supplier's guarantee or certification or with an examination of these substances for contamination. Toxic materials must not be used or held in the physical plant in which raw materials, other ingredients, in-process materials, dietary ingredients, and food-contact surfaces are manufactured and/or exposed, unless those materials are required to maintain clean and sanitary conditions, to conduct laboratory test procedures, to maintain or operate the physical plant or equipment, or for use in the physical plant's operations. Toxic materials must be identified and held in a manner that protects against contamination of raw materials, other ingredients, in-process materials, dietary ingredients, and food-contact surfaces. All relevant federal, state, and local government regulations for the use and holding of these toxic materials must be followed.

Any substances associated with the operation of equipment, such as lubricants, heating fluids, or coolants, should not contact in-process materials or dietary ingredients so as to alter their quality beyond established specifications. Any deviations from this should be evaluated to ensure that there are no detrimental effects upon the suitability for use of the material. When possible, food-grade lubricants and oils should be used.

#### 3.3.2 PEST CONTROL

No animals or pests must be allowed in any area of the physical plant. Guard, guide, or pest-detecting dogs may be allowed in some areas of a plant if the presence of the dogs is unlikely to result in the contamination of materials or food-contact surfaces.

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Effective measures must be taken to exclude pests from the physical plant and to protect against contamination of materials and food-contact surfaces on the premises by pests. The use of pesticides, such as insecticides, fumigants, fungicides, or rodenticides, is permitted only if precautions and restrictions are taken to protect against the contamination of raw materials, other ingredients, in-process materials, dietary ingredients, and food-contact surfaces. Pesticides should be registered and used in accordance with the Federal Insecticide, Fungicide, and Rodenticide Act (7 USC §136 et seq.).

### 3.3.3 SANITATION OF CONTACT SURFACES

All food-contact surfaces including utensils and equipment must be cleaned as frequently as necessary to protect against allergen cross-contact and against contamination of dietary ingredients.

Food-contact surfaces used for manufacturing/processing, packing, or holding low-moisture food must be in a clean, dry, and sanitary condition before use. When the surfaces are wet-cleaned, they must, when necessary, be sanitized and thoroughly dried before subsequent use.

In wet processing, when cleaning is necessary to protect against allergen cross-contact or the introduction of microorganisms into the dietary ingredient, all food-contact surfaces must be cleaned and sanitized before use and after any interruption during which food-contact surfaces may have become contaminated. When equipment and utensils are used in a continuous production operation, the utensils and food-contact surfaces of the equipment must be cleaned and sanitized, as necessary, to prevent contamination.

Single-service articles (e.g., utensils intended for one-time use, paper cups, and paper towels) must be stored, handled, and disposed of in a manner that protects against allergen cross-contact and against contamination of food and food-contact surfaces.

Non-food-contact surfaces of equipment used in the operation of the physical plant must be cleaned in a manner and as frequently as necessary to protect against allergen cross-contact and against contamination of food, food-contact surfaces, and food-packaging materials.

Cleaned and sanitized portable equipment with food-contact surfaces and utensils must be stored in a location and manner that protects food-contact surfaces from allergen cross-contact and contamination.

### 3.3.4 WATER SUPPLY

Potable water at a suitable temperature, and under pressure as needed, must be supplied in all areas where required for the processing of food; for cleaning of equipment, utensils, and food-packaging materials; or for staff sanitary facilities. The potable water must be supplied in a plumbing system free of defects and that protects against contamination or undesirable chemical reaction of raw materials, other ingredients, in-process materials, dietary ingredients, and food-contact surfaces. Any water that contacts food, food-contact surfaces, or food packaging materials must be safe and of adequate sanitary quality.

Potable water must meet, at minimum, all the requirements for drinking water promulgated in the US Environmental Protection Agency's National Primary Drinking Water Regulations (40 CFR Part 141), and any applicable state and local drinking water requirements that are more stringent. For manufacturers outside the United States, potable water meeting equivalent requirements may be acceptable with justification; for example, the drinking water regulations of the European Union (European Commission Directive 98/93/EC) or Japan Drinking Water Quality Standards. Water not meeting such requirements should not be permitted for use in the water purification system for *Purified Water*.

When water used in the manufacturing process is treated by the manufacturer to achieve a defined quality, the treatment process should be qualified and monitored with appropriate acceptance and action criteria.

### 3.3.5 PLUMBING

The plumbing in the physical plant must be of an adequate size and design and be adequately installed and maintained to do the following:

- Carry adequate amounts of water to the required locations throughout the physical plant
- Properly convey sewage and liquid disposable waste from the physical plant
- Avoid constituting a source of contamination to food, water supplies, and contact surfaces, or creating an unsanitary condition
- Provide adequate floor drainage in all areas where floors are subject to flooding-type cleaning or where normal operations release or discharge water or other liquid waste on the floor
- Not allow backflow from or cross connection between piping systems that discharge waste water or sewage and piping systems that carry water used for manufacturing dietary ingredients, for cleaning contact surfaces, or for use in bathrooms or hand-washing facilities

Permanently installed pipework should be appropriately identified. This can be accomplished by identifying individual lines with content and directional flow arrows, documentation of computer control systems, or alternative means.

### 3.3.6 SEWAGE AND TRASH DISPOSAL

Sewage, refuse, and other waste in and from buildings and the immediate surrounding area should be disposed of in a safe, timely, and sanitary manner. Sewage must be disposed of in an adequate sewage system or through other adequate means. Trash and hazardous waste must be conveyed, stored, and disposed of in a manner to minimize the development of odors; minimize the potential for the trash to attract, harbor, or become a breeding place for pests; and protect against contamination of materials, contact surfaces, water supplies, and grounds surrounding the physical plant. Containers and/or pipes for waste material should be clearly identified.

When raw materials, other ingredients, or refuse are unprotected, they must not be handled simultaneously in a receiving, loading, or shipping area if that handling could result in allergen cross-contact or contaminated food.

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#### 3.3.7 BATHROOMS AND HAND-WASHING FACILITIES

Adequate and readily accessible bathrooms and hand-washing facilities must be provided and kept clean and must not be a potential source of contamination of materials and contact surfaces. Hand-washing facilities must be designed to ensure that an employee's hands are not a source of contamination of materials and contact surfaces. The facilities must be designed to provide water at a suitable temperature, as well as soap and sanitizing agents, air driers, or single-service towels. Adequate facilities for showering and/or changing clothes should be provided, when appropriate.

#### 3.3.8 ALLERGEN CROSS-CONTAMINATION REDUCTION CONTROL

Adequate precautions must be taken to reduce the potential for allergen cross-contact of raw materials, other ingredients, in-process materials, dietary ingredients, and contact surfaces with adequate food safety controls and operating practices or effective design of the physical plant, including the separation of operations in which allergen cross-contact is likely to occur, by using one or more of the following means: location, time, partition, air-flow systems, dust-control systems, enclosed systems, or other effective means.

#### 3.3.9 SANITATION SUPERVISORS

One or more employees must be assigned to supervise overall sanitation and must be qualified by education, training, and/or experience to develop and supervise sanitation procedures, using appropriate risk-based assessments and preventative controls.

### 3.4 Equipment, Instruments, and Utensils

Equipment, instruments, and utensils used in the manufacture of dietary ingredients must be of appropriate design, construction, and workmanship to enable them to be suitable for their intended use, to be and suitably located to facilitate operations for their intended use, and to be adequately cleaned and properly maintained to protect against allergen cross-contact and contamination.

Equipment and utensils include but are not limited to the following: equipment used to hold or convey; equipment used to measure (i.e., instruments); equipment using compressed air or gas; equipment used to carry out processes in closed pipes and vessels.

Major equipment and permanently installed processing lines used during the production, packaging, and labeling of dietary ingredients should be appropriately identified. A set of current drawings and/or manuals should be maintained for equipment and critical installations (e.g., instrumentation and utility systems). Equipment should be identified as to its contents and its cleanliness status by appropriate means.

Equipment that is in areas where food is manufactured, processed, packed, or held and that does not come into contact with food must be so constructed that it can be kept in a clean and sanitary condition.

Holding, conveying, and manufacturing systems, including gravimetric, pneumatic, closed, and automated systems, must be of a design and construction that enables them to be maintained in an appropriate clean and sanitary condition.

#### 3.4.1 DESIGN AND CONSTRUCTION

Equipment and utensils must be appropriate in design and construction and used appropriately to do the following:

- Prevent contamination of dietary ingredient with lubricants, fuel, coolants, metal or glass fragments, filth or any other extraneous material, contaminated water, or any other contaminants
- Be corrosion-resistant and made of nontoxic materials if the equipment or utensils contact dietary ingredients
- Withstand the environment in which they are used in manufacturing, cleaning, and sanitizing
- Have seams that are smoothly bonded or maintained to minimize accumulation of dirt, filth, organic material, particles of dietary ingredient, or any other extraneous materials or contaminants, and thus minimize the opportunity for growth of microorganisms and allergen cross-contact
- Constructed so that surfaces that contact dietary ingredients are not corrosive, reactive, additive, or absorptive so as to alter the quality of the dietary ingredient beyond the established requirements
- Must be maintained to protect dietary ingredients from allergen cross-contact and from being contaminated by any source

Automated, mechanical, or electronic equipment that is used to manufacture, package, label, or hold a dietary ingredient must be designed or selected to ensure that the dietary ingredient specifications are consistently met. The suitability of the equipment must be determined by ensuring that the equipment is capable of operating satisfactorily within the operating limits required by the process.

#### 3.4.2 INSTALLATION AND MAINTENANCE

Equipment and utensils must be installed and/or maintained to facilitate cleaning the equipment and utensils and adjacent spaces and to protect dietary ingredients from being contaminated by any source. Equipment and utensils must be maintained in an adequate condition through appropriate cleaning and sanitizing, as necessary. Insofar as necessary, equipment must be taken apart for thorough cleaning.

Each freezer, refrigerator, and cold storage compartment used to store and hold dietary ingredients must do the following:

- Be fitted with an indicating thermometer, temperature-measuring device, or temperature-recording device that shows, indicates, and records or allows for recording by hand the temperature accurately within the compartment

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- Have an automated device for regulating temperature or an automated alarm system to indicate a significant temperature change in a manual operation

Compressed air or other gases introduced mechanically into or onto dietary ingredients or dietary ingredient-contact surfaces, or that are used to clean food-contact surfaces, must be treated in such a way (e.g., proper filtration) that food is not contaminated.

Instruments or controls used in the manufacturing, packaging, labeling, or holding of a dietary ingredient, and instrument controls used to measure, regulate, or record temperatures, pH, water activity, or other conditions and to control or prevent the growth of microorganisms or other contamination, must be accurate and precise, periodically calibrated, adequately maintained, and adequate in number for their designated uses.

### 3.4.3 OPERATION AND CALIBRATION

Control, weighing, measuring, monitoring and test equipment (i.e., instruments and controls), which are critical for assuring the quality of dietary ingredients, must be calibrated according to written procedures and an established schedule. Equipment and instruments used in the manufacture of dietary ingredients must be used only within its qualified operating range. Equipment calibrations should be performed using standards traceable to certified standards, if available.

Instruments and controls used in manufacturing or testing raw materials, other ingredients, in-process material, or dietary ingredients must be calibrated before first use at a frequency specified in writing by the manufacturer of the instrument or control and at established intervals or as otherwise necessary to ensure the accuracy and precision of the instrument and control. Instruments or controls that cannot be adjusted to agree with the reference standard must be repaired or replaced.

Balances used to accurately weigh material should comply with *Balances* ⟨41⟩ and *Weighing on an Analytical Balance* ⟨1251⟩.

Automated, mechanical, or electronic equipment that is used to manufacture, package, label, or hold a dietary ingredient must be routinely calibrated, inspected, or checked to ensure proper performance. The quality unit must approve these calibrations, inspections, or checks. The appropriate controls for automated, mechanical, and electronic equipment (including software for a computer-controlled process) must be established and used to ensure that any changes to the manufacturing, packaging, labeling, holding, or other operations are approved by the quality unit and instituted only by authorized personnel. The appropriate controls must be established and used to ensure that the equipment functions in accordance with its intended use. These controls must be approved by the QC unit.

Deviations from approved standards of calibration on critical instruments should be investigated to determine if these could have had an effect on the quality of in-process materials or dietary ingredients using the equipment since the last successful calibration.

Quality unit personnel must do the following:

- Review and approve all processes for calibrating equipment and instruments
- Periodically review all records for calibration of equipment, instruments, and controls
- Periodically review all records for calibrations, inspections, and checks of automated, mechanical, or electronic equipment
- Review and approve controls to ensure that automated, mechanical, or electronic equipment functions in accordance with its intended use

### 3.4.4 CLEANING AND MAINTENANCE

Equipment, utensils, and any other contact surfaces used to manufacture, package, label, or hold raw materials, other ingredients, in-process materials, or dietary ingredients must be maintained, cleaned, and sanitized as necessary to protect against allergen cross-contact and contamination of raw materials, other ingredients, in-process materials, and dietary ingredients. Equipment and utensils must be taken apart as necessary for thorough maintenance, cleaning, and sanitizing.

Schedules and procedures, including assignment of responsibility, should be established for the preventative maintenance of equipment.

Written procedures should be established for the cleaning of equipment and its subsequent release for use in the manufacture of dietary ingredients. Cleaning procedures should contain enough details to enable operators to clean each type of equipment in a reproducible and effective manner. These procedures should include the following:

- Assignment of responsibility for cleaning of equipment
- Cleaning and sanitizing schedules as needed
- Complete description of the methods and materials, including dilution of cleaning agents used to clean equipment
- Instructions, as needed, for disassembling and reassembling each part of equipment to ensure proper cleaning
- Instructions for the removal or obliteration of previous batch identification
- Instructions for the protection of clean equipment from contamination prior to use, and the maximum time that may elapse between completion of cleaning and use in manufacturing
- Inspection of equipment cleanliness immediately before use
- Establishment of the maximum time that may elapse between the completion of manufacturing processing and equipment cleaning

All contact surfaces must be cleaned and sanitized as necessary before use and after any interruption during which the contact surfaces may have become contaminated. In consecutive operations involving batches of different dietary ingredients, the contact surfaces must be adequately cleaned, and sanitized as needed, to prevent cross-contamination. In consecutive operations involving different batches of the same dietary ingredient, the contact surfaces must be inspected and adequately cleaned and sanitized as necessary to prevent build-up and carry-over of contaminants (e.g., objectionable levels of microorganism). Acceptance criteria for residues and the choice of cleaning procedures and cleaning agents should be defined and justified. The cleaning status of equipment should be stated on the equipment or in close proximity to the equipment.

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Surfaces that do not come into direct contact with raw materials, other ingredients, in-process materials, or dietary ingredients must be cleaned as frequently as necessary to protect against contaminating raw materials, other ingredients, in-process materials, or dietary ingredients.

Effective systems should be used to reduce the potential of non-food-contact surfaces from becoming sources of contamination, such as the use of floor sticky pads between different production areas and a captive shoe policy and program or shoe covers in high-risk areas of operation.

Heat-treated pallets (or plastic pallets) must be used in areas where raw materials, other ingredients, in-process materials, dietary ingredients, and contact surfaces are exposed. An effective pallet cleaning, maintenance, inspection, and management program must be established, documented, and followed.

Contact surfaces used for manufacturing or holding low-moisture raw materials, other ingredients, in-process materials or dietary ingredients must be in a clean, dry, and sanitary condition before use. When the surfaces are wet-cleaned, they must be sanitized, when necessary, and thoroughly dried before subsequent use.

If wet processing is used during manufacturing, all contact surfaces must be cleaned and sanitized as necessary to protect against the introduction of microorganisms into raw materials, other ingredients, in-process materials, or dietary ingredients. When cleaning and sanitizing is necessary, all contact surfaces must be cleaned and sanitized before use and after any interruption during which the contact surface may have become contaminated. In a continuous production operation or in consecutive operations involving different batches of the same dietary ingredient, the contact surfaces must be adequately cleaned and sanitized, as necessary.

Single-service articles (e.g., utensils intended for one-time use, paper cups, and paper towels) must be stored in appropriate containers and handled, dispensed, used, and disposed of in a manner that protects against contamination of raw materials, other ingredients, in-process materials, dietary ingredients, or any contact surface.

Cleaning compounds and sanitizing agents must be adequate for their intended use and safe under their conditions of use.

The portable equipment and utensils that have contact surfaces must be cleaned, sanitized, and stored in a location and manner that protects them from contamination.

### 3.4.5 EQUIPMENT AND INSTRUMENT QUALIFICATION

"Fitness for purpose" must be demonstrated for equipment and instruments. There are many ways of demonstrating that equipment and instruments meet fitness for purpose and are under control, and these can include qualification, calibration, and maintenance. Equipment or instrument qualification activities necessary to establish fitness for purpose may be grouped into four phases: design qualification (DQ), installation qualification (IQ), operational qualification (OQ), and performance qualification (PQ). In order to ensure fitness for purpose, an integrated approach based on a risk assessment should be undertaken.

A variety of equipment and instruments ranging from a simple apparatus to complex computerized systems is used in the dietary ingredient industry to manufacture, package, label, test, and hold dietary ingredients. A risk assessment enables the classification of equipment and instruments to determine the extent of qualification and actions needed to demonstrate fitness for purpose. Generally, the more complex the equipment or instrument or the higher the criticality of its operation, the greater the amount of work required to ensure fitness for purpose.

Requirements for the intended use should be appropriately defined to ensure that the particular needs and technical and operational requirements are met. Qualification activities are described in *Analytical Instrument Qualification* ⟨1058⟩.

When an equipment or instrument undergoes major repairs or modifications, it should be evaluated using change control. Relevant equipment qualification should be repeated to verify that the equipment continues to operate satisfactorily. If equipment is moved to another location, an assessment should be made of what, if any, qualification stage should be repeated.

### 3.4.6 COMPUTERIZED SYSTEMS

Written procedures should be available for the operation and maintenance of computerized systems.

Appropriate IQ and OQ should demonstrate the suitability of the computer hardware and software to perform assigned tasks.

cGMP-related computerized systems must be validated. The depth and scope of validation depends on the diversity, complexity, and criticality of the computerized application. Commercially available software that has been validated may require an abbreviated level of testing. If an existing system was not validated at time of installation, a retrospective validation could be conducted if appropriate documentation is available.

When critical data are being entered manually, an additional check on the accuracy of the entry must be done, either by a second operator or by the system itself (e.g., barcode reader).

Changes to the computerized system must be made according to a change control procedure and should be formally authorized, documented, and tested. Records must be kept of all changes, including modifications and enhancements made to hardware and software. These records should demonstrate that the system is maintained in a validated state.

If system breakdowns or failures would result in the permanent loss of records, a back-up system should be provided. A means of ensuring data protection should be established for all cGMP-related computerized systems. Data can be recorded by a second means in addition to the computer system. Test records should not be written to erasable media not covered by an audit trail, such as thumb drives.

All electronic records must comply with 21 CFR Part 11 (*Electronic Records; Electronic Signatures*).

**3.4.6.1 Computer systems procedures and controls:** cGMP-related computerized systems used to create, modify, maintain, or transmit electronic records must employ procedures and controls designed to ensure the authenticity, integrity, and, when appropriate, the confidentiality of electronic records, and to ensure that the signer cannot readily repudiate the signed record as not genuine. Such procedures and controls must include but are not limited to the following:

- Validation of systems to ensure accuracy, reliability, consistent intended performance, and the ability to discern invalid or altered records

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- The ability to generate accurate and complete copies of records (including controls to prevent omissions in data, such as the system being turned off and data not being captured) for inspection, review, and copying
- Protection of records to enable their accurate and ready retrieval throughout the records retention period
- Limiting system access to authorized individuals to prevent unauthorized access or changes to data
- Use of secure, computer-generated, and time-stamped audit trails to independently record the date and time of operator entries and actions that create, modify, or delete electronic records
- Establishment of and adherence to written policies that hold individuals accountable and responsible for actions initiated under their electronic signatures in order to deter record and signature falsification
- Use of appropriate controls over systems documentation including distribution of, access to, and use of documentation for system operation and maintenance, and revision and change control procedures to maintain an audit trail that documents time-sequenced development and modification of systems documentation

**3.4.6.2 Electronic signatures:** Signed electronic records must contain information associated with the signing that clearly indicates the following: the printed name of the signer, the date and time when the signature was executed, and the meaning (e.g., review, approval, responsibility, or authorship) associated with the signature.

Each electronic signature must be unique to one individual and must not be reused by or reassigned to anyone else. Individuals responsible for electronic signatures must have a specific identification code and password. Persons who used electronic signatures must employ controls to ensure their security and integrity.

## 3.5 Physical Plant and Equipment Documentation

The following documentation must be made and kept for fulfilling the requirements of the physical plant and equipment quality system (see *1. General Provisions*).

### 3.5.1 WRITTEN PROCEDURES

Written procedures must be established and followed for the following:

- Maintaining, cleaning, and sanitizing as necessary, the physical plant and facilities
- Pest control
- Maintaining, cleaning, and sanitizing as necessary, equipment, utensils, and any other contact surfaces that are used to manufacture package, label, or hold raw materials, other ingredients, in-process materials, and dietary ingredients
- Use of cleaning compounds, sanitizing agents, pesticides, and other toxic materials
- Pallet inspection
- Equipment and instrument qualification
- Calibrating instruments and controls used in manufacturing or testing a raw material, other ingredient, in-process material, or dietary ingredient
- Calibrating, inspecting, and checking automated, mechanical, and electronic equipment and instruments
- Operating and maintaining cGMP-related computerized systems

### 3.5.2 RECORDS

Records must be made and kept for the following:

- List of approved cleaning compounds, sanitizing agents, and corresponding safety data sheets
- Drawings for utility systems (e.g., water, steam, gases, compressed air, and heating, ventilation, and air conditioning)
- Physical plant and facility maintenance, cleaning, and sanitizing schedules, and logs of the date of maintenance and any cleaning and sanitizing unless such documentation is kept with the batch record
- Pest control
- Demonstrating that water, when used as a component of a dietary ingredient, meets all federal, state, and local standards of potability. Some intended uses of water in dietary ingredients may require the use of compendial grade *Purified water*
- List of manufacturing equipment and instrumentation
- Drawings and/or manuals for equipment and critical installations
- Documentation in individual equipment logs of the date of use, maintenance, cleaning, and sanitizing of equipment, unless such documentation is kept with the batch record
- Documentation of the controls used to ensure that equipment and instruments function in accordance with their intended use
- Documentation of any calibration and each time the calibration is performed for instruments and controls used in manufacturing or testing a raw material, other ingredient, in-process material, or dietary ingredient
    - The documentation must identify the instrument or control calibrated and date of its calibration, identify the reference standard used including the certification of accuracy of the reference standard and history of recertification of accuracy, identify the calibration method used including appropriate acceptance limits for accuracy and precision of instruments and controls when calibrating, provide the calibration reading(s) found, identify the recalibration method used and reading(s) found if accuracy and/or precision acceptance limits were not met, and initials of the person who performed the calibration or recalibration
- Documentation of pallet inspection and disposition
- Documentation of equipment and instrument qualification

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- Documentation of calibrations, inspections, and checks of automated, mechanical, and electronic equipment
- Back-up file(s) of current and outdated software, as necessary, for retrieval of records that are required to be kept, and of data entered into the computer system used to manufacture, package, label, and hold dietary ingredients

Back-up file(s) (e.g., a hard copy of data entered, tapes, compact disks, flash drives) must be an exact and complete record of the date entered and must be kept secure from alterations, inadvertent erasures, or loss.

# 4. MATERIALS MANAGEMENT OPERATIONS AND CONTROLS

The person(s) authorized to release raw materials, other ingredients, packaging materials, labeling, and dietary ingredients should be specified. No materials should be released or used before the satisfactory completion of evaluation by the quality unit(s) unless appropriate systems are in place to allow for such use (e.g., release under quarantine, see *4.5 Holding, Distribution, and Transportation*).

## 4.1 Establishing Material and In-Process Production Specifications

Specifications must be established for any point, step, or stage in the manufacturing process in which control is necessary to ensure the quality of the dietary ingredient and that the dietary ingredient is packaged and labeled as specified in the master manufacturing and packaging control record.

### 4.1.1 RAW MATERIAL AND OTHER INGREDIENT SPECIFICATIONS

Specifications must be established for each raw material and other ingredient that is used in the manufacture of a dietary ingredient to ensure that the specifications for its purity, strength, and composition are met. The specification for the raw materials and other ingredients should include test specifications for the following:

- Identity (i.e., identification)
- Purity, strength, or composition (i.e., content or assay)
- Limits on contaminants that may adulterate or may lead to adulteration of the finished batch of dietary ingredients
- Applicable specific tests (e.g., peroxide value, anisidine value for oil rancidity)
- Manufacturing processing quality tests (e.g., particle size, bulk density)

Starting materials and processing aids used to manufacture dietary ingredients should be of suitable quality to ensure that the finished dietary ingredient meets appropriate standards of identity, strength, purity, composition, and limits on contaminants.

Other ingredients used in manufacturing dietary ingredient preparations should meet *USP*, *NF*, or *Food Chemical Codex* standards. When such standards do not exist, other ingredients may be used in dietary ingredients if they have been shown to be of acceptable food grade quality using other suitable procedures.

Raw materials and other ingredients either must not contain levels of microorganisms that may render the food injurious to the health of humans, or must be pasteurized or otherwise treated during manufacturing operations so that they no longer contain levels that would cause the dietary ingredient to be adulterated.

Other ingredients may be added to dietary ingredients provided that the other ingredients comply with applicable regulatory requirements and do not interfere with the assay and tests prescribed for determining compliance with the dietary ingredient compendial specification.

Adequate documentation must be provided for the basis for why the raw material and other ingredient specifications will help ensure that the specifications are met for the identity, purity, strength, and composition of the dietary ingredients and for limits on those types of contamination that may adulterate or may lead to adulteration of the finished batch of the dietary ingredient.

Each raw material and other ingredient received from a supplier that is liable to contamination with microorganisms, filth, insect infestation, allergens, aflatoxins, other natural toxins, pesticides, organic impurities (e.g., residual solvents), inorganic impurities (e.g., elemental impurities), foreign substances, or other extraneous substances that may be a source of contamination that is objectionable in view of its intended use must be tested or examined against established specifications for such contamination. The following *USP* chapters should be consulted:

- *Residual Solvents* ⟨467⟩
- *Articles of Botanical Origin* ⟨561⟩, *Test for Aflatoxins* and *Pesticide Residue Analysis*
- *Microbial Enumeration Tests* ⟨2021⟩
- *Absence of Specified Microorganisms* ⟨2022⟩
- *Microbiological Attributes of Nonsterile Nutritional and Dietary Supplements* ⟨2023⟩
- *Elemental Contaminants in Dietary Supplements* ⟨2232⟩

Raw materials and other ingredients susceptible to contamination with aflatoxin or other natural toxins must comply with FDA regulations for poisonous or deleterious substances before these raw materials or other ingredients are incorporated into the dietary ingredient and ultimately finished food.

Raw materials, other ingredients, and rework susceptible to contamination with pests, undesirable microorganisms, or extraneous material must comply with applicable FDA regulations for natural or unavoidable defects if a manufacturer wishes to use the materials in manufacturing food.

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#### 4.1.2 IN-PROCESS PRODUCTION SPECIFICATIONS

Specifications must be established for in-process production for any point, step, or stage in the master production and control record in which control is necessary to help ensure that the specifications are met for the identity, purity, strength, and composition of the dietary ingredients and, as necessary, for limits on those types of contamination that may adulterate or may lead to adulteration of the finished batch of the dietary ingredient.

Adequate documentation must be provided for the basis for why meeting the in-process specifications, in combination with meeting raw material and other ingredient specifications, will help ensure that the specifications are met for the identity, purity, strength, and composition of the dietary ingredients and for limits on those types of contamination that may adulterate or may lead to adulteration of the finished batch of the dietary ingredient. Quality unit personnel must review and approve this documentation.

#### 4.1.3 LABELING AND PACKAGING MATERIAL SPECIFICATIONS

Specifications must be established for labeling (see *Labeling* ⟨7⟩) and packaging materials that come in contact with dietary ingredients (see *Packaging and Storage Requirements* ⟨659⟩, *Containers—Glass* ⟨660⟩, *Plastic Packaging Systems and Their Materials of Construction* ⟨661⟩, *Plastic Materials of Construction* ⟨661.1⟩, *Plastic Packaging Systems for Pharmaceutical Use* ⟨661.2⟩, *Auxiliary Packaging Components* ⟨670⟩, *Containers—Performance Testing* ⟨671⟩). Packaging that may come into contact with dietary ingredients must be safe and suitable for its intended use and must not be reactive or absorptive or otherwise affect the safety or quality of the dietary ingredient.

#### 4.1.4 IN-PROCESS DIETARY INGREDIENT SPECIFICATIONS

Specifications must be established for each dietary ingredient that is manufactured. The specification for the dietary ingredient should include test specifications for the following:

- Identity (i.e., identification) should be established through a validated method that has a high degree of specificity to differentiate between similar dietary ingredients
- Purity, strength, or composition (i.e., content or assay)
- Limits on contaminants that may adulterate or may lead to adulteration of the finished batch of dietary ingredients
- Applicable specific tests (e.g., peroxide value, anisidine value for oil rancidity)
- Applicable performance tests (e.g., particle size)
- Applicable manufacturing quality tests (e.g., content uniformity)

Dietary ingredients that have a *USP* monograph should comply with the monograph standard and other applicable standards in that compendium.

**4.1.4.1 Defect action levels:** Some dietary ingredients, even when produced under cGMPs, contain naturally occurring unavoidable defects that at low levels present no health hazard. The FDA set defect action levels because it is economically impractical to grow, harvest, or process raw products that are totally free of non-hazardous, naturally, and unavoidable defects. Products harmful to consumers are subject to regulatory action whether or not they exceed the defect action levels. The manufacturer, packer, and holder of a dietary ingredient must at all times utilize quality unit operations that reduce natural or unavoidable defects to the lowest level currently feasible. The mixing of a dietary ingredient containing defects at levels that render that dietary ingredient adulterated with another lot of dietary ingredient is not permitted and renders the final dietary ingredient adulterated, regardless of the defect action level of the final dietary ingredient.

#### 4.1.5 RECEIVED IN-PROCESS DIETARY INGREDIENT SPECIFICATIONS

Specifications must be established for an in-process dietary ingredient received from a supplier for packaging and labeling and for its distribution rather than for return to the supplier. The specification must provide sufficient assurance that the in-process dietary ingredient received is adequately identified and consistent with the purchase order or supplier quality agreement.

#### 4.1.6 FINISHED DIETARY INGREDIENT SPECIFICATIONS

Specifications must be established for the packaging and labeling of the finished packaged and labeled dietary ingredients that ensure the correct packaging and labeling was used.

### 4.2 Determining Compliance with Specifications

#### 4.2.1 GENERAL

Quality unit personnel must determine whether the specifications set for raw materials, other ingredients, in-process materials, packaging materials, labeling, in-process dietary ingredients, received in-process dietary ingredients, and finished dietary ingredients are met.

QC (laboratory control) unit personnel must conduct the necessary tests to determine whether the specifications for raw materials, other ingredients, in-process materials, and dietary ingredients comply with specifications. On the basis of adequate process verification, in-process controls, and statistical confidence, a reduced testing (or sampling) plan can be used as an alternative to fully testing of contaminants in every batch of material.

Tests and examinations that are used to determine whether material specifications are met must be appropriate for their intended use, and scientifically validated methods according to *Validation of Compendial Procedures* ⟨1225⟩.

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The QC unit must conduct or review and approve an OOS investigation prior to making a material use and disposition decision on any dietary ingredients. Corrective action plans must be developed and implemented when a dietary ingredient is confirmed to be out-of-specification and a root cause has been identified.

### 4.2.2 RAW MATERIALS AND OTHER INGREDIENTS

Before any raw material or other ingredient is used in manufacturing, at least one appropriate test or examination must be conducted to verify the identity of the raw material or other ingredient and determine whether other applicable raw material and other ingredient specifications are met by doing one of the following:

- Conduct appropriate tests or examinations for all of the test specifications
- Rely on the certificate of analysis from the supplier of the raw material and other ingredient received, provided the following:
  - The supplier is qualified by establishing the reliability of the supplier's certificate of analysis through confirmation of the results of the supplier's tests or examinations, on-site audit, and other appropriate supplier qualification activities
  - The certificate of analysis includes a description of the specific tests or examination methods used (i.e., specific reference to a test procedure that can be performed by an experienced scientist), acceptance criteria for the tests or examinations, and actual results of the tests or examinations
  - Documentation is maintained of how the supplier was qualified
  - The reliability of the supplier's certificate of analysis is periodically re-confirmed (i.e., requalified)
  - Quality unit personnel have reviewed and approved the documentation setting forth the basis for qualification and requalification of the supplier

### 4.2.3 IN-PROCESS PRODUCTION

In-process points, steps, or stages where control is necessary to ensure the quality of the finished batch of dietary ingredients must be monitored to determine whether in-process specifications are met and to detect any deviation or unanticipated occurrence that may result in the failure of the dietary ingredient to meet specifications.

### 4.2.4 DIETARY INGREDIENTS

For every batch of dietary ingredients, the manufacturer must verify that the batch meets product specifications for identity, purity, strength, and composition. Reduced testing (i.e., reduced sampling) of contaminants that may adulterate or may lead to adulteration of the dietary ingredient can be applied to batches that have been identified through a sound statistical sampling plan:

- Established specifications for limits on those types of contamination that may adulterate or that may lead to adulteration of the dietary ingredient would verify that the production process control system is producing a dietary ingredient that meets product specifications
- Appropriate tests or examinations must be conducted to determine compliance with the applicable specifications
- Adequate documentation must be provided of the basis for determining how compliance with reduced testing of contaminants will ensure that the finished batch of dietary ingredients will meet all product specifications for the limits on those types of contamination that may adulterate or that may lead to adulteration of the dietary ingredient
- Quality unit personnel must review and approve all supporting documentation for reduced testing

### 4.2.5 RECEIVED IN-PROCESS DIETARY INGREDIENT

A product received for packaging or labeling as a dietary ingredient and for distribution rather than for return to the supplier must be visually examined and documentation must be provided or obtained through testing and examination to determine whether the in-process product specifications are met.

### 4.2.6 PACKAGING MATERIAL AND LABELING

Before packaging material is used, a visual identification of the containers and closures must be conducted and the supplier's invoice, guarantee, or certification must be reviewed to determine whether the packaging specifications are met, at a minimum.

Before labeling is used, at minimum, a visual identification of the labeling must be conducted and the supplier's invoice, guarantee, or certification must be reviewed to determine whether the labeling specifications are met.

### 4.2.7 FINISHED DIETARY INGREDIENTS

At minimum, a visual examination of the packaging and labeling of the finished dietary ingredient must be conducted to determine whether the packaging used and the label applied was specified in the master packaging control record.

### 4.2.8 DISPOSITION DECISION FOR MATERIALS

For raw materials, other ingredients, in-process materials, packaging materials, labeling, and dietary ingredients that meet specifications, the quality unit may approve the material for use in manufacturing.

For raw materials, other ingredients, in-process materials, packaging materials, labeling, and dietary ingredients that do not meet specifications, the quality unit must reject the material for use in manufacturing, unless quality unit personnel approve a

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treatment, an in-process adjustment, or reprocessing that will ensure the quality of the finished dietary ingredient, and that the dietary ingredient is packaged and labeled as specified in the master packaging record.

For raw materials and other ingredients that do not meet specification(s) for identity (i.e., identification), QC personnel must reject the raw material or other ingredient, which must not be used in manufacturing the dietary ingredient.

For a dietary ingredient received from a supplier for packaging and/or labeling as a dietary ingredient and for distribution rather than for return to the supplier that does not meet specifications, QC personnel must reject the product, which may not be packaged or labeled for distribution as a dietary ingredient.

The quality unit personnel must not approve and release for distribution any batch or reprocessed batch of dietary ingredient in the following situations:

- When any component in the batch does not meet its identity specification
- When all product specifications are not met
- When a dietary ingredient has not been manufactured, packaged, labeled, and held under conditions to prevent adulteration
- When a dietary ingredient was received from a supplier for packaging or labeling as a dietary ingredient and for distribution rather than return to the supplier, but without sufficient assurance provided to adequately identify the product and to determine that the product is consistent with its purchase order

#### 4.2.9 TREATMENTS, IN-PROCESS ADJUSTMENTS, AND REPROCESSING

A rejected dietary ingredient must not be reprocessed, and a rejected component, packaging material, or labeling must not undergo treatment or in-process adjustment to make it suitable for use in the manufacture of a dietary ingredient, unless the following is done:

- Quality unit personnel conduct a material review and make a disposition decision that is based on a scientifically valid reason and approves the reprocessing, treatment, or in-process adjustment
- The reprocessing, treatment, or in-process adjustment will ensure the quality of the finished dietary ingredient and that the dietary ingredient is packaged and labeled as specified in the master production and control record
- All raw materials and other ingredients in the batch met its identity (i.e., identification) specification

For a rejected dietary ingredient received from a supplier for packaging and/or labeling as a dietary ingredient and for distribution rather than for return to the supplier that does not meet specifications, QC personnel must reject the dietary ingredient and the dietary ingredient may not be packaged or labeled for distribution as a dietary ingredient.

Any batch of dietary ingredient that is reprocessed, that contains raw materials and other ingredients that have been treated, or to which in-process adjustments have been made to make them suitable for use in the manufacture of the dietary ingredient must be approved by quality unit personnel before release for distribution.

Prior to approving any batch of dietary ingredient for reprocessing or an in-process adjustment, the quality unit should determine what tests should be performed to verify the effectiveness of the reprocessing/in-process adjustment to ensure that specifications have not been compromised.

Quality unit personnel must not approve and release for distribution any batch or reprocessed batch of dietary ingredient in the following situations:

- When any component in the batch does not meet its identity specification
- When all product specifications have not been met
- When the dietary ingredient has not been manufactured, packaged, labeled, and held under conditions to prevent adulteration
- When the dietary ingredient was received from a supplier for packaging or labeling as a dietary ingredient and for distribution rather than return to the supplier, but without sufficient assurance provided to adequately identify the product and to determine that the product is consistent with its purchase order

### 4.3 Receiving and Release of Materials

#### 4.3.1 RAW MATERIALS, OTHER INGREDIENTS, PACKAGING MATERIALS, AND LABELS

The person responsible for receiving raw materials, other ingredients, packaging materials, or labels must visually examine each immediate container or grouping of immediate containers in a shipment that is received for the appropriate content label, container damage, or broken seals to determine whether the container condition may have resulted in contamination or deterioration of the raw materials, other ingredients, packaging materials, or labels. The person must visually examine the supplier's invoice, guarantee, or certification in a shipment that is received to ensure the raw materials, other ingredients, packaging materials, and labels are consistent with the purchase order.

Raw materials received via bulk truck or railcar must include verification that the vehicle is dedicated to that service or that the vehicle has been properly cleaned and the prior load does not pose a risk of contamination.

Raw materials and other ingredients must be inspected and segregated or otherwise handled as necessary to ascertain that they are clean and suitable for processing into a dietary ingredient and must be stored under conditions that will protect against allergen cross-contact, against contamination, and minimize deterioration. Raw materials must be washed or cleaned as necessary to remove soil or other contamination. Water used for washing, rinsing, or conveying food must be safe and of adequate sanitary quality (i.e., potable water).

The raw materials, other ingredients, packaging materials, and labels must be placed in quarantine before they are used in the manufacture of a dietary ingredient until the following occurs:

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- Representative samples of each unique shipment of raw materials, other ingredients, packaging materials, and labels received are collected, as well as of each unique lot within each unique shipment of raw materials, other ingredients, packaging materials, and labels
- Quality unit personnel review and approve the results of any tests or examinations conducted on raw materials, other ingredients, packaging materials, and labels
- Quality unit personnel approve and release from quarantine the raw materials, other ingredients, packaging materials, and labels for use in the manufacture of a dietary ingredient, including approval of any treatment or in-process adjustments of raw materials, other ingredients, packaging materials, and labels to make them suitable for use in the manufacture of a dietary ingredient

Each unique lot within each unique shipment of raw materials, other ingredients, packaging materials, and labels that is received, and any lot of raw materials and other ingredients that are produced, must be identified in a manner that allows a person to trace the lot to the supplier by the date received; the name and status (e.g., quarantined, approved, or rejected) of the raw material, other ingredient, packaging material, and label; as well as to the dietary ingredient that was manufactured and distributed. This unique identifier must be used whenever recording the disposition of each unique lot within each unique shipment of raw materials, other ingredients, packaging materials, and labels that was received, and any lot of in-process material or dietary ingredient produced.

Raw materials, other ingredients, packaging materials, and labels must be held under conditions that will protect against contamination and deterioration and avoid mix ups.

#### 4.3.2 PRODUCT RECEIVED FOR PACKAGING AND LABELING AS A DIETARY INGREDIENT

The person responsible for receiving product for packaging and labeling as a dietary ingredient and for distribution rather than for return to the supplier must visually examine each immediate container or grouping of immediate containers in a shipment for appropriate content label, container damage, or broken seals to determine whether the container condition may have resulted in contamination or deterioration of the received product. That person must visually examine the supplier's invoice, guarantee, or certification in a shipment of received product to ensure the received product is consistent with the purchase order.

The received product must be placed in quarantine before it is used in the packaging and labeling of a dietary ingredient until the following occurs:

- Representative samples of each unique shipment of received product and of each unique lot within each unique shipment of received product are collected
- Quality unit personnel review and approve results of any tests or examinations conducted on the received product and review and approve the documentation supplied with the received product to determine whether it meets specifications
- Quality unit personnel approve the received product for packaging and labeling as a dietary ingredient and release the received product from quarantine

Each unique lot within each unique shipment of received product must be identified in a manner that allows a person to trace the lot to the supplier by the date received, the name of the received product, packaging material and label, the status of the received product (e.g., quarantined, approved, or rejected), as well as to the dietary ingredient that was packaged and labeled and distributed. This unique identifier must be used whenever recording the disposition of each unique lot within each unique shipment of received product.

Received product must be held under conditions that will protect against contamination and deterioration, and avoid mix-ups.

### 4.4 Representative Samples and Reserve Samples

#### 4.4.1 REPRESENTATIVE SAMPLES

Quality unit personnel must collect representative samples of the following:

- Each unique shipment and each unique lot within each unique shipment of raw materials, other ingredients, packaging material, and labels to be used in manufacturing to determine whether the material meet established specifications
- In-process materials for each manufactured batch at points, steps, or stages in the manufacturing process as specified in the master production and control record where control is necessary to ensure identity, purity, strength, and composition of dietary ingredients, to determine whether in-process materials meet specifications
- Each finished manufactured batch of dietary ingredient before releasing for distribution to verify that the finished batch of dietary ingredient meets specifications
- Each unique shipment and each unique lot within each unique shipment of product received for packaging or labeling as a dietary ingredient and for distribution rather than for return to the supplier to determine whether the received product meets established specifications
- Each lot of packaged and labeled dietary ingredients to determine whether the packaging used and the label applied was specified in the master packaging control record

The representative samples of dietary ingredients must be held in a suitable container–closure system that represents similar barrier properties to the container–closure system in which the dietary ingredient is ultimately sold to the user.

A sampling room or booth must be used to sample raw materials and other ingredients to protect material from allergen cross-contact and contamination.

Written procedures for sampling must be established and followed. The number of containers sampled and the amount of material to be sampled from each container should be based on appropriate criteria such as component variability, statistical

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confidence levels, degree of precision desired, the past quality history of the supplier, and the quantity of material needed for analysis and reserve, when required. Sampling procedures should include the following:

- Containers should be cleaned prior to opening
- Containers should be opened, sampled, and resealed in a manner designed to prevent contamination of their contents and contamination of other raw materials, other ingredients, product containers, or closures
- Containers should be identified so that the following information can be determined: name of the material sampled, the lot number, the container from which the sample was taken, the date on which the sample was taken, and the name of the person who collected the sample

Sampling of botanicals should be in compliance with the provisions in ⟨561⟩.

#### 4.4.2 RESERVE SAMPLES

Quality unit personnel must collect and hold reserve samples of each lot of packaged and labeled dietary ingredients that is distributed, which must be held using a container–closure system that provides essentially the same characteristics to protect against contamination or deterioration as the one in which it is distributed.

Reserve samples of dietary ingredients must be identified with the batch, lot, or control number. The reserve sample of each batch of dietary ingredients must consist of at least twice the quantity necessary for all tests or examinations to determine whether the dietary ingredient meets product specifications and must be collected and retained for at least 1 year past its retest or expiry date.

### 4.5 Holding, Distribution, and Transportation

Written procedures must be established and followed for holding or warehousing and distributing operations.

#### 4.5.1 HOLDING

Raw materials and other ingredients and dietary ingredients must be held under appropriate conditions of temperature, humidity, and light so that the identity, purity, strength, and composition of the raw materials and other ingredients and dietary ingredients are not affected. Raw materials and other ingredients, along with rework, must be held in bulk, or in containers designed and constructed so as to protect against contamination and must be held at such temperature and relative humidity and in such a manner as to prevent the food from becoming contaminated. Frozen raw materials and other ingredients must be kept frozen. If thawing is required prior to use, it must be done in a manner that prevents the raw materials and other ingredients from becoming contaminated. Liquid or dry raw materials and other ingredients received and stored in bulk form must be held in a manner that protects against allergen cross-contact and against contamination. Raw materials and other ingredients that are food allergens and rework that contains food allergens must be identified and held in a manner that prevents allergen cross-contact.

Storage of dietary ingredients must be under conditions that will protect against contamination, as well as the deterioration of the dietary ingredient and the container.

Packaging materials and labeling must be held under appropriate conditions so that they are not adversely affected. Raw materials, other ingredients, dietary ingredients, packaging materials, and labeling must be held under conditions that do not lead to the mix-up or contamination of raw materials, other ingredients, dietary ingredients, packaging materials, and labeling.

In-process material must be identified and held under appropriate conditions that protect against mix-up and contamination. In-process material must be held under appropriate conditions of temperature, humidity, and light to protect against deterioration.

Raw materials and other ingredients and in-process materials that contain food allergens must be clearly identified and held in a manner that prevents allergen cross-contact.

Reserve samples of dietary ingredients must be held in a suitable container–closure system that represents similar barrier properties to the container–closure system in which the dietary ingredient is ultimately sold to the user.

Reserve samples of dietary ingredients must be held in a manner that protects against contamination and deterioration. Reserve samples of dietary ingredients must be held under conditions consistent with the label conditions stated on the product labels. If no storage conditions are recommended on the label (e.g., for in-process dietary ingredients), they must be held under ordinary storage conditions consistent with expected storage conditions for the finished product. Reserve samples of dietary ingredients must be held using the same container–closure system in which packaged and labeled dietary ingredients are distributed. If distributing dietary ingredients that are to be packaged and labeled, a container–closure system that provides essentially the same characteristics to protect against contamination or deterioration must be used.

Reserve samples must be retained for 1 year past the retest or expiry date of the dietary ingredient for use in appropriate investigations.

#### 4.5.2 REJECTED MATERIALS

Any raw material, other ingredients, packaging material, labels, and products received for packaging and labeling as a dietary ingredient and for distribution rather than return to the supplier that is rejected and is unsuitable for use in manufacturing, packaging, and labeling operations must be clearly identified, held, and controlled under a quarantine system for appropriate disposition. The quarantine system for rejected materials must include physical segregation and conspicuous labeling of the material, in addition to any electronic controls.

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#### 4.5.3 RETURNED DIETARY INGREDIENTS

Returned dietary ingredients must be identified and quarantined until quality unit personnel conduct a material review and make a disposition decision.

Returned dietary ingredients must be destroyed or otherwise suitably disposed of unless the outcome of a material review and disposition decision is that QC personnel do one of the following:

- Approve the salvage of the returned dietary ingredient for redistribution
- Approve the returned dietary ingredient for reprocessing

A returned dietary ingredient may be salvaged only if QC personnel conduct a material review and make a disposition decision to allow the salvage.

Any returned dietary ingredients that are reprocessed must meet all dietary ingredient specifications, and QC personnel must approve or reject the release for distribution any returned dietary ingredient that is reprocessed.

If the reason for a dietary ingredient being returned implicates other batches, an investigation of the manufacturing processes and each of those other batches must be conducted to determine compliance with specifications.

#### 4.5.4 DISTRIBUTION

Dietary ingredients must be distributed under conditions that will protect the dietary ingredient against contamination and deterioration. The first manufactured batch of a dietary ingredient should be distributed first. Distributing operations must be designed to facilitate recall of dietary ingredients, if necessary.

#### 4.5.5 TRANSPORTATION OPERATIONS

All transportation operations must be conducted under such conditions and controls necessary to prevent dietary ingredients from becoming adulterated and to protect against deterioration of the dietary ingredient and the container during transportation.

Vehicles and transportation equipment used in transportation operations must meet the following standards:

- Adequately designed and maintained in a sanitary condition for their intended use to prevent raw materials and other ingredients and dietary ingredients from becoming adulterated during transportation operations
- Designed, maintained, and equipped as necessary to provide adequate temperature control to prevent raw materials and other ingredients and dietary ingredients requiring temperature control from becoming adulterated during transportation
- Stored in a manner that prevents it from harboring pests or becoming contaminated in any other manner that could result in raw materials and other ingredients or dietary ingredients for which it will be used becoming adulterated during transportation operations

Responsibility for ensuring that transportation operations are carried out adequately must be assigned to competent supervisory personnel.

Shippers, receivers, loaders, and carriers engaged in transportation must conduct all transportation operations under such conditions and controls necessary to prevent raw materials and other ingredients and dietary ingredients from becoming unsafe during transportation. Such operations include taking the following effective measures:

- Segregation, isolation, or the use of packaging to protect raw materials and other ingredients and dietary ingredients from contamination from other articles in the same load
- Ensure that raw materials and other ingredients and dietary ingredients that require temperature control for safety and quality are transported under adequate temperature control
- Restrict the co-shipment with a non-food item, which might render the raw materials and dietary ingredients to become adulterated through cross-contamination

Shippers must specify to the carrier and the loader all necessary sanitary specifications for the carrier's vehicle and transportation equipment including any specific design specifications (e.g., temperature control) and cleaning procedures.

Considering specifications provided by the shipper when appropriate, loaders must determine if the vehicle or transportation equipment is in appropriate sanitary and operational condition for the transport of raw materials and other ingredients and dietary ingredients.

Upon receipt of raw materials and other ingredients and dietary ingredients, receivers must take steps to adequately assess whether the raw materials and other ingredients and dietary ingredients were not subjected to adverse conditions that may have caused them to become adulterated.

Carriers must ensure that vehicles and transportation equipment meet the shipper's specifications and are otherwise appropriate to prevent raw materials and other ingredients and dietary ingredients from becoming adulterated during transportation operations.

Measures to implement requirements and procedures may be accomplished by the shipper or the carrier or another party involved in transportation operations under a written agreement.

Records should be maintained regarding specifications, agreements, procedures, associated records related to transportation (maintenance, cleaning, previous cargoes, temperature control), and training.

### 4.6 Materials Management Operations and Control Documentation

The following documentation must be made and kept for fulfilling the requirements of the material management controls quality system (see *1. General Provisions*).

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#### 4.6.1 WRITTEN PROCEDURES

Written procedures must be established and followed for the following:

- Preparing, reviewing, and approving material and in-process production specifications
- Supply chain control program
- Material receiving, sampling, testing, and making a disposition decision for materials to be used in manufacturing
- Warehousing maintenance, cleaning, and inventory controls
- Pallet inspection, cleaning, and maintenance
- Implementing a supply-chain control program
- Holding and distributing operations
- Receiving and conducting material reviews and making disposition decisions for returned dietary ingredients

#### 4.6.2 RECORDS

Records must be made and kept for the following:

- Specifications for raw materials, other ingredients, in-process production, packaging materials, labeling, in-process dietary ingredients, received in-process dietary ingredients, and finished dietary ingredients
- List of approved suppliers of raw materials, other ingredients, packaging materials and labeling, and corresponding supplier qualification evaluation reports for the purpose of relying on the supplier's certificate of analysis
- Documentation explaining why meeting in-process specifications, in combination with meeting component specifications, help to ensure that the dietary ingredient meets the specifications for identity, purity, strength, and composition, as well as for limits on those types of contamination that may adulterate or may lead to adulteration of the finished batch of the dietary ingredient
- Documentation explaining why the results of appropriate reduced tests or examinations for dietary ingredients ensure that the dietary ingredient meets all product specifications
- Receiving records (e.g., certificates of analysis, supplier's invoice, and supplier's guarantee) for materials
- Inventory control records documenting material history of use
- Documentation at the time of performance that demonstrates that QC unit personnel determined whether the specifications set for materials to be used in manufacturing were met (i.e, performed the required tests and made a disposition decision for materials)
- Dietary ingredient distribution
- Documentation of any material review and disposition decisions on a returned dietary ingredient
- The results of any testing or examination of returned dietary ingredients to determine compliance with product specifications
- Documentation of the re-evaluation by quality unit personnel of any dietary ingredient that is reprocessed and the determination by quality unit personnel of whether the reprocessed dietary ingredient meets in-process dietary ingredient specifications

## 5. PRODUCTION OPERATIONS AND CONTROLS

All dietary ingredient manufacturing, processing, packing, and holding must be conducted under such conditions and controls that are necessary to minimize the potential for the growth of microorganisms, allergen cross-contact, contamination of food, and deterioration of dietary ingredient. Equipment, containers, and utensils used to convey, hold, or store raw materials, other ingredients, work-in-process, rework, or dietary ingredients must be constructed, handled, and maintained during manufacturing, processing, packing, and holding in a manner that protects against allergen cross-contact and contamination.

### 5.1 Master Production Records

Written master production records must be prepared and followed for each unique formulation of dietary ingredients manufactured, and for each batch size to ensure batch-to-batch uniformity in the finished dietary ingredient. The master production record for each in-process material (i.e., intermediate) and dietary ingredient should be prepared, dated, and signed by one person in production, and then independently checked, dated, and signed by a person in the quality unit.

The master production record may include all manufacturing, packaging, and labeling directions and controls, or it may be separated into two records: one for manufacturing and another for packaging and labeling.

The master production record must do the following:

- Identify in-process production specifications for the points, steps, or stages in the manufacturing process where control is necessary to ensure the quality of the dietary ingredient and that the dietary ingredient is packaged and labeled as specified in the master production and control record
- Establish controls and procedures to ensure that each batch of dietary ingredient that is manufactured meets in-process specifications

The master production record must include the following:

- The name of the intermediate (i.e., in-process material) or dietary ingredient to be manufactured and an identifying document reference code
- The strength, concentration, weight, or measure of each raw material and other ingredient for each batch size

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- A complete list of raw materials and other ingredients to be used, designated by names and codes specific to identify any special quality characteristics
- An accurate statement of the weight or measure of each raw material and other ingredient to be used
  - When the quantity is not fixed, the calculation for each batch size or rate of production should be included
  - Variations to quantities should be included where they are justified
- The identity and weight or measure of each other ingredient and dietary ingredient that will be declared on the finished dietary ingredient (preparation) list of ingredients
- Detailed production instructions, including the following:
  - Sequences to be followed
  - Ranges of process parameters to be used
  - Sampling instructions and in-process controls with their acceptance criteria, when appropriate
  - Time limits for completion of individual processing steps and/or the total process, when appropriate
  - Expected yield ranges at appropriate phases of processing or time
- A statement of theoretical yield of a manufactured intermediate or dietary ingredient (preparation) expected at each point, step, or stage of the manufacturing process where control is needed to ensure the quality of the dietary ingredient, and of the expected yield at the end of manufacturing the dietary ingredient, including the maximum and minimum percentages of theoretical yield beyond which a deviation investigation of a batch is necessary and a material review is conducted and disposition is made
- Where appropriate, special notations and precautions to be followed, or cross-references to them
- The instructions for storage of the intermediate or finished dietary ingredient to assure its suitability for use, including a description of packaging materials and a representative label, or cross-reference to the physical location of the actual or representative label, and special storage conditions with time limits, when appropriate

The master production record must also include written instructions, including the following:

- Specifications for each point, step, or stage in the manufacturing process where control is necessary to ensure the quality of the dietary ingredient and that the dietary ingredient is packaged and labeled as specified in the master production and control record
- Procedures for sampling and cross-reference to procedures for tests and examinations
- Specific actions necessary to perform and verify points, steps, or stages in the manufacturing process where control is necessary to ensure the quality of the dietary ingredient and that the dietary ingredient is packaged and labeled as specified in the master production and control record
  - Such specific actions must include verifying the weight or measure of any raw material and other ingredient and verifying the addition of any raw material and other ingredient
  - For manual operations, such specific actions must include the following:
    - One person weighing or measuring a raw material and other ingredient and another person verifying the weight or measure
    - One person adding the raw material and other ingredient and another person verifying the addition
- Special notations and precautions to be followed
- Corrective action plans for use when a specification is not met

## 5.2 Executed Batch Production Records

An executed batch production record (paper or electronic) must be prepared or generated every time a batch of dietary ingredients is manufactured, and it must include complete information relating to the production and control of each batch. The executed batch production and control record must accurately follow the appropriate master production and control record, and each step in the production of the batch must be performed.

The executed batch production record must include the following:

- The batch, lot, or control number of the dietary ingredient, of each lot of packaged and labeled dietary ingredient from a finished batch of dietary ingredient, and of each lot of dietary ingredient from the finished batch of dietary ingredient that is distributed to another person for packaging or labeling
- The identity of equipment and processing lines used in producing the batch (exceptions can be noted when equipment is dedicated or the identity of the product or processing is self-evident)
- The date and time of the maintenance, cleaning, and sanitizing of the equipment and processing lines used in producing the batch, or a cross-reference to records, such as individual equipment logs, where this information is retained
- The unique identifier that was assigned to each raw material and other ingredient used or, when applicable, to a used product that was received from a supplier for packaging and labeling as a dietary ingredient, packaging, and labeling
- The identity and weight or measure of each raw material and other ingredient used
- A statement of the actual yield and a statement of the percentage of theoretical yield at appropriate phases of processing
- The actual results obtained during any monitoring operation
- The results of any testing or examination performed during the batch production, or a cross-reference to such results
- Documentation that the finished dietary ingredient meets the in-process dietary ingredient and finished dietary ingredient specifications

The executed batch production record must also include documentation at the time of performance of the manufacture of the batch, including the following:

- The date on which each step of the master production and control record was performed

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- The initials of the person performing each step, including initials of the persons responsible for the following:
  - Weighing or measuring each raw material and other ingredient used in the batch
  - Verifying the weight or measure of each raw material and other ingredient used in the batch
  - Adding the raw material and other ingredient to the batch
  - Verifying the addition of raw materials and other ingredients to the batch
- Documentation at the time of performance of packaging and labeling operations, including the following:
  - The unique identifier that was assigned to packaging and labels used, the quantity of the packaging and labels used, and, when label reconciliation is required, reconciliation of any discrepancies between issuance and use of labels
  - An actual or representative label, or a cross-reference to the physical location of the actual or representative label specified in the master manufacturing (or packaging and labeling) record
  - The results of any tests or examinations conducted on a packaged and labeled dietary ingredient, including repackaged or relabeled dietary ingredients, or a cross-reference to the physical location of such results
- Documentation at the time of performance that quality unit personnel did the following:
  - Reviewed the batch production record, including reviewing any monitoring operation required for determining compliance to material specifications and reviewing the results of any tests and examinations, including tests and examinations conducted on raw materials, other ingredients, in-process materials, finished batches of dietary ingredients, and packaged and labeled dietary ingredients
  - Approved or rejected any reprocessing or repackaging
  - Approved and released or rejected the batch for distribution, including any reprocessed batch
  - Approved and released or rejected the packaged and labeled dietary ingredient, including a repackaged or relabeled dietary ingredient
- Documentation at the time of performance of any required material review and disposition decision
- Documentation at the time of performance of any reprocessing

## 5.3 Manufacturing Operations Production and Process Controls

Written procedures must be established and followed for manufacturing operations. Manufacturing processes must be designed and selected to ensure that dietary ingredient specifications are consistently met.

All manufacturing operations must be conducted in accordance with adequate sanitation principles.

All necessary precautions must be taken during the manufacture of a dietary ingredient to prevent contamination of raw materials and other ingredients or dietary ingredients. These precautions include the following:

- Performing manufacturing operations under conditions and controls that protect against the potential for growth of microorganisms and the potential for allergen cross-contact and contamination
- Protecting dietary ingredients from contaminants that may drip, drain, or be drawn into the dietary ingredient
- Washing and cleaning raw materials and other ingredients that contain soil and other contaminants
- Using water that, at a minimum, complies with the applicable federal, state, and local requirements and does not contaminate the dietary ingredient when the water may be used as a raw material or may become another ingredient of the finished batch of dietary ingredient
- Performing chemical, microbiological, or other testing as necessary to prevent the use of contaminated raw materials and other ingredients
- Sterilizing, pasteurizing, freezing, refrigerating, controlling hydrogen-ion concentration (pH), controlling humidity, controlling water activity (aw), or using any other effective means to remove, destroy, or prevent the growth of microorganisms and prevent decomposition
- Washing, cutting, sorting and inspecting, mashing, dewatering, cooling, shredding, extruding, and drying performed in a manner that protects dietary ingredients against allergen cross-contact and against contamination
- Heat blanching when required in the preparation of dietary ingredients capable of supporting microbial growth, effected by heating the food to the required temperature, holding it at this temperature for the required time, and then either rapidly cooling the food or passing it to subsequent manufacturing without delay
  - Growth and contamination by thermophilic microorganisms in blanchers must be minimized by the use of adequate operating temperatures and by periodic cleaning and sanitizing, as necessary
- Treating or maintaining solutions and other similar preparations held and used repeatedly over time in such a manner that they are protected against allergen cross-contact and against contamination, and minimizing the potential for the growth of undesirable microorganisms
- Holding food at risk for the rapid growth of microorganisms of public health significance in a manner that prevents the food from becoming adulterated, such as keeping it at acceptable temperatures
- Processing and maintaining food that relies principally on the control of water activity for preventing the growth of undesirable microorganisms at a safe moisture level
- Monitoring and maintaining food that relies principally on the control of pH for preventing the growth of undesirable microorganisms at or below a pH of 4.6
- Identifying and holding any raw materials and other ingredients or dietary ingredients for which a material review and disposition decision is required and doing so in a manner that protects raw materials and other ingredients or dietary ingredients not under a material review against contamination and mix-ups with those that are under a material review

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- Performing mechanical manufacturing steps (e.g., washing, peeling, trimming, cutting, sorting, inspecting, mashing, shredding, drying, grinding, blending, and sifting) by any effective means to protect the dietary ingredients against contamination, such as the following examples:
  - Cleaning and sanitizing contact surfaces
  - Using temperature controls
  - Using time controls
- Using effective measures to protect against the inclusion of metal or other foreign material in raw materials and other ingredients or dietary ingredients, such as filters or strainers, traps, magnets, or electronic metal detectors
- Segregating and identifying all containers for a specific batch of dietary ingredients to identify their contents and, when necessary, the phase of manufacturing
- Identifying all processing lines and major equipment used during manufacturing to indicate their contents, including the name of the dietary ingredient and the specific batch or lot number and, when necessary, the phase of manufacturing
- Protecting dietary ingredients transported by conveyor against allergen cross-contact and against contamination as necessary

## 5.4 Quality Unit Responsibilities for Production

Quality unit personnel must do the following:

- Review and approve all master production and control records and all modifications to them
- Review and approve all master packaging and labeling records (if separate from master production and control records) and all modifications to them
- Review and approve all batch production-related records
- Review all monitoring required for determining compliance to material specifications
- Conduct any material review and make any required disposition decisions
- Approve or reject any treatments, in-process adjustments, and reprocessing
- Determine whether all in-process production specifications are met
- Determine whether each finished batch of dietary ingredients conforms to its specifications
- Make a disposition decision (i.e., approving and releasing, or rejecting) each finished batch of dietary ingredients, including any reprocessed finished batch of dietary ingredients, for distribution

## 5.5 Disposition Decision for Production

For ingredients that meet specifications and that were manufactured, packaged, labeled, and held under conditions to ensure the quality of the dietary ingredient, the quality unit may approve and release the batch of dietary ingredients for distribution.

The quality unit personnel must not approve and release for distribution any batch or reprocessed batch of dietary ingredient in the following situations:

- Any raw material and other ingredient in the batch does not meet its identity specification
- It does not meet all product specifications
- It has not been manufactured, packaged, labeled, and held under conditions to prevent adulteration
- It was received from a supplier for packaging or labeling as a dietary ingredient and for distribution rather than return to the supplier, but without sufficient assurance to adequately identify the product and to determine that the product is consistent with its purchase order

For dietary ingredients, raw materials, and other ingredients that are adulterated, one of the following must be done:

- Disposed of in a manner that protects against the contamination of other ingredients
- If the adulterated dietary ingredients, raw materials, and other ingredients are capable of being reconditioned, one of the following must be done:
  - Recondition (if appropriate) using a method that has been proven to be effective; or
  - Recondition (if appropriate) and re-examine to find if it is not adulterated before being incorporated into other ingredients

## 5.6 Production Operations and Controls Documentation

The following documentation must be made and kept for fulfilling the requirements of the production operations and controls quality system (see *1. General Provisions*).

### 5.6.1 WRITTEN PROCEDURES

Written procedures for manufacturing operations must be established and followed for the following:

- Preparation and approval of master production and control records
- Cleaning and inspecting manufacturing facilities
- Manufacturing production operations
- Quality unit responsibilities and operations

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#### 5.6.2 RECORDS

Records must be made and kept for manufacturing operations, including the following:

- Executed batch production and control records
- Equipment and production suite cleaning record
- Quality unit operations

# 6. PACKAGING AND LABELING OPERATIONS AND CONTROLS

## 6.1 General

Written procedures must be established and designed to ensure that correct packaging materials and labels are used. Packaging and labeling operations must be designed to prevent mix-ups.

Written procedures should describe the receipt, identification, quarantine, sampling, examination and/or testing and release, and handling of packaging and labeling materials.

Packaging and labeling material should conform to established specifications. Food-packaging materials must be safe and suitable. Those that do not comply with such specifications should be rejected to prevent their use in operations for which they are unsuitable.

Records should be maintained for each shipment of labels and packaging materials that show receipt, examination, or testing, as well as whether the shipment accepted or rejected.

## 6.2 Packaging Materials

#### 6.2.1 GENERAL PROCEDURES

Necessary actions must be taken to determine whether packaging for dietary ingredients meets specifications so that the condition of the packaging will ensure the quality of the dietary ingredients. Containers must provide adequate protection against any deterioration or contamination of the dietary ingredient that may occur during transportation and recommended storage. Food containers must be maintained in an adequate condition through appropriate cleaning and sanitizing, as necessary.

General chapter ⟨659⟩ provides packaging definitions, auxiliary packaging information, and storage condition definitions relevant to the storage and distribution of dietary ingredients, and it should be consulted.

Containers must be clean and, where indicated by the nature of the product, sanitized to ensure that they are suitable for their intended use. These containers must not be reactive, additive, or absorptive so as to alter the quality of the dietary ingredient beyond specified limits.

If containers (e.g., for raw materials and other ingredients, in-process materials, or bulk material) are reused, they should be cleaned in accordance with documented procedures, and all previous labels should be removed or defaced.

#### 6.2.2 TAMPER-EVIDENT PACKAGING

Dietary ingredients should use tamper-evident packaging. A tamper-evident package is one that has an indicator or barrier to entry that, if breached or missing, can reasonably be expected to provide visible evidence to the customer (i.e., dietary supplement manufacturer) that tampering has occurred. To reduce the likelihood of substitution of a tamper-evident feature after tampering, the indicator or barrier to entry must be distinctive by design or by the use of an identifying characteristic (e.g., a pattern, name, registered trademark, logo, or picture). The term “distinctive by design” means that the packaging cannot be duplicated with commonly available materials or through commonly available processes. A tamper-evident package may involve a primary container–closure system, a secondary container or carton system, or any combination of systems intended to provide a visual indication of package integrity. The tamper-evident feature should be designed to remain intact when handled in a reasonable manner during holding, manufacture, and distribution.

## 6.3 Labeling

#### 6.3.1 GENERAL PROCEDURES

Labeling must comply with applicable federal and state labeling regulations for dietary ingredients.

Quantitative label claims for the product must be truthful and accurately reflect the contents of the declared dietary ingredients and/or their marker compounds. There are two classes for label claims:

- Class I (standardized): fortified or fabricated nutrients whose contents have been controlled in some fashion; must meet 100% of the quantitative label claim
- Class II (non-standardized): naturally occurring nutrients, whose constituents occur naturally in an ingredient and whose level is not controlled; must meet at least 80% of the quantitative label claim

Reference to the *USP–NF* on the label or labeling must be appropriate and completely accurate in accordance with *General Notices, 3.20 Indicating Conformance*. When the dietary ingredient monograph title appears on the label, the product must fully conform to the specified compendial standard.

Labeling must comply with applicable federal and state labeling regulations for dietary ingredients.

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For botanicals, the common or usual name and/or the Latin binomial of the botanical species must be listed on the label. The current edition of the American Herbal Product Association's *Herbs of Commerce* should be consulted regarding the appropriate common or usual name of a botanical ingredient. Additionally, the proper Latin binomial name should be provided in parenthesis on the label, except that this name is not required when it is synonymous with the common or usual name for the botanical species. The label must include the plant part(s) used. The terms "whole", "root", "seed", "aerial", and "leaf" are all acceptable terminology.

Labeling for dietary ingredients must comply with applicable allergen labeling requirements as promulgated in the Food Allergen Labeling and Consumer Protection Act (FALCPA).

The label must include a statement of the necessary storage requirements for the dietary ingredient, and it must accurately state the country of origin for any product of foreign origin imported into the United States. The name and place (city, state, zip code) of business of the manufacturer, packer, or distributor also must be located on the label.

#### 6.3.2 RETEST OR EXPIRY DATE

Dietary ingredient labeling must state a retest date or expiry date that is indicative of the date after which the dietary ingredient can no longer meet applicable specifications of identity, strength, quality, and purity when stored under labeled conditions. The expiry date must be supported by suitable stability data.

A written procedure must be designed to ensure that the correct retest date or expiry date is printed on the finished dietary ingredient labeling.

### 6.4 Labeling Issuance and Control

Access to the label storage areas (i.e., label control room) must be limited to authorized personnel. Procedures should be implemented to ensure that labels are stored in a manner that prevents mix-ups and provides proper identification used to reconcile the quantities of labels issued, used, and returned, as well as to evaluate discrepancies found between the number of containers labeled and the number of labels issued and used. Such discrepancies should be investigated, and the investigation should be approved by the quality unit(s).

All excess labels bearing batch numbers or other batch-related printing should be destroyed. Returned labels should be maintained. Obsolete and outdated labels and incorrect labels should be destroyed to ensure that they are not used in any future packaging and labeling operations.

Printing devices used to print labels for packaging operations (e.g, for bulk packaging) should be controlled to ensure that all imprinting conforms to the print specified in the batch production and control record.

Labeling issuance must be designed to prevent mix-ups. Strict control must be exercised over the issuance of labeling for use in finished dietary ingredient labeling operations. Labels should be delivered to packaging lines in a secured container (e.g., locked box or cage).

Labeling materials issued for a batch should be carefully examined for proper identity and conformity to the labeling specifications in the batch packaging record. A printed label or copy of the printed label representative of those used must be included in the batch packaging record.

Procedures should be used to reconcile the quantities of labeling issued, used, and returned, and they should require evaluation of discrepancies found. If discrepancies are found between the quantity of product finished and the quantity of labeling issued and are outside the preset limits based on historical operating data, such discrepancies should be investigated. Label reconciliation is not required for cut or rolled labels if a 100% examination for correct labels is performed by appropriate electronic or electromechanical equipment during or after completion of finishing operations.

Returned labeling should be maintained and sorted in a manner to prevent mix-ups and provide proper identification. All excess labeling bearing lot or control numbers should be destroyed and documented.

### 6.5 Packaging and Labeling Operations

Filling, assembling, packaging, labeling, and other related operations must be performed in a manner that ensures the quality of the dietary ingredient; that the dietary ingredient is protected against allergen cross-contact, contamination, and the growth of undesirable microorganisms; and that the dietary ingredient is packaged and labeled as specified in the master packaging record. This must be done using any effective means, including the following:

- Cleaning and sanitizing all filling and packaging equipment, utensils, and dietary ingredient packaging, as appropriate
- Protecting manufactured dietary ingredients from contamination, particularly airborne contamination
- Using sanitary handling procedures
- Establishing physical or spatial separation of packaging and labeling operations from operations on other raw materials and other ingredients and dietary ingredients to prevent mix-ups
- Identifying filled dietary ingredient containers that are set aside and held in unlabeled conditions for future label operations in order to prevent mix-ups
- Assigning a batch, lot, or control number to each lot of a packaged and labeled dietary ingredient that came from a finished batch of dietary ingredient, as well as each lot of dietary ingredient distributed to another person for packaging or labeling
- Examining a representative sample of each batch of the packaged and labeled dietary ingredient to determine whether the finished dietary ingredient meets its specifications
- Suitably disposing of labels and packaging for dietary ingredients that are obsolete or incorrect to ensure that they are not used in any future packaging and labeling operations

Written procedures must be established and designed to ensure that correct packaging materials and labeling are used. Issuance and use of packaging and labels and reconciliation of any issuance and use discrepancies must be controlled. Before

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packaging and labeling operations begin, packaging and labeling for each batch of dietary ingredients must be examined to determine whether the packaging and labeling conforms to the batch packaging control record. Packaging and labeling materials must be identified by item control number and lot number.

Packaging and labeling facilities must be inspected immediately before use to ensure that all materials not needed for the next packaging operation have been removed, and that packaging and labeling facilities have been properly cleaned. Results of the inspection and cleaning should be documented in the batch packaging control records, the packaging facility log, or other documentation system.

Packaged and labeled dietary ingredients should be examined to ensure that containers and packages in the batch have the correct label. This examination should be part of the packaging operation. Results of these examinations must be recorded in the batch packaging control record.

A reserve sample of each batch of finished dietary ingredients, consisting of at least twice the quantity necessary for finished dietary ingredient specification tests and examinations, must be collected and retained for at least 1 year past its retest or expiry date.

In-process dietary ingredients transported outside the manufacturer's control must be sealed in a manner such that, if the seal is breached or missing, the recipient will be alerted to the possibility that the contents may have been altered.

Any packaged and labeled dietary ingredient that is rejected for distribution must be clearly identified, held, and controlled under a quarantine system for appropriate disposition.

The complete manufacturing history and control of the packaged and labeled dietary ingredient through distribution must be traceable.

## 6.6 Repackaging and Relabeling

Dietary ingredients may be repackaged or relabeled only after quality unit personnel have approved such repackaging or relabeling. A representative sample of each batch of repackaged or relabeled dietary ingredients must be examined to determine whether the repackaged or relabeled dietary ingredients meet all established specifications. Quality unit personnel must approve or reject each batch of repackaged or relabeled dietary ingredients prior to its release for distribution. Repackaged or relabeled ingredients should retain the original retest or expiry date.

*Good Repackaging Practices* ⟨1178⟩ should be consulted.

## 6.7 Packaging and Labeling Operations and Controls Documentation

The following documentation must be made and kept for fulfilling the requirements of the packaging and labeling operations and controls quality system (see *1. General Provisions*).

### 6.7.1 WRITTEN PROCEDURES

Written procedures for packaging and labeling operations must be established and followed for the following:

- Receipt, identification, quarantine, sampling, examination and/or testing and release, and handling of packaging and labeling materials
- Preparation and approval of packaging material and labeling specifications
- Preparation and approval and master batch packaging and labeling control records
- Cleaning and inspecting packaging and labeling facilities
- Filling, assembling, packaging, labeling, and other related operations
- Determination and issuance of finished dietary ingredient retest or expiry date to batch packaging and labeling control records

### 6.7.2 RECORDS

Records for packaging and labeling must be made and kept for the following:

- Packaging and labeling specifications
- Packaging and labeling facility cleaning and inspection records
- Executed batch packaging and labeling control records
- Executed batch repackaging and relabeling control records

# 7. LABORATORY OPERATIONS AND CONTROLS

## 7.1 General

Written procedures must be established and followed for laboratory operations, including written procedures for the tests and examinations to be conducted to determine whether specifications are met.

Adequate laboratory facilities must be used for whatever testing or examinations are necessary to determine whether raw materials, other ingredients, packaging materials, labeling, in-process materials, and dietary ingredients meet specifications.

Laboratory control processes must be established and followed that are reviewed and approved by quality unit personnel, including the following:

- Use of criteria for establishing appropriate specifications
- Use of sampling plans for obtaining representative samples of the following:

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- Raw materials, other ingredients, packing materials, and labels
- In-process materials
- Finished batches of dietary ingredients
- Product that is received for packaging or labeling as a dietary ingredient and for distribution rather than for return to the supplier
- Packaged and labeled dietary ingredients

- Use of criteria for selecting appropriate examination and testing methods
- Use of criteria for selecting standard reference materials used in performing tests and examinations
- Use of test methods and examinations in accordance with established criteria

QC personnel must verify that the laboratory examination and testing methodologies are appropriate for their intended use. QC personnel must identify and use appropriate scientifically validated methods for each established specification for which testing or examination is required to determine whether the specification is met.

Specifications, sampling plans, and test procedures, including changes or modifications to them, should be drafted by the appropriate QC unit personnel and reviewed and approved by the appropriate QA unit personnel. Specifications should be established in accordance with accepted standards and consistent with the manufacturing process.

There also must be documented procedures describing sampling, testing, approval and rejection of materials, and recording and storage of laboratory data.

## 7.2 Reagents and Reference Standards

Reagents and standard solutions should be prepared and labeled following written procedures. “Use by” dates should be established and applied as appropriate for analytical reagents and standard solutions.

Primary reference standards should be obtained as appropriate for the manufacture of dietary ingredients. The source of each primary reference standard must be documented. Records should be maintained of each primary reference standard’s storage and use in accordance with the supplier’s recommendations. Primary reference standards obtained from an officially recognized source (e.g., USP Reference Standard) may be used without testing if stored under conditions consistent with the supplier’s recommendations.

When a primary reference standard is not available from an officially recognized source, an in-house primary standard must be established. Appropriate testing needs to be performed to establish fully the identity and purity of the in-house primary reference standard. Appropriate documentation of this testing should be maintained.

Secondary reference standards should be appropriately prepared, identified, tested, approved, and stored. The suitability of each batch of secondary reference standard should be determined prior to first use by comparing it against a primary reference standard. Each batch of secondary reference standards should be periodically requalified in accordance with a written protocol.

## 7.3 Scientifically Validated Test Procedures

Tests and examinations used to determine whether material specifications are met must be appropriate for their intended use and be scientifically validated methods. Tests methods or procedures must meet proper standards of accuracy and reliability.

Chemical, microbial, or extraneous-material testing procedures must be used when necessary to identify sanitation failures or possible allergen cross-contact and food contamination.

### 7.3.1 VALIDATION OF TEST PROCEDURES

If the test procedure is not in an official compendium, the procedure must be validated according to ⟨1225⟩. Method performance characteristics include specificity, linearity, range accuracy, precision, detection limit, and quantitation limit, and those of interest may vary depending on the type of test: identification, assay, impurities, or performance.

For microbial test procedures, the following *USP* general information chapters should be consulted:

- *Biological Assay Validation* ⟨1033⟩
- *Validation of Alternative Microbiological Methods* ⟨1223⟩
- *Validation of Microbial Recovery from Pharmacopeial Articles* ⟨1227⟩

### 7.3.2 VERIFICATION OF OFFICIAL TEST PROCEDURES

If the test procedure is in an official compendium, such as *USP–NF* or AOAC International’s *Official Methods of Analysis*, the procedure only needs to be verified for its suitability under actual conditions of use, according to *Verification of Compendial Procedures* ⟨1226⟩. Verification requirements should be based on an assessment of the complexity of both the procedure and the material to which the procedure is applied. Verification is not required for basic compendial procedures, such as loss on drying and residue on ignition, or simple instrumental determinations, such as pH measurements.

## 7.4 Test Procedures

Test procedures must have clear and complete descriptions to provide an analyst with directions on how to reproducibly perform the given test. Test procedures should include the following information, as applicable:

- Purpose
- Safety information, if applicable
- Materials and equipment

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- Procedures that include, as applicable:
  - System suitability
  - Preparation of solutions and reagents
  - Preparation of standards and samples
  - Instrumental parameters
  - Calculations and reporting
- Review and approval with approval dates
- Revision history

The tests and examinations can include gross organoleptic analyses, macroscopic analyses, microscopic analyses, chemical (inorganic and organic) analyses, microbiological analyses, DNA-based analyses, and other scientifically valid methods.

Test procedures should be followed and documented at the time of performance, and any departure from established test procedures should be documented and explained.

## 7.5 Transfer of Analytical Test Procedures

Testing materials for compliance to specifications is critical in establishing the quality of the dietary ingredient. The transfer of analytical procedures (TAP), also referred to as "method transfer", is the documented process that qualifies a laboratory (the receiving unit) to use an analytical test procedure that originated in another laboratory (the transferring unit), thus ensuring that the receiving unit has the procedural knowledge and ability to perform the transferred analytical procedure as intended. *Transfer of Analytical Procedures* ⟨1224⟩ summarizes the types of transfers that may occur and outlines the potential raw materials and other ingredients of a transfer protocol, and it should be consulted to qualify a laboratory (both internal and external to the manufacturer) to ensure it has the procedural knowledge and ability to perform the test procedure as intended.

## 7.6 Reduced Testing

### 7.6.1 GENERAL PROCEDURES

Reduced testing or inspection provides a way of reducing the testing or inspection effort on received raw materials, other ingredients, in-process material, and dietary ingredients of high quality, in which all batches are expected to be of the similar quality.

Reduced testing for contaminants should be based on one or more of the following:

- Statistical analysis of an adequate quantity of historical test data
- Statistical confidence in the capability of the manufacturing process as determined by suitable verification
- Ongoing monitoring of the process using recognized statistical process control (SPC) techniques

General control procedures should be established to monitor critical in-process manufacturing attributes that if not properly controlled, could cause a failure to meet identity, strength, purity, composition, or limits on contaminants. Special controls should be used to ensure content uniformity of blended, encapsulated, enrobed, or other specialized dietary ingredients.

The following terms are useful when describing reduced testing:

- "Lot-by-lot inspection" means full inspection of products submitted in a series of lots
- "Reduced sampling inspection" means a sampling inspection procedure in which some lots in a series are accepted without full testing when the sampling results for a stated number of immediately preceding lots meet stated criteria
- "Inspection sampling frequency" means the probability that a lot has been fully tested (e.g., every fifth lot)
- "Disqualification" means failure to qualify for reduced sampling inspection
- "Requalification" means qualification for a resumption of reduced sampling inspection
- "Supplier qualification" means assessment of the supplier's cGMP competence so as to implement reduced sampling inspection
- "Product qualification" means assessment of product (i.e., raw material, other ingredient, in-process material, or dietary ingredient) quality to determine its suitability for reduced sampling inspection

### 7.6.2 REDUCED TESTING REQUIREMENTS

Reduced sampling inspection may be used only when both the supplier's cGMP quality systems and product (i.e., the raw material, other ingredient, in-process material, or dietary ingredient) are qualified. The dietary ingredient certificate of analysis should clearly disclose when reduced testing principles are applied.

- Requirements for supplier qualification are the following:
  - The supplier must have established, documented, and followed effective and applicable cGMP quality systems for managing quality that involves the active participation of personnel
  - An assessment of the supplier's cGMP quality systems must be performed
  - Ongoing assessment of the supplier's cGMP quality systems should be performed on a specified frequency
  - The supplier must not have experienced changes in its cGMP quality systems that adversely affect product quality
- Requirements for product (i.e., raw material, other ingredient, in-process material, or dietary ingredient) qualification are the following:
  - An assessment for product qualification should not be made prior to the assessment of the supplier's cGMP quality systems

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- The preceding 3 or more consecutive lots have been accepted, and results of resubmitted lots are not included

The following is an example of a typical reduced inspection and rotational testing approach that can be performed:

- Conduct lot-by-lot full inspection on the first 3 lots
- If successful, conduct reduced sampling inspection on every 5th lot for a set period of time
- If successful, reduce sampling inspection to every 10th lot, until there is a failure
- Test a minimum of 1 lot every year
- If a failure is encountered, disqualify the supplier and the product, conduct a root cause investigation, and return to full testing of the next 3 lots

#### 7.6.3 RECEIVED RAW MATERIALS, OTHER INGREDIENTS, IN-PROCESS MATERIALS, AND DIETARY INGREDIENTS

On the basis of adequate supplier qualification, process validation, in-process controls, and statistical confidence, a reduced testing plan is an alternative to fully testing every batch of raw materials and other ingredients received from a supplier provided that at least one identity (i.e., identification) test is conducted. Such identification tests must include sufficient specificity to determine the identity of the raw material and other ingredient.

Each batch of raw material and other ingredient received from a supplier must be tested or examined against established specifications for contamination if it is liable to be contaminated with microorganisms, filth, insect infestation, allergens, aflatoxins, other natural toxins, pesticides, organic impurities (e.g., residual solvents), inorganic impurities (e.g., foreign substances), or other extraneous substances that may be a source of contamination that is objectionable in view of its intended use. In such cases, reduced testing of examination must not apply until risk is mitigated by the supplier.

#### 7.6.4 IN-PROCESS MATERIALS AND DIETARY INGREDIENTS

On the basis of process validation, in-process controls, and statistical confidence, a reduced testing plan is an alternative to fully testing every batch of in-process material and dietary ingredients provided that at least one representative measure (i.e., rotational testing) is performed.

### 7.7 Out-of-Specification (OOS) Investigations

Written procedures must be established and followed for investigating deviations or the failure of a batch of material to meet specifications (i.e., OOS investigations), and corrective action plans must be established for use when an established specification is not met. The investigation should extend to other batches that may have been associated with the specific failure or deviation. The procedure should require analysis of the data, assessment of whether a significant problem exists, allocation of the tasks for corrective actions, and conclusions. Any resampling and/or retesting after OOS test results should be performed according to a documented procedure. Regulatory guidance for investigating OOS test results should be consulted.

### 7.8 Stability Testing

A documented on-going testing program must be designed to monitor the stability characteristics of dietary ingredients, and the results must be used to establish appropriate storage conditions and retest or expiry dates for the dietary ingredients. Test procedures used in stability testing must be validated and indicate stability. Stability samples should be stored in container–closure systems that simulate the packaging proposed to distribute the finished dietary ingredient in the marketplace. If multiple container–closure systems are used, stability and expiry date assignment can be satisfied by testing the container–closure system with the least desirable barrier properties. Stability studies should include testing of those attributes of the dietary ingredient that are susceptible to change during storage and that influence the quality of the dietary ingredient.

Ideally the first 3, but at minimum 1, production batches should be placed on the stability monitoring program to establish the product retest or expiry date. Thereafter, at least 1 batch per year of manufactured dietary ingredient (unless none are produced that year) should be added to the stability monitoring program.

As appropriate, the stability storage conditions for temperature and relative humidity (RH) should be as follows:

- Long term = 25 ± 2° and 60% ± 5% RH
- Intermediate = 30 ± 2° and 65% ± 5% RH
- Accelerated = 40 ± 2° and 75% ± 5% RH

Long-term studies also can be performed under the labeled storage conditions, such as at room temperature (ambient conditions) or in a cool, dry place (8°–15° and not more than 40% RH). Alternative storage conditions should be considered for products marketed outside the United States in different climatic zones.

The frequency of testing should be sufficient to establish the stability profile of the dietary ingredient. The following testing frequencies are recommended:

- Long term = 0, 3, 6, 9, 12, 18, 24, and 36 months
- Intermediate = 0, 3, 6, 9, and 12 months
- Accelerated = 0, 1, 2, 3, and 6 months

For long-term studies, the frequency of testing should be sufficient to establish the stability profile of the dietary ingredient. For ingredients intended for storage in a refrigerator, the storage conditions and minimum time period should be as follows:

- Long term = 5 ± 3°; 12 months
- Accelerated = 25 ± 2° and 60% ± 5% RH; 6 months

Accelerated stability studies are designed to increase the rate of chemical degradation or physical change and are intended to evaluate the effect of short-term excursions outside the labeled storage conditions, which might occur during shipping.

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Although accelerated stability studies may not adequately reflect the stability of the product under labeled (e.g., long term) conditions, results from accelerated stability can be used to estimate a preliminary retest or expiry date for a dietary ingredient that should eventually be supported with data from long-term stability studies.

If significant change occurs between 3 and 6 months of testing at the accelerated storage condition, the proposed retest or expiry period should be based on the real time data available during the long-term storage condition. If significant change occurs within the first 3 months of testing at the accelerated storage condition, consideration should be given to address the effect of short-term excursions outside the label storage condition, such as during shipping and handling.

Preliminary shelf-life (expiry) dates can be based on pilot scale batches if the pilot batch is at least one-tenth the size of the commercial batch size and the pilot batches employs a manufacturing process that simulates the process to be used on a commercial manufacturing scale. Bench scale batches should not be used for stability studies.

In certain cases, dietary ingredients being closely related in composition may be grouped together using one representative dietary ingredient formulation with which to obtain stability data to support the retest or expiry date of all dietary ingredient formulations within that group. Under such circumstances, sufficient scientific justification should be provided as to support grouping of the dietary ingredients for stability purposes.

The design of the stability studies may also bracket dietary ingredients in a way that only dietary ingredients on the extremes of certain factors (e.g., strength, package size) are tested. This design assumes that the stability of any intermediate level is represented by the stability of the extremes tested.

Stability studies should be performed for dietary ingredients packaged in different container–closure systems (e.g., HDPE containers, aluminum canisters). In certain cases, stability data using one type of container–closure system may be used to support the stability of the product in a different type of container–closure system (e.g., HDPE versus PET container) with supporting scientific justification regarding the moisture, vapor, and light protection characteristics of the two types of container–closure systems.

Long-term and accelerated (and intermediate as necessary) stability studies should be undertaken according to a prescribed stability protocol. The resultant data must be properly evaluated. The degree of variability of stability data between or within individual batches affects the confidence in the data. The data may show such little degradation and variability that it is apparent the product will meet specifications throughout the proposed retest or expiry date. When the data on a quantitative attribute changes with time and shows significant degradation or significant variability, it is necessary to use formal statistical analysis to determine the retest or expiry date for the dietary ingredient. A stability report should be prepared for the dietary ingredient stating the proposed storage conditions and retest or expiry date of the dietary ingredient(s), along with the stability data and its evaluation.

## 7.9 Laboratory Testing Records

Laboratory control records should include complete data derived from all tests conducted to ensure compliance with established specifications and standards, including examinations and assays, as follows:

- A description of samples received for testing, including material name or source, batch number or other distinctive code, date sample was taken, and, when appropriate, the quantity and date the sample was received for testing
- A statement or reference to each test procedure used
- A statement of the weight or measure of sample used for each test as described by the test procedure
- Data on or cross-reference to the preparation and testing of reference standards, reagents, and standard solutions
- A complete record of all raw data generated during each test, in addition to graphs, charts, and spectra from laboratory instrumentation, properly identified to show the specific material and batch tested
- A record of all calculations performed in connection with the test, including, for example, units of measure, conversion factors, and equivalency factors
- A statement of the test results and how they compare with established acceptance criteria
- The signature of the person who performed each test and the date(s) the tests were performed
- The date and signature of a second person showing that the original records have been reviewed for accuracy, completeness, and compliance with established procedures

A certificate of analysis should be issued for each batch of dietary ingredients. The certificate of analysis should list the name and item code of the dietary ingredient, the batch number, the date of manufacture, the date tested and released, recommended retest date, analytical method reference for each attribute, and the expiry date. The certificate of analysis also should list each test performed, the specific identity of the test procedure, the acceptance limits or criteria, and the results with numerical units, as appropriate. The certificate of analysis should be dated and signed by authorized quality unit personnel. For certificates of analysis issued for external use, the name, address, and telephone number of the manufacturer should be listed.

## 7.10 Laboratory Operations and Controls Documentation

The following documentation must be made and kept for fulfilling the requirements of the laboratory controls quality system (see *1. General Provisions*).

### 7.10.1 WRITTEN PROCEDURES

Written procedures for laboratory operations must be established and followed for the following:

- The tests and examinations conducted to determine whether specifications are met
- Establishment, review, and approval of material specifications
- Preparation, review, and approval of written test procedures

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- Validation and verification of test procedures for their intended use
- Selection, preparation, and handling of reagents and reference standards
- Establishment, review, and approval of sampling plans, including reduced sampling inspections
- Sampling, testing, approval, and rejection of materials, and recording and storage of laboratory data
- Investigation of deviations or OOS test results
- Establishment and implementation of a stability-testing program

### 7.10.2 RECORDS

Records must be made and kept for the following:

- Documentation that established laboratory test and examination procedures are followed
- Documentation that established laboratory methodology is followed, recorded at the time of performance by the person who conducts the testing and examination
- Documentation for laboratory tests and examinations that include the data and results of the testing and examination
- Any modification to an established test procedure
- OOS investigations and corresponding corrective actions
- Test procedure method validation or verification protocols and reports that support test procedure suitability for use
- The periodic calibration of laboratory instruments and controls
- Stability protocols and stability data and reports that support a product's retest or expiry date

## GLOSSARY

**Acceptance criteria:** Predetermined limits (e.g., number, numerical ranges, or other suitable measures) for acceptance of examination or test results.

**Acid foods or acidified foods:** Any foods that have an equilibrium at a pH of 4.6 or below.

**Adequate:** Item/area/system/knowledge that meets basic minimum requirements that are needed to accomplish intended purpose in keeping with good public health practice.

**Adulteration:** A food that contains any poisonous or deleterious substance as specified in §402(a)(1) and (a)(2) of the FD&C Act; that consists of any filthy, putrid, or decomposed substance, or an adulterant that is otherwise unfit for food as specified in 402(a)(3); that has been prepared, packed, or held under insanitary conditions as specified in §402(a)(4); whose its container is composed of any poisonous or deleterious substance that may render the contents injurious to health as specified in §402(a)(6); or that has been intentionally subjected to radiation as specified in §402(a)(7); for which the contents have been altered or that has concealed damage and or inferiority as specified in §402(b)(1-4); that contains a color additive that is unsafe as specified in §402(c); that is a dietary ingredient or contains a dietary ingredient that presents a significant or unreasonable risk of illness or injury as specified in §402(f)(1); or that is a dietary ingredient that has not been prepared, packed, or held under conditions that do not meet current good manufacturing practice regulations as specified in §402(g)(1); or that has not been transported in compliance with regulations as specified in §402(i).

**Adverse event:** Any undesirable experience associated with the use of a food or dietary ingredient in a person.

**Adverse event report:** A direct communication from an identifiable first-hand reporter of an adverse event that includes at least the following information: an identifiable reporter, an identifiable person(s) who experienced the adverse event(s), an identifiable food or dietary ingredient, and one or more adverse events.

**Affiliate:** Any facility that controls, is controlled by, or is under common control with another facility.

**Allergen:** A major food allergen as defined in section 201(qq) of the FD&C Act, and can be any of the following: 1) milk, egg, fish (e.g., bass, flounder, or cod), Crustacean shellfish (e.g., crab, lobster, or shrimp), tree nuts (e.g., almonds, pecans, or walnuts), wheat, peanuts, and soybeans; (2) a food ingredient that contains protein derived from a food specified in 1), except either any highly refined oil derived from a food specified in 1) and any ingredient derived from such highly refined oil, or a food ingredient that is exempt under paragraph (6) and (7) of section 403(w) of the FD&C Act.

**Allergen cross-contact:** The unintentional incorporation of a food allergen into a dietary ingredient.

**Article:** Substances (e.g., raw materials, other ingredients, in-process material, dietary ingredients), consumer products (e.g., foods or dietary ingredients), and materials (e.g., packaging containers and closures and labels).

**Audit:** A planned, systematic, objective, independent, and documented examination (through observation, investigation, records review, discussions with employees of the audited firm, and, as appropriate, sampling and laboratory analysis) to assess whether agreed upon requirements are being met, such as assessing a supplier's food safety processes and procedures.

**Batch:** A specific quantity of a dietary ingredient or other article that is intended to be uniform; that is intended to meet specifications for identity, purity, strength, and composition; and that is produced during a specified time period according to a single manufacturing record during the same cycle of manufacture.

**Batch (or lot) number:** Any distinctive group of letters, numbers, or symbols, or any combination of them, from which the complete history of the manufacturing, packaging, labeling, and/or holding of a batch or lot of dietary ingredients can be determined.

**Blanching:** Except for tree nuts and peanuts, a prepackaging heat treatment of foodstuffs for an adequate time and at an adequate temperature to partially or completely inactivate the naturally occurring enzymes and to effect other physical or biochemical changes in the food.

**Calibration:** The demonstration that a particular instrument or device produces results within specified limits by comparison with those produced by a reference or traceable standard over an appropriate range of measurements.

**Certificate of Analysis:** A document relating specifically to the results of testing a representative sample drawn from the batch of material. The certificate of analysis should list each test performed in accordance with compendial or manufacturer requirements, including reference to the test procedure, the acceptance limits, and the results obtained.

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**Code of Federal Regulations (CFR):** The annual codification of the general and permanent rules and regulations published in the Federal Register by the executive departments and agencies of the US federal government. It is structured into 50 subject matter titles; Title 1 applies to general provisions, while Title 21 applies to food and drugs. Titles are broken down into parts, subparts, sections and paragraphs. For example, 21 CFR 11.10(k)(2) is read as title 21, part 11 (subpart B, not specified here), section 10, paragraph (k)(2). The CFR annual edition is published by the Office of the Federal Register, National Archives and Records Administration, and the Government Publishing Office. In addition to this annual edition, the CFR is published in an unofficial format online in electronic format (www.ecfr.gov), which is updated daily.

**Component:** Any substance intended for use in the manufacture of a dietary ingredient that appears in the finished batch of the dietary ingredient.

**Composition:** The specified mix of product and product-related substances (i.e., specified mix of food ingredients or excipients and dietary ingredients) in a dietary ingredient.

**Contact surface:** Any surface that contacts a raw material, other ingredient, in-process material, or dietary ingredient, and those surfaces for which drainage can occur either to or from the raw material, other ingredient, in-process material, or dietary ingredient, and which occurs during the normal course of operations. Examples of contact surfaces include containers, utensils, tables, contact surface of equipment, and packaging material.

**Contamination:** The undesired introduction of a biological (including allergenic), chemical (including radiological), or microbiological nature, or of foreign matter, into or onto a raw material, other ingredient, in-process material, or dietary ingredient during production, sampling, packaging, repackaging, storage or transport.

**Contract manufacturer:** A manufacturer performing some aspect of manufacturing on behalf of the original manufacturer.

**Corrective action (or correction):** An action that is developed and implemented to ensure that any identified root cause of a nonconformity does not reoccur. Also, an action to identify and correct a problem that occurred during the production of dietary ingredient but without other actions associated with a corrective action procedure (e.g., action to reduce the likelihood that the problem will reoccur, evaluation of all affected food for safety, and prevention of affected food from entering commerce).

**Critical:** A manufacturing process step, process condition, test requirement, or other relevant parameter or item that must be controlled within predetermined criteria to ensure the dietary ingredient meets its specification.

**Critical control point:** A point, step, or procedure in a food process at which control can be applied and is essential to prevent or eliminate a food safety hazard or reduce such hazard to an acceptable level.

**Design qualification (DQ):** The documented collection of activities that defined the functional and operational specifications and intended purpose of the equipment or instrument. DQ states what the user wants the equipment or instrument to do and shows that the selected equipment or instrument is suitable.

**Defect action level:** A level of a non-hazardous, naturally occurring, and unavoidable defect at which the FDA may regard a food product as "unadulterated" and subject to enforcement action under section 402(a)(3) of the FD&C Act.

**Deviation:** Departure from an approved instruction or procedure or from an established standard (i.e., a failure, or OOS result). Deviations can be planned or unplanned (i.e., a nonconformity).

**Dietary ingredient:** An ingredient intended for use or used in a dietary supplement that is a vitamin; a mineral; an herb or other botanical; an amino acid; a dietary substance to supplement the human diet by increasing the total dietary intake; a concentrate, metabolite, constituent, or extract; or a combination of any of the listed ingredients.

**Dietary supplement:** A product (other than tobacco) intended to supplement the diet that bears or contains one or more of the following dietary ingredients: a vitamin; a mineral; an herb or other botanical; an amino acid; a dietary substance to supplement the human diet by increasing the total daily intake; or a concentrate, metabolite, constituent, extract or combination of the aforementioned ingredients. It is intended for ingestion in a tablet, capsule, powder, softgel, gelcap, or liquid form; it is not represented for use as a conventional food or as a sole item of a meal or the diet; it is labeled as a dietary supplement. It does include an article approved as a new drug, certified antibiotic, or licensed biologic, and was marketed as a dietary supplement or food prior to such approval, certification, or license, unless the Secretary of Health and Human Services had issued a regulation finding that the article is unlawful when used as or in a dietary supplement under the conditions of use and dosages set forth in the labeling.

**Disposition decision:** A decision to approve or reject material, QC, and manufacturing related processes or documents, based on a scientifically valid reason. Disposition decisions can be made regarding the release of raw materials, in-process materials, dietary ingredients, packaging materials, labels, and returned dietary ingredients, based on a determination of whether or not established specifications are met, or made regarding reprocessing treatments and in-process adjustments, master production and control records, batch production and control records, repackaging or relabeling, calibrations of an instrument or control, modification or deviations from standard procedures, and whether or not a deviation or unanticipated occurrence during the production and in-process control system could lead to the adulteration of a dietary ingredient.

**Documentation:** Written material related to the requirements of the quality system, including paper and electronic records, that consist of raw data, reports, protocols, and procedures related to manufacturing controls and laboratory testing. Official documents are records that support procedure, product, or the manufacturer's quality systems (e.g., standard operating procedures, master batch records, material specifications, standard test procedures, protocols, reports). Source documents are records that contain original data or information (e.g., forms, notebooks, log books, instrument printouts).

**Equipment:** Devices used to manufacture, package, or hold dietary ingredients, or to maintain or clean the physical plant and devices.

**Environmental pathogen:** A pathogen capable of surviving and persisting within the manufacturing, processing, packing, or holding environment so that it could contaminate food and may result in foodborne illness if that food is consumed without treatment to significantly minimize the environmental pathogen. Examples of environmental pathogens include *Listeria* monocytogenes and *Salmonella* subspecies but do not include the spores of pathogenic spore-forming bacteria.

**Expiry (use by) date:** The date after which the dietary ingredient will no longer meet applicable specifications of identity, strength, quality, and purity when stored under labeled conditions.

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**Facility:** A place, amenity, utility, or piece of equipment provided for a particular purpose. Also, a domestic facility or foreign facility that is required to register under section 415 of the FD&C Act, in accordance with the requirements of 21 CFR Part 1, Subpart H.

**Farm:** A primary production farm is an operation under one management in one general (but not necessarily contiguous) physical location devoted to the growing of crops, the harvesting of crops, the raising of animals (including seafood), or any combination of these activities. In addition to these activities, a primary production farm may pack or hold raw agricultural commodities, may pack or hold processed food, or may manufacture/process food under certain conditions defined in 21 CFR 1.227.

**Federal Food, Drug, and Cosmetic Act (FD&C Act):** The official compilation and codification of the federal statutes of the United States that gives authority to the FDA to oversee the safety of food, drugs, medical devices, and cosmetics (21 USC §§301–392).

**Food:** As defined in section 201(f) of the FD&C Act, 1) articles used for food, dietary supplements, or drink for man or other animals, 2) chewing gum, and 3) articles used for components of any such article. Food includes raw materials, other ingredient, and dietary ingredients.

**Harvesting:** Activities that are traditionally performed on farms for the purpose of removing raw agricultural commodities from the place they were grown or raised and preparing them for use as food. Harvesting applies to farms and farm-mixed-type facilities, and is limited to activities performed on raw agricultural commodities or on processed foods created by drying/dehydrating a raw agricultural commodity without additional manufacturing/processing on a farm. Harvesting does not include activities that transform a raw agricultural commodity into a processed food as defined in §201(gg) of the FD&C Act.

**Hazard:** Any biological (including microbiological hazards such as parasites, environmental pathogens, and other pathogens), chemical (including radiological hazards, substances such as pesticides and drug residues, natural toxins, decomposition, unapproved food or color additives, and food allergens), or physical agent (such as stones, glass, and metal fragments) that has the potential to cause illness or injury.

**Hazard requiring a preventive control:** A known or reasonably foreseeable hazard for which a person knowledgeable about the safe manufacturing, processing, packing, or holding of food would establish and manage one or more preventive controls to significantly minimize or prevent that hazard in food and raw materials and other ingredients. These controls are based on the outcome of a hazard analysis (which includes an assessment of the severity of the illness or injury if the hazard were to occur and the probability that the hazard will occur in the absence of preventive controls), and are managed (e.g., monitoring, corrections or corrective actions, verification, and records) as appropriate to the food, the facility, and the nature of the preventive control and its role in the facility's food safety system.

**Holding:** Storage of food, including incidental activities performed for that storage (e.g., activities performed for the safe or effective storage of food, such as fumigating food during storage, and drying/dehydrating raw agricultural commodities when the drying/dehydrating does not create a distinct commodity). Holding also includes activities performed as a practical necessity for the distribution of food (e.g., blending of the same raw agricultural commodity and breaking down pallets) but does not include activities that transform a raw agricultural commodity into a processed food as defined in §201(gg) of the FD&C Act. (A holding facility could include warehouses, cold storage facilities, storage silos, grain elevators, and liquid storage tanks.)

**Ingredient:** Any substance that is used in the manufacture of a food or dietary ingredient and that is intended to be present in the finished batch of the food or dietary ingredient.

**In-process control (or process control):** Checks performed during production in order to monitor and, if appropriate, to adjust the process and/or to ensure the dietary ingredient conforms to its specification.

**In-process material:** Any material that is fabricated, compounded, blended, ground, extracted, sifted, sterilized, derived by chemical reaction, or processed in any other way for use in the manufacture of a dietary ingredient.

**In-process tests:** Tests that may be performed during the manufacture of the dietary ingredient rather than as part of the formal battery of tests, which are conducted prior to release.

**Instrument (or instrumentation):** A sophisticated measuring device or tool used for analytical or scientific work.

**Installation qualification (IQ):** The documented collection of activities necessary to establish that an equipment or instrument is delivered as designed and specified and is properly installed in the selected environment, and that the environment is suitable for the instrument.

**Known or reasonably foreseeable hazard:** A biological, chemical (including radiological), or physical hazard that is known to be or has the potential to be associated with the facility or the food.

**Label:** A display of written, printed, or graphic matter on the immediate container of a dietary ingredient.

**Labeling:** All labels and other written, printed, or graphic matter on any dietary ingredient or any of its containers or wrappers, or accompanying such dietary ingredient at any time while the dietary ingredient is held for sale after shipment or delivery for shipment in interstate commerce. The term "accompanying" is interpreted liberally to mean more than physical association with the dietary ingredient. It extends to documentation such as posters, tags, pamphlets, circulars, booklets, brochures, and fillers.

**Lot:** A batch or a specific identified portion of a batch (identified by an establishment's specific code) that is intended to be uniform and that is intended to meet specifications for identity, purity, strength, and composition.

**Manufacture:** All operations of receipt of raw materials, production, packaging, labeling, QC testing, release, storage, and distribution of dietary ingredients and related controls.

**Material:** A general term used to denote raw materials, in-process materials, dietary ingredients, and packaging and labeling material.

**Microorganisms:** Yeast, molds, bacteria, viruses, and other microscopic organisms having public health or sanitary concern. These include undesirable species that may have public health significance, that may cause a raw material or dietary ingredient to decompose, that indicate that a raw material or dietary ingredient is contaminated with filth, or that otherwise may cause a raw material a dietary ingredient to be adulterated.

**Monitor:** A means to conduct a planned sequence of observations or measurements to assess whether control measures are operating as intended.

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**Nonconformity:** An event that occurs when a specified requirement is not met or results from an undesirable situation or defect. This covers departure from a procedure, standard or requirement, or the absence of dependability. This typically arises from the inability to meet documented procedures, expectations, or specifications.

**Operational qualification (OQ):** The documented collection of activities necessary to demonstrate that an equipment or instrument will function according to its operational specification testing in the selected environment. OQ demonstrates fitness for the selected use and demonstrates user requirement specifications.

**Other ingredients:** Substances that are added to a dietary ingredient preparation that are not intended or expected to be a dietary ingredient. Other ingredients are substances other than the dietary ingredient(s) that have been appropriately evaluated for safety and are intentionally included in a preparation to do one or more of the following: aid in the manufacture of a dietary supplement; protect, support, or enhance stability, bioavailability, or consumer acceptability; assist in product identification; and/or enhance any other attribute of the overall safety or delivery of the dietary ingredient during storage or use. The term is sometimes used synonymously with the terms "inactive ingredients", "pharmaceutical excipients", and "food ingredients".

**Packing:** Placing food into a container other than packaging the food. Packing includes repacking and activities performed incidental to packing or repacking a food (e.g., activities performed for the safe or effective packing or repacking of that food, such as sorting, culling, grading, and weighing or conveying incidental to packing or repacking), but does not include activities that transform a raw agricultural commodity into a processed food.

**Packaging:** Placing food into a container that transforms a dietary ingredient into a processed food or dietary ingredient.

**Packaging material:** Container and/or closure for raw materials, other ingredients, in-process material, and dietary ingredients.

**Pathogen:** A microorganism of public health significance.

**Performance qualification (PQ):** The documented collection of activities necessary to demonstrate that an equipment or instrument consistently performs according to the specifications defined by the user and is appropriate for the intended use. PQ verifies the fitness for purpose of the equipment or instrument under actual conditions of use.

**Pest:** Any objectionable insect or other animal including but not limited to birds, rodents, flies, mites, and larvae.

**Physical plant:** All or any part of a building or facility used for or in connection with manufacturing, packaging, labeling, or holding of a dietary ingredient.

**Preparation:** Combining, admixing, or diluting one or more dietary ingredients with or without other ingredients. The term is sometimes used synonymously with the term "premix".

**Preventive action:** An action taken to eliminate the cause of potential nonconformities or other undesirable situations in order to prevent occurrence and continuously improve an existing system or process.

**Preventive controls:** Risk-based and reasonably appropriate procedures, practices, and processes that a person knowledgeable about the safe manufacturing, processing, packing, or holding of food would employ to significantly minimize or prevent the hazard identified under the hazard analysis. Preventative controls are consistent with the current scientific understanding of safe food manufacturing, processing, packing, or holding at the time of analysis.

**Preventive controls-qualified individual:** A qualified individual who has successfully completed training in the development and application of risk-based preventive controls at least equivalent to that received in a standardized curriculum recognized as adequate by the FDA or is otherwise qualified through job experience to develop and apply a food safety system.

**Procedure:** A documented description of the operations to be performed, the precautions to be taken, and measures to be applied directly or indirectly related to the manufacture of a dietary ingredient.

**Process evaluation:** A set of tests performed on a process intended to evaluate its capacity to consistently produce the results for which it is intended.

**Product complaint:** Any written, electronic, or oral communication that contains any allegation or expression of concern, for any reason, with the quality of a dietary ingredient that could be related to current manufacturing practice.

**Purity:** The identity and amount of a substance that is the intended substance, typically expressed in units of percentage and determined by using tests for assay or content.

**Qualification:** Action of proving and documenting that equipment or ancillary systems are properly, work correctly, and that actually lead to the expected results. Qualification is part of validation, but the individual qualification steps alone do not constitute process validation.

**Qualified auditor:** A qualified individual who has the technical expertise obtained through education, training, or experience (or a combination thereof) necessary to perform the auditing function and who may be a government employee, including a foreign government employee, or an audit agent of a certification body that is accredited in accordance with ISO 17020 or in accordance with the regulations in 21 CFR part 1, subpart M.

**Qualified individual:** A person who has the education (or combination thereof) necessary to manufacture, process, pack, or hold clean and safe food as appropriate to the individual's assigned duties. A qualified individual may be, but is not required to be, an employee of the establishment.

**Quality:** A standard meaning that the raw materials, in-process materials, packaging materials, labeling and dietary ingredients meet established specifications for identity, purity, strength, and composition, as well as limits on contaminants, and that the dietary ingredient has been manufactured, packaged, labeled, and held under conditions to prevent adulteration.

**Quality assurance (QA):** The sum total of the organized arrangements made with the goal of ensuring that all dietary ingredients are of the quality required for their intended use and that quality systems are maintained.

**Quality assurance personnel (or unit):** Any person, persons, or group within or outside of the firm who is designated by that firm to be responsible for the duties relating to QA.

**Quality control (QC):** A planned and systematic operation or procedure necessary to ensure the quality of the dietary ingredient by checking or testing that specifications are met, and taking all actions necessary to prevent the dietary ingredient from being adulterated.

**Quality control personnel (or unit):** Any person, persons or group, within or outside of the firm, who is designated by that firm to be responsible for the duties relating to QC operations.

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**Quality unit (or personnel):** An organizational unit independent of production, which fulfills both QA and QC responsibilities, which can be in the form of separate QA and QC units or a single individual or group, depending on the size and structure of the organization.

**Quarantine:** The status of materials isolated physically or by other effective means pending a decision on their subsequent disposition decision.

**Raw material:** A general term used to denote starting materials, reagents, and solvents intended for use in the production of in-process materials (or intermediates) or dietary ingredients.

**Records:** Documents stating results achieved or providing evidence of activities performed (e.g., training records, executed batch production and control records, executed batch packaging records, validation reports, laboratory test results forms, laboratory notebooks, equipment logbooks, memos, and emails).

**Reduced testing (or sampling):** A reduced level of testing (or sampling) for a particular specified parameter(s) based on one or more of the following: statistical analysis of an adequate quantity of historical test data, statistical confidence in the capability of the manufacturing process as determined by suitable verification, or ongoing monitoring of the process using recognized SPC techniques.

**Reference standard, primary:** A substance that has been shown by an extensive set of analytical tests to be authentic material that should be of high purity. This standard is obtained from an officially recognized source (i.e., USP Reference Standard).

**Reference standard, secondary:** A substance of established quality and purity, as shown by comparison to a primary reference standard, that is used as a reference standard for routine laboratory analysis.

**Remedial (short term) action:** Action taken to provide temporary control over a situation or condition until permanent corrective actions are implemented (e.g., issuance of a temporary procedure, segregation of material).

**Representative sample:** A sample that consists of an adequate number of units that are drawn based on rational criteria, such as random sampling, and that is intended to ensure that the sample accurately portrays the material being sampled.

**Reprocessing:** The method to introduce clean and uncontaminated raw materials and other ingredients or dietary ingredients back into the manufacturing process and to repeat a step or other appropriate physical manipulation step (e.g., milling, packaging) that is part of the established manufacturing process. Continuation of a manufacturing process step after an in-process control test has shown that the step is incomplete is considered to be part of the normal manufacturing process and not reprocessing.

**Reserve sample:** A representative sample of an article that is held for a designated period of time.

**Retest date:** The date when a material should be re-examined to ensure that it is still suitable for use.

**Reworking:** The method to subject in-process material or dietary ingredients that do not conform to standards or specifications and that have been removed from processing for reasons other than unsanitary conditions to one or more processing steps that are different from the established manufacturing process in order to obtain acceptable quality in-process material or dietary ingredients.

**Root cause:** Any cause that is acted upon by a solution such that the problem does not reoccur.

**Safe-moisture level:** A level of moisture low enough to prevent the growth of undesirable microorganisms in the finished dietary ingredient under the intended conditions of manufacturing, processing, packing, and holding. The safe-moisture level for a dietary ingredient is related to its water activity. Water activity will be considered safe for a dietary ingredient if adequate data are available that demonstrate that the dietary ingredient at or below water activity will not support the growth of undesirable microorganisms.

**Sanitize:** To adequately treat cleaned equipment, containers, utensils, or other cleaned contact surface by a process that is effective in destroying vegetative cells of microorganisms of public health significance (i.e., pathogens), and in substantially reducing numbers of other microorganisms, but without adversely affecting the product or its safety for the consumer.

**Serious adverse event:** An event that results in death, a life-threatening experience, inpatient hospitalization, a persistent or significant disability or incapacity, or a congenital anomaly or birth defect, or that requires a medical or surgical intervention based on a reasonable medical judgment to prevent one of those outcomes.

**Serious adverse event report:** A report that must be submitted to the FDA using the MedWatch form when a manufacturer, packer, or distributor of a dietary ingredient receives any report of a serious adverse event associated with the use of the dietary ingredient in the United States [see §761(a)(3) and (b)(1) of the FD&C Act (21 USC §§379aa-1(a)(3), (b)(1)].

**Signature (signed):** The record of the individual who performed a particular action or review. A signature can be initials, full handwritten signature, or authenticated and secure electronic signature.

**Significantly minimize:** A reduction to an acceptable level, including elimination.

**Specification:** A list of tests, references to test procedures, and appropriate acceptance criteria for the tests described. It establishes the set of criteria to which a material should conform to be considered acceptable for its intended use. "Conformance to specifications" means that the raw material, in-process material, and/or dietary ingredient will meet the listed acceptance criteria when tested according to the listed test procedures. Specifications are critical quality standards that are proposed, justified, and approved by the manufacturer. Material specifications include test specifications for identity, purity, strength, limits on contaminants, and/or performance that define a standard of quality for the material.

**Strength:** The concentration or amount of a dietary ingredient per unit serving of a dietary ingredient. Strength only applies to a dietary ingredient.

**Subsidiary:** Any company that is owned or controlled directly or indirectly by another company.

**Supplier:** The establishment that manufactures/processes the food and raises the animal or grows the food provided to a receiving facility without further manufacturing/processing by another establishment, except for further manufacturing/processing that consists solely of the addition of labeling or similar activity of a *de minimis* nature.

**Supply-chain-applied control:** A preventive control for a hazard in a raw material or other ingredient when the hazard in the raw material or other ingredient is controlled before its receipt.

**Utensil:** A simple tool or device serving a useful purpose.

**Utility system (utility):** A useful system and source of material such as water, steam, gases, compressed air, and heating, ventilation, and air conditioning.

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**Validation:** A documented program that provides a high degree of assurance that a specific process, method, or system will consistently produce a result that meets per-determined acceptance criteria. Also a means in obtaining and evaluating scientific and technical evidence that a control measure, combination of control measures, or the FSP as a whole, when properly implemented, is capable of effectively controlling the identified hazards.

**Validation protocol:** A written plan stating how validation will be conducted and defining acceptance criteria. For example, the protocol for a manufacturing process identifies manufacturing equipment, critical process parameters/operating ranges, product characteristics, sampling, test data to be collected, number of process runs, and acceptable test results.

**Verification:** The application of methods, procedures, tests and other evaluations, in addition to monitoring, to determine whether a control measure or combination of control measures is or has been operating as intended and to establish the validity of the FSP.

**Water activity:** A measure of the free moisture in a substance or material and is the quotient of the water vapor pressure of the substance divided by the vapor pressure of pure water at the same temperature.

**Written procedures for receiving raw materials and other ingredients:** Written procedures that ensure raw materials and other ingredients are received only from suppliers approved by the receiving facility (or, when necessary and appropriate, on a temporary basis from unapproved suppliers whose raw materials or other ingredients are subjected to adequate verification activities before acceptance for use).

**Yield, actual:** The quantity of material or percentage of theoretical yield that is actually produced at any appropriate step of production or during packaging and labeling of a particular dietary ingredient.

**Yield, expected:** The quantity of material or the percentage of theoretical yield anticipated at any appropriate phase of production or during packaging and labeling of a particular dietary ingredient, based on previous laboratory, pilot scale, or manufacturing data.

**Yield, theoretical:** The quantity of material that would be produced at any appropriate step of production or during packaging and labeling of a particular dietary ingredient, based on the quantity of raw materials, other ingredients, packaging, and labeling to be used, in the absence of any loss or error in actual production or packaging and labeling.

## APPENDIX

### Reference Table: Sections of 21 CFR Used in This Chapter

| Section of ⟨2740⟩ | 21 CFR |
|---|---|
| 2. QUALITY MANAGEMENT | |
| 2.2 Organization and Personnel | §§117.4(a)(1) and (2), 111.105(a)–(b), 111.12(a)–(c), 111.12(b), 111.113(c), 117.180(a), 117.180(b)–(d)(1) and (2), 111.13(a), 117.4(b)(2)(d); 111.10(a)(1) and (2), 117.10(a), 111.10(b)(1)–(9), and 117.10(b)(1)–(9) |
| 2.3 Documentation and Records | §§111.605(a)–(c) and 111.610(a)–(b) |
| 2.5 Deviations and Material Reviews | §§111.103, 111.140(b)(1)(3)(i)–(viii), 111.87, 111.113(a)–(b)(1)–(2), 111.77(a), and 111.140(b)(2)(i)–(ii) |
| 2.7 Hazard Analysis and Risk-Based Preventive Controls (HARPC) | §§117.126(a)–(b)(1)–(7), 117.130 (a)–(c)(1)–(2), 117.135(a)–(c)(1)–(6), 117.405(a)(2)(b)–(c), 117.410(a)(1)–(4)(b)(1)–(4)(c)–(d)(1)(i)–(iii)(a–c)(iv)(e), 117.430(a)(f), 117.435(a),(b)(c)(1)–(2)(d), 117.415(a)–(c), 117.475(b)(c)(1)–(18), 111.105(b), 117.139(a)–(b), 117.150(a)(1)(2)(b), 117.155(a)–(b), 117.160(a)–(c), 117.165(a)–(b), 117.170(a)–(f), and 117.190(a)(2)–(6) |
| 2.9 Complaints | §§111.553, 111.570(b)(1)–(2),111.560(a)(1)–(2)(b)(c), 111.135, 117.410(a)(1)–(2), (b)(1)–(4) and (d), 117.420(a), 117.430(a), and 117.425; §111.105(b) |
| 2.12 Quality Management Documentation | §§111.14(b)(1), 111.103, 111.140(b)(1)–(2), 111.553, 111.570(b)(1)(2), 117.305(a)–(f), 117.310(a)7(b), 117.315(a)(1)(b)–(d), and 117.330(a)–(b) |
| 3. Physical Plant and Equipment | |
| 3.1 Grounds Maintenance | §§111.15(a)(1)–(5) and 117.20(a)(1)–(4) |
| 3.2 Physical Plant Design | §§111.20(a)(b), 117.20(b)(1), 111.20(b)(2),111.310, 111.20(c), 117.20(b)(4), 117.20(b)(4)(6)(d), and 111.20(b)(3)(i)–(iv)(5)(7)(d)–(f)(h) |
| 3.3 Physical Plant Sanitation and Facilities | §§111.15(b), 117.35(a)(b)(1)(i)–(iv)(2)(c)(d)(1)–(3)(e)-(f),117.37(a), 111.15(e)–(g)(j), 117.37(b)(1)–(5)(c)(f)(d)(e)(f), 117.80(c)(6), 111.15(g)(h)(i)(j)(k), and 117.20(b)(2) |
| 3.4 Equipment, Instruments, and Utensils | §§111.27(a), 117.40(a)(1)(c)(d),111.27(a)(2)(3)(6)(7)(i)–(v)(4)(5)(d)(2)-(7), 117.40(a)(2)(6)(b)(e)(f), 111.30(a)–(b),117.80(c)(1),117.40(g)–(f), 111.27(b)(c)(d)(1)(4), and 111.605(c) |
| 3.5 Physical Plant and Equipment Documentation | §§111.16, 111.23(b)(c), 111.25(a)-(c), and 111.35(b)(1)(i)(ii)(2)–(4)(6) |
| 4. Materials Management Operations and Controls | |
| 4.1 Establishing Material and In-Process Production Specifications | §§111.70(a)(b)(1)–(3), 117.80(b)(2)–(4), 111.70(c)(1)–(3)(d)(e)(f)(g), and 117.110(a) and (b) |
| 4.2 Determining Compliance with Specifications | §§111.73, 111.75(a)(1)(2)b(1)(2)(c)(1)(2)–(4)(e)f(1)(2)(g)(h)(1), 117.77(a)(b)(c), and 111.90(a)(1)–(2)(c) |

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| Section of ⟨2740⟩ | 21 CFR |
|---|---|
| 4.3 Receiving and Release of Materials | §111.155(a)(b)(c)(1)(d)(1)(2)(e), 111.160(a)(b)(e), 117.80(b)(1), and 111.165(a)(b)(c)(1)–(3)(d)(1)(2)(e) |
| 4.4 Representative Samples and Reserve Samples | §§111.80(a)–(c) and 111.83(b)(2)–(4) |
| 4.5 Holding, Distribution, and Transportation | §§111.453, 111.455, 111.460 (a)–(b), 117.80(b)(5)–(8), 111.465(a)(1)(2)(b), 111.170, 111.370, 111.510, 111.515, 111.520, 111.525, 111.530, 111.470, 1.908(a)(2)(3)(i)(ii)(c)–(e)(1), and 1.912 |
| 4.6 Materials Management Operations and Controls Documentation | §§111.475(b)(1)–(4), 111.503, 111.535(b)(1), 111.95(b)(1)–(4), and 111.140(b)(2) |
| 5. Production Operations and Controls | |
| 5.1 Master Manufacturing Records | §§111.205(a)(b) and 111.210(a)–(g)(h)(1)–(5) |
| 5.2 Executed Batch Production Records | §§111.255(a)–(c) and 111.260(a)–(n) |
| 5.3 Manufacturing Operations Production and Process Controls | §§111.353, 111.355, 111.360, and 117.80(a)(4)(c)(3)–(4)(8)(10)–(12)(14)–(15) |
| 5.4 Quality Unit Responsibilities for Production | §111.123(a)(1)–(6) |
| 5.5 Disposition Decision for Production | §§111.123(b)(1)–(4) and 117.80(c)(9) |
| 6. Packaging and Labeling Operations and Controls | |
| 6.2 Packaging Materials | §§111.410(a) and 117.80(c)(1) |
| 6.4 Labeling Issuance and Control | §§111.415(h) and 111.410(b) |
| 6.5 Packaging and Labeling Operations | §§117.80(c)(13), 111.415(a)–(h), 111.410(b)–(d), 111.83(b)(3)(4), and 111.425 |
| 6.6 Repackaging and Relabeling | §111.420(a)–c) |
| 6.7 Packaging and Labeling Operations and Controls Documentation | §§111.403 and 111.430(b) |
| 7. Laboratory Operations and Controls | |
| 7.1 General | §§111.310(a)–(c), 111.315, and 111.320 |
| 7.3 Scientifically Validated Test Procedures | §§111.75(h)(1), 111.320(a)–(b), and 117.80(a)(5) |
| 7.4 Test Procedures | §111.75(h)(2) |
| 7.7 Out-of-Specification (OOS) Investigations | §111.75 |
| 7.10 Laboratory Operations and Controls Documentation | §111.325(b)(1)(2)(i)–(ii) |

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# ⟨2750⟩ MANUFACTURING PRACTICES FOR DIETARY SUPPLEMENTS

***Change to read:***

## ▲GENERAL PROVISIONS

The aim of this chapter is to establish requirements that address the specific regulatory obligations of the dietary supplement industry and the additional requirements necessary to achieve compliance with the *USP–NF* quality specifications. The chapter covers the process, facilities, and controls to be used in the manufacturing, packaging, labeling, holding, and distributing of dietary supplements consistent with the FDA current good manufacturing practices (cGMP) regulations in title 21, part 111 of the *Code of Federal Regulations* in order to help dietary supplement manufacturers ensure that dietary supplements are consistently produced and controlled to the quality standards appropriate to their intended use and as required by the product specification according to USP standards.

A manufacturing and in-process control system must be designed and implemented to cover all stages of manufacturing, packaging, labeling, holding, and distribution of the dietary supplement to ensure the quality of the dietary supplement and its proper packaging and labeling as specified in the master manufacturing and packaging control records.

An overall scheme of quality systems adopted in the chapter consists of the following:

1. *Quality management system*—This system assures overall compliance with cGMPs and internal procedures and specifications. This system includes the quality control unit and all of its review and approval duties (e.g., change control, reprocessing, batch release, annual record review, validation protocols, and reports, etc.). It includes all product defect evaluations and evaluation of returned and salvaged products. The quality management system must define the quality unit obligations and authorities to ensure that all material use and disposition decisions are independent of any conflict of interest.
2. *Physical plant and equipment system*—This system includes the measures and activities which provide an appropriate physical environment and resources used in the production of the finished products. It includes buildings and facilities along with maintenance; equipment qualifications, calibration, and preventative maintenance; utilities that are not intended to be incorporated into the product such as heating, ventilation, and air-conditioning (HVAC), compressed gases, steam and water systems. The water systems refer to both the “equipment system” and the “material system”.
3. *Materials management system*—This system includes measures and activities to control finished products; components, including water or gases that are incorporated into the product; and containers and closures. It includes validation of computerized inventory control processes, product storage, distribution controls, and records.
4. *Production operations and controls system*—This system includes measures and activities to control the manufacture of finished products, including batch compounding, dosage form production, in-process sampling and testing, and process validation.
5. *Packaging and labeling operations and controls system*—This system includes measures and activities that control the packaging and labeling of finished products. It includes written procedures, label examination and usage, label storage and issuance, packaging and labeling operations controls, and validation of these operations.
6. *Laboratory control system*—This system includes measures and activities related to laboratory procedures, testing, analytical methods development and validation or verification, and the stability program.

Warehouses or other storage facilities for a retailer as well as warehouses or other storage facilities that sell directly to individual consumers must comply with the requirements pertaining to holding dietary supplements. The requirements pertaining to holding dietary supplements do not apply to holding those dietary supplements at a retail establishment for the sole purpose of direct retail sale to individual consumers.

The following is a chapter outline:

1. QUALITY MANAGEMENT
    - 1.1 General Principles
    - 1.2 Organization and Personnel
    - 1.3 Documentation and Records
    - 1.4 Change Control
    - 1.5 Deviations and Material Reviews
    - 1.6 Corrective Actions and Preventative Actions
    - 1.7 Supplier Qualification
    - 1.8 Contract Manufacturers and Contract Laboratories
    - 1.9 Complaints, Serious Adverse Event Reporting, and Recalls
    - 1.10 Internal (Self-Inspection) Audits
    - 1.11 Product Quality Review
    - 1.12 Quality Management Documentation
2. PHYSICAL PLANT AND EQUIPMENT
    - 2.1 Grounds Maintenance
    - 2.2 Physical Plant Design
    - 2.3 Physical Plant Sanitation and Facilities
    - 2.4 Equipment, Instruments, and Utensils
    - 2.5 Physical Plant and Equipment Documentation

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3. MATERIALS MANAGEMENT OPERATIONS AND CONTROLS
- 3.1 Establishing Material and In-Process Production Specifications
- 3.2 Determining Compliance with Specifications
- 3.3 Receiving and Release of Materials
- 3.4 Representative Samples and Reserve Samples
- 3.5 Holding, Distribution, and Transportation
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4. PRODUCTION OPERATIONS AND CONTROLS
- 4.1 Master Manufacturing Records
- 4.2 Executed Batch Production Records
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- 4.4 Quality Unit Responsibilities for Production
- 4.5 Disposition Decision for Production
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5. PACKAGING AND LABELING OPERATIONS AND CONTROLS
- 5.1 General
- 5.2 Packaging Materials
- 5.3 Labeling
- 5.4 Labeling Issuance and Control
- 5.5 Packaging and Labeling Operations
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- 5.7 Quality Unit Responsibilities for Packaging and Labeling Operations
- 5.8 Packaging and Labeling Operations and Controls Documentation

6. LABORATORY OPERATIONS AND CONTROLS
- 6.1 General
- 6.2 Reagents and Reference Standards
- 6.3 Scientifically Validated Test Procedures
- 6.4 Test Procedures
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- 6.6 Reduced Testing
- 6.7 Out-Of-Specification (OOS) Investigations
- 6.8 Stability Testing
- 6.9 Laboratory Testing Records
- 6.10 Quality Control Unit Responsibilities for Laboratory Operations and Controls
- 6.11 Laboratory Operations and Controls Documentation

GLOSSARY

Reference Table: Sections of 21 CFR Used in this Chapter

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# 1. QUALITY MANAGEMENT

## 1.1 General Principles

Quality unit operations must be implemented in manufacturing, packaging, labeling, laboratory, and holding operations for the production of dietary supplements to ensure the quality of the supplements and their packaging and labeling is in accordance with the specifications.

Quality should be the responsibility of all persons involved in manufacturing, testing, packaging, labeling, and holding dietary supplements. An effective system for managing quality that involves the active participation of management and appropriate manufacturing personnel must be established, documented, and followed. Management oversight of quality should encompass the organizational structure, procedures, processes, resources, and activities necessary to ensure that the dietary supplement will meet its intended specifications for quality. All quality-related activities must be defined and documented.

There must be a quality unit(s) that is independent of production (and any other conflicts of interest) and that fulfills both quality assurance (QA) and quality control (QC) responsibilities. This can be in the form of separate QA and QC units or a single individual or group, depending on the size and structure of the organization.

All quality- and manufacturing-related activities must be recorded and reviewed contemporaneously, signed, and dated.

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## 1.2 Organization and Personnel

### 1.2.1 GENERAL

Written procedures must be established and followed for creating and maintaining job position descriptions and listing job responsibilities and required qualifications for education, training, and experience. There must be an organizational chart clearly delineating the structure of the organization and the relationships and relative ranks of its parts and job positions.

### 1.2.2 RESPONSIBILITIES OF THE QUALITY UNIT(S)

A quality unit involved in all quality related matters must be established, which must review all appropriate quality related documents. Quality unit personnel must ensure that manufacturing, packaging, labeling, and holding operations ensure the quality of the dietary supplement and that the dietary supplement is packaged and labeled in accordance with the specifications.

The primary responsibilities of the independent quality unit(s) should not be delegated, and these responsibilities shall be described in writing. The quality unit(s) shall have the primary responsibility and authority for the following:

- Approving the release or rejection of (i.e., making a disposition decision for) all dietary supplements for distribution or in-process materials for use outside the control of the manufacturer;
- Establishing a system to approve the release or rejection of components, in-process materials, packaging materials, and labeling materials;
- Reviewing and approving the release or rejection of products manufactured, packaged, labeled, or held under contract by another company;
- Reviewing and approving all specifications;
- Reviewing and approving all master production, packaging, and labeling instructions;
- Reviewing and approving all procedures impacting the quality of in-process materials and dietary supplements;
- Reviewing completed production records to ensure that no errors have occurred or, if errors have occurred, that they have been investigated and resolved;
- Ensuring that all deviations are properly documented and that critical deviations are investigated and resolved;
- Ensuring that internal audits (self-inspections) are performed;
- Ensuring that corrective and preventative actions as a result of investigations (deviations and complaints) and internal audits are implemented in a timely manner;
- Establishing a system to evaluate and approve suppliers of components, packaging materials, and labeling as well as suppliers of manufacturing and laboratory services (i.e., contract manufacturers and contract laboratories);
- Establishing a system to evaluate and approve changes that potentially impact the quality of dietary supplements;
- Reviewing and approving test method validation protocols and reports;
- Establishing a system to handle complaints and ensure that quality-related complaints are investigated and resolved;
- Establishing effective systems for maintaining and calibrating equipment and instruments;
- Ensuring that materials are properly tested and the results are reported;
- Ensuring that there is stability data to support the shelf-life or expiry date and labeled storage conditions of dietary supplements;
- Performing product quality reviews and trend analysis, as needed;
- Reviewing and approving cGMP training documentations and ensuring the maintenance of these records.

The quality control unit (i.e., quality control laboratory) must have the responsibility for approving or rejecting all laboratory processes procedures, specifications, controls, tests, examinations, and deviations (planned and unplanned) from or modifications to them, that may affect the identity, strength, quality, and purity of the dietary supplement. All responsibilities and procedures applicable to the quality control unit must be in writing.

The quality unit personnel must be identified and qualified to perform quality assurance and/or quality control operations. Any other responsibility that such personnel may have within the company must not interfere or conflict with the ability to make independent judgments about quality.

The designated quality control personnel who conducts a material review and makes the disposition decision must, at the time of performance, document the material review and disposition decision made.

### 1.2.3 RESPONSIBILITIES FOR PRODUCTION OPERATIONS

The responsibilities for production operations must be documented in writing and should include the following:

- Preparing, reviewing, approving, and distributing the instructions for the production of in-process materials and dietary supplements according to written procedures;
- Reviewing all production batch records and ensuring that they are completed and signed;
- Ensuring that production facilities are clean and sanitized, if needed;
- Ensuring that the premises and equipment are maintained and records kept;
- Ensuring that HVAC, dust collection, and other systems are appropriately designed to minimize the potential for cross-contamination;
- Ensuring that, when appropriate, physical and spatial separation are used in combination to minimize the potential for cross-contamination and to prevent mix-ups;
- Ensuring that manufacturing verification/validation protocols and reports are reviewed and approved, if available;
- Evaluating proposed changes in product, process, or equipment; and

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- Ensuring that new and any modified facilities and equipment are qualified through an appropriate documented change control system.

### 1.2.4 PERSONNEL QUALIFICATIONS

Each person engaged in the manufacture of dietary supplements should have the proper education, training, and experience (or any combination thereof) needed to perform the assigned functions. Training should be in the particular operation(s) that the employee performs as they relate to the employee's functions.

Each person responsible for supervising the manufacture of dietary supplements must have the proper education, training, and experience (or any combination thereof) to perform assigned functions in such a manner as to provide assurance that the dietary supplement has the safety, identity, strength, quality, and purity that it is represented to possess.

An adequate number of qualified personnel to perform and supervise the manufacture of dietary supplements should be provided.

Personnel qualifications apply equally to contractors, consultants, and temporary employees. Records should be maintained stating the name, address, qualifications, and type of service provided by contractors and consultants.

### 1.2.5 PERSONNEL TRAINING

Training should be regularly conducted by qualified persons and should cover, at minimum, the particular operations that the employee performs and current good manufacturing practice (GMP) as it relates to the employee's functions. Training requirements should be specified, e.g., read and understand, instructional with trainer, on-the-job training. Training should be periodically assessed by reviewing and approving GMP training documentation and ensuring that these records are maintained. Appropriate documentation of training must be retained by the company, including date of training, the type of training, name of trainer if applicable, the person(s) trained, and any assessment results (e.g., test results).

### 1.2.6 PERSONNEL RESPONSIBILITIES

The company management must take all reasonable measures and precautions to ensure the following:

**1.2.6.1 Preventing microbial contamination:** Any person who, by medical examination, by the person's self acknowledgment, or supervisory observation, is shown to have, or appears to have an illness (including a fever, coughing, and sneezing), infection, open lesion, or any other abnormal source of microbial contamination that could result in microbial contamination of components, in-process material, dietary supplements, or contact surfaces must be excluded from working in any operations that may result in such contamination until the aforementioned conditions no longer exist, or unless conditions such as open lesions, boils, and infected wounds are adequately covered (e.g., by an impermeable cover). Personnel must be instructed to report such conditions to their supervisors.

**1.2.6.2 Hygienic practices:** All persons working in an operation during which adulteration of components, in-process material, dietary supplements, or contact surfaces could occur must use hygienic practices to the extent necessary to protect against such adulteration. These practices include the following:

- Wearing outer garments in a manner that protects against the contamination of components, in-process material, or dietary supplements, or any contact surface;
- Maintaining adequate personal cleanliness;
- Removing cosmetics from parts of the body that may contact components, in-process materials, dietary supplements, or contact surfaces;
- Washing hands thoroughly (and sanitizing if necessary to protect against allergen cross-contact and contamination with microorganisms) in an adequate hand-washing facility before starting work, and at any other time when the hands may have become soiled or contaminated;
- Removing all unsecured jewelry and other objects that might fall into components, in-process materials, dietary supplements, equipment, or packaging materials, and removing hand jewelry that cannot be adequately sanitized during periods in which components, in-process materials, or dietary supplements are manipulated by hand. If hand jewelry and cosmetics cannot be removed, they must be covered by material that is maintained in an intact, clean, and sanitary condition and that effectively protects against contamination of components, in-process materials, dietary supplements, or contact surfaces;
- Maintaining gloves used in handling components, in-process materials, or dietary supplements in an intact, clean, and sanitary condition. The gloves must be of impermeable material;
- Wearing face masks when working within the production and packaging areas;
- Wearing where appropriate, in an effective manner, hair nets, caps, beard covers, or other effective hair restraints;
- Storing clothing or other personal belongings in areas other than where components, in-process materials, dietary supplements, or any contact surface are exposed, where contact surfaces are washed, or where clean work uniforms and gowning are stored;
- Maintaining footwear protocol when working within the production and packaging areas;
- Not eating food, chewing gum, drinking beverages, or using tobacco products in GMP or critical areas where components, in-process materials, dietary supplements, or contact surfaces are exposed or where contact surfaces are washed; and
- Taking any other precautions necessary to protect against allergen cross-contact and contamination of components, in-process materials, dietary supplements, or contact surfaces with microorganisms, allergens, filth, foreign substances, or other extraneous materials, including perspiration, hair, cosmetics, tobacco, chemicals, and medicines applied to the skin. This may include restricting the movement of individuals working in unit operations with known allergens so that those individuals are not allowed to move into non-allergen production areas, and/or color coding of in-process allergen-containing materials to differentiate from non-allergen-containing materials.

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## 1.3 Documentation and Records

### 1.3.1 GENERAL

Clear, complete, accurate records for all operations and procedures should be documented appropriately. [NOTE—see *Good Documentation Guidelines* ⟨1029⟩ for recommendations about appropriate documentation practices.]

All documents related to the manufacture of dietary supplements must be initiated, prepared, reviewed, approved, distributed, and archived according to written procedures. Such documents can be in paper or electronic form. If the firm chooses to maintain its documentation electronically, the resulting documents and related records must be 21 CFR Part 11 compliant.

When entries are made in records, these must be made legibly and indelibly in spaces provided for such entries, directly after performing the activities, and should identify the person making the entry, and the day, and time as needed, when the entry was made. All dates must include day, month, and year in a consistent format. Numerical results must have appropriate numerical units, and calculations must be made with appropriate rounding and significant figures. Corrections to entries must be dated and signed, leaving the original entry still legible. If the reason for the correction is not obvious (e.g., date transcription error), then a statement should be made to provide clarity on the correction.

### 1.3.2 CONTROL OF DOCUMENTATION

The issuance and revision, superseding and withdrawal of all documents must be controlled with maintenance of revision histories. A procedure should be established for retaining all appropriate documentation (e.g., procedures and records). There should be proper control over:

- Standard operating procedures (SOPs) and work instructions;
- Forms: content, revision, review; each form must be associated with one or more SOPs and correspondingly numbered/versioned/dated;
- Master manufacturing records;
- Laboratory testing method;
- Spreadsheets: spreadsheet for specific project—accuracy checked by reviewer, spreadsheet for routine use—validated, access/revision controlled;
- External documents: proprietary documents, guidance documents issued by outside agency;
- Uncontrolled documents;
- For reference only documents;
- Employee notes and memos.

A formal documentation filing system (physical or electronic) should be established, with appropriate security.

### 1.3.3 RECORDS RETENTION

The retention periods for these documents must be specified. Written records must be kept, at minimum, for one (1) year past the shelf life date of the dietary supplement associated with those records. Records must be kept as original records, as true copies (such as photocopies, microfilm, compact disk, or other accurate reproductions of the original records), or as electronic copies. All electronic records must comply with 21 CFR Part 11—*Electronic Records; Electronic Signatures*.

Serious adverse event reports must be kept, at minimum, for six (6) years from the date the report is received by the responsible party.

All records or copies of records required under this general chapter must be readily available during the retention period for inspection by the FDA when requested. If encryption or data reduction techniques are used, suitable equipment to retrieve the information must be made readily available to the FDA.

## 1.4 Change Control

A formal change control system must be established to evaluate all changes that may affect the production and quality of dietary supplements. Written procedures should provide for the identification, documentation, appropriate review, and approval of changes to components, packaging materials, labeling, material specifications, analytical procedures, facilities, utility systems, equipment, computer systems, production operations, and packaging and labeling operations.

Any proposals for GMP relevant changes should be drafted, reviewed, and approved by the appropriate approved units, and reviewed and approved by the quality unit(s).

The potential impact of the proposed change on the quality of the dietary supplement should be evaluated. A change classification system based on the nature and extent of the change (e.g., major, minor) and its impact on the process (e.g., critical, non-critical) should be established with clear definitions. This classification system should be used to determine the level of testing, validation, and documentation needed to justify changes to a system or process. When implementing approved changes, measures should be taken to ensure that all documents affected by the change are revised.

The potential for changes to affect established expiry dates (e.g., changes to product formulation) should be evaluated. If necessary, the dietary supplement produced by the change can be placed on an accelerated stability program and/or added to the stability monitoring program.

Manufactures should require their contract manufactures or packagers to notify them of changes from established production process controls that can affect the quality of the dietary supplement.

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## 1.5 Deviations and Material Reviews

### 1.5.1 DEVIATIONS

Written procedures must be established and followed for documenting all deviations, and for investigating critical deviations. Any deviation from established procedures and standards should be documented and explained. Critical deviations should be investigated, and the investigation and its conclusions should be documented. The investigation should extend to any and all batches of material that may have been associated with the specific failure or deviation. The quality unit must ensure that critical deviations are investigated and resolved. Corrective and preventative actions should be implemented and verified in a timely manner.

### 1.5.2 MATERIAL REVIEW

Written procedures must be established and followed for conducting material reviews and making disposition decisions, and for the quality unit to approve or reject any treatment or in-process adjustment of a component that is rejected, or reprocessing of a dietary supplement that is rejected.

Quality unit personnel must conduct all required material reviews and make all required disposition decisions. Quality unit personnel must conduct a material review and make a disposition decision if:

- A specification is not met for a component, in-process material, packaging, labeling, dietary supplement, a dietary supplement received for packaging and labeling, or finished packaged and labeled dietary supplement;
- A batch record that deviates from the master manufacturing record, including when any step is not completed, and any deviation from the instructions contained therein;
- There is any unanticipated occurrence during manufacturing operations that adulterates or may lead to adulteration and/or misbranding of the component, dietary supplement, or packaging, or could lead to the use of a label not specified in the master manufacturing record;
- Calibration of an instrument or control suggests a problem that may have resulted in a failure to ensure the quality of a batch or batches of a dietary supplement;
- A dietary supplement is returned.

When there is a deviation or unanticipated occurrence during the production and in-process control system that results in or could lead to adulteration of a component, dietary supplement, or packaging, or could lead to the use of a label not specified in the master manufacturing record, quality unit personnel must reject the material unless it approves a treatment, an in-process adjustment, or reprocessing to correct the deviation or unanticipated occurrence.

When an established specification is not met, quality unit personnel must reject the component, in-process material, dietary supplement, packaging, or labeling, unless quality unit personnel approve a treatment, an in-process adjustment, or reprocessing, that will ensure the quality of the finished dietary supplement and that the dietary supplement is packaged and labeled as required in the master manufacturing record.

The quality unit personnel and each qualified individual who provides information relevant to the material review and disposition decision and subsequent approval or rejection of the treatment, in-process adjustment, or reprocessing of the subject material must document performance of the review, by signing and dating in the deviation investigation and material review report at the time of performance.

Deviation investigation and material review reports must include:

- Description of the specific deviation or unanticipated occurrence;
- Description of the root cause investigation;
- Evaluation of whether or not the deviation or unanticipated occurrence has resulted in or could lead to a failure to ensure the quality of the dietary supplement or failure to package and label the dietary supplement as specified in the master manufacturing record;
- Identification of corrective action(s) and preventive action(s) taken with timelines;
- Description of any material reviews, and a scientifically valid reason for any treatment or in-process adjustment of a component or in-process material that is rejected, or any reprocessing of a dietary supplement that is rejected, so as to correct the applicable deviation or unanticipated occurrence, along with the quality unit personnel's approval signature and date, when performed;
- Conclusion(s), including the explanation of the final disposition decision (approval or rejection) made for the subject article; and
- Signatures of individual(s) designated to perform quality unit operations, who conducted the material review and made the disposition decision, and of each qualified individual who provides information relevant to that material review and disposition decision.

Documentation of any material review and disposition decision, or reference to the deviation investigation and material review report, must be included in the appropriate batch production record.

## 1.6 Corrective Actions and Preventative Actions (CAPA)

The CAPA operating plan is the essential instruction by which personnel plan to achieve correction of violative, unacceptable, and improper practices, and to prevent recurrence. CAPAs may arise from many sources, including deviations, customer complaints, investigations, internal audit findings, and external audit findings.

The quality unit must establish, monitor, and maintain a CAPA program. The CAPA program should include a tracking system for deviations, nonconformities, including out-of-specification laboratory results, and CAPAs. All persons must identify deviations

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and nonconformities and notify management and/or the quality unit. Supervisors must determine root causes and develop and implement appropriate remedial action(s), corrective action(s), and/or preventive action(s). The quality unit must monitor CAPAs and manage the CAPA program to assist with adequate development, implementation, and closure of CAPAs.

## 1.7 Supplier Qualification

A written risk-based supplier qualification program, where appropriate, must be established and implemented for components for which the manufacturer has identified a hazard that requires a supply-chain-applied control.

When a supply-chain applied control is applied by an entity other than the manufacturer's supplier (e.g., a component manufacturer applies controls that are under different management than the distributor supplying the component to the manufacturer), the manufacturer must verify the supply-chain control, or obtain documentation of appropriate verification activity from another entity, review and assess the entity's applicable documentation, and document that review and assessment. For the purposes of this section, verification means the application of methods, procedures, tests, and other evaluations, in addition to monitoring, to determine whether a control measure or combination of control measures is or has been operating as intended and to establish the validity of the safety plan [as specified in 21 CFR §117.126(b)].

The supplier qualification program must include:

- Using approved suppliers, and documenting that approval before receiving components from those suppliers;
- Determining appropriate supplier verification activities, including determining the frequency of conducting the activity by considering 21 CFR §§117.410(a)(2), 117.410(d), and 117.425:
    - The hazard analysis of the component, including the nature of the hazard controlled before receipt of the component;
    - The entity or entities that will be applying controls for the hazards requiring a supply-chain-applied control;
    - Supplier performance, including:
        - The supplier's procedures, processes, and practices inherent to the hazards associated with the component;
        - Applicable food safety regulations and information relevant to the supplier's compliance with those regulations, including warning letters or import alert relating to the safety of the component and other regulatory compliance actions related to component safety (or, when applicable, relevant laws and regulations and information relevant to the supplier's compliance with those laws and regulations);
        - The supplier's food safety history relevant to the component that the manufacturer receives from the supplier, including available information about results from testing components for hazards, audit results relating to the safety of the component, and responsiveness of the supplier in correcting problems; and
        - Any other factors as appropriate and necessary, such as storage and transportation practices;
- Conducting one or more supplier qualification activities for components and approving each supplier before using the component:
    - Risked-based supplier qualification activities may include:
        - On-site audits;
        - Sampling and testing of components (certificate of analysis verification);
        - Review of supplier's relevant food safety records; and
        - Other appropriate supplier verification activities based on supplier performance and the risk associated with the component;
- Documenting supplier qualification activities.

The manufacturer's supplier qualification management may require internal cross-functional support from procurement, research and development, manufacturing operations, quality unit operations, and regulatory affairs. Due to limited internal resources, if a large number of components are used by the manufacturer, risk management principles need to be applied to qualify suppliers of components. Quality risk factors to consider include the complexity of the supply chain, component characteristics, and the level of the supplier's technical performance and regulatory compliance. The risk-based supplier qualification program must be written, implemented, and followed.

Supplier qualification activities must be documented. Formal reports should be generated for each component from each supplier summarizing initial qualification activities and analyses, and plans and schedule for continuous monitoring. An approved list of components and suppliers should be readily available to staff. A unique component item code should be assigned for each component from each supplier; however, the manufacturer may identify equivalencies between the same components from different suppliers as long as they meet the same critical specification for identity, strength, purity, composition, and limits on contaminants.

Quality unit personnel must review and approve the documentation setting forth the basis for the qualification of any supplier.

## 1.8 Contract Manufacturers and Contract Laboratories

All contract manufacturers and contract laboratories should comply with the requirements of this general chapter or equivalent regulatory oversight system requirements. Special consideration should be given to the prevention of cross-contamination and to maintaining traceability.

Contract manufacturers and contract laboratories should be evaluated by the contract giver to ensure GMP compliance of the specific operations occurring at the contract sites.

There should be a written and approved contract or quality agreement between the contract giver and the contract acceptor that defines in detail the cGMP responsibilities, including the quality measures, of each party. The contract should include provisions for the contract giver to audit, onsite and/or virtual, the contract acceptor's facilities for compliance with GMPs. Where subcontracting is allowed, the contract acceptor should not pass to a third party any of the work entrusted to it under the contract without the contract giver's prior evaluation and approval of the arrangements.

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Manufacturing and laboratory records should be kept at the site where the activity occurs and be readily available. Changes in the process, equipment, test methods, specifications, or other contractual requirements should not be made unless the contract giver has been informed and has approved the changes.

## 1.9 Complaints, Serious Adverse Event Reporting, and Recalls

### 1.9.1 COMPLAINTS

All quality related complaints, whether received orally or in writing, should be recorded and investigated according to written procedures.

Complaint records should include:

- Name and address of complainant;
- Name and phone number of person submitting the complaint;
- Name of dietary supplement, item code, and lot number;
- Nature of the complaint, including, if known, how the product was used;
- Date the complaint was received;
- Investigation undertaken and initial findings, including dates and identity of person(s) conducting the investigation;
- Action initially taken, including dates and identity of person(s) taking the action;
- Any follow up action taken;
- Response provided to the originator of the complaint, including date response was sent;
- Final findings and decision on product lot; and
- Quality unit personnel review and approval signature.

A qualified person must review and investigate all product complaints to determine whether the product complaint involves a possible failure of a dietary supplement to meet any of its specifications, or any other requirements of this general chapter, including those specifications and other requirements that, if not met, may result in risk of illness or injury.

Quality unit personnel must review and approve decisions about whether to investigate a product complaint and review and approve the findings and follow-up action of any investigation performed.

The review of the product complaint and any investigation performed must extend to all relevant batches and records.

Records of complaints should be retained in order to evaluate trends, product related frequencies, and severity with a view of taking additional, and if appropriate, immediate corrective action.

### 1.9.2 ADVERSE EVENT REPORTING

The manufacturer, packer, or distributor whose name appears on the label of a dietary supplement must maintain records related to dietary supplement adverse event reports that they receive, whether or not the adverse event is serious. The product labeling must include a continuously operating toll free telephone number for reporting adverse events involving the product. [NOTE—Products distributed with labels that do not contain the required telephone number or address are misbranded in the United States.]

All serious adverse event reports associated with the use of a dietary supplement must be submitted to the relevant regulatory agency, with a copy of the dietary supplement label, within 15 business days or within the time required by the regulatory authority after the report is received. [NOTE—In the United States, use MedWatch form FDA 3500A.]

If a retailer's name appears on the label as a distributor, the retailer may, by agreement, authorize the manufacturer or packer of the product to submit the required reports, as long as the retailer directs to the manufacturer or packer all adverse events associated with the product's use that are reported to it through the address or telephone number.

### 1.9.3 RETENTION OF ADVERSE EVENT REPORTING RECORDS

A responsible party must maintain records of any adverse event report it receives, whether serious or not, for 6 years from the date the report is received by the responsible party. Responsible parties must also give the authorized government officials timely access to the records. Responsible parties must maintain records containing the submission of serious-adverse-event reports, records containing submission of new medical information related to serious-adverse event reports, and records containing communications by the responsible party with anyone reporting a serious adverse event to the company. Responsible parties must maintain the records regardless of the source and regardless of any determination that the adverse event was caused by, or associated with, the product.

### 1.9.4 RECALLS

There should be a written procedure that defines the circumstances under which recall of a dietary supplement should be considered. The recall procedure should designate who should be involved in evaluating the information, how a recall should be initiated, who should be informed about the recall, and how the recalled dietary supplement should be treated. In the event of a serious or potentially life-threatening situation, local, national, and/or international regulatory authorities should be informed and regulations followed to ensure proper execution and disposition of the recall under international regulations.

Mock recalls should be performed annually.

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## 1.10 Internal (Self-Inspection) Audits

In order to verify compliance with the requirements of this general chapter, regular internal audits should be performed according to an approved schedule. Audit findings and corrective actions should be documented and brought to the attention of responsible management. Acceptable corrective action should be completed in a timely and effective manner.

## 1.11 Product Quality Review

Regular quality reviews of dietary supplements should be conducted annually with the objective of verifying the consistency of the manufacturing process.

Such review should normally be conducted annually and should include a review of:

- Critical dietary supplement test results;
- All batches that failed to meet established specification(s);
- Critical deviations or nonconformities and related investigations;
- Adequacy of corrective actions;
- Changes to the manufacturing process and analytical procedures;
- Results of the stability monitoring program; and
- Quality-related returns, complaints, and recalls.

The results of this review should be evaluated and an assessment made of whether any additional corrective action or any revalidation should be undertaken. Reasons for such corrective actions should be documented. Acceptable corrective actions should be completed in a timely and effective manner.

## 1.12 Quality Management Documentation

The following documentation must be made and kept for fulfilling the requirements of the Quality Management System (quality system 1).

### 1.12.1 WRITTEN PROCEDURES

Written procedures must be established and followed for:

- Creating and maintaining job position descriptions, listing job responsibilities and required qualifications for education, training and experience;
- Implementing a training program;
- Implementing a documentation and records control management system;
- Implementing a change control program;
- Implementing a deviation management program;
- Conducting material reviews;
- Implementing a corrective action and preventive action management program;
- Implementing a supplier qualification program;
- Implementing contract (manufacturing and laboratory) services program;
- Implementing a complaints handling program;
- Handling recalls;
- Conducting internal audits; and
- Conducting product quality reviews.

### 1.12.2 RECORDS

Records must be made and kept for:

- Organizational chart and job position descriptions; curriculum vitae should be available for all key management individuals in operations and quality;
- Training, including the date of the training, the type of training, name of trainer if applicable, the person(s) trained, and any assessment results (e.g., test results);
- List of and copies of current standard operating procedures, master manufacturing records, and standard test methods;
- List of and reports of changes that affect the production and quality of dietary supplements;
- List of and reports of unanticipated occurrences and deviations from approved instructions or established standards;
- List of and copies of executed batch manufacturing records;
- List of and reports of material reviews;
- List of and reports of corrective actions and preventative actions taken;
- List of approved suppliers of components, packaging materials, and labeling, and corresponding supplier qualification evaluation reports;
- List of approved suppliers of manufacturing and laboratory services, and corresponding evaluation reports, and quality agreements;
- List of complaints and corresponding investigation reports;

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- Recall and mock recall reports;
- Internal (self-inspection) audit schedule and reports; and
- Product quality review reports.

# 2. PHYSICAL PLANT AND EQUIPMENT

## 2.1 Grounds Maintenance

The grounds around the physical plant must be kept in a condition that protects against the contamination of components, in-process material, dietary supplements, and contact surfaces. The methods for adequate ground maintenance include the following:

- Properly storing equipment, removing litter and waste, and cutting weeds or grass within the immediate vicinity of the physical plant so that it does not constitute an attractant, breeding place, or harborage for pests;
- Maintaining roads, yards, and parking lots so that they do not constitute a source of contamination in areas where components, in-process material, dietary supplements, or contact surfaces are exposed;
- Adequately draining areas that may contribute to the contamination of components, in-process materials, dietary supplements, or contact surfaces by seepage, filth, or any other extraneous materials, or by providing a breeding place for pests;
- Adequately operating systems for waste treatment and disposal so that they do not constitute a source of contamination in areas where components, in-process materials, dietary supplements, or contact surfaces are exposed;
- If the physical plant grounds are bordered by grounds not under the operator's control and not maintained in the aforementioned manner, care must be exercised in the plant by inspection, extermination, or other means to exclude pests, dirt, filth, or any other extraneous materials that may be a source of contamination.

## 2.2 Physical Plant Design

Any physical plant used in the manufacture of dietary supplements must be of suitable size, construction and design to facilitate maintenance, cleaning, and sanitizing operations. The physical plant must have adequate space for the orderly placement of equipment and holding of materials as is necessary for maintenance, cleaning, and sanitizing operations, and to prevent contamination and mix-up of materials. The flow of materials and personnel throughout the physical plant should be designed to prevent contamination and mix-up of materials.

### 2.2.1 SEPARATE AND DEFINED AREAS

The physical plant must have and the manufacturer must use separate, defined areas of adequate size or other control systems —such as computerized inventory controls or automated systems of separation, physical or spatial separation—to prevent contamination or allergen cross-contact and mix-up of components, in-process materials, dietary supplements, packaging materials, and labels, during the following operations:

- Receiving, identifying, sampling, holding, and withholding from use materials to be used in manufacturing;
- Separating materials to be used in manufacturing from materials awaiting material review and disposition (i.e., in quarantine);
- Performing production, packaging, labeling, and holding operations;
- Physically separating allergen-containing raw materials from non-allergen raw materials;
- Holding rejected materials before further disposition (e.g., return, reprocessing, or destruction);
- Performing and separating the manufacturing, packaging, labeling, and holding of different product types, including different types of dietary supplements, and other foods, cosmetics, and pharmaceutical products;
- Cleaning and sanitizing contact surfaces;
- Performing and separating chemical and microbiological laboratory analyses and holding of laboratory samples and supplies.

Laboratory areas/operations should normally be separated from production areas. Some laboratory areas, particularly those used for in-process controls, can be located in production areas, provided the operations of the production process do not adversely affect the accuracy of the laboratory measurements, and the laboratory and its operations do not adversely affect the production process.

Dedicated production areas, which can include facilities, air handling equipment and/or process equipment, should be considered when materials of an allergenic nature are involved, unless validated cleaning procedures are established and maintained.

### 2.2.2 DESIGN AND CONSTRUCTION

The physical plant must be designed and constructed in a manner that prevents contamination of materials and contact surfaces by having the following:

- Floors, walls, and ceilings that can be adequately cleaned and kept in good repair;
- Fixtures, ducts, and pipes that do not contaminate materials and contact surfaces by dripping or other leakage, or condensate;

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- Adequate ventilation or environmental control equipment such as airflow systems, including filters, fans, and other air-blowing equipment that minimize odors and vapors (including steam and noxious fumes) in areas where they may cause allergen cross-contact or contamination of materials or contact surfaces, and that are located and operated in a manner that minimizes the potential for allergens, microorganisms, and particulate matter to contaminate materials or contact surfaces;
- Equipment that controls air pressure, temperature, and humidity, as needed to ensure the quality of the dietary supplement; particular attention should be given to areas where components, in-process materials, and dietary supplements are exposed to the environment;
- Aisles or working spaces between equipment and walls that are adequately unobstructed and of adequate width to permit staff to perform their duties and to protect against contamination of materials and contact surfaces with clothing or personal contact;
- Adequate lighting in all areas where material is examined, processed, or held, or where contact surfaces are cleaned; and in hand-washing areas, dressing and locker rooms, and bathrooms;
- Safety-type light bulbs, fixtures, skylights, or other glass or glass-like materials when suspended over exposed materials in any step of manufacture;
- Adequate screening or other protection against pests and insects, where necessary;
- Adequate protection of materials in bulk fermentation vessels by, for example, use of protective coverings; placement in areas where harborages for pests can be eliminated over and around the vessels; placement where staff can check regularly for pests, pest infestation, filth, biofilm, organic material, or any other extraneous materials; or by use of skimming equipment.

## 2.3 Physical Plant Sanitation and Facilities

The physical plant must be kept clean and sanitary, and maintained in repair sufficient enough to prevent materials and contact surfaces from becoming contaminated.

Written procedures should be established assigning responsibility for sanitation and describing the cleaning schedules, methods, equipment, and materials to be used in cleaning buildings and facilities.

Utilities that could affect dietary supplement quality (e.g., purified water, potable water, steam, gases, compressed air, heating, ventilation, and air conditioning) should be qualified and appropriately monitored and action should be taken when acceptance criteria are not met. Drawings for these utility systems should be made and available.

### 2.3.1 CLEANING COMPOUNDS, SANITIZING AGENTS, PESTICIDES, AND OTHER TOXIC MATERIALS

Cleaning compounds and sanitizing agents must be free from undesirable microorganisms and must be safe and adequate under the conditions of use. All toxic materials should be purchased with a safety data sheet and supplier's guarantee or certification. Toxic materials must not be used or held in the physical plant in which components, in-process materials, dietary supplements, and contact surfaces are manufactured or exposed, unless those materials are required to maintain clean and sanitary conditions, to conduct laboratory test procedures, to maintain or operate the physical plant or equipment, or to use in the physical plant's operations. Toxic materials must be identified and held in a manner that protects against contamination of components, in-process materials, dietary supplements, and contact surfaces. All relevant federal, state, and local government regulations for the use and holding of these toxic materials must be followed.

Any substances associated with the operation of equipment, such as lubricants, heating fluids, or coolants, should not contact in-process materials or dietary supplements so as to alter their quality beyond established specifications. Any deviations from this should be evaluated to ensure that there are no detrimental effects upon the suitability for use of the material. When possible, food grade lubricants and oils should be used. When the lubricant used is placed in direct contact with an in-process material (e.g., in the production of soft gelatin capsule film) a food grade is required.

### 2.3.2 PEST CONTROL

Animals or pests must not be allowed in any area of the physical plant. Effective measures must be taken to exclude pests from the physical plant and to protect against contamination of materials and contact surfaces on the premises by pests. The use of pesticides, such as insecticides, fumigants, fungicides, or rodenticides, is permitted only if precautions are taken to protect against the contamination of components, in-process materials, dietary supplements, and contact surfaces. Pesticides should be registered and used in accordance with the Federal Insecticide, Fungicide, and Rodenticide Act.

### 2.3.3 WATER SUPPLY

Potable water at a suitable temperature, and under pressure as needed, must be supplied in a plumbing system free of defects that protects against contamination or undesirable chemical reaction of components, in-process materials, dietary supplements, and contact surfaces. Potable water must meet, at minimum, all the requirements for drinking water promulgated in the U.S. Environmental Protection Agency's National Primary Drinking Water Regulations (40 CFR §141), and any applicable state and local drinking water requirements that are more stringent. For manufacturers outside the U.S., potable water meeting equivalent requirements may be acceptable with justification, for example, the drinking water regulations of the European Union (European Commission Directive 98/93/EC) or Japan Drinking Water Quality Standards. Water not meeting such requirements should not be permitted for use in the water purification system for *Purified Water*.

If water is to be used as a component of a dietary supplement, e.g., when such water contacts components, in-process materials, dietary supplements, or any contact surface, it should be assessed to ensure that the quality of such water does not negatively affect the quality of the dietary supplement over its designated shelf life. Where water used in the manufacturing

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process is treated by the manufacturer to achieve a defined quality, the treatment process should be qualified and monitored with appropriate acceptance and action level criteria.

Water from a private source should be used in a manner such that the water may become a component of the dietary supplement, comply with any state and local requirements, and not contaminate the dietary supplement. Satisfying this requirement may involve performing appropriate water treatment procedures, including filtration, sedimentation, and chlorination.

#### 2.3.4 PLUMBING

The plumbing in the physical plant must be of an adequate size and design and be adequately installed and maintained to:

- Carry sufficient amounts of water to the required locations throughout the physical plant;
- Properly convey sewage and liquid disposable waste from the physical plant;
- Avoid being a source of contamination to components, in-process materials, dietary supplements, or any contact surface, or creating an unsanitary condition;
- Provide adequate floor drainage in all areas where floors are subject to flooding-type cleaning or where normal operations release or discharge water or other liquid waste on the floor; and
- Not allow backflow from, or cross connection between piping systems that discharge waste water or sewage and piping systems that carry water used for manufacturing dietary supplements, for cleaning contact surfaces, or for use in bathrooms or hand-washing facilities.

Permanently installed pipework should be appropriately identified. This can be accomplished by identifying individual lines with content and directional flow arrows, documentation of computer control systems, or alternative means.

#### 2.3.5 SEWAGE AND TRASH DISPOSAL

Sewage, refuse, and other waste in and from buildings and the immediate surrounding area should be disposed of in a safe, timely, and sanitary manner. Sewage must be disposed of into an adequate sewage system. Trash and hazardous waste must be conveyed, stored, and disposed of in a manner to minimize the development of odors; minimize the potential for the trash to attract, harbor, or become a breeding place for pests; and protect against contamination of materials, contact surfaces, water supplies, and grounds surrounding the physical plant. Containers and/or pipes for waste material should be clearly identified.

#### 2.3.6 BATHROOMS AND HAND-WASHING FACILITIES

Adequate and readily accessible bathrooms and hand-washing facilities must be provided and kept clean, and must not be a potential source of contamination of materials and contact surfaces. The facilities must be designed to provide water at a suitable temperature, soap and sanitizing agents, air driers, or single-service towels [cGMP, 21 CFR §111.15(h)–(i)]. Adequate facilities for showering and/or changing clothes should be provided, when appropriate.

#### 2.3.7 ALLERGEN CROSS-CONTAMINATION REDUCTION CONTROL

Adequate precautions must be taken to reduce the potential for allergen cross-contact of components, in-process materials, dietary supplements, and contact surfaces by adequate food safety controls and operating practices or effective design of the physical plant, including the separation of operations in which allergen cross-contact is likely to occur, by one or more of the following means: location, time, partition, air flow systems, dust control systems, enclosed systems, or other effective means [cGMP, 21 CFR §117.20(b)(2)].

#### 2.3.8 SANITATION SUPERVISORS

One or more employees must be assigned to supervise overall sanitation, and must be qualified by education, training, and/or experience to develop and supervise sanitation procedures, using appropriate risk-based assessments and preventative controls.

### 2.4 Equipment, Instruments, and Utensils

Equipment, instruments, and utensils used in the manufacture of dietary supplements must be of appropriate design, construction, and workmanship, to enable them to be suitable for their intended use and suitably located to facilitate operations for their intended use and to be adequately cleaned and properly maintained.

Equipment and utensils include, but are not limited to, the following: equipment used to hold or convey; equipment used to measure (i.e., instruments); equipment using compressed air or gas; equipment used to carry out processes in closed pipes and vessels; and equipment used in automatic, mechanical, or electronic systems.

Major equipment and permanently installed processing lines used during the production, packaging, and labeling of dietary supplements should be appropriately identified. A set of current drawings and/or manuals should be maintained for equipment and critical installations (e.g., instrumentation and utility systems). Equipment should be identified as to its contents and its cleanliness status by appropriate means.

#### 2.4.1 DESIGN AND CONSTRUCTION

Equipment and utensils must be appropriate in design and construction to:

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- Prevent contamination of components, packaging materials, in-process materials, or finished product with lubricants, fuel, coolants, metal or glass fragments, filth or any other extraneous material, contaminated water, or any other contaminants;
- Be corrosion-resistant and made of nontoxic materials if the equipment or utensils contact components, in-process materials, or dietary supplements;
- Withstand the environment in which they are used in manufacturing, cleaning, and sanitizing;
- Must have seams that are smoothly bonded or maintained to minimize accumulation of dirt, filth, biofilm, organic material, particles of components, in-process materials, dietary supplements, or any other extraneous materials or contaminants;
- Constructed so that surfaces that contact components, in-process materials, or dietary supplements are not corrosive, reactive, additive, or absorptive so as to alter the quality of the product beyond the established requirements.

Automated, mechanical, or electronic equipment that is used to manufacture, package, label, or hold a dietary supplement must be designed or selected to ensure that the dietary supplement specifications are consistently met.

The suitability of the equipment must be determined by ensuring that the equipment is capable of operating satisfactorily within the operating limits required by the process.

### 2.4.2 INSTALLATION AND MAINTENANCE

Equipment and utensils must be installed and maintained to facilitate cleaning the equipment and utensils and adjacent spaces, and to protect components, in-process materials, and dietary supplements from being contaminated by any source.

Each freezer, refrigerator, and cold storage compartment used to hold components, in-process materials, or dietary supplements:

- Must be fitted with a calibrated indicating thermometer, temperature-measuring device, or temperature-recording device that shows, indicates, and records, or allows for recording by hand, the temperature accurately within the compartment. If recording by hand, monitoring must occur at a frequency established according to risk management principles.
- Must have an automated device for regulating temperature or an automated alarm system to indicate a significant temperature change in a manual operation.

Compressed air or other gases introduced mechanically into or onto components, in-process materials, dietary supplements, or contact surfaces, or that are used to clean any contact surface, must be treated in such a way (e.g., proper filtration) that the component, in-process material, dietary supplement, or contact surface is not contaminated.

Instruments or controls used in the manufacturing, packaging, labeling, or holding of a dietary supplement, and instrument controls used to measure, regulate, or record temperatures, pH, water activity, or other conditions, and to control or prevent the growth of microorganisms or other contamination must be accurate and precise, calibrated periodically, adequately maintained, and adequate in number for their designated uses.

### 2.4.3 OPERATION AND CALIBRATION

Control, weighing, measuring, monitoring, and test equipment, i.e., instruments and controls, which are critical for assuring the quality of dietary supplements, must be calibrated according to written procedures and an established schedule. Equipment and instruments used in the manufacture of dietary supplements must only be used within its qualified operating range. Equipment calibrations should be performed using compendial reference standards, or standards traceable to certified standards, if available.

Instruments and controls used in manufacturing or testing a component, in-process material, or dietary supplement must be calibrated against a reference standard before first use; at a frequency specified in writing by the manufacturer of the instrument or control, and at routine intervals or as otherwise necessary to ensure the accuracy and precision of the instrument and control.

Instruments or controls that cannot be adjusted to agree with the reference standard must be repaired or replaced.

Balances used to accurately weigh material should comply with *Balances* ⟨41⟩ and *Weighing on an Analytical Balance* ⟨1251⟩. This should include the determination and posting of the minimum accurate weight on all scales and balances used throughout the production and laboratory testing units.

Automated, mechanical, or electronic equipment that is used to manufacture, package, label, or hold a dietary supplement must be routinely calibrated, inspected, or checked to ensure proper performance. The quality unit must periodically approve these calibrations, inspections, or checks.

The appropriate controls for automated, mechanical, and electronic equipment (including software for a computer-controlled process) must be established and used to ensure that any changes to the manufacturing, packaging, labeling, holding, or other operations are approved by the quality unit and instituted only by authorized personnel.

The appropriate controls must be established and used to ensure that the equipment functions in accordance with its intended use. These controls must be approved by the quality control unit.

Deviations from approved standards of calibration on critical instruments should be investigated to determine if these could have had an impact on the quality of in-process materials or dietary supplements using this equipment since the last successful calibration.

Quality unit personnel must:

- Review and approve all processes for calibrating equipment and instruments;
- Periodically review all records for calibration of equipment, instruments, and controls;
- Periodically review all records for calibrations, inspections, and checks of automated, mechanical, or electronic equipment; and
- Review and approve controls to ensure that automated, mechanical, or electronic equipment functions in accordance with its intended use.

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## 2.4.4 CLEANING AND MAINTENANCE

Equipment, utensils, and any other contact surfaces used to manufacture, package, label, or hold components, in-process materials, or dietary supplements must be maintained, cleaned, and sanitized, as necessary, to protect against allergen cross-contact and contamination of components, in-process materials, and dietary supplements. Equipment and utensils must be taken apart as necessary for thorough maintenance, cleaning, and sanitizing.

Schedules and procedures, including assignment of responsibility, should be established for the preventative maintenance of equipment.

Written procedures should be established for cleaning of equipment and its subsequent release for use in the manufacture of dietary supplements. Cleaning procedures should contain sufficient details to enable operators to clean each type of equipment in a reproducible and effective manner. These procedures should include:

- Assignment of responsibility for cleaning of equipment;
- Cleaning schedules, and sanitizing schedules as needed;
- Complete description of the methods and materials, including dilution of cleaning agents used to clean equipment;
- Instructions, as needed, for disassembling and reassembling each part of equipment to ensure proper cleaning;
- Instructions for the removal or obliteration of previous batch identification;
- Instructions for the protection of clean equipment from contamination prior to use, and the maximum time that may elapse between completion of cleaning and use in manufacturing;
- Inspection of equipment cleanliness immediately before use; and
- Establishing the maximum time that may elapse between the completion of manufacturing processing and equipment cleaning.

All contact surfaces must be cleaned and sanitized, as necessary, before use and after any interruption during which the contact surfaces may have become contaminated. In consecutive operations involving batches of different dietary supplements, the contact surfaces must be adequately cleaned, and sanitized as needed, to prevent cross-contamination; in consecutive operations involving different batches of the same dietary supplement, the contact surfaces must be inspected, and adequately cleaned and sanitized, as necessary to prevent buildup and carry-over of contaminants (e.g., objectionable levels of microorganism). Acceptance criteria for residues and the choice of cleaning procedures and cleaning agents should be defined and justified. The cleaning status of equipment should be stated on the equipment.

Surfaces that do not come into direct contact with components, in-process materials, or dietary supplements must be cleaned as frequently as necessary to protect against contaminating components, in-process materials, or dietary supplements.

Effective systems should be used to reduce the potential of non-contact surfaces from becoming sources of contamination, such as floor sticky pads between different production areas and a captive shoe policy and program.

Plastic pallets should be used in high risk areas where components, in-process materials, dietary supplements, and contact surfaces are exposed. At minimum, heat treated pallets must be used in areas where components, in-process materials, dietary supplements, and contact surfaces are exposed. An effective pallet cleaning, maintenance, inspection, and management program must be established, documented, and followed.

Contact surfaces used for manufacturing or holding low-moisture components, in-process materials, or dietary supplements must be in a clean, dry, sanitary condition before use. When the surfaces are wet-cleaned, they must be sanitized, when necessary, and thoroughly dried before subsequent use.

If wet processing is used during manufacturing, all contact surfaces must be cleaned and sanitized, as necessary, to protect against the introduction of microorganisms into components, in-process materials, or dietary supplements. When cleaning and sanitizing is necessary, all contact surfaces must be cleaned and sanitized before use and after any interruption during which the contact surface may have become contaminated. In a continuous production operation or in consecutive operations involving different batches of the same dietary supplement, the contact surfaces must be adequately cleaned and sanitized, as necessary.

Single-service articles (such as utensils intended for one-time use, paper cups, and paper towels) must be stored in appropriate containers; and handled, dispensed, used, and disposed of in a manner that protects against contamination of components, in-process materials, dietary supplements, or any contact surface.

Cleaning compounds and sanitizing agents must be adequate for their intended use and safe under their conditions of use.

The portable equipment and utensils that have contact surfaces must be cleaned, sanitized, and stored in a location and manner that protects them from contamination.

## 2.4.5 EQUIPMENT AND INSTRUMENT QUALIFICATION

Equipment and instruments must be demonstrated to be “fit for purpose.” There are many ways of demonstrating that equipment and instruments are “fit for purpose” and under control, and these can include qualification, calibration, and maintenance. Equipment or instrument qualification activities necessary to establish “fitness for purpose” may be grouped into four phases: design qualification (DQ), installation qualification (IQ), operational qualification (OQ), and performance qualification (PQ). In order to ensure “fitness for purpose”, an integrated approach, based upon a risk assessment, should be undertaken.

A variety of equipment and instruments, ranging from a simple apparatus to complex computerized systems, is used in the dietary supplement industry to manufacture, package, label, test, and hold dietary supplements. The risk assessment enables the classification of equipment and instruments to determine the extent of qualification and actions needed to demonstrate “fitness for purpose.” Generally, the more complex the equipment or instrument, or the higher the criticality of its operation, the greater the amount of work required to ensure “fitness for purpose.”

Requirements for the intended use should be appropriately defined to ensure that the particular needs and technical and operational requirements are met. Qualification activities are described in *Analytical Instrument Qualification* ⟨1058⟩.

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When an equipment or instrument undergoes major repairs or modifications, this should be evaluated using change control. Relevant equipment qualification should be repeated to verify that the equipment continues to operate satisfactorily. If equipment is moved to another location, an assessment should be made of what, if any, qualification stage should be repeated.

### 2.4.6 Computerized Systems

Written procedures should be available for the operation and maintenance of computerized systems.

Appropriate installation qualification and operational qualification should demonstrate the suitability of the computer hardware and software to perform assigned tasks.

GMP related computerized systems must be validated. The depth and scope of validation depends on the diversity, complexity, and criticality of the computerized application. Commercially available software that has been validated may require an abbreviated level of testing. If an existing system was not validated at time of installation, a retrospective validation could be conducted if appropriate documentation is available.

Where critical data are being entered manually, there must be an additional check on the accuracy of the entry. This can be done by a second operator or by the system itself, e.g., barcode reader.

Changes to the computerized system must be made according to a change control procedure and should be formally authorized, documented, and tested. Records must be kept of all change, including modifications and enhancements made to hardware and software. These records should demonstrate that the system is maintained in a validated state.

If system breakdowns or failures would result in the permanent loss of records, a back-up system should be provided. A means of ensuring data protection should be established for all GMP related computerized systems. Data can be recorded by a second means in addition to the computer system.

All electronic records must comply with 21 CFR Part 11—*Electronic Records; Electronic Signatures*.

**2.4.6.1 Computer systems procedures and controls:** GMP related computerized systems used to create, modify, maintain, or transmit electronic records must employ procedures and controls designed to ensure the authenticity, integrity, and, when appropriate, the confidentiality of electronic records, and to ensure that the signer cannot readily repudiate the signed record as not genuine. Such procedures and controls must include, but are not limited to, the following:

- Validation of systems to ensure accuracy, reliability, consistent intended performance, and the ability to discern invalid or altered records;
- The ability to generate accurate and complete copies of records (including controls to prevent omissions in data, e.g., system turned off and data not captured) for inspection, review, and copying;
- Protection of records to enable their accurate and ready retrieval throughout the records retention period;
- Limiting system access to authorized individuals to prevent unauthorized access or changes to data;
- Use of secure, computer-generated, time-stamped audit trails to independently record the date and time of operator entries and actions that create, modify, or delete electronic records;
- Establishment of, and adherence to, written policies that hold individuals accountable and responsible for actions initiated under their electronic signatures in order to deter record and signature falsification;
- Use of appropriate controls over systems documentation including distribution of, access to, and use of documentation for system operation and maintenance; and revision and change control procedures to maintain an audit trail that documents time-sequenced development and modification of systems documentation.

**2.4.6.2 Electronic signatures:** Signed electronic records must contain information associated with the signing that clearly indicates all of the following: the printed name of the signer, the date and time when the signature was executed, and the task performed (such as review, approval, responsibility, or authorship) associated with the signature.

Each electronic signature must be unique to one individual and must not be reused by, or reassigned to, anyone else. Electronic must employ at least two distinct identification components such as an identification code and password. Persons who used electronic signatures must employ controls to ensure their security and integrity.

## 2.5 Physical Plant and Equipment Documentation

The following documentation must be made and kept for fulfilling the requirements of the Physical Plant and Equipment System (quality system 2).

### 2.5.1 Written Procedures

Written procedures must be established and followed for:

- Maintaining, cleaning, and sanitizing, as necessary, the physical plant and facilities;
- Pest control;
- Maintaining, cleaning, and sanitizing, as necessary, equipment, utensils, and any other contact surfaces that are used to manufacture, package, label, or hold components in-process materials, and dietary supplements;
- Use of cleaning compounds, sanitizing agents, pesticides, and other toxic materials;
- Pallet inspection;
- Equipment and instrument qualification;
- Calibrating instruments and controls used in manufacturing or testing a component, in-process material, or dietary supplement;
- Calibrating, inspecting, and checking automated, mechanical, and electronic equipment and instruments; and
- Operating and maintaining GMP related computerized systems.

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#### 2.5.2 RECORDS

Records must be made and kept for:

- List of approved cleaning compounds, sanitizing agents, and corresponding safety data sheets;
- Drawings for utility systems, e.g., water, steam, gases, compressed air, heating, ventilation, and air conditioning;
- Physical plant and facility maintenance, cleaning and sanitizing schedules, and logs of the date of maintenance, cleaning, and sanitizing, as necessary, unless such documentation is kept with the batch record;
- Pest control;
- Demonstrating that water, when used in as a component of a dietary supplement, meets the relevant Compendial requirements;
- List of manufacturing equipment and instrumentation;
- Drawings and/or manuals for equipment and critical installations;
- Documentation, in individual equipment logs, of the date of use, maintenance, cleaning, and sanitizing of equipment, unless such documentation is kept with the batch record;
- Documentation of the controls used to ensure that equipment and instruments function in accordance with their intended use;
- Documentation of any calibration, each time the calibration is performed, for instruments and controls used in manufacturing or testing a component, in-process materials, or dietary supplement:
    - The documentation must identify the instrument or control calibrated and date of calibration, identify the reference standard used including the certification of accuracy of the reference standard and history of recertification of accuracy, identify the calibration method used including appropriate acceptance limits for accuracy and precision of instruments and controls when calibrating, provide the calibration reading(s) found, identify the recalibration method used and reading(s) found if accuracy and/or precision acceptance limits were not met, and initials of the person who performed the calibration or recalibration;
- Documentation of pallet inspection and disposition;
- Documentation of equipment and instrument qualification;
- Documentation of calibrations, inspections, and checks of automated, mechanical, and electronic equipment; and
- Backup file(s) of current and outdated software, as necessary, to retrieve records that are required to be kept, and of data entered into the computer system used to manufacture, package, label, and hold dietary supplements:
    - Backup file(s) (e.g., a hard copy of data entered, tapes, compact disks, flash drives) must be an exact and complete record of the date entered, and must be kept secure from alterations, inadvertent erasures, or loss.

## 3. MATERIALS MANAGEMENT OPERATIONS AND CONTROLS

The persons authorized to release components, packaging materials, labeling, and dietary supplements should be specified. No materials should be released or used before the satisfactory completion of evaluation by the quality unit(s) unless there are appropriate systems in place to allow for such use (e.g., release under quarantine, see distribution procedures).

### 3.1 Establishing Material and In-Process Production Specifications

Specifications must be established for any point, step, or stage in the manufacturing process where control is necessary to ensure the quality of the dietary supplement and that the dietary supplement is packaged and labeled as specified in the master manufacturing and packaging control record.

#### 3.1.1 COMPONENT SPECIFICATIONS

Specifications must be established for each component that is used in the manufacture of a dietary supplement to ensure that the specifications for the purity, strength, and composition of the dietary supplement, manufactured using the components, are met. The specification for the components should include specifications for:

- Identity;
- Purity;
- Content or assay;
- Limits on contaminants that may adulterate or may lead to adulteration of the finished batch of dietary supplements;
- Applicable specific tests (e.g., peroxide value, anisidine value for oil rancidity);
- Physical characteristics impacting manufacturing process (e.g., particle size, bulk density);
- Special claims should also be included in the specifications and be verified by the user of the material. Special claims include gluten-free, no soy, no dairy, etc.

Generally, dietary supplements are prepared from ingredients that meet *USP*, *NF*, or *Food Chemical Codex* standards. Where such standards do not exist, substances may be used in dietary supplements if they have been shown to be of acceptable food grade quality using other suitable procedures.

The purity of water used as a component of a dietary supplement should be appropriately defined to maintain the stability of the dietary supplement throughout its shelf life.

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Ingredients may be added to dietary supplements provided that the ingredients comply with applicable regulatory requirements, and do not interfere with the assay and tests prescribed for determining compliance with the bulk or finished dietary supplement specification.

Adequate documentation must be provided for the basis for why the component specifications will help ensure that the specifications are met for the identity, purity, strength, and composition of the dietary supplements and for limits on those types of contamination that may adulterate or may lead to adulteration of the finished batch of the dietary supplement.

Each component received from a supplier that is liable to contamination with microorganisms, filth, insect infestation, allergens, aflatoxins, other natural toxins, pesticides, organic impurities, residual solvents, inorganic impurities, elemental contaminants, foreign substances, or other extraneous substances that may be a source of contamination that is objectionable in view of its intended use must be tested or examined against established specifications for such contamination. Applicable *USP* general chapters should be used, including the following:

*Residual Solvents* ⟨467⟩
*Articles of Botanical Origin* ⟨561⟩, *Test for Aflatoxins* and *Pesticide Residue Analysis*
*Microbial Enumeration Tests* ⟨2021⟩
*Absence of Specified Microorganisms* ⟨2022⟩
*Microbiological Attributes of Nonsterile Nutritional and Dietary Supplements* ⟨2023⟩
*Elemental Contaminants in Dietary Supplements* ⟨2232⟩

### 3.1.2 IN-PROCESS PRODUCTION SPECIFICATIONS

Specifications must be established for in-process production for any point, step, or stage in the master manufacturing record where control is necessary to help ensure that specifications are met for the identity, purity, strength, and composition of the dietary supplements and, as necessary, for limits on those types of contamination that may adulterate or may lead to adulteration of the finished batch of the dietary supplement.

Adequate documentation must be provided for the basis for why meeting the in-process specifications, in combination with meeting component specifications, will help ensure that the specifications are met for the identity, purity, strength, and composition of the dietary supplements and for limits on those types of contamination that may adulterate or may lead to adulteration of the finished batch of the dietary supplement. Quality unit personnel must review and approve this documentation.

### 3.1.3 LABELING AND PACKAGING MATERIAL SPECIFICATIONS

Specifications must be established for labeling (label specifications, see *Labeling* ⟨7⟩) and packaging materials that come in contact with dietary supplements (packaging specifications, see *Packaging and Storage Requirements* ⟨659⟩, *Containers—Glass* ⟨660⟩, *Plastic Packaging Systems and Their Materials of Construction* ⟨661⟩, *Plastic Materials of Construction* ⟨661.1⟩, *Plastic Packaging Systems for Pharmaceutical Use* ⟨661.2⟩, *Auxiliary Packaging Components* ⟨670⟩, and *Containers—Performance Testing* ⟨671⟩). Packaging that may come into contact with dietary supplements must be safe and suitable for its intended use and must not be reactive or absorptive or otherwise affect the safety or quality of the dietary supplement.

### 3.1.4 IN-PROCESS (BULK) DIETARY SUPPLEMENT SPECIFICATIONS

Specifications must be established for each dietary supplement that is manufactured. The specification for the dietary supplement should include specifications for:

- Identity;
- Purity;
- Strength or composition (i.e., content or assay);
- Limits on contaminants that may adulterate or may lead to adulteration of the finished batch of dietary supplements;
- Applicable specific characteristics (e.g., peroxide value, anisidine value for oil rancidity);
- Performance (i.e., disintegration and/or dissolution);
- Dosage forms quality tests (e.g., tablet friability, weight variation, or content uniformity).

Dietary supplements for which a monograph is provided in the *USP* should comply with the monograph standard and other applicable standards in that compendium.

**Defect action levels:** Some dietary supplements, even when produced under good manufacturing practices, contain naturally occurring unavoidable defects that at low levels present no health hazard. Defect action levels are set because it is economically impractical to grow, harvest, or process raw products that are totally free of non-hazardous, naturally, unavoidable defects. Products harmful to consumers are subject to regulatory action whether or not they exceed the defect action levels. The manufacturer, packer, and holder of a dietary supplement must, at all times, utilize quality unit operations that reduce natural or unavoidable defects to the lowest level currently feasible. The mixing of a dietary supplement containing defects at levels that render that dietary supplement adulterated with another lot of dietary supplement is not permitted and renders the final dietary supplement adulterated, regardless of the defect action level of the final dietary supplement.

### 3.1.5 RECEIVED IN-PROCESS (BULK) DIETARY SUPPLEMENT SPECIFICATIONS

Specifications must be established for an in-process (bulk) dietary supplement received from a supplier for packaging and labeling, and for distribution. The specification must provide sufficient assurance that the in-process (bulk) dietary supplement received is adequately identified, consistent with the purchase order, and the CoA meets agreed-upon specifications or supplier quality agreement.

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### 3.1.6 FINISHED DIETARY SUPPLEMENT SPECIFICATIONS

Specifications must be established for the packaging and labeling of the finished packaged and labeled dietary supplements that ensures that the correct packaging and labeling was used.

## 3.2 Determining Compliance with Specifications

[NOTE—*General Notices* should apply.]

### 3.2.1 GENERAL

Quality unit personnel must determine whether the specifications set for components, in-process materials, packaging materials, labeling, in-process (bulk) dietary supplements, received in-process (bulk) dietary supplements, and finished dietary supplements are met.

Quality control (laboratory control) unit personnel must conduct the necessary tests to determine whether the specifications for components, in-process materials, and dietary supplements comply with specifications. On the basis of adequate process verification, in-process controls, and statistical confidence, a reduced testing (or sampling) plan is an alternative to fully testing every batch of material, provided that:

- For components, at least one identification test or examination with sufficient specificity is conducted;
- For packaging material and labeling, at least one identification test is conducted;
- For dietary supplements, at least one measure representative of the overall quality is performed.

Tests that are used to determine whether material specifications are met must be appropriate for their intended use and must be validated methods according to *Validation of Compendial Procedures* ⟨1225⟩.

The quality control unit must conduct an out-of-specification investigation and corrective action plans must be established for use when an established specification is not met.

### 3.2.2 COMPONENTS

Before any component is used in manufacturing, at least one appropriate test or examination must be conducted to verify the identity of the component, and determine whether other applicable component specifications are established by doing the following:

- Conduct appropriate tests or examinations for all of the test specifications; or
- Rely on the certificate of analysis from the supplier of the component received, provided that:
    - The supplier is qualified by establishing the reliability of the supplier's certificate of analysis through confirmation of the results through independent tests, on-site audit, and other appropriate supplier qualification activities;
    - The certificate of analysis includes a description of the specific tests or examination methods used (i.e., specific reference to a test procedure that can be performed by an experienced scientist); acceptance criteria for the tests or examinations, and actual results of the tests or examinations;
    - Documentation is maintained of how the supplier was qualified;
    - The reliability of the supplier's certificate of analysis is periodically re-confirmed by conducting independent tests for the specifications that consistently confirm the values shown in the certificate of analysis;
    - Quality unit personnel have reviewed and approved the documentation setting forth the basis for qualification and re-qualification of the supplier.

### 3.2.3 IN-PROCESS PRODUCTION

In-process points, steps, or stages where control is necessary to ensure the quality of the finished batch of dietary supplements must be monitored to determine whether in-process specifications are met; and to detect any deviation or unanticipated occurrence that may result in the failure of the dietary supplement to meet specifications.

### 3.2.4 DIETARY SUPPLEMENTS

For a subset of dietary supplement batches (for in-process (bulk) dietary supplements and/or finished dietary supplements) that have been identified through a sound statistical sampling plan (i.e., reduced sampling), or for every batch of dietary supplements, the manufacturer must verify that the batch of dietary supplement meets product specifications for identity, purity, strength, and composition, and for limits on those types of contamination that may adulterate or that may lead to adulteration of the dietary supplement, applicable specific tests, performance, and manufacturing quality tests. To do so:

- One or more established specifications for identity, purity, strength, and composition, and the limits on those types of contamination that may adulterate or that may lead to adulteration of the dietary supplement, applicable specific tests, performance, and manufacturing quality tests, that if tested or examined on the batches of dietary supplement (i.e., reduced testing), would verify that the production process control system is producing a dietary supplement that meets all product specifications;
- Appropriate tests or examinations must be conducted to determine compliance with the applicable specifications;
- Adequate documentation must be provided on the basis for determining how compliance with the test specification(s) selected for reduced testing will ensure that the finished batch of dietary supplement will meet all product specifications for identity, purity, strength, and composition, and for the limits on those types of contamination that may adulterate or

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that may lead to adulteration of the dietary supplement, applicable specific tests, performance, and manufacturing quality tests; and

- Quality unit personnel must review and approve all supporting documentation for reduced testing.

### 3.2.5 RECEIVED IN-PROCESS (BULK) DIETARY SUPPLEMENT

Before packaging or labeling a dietary supplement received for packaging or labeling as a dietary supplement, for distribution rather than for return to the supplier, the product must be visually examined, and documentation must be provided or obtained through testing and examination to determine whether the in-process (bulk) product specifications are met.

### 3.2.6 PACKAGING MATERIAL AND LABELING

Before packaging material is used, at minimum, a visual identification of the containers and closures must be conducted, and the supplier's invoice, guarantee, or certification must be reviewed to determine whether the packaging specifications are met.

Before labeling is used, at minimum, a visual identification of the labeling must be conducted, and the supplier's invoice, guarantee, or certification must be reviewed to determine whether the labeling specifications are met.

### 3.2.7 FINISHED DIETARY SUPPLEMENTS

At minimum, a visual examination of the packaging and labeling of the finished packaged and labeled dietary supplement must be conducted to determine whether the packaging used and the label applied were specified in the master packaging control record.

### 3.2.8 DISPOSITION DECISION FOR MATERIALS

For components, in-process materials, packaging materials, labeling, and dietary supplements that meet specifications, the quality unit may approve the material for use in manufacturing.

For components, in-process materials, packaging materials, labeling, and dietary supplements that do not meet specifications, the quality unit must reject the material for use in manufacturing, unless quality unit personnel approve a treatment, an in-process adjustment, or reprocessing that will ensure the quality of the finished dietary supplement, and that the dietary supplement is packaged and labeled as specified in the master packaging record.

For components that do not meet specification(s) for identity (i.e., identification), quality control personnel must reject the component and the component must not be used in manufacturing the dietary supplement.

For a dietary supplement received from a supplier for packaging and/or labeling as a dietary supplement and for distribution rather than for return to the supplier that does not meet specifications, quality control personnel must reject the product and the product may not be packaged or labeled for distribution as a dietary supplement.

The quality unit personnel must not approve and release for distribution any batch or reprocessed batch of dietary supplement:

- For which any component in the batch does not meet its identity specification;
- That does not meet all product specifications;
- That has not been manufactured, packaged, labeled, and held under conditions to prevent adulteration; and
- That was received from a supplier for packaging or labeling as a dietary supplement, and for distribution rather than return to the supplier, for which sufficient assurance is not provided to adequately identify the product and to determine that the product is consistent with its purchase order.

### 3.2.9 TREATMENTS, IN-PROCESS ADJUSTMENTS, AND REPROCESSING

A rejected dietary supplement must not be reprocessed, and a rejected component or any component, packaging material, or labeling must not undergo treatment or in-process adjustment to make it suitable for use in the manufacture of a dietary supplement, unless:

- Quality unit personnel conduct a material review and make a disposition decision that is based on a scientifically valid reason and approves the reprocessing, treatment, or in-process adjustment;
- The reprocessing, treatment, or in-process adjustment will ensure the quality of the finished dietary supplement and that the dietary supplement is packaged and labeled as specified in the master manufacturing record; and
- All components in the batch met its identity (i.e., identification) specification.

For a rejected dietary supplement received from a supplier for packaging and/or labeling as a dietary supplement and for distribution rather than for return to the supplier that does not meet specifications, quality control personnel must reject the product and the product may not be packaged or labeled for distribution as a dietary supplement.

Any batch of dietary supplement that is reprocessed, that contains components that have been treated, or to which in-process adjustments have been made to make them suitable for use in the manufacture of the dietary supplement must be approved by quality unit personnel before release for distribution.

The quality unit personnel must not approve and release for distribution any batch or reprocessed batch of dietary supplement:

- For which any component in the batch does not meet its identity specification;
- That does not meet all product specifications;
- That has not been manufactured, packaged, labeled, and held under conditions to prevent adulteration; and

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- That was received from a supplier for packaging or labeling as a dietary supplement, and for distribution rather than return to the supplier, for which sufficient assurance is not provided to adequately identify the product and to determine that the product is consistent with its purchase order.

## 3.3 Receiving and Release of Materials

### 3.3.1 COMPONENTS, PACKAGING MATERIALS, AND LABELS

The person responsible for receiving components, packaging, or labels should perform a visual inspection of each truck and examine the containers in the shipment that is received for appropriate content label, container damage, or broken seals to determine whether the container condition may have resulted in contamination or deterioration of the components, packaging materials, or labels. The correct temperature of all refrigerated trailers should be verified prior to unloading. The person must visually examine the supplier's invoice, guarantee, or certification in a shipment that is received to ensure the components, packaging materials, or labels are consistent with the purchase order.

The components, packaging materials, and labels must be placed in quarantine, either physically separated from approved goods or within integrated storage locations strictly controlled through a physical or electronic inventory system, before they are used in the manufacture of a dietary supplement until:

- Representative samples are collected of each unique shipment of components, packaging materials, and labels received, and of each unique lot within each unique shipment of components, packaging materials, and labels;
- Quality unit personnel review and approve the results of any tests or examinations conducted on components, packaging materials, and labels; and
- Quality unit personnel approves the components, packaging materials, and labels for use in the manufacture of a dietary supplement, including approval of any treatment or in-process adjustments of components, packaging materials, and labels to make them suitable for use in the manufacture of a dietary supplement, and releases them from quarantine.

Each unique lot within each unique shipment of components, packaging materials, and labels that is received, and any lot of components that are produced, must be identified in a manner that allows a person to trace the lot to the supplier; the date received; the name of the component, packaging material, and label; the status of the component, packaging material, and label (e.g., quarantined, approved, or rejected); and to the dietary supplement that was manufactured and distributed. This unique identifier must be used whenever recording the disposition of each unique lot within each unique shipment of components, packaging material, and labels that was received, and any lot of components produced.

Components, packaging material, and labels must be held under conditions that will protect against contamination and deterioration, and avoid mix-ups.

### 3.3.2 PRODUCT RECEIVED FOR PACKAGING AND LABELING AS A DIETARY SUPPLEMENT

The person responsible for receiving product that is received for packaging or labeling as a dietary supplement, and for distribution rather than for return to the supplier, must visually examine each immediate container or grouping of immediate containers in a shipment that is received for appropriate content label, container damage, or broken seals to determine whether the container condition may have resulted in contamination or deterioration of the received product. The person must visually examine the supplier's invoice, guarantee, or certification in a shipment of received product to ensure the received product is consistent with the purchase order.

The received product must be placed in quarantine before it is used in the packaging and labeling of a dietary supplement until:

- Representative samples are collected of each unique shipment of received product, and of each unique lot within each unique shipment of received product;
- Quality unit personnel review and approve results of any tests or examinations conducted on the received product, and review and approve the documentation supplied with the received product to determine whether the received product meets specifications; and
- Quality unit personnel approves the received product for packaging and labeling as a dietary supplement, and releases the received product from quarantine.

Each unique lot within each unique shipment of received product must be identified in a manner that allows a person to trace the lot to the supplier; the date received; the name of the received product, packaging material, and label; the status of the received product (e.g., quarantined, approved, or rejected); and to the dietary supplement that was packaged and labeled and distributed. This unique identifier must be used whenever recording the disposition of each unique lot within each unique shipment of received product.

Received product must be held under conditions that will protect against contamination and deterioration, and avoid mix-ups.

## 3.4 Representative Samples and Reserve Samples

### 3.4.1 REPRESENTATIVE SAMPLES

Quality unit personnel must collect representative samples:

- Of each unique shipment, and of each unique lot within each unique shipment, of components, packaging material, and labels to be used in manufacturing to determine whether the materials meet established specifications;

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- Of in-process materials for each manufactured batch at points, steps, or stages in the manufacturing process as specified in the master manufacturing record where control is necessary to ensure identity, purity, strength, and composition of dietary supplements, to determine whether in-process materials meet specifications;
- Of each finished manufactured batch dietary supplement before releasing for distribution to verify that the finished batch of dietary supplement meets specifications;
- Of each unique shipment, and of each unique lot within each unique shipment, of product received for packaging or labeling as a dietary supplement and for distribution rather than for return to the supplier to determine whether the received product meets established specifications; and
- Of each lot of packaged and labeled dietary supplement to determine whether the packaging used and the label applied was specified in the master packaging control record.

Design of the sample room should be consistent with controls necessary to prevent cross-contamination.

Written procedures for sampling must be established and followed. The number of containers sampled, and the amount of material to be sampled from each container, should be based upon appropriate criteria such as component variability, statistical confidence levels, and degree of precision desired, the past quality history of the supplier, and the quantity of material needed for analysis and reserve, when required. Sampling procedures should include:

- Cleaning the containers of components selected for sampling prior to opening;
- Containers should be opened, sampled, and resealed in a manner designed to prevent contamination of their contents and contamination of other components, product containers, or closures; and
- Containers should be identified so that the following information can be determined: name of the material sampled, the lot number, the container from which the sample was taken, the date on which the sample was taken, and the name of the person who collected the sample.

Sampling of botanicals should be in compliance with the provisions in ⟨561⟩.

#### 3.4.2 RESERVE SAMPLES

Quality unit personnel must collect and hold reserve samples:

- Of each lot of packaged and labeled dietary supplements that is distributed, which must be held using the same container-closure system in which the packaged and labeled dietary supplement is distributed;
- Of each lot of dietary supplement that is being distributed to be packaged and labeled, which must be held using a container-closure system that provides essentially the same characteristics to protect against contamination or deterioration as the one in which it is distributed for packaging and labeling elsewhere.

Reserve samples of dietary supplements must be identified with the batch, lot, or control number. The reserve sample of each batch of dietary supplements must consist of at least twice the quantity necessary for all tests or examinations to determine whether or not the dietary supplement meets product specifications, and must be collected and retained for at least one (1) year past its shelf life (expiry) date.

### 3.5 Holding, Distribution, and Transportation

Written procedures must be established and followed for holding or warehousing and distributing operations.

#### 3.5.1 HOLDING

Components and dietary supplements must be held under appropriate conditions of temperature, humidity, and light so that the identity, purity, strength, and composition of the components and dietary supplements are not affected. Temperature and humidity of each warehouse must be monitored and should be mapped where appropriate during both the summer and winter to ensure that required storage conditions are controlled and documented. Packaging materials and labeling must be held under appropriate conditions so that the packaging materials and labeling are not adversely affected. Components, dietary supplements, packaging materials, and labeling must be held under conditions that do not lead to the mix-up or contamination of components, dietary supplements, packaging materials, and labeling.

In-process material must be identified and held under appropriate conditions that protect against mix-up and contamination. In-process material must be held under appropriate conditions of temperature, humidity, and light to protect against deterioration.

Components, in-process materials that contain food allergens must be clearly identified and held in a manner that prevents allergen cross-contact.

Spills of allergen-containing components must be cleaned promptly and assessed to determine the potential for cross-contamination to adjacent materials.

Reserve samples of dietary supplements must be held in a manner that protects against contamination and deterioration. Reserve samples of dietary supplements must be held under conditions consistent with the label conditions stated on the product labels, or if no storage conditions are recommended on the label [e.g., for in-process (bulk) dietary supplements], under ordinary storage conditions consistent with expected storage conditions for the finished product while on display at retail. Reserve samples of dietary supplements must be held using the same container-closure system in which packaged and labeled dietary supplements are distributed, or if distributing dietary supplements to be packaged and labeled, using a container-closure system that provides essentially the same characteristics to protect against contamination or deterioration as the one in which dietary supplements will be distributed.

Reserve samples must be retained for one (1) year past the shelf life (expiry) date of the dietary supplement for use in appropriate investigations.

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### 3.5.2 REJECTED MATERIALS

Any component, packaging material, labels, and product received for packaging and labeling as a dietary supplement and for distribution rather than return to the supplier that is rejected and is unsuitable for use in manufacturing, packaging, and labeling operations, must be clearly identified, held, and controlled under a quarantine system for appropriate disposition. The quarantine system for rejected materials must include physical segregation and conspicuous labeling of the material in addition to electronic controls.

### 3.5.3 RETURNED DIETARY SUPPLEMENTS

Returned dietary supplements must be identified and quarantined until quality unit personnel conduct a material review and make a disposition decision.

Returned dietary supplements must be destroyed, or otherwise suitably disposed of, unless the outcome of a material review and disposition decision is that quality control personnel do the following:

- Approve the salvage of the returned dietary supplement for redistribution; or
- Approve the returned dietary supplement for reprocessing.

A returned dietary supplement may be salvaged only if quality control personnel conduct a material review and make a disposition decision to allow the salvage.

Any returned dietary supplements that are reprocessed must meet all in-process (bulk) dietary supplement specifications, and quality control personnel must approve or reject the release for distribution any returned dietary supplement that is reprocessed.

If the reason for a dietary supplement being returned implicates other batches, an investigation must be conducted of the manufacturing processes and each of those other batches to determine compliance with specifications.

### 3.5.4 DISTRIBUTION

Dietary supplements must be distributed under conditions that will protect the dietary supplement against contamination and deterioration. Distributing operations must be designed to facilitate recall of the dietary supplement, if necessary.

### 3.5.5 TRANSPORTATION OPERATIONS

All transportation operations must be conducted under such conditions and controls necessary to prevent dietary supplements from becoming spoiled or adulterated during transportation.

Vehicles and transportation equipment used in transportation operations must be:

- Designed and maintained, and equipped as necessary to provide adequate temperature control to prevent components and dietary supplements requiring temperature control (e.g., chewable gels, soft gelatin capsules) from becoming spoiled during transportation; and
- Stored in a manner that prevents it from harboring pests or becoming contaminated in any other manner that could result in components or dietary supplements for which it will be used becoming adulterated during transportation operations.

Responsibility for ensuring that transportation operations are carried out adequately must be assigned to competent supervisory personnel.

Shippers, receivers, loaders, and carriers engaged in transportation must conduct all transportation operations under such conditions and controls necessary to prevent components and dietary supplements from becoming unsafe during transportation. Such operations include taking effective measures:

- Such as segregation, isolation, or the use of packaging to protect components and dietary supplements from contamination from other articles in the same load;
- To ensure that components and dietary supplements that requires temperature control for safety and quality are transported under adequate temperature control; and
- Restricting the co-shipment of non-food items that might render the components or finished dietary supplements adulterated through cross-contamination.

Shippers must specify to the carrier and the loader all necessary sanitary specifications for the carrier's vehicle and transportation equipment including any specific design specifications (e.g. temperature control) and cleaning procedures.

Loaders must determine, considering as appropriate specifications provided by the shipper, that the vehicle or transportation equipment is in appropriate sanitary and operational condition for the transport of components and dietary supplements.

Receivers, upon receipt of components and dietary supplements, must take steps to adequately assess that the components and dietary supplements were not subjected to adverse conditions that may have caused them to become adulterated.

Carriers must ensure that vehicles and transportation equipment meet the shipper's specifications and are otherwise appropriate to prevent components and dietary supplements from becoming adulterated during transportation operations.

Measures to implement requirements and procedures may be accomplished by the shipper or the carrier or another party involved in transportation operations under a written agreement.

Records should be maintained regarding specifications, agreements, procedures, associated records related to transportation (maintenance, cleaning, previous cargoes, temperature control), and training.

## 3.6 Quality Unit Responsibilities for Materials Management Operations

Before components, packaging materials, and labeling are used in manufacturing, quality unit personnel must:

- Review all receiving records for components, packaging, and labeling;

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- Conduct any required material review and make any required disposition decision;
- Approve or reject any treatment and in-process adjustments of components, in-process materials, packaging, or labeling to make them suitable for use in the manufacture of a dietary supplement; and
- Approve, and release from quarantine, all components, packaging, and labeling before they are used in manufacturing.

For returned dietary supplements, quality unit personnel must:

- Conduct any required material review and make any required disposition decision, including determining whether tests or examinations are necessary to determine compliance with established product specifications, and review the results of any tests or examinations that are conducted to determine compliance with established product specifications;
- Approve or reject any salvage and redistribution of any returned dietary supplements;
- Approve or reject any reprocessing of any returned dietary supplement; and
- Determine whether the reprocessed dietary supplement meets product specifications and either approve for release, or reject, any returned dietary supplement that is reprocessed.

## 3.7 Materials Management Operations and Controls Documentation

The following documentation must be made and kept for fulfilling the requirements of the Material Management Controls System (quality system 3).

### 3.7.1 WRITTEN PROCEDURES

Written procedures must be established and followed for materials management operations and controls, including:

- Preparing, reviewing, and approving material and in-process production specifications;
- Material receiving, sampling, testing, and making a disposition decision for materials to be used in manufacturing;
- Warehousing maintenance, cleaning, and inventory controls;
- Pallet inspection, cleaning, and maintenance;
- Implementing a supplier qualification program;
- Holding and distributing operations;
- Receiving and conducting material reviews and making disposition decisions for returned dietary supplements.

### 3.7.2 RECORDS

Records must be made and kept for:

- Specifications for components, in-process production, packaging materials, labeling, in-process (bulk) dietary supplements, received in-process (bulk) dietary supplements, and finished dietary supplements;
- List of approved suppliers of components, packaging materials and labeling, and corresponding supplier qualification evaluation reports for the purpose of relying on the supplier's certificate of analysis;
- Documentation for why meeting in-process specifications, in combination with meeting component specifications, helps to ensure the dietary supplement meets the specifications for identity, purity, strength, and composition, and for limits on those types of contamination that may adulterate or may lead to adulteration of the finished batch of the dietary supplement;
- Documentation that justifies why a reduced testing program is appropriate;
- Receiving records (including, certificates of analysis, supplier's invoice, and supplier's guarantee) for components, packaging, and labels, and for products received for packaging or labeling as a dietary supplement, and for distribution rather than for return to the supplier;
- Documentation by the person who performs the required operation, at the time of performance, that the required operation was performed, including:
  - The date that the components, packaging, labeling, or products received for packaging or labeling as a dietary supplement were received;
  - The initials of the person performing the required operation;
  - The results of any tests or examinations conducted on components, packaging, or labeling, and of any visual examination of product received for packaging or labeling as a dietary supplement; and
  - Any material review and disposition decision conducted on components, packaging material, labeling, or products received for packaging and labeling as a dietary supplement;
- Inventory control records documenting material history of use;
- Documentation, at the time of performance, demonstrating that quality control unit personnel determined whether the specifications set for materials to be used in manufacturing were met (i.e., performed the required tests and made a disposition decision for materials);
- Dietary supplement distribution;
- Documentation of any material review and disposition decisions on a returned dietary supplement;
- The results of any testing or examination of returned dietary supplements to determine compliance with product specifications; and

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- Documentation of the reevaluation by quality unit personnel of any dietary supplement that is reprocessed and the determination by quality unit personnel of whether the reprocessed dietary supplement meets in-process (bulk) dietary supplement specifications.

# 4. PRODUCTION OPERATIONS AND CONTROLS

## 4.1 Master Manufacturing Records

Written master manufacturing records must be prepared and followed for each unique formulation of dietary supplements manufactured, and for each batch size, to ensure uniformity in the finished batch from batch to batch.

The master manufacturing record may include all manufacturing, packaging, and labeling directions and controls, or be separated into two records: one for manufacturing and another for packaging and labeling. In some cases, such as in soft gelatin product manufacturing, the gelatin manufacturing record may be separated from that of the fill material used to make a specific final product lot.

The master manufacturing record must:

- Identify in-process production specifications for the points, steps, or stages in the manufacturing process where control is necessary to ensure the quality of the dietary supplement and that the dietary supplement is packaged and labeled as specified in the master manufacturing record; and
- Establish controls and procedures to ensure that each batch of dietary supplement that is manufactured meets in-process specifications.

The batch production record must include:

- The name of the dietary supplement to be manufactured and the strength, concentration, weight, or measure of each dietary ingredient and excipient for each batch size;
- A complete list of components to be used;
- An accurate statement of the weight or measure of each component to be used;
- Any necessary weight or measure adjustment due to actual strength or purity of the dietary ingredients with labeled dietary supplements claims;
- The identity and weight or measure of each dietary ingredient that will be declared on the supplement facts label and the identity of each ingredient that will be declared on the ingredients list of the dietary supplement;
- A statement of any intentional overage amount of a dietary ingredient;
- A statement of theoretical yield of a manufactured dietary supplement expected at each point, step, or stage of the manufacturing process where control is needed to ensure the quality of the dietary supplement, and the expected yield at the end of manufacturing the dietary supplement, including the maximum and minimum percentages of theoretical yield beyond which a deviation investigation of a batch is necessary and material review is conducted and disposition is made; and a description of packaging and a representative label, or cross-reference to the physical location of the actual or representative label.

The master manufacturing record must also include written instructions, including the following:

- Specifications for each point, step, or stage in the manufacturing process where control is necessary to ensure the quality of the dietary supplement and that the dietary supplement is packaged and labeled as specified in the master manufacturing record;
- Procedures for sampling and cross-reference to procedures for tests and examinations;
- Specific actions necessary to perform and verify points, steps, or stages in the manufacturing process where control is necessary to ensure the quality of the dietary supplement and that the dietary supplement is packaged and labeled as specified in the master manufacturing record:
    - Such specific actions must include verifying the weight or measure of any component and verifying the addition of any component; and
    - For manual operations, such specific actions must include:
        - One person weighing or measuring a component and another person verifying the weight or measure; and
        - One person adding the component and another person verifying the addition;
- Special notations and precautions to be followed; and
- Corrective action plans for use when a specification is not met.

Detailed production instructions should also include:

- Sequences to be followed;
- Ranges of process parameters to be used;
- Time limits for completion of individual processing steps or the total process, where appropriate.

The dietary supplement must be formulated with the intent to provide 100% of the quantitative label amount of each dietary ingredient and may include overage inputs as necessary to ensure the product meets label claim at the end of shelf life. The contents of the declared dietary ingredients and/or their marker compounds must be consistent with the strengths stated on the label within the tolerances established as acceptance criteria set in the specifications and the local regulatory requirements.

## 4.2 Executed Batch Production Records

An executed batch production record must be prepared every time a batch of dietary supplements is manufactured, and it must include complete information relating to the production and control of each batch. The executed batch production record

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must accurately follow the appropriate master manufacturing record and each step in the production of the batch must be performed.

The executed batch production record must include:

- The batch, lot, or control number of the dietary supplement; of each lot of packaged and labeled dietary supplement from a finished batch of dietary supplement; and each lot of dietary supplement, from the finished batch of dietary supplement, that is distributed to another person for packaging or labeling;
- The identity of equipment and processing lines used in producing the batch;
- The date and time of the maintenance, cleaning, and sanitizing of the equipment and processing lines used in producing the batch, or a cross-reference to records, such as individual equipment logs, where this information is retained;
- The unique identifier that was assigned to each component, or, when applicable, to a product that was received from a supplier for packaging and labeling as a dietary supplement, packaging and label used;
- The identity and weight or measure of each component used;
- A statement of the actual yield and a statement of the percentage of theoretical yield at appropriate phases of processing;
- The actual results obtained during any monitoring operation;
- The results of any testing or examination performed during the batch production, or a cross-reference to such results;
- Documentation that the finished dietary supplement meets the in-process (bulk) dietary supplement and finished dietary supplement specifications.

The executed batch production record must also include documentation, at the time of performance, of the manufacture of the batch, including:

- The date on which each step of the master manufacturing record was performed;
- The initials of the person performing each step, including initials of the person responsible for:
  - Weighing or measuring each component used in the batch;
  - Verifying the weight or measure of each component used in the batch;
  - Adding the component to the batch; and
  - Verifying the addition of components to the batch;
- Documentation, at the time of performance, of packaging and labeling operations, including:
  - The unique identifier that was assigned to packaging and labels used, the quantity of the packaging and labels used, and, when label reconciliation is required, reconciliation of any discrepancies between issuance and use of labels;
  - An actual or representative label, or a cross-reference to the physical location of the actual or representative label specified in the master manufacturing (or packaging and labeling) record; and
  - The results of any tests or examinations conducted on packaged and labeled dietary supplements, including repackaged or relabeled dietary supplements, or a cross-reference to the physical location of such results;
- Documentation at the time of performance that quality unit personnel:
  - Reviewed the batch production record, including review of any monitoring operation required for determining compliance to material specifications, and review of the results of any tests and examinations, including tests and examinations conducted on components, in-process materials, finished batches of dietary supplements, and packaged and labeled dietary supplements;
  - Approved or rejected any reprocessing or repackaging;
  - Approved and released, or rejected, the batch for distribution, including any reprocessed batch;
  - Approved and released, or rejected, the packaged and labeled dietary supplement, including any repackaged or relabeled dietary supplement;
- Documentation at the time of performance or any required material review and disposition decision; and
- Documentation at the time of performance of any reprocessing.

## 4.3 Manufacturing Operations Production and Process Controls

Written procedures must be established and followed for manufacturing operations. Manufacturing processes must be designed and selected to ensure that dietary supplement specifications are consistently met.

All manufacturing operations must be conducted in accordance with adequate sanitation principles.

All necessary precautions must be taken during the manufacture of a dietary supplement to prevent contamination of components or dietary supplements. These precautions include:

- Performing manufacturing operations under conditions and controls that protect against the potential for growth of microorganisms; the potential for allergen cross-contact and contamination; and the potential for the adulteration of raw materials, in-process materials, and finished product;
- Washing and cleaning components that contain soil and other contaminants;
- Using water that, at a minimum, complies with the applicable federal, state, and local requirements and does not contaminate the dietary supplement when the water may become a component of the finished batch of dietary supplement;
- Performing chemical, microbiological, or other testing, as necessary to prevent the use of contaminated components;
- Sterilizing, pasteurizing, freezing, refrigerating, controlling hydrogen-ion concentration (pH), controlling humidity, controlling water activity ($a_w$), or using any other effective means to remove, destroy, or prevent the growth of microorganisms and prevent decomposition;
- Holding components and dietary supplements that can support the rapid growth of microorganisms of public health significance in a manner that prevents the components and dietary supplements from becoming adulterated;

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- Identifying and holding any components or dietary supplements, for which a material review and disposition decision is required, in a manner that protects components or dietary supplements that are not under a material review against contamination and mix-up with those that are under a material review;
- Performing mechanical manufacturing steps (such as cutting, sorting, inspecting, shredding, drying, grinding, blending, and sifting) by any effective means to protect the dietary supplements against contamination by, for example:
  - Cleaning and sanitizing contact surfaces;
  - Using temperature controls; and
  - Using time controls;
- Using effective measures to protect against the inclusion of metal or other foreign material in components or dietary supplements, by, for example, filters or strainers, traps, magnets, or electronic metal detectors;
- Segregating and identifying all containers for a specific batch of dietary supplements to identify their contents and, when necessary, the phase of manufacturing; and
- Identifying all processing lines and major equipment used during manufacturing to indicate their contents, including the name of the dietary supplement and the specific batch or lot number and, when necessary, the phase of manufacturing.

## 4.4 Quality Unit Responsibilities for Production

Quality unit personnel must:

- Review and approve all master manufacturing records (MMR) and all modifications to them;
- Review and approve all master packaging and labeling records (if separate from the MMR) and all modifications to them;
- Review and approve all batch production-related records;
- Review all monitoring required for determining compliance to material specifications;
- Conduct any material review and make any required disposition decisions;
- Approve or reject any treatments, in-process adjustments, and reprocessing;
- Determine whether all in-process production specifications are met;
- Determine whether each finished batch of dietary supplements conforms to its specifications; and
- Make a disposition decision (approving and releasing, or rejecting) each finished batch of dietary supplements, including any reprocessed finished batch of dietary supplements, for distribution.

## 4.5 Disposition Decision for Production

For supplements that meet specifications and that were manufactured, packaged, labeled, and held under conditions to ensure the quality of the dietary supplement, the quality unit may approve and release the batch of dietary supplements for distribution.

The quality unit personnel must not approve and release for distribution any batch or reprocessed batch of dietary supplement:

- For which any component in the batch does not meet its identity specification;
- That does not meet all product specifications;
- That has not been manufactured, packaged, labeled, and held under conditions to prevent adulteration; and
- That was received from a supplier for packaging or labeling as a dietary supplement, and for distribution rather than return to the supplier, for which sufficient assurance is not provided to adequately identify the product and to determine that the product is consistent with its purchase order.

## 4.6 Production Operations and Controls Documentation

The following documentation must be made and kept for fulfilling the requirements of the Production Operations and Controls System (quality system 4).

### 4.6.1 WRITTEN PROCEDURES

Written procedures for manufacturing operations must be established and followed for:

- Preparing and approving master manufacturing records and production instructions;
- Cleaning and inspecting manufacturing facilities;
- Conducting manufacturing production operations; and
- Quality unit responsibilities and operations.

### 4.6.2 RECORDS

Records must be made and kept for manufacturing operations, including:

- Master manufacturing records and executed batch production records;
- Equipment and production suite cleaning records; and
- Documentation of any material reviews and disposition decisions by the quality unit.

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# 5. PACKAGING AND LABELING OPERATIONS AND CONTROLS

## 5.1 General

There must be written procedures designed to ensure that correct packaging materials and labels are used. Packaging and labeling operations must be designed to prevent mix-ups.

There should be written procedures describing the receipt, identification, quarantine, sampling, examination and/or testing and release, and handling of packaging and labeling materials.

Packaging and labeling material should conform to established specifications. Those that do not comply with such specifications should be rejected to prevent their use in operations for which they are unsuitable.

Records should be maintained for each shipment of labels and packaging materials showing receipt, examination or testing, and whether accepted or rejected.

## 5.2 Packaging Materials

### 5.2.1 GENERAL

Necessary actions must be taken to determine whether packaging for dietary supplements meets specifications so that the condition of the packaging will ensure the quality of the dietary supplements [cGMP, 21 CFR §111.410(a)]. Containers must provide adequate protection against deterioration or contamination of the dietary supplement that may occur during transportation and recommended storage.

Chapter ⟨659⟩ provides packaging definitions, auxiliary packaging information, and storage condition definitions relevant to the storage and distribution of dietary supplements, and should be consulted.

Containers must be clean and, where indicated by the nature of the product, sanitized to ensure that they are suitable for their intended use. These containers must not be reactive, additive, or absorptive so as to alter the quality of the dietary supplement beyond specified limits.

If containers (e.g., for components, in-process materials, or bulk material) are re-used, they should be cleaned in accordance with documented procedures and all previous labels should be removed or defaced.

### 5.2.2 TAMPER-RESISTANT PACKAGING

Dietary supplements for retail sale must be packaged in a tamper-resistant package. A tamper-resistant package is one having an indicator or barrier to entry which, if breached or missing, can reasonably be expected to provide visible evidence to consumers that tampering has occurred. To reduce the likelihood of substitution of a tamper-resistant feature after tampering, the indicator or barrier to entry must be distinctive by design or by the use of an identifying characteristic (e.g., a pattern, name, registered trademark, logo, or picture). The term “distinctive by design” means that the packaging cannot be duplicated with commonly available materials or through commonly available processes. A tamper-resistant package may involve a primary container–closure system, a secondary container or carton system, or any combination of systems intended to provide a visual indication of package integrity. The tamper-resistant feature should be designed to remain intact when handled in a reasonable manner during manufacture and distribution, or held at retail display.

## 5.3 Labeling

### 5.3.1 GENERAL

Labeling must comply with applicable federal and state labeling regulations or dietary supplements.

Quantitative label claims for the product must be truthful and accurately reflect the contents of the declared dietary ingredients and/or their marker compounds.

Reference to the *USP–NF* on the label or labeling must be appropriate and completely accurate in accordance with the *General Notices, 3.20 Indicating Conformance*. When the dietary supplement product monograph title appears on the label, the product must fully conform to the specified compendial standard.

Labeling must comply with applicable labeling regulations for dietary supplements, including, but not limited to, listing:

- The term “dietary supplement” or “supplement”;
- The quantity of each dietary ingredient and the correct reference daily intake, listed as percent daily value, as necessary;
- The common or usual name of each ingredient in descending order of predominance by weight, except that dietary ingredients listed in the nutrition label supplement facts need not be repeated in the ingredient list; incidental additives including water, present in a dietary supplement at insignificant levels are exempted from this requirement;
- With respect to a proprietary blend of ingredients, the total quantity of all ingredients in the blend is provided in order of predominance by weight and identified by the term “proprietary blend” or other appropriately descriptive term;
- Products making a claim of health benefit (including statements, symbols, or vignettes) do so in a manner that is consistent with the applicable regulatory requirements; health benefit claims include structure/function claims, health claims, and qualified health claims.

For botanicals, the common or usual name and/or the Latin binomial of the botanical species must be listed on the label. The American Herbal Product Association’s publication, *Herbs of Commerce*, current edition, should be consulted regarding the appropriate common or usual name of a botanical ingredient. Additionally, the proper Latin binomial name should be provided in parenthesis on the label, except that this name is not required when it is synonymous with the common or usual name for

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the botanical species. The label must include the plant part(s) used. The terms "whole", "root", "seed", "aerial", and "leaf" are all acceptable terminology.

Labels for dietary supplements containing iron or iron salts for use as an iron source must include the following required cautionary statement: "WARNING: Accidental overdose of iron-containing products is a leading cause of fatal poisoning in children under 6. Keep this product out of reach of children. In case of accidental overdose, call a doctor or poison control center immediately."

Dietary supplements that contain over 250 mg of elemental iron in a single package must comply with the Consumer Product Safety Commission's regulations for child resistant packaging.

Labeling for dietary supplements must comply with applicable allergen labeling requirements as promulgated in the Food Allergen Labeling and Consumer Protection Act (FALCPA).

The label must include a statement of the necessary storage requirements for the dietary supplement.

The label must accurately state the country of origin for any product of foreign origin imported into the United States.

The name and place (city, state, zip code) of business of the manufacturer, packer, or distributor must be located to the right of the principle display panel. When the name appearing on the label is not that of the actual manufacturer, the name should be qualified in a manner to accurately reflect this relationship (e.g., "Manufactured for ___", "Distributed by ___"). The label must include a domestic address or phone number through which an adverse event report for a dietary supplement may be received.

#### 5.3.2 SHELF LIFE (EXPIRY) DATE

Dietary supplement labeling must state a shelf life (expiry) date that is indicative of the date before which the dietary supplement is ensured to meet applicable specifications of identity, strength, quality, and purity when stored under labeled conditions. The shelf life (expiry) date must be supported by suitable stability data.

There must be a written procedure designed to ensure that the correct shelf life (expiry) date is printed on the finished dietary supplement labeling.

#### 5.3.3 TAMPER-RESISTANT LABELING

Each retail package of a dietary supplement covered by this section must bear a statement that is prominently placed so that consumers are alerted to the specific tamper-resistant feature of the package. The labeling statement should be so placed that it will be unaffected if the tamper-resistant feature of the packaging is breached or missing. If the tamper-resistant feature chosen to meet the requirement above is one that uses an identifying characteristic, that characteristic should be referred to in the labeling statement. For example, the labeling statement on a bottle with a shrink band could say "For your protection, this bottle has an imprinted seal around the neck."

### 5.4 Labeling Issuance and Control

Access to the label storage areas (i.e., label control room) must be limited to authorized personnel. Procedures for labeling issuance and control should be established to ensure that labels are stored in a manner that prevents mix-ups and provides proper identification used to reconcile the quantities of labels issued, used, and returned, and to evaluate discrepancies found between the number of containers labeled and the number of labels issued and used. Such discrepancies should be investigated, and the investigation should be approved by the quality unit(s).

All excess labels bearing batch numbers or other batch-related printing should be destroyed. Returned labels should be maintained. Obsolete and outdated labels, and incorrect labels should be destroyed to ensure that they are not used in any future packaging and labeling operations.

Printing devices used to print labels for packaging operations (e.g., for bulk packaging) should be controlled to ensure that all imprinting conforms to the print specified in the batch production record.

Labeling issuance must be designed to prevent mix-ups. Strict control must be exercised over issuance of labeling for use in finished dietary supplement labeling operations. Labels should be delivered to packaging lines in a secured container (e.g., locked box or cage).

Labeling materials issued for a batch should be carefully examined for proper identity and conformity to the labeling specifications in the batch packaging record. A printed label or copy of the printed label representative of those used must be included in the batch packaging record.

Procedures should be used to reconcile the quantities of labeling issued, used, and returned, and should require evaluation of discrepancies found. If discrepancies are found between the quantity of product finished and the quantity of labeling issued and are outside preset limits based on historical operating data, such discrepancies should be investigated.

If a 100-percent examination for correct labels has been performed by appropriate electronic or electromechanical equipment during or after completion of finishing operations, then label reconciliation is not required for cut or rolled labels.

Returned labeling should be maintained and sorted in a manner to prevent mix-ups and provide proper identification. All excess labeling bearing lot or control numbers should be destroyed and documented.

### 5.5 Packaging and Labeling Operations

Filling, assembling, packaging, labeling, and other related operations must be performed in a manner that ensures the quality of the dietary supplement, and that the dietary supplement is packaged and labeled as specified in the master packaging record. This must be done using any effective mean including the following:

- Cleaning and sanitizing all filling and packaging equipment, utensils, and dietary supplement packaging, as appropriate;
- Protecting manufactured dietary supplements from contamination, particularly airborne contamination;

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- Using sanitary handling procedures;
- Establishing physical or spatial separation of packaging and labeling operations from operations on other components and dietary supplements to prevent mix-ups;
- Identifying, by any effective means, filled dietary supplement containers that are set aside and held in unlabeled conditions for future label operations, to prevent mix-ups;
- Assigning a batch, lot, or control number to each lot of packaged and labeled dietary supplement from a finished batch of dietary supplement; and each lot of dietary supplement, that you distribute to another person for packaging or labeling;
- Examining a representative sample of each batch of the packaged and labeled dietary supplement to determine whether the finished dietary supplement meets its specifications; and
- Suitably disposing of labels and packaging for dietary supplements that are obsolete or incorrect to ensure that they are not used in any future packaging and labeling operations.

There must be written procedures designed to ensure that correct packaging materials and labeling are used. Issuance and use of packaging and labels and reconciliation of any issuance and use discrepancies must be controlled. Before packaging and labeling operations begin, packaging and labeling for each batch of dietary supplements must be examined to determine whether the packaging and labeling conforms to the batch packaging control record. Packaging and labeling materials must be identified by item control number and lot number.

Packaging and labeling facilities must be inspected immediately before use to ensure that all materials not needed for the next packaging operation have been removed, and that packaging and labeling facilities have been properly cleaned. Results of the inspection and cleaning should be documented in the batch packaging control records, the packaging facility log, or other documentation system.

Packaged and labeled dietary supplements should be examined to ensure that containers and packages in the batch have the correct label. This examination should be part of the packaging operation. Results of these examinations must be recorded in the batch packaging control record.

A reserve sample of each batch of finished dietary supplements, consisting of at least twice the quantity necessary for finished dietary supplement specification tests and examinations, must be collected and retained for at least one (1) year past its shelf life (expiry) date.

In-process dietary supplements that are transported outside the manufacturer's control must be sealed in a manner such that, if the seal is breached or missing, the recipient will be alerted to the possibility that the contents may have been altered.

Any packaged and labeled dietary supplement that is rejected for distribution must be clearly identified, held, and controlled under a quarantine system for appropriate disposition.

The complete manufacturing history and control of the packaged and labeled dietary supplement through distribution must be traceable.

## 5.6 Repackaging and Relabeling

Dietary supplements may be repackaged or relabeled only after quality unit personnel have approved such repackaging or relabeling. A representative sample of each batch of repackaged or relabeled dietary supplements must be examined to determine whether the repackaged or relabeled dietary supplements meet all established specifications. Quality unit personnel must approve or reject each batch of repackaged or relabeled dietary supplements prior to its release for distribution.

*Good Repackaging Practices* ⟨1178⟩ should be consulted.

## 5.7 Quality Unit Responsibilities for Packaging and Labeling Operations

For packaging and labeling operations, quality unit personnel must:

- Review the results of any visual examination and documentation to ensure that specifications for product received from a supplier, for packaging and labeling as a return to the supplier, is met;
- Approve, and release from quarantine, all products received for packaging and labeling as a dietary supplement, and for distribution rather than for return to the supplier, before they are used for packaging and labeling;
- Review and approve all records for packaging and labeling operations;
- Determine whether the finished packaged and labeled dietary supplement conforms to established specifications;
- Conduct any required material review and make any required disposition decisions;
- Approve or reject any repackaging of a packaged dietary supplement;
- Approve or reject any relabeling of a packaged and labeled dietary supplement; and
- Approve for release, or reject, any packaged and labeled dietary supplement, including repackaged or relabeled dietary supplements, for distribution.

## 5.8 Packaging and Labeling Operations and Controls Documentation

The following documentation must be made and kept for fulfilling the requirements of the Packaging and Labeling Operations and Controls System (quality system 5).

### 5.8.1 WRITTEN PROCEDURES

Written procedures for packaging and labeling operations must be established and followed for:

- Receipt, identification, quarantine, sampling, examination and/or testing and release, and handling of packaging and labeling materials;

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- Preparation and approval of packaging material and labeling specifications;
- Preparation and approval and master batch packaging and labeling control records;
- Cleaning and inspecting packaging and labeling facilities;
- Filling, assembling, packaging, labeling, and other related operations; and
- Determination and issuance of finished dietary supplement shelf-life (expiry) date to batch packaging and labeling control records.

5.8.2 RECORDS

Records for packaging and labeling must be made and kept for:

- Packaging and labeling specifications;
- Packaging and labeling facility cleaning and inspection records;
- Executed batch packaging and labeling control records; and
- Executed batch repackaging and relabeling control records.

# 6. LABORATORY OPERATIONS AND CONTROLS

## 6.1 General

Written procedures must be established and followed for laboratory operations, including written procedures for the tests and examinations to be conducted to determine whether specifications are met.

Adequate laboratory facilities must be used for whatever testing or examinations are necessary to determine whether components, packaging materials, labeling, in-process materials, and dietary supplements meet specifications.

Laboratory control processes must be established and followed that are reviewed and approved by quality unit personnel, including the following:

- Criteria to be used for establishing appropriate specifications;
- Sampling plans for obtaining representative samples of:
  - Components, packing materials, and labels;
  - In-process materials;
  - Finished batches of dietary supplements;
  - Product that is received for packaging or labeling as a dietary supplement, and for distribution rather than for return to the supplier; and
  - Packaged and labeled dietary supplements;
- Use of criteria for selecting appropriate examination and testing methods;
- Use of criteria for selecting standard reference materials used in performing tests and examinations; and
- Use of test methods and examinations in accordance with established criteria.

Quality control personnel must verify that the laboratory examination and testing methodologies are appropriate for their intended use. Quality control personnel must identify and use appropriate scientifically validated methods for each established specification for which testing or examination is required to determine whether the specification is met.

Specifications, sampling plans, and test procedures, including changes or modifications to them, should be drafted by the appropriate quality control unit personnel and reviewed and approved by the appropriate quality assurance unit personnel. Specifications should be established in accordance with accepted standards and consistent with the manufacturing process.

There must be documented procedures describing sampling, testing, approval and rejection of materials, and recording and storage of laboratory data.

## 6.2 Reagents and Reference Standards

Reagents and standard solutions should be prepared and labeled following written procedures. “Use by” dates should be established and applied as appropriate for analytical reagents and standard solutions.

Primary reference standards should be obtained as appropriate for the manufacture of dietary supplements. The source of each primary reference standard must be documented. Records should be maintained of each primary reference standard’s storage and use in accordance with the supplier’s recommendations. Primary reference standards obtained from an officially recognized source, e.g., USP Reference Standards, may be used without testing if stored under conditions consistent with the supplier’s recommendations.

Where a primary reference standard is not available from an officially recognized source, an “in-house primary standard” must be established. Appropriate testing needs to be performed to establish fully the identity and purity of the “in-house primary reference standard”. Appropriate documentation of this testing should be maintained.

Secondary reference standards should be appropriately prepared, identified, tested, approved, and stored. The suitability of each batch of secondary reference standard should be determined prior to first use by comparing against a primary reference standard. Each batch of secondary reference standards should be periodically requalified in accordance with a written protocol.

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## 6.3 Scientifically Validated Test Procedures

Tests and examinations that are used to determine whether material specifications are met must be scientifically validated methods, and appropriate for their intended use. Tests methods or procedures must meet proper standards of accuracy and reliability.

### 6.3.1 VALIDATION OF TEST PROCEDURES

If the test procedure is not in an official compendium, the procedure must be validated according to ⟨1225⟩. Method performance characteristics include specificity, linearity, range accuracy, precision, detection limit, and quantitation limit, and those of interest may vary depending on the type of test: identification, assay, impurities, or performance.

For microbial test procedures, the following *USP* general chapters should be consulted:

- *Biological Assay Validation* ⟨1033⟩
- *Validation of Alternative Microbiological Methods* ⟨1223⟩
- *Validation of Microbial Recovery from Pharmacopeial Articles* ⟨1227⟩

For performance test procedures, the following *USP* general chapter should be consulted:

- *The Dissolution Procedure: Development and Validation* ⟨1092⟩

### 6.3.2 VERIFICATION OF OFFICIAL TEST PROCEDURES

If the test procedure is in an official compendium, such as *USP–NF* or AOAC International *Official Methods of Analysis*, the procedure only needs to be verified for its suitability under actual conditions of use, according to *Verification of Compendial Procedures* ⟨1226⟩. Verification requirements should be based on an assessment of the complexity of both the procedure and the material to which the procedure is applied. Verification is not required for basic compendial procedures, such as loss on drying, residue on ignition, and simple instrumental determinations, such as pH measurements.

## 6.4 Test Procedures

Test procedures must have clear, complete descriptions, so as to provide an analyst with directions on how to reproducibly perform the given test. Test procedures should include the following information, as applicable:

- Purpose;
- Safety information, if applicable;
- Materials and equipment;
- Procedure, as applicable:
    - System suitability;
    - Preparation of solutions and reagents;
    - Preparation of standards and samples;
    - Instrumental parameters; and
    - Calculations and reporting;
- Review and approval with approval dates;
- Revision history.

The tests and examinations can include gross organoleptic analyses, macroscopic analyses, microscopic analyses, chemical (inorganic and organic) analyses, microbiological analyses, DNA-based analyses, and other scientifically valid methods.

Test procedures should be followed and documented at the time of performance, and any departure from established test procedures should be documented and explained.

All validated procedures must have backup data that is readily available for review and audit.

## 6.5 Transfer of Analytical Test Procedures

Testing materials for compliance to specifications is critical in establishing the quality of the dietary supplement. The transfer of analytical procedures (TAP), also referred to as method transfer, is the documented process that qualifies a laboratory (the receiving unit) to use an analytical test procedure that originated in another laboratory (the transferring unit), thus ensuring that the receiving unit has the procedural knowledge and ability to perform the transferred analytical procedure as intended. *USP* general chapter *Transfer of Analytical Procedures* ⟨1224⟩ summarizes the types of transfers that may occur and outlines the potential components of a transfer protocol, and should be consulted to qualify a laboratory (both internal and external to the manufacturer) to ensure it has the procedural knowledge and ability to perform the test procedure as intended.

## 6.6 Reduced Testing

### 6.6.1 GENERAL

Reduced testing or inspection provides a way of reducing the testing or inspection effort on received components, in-process material, and dietary supplements of high quality, in which all batches are expected to be of the similar quality.

Reduced testing for a particular specified parameter(s) should be based upon one or more of the following:

- Statistical analysis of an adequate quantity of historical test data;

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- Statistical confidence in the capability of the manufacturing process as determined by suitable verification; and
- Ongoing monitoring of the process using recognized statistical process control (SPC) techniques.

Control procedures should be established to monitor the output of manufacturing processes that may cause variability for in-process material and dietary supplements, which may include, but are not limited to: clarity, completeness, or pH of solution; blend uniformity; friability; weight variation or content uniformity; and disintegration or dissolution time.

The following terms are useful when describing reduced testing:

- Lot-by-lot inspection means inspection of products submitted in a series of lots;
- Reduced sampling inspection means a sampling inspection procedure in which some lots in a series are accepted without full testing when the sampling results for a stated number of immediately preceding lots meet stated criteria;
- Inspection sampling frequency means probability that a lot is fully tested (e.g., every fifth lot);
- Disqualification means failure to qualify for reduced sampling inspection;
- Requalification means qualification for a resumption of reduced sampling inspection;
- Supplier qualification means assessment of the supplier's GMP competence so as to implement reduced sampling inspection;
- Product qualification means assessment of product (i.e., component, in-process material, or dietary supplement) quality to determine its suitability for reduced sampling inspection.

### 6.6.2 REDUCED TESTING REQUIREMENTS

Reduced sampling inspection may only be used when both the supplier's GMP quality systems and product (i.e., the component, in-process material, or dietary supplement) are qualified. See *1.7 Supplier Qualification* for guidance.

- Requirements for supplier qualification:
  - The supplier must have established, documented, and followed effective and applicable GMP quality systems for managing quality that involves the active participation of personnel;
  - An assessment of the supplier's GMP quality systems must be performed;
  - Ongoing assessment of the supplier's GMP quality systems should be performed on a specified frequency; and
  - The supplier must not have experienced changes in its GMP quality systems that adversely affect product quality.
- Requirements for product (i.e., component, in-process material or dietary supplement) qualification:
  - An assessment for product qualification should not be made prior to the assessment of the supplier's GMP quality systems;
  - The preceding three (3), at minimum, or more consecutive lots have been accepted, and results of resubmitted lots are not included.

The following are examples of typical reduced inspection and rotational testing that can be performed:

- Reduced testing, for example, a typical approach:
  - Conduct lot-by-lot full inspection on the first 3 lots;
  - If successful, conduct reduced sampling inspection on every fifth (5th) lot for a set period of time;
  - If successful, reduce sampling inspection to every tenth (10th) lot, until there is a failure;
  - Test a minimum of 1 lot every year; and
  - If a failure is encountered, disqualify the supplier and its product, and conduct a root cause investigation and return to full testing of the next 3 lots.
- Rotational testing, for example, a typical approach:
  - Establish testing levels, e.g., I, II, III, specifying what tests on the product specification are to be conducted on a given lot in sequence (i.e., I, II, III), such that all test on the product specification are tested at least once on every 3 lots;
  - A subset of tests should be selected for identification, potency, limit of contaminants, and performance;
  - Tests for key potential contaminants are conducted for every lot; and
  - Some critical tests are conducted for every lot.

### 6.6.3 RECEIVED COMPONENTS, IN-PROCESS MATERIALS, AND DIETARY SUPPLEMENTS

On the basis of adequate supplier qualification, process validation, in-process controls, and statistical confidence, a reduced testing plan is an alternative to fully testing every batch of components received from a supplier provided that at least one identity (i.e., identification) test is conducted. Such identification tests must include sufficient specificity to determine the identity of the component.

Each batch of components received from a supplier that is liable to be contaminated with microorganisms, filth, insect infestation, allergens, aflatoxins, other natural toxins, pesticides, organic impurities (e.g., residual solvents), and/or inorganic impurities (e.g., foreign substances, or other extraneous substances that may be a source of contamination that is objectionable in view of its intended use) must be tested or examined against established specifications for such contamination. In such cases, reduced testing of examination must not apply until risk is mitigated by the supplier.

### 6.6.4 IN-PROCESS MATERIALS AND DIETARY SUPPLEMENTS

On the basis of process validation, in-process controls, and statistical confidence, a reduced testing plan is an alternative to fully testing every batch of in-process material and dietary supplements provided that at least one representative measure (i.e., rotational testing) is performed.

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## 6.7 Out-Of-Specification (OOS) Investigations

Written procedures must be established and followed for investigating critical deviations or the failure of a batch of material to meet specifications (i.e., out-of-specification investigations), and corrective action plans must be established for use when an established specification is not met. The investigation should extend to other batches that may have been associated with the specific failure or deviation. The procedure should require analysis of the data, assessment of whether a significant problem exists, allocation of the tasks for corrective actions, and conclusions. Any resampling and/or retesting after OOS test results should be performed according to a documented procedure. Regulatory guidance for investigating OOS test results should be consulted.

## 6.8 Stability Testing

A documented on-going testing program must be designed to monitor the stability characteristics of dietary supplements, and the results must be used to establish appropriate storage conditions and shelf life (expiry) dates for the dietary supplements. Test procedures used in stability testing must be validated. Stability samples should be stored in container-closure systems that simulate the packaging proposed to distribute the finished dietary supplement in the marketplace. Stability studies should include testing of those attributes of the dietary supplement that are susceptible to change during storage and that influence the quality of the dietary supplement.

Ideally the first three (3), but at minimum one (1), production batch(s) should be placed on the stability monitoring program to establish the product shelf life (expiry) date. Ideally, thereafter, at least one (1) batch per year of manufactured dietary supplement (unless none is produced that year) should be added to the stability monitoring program.

As appropriate, the stability storage conditions for temperature and relative humidity (RH) should be as follows:

- Long term = 25 ± 2° and 60 ± 5% RH;
- Intermediate = 30 ± 2° and 65 ± 5% RH;
- Accelerated = 40 ± 2° and 75 ± 5% RH.

Long term studies can also be performed under the labeled storage conditions, e.g., at room temperature (ambient conditions), or in a cool, dry place (8°–15° and NMT 40% RH). Alternative storage conditions should be considered for products marketed outside the United States in different climatic zones.

The frequency of testing should be sufficient to establish the stability profile of the dietary supplement. The following testing frequencies are recommended:

- Long term = 0, 3, 6, 9, 12, 18, and 24 months;
- Intermediate = 0, 3, 6, 9, and 12 months;
- Accelerated = 0, 1, 2, 3, and 6 months.

For long term studies, frequency of testing should be sufficient to establish the stability profile of the dietary supplement.

Accelerated stability studies are designed to increase the rate of chemical degradation or physical change and are intended to evaluate the effect of short-term excursions outside the labeled storage conditions such as might occur during shipping. Although accelerated stability studies may not adequately reflect the stability of the product under labeled (e.g., long term) conditions, results from accelerated stability can be used to estimate a preliminary shelf life (expiry) date for a dietary supplement that should eventually be supported with data from long term stability studies.

Preliminary shelf life (expiry) dates can be based on pilot scale batches if the pilot batch is at least one-tenth (1/10th) the size of the commercial batch size and the pilot batch employs a manufacturing process that simulates the process to be used on a commercial manufacturing scale. Bench scale batches should not be used for stability studies.

In certain cases, dietary supplements being closely related in composition may be grouped together using one representative dietary supplement formulation with which to obtain stability data to support the shelf life (expiry) date of all dietary supplement formulations within that group. Under such circumstances, sufficient scientific justification should be provided as to support grouping of the dietary supplements for stability purposes.

The design of the stability studies may also bracket dietary supplements such that only dietary supplements on the extremes of certain factors, e.g., strength, package size, are tested. This design assumes that the stability of any intermediate level is represented by the stability of the extremes tested.

Stability studies should be performed for dietary supplements packaged in different container–closure systems [e.g., high density polyethylene (HDPE) bottles, polyethylene terephthalate (PET) bottles, blister packs]. In certain cases, stability data using one type of container–closure system may be used to support stability of the product in a different type of container–closure system (e.g., HDPE versus PET bottles) with supporting scientific justification regarding the moisture, vapor, and light protection characteristics of the two types of container–closure systems.

Long term and accelerated (and intermediate) stability studies should be undertaken according to a prescribed stability protocol. The resultant data must be properly evaluated. The degree of variability of stability data between or within individual batches affects the confidence in the data. The data may show little degradation and little variability such that it is apparent from looking at the data that the product will meet specifications throughout the proposed shelf-life (expiry) date. When the data on a quantitative attribute changes with time and shows significant degradation or significant variability, it is necessary to use formal statistical analysis to determine the shelf life (expiry) date for the dietary supplement. A stability report should be prepared for the dietary supplement stating the proposed storage conditions and shelf life (expiry) date of the dietary supplement(s), along with the stability data and its evaluation.

## 6.9 Laboratory Testing Records

Laboratory control records should include complete data derived from all tests conducted to ensure compliance with established specifications and standards, including examinations and assays, as follows:

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- A description of samples received for testing, including material name or source, batch number or other distinctive code, date sample was taken, and, where appropriate, the quantity and date the sample was received for testing;
- A statement or reference to each test procedure used;
- A statement of the weight or measure of sample used for each test as described by the test procedure;
- Data on or cross-reference to the preparation and testing of reference standards, reagents, and standard solutions;
- A complete record of all raw data generated during each test, in addition to graphs, charts, and spectra from laboratory instrumentation, properly identified to show the specific material and batch tested;
- A record of all calculations performed in connection with the test, including, for example, units of measure, conversion factors, and equivalency factors;
- A statement of the test results and how they compare with established acceptance criteria;
- The signature of the person who performed each test and the date(s) the tests were performed; and
- The date and signature of a second person showing that the original records have been reviewed for accuracy, completeness, and compliance with established procedures.

A certificate of analysis (CoA) should be issued for each batch of dietary supplements. The certificate of analysis should list the name and item code of the dietary supplement, the batch number, the release date, and the expiry date. The CoA should list each test performed, the specific identity of the test procedure, the acceptance limits or criteria, and the results with numerical units, as appropriate. The CoA should be dated and signed by authorized quality unit personnel. For CoA issued for external use, the CoA should list the name, address, and telephone number of the manufacturer.

## 6.10 Quality Control Unit Responsibilities for Laboratory Operations and Controls

For laboratory operations, quality control unit personnel must:

- Review and approve all laboratory control processes associated with the production and process control system;
- Ensure that all tests and examinations required to determine compliance to specifications are conducted; and
- Review and approve the results of all tests and examinations required to determine compliance to specifications.

## 6.11 Laboratory Operations and Controls Documentation

The following documentation must be made and kept for fulfilling the requirements of the laboratory controls system (quality system 6).

### 6.11.1 WRITTEN PROCEDURES

Written procedure for laboratory operations must be established and followed for:

- The tests and examinations conducted to determine whether specifications are met;
- Establishing, reviewing, and approving material specifications;
- Preparation, review, and approval of written test procedures;
- Validating and verifying test procedures for their intended use;
- Selection, preparation, and handling of reagents and reference standards;
- Establishing, reviewing, and approving sampling plans, including reduced sampling inspections;
- Sampling, testing, approval, and rejection of materials, and recording and storage of laboratory data;
- Investigating critical deviations or out-of-specification test results; and
- Establishment and implementation of a stability testing program.

### 6.11.2 RECORDS

Records must be made and kept for:

- Documentation that established laboratory test and examination procedures are followed;
- The person who conducts the testing and examination must document, at the time of performance, that established laboratory methodology is followed;
- Documentation for laboratory tests and examinations must include the data and results of the testing and examination;
- Any modification to an established test procedure;
- Out-of-specification (OOS) investigations and corresponding corrective actions;
- Test procedure method validation or verification protocols and reports, supporting test procedure suitability for use;
- The periodic calibration of laboratory instruments and controls;
- Stability protocols and stability data and reports, supporting a product's shelf life (expiry) date.

# GLOSSARY

**Acceptance criteria:** Predetermined limits (e.g., number, numerical ranges, or other suitable measures) for acceptance of examination or test results.

**Adequate:** Item/area/system/knowledge that meets basic minimum requirements that are needed to accomplish intended purpose.

**Adulteration:** A food shall be deemed to be adulterated if it contains any poisonous or deleterious substance as specified in §402(a)(1)–(2) of the FFDCA: or if it consists of any filthy, putrid, or decomposed substance, or if it is otherwise unfit for food

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as specified in §402(a)(3) of the FFDCA; or if it has been prepared, packed, or held under insanitary conditions as specified in §402(a)(4) of the FFDCA; or if its container is composed of any poisonous or deleterious substance which may render the contents injurious to health as specified in §402(a)(6) of the FFDCA; or if it has been intentionally subjected to radiation as specified in §402(a)(7) of the FFDCA; or if the contents have been altered, or if damage and or inferiority has been concealed as specified in §402(b)(1)–(4); or if it contains a color additive which is unsafe as specified in §402(c); or if it is a dietary supplement or contains a dietary ingredient that presents a significant or unreasonable risk of illness or injury as specified in §402(f)(1); or if it is a dietary supplement that has been prepared, packed, or held under conditions that do not meet current good manufacturing practice regulations as specified in §402(g)(1); or if it has not been transported in compliance with regulations as specified in §402(i).

**Adverse event:** Any undesirable experience associated with the use of a dietary supplement in a person.

**Adverse event report:** A direct communication from an identifiable first-hand reporter of an adverse event that includes at least the following information: an identifiable reporter, an identifiable person(s) who experienced the adverse event(s), an identifiable dietary supplement, and one or more adverse events.

**Allergen cross-contact:** The unintentional incorporation of a food allergen into a dietary supplement.

**Allergen:** A major food allergen as defined in §201(qq) of the FFDCA, means any of the following: (1) milk, egg, fish (e.g., bass, flounder, or cod), Crustacean shellfish (e.g., crab, lobster, or shrimp), tree nuts (e.g., almonds, pecans, or walnuts), wheat, peanuts, and soybeans; (2) a food ingredient that contains protein derived from a food specified in (1), except (A) any highly refined oil derived from a food specified in (1) and any ingredient derived from such highly refined oil, and (B) a food ingredient that is exempt under paragraphs (6) and (7) of §403(w) of the FFDCA.

**Article:** Includes substances (such as components, in-process material, dietary ingredients), products (such as dietary supplements), and materials (such as packaging containers and closures, and labels).

**Audit:** A planned, systematic, objective, independent, and documented examination (through observation, investigation, records review, discussions with employees of the audited firm, and, as appropriate, sampling and laboratory analysis) to assess whether agreed-upon requirements are being met.

**Batch:** A specific quantity of a dietary supplement or other article that is intended to be uniform; that is intended to meet specifications for identity, purity, strength, and composition; and that is produced during a specified time period according to a single manufacturing record during the same cycle of manufacture.

**Batch (or lot) number:** Any distinctive group of letters, numbers, or symbols, or any combination of them, from which the complete history of the manufacturing, packaging, labeling, and/or holding of a batch or lot of dietary supplements can be determined.

**Calibration:** The demonstration that a particular instrument or device produces results within specified limits by comparison with those produced by a reference or traceable standard over an appropriate range of measurements.

**Can:** Used to indicate a possibility or a capability.

**Certificate of analysis (CoA):** A document relating specifically to the results of testing a representative sample drawn from the batch of material. The CoA should list each test performed in accordance with compendial or manufacturer requirements, including reference to the test procedure, the acceptance limits, and the results obtained.

**Code of Federal Regulations:** The *Code of Federal Regulations* (CFR) annual edition is the codification of the general and permanent rules and regulations published in the Federal Register by the executive departments and agencies of the federal government of the United States. It is structured into 50 subject matter titles; title 1 applies to general provisions, and title 21 applies to food and drugs. Titles are broken down into parts, subparts, sections, and paragraphs. For example, 21 CFR §11.10(k)(2) would be read as title 21, part 11 (subpart B, not specified here), section 10, paragraph (k)(2). The CFR annual edition is published by the Office of the Federal Register, National Archives and Records Administration and the Government Publishing Office. In addition to this annual edition, the CFR is published in an unofficial format online in electronic format (eCFR), which is updated daily.

**Component:** Any substance intended for use in the manufacture of a dietary supplement, including those that may not appear in the finished batch of the dietary supplement.

**Composition:** The specified mix of product and product-related substances, i.e., specified mix of food ingredients or excipients and dietary ingredients in a dietary supplement.

**Contact surface:** Any surface that contacts a component, in-process material, or dietary supplement, and those surfaces from which drainage onto the component or dietary supplement, or onto the surfaces that contact the component or dietary supplement, occurs during the normal course of operations. Examples of contact surfaces include containers, utensils, tables, contact surface of equipment, and packaging.

**Contamination:** The undesired introduction of a chemical or microbiological nature, or of foreign matter, into or onto a component, in-process material, or dietary supplement during production, sampling, packaging, repackaging, storage, or transport.

**Contract manufacturer:** A manufacturer performing some aspect of manufacturing on behalf of the original manufacturer.

**Corrective action:** An action that is developed and implemented to ensure that any identified root cause of a nonconformity does not recur.

**Critical:** Describes a manufacturing process step, process condition, test requirement, or other relevant parameter or item that must be controlled within predetermined criteria to ensure the dietary supplement meets its specification.

**Design qualification (DQ):** DQ is the documented collection of activities that defined the functional and operational specifications and intended purpose of the equipment or instrument. DQ states what the user wants the equipment or instrument to do and shows that the selected equipment or instrument is suitable.

**Defect action level:** A level of a non-hazardous, naturally occurring, unavoidable defect at which FDA may regard a food product "unadulterated" and subject to enforcement action under §402(a)(3) of the FFDCA.

**Deviation:** Departure from an approved instruction or procedure, or from an established standard (i.e., a failure, or an out of specification result). Deviations can be planned, or unplanned (i.e., a nonconformity).

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**Dietary ingredient:** An ingredient intended for use or used in a dietary supplement that is: a vitamin; a mineral; an herb or other botanical; an amino acid; a dietary substance for use by man to supplement the diet by increasing the total dietary intake, or a concentrate, metabolite, constituent, extract; or a combination of the aforementioned ingredients.

**Dietary supplement:** A product (other than tobacco) intended to supplement the diet that bears or contains one or more of the following dietary ingredients: a vitamin; a mineral; an herb or other botanical; an amino acid; a dietary substance for use by man to supplement the diet by increasing the total daily intake; or a concentrate, metabolite, constituent, extract or combination of the aforementioned ingredients; that is intended for ingestion in a tablet, capsule, powder, softgel, gelcap, or liquid form; that is not represented for use as a conventional food or as a sole item of a meal or the diet; and is labeled as a dietary supplement; and does include an article approved as a new drug, certified antibiotic, or licensed biologic, and was prior to such approval, certification, or license, marketed as a dietary supplement or food unless the Secretary of Health and Human Services has issued a regulation finding that the article, when used as or in a dietary supplement under the conditions of use and dosages set forth in the labeling for such dietary supplement is unlawful.

**Disposition decision:** A decision to approve or reject material, quality control, and manufacturing related processes or documents, based on a scientifically valid reason. Disposition decisions can be made regarding release of components, in-process materials, dietary supplements, packaging materials, labels, returned dietary supplements, based on a determination of whether or not established specifications are met; or regarding reprocessing treatments and in-process adjustments, master manufacturing records and batch production records, repackaging or relabeling, calibrations of an instrument or control, modification or deviations from standard procedures, and whether or not a deviation or unanticipated occurrence during the production and in-process control system could lead to adulteration of a dietary supplement.

**Documentation:** Written material related to the requirements of the quality system, including paper and electronic records, that consist of raw data, reports, protocols, and procedures related to manufacturing controls and laboratory testing. Official documents are records that support procedures, products, or the manufacturer's quality systems (e.g., standard operating procedures, master batch records, material specifications, standard test procedures, protocols, reports). Source documents are records that contain original data or information (e.g., forms, notebooks, logbooks, instrument printouts).

**Equipment:** Devices used to manufacture, package, or hold dietary supplements, or to maintain or clean the physical plant and devices.

**Facility:** A place, amenity, utility, or piece of equipment provided for a particular purpose.

**Federal Food, Drug, and Cosmetic Act:** Federal Food, Drug and Cosmetic Act (FFDCA or FD&C Act) [United States Code (USC) title 21, chapter 9] is the official compilation and codification of the federal statutes of the US that gives authority to the Food and Drug Administration (FDA) oversee the safety of food, drugs, medical devices and cosmetics in the US.

**Ingredient:** Any substance that is used in the manufacture of a dietary supplement and that is intended to be present in the finished batch of the dietary supplement.

**In-process control (or process control):** Checks performed during production in order to monitor and, if appropriate, to adjust the process and/or to ensure the dietary supplement conforms to its specification.

**In-process material:** Any material that is fabricated, compounded, blended, ground, extracted, sifted, sterilized, derived by chemical reaction, or processed in any other way for use in the manufacture of a dietary supplement.

**In-process test:** A test that may be performed during the manufacture of the dietary supplement, rather than as part of the formal battery of tests that are conducted prior to release.

**Instrument (or instrumentation):** A sophisticated measuring device or tool used for analytical or scientific work.

**Installation qualification (IQ):** The documented collection of activities necessary to establish that an equipment or instrument is delivered as designed and specified, is properly installed in the selected environment, and that the environment is suitable for the instrument.

**Label:** A display of written, printed, or graphic matter upon the immediate container of a dietary supplement.

**Labeling:** All labels and other written, printed, or graphic matter upon any dietary supplement or any of its containers, or wrappers, or accompanying such dietary supplement at any time while the dietary supplement is held for sale after shipment or delivery for shipment in interstate commerce. The term "accompanying" is interpreted liberally to mean more than physical association with the dietary supplement. It extends to posters, tags, pamphlets, circulars, booklets, brochures, fillers, etc.

**Lot:** A batch, or a specific identified portion of a batch, that is intended to be uniform and that is intended to meet specifications for identity, purity, strength, and composition.

**Manufacture:** All operations of receipt of components, production, packaging, labeling, quality control testing, release, storage, and distribution of dietary supplements and related controls.

**Material:** A general term used to denote components, in-process materials, dietary supplements, and packaging and labeling material.

**May:** Used to indicate a permission.

**Microorganism:** Any yeasts, molds, bacteria, viruses, or other microscopic organisms having public health or sanitary concern. This includes undesirable species that may have public health significance; that may cause a component or dietary supplement to decompose; that indicate that a component or dietary supplement is contaminated with filth; or that otherwise may cause a component a dietary supplement to be adulterated.

**Must:** Used to state a requirement.

**Nonconformity:** An event that occurs when a specified requirement is not met or results from an undesirable situation or defect. This covers departure from a procedure, standard or requirement, or the absence of dependability. This typically arises from the inability to meet documented procedures, expectations or specifications.

**Operational qualification (OQ):** OQ is the documented collection of activities necessary to demonstrate that an equipment or instrument will function according to its operational specification testing in the selected environment. OQ demonstrates fitness for the selected use and demonstrates user requirement specifications.

**Packaging material:** Container and/or closure for components, in-process material and dietary supplements.

**Performance qualification (PQ):** PQ is the documented collection of activities necessary to demonstrate that an equipment or instrument consistently performs according to the specifications defined by the user, and is appropriate for the intended use. PQ verifies the fitness for purpose of the equipment or instrument under actual conditions of use.

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**Pest:** Any objectionable insect or other animal including, but not limited to, birds, rodents, flies, mites, and larvae.

**Physical plant:** All or any part of a building or facility used for or in connection with manufacturing, packaging, labeling, or holding of a dietary supplement.

**Preventive action:** An action taken to eliminate the cause of potential nonconformities or other undesirable situations in order to prevent occurrence, and continuously improve an existing system or process.

**Preventive controls qualified individual (PCQI):** A qualified individual who has successfully completed training in the development and application of risk-based preventive controls at least equivalent to that received under a standardized curriculum recognized as adequate by FDA or is otherwise qualified through job experience to develop and apply a food safety system.

**Procedure:** A documented description of the operations to be performed, the precautions to be taken, and measures to be applied directly or indirectly related to the manufacture of a dietary supplement.

**Process evaluation:** A set of tests performed on a process intended to evaluate its capacity to consistently produce the results that it is intended for.

**Product complaint:** Any communication that contains any allegation, written, electronic, or oral, expressing concern, for any reason, with the quality of a dietary supplement, that could be related to current manufacturing practice.

**Purity:** The identity and amount of a substance that is the intended substance, typically expressed in units of percentage, and determined using tests for assay or content.

**Qualification:** Action of proving and documenting that equipment or ancillary systems are properly, work correctly, and actually lead to the expected results. Qualification is part of validation, but the individual qualification steps alone do not constitute process validation.

**Quality:** Means that the components, in-process materials, packaging materials, labeling, and dietary supplements meet established specifications for identity, purity, strength and composition, and limits on contaminants, and that the dietary supplement has been manufactured, packaged, labeled, and held under conditions to prevent adulteration.

**Quality assurance:** The sum total of the organized arrangements made with the goal of ensuring that all dietary supplements are of the quality required for their intended use and that quality systems are maintained.

**Quality assurance personnel (or unit):** Any person, persons or group, within or outside of the firm who is designated by the firm to be responsible for the duties relating to quality assurance.

**Quality control:** A planned and systematic operation or procedure necessary to ensure the quality of the dietary supplement, by checking or testing that specifications are met.

**Quality control personnel (or unit):** Any person, persons or group, within or outside of the firm, who is designated by the firm to be responsible for the duties relating to quality control operations.

**Quality unit (or personnel):** An organizational unit independent of production, which fulfills both quality assurance and quality control responsibilities, which can be in the form of separate quality assurance and quality control units or a single individual or group, depending upon the size and structure of the organization.

**Quarantine:** The status of materials isolated physically or by other effective means pending a decision on their subsequent disposition decision.

**Records:** Records are documents stating results achieved or providing evidence of activities performed, e.g., training records, executed batch production records, executed batch packaging records, validation reports, laboratory test results forms, laboratory notebooks, equipment logbooks, memos, and emails.

**Reduced testing (**or **sampling):** A reduced level of testing (or sampling) for a particular specified parameter(s) based upon one or more of the following: statistical analysis of an adequate quantity of historical test data; statistical confidence in the capability of the manufacturing process as determined by suitable verification; or ongoing monitoring of the process using recognized statistical process control (SPC) techniques.

**Reference standard, primary:** A substance that has been shown by an extensive set of analytical tests to be authentic material that should be of high purity. This standard is obtained from an officially recognized source, i.e., USP RS.

**Reference standard, secondary:** A substance of established quality and purity, as shown by comparison to a primary reference standard, used as a reference standard for routine laboratory analysis.

**Remedial (short term) action:** Action taken to provide temporary control over a situation or condition until permanent corrective actions are implemented (e.g., issuance of a temporary procedure, segregation of material).

**Representative sample:** A sample that consists of an adequate number of units that are drawn based on rational criteria, such as random sampling, and that is intended to ensure that the sample accurately portrays the material being sampled.

**Reprocessing:** Introducing clean and uncontaminated components or dietary supplements, back into the manufacturing process and repeating a step or other appropriate physical manipulation step (e.g., milling, packaging) that are part of the established manufacturing process. Continuation of a manufacturing process step after an in-process control test has shown that the step is incomplete is considered to be part of the normal manufacturing process and not reprocessing.

**Responsible party:** The person who submits the registration under section 415(a) of the FD&C Act (21 USC 350(d)) for a food facility that is required to register under section 415(a), at which such article of food is manufactured, processed, packed, or held. Persons who are required to submit a facility registration under section 415 of the FD&C Act are the owner, operator, or agent in charge of a domestic or foreign facility engaged in manufacturing, processing, packing, or holding food for consumption in the United States. “Person” is defined in section 201(e) of the FD&C Act (21 USC 321(e)) as including individuals, partnerships, corporations, and associations.

**Reworking:** Subjecting in-process material or dietary supplements that do not conform to standards or specifications and that have been removed from processing for reasons other than unsanitary conditions, to one or more processing steps that are different from the established manufacturing process to obtain acceptable quality in-process material or dietary supplement.

**Reserve sample:** A representative sample of an article that is held for a designated period of time.

**Risk:** The combination of the probability of occurrence of harm and the severity of that harm.

**Risk assessment:** A systematic process of organizing information to support a risk decision to be made within a risk management process. It consists of the identification of hazards and the analysis and evaluation of risks associated with exposure to those hazards.

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**Risk control:** Actions implementing risk management decisions to reduce risk and/or accept risks in order to reduce risk to an acceptable level.

**Risk management (quality):** A systematic process for the assessment, control, communication, and review of risks to the quality of the dietary supplement throughout the dietary supplement's shelf life. Risk management tools that might be used in quality risk management include facilitation methods (e.g., flowcharts, check sheets, process mapping, cause and effect diagrams); failure mode effects analysis (FMEA); failure mode, effects, and criticality analysis (FMECA); fault tree analysis (FTA); hazard analysis and critical control points (HACCP); and supporting statistical tools (e.g., control charts, histograms, pareto charts, process capability analysis, design of experiments).

**Root cause:** Any cause that is acted upon by a solution such that the problem does not recur.

**Sanitize:** To adequately treat cleaned equipment, containers, utensils, or other cleaned contact surface by a process that is effective in destroying vegetative cells of microorganisms of public health significance, and in substantially reducing numbers of other microorganisms, but without adversely affecting the product or its safety for the consumer.

**Serious adverse event:** An event that results in death, a life-threatening experience, inpatient hospitalization, a persistent or significant disability or incapacity, or a congenital anomaly or birth defect; or requires based on a reasonable medical judgment, a medical or surgical intervention to prevent an outcome previously described.

**Serious adverse event report:** A report that must be submitted to the FDA using the MedWatch form when a manufacturer, packer, or distributor of a dietary supplement receives any report of a serious adverse event associated with the use of the dietary supplement in the United States. (See §761(a)(3) and (b)(1) of the FD&C Act [21 USC §379aa–1(a)(3) and (b)(1)]).

**Shelf-life (use by** or **expiry) date:** The date before which the dietary supplement is ensured to meet applicable specifications of identity, strength, quality, and purity, when stored under labeled conditions.

**Should:** Used to indicate a recommendation.

**Signature (signed):** The record of the individual who performed a particular action or review. This record can be initials, full handwritten signature, or authenticated and secure electronic signature.

**Specification:** A list of tests, references to test procedures, and appropriate acceptance criteria for the tests described. It establishes the set of criteria to which a material should conform to be considered acceptable for its intended use. "Conformance to specifications" means that the component, in-process material, and/or dietary supplement, when tested according to the listed test procedures, will meet the listed acceptance criteria. Specifications are critical quality standards that are proposed, justified and approved by the manufacturer. Material specifications include test specifications for identity, purity, strength, limits on contaminants, and/or performance that define a standard of quality for the material.

**Strength:** The concentration or amount of a dietary ingredient per unit serving of a dietary supplement. Strength only applies to a dietary supplement.

**Utensil:** A simple tool or device serving a useful purpose.

**Utility system (utility):** A useful system and source of material such as water, steam, gases, compressed air, heating, ventilation, and air conditioning (HVAC).

**Validation:** A documented program that provides a high degree of assurance that a specific process, method, or system will consistently produce a result meeting predetermined acceptance criteria.

**Validation protocol:** A written plan stating how validation will be conducted and defining acceptance criteria. For example, the protocol for a manufacturing process identifies manufacturing equipment, critical process parameters/operating ranges, product characteristics, sampling, test data to be collected, number of validation runs, and acceptable test results.

**Water activity ($a_w$):** A measure of the free moisture in a component or dietary supplement and is the quotient of the water vapor pressure of the substance divided by the vapor pressure of pure water at the same temperature.

**Yield, actual:** The quantity of material or percentage of theoretical yield that is actually produced at any appropriate step of production, or packaging and labeling of a particular dietary supplement.

**Yield, expected:** The quantity of material or the percentage of theoretical yield anticipated at any appropriate phase of production, or packaging and labeling, based on previous laboratory, pilot scale, or manufacturing data.

**Yield, theoretical:** The quantity of material that would be produced at any appropriate step of manufacture or packaging and labeling of a particular dietary supplement, based on the quantity of components, packaging labeling to be used, in the absence of any loss or error in actual production.

**Reference Table: Sections of 21 CFR Used in this Chapter**

| ⟨2750⟩ | 21 CFR |
|---|---|
| 1. QUALITY MANAGEMENT | |
| 1.2 Organization and Personnel | §§111.105(a), 111.12(a)–(c), 111.113(a)–(c), 111.14(b)(2),111.10(a)(1)&(2), and 111.10(b)(1)–(9) |
| 1.3 Documentation and Records | §§111.605(a)–(c) and 111.610(a)–(b) |
| 1.5 Deviations and Material Reviews | §§111.103, 111.140(b)[(1)–(2)(i)–(ii)&(3)], 111.87, 111.113(a)–(b), and 111.77(a) |
| 1.7 Supplier Qualification | §§117.405(a)–(c), 117.126(b), 117.410(a)(1)–(2), (b)(1)–(4)&(d), 117.420(a), 117.430(a), 117.425, and 111.105(b) |
| 1.9 Complaints, Serious Adverse Event Reporting, and Recalls | §§111.553, 111.570(b)(1)–(2), 111.560(a)(1)–(2)(b)&(c), and 111.135 |
| 1.12 Quality Management Documentation | §§111.14(b)(1), 111.103, 140(b)(1)–(2), 111.553, and 111.570(b)(1)–(2) |
| 2. PHYSICAL PLANT AND EQUIPMENT | |
| 2.1 Grounds Maintenance | §111.15(a)(1)–(5) |
| 2.2 Physical Plant Design | §111.20 |

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**Reference Table: Sections of 21 CFR Used in this Chapter** *(continued)*

| 〈2750〉 | 21 CFR |
|---|---|
| 2.3 Physical Plant Sanitation and Facilities | §§111.15 and 117.20(b)(2) |
| 2.4 Equipment, Instruments, and Utensils | §§111.27(a)–(d), 111.30(a)–(d), 111.117(a)–(d), and 111.605(c) |
| 2.5 Physical Plant and Equipment Documentation | §§111.16, 111.23, 111.25(a)–(c), and 111.35(b)(1)–(6) |
| 3. MATERIALS MANAGEMENT AND CONTROLS | |
| 3.1 Establishing Material and In-Process Production Specifications | §111.70(a)–(g) |
| 3.2 Determining Compliance with Specifications | §111.73, 111.105(d)–(h), 111.75(a)–(i), 111.77(a)–(c), 111.123(b)(1)–(4), and 111.90 |
| 3.3 Receiving and Release of Materials | §§111.155, 111.160, and 111.165 (a)–(e) |
| 3.4 Representative Samples and Reserve Samples | §§111.80 and 111.83 |
| 3.5 Holding, Distribution, and Transportation | §§111.453, 111.455, 111.460 (a)–(b), 117.80(b)(8), 111.465(a)–(b), 111.170, 111.370, 111.510, 111.515, 111.520, 111.525, 111.470, 1.908, 1.906, 1.912 |
| 3.6 Quality Unit Responsibilities for Materials Management Operations | §§111.120 and 111.130(a)–(d) |
| 3.7 Materials Management Operations and Controls Documentation | §§111.180(b)(1)–(3), 111.475(b), 111.503, 111.535(b)(1), 111.95(b)(1)–(4), and 111.140(b)(2) |
| 4. PRODUCTION OPERATIONS AND CONTROLS | |
| 4.1 Master Manufacturing Records | §§111.205(b) and 111.210(a)–(h) |
| 4.2 Executed Batch Production Records | §§111.255(a)–(c) and 111.260(a)–(n) |
| 4.3 Manufacturing Operations Production and Process Controls | §§111.355, 111.360, and 111.365 |
| 4.4 Quality Unit Responsibilities for Production | §111.123 |
| 4.5 Disposition Decision for Production | §111.123(b)(1)–(4) |
| 5. PACKAGING AND LABELING OPERATIONS AND CONTROLS | |
| 5.2 Packaging Materials | §111.410(a) |
| 5.4 Labeling Issuance and Control | §111.410(b) |
| 5.5 Packaging and Labeling Operations | §§111.415(a)–(h), 111.410, 111.83(b)(3)&(4), and 111.425 |
| 5.6 Repackaging and Relabeling | §111.420(a)–(c) |
| 5.7 Quality Unit Responsibilities for Packaging and Labeling Operations | §111.127(a)–(h) |
| 6. LABORATORY OPERATIONS AND CONTROLS | |
| 6.1 General | §§111.310(a)–(c) and 111.315 |
| 6.10 Quality Control Unit Responsibilities for Laboratory Operations and Controls | §111.110(a)–(c) |
| 6.11 Laboratory Operations and Controls Documentation | §§111.325(b)(1), 111.325(b)(1)–(2), and 111.325(b)(2)(i)–(ii)▲ (USP 1-Aug-2021) |

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# ALCOHOLOMETRIC TABLE

***Change to read:***

## ▲INTRODUCTION

Ethanol ($C_2H_5OH$) content of water mixtures is based on specific gravity (also known as relative density) and is calculated using the *Alcoholometric Table*. The information in the table is based on a method and specifications that were developed from regulations from the Bureau of Alcohol, Tobacco, Firearms and Explosives (ATF). The table is based on the measurement of alcohol at 15.56° (60° F) as standard temperature for alcohol determination. When alcohol and water are mixed together, the volume of the mixture is invariably less than the sum of the initial volumes, and the degree of contraction varies with the proportion of alcohol present. Therefore, the specific gravities of alcohol and water mixtures in all proportions and at various temperatures have been established experimentally.

Relevant values have been reproduced in the *Alcoholometric Table* to help stakeholders perform the *Specific Gravity* test in air at 25°, if a stable 15.56° is not achievable. The table includes the specific gravities and the corresponding percentages of alcohol by weight and by volume (v/v) for the aqueous alcoholic mixtures most commonly used in compendial applications. Comprehensive values can be found in other sources (*1*). The specific gravities are reduced to air values and represent the ratio of the weight of a given volume of spirit to the weight of the same volume of water at 15.56°, under the same atmospheric conditions.

Therefore, the objective is for the user to determine the specific gravity of a sample mixture of alcohol and water in air at 25°. Then, the user locates the specific gravity value in either column *1* or *5*, depending on whether the percentage of ethanol is required to be known v/v (columns *3* or *8*) or by weight (columns *4* or *7*). For example, a specific gravity value of 0.9480 in column *5* corresponds to a solution that contains 33% alcohol by weight or 39.59% alcohol v/v. Similarly, a specific gravity value of 0.8092 in column *1* corresponds to a solution that contains 95% alcohol ▲v/v▲ (ERR 1-Dec-2021) or 92.42% alcohol ▲by weight.▲ (ERR 1-Dec-2021) If the user is able to determine the specific gravity of a sample mixture of alcohol and water in air at 15.56°, then the specific gravity value in either column *2* or *6* can be used to find the respective percentage of ethanol v/v (columns *3* or *8*) or by weight (columns *4* or *7*). If the measured specific gravity falls between two values in the table, a simple linear interpolation or a spline fit can be applied to determine the percentage of ethanol.

This Alcoholometric Table is only valid for ethanol–water mixtures. It does not apply if other components are added. For example, this table is not applicable for hand sanitizer solutions.

The following table is based on data appearing in the *Bulletin of the Bureau of Standards*, a publication of the National Bureau of Standards (now the National Institute of Standards and Technology) (*2*).▲ (USP 1-Dec-2021)

| (1) | (2) | (3) | (4) | (5) | (6) | (7) | (8) |
|---|---|---|---|---|---|---|---|
| Specific gravity in air | | Percentage of ethanol ($C_2H_5OH$) | | Specific gravity in air | | Percentage of ethanol ($C_2H_5OH$) | |
| At 25°/25° | At 15.56°/15.56° | By volume (v/v) at 15.56° | By weight | At 25°/25° | At 15.56°/15.56° | By weight | By volume (v/v) at 15.56° |
| 1.0000 | 1.0000 | 0 | 0.00 | 1.0000 | 1.0000 | 0 | 0.00 |
| 0.9985 | 0.9985 | 1 | 0.80 | 0.9981 | 0.9981 | 1 | 1.26 |
| 0.9970 | 0.9970 | 2 | 1.59 | 0.9963 | 0.9963 | 2 | 2.51 |
| 0.9956 | 0.9956 | 3 | 2.39 | 0.9945 | 0.9945 | 3 | 3.76 |
| 0.9941 | 0.9942 | 4 | 3.19 | 0.9927 | 0.9928 | 4 | 5.00 |
| 0.9927 | 0.9928 | 5 | 4.00 | 0.9911 | 0.9912 | 5 | 6.24 |
| 0.9914 | 0.9915 | 6 | 4.80 | 0.9894 | 0.9896 | 6 | 7.48 |
| 0.9901 | 0.9902 | 7 | 5.61 | 0.9879 | 0.9881 | 7 | 8.71 |
| 0.9888 | 0.9890 | 8 | 6.42 | 0.9863 | 0.9867 | 8 | 9.94 |
| 0.9875 | 0.9878 | 9 | 7.23 | 0.9848 | 0.9852 | 9 | 11.17 |
| 0.9862 | 0.9866 | 10 | 8.05 | 0.9833 | 0.9839 | 10 | 12.39 |
| 0.9850 | 0.9854 | 11 | 8.86 | 0.9818 | 0.9825 | 11 | 13.61 |
| 0.9838 | 0.9843 | 12 | 9.68 | 0.9804 | 0.9812 | 12 | 14.83 |
| 0.9826 | 0.9832 | 13 | 10.50 | 0.9789 | 0.9799 | 13 | 16.05 |
| 0.9814 | 0.9821 | 14 | 11.32 | 0.9776 | 0.9787 | 14 | 17.26 |
| 0.9802 | 0.9810 | 15 | 12.14 | 0.9762 | 0.9774 | 15 | 18.47 |
| 0.9790 | 0.9800 | 16 | 12.96 | 0.9748 | 0.9763 | 16 | 19.68 |
| 0.9778 | 0.9789 | 17 | 13.79 | 0.9734 | 0.9751 | 17 | 20.88 |
| 0.9767 | 0.9779 | 18 | 14.61 | 0.9720 | 0.9738 | 18 | 22.08 |
| 0.9756 | 0.9769 | 19 | 15.44 | 0.9706 | 0.9726 | 19 | 23.28 |

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| (1) | (2) | (3) | (4) | (5) | (6) | (7) | (8) |
|---|---|---|---|---|---|---|---|
| Specific gravity in air | | Percentage of ethanol ($C_2H_5OH$) | | Specific gravity in air | | Percentage of ethanol ($C_2H_5OH$) | |
| At 25°/25° | At 15.56°/15.56° | By volume (v/v) at 15.56° | By weight | At 25°/25° | At 15.56°/15.56° | By weight | By volume (v/v) at 15.56° |
| 0.9744 | 0.9759 | 20 | 16.27 | 0.9692 | 0.9714 | 20 | 24.47 |
| 0.9733 | 0.9749 | 21 | 17.10 | 0.9677 | 0.9701 | 21 | 25.66 |
| 0.9721 | 0.9739 | 22 | 17.93 | 0.9663 | 0.9688 | 22 | 26.85 |
| 0.9710 | 0.9729 | 23 | 18.77 | 0.9648 | 0.9675 | 23 | 28.03 |
| 0.9698 | 0.9719 | 24 | 19.60 | 0.9633 | 0.9662 | 24 | 29.21 |
| 0.9685 | 0.9708 | 25 | 20.44 | 0.9617 | 0.9648 | 25 | 30.39 |
| 0.9673 | 0.9697 | 26 | 21.29 | 0.9601 | 0.9635 | 26 | 31.56 |
| 0.9661 | 0.9687 | 27 | 22.13 | 0.9585 | 0.9620 | 27 | 32.72 |
| 0.9648 | 0.9676 | 28 | 22.97 | 0.9568 | 0.9605 | 28 | 33.88 |
| 0.9635 | 0.9664 | 29 | 23.82 | 0.9551 | 0.9590 | 29 | 35.03 |
| 0.9622 | 0.9653 | 30 | 24.67 | 0.9534 | 0.9574 | 30 | 36.18 |
| 0.9609 | 0.9641 | 31 | 25.52 | 0.9516 | 0.9558 | 31 | 37.32 |
| 0.9595 | 0.9629 | 32 | 26.38 | 0.9498 | 0.9541 | 32 | 38.46 |
| 0.9581 | 0.9617 | 33 | 27.24 | 0.9480 | 0.9524 | 33 | 39.59 |
| 0.9567 | 0.9604 | 34 | 28.10 | 0.9461 | 0.9506 | 34 | 40.72 |
| 0.9552 | 0.9590 | 35 | 28.97 | 0.9442 | 0.9488 | 35 | 41.83 |
| 0.9537 | 0.9576 | 36 | 29.84 | 0.9422 | 0.9470 | 36 | 42.94 |
| 0.9521 | 0.9562 | 37 | 30.72 | 0.9402 | 0.9451 | 37 | 44.05 |
| 0.9506 | 0.9548 | 38 | 31.60 | 0.9382 | 0.9432 | 38 | 45.15 |
| 0.9489 | 0.9533 | 39 | 32.48 | 0.9362 | 0.9412 | 39 | 46.24 |
| 0.9473 | 0.9517 | 40 | 33.36 | 0.9341 | 0.9392 | 40 | 47.33 |
| 0.9456 | 0.9501 | 41 | 34.25 | 0.9320 | 0.9372 | 41 | 48.41 |
| 0.9439 | 0.9485 | 42 | 35.15 | 0.9299 | 0.9352 | 42 | 49.48 |
| 0.9421 | 0.9469 | 43 | 36.05 | 0.9278 | 0.9331 | 43 | 50.55 |
| 0.9403 | 0.9452 | 44 | 36.96 | 0.9256 | 0.9310 | 44 | 51.61 |
| 0.9385 | 0.9434 | 45 | 37.87 | 0.9235 | 0.9289 | 45 | 52.66 |
| 0.9366 | 0.9417 | 46 | 38.78 | 0.9213 | 0.9268 | 46 | 53.71 |
| 0.9348 | 0.9399 | 47 | 39.70 | 0.9191 | 0.9246 | 47 | 54.75 |
| 0.9328 | 0.9380 | 48 | 40.62 | 0.9169 | 0.9225 | 48 | 55.78 |
| 0.9309 | 0.9361 | 49 | 41.55 | 0.9147 | 0.9203 | 49 | 56.81 |
| 0.9289 | 0.9342 | 50 | 42.49 | 0.9124 | 0.9181 | 50 | 57.83 |
| 0.9269 | 0.9322 | 51 | 43.43 | 0.9102 | 0.9159 | 51 | 58.84 |
| 0.9248 | 0.9302 | 52 | 44.37 | 0.9079 | 0.9137 | 52 | 59.85 |
| 0.9228 | 0.9282 | 53 | 45.33 | 0.9056 | 0.9114 | 53 | 60.85 |
| 0.9207 | 0.9262 | 54 | 46.28 | 0.9033 | 0.9092 | 54 | 61.85 |
| 0.9185 | 0.9241 | 55 | 47.25 | 0.9010 | 0.9069 | 55 | 62.84 |
| 0.9164 | 0.9220 | 56 | 48.21 | 0.8987 | 0.9046 | 56 | 63.82 |
| 0.9142 | 0.9199 | 57 | 49.19 | 0.8964 | 0.9024 | 57 | 64.80 |
| 0.9120 | 0.9177 | 58 | 50.17 | 0.8941 | 0.9001 | 58 | 65.77 |
| 0.9098 | 0.9155 | 59 | 51.15 | 0.8918 | 0.8978 | 59 | 66.73 |
| 0.9076 | 0.9133 | 60 | 52.15 | 0.8895 | 0.8955 | 60 | 67.79 |
| 0.9053 | 0.9111 | 61 | 53.15 | 0.8871 | 0.8932 | 61 | 68.64 |
| 0.9030 | 0.9088 | 62 | 54.15 | 0.8848 | 0.8909 | 62 | 69.59 |

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| (1) | (2) | (3) | (4) | (5) | (6) | (7) | (8) |
|---|---|---|---|---|---|---|---|
| Specific gravity in air | | Percentage of ethanol ($C_2H_5OH$) | | Specific gravity in air | | Percentage of ethanol ($C_2H_5OH$) | |
| At 25°/25° | At 15.56°/15.56° | By volume (v/v) at 15.56° | By weight | At 25°/25° | At 15.56°/15.56° | By weight | By volume (v/v) at 15.56° |
| 0.9006 | 0.9065 | 63 | 55.17 | 0.8824 | 0.8886 | 63 | 70.52 |
| 0.8983 | 0.9042 | 64 | 56.18 | 0.8801 | 0.8862 | 64 | 71.46 |
| 0.8959 | 0.9019 | 65 | 57.21 | 0.8777 | 0.8839 | 65 | 72.38 |
| 0.8936 | 0.8995 | 66 | 58.24 | 0.8753 | 0.8815 | 66 | 73.30 |
| 0.8911 | 0.8972 | 67 | 59.28 | 0.8729 | 0.8792 | 67 | 74.21 |
| 0.8887 | 0.8948 | 68 | 60.33 | 0.8706 | 0.8768 | 68 | 75.12 |
| 0.8862 | 0.8923 | 69 | 61.38 | 0.8682 | 0.8745 | 69 | 76.02 |
| 0.8837 | 0.8899 | 70 | 62.44 | 0.8658 | 0.8721 | 70 | 76.91 |
| 0.8812 | 0.8874 | 71 | 63.51 | 0.8634 | 0.8697 | 71 | 77.79 |
| 0.8787 | 0.8848 | 72 | 64.59 | 0.8609 | 0.8673 | 72 | 78.67 |
| 0.8761 | 0.8823 | 73 | 65.67 | 0.8585 | 0.8649 | 73 | 79.54 |
| 0.8735 | 0.8797 | 74 | 66.77 | 0.8561 | 0.8625 | 74 | 80.41 |
| 0.8709 | 0.8771 | 75 | 67.87 | 0.8537 | 0.8601 | 75 | 81.27 |
| 0.8682 | 0.8745 | 76 | 68.98 | 0.8512 | 0.8576 | 76 | 82.12 |
| 0.8655 | 0.8718 | 77 | 70.10 | 0.8488 | 0.8552 | 77 | 82.97 |
| 0.8628 | 0.8691 | 78 | 71.23 | 0.8463 | 0.8528 | 78 | 83.81 |
| 0.8600 | 0.8664 | 79 | 72.38 | 0.8439 | 0.8503 | 79 | 84.64 |
| 0.8572 | 0.8636 | 80 | 73.53 | 0.8414 | 0.8479 | 80 | 85.46 |
| 0.8544 | 0.8608 | 81 | 74.69 | 0.8389 | 0.8454 | 81 | 86.28 |
| 0.8516 | 0.8580 | 82 | 75.86 | 0.8364 | 0.8429 | 82 | 87.08 |
| 0.8487 | 0.8551 | 83 | 77.04 | 0.8339 | 0.8404 | 83 | 87.89 |
| 0.8458 | 0.8522 | 84 | 78.23 | 0.8314 | 0.8379 | 84 | 88.68 |
| 0.8428 | 0.8493 | 85 | 79.44 | 0.8288 | 0.8354 | 85 | 89.46 |
| 0.8397 | 0.8462 | 86 | 80.66 | 0.8263 | 0.8328 | 86 | 90.24 |
| 0.8367 | 0.8432 | 87 | 81.90 | 0.8237 | 0.8303 | 87 | 91.01 |
| 0.8335 | 0.8401 | 88 | 83.14 | 0.8211 | 0.8276 | 88 | 91.77 |
| 0.8303 | 0.8369 | 89 | 84.41 | 0.8184 | 0.8250 | 89 | 92.52 |
| 0.8271 | 0.8336 | 90 | 85.69 | 0.8158 | 0.8224 | 90 | 93.25 |
| 0.8237 | 0.8303 | 91 | 86.99 | 0.8131 | 0.8197 | 91 | 93.98 |
| 0.8202 | 0.8268 | 92 | 88.31 | 0.8104 | 0.8170 | 92 | 94.70 |
| 0.8167 | 0.8233 | 93 | 89.65 | 0.8076 | 0.8142 | 93 | 95.41 |
| 0.8130 | 0.8196 | 94 | 91.03 | 0.8048 | 0.8114 | 94 | 96.10 |
| 0.8092 | 0.8158 | 95 | 92.42 | 0.8020 | 0.8086 | 95 | 96.79 |
| 0.8053 | 0.8118 | 96 | 93.85 | 0.7992 | 0.8057 | 96 | 97.46 |
| 0.8011 | 0.8077 | 97 | 95.32 | 0.7962 | 0.8028 | 97 | 98.12 |
| 0.7968 | 0.8033 | 98 | 96.82 | 0.7932 | 0.7998 | 98 | 98.76 |
| 0.7921 | 0.7986 | 99 | 98.38 | 0.7902 | 0.7967 | 99 | 99.39 |
| 0.7871 | 0.7936 | 100 | 100.00 | 0.7871 | 0.7936 | 100 | 100.00 |

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2. Osborne N S, McKelvy E C, Bearce H W. Density and thermal expansion of ethyl alcohol and of its mixtures with water. *Bulletin of the Bureau of Standards*. 1913; 9:327–474.

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# Articles of Incorporation

In May of 1900, the USP Board of Trustees was directed by the Convention to incorporate the USP organization under the laws of the District of Columbia. Because the District of Columbia required that the majority of officers subscribing to the Certificate of Incorporation be residents, the filing was slightly delayed in order to appoint appropriate representatives. Nevertheless, the articles of incorporation were prepared, appropriately signed, and finally filed on July 11th of that year. The original certificate reads as follows:

# Certification of Incorporation

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This is to certify that we, whose names are hereunto subscribed, citizens of the United States, of full age, and a majority citizens of the District of Columbia, do associate ourselves together pursuant to the provisions of sections 545–552 inclusive of the Revised Statutes of the United States relating to the District of Columbia and of the Act of Congress to amend the same, approved the twenty-third day of April 1884, under the corporate name of The United States Pharmacopoeial Convention.

This Association is organized for a period of nine hundred and ninety-nine years. The particular objects and business of this Association are the encouragement and promotion of the science and art of medicine and pharmacy by selecting by research and experiment and other proper methods and by naming such materials as may be properly used as medicines and drugs with formulas for their preparation; by establishing one uniform standard and guide for the use of those engaged in the practice of medicine and pharmacy in the United States whereby the identity, strength, and purity of all such medicines and drugs may be accurately determined, and for other like and similar purposes; and by printing and distributing at suitable intervals such formulas and the results of such and similar selections, names and determinations among the members of this Association, pharmacists, and physicians generally in the United States and others interested in pharmacy and medicine.

The management and control of the affairs, funds, and property of this Association for the first year of its existence shall be vested in a Board of Trustees consisting of the seven following persons:

Albert E. Ebert
Samuel A.D. Sheppard
William S. Thompson
Charles E. Dohme
George W. Sloan
Horatio C. Wood
Charles Rice

In testimony whereof we have hereunto set our hands and affixed our seals this seventh day of July, 1900.

| | | | |
|---|---|---|---|
| William S. Thompson | [SEAL] | William M. Mew | [SEAL] |
| G. Lloyd Magruder | [SEAL] | Frank M. Criswell | [SEAL] |
| John T. Winter | [SEAL] | Murray G. Motter | [SEAL] |
| Thomas C. Smith | [SEAL] | | |

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# ATOMIC WEIGHTS

**Standard Atomic Weights of the Elements, Recommended by the Commission on Atomic Weights and Isotopic Abundances of the International Union of Pure and Applied Chemistry**

This table can be found at http://www.sbcs.qmul.ac.uk/iupac/AtWt/.

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- ⟨1163⟩ *Quality Assurance in Pharmaceutical Compounding*
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Product Related

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# Chapter Charts

The lists that follow are intended to be a guide to the chapters in this publication. They may not be all-inclusive, and they are not intended to describe expectations for articles or limit the application of tests to any article in the *USP–NF*.

**Official Articles**

- *Non-Complex Drug Substances*
- *Biotechnology-Derived Drug Substances*
- *Excipients*
- *Non-Complex Active Drug Products*
- *Biotechnology-Derived Drug Products*
- *Vaccines*
- *Blood and Blood Products*
- *Gene and Cell Therapy Products*
- *Dietary Supplement Ingredients*
- *Dietary Supplement Products*
- *Compounding—Substance/Preparation/Practice*
- *Medical Devices*

**Generally Applicable**

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  - ⟨1010⟩ *Analytical Data—Interpretation and Treatment*
  - ⟨1029⟩ *Good Documentation Guidelines*
  - ⟨1039⟩ *Chemometrics*
  - ⟨1058⟩ *Analytical Instrument Qualification*
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# DESCRIPTION AND RELATIVE SOLUBILITY

## Description and Relative Solubility of USP and NF Articles

The "description" and "solubility" statements pertaining to an article (formerly included in the individual monograph) are general in nature. The information is provided for those who use, prepare, and dispense drugs, solely to indicate descriptive and solubility properties of an article complying with monograph standards. The properties are not in themselves standards or tests for purity even though they may indirectly assist in the preliminary evaluation of the integrity of an article.

## Taste and Odor

Organoleptic characteristics are indicated in many instances because they may be useful and descriptive properties of substances. However, they are not meant to be applied as tests for identifying materials.

The inclusion of odor or taste among other descriptive properties may aid in identifying the causative agent following accidental exposure to or contact with a substance. This information is provided as a warning or to make an individual aware of sensations that may be encountered. The use of odor or taste as a test for identification or content is strongly discouraged.

The characteristic odor of a volatile substance becomes apparent immediately on opening a container of it. The odor may be agreeable (e.g., Peppermint Oil), unpleasant (e.g., Sulfur Dioxide), or potentially hazardous on prolonged exposure (e.g., Coal Tar). Moreover, an unexpected odor may be encountered if the characteristics of a substance are not known or if a container is incorrectly labeled. Consequently, containers of such substances should be opened cautiously, preferably in a well-ventilated fume hood. A characteristic taste or sensation produced in the oral cavity likewise is apparent if traces of residue materials on fingers are inadvertently brought into contact with the tongue or adjacent mucosal tissues.

## Solubility

Only where a special, quantitative solubility test is given in the individual monograph, and is designated by a test heading, is it a test for purity.

The approximate solubilities of Pharmacopeial and National Formulary substances are indicated by the descriptive terms in the accompanying table. The term "miscible" as used in this Pharmacopeia pertains to a substance that yields a homogeneous mixture when mixed in any proportion with the designated solvent.

| Descriptive Term | Parts of Solvent Required for 1 Part of Solute |
|---|---|
| Very soluble | Less than 1 |
| Freely soluble | From 1 to 10 |
| Soluble | From 10 to 30 |
| Sparingly soluble | From 30 to 100 |
| Slightly soluble | From 100 to 1000 |
| Very slightly soluble | From 1000 to 10,000 |
| Practically insoluble, or Insoluble | 10,000 and over |

Soluble Pharmacopeial and National Formulary articles, when brought into solution, may show traces of physical impurities, such as minute fragments of filter paper, fibers, and other particulate matter, unless limited or excluded by definite tests or other specifications in the individual monographs.

**Abacavir Sulfate:** White to off-white powder. Soluble in water, in ethyl acetate, in absolute alcohol, and in methanol.

**Abiraterone Acetate:** White to off-white powder. Freely soluble in methylene chloride, in tetrahydrofuran, and in toluene; soluble in methanol, in ethanol, in ethyl acetate, in isobutyl methyl ketone, in *N,N*-dimethylformamide, and in acetone; sparingly soluble in acetonitrile and in dimethyl sulfoxide; slightly soluble in hexane; very slightly soluble in 0.1 N hydrochloric acid; practically insoluble in aqueous media over a wide range of pH values.

**Acacia:** Is practically odorless and produces a mucilaginous sensation on the tongue. Insoluble in alcohol. Optical rotation varies depending on the source of Acacia. For example, specific rotation values, calculated on the anhydrous basis and determined on a 1.0% (w/v) solution, usually are between −25° and −35° for *Acacia senegal* and between +35° and +60° for *Acacia seyal*. *NF category:* Emulsifying agent; suspending and/or viscosity-increasing agent; wet binder.

**Acamprosate Calcium:** A white or almost-white powder. Freely soluble in water; practically insoluble in alcohol and in methylene chloride.

**Acebutolol Hydrochloride:** White or almost white, crystalline powder. Soluble in alcohol and in water; very slightly soluble in acetone and in methylene chloride; practically insoluble in ether. Melts at about 141° to 144°.

**Acesulfame Potassium:** A white, crystalline powder or colorless crystals. Soluble in water; very slightly soluble in acetone and in alcohol. *NF category:* Sweetening agent.

**Acetaminophen:** White, odorless, crystalline powder, having a slightly bitter taste. Freely soluble in alcohol; soluble in boiling water and in 1 N sodium hydroxide.

**Acetazolamide:** White to faintly yellowish-white, crystalline, odorless powder. Sparingly soluble in practically boiling water; slightly soluble in alcohol; very slightly soluble in water.

**Acetic Acid:** Clear, colorless liquid, having a strong, characteristic odor, and a sharply acid taste. Specific gravity is about 1.045. Miscible with water, with alcohol, and with glycerin. *NF category:* pH modifier (acidifying agent/alkalizing agent/buffering agent).

**Glacial Acetic Acid:** Clear, colorless liquid, having a pungent, characteristic odor and, when well diluted with water, an acid taste. Boils at about 118°. Specific gravity is about 1.05. Miscible with water, with alcohol, and with glycerin. *NF category:* pH modifier (acidifying agent/alkalizing agent/buffering agent).

**Acetohydroxamic Acid:** White, slightly hygroscopic, crystalline powder. Melts, after drying at about 80° for 2 to 4 hours, at about 88°. Freely soluble in water and in alcohol; very slightly soluble in chloroform.

**Acetone:** Transparent, colorless, mobile, volatile liquid, having a characteristic odor. A solution (1 in 2) is neutral to litmus. Miscible with water, with alcohol, with ether, with chloroform, and with most volatile oils. *NF category:* Solvent.

**Acetylcholine Chloride:** White or off-white crystals or crystalline powder. Very soluble in water; freely soluble in alcohol; insoluble in ether. Is decomposed by hot water and by alkalies.

**Acetylcysteine:** White, crystalline powder, having a slight acetic odor. Freely soluble in water and in alcohol; practically insoluble in chloroform and in ether.

**Acetyltributyl Citrate:** Clear, practically colorless, oily liquid. Freely soluble in alcohol, in isopropyl alcohol, in acetone, and in toluene; insoluble in water. *NF category:* Plasticizer.

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**Acetyltriethyl Citrate:** Clear, practically colorless, oily liquid. Freely soluble in alcohol, in isopropyl alcohol, in acetone, and in toluene; insoluble in water. *NF category:* Plasticizer.

**Acitretin:** Yellow or greenish, crystalline powder. Sparingly soluble in tetrahydrofuran; slightly soluble in acetone and in alcohol; very slightly soluble in cyclohexane; practically insoluble in water.

**Acyclovir:** White to off-white, crystalline powder. Melts at temperatures higher than 250°, with decomposition. Soluble in diluted hydrochloric acid; slightly soluble in water; insoluble in alcohol.

**Adapalene:** White or almost white powder. Soluble in tetrahydrofuran; sparingly soluble in ethanol; practically insoluble in water.

**Ademetionine Disulfate Tosylate:** White powder. Freely soluble in water.

**Adenine:** White crystals or crystalline powder. Is odorless and tasteless. Sparingly soluble in boiling water; slightly soluble in alcohol; very slightly soluble in water; practically insoluble in ether and in chloroform.

**Adenosine:** White, odorless, crystalline powder. Slightly soluble in water; practically insoluble in alcohol. Melts at about 235°.

**Adipic Acid:** A white, crystalline powder. Freely soluble in alcohol and in methanol; soluble in boiling water and in acetone; slightly soluble in water. *NF category:* pH modifier (acidifying agent/alkalizing agent/buffering agent); flavors and fragrance.

**Agar:** Odorless or has a slight odor, and produces a mucilaginous sensation on the tongue. Soluble in boiling water; insoluble in cold water. *NF category:* Suspending and/or viscosity-increasing agent; emulsifying agent; suppository base; wet binder.

**Alamic Acid:** *NF category:* Suspending and/or viscosity-increasing agent.

**Alanine:** White, odorless crystals or crystalline powder, having a slightly sweet taste. Freely soluble in water; slightly soluble in 80% alcohol; insoluble in ether.

**Albendazole:** White to faintly yellowish powder. Freely soluble in anhydrous formic acid; very slightly soluble in ether and in methylene chloride; practically insoluble in alcohol and in water.

**Albumin Human:** Practically odorless, moderately viscous, clear, brownish fluid.

**rAlbumin Human:** Clear, slightly viscous, and colorless to yellow amber in color. *NF category:* Vehicle (sterile).

**Albuterol:** White, crystalline powder. Soluble in alcohol; sparingly soluble in water. Melts at about 156°.

**Albuterol Sulfate:** White or practically white powder. Freely soluble in water; slightly soluble in alcohol, in chloroform, and in ether.

**Alcohol:** Clear, colorless, mobile, volatile liquid. Has a characteristic odor and produces a burning sensation on the tongue. Is readily volatilized even at low temperatures, and boils at about 78°. Is flammable. Miscible with water and with practically all organic solvents. *NF category:* Solvent; antimicrobial preservative.

**Dehydrated Alcohol:** Clear, colorless, mobile, volatile liquid. Has a characteristic odor and produces a burning sensation on the tongue. Is readily volatilized even at low temperatures, and boils at about 78°. Is flammable. Miscible with water and with practically all organic solvents.

**Diluted Alcohol:** Clear, colorless, mobile liquid, having a characteristic odor and producing a burning sensation on the tongue. *NF category:* Solvent.

**Rubbing Alcohol:** Transparent, colorless, or colored as desired, mobile, volatile liquid. Has an extremely bitter taste and, in the absence of added odorous constituents, a characteristic odor. Is flammable.

**Alendronate Sodium:** White, free-flowing powder. Soluble in water; very slightly soluble in dimethyl sulfoxide, in methyl alcohol, and in propylene glycol; practically insoluble in acetone, in acetonitrile, in alcohol, in chloroform, and in isopropyl alcohol.

**Alfadex:** White or almost white, amorphous or crystalline powder. Freely soluble in water and in propylene glycol; practically insoluble in ethanol and in methylene chloride. *NF category:* Chelating and/or complexing agent.

**Alfentanil Hydrochloride:** White to almost white powder. Freely soluble in methanol, in alcohol, and in chloroform; soluble in water; sparingly soluble in acetone. Melting point range, crystals from acetone: 136°–143° (anhydrous) and reported as crystals from aqueous hydrochloric acid: 116°–126° (monohydrate).

**Alfentanil Injection:** Clear, colorless solution.

**Alfuzosin Hydrochloride:** White to almost white powder, slightly hygroscopic. Freely soluble in water; sparingly soluble in alcohol; practically insoluble in methylene chloride.

**Alginic Acid:** White to yellowish white, fibrous powder. Is odorless, or practically odorless, and is tasteless. Soluble in alkaline solutions; insoluble in water and in organic solvents. *NF category:* Suspending and/or viscosity-increasing agent; wet binder; disintegrant; film-forming agent; release-modifying agent.

**Alkyl (C12-15) Benzoate:** Clear, practically colorless, oily liquid. Soluble in acetone, in alcohol, in isopropyl alcohol, in ethyl acetate, in isopropyl myristate, in isopropyl palmitate, in lanolin, in mineral oil, in vegetable oils, and in volatile silicones; insoluble in water, in glycerin, and in propylene glycol. *NF category:* Vehicle (oleaginous); emollient.

**Allantoin:** White, crystalline powder. Slightly soluble in water; very slightly soluble in alcohol. Melts at about 225°, with decomposition.

**Allopurinol:** Fluffy white to off-white powder, having only a slight odor. Soluble in solutions of potassium and sodium hydroxides; very slightly soluble in water and in alcohol; practically insoluble in chloroform and in ether.

**Allyl Isothiocyanate:** Colorless to pale yellow, very refractive, liquid. Pungent irritating odor, acrid taste. [**Caution**—*Lachrymator.*] Miscible with alcohol, with carbon disulfide, and with ether. Slightly soluble in water.

**Almond Oil:** Clear, pale straw-colored or colorless, oily liquid, having a bland taste. Remains clear at –10°, and does not congeal until cooled to almost –20°. Slightly soluble in alcohol. Miscible with ether, with chloroform, with benzene, and with solvent hexane. *NF category:* Flavors and fragrance; vehicle (oleaginous); emollient; solvent.

**Almotriptan Malate:** White to slightly yellow, crystalline powder. Soluble in water; slightly soluble in methanol and in *N*,*N*-dimethylformamide; very slightly soluble in acetone, in acetonitrile, in ethanol, in ethyl acetate, in propanol, in tetrahydrofuran, and in toluene; practically insoluble in chloroform and in methylene chloride.

**Aloe:** Has a characteristic, somewhat sour and disagreeable, odor.

**Alosetron Hydrochloride:** White to beige solid. Very soluble in water and in 0.1 M hydrochloric acid; freely soluble in pH 6 phosphate buffer; soluble in pH 8 phosphate buffer; sparingly soluble in methanol.

**Alprazolam:** A white to off-white, crystalline powder. Melts at about 225°. Freely soluble in chloroform; soluble in alcohol; sparingly soluble in acetone; slightly soluble in ethyl acetate; insoluble in water.

**Alprostadil:** A white to off-white, crystalline powder. Melts at about 110°. Freely soluble in alcohol; soluble in water and

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in acetone; slightly soluble in ethyl acetate; very slightly soluble in chloroform and in ether.

**Altretamine:** White, crystalline powder. Soluble in chloroform; insoluble in water.

**Ammonium Alum:** Large, colorless crystals, crystalline fragments, or white powder. Is odorless, and has a sweetish, strongly astringent taste. Its solutions are acid to litmus. Very soluble in boiling water; freely soluble in water and in glycerin; insoluble in alcohol.

**Potassium Alum:** Large, colorless crystals, crystalline fragments, or white powder. Is odorless, and has a sweetish, strongly astringent taste. Its solutions are acid to litmus. Very soluble in boiling water; freely soluble in water and in glycerin; insoluble in alcohol.

**Aluminum Acetate Topical Solution:** Clear, colorless liquid having a faint odor of acetic acid, and a sweetish, astringent taste. Specific gravity is about 1.02.

**Aluminum Chloride:** White, or yellowish-white, deliquescent, crystalline powder. Is practically odorless, and has a sweet, very astringent taste. Its solutions are acid to litmus. Very soluble in water; freely soluble in alcohol; soluble in glycerin.

**Aluminum Hydroxide Gel:** White, viscous suspension, from which small amounts of clear liquid may separate on standing.

**Dried Aluminum Hydroxide Gel:** White, odorless, tasteless, amorphous powder. Soluble in dilute mineral acids and in solutions of fixed alkali hydroxides; insoluble in water and in alcohol.

**Aluminum Monostearate:** Fine, white to yellowish-white, bulky powder, having a faint, characteristic odor. Insoluble in water, in alcohol, and in ether. *NF category:* Suspending and/or viscosity-increasing agent; emollient.

**Aluminum Oxide:** Occurs as a white or almost white, amorphous powder. It is very slightly soluble in dilute mineral acids and in solutions of alkali hydroxides. It is practically insoluble in water. *NF category:* Coloring agent.

**Aluminum Phosphate Gel:** White, viscous suspension from which small amounts of water separate on standing.

**Aluminum Subacetate Topical Solution:** Clear, colorless or faintly yellow liquid, having an odor of acetic acid and an acid reaction to litmus. Gradually becomes turbid on standing, through separation of a more basic salt.

**Aluminum Sulfate:** White, crystalline powder, shining plates, or crystalline fragments. Is stable in air. Is odorless, and has a sweet taste, becoming mildly astringent. Freely soluble in water; insoluble in alcohol.

**Amantadine Hydrochloride:** White or practically white, crystalline powder, having a bitter taste. Freely soluble in water; soluble in alcohol and in chloroform.

**Amifostine:** White, crystalline powder. Freely soluble in water.

**Amikacin:** White, crystalline powder. Sparingly soluble in water.

**Amikacin Sulfate:** White, crystalline powder. Freely soluble in water.

**Amiloride Hydrochloride:** Yellow to greenish-yellow, odorless or practically odorless powder. Freely soluble in dimethyl sulfoxide; sparingly soluble in methanol; slightly soluble in water; insoluble in ether, in ethyl acetate, in acetone, and in chloroform.

**Amino Methacrylate Copolymer:** Colorless to yellowish granules or white powder. Soluble in acetone, in isopropyl alcohol, and in diluted acids; practically insoluble in water. The solutions are clear to slightly cloudy. *NF category*: Coating agent; polymer membrane; wet binder; diluent; film-forming agent.

**Aminobenzoate Potassium:** White, crystalline powder. The pH of a 1 in 100 solution in water is about 7. Very soluble in water; soluble in alcohol; practically insoluble in ether.

**Aminobenzoic Acid:** White or slightly yellow, odorless crystals or crystalline powder. Discolors on exposure to air or light. Freely soluble in alcohol and in solutions of alkali hydroxides and carbonates; sparingly soluble in ether; slightly soluble in water and in chloroform. *NF category:* Free radical scavenger.

**Aminocaproic Acid:** Fine, white, crystalline powder. Is odorless, or practically odorless. Its solutions are neutral to litmus. Melts at about 205°. Freely soluble in water, in acids, and in alkalies; slightly soluble in methanol and in alcohol; practically insoluble in chloroform and in ether.

**Aminohippuric Acid:** White, crystalline powder. Discolors on exposure to light. Melts at about 195°, with decomposition. Freely soluble in alkaline solutions, with some decomposition, and in diluted hydrochloric acid; sparingly soluble in water and in alcohol; very slightly soluble in benzene, in carbon tetrachloride, in chloroform, and in ether.

**Aminolevulinic Acid Hydrochloride:** White to off-white, odorless, crystalline solid. Very soluble in water; slightly soluble in methanol and in ethanol; practically insoluble in chloroform, in hexane, and in mineral oil.

**Aminophylline:** White or slightly yellowish granules or powder, having a slight ammoniacal odor and a bitter taste. Upon exposure to air, it gradually loses methamphetamine and absorbs carbon dioxide with the liberation of free theophylline. Its solutions are alkaline to litmus. One g dissolves in 25 mL of water to give a clear solution; 1 g dissolved in 5 mL of water crystallizes upon standing, but redissolves when a small amount of ethylenediamine is added. Insoluble in alcohol and in ether.

**Aminophylline Tablets:** May have a faint ammoniacal odor.

**Aminosalicylic Acid:** White or practically white, bulky powder, that darkens on exposure to light and air. Is odorless, or has a slight acetous odor. Soluble in alcohol; slightly soluble in water and in ether; practically insoluble in benzene.

**Amiodarone Hydrochloride:** White or almost white, fine, crystalline powder. Freely soluble in methylene chloride; soluble in methanol; sparingly soluble in alcohol; very slightly soluble in water.

**Amitriptyline Hydrochloride:** White or practically white, odorless or practically odorless, crystalline powder or small crystals. Freely soluble in water, in alcohol, in chloroform, and in methanol; insoluble in ether.

**Amlodipine Besylate:** A white or almost white powder. Freely soluble in methanol; sparingly soluble in alcohol; slightly soluble in 2-propanol and in water.

**Strong Ammonia Solution:** Clear, colorless liquid, having an exceedingly pungent, characteristic odor. Specific gravity is about 0.90. *NF category:* pH modifier (acidifying agent/alkalizing agent/buffering agent).

**Aromatic Ammonia Spirit:** Practically colorless liquid when recently prepared, but gradually acquiring a yellow color on standing. Has the taste of ammonia, has an aromatic and pungent odor, and is affected by light. Specific gravity is about 0.90.

**Ammonio Methacrylate Copolymer:** Colorless, clear to white-opaque granules or a white powder, both with a faint amine-like odor. Soluble to freely soluble in methanol, in alcohol, and in isopropyl alcohol, each of which contains small amounts of water; soluble to freely soluble in acetone, in ethyl acetate, and in methylene chloride. The solutions are clear to slightly cloudy. Insoluble in petroleum ether and in water. *NF category:* Coating agent; wet binder; polymer membrane; diluent; film-forming agent.

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**Ammonio Methacrylate Copolymer Dispersion:** Milky-white liquids of low viscosity with a faint characteristic odor. Miscible with water in any proportion, the milky-white appearance being retained. A clear or slightly cloudy solution is obtained on mixing one part with five parts of acetone, alcohol, or isopropyl alcohol. When mixed with methanol in a ratio of 1:5, Ammonio Methacrylate Copolymer Dispersion Type A dissolves completely, and Ammonio Methacrylate Copolymer Dispersion Type B dissolves only partially. *NF category:* Coating agent; polymer membrane; wet binder; diluent; film-forming agent.

**Ammonium Carbonate:** White powder, or hard, white or translucent masses, having a strong odor of ammonia, without empyreuma, and a sharp, ammoniacal taste. Its solutions are alkaline to litmus. On exposure to air, it loses ammonia and carbon dioxide, becoming opaque, and is finally converted into friable porous lumps or a white powder of ammonium bicarbonate. Freely soluble in water, but is decomposed by hot water. *NF category:* pH modifier (acidifying agent/alkalizing agent/buffering agent).

**Ammonium Chloride:** Colorless crystals or white, fine or coarse, crystalline powder. Has a cool, saline taste, and is somewhat hygroscopic. Freely soluble in water and in glycerin, and even more so in boiling water; sparingly soluble in alcohol. *NF category:* pH modifier (acidifying agent/alkalizing agent/buffering agent).

**Ammonium Glycyrrhizate:** White or yellowish-white, hygroscopic powder. It is slightly soluble in water; very slightly soluble in anhydrous ethanol; practically insoluble in acetone. It dissolves in dilute solutions of acids and of alkali hydroxides. *NF category:* Flavors and fragrance; wetting and/or solubilizing agent.

**Ammonium Molybdate:** Colorless or slightly greenish or yellowish crystals. Soluble in water; practically insoluble in alcohol.

**Ammonium Phosphate:** Colorless or white granules or powder, having a saline taste. Freely soluble in water; practically insoluble in acetone and in alcohol. *NF category:* pH modifier (acidifying agent/alkalizing agent/buffering agent).

**Ammonium Sulfate:** Colorless or white crystals or granules that decompose at temperatures above 280°. One g is soluble in about 1.5 mL of water. It is insoluble in alcohol. The pH of a 0.1 M solution is between 4.5 and 6.0.

**Amobarbital Sodium:** White, friable, granular powder. Is odorless, has a bitter taste, and is hygroscopic. Its solutions decompose on standing, heat accelerating the decomposition. Very soluble in water; soluble in alcohol; practically insoluble in ether and in chloroform.

**Amodiaquine:** Very pale yellow to light tan-yellow, odorless powder. Sparingly soluble in 1.0 N hydrochloric acid; slightly soluble in alcohol; practically insoluble in water.

**Amodiaquine Hydrochloride:** Yellow, crystalline powder. Is odorless and has a bitter taste. Soluble in water; sparingly soluble in alcohol; very slightly soluble in benzene, in chloroform, and in ether.

**Amoxapine:** White to yellowish crystalline powder. Freely soluble in chloroform; soluble in tetrahydrofuran; sparingly soluble in methanol and in toluene; slightly soluble in acetone; practically insoluble in water.

**Amoxicillin:** White, practically odorless, crystalline powder. Slightly soluble in water and in methanol; insoluble in benzene, in carbon tetrachloride, and in chloroform.

**Amphetamine Sulfate:** White, odorless, crystalline powder, having a slightly bitter taste. Its solutions are acid to litmus, having a pH of 5 to 6. Freely soluble in water; slightly soluble in alcohol; practically insoluble in ether.

**Amphotericin B:** Yellow to orange powder; odorless or practically so. Soluble in dimethylformamide, in dimethyl sulfoxide, and in propylene glycol; slightly soluble in methanol; insoluble in water, in anhydrous alcohol, in ether, in benzene, and in toluene.

**Amphotericin B for Injection:** It yields a colloidal dispersion in water.

**Ampicillin:** White, practically odorless, crystalline powder. Slightly soluble in water and in methanol; insoluble in benzene, in carbon tetrachloride, and in chloroform.

**Ampicillin Sodium:** White to off-white, odorless or practically odorless, crystalline powder. Is hygroscopic. Very soluble in water and in isotonic sodium chloride and dextrose solutions.

**Amprolium** ($C_{14}H_{19}ClN_4 \cdot HCl$)**:** White to light yellow powder. Freely soluble in water, in methanol, in alcohol, and in dimethylformamide; sparingly soluble in dehydrated alcohol; practically insoluble in isopropyl alcohol, in butyl alcohol, and in acetone.

**Amyl Nitrite:** Clear, yellowish liquid, having a peculiar, ethereal, fruity odor. Is volatile even at low temperatures, and is flammable. Boils at about 96°. Practically insoluble in water. Miscible with alcohol and with ether.

**Amylene Hydrate:** Clear, colorless liquid, having a camphoraceous odor. Its solutions are neutral to litmus. Freely soluble in water. Miscible with alcohol, with chloroform, with ether, and with glycerin. *NF category:* Solvent.

**Anagrelide Hydrochloride:** Off-white to pale pinkish powder. Sparingly soluble in dimethylsulfoxide and in dimethylformamide; very slightly soluble in water.

**Anastrozole:** White to off-white crystalline powder. Very soluble in acetonitrile; freely soluble in methanol, in acetone, in alcohol, and in tetrahydrofuran.

**Anethole:** Colorless or faintly yellow liquid at or above 23°. Has a sweet taste and the aromatic odor of anise. Is affected by light. Freely soluble in alcohol; very slightly soluble in water. Readily miscible with ether and with chloroform. *NF category:* Flavors and fragrance.

**Anise Oil:** Clear, colorless to light yellow in the liquid state having a powerful and characteristic licorice-like scent. Congeals between 15° and 19°. Miscible with alcohol. *NF category:* Flavors and fragrance.

**Antazoline Phosphate:** White to off-white, crystalline powder, having a bitter taste. Soluble in water; sparingly soluble in methanol; practically insoluble in benzene and in ether.

**Anthralin:** Yellowish-brown, crystalline powder. Is odorless and tasteless. Soluble in chloroform, in acetone, in benzene, and in solutions of alkali hydroxides; slightly soluble in alcohol, in ether, and in glacial acetic acid; insoluble in water.

**Anticoagulant Citrate Dextrose Solution:** Clear, colorless, odorless liquid. Is dextrorotatory.

**Anticoagulant Citrate Phosphate Dextrose Solution:** Clear, colorless to slightly yellow, odorless liquid. Is dextrorotatory.

**Anticoagulant Sodium Citrate Solution:** Clear and colorless liquid.

**Antimony Potassium Tartrate:** Colorless, odorless, transparent crystals, or white powder. The crystals effloresce upon exposure to air and do not readily rehydrate even on exposure to high humidity. Its solutions are acid to litmus. Freely soluble in boiling water; soluble in water and in glycerin; insoluble in alcohol.

**Antipyrine:** Colorless crystals, or white, crystalline powder. Is odorless and has a slightly bitter taste. Its solutions are neutral to litmus. Very soluble in water; freely soluble in alcohol and in chloroform; sparingly soluble in ether.

**Apomorphine Hydrochloride:** Minute, white or grayish-white, glistening crystals or white powder. Is odorless. It gradually acquires a green color on exposure to light and air. Its solutions are neutral to litmus. Soluble in water at 80°;

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sparingly soluble in water and in alcohol; very slightly soluble in chloroform and in ether.

**Apraclonidine Hydrochloride:** White to off-white, odorless to practically odorless powder. Soluble in methanol; sparingly soluble in water and in alcohol; insoluble in chloroform, in ethyl acetate, and in hexanes.

**Aprepitant:** White to off-white powder. Sparingly soluble in alcohol; slightly soluble in acetonitrile; practically insoluble in water.

**Arginine:** White, practically odorless crystals. Freely soluble in water; sparingly soluble in alcohol; insoluble in ether.

**Arginine Hydrochloride:** White crystals or crystalline powder, practically odorless. Freely soluble in water.

**Aripiprazole:** A white to off-white, crystalline powder. Freely soluble in dichloromethane; sparingly soluble in toluene; insoluble in methanol and in water.

**Aromatic Elixir:** *NF category:* Vehicle (flavored and/or sweetened).

**Articaine Hydrochloride:** White or almost white, crystalline powder. Freely soluble in water and in alcohol.

**Ascorbic Acid:** White or slightly yellow crystals or powder. On exposure to light it gradually darkens. In the dry state, is reasonably stable in air, but in solution rapidly oxidizes. Melts at about 190°. Freely soluble in water; sparingly soluble in alcohol; insoluble in chloroform, in ether, and in benzene. *NF category:* Antioxidant.

**Ascorbyl Palmitate:** White to yellowish white powder, having a characteristic odor. Soluble in alcohol; very slightly soluble in water and in vegetable oils. *NF category:* Antioxidant.

**Asparagine:** White crystals or a crystalline powder. Soluble in water; practically insoluble in alcohol and in ether. Its solutions are acid to litmus. It melts at about 234°.

**Aspartame:** White, odorless, crystalline powder, having a sweet taste. Sparingly soluble in water; slightly soluble in alcohol. Melts at about 246°. The pH of an 8 in 1000 solution is about 5. *NF category:* Sweetening agent.

**Aspartame Acesulfame:** White, odorless, crystalline powder. Slightly soluble in water and in ethanol. *NF category:* Sweetening agent.

**Aspartic Acid:** White or almost white, crystalline powder, or colorless crystals. Soluble in dilute solutions of alkali hydroxides and in dilute mineral acids; slightly soluble in water; practically insoluble in alcohol and in ether.

**Aspirin:** White crystals, commonly tabular or needle-like, or white, crystalline powder. Is odorless or has a faint odor. Is stable in dry air; in moist air it gradually hydrolyzes to salicylic and acetic acids. Freely soluble in alcohol; soluble in chloroform and in ether; sparingly soluble in absolute ether; slightly soluble in water.

**Atazanavir Sulfate:** White to pale-yellow, crystalline powder. Soluble in methanol and in ethyl alcohol; slightly soluble in water; practically insoluble in heptane.

**Atenolol:** White or practically white, odorless powder. Melting point 146°–148° (crystals from ethyl acetate). Freely soluble in methanol; sparingly soluble in alcohol; slightly soluble in water and in isopropanol.

**Atomoxetine Hydrochloride:** White to practically white solid. Sparingly soluble in water.

**Atorvastatin Calcium:** White to off-white powder. Soluble to freely soluble in methanol; slightly soluble in alcohol; insoluble to very slightly soluble in distilled water, in pH 7.4 phosphate buffer, and in acetonitrile; insoluble in aqueous solutions of pH 4 and below.

**Atovaquone:** Yellow powder. Freely soluble in *N*-methyl-2-pyrrolidone and in tetrahydrofuran; soluble in chloroform; sparingly soluble in acetone, in di- *n*-butyl adipate, in dimethyl sulfoxide, and in polyethylene glycol 400; slightly soluble in alcohol, in 1,3-butanediol, in ethyl acetate, in glycerin, in octanol, and in polyethylene glycol 200; very slightly soluble in 0.1 N sodium hydroxide; insoluble in water.

**Atracurium Besylate:** White to yellowish white powder, slightly hygroscopic. Very soluble in acetonitrile, in alcohol, and in methylene chloride; soluble in water.

**Atropine:** White crystals, usually needle-like, or white, crystalline powder. Its saturated solution is alkaline to phenolphthalein TS. Is optically inactive, but usually contains some levorotatory hyoscyamine. Freely soluble in alcohol and in chloroform; soluble in glycerin and in ether; slightly soluble in water; sparingly soluble in water at 80°.

**Atropine Sulfate:** Colorless crystals, or white, crystalline powder. Odorless; effloresces in dry air; is slowly affected by light. Very soluble in water; freely soluble in alcohol and even more so in boiling alcohol; freely soluble in glycerin.

**Activated Attapulgite:** Cream-colored, micronized, nonswelling powder, free from gritty particles. The high heat treatment used in its preparation causes it to yield only moderately viscous aqueous suspensions, its dispersion consisting mainly of particle groups. Insoluble in water. *NF category:* Suspending and/or viscosity-increasing agent.

**Colloidal Activated Attapulgite:** Cream-colored, micronized, nonswelling powder, free from gritty particles. Yields viscous aqueous suspensions, as a result of dispersion into its constituent ultimate particles. Insoluble in water. *NF category:* Suspending and/or viscosity-increasing agent.

**Aurothioglucose:** Yellow, odorless or practically odorless powder. Is stable in air. An aqueous solution is unstable on long standing. The pH of its 1 in 100 solution is about 6.3. Freely soluble in water; practically insoluble in acetone, in alcohol, in chloroform, and in ether.

**Azathioprine:** Pale yellow, odorless powder. Soluble in dilute solutions of alkali hydroxides; sparingly soluble in dilute mineral acids; very slightly soluble in alcohol and in chloroform; insoluble in water.

**Azathioprine Sodium for Injection:** Bright yellow, hygroscopic, amorphous mass or cake.

**Azelastine Hydrochloride:** White or almost white, crystalline powder. Soluble in ethanol and in methylene chloride; sparingly soluble in water.

**Azithromycin:** White or almost white powder. Freely soluble in anhydrous ethanol and in methylene chloride; practically insoluble in water.

**Aztreonam:** White, odorless, crystalline powder. Soluble in dimethylformamide and in dimethyl sulfoxide; slightly soluble in methanol; very slightly soluble in dehydrated alcohol; practically insoluble in ethyl acetate, in chloroform, and in toluene.

**Bacampicillin Hydrochloride:** White or practically white powder. Is hygroscopic. Freely soluble in alcohol and in chloroform; soluble in methylene chloride and in water; very slightly soluble in ether.

**Bacitracin:** White to pale buff powder, odorless or having a slight odor. Is hygroscopic. Its solutions deteriorate rapidly at room temperature. Is precipitated from its solutions and is inactivated by salts of many of the heavy metals. Freely soluble in water; soluble in alcohol, in methanol, and in glacial acetic acid, the solution in the organic solvents usually showing some insoluble residue; insoluble in acetone, in chloroform, and in ether.

**Bacitracin Zinc:** White to pale tan powder, odorless or having a slight odor. Is hygroscopic. Sparingly soluble in water.

**Baclofen:** White to off-white, crystalline powder. Is odorless or practically so. Slightly soluble in water; very slightly soluble in methanol; insoluble in chloroform.

**Balsalazide Disodium:** Orange to yellow powder. Freely soluble in water and in isotonic saline; sparingly soluble in

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methanol and in alcohol; practically insoluble in all other organic solvents.

**Adhesive Bandage:** The compress of Adhesive Bandage is substantially free from loose threads or ravelings. The adhesive strip may be perforated, and the back may be coated with a water-repellent film.

**Gauze Bandage:** One continuous piece, tightly rolled, in various widths and lengths and substantially free from loose threads and ravelings.

**Barium Sulfate:** Fine, white, odorless, tasteless, bulky powder, free from grittiness. Practically insoluble in water, in organic solvents, and in solutions of acids and of alkalies.

**Barium Sulfate for Suspension:** White or colored, bulky or granular powder.

**Beclomethasone Dipropionate:** White to cream white, odorless powder. Very soluble in chloroform; freely soluble in acetone and in alcohol; very slightly soluble in water.

**Behenoyl Polyoxylglycerides:** Waxy solid or fine powder. Soluble in methylene chloride; insoluble in alcohol; dispersible in water. *NF category:* Lubricant; emulsifying agent; wetting and/or solubilizing agent.

**Belladonna Leaf:** When moistened, its odor is slight, somewhat tobacco-like. Its taste is somewhat bitter and acrid.

**Benazepril Hydrochloride:** White to off-white, crystalline powder. Soluble in water, in methanol, and in alcohol.

**Bendamustine Hydrochloride:** White or almost-white powder. Freely soluble in methanol; slightly soluble in isopropyl alcohol, in acetonitrile, and in tetrahydrofuran.

**Bendroflumethiazide:** White to cream-colored, finely divided, crystalline powder. Is odorless, or has a slight odor. Melts at about 220°. Freely soluble in alcohol and in acetone; practically insoluble in water.

**Benoxinate Hydrochloride:** White, or slightly off-white, crystals or crystalline powder. Is odorless, or has a slight characteristic odor, has a salty taste, and exhibits local anesthetic properties when placed upon the tongue. Its solutions are neutral to litmus, and it melts at about 158°. Very soluble in water; freely soluble in chloroform and in alcohol; insoluble in ether.

**Bentonite:** Very fine, odorless, pale buff or cream-colored to grayish powder, free from grit. Has a slightly earthy taste. Is hygroscopic. Insoluble in water, but swells to approximately twelve times its volume when added to water; insoluble in, and does not swell in, organic solvents. *NF category:* Suspending and/or viscosity-increasing agent.

**Purified Bentonite:** Odorless, tasteless, fine (micronized) powder or small flakes that are creamy when viewed on their flat surfaces and tan to brown when viewed on their edges. Insoluble in water and in alcohol. Swells when added to water or glycerin. *NF category:* Suspending and/or viscosity-increasing agent.

**Bentonite Magma:** *NF category:* Suspending and/or viscosity-increasing agent.

**Benzaldehyde:** Colorless, strongly refractive liquid. Is affected by light. Slightly soluble in water. Miscible with alcohol, with ether, and with fixed and volatile oils. The specific gravity is 1.041–1.046 at 25° (see *Specific Gravity* ⟨841⟩), and the refractive index is 1.544–1.546 at 20° (see *Refractive Index* ⟨831⟩). *NF category:* Flavors and fragrance.

**Benzaldehyde Elixir, Compound:** *NF category:* Flavored and/or sweetened vehicle.

**Benzalkonium Chloride:** White or yellowish-white, thick gel or gelatinous pieces. Usually has a mild, aromatic odor. Its aqueous solution has a bitter taste, foams strongly when shaken, and usually is slightly alkaline. Very soluble in water and in alcohol. Anhydrous form freely soluble in benzene, and slightly soluble in ether. *NF category:* Antimicrobial preservative; wetting and/or solubilizing agent; emulsifying agent.

**Benzalkonium Chloride Solution:** Clear liquid; colorless or slightly yellow unless a color has been added. Has an aromatic odor and a bitter taste. *NF category:* Antimicrobial preservative.

**Benzethonium Chloride:** White crystals, having a mild odor. Its solution (1 in 100) is slightly alkaline to litmus. Soluble in water, in alcohol, and in chloroform; slightly soluble in ether. *NF category:* Antimicrobial preservative; wetting and/or solubilizing agent.

**Benzethonium Chloride Solution:** Odorless, clear liquid, slightly alkaline to litmus.

**Benzethonium Chloride Tincture:** Clear liquid, having the characteristic odor of acetone and of alcohol.

**Benzocaine:** Small, white crystals or white, crystalline powder. Is odorless, is stable in air, and exhibits local anesthetic properties when placed upon the tongue. Freely soluble in alcohol, in chloroform, and in ether; sparingly soluble in almond oil and in olive oil; very slightly soluble in water. Dissolves in dilute acids.

**Benzoic Acid:** White crystals, scales, or needles. Has a slight odor, usually suggesting benzaldehyde or benzoin. Somewhat volatile at moderately warm temperatures. Freely volatile in steam. Freely soluble in alcohol, in chloroform, and in ether; slightly soluble in water. *NF category:* Antimicrobial preservative.

**Benzoin:** Sumatra Benzoin has an aromatic and balsamic odor. When heated it does not emit a pinaceous odor. When Sumatra Benzoin is digested with boiling water, the odor suggests cinnamates or storax. Its taste is aromatic and slightly acrid. Siam Benzoin has an agreeable, balsamic, vanilla-like odor. Its taste is aromatic and slightly acrid.

**Benzonatate:** Clear, pale yellow, viscous liquid, having a faint, characteristic odor. Has a bitter taste, and exhibits local anesthetic properties when placed upon the tongue. Miscible with water in all proportions. Freely soluble in chloroform, in alcohol, and in benzene.

**Hydrous Benzoyl Peroxide:** White, granular powder, having a characteristic odor. Soluble in acetone, in chloroform, and in ether; sparingly soluble in water and in alcohol.

**Benzoyl Peroxide Gel:** A soft, white gel, having a characteristic odor.

**Benzoyl Peroxide Lotion:** White, viscous, creamy lotion, having a characteristic odor.

**Benzphetamine Hydrochloride:** White to off-white, crystalline powder. Freely soluble in alcohol and soluble in chloroform.

**Benztropine Mesylate:** White, slightly hygroscopic, crystalline powder. Very soluble in water; freely soluble in alcohol; very slightly soluble in ether.

**Benzyl Alcohol:** Clear, colorless, oily liquid. Boils at about 206°, without decomposition. Is neutral to litmus. Freely soluble in 50% alcohol; sparingly soluble in water. Miscible with alcohol, with ether, and with chloroform. The specific gravity is between 1.042 and 1.047. *NF category:* Antimicrobial preservative; solvent.

**Benzyl Benzoate:** Clear, colorless, oily liquid having a slight aromatic odor and producing a sharp, burning sensation on the tongue. Practically insoluble in water and in glycerin. Miscible with alcohol, with ether, and with chloroform. *NF category:* Solvent; emulsifying agent; plasticizer.

**Beta Carotene:** Red or reddish-brown to violet-brown crystals or crystalline powder. Soluble in carbon disulfide, in benzene, and in chloroform; sparingly soluble in ether, in solvent hexane, and in vegetable oils; practically insoluble in methanol and in alcohol; insoluble in water, in acids, and in alkalies.

**Betadex:** White, practically odorless, fine crystalline powder having a slightly sweet taste. Sparingly soluble in

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water. *NF category:* Sequestering agent; chelating and/or complexing agent.

**Betadex Sulfobutyl Ether Sodium:** White to off-white, amorphous powder. Freely soluble in water; sparingly soluble in methanol; practically insoluble in ethanol, in *n*-hexane, in 1-butanol, in acetonitrile, in 2-propanol, and in ethyl acetate. *NF category:* Chelating and/or complexing agent; sequestering agent; wetting and/or solubilizing agent.

**Betahistine Hydrochloride:** White to almost yellow, crystalline powder. Very hygroscopic. Melts between 151° and 154°. Very soluble in water; freely soluble in alcohol; practically insoluble in isopropyl alcohol.

**Betaine Hydrochloride:** White, crystalline powder. Soluble in water and in alcohol; practically insoluble in chloroform and in ether.

**Betamethasone:** White to practically white, odorless, crystalline powder. Melts at about 240°, with some decomposition. Sparingly soluble in acetone, in alcohol, in dioxane, and in methanol; very slightly soluble in chloroform and in ether; insoluble in water.

**Betamethasone Acetate:** White to creamy white, odorless powder. Sinters and resolidifies at about 165°, and remelts at about 200° or 220°, with decomposition (see *Melting Range or Temperature* ⟨741⟩). Freely soluble in acetone; soluble in alcohol and in chloroform; practically insoluble in water.

**Betamethasone Dipropionate:** White to cream-white, odorless powder. Freely soluble in acetone and in chloroform; sparingly soluble in alcohol; insoluble in water.

**Betamethasone Sodium Phosphate:** White to practically white, odorless powder. Is hygroscopic. Freely soluble in water and in methanol; practically insoluble in acetone and in chloroform.

**Betamethasone Valerate:** White to practically white, odorless powder. Melts at about 190°, with decomposition. Freely soluble in acetone and in chloroform; soluble in alcohol; slightly soluble in benzene and in ether; practically insoluble in water.

**Betaxolol Hydrochloride:** White, crystalline powder. Freely soluble in water, in alcohol, in chloroform, and in methanol.

**Bethanechol Chloride:** Colorless or white crystals or white, crystalline powder, usually having a slight, amine-like odor. Is hygroscopic. Exhibits polymorphism, and of two crystalline forms observed, one melts at about 211° and the other melts at about 219°. Freely soluble in water and in alcohol; insoluble in chloroform and in ether.

**Bicalutamide:** Fine, white to off-white powder. Freely soluble in tetrahydrofuran and in acetone; soluble in acetonitrile; sparingly soluble in methanol; slightly soluble in alcohol.

**Biotin:** Practically white, crystalline powder. Very slightly soluble in water and in alcohol; insoluble in other common organic solvents.

**Bisacodyl:** White to off-white, crystalline powder, in which the number of particles having a longest diameter smaller than 50 µm predominate. Soluble in chloroform and in benzene; sparingly soluble in alcohol and in methanol; slightly soluble in ether; practically insoluble in water.

**Milk of Bismuth:** Thick, white, opaque suspension that separates on standing. Is odorless and practically tasteless. Miscible with water and with alcohol.

**Bismuth Citrate:** White, amorphous or crystalline powder. Stable in air. Melts at about 300°, with decomposition. Soluble in ammonia TS and in solutions of alkali citrates; insoluble in water and in alcohol.

**Bismuth Subcarbonate:** White or almost white powder. Practically insoluble in water, in alcohol, and in ether. Dissolves in dilute acids with effervescence.

**Bismuth Subgallate:** Amorphous, bright yellow powder. Is odorless and tasteless. Is stable in air, but is affected by light. Dissolves readily with decomposition in warm, moderately dilute hydrochloric, nitric, or sulfuric acid; readily dissolved by solutions of alkali hydroxides, forming a clear, yellow liquid that rapidly assumes a deep red color. Practically insoluble in water, in alcohol, in chloroform, and in ether; insoluble in very dilute mineral acids.

**Bismuth Subnitrate:** White, slightly hygroscopic powder. Practically insoluble in water and in alcohol; readily dissolved by hydrochloric acid or by nitric acid.

**Bismuth Subsalicylate:** Fine to off-white, microcrystalline, odorless, tasteless powder. Practically insoluble in water, in alcohol, and in ether. Reacts with alkalies and mineral acids.

**Bisoprolol Fumarate:** White, crystalline powder. Very soluble in water and in methanol; freely soluble in chloroform, in glacial acetic acid, and in alcohol; slightly soluble in acetone and in ethyl acetate.

**Bleomycin Sulfate:** Cream-colored, amorphous powder. Very soluble in water.

**Boric Acid:** Colorless, odorless scales of a somewhat pearly luster, or crystals, or white powder that is slightly unctuous to the touch. Is stable in air. Freely soluble in glycerin, in boiling water, and in boiling alcohol; soluble in water and in alcohol. *NF category:* pH modifier (acidifying agent/alkalizing agent/buffering agent); antimicrobial preservative.

**Bretylium Tosylate:** White, crystalline powder. Is hygroscopic. Freely soluble in water, in methanol, and in alcohol; practically insoluble in ether, in ethyl acetate, and in hexane.

**Brimonidine Tartrate:** White, or slightly yellowish, or slightly brownish powder. Soluble in water; practically insoluble in anhydrous ethanol and in toluene.

**Brinzolamide:** White or almost white powder. Slightly soluble in alcohol and in methanol; insoluble in water.

**Bromocriptine Mesylate:** White or slightly colored, fine crystalline powder, odorless or having a weak, characteristic odor.

**Bromodiphenhydramine Hydrochloride:** White to pale buff, crystalline powder, having no more than a faint odor. Freely soluble in water and in alcohol; soluble in isopropyl alcohol; insoluble in ether and in solvent hexane.

**Brompheniramine Maleate:** White, odorless, crystalline powder. Freely soluble in water; soluble in alcohol and in chloroform; slightly soluble in ether and in benzene.

**Budesonide:** White to off-white, odorless, crystalline powder. Freely soluble in chloroform; sparingly soluble in alcohol; practically insoluble in water and in heptane.

**Bumetanide:** Practically white powder. Soluble in alkaline solutions; slightly soluble in water.

**Bupivacaine Hydrochloride:** White, odorless, crystalline powder. Melts at about 248°, with decomposition. Freely soluble in water and in alcohol; slightly soluble in chloroform and in acetone.

**Bupivacaine Hydrochloride Injection:** Clear, colorless solution.

**Bupivacaine Hydrochloride and Epinephrine Injection:** Clear, colorless solution.

**Bupropion Hydrochloride:** White powder. Soluble in water, in 0.1 N hydrochloric acid, and in alcohol.

**Buspirone Hydrochloride:** White crystalline powder. Very soluble in water; freely soluble in methanol and in methylene chloride; sparingly soluble in ethanol and in acetonitrile; very slightly soluble in ethyl acetate; practically insoluble in hexanes.

**Busulfan:** White, crystalline powder. Sparingly soluble in acetone; slightly soluble in alcohol; very slightly soluble in water.

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**Butabarbital Sodium:** White powder, having a bitter taste. Freely soluble in water and in alcohol; practically insoluble in absolute ether.

**Butalbital:** White, crystalline, odorless powder, having a slightly bitter taste. Is stable in air. Its saturated solution is acid to litmus. Freely soluble in alcohol, in ether, and in chloroform; soluble in boiling water, and in solutions of fixed alkalies and alkali carbonates; slightly soluble in cold water.

**Butamben:** White, crystalline powder. Is odorless and tasteless. Soluble in dilute acids, in alcohol, in chloroform, in ether, and in fixed oils; very slightly soluble in water. Is slowly hydrolyzed when boiled with water.

**Butane:** Colorless, flammable gas (boiling temperature is about –0.5°). One volume of water dissolves 0.15 volume, and 1 volume of alcohol dissolves 18 volumes at 17° and 770 mm; 1 volume of ether or chloroform at 17° dissolves 25 or 30 volumes, respectively. Vapor pressure at 21° is about 1620 mm of mercury (17 psig). *NF category:* Propellant.

**Butoconazole Nitrate:** White to off-white, crystalline powder. Melts at about 160°. Sparingly soluble in methanol; slightly soluble in acetonitrile, in acetone, in dichloromethane, and in tetrahydrofuran; very slightly soluble in ethyl acetate; practically insoluble in water.

**Butorphanol Tartrate:** White powder. Its solutions are slightly acidic. Melts between 217° and 219°, with decomposition. Soluble in dilute acids; sparingly soluble in water; slightly soluble in methanol; insoluble in alcohol, in chloroform, in ethyl acetate, in ethyl ether, and in hexane.

**Butyl Alcohol:** Clear, colorless, mobile liquid, having a characteristic, penetrating vinous odor. Soluble in water. Miscible with alcohol, with ether, and with many other organic solvents. *NF category:* Solvent.

**Butylene Glycol:** Clear, colorless, hygroscopic, viscous liquid. It is miscible with water, with acetone, and with ether in all proportions; immiscible with fixed oils. It dissolves most essential oils and synthetic flavoring substances. *NF category*: Solvent; wetting and/or solubilizing agent.

**Butyl Palmitostearate:** Colorless or pale yellowish waxy solid at temperatures below 17°–24° or colorless or pale yellowish liquid at temperatures of 17°–24° or above. Soluble in acetone, in alcohol, in ether, in mineral oils, and in vegetable oils; practically insoluble in water and in propylene glycol. *NF category:* Emulsifying and/or solubilizing agent; flavors and fragrance; plasticizer.

**Butyl Stearate:** Colorless or pale yellowish waxy solid at temperatures below 19°–24° or colorless or pale yellowish liquid at temperatures of 19°–24° or above. Soluble in acetone, in alcohol, in ether, in mineral oils, and in vegetable oils; practically insoluble in water and in propylene glycol. *NF category:* Emulsifying and/or solubilizing agent; flavors and fragrance; plasticizer.

**Butylated Hydroxyanisole:** White or slightly yellow, waxy solid, having a faint, characteristic odor. Freely soluble in alcohol, in propylene glycol, in chloroform, and in ether; insoluble in water. *NF category:* Antioxidant.

**Butylated Hydroxytoluene:** White, crystalline solid, having a faint, characteristic odor. Freely soluble in alcohol, in chloroform, and in ether; insoluble in water and in propylene glycol. *NF category:* Antioxidant.

**Butylparaben:** Small, colorless crystals or white powder. Freely soluble in acetone, in alcohol, in ether, and in propylene glycol; very slightly soluble in water and in glycerin. *NF category:* Antimicrobial preservative.

**Cabergoline:** White or almost white, crystalline or amorphous powder. Freely soluble in alcohol (96%); slightly soluble in 0.1 M hydrochloric acid; very slightly soluble in hexane; practically insoluble in water.

**Caffeine:** White powder or white, glistening needles, usually matted together. Is odorless and has a bitter taste. Its solutions are neutral to litmus. The hydrate is efflorescent in air. Freely soluble in chloroform; sparingly soluble in water and in alcohol; slightly soluble in ether.

**Calamine:** Pink, odorless, practically tasteless, fine powder. Soluble in mineral acids; insoluble in water.

**Calcipotriene:** White or almost white crystalline powder. Freely soluble in alcohol; slightly soluble in methylene chloride; practically insoluble in water.

**Calcitriol:** White or almost white crystals. Freely soluble in alcohol; soluble in ether and in fatty oils; practically insoluble in water. It is sensitive to air, heat, and light.

**Calcium Acetate:** White, odorless or almost odorless, hygroscopic, crystalline powder. When heated to above 160°, it decomposes to calcium carbonate and acetone. Freely soluble in water; slightly soluble in methanol; practically insoluble in acetone, in dehydrated alcohol, and in benzene. *NF category:* Antimicrobial preservative; sequestering agent.

**Calcium Ascorbate:** White to slightly yellow, practically odorless powder. Freely soluble in water (approximately 50 g per 100 mL); slightly soluble in alcohol; insoluble in ether.

**Calcium Carbonate:** Fine, white, odorless, tasteless, microcrystalline powder. Is stable in air. Practically insoluble in water. Its solubility in water is increased by the presence of any ammonium salt or of carbon dioxide. The presence of any alkali hydroxide reduces its solubility. Insoluble in alcohol. Dissolves with effervescence in 1 N acetic acid, in 3 N hydrochloric acid, and in 2 N nitric acid. *NF category:* Diluent; pH modifier (acidifying agent/alkalizing agent/buffering agent); coating agent; wet binder.

**Calcium Chloride:** White, hard, odorless fragments or granules. Is deliquescent. Very soluble in boiling water; freely soluble in water, in alcohol, and in boiling alcohol. *NF category:* Desiccant; antimicrobial preservative.

**Calcium Citrate:** White, odorless, crystalline powder. Freely soluble in diluted 3 N hydrochloric acid and in diluted 2 N nitric acid; slightly soluble in water; insoluble in alcohol.

**Calcium Gluceptate:** White to faintly yellow, amorphous powder. Is stable in air, but the hydrous forms may lose part of their water of hydration on standing. Freely soluble in water; insoluble in alcohol and in many other organic solvents.

**Calcium Gluconate:** White, crystalline, odorless, tasteless granules or powder. Is stable in air. Its solutions are neutral to litmus. Freely soluble in boiling water; sparingly (and slowly) soluble in water; insoluble in alcohol.

**Calcium Hydroxide:** White powder. Has an alkaline, slightly bitter taste. Soluble in glycerin and in syrup; slightly soluble in water; very slightly soluble in boiling water; insoluble in alcohol. *NF category:* pH modifier (acidifying agent/alkalizing agent/buffering agent).

**Calcium Hydroxide Solution:** Clear, colorless liquid having an alkaline taste. Is alkaline to litmus.

**Calcium Lactate:** White, practically odorless granules or powder. The pentahydrate is somewhat efflorescent and at 120° becomes anhydrous. The pentahydrate is soluble in water; it is practically insoluble in alcohol. *NF category:* pH modifier (acidifying agent/alkalizing agent/buffering agent); antimicrobial preservative; wet binder.

**Calcium Levulinate:** White, crystalline or amorphous, powder, having a faint odor suggestive of burnt sugar. Has a bitter, salty taste. Freely soluble in water; slightly soluble in alcohol; insoluble in ether and in chloroform.

**Calcium Pantothenate:** Slightly hygroscopic, white powder. Is odorless and has a bitter taste. Freely soluble in water; soluble in glycerin; practically insoluble in alcohol, in chloroform, and in ether.

**Racemic Calcium Pantothenate:** White, slightly hygroscopic powder, having a faint, characteristic odor, and a bitter taste. Is stable in air. Its solutions are neutral or alkaline to litmus. Is optically inactive. Freely soluble in water; soluble

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in glycerin; practically insoluble in alcohol, in chloroform, and in ether.

**Dibasic Calcium Phosphate:** White, odorless, tasteless powder. Is stable in air. Soluble in 3 N hydrochloric acid and in 2 N nitric acid; practically insoluble in water; insoluble in alcohol. *NF category:* Diluent.

**Tribasic Calcium Phosphate:** White, odorless, tasteless powder. Is stable in air. Freely soluble in 3 N hydrochloric acid and in 2 N nitric acid; practically insoluble in water; insoluble in alcohol. *NF category:* Diluent; pH modifier (acidifying agent/alkalizing agent/buffering solution); glidant and/or anticaking agent.

**Calcium Polycarbophil:** White to creamy white powder. Insoluble in water, in dilute acids, in dilute alkalies, and in common organic solvents.

**Calcium Propionate:** Occurs as a white crystalline solid. One g dissolves in about 3 mL of water. *NF category:* Antimicrobial preservative.

**Calcium Saccharate:** White, odorless, tasteless, crystalline powder. Soluble in dilute mineral acids and in solutions of calcium gluconate; slightly soluble in boiling water; very slightly soluble in alcohol, and in cold water; practically insoluble in ether and in chloroform.

**Calcium Silicate:** White to off-white, free-flowing powder that remains so after absorbing relatively large amounts of water or other liquids. Insoluble in water. Forms a gel with mineral acids. *NF category:* Glidant and/or anticaking agent.

**Calcium Stearate:** Fine, white to yellowish-white, bulky powder having a slight, characteristic odor. Is unctuous, and is free from grittiness. Insoluble in water, in alcohol, and in ether. *NF category:* Lubricant.

**Calcium Sulfate:** Fine, white to slightly yellow-white, odorless powder. Soluble in 3 N hydrochloric acid; slightly soluble in water. *NF category:* Desiccant; diluent.

**Calcium Undecylenate:** Fine, white powder, having a characteristic odor and no grit. Slightly soluble in hot alcohol; practically insoluble in water, in ether, in chloroform, in acetone, and in cold alcohol.

**Camphor:** Colorless or white crystals, granules, or crystalline masses; or colorless to white, translucent, tough masses. Has a penetrating, characteristic odor and a pungent, aromatic taste. Specific gravity is about 0.99. Slowly volatilizes at ordinary temperatures. Slightly soluble in water; very soluble in alcohol, in chloroform, and in ether; freely soluble in carbon disulfide, in solvent hexane, and in fixed and volatile oils.

***Change to read:***

**Candelilla Wax:** A hard, yellowish-brown-opaque to translucent wax. Its specific gravity is about 0.983. Soluble in chloroform and in toluene; insoluble in water. ▲*NF category:* Coating agent; colloid stabilizing agent; emollient; emulsifying agent; film-forming agent; ointment base; stiffening agent; vehicle.▲ (NF 1-Dec-2023)

**Candesartan Cilexetil:** White to off-white powder. Sparingly soluble in methanol; practically insoluble in water.

**Canola Oil:** Clear, pale yellow, slightly viscous liquid. Practically insoluble in water and in alcohol. Miscible with light petroleum (bp: 40° to 60°). *NF category:* Solvent; emollient; vehicle (oleaginous).

**Capecitabine:** White to off-white crystalline powder. Freely soluble in methanol; soluble in acetonitrile and in alcohol; sparingly soluble in water.

**Capreomycin Sulfate:** White to practically white, amorphous powder. Freely soluble in water; practically insoluble in most organic solvents.

**Caprylic Acid:** Clear, colorless or slightly yellowish, oily liquid. Very soluble in acetone and in alcohol; very slightly soluble in water. It dissolves in dilute solutions of alkali hydroxides. *NF category:* Emulsifying agent.

**Caprylocaproyl Polyoxylglycerides:** Pale yellow, oily liquids. Dispersible in hot water; freely soluble in methylene chloride. *NF category:* Ointment base; solvent; wetting and/or solubilizing agent; emulsifying agent.

**Capsaicin:** Off-white powder. Melts at about 65°. Soluble in alcohol, in benzene, in chloroform; slightly soluble in carbon disulfide; practically insoluble in cold water.

**Capsicum Oleoresin:** Dark red, oily liquid. Soluble in alcohol, in acetone, in ether, in chloroform, and in volatile oils; soluble with opalescence in fixed oils.

**Captopril:** White to off-white, crystalline powder, which may have a characteristic, sulfide-like odor. Melts in the range of 104° to 110°. Freely soluble in water, in methanol, in alcohol, and in chloroform.

**Caramel:** Thick, dark brown liquid having the characteristic odor of burnt sugar, and a pleasant, bitter taste. One part dissolved in 1000 parts of water yields a clear solution having a distinct yellowish-orange color. The color of this solution is not changed and no precipitate is formed after exposure to sunlight for 6 hours. When spread in a thin layer on a glass plate, it appears homogeneous, reddish-brown, and transparent. Miscible with water. Soluble in dilute alcohol up to 55% (v/v). Immiscible with ether, with chloroform, with acetone, with benzene, and with solvent hexane. *NF category:* Coloring agent.

**Carbachol:** White powder. Freely soluble in water; sparingly soluble in alcohol; practically insoluble in chloroform and in ether.

**Carbamazepine:** White to off-white powder. It exhibits polymorphism. Soluble to very slightly soluble in alcohol and in acetone; practically insoluble in water.

**Carbamide Peroxide Topical Solution:** Clear, colorless, viscous liquid, having a characteristic odor and taste.

**Carbidopa:** White to creamy white, odorless or practically odorless, powder. Freely soluble in 3 N hydrochloric acid; slightly soluble in water, and in methanol; practically insoluble in alcohol, in acetone, in chloroform, and in ether.

**Carbinoxamine Maleate:** White, odorless, crystalline powder. Very soluble in water; freely soluble in alcohol and in chloroform; very slightly soluble in ether.

**Carbol-Fuchsin Topical Solution:** Dark purple liquid, which appears purplish red when spread in a thin film.

**Carbomer 910:** White, fluffy powder, having a slight, characteristic odor. Is hygroscopic. The pH of a 1 in 100 dispersion is about 3. When neutralized with alkali hydroxides or with amines, it dissolves in water, in alcohol, and in glycerin. *NF category:* Suspending and/or viscosity-increasing agent.

**Carbomer 934:** See *Carbomer 910.*

**Carbomer 934P:** See *Carbomer 910.*

**Carbomer 940:** See *Carbomer 910.*

**Carbomer 941:** See *Carbomer 910.*

**Carbomer 1342:** See *Carbomer 910.*

**Carbomer Copolymer:** White, hygroscopic powder. It swells in water when a dispersion of it is neutralized with sodium hydroxide to a pH within the range of 7.3 to 7.8. *NF category:* Emulsifying agent; suspending and/or viscosity-increasing agent; wet binder; polymers for ophthalmic use; release-modifying agent.

**Carbomer Homopolymer:** White, fluffy hygroscopic powder, having a slight, characteristic odor. The pH of a 1 in 100 dispersion in water is about 3. When neutralized with alkali hydroxides or with amines, it swells giving the appearance of dissolving in water; when neutralized with lower amines and alkanolamines, it swells giving the appearance of dissolving in methanol or glycerin; when neutralized with ethoxylated long-chain ($C_{14}$–$C_{18}$) amines, it swells giving the appearance of dissolving in ethanol. *NF*

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*category:* Wet binder; suspending and/or viscosity-increasing agent; emulsifying agent; polymers for ophthalmic use; release-modifying agent.

**Carbomer Interpolymer:** White, hygroscopic powder. It swells in water when a dispersion of it is neutralized with sodium hydroxide to a pH within the range of 5.5 to 9. *NF category:* Emulsifying agent; suspending and/or viscosity increasing agent; wet binder; polymers for ophthalmic use; release-modifying agent.

**Carbon Dioxide:** Odorless, colorless gas. Its solutions are acid to litmus. One L at 0° and at a pressure of 760 mm of mercury weighs 1.977 g. One volume dissolves in about 1 volume of water. *NF category:* Air displacement; propellant.

**Carboprost Tromethamine:** White to off-white powder. Soluble in water.

**Carboxymethylcellulose Calcium:** White to yellowish-white powder. Is hygroscopic. Practically insoluble in alcohol, in acetone, in ether, in chloroform, and in benzene. It swells with water to form a suspension; the pH of the suspension, obtained by shaking 1 g with 100 mL of water, is between 4.5 and 6.0. *NF category:* Suspending and/or viscosity-increasing agent; coating agent; emulsifying agent; film-forming agent; disintegrant.

**Carboxymethylcellulose Sodium:** White to cream-colored powder or granules. The powder is hygroscopic. Is easily dispersed in water to form colloidal solutions. Insoluble in alcohol, in ether, and in most other organic solvents. *NF category:* Coating agent; suspending and/or viscosity-increasing agent; wet binder; film-forming agent; release-modifying agent; disintegrant.

**Carboxymethylcellulose Sodium 12:** Colorless or white to off-white powder or granules. Is odorless. Water solubility depends on degree of substitution (easily dispersed in water at all temperatures, forming a clear, colloidal solution). Insoluble in acetone, in alcohol, in ether, and in toluene. *NF category:* Suspending and/or viscosity-increasing agent.

**Enzymatically-Hydrolyzed Carboxymethylcellulose Sodium:** White or slightly yellowish or grayish, odorless, slightly hygroscopic granular or fibrous powder. Soluble in water; insoluble in alcohol. *NF category:* Coating agent; suspending and/or viscosity-increasing agent; film-forming agent.

**Low-Substituted Carboxymethylcellulose Sodium:** A white or almost white powder or short fibers. Practically insoluble in acetone, in alcohol, and in toluene. It swells in water to form a gel.

**Carisoprodol:** White, crystalline powder, having a mild, characteristic odor and a bitter taste. Freely soluble in alcohol, in chloroform, and in acetone; very slightly soluble in water.

**Carmellose:** White powder. Practically insoluble in ethanol (99.5%). Swells with water to form a suspension. Becomes viscous in sodium hydroxide TS. Is hygroscopic. *NF category:* Suspending and/or viscosity-increasing agent; polymers for ophthalmic use.

**Carmustine:** Light yellow powder. Freely soluble in ether.

**Carprofen:** White crystalline powder. Freely soluble in ether, in acetone, in ethyl acetate, and in sodium hydroxide TS or sodium carbonate TS; practically insoluble in water.

**Carrageenan:** Yellowish or tan to white, coarse to fine powder. Is practically odorless and has a mucilaginous taste. Soluble in water at a temperature of about 80°, forming a viscous, clear or slightly opalescent solution that flows readily. Disperses in water more readily if first moistened with alcohol, with glycerin, or with a saturated solution of sucrose in water. *NF category:* Suspending and/or viscosity-increasing agent; release-modifying agent.

**Carvedilol:** White or nearly white, crystalline powder. Slightly soluble in alcohol; practically insoluble in water and in dilute acids.

**Casanthranol:** Light tan to brown, amorphous, hygroscopic powder. Freely soluble in water, with some residue; partially soluble in methanol and in hot isopropyl alcohol; practically insoluble in acetone.

**Cascara Sagrada:** Has a distinct odor and a bitter and slightly acrid taste.

**Castor Oil:** Pale yellowish or almost colorless, transparent, viscid liquid. Has a faint, mild odor; is free from foreign and rancid odor; and has a bland, characteristic taste. Soluble in alcohol. Miscible with dehydrated alcohol, with glacial acetic acid, with chloroform, and with ether. *NF category:* Plasticizer; emollient; solvent; vehicle.

**Hydrogenated Castor Oil:** White, crystalline wax. Insoluble in water and in most common organic solvents. *NF category:* Stiffening agent; lubricant.

**Cefaclor:** White to off-white, crystalline powder. Slightly soluble in water; practically insoluble in methanol, in chloroform, and in benzene.

**Cefadroxil:** White to off-white, crystalline powder. Slightly soluble in water; practically insoluble in alcohol, in chloroform, and in ether.

**Cefazolin:** White to slightly off-white, odorless, crystalline powder. Melts at about 198° to 200°, with decomposition. Soluble in dimethylformamide and in pyridine; sparingly soluble in acetone; slightly soluble in alcohol, in methanol, and in water; very slightly soluble in ethyl acetate, in isopropyl alcohol, and in methyl isobutyl ketone; practically insoluble in benzene, in chloroform, in ether, and in methylene chloride.

**Cefazolin Sodium:** White to off-white, practically odorless, crystalline powder, or white to off-white solid. Freely soluble in water, in saline TS, and in dextrose solutions; very slightly soluble in alcohol; practically insoluble in chloroform and in ether.

**Cefdinir:** White to light-yellow crystalline powder. Sparingly soluble in 0.1 M phosphate buffer (pH 7) solution; practically insoluble in water, in alcohol, and in diethyl ether.

**Cefepime Hydrochloride:** White to off-white, crystalline, nonhygroscopic solid. Freely soluble in water.

**Cefepime for Injection:** White to pale yellow powder. Freely soluble in water.

**Cefixime:** White to light yellow, crystalline powder. Soluble in methanol and in propylene glycol; slightly soluble in alcohol, in acetone, and in glycerin; very slightly soluble in 70% sorbitol and in octanol; practically insoluble in ether, in ethyl acetate, in hexane, and in water.

**Cefmetazole Sodium:** White solid. Very soluble in water and in methanol; soluble in acetone; practically insoluble in chloroform.

**Cefoperazone Sodium:** White to pale buff crystalline powder. Freely soluble in water; soluble in methanol; slightly soluble in dehydrated alcohol; insoluble in acetone, in ethyl acetate, and in ether.

**Cefotaxime Sodium:** Off-white to pale yellow crystalline powder. Freely soluble in water; practically insoluble in organic solvents.

**Cefoxitin Sodium:** White to off-white, granules or powder, having a slight characteristic odor. Is somewhat hygroscopic. Very soluble in water; soluble in methanol; sparingly soluble in dimethylformamide; slightly soluble in acetone; insoluble in ether and in chloroform.

**Cefpodoxime Proxetil:** White to light brownish-white powder. Odorless or having a faint odor, and has a bitter taste. Freely soluble in dehydrated alcohol; soluble in acetonitrile and in methanol; slightly soluble in ether; very slightly soluble in water.

**Ceftazidime:** White to cream-colored, crystalline powder. Soluble in alkali and in dimethyl sulfoxide; slightly soluble in dimethylformamide, in methanol, and in water; insoluble in

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acetone, in alcohol, in chloroform, in dioxane, in ether, in ethyl acetate, and in toluene.

**Ceftiofur Hydrochloride:** White to beige powder. Sparingly soluble in dimethylformamide and in ethanol; slightly soluble in 0.1 N hydrochloric acid; very slightly soluble in tetrahydrofuran; practically insoluble in ethyl acetate.

**Ceftiofur Sodium:** White to beige powder. Freely soluble in propylene glycol; soluble in water; slightly soluble in tetrahydrofuran, in dehydrated alcohol, and in acetone; insoluble in methylene chloride.

**Ceftizoxime Sodium:** White to pale yellow crystalline powder. Freely soluble in water.

**Ceftriaxone Sodium:** White to yellowish-orange crystalline powder. Freely soluble in water; sparingly soluble in methanol; very slightly soluble in alcohol.

**Cefuroxime Axetil:** White to almost white powder. The amorphous form is freely soluble in acetone; soluble in chloroform, in ethyl acetate, and in methanol; slightly soluble in dehydrated alcohol; insoluble in ether and in water. The crystalline form is freely soluble in acetone; sparingly soluble in chloroform, in ethyl acetate, and in methanol; slightly soluble in dehydrated alcohol; insoluble in ether and in water.

**Cefuroxime Sodium:** White or faintly yellow powder. Freely soluble in water; soluble in methanol; very slightly soluble in alcohol, in ether, in ethyl acetate, and in chloroform.

**Celecoxib:** White or almost white, crystalline or amorphous powder. Soluble to freely soluble in ethanol; soluble in methylene chloride; practically insoluble in water.

**Cellaburate:** Fine white or almost white powder or granules. Available in a range of viscosities, acetyl and butyl contents. Slightly hygroscopic; soluble in acetone, in methylene chloride, in pyridine, and in dimethyl sulfoxide; practically insoluble in water and in alcohol. *NF category:* Coating agent; polymer membrane; film-forming agent; release-modifying agent; diluent.

**Cellacefate:** Free-flowing, white powder. May have a slight odor of acetic acid. Soluble in acetone and in dioxane; insoluble in water and in alcohol. *NF category:* Coating agent; film-forming agent.

**Cellulose Acetate:** Fine, white powder or free-flowing pellets. Available in a range of viscosities and acetyl contents. High viscosity, which reflects high molecular weight, decreases solubility slightly. High acetyl content cellulose acetates generally have more limited solubility in commonly used organic solvents than low acetyl content cellulose acetates, but are more soluble in methylene chloride. All acetyl content cellulose acetates are soluble in dioxane and in dimethylformamide; insoluble in alcohol and in water. *NF category:* Coating agent; polymer membrane; film-forming agent; diluent.

**Microcrystalline Cellulose:** Fine, white or almost white powder. It consists of free-flowing, nonfibrous particles. Practically insoluble in sodium hydroxide solution (1 in 20); insoluble in water, in dilute acids, and in most organic solvents. *NF category:* Diluent; disintegrant; wet binder; suspending and/or viscosity-increasing agent.

**Silicified Microcrystalline Cellulose:** White or almost white, very fine to moderately fine powder. It is a free-flowing material that may be compacted into self-binding tablets that disintegrate rapidly in water. Slightly soluble in sodium hydroxide solution (1 in 20); practically insoluble in water, in acetone, in ethanol, in toluene, and in diluted acid. *NF category:* Diluent; disintegrant; wet binder.

**Microcrystalline Cellulose and Carboxymethylcellulose Sodium:** Tasteless, odorless, white to off-white, coarse to fine powder. Swells in water, producing, when dispersed, a white, opaque dispersion or gel. Insoluble in organic solvents and in dilute acids. *NF category:* Suspending and/or viscosity-increasing agent.

**Oxidized Cellulose:** In the form of gauze or lint. Is slightly off-white in color, is acidic to the taste, and has a slight, charred odor. Soluble in dilute alkalies; insoluble in water and in acids.

**Oxidized Regenerated Cellulose:** A knit fabric, usually in the form of sterile strips. Slightly off-white, having a slight odor. Soluble in dilute alkalies; insoluble in water and in dilute acids.

**Powdered Cellulose:** White or almost white powder. Exhibits degrees of fineness ranging from a free-flowing dense powder to a coarse, fluffy, nonflowing material. Slightly soluble in sodium hydroxide solution (1 in 20); insoluble in water, in dilute acids, and in nearly all organic solvents. *NF category:* Filtering aid; sorbent; diluent; disintegrant; glidant and/or anticaking agent; suspending and/or viscosity-increasing agent.

**Cephalexin:** White to off-white, crystalline powder. Slightly soluble in water; practically insoluble in alcohol, in chloroform, and in ether.

**Cephalexin Hydrochloride:** White to off-white, crystalline powder. Soluble to the extent of 10 mg per mL in water, in acetone, in acetonitrile, in alcohol, in dimethylformamide, and in methanol; practically insoluble in chloroform, in ether, in ethyl acetate, and in isopropyl alcohol.

**Cephalothin Sodium:** White to off-white, practically odorless, crystalline powder. Freely soluble in water, in saline TS, and in dextrose solutions; insoluble in most organic solvents.

**Cephapirin Benzathine:** White, crystalline powder. Soluble in 0.1 N hydrochloric acid; practically insoluble in water, in ether, and in toluene; insoluble in alcohol.

**Cephapirin Sodium:** White to off-white, crystalline powder, odorless or having a slight odor. Very soluble in water; insoluble in most organic solvents.

**Cephradine:** White to off-white, crystalline powder. Sparingly soluble in water; very slightly soluble in alcohol and in chloroform; practically insoluble in ether.

**Cetirizine Hydrochloride:** White to almost white powder. Freely soluble in water; practically insoluble in acetone and in methylene chloride.

**Cetostearyl Alcohol:** Unctuous, white flakes or granules, having a faint, characteristic odor, and a bland, mild taste. Soluble in alcohol and in ether; insoluble in water. *NF category:* Stiffening agent; emollient; emulsifying agent; suspending and/or viscosity-increasing agent.

**Cetrimonium Bromide:** A white to creamy white, voluminous, free-flowing powder, with a characteristic faint odor and bitter, soapy taste. Freely soluble in water and in alcohol; practically insoluble in ether. *NF category:* Antimicrobial preservative.

**Cetyl Alcohol:** Unctuous, white flakes, granules, cubes, or castings. Has a faint characteristic odor and a bland, mild taste. Usually melts in the range between 45° and 50°. Soluble in alcohol and in ether, the solubility increasing with an increase in temperature; insoluble in water. *NF category:* Stiffening agent; coating agent; emulsifying agent.

**Cetyl Esters Wax:** White to off-white, somewhat translucent flakes, having a crystalline structure and a pearly luster when caked. Has a faint odor and a bland, mild taste, free from rancidity, and has a specific gravity of about 0.83 at 50°. Soluble in boiling alcohol, in ether, in chloroform, and in fixed and volatile oils; slightly soluble in cold solvent hexane; practically insoluble in cold alcohol; insoluble in water. *NF category:* Stiffening agent; emollient.

**Cetyl Palmitate:** White crystals or flakes. Freely soluble in alcohol and in ether; practically insoluble in water. *NF category:* Stiffening agent.

**Cetylpyridinium Chloride:** White to almost-white powder, having a slight, characteristic odor. Very soluble in

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water, in alcohol, and in chloroform; slightly soluble in benzene and in ether. *NF category:* Antimicrobial preservative; wetting and/or solubilizing agent; emulsifying agent.

**Cetylpyridinium Chloride Topical Solution:** Clear liquid. Is colorless unless a color has been added; has an aromatic odor and a bitter taste.

***Add the following:***

▲**Cevimeline Hydrochloride:** White to off-white, crystalline powder. Very soluble in water; freely soluble in alcohol and in chloroform; practically insoluble in ether.▲ (USP 1-Dec-2023)

**Activated Charcoal:** Fine, black, odorless, tasteless powder, free from gritty matter. *NF category:* Sorbent.

**Chitosan:** White or almost white powder or granules. Soluble in aqueous solutions of glycolic acid, of formic acid, of acetic acid, of hydrochloric acid, and of lactic acid; practically insoluble in organic solvents and in water. *NF category:* Coating agent; film-forming agent; suspending and/or viscosity-increasing agent; vehicle (solid carrier).

**Chloral Hydrate:** Colorless, transparent, or white crystals having an aromatic, penetrating, and slightly acrid odor, and a slightly bitter, caustic taste. Melts at about 55°, and slowly volatilizes when exposed to air. Very soluble in water and in olive oil; freely soluble in alcohol, in chloroform, and in ether.

**Chlorambucil:** Off-white, slightly granular powder. Freely soluble in acetone; soluble in dilute alkali; very slightly soluble in water.

**Chloramphenicol:** Fine, white to grayish-white or yellowish-white, needle-like crystals or elongated plates. Its solutions are practically neutral to litmus. Is reasonably stable in neutral or moderately acid solutions. Its alcohol solution is dextrorotatory and its ethyl acetate solution is levorotatory. Freely soluble in alcohol, in propylene glycol, in acetone, and in ethyl acetate; slightly soluble in water.

**Chloramphenicol Palmitate:** Fine, white, unctuous, crystalline powder, having a faint odor and a bland, mild taste. Freely soluble in acetone and in chloroform; soluble in ether; sparingly soluble in alcohol; very slightly soluble in solvent hexane; insoluble in water.

**Chloramphenicol Sodium Succinate:** Light yellow powder. Freely soluble in water and in alcohol.

**Chlordiazepoxide:** Yellow, practically odorless, crystalline powder. Is sensitive to sunlight. Melts at about 240°. Sparingly soluble in chloroform and in alcohol; insoluble in water.

**Chlordiazepoxide Hydrochloride:** White or practically white, odorless, crystalline powder. Is affected by sunlight. Soluble in water; sparingly soluble in alcohol; insoluble in solvent hexane.

**Chlorhexidine Acetate:** A white or almost white, microcrystalline powder. Soluble in alcohol; sparingly soluble in water; slightly soluble in glycerol and in propylene glycol.

**Chlorhexidine Gluconate Solution:** Almost colorless or pale yellow, clear liquid. Miscible with glacial acetic acid and with water; miscible with three times its volume of acetone and with five times its volume of dehydrated alcohol; further addition of acetone or dehydrated alcohol yields a white turbidity.

**Chlorhexidine Hydrochloride:** White or almost white, crystalline powder. Sparingly soluble in propylene glycol and in water; very slightly soluble in alcohol.

**Chlorobutanol:** Colorless to white crystals, having a characteristic, somewhat camphoraceous, odor and taste. Anhydrous form melts at about 95°, and hydrous form melts at about 76°. Freely soluble in alcohol, in ether, in chloroform, and in volatile oils; soluble in glycerin; slightly soluble in water. *NF category:* Antimicrobial preservative; plasticizer.

**Chlorocresol:** Colorless or practically colorless crystals or crystalline powder, having a characteristic, nontarry odor. Is volatile in steam. Very soluble in alcohol; soluble in ether, in terpenes, in fixed oils, and in solutions of alkali hydroxides; slightly soluble in water and more soluble in hot water. *NF category:* Antimicrobial preservative.

**Chloroprocaine Hydrochloride:** White, crystalline powder. Is odorless, and is stable in air. Its solutions are acid to litmus. Exhibits local anesthetic properties when placed upon the tongue. Soluble in water; slightly soluble in alcohol; very slightly soluble in chloroform; practically insoluble in ether.

**Chloroquine:** White or slightly yellow, crystalline powder. Is odorless, and has a bitter taste. Soluble in dilute acids, in chloroform, and in ether; very slightly soluble in water.

**Chloroquine Phosphate:** White, crystalline powder. Is odorless, has a bitter taste, and is discolored slowly on exposure to light. Its solutions have a pH of about 4.5. Exists in two polymorphic forms, one melting between 193° and 195° and the other between 210° and 215° (see *Melting Range or Temperature* ⟨741⟩); mixture of the forms melts between 193° and 215°. Freely soluble in water; practically insoluble in alcohol, in chloroform, and in ether.

**Chlorothiazide:** White or practically white, crystalline, odorless powder. Melts at about 340°, with decomposition. Freely soluble in dimethylformamide and in dimethyl sulfoxide; slightly soluble in methanol and in pyridine; practically insoluble in ether, in benzene, and in chloroform; very slightly soluble in water.

**Chloroxylenol:** White crystals or crystalline powder, having a characteristic odor. Is volatile in steam. Freely soluble in alcohol, in ether, in terpenes, in fixed oils, and in solutions of alkali hydroxides; very slightly soluble in water. *NF category:* Antimicrobial preservative.

**Chlorpheniramine Maleate:** White, odorless, crystalline powder. Its solutions have a pH between 4 and 5. Freely soluble in water; soluble in alcohol and in chloroform; slightly soluble in ether and in benzene.

**Chlorpromazine:** White, crystalline solid, having an amine-like odor. Darkens on prolonged exposure to light. Melts at about 60°. Freely soluble in alcohol, in benzene, in chloroform, in ether, and in dilute mineral acids; practically insoluble in water and in dilute alkali hydroxides.

**Chlorpromazine Hydrochloride:** White or slightly creamy white, odorless, crystalline powder. Darkens on prolonged exposure to light. Very soluble in water; freely soluble in alcohol and in chloroform; insoluble in ether and in benzene.

**Chlorpropamide:** White, crystalline powder, having a slight odor. Soluble in alcohol; sparingly soluble in chloroform; practically insoluble in water.

**Chlortetracycline Hydrochloride:** Yellow, crystalline powder. Is odorless, and has a bitter taste. Is stable in air, but is slowly affected by light. Soluble in solutions of alkali hydroxides and carbonates; sparingly soluble in water; slightly soluble in alcohol; practically insoluble in acetone, in chloroform, in dioxane, and in ether.

**Chlorthalidone:** White to yellowish-white, crystalline powder. Melts at a temperature above 215°, with decomposition. Soluble in methanol; slightly soluble in alcohol; practically insoluble in water, in ether, and in chloroform.

**Chlorzoxazone:** White or practically white, practically odorless, crystalline powder. Soluble in solutions of alkali hydroxides and ammonia; sparingly soluble in alcohol, in isopropyl alcohol, and in methanol; slightly soluble in water.

**Cholecalciferol:** White, odorless crystals. Is affected by air and by light. Melts at about 85°. Soluble in alcohol, in chloroform, and in fatty oils; insoluble in water.

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**Cholesterol:** White or faintly yellow, practically odorless, pearly leaflets, needles, powder, or granules. Acquires a yellow to pale tan color on prolonged exposure to light. Soluble in acetone, in chloroform, in dioxane, in ether, in ethyl acetate, in solvent hexane, and in vegetable oils; sparingly soluble in dehydrated alcohol; slightly (and slowly) soluble in alcohol; insoluble in water. *NF category:* Emulsifying agent; emollient.

**Cholestyramine Resin:** White to buff-colored, hygroscopic, fine powder. Is odorless or has not more than a slight amine-like odor. Insoluble in water, in alcohol, in chloroform, and in ether.

**Choline Bitartrate:** White, hygroscopic, crystalline powder. Clear, colorless liquid in solution. Melts between 148° and 153°. Is odorless, or may have a faint trimethylamine odor. Freely soluble in water; slightly soluble in alcohol; insoluble in ether and in chloroform.

**Choline Chloride:** Colorless or white crystals or crystalline powder, usually having a slight odor of trimethylamine. Clear and colorless in solution. Hygroscopic. Soluble in alcohol and in water.

**Sodium Chromate Cr 51 Injection:** Clear, slightly yellow solution.

**Chromic Chloride:** Dark green, odorless, slightly deliquescent crystals. Soluble in water and in alcohol; slightly soluble in acetone; practically insoluble in ether.

**Chymotrypsin:** White to yellowish-white, crystalline or amorphous, odorless, powder. An amount equivalent to 100,000 USP Units is soluble in 10 mL of water and in 10 mL of saline TS.

**Ciclopirox:** White to slightly yellowish-white, crystalline powder. Freely soluble in ethanol and in methylene chloride; soluble in ether; slightly soluble in water.

**Ciclopirox Olamine:** White to slightly yellowish-white, crystalline powder. Very soluble in alcohol and in methylene chloride; slightly soluble in water; practically insoluble in cyclohexane.

**Cidofovir:** White to off-white, crystalline powder. Soluble in 0.1 M sodium hydroxide; sparingly soluble in water; practically insoluble in methanol and in ethanol.

**Cilastatin Sodium:** White to tan-colored powder. Soluble in water and in methanol.

**Cilostazol:** White to off-white crystals. Freely soluble in chloroform; slightly soluble in methanol and in alcohol; practically insoluble in water.

**Cimetidine:** White to off-white, crystalline powder; odorless, or having a slight mercaptan odor. Freely soluble in methanol; soluble in alcohol and in polyethylene glycol 400; sparingly soluble in isopropyl alcohol; slightly soluble in water and in chloroform; practically insoluble in ether.

**Ciprofloxacin Hydrochloride:** Faintly yellowish to light yellow crystals. Sparingly soluble in water; slightly soluble in acetic acid and in methanol; very slightly soluble in dehydrated alcohol; practically insoluble in acetone, in acetonitrile, in ethyl acetate, in hexane, and in methylene chloride.

**Cisapride:** White or almost white powder. Freely soluble in dimethylformamide; soluble in methylene chloride; sparingly soluble in methanol; practically insoluble in water.

**Cisatracurium Besylate:** White to pale yellow hygroscopic powder. Very soluble in acetonitrile; freely soluble in chloroform and in methylene chloride; soluble in water; slightly soluble in methanol.

**Citalopram Hydrobromide:** White to almost white, crystalline powder. Soluble in alcohol; sparingly soluble in water and in dehydrated alcohol.

**Anhydrous Citric Acid:** Colorless, translucent crystals, or white, granular to fine, crystalline powder. Melts at about 153°, with decomposition. Very soluble in water; freely soluble in alcohol; very slightly soluble in ether. *NF category:* pH modifier (acidifying agent/alkalizing agent/buffering agent).

**Citric Acid Monohydrate:** Colorless, translucent crystals, or white, granular to fine, crystalline powder. Efflorescent in dry air. Very soluble in water; freely soluble in alcohol; very slightly soluble in ether. *NF category:* pH modifier (acidifying agent/alkalizing agent/buffering agent); antioxidant; chelating and/or complexing agent.

**Clarithromycin:** White to off-white, crystalline powder. Soluble in acetone; slightly soluble in dehydrated alcohol, in methanol, and in acetonitrile, and in phosphate buffer at pH values of 2 to 5; practically insoluble in water.

**Clavulanate Potassium:** White to off-white powder. Is moisture-sensitive. Freely soluble in water, but stability in aqueous solution is not good, optimum stability at a pH of 6.0 to 6.3; soluble in methanol, with decomposition.

**Clemastine Fumarate:** White to off-white, odorless powder. Its solutions are acid to litmus. Slightly soluble in methanol; very slightly soluble in water, and in chloroform.

**Clenbuterol Hydrochloride:** White or almost white, crystalline powder. Soluble in water and in alcohol; slightly soluble in acetone. It melts with decomposition at 173°.

**Clidinium Bromide:** White to nearly white, practically odorless, crystalline powder. Is optically inactive. Melts at about 242°. Soluble in water and in alcohol; slightly soluble in benzene and in ether.

**Clindamycin Hydrochloride:** White or practically white, crystalline powder. Is odorless or has a faint mercaptan-like odor. Is stable in the presence of air and light. Its solutions are acidic and are dextrorotatory. Freely soluble in water, in dimethylformamide, and in methanol; soluble in alcohol; practically insoluble in acetone.

**Clindamycin Palmitate Hydrochloride:** White to off-white amorphous powder, having a characteristic odor. Very soluble in ethyl acetate and in dimethylformamide; freely soluble in water, in benzene, in ether, in chloroform, and in alcohol.

**Clindamycin Phosphate:** White to off-white, hygroscopic, crystalline powder. Is odorless or practically odorless, and has a bitter taste. Freely soluble in water; slightly soluble in dehydrated alcohol; very slightly soluble in acetone; practically insoluble in chloroform, in benzene, and in ether.

**Clioquinol:** Voluminous, spongy, yellowish-white to brownish-yellow powder, having a slight, characteristic odor. Darkens on exposure to light. Melts at about 180°, with decomposition. Soluble in hot ethyl acetate and in hot glacial acetic acid; practically insoluble in water and in alcohol.

***Add the following:***

▲**Clobazam:** White or almost white, crystalline powder. Freely soluble in methylene chloride; sparingly soluble in ethanol; slightly soluble in water.▲ (USP 1-Dec-2023)

**Clobetasol Propionate:** White to cream, crystalline powder. Soluble in acetone, in dimethyl sulfoxide, in chloroform, in methanol, and in dioxane; sparingly soluble in ethanol; slightly soluble in benzene and in diethyl ether; practically insoluble in water.

**Clocortolone Pivalate:** White to yellowish-white, odorless powder. Melts at about 230°, with decomposition. Freely soluble in chloroform and in dioxane; soluble in acetone; sparingly soluble in alcohol; slightly soluble in benzene and in ether.

**Clofazimine:** Dark red crystals. Melts at about 217°, with decomposition. Soluble in chloroform and in benzene; sparingly soluble in alcohol, in acetone, and in ethyl acetate; practically insoluble in water.

**Clomiphene Citrate:** White to pale yellow, essentially odorless powder. Freely soluble in methanol; sparingly soluble

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in alcohol; slightly soluble in water and in chloroform; insoluble in ether.

**Clomipramine Hydrochloride:** White to faintly yellow, crystalline powder. Freely soluble in water and in methylene chloride; soluble in alcohol; practically insoluble in ethyl ether and in hexane.

**Clonazepam:** Light yellow powder, having a faint odor. Sparingly soluble in acetone and in chloroform; slightly soluble in alcohol and in ether; insoluble in water.

**Clonidine:** White to almost white, crystalline powder. Melting point is about 130°. Freely soluble in methanol and in alcohol.

**Clopidogrel Bisulfate:** White to off-white powder. Freely soluble at pH 1; practically insoluble at neutral pH.

**Cloprostenol Sodium:** White or almost white, amorphous powder. Is hygroscopic. Freely soluble in water, in alcohol, and in methanol; practically insoluble in acetone.

**Clorazepate Dipotassium:** Light yellow, crystalline powder. Darkens on exposure to light. Soluble in water but, upon standing, may precipitate from the solution; slightly soluble in alcohol and in isopropyl alcohol; practically insoluble in acetone, in benzene, in chloroform, in ether, and in methylene chloride.

**Clorsulon:** White to off-white powder. Freely soluble in acetonitrile and in methanol; slightly soluble in water; very slightly soluble in methylene chloride.

**Clotrimazole:** White to pale yellow, crystalline powder. Melts at about 142°, with decomposition. Freely soluble in methanol, in chloroform, and in alcohol; freely soluble to soluble in acetone; practically insoluble in water.

**Cloxacillin Benzathine:** White or almost white, almost odorless, crystals or crystalline powder. Soluble in chloroform and in methanol; sparingly soluble in acetone; slightly soluble in water, in alcohol, and in isopropyl alcohol.

**Cloxacillin Sodium:** White, odorless, crystalline powder. Freely soluble in water; soluble in alcohol; slightly soluble in chloroform.

**Clozapine:** Yellow, crystalline powder. Soluble in chloroform, in acetone, and in alcohol; sparingly soluble in acetonitrile; insoluble in water.

**Coal Tar:** Nearly black, viscous liquid, heavier than water, having a characteristic, naphthalene-like odor, and producing a sharp, burning sensation on the tongue. Slightly soluble in water, to which it imparts its characteristic odor and taste and a faintly alkaline reaction; partially soluble in acetone, in alcohol, in carbon disulfide, in chloroform, in ether, in methanol, and in solvent hexane; soluble in benzene and nitrobenzene.

**Cocaine:** Colorless to white crystals or white, crystalline powder. Is levorotatory in 3 N hydrochloric acid solution. Its saturated solution is alkaline to litmus. Very soluble in warm alcohol; freely soluble in alcohol, in chloroform, and in ether; soluble in olive oil; sparingly soluble in mineral oil; slightly soluble in water.

**Cocaine Hydrochloride:** Colorless crystals or white, crystalline powder. Very soluble in water; freely soluble in alcohol; soluble in chloroform and in glycerin; insoluble in ether.

**Cocoa Butter:** Yellowish-white solid, having a faint, agreeable odor, and a bland, chocolate-like taste if the cocoa butter is obtained by pressing. If obtained by extraction, the taste is bland. Is usually brittle at temperatures below 25°. Freely soluble in ether and in chloroform; soluble in boiling dehydrated alcohol; slightly soluble in alcohol. *NF category:* Suppository base.

**Coconut Oil:** Clear, white to light yellow-tan, viscous liquid. Freely soluble in methylene chloride and in light petroleum (bp: 65° to 70°); very slightly soluble in alcohol; practically insoluble in water. *NF category:* Coating agent; emulsifying agent; emollient; ointment base.

**Hydrogenated Coconut Oil:** White to yellowish, fatty solid to semi-solid. Freely soluble in ether; very slightly soluble in alcohol; practically insoluble in water. *NF category:* Coating agent; lubricant; wet binder.

**Cod Liver Oil:** Thin, oily liquid, having a characteristic, slightly fishy but not rancid odor, and a fishy taste. Freely soluble in ether, in chloroform, in carbon disulfide, and in ethyl acetate; slightly soluble in alcohol.

**Codeine:** Colorless or white crystals or white, crystalline powder. It effloresces slowly in dry air, and is affected by light. In acid or alcohol solutions it is levorotatory. Its saturated solution is alkaline to litmus. Very soluble in chloroform; freely soluble in alcohol; sparingly soluble in ether; slightly soluble in water. When heated in an amount of water insufficient for complete solution, it melts to oily drops that crystallize on cooling.

**Codeine Phosphate:** Fine, white, needle-shaped crystals, or white, crystalline powder. Is odorless. Is affected by light. Its solutions are acid to litmus. Very soluble in hot water; freely soluble in water; slightly soluble in alcohol but more so in boiling alcohol.

**Codeine Sulfate:** White crystals, usually needle-like, or white, crystalline powder. Is affected by light. Freely soluble in water at 80°; soluble in water; very slightly soluble in alcohol; insoluble in chloroform and in ether.

**Colchicine:** Pale yellow to pale greenish-yellow, amorphous scales, or powder or crystalline powder. Is odorless or nearly so, and darkens on exposure to light. Freely soluble in alcohol and in chloroform; soluble in water; slightly soluble in ether.

**Colestipol Hydrochloride:** Yellow to orange beads. Swells but does not dissolve in water or dilute aqueous solutions of acid or alkali. Insoluble in the common organic solvents.

**Colistimethate Sodium:** White to slightly yellow, odorless, fine powder. Freely soluble in water; soluble in methanol; insoluble in acetone and in ether.

**Colistin Sulfate:** White to slightly yellow, odorless, fine powder. Freely soluble in water; slightly soluble in methanol; insoluble in acetone and in ether.

**Collodion:** Clear, or slightly opalescent, viscous liquid. Is colorless, or slightly yellowish, and has the odor of ether.

**Flexible Collodion:** Clear, or slightly opalescent, viscous liquid. Is colorless or slightly yellow, and has the odor of ether. The strong odor of camphor becomes noticeable as the ether evaporates.

**Copovidone:** White to yellowish-white powder or flakes. Is hygroscopic. Freely soluble in water, in alcohol, and in methylene chloride; practically insoluble in ether. *NF category:* Wet binder; coating agent; film-forming agent.

**Corn Oil:** Clear, light yellow, oily liquid, having a faint, characteristic odor and taste. Slightly soluble in alcohol. Miscible with ether, with chloroform, with benzene, and with solvent hexane. *Specific Gravity* ⟨841⟩: Between 0.914 and 0.921. *NF category:* Solvent; vehicle (oleaginous).

**Corn Syrup:** Clear, white to light yellow, viscous liquid. Is miscible in all proportions with water. *NF category:* Suspending and/or viscosity-increasing agent; sweetening agent; diluent; wet binder; tonicity agent.

**Corn Syrup Solids:** Sweet, white to light yellow powder or granules. Soluble in water. *NF category:* Coating agent; vehicle (flavored and/or sweetened and solid carrier); humectant; suspending and/or viscosity-increasing agent; sweetening agent; diluent; wet binder; tonicity agent.

**Corticotropin Injection:** Colorless or light straw-colored liquid.

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**Corticotropin for Injection:** White or practically white, soluble, amorphous solid having the characteristic appearance of substances prepared by freeze-drying.

**Repository Corticotropin Injection:** Colorless or light straw-colored liquid, which may be quite viscid at room temperature. Is odorless or has an odor of an antimicrobial agent.

**Cortisone Acetate:** White or practically white, odorless, crystalline powder. Is stable in air. Melts at about 240°, with some decomposition (see *Melting Range or Temperature* ⟨741⟩). Freely soluble in chloroform; soluble in dioxane; sparingly soluble in acetone; slightly soluble in alcohol; insoluble in water.

**Purified Cotton:** White, soft, fine filament-like hairs appearing under the microscope as hollow, flattened, and twisted bands, striate and slightly thickened at the edges. Is practically odorless and practically tasteless. Soluble in ammoniated cupric oxide TS; insoluble in ordinary solvents.

**Cottonseed Oil:** Pale yellow, oily liquid. Is odorless or nearly so, and has a bland taste. At temperatures below 10° particles of solid fat may separate from the Oil, and at about 0° to –5° the Oil becomes a solid or nearly so. *Specific Gravity* ⟨841⟩: Between 0.915 and 0.921. Slightly soluble in alcohol. Miscible with ether, with chloroform, with solvent hexane, and with carbon disulfide. *NF category:* Solvent; vehicle (oleaginous).

**Hydrogenated Cottonseed Oil:** A white mass or powder that melts to a clear, pale yellow liquid when heated. Freely soluble in methylene chloride and in toluene; very slightly soluble in alcohol; practically insoluble in water.

**Creatinine:** White crystals or crystalline powder; odorless. Soluble in water; slightly soluble in alcohol; practically insoluble in acetone, in ether, and in chloroform. *NF category:* Bulking agent.

**Cresol:** Colorless, or yellowish to brownish-yellow, or pinkish, highly refractive liquid, becoming darker with age and on exposure to light. Has a phenol-like, sometimes empyreumatic odor. A saturated solution of it is neutral or only slightly acid to litmus. Sparingly soluble in water, usually forming a cloudy solution; dissolves in solutions of fixed alkali hydroxides. Miscible with alcohol, with ether, and with glycerin. *NF category:* Antimicrobial preservative.

**Cromolyn Sodium:** White, odorless, crystalline powder. Is tasteless at first, with a slightly bitter aftertaste. Is hygroscopic. Soluble in water; insoluble in alcohol and in chloroform.

**Cromolyn Sodium for Inhalation:** White to creamy white, odorless, hygroscopic, and very finely divided powder.

**Croscarmellose Sodium:** White, free-flowing powder. Partially soluble in water; insoluble in alcohol, in ether, and in other organic solvents. *NF category:* Disintegrant.

**Crospovidone:** White to creamy-white, hygroscopic powder, having a faint odor. Insoluble in water and in ordinary organic solvents. *NF category:* Disintegrant.

**Crotamiton:** Colorless to slightly yellowish oil, having a faint amine-like odor. Soluble in alcohol and in methanol.

**Cupric Chloride:** Bluish green, deliquescent crystals. Freely soluble in water; soluble in alcohol; slightly soluble in ether.

**Cupric Sulfate** ($CuSO_4 \cdot 5H_2O$)**:** Deep blue, triclinic crystals or blue, crystalline granules or powder. It effloresces slowly in dry air. Its solutions are acid to litmus. Very soluble in boiling water; freely soluble in water and in glycerin; slightly soluble in alcohol.

**Cupric Sulfate (anhydrous):** A white or grayish-white powder. Soluble in water; insoluble in alcohol.

**Cyanocobalamin:** Dark red crystals or amorphous or crystalline red powder. In the anhydrous form, it is very hygroscopic and when exposed to air it may absorb about 12% of water. Soluble in alcohol; sparingly soluble in water; insoluble in acetone, in chloroform, and in ether.

**Cyclizine Hydrochloride:** White, crystalline powder or small, colorless crystals. Is odorless or nearly so, and has a bitter taste. Melts indistinctly at about 285°, with decomposition. Sparingly soluble in chloroform; slightly soluble in water and in alcohol; insoluble in ether.

**Cyclobenzaprine Hydrochloride:** White to off-white, odorless, crystalline powder. Freely soluble in water, in alcohol, and in methanol; sparingly soluble in isopropanol; slightly soluble in chloroform and in methylene chloride; insoluble in hydrocarbons.

**Cyclopentolate Hydrochloride:** White, crystalline powder, which upon standing develops a characteristic odor. Its solutions are acid to litmus. Melts at about 138°, the melt appearing opaque. Very soluble in water; freely soluble in alcohol; insoluble in ether.

**Cyclophosphamide:** White, crystalline powder. Liquefies upon loss of its water of crystallization. Soluble in water and in alcohol.

**Cycloserine:** White to pale yellow, crystalline powder. Is odorless or has a faint odor. Is hygroscopic and deteriorates upon absorbing water. Its solutions are dextrorotatory. Freely soluble in water.

**Cyclosporine:** White to almost white powder. Soluble in acetone, in alcohol, in methanol, in ether, in chloroform, and in methylene chloride; slightly soluble in saturated hydrocarbons; practically insoluble in water.

**Cyproheptadine Hydrochloride:** White to slightly yellow, odorless or practically odorless, crystalline powder. Freely soluble in methanol; soluble in chloroform; sparingly soluble in alcohol; slightly soluble in water; practically insoluble in ether.

**Cysteine Hydrochloride:** White crystals or crystalline powder. Soluble in water, in alcohol, and in acetone.

**Cytarabine:** Odorless, white to off-white, crystalline powder. Freely soluble in water; slightly soluble in alcohol and in chloroform.

**Dactinomycin:** Bright red, crystalline powder. Is somewhat hygroscopic and is affected by light and by heat. Freely soluble in alcohol; soluble in water at 10° and slightly soluble in water at 37°; very slightly soluble in ether.

**Dalfampridine:** White powder. Soluble in water, in methanol, in acetone, in tetrahydrofuran, in isopropanol, in acetonitrile, in *N*,*N*-dimethylformamide, in dimethylsulfoxide, and in alcohol.

**Danazol:** White to pale yellow, crystalline powder. Melts at about 225°, with some decomposition. Freely soluble in chloroform; soluble in acetone; sparingly soluble in alcohol and in benzene; slightly soluble in ether; practically insoluble or insoluble in water and in hexane.

**Dantrolene Sodium:** Fine orange to orange-brown powder. Sparingly soluble in dimethylformamide and in glycerine; sparingly soluble to practically insoluble in acetone.

**Dapagliflozin Propanediol:** White to off-white powder which may contain lumps. Freely soluble in methanol, in tetrahydrofuran, in ethanol, and in acetone; soluble in acetonitrile; slightly soluble in water.

**Dapsone:** White or creamy white, crystalline powder. Is odorless and has a slightly bitter taste. Soluble in acetone and in dilute mineral acids; sparingly soluble in alcohol; very slightly soluble in water.

**Daunorubicin Hydrochloride:** Orange-red, crystalline, hygroscopic powder. Freely soluble in water and in methanol; slightly soluble in alcohol; very slightly soluble in chloroform; practically insoluble in acetone.

**Deferoxamine Mesylate:** White to off-white powder. Freely soluble in water; slightly soluble in methanol.

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**Dehydroacetic Acid:** White or nearly white, crystalline powder. Soluble in aqueous solutions of alkalies; very slightly soluble in water. One g of sample dissolves in about 35 mL of alcohol and in 5 mL of acetone. *NF category:* Antimicrobial preservative.

**Dehydrocholic Acid:** White, fluffy, odorless powder, having a bitter taste. Soluble in glacial acetic acid and in solutions of alkali hydroxides and carbonates; sparingly soluble in chloroform (the solutions in alcohol and in chloroform usually are slightly turbid); slightly soluble in alcohol and in ether; practically insoluble in water.

**Demecarium Bromide:** White or slightly yellow, slightly hygroscopic, crystalline powder. Freely soluble in water and in alcohol; soluble in ether; sparingly soluble in acetone.

**Demeclocycline:** Yellow, crystalline, odorless powder, having a bitter taste. Soluble in alcohol; sparingly soluble in water. Dissolves readily in 3 N hydrochloric acid and in alkaline solutions.

**Demeclocycline Hydrochloride:** Yellow, crystalline, odorless powder, having a bitter taste. Sparingly soluble in water and in solutions of alkali hydroxides and carbonates; slightly soluble in alcohol; practically insoluble in acetone and in chloroform.

**Denatonium Benzoate:** Very soluble in chloroform and in methanol; freely soluble in water and in alcohol; very slightly soluble in ether. *NF category:* Alcohol denaturant; flavors and fragrance.

**Desipramine Hydrochloride:** White to off-white, crystalline powder. Melts at about 213°. Freely soluble in methanol and in chloroform; soluble in water and in alcohol; insoluble in ether.

**Desloratadine:** White to light pink-colored powder. Very soluble in ethanol and in propylene glycol; soluble in dichloromethane; slightly soluble in water.

**Desmopressin Acetate:** White, fluffy powder. Soluble in water, in alcohol, and in acetic acid.

**Desogestrel:** White or almost-white, crystalline powder. Very soluble in methanol; freely soluble in anhydrous ethanol and in methylene chloride; practically insoluble in water.

**Desonide:** White powder or crystal. Soluble in chloroform; sparingly soluble in ethanol and in acetone; practically insoluble in water.

**Desoximetasone:** White to practically white, odorless, crystalline powder. Freely soluble in alcohol, in acetone, and in chloroform; insoluble in water.

**Deoxycholic Acid:** Occurs as a white, crystalline powder. Freely soluble in alcohol; soluble in acetone and in solutions of alkali hydroxides and carbonates; slightly soluble in chloroform and in ether; practically insoluble in water. *NF category:* Emulsifying agent.

**Desvenlafaxine:** White to off-white, crystalline powder. Sparingly soluble in dimethyl sulfoxide.

**Desvenlafaxine Fumarate:** White to off-white powder. Soluble in methanol; slightly soluble in water.

**Desvenlafaxine Succinate:** White to creamish, crystalline powder. Sparingly soluble in dimethyl sulfoxide, in methanol, and in water.

**Dexamethasone:** White to practically white, odorless, crystalline powder. Is stable in air. Melts at about 250°, with some decomposition. Sparingly soluble in acetone, in alcohol, in dioxane, and in methanol; slightly soluble in chloroform; very slightly soluble in ether; practically insoluble in water.

**Dexamethasone Acetate:** Clear, white to off-white, odorless powder. Freely soluble in methanol, in acetone, and in dioxane; practically insoluble in water.

**Dexamethasone Sodium Phosphate:** White or slightly yellow, crystalline powder. Is odorless or has a slight odor of alcohol, and is exceedingly hygroscopic. Freely soluble in water; slightly soluble in alcohol; very slightly soluble in dioxane; insoluble in chloroform and in ether.

**Dexbrompheniramine Maleate:** White, odorless, crystalline powder. Exists in two polymorphic forms, one melting between 106° and 107° and the other between 112° and 113°. Mixtures of the forms may melt between 105° and 113°. The pH of a solution (1 in 100) is about 5. Freely soluble in water; soluble in alcohol and in chloroform.

**Dexchlorpheniramine Maleate:** White, odorless, crystalline powder. Freely soluble in water; soluble in alcohol and in chloroform; slightly soluble in benzene and in ether.

**Dexmedetomidine Hydrochloride:** White or almost white powder. Freely soluble in water, and has a pKa of 7.1.

**Dexpanthenol:** Clear, viscous, somewhat hygroscopic liquid, having a slight, characteristic odor. Some crystallization may occur on standing. Freely soluble in water, in alcohol, in methanol, and in propylene glycol; soluble in chloroform and in ether; slightly soluble in glycerin.

**Dextran 1:** A white to off-white powder. Is hygroscopic. Very soluble in water; sparingly soluble in alcohol.

**Dextrates:** Free-flowing, porous, white, odorless, spherical granules consisting of aggregates of microcrystals, having a sweet taste and producing a cooling sensation in the mouth. May be compressed directly into self-binding tablets. Freely soluble in water (heating increases its solubility in water); soluble in dilute acids and alkalies and in basic organic solvents such as pyridine; insoluble in the common organic solvents. *NF category:* Sweetening agent; diluent; wet binder.

**Dextrin:** Free-flowing, white, yellow, or brown powder. Its solubility in water varies; it is usually very soluble, but often contains an insoluble portion. *NF category:* Suspending and/or viscosity-increasing agent; diluent; wet binder; film-forming agent; stiffening agent.

**Dextroamphetamine Sulfate:** White, odorless, crystalline powder. Soluble in water; slightly soluble in alcohol; insoluble in ether.

**Dextromethorphan:** Practically white to slightly yellow, odorless, crystalline powder. Eleven mg of Dextromethorphan is equivalent to 15 mg of dextromethorphan hydrobromide monohydrate. Freely soluble in chloroform; practically insoluble in water.

**Dextromethorphan Hydrobromide:** Practically white crystals or crystalline powder, having a faint odor. Melts at about 126°, with decomposition. Freely soluble in alcohol and in chloroform; sparingly soluble in water; insoluble in ether.

**Dextrose:** Colorless crystals or white, crystalline or granular powder. Is odorless, and has a sweet taste. Very soluble in boiling water; freely soluble in water; soluble in boiling alcohol; slightly soluble in alcohol. *NF category:* Sweetening agent; tonicity agent; vehicle (flavored and/or sweetened); diluent.

**Dextrose Excipient:** Colorless crystals or white, crystalline or granular powder. Is odorless and sweet-tasting. Very soluble in boiling water; freely soluble in water; sparingly soluble in boiling alcohol; slightly soluble in alcohol. *NF category:* Sweetening agent; diluent.

**Diacetylated Monoglycerides:** Clear liquid. Very soluble in 80% (w/w) aqueous alcohol, in vegetable oils, and in mineral oils; sparingly soluble in 70% alcohol. *NF category:* Plasticizer.

**Diatrizoate Meglumine:** White, odorless powder. Freely soluble in water.

**Diatrizoate Meglumine Injection:** Clear, colorless to pale yellow, slightly viscous liquid.

**Diatrizoate Meglumine and Diatrizoate Sodium Injection:** Clear, colorless to pale yellow, slightly viscous liquid. May crystallize at room temperature or below.

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**Diatrizoate Sodium:** White, odorless powder. Soluble in water; slightly soluble in alcohol; practically insoluble in acetone and in ether.

**Diatrizoate Sodium Injection:** Clear, colorless to pale yellow, slightly viscous liquid.

**Diatrizoate Sodium Solution:** Clear, pale yellow to light brown liquid.

**Diatrizoic Acid:** White, odorless powder. Soluble in dimethylformamide and in alkali hydroxide solutions; very slightly soluble in water and in alcohol.

**Diazepam:** Off-white to yellow, practically odorless, crystalline powder. Freely soluble in chloroform; soluble in alcohol; practically insoluble in water.

**Diazoxide:** White or cream-white crystals or crystalline powder. Very soluble in strong alkaline solutions; freely soluble in dimethylformamide; sparingly soluble to practically insoluble in water and in most organic solvents.

**Dibucaine:** White to off-white powder, having a slight, characteristic odor. Darkens on exposure to light. Soluble in 1 N hydrochloric acid and in ether; slightly soluble in water.

**Dibucaine Hydrochloride:** Colorless or white to off-white crystals or white to off-white, crystalline powder. Is odorless, is somewhat hygroscopic, and darkens on exposure to light. Its solutions have a pH of about 5.5. Freely soluble in water, in alcohol, in acetone, and in chloroform.

**Dibutyl Phthalate:** A clear, oily liquid, colorless or very slightly yellow. Practically insoluble in water. Miscible with alcohol and with ether. *NF category:* Film-forming agent; solvent.

**Dibutyl Sebacate:** Colorless, oily liquid of very mild odor. Soluble in alcohol, in isopropyl alcohol, and in mineral oil; very slightly soluble in propylene glycol; practically insoluble in water and in glycerin. *NF category:* Plasticizer.

**Dichlorphenamide:** White or practically white, crystalline compound. Soluble in dilute solutions of alkali hydroxides and carbonates; very slightly soluble in water.

**Diclazuril:** White to yellow powder. Sparingly soluble in dimethylformamide; practically insoluble in water, in alcohol, and in methylene chloride.

**Diclofenac Potassium:** White to off-white or slightly yellowish crystalline powder, slightly hygroscopic. Freely soluble in methanol; soluble in alcohol; sparingly soluble in water; slightly soluble in acetone.

**Diclofenac Sodium:** White to off-white, hygroscopic, crystalline powder. Melts at about 284°. Freely soluble in methanol; soluble in ethanol; sparingly soluble in water; practically insoluble in chloroform and in ether.

**Dicloxacillin Sodium:** White to off-white, crystalline powder. Freely soluble in water.

**Dicyclomine Hydrochloride:** Fine, white, crystalline powder. Is practically odorless and has a very bitter taste. Freely soluble in alcohol and in chloroform; soluble in water; very slightly soluble in ether.

**Dicyclomine Hydrochloride Injection:** Colorless solution, which may have the odor of a preservative.

**Didanosine:** White to off-white, crystalline powder. Very soluble in dimethyl sulfoxide; practically insoluble or insoluble in acetone and in methanol.

**Diethanolamine:** White or clear, colorless crystals, deliquescing in moist air; or colorless liquid. Miscible with water, with alcohol, with acetone, with chloroform, and with glycerin. Slightly soluble to insoluble in benzene, in ether, and in petroleum ether. *NF category:* Emulsifying agent; pH modifier (acidifying agent/alkalizing agent/buffering agent).

**Diethylcarbamazine Citrate:** White, crystalline powder. Melts at about 136°, with decomposition. Is odorless or has a slight odor; is slightly hygroscopic. Very soluble in water; sparingly soluble in alcohol; practically insoluble in acetone, in chloroform, and in ether.

**Diethylene Glycol Monoethyl Ether:** Clear, colorless liquid. Is hygroscopic. Miscible with water, with acetone, and with alcohol; partially miscible with vegetable oils; immiscible with mineral oils. Specific gravity about 0.991. *NF category:* Ointment base; solvent; emulsifying agent.

**Diethylene Glycol Stearates:** White or almost white, waxy solid. Soluble in acetone and in hot alcohol; practically insoluble in water. *NF category:* Emulsifying agent.

**Diethyl Phthalate:** Colorless, practically odorless, oily liquid. Insoluble in water. Miscible with alcohol, with ether, and with other usual organic solvents. *NF category:* Plasticizer; film-forming agent; solvent.

**Diethyl Sebacate:** A colorless to slightly yellow liquid. Miscible with alcohol, with ether, with other organic solvents, and with most fixed oils; insoluble or practically insoluble in water. *NF category*: Flavors and fragrance.

**Diethylpropion Hydrochloride:** White to off-white, fine crystalline powder. Is odorless, or has a slight characteristic odor. It melts at about 175°, with decomposition. Freely soluble in water, in chloroform, and in alcohol; practically insoluble in ether.

**Diethylstilbestrol:** White, odorless, crystalline powder. Soluble in alcohol, in chloroform, in ether, in fatty oils, and in dilute alkali hydroxides; practically insoluble in water.

**Diethyltoluamide:** Colorless liquid, having a faint, pleasant odor. Boils at about 111° under a pressure of 1 mm of mercury. Practically insoluble in water and in glycerin. Miscible with alcohol, with isopropyl alcohol, with ether, with chloroform, and with carbon disulfide.

**Diflorasone Diacetate:** White to pale yellow, crystalline powder. Soluble in methanol and in acetone; sparingly soluble in ethyl acetate; slightly soluble in toluene; very slightly soluble in ether; insoluble in water.

**Diflunisal:** White to off-white, practically odorless powder. Freely soluble in alcohol and in methanol; soluble in acetone and in ethyl acetate; slightly soluble in chloroform, in carbon tetrachloride, and in methylene chloride; insoluble in hexane and in water.

**Digitoxin:** White or pale buff, odorless, microcrystalline powder. Sparingly soluble in chloroform; slightly soluble in alcohol; very slightly soluble in ether; practically insoluble in water.

**Digoxin:** Clear to white, odorless crystals or white, odorless crystalline powder. Freely soluble in pyridine; slightly soluble in diluted alcohol and in chloroform; practically insoluble in water and in ether.

**Dihydroergotamine Mesylate:** White to slightly yellowish powder, or off-white to faintly red powder, having a faint odor. Soluble in alcohol; slightly soluble in water and in chloroform.

**Dihydrostreptomycin Sulfate:** White or almost white, amorphous or crystalline powder. Amorphous form is hygroscopic. Freely soluble in water; practically insoluble in acetone, in chloroform, and in methanol.

**Dihydroxyacetone:** White to off-white crystalline powder. The monomeric form is freely soluble in water, in alcohol, and in ether. The dimeric form is freely soluble in water; soluble in alcohol; and sparingly soluble in ether.

**Dihydroxyaluminum Aminoacetate:** White, odorless powder having a faintly sweet taste. Soluble in dilute mineral acids and in solutions of fixed alkalies; insoluble in water and in organic solvents.

**Dihydroxyaluminum Aminoacetate Magma:** White, viscous suspension, from which small amounts of water may separate on standing.

**Dihydroxyaluminum Sodium Carbonate:** Fine, white, odorless powder. Soluble in dilute mineral acids with the evolution of carbon dioxide; practically insoluble in water and in organic solvents.

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**Diloxanide Furoate:** White or almost white, crystalline powder. Freely soluble in chloroform; slightly soluble in alcohol and in ether; very slightly soluble in water.

**Diltiazem Hydrochloride:** White, odorless, crystalline powder or small crystals. Freely soluble in chloroform, in formic acid, in methanol, and in water; sparingly soluble in dehydrated alcohol; insoluble in ether. Melts at about 210°, with decomposition.

**Dimenhydrinate:** White, crystalline, odorless powder. Freely soluble in alcohol and in chloroform; sparingly soluble in ether; slightly soluble in water.

**Dimercaprol:** Colorless or practically colorless liquid, having a disagreeable, mercaptan-like odor. Soluble in water, in alcohol, in benzyl benzoate, and in methanol.

**Dimercaprol Injection:** Yellow, viscous solution having a pungent, disagreeable odor. Specific gravity is about 0.978.

**Dimethicone:** A clear, colorless, and odorless liquid. Soluble in chlorinated hydrocarbons, in benzene, in toluene, in xylene, in *n*-hexane, in petroleum spirits, in ether, and in amyl acetate; very slightly soluble in isopropyl alcohol; insoluble in water, in methanol, in alcohol, and in acetone. *NF category:* Antifoaming agent; water-repelling agent; adhesive; emollient.

***Add the following:***

▲**Dimethyl Fumarate:** White to off-white, crystalline powder. Soluble in methanol; slightly soluble in water.▲ (USP 1-Dec-2023)

**Dimethyl Sulfoxide:** Clear, colorless, odorless, hygroscopic liquid. Melts at about 18.4°. Boils at about 189°. Soluble in water; practically insoluble in acetone, in alcohol, in benzene, in chloroform, and in ether. *NF category:* Solvent.

**Dinoprostone:** White to off-white, crystalline powder. Freely soluble in acetone, in alcohol, in ether, in ethyl acetate, in isopropyl alcohol, in methanol, and in methylene chloride; soluble in toluene and in diisopropyl ether; practically insoluble in hexanes.

**Dinoprost Tromethamine:** White to off-white, crystalline powder. Very soluble in water; freely soluble in dimethylformamide; soluble in methanol; slightly soluble in chloroform.

**Dioxybenzone:** Yellow powder. Freely soluble in alcohol and in toluene; practically insoluble in water.

**Diphenhydramine Hydrochloride:** White, odorless, crystalline powder. Slowly darkens on exposure to light. Its solutions are practically neutral to litmus. Freely soluble in water, in alcohol, and in chloroform; sparingly soluble in acetone; very slightly soluble in benzene and in ether.

**Diphenoxylate Hydrochloride:** White, odorless, crystalline powder. Its saturated solution has a pH of about 3.3. Freely soluble in chloroform; soluble in methanol; sparingly soluble in alcohol and in acetone; slightly soluble in water and in isopropanol; practically insoluble in ether and in solvent hexane.

**Dipivefrin Hydrochloride:** White, crystalline powder or small crystals, having a faint odor. Very soluble in water.

**Dipyridamole:** Intensely yellow, crystalline powder or needles. Very soluble in methanol, in alcohol, and in chloroform; slightly soluble in water; very slightly soluble in acetone and in ethyl acetate.

**Disopyramide Phosphate:** White or practically white, odorless powder. Melts at about 205°, with decomposition. Freely soluble in water; slightly soluble in alcohol; practically insoluble in chloroform and in ether.

**Disulfiram:** White to off-white, odorless, crystalline powder. Soluble in acetone, in alcohol, in carbon disulfide, and in chloroform; very slightly soluble in water.

**Divalproex Sodium:** White to off-white powder. Very soluble in chloroform; freely soluble in methanol and in ethyl ether; soluble in acetone; practically insoluble in acetonitrile.

**Dobutamine Hydrochloride:** White to practically white, crystalline powder. Soluble in pyridine; sparingly soluble in water, in alcohol, and in methanol.

**Docetaxel:** White or almost white, crystalline powder. Freely soluble in acetone; soluble in methanol; practically insoluble in water.

**Docusate Calcium:** White, amorphous solid, having the characteristic odor of octyl alcohol. It is free of the odor of other solvents. Very soluble in alcohol, in polyethylene glycol 400, and in corn oil; very slightly soluble in water.

**Docusate Potassium:** White, amorphous solid, having a characteristic odor suggestive of octyl alcohol. Very soluble in solvent hexane; soluble in alcohol and in glycerin; sparingly soluble in water.

**Docusate Sodium:** White, wax-like, plastic solid, having a characteristic odor suggestive of octyl alcohol, but no odor of other solvents. Very soluble in solvent hexane; freely soluble in alcohol and in glycerin; sparingly soluble in water. *NF category:* Wetting and/or solubilizing agent.

**Dofetilide:** White to off-white powder. Soluble in 0.1 N sodium hydroxide, in acetone, and in 0.1 N hydrochloric acid; very slightly soluble in water and in isopropyl alcohol.

**Dolasetron Mesylate:** White to off-white powder. Freely soluble in water and in propylene glycol; slightly soluble in alcohol and in saline TS.

**Donepezil Hydrochloride:** White to cream-colored, crystalline powder. Some polymorphic forms are very hygroscopic. Freely soluble in chloroform; soluble in water and in glacial acetic acid; slightly soluble in alcohol and in acetonitrile; practically insoluble in ethyl acetate and in *n*-hexane.

**Dopamine Hydrochloride:** White to off-white, crystalline powder. May have a slight odor of hydrochloric acid. Melts at about 240°, with decomposition. Freely soluble in water and in aqueous solutions of alkali hydroxides; soluble in methanol; insoluble in ether and in chloroform.

**Dorzolamide Hydrochloride:** White to off-white, crystalline powder. Soluble in water.

**Doxapram Hydrochloride:** White to off-white, odorless, crystalline powder. Melts at about 220°. Soluble in water and in chloroform; sparingly soluble in alcohol; practically insoluble in ether.

**Doxazosin Mesylate:** White to tan-colored powder. Freely soluble in formic acid; very slightly soluble in methanol and in water.

**Doxepin Hydrochloride:** White or almost white, crystalline powder. Freely soluble in water, in alcohol, and in methylene chloride.

**Doxercalciferol:** White to off-white powder or crystalline solid. Soluble in *N*,*N*-dimethylformamide; sparingly soluble in methanol; slightly soluble in acetonitrile; practically insoluble in water.

**Doxorubicin Hydrochloride:** Red-orange, hygroscopic, crystalline or amorphous powder. Soluble in water, in isotonic sodium chloride solution, and in methanol; practically insoluble in chloroform, in ether, and in other organic solvents.

**Doxycycline:** Yellow, crystalline powder. Freely soluble in dilute acid and in alkali hydroxide solutions; very slightly soluble in alcohol and in water; practically insoluble in chloroform and in ether.

**Doxycycline Hyclate:** Yellow, crystalline powder. Soluble in water and in solutions of alkali hydroxides and carbonates; slightly soluble in alcohol; practically insoluble in chloroform and in ether.

**Doxylamine Succinate:** White or creamy white powder, having a characteristic odor. Very soluble in water; freely

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soluble in chloroform and in alcohol; very slightly soluble in ether.

**Dronabinol:** Light yellow resinous oil that is sticky at room temperature and hardens upon refrigeration. Insoluble in water.

**Dronedarone Hydrochloride:** A white to off-white, fine powder. Freely soluble in methylene chloride and in methanol; practically insoluble in water.

**Droperidol:** White to light tan, amorphous or microcrystalline powder. Freely soluble in chloroform; slightly soluble in alcohol and in ether; practically insoluble in water. Melts at about 145°.

**Drospirenone:** White to off-white powder. Freely soluble in methylene chloride; soluble in acetone and in methanol; sparingly soluble in ethyl acetate and in alcohol; practically insoluble in hexane and in water.

**Duloxetine Hydrochloride:** White to brownish-white solid. Freely soluble in methanol; sparingly soluble in water; practically insoluble in hexane.

**Dutasteride:** White to pale-yellow powder. Soluble in ethanol and methanol; slightly soluble in polyethylene glycol 400; and insoluble in water.

**Dyclonine Hydrochloride:** White crystals or white crystalline powder, which may have a slight odor. Exhibits local anesthetic properties when placed upon the tongue. Soluble in water, in acetone, in alcohol, and in chloroform.

**Dydrogesterone:** White to pale yellow, crystalline powder. Sparingly soluble in alcohol; practically insoluble in water.

**Dyphylline:** White, odorless, extremely bitter, amorphous or crystalline solid. Freely soluble in water; sparingly soluble in alcohol and in chloroform; practically insoluble in ether.

**Ecamsule Solution:** Clear yellow liquid.

**Echothiophate Iodide:** White, crystalline, hygroscopic solid having a slight mercaptan-like odor. Its solutions have a pH of about 4. Freely soluble in water and in methanol; soluble in dehydrated alcohol; practically insoluble in other organic solvents.

**Echothiophate Iodide for Ophthalmic Solution:** White, amorphous powder.

**Econazole Nitrate:** White or practically white, crystalline powder, having not more than a slight odor. Soluble in methanol; sparingly soluble in chloroform; slightly soluble in alcohol; very slightly soluble in water and in ether.

**Edetate Calcium Disodium:** White, crystalline granules or white, crystalline powder. Is odorless, is slightly hygroscopic, and has a faint, saline taste. Is stable in air. Freely soluble in water. *NF category:* Chelating and/or complexing agent.

**Edetate Disodium:** White, crystalline powder. Soluble in water. *NF category:* Chelating and/or complexing agent; transfer ligand.

**Edetic Acid:** White, crystalline powder. Melts above 220°, with decomposition. Soluble in solutions of alkali hydroxides; very slightly soluble in water. *NF category:* Chelating and/or complexing agent.

**Edrophonium Chloride:** White, odorless, crystalline powder. Its solution (1 in 10) is practically colorless. Very soluble in water; freely soluble in alcohol; insoluble in chloroform and in ether.

**Efavirenz:** White to slightly pink crystalline powder. Soluble in methanol; practically insoluble in water.

**Emedastine Fumarate:** White to faintly yellow, crystalline powder. Soluble in water.

**Emtricitabine:** White to almost white crystalline powder. Freely soluble in methanol and in water; practically insoluble in dichloromethane.

**Enalapril Maleate:** Off-white, crystalline powder. Melts at about 144°. Freely soluble in methanol and in dimethylformamide; soluble in alcohol; sparingly soluble in water; slightly soluble in semipolar organic solvents; practically insoluble in nonpolar organic solvents.

**Enalaprilat:** White to nearly white, hygroscopic, crystalline powder. Sparingly soluble in methanol and in dimethylformamide; slightly soluble in water and in isopropyl alcohol; very slightly soluble in acetone, in alcohol, and in hexane; practically insoluble in acetonitrile and in chloroform.

**Enflurane:** Clear, colorless, stable, volatile liquid, having a mild, sweet odor. Is nonflammable. Slightly soluble in water. Miscible with organic solvents, with fats, and with oils.

**Enrofloxacin:** Pale yellow to light yellow crystalline powder. Very slightly soluble in water at pH 7.

**Entacapone:** Greenish yellow to yellow powder. Sparingly soluble in acetone and in methanol; slightly soluble in ethanol, chloroform, isopropanol, and ether; very slightly soluble in toluene; practically insoluble in water.

**Entecavir:** White to off-white powder. Practically insoluble to slightly soluble in water; practically insoluble in anhydrous ethanol and in heptane.

**Ephedrine:** Unctuous, practically colorless solid or white crystals or granules. Gradually decomposes on exposure to light. Melts between 33° and 40°, the variability in the melting point being the result of differences in the moisture content, anhydrous Ephedrine having a lower melting point than the hemihydrate of Ephedrine. Its solutions are alkaline to litmus. Soluble in water, in alcohol, in chloroform, and in ether; sparingly and slowly soluble in mineral oil, the solution becoming turbid if the Ephedrine contains more than about 1% of water.

**Ephedrine Hydrochloride:** Fine, white, odorless crystals or powder. Is affected by light. Freely soluble in water; soluble in alcohol; insoluble in ether.

**Ephedrine Sulfate:** Fine, white, odorless crystals or powder. Darkens on exposure to light. Freely soluble in water; sparingly soluble in alcohol.

**Ephedrine Sulfate Nasal Solution:** Clear, colorless solution. Is neutral or slightly acid to litmus.

**Epinephrine:** White to practically white, odorless, microcrystalline powder or granules, gradually darkening on exposure to light and air. With acids, it forms salts that are readily soluble in water, and the base may be recovered by the addition of ammonia water or alkali carbonates. Its solutions are alkaline to litmus. Very slightly soluble in water and in alcohol; insoluble in ether, in chloroform, and in fixed and volatile oils.

**Epinephrine Injection:** Practically colorless, slightly acid liquid. Gradually turns dark on exposure to light and air.

**Epinephrine Inhalation Solution:** Practically colorless, slightly acid liquid. Gradually turns dark on exposure to light and air.

**Epinephrine Nasal Solution:** Nearly colorless, slightly acid liquid. Gradually turns dark on exposure to light and air.

**Epinephrine Ophthalmic Solution:** Colorless to faint yellow solution. Gradually turns dark on exposure to light and air.

**Epinephrine Bitartrate:** White, or grayish-white or light brownish-gray, odorless, crystalline powder. Slowly darkens on exposure to air and light. Its solutions are acid to litmus, having a pH of about 3.5. Freely soluble in water; slightly soluble in alcohol; practically insoluble in chloroform and in ether.

**Epinephrine Bitartrate for Ophthalmic Solution:** White to off-white solid.

**Epirubicin Hydrochloride:** Orange-red powder. Soluble in water and in methanol; slightly soluble in anhydrous ethanol; practically insoluble in acetone.

**Eprinomectin:** White to off-white powder. Insoluble in cold water.

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**Ergocalciferol:** White, odorless crystals. Is affected by air and by light. Soluble in alcohol, in chloroform, in ether, and in fatty oils; insoluble in water.

**Ergocalciferol Oral Solution:** Clear liquid having the characteristics of the solvent used in preparing the Solution.

**Ergoloid Mesylates:** White to off-white, microcrystalline or amorphous, practically odorless powder. Soluble in methanol and in alcohol; sparingly soluble in acetone; slightly soluble in water.

**Ergonovine Maleate:** White to grayish-white or faintly yellow, odorless, microcrystalline powder. Darkens with age and on exposure to light. Sparingly soluble in water; slightly soluble in alcohol; insoluble in ether and in chloroform.

**Ergotamine Tartrate:** Colorless crystals or white to yellowish-white, crystalline powder. Is odorless. Melts at about 180°, with decomposition. One g dissolves in about 3200 mL of water; in the presence of a slight excess of tartaric acid 1 g dissolves in about 500 mL of water. Slightly soluble in alcohol.

**Erythorbic Acid:** White or slightly yellow crystals or powder. It gradually darkens when exposed to light. In the dry state, it is reasonably stable in air, but in solution, it rapidly deteriorates in the presence of air. It melts between 164° and 171° with decomposition. One g is soluble in about 2.5 mL of water and in about 20 mL of alcohol. Slightly soluble in glycerin. *NF category:* Antimicrobial preservative; antioxidant.

**Erythritol:** White or almost white, crystalline powder or free-flowing granules. It is stable to heat and is nonhygroscopic. Freely soluble in water; very slightly soluble in alcohol. *NF category:* Humectant; sweetening agent; diluent.

**Erythromycin:** White or slightly yellow, crystalline powder. Is odorless or practically odorless. Soluble in alcohol, in chloroform, and in ether; slightly soluble in water.

**Erythromycin Ethylsuccinate:** White or slightly yellow crystalline powder. Is odorless or practically odorless, and is practically tasteless. Freely soluble in alcohol, in chloroform, and in polyethylene glycol 400; very slightly soluble in water.

**Erythromycin Lactobionate for Injection:** White or slightly yellow crystals or powder, having a faint odor. Its solution (1 in 20) is neutral or slightly alkaline. Freely soluble in water, in alcohol, and in methanol; slightly soluble in acetone and in chloroform; practically insoluble in ether.

**Erythromycin Stearate:** White or slightly yellow crystals or powder. Is odorless or may have a slight, earthy odor, and has a slightly bitter taste. Soluble in alcohol, in chloroform, in methanol, and in ether; practically insoluble in water.

**Esmolol Hydrochloride:** White to off-white crystalline powder. Very soluble in water; freely soluble in alcohol.

**Escitalopram Oxalate:** Fine, white to slightly yellow powder. Freely soluble in methanol and in dimethyl sulfoxide; sparingly soluble in water and in alcohol; very slightly soluble in ethyl acetate and in isopropyl alcohol; insoluble in heptane.

**Esomeprazole Magnesium:** White to slightly colored powder. Soluble in methanol; slightly soluble in water; practically insoluble in heptane.

**Esomeprazole Strontium:** White to almost-white, crystalline powder. Soluble in water.

**Estazolam:** White to pale yellowish-white crystal. Soluble in methanol and in acetic anhydride; sparingly soluble in ethanol; practically insoluble in water and in ether.

**Estradiol:** White or creamy white, small crystals or crystalline powder. Is odorless, and is stable in air. Is hygroscopic. Soluble in alcohol, in acetone, in dioxane, and in solutions of fixed alkali hydroxides; slightly soluble in chloroform; sparingly soluble in vegetable oils; practically insoluble in water.

**Estradiol Benzoate:** White to off-white, crystalline powder. Soluble in alcohol and in acetone; slightly soluble in diethyl ether; insoluble in water.

**Estradiol Cypionate:** White to practically white, crystalline powder. Is odorless or has a slight odor. Soluble in alcohol, in acetone, in chloroform, and in dioxane; sparingly soluble in vegetable oils; insoluble in water.

**Estradiol Valerate:** White, crystalline powder. Is usually odorless but may have a faint, fatty odor. Soluble in castor oil, in methanol, in benzyl benzoate, and in dioxane; sparingly soluble in sesame oil and in peanut oil; practically insoluble in water.

**Estriol:** White to practically white, odorless, crystalline powder. Melts at about 280°. Soluble in acetone, in chloroform, in dioxane, in ether, and in vegetable oils; sparingly soluble in alcohol; insoluble in water.

**Conjugated Estrogens:** Conjugated Estrogens obtained from natural sources is a buff-colored, amorphous powder, odorless or having a slight, characteristic odor. The synthetic form is a white to light buff, crystalline or amorphous powder, odorless or having a slight odor.

**Esterified Estrogens:** White or buff-colored, amorphous powder, odorless or having a slight, characteristic odor.

**Estrone:** Small, white crystals or white to creamy white, crystalline powder. Is odorless, and is stable in air. Melts at about 260°. Soluble in alcohol, in acetone, in dioxane, and in vegetable oils; slightly soluble in solutions of fixed alkali hydroxides; practically insoluble in water.

**Estropipate:** White to yellowish-white, fine, crystalline powder. Is odorless, or may have a slight odor. Melts at about 190° to a light brown, viscous liquid, which solidifies on further heating and finally melts at about 245°, with decomposition. Soluble in warm water; very slightly soluble in water, in alcohol, in chloroform, and in ether.

**Eszopiclone:** White or light-yellow crystalline solid. Freely soluble in methylene chloride; soluble in pH 3.2 phosphate buffer and in dilute mineral acids; slightly soluble in alcohol; very slightly soluble in water.

**Ethacrynic Acid:** White or practically white, odorless or practically odorless, crystalline powder. Freely soluble in alcohol, in chloroform, and in ether; very slightly soluble in water.

**Ethambutol Hydrochloride:** White, crystalline powder. Freely soluble in water; soluble in alcohol and in methanol; slightly soluble in ether and in chloroform.

**Ethchlorvynol:** Colorless to yellow, slightly viscous liquid, having a characteristic pungent odor. Darkens on exposure to light and air. Immiscible with water; miscible with most organic solvents.

**Ether:** Colorless, mobile, volatile liquid, having a characteristic sweet, pungent odor. Is slowly oxidized by the action of air and light, with the formation of peroxides. It boils at about 35°. Soluble in water and in hydrochloric acid. Miscible with alcohol, with benzene, with chloroform, with solvent hexane, with methylene chloride, and with fixed and volatile oils.

**Ethinyl Estradiol:** White to creamy white, odorless, crystalline powder. Soluble in alcohol, in chloroform, in ether, in vegetable oils, and in solutions of fixed alkali hydroxides; insoluble in water.

**Ethiodized Oil Injection:** Straw-colored to amber-colored, oily liquid. It may possess an alliaceous odor. Soluble in acetone, in chloroform, in ether, and in solvent hexane; insoluble in water.

**Ethionamide:** Bright yellow powder, having a faint to moderate sulfide-like odor. Soluble in methanol; sparingly soluble in alcohol and in propylene glycol; slightly soluble in water, in chloroform, and in ether.

**Ethopabate:** White to pinkish-white, odorless or practically odorless powder. Soluble in acetonitrile, in acetone, in dehydrated alcohol, and in methanol; sparingly soluble in isopropyl alcohol, in dioxane, in ethyl acetate, and in

methylene chloride; slightly soluble in ether; very slightly soluble in water.

**Ethosuximide:** White to off-white, crystalline powder or waxy solid, having a characteristic odor. Freely soluble in water and in chloroform; very soluble in alcohol and in ether; very slightly soluble in solvent hexane.

**Ethotoin:** White, crystalline powder. Freely soluble in dehydrated alcohol and in chloroform; soluble in ether; insoluble in water.

**Ethyl Acetate:** Transparent, colorless liquid, having a fragrant, refreshing, slightly acetous odor, and a peculiar, acetous, burning taste. Soluble in water. Miscible with alcohol, with ether, with fixed oils, and with volatile oils. *NF category:* Flavors and fragrance; solvent.

**Ethyl Acrylate and Methyl Methacrylate Copolymer Dispersion:** Milky-white liquid of low viscosity with a faint, characteristic odor. It is miscible with water in any proportion; the milky-white appearance is retained. A clear or slightly opalescent, viscous solution is obtained on mixing one part with five parts of acetone, alcohol, or isopropyl alcohol; the polymer substance first precipitates, but then dissolves in the excess organic solvent. When mixed with 1 N sodium hydroxide in a ratio of 1:2, the dispersion does not dissolve; the milky-white appearance is retained. *NF category:* Coating agent; polymer membrane; wet binder; film-forming agent; diluent.

**Ethyl Maltol:** White, crystalline powder having a cotton-candy odor and a sweet, fruit-like flavor in dilute solution. One g dissolves in about 55 mL of water, 10 mL of alcohol, 17 mL of propylene glycol, and 5 mL of chloroform. It melts at about 90°. *NF category:* Vehicle (flavored and/or sweetened); flavors and fragrance.

**Ethyl Oleate:** Mobile, practically colorless liquid, having an agreeable taste. Insoluble in water. Miscible with vegetable oils, with mineral oil, with alcohol, and with most organic solvents. *NF category:* Vehicle (oleaginous); solvent.

**Ethyl Vanillin:** Fine, white or slightly yellowish crystals. Its taste and odor are similar to the taste and odor of vanillin. Is affected by light. Its solutions are acid to litmus. Freely soluble in alcohol, in chloroform, in ether, and in solutions of alkali hydroxides; sparingly soluble in water at 50°. *NF category:* Flavors and fragrance.

**Ethylcellulose:** Free-flowing, white to light tan powder. It forms films that have a refractive index of about 1.47. Its aqueous suspensions are neutral to litmus. Ethylcellulose containing less than 46.5% of ethoxy groups is freely soluble in tetrahydrofuran, in methyl acetate, in chloroform, and in mixtures of aromatic hydrocarbons with alcohol; Ethylcellulose containing not less than 46.5% of ethoxy groups is freely soluble in alcohol, in methanol, in toluene, in chloroform, and in ethyl acetate; insoluble in water, in glycerin, and in propylene glycol. *NF category:* Coating agent; wet binder; flavors and fragrance; film-forming agent; polymer membrane; release-modifying agent; suspending and/or viscosity-increasing agent.

**Ethylcellulose Dispersion Type B:** Off-white and slightly viscous liquid. Soluble in alcohol, in methyl alcohol, in tetrahydrofuran, and in ethyl acetate; insoluble in water and in chloroform. *NF category:* Coating agent; film-forming agent; polymer membrane; release-modifying agent.

**Ethylenediamine:** Clear, colorless or only slightly yellow liquid, having an ammonia-like odor and a strong alkaline reaction. Miscible with water and with alcohol.

**Ethylene Glycol Stearates:** White or almost white, waxy solid. Soluble in acetone and in hot alcohol; practically insoluble in water. *NF category:* Emulsifying agent; emollient.

**Ethylene Glycol and Vinyl Alcohol Graft Copolymer:** White or slightly yellowish powder. Very soluble in water; practically insoluble in anhydrous alcohol, and in acetone. It dissolves in dilute acids and dilute solutions of alkali hydroxides. *NF category:* Coating agent; wet binder; film-forming agent.

**Ethylparaben:** Small, colorless crystals or white powder. Freely soluble in acetone, in alcohol, in ether, and in propylene glycol; slightly soluble in water and in glycerin. *NF category:* Antimicrobial preservative.

**Ethylparaben Sodium:** White or almost-white, hygroscopic, crystalline powder. Freely soluble in water; soluble in anhydrous alcohol; practically insoluble in methylene chloride. *NF category:* Antimicrobial preservative.

**Ethynodiol Diacetate:** White, odorless, crystalline powder. Is stable in air. Very soluble in chloroform; freely soluble in ether; soluble in alcohol; sparingly soluble in fixed oils; insoluble in water.

**Etidronate Disodium:** White powder, which may contain lumps. Freely soluble in water; practically insoluble in alcohol.

**Etomidate:** White or almost white powder. Freely soluble in alcohol and in methylene chloride; very slightly soluble in water.

**Etoposide:** Fine, white to off-white, crystalline powder. Sparingly soluble in methanol; slightly soluble in alcohol, in chloroform, in ethyl acetate, and in methylene chloride; very slightly soluble in water.

**Etoposide Phosphate:** White powder. Freely soluble in water; soluble in dimethylsulfoxide.

**Eucalyptus Oil:** Colorless or pale yellow liquid. One mL of eucalyptus oil dissolves in 5 mL of 70% alcohol. Insoluble in cold water. *NF category*: Flavors and perfumes.

**Eugenol:** Colorless or pale yellow liquid, having a strongly aromatic odor of clove and a pungent, spicy taste. Upon exposure to air, it darkens and thickens. Is optically inactive. Slightly soluble in water. Miscible with alcohol, with chloroform, with ether, and with fixed oils.

**Exemestane:** White to slightly yellow, crystalline powder. Freely soluble in *N*,*N*-dimethylformamide; soluble in methanol; insoluble in water.

**Ezetimibe:** White powder. Freely soluble in alcohol; soluble in acetonitrile; insoluble in aqueous solvents and nonpolar solvents like hexane.

**Famciclovir:** A white to pale yellow solid. Freely soluble in methanol and in acetone; sparingly soluble in ethanol and in isopropyl alcohol.

**Famotidine:** White to pale yellowish-white, crystalline powder. Is sensitive to light. Freely soluble in dimethylformamide and in glacial acetic acid; slightly soluble in methanol; very slightly soluble in water; practically insoluble in acetone, in alcohol, in chloroform, in ether, and in ethyl acetate.

**Hard Fat:** White mass; almost odorless and free from rancid odor; greasy to the touch. On warming, melts to give a colorless or slightly yellowish liquid. When the molten material is shaken with an equal quantity of hot water, a white emulsion is formed. Freely soluble in ether; slightly soluble in alcohol; practically insoluble in water. *NF category:* Stiffening agent; suppository base.

**Felbamate:** White to off-white powder. Freely soluble in dimethyl sulfoxide; sparingly soluble in methanol; slightly soluble in acetonitrile; very slightly soluble in water.

**Felodipine:** Light yellow to yellow, crystalline powder. Freely soluble in acetone and in methanol; very slightly soluble in heptane; insoluble in water.

**Fenbendazole:** White to off-white powder. Sparingly soluble in dimethylformamide; very slightly soluble in methanol; practically insoluble in water.

**Fenofibrate:** White or almost white, crystalline powder. Very soluble in methylene chloride; slightly soluble in alcohol; practically insoluble in water.

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**Fenoldopam Mesylate:** White to off-white powder. Soluble in water.

**Fenoprofen Calcium:** White, crystalline powder. Slightly soluble in *n*-hexanol, in methanol, and in water; practically insoluble in chloroform.

**Fentanyl Citrate:** White, crystalline powder or white, glistening crystals. Melts at about 150°, with decomposition. Soluble in methanol; sparingly soluble in water; slightly soluble in chloroform.

**Ferric Oxide:** Powder exhibiting two basic colors (red and yellow), or other shades produced on blending the basic colors. Insoluble in water and in organic solvents; dissolves in hydrochloric acid upon warming, a small amount of insoluble residue usually remaining. *NF category:* Coloring agent.

**Ferric Subsulfate Solution:** Reddish-brown liquid, odorless or nearly so. Acid to litmus, and is affected by light. Specific gravity is about 1.548.

**Ferric Sulfate:** Grayish-white or yellowish powder or fawn-colored pearls. Hygroscopic. Slightly soluble in water and in ethanol (96%); practically insoluble in acetone and in ethyl acetate. Hydrolyzes slowly in aqueous solution.

**Ferrosoferric Oxide:** Black powder. Dissolves in hydrochloric acid upon warming, a small amount of insoluble residue usually remaining; insoluble in water and in organic solvents. *NF category:* Coloring agent.

**Ferrous Fumarate:** Reddish-orange to red-brown, odorless powder. May contain soft lumps that produce a yellow streak when crushed. Slightly soluble in water; very slightly soluble in alcohol. Its solubility in dilute hydrochloric acid is limited by the separation of fumaric acid.

**Ferrous Gluconate:** Yellowish-gray or pale greenish-yellow, fine powder or granules, having a slight odor resembling that of burned sugar. Its solution (1 in 20) is acid to litmus. Soluble in water, with slight heating; practically insoluble in alcohol.

**Ferrous Sulfate:** Pale, bluish-green crystals or granules. Is odorless and is efflorescent in dry air. Oxidizes readily in moist air to form brownish yellow basic ferric sulfate. Its solution (1 in 10) is acid to litmus, having a pH of about 3.7. Very soluble in boiling water; freely soluble in water; insoluble in alcohol.

**Dried Ferrous Sulfate:** Grayish-white to buff-colored powder, consisting primarily of $FeSO_4 \cdot H_2O$ with varying amounts of $FeSO_4 \cdot 4H_2O$. Slowly soluble in water; insoluble in alcohol.

**Fexofenadine Hydrochloride:** White to off-white powder. Freely soluble in methanol; very slightly to slightly soluble in water; very slightly soluble in acetone.

**Finasteride:** White to off-white, crystalline solid. Melts at about 257°. Freely soluble in chloroform and in alcohol; very slightly soluble in water.

**Fingolimod Hydrochloride:** White to practically white powder. Freely soluble in water and in alcohol. Soluble in propylene glycol.

**Fish Oil Containing Omega-3 Acids:** Pale yellow liquid. Very soluble in acetone and in heptane; slightly soluble in anhydrous alcohol; practically insoluble in water.

**Flavoxate Hydrochloride:** White or almost white, crystalline powder. Slightly soluble in alcohol, in water, and in methylene chloride.

**Flecainide Acetate:** White to slightly off-white, crystalline powder. Freely soluble in alcohol; soluble in water. $pK_a$ is 9.3.

**Fluconazole:** White or almost white, crystalline powder. Freely soluble in methanol; soluble in alcohol and in acetone; sparingly soluble in isopropanol and in chloroform; slightly soluble in water; very slightly soluble in toluene.

**Flucytosine:** White to off-white, crystalline powder. Is odorless or has a slight odor. Sparingly soluble in water; slightly soluble in alcohol; practically insoluble in chloroform and in ether.

**Fludarabine Phosphate:** White to off-white, crystalline, hygroscopic powder. Freely soluble in dimethylformamide; slightly soluble in water and in 0.1 M hydrochloric acid; practically insoluble in ethanol.

**Fludrocortisone Acetate:** White to pale yellow crystals or crystalline powder. Is odorless or practically odorless. Is hygroscopic. Sparingly soluble in alcohol and in chloroform; slightly soluble in ether; insoluble in water.

**Flumazenil:** White to off-white powder. Slightly soluble in acidic aqueous solutions; practically insoluble in water.

**Flunisolide:** White to creamy-white, crystalline powder. Melts at about 245°, with decomposition. Soluble in acetone; sparingly soluble in chloroform; slightly soluble in methanol; practically insoluble in water.

**Flunixin Meglumine:** White to off-white crystalline powder. Soluble in water, in alcohol, and in methanol; practically insoluble in ethyl acetate.

**Fluocinolone Acetonide:** White or practically white, odorless, crystalline powder. Is stable in air. Melts at about 270°, with decomposition. Soluble in methanol; slightly soluble in ether and in chloroform; insoluble in water.

**Fluocinonide:** White to cream-colored, crystalline powder, having not more than a slight odor. Sparingly soluble in acetone and in chloroform; slightly soluble in alcohol, in methanol, and in dioxane; very slightly soluble in ether; practically insoluble in water.

**Fluorescein:** Yellowish-red to red, odorless powder. Soluble in dilute alkali hydroxides; insoluble in water.

**Fluorescein Sodium:** Orange-red, hygroscopic, odorless powder. Freely soluble in water; sparingly soluble in alcohol.

**Fluorescein Sodium Ophthalmic Strip:** Each Strip is a dry, white piece of paper, one end of which is rounded and is uniformly orange-red in color because of the fluorescein sodium impregnated in the paper.

**Fluorometholone:** White to yellowish-white, odorless, crystalline powder. Melts at about 280°, with some decomposition. Slightly soluble in alcohol; very slightly soluble in chloroform; practically insoluble in water and in ether.

**Fluorouracil:** White to practically white, practically odorless, crystalline powder. Decomposes at about 282°. Sparingly soluble in water; slightly soluble in alcohol; practically insoluble in chloroform and in ether.

**Fluoxetine Hydrochloride:** White to off-white crystalline powder. Freely soluble in alcohol and in methanol; sparingly soluble in water and in dichloromethane; practically insoluble in ether.

**Fluoxymesterone:** White or practically white, odorless, crystalline powder. Melts at about 240°, with some decomposition. Sparingly soluble in alcohol; slightly soluble in chloroform; practically insoluble in water.

**Fluphenazine Decanoate:** Pale yellow to yellow-orange, viscous liquid or yellow solid. Slowly crystallizes at room temperature. Melts at about 30°–32°. Very soluble in chloroform, in ether, in cyclohexane, in dehydrated alcohol, and in methylene chloride; freely soluble in methanol; insoluble in water.

**Fluphenazine Enanthate:** Pale yellow to yellow-orange, clear to slightly turbid, viscous liquid, having a characteristic odor. Is unstable in strong light, but stable to air at room temperature. Freely soluble in alcohol, in chloroform, and in ether; insoluble in water.

**Fluphenazine Hydrochloride:** White or nearly white, odorless, crystalline powder. Melts, within a range of 5°, at a temperature above 225°. Freely soluble in water; slightly soluble in alcohol, in chloroform, and in methylene chloride; practically insoluble in benzene and in ether.

**Flurandrenolide:** White to off-white, fluffy, crystalline powder. Is odorless. Freely soluble in chloroform; soluble in

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methanol; sparingly soluble in alcohol; practically insoluble in water and in ether.

**Flurazepam Hydrochloride:** Off-white to yellow, crystalline powder. Is odorless, or has a slight odor, and its solutions are acid to litmus. Melts at about 212°, with decomposition. Freely soluble in water and in alcohol; slightly soluble in isopropyl alcohol and in chloroform.

**Flurbiprofen:** White, crystalline powder. Freely soluble in acetone, in dehydrated alcohol, in ether, and in methanol; soluble in acetonitrile; practically insoluble in water. Optically inactive (1 in 50 solution in dehydrated alcohol).

**Flutamide:** Pale yellow, crystalline powder. Freely soluble in acetone, in ethyl acetate, and in methanol; soluble in chloroform and in ether; practically insoluble in mineral oil, in petroleum ether, and in water.

**Fluticasone Propionate (micronized):** Fine, white powder.

**Fluvastatin Sodium:** White to pale yellow, brownish-pale yellow, or reddish-pale yellow, hygroscopic powder. Soluble in alcohol, in methanol, and in water.

**Fluvoxamine Maleate:** White to off-white, crystalline powder. Freely soluble in alcohol and in chloroform; sparingly soluble in water; and practically insoluble in diethyl ether.

**Folic Acid:** Yellow, yellow-brownish, or yellowish-orange, odorless, crystalline powder. It readily dissolves in dilute solutions of alkali hydroxides and carbonates. Soluble in hot, 3 N hydrochloric acid, in hot, 2 N sulfuric acid, in hydrochloric acid, and in sulfuric acid, yielding very pale yellow solutions; very slightly soluble in water; insoluble in alcohol, in acetone, in chloroform, and in ether.

**Folic Acid Injection:** Clear, yellow to orange-yellow, alkaline liquid.

**Formaldehyde Solution:** Clear, colorless or practically colorless liquid, having a pungent odor. The vapor from it irritates the mucous membrane of the throat and nose. On long standing, especially in the cold, it may become cloudy because of the separation of paraformaldehyde. This cloudiness disappears when the solution is warmed. Miscible with water and with alcohol.

**Formoterol Fumarate Dihydrate:** White or almost white or slightly yellow powder. Freely soluble in dimethyl sulfoxide and in acetic acid; soluble in methanol; slightly soluble in water and in 2-propanol; practically insoluble in acetonitrile and in diethyl ether.

**Fosamprenavir Calcium:** White to cream-colored solid. Soluble in water, in ethanol, in dimethyl sulfoxide (DMSO), and in dimethyl formamide.

**Foscarnet Sodium:** White to almost white, crystalline powder. Soluble in water; practically insoluble in alcohol.

**Fosphenytoin Sodium:** White to pale yellow solid. Freely soluble in water.

**Fructose:** Colorless crystals or as a white, crystalline powder. Is odorless, and has a sweet taste. Freely soluble in water; soluble in alcohol and in methanol. *NF category:* Sweetening agent; diluent; flavors and fragrance.

**Basic Fuchsin:** Dark green powder or greenish glistening crystalline fragments, having a bronze-like luster and not more than a faint odor. Soluble in water, in alcohol, and in amyl alcohol; insoluble in ether.

**Fulvestrant:** White powder. Freely soluble in alcohol.

**Fumaric Acid:** White, odorless granules or crystalline powder. Soluble in alcohol; slightly soluble in water and in ether; very slightly soluble in chloroform. *NF category:* pH modifier (acidifying agent/alkalizing agent/buffering agent); flavors and fragrance; antioxidant.

**Furazolidone:** Yellow, odorless, crystalline powder. Is tasteless at first, then a bitter aftertaste develops. Practically insoluble in water, in alcohol, and in carbon tetrachloride.

**Furosemide:** White to slightly yellow, odorless, crystalline powder. Freely soluble in acetone, in dimethylformamide, and in solutions of alkali hydroxides; soluble in methanol; sparingly soluble in alcohol; slightly soluble in ether; very slightly soluble in chloroform; practically insoluble in water.

**Furosemide Injection:** Clear, colorless solution.

**Gabapentin:** White to off-white, crystalline solid. Freely soluble in water and in alkaline and acidic solutions.

**Gadobutrol:** White or almost-white powder. Anhydrous form may be hygroscopic. Freely soluble in water; practically insoluble in absolute alcohol and in heptane.

**Gadodiamide:** White, odorless powder. Freely soluble in water and in methanol; soluble in ethyl alcohol; slightly soluble in acetone and in chloroform.

**Gadoteridol:** White to off-white, crystalline, odorless powder. Freely soluble in water and in methyl alcohol; soluble in isopropyl alcohol. Melts at about 300°.

**Galactose:** A white, crystalline or finely granulated powder. Soluble in water; very slightly soluble in alcohol. *NF category:* Sweetening agent; chelating and/or complexing agent.

**Galantamine Hydrobromide:** White to almost white powder. Soluble in 0.1 N sodium hydroxide; sparingly soluble in water; very slightly soluble in alcohol; insoluble in *n*-propanol.

**Gamma Cyclodextrin:** White or almost white, amorphous or crystalline powder. Freely soluble in water and in propylene glycol; very slightly soluble in alcohol. *NF category:* Sequestering agent; chelating and/or complexing agent.

**Ganciclovir:** White to off-white, crystalline powder.

**Ganciclovir for Injection:** White to off-white powder. Soluble in water.

**Petrolatum Gauze:** The petrolatum recovered by draining in the *Assay* is a white or faintly yellowish, unctuous mass, transparent in thin layers even after cooling to 0°.

**Gelatin:** Sheets, flakes, or shreds, or coarse to fine powder. Is faintly yellow or amber in color, the color varying in depth according to the particle size. Has a slight, characteristic bouillon-like odor in solution. Is stable in air when dry, but is subject to microbic decomposition when moist or in solution. Gelatin has any suitable strength that is designated by Bloom Gelometer number. Type A Gelatin exhibits an isoelectric point between pH 7 and pH 9, and Type B Gelatin exhibits an isoelectric point between pH 4.7 and pH 5.2. Soluble in hot water, in 6 N acetic acid, and in a hot mixture of glycerin and water; insoluble in cold water, but swells and softens when immersed in it, gradually absorbing from 5 to 10 times its own weight of water, in alcohol, in chloroform, in ether, and in fixed and volatile oils. *NF category:* Coating agent; suspending and/or viscosity-increasing agent; wet binder; capsule shell; colloid stabilizing agent; film-forming agent.

**Absorbable Gelatin Film:** Light amber, transparent, pliable film which becomes rubbery when moistened. Insoluble in water.

**Absorbable Gelatin Sponge:** Light, nearly white, nonelastic, tough, porous, hydrophilic solid. Insoluble in water.

**Gellan Gum:** Off-white powder. Soluble in hot or in cold deionized water. *NF category:* Suspending and/or viscosity-increasing agent.

**Gemcitabine Hydrochloride:** White to off-white solid. Soluble in water; slightly soluble in methanol; practically insoluble in alcohol and in polar organic solvents.

**Gemfibrozil:** White, waxy, crystalline solid. Soluble in alcohol, in methanol, and in chloroform; practically insoluble in water.

**Gemifloxacin Mesylate:** A white to light brown solid. Freely soluble at neutral pH.

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**Gentamicin Sulfate:** White to buff powder. Freely soluble in water; insoluble in alcohol, in acetone, in chloroform, in ether, and in benzene.

**Gentamicin Injection:** Clear, slightly yellow solution, having a faint odor.

**Gentian Violet:** Dark green powder or greenish, glistening pieces having a metallic luster, and having not more than a faint odor. Soluble in alcohol, in glycerin, and in chloroform; sparingly soluble in water; insoluble in ether.

**Gentian Violet Cream:** Dark purple, water-washable cream.

**Gentian Violet Topical Solution:** Purple liquid, having a slight odor of alcohol. A dilution (1 in 100), viewed downward through 1 cm of depth, is deep purple in color.

**Powdered Asian Ginseng Extract:** Pale yellow-brown, hygroscopic, powdery or easily pulverizable mass. Soluble in water, forming a slightly cloudy solution.

**Glaze, Pharmaceutical:** Denatured alcohol solution. *NF category:* Coating agent; film-forming agent.

**Glimepiride:** White to almost white powder. Soluble in dimethylformamide; sparingly soluble in methylene chloride; slightly soluble in methanol; practically insoluble in water.

**Glipizide:** White to off-white powder. Freely soluble in dimethylformamide; soluble in 0.1 N sodium hydroxide; slightly soluble in methylene chloride.

**Glucagon:** Fine, white or faintly colored, crystalline powder. Is practically odorless and tasteless. Soluble in dilute alkali and acid solutions; insoluble in most organic solvents.

**Glucagon for Injection:** White, odorless powder.

**Gluconolactone:** Fine, white, practically odorless, crystalline powder. Melts at about 153°, with decomposition. Freely soluble in water; sparingly soluble in alcohol; insoluble in ether.

**Liquid Glucose:** Colorless or yellowish, thick, syrupy liquid. Odorless or nearly odorless, and has a sweet taste. Sparingly soluble in alcohol. Miscible with water. *NF category:* Wet binder; coating agent; sweetening agent.

**L-Glutamic Acid Hydrochloride:** A white, crystalline powder. 1 g dissolves in about 3 mL of water. It is almost insoluble in alcohol and in ether. Its solutions are acid to litmus. *NF category*: Flavors and fragrance.

**Glutamine:** White crystals or crystalline powder. Soluble in water; practically insoluble in alcohol and in ether.

**Glutaral Concentrate:** Clear, colorless or faintly yellow liquid, having a characteristic, irritating odor.

**Glycerin:** Clear, colorless, syrupy liquid, having a sweet taste. Has not more than a slight characteristic odor, which is neither harsh nor disagreeable. Is hygroscopic. Its solutions are neutral to litmus. Insoluble in chloroform, in ether, and in fixed and volatile oils. Miscible with water and with alcohol. *NF category:* Humectant; plasticizer; solvent; tonicity agent; antimicrobial preservative; emollient; sweetening agent.

**Glyceryl Dibehenate:** Fine powder, having a faint odor. Melts at about 70°. Soluble in chloroform; practically insoluble in water and in alcohol. *NF category*: Lubricant; coating agent; suspending and/or viscosity-increasing agent; wet binder.

**Glyceryl Distearate:** Hard, waxy mass or powder or white or almost white flakes. Soluble in methylene chloride and in tetrahydrofuran; slightly soluble in hot alcohol; insoluble in water. *NF category:* Emulsifying agent.

**Glyceryl Mono and Dicaprylate:** Colorless or slightly yellow, oily liquid or semisolid. Very soluble in alcohol; freely soluble in methylene chloride; practically insoluble in water. *NF category:* Lubricant; emulsifying agent.

**Glyceryl Mono and Dicaprylocaprate:** Colorless or slightly yellow, oily liquid or semisolid. Very soluble in alcohol; freely soluble in methylene chloride; practically insoluble in water. *NF category:* Lubricant; emulsifying agent.

**Glyceryl Monocaprylate:** Colorless or slightly yellow, oily liquid or semisolid. Very soluble in alcohol; freely soluble in methylene chloride; practically insoluble in water. *NF category*: Lubricant; emulsifying agent.

**Glyceryl Monocaprylocaprate:** Colorless or slightly yellow, oily liquid or semisolid. Very soluble in alcohol; freely soluble in methylene chloride; practically insoluble in water. *NF category*: Lubricant; emulsifying agent.

**Glyceryl Monolinoleate:** Amber, oily liquids that may be partially solidified at room temperature. Freely soluble in methylene chloride; soluble in tetrahydrofuran; practically insoluble in water. *NF category:* Emulsifying agent.

**Glyceryl Monooleate:** Amber, oily liquids that may be partially solidified at room temperature. Freely soluble in methylene chloride; soluble in tetrahydrofuran; practically insoluble in water. *NF category:* Emulsifying agent; emollient; release-modifying agent.

**Glyceryl Monostearate:** White to yellowish wax-like solid; or white to yellowish wax-like beads, flakes, or powder. Slight, agreeable, fatty odor and taste. Is affected by light. Dissolves in hot organic solvents such as alcohol, minerals or fixed oils, benzene, ether, and acetone. Insoluble in water, but it may be dispersed in hot water with the aid of a small amount of soap or other suitable surface-active agent. *NF category:* Emulsifying agent; emollient; release-modifying agent; lubricant.

**Glyceryl Tricaprylate:** Clear, colorless to pale-yellow liquid. Very soluble in alcohol; freely soluble in methylene chloride; practically insoluble in water. *NF category*: Lubricant; emulsifying agent; vehicle (solid carrier).

**Glyceryl Tristearate:** White, solid, microcrystalline powder. Soluble in hot alcohol, in acetone, and in chloroform; very slightly soluble in cold alcohol, in ether, and in petroleum ether; insoluble in water. *NF category:* Lubricant; emulsifying agent.

**Glycine:** White, odorless, crystalline powder, having a sweetish taste. Its solutions are acid to litmus. Freely soluble in water; very slightly soluble in alcohol and in ether. *NF category:* Bulking agent; disintegrant; wetting and/or solubilizing agent; pH modifier (acidifying agent/alkalizing agent/buffering agent).

**Glycopyrrolate:** White, odorless, crystalline powder. Soluble in water and in alcohol; practically insoluble in chloroform and in ether.

**Gonadorelin Acetate:** White to slightly yellowish powder. Soluble in water; sparingly soluble in methanol.

**Chorionic Gonadotropin:** White or practically white, amorphous powder. Freely soluble in water.

**Chorionic Gonadotropin for Injection:** White or practically white, amorphous solid having the characteristic appearance of substances prepared by freeze-drying.

**Gramicidin:** White or practically white, odorless, crystalline powder. Soluble in alcohol; insoluble in water.

**Granisetron:** Off-white to pale-yellow powder. Soluble to freely soluble in methanol and in methylene chloride.

**Granisetron Hydrochloride:** White or almost white powder. Freely soluble in water; sparingly soluble in methylene chloride; slightly soluble in methanol.

**Green Soap:** Soft, unctuous, yellowish-white to brownish or greenish yellow, transparent to translucent mass. Has a slight, characteristic odor, often suggesting the oil from which it was prepared. Its solution (1 in 20) is alkaline to bromothymol blue TS.

**Griseofulvin:** White to creamy white, odorless powder, in which particles of the order of 4 µm in diameter predominate. Soluble in acetone, in dimethylformamide, and in chloroform; sparingly soluble in alcohol; very slightly soluble in water.

**Guaifenesin:** White to slightly gray, crystalline powder. May have a slight characteristic odor. Soluble in water, in

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alcohol, in chloroform, and in propylene glycol; sparingly soluble in glycerin.

**Guanabenz Acetate:** White or almost white powder having not more than a slight odor. Soluble in alcohol and in propylene glycol; sparingly soluble in water and in 0.1 N hydrochloric acid.

**Guanethidine Monosulfate:** White to off-white, crystalline powder. Very soluble in water; sparingly soluble in alcohol; practically insoluble in chloroform.

***Add the following:***

▲**Guanfacine Hydrochloride:** White to off-white, crystalline powder. Sparingly soluble in water, in methanol, and in ethanol; slightly soluble in acetone.▲ (USP 1-Dec-2023)

**Guanidine Hydrochloride:** White crystalline powder. Freely soluble in water and in alcohol. *NF category*: Chaotropic agent; wetting and/or solubilizing agent.

**Guar Gum:** White to yellowish-white, practically odorless powder. Dispersible in hot or cold water, forming a colloidal solution. *NF category:* Suspending and/or viscosity-increasing agent; wet binder; polymers for ophthalmic use; release-modifying agent; disintegrant.

**Gutta Percha:** Lumps or blocks of variable size; externally brown or grayish-brown to grayish-white in color; internally reddish yellow or reddish gray and having a laminated or fibrous appearance. Is flexible but only slightly elastic. Has a slight, characteristic odor and a slight taste. Soluble in chloroform; partly soluble in benzene, in carbon disulfide, and in turpentine oil; insoluble in water.

**Halazone:** White, crystalline powder, having a characteristic chlorine-like odor. Is affected by light. Melts at about 194°, with decomposition. Soluble in glacial acetic acid; very slightly soluble in water and in chloroform. Dissolves in solutions of alkali hydroxides and carbonates with the formation of a salt.

**Halazone Tablets for Solution:** Soluble in water.

**Halcinonide:** White to off-white, odorless, crystalline powder. Soluble in acetone and in chloroform; slightly soluble in alcohol and in ethyl ether; insoluble in water and in hexanes.

**Halobetasol Propionate:** White to off-white powder. Freely soluble in dichloromethane and in acetone; practically insoluble in water.

**Haloperidol:** White to faintly yellowish, amorphous or microcrystalline powder. Its saturated solution is neutral to litmus. Soluble in chloroform; sparingly soluble in alcohol; slightly soluble in ether; practically insoluble in water.

**Haloperidol Decanoate:** A white or almost white powder. Very soluble in alcohol, in methanol, and in methylene chloride; practically insoluble in water.

**Halothane:** Colorless, mobile, nonflammable, heavy liquid, having a characteristic odor resembling that of chloroform. Its taste is sweet and produces a burning sensation. Slightly soluble in water. Miscible with alcohol, with chloroform, with ether, and with fixed oils.

**Helium:** Colorless, odorless, tasteless gas, which is not combustible and does not support combustion. Very slightly soluble in water. At 0° and at a pressure of 760 mm of mercury, 1000 mL of the gas weighs about 180 mg.

**Heparin Sodium:** White or pale-colored, amorphous powder. Is odorless or practically so, and is hygroscopic. Soluble in water.

**Hexachlorophene:** White to light tan, crystalline powder. Is odorless or has only a slight, phenolic odor. Freely soluble in acetone, in alcohol, and in ether; soluble in chloroform and in dilute solutions of fixed alkali hydroxides; insoluble in water.

**Hexachlorophene Liquid Soap:** Clear, amber-colored liquid, having a slight, characteristic odor. Its solution (1 in 20) is clear and has an alkaline reaction.

**Hexylene Glycol:** Clear, colorless, viscous liquid. Absorbs moisture when exposed to moist air. Miscible with water and with many organic solvents, including alcohol, ether, chloroform, acetone, and hexanes. *NF category:* Humectant; solvent.

**Histamine Phosphate:** Colorless, odorless, long prismatic crystals. Is stable in air but is affected by light. Its solutions are acid to litmus. Freely soluble in water.

**Histidine:** White, odorless crystals, having a slightly bitter taste. Soluble in water; very slightly soluble in alcohol; insoluble in ether.

**Homatropine Hydrobromide:** White crystals, or white, crystalline powder. Slowly darkens on exposure to light. Freely soluble in water; sparingly soluble in alcohol; slightly soluble in chloroform; insoluble in ether. Melts between 214° and 217°, with slight decomposition.

**Homatropine Methylbromide:** White, odorless powder. Slowly darkens on exposure to light. Melts at about 190°. Very soluble in water; freely soluble in alcohol and in acetone containing about 20% of water; practically insoluble in ether and in acetone.

**Hydralazine Hydrochloride:** White to off-white, odorless, crystalline powder. Melts at about 275°, with decomposition. Soluble in water; slightly soluble in alcohol; very slightly soluble in ether.

**Hydrochloric Acid:** Colorless, fuming liquid having a pungent odor. It ceases to fume when it is diluted with 2 volumes of water. Specific gravity is about 1.18. *NF category:* pH modifier (acidifying agent/alkalizing agent/buffering agent).

**Diluted Hydrochloric Acid:** Colorless, odorless liquid. Specific gravity is about 1.05. *NF category:* pH modifier (acidifying agent/alkalizing agent/buffering agent).

**Hydrochlorothiazide:** White, or practically white, practically odorless, crystalline powder. Freely soluble in sodium hydroxide solution, in *n*-butylamine, and in dimethylformamide; very slightly soluble in water; sparingly soluble in methanol; insoluble in ether, in chloroform, and in dilute mineral acids.

**Hydrocodone Bitartrate:** Fine, white crystals or a crystalline powder. Is affected by light. Soluble in water; slightly soluble in alcohol; insoluble in ether and in chloroform.

**Hydrocortisone:** White to practically white, odorless, crystalline powder. Melts at about 215°, with decomposition. Sparingly soluble in acetone and in alcohol; slightly soluble in chloroform; very slightly soluble in water and in ether.

**Hydrocortisone Acetate:** White to practically white, odorless, crystalline powder. Melts at about 220°, with decomposition. Slightly soluble in alcohol and in chloroform; insoluble in water.

**Hydrocortisone Butyrate:** White to practically white, practically odorless, crystalline powder. Freely soluble in chloroform; soluble in methanol, in alcohol, and in acetone; slightly soluble in ether; practically insoluble in water.

**Hydrocortisone Sodium Phosphate:** White to light yellow, odorless or practically odorless, powder. Is exceedingly hygroscopic. Freely soluble in water; slightly soluble in alcohol; practically insoluble in chloroform, in dioxane, and in ether.

**Hydrocortisone Sodium Succinate:** White or nearly white, odorless, hygroscopic, amorphous solid. Very soluble in water and in alcohol; very slightly soluble in acetone; insoluble in chloroform.

**Hydroflumethiazide:** White to cream-colored, finely divided, odorless, crystalline powder. Freely soluble in acetone; soluble in alcohol; very slightly soluble in water.

**Hydrogen Peroxide Concentrate:** Clear, colorless liquid. Is acid to litmus. Slowly decomposes, and is affected by light.

**Hydrogen Peroxide Solution:** Clear, colorless liquid, odorless, or having an odor resembling that of ozone. Is acid

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to litmus and to the taste and produces a froth in the mouth. Rapidly decomposes when in contact with many oxidizing as well as reducing substances. When rapidly heated, it may decompose suddenly. Is affected by light. Specific gravity is about 1.01.

**Hydromorphone Hydrochloride:** Fine, white or practically white, odorless, crystalline powder. Is affected by light. Freely soluble in water; sparingly soluble in alcohol; practically insoluble in ether.

**Hydroquinone:** Fine white needles. Darkens upon exposure to light and air. Freely soluble in water, in alcohol, and in ether.

**Hydroxocobalamin:** Dark red crystals or red crystalline powder. Is odorless, or has not more than a slight acetone odor. The anhydrous form is very hygroscopic. Sparingly soluble in water, in alcohol, and in methanol; practically insoluble in acetone, in ether, in chloroform, and in benzene.

**Hydroxyamphetamine Hydrobromide:** White, crystalline powder. Its solutions are slightly acid to litmus, having a pH of about 5. Freely soluble in water and in alcohol; slightly soluble in chloroform; practically insoluble in ether.

**Hydroxychloroquine Sulfate:** White or practically white, crystalline powder. Is odorless, and has a bitter taste. Its solutions have a pH of about 4.5. Exists in two forms, the usual form melting at about 240° and the other form melting at about 198°. Freely soluble in water; practically insoluble in alcohol, in chloroform, and in ether.

**Hydroxyethyl Cellulose:** White to light tan, practically odorless and tasteless, hygroscopic powder. Soluble in hot water and in cold water, giving a colloidal solution; practically insoluble in alcohol and in most organic solvents. *NF category:* Suspending and/or viscosity-increasing agent; coating agent; film-forming agent; polymers for ophthalmic use; wet binder.

**Hydroxyprogesterone Caproate:** White or creamy white, crystalline powder. Is odorless or has a slight odor. Soluble in ether; slightly soluble in benzene; insoluble in water.

**Hydroxypropyl Betadex:** White or almost white, amorphous or crystalline powder. Freely soluble in water and in propylene glycol. *NF category:* Sequestering agent; chelating and/or complexing agent; release-modifying agent.

**Hydroxypropyl Cellulose:** White to cream-colored, practically odorless and tasteless, granular solid or powder. Is hygroscopic after drying. Soluble in cold water, in alcohol, in chloroform, and in propylene glycol, giving a colloidal solution; insoluble in hot water. *NF category:* Coating agent; suspending and/or viscosity-increasing agent; emulsifying agent; film-forming agent; release-modifying agent; wet binder.

**Low-Substituted Hydroxypropyl Cellulose:** White to yellowish-white, practically odorless and tasteless, fibrous or granular powder. Is hygroscopic. Practically insoluble in alcohol and in ether. Dissolves in a solution of sodium hydroxide (1 in 10), and produces a viscous solution. Swells in water, in sodium carbonate TS, and in 2 N hydrochloric acid. The pH of the suspension, obtained by shaking 1.0 g with 100 mL of water, is between 5.0 and 7.5. *NF category:* Disintegrant; wet binder.

**Hydroxyurea:** White to off-white powder. Is somewhat hygroscopic, decomposing in the presence of moisture. Melts at a temperature exceeding 133°, with decomposition. Freely soluble in water and in hot alcohol.

**Hydroxyzine Hydrochloride:** White, odorless powder. Melts at about 200°, with decomposition. Very soluble in water; soluble in chloroform; slightly soluble in acetone; practically insoluble in ether.

**Hydroxyzine Pamoate:** Light yellow, practically odorless powder. Freely soluble in dimethylformamide; practically insoluble in water and in methanol.

**Hymetellose:** A white, yellowish-white or grayish-white powder or granules. Hygroscopic after drying. Dissolves in cold water, giving a colloidal solution. Insoluble in hot water, in acetone, in alcohol, in ether, and in toluene.

**Hyoscyamine:** White, crystalline powder. Is affected by light. Its solutions are alkaline to litmus. Freely soluble in alcohol, in chloroform, and in dilute acids; sparingly soluble in ether; slightly soluble in water and in benzene.

**Hyoscyamine Hydrobromide:** White, odorless crystals or crystalline powder. The pH of a solution (1 in 20) is about 5.4. Is affected by light. Freely soluble in water, in alcohol, and in chloroform; very slightly soluble in ether.

**Hyoscyamine Sulfate:** White or almost white, crystalline powder or colorless needles. Is deliquescent and is affected by light. The pH of a solution (1 in 100) is about 5.3. Very soluble in water; freely soluble in alcohol; practically insoluble in ether. Melts at a temperature not less than 200°.

**Hypophosphorous Acid:** Colorless or slightly yellow, odorless liquid. Specific gravity is about 1.13. *NF category:* Antioxidant.

**Hypromellose:** White to slightly off-white, fibrous or granular powder. Swells in water and produces a clear to opalescent, viscous, colloidal mixture. Insoluble in dehydrated alcohol, in ether, and in chloroform. *NF category:* Coating agent; suspending and/or viscosity-increasing agent; wet binder; polymers for ophthalmic use; capsule shell; emulsifying agent; film-forming agent; release-modifying agent.

**Hypromellose 2208:** White to slightly off-white, fibrous or granular powder. Swells in water and produces a clear to opalescent, viscous, colloidal mixture. Insoluble in dehydrated alcohol, in ether, and in chloroform. *NF category:* Coating agent; suspending and/or viscosity-increasing agent; tablet binder.

**Hypromellose 2906:** White to slightly off-white, fibrous or granular powder. Swells in water and produces a clear to opalescent, viscous, colloidal mixture. Insoluble in dehydrated alcohol, in ether, and in chloroform. *NF category:* Coating agent; suspending and/or viscosity-increasing agent; tablet binder.

**Hypromellose 2910:** White to slightly off-white, fibrous or granular powder. Swells in water and produces a clear to opalescent, viscous, colloidal mixture. Insoluble in dehydrated alcohol, in ether, and in chloroform. *NF category:* Coating agent; suspending and/or viscosity-increasing agent; tablet binder.

**Hypromellose Acetate Succinate:** White to yellowish-white powder or pills. Odorless, or has a faint, acetic acid-like odor, and tasteless. Practically insoluble in water, in dehydrated alcohol, in xylene, and in hexane. On the addition of a mixture of dehydrated alcohol and dichloromethane (1:1) or acetone, a clear or turbid viscous liquid is produced. Dissolves in 1 N sodium hydroxide. Slightly hygroscopic. *NF category:* Coating agent; wet binder; film-forming agent.

**Hypromellose Phthalate:** White powder or granules. Is odorless and tasteless. Practically insoluble in water, in dehydrated alcohol, and in hexane. Produces a viscous solution in a mixture of methanol and dichloromethane (1:1), or in a mixture of dehydrated alcohol and acetone (1:1). Dissolves in 1 N sodium hydroxide. *NF category:* Coating agent; film-forming agent.

**Ibandronate Sodium:** White to off-white powder. Freely soluble in water; practically insoluble in methanol and in organic solvents.

**Ibuprofen:** White to off-white, crystalline powder, having a slight, characteristic odor. Very soluble in alcohol, in methanol, in acetone, and in chloroform; slightly soluble in ethyl acetate; practically insoluble in water.

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**Ibutilide Fumarate:** White to off-white powder. Freely soluble in water; sparingly soluble in methanol.

**Ichthammol:** Reddish-brown to brownish-black, viscous fluid, having a strong, characteristic, empyreumatic odor. Miscible with water, with glycerin, and with fixed oils and fats. Partially soluble in alcohol and in ether.

**Idarubicin Hydrochloride:** Red-orange to red-brown powder. Soluble in methanol; slightly soluble in water; insoluble in acetone and in ethyl ether.

**Idoxuridine:** White, crystalline, practically odorless powder. Slightly soluble in water and in alcohol; practically insoluble in chloroform and in ether.

**Ifosfamide:** White, crystalline powder. Melts at about 40°. Very soluble in alcohol, in ethyl acetate, in isopropyl alcohol, in methanol, and in methylene chloride; freely soluble in water; very slightly soluble in hexanes.

**Imidurea:** White, odorless, tasteless powder. Soluble in water and in glycerin; sparingly soluble in propylene glycol; insoluble in most organic solvents. *NF category:* Antimicrobial preservative.

**Imipenem:** White to tan-colored crystalline powder. Slightly soluble in water and in methanol.

**Imipramine Hydrochloride:** White to pale yellow powder. Freely soluble in water and in alcohol; insoluble in ether and in benzene.

**Imipramine Pamoate:** Fine, yellow powder. Soluble in acetone, in chloroform, in carbon tetrachloride, in ethanol, and in ether; insoluble in water.

**Imiquimod:** White or almost white, powder or crystalline powder. Very slightly soluble in methanol; practically insoluble in water and in acetone.

**Inamrinone:** Pale yellow to tan powder. It is odorless or has a faint odor. Slightly soluble in methanol; practically insoluble or insoluble in chloroform and in water.

**Indapamide:** White to off-white, crystalline powder. Melts between 167° and 170°. Soluble in methanol, in alcohol, in acetonitrile, in glacial acetic acid, and in ethyl acetate; very slightly soluble in ether and in chloroform; practically insoluble in water.

**Indigotindisulfonate Sodium:** Dusky, purplish-blue powder, or blue granules having a coppery luster. Is affected by light. Its solutions have a blue or bluish purple color. Slightly soluble in water and in alcohol; practically insoluble in most other organic solvents.

**Indinavir Sulfate:** White or almost white, hygroscopic powder. Freely soluble in water; soluble in methanol; practically insoluble in heptane.

**Indocyanine Green:** Olive-brown, dark green, blue-green, dark blue, or black powder. Is odorless or has a slight odor. Its solutions are deep emerald-green in color. The pH of a solution (1 in 200) is about 6. Its aqueous solutions are stable for about 8 hours. Soluble in water and in methanol; practically insoluble in most other organic solvents.

**Indomethacin:** Pale yellow to yellow-tan, crystalline powder, having not more than a slight odor. Is sensitive to light. Melts at about 162°. Exhibits polymorphism. Sparingly soluble in alcohol, in chloroform, and in ether; practically insoluble in water.

**Inositol:** White or almost white, crystalline powder. Very soluble in water; practically insoluble in alcohol absolute and in ether. *NF category:* Humectant.

**Insulin:** White or practically white crystals. Soluble in solutions of dilute acids and alkalies.

**Insulin Injection:** The Injection containing, in each mL, not more than 100 USP Units is a clear, colorless or almost colorless liquid; the Injection containing, in each mL, 500 Units may be straw-colored. Contains between 0.1% and 0.25% (w/v) of either phenol or cresol. Contains between 1.4% and 1.8% (w/v) of glycerin.

**Insulin Lispro:** White or practically white crystals. Soluble in solutions of dilute acids and alkalies.

**Isophane Insulin Suspension:** White suspension of rod-shaped crystals, free from large aggregates of crystals following moderate agitation. Contains either (1) between 1.4% and 1.8% (w/v) of glycerin, between 0.15% and 0.17% (w/v) of metacresol, and between 0.06% and 0.07% (w/v) of phenol; or (2) between 1.4% and 1.8% (w/v) of glycerin and between 0.20% and 0.25% (w/v) of phenol. Contains between 0.15% and 0.25% (w/v) of dibasic sodium phosphate. When examined microscopically, the insoluble matter in the Suspension is crystalline, and contains not more than traces of amorphous material.

**Insulin Zinc Suspension:** Practically colorless suspension of a mixture of characteristic crystals predominantly between 10 and 40 µm in maximum dimension and many particles that have no uniform shape and do not exceed 2 µm in maximum dimension. Contains between 0.15% and 0.17% (w/v) of sodium acetate, between 0.65% and 0.75% (w/v) of sodium chloride, and between 0.09% and 0.11% (w/v) of methylparaben.

**Extended Insulin Zinc Suspension:** Practically colorless suspension of a mixture of characteristic crystals the maximum dimension of which is predominantly between 10 and 40 µm. Contains between 0.15% and 0.17% (w/v) of sodium acetate, between 0.65% and 0.75% (w/v) of sodium chloride, and between 0.09% and 0.11% (w/v) of methylparaben.

**Prompt Insulin Zinc Suspension:** Practically colorless suspension of particles that have no uniform shape and the maximum dimension of which does not exceed 2 µm. Contains between 0.15% and 0.17% (w/v) of sodium acetate, between 0.65% and 0.75% (w/v) of sodium chloride, and between 0.09% and 0.11% (w/v) of methylparaben.

**Inulin:** White, friable, chalk-like, amorphous, odorless, tasteless powder. Soluble in hot water; slightly soluble in cold water and in organic solvents. *NF category:* Sweetening agent; wet binder.

**Invert Sugar:** Colorless to pale yellow liquid. Miscible with water, with glycerin, and with glycols. *NF category*: Sweetening agent; diluent; wet binder.

**Iodine:** Heavy, grayish-black plates or granules, having a metallic luster and a characteristic odor. Freely soluble in carbon disulfide, in chloroform, in carbon tetrachloride, and in ether; soluble in alcohol and in solutions of iodides; sparingly soluble in glycerin; very slightly soluble in water.

**Iodine Topical Solution:** Transparent, reddish-brown liquid, having the odor of iodine.

**Strong Iodine Solution:** Transparent liquid having a deep brown color and having the odor of iodine.

**Iodine Tincture:** Transparent liquid having a reddish-brown color and the odor of iodine and of alcohol.

**Sodium Iodide I 123 Capsules:** Capsules may contain a small amount of solid or solids, or may appear empty.

**Sodium Iodide I 123 Solution:** Clear, colorless solution. Upon standing, both the Solution and the glass container may darken as a result of the effects of the radiation.

**Iodinated I 125 Albumin Injection:** Clear, colorless to slightly yellow solution. Upon standing, both the Albumin and the glass container may darken as a result of the effects of the radiation.

**Iodinated I 131 Albumin Injection:** Clear, colorless to slightly yellow solution. Upon standing, both the Albumin and the glass container may darken as a result of the effects of the radiation.

**Sodium Iodide I 131 Capsules:** May contain a small amount of solid or solids, or may appear empty.

**Sodium Iodide I 131 Solution:** Clear, colorless solution. Upon standing, both the Solution and the glass container may darken as a result of the effects of the radiation.

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**Iodipamide:** White, practically odorless, crystalline powder. Slightly soluble in alcohol; very slightly soluble in water, in chloroform, and in ether.

**Iodipamide Meglumine Injection:** Clear, colorless to pale yellow, slightly viscous liquid.

**Iodixanol:** White to off-white, amorphous, odorless, hygroscopic powder. Freely soluble in water.

**Iodoform:** Lustrous greenish yellow powder, or lustrous crystals. It is slightly volatile even at ordinary temperatures, and distills slowly with steam. Freely soluble in ether and in chloroform; soluble in boiling alcohol; sparingly soluble in alcohol, in glycerin, and in olive oil; practically insoluble in water. Melts to a brown liquid at about 115°, and decomposes at a higher temperature, emitting vapors of iodine.

**Iodoquinol:** Light yellowish to tan, microcrystalline powder not readily wetted by water. Is odorless or has a faint odor; is stable in air. Melts with decomposition. Sparingly soluble in alcohol and in ether; practically insoluble in water.

**Iohexol:** White to off-white, hygroscopic, odorless powder. Very soluble in water and in methanol; practically insoluble or insoluble in ether and in chloroform.

**Iohexol Injection:** Clear, colorless to pale yellow liquid.

**Iopamidol:** Practically odorless, white to off-white powder. Very soluble in water; sparingly soluble in methanol; practically insoluble in alcohol and in chloroform.

**Iopromide:** White to slightly yellow powder. Freely soluble in water and in dimethyl sulfoxide; practically insoluble in alcohol, in acetone, and in ether.

**Iothalamate Meglumine Injection:** Clear, colorless to pale yellow, slightly viscous liquid.

**Iothalamate Meglumine and Iothalamate Sodium Injection:** Clear, colorless to pale yellow, slightly viscous liquid.

**Iothalamic Acid:** White, odorless powder. Soluble in solutions of alkali hydroxides; slightly soluble in water and in alcohol.

**Ioxilan:** White to off-white, practically odorless powder. Soluble in water and in methanol.

**Ioxilan Injection:** Clear, colorless to pale yellow liquid.

**Powdered Ipecac:** Pale brown, weak yellow, or light olive-gray powder.

**Ipratropium Bromide:** White to off-white, crystalline powder. Freely soluble in methanol; soluble in water; slightly soluble in alcohol.

**Irbesartan:** White to off-white, crystalline powder. Slightly soluble in alcohol and in methylene chloride; practically insoluble in water.

**Irinotecan Hydrochloride:** Pale yellow to yellow crystalline powder. Slightly soluble in water and in organic solvents.

**Iron Dextran Injection:** Dark brown, slightly viscous liquid.

**Isobutane:** Colorless, flammable gas (boiling temperature is about –11°). Vapor pressure at 21° is about 2950 mm of mercury (31 psig). *NF category:* Propellant.

**Isobutyl Alcohol:** Clear, colorless, mobile liquid. Soluble in water. Miscible with alcohol, with ether, and with many other organic solvents. *NF category*: Flavors and fragrance; solvent.

**Isoflurane:** Clear, colorless, volatile liquid, having a slight odor. Boils at about 49°. Insoluble in water. Miscible with common organic solvents and with fats and oils.

**Isoleucine:** White, practically odorless crystals, having a slightly bitter taste. Soluble in water; slightly soluble in hot alcohol; insoluble in ether.

**Isometheptene Mucate:** White, crystalline powder. Freely soluble in water; soluble in alcohol; practically insoluble in chloroform and in ether.

**Isoniazid:** Colorless or white crystals or white, crystalline powder. Is odorless and is slowly affected by exposure to air and light. Freely soluble in water; sparingly soluble in alcohol; slightly soluble in chloroform; very slightly soluble in ether.

**Isoniazid Injection:** Clear, colorless to faintly greenish-yellow liquid. Gradually darkens on exposure to air and light. Tends to crystallize at low temperatures.

**Isopropamide Iodide:** White to pale yellow, crystalline powder, having a bitter taste. Freely soluble in chloroform and in alcohol; sparingly soluble in water; very slightly soluble in benzene and in ether.

**Isopropyl Alcohol:** Transparent, colorless, mobile, volatile liquid, having a characteristic odor and a slightly bitter taste. Is flammable. Miscible with water, with alcohol, with ether, and with chloroform. *NF category:* Solvent.

**Azeotropic Isopropyl Alcohol:** Transparent, colorless, mobile, volatile liquid, having a characteristic odor and a slightly bitter taste. Is flammable. Miscible with water, with alcohol, with ether, and with chloroform.

**Isopropyl Isostearate:** Clear, colorless to pale-yellow liquid. Miscible with alcohol and with fatty oils. Insoluble in water. *NF category*: Emollient; emulsifying agent.

**Isopropyl Myristate:** Clear, practically colorless, oily liquid. Is practically odorless, and congeals at about 5°. Freely soluble in 90% alcohol; insoluble in water, in glycerin, and in propylene glycol. Miscible with most organic solvents and with fixed oils. *NF category:* Vehicle (oleaginous); emollient; solvent.

**Isopropyl Palmitate:** Colorless, mobile liquid having a very slight odor. Soluble in acetone, in castor oil, in chloroform, in cottonseed oil, in ethyl acetate, in alcohol, and in mineral oil; insoluble in water, in glycerin, and in propylene glycol. *NF category:* Vehicle (oleaginous); emollient; solvent.

**Isoproterenol Hydrochloride:** White to practically white, odorless, crystalline powder, having a slightly bitter taste. Gradually darkens on exposure to air and light. Its solutions become pink to brownish pink on standing exposed to air, doing so almost immediately when rendered alkaline. Its solution (1 in 100) has a pH of about 5. Freely soluble in water; sparingly soluble in alcohol and less soluble in dehydrated alcohol; insoluble in chloroform and in ether.

**Isoproterenol Hydrochloride Injection:** Colorless or practically colorless liquid, gradually turning dark on exposure to air and light.

**Isosorbide Concentrate:** Colorless to slightly yellow liquid. Soluble in water and in alcohol.

**Diluted Isosorbide Dinitrate:** Ivory-white, odorless powder. [NOTE—Undiluted isosorbide dinitrate occurs as white, crystalline rosettes.] Undiluted isosorbide dinitrate is very soluble in acetone; freely soluble in chloroform; sparingly soluble in alcohol; very slightly soluble in water.

**Isostearyl Isostearate:** Colorless to slightly yellow, oily liquid. *Specific Gravity* ⟨841⟩ : 0.840–0.880 at 20°. Soluble in methylene chloride and in mineral oil; insoluble in water and in alcohol. *NF category*: Emollient; emulsifying agent.

**Isotretinoin:** Yellow crystals. Soluble in chloroform; sparingly soluble in alcohol, in isopropyl alcohol, and in polyethylene glycol 400; practically insoluble in water.

**Isoxsuprine Hydrochloride:** White, odorless, crystalline powder, having a bitter taste. Melts at about 200°, with decomposition. Sparingly soluble in alcohol; slightly soluble in water.

**Isradipine:** Yellow, fine crystalline powder.

**Itraconazole:** A white or almost white powder. Freely soluble in methylene chloride; sparingly soluble in tetrahydrofuran; very slightly soluble in alcohol; practically insoluble in water.

**Ivermectin:** White to yellowish-white, crystalline powder. Slightly hygroscopic. Freely soluble in methanol and in methylene chloride; soluble in acetone and in acetonitrile; practically insoluble in hexane and in water.

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**Ixabepilone:** White or almost white powder. Slightly soluble in acetonitrile.

**Juniper Tar:** Dark brown, clear, thick liquid, having a tarry odor and a faintly aromatic, bitter taste. Sparingly soluble in solvent hexane; very slightly soluble in water. One volume dissolves in 9 volumes of alcohol. Dissolves in 3 volumes of ether, leaving only a slight, flocculent residue. Miscible with amyl alcohol, with chloroform, and with glacial acetic acid.

**Kanamycin Sulfate:** White, odorless, crystalline powder. Freely soluble in water; insoluble in acetone, in ethyl acetate, and in benzene.

**Kaolin:** Soft, white or yellowish-white powder or lumps. Has an earthy or clay-like taste and, when moistened with water, assumes a darker color and develops a marked clay-like odor. Insoluble in water, in cold dilute acids, and in solutions of alkali hydroxides. *NF category:* Diluent; suspending and/or viscosity-increasing agent.

**Ketamine Hydrochloride:** White, crystalline powder, having a slight, characteristic odor. Freely soluble in water and in methanol; soluble in alcohol; sparingly soluble in chloroform.

**Ketorolac Tromethamine:** White to off-white, crystalline powder. Melts between 165° and 170°, with decomposition. Freely soluble in water and in methanol; slightly soluble in alcohol, in dehydrated alcohol, and in tetrahydrofuran; practically insoluble in acetone, in dichloromethane, in toluene, in ethyl acetate, in dioxane, in hexane, in butyl alcohol, and in acetonitrile.

**Labetalol Hydrochloride:** White to off-white powder. Melts at about 180°, with decomposition. Soluble in water and in alcohol; insoluble in ether and in chloroform.

**Lacosamide:** White to light yellow powder. Freely soluble in methanol; soluble in anhydrous ethanol; sparingly soluble in water; slightly soluble in acetonitrile; and practically insoluble in heptane.

**Alpha-Lactalbumin:** Free-flowing, slightly hygroscopic light cream-colored powder. Freely soluble in water; soluble in wide pH ranges; insoluble in methanol, in alcohol, in ether, and in acetone. *NF category:* Bulking agent; coating agent; emulsifying agent; stiffening agent; suspending and/or viscosity-increasing agent; diluent; wet binder; vehicle (solid carrier); pH modifier (acidifying agent/alkalizing agent/buffering agent); chelating and/or complexing agent.

**Lactic Acid:** Colorless or yellowish, practically odorless, syrupy liquid. Is hygroscopic. When it is concentrated by boiling, lactic acid lactate is formed. Specific gravity is about 1.20. Insoluble in chloroform. Miscible with water, with alcohol, and with ether. *NF category:* pH modifier (acidifying agent/alkalizing agent/buffering agent).

**Lactitol:** A white or light brown, odorless crystal. Has a mild, sweet taste, and no aftertaste. *NF category:* Flavors and fragrance; diluent; sweetening agent.

**Lactobionic Acid:** White or almost white, crystalline powder with a melting point of about 125° with decomposition. Freely soluble in water; slightly soluble in glacial acetic acid, in anhydrous ethanol, and in methanol. *NF category:* Antioxidant.

**Anhydrous Lactose:** White or almost white powder. Freely soluble in water; practically insoluble in alcohol. *NF category:* Diluent; carrier.

**Lactose Monohydrate:** White, free-flowing powder. Freely but slowly soluble in water; practically insoluble in alcohol. *NF category:* Diluent; carrier; wet binder.

**Lactulose Concentrate:** Colorless to amber syrupy liquid, which may exhibit some precipitation and darkening upon standing. Miscible with water.

**Lamivudine:** White to off-white solid. Soluble in water. Melts at about 176°.

**Lamotrigine:** White to pale cream-colored powder. Slightly soluble in 0.1 N hydrochloric acid, in acetone, and in methanol; very slightly soluble in water.

**Lanolin:** Yellow, tenacious, unctuous mass, having a slight, characteristic odor. Freely soluble in ether and in chloroform; soluble in hot alcohol; sparingly soluble in cold alcohol; insoluble in water, but mixes without separation with about twice its weight of water. *NF category:* Emulsifying agent; ointment base.

**Hydrogenated Lanolin:** White or pale yellow, unctuous substance. Soluble in boiling dehydrated alcohol and in light petroleum; insoluble in water. *NF category*: Emulsifying agent; emollient; humectant; ointment base.

**Lanolin Alcohols:** Hard, waxy, amber solid, having a characteristic odor. Freely soluble in chloroform, in ether, and in petroleum ether; slightly soluble in alcohol; insoluble in water. *NF category:* Emulsifying agent; ointment base.

**Lansoprazole:** White to brownish-white powder. Freely soluble in dimethylformamide; practically insoluble in water. Melts at about 166°, with decomposition.

**Latanoprost:** Colorless to slightly yellow oil. Very soluble in acetonitrile; freely soluble in acetone, in ethanol, in ethyl acetate, in isopropanol, in methanol, and in octanol; practically insoluble in water.

**Lauric Acid:** White or faintly yellow, somewhat glossy, crystalline solid or powder. Very soluble in alcohol, in ether, and in methanol; soluble in acetone; practically insoluble in water. *NF category*: Antifoaming agent; emulsifying agent; lubricant.

**Lauroyl Polyoxylglycerides:** Pale yellow semi-solids. Freely soluble in methylene chloride. Dispersible in hot water. *NF category*: Ointment base; solvent; emulsifying agent; wetting and/or solubilizing agent.

**Lecithin:** The consistency of both natural grades and refined grades of lecithin may vary from plastic to fluid, depending upon free fatty acid and oil content, and upon the presence or absence of other diluents. Its color varies from light yellow to brown, depending on the source, on crop variations, and on whether it is bleached or unbleached. It is odorless or has a characteristic, slight nut-like odor and a bland taste. Practically insoluble in water, but it readily hydrates to form emulsions. The oil-free phosphatides are soluble in fatty acids, but are practically insoluble in fixed oils. When all phosphatide fractions are present, lecithin is sparingly soluble in alcohol and practically insoluble in acetone. *NF category:* Emulsifying agent; emollient.

**Leflunomide:** White to almost white powder. Freely soluble in methanol, in alcohol, in 2-propanol, in ethyl acetate, in acetone, and in acetonitrile; practically insoluble in water.

**Letrozole:** White to yellowish, crystalline powder. Freely soluble in dichloromethane; slightly soluble in alcohol; practically insoluble in water.

**Leucine:** White, practically odorless, tasteless crystals. Sparingly soluble in water; insoluble in ether. *NF category:* Flavors and fragrance.

**Leucovorin Calcium:** Yellowish-white or yellow powder. Slightly soluble to freely soluble in water; practically insoluble in alcohol.

**Leucovorin Calcium Injection:** Clear, yellowish solution.

**Levamisole Hydrochloride:** White or almost white, crystalline powder. Freely soluble in water; soluble in alcohol; slightly soluble in methylene chloride; practically insoluble in ether.

**Levetiracetam:** White to almost white powder. Very soluble in water; soluble in acetonitrile; practically insoluble in hexane.

**Levmetamfetamine:** Clear, practically colorless liquid.

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**Levobunolol Hydrochloride:** White crystalline, odorless powder. Soluble in water and in methanol; slightly soluble in alcohol and in chloroform.

**Levocarnitine:** White crystals or crystalline powder. Hygroscopic. Freely soluble in water, and in hot alcohol; practically insoluble in acetone, in ether, and in benzene.

**Levocetirizine Dihydrochloride:** White to almost white powder. Freely soluble in water and in methanol.

**Levodopa:** White to off-white, odorless, crystalline powder. In the presence of moisture, is rapidly oxidized by atmospheric oxygen and darkens. Freely soluble in 3 N hydrochloric acid; slightly soluble in water; insoluble in alcohol.

**Levofloxacin:** Light yellowish-white to yellow-white crystals or crystalline powder. Soluble in dimethylsulfoxide and in acetic acid; sparingly soluble in water, in acetone, and in methanol; practically insoluble in glycerin and in *n*-octanol.

**Levonordefrin:** White to buff-colored, odorless, crystalline solid. Melts at about 210°. Freely soluble in aqueous solutions of mineral acids; slightly soluble in acetone, in chloroform, in alcohol, and in ether; practically insoluble in water.

**Levonorgestrel:** White or practically white, odorless powder. Soluble in chloroform; slightly soluble in alcohol; practically insoluble in water.

**Levorphanol Tartrate:** Practically white, odorless, crystalline powder. Sparingly soluble in water; slightly soluble in alcohol; insoluble in chloroform and in ether. Melts, in a sealed tube, at about 110°, with decomposition.

**Levothyroxine Sodium:** Light yellow to buff-colored, odorless, tasteless, hygroscopic powder. Is stable in dry air but may assume a slight pink color upon exposure to light. The pH of a saturated solution is about 8.9. Soluble in solutions of alkali hydroxides and in hot solutions of alkali carbonates; slightly soluble in alcohol; very slightly soluble in water; insoluble in acetone, in chloroform, and in ether.

**Lidocaine:** White or slightly yellow, crystalline powder. Has a characteristic odor and is stable in air. Very soluble in alcohol and in chloroform; freely soluble in benzene and in ether; practically insoluble in water. Dissolves in oils.

**Lidocaine Hydrochloride:** White, odorless, crystalline powder, having a slightly bitter taste. Very soluble in water and in alcohol; soluble in chloroform; insoluble in ether.

**Lincomycin Hydrochloride:** White or practically white, crystalline powder. Is odorless or has a faint odor. Is stable in the presence of air and light. Its solutions are acid and dextrorotatory. Freely soluble in water; soluble in dimethylformamide; very slightly soluble in acetone.

**Lincomycin Hydrochloride Soluble Powder:** White to off-white, or light tan free-flowing, fine powder.

**Lindane:** White, crystalline powder, having a slight, musty odor. Freely soluble in chloroform; soluble in dehydrated alcohol; sparingly soluble in ether; slightly soluble in ethylene glycol; practically insoluble in water.

**Linezolid:** White to off-white or cream-colored, crystalline powder. Sparingly soluble in acetone, in ethanol, and in methanol; very slightly soluble in water.

**Linoleoyl Polyoxylglycerides:** Amber, oily liquids. May develop deposit after prolonged storage periods at 20°. Freely soluble in methylene chloride; practically insoluble but dispersible in water. *NF category:* Ointment base; solvent; emulsifying agent; wetting and/or solubilizing agent.

**Liothyronine Sodium:** Light tan, odorless, crystalline powder. Slightly soluble in alcohol; very slightly soluble in water; practically insoluble in most other organic solvents.

**Lisinopril:** White, crystalline powder. Melts at about 160°, with decomposition. Soluble in water; sparingly soluble in methanol; practically insoluble in alcohol, in acetone, in acetonitrile, and in chloroform.

**Lithium Carbonate:** White, granular, odorless powder. Sparingly soluble in water; very slightly soluble in alcohol. Dissolves, with effervescence, in dilute mineral acids.

**Lithium Citrate:** White, odorless, deliquescent powder or granules, having a cooling, faintly alkaline taste. Freely soluble in water; slightly soluble in alcohol.

**Lomustine:** Yellow crystalline powder. Freely soluble in acetone, and in methylene chloride; soluble in ethanol; practically insoluble in water.

**Loperamide Hydrochloride:** White to slightly yellow powder. Melts at about 225°, with some decomposition. Freely soluble in methanol and in chloroform; slightly soluble in water and in dilute acids; very slightly soluble in isopropyl alcohol.

**Lopinavir:** White powder. Freely soluble in methanol and alcohol; soluble in isopropanol; practically insoluble in water.

**Loratadine:** White to off-white powder. Freely soluble in acetone, in chloroform, in methanol, and in toluene; insoluble in water.

**Lorazepam:** White or practically white, practically odorless powder. Sparingly soluble in alcohol; slightly soluble in chloroform; insoluble in water.

**Losartan Potassium:** White to off-white powder. Freely soluble in water; sparingly soluble in isopropyl alcohol; slightly soluble in acetonitrile.

**Lovastatin:** White to off-white, crystalline powder. Freely soluble in chloroform; soluble in acetone, in acetonitrile, and in methanol; sparingly soluble in alcohol; practically insoluble in hexane; insoluble in water.

**Loxapine Succinate:** White to yellowish, crystalline powder. Is odorless.

**Lufenuron:** White or pale yellow powder. Freely soluble in acetonitrile; soluble in dehydrated alcohol; practically insoluble in water.

**Lumefantrine:** Yellow crystalline powder. Freely soluble in *N,N*-dimethylformamide, in chloroform, and in ethyl acetate; soluble in dichloromethane; slightly soluble in ethanol and in methanol; practically insoluble in water.

**Lutein:** Red, crystalline powder. Soluble in ethanol, in ethyl acetate, and in methylene chloride; partially soluble in hexane.

**Lysine Acetate:** White, odorless crystals or crystalline powder, having an acid taste. Freely soluble in water.

**Lysine Hydrochloride:** White, odorless powder. Freely soluble in water. *NF category:* pH modifier (acidifying agent/alkalizing agent/buffering agent).

**Mafenide Acetate:** White to pale yellow, crystalline powder. Freely soluble in water.

**Magaldrate:** White, odorless, crystalline powder. Soluble in dilute solutions of mineral acids; insoluble in water and in alcohol.

**Milk of Magnesia:** White, opaque, more or less viscous suspension from which varying proportions of water usually separate on standing. pH is about 10.

**Magnesium Aluminometasilicate:** White powder or granules with an amorphous structure. Very slightly soluble in acids; practically insoluble or insoluble in water, in alcohol, and in alkalies.

**Magnesium Aluminosilicate:** White powder or granules with an amorphous structure. Very slightly soluble in acids; practically insoluble or insoluble in water, in alcohol, and in alkalies.

**Magnesium Aluminum Silicate:** Odorless, tasteless, fine (micronized) powder, small cream to tan granules, or small flakes that are creamy when viewed on their flat surfaces and tan to brown when viewed on their edges. Insoluble in water and in alcohol. Swells when added to water or glycerin. *NF category:* Suspending and/or viscosity-increasing agent; disintegrant.

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**Magnesium Carbonate:** Light, white, friable masses or bulky, white powder. Is odorless, and is stable in air. Practically insoluble in water to which, however, it imparts a slightly alkaline reaction; insoluble in alcohol, but is dissolved by dilute acids with effervescence. *NF category:* Diluent.

**Magnesium Chloride:** Colorless, odorless, deliquescent flakes or crystals, which lose water when heated to 100° and lose hydrochloric acid when heated to 110°. Very soluble in water; freely soluble in alcohol.

**Magnesium Citrate Oral Solution:** Colorless to slightly yellow, clear, effervescent liquid, having a sweet, acidulous taste and a lemon flavor.

**Magnesium Gluconate:** Colorless crystals or white powder or granules. Is odorless and tasteless. Freely soluble in water; very slightly soluble in alcohol; insoluble in ether.

**Magnesium Hydroxide:** Bulky, white powder. Soluble in dilute acids; practically insoluble in water and in alcohol.

**Magnesium Oxide:** Very bulky, white powder or relatively dense, white powder or granulated powder. Soluble in dilute acids; practically insoluble in water; insoluble in alcohol. *NF category:* Emulsifying agent; glidant and/or anticaking agent; diluent.

**Magnesium Phosphate:** White, odorless, tasteless powder. Soluble in diluted mineral acids; practically insoluble in water.

**Magnesium Salicylate:** White, odorless, efflorescent, crystalline powder. Freely soluble in methanol; soluble in alcohol and in water; slightly soluble in ether.

**Magnesium Silicate:** Fine, white, odorless, tasteless powder, free from grittiness. Insoluble in water and in alcohol. Is readily decomposed by mineral acids. *NF category:* Glidant and/or anticaking agent.

**Magnesium Stearate:** Very fine, light, white powder, slippery to touch. Insoluble in water, in alcohol, and in ether. *NF category:* Lubricant.

**Magnesium Sulfate:** Small, colorless crystals, usually needle-like, with a cooling, saline, bitter taste. It effloresces in warm, dry air. Very soluble in boiling water; freely soluble in water; freely (and slowly) soluble in glycerin; sparingly soluble in alcohol.

**Magnesium Trisilicate:** Fine, white, odorless, tasteless powder, free from grittiness. Insoluble in water and in alcohol. Is readily decomposed by mineral acids. *NF category:* Glidant and/or anticaking agent.

**Malathion:** Clear, colorless, or slightly yellowish liquid, having a characteristic odor. Congeals at about 2.9°. Slightly soluble in water. Miscible with alcohols, with esters, with ketones, with ethers, with aromatic and alkylated aromatic hydrocarbons, and with vegetable oils.

**Maleic Acid:** White, crystalline powder. Freely soluble in water and in alcohol; sparingly soluble in ether. *NF category:* pH modifier (acidifying agent/alkalizing agent/buffering agent).

**Malic Acid:** White or practically white, crystalline powder or granules, having a strongly acid taste. Melts at about 130°. Very soluble in water; freely soluble in alcohol. *NF category:* pH modifier (acidifying agent/alkalizing agent/buffering agent); flavors and fragrance; antioxidant, chelating and/or complexing agent.

**Maltitol:** White, crystalline powder. Very soluble in water; practically insoluble in ethanol. *NF category:* Humectant; sweetening agent; diluent; coating agent.

**Maltodextrin:** White, hygroscopic powder or granules. Freely soluble or readily dispersible in water; slightly soluble to insoluble in anhydrous alcohol. *NF category:* Coating agent; suspending and/or viscosity-increasing agent; diluent; wet binder.

**Maltol:** A white, crystalline powder having a characteristic caramel-butterscotch odor, suggestive of a fruity-strawberry aroma in dilute solution. One g dissolves in about 82 mL of water, in 21 mL of alcohol, in 80 mL of glycerin, and in 28 mL of propylene glycol. *NF category:* Flavors and fragrance.

**Maltose:** Maltose occurs in either the anhydrous state or as a monohydrate. It is a white, crystalline powder, odorless, and has a sweet taste. Very slightly soluble in ethanol; freely soluble in water; slightly soluble in methanol; practically insoluble in ether. *NF category:* Sweetening agent; diluent; disintegrant; wet binder.

**Mandelic Acid:** White to light yellow powder or crystalline solid. Soluble in alcohol, in isopropyl alcohol; sparingly soluble in water. *NF category*: Antimicrobial preservative.

**Manganese Chloride:** Large, irregular, pink, odorless, translucent crystals. Soluble in water and in alcohol; insoluble in ether.

**Manganese Sulfate:** Pale red, slightly efflorescent crystals, or purple, odorless powder. Soluble in water; insoluble in alcohol.

**Mannitol:** White, crystalline powder or free-flowing granules. Is odorless and has a sweet taste. Freely soluble in water; soluble in alkaline solutions; slightly soluble in pyridine; very slightly soluble in alcohol; practically insoluble in ether. *NF category:* Sweetening agent; tonicity agent; bulking agent; diluent; plasticizer.

**Maprotiline Hydrochloride:** Fine, white to off-white, crystalline powder. Is practically odorless. Freely soluble in methanol and in chloroform; slightly soluble in water; practically insoluble in isooctane.

**Mebendazole:** White to slightly yellow powder. Is almost odorless. Melts at about 290°. Freely soluble in formic acid; practically insoluble in water, in dilute solutions of mineral acids, in alcohol, in ether, and in chloroform.

**Mechlorethamine Hydrochloride:** White, crystalline powder. Is hygroscopic.

**Meclizine Hydrochloride:** White or slightly yellowish, crystalline powder. Has a slight odor and is tasteless. Slightly soluble in dilute acids and in alcohol; practically insoluble in water and in ether; freely soluble in chloroform, in pyridine, and in acid-alcohol-water mixtures.

**Meclofenamate Sodium:** A white to creamy white, odorless to almost odorless, crystalline powder. Freely soluble in water, the solution sometimes being somewhat turbid due to partial hydrolysis and absorption of carbon dioxide; soluble in methanol; slightly soluble in chloroform; practically insoluble in ether. The solution is clear above pH 11.5.

**Medroxyprogesterone Acetate:** White to off-white, odorless, crystalline powder. Melts at about 205°. Is stable in air. Freely soluble in chloroform; soluble in acetone and in dioxane; sparingly soluble in alcohol and in methanol; slightly soluble in ether; insoluble in water.

**Mefenamic Acid:** White to off-white, crystalline powder. Melts at about 230°, with decomposition. Soluble in solutions of alkali hydroxides; sparingly soluble in chloroform; slightly soluble in alcohol and in methanol; practically insoluble in water.

**Mefloquine Hydrochloride:** White or slightly yellow, crystalline powder. It exhibits polymorphism. Freely soluble in methanol; soluble in alcohol; very slightly soluble in water.

**Megestrol Acetate:** White to creamy white, tasteless and essentially odorless, crystalline powder. Very soluble in chloroform; soluble in acetone; sparingly soluble in alcohol; slightly soluble in ether and in fixed oils; insoluble in water. Is unstable under aqueous conditions at pH 7 or above.

**Meglumine:** White to faintly yellowish-white, odorless crystals or powder. Freely soluble in water; sparingly soluble in alcohol.

**Melengestrol Acetate:** White to light yellow, crystalline powder. Freely soluble in chloroform and in ethyl acetate; slightly soluble in alcohol; insoluble in water.

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**Meloxicam:** Pale yellow powder. Soluble in dimethylformamide; slightly soluble in acetone; very slightly soluble in methanol and in alcohol; practically insoluble in water.

**Melphalan:** Off-white to buff powder, having a faint odor. Melts at about 180°, with decomposition. Soluble in dilute mineral acids; slightly soluble in alcohol and in methanol; practically insoluble in water, in chloroform, and in ether.

**Memantine Hydrochloride:** White to off-white, colored powder. Slightly soluble in water.

**Menadione:** Bright yellow, crystalline, practically odorless powder. Is affected by sunlight. Soluble in vegetable oils; sparingly soluble in chloroform and in alcohol; practically insoluble in water.

**Menthol:** Colorless, hexagonal crystals, usually needle-like, or in fused masses, or crystalline powder. Has a pleasant, peppermint-like odor. Very soluble in alcohol, in chloroform, in ether, and in solvent hexane; freely soluble in glacial acetic acid, in mineral oil, and in fixed and volatile oils; slightly soluble in water. *NF category:* Flavors and fragrance.

**Meperidine Hydrochloride:** Fine, white, crystalline, odorless powder. The pH of a solution (1 in 20) is about 5. Very soluble in water; soluble in alcohol; sparingly soluble in ether.

**Mephobarbital:** White, odorless, crystalline powder, having a bitter taste. Its saturated solution is acid to litmus. Soluble in chloroform and in solutions of fixed alkali hydroxides and carbonates; slightly soluble in water, in alcohol, and in ether.

**Mepivacaine Hydrochloride:** White, odorless, crystalline solid. The pH of a solution (1 in 50) is about 4.5. Freely soluble in water and in methanol; very slightly soluble in chloroform; practically insoluble in ether.

**Meprobamate:** White powder, having a characteristic odor and a bitter taste. Freely soluble in acetone and in alcohol; slightly soluble in water; practically insoluble or insoluble in ether.

**Mercaptopurine:** Yellow, odorless or practically odorless, crystalline powder. Melts at a temperature exceeding 308°, with decomposition. Soluble in hot alcohol and in dilute alkali solutions; slightly soluble in 2 N sulfuric acid; insoluble in water, in acetone, and in ether.

**Ammoniated Mercury:** White, pulverulent pieces or white, amorphous powder. Is odorless, and is stable in air, but darkens on exposure to light. Readily soluble in warm hydrochloric, nitric, and acetic acids; insoluble in water, and in alcohol.

**Meropenem:** Colorless to white or light yellow crystals or crystalline powder. Soluble in dimethylformamide and in 5% dibasic potassium phosphate solution; sparingly soluble in water and in 5% monobasic potassium phosphate solution; practically insoluble in alcohol, in acetone, in methylene chloride, and in ether.

**Mesalamine:** Light tan to pink colored, needle-shaped crystals. Color may darken on exposure to air. Is odorless or may have a slight characteristic odor. Soluble in dilute hydrochloric acid and in dilute alkali hydroxides; slightly soluble in water; very slightly soluble in methanol, in dehydrated alcohol, and in acetone; practically insoluble in *n*-butyl alcohol, in chloroform, in ether, in ethyl acetate, in *n*-hexane, in methylene chloride, and in *n*-propyl alcohol.

**Mesna:** White or slightly yellow crystalline powder; hygroscopic. Freely soluble in water; slightly soluble in alcohol; practically insoluble in cyclohexane.

**Mestranol:** White to creamy white, odorless, crystalline powder. Freely soluble in chloroform; soluble in dioxane; sparingly soluble in dehydrated alcohol; slightly soluble in methanol; insoluble in water.

**Metaproterenol Sulfate:** White to off-white, crystalline powder. Freely soluble in water.

**Metaxalone:** White to almost-white, crystalline powder. Freely soluble in chloroform; soluble in methanol and in alcohol; practically insoluble in ether and in water.

**Metformin Hydrochloride:** White, crystalline powder. Freely soluble in water; slightly soluble in alcohol; practically insoluble in acetone and in methylene chloride.

**Methacholine Chloride:** Colorless or white crystals, or white, crystalline powder. Is odorless or has a slight odor, and is very hygroscopic. Its solutions are neutral to litmus. Very soluble in water; freely soluble in alcohol and in chloroform.

**Methacrylic Acid and Ethyl Acrylate Copolymer Dispersion:** Milky-white liquid of low viscosity. It is miscible with water in any proportion; the milky-white appearance is retained. A clear or slightly opalescent, viscous solution is obtained on mixing one part with five parts of acetone, alcohol, or isopropyl alcohol; the polymer substance is first precipitated, but then dissolves in the excess organic solvent. A clear or slightly opalescent, viscous solution is obtained on mixing one part with two parts of 1 N sodium hydroxide. *NF category:* Coating agent; film-forming agent; diluent; wet binder.

**Methacrylic Acid and Ethyl Acrylate Copolymer:** White powder having a faint, characteristic odor. Soluble to freely soluble in methanol, in alcohol, in isopropyl alcohol, and in acetone, each of which contains not less than 3% of water; soluble in diluted alkali, in simulated intestinal fluid TS, and in buffer solutions of pH 7 and above; insoluble in water, in diluted acids, in simulated gastric fluid TS, and in buffer solutions of up to pH 5. The solubility between pH 5.5 and pH 7 depends on the content of methacrylic acid units in the copolymer. *NF category:* Coating agent; film-forming agent.

**Partially-Neutralized Methacrylic Acid and Ethyl Acrylate Copolymer:** White or almost white, free-flowing powder. Freely soluble in alcohol, in methanol, and in a 40 g/L solution of sodium hydroxide; soluble in solutions at pH values above pH 5.5 under salt formation; practically insoluble in ethyl acetate and in acidic aqueous solutions. *NF category:* Coating agent; film-forming agent.

**Methacrylic Acid and Methyl Methacrylate Copolymer:** White powder having a faint, characteristic odor. Soluble to freely soluble in methanol, in alcohol, in isopropyl alcohol, and in acetone, each of which contains not less than 3% of water; soluble in diluted alkali, in simulated intestinal fluid TS, and in buffer solutions of pH 7 and above; insoluble in water, in diluted acids, in simulated gastric fluid TS, and in buffer solutions of up to pH 5. The solubility between pH 5.5 and pH 7 depends on the content of methacrylic acid units in the copolymer. *NF category:* Coating agent; film-forming agent.

**Methacycline Hydrochloride:** Yellow to dark yellow, crystalline powder. Soluble in water.

**Methadone Hydrochloride:** Colorless crystals or white, crystalline, odorless powder. Freely soluble in alcohol and in chloroform; soluble in water; practically insoluble in ether and in glycerin.

**Methadone Hydrochloride Oral Concentrate:** Clear to slightly hazy, syrupy liquid.

**Methamphetamine Hydrochloride:** White crystals or white, crystalline powder. Is odorless or practically so. Its solutions have a pH of about 6. Freely soluble in water, in alcohol, and in chloroform; very slightly soluble in absolute ether.

**Methazolamide:** White or faintly yellow, crystalline powder having a slight odor. Melts at about 213°. Soluble in dimethylformamide; slightly soluble in acetone; very slightly soluble in water and in alcohol.

**Methenamine:** Colorless, lustrous crystals or white, crystalline powder. Is practically odorless. When brought into contact with fire, it readily ignites, burning with a smokeless flame. It sublimes at about 260°, without melting. Its solutions

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are alkaline to litmus. Freely soluble in water; soluble in alcohol and in chloroform.

**Methenamine Mandelate:** White, crystalline powder. Has a sour taste and is practically odorless. Its solutions have a pH of about 4. Melts at about 127°, with decomposition. Very soluble in water; soluble in alcohol and in chloroform; slightly soluble in ether.

**Methimazole:** White to pale buff, crystalline powder, having a faint, characteristic odor. Its solutions are practically neutral to litmus. Freely soluble in water, in alcohol, and in chloroform; slightly soluble in ether.

**Methionine:** White crystals, having a characteristic odor and taste. Soluble in water, in warm dilute alcohol, and in dilute mineral acids; insoluble in ether, in absolute alcohol, in benzene, and in acetone (L-form). *NF category:* Flavors and fragrance; pH modifier (acidifying agent/alkalizing agent/buffering agent); antioxidant.

**Methocarbamol:** White powder, odorless, or having a slight characteristic odor. Melts at about 94°, or, if previously ground to a fine powder, melts at about 90°. Soluble in alcohol only with heating; sparingly soluble in water and in chloroform; insoluble in benzene and in *n*-hexane.

**Methohexital:** White to faintly yellowish-white, crystalline, odorless powder. Slightly soluble in alcohol, in chloroform, and in dilute alkalies; very slightly soluble in water.

**Methohexital Sodium for Injection:** White to off-white, hygroscopic powder. Is essentially odorless.

**Methotrexate:** Orange-brown, or yellow, crystalline powder. Freely soluble in dilute solutions of alkali hydroxides and carbonates; slightly soluble in 6 N hydrochloric acid; practically insoluble in water, in alcohol, in chloroform, and in ether.

**Methotrimeprazine:** Fine, white, practically odorless, crystalline powder. Melts at about 126°. Freely soluble in chloroform, in ether, and in boiling alcohol; sparingly soluble in methanol and in alcohol at 25°; practically insoluble in water.

**Methoxsalen:** White to cream-colored, fluffy, needle-like crystals. Is odorless. Freely soluble in chloroform; soluble in boiling alcohol, in acetone, in acetic acid, in propylene glycol, and in benzene; sparingly soluble in boiling water and in ether; practically insoluble in water.

**Methoxsalen Topical Solution:** Clear, colorless liquid.

**Methoxyflurane:** Clear, practically colorless, mobile liquid, having a characteristic odor. Boils at about 105°. Miscible with alcohol, with acetone, with chloroform, with ether, and with fixed oils.

**Methsuximide:** White to grayish white, crystalline powder. Is odorless, or has not more than a slight odor. Very soluble in chloroform; freely soluble in alcohol and in ether; slightly soluble in hot water.

**Methyclothiazide:** White or practically white, crystalline powder. Is odorless, or has a slight odor. Freely soluble in acetone and in pyridine; sparingly soluble in methanol; slightly soluble in alcohol; very slightly soluble in water, in chloroform, and in benzene.

**Methyl Acrylate, Methyl Methacrylate and Methacrylic Acid (7:3:1) Copolymer 280000 Dispersion:** Milky-white liquid of low viscosity. It is miscible with water in any proportion; the milky-white appearance is retained. A clear or slightly opalescent, viscous solution is obtained on mixing one part of the dispersion with five parts of acetone, ethanol or isopropyl alcohol; the polymer substance is first precipitated, but then dissolves in the excess organic solvent. A clear or slightly opalescent solution is obtained on mixing one part of the dispersion with two parts of 1 N sodium hydroxide. *NF category*: Coating agent; diluent; film-forming agent; wet binder.

**Methyl Alcohol:** Clear, colorless liquid, having a characteristic odor. Is flammable. Miscible with water, with alcohol, with ether, with benzene, and with most other organic solvents. *NF category:* Solvent.

**Methyl Benzylidene Camphor:** A white, fine crystalline powder. Very soluble in chloroform; freely soluble in alcohol; practically insoluble in water.

**Methyl Isobutyl Ketone:** Transparent, colorless, mobile, volatile liquid, having a faint ketonic and camphoraceous odor. Slightly soluble in water. Miscible with alcohol, with ether, and with benzene. *NF category:* Alcohol denaturant; solvent.

**Methyl Salicylate:** Colorless, yellowish, or reddish liquid, having the characteristic odor and taste of wintergreen. It boils between 219° and 224°, with some decomposition. Soluble in alcohol and in glacial acetic acid; slightly soluble in water. *NF category:* Flavors and fragrance.

**Methylbenzethonium Chloride:** White, hygroscopic crystals, having a mild odor. Its solutions are neutral or slightly alkaline to litmus. Very soluble in water, in alcohol, and in ether; practically insoluble in chloroform.

**Methylcellulose:** White, fibrous powder or granules. Its aqueous suspensions are neutral to litmus. It swells in water and produces a clear to opalescent, viscous, colloidal suspension. Soluble in glacial acetic acid and in a mixture of equal volumes of alcohol and chloroform; insoluble in alcohol, in ether, and in chloroform. *NF category:* Coating agent; suspending and/or viscosity-increasing agent; wet binder; emulsifying agent; film-forming agent; disintegrant.

**Methyldopa:** White to yellowish-white, odorless, fine powder, which may contain friable lumps. Very soluble in 3 N hydrochloric acid; sparingly soluble in water; slightly soluble in alcohol; practically insoluble in ether.

**Methylene Blue:** Dark green or blue crystals or crystalline powder having a bronze-like luster. Is odorless or practically so, and is stable in air. Its solutions in water and in alcohol are deep blue in color. Slightly soluble in water and in alcohol. *NF category:* Free radical scavenger.

**Methylene Chloride:** Clear, colorless, mobile liquid, having an odor resembling that of chloroform. Miscible with alcohol, with ether, and with fixed and volatile oils. *NF category:* Solvent.

**Methylergonovine Maleate:** White to pinkish-tan, microcrystalline powder. Is odorless. Slightly soluble in water and in alcohol; very slightly soluble in chloroform and in ether.

**Methylnaltrexone Bromide:** White to almost-white crystalline powder. Freely soluble in dimethylsulfoxide; soluble in dimethylformamide and in water; slightly soluble in methanol; very slightly soluble in ethanol; practically insoluble in toluene.

**Methylparaben:** White, crystalline powder or colorless crystals. Freely soluble in alcohol and in methanol; slightly soluble in water. *NF category:* Antimicrobial preservative.

**Methylparaben Sodium:** White, hygroscopic powder. Freely soluble in water; sparingly soluble in alcohol; insoluble in fixed oils. *NF category:* Antimicrobial preservative.

**Methylphenidate Hydrochloride:** White, odorless, fine, crystalline powder. Its solutions are acid to litmus. Freely soluble in water and in methanol; soluble in alcohol; slightly soluble in chloroform and in acetone.

**Methylprednisolone:** White to practically white, odorless, crystalline powder. Melts at about 240°, with some decomposition (see *Melting Range or Temperature* ⟨741⟩). Sparingly soluble in alcohol, in dioxane, and in methanol; slightly soluble in acetone and in chloroform; very slightly soluble in ether; practically insoluble in water.

**Methylprednisolone Acetate:** White or practically white, odorless, crystalline powder. Melts at about 225°, with some decomposition (see *Melting Range or Temperature* ⟨741⟩).

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Soluble in dioxane; sparingly soluble in acetone, in alcohol, in chloroform, and in methanol; slightly soluble in ether; practically insoluble in water.

**Methylprednisolone Hemisuccinate:** White or nearly white, odorless or nearly odorless, hygroscopic solid. Freely soluble in alcohol; soluble in acetone; very slightly soluble in water.

**Methylprednisolone Sodium Succinate:** White or nearly white, odorless, hygroscopic, amorphous solid. Very soluble in water and in alcohol; very slightly soluble in acetone; insoluble in chloroform.

**Methylpyrrolidone:** A clear, colorless to very slightly yellow liquid. Miscible with water and with most organic solvents including alcohol, ketones, and aromatic and chlorinated hydrocarbons. Boiling point: about 202°. Refractive index: about 1.469. *NF category:* Solvent.

**Methylsulfonylmethane:** White powder or flake crystal. Melts at about 109°. Freely soluble in water, in methanol, in alcohol, and in acetone; sparingly soluble in ether.

**Methyltestosterone:** White or creamy white crystals or crystalline powder. Is odorless and is stable in air, but is slightly hygroscopic. Is affected by light. Soluble in alcohol, in methanol, in ether, and in other organic solvents; sparingly soluble in vegetable oils; practically insoluble in water.

**Methysergide Maleate:** White to yellowish-white or reddish-white, crystalline powder. Is odorless or has not more than a slight odor. Slightly soluble in water and in alcohol; very slightly soluble in chloroform; practically insoluble in ether.

**Metoclopramide Hydrochloride:** White or practically white, crystalline, odorless or practically odorless powder. Very soluble in water; freely soluble in alcohol; sparingly soluble in chloroform; practically insoluble in ether.

**Metoprolol Succinate:** White to off-white powder. Freely soluble in water; soluble in methanol; sparingly soluble in alcohol; slightly soluble in isopropyl alcohol.

**Metoprolol Tartrate:** White, crystalline powder. Very soluble in water; freely soluble in methylene chloride, in chloroform, and in alcohol; slightly soluble in acetone; insoluble in ether.

**Metronidazole:** White to pale yellow, odorless crystals or crystalline powder. Is stable in air, but darkens on exposure to light. Soluble in dilute hydrochloric acid (1 in 2); sparingly soluble in water and in alcohol; slightly soluble in ether and in chloroform.

**Metronidazole Benzoate:** White to slightly yellow, crystalline powder. Freely soluble in methylene chloride; soluble in acetone; slightly soluble in alcohol; very slightly soluble in ethyl ether; practically insoluble in water.

**Metyrapone:** White to light amber, fine, crystalline powder, having a characteristic odor. Darkens on exposure to light. Soluble in methanol and in chloroform; sparingly soluble in water. It forms water-soluble salts with acids.

**Mexiletine Hydrochloride:** White powder. Freely soluble in dehydrated alcohol and in water; slightly soluble in acetonitrile; practically insoluble in ether. Optically inactive (1 in 20 solution in water).

**Mibolerone:** White to off-white powder. Slightly soluble in chloroform, in dioxane, and in methylene chloride; practically insoluble in water (0.0454 mg per mL at 37°).

**Micafungin Sodium:** White to almost white amorphous powder. Freely soluble in water, in isotonic sodium chloride, in *N,N*-dimethylformamide, and in dimethyl sulfoxide; slightly soluble in methyl alcohol; practically insoluble in acetonitrile, in alcohol, in acetone, in diethyl ether, and in *n*-hexane.

**Miconazole:** White to pale cream powder. Melts in the range of 78° to 88°. May exhibit polymorphism. Freely soluble in alcohol, in methanol, in isopropyl alcohol, in acetone, in propylene glycol, in chloroform, and in dimethylformamide; soluble in ether; insoluble in water.

**Miconazole Nitrate:** White or practically white, crystalline powder, having not more than a slight odor. Melts in the range of 178° to 183°, with decomposition. Freely soluble in dimethyl sulfoxide; soluble in dimethylformamide; sparingly soluble in methanol; slightly soluble in alcohol, in chloroform, and in propylene glycol; very slightly soluble in water and in isopropyl alcohol; insoluble in ether.

**Midazolam:** White or yellowish powder. The hydrochloride salt of midazolam is soluble in aqueous solutions. Insoluble in water.

**Midodrine Hydrochloride:** White crystalline powder. Soluble in water; sparingly soluble in methanol.

**Milrinone:** White to tan, crystalline solid. Is hygroscopic. Freely soluble in dimethyl sulfoxide; very slightly soluble in methanol; practically insoluble in water and in chloroform.

**Mineral Oil:** Colorless, transparent, oily liquid, free or practically free from fluorescence. Is odorless and tasteless when cold, and develops not more than a faint odor of petroleum when heated. Soluble in volatile oils; insoluble in water and in alcohol. Miscible with most fixed oils but not with castor oil. *NF category:* Solvent; vehicle (oleaginous); emollient.

**Light Mineral Oil:** Colorless, transparent, oily liquid, free, or practically free, from fluorescence. Is odorless and tasteless when cold, and develops not more than a faint odor of petroleum when heated. Soluble in volatile oils; insoluble in water and in alcohol. Miscible with most fixed oils, but not with castor oil. *NF category:* Vehicle (oleaginous); lubricant; emollient; solvent.

**Minocycline Hydrochloride:** Yellow, crystalline powder. Soluble in solutions of alkali hydroxides and carbonates; sparingly soluble in water; slightly soluble in alcohol; practically insoluble in chloroform and in ether.

**Minoxidil:** White to off-white, crystalline powder. Melts in the approximate range of between 248° and 268°, with decomposition. Soluble in alcohol and in propylene glycol; sparingly soluble in methanol; slightly soluble in water; practically insoluble in chloroform, in acetone, in ethyl acetate, and in hexane.

**Mirtazapine:** White to creamy white, crystalline powder. Freely soluble in methanol and in toluene; soluble in ethyl ether; sparingly soluble in *n*-hexane; practically insoluble in water.

**Misoprostol:** Clear, colorless or light yellow viscous liquid. Very slightly soluble in water.

**Mitomycin:** Blue-violet, crystalline powder. Soluble in acetone, in methanol, in butyl acetate, and in cyclohexanone; slightly soluble in water.

**Mitotane:** White, crystalline powder, having a slight, aromatic odor. Soluble in alcohol, in ether, in solvent hexane, and in fixed oils and fats; practically insoluble in water.

**Mitoxantrone Hydrochloride:** Dark blue powder. Sparingly soluble in water; slightly soluble in methanol; practically insoluble in acetone, in acetonitrile, and in chloroform.

**Modafinil:** White to off-white, crystalline powder. Sparingly soluble in methanol; slightly soluble in absolute alcohol; very slightly soluble in water.

**Moexipril Hydrochloride:** White to off-white powder. Soluble in water, in alcohol, and in methanol.

**Mometasone Furoate:** White to off-white powder. Melts at about 220°, with decomposition. Soluble in acetone and in methylene chloride.

**Monensin Sodium:** Off-white to tan, crystalline powder. Soluble in chloroform and in methanol; slightly soluble in water; practically insoluble in solvent hexane.

**Mono- and Di-glycerides:** Vary in consistency from yellow liquids, through ivory-colored plastics, to ivory white-colored solids (bead or flake forms). Soluble in alcohol, in ethyl acetate,

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in chloroform, and in other chlorinated hydrocarbons; insoluble in water. *NF category:* Emulsifying agent.

**Monobenzone Ointment:** Dispersible with, but not soluble in, water.

**Monoethanolamine:** Clear, colorless, moderately viscous liquid, having a distinctly ammoniacal odor. Miscible with water, with acetone, with alcohol, with glycerin, and with chloroform. Immiscible with ether, with solvent hexane, and with fixed oils, although it dissolves many essential oils. *NF category:* Emulsifying agent; pH modifier (acidifying agent/alkalizing agent/buffering agent).

**Monoglyceride Citrate:** Soft white to ivory-colored, waxy solid with a lard-like consistency and bland odor. Dispersible in most common fat solvents and in alcohol. Insoluble in water.

**Monosodium Glutamate:** White, practically odorless, free-flowing crystals or crystalline powder. Freely soluble in water; sparingly soluble in alcohol. May have either a slightly sweet or a slightly salty taste. *NF category:* Flavors and fragrance; pH modifier (acidifying agent/alkalizing agent/buffering agent).

**Monothioglycerol:** Colorless or pale yellow, viscous liquid, having a slight sulfidic odor. Is hygroscopic. Freely soluble in water; insoluble in ether. Miscible with alcohol. *NF category:* Antioxidant; antimicrobial preservative.

**Montelukast Sodium:** White or almost white, hygroscopic powder. Freely soluble to very soluble in alcohol; freely soluble in water and in methylene chloride.

**Morantel Tartrate:** A white or pale yellow, crystalline powder. Very soluble in water and in alcohol; practically insoluble in ethyl acetate.

**Morphine Sulfate:** White, feathery, silky crystals, cubical masses of crystals, or white, crystalline powder. Is odorless, and when exposed to air it gradually loses water of hydration. Darkens on prolonged exposure to light. Freely soluble in hot water; soluble in water; slightly soluble in alcohol but more so in hot alcohol; insoluble in chloroform and in ether.

**Moxidectin:** White to pale yellow powder. Very soluble in alcohol; slightly soluble in hexane; practically insoluble in water.

**Moxifloxacin Hydrochloride:** Slightly yellow to yellow powder or crystals. Soluble in 0.1 N sodium hydroxide; sparingly soluble in water and in methanol; slightly soluble in 0.1 N hydrochloric acid, in dimethylformamide, and in alcohol; practically insoluble in methylene chloride, in acetone, in ethyl acetate, and in toluene; insoluble in *tert*-butyl methyl ether and *n*-heptane.

**Mupirocin:** White to off-white, crystalline solid. Freely soluble in acetone, in chloroform, in dehydrated alcohol, and in methanol; slightly soluble in ether; very slightly soluble in water.

**Mycophenolate Mofetil:** White or almost white, crystalline powder. Its melting range is between 94° and 98°. Freely soluble in acetone; soluble in methanol; sparingly soluble in dehydrated alcohol; slightly soluble in water.

**Mycophenolate Sodium:** White to off-white crystalline powder. Slightly soluble in water; practically insoluble in 0.1 N hydrochloric acid.

**Myristic Acid:** Hard, white or faintly yellow, somewhat glossy, crystalline solid or white or yellow-white powder. Soluble in alcohol, in chloroform, and in ether; practically insoluble in water. *NF category:* Antifoaming agent; emulsifying agent; lubricant.

**Nabumetone:** A white, or almost white, crystalline powder. Freely soluble in acetone; sparingly soluble in alcohol and in methanol; practically insoluble in water.

**Nadolol:** White to off-white, practically odorless, crystalline powder. Freely soluble in alcohol and in methanol; soluble in water at pH 2; slightly soluble in chloroform, in methylene chloride, in isopropyl alcohol, and in water (between pH 7 and pH 10); insoluble in acetone, in benzene, in ether, in hexane, and in trichloroethane.

**Nafcillin Sodium:** White to yellowish-white powder, having not more than a slight characteristic odor. Freely soluble in water and in chloroform; soluble in alcohol.

**Naloxone Hydrochloride:** White to slightly off-white powder. Its aqueous solution is acidic. Soluble in water, in dilute acids, and in strong alkali; slightly soluble in alcohol; practically insoluble in ether and in chloroform.

**Naloxone Hydrochloride Injection:** Clear, colorless liquid.

**Nandrolone Decanoate:** Fine, white to creamy white, crystalline powder. Is odorless, or may have a slight odor. Soluble in chloroform, in alcohol, in acetone, and in vegetable oils; practically insoluble in water.

**Naphazoline Hydrochloride:** White, crystalline powder. Freely soluble in water; soluble in alcohol.

**Naproxen:** White to off-white, practically odorless, crystalline powder. Soluble in chloroform, in dehydrated alcohol, and in alcohol; sparingly soluble in ether; practically insoluble in water.

**Naproxen Sodium:** White to creamy crystalline powder. Soluble in water and in methanol; sparingly soluble in alcohol; very slightly soluble in acetone; and practically insoluble in chloroform and in toluene. Melts at about 255°, with decomposition.

**Narasin:** White to off-white, crystalline powder. Melts at about 217°, with decomposition. Soluble in methanol and in water.

**Naratriptan Hydrochloride:** White to pale yellow solid. Soluble in water.

**Natamycin:** Off-white to cream-colored powder, which may contain up to 3 moles of water. Soluble in glacial acetic acid and in dimethylformamide; slightly soluble in methanol; practically insoluble in water.

**Nateglinide:** White powder. Freely soluble in methanol and in alcohol; soluble in ether; sparingly soluble in acetonitrile and in octanol; practically insoluble in water.

**Nefazodone Hydrochloride:** Nonhygroscopic, white powder. Freely soluble in chloroform; soluble in propylene glycol; slightly soluble in polyethylene glycol and in water.

**Neohesperidin Dihydrochalcone:** White or yellowish-white powder. Freely soluble in dimethyl sulfoxide; soluble in methanol; practically insoluble in water and in methylene chloride. *NF category:* Sweetening agent; flavors and fragrance.

**Neomycin Sulfate:** White to slightly yellow powder, or cryodesiccated solid. Is odorless or practically so and is hygroscopic. Its solutions are dextrorotatory. Freely soluble in water; very slightly soluble in alcohol; insoluble in acetone, in chloroform, and in ether.

**Nevirapine:** White to off-white, odorless to nearly odorless, crystalline powder. Slightly soluble in alcohol and in methanol; practically insoluble in water. Hydrous form also slightly soluble in propylene glycol.

**Niacin:** White crystals or crystalline powder. Is odorless, or has a slight odor. Melts at about 235°. Freely soluble in boiling water, in boiling alcohol, and in solutions of alkali hydroxides and carbonates; sparingly soluble in water; practically insoluble in ether.

**Niacinamide:** White, crystalline powder. Is odorless or practically so, and has a bitter taste. Its solutions are neutral to litmus. Freely soluble in water and in alcohol; soluble in glycerin.

**Nifedipine:** Yellow powder. Is affected by exposure to light. Freely soluble in acetone; practically insoluble in water.

**Nilotinib:** White to slightly yellowish or slightly greenish-yellowish solid. Decomposes prior to melting.

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Slightly soluble in ethanol, in methanol, and in isopropanol; very slightly soluble in water.

**Nilutamide:** White or almost-white powder. Freely soluble in acetone and in ethyl acetate; soluble in methanol and in dichloromethane; sparingly soluble in absolute alcohol; very slightly soluble in water.

**Nimodipine:** Light yellow or yellow, crystalline powder, affected by light. Freely soluble in ethyl acetate; sparingly soluble in alcohol; practically insoluble in water. Exhibits polymorphism.

**Nitric Acid:** Highly corrosive fuming liquid, having a characteristic, highly irritating odor. Stains animal tissues yellow. Boils at about 120°. Specific gravity is about 1.41. *NF category:* pH modifier (acidifying agent/alkalizing agent/buffering agent).

**Nitrofurantoin:** Lemon-yellow, odorless crystals or fine powder. Has a bitter aftertaste. Soluble in dimethylformamide; very slightly soluble in water and in alcohol.

**Nitrofurazone:** Lemon yellow, odorless, crystalline powder. Darkens slowly on exposure to light. Melts at about 236°, with decomposition. Soluble in dimethylformamide; slightly soluble in propylene glycol and in polyethylene glycol mixtures; very slightly soluble in alcohol and in water; practically insoluble in chloroform and in ether.

**Nitrofurazone Ointment:** Yellow, opaque, and water-miscible, and has ointment-like consistency.

**Nitrogen:** Colorless, odorless, tasteless gas. Is nonflammable and does not support combustion. One L at 0° and at a pressure of 760 mm of mercury weighs about 1.251 g. One volume dissolves in about 65 volumes of water and in about 9 volumes of alcohol at 20° and at a pressure of 760 mm of mercury. *NF category:* Air displacement; propellant.

**Diluted Nitroglycerin:** When diluted with lactose, it is a white, odorless powder. When diluted with propylene glycol or alcohol, it is a clear, colorless, or pale yellow liquid. [NOTE—Undiluted nitroglycerin occurs as a white to pale yellow, thick, flammable, explosive liquid.] Undiluted nitroglycerin is soluble in methanol, in alcohol, in carbon disulfide, in acetone, in ethyl ether, in ethyl acetate, in glacial acetic acid, in benzene, in toluene, in nitrobenzene, in phenol, in chloroform, and in methylene chloride; slightly soluble in water.

**Nitromersol:** Brownish yellow to yellow granules or brownish yellow to yellow powder. Is odorless and tasteless and is affected by light. Soluble in solutions of alkalies and of ammonia by opening of the anhydride ring and the formation of a salt; very slightly soluble in water, in alcohol, in acetone, and in ether.

**Nitromersol Topical Solution:** Clear, reddish-orange solution. Is affected by light.

**Nitrous Oxide:** Colorless gas, without appreciable odor or taste. One L at 0° and at a pressure of 760 mm of mercury weighs about 1.97 g. One volume dissolves in about 1.4 volumes of water at 20° and at a pressure of 760 mm of mercury. Freely soluble in alcohol; soluble in ether and in oils. *NF category:* Propellant.

**Nizatidine:** Off-white to buff crystalline solid. Freely soluble in chloroform; soluble in methanol; sparingly soluble in water.

**Nonoxynol 9:** Clear, colorless to light yellow, viscous liquid. Soluble in water, in alcohol, and in corn oil. *NF category:* Wetting and/or solubilizing agent.

**Norepinephrine Bitartrate:** White or faintly gray, odorless, crystalline powder. Slowly darkens on exposure to air and light. Its solutions are acid to litmus, having a pH of about 3.5. Freely soluble in water; slightly soluble in alcohol; practically insoluble in chloroform and in ether. Melts between 98° and 104°, without previous drying of the specimen, the melt being turbid.

**Norepinephrine Bitartrate Injection:** Colorless or practically colorless liquid, gradually turning dark on exposure to air and light.

**Norethindrone:** White to creamy white, odorless, crystalline powder. Is stable in air. Soluble in chloroform and in dioxane; sparingly soluble in alcohol; slightly soluble in ether; practically insoluble in water.

**Norethindrone Acetate:** White to creamy white, odorless, crystalline powder. Very soluble in chloroform; freely soluble in dioxane; soluble in ether and in alcohol; practically insoluble in water.

**Norflurane:** Non-flammable hydrofluorocarbon (HFC) and haloalkane refrigerant used as a propellant in metered dose inhalers. Clear and colorless liquefied gas under pressure.

**Norgestimate:** White to pale yellow powder. Very to freely soluble in methylene chloride; sparingly soluble in acetonitrile; insoluble in water.

**Norgestrel:** White or practically white, practically odorless, crystalline powder. Freely soluble in chloroform; sparingly soluble in alcohol; insoluble in water.

**Norelgestromin:** White to off-white powder. Freely soluble in acetone, in ethanol, in methanol, and in dimethylformamide; sparingly soluble in methylene chloride and in iso-propanol; slightly soluble in acetonitrile; practically insoluble in water.

**Nortriptyline Hydrochloride:** White to off-white powder, having a slight, characteristic odor. Its solution (1 in 100) has a pH of about 5. Soluble in water and in chloroform; sparingly soluble in methanol; practically insoluble in ether, in benzene, and in most other organic solvents.

**Noscapine:** Fine, white or practically white, crystalline powder. Freely soluble in chloroform; soluble in acetone; slightly soluble in alcohol and in ether; practically insoluble in water.

**Novobiocin Sodium:** White or yellowish-white, odorless, hygroscopic, crystalline powder. Freely soluble in water, in alcohol, in methanol, in glycerin, and in propylene glycol; slightly soluble in butyl acetate; practically insoluble in acetone, in chloroform, and in ether.

**Nystatin:** Yellow to light tan powder. Is hygroscopic, and is affected by long exposure to light, heat, and air. Freely soluble in dimethylformamide and in dimethyl sulfoxide; very slightly soluble in methanol; practically insoluble or insoluble in water, in alcohol, in *n*-propyl alcohol, in *n*-butyl alcohol, in chloroform, and in ether.

**Octoxynol 9:** Clear, pale yellow, viscous liquid, having a faint odor and a bitter taste. Soluble in benzene and in toluene; practically insoluble in solvent hexane. Miscible with water, with alcohol, and with acetone. *NF category:* Wetting and/or solubilizing agent.

**Octyldodecanol:** Clear water-white, free-flowing liquid. Soluble in alcohol and in ether; insoluble in water. *NF category:* Vehicle (oleaginous); emollient; emulsifying agent.

**Octyl Methoxycinnamate:** Pale yellow oil. Insoluble in water.

**Ofloxacin:** Pale yellowish-white to light yellowish-white crystals or crystalline powder. Sparingly soluble in chloroform; slightly soluble in alcohol, in methanol, and in water.

Hydrophilic Ointment:*NF category:* Ointment base.

White Ointment:*NF category:* Ointment base.

Yellow Ointment:*NF category:* Ointment base.

**Olanzapine:** A yellow crystalline solid. Soluble in *n*-propanol; sparingly soluble in acetonitrile; slightly soluble in methanol and in dehydrated alcohol; practically insoluble in water.

**Oleic Acid:** Colorless to pale yellow, oily liquid when freshly prepared, but on exposure to air it gradually absorbs oxygen and darkens. Has a characteristic, lard-like odor and taste. When strongly heated in air, it is decomposed with the

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production of acrid vapors. Practically insoluble in water. Miscible with alcohol, with chloroform, with ether, with benzene, and with fixed and volatile oils. *NF category:* Emulsifying agent.

**Oleovitamin A and D:** Yellow to red, oily liquid, practically odorless or having a fish-like odor, and having no rancid odor or taste. Is a clear liquid at temperatures exceeding 65°, and may crystallize on cooling. Is unstable in air and in light. Very soluble in ether and in chloroform; soluble in dehydrated alcohol and in vegetable oils; insoluble in water and in glycerin.

**Oleovitamin A and D Capsules:** The oil contained in Oleovitamin A and D Capsules is a yellow to red, oily liquid, practically odorless or having a fish-like odor, and having no rancid odor or taste. Is a clear liquid at temperatures exceeding 65°, and may crystallize on cooling. Is unstable in air and in light.

**Oleoyl Polyoxylglycerides:** Amber, oily liquids. May develop deposit after prolonged storage at 20°. Freely soluble in methylene chloride; practically insoluble but dispersible in water. *NF category:* Ointment base; solvent; emulsifying agent; wetting and/or solubilizing agent.

**Oleyl Alcohol:** Clear, colorless to light yellow, oily liquid. Has a faint characteristic odor and a bland taste. Soluble in alcohol, in ether, in isopropyl alcohol, and in light mineral oil; insoluble in water. *NF category:* Emollient; emulsifying agent.

**Oleyl Oleate:** Clear, colorless to light yellow liquid. Has a faint characteristic odor. Slightly soluble in alcohol. Miscible with chloroform and with ether. *NF category:* Emollient; emulsifying agent.

**Olive Oil:** Pale yellow, or light greenish-yellow, oily liquid, having a slight, characteristic odor and taste, with a faintly acrid aftertaste. Slightly soluble in alcohol. Miscible with ether, with chloroform, and with carbon disulfide. *Specific Gravity* ⟨841⟩: Between 0.910 and 0.915. *NF category:* Methacrylic Acid and Vehicle (oleaginous).

**Olmesartan Medoxomil:** White to off-white crystalline powder. Sparingly soluble in methanol; practically insoluble in water.

**Olopatadine Hydrochloride:** White crystalline powder. Very soluble in formic acid; sparingly soluble in water; very slightly soluble in dehydrated alcohol.

**Omeprazole:** White to off-white powder. Soluble in dichloromethane; sparingly soluble in methanol and in alcohol; very slightly soluble in water.

**Omeprazole Magnesium:** White to off-white powder. Sparingly soluble in methanol; slightly soluble in alcohol; very slightly soluble in water and in dichloromethane.

**Ondansetron:** White to off-white powder. Very soluble in acid solutions; sparingly soluble in water.

**Ondansetron Hydrochloride:** White to off-white powder. Soluble in methanol; sparingly soluble in water and in alcohol; slightly soluble in isopropyl alcohol and in dichloromethane; very slightly soluble in acetone, in chloroform, and in ethyl acetate.

**Opium:** Has a very characteristic odor and a very bitter taste.

**Powdered Opium:** Light brown or moderately yellowish-brown powder.

**Orbifloxacin:** White to pale yellow crystals or crystalline powder. Odorless. Soluble in acetic acid; very slightly soluble in methanol, in water, and in chloroform; practically insoluble in ethanol and in diethyl ether.

**Orlistat:** White to off-white fine powder or fine powder with lumps. Freely soluble in chloroform; very soluble in methanol and in alcohol; practically insoluble in water.

**Orphenadrine Citrate:** White, practically odorless, crystalline powder, having a bitter taste. Sparingly soluble in water; slightly soluble in alcohol; insoluble in chloroform, in benzene, and in ether.

**Oseltamivir Phosphate:** White to off-white powder. Freely soluble in water; soluble in methanol, in dimethyl sulfoxide, and in propylene glycol; sparingly soluble in dimethylformamide; slightly soluble in alcohol; very slightly soluble in isopropyl alcohol and in polyethylene glycol 400; practically insoluble in acetonitrile, in acetone, in dichloromethane, and in *n*-hexane.

**Oxacillin Sodium:** Fine, white, crystalline powder, odorless or having a slight odor. Freely soluble in water, in methanol, and in dimethyl sulfoxide; slightly soluble in absolute alcohol, in chloroform, in pyridine, and in methyl acetate; insoluble in ethyl acetate, in ether, in benzene, and in ethylene chloride.

**Oxacillin Sodium for Injection:** Fine, white, crystalline powder, odorless or having a slight odor. Freely soluble in water, in methanol, and in dimethyl sulfoxide; slightly soluble in absolute alcohol, in chloroform, in pyridine, and in methyl acetate; insoluble in ethyl acetate, in ether, in benzene, and in ethylene chloride.

**Oxaliplatin:** White to off-white crystalline powder. Slightly soluble in water; very slightly soluble in methanol; practically insoluble in alcohol.

**Oxandrolone:** White, odorless, crystalline powder. Is stable in air, but darkens on exposure to light. Melts at about 225°. Freely soluble in chloroform; sparingly soluble in alcohol and in acetone; practically insoluble in water.

**Oxaprozin:** White to yellowish-white, crystalline powder.

**Oxazepam:** Creamy white to pale yellow powder. Is practically odorless. Slightly soluble in alcohol and in chloroform; very slightly soluble in ether; practically insoluble in water.

**Oxcarbazepine:** Light orange to creamish white or off-white powder. Soluble in acetic acid; sparingly soluble in chloroform; practically insoluble in water.

**Oxfendazole:** White or almost white powder. Slightly soluble in alcohol and in methylene chloride; practically insoluble in water.

**Oxprenolol Hydrochloride:** White, crystalline powder. Freely soluble in alcohol, in chloroform, and in water; sparingly soluble in acetone; practically insoluble in ether.

**Oxtriphylline:** White, crystalline powder, having an amine-like odor. A solution (1 in 100) has a pH of about 10.3. Freely soluble in water and in alcohol; very slightly soluble in chloroform.

**Oxybenzone:** Pale yellow powder. Freely soluble in alcohol and in toluene; practically insoluble in water.

**Oxybutynin Chloride:** White, crystalline, practically odorless powder. Very soluble in methanol and in chloroform; freely soluble in water and in alcohol; soluble in acetone; slightly soluble in ether; very slightly soluble in hexane.

**Oxycodone Hydrochloride:** White to off-white, hygroscopic crystals or powder. Is odorless. Soluble in water; slightly soluble in alcohol.

**Oxygen:** Colorless, odorless, tasteless gas, which supports combustion more energetically than does air. One L at 0° and at a pressure of 760 mm of mercury weighs about 1.429 g. One volume dissolves in about 32 volumes of water and in about 7 volumes of alcohol at 20° and at a pressure of 760 mm of mercury.

**Oxymetazoline Hydrochloride:** White to practically white, fine crystalline powder. Is hygroscopic. Melts at about 300°, with decomposition. Soluble in water and in alcohol; practically insoluble in benzene, in chloroform, and in ether.

**Oxymetholone:** White to creamy white, crystalline powder. Is odorless, and is stable in air. Freely soluble in chloroform; soluble in dioxane; sparingly soluble in alcohol; slightly soluble in ether; practically insoluble in water.

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**Oxymorphone Hydrochloride:** White or slightly off-white, odorless powder. Darkens on exposure to light. Its aqueous solutions are slightly acidic. Freely soluble in water; sparingly soluble in alcohol and in ether.

**Oxyquinoline Sulfate:** Yellow powder. Melts at about 185°. Very soluble in water; freely soluble in methanol; slightly soluble in alcohol; practically insoluble in acetone and in ether. *NF category:* Chelating and/or complexing agent.

**Oxytetracycline:** Pale yellow to tan, odorless, crystalline powder. Is stable in air, but exposure to strong sunlight causes it to darken. It loses potency in solutions of pH below 2, and is rapidly destroyed by alkali hydroxide solutions. Freely soluble in 3 N hydrochloric acid and in alkaline solutions; sparingly soluble in alcohol; very slightly soluble in water.

**Oxytetracycline Hydrochloride:** Yellow, odorless, crystalline powder, having a bitter taste. Is hygroscopic. Decomposes at a temperature exceeding 180°, and exposure to strong sunlight or to temperatures exceeding 90° in moist air causes it to darken. Its potency is diminished in solutions having a pH below 2, and is rapidly destroyed by alkali hydroxide solutions. Freely soluble in water, but crystals of oxytetracycline base separate as a result of partial hydrolysis of the hydrochloride; sparingly soluble in alcohol and in methanol, and even less soluble in dehydrated alcohol; insoluble in chloroform and in ether.

**Paclitaxel:** White to off-white powder. Soluble in alcohol; insoluble in water.

**Padimate O:** A light yellow, mobile liquid having a faint, aromatic odor. Soluble in alcohol, in isopropyl alcohol, and in mineral oil; practically insoluble in water, in glycerin, and in propylene glycol.

**Paliperidone:** White to yellow powder. Sparingly soluble in 0.1 N hydrochloric acid and in methylene chloride; slightly soluble in *N,N*-dimethylformamide and in tetrahydrofuran; practically insoluble in water, in 0.1 N sodium hydroxide, and in hexane.

**Palm Oil:** White to yellowish, fatty solid to semisolid. Insoluble in water. *NF category:* Coating agent; emulsifying agent.

**Hydrogenated Palm Oil:** White to yellowish, fatty solid to semi-solid. Freely soluble in ether; very slightly soluble in alcohol; practically insoluble in water. *NF category:* Coating agent; lubricant; wet binder.

**Palm Kernel Oil:** White to yellowish, fatty solid. Insoluble in water. *NF category:* Coating agent; emulsifying agent; suppository base.

**Palmitic Acid:** Hard, white or faintly yellow, somewhat glossy crystalline solid, or white or yellowish-white powder. It has a slight characteristic odor and taste. Soluble in alcohol, in ether, and in chloroform; practically insoluble in water. *NF category:* Antifoaming agent; emulsifying agent; lubricant.

**Palonosetron Hydrochloride:** White to off-white, crystalline powder. Freely soluble in water; soluble in propylene glycol; slightly soluble in ethanol; very slightly soluble to slightly soluble in isopropyl alcohol.

**Pamidronate Disodium:** White, crystalline powder. Soluble in water and in 2 N sodium hydroxide; sparingly soluble in 0.1 N hydrochloric acid and in 0.1 N acetic acid; practically insoluble in organic solvents.

**Pancreatin:** Cream-colored, amorphous powder, having a faint, characteristic, but not offensive odor. It hydrolyzes fats to glycerol and fatty acids, changes protein into proteoses and derived substances, and converts starch into dextrins and sugars. Its greatest activities are in neutral or faintly alkaline media; more than traces of mineral acids or large amounts of alkali hydroxides make it inert. An excess of alkali carbonate also inhibits its action.

**Pancrelipase:** Cream-colored, amorphous powder, having a faint, characteristic, but not offensive odor. Pancrelipase hydrolyzes fats to glycerol and fatty acids, changes protein into proteoses and derived substances, and converts starch into dextrins and sugars. Its greatest activities are in neutral or faintly alkaline media; more than traces of mineral acids or large amounts of alkali hydroxides make it inert. An excess of alkali carbonate also inhibits its action.

**Pancrelipase Capsules:** The contents of Capsules conform to the *Description* under *Pancrelipase*, except that the odor may vary with the flavoring agent used.

**Pancuronium Bromide:** White, yellowish-white, or slightly pink, crystalline powder. Is hygroscopic. Freely soluble in water, in methylene chloride, and in alcohol.

**Panthenol:** White to creamy white, crystalline powder having a slight, characteristic odor. Freely soluble in water, in alcohol, and in propylene glycol; soluble in chloroform and in ether; slightly soluble in glycerin.

**Pantoprazole Sodium:** White to off-white powder. Freely soluble in water, in methanol, and in dehydrated alcohol; practically insoluble in hexane and in dichloromethane.

**Papain:** White to light tan, amorphous powder. Soluble in water, the solution being colorless to light yellow and more or less opalescent; practically insoluble in alcohol, in chloroform, and in ether.

**Papaverine Hydrochloride:** White crystals or white, crystalline powder. Is odorless, and has a slightly bitter taste. Is optically inactive. Its solutions are acid to litmus. Melts at about 220°, with decomposition. Soluble in water and in chloroform; slightly soluble in alcohol; practically insoluble in ether.

**Parachlorophenol:** White or pink crystals having a characteristic phenolic odor. When undiluted, it whitens and cauterizes the skin and mucous membranes. Melts at about 42°. Very soluble in alcohol, in glycerin, in chloroform, in ether, and in fixed and volatile oils; soluble in petrolatum; sparingly soluble in water and in liquid petrolatum.

**Paraffin:** Colorless or white, more or less translucent mass showing a crystalline structure. Is odorless and tasteless, and is slightly greasy to the touch. Freely soluble in chloroform, in ether, in volatile oils, and in most warm fixed oils; slightly soluble in dehydrated alcohol; insoluble in water and in alcohol. *NF category:* Stiffening agent; ointment base.

**Synthetic Paraffin:** Very hard, white, practically tasteless and odorless wax. Contains mostly long-chain, unbranched, saturated hydrocarbons, with a small amount of branched hydrocarbons. Is represented by the formula $C_nH_{2n2}$, in which *n* may range from 20 to about 100. The average molecular weight may range from 400 to 1400. Slightly soluble in aromatic and normal paraffinic solvents; very slightly soluble in aliphatic, oxygenated, and halogenated hydrocarbon solvents; insoluble in water. *NF category:* Stiffening agent.

**Paraldehyde:** Colorless, transparent liquid. Has a strong, characteristic but not unpleasant or pungent odor, and a disagreeable taste. Specific gravity is about 0.99. Soluble in water, but less soluble in boiling water. Miscible with alcohol, with chloroform, with ether, and with volatile oils.

**Paricalcitol:** White to almost white powder. Soluble in alcohol; insoluble in water.

**Paromomycin Sulfate:** Creamy white to light yellow powder. Is odorless or practically odorless, and is very hygroscopic. Very soluble in water; insoluble in alcohol, in chloroform, and in ether.

**Paroxetine Hydrochloride:** White to off-white solid. Soluble in methanol and in alcohol; slightly soluble in water.

**Peanut Oil:** Colorless or pale yellow, oily liquid with a bland taste. May have a characteristic, nutty odor. Very slightly soluble in alcohol. Miscible with ether, with chloroform, and with carbon disulfide. *Specific Gravity* ⟨841⟩: Between 0.912 and 0.920. *Refractive Index* ⟨831⟩: Between 1.462 and 1.464 at 40°. *NF category:* Solvent; vehicle (oleaginous).

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**Pectin:** Coarse or fine powder, yellowish-white in color, almost odorless, and having a mucilaginous taste. Soluble in 20 parts of water, forming a viscous, opalescent, colloidal solution that flows readily and is acid to litmus; practically insoluble in alcohol or in diluted alcohol and in other organic solvents. Pectin dissolves in water more readily if first moistened with alcohol, glycerin, or simple syrup, or if first mixed with 3 or more parts of sucrose. *NF category:* Suspending and/or viscosity-increasing agent; emulsifying agent; film-forming agent.

**Pemetrexed Disodium:** White or almost white powder. Freely soluble in water; very slightly soluble in dehydrated alcohol; practically insoluble in methylene chloride.

**Penicillamine:** White or practically white, crystalline powder, having a slight, characteristic odor. Freely soluble in water; slightly soluble in alcohol; insoluble in chloroform and in ether.

**Penicillin G Benzathine:** White, odorless, crystalline powder. Sparingly soluble in alcohol; very slightly soluble in water.

**Penicillin G Potassium:** Colorless or white crystals, or white, crystalline powder. Is odorless or practically so, and is moderately hygroscopic. Its solutions are dextrorotatory. Its solutions retain substantially full potency for several days at temperatures below 15°, but are rapidly inactivated by acids, by alkali hydroxides, by glycerin, and by oxidizing agents. Very soluble in water, in saline TS, and in dextrose solutions; sparingly soluble in alcohol.

**Penicillin G Procaine:** White crystals or white, very fine, microcrystalline powder. Is odorless or practically odorless, and is relatively stable in air. Its solutions are dextrorotatory. Is rapidly inactivated by acids, by alkali hydroxides, and by oxidizing agents. Soluble in alcohol and in chloroform; slightly soluble in water.

**Penicillin G Sodium:** Colorless or white crystals or white to slightly yellow, crystalline powder. Is odorless or practically odorless, and is moderately hygroscopic. Its solutions are dextrorotatory. Is relatively stable in air, but is inactivated by prolonged heating at about 100°, especially in the presence of moisture. Its solutions lose potency fairly rapidly at room temperature, but retain substantially full potency for several days at temperatures below 15°. Its solutions are rapidly inactivated by acids, by alkali hydroxides, by oxidizing agents, and by penicillinase.

**Penicillin V:** White, odorless, crystalline powder. Freely soluble in alcohol and in acetone; very slightly soluble in water; insoluble in fixed oils.

**Penicillin V Potassium:** White, odorless, crystalline powder. Very soluble in water; slightly soluble in alcohol; insoluble in acetone.

**Pentamidine Isethionate:** White or almost white powder or colorless crystals, hygroscopic. Freely soluble in water; sparingly soluble in alcohol; practically insoluble in methylene chloride.

**Pentazocine:** White or very pale, tan-colored powder. Freely soluble in chloroform; soluble in alcohol, in acetone, and in ether; sparingly soluble in benzene and in ethyl acetate; practically insoluble in water.

**Pentazocine Hydrochloride:** White, crystalline powder. It exhibits polymorphism, one form melting at about 254° and the other at about 218°. Freely soluble in chloroform; soluble in alcohol; sparingly soluble in water; very slightly soluble in acetone and in ether; practically insoluble in benzene.

**Pentetic Acid:** White, odorless or almost odorless powder. Melts with foaming and degradation at 220°. *NF category:* Chelating and/or complexing agent; antimicrobial preservative; sequestering agent.

**Pentobarbital:** White to practically white, fine, practically odorless powder. May occur in a polymorphic form that melts at about 116°. This form gradually reverts to the more stable higher-melting form upon being heated at about 110°. Very soluble in alcohol, in methanol, in ether, in chloroform, and in acetone; soluble in benzene; very slightly soluble in water and in carbon tetrachloride.

**Pentobarbital Sodium:** White, crystalline granules or white powder. Is odorless or has a slight characteristic odor, and has a slightly bitter taste. Its solutions decompose on standing, heat accelerating the decomposition. Very soluble in water; freely soluble in alcohol; practically insoluble in ether.

**Pentoxifylline:** White to almost white crystalline powder. Freely soluble in chloroform and in methanol; soluble in water; sparingly soluble in alcohol; slightly soluble in ether.

**Peppermint:** Has an aromatic, characteristic odor and a pungent taste, and produces a cooling sensation in the mouth. *NF category:* Flavors and fragrance.

**Peppermint Oil:** Colorless or pale yellow liquid, having a strong, penetrating, characteristic odor and a pungent taste, followed by a sensation of cold when air is drawn into the mouth. *NF category:* Flavors and fragrance.

**Peppermint Spirit:** A clear, colorless liquid with a peppermint fragrance. Freely soluble in methanol and in diethyl ether; soluble in water. *NF category:* Flavors and fragrance.

**Peppermint Water:** *NF category:* Vehicle (flavored and/or sweetened).

**Perflubron:** Clear, colorless, practically odorless liquid.

**Pergolide Mesylate:** White to off-white powder. Sparingly soluble in methanol; slightly soluble in water, in dehydrated alcohol, and in chloroform; very slightly soluble in acetone; practically insoluble in ether.

**Perindopril Erbumine:** White or off-white, crystalline powder; freely soluble in water and in alcohol; sparingly soluble in methylene chloride.

**Perphenazine:** White to creamy white, odorless powder. Freely soluble in alcohol and in chloroform; soluble in acetone; practically insoluble in water.

**Petrolatum:** Unctuous yellowish to light amber mass, having not more than a slight fluorescence even after being melted. Is transparent in thin layers. Is free or practically free from odor and taste. Freely soluble in benzene, in carbon disulfide, in chloroform, and in turpentine oil; soluble in ether, in solvent hexane, and in most fixed and volatile oils; practically insoluble in cold and hot alcohol and in cold dehydrated alcohol; insoluble in water. *NF category:* Ointment base; emollient.

**Hydrophilic Petrolatum:** *NF category:* Ointment base.

**White Petrolatum:** White or faintly yellowish, unctuous mass, transparent in thin layers even after cooling to 0°. Freely soluble in benzene, in carbon disulfide, and in chloroform; soluble in ether, in solvent hexane, and in most fixed and volatile oils; slightly soluble in cold or hot alcohol, and in cold dehydrated alcohol; insoluble in water. *NF category:* Ointment base.

**Phenazopyridine Hydrochloride:** Light or dark red to dark violet, crystalline powder. Is odorless, or has a slight odor. Melts at about 235°, with decomposition. Slightly soluble in water, in alcohol, and in chloroform.

**Phendimetrazine Tartrate:** White, odorless, crystalline powder. Freely soluble in water; sparingly soluble in warm alcohol; insoluble in chloroform, in acetone, in ether, and in benzene. Phendimetrazine base is extracted by organic solvents from alkaline solution.

**Phenelzine Sulfate:** White to yellowish white powder, having a characteristic odor. Freely soluble in water; practically insoluble in alcohol, in chloroform, and in ether.

**Pheniramine Maleate:** White, crystalline powder having a faint amine-like odor. Soluble in water and in alcohol.

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**Phenobarbital:** White, odorless, glistening, small crystals, or white, crystalline powder, which may exhibit polymorphism. Is stable in air. Its saturated solution has a pH of about 5. Soluble in alcohol, in ether, and in solutions of fixed alkali hydroxides and carbonates; sparingly soluble in chloroform; very slightly soluble in water.

**Phenobarbital Sodium:** Flaky crystals, or white, crystalline granules, or white powder. Is odorless, has a bitter taste, and is hygroscopic. Its solutions are alkaline to phenolphthalein TS, and decompose on standing. Very soluble in water; soluble in alcohol; practically insoluble in ether and in chloroform.

**Phenol:** Colorless to light pink, interlaced or separate, needle-shaped crystals, or white to light pink, crystalline mass. Has a characteristic odor. Is liquefied by warming and by the addition of 10% of water. Boils at about 182°, and its vapor is flammable. Gradually darkens on exposure to light and air. Very soluble in alcohol, in glycerin, in chloroform, in ether, and in fixed and volatile oils; soluble in water; sparingly soluble in mineral oil. *NF category:* Antimicrobial preservative.

**Liquefied Phenol:** Colorless to pink liquid, which may develop a red tint upon exposure to air or light. Has a characteristic, somewhat aromatic odor. It whitens and cauterizes the skin and mucous membranes. Specific gravity is about 1.065. Miscible with alcohol, with ether, and with glycerin. A mixture of equal volumes of Liquefied Phenol and glycerin is miscible with water.

**Camphorated Phenol Topical Gel:** Clear, colorless, oily gel.

**Phenolsulfonphthalein:** A bright-red to dark-red, crystalline powder. Slightly soluble in alcohol; very slightly soluble in water.

**Phenoxyethanol:** A colorless, slightly viscous liquid. Slightly soluble in water, in peanut oil, and in olive oil. Miscible with acetone, with alcohol, and with glycerol. *NF category:* Antimicrobial preservative.

**Phentermine Hydrochloride:** White, odorless, hygroscopic, crystalline powder. Soluble in water and in the lower alcohols; slightly soluble in chloroform; insoluble in ether.

**Phentolamine Mesylate:** White or off-white, odorless, crystalline powder. Its solutions are acid to litmus, having a pH of about 5, and slowly deteriorate. Melts at about 178°. Freely soluble in water and in alcohol; slightly soluble in chloroform.

**Phenylalanine:** White, odorless crystals, having a slightly bitter taste. Sparingly soluble in water; very slightly soluble in methanol, in alcohol, and in dilute mineral acids.

**Phenylbenzimidazole Sulfonic Acid:** White to ivory-colored, odorless powder. Soluble in alcohol; practically insoluble in oily solvents and in water. Its salts are freely soluble in water.

**Phenylbutazone:** White to off-white, odorless, crystalline powder. Freely soluble in acetone and in ether; soluble in alcohol; very slightly soluble in water.

**Phenylephrine Bitartrate:** White or almost white powder or colorless crystals. Freely soluble in water.

**Phenylephrine Hydrochloride:** White or practically white, odorless crystals, having a bitter taste. Freely soluble in water and in alcohol.

**Phenylephrine Hydrochloride Nasal Solution:** Clear, colorless or slightly yellow, odorless liquid. Is neutral or acid to litmus.

**Phenylephrine Hydrochloride Ophthalmic Solution:** Clear, colorless or slightly yellow liquid, depending on the concentration.

**Phenylethyl Alcohol:** Colorless liquid, having a rose-like odor and a sharp, burning taste. Very soluble in alcohol, in fixed oils, in glycerin, and in propylene glycol; sparingly soluble in water; slightly soluble in mineral oil. *NF category:* Antimicrobial preservative.

**Phenylmercuric Acetate:** White to creamy white, crystalline powder, or small white prisms or leaflets. Is odorless. Soluble in alcohol and in acetone; slightly soluble in water. *NF category:* Antimicrobial preservative.

**Phenylmercuric Nitrate:** White, crystalline powder. Is affected by light. Its saturated solution is acid to litmus. Slightly soluble in alcohol and in glycerin; very slightly soluble in water. It is more soluble in the presence of either nitric acid or alkali hydroxides. *NF category:* Antimicrobial preservative.

**Phenylpropanolamine Hydrochloride:** White, crystalline powder, having a slight aromatic odor. Is affected by light. Freely soluble in water and in alcohol; insoluble in ether.

**Phenyltoloxamine Citrate:** White, crystalline powder. Very soluble in boiling water; slightly soluble in cold water and in alcohol; practically insoluble in cold acetone, in ethyl ether, and in toluene.

**Phenytoin:** White, odorless powder. Melts at about 295°. Soluble in hot alcohol; slightly soluble in cold alcohol, in chloroform, and in ether; practically insoluble in water.

**Phenytoin Sodium:** White, odorless powder. Is somewhat hygroscopic and on exposure to air gradually absorbs carbon dioxide. Freely soluble in water, the solution usually being somewhat turbid due to partial hydrolysis and absorption of carbon dioxide; soluble in alcohol; practically insoluble in ether and in chloroform.

**Phosphoric Acid:** Colorless, odorless liquid of syrupy consistency. Specific gravity is about 1.71. Miscible with water and with alcohol. *NF category:* pH modifier (acidifying agent/alkalizing agent/buffering agent).

**Diluted Phosphoric Acid:** Clear, colorless, odorless liquid. Specific gravity is about 1.057. *NF category:* pH modifier (acidifying agent/alkalizing agent/buffering agent).

**Physostigmine Salicylate:** White, shining, odorless crystals or white powder. Acquires a red tint when exposed to heat, light, air, or contact with traces of metals for long periods. Melts at about 184°. Freely soluble in chloroform; soluble in alcohol; sparingly soluble in water; slightly soluble in ether.

**Phytonadione:** Clear, yellow to amber, very viscous, odorless or practically odorless liquid, having a specific gravity of about 0.967. Is stable in air, but decomposes on exposure to sunlight. Soluble in dehydrated alcohol, in benzene, in chloroform, in ether, and in vegetable oils; slightly soluble in alcohol; insoluble in water.

**Pilocarpine:** A viscous, oily liquid, or crystals melting at about 34°. Exceedingly hygroscopic. Soluble in water, in alcohol, and in chloroform; sparingly soluble in ether and in benzene; practically insoluble in petroleum ether.

**Pilocarpine Hydrochloride:** Colorless, translucent, odorless, faintly bitter crystals. Is hygroscopic and is affected by light. Its solutions are acid to litmus. Very soluble in water; freely soluble in alcohol; slightly soluble in chloroform; insoluble in ether.

**Pilocarpine Nitrate:** Shining, white crystals. Is stable in air but is affected by light. Its solutions are acid to litmus. Freely soluble in water; sparingly soluble in alcohol; insoluble in chloroform and in ether.

**Pimozide:** White, crystalline powder. Freely soluble in chloroform; slightly soluble in ether and in alcohol; insoluble in water.

**Pindolol:** White to off-white, crystalline powder, having a faint odor. Slightly soluble in methanol; very slightly soluble in chloroform; practically insoluble in water.

**Pioglitazone Hydrochloride:** White crystals or crystalline powder. Soluble in dimethylformamide; slightly soluble in dehydrated alcohol; very slightly soluble in acetone and in acetonitrile; practically insoluble in water; insoluble in ether.

**Piperacillin:** White to off-white, crystalline powder. Very soluble in methanol; slightly soluble in isopropyl alcohol; very

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slightly soluble in ethyl acetate and practically insoluble to very slightly soluble in water.

**Piperacillin Sodium:** White to off-white solid. Freely soluble in water and in alcohol.

**Piperazine:** White to slightly off-white lumps or flakes, having an ammoniacal odor. Soluble in water and in alcohol; insoluble in ether.

**Piperazine Citrate:** White, crystalline powder, having not more than a slight odor. Its solution (1 in 10) has a pH of about 5. Soluble in water; insoluble in alcohol and in ether.

**Piperazine Dihydrochloride:** White crystalline powder. Soluble in water.

**Piperazine Phosphate:** White crystalline powder. Sparingly soluble in water; practically insoluble in alcohol.

**Piroxicam:** Off-white to light tan or light yellow, odorless powder. Forms a monohydrate that is yellow. Slightly soluble in alcohol and in aqueous alkaline solutions; very slightly soluble in water, in dilute acids, and in most organic solvents.

**Plantago Seed:** All varieties are practically odorless and have a bland, mucilaginous taste.

**Podophyllum:** Has a slight odor and a disagreeably bitter and acrid taste.

**Podophyllum Resin:** Amorphous powder, varying in color from light brown to greenish yellow, turning darker when subjected to a temperature exceeding 25° or when exposed to light. Has a slight, peculiar, faintly bitter taste. Its alcohol solution is acid to moistened litmus paper. Soluble in alcohol with a slight opalescence; partially soluble in ether and in chloroform.

**Polacrilin Potassium:** White to off-white, free-flowing powder. Has a faint odor or is odorless. Insoluble in water and in most liquids. *NF category:* Disintegrant.

**Polidocanol:** White, waxy solid in appearance and texture at 15°–20°. Polidocanol is hygroscopic. Soluble in water and in alcohol; practically insoluble in light petroleum.

**Poloxalene:** Colorless or pale yellow liquid. Soluble in water, in chloroform, and in ethylene dichloride.

**Poloxamer:** *NF category:* Wetting and/or solubilizing agent; emulsifying agent; lubricant.

**Poloxamer 124:** Colorless liquid, having a mild odor. When solidified, it melts at about 16°. Freely soluble in water, in alcohol, in isopropyl alcohol, in propylene glycol, and in xylene.

**Poloxamer 188:** White, prilled or cast solid. Is odorless, or has a very mild odor. Melts at about 52°. Freely soluble in water and in alcohol.

**Poloxamer 237:** White, prilled or cast solid. Is odorless, or has a very mild odor. Melts at about 49°. Freely soluble in water and in alcohol; sparingly soluble in isopropyl alcohol and in xylene.

**Poloxamer 338:** White, prilled or cast solid. Is odorless, or has a very mild odor. Melts at about 57°. Freely soluble in water and in alcohol; sparingly soluble in propylene glycol.

**Poloxamer 407:** White, prilled or cast solid. Is odorless, or has a very mild odor. Melts at about 56°. Freely soluble in water, in alcohol, and in isopropyl alcohol.

**Polycarbophil:** White to creamy white granules, having a characteristic, ester-like odor. Swells in water to a range of volumes, depending primarily on the pH. Insoluble in water, in dilute acids, in dilute alkalies, and in common organic solvents. *NF category:* Emulsifying agent; suspending and/or viscosity-increasing agent; wet binder.

**Hydrogenated Polydecene:** Clear, colorless, odorless, tasteless liquid. Very slightly soluble in water. *NF category:* Emollient; ointment base; solvent; vehicle (oleaginous).

**Polydextrose:** Off-white to light tan-colored solid. Very soluble in water; soluble in alcohol; slightly soluble in glycerin and in propylene glycol. *NF category:* Bulking agent; humectant; coating agent; suspending and/or viscosity-increasing agent; diluent.

**Hydrogenated Polydextrose:** Off-white to light tan-colored solid. Very soluble in water; soluble in alcohol; slightly soluble in glycerin and in propylene glycol. *NF category:* Bulking agent; coating agent; humectant; suspending and/or viscosity-increasing agent; wet binder.

**Polyethylene Glycol:** Polyethylene Glycol is usually designated by a number that corresponds approximately to its average molecular weight. As the average molecular weight increases, the water solubility, vapor pressure, hygroscopicity, and solubility in organic solvents decrease, while congealing temperature, specific gravity, flash point, and viscosity increase. Liquid grades occur as clear to slightly hazy, colorless or practically colorless, slightly hygroscopic, viscous liquids, having a slight, characteristic odor, and a specific gravity at 25° of about 1.12. Solid grades occur as practically odorless and tasteless, white, waxy, plastic material having a consistency similar to beeswax, or as creamy white flakes, beads, or powders. The accompanying table states the approximate congealing temperatures that are characteristic of commonly available grades. Liquid grades are miscible with water; solid grades are freely soluble in water; and all are soluble in acetone, in alcohol, in chloroform, in ethylene glycol monoethyl ether, in ethyl acetate, and in toluene; all are insoluble in ether and in hexane. *NF category:* Coating agent; plasticizer; solvent; suppository base; lubricant; film-forming agent; ointment base; diluent.

| Nominal Molecular Weight Polyethylene Glycol | Approximate Congealing Temperature (°) |
|---|---|
| 300 | –11 |
| 400 | 6 |
| 600 | 20 |
| 900 | 34 |
| 1000 | 38 |
| 1450 | 44 |
| 4500 | 58 |
| 8000 | 60 |

**Polyethylene Glycol 3350:** White granular, powder, or flake. Approximate congealing temperature: 56°. Freely soluble in water; soluble in acetone, in alcohol, in chloroform, in ethylene glycol monoethyl ether, in ethyl acetate, and in toluene; insoluble in ether and in hexane. *NF category:* Coating agent; diluent; film-forming agent; lubricant; ointment base; plasticizer; solvent; suppository base.

**Polyethylene Glycol Monomethyl Ether:** Polyethylene Glycol Monomethyl Ether is usually designated by a number that corresponds approximately to its average molecular weight. As the average molecular weight increases, the water solubility, vapor pressure, hygroscopicity, and solubility in organic solvents decrease, while congealing temperature, specific gravity, flash point, and viscosity increase. Liquid grades occur as clear to slightly hazy, colorless or practically colorless, slightly hygroscopic, viscous liquids, having a slight, characteristic odor, and a specific gravity at 25° of about 1.09–1.10. Solid grades occur as practically odorless and tasteless, white, waxy, plastic material having a consistency similar to beeswax, or as creamy white flakes, beads, or powders. The accompanying table states the approximate congealing temperatures that are characteristic of commonly available grades. Liquid grades are miscible with water; solid grades are freely soluble in water; and all are soluble in acetone, in alcohol, in chloroform, in ethylene glycol monoethyl ether, in

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ethyl acetate, and in toluene; all are insoluble in ether and in hexane. *NF category:* Ointment base; solvent; plasticizer.

| Nominal Molecular Weight Polyethylene Glycol Monomethyl Ether | Approximate Congealing Temperature (°) |
|---|---|
| 350 | –7 |
| 550 | 17 |
| 750 | 28 |
| 1000 | 35 |
| 2000 | 51 |
| 5000 | 59 |
| 8000 | 60 |
| 10000 | 61 |

**Polyethylene Oxide:** Polyethylene oxide resins are high molecular weight polymers having the common structure:

$$(–O–CH_2CH_2–)_n$$

in which *n*, the degree of polymerization, varies from about 2000 to over 100,000. Polyethylene oxide, being a polyether, strongly hydrogen, bonds with water. It is nonionic and undergoes salting-out effects associated with neutral molecules in solutions of high dielectric media. Salting-out effects manifest themselves in depressing the upper temperature limit of solubility, and in reducing the viscosity of both dilute and concentrated solutions of the polymers. All molecular weight grades are powdered or granular solids. They are soluble in water but, because of the high solution viscosities obtained (see *table*), solutions over 1% in water may be difficult to prepare. The water solubility, hygroscopicity, solubility in organic solvents, and melting point do not vary in the specified molecular weight range. At room temperature polyethylene oxide is miscible with water in all proportions. At concentrations of about 20% polymer in water, the solutions are nontacky, reversible, elastic gels. At higher concentrations, the solutions are tough, elastic materials with the water acting as a plasticizer. Polyethylene oxide is also freely soluble in acetonitrile, in ethylene dichloride, in trichloroethylene, and in methylene chloride. Heating may be required to obtain solutions in many other organic solvents. It is insoluble in aliphatic hydrocarbons, in ethylene glycol, in diethylene glycol, and in glycerol. *NF category:* Suspending and/or viscosity-increasing agent; wet binder; coating agent; release-modifying agent.

| Approximate Molecular Weight | Typical Solution Viscosity (cps), 25° | |
|---|---|---|
| | 5% Solution | 1% Solution |
| 100,000 | 40 | |
| 200,000 | 100 | |
| 300,000 | 800 | |
| 400,000 | 3000 | |
| 600,000 | 6000 | |
| 900,000 | 15,000 | |
| 4,000,000 | | 3500 |
| 5,000,000 | | 5500 |

**Polyethylene 50 Stearate:** *NF category:* Emulsifying and/or solubilizing agent.

**Polyglyceryl 3 Diisostearate:** Viscous liquid. Soluble in alcohol, in methylene chloride, in mineral oil, and in vegetable oils; insoluble in water. *NF category:* Emulsifying agent; ointment base.

**Polyglyceryl Dioleate:** Viscous liquid. Soluble in methylene chloride, in mineral oil, and in vegetable oils; sparingly soluble in alcohol; insoluble in water. *NF category:* Emulsifying agent.

**Polyisobutylene:** Low molecular-weight grades are soft and gummy; high molecular-weight grades are tough and elastic. All grades are light in color, odorless, and tasteless. Soluble in diisobutylene, in toluene, and in chloroform; insoluble in water. *NF category:* Adhesive.

**Polymyxin B Sulfate:** White to buff-colored powder. Is odorless or has a faint odor. Freely soluble in water; slightly soluble in alcohol.

**Polyoxyl Lauryl Ether:** A material with 3–5 oxyethylene units per molecule is a colorless liquid. Soluble or dispersible in alcohol; practically insoluble in water and in hexane. A material with 9–23 oxyethylene units per molecule is a white, waxy mass. Soluble or dispersible in water; soluble in alcohol; practically insoluble in hexane. *NF category:* Emulsifying agent; wetting and/or solubilizing agent.

**Polyoxyl Oleate:** A slightly yellowish, viscous liquid. Dispersible in water and in oils. Soluble in alcohol and in isopropyl alcohol. Miscible with fatty oils and with waxes. Its refractive index is about 1.466.

**Polyoxyl 10 Oleyl Ether:** White, soft semisolid, or pale yellow liquid, having a bland odor. Soluble in water and in alcohol. Dispersible in mineral oil and in propylene glycol, with possible separation on standing. *NF category:* Emulsifying agent; lubricant; wetting and/or solubilizing agent.

**Polyoxyl 15 Hydroxystearate:** Yellowish to white waxy mass. Very soluble in water; soluble in alcohol and in 2-propanol; insoluble in mineral oil. It solidifies at 25°. *NF category:* Wetting and/or solubilizing agent; vehicle (oleaginous); lubricant; emulsifying agent.

**Polyoxyl 20 Cetostearyl Ether:** Cream-colored, waxy, unctuous mass, melting, when heated, to a clear brownish-yellow liquid. Soluble in water, in alcohol, and in acetone; insoluble in solvent hexane. *NF category:* Wetting and/or solubilizing agent; emulsifying agent; lubricant.

**Polyoxyl 35 Castor Oil:** Yellow, oily liquid, having a faint, characteristic odor and a somewhat bitter taste. Very soluble in water, producing a practically odorless and colorless solution; soluble in alcohol and in ethyl acetate; insoluble in mineral oils. *NF category:* Wetting and/or solubilizing agent; emulsifying agent; lubricant.

**Polyoxyl 40 Hydrogenated Castor Oil:** White to yellowish paste or pasty liquid, having a faint odor and a slight taste. Very soluble in water, producing a practically tasteless, odorless, and colorless solution; soluble in alcohol and in ethyl acetate; insoluble in mineral oils. *NF category:* Wetting and/or solubilizing agent; emulsifying agent; lubricant.

**Polyoxyl Stearate:** White or slightly yellowish waxy mass. Soluble in alcohol and in isopropyl alcohol. Polyoxyl stearate corresponding to a product with 6–8 units of ethylene oxide per molecule is soluble in fatty oils and in waxes; practically insoluble in water. Polyoxyl stearate corresponding to a product with 20–100 units of ethylene oxide per molecule is soluble in water; practically insoluble in fatty oils and in waxes. *NF category:* Emulsifying agent; wetting and/or solubilizing agent.

**Polyoxyl Stearyl Ether:** A white to yellowish-white, waxy, unctuous mass, pellets, microbeads, or flakes. Polyoxyl Stearyl Ether with 2 oxyethylene units per molecule is soluble in alcohol, with heating, and in methylene chloride; practically insoluble in water. Polyoxyl Stearyl Ether with 10 oxyethylene units per molecule is soluble in water and in alcohol. Polyoxyl

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Stearyl Ether with 20 oxethylene units per molecule is soluble in water, in alcohol, and in methylene chloride. After melting, it solidifies at about 45°. *NF category:* Emulsifying agent; wetting and/or solubilizing agent.

**Polypropylene Glycol 11 Stearyl Ether:** Colorless or pale yellow, clear or slightly turbid liquid. Soluble in ethanol (96%), in mineral oils, and in 2-propanol; practically insoluble in water, in propylene glycol, and in glycerol. Relative density: about 0.94 at 25°. Refractive index: about 1.448 at 25°. *NF category:* Emollient; solvent; vehicle.

**Polysorbate 20:** Lemon to amber liquid having a faint characteristic odor. Soluble in water, in alcohol, in ethyl acetate, in methanol, and in dioxane; insoluble in mineral oil. *NF category:* Wetting and/or solubilizing agent; emulsifying agent; lubricant; suspending and/or viscosity-increasing agent.

**Polysorbate 40:** Yellow liquid having a faint, characteristic odor. Soluble in water and in alcohol; insoluble in mineral oil and in vegetable oils. *NF category:* Wetting and/or solubilizing agent; emulsifying agent; lubricant; suspending and/or viscosity-increasing agent.

**Polysorbate 60:** Lemon- to orange-colored, oily liquid or semi-gel having a faint, characteristic odor. Soluble in water, in ethyl acetate, and in toluene; insoluble in mineral oil and in vegetable oils. *NF category:* Emulsifying agent; lubricant; suspending and/or viscosity-increasing agent; wetting and/or solubilizing agent.

**Polysorbate 80:** Lemon- to amber-colored, oily liquid having a faint, characteristic odor and a warm, somewhat bitter taste. Very soluble in water, producing an odorless and practically colorless solution; soluble in alcohol and in ethyl acetate; insoluble in mineral oil. *NF category:* Wetting and/or solubilizing agent; emulsifying agent; lubricant; suspending and/or viscosity-increasing agent.

**Polyvinyl Acetate:** White or off-white powder or colorless granules or beads. Freely soluble in ethyl acetate; soluble in alcohol, in acetone, and in chloroform; practically insoluble in water. It is hygroscopic and swells in water. *NF category:* Coating agent; desiccant; wet binder; film-forming agent.

**Polyvinyl Acetate Dispersion:** Opaque, white or off-white, slightly viscous liquid. Miscible with water and with ethanol. It is sensitive to spoilage by microbial contaminants. *NF category:* Coating agent; film-forming agent; release-modifying agent.

**Polyvinyl Acetate Phthalate:** Free-flowing white powder. May have a slight odor of acetic acid. Soluble in methanol and in alcohol; insoluble in water, in methylene chloride, and in chloroform. *NF category:* Coating agent; film-forming agent.

**Polyvinyl Alcohol:** White to cream-colored granules, or white to cream-colored powder. Is odorless. Freely soluble in water at room temperature. Solution may be effected more rapidly at somewhat higher temperatures. *NF category:* Suspending and/or viscosity-increasing agent; coating agent; film-forming agent; polymers for ophthalmic use.

**Sulfurated Potash:** Irregular, liver-brown pieces when freshly made, changing to a greenish yellow. Has an odor of hydrogen sulfide and a bitter, acrid, and alkaline taste, and decomposes on exposure to air. A solution (1 in 10) is light brown in color and is alkaline to litmus. Freely soluble in water, usually leaving a slight residue. Alcohol dissolves only the sulfides.

**Potassium Acetate:** Colorless, monoclinic crystals or white, crystalline powder having a saline and slightly alkaline taste. Is odorless, or has a faint acetous odor. Deliquesces on exposure to moist air. Very soluble in water; freely soluble in alcohol.

**Potassium Alginate:** White to yellow, fibrous or granular powder. Dissolves in water to form a viscous, colloidal solution; insoluble in alcohol and in hydroalcoholic solutions in which the alcohol content is greater than 30% by weight; insoluble in chloroform, in ether, and in acids having a pH lower than about 3. *NF category:* Emulsifying agent; suspending and/or viscosity-increasing agent.

**Potassium Benzoate:** White, odorless, or practically odorless, granular or crystalline powder. Is stable in air. Freely soluble in water; soluble in 90% alcohol; sparingly soluble in alcohol. *NF category:* Antimicrobial preservative; lubricant.

**Potassium Bicarbonate:** Colorless, transparent, monoclinic prisms or as a white, granular powder. Is odorless, and is stable in air. Its solutions are neutral or alkaline to phenolphthalein TS. Freely soluble in water; practically insoluble in alcohol. *NF category:* pH modifier (acidifying agent/alkalizing agent/buffering agent).

**Potassium Bitartrate:** Colorless or slightly opaque crystals, or white, crystalline powder. A saturated solution is acid to litmus. Soluble in boiling water; slightly soluble in water; very slightly soluble in alcohol.

**Potassium Bromide:** White, crystalline powder or colorless, cubical crystals. Freely soluble in water and in glycerol; slightly soluble in alcohol.

**Potassium Chloride:** Colorless, elongated, prismatic, or cubical crystals, or white, granular powder. Is odorless, has a saline taste, and is stable in air. Its solutions are neutral to litmus. Freely soluble in water; insoluble in alcohol. *NF category:* Tonicity agent.

**Potassium Citrate:** Transparent crystals or white, granular powder. Is odorless, has a cooling, saline taste, and is deliquescent when exposed to moist air. Freely soluble in water; very slightly soluble in alcohol. *NF category:* pH modifier (acidifying agent/alkalizing agent/buffering agent); chelating and/or complexing agent.

**Potassium Gluconate:** White to yellowish-white, crystalline powder or granules. Is odorless, has a slightly bitter taste, and is stable in air. Its solutions are slightly alkaline to litmus. Freely soluble in water; practically insoluble in dehydrated alcohol, in ether, in benzene, and in chloroform.

**Potassium Hydroxide:** White or practically white, fused masses, or small pellets, or flakes, or sticks, or other forms. Is hard and brittle and shows a crystalline fracture. Exposed to air, it rapidly absorbs carbon dioxide and moisture, and deliquesces. Very soluble in boiling alcohol; freely soluble in water, in alcohol, and in glycerin. *NF category:* pH modifier (acidifying agent/alkalizing agent/buffering agent).

**Potassium Iodide:** Hexahedral crystals, either transparent and colorless or somewhat opaque and white, or a white, granular powder. Is slightly hygroscopic. Its solutions are neutral or alkaline to litmus. Very soluble in water and even more soluble in boiling water; freely soluble in glycerin; soluble in alcohol.

**Potassium Iodide Oral Solution:** Clear, colorless, odorless liquid, having a characteristic, strongly salty taste. Is neutral or alkaline to litmus. Specific gravity is about 1.70.

**Potassium Metabisulfite:** White or colorless, free-flowing crystals, crystalline powder, or granules, usually having an odor of sulfur dioxide. Gradually oxidizes in air to the sulfate. Its solutions are acid to litmus. Soluble in water; insoluble in alcohol. *NF category:* Antioxidant; antimicrobial preservative.

**Potassium Metaphosphate:** White, odorless powder. Soluble in dilute solutions of sodium salts; insoluble in water. *NF category:* pH modifier (acidifying agent/alkalizing agent/buffering agent).

**Potassium Nitrate:** White, crystalline powder or colorless crystals. Very soluble in boiling water; freely soluble in water; soluble in glycerin; practically insoluble in alcohol.

**Potassium Permanganate:** Dark purple crystals, almost opaque by transmitted light and of a blue metallic luster by reflected light. Its color is sometimes modified by a dark

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bronze-like appearance. Is stable in air. Freely soluble in boiling water; soluble in water.

**Dibasic Potassium Phosphate:** Colorless or white, somewhat hygroscopic, granular powder. The pH of a solution (1 in 20) is about 8.5 to 9.6. Freely soluble in water; very slightly soluble in alcohol. *NF category:* pH modifier (acidifying agent/alkalizing agent/buffering agent).

**Monobasic Potassium Phosphate:** Colorless crystals or white, granular or crystalline powder. Is odorless, and is stable in air. The pH of a solution (1 in 100) is about 4.5. Freely soluble in water; practically insoluble in alcohol. *NF category:* pH modifier (acidifying agent/alkalizing agent/buffering agent).

**Potassium Sodium Tartrate:** Colorless crystals or white, crystalline powder, having a cooling, saline taste. As it effloresces slightly in warm, dry air, the crystals are often coated with a white powder. Freely soluble in water; practically insoluble in alcohol.

**Potassium Sorbate:** White crystals or powder, having a characteristic odor. Melts at about 270°, with decomposition. Freely soluble in water; soluble in alcohol. *NF category:* Antimicrobial preservative.

**Povidone:** White to slightly creamy white powder. Is hygroscopic. Freely soluble in water, in methanol, and in alcohol; slightly soluble in acetone; practically insoluble in ether. *NF category:* Suspending and/or viscosity-increasing agent; wet binder; polymers for ophthalmic use.

**Povidone-Iodine:** Yellowish-brown to reddish-brown, amorphous powder, having a slight, characteristic odor. Its solution is acid to litmus. Soluble in water and in alcohol; practically insoluble in chloroform, in carbon tetrachloride, in ether, in solvent hexane, and in acetone.

**Povidone-Iodine Topical Aerosol Solution:** The liquid obtained from Povidone-Iodine Topical Aerosol Solution is transparent, having a reddish brown color.

**Pralidoxime Chloride:** White to pale-yellow, crystalline powder. Is odorless and is stable in air. Freely soluble in water.

**Pramipexole Dihydrochloride:** White to almost white crystalline powder. Freely soluble in water; soluble in methanol; slightly soluble in alcohol; practically insoluble in methylene chloride.

**Pramoxine Hydrochloride:** White to practically white, crystalline powder, having a numbing taste. May have a slight aromatic odor. The pH of a solution (1 in 100) is about 4.5. Freely soluble in water and in alcohol; soluble in chloroform; very slightly soluble in ether.

**Prasugrel Hydrochloride:** White to almost-white powder. Soluble in water; slightly soluble in acetonitrile; practically insoluble in ethyl acetate.

**Pravastatin Sodium:** White to yellowish white, hygroscopic powder. Freely soluble in water and in methanol; soluble in dehydrated alcohol; practically insoluble in acetonitrile and in chloroform.

**Praziquantel:** White or practically white, crystalline powder; odorless or having a faint characteristic odor. Freely soluble in alcohol and in chloroform; very slightly soluble in water.

**Prazosin Hydrochloride:** White to tan powder. Slightly soluble in water, in methanol, in dimethylformamide, and in dimethylacetamide; very slightly soluble in alcohol; practically insoluble in chloroform and in acetone.

**Prednicarbate:** White to almost white, crystalline powder. Freely soluble in acetone and in alcohol; sparingly soluble in propylene glycol; practically insoluble in water.

**Prednisolone:** White to practically white, odorless, crystalline powder. Melts at about 235°, with some decomposition (see *Melting Range or Temperature* ⟨741⟩). Soluble in methanol and in dioxane; sparingly soluble in acetone and in alcohol; slightly soluble in chloroform; very slightly soluble in water.

**Prednisolone Acetate:** White to practically white, odorless, crystalline powder. Melts at about 235°, with some decomposition (see *Melting Range or Temperature* ⟨741⟩). Slightly soluble in acetone, in alcohol, and in chloroform; practically insoluble in water.

**Prednisolone Hemisuccinate:** Fine, creamy white powder with friable lumps; practically odorless. Melts at about 205°, with decomposition. Freely soluble in alcohol; soluble in acetone; very slightly soluble in water.

**Prednisolone Sodium Phosphate:** White or slightly yellow, friable granules or powder. Is odorless or has a slight odor. Is slightly hygroscopic. Freely soluble in water; soluble in methanol; slightly soluble in alcohol and in chloroform; very slightly soluble in acetone and in dioxane.

**Prednisolone Sodium Succinate for Injection:** Creamy white powder with friable lumps, having a slight odor.

**Prednisolone Tebutate:** White to slightly yellow, free-flowing powder, which may show some soft lumps. Is odorless or has not more than a moderate, characteristic odor. Is hygroscopic. Freely soluble in chloroform and in dioxane; soluble in acetone; sparingly soluble in alcohol and in methanol; very slightly soluble in water.

**Prednisone:** White to practically white, odorless, crystalline powder. Melts at about 230°, with some decomposition (see *Melting Range or Temperature* ⟨741⟩). Slightly soluble in alcohol, in chloroform, in dioxane, and in methanol; very slightly soluble in water.

**Pregabalin:** White to off-white, crystalline solid. Freely soluble in water and in both basic and acidic aqueous solutions.

**Prilocaine:** White or almost white powder or crystal aggregates. Very soluble in alcohol and in acetone; slightly soluble in water.

**Prilocaine Hydrochloride:** White, odorless, crystalline powder, having a bitter taste. Freely soluble in water and in alcohol; slightly soluble in chloroform; very slightly soluble in acetone; practically insoluble in ether.

**Primaquine Phosphate:** Orange-red, crystalline powder. Is odorless and has a bitter taste. Its solutions are acid to litmus. Melts at about 200°. Soluble in water; insoluble in chloroform and in ether.

**Primidone:** White, crystalline powder. Is odorless and has a slightly bitter taste. Slightly soluble in alcohol; very slightly soluble in water and in most organic solvents.

**Probenecid:** White or practically white, fine, crystalline powder. Is practically odorless. Soluble in dilute alkali, in chloroform, in alcohol, and in acetone; practically insoluble in water and in dilute acids.

**Procainamide Hydrochloride:** White to tan, crystalline powder. Is odorless. Its solution (1 in 10) has a pH between 5 and 6.5. Very soluble in water; soluble in alcohol; slightly soluble in chloroform; very slightly soluble in benzene and in ether.

**Procainamide Hydrochloride Injection:** Colorless, or having not more than a slight yellow color.

**Procaine Hydrochloride:** Small, white crystals or white, crystalline powder. Is odorless. Exhibits local anesthetic properties when placed on the tongue. Freely soluble in water; soluble in alcohol; slightly soluble in chloroform; practically insoluble in ether.

**Procaine Hydrochloride Injection:** Clear, colorless liquid.

**Prochlorperazine:** Clear, pale yellow, viscous liquid. Is sensitive to light. Freely soluble in alcohol, in chloroform, and in ether; very slightly soluble in water.

**Prochlorperazine Edisylate:** White to very light yellow, odorless, crystalline powder. Its solutions are acid to litmus.

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Freely soluble in water; very slightly soluble in alcohol; insoluble in ether and in chloroform.

**Prochlorperazine Maleate:** White or pale yellow, practically odorless, crystalline powder. Its saturated solution is acid to litmus. Slightly soluble in warm chloroform; practically insoluble in water and in alcohol.

**Procyclidine Hydrochloride:** White, crystalline powder, having a moderate, characteristic odor. Melts at about 225°, with decomposition. Soluble in water and in alcohol; insoluble in ether and in acetone.

**Progesterone:** White or creamy white, odorless, crystalline powder. Is stable in air. Soluble in alcohol, in acetone, and in dioxane; sparingly soluble in vegetable oils; practically insoluble in water.

**Proguanil Hydrochloride:** White, crystalline powder. Sparingly soluble in alcohol; slightly soluble in water; practically insoluble in methylene chloride.

**Proline:** White, odorless crystals, having a slightly sweet taste. Freely soluble in water and in absolute alcohol; insoluble in ether, in butanol, and in isopropanol.

**Promazine Hydrochloride:** White to slightly yellow, practically odorless, crystalline powder. It oxidizes upon prolonged exposure to air and acquires a blue or pink color. Freely soluble in water and in chloroform.

**Promethazine Hydrochloride:** White to faint yellow, practically odorless, crystalline powder. Slowly oxidizes, and acquires a blue color, on prolonged exposure to air. Freely soluble in water, in hot dehydrated alcohol, and in chloroform; practically insoluble in ether, in acetone, and in ethyl acetate.

**Propafenone Hydrochloride:** White powder. Soluble in methanol and in hot water; slightly soluble in alcohol and in chloroform; very slightly soluble in acetone; insoluble in diethyl ether and in toluene.

**Propane:** Colorless, flammable gas (boiling temperature is about −42°). One hundred volumes of water dissolves 6.5 volumes at 17.8° and 753 mm pressure; 100 volumes of anhydrous alcohol dissolves 790 volumes at 16.6° and 754 mm pressure; 100 volumes of ether dissolves 926 volumes at 16.6° and 757 mm pressure; 100 volumes of chloroform dissolves 1299 volumes at 21.6° and 757 mm pressure. Vapor pressure at 21° is about 10290 mm of mercury (108 psig). *NF category:* Propellant.

**Propanediol:** Clear, colorless, hygroscopic liquid. *Specific Gravity* ⟨841⟩, *Method I*: 1.040–1.065. Soluble in water, in alcohol, in methyl alcohol, in isopropyl alcohol, in butyl alcohol, in acetone, in propylene glycol, and miscible with many polar solvents. *NF category:* Humectant; solvent; wetting and/or solubilizing agent.

**Propantheline Bromide:** White or practically white crystals. Is odorless and has a bitter taste. Melts at about 160°, with decomposition. Very soluble in water, in alcohol, and in chloroform; practically insoluble in ether and in benzene.

**Proparacaine Hydrochloride:** White to off-white, or faintly buff-colored, odorless, crystalline powder. Its solutions are neutral to litmus. Soluble in water, in warm alcohol, and in methanol; insoluble in ether and in benzene.

**Proparacaine Hydrochloride Ophthalmic Solution:** Colorless or faint yellow solution.

**Propionic Acid:** Oily liquid having a slight pungent, rancid odor. Miscible with water and with alcohol and various other organic solvents. *NF category:* pH modifier (acidifying agent/alkalizing agent/buffering agent); antimicrobial preservative; antioxidant.

**Propofol:** Clear, colorless to slightly yellowish liquid. Very soluble in methanol and in ethanol; slightly soluble in cyclohexane and in isopropyl alcohol; very slightly soluble in water.

**Propranolol Hydrochloride:** White to off-white, crystalline powder. Is odorless and has a bitter taste. Melts at about 164°. Soluble in water and in alcohol; slightly soluble in chloroform; practically insoluble in ether.

**Propyl Gallate:** White, crystalline powder having a very slight, characteristic odor. Freely soluble in alcohol; slightly soluble in water. *NF category:* Antioxidant.

**Propylene Glycol:** Clear, colorless, viscous liquid having a slight, characteristic taste. Is practically odorless. Absorbs moisture when exposed to moist air. Miscible with water, with acetone, and with chloroform. Soluble in ether and will dissolve many essential oils, but is immiscible with fixed oils. *NF category:* Humectant; plasticizer; solvent; antimicrobial preservative.

**Propylene Glycol Alginate:** White to yellowish fibrous or granular powder. Practically odorless and tasteless. Soluble in water, in solutions of dilute organic acids, and, depending on the degree of esterification, in hydroalcoholic mixture containing up to 60% by weight of alcohol to form stable, viscous colloidal solutions at a pH of 3. *NF category:* Suspending and/or viscosity-increasing agent; emulsifying agent.

**Propylene Glycol Diacetate:** Clear, colorless liquid having a mild, fruity odor. Soluble in water. *NF category*: Emulsifying agent; solvent.

**Propylene Glycol Dicaprylate/Dicaprate:** Clear, colorless or slightly yellow oily liquid at 20°. Soluble in fatty oils and in light petroleum; slightly soluble in dehydrated alcohol; practically insoluble in water. *NF category:* Emulsifying agent; vehicle.

**Propylene Glycol Dilaurate:** Clear, oily liquid at 20°. Colorless or slightly yellow. Very soluble in alcohol, in methanol, and in methylene chloride; practically insoluble in water. *NF category:* Emollient; emulsifying agent.

**Propylene Glycol Monocaprylate:** Clear, colorless, or slightly yellow, oily liquid at 20°. Very soluble in alcohol, in chloroform, and in methylene chloride; practically insoluble in water. *NF category:* Vehicle; emulsifying agent; diluent.

**Propylene Glycol Mono and Dicaprylate:** Clear, colorless, or slightly yellow, oily liquid at 20°. Very soluble in alcohol, in chloroform, and in methylene chloride; practically insoluble in water. *NF category*: Vehicle (solid carrier); emulsifying agent; diluent.

**Propylene Glycol Monolaurate:** Clear, oily liquid at 20°. Colorless or slightly yellow. Very soluble in alcohol, in methanol, and in methylene chloride; practically insoluble in water. *NF category:* Emollient; emulsifying agent.

**Propylene Glycol Monostearate:** White, wax-like solid or as white, wax-like beads or flakes. Has a slight, agreeable, fatty odor and taste. Soluble in organic solvents such as alcohol, mineral or fixed oils, benzene, ether, and acetone; insoluble in water, but may be dispersed in hot water with the aid of a small amount of soap or other suitable surface-active agent. *NF category:* Emulsifying agent.

**Propylhexedrine:** Clear, colorless liquid, having a characteristic, amine-like odor. Volatilizes slowly at room temperature. Absorbs carbon dioxide from the air, and its solutions are alkaline to litmus. Boils at about 205°. Very slightly soluble in water. Miscible with alcohol, with chloroform, and with ether.

**Propylparaben:** Small, colorless crystals or white powder. Freely soluble in alcohol and in ether; slightly soluble in boiling water; very slightly soluble in water. *NF category:* Antimicrobial preservative.

**Propylparaben Sodium:** White powder. Is odorless and hygroscopic. Freely soluble in water; sparingly soluble in alcohol; insoluble in fixed oils. *NF category:* Antimicrobial preservative.

**Propylthiouracil:** White, powdery, crystalline substance. Is starch-like in appearance and to the touch, and has a bitter taste. Soluble in ammonium hydroxide and in alkali

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hydroxides; sparingly soluble in alcohol; slightly soluble in water, in chloroform, and in ether.

**Protamine Sulfate Injection:** Colorless solution, which may have the odor of a preservative.

**Protriptyline Hydrochloride:** White to yellowish powder. Is odorless, or has not more than a slight odor. Melts at about 168°. Freely soluble in water, in alcohol, and in chloroform; practically insoluble in ether.

**Pseudoephedrine Hydrochloride:** Fine, white to off-white crystals or powder, having a faint characteristic odor. Very soluble in water; freely soluble in alcohol; slightly soluble in chloroform.

**Pseudoephedrine Sulfate:** White crystals or crystalline powder. Is odorless. Freely soluble in alcohol.

**Pullulan:** White powder. Freely soluble in water; practically insoluble in dehydrated alcohol. *NF category:* Coating agent; plasticizer; polymer membrane; sequestering agent; suspending and/or viscosity-increasing agent; wetting and/or solubilizing agent; bulking agent; diluent; disintegrant; wet binder; capsule shell; film-forming agent.

**Pumice:** Very light, hard, rough, porous, grayish masses or gritty, grayish powder. Is odorless and tasteless, and is stable in air. Practically insoluble in water; is not attacked by acids.

**Pyrantel Pamoate:** Yellow to tan solid. Soluble in dimethyl sulfoxide; slightly soluble in dimethylformamide; practically insoluble in water and in methanol.

**Pyrazinamide:** White to practically white, odorless or practically odorless, crystalline powder. Sparingly soluble in water; slightly soluble in alcohol, in ether, and in chloroform.

**Pyrethrum Extract:** Pale yellow liquid having a bland, flowery odor. Soluble in mineral oil and in most organic solvents; insoluble in water. *Pyrethrins I* denotes the group containing pyrethrin 1, cinerin 1, and jasmolin 1; *Pyrethrins II* denotes the group containing pyrethrin 2, cinerin 2, and jasmolin 2.

**Pyridostigmine Bromide:** White or practically white, crystalline powder, having an agreeable, characteristic odor. Is hygroscopic. Freely soluble in water, in alcohol, and in chloroform; slightly soluble in solvent hexane; practically insoluble in ether.

**Pyridoxine Hydrochloride:** White to practically white crystals or crystalline powder. Is stable in air, and is slowly affected by sunlight. Its solutions have a pH of about 3. Freely soluble in water; slightly soluble in alcohol; insoluble in ether.

**Pyrilamine Maleate:** White, crystalline powder, usually having a faint odor. Its solutions are acid to litmus. Very soluble in water; freely soluble in alcohol and in chloroform; slightly soluble in ether and in benzene.

**Pyrimethamine:** White, odorless, crystalline powder. Slightly soluble in acetone, in alcohol, and in chloroform; practically insoluble in water.

**Quazepam:** Off-white to yellowish powder.

**Quetiapine Fumarate:** White to off-white crystalline powder. Soluble in 0.1 N hydrochloric acid; slightly soluble in water, in alcohol, and in methanol.

**Quinapril Hydrochloride:** White to off-white powder, with a pink cast at times. Freely soluble in aqueous solvents.

**Quinidine Gluconate:** White powder. Is odorless and has a very bitter taste. Freely soluble in water; slightly soluble in alcohol.

**Quinidine Sulfate:** Fine, needle-like, white crystals, frequently cohering in masses, or fine, white powder. Is odorless, and darkens on exposure to light. Its solutions are neutral or alkaline to litmus. Soluble in alcohol; sparingly soluble in chloroform; slightly soluble in water; insoluble in ether.

**Quinine Sulfate:** White, fine, needle-like crystals, usually lusterless, making a light and readily compressible mass. Is odorless. It darkens on exposure to light. Its saturated solution is neutral or alkaline to litmus. Freely soluble in alcohol at 80°, and in a mixture of 2 volumes of chloroform and 1 volume of dehydrated alcohol; sparingly soluble in water at 100°; slightly soluble in water, in alcohol, and in chloroform; very slightly soluble in ether.

**Racemethionine:** Almost white, crystalline powder or small flakes. Sparingly soluble in water; very slightly soluble in alcohol. It dissolves in dilute acids and in dilute solutions of alkali hydroxides. It melts at about 270°. *NF category:* Antioxidant; pH modifier (acidifying agent/alkalizing agent/buffering agent); flavors and fragrance.

**Racepinephrine:** White to nearly white, crystalline, odorless powder, gradually darkening on exposure to light and air. With acids, it forms salts that are readily soluble in water, and the base may be recovered by the addition of ammonium hydroxide. Very slightly soluble in water and in alcohol; insoluble in ether, in chloroform, and in fixed and volatile oils.

**Racepinephrine Hydrochloride:** Fine, white, odorless powder. Darkens on exposure to light and air. Its solutions are acid to litmus. Melts at about 157°. Freely soluble in water; sparingly soluble in alcohol.

**Raloxifene Hydrochloride:** Almost white to pale yellow powder. Freely soluble in dimethylsulfoxide; slightly soluble to sparingly soluble in methanol; practically insoluble to very slightly soluble in water; practically insoluble in ether and in ethyl acetate.

**Raltegravir Potassium:** White to off-white powder. Soluble in water; slightly soluble in methanol; very slightly soluble in acetonitrile and in ethanol; insoluble in isopropyl alcohol.

**Ramipril:** White to almost white crystalline powder. Freely soluble in methanol; sparingly soluble in water.

**Ranitidine Hydrochloride:** White to pale yellow, crystalline, practically odorless powder. Is sensitive to light and moisture. Melts at about 140°, with decomposition. Very soluble in water; sparingly soluble in alcohol.

**Regorafenib:** White to pink or brownish powder. Sparingly soluble in acetone; slightly soluble in acetonitrile, in methanol, in ethanol, and in ethyl acetate; practically insoluble in water and in heptane.

**Fully Hydrogenated Rapeseed Oil:** White, waxy solid. Insoluble in water and in alcohol. *NF category:* Coating agent; stiffening agent.

**Superglycerinated Fully Hydrogenated Rapeseed Oil:** White solid. Insoluble in water and in alcohol. *NF category:* Coating agent; emulsifying agent; stiffening agent.

**Purified Rayon:** White, lustrous or dull, fine, soft, filamentous fibers, appearing under the microscope as round, oval, or slightly flattened translucent rods, straight or crimped, striate and with serrate cross-sectional edges. Is practically odorless and practically tasteless. Very soluble in ammoniated cupric oxide TS and in dilute sulfuric acid (3 in 5); insoluble in ordinary solvents.

**Repaglinide:** White to off-white solid. Melts at about 132° to 136°. Soluble in methanol.

**Reserpine:** White or pale buff to slightly yellowish, odorless, crystalline powder. Darkens slowly on exposure to light, but more rapidly when in solution. Freely soluble in acetic acid and in chloroform; slightly soluble in benzene; very slightly soluble in alcohol and in ether; insoluble in water.

**Resorcinol:** White, or practically white, needle-shaped crystals or powder. Has a faint, characteristic odor and a sweetish, followed by a bitter, taste. Acquires a pink tint on exposure to light and air. Its solution (1 in 20) is neutral or acid to litmus. Freely soluble in water, in alcohol, in glycerin, and in ether; slightly soluble in chloroform.

**Ribavirin:** White, crystalline powder. Freely soluble in water; slightly soluble in dehydrated alcohol.

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**Riboflavin:** Yellow to orange-yellow, crystalline powder having a slight odor. Melts at about 280°. Its saturated solution is neutral to litmus. When dry, it is not appreciably affected by diffused light, but when in solution, light induces quite rapid deterioration, especially in the presence of alkalies. Soluble in dilute solutions of alkalies; very slightly soluble in water, in alcohol, and in isotonic sodium chloride solution; insoluble in ether and in chloroform.

**Riboflavin 5′-Phosphate Sodium:** Fine, orange-yellow, crystalline powder, having a slight odor. Sparingly soluble in water. When dry, it is not affected by diffused light, but when in solution, light induces deterioration rapidly. Is hygroscopic.

**Rifabutin:** Amorphous red-violet powder. Soluble in chloroform and in methanol; sparingly soluble in alcohol; very slightly soluble in water.

**Rifampin:** Red-brown, crystalline powder. Freely soluble in chloroform; soluble in ethyl acetate and in methanol; very slightly soluble in water.

**Riluzole:** White to slightly yellow powder or crystalline powder. Freely soluble in acetonitrile, in alcohol, and in methylene chloride; slightly soluble in hexane; very slightly soluble in water.

**Rimexolone:** White to off-white powder. Freely soluble in chloroform; sparingly soluble in methanol.

**Riociguat:** White or yellowish powder. Very slightly soluble in anhydrous ethanol; practically insoluble in water and in heptane.

**Risedronate Sodium:** White to off-white powder. Soluble in water and in aqueous solutions; insoluble in common organic solvents.

**Risperidone:** White or almost white powder. Soluble in methylene chloride; sparingly soluble in alcohol; practically insoluble in water.

**Ritonavir:** White to light tan powder. Freely soluble in methanol and in methylene chloride; very slightly soluble in acetonitrile; practically insoluble in water.

**Rivaroxaban:** White to yellow powder. Slightly soluble in organic solvents (e.g., acetone, polyethylene glycol 400); practically insoluble in water and in aqueous media.

**Rivastigmine Tartrate:** White to off-white powder. Very soluble in water and in methanol; very slightly soluble in ethyl acetate.

**Rizatriptan Benzoate:** White to almost white crystalline powder. Soluble in water; sparingly soluble in alcohol; slightly soluble in methylene chloride.

**Rocuronium Bromide:** Almost white or pale yellow. Slightly hygroscopic powder. Freely soluble in water and in dehydrated alcohol.

**Roflumilast:** White to off-white powder. Freely soluble in acetone; sparingly soluble in ethanol; practically insoluble in water and in hexane.

**Ropinirole Hydrochloride:** Pale cream to yellow powder. Soluble in water.

**Ropivacaine:** White to off-white powder. Freely soluble in methylene chloride; soluble in ethanol; practically insoluble in water.

**Ropivacaine Hydrochloride:** White, crystalline powder. Soluble in water.

**Rose Oil:** Colorless or yellow liquid, having the characteristic odor and taste of rose. At 25° is a viscous liquid. Upon gradual cooling, changes to a translucent, crystalline mass, easily liquefied by warming. *NF category:* Flavors and fragrance.

**Rose Water Ointment:** *NF category:* Ointment base.

**Stronger Rose Water:** Practically colorless and clear, having the pleasant odor and taste of fresh rose blossoms. Is free from empyreuma, mustiness, and fungal growths. *NF category:* Flavors and fragrance.

**Rosiglitazone Maleate:** White to off-white solid. Sparingly soluble in alcohol; slightly soluble in methylene chloride; practically insoluble to very slightly soluble in water.

**Rosuvastatin Calcium:** A white to off-white, amorphous, hydrophilic powder. Soluble in dimethyl formamide, in dimethyl sulfoxide, in acetone, and in acetonitrile; sparingly soluble in methanol; sparingly soluble to slightly soluble in water; slightly soluble in ethanol.

**Rotigotine:** White or light-brownish powder. Soluble in anhydrous alcohol; very slightly soluble in heptane; practically insoluble in water.

**Rufinamide:** White, crystalline neutral powder. Slightly soluble in tetrahydrofuran and in methanol; very slightly soluble in alcohol and in acetonitrile; practically insoluble in water.

**Saccharin:** White crystals or white, crystalline powder. Is odorless or has a faint, aromatic odor. In dilute solution, it is intensely sweet. Its solutions are acid to litmus. Soluble in boiling water; sparingly soluble in alcohol; slightly soluble in water, in chloroform, and in ether. Is readily dissolved by dilute solutions of ammonia, by solutions of alkali hydroxides, and by solutions of alkali carbonates with the evolution of carbon dioxide. *NF category:* Sweetening agent.

**Saccharin Calcium:** White crystals or white, crystalline powder. Is odorless, or has a faint, aromatic odor, and has an intensely sweet taste even in dilute solutions. Its dilute solution is about 300 times as sweet as sucrose. Freely soluble in water. *NF category:* Sweetening agent.

**Saccharin Sodium:** White crystals or white, crystalline powder. Is odorless, or has a faint, aromatic odor, and has an intensely sweet taste even in dilute solutions. Its dilute solution is about 300 times as sweet as sucrose. When in powdered form, it usually contains about one-third the theoretical amount of water of hydration as a result of efflorescence. Freely soluble in water; sparingly soluble in alcohol. *NF category:* Sweetening agent.

**Saccharin Sodium Oral Solution:** Clear, colorless, odorless liquid, having a sweet taste.

**Safflower Oil:** Light yellow oil. Thickens and becomes rancid on prolonged exposure to air. Insoluble in water. Miscible with ether and with chloroform. *NF category:* Vehicle (oleaginous); emollient; solvent.

**Salicylamide:** White, practically odorless, crystalline powder. Freely soluble in ether and in solutions of alkalies; soluble in alcohol and in propylene glycol; slightly soluble in water and in chloroform.

**Salicylic Acid:** White crystals, usually in fine needles, or fluffy, white, crystalline powder. Has a sweetish, followed by an acrid, taste and is stable in air. The synthetic form is white and odorless. When prepared from natural methyl salicylate, it may have a slightly yellow or pink tint, and a faint, mint-like odor. Freely soluble in alcohol and in ether; soluble in boiling water; sparingly soluble in chloroform; slightly soluble in water and in benzene.

**Salmeterol Xinafoate:** White to off-white powder. Soluble in methanol; slightly soluble in alcohol, in isopropanol, and in chloroform; practically insoluble in water (pH 8), and in saline solution (0.9% w/w).

**Scopolamine Hydrobromide:** Colorless or white crystals or white, granular powder. Melts at about 197°, with decomposition. Is odorless, and slightly efflorescent in dry air. Freely soluble in water; soluble in alcohol; slightly soluble in chloroform; insoluble in ether.

**Secobarbital:** White, amorphous or crystalline, odorless powder, having a slightly bitter taste. Its saturated solution has a pH of about 5.6. Freely soluble in alcohol, in ether, and in solutions of fixed alkali hydroxides and carbonates; soluble in chloroform; very slightly soluble in water.

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**Secobarbital Sodium:** White powder. Is odorless, has a bitter taste, and is hygroscopic. Its solutions decompose on standing, heat accelerating the decomposition. Very soluble in water; soluble in alcohol; practically insoluble in ether.

**Selegiline Hydrochloride:** White, odorless, crystalline powder. Freely soluble in water, in chloroform, and in methanol.

**Selenium Sulfide:** Reddish-brown to bright orange powder, having not more than a faint odor. Practically insoluble in water and in organic solvents.

**Sennosides:** Brownish powder.

**Serine:** White, odorless crystals, having a sweet taste. Soluble in water; practically insoluble in absolute alcohol and in ether.

**Sertraline Hydrochloride:** White or off-white crystalline powder. Sparingly soluble or slightly soluble in absolute alcohol; slightly soluble in water and in isopropanol; and slightly or very slightly soluble in acetone.

**Sesame Oil:** Pale yellow, oily liquid. Is practically odorless, and has a bland taste. Slightly soluble in alcohol. Miscible with ether, with chloroform, with solvent hexane, and with carbon disulfide. *NF category:* Solvent, vehicle (oleaginous).

**Sevoflurane:** Clear, colorless, volatile, nonflammable liquid. Slightly soluble in water. Miscible with alcohol, with chloroform, and with ether.

**Shellac:** *Orange Shellac*—Thin, hard, brittle, transparent, pale lemon-yellow to brownish orange flakes, having little or no odor; *Bleached Shellac*—Opaque, amorphous cream to yellow granules or coarse powder, having little or no odor. Soluble (very slowly) in alcohol, 85% to 95% (w/w), in ether, 13% to 15%, in benzene, 10% to 20%, in petroleum ether, 2% to 6%; soluble in aqueous solutions of ethanolamines, alkalies, and borax; sparingly soluble in oil of turpentine; insoluble in water. *NF category:* Coating agent; film-forming agent; release-modifying agent.

**Sildenafil Citrate:** White or almost white slightly hygroscopic crystalline powder. Slightly soluble in water and in methanol; practically insoluble in hexane.

**Dental-Type Silica:** Fine, white, hygroscopic, odorless, amorphous powder, in which the diameter of the average particles ranges between 0.5 and 40 µm. Soluble in hot solutions of alkali hydroxides; insoluble in water, in alcohol, and in acid (except hydrofluoric acid). *NF category:* Glidant and/or anticaking agent; suspending and/or viscosity-increasing agent.

**Hydrophobic Colloidal Silica:** Light, fine, white or almost white, amorphous powder, not wettable by water. Dissolves slowly in hot solutions of alkali hydroxides. Practically insoluble in water and in mineral acids, except hydrofluoric acid. *NF category:* Glidant and/or anticaking agent; suspending and/or viscosity-increasing agent.

**Purified Siliceous Earth:** Very fine, white, light gray, or pale buff mixture of amorphous powder and lesser amounts of crystalline polymorphs, including quartz and cristobalite. Is gritty, readily absorbs moisture, and retains about four times its weight of water without becoming fluid. Insoluble in water, in acids, and in dilute solutions of the alkali hydroxides. *NF category:* Filtering aid; sorbent.

**Silicon Dioxide:** Fine, white, hygroscopic, odorless, amorphous powder, in which the diameter of the average particles ranges between 2 and 10 µm. Soluble in hot solutions of alkali hydroxides; insoluble in water, in alcohol, and in other organic solvents. *NF category:* Desiccant; suspending and/or viscosity-increasing agent.

**Colloidal Silicon Dioxide:** Light, white, nongritty powder of extremely fine particle size (about 15 nm). Soluble in hot solutions of alkali hydroxides; insoluble in water and in acid (except hydrofluoric). *NF category:* Glidant and/or anticaking agent; suspending and/or viscosity-increasing agent; disintegrant.

**Silver Nitrate:** Colorless or white crystals. The pH of its solutions is about 5.5. On exposure to light in the presence of organic matter, it becomes gray or grayish black. Very soluble in water and even more so in boiling water; freely soluble in boiling alcohol; sparingly soluble in alcohol; slightly soluble in ether.

**Toughened Silver Nitrate:** White, crystalline masses generally molded as pencils or cones. It breaks with a fibrous fracture. Its solutions are neutral to litmus. It becomes gray or grayish black upon exposure to light. Soluble in water to the extent of its nitrate content (there is always a residue of silver chloride); partially soluble in alcohol; slightly soluble in ether.

**Simethicone:** Translucent, gray, viscous fluid. The liquid phase is soluble in chloroform, in ether, and in benzene, but silicon dioxide remains as a residue in these solvents. Insoluble in water and in alcohol. *NF category:* Antifoaming agent; water-repelling agent; diluent.

**Simvastatin:** White to off-white powder. Freely soluble in chloroform, in methanol, and in alcohol; sparingly soluble in propylene glycol; very slightly soluble in hexane; practically insoluble in water.

**Sitagliptin Phosphate:** White or almost white powder. Soluble in water; very slightly soluble in anhydrous ethanol; practically insoluble in heptane.

**Soda Lime:** White or grayish-white granules. May have a color if an indicator has been added. *NF category:* Sorbent, carbon dioxide.

**Sodium Acetate:** Colorless, transparent crystals, or white, granular crystalline powder, or white flakes. Is odorless or has a faint acetous odor, and has a slightly bitter, saline taste. Is efflorescent in warm, dry air. Very soluble in water; soluble in alcohol. *NF category:* Flavors and fragrance; pH modifier (acidifying agent/alkalizing agent/buffering agent); antimicrobial preservative; transfer ligand.

**Sodium Alginate:** Practically odorless and tasteless, coarse or fine powder, yellowish white in color. Soluble in water, forming a viscous, colloidal solution; insoluble in alcohol and in hydroalcoholic solutions in which the alcohol content is greater than about 30% by weight, in chloroform, in ether, and in acids when the pH of the resulting solution becomes lower than about 3. *NF category:* Suspending and/or viscosity-increasing agent; film-forming agent; release-modifying agent; disintegrant; wet binder.

**Sodium Ascorbate:** White or very faintly yellow crystals or crystalline powder. Is odorless or practically odorless. Is relatively stable in air. On exposure to light it gradually darkens. Freely soluble in water; very slightly soluble in alcohol; insoluble in chloroform and in ether. *NF category:* Antioxidant.

**Sodium Benzoate:** White, odorless or practically odorless, granular or crystalline powder. Is stable in air. Freely soluble in water; soluble in 90% alcohol; sparingly soluble in alcohol. *NF category:* Antimicrobial preservative; lubricant.

**Sodium Bicarbonate:** White, crystalline powder. Is stable in dry air, but slowly decomposes in moist air. Its solutions, when freshly prepared with cold water, without shaking, are alkaline to litmus. The alkalinity increases as the solutions stand, as they are agitated, or as they are heated. Soluble in water; insoluble in alcohol. *NF category:* pH modifier (acidifying agent/alkalizing agent/buffering agent).

**Sodium Borate:** Colorless, transparent crystals or white, crystalline powder. Is odorless. Its solutions are alkaline to phenolphthalein TS. As it effloresces in warm, dry air, the crystals are often coated with white powder. Freely soluble in boiling water and in glycerin; soluble in water; insoluble in alcohol. *NF category:* pH modifier (acidifying agent/alkalizing agent/buffering agent); antimicrobial preservative; emulsifying agent.

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**Sodium Bromide:** White, crystalline powder or colorless, cubical crystals. Freely soluble in water; soluble in alcohol.

**Sodium Butyrate:** Clear, colorless, hygroscopic powder. Soluble in water and in methanol. Melting range is about 250° to 253°.

**Sodium Caprylate:** A white, crystalline powder. Very soluble or freely soluble in water; freely soluble in acetic acid; sparingly soluble in alcohol; practically insoluble in acetone.

**Sodium Carbonate:** Colorless crystals, or white, crystalline powder or granules. Is stable in air under ordinary conditions. When exposed to dry air above 50°, the hydrous salt effloresces and, at 100°, becomes anhydrous. Very soluble in boiling water; freely soluble in water. *NF category:* pH modifier (acidifying agent/alkalizing agent/buffering agent).

**Sodium Cetostearyl Sulfate:** A white or pale yellow, amorphous or crystalline powder. Soluble in hot water giving an opalescent solution; slightly soluble in alcohol; practically insoluble in cold water. *NF category:* Emulsifying agent.

**Sodium Chloride:** Colorless, cubic crystals or white crystalline powder. Has a saline taste. Freely soluble in water; soluble in glycerin; slightly soluble in alcohol. *NF category:* Tonicity agent; diluent.

**Sodium Chloride Inhalation Solution:** Clear, colorless solution.

**Bacteriostatic Sodium Chloride Injection:** Clear, colorless solution, odorless or having the odor of the bacteriostatic substance. *NF category:* Vehicle (sterile).

**Sodium Chloride Irrigation:** Clear, colorless solution.

**Sodium Citrate:** Colorless crystals, or white, crystalline powder. Hydrous form very soluble in boiling water; freely soluble in water; insoluble in alcohol. *NF category:* pH modifier (acidifying agent/alkalizing agent/buffering agent); sequestering agent; transfer ligand.

**Sodium Citrate and Citric Acid Oral Solution:** Clear solution having the color of any added preservative or flavoring agents.

**Sodium Dehydroacetate:** White or practically white, odorless powder, having a slight characteristic taste. Freely soluble in water, in propylene glycol, and in glycerin. *NF category:* Antimicrobial preservative.

**Sodium Fluoride:** White, odorless powder. Soluble in water; insoluble in alcohol.

**Sodium Formaldehyde Sulfoxylate:** White crystals or hard, white masses, having the characteristic odor of garlic. Freely soluble in water; slightly soluble in alcohol, in ether, in chloroform, and in benzene. *NF category:* Antioxidant.

**Sodium Hydroxide:** White, or practically white, fused masses, in small pellets, in flakes, or sticks, and in other forms. Is hard and brittle and shows a crystalline fracture. Exposed to the air, it rapidly absorbs carbon dioxide and moisture. Freely soluble in water and in alcohol. *NF category:* pH modifier (acidifying agent/alkalizing agent/buffering agent).

**Sodium Hypochlorite Solution:** Clear, pale greenish-yellow liquid, having the odor of chlorine. Is affected by light.

**Sodium Iodide:** Colorless, odorless crystals, or white, crystalline powder. Is deliquescent in moist air, and develops a brown tint upon decomposition. Very soluble in water; freely soluble in alcohol and in glycerin.

**Sodium Lactate Solution:** Clear, colorless or practically colorless, slightly viscous liquid, odorless or having a slight, not unpleasant odor. Miscible with water. *NF category:* pH modifier (acidifying agent/alkalizing agent/buffering agent); antimicrobial preservative; humectant; flavors and fragrance.

**Sodium Lauroyl Sarcosinate:** White or almost white powder. Freely soluble in water, giving a clear or slightly hazy solution; sparingly soluble in alcohol. *NF category*: Emulsifying agent; surfactant; wetting and/or solubilizing agent.

**Sodium Lauryl Sulfate:** Small, white or light yellow crystals having a slight, characteristic odor. Freely soluble in water, forming an opalescent solution. *NF category:* Emulsifying agent; lubricant; wetting and/or solubilizing agent.

**Sodium Metabisulfite:** White crystals or white to yellowish, crystalline powder, having the odor of sulfur dioxide. Freely soluble in water and in glycerin; slightly soluble in alcohol. *NF category:* Antioxidant; antimicrobial preservative.

**Sodium Monofluorophosphate:** White to slightly gray, odorless powder. Freely soluble in water.

**Sodium Nitrite:** White to slightly yellow, granular powder, or white or practically white, opaque, fused masses or sticks. Has a mild, saline taste and is deliquescent in air. Its solutions are alkaline to litmus. Freely soluble in water; sparingly soluble in alcohol.

**Sodium Nitrite Injection:** Clear, colorless liquid.

**Sodium Nitroprusside:** Reddish-brown, practically odorless, crystals or powder. Freely soluble in water; slightly soluble in alcohol; very slightly soluble in chloroform; insoluble in benzene.

**Dibasic Sodium Phosphate (*dried*):** White powder that readily absorbs moisture. Freely soluble in water; insoluble in alcohol. *NF category:* pH modifier (acidifying agent/alkalizing agent/buffering agent); chelating and/or complexing agent.

**Dibasic Sodium Phosphate (*heptahydrate*):** Colorless or white, granular or caked salt. Effloresces in warm, dry air. Its solutions are alkaline to phenolphthalein TS, a 0.1 M solution having a pH of about 9. Freely soluble in water; very slightly soluble in alcohol. *NF category:* Buffering agent.

**Monobasic Sodium Phosphate:** Colorless crystals or white, crystalline powder. Is odorless and is slightly deliquescent. Its solutions are acid to litmus and effervesce with sodium carbonate. Freely soluble in water; practically insoluble in alcohol. *NF category:* pH modifier (acidifying agent/alkalizing agent/buffering agent); chelating and/or complexing agent.

**Tribasic Sodium Phosphate:** The formula for a crystalline material is approximately $4(Na_3PO_4 \cdot 12H_2O)NaOH$. It occurs as white, odorless crystals or granules or as a crystalline powder. Freely soluble in water; insoluble in alcohol. The pH of a 1 in 100 solution is between 11.5 and 12.0.

**Sodium Picosulfate:** White or almost white crystalline powder. Freely soluble in water; slightly soluble in alcohol.

**Sodium Polystyrene Sulfonate:** Golden brown, fine powder. Is odorless and has a characteristic taste. Insoluble in water.

**Sodium Propionate:** Colorless, transparent crystals or granular, crystalline powder. Is odorless, or has a faint acetic-butyric odor and is deliquescent in moist air. Very soluble in water; soluble in alcohol. *NF category:* Antimicrobial preservative.

**Sodium Salicylate:** Amorphous or microcrystalline powder or scales. Is colorless, or has not more than a faint, pink tinge. Is odorless, or has a faint, characteristic odor, and is affected by light. A freshly made solution (1 in 10) is neutral or acid to litmus. Very soluble in boiling water and in boiling alcohol; freely (and slowly) soluble in water and in glycerin; slowly soluble in alcohol.

**Sodium Starch Glycolate:** White, tasteless, odorless, relatively free-flowing powder; available in several different viscosity grades. A 2% (w/v) dispersion in cold water settles, on standing, in the form of a highly hydrated layer. *NF category:* Disintegrant.

**Sodium Stearate:** Fine, white powder, soapy to the touch, usually having a slight, tallow-like odor. Is affected by light. Its solutions are alkaline to phenolphthalein TS. Slowly soluble in cold water and in cold alcohol; readily soluble in hot water and

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in hot alcohol. *NF category:* Emulsifying agent; glidant and/or anticaking agent; stiffening agent; lubricant.

**Sodium Stearyl Fumarate:** Fine, white powder. Slightly soluble in methanol; practically insoluble in water. *NF category:* Lubricant.

**Sodium Succinate:** White crystals or white powder. Freely soluble in water. *NF category:* Flavors and fragrance; pH modifier.

**Sodium Sulfate:** Large, colorless, odorless, transparent crystals, or a granular powder. Effloresces rapidly in air, liquefies in its water of hydration at about 33°, and loses all of its water of hydration at about 100°. Freely soluble in water; soluble in glycerin; insoluble in alcohol.

**Sodium Sulfite:** Colorless crystals. Freely soluble in water; very slightly soluble in alcohol. *NF category:* Antioxidant; antimicrobial preservative.

**Sodium Tartrate:** Transparent, colorless, odorless crystals. Freely soluble in water; insoluble in alcohol. *NF category:* Sequestering agent; transfer ligand.

**Sodium Thiosulfate:** Large, colorless crystals or coarse, crystalline powder. Is deliquescent in moist air and effloresces in dry air at temperatures exceeding 33°. Its solutions are neutral or faintly alkaline to litmus. Very soluble in water; insoluble in alcohol. *NF category:* Antioxidant.

**Solifenacin Succinate:** White to pale, yellowish-white crystal or crystalline powder. Freely soluble in water and in methanol.

**Sorafenib Tosylate:** White or slightly yellowish or brownish powder. Soluble in PEG 400; slightly soluble in ethanol; practically insoluble in aqueous media and in heptane.

**Sorbic Acid:** Free-flowing, white, crystalline powder, having a characteristic odor. Soluble in alcohol and in ether; slightly soluble in water. *NF category:* Antimicrobial preservative.

**Sorbitan Monolaurate:** Yellow to amber-colored, oily liquid, having a bland, characteristic odor. Soluble in mineral oil; slightly soluble in cottonseed oil and in ethyl acetate; insoluble in water. *NF category:* Emulsifying agent; lubricant; suspending and/or viscosity-increasing agent; wetting and/or solubilizing agent.

**Sorbitan Monooleate:** Viscous, yellow to amber-colored, oily liquid, having a bland, characteristic odor. Insoluble in water and in propylene glycol. Miscible with mineral and vegetable oils. *NF category:* Emulsifying agent; lubricant; suspending and/or viscosity-increasing agent; wetting and/or solubilizing agent.

**Sorbitan Monopalmitate:** Cream-colored, waxy solid having a faint fatty odor. Soluble in warm absolute alcohol; soluble, with haze, in warm peanut oil and in warm mineral oil; insoluble in water. *NF category:* Emulsifying agent; lubricant; suspending and/or viscosity-increasing agent; wetting and/or solubilizing agent.

**Sorbitan Monostearate:** Cream-colored to tan, hard, waxy solid, having a bland odor and taste. Soluble, with haze, above 50° in mineral oil and in ethyl acetate; insoluble in cold water and in acetone. Dispersible in warm water. *NF category:* Emulsifying agent; lubricant; suspending and/or viscosity-increasing agent; wetting and/or solubilizing agent.

**Sorbitan Sesquioleate:** Viscous, yellow to amber-colored, oily liquid. Soluble in alcohol, in isopropyl alcohol, in cottonseed oil, and in mineral oil; insoluble in water and in propylene glycol. *NF category:* Emulsifying agent; lubricant; suspending and/or viscosity-increasing agent; wetting and/or solubilizing agent.

**Sorbitan Trioleate:** Yellow to amber-colored, oily liquid. Soluble in methyl alcohol, in alcohol, in isopropyl alcohol, in corn oil, in cottonseed oil, and in mineral oil; insoluble in water, in ethylene glycol, and in propylene glycol. *NF category:* Emulsifying agent; lubricant; suspending and/or viscosity-increasing agent; wetting and/or solubilizing agent.

**Sorbitol:** D-Sorbitol occurs as white granules, powder, or crystalline masses. Is odorless, and has a sweet taste with a cold sensation. Very soluble in water; sparingly soluble in alcohol; and practically insoluble in ethyl ether. Is hygroscopic. *NF category:* Humectant; sweetening agent; diluent; plasticizer.

**Sorbitol Solution:** Clear, colorless, syrupy liquid. Is odorless and has a sweet taste. It sometimes separates into crystalline masses. Miscible with water, with alcohol, with glycerin, and with propylene glycol. Is neutral to litmus. *NF category:* Sweetening agent; vehicle (flavored and/or sweetened).

**Sorbitol Sorbitan Solution:** A clear, colorless to pale yellow, syrupy liquid. Is odorless and has a sweet taste. Insoluble in mineral oil and in vegetable oil. Miscible with water, with alcohol, with glycerin, and with propylene glycol. *NF category:* Humectant; plasticizer.

**Sotalol Hydrochloride:** White, crystalline powder. It is hydrophilic. Soluble in water, in propylene glycol, and in ethanol; slightly soluble in chloroform.

**Soybean Oil:** Clear, pale yellow, oily liquid having a characteristic odor and taste. Insoluble in water. Miscible with ether and with chloroform. *Specific Gravity* ⟨841⟩: between 0.916 and 0.922. *Refractive Index* ⟨831⟩: between 1.465 and 1.475. *NF category:* Vehicle (oleaginous); solvent.

**Hydrogenated Soybean Oil:** A white mass or powder that melts to a clear, pale yellow liquid when heated. Freely soluble in methylene chloride, in hexane after heating, and in toluene; very slightly soluble in alcohol; practically insoluble in water. *NF category:* Emollient.

**Spectinomycin Hydrochloride:** White to pale-buff crystalline powder. Freely soluble in water; practically insoluble in alcohol, in chloroform, and in ether.

**Spironolactone:** Light cream-colored to light tan, crystalline powder. Has a faint to mild mercaptan-like odor; is stable in air. Freely soluble in benzene and in chloroform; soluble in ethyl acetate and in alcohol; slightly soluble in methanol and in fixed oils; practically insoluble in water.

**Squalane:** Colorless, practically odorless transparent oil. Slightly soluble in acetone; very slightly soluble in absolute alcohol; insoluble in water. Miscible with ether and with chloroform. *NF category:* Ointment base; vehicle (oleaginous).

**Stannous Chloride:** White, crystalline powder or colorless crystals, efflorescent in air. Freely soluble in water (the solution becomes cloudy after standing or on dilution) and in alcohol. Dissolves in dilute hydrochloric acid. *NF category:* Emulsifying agent; antioxidant; reducing agent.

**Stannous Fluoride:** White, crystalline powder, having a bitter, salty taste. Melts at about 213°. Freely soluble in water; practically insoluble in alcohol, in ether, and in chloroform. *NF category:* Reducing agent.

**Stanozolol:** Odorless, crystalline powder, occurring in two forms: as needles, melting at about 155°, and as prisms, melting at about 235°. Soluble in dimethylformamide; sparingly soluble in alcohol and in chloroform; slightly soluble in ethyl acetate and in acetone; very slightly soluble in benzene; insoluble in water.

**Star Anise Oil:** Clear, colorless to light yellow in the liquid state having a powerful and characteristic licorice–like scent. Congeals between 15° and 19°. Miscible with alcohol. *NF category*: Flavors and fragrance.

**Corn Starch:** Irregular, angular, white masses (which may be bleached) or fine powder. Is odorless, and has a slight, characteristic taste. Insoluble in cold water and in alcohol. *NF category:* Diluent; disintegrant; wet binder; suspending and/or viscosity-increasing agent.

**Hydroxypropyl Corn Starch:** White or slightly yellowish powder. Practically insoluble in cold water and in alcohol. *NF*

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*category:* Diluent; disintegrant; wet binder; emulsifying agent; suspending and/or viscosity-increasing agent.

**Pregelatinized Hydroxypropyl Corn Starch:** White or slightly yellowish powder. It swells in water and produces a clear or translucent, viscous, colloidal mixture. *NF category:* Suspending and/or viscosity-increasing agent; diluent; disintegrant; wet binder; emulsifying agent.

**Pea Starch:** White or almost white, very fine powder. Practically insoluble in cold water and in alcohol. *NF category:* Suspending and/or viscosity-increasing agent; diluent; disintegrant; wet binder.

**Hydroxypropyl Pea Starch:** White or slightly yellowish powder. Practically insoluble in cold water and in alcohol. *NF category:* Suspending and/or viscosity-increasing agent; diluent; disintegrant; wet binder; emulsifying agent.

**Pregelatinized Hydroxypropyl Pea Starch:** White or slightly yellowish powder. It swells in water and produces a clear or translucent, viscous, colloidal mixture. *NF category:* Suspending and/or viscosity-increasing agent; wet binder; diluent; disintegrant.

**Potato Starch:** Irregular, angular, white masses (which may be bleached) or fine powder. Is odorless, and has a slight, characteristic taste. Insoluble in cold water and in alcohol. *NF category:* Suspending and/or viscosity-increasing agent; diluent; disintegrant; wet binder.

**Hydroxypropyl Potato Starch:** White or slightly yellowish powder. Practically insoluble in cold water and in alcohol. *NF category:* Suspending and/or viscosity-increasing agent; diluent; disintegrant; wet binder; emulsifying agent.

**Pregelatinized Hydroxypropyl Potato Starch:** White or slightly yellowish powder. It swells in water and produces a clear or translucent, viscous, colloidal mixture. *NF category:* Suspending and/or viscosity-increasing agent; wet binder; diluent; disintegrant.

**Pregelatinized Starch:** Moderately coarse to fine, white to off-white powder. Is odorless and has a slight, characteristic taste. Slightly soluble to soluble in cold water; insoluble in alcohol. *NF category:* Wet binder; diluent; disintegrant; release-modifying agent.

**Pregelatinized Modified Starch:** Moderately coarse to fine, white to off-white powder. Is odorless and has a slight, characteristic taste. Soluble to slightly soluble in cold water; insoluble in alcohol. *NF category:* Coating agent; diluent; disintegrant; wet binder; release-modifying agent.

**Tapioca Starch:** Irregular, angular, white to pale yellow masses (which may be bleached) or fine powder. Insoluble in cold water and in alcohol. *NF category:* Suspending and/or viscosity-increasing agent; diluent; disintegrant; wet binder.

**Wheat Starch:** Irregular, angular, white masses (which may be bleached) or fine powder. Is odorless and has a slight, characteristic taste. Insoluble in cold water and in alcohol. *NF category:* Suspending and/or viscosity-increasing agent; diluent; disintegrant; wet binder.

**Hydrogenated Starch Hydrolysate:** Concentrated, aqueous solution or spray-dried or dried powder. Very soluble in water; insoluble in alcohol. *NF category:* Sweetening agent; humectant; diluent; wet binder.

**Stavudine:** White to off-white, crystalline powder. Soluble in water, in dimethylacetamide, and in dimethyl sulfoxide; sparingly soluble in methanol, in alcohol, and in acetonitrile; slightly soluble in dichloromethane; insoluble in hexane.

**Stearic Acid:** Hard, white or faintly yellowish, somewhat glossy and crystalline solid, or white or yellowish-white powder. Its odor and taste are slight, suggesting tallow. Freely soluble in chloroform and in ether; soluble in alcohol; practically insoluble in water. *NF category:* Emulsifying agent; lubricant.

**Purified Stearic Acid:** Hard, white or faintly yellowish, somewhat glossy and crystalline solid, or white or yellowish-white powder. Its odor and taste are slight, suggesting tallow. Freely soluble in chloroform and in ether; soluble in alcohol; practically insoluble in water. *NF category:* Lubricant.

**Stearoyl Polyoxylglycerides:** Pale yellow, waxy solids. Dispersible in warm water and in warm paraffin. Freely soluble in methylene chloride; soluble in warm methanol. *NF category:* Ointment base; solvent; emulsifying agent; wetting and/or solubilizing agent.

**Stearyl Alcohol:** Unctuous, white flakes or granules. Has a faint, characteristic odor and a bland, mild taste. Soluble in alcohol and in ether; insoluble in water. *NF category:* Stiffening agent.

**Storax:** Semiliquid, grayish to grayish-brown, sticky, opaque mass depositing on standing a heavy dark brown layer (Levant Storax); or semisolid, sometimes a solid mass, softened by gently warming (American Storax). Is transparent in thin layers, has a characteristic odor and taste, and is more dense than water. Soluble, usually incompletely, in an equal weight of warm alcohol, in acetone, in carbon disulfide, and in ether, some insoluble residue usually remaining; insoluble in water.

**Streptomycin Sulfate:** White or practically white powder. Is odorless or has not more than a faint odor. Is hygroscopic, but is stable in air and on exposure to light. Its solutions are acid to practically neutral to litmus. Freely soluble in water; very slightly soluble in alcohol; practically insoluble in chloroform.

**Succinic Acid:** White, odorless crystals. Freely soluble in boiling water; soluble in water, in alcohol, and in glycerin. *NF category:* pH modifier (acidifying agent/alkalizing agent/buffering agent).

**Succinylcholine Chloride:** White, odorless, crystalline powder. Its solutions have a pH of about 4. The dihydrate form melts at about 160°; the anhydrous form melts at about 190°, and is hygroscopic. Freely soluble in water; slightly soluble in alcohol and in chloroform; practically insoluble in ether.

**Sucralfate:** White or almost-white amorphous powder. Soluble in a dilute solution of mineral acids or alkali hydroxides; practically insoluble in water, in alcohol, and in methylene chloride.

**Sucralose:** White to off-white, crystalline powder. Freely soluble in water, in methanol, and in alcohol; slightly soluble in ethyl acetate. *NF category:* Sweetening agent.

**Sucrose:** White, crystalline powder or lustrous, dry, colorless or white crystals. Very soluble in water; slightly soluble in alcohol; practically insoluble in dehydrated alcohol. *NF category:* Coating agent; sweetening agent; diluent; suspending and/or viscosity-increasing agent; wet binder.

**Sucrose Diacetate Hexaisobutyrate:** Colorless or pale yellow, clear or slightly turbid liquid. Freely soluble in acetone, in ethanol, in ethyl lactate, in benzyl alcohol, in triacetin, and in medium-chain triglycerides; soluble in ethyl acetate; very slightly soluble in water. *NF category:* Wet binder; plasticizer; ointment base; diluent.

**Sucrose Palmitate:** White or almost white, unctuous powder. Sparingly soluble in ethanol (96%); very slightly soluble in water. *NF category:* Suspending and/or viscosity-increasing agent; emulsifying agent.

**Sucrose Octaacetate:** White, practically odorless powder, having an intensely bitter taste. Is hygroscopic. Very soluble in methanol and in chloroform; soluble in alcohol and in ether; very slightly soluble in water. *NF category:* Alcohol denaturant.

**Sucrose Stearate:** White or almost white, unctuous powder. Sparingly soluble in ethanol (96%); very slightly soluble in water. *NF category:* Emulsifying agent; lubricant.

**Sufentanil Citrate:** White powder. Freely soluble in methanol; soluble in water; sparingly soluble in acetone, in alcohol, and in chloroform. Melts between 133° and 140°.

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**Compressible Sugar:** Practically white, crystalline, odorless powder, having a sweet taste. Is stable in air. The sucrose portion of Compressible Sugar is very soluble in water. *NF category:* Sweetening agent; diluent.

**Confectioner's Sugar:** Fine, white, odorless powder, having a sweet taste. Is stable in air. The sucrose portion of Confectioner's Sugar is soluble in cold water. Confectioner's Sugar is freely soluble in boiling water. *NF category:* Sweetening agent; diluent; coating agent.

**Sugar Spheres:** Hard, brittle, free-flowing, spherical masses ranging generally in size from 10- to 60-mesh. Usually white, but may be colored. Solubility in water varies according to the sugar-to-starch ratio. *NF category:* Vehicle (solid carrier); diluent.

**Sulbactam Sodium:** White to off-white, crystalline powder. Freely soluble in water and in dilute acid; sparingly soluble in acetone, in ethyl acetate, and in chloroform.

**Sulconazole Nitrate:** White to off-white, crystalline powder. Melts at about 130°, with decomposition. Freely soluble in pyridine; sparingly soluble in methanol; slightly soluble in alcohol, in chloroform, in acetone, and in methylene chloride; very slightly soluble in water, in toluene, and in dioxane.

**Sulfacetamide Sodium:** White, crystalline powder. Is odorless and has a bitter taste. Freely soluble in water; sparingly soluble in alcohol; practically insoluble in chloroform and in ether.

**Sulfadiazine:** White or slightly yellow powder. Is odorless or nearly odorless and is stable in air, but slowly darkens on exposure to light. Freely soluble in dilute mineral acids, in solutions of potassium and sodium hydroxides, and in ammonia TS; sparingly soluble in alcohol and in acetone; slightly soluble in human serum at 37°; practically insoluble in water.

**Silver Sulfadiazine:** White to creamy-white, crystalline powder, odorless to having a slight odor. Is stable in air, but turns yellow on exposure to light. Freely soluble in 30% ammonium solution; slightly soluble in acetone; practically insoluble in alcohol, in chloroform, and in ether. Decomposes in moderately strong mineral acids.

**Sulfadiazine Sodium:** White powder. On prolonged exposure to humid air it absorbs carbon dioxide with the liberation of sulfadiazine and becomes incompletely soluble in water. Its solutions are alkaline to phenolphthalein. Is affected by light. Freely soluble in water; slightly soluble in alcohol.

**Sulfadimethoxine:** Practically white, crystalline powder. Soluble in 2 N sodium hydroxide; sparingly soluble in 2 N hydrochloric acid; slightly soluble in alcohol, in ether, in chloroform, and in hexane; practically insoluble in water.

**Sulfamethazine:** White to yellowish-white powder, which may darken on exposure to light. Has a slightly bitter taste and is practically odorless. Soluble in acetone; slightly soluble in alcohol; very slightly soluble in water and in ether.

**Sulfamethizole:** White crystals or powder, having a slightly bitter taste. Is practically odorless, and has no odor of hydrogen sulfide. Freely soluble in solutions of ammonium, potassium, and sodium hydroxides; soluble in dilute mineral acids and in acetone; sparingly soluble in alcohol; very slightly soluble in water, in chloroform, and in ether; practically insoluble in benzene.

**Sulfamethoxazole:** White to off-white, practically odorless, crystalline powder. Freely soluble in acetone and in dilute solutions of sodium hydroxide; sparingly soluble in alcohol; practically insoluble in water, in ether, and in chloroform.

**Sulfapyridine:** White or faintly yellowish-white crystals, granules, or powder. Is odorless or practically odorless, and is stable in air, but slowly darkens on exposure to light. Freely soluble in dilute mineral acids and in solutions of potassium and sodium hydroxides; sparingly soluble in acetone; slightly soluble in alcohol; very slightly soluble in water.

**Sulfasalazine:** Bright yellow or brownish-yellow, odorless, fine powder. Melts at about 255°, with decomposition. Soluble in aqueous solutions of alkali hydroxides; very slightly soluble in alcohol; practically insoluble in water, in ether, in chloroform, and in benzene.

**Sulfathiazole:** Fine, white or faintly yellowish-white, practically odorless powder. Soluble in acetone, in dilute mineral acids, in solutions of alkali hydroxides, and in 6 N ammonium hydroxide; slightly soluble in alcohol; very slightly soluble in water.

**Sulfisoxazole:** White to slightly yellowish, odorless, crystalline powder. Soluble in boiling alcohol and in 3 N hydrochloric acid; very slightly soluble in water.

**Sulfisoxazole Acetyl:** White or slightly yellow, crystalline powder. Sparingly soluble in chloroform; slightly soluble in alcohol; practically insoluble in water.

**Precipitated Sulfur:** Very fine, pale yellow, amorphous or microcrystalline powder. Is odorless and tasteless. Very soluble in carbon disulfide; slightly soluble in olive oil; very slightly soluble in alcohol; practically insoluble in water.

**Sublimed Sulfur:** Fine, yellow, crystalline powder, having a faint odor and taste. Sparingly soluble in olive oil; practically insoluble in water and in alcohol.

**Sulfur Dioxide:** Colorless, nonflammable gas, possessing a strong, suffocating odor characteristic of burning sulfur. Under pressure, it condenses readily to a colorless liquid that boils at –10° and has a density of approximately 1.5. At 20° and at standard pressure, approximately 36 volumes dissolve in 1 volume of water and approximately 114 volumes dissolve in 1 volume of alcohol. Soluble also in ether and in chloroform. *NF category:* Antioxidant; antimicrobial preservative.

**Sulfuric Acid:** Clear, colorless, oily liquid. Miscible with water and with alcohol with the generation of much heat. Is very caustic and corrosive. Specific gravity is about 1.84. *NF category:* pH modifier (acidifying agent/alkalizing agent/buffering agent).

**Sulindac:** Yellow, crystalline powder, which is odorless or practically so. Slightly soluble in methanol, in alcohol, in acetone, and in chloroform; very slightly soluble in isopropanol and in ethyl acetate; practically insoluble in hexane and in water.

**Sulisobenzone:** Light tan powder, with a melting point of about 145°. Freely soluble in methanol, in alcohol, and in water; sparingly soluble in ethyl acetate.

**Sumatriptan:** White to pale yellow powder. Very slightly soluble in water.

**Sumatriptan Succinate:** White or almost white powder. Freely soluble in water; sparingly soluble in methanol; practically insoluble in methylene chloride.

**Sunflower Oil:** Clear, light yellow liquid. It is miscible with petroleum at a boiling point of 40°–60°. Practically insoluble in water and in alcohol. *Refractive Index* ⟨831⟩: 1.472–1.474 at 25°. *Specific Gravity* ⟨841⟩: 0.914–0.924 at 20°. *NF category*: Coating agent; emollient; solvent; diluent; vehicle (oleaginous).

**Syrup:** *NF category:* Sweetening agent; wet binder; vehicle.

**Tacrolimus:** White crystals or white crystalline powder. Very soluble in methanol; freely soluble in *N,N*-dimethylformamide, and in alcohol; practically insoluble in water.

**Tadalafil:** White or almost white powder. Freely soluble in dimethyl sulfoxide; slightly soluble in methylene chloride; practically insoluble in water.

**Tagatose:** White or almost white crystals, having a sweet taste. Very soluble in water; very slightly soluble in alcohol. *NF category:* Sweetening agent; humectant.

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**Talc:** Very fine, white or grayish-white, crystalline powder. Is unctuous, adheres readily to the skin, and is free from grittiness. *NF category:* Glidant and/or anticaking agent; lubricant; diluent.

**Tamoxifen Citrate:** White, fine, crystalline powder. Soluble in methanol; very slightly soluble in water, in acetone, in chloroform, and in alcohol. Melts at about 142°, with decomposition.

**Tamsulosin Hydrochloride:** White or almost white crystalline powder. Melts with decomposition at approximately 230°. Freely soluble in formic acid; sparingly soluble in methanol; slightly soluble in water and in dehydrated alcohol; practically insoluble in ether.

**Tannic Acid:** Amorphous powder, glistening scales, or spongy masses, varying in color from yellowish-white to light brown. Is odorless or has a faint, characteristic odor, and has a strongly astringent taste. Very soluble in water, in acetone, and in alcohol; freely soluble in diluted alcohol; slightly soluble in dehydrated alcohol; practically insoluble in benzene, in chloroform, in ether, and in solvent hexane; 1 g dissolves in about 1 mL of warm glycerin.

**Tartaric Acid:** Colorless or translucent crystals or white, fine to granular, crystalline powder. Is odorless, has an acid taste, and is stable in air. Very soluble in water; freely soluble in alcohol. *NF category:* pH modifier (acidifying agent/alkalizing agent/buffering agent); flavors and fragrance; sequestering agent.

**Taurine:** White crystals or crystalline powder. Soluble in water.

**Tazobactam:** White to pale yellow, nonhygroscopic, crystalline powder. Soluble in dimethylformamide; slightly soluble in water, in methanol, in acetone, and in alcohol; very slightly soluble in ethyl acetate, in ethyl ether, and in chloroform; insoluble in hexane.

**Technetium Tc 99m Aggregated Albumin Injection:** Milky suspension, from which particles settle upon standing.

**Technetium Tc 99m Pentetate Injection:** Clear, colorless solution.

**Sodium Pertechnetate Tc 99m Injection:** Clear, colorless solution.

**Technetium Tc 99m (Pyro- and trimeta-) Phosphates Injection:** Clear solution.

**Technetium Tc 99m Sulfur Colloid Injection:** Colloidal dispersion. Slightly opalescent, colorless to light tan liquid.

**Telmisartan:** White or slightly yellowish, crystalline powder. Sparingly soluble in methylene chloride; slightly soluble in methanol; practically insoluble in water. It dissolves in 1 M sodium hydroxide.

**Temazepam:** White or nearly white, crystalline powder. Sparingly soluble in alcohol; very slightly soluble in water. Melts between 157° and 163°, within a 3° range.

**Temozolomide:** White to light pink/light tan powder. Soluble in dimethyl sulfoxide; sparingly soluble in water; practically insoluble in toluene.

**Teniposide:** White to off-white, crystalline powder. Very soluble in acetone and in dimethylformamide; slightly soluble in methanol; insoluble in water and in ether.

**Terazosin Hydrochloride:** White to pale yellow, crystalline powder. Freely soluble in isotonic saline solution; soluble in methanol and in water; slightly soluble in alcohol and in 0.1 N hydrochloric acid; very slightly soluble in chloroform; practically insoluble in acetone and in hexanes.

**Terbinafine Hydrochloride:** White or off-white powder. Freely soluble in dehydrated alcohol and in methanol; slightly soluble in acetone; very slightly or slightly soluble in water.

**Terbutaline Sulfate:** White to gray-white, crystalline powder. Is odorless or has a faint odor of acetic acid. Soluble in water and in 0.1 N hydrochloric acid; slightly soluble in methanol; insoluble in chloroform.

**Terconazole:** White to off-white powder. Freely soluble in methylene chloride; soluble in acetone; sparingly soluble in alcohol; practically insoluble in water. It exhibits polymorphism.

**Terpin Hydrate:** Colorless, lustrous crystals or white powder. Has a slight odor, and effloresces in dry air. A hot solution (1 in 100) is neutral to litmus. When dried in vacuum at 60° for 2 hours, it melts at about 103°. Very soluble in boiling alcohol; soluble in alcohol; sparingly soluble in boiling water; slightly soluble in water, in chloroform, and in ether.

**Testosterone:** White or slightly creamy white crystals or crystalline powder. Is odorless, and is stable in air. Freely soluble in dehydrated alcohol and in chloroform; soluble in dioxane and in vegetable oils; slightly soluble in ether; practically insoluble in water.

**Testosterone Cypionate:** White or creamy white, crystalline powder. Is odorless or has a slight odor, and is stable in air. Freely soluble in alcohol, in chloroform, in dioxane, and in ether; soluble in vegetable oils; insoluble in water.

**Testosterone Enanthate:** White or creamy white, crystalline powder. Is odorless or has a faint odor characteristic of heptanoic acid. Very soluble in ether; soluble in vegetable oils; insoluble in water.

**Testosterone Propionate:** White or creamy white crystals or crystalline powder. Is odorless and is stable in air. Freely soluble in alcohol, in dioxane, in ether, and in other organic solvents; soluble in vegetable oils; insoluble in water.

**Tetracaine:** White or light yellow, waxy solid. Soluble in alcohol, in ether, in benzene, and in chloroform; very slightly soluble in water.

**Tetracaine Hydrochloride:** Fine, white, crystalline, odorless powder. Has a slightly bitter taste followed by a sense of numbness. Its solutions are neutral to litmus. Melts at about 148°, or may occur in either of two other polymorphic modifications that melt at about 134° and 139°, respectively. Mixtures of the forms may melt within the range of 134° to 147°. Is hygroscopic. Very soluble in water; soluble in alcohol; insoluble in ether and in benzene.

**Tetracycline:** Yellow, odorless, crystalline powder. Is stable in air, but exposure to strong sunlight causes it to darken. It loses potency in solutions of pH below 2, and is rapidly destroyed by alkali hydroxide solutions. Freely soluble in dilute acid and in alkali hydroxide solutions; sparingly soluble in alcohol; very slightly soluble in water; practically insoluble in chloroform and in ether.

**Tetracycline Hydrochloride:** Yellow, odorless, crystalline powder. Is moderately hygroscopic. Is stable in air, but exposure to strong sunlight in moist air causes it to darken. It loses potency in solution at a pH below 2, and is rapidly destroyed by alkali hydroxide solutions. Soluble in water and in solutions of alkali hydroxides and carbonates; slightly soluble in alcohol; practically insoluble in chloroform and in ether.

**Tetrahydrozoline Hydrochloride:** White, odorless solid. Melts at about 256°, with decomposition. Freely soluble in water and in alcohol; very slightly soluble in chloroform; practically insoluble in ether.

**Thalidomide:** White to off-white powder. Very soluble in dimethyl sulfoxide; sparingly soluble in ethanol and in water.

**Theophylline:** White, odorless, crystalline powder, having a bitter taste. Is stable in air. Freely soluble in solutions of alkali hydroxides and in ammonia; sparingly soluble in alcohol, in chloroform, and in ether; slightly soluble in water, but more soluble in hot water.

**Theophylline Sodium Glycinate:** White, crystalline powder having a slight ammoniacal odor and a bitter taste. Freely soluble in water; very slightly soluble in alcohol; practically insoluble in chloroform.

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**Thiabendazole:** White to practically white, odorless or practically odorless powder. Slightly soluble in acetone and in alcohol; very slightly soluble in chloroform and in ether; practically insoluble in water.

**Thiamine Hydrochloride:** White crystals or crystalline powder, usually having a slight, characteristic odor. When exposed to air, the anhydrous product rapidly absorbs about 4% of water. Melts at about 248°, with some decomposition. Freely soluble in water; soluble in glycerin; slightly soluble in alcohol; insoluble in ether and in benzene.

**Thiamine Mononitrate:** White crystals or crystalline powder, usually having a slight, characteristic odor. Sparingly soluble in water; slightly soluble in alcohol; very slightly soluble in chloroform.

**Thimerosal:** Light cream-colored, crystalline powder, having a slight characteristic odor. Is affected by light. The pH of a solution (1 in 100) is about 6.7. Freely soluble in water; soluble in alcohol; practically insoluble in ether. *NF category:* Antimicrobial preservative.

**Thimerosal Topical Solution:** Clear liquid, having a slight characteristic odor. Is affected by light.

**Thimerosal Tincture:** Transparent, mobile liquid, having the characteristic odor of alcohol and acetone. Is affected by light.

**Thioguanine:** Pale yellow, odorless or practically odorless, crystalline powder. Freely soluble in dilute solutions of alkali hydroxides; insoluble in water, in alcohol, and in chloroform.

**Thiopental Sodium:** White to off-white, crystalline powder, or yellowish-white to pale greenish-yellow, hygroscopic powder. May have a disagreeable odor. Its solutions are alkaline to litmus. Its solutions decompose on standing, and on boiling precipitation occurs. Soluble in water and in alcohol; insoluble in benzene, in absolute ether, and in solvent hexane.

**Thiopental Sodium for Injection:** White to off-white, crystalline powder, or yellowish-white to pale greenish-yellow, hygroscopic powder. May have a disagreeable odor. Its solutions are alkaline to litmus. Its solutions decompose on standing, and on boiling precipitation occurs.

**Thioridazine:** White to slightly yellow, crystalline or micronized powder, odorless or having a faint odor. Very soluble in chloroform; freely soluble in dehydrated alcohol and in ether; practically insoluble in water.

**Thioridazine Hydrochloride:** White to slightly yellow, granular powder, having a faint odor and a very bitter taste. Freely soluble in water, in methanol, and in chloroform; insoluble in ether.

**Thiostrepton:** White to off-white, crystalline solid. Soluble in glacial acetic acid, in chloroform, in dimethylformamide, in dimethyl sulfoxide, in dioxane, and in pyridine; practically insoluble in water, in the lower alcohols, in nonpolar organic solvents, and in dilute aqueous acids or alkali.

**Thiotepa:** Fine, white, crystalline flakes, having a faint odor. Freely soluble in water, in alcohol, in chloroform, and in ether.

**Thiotepa for Injection:** White powder.

**Thiothixene:** White to tan, practically odorless crystals. Is affected by light. Very soluble in chloroform; slightly soluble in methanol and in acetone; practically insoluble in water.

**Threonine:** White, odorless crystals, having a slightly sweet taste. Freely soluble in water; insoluble in absolute alcohol, in ether, and in chloroform.

**Thymol:** Colorless, often large, crystals, or white, crystalline powder, having an aromatic, thyme-like odor and a pungent taste. Is affected by light. Its alcohol solution is neutral to litmus. Freely soluble in alcohol, in chloroform, in ether, and in olive oil; soluble in glacial acetic acid and in fixed and volatile oils; very slightly soluble in water. *NF category:* Antimicrobial preservative; flavors and fragrance; antioxidant.

**Thyroid:** Yellowish to buff-colored, amorphous powder, having a slight, characteristic, meat-like odor and a saline taste.

**Tiagabine Hydrochloride:** White to off-white powder. Freely soluble in methanol and in alcohol; soluble in isopropanol; very slightly soluble in chloroform; sparingly soluble in water; practically insoluble in *n*-heptane.

**Tiamulin:** A sticky, translucent yellowish mass, slightly hygroscopic. Very soluble in dichloromethane; freely soluble in dehydrated alcohol; practically insoluble in water.

**Ticagrelor:** White or off-white to pale pink powder. Freely soluble in methanol; soluble in ethanol; practically insoluble in water.

**Ticarcillin Disodium:** White to pale yellow powder, or white to pale yellow solid. Freely soluble in water.

**Tigecycline:** Orange powder. Freely soluble in water; soluble in 1-octanol; slightly soluble in methanol, in ethanol, and in isopropyl alcohol.

**Tiletamine Hydrochloride:** White to off-white, crystalline powder. Freely soluble in water and in 0.1 N hydrochloric acid; soluble in methanol; slightly soluble in chloroform; practically insoluble in ether.

**Tilmicosin:** White to off-white, amorphous solid. Slightly soluble in water and in *n*-hexane.

**Timolol Maleate:** White to practically white, odorless or practically odorless powder. Soluble in water, in alcohol, and in methanol; sparingly soluble in chloroform and in propylene glycol; insoluble in ether and in cyclohexane.

**Tinidazole:** Almost white or pale yellow, crystalline powder. Soluble in acetone and in methylene chloride; sparingly soluble in methanol; practically insoluble in water.

**Tiotropium Bromide:** White to yellowish-white powder or crystals. Soluble to sparingly soluble in methanol and in water.

**Titanium Dioxide:** White, odorless, tasteless powder. Its 1 in 10 suspension in water is neutral to litmus. Insoluble in water, in hydrochloric acid, in nitric acid, and in 2 N sulfuric acid. Dissolves in hydrofluoric acid and in hot sulfuric acid. Is rendered soluble by fusion with potassium bisulfate or with alkali carbonates or hydroxides. *NF category:* Coating agent.

**Tizanidine Hydrochloride:** Almost white to slightly yellow, crystalline powder. Slightly soluble in water and in methanol.

**Tobramycin:** White to off-white, hygroscopic powder. Freely soluble in water; very slightly soluble in alcohol; practically insoluble in chloroform and in ether.

**Tobramycin Sulfate Injection:** Clear, colorless solution.

**Tocopherol:** Clear, colorless to yellow, yellowish-brown, or greenish-yellow, viscous oil. Is odorless. Soluble in oils, in fats, in acetone, in alcohol, in chloroform, in ether, and in alcohol; insoluble in water. *NF category:* Antioxidant.

**Tocopherols Excipient:** Brownish-red to red, clear, viscous oil, having a mild, characteristic odor and taste. May show a slight separation of waxlike constituents in microcrystalline form. Oxidizes and darkens slowly in air and on exposure to light, particularly in alkaline media. Soluble in alcohol; insoluble in water. Miscible with acetone, with chloroform, with ether, and with vegetable oils. *NF category:* Antioxidant.

**Tolazamide:** White to off-white, crystalline powder, odorless or having a slight odor. Melts with decomposition in the approximate range of 161° to 173°. Freely soluble in chloroform; soluble in acetone; slightly soluble in alcohol; very slightly soluble in water.

**Tolazoline Hydrochloride:** White to off-white, crystalline powder. Its solutions are slightly acid to litmus. Freely soluble in water and in alcohol.

**Tolbutamide:** White, or practically white, crystalline powder. Is slightly bitter and practically odorless. Soluble in alcohol and in chloroform; practically insoluble in water.

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**Tolcapone:** Yellow, fine powder or fine powder with lumps. Freely soluble in acetone and in tetrahydrofuran; soluble in methanol and in ethyl acetate; sparingly soluble in chloroform and in dichloromethane; insoluble in water and in *n*-hexane.

**Tolmetin Sodium:** Light yellow to light orange, crystalline powder. Freely soluble in water and in methanol; slightly soluble in alcohol; very slightly soluble in chloroform.

**Tolnaftate:** White to creamy white, fine powder, having a slight odor. Freely soluble in acetone and in chloroform; sparingly soluble in ether; slightly soluble in alcohol; practically insoluble in water.

**Tolterodine Tartrate:** White or almost white, crystalline powder. Sparingly soluble in water; slightly soluble in anhydrous ethanol; practically insoluble in heptane.

**Tolu Balsam:** Brown or yellowish-brown, plastic solid, transparent in thin layers and brittle when old, dried, or exposed to cold temperatures. Has a pleasant, aromatic odor resembling that of vanilla, and a mild, aromatic taste. Soluble in alcohol, in chloroform, and in ether, sometimes with slight residue or turbidity; practically insoluble in water and in solvent hexane. *NF category:* Flavors and perfumes.

**Topiramate:** White to off-white powder. Freely soluble in dichloromethane.

**Torsemide:** White to off-white, crystalline powder. Slightly soluble in 0.1 N sodium hydroxide, in 0.1 N hydrochloric acid, in alcohol, and in methanol; very slightly soluble in acetone and in chloroform; practically insoluble in water and in ether.

**Tragacanth:** Is odorless, and has an insipid, mucilaginous taste. *NF category:* Suspending and/or viscosity-increasing agent.

**Tramadol Hydrochloride:** White, crystalline powder. Freely soluble in water and in methanol; very slightly soluble in acetone.

**Trandalopril:** White or almost white powder. Freely soluble in methylene chloride; sparingly soluble in absolute alcohol; practically insoluble in water.

**Tranexamic Acid:** White, crystalline powder. Freely soluble in water and in glacial acetic acid; practically insoluble in acetone and in alcohol.

**Tranylcypromine Sulfate:** White or almost white crystalline powder. Freely soluble in water; very slightly soluble in alcohol and in ether; practically insoluble in chloroform.

**Travoprost:** Clear, colorless, viscous oil. Insoluble in water.

**Trazodone Hydrochloride:** White to off-white, crystalline powder. Sparingly soluble in chloroform and in water. Melts between 231° and 234° when the melting point determination is carried out in an evacuated capillary tube; otherwise melts with decomposition over a broad range below 230°.

**Trehalose:** White, odorless, nonhygroscopic crystalline powder. Soluble in water, solubility increases with temperature; practically insoluble in dehydrated alcohol. Trehalose is typically used in the dihydrate form. *NF category:* Bulking agent; sweetening agent; diluent; disintegrant; wet binder; vehicle.

**Tretinoin:** Yellow to light-orange, crystalline powder. Slightly soluble in alcohol, in chloroform, and in methanol; insoluble in water.

**Triacetin:** Colorless, somewhat oily liquid having a slight, fatty odor and a bitter taste. Soluble in water; slightly soluble in carbon disulfide. Miscible with alcohol, with ether, and with chloroform. *NF category:* Plasticizer; humectant; solvent.

**Triamcinolone:** White or practically white, odorless, crystalline powder. Slightly soluble in alcohol and in methanol; very slightly soluble in water, in chloroform, and in ether.

**Triamcinolone Acetonide:** White to cream-colored, crystalline powder, having not more than a slight odor. Sparingly soluble in dehydrated alcohol, in chloroform, and in methanol; practically insoluble in water.

**Triamcinolone Diacetate:** Fine, white to off-white, crystalline powder, having not more than a slight odor. Soluble in chloroform; sparingly soluble in alcohol and in methanol; slightly soluble in ether; practically insoluble in water.

**Triamcinolone Hexacetonide:** White to cream-colored powder. Soluble in chloroform; slightly soluble in methanol; practically insoluble in water.

**Triamterene:** Yellow, odorless, crystalline powder. Soluble in formic acid; sparingly soluble in methoxyethanol; very slightly soluble in acetic acid, in alcohol, and in dilute mineral acids; practically insoluble in water, in benzene, in chloroform, in ether, and in dilute alkali hydroxides.

**Triazolam:** White to off-white, practically odorless, crystalline powder. Soluble in chloroform; slightly soluble in alcohol; practically insoluble in ether and in water.

**Tributyl Citrate:** Clear, practically colorless, oily liquid. Freely soluble in alcohol, in isopropyl alcohol, in acetone, and in toluene; insoluble in water. *NF category:* Plasticizer.

**Trichlorfon:** White crystalline powder. Very soluble in methylene chloride; freely soluble in acetone, in alcohol, in benzene, in chloroform, in ether, and in water; very slightly soluble in hexane and in pentane. Decomposed by alkali. Melts at about 78° with decomposition.

**Trichlormethiazide:** White or practically white, crystalline powder. Is odorless, or has a slight characteristic odor. Melts at about 274°, with decomposition. Freely soluble in acetone; soluble in methanol; sparingly soluble in alcohol; very slightly soluble in water, in ether, and in chloroform.

**Trichloromonofluoromethane:** Clear, colorless gas, having a faint, ethereal odor. Its vapor pressure at 25° is about 796 mm of mercury (1 psig). *NF category:* Propellant.

**Triclabendazole:** White to off-white crystalline powder. Soluble in acetone; sparingly soluble in ethanol; practically insoluble in water.

**Triclocarban:** White powder. Freely soluble in ethanol, in acetone, and in polyethylene glycol; slightly soluble in acetonitrile and in propylene glycol; insoluble in water.

**Triclosan:** Fine, whitish, crystalline powder. Melts at about 57°. Soluble in methanol, in alcohol, and in acetone; slightly soluble in hexane; practically insoluble in water.

**Trientine Hydrochloride:** White to pale yellow, crystalline powder, hygroscopic. Freely soluble in water; soluble in methanol; slightly soluble in alcohol; insoluble in chloroform and in ether.

**Triethyl Citrate:** Practically colorless, oily liquid. Soluble in water. Miscible with alcohol and with ether. *NF category:* Plasticizer; solvent.

**Trifluoperazine Hydrochloride:** White to pale yellow, crystalline powder. Is practically odorless, and has a bitter taste. Melts at about 242°, with decomposition. Freely soluble in water; soluble in alcohol; sparingly soluble in chloroform; insoluble in ether and in benzene.

**Triflupromazine:** Viscous, light amber-colored, oily liquid, which crystallizes on prolonged standing into large, irregular crystals. Practically insoluble in water.

**Triflupromazine Hydrochloride:** White to pale tan, crystalline powder, having a slight, characteristic odor. Melts between 170° and 178°. Soluble in water, in alcohol, and in acetone; insoluble in ether.

**Trifluridine:** Odorless, white powder appearing under the microscope as rodlike crystals; melts at 175°, with sublimation.

**Medium-Chain Triglycerides:** Colorless or slightly yellowish, oily liquid. Practically insoluble in water. Miscible with alcohol, with methylene chloride, with hexane, and with fatty oils. The refractive index is 1.440–1.452 at 20° (see

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*Refractive Index* ⟨831⟩). *NF category:* Emulsifying agent; solvent; suspending and/or viscosity-increasing agent.

**Trihexyphenidyl Hydrochloride:** White or slightly off-white, crystalline powder, having not more than a very faint odor. Melts at about 250°. Soluble in alcohol and in chloroform; slightly soluble in water.

**Trimeprazine Tartrate:** White to off-white, odorless, crystalline powder. Freely soluble in water and in chloroform; soluble in alcohol; very slightly soluble in ether and in benzene.

**Trimethobenzamide Hydrochloride:** White, crystalline powder having a slight phenolic odor. Soluble in water and in warm alcohol; insoluble in ether and in benzene.

**Trimethoprim:** White to cream-colored, odorless crystals, or crystalline powder. Soluble in benzyl alcohol; sparingly soluble in chloroform and in methanol; slightly soluble in alcohol and in acetone; very slightly soluble in water; practically insoluble in ether and in carbon tetrachloride.

**Trimethoprim Sulfate:** White to off-white, crystalline powder. Soluble in water, in alcohol, in dilute mineral acids, and in fixed alkalies.

**Trimipramine Maleate:** White to almost white crystalline powder. Slightly soluble in water and in alcohol.

**Tripelennamine Hydrochloride:** White, crystalline powder. Slowly darkens on exposure to light. Its solutions are practically neutral to litmus. Freely soluble in water, in alcohol, and in chloroform; slightly soluble in acetone; insoluble in benzene, in ether, and in ethyl acetate.

**Triprolidine Hydrochloride:** White, crystalline powder, having no more than a slight, but unpleasant, odor. Its solutions are alkaline to litmus, and it melts at about 115°. Soluble in water, in alcohol, and in chloroform; insoluble in ether.

**Trolamine:** Colorless to pale yellow, viscous, hygroscopic liquid having a slight, ammoniacal odor. Soluble in chloroform. Miscible with water and with alcohol. *NF category:* Emulsifying agent; pH modifier (acidifying agent/alkalizing agent/buffering agent).

**Tromethamine:** White, crystalline powder, having a slight, characteristic odor. Freely soluble in water.

**Tropicamide:** White or practically white, crystalline powder, odorless or having not more than a slight odor. Freely soluble in chloroform and in solutions of strong acids; slightly soluble in water.

**Trospium Chloride:** Colorless or white to slightly yellow crystalline powder. Very soluble in water; freely soluble in methanol.

**Crystallized Trypsin:** White to yellowish white, odorless, crystalline or amorphous powder.

**Tryptophan:** White to slightly yellowish-white crystals or crystalline powder, having a slightly bitter taste. Soluble in hot alcohol and in dilute hydrochloric acid.

**Tylosin:** White to buff-colored powder. Freely soluble in methanol; soluble in alcohol, in amyl acetate, in chloroform, and in dilute mineral acids; slightly soluble in water.

**Tylosin Tartrate:** Almost white or slightly yellow, hygroscopic powder. Freely soluble in water and in dichloromethane; slightly soluble in alcohol. It dissolves in dilute solutions of mineral acids.

**Tyloxapol:** Viscous, amber liquid, having a slight, aromatic odor. May exhibit a slight turbidity. Slowly but freely miscible with water. Soluble in glacial acetic acid, in benzene, in toluene, in carbon tetrachloride, in chloroform, and in carbon disulfide. *NF category:* Wetting and/or solubilizing agent.

**Tyrosine:** White, odorless, tasteless crystals or crystalline powder. Very slightly soluble in water; insoluble in alcohol and in ether.

**Ubidecarenone:** Yellow to orange, crystalline powder. Melts at about 48°. Soluble in ether; very slightly soluble in dehydrated alcohol; practically insoluble in water.

**Undecylenic Acid:** Clear, colorless to pale yellow liquid having a characteristic odor. Practically insoluble in water. Miscible with alcohol, with chloroform, with ether, with benzene, and with fixed and volatile oils.

**Urea:** Colorless to white, prismatic crystals, or white, crystalline powder, or small white pellets. Is practically odorless, but may gradually develop a slight odor of ammonia upon long standing. Its solutions are neutral to litmus. Freely soluble in water and in boiling alcohol; practically insoluble in chloroform and in ether.

**Urea C 13:** See *Urea*.

**Ursodiol:** White or almost white, crystalline powder. Freely soluble in alcohol and in glacial acetic acid; sparingly soluble in chloroform; slightly soluble in ether; practically insoluble in water.

**Valacyclovir Hydrochloride:** White to off-white powder. Soluble in water; insoluble in dichloromethane.

**Powdered Valerian Extract:** Brown, hygroscopic, powdery or easily pulverizable mass. Soluble in water to form a slightly cloudy solution; sparingly soluble in 70 percent alcohol; practically insoluble in alcohol.

**Valganciclovir Hydrochloride:** White to off-white powder. Very slightly soluble in alcohol; practically insoluble in 2-propanol, in hexane, in acetone, and in ethyl acetate.

**Valine:** White, odorless, tasteless crystals. Soluble in water; practically insoluble in ether, in alcohol, and in acetone.

**Valproic Acid:** Colorless to pale yellow, slightly viscous, clear liquid, having a characteristic odor. Refractive index: about 1.423 at 20°. Freely soluble in 1 N sodium hydroxide, in methanol, in alcohol, in acetone, in chloroform, in benzene, in ether, and in *n*-heptane; slightly soluble in water and in 0.1 N hydrochloric acid.

**Valrubicin:** Orange to orange-red, crystalline powder. Soluble in methylene chloride, in dehydrated alcohol, in methanol, and in acetone; very slightly soluble in water, in hexane, and in petroleum ether.

**Valsartan:** White or almost white, hygroscopic powder. Freely soluble in anhydrous ethanol; sparingly soluble in methylene chloride; practically insoluble in water.

**Vancomycin Hydrochloride:** White, almost white, or tan to brown, free-flowing powder, odorless, and having a bitter taste. Freely soluble in water; insoluble in ether and in chloroform.

**Vanillin:** Fine, white to slightly yellow crystals, usually needle-like, having an odor and taste suggestive of vanilla. Is affected by light. Its solutions are acid to litmus. Freely soluble in alcohol, in chloroform, in ether, and in solutions of the fixed alkali hydroxides; soluble in glycerin and in hot water; slightly soluble in water. *NF category:* Flavors and fragrance.

**Vardenafil Hydrochloride:** White or slightly brown or yellow powder. Freely soluble in anhydrous alcohol; slightly soluble in water; practically insoluble in heptane.

**Vasopressin Injection:** Clear, colorless or practically colorless liquid, having a faint, characteristic odor.

**Vecuronium Bromide:** White or creamy white crystals, or a crystalline powder. Sparingly soluble in alcohol; slightly soluble in water and in acetone.

**Hydrogenated Vegetable Oil:** Type I Hydrogenated Vegetable Oil—Fine, white powder, beads, or small flakes. Type II Hydrogenated Vegetable Oil—Plastic (semi-solid) or flakes having a softer consistency than Type I. Soluble in hot isopropyl alcohol, in hexane, and in chloroform; insoluble in water. *NF category:* Hydrogenated Vegetable Oil—Wet binder; Type I Hydrogenated Vegetable Oil—Lubricant; Type II Hydrogenated Vegetable Oil—Ointment base.

**Venlafaxine Hydrochloride:** Off-white to white crystalline powder. Soluble in methanol and in water.

**Verapamil Hydrochloride:** White or practically white, crystalline powder. Is practically odorless and has a bitter taste.

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Freely soluble in chloroform; soluble in water; sparingly soluble in alcohol; practically insoluble in ether.

**Vigabatrin:** White or almost white powder. Freely soluble in water; slightly soluble in methanol; very slightly soluble in alcohol and in chloroform; practically insoluble in methylene chloride; insoluble in hexane and in toluene.

**Vinblastine Sulfate:** White or slightly yellow, odorless, amorphous or crystalline powder. Is hygroscopic. Freely soluble in water.

**Vincristine Sulfate:** White to slightly yellow, odorless, amorphous or crystalline powder. Is hygroscopic. Freely soluble in water; soluble in methanol; slightly soluble in alcohol.

**Vincristine Sulfate for Injection:** Yellowish-white solid, having the characteristic appearance of products prepared by freeze-drying.

**Vinorelbine Tartrate:** White to yellow or light brown, amorphous powder. Freely soluble in water.

**Vitamin A:** In liquid form, a light-yellow to red oil that may solidify upon refrigeration. In solid form, has the appearance of any diluent that has been added. May be practically odorless or may have a mild fishy odor, but has no rancid odor or taste. Is unstable to air and light. In liquid form, very soluble in chloroform and in ether; soluble in absolute alcohol and in vegetable oils; insoluble in water and in glycerin. In solid form, may be dispersible in water.

**Vitamin E:** Practically odorless and tasteless. The alpha tocopherols and alpha tocopheryl acetates occur as clear, yellow, or greenish yellow, viscous oils. *d*-Alpha tocopheryl acetate may solidify in the cold. Alpha tocopheryl acid succinate occurs as a white powder; the *d*-isomer melts at about 75°, and the *dl*-form melts at about 70°. The alpha tocopherols are unstable to air and light, particularly when in alkaline media. The esters are stable to air and light, but are unstable to alkali; the acid succinate is also unstable when held molten. Alpha tocopheryl acid succinate is very soluble in chloroform; soluble in alcohol, in ether, in acetone, and in vegetable oils; slightly soluble in alkaline solutions; insoluble in water. The other forms of Vitamin E are insoluble in water; soluble in alcohol; miscible with ether, with acetone, with vegetable oils, and with chloroform. *NF category:* Antioxidant; plasticizer.

**Vitamin E Preparation:** The liquid forms are clear, yellow to brownish red, viscous oils. The solid forms are white to tan-white granular powders. The liquid forms are soluble in alcohol; insoluble in water. Miscible with ether, with acetone, with vegetable oils, and with chloroform. The solid forms disperse in water to give cloudy suspensions.

**Voriconazole:** White to almost white powder. Freely soluble in acetone and in methylene chloride; very slightly soluble in water.

**Warfarin Sodium:** White, odorless, amorphous or crystalline powder, having a slightly bitter taste. Is discolored by light. Very soluble in water; freely soluble in alcohol; very slightly soluble in chloroform and in ether.

**Water for Injection:** Clear, colorless, odorless liquid. *NF category:* Solvent; pharmaceutical water.

**Bacteriostatic Water for Injection:** Clear, colorless liquid, odorless or having the odor of the antimicrobial substance. *NF category:* Vehicle (sterile); pharmaceutical water.

**Sterile Water for Inhalation:** Clear, colorless solution.

**Sterile Water for Injection:** Clear, colorless, odorless liquid. *NF category:* Solvent; pharmaceutical water.

**Sterile Water for Irrigation:** Clear, colorless, odorless liquid. *NF category:* Solvent; pharmaceutical water.

**Purified Water:** Clear, colorless, odorless liquid. *NF category:* Solvent; pharmaceutical water.

**Carnauba Wax:** Light brown to pale yellow, moderately coarse powder or flakes, possessing a characteristic bland odor, and free from rancidity. Specific gravity is about 0.99. Freely soluble in warm benzene; soluble in warm chloroform and in warm toluene; slightly soluble in boiling alcohol; insoluble in water. *NF category:* Coating agent.

**Emulsifying Wax:** Creamy white, wax-like solid, having a mild, characteristic odor. Freely soluble in ether, in chloroform, in most hydrocarbon solvents, and in aerosol propellants; soluble in alcohol; insoluble in water. *NF category:* Emulsifying agent; wetting and/or solubilizing agent; stiffening agent.

**Microcrystalline Wax:** White or cream-colored, odorless, waxy solid. Soluble in chloroform, in ether, in volatile oils, and in most warm fixed oils; sparingly soluble in dehydrated alcohol; insoluble in water. *NF category:* Coating agent; stiffening agent.

**White Wax:** Yellowish-white solid, somewhat translucent in thin layers. Has a faint, characteristic odor, and is free from rancidity. Specific gravity is about 0.95. Sparingly soluble in cold alcohol; insoluble in water. Boiling alcohol dissolves the cerotic acid and a portion of the myricin, which are constituents of White Wax. Completely soluble in chloroform, in ether, and in fixed and volatile oils. Partly soluble in cold benzene and in cold carbon disulfide; completely soluble in these liquids at about 30°. *NF category:* Stiffening agent.

**Yellow Wax:** Solid varying in color from yellow to grayish brown. Has an agreeable, honey-like odor. Is somewhat brittle when cold, and presents a dull, granular, noncrystalline fracture when broken. It becomes pliable from the heat of the hand. Specific gravity is about 0.95. Sparingly soluble in cold alcohol; insoluble in water. Boiling alcohol dissolves the cerotic acid and a portion of the myricin, that are constituents of Yellow Wax. Soluble in chloroform, in ether, in fixed oils, and in volatile oils; sparingly soluble in cold benzene and in cold carbon disulfide; soluble in these liquids at about 30°. *NF category:* Stiffening agent.

**Wheat Bran:** Light tan powder having a characteristic aroma. Practically insoluble in cold water and in alcohol. Available in a variety of particle sizes depending upon the degree of milling to which it is subjected. Color and flavor development variable, depending on the extent to which it is heat-stabilized.

**Xanthan Gum:** Cream-colored powder. Its solutions in water are neutral to litmus. Soluble in hot or cold water. *NF category:* Suspending and/or viscosity-increasing agent; film-forming agent; polymers for ophthalmic use; release-modifying agent.

**Xylazine:** Colorless to white crystals. Sparingly soluble in dilute acid, in acetone, and in chloroform; insoluble in dilute alkali.

**Xylazine Hydrochloride:** Colorless to white crystals. Sparingly soluble in dilute acid, in acetone, and in methanol; insoluble in dilute alkali.

**Xylitol:** White crystals or crystalline powder. It has a sweet taste and produces a cooling sensation in the mouth. One g dissolves in about 0.65 mL of water. Sparingly soluble in alcohol. Crystalline xylitol has a melting range between 92° and 96°. *NF category:* Coating agent; emollient; humectant; sweetening agent; diluent.

**Xylometazoline Hydrochloride:** White to off-white, odorless, crystalline powder. Melts above 300°, with decomposition. Freely soluble in alcohol; soluble in water; sparingly soluble in chloroform; practically insoluble in benzene and in ether.

**Xylose:** Colorless needles or white, crystalline powder. Is odorless, and has a slightly sweet taste. Very soluble in water; slightly soluble in alcohol.

**Yohimbine Hydrochloride:** White to yellow powder. Melts at about 295°, with decomposition. Soluble in boiling water; slightly soluble in water and in alcohol.

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**Zaleplon:** White to off-white powder. Sparingly soluble in alcohol; slightly soluble in propylene glycol; practically insoluble in water.

**Zanamivir:** White, crystalline powder. Sparingly soluble in water; insoluble in acetone, in acetonitrile, in alcohol, and in isopropyl alcohol.

**Zein:** White to yellow powder. Soluble in aqueous alcohols, in glycols, in ethylene glycol ethyl ether, in furfuryl alcohol, in tetrahydrofurfuryl alcohol, in aqueous alkaline solutions of pH 11.5 or greater, and in acetone-water mixtures between the limits of 60% and 80% of acetone by volume; insoluble in water, in acetone, and in all anhydrous alcohols except methanol. *NF category:* Coating agent; wet binder.

**Zidovudine:** White to yellowish powder. Melts at about 124°. Exhibits polymorphism. Soluble in alcohol; sparingly soluble in water.

**Zileuton:** White to off-white powder.

**Zinc Acetate:** White crystals or granules, having a slight acetous odor and an astringent taste. Is slightly efflorescent. Freely soluble in water and in boiling alcohol; slightly soluble in alcohol. *NF category:* Emollient.

**Zinc Chloride:** White or practically white, odorless, crystalline powder, or white or practically white crystalline granules. May also be in porcelain-like masses or molded into cylinders. Is very deliquescent. A solution (1 in 10) is acid to litmus. Very soluble in water; freely soluble in alcohol and in glycerin. Its solution in water or in alcohol is usually slightly turbid, but the turbidity disappears when a small quantity of hydrochloric acid is added.

**Zinc Gluconate:** White or practically white powder or granules. Soluble in water; very slightly soluble in alcohol.

**Zinc Oxide:** Very fine, odorless, amorphous, white or yellowish white powder, free from gritty particles. It gradually absorbs carbon dioxide from air. Soluble in dilute acids; insoluble in water and in alcohol. *NF category:* Antimicrobial preservative; coating agent.

**Zinc Stearate:** Fine, white, bulky powder, free from grittiness. Has a faint, characteristic odor. Is neutral to moistened litmus paper. Insoluble in water, in alcohol, and in ether. *NF category:* Lubricant.

**Zinc Sulfate:** Colorless, transparent prisms, or small needles. May occur as a white, granular, crystalline powder. Is odorless and is efflorescent in dry air. Its solutions are acid to litmus. Very soluble in water (heptahydrate); freely soluble in water (monohydrate) and in glycerin (heptahydrate); practically insoluble in alcohol (monohydrate); insoluble in alcohol (heptahydrate).

**Zinc Undecylenate:** Fine, white powder. Practically insoluble in water and in alcohol.

**Ziprasidone Hydrochloride (anhydrous):** White to slightly pink powder. Soluble in anhydrous formic acid; insoluble in *n*-hexane.

**Ziprasidone Hydrochloride (monohydrate):** White to slightly pink powder. Slightly soluble in methanol and in methylene chloride; practically insoluble in water.

**Zolazepam Hydrochloride:** White to off-white, crystalline powder. Freely soluble in water and in 0.1 N hydrochloric acid; soluble in methanol; slightly soluble in chloroform; practically insoluble in ether.

**Zolmitriptan:** White to off-white, crystalline powder. Freely soluble to soluble in methanol; soluble in water; sparingly soluble in dichloromethane; practically insoluble in toluene.

**Zolpidem Tartrate:** White to off-white powder, hygroscopic. Sparingly soluble in methanol; slightly soluble in water; practically insoluble in methylene chloride.

**Zonisamide:** White to off-white powder. Freely soluble in dimethylformamide; soluble in methanol.

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[1] For complex see *Chapter Guide: Biotechnology-Derived Drug Substances*.
[2] For noncomplex Minerals, Amino Acids, and Metabolites, see *Chapter Guide: Non-Complex Drug Substances*.
[3] See also *Chapter Guide: Non-Complex Drug Substances*

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# General Information

The chapters in this section are information, and aside from excerpts given herein from Federal Acts and regulations that may be applicable, they contain no standards, tests, assays, nor other mandatory specifications, with respect to any Pharmacopeial articles. The excerpts from pertinent Federal Acts and regulations included in this section are placed here inasmuch as they are not of Pharmacopeial authorship. Revisions of the federal requirements that affect these excerpts will be included in *USP Supplements* as promptly as practical. The official requirements for Pharmacopeial articles are set forth in the *General Notices,* the individual monographs, and the *General Tests and Assays* chapters of this *Pharmacopeia.*

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# GENERAL NOTICES AND REQUIREMENTS

The *General Notices and Requirements* section (the *General Notices*) presents the basic assumptions, definitions, and default conditions for the interpretation and application of the *United States Pharmacopeia* (*USP*) and the *National Formulary* (*NF*).

Requirements stated in these *General Notices* apply to all articles recognized in the *USP* and *NF* (the "compendia") and to all general chapters unless specifically stated otherwise.

# 1. TITLE AND REVISION

The full title of this joint compendium is *The Pharmacopeia of the United States of America* and the *National Formulary* (*USP–NF*). Although *USP* and *NF* are published together and share these *General Notices*, they are separate compendia. Where the terms "*USP*", "*NF*", or "*USP–NF*" are used without further qualification, they refer to the currently official standard.

New and revised content is added to the *USP–NF* periodically (see section *2.10 Official Text*). Routine revisions are published three times a year, on the following dates, corresponding with three "issues":

*USP–NF* 2021 Issue 1: Published November 1, 2020
*USP–NF* 2021 Issue 2: Published February 1, 2021
*USP–NF* 2021 Issue 3: Published June 1, 2021

"Issues" represent dates on which new and revised content is added to the *USP–NF*. The *USP–NF* is in continuous revision, with standards having official dates that correspond to their specific revision histories.

*Change to read:*

# 2. OFFICIAL STATUS AND LEGAL RECOGNITION

### 2.10. Official Text

*Official text* of the *USP* and *NF* is published online at https://online.uspnf.com.

Routine revisions are published in the *USP–NF* and become official on the date indicated, usually six months after publication.

Accelerated Revisions, published periodically in the *USP–NF*, are designed to make revisions official more quickly than through the routine process for publishing standards in the *USP–NF*. Accelerated Revisions may also be published on the *Official Text* section of USP's website (www.uspnf.com/official-text). Accelerated Revisions supersede previously published content and become official on the date indicated.

*Interim Revision Announcements* are Accelerated Revisions to *USP* and *NF* that contain revisions and their official dates.

*Revision Bulletins* are Accelerated Revisions to official text or postponements that require expedited publication. They generally are official immediately unless otherwise specified in the *Revision Bulletin*.

*Errata* are Accelerated Revisions representing corrections to items erroneously published.

Periodically, a non-official volume of associated revisions and additions to the *USP–NF* is published in print or other media. This volume is intended to serve as a historical reference document and is not considered official text.

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### 2.20. Official Articles

An *official article* is an article that is recognized in *USP* or *NF*. An article is deemed to be recognized and included in a compendium when a monograph for the article is published in the compendium and an official date is generally or specifically assigned to the monograph.

The title specified in a monograph is the *official title* for such article. Other names considered to be synonyms of the *official titles* may not be used as substitutes for *official titles*. ▲For a biologic product licensed under the Public Health Service Act, the *official title* shall be the title specified in the relevant monograph plus any prefix and/or suffix designated by the FDA unless otherwise specified in the applicable monograph.▲ (USP 1-Dec-2022) For drug products that incorporate a sensor to detect that the product has been administered, the *official title* shall be the title specified in the relevant drug product monograph plus the words "with sensor".

*Official articles* include both *official substances* and *official products*. An *official substance* is a drug substance, excipient, dietary ingredient, other ingredient, or component of a finished device for which the monograph title includes no indication of the nature of the finished form.

An *official product* is a drug product, dietary supplement, compounded preparation, or finished device for which a monograph is provided.

### 2.30. Legal Recognition

The *USP* and *NF* are recognized in the laws and regulations of many countries throughout the world. Regulatory authorities may enforce the standards presented in the *USP* and *NF*, but because recognition of the *USP* and *NF* may vary by country, users should understand applicable laws and regulations. In the United States under the Federal Food, Drug, and Cosmetic Act (FDCA), both *USP* and *NF* are recognized as official compendia. A drug with a name recognized in *USP–NF* must comply with compendial identity standards or be deemed adulterated, misbranded, or both. See, e.g., FDCA § 501(b) and 502(e)(3)(b); also U.S. Food and Drug Administration (FDA) regulations, 21 CFR § 299.5(a&b). To avoid being deemed adulterated, such drugs must also comply with compendial standards for strength, quality, and purity, unless labeled to show all respects in which the drug differs. See, e.g., FDCA § 501(b) and 21 CFR § 299.5(c). In addition, to avoid being deemed misbranded, drugs recognized in *USP–NF* must also be packaged and labeled in compliance with compendial standards. See FDCA § 502(g).

A dietary supplement represented as conforming to specifications in *USP* will be deemed a misbranded food if it fails to so conform. See FDCA § 403(s)(2)(D).

Enforcement of *USP* standards is the responsibility of FDA and other government authorities in the U.S. and elsewhere. USP has no role in enforcement.

# 3. CONFORMANCE TO STANDARDS

Official

### 3.10. Applicability of Standards

Standards for an article recognized in the compendia (*USP–NF*) are expressed in the article's monograph, applicable general chapters, and *General Notices*. The identity, strength, quality, and purity of an article are determined by the official tests, procedures, and acceptance criteria, and other requirements incorporated in the monograph, in applicable general chapters, or in the *General Notices*.

In a monograph, requirements for official articles are indicated in two ways: (1) for certain monographs, after the official title, the primarily informational portions of the text appear first, followed by the text comprising requirements, the latter section of the monograph being introduced by a double arrow symbol »; and (2) for monographs more recently redesigned, requirements appear in the official title and in any text beginning with the section titled "Definition". In either format, information that appears: (1) after the title but before the double arrow symbol; (2) after the title but before the section titled "Definition"; or (3) in or after the section titled "Auxiliary Information" is provided for informational purposes. See *Preface* for information related to updates to Chemical Information.

Where conformance to any specifications represented by Chemical Information is critical to establishing identity, purity, potency, or another quality attribute of an official article, the monograph will include such specifications in the text that states requirements.

"Applicable general chapters" means general chapters numbered below 1000 or above 2000 that are made applicable to an article through reference in *General Notices*, a monograph, or another applicable general chapter numbered below 1000. Where the requirements of a monograph differ from the requirements specified in these *General Notices* or an applicable general chapter, the monograph requirements apply and supersede the requirements of the *General Notices* or applicable general chapters, whether or not the monograph explicitly states the difference.

General chapters numbered 1000 to 1999 are for informational purposes only. They contain no mandatory tests, assays, or other requirements applicable to any official article, regardless of citation in a general chapter numbered below 1000, a monograph, or these *General Notices*. General chapters numbered above 2000 apply only to articles that are intended for use as dietary ingredients and dietary supplements. General chapter citations in *NF* monographs refer to *USP* general chapters.

Early adoption of revised standards in advance of the official date is allowed by USP unless specified otherwise at the time of publication. Where revised standards for an existing article have been published as final approved "official text" (as approved in section *2.10 Official Text*) but have not yet reached the official date (6 months after publication, unless otherwise specified; see "official date", section *2.20 Official Articles*), compliance with the revised standard shall not preclude a finding or indication of conformance with

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compendial standards, unless USP specifies otherwise by prohibiting early adoption in a particular standard.

The standards in the relevant monograph, general chapter(s), and *General Notices* apply at all times in the life of the article from production to expiration. It is also noted that the manufacturer's specifications, and manufacturing practices (e.g., Quality by Design, Process Analytical Technology, and Real Time Release Testing initiatives), generally are followed to ensure that the article will comply with compendial standards until its expiration date, when stored as directed. Every compendial article in commerce shall be so constituted that when examined in accordance with these assays and test procedures, it meets all applicable pharmacopeial requirements (*General Notices*, monographs, and general chapters). Thus, any official article is expected to meet the compendial standards if tested, and any official article actually tested as directed in the relevant monograph must meet such standards to demonstrate compliance.

Some tests, such as those for *Dissolution* and *Uniformity of Dosage Units*, require multiple dosage units in conjunction with a decision scheme. These tests, albeit using a number of dosage units, are in fact one determination. These procedures should not be confused with statistical sampling plans. The similarity to statistical procedures may seem to suggest an intent to make inference to some larger group of units, but in all cases, statements about whether the compendial standard is met apply only to the units tested. Repeats, replicates, statistical rejection of outliers, or extrapolations of results to larger populations, as well as the necessity and appropriate frequency of batch testing, are neither specified nor proscribed by the compendia; such decisions are based on the objectives of the testing. Frequency of testing and sampling are left to the preferences or direction of those performing compliance testing, and other users of *USP–NF*, including manufacturers, buyers, or regulatory authorities.

Official products are prepared according to recognized principles of good manufacturing practice and from ingredients that meet *USP* or *NF* standards, where standards for such ingredients exist (for dietary supplements, see section *3.10.20 Applicability of Standards to Medical Devices, Dietary Supplements, and Their Components and Ingredients*).

Official substances are prepared according to recognized principles of good manufacturing practice and from ingredients complying with specifications designed to ensure that the resultant substances meet the requirements of the compendial monographs.

**3.10.10. Applicability of Standards to Drug Products, Drug Substances, and Excipients**

The applicable *USP* or *NF* standard applies to any article marketed in the United States that (1) is recognized in the compendium and (2) is intended or labeled for use as a drug or as an ingredient in a drug. Such articles (drug products, drug substances, and excipients) include both human drugs (whether dispensed by prescription, "over the counter", or otherwise), as well as animal drugs. The applicable standard applies to such articles whether or not the added designation "USP" or "NF" is used. The standards apply equally to articles bearing the official titles or names derived by transposition of the definitive words of official titles or transposition in the order of the names of two or more drug substances in official titles, or where there is use of synonyms with the intent or effect of suggesting a significant degree of identity with the official title or name.

**3.10.20. Applicability of Standards to Medical Devices, Dietary Supplements, and Their Components and Ingredients**

An article recognized in *USP* or *NF* shall comply with the compendial standards if the article is a medical device, component intended for a medical device, dietary supplement, dietary ingredient, or other ingredient that is intended for incorporation into a dietary supplement, and is labeled as conforming to the *USP* or *NF*.

Generally, dietary supplements are prepared from ingredients that meet *USP*, *NF*, or *Food Chemicals Codex* standards. Where such standards do not exist, substances may be used in dietary supplements if they have been shown to be of acceptable food grade quality using other suitable procedures.

**3.10.30. Applicability of Standards to the Practice of Compounding**

*USP* compounding practice standards, *Pharmaceutical Compounding—Nonsterile Preparations* ⟨795⟩ and *Pharmaceutical Compounding—Sterile Preparations* ⟨797⟩, as appropriate, apply to compounding practice or activity regardless of whether a monograph exists for the compounded preparation or these chapters are referenced in such a monograph. In the United States, ⟨795⟩ and ⟨797⟩ are not applicable to drugs compounded by entities registered with FDA as outsourcing facilities as defined by FDCA § 503B, because such facilities are required to comply with FDA's current good manufacturing practice requirements. Compounded preparations, including drug products compounded by outsourcing facilities, may also be subject to applicable monographs; see section *2.20 Official Articles* and section *4.10 Monographs*.

**3.10.40. Applicability of Global Health Monographs**

Global Health Monographs (GHM) do not apply to products marketed in the United States. Monographs in this section of the *USP–NF* are not considered "applicable" *USP–NF* monographs for use in pharmacy compounding in the United States under the Federal Food, Drug, and Cosmetic Act. GHMs are provided for possible use outside of the United States, at the discretion of individual government authorities.

Applicability of a GHM, in whole or in part, along with the applicability of associated *General Notices* and general chapter requirements, in whole or in part, is at the discretion of individual government authorities.

Where a product purports to comply with the requirements of an official GHM, it is recommended that the product label or labeling clearly specify conformance to a GHM, e.g., "USP-GHM" or "USP–GH". The designations, "USP–NF", "USP", and "NF" should be used only to indicate conformance to USP or NF monographs outside of GHMs (see section *3.20 Indicating Conformance*).

The designation "USP–GHM" or "USP–GH" on the label may not and does not constitute an endorsement by USP and does not represent assurance by USP that the article is known to comply with the relevant standards. USP may seek legal redress if an article purports to be or is represented as an official GHM article and such claim is determined by USP not to be made in good faith.

Enforcement of GHM requirements is the responsibility of government authorities outside of the U.S. USP has no role in enforcement.

## 3.20. Indicating Conformance

A drug product, drug substance, or excipient may use the designation "USP" or "NF" in conjunction with its official title or elsewhere on the label only when (1) a monograph is provided in the specified compendium and (2) the article complies with the identity prescribed in the specified compendium.

When a drug product, drug substance, compounded preparation, or excipient differs from the relevant *USP* or *NF* standard of strength, quality, or purity, as determined by the application of the tests, procedures, and acceptance criteria set forth in the relevant compendium, its difference shall be plainly stated on its label.

Official

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When a drug product, drug substance, compounded preparation, or excipient fails to comply with the identity prescribed in *USP* or *NF* or contains an added substance that interferes with the prescribed tests and procedures, the article shall be designated by a name that is clearly distinguishing and differentiating from any name recognized in *USP* or *NF*.

A medical device, dietary supplement, or ingredient or component of a medical device or dietary supplement may use the designation "USP" or "NF" in conjunction with its official title or elsewhere on the label only when (1) a monograph is provided in the specified compendium and (2) the article complies with the monograph standards and other applicable standards in that compendium.

The designation "USP" or "NF" on the label may not and does not constitute an endorsement by USP and does not represent assurance by USP that the article is known to comply with the relevant standards. USP may seek legal redress if an article purports to be or is represented as an official article in one of USP's compendia and such claim is determined by USP not to be made in good faith.

The designation "USP–NF" may be used on the label of an article provided that the label also bears a statement such as "Meets *NF* standards as published by USP", indicating the particular compendium to which the article purports to apply.

When the letters "USP", "NF", or "USP–NF" are used on the label of an article to indicate compliance with compendial standards, the letters shall appear in conjunction with the official title of the article. The letters are not to be enclosed in any symbol such as a circle, square, etc., and shall appear in capital letters.

If a dietary supplement does not comply with all applicable compendial requirements but contains one or more dietary ingredients or other ingredients that are recognized in *USP* or *NF*, the individual ingredient(s) may be designated as complying with *USP* or *NF* standards or being of *USP* or *NF* quality provided that the designation is limited to the individual ingredient(s) and does not suggest that the dietary supplement complies with *USP* standards.

# 4. MONOGRAPHS AND GENERAL CHAPTERS

Official

## 4.10. Monographs

Monographs set forth the article's name, definition, specification, and other requirements related to packaging, storage, and labeling. The specification consists of tests, procedures, and acceptance criteria that help ensure the identity, strength, quality, and purity of the article. For general requirements relating to specific monograph sections, see section *5. Monograph Components*.

Because monographs may not provide standards for all relevant characteristics, some official substances may conform to the *USP* or *NF* standard but differ with regard to nonstandardized properties that are relevant to their use in specific preparations. To assure substitutability in such instances, users may wish to ascertain functional equivalence or determine such characteristics before use.

### 4.10.10. Applicability of Test Procedures

A single monograph may include more than one test, procedure, and/or acceptance criterion for the same attribute. Unless otherwise specified in the monograph, all tests are requirements. In some cases, monograph instructions allow the selection of tests that reflect attributes of different manufacturers' articles, such as different polymorphic forms, impurities, hydrates, and dissolution. Monograph instructions indicate the tests, procedures, and/or acceptance criteria to be used and the required labeling.

The order in which the tests are listed in the monograph is based on the order in which they are approved by the relevant Expert Committee for inclusion in the monograph. Test 1 is not necessarily the test for the innovator or for the reference product. Depending on monograph instructions, a labeling statement is not typically required if Test 1 is used.

### 4.10.20. Acceptance Criteria

The acceptance criteria allow for analytical error, for unavoidable variations in manufacturing and compounding, and for deterioration to an extent considered acceptable under practical conditions. The existence of compendial acceptance criteria does not constitute a basis for a claim that an official substance that more nearly approaches 100% purity "exceeds" compendial quality. Similarly, the fact that an article has been prepared to tighter criteria than those specified in the monograph does not constitute a basis for a claim that the article "exceeds" the compendial requirements.

An official product shall be formulated with the intent to provide 100% of the quantity of each ingredient declared on the label. Where the minimum amount of a substance present in a dietary supplement is required by law to be higher than the lower acceptance criterion allowed for in the monograph, the upper acceptance criterion contained in the monograph may be increased by a corresponding amount.

The acceptance criteria specified in individual monographs and in the general chapters for compounded preparations are based on such attributes of quality as might be expected to characterize an article compounded from suitable bulk drug substances and ingredients, using the procedures provided or recognized principles of good compounding practice, as described in these compendia.

## 4.20. General Chapters

Each general chapter is assigned a number that appears in angle brackets adjacent to the chapter name (e.g., *Chromatography* ⟨621⟩). General chapters may contain the following:

- Descriptions of tests and procedures for application through individual monographs,
- Descriptions and specifications of conditions and practices for pharmaceutical compounding,
- General information for the interpretation of the compendial requirements,

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- Descriptions of general pharmaceutical storage, dispensing, and packaging practices, or
- General guidance to manufacturers of official substances or official products.

When a general chapter is referenced in a monograph, acceptance criteria may be presented after a colon.

Some chapters may serve as introductory overviews of a test or of analytical techniques. They may reference other general chapters that contain techniques, details of the procedures, and, at times, acceptance criteria.

# 5. MONOGRAPH COMPONENTS

Official

### 5.10. Molecular Formula

The use of the molecular formula for the official substance(s) named in defining the required strength of a compendial article is intended to designate the chemical entity or entities, as given in the complete chemical name of the article, having absolute (100%) purity.

### 5.20. Added Substances

Added substances are presumed to be unsuitable for inclusion in an official article and therefore prohibited, if their presence impairs the bioavailability, therapeutic efficacy, or safety of the official article; or they interfere with the assays and tests prescribed for determining compliance with the compendial standards (see section *3.20 Indicating Conformance*).

The air in a container of an official article may, where appropriate, be evacuated or be replaced by carbon dioxide, helium, argon, or nitrogen, or by a mixture of these gases. The use of such gas need not be declared in the labeling.

**5.20.10. Added Substances in Official Substances**

Official substances may contain only the specific added substances that are permitted by the individual monograph. Such added substances shall not exceed the quantity required for providing their intended effect. Where such addition is permitted, the label shall indicate the name(s) and amount(s) of any added substance(s).

**5.20.20. Added Substances (Excipients and Ingredients) in Official Products**

Suitable substances and excipients such as antimicrobial agents, pharmaceutical bases, carriers, coatings, flavors, preservatives, stabilizers, and vehicles may be added to an official product to enhance its stability, usefulness, or elegance, or to facilitate its preparation, unless otherwise specified in the individual monograph.

Added substances and excipients employed solely to impart color may be incorporated into official products other than those intended for parenteral or ophthalmic use, in accordance with the regulations pertaining to the use of colors issued by the FDA, provided such added substances or excipients are otherwise appropriate in all respects. (See also *Injections and Implanted Drugs Products* ⟨1⟩*, Product Quality Tests Common to Parenteral Dosage Forms, Specific Tests, Vehicles and added substances, Added substances*.)

The proportions of the substances constituting the base in ointment and suppository products and preparations may be varied to maintain a suitable consistency under different climatic conditions, provided that the concentrations of drug substances are not varied and provided that the bioavailability, therapeutic efficacy, and safety of the preparation are not impaired.

***5.20.20.1. In Compounded Preparations***

Compounded preparations for which a complete composition is given shall contain only the ingredients named in the formulas unless specifically exempted herein or in the individual monograph. Deviation from the specified processes or methods of compounding, although not from the ingredients or proportions thereof, may occur provided that the finished preparation conforms to the relevant standards and to preparations produced by following the specified process.

Where a monograph for a compounded preparation calls for an ingredient in an amount expressed on the dried basis, the ingredient need not be dried before use if due allowance is made for the water or other volatile substances present in the quantity taken.

Specially denatured alcohol formulas are available for use in accordance with federal statutes and regulations of the Internal Revenue Service. A suitable formula of specially denatured alcohol may be substituted for Alcohol in the manufacture of official preparations intended for internal or topical use, provided that the denaturant is volatile and does not remain in the finished product. A preparation that is intended for topical application to the skin may contain specially denatured alcohol, provided that the denaturant is either a usual ingredient in the preparation or a permissible added substance; in either case the denaturant shall be identified on the label of the topical preparation. Where a process is given in the individual monograph, any preparation compounded using denatured alcohol shall be identical to that prepared by the monograph process.

***5.20.20.2. In Dietary Supplements***

Additional ingredients may be added to dietary supplement products provided that the additional ingredients (1) comply with applicable regulatory requirements, and (2) do not interfere with the assays and tests prescribed for determining compliance with compendial standards.

### 5.30. Description and Solubility

Only where a quantitative solubility test is given in a monograph and is designated as such is it a test for purity.

A monograph may include information regarding the article's description. Information about an article's "description and solubility" also is provided in the reference table *Description and Relative Solubility of USP and NF Articles*. The reference table merely denotes the properties of articles

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that comply with monograph standards. The reference table is intended primarily for those who use, prepare, and dispense drugs and/or related articles. Although the information provided in monographs and the information in the reference table may indirectly assist in the preliminary evaluation of an article, it is not intended to serve as a standard or test for purity.

The approximate solubility of a compendial substance is indicated by one of the following descriptive terms:

| Descriptive Term | Parts of Solvent Required for 1 Part of Solute |
|---|---|
| Very soluble | Less than 1 |
| Freely soluble | From 1 to 10 |
| Soluble | From 10 to 30 |
| Sparingly soluble | From 30 to 100 |
| Slightly soluble | From 100 to 1,000 |
| Very slightly soluble | From 1,000 to 10,000 |
| Practically insoluble, or Insoluble | Greater than or equal to 10,000 |

## 5.40. Identification

A compendial test titled *Identification* is provided as an aid in verifying the identity of articles as they are purported to be, e.g., those taken from labeled containers, and to establish whether it is the article named in *USP–NF*. The *Identification* test for a particular article may consist of one or more procedures. When a compendial *Identification* test is undertaken, all requirements of all specified procedures in the test must be met to satisfy the requirements of the test. Failure of an article to meet all the requirements of a prescribed *Identification* test (i.e., failure to meet the requirements of all of the specified procedures that are components of that test) indicates that the article is mislabeled and/or adulterated.

## 5.50. Assay

Assay tests for compounded preparations are not intended for evaluating a compounded preparation before dispensing, but instead are intended to serve as the official test in the event of a question or dispute regarding the preparation's conformance to official standards.

### 5.50.10. Units of Potency (Biological)

For substances that cannot be completely characterized by chemical or physical means or that need confirmation of functionality or tertiary structure, it may be necessary to express quantities of biological activity in units of biological potency, each defined by an authoritative, designated reference standard. In cases where international reference materials have been discontinued, international units of potency may be defined in terms of molecular mass, such as in the cases of vitamins A, D, and E.

Where available, World Health Organization (WHO) international biological standards define the International Units (IU). *USP* monographs refer to the units assigned by USP Reference Standards either directly as International Units (IU) or as "USP Units". For some biological products, units of potency are value assigned against a corresponding U.S. Standard established by FDA, whether or not International Units or USP Units have been defined (see *Biologics* ⟨1041⟩). Note that product-related labeling, e.g., on containers, need not use the full phrase "USP [product name] Units" that appears in many *USP* monograph labeling sections. The term "USP Units" can be used on product labeling consistent with USP compendial requirements, provided it is clear from the context that the potency is stated in terms of USP [product name] Units. In such circumstances it should be clear that "USP Units" and "USP [product name] Units" share the same meaning.

## 5.60. Impurities and Foreign Substances

Tests for the presence of impurities and foreign substances are provided to limit such substances to amounts that are unobjectionable under conditions in which the article is customarily employed (see also *Impurities in Drug Substances and Drug Products* ⟨1086⟩).

Nonmonograph tests and acceptance criteria suitable for detecting and controlling impurities that may result from a change in the processing methods or that may be introduced from external sources should be employed in addition to the tests provided in the individual monograph, where the presence of the impurity is inconsistent with applicable good manufacturing practices or good pharmaceutical practices.

### 5.60.10. Other Impurities in *USP* and *NF* Articles

If a *USP* or *NF* monograph includes an assay or organic impurity test based on chromatography, other than a test for residual solvents, and that monograph procedure does not detect an impurity present in the substance, the amount and identity of the impurity, where both are known, shall be stated in the labeling (certificate of analysis) of the official substance, under the heading *Other Impurity(ies)*.

The presence of any unlabeled other impurity in an official substance is a variance from the standard if the content is 0.1% or greater. The sum of all *Other Impurities* combined with the monograph-detected impurities may not exceed 2.0% (see *Ordinary Impurities* ⟨466⟩), unless otherwise stated in the monograph.

The following categories of drug substances are excluded from *Other Impurities* requirements:

- Fermentation products and semi-synthetics derived therefrom,
- Radiopharmaceuticals,
- Biologics,
- Biotechnology-derived products,
- Peptides,
- Herbals, and
- Crude products of animal or plant origin.

Any substance known to be toxic shall not be listed under *Other Impurities*.

### 5.60.20. Residual Solvents in *USP* and *NF* Articles

All *USP* and *NF* articles are subject to relevant control of residual solvents, even when no test is specified in the individual monograph. If solvents are used during production, they must be of suitable quality. In addition, the toxicity and residual level of each solvent shall be taken into consideration, and the solvents limited according to the principles defined and the requirements specified in *Residual Solvents* ⟨467⟩, using the general methods presented therein or other suitable methods.

### 5.60.30. Elemental Impurities in USP Drug Products and Dietary Supplements

Elemental impurities in official drug products are controlled according to the principles defined and requirements specified in *Elemental Impurities—Limits* ⟨232⟩. Elemental contaminants in official dietary supplements are controlled according to the principles defined and requirements specified in *Elemental Contaminants in Dietary Supplements* ⟨2232⟩.

## 5.70. Performance Tests

Where content uniformity determinations have been made using the same analytical methodology specified in the *Assay*, with appropriate allowances made for differences in sample

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preparation, the average of all of the individual content uniformity determinations may be used as the *Assay* value.

### 5.80. USP Reference Standards

USP Reference Standards are authentic specimens that have been approved as suitable for use in *USP* or *NF* tests and assays (see *USP Reference Standards* ⟨11⟩). Where *USP* or *NF* tests or assays call for the use of a USP Reference Standard, only those results obtained using the specified USP Reference Standard are conclusive. Where a procedure calls for the use of a compendial article rather than for a USP Reference Standard as a material standard of reference, a substance meeting all of the compendial monograph requirements for that article shall be used. If any new *USP* or *NF* standard requires the use of a new USP Reference Standard that is not yet available, that portion of the standard containing the requirement shall not be official until the specified USP reference material is available.

Announcements of the availability of new USP Reference Standards and announcements of tests or procedures that are held in abeyance pending availability of required USP Reference Standards are also available on the "Official Text" tab of USP's website (www.uspnf.com). Unless otherwise directed in the procedure in the individual monograph or in a general chapter, USP Reference Standards are to be used in accordance with the instructions on the label of the Reference Standard.

# 6. TESTING PRACTICES AND PROCEDURES

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### 6.10. Safe Laboratory Practices

In performing compendial procedures, safe laboratory practices shall be followed, including precautionary measures, protective equipment, and work practices consistent with the chemicals and procedures used. Before undertaking any procedure described in the compendia, the analyst should be aware of the hazards associated with the chemicals and the techniques and means of protecting against them. These compendia are not designed to describe such hazards or protective measures.

### 6.20. Automated Procedures

Automated and manual procedures employing the same basic chemistry are considered equivalent provided the automated system is properly qualified as being suitable to execute the compendial manual method and the analytical procedure is verified under the new equipment conditions.

### 6.30. Alternative and Harmonized Methods and Procedures

An alternative method or procedure is defined as any method or procedure other than the compendial method or procedure for the article in question. The alternative method or procedure must be fully validated (see *Validation of Compendial Procedures* ⟨1225⟩) and must produce comparable results to the compendial method or procedure within allowable limits established on a case-by-case basis. Alternative methods or procedures can be developed for any one of a number of reasons not limited to simplification of sample preparation, enhanced precision and accuracy, improved (shortened) run time, or being better suited to automation than the compendial method or procedure. Only those results obtained by the methods and procedures given in the compendia are conclusive.

For evaluation as a potential replacement or addition to the standard, alternative methods and procedures should be submitted to USP (see section *4.10 Monographs*).

Certain general chapters contain a statement that the text in question is harmonized with the corresponding text of the *European Pharmacopoeia* and/or the *Japanese Pharmacopoeia* and that these texts are interchangeable. Therefore, if a substance or preparation is found to comply with a requirement using an interchangeable method or procedure from one of these pharmacopeias, it should comply with the requirements of the *USP–NF*. When a difference appears, or in the event of dispute, only the result obtained by the method and/or procedure given in the *USP–NF* is conclusive.

### 6.40. Dried, Anhydrous, Ignited, or Solvent-Free Basis

All calculations in the compendia assume an "as-is" basis unless otherwise specified.

Test procedures may be performed on the undried or unignited substance and the results calculated on the dried, anhydrous, or ignited basis, provided a test for *Loss on Drying*, or *Water Determination*, or *Loss on Ignition*, respectively, is given in the monograph. Where the presence of moisture or other volatile material may interfere with the procedure, previous drying of the substance is specified in the individual monograph and is obligatory.

The term "solvent-free" signifies that the calculation shall be corrected for the presence of known solvents as determined using the methods described in ⟨467⟩ unless a test for limit of organic solvents is provided in the monograph.

The term "previously dried" without qualification signifies that the substance shall be dried as directed under *Loss on Drying* ⟨731⟩ or *Water Determination* ⟨921⟩ (gravimetric determination).

Where drying in vacuum over a desiccant is directed, a vacuum desiccator, a vacuum drying pistol, or other suitable vacuum drying apparatus shall be used.

#### 6.40.10. Ignite to Constant Weight

"Ignite to constant weight" means that ignition shall be continued at 800 ± 25°, unless otherwise indicated, until two consecutive weighings, the second of which is taken after an additional period appropriate to the nature and quantity of the residue, do not differ by more than 0.50 mg per g of substance taken.

#### 6.40.20. Dried to Constant Weight

"Dried to constant weight" means that drying shall be continued until two consecutive weighings, the second of which is taken after an additional drying period appropriate to

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the nature and quantity of the residue, do not differ by more than 0.50 mg per g of substance taken.

## 6.50. Preparation of Solutions

### 6.50.10. Filtration

Where a procedure gives direction to "filter" without further qualification, the liquid shall be passed through suitable filter paper or equivalent device until the filtrate is clear. Due to the possibility of filter effects, the initial volumes of a filtrate may be discarded.

### 6.50.20. Solutions

Unless otherwise specified, all solutions shall be prepared with Purified Water. Solutions for quantitative measures shall be prepared using accurately weighed or accurately measured analytes (see section *8.20 About*).

An expression such as "(1 in 10)" means that 1 part *by volume* of a liquid shall be diluted with, or 1 part *by weight* of a solid shall be dissolved in, a sufficient quantity of the diluent or solvent to make the volume of the finished solution 10 parts *by volume*. For example, a 1 in 10 solution is prepared by diluting 1 mL of a liquid or dissolving 1 g of a solid in sufficient solvent to make 10 mL of the solution. An expression such as "(20:5:2)" means that the respective numbers of parts, by volume, of the designated liquids shall be mixed, unless otherwise indicated.

#### *6.50.20.1. Adjustments to Solutions*

When a specified concentration is called for in a procedure, a solution of other normality or molarity may be used, provided that allowance is made for the difference in concentration and that the change does not increase the error of measurement.

Proportionately larger or smaller quantities than the specified weights and volumes of assay or test substances and Reference Standards may be taken, provided the measurement is made with at least equivalent accuracy.

Unless otherwise indicated, analyte concentrations shall be prepared to within ten percent (10%) of the indicated value. In the case in which a procedure is adapted to the working range of an instrument, solution concentrations may differ from the indicated value by more than ten percent (10%), with appropriate changes in associated calculations. Any changes shall fall within the validated range of the instrument.

When adjustment of pH is indicated with either an acid or base and the concentration is not indicated, appropriate concentrations of that acid or base may be used.

#### *6.50.20.2. Test Solutions*

Information on Test Solutions (TS) is provided in the *Test Solutions* portion of the *Reagents, Indicators, and Solutions* section of the *USP–NF*. Use of an alternative Test Solution or a change in the Test Solution used may require validation.

#### *6.50.20.3. Indicator Solutions*

Where a procedure specifies the use of an indicator TS, approximately 0.2 mL, or 3 drops, of the solution shall be added unless otherwise directed.

## 6.60. Units Necessary to Complete a Test

Unless otherwise specified, a sufficient number of units to ensure a suitable analytical result shall be taken.

### 6.60.10. Tablets

Where the procedure of a Tablet monograph directs to weigh and finely powder not fewer than a given number of Tablets, a counted number of Tablets shall be weighed and reduced to a powder. The portion of the powdered Tablets taken shall be representative of the whole Tablets and shall, in turn, be weighed accurately.

### 6.60.20. Capsules

Where the procedure of a Capsule monograph gives direction to remove, as completely as possible, the contents of not fewer than a given number of the Capsules, a counted number of Capsules shall be carefully opened and the contents quantitatively removed, combined, mixed, and weighed accurately. The portion of mixed Capsules contents taken shall be representative of the contents of the Capsules and shall, in turn, be weighed accurately.

## 6.70. Reagents

The proper conduct of the compendial procedures and the reliability of the results depend, in part, upon the quality of the reagents used in the performance of the procedures. Unless otherwise specified, reagents conforming to the specifications set forth in the current edition of *Reagent Chemicals* published by the American Chemical Society (ACS) shall be used. Where such ACS reagent specifications are not available or where the required purity differs, compendial specifications for reagents of acceptable quality are provided (see the *Reagents, Indicators, and Solutions* section of the *USP–NF*). Reagents not covered by any of these specifications should be of a grade suitable to the proper performance of the method of assay or test involved.

Listing of these reagents, including the indicators and solutions employed as reagents, in no way implies that they have therapeutic utility; furthermore, any reference to *USP* or *NF* in their labeling shall include also the term "reagent" or "reagent grade". USP may supply reagents if they otherwise may not be generally commercially available.

## 6.80. Equipment

Unless otherwise specified, a specification for a definite size or type of container or apparatus in a procedure is given solely as a recommendation. Other dimensions or types may be used if they are suitable for the intended use.

### 6.80.10. Apparatus for Measurement

Where volumetric flasks or other exact measuring, weighing, or sorting devices are specified, this or other equipment of at least equivalent accuracy shall be employed.

#### *6.80.10.1. Pipet/Pipette*

Where a pipet/pipette is specified, a suitable buret may be substituted. Where a "to contain" pipet/pipette is specified, a suitable volumetric flask may be substituted.

#### *6.80.10.2. Light Protection*

Where low-actinic or light-resistant containers are specified, either containers specially treated to protect contents from light or clear containers that have been rendered opaque by application of a suitable coating or wrapping may be used.

### 6.80.20. Instrumental Apparatus

An instrument may be substituted for the specified instrument if the substitute uses the same fundamental principles of operation and is of equivalent or greater sensitivity and accuracy. These characteristics shall be qualified as appropriate. Where a particular brand or source of a material, instrument, or piece of equipment, or the name and address of a manufacturer or distributor, is mentioned (ordinarily in a footnote), this identification is furnished solely for informational purposes as a matter of convenience, without implication of approval, endorsement, or certification.

#### *6.80.20.1. Chromatographic Tubes and Columns*

The term "diameter" refers to internal diameter (ID).

#### *6.80.20.2. Tubing*

The term "diameter" refers to outside diameter (OD).

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***6.80.20.3. Steam Bath***

Where use of a steam bath is directed, use actively flowing steam or another regulated heat source controlled at an equivalent temperature.

***6.80.20.4. Water Bath***

A water bath requires vigorously boiling water unless otherwise specified.

**6.80.30. Temperature Reading Devices**

Temperature reading devices suitable for pharmacopeial tests conform to specifications that are traceable to a National Institute of Standards and Technology (NIST) standard or equivalent. Temperature reading devices may be of the liquid-in-glass type or an analog or digital temperature indicator type, such as a resistance temperature device, thermistor, or thermocouple. Standardization of thermometers is performed on an established testing frequency with a temperature standard traceable to NIST. For example, refer to the current issue of American Society of Testing and Materials (ASTM) standards E1 for liquid-in-glass thermometers.

# 7. TEST RESULTS

## 7.10. Interpretation of Requirements

Analytical results observed in the laboratory (or calculated from experimental measurements) are compared with stated acceptance criteria to determine whether the article conforms to compendial requirements.

The reportable value, which often is a summary value for several individual determinations, is compared with the acceptance criteria. The reportable value is the end result of a completed measurement procedure, as documented.

Where acceptance criteria are expressed numerically herein through specification of an upper and/or lower limit, permitted values include the specified values themselves, but no values outside the limit(s). Acceptance criteria are considered significant to the last digit shown.

**7.10.5. Nominal Concentrations in Equations**

Where a "nominal concentration" is specified, calculate the concentration based on the label claim. In assay procedures, water correction is typically stated in the Definition and on the label of the USP Reference Standard. For other procedures, correction for assayed content, potency, or both is made prior to using the concentration in the equation provided in the monograph.

**7.10.10. Equivalence Statements in Titrimetric Procedures**

The directions for titrimetric procedures conclude with a statement of the weight of the analyte that is equivalent to each mL of the standardized titrant. In such an equivalence statement, the number of significant figures in the concentration of the titrant should be understood to correspond to the number of significant figures in the weight of the analyte. Corrections to calculations based on the blank determination are to be made for all titrimetric assays where appropriate (see *Titrimetry* ⟨541⟩).

## 7.20. Rounding Rules

The observed or calculated values shall be rounded off to the number of decimal places that is in agreement with the limit expression. Numbers should not be rounded until the final calculations for the reportable value have been completed. Intermediate calculations (e.g., slope for linearity) may be rounded for reporting purposes, but the original (not rounded) value should be used for any additional required calculations. Acceptance criteria are fixed numbers and are not rounded.

When rounding is required, consider only one digit in the decimal place to the right of the last place in the limit expression. If this digit is smaller than 5, it is eliminated and the preceding digit is unchanged. If this digit is equal to or greater than 5, it is eliminated and the preceding digit is increased by 1.

**Illustration of Rounding Numerical Values for Comparison with Requirements**

| Compendial Requirement | Unrounded Value | Rounded Result | Conforms |
|---|---|---|---|
| Assay limit ≥98.0% | 97.96% | 98.0% | Yes |
| | 97.92% | 97.9% | No |
| | 97.95% | 98.0% | Yes |
| Assay limit ≤101.5% | 101.55% | 101.6% | No |
| | 101.46% | 101.5% | Yes |
| | 101.45% | 101.5% | Yes |
| Limit test ≤0.02% | 0.025% | 0.03% | No |
| | 0.015% | 0.02% | Yes |
| | 0.027% | 0.03% | No |
| Limit test ≤3 ppm | 3.5 ppm | 4 ppm | No |
| | 3.4 ppm | 3 ppm | Yes |
| | 2.5 ppm | 3 ppm | Yes |

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# 8. TERMS AND DEFINITIONS

### 8.10. Abbreviations

- RS refers to a USP Reference Standard.
- CS refers to a Colorimetric Solution.
- TS refers to a Test Solution.
- VS refers to a Volumetric Solution that is standardized in accordance with directions given in the individual monograph or in the *Reagents, Indicators, and Solutions* section of *USP–NF*.

### 8.20. About

"About" indicates a quantity within 10%.

If the measurement is stated to be "accurately measured" or "accurately weighed", follow the statements in *Volumetric Apparatus* ⟨31⟩ and *Balances* ⟨41⟩, respectively.

### 8.30. Alcohol Content

Percentages of alcohol, such as those under the heading *Alcohol Content*, refer to percentage by volume of $C_2H_5OH$ at 15.56°. Where a formula, test, or assay calls for alcohol, ethyl alcohol, or ethanol, the *USP* monograph article Alcohol shall be used. Where reference is made to "$C_2H_5OH$", absolute (100%) ethanol is intended. Where a procedure calls for dehydrated alcohol, alcohol absolute, or anhydrous alcohol, the *USP* monograph article Dehydrated Alcohol shall be used.

### 8.40. Atomic Weights

Atomic weights used in computing molecular weights and the factors in the assays and elsewhere are those established by the IUPAC Commission on Isotopic Abundances and Atomic Weights.

### 8.50. Blank Determinations

Where it is directed that "any necessary correction" be made by a blank determination, the determination shall be conducted using the same quantities of the same reagents treated in the same manner as the solution or mixture containing the portion of the substance under assay or test, but with the substance itself omitted.

### 8.60. Concomitantly

"Concomitantly" denotes that the determinations or measurements are to be performed in immediate succession.

### 8.70. Desiccator

The instruction "in a desiccator" indicates use of a tightly closed container of suitable size and design that maintains an atmosphere of low moisture content by means of a suitable desiccant such as anhydrous calcium chloride, magnesium perchlorate, phosphorus pentoxide, or silica gel. See also section *8.220 Vacuum Desiccator*.

### 8.80. Logarithms

Logarithms are to the base 10.

### 8.90. Microbial Strain

A microbial strain cited and identified by its American Type Culture Collection (ATCC) catalog number shall be used directly or, if subcultured, shall be used not more than five passages removed from the original strain.

### 8.100. Negligible

"Negligible" indicates a quantity not exceeding 0.50 mg.

### 8.110. NLT/NMT

"NLT" means "not less than". "NMT" means "not more than."

### 8.120. Odor

"Odorless", "practically odorless", "a faint characteristic odor", and variations thereof indicate evaluation of a suitable quantity of freshly opened material after exposure to the air for 15 minutes. An odor designation is descriptive only and should not be regarded as a standard of purity for a particular lot of an article.

### 8.130. Percent

"Percent" used without qualification means:

- For mixtures of solids and semisolids, percent weight in weight;
- For solutions or suspensions of solids in liquids, percent weight in volume;
- For solutions of liquids in liquids, percent volume in volume;
- For solutions of gases in liquids, percent weight in volume.

For example, a 1 percent solution is prepared by dissolving 1 g of a solid or semisolid, or 1 mL of a liquid, in sufficient solvent to make 100 mL of the solution.

### 8.140. Percentage Concentrations

Percentage concentrations are expressed as follows:

- *Percent Weight in Weight* (w/w) is defined as the number of g of a solute in 100 g of solution.
- *Percent Weight in Volume* (w/v) is defined as the number of g of a solute in 100 mL of solution.
- *Percent Volume in Volume* (v/v) is defined as the number of mL of a solute in 100 mL of solution.

### 8.150. Pressure

Pressure is determined by use of a suitable manometer or barometer calibrated in terms of the pressure exerted by a column of mercury of the stated height.

### 8.160. Reaction Time

Reaction time is 5 minutes unless otherwise specified.

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### 8.170. Specific Gravity

Specific gravity is the weight of a substance in air at 25° divided by the weight of an equal volume of water at the same temperature.

### 8.180. Temperatures

Temperatures are expressed in centigrade (Celsius) degrees, and all measurements are made at 25° unless otherwise indicated. Where moderate heat is specified, any temperature not higher than 45° (113° F) is indicated.

### 8.190. Time

Unless otherwise specified, rounding rules, as described in section *7.20 Rounding Rules*, apply to any time specified.

### 8.200. Transfer

"Transfer" indicates a quantitative manipulation.

### 8.210. Vacuum

"Vacuum" denotes exposure to a pressure of less than 20 mm of mercury (2.67 kPas), unless otherwise indicated.

### 8.220. Vacuum Desiccator

"Vacuum desiccator" indicates a desiccator that maintains a low-moisture atmosphere at a reduced pressure of not more than 20 mm of mercury (2.67 kPas) or at the pressure designated in the individual monograph.

### 8.230. Water

#### 8.230.10. Water as an Ingredient in an Official Product

As an ingredient in an official product, water meets the requirements of the appropriate water monograph in *USP* or *NF*.

#### 8.230.20. Water in the Manufacture of Official Substances

When used in the manufacture of official substances, water shall meet the requirements for drinking water as set forth in the U.S. Environmental Protection Agency National Primary Drinking Water Regulations or in the drinking water regulations of the European Union or of Japan, or in the World Health Organization's Guidelines for Drinking Water Quality. Additional specifications may be required in monographs.

#### 8.230.30. Water in a Compendial Procedure

When water is called for in a compendial procedure, the *USP* monograph article Purified Water shall be used unless otherwise specified. Definitions for other types of water are provided in *Reagents, Indicators, and Solutions* and in *Water for Pharmaceutical Purposes* ⟨1231⟩.

### 8.240. Weights and Measures

In general, weights and measures are expressed in the International System of Units (SI) as established and revised by the *Conférence générale des poids et mesures*. For compendial purposes, the term "weight" is considered to be synonymous with "mass".

Molality is designated by the symbol *m* preceded by a number that represents the number of moles of the designated solute contained in 1 kilogram of the designated solvent.

Molarity is designated by the symbol M preceded by a number that represents the number of moles of the designated solute contained in an amount of the designated solvent that is sufficient to prepare 1 liter of solution.

Normality is designated by the symbol N preceded by a number that represents the number of equivalents of the designated solute contained in an amount of the designated solvent that is sufficient to prepare 1 liter of solution.

The symbol for degrees (°) without a qualifying unit of measure represents degrees Celsius.

Chart of Symbols and Prefixes commonly employed for SI metric units and other units:

| | Units | Symbol | Notes |
|---|---|---|---|
| **Length** | | | |
| | meter | m | |
| | centimeter | cm | |
| | millimeter | mm | |
| | micrometer | µm | Previously referred to as a micron |
| | nanometer | nm | Previously the symbol mµ (for millimicron) was used |
| | Ångström | Å | Equal to 0.1 nm |
| **Mass** | | | |
| | kilogram | kg | |
| | gram | g | |
| | milligram | mg | |
| | microgram | µg | The symbol µg is used in the *USP* and *NF* to represent micrograms, but micrograms may be represented as "mcg" for labeling and prescribing purposes. The term "gamma", symbolized by γ, frequently is used to represent micrograms in biochemical literature. |
| | nanogram | ng | |
| | picogram | pg | |
| | dalton | Da | Also referred to as the unified atomic mass unit and is equal to 1/12 times the mass of the free carbon 12 atom. |
| | kilodalton | kDa | |
| **Time** | | | |
| | second | s | |
| | minute | min | |
| | hour | h | |
| **Volume** | | | |
| | liter | L | 1 L is equal to 1000 $cm^3$ (cubic centimeters) |
| | deciliter | dL | |
| | milliliter | mL | 1 mL is equal to 1 $cm^3$, sometimes referred to as cc |
| | microliter | µL | |
| **Temperature** | | | |
| | Celsius | °C | |
| **Amount of Substance** | | | |
| | mole | mol | Historically referred to as gram-molecular weight or gram-atomic weight |
| | millimole | mmol | |

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| | Units | Symbol | Notes |
|---|---|---|---|
| | micromole | µmol | |
| | femtomole | fmol | |
| | equivalent | Eq | Also referred to as gram-equivalent weight. It is used in the calculation of substance concentration in units of normality. This unit is no longer preferred for use in analytical chemistry or metrology. |
| | milli equivalent | mEq | |
| | osmole | Osmol | Osmotic pressure of a solution, related to substance concentration. |
| | milliosmole | mOsmol | |
| **Pressure** | | | |
| | pascal | Pa | |
| | kilopascal | kPa | |
| | pounds per square inch | psi | |
| | millimeter of mercury | mmHg | Equal to 133.322 Pa |
| **Electrical units** | | | |
| | ampere | A | |
| | volt | V | |
| | millivolt | mV | |
| | hertz | Hz | Unit of frequency |
| | kilohertz | kHz | |
| | megahertz | MHz | |
| | electron volt | eV | |
| | kilo-electron volt | keV | |
| | mega-electron volt | MeV | |

| | Units | Symbol | Notes |
|---|---|---|---|
| **Radiation** | | | |
| | becquerel | Bq | SI unit of activity for radionuclides |
| | kilobecquerel | kBq | |
| | megabecquerel | MBq | |
| | gigabecquerel | GBq | |
| | curie | Ci | Non-SI unit of activity for radionuclides |
| | millicurie | mCi | |
| | microcurie | µCi | |
| | nanocurie | nCi | |
| **Other** | | | |
| | acceleration due to gravity | g | Used to express rate of centrifugation |
| | revolutions per minute | rpm | Used to express rate of centrifugation |

**Selected SI Prefixes**

| Name | Symbol | Factor |
|---|---|---|
| giga | G | $10^{9}$ |
| mega | M | $10^{6}$ |
| kilo | k | $10^{3}$ |
| deci | d | $10^{-1}$ |
| centi | c | $10^{-2}$ |
| milli | m | $10^{-3}$ |
| micro | µ | $10^{-6}$ |
| nano | n | $10^{-9}$ |
| pico | p | $10^{-12}$ |
| femto | f | $10^{-15}$ |

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# 9. PRESCRIBING AND DISPENSING

### 9.10. Use of Metric Units

Prescriptions for compendial articles shall be written to state the quantity and/or strength desired in metric units unless otherwise indicated in the individual monograph [see also section *5.50.10 Units of Potency (Biological)* above]. If an amount is prescribed by any other system of measurement, only an amount that is the metric equivalent of the prescribed amount shall be dispensed. Abbreviations for the terms "Units" or "International Units" shall not be used for labeling or prescribing purposes. Apothecary unit designations on labels and labeling shall not be used.

### 9.20. Changes in Volume

In the dispensing of prescription medications, slight changes in volume owing to variations in room temperatures may be disregarded.

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# 10. PRESERVATION, PACKAGING, STORAGE, AND LABELING

### 10.10. Packaging and Storage

All articles in *USP* or *NF* are subject to the packaging and storage requirements specified in *Packaging and Storage Requirements* ⟨*659*⟩, unless different requirements are provided in an individual monograph.

### 10.20. Labeling

All articles in *USP* or *NF* are subject to the labeling requirements specified in *Labeling* ⟨*7*⟩, unless different requirements are provided in an individual monograph.

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# Half-Lives of Selected Radionuclides

This table can be found at http://www.nist.gov/pml/data/halflife-html.cfm.

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# INTRINSIC VISCOSITY TABLE

This table is based on data appearing in Table 3 of ASTM Test Method D 1795 (publication of the American Society for Testing and Materials).

**Intrinsic Viscosity, [η]c, at Different Values of Relative Viscosity, $\eta_{rel}$[A,B]**

| | [η]c | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| $\eta_{rel}$ | **0.00** | **0.01** | **0.02** | **0.03** | **0.04** | **0.05** | **0.06** | **0.07** | **0.08** | **0.09** |
| 1.1 | 0.098 | 0.106 | 0.115 | 0.125 | 0.134 | 0.143 | 0.152 | 0.161 | 0.170 | 0.180 |
| 1.2 | 0.189 | 0.198 | 0.207 | 0.216 | 0.225 | 0.233 | 0.242 | 0.250 | 0.259 | 0.268 |
| 1.3 | 0.276 | 0.285 | 0.293 | 0.302 | 0.310 | 0.318 | 0.326 | 0.334 | 0.342 | 0.350 |
| 1.4 | 0.358 | 0.367 | 0.375 | 0.383 | 0.391 | 0.399 | 0.407 | 0.414 | 0.422 | 0.430 |
| 1.5 | 0.437 | 0.445 | 0.453 | 0.460 | 0.468 | 0.476 | 0.484 | 0.491 | 0.499 | 0.507 |
| 1.6 | 0.515 | 0.522 | 0.529 | 0.536 | 0.544 | 0.551 | 0.558 | 0.566 | 0.573 | 0.580 |
| 1.7 | 0.587 | 0.595 | 0.602 | 0.608 | 0.615 | 0.622 | 0.629 | 0.636 | 0.642 | 0.649 |
| 1.8 | 0.656 | 0.663 | 0.670 | 0.677 | 0.683 | 0.690 | 0.697 | 0.704 | 0.710 | 0.717 |
| 1.9 | 0.723 | 0.730 | 0.736 | 0.743 | 0.749 | 0.756 | 0.762 | 0.769 | 0.775 | 0.782 |
| | | | | | | | | | | |
| 2.0 | 0.788 | 0.795 | 0.802 | 0.809 | 0.815 | 0.821 | 0.827 | 0.833 | 0.840 | 0.846 |
| 2.1 | 0.852 | 0.858 | 0.864 | 0.870 | 0.876 | 0.882 | 0.888 | 0.894 | 0.900 | 0.906 |
| 2.2 | 0.912 | 0.918 | 0.924 | 0.929 | 0.935 | 0.941 | 0.948 | 0.953 | 0.959 | 0.965 |
| 2.3 | 0.971 | 0.976 | 0.983 | 0.988 | 0.994 | 1.000 | 1.006 | 1.011 | 1.017 | 1.022 |
| 2.4 | 1.028 | 1.033 | 1.039 | 1.044 | 1.050 | 1.056 | 1.061 | 1.067 | 1.072 | 1.078 |
| 2.5 | 1.083 | 1.089 | 1.094 | 1.100 | 1.105 | 1.111 | 1.116 | 1.121 | 1.126 | 1.131 |
| 2.6 | 1.137 | 1.142 | 1.147 | 1.153 | 1.158 | 1.163 | 1.169 | 1.174 | 1.179 | 1.184 |
| 2.7 | 1.190 | 1.195 | 1.200 | 1.205 | 1.210 | 1.215 | 1.220 | 1.225 | 1.230 | 1.235 |
| 2.8 | 1.240 | 1.245 | 1.250 | 1.255 | 1.260 | 1.265 | 1.270 | 1.275 | 1.280 | 1.285 |
| 2.9 | 1.290 | 1.295 | 1.300 | 1.305 | 1.310 | 1.314 | 1.319 | 1.324 | 1.329 | 1.333 |
| | | | | | | | | | | |
| 3.0 | 1.338 | 1.343 | 1.348 | 1.352 | 1.357 | 1.362 | 1.367 | 1.371 | 1.376 | 1.381 |
| 3.1 | 1.386 | 1.390 | 1.395 | 1.400 | 1.405 | 1.409 | 1.414 | 1.418 | 1.423 | 1.427 |
| 3.2 | 1.432 | 1.436 | 1.441 | 1.446 | 1.450 | 1.455 | 1.459 | 1.464 | 1.468 | 1.473 |
| 3.3 | 1.477 | 1.482 | 1.486 | 1.491 | 1.496 | 1.500 | 1.504 | 1.508 | 1.513 | 1.517 |
| 3.4 | 1.521 | 1.525 | 1.529 | 1.533 | 1.537 | 1.542 | 1.546 | 1.550 | 1.554 | 1.558 |
| 3.5 | 1.562 | 1.566 | 1.570 | 1.575 | 1.579 | 1.583 | 1.587 | 1.591 | 1.595 | 1.600 |
| 3.6 | 1.604 | 1.608 | 1.612 | 1.617 | 1.621 | 1.625 | 1.629 | 1.633 | 1.637 | 1.642 |
| 3.7 | 1.646 | 1.650 | 1.654 | 1.658 | 1.662 | 1.666 | 1.671 | 1.675 | 1.679 | 1.683 |
| 3.8 | 1.687 | 1.691 | 1.695 | 1.700 | 1.704 | 1.708 | 1.712 | 1.715 | 1.719 | 1.723 |
| 3.9 | 1.727 | 1.731 | 1.735 | 1.739 | 1.742 | 1.746 | 1.750 | 1.754 | 1.758 | 1.762 |
| | | | | | | | | | | |
| 4.0 | 1.765 | 1.769 | 1.773 | 1.777 | 1.781 | 1.785 | 1.789 | 1.792 | 1.796 | 1.800 |
| 4.1 | 1.804 | 1.808 | 1.811 | 1.815 | 1.819 | 1.822 | 1.826 | 1.830 | 1.833 | 1.837 |
| 4.2 | 1.841 | 1.845 | 1.848 | 1.852 | 1.856 | 1.859 | 1.863 | 1.867 | 1.870 | 1.874 |
| 4.3 | 1.878 | 1.882 | 1.885 | 1.889 | 1.893 | 1.896 | 1.900 | 1.904 | 1.907 | 1.911 |
| 4.4 | 1.914 | 1.918 | 1.921 | 1.925 | 1.929 | 1.932 | 1.936 | 1.939 | 1.943 | 1.946 |
| 4.5 | 1.950 | 1.954 | 1.957 | 1.961 | 1.964 | 1.968 | 1.971 | 1.975 | 1.979 | 1.982 |
| 4.6 | 1.986 | 1.989 | 1.993 | 1.996 | 2.000 | 2.003 | 2.007 | 2.010 | 2.013 | 2.017 |

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**Intrinsic Viscosity, [η]c, at Different Values of Relative Viscosity, $\eta_{rel}^{A,B}$** *(continued)*

| | [η]c | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| $\eta_{rel}$ | **0.00** | **0.01** | **0.02** | **0.03** | **0.04** | **0.05** | **0.06** | **0.07** | **0.08** | **0.09** |
| 4.7 | 2.020 | 2.023 | 2.027 | 2.030 | 2.033 | 2.037 | 2.040 | 2.043 | 2.047 | 2.050 |
| 4.8 | 2.053 | 2.057 | 2.060 | 2.063 | 2.067 | 2.070 | 2.073 | 2.077 | 2.080 | 2.083 |
| 4.9 | 2.087 | 2.090 | 2.093 | 2.097 | 2.100 | 2.103 | 2.107 | 2.110 | 2.113 | 2.116 |
| | | | | | | | | | | |
| 5.0 | 2.119 | 2.122 | 2.125 | 2.129 | 2.132 | 2.135 | 2.139 | 2.142 | 2.145 | 2.148 |
| 5.1 | 2.151 | 2.154 | 2.158 | 2.160 | 2.164 | 2.167 | 2.170 | 2.173 | 2.176 | 2.180 |
| 5.2 | 2.183 | 2.186 | 2.190 | 2.192 | 2.195 | 2.197 | 2.200 | 2.203 | 2.206 | 2.209 |
| 5.3 | 2.212 | 2.215 | 2.218 | 2.221 | 2.224 | 2.227 | 2.230 | 2.233 | 2.236 | 2.240 |
| 5.4 | 2.243 | 2.246 | 2.249 | 2.252 | 2.255 | 2.258 | 2.261 | 2.264 | 2.267 | 2.270 |
| 5.5 | 2.273 | 2.276 | 2.279 | 2.282 | 2.285 | 2.288 | 2.291 | 2.294 | 2.297 | 2.300 |
| 5.6 | 2.303 | 2.306 | 2.309 | 2.312 | 2.315 | 2.318 | 2.320 | 2.324 | 2.326 | 2.329 |
| 5.7 | 2.332 | 2.335 | 2.338 | 2.341 | 2.344 | 2.347 | 2.350 | 2.353 | 2.355 | 2.358 |
| 5.8 | 2.361 | 2.364 | 2.367 | 2.370 | 2.373 | 2.376 | 2.379 | 2.382 | 2.384 | 2.387 |
| 5.9 | 2.390 | 2.393 | 2.396 | 2.400 | 2.403 | 2.405 | 2.408 | 2.411 | 2.414 | 2.417 |
| | | | | | | | | | | |
| 6.0 | 2.419 | 2.422 | 2.425 | 2.428 | 2.431 | 2.433 | 2.436 | 2.439 | 2.442 | 2.444 |
| 6.1 | 2.447 | 2.450 | 2.453 | 2.456 | 2.458 | 2.461 | 2.464 | 2.467 | 2.470 | 2.472 |
| 6.2 | 2.475 | 2.478 | 2.481 | 2.483 | 2.486 | 2.489 | 2.492 | 2.494 | 2.497 | 2.500 |
| 6.3 | 2.503 | 2.505 | 2.508 | 2.511 | 2.513 | 2.516 | 2.518 | 2.521 | 2.524 | 2.526 |
| 6.4 | 2.529 | 2.532 | 2.534 | 2.537 | 2.540 | 2.542 | 2.545 | 2.547 | 2.550 | 2.553 |
| 6.5 | 2.555 | 2.558 | 2.561 | 2.563 | 2.566 | 2.568 | 2.571 | 2.574 | 2.576 | 2.579 |
| 6.6 | 2.581 | 2.584 | 2.587 | 2.590 | 2.592 | 2.595 | 2.597 | 2.600 | 2.603 | 2.605 |
| 6.7 | 2.608 | 2.610 | 2.613 | 2.615 | 2.618 | 2.620 | 2.623 | 2.625 | 2.627 | 2.630 |
| 6.8 | 2.633 | 2.635 | 2.637 | 2.640 | 2.643 | 2.645 | 2.648 | 2.650 | 2.653 | 2.655 |
| 6.9 | 2.658 | 2.660 | 2.663 | 2.665 | 2.668 | 2.670 | 2.673 | 2.675 | 2.678 | 2.680 |
| | | | | | | | | | | |
| 7.0 | 2.683 | 2.685 | 2.687 | 2.690 | 2.693 | 2.695 | 2.698 | 2.700 | 2.702 | 2.705 |
| 7.1 | 2.707 | 2.710 | 2.712 | 2.714 | 2.717 | 2.719 | 2.721 | 2.724 | 2.726 | 2.729 |
| 7.2 | 2.731 | 2.733 | 2.736 | 2.738 | 2.740 | 2.743 | 2.745 | 2.748 | 2.750 | 2.752 |
| 7.3 | 2.755 | 2.757 | 2.760 | 2.762 | 2.764 | 2.767 | 2.769 | 2.771 | 2.774 | 2.776 |
| 7.4 | 2.779 | 2.781 | 2.783 | 2.786 | 2.788 | 2.790 | 2.793 | 2.795 | 2.798 | 2.800 |
| 7.5 | 2.802 | 2.805 | 2.807 | 2.809 | 2.812 | 2.814 | 2.816 | 2.819 | 2.821 | 2.823 |
| 7.6 | 2.826 | 2.828 | 2.830 | 2.833 | 2.835 | 2.837 | 2.840 | 2.842 | 2.844 | 2.847 |
| 7.7 | 2.849 | 2.851 | 2.854 | 2.856 | 2.858 | 2.860 | 2.863 | 2.865 | 2.868 | 2.870 |
| 7.8 | 2.873 | 2.875 | 2.877 | 2.879 | 2.881 | 2.884 | 2.887 | 2.889 | 2.891 | 2.893 |
| 7.9 | 2.895 | 2.898 | 2.900 | 2.902 | 2.905 | 2.907 | 2.909 | 2.911 | 2.913 | 2.915 |
| | | | | | | | | | | |
| 8.0 | 2.918 | 2.920 | 2.922 | 2.924 | 2.926 | 2.928 | 2.931 | 2.933 | 2.935 | 2.937 |
| 8.1 | 2.939 | 2.942 | 2.944 | 2.946 | 2.948 | 2.950 | 2.952 | 2.955 | 2.957 | 2.959 |
| 8.2 | 2.961 | 2.963 | 2.966 | 2.968 | 2.970 | 2.972 | 2.974 | 2.976 | 2.979 | 2.981 |
| 8.3 | 2.983 | 2.985 | 2.987 | 2.990 | 2.992 | 2.994 | 2.996 | 2.998 | 3.000 | 3.002 |
| 8.4 | 3.004 | 3.006 | 3.008 | 3.010 | 3.012 | 3.015 | 3.017 | 3.019 | 3.021 | 3.023 |
| 8.5 | 3.025 | 3.027 | 3.029 | 3.031 | 3.033 | 3.035 | 3.037 | 3.040 | 3.042 | 3.044 |
| 8.6 | 3.046 | 3.048 | 3.050 | 3.052 | 3.054 | 3.056 | 3.058 | 3.060 | 3.062 | 3.064 |

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**Intrinsic Viscosity, [η]c, at Different Values of Relative Viscosity, $\eta_{rel}$[A,B]** *(continued)*

| | [η]c | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| $\eta_{rel}$ | **0.00** | **0.01** | **0.02** | **0.03** | **0.04** | **0.05** | **0.06** | **0.07** | **0.08** | **0.09** |
| 8.7 | 3.067 | 3.069 | 3.071 | 3.073 | 3.075 | 3.077 | 3.079 | 3.081 | 3.083 | 3.085 |
| 8.8 | 3.087 | 3.089 | 3.092 | 3.094 | 3.096 | 3.098 | 3.100 | 3.102 | 3.104 | 3.106 |
| 8.9 | 3.108 | 3.110 | 3.112 | 3.114 | 3.116 | 3.118 | 3.120 | 3.122 | 3.124 | 3.126 |
| | | | | | | | | | | |
| 9.0 | 3.128 | 3.130 | 3.132 | 3.134 | 3.136 | 3.138 | 3.140 | 3.142 | 3.144 | 3.146 |
| 9.1 | 3.148 | 3.150 | 3.152 | 3.154 | 3.156 | 3.158 | 3.160 | 3.162 | 3.164 | 3.166 |
| 9.2 | 3.168 | 3.170 | 3.172 | 3.174 | 3.176 | 3.178 | 3.180 | 3.182 | 3.184 | 3.186 |
| 9.3 | 3.188 | 3.190 | 3.192 | 3.194 | 3.196 | 3.198 | 3.200 | 3.202 | 3.204 | 3.206 |
| 9.4 | 3.208 | 3.210 | 3.212 | 3.214 | 3.215 | 3.217 | 3.219 | 3.221 | 3.223 | 3.225 |
| 9.5 | 3.227 | 3.229 | 3.231 | 3.233 | 3.235 | 3.237 | 3.239 | 3.241 | 3.242 | 3.244 |
| 9.6 | 3.246 | 3.248 | 3.250 | 3.252 | 3.254 | 3.256 | 3.258 | 3.260 | 3.262 | 3.264 |
| 9.7 | 3.266 | 3.268 | 3.269 | 3.271 | 3.273 | 3.275 | 3.277 | 3.279 | 3.281 | 3.283 |
| 9.8 | 3.285 | 3.287 | 3.289 | 3.291 | 3.293 | 3.295 | 3.297 | 3.298 | 3.300 | 3.302 |
| 9.9 | 3.304 | 3.305 | 3.307 | 3.309 | 3.311 | 3.313 | 3.316 | 3.318 | 3.320 | 3.321 |
| | | | | | | | | | | |
| | **0.0** | **0.1** | **0.2** | **0.3** | **0.4** | **0.5** | **0.6** | **0.7** | **0.8** | **0.9** |
| 10 | 3.32 | 3.34 | 3.36 | 3.37 | 3.39 | 3.41 | 3.43 | 3.45 | 3.46 | 3.48 |
| 11 | 3.50 | 3.52 | 3.53 | 3.55 | 3.56 | 3.58 | 3.60 | 3.61 | 3.63 | 3.64 |
| 12 | 3.66 | 3.68 | 3.69 | 3.71 | 3.72 | 3.74 | 3.76 | 3.77 | 3.79 | 3.80 |
| 13 | 3.80 | 3.83 | 3.85 | 3.86 | 3.88 | 3.89 | 3.90 | 3.92 | 3.93 | 3.95 |
| 14 | 3.96 | 3.97 | 3.99 | 4.00 | 4.02 | 4.03 | 4.04 | 4.06 | 4.07 | 4.09 |
| 15 | 4.10 | 4.11 | 4.13 | 4.14 | 4.15 | 4.17 | 4.18 | 4.19 | 4.20 | 4.22 |
| 16 | 4.23 | 4.24 | 4.25 | 4.27 | 4.28 | 4.29 | 4.30 | 4.31 | 4.33 | 4.34 |
| 17 | 4.35 | 4.36 | 4.37 | 4.38 | 4.39 | 4.41 | 4.42 | 4.43 | 4.44 | 4.45 |
| 18 | 4.46 | 4.47 | 4.48 | 4.49 | 4.50 | 4.52 | 4.53 | 4.54 | 4.55 | 4.56 |
| 19 | 4.57 | 4.58 | 4.59 | 4.60 | 4.61 | 4.62 | 4.63 | 4.64 | 4.65 | 4.66 |

[A] Swedish Method CCA 27:57, Karin Wilson, *Svensk Papperstidning,* Vol. 60, 1957, pp. 513 to 521.
[B] Derived from the equation: $\eta_{rel} - 1 = \eta_{sp} = [\eta]ce^{k'[\eta]c}$, where $k' = 0.30$.

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# Chapter Guide: Medical Devices

To return to the Chapter Charts main list, *click here*.

- ⟨691⟩ *Cotton*
- ⟨861⟩ *Sutures—Diameter*
- ⟨871⟩ *Sutures—Needle Attachment*

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# Chapter Guide: Microbiology Products

To return to the Chapter Charts main list, *click here*.

Nonsterile Products

Microbial Enumeration

- ⟨61⟩ *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests*
- ⟨610⟩ *Alternative Microbiological Sampling Methods for Nonsterile Inhaled and Nasal Products*
- ⟨1111⟩ *Microbiological Examination of Nonsterile Products: Acceptance Criteria for Pharmaceutical Preparations and Substances for Pharmaceutical Use*
- ⟨2021⟩ *Microbial Enumeration Tests—Nutritional and Dietary Supplements*
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Absence of Objectionable Microorganisms

- ⟨60⟩ *Microbiological Examination of Nonsterile Products: Tests for Burkholderia Cepacia Complex*
- ⟨62⟩ *Microbiological Examination of Nonsterile Products: Tests for Specified Microorganisms*
- ⟨63⟩ *Mycoplasma Tests*
- ⟨610⟩ *Alternative Microbiological Sampling Methods for Nonsterile Inhaled and Nasal Products*
- ⟨1111⟩ *Microbiological Examination of Nonsterile Products: Acceptance Criteria for Pharmaceutical Preparations and Substances for Pharmaceutical Use*
- ⟨1113⟩ *Microbial Characterization, Identification, and Strain Typing*
- ⟨1115⟩ *Bioburden Control of Nonsterile Drug Substances and Products*
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Sterility Tests

- ⟨71⟩ *Sterility Tests*
- ⟨1071⟩ *Rapid Microbial Tests for Release of Sterile Short-Life Products: A Risk-Based Approach*
- ⟨1113⟩ *Microbial Characterization, Identification, and Strain Typing*
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- ⟨1113⟩ *Microbial Characterization, Identification, and Strain Typing*
- ⟨1229.17⟩ *Mycoplasma Sterilization*

Aseptic Processing

- ⟨1113⟩ *Microbial Characterization, Identification, and Strain Typing*
- ⟨1116⟩ *Microbiological Control and Monitoring of Aseptic Processing Environments*
- ⟨1208⟩ *Sterility Testing—Validation of Isolator Systems*
- ⟨1211⟩ *Sterility Assurance*
- ⟨1228⟩ *Depyrogenation*
- ⟨1228.1⟩ *Dry Heat Depyrogenation*
- ⟨1228.3⟩ *Depyrogenation by Filtration*
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- ⟨1228.5⟩ *Endotoxin Indicators for Depyrogenation*
- ⟨1229⟩ *Sterilization of Compendial Articles*
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- ⟨1229.4⟩ *Sterilizing Filtration of Liquids*
- ⟨1229.12⟩ *New Sterilization Methods*
- ⟨1229.14⟩ *Sterilization Cycle Development*
- ⟨1229.17⟩ *Mycoplasma Sterilization*
- ⟨1229.18⟩ *Viral Clearance Methods*

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- ⟨1211⟩ *Sterility Assurance*
- ⟨1228⟩ *Depyrogenation*
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- ⟨1228.4⟩ *Depyrogenation by Rinsing*
- ⟨1228.5⟩ *Endotoxin Indicators for Depyrogenation*
- ⟨1229⟩ *Sterilization of Compendial Articles*

[1] For endotoxins limits, see ⟨85⟩ *Bacterial Endotoxins Test*.

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- ⟨1229.3⟩ *Monitoring of Bioburden*
- ⟨1229.4⟩ *Sterilizing Filtration of Liquids*
- ⟨1229.14⟩ *Sterilization Cycle Development*
- ⟨1229.17⟩ *Mycoplasma Sterilization*
- ⟨1229.18⟩ *Viral Clearance Methods*

Assembly[2]

Assembly

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- ⟨1207.3⟩ *Package Seal Quality Test Technologies*
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- ⟨1228.1⟩ *Dry Heat Depyrogenation*
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- ⟨1229⟩ *Sterilization of Compendial Articles*
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Terminal Sterilization

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- ⟨1222⟩ *Terminally Sterilized Pharmaceutical Products—Parametric Release*
- ⟨1228⟩ *Depyrogenation*
- ⟨1228.1⟩ *Dry Heat Depyrogenation*
- ⟨1228.4⟩ *Depyrogenation by Rinsing*
- ⟨1228.5⟩ *Endotoxin Indicators for Depyrogenation*
- ⟨1229⟩ *Sterilization of Compendial Articles*
- ⟨1229.12⟩ *New Sterilization Methods*
- ⟨1229.13⟩ *Sterilization-in-Place*
- ⟨1229.14⟩ *Sterilization Cycle Development*
- ⟨1229.17⟩ *Mycoplasma Sterilization*
- ⟨1229.18⟩ *Viral Clearance Methods*

Moist Heat

- ⟨55⟩ *Biological Indicators—Resistance Performance Tests*
- ⟨56⟩ *Methods for Determination of Resistance of Microorganisms to Sterilization Processes*
- ⟨1211⟩ *Sterility Assurance*
- ⟨1228⟩ *Depyrogenation*
- ⟨1229⟩ *Sterilization of Compendial Articles*
- ⟨1229.1⟩ *Steam Sterilization by Direct Contact*
- ⟨1229.2⟩ *Moist Heat Sterilization of Aqueous Liquids*
- ⟨1229.3⟩ *Monitoring of Bioburden*
- ⟨1229.5⟩ *Biological Indicators for Sterilization*
- ⟨1229.9⟩ *Physicochemical Integrators and Indicators for Sterilization*
- ⟨1229.13⟩ *Sterilization-in-Place*
- ⟨1229.14⟩ *Sterilization Cycle Development*

Dry Heat

- ⟨55⟩ *Biological Indicators—Resistance Performance Tests*
- ⟨1211⟩ *Sterility Assurance*
- ⟨1228⟩ *Depyrogenation*
- ⟨1228.1⟩ *Dry Heat Depyrogenation*

[2] For BFS, FFS, and SFS, see ⟨1116⟩ *Microbiological Control and Monitoring of Aseptic Processing Environments*.

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- ⟨1228.5⟩ *Endotoxin Indicators for Depyrogenation*
- ⟨1229⟩ *Sterilization of Compendial Articles*
- ⟨1229.5⟩ *Biological Indicators for Sterilization*
- ⟨1229.8⟩ *Dry Heat Sterilization*
- ⟨1229.13⟩ *Sterilization-in-Place*
- ⟨1229.14⟩ *Sterilization Cycle Development*

Radiation

- ⟨55⟩ *Biological Indicators—Resistance Performance Tests*
- ⟨1211⟩ *Sterility Assurance*
- ⟨1228⟩ *Depyrogenation*
- ⟨1228.3⟩ *Depyrogenation by Filtration*
- ⟨1229⟩ *Sterilization of Compendial Articles*
- ⟨1229.3⟩ *Monitoring of Bioburden*
- ⟨1229.5⟩ *Biological Indicators for Sterilization*
- ⟨1229.10⟩ *Radiation Sterilization*
- ⟨1229.14⟩ *Sterilization Cycle Development*

EtO

- ⟨55⟩ *Biological Indicators—Resistance Performance Tests*
- ⟨1211⟩ *Sterility Assurance*
- ⟨1228⟩ *Depyrogenation*
- ⟨1229⟩ *Sterilization of Compendial Articles*
- ⟨1229.5⟩ *Biological Indicators for Sterilization*
- ⟨1229.7⟩ *Gaseous Sterilization*
- ⟨1229.9⟩ *Physicochemical Integrators and Indicators for Sterilization*
- ⟨1229.13⟩ *Sterilization-in-Place*
- ⟨1229.14⟩ *Sterilization Cycle Development*

Liquid Phase

- ⟨1229.6⟩ *Liquid-Phase Sterilization*
- ⟨1229.13⟩ *Sterilization-in-Place*
- ⟨1229.14⟩ *Sterilization Cycle Development*

Vapor Phase

- ⟨1229.11⟩ *Vapor Phase Sterilization*
- ⟨1229.13⟩ *Sterilization-in-Place*
- ⟨1229.14⟩ *Sterilization Cycle Development*

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# Mission and Preface

This section provides background information on the United States Pharmacopeial Convention (USP), as well as general information about the *United States Pharmacopeia* and the *National Formulary (USP–NF)*. Whether a document is official or not official is not linked to when a specific publication becomes official, but is based on the individual document. USP publishes the *USP–NF* on a set schedule, and each individual document is associated with its indicated official date. Learn more about USP's document-centric official dates on our website (www.uspnf.com/new-uspnf-online-faqs/end-user#irg1).

## MISSION STATEMENT

*USP–NF* is published in continuing pursuit of the mission of USP: *To improve global health through public standards and related programs that help ensure the quality, safety, and benefit of medicines and foods.*

## HISTORY

USP has a rich history, dating back to 1820, when 11 physicians met in the Senate Chamber of the U.S. Capitol building to establish a pharmacopeia for the United States. Learn more about USP's history and major milestones on the USP website (https://www.usp.org/about/usp-timeline).

## CONTENT OF *USP–NF*

*USP–NF* contains official substance (ingredient) and product monographs for official articles recognized in *USP–NF* (see *General Notices 2.20 Official Articles*). *USP–NF* also includes monographs for compounded preparations. With few exceptions, such as articles covered by *Global Health* monographs, the intent is that all articles for which monographs are provided in *USP–NF* are legally marketed in the United States or are contained in legally marketed articles. *Global Health* monographs are provided for articles that are not approved or legally marketed in the United States, but that have been approved by a stringent regulatory authority [as defined by the World Health Organization (WHO)] and are used for essential purposes in other parts of the world (see *General Notices 3.10.40 Applicability of Global Health Monographs*).

A *USP–NF* monograph for an official substance, product, or preparation may consist of various components, including the article's name; definition; packaging, storage, and other requirements; and a specification. General chapters provide frequently cited procedures, sometimes with acceptance criteria, in order to compile into one location repetitive information that is applicable to many monographs. See *General Notices 3.10 Applicability of Standards* for more information about standards contained in *USP–NF* monographs and general chapters.

The *USP Admissions List* section of the *USP–NF* indicates those new and revised monographs and general chapters as well as any omissions that published on a specific delivery date.

**Revisions to *USP–NF*—**

*USP–NF* is continuously revised by an exceptional process of public involvement and substantial interaction between USP and its stakeholders, both domestically and internationally. Revisions are presented in the *USP–NF* three times per year in November, February, and June. *Accelerated Revisions* [including *Errata, Interim Revision Announcements* (*IRA*s), and *Revision Bulletins*] are published periodically and with greater frequency.

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***Regular Revisions***—USP's regular revision process calls for publication of a proposed revision in the *Pharmacopeial Forum (PF)* for a 90-day notice and comment period and, after the revision is approved by the relevant USP Expert Committee, publication in the *USP–NF*.

***Accelerated Revisions***—The *Accelerated Revision* process is used to make revisions to *USP–NF* official more quickly than through USP's *Regular Revision* process. Learn more about accelerated revisions and the criteria for and implementation thereof on the USP website (http://www.uspnf.com/official-text).

### Modification of Compendial References—

USP and its Expert Committees periodically deem it necessary to modify general chapter titles or similar text that may be referenced in other standards throughout the *USP–NF*. When this occurs, USP staff undertake a rigorous process for identifying and updating such references. These updates may occur through a regular revision, or, in cases in which an update appears to present no significant change in the affected standard, through a direct update of the reference in that standard without providing an opportunity for notice and comment. In all cases, USP will publish on its website a notice indicating the source change, any resulting references, and whether those references will be updated through a regular revision or a direct update.

### Updating Chemical Information—

Updates to the *Chemical Information* section at the beginning of monographs occur on an ongoing basis. Chemical names and molecular weights may also be reviewed and updated when a monograph undergoes revision to match the *United States Adopted Names* (*USAN*) or the original source of chemical information. Chemical structures are updated on a continuous basis.

Chemical names typically reflect the naming conventions at the time of the monograph development or revision. If the nomenclature rules of CAS or IUPAC are significantly changed, the chemical names can be revised or added to implement those rules. Molecular weights are derived from the chemical formula and are based on the table of atomic weights as published by the IUPAC. When the IUPAC recommended values are changed to reflect the isotopic composition of normal terrestrial material, it is understood that the changes in molecular weights will be made in due course.

Graphical representation of the chemical compound structures is intended as a visual aid to help establish chemical identity and is understood to represent one of many possible ways to depict the molecule. Addition of a graphical representation or changes in such representation, that result in the same chemical information, e.g., a flipped chiral molecule or adding a molecule structure, may be introduced outside of the revision process. It is also understood that in the case of tautomerism, the molecule depicted may be one of the tautomers, but it is intended to represent all isomers in equilibrium. Stereogenic centers depicted with plain bonds imply mixtures of pertinent stereomers—enantiomer, diastereomers, epimers (anomers), etc. (see *General Notices 3.10 Applicability of Standards*).

### Shading and Symbols—

Shading is used to identify text that has been modified, added, or deleted since it was last published. Symbols identify the beginning and end of each revision or nonharmonized text. The following table summarizes the types of symbols and the associated subscripts used in USP publications:

| Revision Type | Revision Symbol and Text |
|---|---|
| In Process Regular Revision | ▲▲(USP 1-May-2023) |
| | ▲▲(NF 1-May-2023) |
| | ▲▲(GH 1-May-2023) |
| Interim Revision Announcement | ▲▲(IRA 1-Jul-2023) |
| Revision Bulletin | ▲▲(RB 1-Jul-2023) |
| Harmonization | ◆◆ |
| Errata | ▲▲(ERR 1-Jul-2023) |
| Chapter References | ▲▲(CN 1-Jul-2023) |
| | ▲▲(Official 1-Jul-2023) |
| Pending | ▲▲(TBD) |

### *Commentary*—

For revisions that are published for public review and comment in *PF*, the proposal may advance to official status or be republished in *PF* for further notice and comment. If comments are received, they are considered and incorporated as appropriate by the Expert Committee(s). In cases where proposals advance to official status without republication in *PF*, a summary of comments received and the appropriate Expert Committee's responses are published in the *Commentary* section of the USP website at the time the revision is published (see Rules and Procedures of the 2020-2025 Council of Experts, Section 9.05 Publication in the Pharmacopeial Forum).

The *Commentary* is not part of the official text and is not intended to be enforceable by regulatory authorities. Rather, it explains the basis of the Expert Committee's response to public comments. If there is a difference between the contents of the *Commentary* and the official text, the official text prevails. In case of a dispute or question of interpretation, the language of the official text, alone and independent of the *Commentary*, shall prevail.

### *USP-NF* Presentations—

See *General Notices 2.10 Official Text* for more information about *USP–NF* product formats. The *USP-NF* is available online. *USP43–NF38 (published November 1, 2019)* was the last publication made available in print or on a flash drive.

### *USP–NF Translations*—

Translations of the *USP–NF* are available in Spanish, Russian, and Chinese. The Spanish translation is current; other translations are based on previous revisions of the *USP–NF*.

### *USP Reference Standards*—

The use of USP Reference Standards promotes uniform quality of drugs and supports reliability and consistency by those performing compliance testing and other users of *USP–NF*, including manufacturers, buyers, and regulatory authorities. USP Reference Standards are referenced in specific procedures in both monographs and general chapters. USP advances this material via careful characterization studies and collaborative testing, followed by review and approval of the compendial use of the reference material by Expert Committees of the Council of Experts. This program benefits from the widespread voluntary contribution of suitable materials and test data from pharmaceutical manufacturers. The *USP Catalog*, which lists the collection of USP Reference Standards, and more information about use and storage, can

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be accessed on USP's website (https://www.usp.org/reference-standards/reference-standards-catalog).

# USP GOVERNING, STANDARDS-SETTING, AND ADVISORY BODIES

USP's governing, standards-setting, and advisory bodies include the USP Convention, the Board of Trustees, the Council of Experts and its Expert Committees, Expert Panels, Subcommittees, Joint Standard-Setting Subcommittees, and staff. Additional volunteer bodies include Stakeholder Forums and Project Teams, which offer stakeholders the opportunity to contribute, through advice and recommendations, to the advancement of USP's standards and processes. Learn about the composition and work of the USP Convention, Board of Trustees, Expert Committees, and Expert Panels on the USP website (https://www.usp.org/about/convention-membership). Learn more about Stakeholder Forums and Project Teams on the USP website (https://www.usp.org/get-involved/provide-input/stakeholder-forums). A listing of all current Voting Delegates of the USP Convention and members and Government Liaisons to the Council of Experts and its Expert Committees can be found on the USP website (https://www.usp.org/about/convention-membership, https://www.usp.org/about/volunteer-experts/expert-committees).

**Working with the Food and Drug Administration—**

USP works with the Secretary of the Department of Health and Human Services, and the principal agency in the Department for this work is the Food and Drug Administration (FDA). USP works in many ways with the agency, but the primary interaction is through the Government Liaison Program. The Government Liaison Program allows representatives of FDA and other government agencies to participate in Expert Committee, Expert Panel, and Subcommittee and related meetings, enabling interactions between government staff and Expert volunteer bodies. Staff in the FDA Centers who are responsible for review of compendial activities provide specific links and opportunities for exchange of comments. The Office of Policy for Pharmaceutical Quality in the Center for Drug Evaluation and Research provides a primary compendial point of contact between FDA and USP.

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# RULES AND PROCEDURES

**Governing Documents—**

*USP–NF* standards are recognized widely because they are authoritative and science-based and are established by a transparent and credible process. See the *Articles of Incorporation* section in this publication; the Bylaws (https://www.usp.org/about/convention-membership/bylaws), and the *Rules and Procedures of the Council of Experts* (https://www.usp.org/about/leadership/policies-rules). Collectively, these documents serve USP volunteers and staff as the governing principles for USP's standards-setting activities.

# LEGAL RECOGNITION

**Recognition of *USP–NF*—**

*USP–NF* is recognized by law and custom in many countries throughout the world. In the United States, the Federal Food, Drug, and Cosmetic Act (FD&C Act) defines the term "official compendium" as the official *USP*, the official *NF*, the official *Homeopathic Pharmacopeia of the United States,* or any supplement to them. *USP–NF* standards play a role in the

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adulteration and misbranding provisions of the FD&C Act, which apply as well to biologics, a subset of drugs, under the Public Health Service Act (see *General Notices 2.30 Legal Recognition*).

FDA requires that names for articles that are not official must be clearly distinguished and differentiated from any name recognized in an official compendium. Drugs with a name recognized in *USP–NF* also will be considered misbranded unless they meet compendial standards for packaging and labeling (see *General Notices 3.10.10 Applicability of Standards to Drug Products, Drug Substances, and Excipients* for more information).

**Drugs—**

USP's goal is to have substance and drug product monographs in *USP–NF* for FDA-approved drugs in the United States, including chemical and biologic medicines, and their ingredients. USP also provides monographs in *USP–NF* for legally marketed therapeutic products not approved by FDA, e.g., pre-1938 drugs, over-the-counter (OTC) drugs marketed under FDA's OTC Monograph system, dietary supplements, and compounded preparations. The *Global Health* section of *USP–NF* contains monographs for articles that are not approved or legally marketed in the United States, but that have been approved by a stringent regulatory authority (as defined by WHO) and are used for essential purposes in other parts of the world. Conformance with a *USP–NF* monograph, if applicable, is required at all times in the life of an article from production to expiration.

**Biologics—**

In the United States, biologics are considered to be a subset of drugs, whether they were approved by FDA under the FD&C Act [and received a new drug application (NDA)] or under the Public Health Service Act [PHS Act, where they receive a biologics license application (BLA)]. As of March 23, 2020, applications for biological products approved under the FD&C Act are deemed to be licenses for the biological products under the PHS Act. All PHS Act biologics are subject to the applicable drug regulatory requirements of the FD&C Act, which means they are required to comply with the adulteration and misbranding provisions of the FD&C Act, including *USP–NF* compendial requirements, to the extent that such requirements apply to a particular biologic product. This is equally so for biologics approved under the long-standing PHS Act "351(a)" pathway, as well as the new "351(k)" pathway for biosimilars added by the 2010 healthcare reform legislation (Biologics Price Competition and Innovation Act, Title VII, Subtitle A of the Patient Protection and Affordable Care Act, Public Law 111-148).

**Dietary Supplements—**

The Dietary Supplement Health and Education Act of 1994 amendments to the FD&C Act provide that a dietary supplement may be deemed a misbranded food if it is covered by the specifications of an official compendium (e.g., *USP–NF*), is represented as conforming to the specifications of an official compendium, and fails to so conform. This contrasts with pharmaceutical products, wherein conformance to applicable compendial standards is mandatory, whether or not the product claims to conform.

**Compounding—**

USP provides general chapters and monographs for compounded preparations, as well as monographs for bulk substances used in compounding. USP standards are recognized in various provisions of the FD&C Act and such provisions do not differentiate between manufactured and compounded medicines. Moreover, the FD&C Act and the 2013 Drug Quality and Security Act specifically reference USP standards for compounding. Learn more about compounding on the USP website (https://www.usp.org/compounding).

**Medical Devices—**

Section 201(h) of the FD&C Act defines a device as an instrument, apparatus, similar article, or component thereof recognized in *USP–NF*. Section 502(e) of the FD&C Act defines the established name of a device in the absence of an FDA designation of the official name as the official title in an official compendium. Despite these statutory provisions, there is no comparable recognition of USP's role in establishing compendial standards for medical devices as exists for drugs and biologics. Under authority granted by the Food and Drug Administration Modernization Act of 1997, the Center for Devices and Radiological Health recognizes national and international standards, including some *USP* tests and assays, for medical devices.

**Nomenclature—**

For information on the nomenclature development process, the Nomenclature and Labeling Expert Committee, and USP's work with United States Adopted Names (USAN), see the USP website (https://www.usp.org/expert-committees/nomenclature-and-labeling-expert-committee-work-plan).

**Chemical Names and CAS Registry Numbers—**

Chemical subtitles given in the monographs are CA Index Names used by CAS, a division of the American Chemical Society. CAS Registry Number® and CAS RN® are registered trademarks of the American Chemical Society. They are provided only in monographs in which the titles specify substances that are definable chemical entities. The first subtitle is the inverted form of the systematic chemical name developed by CAS for the purpose of the Collective Index (CI). The second subtitle, given in uninverted form, is a preferred IUPAC name (PIN) sanctioned and used by the International Union of Pure and Applied Chemistry (IUPAC). Preferred IUPAC names also are used by the WHO. Occasionally a third subtitle is supplied for historical reasons or when the synonym uses an alternative, but equivalent, naming convention. Monographs with chemical subtitles also generally carry CAS registry numbers. These numbers function independently of nomenclature as invariant numerical designators of unique, unambiguous chemical substances in the CAS Registry℠, and thus are convenient and widely used.

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# HARMONIZATION ACTIVITIES

USP participates in several collaborative activities with global pharmacopeias in both bilateral and multilateral settings. Examples of USP's current activities include the following.

**Pharmacopeial Discussion Group—**

USP harmonizes pharmacopeial excipient monographs and general chapters through the Pharmacopeial Discussion Group (PDG), which includes representatives from the European, Japanese, and United States pharmacopeias, and WHO (as an observer). According to the PDG definition, "a pharmacopeial general chapter or other pharmacopeial document is harmonized when a pharmaceutical substance or preparation tested by the harmonized procedure yields the same results, and the same accept/reject decision is reached." Information regarding PDG, including history, purpose, the PDG working procedure, and lists of monographs and general chapters that have completed stages 1–4 of the pharmacopeial harmonization process, resulting in an approved USP Stage 4 Harmonization text, is available on USP's website (https://www.usp.org/harmonized-standards).

**International Meeting of the World Pharmacopeias—**

USP works with WHO and global pharmacopeial partners in this WHO-led forum. Its initial focus was the strategy and establishment of Good Pharmacopoeial Practices (GPhP) as a set of guiding principles for the appropriate establishment of pharmacopeial standards. Following the completion of GPhP in 2016, the IMWP has primarily served as a venue for information and knowledge exchange across global pharmacopeias and recently served as a forum for rapid coordination of pharmacopeias to respond to public health emergencies, as was recently the case for the COVID-19 pandemic.

**Adopt Agreements—**

USP grants the rights to copy USP standards for use in other pharmacopeias through this formal mechanism.

**Bilateral Agreements—**

USP partners with pharmacopeias for the joint development of pharmacopeial standards using this informal process.

**USP Exchange Programs—**

USP regularly welcomes the exchange of scientific personnel through this program, with the goal of sharing scientific knowledge among global organizations involved in standards setting and the effective use of standards.

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# OTHER USP COMPENDIA

***USP Compounding Compendium—***

The *USP Compounding Compendium* is an online compendium that includes all compounding-related general chapters from the *USP–NF* as well as the supporting general chapters that are referenced in the compounding general chapters and in *USP–NF General Notices*. The purpose of the *USP Compounding Compendium* is to provide compounding practitioners with convenient access to associated general chapters.

***USP Herbal Medicines Compendium—***

The *USP Herbal Medicines Compendium (HMC)* is an online compendium that helps ensure the quality of the herbal ingredients used in herbal medicines. *HMC* monographs provide quality specifications—tests, procedures, and acceptance criteria—with validated analytical procedures and allied reference materials that aid in conformity assessment. *HMC* can help ingredient manufacturers, herbal product manufacturers, regulatory agencies, and other stakeholders to assess conformance of herbal medicinal ingredients with independent public standards and to control the quality of articles moving in international commerce. The *HMC* is available at https://hmc.usp.org.

***USP Dietary Supplements Compendium—***

The *Dietary Supplements Compendium* is an online compendium that combines *USP–NF* standards for dietary supplements, standards and information from the *Food Chemicals Codex*, regulatory and industry documents, and other tools and resources.

***Food Chemicals Codex—***

The *Food Chemicals Codex* (*FCC*) is an online compendium of internationally recognized monograph standards and tests for the purity and quality of food ingredients, e.g., preservatives, flavorings, colorings, and nutrients. *FCC* is

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published every two years, with supplements every six months. Proposed revisions to *FCC* are available for public viewing and comment through the *FCC Forum*. The *FCC Forum* can be accessed free of charge at http://www.foodchemicalscodex.org.

# OTHER USP RESOURCES

***Chromatographic Columns—***

This comprehensive reference, previously titled *Chromatographic Reagents*, provides detailed information needed to conduct chromatographic procedures found in *USP–NF*. *Chromatographic Columns* lists the brand names of the column reagents cited in every proposal for new or revised gas- or liquid-chromatographic analytical procedures that have been published in *PF* since 1980. *Chromatographic Columns* also helps to track which column reagents were used to validate analytical procedures that have become official. The branded column reagents list is updated bimonthly and maintained on USP's website.

***USP Dictionary—***

The *USP Dictionary of USAN and International Drug Names* is an online resource containing the most up-to-date United States Adopted Names of drugs; official *USP–NF* names; nonproprietary, brand, and chemical names; graphic formulas; molecular formulas and weights; CAS registry numbers and code designations; drug manufacturers; and pharmacologic and therapeutic categories. The *Dictionary* helps to ensure the accuracy of the following: product labeling; reports, articles, and correspondence; FDA regulatory filings; and pharmaceutical package inserts. It is published annually. For more information about the *Dictionary* see the USP website (https://www.usp.org/products/usp-dictionary).

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# NF Admissions

## New Articles Appearing in This Issue

There are no new articles appearing in this issue.

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# NF Annotated List

## General Notices, Monographs, General Chapters, Reagents, and Tables Affected by Changes Appearing in *NF 2023, Issue 3*

*Note—In the list below, if a section is new or if a subsection is added to or deleted from an existing section, it is labeled as such in parentheses after the section or subsection name. Items on this list that appear without the designation "new", "added", or "deleted" are items in which changes have been made to existing official text.*

### Reference Tables (*NF 2023, Issue 3*)

**Excipients**

Candelilla Wax

### Monographs (*NF 2023, Issue 3*)

Candelilla Wax
- SPECIFIC TESTS
  - *Melting Range or Temperature, Procedure for Class II*

Cetostearyl Alcohol
- CHEMICAL INFORMATION
- IMPURITIES
  - *Limit of Related Fatty Alcohols*
- SPECIFIC TESTS
  - *Fats and Fixed Oils, Iodine Value* (deleted)
- ADDITIONAL REQUIREMENTS
  - *USP Reference Standards*

Sucralose
- ASSAY
- IMPURITIES
  - *Limit of Methanol* and *Limit of Hydrolysis Products*
- SPECIFIC TESTS
  - *Optical Rotation, Specific Rotation*

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# Chapter Guide: Non-Complex Active Drug Products

To return to the Chapter Charts main list, *click here*.

Universal Tests

Description

- ⟨1⟩ *Injections and Implanted Drug Products (Parenterals)—Product Quality Tests*
- ⟨2⟩ *Oral Drug Products—Product Quality Tests*
- ⟨3⟩ *Topical and Transdermal Drug Products—Product Quality Tests*
- ⟨4⟩ *Mucosal Drug Products—Product Quality Tests*
- ⟨5⟩ *Inhalation and Nasal Drug Products—General Information and Product Quality Tests*
- ⟨7⟩ *Labeling*
- ⟨1121⟩ *Nomenclature*
- ⟨1821⟩ *Radioactivity—Theory and Practice*
- ⟨1823⟩ *Positron Emission Tomography Drugs—Information*

Identification

- ⟨1⟩ *Injections and Implanted Drug Products (Parenterals)—Product Quality Tests*
- ⟨2⟩ *Oral Drug Products—Product Quality Tests*
- ⟨3⟩ *Topical and Transdermal Drug Products—Product Quality Tests*
- ⟨4⟩ *Mucosal Drug Products—Product Quality Tests*
- ⟨5⟩ *Inhalation and Nasal Drug Products—General Information and Product Quality Tests*
- ⟨191⟩ *Identification Tests—General*
- ⟨197⟩ *Spectroscopic Identification Tests*
- ⟨201⟩ *Thin-Layer Chromatographic Identification Test*
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- ⟨1735⟩ *X-Ray Fluorescence Spectrometry—Theory and Practice*
- ⟨1736⟩ *Applications of Mass Spectrometry*
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- ⟨2⟩ *Oral Drug Products—Product Quality Tests*
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- ⟨415⟩ *Medical Gases Assay*
- ⟨451⟩ *Nitrite Titration*
- ⟨501⟩ *Salts of Organic Nitrogenous Bases*
- ⟨541⟩ *Titrimetry*
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- ⟨621⟩ *Chromatography*
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Organic

- ⟨1⟩ *Injections and Implanted Drug Products (Parenterals)—Product Quality Tests*
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- ⟨4⟩ *Mucosal Drug Products—Product Quality Tests*
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- ⟨461⟩ *Nitrogen Determination*
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- ⟨761⟩ *Nuclear Magnetic Resonance Spectroscopy*
- ⟨781⟩ *Optical Rotation*
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- ⟨1761⟩ *Applications of Nuclear Magnetic Resonance Spectroscopy*
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- ⟨1854⟩ *Mid-Infrared Spectroscopy—Theory and Practice*
- ⟨1856⟩ *Near-Infrared Spectroscopy—Theory and Practice*
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Inorganic

- ⟨1⟩ *Injections and Implanted Drug Products (Parenterals)—Product Quality Tests*
- ⟨2⟩ *Oral Drug Products—Product Quality Tests*
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- ⟨4⟩ *Mucosal Drug Products—Product Quality Tests*
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- ⟨281⟩ *Residue on Ignition*
- ⟨730⟩ *Plasma Spectrochemistry*
- ⟨733⟩ *Loss on Ignition*
- ⟨735⟩ *X-Ray Fluorescence Spectrometry*
- ⟨821⟩ *Radioactivity*
- ⟨823⟩ *Positron Emission Tomography Drugs for Compounding, Investigational, and Research Uses*

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- ⟨1⟩ *Injections and Implanted Drug Products (Parenterals)—Product Quality Tests*
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- ⟨3⟩ *Topical and Transdermal Drug Products—Product Quality Tests*
- ⟨4⟩ *Mucosal Drug Products—Product Quality Tests*
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- ⟨821⟩ *Radioactivity*
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- ⟨41⟩ *Balances*
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- ⟨1228.4⟩ *Depyrogenation by Rinsing*
- ⟨1229.13⟩ *Sterilization-in-Place*
- ⟨1229.15⟩ *Sterilizing Filtration of Gases*
- ⟨1251⟩ *Weighing on an Analytical Balance*

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- ⟨891⟩ *Thermal Analysis*
- ⟨921⟩ *Water Determination*
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Ophthalmic[1]

- ⟨755⟩ *Minimum Fill*
- ⟨771⟩ *Ophthalmic Products—Quality Tests*
- ⟨785⟩ *Osmolality and Osmolarity*
- ⟨789⟩ *Particulate Matter in Ophthalmic Solutions*
- ⟨791⟩ *pH*
- ⟨905⟩ *Uniformity of Dosage Units*
- ⟨1085⟩ *Guidelines on Endotoxins Test*
- ⟨1099⟩ *Limit on Number of Large Deviations when Assessing Content Uniformity in Large Samples* (postponed indefinitely)
- ⟨1771⟩ *Ophthalmic Products—Performance Tests*

Nasal

[1] See *Microbiology Products*.

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- ⟨60⟩ *Microbiological Examination of Nonsterile Products: Tests for Burkholderia Cepacia Complex*
- ⟨61⟩ *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests*
- ⟨62⟩ *Microbiological Examination of Nonsterile Products: Tests for Specified Microorganisms*
- ⟨432⟩ *Determination of Zeta Potential by Electrophoretic Light Scattering*
- ⟨601⟩ *Inhalation and Nasal Drug Products: Aerosols, Sprays, and Powders—Performance Quality Tests*
- ⟨604⟩ *Leak Rate*
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- ⟨905⟩ *Uniformity of Dosage Units*
- ⟨1099⟩ *Limit on Number of Large Deviations when Assessing Content Uniformity in Large Samples* (postponed indefinitely)
- ⟨1603⟩ *Good Cascade Impactor Practices*

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- ⟨697⟩ *Container Content for Injections*
- ⟨729⟩ *Globule Size Distribution in Lipid Injectable Emulsions*
- ⟨787⟩ *Subvisible Particulate Matter in Therapeutic Protein Injections*
- ⟨788⟩ *Particulate Matter in Injections*
- ⟨790⟩ *Visible Particulates in Injections*
- ⟨791⟩ *pH*
- ⟨905⟩ *Uniformity of Dosage Units*
- ⟨1085⟩ *Guidelines on Endotoxins Test*
- ⟨1099⟩ *Limit on Number of Large Deviations when Assessing Content Uniformity in Large Samples* (postponed indefinitely)
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- ⟨1211⟩ *Sterility Assurance*
- ⟨1228⟩ *Depyrogenation*
- ⟨1228.1⟩ *Dry Heat Depyrogenation*
- ⟨1228.3⟩ *Depyrogenation by Filtration*
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- ⟨1228.5⟩ *Endotoxin Indicators for Depyrogenation*
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- ⟨1229.3⟩ *Monitoring of Bioburden*
- ⟨1229.4⟩ *Sterilizing Filtration of Liquids*
- ⟨1229.14⟩ *Sterilization Cycle Development*
- ⟨1229.15⟩ *Sterilizing Filtration of Gases*
- ⟨1229.18⟩ *Viral Clearance Methods*
- ⟨1788⟩ *Methods for the Determination of Subvisible Particulate Matter*
- ⟨1788.1⟩ *Light Obscuration Method for the Determination of Subvisible Particulate Matter*
- ⟨1788.2⟩ *Membrane Microscope Method for the Determination of Subvisible Particulate Matter*
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- ⟨1787⟩ *Measurement of Subvisible Particulate Matter in Therapeutic Protein Injections*

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- ⟨60⟩ *Microbiological Examination of Nonsterile Products: Tests for Burkholderia Cepacia Complex*
- ⟨61⟩ *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests*
- ⟨62⟩ *Microbiological Examination of Nonsterile Products: Tests for Specified Microorganisms*
- ⟨301⟩ *Acid-Neutralizing Capacity*
- ⟨432⟩ *Determination of Zeta Potential by Electrophoretic Light Scattering*
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- ⟨701⟩ *Disintegration*
- ⟨705⟩ *Quality Attributes of Tablets Labeled as Having a Functional Score*
- ⟨711⟩ *Dissolution*

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- ⟨724⟩ *Drug Release*
- ⟨755⟩ *Minimum Fill*
- ⟨905⟩ *Uniformity of Dosage Units*
- ⟨1005⟩ *Acoustic Emission*
- ⟨1087⟩ *Intrinsic Dissolution Testing Procedures for Rotating Disk and Stationary Disk*
- ⟨1088⟩ *In Vitro and In Vivo Evaluation of Oral Dosage Forms*
- ⟨1090⟩ *Assessment of Solid Oral Drug Product Performance and Interchangeability, Bioavailability, Bioequivalence, and Dissolution*
- ⟨1092⟩ *The Dissolution Procedure: Development and Validation*
- ⟨1094⟩ *Capsules—Dissolution Testing and Related Quality Attributes*
- ⟨1099⟩ *Limit on Number of Large Deviations when Assessing Content Uniformity in Large Samples* (postponed indefinitely)
- ⟨1216⟩ *Tablet Friability*
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- ⟨60⟩ *Microbiological Examination of Nonsterile Products: Tests for Burkholderia Cepacia Complex*
- ⟨61⟩ *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests*
- ⟨62⟩ *Microbiological Examination of Nonsterile Products: Tests for Specified Microorganisms*
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- ⟨604⟩ *Leak Rate*
- ⟨724⟩ *Drug Release*
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- ⟨60⟩ *Microbiological Examination of Nonsterile Products: Tests for Burkholderia Cepacia Complex*
- ⟨61⟩ *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests*
- ⟨62⟩ *Microbiological Examination of Nonsterile Products: Tests for Specified Microorganisms*
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- ⟨602⟩ *Propellants*
- ⟨604⟩ *Leak Rate*
- ⟨791⟩ *pH*
- ⟨905⟩ *Uniformity of Dosage Units*
- ⟨1601⟩ *Products for Nebulization—Characterization Tests*
- ⟨1602⟩ *Spacers and Valved Holding Chambers Used With Inhalation Aerosols—Characterization Tests*
- ⟨1603⟩ *Good Cascade Impactor Practices*

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- ⟨755⟩ *Minimum Fill*
- ⟨905⟩ *Uniformity of Dosage Units*
- ⟨1004⟩ *Mucosal Drug Products—Performance Tests*
- ⟨1099⟩ *Limit on Number of Large Deviations when Assessing Content Uniformity in Large Samples* (postponed indefinitely)

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# Chapter Guide: Non-Complex Drug Substances

Classification according to ICH Q6 Guidelines: https://www.ich.org/page/quality-guidelines
To return to the Chapter Charts main list, *click here*.

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- Not an official requirement and for informational purposes only. See *Description and Solubility*. [NOTE—This is not official text. See *General Notices 3.10*.]
- ⟨7⟩ *Labeling*
- ⟨659⟩ *Packaging and Storage Requirements*

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- ⟨191⟩ *Identification Tests—General*
- ⟨193⟩ *Identification—Tetracyclines*
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- ⟨202⟩ *Identification of Fixed Oils by Thin-Layer Chromatography*
- ⟨203⟩ *High-Performance Thin-Layer Chromatography Procedure for Identification of Articles of Botanical Origin*
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- ⟨735⟩ *X-Ray Fluorescence Spectrometry*
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- ⟨857⟩ *Ultraviolet-Visible Spectroscopy*
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- ⟨1064⟩ *Identification of Articles of Botanical Origin by High-Performance Thin-Layer Chromatography Procedure*
- ⟨1730⟩ *Plasma Spectrochemistry—Theory and Practice*
- ⟨1735⟩ *X-Ray Fluorescence Spectrometry—Theory and Practice*
- ⟨1736⟩ *Applications of Mass Spectrometry*
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- ⟨1858⟩ *Raman Spectroscopy—Theory and Practice*

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- ⟨11⟩ *USP Reference Standards*
- ⟨81⟩ *Antibiotics—Microbial Assays*
- ⟨351⟩ *Assay for Steroids*
- ⟨391⟩ *Epinephrine Assay*
- ⟨425⟩ *Iodometric Assay—Antibiotics*
- ⟨451⟩ *Nitrite Titration*
- ⟨511⟩ *Single-Steroid Assay*
- ⟨541⟩ *Titrimetry*
- ⟨621⟩ *Chromatography*
- ⟨735⟩ *X-Ray Fluorescence Spectrometry*
- ⟨736⟩ *Mass Spectrometry*
- ⟨761⟩ *Nuclear Magnetic Resonance Spectroscopy*
- ⟨801⟩ *Polarography*

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- ⟨852⟩ *Atomic Absorption Spectroscopy*
- ⟨853⟩ *Fluorescence Spectroscopy*
- ⟨854⟩ *Mid-Infrared Spectroscopy*
- ⟨856⟩ *Near-Infrared Spectroscopy*
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- ⟨858⟩ *Raman Spectroscopy*
- ⟨1223.1⟩ *Validation of Alternative Methods to Antibiotic Microbial Assays*
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- ⟨1736⟩ *Applications of Mass Spectrometry*
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- ⟨1853⟩ *Fluorescence Spectroscopy—Theory and Practice*
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- ⟨1856⟩ *Near-Infrared Spectroscopy—Theory and Practice*
- ⟨1857⟩ *Ultraviolet-Visible Spectroscopy—Theory and Practice*
- ⟨1858⟩ *Raman Spectroscopy—Theory and Practice*

Impurities

Organic Impurities

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- ⟨621⟩ *Chromatography*
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- ⟨858⟩ *Raman Spectroscopy*
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- ⟨1852⟩ *Atomic Absorption Spectroscopy—Theory and Practice*
- ⟨1853⟩ *Fluorescence Spectroscopy—Theory and Practice*
- ⟨1854⟩ *Mid-Infrared Spectroscopy—Theory and Practice*
- ⟨1856⟩ *Near-Infrared Spectroscopy—Theory and Practice*
- ⟨1857⟩ *Ultraviolet-Visible Spectroscopy—Theory and Practice*
- ⟨1858⟩ *Raman Spectroscopy—Theory and Practice*

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- ⟨206⟩ *Aluminum*
- ⟨211⟩ *Arsenic*
- ⟨221⟩ *Chloride and Sulfate*
- ⟨232⟩ *Elemental Impurities—Limits*
- ⟨233⟩ *Elemental Impurities—Procedures*
- ⟨241⟩ *Iron*
- ⟨251⟩ *Lead*
- ⟨261⟩ *Mercury*
- ⟨281⟩ *Residue on Ignition*
- ⟨291⟩ *Selenium*
- ⟨471⟩ *Oxygen Flask Combustion*
- ⟨630⟩ *Visual Comparison*
- ⟨730⟩ *Plasma Spectrochemistry*
- ⟨733⟩ *Loss on Ignition*
- ⟨735⟩ *X-Ray Fluorescence Spectrometry*

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- ⟨941⟩ *Characterization of Crystalline and Partially Crystalline Solids by X-Ray Powder Diffraction (XRPD)*

Residual Solvents

- ⟨467⟩ *Residual Solvents*
- ⟨621⟩ *Chromatography*
- ⟨731⟩ *Loss on Drying*
- ⟨1467⟩ *Residual Solvents—Verification of Compendial Procedures and Validation of Alternative Procedures*

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- ⟨301⟩ *Acid-Neutralizing Capacity*
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- ⟨1079⟩ *Risks and Mitigation Strategies for the Storage and Transportation of Finished Drug Products*
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# Preface

This section contains monographs for articles which are not currently legally marketed in the United States, but which have been approved by a stringent regulatory authority as defined by the World Health Organization and are used for essential purposes in other parts of the world. Selection and prioritization of new entries to this section will be accomplished in close collaboration with stakeholders throughout the global health community. These monographs are not applicable to articles marketed for use in the United States.

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# USP Admissions

## New Articles Appearing in *USP 2023, Issue 3*

### GENERAL CHAPTERS

⟨314⟩ Molecular Weight Determination for Copolymers Containing Alkyl Methacrylate or Alkyl Acrylate
⟨901⟩ Detection of Asbestos in Pharmaceutical Talc
⟨1079.3⟩ Monitoring Devices—Time, Temperature, and Humidity
⟨1504⟩ Quality Attributes of Starting Materials for the Chemical Synthesis of Therapeutic Peptides
⟨1567⟩ Pyrrolizidine Alkaloids as Contaminants
⟨1604⟩ Presentation of Aerodynamic Particle Size Distribution (ASPD) Measurement Data for Orally Inhaled Products
⟨1901⟩ Theory and Practice of Asbestos Detection in Pharmaceutical Talc

### REAGENTS, INDICATORS, AND SOLUTIONS

Gamma-Decalactone
Glycolide
DL-Lactide
Alpha Tocopherol

### USP MONOGRAPHS

Acetazolamide Extended-Release Capsules
Bivalirudin
Bivalirudin for Injection
Cisplatin Injection
Clobazam
Dapagliflozin Propanediol
Dimethyl Fumarate
Dimethyl Fumarate Delayed-Release Capsules
Gabapentin Compounded Cream

### DIETARY SUPPLEMENTS

Bromelain
Cranberry Fruit Juice Dry Extract
Cranberry Fruit Juice Dry Extract Capsules
Inositol Niacinate

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# USP Annotated List

## General Notices, Monographs, General Chapters, Reagents, and Tables Affected by Changes Appearing in *USP 2023, Issue 3*

*Note—This list only contains In-Process Revision file types. In the lists below, if a section is new or if a subsection is added to or deleted from an existing section, it is labeled as such in parentheses after the section or subsection name. Items on this list that appear without the designation "new", "added", or "deleted" are items in which changes have been made to existing official text. The USP Admissions and Annotated lists are updated three times a year: Issue 1 (November), Issue 2 (February), and Issue 3 (June). These documents are static and will not be updated with the monthly revision postings. Although the Annotated List itself carries an official date, each Documentary Standard that is listed may have a different official date. Please open each standard to see its official date. For more questions on official statuses, please visit our Frequently Asked Questions (*https://www.usp.org/frequently-asked-questions/identifying-official-text*).*

### General Chapters

***Chemical Tests and Assays***

- ⟨314⟩ Molecular Weight Determination for Copolymers Containing Alkyl Methacrylate or Alkyl Acrylate (new)
- ⟨581⟩ Vitamin D Assay
  - ASSAY

***Physical Tests and Determinations***

- ⟨670⟩ Auxiliary Packaging Components
  - TITLE
  - PHARMACEUTICAL COIL
  - DESICCANT
  - ODOR ADSORBENT (added)
- ⟨831⟩ Refractive Index
- ⟨901⟩ Detection of Asbestos in Pharmaceutical Talc (new)

***General Information***

- ⟨1079⟩ Risks and Mitigation Strategies for the Storage and Transportation of Finished Drug Products
  - 4. RISK MITIGATION CATEGORIES AS QMS ELEMENTS
- ⟨1079.3⟩ Monitoring Devices—Time, Temperature, and Humidity (new)
- ⟨1094⟩ Capsules—Dissolution Testing and Related Quality Attributes
- ⟨1118⟩ Monitoring Devices—Time, Temperature, and Humidity (deleted)
- ⟨1504⟩ Quality Attributes of Starting Materials for the Chemical Synthesis of Therapeutic Peptides (new)
- ⟨1567⟩ Pyrrolizidine Alkaloids as Contaminants (new)
- ⟨1604⟩ Presentation of Aerodynamic Particle Size Distribution (APSD) Measurement Data for Orally Inhaled Products (new)
- ⟨1724⟩ Semisolid Drug Products—Performance Tests
- ⟨1901⟩ Theory and Practice of Asbestos Detection in Pharmaceutical Talc (new)
- ⟨1912⟩ Measurement of Yield Stress of Semisolids

### Reagents, Indicators, and Solutions

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- Bromelain
- Gamma-Decalactone (new)
- Glycolide (new)
- Guanine Hydrochloride
- DL-Lactide (new)
- Methylbenzothiazolone Hydrazone Hydrochloride
- Alpha Tocopherol (new)
- Water, Deaerated

### Reference Tables

**Description and Relative Solubility of USP and NF Articles**

- Candelilla Wax
- Cevimeline Hydrochloride (new)
- Clobazam (new)
- Dimethyl Fumarate (new)
- Guanfacine Hydrochloride (new)

**Excipients of USP and NF Articles**

- Candelilla Wax

## Monographs (*USP 2023, Issue 3*)

- Acetazolamide Extended-Release Capsules (new)
- Betamethasone Acetate
  - CHEMICAL INFORMATION
  - IDENTIFICATION
  - ASSAY
  - IMPURITIES
    - *Ordinary Impurities* (deleted) and *Organic Impurities* (added)
  - ADDITIONAL REQUIREMENTS
    - *USP Reference Standards*
- Bivalirudin (new)
- Bivalirudin for Injection (new)
- Calcium Pantothenate
  - ASSAY
  - IMPURITIES (added)
- Carbamazepine
  - CHEMICAL INFORMATION
  - IDENTIFICATION
    - *Spectroscopic Identification Tests, Infrared Spectroscopy, Test A*
  - ASSAY
  - IMPURITIES
    - *Organic Impurities*
  - ADDITIONAL REQUIREMENTS
    - *USP Reference Standards*
- Cefoperazone Sodium
  - CHEMICAL INFORMATION
  - ASSAY
  - SPECIFIC TESTS
    - *Sterility Tests* (deleted) and *Bacterial Endotoxins Test* (deleted)
  - ADDITIONAL REQUIREMENTS
    - *Labeling* (deleted)
- Cisplatin Injection (new)

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Clobazam (new)
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Dalteparin Sodium
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  - *Molecular Weight Distribution and Weight-Average Molecular Weight, Test B* and *Identification Tests—General, Sodium, Test D*
- IMPURITIES
  - *Limit of Nitrites*
- SPECIFIC TESTS
  - *Bacterial Endotoxins Test*
- ADDITIONAL REQUIREMENTS
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Dapagliflozin Propanediol (new)
Dimethyl Fumarate (new)
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Sodium Fluoride F 18 Injection
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- ASSAY
- PURITY
  - *Radiochemical Purity*
- IMPURITIES
  - *Radionuclidic Impurities*
- SPECIFIC TESTS
  - *Sterility Tests*
- ADDITIONAL REQUIREMENTS
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- DEFINITION
- IDENTIFICATION
- ASSAY
- IMPURITIES
  - *Organic Impurities*
- ADDITIONAL REQUIREMENTS
  - *USP Reference Standards*

Flurbiprofen Tablets
- ASSAY
- PERFORMANCE TESTS
- IMPURITIES
- ADDITIONAL REQUIREMENTS

Gabapentin Compounded Cream (new)
Glucagon
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- DEFINITION
- IDENTIFICATION
- ASSAY
- PRODUCT-RELATED SUBSTANCES AND IMPURITIES
- PROCESS-RELATED IMPURITIES AND OTHER COMPONENTS (added)
- SPECIFIC TESTS
  - *Peptide Mapping* (added), *Mass Spectral Analysis* (added), *Microbial Enumeration Tests* (added), and *Water Determination, Method Ic*

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- ASSAY
- IMPURITIES
  - *Organic Impurities* (added)
- SPECIFIC TESTS
  - *Composition* (added)

Isopropyl Alcohol
- ASSAY
- SPECIFIC TESTS
  - *Ultraviolet Absorption* (added)

Methylprednisolone
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- IMPURITIES
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- ADDITIONAL REQUIREMENTS
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Cranberry Fruit Juice Dry Extract Capsules (new)
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ASSAY
SPECIFIC TESTS
*Optical Rotation, Specific Rotation* and *Water Determination, Method Ia*
Inositol Niacinate (new)

*Lacticaseibacillus rhamnosus*
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*Nucleic Acid-Based Identification, Test B*
ADDITIONAL REQUIREMENTS
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TITLE
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COMPOSITION
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*HPTLC for Articles of Botanical Origin, Test A*
COMPOSITION

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# Chapter Guide: Vaccines

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- ⟨788⟩ *Particulate Matter in Injections*
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- ⟨1660⟩ *Evaluation of the Inner Surface Durability of Glass Containers*
- ⟨1661⟩ *Evaluation of Plastic Packaging Systems for Pharmaceutical Use and Their Materials of Construction*
- ⟨1663⟩ *Assessment of Extractables Associated with Pharmaceutical Packaging/Delivery Systems*
- ⟨1664⟩ *Assessment of Drug Product Leachables Associated with Pharmaceutical Packaging/Delivery Systems*
- ⟨1776⟩ *Image Analysis of Pharmaceutical Systems*
- ⟨1787⟩ *Measurement of Subvisible Particulate Matter in Therapeutic Protein Injections*
- ⟨1788⟩ *Methods for the Determination of Subvisible Particulate Matter*
- ⟨1788.1⟩ *Light Obscuration Method for the Determination of Subvisible Particulate Matter*
- ⟨1788.2⟩ *Membrane Microscope Method for the Determination of Subvisible Particulate Matter*
- ⟨1788.3⟩ *Flow Imaging Method for the Determination of Subvisible Particulate Matter*

Description

- ⟨791⟩ *pH*
- ⟨1041⟩ *Biologics*

Safety

- ⟨11⟩ *USP Reference Standards*
- ⟨63⟩ *Mycoplasma Tests*
- ⟨71⟩ *Sterility Tests*
- ⟨85⟩ *Bacterial Endotoxins Test*
- ⟨88⟩ *Biological Reactivity Tests, In Vivo*
- ⟨90⟩ *Fetal Bovine Serum—Quality Attributes and Functionality Tests*
- ⟨151⟩ *Pyrogen Test*
- ⟨341⟩ *Antimicrobial Agents—Content*
- ⟨1024⟩ *Bovine Serum*
- ⟨1071⟩ *Rapid Microbial Tests for Release of Sterile Short-Life Products: A Risk-Based Approach*
- ⟨1085⟩ *Guidelines on Endotoxins Test*
- ⟨1113⟩ *Microbial Characterization, Identification, and Strain Typing*
- ⟨1121⟩ *Nomenclature*
- ⟨1211⟩ *Sterility Assurance*
- ⟨1228⟩ *Depyrogenation*

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- ⟨1228.1⟩ *Dry Heat Depyrogenation*
- ⟨1228.3⟩ *Depyrogenation by Filtration*
- ⟨1228.4⟩ *Depyrogenation by Rinsing*
- ⟨1228.5⟩ *Endotoxin Indicators for Depyrogenation*
- ⟨1229⟩ *Sterilization of Compendial Articles*
- ⟨1229.4⟩ *Sterilizing Filtration of Liquids*
- ⟨1229.14⟩ *Sterilization Cycle Development*
- ⟨1229.15⟩ *Sterilizing Filtration of Gases*
- ⟨1229.16⟩ *Prion Inactivation*
- ⟨1229.17⟩ *Mycoplasma Sterilization*
- ⟨1229.18⟩ *Viral Clearance Methods*
- ⟨1234⟩ *Vaccines for Human Use—Polysaccharide and Glycoconjugate Vaccines*
- ⟨1237⟩ *Virology Test Methods*

Assay

- ⟨111⟩ *Design and Analysis of Biological Assays*
- ⟨621⟩ *Chromatography*
- ⟨736⟩ *Mass Spectrometry*
- ⟨761⟩ *Nuclear Magnetic Resonance Spectroscopy*
- ⟨852⟩ *Atomic Absorption Spectroscopy*
- ⟨853⟩ *Fluorescence Spectroscopy*
- ⟨854⟩ *Mid-Infrared Spectroscopy*
- ⟨855⟩ *Nephelometry and Turbidimetry*
- ⟨856⟩ *Near-Infrared Spectroscopy*
- ⟨857⟩ *Ultraviolet-Visible Spectroscopy*
- ⟨1030⟩ *Biological Assay Chapters—Overview and Glossary*
- ⟨1032⟩ *Design and Development of Biological Assays*
- ⟨1033⟩ *Biological Assay Validation*
- ⟨1034⟩ *Analysis of Biological Assays*
- ⟨1044⟩ *Cryopreservation of Cells*
- ⟨1052⟩ *Biotechnology Derived Articles—Amino Acid Analysis*
- ⟨1053⟩ *Capillary Electrophoresis*
- ⟨1054⟩ *Biotechnology Derived Articles—Isoelectric Focusing*
- ⟨1055⟩ *Biotechnology Derived Articles—Peptide Mapping*
- ⟨1056⟩ *Biotechnology Derived Articles—Polyacrylamide Gel Electrophoresis*
- ⟨1057⟩ *Biotechnology Derived Articles—Total Protein Assay*
- ⟨1105⟩ *Immunological Test Methods—Surface Plasmon Resonance*
- ⟨1234⟩ *Vaccines for Human Use—Polysaccharide and Glycoconjugate Vaccines*
- ⟨1235⟩ *Vaccines for Human Use—General Considerations*
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- ⟨1736⟩ *Applications of Mass Spectrometry*
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- ⟨1854⟩ *Mid-Infrared Spectroscopy—Theory and Practice*
- ⟨1856⟩ *Near-Infrared Spectroscopy—Theory and Practice*
- ⟨1857⟩ *Ultraviolet-Visible Spectroscopy—Theory and Practice*

Physicochemical

- ⟨432⟩ *Determination of Zeta Potential by Electrophoretic Light Scattering*
- ⟨507⟩ *Protein Determination Procedures*
- ⟨621⟩ *Chromatography*
- ⟨736⟩ *Mass Spectrometry*
- ⟨761⟩ *Nuclear Magnetic Resonance Spectroscopy*
- ⟨786⟩ *Particle Size Distribution Estimation by Analytical Sieving*
- ⟨791⟩ *pH*
- ⟨852⟩ *Atomic Absorption Spectroscopy*
- ⟨853⟩ *Fluorescence Spectroscopy*
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- ⟨856⟩ *Near-Infrared Spectroscopy*

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- ⟨857⟩ *Ultraviolet-Visible Spectroscopy*
- ⟨1052⟩ *Biotechnology Derived Articles—Amino Acid Analysis*
- ⟨1053⟩ *Capillary Electrophoresis*
- ⟨1054⟩ *Biotechnology Derived Articles—Isoelectric Focusing*
- ⟨1055⟩ *Biotechnology Derived Articles—Peptide Mapping*
- ⟨1056⟩ *Biotechnology Derived Articles—Polyacrylamide Gel Electrophoresis*
- ⟨1057⟩ *Biotechnology Derived Articles—Total Protein Assay*
- ⟨1065⟩ *Ion Chromatography*
- ⟨1084⟩ *Glycoprotein and Glycan Analysis—General Considerations*
- ⟨1234⟩ *Vaccines for Human Use—Polysaccharide and Glycoconjugate Vaccines*
- ⟨1238⟩ *Vaccines for Human Use—Bacterial Vaccines*
- ⟨1430.1⟩ *Analytical Methodologies Based on Scattering Phenomena—Static Light Scattering*
- ⟨1430.2⟩ *Analytical Methodologies Based on Scattering Phenomena—Light Diffraction Measurements of Particle Size*
- ⟨1430.3⟩ *Analytical Methodologies Based on Scattering Phenomena—Dynamic Light Scattering*
- ⟨1430.4⟩ *Analytical Methodologies Based on Scattering Phenomena—Electrophoretic Light Scattering (Determination of Zeta Potential)*
- ⟨1430.5⟩ *Analytical Methodologies Based on Scattering Phenomena—Small Angle X-Ray Scattering and Small Angle Neutron Scattering*
- ⟨1430.6⟩ *Analytical Methodologies Based on Scattering Phenomena—Particle Counting via Light Scattering*
- ⟨1430.7⟩ *Analytical Methodologies Based on Scattering Phenomena—Nephelometry And Turbidimetry*
- ⟨1736⟩ *Applications of Mass Spectrometry*
- ⟨1852⟩ *Atomic Absorption Spectroscopy—Theory and Practice*
- ⟨1853⟩ *Fluorescence Spectroscopy—Theory and Practice*
- ⟨1854⟩ *Mid-Infrared Spectroscopy—Theory and Practice*
- ⟨1856⟩ *Near-Infrared Spectroscopy—Theory and Practice*
- ⟨1857⟩ *Ultraviolet-Visible Spectroscopy—Theory and Practice*

Impurities

Process Related

- ⟨81⟩ *Antibiotics—Microbial Assays*
- ⟨90⟩ *Fetal Bovine Serum—Quality Attributes and Functionality Tests*
- ⟨111⟩ *Design and Analysis of Biological Assays*
- ⟨509⟩ *Residual DNA Testing*
- ⟨621⟩ *Chromatography*
- ⟨731⟩ *Loss on Drying*
- ⟨736⟩ *Mass Spectrometry*
- ⟨761⟩ *Nuclear Magnetic Resonance Spectroscopy*
- ⟨921⟩ *Water Determination*
- ⟨922⟩ *Water Activity*
- ⟨1024⟩ *Bovine Serum*
- ⟨1052⟩ *Biotechnology Derived Articles—Amino Acid Analysis*
- ⟨1053⟩ *Capillary Electrophoresis*
- ⟨1054⟩ *Biotechnology Derived Articles—Isoelectric Focusing*
- ⟨1055⟩ *Biotechnology Derived Articles—Peptide Mapping*
- ⟨1056⟩ *Biotechnology Derived Articles—Polyacrylamide Gel Electrophoresis*
- ⟨1057⟩ *Biotechnology Derived Articles—Total Protein Assay*
- ⟨1084⟩ *Glycoprotein and Glycan Analysis—General Considerations*
- ⟨1102⟩ *Immunological Test Methods—General Considerations*
- ⟨1103⟩ *Immunological Test Methods—Enzyme-Linked Immunosorbent Assay (ELISA)*
- ⟨1104⟩ *Immunological Test Methods—Immunoblot Analysis*
- ⟨1126⟩ *Nucleic Acid-Based Techniques—Extraction, Detection, and Sequencing*
- ⟨1127⟩ *Nucleic Acid-Based Techniques—Amplification*
- ⟨1130⟩ *Nucleic Acid-Based Techniques—Approaches for Detecting Trace Nucleic Acids (Residual DNA Testing)*
- ⟨1229.16⟩ *Prion Inactivation*
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- ⟨1229.18⟩ *Viral Clearance Methods*
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- ⟨1238⟩ *Vaccines for Human Use—Bacterial Vaccines*
- ⟨1736⟩ *Applications of Mass Spectrometry*
- ⟨1761⟩ *Applications of Nuclear Magnetic Resonance Spectroscopy*

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- ⟨1776⟩ *Image Analysis of Pharmaceutical Systems*

Product Related

- ⟨111⟩ *Design and Analysis of Biological Assays*
- ⟨621⟩ *Chromatography*
- ⟨736⟩ *Mass Spectrometry*
- ⟨761⟩ *Nuclear Magnetic Resonance Spectroscopy*
- ⟨1102⟩ *Immunological Test Methods—General Considerations*
- ⟨1103⟩ *Immunological Test Methods—Enzyme-Linked Immunosorbent Assay (ELISA)*
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- ⟨1229.17⟩ *Mycoplasma Sterilization*
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- ⟨1234⟩ *Vaccines for Human Use—Polysaccharide and Glycoconjugate Vaccines*
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- ⟨1238⟩ *Vaccines for Human Use—Bacterial Vaccines*
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